Studies in Natural Products Chemistry

Atta-ur-Rahman, FRS
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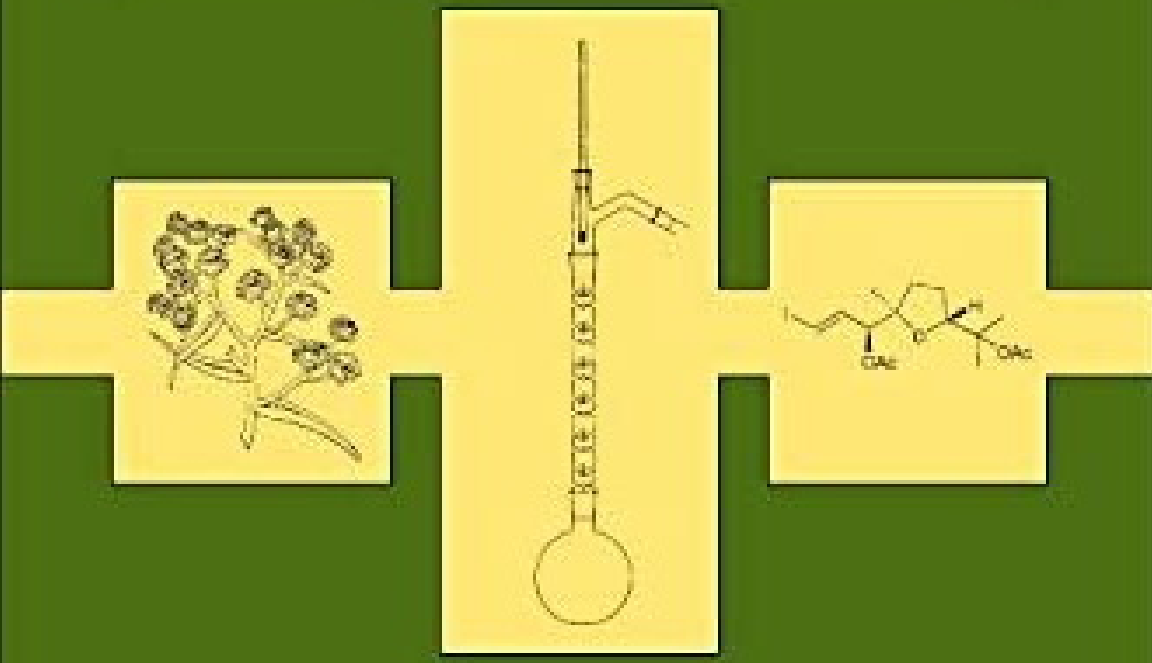

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Preface

More than half of the drugs used in modern medicine are derived from natural sources. Natural product chemistry continues to expose new pharmacophores and new mechanisms of action against various diseases. Volume 40 of "Studies in Natural Product Chemistry" presents many interesting classes of natural products with exciting biological activities and interesting synthetic approaches to their challenging structures.

Chapters 1 and 2 of this book cover the role of triterpenoids and brassinosteroids against cancer. In Chapter 1 (Tundis *et al.*), recent work on the anticancer potential of triterpenoids and their derivatives is discussed and structure–activity relationships reviewed. Bajguz *et al.*, in Chapter 2, present brassinosteroids that are known to inhibit cancer cells as well as for their antiviral activities. Brassinosteroids are polyhydroxylated derivatives of 5α-cholestane that are involved in the promotion of plant growth and development. Their functions include regulation of gene expression, cell division and expansion, differentiation, programmed cell death, and homeostasis. Their potential as new lead drugs with anticancer antiviral properties is discussed. Marco-Contelles *et al.* present the use of [3,3]-sigmatropic rearrangements for the transformations of propargylic esters to key intermediates in Chapter 3. Chapter 4 (Moyano and Companyó) covers different strategies for the catalytic synthesis of 3, 3-disubstituted oxindoles, which are intermediates used for the synthesis of indole alkaloids, having different biological activities. Abyssomicin C was isolated from the marine actinomycete *Verrucosispora* AB-18-032, collected from Japanese sea at 289 m depth. It is the first natural product acting as inhibitor of the enzymes involved in *p*ABA and folic acid biosynthesis. Synthetic approaches to abyssomicins are presented in Chapter 5 by Savic. Chang and Song, in Chapter 6, have discussed the origin and properties of lignans, including the spectroscopic properties of dibenzo cyclooctadiene lignan and synthetic approaches to these compounds.

Chapter 7 by Andrade and co-workers focuses on anti-inflammatory compounds of small molecular weight obtained from marine organisms: sponges, gorgonians, mollusks, echinoderms. Gupta and co-workers, in Chapter 8, also cover bioactive compounds with anticancer activity from marine organisms. Shukla *et al.*, in Chapter 9, have reviewed the isolation, biological properties, and synthesis of 14b-hydroxypregnane derivatives found in *Caralluma adscendens* var. *fimbriata* and *Hoodia gordonii*. There has been much interest in lectins and their anticancer activities in recent years. Chapter 10 by Rao *et al.* involves a discourse on lectins that have specificity for various

carbohydrates and thus are able to target cancer cells. Lycopenes are known to neutralize toxic free radicals in the body. However, they cannot be synthesized by the human body and must be provided in the diet. The analysis of lycopenes by spectroscopic and chromatographic methods is presented by Caceres *et al*., in Chapter 11, as well as computational modelling of their structures that could be important in the development of food products.

In Chapter 12 by Goodger and Woodrow, the structure–activity relationships of various plant-based glucose esters of monoterpenoids are described. Their biological activities, commercial potential as therapeutics, functional roles in plants such as biotic and abiotic stress and information available on their biosynthesis are presented.

Cordyceps are a type of fungi producing various medicinally active metabolites. However, their inclusion in diet faces quality control concerns. The review by Prasain in Chapter 13 highlights the recent advances in profiling the bioactive compounds from *Cordyceps sinensis*, their pharmacological properties and quality control aspects. Chapter 14 by Abouzid and Mohamed presents the hepatoprotective role of silymarin—an isomeric mixture of seven flavonolignans (silybin A, silybin B, isosilybin A, isosilybin B, silychristin A, silychristin B and silydianin, and a flavonoid taxifolin); the structure–activity relationship is also discussed. Finally, Chapter 15 by Kusumawati and Indrayanto discusses the role of different plant-based antioxidants in cosmetics.

The rich material on bioactive compounds from terrestrial and marine organisms contained in the present volume should be of great interest to a large number of scientists interested in the treasure house of new exciting pharmacophores offered by nature.

I would like to thank Ms. Taqdees Malik, Ms. Darshna Kumari, and Ms. Humaira Hashmi for their assistance in the preparation of this volume. I am also grateful to Mr. Mahmood Alam for the editorial assistance.

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Chapter 1

Recent Insights into the Emerging Role of Triterpenoids in Cancer Therapy: Part I

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Chapter Outline

INTRODUCTION

Cancer is a leading cause of death worldwide, accounting for 7.6 million deaths (around 13% of all deaths in 2008) [1]. Lung, stomach, liver, colon, and breast cancers cause the most cancer deaths each year. About 30% of cancer deaths are due to the five leading behavioral and dietary risks: high body mass index, low fruit and vegetable intake, lack of physical activity, tobacco use, and alcohol use. Tobacco use is the most important risk factor for cancer, causing 22% of global cancer deaths and 71% of global lung cancer deaths. Cancer-causing viral infections, such as hepatitis B virus, hepatitis C virus, and human papilloma virus, are responsible for up to 20% of cancer deaths in low- and middle-income countries. About 70% of all cancer deaths in 2008 occurred in low- and middle-income countries. Deaths from cancer worldwide are projected to continue rising, with an estimated 13.1 million deaths in 2030 [1].

Despite the discovery of many drugs, the search for new anticancer agents is still necessary to increase the range available and to find less toxic and more effective drugs. Natural products have been a rich source of agents of value in medicine. In addition, many new natural compounds isolated from

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plant sources have been considered prototypes, leads, or heads of series and their later structural modification has afforded compounds with pharmacological activity and extraordinary therapeutic possibilities.

Terpenoids represent a large class of natural compounds that are classified according to the number of isoprene units [2]. Their biosynthesis proceeds via the isoprenoid pathway in which three isoprene units are linked in a head-to-tail manner to each other, resulting in the 15 C-atom molecule farnesyl pyrophosphate. Two farnesyl pyrophosphates are subsequently linked in a tail-to-tail manner to give a compound of 30 carbon atoms, called squalene [3]. Squalene is oxidized to oxidosqualene, which is the common starting point for cyclization reactions in triterpenoid biosynthesis. Oxidosqualene is converted to cyclic derivatives via protonation and epoxide ring opening, which creates a carbocation that can undergo several types of cyclization reactions. Many different kinds of cyclases have been described, and their mechanisms of action are well documented in the biosynthesis of triterpenoids [4]. To date, approximately 20,000 triterpenoids have been identified from the various parts of medicinal plants [5]. These secondary plant metabolites are constituents of the cell membrane, and they play important roles in cell membrane defense, and in the biological efficiency and fluidity of the cell membrane. The diversity of skeletal structures, isolation challenge, and several biological and pharmacological activities are the driving forces for research work on these phytochemicals. In particular, they have attracted great attention for their antineoplastic and antiproliferative agents in various cancer cell lines.

In this chapter, we have made an attempt to discuss the recent developments in the antitumor properties of natural triterpenoids with oleanane, dammarane, hopane, lanostane, and ursane skeletons and their synthetic derivatives with a special focus on the structure–activity relationship (SAR).

MECHANISMS OF THE ANTITUMOR EFFECT OF TRITERPENOIDS

The Oleanane Group

Oleanane-type pentacyclic triterpenoids are abundant in the plant kingdom [6]. Several *in vitro* and *in vivo* studies have demonstrated the potential antitumor properties of this class of triterpenoids.

Oleanolic Acid and Derivatives

Among them, oleanolic acid (**1**) was isolated as free acid or aglycones of triterpenoid saponins in almost 190 species of 60 branches of food, medicinal herbs, and other plants [7]. It was demonstrated to possess antiinflammatory activity, protective action against toxic injury to the liver, collagen synthesis-inducing properties, and differentiation-inducing activity in leukemia or teratocarcinoma cells [8]. In recent years, it was found that oleanolic acid (**1**) had marked

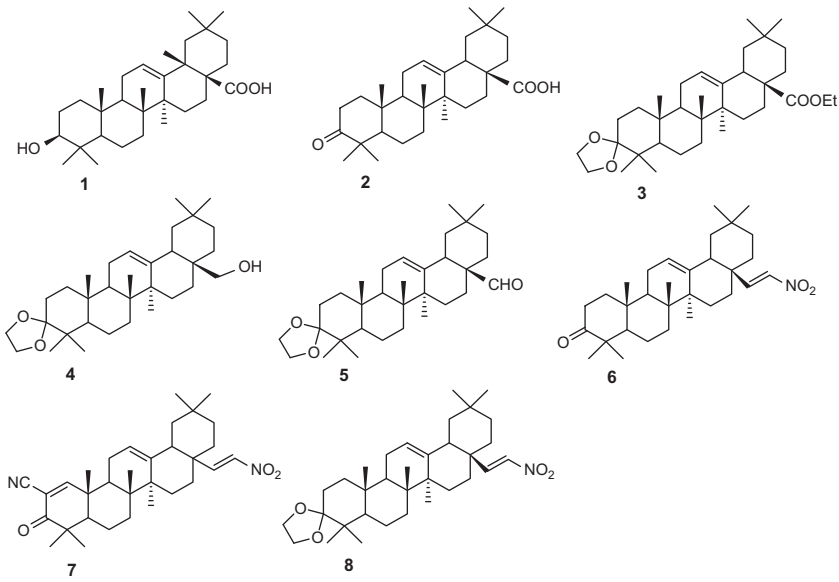

FIGURE 1 Chemical structures of compounds **1**–**8**.

antitumor effects on many cancer cell lines. On the basis of the bioactivity of **1**, various research groups have tried to synthesize derivatives that could be more active than the parent compound (Fig. 1).

Oleanane derivatives were synthesized and tested for their cytotoxic activity against HepG2 and Col-02 cell lines [9]. SAR analysis of these derivatives indicated that the keto-derivative **2** is a little more active than **1**. Acetylation of the C3 keto group (**3–5**) diminished the inhibitory activity compared to **2**. The introduction of a nitro group at C17 of **1** increased the potency by three times compared to **1** (**6**: GI_{50} values of 1.30 and 4.18 μM against HepG2 and Col-02 cell lines, respectively). These results indicated that the nitro moiety was important for the retention of cytotoxic activity. Nitro derivatives **6–8** with modified ring A exhibited moderate-to-good inhibitory activity. Compound **7** showed the best activity (GI_{50} values of 1.75 and 0.71 μM against HepG2 and Col-02, respectively). Both the structures 1-en-2-cyano-3-oxo in ring A and nitro group at C17 are important in the retention of inhibition against HepG2 and Col-02 cell lines. In this study, the positive control taxol exerted 50% inhibition of cell growth (GI_{50}) values of 0.05 and 0.03 μM against HepG2 and Col-02, respectively.

Glycyrrhetinic Acid and Derivatives

Glycyrrhetinic acid (**9**) is the triterpenoid aglycone of glycyrrhizin, the principal licorice component. When this saponin is orally administered, glycyrrhizin is hydrolyzed to the pharmacologically active **9**. The *in vitro* and *in vivo*

growth-inhibiting activity of **9** was reported in various experimental cancer models [10,11]. Compound **9** displays both cytostatic and cytotoxic effects in cancer cells, depending on the concentrations, cell lines, and durations of treatment. Cytostatic effects are mediated through the arrest of cancer cells in the G1 phase of the cell cycle. The **9**-induced cytotoxic effects on cancer cells occur at higher doses and relate to proapoptotic stimuli [12–14].

Glycyrrhetinic acid derivatives were obtained by the introduction of a cyano group at the position of C2 and its methyl ester at C30 carboxylic acid group, besides two isomers, 2-cyano-3,12-dioxooleana-1,12-dien-30-oic acid and methyl 2-cyano-3,12-dioxooleana-1,12-dien-30-oate, with a keto group at C12 [15]. SAR analysis revealed that the introduction of a cyano group significantly increased antileukemia effects compared with the parental compound; switching the keto group from C11 to C12 increased the activity of cell growth inhibition about fivefold and apoptosis induction about 10-fold; the addition of methyl ester further increased cell growth inhibition and apoptosis induction abilities.

The study of the cytotoxicity of a series of aminoalkyl-substituted derivatives synthesized starting from glycyrrhetinic acid methyl ester revealed that a free carboxylic group in position C30 results in a lower inhibition of cell growth compared to the corresponding esters; esterification results in an approximately threefold higher cytotoxicity; and additional chloroacetylation affords an analog showing a ca. 10-fold higher cytotoxicity than the parent compound **9** [16]. A compound carrying a diaminohexyl chain was the most active derivative (IC_{50} values 0.59–3.0 μM). Other derivatives of **9** differing in lipophilicity and structure at ring A were synthesized and screened for their cytotoxicity [17]. Neither configurational inversion at position C2 nor structural modifications at position C3 led to higher cytotoxicity. The introduction of at least one nitrogen-containing substituent, however, was quite promising. Thus, derivatives possessing a primary amino group showed the highest activities while substitution reduced cytotoxicity. In addition, introduction of sulfur decreased cytotoxicity. Structural modification of ring A, that is, expanding or ring opening by and large decreased cytotoxicity. It seems that the presence of an intact ring A is essential for cytotoxicity. Attachment of a diaminoalkyl moiety at position C3 of a lipophilic ester of glycyrrhetinic acid (**9**) has an increasing effect on the cytotoxicity (Fig. 2).

The glycyrrhetinic acid derivative, *N*-(2-{3-[3,5-bis(trifluoromethyl)-phenyl]ureido}ethyl)-glycyrrhetinamide, demonstrated inhibitory concentration 50% (IC_{50}) values in single-digit micromolarity in a panel of cancer cell lines including U373, T98G, Hs683, A549, MCF-7, PC-3, SKMEL-28, and B16F10 cell line. This compound showed cytostatic and not cytotoxic effects in cancer cells. These cytostatic effects occur, at least partly, through the targeting of a dozen kinases that are implicated in cancer cell proliferation and in the control of the actin cytoskeleton organization [18]. Glycyrrhetinic acid (**9**) is less efficient than this compound in inhibiting proteasome activity and it is cytotoxic, at least partly through the activation of proapoptotic processes.

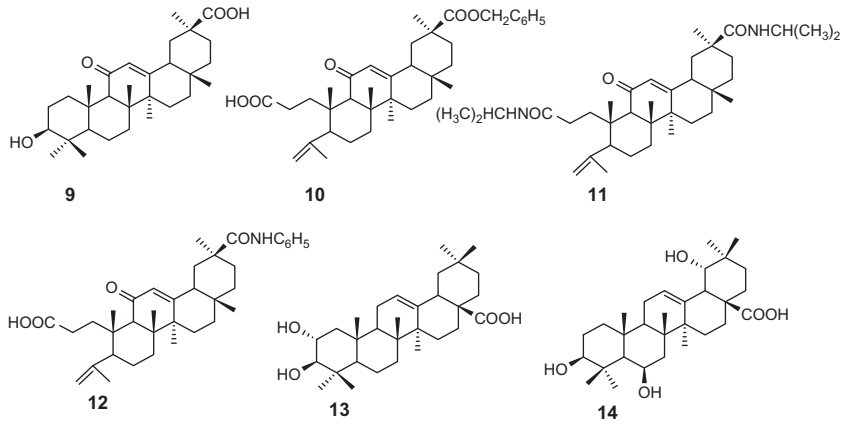

FIGURE 2 Chemical structures of compounds **9**–**14**.

In a series of 26 glycyrrhetinic acid derivatives, compounds **10**, **11**, and **12** are the most potent compounds inhibiting cell growth of NTUB1 cell line with IC_{50} values of 2.34, 4.76, and 3.31 μM, respectively [19]. Exposure of NTUB1 cells to **11** significantly increased the production of reactive oxygen species (ROS). Moreover, the treatment of NTUB1 cell line with **11** did not induce cell cycle arrest but was accompanied by an increase in apoptotic cell death in a dose-dependent manner after 24 h. Mitochondrial membrane potential decreased significantly in a dose-dependent manner when the NTUB1 cells were exposed to **11**. The marked collapse of the mitochondrial membrane potential suggested that dysfunction of the mitochondria may be involved in the oxidative burst and apoptosis induced by **11**. Western blot analysis showed that NTUB1 cells treated with **11** increased the level of p53 in a dose-dependent manner. These results suggested that **11** induced a mitochondrial-mediated apoptosis in NTUB1 cells through activation of p53, an important regulator of apoptosis induced by ROS. In a recent work, doxorubicin-loaded glycyrrhetinic acid-modified alginate nanoparticles were prepared for liver-targeted drug delivery. This formulation showed great potential in liver cancer-targeted therapy [20]. In another recent study, the pharmacokinetics and antitumor properties of docetaxel liposomes surface-modified with glycyrrhetinic acid (**9**) as a new hepatocyte-targeted delivery vehicle were investigated [21]. The size of this formulation was about 90 nm with negative charge and the entrapment efficiency was more than 95%. Glycyrrhetinic acid-modified liposomes had the specific receptor-mediated cellular endocytosis to hepatocytes, and the uptake ratio of hepatocytes to nonparenchymal cells was 2.28 times. The tumor inhibitory *in vitro* was 2.03 times of docetaxel liposomes. The preliminary cellular uptake test showed the receptor-mediated endocytosis and enhanced hepatocyte target for glycyrrhetinic acid-modified liposomes.

Compared with the unmodified liposome, the new formulation possessed better antitumor activity and unchanged pharmacokinetic behavior.

Maslinic Acid

Maslinic acid (**13**) significantly enhanced tumor necrosis factor α (TNFα)-induced inhibition of pancreatic cancer cell proliferation, invasion, and potentiated TNFα-induced cell apoptosis by suppressing TNFα-induced nuclear factor-κB (NF-κB) activation in a dose- and time-dependent manner [22]. Maslinic acid (**13**) inhibited TNFα-induced IBa degradation, p65 phosphorylation, and nuclear translocation, and decreased the expression levels of NF-κB-regulated genes, including genes involved in tumor cell proliferation (cyclin D1, COX2, and c-Myc), invasion (MMP-9 and ICAM-1 (Intercellular Adhesion Molecule 1)), and angiogenesis (vascular endothelial growth factor (VEGF)). Moreover, maslinic acid demonstrated proapoptotic effects in several cell lines. In a recent study, the effects of maslinic acid (**13**) on the metastatic capacity of prostate cancer cells were examined. Maslinic acid downregulated both basal and EGF-stimulated secretion of matrix metallopeptidase (MMP)-9, MMP-2, urokinase-type plasminogen activator, VEGF, and tissue inhibitors of metalloproteinases (TIMP)-1, as well as the expression of uPA receptors, intercellular adhesion molecules, vascular cell adhesion molecules, and E-cadherin, while increasing TIMP-2 secretion. Maslinic acid (**13**) reduced the levels of hypoxia-inducible factor-1α (HIF-1α) protein and mRNA; the reduction was accompanied by reduced stability, nuclear levels, and transcriptional activity of HIF-1α. Maslinic acid markedly inhibited the migration, invasion, and adhesion of DU145 prostate cancer cells. In *in vivo* models (athymic nu/nu mouse model), maslinic acid (**13**) significantly suppressed pancreatic tumor growth, induced tumor apoptosis, and inhibited NF-κB-regulated antiapoptotic gene expression, such as Survivin and Bcl-xl.

Others

A new oleanolic triterpene, 3β,6β,19α-trihydroxy-12-oleanen-28-oic acid (**14**), obtained from the stem bark of *Uncaria macrophylla* exhibited weak activities against two cancer cell lines of MCF-7 and HepG2 with IC_{50} values of 78.2 and 73.9 μg/ml, while those of cisplatin used as positive control were 7.5 and 8.2 μg/ml, respectively[23].

A moderate antiproliferative activity was found for two new 13,28-epoxy oleanane-type triterpenoids, namely heterogenoside E and F, isolated from *Lysimachia heterogenea*, against Hela, KB-3-1, and HepG2 cells, and seven new oleanane-type triterpenoids, named fatsicarpains A–G, isolated from *Fatsia polycarpa*, against HepG2 2.2.15 and AGS cell lines [24,25]. IC_{50} values of 1.14–2.62 μM against HT-29, HePG2, BGC-823, A549, and A375 tumor cell lines were found for another three new oleanane-type triterpenoid saponins, namely clethroidosides C, D, and F, isolated from *L. clethroides* together with clethroidoside A, B, E, and G [26]. Clethroidoside E showed selective cytotoxic activity against HT-29, HePG2, and BGC-823 cell lines (IC_{50} values of 1.40–8.05 μM). Generally, all isolated compounds exhibited

a more potent cytotoxic activity than paclitaxel (IC_{50} values in the range 3.29×10^{-3} to 4.9×10^{-3} μM).

IC_{50} values in the range of 11.4–15.9 μM against HCT 116 cell line and IC_{50} values in the range 9.2–19.7 μM against HT-29 demonstrated three unusual oleanane-type saponins, isolated from *Arenaria montana* [27].

The Dammarane Group

Dammarane-type triterpenoids began to draw attention when several publications showed their anticancer activities toward a variety of cancers. This paragraph reports the most recent studies on the bioactivity of this class of triterpenoids.

Bacopaside É (**15**) and bacopaside VII (**16**), new dammarane triterpene saponins isolated from *n*-BuOH fraction of *Bacopa monniera*, showed cytotoxicity against MDA-MB-231, SHG-44, HCT-8, A-549, and PC-3M human tumor cell lines. Moreover, both compounds significantly inhibited breast cancer cell line MDA-MB-231 adhesion, and migration and Matrigel invasion *in vitro* at the concentration of 50 μmol/l. In *in vivo* models, compounds **15** and **16** showed 90.52% and 84.13% inhibition in mouse implanted with sarcoma S180 at the concentration of 50 μmol/kg [28] (Fig. 3).

More recently, among the novel dammarane-type sapogenins isolated from *Panax ginseng* berry, the compound 20(*R*)-20-methoxyl-dammarane-3β,12β,25-triol (**17**) showed antiproliferative activity against HepG2, Colon205, and HL-60 tumor cell lines with IC_{50} values of 8.78, 8.64, and 3.98 μM, respectively [29]. Other interesting new dammarane-type glycosides, namely ginsenosides SL1–SL3, and 11 known compounds were isolated from the heat-processed leaves of *P. ginseng* [30]. Ginsenosides Rh3 (**18**) and Rk2 (**19**) exhibited potent effects against human leukemia HL-60 cells with IC_{50} values of 0.8 and 0.9 μM, respectively. In addition, ginsenosides SL3 (**20**), 20*S*-Rg2 (**21**), F4 (**22**), and 20*S*-Rh2 (**23**) displayed good activity with IC_{50} values of 9.0, 9.0, 7.5, and 8.2 μM, respectively. SAR analysis suggested that variations in structures of ginsenosides, especially the side chain, influenced the cytotoxic activity against HL-60 cells. In particular, *S* stereospecificity at C20 in cytotoxic action against HL-60 cells was observed since, of the two isomeric pairs at C20 of ginsenoside Rg2 (**21**, **24**) and ginsenoside Rh2 (**23**, **25**), only the 20*S* isomers (**21**, **23**) were strongly active. It is notable that oral administration of ginsenosides such as Rg1, Rg3, and Rh2 has been reported to show anticancer activity [31–33].

A moderate cytotoxic activity was found for dammarane-type saponins isolated from *Gynostemma pentaphyllum* [34,35].

Centella asiatica is a traditional herbal medicine used in Asiatic countries. Experimental and clinical investigations showed that this plant, characterized mainly by pentacyclic triterpenes as secondary metabolites, had a wide spectrum of medicinal effects, for example, its usefulness in the treatment of venous

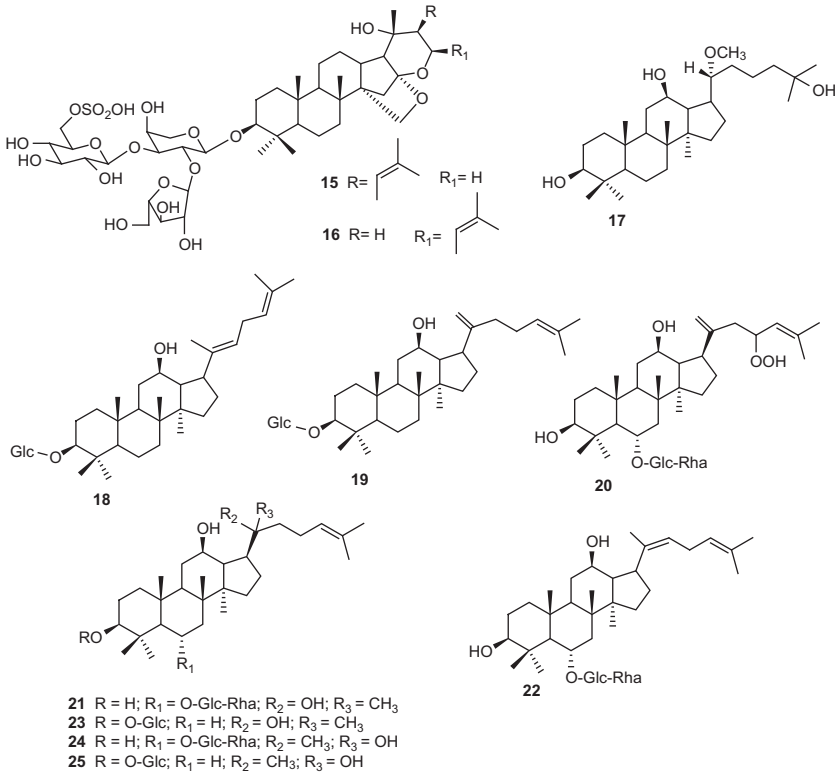

FIGURE 3 Chemical structures of compounds **15**–**25**.

insufficiency, striae gravidarum, and wound-healing disturbances [36–38]. Recently, a phytochemical study evidenced the presence of two new dammarane monodesmosides, centellosides A and B, in whole plants of *C. asiatica* that showed mild *in vitro* cytotoxicity against HepG2 and K562 cells. [39].

Panaxadiol (**26**), one of the protopanaxadiol-type of ginsenoside with a dammarane skeleton, also demonstrated antitumor properties when coadministrated with cyclophosphamide, 5-fluorouracil, and epicatechinin in human cancer cell lines [40–42]. Three panaxadiol derivatives, such as 3β-acetoxy-panaxadiol, 3β-palmitic acid aceloxy-panaxadiol, and 3β-octadecanoic-panaxadiol, have been reported to possess potent antitumor activity. Recently, some unsaturated fatty acid ester and amino acid ester groups were modified on the panaxadiol backbone resulting in a series of panaxadiol derivatives [43,44]. The synthesized analogs were tested for their antiproliferative activity in ES-2, U2-OS, HepG2, and A549 cell lines. The most promising compounds in this series were **27**–**33**, which showed stronger antiproliferative activities than the parent compound panaxadiol (**26**). Against ES-2 cells, compound **27** exhibited the most potent activity with an IC_{50} value of 4.26 μM. They showed almost similar activity

regardless of the substituents (unsaturated fatty acyl groups vs. amino acyl groups). For the osteosarcoma cells U2-OS, compounds **27**, **28**, and **33** exhibited more potent antitumor activity than **26** (IC_{50}, 26.81 μM), with IC_{50} values of 14.71, 13.67, and 14.44 μM, respectively. The results indicated that most amino acid-acylated panaxadiol derivatives showed stronger activity than the unsaturated fatty acid-acylated **26** compounds. From the results of the antitumor tests in HepG2, more significant differences were distinguishable between the two kinds of acyl groups. Derivatives **28**–**33** exhibited stronger activities than **26** (IC_{50}, 37.91 μM) with IC_{50} values in the range 5.03–18.75 μM. The data from antitumor tests in A549 cells showed similar results as in the HepG2 cells.

Previously, several derivatives obtained by modifying dammarenolic acid (**34**) were examined for their inhibitory effects on the induction of Epstein–Barr virus early antigen (EBV-EA) by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) in Raji cells, a known primary screening test for antitumor promoters [45]. Dammarenolic acid (**34**) and its two reduction products **35** and **36** exhibited the highest inhibitory effects against EBV-EA with IC_{50} values of 226, 208, and 212 mol ratio/32 pmol TPA, respectively. Dammarane-type triterpenoids with a linear side chain at C17 exhibited more potent inhibitory effects than those with a cyclic side chain, and 3-oxo groups exerted almost no influence on the activity when compared with 3β-OH groups. Conjugation with L-amino acids at C3 reduced activity, while reduction to alcohol and aldehyde at C3 enhanced activity. Compounds were analyzed for their potential cytotoxicity against HL60 and CRL1579 cell lines and it appears that reduction and conjugation with amino acids of **34** are generally not responsible for the improvement of cytotoxicity against HL60 cell line. On the contrary, reduction and conjugation with amino acids enhance cytotoxic activity against CRL1579 cell line (Fig. 4).

Ginsenosides are classified into two groups according to their different aglycones: dammarane-type and oleanane-type. Among the first group, 20(*S*)-ginsenoside Rg3 (Rg3) was shown to inhibit the growth of several tumor cells such as prostate cancer cells, Lewis lung cancer cells, colon cancer cells, and B16 melanoma cells [46–49]. A recent *in vivo* study in Beagle dogs examined the potential subchronic toxicity of Rg3 by repeated intramuscular administration over a 26-week period as a part of the preclinical safety evaluation program for this compound, taking into account that Rg3 formulations have displayed poor bioavailability, and the long-term *in vivo* toxicity of Rg3 is still unknown [50,51]. This study concluded that Rg3 is nontoxic and well tolerated up to a 7.20 mg/kg/day intramuscularly injected dosage level.

Extensive pharmacokinetic studies indicated that many active ginsenosides have very poor oral bioavailability, which was attributed to poor oral absorption [52,53]. Moreover, the poor bioavailability of ginsenosides makes it hard for their potency to be demonstrated *in vivo* [54]. The same impediments that will unequivocally demonstrate their clinical effectiveness hinder the entry of these compounds into clinical trials. A further difficulty is that these

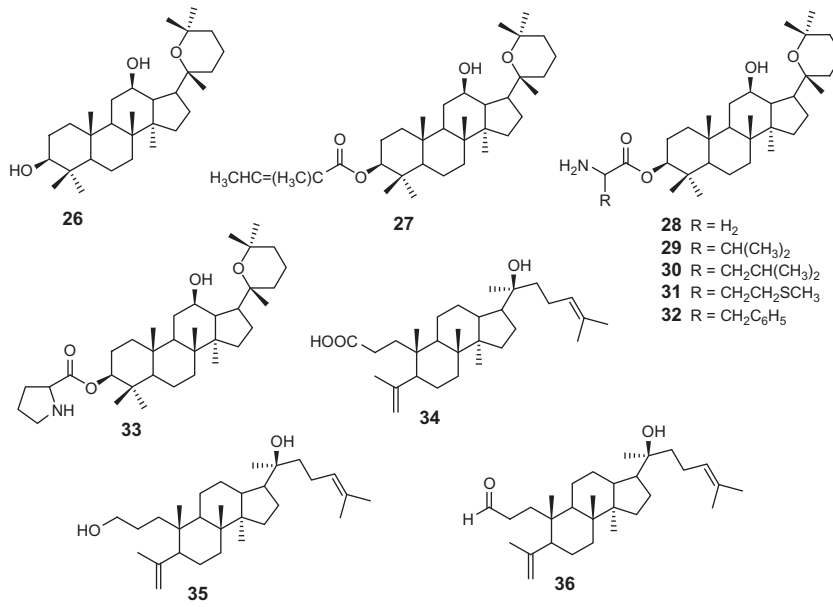

FIGURE 4 Chemical structures of compounds **26–36**.

compounds are not easily detected in plasma. In fact, one of the prerequisites for carrying out pharmacokinetic studies is the availability of a reliable and sensitive bioanalytical method for monitoring the analytes in plasma samples. For example, Qian *et al*. [55] and Xie *et al*. [56] tried to monitor the plasma concentration of Rh2 as a metabolite of ginsenoside Rg3, but generally no Rh2 could be detected.

So, many studies focused mainly on the development of an adequate analytical method, rather than on detailed pharmacokinetic studies. Yang *et al*. [57] demonstrated that Rh2 is a good substrate of P-glycoprotein (P-gp), and the inhibition of P-gp can significantly enhance its oral bioavailability.

The Hopane Group

Among hopane triterpenes, one of the most promising compounds is celastrol (**37**). This triterpene was extensively investigated in both *in vitro* and *in vivo* tumor models [58]. Its anticancer effects are attributed to many signaling molecules including the NF-κB, inducible nitric oxide synthase (iNOS), VEGF receptors, heat-shock proteins, potassium and calcium channels, and immunoglobulin Fc epsilon receptor I [59–64].

Celastrol (**37**) showed a dose- and time-dependent growth inhibition of A549 cells with an IC_{50} of 2.12 μM at 48 h of treatment [64]. Apoptosis

was characterized by cleavage of caspase-9, caspase-8, caspase-3, and PARP protein, increase in Fas and FasL expression, and a reduction in the MMP.

Furthermore, celastrol (**37**) induced the release of cytochrome *c*, upregulated the expression of proapoptotic Bax, downregulated antiapoptotic Bcl-2, and inhibited Akt phosphorylation. In the same year, it was demonstrated that celastrol (**37**) exhibited *in vitro* antitumor activity against a panel of human breast cancer cell lines, with selectivity toward those overexpressing receptor tyrosine kinase ErbB2 [65]. Celastrol (**37**) strongly synergized with ErbB2-targeted therapeutics trastuzumab and lapatinib, producing higher cytotoxicity with lower doses of celastrol. Celastrol (**37**) significantly retarded the rate of growth of ErbB2-overexpressing human breast cancer cells in a mouse xenograft model with only minor systemic toxicity.

This triterpene was shown to be a potent inhibitor of hypoxia-induced angiogenic and metastatic activity as shown by a decrease in the proliferation of both endothelial and cancer cells, blocking of migration as well as of tube formation of endothelial cells, and by inhibition of cancer cell invasion under hypoxic conditions [66]. Moreover, celastrol (**37**) decreased HIF-1α mRNA levels under both normoxia and hypoxia and inhibited hypoxia-induced accumulation of nuclear HIF-1α protein. Meanwhile, inhibition of nuclear HIF-1α protein levels was accompanied by a reduction in the transcriptional activity of HIF-1α target genes, including VEGF. In addition, the inhibitory effect of celastrol (**37**) on HIF-1α protein was partly due to its suppression of HSP90 activity.

Administered subcutaneously to mice bearing human prostate cancer (PC-3 cell) xenografts, celastrol (**37**) (2 mg/kg/day) significantly reduced the volume and the weight of solid tumors and decreased tumor angiogenesis [67]. The inhibition of VEGF-induced proliferation, migration, invasion, and capillary-like structure formation by primary cultured human umbilical endothelial cells (HUVECs) in a dose-dependent manner was demonstrated. Further, **37** abrogated VEGF-induced sprouting of vessels from aortic rings and inhibited vascular formation in the Matrigel plug assay *in vivo*. To understand the molecular mechanism of these activities, the signaling pathways in treated HUVECs and PC-3 tumor cells were examined. Celastrol (**37**) suppressed the VEGF-induced activation of Akt, mammalian target of rapamycin (mTOR), and ribosomal protein S6 kinase (P70S6K). In addition, it was found that **37** inhibited the proliferation of prostate cancer cells and induced apoptosis, and these effects correlated with the extent of inhibition of Akt/mTOR/P70S6 kinase signaling. Taken together, these results suggest that celastrol (**37**) targets the Akt/mTOR/P70S6K pathway, which leads to the suppression of tumor growth and angiogenesis.

Celastrol (**37**) combined with tumor necrosis factor-related apoptosis-inducing ligand (TRAIL/Apo-2L) exhibited significant synergistic anticancer activities [68]. In particular, celastrol upregulated death receptor 4 (DR4) and 5 (DR5) expression at mRNA, total protein and cell surface levels, and

the specific knockdown using DR4- or DR5-targeting siRNA transfection attenuates the PARP cleavage caused by the combination of celastrol and TRAIL/Apo-2L, denoting the critical roles of DR induction in this sensitization. Of note is that although celastrol (**37**) activates p38 mitogen-activated protein kinases (p38 MAPK), SB203580, a specific inhibitor of p38, fails to interrupt celastrol-induced DR4 expression and the enhanced apoptosis caused by celastrol plus TRAIL/Apo-2L. In addition, the protein expression of Mcl-1 and FLIP, two critical antiapoptotic factors, is not decreased upon celastrol (**37**) treatment under our experimental conditions. Taken together, this study demonstrates that the enhanced mRNA and protein expression of DR4 and DR5 play prominent roles in the sensitization of celastrol (**37**) to TRAIL/Apo-2L-induced apoptosis, in a p38 MAPK-independent manner.

Overall, these studies may provide new insight into the mechanisms of the antitumor action of celastrol (**37**), which may represent an interesting candidate for the development of novel chemotherapeutic cancer agents. Therefore, much more attention should be directed to the use of celastrol and its formulations. Since a better understanding of pharmacokinetics has made precise administration of this triterpenoid, exhaustive pharmacokinetics data of **37** are in great demand to achieve the best therapeutic effects and the least toxicity. In a recent study, a formulation of Thunder God Vine (TGV) tablets containing compound **37** displayed a considerably high bioavailability [69]. After oral administration in rats, the absolute bioavailability of **37** significantly increased from 17.06% for pure celastrol (**37**) to 94.19% for TGV tablets containing equivalent celastrol. TGV is a complex mixture that contains more than 100 compounds such as diterpenes, triterpenes, sesquiterpenoids, and alkaloids. It is supposed that conversion from celastrol-like components in TGV tablets or the influence of other constituents on the solubility of **37** may contribute to the increase in oral bioavailability. As for gender difference, female rats showed a significantly better absorption of celastrol (**37**) than males.

The Lanostane Group

Ganodermanontriol and Its Isomers

Colorectal cancer is one of the most common cancers in men and women worldwide. Previous molecular studies have revealed that deregulation of the β-catenin signaling pathway plays a crucial role in the progression of colorectal cancer. Therefore, modulation of the β-catenin pathway offers a strategy to control colorectal cancer progression. The mushroom *Ganoderma lucidum* is a rich source of lanostane triterpenes with anticancer properties. Ganodermanontriol (**38**), a purified triterpene from *G. lucidum*, inhibited proliferation of HCT-116 and HT-29 colon cancer cells without any significant effect on cell viability [70]. Moreover, compound **38** inhibited transcriptional

activity of β-catenin and protein expression of its target gene cyclin D1 in a dose-dependent manner. A marked inhibition effect was also seen on Cdk-4 and PCNA expression, whereas expression of Cdk-2, p21, and cyclin E was not affected by the **38** treatment. In addition, ganodermanontriol (**38**) caused a dose-dependent increase in the protein expression of E-cadherin and β-catenin in HT-29 cells. Finally, ganodermanontriol (**38**) suppressed tumor growth in a xenograft model of human colon adenocarcinoma cells HT-29 implanted in nude mice without any side effects and inhibited expression of cyclin D1 in tumors.

Ganodermanontriol (**38**) and its stereoisomeric triols were examined for their relative effectiveness in the inhibition and proliferation of breast cancer cells MCF-7 and MDA-MB-231 [71]. The most active was **38** with an IC_{50} value of 5.8 μM at 72 h in the proliferation of MCF-7 cancer cells. An IC_{50} value of 9.7 μM was obtained for the MDA-MB-231 cell line. Isomers were less active (**39**: IC_{50} values of 24.1 and 33.8 μM; **40**: IC_{50} values of 16.3 and 36.7 μM; **41**: IC_{50} values of 24.1 and 11.3 μM, against MCF-7 and MDA-MB-231 cell lines, respectively).

Others

Moderate cancer cell growth inhibitory properties against murine P388 leukemia, murine L1210 leukemia, human HL-60 leukemia, and human KB epidermoid carcinoma cell lines were found for an unusual lanostane-type triterpenoid, spiroinonotsuoxodiol, and two lanostane-type triterpenoids, inonotsudiol A and inonotsuoxodiol A, isolated from the sclerotia of *Inonotus obliquus* [72]. Inonotsuoxodiol A was the most active against P388 and HL-60 cell lines with IC_{50} values of 15.2 and 17.7 μM, respectively. Spiroinonotsuoxodiol was shown to inhibit L1210 cell growth with an IC_{50} value of 12.5 μM.

Six lanostane-type triterpene acids, isolated from *Poria cocos*, and their methyl ester and hydroxy derivatives were evaluated for their cytotoxic activities against HL60, A549, CRL1579, NIH:OVCAR-3, SK-BR-3, DU145, AZ521, and PANC-1 cancer cell lines. The hydroxy derivative of poricoic acid A, poricotriol A (**42**), exhibited potent cytotoxicity against six cell lines (IC_{50} values of 1.2–5.5 μM) and was studied for the induction of apoptosis in HL60 and A549 cells and the mechanisms of apoptotic cell death [73]. Poricotriol A (**42**) was shown to induce typical apoptotic cell death in HL60 and A549 cells on evaluation of its apoptosis-inducing activity by flow cytometric analysis. Western blot analysis in HL60 cells showed that poricotriol A activated caspases-3, -8, and -9, while increasing the ratio of Bax/Bcl-2. This suggested that poricotriol A (**42**) induced apoptosis via both mitochondrial and DR pathways in HL60. On the other hand, poricotriol A (**42**) did not activate caspases-3, -8, and -9, but induced translocation of apoptosis-inducing factor (AIF) from mitochondria and increased the ratio of Bax/Bcl-2 in A549.

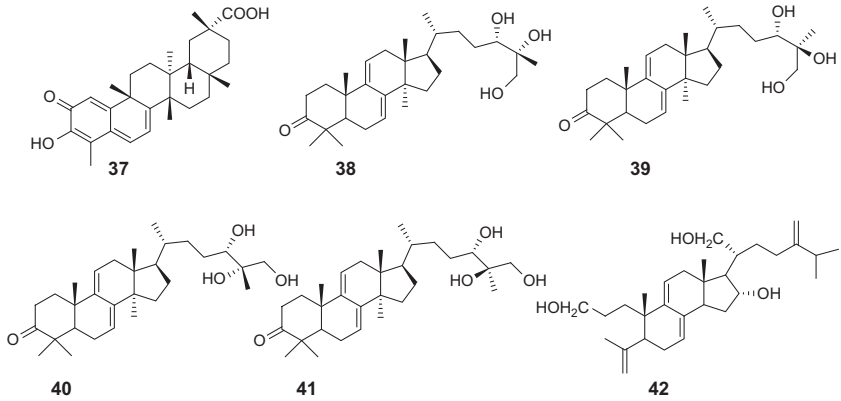

FIGURE 5 Chemical structures of compounds **37**–**42**.

This suggested that poricotriol A (**42**) induced apoptosis via the mitochondrial pathway mostly by translocation of AIF, independently of the caspase pathway in A549. Furthermore, poricotriol A (**42**) was shown to possess high selective toxicity in lung cancer cells since it exhibited only weak cytotoxicity against a normal lung cell line (WI-38) (Fig. 5).

Fasciculols H and I, isolated from Chinese mushroom *Naematoloma fasciculare*, were assessed for their inhibitory effects against glioma and NF-κB. A weak inhibitory activity of human glioma cell line U87 was found (inhibition rate of 13.1% at concentrations of 100 μM) [74]. Fasciculol H also exhibited a weak inhibitory activity against NF-κB (inhibition rate of 16.3%). NF-κB is a heterodimeric protein composed of different combinations of members of the Rel family of transcription factors, involved mainly in stress-induced, immune, and inflammatory responses. In addition, these molecules play important roles during the development of certain hemopoietic cells, keratinocytes, and lymphoid organ structures. More recently, NF-κB family members have been implicated in neoplastic progression and the formation of neuronal synapses. NF-κB is also an important regulator in programmed cell death and proliferation control, and is critical in tumorigenesis. Previously, a new ganoderic acid, 7-*O*-ethyl ganoderic acid O, was isolated and purified from fermented mycelia of *G. lucidum* [75]. This compound, which was characterized by a rare ethoxyl group at C7, exhibited antiproliferative activity against 95D and HeLa human cancer cell lines (IC_{50} values of 46.7 and 59.1 μM, respectively).

The Ursane Group

In recent years, some triterpenoids belonging to the ursane group, such as boswellic acids and ursolic acid, have drawn attention for their anticancer potential.

Boswellic Acid and Derivatives

3-Acetyl-11-keto-β-boswellic acid (**43**) has been shown to inhibit the growth of a wide variety of tumor cells, including glioma, colon cancer, leukemia, hepatocellular carcinoma, and prostate cancer [76]. Although 3-acetyl-11-keto-β-boswellic acid (**43**) can inhibit the growth of colorectal cancer cells, whether it can inhibit the growth and metastasis of colorectal cancer in an orthotopic mouse model is not known. Yadav *et al.* [76] evaluated the activity of the orally administered boswellic acid analog **43** in a mouse xenograft model of human colorectal cancer, demonstrating that **43** not only reduced tumor growth but also suppressed distant metastasis and ascites, which strongly correlated with the inhibition of numerous biomarkers linked to inflammation, proliferation, invasion, angiogenesis, and metastasis. Previously, 3-acetyl-11-keto-β-boswellic acid (**43**) had also shown activity against pancreatic cancer, one of the most lethal cancers [77]. In particular, **43** inhibited the proliferation of four different pancreatic cancer cell lines, namely AsPC-1, PANC-28, MIA PaCa-2 with K-Ras and p53 mutations, and BxPC-3 with wild-type K-Ras and p53 mutation. These effects correlated with an inhibition of constitutively active NF-κB and suppression of NF-κB-regulating gene expression. The compound **43** also induced apoptosis and sensitized the cells to the apoptotic effects of gemcitabine. In the orthotopic nude mouse model of PaCa, p.o. administration of **43** alone (100 mg/kg) significantly inhibited the tumor growth; this activity was enhanced by gemcitabine. In addition, 3-acetyl-11-keto-β-boswellic acid (**43**) inhibited the metastasis of the PaCa to the spleen, liver, and lungs. This correlated with decreases in Ki-67, a biomarker of proliferation, and CD31, a biomarker of microvessel density, in the tumor tissue. 3-Acetyl-11-keto-β-boswellic acid (**43**) produced significant decreases in the expression of NF-κB-regulating genes in the tissues. Immunohistochemical analysis also showed that **43** downregulated the expression of cyclooxygenase 2 (COX2), MMP-9, CXCR-4 (C-X-C chemokine receptor type 4), and VEGF in the tissues. Overall, these results demonstrate that **43** can suppress the growth and metastasis of human pancreatic tumors in an orthotopic nude mouse model that correlates with modulation of multiple targets [77] (Fig. 6).

A semisynthetic modification of **43** led to propionyloxy-11-keto-β-boswellic (**44**) acid that induced apoptosis in HL-60 cells by inhibiting topoisomerases I and II at lower concentration than **43** [78]. This study pointed toward the potentiating role of acyl groups at the 3-α-hydroxy position in the anticancer potential of boswellic acid. Successively, considering the increment in anticancer potential by **44**, two derivatives butyryloxy (**45**) and hexanoyloxy (**46**) were synthesized and studied for their potential antiproliferative activity [79]. Against the HL-60 cell line, derivative **46** was found to be more active (IC_{50} value of 4.5 μg/ml) than **45** (IC_{50} value of 7.7 μg/ml), which was comparatively more active than **44** (IC_{50} value of 8.7 μg/ml). Moreover, at low concentrations, **46** was found to induce apoptosis in HL-60 cells and to arrest cells in the G2/M phase of the cell cycle.

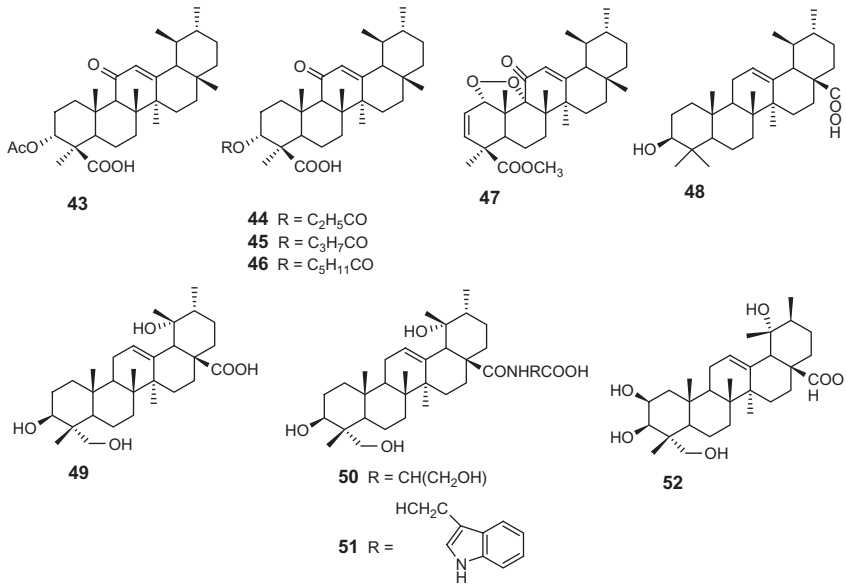

FIGURE 6 Chemical structures of compounds **43**–**52**.

The induction of apoptosis by **46** was found to involve the mitochondrial pathway, resulting in the loss of MMP, release of proapoptotic factors from mitochondria, and the activation of upstream and downstream caspases. Furthermore, **46** was found to inhibit the catalytic activity of topoisomerases I and II at low doses. The *in vivo* anticancer studies of **46** were performed in ascitic (EAC) and solid (EAT and S-180) murine tumor models. The obtained results revealed a low dose inhibitory effect of **46** on both tumor models. Overall, these established **46** to be a potentially better anticancer compound than other previously known boswellic acids, including **44**. Previously, another research group had obtained an endoperoxide (**47**) starting from **43** [80]. The compound induced apoptosis and showed an average IC_{50} value of 0.4–4.5 μM against a panel of 15 tumor cell lines (518A2, 8505C, A253, A2780, A431, A549, DLD-1, FaDu, HCT-116, HCT-8, HT-29, LIPO, MCF-7, SW1736, and SW480).

Cyano-enone-modified methyl boswallates was shown to be a potent inhibitor of nitric oxide (NO) production induced by IFN-γ in elicited mouse primary macrophages; an inhibitor of DNA synthesis; an inducer of caspase 8 and caspase 3 activation and thereby apoptosis in A549 cells; an inducer of adipogenic differentiation in 3T3-L1 cells; and has antitumor activity on tumor xenografts in a mouse model [81]. 2-Cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO) and its methyl ester (CDDO-Me) were used as positive controls. Both these compounds were more potent in inhibiting NO production ($IC_{50} \leq 1$ nM) than cyano-enone-modified methyl boswallates.

Moreover, this derivative showed antiproliferative activity with IC_{50} values in the range 0.15–0.7 μM against A549, A431, HT1080, HepG2, HeLa, HCT116, MCF-7, T47D, T84, CaCO2, JEG, PC3, Jurkat, HL-60, THP-1, and K562 cell lines.

Pharmacokinetic studies have demonstrated that the systemic absorption of boswellic acids, especially compounds **43** and **44**, was very low in both animals and humans, rationalizing the efforts to overcome their low oral bioavailability [82,83]. Hüsch *et al*. [84] recently proposed a lecithin delivery form (Phytosome®) to enhance the oral bioavailability of these compounds.

Ursolic Acid

Ursolic acid (**48**), which was identified in several medicinal plants, demonstrated the inhibition of cell growth and induction of apoptosis in several cancer models [85–88]. Different apoptotic pathways, such as activating caspases; inhibiting protein tyrosine kinase, signal transducer and activator of transcription 3 (STAT3), and NF-κB activities; inhibiting DNA replication; inducing Ca^{2+} release; activating c-Jun N-terminal protein kinase (JNK); downregulating antiapoptotic genes; inhibiting COX2 and iNOS; and suppressing MMP-9, were described [89–93]. Furthermore, **48** has been shown in *in vivo* studies to suppress tumor invasion, to be chemopreventive, and to inhibit experimental metastasis of esophageal carcinoma [94–97].

Ursolic acid (**48**) was recently demonstrated to potentiate TRAIL-induced apoptosis in cancer cells [98]. TRAIL is one such apoptosis-inducing cytokine that has shown promise as an anticancer agent. TRAIL selectively induces apoptosis in tumor cells but not in normal cells. Five receptors have been identified for TRAIL. However, only DR4 (TRAIL-R1) and DR5 (TRAIL-R2) have cytoplasmic death domains that activate the apoptotic machinery upon TRAIL binding [99]. Compound **48** downregulated cell survival proteins and induced the cell surface expression of both TRAIL receptors DR4 and DR5. The induction of DRs was independent of p53 because **48** induced DR4 and DR5 in HCT116 p53/cells; however, it was dependent on JNK because **48** induced JNK, and its pharmacological inhibition abolished the induction of the receptors. The downregulation of survival proteins and upregulation of the DRs required ROS because **48** induced ROS, and its quenching abolished the effect of this triterpene. Also, potentiation of TRAIL-induced apoptosis by **48** was significantly reduced by both ROS quenchers and JNK inhibitors. In addition, **48** was found to induce the expression of DRs, downregulate cell survival proteins, and activate JNK in orthotopically implanted human colorectal cancer in a nude mouse model. Overall, these results showed that **48** potentiates TRAIL-induced apoptosis through activation of ROS and JNK-mediated upregulation of DRs and downregulation of decoy receptor 2 and cell survival proteins.

Ursolic acid (**48**) inhibited constitutive and TNF-α-induced activation of NF-κB in DU145 and LNCaP cells in a dose-dependent manner [100].

The suppression was mediated through the inhibition of constitutive and TNF-α-induced IκB kinase activation, phosphorylation of IκBα and p65, and NF-κB-dependent reporter activity. Furthermore, **48** suppressed both constitutive and inducible STAT3 activation in prostate cancer cells, concomitant with the suppression of activation of upstream kinases (Src and JAK2) and STAT3-dependent reporter gene activity. Ursolic acid (**48**) also downregulated the expression of various NF-κB- and STAT3-regulated gene products involved in proliferation, survival, and angiogenesis, and induced apoptosis in both cells lines as evidenced by DNA fragmentation and annexin V staining.

Ursolic acid (**48**) inhibited growth of BGC-803 cells *in vitro* in a dose- and time-dependent manner and arrested tumor cells from xenograft to G0/G1 stage *in vivo* [101]. The apoptotic rate was significantly increased in tumor cells both *in vitro* and *in vivo*. Ursolic acid activated caspases-3, -8, and -9 and downregulated the expression of Bcl-2 in BGC-803 cells. Previously, **48** had shown antitumor activity in a mouse model of postmenopausal breast cancer, and these findings are supported by its inhibitory effects on an MMTV-Wnt-1 mammary tumor cell line *in vitro* [102]. Ovariectomized C57BL/6 mice ($n = 40$) were randomized to receive a control diet (AIN-93G) or a diet supplemented with **48** at one of three doses: 0.05%, 0.10%, or 0.25% (≈54, 106, or 266 mg/kg body weight/day, respectively). After 3 weeks, syngeneic MMTV-Wnt-1 mammary tumor cells were injected in the mammary fat pad, and mice continued on their respective diets for 5 more weeks. All doses of **48** decreased tumor cell proliferation. Modulation of Akt/mTOR signaling and induction of apoptosis appeared to mediate these effects on tumor growth. Ursolic acid potently disrupted cell cycle progression and induced necrosis in a clonal MMTV-Wnt-1 mammary tumor cell line *in vitro*.

One of the problems that hinders the administration of ursolic acid is its poor solubility. In order to obtain a high bioavailability, targeting effect, and stability, and an intravenous (i.v.) administration, especially in antitumor formulation, compound **48** phospholipid nanopowders (**48**-PL-NP) were evaluated [103]. The results showed that the entrapment efficiency was up to 86.0%, and the drug loading was 12.8%. After i.v. administration of **48**-PL-NP with low, middle, and high doses, ursolic acid concentration in the livers of mice increased during the tested period and was highest in the tested organs after 4 h. Therefore, with this formulation ursolic acid was shown to have a high bioavailability and targeted antitumor effect (Table 1).

Others

Another interesting potential antitumor triterpene belonging to the ursane group is rotundic acid (**49**). This compound showed cytotoxicity against A375, HepG2, and NCI-H446 cell lines with IC_{50} values of 16.58, 7.33, and

TABLE 1 *In Vitro* Cytotoxic Activity (IC_{50}, GI_{50}, EC_{50}, ID_{50}) of Triterpenoids Against Human Tumor Cell Lines

Compound	Cell Line	IC_{50}, GI_{50}, EC_{50}, ID_{50} (μM)	References
Oleanolic acid (**1**)	HepG2	70	[12]
3-Oxo-12-en-28-(20-nitro-vinyl)-olean (**6**)	HepG2, Col-02	1.3, 4.2	[9]
2-Cyano-3-oxo-28-(β-nitro)vinyllolean-1,12(13)-diene (**7**)	HepG2, Col-02	1.7, 0.7	[9]
N-(2-{3-[3,5-bis(trifluoromethyl)-phenyl]ureido}ethyl)-glycyrrhetinamide	U373, T98G, Hs683, A549, MCF-7, PC-3, SKMEL-28, B16F10	4–12	[18]
Glycyrrhetinic acid (**9**)	HepG2, LNCaP	80 μM, 0.5 mg/ml	[12,13]
3,4-Seco-11-oxo-18β-olean-4(23),12-dien-3,30-dioic acid 3-methyl,30-benzyl ester (**10**)	NTUB1	2.3	[19]
3,4-Seco-11-oxo-18β-olean-4(23),12-dien-3,30-diisopropyl carbamate (**11**)	NTUB1	4.8	[19]
Methyl 30-phenylcarbamoyl-11-oxo-18β-olean-3,4-secoolean-4 (23),12-dien-3-oate (**12**)	NTUB1	3.3	[19]
3β,6β,19α-Trihydroxy-12-oleanen-28-oic acid (**14**)	MCF-7, HepG2	78.2, 73.9 μg/ml	[23]
Clethroidoside C	HT29, HePG2, BGC-823, A549, A375	1.1–2.6	[26]
Clethroidoside D	HT29, HePG2, BGC-823, A549, A375	1.4–2.4	[26]
Clethroidoside E	HT29, HePG2, BGC-823, A549, A375	1.40 to >10	[26]
Clethroidoside F	HT29, HePG2, BGC-823, A549, A375	1.3–2.3	[26]
Bacopaside E (**15**)	MDA-MB-231, SHG-44, HCT-8, A549, PC-3M	8.9–13.9	[28]

Continued

TABLE 1 *In Vitro* Cytotoxic Activity (IC_{50}, GI_{50}, EC_{50}, ID_{50}) of Triterpenoids Against Human Tumor Cell Lines—Cont'd

Compound	Cell Line	IC_{50}, GI_{50}, EC_{50}, ID_{50} (μM)	References
Bacopaside VII (**16**)	MDA-MB-231, SHG-44, HCT-8, A549, PC-3M	9.7–15.9	[28]
20(*R*)-20-Methoxyl-dammarane-3β,12β,25-triol (**17**)	HepG2, Colon205, HL-60	4.0–8.8	[29]
Ginsenoside Rh3 (**18**)	HL-60	0.8	[30]
Ginsenoside Rk2 (**19**)	HL-60	0.9	[30]
Ginsenoside SL3 (**20**)	HL-60	9.0	[30]
Ginsenoside 20*S*-Rg2 (**21**)	HL-60	9.0	[30]
Ginsenoside F4 (**22**)	HL-60	7.5	[30]
Ginsenoside 20*S*-Rh2 (**23**)	HL-60	8.2	[30]
Panaxadiol (**26**)	ES-2, U2-OS, HepG2, A549	5.6–43.1	[43,44]
Panaxadiol-3-yl 2-methylbut-2-enoate (**27**)	ES-2, U2-OS, HepG2, A549	4.3 to >50	[43,44]
Panaxadiol-3-yl 2-aminoacetate (**28**)	ES-2, U2-OS, HepG2, A549	9.9–14.0	[43,44]
Panaxadiol-3-yl 2-amino-3-methylbutanoate (**29**)	ES-2, U2-OS, HepG2, A549	9.3–31.8	[43,44]
Panaxadiol-3-yl 2-amino-4-methylpentanoate (**30**)	ES-2, U2-OS, HepG2, A549	5.0–24.9	[43,44]
Panaxadiol-3-yl 2-amino-4-(methylthio)butanoate (**31**)	ES-2, U2-OS, HepG2, A549	11.7–22.4	[43,44]
Panaxadiol-3-yl 2-amino-3-phenylpropanoate (**32**)	ES-2, U2-OS, HepG2, A549	8.8–18.6	[43,44]

Panaxadiol-3-yl pyrrolidine-2-carboxylate (**33**)	ES-2, U2-OS, HepG2, A549	7.6–16.7	[43,44]
Dammarenolic acid (**34**)	HL60, CRL1579	13.5, >100	[45]
(20*S*)-3,4-Secodammara-4(28),24-diene-3,20-diol (**35**)	HL60, CRL1579	21.7, 80.8	[45]
(20*S*)-20-Hydroxy-3,4-secodammara-4(28),24-dien-3-al (**36**)	HL60, CRL1579	17.7, 13.4	[45]
Celastrol (**37**)	A549	2.12	[64–66]
Ganodermanontriol (**38**)	MCF-7, MDA-MB-231	5.8, 9.7	[71]
(10*S*,13*R*,14*R*,17*R*)-4,4,10,13,14-Pentamethyl-17-((2*R*,5*R*,6*S*)-5,6,7-trihydroxy-6-methylheptan-2-yl)-4,5,6,10,12,13,14,15,16,17-decahydro-1H-cyclopenta[*a*]phenanthren-3(2H)-one (**39**)	MCF-7, MDA-MB-231	24.1, 33.8	[71]
(10*S*,13*R*,14*R*,17*R*)-4,4,10,13,14-Pentamethyl-17-((2*R*,5*S*,6*S*)-5,6,7-trihydroxy-6-methylheptan-2-yl)-4,5,6,10,12,13,14,15,16,17-decahydro-1H-cyclopenta[*a*]phenanthren-3(2H)-one (**40**)	MCF-7, MDA-MB-231	16.3, 36.7	[71]
(10*S*,13*R*,14*R*,17*R*)-4,4,10,13,14-Pentamethyl-17-((2*R*,5*R*,6*R*)-5,6,7-trihydroxy-6-methylheptan-2-yl)-4,5,6,10,12,13,14,15,16,17-decahydro-1H-cyclopenta[*a*]phenanthren-3(2H)-one (**41**)	MCF-7, MDA-MB-231	24.1, 11.3	[71]
Poricotriol A (**42**)	HL-60, A549, CRL1579, NIH:OVCAR-3, SK-BR-3, DU145, AZ521, PANC-1	1.4–26.3	[73]
Propionyloxy-11-keto-β-boswellic (**44**)	HL-60	8.7 μg/ml	[79]
Butyryloxy-11-keto-β-boswellic (**45**)	HL-60	7.7 μg/ml	[79]
Hexanoyloxy-11-keto-β-boswellic (**46**)	HL-60	4.5 μg/ml	[79]

Continued

TABLE 1 *In Vitro* Cytotoxic Activity (IC_{50}, GI_{50}, EC_{50}, ID_{50}) of Triterpenoids Against Human Tumor Cell Lines—Cont'd

Compound	Cell Line	IC_{50}, GI_{50}, EC_{50}, ID_{50} (μM)	References
Methyl 2,3-dihydro-1α,9α-peroxo-11-oxo-urs-12-en-24-oate (**47**)	518A2, 8505C, A253, A2780, A431, A549, DLD-1, FaDu, HCT-116, HCT-8, HT-29, LIPO, MCF-7, SW1736, SW480	0.4–4.5	[80]
Cyano-enone of methyl boswellates	A549, A431, HT1080, HepG2, HeLa, HCT116, MCF-7, T47D, T84, CaCO2, JEG, PC3, Jurkat, HL-60, THP-1, K562	0.15–0.7	[81]
Ursolic acid (**48**)	HepG2	20	[12,85–93,98]
Rotundic acid (**49**)	A375, HepG2, NCI-H446	7.3–16.6	[104]
Methyl *N*-[3β,23-diacetoxy-19α-hydroxy-urs-12-en-28-oyl]-2-amino-3-hydroxypropionate (**50**)	A375, HepG2, NCI-H446	3.4–6.0	[104]
N-[3β,19α,23-Trihydroxyurs-12-en-28-oyl]-2-amino-3-(1H-indol-3-yl)propionic acid (**51**)	A375, HepG2, NCI-H446	6.1–11.3	[104]
2α,3α,19β,23β-Tetrahydroxyurs-12-en-28-oic acid (**52**)	HepG2, A2780, HCT116	19.5–29.7 μg/ml	[105]

11.40 μM, respectively [103]. In order to improve its bioactivity, the synthesis of eight new amino acid derivatives of **49** at the 28-COOH position was realized [104].

Among the synthesized derivatives, compound **50** showed the best inhibition activity against the three tested tumor cell lines with IC_{50} values <10 μM (5.99, 3.41, and 3.84 μM against A375, HepG2, and NCI-H446, respectively). For this compound, we can suppose that hydroxyl groups can increase water solubility, which might enhance its bioactivity. The cytotoxic activity of compound **51** was significantly higher than that of **49** on the A375 cell line with an IC_{50} value of 8.03 μM.

2α,3α,19β,23β-Tetrahydroxyurs-12-en-28-oic acid (**52**), a novel triterpenoid, was isolated from the leaves of *Sinojackia sarcocarpa*, and its anticancer activity was investigated both *in vitro* and *in vivo* [105]. Compound **52** possessed potent tumor-selective toxicity *in vitro*. It exhibited significantly high cytotoxicity against cancer cell lines A2780 and HepG2. Moreover, **52** showed a dose-dependent inhibitory effect on A2780 ovary tumor growth *in vivo* in nude mice. Compound **52** induced a dose-dependent apoptosis and G2/M cell cycle arrest in A2780 and HepG2 cells. The **52**-induced cell cycle arrest was accompanied by a downregulation of Cdc2.

The apoptosis induced by **52** was evident from the induction of DNA fragmentation, release of cytoplasmic cytochrome *c* from mitochondria, activation of caspases, downregulation of Bcl-2, and upregulation of Bax (Table 2).

CONCLUDING REMARKS

Triterpenes comprise one of the most interesting groups of natural products because of their high potential as pharmacological agents. Biological and phytochemical research carried out during the last few years confirms the potential antitumor properties of this class of secondary metabolites, which are widely distributed in medicinal plants and plant foods.

Several structural types of triterpenes have been shown to interfere with cell-signaling pathways and thus have potential anticancer value. This promising antitumor activity was verified not only through *in vitro* studies but also through *in vivo* studies using various animal models.

Almost all triterpenes induce apoptosis in tumor cells; they are preferred drugs in the treatment of cancer, because eliminating tumor cells by apoptosis is helpful in lowering side effects in patients by avoiding necrosis. Another important aspect could be the synergism of action since some of these compounds exhibited an increase in their bioactivity when coadministered with other drugs. Further investigations of the reported triterpenes to better understand their mechanism of action and their adverse effects are necessary for their inclusion in tumor therapies in the future.

TABLE 2 Triterpenoid *In Vitro* and *In Vivo* Studies

	In Vivo Studies		*In Vitro* Studies		
Compound	***Models***	***Effects***	***Cells***	***Effects***	**References**
Maslinic acid (**13**)	Athymic nu/nu mouse	Suppression of tumor growth; induction of apoptosis	DU145	Inhibition of metastasis	[22]
Bacopaside E (**15**)	Mouse implanted with sarcoma S180	Inhibition of tumor growth			[28]
Bacopaside VII (**16**)	Mouse implanted with sarcoma S180	Inhibition of tumor growth			[28]
20(*S*)-Ginsenoside Rg3 (**Rg3**)	Mouse	Inhibition of tumor metastasis; block of angiogenesis			[50,51]
Dammarenolic acid (**34**)			Raji	Inhibition of EBV-EA activation	[45]
(20*S*)-3,4-Secodammara-4 (28),24-diene-3,20-diol (**35**)			Raji	Inhibition of EBV-EA activation	[45]
(20*S*)-20-Hydroxy-3,4-secodammara-4 (28),24-dien-3-al (**36**)			Raji	Inhibition of EBV-EA activation	[45]

Celastrol (**37**)	Mouse	Reduction in volume and weight of solid tumors; decrease in angiogenesis	A549	Induction of apoptosis by different pathways	[64–67]
Ganodermanontriol (**38**)	Xenograft model of human colon adenocarcinoma cells HT-29 implanted in nude mice	Suppression of tumor growth; inhibition of expression of cyclin D1	HT-29	Inhibition of transcriptional activity of β-catenin and protein expression of cyclin D1; inhibition of Cdk-4 and PCNA expression; increase in protein expression of E-cadherin and β-catenin	[70,71]
Poricotriol A (**42**)			HL-60, A549, CRL1579, NIH: OVCAR-3, SK-BR-3, DU145, AZ521, PANC-1	Induction of apoptosis	[73]
3-Acetyl-11-keto-β-boswellic acid (**43**)	AsPC-1, PANC-28, MIA PaCa-2 cells; mouse xenograft model of human colorectal cancer; orthotopic nude mouse model of PaCa	Reduction of tumor growth; inhibition of cancer cells; suppression of distant metastasis and ascites; induction of apoptosis	AsPC-1, BxPC-3, MIA PaCa-2, PANC-28	Induction of apoptosis; suppression of NF-κB gene expression	[76,77]
Hexanoyloxy-11-keto-β-boswellic (**46**)	Ascitic (EAC) and solid (EAT and S-180) murine tumor;	Inhibition of tumor growth	Induction of apoptosis		[79]

Continued

TABLE 2 Triterpenoid *In Vitro* and *In Vivo* Studies—Cont'd

	In Vivo Studies		*In Vitro* Studies		
Compound	*Models*	*Effects*	*Cells*	*Effects*	**References**
Cyano-enone of methyl boswellates	Xenograft mouse model	Inhibition of tumor growth	A549	Inhibition of NO; inhibition of DNA synthesis; induction of apoptosis; induction of adipogenesis differentiation	[81]
Ursolic acid (**48**)	Mouse; mouse model of postmenopausal breast cancer	Suppression of tumor invasion; inhibition of metastasis; chemoprevention		Induction of apoptosis by different pathways	[85–97,102]
2α,3α,19β,23β-Tetrahydroxyurs-12-en-28-oic acid (**52**)	Nude mouse	Inhibitory effect on A2780 ovary tumor		Induction of apoptosis by different pathways	[105]

ABBREVIATIONS

AIF	apoptosis-inducing factor
CDDO	2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid
COX2	cyclooxygenase 2
CXCR-4	C-X-C chemokine receptor type 4
DR	death receptor
EBV-EA	Epstein–Barr virus early antigen
EC_{50}	effective concentration 50%
GI_{50}	50% inhibition of cell growth
HUVEC	human umbilical endothelial cell
IC_{50}	inhibitory concentration 50%
ICAM-1	Intercellular Adhesion Molecule 1
ID_{50}	infective dose 50%
iNOS	inducible nitric oxide synthase
JNK	c-Jun N-terminal protein kinase
MMP-9	matrix metallopeptidase 9
NF-κB	nuclear factor-κB
NO	nitric oxide
Rg3	20(*S*)-ginsenoside Rg3
ROS	reactive oxygen species
SAR	structure–activity relationship
STAT	signal transducer and activator of transcription
TGV	Thunder God Vine
TPA	12-*O*-tetradecanoylphorbol-13-acetate
TRAIL	tumor necrosis factor-related apoptosis-inducing ligand
VEGF	vascular endothelial growth factor

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Chapter 2

Recent Advances in Medicinal Applications of Brassinosteroids, a Group of Plant Hormones

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Chapter Outline

INTRODUCTION

Plants, plant parts, and derived oils and extracts have been used for thousands of years as one of the main source of bioactive substances for therapeutic purpose. Recent records reported that medicinal herbs are used by 80% of the people living in rural areas as primary healthcare system. In spite of the recent domination of the synthetic chemistry as a method to discover and produce drugs, the potential of bioactive plants or their extracts to provide new and novel products for disease treatment and prevention is still enormous. Compared with chemical synthesis, plant-derived natural products represent an

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attractive source of biologically active agents as they are natural and available at affordable prices. Such plant-derived natural products are the main focus of many scientists to develop new medication for different diseases such as cancer and microbial infection [1]. One of the interest bioactive natural compounds is brassinosteroids (BRs). In the remainder of this chapter, the medicinal applications of BRs are discussed in more details.

BRs, a class of plant-specific steroid hormones characterized by their polyhydroxylated sterol structure, were first isolated and purified from *Brassica napus* pollen in 1979 [2]. The chemical structure of brassinolide (BL), and that of the second steroidal plant hormone, castasterone (CS) discovered in 1982 [3], was found to be similar to that of ecdysone, the insect molting steroid hormone (ecdysteroids) and mammalian steroids. Natural BRs have 5α-cholestane skeleton, and their structural variations come from the kind and orientation of oxygenated functions in A- and B-ring. They are divided into free (64) and conjugated (5) compounds (Fig. 1) [4,5]. BRs function in multiple developmental and physiological processes, including vascular differentiation, reproductive development, and photomorphogenesis. More recently, interactions of BRs with other plant hormones, such as auxins, abscisic acid, gibberellins, and ethylene, have also been found to play a major role in plant stress alleviation. Furthermore, ability of BRs to boost antioxidant system of plants is extensively used to confer resistance in plants against a variety of abiotic stresses, such as thermal, drought, heavy metal, pesticides, and salinity. There is ample evidence from both field experiments and greenhouse trials demonstrating the protective effects of exogenous BRs against a fairly broad range of fungal, viral, and bacterial pathogens exhibiting diverse parasitic habits [6]. Over the past decade, molecular genetic studies using *Arabidopsis thaliana* and rice (*Oryza sativa*) as model plants have identified numerous genes involved in BR biosynthesis and gene regulation. Coupled with more recent biochemical approaches, these studies have provided fascinating insights into the various aspects of plant steroid signaling, ranging from BR perception at the cell surface to activation of transcription factors (TFs) in the nucleus [7]. BRs are key components in aims to improve the productivity and quality of agricultural products, such as seeds of rice and other cereal species. However, the practical application of BR research in agriculture has not yet been fully explored. At present, our knowledge of the effects of BRs in animals is still rather fragmentary. Moreover, it is known that BRs have an anabolic action and anticancer and antiproliferative properties in animals.

SOURCE OF BRs

BRs occur at low concentrations throughout the plant kingdom. Since the discovery of BL in 1979, 69 chemically different BRs have been isolated from 61 species of embryophytes: 53 angiosperms (12 monocotyledonous and 41 dicotyledonous plants), 6 gymnosperms, 1 pteridophyte (*Equisetum arvense*), and 1 bryophyte (*Marchantia polymorpha*). In addition, BRs have also been

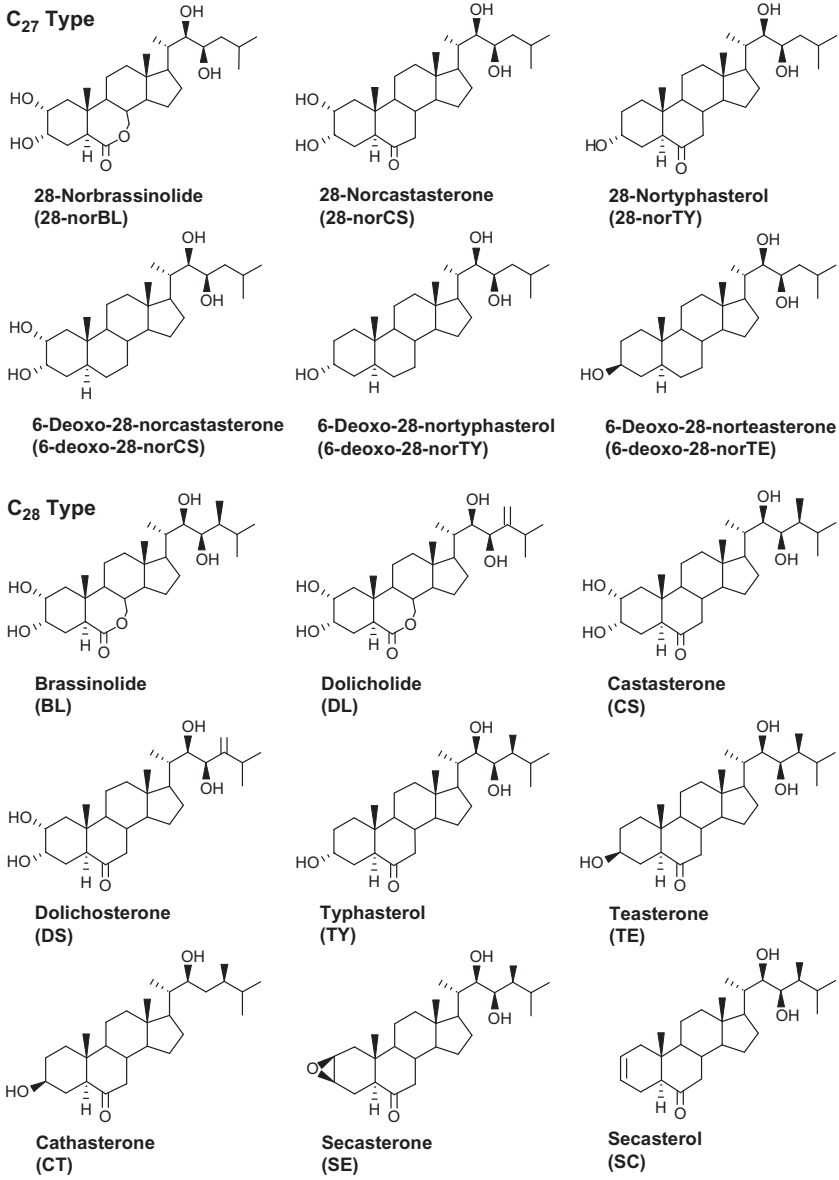

FIGURE 1—Cont'd

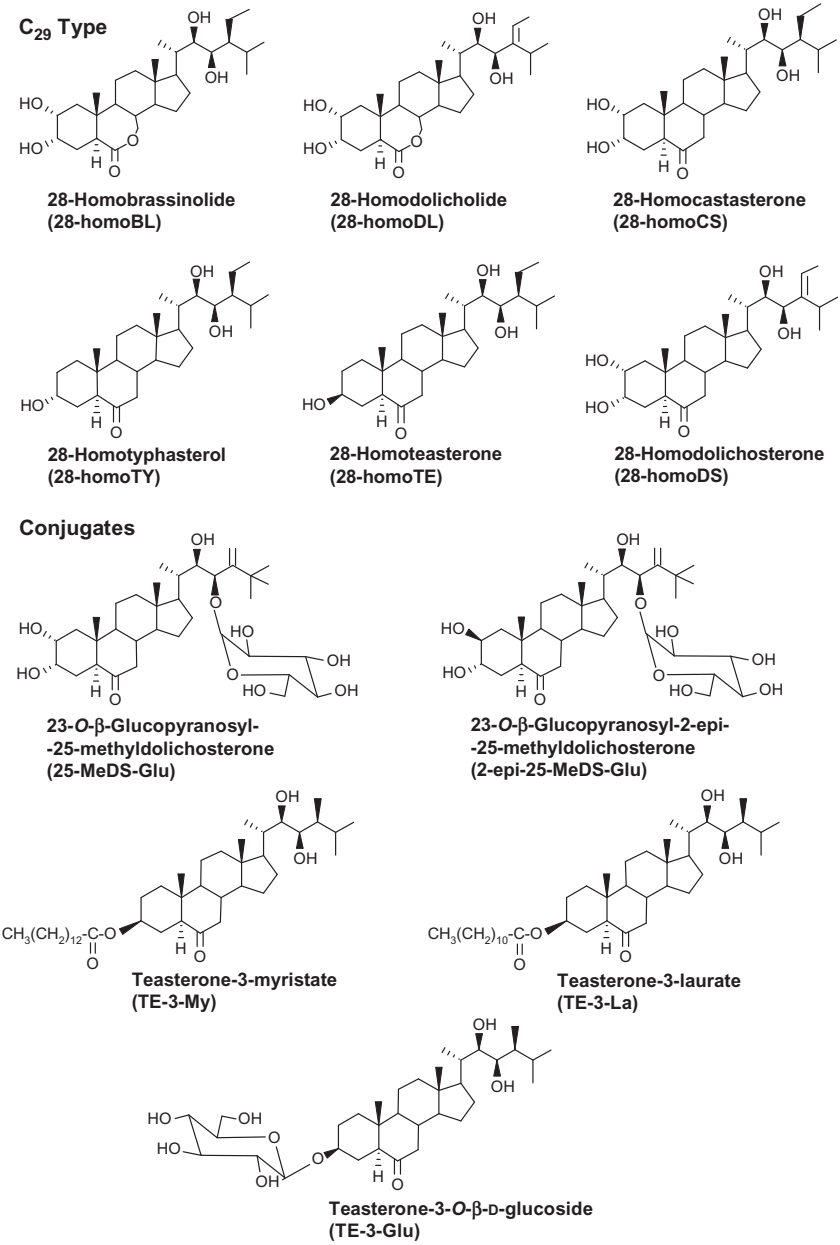

FIGURE 1 Chemical structures of the most important free (C_{27}, C_{28}, and C_{29} type) and conjugated brassinosteroids.

discovered in two species of single-celled green freshwater algae (Chlorophyta) (*Chlorella vulgaris* and *Hydrodictyon reticulatum*) and in the marine brown alga *Cystoseira myrica*. BRs have been detected in all organs of higher plants, such as pollen, anthers, seeds, leaves, stems, roots, flowers, and grain. They also occur in the insect and crown galls of *Castanea crenata*, *Distylium racemosum*, or *Catharanthus roseus*. These plants have higher levels of BRs than the normal tissues. Furthermore, young growing tissues contain higher levels of BRs than mature tissues. Pollen and immature seeds are the richest sources of BRs with ranges of 1–100 $\mu g\ kg^{-1}$ fresh weight, while shoots and leaves usually have lower amounts of 0.01–0.1 $\mu g\ kg^{-1}$ fresh weight [4].

TOXICITY OF BRs

24-Epibrassinolide, the most widely used BR in agriculture, has a favorable safety profile. The median lethal dose (LD_{50}) of this compound is higher than 1000 mg/kg in mice and higher than 2000 mg/kg in Wistar rats when applied orally or subcutaneously. In concentration of 0.01%, 24-epibrassinolide did not irritate mucous membranes of rabbit eyes. The fish toxicity, TML48 (fish test on carp treated with tetramethyllead (TML) for 48 h), for carp was higher than 10 ppm, and the Ames test on mutagenicity was negative and 0.01% solution caused no eye irritation in the rabbit [8]. 28-Homobrassinolide was administered by oral gavage at doses of 0, 100, and 1000 mg/kg body weight in water during the gestation days of 6 through 15 in groups of 20 mated females of Wistar rats. Maternal and embryo–fetal toxicities were analyzed by studying the effects such as clinical signs, mortality/morbidity, abortions, body weight, feed consumption, and pregnancy data, gravid uterine weights, implantation losses, litter size, external, visceral, and skeletal malformations. No treatment-related effect was observed on any of the maternal/fetal end points in any dose group. From the results, it can be concluded that 28-homobrassinolide is nonteratogenic at doses as high as up to 1000 mg/kg body weight in Wistar rats [9]. While some potential pharmacological applications of BRs have been suggested, in order to determine their potential uses, additional mode of action studies are needed to explain how they interact with animal regulatory pathways.

SIMILARITY OF BRs TO ANIMAL STEROID HORMONES

What is particularly intriguing about BRs is their similarity to animal steroid hormones (Fig. 2). All plant and animal steroid hormones are cholesterol derivatives, synthesized by the organisms. Physiologic effects of steroid hormones are necessary for regulation of growth, development, and homeostasis. The majority of steroid hormones retain the four-ring core of cholesterol with one exception of vitamin D, where B-ring is broken. In vertebrates, steroids are a very large group of hormones which includes sex hormones (e.g., testosterone, estradiol), glucocorticoids (e.g., cortisol), metabolites of vitamin D_3,

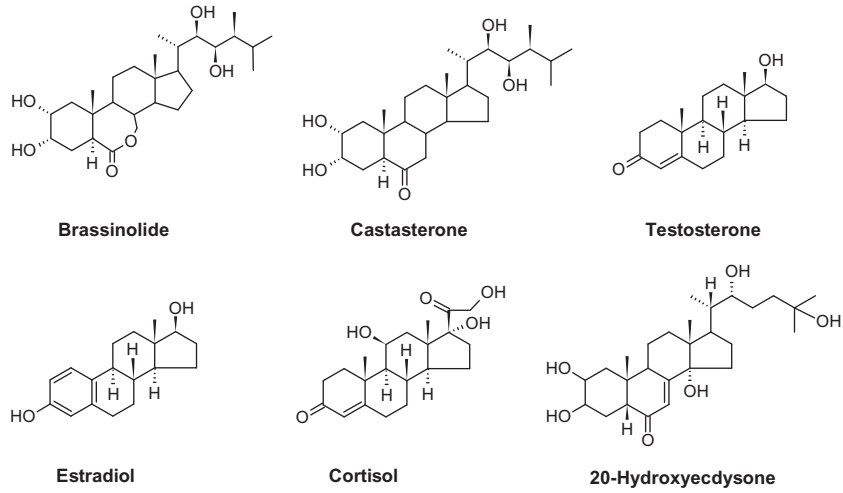

FIGURE 2 Brassinosteroids (brassinolide, castasterone) have structural similarities to animal steroid hormones (testosterone, estradiol, cortisol, 20-hydroxyecdysone).

and neurosteroids. In insects, ecdysteroids are absolutely necessary for development, growth, and molting. One of the genes coding for an enzyme in the BR biosynthetic pathway has significant similarity to an enzyme used in the synthesis of testosterone and related steroids. It is plausible that their evolutionary origin predated the plant–animal split. From an evolutionary perspective, it will be quite interesting to see how these pathways compare with animal steroid signal transduction pathways [10].

BL, the most important BR in plants, has been shown to be synthesized via two pathways from campesterol (early and late C-6 oxidation pathways) (Fig. 3). Sterols are synthesized via the nonmevalonate pathway in lower plants or the mevalonate pathway of isoprenoid metabolism in higher plants. Campesterol, one of the major plant sterols, is the precursor of BRs, which is primarily derived from isopentenyl diphosphate. Although metabolic experiments with labeled C_{27} BRs have not yet been performed, the natural occurrence of C_{27} BRs in tomato (6-deoxo-28-norCT, 6-deoxo-28-norTE, 6-deoxo-28-norTY, 6-deoxo-28-norCS, and 28-norCS) suggests an *in vivo* biosynthetic pathway to 6-deoxo-28-norCT. Based on these findings, a biosynthetic pathway of C_{27} BRs has been suggested: cholestanol → 6-deoxo-28-norCT → 6-deoxo-28-norTE → 3-dehydro-6-deoxo-28-norTE → 6-deoxo-28-norTY → 6-deoxo-28-norCS → 28-norCS in tomato seedlings. The cell cultures produced representatives of C_{28} BRs, such as CT, TE, 3-DT, TY, CS, and BL. The levels of BRs in cell cultures of *C. roseus* have been found to be comparable to those of BRs-rich plant tissues such as pollen and immature seeds. The reactions, named the early C-6 oxidation pathway for C_{28} BRs, have been demonstrated in Fig. 3. The occurrence of 6-deoxoBRs

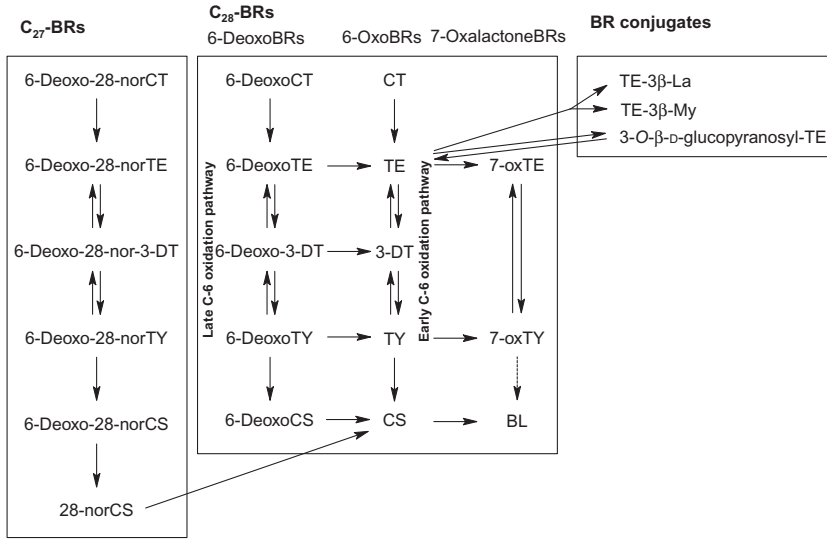

FIGURE 3 Brassinosteroid biosynthetic pathways in plants (CT, cathasterone; TE, teasterone; 3-DT, 3-dehydroTE; TY, typhasterol; CS, castasterone; BL, brassinolide; La, laurate; My, myristate).

such as 6-deoxoCS, 6-deoxoTE, and 6-deoxotyphasterol in several plants suggested that the parallel or/and alternative BR biosynthetic route exists. This late C-6 oxidation pathway for C_{28} BRs in *A. thaliana*, *C. roseus*, *Lycopersicon esculentum*, *C. vulgaris*, and *M. polymorpha* has been investigated. The conversion of 6-deoxoCS to CS via 6α-hydroxyCS has been found in *A. thaliana*. In addition to the early and late C-6 oxidation pathways of C_{28} BRs, cross-links between both branches exist. Conversion of CS to BL is the final biosynthetic step of BRs. Unfortunately, the biosynthesis of C_{29} BRs is still unclear [11,12].

Similar to animal steroid hormones, BRs regulate the expression of specific plant genes and complex physiological responses involved in growth, partly via interactions with other hormones setting the frame for BR responses. Both animals and plants use steroids as signaling molecules to regulate a variety of growth and developmental processes; however, their signaling mechanisms are quite different. During the early events of BR signaling (Fig. 4), BR-insensitive 1 (BRI1) perceives BRs, thus inducing dissociation of the inhibitory protein kinase inhibitor 1, which results in association with and transphosphorylation of the coreceptor BRI1-associated receptor kinase 1. BR signal kinases mediate signal transduction from BRI1 to bri1-suppressor 1 (BSU1) phosphatase through association with and phosphorylation of BSU1. BSU1 positively regulates BR signaling by dephosphorylating the negative regulator brassinosteroid-insensitive 2 (BIN2). This process facilitates accumulation of unphosphorylated brassinazole-resistant 1 (BZR1) and bri1-EMS-Suppressor

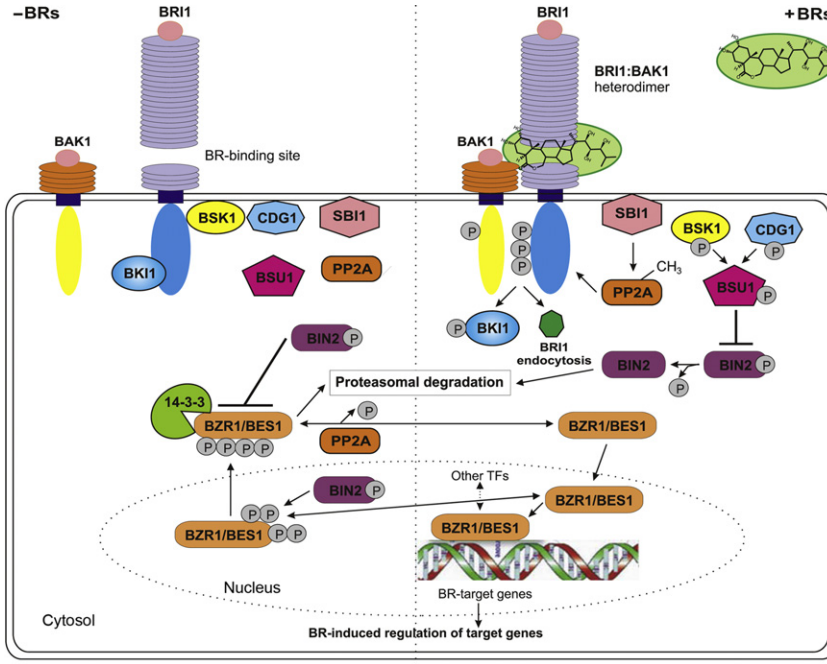

FIGURE 4 Model of brassinosteroid signaling in plants.

1 (BES1) in the nucleus, which directly or indirectly activate the expression of BR-responsive genes and regulate plant growth. BZR1 is also responsible for the negative feedback of BR biosynthetic genes such as constitutive photomorphogenesis and dwarfism by directly repressing transcription. BZR1 and BES1 are major TFs in the BR-signaling pathway. BES1 is 89% identical to BZR1 and was also named BZR2. BZR1 binds to the BR-responsive element (BRRE, CGTGT/CG) and mainly represses gene expression. BES1 binds to E-box by interacting with BIM1 or MYB30 (TFs) to promote target gene expression. BZR1 could also bind to E-box and BES1 to BRRE, so the functions of the family members may overlap. These are key TFs activating the BR-signaling pathway in plants. Protein phosphatase 2A dephosphorylates BZR1 and also BRI1 in mediating BR signaling. BRI1 degradation depends on PP2A-mediated dephosphorylation that is specified by methylation of the phosphatase, thus leading to the termination of BR signaling [7,12].

While animal steroids are mainly recognized by intracellular steroid receptors that provide a fast track for the chemical signals to move into the nucleus for controlling gene activities, plants steroids are perceived by a transmembrane receptor kinase that initiates a phosphorylation-mediated signal transduction pathway that includes inactivation of glycogen synthase kinase 3 (GSK3)-like kinase brassinosteroid-insensitive 2 (BIN2) by dephosphorylation at a conserved

phosphotyrosine residue pTyr200, therefore allowing for the accumulation of transcriptional factors BZR1 and BES1 in the nucleus. This signal transduction pathway shares a striking parallel with animal Wnt signaling. In mammals, Wnt binds to its receptor, Frizzled, causing inhibition of GSK-3β kinase activity and subsequent accumulation of β-catenin in the nucleus, where it directly affects the transcription of target genes. Although BIN2 is homologous to GSK-3β, the transcriptional factors BZR1 and BES1 are unique plant proteins that do not share sequence similarity with β-catenin, one of the key molecules in Wnt signaling. BR signaling in plants resembles the Wnt pathway and is mediated by GSK3-like kinase [7,12,13].

ANABOLIC EFFECT OF BRs

Anabolic steroids, officially known as anabolic–androgen steroids or colloquially simply as "steroids," are drugs which mimic the effects of the male sex hormones testosterone and dihydrotestosterone. They increase protein synthesis within cells, which results in the buildup of cellular tissue, especially in muscles. Androgens are required for the maintenance of normal sexual activity in adulthood and for enhancing muscle growth and lean body mass in adolescents and adults. Androgens have anabolic activity in prostate, bone, muscle, and hair follicles of the scalp and skin. However, there are health risks associated with long-term use or excessive doses of anabolic steroids. Anabolic androgenic steroids have profound effects on male cardiovascular, metabolic, and reproductive systems. These effects include harmful changes in cholesterol levels (increased low-density lipoprotein and decreased high-density lipoprotein), acne, high blood pressure, liver damage (mainly with oral steroids), and dangerous changes in the structure of the left ventricle of the heart [14–16].

28-Homobrassinolide presents very low androgenic activity when tested in the Hershberger assay and improved physical fitness of untrained healthy rats [17]. It stimulated protein synthesis and inhibited protein degradation in L6 rat skeletal muscle cells (EC_{50} 4 μM; EC_{50} is a median effective concentration of test substance affecting 50% of the test population during a specified period of time) mediated in part by PI3K/Akt (phosphatidylinositol 3-kinase/protein kinase B) signaling pathway. Oral administration of BR (20 or 60 mg/kg/day for 24 days) to healthy rats fed normal diet (protein content 23.9%) increased food intake, body weight gain, lean body mass, and gastrocnemius muscle mass as compared with vehicle-treated controls. In the gastrocnemius muscle of castrated animals, BR treatment significantly increased the number of type IIa and IIb fibers and the cross-sectional area of type I and type IIa fibers. Although BR produced anabolic effects in animals similar to androgens, they seemed to be pharmacologically different. Also BR has low or no significant binding to the androgen receptor and did not modulate plasma testosterone levels. It suggests that BRs may exert their anabolic effect

through an androgen-independent mechanism by stimulating protein synthesis and inhibited protein degradation in muscle cells, in part by inducing PI3K/Akt signaling.

The PI3K/Akt/mTOR pathway is a crucial intercellular regulator of muscle hypertrophy. Activation of PI3K by upstream ligands such as IGF-1 or IGF-2 phosphorylates the membrane phospholipids and creates a lipid binding site for Akt, which in turn increases protein synthesis and suppresses proteolytic activity and gene expression of the proteolytic genes. BRs activated PI3K/Akt signaling pathway as evident by increased Akt phosphorylation *in vitro*. However, they exert significant activation of Akt after 1 h, a much slower response than that produced by IGF-1, which phosphorylates Akt within 10 min [18].

ANTICANCER AND ANTIPROLIFERATIVE PROPERTIES OF BRs

BRs are able to disturb cell cycling in breast and prostate cancer cell lines. In the MCF-7 breast cancer cell model (estrogen receptor-α-positive), the most widely used experimental system to study breast cancer, the typical growth inhibitory response of antiestrogens is manifested by similar reductions in the proportions of cells synthesizing DNA (S phase) after the antiestrogen treatment and a corresponding increase in the proportions of cells in G_0/G_1 phase. Using flow cytometry, it was shown that treatment of breast (MCF-7/MDA-MB-468) and prostate (LNCaP/DU-145) cancer cell lines with 28-homocastasterone and 24-epibrassinolide blocked the cell cycle in the G_1 phase, with concomitant reductions in the percentages of cells in the S phase. BR also induced apoptosis in MDA-MB-468 (estrogen receptor-α-negative), LNCaP (androgen-sensitive), and slightly in the DU-145 cells (androgen-insensitive). These results proved that natural BRs, at micromolar concentrations, can inhibit the growth of several human cancer cell lines without affecting the growth of normal cells [19].

BRs, that is, 28-homobrassinolide and 24-epibrassinolide, were also examined in different normal and cancer cell lines in primary endothelial cells *in vitro*. Natural and synthetic BRs caused growth inhibition, cell cycle arrest, and initiation of apoptosis in many different cancer cell lines: the T-lymphoblastic leukemia CEM, breast carcinoma MCF-7, lung carcinoma A549, chronic myeloid leukemia K562, multiple myeloma RPMI 8226, cervical carcinoma HeLa, malignant melanoma G361, and osteosarcoma HOS cell lines. The inhibition of proliferation and migration of human endothelial cells has been demonstrated by BRs. Estrogen- and androgen-sensitive and insensitive breast and prostate cancer cell lines were also shown to respond differently to treatment with BRs. Most breast cancers consist of a mixture of estrogen-sensitive and estrogen-insensitive cells, and the key to the control of breast cancer seems to lie in the elimination of both cell types. Hormone-sensitive cell lines were more susceptible to treatment with BRs. Furthermore, a cytotoxic effect of natural BRs was observed in cancer cells, but not in

untransformed human fibroblasts, suggesting that BRs induce different responses in cancer and normal cells. Cytotoxic activity of BRs has been tested *in vitro* by Calcein AM assay. IC_{50} (median inhibition concentration causing a 50% reduction in a nonquantal measurement for the test population) values were estimated for human breast adenocarcinoma cell lines (MCF-7–estrogen-sensitive, MDA-MB-468–estrogen-insensitive), human acute lymphoblastic leukemia cell line (CEM), and human myeloma cell line (RPMI 8226) [19,20].

It has been reported that 24-epibrassinolide has possible effect on cultured hybridoma mouse cells [21]. Typical effects of 24-epibrassinolide were (a) increase the mitochondrial membrane potential, (b) reduce intracellular antibody levels, (c) increase the proportions of cells in G_0/G_1 phase, (d) reduce the proportions of cells in S phase, and (e) increase the proportions of viable hybridoma mouse cells at subnanomolar concentrations 10^{-13} and 10^{-12} M.

ANTIANGIOGENIC PROPERTIES OF BRs

Angiogenesis is the physiological process involving the growth of new blood vessels from preexisting vessels, as well as for growth of solid tumors and for metastasis. Endothelial cells play an important role in angiogenesis. These cells could be a target for antiangiogenic therapy because they are nontransformed and easily accessible to antiangiogenic agents. Endothelial cells are also unlikely to acquire drug resistance, because these cells are genetically stable, homogenous, and have a low mutation rate. There are many natural products that inhibit angiogenesis, for example, capsaicin (an active component of chilli peppers), erianin (product isolated from *Dendrobium chrysotoxum*), honokiol (from seed cone of *Magnolia grandiflora*), and glucocorticoids (dexamethasone and cortisone) [22,23].

BRs, that is, 24-epibrassinolide or 28-homocastasterone, have been used to present antiangiogenic activity. They inhibited growth of human microvascular endothelial (HMEC-1) and reduced migration of umbilical vein endothelial cells (HUVEC). Flow cytometric analysis showed that treatment with BRs only slightly increased the proportion of cells in the $subG_1$ (apoptotic) fraction in HMEC-1 cells when compared to the untreated controls. This antiangiogenic activity of BRs, along with their antiproliferative activity, suggests that these plant hormones might become important for the development of new anticancer drugs [24].

ANTIVIRAL PROPERTIES OF BRs

Natural BRs, BL and 28-homocastasterone, and synthetic BR [(22*S*,23*S*)-3β-bromo-5α,22,23-trihydroxystigmastan-6-one] display antiviral activity against poliovirus (PV), herpes simplex virus (HSV) type-1 and HSV-2, measles virus (MV), vesicular stomatitis virus (VSV), and the arenaviruses: Junin (JUNV),

Tacaribe (TCRV), and Pichinde (PICV) (Table 1) [25–27]. Viruses which have been tested for their susceptibility to BRs comprised four RNA viral families, *Paramyxoviridae*, *Arenaviridae*, *Picornaviridae*, *Rhabdoviridae*, and one DNA virus family *Herpesviridae*. MV, a member of *Paramyxoviridae*, causes an acute respiratory infection and immunosuppression. Despite the generalized use of an effective live attenuated vaccine, MV continues to contribute to high infant mortality in underdeveloped countries. JUNV, TCRV, and PICV are members of the *Arenaviridae* family. JUNV causes a severe disease in humans known as Argentine hemorrhagic fever. PV, a member of the *Picornaviridae* family, still represents a relevant health problem in some countries of the world, despite a dramatic decrease in its incidence as a result of intensive vaccination programs in developed countries. VSV, a member of the *Rhabdoviridae* family, produces a vesicular disease in cattle, horses, swine, and lambs in foot-and-mouth disease-free countries, causing thousands of outbreaks every year [28]. HSV-1 and HSV-2 are serious human pathogens. HSV-1 is normally associated with orofacial infections and encephalitis, whereas HSV-2 usually causes genital infections and can be transmitted from infected mothers to neonates [29].

The replication of HSV type 1 in Vero cells has been inhibited in the presence of (22*S*,23*S*)-3β-bromo-5α,22,23-trihydroxystigmastan-6-one. It was determined that a synthetic BR derivative would act synergistically with low concentrations of acyclovir (ACV) and moderate concentrations of foscarnet (FOS) against HSV. The best drug combination tested in this study resulted in an increase of 29.3% and 47.2% in antiviral activity for ACV (0.036 μM) and

TABLE 1 Antiviral Activity of Natural BRs Against Several RNA and DNA Viruses [25]

	Inhibition (%)	
Viruses	*1 μM Brassinolide*	*40 μM 28-Homocastasterone*
PV type I	96	85
VSV Indiana strain	100	23
JUNV IV_{4454} strain	74	79
TCRV $TRLV_{11573}$ strain	55	99
PICV AN_{3739} strain	67	98
HSV-1 F strain (tk^+)	96	50
HSV-1 B2006 strain (tk^-)	100	35
HSV-2 G strain (tk^+)	98	48
MV Brasil/001/91	100	50

FOS (37.5 μM) in the presence of 14.8 and 6.9 μM of BR derivative, respectively [30]. Furthermore, synthetic BR affected the late event of the virus growth cycle and inhibited virus protein synthesis and viral mature particle formation [31]. *In vitro* and *in vivo* antiherpetic activity of synthetic BRs analogues, that is, (22*S*,23*S*)-3β-bromo-5α,22,23-trihydroxystigmastan-6-one, (22*S*,23*S*)-5α-fluoro-3β-22,23-trihydroxystigmastan-6-one, and (22*S*,23*S*)-3β-5α,22,23-trihydroxystigmastan-6-one, has been reported in human conjunctive cell lines (IOBA-NHC) [32]. These compounds prevented HSV-1 multiplication in NHC cells when added after infection with no cytotoxicity. Evaluation of *in vivo* antiherpetic activity of these compounds was also performed using the murine herpetic stromal keratitis (HSK) [33]. Administration of BR derivatives to the eyes of mice at 1, 2, and 3 days postinfection delayed and reduced the incidence of HSK; however, viral titers of eye washes were not diminished in BR-treated mice, suggesting that the compounds do not exert a direct antiviral effect but rather may play a role in immune-mediated stromal inflammation. This hypothesis was further supported by *in vitro* studies that demonstrated that BR derivative, (22*S*,23*S*)-3β-bromo-5α,22,23-trihydroxystigmastan-6-one, modulates the response of epithelial and immune cells to HSV-1 infection through the induction or inhibition of cytokine production, depending on the cell type involved.

Twenty-seven BR derivatives have been tested for antiviral activity against MV via a virus yield reduction assay [34]. MV/BRAZIL/001/91, an attenuated strain of MV, was used in the virus inhibition assays. Only the following compounds: [(22*S*,23*S*)-3β-bromo-5α,22,23-trihydroxystigmastan-6-one], [(22*R*,23*R*)-2α,3α,22,23-tetrahydroxy-β-homo-7-oxa-stigmastan-6-one], [(22*R*,23*R*)-3β-fluoro-22,23-dihydroxystigmastan-6-one], [(22*S*,23*S*)-3β-fluoro-5α,22,23-trihydroxystigmastan-6-one], and [(22*S*,23*S*)-5α-fluor-3β,22,23-trihydroxystigmastan-6-one], with selectivity indexes (SI) of 40, 57, 31, 37, and 53, have an antiviral activity against MV. The SI values of derivatives of BRs were higher than those obtained with the reference drug ribavirin. The EC_{50} values for the active compounds, as determined by virus yield reduction assay, ranged from 1 to 8 μM. Cytotoxicity of these compounds was measured in stationary cells and ranged from 43 to 427 μM. Given that the compounds were only effective at millimolar concentrations, they will not be clinically useful because of the high doses that may be required to achieve an antiviral effect. The mechanism of inhibition of MV replication is unknown for BRs, probably they inhibited virus replication after virus adsorption.

NEUROPROTECTIVE ACTION OF BRs

BRs play an essential role in plant response to oxidative stress. It was shown that exogenous application of BRs modified antioxidant enzymes, such as superoxide dismutase, catalase, glutathione peroxidase, and ascorbate peroxidase, and nonenzymatic antioxidants, such as ascorbic acid, tocopherols,

carotenoids, and glutathione, in plants under different stress conditions. Vitamins C, E, and glutathione react directly or via enzyme catalysis with OH, H_2O_2, or O_2, while carotenes directly operate as effective quenchers of reactive intermediary forms of oxygen [6,35]. In animal and human organisms, reactive oxygen species can damage the normal cellular functions and can cause atherosclerosis in vessels or malignant growth in other tissues and aging processes [36]. It was shown that CS, one of the most active BR, acts against H_2O_2-induced DNA damage in human blood lymphocytes [37]. CS at 10^{-9} M concentration proved to be effective in diminishing the DNA damage by 89%.

Several neurodegenerative diseases, for example, Parkinson's disease, are associated with oxidative stress [38]. Parkinson's disease is characterized by the selective degeneration of nigrostriatal dopaminergic neurons, resulting in dopamine depletion [39]. Numerous studies have demonstrated that, in *postmortem* samples of *substantia nigra pars compacta*, DAergic neurons exhibit markers of oxidative stress, such as lipid peroxidation, DNA oxidative damage, and carbonyl modifications of soluble proteins [40]. BRs occur in many parts of plants which suggest their bright prospects in traditional medicines. The occurrence of BRs, that is, BL, 24-epiBL, CS, and 28-norCS, in broad bean (*Vicia faba*) seeds has been reported [4]. This plant also contains L-3,4-dihydroxyphenylalanine (L-dopa), the amino acid precursor of dopamine, which is the most effective symptomatic treatment of Parkinson's disease. Clinical reports indicate that consumption of broad bean has beneficial effects on Parkinson's disease patients. However, L-dopa concentrations in broad bean are not sufficient to explain the magnitude of the responses observed in Parkinson's disease patients and raise the possibility that other compounds from broad bean, such as BRs, may complement the L-dopa effect by their antioxidative activities [41,42]. Studies in Refs. [43] and [44] indicate the ability of 24-epibrassinolide to protect neuronal PC12 cells from 1-methyl-4-phenylpyridinium (MPP^+)-induced oxidative stress and consequent apoptosis in dopaminergic neurons. MPP^+ is the active metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, a toxin serving extensively to reproduce Parkinson's disease in animal models. 24-Epibrassinolide reduces the levels of intracellular reactive oxygen species and modulates superoxide dismutase, catalase, and glutathione peroxidase activities. The antioxidative properties of 24-epibrassinolide lead to the inhibition of MPP^+-induced apoptosis by reducing DNA fragmentation as well as the Bax/Bcl-2 protein ratio and cleaved caspase-3. These studies clearly demonstrate that BRs are new, efficient, protective molecules against MPP^+-induced oxidative stress and might thus be regarded as novel agents in complementary and/or preventive therapies of neurodegenerative diseases.

CONCLUDING REMARKS

Being natural, nontoxic, nonhazardous, biosafe, antigenotoxic, and plant products, BRs have been implicated in various medicinal applications. BRs are highly concentrated in many parts of plants which further suggest their bright

prospects in traditional medicines. Although it is too early to predict their clinical utility, but due to their antiviral, anticancer, and neuroprotective properties, BRs are projected as potential drugs against several RNA and DNA viruses. However, the mechanism underlying the antiviral mode of action of BRs with host cell signal transduction system must also be further explored. The action mechanisms of BRs in animal cells are still largely unknown, but it seems possible that BRs may interact with one or more of the numerous steroid-binding proteins. It also seems possible that BRs induce multiple effects, both steroid receptor-dependent and independent. BRs may prove to be promising leads for the development of new generation of natural drugs.

ABBREVIATIONS

ACV	acyclovir
BES1	bri1-EMS-Suppressor 1
BIN2	brassinosteroid-insensitive 2
BL	brassinolide
BR	brassinosteroid
BRI1	BR-insensitive 1
BSU1	bri1-suppressor 1
BZR1	brassinazole-resistant 1
CS	castasterone
EC_{50}	median effective concentration of test substance affecting 50% of the test population during a specified period of time, such as growth
FOS	foscarnet
GSK3	glycogen synthase kinase 3
HSK	herpetic stromal keratitis
HSV	herpes simplex virus
IC_{50}	median inhibition concentration causing a 50% reduction in a nonquantal measurement (such as movement) for the test population
JUNV	Junin virus
LD_{50}	the dosage (mg/kg body weight) causing death in 50% of exposed animals
L-dopa	L-3,4-dihydroxyphenylalanine
MPP^+	1-methyl-4-phenylpyridinium
MV	measles virus
PI3K/Akt	phosphatidylinositol 3-kinase/protein kinase B
PICV	Pichinde virus
PP2A	protein phosphatase 2A
PV	poliovirus
SI	selectivity indexes
TCRV	Tacaribe virus
TF	transcription factor

TML48 fish test on carp treated with tetramethyllead (TML) for 48 h
VSV vesicular stomatitis virus

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Chapter 3

Synthesis of Bioactive Natural Products by Propargylic Carboxylic Ester Rearrangements

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Chapter Outline

INTRODUCTION

Life on earth, which is undergoing incessant change through evolution and human-operated transformation, needs some permanent tools to flourish. Organic synthesis provides competitive methods for constructing a novel world of purely synthetic compounds and natural products. Organic synthesis has proved to be one of the most potent driving forces of life support and sustainable development. It is an incontrovertible fact that organic synthesis pivots on the scientific hinge of molecular rearrangements [1], and

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consequently, many complex natural products have undergone the process of synthesis to an unfathomable extent during the last decades [2].

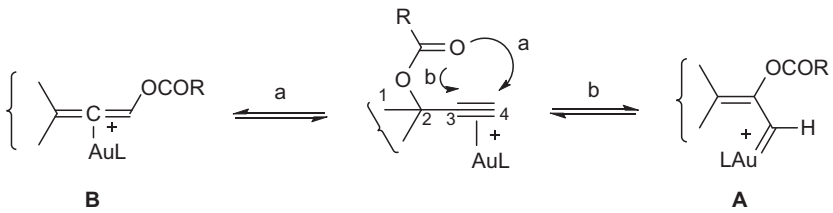
The book focuses on the synthesis of complex bioactive molecules, and this chapter deals with a set of selected organized molecules of personal choice for the synthesis of bioactive natural products.

Our choice has been directed toward [3,3]-sigmatropic rearrangement [3]; it continues to provide excellent opportunities for reaching high chemical diversity in efficient synthetic schemes. [3,3]-Sigmatropic rearrangements, such as the Cope [4] and Claisen [5] rearrangements, are among the most basic and useful transformations in synthetic organic chemistry. These remarkable rearrangements are effective procedures for the synthesis of quaternary, sterically hindered chiral centers and for the stereoselective formation of carbon–carbon and carbon–heteroatom bonds. In addition, [3,3]-sigmatropic rearrangements can be easily integrated in, and adapted to, cascade processes as a simple method to prepare complex molecules in atom-efficient reactions.

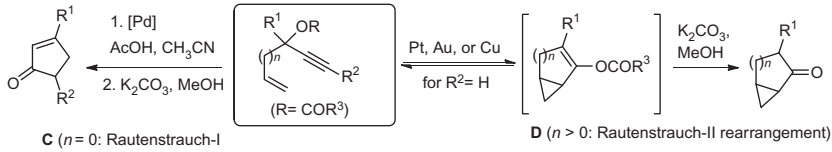
Consequently, in the milieu of [3,3]-sigmatropic rearrangements, we focus on the transformations of propargylic carboxylic esters, whose rearrangements, in different experimental conditions, have been largely exploited for the synthesis of interesting key and useful intermediates [6–13]. The rearrangements can be promoted by protic acids, Lewis acids, and noble metals, such as Pt, Au, or Cu salts, or complexes, which have recently emerged as the most popular and potent electrophilic activators of alkynes toward a number of nucleophilic agents, under homogeneous conditions [11].

The reactivity of propargylic esters leads, by 1,2-acyl migration, to the formation of α-acyloxy-α,β-carbenes **A**, or via 1,3-acyl shift through [3,3]-sigmatropic rearrangements, to allene–metal complexes **B**, giving rise to a carbene-type, or to an allene activation, respectively (Scheme 1). Furthermore, the presence of additional unsaturated groups in these intermediates has been used with success to yield complex structures taking advantage of the rich reactivity embedded in the cycloisomerization of enynes or allenes [12].

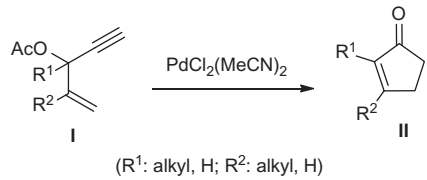
Aiming at propargylic esters, the transition metal-promoted rearrangements of propargylic esters through a 1,2-acyl migration, such as the Rautenstrauch-I and Rautenstrauch-II rearrangements, are described. The Rautenstrauch-I rearrangement at its nascent phase was the palladium-



SCHEME 1 1,2-Acyl migration or 1,3-acyl shifts through [3,3]-sigmatropic rearrangements from propargylic esters.



SCHEME 2 Rearrangement of propargyl derivatives: Rautenstrauch-I and Rautenstrauch-II rearrangements.



SCHEME 3 Rautenstrauch-I rearrangement.

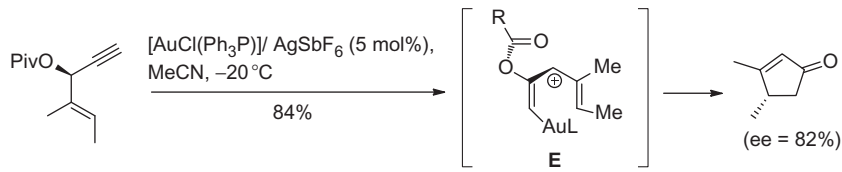
catalyzed cyclization of 3-acyloxy-1,4-enynes **I** to give α,β-unsaturated cyclopentenones (**C**) [14] (Scheme 2). A particular case of the Rautenstrauch-I rearrangement that has evolved into an independent and completely different type of transformation is the transition metal-catalyzed rearrangement using palladium [14], platinum [15], gold [16], and copper as catalysts [17] in the rearrangement of $(n-1)$-acyloxy-1,*n*-enynes $(n>4)$ **I** for the synthesis of bicyclo[$(n-1)$.1.0]-2-enol esters (**D**), an extremely useful type of synthetic intermediate for the preparation of natural products (Scheme 2).

Finally, continuing with the rearrangement of propargylic esters, we describe selected examples of the 1,3-acyl migration or the [3,3]-sigmatropic rearrangement of propargylic esters, a process that plays a prominent role in this context and has been extensively used in the last few years for interesting chemical transformations and for the synthesis of natural products [18].

SYNTHESIS OF BIOACTIVE NATURAL PRODUCTS BY REARRANGEMENT OF PROPARGYLIC ESTERS VIA 1,2-ACYL MIGRATION

Substituted cyclopentenones are very well-known, useful key intermediates in the total synthesis of natural products. Consequently, a number of synthetic strategies have been designed and proposed for the preparation of this five-membered ring system, such as the aldol reaction [19], the Pauson–Khand reaction [20,21], and the Rautenstrauch rearrangement [14].

Rautenstrauch described the Pd(II)-catalyzed rearrangement of 1,4-enynes **I** substituted with a carboxylate group at the propargylic position as a practical method for the synthesis of cyclopentenones of type **II** (Scheme 3) [14]. The method proceeds with high chirality transfer, giving rise to enantiomerically



SCHEME 4 Synthesis of (*S*)-3,4-dimethylcyclopent-2-enone by gold-mediated cyclization of (*R*)-2-methylpent-1-en-4-yn-3-yl pivalate.

pure cyclopentenones from the corresponding optically pure propargylic esters (Scheme 4) [22].

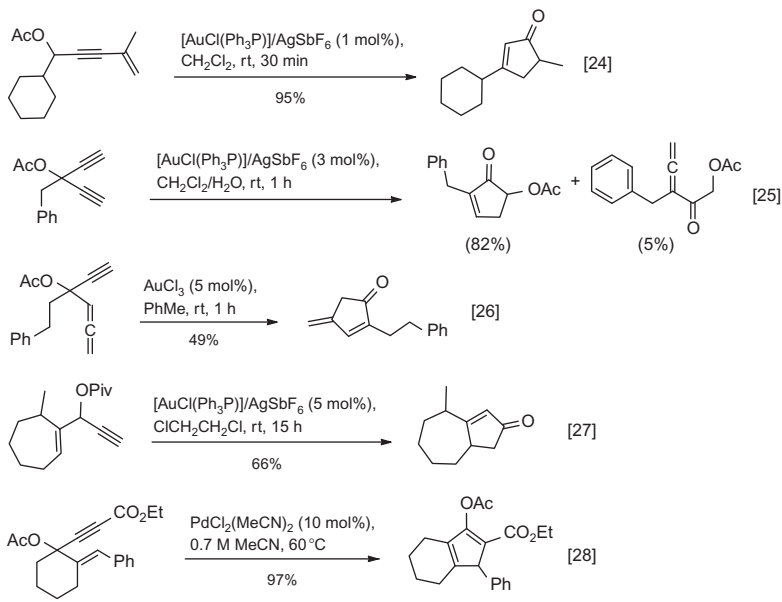
This result was inconsistent with a metallocarbenoid intermediate. It was elegantly explained and rationalized by a center-to-helix chirality transfer, supported by computational analysis [23]. The reaction was proposed to proceed via 1,2-acyl migration to form a pentadienyl cation **E** intermediate that displays a helical nature, which indeed preserves the chiral information. Then, this key intermediate undergoes electrocyclization to form a cyclic intermediate that suffers demetallation to yield a cyclopentadiene bearing an enol ester moiety whose basic hydrolysis should finally afford the cyclopentenone (Scheme 4) [22]. The calculations confirm that the chiral information present in the original enyne is conveyed to the pentadienyl cation, where it is stored in the helicity of the main carbon chain. The concurrent existence of a barrier to cyclization lower than that corresponding to the inversion of the helix and ester rotation ensures the faithful transfer of chiral information to the final product [23].

The scope of this Rautenstrauch-I rearrangement has been subsequently applied in a number of laboratories, which have published their reports in the last few years (Scheme 5) [24–28].

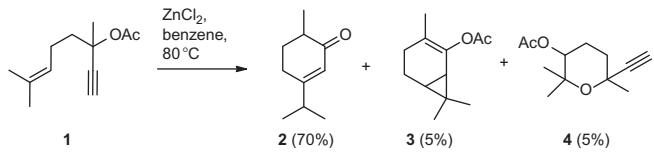
It has been observed that the type of substituents at the terminal carbons in the alkyne and/or the alkene moieties of the enyne have important effects on the general reactivity trend of this rearrangement [28]. Thus, precursors with carboxylic esters, or aryl groups, at the terminal carbon of the alkyne rearrange to give enol ester compared with those bearing alkyl substituents [28].

An impressive development has been observed in the homogeneous gold catalyst's activation of alkynes in the past 10 years [29]. Marco-Contelles and Malacria jointly published their work on the $PtCl_2$-mediated cycloisomerization of unsaturated propargylic esters [10,15,16,29,30]; they proposed a definite "Rautenstrauch-II rearrangement" reaction to highlight the importance of, and the clear differences between, this rearrangement and the Rautenstrauch-I rearrangement (see earlier) [14], which has two precedents in the literature.

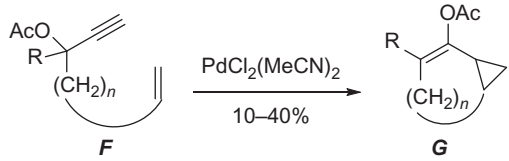
Ohloff proposed that the $ZnCl_2$-catalyzed ring closure of 3,7-dimethyloct-6-en-1-yn-3-yl acetate (**1**) affords a complex reaction mixture of compounds **2**–**4**, 3,7,7-trimethylbicyclo[4.1.0]hept-2-en-2-yl acetate (**3**) being isolated in poor yield [31] (Scheme 6).



SCHEME 5 Examples of the Rautenstrauch-I rearrangement on propargylic esters leading to substituted cyclopentenones.



SCHEME 6 $ZnCl_2$-mediated Rautenstrauch-II rearrangement of 3,7-dimethyloct-6-en-1-yn-3-yl acetate (**1**).



(1)

(Refs. [14] and [32])

Some years later, Rautenstrauch [14] described the formation of compounds of type **3** in a similar $PdCl_2$-catalyzed reaction [32], stating:

*"...$PdCl_2$-(MeCN)$_2$ also catalyzes the cyclization of homologous of **F** ($n=2$ and 3; R = alkyl) to bicyclic acetates **G** (see Eq. 1), yields for five examples being 10-40%. It was reasoned that chelation of Pd(II) probably channels the intermediate toward cyclization, but it is inherently poor for $n=2$ and 3 and optimal for $n=0$ (i.e. **1**) and for $n=1$. The latter case and further variants are under study."*

This attractive transition metal-catalyzed rearrangement of a propargylic carboxylic ester, very surprisingly, remained neglected, until it was rediscovered by serendipity in Marco-Contelles' laboratory in Madrid, in the course of the $PtCl_2$-catalyzed transformation of 4-ethynyl-octa-1,7-dien-4-carboxylates of type **5**, a reaction that gave a mixture of compounds **6** and **7** (Scheme 7) [15]. Note that the structure of the precursor **5** yielded the preferred Rautenstrauch-II rearrangement of the 6-hepten-1-yn-3-carboxylate, and the first example of a similar reaction on a 5-hexen-1-yn-3-carboxylate affording bicyclo[3.1.0]hexenol acetate (**7**) [15].

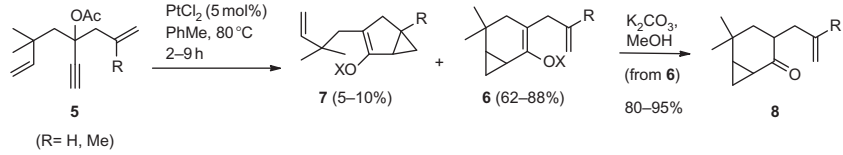
Consequently, it is clear that the Rautenstrauch-II is a particular case reaction of the Rautenstrauch-I rearrangement, showing that the elongation of the tether connecting the double bond and the propargylic motif produces different types of final products, such as the synthetically useful bicyclo[n.1.0]enol carboxylates [15].

It is important to highlight that the basic methanolysis of these enol esters in compounds of type 6 gave the corresponding bicyclo[n.1.0]ketones (**8**) in good yields (Scheme 7) just in a two-steps [15]. This method can be considered as an equivalent of the cycloisomerization of a-diazocarbonyl species [33], with notable advantages.

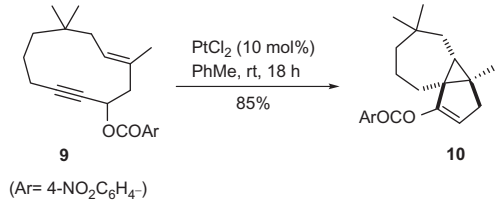
After this preliminary communication [15], a number of reports have been published showing the scope and generality of this Rautenstrauch-II rearrangement.

Very recently, the use of *N*-heterocyclic carbenes, such as [IPr(AuCl)] $AgBF_4$, has been shown in the gold(I)-catalyzed cycloisomerization of precursor **5** [34]. This report shows that very simple modifications of the catalytic system can affect the mode of these cycloisomerization reactions.

Similarly, the $PtCl_2$-catalyzed transannular cycloisomerization of 1,5-enynes of type **9** is a highly stereocontrolled way to access tricyclic derivatives (**10**) (Scheme 8) [35], showing the power of this synthetic method.



SCHEME 7 The rediscovery of Rautenstrauch-II rearrangement.

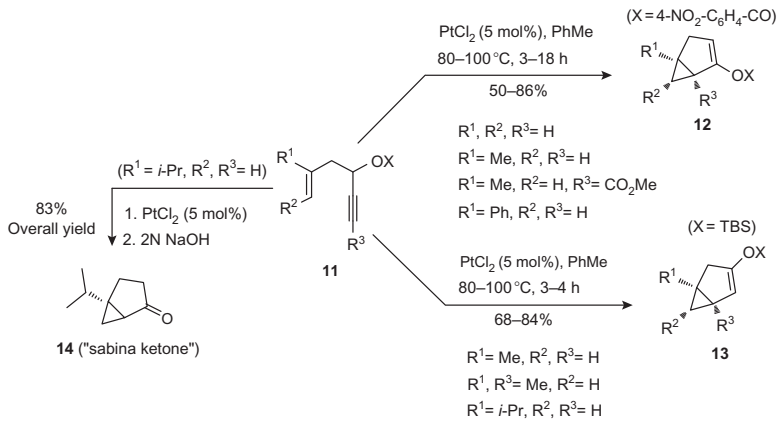


SCHEME 8 $PtCl_2$-catalyzed transannular cycloisomerization of 1,5-enynes.

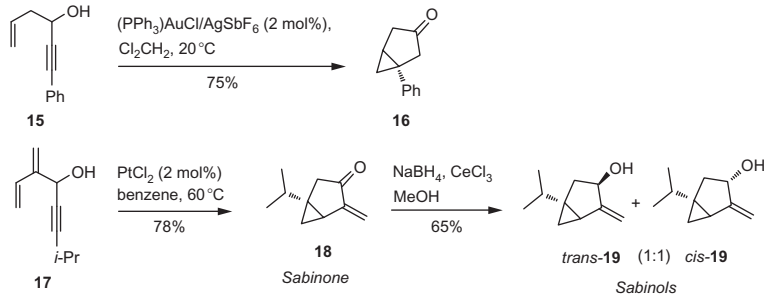
The same group has also reinvestigated the previously reported $PtCl_2$-catalyzed cycloisomerization of an 1,5-enyne embedded in precursor **6** [15] (Scheme 7), by extending it to other secondary 5-en-1-yn-3-ols (**11**) (Scheme 9) [36].

As shown in Scheme 9, the expected bicyclo[3.1.0]enol-4-nitrobenzoates **12** were obtained in excellent yields using precursors substituted at the alkene and/or at the acetylene. However, a more relevant finding described here was the $PtCl_2$ cycloisomerization reaction of free alcohols or *t*-butyldimethylsilyl ethers, because the corresponding silyloxy enol ethers **13** were obtained in good yields from compounds of type **11** (Scheme 9). Note that in this case, the preferred 1,2-hydrogen shift leaves the silyloxy moiety at the same position, while for precursors bearing ester groups, the acyloxy moiety migrates first affording the rearranged enol ester. Again, the change in the type of *O*-protecting group allowed the authors to favor the migration of one of the possible functional groups and, consequently, the preparation of different bicyclo[3.1.0]hexane ketones from unsaturated propargylic precursors [36]. In addition, in this work, the authors described the synthesis of *sabina ketone* (**14**) (Scheme 9), a natural product and a key intermediate for the synthesis of *sabinene* [37], starting with the appropriate precursor after cycloisomerization and basic hydrolysis of the enol ester [35,36]. In a recent paper, the same team has reported the synthesis of polycyclic derivatives incorporating a cyclopropyl group from propargylic acetates using platinum(II) and gold(I/III) as catalysts [38].

In an independent work, it was reported that the Rautenstrauch-II rearrangement of an acetylated hydroxylated 1,5-enyne can be efficiently catalyzed by AuCl [39]. These authors have also analyzed in depth the metal-promoted cycloisomerization of hydroxylated enynes (see for instance the case of compound **15** affording ketone **16** (Scheme 10)), including the stereogenic consequences.



SCHEME 9 $PtCl_2$-catalyzed cycloisomerization of 5-en-1-yn-3-ol systems.



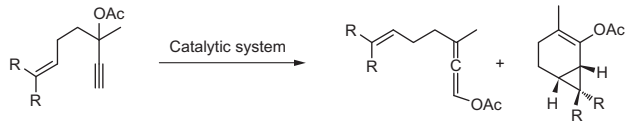
SCHEME 10 Metal-catalyzed cycloisomerization of hydroxylated enynes.

The synthetic power of these transformations has been exemplified in the synthesis of *sabinone* **18** and the two isomeric *sabinols* **19** (Scheme 10), natural products found in plants of various *Artemisia*, *Juniperus*, and *Thuja* species [40], from propargyl alcohol **17** after treatment with catalytic amounts of $PtCl_2$ using benzene as solvent, a result that shows that an additional double bond in the precursor does not interfere with the rearrangement of a hex-1-en-5-yn-4-ol.

In the meantime, and independently, Marco-Contelles and Anjum in Madrid have explored different aspects of the Rautenstrauch-II rearrangement, trying to investigate the scope and generality of the process [41]. In 2002, they prepared new 1,6-enyne derivatives, simplifying the structure of their former precursors **6** (Scheme 7) [15], incorporating a methyl substituent at the quaternary propargylic center, fixing in two methylene groups the tether connecting the unsaturated double bond with the quaternary center, and moving the *gem*-dimethyl groups to the terminal carbon of the alkene moiety. New and electronic different acyloxy groups such as the acetyloxy, benzoyloxy, trichloroacetyloxy, 3,4,5-trimethoxybenzoyloxy, and carbonate were also tested in the rearrangement process. Not unexpectedly, the $PtCl_2$-catalyzed Rautenstrauch-II rearrangement of these diversely substituted propargylic esters gave mixtures of 3,7,7-trimethylbicyclo[4.1.0]hept-2-en-2-yl carboxylic esters and allenes (3,7-dimethylocta-1,2,6-trien-1-yl) (**21**), not previously observed or detected [41].

Note that in the usual experimental conditions, and from all these derivatives, precursor **20** gave the best results, affording a mixture of the allene type of compounds **21** and the expected Rautenstrauch product **22** in moderate yield (Scheme 11). Very interestingly, compound **23**, without the methyl groups at the terminal alkene bond, afforded the enol acetate **24** in almost quantitative chemical yield (Scheme 11) [41].

Subsequently, other authors have also prepared and subjected the same compounds **20** and **23** to metal-transition-catalyzed cycloisomerization, confirming the results of Marco-Contelles and Anjum [41] but improving the chemical yields, since $AuCl_3$ worked much more efficiently than $PtCl_2$, giving



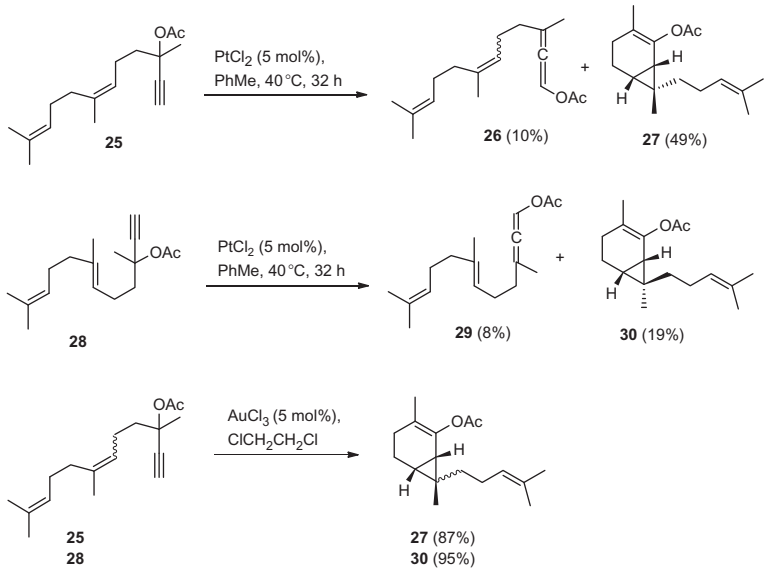
20 (R= Me) $PtCl_2$ (5 mol%), toluene, 40 °C — **21** (8%) — **22** (54%) [41]

20 (R= Me) $AuCl_3$ (5 mol%), 1,2-dichloroethane, rt — **22** (98%) [42]

23 (R= H) $PtCl_2$ (5 mol%), toluene, 40 °C — **24** (99%) [41]

23 (R= H) $AuCl_3$ (5 mol%), toluene, 80 °C — **24** (79%) [43]

SCHEME 11 Metal-catalyzed cycloisomerization of new propargylic acetates.



SCHEME 12 Transition metal cycloisomerization of (*Z*)-3,7,11-trimethyldodeca-6,10-dien-1-yn-3-yl acetate (**25**) and (*E*)-3,7,11-trimethyldodeca-6,10-dien-1-yn-3-yl acetate (**28**).

clean reactions leading to compounds **22** and **24**, respectively, without the allene secondary by-product [42,43].

Starting with commercial neryl acetone, Marco-Contelles and Anjum have synthesized propargylic acetate **25** and subjected it to cyclization with $PtCl_2$ (5 mol%) to give allene **26** and enol ester **27**, in 10% and 49% yields, respectively (Scheme 12) [41]. Similar results were obtained from compound **28**, prepared from geranyl acetone, which furnished compounds **29** and **30** with the opposite stereochemistry at the double bond in the allene derivative and at the cyclopropyl ring in the enol ester (Scheme 12) [41]. Similarly, and based on Marco-Contelles' work [41], other authors [42,43] have prepared identical propargylic acetates and found that when using $AuCl_3$ as catalyst, the yields for compounds **27** and **30** were higher (Scheme 12).

These results clearly confirm that the transition metal-promoted cycloisomerization process of trisubstituted alkenes is achievable, and it can proceed stereospecifically translating the geometry of the double bond on the reacting alkene into the relative configuration on the cyclopropane ring. The *Z* isomer affords the *cis*-isomer, while the *E* isomer leads to the corresponding *trans*-derivative.

The synthesis of compounds **22** (Scheme 11) and **27**/**30** (Scheme 12) constitutes a simple and efficient entry to the carene type of natural products [40].

Basic hydrolysis (K_2CO_3, MeOH) of compound **22** gave *2-carvone* **31** [41,42], which was next converted into *2-carene* **32** (Fig. 1) [43]. Under basic hydrolysis followed by vinyl triflate formation and reduction, compound **27** afforded the *episesquicarene* **33** (Fig. 1) [42,43], and product **30** provided the *sesquicarene* **34** (Fig. 1) [43].

Recently, Schlecker and Fürstner reported [44] the gold-catalyzed synthesis of *sesquisabinene* and *sesquithujene* terpenoids and the formal total synthesis of *cedrene* and *cedrol* [45].

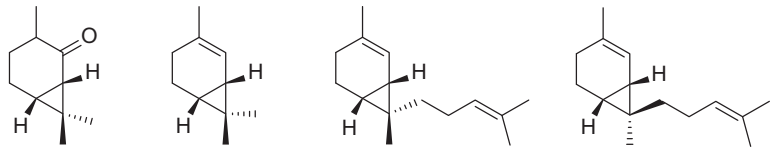
In these synthetic sequences, compounds **37** and **40** played a key role. These epimeric ketones were readily prepared from enantiomerically pure propargylic *p*-nitrobenzoates **35** and **38**, by the Rautenstrauch-II rearrangement followed by basic hydrolysis of the intermediate enol esters **36** and **39** (Scheme 13).

The first target, the aggregation pheromone (*R*,*Z*)-2-methyl-6-((1*S*,5*S*)-4-methylenebicyclo[3.1.0]hexan-1-yl)hept-2-en-1-ol (**42**) produced by *Eysarcoris lewisi* (Distant), which caused severe damage in rice fields in Japan [46], has been synthesized from ketone **37** as shown in Scheme 14 via intermediate **41**.

Essential oils *cis-sesquisabinene* (**43**) [47], *trans-sesquisabinene* (**44**) [48], and 7-epi-*sesquithujene* (**45**) [49] have been also efficiently obtained from ketone **37** as shown in Scheme 15.

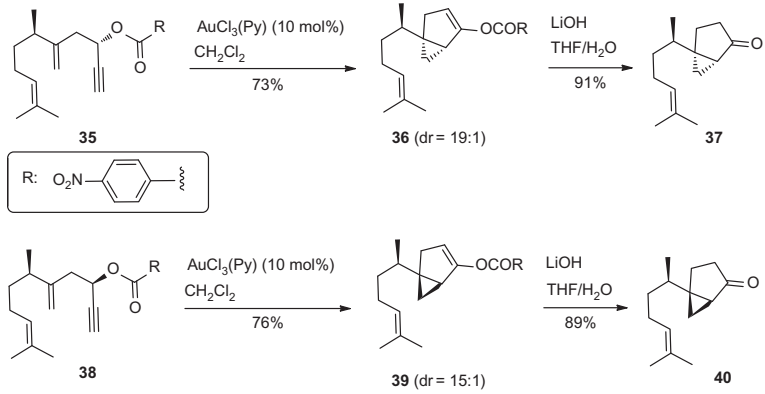
Using a similar synthetic approach, but starting from ketone **40** (Scheme 13), related natural products **46** [49], **47** [48], and **48** [47] (Fig. 2) have also been geared up [44].

Epoxysesquithujene **50**, isolated from *Valeriana hardwickii* var. *hardwickii*, an aromatic Himalayan herb [50], was obtained from ketone **40** by epoxidation,

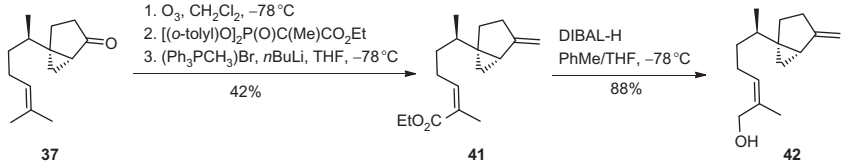


31 *2-Carvone* **32** *2-Carene* **33** *Episesquicarene* **34** *Sesquicarene*

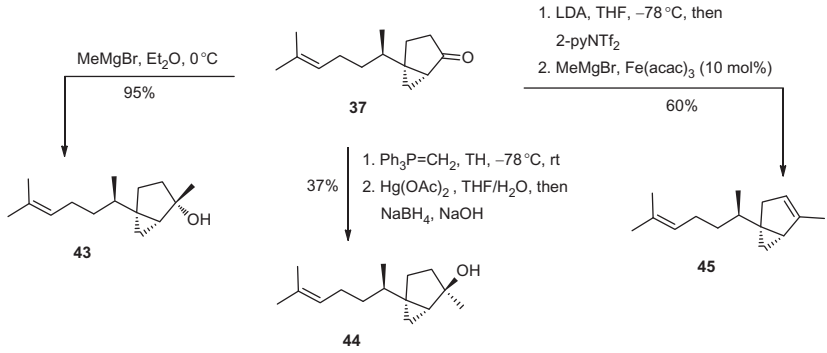
FIGURE 1 Natural products bearing the *carene* skeleton.



SCHEME 13 Synthesis of (1*R*,5*S*)-5-((*R*)-6-methylhept-5-en-2-yl)bicyclo[3.1.0]hexan-2-one (**37**) and (1*R*,5*R*)-5-((*R*)-6-methylhept-5-en-2-yl)bicyclo[3.1.0]hexan-2-one (**40**) from propargylic esters **35** and **38** by Rautenstrauch-II rearrangement.



SCHEME 14 Synthesis of (*R*,*Z*)-2-methyl-6-((1*S*,5*S*)-4-methylenebicyclo[3.1.0]hexan-1-yl)hept-2-en-1-ol (**42**) from (1*R*,5*S*)-5-((*R*)-6-methylhept-5-en-2-yl)bicyclo[3.1.0]hexan-2-one (**37**).



SCHEME 15 Synthesis of essential oils **43–45** from (1*R*,5*S*)-5-((*R*)-6-methylhept-5-en-2-yl) bicyclo[3.1.0]hexan-2-one (**37**).

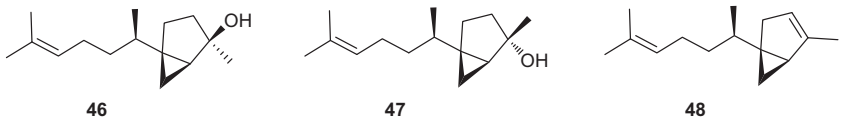
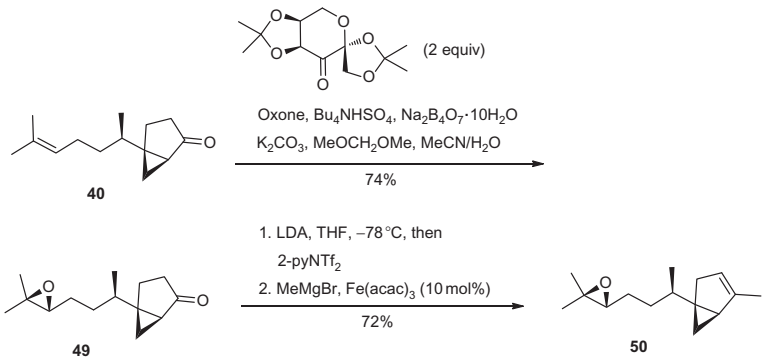
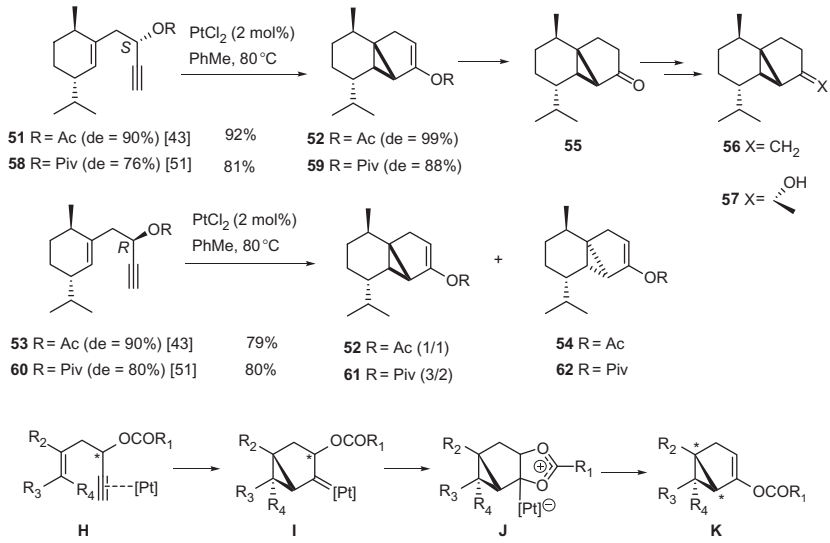


FIGURE 2 Structure of essential oils *cis-sesquisabinene* (**43**) [47], *trans-sesquisabinene* (**44**) [48], and 7-epi-*sesquithujene* (**45**) [49].



SCHEME 16 Synthesis of essential oil **50** from (1*S*,5*R*)-5-((*R*)-6-methylhept-5-en-2-yl)bicyclo [3.1.0]hexan-2-one (**40**).



SCHEME 17 Rautenstrauch-II rearrangement of enantiomerically pure propargylic carboxylates en route to (−)-β-*cubebene* **56** and (−)-*cubebol* **57**.

transformation of the carbonyl group of the resulting ketone **49** into the corresponding triflate, and Grignard reaction with methylmagnesium bromide (Scheme 16).

In a project directed toward the synthesis of (−)-β-*cubebene* **56** and (−)-*cubebol* **57** (Scheme 17), Hannen and Fürstner synthesized compounds (*S*)-**51** and (*R*)-**53** from (*R*)-(−)-carvone [43]. While acetate (*S*)-**51** afforded diastereomerically pure enol acetate **52** in a clean Rautenstrauch-II rearrangement promoted by $PtCl_2$ (2 mol%), under the same experimental conditions, propargylic acetate (*R*)-**52** gave a mixture of compounds **52** and **54** (79% yield) in a 1:1 ratio. Compound **54** was the epimer of acetate **52** at the newly

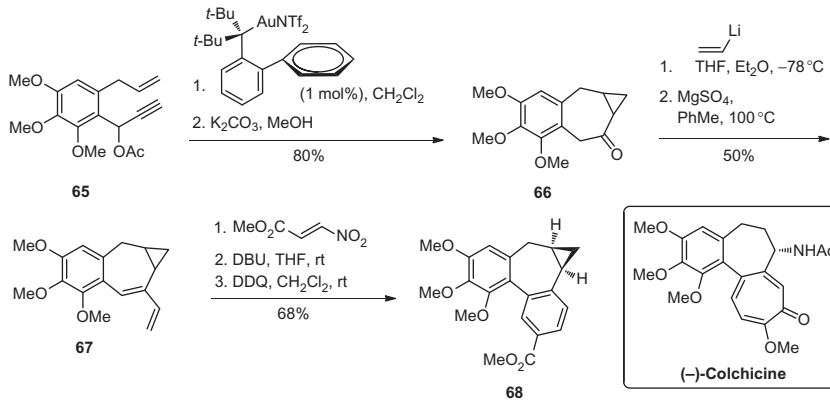
formed stereocenters. Enol acetate **52** was subjected to methanolysis to give ketone **55** that, after standard transformations, afforded natural *cubebenes* **56** and **57** (Scheme 17) [43].

Fehr and colleagues at Firmenich (Switzerland) have also described the synthesis of (−)-*cubebol* **57** from a related enol carboxylate **61** obtained through a similar synthetic approach, by $PtCl_2$-promoted cycloisomerization of enantiomerically pivalate (*S*)-**58** (Scheme 17) [51]. They also observed that a diastereomerically enriched mixture of epimer (*R*)-**60** gave, in addition to the previously isolated pivalate **61**, a new enol pivalate **62** that turned out to be its epimer at the newly formed stereocenter. In addition, these authors have shown that inexpensive $[Cu(CH_3CN)_4](BF_4)$ efficiently catalyzes all these Rautenstrauch-II rearrangements [51].

These results supported the previously advanced mechanistic proposal by Soriano and Marco-Contelles and unequivocally confirmed that the chirality at the propargylic center determined the stereochemical outcome of the Rautenstrauch-II rearrangement and that the cyclopropanation step to form a cyclopropyl-metallocarbenoid intermediate (**I**, Scheme 17) precedes the stepwise rearrangement event [52].

Hanna and colleagues have reported [53] the synthesis of a new class of allocolchicinoids, as synthetic analogs of the important antimitotic (−)-colchicine in which the tropolone ring has been replaced by a benzene nucleus, showing tubulin polymerization inhibition [54]. Allocolchicinoids are analogs of the important antimitotic (−)-colchicine in which the tropolone ring has been replaced by a benzene nucleus, showing tubulin polymerization inhibition.

The Au-catalyzed Rautenstrauch-II rearrangement of the 1,7-enyne propargylic acetate **65** afforded the seven-membered ring fused to a cyclopropane derivative **66** that, after transformation into intermediate **67** followed by a Diels–Alder/aromatization sequence, provided compound **68** (Scheme 18) [53].



SCHEME 18 Hanna's approach to allocolchicinoids via Rautenstrauch-II rearrangement of propargylic acetate **65**.

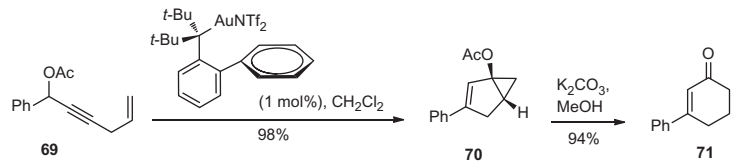
SYNTHESIS OF BIOACTIVE NATURAL PRODUCTS BY REARRANGEMENT OF PROPARGYLIC ESTERS VIA 1,3-ACYL MIGRATION: [3,3]-SIGMATROPIC REARRANGEMENT OF PROPARGYLIC ESTERS

The most significant limiting factor for the intramolecular Rautenstrauch-II rearrangement concerns the substitution at the terminal acetylene carbon in hept-6-en-1-yne derivatives. In these cases, the rearrangement proceeds through a 1,3-acyl migration to afford [3,3]-sigmatropic rearrangement products. Now we highlight the most interesting and significant results related to this topic.

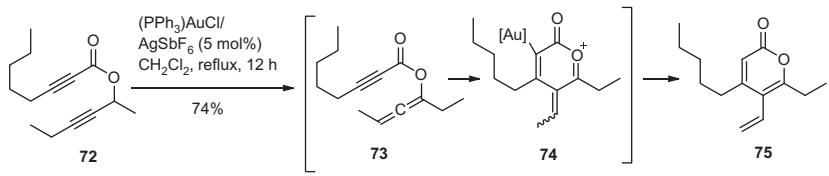
An efficient approach to the synthesis of bicyclo [3.1.0] hexenes has been reported that proceeds via gold(I)-catalyzed cycloisomerization of 5-en-2-yn-1-yl acetates through a [3,3]-sigmatropic rearrangement [55]. As shown, gold(I)-catalyzed isomerization of 1-phenylhex-5-en-2-yn-1-yl acetate (**69**) affords intermediate **70**, which after basic hydrolysis leads to 5,6-dihydro-[1,1′-biphenyl]-3(4*H*)-one (**71**) (Scheme 19). As reported, this method provides a new entry to 2-cycloalken-1-ones, key building blocks in a number of total syntheses.

The [3,3]-sigmatropic rearrangement of propargyl propiolates promoted by a late transition metal has been investigated [56] for the synthesis of diverse α-pyrones, which are heterocyclic core elements present in a number of bioactive molecules. In the example shown in Scheme 20, starting with compound **72**, the presumed resulting enyne allene **73** evolves through the oxocarbenium intermediate **74** to yield vinyl α-pyrone **75** [56].

In the context of a project targeted to the synthesis of the potent antitumor natural product *cortistatin* [57], the gold(I)-catalyzed tandem [3,3]-sigmatropic



SCHEME 19 Gold(I)-catalyzed isomerization of 1-phenylhex-5-en-2-yn-1-yl acetate (**69**) followed by basic hydrolysis to give 5,6-dihydro-[1,1′-biphenyl]-3(4*H*)-one (**71**).

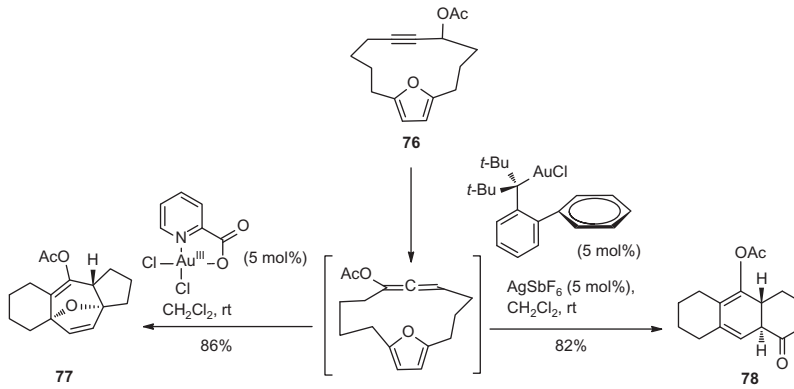


SCHEME 20 Gold(I) cycloisomerization of propargylic propiolate **72**.

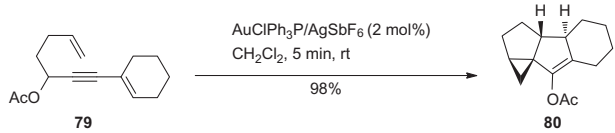
rearrangement plus transannular [4 + 3]-cycloaddition of propargylic acetate **76** has been described [58]. Very intriguingly, depending on the catalyst, the reaction affords different products such as **77** or **78** (Scheme 21). A gold(I)-catalyzed [3,3]-sigmatropic rearrangement, followed by a metallo-Nazarov reaction and ending with an electrophilic cyclopropanation reaction, has been reported for the efficient synthesis of polycyclic compounds from propargylic acetates [59].

In Scheme 22, we show one of the reported examples describing the easy formation of compound **80** from precursor **79** [59].

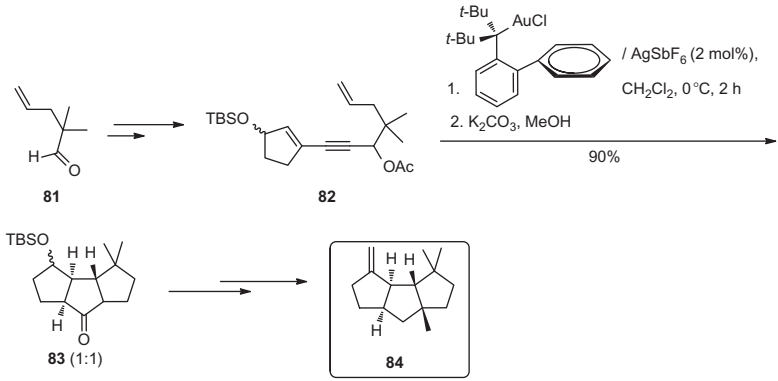
This chemistry has been used as a key step in a new total synthesis [60] of $\Delta^{8\ (12)}$-capnellene **39**, a marine sesquiterpene isolated from *Capnella imbricata* and supposed to be a biosynthetic precursor of some capnellenols showing antibacterial and antitumor activities [61]. Starting with 2,2-dimethylpent-4-enal (**81**), propargylic acetate **82** was obtained in six steps in 57% yield, as a 1/1 mixture of diastereoisomers that, after gold(I)-mediated cycloisomerization followed by basic hydrolysis, gave the expected mixture of tricyclic ketones **83** in 90% yield (Scheme 23). From this key intermediate, and following a standard synthetic sequence, $\Delta^{8\ (12)}$-capnellene **84** was finally obtained in a 15-step synthesis with an overall yield of 17%—one of the most efficient yields reported to date, as highlighted by the authors [61].



SCHEME 21 Gold(I) cycloisomerization of propargylic acetate **76**.



SCHEME 22 Tandem gold(I)-catalyzed cyclization/electrophilic cyclopropanation of propargylic acetate **79**.



SCHEME 23 Synthesis of capnellene (**84**) from 2,2,-dimethylpent-4-enal (**81**) via gold(I)-catalyzed cyclization/electrophilic cyclopropanation of propargylic acetate **82**.

CONCLUSIONS

In this chapter, we have emphasized and summarized the recently reported successful transition metal (Au, Pt, Cu)-promoted intramolecular cyclization of unsaturated propargylic carboxylic esters. This nifty approach leads to highly functionalized synthetic intermediates for the preparation of biologically important natural products. Novel transformations catalyzed by transition metal complexes (Pt, Au) have reconfigured the classical scenario of C—C and C—X bond forming reactions. The use of these innovative tools in the total synthesis of "small molecules" will render a modular and flexible approach where structural diversity can be installed at two central stages of the synthetic sequence: in the selection of a wide variety of simple starting building blocks, and second, the smart chemical edition of a common core targeting a major accuracy in the search of a bioactivity profile.

Electrophilic transition metals Pt(II) and Au(I, III) exhibit significant efficacy in catalyzing the formation of carbon–carbon and carbon–heteroatom bonds and promoting a growing variety of organic transformations of unsaturated precursors. These reactions provide an atom-efficient entry into functionalized cyclic and acyclic scaffolds useful for the synthesis of natural and nonnatural products under mild conditions, with excellent chemoselectivity and high synthetic efficiency. These transformations show a high versatility as they are strongly substrate-dependent processes and may yield a wide diversity of molecular scaffolds.

The transition metal-catalyzed intramolecular cycloisomerization of propargylic carboxylates provides functionalized bicyclo[n.1.0]enol esters in a very diastereoselective manner and, depending on the structure, with partial or complete transfer of chirality from enantiomerically pure precursors. The subsequent methanolysis gives bicyclo[n.1.0]ketones, resulting in a very

efficient two-step protocol for the syntheses of α,β-unsaturated cyclopropyl ketones, key intermediates for the preparation of natural products.

The results from mechanistic computational studies suggest that the reaction probably supervenes through cyclopropyl metallocarbenoids, formed by *endo*-cyclopropanation, that undergo a 1,2-acyl migration.

ACKNOWLEDGMENTS

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Chapter 4

Catalytic Asymmetric Strategies for the Synthesis of 3,3-Disubstituted Oxindoles

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Chapter Outline

INTRODUCTION

Chiral 3,3-disubstituted-2-oxindole substructures are present in a large number of alkaloid natural products that in many instances exhibit interesting biological properties [1]. The molecular architectures of these compounds encompass a wide range of structural and functional group diversity, as can be seen in the selected examples depicted in Fig. 1 (3-hydroxyindolin-2-one alkaloids) and Fig. 2 (spirocyclic oxindole-derived alkaloids).

In view of the remarkable biological properties of many oxindole-derived alkaloids, it is not surprising that these compounds and their derivatives

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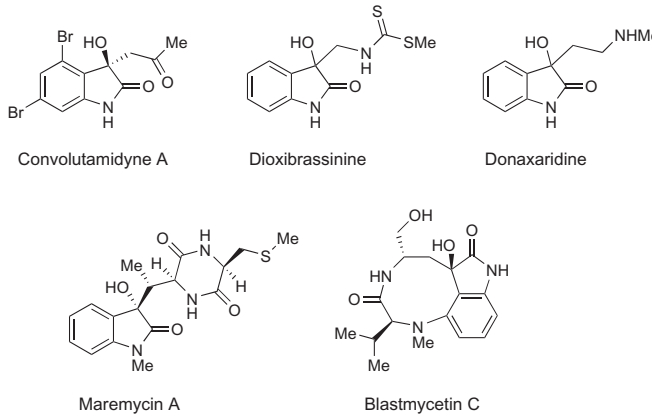


FIGURE 1 Representative 3-hydroxyindolin-2-one alkaloids.

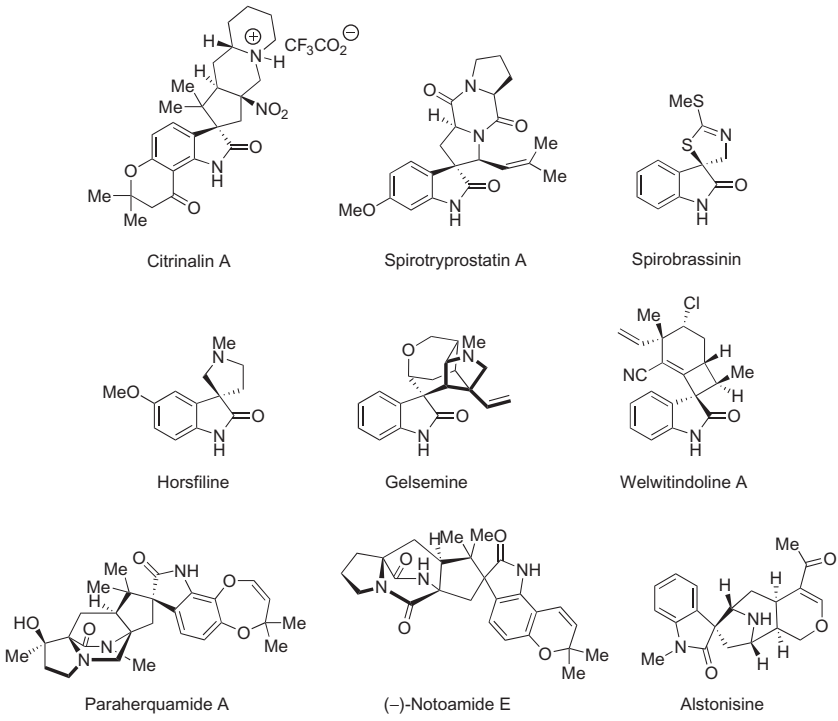


FIGURE 2 Some spirooxindole alkaloids.

constitute important targets in the development of drug candidates [2]. Some representative examples are shown in Fig. 3.

It is also worth noting that 3,3-disubstituted oxindoles serve as key intermediates in the total syntheses of complex indole-containing alkaloids [3].

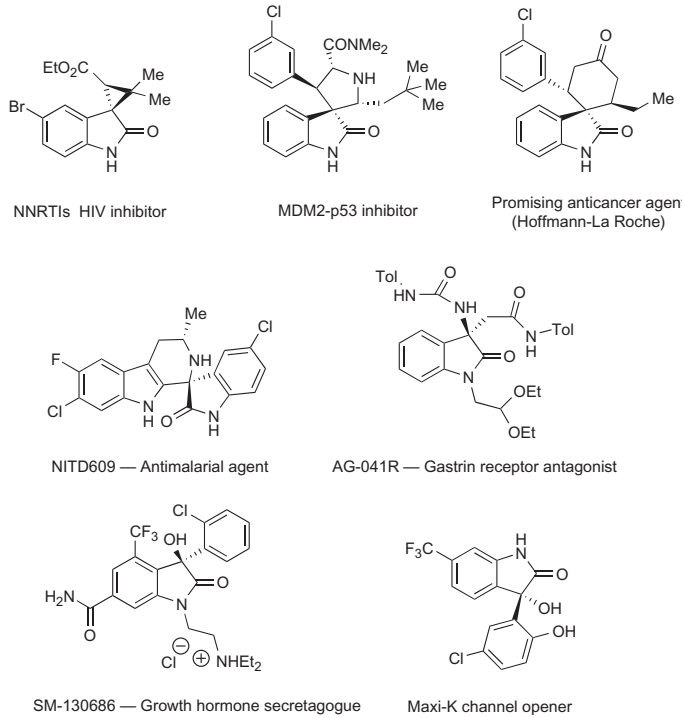


FIGURE 3 Selected bioactive nonnatural chiral oxindole derivatives.

All of these factors have contributed to draw the interest of the synthetic community toward the development of efficient and selective methods for the construction of oxindole skeletons, either in racemic [4,5] or in asymmetric [6] fashion.

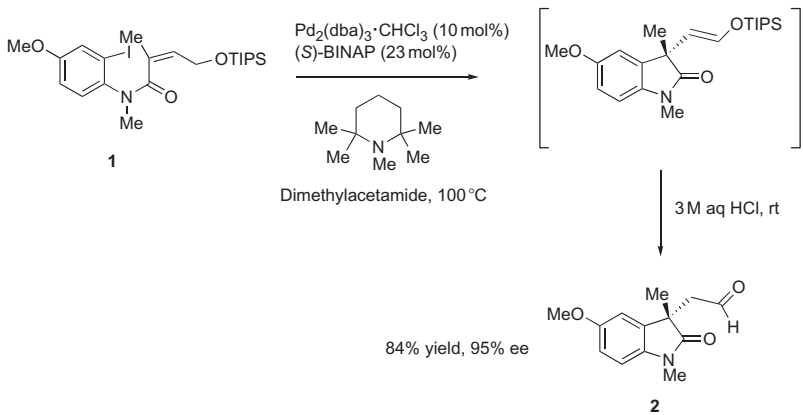
Nowadays, asymmetric catalysis (transition metal-mediated catalysis, biocatalysis, or organocatalysis) constitutes the most actively pursued strategy for the preparation of chiral compounds in highly enantiopure fashion, and accordingly the enantioselective synthesis of chiral oxindoles has been mainly achieved by asymmetric catalytic methods. The aim of this review is to highlight the most important catalytic asymmetric methods relevant to the synthesis of chiral 3,3-disubstituted oxindoles, an area of research whose growth continues unabated. The literature is covered until articles published online in July 2012, and due to both space limitations and the recent publication of a comprehensive review on the catalytic asymmetric synthesis of spirocyclic oxindoles by Franz and coworkers [6c], we will not deal presently with this subtopic. Mechanistic aspects are not explicitly discussed in this review, and the interested reader should consult the references for more information in this subject.

STRATEGIES BASED ON CYCLIZATION REACTIONS FOR THE CONSTRUCTION OF THE LACTAM RING

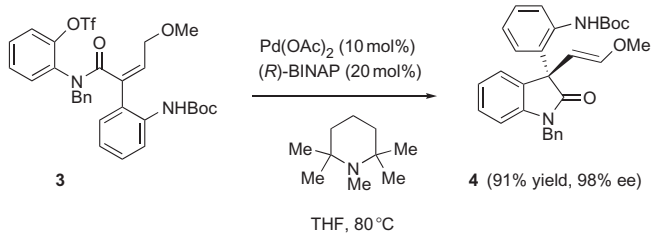
The first practical catalytic asymmetric method for the enantioselective synthesis of chiral oxindoles was developed in the past decade of the past century by Overman and coworkers in the context of the total syntheses of the alkaloids (−)-physostigmine and (−)-physovenine [7]. Overman's approach relied on the intramolecular Heck reaction of the (*Z*)-(2-methyl)-2-butenanilide **1**, which was catalyzed by a palladium(0) complex formed *in situ* from commercially available (*S*)-BINAP and $Pd_2(dba)_3$ to afford the 3,3-disubstituted oxindole **2** with excellent enantioselectivity (Scheme 1).

Subsequently, Overman's group found that this approach could also be applied to the enantioselective synthesis of 3-alkyl-3-aryloxindoles (see Scheme 2 for a representative example) [8].

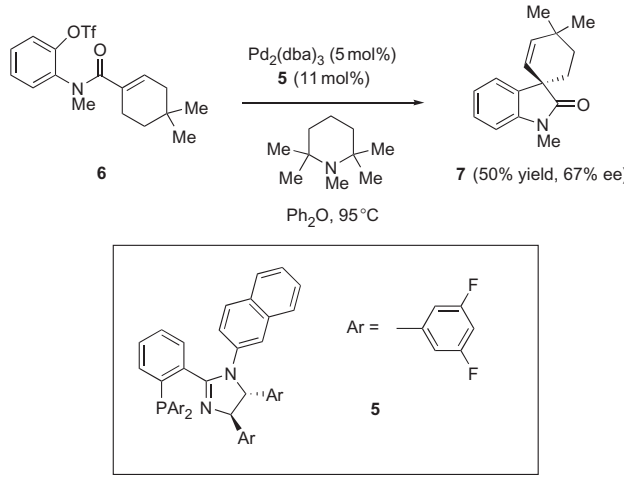
The applicability of intramolecular Heck reactions in the asymmetric synthesis of spirocyclic oxindoles was examined by Busacca *et al*. [9]. Good enantioselectivities (up to 87% ee) could be obtained by means of the phosphino-imidazoline ligand **5** (see Scheme 3 for a representative example).



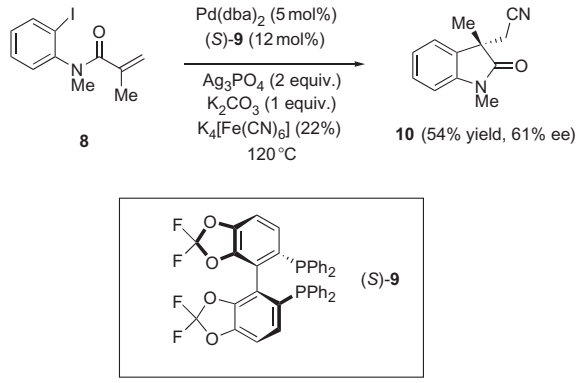
SCHEME 1 Enantioselective Heck cyclization of anilide **1**.



SCHEME 2 Enantioselective synthesis of 3-alkyl-3-aryloxindoles.



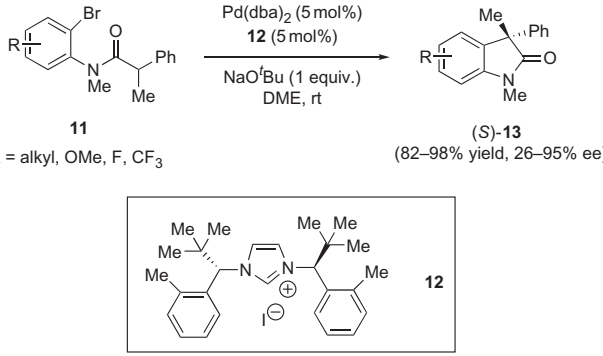
SCHEME 3 Asymmetric synthesis of spirocyclic oxindoles by Heck cyclization.



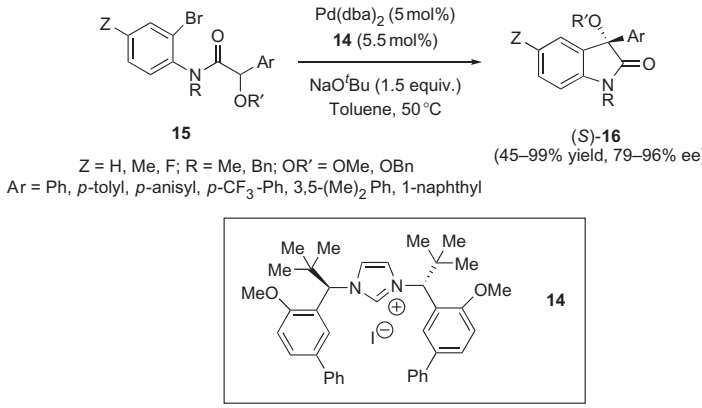
SCHEME 4 Asymmetric tandem Heck cyclization–cyanation reaction.

The tandem Heck cyclization–cyanation reaction of the anilide **8** was achieved by Zhu and coworkers by using the axially chiral DifluorPhos ligand (*S*)-**9**, albeit with moderate yield and enantioselectivity (Scheme 4) [10].

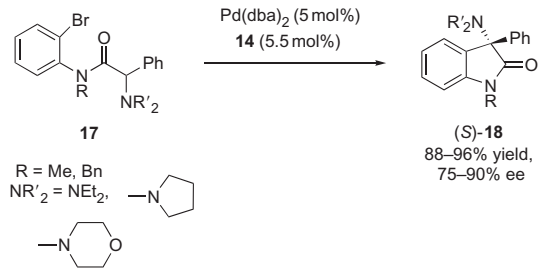
The palladium-catalyzed asymmetric synthesis of 3,3-disubstituted oxindoles by intramolecular amide α-arylation has received much attention. Following the seminal work of Hartwig and coworkers, which discovered that by using chiral NHC ligands they could perform this reaction at room temperature with moderate enantioselectivities (40–71% ee) [11], subsequent research focused on modifying the structure of the NHC ligand in order to ameliorate the stereoselectivity of the process [12]. In 2007, Kündig and coworkers were able to achieve high enantioselectivities (up to 95% ee) in the cyclization of *o*-bromo(α-aryl)propanilides **11** by means of the sterically hindered NHC derived from the chiral imidazolium iodide **12** (Scheme 5) [13].



SCHEME 5 Asymmetric intramolecular arylation of anilides **11**.



SCHEME 6 Cyclization approach to chiral 3-alkoxy-3-aryloxindoles.



SCHEME 7 Cyclization approach to chiral 3-amino-3-phenyloxindoles.

The related chiral NHC ligand derived from **14** was successfully applied to the asymmetric synthesis of chiral 3-alkoxy-3-aryloxindoles **16** (Scheme 6) and of chiral 3-aminooxindoles **18** (Scheme 7) [14].

More recently, Murakami *et al.* [15] have developed chiral NHC ligands having a 2,2′-bisquinoline-based C_2-symmetric skeleton such as **19** that can

also be applied to the palladium-catalyzed α-arylation of *o*-bromo(α-aryl)propanilides **11** with good yields and enantioselectivities (Fig. 4).

In 2010, Dorta and coworkers [16] reported a new NHC–Pd-catalyzed asymmetric α-arylation of anilides that gave access to 3-allyl-3-aryloxindoles **22** in high yields and good enantioselectivities (up to 94% ee; Scheme 8).

More recently, Dorta's research group has achieved the asymmetric synthesis of 3-fluoro-3-aryloxindoles **24** by the direct enantioselective α-arylation of fluorinated amides **23** [17]. The optimal catalyst was also a chiral NHC–palladium complex (**25**). As it can be seen in Scheme 9, the yields are from moderate to good but the enantioselectivity of the process is excellent (from 82% to >99% ee).

The catalytic enantioselective intramolecular reaction of α-ketoanilides **26**, leading to 3-hydroxy-2-oxindoles with an arylic tetrasubstituted carbon center (**27**) with impressive yields and enantioselectivities, has been achieved by Yin *et al.* by palladium catalysis in the presence of the DifluorPhos ligand (*R*)-**9** (Scheme 10) [18].

It is worth noting that in this process the triethylamine is utilized both as a stoichiometric reductant and as a base, thus avoiding the use of stoichiometric

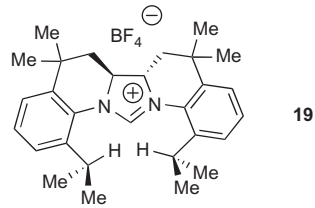
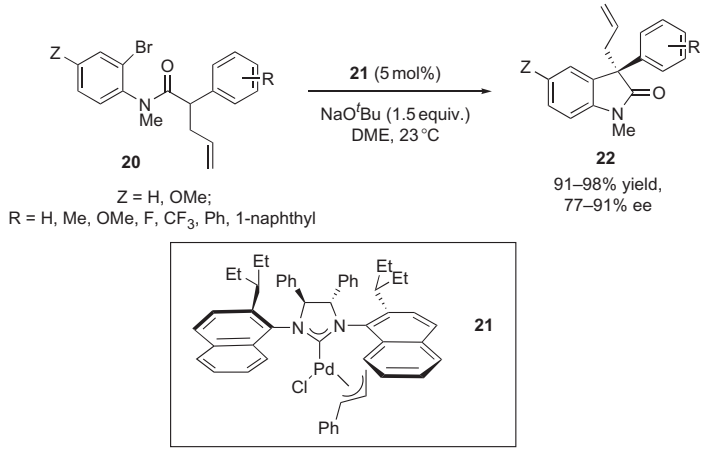
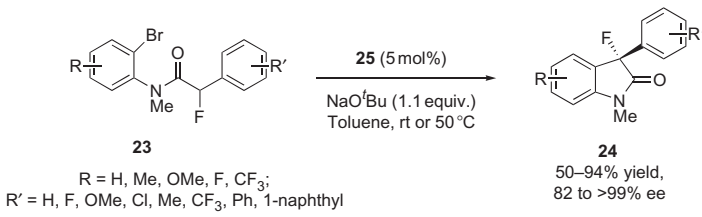


FIGURE 4 Chiral imidazolidinium tetrafluoroborate salt developed by Murakami.



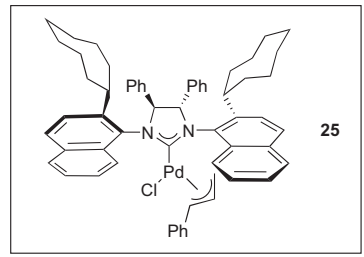
SCHEME 8 Asymmetric 3-allyl-3-aryloxindole synthesis by palladium-catalyzed α-arylation.



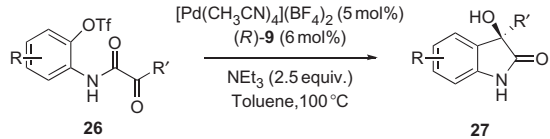
23

R = H, Me, OMe, F, CF_3;
R′ = H, F, OMe, Cl, Me, CF_3, Ph, 1-naphthyl

24
50–94% yield,
82 to >99% ee



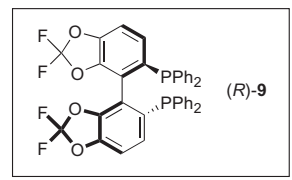
SCHEME 9 Asymmetric synthesis of 3-fluoro-aryloxindoles by enantioselective α-arylation.



26

R = H, Me, OMe, Cl
R′ = aryl, 2-thienyl, 2-indolyl, alkyl, vinyl

27
55–93% yield,
82–99% ee

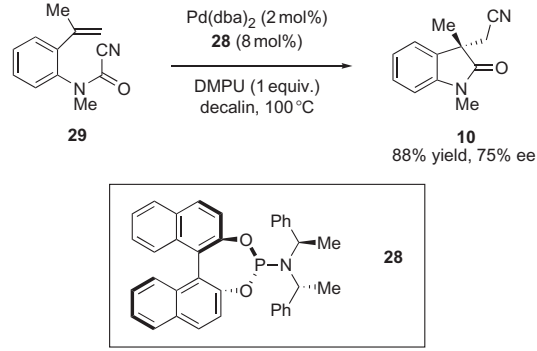


SCHEME 10 Catalytic enantioselective intramolecular arylation of α-ketoamides.

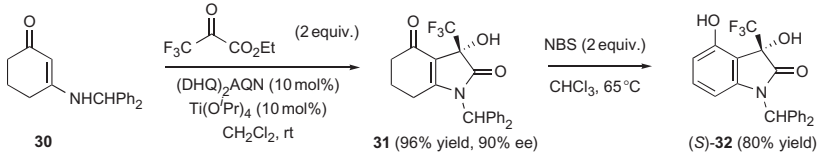
amounts of palladium. A somewhat less practical copper-catalyzed version of this process had been previously developed by Kanai's and Shibasaki's groups [19].

In 2008, Takemoto and coworkers disclosed an enantioselective palladium-catalyzed intramolecular cyanoamidation reaction that constituted a new entry to the 3-alkyl-3-cyanomethyl-2-oxindole subunit (see Scheme 11 for an example) [20]. The combination of $Pd(dba)_2$, the optically active phosphoramidite **28**, and *N*,*N*-dimethylpropylene urea as a polar additive provided the best conditions. It is worth noting that this approach is complementary to the tandem Heck cyanation reaction described by Zhu [10] (see Scheme 4). For a range of methylcyanoformanides derived from (*o*-alkenyl)anilines, the yields ranged from moderate to excellent (43–100% yield) and enantioselectivities were good (68–86% ee).

It should be noted, however, that two reports by Reddy and Douglas, published in 2010 and describing the application of this strategy to the total syntheses of the



SCHEME 11 Enantioselective intramolecular cyanoamidation.



$(DHQ)_2AQN$ = hydroquinine anthraquinone-1,4-diyl ether, NBS = *N*-bromosuccinimide

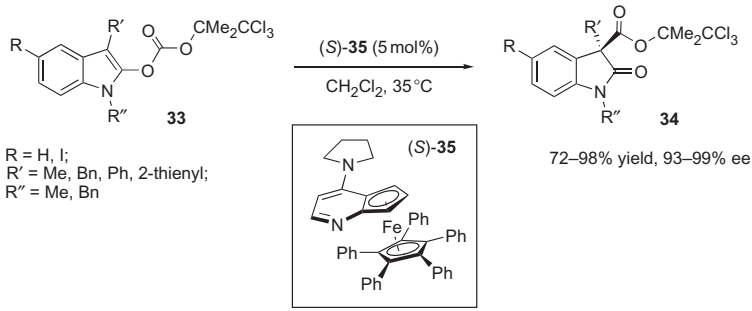
SCHEME 12 Asymmetric synthesis of the 3-hydroxy-3-trifluoromethyloxindole (*S*)-**32**.

indole alkaloids (+)-horsfiline, (−)-coerulescine, and (−)-esermethole [21], were subsequently withdrawn at the request of the corresponding author (C. J. D.) [22].

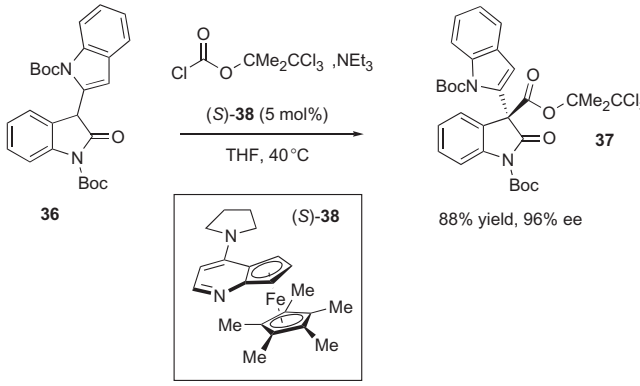
Finally, the asymmetric construction of bicyclic 3-trifluoromethyl-3-hydroxy-γ-lactam derivatives, that in some instances can be transformed into medicinally attractive 3-hydroxy-3-trifluoromethyloxindoles, was achieved with moderate to good enantioselectivities by Shibata *et al*., by means of an enantioselective enamine–trifluoropyruvate condensation–cyclization reaction catalyzed by a dihydroquinine-derived Sharpless ligand/Ti(IV) complex (Scheme 12) [23]. An interesting feature of this approach is that by employing suitable pseudoenantiomeric *Cinchona* alkaloids as catalyst precursors, both enantiomers of the trifluoromethylated heterocycles can be obtained selectively. Thus, the use of the dihydroquinidine anthraquinone 1,4-diyl ether $(DHQD)_2AQN$ as a chiral ligand in the reaction of **30** with trifluoroacetyl pyruvate afforded *ent*-**31** (*R* absolute configuration) in 98% yield and 89% ee.

ASYMMETRIC REARRANGEMENTS OF *O*-CARBONYLATED OXINDOLES AND RELATED PROCESSES

The first example of a catalytic asymmetric Steglich-type rearrangement [24] of *O*-carbonylated oxindoles **33** to 3-alkoxycarbonyl-3-substituted oxindoles **34**, catalyzed by the planar chiral nucleophilic catalyst **35**, was reported by Hills and Fu (Scheme 13) [25].



SCHEME 13 Steglich-type rearrangement of *O*-carbonylated oxindoles.

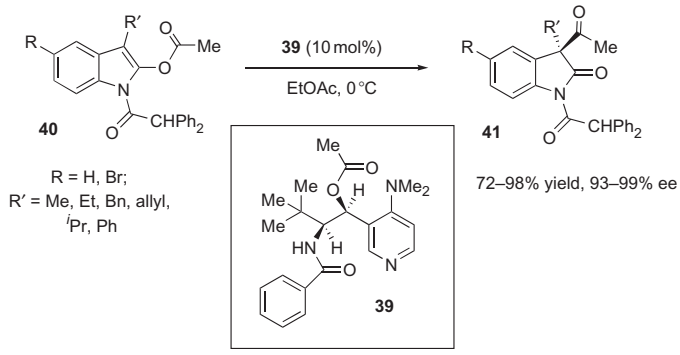


SCHEME 14 Asymmetric rearrangement step in the total synthesis of (+)-gliocladine C.

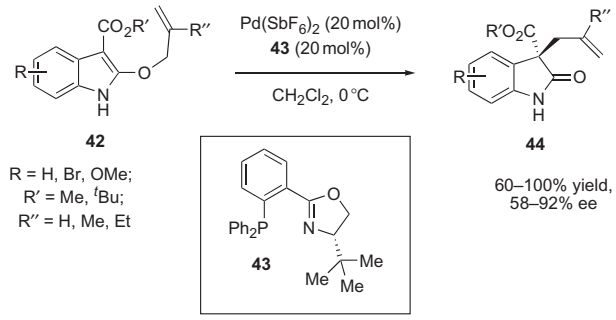
The usefulness of this approach can be exemplified by a key step in Overman's total synthesis of (+)-gliocladine C (Scheme 14) [26]. In this case, the *O*-carbonylation of the racemic oxindole precursor **36** and the subsequent rearrangement to **37** (by means of Fu's catalyst (*S*)-**38**) [24,25] could be performed in a two-step, one-pot sequence with excellent yield and enantioselectivity.

On the other hand, the Steglich-like rearrangement of *O*-acylated 3-substituted oxindoles appears to be more challenging [24]. Up to now, the best results have been obtained by Vedejs and coworkers by means of the central chiral nucleophilic catalyst **39** (Scheme 15) [27].

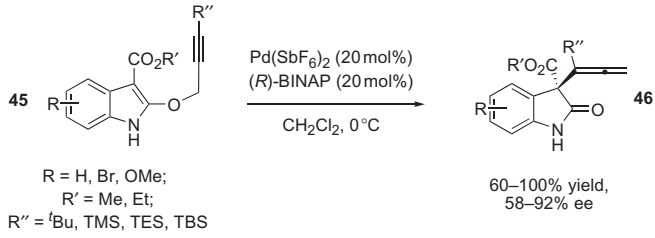
The Meerwein–Eschenmoser Claisen rearrangement of 3-alkoxycarbonyl-*O*-allyloxindoles **42** was shown by Linton and Kozlowski [28] to be efficiently and stereoselectively catalyzed by a Pd(II) complex derived from the phosphinooxazoline ligand **43** (Scheme 16). More recently, Kozlowski and coworkers [29] have disclosed that a similar catalyst derived from commercially available BINAP is able to promote the enantioselective Saucy–Marbet Claisen rearrangement of *O*-propargylated oxindoles **45** (Scheme 17).



SCHEME 15 Organocatalytic asymmetric rearrangement of indolyl acetates.



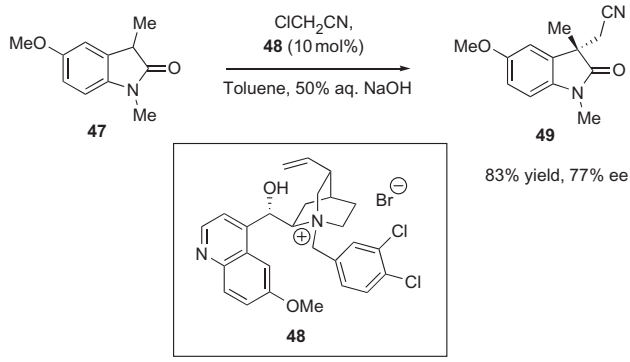
SCHEME 16 Enantioselective, Pd(II)-catalyzed Meerwein–Eschenmoser Claisen rearrangement.



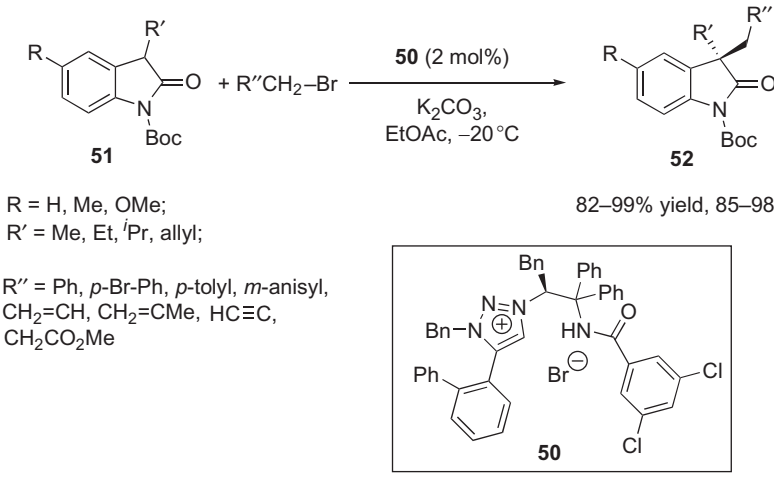
SCHEME 17 Enantioselective, Pd(II)-catalyzed Saucy–Marbet Claisen rearrangement.

3-SUBSTITUTED OXINDOLES AS NUCLEOPHILES: ALKYLATION, CONJUGATE ADDITION, ALDOL, AND MANNICH REACTIONS

The catalytic asymmetric alkylation of a 3-substituted oxindole was achieved by Lee and Wong by means of phase-transfer catalysis (PTC), in the context of a formal total synthesis of (−)-esethermole [30]. The alkylation of **47** with chloroacetonitrile was performed under PTC conditions with the aid of the



SCHEME 18 First asymmetric PTC-mediated alkylation of a 3-substituted oxindole.

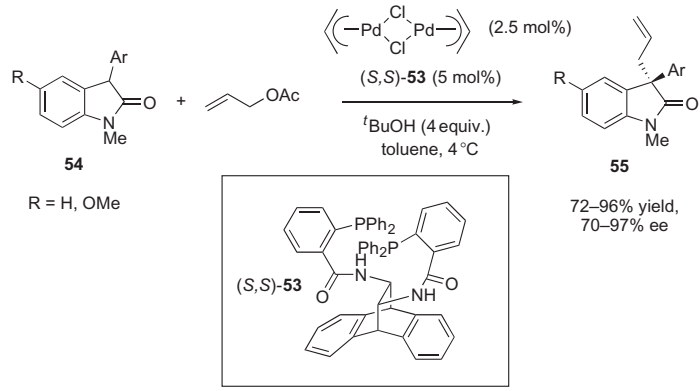


SCHEME 19 Chiral triazolium salt-catalyzed asymmetric alkylation of oxindoles.

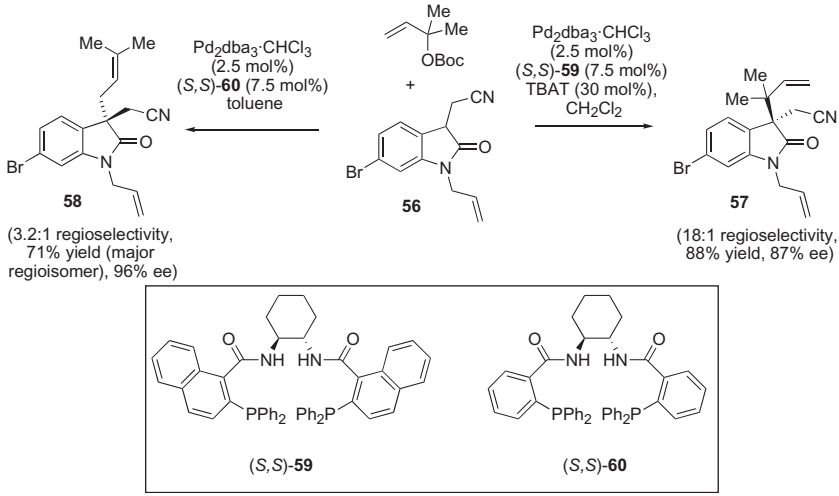
quinidine-derived quaternary ammonium salt catalyst **48** and furnished the 3,3-disubstituted oxindole **49** in 77% ee (Scheme 18).

Remarkably, only another example of the application of PTC catalysis to the asymmetric alkylation of oxindoles by alkyl halides has been reported up to now. In 2010, Ooi and coworkers synthesized and tested the chiral triazolium salt **50**. Under PTC conditions, this compound catalyzed the asymmetric alkylation of a series of 3-substituted *N*-Boc oxindoles **51** with reactive alkyl bromides with excellent yields and enantioselectivities (Scheme 19) [31].

The palladium- and molybdenum-catalyzed asymmetric alkylation of oxindoles, on the other hand, has been extensively investigated, mainly by Trost and coworkers. In 2005, Trost and Fredriksen demonstrated that the complex derived from dimeric allylpalladium chloride and the chiral diphosphine (*S*,*S*)-**53** could affect the enantioselective allylation of *N*-methyl-3-aryloxindoles **54** with good yields and enantioselectivities (Scheme 20) [32,33].



SCHEME 20 Palladium-catalyzed asymmetric allylation of 3-aryloxindoles.

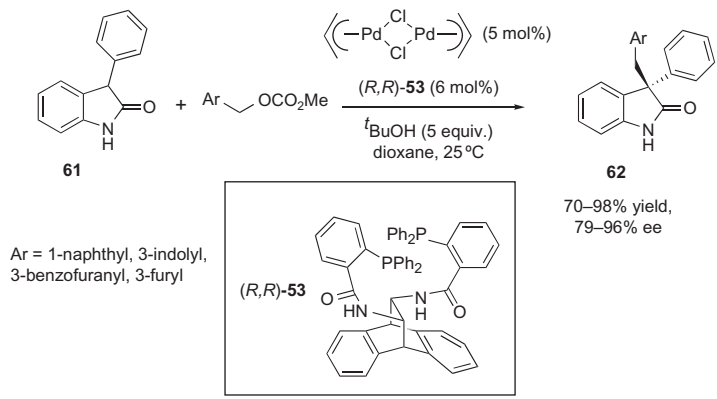


SCHEME 21 Regioselective Pd-catalyzed asymmetric reverse prenylation and prenylation (TBAT, tetrabutylammonium difluorotriphenylsilicate).

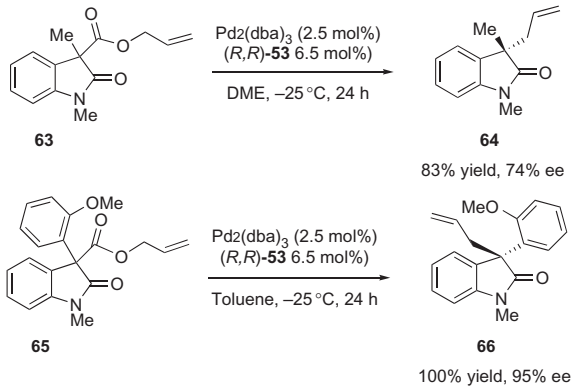
More recently, Trost *et al*. have found that both the regio- and the stereoselectivity of palladium-catalyzed prenylation and geranylation of 3-substituted oxindoles can be controlled by structural modification of the chiral diphosphine ligand [34]. Thus, compound **56** can afford preferentially the reverse prenylated product **57** or the prenylated product **58** (with opposite sense of asymmetric induction) depending on the ligand being used in the reaction (Scheme 21).

Ligand **53** is also competent in the palladium-catalyzed asymmetric benzylation of 3-aryloxindoles, as evinced by Trost and Czabaniuk (Scheme 22) [35].

In 2011, Taylor and coworkers developed an asymmetric decarboxylative palladium-catalyzed allylation of 3-alkyl- and 3-aryloxindoles, again using the diphosphine (*R*,*R*)-**53** as a chiral ligand [36]. An intriguing substrate-dependant



SCHEME 22 Asymmetric Pd-catalyzed benzylation of 3-phenyloxindole.

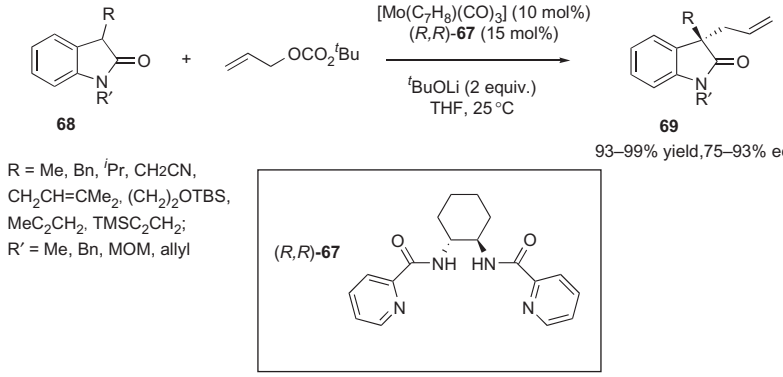


SCHEME 23 Asymmetric decarboxylative allylation of oxindoles.

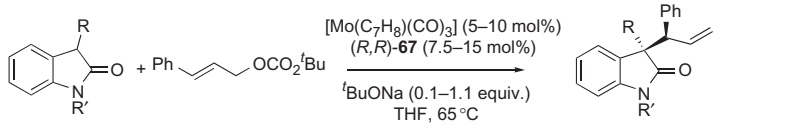
reversal in stereoselectivity was observed by these authors, whereby the size of the 3-substituent determines the facial enantioselectivity in the allylation step (Scheme 23).

While as we have seen 3-aryloxindoles can be efficiently allylated to form quaternary stereocenters at the three-position with excellent levels of stereocontrol by means of palladium catalysis, the same reaction with 3-alkyloxindoles takes place with low enantioselectivities. However, Trost and Zhang found that the molybdenum-catalyzed asymmetric allylation, which is known to proceed via an inner sphere mechanism of coordination of nucleophile and reductive elimination, can effect this reaction with high enantioselectivities [37]. The catalyst formed *in situ* from a suitable molybdenum source and the chiral ligand **67** provided the best results, both in terms of yields and enantioselectivity (Scheme 24).

The same molybdenum catalyst was subsequently found to enable the construction of vicinal quaternary and tertiary stereocenters by allylation of 3-aryl or 3-alkyloxindoles with *trans*-cinnamyl carbonates (Scheme 25) [38].



SCHEME 24 Asymmetric allylation of 3-alkyloxindoles.



70 (R = aryl, R′ = Me)
71 (R = alkyl, R′ = CO_2Me)

72 (R = aryl, R′ = Me):
up to >19:1 branched/linear, up to 19:1 dr,
63–95% yield, 89–97% ee

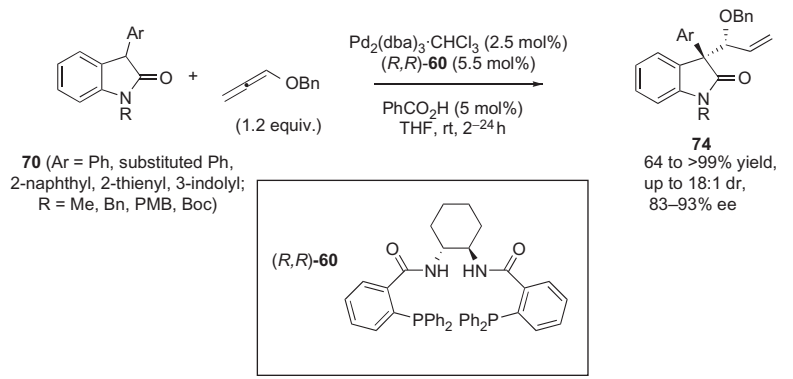
73 (R = alkyl, R′ = CO_2Me):.
up to >19:1 branched/linear, up to >19:1 dr,
83–90% yield, 96–99% ee

SCHEME 25 Regio-, diastereo-, and enantioselective allylation of 3-substituted oxindoles.

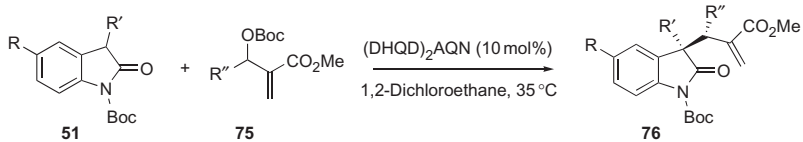
More recently, Trost and coworkers have found that allenes can be used to generate the reactive (π-allyl)palladium intermediate in the presence of an acid cocatalyst and that this system is compatible with the use of *N*-protected 3-aryloxindoles **70** as nucleophiles [39]. In the presence of the chiral ligand **60**, this hydrocarbonation reaction also provides 3,3-disubstituted oxindoles with two vicinal stereocenters, one of them being quaternary, in excellent yields and regio- and stereoselectivities (Scheme 26).

The asymmetric organocatalytic allylation of *N*-Boc-3-substituted oxindoles **51** by Morita–Baylis–Hillman (MBH) carbonates **75** was reported by Chen and coworkers [40]. This reaction, catalyzed by commercially available Sharpless $(DHQD)_2AQN$ ligand, affords multifunctional oxindoles **76** with adjacent quaternary and tertiary stereocenters with very good diastereo- and enantioselectivities (Scheme 27).

The catalytic asymmetric nucleophilic addition of 3-substituted oxindoles to Michael acceptors has become a very popular method for the construction of a quaternary stereocenter at the C3 position of the oxindole skeleton. Using cinnamaldehyde derivatives as Michael acceptors, this approach was pioneered by Melchiorre and coworkers [41]. The authors hypothesized that a key factor to improve the stereocontrol of the process was to use a bifunctional amine–thiourea



SCHEME 26 Palladium-catalyzed asymmetric addition of oxindoles and allenes.



R = H, Me, F; R′ = Ph, *p*-tolyl, *p*-F-Ph, Bn.

R″ = Ph, substituted Ph, 2-thienyl.

50–98% yield, 63:37–92:8 dr, 86–97% ee

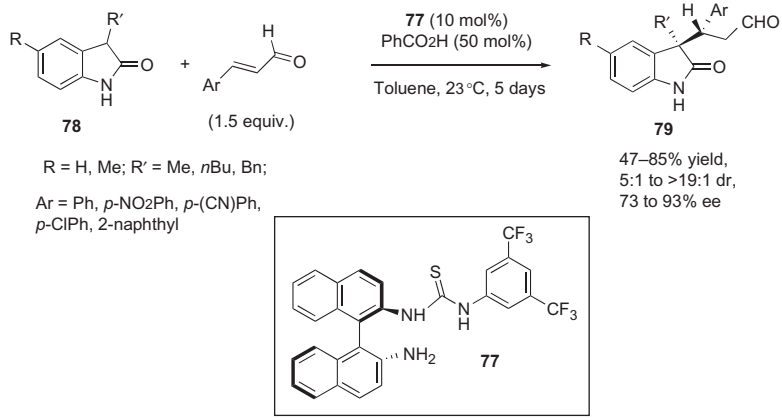
SCHEME 27 Asymmetric allylic alkylation of *N*-Boc-3-substituted oxindoles with MBH carbonates.

catalysts, able to activate both the aldehyde (via iminium ion formation) and the oxindole nucleophile (by hydrogen bonding with the enol oxygen). This concept proved to be successful, and with the aid of benzoic acid as an additive, the BINAM-thiourea catalyst **77** afforded the Michael adducts of the free 3-alkyloxindoles **78** with good yields and diastereoselectivities and with good to excellent enantioselectivity (Scheme 28).

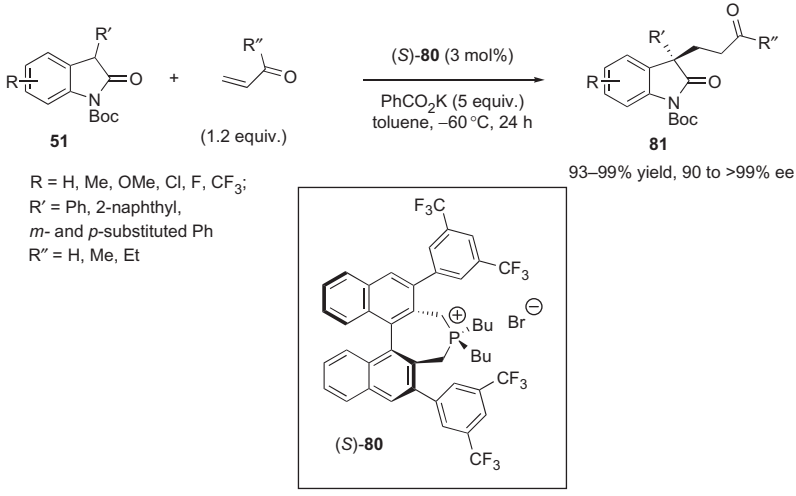
Shortly afterward, Maruoka and coworkers reported that the phosphonium salt **80**, under phase-transfer conditions, catalyzed the Michael addition of *N*-Boc-3-aryloxindoles **51** to acrolein, methyl vinyl ketone, and ethyl vinyl ketone with outstanding enantioselectivities (Scheme 29) [42].

At the end of 2009, Cheng *et al.* disclosed that a bifunctional tertiary amine–thiourea catalyst (**82**) can also be used to promote the Michael addition of *N*-Boc-3-aryloxindoles **51** to vinyl ketones (and also to phenyl vinyl sulfone, 82–90% ee), with moderate to good enantioselectivities (Scheme 30) [43,44].

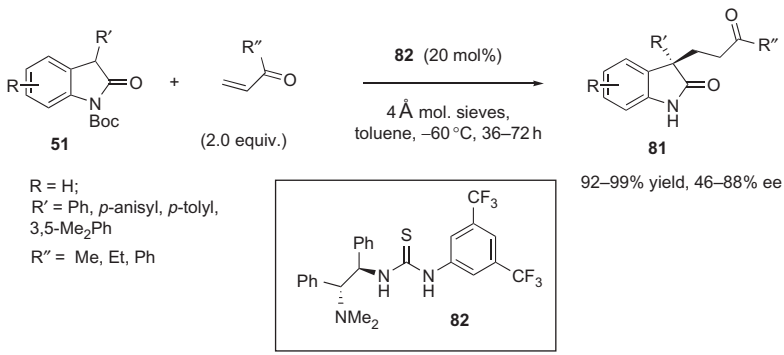
In 2010, Melchiorre and coworkers found that 9-amino(9-deoxy)-*epi*-dihydroquinine **83** could catalyze (via iminium catalysis activation and with benzoic acid as a cocatalyst) the Michael addition of *N*-Boc-3-aryl- and 3-alkyloxindoles to cyclic enones, with good yields, diastereo- and enantioselectivities [45]. An advantage of this approach is that when using the



SCHEME 28 Asymmetric organocatalytic Michael addition of unprotected 3-alkyloxindoles to cinnamaldehydes.



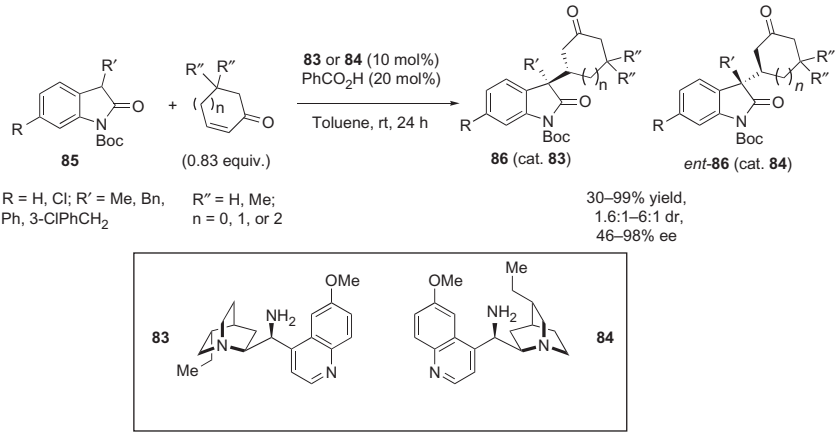
SCHEME 29 PTC of asymmetric Michael addition of oxindoles to unsubstituted unsaturated carbonyl compounds.



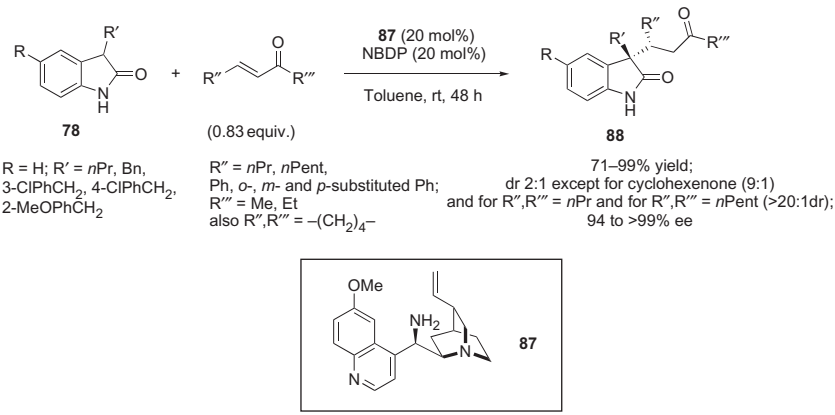
SCHEME 30 Tertiary amine–thiourea catalysis of the Michael addition of *N*-Boc-3-aryloxindoles to vinyl ketones.

quasienantiomeric dihydroquinidine-derived catalyst **84**, the opposite enantiomers became the major products (Scheme 31).

The application of primary amines in the iminium activation catalysis of the Michael addition of unprotected 3-alkyloxindoles **78** to enones was studied by Wang and coworkers [46]. They found that quinidine catalyst **87** (in the presence of equimolar amounts of *N*-Boc-D-phenylglycine, NBDP) afforded the desired products **88** in good yields and excellent enantioselectivities, although the diastereoselectivity of the process was generally very low (Scheme 32; 2:1 dr for the vast majority of examples). On the other hand, secondary amine catalysis for this kind of process is less efficient [47].



SCHEME 31 Primary amine catalysis of the Michael addition of *N*-Boc-3-substituted oxindoles to cyclic enones.

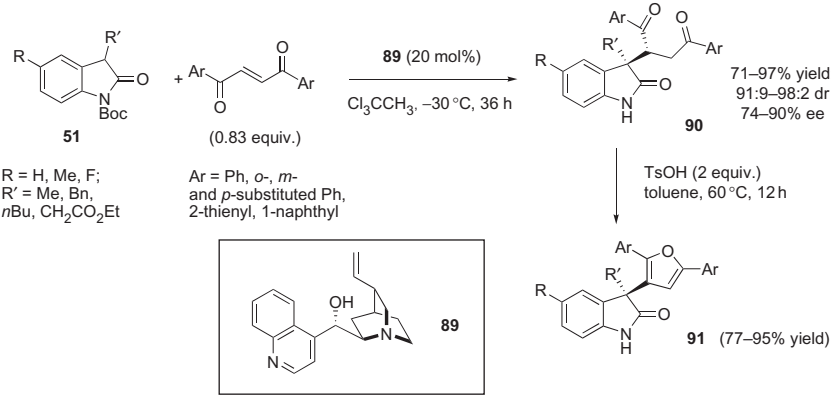


SCHEME 32 Primary amine catalysis of the Michael addition of unprotected 3-alkyloxindoles to acyclic enones.

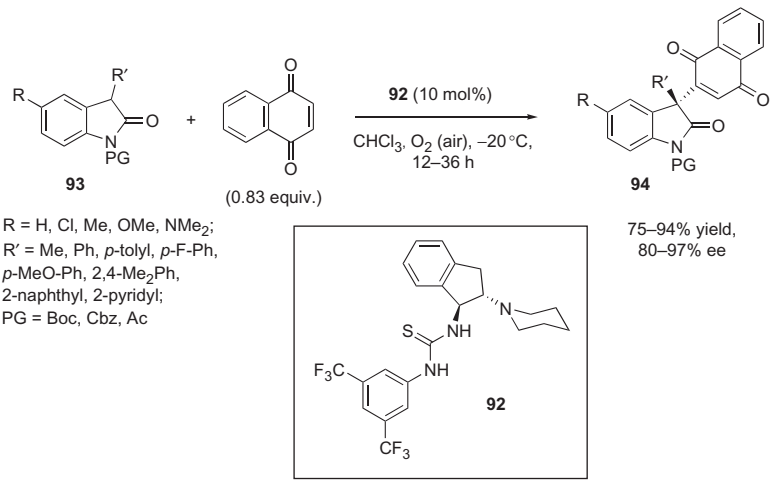
Recently, Yuan and coworkers have reported on the organocatalytic asymmetric Michael addition of *N*-Boc-3-alkyloxindoles **51** to symmetric (*E*)-1,4-diaryl-2-buten-1,4-diones. In this case, readily available cinchonine **89**, probably acting as a bifunctional chiral base, was able to catalyze the reaction with good yields and diastereoselectivities, and with moderate to good (up to 90% ee) enantioselectivities (Scheme 33) [48]. The resulting products **90** are immediate precursors of 3-furanyl-3-alkyloxindole derivatives **91**.

Also very recently, Wang and coworkers described a novel method for the organocatalytic, enantioselective, aerobic oxidative Michael addition of 3-substituted oxindoles to naphthoquinones that takes place under mild conditions and in the presence of an indane tertiary amine–thiourea bifunctional catalyst **92** [49]. The rearomatized products **94** are isolated in good yields and enantioselectivities (Scheme 34) [50].

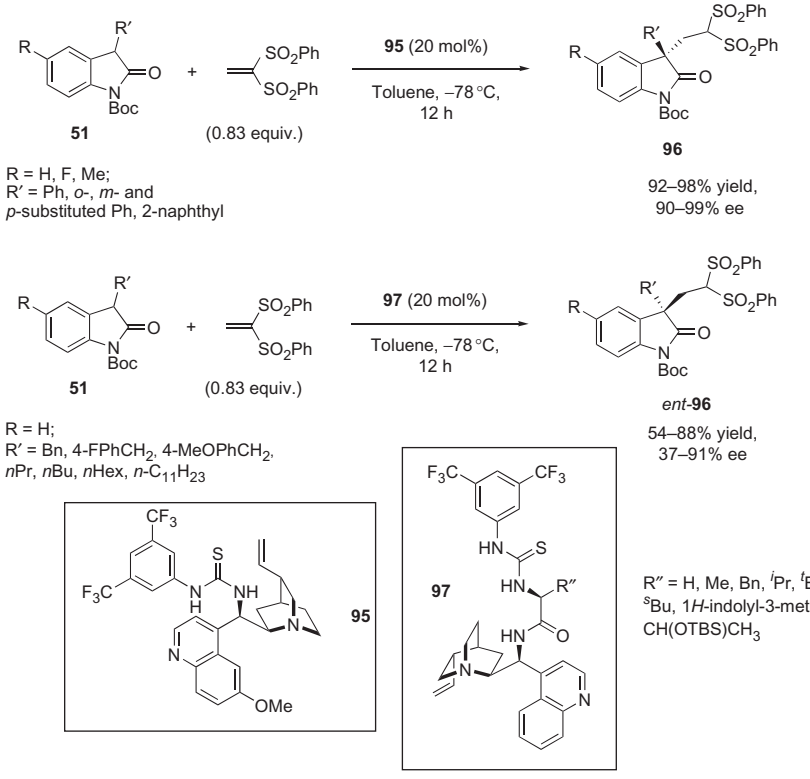
Shortly afterward, Cheng's seminal report of organocatalytic asymmetric Michael additions of oxindoles to vinyl sulfone [43], Zhu and Lu found that the quinidine-derived tertiary amine–thiourea **95** was a suitable catalyst for the enantioselective Michael addition of *N*-Boc-3-aryloxindoles to 1,1-bis(phenylsulfonyl)ethylene. On the other hand, this catalyst afforded very low enantioselectivities with *N*-Boc-3-alkyloxindoles; for these substrates, the novel trifunctional catalysts **97**, containing cinchonidine, primary amino acid, and thiourea units, were devised and tested successfully, although a different catalyst had, in general, to be used for each oxindole (Scheme 35) [51]. In 2011, Kim and coworkers disclosed a similar approach, applicable to both *N*-Boc-3-aryloxindoles and *N*-Boc-3-alkyloxindoles as starting products that relied on the use of bifunctional tertiary amine–thiourea or tertiary amine–squaramide catalysts exhibiting both central and axial chirality (Scheme 36) [52]. The high synthetic versatility of the gem(disulfone) moiety [53] is an added value of these processes.



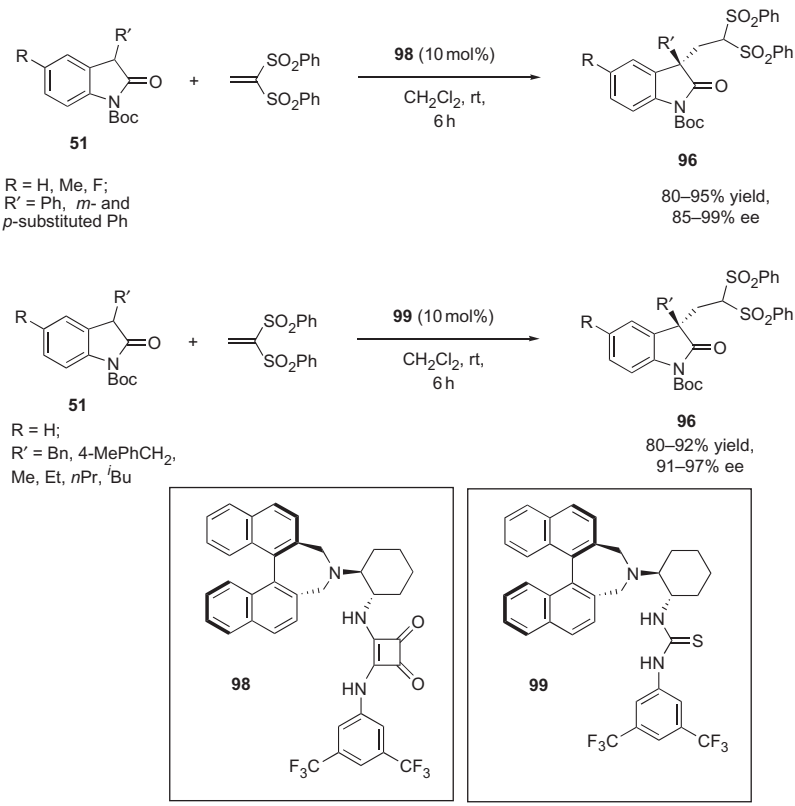
SCHEME 33 Chiral base-catalyzed asymmetric Michael addition of *N*-Boc-3-alkyloxindoles to (*E*)-1,4-diaryl-2-buten-1,4-diones.



SCHEME 34 Organocatalytic asymmetric 3-functionalization of oxindoles through oxidative Michael additions to naphthoquinones.



SCHEME 35 Asymmetric organocatalytic Michael addition of *N*-Boc-3-substituted oxindoles to 1,1-bis(phenylsulfonyl)ethylene (Lu's approach).

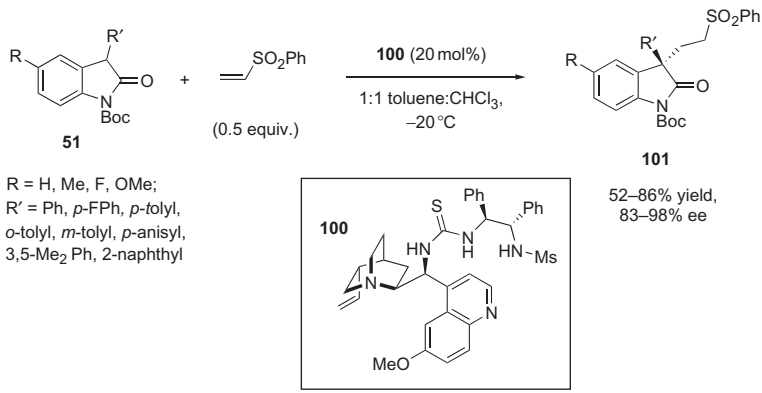


SCHEME 36 Asymmetric organocatalytic Michael addition of *N*-Boc-3-substituted oxindoles to 1,1-bis(phenylsulfonyl)ethylene (Kim's approach).

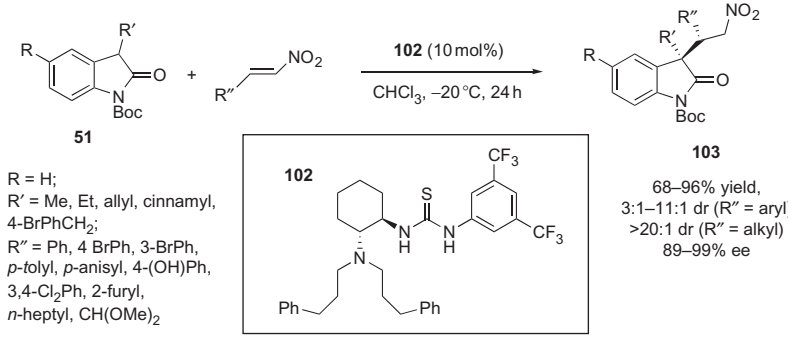
On the other hand, the Michael addition of *N*-Boc-3-aryloxindoles to phenyl vinyl sulfone was investigated by Zhao *et al*. [54]. Again, a bifunctional tertiary amine–thiourea (**100**, derived from quinine and (*S*,*S*)-1,2-diphenylethanediamine) was found to be optimal catalyst for this transformation (Scheme 37).

Much effort has been devoted to the catalytic asymmetric Michael addition of 3-substituted oxindoles to nitroalkenes. Barbas III and coworkers were the first to demonstrate that tertiary amine–thiourea bifunctional catalysts such as **102** gave good to excellent yields, diastereo- and enantioselectivities in the addition of *N*-Boc-3-alkyloxindoles to α-nitroalkenes (Scheme 38) [55]. The diastereoselectivity of the process is dependent on the nature of the β-substituent of the oxindole. Thus, arylnitroalkenes react with variable diastereoselectivities (from 3:1 to 11:1), while alkylnitroalkenes afford essentially only one diastereomer (dr > 20:1).

Subsequently, several bifunctional tertiary amine–thiourea or tertiary amine–squaramide bifunctional catalysts have been found to promote the



SCHEME 37 Organocatalytic enantioselective Michael addition of *N*-Boc-3-aryloxindoles to phenyl vinyl sulfone.

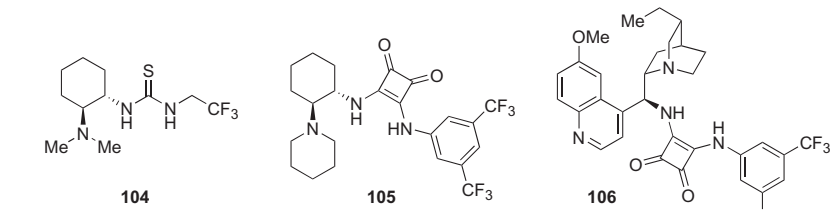


SCHEME 38 Tertiary amine–thiourea catalysis of the Michael addition of *N*-Boc-3-alkyloxindoles to nitroalkenes.

Michael addition of 3-substituted oxindoles to nitroalkenes, but in general, the results are not better than those of Barbas III (Fig. 5) [56–59].

Transition metal-based chiral catalysts can also be used for this reaction. In 2009, and almost simultaneously with Barbas III organocatalytic approach [55], Matsunaga, Shibasaki and coworkers reported on the use of homodinuclear $[Mn(III)]_2$–Schiff base complexes (**107**) for catalytic asymmetric Michael additions of *N*-Boc-3-alkyloxindoles to β-aryl- or β-vinyl-substituted nitroalkenes (Scheme 39) [60]. The results were excellent both in terms of yields and estereoselectivities. Very recently, this approach has been put to use by the same research groups in the catalytic asymmetric total syntheses of dimeric hexahydropyrroloindoline-derived alkaloids through a double Michael reaction of bis-3,3′-(*N*-Boc-oxindole) with nitroethylene [61].

In 2011, Yuan and coworkers disclosed the use of the chiral $Ni(OAc)_2$ diamine complex **108** for the addition of *N*-protected 3-alkyloxindoles to β-aryl nitroalkenes (Scheme 40) [62].



104

Luo and Cheng, 2010 (Ref. [56])
65–99% yield
1:1 to 19:1 dr
53–98% ee

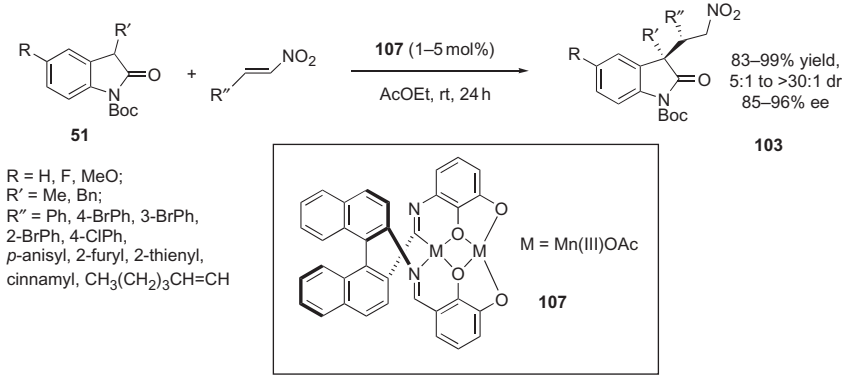
105

Du, 2010 (Ref. [57])
33–97% yield
74:26 to 98:2 dr
44–88% ee

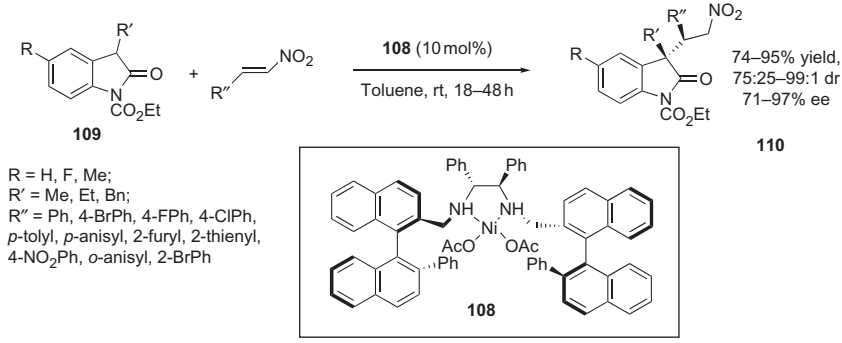
106

Kanger, 2012 (Ref. [58])
90–99% yield
5:1 to 11:1 dr
76–92% ee
(addition of unprotected 3-chlorooxindoles to β-nitrostyrenes)

FIGURE 5 Tertiary amine–hydrogen bond donor bifunctional catalysts for the asymmetric Michael addition of 3-substituted oxindoles to nitroalkenes.



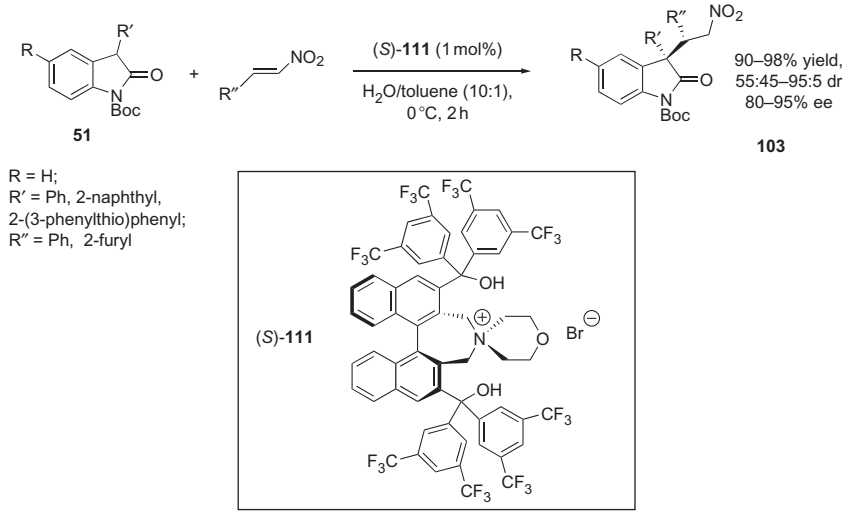
SCHEME 39 Catalytic asymmetric Michael addition of *N*-Boc-3-alkyloxindoles to nitroalkenes using a $Mn_2(OAc)_2$–Schiff base complex.



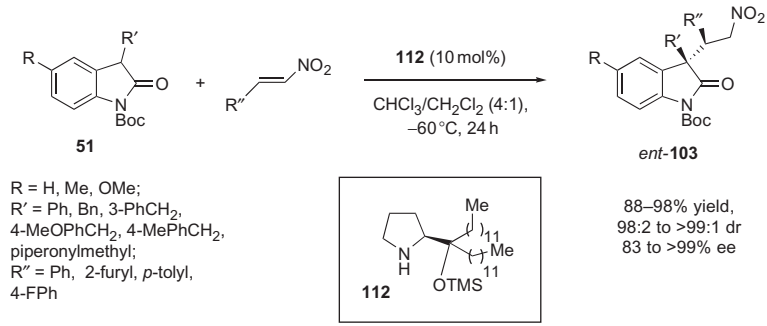
SCHEME 40 Catalytic asymmetric Michael addition of *N*-ethoxycarbonyl 3-alkyloxindoles to nitroalkenes using a $Ni(OAc)_2$–diamine complex.

PTC can also be applied to these enantioselective Michael additions, as demonstrated by Maruoka and coworkers [63]. In the presence of the bifunctional chiral ammonium salt **111**, *N*-Boc-3-aryloxindoles were added to a variety of nitroalkenes with good diastereo- and enantioselectivities (Scheme 41). A single example with *N*-Boc-3-methyloxindole and β-nitrostyrene and reactants gave low yield (34% yield) and poor stereocontrol (20% ee for the major diastereomer in a 70:30 diastereomer mixture).

Very recently, Enders and coworkers [64] have found that a chiral secondary amine, the pyrrolidine **112** developed by Palomo [65], is also able to catalyze the Michael addition of *N*-Boc-3-substituted oxindoles **51** to β-arylnitroalkenes, with remarkable yields, diastereo- and enantioselectivities (Scheme 42). The proposed mechanism for this intriguing process involves



SCHEME 41 PT-catalyzed asymmetric Michael addition of oxindoles to nitroalkenes.



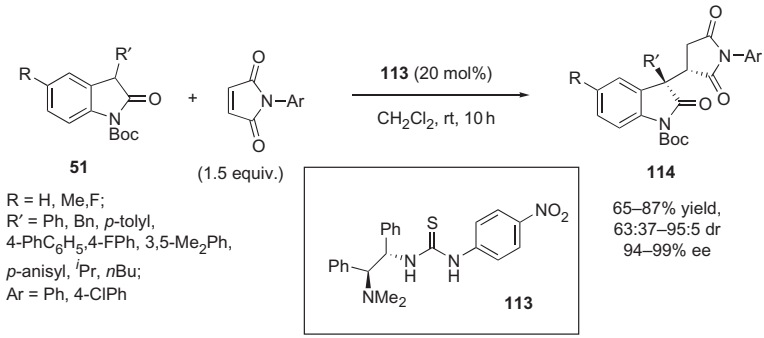
SCHEME 42 Asymmetric Michael addition of *N*-Boc-3-substituted oxindoles to β-nitrostyrenes catalyzed by a chiral secondary amine.

the activation of both the enol form of the oxindole and the nitro group of the Michael acceptor through hydrogen bonds.

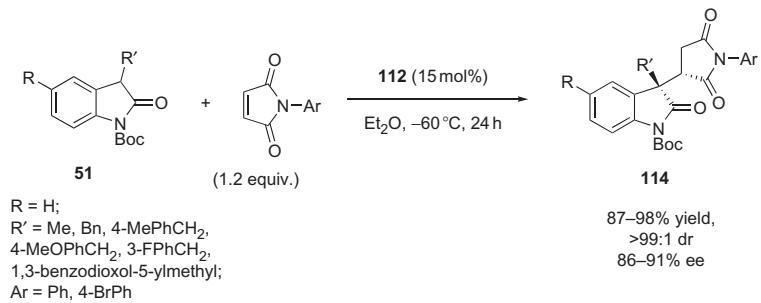
A highly diastereo- and enantioselective Michael addition of *N*-Boc-3-substituted oxindoles **51** to *N*-aryl succinimides promoted by a chiral bifunctional tertiary amine–thiourea catalyst (**113**) was investigated for the first time by Yuan and coworkers, the results being reported at the beginning of 2010 (Scheme 43) [66].

Most recently, Enders and coworkers found that the same chiral secondary amine **112**, used as a catalyst for the addition of *N*-Boc-3-substituted oxindoles to nitroalkenes (see Scheme 42) [64], could also be successfully used for the addition of *N*-Boc-3-alkyloxindoles to *N*-arylmaleimides, with total diastereoselectivity but with somewhat lower enantioselectivity (up to 91% ee; Scheme 44) [67].

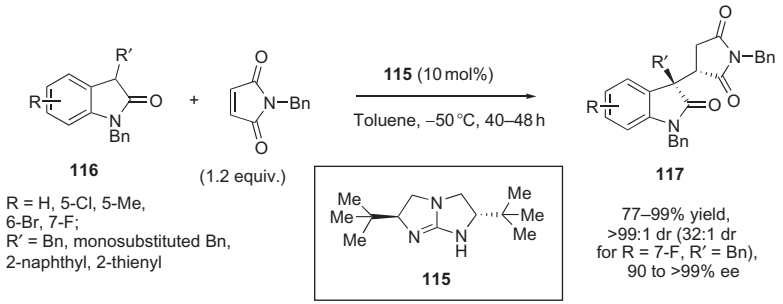
Also in 2012, Tan, Jiang, and coworkers used the chiral bicyclic guanidine catalyst **115** for the Michael addition of *N*-benzyl 3-substituted oxindoles **116** to maleimides, obtaining the products in excellent diastereoselectivities (Scheme 45) [68].



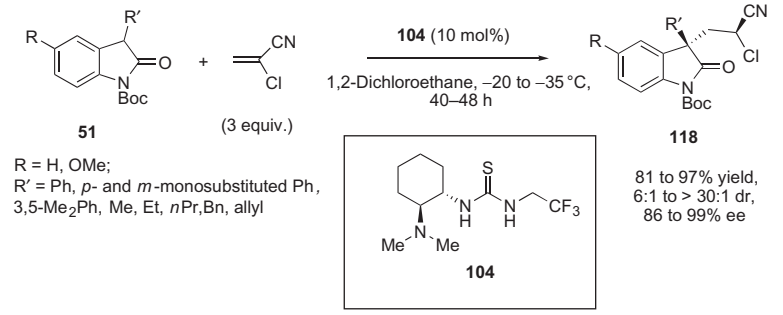
SCHEME 43 Asymmetric Michael addition of *N*-Boc-3-substituted oxindoles to maleimides catalyzed by a tertiary amine–thiourea bifunctional catalyst.



SCHEME 44 Asymmetric Michael addition of *N*-Boc-3-alkyloxindoles to maleimides catalyzed by a chiral secondary amine.



SCHEME 45 Asymmetric Michael addition of *N*-benzyl 3-substituted oxindoles to maleimides catalyzed by a chiral bicyclic guanidine.

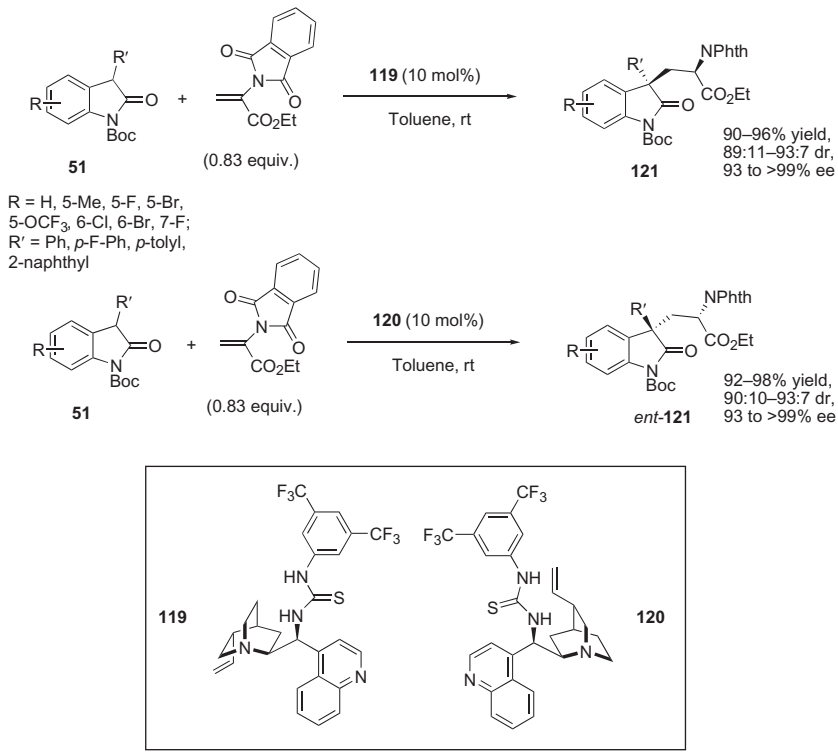


SCHEME 46 Asymmetric Michael addition of *N*-Boc-3-substituted oxindoles to chloroacrylonitrile catalyzed by a chiral tertiary amine–thiourea catalyst.

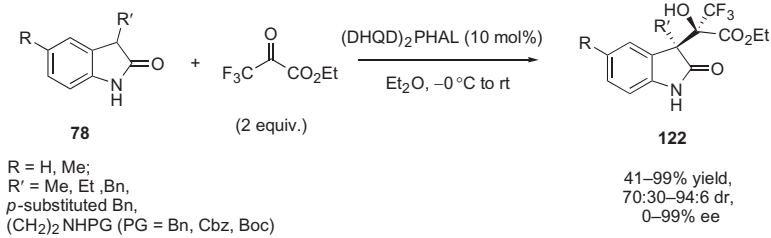
The Michael addition of *N*-Boc-3-substituted oxindoles **51** to 2-chloroacrylonitrile allows for the simultaneous construction of 1,3-nonadjacent stereocenters and has been achieved by Luo and coworkers by means of the bifunctional catalyst **104** (Scheme 46). The resulting adducts **118** are convenient precursors of chiral pyrroloindolines that constitute the core structures of many indole alkaloids [69].

Subsequently, the cinchonidine-derived thiourea catalyst **119** was found by Chen, Xiao and coworkers to promote the asymmetric Michael addition of *N*-Boc-3-aryloxindoles to ethyl 2-phthalymidoacrylate, also leading to the stereocontrolled construction of nonadjacent 1,3-stereocenters [70]. The reaction with *N*-Boc-3-benzyloxindole gave a nearly 1:1 mixture of diastereomers. An advantage of this approach is that changing the catalyst to its cinchonine-derived quasienantiomer **120** the enantiomers of the final adducts can be produced with even slightly better stereoselectivities (Scheme 47).

Catalytic asymmetric aldol additions of unprotected 3-substituted oxindoles **78** were first described by Toru, Shibata and coworkers, using ethyl trifluoropyruvate as the carbonyl acceptor, and commercially available Sharpless $(DHQD)_2PHAL$ ligand as a chiral base catalyst. The two new



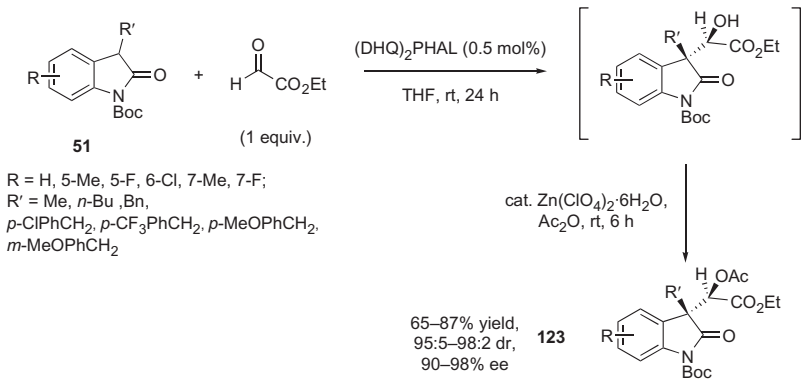
SCHEME 47 Asymmetric Michael addition of *N*-Boc-3-substituted oxindoles to ethyl 2-phthalymidoacrylate, catalyzed by chiral tertiary amine–thiourea catalysts.



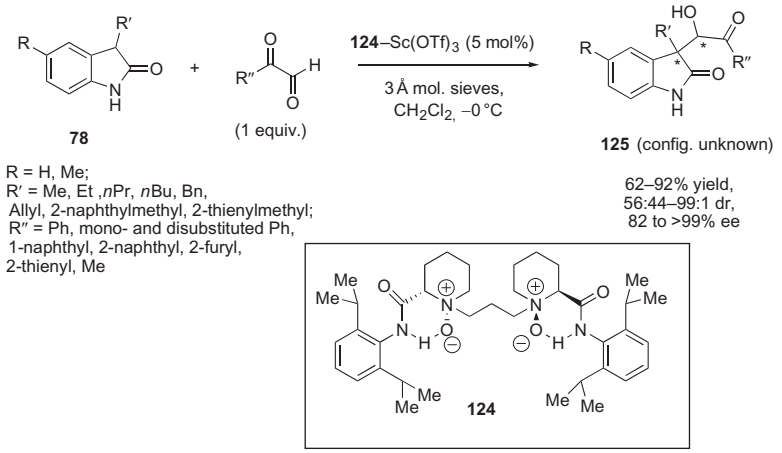
SCHEME 48 Organocatalytic asymmetric aldol reaction between unprotected 3-alkyloxindoles and ethyl trifluoropyruvate.

stereogenic centers in the products **122** were created with acceptable to excellent stereoselectivities (Scheme 48) [71].

In 2011, Bencivenni and coworkers found that the direct aldol reaction between *N*-Boc-3-alkyloxindoles and ethyl glyoxylate could be performed in good yields and excellent diastereo- and enantioselectivities by using the quasienantiomeric $(DHQ)_2PHAL$ ligand, again as a chiral base catalyst (Scheme 49) [72]. As it can be seen, the stereochemical sense of induction



SCHEME 49 Organocatalytic asymmetric aldol reaction between *N*-Boc-3-alkyloxindoles and ethyl glyoxylate.



SCHEME 50 Scandium(III)-catalyzed asymmetric aldol reaction between unprotected 3-alkyloxindoles and glyoxal derivatives.

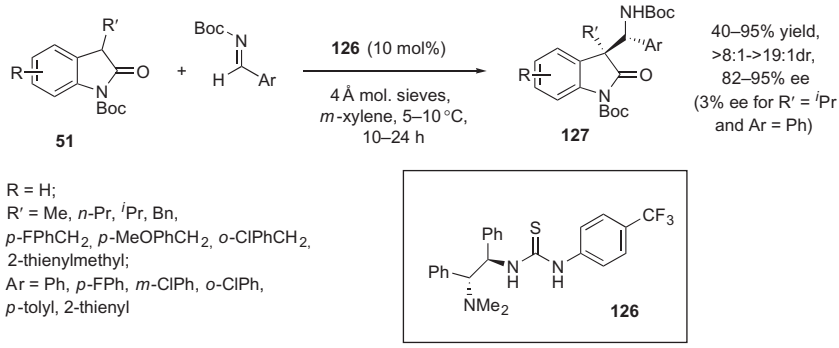
at the C3-quaternary carbon is opposite to that observed by Toru and Shibata, as it is logical to expect. The aldol adducts were too unstable to be isolated and were converted *in situ* to the corresponding acetates **123**.

In 2010, Kobayashi *et al.* reported that the aldol addition of *N*-Boc-3-methyloxindole to formaldehyde afforded the corresponding hydroxymethylated product in 59% yield and 82% ee under catalysis from a chiral bipyridine–scandium(III) complex [73]. Subsequently, Feng and coworkers were able to perform the enantioselective aldol addition of unprotected 3-substituted oxindoles to a variety of glyoxal derivatives, using in this case a chiral scandium(III)–*N*, *N*′-dioxide complex (**124**) as the catalyst (Scheme 50) [74]. The direct catalytic

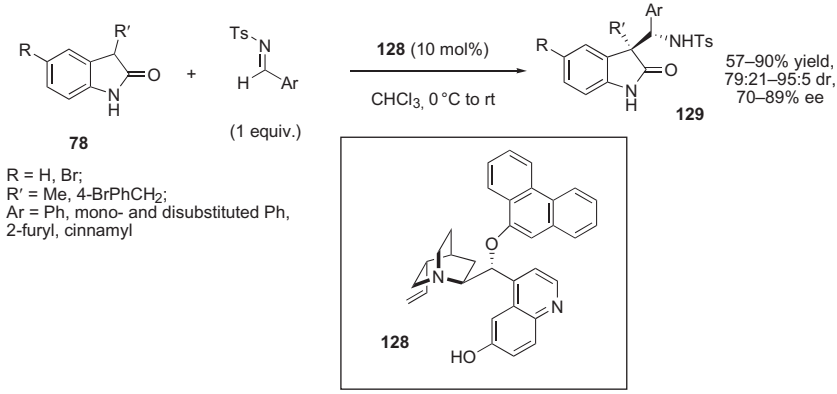
enantioselective aldol reaction between 3-substituted oxindoles and simple aldehydes or ketones has therefore not yet been achieved in a general fashion.

In 2008, Chen and coworkers disclosed the first examples of asymmetric Mannich reactions between *N*-Boc-3-alkyloxindoles and *N*-Boc imines derived from aromatic aldehydes [75]. A suitable catalyst was the bifunctional tertiary amine–thiourea **126**, and the Mannich adducts **127** were obtained with good yields and diastereoselectivities and (generally) with good enantioselectivities (Scheme 51).

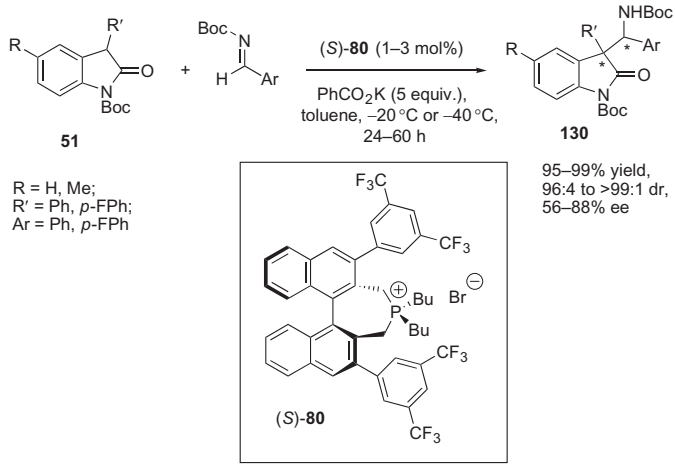
Subsequently, Liu, Chen, and coworkers developed an asymmetric *anti*-selective Mannich reaction of unprotected 3-alkyloxindoles **78** with aromatic *N*-tosyl aldimines. Under catalysis by the cupreine derivative **128**, the reaction took place in moderate to good yields, affording the Mannich adducts **129** in moderate to good diastereoselectivity and with up to 89% ee (Scheme 52) [76].



SCHEME 51 Tertiary amine–thiourea bifunctional catalysis of the Mannich reaction between *N*-Boc-3-alkyloxindoles and aromatic *N*-Boc imines.



SCHEME 52 Organocatalytic asymmetric *anti*-selective Mannich reaction of unprotected 3-alkyloxindoles with aromatic *N*-tosyl aldimines.



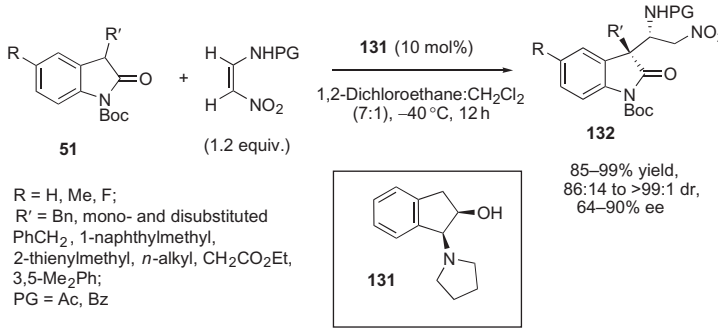
SCHEME 53 Chiral PT-catalyzed Mannich reaction between *N*-Boc-3-aryloxindoles and aromatic *N*-Boc imines.

It should be noted that in the 2009 paper by Maruoka and coworkers [42] concerning the use of the chiral phosphonium salt (*S*)-**80** in the asymmetric Michael addition of *N*-Boc-3-aryloxindoles to unsaturated carbonyl compounds (see Scheme 29), the authors described that the same phase-transfer catalyst could also be used for asymmetric Mannich reactions between *N*-Boc-3-aryloxindoles and *N*-Boc imines derived from benzaldehyde and 4-fluorobenzaldehyde, with good yields and diastereoselectivities, but with moderate to good enantioselectivities (Scheme 53). It is worth noting that *N*-Boc-3-aryloxindoles gave very poor results under the conditions of Scheme 51 [75]. The stereochemistry of the adducts **130**, however, could not be determined.

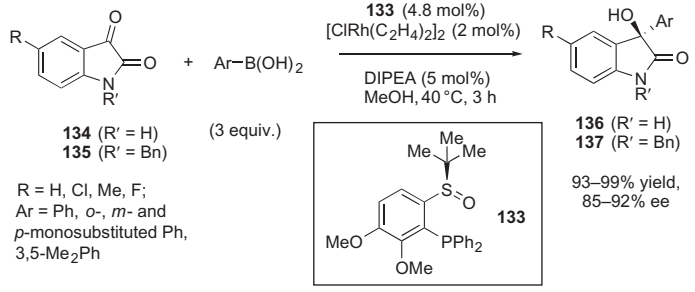
In 2011, an alternative strategy for the asymmetric synthesis of 3-(α-alkylamino)oxindoles (that can be regarded formally as the Mannich adducts derived from 3-substituted oxindoles and β-nitroacetaldehyde imines), based on the enantioselective Michael addition of *N*-Boc-3-substituted oxindoles **51** to protected 2-amino-1-nitroethenes, was developed by Yuan and coworkers [77]. The authors found that this reaction was catalyzed by the readily available amino-indanol derivative **131**, affording the desired products **132** in very high yields (up to 99%), with excellent diastereoselectivities (up to >99:1 dr) and with good enantioselectivities (Scheme 54).

ADDITION OF CARBON NUCLEOPHILES TO ISATINS OR TO ISATIN IMINES

Transition metal-catalyzed enantioselective arylation of isatins was pioneered in 2006 through the independent reports of Feringa and coworkers [78] and Hayashi and coworkers [79]. Both groups used Rh(I) catalysts containing a



SCHEME 54 Amino-indanol-catalyzed asymmetric Michael addition of *N*-Boc-3-substituted oxindoles to protected 2-amino-1-nitroethenes.



SCHEME 55 Rh(I)-phosphine-catalyzed asymmetric arylation of isatins.

chiral phosphine ligand. Hayashi, with a catalyst derived from the dimeric chlororhodium–bis(ethylene) complex $[ClRh(C_2H_4)_2]_2$ and the binaphthyl (*R*)-MeO-MOP ligand, obtained high enantioselectivities with both unprotected and *N*-PMP protected isatins (up to 87% ee) [79], while Feringa, with a chiral phosphoramidite ligand, achieved a maximum enantiomeric excess of 55% [78]. Very recently, Liao and coworkers have found a Rh(I) complex derived from the chiral sulfoxide phosphine ligand **133**, in the presence of ethyl diisopropylamine (DIPEA) as a basic cocatalyst, gave even higher yields and enantioselectivities in this process (Scheme 55) [80].

In 2011, Shi, Li, and coworkers demonstrated that the chiral cationic NHC–Pd(II) diaqua complex **138** (Fig. 6) could also be used to catalyze the asymmetric addition of arylboronic acids to a variety of *N*-protected isatins [81]. The yields were satisfactory (79–92%), but the enantiomeric purities of the resulting 3-aryl-3-hydroxyoxindoles were only moderate (50–87% ee).

The addition of aryl(trimethoxy)silanes and vinyl(trimethoxy)silanes to isatins could be rendered enantioselective by Shibasaki and coworkers, by means of the chiral diphosphine–Cu(I) complex derived from ligand **139** and $CuF{\cdot}3P(4\text{-}FPh)_3{\cdot}2EtOH$ as the copper source [82]. As it can be seen from Fig. 7, the structure of the ligand is very complex. Moreover, the reaction

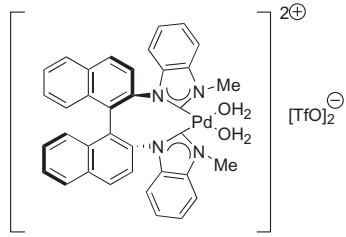


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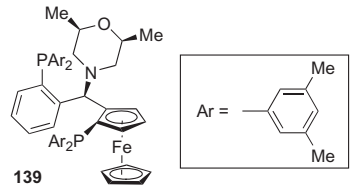
FIGURE 6 Chiral cationic *N*-heterocyclic carbene–Pd(II) complex for catalytic asymmetric arylation of isatins.



139

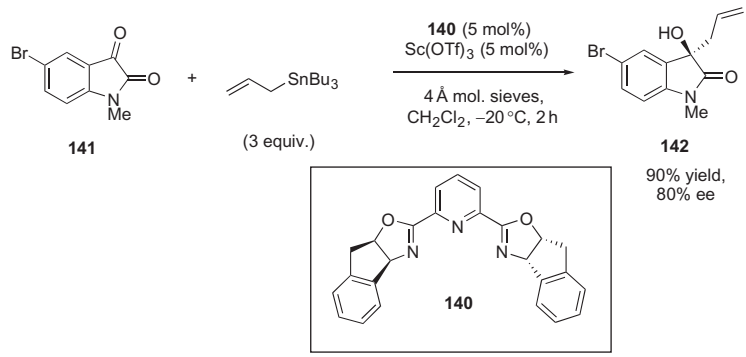
FIGURE 7 Chiral ferrocene-derived ligand for the enantioselective arylation and vinylation of *N*-protected isatins.

required the use of 3 mol. equiv. of ZnF_2 as a cocatalyst, and good enantioselectivities (81–97% ee) could be only achieved when a bulky *N*-protecting group di(*p*-methoxyphenyl)phenylmethyl (DMTr) was present in the isatin. For all of these reasons, this method is of rather low practical value.

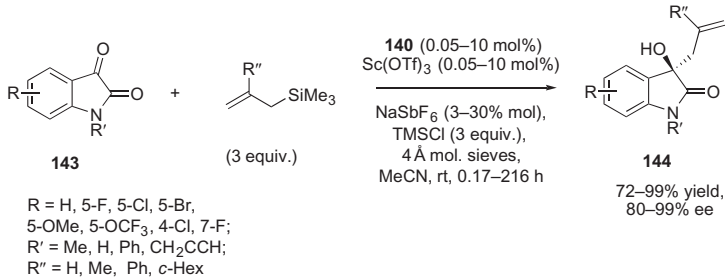
The catalytic asymmetric allylation of isatins was pioneered by Takayama *et al.*, who reported that the reaction of tetraallylstannane with isatin itself afforded (*S*)-3-allyl-3-hydroxyisatin with 42% ee under catalysis from a complex derived from (*R*)-BINOL and titanium(tetraisopropoxide) [83]. This result was somewhat improved by Franz and coworkers. With the aid of a scandium(IIII) catalyst derived from the chiral pybox ligand **140**, the addition of allyltributylstannane to *N*-methyl-3-bromoisatin **141** at −20 °C gave the (*R*)-configured addition product **142** in 90% yield and 80% ee (Scheme 56) [84]. In the same conditions, several other C-nucleophiles (indole, *N*, *N*-dimethyl-3-methoxyaniline, 2-methoxyfuran, and the TMS enol ether of acetophenone) gave the corresponding adducts with good yields (80–90%) and enantioselectivities (95–99% ee) [84].

Very recently, Franz's research group has uncovered a very general method for the enantioselective allylation of isatins, either protected or unprotected, by using allylsilanes and the same catalyst derived from **140** and scandium(III) trilate (Scheme 57) [85].

The first and only enantioselective allylation of isatins that does not require a stoichiometric amount of an allylmetal reagent was described by Krische and coworkers [86]. In Krische's protocol, isopropyl alcohol-mediated transfer



SCHEME 56 Scandium(III)-catalyzed allylation of *N*-methyl-5-bromoisatin.



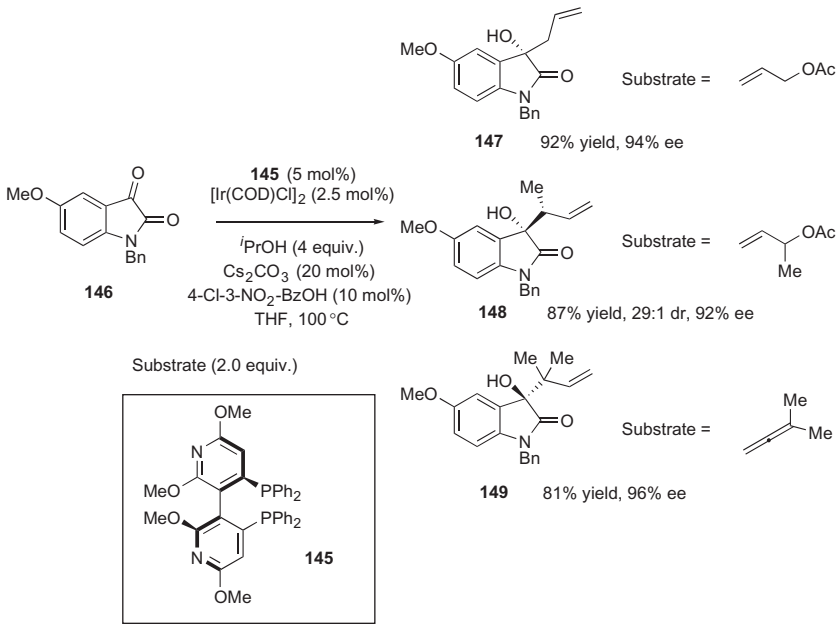
SCHEME 57 Scandium(III)-catalyzed allylation of isatins.

hydrogenation of substituted isatins in the presence of allyl acetate, α-methylallyl acetate, or 1,1-dimethylallene took place enantioselectively in the presence of a chiral cyclometallated iridium(III) catalyst (generated *in situ* from $[Ir(COD)Cl]_2$, biphep ligand **145**, and 4-chloro-3-nitrobenzoic acid), provided products of allylation, crotylation, and reverse prenylation, respectively (Scheme 58).

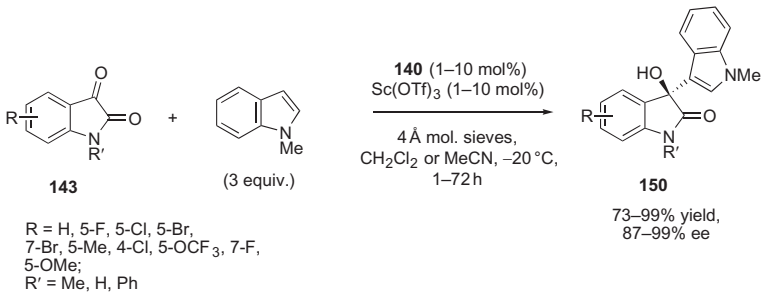
The catalytic asymmetric addition of indoles to isatins was first reported by Franz, in the same paper describing the addition of allyltributylstannane and other C-nucleophiles (see Scheme 56) [84]. The scandium(III) catalyst derived from the pybox ligand **140** promoted the addition of *N*-methyl indole to a variety of *N*-protected and -unprotected isatins **143** with excellent results both in terms of yield and enantioselectivity (Scheme 59).

Shortly afterward, Wang, Li, and coworkers [87] and Chimni and coworker [88] independently and almost simultaneously described two very similar approaches for the asymmetric addition of unprotected indoles to isatins. Wang and Li used a 1:1 mixture of cupreine **151** and acetic acid as catalyst, and Chauhan and Chimni used 9-(*O*-benzyl)cupreine **152** (Fig. 8). Chimni's catalyst gave somewhat better results.

The asymmetric aldol addition of ketones to isatins was pioneered by Tomasini and coworkers [89]. The addition of acetone to isatin, catalyzed



SCHEME 58 Iridium-catalyzed allylation, crotylation, and reverse prenylation of *N*-benzyl isatin **147**.



SCHEME 59 Scandium(III)-catalyzed indole addition to isatins.

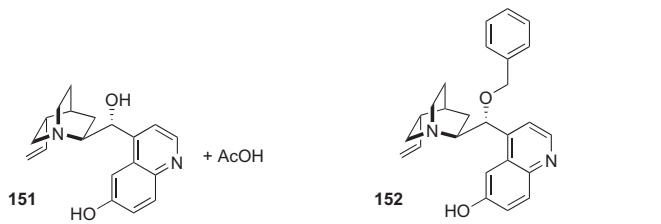


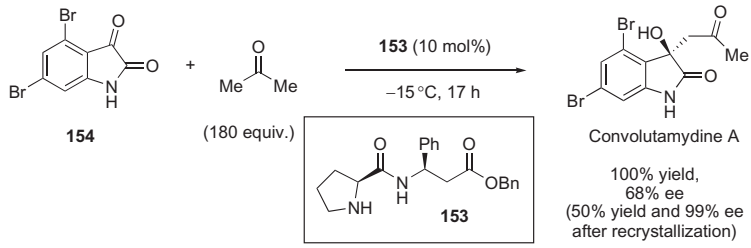
FIGURE 8 Cupreine-based catalysts for enantioselective indole addition to isatins.

by L-proline, took place with low enantioselectivity (33% ee). The prolinamide-derived catalyst **153**, however, gave much better results and was subsequently applied to the synthesis of (*R*)-convolutamydine A by addition of acetone to 4,6-dibromoisatin **154** (Scheme 60) [90].

Up to date (see Ref. [6a] for other approaches), the highest enantioselectivity for this reaction (97% ee) has been achieved by Nakamura and coworkers, by means of another prolinamide-derived catalyst (Fig. 9) [91].

In 2010, Zhao and coworkers [92] found that the quinidine-derived tertiary amine–thiourea **95** was a rather general catalyst for the asymmetric cross-aldol reactions between unprotected isatins **143** and ketones (Scheme 61) [93].

The same catalyst **95** gives moderate to good yields (19–98%) with moderate to good enantioselectivities (30–97% ee) in the asymmetric cross-aldol reaction of isatins **143** with α,β-unsaturated methyl ketones, as shown by Wang and coworkers (Scheme 62) [94]. The quasienantiomeric catalyst derived from quinine (**157**) can be used to access the enantiomeric aldol



SCHEME 60 Prolinamide-catalyzed synthesis of convolutamydine A.

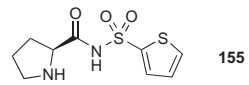
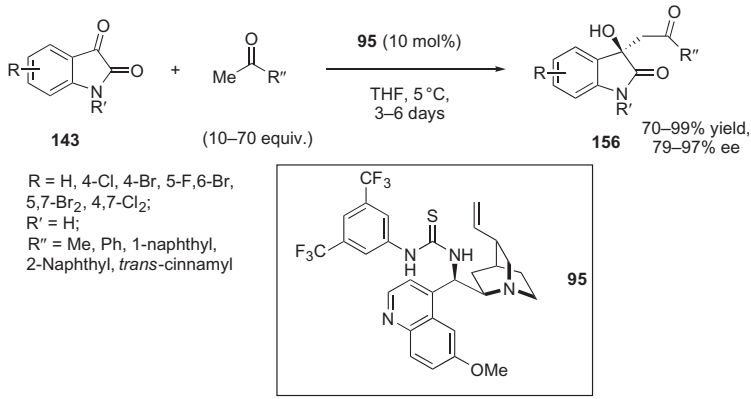
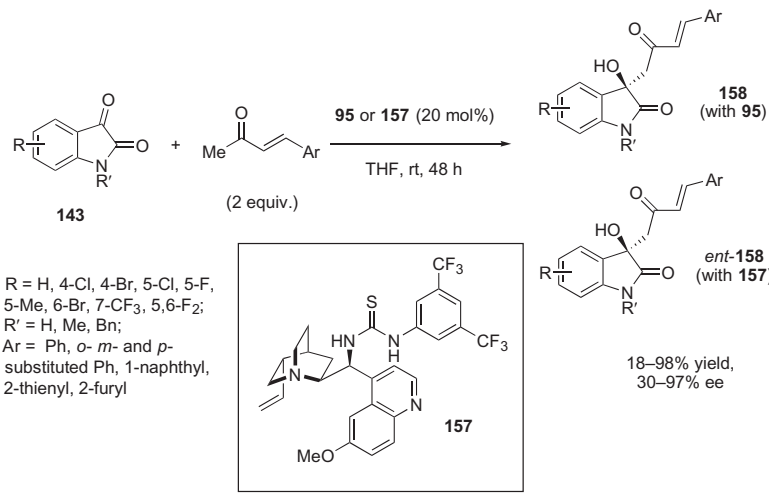


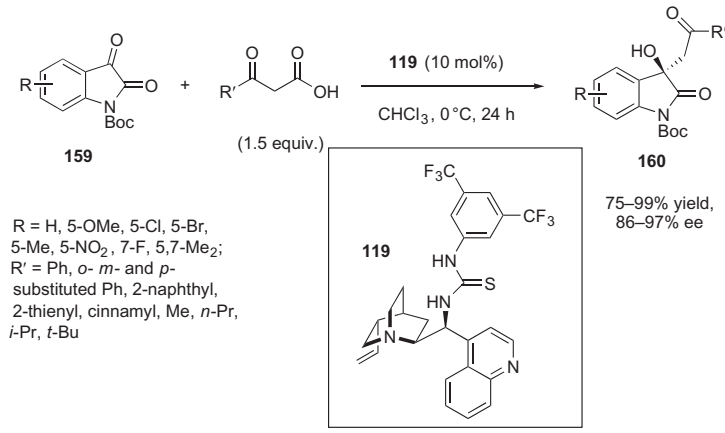
FIGURE 9 Nakamura's catalyst for the synthesis of convolutamydine A.



SCHEME 61 Quinidine thiourea-catalyzed cross-aldol reactions between ketones and isatins.



SCHEME 62 Quinidine- and quinine thiourea-catalyzed cross-aldol reactions between unsaturated methyl ketones and isatins.



SCHEME 63 Catalytic asymmetric decarboxylative addition of β-ketoacids to isatins.

products *ent*-**158**. The asymmetric aldol reaction between isatins and an ynone (4-phenyl-3-butyn-2-one) takes place also with a tertiary amine–thiourea bifunctional catalyst derived from *trans*-1,2-cyclohexanediamine, albeit with moderate stereocontrol (56–71% ee), as reported by Kang *et al*. [95].

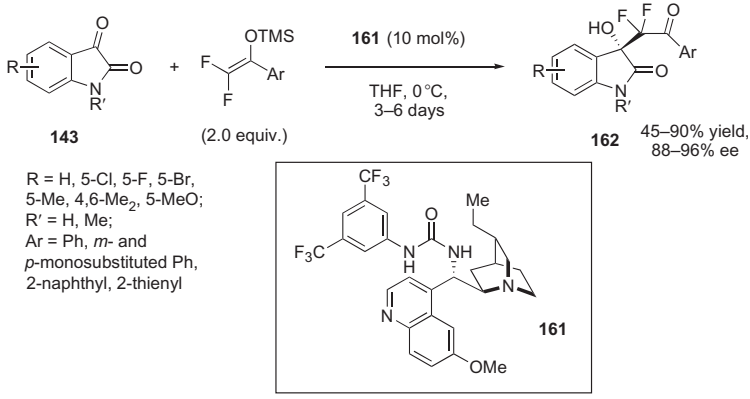
The first highly enantioselective decarboxylative addition of β-ketoacids to *N*-Boc-protected isatins (**159**), mediated by another bifunctional tertiary amine–thiourea catalyst (**119**, derived from cinchonidine), has been recently developed by Lu and coworkers (Scheme 63) [96].

A tertiary amine–urea bifunctional catalyst (**161**, derived from 9-aminodihydroquinidine), afforded good results in the asymmetric addition of

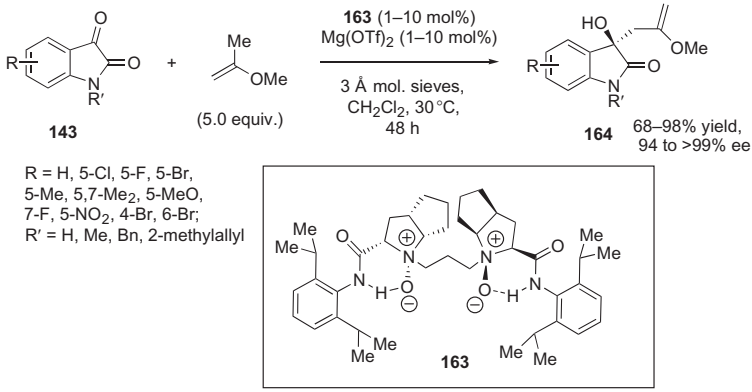
difluoroenoxysilanes to isatins **143**, providing a very attractive and highly enantioselective entry to 3-difluoroalkyl-3-hydroxyoxindoles (Scheme 64) [97].

An indirect method for the enantioselective preparation of isatine–acetone aldol adducts relies on the catalytic asymmetric hetero-ene reaction of isatins **143** with 2-methoxypropene, described by Feng and coworkers [98]. The catalyst of choice for this reaction was the magnesium(II) complex derived from magnesium triflate and the C_2-symmetric *N*,*N*′-dioxide ligand **163** (Scheme 65). The resulting enol ether adducts **164** were easily hydrolyzed by aqueous hydrochloric acid to the corresponding ketones; thus, starting from 4,6-dibromoisatin **154**, (*R*)-convolutamydine A was obtained in 97% and 85% global yield (two steps) by this procedure (compare with Scheme 60).

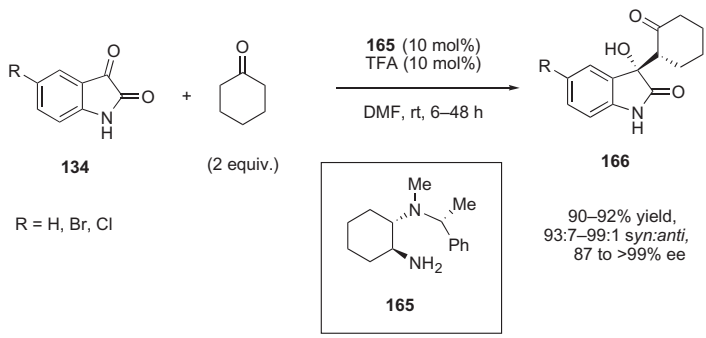
From the examples shown in Schemes 60–65, it is clear that excellent degrees of stereocontrol can be achieved in the aldol reaction of isatins with methyl ketones by asymmetric catalysis. When using more substituted



SCHEME 64 Organocatalytic asymmetric synthesis of 3-difluoroalkyl-3-hydroxyoxindoles.



SCHEME 65 Catalytic asymmetric ene reaction of isatins with 2-methoxypropene.



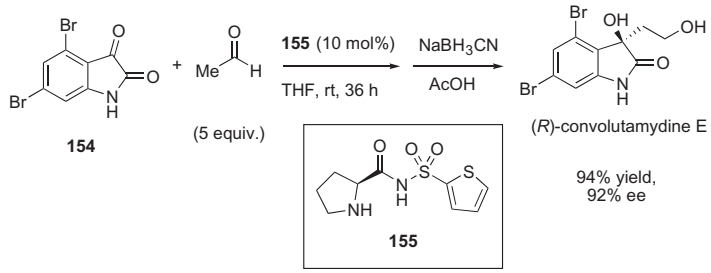
SCHEME 66 Asymmetric organocatalytic aldol reaction between isatins and cyclohexanone.

ketones, however, the reaction becomes more difficult, and the issue of the *anti:syn* diastereoselectivity appears. Up to now, good yields and stereoselectivities have only been achieved for cyclic ketones. In 2010, Singh and coworkers reported that enamine catalysis with the chiral primary amine **165** in the presence of trifluoroacetic acid gave good results in the aldol reaction of three different unprotected isatins **134** and cyclohexanone (Scheme 66) [99].

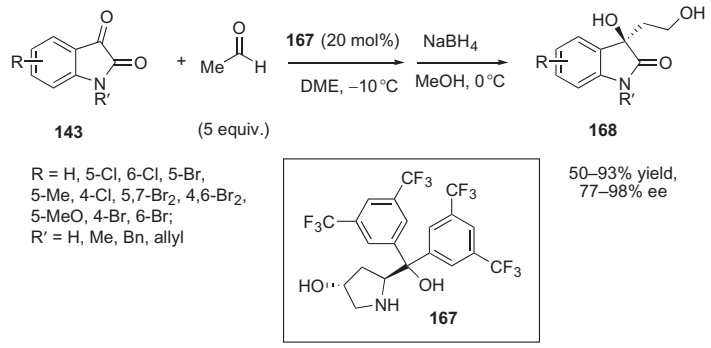
More recently, Sun, Li, and coworkers have shown that a simpler, suitable catalyst for this reaction is the 1:1 mixture between enantiomerically pure *trans*-1,2-cyclohexanediamine and hexanedioic acid that affords the opposite *anti*-diastereomers (up to 20:1 dr) in good yields (up to 90% global yield) and enantioselectivity (up to 99% ee) [100]. The (3*R*,2′*S*)-configured product is obtained starting from the (*R*,*R*)-enantiomer of the diamine, and the reaction can be extended, with lower enantioselectivities to other cyclic ketones (tetrahydropyran-4-one, cyclopentanone). Shortly afterward, Yanagisawa *et al*. have disclosed an alternative strategy, based on a chiral Lewis acid tin (II) catalyst that promotes the Mukaiyama-type reaction between *N*-benzylisatins **135** and enol trifluoroacetates or trichloroacetates of aromatic ketones; this reaction takes place with good yields and enantioselectivities, but with moderate diastereoselectivities (51:49 to 82:18 dr), and the stereochemistry of the adducts was not determined [101].

Nakamura's prolinamide catalyst **155**, which gave very good enantioselectivity in the aldol reaction between 4,6-dibromoisatin **154** and acetone [91a], was shown to be also efficient in the aldol addition of acetaldehyde to the same compound [102]. After *in situ* reduction of the aldol, (*R*)-convolutamydine E was isolated in 94% yield and 92% ee (Scheme 67). Three other aldehydes (propanal, butanal, 3-phenylpropanal) were used in the same reaction, with variable diastereoselectivities (70:30 to >98:2 dr) and good enantioselectivities (90–98% ee of the major diastereomer).

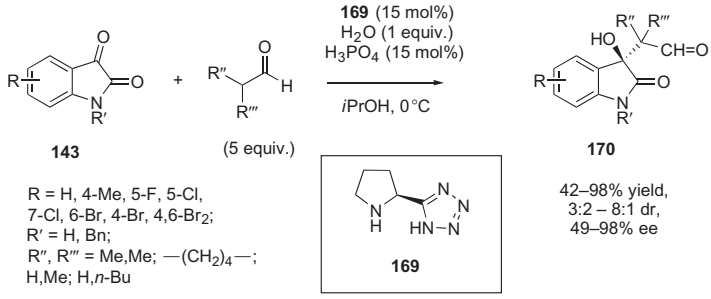
A drawback of this approach is that the scope of the substrate is very limited, since high enantioselectivities are limited to 4-halogenated or 4,6-dihalogenated isatins. Subsequently, Yuan and coworkers demonstrated that



SCHEME 67 Organocatalytic enantioselective synthesis of convolutamydine E.



SCHEME 68 Organocatalytic asymmetric aldol reaction between acetaldehyde and isatins.



SCHEME 69 Organocatalytic asymmetric aldol reaction between α-substituted aldehydes and isatins.

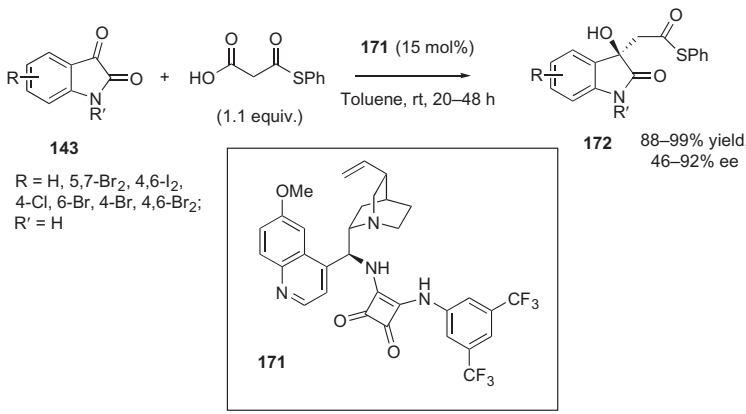
the 4-hydroxyprolinol derivative **167** was a rather general catalyst for the asymmetric aldol reaction between unprotected and protected isatins **143** and acetaldehyde (Scheme 68) [103].

Up to now, the most general catalyst for the asymmetric aldol reaction between isatins and α-substituted aldehydes is (*S*)-pyrrolidine tetrazole **169**, that as described by Wang and coworkers [104], was able to promote the formation of two contiguous quaternary centered 3-substituted 3-hydroxyoxindoles **170** with good to excellent diastereo- and enantioselectivities (Scheme 69).

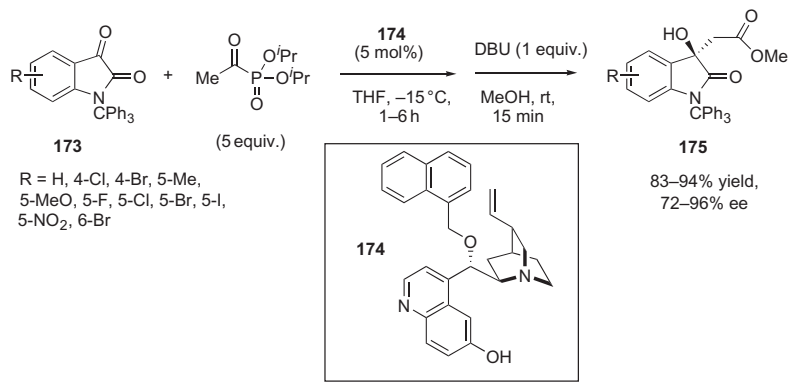
The catalytic enantioselective direct aldol reaction between isatins and acetates is a difficult task, due to the low acidities of the α-protons of esters that prevents the formation of ester enolates with small quantities of bases. A solution to this problem was uncovered by Nakamura, Shibata and coworkers, by means of a decarboxylative addition of malonic acid half thioesters to unprotected isatins, catalyzed by the tertiary amine–squaramide bifunctional quinine derivative **171** (Scheme 70) [105].

Another approach to the asymmetric acetate aldol addition to isatins was subsequently uncovered by Zhao and coworkers. These authors found that diisopropyl acetylphosphonate was a more acidic acetate surrogate, whose aldol addition to *N*-trityl isatins **173** could take place with high enantioselectivities when promoted by the cupreidine (1-naphthylmethyl)ether catalyst **174**. The resulting adducts were not isolated, but submitted *in situ* to methanolysis in the presence of base to provide the desired methyl acetate aldol adducts **175**, of (*R*) absolute configuration (Scheme 71) [106]. An advantage of this approach is the facile elimination of the *N*-trityl protecting group (Et_3SiH, TFA, CH_2Cl_2, rt).

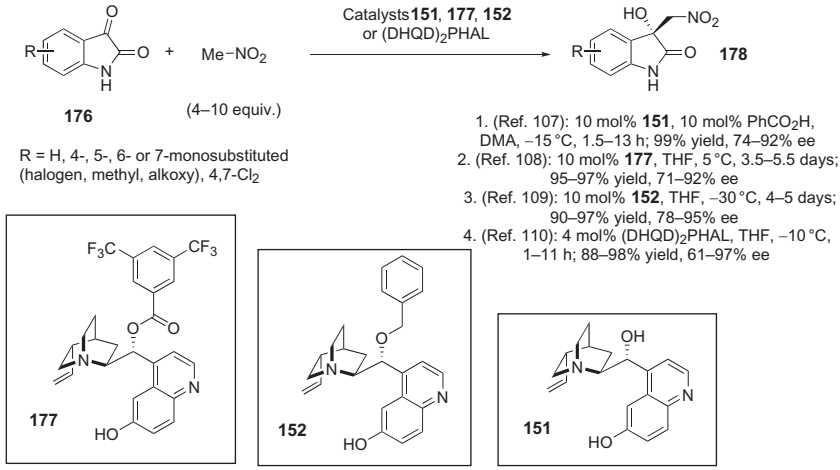
In 2011, four research groups disclosed, almost simultaneously and independently, asymmetric organocatalytic approaches to the Henry (nitroaldol) reactions of isatins. In all of them, nitromethane was generally used as the nucleophile, unprotected isatins **176** gave the best results, *Cinchona* alkaloid-derived chiral bases (cupreine **151** plus benzoic acid [107], cupreine bis-(3,5-trifluoromethyl)benzoate **177** [108], cupreine benzyl ether **152** [109], and Sharpless ligand $(DHQD)_2PHAL$ [110]) were chosen as catalysts, and the major enantiomers of the Henry adducts **178** with nitromethane had an absolute (*R*) configuration. All of these findings are summarized in Scheme 72. In all instances, good enantioselectivities were achieved, and the Henry adduct from isatin themself was used in the first two references as starting material for the synthesis of (*S*)-spirobrassinin [107,108].



SCHEME 70 Organocatalytic enantioselective decarboxylative addition of malonic acid half thiosters to isatins.



SCHEME 71 Organocatalytic enantioselective formal aldol addition of methyl acetate to isatins.

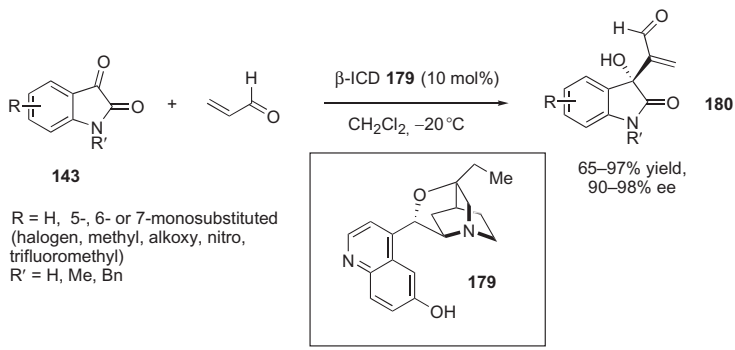


SCHEME 72 Organocatalytic asymmetric Henry reaction of unprotected isatins.

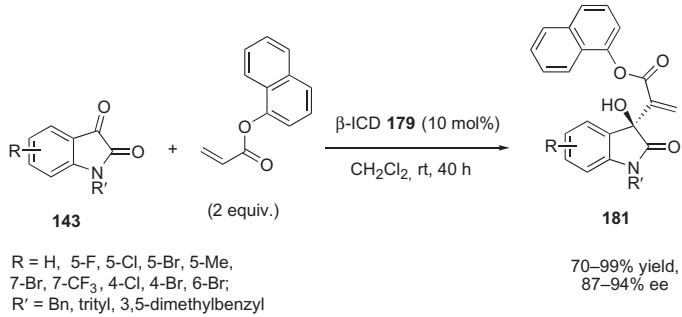
Wang's group (Shanghai) also used nitroethane and nitropropane as nucleophiles in three examples, with variable diastereoselectivities (3:1, 7:1, and 10:1 dr) and good enantioselectivities (84%, 93%, and 88% ee for the major diastereomer, respectively) [107].

The use of isatins **143** as electrophilic components in catalytic asymmetric MBH reactions was first disclosed by Zhou and coworkers. Using acrolein as the pronucleophile and β-isocupreidine (β-ICD, **179**) as a catalyst, the MBH adducts **180** were obtained with excellent enantioselectivities (Scheme 73) [111].

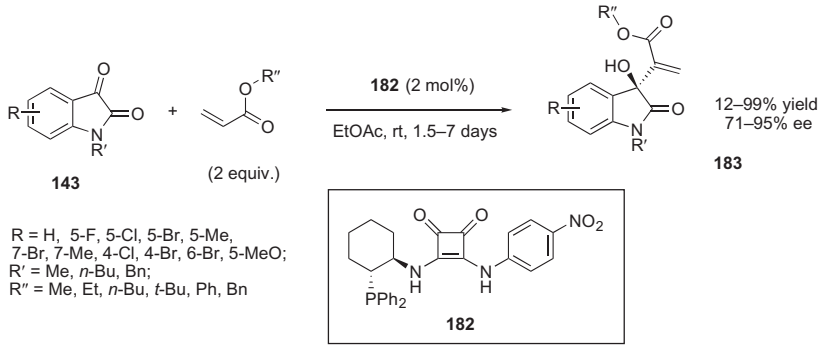
Simultaneously with Zhou and coworkers, Guan, Wie, and Shi reported that β-isocupreidine **179** could also be used to catalyze the MBH reaction of protected isatins with 1-naphthyl acrylate (Scheme 74) [112]. Shortly afterward, and using the same catalyst, Lu and coworkers found that very good results (61–96% yield, 85–96% ee) were obtained with benzyl acrylate as



SCHEME 73 Organocatalytic asymmetric Morita–Baylis–Hillman reaction of isatins with acrolein.



SCHEME 74 Organocatalytic asymmetric MBH reaction of isatins with naphthyl acrylate.



SCHEME 75 Chiral phosphine-squaramide-catalyzed asymmetric MBH reaction of isatins.

the pronucleophile in the MBH reaction [113]. In this case, the sense of asymmetric induction was the same that in the case of acrolein (adducts **180**) and opposite to that of naphthyl acrylate (adducts **181**).

More recently, Wu and coworkers have developed chiral phosphine-squaramide catalysts such as **182** for the asymmetric MBH reaction between *N*-protected isatins and acrylic acid esters (Scheme 75) [114]. The reaction

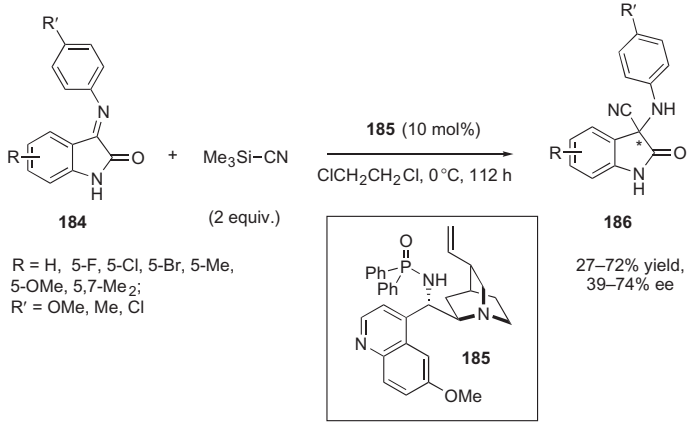
takes place in very good yields, and with moderate to good enantioselectivities (71–95% ee), although the enantiomeric purity of the adducts **183** can be generally increased up to 98–99% ee after a single recrystallization [115].

In 2011, Xu, Wang, and coworkers reported that quinine was able to promote the phospho-aldol reaction of *N*-alkylated isatins with diphenyl phosphite, in good to excellent yields (up to 99%) and with moderate enantioselectivity (25–73% ee) [116]. The absolute configuration of the adducts could not be determined.

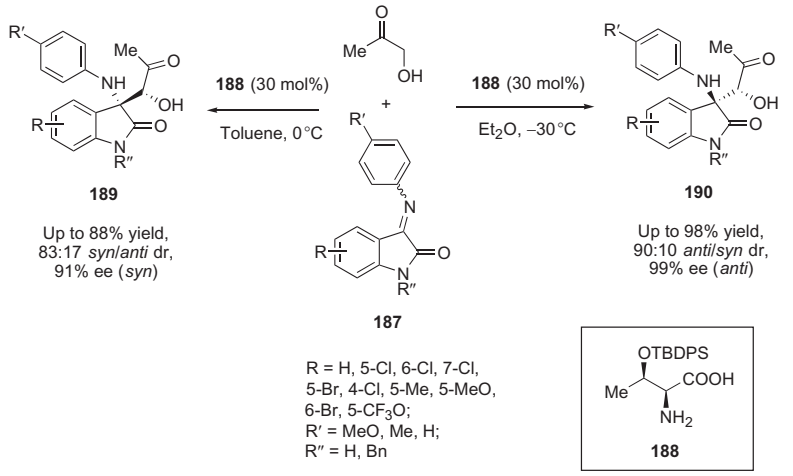
In 2010, the α-cyanoamination (Strecker reaction) of unprotected isatins with trimethylsilyl cyanide (TMSCN) was developed by Zhou and coworkers. Starting from preformed *N*-arylketimines of isatins (**184**) and with the aid of the quinidine-derived bifunctional catalyst **185**, the quaternary α-aminonitriles **186**, of unknown absolute configuration, were obtained with moderate yields and enantioselectivities (Scheme 76) [117].

The first catalytic asymmetric aza-aldol (Mannich) reaction between *N*-arylketimines derived from isatines (**187**) and ketones (hydroxyacetone) was disclosed by Guo *et al*. at the beginning of 2012. The reaction was catalyzed by the *O*-silylated threonine **188** and showed an interesting solvent effect on the diastereoselectivity: while in toluene the major diastereomers were the *syn* adducts **189**, in diethyl ether the *anti* adducts **190** were preferentially obtained (Scheme 77) [118]. This change in the diastereoselectivity could be traced to a solvent-dependent equilibrium between the (*E*)- and (*Z*)-isomers of the ketimines.

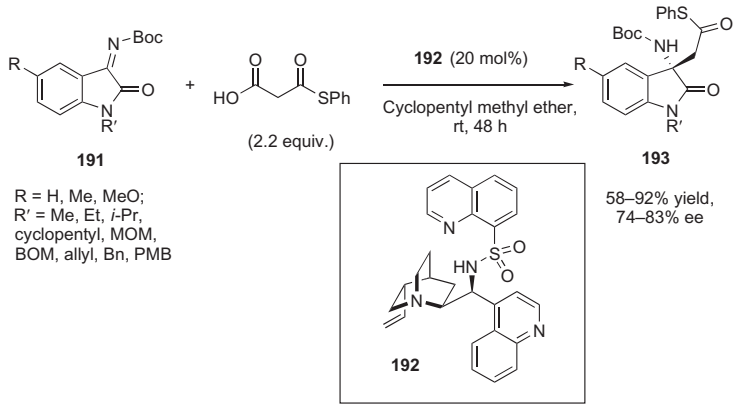
Subsequently, Nakamura, Shibata, and coworkers, in an extension of their work on the decarboxylative addition of malonic acid half thioesters to unprotected isatins (see Scheme 70) [105], have found that when *N*-Boc ketimines **191** as used as substrates, the decarboxylative aza-aldol reaction is efficiently catalyzed by the new cinchonidine-derived *N*-heterosulfonyl amide **192**, affording the Mannich adducts **193** with good enantioselectivities (Scheme 78) [119].



SCHEME 76 Organocatalytic asymmetric cyanide addition to isatin-derived ketimines.



SCHEME 77 Stereoselective Mannich reaction of isatin imines with hydroxyacetone.

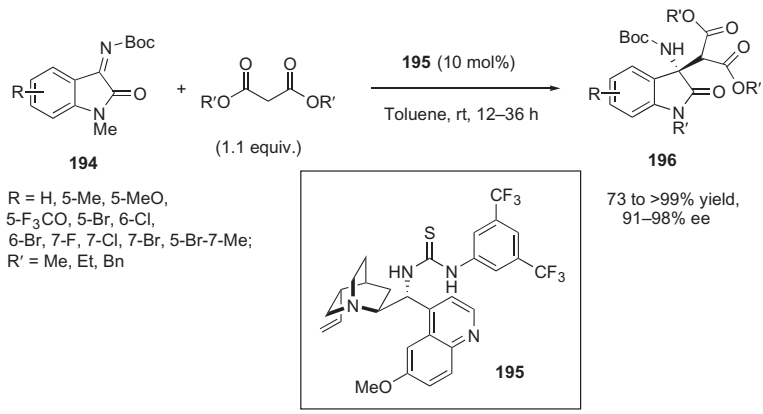


SCHEME 78 Organocatalytic enantioselective decarboxylative Mannich reaction of isatins.

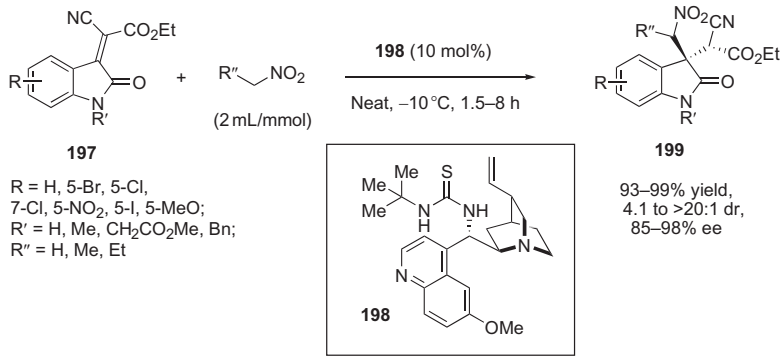
Almost simultaneously, Wang and coworkers reported that the enantioselective addition of 1,3-dicarbonyl compounds (malonates, 1,3-pentanedione, 1,3-diphenyl-1,3-propanedione, α-(ethoxycarbonyl)acetophenone, 2-(ethoxycarbonyl)cyclopentanone) to *N*-Boc ketimines derived from *N*-methyl isatins (**194**) was catalyzed by the quinine-derived thiourea **195** [120]. In Scheme 79, the results for the addition of malonates are summarized.

ASYMMETRIC MICHAEL ADDITIONS TO ISATIN-DERIVED ELECTRON-DEFICIENT ALKENES AND RELATED PROCESSES

Isatylidene compounds derived from the condensation of oxindoles with active methylene compounds such as cyanoacetates or malonodinitrile are



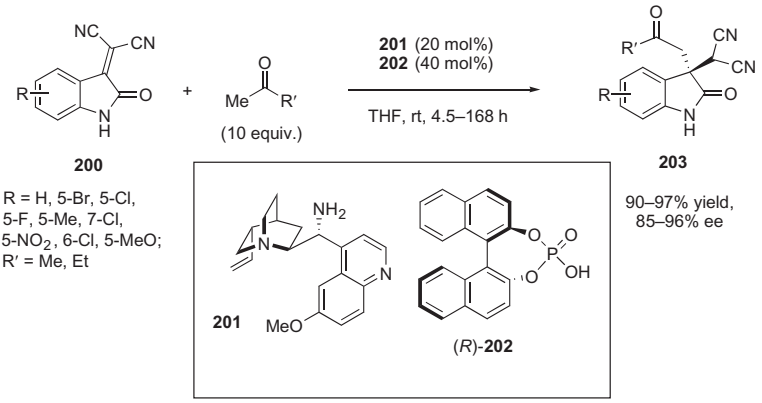
SCHEME 79 Organocatalytic enantioselective addition of malonates to *N*-Boc ketimines of isatins.



SCHEME 80 Organocatalytic enantioselective Michael addition of nitroalkanes to isatylidene cyanoacetates.

highly electrophilic compounds that can undergo Michael additions to afford 3,3′-disubstituted chiral oxindoles. The successful realization of a catalytic asymmetric version of this process was first achieved by Li, Wang, and coworkers. These authors found that the enantioselective Michael addition of nitroalkanes to isatylidene cyanoacetates **197** was efficiently catalyzed by the quinidine-derived bifunctional amine–thiourea compound **198** [121]. Notably, in spite that three new stereogenic centers are created, the reaction took place with good diastereoselectivity (4:1 to >20:1 dr) and enantioselectivity (85–98% ee, Scheme 80).

Subsequently, the same research groups reported that the catalytic enantioselective Michael addition of acetone and butanone to isatylidene malonodinitriles **200** could be achieved in the presence of a mixture of 9-amino-9-deoxyquinine **201** and the (*R*)-BINOL-derived phosphoric acid **202**, with good yields and enantioselectivities (Scheme 81) [122]. In the case of acetone, slightly better results (88% to >99% ee) were obtained when the reaction was performed in a direct



SCHEME 81 Organocatalytic enantioselective Michael addition of ketones to isatylidene malonodinitriles.

three-component fashion, generating *in situ* the isatylidenes **200** from the corresponding isatines and malonodinitrile.

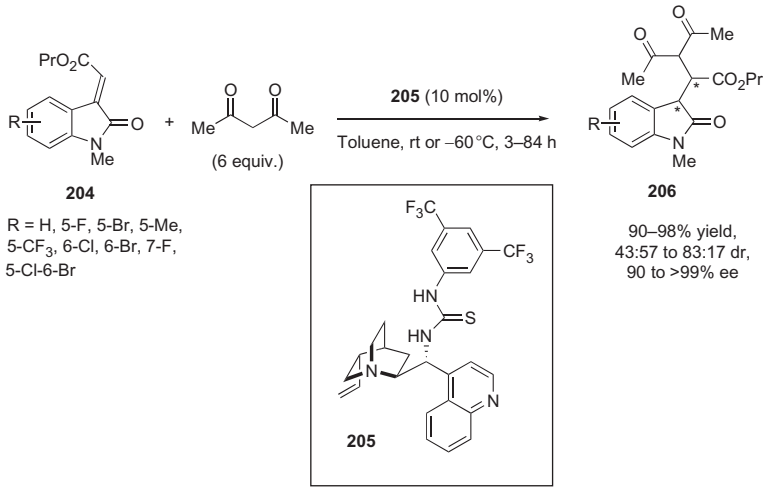
The same Michael addition was almost simultaneously disclosed by Wang, Tao, and coworkers, using essentially the same substrates (isatylidene malonodinitriles, acetone, and butanone) and very similar reaction conditions. The catalyst used in this case was readily available 9-amino-9-deoxy-*epi*-quinidine (20 mol %), in the presence of L-camphorsulfonic acid (40 mol%), and the reactions were run in dichloromethane at room temperature. The Michael adducts **203**, with the same absolute configuration, were obtained in good yields (81–99%) and enantioselectivities (94% to >99% ee) [123].

On the other hand, isatylidenes containing only one electron-withdrawing substituent undergo Michael addition at the exocyclic ylidene carbon, leading to the formation of 3-monosubstituted oxindoles. An asymmetric organocatalytic version of this reaction, using propyl 2-(2-oxoindolin-3-ylidene)acetates **204** as Michael acceptors and 1,3-pentanodione as the nucleophile, was reported by Xiao and coworkers [124]. The reaction was catalyzed by the cinchonidine-derived tertiary amine–thiourea **205** and took place with good yields and enantioselectivities (Scheme 82).

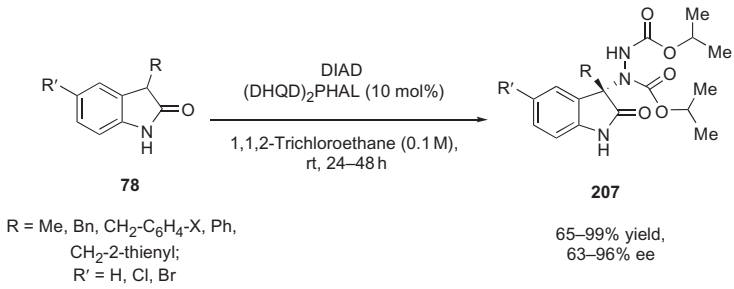
Probably due to the easy epimerization of 3-monosubstituted oxindoles, the diastereoselectivity was only moderate (from 52:48 to 83:17 dr), and the stereochemistry of the adducts **206** was not ascertained.

AMINATION, HYDROXYLATION, AND HALOGENATION REACTIONS OF 3-SUBSTITUTED OXINDOLES

3-Amino-2-oxindole compounds bearing a chiral quaternary carbon center at C3 have been extensively investigated and recognized as a key structure in a variety of biologically active compounds as well as challenging targets for



SCHEME 82 Organocatalytic Michael addition of acetylacetone to 3-ylidineoxindoles.



SCHEME 83 Organocatalytic α-amination of *N*-unprotected 2-oxindoles.

medicinal chemistry and synthetic organic chemistry. Therefore, since 2009, several methodologies have been reported to achieve the amination of 3-substituted oxindoles. In all the examples, only two sources of nitrogen have been employed: either azodicarboxylate derivatives or nitrosobenzene.

The first example of amination of *N*-unprotected 3-subtituted-2-oxindoles **78** employing diisopropyl azodicarboxylate (DIAD) was reported by Liu, Chen, and coworkers (Scheme 83) [125]. Employing the commercially available Sharpless ligand $(DHQD)_2PHAL$ as catalyst, with 1.0 equivalent of DIAD, several aminated 3-subtituted oxindoles **207** were obtained with excellent yields and enantioselectivities. Only the amination product derived from 3-phenyl-2-oxindole was obtained with acceptable yield (74%) but in moderate enantiopurity (63% ee), presumably due to its high reactivity toward electrophiles in the background noncatalyzed reaction. It should be noticed that the amination catalyzed by the quasienantiomeric ligand $(DHQ)_2PHAL$ also proceeded in excellent yield and enantioselectivity, demonstrating that both

enantiomers (**207** or *ent*-**207**) can be obtained upon the choice of the appropriate catalyst.

Soon after, closely related protocols were published [126], with similar results.

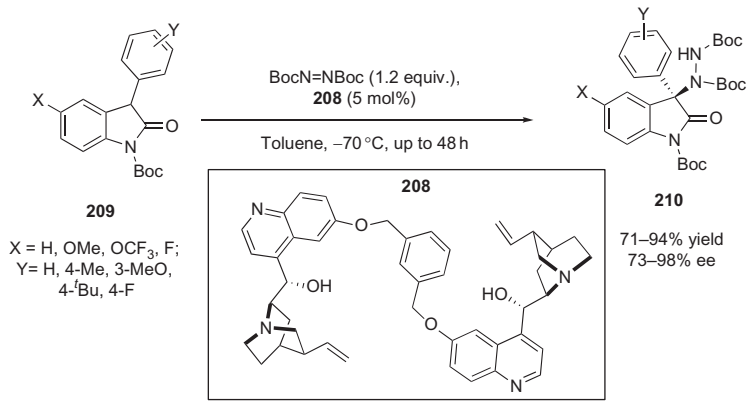
In 2010, Matsunaga, Shibasaki and coworkers [127] published the amination of 3-substituted oxindoles with di-*tert*-butyl azodicarboxylate catalyzed by an homodinuclear Ni_2–Schiff base complex with excellent results (up to 99% yield, up to 99% ee). Moreover, the authors demonstrated the synthetic utility of their process, obtaining from an aminated product a key intermediate in the synthesis of AG-041R, a gastrin/CCK-B receptor agonist. To overcome the substrate limitation in the aryloxindole examples present in all the methodologies reported until then, Barbas III and coworkers developed a new dimeric quinidine-derived catalyst (**208**) that allowed the organocatalytic amination of a wide range of *N*-Boc-3-aryloxindoles **209** in excellent results (Scheme 84) [128].

On the other hand, nitrosobenzene was used by different groups in order to achieve the oxyamination of oxindoles. Nitrosobenzene has two electrophilic atoms: the oxygen (aminooxylation reaction) or the nitrogen atom (oxyamination reaction). Therefore, to develop a synthetically useful reaction, the appropriate choice of the catalyst is crucial in order to obtain only one regioisomer.

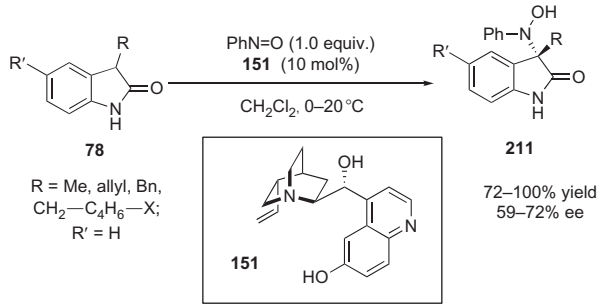
In 2010, Liu, Chen, and coworkers [129] reported the oxyamination reaction of *N*-unprotected 3-substituted oxindoles **78** catalyzed by cupreine **151** (Scheme 85).

Despite the total regioselectivity of the reaction (only oxyaminated products **211** were obtained in all the examples), the enantioselectivities were quite low (up to 72% ee). Interestingly, reductive cleavage of the N—O bond with Zn in AcOH furnishes the aminated product in good yield maintaining the enantiomeric purity (Scheme 86).

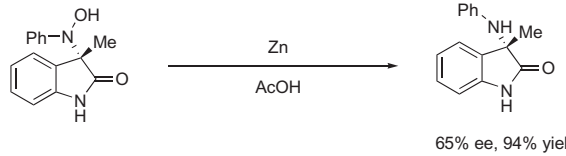
Subsequently, two related protocols were published employing as catalysts bifunctional tertiary amine–thioureas [130], with similar or slightly improved



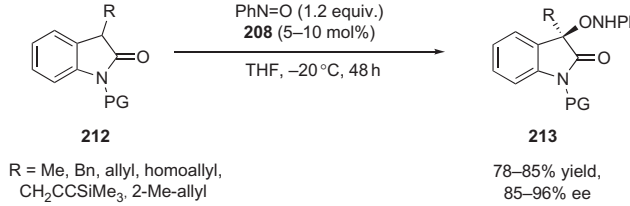
SCHEME 84 Highly enantioselective organocatalytic α-amination of aryloxindoles.



SCHEME 85 Asymmetric organocatalytic *N*-nitroso-aldol reaction of oxindoles.



SCHEME 86 Reductive cleavage of the N—O bond in oxyaminated oxindoles.



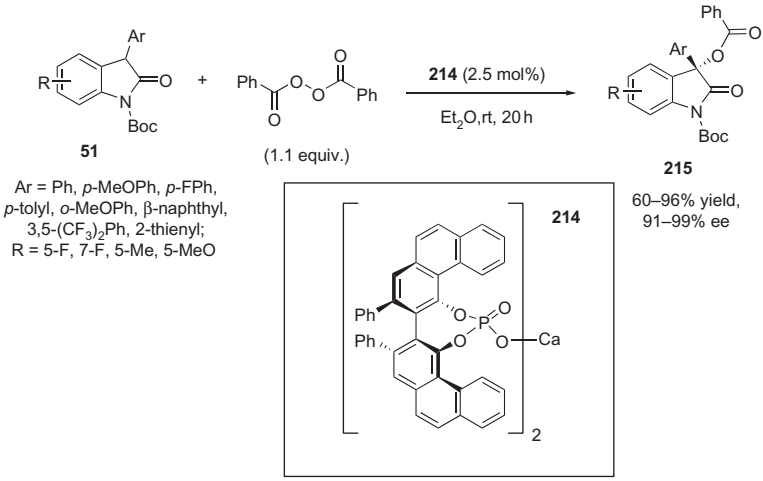
SCHEME 87 Enantioselective aminooxygenation of oxindoles.

results. Finally, in 2011, Feng and coworkers [131] reported an organometallic version of this reaction catalyzed by a $Sc(OTf)_3$/*N*,*N*′-dioxide complex, with excellent results in terms of yield and enantioselectivities (up to 95% yield, up to 95% ee).

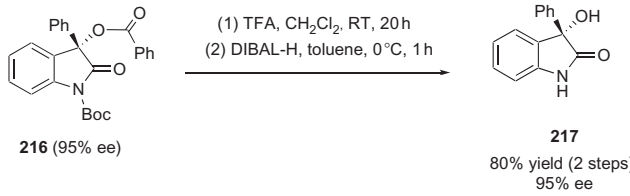
The first protocol developed for the introduction of an oxygen atom at the C3 position of an oxindole was reported by Barbas' group [132]. Employing as catalyst the newly designed dimeric quinidine-derived compound **208** (see Scheme 84), and nitrosobenzene as an electrophilic oxygen source, several *N*-protected 3-substituted oxindoles were aminooxylated in good yields and enantioselectivities (Scheme 87).

Presumably, the *O*-selectivity observed takes place as a result of the directing effect of a hydroxyl group of catalyst that engages in a hydrogen bond with the nitrogen atom of PhNO, enabling the oxindole enolate to attack at the electrophilic oxygen atom.

In 2011, Antilla and coworkers [133] reported a highly enantioselective catalytic benzoyloxylation of *N*-Boc-3-aryloxindoles catalyzed by a chiral



SCHEME 88 Highly enantioselective benzoyloxylation of 3-aryloxindoles.

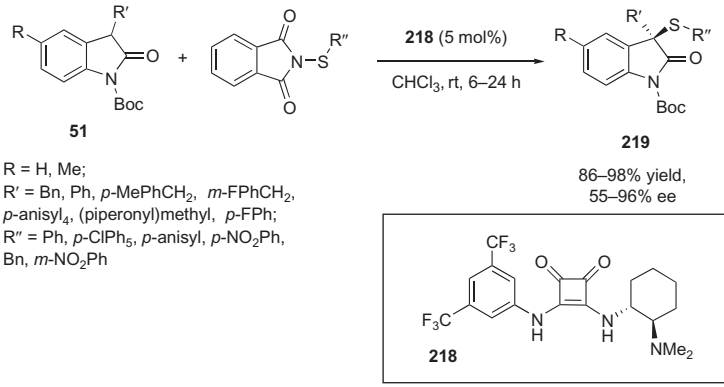


SCHEME 89 Enantioselective preparation of 3-hydroxy-3-phenyl-2-oxindole **217**.

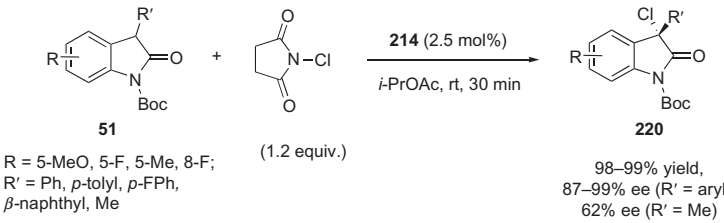
VAPOL-derived calcium phosphate (Scheme 88). Using readily available benzoyl peroxide as a benzoyloxylation reagent together with the chiral phosphoric acid salt dimer **214**, a series of 3-aryloxindoles were benzoyloxylated in mild conditions with excellent yields and enantioselectivities. Moreover, in a short two steps sequence, the authors transformed the benzoyloxylated product **216** to a known 3-hydroxy-2-oxindole (**217**), demonstrating the synthetic utility of this methodology (Scheme 89).

Although many oxindoles bearing a sulfenyl group at the stereogenic C3 position have been found to possess anticancer, antifungal or antitubercular activities, a direct and asymmetric protocol to construct oxindole derivatives bearing a sulfur-containing quaternary stereocenter has remained elusive until 2012. Recently, Enders and coworkers [134] published an organocatalytic sulfenylation of *N*-Boc-3-substituted oxindoles **51** with *N*-(sulfenyl)phthalimides catalyzed by a chiral squaramide **218** (Scheme 90).

A wide set of oxindoles were subjected to sulfenylation such as 3-benzyl, 3-aryl, and 3-heteroaryl oxindoles, obtaining in all the cases the final product in excellent results in terms of yield and enantioselectivities. The nature of the aryl group in the sulfenyl moiety did not affect the excellent results, but when



SCHEME 90 Organocatalytic synthesis of 3-sulfenilated *N*-Boc-protected oxindoles.



SCHEME 91 Chiral calcium VAPOL phosphate-mediated asymmetric chlorination of 3-substituted *N*-Boc oxindoles.

the reaction was run with *N*-(benzylsulfenyl)phthalimide, the enantiomeric purity of the final product dropped dramatically (55% ee).

Almost at the same time, two independent groups found very similar organocatalytic protocols using as catalysts different *Cinchona* alkaloid derivatives [135]. In both cases, the final products were synthesized with excellent results. Finally, Feng and coworkers [136] developed a metal-catalyzed version of this reaction. Using the cooperative catalysis of a chiral *N*,*N*′-dioxide–$Sc(OTf)_3$ complex and a Brønsted base, they performed the sulfenylation of unprotected 3-subtituted oxindoles again with high levels of enantioselectivities (up to 99% ee) and excellent yields (up to 97%).

Although the enantioselective construction of a carbon–halogen center is a topical area in asymmetric catalysis due to the fact that the halogen atoms attached to a chiral stereocenter can serve as a linchpin for further stereospecific manipulations and, moreover, an important number of pharmaceutical agents contain chiral centers with halogen substitution (such as fluoro or chloro), it was not until 2011 that Antilla and coworkers [137] reported the first asymmetric chlorination of *N*-Boc-3-substituted oxindoles **51** (Scheme 91).

Employing as a catalyst the novel chiral calcium VAPOL-derived phosphate salt (**214**, see Scheme 88) and *N*-chlorosuccinimide as a source of

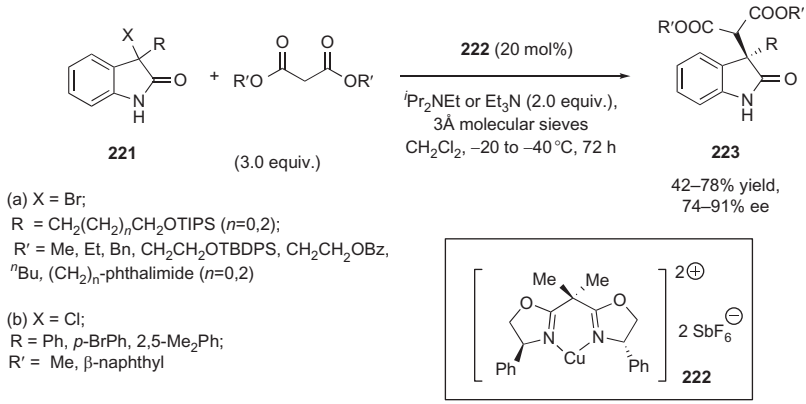
electrophilic chlorine, a wide set of *N*-Boc-3-aryloxindoles were chlorinated with almost total enantioselectivity and quantitative yields. When the reaction was tested with *N*-Boc-3-methyl oxindole, although the yield was maintained almost quantitative (98% yield), the enantioselectivity dropped to 62% ee.

Shortly afterward, Shi and coworkers published that a complex derived from an axially chiral BINIM ligand and Ni(II) catalyzed the asymmetric chlorination of 3-substituted oxindoles [138], as well as a metal-free version [139] of this reaction utilizing *O*-benzoylquinidine as a catalyst. While in the organocatalytic approach the results ranged from excellent for 3-aryloxindoles (up to 93% ee) to poor for 3-alkyl oxindoles (22% ee), in the Ni-catalyzed one, the final products were obtained with moderate to good yields (up to 99%) but in lower enantioselectivities (7–88% ee).

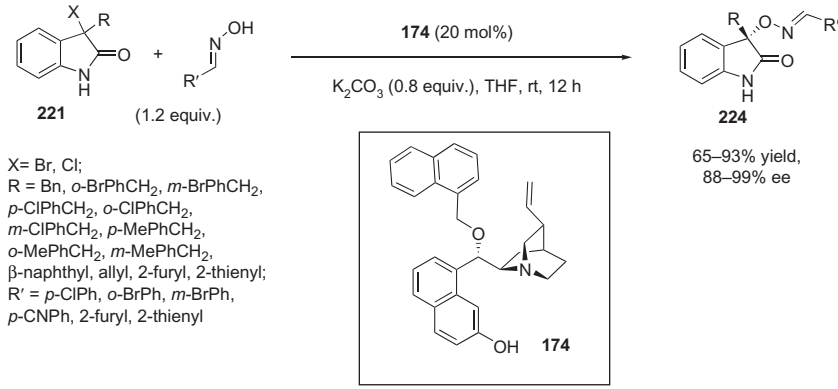
NUCLEOPHILIC SUBSTITUTION REACTIONS OF FUNCTIONALIZED 3-SUBSTITUTED OXINDOLES

Up to now, in the vast majority of reactions discussed in this chapter for the stereoselective functionalization of an existing 3-substituted oxindole, the oxindole acts as a nucleophile at C3; the opposite approach will be disclosed in this section, where the oxindole moiety is employed as the electrophilic partner at C3.

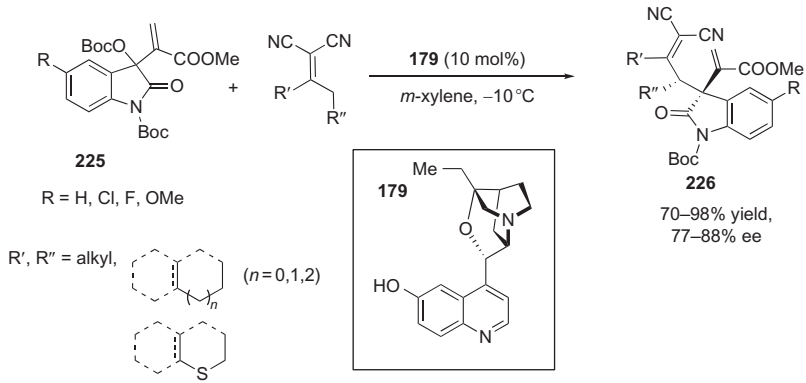
The first example of an enantioselective stereoablative metal-catalyzed alkylation of 3-halooxindoles was reported by Stoltz and coworkers [140] (Scheme 92). The treatment of racemic 3-bromo- or 3-chloro-3-subtituted oxindoles **221** with an organic base such as triethylamine or DIPEA promotes the *in situ* formation of a highly reactive *o*-azaxylylene intermediate that undergoes stereoselective malonate alkylation catalyzed by a chiral bis(oxazoline)–Cu(II) species (**222**). Both 3-alkyl- and 3-aryl-3-bromo-2-oxindoles were alkylated, with results ranging from good to excellent (up to 78% yield, up to 91% ee).



SCHEME 92 Enantioselective stereoablative alkylation of 3-halooxindoles.



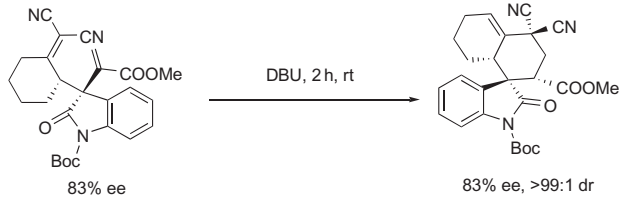
SCHEME 93 Organocatalytic stereoablative hydroxylation of 3-halooxindoles.



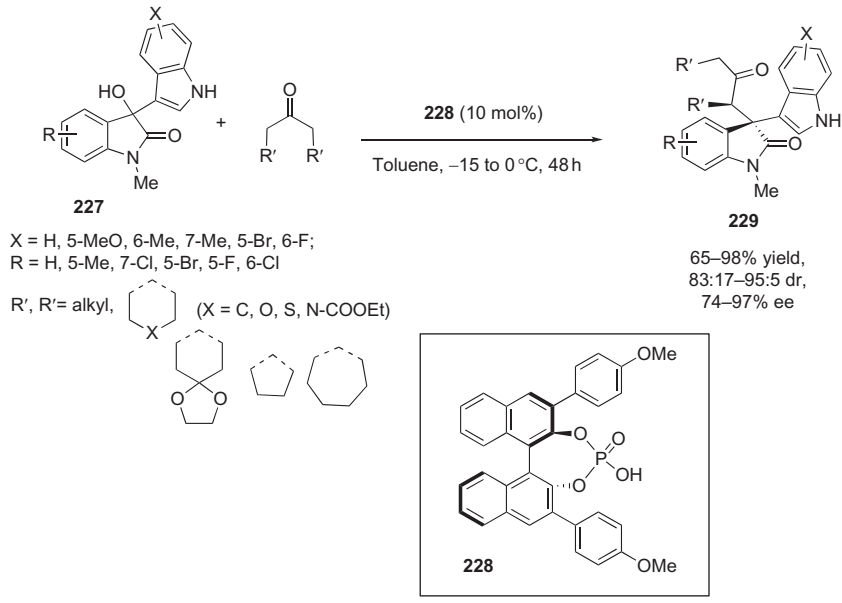
SCHEME 94 Asymmetric allylic alkylation of Morita–Baylis–Hillman carbonates derived from isatins.

In the wake of the pioneering work by Stoltz, Yuan, and coworkers [141] have recently developed the first organocatalytic stereoablative process for the hydroxylation of 3-halooxindoles (Scheme 93). Catalyzed by the cupreidine (1-naphthylmethyl) ether **174** and using aromatic oximes as hydroxylating agents, a wide variety of substrates **221** were tested to afford the hydroxylated 3-substituted oxindoles **224** in high yields (up to 93%) and enantioselectivities (up to 99% ee) under mild conditions. Notably, the known 3-benzyl-3-hydroxyl-2-oxindole was obtained after reductive cleavage of the O—N bond without loss of enantiopurity.

On the other hand, Chen *et al.* [142] developed the Lewis base-catalyzed asymmetric allylic alkylation of racemic MBH carbonates derived from isatins (**225**), giving access to 3,3-disubstituted oxindoles **226** in enantioselective fashion (Scheme 94). Employing as alkylating agents the allyl anions of α,α-dicyanoolefins derived from cyclic and acyclic ketones and β-isocupreidine



SCHEME 95 Intramolecular Michael addition of adducts **226**.



SCHEME 96 Direct asymmetric α-alkylation of ketones with 3-hydroxy-2-oxindoles.

179 as a chiral Lewis base catalyst, they achieved the synthesis of oxindoles of high-molecular complexity with good yields (up to 98%), excellent diastereoselectivity (up to >99:1 dr), and good enantioselectivities (up to 88% ee).

Moreover, the treatment of adducts **226** with a tertiary amine such as DBU promotes an intramolecular Michael addition to furnish a tetracyclic oxindole without loss of enantiopurity (see Scheme 95 for an example).

Subsequently, Chen's group extended this methodology employing as nucleophiles α-angelica lactone [143] and protected hydroxylamines [144]. Later on, Lu and coworkers [145] used simple nitroalkanes as nucleophiles. In these three instances, β-isocupreidine **179** was chosen as the best catalyst.

In order to perform a metal-free α-alkylation of unmodified symmetrical ketones with an activated alcohol, Peng and coworkers [146] chose an isatin-derived 3-indolyl-2-oxindoles **227** as alkylating agents (Scheme 96). Under catalysis by the chiral BINOL-derived phosphoric acid **228**, cyclic

and acyclic ketones were successfully alkylated with the 3-hydroxyl-2-oxindole derivatives **227**. As a result, a wide collection of 3,3-disubstituted oxindoles **229** with high-molecular complexity were synthesized with excellent yields (up to 98%), diastereoselectivities (up to 95:5 dr), and enantioselectivities (up to 97% ee).

CONCLUDING REMARKS

Molecules containing an oxindole core subunit, either of natural or of synthetic origin, are attractive synthetic targets due to their biological activities. In the past decade, but more particularly in the past 3 years, we have witnessed numerous advances in the field of catalytic enantioselective synthesis of chiral 3,3-disubstituted oxindoles. In spite of this impressive progress, there are still significant challenges in the design and development of efficient synthetic methods that allow the stereocontrolled preparation of this important class of compounds. For instance, the application of the catalytic enantioselective reactions described in this chapter to the total synthesis of oxindole natural products has been actually achieved only in a very limited extent. Is therefore necessary to extend the range of applicability of protocols based in both transition metal- and organocatalyzed reactions. These developments will have relevant applications for the asymmetric synthesis of complex natural products and for the preparation of new pharmaceutically active compounds. Research in this field remains unabated, as exemplified by the several reports that have appeared in the literature after the completion and during the review of this chapter [147].

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Chapter 5

Abyssomicins: Isolation, Properties, and Synthesis

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Chapter Outline

INTRODUCTION

Perhaps one of the most significant scientific achievements of the twentieth century is the discovery of antibacterials and their use in therapies of infectious diseases. These substances saved countless lives and helped to control the diseases that were main cause of mortality for many years. The success of antibacterial agents is based essentially on their ability to act selectively against bacteria as a result of structural and biochemical differences between prokaryotic and eukaryotic cells. The term antibiotic was coined by Waksman and was used to describe substances produced by microbes capable of preventing the growth of other microbes [1]. The modern era of these molecules is usually associated with the work of Erlich and Fleming [2]. Erlich's work

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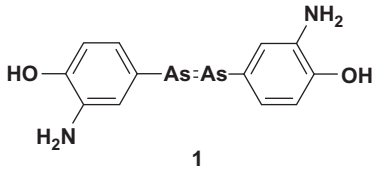
on sexually transmitted diseases eventually produced salvarsan **1**, the antibacterial drug used in treatment of syphilis. A little latter, Fleming unveiled his observations on antimicrobial effect of *Penicillium* strain which ultimately led to isolation, production, and synthesis of penicillin G **2**.



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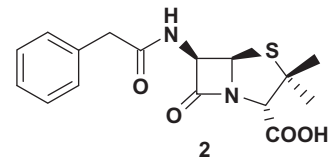


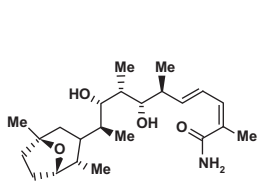
2

This initial discovery of substances with antibacterial properties prompted intensive activity in this area and period between 1950s and 1970s is usually regarded as a golden era of antibiotics. In those few decades, a range of structurally different and potent classes of compounds was developed, eventually resulting in around 200 substances currently available in the drug market, that generated, in 2009, global sales of US$ 42 billion [3]. Even long before the extensive application of these medicines, some reports suggested that bacterial cells could develop resistance to the lethal effect of antibiotics [4]. Consequently, therapies may fail and common infections could become serious threat. Extensive, sometimes inappropriate, use of antibiotics, the multiple resistance, and appearance of new bacterial pathogens exacerbated this problem and, as a result, there is almost constant need for new, safe, and effective compounds. Even today, infectious diseases are the second major cause of death worldwide, or the third in developed countries and, therefore, it is not surprising that this area still attracts much attention from medicinal chemists in particular and chemists in general [5]. Natural sources, specifically microbial secondary metabolites, are currently the most important supply of new antibiotics. Of around 20,000 of these compounds, more than 40% are produced by actinobacteria, which represent the major source of antibiotic substances of interest to the pharmaceutical industry [6].

SECONDARY METABOLITES FROM ACTINOMYCETES

Actinomycetes are one of the most important sources of naturally occurring antimicrobial agents [6,7]. The discovery of streptomycin, produced by genus *Streptomyces*, marked the beginning of intensive research in the area of these diverse Gram-positive bacteria [8]. Actinobacteria are a prolific source of structurally diverse metabolites belonging to a various classes of organic compounds such as terpenes, polyketides, peptides, butenolides, and macrolides with a wide range of biological properties. It is estimated that the top 10 cm of global soil contains 10^{25}–10^{26} actinomycetes, of which only 10^{7} have been screened for antibiotic production, making them very attractive sources of new biologically important compounds [7b]. Actinomycetes are involved in

creating around 50% of known antibiotics, and the structural complexity and diversity of compounds of this origin is best illustrated by some of the recently isolated natural products such as **3–8** [9].



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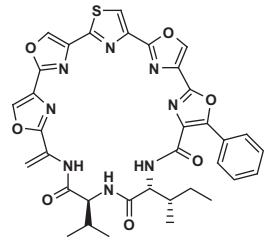

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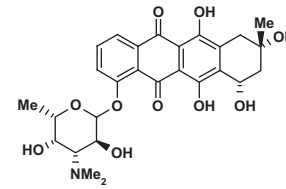

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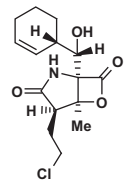
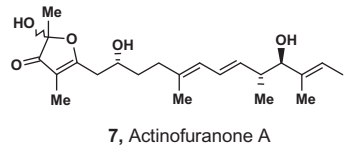


6, Salinosporamide A



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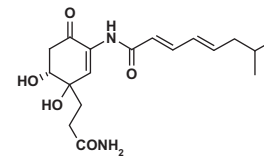


8, Daryamides A

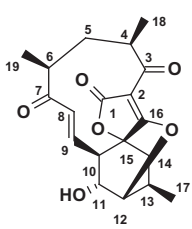
Apart from the terrestrial environment, actinobacteria inhabit the marine ecosystem as well. Around 10% of the sea bacteria are actinomycetes. They are usually isolated from sediments but also from free swimming and sessile vertebrates and invertebrates. Important biochemical processes involving non-ribosomal polyketide synthetase and polyketide synthetase are functional in these bacteria and are the hallmarks of secondary metabolite production. The currently underexplored and exciting world of the marine actinomycetes offers further opportunity to discover new genera and, consequently, new metabolites with distinct biological properties [10]. Actinobacteria have been a source of abyssomicins, a new class of unique polycyclic natural products with potent antibacterial activity, which are the subject of this chapter.

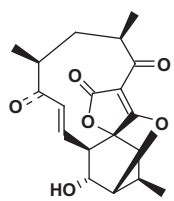
ISOLATION, STRUCTURE, BIOSYNTHESIS, AND BIOLOGICAL PROPERTIES OF ABYSSOMICINS

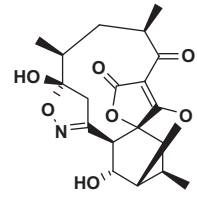
Isolation and Structures

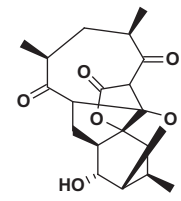
The first reported abyssomicins, abyssomicin B, C, and D, were isolated from marine actinomycetes *Verrucosispora* strain AB 18-032 found in a sediment sample in the Japanese sea at a depth of 289 m [11]. Their discovery has been the outcome of the search for secondary metabolites possessing inhibitory effect on *para*-aminobenzoic acid (*p*ABA) biosynthesis. By using an agar-plate diffusion assay, it was shown that some of these initially discovered compounds inhibited *p*ABA biosynthesis, a biosynthetic pathway found in

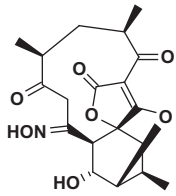
many microorganisms but not in humans. A few years later, additional members of this class of compounds were reported. Abyssomicin I was found in soil-derived *Streptomyces* strain (CHI39) [12].

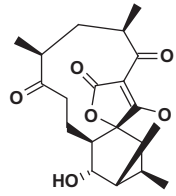


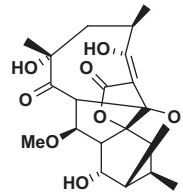


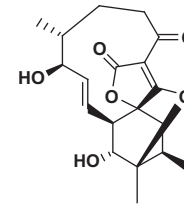


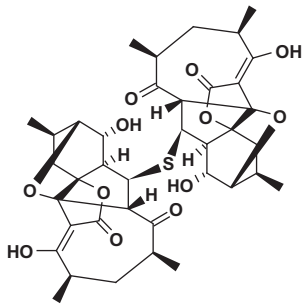


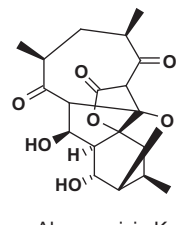


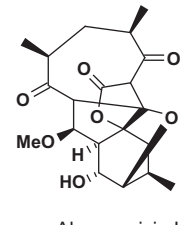












The most significant structural variation, compared to other members of the family, is a different methylation pattern. Abyssomicin E was isolated from *Streptomyces* sp. (HKI0381) and is most closely related to abyssomicin D [13]. Both compounds lack typical tetronic moiety and also the conjugated ketone functionality. Similarly, abyssomicins G and H, isolated from already mentioned *Verrucosispora* strain AB 18-032, are devoid of the conjugated ketone moiety, while the former possesses also an oxime functionality [14]. Interestingly, *atrop*-abyssomicin C was first discovered during the synthesis of abyssomicin C [15]. The work directed toward the synthesis of the natural product afforded the compound whose spectroscopic data did not completely match with that of the previously reported abyssomicin C. During the NMR experimentation with the synthesized derivative, the isomerization process was observed leading to 2:1 mixture in favor of the natural product [15b].

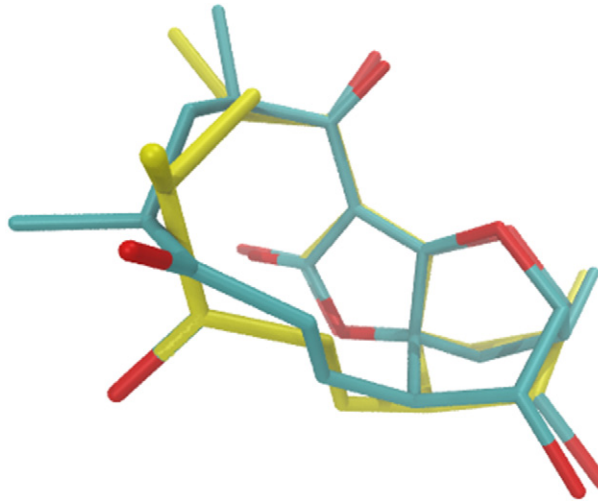
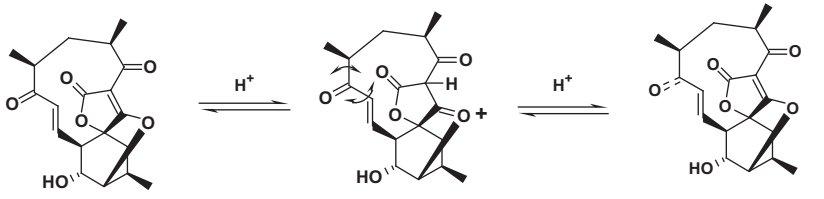


FIGURE 1 Overlaid structures of *atrop*-abyssomicin C (yellow) and abyssomicin C (cyan).

Further study employing X-ray crystallographic analysis revealed that the synthesized compound was a diastereomeric atropisomer named *atrop*-abyssomicin C. The most prominent structural deviations between the two lie in the region of the conjugated ketone functionality (Fig. 1). While in natural product (Fig. 1, cyan) the carbonyl at C(9) adopts *s-trans* conformation in the synthetic compound (Fig. 1, yellow), the orientation is *s-cis*. Consequently, these conformational variations influence the electrophilic properties of the α,β-unsaturated ketone making *atrop*-abyssomicin C better Michael acceptor due to a higher degree of the conjugation. Additional structural divergences are present in the region of the methyl C(19) and the carbonyl C(3) residue. Further inspection of the X-ray structures revealed a certain strain within the macrocyclic ring in the region of the conjugated C(8)═C(9) bond and the tetronic C(2) atom [15b].

Inspection of the abyssomicin C and *atrop*-abyssomicin C structural properties did not reveal any obvious steric effects accountable for the observed atropisomerism, and therefore, a further study of the relationship between the two was carried out [15b]. Initial attempts to convert *atrop*-abyssomicin C to abyssomicin C under thermal conditions failed and were eventually achieved at reflux in 1,2-dichlorobenzen at 180 °C to afford a 1:1 mixture of the isomers at equilibrium. In contrast, isomerization promoted by catalytic etheral HCl efficiently produced the isomers in ratios depending on the reaction conditions. This study proposed several possible mechanistic pathways for the interconversion of the atropisomers under acidic conditions, while the authors favored the one outlined in Scheme 1 [15b].



Abyssomicin C *Atrop*-abyssomicin C

SCHEME 1

Under the acidic conditions, the tetronic C(2) is protonated generating the oxocarbenium ion **9**. This process transforms the already deformed and partially pyramidalized C(2)sp^2 carbon, as revealed by X-ray analysis, into a sp^3 carbon relieving strain in the macrocyclic moiety and facilitating the rotation around the requisite C(7)—C(8) bond. Interestingly, the presence of *atrop*-abyssomicin C in natural material and its production by *Verrucosispora* AB-18-932 were established after completion of its synthesis [14]. Reconsideration of experimental data from cultivation which led to the discovery of abyssomicin C confirmed the presence of *atrop*-abyssomicin C as well, which, in fact, was the main component, but was suggested to rearrange to its isomer by using acidic HPLC conditions during the purification procedure [14].

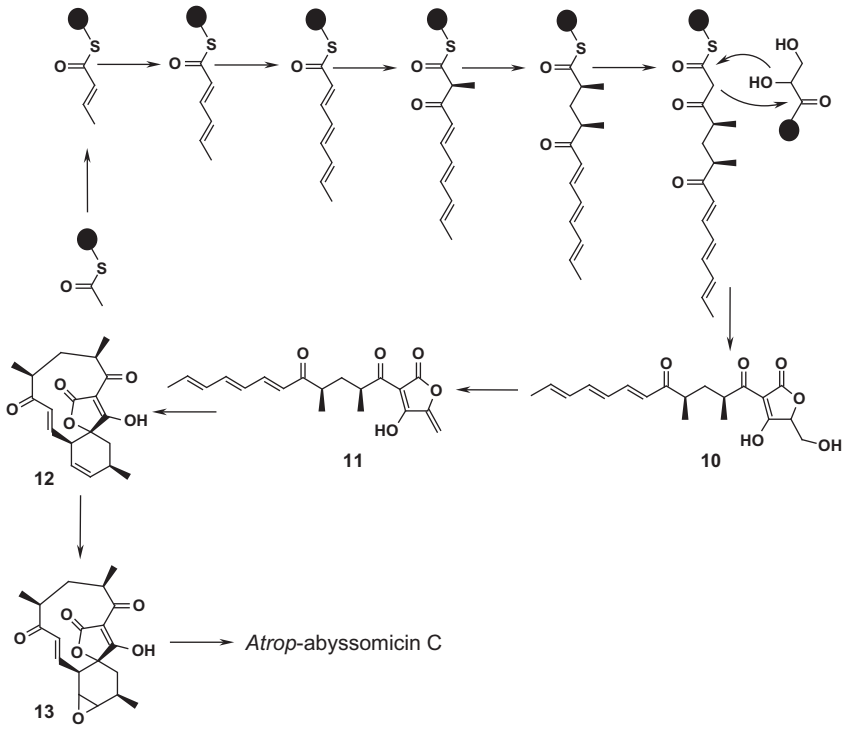
The most recent additions to the abyssomicin family, abyssomicin J, K, and L reported in 2012, were isolated from sediment-derived actinomycete *Verrucosispora* strain MS100128 [16]. While abyssomicin K and L possess structural properties found in congeners E and D, abyssomicin J has a unique dimeric structure. It has been shown that this compound, as a major component, could be formed in reaction of abyssomicin C and Na_2S [16]. Similarly, a chemical relationship was demonstrated for abyssomicin C and derivatives K and L as well.

Biosynthesis

The biosynthesis of *atrop*-abyssomicin C has been investigated by means of feeding studies using ^{13}C-labeled building blocks and identification of abyssomicin biosynthetic gene cluster [17]. It has been proposed that *atrop*-abyssomicin C is synthesized as a linear polyketide chain from five acetates, two propionates, and the glycolytic pathway metabolite, followed by formation of tetronate, Diels–Alder cycloaddition, and oxygenation.

Involvement of a polyketide biosynthetic pathway has been confirmed by feeding growing *Verrucosispora* AB-18-932 cultures with [1-^{13}C]acetate, [1,2-^{13}C]acetate, and [1-^{13}C]propionate. Upon feeding with [1-^{13}C]acetate, the isolated *atrop*-abyssomicin C was analyzed by ^{13}C NMR and the results suggested incorporation of ^{13}C carbons into C(1), C(7), C(9), C(11), and

C(13) positions. Related experiments with [1,2-^{13}C]acetate confirmed the previous results and incorporation was found in C(1)/C(2), C(7)/C(8), C(9)/C(10), and C(11)/C(12) positions. When the [1-^{13}C]propionate building block was used for feeding studies, increased ^{13}C signals were observed for C(3) and C(5) of *atrop*-abyssomicin C. These experiments demonstrated assembly of five acetate and two propionate units as building blocks but left unassigned carbons C(14)–C(16). Attempts to use labeled glycerol, serine, alanine, and succinic acid, putative C_3 precursors, did not yield ^{13}C-labeled *atrop*-abyssomicin C. Remarkably, only the use of [1,2-^{13}C]glucose led to the incorporation of C_3 unit into the backbone of the natural product, indicated that this fragment originated from the glycolytic pathway. The proposed biosynthesis is outlined in Scheme 2. Although the gene encoding Diels–Alderase was not found, the spiro compound **12** was proposed to be formed via intramolecular Diels–Alder reaction of the linear tetronic derivative **11**. Further reactions catalyzed by monoamine oxidase to form epoxide **13** and the ring opening via the intramolecular nucleophilic addition of the enol moiety afford *atrop*-abyssomicin C.



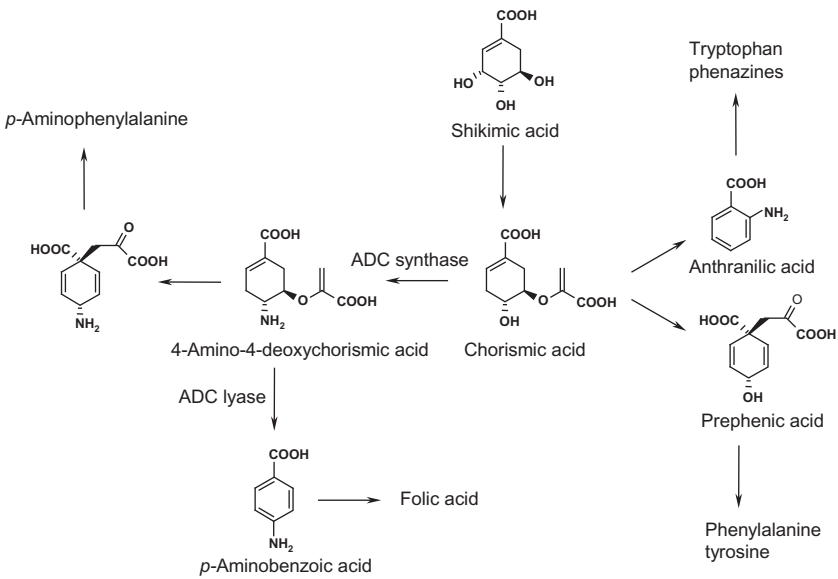
SCHEME 2

Biological Properties

As already mentioned, abyssomicins were discovered during the search for new natural products which inhibit biosynthesis of *p*ABA, an important component in tetrahydrofolate production. The biosynthetic route producing *p*ABA is part of the shikimic acid pathway leading to chorismic acid (Scheme 3) [18].

At the chorismate branch point, evolving routes afford aromatic amino acids and *p*ABA. Two key enzymes in *p*ABA biosynthesis are 4-amino-4-deoxychorismate (ADC) synthase responsible for converting chorismate into ADC and ADC lyase which catalyses the elimination reaction of ADC to produce *p*ABA [19]. ADC synthase is an enzyme complex formed from two components, the products of *pab*A and *pab*B genes. In order to rationalize the established biological properties of abyssomicins, their activity has been investigated on the molecular level. Interestingly, the structure of abyssomicin C and *atrop*-abyssomicin C, actually their oxabicyclo[2.2.2]octane ring, resembles the conformation of chorismate, suggesting that these natural products are substrate mimetics (Fig. 2) [20].

Detailed investigation of *atrop*-abyssomicin C activity revealed the ADC synthase *pab*B subunit of *Bacillus subtilis* as a key molecular target [21]. Its Cys-263 amino acid, acting as an S-nucleophile, binds covalently to the natural product by exploiting the reactivity of the conjugated ketone functionality. But the mechanism of this reaction is slightly more complex, as showed by studying the reactions of 2-sulfanylethanol or N-acetylcystein and *atrop*-abyssomicin C [21]. The initial attack of Cys-263 onto the conjugated ketone



SCHEME 3

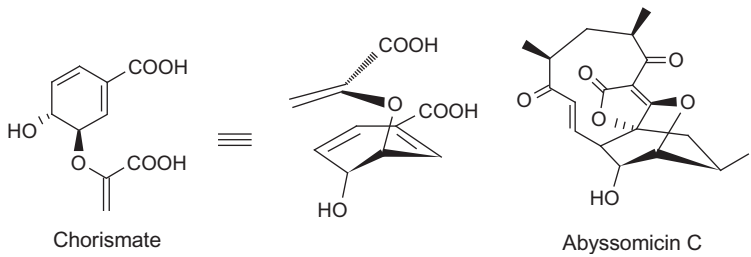
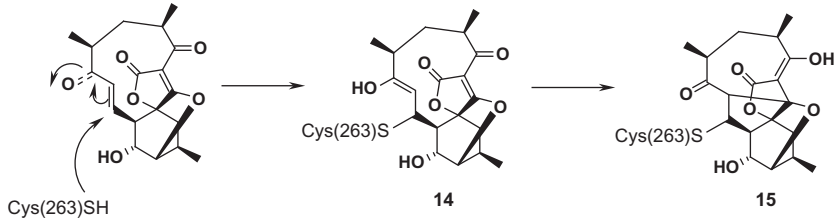


FIGURE 2 Comparison of the conformations of chorismic acid and abyssomicin C.

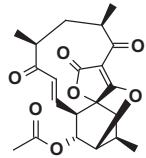
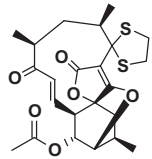
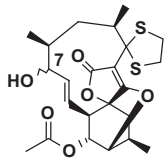
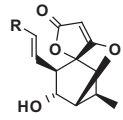


SCHEME 4

produces **14** and this process is followed by Michael addition of the generated enolate/enol onto the tetronic moiety affording, as a final product, compound **15**, derivative of abyssomicin D (Scheme 4).

The antibacterial properties of the abyssomicin family members have been intensively investigated [11–13,15,16,22]. However, in these tests, only abyssomicins possessing the α,β-unsaturated ketone functionality, with the exception of abyssomicin J, showed activity. Abyssomicin C demonstrated antibiotic activity against Gram-positive bacteria including pathogenic *Staphylococcus aureus* strains. The minimal inhibition concentration (MIC) against methicillin-resistant *S. aureus* and multiresistant bacteria including vancomycin-resistant *S. aureus* strain were 4 and 13 μg/mL, respectively. A brief study of the biological activity of some abyssomicins and their derivatives, outlined in Table 1, demonstrated the importance of the α,β-unsaturated ketone functionality for activity and revealed superior biological features of *atrop*-abyssomicin C (entry b, Table 1) as a result of its more reactive Michael acceptor moiety [15b]. Interestingly, acylated abyssomicin C, compound **16** (entry c, Table 1), was as potent as the parent abyssomicin C (entry a, Table 1) suggesting that the role of the hydroxy moiety is not detrimental in shaping their biological profile. Other tested compounds (entries e–i, Table 1), including simplified derivatives which lack the conjugated ketone, did not show any activity. Some compounds, such as **21** and **22** (entries h and i, Table 1), possess the requisite conjugation but were not biologically active, suggesting that the stereochemical aspect of the macrocyclic ring is important in orientating the Michael acceptor functionality correctly.

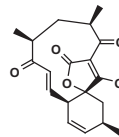
TABLE 1 MICs of Abyssomicins and Derivatives Against Methicillin-Resistant *S. aureuas*

Entry	Compound		MIC (μM)
a	Abyssomicin C		20
b	*atrop*-Abyssomicin C		15
c		**16**	20
d		**17**	70
e		**18** C(7)-(*R*)	> 500
f		**19** C(7)-(*S*)	> 500
g		**20** R = H	> 500
h		**21** R = COOMe	> 500
i		**22** R = $C(O)CH_3$	> 500

The antimycobacterial efficacy of the abyssomicins and some of their derivatives has been investigated against *Mycobacterium smegmatis*, *Mycobacterium bovis*, and *Mycobacterium tuberculosis* (Table 2) [23]. Naturally occurring abyssomicin C and *atrop*-abyssomicin C (entries a and c, Table 2) exhibited low micromolar inhibition, in particular against *M. tuberculosis*, while their antipodes (entries b and d, Table 2) were slightly less active. The compounds **23** and **24** (entries e and f, Table 2), although possessing the enone fragment, were significantly less efficacious, confirming the importance of the oxabicyclo[2.2.2]octan fragment, which is likely to mimic chorismate, for the biological activity.

The fact that abyssomicin J showed inhibitory activity (MIC: 3.125 μg/mL) against Bacille Calmette Guerin, an attenuated strain of the bovine tuberculosis

TABLE 2 MICs of Abyssomicins and Derivatives Against Mycobacteria

		MIC μg/mL (μM)		
Entry	Compound	M. smegmatis mc²155	M. bovis BCG	M. tuberculosis
a	(–)-abyssomicin C	10 (29)	2.5 (7.2)	1.2 (3.6)
b	(+)-abyssomicin C	40 (110)	20 (58)	nd
c	(–)-*atrop*-abyssomicin C	20 (58)	5.0 (14)	2.5 (7.2)
d	(+)-*atrop*-abyssomicin C	40 (110)	10 (29)	nd
e	 **23** (–)-isomer	> 80 (230)	40 (120)	nd
f	**24** (+)-isomer	> 80 (230)	40 (120)	nd

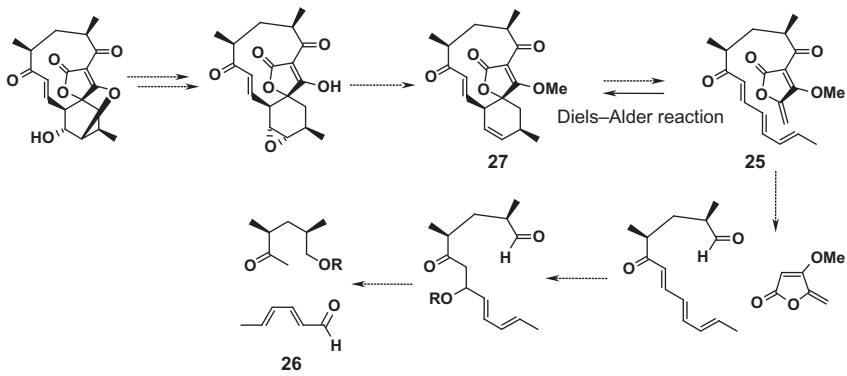
bacillus *M. bovis*, was quite surprising, since this compound does not possess the conjugated ketone pharmacophore [16]. To address this departure from the theory of the Michael-acceptor-related biological activity, the authors proposed *in situ* formation of the requisite functionality via the thioether oxidation/reverse Michael addition sequence promoted, most likely, by P450 enzymes. This hypothesis was experimentally supported [16], namely, oxidation of abyssomicin J with the Oxone furnished abyssomicin C via the sulfoxide/sulfon intermediates suggesting a prodrug propensity of the dimeric thioether.

SYNTHESIS OF ABYSSOMICINS

The fascinating structural arrangement and biological profile of abyssomicin family members caught attention from synthetic chemists almost immediately after report on their isolation and a number of research teams initiated programs toward development of their total synthesis [24]. The common structural features of these molecules are the tetronate-fused oxabicyclo[2.2.2]octane and the 11-membered macrocyclic ring. An additional element contributing to the structural complexity is the presence of a several chiral centers. Although these properties make abyssomicins challenging synthetic targets, in recent years, several routes for their preparations have been developed.

Approach Based on an Intramolecular Diels–Alder Reaction

The first synthesis of abyssomicin C was reported in 2005 by Sorensen's group [25]. Their approach was based on an intramolecular Diels–Alder reaction,

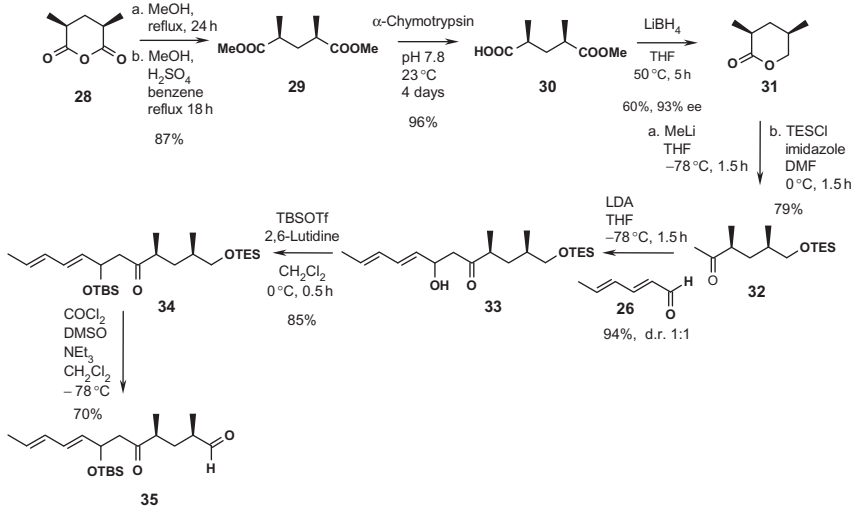


SCHEME 5

creating the spirotetronic moiety and the macrocyclic ring in a single operation (Scheme 5). The cycloaddition step involving the tetronate exocyclic double bond was expected to establish majority of the structural properties, including the cyclohexene double bond as a valuable functionality in further elaboration of the product. The reactivity of the simple tetronates in the cycloadditions has been investigated mainly in the intermolecular Diels–Alder reactions [26a–c], while some related compounds were also studied in intramolecular 1,3-dipolar cycloadditions [26d]. Interestingly, these substrates proved to be reactive dienophiles, showing excellent site selectivity leading to the formation of the spyrocyclic compounds. According to the retrosynthetic plan (Scheme 5), the linear cycloaddition precursor **25** would be created starting from commercial aldehyde **26**. Elongation of the hydrocarbon chain of the aldehyde would be achieved via an aldol condensation followed by the addition of a lithiated tetronate. The transformation of cycloadduct **27**, generated via an intramolecular Diels–Alder reaction from **25**, would lead to the natural abyssomicin C via the epoxidation/cyclization sequence involving the cyclohexene double bond and the tetronic moiety.

The synthesis was initiated with the transformation of dimethylglutaric acid anhydride **28** into the *meso* diester **29** using standard esterification procedures (Scheme 6). Desymmetrization of **29** was achieved using chymotrypsin-promoted hydrolysis to afford monoester **30** in excellent yield. The following step, a chemoselective reduction employing $LiBH_4$, afforded lactone **31** in moderate yield and in 93% e.e.

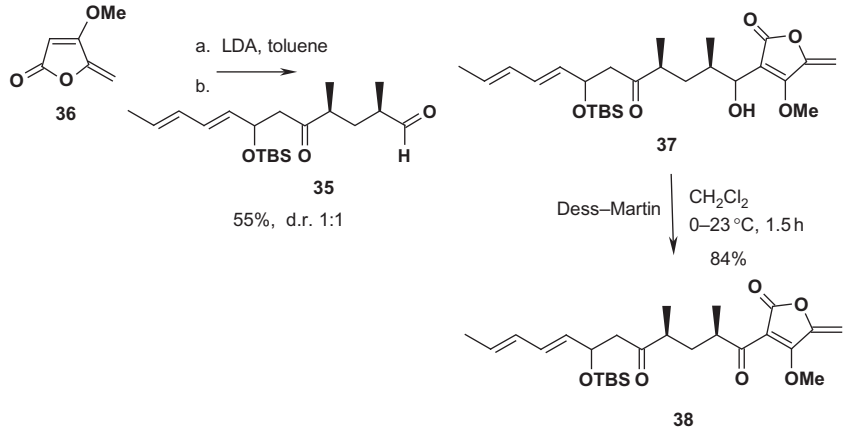
Reaction of the lactone functionality with MeLi followed by protection with triethylsilyl chloride (TESCl) produced ketone **32**, the substrate for the aldol condensation involving *trans, trans*-2,4-hexadienal **26**. Kinetically created enolate from ketone **32** reacted with hexadienal to afford the aldol product **33** in an excellent 94% yield. The fact that this reaction yielded a 1:1 mixture of diastereomers was not significant since the strategy required installation of



SCHEME 6

the double bond and the conjugated triene formation immediately before the cycloaddition step. Routine *tert*-butyldimethylsilyl (TBDMS) protection of the secondary alcohol **33** furnished compound **34**. Finally, the chemoselective deprotection/oxidation sequence of the triethylsilyl (TES) protected primary alcohol under the typical Swern conditions produced efficiently aldehyde **35** [27].

The next stage of the synthesis was the introduction of the tetronic moiety onto the aldehyde **35**. This was achieved in a few steps using known methyl tetronate **36** (Scheme 7) [28]. Treatment of tetronate **36** with lithium diisopropylamide (LDA) generated the lithiated intermediate which reacted with aldehyde **35** to produce compound **37**. This reaction was carried out on a gram scale with variable yields (35–55%) affording an equimolar amount of two diastereomers and, although not very efficient, led to the compound possessing all atoms present in naturally occurring abyssomicin C. The product **37** comprising four diastereomers was further oxidized employing Dess–Martin periodinane to afford **38** as a mixture of two diastereomers in 84% yield. Having reached this stage, it was now necessary to generate a conjugated trienone functionality which would act as a dienophile component in the intramolecular Diels–Alder reaction onto the tetronic moiety. As a key step of the proposed synthesis, this would create the spirotetronic fragment and also the macrocyclic ring in a single operation. Some preliminary studies suggested that the trienone compound is rather unstable but can be prepared by treating diene **38** with catalytic amount (5 mol%) of $Sc(OTf)_3$. The crude triene **25** then underwent the thermally promoted Diels–Alder reaction to afford the expected cycloadduct **27** in 79% yield with high degree of diastereoselectivity.



SCHEME 7

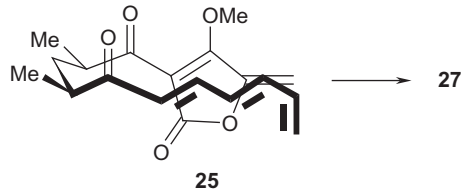
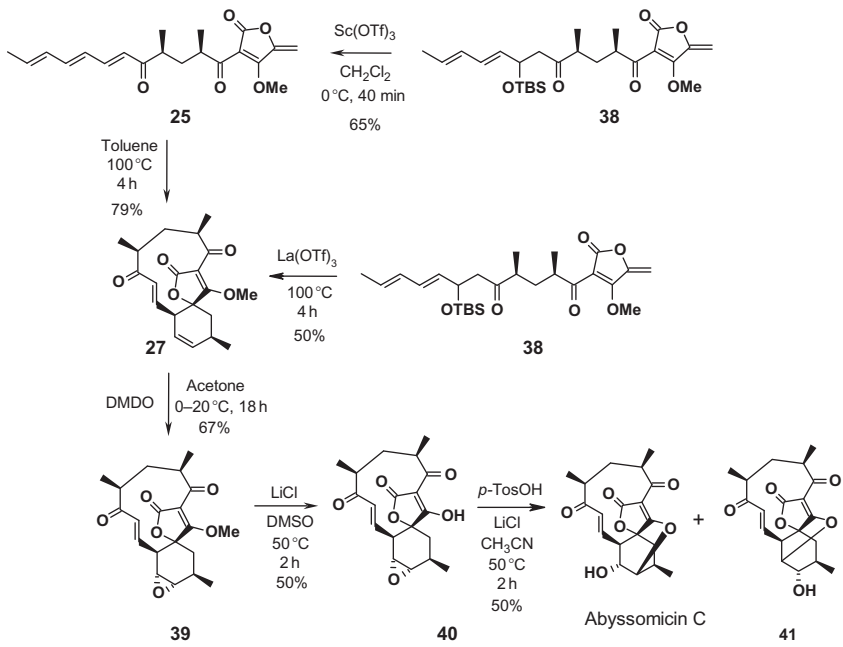


FIGURE 3 Transition state for the intramolecular Diels–Alder reaction of **25**.

This two-step elimination/cycloaddition procedure was further optimized by employing $La(OTf)_3$ as a Lewis acid permitting both transformations to be carried out as an one-pot process. Although the yields are similar, the later method avoids isolation and handling of unstable triene **25**. The cycloaddition step performed either way showed a high degree of diastereoselectivity, affording a single stereoisomer **27**. Computational study of the cycloaddition step at the HF/3-21G level of theory recognized transition state depicted in Fig. 3 as the lowest energy state in which the tetronate and the adjacent acyl carbonyls are in *anti* position [25].

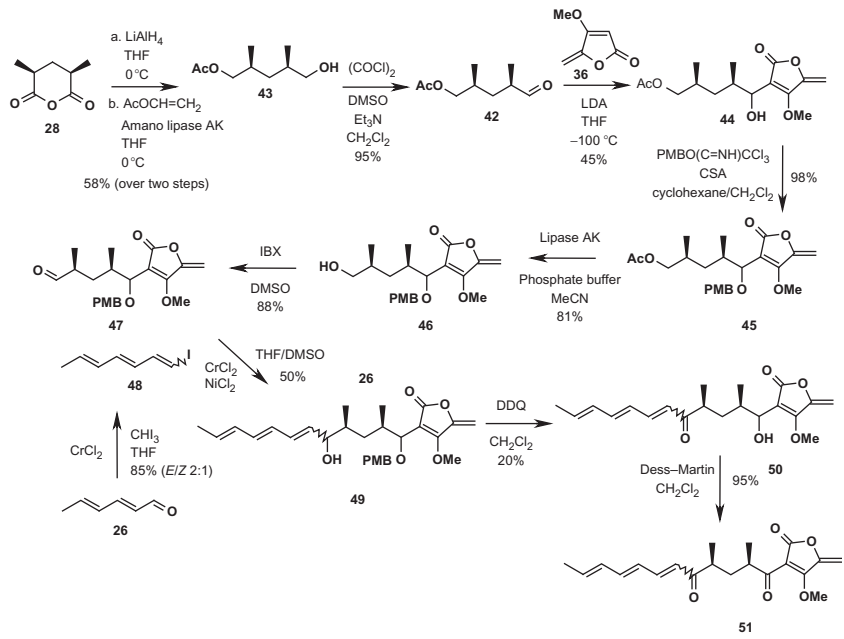
Finally, the resultant cycloadduct **27** was transformed to abyssomicin C in three straightforward steps (Scheme 8). Chemoselective oxidation of the isolated double bond was accomplished using dimethyldioxirane (DMDO) in acetone as a solvent to produce **39**, while the following demethylation of the vinylether moiety was successfully accomplished employing LiCl/dimethyl sulphoxide (DMSO). In a final step, compound **40** was exposed to acidic conditions (Scheme 8) which promoted the epoxide ring opening via cyclization to produce naturally occurring abyssomicin C. The natural product was accompanied by a by-product proposed to be compound **41**, whose conclusive structural assignment was not reported.



SCHEME 8

A very similar strategy to that described by Sorensen *et al*., also based on the intramolecular Diels–Alder reaction, has been exploited by Couladouros *et al*. and led to the development of formal synthesis of abyssomicin C reported in 2006 [29]. The synthesis commenced with preparation of aldehyde **42** starting from *meso* anhydride **28** (Scheme 9). Reduction of **28** with $LiAlH_4$ followed by enzymatic monoacetylation afforded alcohol **43** in 58% yield which was oxidized under Swern conditions to produce aldehyde **42**. Functionalization of the tetronate **36** was achieved under standard basic conditions. Reaction of **36** and LDA generated 2-Li-tetronate which, upon addition of aldehyde **42**, afforded product **44** as a 1:1 mixture of diastereomers in moderate yield and with incomplete conversion. Protection of the secondary alcohol as *para*-methoxybenzyl (PMB) ether proceeded in excellent yield furnishing **45**. An enzyme-promoted deacetylation of **45** liberated alcohol functionality, which was oxidized with 2-iodoxybenzoic acid (IBX) to produce aldehyde **47**. Extension of the tetronate side chain and the introduction of a triene moiety were accomplished via Nozaki–Hiyama–Kishi reaction. The substrate **48** was prepared from commercial aldehyde **26**.

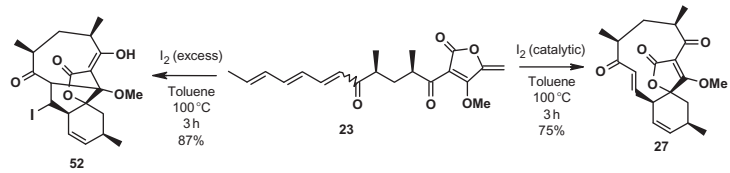
Various reaction conditions were used for transformation **26** → **48**, but the best results were obtained with $CrCl_2/CHI_3$/tetrahydrofurane (THF) bearing 2:1 *E*/*Z* mixture of **48** in 85%. Attempts to alter the *E*/*Z* ratio by isomerization did not bring about any improvement. Thus, the stereoisomeric mixture of



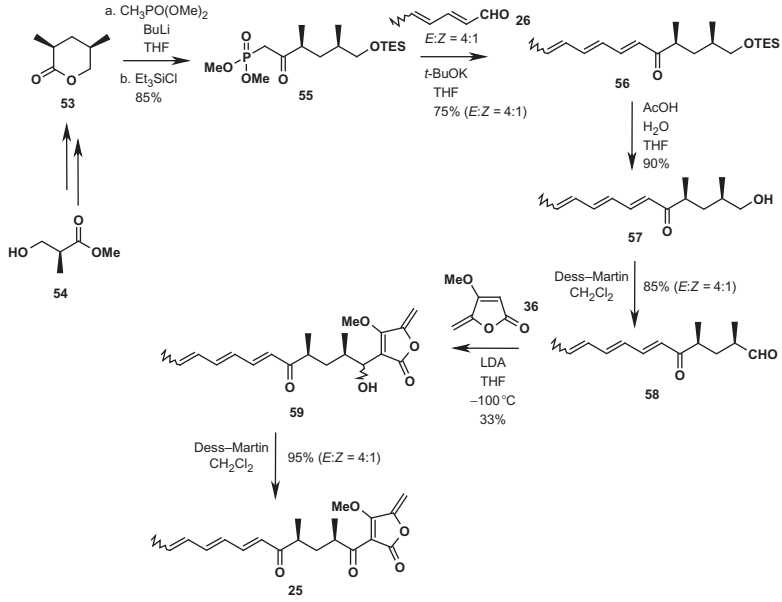
SCHEME 9

vinyliodide **48** coupled with aldehyde **47** under Nozaki–Hiyama–Kishi conditions afforded **49**, possessing all necessary carbons to build the abyssomicin skeleton, in 50% yield. At this stage, it only remained to alter the oxidation state of the protected and the unprotected alcohol groups before studying the key cycloaddition step. Exposing compound **49** to 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) led to two simultaneous processes, oxidation of the allyl alcohol and deprotection of the *para*-methoxybenzyl (PMB) ether to produce **50** in only 20% yield. This low yield prompted Couladouros group to investigate processes with TBDMS-protecting group in place of PMB which proved to be more efficient. Compound **50** was further oxidized using Dess–Martin reagent to afford diketone **51** isolated as a mixture of two diastereomers due to the stereochemistry of the double bond. The oxidation of two secondary alcohol functions significantly decreased structural complexity of the product which arose from the fact that compound **49** possessed the two diastereomeric centers and the *E*/*Z* double bond.

The key step for construction of the abyssomicin skeleton, Diels–Alder reaction, was carried out in toluene at 100 °C and in the presence of a catalytic amount of I_2 to give cleanly cycloadduct **27** in 75% yield (Scheme 10). This cycloadduct according to the previously published Sorensen synthesis can be transformed to abyssomicin C and, thus, it renders work of Couladouros group formal synthesis of this natural product. The cycloaddition reaction carried out



SCHEME 10



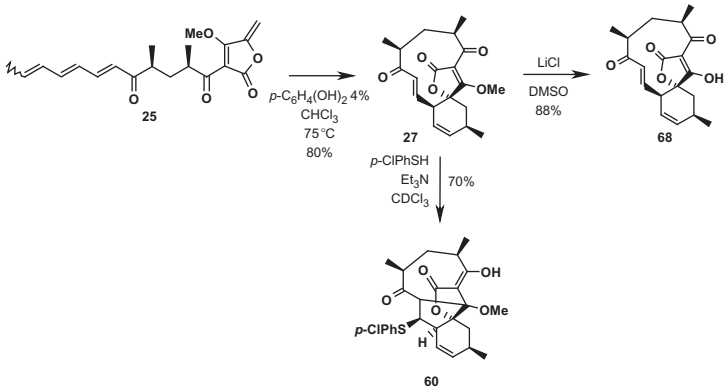
SCHEME 11

with an excess of I_2 under the same conditions afforded product **52** with the carbon skeleton of abyssomicin D.

Snider's approach toward the synthesis of abyssomicin C also relied on the intramolecular Diels–Alder reaction for constructing the abyssomicin core structure and, in general, resembles the previously discussed synthesis (Scheme 11) [30]. The manuscript with these results was published in 2005 almost in the same time with Sorensen's synthesis of abyssomicin C. Synthesis of lactone **53** was accomplished in several steps starting from ester **54** according to the literature procedure [31]. The lactone was transformed to keto phosphonate which was *in situ* protected to afford compound **55** in 85% yield. Wittig olefination of the 2,4-hexadienal, used as a mixture of isomers, produced conjugated trienone **56** in 75% yield. Routine acidic deprotection followed by Dess–Martin oxidation of the alcohol afforded aldehyde **58** in 76% yield over two steps. Deprotonation of tetronic derivative **36** was performed at −100 °C in the presence of LDA to generate 2-lithio derivative

which was allowed to react with aldehyde **58**. The reaction did not proceed with full conversion and yielded only 33% of the expected **59** as a mixture of four diastereomers. Fortunately, the following Dess–Martin oxidation of the secondary alcohol decreased structural complexity to give compound **25** in excellent yield as a 4:1 mixture of *E*/*Z* isomers. Interestingly, it was observed that compound **25** underwent Diels–Alder reaction under very mild conditions, in $CDCl_3$ at room temperature, albeit with only 40% conversion after 1 week, suggesting that the same process is likely during the biosynthesis of abyssomicins. On the other hand, performing the reaction in sealed tube at 75 °C in $CHCl_3$ containing 4% of hydroquinone for 2 days afforded a single product **27** in 80% (based on conversion for *E*-**25**) accompanying with 10% *Z*-**25**. The following removal of the methyl substituent from the tetronic moiety was efficiently accomplished by LiCl/DMSO to yield compound **68**. Unfortunately, all attempts to form epoxide via *meta*-chloroperoxybenzoic acid (MCPBA) or DMDO oxidation produced a complex mixture in which the cyclohexene double bond was preserved while the enone was oxidized. As a result of this unwanted chemoselectivity, further efforts of Snider's group were directed toward protection of the enone functionality via a nucleophilic addition (Scheme 12).

The reaction of a thiol and the conjugated ketone was expected to remove temporarily the reactive enone but, in the same time, would latter allow straightforward restoration of this key moiety via an oxidation/elimination sequence. Indeed, the reaction of **27** with *p*-ClPhSH proceeded via addition of the nucleophile on the enone but, unfortunately, did not stop at this stage. The generated enolate reacted further in an intramolecular Michael reaction onto the tetronate to produce compound **60** with the carbon skeleton of abyssomicin D/E. Although compound **27** was not transformed into the natural product by Snider's group, this advanced intermediate can be elaborated into the abyssomicin C as demonstrated by Sorensen.

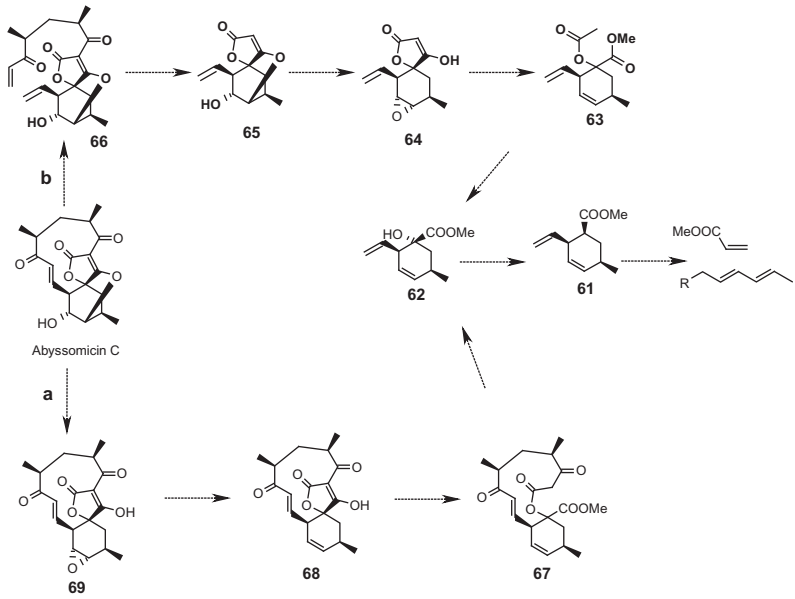


SCHEME 12

A concise enantioselective synthesis of abyssomicin C completed by Sorensen's group and related formal synthesis by Snider's and Couladouros groups successfully explored a biomimetic approach based on the intramolecular Diels–Alder reaction. This key step proved very efficient, creating the spirotetronic and macrocycle fragments with three new chiral centers in a single step and in a highly diastereoselective manner.

Approach Based on Intermolecular Diels–Alder Reaction/Ring-Closing Metathesis

The second total synthesis of abyssomicin C developed by Nicolaou group, reported in 2006, was based on the Lewis-acid-templated Diels–Alder reaction for the construction of a highly functionalized cyclohexane ring and latter ring-closing metathesis (RCM) to create the macrocyclic fragment [15]. The retrosynthetic plan, outlined in Scheme 13, proposed two similar synthetic strategies. They have a common intermediate, functionalized cyclohexene compound **61**, which could be synthesized via Diels–Alder reaction. Hydroxylation of the cycloadduct **61** creates compound **62** which possesses all necessary functional groups for further elaboration. Both routes propose to use a Dieckmann condensation in a strategically slightly different manner to construct tetronic moiety (**67** → **68** route a; **63** → **64**, route b; Scheme 13), while the RCM would establish the macrocyclic ring (**62** → **67**, route a;



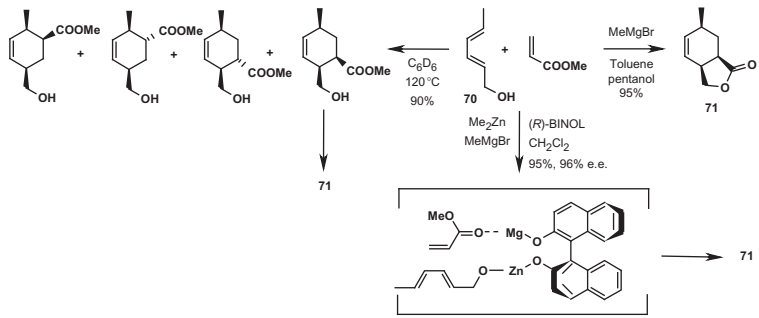
SCHEME 13

66 → abyssomicin C, route b; Scheme 9). Oxabicyclo[2.2.2]octane system could be assembled by epoxidation of the cyclohexane double bond and the subsequent ring opening via nucleophilic attack of the enol moiety.

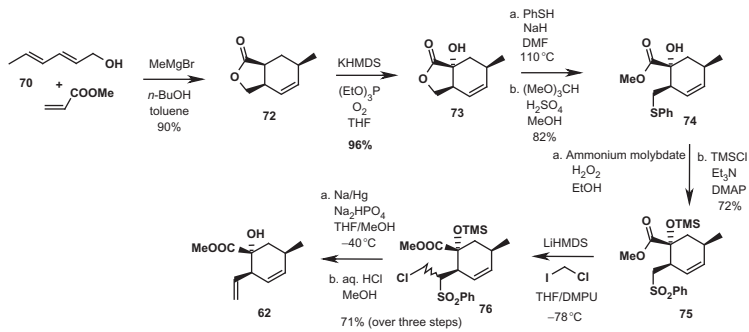
The Lewis acid-templated intermolecular Diels–Alder reaction exploited in the synthesis of abyssomicin C was initially reported by Ward [32a,b]. The thermal reaction of diene **70** and methyl acrylate generally affords a mixture of four products, but the same reactants in the presence of MeMgBr produce a single product, compound **71**, highly regio- and diastereoselectively (Scheme 14).

In addition, the author also reported highly enantioselective variant of this transformation [32b]. The use of two Lewis acids, Me_2Zn and MeMgBr, and (*R*)-1,1′-bi-2-naphthol (BINOL) as a chiral ligand afforded the cycloadduct **71** in excellent yield and with high degree of stereoselectivity (Scheme 14). It was proposed that the Lewis acids tethered both reactants to BINOL creating a chiral environment responsible for the observed enantioselectivity. The reaction was carried out with and without substoichiometric amounts of Lewis acids/BINOL, and although the yields and e.e. were comparable, the former one required a significantly longer reaction time (14 h vs. 1 h).

Initial efforts of the Nicolaou group resulted in successful synthesis of racemic **62** (Scheme 15). It began with the Diels–Alder reaction of diene **70**



SCHEME 14



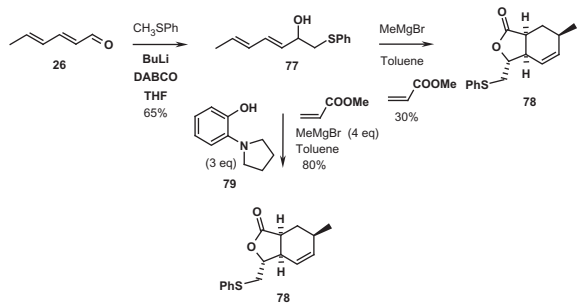
SCHEME 15

and methyl acrylate according to Ward's procedure. The cycloadduct **72** was then hydroxylated under basic conditions using oxygen to produce **73** in excellent yield. All attempts to open the lacton ring with aqueous LiOH resulted only in isolation of the starting material presumably due to cyclization of the initially obtained hydroxyacid. This problem was solved in reaction of lactone **73** with thiophenoxyde ion generated *in situ* to afford compound **74** in 82% yield. The following sequence was designed to introduce the exocyclic vinyl functionality and started with oxidation of sulfide **74** to sulfone **75**. Alkylation of the sulfone-activated methylene group with ICH_2Cl produced a mixture of diastereomers **76**, which upon reduction and deprotection yielded the desired derivative **62** in 71% overall yield.

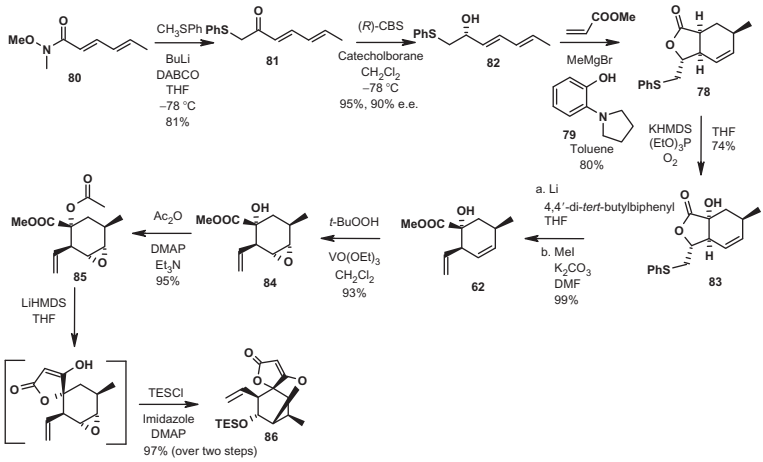
Although the target molecule was successfully prepared and the route was likely to provide an access to chiral **62** via the already mentioned Lewis acid tethered Diels–Alder reaction, inefficiency of the synthesis and the use of stoichiometric BINOL/Me_2Zn in the Ward's enantioselective cycloadditions prompted reevaluation of the approach delineated in Scheme 15. The redesigned route started with synthesis of diene **77**, prepared from aldehyde **26** and lithiothioanisole in 65% yield (Scheme 16). Subsequent cycloaddition step was quite inefficient, which, performed as previously explained, produced cyclohexene **78** in only 30% yield.

Addition of alcohols such as *n*-BuOH or *i*-PrOH, which have been shown to have beneficial effect, did not result in any improvement. Similar results were obtained when various Lewis acids were tested in this transformation. This unexpected outcome provoked further study of the cycloaddition reaction. The fact that the enantioselective cycloadditions utilizing BINOL/Me_2Zn/MeMgBr were more efficient than the simple templated Diels–Alder reactions prompted authors to investigate the effect of various bisphenols and related compounds.

After screening several of these, the optimal conditions (Scheme 16) employed aminophenol **79** (3 eq) and MeMgBr (4 eq) producing within 12 h product **78** in 80% yield. Having resolved the efficiency of the cycloaddition step, it remained to develop enantioselective synthesis of the spirotetronic core.



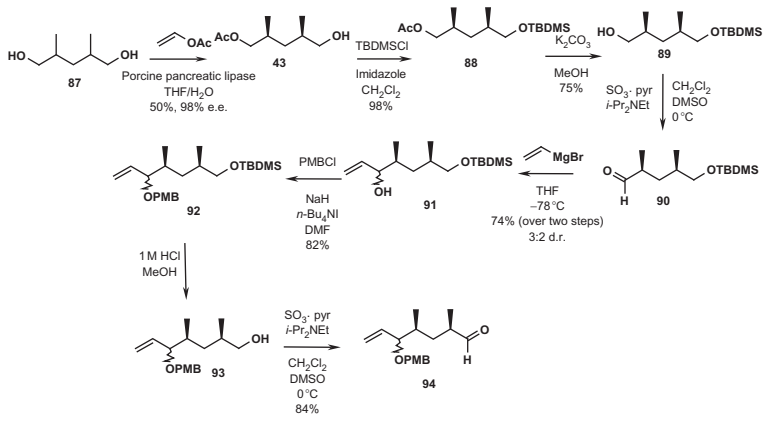
SCHEME 16



SCHEME 17

The process commenced with the preparation of ketone **81** by reacting the Weinreb amide **80** and lithiothioanisole (Scheme 17). Corey–Bakshi–Shibata reduction of the ketone **81** afforded hydroxy compound **82** in 95% yield and 90% e.e. [33]. In the following step, diene **82** was subjected to the modified Diels–Alder conditions, using aminoalcohol **79** and MeMgBr, producing desired cyclohexene derivative **78** in 80% yield. α-Hydroxylation afforded hydroxylactone **83**. The early attempts to promote the elimination in **83** to introduce an important double bond used a two-step procedure, oxidation to the sulfon-reduction with Na(Hg) or $Mg/HgCl_2$, but with no success. The more direct reductive elimination promoted by the lithium di-*tert*-butylbiphenylide radical anion introduced the double bond with concomitant carboxylate ion, which was methylated *in situ*. This sequence provided access to **62**, the cyclohexene derivative possessing all necessary functional groups with correct stereochemistry, which led to the spirotetronic product **86** in straightforward manner. Thus a vanadium-directed epoxidation, executed with high degree of stereo- and regioselectivity, produced epoxide **84** in 93% yield. Interestingly, the best results were obtained with catalytic amount of $VO(OEt)_3$ instead of usually employed $VO(acac)_2$ [34]. The following routine acetylation of the tertiary alcohol afforded ester **85** and finally set the stage for creating tetronic moiety via Dieckmann cyclization. Treating **85** with lithium hexamethyldisilazide (LiHMDS) at −78 °C generated anion which reacted intramoleculary with the proximal ester group to produce the tetronic core. Instead of isolating the product, it was further refluxed to promote highly regioselective cyclization of the tetronic enol onto the epoxide. Subsequent *in situ* protection with TESCl gave the final spiro-product **86** in excellent yield.

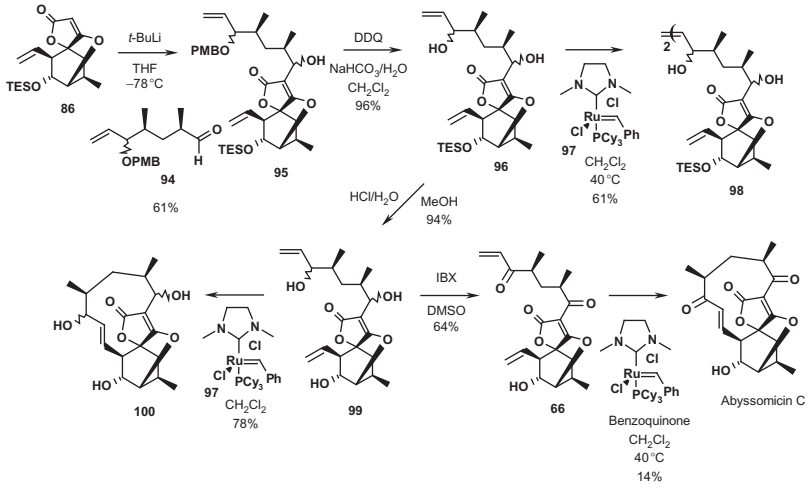
With compound **86** in hand, it was then necessary to synthesize the upper part of the abyssomicin C structure, which would be used in constructing the



SCHEME 18

macrocyclic ring. The C-7 fragment was prepared starting from *meso*-diol **87** [35]. Desymmetrization of this diol using porcine pancreatic lipase (PPA) in the presence of vinyl acetate afforded monoacetate **43** with 98% e.e. (Scheme 18). A further routine protection/deprotection sequence produced alcohol **89** in overall 74% yield. Parikh–Doering oxidation followed by vinylation of the aldehyde gave alcohol **91** in overall 74% yield and as a 3:2 mixture of diastereomers [36]. The secondary alcohol was protected as a PMB-ester, while deprotection of the primary alcohol functionality and the subsequent oxidation afforded the C-7 functionalized fragment **94** required for installing the macrocyclic ring.

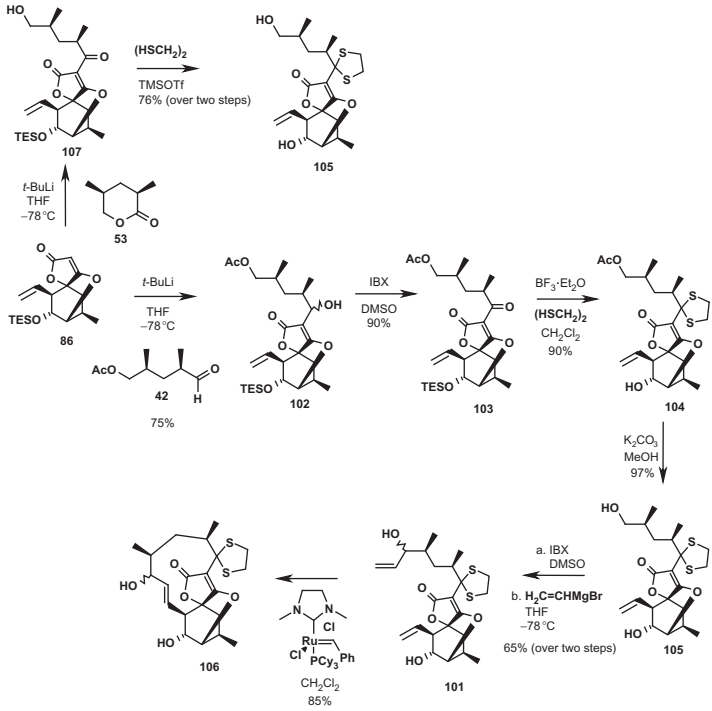
Completion of the synthetic routes for compounds **86** and **94** progressed the synthesis of abyssomicin C to the final stages, which explored methodologies to couple these two fragments (Scheme 19). Thus, treatment of **86** with *t*-BuLi resulted in lithiation of the tetronic C(2) atom, which, upon addition of aldehyde **94**, yielded product **95** as a mixture of diastereomers. The following deprotection using DDQ released the secondary alcohol to afford **96** in almost quantitative yield again as a mixture of diastereomers. Unfortunately, attempts to perform an intramolecular RCM on **96** using the second generation Grubb's catalysts **97** resulted only in dimerization. In order to decrease the steric constraints near to the reacting double bond, the TES group was removed and the product, compound **99**, was subjected to the RCM conditions. The cyclization took place to give macrocyclic product **100** in 78% yield. At this stage, a step from the natural product, it was expected to achieve a selective oxidation of two allylic alcohols to complete the synthesis. Unfortunately, the allyl alcohol selective oxidizing reagents afforded only monooxidized product, while stronger oxidants led also to the oxidation of the nonactivated C(11) alcohol functionality. A slightly modified route where the oxidation step was carried out prior to the metathesis reaction proved to be more efficient. Thus, the oxidation of the allylic alcohols with *o*-iodoxybenzoic acid (IBX) produced diketone **66** in 64% yield. The subsequent metathesis performed with the second



SCHEME 19

generation Grubb's catalyst and in the presence of bezoquinone to suppress a double bond isomerization did afford abyssomicin C but only in 14% yield as a 2:1 inseparable mixture with the starting **66**.

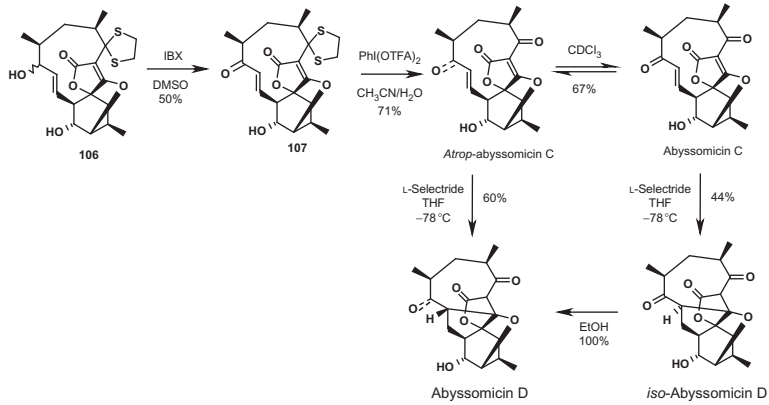
In order to improve the efficiency of the final steps, the authors embarked on modifying the route, assuming that the presence of two additional sp^2 carbon atoms in **66**, compared to **99**, influenced the RCM process. It was speculated that protecting one keto group of the cyclization precursor **66** would favor RCM and prevent some side reactions. Since the carbonyl group conjugated with the tetronic moiety is away from the double bond involved in the cyclization and therefore it is less likely to interfere with the RCM, it was selected for this transformation. The synthetic route of desired compound **101** was initiated with the functionalization of the tetronic ring of **86** under basic conditions and in the presence of aldehyde **42** to afford alcohol **102** (Scheme 20). The IBX oxidation of this alcohol cleanly produced ketone **103** in 90% yield. The following step, protection of the ketone in the form of a thioketal, was performed under standard conditions with similar efficacy. With the first part of the plan completed, it remained only to transform the terminal acetate in order to introduce the double bond, which would set a stage to execute RCM. This was achieved in several steps: hydrolysis of the acetate, oxidation of the alcohol using IBX, and, finally, addition of vinyl-Grignard reagent onto the aldehyde to produce compound **101** in overall 63% yield. An alternative, slightly shorter route was also created for the preparation of compound **105**. The *in situ* generated lithio derivative of **86** on reaction with lactone **53** produced **107** whose direct protection led to **105** in good overall yield (76%). Compound **101** was then subjected to the RCM conditions and the product **106**, with the *trans* geometry of the double bond, was isolated in 85% yield



SCHEME 20

as a 2:3 mixture of C(7)-(*R*) and C(7)-(*S*). The compounds possessing *trans* double bond incorporated into the medium-sized ring are known to show atropisomerism, but alkene **106** was isolated as a single atropisomer, as evidenced by NOE experiment [37]. The observed product was formed via approach from the *re* face of the C(10)-double bond during the RCM reaction.

Compound **106** was oxidized using IBX, a reagent frequently employed for these purposes during the development of the synthetic route, to give conjugated ketone **107** (Scheme 21). The final step, deprotection of the thioketal functionality, was carried out using $PhI(OTFA)_2$ to yield the product whose spectroscopic data did not match perfectly with that of the reported natural products. This surprising result prompted extensive NMR studies of the product and resulted in discovery that the synthesized product, later named *atrop*-abyssomicin, in $CDCl_3$ isomerized to the natural product, eventually leading to a 2:1 equilibrium mixture in favor of abyssomicin C. The differences between the two are discussed in Isolation and structures. Furthermore, reduction of *atrop*-abyssomicin C with L-selectride in THF as a solvent produced an additional member of the abyssomicin family, abyssomicin D. Interestingly, the same reaction with abyssomicin C afforded a mixture of products with



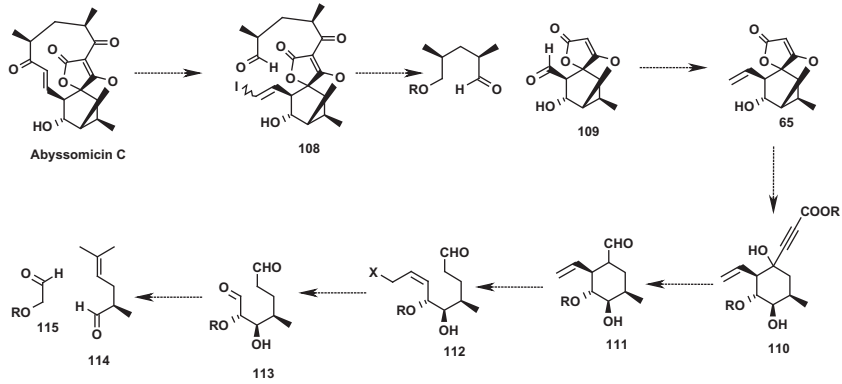
SCHEME 21

iso-abyssomicin D as a major product isolated in 44% yield. On an attempt to prepare a crystal for X-ray analysis, complete isomerization of *iso*-abyssomicin D to abyssomicin D was observed when ethanol was used as a solvent.

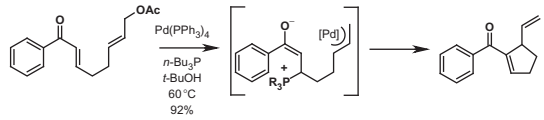
Similar to Sorensen synthesis and the related formal synthesis, Nicolaou's synthesis of abyssomicin C was based on the Diels–Alder reaction, although tactically in a different manner. It employed the intermolecular Lewis acid-templated Diels–Alder reaction as a way to access highly functionalized cyclohexene fragment. The substitution pattern of the cyclohexene derivative permitted straightforward introduction of the tetronic moiety via Dieckmann condensation and further attachment of the side chain. A macrocyclization to form 11-membered ring was accomplished by an RCM reaction. An additional significant contribution of this work to our knowledge of abyssomicin natural products is the discovery of *atrop*-abyssomicin C and a detailed study of atropisomerism in these compounds.

Approach Based on Pd-Catalyzed Alkylation/Nozaki–Hiyama–Kishi Reaction

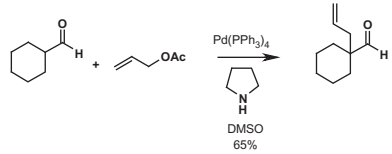
A third reported synthesis of the abyssomicin class of compounds, namely, *atrop*-abyssomicin C and abyssomicin H, by Saicic and Bihelovic appeared in the literature in 2012 [38]. It has many specific features compared to the above discussed approaches. While the Sorensen and Nicolaou syntheses relied on Diels–Alder reaction as one of the key steps for constructing the functionalized cyclohexane fragment of the abyssomicin structure, the Saicic–Bihelovic approach is based on combining organocatalysis and Pd-catalyzed allylation processes to create a suitable substituted cyclohexane **111** (Scheme 22). According to their retrosynthetic analysis, the macrocyclization step would employ Nozaki–Hiyama–Kishi reaction (**108** → abyssomicin). Further disconnection led to compound **110** which was to be used to create the tetronic moiety with concomitant



SCHEME 22



SCHEME 23



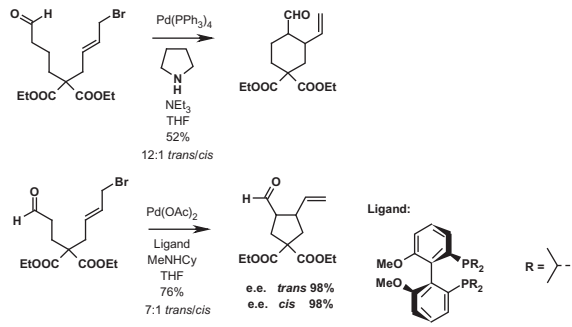
SCHEME 24

cyclization involving the secondary alcohol functionality to form bicyclo[2.2.2] octan. The key intermediate for the synthesis of highly functionalized cyclohexane ring via organocatalysis/Pd-catalyzed allylation, compound **112**, was expected to be accessible via the aldol condensation reaction (**115/114** → **113** → **112**).

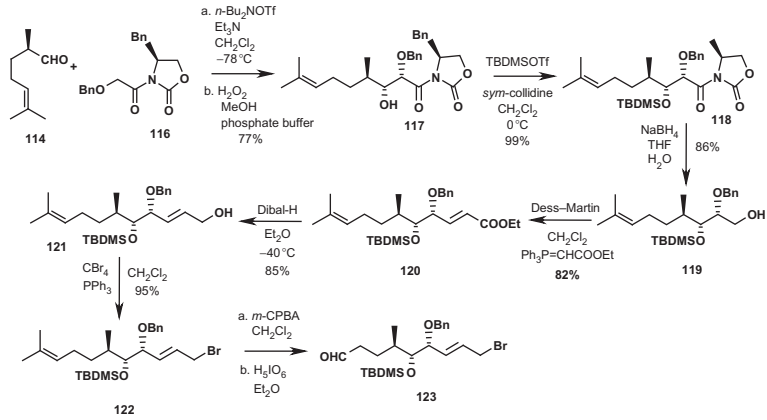
The idea of combining organocatalysis and Pd-mediated allylation has been explored previously in the synthesis of cyclopentanes (Scheme 23) [39]. In this ingenious strategy involving two simultaneous processes, the Pd-catalyst serves to generate π-allyl Pd-intermediate, while the organocatalyst activates the masked nucleophile allowing a smooth reaction between the two.

Variation of this methodology provided an elegant method for the allylation of aldehydes and ketones without the need for strong bases [40]. The generated π-allyl Pd-intermediate reacted with the prepared enamine from carbonyl compound and pyrrolidine *in situ* to afford allylated product (Scheme 24).

The intramolecular variant of this methodology proved to be an efficient method for the synthesis of 5- and 6-membered rings (Scheme 25) [41]. The



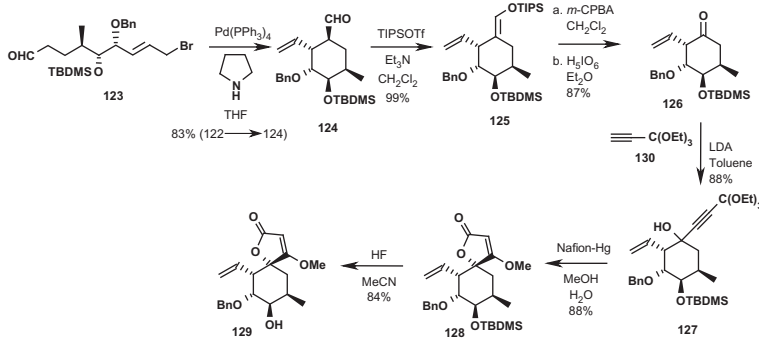
SCHEME 25



SCHEME 26

products were usually isolated as a mixture of *cis* and *trans* isomers, generally with the *trans* isomer predominating. Performing this reaction in the presence of a chiral ligand additionally enriched the methodology by enabling a highly stereoselective synthesis of cyclopentane derivatives (Scheme 25) [41].

Work on the abyssomicin structure commenced with the aldol condensation of citronellal **114** and benzyloxyacetate-derived oxazolidinone **116** to produce aldol **117** as a single diastereomer (Scheme 26). Protection of the alcohol functionality and removal of the oxazolidinone auxiliary under reductive conditions furnished alcohol **119** in excellent yield. A one-pot procedure incorporating Dess–Martin oxidation/Wittig reaction afforded the conjugated ester, which was then reduced to give the allylic alcohol **121**. Transformation of the allyl alcohol into the allylbromide **122** was accomplished using CBr_4/PPh_3 in 95% yield. The final step in the synthesis of aldehyde **123**, required for the preparation of the key cyclohexane intermediate, was carried out

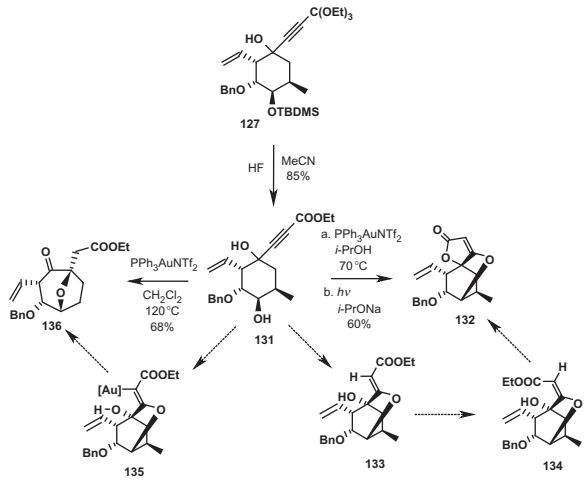


SCHEME 27

employing MCPBA/H_5IO_6 leading to the double bond cleavage and introduction of the aldehyde moiety.

At this point, the crucial cyclization to build a highly functionalized cyclohexane derivative was studied (Scheme 27). Initial work was carried out with the ester enolate (the ester group in place of the aldehyde in **123**) under a variety of conditions but with no success. Even aldehyde **123** did not cyclize under the conditions used for the related reactions [42]. Fortunately, an experiment employing aldehyde **123** and Pd-catalyst in the presence of piperidine efficiently furnished cyclohexane derivative **124**. Following successful synthesis of the functionalized cyclohexane ring, it was now necessary to assemble the spirotetronic moiety. Initial experiments based on α-hydroxylatation of the aldehyde **124** did not give expected results, mainly due to stereoselectivity of the process and stability of the product. Therefore, this approach had to be abandoned. The modified route, outlined in Scheme 27, began with transformation of the aldehyde **124** into ketone **125** using a two-step procedure, formation of TIPS-protected enol **125** and oxidative cleavage of the double bond, to form **126** in 87% yield. This ketone reacted with the propargyl anion generated from **130** to give product **127** designed to allow straightforward access to the spirotetronic derivative. Thus, when treated with Nafion-Hg, **127** produced spyro compound **128** in excellent yield and was further easily deprotected producing compound **129**. At this stage, another drawback necessitated altering the route. Namely, compound **129** could not be cyclized into the tricyclic compound, which would have been formed by the nucleophilic addition of the alcohol onto the tetronate conjugated system. This was attributed to the necessary but unfavorable a boat-like transition state, which would allow access of the nucleophilic alcohol to the electrophilic tetronate carbon.

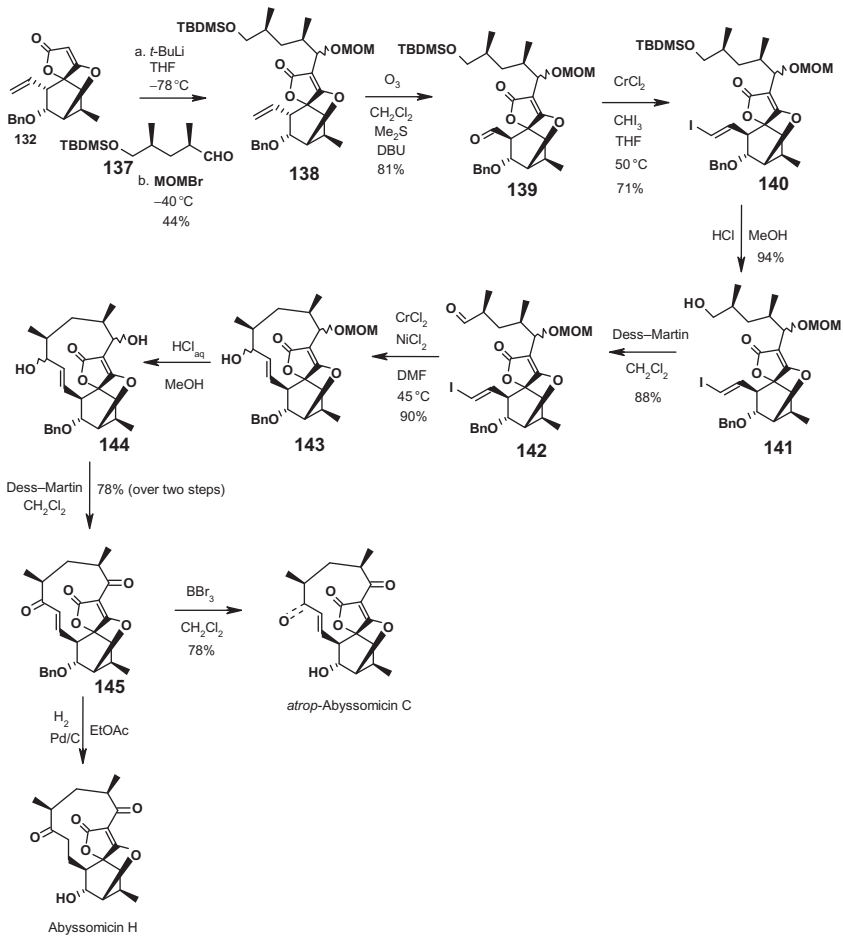
Fortunately, the strategy was slightly adapted using the already prepared compound **127**. Thus, compound **127** was transformed into the propargyl ester **131** in 85% yield upon treating with HF in MeCN (Scheme 28). The cyclization processes of **131** in the presence of Au-catalyst, after some



SCHEME 28

experimentation, finally afforded tricyclic compound **132**. The most efficient was the Gagosz catalyst, an air stable and exceedingly active compound, which has been shown to promote various processes [43]. The ester **131** was subjected to the Gagosz Au(I) catalyst at 120 °C in CH_2Cl_2 as a solvent to afford product **136** in 68% yield. Although this was not the desired compound, it was proposed that its formation involved intermediate **135**. This prompted further exploration of the reaction conditions and led to the synthesis of *Z*-configured **133**. In order to locate the ester group in the correct position for cyclization to take place, it was necessary to isomerize **133** into **134** and this was achieved via irradiation of **133** in the presence of a catalytic amount of alkoxide. The cyclization then spontaneously occurred to produce tricyclic compound **132** and, in the same time, shifted *Z*-**133**–*E*-**134** equilibrium toward formation of *E*-isomer **134**.

This completed the synthesis of the key compound **132** and permitted an examination of the formation of the macrocyclic ring. Aldehyde **137** was utilized to attach a side chain onto the tetronic moiety (Scheme 29). Li-tetronate was generated under typical conditions using *t*-BuLi and upon the reaction with **132** and subsequent protection furnished compound **138** as a mixture of diastereomers in moderate yield. The following ozonolysis of the terminal C═C bond and subsequent base-promoted isomerization to correct the stereochemistry cleanly afforded aldehyde **139** (81%). The aldehyde was then subjected to Takai reaction to produce vinyliodide **140** in 71% yield. To explore the potential of Nozaki–Hiyama–Kishi reaction in the macrocyclization, as proposed by the retrosynthetic analysis (Scheme 22), it was now necessary to install an aldehyde functionality on the opposite end of the molecule. This was accomplished simply by removal of the TBDMS-protecting group and



SCHEME 29

oxidation of the primary alcohol with Dess–Martin periodinane. Nozaki–Hiyama–Kishi reaction, used previously in macrocyclization transformations [44], with substrate **142** showed remarkable efficiency. It was carried out in DMF as solvent and at a slightly elevated temperature to produce 11-membered macrocycle **143** as a mixture of four diastereomers in 90% or 96% yield if based on recovered starting material. Removal of the methoxymethyl (MOM) protection group and oxidation of the both alcohol functionalities simultaneously produced diketone **145**. Final step, removal of the benzyl (Bn) group, was accomplished by BBr_3 to produce, as showed by the NOESY experiment, *atrop*-abyssomicin C, while deprotection employing H_2 Pd/C cleaved the Bn group with concomitant reduction of the conjugated double bond to afford abyssomicin H.

The Saicic–Bihelovic synthesis of abyssomicin natural products is unique in its approach and does not rely on Diels–Alder reaction, which has been extensively employed by other groups. Dual catalysis formation of the highly functionalized six-membered ring, developed in their laboratories, was utilized for the preparation of the key cyclohexane derivative. Further elaboration of this compound, installation of the tetronic fragment by Au-catalysis followed by an intramolecular cyclization, elegantly secured bicyclo[2.2.2] octane, while final steps, based on Nozaki–Hiyama–Kishi reaction, proved to be an excellent strategic solution for the macrocyclization process.

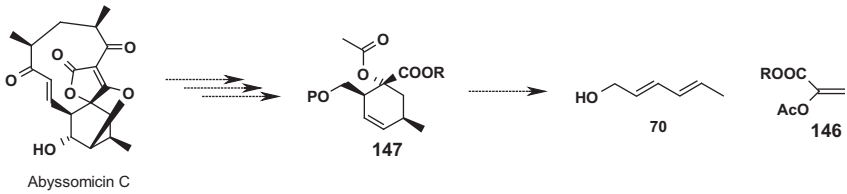
SYNTHETIC STUDIES

Apart from the above described synthetic routes, several reports appeared in the literature discussing various issues related to the synthesis of abyssomicin natural products.

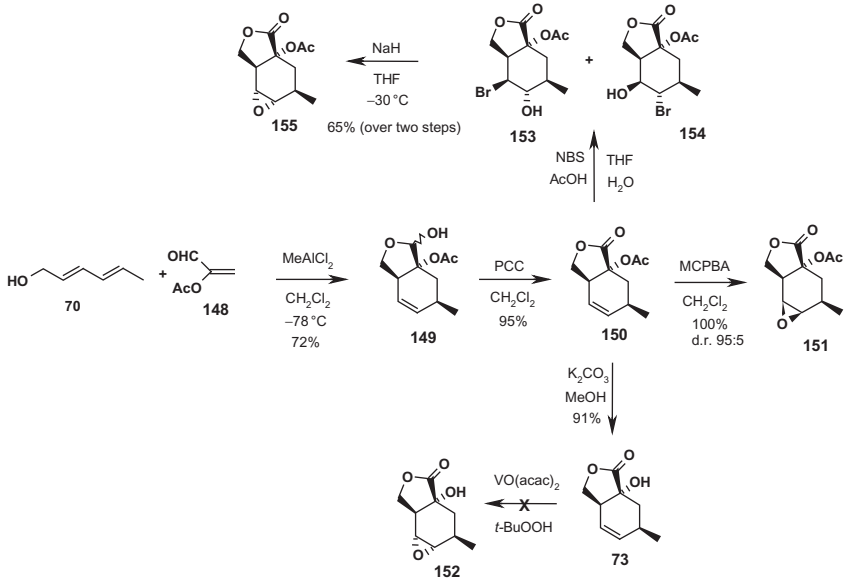
As a central transformation toward synthesis of the spirotetronic core of abyssomicins, the Georgiadis group investigated intermolecular Diels–Alder reaction of the dienol **70** and 2-acetoxyacrylates **146** (Scheme 30) [45]. Their strategy, reported in 2005, was based on Ward's variant of the Diels–Alder reaction, methodology which was later fully exploited by Nicolaou's group in the abyssomicin C/*atrop*-abyssomicin C synthesis.

Soon after initiating this work, the Georgiadis group faced many problems and all attempts to react diene **70**, as O-protected derivative, and acetoxy acrylic ester or related acrolein derivative under thermal conditions or Lewis acid catalysis failed. Performing the reaction in an intramolecular fashion by using dienol ester of acrylic derivative did not result in any improvement.

This was attributed to the effect of the acetoxy group since the same diene reacts with acrolein or methylacrylate to afford the expected cycloadduct [46]. The previously described Ward conditions of Lewis acid-templated intermolecular Diels–Alder reaction employing Me_2AlCl or $MgBr_2$ also failed initially, but their further exploration resulted in success. The use of $MeAlCl_2$ as a Lewis acid and the precise order of addition of reactants were key elements in the successful synthesis of **149** (Scheme 31). Lactol **149** was oxidized using pyridinium chlorochromate (PCC) to afford lactone **150** in almost quantitative yield. Oxidation of the double bond by MCPBA proceeded in a highly diastereoselective manner producing epoxide **151** in excellent yield, but unfortunately, with the undesired stereochemistry. Therefore, the acetate functionality was hydrolyzed and the oxidation was attempted with $VO(acac)_2$/*t*-BuOOH with hope that the hydroxy group might control the approach of the oxidizing reagent [47]. As this did not take place as expected, an indirect method afforded desired epoxide by two-step procedure, bromohydrin formation followed by a base-promoted cyclization. The first step was carried out in a mixture of AcOH/THF/H_2O with NBS to yield, according to the authors, only two diastereomers in an undisclosed ratio. The mixture of products was then treated with



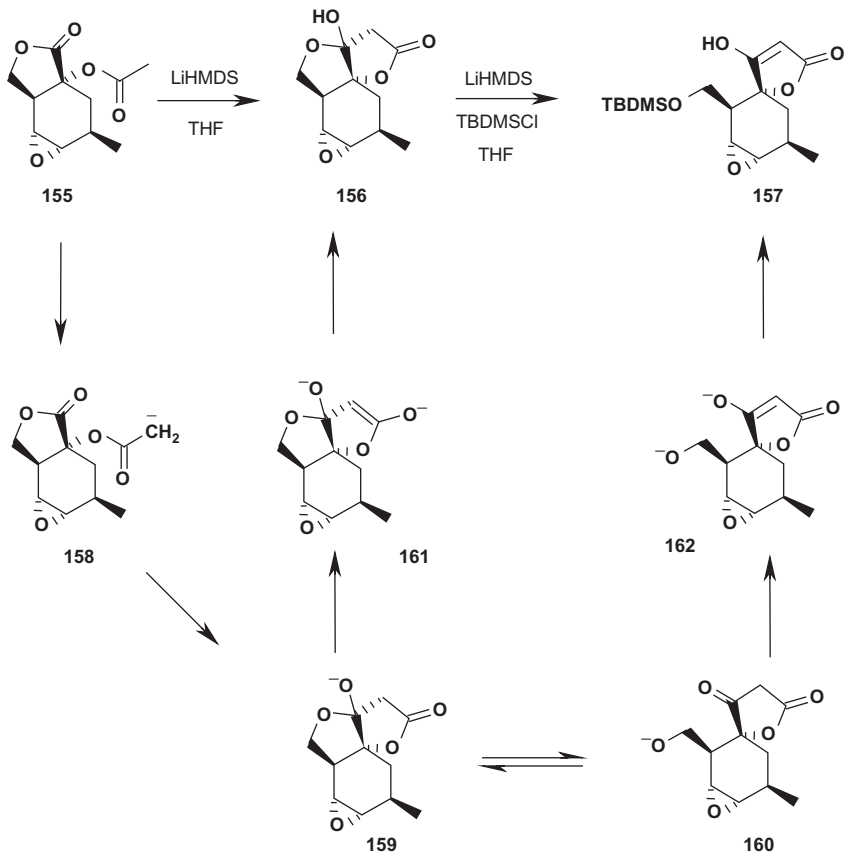
SCHEME 30



SCHEME 31

NaH in THF at −30 °C to furnish desired epoxide **155** in good overall yield. Successful preparation of the correctly substituted cyclohexane derivative **155** moved the synthesis to the next stage, installation of the tetronic moiety. Dieckmann condensation was carried out in THF using LiHDMS as a base to afford product **156** (Scheme 32). Although the tetronic structure was generated in a protected form, lactol **156** was resistant to opening of the hemiketal group. Exploration of the reaction conditions disclosed that when **156** was treated in precise order with two equivalents of LiHMDS and two equivalents of TBDMSCl desired **157** could be formed.

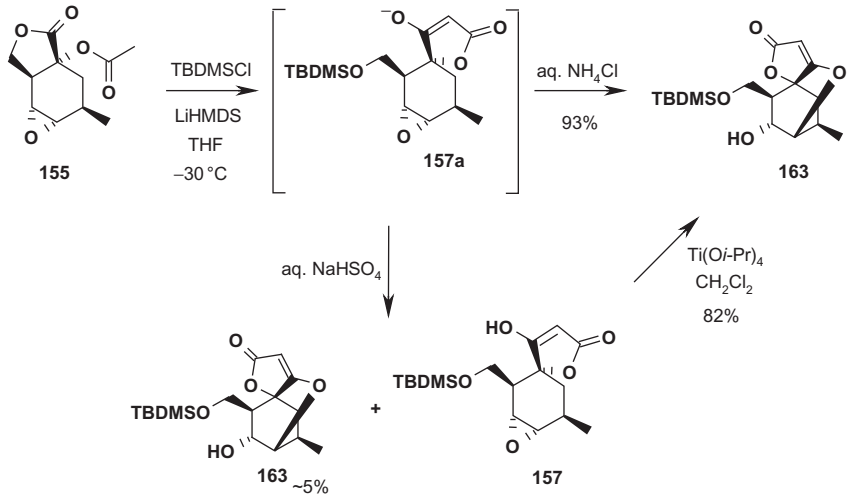
Combining reaction conditions for transformations **155** → **156** → **157** led to the direct synthesis of tetronic derivative **157** from lactone **155**. This process necessitated addition of two equivalents of the base into the solution of



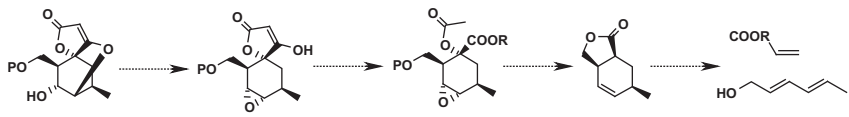
SCHEME 32

155 and TBDMSCl in THF at −30 °C. After acidic work up, compound **157** was obtained as a major component (yield not reported). The authors rationalized the observed results as outlined in Scheme 32. Initial formation of anion **158** led to the cyclization to produce **159** equilibrated with **160**. The second molecule of base generated dianion either **161** or **162**, but faster formation of **162** and its rapid reaction with TBDMSCl shifted equilibrium toward product **157**.

During this study and synthesis of compound **157**, formation of small quantities of compound **163** was also observed upon acidic work up. It was further discovered that crude **157** produced the target compound **163** when exposed to Ti(O*i*-Pr)$_4$ in CH_2Cl_2 in 82% yield (Scheme 33). All these results allowed the Georgiadis group to design experiments that would merge the Dieckmann condensation/TBDMS trapping/ring opening of the epoxide and, therefore, the direct transformation **155** → **163**. Thus, after initial reaction of **155** with LiHMDS/TBDMSCl, the work up involving aqueous NH_4Cl



SCHEME 33



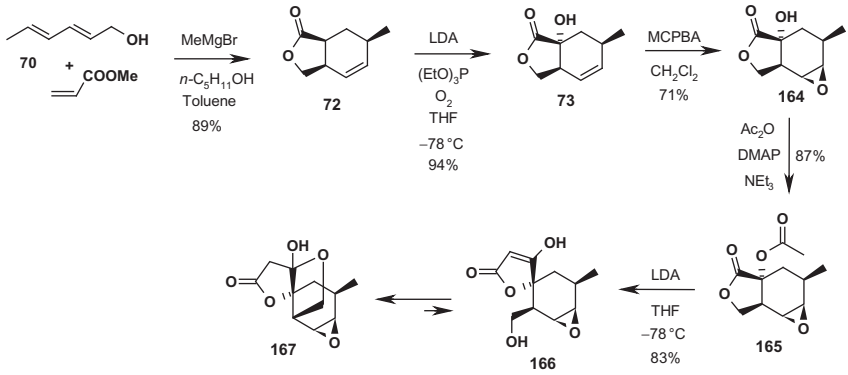
SCHEME 34

furnished **163** in excellent 93% yield. This completed the synthesis of the core tricyclic structure of abyssomicin in impressive 5 steps and 42% overall yield.

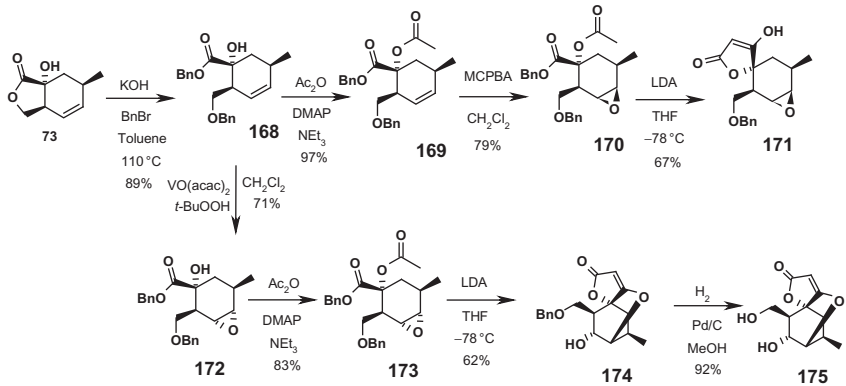
The core tricyclic structure was the research subject of the Maier's group as well [48]. Actually, their report from 2005 discussing the potential application of Diels–Alder reaction was the first one considering the Lewis acid-templated intermolecular [4 + 2] cycloaddition reaction to construct the cyclohexene ring related to the abyssomicin C synthesis (Scheme 34) [48a].

The synthesis started with Ward modification of Diels–Alder reaction to produce **72** followed by hydroxylation with molecular oxygen as discussed above (Scheme 35). The obtained product **73** was epoxidized under typical conditions employing MCPBA to afford compound **164** in 71% yield. Acetylation of the alcohol group furnished acetate **165** and set a stage for Dieckmann condensation and introduction of the tetronate. The condensation proceeded smoothly at −78 °C in the presence of LDA as a base, but initial formation of the tetronic moiety was followed by a nucleophilic addition of the generated primary alcohol onto the conjugated double bond to afford hemiketal **167**.

At this stage, the synthetic route was slightly altered to prevent formation of **167**. Thus, lactone **73** was hydrolyzed under basic conditions and



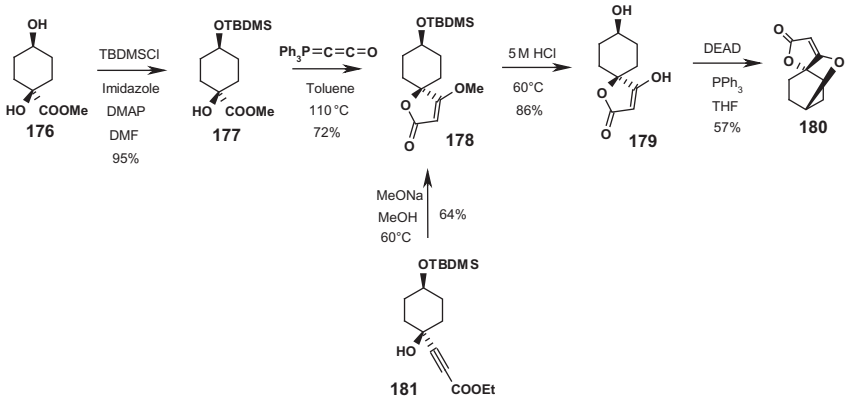
SCHEME 35



SCHEME 36

subsequently the released functionalities alkylated with benzyl bromide to produce **168** in 83% yield (Scheme 36). Routine acetylation and epoxydation of **168** yielded compound **170** in good overall yield. Dieckmann reaction of compound **170**, as expected, led to the epoxytetronate derivative **171**.

However, compound **171** proved to be resistant to various reaction conditions (BF_3/Et_2O, CH_2Cl_2; $Ti(O\textit{i}\text{-}Pr)_4/CH_2Cl_2$) designed to promote an intramolecular ring opening of the epoxide by the enol-tetronate. Based on these difficulties, the authors assigned stereochemistry of **171** as depicted, positioning the nucleophilic enol and the epoxide *syn*. To reverse orientation of the three-membered ring and to secure the right relationship between the tetronate and the epoxide in order to promote S_N2 process, the authors turned attention to a directed epoxidation using $VO(acac)_2/H_2O_2$ [47]. Using these conditions, the epoxide was formed *syn* to the guiding alcohol function and this finally set a stage for preparing the core bicyclic structure of abyssomicin C. Routine acylation of **172** afforded **173**. The basic conditions of the Dieckmann



SCHEME 37

condensation, leading to the formation of tetronate, promoted further transformation, the regioselective epoxide ring opening to furnish compound **174** in 62% yield. Removal of the Bn group was accomplished using H_2, Pd/C yielding the target compound **175**.

In addition to the above work, Maier *et al*. reported synthesis of a simple tetronic derivatives condensed to bicyclo[2.2.2]octane (Scheme 37) [48b]. Double protected spirotetronate **178** obtained via two different routes afforded compound **179** after exposure to acidic conditions. The following cyclization involving the enol and the alcohol moieties under Mitsunobu conditions produced compound **180** in 57% yield.

CONCLUSION

The resistance that Gram-positive bacteria develop toward typical antibiotics necessitates search for new targets and lead molecules. In this regard, the discovery of abyssomicins is of great significance since these molecules are the first to inhibit biosynthesis of *p*ABA, the pathway essential for many microorganisms but absent in humans. In recent years, several synthetic routes for abyssomicins and some model system studies appeared in the literature. Strategically, many of them were based on the synthesis of a highly substituted cyclohexene in early stages and its further elaboration to build the oxabicyclo core and then the final target via the macrocyclization. Intermolecular Diels–Alder reaction was utilized for the initial task of assembling a cyclohexene derivative, but particularly attractive is its use in later stages in an intramolecular fashion. Such an approach has created the spirotetronic fragment and the macrocyclic ring in a single operation highly diastereoselectively. An alternative method to the strategies based on Diels–Alder reaction employs an attractive dual catalysis, combining organocatalysis/Pd-mediated allylation, to access a cyclohexane derivative suitable for further transformation.

Particularly impressive in this approach is efficient macrocyclization utilizing the Nozaki–Hiyama–Kishi reaction.

The developed synthetic routes provide access to some of the naturally occurring abyssomicins, but they may also be applied for preparation of their derivatives. This will hopefully promote further exploration of this interesting class of compounds and perhaps may lead to related derivatives with better biological profiles.

ABBREVIATIONS

ADC	4-amino-4-deoxychorismate
BINOL	1,1′-bi-2-naphthol
Bn	benzyl
DABCO	1,4-diazabicyclo[2.2.2]octane
DDQ	2,3-dichloro-5,6-dicyano-1,4-benzoquinone
DibalH	diisobutylaluminium hydride
DMDO	dimethyldioxirane
DMF	dimethylformamide
DMPU	*N*,*N*′-dimethylpropylene urea
DMSO	dimethyl sulphoxide
IBX	2-iodoxybenzoic acid
LDA	lithium diisopropylamide
LiHMDS	lithium hexamethyldisilazide
MCPBA	*meta*-chloroperoxybenzoic acid
MIC	minimal inhibition concentration
MOM	methoxymethyl
NBS	*N*-bromosuccinimide
PCC	pyridinium chlorochromate
***p*ABA**	*para*-aminobenzoic acid
PMB	*para*-methoxybenzyl
PPA	porcine pancreatic lipase
RCM	ring-closing metathesis
TBDMS	*tert*-butyldimethylsilyl
TES	triethylsilyl
Tf	triflate
THF	tetrahydrofurane
TIPS	triisopropylsilyl

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Chapter 6

Progress in the Syntheses of Dibenzocyclooctadiene Lignans

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Chapter Outline

INTRODUCTION

The lignans comprise a class of natural products which are phenylpropanoid dimers where the phenylpropane units are linked by the central carbon of their side chains [1–8]. They are distributed widely in vascular plants and are related biochemically to phenylalanine metabolism. Many lignans show important biological activities, such as antitumor, antimitotic, anti-HIV, and antiviral activities [9,10].

The term lignan, reflecting the woody tissue from which many examples derive, was introduced in 1936 by Haworth in a review of natural resins [11]. Later in 1978, lignan was redefined by Gottlieb as the dimers of cinnamyl alcohols and/or cinnamic acids [12]. However, Haworth's definition has been widely used and was adopted by the IUPAC recommendations 2000 [13]. For nomenclature purpose, the C_6C_3 unit is treated as propylbenzene and numbered from 1 to 6 in the ring starting from the propyl group and with the propyl group numbered from 7 to 9 starting from the benzene ring. With the second C_6C_3 unit, the numbers are primed (Fig. 1). Thus, all lignans have a C-8 to C-8′ bond. Apart from the C8–C8′ linkage, other positions of lignans

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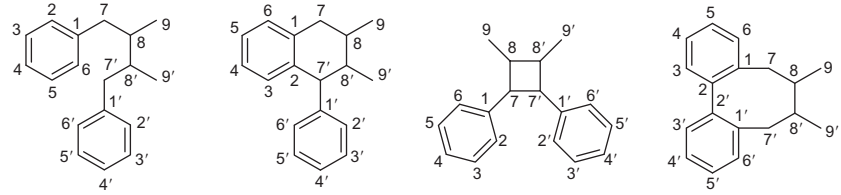


FIGURE 1 Basic carbon skeletons of lignans.

may be bridged by C—C bond (2–7′, 7–7′, 2–2′) formation to create additional rings, and accordingly, there are four classes of lignans, namely, acyclic lignan **I**, 2,7′-cyclolignan **II**, 7,7′-cyclolignan **III**, and 2,2′-cyclolignan **IV** (Fig. 1).

Natural products biosynthetically related to but distinct from lignans are neolignans and norlignans. Neolignans are composed of two phenylpropane units linked in a manner other than C8–C8′ [14]. The linkage with neolignans may include C-8 or C-8′ and does not restrict to direct carbon–carbon bond. Compounds where an ether oxygen atom provides the linkage between the two C_6C_3 units are also included as neolignans and are named as oxyneolignan. Norlignans refer to natural products with 1,3-diphenylpentane carbon skeletons found in many conifers and some monocotyledonous plants [15]. These two classes of natural products, as well as lignans of the subgroups acyclic lignan **I**, 2,7′-cyclolignan **II**, 7,7′-cyclolignan **III** (Fig. 1), are not covered in this review. Emphasis will be addressed on 2,2′-cyclolignan **IV** (Fig. 1). 2,2′-Cyclolignans are characterized by a dibenzocyclooctadiene moiety, thus the name dibenzocyclooctadiene lignan is well accepted and will be used throughout the rest of this chapter.

STRUCTURAL CHARACTERISTICS AND CLASSIFICATIONS OF DIBENZOCYCLOOCTADIENE LIGNANS

Up until now, more than 300 dibenzocyclooctadiene lignans have been isolated, most of which from the genera *Schisandra* and *Kadsura* of the plant family Schisandraceae [16]. They have been found to exhibit a variety of important biological activities such as antihepatitis [17], antitumor [18], anti-HIV [19], antidiabetes [20], antioxidant [21], and neuroprotective effects [22].

The structural complexity of dibenzocyclooctadiene lignans arise not only from the different oxidation levels and chiral centers of the side chain but also from the axial chirality [23] incurred by the presence of substituents on C3 and C3′ as well as the eight-membered ring, which leads to high energy barrier for rotation around the biaryl bridge.

Gottlieb *et al*. [24] studied the conformations of dibenzocyclooctadiene systems utilizing X-ray and ^{1}H- and ^{13}C NMR spectroscopy. They found that the cyclooctadiene rings always exist in conformations similar to Anet and

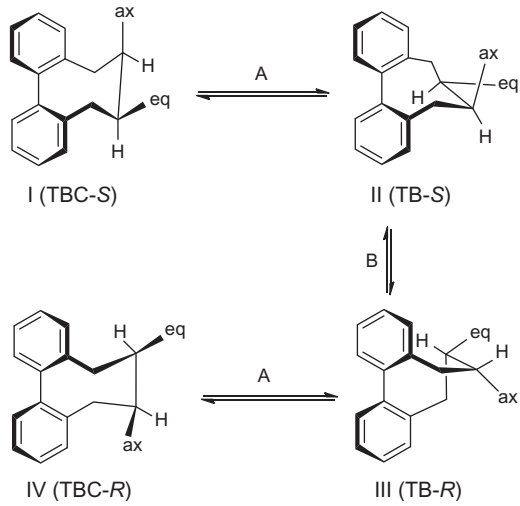
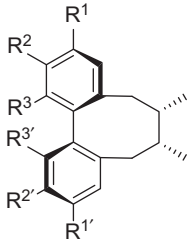


FIGURE 2 Interconversion of TB and TBC conformations of dibenzocyclooctadiene.

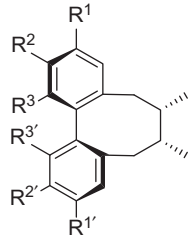
Yavari's twist boat (TB) or twist-boat-chair (TBC) of the parent cyclooctadiene ring (Fig. 2) [25]. The eight-membered ring conformation change (process A) has a barrier no higher than 60 kJ/mol and should be fast at room temperature. On the other hand, process B, which involves biaryl rotation, has a high energy barrier ($\geq$145 kJ/mol) because of substitution of the aromatic rings *ortho* to the biaryl bond (the substituents are omitted for simplicity). For all practical purposes, at room temperature, process B does not occur and biphenyl rotamers are isolatable.

Most of the dibenzocyclooctadiene lignans isolated so far possess 3,4,5(3′,4′,5′)-trioxygenated phenyl rings, mainly in the form of 3,4,5(3′,4′,5′)-trimethoxyphenyl or 3(3′)-methoxy-4,5(4′,5′)-methylenedioxyphenyl. Substitution patterns of 3(3′)-hydroxy-4,5(4′,5′)-dimethoxy, as well as 3(3′)-acyloxy-4,5(4′,5′)-methylenedioxy, are also commonly encountered. Particularly, most compounds of the dibenzocyclooctadiene-9,9′-lactone subgroup lack a 3-oxygen substituent, having a 4,5-methylenedioxy substituted C_6 unit (*vide infra*).

Apart from the substitution pattern of the biaryl unit and the absolute configuration of the biaryl axis, the structural variation of dibenzocyclooctadiene lignan mainly comes from the oxidation level, substitution pattern, and configuration of stereocenters along the aliphatic bridge. Accordingly, the majority of dibenzocyclooctadiene lignans can be classified into 11 subgroups [1–8,13,16,17,18b,d,19,20,21d,22b,d,26–53], including non-oxygen-substituted dibenzocyclooctadiene lignans (Fig. 3), C7(C7′)-oxygen-substituted dibenzocyclooctadiene lignans (Fig. 4), C8′-hydroxy dibenzocyclooctadiene lignans (Fig. 5), C7′,C8′ (C7,C8)-dioxygen-substituted dibenzocyclooctadiene lignans (Fig. 6), C7,C7′-dioxygen-substituted dibenzocyclooctadiene lignans (Fig. 7), C7,C7′,C8′-trioxygen-substituted dibenzocyclooctadiene lignans (Fig. 8), C7,



$R^1 = R^2 = R^3 = R^{1'} = R^{2'} = R^{3'} = OMe$. (+)-deoxyschisandrin (wuweizisu A) **1**
$R^1,R^2 = OCH_2O$; $R^3 = R^{1'} = R^{2'} = R^{3'} = OMe$. γ–schisandrin (wuweizisu B) **2**
$R^1,R^2 = R^{1'},R^{2'} = OCH_2O$; $R^3 = R^{3'} = OMe$. (+)-wuweizisu C (schisandrin C) **3**
$R^1 = OH$; $R^2 = R^3 = R^{1'} = R^{2'} = R^{3'} = OMe$. (+)-gomisin K_2 **4**
$R^1 = R^2 = R^{1'} = R^{2'} = R^{3'} = OMe$; $R^3 = OH$. (+)-gomisin K_3 **5**
$R^1 = R^2 = R^{3'} = OMe$; $R^3 = OH$; $R^{1'},R^{2'} = OCH_2O$. (+)-gomisin M_1 **6**
$R^1 = R^2 = R^{3'} = OMe$; R^3 = angeloyloxy; $R^{1'},R^{2'} = CH_2$. (+)-angeloylgomisin M_1 **7**
$R^1 = R^2 = R^3 = OMe$; $R^{1'},R^{2'} = OCH_2O$; $R^{3'} = OH$. (+)-gomisin M_2 **8**
$R^1 = R^2 = R^3 = R^{3'} = OMe$; $R^{1'},R^{2'} = OCH_2O$. (+)-kadsuranin **9**
$R^1,R^2 = OCH_2O$; $R^3 = R^{1'} = R^{2'} = R^{3'} = OMe$. (+)-isokadsuranin **10**
$R^1,R^2 = OCH_2O$; R^3 = angeloyloxy; $R^{1'} = R^{2'} = R^{3'} = OMe$. (+)-kadsutherin **11**
$R^1 = R^2 = R^{1'} = R^{2'} = R^{3'} = OMe$; $R^3 = OAc$. (+)-acetylschisanhenol **12**
$R^1,R^2 = OCH_2O$; $R^3 = R^{1'} = R^{2'} = OMe$; $R^{3'} = OH$. (+)-schisanhenol B **13**
$R^1 = R^{3'} = OH$; $R^2 = R^3 = R^{1'} = R^{2'} = OMe$. arisanschinin F **14**
$R^1 = R^2 = R^{1'} = R^{2'} = R^{3'} = OMe$; R^3 = tigloyloxy. (+)-14-tigloylgomisin K_3 **15**
$R^1 = R^2 = R^{1'} = R^{2'} = R^{3'} = OMe$; R^3 = angeloyloxy. angeloyl-(+)-gomisin K_3 **16**
$R^1 = R^2 = R^{1'} = R^{2'} = R^{3'} = OMe$; $R^3 = OBz$. neglschisandrin D **17**



$R^1,R^2 = R^{1'},R^{2'} = OCH_2O$; $R^3 = R^{3'} = OMe$. (–)-wuweizisu C **18**
$R^1 = R^{1'} = OH$; $R^2 = R^3 = R^{2'} = R^{3'} = OMe$. (–)-gomisin J **19**
$R^1 = OH$; $R^2 = R^3 = R^{1'} = R^{2'} = R^{3'} = OMe$. (–)-gomisin K_1 **20**
$R^1 = R^2 = R^{3'} = OMe$; $R^3 = OH$; $R^{1'},R^{2'} = OCH_2O$. (–)-gomisin L_1 **21**
$R^1 = OH$; $R^2 = R^3 = R^{3'} = OMe$; $R^{1'},R^{2'} = OCH_2O$. (–)-gomisin L_2 **22**
$R^1,R^2 = OCH_2O$; $R^3 = R^{1'} = R^{2'} = R^{3'} = OMe$. (–)-gomisin N **23**
$R^1 = R^2 = R^{2'} = R^{3'} = OMe$; $R^3 = R^{1'} = OH$. (+)-rubrisandrin A a **24**
$R^1 = R^{3'} = OH$; $R^2 = R^3 = R^{1'} = R^{2'} = OMe$. (+)-rubrisandrin A b **25**
$R^1 = R^2 = R^{1'} = R^{2'} = OMe$; $R^3 = R^{3'} = OH$. (+)-rubrisandrin B **26**
$R^1 = R^2 = R^3 = R^{3'} = OMe$; $R^{1'},R^{2'} = OCH_2O$. (–)-rubschisandrin **27**
$R^1 = R^3 = R^{1'} = OH$; $R^2 = R^{2'} = R^{3'} = OMe$. marlignan A **28**
$R^1 = R^2 = R^{2'} = OMe$; $R^3 = R^{1'} = R^{3'} = OH$. marlignan B **29**
$R^1 = R^{1'} = R^{2'} = OMe$; $R^2 = R^3 = R^{3'} = OH$. marlignan C **30**
$R^1 = R^3 = R^{2'} = R^{3'} = OMe$; $R^2 = R^{1'} = OH$. schineglignan A **31**
$R^1 = R^2 = R^3 = R^{2'} = OMe$; $R^{1'} = R^{3'} = OH$. schineglignan B **32**
$R^1 = R^{3'} = OH$; $R^2 = R^3 = R^{1'} = R^{2'} = OMe$. arisanschinin G **33**

FIGURE 3 Non-oxygen-substituted dibenzocyclooctadiene lignans.

C7′-epoxy dibenzocyclooctadiene lignans (Fig. 9), C7,C9′-epoxy dibenzocyclooctadiene lignans (Fig. 10), dibenzocyclooctadiene-9,9′-lactones (Fig. 11), C7,C2′-epoxy dibenzocyclooctadiene lignans (Fig. 12), and C3,C2′-methylene-epoxy dibenzocyclooctadiene lignans (Fig. 13). Among these, the C3, C2′-methylene-epoxy dibenzocyclooctadiene lignan subgroup compounds are formed by oxidative coupling between an aromatic carbon (C2′) and a methoxy carbon (C3-methoxy carbon). Like C7,C2′-epoxy dibenzocyclooctadiene lignans, the C6′ structural unit loses its aromaticity. Compounds **319–323** formed by oxidative cleavage of the C4′–C5′ bond, as well as the C8–C2′-linked compounds **324–326**, are also included as dibenzocyclooctadiene lignans (Fig. 14).

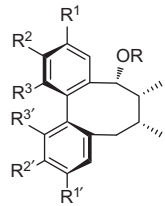
DIBENZOCYCLOOCTADIENE LIGNAN SYNTHESES

As shown in Scheme 1, the core structure of dibenzocyclooctadiene lignan derivatives can be obtained by two major pathways, which differ in the order of biaryl coupling and side-chain condensation. In route A, an intermolecular

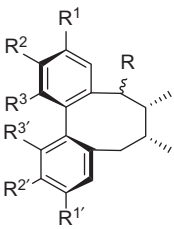
biaryl coupling is carried out prior to eight-membered ring closure, whereas in route B, condensation of the side chain gives rise to the formation of a dibenzylbutane derivative from which an intramolecular biaryl coupling would eventually close the eight-membered ring. A key to the total synthesis of dibenzocyclooctadiene lignans is the controlment of the stereochemical outcome of the side chains as well as the challenging biaryl unit [23,54].

Intermolecular Biphenyl Coupling

This strategy proceeds by aryl–aryl coupling to form the biphenyl compound, followed by an intramolecular condensation reaction between the two aliphatic side chains (Route A of Scheme 1). The Ullmann reaction, coupling of aryl halides in the presence of Cu, Ni, or Pd, is a classical method to synthesize the biphenyl derivatives [55]. In their synthesis of (±)-deoxyshizandrin **1**, Carroll [56] and coworkers found that Ullmann coupling of the benzoate derivative **327** of 3-iodo-2,6-dimethoxyphenol gave

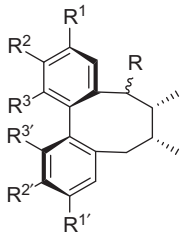


R^1 = OH, R^2 = R^3 = $R^{1'}$ = $R^{2'}$ = OMe; $R^{3'}$ = angeloyloxy; R = H. (+)-schisantherin F **34**
R^1,R^2 = OCH_2O; R^3 = $R^{1'}$ = $R^{2'}$ = $R^{3'}$ = OMe; R = Ac. (–)-kadsurin **35**
R^1,R^2 = OCH_2O; R^3 = $R^{1'}$ = $R^{2'}$ = OMe; $R^{3'}$ = OH; R = Ac. (+)-acetylbinankadsurin A **36**
R^1,R^2 = OCH_2O; R^3 = $R^{1'}$ = $R^{2'}$ = OMe; $R^{3'}$ = OH; R = angeloyl. (+)-angeloyl binankadsurin A **37**
R^1, R^2 = OCH_2O; R^3 = $R^{1'}$ = $R^{2'}$ = OMe; $R^{3'}$ = OH; R = hexanoyl. (+)-caproyl binankadsurin A **38**
R^1, R^2 = OCH_2O; R^3 = $R^{1'}$ = $R^{2'}$ = OMe; $R^{3'}$ = OH; R = H. (–)-binankadsurin A **39**
R^1, R^2 = OCH_2O; R^3 = $R^{1'}$ = $R^{2'}$ = $R^{3'}$ = OMe; R = Me. schilancifolignan A **40**
R^1, R^2 = OCH_2O; R^3 = $R^{2'}$ = $R^{3'}$ = OMe; $R^{1'}$ = OH; R = Me. schilancifolignan B **41**
R^1, R^2 = OCH_2O; R^3 = $R^{2'}$ = $R^{3'}$ = OMe; $R^{1'}$ = OH; R = Ac. schilancifolignan C **42**
R^1 = R^2 = R^3 = $R^{1'}$ = $R^{2'}$ = OMe; $R^{3'}$ = Isovaleryloxy; R = H. tiegusanin A **43**
R^1 = R^2 = R^3 = $R^{1'}$ = $R^{2'}$ = OMe; $R^{3'}$ = Isobutyryloxy; R = H. tiegusanin B **44**
R^1 = R^2 = $R^{1'}$ = $R^{2'}$ = $R^{3'}$ = OMe; R^3 = OBz; R = H. tiegusanin C **45**
R^1,R^2 = OCH_2O; R^3 = $R^{1'}$ = $R^{2'}$ = $R^{3'}$ = OMe; R = cinnamoyl. kadsuphilin A **46**
R^1,R^2 = OCH_2O; R^3 = $R^{1'}$ = $R^{2'}$ = OMe; $R^{3'}$ = OH; R = cinnamoyl. 1-demethylkadsuphilin A **47**
R^1 = R^2 = R^3 = $R^{1'}$ = $R^{2'}$ = $R^{3'}$ = OMe; R = Bz. schizanrin L **48**
R^1,R^2 = OCH_2O; R^3 = $R^{2'}$ = $R^{3'}$ = OMe; $R^{1'}$ = OH, R = H. kadsuralignan A **49**
R^1,R^2 = OCH_2O; R^3 = $R^{1'}$ = $R^{2'}$ = $R^{3'}$ = OMe; R = *cis*-cinnamoyl. polysperlignan F **50**
R^1,R^2 = OCH_2O; R^3 = $R^{1'}$ = $R^{2'}$ = OMe; $R^{3'}$ = angeloyl; R = H. kadsuralignan I **51**
R^1,R^2 = OCH_2O; R^3 = $R^{1'}$ = $R^{2'}$ = OMe; $R^{3'}$ = 2-methylbutanoyl; R = H. kadsuralignan J **52**
R^1,R^2 = OCH_2O; R^3 = $R^{1'}$ = $R^{2'}$ = OMe; $R^{3'}$ = Bz; R = H. kadsuralignan K **53**
R^1,R^2 = OCH_2O; R^3 = $R^{1'}$ = $R^{2'}$ = OMe; $R^{3'}$ = OH; R = $COC(CH_3)$=$CHCH(OCH_3)_2$. kadsuralignan G **54**
R^1 = OH; R^2 = R^3 = $R^{1'}$ = $R^{2'}$ = OMe; $R^{3'}$ = 2-methylbutanoyl; R = H. kadsuralignan L **55**
R^1,R^2 = OCH_2O; R^3 = $R^{1'}$ = $R^{2'}$ = OMe; $R^{3'}$ = OH; R = tigloyl. propinquain G **56**
R^1 = OH; R^2 = R^3 = $R^{1'}$ = $R^{2'}$ = $R^{3'}$ = OMe; R = H. propinquain H **57**
R^1 = R^2 = R^3 = $R^{1'}$ = $R^{2'}$ = OMe; $R^{3'}$ = angeloyloxy; R = H. methyl schisantherin F **58**
R^1,R^2 = OCH_2O; R^3 = $R^{1'}$ = $R^{2'}$ = OMe; $R^{3'}$ = OH; R = butanoyl. (+)-butyryl binankadsurin A **59**
R^1,R^2 = OCH_2O; R^3 = $R^{1'}$ = $R^{2'}$ = OMe; $R^{3'}$ = OH; R = isobutanoyl. (+)-isobutyroyl binankadsurin A **60**
R^1,R^2 = OCH_2O; R^3 = $R^{1'}$ = $R^{2'}$ = OMe; $R^{3'}$ = OH; R = isovaleroyl. (+)-isovaleroyl binankadsurin A **61**
R^1,R^2 = OCH_2O; R^3 = $R^{1'}$ = $R^{2'}$ = OMe; $R^{3'}$ = OH; R = Bz. (–)-benzoyl binankadsurin A **62**
R^1,R^2 = OCH_2O; R^3 = $R^{1'}$ = $R^{2'}$ = $R^{3'}$ = OMe; R = isovaleroyl. (+)-heteroclitin A **63**
R^1,R^2 = OCH_2O; R^3 = $R^{1'}$ = $R^{2'}$ = $R^{3'}$ = OMe; R = angeloyl. (+)-heteroclitin B **64**
R^1,R^2 = OCH_2O; R^3 = $R^{1'}$ = $R^{2'}$ = $R^{3'}$ = OMe; R = tigloyl. (–)-heteroclitin C **65**

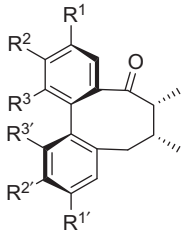


R^1,R^2 = $R^{1'}$,$R^{2'}$ = OCH_2O; R^3 = $R^{3'}$ = OMe; R = OH. (–)-schisanligaol D **66**
R^1,R^2 = OCH_2O; R^3 = OH; $R^{1'}$ = $R^{2'}$ = $R^{3'}$ = OMe; R = β-cinnamoyloxy. (+)-schisanrin A **67**

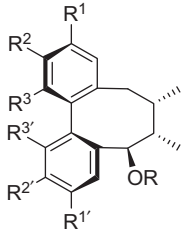
FIGURE 4—Cont'd



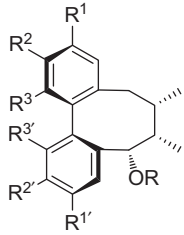
$R^1,R^2 = OCH_2O$; $R^3 = R^{1'} = R^{2'} = OMe$; $R^{3'} = OH$; R = α-OAc. (+)-schisantherin O **68**
$R^1,R^2 = OCH_2O$; $R^3 = R^{1'} = R^{2'} = OMe$; $R^{3'} = OH$; R = β-OH. (+)-kadsuphilol A **69**
$R^1 = R^3 = R^{1'} = R^{2'} = OMe$; $R^2 = R^{3'} = OH$; R = β-cinnamoyloxy. (+)-kadsuphilol D **70**
$R^1,R^2 = OCH_2O$; $R^3 = R^{1'} = R^{2'} = R^{3'} = OMe$; R = α-OAc. ananolignan A **71**
$R^1,R^2 = OCH_2O$; $R^3 = R^{1'} = R^{2'} = OMe$; $R^{3'} = OAc$; R = β-OH. polysperlignan K **72**
$R^1,R^2 = OCH_2O$; $R^3 = R^{2'} = R^{3'} = OMe$; $R^{1'} = OH$; R = β-OH. kadsuralignan E **73**



$R^1 = R^2 = R^3 = R^{1'} = R^{2'} = R^{3'} = OMe$. (–)-schisanlignone A **74**
$R^1 = OH$; $R^2 = R^3 = R^{1'} = R^{2'} = R^{3'} = OMe$. (–)-schisanlignone B **75**
$R^1 = R^2 = R^3 = R^{3'} = OMe$; $R^{1'},R^{2'} = OCH_2O$. (–)-schisanlignone C **76**
$R^1,R^2 = R^{1'},R^{2'} = OCH_2O$; $R^3 = R^{3'} = OMe$. (–)-schisanlignone D **77**
$R^1,R^2 = OCH_2O$; $R^3 = R^{1'} = R^{2'} = OMe$; $R^{3'} = OBz$. (–)-schisanlignone E **78**



$R^1,R^2 = OCH_2O$; $R^3 = R^{1'} = R^{2'} = R^{3'} = OMe$; R = H. (–)-gomisin O **79**
$R^1,R^2 = OCH_2O$; $R^3 = R^{1'} = R^{2'} = R^{3'} = OMe$; R = tigloyl. (–)-tigloylgomisin O **80**
$R^1,R^2 = OCH_2O$; $R^3 = R^{1'} = R^{2'} = R^{3'} = OMe$; R = angeloyl. (+)-angeloylgomisin O **81**
$R^1 = R^2 = R^3 = R^{3'} = OMe$; $R^{1'},R^{2'} = OCH_2O$; R = H. isogomisin O **82**
$R^1 = R^2 = R^3 = R^{3'} = OMe$; $R^{1'},R^{2'} = OCH_2O$; R = Me. methyl isogomisin O **83**
$R^1 = R^2 = R^3 = R^{3'} = OMe$; $R^{1'},R^{2'} = OCH_2O$; R = angeloyl. (+)-angeloylisogomisin O **84**
$R^1 = R^2 = R^3 = R^{3'} = OMe$; $R^{1'},R^{2'} = OCH_2O$; R = Bz. (–)-benzoylolylisogomisin O**85**
$R^1,R^2 = R^{1'},R^{2'} = OCH_2O$; $R^3 = R^{3'} = OMe$; R = H. (–)-gomisin R **86**
$R^1,R^2 = R^1,R^{2'} = CH_2$; $R^3 = R^{3'} = Me$; R = Ac. (–)-acetylgomisin R **87**
$R^1,R^2 = R^{1'},R^{2'} = OCH_2O$; $R^3 = R^{3'} = OMe$; R = Me. methylgomisin R **88**
$R^1,R^2 = R^{1'},R^{2'} = OCH_2O$; $R^3 = R^{3'} = OMe$; R = angeloyl. (+)-angeloylgomisin R **89**
$R^1,R^2 = OCH_2O$; $R^3 = R^{1'} = R^{2'} = R^{3'} = OMe$; R = Ac. (–)-rubschisantherin **90**
$R^1,R^2 = R^{1'},R^{2'} = OCH_2O$; $R^3 = R^{3'} = OMe$; R = Bz. (–)-interiotherin A **91**
$R^1 = OH$; $R^2 = R^3 = R^{1'} = R^{2'} = R^{3'} = OMe$; R = H. (–)-gomisin U **92**
$R^1 = OH$; $R^2 = R^3 = R^{1'} = R^{2'} = R^{3'} = OMe$; R = Bz. (–)-benzoylgomisin U **93**
$R^1,R^2 = OCH_2O$; $R^3 = OH$; $R^{1'} = R^{2'} = R^{3'} = OMe$; R = Me. wilsonilignan C **94**
$R^1 = R^2 = R^3 = R^{2'} = R^{3'} = OMe$; $R^{1'} = OAc$; R = Me. rubrilignan A **95**
$R^1 = OAc$; $R^2 = R^3 = R^{1'} = R^{2'} = R^{3'} = OMe$; R = Me. rubrilignan B **96**
$R^1,R^2 = OCH_2O$; $R^3 = R^{2'} = R^{3'} = OMe$; $R^{1'} = OAc$; R = H. gomisin S **97**
$R^1 = OH$; $R^2 = R^3 = R^{3'} = OMe$; $R^{1'},R^{2'} = OCH_2O$; R = Me. 12-demethylwuweilignan I **98**
$R^1 = R^{1'} = OH$; $R^2 = R^3 = R^{2'} = R^{3'} = OMe$; R = H. marlignan D **99**
$R^1 = R^{1'} = OH$; $R^2 = R^3 = R^{2'} = R^{3'} = OMe$; R = Ac. marlignan E **100**
$R^1 = R^{1'} = OH$; $R^2 = R^3 = R^{2'} = R^{3'} = OMe$; R = Me. marlignan F **101**
$R^1 = R^{1'} = OH$; $R^2 = R^3 = R^{2'} = R^{3'} = OMe$; R = Bz. marlignan G **102**
$R^1 = R^2 = R^3 = R^{1'} = R^{2'} = R^{3'} = OMe$; R = Ac. marlignan I **103**
$R^1 = R^2 = R^3 = R^{1'} = R^{2'} = R^{3'} = OMe$; R = Et. marlignan J **104**



$R^1 = OH$; $R^2 = R^3 = R^{1'} = R^{2'} = R^{3'} = OMe$; R = H. (–)-gomisin S **105**
$R^1,R^2 = R^{1'},R^{2'} = OCH_2O$; $R^3 = R^{3'} = OMe$; R = H. 6-epigomisin **106**
$R^1,R^2 = OCH_2O$; $R^3 = R^{1'} = R^{2'} = R^{3'} = OMe$; R = H. (–)-epigomisin O **107**
$R^1,R^2 = OCH_2O$; $R^3 = R^{2'} = R^{3'} = OMe$; $R^{1'} = OH$; R = H. (+)-kadsuphilin B **108**
$R^1,R^2 = OCH_2O$; $R^3 = OH$; $R^{1'} = R^{2'} = R^{3'} = OMe$; R = H. wilsonilignan A **109**
$R^1,R^2 = OCH_2O$; $R^3 = R^{1'} = R^{2'} = OMe$; $R^{3'} = OH$; R = H. wilsonilignan B **110**

FIGURE 4—Cont'd

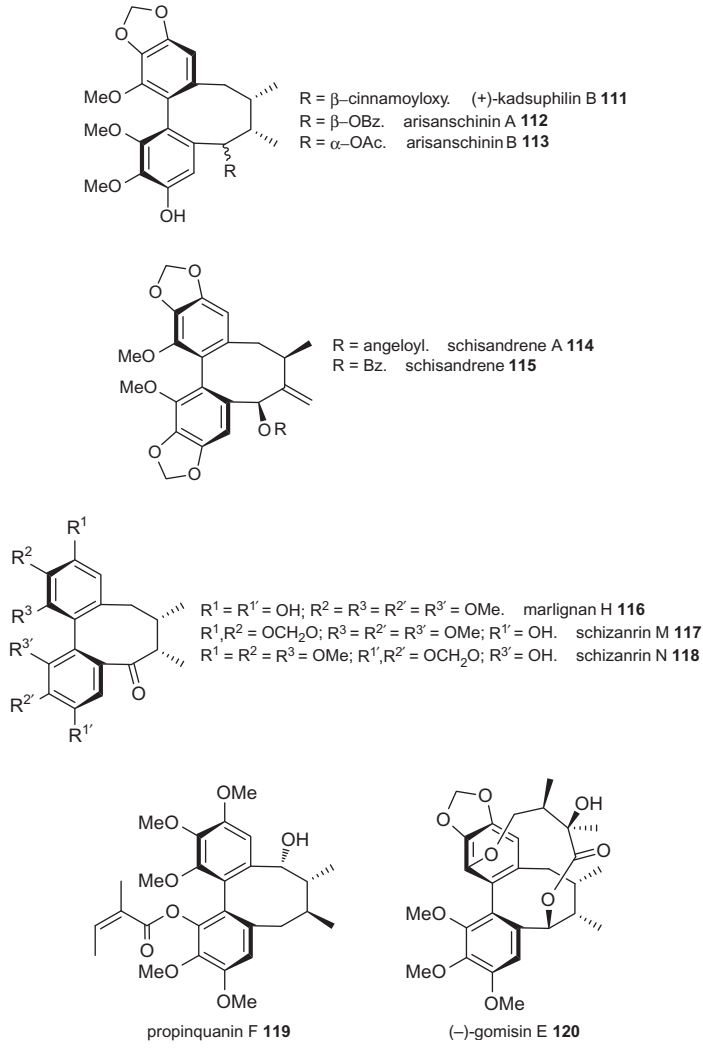
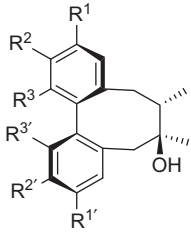
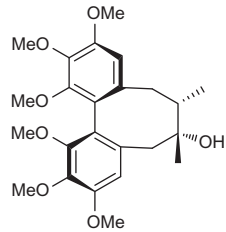


FIGURE 4 C7(C7′)-oxygen-substituted dibenzocyclooctadiene lignans.

biphenyldiol **328** in good yield (Scheme 2). In contrast, Ullmann reaction of the acetate and benzyl derivatives gave unsatisfactory results. A major reaction with these substrates was reductive removal of iodine. Oxidative cleavage of the bisolefin **330**, formed by Claisen rearrangement of the bismethallyl ether **329** of biphenyldiol **328**, gave bisacetonylbiphenyl **331**. Intramolecular reductive coupling of **331**, followed by hydrogenation of the resulting olefin,



$R^1 = R^2 = R^3 = R^{1'} = R^{2'} = R^{3'} = OMe$. (+)-schisandrin [(+)-wuweizichun A] **121**
$R^1,R^2 = OCH_2O$; $R^3 = R^{1'} = R^{2'} = R^{3'} = OMe$. (+)-gomisin A [wuweizichun B] **122**
$R^1 = R^2 = R^{1'} = R^{2'} = R^{3'} = OMe$; $R^3 = OH$. (+)-gomisin H **123**
$R^1 = OH$; $R^2 = R^3 = R^{1'} = R^{2'} = R^{3'} = OMe$. (+)-gomisin T **124**
$R^1 = R^2 = R^{1'} = R^{2'} = R^{3'} = OMe$; R^3 = angeloyloxy. (+)-angeloylgomisin H **125**
$R^1 = R^2 = R^{1'} = R^{2'} = R^{3'} = OMe$; R^3 = tigloyloxy. (+)-tigloylgomisin H **126**
$R^1 = R^2 = R^{1'} = R^{2'} = R^{3'} = OMe$; $R^3 = OBz$. (+)-benzoylgomisin H **127**
$R^1 = R^2 = R^{1'} = R^{2'} = R^{3'} = OMe$; R^3 = cinnamoyloxy. neglschisandrin C **128**
$R^1 = R^3 = OH$; $R^2 = R^{1'} = R^{2'} = R^{3'} = OMe$. marlignan K **129**
$R^1 = R^{1'} = OH$; $R^2 = R^3 = R^{2'} = R^{3'} = OMe$. marlignan L **130**

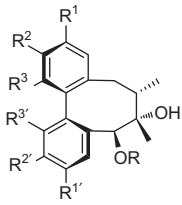


(+)-isoschisandrin **131**

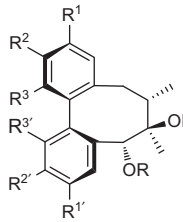
FIGURE 5 C8′-hydroxy dibenzocyclooctadiene lignans.

gave the target molecule **1** as a major component (46% over the two steps). The *trans* isomer **332** with a TBC conformation was obtained as a minor product (16%), which was believed to have been formed through double bond isomerization to a trisubstituted position prior to hydrogenation.

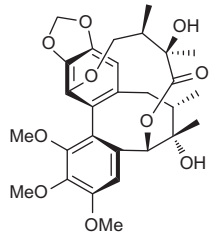
The side chains of the biphenyl derivatives can be cyclized by various intramolecular coupling strategies to close the eight-membered ring of lignan structures. Molander [57] has utilized a SmI_2 promoted 8-endo ketyl-olefin cyclization as a key step in the total synthesis of (+)-isoschizandrin **131** (Scheme 3). Standard Ullmann coupling reaction of bromoaldehyde **333** provided the biaryl dialdehyde **334**, Cannizzaro reaction of which followed by DCC coupling produced the racemic seven-membered lactone (±)-**335**. This lactone underwent kinetic resolution on treatment with (*R*)-2-methyl-CBS-oxazaborolidine to provide the desired lactone (+)-**335** in 45% yield (out of a possible 50%) with 98% enantiomeric excess (ee) as determined by chiral HPLC. DIBAL-H reduction of the enantiomerically enriched lactone at −78 °C revealed aldehyde (+)-**336**, which was further converted into alkenyl ketone (+)-**337**. The synthesis was finalized by treatment of ketoolefin (+)-**337** with 2.2 equiv. of samarium(II) iodide and HMPA in THF along with 2 equiv. of *t*-BuOH, which gave the natural product (+)-isoschizandrin **131** with a dr >18:1 and with no loss of ee (98%). The intramolecular nucleophilic substitution of the side chains of biphenyl derivatives can provide the corresponding eight-membered ring biphenyl compounds. Xie [58] and coworkers synthesized the key intermediate, tetraester **339** available from gallic



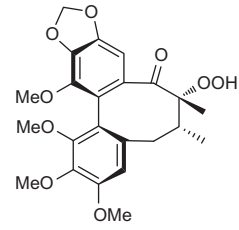
$R^1,R^2 = OCH_2O$; $R^3 = R^{1'} = R^{2'} = R^{3'} = OMe$; R = angeloyl. (–)-gomisin B [(–)-schisantherin B] **132**
$R^1,R^2 = OCH_2O$; $R^3 = R^{1'} = R^{2'} = R^{3'} = OMe$; R = Bz. (–)-gomisin C [(–)-schisantherin A] **133**
$R^1 = R^2 = R^3 = R^{3'} = OMe$; $R^{1'},R^{2'} = OCH_2O$; R = angeloyl. (–)-gomisin F **134**
$R^1 = R^2 = R^3 = R^{3'} = OMe$; $R^{1'},R^{2'} = OCH_2O$; R = Bz. (–)-gomisin G **135**
$R^1,R^2 = OCH_2O$; $R^3 = R^{1'} = R^{2'} = R^{3'} = OMe$; R = tigloyl. (–)-schisantherin C **136**
$R^1,R^2 = R^{1'},R^{2'} = OCH_2O$; $R^3 = R^{3'} = OMe$; R = Bz. (–)-schisantherin D **137**
$R^1 = OH$; $R^2 = R^3 = R^{1'} = R^{2'} = R^{3'} = OMe$; R = Bz. (–)-schisantherin E **138**
$R^1 = R^2 = R^3 = R^{1'} = R^2 = R^{3'} = OMe$; R = Bz. (–)-benzoylgomisin Q **139**
$R^1,R^2 = R^{1'},R^{2'} = OCH_2O$; $R^3 = R^{3'} = OMe$; R = angeloyl. (+)-interiotherin B **140**
$R^1,R^2 = OCH_2O$; $R^3 = R^{1'} = R^{2'} = OMe$; $R^{3'} = OH$; R = angeloyl. schirubrisin A **141**
$R^1,R^2 = OCH_2O$; $R^3 = R^{1'} = R^{2'} = OMe$; $R^{3'} = OH$; R = Bz. schirubrisin B **142**
$R^1,R^2 = OCH_2O$; $R^3 = R^{2'} = R^{3'} = OMe$; $R^{1'} = OH$; R = angeloyl. schirubrisin C **143**
$R^1 = R^2 = R^3 = OMe$; $R^{1'},R^{2'} = OCH_2O$; $R^{3'} = OH$; R = angeloyl. schirubrisin D **144**



$R^1,R^2 = OCH_2O$; $R^3 = R^{1'} = R^{2'} = R^{3'} = OMe$; R = tigloyl. (–)-tigloylgomisin P **145**
$R^1,R^2 = OCH_2O$; $R^3 = R^{1'} = R^{2'} = R^{3'} = OMe$; R = angeloyl. (–)-angeloylgomisin P **146**
$R^1,R^2 = OCH_2O$; $R^3 = R^{1'} = R^{2'} = R^{3'} = OMe$; R = Bz. (–)-benzoylgomisin P **147**



(–)-gomisin D **148**

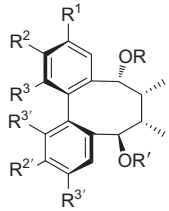


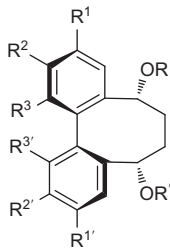
tiegusanin M **149**

FIGURE 6 C7′,C8′(C7,C8)-dioxygen-substituted dibenzocyclooctadiene lignans.

acid via an Ullmann coupling reaction, using this approach. Further elaboration provided (±)-wuweizisu C **340** (Scheme 4). Robin *et al*. [59] applied an intramolecular aldol condensation as a key step to close the eight-membered ring in their asymmetric total synthesis of (−)-steganone **280** (Scheme 5). Alkylation of the lithium enolate of the optically active precursor **341** prepared from L-glutamic acid, with 3,4,5-trimethoxybenzyl bromide afforded **342** as a single diastereoisomer. Compound **342** was transformed into the optically active lactone **344** in four steps. Iodination and Ullmann coupling reaction with 2-bromo-4,5-methylenedioxybenzaldehyde gave **346**, which was set for the key intramolecular aldol condensation. Jones' oxidation of the aldol product, followed by decarboxylation, gave **347** along with its atropisomer. Compound **347** could be transformed into (−)-steganone **280** over four steps via thermal isomerization of pure (+)-isosteganone **281**. Similar treatment of the mixture of **347** and its atropisomer also afforded the thermodynamically favored (−)-steganone **280**.

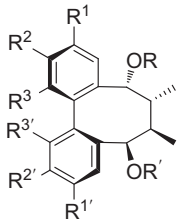
$R^1,R^2 = R^{1'},R^{2'} = OCH_2O$; $R^3 = R^{3'}$ = OMe; R = H; R′ = angeloyl. (+)-schisantherin L **150**
$R^1,R^2 = R^{1'},R^{2'} = OCH_2O$; $R^3 = R^{3'}$ = OMe; R = Ac; R′ = angeloyl. (+)-acetylschisantherin L **151**
$R^1,R^2 = R^{1'},R^{2'} = OCH_2O$; $R^3 = R^{3'}$ = OMe; R = tigloyl; R′ = angeloyl. (+)-schisantherin M **152**
$R^1,R^2 = R^{1'},R^{2'} = OCH_2O$; $R^3 = R^{3'}$ = OMe; R = R′ = angeloyl. (+)-schisantherin N **153**
$R^1,R^2 = OCH_2O$; $R^3 = R^{1'} = R^{2'} = R^{3'}$ = OMe; R = Ac; R′ = angeloyl. (+)-interiotherin C **154**
$R^1,R^2 = R^{1'},R^{2'} = OCH_2O$; $R^3 = R^{3'}$ = OMe; R = R′ = Bz. (–)-angustifolin A **155**
$R^1,R^2 = R^{1'},R^{2'} = OCH_2O$; $R^3 = R^{3'}$ = OMe; R = Ac; R′ = Bz. (–)-angustifolin B **156**
$R^1,R^2 = R^{1'},R^{2'} = OCH_2O$; $R^3 = R^{3'}$ = OMe; R = H; R′ = Bz. (–)-angustifolin C **157**
$R^1 = R^2 = R^3 = R^{1'} = R^{2'} = R^{3'}$ = OMe; R = R' = Ac. (*dl*)-angustifolin D **158**
$R^1,R^2 = OCH_2O$; $R^3 = R^{1'} = R^{2'} = R^{3'}$ = OMe; R = H; R′ = tigloyl. (–)-ananosin A **159**
$R^1,R^2 = OCH_2O$; $R^3 = R^{1'} = R^{2'}$ = OMe; $R^{3'}$ = OH; R = Bz; R′ = Ac. (+)-kadsuphilol B **160**
$R^1 = R^2 = R^3 = R^{1'} = R^{2'}$ = OMe; $R^{3'}$ = OH; R = H; R′ = Ac. kadsuphilol I **161**
$R^1,R^2 = OCH_2O$; $R^3 = R^{1'} = R^{2'}$ = OMe; $R^{3'}$ = OH; R = Ac; R′ = Bz. kadsuphilol P **162**
$R^1 = R^2 = R^3 = R^{1'} = R^{2'}$ = OMe; $R^{3'}$ = OH; R = Ac; R′ = Bz. kadsuphilol Q **163**
$R^1 = R^2 = R^3 = R^{1'} = R^{2'}$ = OMe; $R^{3'}$ = OH; R = Ac; R′ = H. (+)-kadangustin A **164**
$R^1,R^2 = R^{1'},R^{2'} = OCH_2O$; $R^3 = R^{3'}$ = OMe; R = Ac; R′ = H. (+)-kadangustin B **165**
$R^1 = R^2 = R^3 = R^{1'} = R^{2'}$ = OMe; $R^{3'}$ = OH; R = Ac; R′ = cinnamoyl. (–)-kadangustin D **166**
$R^1 = R^2 = R^3 = R^{1'} = R^{2'}$ = OMe; $R^{3'}$ = OH; R = Ac; R′ = Bz. kadangustin E **167**
$R^1 = R^2 = R^3 = R^{1'} = R^{2'}$ = OMe; $R^{3'}$ = OH; R = Ac; R′ = angeloyl. (+)-kadangustin F **168**
$R^1 = R^2 = R^3 = R^{1'} = R^{2'}$ = OMe; $R^{3'}$ = OH; R = Ac; R′ = tigloyl. (+)-kadangustin G **169**
$R^1,R^2 = OCH_2O$; $R^3 = R^{1'} = R^{2'} = R^{3'}$ = OMe; R = H; R′ = Ac. ananolignan E **170**
$R^1,R^2 = OCH_2O$; $R^3 = R^{1'} = R^{2'} = R^{3'}$ = OMe; R = R′ = Ac. ananolignan F **171**
$R^1,R^2 = OCH_2O$; $R^3 = R^{1'} = R^{2'} = R^{3'}$ = OMe; R = propanoyl; R′ = Ac. ananolignan G **172**
$R^1,R^2 = OCH_2O$; $R^3 = R^{1'} = R^{2'} = R^{3'}$ = OMe; R = isobutanoyl; R′ = Ac. ananolignan H **173**
$R^1,R^2 = OCH_2O$; $R^3 = R^{1'} = R^{2'} = R^{3'}$ = OMe; R = butanoyl; R′ = Ac. ananolignan I **174**
$R^1,R^2 = OCH_2O$; $R^3 = R^{1'} = R^{2'} = R^{3'}$ = OMe; R = 2-methylbutanoyl; R′ = Ac. ananolignan J **175**
$R^1,R^2 = OCH_2O$; $R^3 = R^{1'} = R^{2'} = R^{3'}$ = OMe; R = Bz; R′ = Ac. ananolignan K **176**
$R^1,R^2 = OCH_2O$; $R^3 = R^{1'} = R^{2'} = R^{3'}$ = OMe; R = Ac; R′ = tigloyl. ananolignan L **177**
$R^1,R^2 = OCH_2O$; $R^3 = R^{1'} = R^{2'} = R^{3'}$ = OMe; R = isobutanoyl; R′ = angeloyl. ananolignan M **178**
$R^1,R^2 = OCH_2O$; $R^3 = R^{1'} = R^{2'} = R^{3'}$ = OMe; R = butanoyl; R′ =angeloyl. ananolignan N **179**
$R^1,R^2 = OCH_2O$; $R^3 = R^{1'} = R^{2'}$ = OMe; $R^{3'}$ = OH; R = cinnamoyl; R′ =angeloyl. tiegusanin K **180**
$R^1,R^2 = OCH_2O$; $R^3 = R^{1'} = R^{2'} = R^{3'}$ = OMe; R = R′ = angeloyl. polysperlignan A **181**
$R^1,R^2 = OCH_2O$; $R^3 = R^{1'} = R^{2'} = R^{3'}$ = OMe; R = tigloyl; R′ = Ac. polysperlignan B **182**
$R^1,R^2 = OCH_2O$; $R^3 = R^{1'} = R^{2'} = R^{3'}$ = OMe; R = tigloyl; R′ = Bz. polysperlignan C **183**
$R^1,R^2 = OCH_2O$; $R^3 = R^{1'} = R^{2'} = R^{3'}$ = OMe; R = tigloyl; R′ = angeloyl. polysperlignan D **184**
$R^1,R^2 = OCH_2O$; $R^3 = R^{1'} = R^{2'} = R^{3'}$ = OMe; R = angeloyl; R′ = cinnamoyl. polysperlignan E **185**
$R^1,R^2 = OCH_2O$; $R^3 = R^{1'} = R^{2'}$ = OMe; $R^{3'}$ = Oangeloyl; R = H; R′ = angeloyl. polysperlignan G **186**
$R^1,R^2 = OCH_2O$; $R^3 = R^{1'} = R^{2'}$ = OMe; $R^{3'}$ = Oangeloyl; R = H; R′ = tigloyl. polysperlignan H **187**
$R^1 = R^2 = R^3 = R^{1'} = R^{2'}$ = OMe; $R^{3'}$ = Oangeloyl; R = H; R′ = angeloyl. polysperlignan I **188**
$R^1 = R^2 = R^3 = R^{1'} = R^{2'}$ = OMe; $R^{3'}$ = Oangeloyl; R = H; R′ = tigloyl. polysperlignan J **189**
$R^1,R^2 = OCH_2O$; $R^3 = R^{1'} = R^{2'} = R^{3'}$ = OMe; R = H; R′ = Bz. ananonin A **190**
$R^1,R^2 = OCH_2O$; $R^3 = R^{1'} = R^{2'} = R^{3'}$ = OMe; R = Ac; R′ = Bz. ananonin B **191**
$R^1,R^2 = OCH_2O$; $R^3 = R^{1'} = R^{2'} = R^{3'}$ = OMe; R = propanoyl; R′ = Bz. ananonin C **192**
$R^1,R^2 = OCH_2O$; $R^3 = R^{1'} = R^{2'} = R^{3'}$ = OMe; R = butanoyl; R′ = Bz. ananonin D **193**
$R^1,R^2 = OCH_2O$; $R^3 = R^{1'} = R^{2'} = R^{3'}$ = OMe; R = 2-methylpropanoyl; R′ = Bz. ananonin E **194**
$R^1,R^2 = OCH_2O$; $R^3 = R^{1'} = R^{2'} = R^{3'}$ = OMe; R = 2-methylbutanoyl; R′ = Bz. ananonin F **195**
$R^1,R^2 = OCH_2O$; $R^3 = R^{1'} = R^{2'} = R^{3'}$ = OMe; R = 2-methylbutanoyl; R′ = angeloyl. ananonin G **196**
$R^1,R^2 = OCH_2O$; R^3 = H; $R^{1'} = R^{2'} = R^{3'}$ = OMe; R = Ac; R′ = angeloyl. ananonin H **197**
$R^1,R^2 = OCH_2O$; R^3 = H; $R^{1'} = R^{2'} = R^{3'}$ = OMe; R = propanoyl; R′ = angeloyl. ananonin I **198**
$R^1,R^2 = OCH_2O$; R^3 = H; $R^{1'} = R^{2'} = R^{3'}$ = OMe; R = 2-methylbutanoyl; R′ = angeloyl. ananonin J **199**
R^1 = OH; $R^2 = R^3 = R^{1'} = R^{2'} = R^{3'}$ = OMe; R = R′ =Ac. ananonin K **200**
R^1 = OH; $R^2 = R^3 = R^{1'} = R^{2'} = R^{3'}$ = OMe; R = Ac; R′ = angeloyl. ananonin L **201**
R^1 = OH; $R^2 = R^3 = R^{1'} = R^{2'} = R^{3'}$ = OMe; R = H; R′ = Bz. ananonin M **202**
R^1 = OH; $R^2 = R^3 = R^{1'} = R^{2'} = R^{3'}$ = OMe; R = Ac; R′ = Bz. ananonin N **203**



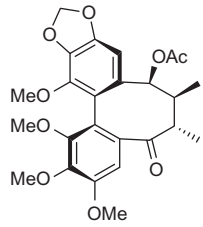


$R^1,R^2 = R^{1'},R^{2'} = OCH_2O$; $R^3 = R^{3'}$ = OMe; R = R′ = H. (+)-schisantherin P **204**
$R^1,R^2 = OCH_2O$; $R^3 = R^{1'} = R^{2'} = R^{3'}$ = OMe. R = R′ = H. ananolignan C **205**
$R^1,R^2 = OCH_2O$; $R^3 = R^{1'} = R^{2'} = R^{3'}$ = OMe. R = Ac; R′ = H. ananolignan D **206**

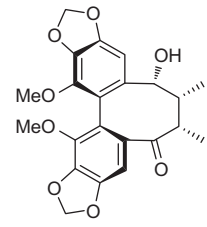
FIGURE 7—Cont'd



R^1 = OH; R^2 = R^3 = $R^{1'}$ = $R^{2'}$ = OMe; $R^{3'}$ = angeloyloxy; R = H; R′ = Ac. (+)-propinquanin D **207**
R^1 = R^2 = R^3 = $R^{1'}$ = $R^{2'}$ = OMe; $R^{3'}$ = tigloyloxy; R = H; R′ = Ac. schisantherin K **208**
R^1,R^2 = OCH_2O; R^3 = $R^{1'}$ = $R^{2'}$ = $R^{3'}$ = OMe; R = Bz; R′ = angeloyl. tiegusanin H **209**
R^1,R^2 = OCH_2O; R^3 = $R^{1'}$ = $R^{2'}$ = $R^{3'}$ = OMe; R = propanoyl; R′ = angeloyl. tiegusanin I **210**
R^1,R^2 = OCH_2O; R^3 = $R^{1'}$ = $R^{2'}$ = $R^{3'}$ = OMe; R = R′ = tigloyl. tiegusanin J **211**



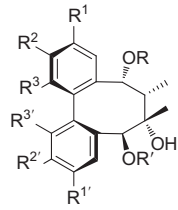
ananolignan B **212**



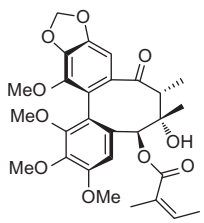
schisantherin Q **213**

FIGURE 7 C7,C7′-dioxygen-substituted dibenzocyclooctadiene lignans.

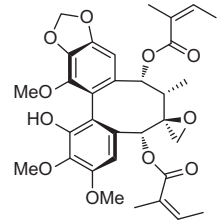
Robin's synthesis of (−)-steganone **280** (Scheme 5) used an Ullmann coupling reaction between two different aryl halides. The homocoupling of a single aryl halide provides the symmetrical biphenyl derivative, while coupling of two different aryl halides gives a mixture of three possible biphenyl derivatives. In order to avoid homocoupling in the Ullmann reaction of two different aryl halides, one aryl halide is first converted to an aryl metal derivative by reaction with metallic copper. The coupling of the aryl copper intermediate with the second aryl halide occurs at low temperature to give the corresponding nonsymmetrical biphenyl derivative. The reaction conditions of such biaryl coupling protocol are mild and work well even for sterically hindered aromatic halides with *ortho* substituents. Such a strategy was applied by Ziegler [60] and coworkers for the synthesis of (±)-steganacin **269** (Scheme 6). Metal–halogen exchange of **348** provided the corresponding organolithium, which, upon treatment with $CuI{\cdot}P(OEt)_3$ complex, produced the organocopper reagent **349**. Coupling of organometallic intermediate **349** with *N*-cyclohexyl-2-iodo-3,4,5-trimethoxybenzylideneamine **350** provided imine **351**, which was transformed into **352** over five steps. Intramolecular alkylation of **352** was effected by potassium *tert*-butoxide, affording the keto-diester **353** in 73% yield. Decarboxylation of diester **353**, and following a similar strategy as applied by Robin *et al*. for the total synthesis of (−)-steganone (Scheme 5) afforded (±)-steganone **280**, reduction of which gave a mixture of steganol and episteganol (structure not shown). Acetylation of steganol then provided (±)-steganacin **269**.



$R^1,R^2 = OCH_2O$; $R^3 = R^{1'} = R^{2'} = R^{3'} = OMe$; R = Ac; R′ =angeloyl. (–)-kadsurarin **214**
R^1 = OH; $R^2 = R^3 = R^{1'} = R^{2'} = R^{3'} = OMe$; R = hexanoyl; R′ = tigloyl. (–)-propinquanin A **215**
$R^1 = R^{3'}$ = OH; $R^2 = R^3 = R^{1'} = R^{2'} = OMe$; R = hexanoyl; R′ = Bz. (–)-propinquanin B **216**
$R^1,R^2 = OCH_2O$; R^3 = OH; $R^{1'} = R^{2'} = R^{3'} = OMe$; R = propanoyl; R′ = angeloyl. (+)-propinquanin C **217**
$R^1,R^2 = OCH_2O$; $R^3 = R^{3'}$ = H; $R^{1'} = R^{2'} = OMe$; R = R′ = angeloyl. propinquanin E **218**
R^1 = OH; $R^2 = R^3 = R^{1'} = R^{2'} = R^{3'} = OMe$; R = tigloyl; R′ = angeloyl. propinquain E **219**
R^1 = OH; $R^2 = R^3 = R^{1'} = R^{2'} = R^{3'} = OMe$; R = propanoyl; R′ = angeloyl. propinquain F **220**
$R^1,R^2 = OCH_2O$; R^3 = OH; $R^{1'} = R^{2'} = R^{3'} = OMe$; R = Ac; R′ = angeloyl. (+)-schisantherin G **221**
R^1 = OH; $R^2 = R^3 = R^{1'} = R^{2'} = R^{3'} = OMe$; R = hexanoyl; R′ = Bz. (-)-schisantherin I **222**
$R^1,R^2 = OCH_2O$; R^3= $R^{1'} = R^{2'} = R^{3'} = OMe$; R = isovaleryl; R′ = Bz. schisantherin J **223**
$R^1,R^2 = OCH_2O$; $R^3 = R^{1'} = R^{2'} = R^{3'} = OMe$; R = Ac; R′ = Bz. (+)-schisanrin F **224**
$R^1,R^2 = OCH_2O$; $R^3 = R^{1'} = R^{2'}$ =OMe; $R^{3'}$ = OH; R = Ac; R′ = angeloyl. (+)-schisanrin G **225**
$R^1 = R^2 = R^3 = R^{1'} = R^{2'} = R^{3'} = OMe$; R = Ac; R′ = Bz. (+)-schisanrin H **226**
$R^1,R^2 = OCH_2O$; R^3 = OH; $R^{1'} = R^{2'} = R^{3'} = OMe$; R = R′ = Bz. schizanrin I **227**
$R^1,R^2 = OCH_2O$; R^3 = OH; $R^{1'} = R^{2'} = R^{3'} = OMe$; R = R′ = angeloyl. schizanrin J **228**
$R^1 = R^2 = R^3 = R^{1'} = R^{2'} = OMe$; $R^{3'}$ = OH; R = Ac; R′ = cinnamoyl. (-)-kadangustin C **229**
$R^1,R^2 = OCH_2O$; $R^3 = R^{1'} = R^{2'} = R^{3'} = OMe$; R = R′ = Ac. kadsuralignan B **230**
$R^1,R^2 = OCH_2O$; $R^3 = R^{1'} = R^{2'} = R^{3'} = OMe$; R = R′ = Bz. tiegusanin D **231**
$R^1,R^2 = OCH_2O$; $R^3 = R^{1'} = R^{2'} = R^{3'} = OMe$; R = hexanoyl; R′ = Bz. tiegusanin E **232**
$R^1,R^2 = OCH_2O$; $R^3 = R^{1'} = R^{2'} = R^{3'} = OMe$; R = Bz; R′ = cinnamoyl. tiegusanin F **233**
$R^1,R^2 = OCH_2O$; $R^3 = R^{1'} = R^{2'} = R^{3'} = OMe$; R = cinnamoyl; R′ = angeloyl. tiegusanin G **234**
$R^1,R^2 = OCH_2O$; R^3= $R^{1'} = R^{2'}$= OMe; $R^{3'}$ = OH; R = Ac; R′ = Bz. kadsuphilin C **235**
$R^1,R^2 = OCH_2O$; R^3= $R^{1'} = R^{2'}$= OMe; $R^{3'}$ = OH; R = Ac; R′ = H. kadsuphilin D **236**
$R^1 = R^2 = R^3 = R^{1'} = R^{2'}$= OMe; $R^{3'}$ = OH; R = Ac; R′ = Bz. kadsuphilin E **237**
$R^1,R^2 = OCH_2O$; R^3= $R^{1'} = R^{2'}$= OMe; $R^{3'}$ = OH; R = Bz; R′ = H. kadsuphilin F **238**
$R^1,R^2 = OCH_2O$; R^3= $R^{1'} = R^{2'}$= OMe; $R^{3'}$ = OH; R = angeloyl; R′ = H. kadsuphilin H **239**
$R^1 = R^2 = R^3 = R^{1'} = R^{2'}$= OMe; $R^{3'}$ = OH; R = Ac; R′ = angeloyl. kadsuphilol J **240**
$R^1,R^2 = OCH_2O$; R^3= $R^{1'} = R^{2'}$= OMe; $R^{3'}$ = OH; R = H; R′ = Bz. kadsuphilol K **241**
$R^1,R^2 = OCH_2O$; R^3 = OH; $R^{1'} = R^{2'} = R^{3'}$ = OMe; R = cinnamoyl; R′ = Ac. kadsuphilol L **242**
$R^1,R^2 = OCH_2O$; R^3= $R^{1'} = R^{2'}$= OMe; $R^{3'}$ = OH; R = R′ = angeloyl. kadsuphilol R **243**
$R^1,R^2 = OCH_2O$; R^3= $R^{1'} = R^{2'}$= OMe; $R^{3'}$ = OH; R = propanoyl; R′ = angeloyl. kadsuphilol S **244**
$R^1,R^2 = OCH_2O$; R^3= $R^{1'} = R^{2'}$= OMe; $R^{3'}$ = OH; R = angeloyl; R′ = Bz. kadsuphilol T **245**
R^1 = OH; $R^2 = R^3 = R^{1'} = R^{2'} = R^{3'} = OMe$; R = angeloyl; R′ = Bz. intermedin A **246**
$R^1,R^2 = OCH_2O$; R^3 = OH; $R^{1'} = R^{2'} = R^{3'} = OMe$; R = Bz; R′ = Ac. heteroclitalignan A **247**
$R^1,R^2 = OCH_2O$; $R^3 = R^{1'} = R^{2'} = R^{3'} = OMe$; R = propanoyl; R′ = angeloyl. heteroclitalignan B **248**
$R^1,R^2 = OCH_2O$; $R^3 = R^{1'} = R^{2'} = R^{3'} = OMe$; R = Bz; R′ = Ac. heteroclitalignan D **249**



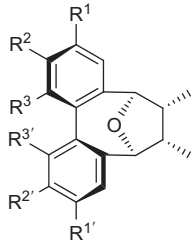
schizanrin K **250**



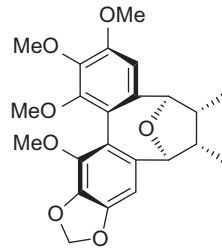
(+)-kadsuphilol C **251**

FIGURE 8 C7,C7′,C8′-trioxygen-substituted dibenzocyclooctadiene lignans.

The Kumada [61], Negishi [62], Stille [63], and Suzuki [64] cross-coupling reactions are more versatile and widely used alternatives for aryl–aryl bond formation. These coupling reactions are used to synthesize various nonsymmetrical biphenyl derivatives in the presence of nickel or palladium complexes as catalyst. In the Kumada cross-coupling reaction, a Grignard reagent is reacted with an aryl halide catalyzed by a Ni- or Pd-complex to yield a biphenyl compound. Aryl halides substituted with electron withdrawing groups, such as RC═O, CO_2R, and NO_2, fail to react with the Grignard reagent. In addition, the coupling of *ortho*-substituted aryl halides gives low yields because of steric hindrance. In the related Negishi cross-coupling

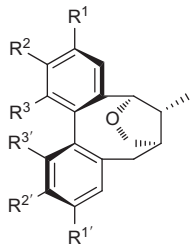


$R^1,R^2 = R^{1'},R^{2'} = OCH_2O$; $R^3 = R^{3'}$ = OMe.
3,3′-dimethoxy-4,5:4′5′-bismethylenedioxy-7,7′-epoxy-2,2′-cyclolignan **252**
$R^1,R^2 = OCH_2O$; $R^3 = R^{1'} = R^{2'} = R^{3'}$ = OMe. (+)-kadsulignan L **253**
$R^1,R^2 = OCH_2O$; $R^3 = R^{2'} = R^{3'}$ = OMe; $R^{1'}$ = OH. (+)-kadsulignan M **254**
$R^1 = R^2 = R^3 = R^{1'} = R^{2'} = R^{3'}$ = OMe. kadsulignan N **255**

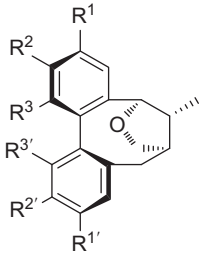


(+)-neokadsuranin **256**

FIGURE 9 C7,C7′-epoxy dibenzocyclooctadiene lignans.



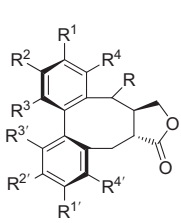
$R^1 = R^2 = R^3 = R^{1'} = R^{2'} = R^{3'}$ = OMe. pyramidatin A **257**
$R^1 = R^2 = R^3 = R^{1'} = R^{2'}$ = OMe; $R^{3'}$ = OH. pyramidatin C **258**
$R^1 = R^2 = R^{1'} = R^{2'} = R^{3'}$ = OMe; R^3 = OH. pyramidatin D **259**
$R^1,R^2 = R^{1'},R^{2'} = OCH_2O$; $R^3 = R^{3'}$ = OMe. pyramidatin H **260**



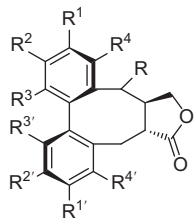
$R^1 = R^2 = R^3 = R^{2'} = R^{3'}$ = OMe; $R^{1'}$ = OH. pyramidatin B **261**
$R^1 = R^2 = R^{3'}$ = OMe; R^3 = OH; $R^{1'},R^{2'} = OCH_2O$. pyramidatin F **262**
$R^1 = R^2 = R^3 = R^{3'}$ = OMe; $R^{1'},R^{2'} = OCH_2O$. pyramidatin E **263**
$R^1 = R^2 = R^3 = R^{2'} = OMe$; $R^{1'} = R^{3'}$ = OH. pyramidatin G **264**

FIGURE 10 C7,C9′-epoxy dibenzocyclooctadiene lignans.

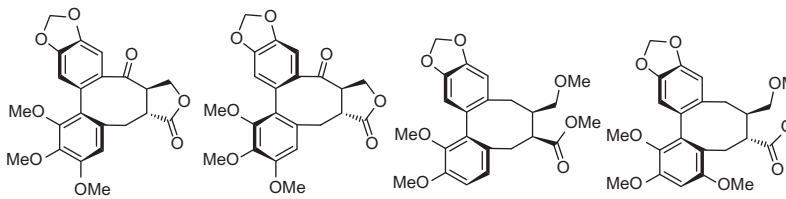
reaction, an aryl zinc reagent is coupled with an aryl halide or aryl triflate catalyzed by nickel or palladium complex. Because the aryl zinc complex is a milder reagent compared with the Grignard reagent, the Negishi coupling reaction is compatible with many functional groups including ketones, esters, amines, and nitriles. The biphenyl aldehyde **355**, a key intermediate in the



$R^1,R^2 = OCH_2O$; $R^3 = R^4 = R^{4'} = H$; $R^{1'} = R^{2'} = R^{3'} = OMe$; R = H. (–)-stegane **265**
$R^1 = R^2 = R^4 = R^{1'} = R^{2'} = R^{4'} = OMe$; $R^3 = R^{3'} = H$; R = H. (+)-steganolide A **266**
$R^1,R^2 = OCH_2O$; $R^3 = R^4 = R^{1'} = R^{4'} = H$; $R^{2'} = R^{3'} = OMe$; R = H. (–)-10-demethoxystegane **267**
$R^1 = R^2 = R^{1'} = R^{2'} = R^{4'} = OMe$; $R^3 = R^4 = R^{3'} = H$; R = H. (+)-neoisostegane **268**
$R^1,R^2 = OCH_2O$; $R^3 = R^4 = R^{4'} = H$; $R^{1'} = R^{2'} = R^{3'} = OMe$; R = α-OAc. (–)-steganacin **269**
$R^1,R^2 = OCH_2O$; $R^3 = R^4 = R^{4'} = H$; $R^{1'} = R^{2'} = R^{3'} = OMe$; R = β-OAc. (–)-episteganacin **270**
$R^1,R^2 = OCH_2O$; $R^3 = R^4 = R^{4'} = H$; $R^{1'} = R^{2'} = R^{3'} = OMe$; R = α-angeloyloxy. (–)-steganangin **271**
$R^1,R^2 = OCH_2O$; $R^3 = R^4 = R^{4'} = H$; $R^{1'} = R^{2'} = R^{3'} = OMe$; R = α-OH. (–)-steganol **272**
$R^1,R^2 = OCH_2O$; $R^3 = R^4 = R^{4'} = H$; $R^{1'} = R^{2'} = R^{3'} = OMe$; R = β-OH. (–)-episteganol **273**
$R^1 = R^4 = R^{3'} = R^{4'} = H$; $R^2 = R^3 = OMe$; $R^{1'},R^{2'} = OCH_2O$; R = β-angeloyloxy. (–)-araliangine **274**
$R^1 = R^2 = R^{2'} = R^{3'} = OMe$; $R^3 = R^4 = R^{1'} = R^{4'} = H$; R = β-angeloyloxy. (+)-steganolide B **275**
$R^1 = R^2 = R^{2'} = R^{3'} = OMe$; $R^3 = R^4 = R^{1'} = R^{4'} = H$; R = β-tigloyloxy. (+)-steganolide C **276**
$R^1,R^2 = OCH_2O$; $R^3 = R^4 = R^{4'} = H$; $R^{1'} = R^{2'} = R^{3'} = OMe$; R = β-angeloyloxy. (–)-episteganangin **277**

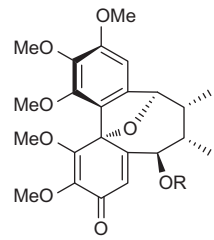


$R^1,R^2 = OCH_2O$; $R^3 = R^4 = R^{4'} = H$; $R^{1'} = R^{2'} = R^{3'} = OMe$; R = H. (–)-isostegane **278**
$R^1,R^2 = OCH_2O$; $R^3 = R^4 = R^{4'} = H$; $R^{1'} = R^{2'} = R^{3'} = OMe$; R = α-OAc. (–)-isosteganacin **279**

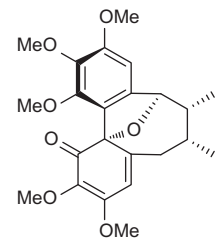


(–)-steganone **280** (+)-isosteganone **281** steganoate A **282** steganoate B **283**

FIGURE 11 Dibenzocyclooctadiene-9,9′-lactones.



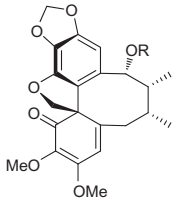
R = H. (–)-kadsulignan A **284**
R = Ac. (–)-kadsulignan B **285**



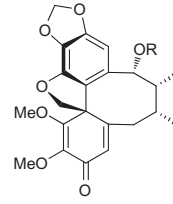
kadsuralignan D **286**

FIGURE 12 C7,C2′-epoxy dibenzocyclooctadiene lignans.

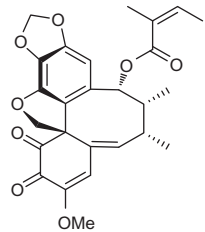
total synthesis of (−)-steganone **280** by Larson and Raphael [65], was obtained through Negishi cross-coupling of methylenedioxyphenylzinc chloride **354** and iodobenzylideneamine **350** (Scheme 7). In the Stille reaction, an aryl tin derivative is used as the organometallic reagent for the cross-coupling reaction. The neutral reaction conditions in the Stille reaction can be applied to a wide range of substrates having a variety of functional groups. However, the organotin reagents and tin byproducts are quite toxic. Since first



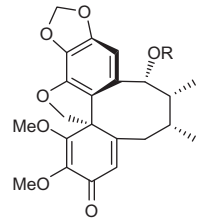
R = H. oxokadsurane **287**
R = propanoyl. (–)-kadsulignan I **288**
R = isovaleryl. (–)-schiarisanrin A **289**
R = Ac. (–)-schiarisanrin B **290**
R = Bz. (–)-schiarisanrin C **291**
R = cinnamoyl. (–)-schiarisanrin D **292**
R = angeloyl. (–)-schiarisanrin E **293**
R = butanoyl. (–)-kadsulignan H **294**
R = tigloyl. heteroclitin H **295**



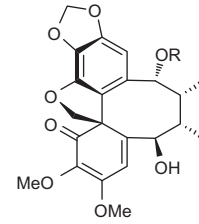
R = H, spirodienone **296**
R = angeloyl. (–)-interiorin **297**
R = tigloyl. (–)-interiorin B **298**
R = Ac. (–)-interiorin C **299**
R = Bz. (–)-interiorin D **300**



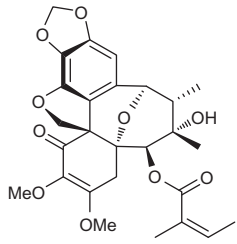
(–)-interiotherin D **301**



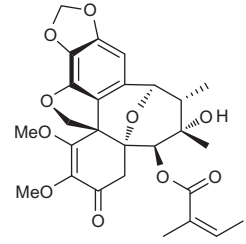
R = methacryloyl. kadsutherin D **302**
R = angeloyl. (+)-isointeriorin **303**



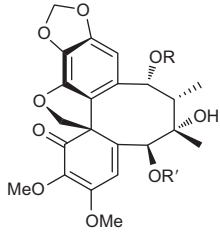
R = isovaleryl. (+)-isovaleryloxokadsuranol **304**
R = angeloyl. (–)-heteroclitin E **305**



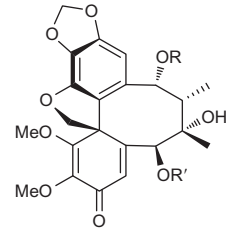
(+)-kadsuphilol G **306**



(+)-kadsuphilol H **307**



R = Bz; R′ = Ac. (–)-kadsulignan C **308**
R = R′ = angeloyl. (–)-kadsulignan D **309**
R = cinnamoyl; R′ = Ac. kadsulignan P **310**
R = angeloyl; R′ = Bz. kadsuphilol E **311**
R = Bz; R′ = angeloyl. kadsuphilol F **312**
R = Ac; R′ = angeloyl. kadsuphilol M **313**



R = Bz; R′ = Ac. (–)-kadsulignan E **314**
R = Ac; R′ = angeloyl. (–)-kadsulignan F **315**
R = R′ = angeloyl. (–)-kadsulignan G **316**
R = cinnamoyl; R′ = Ac. kadsulignan O **317**
R = propanoyl; R′ = angeloyl. heteroclitalignan C **318**

FIGURE 13 C3,C2′-methylene-epoxy dibenzocyclooctadiene lignans.

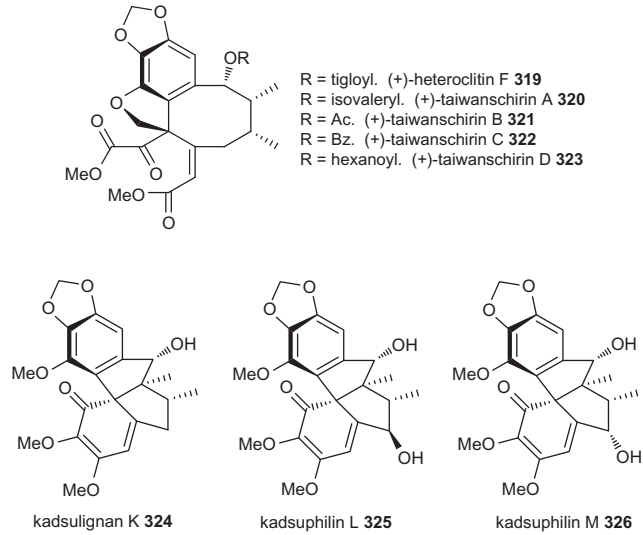
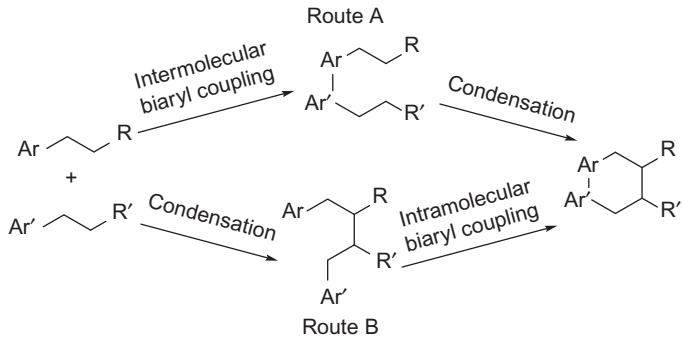
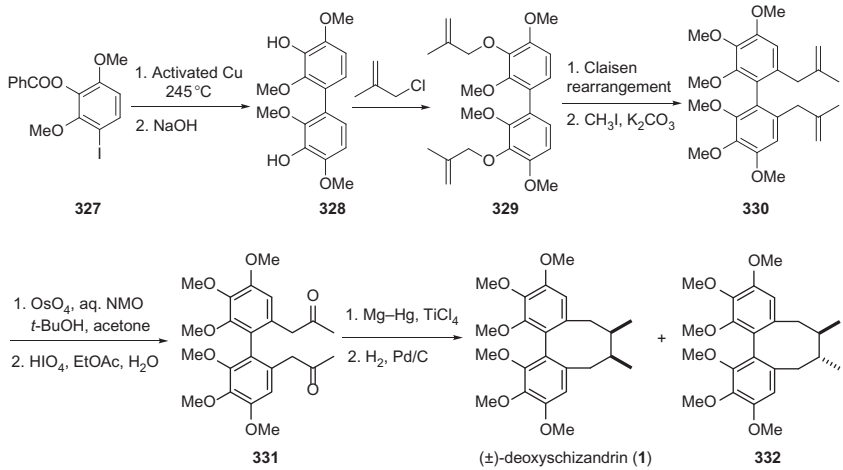


FIGURE 14 Others.

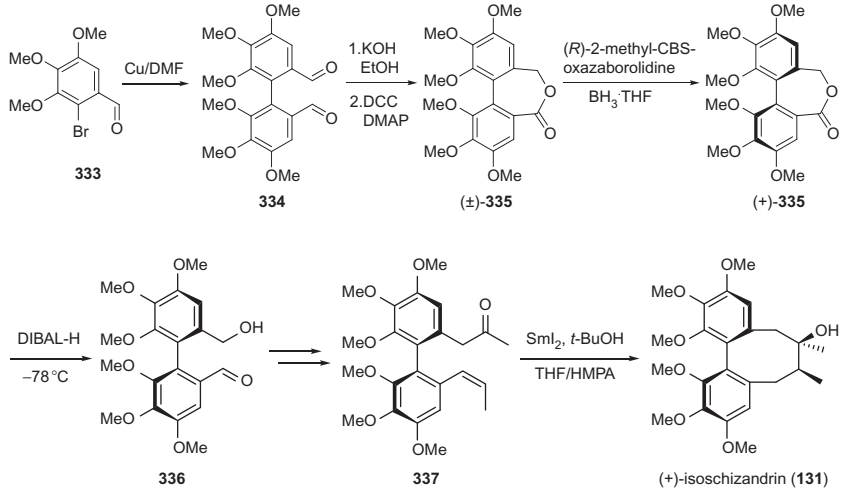


SCHEME 1 Strategies for the synthesis of dibenzocyclooctadiene lignans.

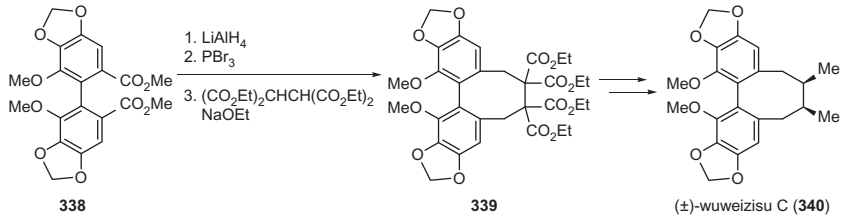
being published in 1979, the Suzuki cross-coupling reaction of organoboron compounds and organic halides or pseudohalides has become one of the most efficient methods for the construction of carbon–carbon bonds. The key advantages of the Suzuki coupling are the mild reaction conditions and the commercial availability of the diverse boronic acids that are environmentally safer than the other organometallic reagents. They are stable toward air and moisture, tolerate a broad range of functional groups. In addition, the handling and removal of boron-containing byproducts is easier when compared with other organometallic reagents, especially in a large-scale synthesis. Phosphane-based palladium catalysts are generally used in the Suzuki reaction. Typically, an aqueous solution of a weak base, such as Na_2CO_3, K_3PO_4, or $Ba(OH)_2$, is used as the reaction solvent. The Suzuki reaction gives



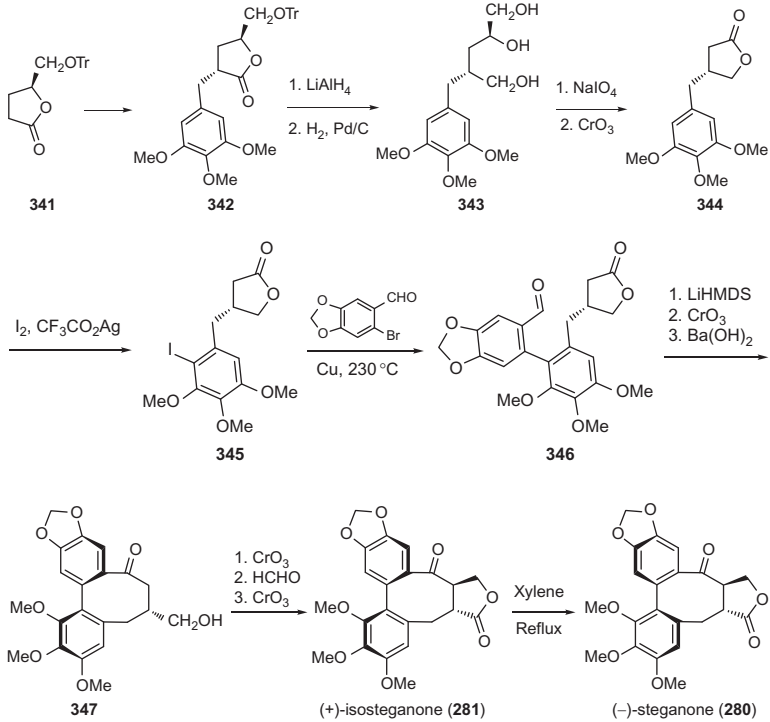
SCHEME 2 Synthesis of (±)-deoxyshizandrin (**1**) by Carroll *et al*.



SCHEME 3 Total synthesis of (+)-isoschizandrin (**131**) by Molander utilizing a samarium(II) iodide-promoted 8-endo ketyl-olefin cyclization.



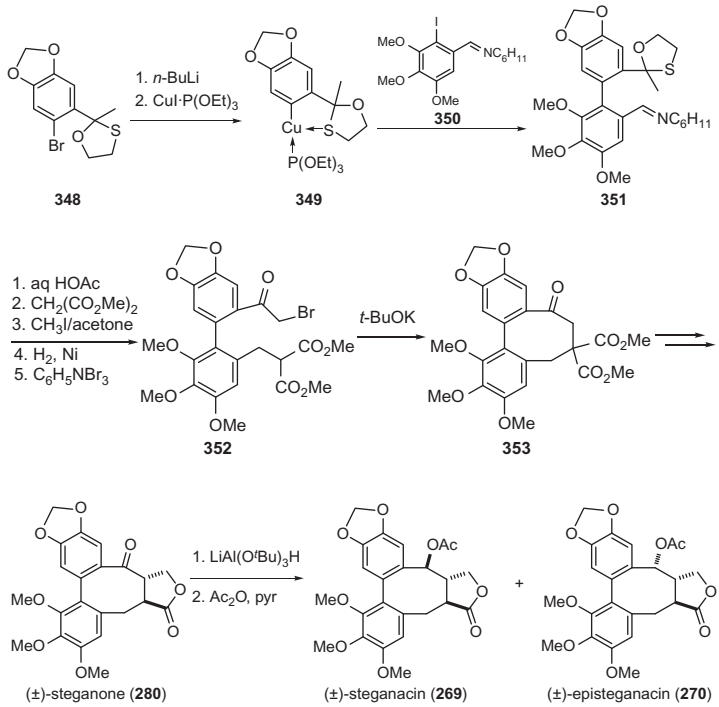
SCHEME 4 Synthesis of (±)-wuweizisu C (**340**) by Xie *et al*.



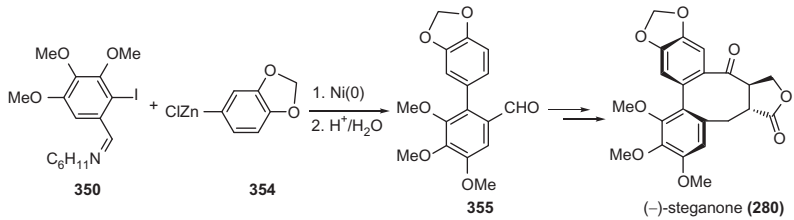
SCHEME 5 Asymmetric total synthesis of (−)-steganone (**280**) by Robin *et al.*

high yields of biphenyl derivatives even with highly sterically hindered substrates. The enantiopure biaryl derivative **358**, synthesized through a Suzuki cross-coupling reaction between the optically active chromium complex **356** and boronic acid **357**, was a key intermediate in the total synthesis of (−)-steganone **280** by Uemura *et al.* [66] and Molander *et al.* [67] (Scheme 8), respectively.

Meyers and Himmelsbach [68] also developed a substrate-mediated approach for the synthesis of chiral biphenyls with oxazolines as chiral auxiliary. Nucleophilic aromatic substitution of the *o*-methoxy group to the chiral oxazoline of **360** by aryl Grignard reagent from **361** and **363** resulted in the formation of biaryl coupling products **362a**/**362b** and **364a**/**364b** in good diastereoselectivity and combined yield (Scheme 9). Further manipulation of **362a** and **364a** provided the natural product (−)-steganone **280** [69], (−)-schizandrin **121**, and (−)-isoschizandrin **131** [70], respectively. Recently, this strategy was applied by RajanBabu [71] and coworkers to synthesize a series of dibenzocyclooctadiene lignans, including the natural products gomisin E **120**, gomisin O **79**, interiotherin A **91**, and angeloylgomisin R **89**. Similarly, Sargent *et al.* [72] reported the synthesis of binaphthoquinone natural products. Chang *et al.* [73] found that the oxazoline-mediated Ullmann reaction

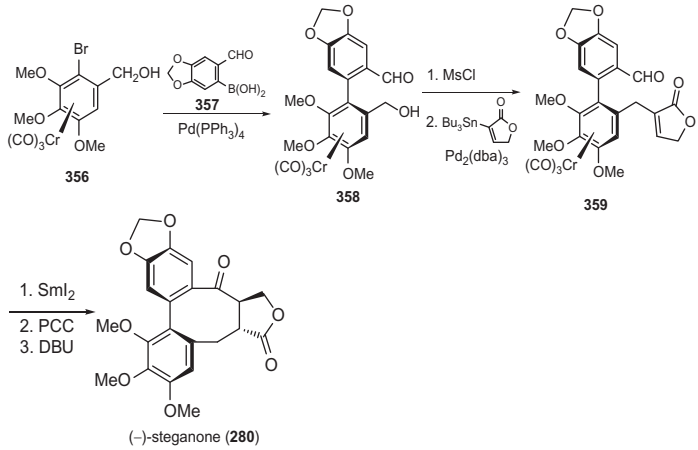


SCHEME 6 Synthesis of (±)-steganacin **269** by Ziegler *et al*.

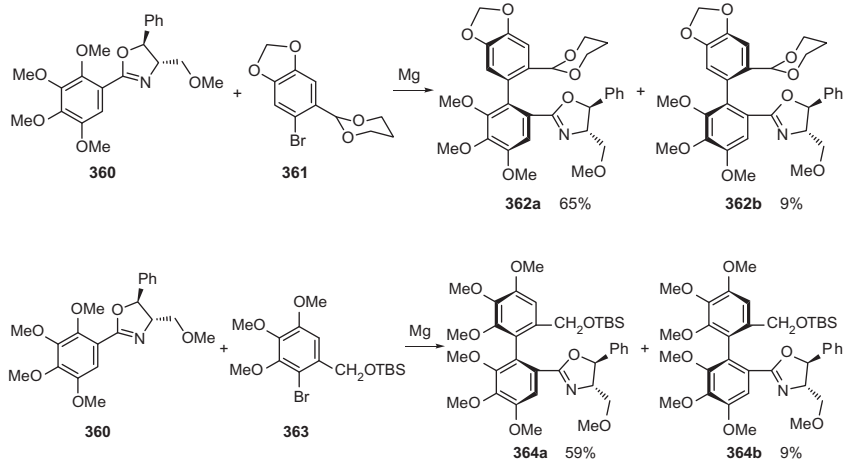


SCHEME 7 Synthesis of (−)-steganone **280** by Larson and Raphael using the Negishi cross-coupling reaction.

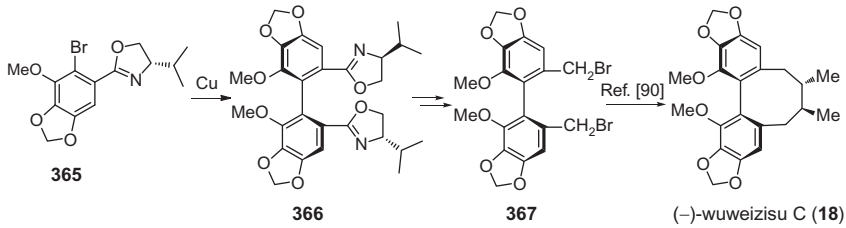
of **365** afforded biaryl derivative **366** in 68% yield and >99% de (Scheme 10). Following known literature procedure [58] for the synthesis of racemic wuweizisu C, the dibromide **367**, obtained from **366**, could be transformed into (−)-wuweizisu C **18**. Recently, Lin *et al*. [74] reported the first example of a nickel-catalyzed asymmetric Ullmann coupling of bis-*ortho*-substituted arylhalides. In the presence of the chiral BINOL-based monodentate phosphoramidite ligand **368**, atropoenantioselective Ullmann coupling of 2-bromo-3,4,5-trimethoxybenzaldehyde **333** gave biaryl dial **334** in 67% isolated yield and 68% ee (Scheme 11). The ee value of **334** could be



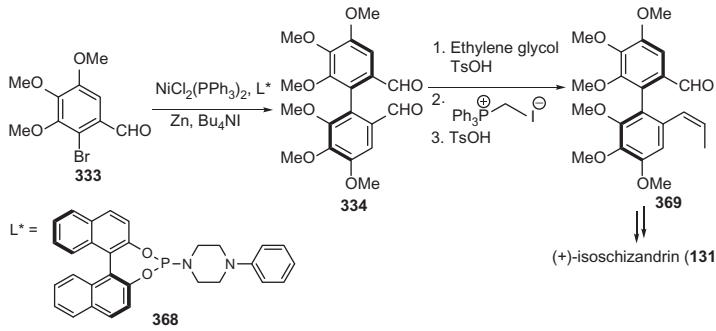
SCHEME 8 Total synthesis of (−)-steganone (**280**) by Molander *et al*.



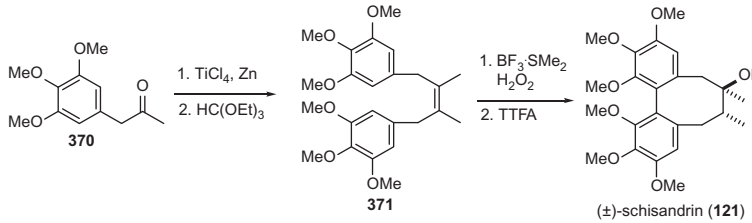
SCHEME 9 Synthesis of chiral biphenyls **362** and **364** by Meyers *et al*.



SCHEME 10 Total synthesis of (−)-wuweizisu C **18** by Chang *et al*.



SCHEME 11 Catalytic asymmetric Ullmann coupling for the synthesis of axially chiral tetra-*ortho*-substituted biaryl dials and a formal total synthesis of (+)-isoschizandrin **131** by Lin *et al*.



SCHEME 12 Total synthesis of (±)-schisandrin **121** by Chang and Xie.

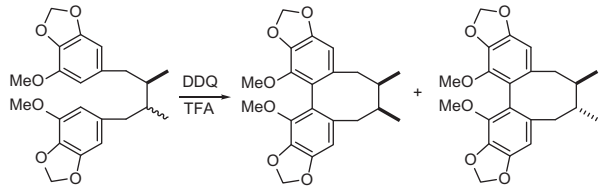
improved to 98% by recrystallization. Starting from the enantiomerically enriched biaryl dial **334** (98% ee), a formal synthesis of (+)-isoschizandrin **131** was accomplished [57].

Intramolecular Biphenyl Coupling

According to biogenesis theory, biosynthesis is the most efficient process with respect to energy consumption and material throughput. In nature, the mechanism of the enzyme-catalyzed formation of the dibenzocyclooctadiene series of lignans is believed to proceed through an oxidative coupling process via radical cation intermediates generated from the phenyl groups of acyclic lignans [8,75,76].

The dibenzylbutane and dibenzylbutyrolactone subgroups of acyclic lignans [1–8] are not only abundant and diverse natural products with various biological activities, but they are also key precursors for the syntheses of the dibenzocyclooctadiene series of lignans. Chang and Xie [77] synthesized the dibenzylbutene intermediate **371** and its *E* isomer (structure not shown) via Ti-induced reductive dimerization of aryl acetone **370** (Scheme 12). **371** is an oil, while its *E* isomer is a solid at ambient temperature, thus the two compounds can be easily separated through crystallization followed by

chromatography. Hydroboration–oxidation on **371** and a thallium(III) trifluoroacetate-induced intramolecular oxidative coupling produced (±)-schisandrin **121**. The same authors also reported the synthesis of (±)-deoxyschisandrin **1** by intramolecular oxidative coupling of the corresponding 1,4-diaryl-2,3-dimethylbutane precursor with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) [78]. Tobinaga *et al*. [79] used a similar oxidative aryl–aryl coupling strategy in their synthesis of (±)-schizandrin **121**, (±)-gomisin A **122**, and stereoisomers. Recently, we [80] carried out a DDQ-mediated oxidative coupling on the diastereomeric mixture **372** (Scheme 13). The reaction resulted in the formation of (±)-wuweizisu C **340** and the stereoisomer (±)-**373**, which could be separated by column chromatography and the structures confirmed by X-ray diffraction (Fig. 15). The other regioisomers were not formed in that they have relatively higher potential energy, while the regioisomer with the two methoxy group *ortho* to the biphenyl bond has the lowest potential energy according to theoretical calculation [81]. Coy *et al*. [82] adopted a palladium-catalyzed direct intramolecular biaryl coupling by C—H activation to transform the isomeric dibenzylbutane lignans **374a** and **374b** into



SCHEME 13 DDQ-mediated oxidative coupling of **372**.

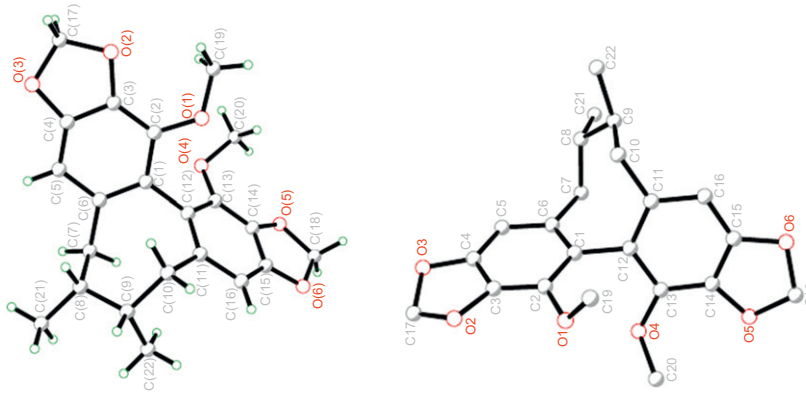
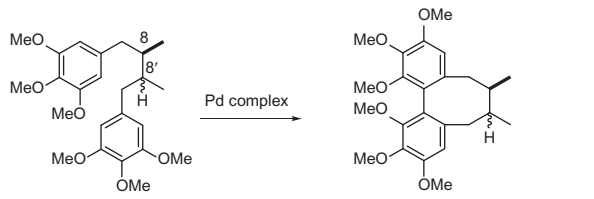


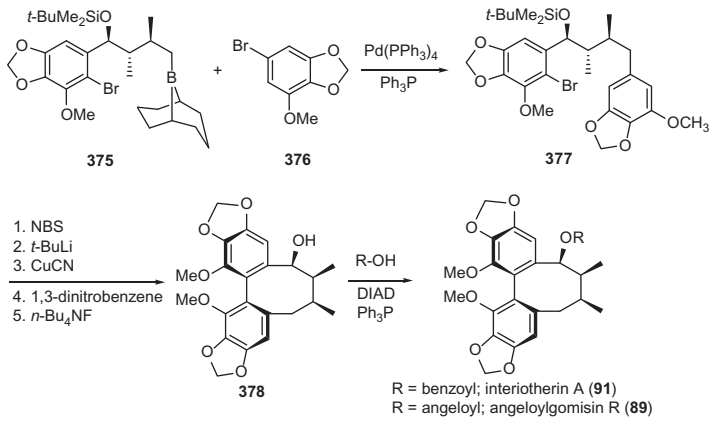
FIGURE 15 X-ray structure of (±)-wuweizisu C **340** and (±)-**373**.

the corresponding dibenzocyclooctane lignans *cis*- and *trans*-deoxyschizandrin **1** and **332**, respectively (Scheme 14). Coleman *et al*. [83] reported the first asymmetric total synthesis of interiotherin A **91** and angeloylgomisin R **89** (Scheme 15). Suzuki–Miyaura coupling of borane **375** with aryl bromide **376** occurred smoothly to afford 1,4-diarylbutane **377**. Regioselective bromination, followed by an atropdiastereoselective, copper-promoted biaryl coupling afforded, after removal of the silyl ether, the parent dibenzocyclooctadiene ring system **378**. Alcohol **378** underwent a Mitsunobu reaction with benzoic acid to afford interiotherin A **91**. In a similar manner, reaction of alcohol **378** with angelic acid under Mitsunobu reaction conditions gave angeloylgomisin R **89**. Starting with the corresponding trimethoxyphenyl bromide precursor, the asymmetric synthesis of gomisin O **79** and gomisin E **120** could be realized following similar sequence of transformations [83b].

The Stobbe reaction, condensation of dialkyl succinate and its derivative with carbonyl compounds in the presence of a base, is ideally suited to assembling of the basic carbon skeleton of lignans. Starting from the condensation product **379**, Wakamatsu *et al*. [84] synthesized the dibenzocyclooctatriene-9,9′-lactone **383** by a highly efficient iron(III) perchlorate-mediated



SCHEME 14 Synthesis of *cis*-deoxyschizandrin **1** and *trans*-deoxyschizandrin **332** by Coy *et al*.



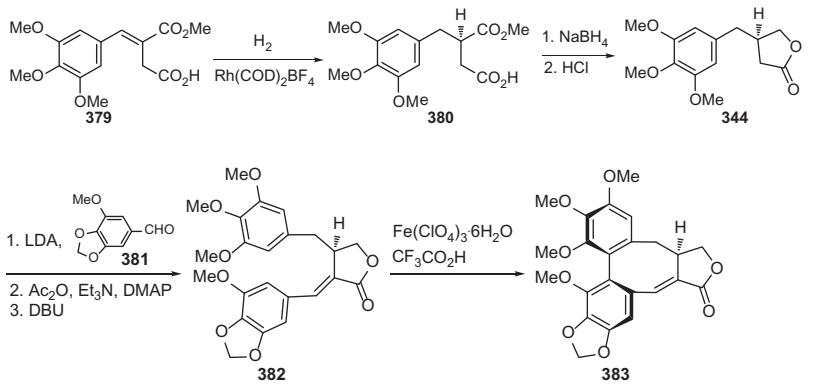
SCHEME 15 Asymmetric total synthesis of interiotherin A **91** and gomisin R **89** by Coleman *et al*.

intramolecular oxidative coupling of α-benzylidenebutyrolactone **382** (Scheme 16). Lactone **383** is a valuable precursor to access naturally occurring dibenzocyclooctadiene lignans, for example, kadsurin **35** [84b]. In a similar manner, Wakamatsu and coworkers have also accomplished the total synthesis of the natural products (−)-wuweizisu C **18**, (−)-gomisin J **19**, (−)-gomisin N **23**, (±)-γ-schizandrin **2**, (+)-schizandrin **121**, (−)-deoxyschizandrin **1**, (+)-gomisin A **122**, (+)-isoschizandrin **131**, kadsurin **35**, and a variety of related metabolites of schizandrin [85]. The strategy of intramolecular oxidative coupling of dibenzylbutyrolactone derivatives for the synthesis of dibenzocyclooctadiene-9,9′-lactones has also been utilized by Waldvogel *et al*. [86], Ward *et al*. [87], and Robin *et al*. [88].

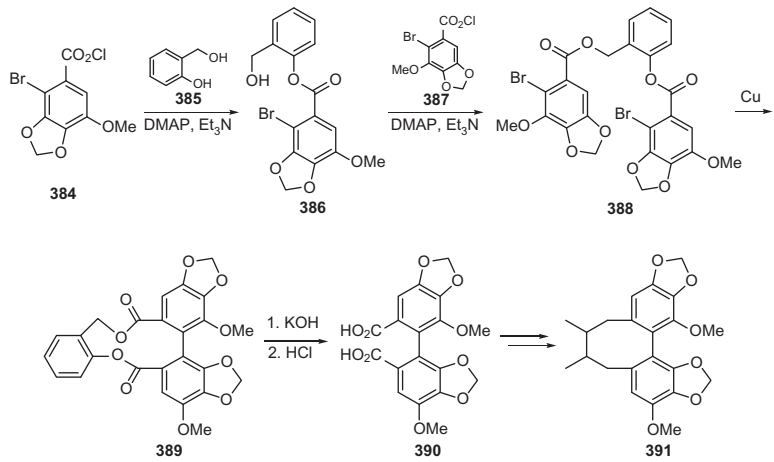
As indicated previously, the intermolecular Ullmann coupling of two different aryl halides gives a mixture of three possible biphenyl derivatives. In addition to the alternative biaryl coupling strategies mentioned earlier which relied on transformation of one of the coupling partners into an organometallic derivative prior to the coupling reaction, the intramolecular Ullmann coupling, a so-called template reaction, has also been used for the synthesis of nonsymmetrical biphenyl derivatives [89,90]. In Chang's synthesis of the nonsymmetrical biaryl diacid **390** relating to the dibenzocyclooctadiene lignan **391** [89], two different benzoyl chlorides **384** and **387** were sequentially linked to salicyl alcohol (the template) to generate a bridged diarylhalide **388** (Scheme 17). An intramolecular Ullmann coupling was then carried out to give the cyclic product **389**, which upon removal of the salicyl bridge afforded **390** in good yield.

Other Methods

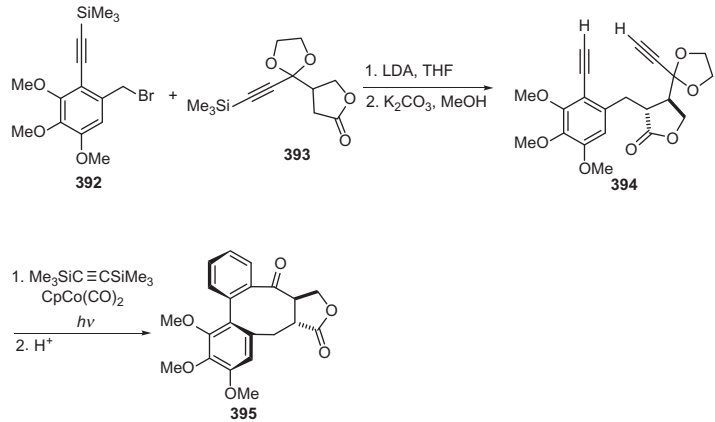
Motherwell [91] and coworkers have developed a cobalt-mediated [2 + 2 + 2] cycloaddition reaction of a tethered deca-1,9-diyne for the synthesis of steganone analogues. Nucleophilic attack of aryl bromide **392** with the lithium enolate of lactone **393** provided, after desilylation, the diyne **394** as a single



SCHEME 16 Synthesis of dibenzocyclooctatriene-9,9′-lactone **383** by Wakamatsu *et al*.



SCHEME 17 Synthesis of biaryl diacid **390** relating to dibenzocyclooctadiene lignan **391** by Chang *et al.*



SCHEME 18 Synthesis of steganone analogue **395** by Motherwell *et al.*

diastereoisomer with the required *trans* stereochemistry round the γ-lactone (Scheme 18). The key cobalt-mediated [2+2+2] cycloaddition reaction was carried out under UV irradiation to give the desired biaryl **395** possessing the carbocyclic steganone core. This strategy provides a new entry, although has not yet been applied to the total synthesis of dibenzocyclooctadiene lignan natural products.

CONCLUSION

The chemistry of dibenzocyclooctadiene lignans has developed very quickly in recent years. More than 160 new products have been isolated since 2005,

the year when we wrote our first review article on this class of natural products [26], which nearly doubled the amount previously reported. Dibenzocyclooctadiene lignan natural products have received much attention due to their promising pharmacological properties and complex structural architectures. More and more natural dibenzocyclooctadiene lignans and analogues have been synthesized for their biological activity evaluation. Quite a number of reliable strategies for the atroposelective construction of the biaryl axis have been developed. However, the syntheses of many subgroups of these natural products still remain unexplored. There is an ongoing need for the development of more efficient methodologies for the controlment of axial chirality, as well as the stereogenic centers of the side chains during total synthesis, which still remains a challenge for organic chemists.

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ABBREVIATIONS

Ac	acetyl
BINOL	1,1′-bi-2-naphthol
Bu	butyl
Bz	benzoyl
CBS	Corey–Bakshi–Shibata
COD	1,5-cyclooctadiene
Cp	cyclopentadienyl
dba	dibenzylideneacetone
DBU	1,8-diazabicyclo[5,4,0]undec-7-ene
DCC	dicyclocarbodiimide
DDQ	2,3-dichloro-5,6-dicyano-1,4-benzoquinone
de	diastereomeric excess
DIAD	diisopropyl azodicarboxylate
DIBAL-H	diisobutylaluminium hydride
DMAP	4-dimethylaminopyridine
DMF	*N*, *N*-dimethylformamide
dr	diastereomeric ratio
ee	enantiomeric excess
Et	ethyl
HMPA	hexamethylphosphoramide
HPLC	high-pressure liquid chromatography
LDA	lithium diisopropylamide
LiHMDS	lithium hexamethyl disilazide

Ms	methanesulfonyl
NBS	*N*-bromosuccinimide
NMO	*N*-methylmorpholine-*N*-oxide
PCC	pyridinium chlorochromate
pyr	pyridine
tert	tertiary
TFA	trifluoroacetic acid
THF	tetrahydrofuran
Ts	tosyl
Tr	trityl
TTFA	thallium(III) trifluoroacetate
UV	ultraviolet

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Chapter 7

Lessons from the Sea: Distribution, SAR, and Molecular Mechanisms of Anti-inflammatory Drugs from Marine Organisms

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Chapter Outline

INTRODUCTION

For many years, nature has been a source of molecules that display a number of physiological activities, some of them being exploited for the treatment of several pathological conditions, such as pain, cancer, inflammation, and autoimmune diseases. While plants have been the main source of new drugs for a

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long time [1], this trend is now shifting toward alternative sources of bioactive molecules, such as marine organisms and species living in extreme habitats.

This interest in natural products is a consequence of the success of natural molecules as biologically active agents, which, in part, arises from their complex chirality, allowing them to bind to complex proteins and other three-dimensional biological targets [2]. This property adds to other interesting features, such as complex ring systems and the number of heteroatoms and aromatic rings. For this reason, natural products have been addressed as "privileged structures," as their molecular scaffolds are able to accommodate several pharmacophores, hence displaying multiple biological activities [3].

PHYSIOPATHOLOGY OF INFLAMMATION

Inflammation is a complex process occurring in many animals that constitutes one of the first lines of defense against a number of stimuli that are perceived as harmful, such as bacteria, trauma, and irritants. While acute inflammatory processes may serve to protect the organism, deregulated or chronic inflammatory processes are the base of a number of pathological conditions that include asthma, rheumatoid arthritis, inflammatory bowel diseases, among many others. Typical symptoms of inflammation include pain, redness, heat, and swelling of the affected areas and can lead to the loss of function.

Inflammation is a process tightly regulated by several chemical mediators that include cytokines, eicosanoids, platelet-activating factor (PAF), nitric oxide (NO), and neuropeptides, among many others. Eicosanoids, comprising prostaglandins, prostacyclins, thromboxanes, and leukotrienes, play a major role in inflammation. These molecules are active at very low concentrations and constitute a family of oxygenated fatty acid derivatives obtained from arachidonic acid *via* cyclooxygenases (COXs) and lipoxygenases (LOXs). While COXs are mainly responsible for the production of prostaglandins and thromboxanes, LOXs mediate the synthesis of 5-hydroperoxyeicosatetraenoic acid (5-HPETE), which, in turn, originates leukotrienes and other mediators.

Several different approaches for the management of inflammatory conditions can be designed, ranging from upstream inhibition of arachidonic acid synthesis *via* phospholipase A_2 (PLA_2) inhibition to downstream modulation of pro-inflammatory cytokines.

MOLECULAR TARGETS IN INFLAMMATION

Phospholipases A_2

PLA_2s are a group of enzymes responsible for the hydrolysis of membrane phospholipids into arachidonic acid and have been increasingly regarded as an interesting target in anti-inflammatory drug discovery [4–9].

These enzymes can be found in almost all types of cells and hydrolyze the 2-acyl ester bond of 1,2-diacyl-sn-3-glycerophospholipids, of which arachidonic acid is an example (Figs. 1 and 2). The several PLA_2s described so far

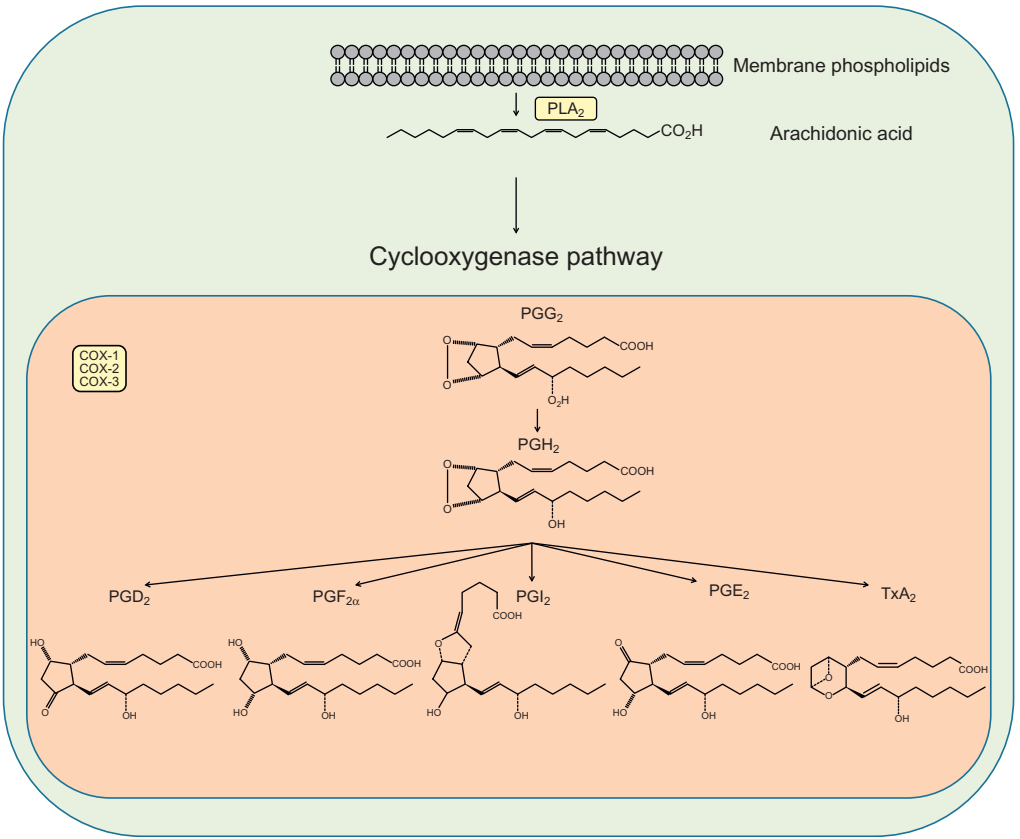

FIGURE 1 COX-pathway. PLA_2 hydrolyzes membrane phospholipids, originating arachidonic acid. COX is one of the enzymes metabolizing this fatty acid, which will originate prostaglandins.

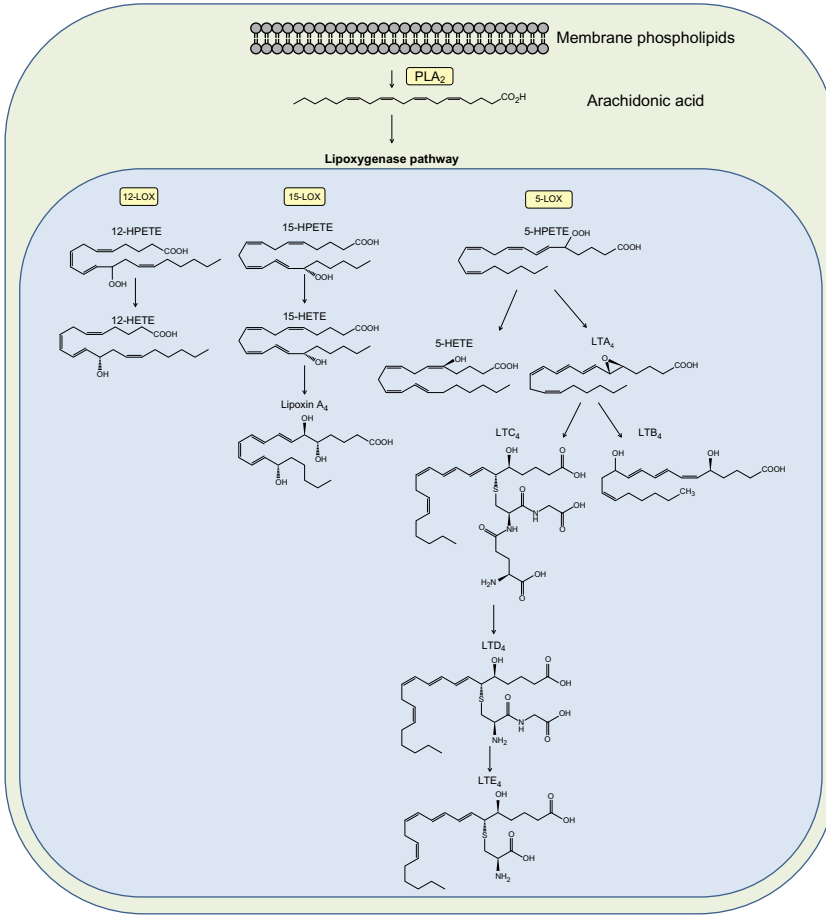

FIGURE 2 LOX-pathway. PLA_2 hydrolyzes membrane phospholipids, originating arachidonic acid. LOX is one of the enzymes metabolizing this fatty acid, which will originate leukotrienes and other molecules.

(over 15) are sorted into four families: cytosolic phospholipases A_2 ($cPLA_2$s), secreted phospholipasesA_2 ($sPLA_2$s), lipoprotein-associated phospholipases A_2 ($LpPLA_2$s), and calcium-independent PLA_2s. Notably, $LpPLA_2$s display anti-inflammatory properties, as they are able to degrade pro-inflammatory molecules, such as PAFs [4]. In the particular case of inflammation, $cPLA_2$s are very important for the synthesis of eicosanoids, being critical for the signaling pathways involving these bioactive lipids.

While the role of $sPLA_2$s in inflammation remains poorly understood, it has been suggested that $sPLA_2$s induce an increase of $cPLA_2$-dependent eicosanoid release and that they synergize with other pro-inflammatory mediators [4].

cPLA$_2$s are calcium-dependent enzymes activated by extracellular stimulations, such as pathogens, tissue injury, or physical or chemical stresses. Noteworthy, cPLA$_2$s are the only PLA$_2$s with specificity for arachidonic acid at the phospholipase sn-2 position.

A detailed description of the chemical mediators involved in the inflammatory cascade is beyond the scope of this work. Briefly, arachidonic acid is formed after PLA$_2$-mediated hydrolysis of membrane phospholipids. After this point, several different routes can be followed, metabolization by COX or LOX being the most relevants for this work.

In addition to the above-mentioned arachidonic acid, the action of PLA$_2$ on membrane phospholipids originates yet another set of molecules: lysophospholipids. These compounds, precursors of PAFs, are not deprived of biological activity, as they are involved in the secretion of histamine by mast cells and in the activation of leukocytes [10–12].

COX Enzymes and Prostanoids

Arachidonic acid is a ω-6 polyunsaturated fatty acid (PUFA) with an important role in both homeostasis and pathological conditions. In its free form, arachidonic acid is a substrate to COXs, also known as prostaglandin H synthases, being readily oxidized and originating prostaglandin G$_2$ (PGG$_2$) and then prostaglandin H$_2$ (PGH$_2$) (Fig. 1). Three isoforms of COX are known: COX-1, COX-2, and COX-3.

While COX-1 is constitutively expressed in most mammalian tissues, being responsible for the physiological production of prostanoids, COX-2 is usually not detectable in normal tissues and can be induced by either mitogenic and/or pro-inflammatory stimuli [13], which cause an elevated production of prostanoids, thus triggering inflammatory effects [14]. For this reason, COX-2 is widely targeted for the treatment of inflammation, while nonspecific inhibition of COX-1 is usually responsible for the side effects of anti-inflammatory therapy. In fact, the first generation of nonsteroidal anti-inflammatory drugs suffer from strong side effects as consequence of their inability to distinguish between the two isoforms. Nowadays, COX-2-selective drugs are able to prevent most of these side effects. Recently, a third isoform of COX was described: COX-3. In fact, this isoform results from the same gene of COX-1 but with alternative splicing that leads to the retention of intron 1 in its mRNA. For this reason, some authors claim that it should be named COX-1b instead [14].

Regardless of the COX isoform involved, in all of the cases, arachidonic acid is converted into PGG$_2$, which subsequently originates PGH$_2$ *via* the peroxidase activity of COX. From this point forward, several different molecules can be synthesized according to the enzymes involved in the reaction (Fig. 1). The distinct distribution of these enzymes in the tissues leads to different biological effects of prostanoids, which include vasodilatation (PGD$_2$, PGE$_2$, PGI$_2$), gastric cytoprotection (PGI$_2$), as well as fever mediation, pain sensitivity, and

inflammation (PGE_2) [15,16]. Other functions include involvement in the contraction of bronchial airways and regulation of platelet aggregation (TXA_2) [16].

As it will be presented later, arachidonic acid can also be metabolized by LOX, thus originating hydroxyeicosatetraenoic acids (HETE), leukotrienes, and lipoxins.

LOX

LOXs are an enzyme super family that have PUFAs as substrates. These enzymes are responsible for several biological functions in cells, which include signaling, structural changes, and pathological changes, according to the products obtained. LOXs can be found in plants and animals and are absent in yeast, while their presence in insects is not yet consensual. Nowadays, several LOXs are known to occur in humans, 5-LOX, 12-LOX, and 15-LOX being the most important in physiological and pathological processes [17].

In the particular case of inflammatory processes, arachidonic acid is a substrate for LOX, originating 5-HPETE, which is spontaneously converted to 5-HETE that, in turn, is converted to LTA_4 and then sequentially to LTC_4, LTD_4, and LTE_4 (Fig. 2).

NOS Enzymes and NO

NO is a signaling molecule involved in the regulation of several physiological and pathological processes. As it is a gas, it has some unique characteristics. The biological properties of NO can result from a direct effect or instead a consequence of the formation of reactive nitrogen species, such as peroxynitrite generated by reaction with superoxide anion. Several physiological processes are regulated by NO, including blood pressure, host defense, and platelets function.

In the particular case of inflammation, NO can enhance the production of tumor necrosis factor α (TNF-α) [18] and interleukin 1 (IL-1)β [19] and hence acts synergically in the production of inflammatory mediators.

NO is synthesized from L-arginine in a reaction that also yields L-citrulline and requires NADPH and O_2 as co-substrates and (6R)-tetrahydrobiopterin (BH_4), FAH, FMN, and iron protoporphyrin IX as cofactors. The main enzyme involved in the synthesis of NO is nitric oxide synthase (NOS). Three different NOS isoforms are known. The neuronal nitric oxide synthase (NOS I) is expressed predominantly in neurons in the brain and peripheral nervous system [20]. Other tissue-dependent isoform of this enzyme is the endothelial nitric oxide synthase (NOS III) that, as it can be easily inferred by the name, is found mainly in endothelial cells. Both NOS I and NOS III isoforms are usually described as being constitutively expressed in resting cells, where they exert physiological actions. When certain stimuli are triggered, mostly related with an increase of the intracellular concentration of Ca^{2+}, the enzymes are activated and the synthesis of NO takes place [21].

The third isoform is inducible nitric oxide synthase (iNOS, NOS II) and is responsible for many of the deleterious effects experienced in some pathological conditions, such as inflammation [21,22].

The exposure to microbial products, such as lipopolysaccharide (LPS) and double-stranded RNA, or to pro-inflammatory cytokines, such as IL-1, TNF-α, and interferon-γ, induces the expression of iNOS gene in various inflammatory and tissue cells. Binding of calmodulin to iNOS is tight even at low Ca^{2+} concentrations, and therefore, iNOS is also called as calcium-independent NOS. It can constantly produce high levels of NO for prolonged periods [21,22]. The ability to prevent NO production following a pro-inflammatory stimulus is commonly used to screen compounds for anti-inflammatory activity.

NF-κB

Nuclear factor κB (NF-κB) is a ubiquitous and inducible transcription factor. It is one of the most important regulators of the inflammatory response and immune system, being conserved across several species. It is also involved in cell proliferation and apoptosis [23].

Under basal conditions, this transcription factor is located in the cytoplasm, in an inactive form that is a consequence of the binding of a group of inhibitory proteins, named IkB, which include IkBα, IkBβ, IkBε, and IkBγ.

In the particular case of mammalian cells, five NF-κB family members are known: RelA/p65, RelB, c-Rel, p50/p105 (NF-κB1), and p52/p100 (NF-κB2). In turn, NF-κB is composed by homodimers and heterodimers of different proteins of the Rel family; however, the most common forms of NF-κB are dimeric complexes of p50/RelA (p50/p65) [24,25].

Following phosphorylation by the IkB kinase complex (IKK), IKB is marked for degradation *via* the proteasome, thus leaving NF-κB free. Once free from IκB, NF-κB translocates to the nucleus, where it binds to the promoter region of several genes, thus exerting a number of actions (Fig. 3) [26]. Target genes include cytokines, COX-2, iNOS, proteases, and many others. At this point, it is important to highlight that nowadays, two different activation pathways for NF-κB are known: the canonical and the alternative pathways. The proteasome degradation of the inhibitor complex IkB takes place in both cases, but different stimuli trigger this action. While in the classical canonical pathway, NF-κB is activated by a number of pro-inflammatory signals, such as cytokines and activation of toll-like receptors, and in the alternative pathway, the activation of B-cells is required.

The knowledge of the events that take place in NF-κB activation allows the identification of distinct mechanisms by which inhibition can occur: prevention of the IKK complex activation, modulation of proteasome activity, and modulation of NF-κB translocation to the nucleus/prevention of binding to DNA. As it will be presented later, in the case of molecules of marine origin, several mechanisms of action are found.

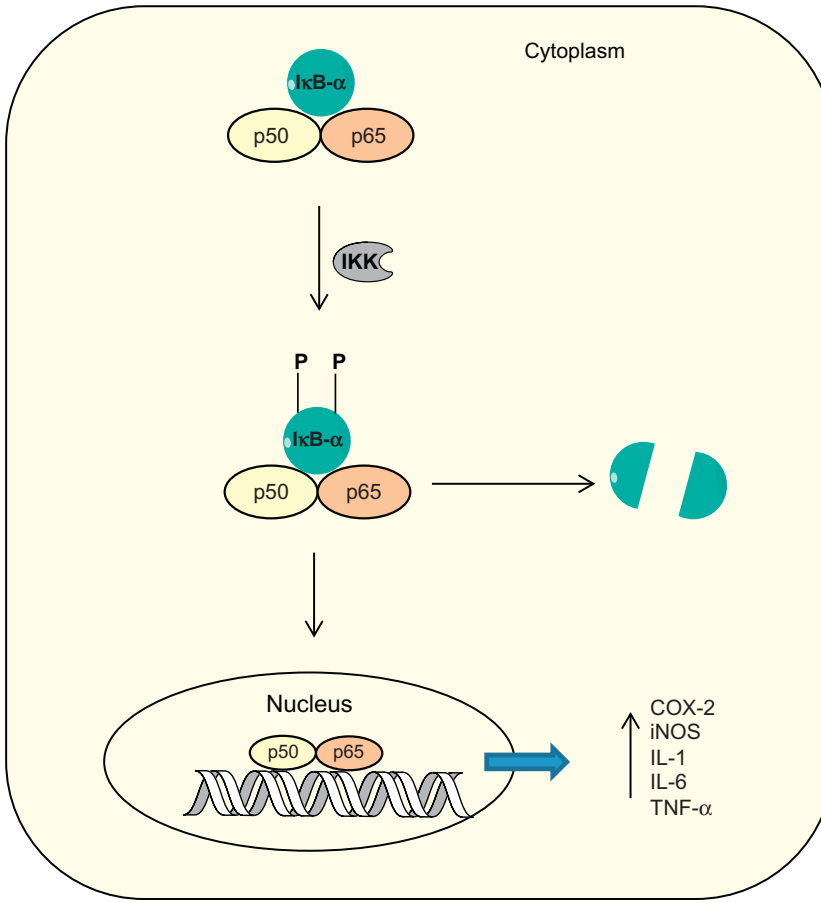

FIGURE 3 Representation of NF-κB activation. Under basal conditions, NF-κB is inhibited by IκB-α that, upon phosphorylation by IKK, is marked for degradation by the 26s proteasome complex, thus leaving NF-κB free to be translocated to the nucleus where it modulates gene expression.

Due to the relation established between NF-κB and some types of cancer [27], modulation of this transcription factor can also be one of the targets in cancer chemotherapy.

PPARγ

Peroxisome proliferator-activated receptor γ (PPARγ) is a transcription factor that belongs to the nuclear receptor family of ligand-activated transcription factors. It forms heterodimers with the retinoid X receptor and then exerts its effects by binding to a DNA element, known as the PPAR response

element. Several physiological and pathological events are PPAR-mediated, and the effect depends on the cell type [28].

PPAR is involved in several pathways, which include lipid homeostasis, proliferation and differentiation. In particular, activation of this transcription factor in macrophages results in anti-inflammatory effects, such as decreased cytokine production or iNOS expression. These effects can be the result of direct negative modulation of target genes or, alternatively, of the prevention of NF-κB activation [28–30].

ANTI-INFLAMMATORY MARINE DRUGS

Marine sources continue to surprise us with their remarkable chemistry and structural diversity, which frequently results in interesting biological properties [31,32]. Most of the bioactive metabolites found belong to the class of terpenes, alkaloids, macrolides, cyclopeptides, and depsipeptides. Terpenes, one of the most common classes of natural products, are further grouped into different subclasses according to the number of carbons in their structures, which, in turn, is a consequence of their respective biosynthetic pathways. As so, they are classified as monoterpenes (C_{10}), sesquiterpenes (C_{15}), diterpenes (C_{20}), sesterterpenes (C_{25}), triterpenes (C_{30}), and so on. Regarding bioactive terpenes from marine animals, as it will be presented later, most of the compounds are sesquiterpenes or sesterterpenes.

Sesquiterpenes

Avarol

Avarol is a sesquiterpene hydroquinone isolated from the sponge *Dysidea avara*. Avarone is the corresponding quinone compound (Fig. 4).

Early experiments using the paw and ear edema model revealed that both compounds display anti-inflammatory activity at concentrations below 10 mg/kg, a potency comparable to that of indomethacin. Inhibition of leukotriene B_4 (LTB_4) and thromboxane B_2 release in human rat peritoneal leukocytes was also reported [33].

Recently, avarol showed to inhibit TNF-α generation in human monocytes (IC_{50} of 1 μM) and NF-κB activation. In an animal model, IL-1β, PGE_2, and LTB_4 production was also inhibited [34].

Bolinaquinone

Bolinaquinone is a very interesting compound obtained from *Dysidea* sp. and closely related to avarol and avarone due to the fact that it contains both a quinone and a methoxylated hydroquinone moiety, which are connected to a *trans*-decalin terpene unit in a rearranged drimane skeleton (Fig. 4). This compound was first described by Giannini *et al*. in 2001, and at the time,

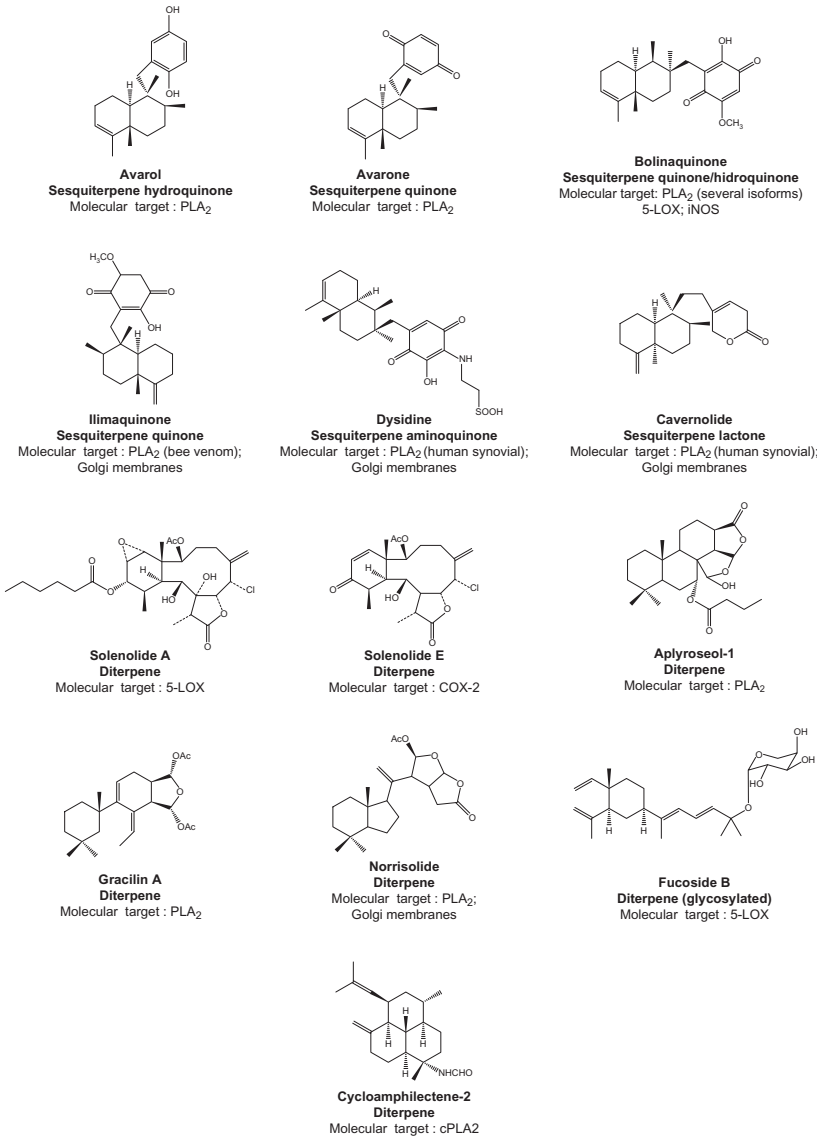

FIGURE 4 Structures of some sesquiterpenes and diterpenes mentioned in this study. Ac, acetate.

the team also described its inhibitory capacity against several types of PLA_2: group I (porcine pancreas), group II (human synovial), and group III (bee venom), without effect on cytosolic PLA_2 [35]. It is important to highlight that the potency of bolinaquinone on group II enzymes is superior to that of manoalide, a sesterterpene that will be presented later and that is frequently

used as a reference compound for anti-inflammatory drugs of marine origin. When at concentrations between 1 and 2 μM, this terpenoid was able to prevent LTB_4 release in human neutrophils, as consequence of 5-LOX inhibition [36]. *In vivo* experiments showed that this compound successfully prevented the increase of PGE_2 levels, and interestingly, only PGE_2 concentration in the inflammation site was altered, with the authors proving that serum and stomach levels remained unchanged. This shows the selectivity of this compound for inflammation sites, thus not affecting physiologic concentrations of the mediator in the organs. The authors showed that while inhibition of NO was a consequence of the inhibition of iNOS expression, the same was not true for PGE_2 and COX-2. In fact, the registered inhibition of PGE_2 was thought to be a consequence of $sPLA_2$ inhibition [36]. In another study, the molecular mechanism of human group IIA PLA_2 inhibition by bolinaquinone was investigated. Spectroscopic techniques, biosensors analysis, mass spectrometry, and molecular docking revealed that competitive inhibition takes place, also showing the importance of the quinone moiety, which chelates the catalytic Ca^{2+} ion in the active site of the enzyme [7].

Ilimaquinone

Another sesquiterpene quinone, ilimaquinone (Fig. 4), was obtained from the sponge *Hippospongia metachromia* in a bioguided isolation of antimicrobial products [37]. Preliminary studies pointed to an interesting anti-inflammatory activity, mainly related to its ability to inhibit PLA_2 in a bee venom model. Curiously, it seems that the evaluation of the anti-inflammatory activity of this molecule has been abandoned when other biological properties were discovered, namely, the ability to induce vesiculation and break down of golgi membranes, thus being an inhibitor of protein synthesis [38]. In fact, this mechanism has been extensively studied, and nowadays, ilimaquinone is a well-known inhibitor used in this area [39–44]. Another compound that displays a similar effect toward Golgi membranes is norrisolide that, besides its well-established anti-inflammatory properties, has also been found to be a novel Golgi-vesiculating agent with important biochemical and pharmacological implications [45].

Other biological activities have been ascribed to ilimaquinone, namely, anticancer properties against several human cancer cell lines [46].

Dysidine

Dysidine, a sesquiterpene aminoquinone (Fig. 4), was isolated from *Dysidea* sp. This compound displayed human synovial PLA_2 inhibitory effect, reaching ~75% at 10 μM. No significant inhibition of the snake *Naja naja* venom or porcine pancreas PLA_2 was found, although partial inhibition of bee venom was described [35]. In the same work, two other compounds were found,

dysidenones A and B, along with bolinaquinone. A 1:1 mixture of dysidenones A and B displayed about 45% inhibition of human synovial PLA_2 at 10 μM.

Apart from its anti-inflammatory properties, dysidine has also been associated to other biological activities, such as activation of insulin signaling pathways and insulin-sensitizing activity [47].

Dysidiotronic Acid

Dysidiotronic acid is a sesquiterpene analog of manoalide that lacks the pyranofuranone ring. This compound was tested against a panel of several PLA_2, namely, *N. naja* venom, porcine pancreas, human synovial and bee venom. The compound displayed significant selectivity against the human enzyme, its IC_{50} being lower than that of manoalide (2.6 and 3.9 μM, respectively) [48].

Subsequent works studied the effect of dysidiotronic acid on the levels of NO, cytokines, and prostanoids in RAW 264.7 cell line model [49]. Dysidiotronic acid effectively inhibited the production of NO (IC_{50} of 1.7 μM), PGE_2 (IC_{50} was 1.1 μM), and TNF-α (1.3 μM). Regarding NO production, the authors proved that the activity of the compound was related to the downregulation of iNOS expression, rather than the inhibition of the enzyme. In the case of PGE_2, the mechanism of action was different, as the levels of this PG were reduced even in the post-induction phase, which was related to PLA_2 inhibition [49].

Cavernolide

The sesquiterpene lactone cavernolide (Fig. 4) is a molecule that can be obtained from the sponge *Fasciospongia cavernosa*. This molecule inhibited PLA_2 in several assays, including secretory PLA_2 obtained from *N. naja* venom, porcine pancreas, human synovial, and bee venom. This sesquiterpene inhibited $sPLA_2$ activation (IC_{50} of 8.8 μM) in human systems. TNF-α, NO, and PGE_2 production was also inhibited at concentrations below 10 μM. Further experiments showed that this molecule also inhibited the expression of iNOS and COX-2, the effect being more pronounced with the former [8].

Diterpenes

Diterpenes are usually distributed in sponges species from the orders Dendroceratida and Dictyoceratida and, frequently, in nudibranchs that predate upon these sponges.

Solenolides A–F

Six diterpenoids, named after the genus as solenolides A–F, were obtained from sponges of the genus *Solenopodium*. These compounds display a polycyclic moiety with a lactone function (Fig. 4) [50]. All of the active compounds

exerted their effects by interfering with arachidonic acid metabolism; however, different mechanisms were found: while solenolide A was able to inhibit 5-LOX, solenolide E acted as a COX inhibitor.

Aplyroseol-1, Gracilin A, and Acetoxytetrahydroaplysulfurin-1

Sponges from *Aplysilla* sp. have been used for the isolation of aplyroseol-1 (Fig. 4), gracilin A (Fig. 4), and 12-acetoxytetrahydroaplysulfurin-1 [51]. These compounds proved to be active in inhibiting PLA_2 at 2 μg/mL, displaying over 60% inhibition [9]. The same range of activity was found for norrisolide (Fig. 4), a diterpene isolated from the nudibranch *Chromodoris norrisi* [52] that is thought to be a consequence of the predation on *Aplysilla polyrhaphis* [53].

Fucosides A–D

The soft coral *Eunicea fusca* is the source of some unusual glycosylated diterpenes: fucosides A–D. These compounds, displaying lobane and eremophilene-like skeletons, were identified for the first time by the Fenical group in 1991 [54]. Posterior studies showed that these compounds, notably fucoside B (Fig. 4), were able to inhibit 5-LOX in both murine and human models (IC_{50} of about 9 μM) [55,56]. Interestingly, it is now known that fucoside B's aglycone, fuscol, is produced by a symbiont alga of the coral.

Recently, a new compound of this series, fucoside E, was described in the same species. Studies involving the ear edema model revealed an activity of the same magnitude of that of fucoside B, both compounds displaying over 80% of ear edema inhibition at a concentration of 0.5 mg/ear, thus surpassing indomethacin [57].

Cycloamphilectenes

The order Halichondrida has been a prolific source of bioactive compounds, mainly in the area of cancer therapeutics [32,58]. For example, halichondrine B has been shown to influence cell cycle and induces cell death in the A549 lung cancer cell line (IC_{50} around 3 nM) [58].

Sponges of the genus *Axinella* are sources of a group of compounds named cycloamphilectenes, which are diterpenes characterized by tri- or tetracyclic skeletons that can display several functional groups, such as isonitrile, isothiocyanate, and isocyanate. They were first described in 1987 in *Halichondria* sp. [59] and have then been found in other organisms, such as *Axinella* sp. [60].

Cycloamphilectene 1–6 were tested for their ability to prevent zymosan-induced generation of NO, TNF-α PG and expression of COX-2 and iNOS in mouse peritoneal macrophages [61]. Overall, at 10 μM, all of the compounds were able to inhibit the production of NO by over 70%; in the case of PGE_2, the values of inhibition ranged from 37% for cycloamphilectene-3 to 70% with cycloamphilectene-4. These two compounds, together with cycloamphilectene-5 and cycloamphilectene-6, inhibited 50%, or more,

TNF-α production. Concerning protein expression, cycloamphilectene-2 (Fig. 4), -4, and -6 were tested for their ability to prevent zymosan-mediated increase of COX-2 and iNOS. While no activity was found for COX-2, all compounds decreased the levels of iNOS, cycloamphilectene-6 being the most potent. The authors showed that while the reduction of NO levels was a consequence of the inhibition of iNOS expression, the decrease of PGE_2 resulted from COX-2 inhibition rather than expression [61]. In the case of cycloamphilectene-2, prevention of NF-κB activation was also found. This anti-inflammatory activity was confirmed in *in vivo* studies using the ear edema model, 200 μg/ear displaying activity comparable to that of indomethacin. It is interesting to realize that cycloamphilectene-2 is the only compound presenting an exocyclic methylene group.

Sesterterpenes

Sesterterpenes constitute one of the most prolific classes of marine natural products in what concerns to their biological properties, namely, anti-inflammatory and anticancer.

The leading compound of this class is manoalide (Fig. 5), a sesterterpene with a γ-hydroxybutenolide group that has launched the discovery of anti-inflammatory molecules from marine sources in the 1980s. Manoalide is a nonspecific inhibitor able to inhibit human $sPLA_2$ ($IC_{50} = 1.7$ μM), $cPLA_2$ ($IC_{50} = 10$ μM), and snake venom $sPLA_2$ ($IC_{50} = 30$ nM) [62].

The anti-inflammatory properties of this compound were discovered simultaneously by the groups of Dennis [63] and Jacobs [64] at the universities of San Diego and Santa Barbara, respectively. Despite manoalide's excellent activity, clinical trials were discontinued due to problems regarding formulation.

Since then several manoalide derivatives have been described, such as cacospongionolides, petrosaspongiolides, luffariellolides and luffariellins, among others. These compounds have a γ-hydroxybutenolide moiety in common, which seems to be critical for their anti-inflammatory activity, as all of them are irreversible inhibitors of PLA_2.

Variabilin

Variabilin is a sesterterpene (Fig. 5) first isolated from the sponge *Ircinia variabilis*, although it can be found in other species, such as *Hemimycale columella*.

This compound displayed significant PLA_2 inhibitory effect in a model of human synovial $sPLA_2$. Curiously, several other sources of $sPLA_2$ were tested (snake venom $sPLA_2$, bee venom, porcine pancreas, among others), but the human isoform was the one displaying the highest susceptibility. Direct inhibition of 5-LOX, COX-2, COX-1 was investigated in cell-free systems, but no activity was found [65].

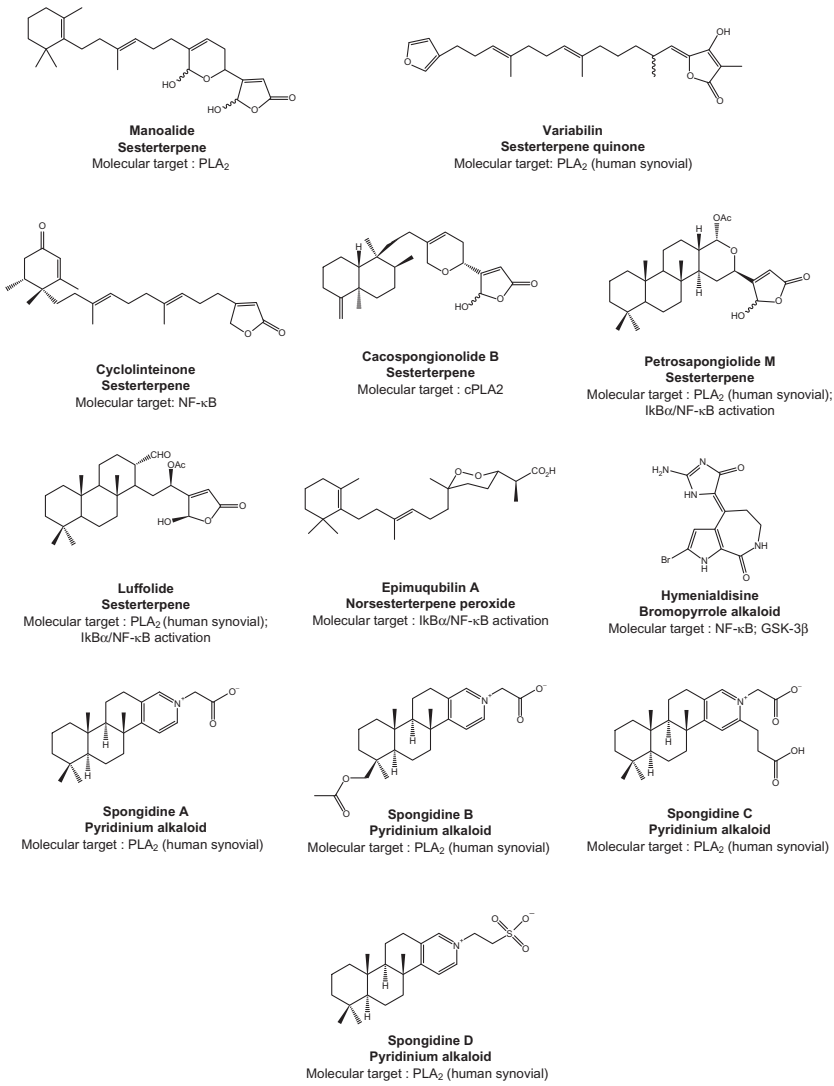

FIGURE 5 Structures of some sesterterpenes and alkaloids mentioned in this work. Ac, acetate.

Topical administration in the mouse ear edema model using phorbol myristate acetate (PMA) in the range of 30–45 mg/kg prevented carrageenan-induced edema and in the mouse air pouch it was able to lower the levels of PGE_2 and LTB_4. It is interesting to highlight that when arachidonic acid was used as pro-inflammatory stimulus, instead of PMA, no activity was found. These results point to a mechanism of action that takes place upstream in arachidonic acid metabolism [65]. It is probably no coincidence that variabilin shares some

chemical traits with manoalide, namely, γ-hydroxybutenolide ring, which is known to be critical for the anti-inflammatory activity, as referred earlier.

Cyclolinteinone

Cyclolinteinone (Fig. 5) is a compound found in Caribbean sponges from *Cacospongia* genus. The J774 murine macrophage cell line was used to evaluate the anti-inflammatory potential of this sesterterpene. Overall, this molecule was able to prevent LPS-induced increase of COX-2 and iNOS and, hence, to control the production of prostaglandins and NO. The effect upon COX-2 and iNOS was proved to be a consequence of the inhibition of NF-κB activation in an electrophoretic mobility shift assay (EMSA). The addition of cyclolinteinone to the cells after incubation with LPS, when the inflammatory cascade was already active, resulted in no activity, thus discharging the possibility of direct enzyme inhibition [66].

Cacospongionolide B

Cacospongionolide B (Fig. 5) is a sesterterpene first isolated from the sponge *Cacospongia mollior* [67] that has been the first of an increasing series of analogue compounds displaying marked biological properties, such as anticancer [68]. In particular, cacospongionolide B has been increasingly regarded as a potent anti-inflammatory drug and has already been found in other species, such as *F. cavernosa* [69].

Early studies showed the selective inhibition of this compound (10 μM) against sPLA2 when compared to $cPLA_2$, the potency toward group II enzymes being comparable to that of manoalide. These results were confirmed in *in vivo* experiments. This compound successfully prevented the inflammatory process in mouse paw and ear edema model [70].

Synthetic analogues of cacospongionolide B have been synthesized and have highlighted the importance of the flexible aliphatic region between the decalin and the side chain portions, as shown by the lower inhibitory activity of conformation-restricted analogues [71].

Petrosaspongiolide M

Petrosaspongiolide M (Fig. 5) was isolated from the sponge *Petrosaspongia nigra* in 1998, and at the time, the high inhibitory capacity of this molecule toward human synovial PLA_2 ($IC_{50} = 1.6$ μM) was noticed [72]. A series of analogues was isolated (petrosaspongiolide M–R) and some structure–activity relationships (SARs) could be pointed. The importance of the tricyclic system was shown as result of the lower activity of 21-acetoxy derivatives. The already known importance of the aldehyde function in C-24 was also confirmed by the fact that luffolide (Fig. 5), a related compound, displayed higher activity [72]. The mechanism of action involves a noncovalent recognition of the enzyme. Once again, the importance of the γ-hydroxybutenolide ring is highlighted.

Subsequent studies showed that the inhibition of NO, PGE_2, and TNF-α in the mouse air pouch injected with zymosan resulted from the negative modulation of iNOS and COX-2. At 1 μM, petrosaspongiolide M successfully prevented NF-κB binding to DNA as consequence of the inhibition of IkBα phosphorylation.

Acetylated petrosaspongiolide M analogues, such as the suicide inhibitor 25-acetyl-petrosaspongiolide M, have been used and showed that PLA_2 also displays a esterase-like activity, being capable of differentiating between acetylation in different carbons, which can be particularly important for the design of anti-inflammatory pro-drugs [6].

Epimuqubilin A

Epimuqubilin A is a terpenoid with very interesting chemical and biological properties, due to the fact that it is a norsesterterpene peroxide (Fig. 5). These peroxides have a 2-substituted propionic acid or methyl propionate group attached to a 1,2-dioxane ring at C-3. This compound can be obtained from *Latrunculia* sp. and displays promising anti-inflammatory properties.

The macrophage cell line RAW 264.7 was used in the evaluation of the effect of epimuqubilin A on the most representative mediators involved in inflammatory processes. The IC_{50} for the inhibition of NO production was about 7.5 μM and other closely related compounds from the same species, such as muqubilone B and epimuqubilin B, also displayed inhibitory activity [73]. Subsequent studies showed that the reduction of NO production was a consequence of the downregulation of the levels of iNOS, which could be noticed starting at 5 μM and reaching its maximum at 10 μM [74]. The expression of COX-2 was also suppressed by epimuqubilin A in the same concentration range. Apart from these changes in protein levels, mRNA was also diminished. The effect of this compound on the phosphorylation of several MAPKs (p-p38, p-SAPK/JNK, p-ERK1/2) was studied, but no effect could be found. The above-mentioned effect on the expression levels of COX-2 and iNOS was then proved to result from the inhibition of NF-κB binding to DNA, which, in turn, was shown to result from the prevention of IkBα degradation [74].

Amino Acid Derivatives

Alkaloids

While alkaloids constitute one of the classes of natural products displaying more biological properties, anti-inflammatory alkaloids are not so common.

Hymenialdisine

Hymenialdisine is an alkaloid with a bromopyrrole scaffold (Fig. 5) that has been obtained from the sponge *Axinella verrucosa*. The effect of hymenialdisine on NF-κB was investigated by luciferase reporter assays and also

EMSA, which confirmed the direct and specific interaction of this molecule with this transcription factor. This compound also inhibited the production of PGE_2 in rheumatoid synovial fibroblasts activated with IL-1 in a concentration-dependent manner (IC_{50} of 1 μM) [75]. Posterior studies confirmed this mechanism of action and also revealed its inhibitory effect on iNOS gene expression, proteoglycan synthesis, [76] and glycogen synthase kinase 3 β (GSK-3β) [77].

Semi-synthetic derivatives of hymenialdisine were obtained, and SAR studies have highlighted the importance of the secondary nitrogen in the five-membered ring of guanidine [78].

Spongidines A–D

Four pyridinium alkaloids have been isolated from the sponge *P. nigra* and were named spongidines A–D. Their structure can be found in Fig. 5. Several PLA_2 were tested, namely from *N. naja* venom, porcine pancreas, human synovial, and bee venom. While no inhibitory activity was found toward $cPLA_2$, pyridinium alkaloids significantly inhibited human synovial PLA_2 [79].

Other Amino Acid Derivatives

Ascidians have been a prolific source of amino acid derivatives, which include cyclic peptides, alkaloids, and other compounds.

Herdmanines A–D have been isolated from the ascidian *Herdmania momus*. Interestingly, herdmanines A–C display a D-arginine moiety that, despite being described before in natural sources, is usually associated with bacterial metabolism [80]. For this reason, these amino acid derivatives are thought to be the result of the symbiosis of *H. momus* with microorganisms.

Herdmanines C and D (Fig. 6) were able to inhibit the production of NO in LPS-stimulated RAW 264.7 cells (IC_{50} of 96 and 9 μM, respectively) [81]. The same study showed that the reduction of the levels of NO resulted from the downregulation of the respective gene, as indicated by the levels of mRNA. The same trend was found for prostaglandins and COX-2, whose mRNA levels were also diminished. Inhibition of interleukin 6 (IL-6) production was also found [81].

Sterols

Sulfated sterols are commonly found in sponges and echinoderms [82].

Sterols obtained from marine sources usually display some unique structural characteristics that are often absent in terrestrial sources. Such is the case of the recently obtained chlorinated and iodinated topsentiasterol sulfates from *Topsentia* sp. Frequently, this unique chemistry results in unique biological properties.

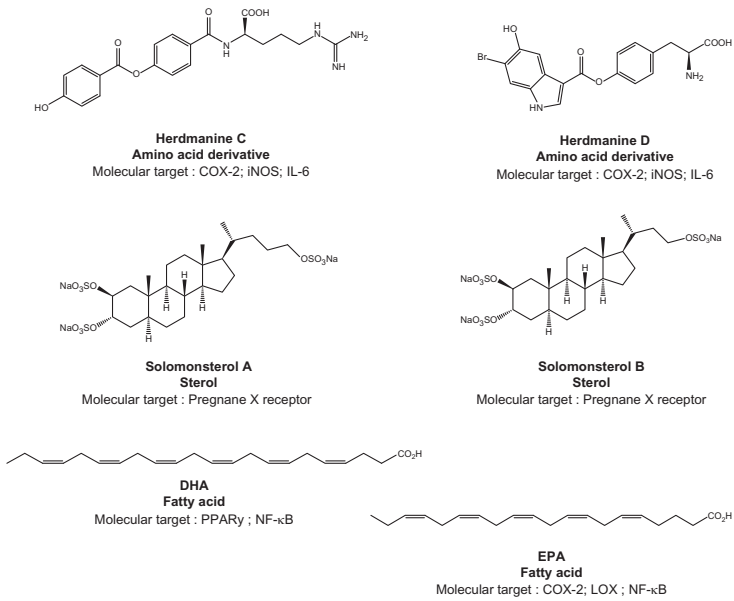

FIGURE 6 Structures of some amino acids derivatives, sterols, and fatty acids mentioned in this work. DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid.

Solomonsterol A and solomonsterol B (Fig. 6) were tested for their ability to prevent LPS-induced increase of mRNA of pro-inflammatory interleukins in RAW 264.7 cell line. Both compounds lead to 50% inhibition of IL-1β mRNA levels at 50 μM, while no effect could be noticed for TNF-α [83]. Further studies identified an agonistic effect on human pregnane-X receptor, but no activity on the farnesoid-X receptor. The authors conducted docking studies that revealed sulfate group as being involved in the hydrogen bonds providing the complex increased stability [83].

Fatty Acids

Increasing information points to the possible use of unsaturated fatty acids (FA) as modulators of inflammatory processes [84].

One striking difference between FA and all of the above-mentioned molecules is due to the fact that FA are present in our diet and play several physiological roles, being the endogenous ligands to a number of receptors.

High dietary contents of PUFAs have been shown to alter cell membrane composition, which normally contains high amounts of the ω-6 PUFA arachidonic acid that, as presented before, is pivotal for the subsequent steps of inflammatory processes. As so, it is clear that reduced levels of arachidonic acid in cell membranes can ameliorate inflammatory conditions. In fact, several studies have shown that high oral intake of the ω-3 PUFA

eicosapentaenoic acid (EPA) (Fig. 6) and docosahexaenoic acid (DHA) (Fig. 6) results in their increased incorporation in biological membranes, accompanied by an inverse decrease in the levels of arachidonic acid [85,86]. This reduction on the content of membrane arachidonic acid also results in decreased levels of some prostaglandins and leukotrienes, due to lower amounts of the substrate. This effect is further propagated if we consider that EPA itself can be a substrate to COX and LOX, the resulting products being up to 100 times less potent than LTB_4 [87], some of them even antagonizing arachidonic acid-derived eicosanoids [88]. Other mechanisms of the anti-inflammatory activity of fatty acids, such as resolvins, protectins, and GPR120, have been reviewed recently [88].

Several PUFA are endogenous ligands of PPARγ and it has been shown that exogenous administration of some of these compounds, notably DHA, could result in decreased levels of TNF-α and IL-6 [28,29].

PUFA are also known to interfere with NF-κB, the "master switch" orchestrating most of the inflammatory events, although the exact mechanism is still not consensual. Given the fact that many studies addressing anti-inflammatory drugs use the LPS-activated macrophages system, it is relevant to highlight that EPA and DHA effectively prevent LPS-mediated increase of COX-2, iNOS, and several interleukines. This activity is thought to be the result of insufficient IκB phosphorylation.

CONCLUDING REMARKS

There has been increasing awareness of the pivotal role of natural products in the treatment of several pathological conditions, including inflammation. Among natural sources, marine organisms have proved to be excellent providers of molecules with unique chemistry, which is often translated in potent biological activities.

In the particular case of inflammation, the most promising compounds are terpenes and several mechanisms of action are described, ranging from the direct inhibition of the synthesis of inflammatory mediators to the modulation of NF-κB. However, according to the available literature, a major part of these compounds exerts anti-inflammatory activity *via* PLA_2 inhibition.

This area of research is expected to grow in the next few years, thus adding to our knowledge drugs of marine origin that can be used as medicines, with molecules increasingly potent and selective. The study of species living in extreme habitats is also expected to provide yet new compounds with novel biological properties.

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ABBREVIATIONS

5-HETE	5-hydroxyeicosatetraenoic acid
5-HPETE	5-hydroperoxyeicosatetraenoic acid
COX	cyclooxygenase
cPLA$_2$	cytosolic phospholipase A$_2$
DHA	docosahexaenoic acid
EMSA	electrophoretic mobility shift assay
EPA	eicosapentaenoic acid
GSK-3β	glycogen synthase kinase 3 beta
IL-1	interleukin 1
IL-6	interleukin-6
iNOS	inducible nitric oxide synthase
LOX	lipoxygenase
LpPLA$_2$	lipoprotein-associated phospholipase A$_2$
LPS	lipopolysaccharide
LTA$_4$	leukotriene A$_4$
LTC$_4$	leukotriene C$_4$
LTD$_4$	leukotriene D$_4$
LTE$_4$	leukotriene E$_4$
NF-κB	nuclear factor κB
NO	nitric oxide
PAF	platelet-activating factor
PG	prostaglandin
PGD$_2$	prostaglandin D$_2$
PGE$_2$	prostaglandin E$_2$
PGG$_2$	prostaglandin G$_2$
PGH$_2$	prostaglandin H$_2$
PMA	phorbol myristate acetate
PPARγ	peroxisome proliferator-activated receptor γ
PUFAs	polyunsaturated fatty acids
sPLA$_2$	secreted phospholipase A$_2$
TNF-α	tumor necrosis factor α

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Chapter 8

Marine Resource: A Promising Future for Anticancer Drugs

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Chapter Outline

INTRODUCTION

Cancer

Cancer is a group of diseases characterized by uncontrolled growth and spread of abnormal cells. All types of cancers involve malfunction of genes that control cell growth and division. Among them, only about 5% of all cancers are strongly hereditary. However, most cancers do not result from inherited genes but from damage to genes occurring during one's lifetime. Genetic damage may result from internal factors, such as hormones or the metabolism of nutrients within

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cells, or external factors, such as tobacco, chemicals, and excessive exposure to sunlight. These causal factors may act together or in sequence to initiate or promote the development of cancer. Research reveals that 10 or more years often pass between exposure to external factors and disease diagnosis. According to the American Cancer Society in "2012," about 173,200 cancer deaths will result due to tobacco use. Further scientific evidences indicates about one-third of the 577,190 cancer deaths, expected to occur in 2012, will be related to overweight or obesity, physical inactivity, and poor nutrition. Irony is, all cancers caused by cigarette smoking and heavy use of alcohol, obesity, and malnutrition could be prevented completely, if diagnosed early. In addition, many of more than 2 million skin cancers associated with infectious agents, such as hepatitis B virus, human papillomavirus, human immunodeficiency virus (HIV), *Helicobacter pylori* (*H. pylori*), and others, could be prevented through behavioral changes, vaccines, or antibiotics. Regular screening examinations by a health care professional can result in the detection and removal of precancerous growths, as well as the diagnosis of cancers at an early stage, when they are most treatable. Cancers of the cervix, colon, and rectum can be prevented by removal of precancerous tissue. Early diagnosis of the breast, colon, rectum, cervix, prostate, oral cavity, and skin are treatable. A heightened awareness of changes in the breast or skin may also result in detection of these tumors at earlier stages. Cancer is the second most common cause of death in the United States, exceeded only by heart disease, accounting for nearly 1 of every 4 deaths. However, the 5-year relative survival rate for all cancers diagnosed between 2001 and 2007 has gone up to 67%, from 49% in 1975–1977. The improvement in survival reflects both progresses in diagnosis of certain cancers at an early stage, awareness enhancement, and improvements in analysis of tumor registry data [1].

Treatment of Cancer

Most cancer treatments rely heavily on chemotherapy; however, chemotherapy has limitations. Chemotherapeutic drugs lack selectivity meaning they can also kill normal cells. They might cause multi-drug resistance as well. These problems result in serious side effects, immunity suppression, and poor treatment outcomes. Scientists are making great efforts to overcome the problems caused by chemotherapy. Natural products provide a major source of new drug candidates. It is estimated that there are at least 250,000 species of higher plants around the world, while marine organisms are widely distributed over 70% of the earth's surface. All of these species are able to produce secondary metabolites with different activities due to defense, competition, or a number of other environmental reasons. From the 1940s to 2006, 175 anticancer drugs were discovered, among which 14% are natural products and 43% are derived from natural products by semisynthesis or using their pharmacophore [1].

Marine Biodiversity

Currently, more than 30 compounds of marine origin are in different phases of clinical study for the treatment of different types of cancers. Of importance

are ixabepilone (epothilone B from *Sorangium cellulosum*), romidepsin (depsipeptide from *Chromobacterium violaceum*), and the dibenzodiazepine ECO-4601 (from *Micromonospora* sp.), besides the other new natural compounds that could be developed into new drugs or leads, through further modifications to make them more potent, more selective and less toxic than currently available anticancer drugs. Author has predicted approximately 6 lakhs novel chemicals in marine organisms, out of which more than 90% are undiscovered. According to their estimated, a total of 55–214 new anticancer drugs are expected to reach the market, sourced primarily from marine sources. Besides, a lot of structurally and pharmacologically important substances have been isolated with novel antimicrobial, antitumor, and anti-inflammatory properties [2–5].

Marine biodiversity is a resource of enormous importance to human society that provides critical ecosystem services. Over the evolutionary time, marine organism has defined the chemistry of the oceans and atmosphere forming a unique ecosystem and weaving a complex food web. Thousands of different species of bacteria, fungi, and viruses exist in this web, playing highly diverse roles in terms of ecology and biochemistry, in one of the most unique ecosystems. The diversity of species comprising marine ecosystem to so rich that each drop of water taken from the ocean contains microbial species unknown to humans in 9:1 ratio [2]. Ocean represents a rich resource for novel compounds with great potential as pharmaceutical, nutritional supplements, cosmetics, agrichemicals and enzymes, and more usage where each of these marine bioproducts has a strong potential market value [6].

The chapter highlights marine pharmacology with respect to anticancer drug molecules which have shown potential to become drug or is in pipeline to become clinical drug. The information collected covers published preclinical pharmacology of marine chemicals derived from a diverse group of marine animals, algae, fungi and bacteria, cyanobacteria, and others. The study covered includes integrated effort by investigators from Argentina, Australia, Brazil, Belgium, Canada, China, France, Germany, India, Indonesia, Israel, Italy, Japan, Mexico, Morocco, the Netherlands, New Zealand, Norway, Panama, the Philippines, Portugal, Russia, Slovenia, South Korea, Spain, Thailand, Turkey, United Kingdom, and the United States. These countries have contributed numerous chemical leads for the continued global search for novel therapeutic agents with broad anticancer activity. Their investigation has predicted that among the quarter of a million plant species estimated to be present, only small fraction of them have been studied, which has yielded $>$160,000 natural compounds, a number that is growing at a rate of $>$10,000 compounds per year [7,8] along with investigated marine resources. Research has further revealed approximately 30,000 genes in our genome, thereby implying that there could be at least that many number of proteins and protein–protein interactions which could be the targets for a potential drug. At present, $\sim$600 different molecular targets have been identified and 54 marine

compounds have been reported to act on a variety of these molecular targets probably belonging to several pharmacological classes [9].

Phytomedicine: An Important Contribution of Terrestrial Plants

Natural products have been a rich source of agents of value to medicine. In the past, plant secondary metabolites have proved to be an excellent reservoir of new medical compounds. Many anticancer agents have been isolated from various plant sources like *Catharanthus roseus*, *Podophyllum* species, *Taxus brevifolia*, *Camptotheca acuminate*, *Betula alba*, *Cephalotaxus species*, *Erythroxylum pervillei*, *Curcuma longa*, *Ipomoeca batatas*, *Centaurea schischkinii*, and many others. Scientists are still attempting to explore the bioavailability of anticancerous compounds in unexplored plant species. Historical experiences and success with plants as therapeutic tools have helped to introduce single chemical entities in modern day medicine. In the early days of drug discovery plants, the primary sources of medicines were especially those with ethnopharmacological uses [7].

According to Gordaliza [10] more than half of currently available drugs are natural compounds or are related to them, and in case of cancer this proportion surpasses 60%. Additionally, many new natural compounds of diverse structures, isolated from plant sources, have been considered prototypes, leads, or heads of series and their later structural modification has afforded compounds with pharmacological activity and extraordinary therapeutic possibilities [9–12].

The search for improved cytotoxic agents (more potent, more selective, and less toxic) continues to be an important line in the discovery of modern anticancer drugs. Kintzios (2006) suggested that the huge structural diversity of natural compounds and their bioactivity potential isolated from plants, marine flora, and microorganism could lead to several products which can serve as "lead" compounds [13]. These "lead" compounds can be improved of their therapeutic potential by molecular modification of the functional groups of lead compounds by semisynthesis, which may generate structural analogues with greater pharmacological activity and with fewer side effects. These processes, complemented with high-throughput screening protocols, combinatorial chemistry, computational chemistry, and bioinformatics, will result in compounds that are far more efficient than those currently being used in clinical practice [12,14]. For example, a phytochemical and simple structured molecule, curcumin, obtained from the dried rhizomes of *C. longa*, have antioxidant, antiproliferative, antiangiogenic, and antitumorigenic properties, but low potency and poor bioavailability does not qualify it as a drug. None the less, low-molecular weight, less toxicity, and significant antineoplastic activity of curcumin, makes this molecule a good natural lead compound for the collection of potential chemotherapeutic derivatives or analogues [9,13,15–17].

An analysis of the origin of the drugs developed between 1981 and 2002 showed that natural products or natural product-derived drugs comprised 28% of all new chemical entities (NCEs) launched onto the market. In addition, 24% of these NCEs were synthetic or natural mimic compounds, based on the study of pharmacophores related to natural products [18,19]. This combined percentage (52% of all NCEs) indicated natural products as important sources for new drugs and further are also good lead compounds suitable for further modification during drug development. Another study found that natural products or related substances accounted for 40%, 24%, and 26%, respectively, of the top 35 worldwide ethical drug sales from 2000, 2001, and 2002 [20]. Of these natural product-based drugs, paclitaxel (ranked at 25 in 2000), an anticancer drug, had sales of $1.6 billion in 2000. The sales of two categories of plant-derived cancer chemotherapeutic agents namely, the taxanes (paclitaxel and docetaxel) and the camptothecin derivatives (irinotecan and topotecan), were responsible for approximately one-third of the total anticancer drug sales worldwide in 2002 [21,22]. One of the primary reason for the success of natural product as drug molecule is that they have been elaborated within living systems, they are often perceived as showing more "drug-likeness and biological friendliness than totally synthetic molecules," making them good candidates for further drug development [23,24].

In fact, a recent analysis had showed that the uses of 80% of 122 plant-derived drugs were related to their original ethnopharmacological purposes [13]. One hundred and eighty-seven plant species, belonging to 102 genera and 61 families have been identified as an active or promising source of phytochemicals with antitumor properties. Among them, only 15 species (belonging to ten genera and nine families) are either active against cancer cell lines or exhibit chemotherapeutic properties on tumor-bearing animals under experimental conditions [9].

Although, many countries world over are effectively collaborating in this field, it has been felt that, there is a widening gap between natural product researchers in countries rich in biodiversity and drug discovery scientists immersed in proteomics and high-throughput screening. Despite annual increase in funding for drug discovery and development, a decrease in the introduction of number of new medicines to the world market, has been observed [18–22]. This raises questions about whether the previously most successful source of drugs (plant-based natural products) has any place in modern drug discovery. Are all the plant sources exhausted? Does this inspire us to look for alternative more promising sources?

Unfortunately, many of the "wonder drugs" generated over the past several decades have become less useful due to the development of drug resistance. Similarly, some forms of cancer have evolved multiple drug resistance, making virtually all drug treatments ineffective. Many pathogenic bacteria, once susceptible to antibiotics, have developed sophisticated biochemical methods to escape the effects of these drugs. Drug-resistant

mycobacterium, the pathogen that causes tuberculosis, for example, is almost totally resistant to the present arsenal of antibiotic drugs. Today, some infections are produced by bacteria that are immune to all known antibiotics. This has prompted researchers to look for newer areas having biodiversity with genetic uniqueness [23].

Despite advances in molecular biology and in computer-assisted drug design, nature still provides the foundation for the discovery of new medically important compounds. It is continually surprising us with new chemical structures having diverse and intricate unusual carbon skeleton with powerful cytotoxic properties (gymnastatin G from *Gymnascella dankaliensis* and hopeanol from *Hopea exalata*) [24]. The sponge-derived fungus *G. dankaliensis* is the source of several cytostatic metabolites, including gymnastatin G, which has an unusual bicyclo[3.3.1] nonane ring system [25]. The highly cytotoxic hopeanol, from the bark of *H. exalata*, has a new carbon skeleton [26]. Early investigations in this area, contributed greatly by doing the broad survey of marine life for novel natural products with useful biological properties; however, these initial efforts clearly prioritized description of unique structural chemistry rather than discovering drugs or drug leads. However, this important baseline information established the fact that oceans reserves are the goldmine of diverse, unusual and intricate molecules. Efforts became more serious and focused through a series of agency-supported programs, with the National Cooperative Drug Discovery Program of the National Cancer Institute (NCI) playing a key role. This inspired program recognized that the rich chemistry of marine organisms was not translating into useful drug leads largely because of poorly developed connections between academic researchers and the pharmaceutical industry. By forging collaborative interactions between groups of academic investigators and major pharmaceutical companies as well as the NCI, a critical mass of natural product materials, modern assays and development know-how could be assembled, which has translated into several clinical trial agents. This brief perspective is intended to showcase several marine natural products (MNPs) or derivatives which are advancing through anticancer clinical trials and which illustrate the success of modern academic–industry–government collaborations [27,28].

Marine Resource: A Wonder Reservoir

The marine resources are nowadays widely studied because of numerous reasons. One of the reason being vastness of the oceans, covering more than 70% of the world surface harboring 34 of the 36 known living phyla, with more than 300,000 known species of fauna and flora probably containing 80% of world's plant and animals species [29]. The rationale of searching for drugs from marine environment stems from the fact that marine plants and animals have adapted to all sorts of marine environments and these creatures are constantly under tremendous selection pressure including space competition,

predation, surface fouling, and reproduction. It covers a wide thermal, pressure, and nutrient ranges comprising extensive photic and nonphotic zones. This selection pressure has facilitated extensive specification at all phylogenetic levels, from microorganism to mammals. As a result of this intense competition, a high percentage of species have evolved chemical means by which to defend against predation, overgrowth by competing species, or conversely, to subdue motile prey species for ingestion. These chemical adaptations generally take the form of the so-called "secondary metabolites," and involve such well-known chemical classes as terpenoids, alkaloids, polyketides, peptides, shikimic acid derivatives, sugars, steroids, and a multitude of mixed biogenesis metabolites. In addition, and unique to the marine environment, is the relatively common utilization of covalently bound halogen atoms in secondary metabolites, mainly chlorine and bromine, presumably due to their ready availability in seawater.

In past few decades, approximately 300 patents on bioactive MNP have been issued. So far, more than 10,000 compounds have been isolated from marine organisms. Out of which over 6000 are structurally unprecedented. The chemical richness of the marine sources is so high that only during the past decade, over 4200 novel compounds have been isolated from shallow waters to 900-m depths of the sea from various marine animals like tunicates, sponges, soft corals, bryozoans, sea slugs, and marine organisms marine samples [30–32].

Marine Chemical Weapons for Human Welfare

To understand the link between marine chemical warfare and human health, it is crucial to study chemical ecology in the oceans. Many sessile invertebrates such as sponges, corals, and tunicates feed by filtering seawater. Since, seawater contains high concentrations of bacteria; these organisms produce antibiotics to defend themselves from potentially harmful microorganisms. Thus, the production of antibacterial compounds by filter feeders such as sponges provides a possible link between chemical defense for sponges and antibiotics for use in humans. However, why should a sponge produce anticancer drugs or why a coral should produce a compound useful in the treatment of arthritis? In the scenario of two encrusting sponges growing together, the sponge that will win the race of competition for space is the one that produces the chemical most effective at killing the rapidly dividing cells of the neighboring sponge. The ability of chemical to kill rapidly dividing cells is the hallmark of chemotherapy. Anticancer drugs often act by killing the rapidly dividing cells of a tumor but generally do not harm "normal" healthy cells. These ideas provide a connection between marine chemical warfare and the possible application of MNPs in medicine. Studying chemical ecology of marine organisms by exploring these secondary metabolites will help to develop drugs to treat various life threatening diseases. Natural product released into the water is rapidly diluted and therefore need to be highly potent to have any effect. For this reason, some

of these molecules are highly toxic (up to the nanomolars levels). Because of the immense biological diversity in the sea, as a whole, chemical entities exist in the ocean with biological activities that may be useful in the quest of finding drugs and molecular targets for drugs with greater efficacy and specificity for the treatment of many human diseases [33,34].

MARINE MACROORGANISMS

Sponges

Sponges are often studied because of their wealth of metabolites, which display biological activity. So far, an estimated 15,000 species have been described, but a higher diversity is expected in the oceanic reservoir, particularly the tropical sponges known for their colorful appearances and their morphological plasticity, ranging in size from a few mm to >1 m. Sponges are diploblastic metazoans that lack true tissues or organs. In spite of their simple organization, genome sequencing has revealed genes encoding function that are highly homologous to those of their vertebrate analogues.

Marine sponges belonging to the genus *Ircinia* are known to be a very rich source of terpenoids, several of which have shown a wide variety of biological activities. Variabilins, which are polyprenyl-hydroquinones, possess analgesic, and anti-inflammatory properties. Among the halogenated alkaloids, bromoalkaloids, form the most widely distributed group of natural compounds, in marine sponges, are significantly rarer in prokaryotic microplants and animals [35].

Components of marine sponges are known to modulate various biological activities and have anti-inflammatory, antifungal, and anticancer effects. These *in vitro* activities imply that marine products may be potential therapeutic agents. Polyacetylenic alcohols, including (35,145)-petrocortyre A, purified from the marine sponge *Petrosia* sp., are biologically active lipid compound, having similar structure to a long carbon chain compounds such as sphingolipids, possess cytotoxic activity against a small panel of human solid tumor cell liner by inhibiting DNA replication [36].

Tunicates

The Urochordata (Tunicata) are commonly known as "sea squirts." They are all sessile as adults and possess tunic attached to the substrate by a small holdfast and standing upright. It has two openings, an inhalant siphon and an exhalent siphon. The blood of tunicates is normally clear and often contains extremely high quantities of vanadium, a rare element normally occurring in very small quantities in seawater its presence in the organism in such high quantity is still a mystery [37,38].

Many of them are known to be a rich source of chemically diverse secondary metabolites with remarkable biological activities. They often exhibit

potent anticancer activities, so they are considered unusual cytotoxic metabolites. Perhaps, this property has limited the antimalarial potential of the pyridoacridones, isolated from *Cystodytes dellechiajei*, and of bistramides, isolated from *Lissoclinum bistreatum*, as they possessed very narrow therapeutic indices [39].

Iejimalides obtained from a marine tunicate *Eudistomacf rigida*, are unique 24-membered polyene macrolides having two methoxy groxy, four dienes units, and an *N*-formyl-L-serine terminus exhibiting potent cytotoxic activity *in vitro*. Aromatic alkaloids possessing polysulfide structures have been isolated from ascidians of the genera Lissoclinum, Eudistoma, and Polycitor. These compounds have shown various biological activities like antifungal, antibacterial, cytotoxicity, antimalarial activity, and inhibition of protein kinase C [40].

Seaweeds

The term seaweed refers to the large marine alga that grows almost exclusively in the shallow waters at the edge of the world's oceans. They provide home and food for many different sea animals. Seaweed draws an extraordinary wealth of mineral elements from the sea which includes sodium, calcium, magnesium, potassium, chlorine, sulfur, and phosphorus; the micronutrients include iodine, iron, zinc, copper, selenium, molybdenum, fluoride, manganese, boron, nickel, and cobalt. It also contains several vitamins like carotenes (provitamin A); vitamin C, B_{12} along with higher proportion of essential fatty acids than land plants. Seaweeds provide a rich source of structurally diverse secondary metabolites which includes terpenes, acetogenins, alkaloids, and polyphenolics, with many of these compounds being halogenated. The functions of these secondary metabolites are defense against herbivores, fouling organisms, and pathogens; they also play a role in reproduction, protection from UV radiation and as allelopathic agents. Chemical defense mechanisms that inhibit biofilm development are a common occurrence in seaweeds, with many secondary metabolites produced by seaweeds having bacteriocidal or bacteriostatic properties. Physical stress such as desiccation, UV and visible light, and nutrient availability can alter the secondary metabolites in seaweeds. Some of the active algal specimens are *Laminaria angustata* var. langissima, *L. japonica*, *L. japonica* var. *ochotencs*, *Ecklonia cava* and *Eisenia bicyclis*, and the green seaweed *Monostroma nitidum* [41–43]. The number and diversity of studies related to toxicity of marine algae are high. The first report on toxicity research says that the biological activity of the compound caulerpicine, isolated from caulerpa species was found to be toxic to mice.

An antitumor compound was isolated from *Portieria hornemannii* and *Ulva fasciata* produces a novel sphingosine derivative which has been found to have antiviral activity *in vivo*. A cytotoxic metabolite, stypoldione, which inhibits microtubule polymerization and thereby preventing mitotic spindle

formation, has been isolated from tropical brown alga, *Stypodium zonale*. *P. hornemannii* was found to be a novel source of cytotoxic pentahalogenated monoterpene, halomon, which exhibited one of the most extreme of differential cytotoxicity in the screening conducted by the NCI, USA. Haloman has been selected for preclinical drug development since this compound shows toxicity to brain, renal, and colon tumor cell lines and preliminary *in vivo* evaluations have been encouraging. An iodinated novel nucleoside has been isolated from *Hyprea volitiae*, which is a potent and specific inhibitor of adenosine kinase [44–46].

MARINE MICROORGANISM

Whereas the oceans are vast and constitute three-fourth of the world's surface, the majority of this species diversity is found in the ocean fringe. This slender land–sea interface with its high concentration of species is among the most biodiverse and productive environments on the planet. Deep ocean thermal vent communities represent another highly biodiverse and productive habitat, albeit to limited extent. By contrast, open ocean waters are generally low in nutrients and have been likened to deserts in terms of biomass and species diversity, although recent evidence suggests the existence of substantial microbial diversity in pelagic waters [28]. It is estimated that $<1\%$ of the earth's surface, the narrow ocean fringe, and the known deep sea vent communities, are home to a majority of the world's species, and thus constitute the most species rich and biologically productive regions of the world. The intense concentration of species coexisting in these limited extent habitats necessarily makes them highly competitive and complex.

Marine bacteria constitute $\sim 10\%$ of the living biomass carbon of the biosphere and they represent dramatically different environment than their terrestrial counterpart. These bacteria originate mainly in sediments but are also found in open oceans and in association with the marine organisms. It was surprising to find that many bioactive compounds, reported from marine invertebrates are produced by their microbial symbionts. Competition among microbes for space and nutrients in the marine environment is the driving force behind the production of such precious antibiotics and other useful pharmaceuticals. Interestingly, microorganisms associated with marine invertebrates are proved valuable candidates for drug discovery program. Vast resources enriched with chemicals, the marine florae are largely unexplored for anticancer lead compounds. Obligate marine fungi are still an unexplored resource, although marine facultative fungi, have been studied due to their production of new metabolites which are not found in terrestrial fungi. Recently, more interest has been generated on studying biologically active metabolites from higher fungi (Basidiomycetes), endophytic fungi, and filamentous fungi from marine habitats and the symbiotic lichens. Sorbicilactone-A, novel alkaloids

was reported from sponge (*Ircinia fasciculata*) associated fungus, *Penicillium chrysogenum* [47–51].

SYMBIONTS

Microorganisms associated with marine invertebrates are reported to be involved in the production of bioactive molecules. Though these bioactive compounds may be important for epibiotic defense of marine invertebrate hosts, they also have significant medical and industrial applications.

Marine sponges and the microbes residing within them are important from ecological viewpoint also. Sponges are important members of shallow and deepwater reef communication with nutrition supplied by photosynthetic symbionts often allowing them to compete with other benthic organisms such as corals. In some cases, the active metabolites are produced by the microbes, rather than the sponge itself. Sponges and their associated microorganisms are therefore receiving much attention from pharmaceutical companies. Convincing evidence for the involvement of microorganisms in natural product synthesis has been compiled impressively for the tropical sponges *Dysidea herbacea* and *Theonella swinhoei*, in which the producing microbe is a cyanobacterium in the former and a bacterium in the latter [52–54].

Sponges harbor a rich diversity of microorganism in their tissues and in some case constitute up to 40% of the biomass, for example, the Mediterranean sponge *Aplysina aerophoba*. Sponge-associated bacteria are capable of producing antibacterial metabolites. Surface-associated bacteria with sponge *Ircinia ramosa* is one such example. Several bacteria activated from tunicate have yielded natural products, for example, andrimid and the moramides isolated from a *Pseudomonas fluorescens* strain and it has been proved that it synthesized by a tunicate—associated *Enterococcus faecium*.

The epibiotic bacteria in seaweed play a protective role by releasing secondary metabolites into the surrounding seawater that help in preventing extensive fouling of the surface. Epibiotic bacteria are therefore attracting attention as a source of new natural products. The proportion of active bacteria associated with marine invertebrates (20%) and seaweeds (11%) is higher than that isolated from seawater (7%) and sediments (5%) [55–60]. The exploitation of marine actinomycetes as a source for novel secondary metabolites production is in its infancy. There is a tremendous potential for the isolation of novel secondary metabolites from marine actinomycetes. In this respect, future success relies upon our ability to isolate novel actinomycetes from marine environments.

Pharmacological investigations of marine organisms are relatively new and have been founded on the establishment of unprecedented "scientific bridges" between the marine and pharmaceutical sciences. Today, approximately one-half of all cancer drug discovery focuses on marine organisms, and forecasts for the future are bright. During the late 1970s, early investigators demonstrated, unequivocally, that marine plants and animals were genetically and biochemically unique. NCI took the initiative in a big way in 1985

and began a new program in which extracts from plants, animals and microorganisms (increasingly, those of marine origin) were screened against a panel of 60 human cancer cell lines, including those from solid tumors (lung, colon, skin, kidney, ovary, brain, breast, and prostate), as well as several leukemias. Since then in the past 30 years, more than 300 patents have been issued covering potential anticancer agents from the sea. Despite having two major limitations: Collection of material from an often hostile environments and lower concentrations of metabolite that are present in the organisms. For example, a 1000-kg sample of wild (and still damp) *B. neritina* from the waters off Southern California might yield 1.5 g of bryostatin, and perhaps 2 g of other members of the family [61].

The promising anticancer clinical candidates like salinosporamide A and bryostatin only hint at the incredible wealth of drug leads hidden just beneath the ocean surface. Salinosporamide A is isolated from marine bacteria that is currently in several phase I clinical trials for the treatment of drug-resistant multiple myelomas and three other types of cancers [62].

Marine Pharmacology

Marine ecosystems have biological diversity higher than in tropical rain forests. They are taxonomically diverse, largely productive, biologically active, and chemically unique offering a great scope for discovery of new anticancer drugs. Research has revealed the active components in algal polysaccharides are mainly sulfated ones which can enhance the innate immune response by promoting the tumoricidal activities of macrophages and natural killer cells.

Marine drug discovery programs have been applied to selected, difficult to treat diseases that have eluded cures for decades. New drug leads have been identified with potent immunosuppressant, anti-inflammatory properties, and with significant anticancer potential. Although most of these compounds have not been suitable for drug development, they have already found utility as "pharmacological probes" providing selective pharmacological properties that can be used to explore the foundations of disease. For example, cone snails inject incredibly potent peptide toxins (the conotoxins) to immobilize prey fish [63]. Lionfish spines carry lethal protein venom to the unwary. Zooanthids from a tide pool in Oahu, HI possess an extraordinarily toxic polyketide, "palytoxin" making them unpalatable to potential predators [64]. Microalgae produce incredibly potent alkaloidal neurotoxins such as saxitoxin and polyketide neurotoxins such as the brevetoxins [65].

Recent technological and methodological advances in structure elucidation, organic synthesis, and biological assays have resulted in the isolation and clinical evaluation of various novel anticancer agents. These compounds range in structural class from simple linear peptides, such as dolastatin 10, to complex macrocyclic polyethers, such as halichondrin B, equally as diverse

are the molecular modes of action by which these molecules impart their biological activity [66].

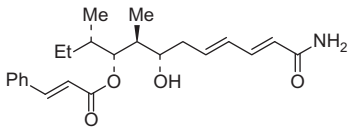
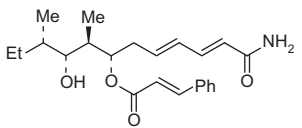
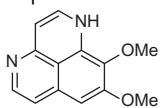
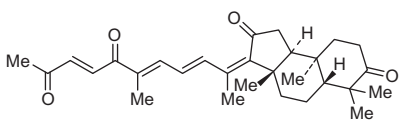
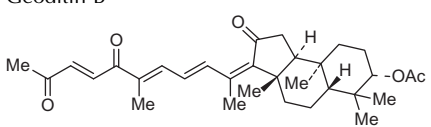
An exciting "marine pipeline" of new anticancer clinical and preclinical agents has emerged from intense efforts over the past decade to more effectively explore the rich chemical diversity offered by marine life (Table 1). However, unlike the long-standing historical medical uses of terrestrial plants, marine organisms have a shorter history of utilization in the treatment and/or prevention of human disease.

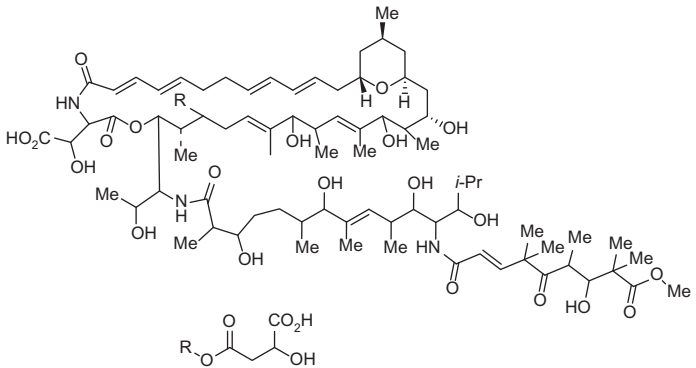
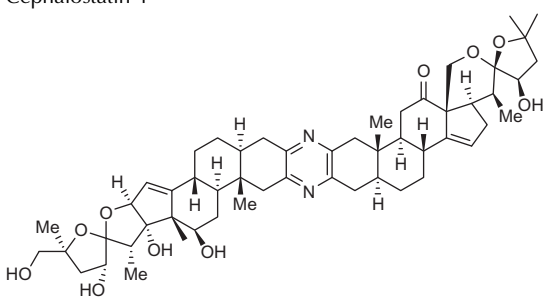
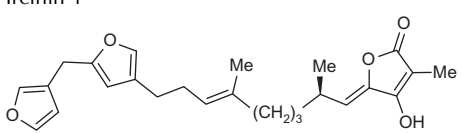
Induction of apoptosis is one of the active strategies to arrest proliferation of cancer cells. The apoptosis-inducing effect of plant extracts may be attributed to upregulated immune surveillance, increased macrophage, and activations of death-inducing signal complex. Many chemopreventive agents exert their anticarcinogenic effects by inducing apoptosis. Radiation and chemical agents like tamoxifen, capable of inducing apoptosis, have been used to treat cancer [199–201]. Research has shown marine phytochemicals also can activate the macrophages and induce apoptosis. Fucoidan from *Laminaria japonica* can restore the immune functions of immunosuppressed mice, and it is an immunomodulator acting directly on macrophage and T lymphocyte. It can also promote the recovery of immunologic function in irradiated rats [202].

Among the first bioactive compounds from marine sources, spongouridine and spongothymidine from the Caribbean sponge (*Cryptotheca crypta*), were isolated serendipitously in the early 1950s [203]. They were approved as an anticancer drug (cytosine arabinoside, Ara-C) and an antiviral drug (adenine arabinoside, Ara-A), respectively, 15 years later [204]. The secondary metabolites of marine organisms have been studied extensively over the past 30 years, since a small number of academic chemists began to isolate and elucidate novel compounds from marine sources in the 1970s. Drug discovery research from marine organisms has been accelerating and it now involves interdisciplinary research including biochemistry, biology, ecology, organic chemistry, and pharmacology [205,206]. Consequently, a compound based on new chemical template has been developed and launched in 2004, while numerous other candidates are in clinical trials (Table 2) [202,203,207].

Didemnin B, isolated from a tunicate over 20 years ago, was the first MNP to enter human clinical trials against cancer and led the way for a plethora of drug candidates isolated from marine organisms. The supply of marine metabolites being tested in the clinical trials is currently provided by several means: open aquaculture of the invertebrates (ET-743 and bryostatin), total synthesis (ziconotide, discodermolide, dolastatin 10, dehydrodidemnin, hemiasterlin), semisynthesis (halichondrin B derivative, ET-743), and fermentation of producing microbes (thiocoraline) [208]. In general, fermentation has been the most successful method for production of natural products (penicillin, clavulanic acid, erythromycin, etc.), especially if the compounds proceed through clinical trials and are needed on a commercial scale (>kg) [12].

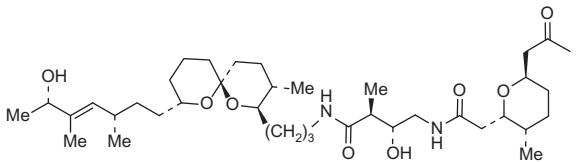
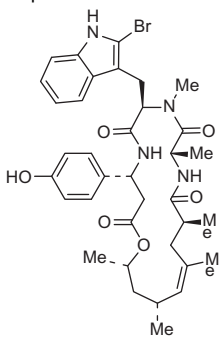
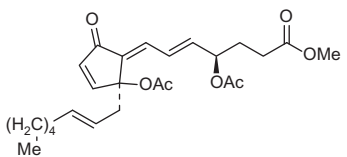
TABLE 1 Marine Anticancer Clinical and Preclinical Agents

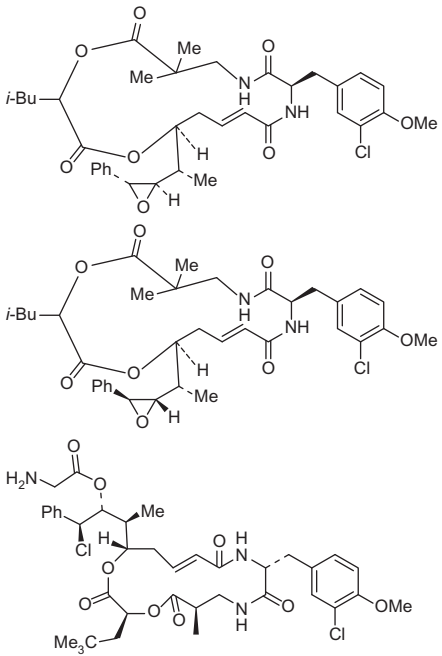
No.	Source of Organism	Compounds	Chemical Class	Molecular Target	References
1	*Bacillus laterosporus*	Basiliskamides A  Basiliskamides B 	Cyclic decapeptide antibiotics Polyketide	Unknown	[67]
2	*Aaptos aaptos* Sponge	Aaptamine 	Alkaloid	HU osteosarcoma cell line	[68]
3	*Geodia japonica* Sponge	Geoditin A  Geoditin B 	Triterpene	HU leukemia cell line	[69]

4	*Chondropsis* sp. Sponge	Chondropsin A 	Macrolide	NCI 60 tumor cell line panel	[70]
5	*Cephalodiscus gilchristi* Tube worm	Cephalostatin 1 	Steroid	HU Jurkat T cell line HU Jurkat T cell line	[71]
6	*Sarcotragus* sp. Sponge	Ircinin-1 	Sesterterpene	HU melanoma cell line	[72]

Continued

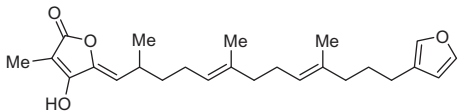
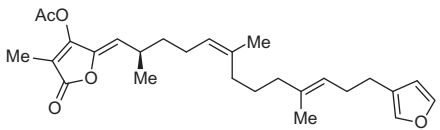
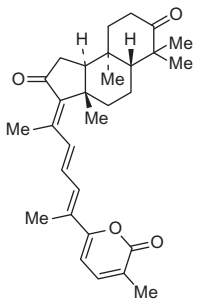
TABLE 1 Marine Anticancer Clinical and Preclinical Agents—Cont'd

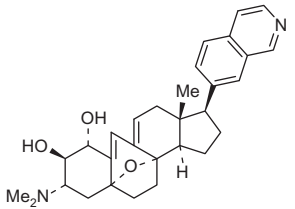
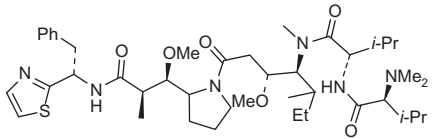
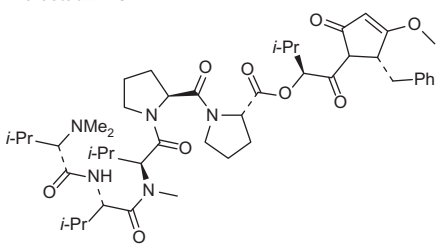
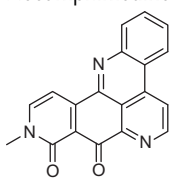
No.	Source of Organism	Compounds	Chemical Class	Molecular Target	References
7	*Tridemnum cyclops* Ascidian	Bistramide A 	Polyketide		[73,74]
8	*Jaspis splendens* Sponge	Jasplakinolide 	Depsipeptide	HU breast and lung cancer cell lines	[75]
9	*Clavularia viridis* Soft coral	Clavulone II 	Prostanoid	HU leukemia cell line	[76]

10	*Cyanobacterium Nostoc* sp. Bacterium	Cryptophycins 52, 53, and 309 	Depsipeptide	HU and MU glutathione metabolism	[77]
11	*Haplosclerid* Sponge	Alkylpyridinium salts	Alkaloid	HU adenocarcinoma cell lines	[78]

Continued

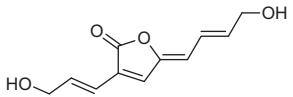
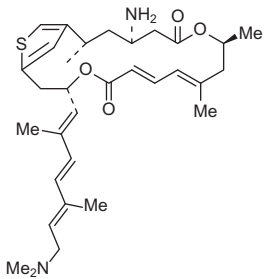
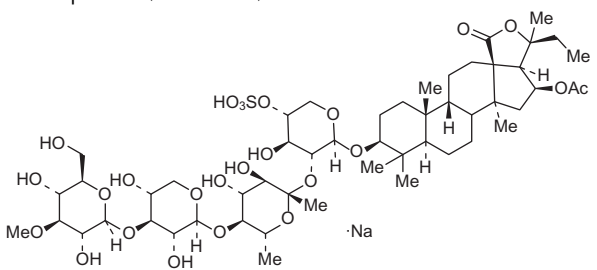
TABLE 1 Marine Anticancer Clinical and Preclinical Agents—Cont'd

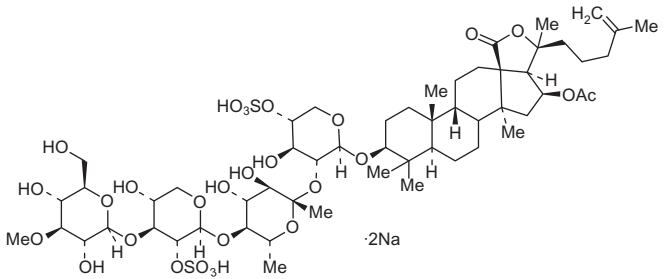
No.	Source of Organism	Compounds	Chemical Class	Molecular Target	References
12	*Ircinia strobilina* Sponge	Strobilinin  Felixinin 	Sesterterpene	HU tumor cell line	[79]
13	*Geodia japonica* Sponge	Stellettin A 	Triterpene	HU leukemia cell line	[80]

14	*Corticium simplex* Sponge	Cortistatin A 	Alkaloid	HU normal and tumor cell lines	[81]
15	Sea hares *Dolabella auricularia*/ *Symploca* sp. Mollusk/ cyanobacterium	Dolastatin 10 and  Dolastatin 15 	Simple linear peptides	Tubulin (Dolastatin 10; withdrawn from antitumor clinical trials due to no significant activity was seen in a phase II trial against metastatic melanoma)	[82]
16	*Xestospongia* cf. *carbonaria* Sponge	Neoamphimedine 	Alkaloid	HA and HU tumor cell lines	[83]

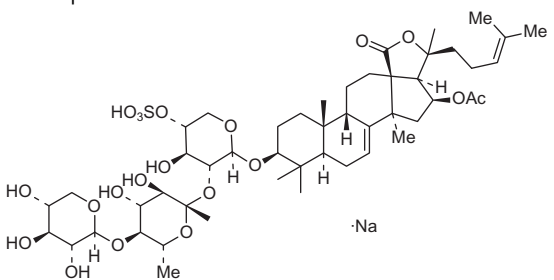
Continued

TABLE 1 Marine Anticancer Clinical and Preclinical Agents—Cont'd

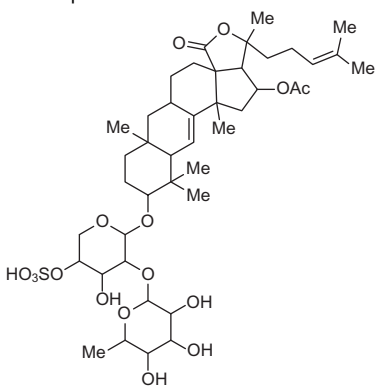
No.	Source of Organism	Compounds	Chemical Class	Molecular Target	References
17	*Lissoclinum tunicate*	Lissoclinolide 	Fatty acid	NCI 60 tumor cell line panel	[84]
18	*Mycale* sp. Sponge	Pateamine A 	Macrolide	HU T cell leukemia	[85]
19	*Pentacta quadrangularis* Sea cucumber	Philinopside A (sodium salt)  Philinopside B	Saponin	HU adenocarcinoma cell lines	[86]



Philinopside C



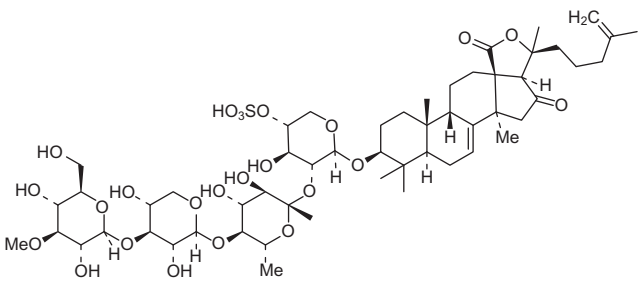
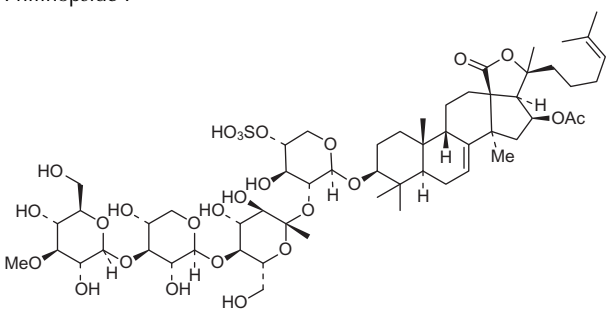
Philinopside D

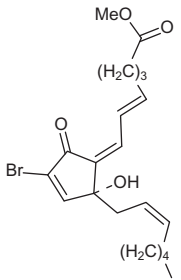
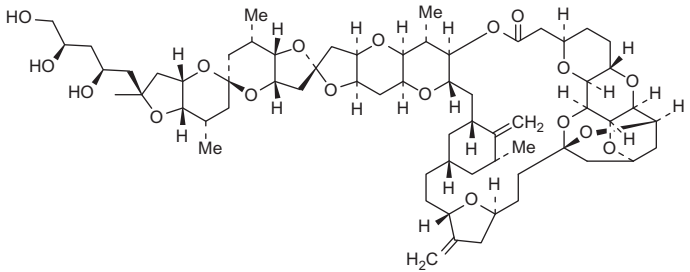
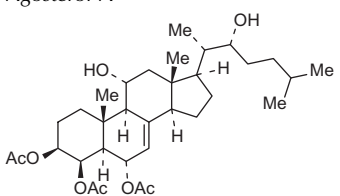


Philinopside E

Continued

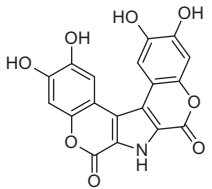
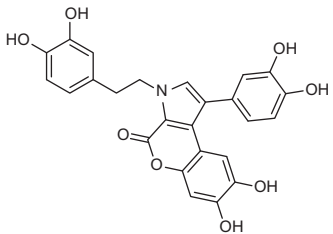
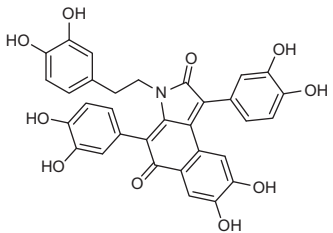
TABLE 1 Marine Anticancer Clinical and Preclinical Agents—Cont'd

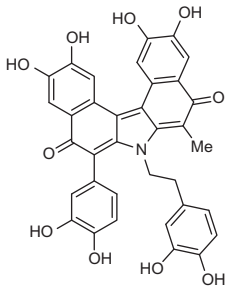
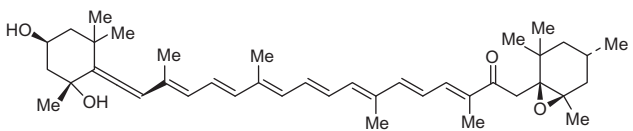
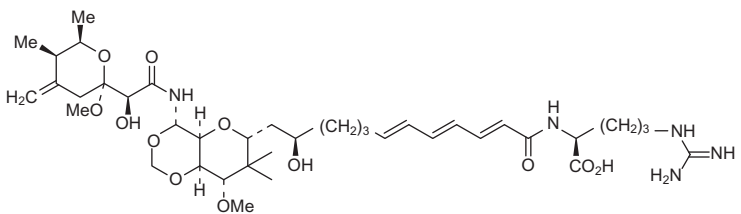
No.	Source of Organism	Compounds	Chemical Class	Molecular Target	References
		 Philinopside F			
					

20	*Clavularia viridis* Soft coral	Bromovulone III 	Prostanoid	HU hepatocarcinoma cell line Hormone-resistant prostate cancer cell line	[87,88]
21	*Lissodermis* sp. Sponge/synthetic analogue E7389	Halichondrin B 	Complex macrocyclic polyethers	HU tumor cell lines and molecular modeling	[69]
22	*Acanthodendrilla* sp. Sponge	Agosterol A 	Steroid	HU epidermoid carcinoma cell sublines Insect cell expression of MRP1 wild type or mutants	[89]

Continued

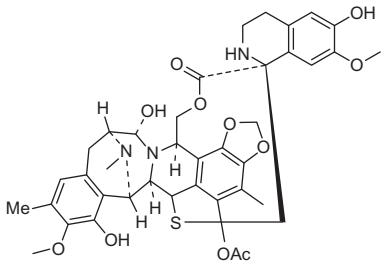
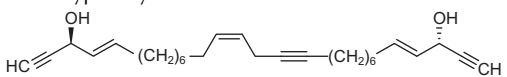
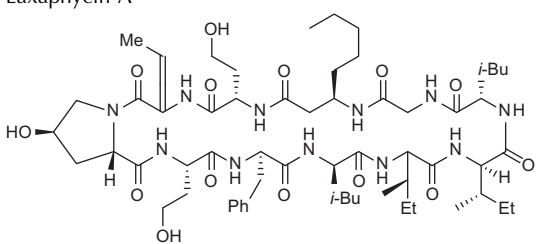
TABLE 1 Marine Anticancer Clinical and Preclinical Agents—Cont'd

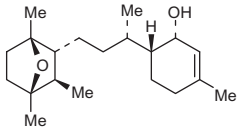
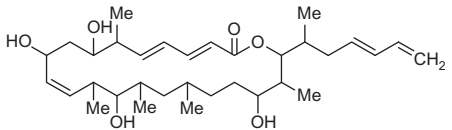
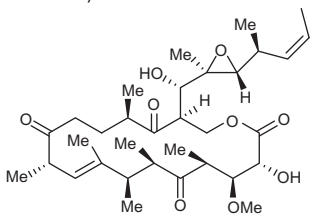
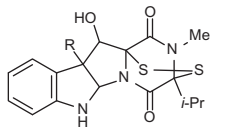
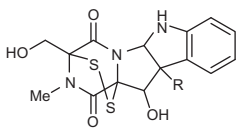
No.	Source of Organism	Compounds	Chemical Class	Molecular Target	References
23	*Iotrochota baculifera* Ascidian/ synthetic	Ningalin A  B  C 	Alkaloid	HU leukemia and breast cancer cell lines	[90]

		D 			
24	*Cladosiphon okamuranus* and other brown seaweed	Fucoxanthinol 	Carotenoid	HU leukemia, breast, and colon tumor cell lines	[91]
25	*Theonella* sp. Sponge	Onnamide A 	Polyketide	HU leukemia cell line	[92]

Continued

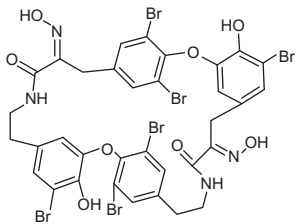
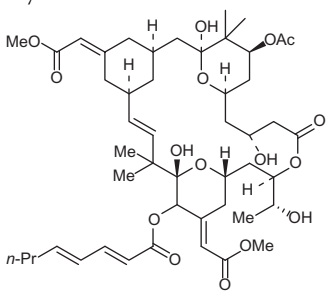
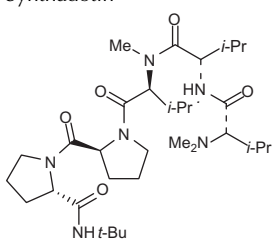
TABLE 1 Marine Anticancer Clinical and Preclinical Agents—Cont'd

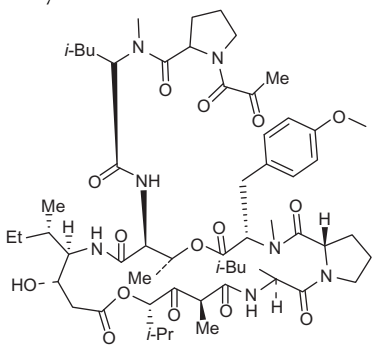
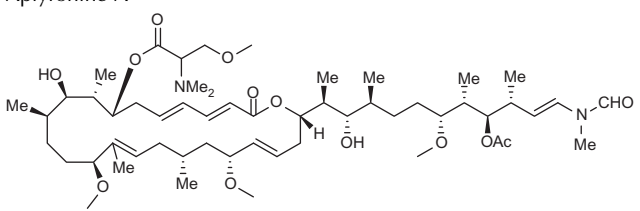
No.	Source of Organism	Compounds	Chemical Class	Molecular Target	References
26	*Ecteinascidia turbinate* Tunicate; possible bacterial source	Ecteinascidin 743 	Tetrahydro-isoquinolone alkaloid	HU melanoma cell lines NIH 3T3 fibroblasts and HU	[93]
27	*Petrosia* sp. Sponge	Dideoxypetrosynol A 	Polyacetylene fatty acid	HU monocytic leukemia	[94]
28	*Lyngbya majuscula* Bacterium	Laxaphycin A 	Cyclic peptides	HU lymphoblastic cell lines	[95]

29	*Laurencia intricata* Alga	Laurenditerpenol 	Diterpene	Breast tumor cell-based reporter assay	[96]
30	*Spongia* sp. Sponge	Dictyostatin-1 	Macrolide	HU ovarian adenocarcinoma cell lines	[97]
31	*M. adhaerens* Sponge	13-Deoxytedanolide 	Macrolide	HU ovarian adenocarcinoma cell lines	[98]
32	*Leptoshaeria* sp. Fungus	Leptosins C  	Alkaloid	HU lymphoblastoid cell line	[99]

Continued

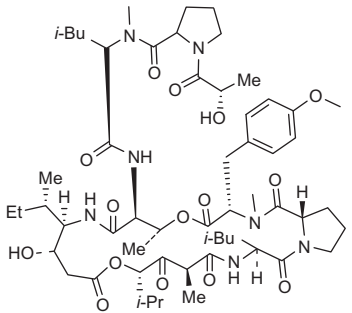
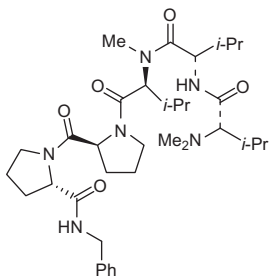
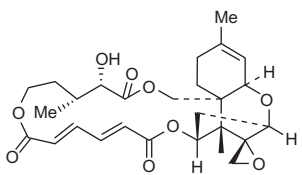
TABLE 1 Marine Anticancer Clinical and Preclinical Agents—Cont'd

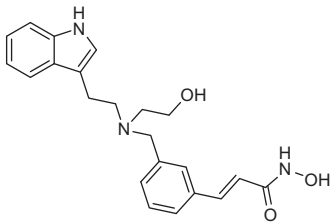
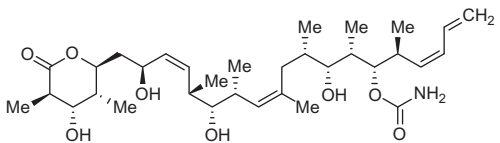
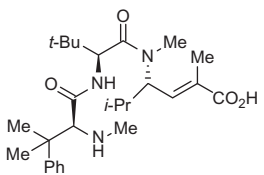
No.	Source of Organism	Compounds	Chemical Class	Molecular Target	References
33	*Ianthella basta* Sponge	Bastadin 6 	Alkaloid	Assessment of DNA binding	[100]
34	*Bugula neritina* Bryozoan	Bryostatin 1 	Macrocyclic lactone	HU prostate cancer cell lines HU cervical carcinoma cells HU monocytic cell lines MU keratinocytes HU Jurkat T cell line	[101–104]
35	*Dolabella auricularia*/ *Symploca* sp. Synthetic analogue	Synthadotin 	Linear peptide	Tubulin	[105]

36	*Tridemnum solidum* Tunicate, synthetic; possible bacterial/ cyanobacterial source	Dehydrodidemnin B 	Cyclic depsipeptide	HU adenocarcinoma and colon carcinoma cell lines HUVECs, HU ovarian carcinoma, and angiogenesis assay MU fibroblast cell line HU thyroid cancer cells Fas-positive and deficient HU leukemia cell line	[106]
37	*Aplysia kurodai* Sea hare	Aplyronine A 	Macrolide	Tumor cell line HeLa S3 P388 leukemia	[107]

Continued

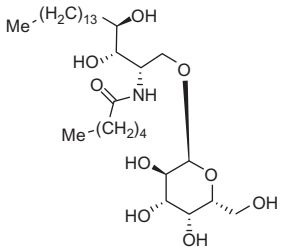
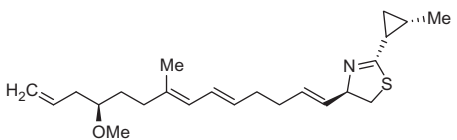
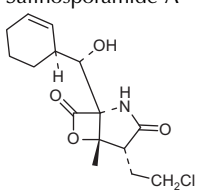
TABLE 1 Marine Anticancer Clinical and Preclinical Agents—Cont'd

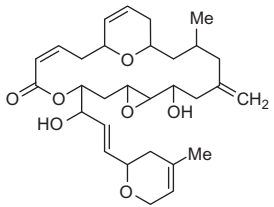
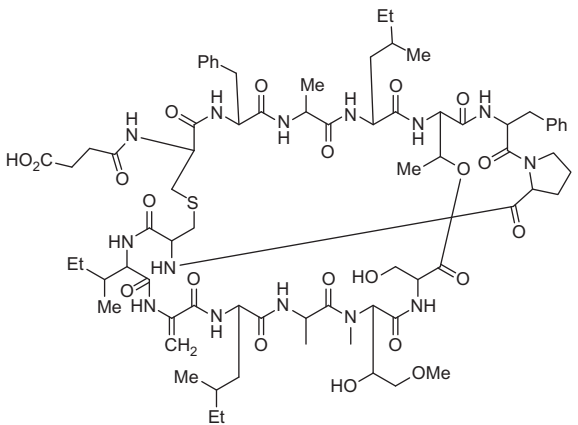
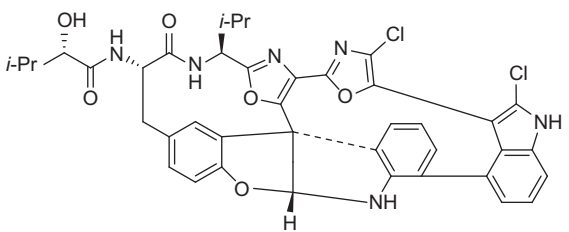
No.	Source of Organism	Compounds	Chemical Class	Molecular Target	References
38	*Tridemnum solidum* Tunicate	Didemnin B 	Cyclic depsipeptide	FK-506 bp Molecular dynamics simulations	[108] (first marine natural product entered in to clinical trial as an antitumor agent but discontinue in IInd phase)
39	*Dolabella auricularia/ Symploca* sp. Synthetic analogue	Cemadotin; LU103793, (dolastatin 15 derivative) 	Linear peptide	Inhibition of tubulin polymerization	[82]
40	*Myrothecium roridum*	Verrucarin A 	Macrolide	HU leukemia cell lines	[109]

41	*Psammaplysilla* sp. Sponge, synthetic	NVP-LAQ824 (Psammaplin derivative) 	Indolic cinnamyl hydroxamate	Human fibroblast cell line	[110]
42	*Discodermia dissoluta* Caribbean sponge	Discodermolide 	Polyhydroxylated lactone	Direct photoaffinity labeling HU tumor cell lines HU lung, colon, breast, and cervical carcinoma	[111–113]
43	*Cymbastella* sp. Synthetic analogue of sponge metabolite	HTI-286; (Hemiasterlin derivative) 	Linear peptide	Molecular docking experiments	[114]

Continued

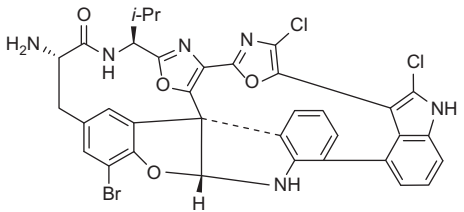
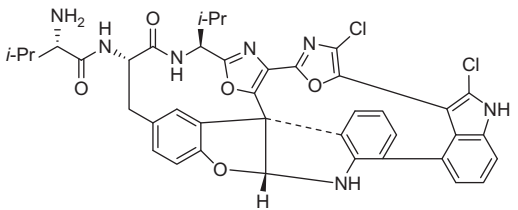
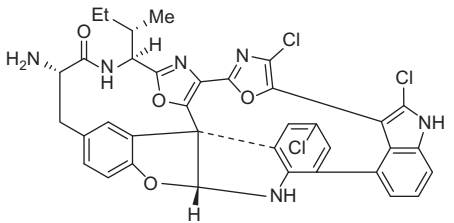
TABLE 1 Marine Anticancer Clinical and Preclinical Agents—Cont'd

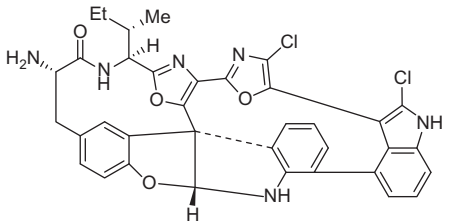
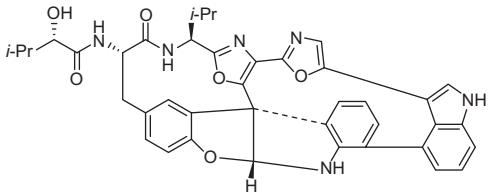
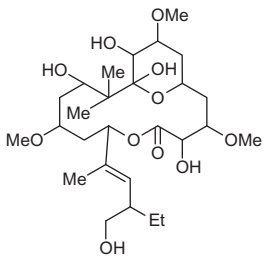
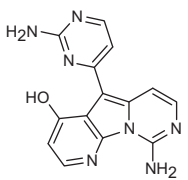
No.	Source of Organism	Compounds	Chemical Class	Molecular Target	References
44	*Agelas mauritianus* Sponge, synthetic	KRN-7000  (Agelasphin derivative)	a-Galacosylceramide	Melanoma B16 hepatic metastasis	[115]
45	*Lyngbya majuscula* Cyanobacterium	Curacin A 	Thiazole lipid	MCF-7 human breast carcinoma cells	[116]
46	*Salinospora* sp. Bacterium	Salinosporamide A 	Bicyclic γ-lactam-β-lactone	HU tumor cell lines	[117]

47	*Cacospongia mycofijiensis* Sponge	Laulimalide 	Macrolide	Microtubule-binding agents	[118]
48	*Didemnin cucliferum/ Polysyncration lithostrotum* Tunicates	Vitilevuamide 	Cyclic peptide	Tubulin interactive agent	[119]
49	*Diazona angulata* Tunicate	Diazonamide A 	Cyclic peptide	HU breast, prostate and lung tumor cell lines	[120]

Continued

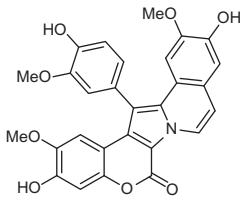
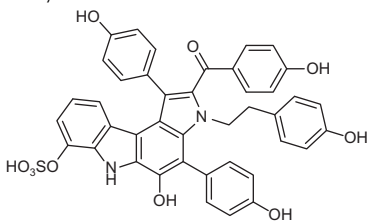
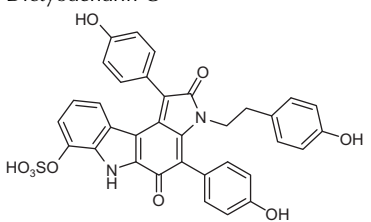
TABLE 1 Marine Anticancer Clinical and Preclinical Agents—Cont'd

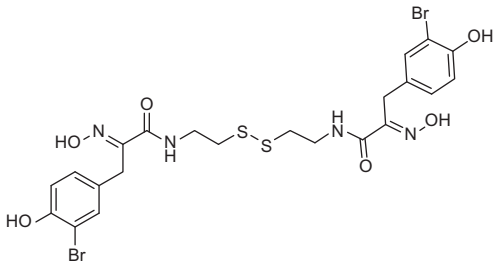
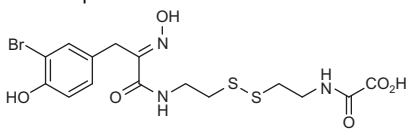
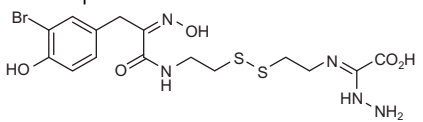
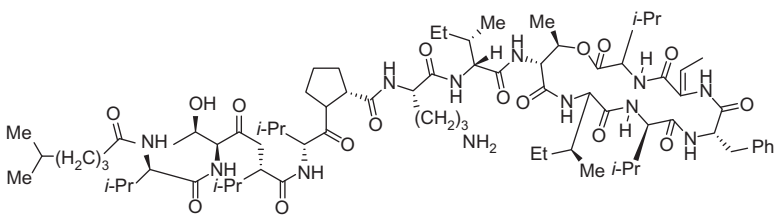
No.	Source of Organism	Compounds	Chemical Class	Molecular Target	References
		Diazonamide B 			
		Diazonamide C 			
		Diazonamide D 			

		Diazonamide E  Diazonamide AB 			
50	*Mycale hentscheli* Sponge	Peloruside A 	Macrocyclic lactone	HA and HU tumor cell line	[121]
51	*Kirkpatrickia variolosa* Sponge	Variolin B 	Heterocyclic alkaloid	HU colon, leukemia, and ovarian cell lines	[122]

Continued

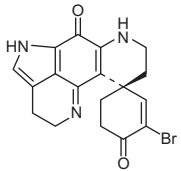
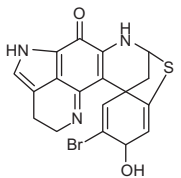
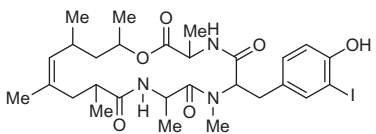
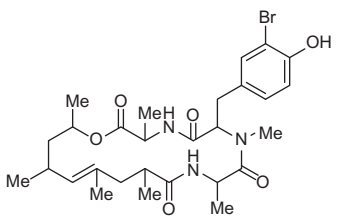
TABLE 1 Marine Anticancer Clinical and Preclinical Agents—Cont'd

No.	Source of Organism	Compounds	Chemical Class	Molecular Target	References
52	*Lamellaria* sp. Mollusk and various soft corals	Lamellarin D 	Pyrrole alkaloid	HU and MU tumor cell lines Topoisomerase I/ mitochondria	[123]
53	*Dictyodendrilla verongiformis* Sponge	Dictyodendrin B  Dictyodendrin C 	Pyrrolocarbazole derivatives	First telomerase inhibitors of marine origin	[124]

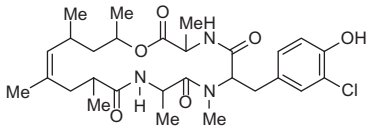
54	Various verongid sponges (e.g., *Psammaplysilla* sp.)	Psammaplin A  Psammaplin F  Psammaplin G 	Symmetrical bromotyrosine disulfide Degraded cysteine dimer	Fibroblast cell line L-929 cytotoxic activity against the RAW264.7	[110]
55	Sacoglossan mollusk *Elysia rufescens* following grazing by the mollusk on a green macroalga and *Bryopsis* sp. also occurs in the alga	Kahalalide F 	Cyclic depsipeptide	Selectivity for tumor cells with high lysosomal activity such as prostate tumors	[75,125]

Continued

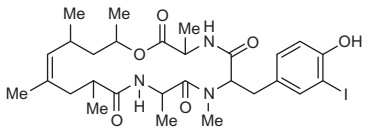
TABLE 1 Marine Anticancer Clinical and Preclinical Agents—Cont'd

No.	Source of Organism	Compounds	Chemical Class	Molecular Target	References
56	*Latrunculia* sp. Alaskan sponge	Discorhabdins (discorhabdin Y)  and dihydrodiscorhabdin B 	A new class of pyrroloiminoquinone alkaloids	Cytotoxicity against P388 and A549 cell lines	[126]
57	*Cymbastela* sp. Sponge	Geodiamolides A–F, hemiasterlin (hemiasterlins A and B), and other geodiamolides and criamides) A  B 	Peptides	HU breast cancer cell lines	[127]

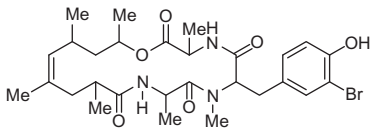
C



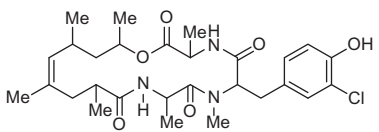
D

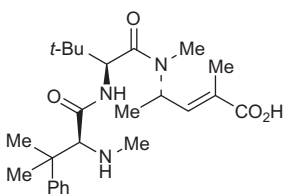


E



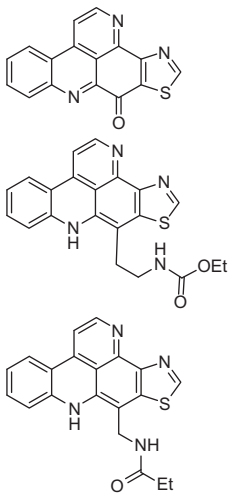
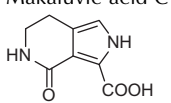
F

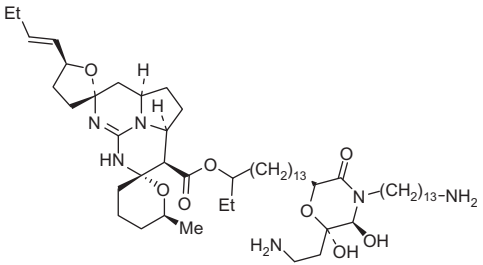
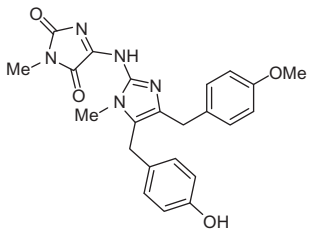
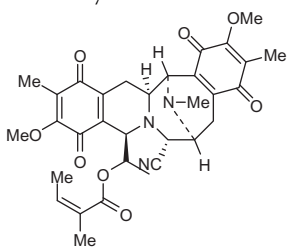


58	*Hemiasterella minor* Sponge	HTI-286  Hemiasterlin also contained jaspamide and geodiamolide	Tripeptide	Molecular docking experiments	[114]

Continued

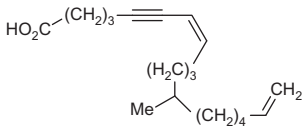
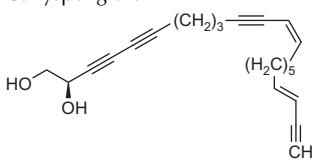
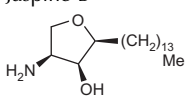
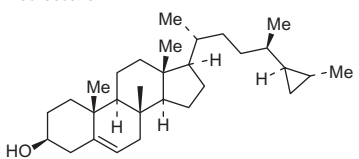
TABLE 1 Marine Anticancer Clinical and Preclinical Agents—Cont'd

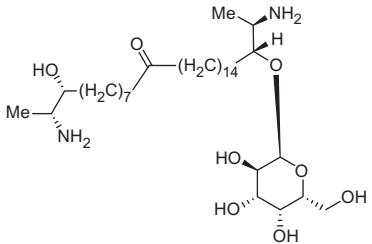
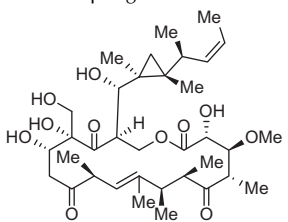
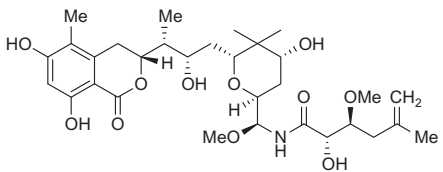
No.	Source of Organism	Compounds	Chemical Class	Molecular Target	References
59	*Oceanapia sagittaria* Sponge	Kuanoniamine A–C 	Alkaloid	MCF-7	[128]
60	*Strongylodesma aliwaliensis*	Makaluvic acid C 	Alkaloid	WHC01 WHC06 KYSE30	[129]

61	*Monanchora pulchra*	Monanchocidin 	Alkaloid	THP-1 HeLa	[130]
62	*Leucetta chagosensis*	Naamidine A 	Alkaloid	A-431	[131]
63	*Xestospongia* sp.	Renieramycin M 	Alkaloid	NSCLC	[132]

Continued

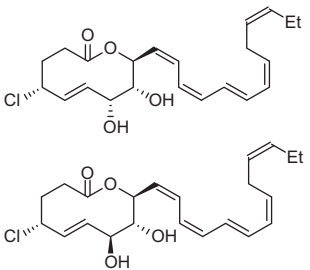
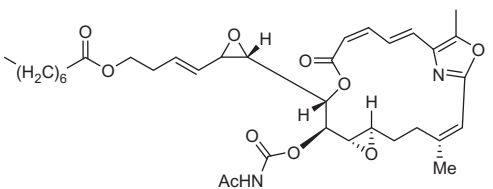
TABLE 1 Marine Anticancer Clinical and Preclinical Agents—Cont'd

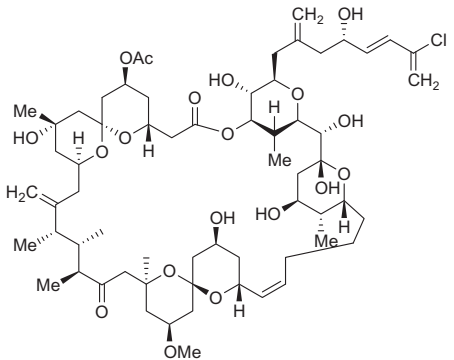
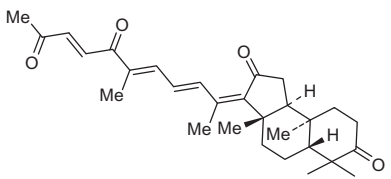
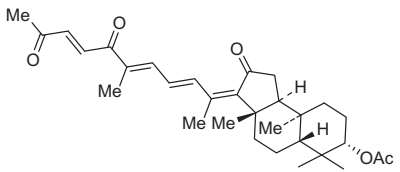
No.	Source of Organism	Compounds	Chemical Class	Molecular Target	References
64	*Stelletta* sp.	(*Z*)-stellettic acid C 	Lipid	U937	[133]
65	*Callyspongia* sp.	Callyspongidiol 	Lipid	HL-60	[134]
66	*Jaspis* sp.	Jaspine B 	Lipid	SK-Mel28	[135]
67	*Lanthella* sp.	Petrosterol 	Lipid	HL-60	[136]

68	*Rhizochalina incrustata*	Rhizochalin 	Lipid	THP-1 HeLa SNU-C4	[137]
69	*Candidaspongia* sp.	Candidaspongiolide 	macrolide	U251 HCT-116	[138]
70	*Ircinia ramose*	Irciniastatin A 	Macrolide	Jurkat T	[139]

Continued

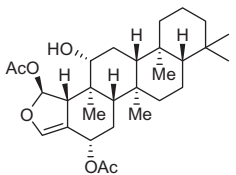
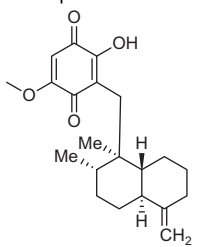
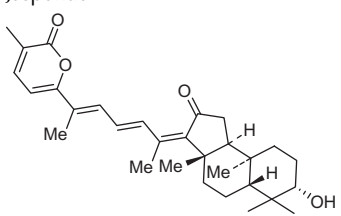
TABLE 1 Marine Anticancer Clinical and Preclinical Agents—Cont'd

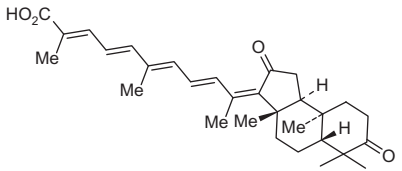
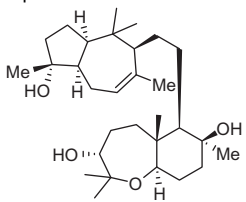
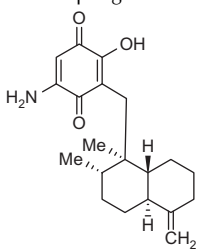
No.	Source of Organism	Compounds	Chemical Class	Molecular Target	References
71	*Negombata magnifica*	Latrunculins A and B 	Macrolide	MKN45 NUGC-4	[140]
72	*Fascaplysinopsis* sp.	Salarin C 	Macrolide	K562	[141]

73	*Spongia*	Spongistatin 1 		Jurkat T	[142]
74	*Geodia japonica*	Geoditin A 	Terpenoid	HL-60 HT29	[143,144]
75	*Geodia japonica*	Geoditin B 	Terpenoid	HL-60	[143]

Continued

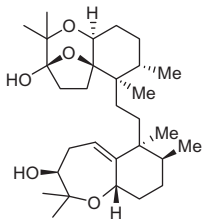
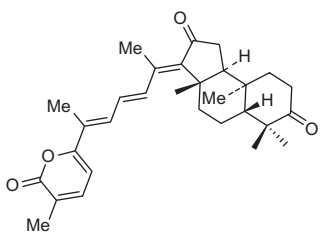
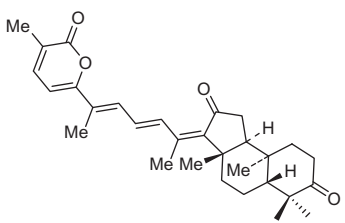
TABLE 1 Marine Anticancer Clinical and Preclinical Agents—Cont'd

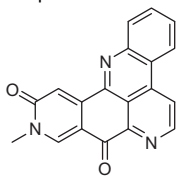
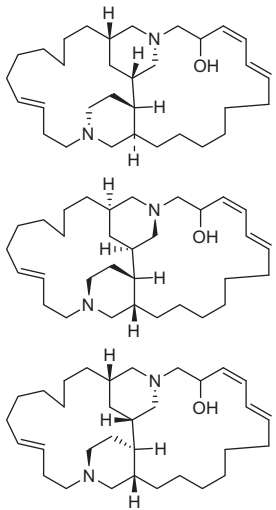
No.	Source of Organism	Compounds	Chemical Class	Molecular Target	References
76	*Hyrtios* sp.	Heteronemin 	Terpenoid	K562 Jurkat T	[145]
77	*Hippospongia metachromia*	Ilimaquinone 	Terpenoid	PC3	[146]
78	*Jaspis* sp.	Jaspolide B 	Terpenoid	Bel-7402 HepG2	[147]

79	*Rhabdastrella globostellata*	Rhabdastrellic acid-A 	Terpenoid	HL-60	[148]
80	*Callyspongia siphonella*	Sipholenol A 	Terpenoid	KB-3-1 KB-C2 KB-V1	[149]
81	*Dactylospongia elegans*	Smenospongine 	Terpenoid	K56	[150]

Continued

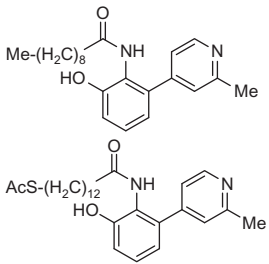
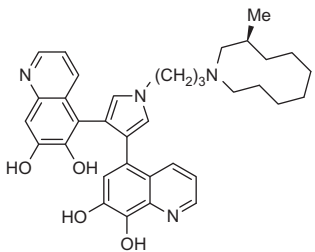
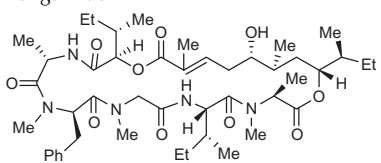
TABLE 1 Marine Anticancer Clinical and Preclinical Agents—Cont'd

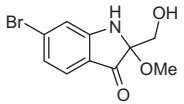
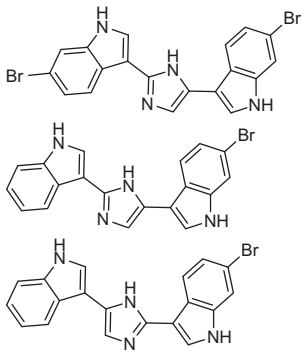
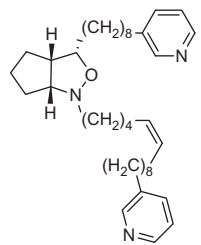
No.	Source of Organism	Compounds	Chemical Class	Molecular Target	References
82	*Axinella* sp.	Sodwanone V 	Terpenoid	MDA-MB-231	[151]
83	*Geodia japonica*	Stellettin A 	Terpenoid	HL-60	[143]
84	*Geodia japonica*	Stellettin B 	Terpenoid	HL-60	[143]

85	*Amphimedon* sp.	Amphimedine 	Alkaloid	U-87MG, U-373Mg, J82, HCT-15, LoVo, and A549	[152]
86	*Arenosclera brasiliensis*	Arenosclerin A–C 	Alkaloid	HL-60, B16, U138, and L-929	[153]

Continued

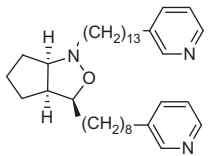
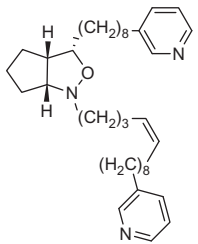
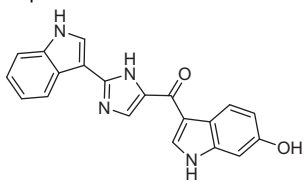
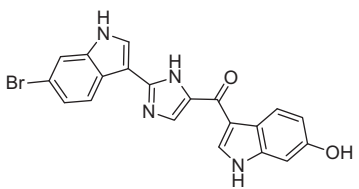
TABLE 1 Marine Anticancer Clinical and Preclinical Agents—Cont'd

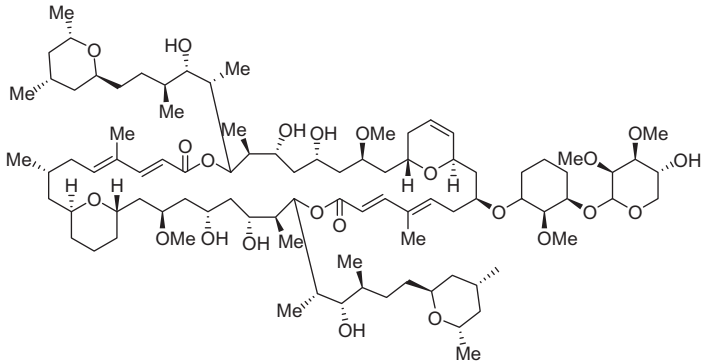
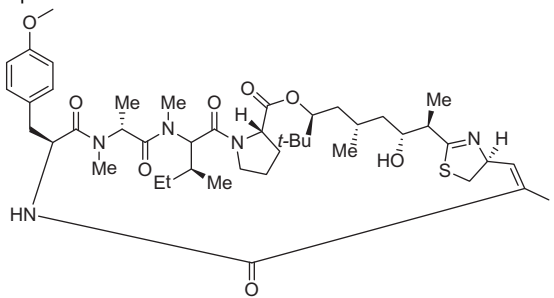
No.	Source of Organism	Compounds	Chemical Class	Molecular Target	References
87	*Echinoclathria* sp.	Echinoclathrines A–C 	Alkaloid	P388 and A549m HT29	[152]
88	*Haliclona tulearensis*	Halitulin 	Alkaloid	P388, A549, and MEL-28	[154]
89	*Agelas longissima*, *Homaxinella*	Longamide 	Alkaloid	P388	[155,156]

90	*Iotrochota purpurea*	Matemone 	Alkaloid	NSCLC-N6 L16, Mia PaCa-2, and DU145	[157,158]
91	*Spongsorites ruetzleri*	Nortopsentins A, B, and C 	Alkaloid	P388	[159]
92	*Amphimedon* sp.	Pyrinodemin A–D 	Alkaloid	L1210 and KB	[160,161]

Continued

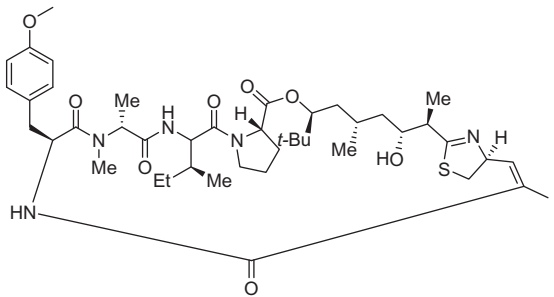
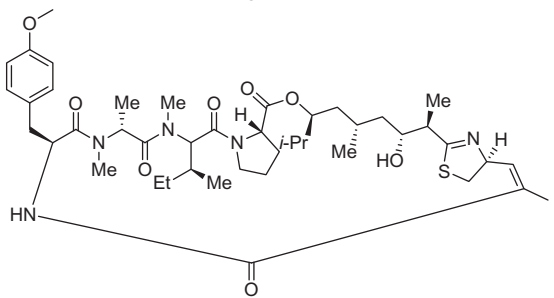
TABLE 1 Marine Anticancer Clinical and Preclinical Agents—Cont'd

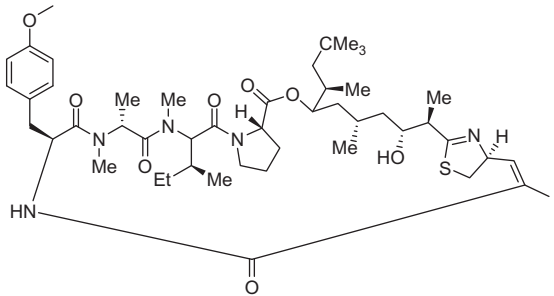
No.	Source of Organism	Compounds	Chemical Class	Molecular Target	References
		 			
93	*Rhaphisia lacazei*	Topsentine B1 and B2  	Alkaloid	NSCLC-N6	[162]

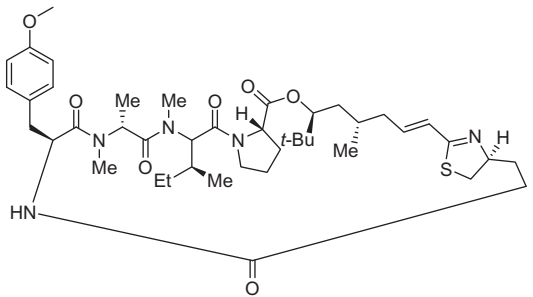
94	*Geitlerinema*	Ankaraholide A 	Glycosilated swinholide	NCI-H-460 lung tumor MDA-MB-435 breast carcinoma KB oral epidermoid cancer and LoVo colon cancer	[163,164]
95	*Lyngbya majuscule*, *Lyngbya bouilloni*, and *Lyngbya sordida*	Apratoxin A–G 	Cyclic depsipeptide	U2OS osteosarcoma, HT29 colon adenocarcinoma, and HeLa cervical carcinoma	[165]

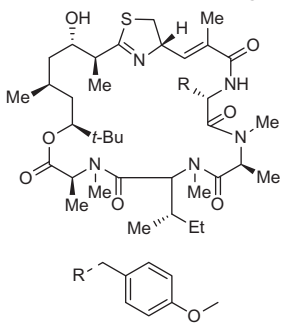
Continued

TABLE 1 Marine Anticancer Clinical and Preclinical Agents—Cont'd

No.	Source of Organism	Compounds	Chemical Class	Molecular Target	References
					
					

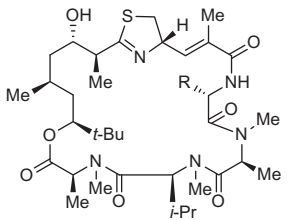
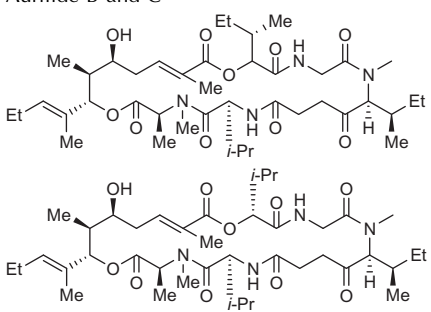


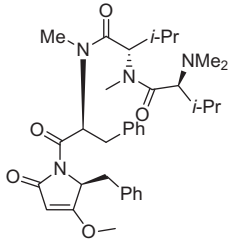
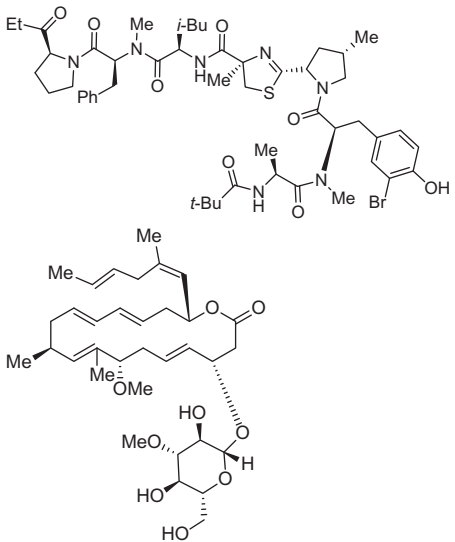




Continued

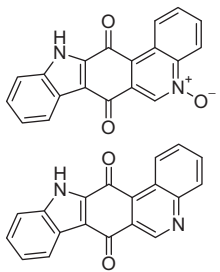
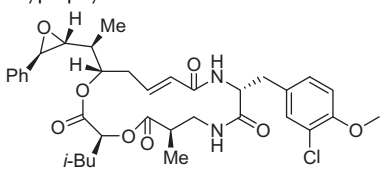
TABLE 1 Marine Anticancer Clinical and Preclinical Agents—Cont'd

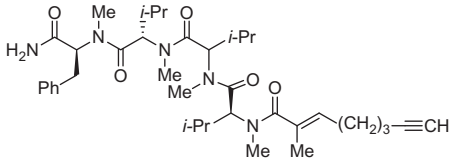
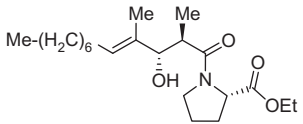
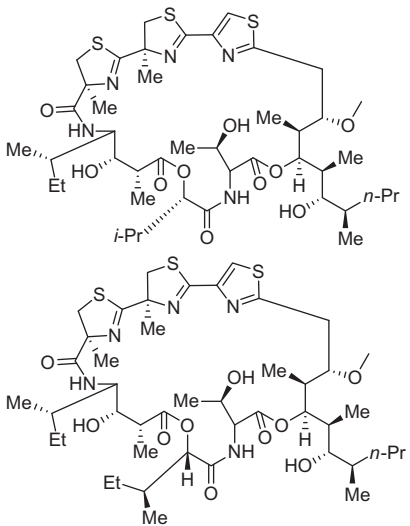
No.	Source of Organism	Compounds	Chemical Class	Molecular Target	References
					
96	*Lyngbya majuscula*	Aurilide B and C 	Cyclic depsipeptide	H-460 lung tumor NCI-H-460 lung tumor	[166]

97	*Symploca* sp.	Belamide A 	Linear tetrapeptide	HCT-116 colon cancer	[167]
98	*Lyngbya* sp.	Bisebromoamide and Biselyngbyaside 	Peptide Glicomacrolide	HeLa S3 epithelial carcinoma HeLa S3 epithelial carcinoma, SNB-78 central nervous system cancer, and NCI H522 lung cancer	[168,169]

Continued

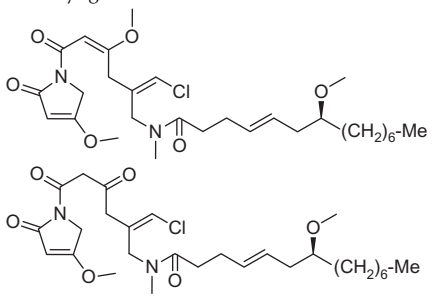
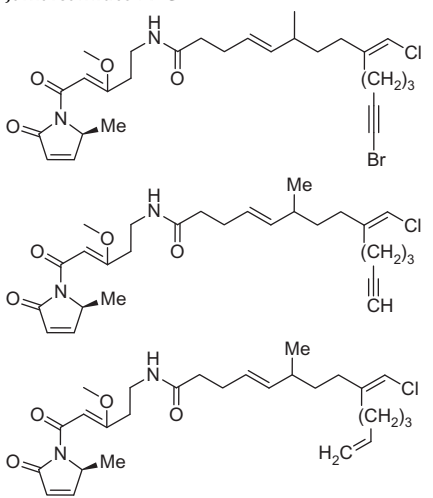
TABLE 1 Marine Anticancer Clinical and Preclinical Agents—Cont'd

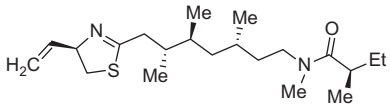
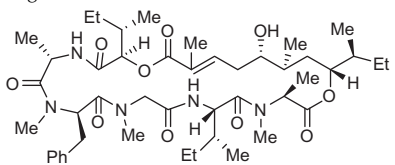
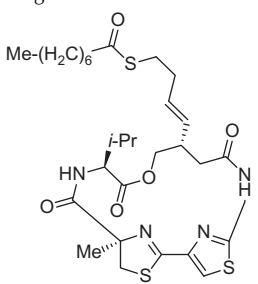
No.	Source of Organism	Compounds	Chemical Class	Molecular Target	References
99	*Calothrix*	Calothrixin A and B 	Pentacyclic indolophenanthridine	HeLa epithelial carcinoma Leukemia CEM HeLa epithelial carcinoma	[170–172]
100	*Nostoc* spp.	Cryptophycin 1 	Cyclic depsipeptide	Leukemia U937, CCRF-CEM, and HL-60 Colon carcinoma HT29, GC3, and Caco-2 Mammary carcinoma MCF-7 and MDA-MB-231 Cervical carcinoma HeLa	[173]

101	*Lyngbya majuscula*	Dragonamide 	Lipopeptide	A549 lung epithelial adenocarcinoma, HT29 colon adenocarcinoma, and MEL-28 melanoma	[174]
102	*Oscillatoria margaritifera*	Ethyl tumonoate A 	Peptide	H-460 lung cancer	[175]
103	Assemblage of *Lyngbya majuscule* and *Phormidium* gracile	Hoiamide A and B 	Cyclic depsipeptide	H-460 lung cancer	[176]

Continued

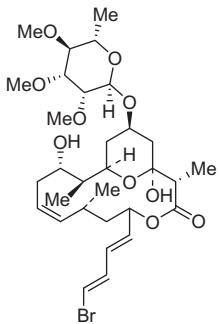
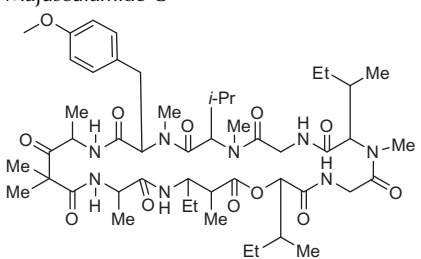
TABLE 1 Marine Anticancer Clinical and Preclinical Agents—Cont'd

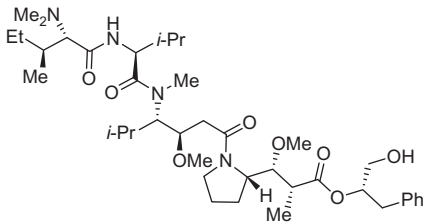
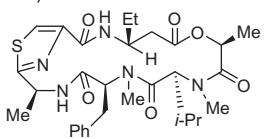
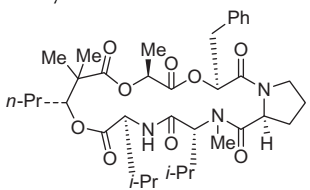
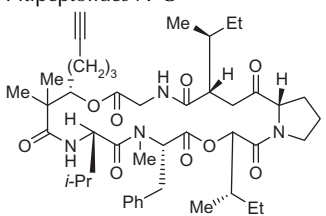
No.	Source of Organism	Compounds	Chemical Class	Molecular Target	References
104	*Lyngbya majuscula*	Isomalyngamide A and A-1 	Fatty acid amides	Breast cancer MCF-7 and MDA-MB-231	[177]
105	*Lyngbya majuscula*	Jamaicamides A–C 	Polyketide–peptides	H-460 lung cancer	[178]

106	*Lyngbya majuscula*	Kalkitoxin 	Lipopeptide	HCT-116 colon	[179]
107	*Lyngbya majuscula*	Lagunamide C 	Cyclic depsipeptide	Colorectal cancer HCT-8 and ovary cancer SK-OV	[180]
108	*Symploca* sp.	Largazole 	Cyclic depsipeptide	Osteosarcoma A549 lung cancer and HCT-116 colorectal carcinoma A549 lung cancer and HCT-116 colorectal carcinoma	[181,182]

Continued

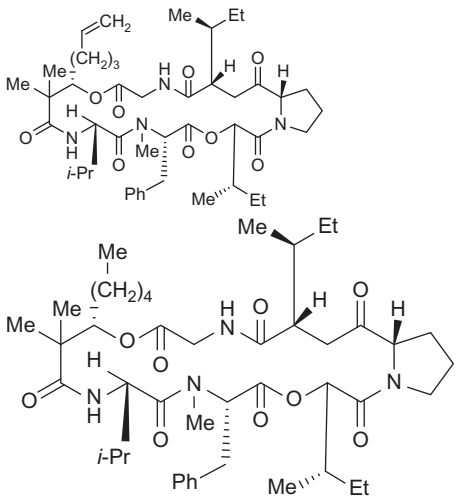
TABLE 1 Marine Anticancer Clinical and Preclinical Agents—Cont'd

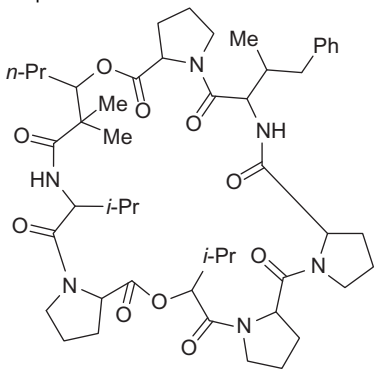
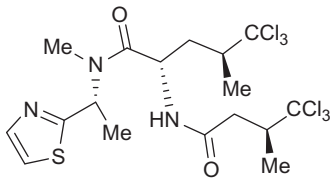
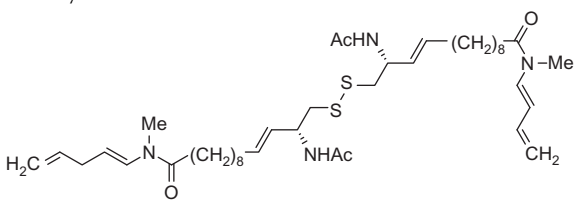
No.	Source of Organism	Compounds	Chemical Class	Molecular Target	References
109	*Lyngbya* sp.	Lyngbyaloside 	Glicomacrolide	KB nasopharyngeal carcinoma and LoVo colon adenocarcinoma	[183]
110	*Lyngbya majuscula*	Majusculamide C 	Cyclic depsipeptide	Lung cancer NCI-H-460, colorectal cancer KM20L2, and glioblastoma SF-295	[184]

111	*Symploca hydnoides*	Malevamide D 	Peptide ester	Lung cancer A549, colon cancer HT29, and melanoma MEL-28	[185]
112	*Lyngbya confervoides*	Obyanamide 	Cyclic depsipeptide	KB oral epidermoid cancer and LoVo colon cancer	[186]
113	*Lyngbya majuscula*	Palmyramide A 	Cyclic depsipeptide	H-460 lung cancer	[187]
114	*Lyngbya majuscula*	Pitipeptolides A–C 	Cyclic depsipeptides	Nonspecified LoVo colon cancer MTT HT29 colon adenocarcinoma and MCF-7 breast cancer HT29 colon adenocarcinoma	[188,189]

Continued

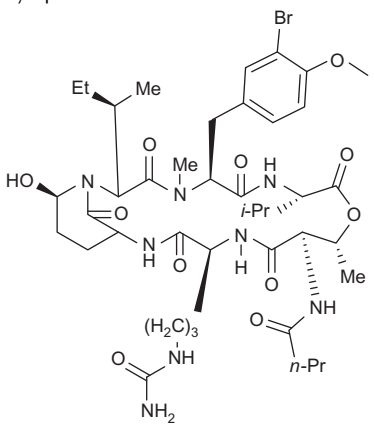
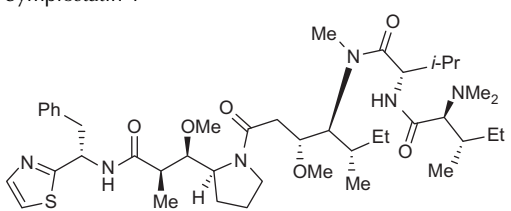
TABLE 1 Marine Anticancer Clinical and Preclinical Agents—Cont'd

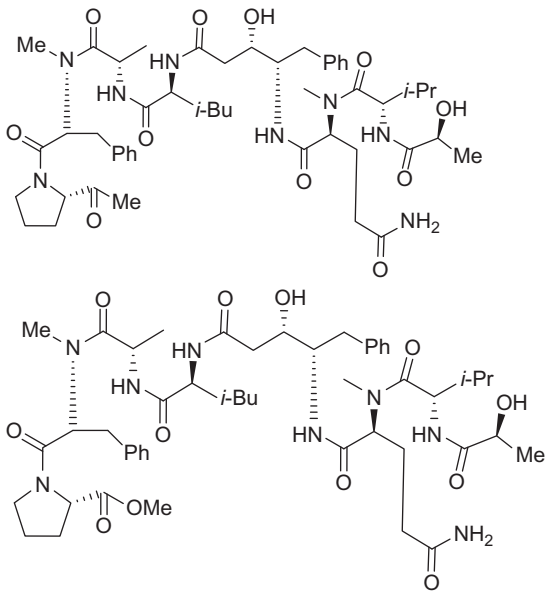
No.	Source of Organism	Compounds	Chemical Class	Molecular Target	References
				and MCF-7 breast cancer	

115	*Lyngbya majuscula*	Pitiprolamide 	Cyclic depsipeptide	HCT-116 colorectal carcinoma and MCF-7 breast	[188]
116	*Lyngbya majuscula*	Pseudodysidenin 	Lipopeptide	A549 lung adenocarcinoma and HT29 colon	[190]
117	*Lyngbya majuscula*	Somocystinamide A 	Lipopeptide	Jurkat and CEM leukemia, A549 lung carcinoma, Molt4 T leukemia, M21 melanoma, and U266 myeloma	[176]

Continued

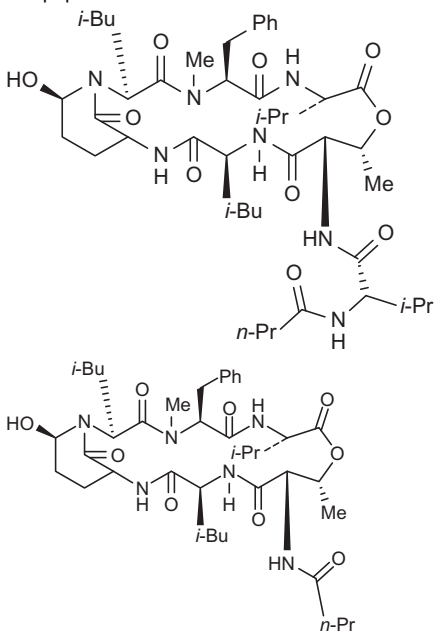
TABLE 1 Marine Anticancer Clinical and Preclinical Agents—Cont'd

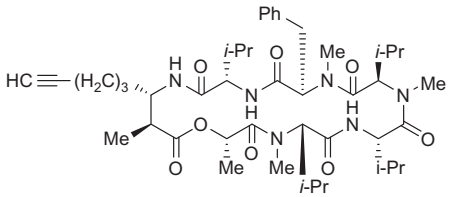
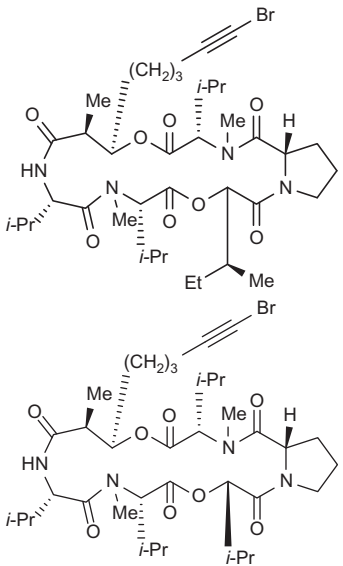
No.	Source of Organism	Compounds	Chemical Class	Molecular Target	References
118	*Symploca* sp.	Symplocamide 	Cyclic peptide	H-460 lung cancer	[191]
119	*Symploca hydnoides*	Symplostatin 1 	Linear pentapeptide	MDA-MB-435 breast carcinoma and NCI/ADR ovarian carcinoma	[192]

120	*Symploca* sp.	Tasiamide A and B		Cyclic peptide, peptide	KB oral epidermoid cancer and LoVo colon cancer [193,194]

Continued

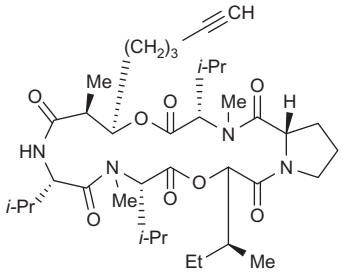
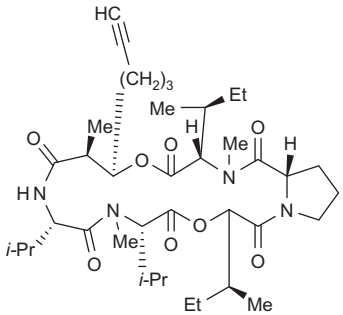
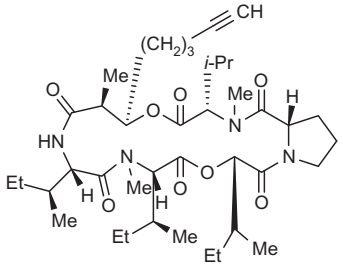
TABLE 1 Marine Anticancer Clinical and Preclinical Agents—Cont'd

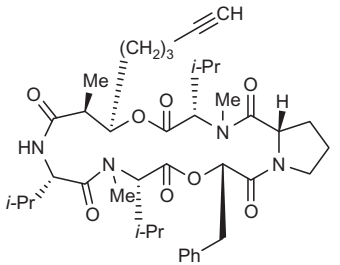
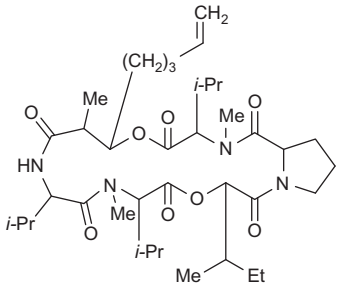
No.	Source of Organism	Compounds	Chemical Class	Molecular Target	References
121	*Symploca* sp.	Tasipeptins A and B 	Cyclic depsipeptides	KB oral epidermoid cancer	[195]

122	*Lyngbya* sp.	Ulongapeptin 	Cyclic depsipeptide	Nonspecified KB oral epidermoid cancer	[196]
123	*Symploca* cf. *hydnoides*	Veraguamides A–G 	Cyclic depsipeptides	H-460 lung cancer	[197]

Continued

TABLE 1 Marine Anticancer Clinical and Preclinical Agents—Cont'd

No.	Source of Organism	Compounds	Chemical Class	Molecular Target	References
					
					
					

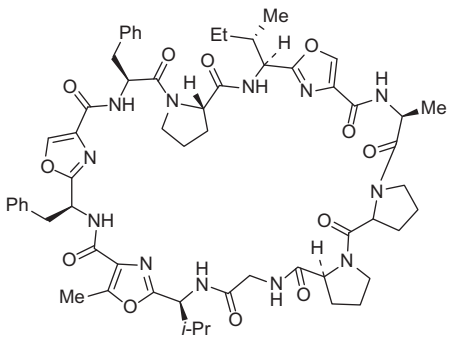
124	*Lyngbya sordida*	Wewakazole		Cyclic dodecapeptide	H-460 lung cancer	[198]

TABLE 2 Ongoing Clinical/Total Trials as Reported at www.clinicaltrials.gov

Clinical Status	Compound Name	Marine Organism	Chemical Class	Molecular Target
FDA approved	Cytarabine (Ara-C)	Sponge	Nucleoside	DNA polymerase
	Eribulin mesylate (E7389)	Sponge	Macrolide	Microtubules
	Trabectedin (ET-743) (EU registered only)	Tunicate	Alkaloid	Minor groove of DNA
	Brentuximab vedotin (SGN-35)	Mollusk	Antibody drug conjugate (MM auristatin E)	CD30 and microtubules
Phase III	Plitidepsin	Tunicate	Depsipeptide	Rac1 and JKK activation
Phase II	Plinabulin (NPI2358)	Fungus	Diketopiperazine	Microtubules and JNK stress protein
	PM00104	Mollusk	Alkaloid	DNA binding
	Elisidepsin	Mollusk	Depsipeptide	Plasma membrane fluidity
	PM01183	Tunicate	Alkaloid	Minor groove of DNA
	CDX-011	Mollusk	Antibody drug conjugate (MM auristatin E)	Glycoprotein NMB and microtubules
Phase I	Marizomib (Salinosporamide A; PI-0052)	Bacterium	Beta-lactone-gamma lactam	20S proteosome
	SGN-75	Mollusk	Antibody drug conjugate (MM auristatin E)	ASG-5 and microtubules
	ASG-5ME	Mollusk	Antibody drug conjugate (MM auristatin E)	ASG-5 and microtubules

Production of secondary metabolites might be highly dependent on the culture conditions and the origin of the strains. For example the lignicolous fungus *Leptosphaeria oraemaris* (Pleosporaceae) yielded leptosphaerin when cultured on specific medium [209,14]. A further study of the same fungal

species when grown in different culture conditions yielded none of the previously found metabolites, but the polyketides, leptosphaerolide, its *o*-dihydroquinone derivative, and leptosphaerodione [210]. To produce these metabolites and to maximize the potential chemical diversity, they need to be grown in various nutrient limited media. For example, media for *Penicillium* spp. that are deficient in carbon can produce penicillins, those that are phosphorus-limited can produce cephalosporins and vancomycin, and those that are nitrogen limited can produce carbapenems [211].

Media-Dependent Synthesis

The marine florae are rich in medicinally potent chemicals, predominantly belonging to polyphenols, and sulfated polysaccharides for antioxidant and anticancer activity. The ecology of MNPs has shown that many of these compounds function as chemical weapons and have evolved into highly potent inhibitors of physiological processes in the prey, predators, or competitors of the marine organisms that use them.

Sulfated polysaccharides can enhance the adaptive immune responses [212–214]. Recent studies have implicated that sulfated polysaccharides recognize a range of cell adhesion systems. They can bind to CD2, CD3, and CD4 in T lymphocytes and enhance the proliferative response of T lymphocytes [215,216]. Compared with other sulfated polysaccharides, fucoidans have been increasingly investigated in recent years to develop as drug or functional food [217,218]. They represent one of the sulfated polysaccharides (sulfated L-fucose) derived from cell wall of brown algae [219,220]. The type of fucoidan, its sulfation, molecular weight, and the conformation of its sugar residues vary with the seaweed species [212,221].

Sulfation is critical for fucoidan activity *in vivo*. In particular, desulfated fucoidan fails to promote angiogenesis *in vitro* [222] or to induce immature $CD34^+$ cell mobilization *in vivo* [223]. They can disrupt heparan sulfate-growth factor/cytokine complexes and can substitute for cell-surface heparan sulfates in stabilizing the growth factor/growth factor receptor interaction. Fucoidans have diverse biological properties, ranging from relatively simple mechanical support functions to more intricate effects on cellular processes [224] and binding proteins such as adhesion proteins [225], growth factors [226], cytokines [227], and a variety of enzymes, including coagulation proteases [228].

The microbial toxins are useful in neurophysiological and neuropharmacological studies. For example, bacteria present in *Noctiluca scintillans* are responsible for causing red tides. The major metabolite, macrolactin-A, inhibits B16-F10 murine melanoma cancer cells, mammalian herpes simplex virus (types I and II) and protects T lymphocytes against HIV replication [229].

Kahalalide F is a depsipeptide isolated from the mollusk *Elysia rufescens* from Hawaii and the compound is believed to be synthesized by microbes associated with the animal. Kahalalide F induces cytotoxicity and blocks the cell cycle in G1 phase in a p53-independent manner. *In vitro*, Kahalalide F

displays activity against solid tumors with an interesting pattern of selectivity in prostate cancer cell lines. Marine blue-green algae (cyanobacteria) are considered to be one of the potential organisms which can be the richest sources of known and novel bioactive compounds including toxins with potential for pharmaceutical applications [230,231]. Some of the marine cyanobacteria appear to be potential sources for large-scale production of vitamins (B complex, E) of commercial interest. Scytonemin is a protein serine/threonine kinase inhibitor [33], isolated from the cyanobacterium *Stigonema* sp. and this compound is a yellow-green ultraviolet sunscreen pigment, known to be present in the extracellular sheaths of different genera of aquatic and terrestrial blue-green algae. Scytonemin regulates mitotic spindle formation as well as enzyme kinases involved in cell cycle control and the compound also inhibits proliferation of human fibroblasts and endothelial cells. Thus, scytonemin may provide an excellent drug as protein kinase inhibitors to have antiproliferative and anti-inflammatory activities [232].

More than 50% of the marine cyanobacteria are potentially exploitable for extracting bioactive substances which are effective in either killing the cancer cells by inducing apoptotic death or affecting the cell signaling through activation of the members of protein kinase C family of signaling enzymes. The cell extracts of *Calothrix* isolates inhibit the growth *in vitro* of a chloroquine-resistant strain of the malaria parasite, *Plasmodium falciparum*, and of human HeLa cancer cells in a dose-dependent manner [233].

In recent times, the most significant discoveries are of borophycin, cryptophycin 1 and 8, and cyanovirin. Borophycin is a boron-containing metabolite, isolated from marine cyanobacterial strains of *Nostoc linckia* and *N. spongiaeforme* var. *tenue* [18]. The compound exhibits potent cytotoxicity against human epidermoid carcinoma (LoVo) and human colorectal adenocarcinoma (KB) cell lines [234]. Borophycin is related both to the boron-containing boromycins isolated from a terrestrial strain of *Streptomyces antibioticus* and to the aplasmomycins isolated from a marine strain of *Streptomyces griseus* (actinomycetes) [235].

There are many reports on the immunomodulating and antitumor activities of algae [236–247]. An extract from the brown seaweed *Sargassum thunbergii* has shown antitumor activity [248] and inhibition of tumor metastasis in the rat mammary adenocarcinoma cell (13762 MAT) [249]. Moreover, low-molecular weight fucoidan isolated from *Ascophyllum nodosum* shows an antiproliferative effect on both normal and malignant cells, including fibroblasts (Hamster Kidney Fibroblast CCL39), sigmoid colon adenocarcinoma cells (COLO320 DM), and smooth muscle cells [250]. Fucoidans exhibit antitumor, anticancer, antimetastatic, and fibrinolytic properties in mice [248,251]. Stypolactone, isolated from *Stypodium* sp. is a potent cytotoxic metabolite, which halts mitotic spindle formation [252].

The majority of compounds isolated from marine cyanobacteria originate from the filamentous order Nostocales, in particular from the members of

the genera Lyngbya, Oscillatoria, and Symploca. Many of these metabolites are modified peptides or peptide–polyketide hybrids exhibiting antitumor activities [253].

Largazole was isolated from a cyanobacterial sample of a *Symploca* sp. collected from Key Largo, Florida Keys [181]. Its structure is remarkable as it is the first cyanobacterial natural product containing a thioester.

The structural and biosynthetic variations of secondary metabolites isolated from marine actinomycetes, as exemplified by the marinopyrroles, the marineosins, and the ammosamides, exceeds the breadth displayed by cyanobacterial compounds. The marinopyrroles A and B, produced by a *Streptomyces* sp. isolated from a marine sediment sample collected in La Jolla, CA, are heavily halogenated bispyrroles, whose monomeric building blocks are connected via a unique, rotationally stable N,C-biaryl axis [254].

The ammosamides A and B, derived from a deep-sea sediment sample *Streptomyces* strain collected in the Bahamas, are chlorinated tricyclic pyrroloquinoline alkaloids, structurally related to the microbial and sponge metabolites lymphostin and batzelline A, respectively [255].

The marine environment consists of highly complex microbial communities and many invertebrates are associated with large amounts of epibiotic and endobiotic microorganisms [256,257].

Several potentially interesting marine compounds are in preclinical development. Discodermolide, a metabolite of the deep-sea sponge *Discodermia dissoluta*, was collected in the waters of the Bahamas, and it was shown to induce microtubule stabilization [258]. Halichondrin B, first isolated from the sponge *Halichondria okadai* in Japan, has shown *in vivo* activity against melanoma and leukemia. It is being currently obtained from the New Zealand deepwater sponge *Lissodendoryx*. Isogranulatimide, an aromatic alkaloid extracted from the Brazilian tunicate, ascidian *Didemnum granulation*, acts as a G2 checkpoint inhibitor. Its synthesis has been fully accomplished and several analogues are being developed. Three marine compounds that have entered clinical evaluation, didemnin B, aplidine, and ecteinascidin 743 (ET-743), are derived from tunicates. Didemnin B is a cyclic depsipeptide isolated from the tunicate *Tridemnum solidum* and was the first to enter phase I and II studies [259].

Several antitumor MNPs, derived mainly from marine sponges or molluscs and bryozoans and cyanobacteria, exhibit potent antimitotic properties. Several have advanced to phase I and II clinical trials and, perhaps not coincidently, many such compounds block progression of dividing cancer cells through M phase by targeting the same protein (tubulin) in ways similar to the clinical drugs vincristine, vinblastine, and paclitaxel. Although none has yet been approved as a drug a wide range of these chemotypes function by modulate tubulin dynamics. Important differences have been reported in their mechanisms of action, and this in itself has been tremendously informative for understanding the complex interaction of microtubule proteins with

antimitotic drugs. Several other candidate compounds are presently in the pipeline, and MNPs are being evaluated in phase I–III clinical trials in the United States and in Europe for the treatment of various cancers [203,32] (Table 2).

Avenues for New Targets

This new wave of natural product-derived experimental agents is offering us the opportunity to evaluate not only totally new chemical classes of anticancer agents but also novel and potentially relevant mechanisms of action.

The application of molecular genetics techniques has permitted manipulation of biosynthetic pathways for the generation of novel lead compounds directed at the new targets arising from genomics projects. The exploitation of structural chemical databases comprising a broad variety of chemotypes, in conjunction with databases on target genes and proteins, should facilitate the creation of NCEs through computational molecular modeling for pharmacological evaluation [260–263].

Marine compounds, for instance, can interfere with very relevant intracellular targets such as signal transduction, microtubule stabilization, or new forms of interaction with DNA. They can be extremely potent in culture, with IC_{50} value in tumor cell lines in the nanogram range [264,265]. However, these compounds can be very toxic to normal tissues as well. But the challenge of finding more selective anticancer agents with a more favorable therapeutic index are still to be achieved. Furthermore, manipulation of microbial biosynthetic pathways making the use of genetic engineering has allowed the production of interesting molecules not generated naturally. These unique sources of novel compounds will certainly make anticancer drug discovery even more challenging in the years to come [266–268].

Cancer and Antioxidant

More than 40,000 different species of phytoplankton, 680 species of marine algae belonging to Rhodophyta, Phaeophyta, and Chlorophyta commonly known as red, brown, and green seaweeds, respectively, and 71 mangrove plant species have been documented in the global marine biotope. They provide essential fatty acids, ionic trace minerals, vitamins, enzymes, bioflavonoids, amino acids, and other nutrients, reportedly acting as free radical scavengers, antimicrobial, and anticancer agents [269,270].

Studies have shown a positive correlation between the increased dietary intake of natural antioxidants and the reduced coronary heart disease, cancer mortality, as well as longer life expectancy [271,272]. Marine organisms are natural metal chelators with high antioxidant activity that may be successfully used to prevent a variety of toxic metal ion-induced organ dysfunctions [273]. A close association between anticarcinogenic activity and antioxidant activity

has been reported in a chemically induced mouse carcinoma system with low-molecular weight polyphenols [274–277].

It is estimated that a typical human cell experiences about 10,000 oxidative "hits" to its DNA each day. DNA repair enzymes remove most of the damage. Oxidative lesions to DNA accumulate with age and so does the risk of cancer [278]. A marker of mutagenic DNA damage will be useful in the estimation of cancer risk of various populations and in monitoring the effects of chemoprevention, much of which oxidative in nature. The American Cancer Society estimated that by 2050, the disease is expected to grow further as 27 million new cancer cases and 17.5 million cancer deaths will be simply due to the growth and aging of the population [1].

Diet and Cancer

Use of phytosubstances to improve or enhance their effects with safety in foods is significantly focused in daily food. The activities of diverse constituents vary in their ability by quenching effects against active free radical oxygen by carotenes and cryptoxanthins, and polyphenols and flavonoids, by inhibition of absorption into small intestine by dietary fibers, or by regulation on efflux and influx of ions in cell membranes by minerals to inhibit tumors [279–281].

Nutritional Values and Anticancer Effects

Marine plants play an important role to fulfill the requirement of food and nutrition for rectifying the human ailments. Most diets that are protective against cancer are mainly made up from foods of plant origin. Higher consumption of several plant foods probably protects against cancers. The "plant-based" diets give more emphasis to those plant foods that are high in nutrients, high in dietary fiber (and so in non-starch polysaccharides), and low in energy density. Non-starchy vegetables and fruits probably protect against some cancers [282]. Seaweeds are used extensively for human consumption and they contain other interesting components or traditional medicinal value with curative powers for a variety of diseases (tuberculosis, arthritis, colds, influenza, cancer, etc.). Most people utilize seaweed products daily in the form of processed food items like processed dairy, meat, and fruit products and domestic commodities like paint, toothpaste, solid air fresheners, cosmetics, and so forth. Seaweeds are excellent source of vitamins A, B1, B12, C, D, E, riboflavin, niacin, pantothenic acid and folic acid as well as minerals such as Ca, P, Na, and K. Their amino acid content is well balanced, containing most of the essential amino acids needed for life and health. They have more than 54 trace elements required for human body's physiological functions in quantities greatly exceeding vegetables and other land plants [283–285].

Seaweeds are important sources of protein, iodine, vitamins, and minerals and hence, their metabolites have shown promising activities against cancer incidences [286]. The seaweeds also contain high amounts of polyphenols such as catechin, epicatechin, epigallocatechin gallate, and gallic acid, as reported in *Halimeda* sp. (Chlorophyceae) [287]. In the past three decades, many researchers have worked on the antioxidant, antitumor, and immunomodulating activities of seaweeds [238]. Edible seaweed like *Palmaria palmate* is shown to be effective antioxidant, capable of inhibiting cancer cell proliferation [288]. Algae have gained special interest owing to their biological properties.

Research has shown that a sulfated polysaccharide purified from the same algal species selectively and dose dependently suppresses the proliferation of the cancer cell lines *in vitro* [289].

The crude phlorotannins extracted from brown algae have inhibitory effects on Hyaluronidase [290]. The half maximal inhibition (IC_{50}) values of crude phlorotannins of *E. bicyclis* and *E. kurome*, two terrestrial polyphenols (catechin, EGCG), inhibit four times stronger than that by an anti-allergic drug (DSCG) [291].

Polyphenolic compounds inhibit cancer cells by xenobiotic-metabolizing enzymes that alter metabolic activation of potential carcinogens, while some flavonoids can also alter hormone production and inhibit aromatase to prevent the development of cancer cells [292]. The mechanism of action of anticancer activity of phenolics is by disturbing the cellular division during mitosis at the telophase stage. Phenolics reduce the amount of cellular protein and mitotic index, and the colony formation during cell proliferation of cancer cells [293]. Several studies exhibited a close relationship between antioxidant activities and total phenolic content [294–296].

Screening Strategies

Screening strategies are continuing to evolve, probing new ideas and knowledge of cancer, and introducing high-throughput screening and new analytic methodologies. High-throughput screening methods have enabled more sophisticated mechanism-based screening, and subsequently move prefractionation and peak library generation.

These "prior-to-screening" purifications have the consequence of reducing the complexity of screening materials, increasing the titer of low abundance components, segregating unwanted substances into discrete fractions, and generally speeding up the time line from detection of a primary screening hit to identification of a molecular structure for the active substance. It can generally be concluded that contemporary screening protocols in natural products chemistry are using chromatographic purification steps, sometimes producing pure compounds, before biological or enzymatic bioassay. Coupled to these more effective paradigms for screening are new assays that evaluate

natural products in more detailed, refined, and novel ways. For example, detailed knowledge of the cellular mechanisms controlling proliferation has yielded numerous targets for mechanism-based anticancer screens [297].

Targets of high pharmacological relevance are G-protein-coupled receptors, tyrosine kinase receptors, nuclear hormone receptors, ion channels, proteases, kinases, phosphatases, and transporter molecules. Tests on the genome, transcriptome, or proteome level will become more and more important as more information pertaining to this area is accumulated. The detection of a reaction on the molecular level could be done by biochemical assays (e.g., spectrophotometric measurement of the product of an enzymatic reaction), ligand binding assays (readout by labeling with a tracer), or functional assays (reporter gene assays quantifying the expression level of a specific reporter gene product, second messenger assays and two hybrid assays for measuring protein–protein interactions). Fluorescence-based assay technologies, isotopic labeling, colorimetry, and chemoluminescence are very often used as detection methods. Cell-based assays are more complex and more physiologically relevant than tests on the molecular level. On the other hand, they are still labor intensive and more difficult to validate than molecular assays [32].

Funding agencies have demanded that the search for new drugs be carried out in conjunction with function-based bioassays. These assays have been either traditional ones, such as antimicrobial and cytostatic assays, or focused on functional, cellular, or biochemical assays associated with specific disease states. The majority of present day assays tend to focus on targets such as apoptosis, mitosis, or specific proteins involved in cell cycle control or signal transduction.

During the past decade, pharmaceutical companies have led the search for compounds effective against individual proteins, mostly those in signal transduction pathways that are associated with either cancer or other humandisease states. There is an urgent need to develop assays that are mechanism-based, high-throughput, molecular-target-oriented, and combinatorial chemistry linked [297] for the new anticancer drug in pipeline.

A novel pharmacology paradigm has been developed by Subramanian *et al*. [298] which quickly and efficiently move prospective anticancer drugs from the discovery phase through pharmacology testing and into therapeutic trial assessment. Following discovery, the drug is first assessed in a clonogenic assay which determines the cytotoxic effect of different concentrations of the drug at three different exposure durations: 2, 24 h, and continuous (168 h). Second, pharmacokinetic information is obtained in both plasma and tumor for the drug administered at the maximum tolerated dose given intravenously. The first study defines the time–concentration profile required to obtain a specific cell survival for the tumor cells and the second study determines the concentration–time profile that can be obtained in both plasma and tumor at the maximum tolerated dose of the drug. The integration of this information determines whether a successful therapeutic trial is possible.

Proteomics based mechanism of action study is initiated once a drug shows promising therapeutic efficacy. Two drugs have been assessed in this paradigm: salicortin and fascaplysin A [299].

Limitations

A long-standing and perplexing question in MNPs chemistry has been the identification of the metabolite producing organism or potentially metabolite biotransforming organism, in systems involving an invertebrate host and symbiotic microorganisms. The increasingly interdisciplinary field of MNP chemistry thus strongly impacts future developments in medicine, chemistry, and biology.

The use of these potential drugs, however, has largely been hampered by the low abundance of the natural producers and/or the low concentrations of the compounds of interest. Furthermore, the highly complex structures of many of these metabolites make synthetic approaches for their development at least economically daunting [300].

Many marine organisms are found in remote locations and can require large sum of money just to travel to and from these locations. Additional expenses including the specialized services of divers, submersibles, and the personals safety and costs are enormously high. An example of the prohibitive costs associated with collection of marine organisms is that a ship and submersible costs $14,500 per day.

Only a few marine bacteria can be isolated under laboratory conditions and there is an urgent need to develop new culture techniques to isolate slow-growing bacteria and also to isolate the bacteria that are unique in production of novel natural products [203].

The procurement or manufacture of quantities of rare compounds from marine sources to ensure a sustainable supply is a bottleneck. For example, the chemically versatile marine sponges, the source of many developmental compounds such as discodermolide and hemiasterlin, are primitive metazoans that live almost exclusively in marine habitats. Sponges and their microbial fauna are largely nonculturable, and the valuable compounds they produce must be extracted and purified from specimens collected by hand using scuba diving from shallow to deep waters, or sometimes with the aid of submersibles equipped with robotic arms. The identification of the biosynthetic origin of a natural product derived from complex assemblage of marine organisms is still a challenge. This is particularly true for bacterial symbionts, which have to date eluded laboratory cultivation. Nevertheless, interest in the remarkable properties of MNPs remained high enough to inspire innovative solutions to the supply problem based on a case-by-case basis, ranging from aquaculture of marine invertebrates to semisynthesis [66].

Future Strategies

The sequencing of the genome of a microorganism that has been identified as a potent producer of bioactive compounds allows the identification of the

gene clusters involved in the pathways for the production of these natural compounds. Polyketide synthases (PKSs) are a class of enzymes that are involved in the biosynthesis of secondary metabolites such as erythromycin, rapamycin, tetracycline, lovastatin, and resveratrol. Polyketide biosynthetic genes from bacteria and fungi have been cloned, sequenced, and expressed in heterologous hosts. Some marine sponge-associated bacteria with antimicrobial assets are also detected to have PKSs gene cluster and investigation is underway to explore them. Deep-sea hydrothermal vent microorganisms are also reported to produce unusual bioactive metabolites. The sequencing of genomes from cultivable microorganisms, chromosomal DNA may be used to generate genomic libraries. Large genomic DNA fragments may be directly isolated from the sample and cloned into suitable host vector systems. Establishment of comprehensive gene libraries representing maximum genome sequences of the sample will provide for the biosynthetic machinery of a microflora for further manipulation and understanding [301].

The biomedical potential of marine bacterial agents, which should be amenable to biotechnological production, might help overcome the problems of supply and sustainability encountered with macroorganisms from the sea.

An identification of metabolite producing organism in a symbiotic system has been a difficult task to perform. Few successes following isolation of various cell populations by centrifugation, sieving, or fluorescence-activated cell sorting, and then chemically analyzing these samples for metabolites of interest have been done. For example, in the case of the sponge *D. herbacea* and its symbiotic cyanobacterium *Oscillatoria spongellae*, this has been accomplished on a couple of occasions and generally supports metabolic trends observed from working with pure strains of cyanobacteria [301,302]. The cyanobacterial cells were found to contain a series of highly distinctive chlorinated peptides, previously isolated from work with the intact sponge, and which have strong structural precedence in metabolites isolated from the free-living cyanobacterium *Lyngbya majuscula* [303]. Alternatively, a similar approach with the tunicate *Lissoclinum patella*, which harbors an abundance of the cyanobacterium *Prochloron* spp., yielded equivocal results for a series of distinctive cyclic peptides which were found to be associated with both the cyanobacterial and tunicate cells [304].

Hence, new approaches are needed which truly show the genetic and biochemical ability of a particular cell type to synthesize a metabolite of interest [305]. A genetic approach has also been applied to this tunicate cyanobacteria symbiosis which showed that *Prochloron* spp. possess nonribosomal peptide synthetases (NRPS), but it could not convincingly showed the relevance of NRPS genes are relevant to the biosynthesis of cyclic peptides associated with this source by the cultures [306].

Another work in Newman and Sanader [4] laboratory has made a contribution in this regard. Cloning of the gene cluster responsible for the biosynthesis of the chlorinated peptide barbamide led to the identification of a gene

sequence encoding the production of a putative halogenase that converts an unactivated methyl group to a trichloromethyl functionality. Because an identical trichloromethyl group is present in metabolites isolated from *D. herbacea*, typified by dysidenin, it was reasoned that the symbiotic cyanobacterium *O. spongellae* should also contain a related genetic element. Subsequently, using primers designed to conserved sections of the halogenase were successful in cloning a homologous gene from the cyanobacteria-laden sponge tissue, and this was fluorescently labeled to provide a probe of mRNA expression in intact sponge tissue. Thin sections of sponge cyanobacteria tissue were incubated with this gene probe, washed to remove nonspecific probe binding, and visualized by fluorescent microscopy. This established that the biosynthetic capacity to produce chlorinated peptides resided within the cyanobacterial cells [26].

Further, the application of modern microbial genetics allows for the rational design of recombinant organisms [307] to produce novel "unnatural" natural products by combinatorial biosynthesis [308] and total *in vitro* (bio) synthesis [309].

The development of advanced analytical and preparative methods will help in extending the value of marine bacteria in isolating and identifying the products they synthesize. Improvements of analytical tools will facilitate the identification of the natural source of a certain compound even from complex microbial consortia.

Efforts are also being made to improve the efficiency of the drug discovery process by using high-throughput chemistry complementing with high-throughput screening [299].

Many complementary tools are now available for structural modification for increasing the possibility of obtaining a new drug. Preparing analogues and studying the structure–activity relationship for enhanced selective activity are being tried out. Furthermore, natural product MALDI-I (np-MALDI) was applied to rapidly identify unknown metabolites in a specific producer [310]. The application of MALDI-TOF-MS even allows the analysis of complex bacterial mixtures and to taxonomically identify individual microorganisms based on their mass fingerprints (phyloproteomics), a technique which has already been applied to probe the phylogenetic and chemical diversity of bacteria in marine sponges. Future applications of np-MALDI might thus have a high impact on directed drug discovery programs in future [311].

In this sense, the application of combinatorial chemistry [312] will help in the generation of libraries [260,261] of analogous molecules of natural compounds which can be transformed into lead molecules for the preparation of new, more potent, and less toxic compounds as probable drug candidate. Additionally, manipulation of metabolic pathways will also help to improve the production of a given natural metabolite [313].

An assay for identifying the cellular targets using a semisynthetic immune-affinity fluorescent probe offers a great promise for future. The simple

one-step preparation of ammosamide-based fluorescent probe that selectively binds myosin facilitates future investigations on a variety of myosin-regulated cellular processes and may thus significantly impact further developments of chemotherapeutics targeting this protein.

This new imaging technique has now been utilized to locate phytochemical marker molecules within certain cyanobacteria, even in complex mixtures with other cyanobacteria and to resolve the spatial distribution of molecules in heterogeneous assemblages, such as the sponge *D. herbacea*, thereby illuminating the existence of microenvironments within sponge tissues.

The strong interplay of classical natural product chemistry with modern microbial genetics and bioinformatics will thus help to overcome supply and sustainability issues from the past and to promote marine bacterial secondary metabolites to a well-recognized alternative for future drug discovery programs [314].

Fascinating chemical structures, marine microbial natural products continue to serve as the inspiration for the discovery of new biosynthetic enzymes that may serve as valuable biocatalysts for the fast and effective generation of derivative libraries important in the drug discovery process [315,316].

In future, few very specific strategies to overcome limitation of present day drug discovery from marine sources could be:

a. Harnessing marine bacterial enzymes to total biosynthesis

The extensive work associated with cloning, heterologous expression, and purification of a large number of biosynthetic enzymes, however, constitutes a major drawback of the total biosynthesis of complex natural products. The combination of modern synthetic methods with powerful biocatalysts, in particular when dealing with (stereo)chemically demanding reactions, might be the most effective future approach to synthesize natural products in the chemical laboratory, as recently exemplified by the first stereoselective total synthesis of the antiproliferative polyketide (*R*)-aureothin [317].

The remarkable capabilities of these microbes to produce chemically unique bioactive molecules are supported by the characterization of a wealth of intriguing new structures through the application of classical screening and isolation techniques. These microbes generally organize their biosynthetic genes for each secondary metabolite in compact clusters, making it practical to trace and clone the respective pathways in order to create effective heterologous producers. The assembly line logic of the biosynthetic enzymes furthermore facilitates the manipulation of the encoding genes, allowing the generation of even increased metabolic diversity. Using this metabolic engineering approach, the rational design of novel analogues for biomedical applications has become possible. Furthermore, the powerful and meanwhile quite affordable genome sequencing of the most promising bacterial strains paves the way to discover and mine orphan biosynthetic clusters encoding as yet unexpressed novel chemistry [294].

b. Target-based delivery

In addition, the growing understanding of the modes of action and exquisite selectivities of many natural products should allow the design of other antibody–prodrug conjugates. If discrete tumor cell antigens can be recognized, and humanized antibodies then produced [318].

c. Novel gene targets

The information provided by the Human Genome Project will undoubtedly identify gene targets for novel anticancer drugs, and the pharmaceutical industry will attempt to obtain these drugs through the use of combinatorial chemistry and high-throughput screening technologies [318, 319].

d. Combinatorial chemistry

Nature has already carried out the combinatorial chemistry; all we have to do is refine the structures. Good example was provided by the recent work of Nicolaou and colleagues [320], in which the basic skeleton of the cytotoxic MNP sarcodictyin was attached to a solid support, and this was used as the template for the production of a 100-compound library. Many of these new analogues had much greater cytotoxicities than the parent compound, and several were up to 50 times more potent than wonder drug Taxol. This coupling of synthetic chemistry with new technologies will take advantage of nature's gifts without exploitation of the often delicate ecologies that support the parent organisms, and will ensure that natural products make as many future contributions to cancer chemotherapy as they have in the past 35 years [320].

e. Innovation methods for procurement

The discovery of selective and potent therapeutic activity in a rare MNP can drive innovative methods for its procurement, including production by aquaculture, semisynthesis, synthesis, and the development of synthetic analogues with more manageable properties. Novel approaches to translational medicine, which unite MNPs chemists and pharmacologists with investigators in medical schools and institutes of pharmaceutical sciences, will be important in accelerating the progression of MNPs from their discovery to the laboratory bench-top and to the clinic [318].

f. Concerted efforts

Successful collaborations between academic institutes and pharmaceutical companies to provide mutual benefits that each party seeks taking advantages of each other's capacities. For example, academic programs gain access capacities for sophisticated screening, pharmacological evaluation, and advancement of leads to *in vivo* models, whereas industry gains high-value leads while evading the high risk associated with marine drug discovery [318].

CONCLUSION

Biodiversity translates to genetic uniqueness, which in turn results in the expression of diverse biochemical processes producing metabolic products

which, in their natural settings, primitive societies, interacted with their environments on a trial and error basis and recognized that plants contained "medicines" for a wide variety of maladies. This knowledge, often referred to as "ethnomedicine," was carefully documented and handed down through the centuries. Within the past 200 years, these curative, natural substances have been the focus of great interest leading ultimately to the development of the today's pharmaceutical industry. The process of drug discovery continues today at a pace greater than ever before. And although sophisticated new approaches are applied, nature continues to provide the biochemical insight forming the foundation of many newly developed drugs.

Marine florae are potential source of anticancer compounds, but they are least explored. Of the anticancer compounds extracted so far, the marine algae have contributed 65.63%, the mangroves 28.12%, and the bacteria 6.25%. Owing to a diverse chemical ecology, the marine organisms, especially marine flora, have a great promise for providing potent, cheaper, and safer anticancer drugs, which deserve an extensive investigation.

Drugs developed from marine sources give us novel mechanisms to fight some of the most debilitating diseases encountered today including: HIV, osteoporosis, Alzheimer's disease, and cancer. Although the costs associated with developing drugs from marine sources have been prohibitive in the past, the development of new technology and a greater understanding of marine organisms and their ecosystem are allowing us to further develop our research into this area of drug development.

Tremendous advances in marine molecular biology, genome sequencing, and bioinformatics have paved the way to fully exploit the biomedical potential of marine bacterial products. Combination of novel structures and for some, novel mechanisms of action, is translating into new methods by which to treat cancer, and ultimately, improved outcomes, particularly for patients with solid tumors of the lung, breast, colon, or prostate. Past decade has seen an ever evolving strategy for the screening and discovery of new anticancer leads from nature, and this is proving effective. From former times of evaluation of crude extracts by *in vivo* screens, to current evaluation of peak or prefractionated libraries in mechanism-based assays, the level of sophistication and success has steadily improved. In addition, unique biosynthetic enzymes discovered from bacteria from the sea have begun to emerge as powerful biocatalysts in medicinal chemistry and total synthesis.

The remarkably high hit rates of marine compounds in screening for drug leads were often cited as justification for the search and, indeed, notable discoveries were made of compounds that profoundly affect the cell cycle and cellular metabolism. If anything, MNP chemistry has built a legacy of discovery of biomedical probes shown by examples like okadaic acid (a phosphatase inhibitor produced by dinoflagellates) and xestospongin C (an intracellular blocker of calcium release from a marine sponge).

Approaches in the past which have largely screened crude extracts for biological activity have allowed a rich harvest of "low hanging fruit." With

effective prefractionation strategies broadly in utilization, rich repertoires of diverse biological assays are now available. Highly effective nuclear magnetic resonance and mass spectrometry methods well suited to solve complex structures on vanishingly small quantities of a compound and synthetic methods. With good funding, involvement of investigators from diverse fields and advancement in technology, we can expect the next decade to yield an even more bountiful crop of new clinical agents from the sea. Nevertheless, several marine-derived agents are now approved, most as "first-in-class" drugs, with five of seven appearing in the past few years. Additionally, there is a rich pipeline of clinical and preclinical marine compounds to suggest their continued application in human medicine. Understanding of how these agents are biosynthetically assembled has accelerated in recent years, especially through interdisciplinary approaches, and innovative manipulations and reengineering of some of these gene clusters are yielding novel agents of enhanced pharmaceutical properties compared with the natural product. It was estimated that 118 MNPs are currently in preclinical trials, 22 MNPs in clinical trials, and 3 MNPs on the market.

Very interesting predictions have emerged out of studies done by researchers in this area:

i. The global market for marine-derived drugs by type was around $4.8 billion in 2011, which is expected to be around $5.3 billion in 2012. This global market is forecasted to reach $8.6 billion by 2016 at a compound annual growth rate (CAGR) of 12.5% for the 5-year period of 2011–2016.

ii. The market for sponges was $3 billion in 2010 and is expected to reach around $3.2 billion in the year 2012. BCC has forecasted that this market will reach nearly $4 billion by 2016 at a CAGR of 5% for a 5-year period.

iii. Mollusk is the fastest growing market and is expected to grow from $69.4 million in 2011 to $490.1 million by 2016 at a CAGR of 47.8% [321].

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Chapter 9

14β-Hydroxypregnanes from Succulent Plants Belonging to *Hoodia gordonii* and *Caralluma* Genus: Extraction, Biological Activities, and Synthesis

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Chapter Outline

INTRODUCTION

Plants are vital to humans for several reasons, for example, photosynthesis and sources of food. Plants also represent more or less an infinite source of bioactive natural products. In this context, we focus on succulent plants,

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especially the genera *Caralluma* and *Hoodia* belonging to subfamily Asclepiadoideae in the family of Apocynaceae. *Carallumas* grow widely in India, Pakistan, Sri Lanka, the Canary Islands, Southern Europe, and Afghanistan, and *Hoodia* is mainly found in the Kalahari desert in Southern Africa. These plants especially *Caralluma adscendens* var. *fimbriata* and *Hoodia gordonii* received a growing interest in the past 20 years because it was claimed that the latter presented appetite-suppressant and weight loss properties. The bioactive compounds that were isolated from *C. adscendens* var. *fimbriata* and *H. gordonii* were glycosteroids, specifically 14β-hydroxypregnane derivatives. It is known since a long time that the Bushmen tribes of Southern Africa chew on fresh sap of *H. gordonii* during their long hunting trips across the Kalahari desert in order to reduce hunger and increase energy. Therefore, it was claimed that these succulent plants could represent a new help for fighting obesity, which is one of the major health problems in the twenty-first century. Indeed, worldwide, more than 300 million people are obese, one billion are overweight, and the increase in childhood obesity is a worrisome problem. In the present review, the isolation of 14β-hydroxypregnane derivatives from *C. adscendens* var. *fimbriata* and *H. gordonii* will be described followed by a description of their biological activities. Finally, synthetic approaches of glycosteroids and aglycones as well as total synthesis of the latter will be presented.

ISOLATION OF 14β-HYDROXYPREGNANE GLYCOSIDES FROM *CARALLUMA* SPECIES

Plants from the *Caralluma* genus are a group of perennial, tender, succulent plants belonging to the family Apocynaceae, subfamily Asclepiadoideae. Succulent plants in the Apocyanaceae family are known by the basioname of Stapelia, which are further divided into two subgroups. Trichocaulon subgroup consists of five genera of succulent plants such as *Hoodia*, *Lavrania* (also known as smooth trichocaulon), *Pseudolitos*, *Larryleachia*, and *Whitesloanea*. The second subgroup, namely, *Caralluma* subgroup, encompasses 18 genera of succulents, the genus *Caralluma* being one of them. Plants from the genus *Caralluma* are distributed throughout Africa and Asia. This genus consists of 260 species [1,2]. Plants in this group vary from thin, recumbent stems from 0.5 to 1.5 in. thick to erect-growing clumpy stems up to 8 in. tall. Mostly, the stems are angular with rudimentary leaves. Flowers of *Caralluma* are star shaped with fleshy texture and emit a foul smell (Fig. 1). Commonly, the flowers appear during late summer and are mainly yellow, red, purple, or black in color. They are 1–2 in. across and are borne near the base of the stem [2–4].

Several phytochemical studies on various species of *Caralluma* have been carried out during the past 25 years. While most of the studied species of *Caralluma* have been reported to contain a wide range of pregnane glycosides, there are some reports of fatty acids, sterols, flavonoids, and

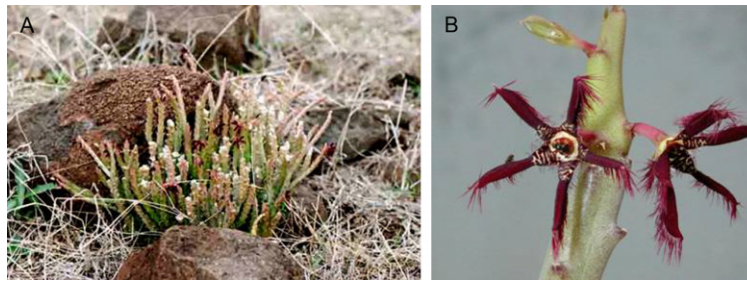


FIGURE 1 Morphology of *Caralluma fimbriata*: (A) *C. fimbriata* and (B) flowers of *C. fimbriata. (photo credit: Vaishali Joshi, NCNPR, University of Mississippi).*

megastigmane glycosides from *Caralluma arabica*, *Caralluma edulis*, *Caralluma nagevensis*, and *Caralluma tuberculata* [5–8]. The most challenging aspect in the characterization of phytochemicals is the structure elucidation of the isolated constituents. In case of pregnane glycosides, identifying the sugar moieties of a glycoside and establishing the structure of glycoside chains were cumbersome. Furthermore, determining the configuration at chiral centers of aglycone was a daunting task for natural products chemists. Recent advances in 2D-nuclear magnetic resonance (NMR) spectroscopy, high-resolution mass spectrometry, high-performance liquid chromatography coupled with mass spectrometer (HPLC-MS), and circular dichroic (CD) spectroscopy have enabled elucidation of complex glycoside structures along with the stereochemical features. ^{1}H-^{1}H COSY HMBC and NOESY NMR experiments are helpful in characterizing the glycoside chains and substitutions on the sugar moieties of a glycoside. Applications of X-ray diffraction (XRD) and electronic circular cichroic spectroscopy are helpful in acquiring information about the absolute configurations at chiral centers of a molecule. For most part, sugar analyses of a glycoside have been carried out by acid hydrolysis of the glycoside followed by characterization of sugars. When authentic reference standards of sugars are available, a simpler method such as thin layer chromatography can help in the identification of sugars. However, determination of absolute configurations of sugars (such as identifying D-glucose over L-glucose) involves application of specialized methods such as gas chromatograph coupled with mass spectrometer (GC-MS) and HPLC-MS. GC-MS analysis of alditol acetates of sugars has been useful in identifying sugar moieties of glycosides [9]. Further conversion of an aldose sugar to the respective acetylated thiazolidine derivatives via reaction with L-cysteine methyl ester hydrochloride has been used as a successful approach for the determination of absolute configuration of sugars [10–12]. These techniques have enabled the characterization of several pregnanes and pregnane glycosides from various species of *Caralluma*.

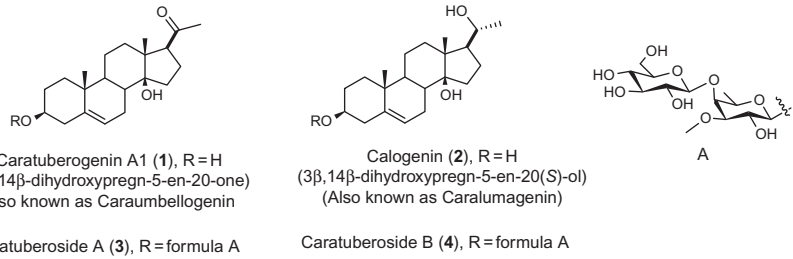


FIGURE 2 Structures of compounds **1–4**.

One of the earliest reports of isolation of constituents from *C. tuberculata* reported the presence of glycosides containing 3β,14β-dihydroxypregn-5-en-20-one (caratuberogenin A1, **1**) and pregn-5-ene-3β,14β,20-triol (calogenin, **2**) as the aglycones. These glycosides were named caratuberoside A (**3**) and B (**4**), respectively (Fig. 2) [13]. Typically, these pregnane aglycones consist of a 21-carbon skeleton with oxygenated functional groups at C-3, C-14, and C-17. Apart from the β-methyl attachment at the ring junctions, a characteristic of steroid skeleton, the presence of β-OH substitutions at C-3 and C-14 is common among most of the glycosides reported from *Caralluma*.

Caratuberoside A2 (**5**) has also been reported from *C. tuberculata*, which consists of 3β,14β-dihydroxypregn-5-en-20-one as the aglycone. However, the glycosylation in this case was seen at the C-14 instead of the commonly observed C-3 position [8]. Another species, *C. umbellata*, which is indigenous to southern India, has shown the presence of pregnane glycosides containing caraumbellogenin (3β,14β-dihydroxypregn-5-en-20-one), and calogenin as aglycones [14]. Some of these constituents from *C. umbellata*, namely, caraumbelloside IV (**6**) and caraumbelloside V (**7**) also contained a benzoyl substitution on the sugar moieties [15,16]. Esterification at the free hydroxy groups on pregnane skeleton is commonly reported by isolation of many more glycosides, wherein acetylation and benzoylation at the hydroxy functionalities at C-12, C-14, C-15, and C-20 are reported. Examples of such phytochemicals include the glycosteroids reported from *Caralluma retrospiciens* (**8**, **9**) [17], *Caralluma penicillata* (**10–16**) [18,19], and *Caralluma stalagmifera* (**17–20**) [20] (Fig. 3). In some cases, glycosides bearing methylbutyryl, isovaleroyl, *o*-aminobenzoyl, and nicotinoyl ester substitutions at C-20 of the aglycone have also been reported from *Caralluma dalzieii*. Structures of these glycosides (**21–24**) are shown in Fig. 4 [21].

Apart from the formation of esters, free hydroxy groups on the pregnane aglycones of *Caralluma* also present an opportunity for glycosylation reactions, giving rise to bisdesmosides. This is evident from various bisdesmosidic pregnane glycosides isolated from various species of this genus. The bisdesmosides usually contain one glycosidic chain containing one or more sugars attached at C-3 of pregnane skeleton, while the second glycosidic linkage can be located at one of the hydroxyl groups at C-12, C-14, C-15, or C-20.

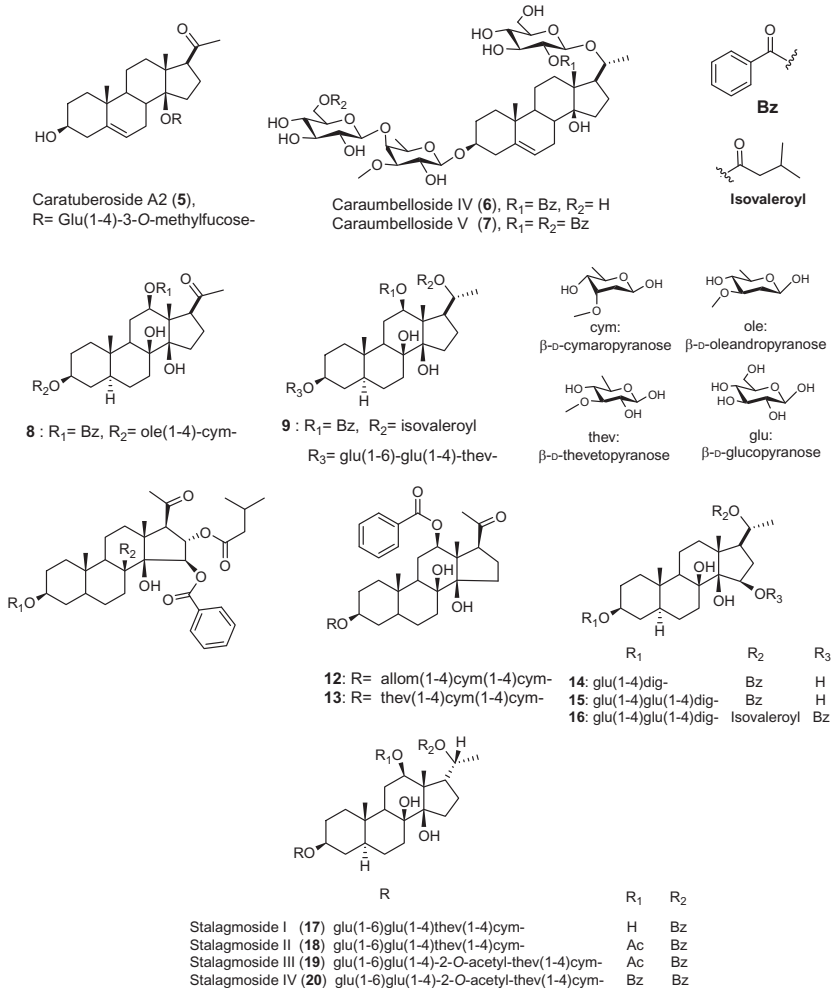
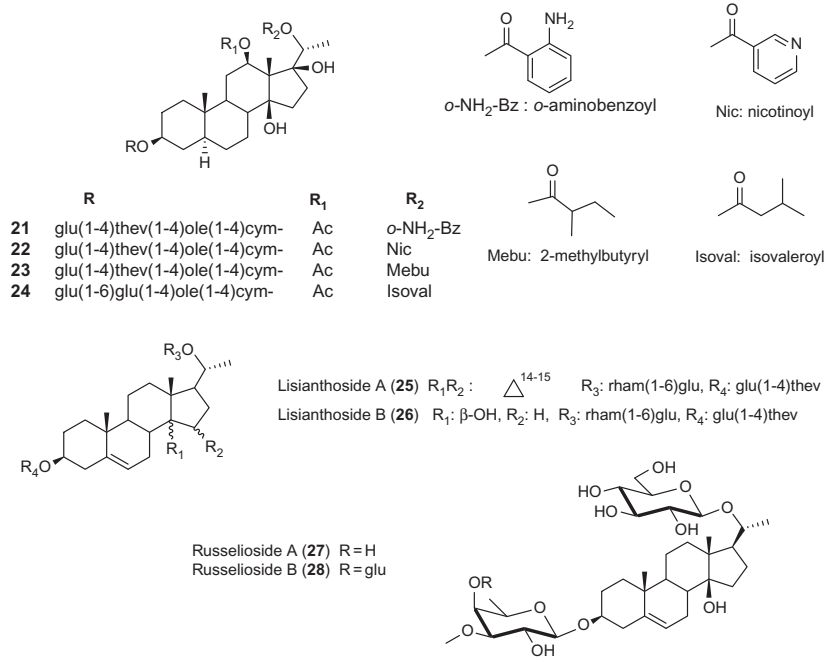


FIGURE 3 Structures of compounds **5**–**20**.

A few examples of the bisdesmosides include Lasianthosides A (**25**) and B (**26**) from *Caralluma lasiantha* [22], and Russeliosides A and B (**27**, **28**) from *C. russeliana* [23].

Recently, *Caralluma fimbriata* has gained popularity as a botanical supplement due to its proposed appetite-suppressant activity. Close examination of the botany of this plant reveals that *C. fimbriata* is in fact a variety of *C. adscendens*, which is indigenous to the central and southern parts of India, Nepal, and Sri Lanka. This species consists of six different varieties, namely, *C. adscendens* var. *adscendens*, *C. adscendens* var. *attenuata*, *C. adscendens* var. *carinata*, *C. adscendens* var. *fimbriata*, *C. adscendens* var. *geniculata*, and *C. adscendens* var. *gracilis*. All of these varieties share same geographical



	R	R_1	R_2
21	glu(1-4)thev(1-4)ole(1-4)cym-	Ac	*o*-NH_2-Bz
22	glu(1-4)thev(1-4)ole(1-4)cym-	Ac	Nic
23	glu(1-4)thev(1-4)ole(1-4)cym-	Ac	Mebu
24	glu(1-6)glu(1-4)ole(1-4)cym-	Ac	Isoval

FIGURE 4 Structures of compounds **21–28**.

habitat and are differentiated from each other based on the shapes of their stems and sizes of flowers [24]. *C. adscendens* var. *fimbriata* is commonly known as *C. fimbriata* and has achieved commercial reputation as a possible aid in weight loss. However, there have been very few studies exploring systematic chemical composition of *C. fimbriata*. Sawant *et al*. had reported the isolation of a waxy solid hydrocarbon compound, pentatriacontane from *C. fimbriata* [25,26]. Recently, Kunert *et al*. reported the isolation of 13 pregnane glycosides from *C. fimbriata* (compounds **29–41**) (Fig. 5). Aglycones for all of the glycosides bore an esterification at the 12-β-hydroxy group, while the aglycone for compounds **38–41** showed the presence of an ester at C-19 methyl group. Glycosidic chains for all of the glycosides were attached at C-3 [27]. In another study reported in 2011, two pregnanes (**42**, **43**) and a pregnane glycoside (**44**) have been reported from *C. adscendens* var. *gracilis* [28].

ISOLATION OF 14β-HYDROXYPREGNANE GLYCOSIDES FROM *H. GORDONII*

There are around 2000 species of plants that belong to the family Apocynaceae. They are split into 300 genera, about 50% of which are succulent plants [24,29,30]. *Hoodia* are bitter-tasting cactus-like plants. Their tall, leafless,

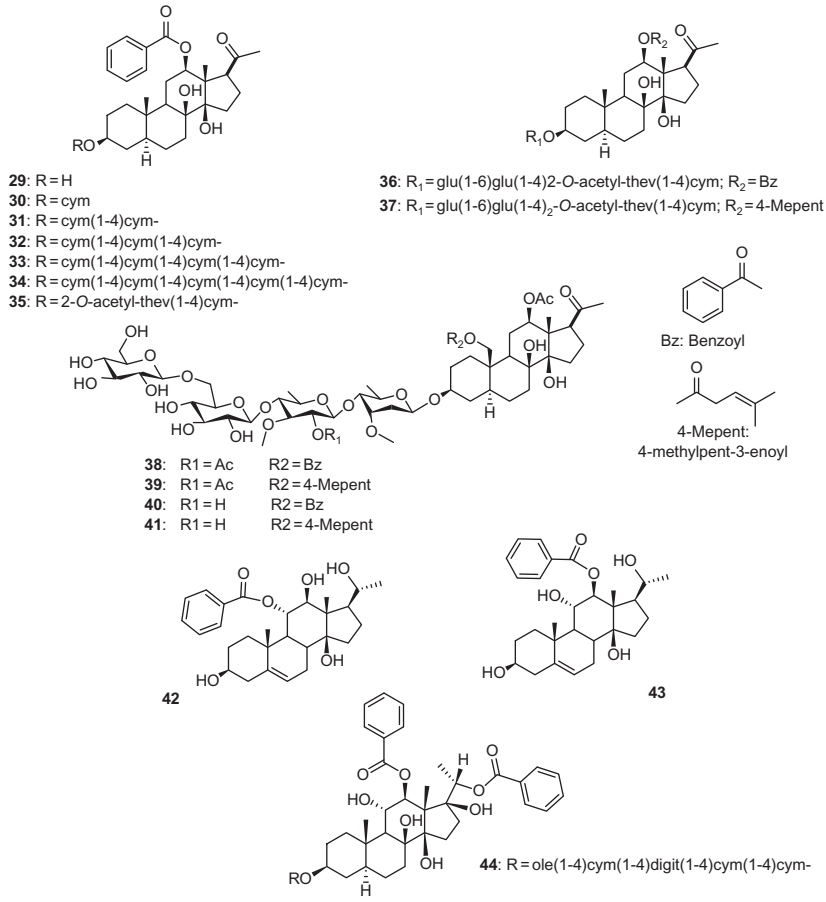


FIGURE 5 Structures of compounds **29**–**44**.

swollen, spiny stems, with 10–35 rows of spiny tubercles, give them an appearance similar to the cacti, which is why they are often mistakenly called "African Hoodia Cactus." In 1773, the first species of *Hoodia*, *H. pilifera* was discovered by Thunberg. Due to its smaller flowers (<15 mm diameter) and distinctly lobed corolla, *H. pilifera* was initially placed in the traditionally known *Trichocaulon* genus. However, as the species with bigger flowers were later discovered, the genus *Trichocaulon* was replaced with the name *Hoodia* [31].

Hoodia thrives in extremely hot weather and takes many years to mature. In the early stages of growth, only one small stem is produced. Later as the plant grows, it starts forming branches. At maturity, these plants can have as many as 50 individual branches and weigh as much as 66 lbs. Under ideal growing conditions, a *Hoodia* plant can attain a height of about 1 m. The flowering season starts around August. Flowers are borne on or near

the terminal apex (top part of the plant). These flowers resemble petunia flowers and are red, purple to brown, or mottled dark yellow in color, depending on the species. A full-blown flower can reach diameter of 70–75 mm and has a typical carrion-like odor (similar to rotten meat). The mature *Hoodia* plants produce seeds around October and November. These seeds bear capsules, which resemble in shape to an antelope or a goat horn. Because of this unique appearance of the seeds, *Hoodia* has the Afrikaans common name of *Bokhorings* [31,32]. The light brown, flat-shaped seeds of *Hoodia* have a pappus of fluffy hair attached to one end, which acts as a parachute when the seed pod splits open. With the wind, these seeds are blown away from the parent plant where they can establish themselves. Pollination by flies is also an alternate way for propagation. It has been observed that the flies are attracted by the typical odor given off by the flowers and thus act as the ecological carriers for *Hoodia* seeds. Usually, a young *Hoodia* plant begins its development under the protection of a nurse plant, which protects it from extreme climatic conditions [24,32]. Kalahari deserts in Africa host a wide range of taxa of *Hoodia* genus. There are about 16 species of this desert plant, and they occur mainly in the summer rainfall regions in Angola, Botswana, Namibia, and South Africa as well as the winter rainfall regions in Namibia [24]. However, the major centers of diversity for *Hoodia* are in Namibia (11 species) and South Africa (9 species). Due to harsh climatic conditions, slow growth, and overexploitation for commercial purposes, most of the *Hoodia* species have been classified as "threatened" by the CITES (Convention on International Trade in Endangered Species of wild Fauna and Flora) [24,32,33]. Because of the claims of appetite-suppressant properties, *H. gordonii* has become a popular weight control herb (Fig. 6) [30,33]. However, knowledge about the chemical constitution of *H. gordonii* was very limited until recently.

In 1997, studies conducted in South Africa reported the presence of a pregnane glycoside, P57AS3 (**45**), from *H. gordonii* [34,35] (Fig. 7). P57 consists of 14-hydroxy-12-[(2*E*)-2-methyl-1-oxo-2-butenyloxy]-(3β,12β,14β)-pregn-5-en-20-one as the aglycone moiety. The sugars attached at the C-3 position of this aglycone are β-D-cymaropyranose (2,6-dideoxy-3-*O*-methyl-β-D-ribohexopyranose) and β-D-thevetopyranose (6-deoxy-3-*O*-methyl-β-D-glucopyranose) [12,36]. Shukla *et al.* later reported the isolation of the aglycone for P57AS3 from the extract of *H. gordonii*. Structure of this aglycone was established based on NMR spectroscopy and XRD studies. This compound was named as Hoodigogenin A (**46**) [37]. Further extensive phytochemical studies performed at the University of Mississippi, USA have resulted in characterization of several pregnane glycosides from *H. gordonii* [11,12,38].

Most of the reported glycosides comprise Hoodigogenin A and Calogenin as aglycones. Hoodigosides A–K (**47**–**57**) and Hoodigoside W (**58**) consisted of Hoodigogenin A as the aglycone, while the glycoside linkages were attached at the C-3 of aglycone [12] (Fig. 7). Furthermore, 13 Calogenin bisdesmosides, Hoodigosides L–V, and Hoodigosides Y–Z (**59**–**71**) were isolated

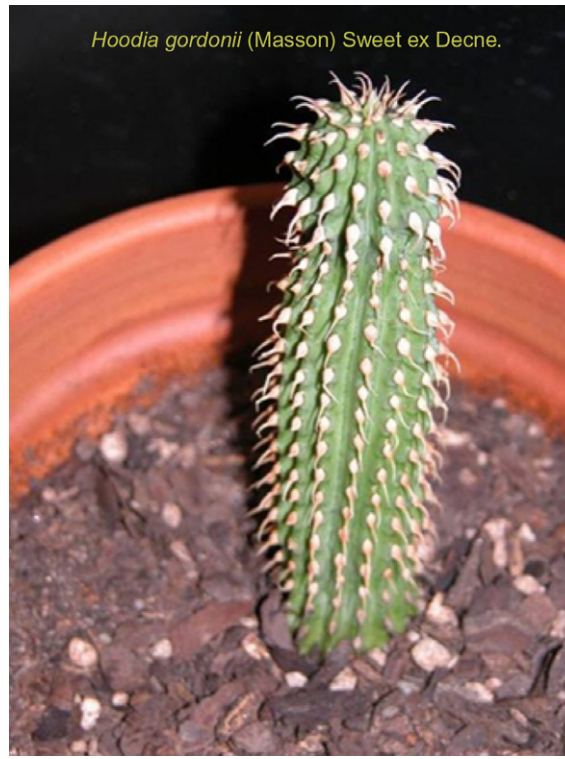


FIGURE 6 Morphology of *Hoodia gordonii*. *(photo credit: Vaishali Joshi, NCNPR, University of Mississippi).*

from methanolic extract of *H. gordonii*, wherein the glycosidic chains were attached at C-3 and C-20 of the calogenin aglycone. Hoodigoside X (**72**) was observed to be a glycoside of isoramanone [11]. Recently, Shukla *et al.* have also reported the characterization of two novel 5 (6→7)*abeo*-pregnane bisdesmosides, Hoodistanalosides A and B (**73**–**74**), which consist of a five-membered ring B of the pregnane aglycone resulting in formation of an aldehyde at C-6 (Fig. 8) [38].

BIOLOGICAL ACTIVITIES

Caralluma Species

Various plants from *Caralluma* genus have been used for centuries, by the tribes in India, Arabian subregions, and eastern parts of Africa. They have been used as a part of food and have also been claimed to possess various medicinal properties. Plants from *Caralluma* genus are considered edible

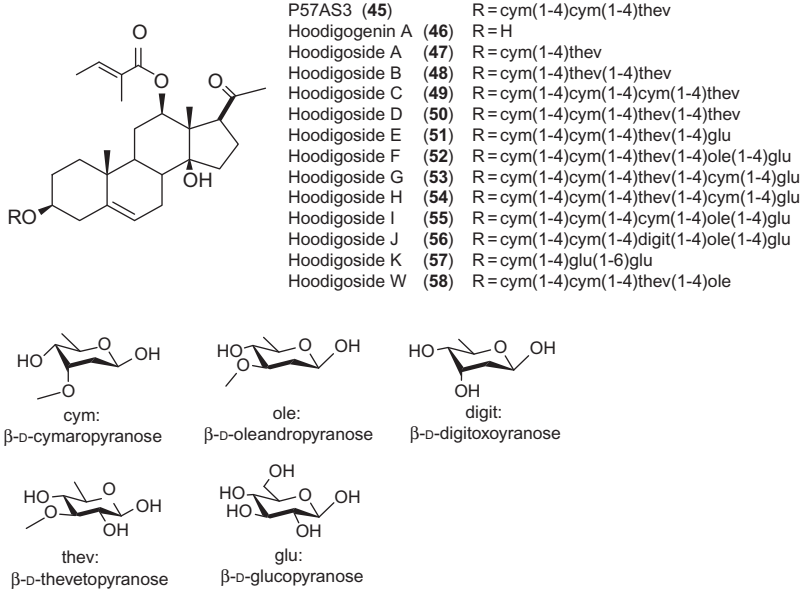
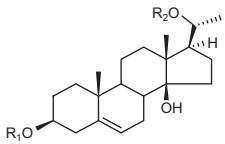
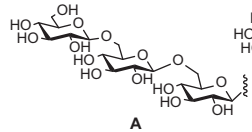
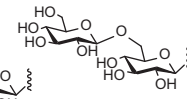


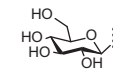
FIGURE 7 Structures of compounds **45–58**.

and possess history of safe use as food ingredients by natives of India and Eastern Africa [39,40]. Some of the folklore uses of *Caralluma* include treatment of tuberculosis [41], aphrodisiac, analgesic, antiemetic [42], treatment of maigraine, and diabetes [27]. *C. adscendens* var. *fimbriata* Roxb. is found in the hilly regions of the western states of India at altitudes of up to 600 m [4]. In India, it is also known by the common names like *Ranshabar*, *Makad Shenguli*, *Kullee Mooliyan*, and *Yungmaphallottama* [43]. Tribal people in Southern parts of India use *C. fimbriata* as a vegetable in their daily diet. Aerial parts of *C. fimbriata* are eaten raw, cooked with spices to make a curry, or are occasionally preserved as pickle. It is also used by the Indian tribes as famine food. In the arid and semi-arid regions of India, *C. fimbriata* is consumed as a substitute of food, in times of drought and scarcity of food [40]. In the Indian traditional system of medicines (Ayurveda), juice of *C. fimbriata* is mixed with black pepper (*Piper nigrum*) and is used for the treatment of migraine as well as diabetes [39]. Recently, *C. fimbriata* was introduced into the US market as a dietary supplement for weight loss. With the increased popularity of the African succulent plant *H. gordonii*, botanical supplements containing *C. fimbriata* have been widely publicized as the "Indian *Hoodia*." Taking into account the folklore use of *C. fimbriata* as a famine food, it has been claimed to possess appetite-suppressant qualities. *C. fimbriata* extracts have been evaluated for efficacy and safety by human clinical study. Only two reports of clinical study are available. A randomized clinical study conducted in India using 50 human subjects proposed that *C. fimbriata* extracts

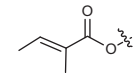


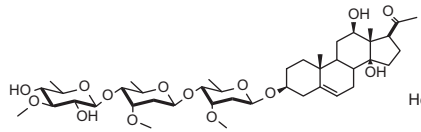


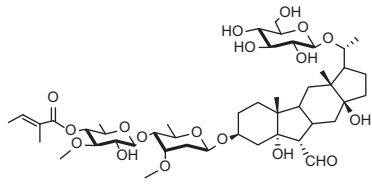




		R_1	R_2
Hoodigoside L	(**59**)	cym(1-4)thev(4-tigl)	A
Hoodigoside M	(**60**)	cym(1-4)thev	A
Hoodigoside N	(**61**)	cym(1-4)thev	H
Hoodigoside O	(**62**)	cym(1-4)thev(4-tigl)	B
Hoodigoside P	(**63**)	cym(1-4)ole(4-tigl)	A
Hoodigoside Q	(**64**)	cym(1-4)cym(1-4)thev(4-tigl)	A
Hoodigoside R	(**65**)	cym(1-4)cym(1-4)ole(4-tigl)	A
Hoodigoside S	(**66**)	cym(1-4)cym(1-4)cym(1-4)ole(4-tigl)	A
Hoodigoside T	(**67**)	cym(1-4)cym(1-4)cym(1-4)ole(4-tigl)	B
Hoodigoside U	(**68**)	cym(1-4)cym(1-4)cym(1-4)cym(4-tigl)	A
Hoodigoside V	(**69**)	ole(1-4)thev(4-tigl)	C
Hoodigoside Y	(**70**)	cym(1-4)thev	C
Hoodigoside Z	(**71**)	cym(1-4)cym(1-4)thev(4-tigl)	C







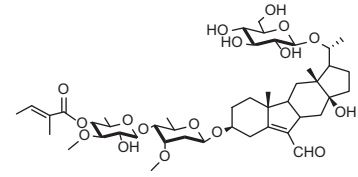


FIGURE 8 Structures of compounds **59**–**74**.

have appetite-reducing activity [44]. Hydroalcoholic extract of the aerial parts of *C. fimbriata* was tested at 1-g daily doses given prior to meals to 25 obese individuals with a body mass index (BMI) > 26 [45]. A placebo group of 25 obese individuals was used for comparison. All subjects were advised to walk for 30 min in the morning and evening. At the end of 1-month trial, the test group reported reduction in body weight by about 3% and reduction in the BMI by a scale of 1. A second clinical study was performed at the Western Geriatric Research Institute, Los Angeles, USA [45]. Under similar study parameters and doses of 1 g daily, it was reported that 83% of test subjects lost 1% or more of body weight with a decrease of 1 point on the BMI scale. However, there have not been any scientific reports analyzing the safety and toxicity of this plant.

Various species of *Caralluma* have also been studied for antihyperglycemic activity. *C. edulis* has been evaluated for its antidiabetic properties [46,47].

The fresh juice of *C. tuberculata* was evaluated for hypoglycemic activity *in vivo*. Along with reduction in the blood glucose levels, it also showed improvement in glucose tolerance [48]. *C. attenuata* has also showed significant antihyperglycemic activity [47,49]. In a similar study, oral administration of alcoholic extract of *Caralluma sinaica* at increasing dosing levels (50, 100, 150, and 200 mg/kg.) in healthy rabbits caused significant reduction in blood glucose levels ($P < 0.01$) [50].

Pregnane glycosides isolated from various *Caralluma* species have been assessed for potential antiparasitic activity. Acylated glycosteroids from *C. penicillata*, *C. tuberculata*, and *C. russelliana* showed antitrypanosomal and antiplasmodial activity using *in vitro* assay methodologies. It was observed that the presence of acetylation on the aglycone or sugar moieties was a required structural parameter for antitrypanosomal activity [51,52].

Six pregnane glycosides from *C. tuberculata* also exhibited weak to moderate antimalarial activity and moderate antitrypanosomal activity [53]. Furthermore, cytotoxic potential of *C. tuberculata* was evaluated in a study involving conjoined administration with known anticancer drug cyclophosphamide. It was observed that use of *C. tuberculata* extract reduced the effectiveness of cyclophosphamide at DNA level, while causing further inhibition of RNA synthesis. This resulted in increased cytotoxicity [54]. In another study, glycosteroids from *C. dalzielii* were reported to possess antiproliferative activity when tested against various cell lines such as murine monocyte/macrophage (J774.A1), human epithelial kidney (HEK-293), and murine fibrosarcoma (WEHI-164) cell lines 31 [20]. *C. tuberculata* and *C. arabica* have exhibited protective effects on gastric mucosa. The ethanolic extract of *C. tuberculata* was screened for its potential gastroprotective action against injuries caused by 80% ethanol, 0.2 M NaOH, hypertonic saline, and indomethacin [54,55]. Several studies have also reported anti-inflammatory, antihyperglycemic, antinociceptive, and antiulcer properties of *C. arabica* [56–58]. Extracts of *C. stalagmifera* and *C. attenuata* are reported to possess anti-inflammatory activity [59]. Similarly, *C. umbellata* has also been evaluated for anti-inflammatory and antinociceptive activity [3].

Hoodia gordonii

Hoodia was not well known to the western world until recently. However, traditional uses of *Hoodia* are dated many centuries back, when it became an integral part of diet for the San tribes, in the Kalahari Desert. The San people, a tribe of hunters in Kalahari, have used the sap of *H. gordonii* for curbing the sensations of hunger and thirst, during the long hunting trips in the desert. Various other folkloric uses such as cure for abdominal cramps, hemorrhoids, tuberculosis, indigestion, hypertension, and diabetes have been recorded among Anikhwe tribes (Northern Botswana), Hai Om (Northern Namibia), Khomani (Northwestern S. Africa), and the Xun and Khwe tribes (Angola)

[60–62]. First scientific report substantiating the claims of appetite-suppressant activity of *Hoodia* was published in 1997, wherein it was found that extracts of *Hoodia* species, particularly *H. gordonii* and *H. pilifera*, possessed appetite-suppressant effects [63,64]. Fractionation of the extracts coupled with *in vivo* studies in rats led to the isolation of pregnane glycoside P57AS3 (Fig. 5). Upon administration of *Hoodia* extracts or P57AS3 (also referred to as P57), significant reduction in weight and body mass was observed [61]. Further patents for application of *H. gordonii* extracts for antidiabetic action and prevention of gastric damage caused by aspirin have also been granted [65,66]. In a supportive study by Tulp *et al*., which was published as a conference abstract, 2% (w/v) aqueous homogenate of dehydrated *Hoodia* sp. was administered to lean and obese mice. It resulted in a decreased food intake within 48 h. The body weight of the obese mice, initially twice the weight of the lean mice, decreased to near-normal weight after 2–3 weeks of dosing with *Hoodia*, and the body fat percentages in lean as well as obese mice (in treatment group) were decreased compared to mice in control group, which were fed a normal diet [67]. The appetite-suppressant potential of *H. gordonii* was further validated in 2007 when oral administration of P57 to female rats using five dosing levels (6.25–50 mg/kg) was performed for 3 consecutive days, followed by monitoring of weight loss for 8 days. Fenfluramine, a pharmaceutical antiobesity drug, was used as a positive control for comparison. While both fenfluramine and P57 administration resulted in decreased food intake, a higher body mass gain was noted for fenfluramine compared to that for P57 [68].

Although above reports demonstrated the appetite-suppressant activity of *H. gordonii*, the exact mechanism of action and the pharmacological target(s) responsible for the above effect have been elusive. An *in vitro* study by MacLean *et al*. attempted to address this issue in 2004 [36]. It was postulated that P57 acts at the hypothalamus in midbrain, which regulates food intake. An intracerebroventricular (i.c.v.) administration of P57 in rats led to increase in the levels of ATP in hypothalamus neurons. P57 injections into the third ventricle of hypothalamus *in vivo*, at doses of 0.4–40 ηM, reduced 24-h food intake by rats up to 60%. Subsequent experiments also showed that in rats fed with a low-calorie for 4 days, the content of ATP in the hypothalamii fell by 30–150%. This effect was blocked by i.c.v. injections of P57. The neurons of hypothalamus regulate appetite-sensing mechanisms by using ATP as one of the key signaling molecules. At a state of food deprivation or reduced food intake, the ATP content in hypothalamus is reduced, while a full stomach leads to increase in ATP levels [36]. Though this mechanism of action for P57 contends increased ATP production as energy-sensing signaling mechanism of CNS, there has been no further evidence to establish the exact molecular target for P57, which may be responsible for the altered levels of ATP. Furthermore, this CNS-mediated mechanism of action does not preclude peripheral actions of *H. gordonii*. In Ross 308 broiler chickens, daily supplementation with a *H. gordonii* meal

(300 mg) had no effect on feed intake, digestibility, and growth but reduced fat pad weights by 40%. Since the carcass quality and feed efficiency are reduced by excessive fat, *H. gordonii* supplementation in chicken diet may have a beneficial effect. However, further investigation is needed to establish the mechanism for this activity [69].

A double-blind, placebo-controlled clinical study of *H. gordonii* extract was conducted by Phytopharm, a European nutraceutical company, based in England. In 2001, this clinical study claimed efficacy of *H. gordonii* extract to suppress appetite in humans upon oral administration [70]. Effect of purified P57 compound in 19 overweight male individuals was evaluated, as compared to a placebo group of 19 overweight males. It was observed that the treatment group achieved a 30% reduction in calorie intake as compared to the placebo group. However, the exact dosing of P57 and details of safety tolerability and pharmacokinetic properties of P57 could not be conclusively established from this study [71]. The Phytopharm press release relating to this study has been removed from their Web site (http://www.phytopharm.co.uk). In another randomized, double-blind clinical trial, scientists at Unilever tested the efficacy of purified *H. gordonii* extract containing 79.3% w/w steroidal glycosides in 15 overweight women [72]. It was observed that oral administration of 2.2 g *H. gordonii*-purified extract per day did not lead to significant changes in body weight and energy intake in the treatment group as compared to the placebo group. Moreover, the treatment group receiving *H. gordonii* extract reported adverse reactions such as nausea, vomiting, changes in touch sensations, and increased blood pressure, pulse, bilirubin, and alkaline phosphatase levels [72]. It is noteworthy that the results of the two clinical trials above are not directly comparable. Clinical trial by Phytopharm involved administration of purified P57 glycoside as the test compound, while scientists at Unilever tested a standardized extract containing steroid glycosides and other unknown constituents. Pharmacological activity of a plant or plant extract is exerted by the presence of specific active constituents in the same. In this case, clinical study by Phytopharm proposed P57 as the active steroidal glycoside from *H. gordonii*. However, no synergistic effect was observed due to the presence of several steroid glycosides (79.3% w/w) in the extract tested by Unilever. Further, the outcome of this study did not report significant weight loss in the test group [72]. Thus, scarcity of clinical studies, conflicting results, and inaccessibility of the data from these studies raise more questions regarding use of *Hoodia* as antiobesity supplement in humans. On the contrary, there are a wide number of products in the US market, which claim to contain authentic *H. gordonii*, and are sold as over-the-counter botanical supplements. Analysis of these products revealed that many of the marketed products did not contain P57, the proposed active constituent and chemical marker for establishing the authenticity of *H. gordonii* [63,73]. This has led to concerns about authenticity, quality, and safety of the products containing *Hoodia*. Consequently, a few safety and toxicity assessment studies have been

performed on *H. gordonii*. Hoodigosides A–K and P57 were evaluated for cytotoxic and antioxidant properties *in vitro*. The compounds were not cytotoxic and did not show any inhibition of growth against a panel of cell lines including SK-MEL, KB, BT-549, SKOV-3, VERO, and LLC-PK1 up to the highest concentration of 25 μg/mL. No antioxidant activity was recorded in HL-60 cells, and intracellular reactive oxygen species generation was not inhibited by any of the tested compounds [12]. Extract of *H. gordonii* was tested for genotoxicity using *in vitro* assay techniques and was found to be nongenotoxic at up to 400 mg/kg dosing [74]. Prenatal developmental toxicity studies have also been performed to evaluate effects of *H. gordonii* consumption on fetal development in mouse as well as rabbit [75,76]. Oral administration of *H. gordonii* extract at 50 mg/kg/day in gestational mice caused significant reduction in feed intake and body weight, while at doses more than 15 mg/kg/day, weight of uterus, fetal weights, and ossification of bones were affected [75]. Similarly, reduction in feed intake and body weight was observed in rabbits treated with 6 and 12 mg/kg/day of *H. gordonii* extract. However, there were no observed effects on fetal weight and development at all the tested doses [76].

SYNTHETIC APPROACHES TO THE AGLYCONES OF 14β-HYDROXYPREGNANE GLYCOSIDES

We focus on the synthesis of 14β-hydroxypregnane derivatives bearing an A/B trans ring junction and (or) a $\Delta^{5,6}$ double bond. Compounds bearing an A/B *cis* skeleton are mainly found in cardenolides and their synthesis was recently reviewed by Heasley [77].

As it was shown above, the main aglycones found in *Caralluma* (caraumbellogenin, caralumagenin, calogenin) and *H. gordonii* (isoramanone, digipurpurogenin I, boucerin) are characterized by a 14β-hydroxy pregnane skeleton bearing a $\Delta^{5,6}$ double bond, a 3β-hydroxy group, a ketone, or a hydroxyl group at position 20 and a 12β-hydroxyl group or no 12β-hydroxyl group (Fig. 9).

The introduction of a 14β-hydroxy group on a pregnane skeleton was pioneered by Bladon *et al*. [78]. These authors showed that the treatment of hecogenin acetate **80** which is commercially available by UV irradiation promoted a Norrish reaction affording lumihecogenin acetate **81** which on further irradiation underwent Paterno Büchi cyclization to photohecogenin acetate **82**. After chromic oxidation of compound **81**, the 14-hydroxy derivative was obtained and a 14α configuration was assigned by Bladon *et al*. However, almost 20 years later, Welzel *et al*. examined carefully the photochemical reaction of hecogenin acetate [79]. They observed that the irradiation length can be shortened (25 min instead of 9 h) and that the addition of dilute acetic acid to lumihecogenin acetate **81** promoted a Prins reaction yielding a mixture of 12β,14β-dihydroxy pregnane **83** and 12α,14β-dihydroxy pregnane **84**, the

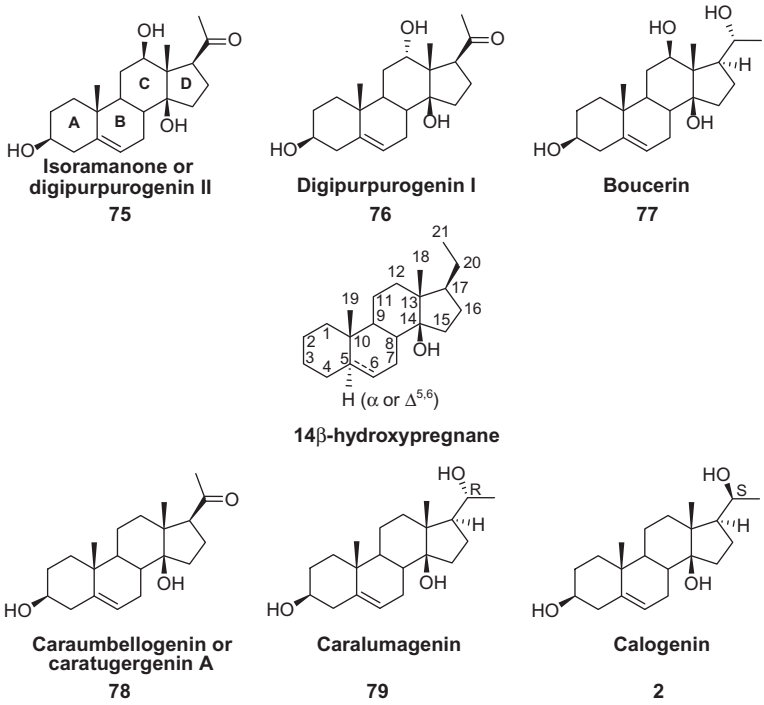
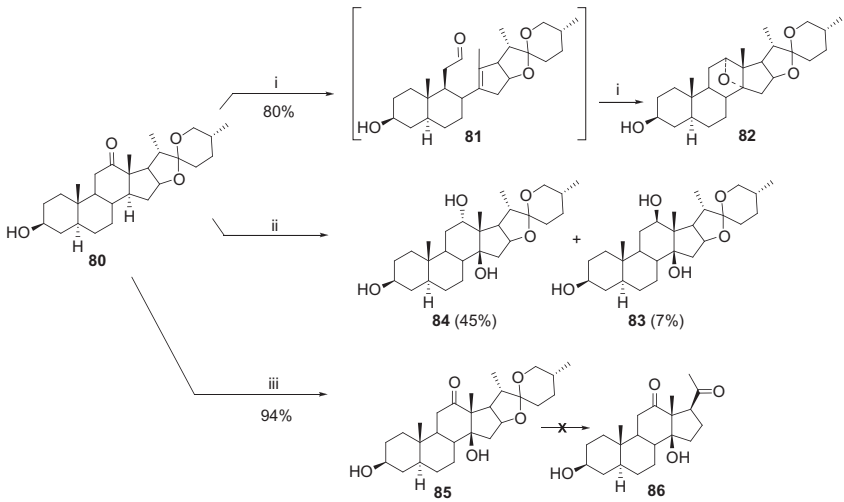


FIGURE 9 Aglycones of glycosteroids extracted from *C. fimbriata* and *H. gordonii*.

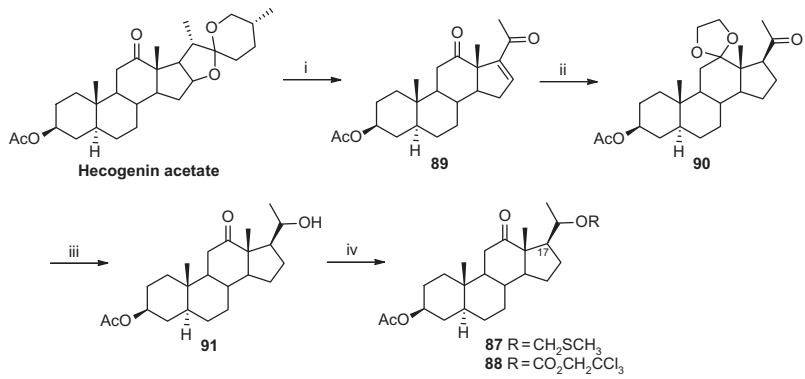
latter being the major compound. These results were improved by Fuchs *et al.* who were able to obtain the keto derivative **85** in 94% yield after a reaction sequence Norrish–Prins–Jones oxidation [80]. Unfortunately, the 14β-hydroxy group was unstable to the reaction conditions necessary (Ac_2O, >155 °C) for spiroketal ring opening leading exclusively to a complex mixture of compounds and not to the desired 14β-hydroxy derivative **86** (Scheme 1).

Thus, the Norrish–Prins reaction starting from hecogenin acetate did not allow an access to 14β-hydroxypregnane derivatives bearing either a carbonyl group or a hydroxyl group at position 17.

To address this drawback, Welzel *et al.* decided to study the Norrish–Prins reaction starting from pregnane derivatives **87** and **88** bearing a hydroxy protected group at position 17 [81]. The latter were obtained by the degradation of hecogenin acetate spiroketal moiety according to know procedures [82]. They were able to optimize the reaction sequence so that the 12-keto pregnane derivative **89** is now available in 67% overall yield [83]. After catalytic hydrogenation of the $\Delta^{16,17}$ double bond, a regioselective protection of the 12-keto group was achieved by addition of ethylene glycol in the presence of BF_3Et_2O affording compound **90**. Finally, a stereoselective reduction of the 20-keto group using Luche conditions yielded the pregnane derivative



SCHEME 1 i: h, quartz, dioxane, 9 h, 25°C; ii: (a) h, quartz, dioxane, 25 min, 25°C; (b) AcOH, H_2O; iii: (a) h, quartz, dioxane, 25 min, 25°C; (b) AcOH, H_2O; (c) Jones oxidation.



SCHEME 2 i: (a) NH_4Cl, pyridine, Ac_2O, 15 h, reflux; (b) CrO_3, AcOH, H_2O; (c) AcOH, 1 h, reflux (overall yield: 67%); ii: (a) H_2, Pd/C, EtOH, 1 h, rt (81%); (b) ethylene glycol, $BF_3{\cdot}Et_2O$, CH_2Cl_2, 3 h, rt (87%); iii: (a) $NaBH_4$, $CeCl_3{\cdot}7H_2O$, THF/MeOH (2/1), 2 h, rt (100%); (b) acetone, APTS, 2 h, reflux (69%); iv: for compound **87**: DMSO, Ac_2O, AcOH, 4 h rt; for compound **88**: $ClCO_2CCl_3$, py, 16 h, rt.

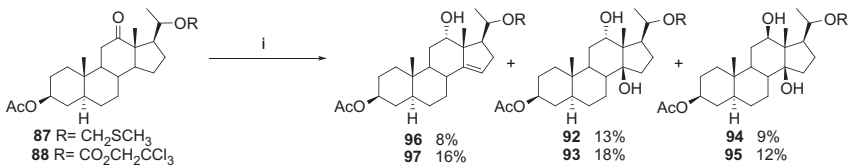
91 after acidic treatment [84]. The protection of the 17-hydroxy group was carried out under conventional reaction conditions to deliver compounds **87** and **88** (Scheme 2).

Starting from compounds **87** and **88**, the Norrish–Prins reaction afforded a complex mixture of 14β-hydroxy pregnane derivatives bearing either a 12α-hydroxy group (compounds **92** and **93**) or a 12β-hydroxy group (compounds **94** and **95**) and compounds **96** and **97** bearing a 12α-hydroxy group and a

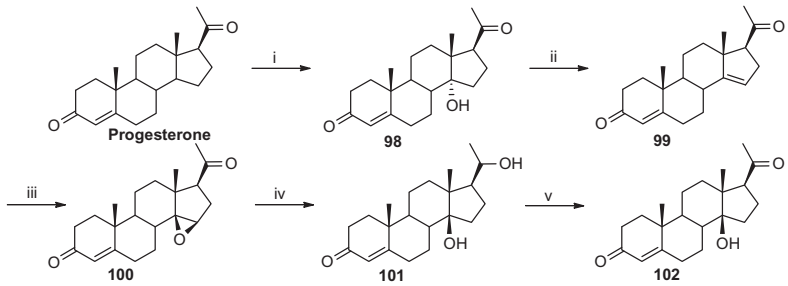
$\Delta^{14,15}$ double bond. At this stage, it has to be noted that compounds **94** and **95** could be considered as closely related analogs of boucerin bearing an A/B trans ring junction instead of a $\Delta^{5,6}$ double bond (Scheme 3).

Another methodology for the obtention of 14β-hydroxy pregnane derivatives was set up by Templeton *et al.* starting from progesterone [85]. The incubation of the latter with *Mucor griseocyanus* (+) 1027a gave 14α-hydroxyprogesterone **98** which was dehydrated in the presence of pTsOH to afford the pregnane derivative **99**. The successive treatment of compound **99** with *N*-bromoacetamide and potassium hydroxide delivered the epoxide **100**. Ring opening of the epoxide with excess $LiEt_3BH$ gave diol **101** which was directly oxidized with pyridinium dichromate to yield 14β-hydroxyprogesterone **102**. The conversion of 14α-hydroxyprogesterone **98** to 14β-hydroxyprogesterone **102** can be carried out without isolation of intermediates in 32% overall yield (Scheme 4).

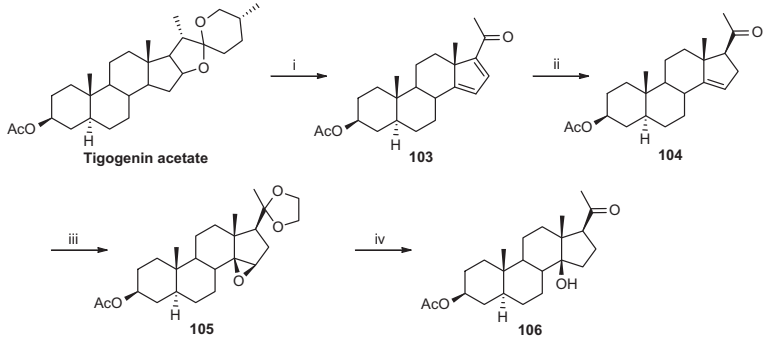
Yoshii *et al.* [86] developed also an efficient route to 14β-hydroxy pregnane derivatives, starting from compound **103** easily available from tigogenin acetate [77–79,87]. After reduction of the electrophilic double bond with $n Bu_3SnH$ [80,87], the resulting compound **104** was treated with 2-ethyl-2-methyl-dioxolane and then with *N*-bromoacetamide to generate an unstable bromohydrin which was dehydrobrominated by refluxing in methanol in the presence of K_2CO_3 to give epoxide **105**. Finally, reduction with $LiAlH_4$ afforded, after acidic treatment, the 14β-hydroxypregnane derivative **106** (Scheme 5).



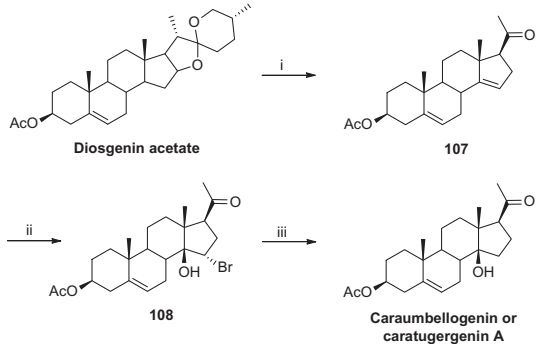
SCHEME 3 i: (a) hυ, quartz, CH_2Cl_2, 1 h; (b) $AcOH/H_2O/TFA$ (2.5/1/0.6), 4 h.



SCHEME 4 i: *Mucor griseocyanus* (+) 1207a; ii: pTsOH, C_6H_6; iii: (a) NBA, acetone, H_2O, $HClO_4$; (b) KOH; iv: $LiEt_3BH$, THF; v: PDC, DMF (overall yield: 32%).



SCHEME 5 i: see Ref. [87]; ii: nBu$_3$SnH, xylene (60%), reflux; iii: (a) 2-ethyl-2-methyl-1,3-dioxolane, pTsOH, benzene reflux; (b) NBA, acetone, AcOH, 0 °C, 15 min; (c) K_2CO_3, MeOH (overall yield: 55%); iv: (a) $LiAlH_4$, Et_2O reflux (80%); (b) AcOH 50%, MeOH 65 °C, 30 min.



SCHEME 6 i: see Ref. [87]; ii: *N*-bromoacetamide, acetone, AcOH 0 °C, 1 h; iii: Raney Ni, MeOH (overall yield from **107**: 35%).

To the best of our knowledge, the methods described above are the only ones that allow an access to 14β-hydroxypregane derivatives.

The utilization of these different methodologies allowed to develop synthetic routes to (functionalized) aglycones of glycosteroids extracted from *C. fimbriata* and *H. gordonii* as well as the total synthesis of P57.

Synthesis of Caraumbellogenin [86]

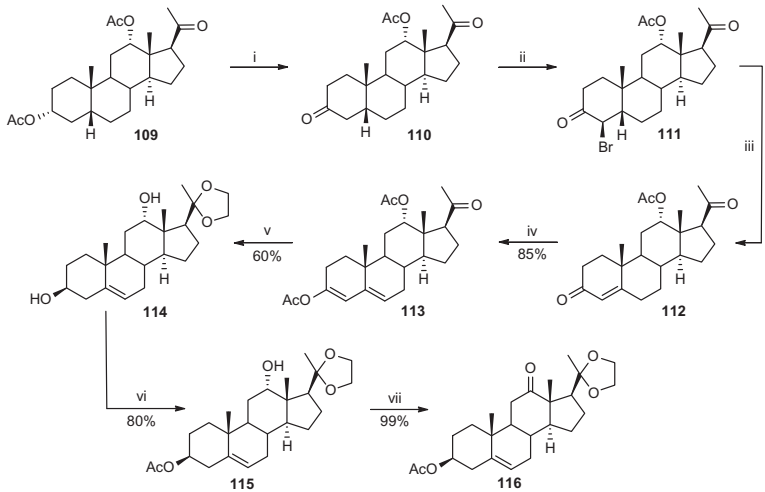
Yoshii *et al.* showed that the treatment of compound **107** (easily available from disogenin [88]) with NBA afforded also an unstable bromohydrine **108** which was subjected to hydrogenolysis with Raney Ni yielding caraumbellogenin (or caratubersin), the aglycone of some glycosteroids extracted from *C. fimbriata* (three steps from diosgenin acetate, 35% overall yield) (Scheme 6 [86]).

SYNTHESIS OF HOODIGOGENIN A AND P57

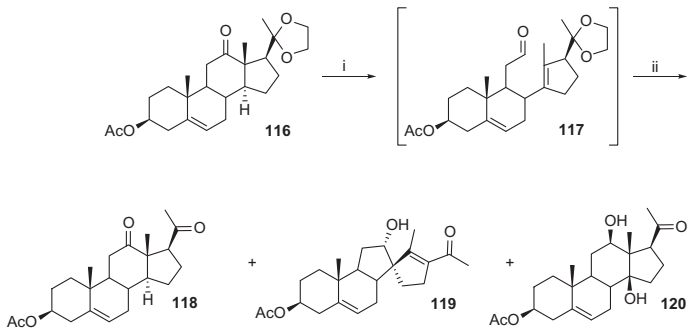
Synthesis of Hoodigogenin A [89]

Miesch *et al*. developed the first synthesis of Hoodigogenin A, the aglycone of the bioactive glycosteroid P57AS3. For that purpose, they started from the commercially available compound α,α′-diacetoxy-3,12 pregnanone **109**. After a regioselective deprotection of the 3α-acetoxy group and oxidation of the resulting hydroxy group, a regioselective α-bromination of the diketo derivative **110** yielded stereospecifically the β-bromoketo derivative **111**. A dehydrobromination reaction led to 12α-acetoxy-progesterone **112**. The latter was treated with acetic anhydride and acetyl chloride to give readily the dienol acetate **113**. After protection of the carbonyl group with ethylene glycol, the dienol acetate was reduced in the presence of $NaBH_4$ followed by a KOH-promoted deprotection of the 12α-acetate group, allowing the introduction of the double bond in the B ring and yielding the unstable diol **114**. A regioselective protection of the 3β-hydroxyl group gave compound **115**, which was subjected to a Dess–Martin periodane oxidation to afford the keto derivative **116** (Scheme 7).

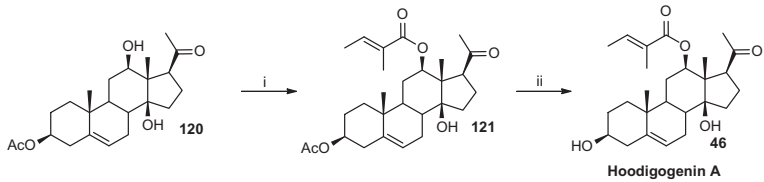
Compound **116** was then suitable to undergo a Norrish type I reaction. Indeed, the photolysis of the latter, which was carried out in a quartz apparatus with a 125 W high-pressure mercury lamp, led readily to the formation of



SCHEME 7 i: (a) K_2CO_3, MeOH, 25 °C 1 h (83%, formation of compound **1a**); (b) CrO_3, acetone, 25 °C, 1 h (quant.); ii: Br_2, AcOH, 25 ° C, 1.5 h; iii: LiCl, DMF, reflux, 1 h (67% for the 2 steps); iv: AcCl, Ac_2O, reflux 50 °C, 1 h (85%); v: (a) ethylene glycol, toluene, reflux, 3 h (quant.); (b) $NaBH_4$, MeOH/THF (1/1), 25 °C, 1.5 h; (c) KOH, MeOH, reflux, 1 h (60%); vi: Ac_2O, pyridine, DMAP, CH_2Cl_2, 25 °C, 2.5 h (80%); vii: Dess–Martin reagent, CH_2Cl_2, 0–25 °C, 1 h (99%).



SCHEME 8 i: hυ, CH_2Cl_2, 25 °C, 30 min (quant.); ii: AcOH/H_2O/TFA (2.5/1/0.6), THF, 25 °C, 1.5 h [**118** (19%), **119** (15%), **120** (25%)].



SCHEME 9 i: tigloyl chloride, pyridine, DMAP, CH_2Cl_2, reflux, 7 h (80%); ii: K_2CO_3, AcOH, 25 °C, 2 h (69%).

the unstable aldehyde **117** which was directly submitted to a Prins reaction leading to a mixture of three compounds: compound **118** that resulted from a deprotection of the dioxolane **116**, the spiro derivative **119,** and the desired compound **120**, which was isolated in 25% yield (Scheme 8).

Finally, the protection of the 12β-hydroxy group with tigloyl chloride in the presence of pyridine and DMAP afforded compound **121**. Deprotection of the 3β-acetate group gave Hoodigogenin A **46** (10 steps; 3% overall yield) (Scheme 9).

Synthesis of P57

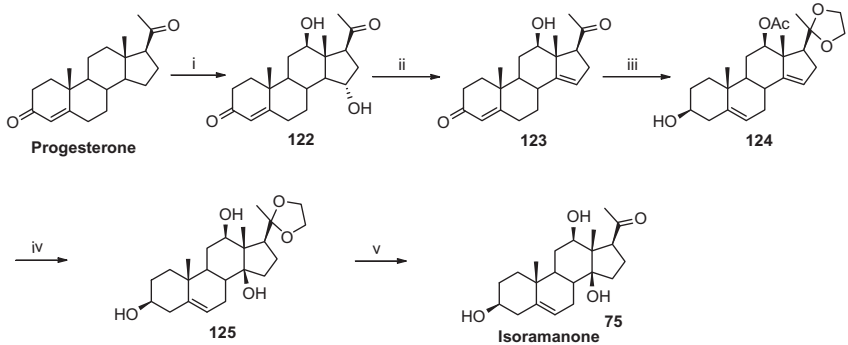
Van Heerden Synthesis [90]

In 1998, Van Heerden *et al*. disclosed in a patent the first synthesis of P57. Their aim was to introduce the 14β-hydroxy group via a 14,15β-oxirane ring opening. Starting from progesterone, incubation with *Calonectria decora* allowed the introduction of the 12β-hydroxy group as well as the introduction of the 15α-hydroxy group to give diol **122**. The introduction of the $\Delta^{14,15}$ double bond was carried out by regioselective tosylation followed by a thermal elimination of the tosylate to afford compound **123**. After isomerization of the $\Delta^{4,5}$ double bond under usual reaction conditions and protection of the 20-keto group, the resulting compound **124** was submitted to a tentative

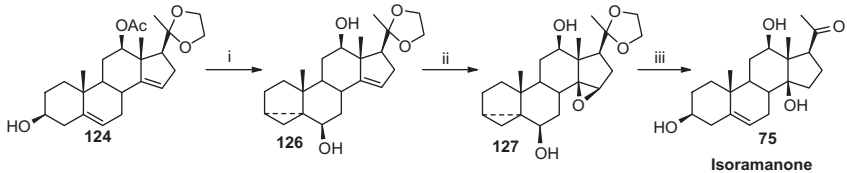
regioselective epoxidation of the $\Delta^{14,15}$ double bond. However, a complex mixture of compounds was obtained from which it was possible to isolate the desired 14β-hydroxy derivative **125** in very low yield after ring opening of the epoxide with $LiAlH_4$. Finally, after deprotection of the dioxolane group, isoramanone **75** was isolated (11 steps; 0.15% overall yield) (Scheme 10).

To improve the lack of selectivity during the epoxidation step, Van Heerden *et al*. developed an alternative synthesis, namely the protection of the $\Delta^{5,6}$ double bond by a cyclopropyl derivative. Thus, after transformation of the dioxolane **124** into the cyclopropyl derivative **126**, an epoxidation of the $\Delta^{14,15}$ double bond delivered the desired epoxide **127** in moderate yield. Ring opening of the epoxide was carried out in the presence of $LiAlH_4$ to yield the desired 14β-hydroxypregnane derivative which by an acidic treatment gave isoramanone **75** (12 steps; 0.2% overall yield) (Scheme 11).

The synthesis of the pyranoside derivatives **128** and **129** was carried out starting from methyl-α-D-glucoside **130**. For that purpose, the latter was transformed into the benzylidene derivative **131** which after NBS treatment



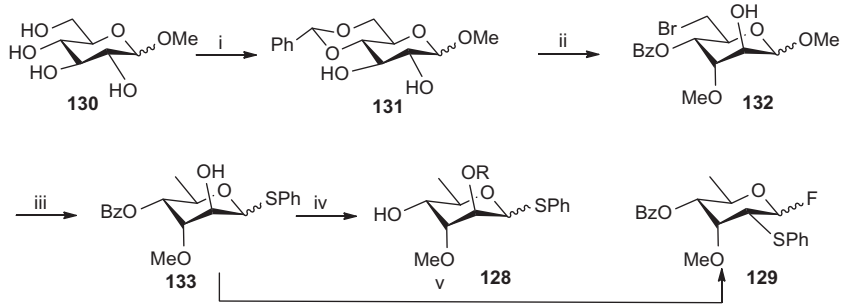
SCHEME 10 i: *Calonectria decora*, 5 d, 36 °C (45%); ii: (a) TsCl, pyridine, 15 h, 0 °C (92%); (b) collidine, 3 h, 150 °C (78%); iii: (a) AcCl, Ac_2O, reflux, 2 h (93%); (b) ethylene glycol, APTS (cat.), reflux, 16 h (53%); (c) $NaBH_4$, EtOH, 15 h, rt (80%); iv: (a) $LiAlH_4$, THF, 24 h, rt (90%); (b) NBA, acetone, AcOH, H_2O, 0 °C, 15 min (69%); (c) $LiAlH_4$, THF, 24 h, rt (2.1%); (d) $LiAlH_4$, THF, 48 h, reflux (83%); v: AcOH, H_2O (89%).



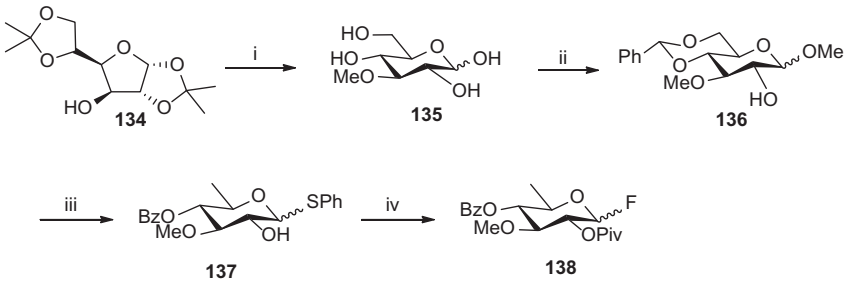
SCHEME 11 i: (a) TsCl, pyridine, 24 h, rt (84%); (b) KOAc, acetone, H_2O, 16 h, reflux (61%); ii: (a) $LiAlH_4$, THF, 4 h, rt (83%); (b) NBA, acetone, AcOH, H_2O, 15 min, 0 °C (51%); iii: (a) $LiAlH_4$, THF, 7 h, reflux (53%); (b) acetone, HCl, 2 h, 60 °C (14%).

yielded the bromo derivative **132**. The phenylthioaltroside **133** was obtained by successive NBS bromination, $NaBH_4$ reduction, and phenylthiotrimethylsilane treatment of compound **132**. Finally, the fluorocymaropyranoside **129** was generated by DAST treatment of compound **133** and the activated monosaccharide **128** was obtained by successive protection and deprotection (Scheme 12).

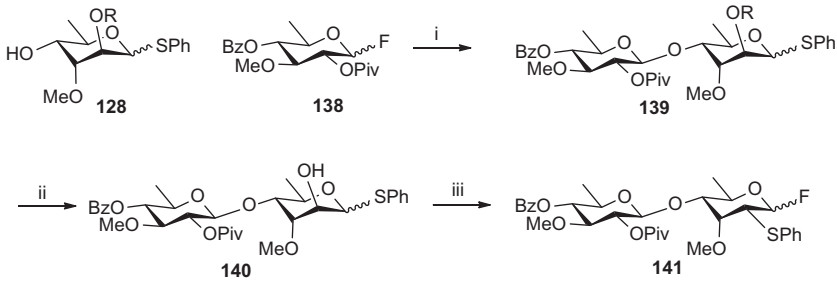
Using conventional reaction conditions, the diisopropylidene α-D-glucofuranoside **134** was transformed into the pyranoside derivative **135**. The latter was submitted to the reaction conditions described above to generate the thiophenylderivative **137** via the benzilidene derivative **136**. Finally, after protection the hydroxyl group, the fluoro thevetose **138** was obtained after DAST treatment (Scheme 13).



SCHEME 12 i: (a) C_6H_4CHO, $ZnCl_2$, 24 h, rt (72%); (b) TsCl, pyr, 0 °C then rt 48 h (60%); (c) Na, MeOH, 110 °C, 48 h (52%); ii: (a) NBS, $BaCO_3$, CCl_4, 75 °C (69%); iii: (a) $NaBH_4$, H_2O, $NiCl_2$, EtOH, 0 °C then 75 °C, 1 h (72%); (b) $C_6H_4SSiMe_3$, TMSOTf, CH_2Cl_2, rt, 6 h (63%); iv: (a) *t*$BuMe_2SiCl$, imidazole, pyr (80%); (b) NaOMe, rt, 4 h (75%); v: DAST, CH_2Cl_2, 0 °C (90%).



SCHEME 13 i: (a) NaH, MeI, THF, 24 h (83%); (b) AcOH 50%, reflux, 18 h (50%); ii: (a) MeOH, HCl, reflux, 4 h (95%); (b) C_6H_4CHO, $ZnCl_2$ (60%); iii: (a) NBS, $BaCO_3$, CCl_4, reflux (70%); (b) $NaBH_4$, H_2O, $NiCl_2$, EtOH, 0 °C then 75 °C 1 h (70%); (c) $C_6H_4SSiMe_3$, TMSOTf, CH_2Cl_2 (60%); iv: (a) *t*BuCOCl, pyr (80%); (b) NBS, DAST, CH_2Cl_2 (45%).



SCHEME 14 i: $SnCl_2$, AgOTf, CH_2Cl_2, −15 °C, 16 h (15%); ii: TBAF, THF (60%); iii: DAST, CH_2Cl_2.

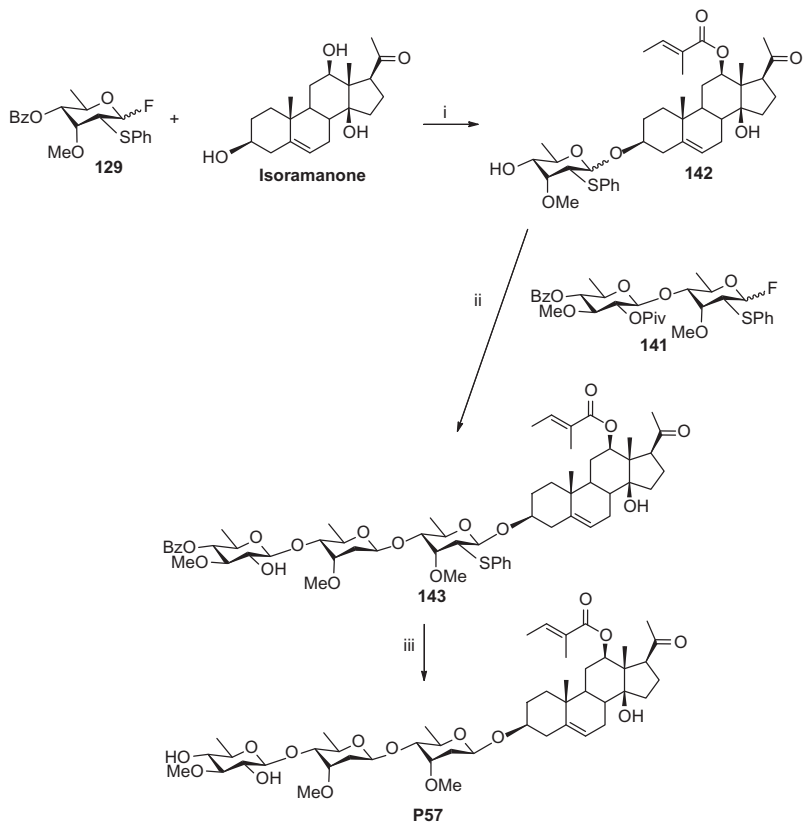
The coupling of cymarose derivative **128** with thevetose derivative **138** was carried out in the presence of $SnCl_2$ and AgOTf, affording the disaccharide **139**. Deprotection of the TBS ether gave the corresponding alcohol **140** which was treated with DAST to deliver the fluorodisaccharide **141** (Scheme 14).

The coupling of fluorocymaropyranoside **129** with isoramanone yielded compound **142**. To complete the trisaccharide unit, the coupling of compound **142** with disaccharide **141** was carried out to deliver compound **143**. Finally, P57 was obtained after a Ni Raney thiol reduction followed by a benzoyl deprotection. However, no experimental data and no yields were given in the patent (Scheme 15).

Yu Synthesis [91]

In 2012, Yu *et al*. achieved the total synthesis of P57 starting from digoxin, a commercially available cardiotonic glycosteroid. After protection of the 12β-hydroxy group, an acidic hydrolysis induced the cleavage of the sugar moiety to give, after protection of the resulting 3β-hydroxy derivative as a TBDPS ether, compound **144**. An ozonolysis of the electrophilic double bond followed by Zn acetic acid reduction and protection of the resulting carbonyl group generated dioxolane **145**. A TBAF deprotection of the silyl ether and subsequent Dess–Martin oxidation generated the keto derivative **146**. The introduction of the $\Delta^{5,6}$ double bond was achieved by addition of LiHMDS and TMSCl at low temperature following by an oxidation of the resulting enolate, yielding the desired α,β-unsaturated ketone **147** and the $\Delta^{1,2}$ isomer **148**. The introduction of the $\Delta^{5,6}$ double bond was carried by the treatment of compound **147** with potassium *t*-butylate affording after $LiAlH_4$ reduction and regioselective protection of the resulting 3β-hydroxy group as a TIPS ether, compound **149**. The addition of tiglic acid in the presence of Yamaguchi reagent followed by a deprotection of the silyl ether with TBAF and deprotection of the dioxolane gave Hoodigogenin A **46** (16 steps: overall yield: 10%) (Scheme 16).

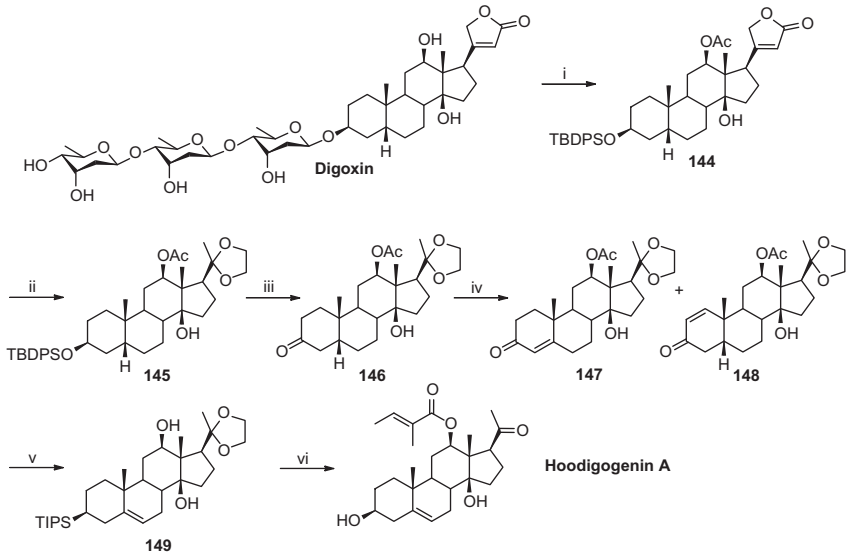
The introduction of the glycosidic side chain was carried out as follows: the 3β-hydroxy group of compound **150**, easily available from α-D-glucopyranoside, was methylated and the resulting methoxy derivative was submitted to a ring



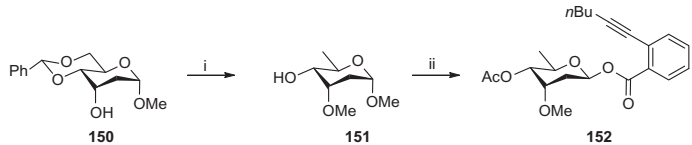
SCHEME 15 i: (a) $SnCl_2$, Et_2O, 3 days, −15 °C (15%); TigCl, pyr, NaOMe (yield is not indicated); ii: no experimental details and no yield are indicated; iii: Raney Ni, NaOMe (yield is not indicated).

opening–reduction process to give methyl α-D-cymaropyranoside **151**. After acetylation of the 4-hydroxy group and hydrolysis with 80% acetic acid, the resulting cymaropyranoside was condensed with *o*-hexynylbenzoate to generate the cymarosyl *o*-hexynylbenzoate **152** (Scheme 17).

On the other hand, the glucofuranoside derivative **153** was transformed into 2,4-di-*O*-benzoyl-thevetose **154** by successive treatment with acetic acid and benzoyl chloride followed by a selective deprotection of the anomeric benzoate in the presence of NH_3 in THF/MeOH. After condensation of *o*-hexynylbenzoic acid with compound **154**, the resulting thevetosyl donor **155** was coupled to compound **151** in the presence of $Ph_3PAuOTf$ as catalyst to generate compound **156** as a mixture of two isomers. A reductive deprotection followed by acetylation gave compound **157** which afforded the desired disaccharide **158** after selective cleavage of anomeric methyl group and condensation with *o*-hexynylbenzoic acid (Scheme 18).

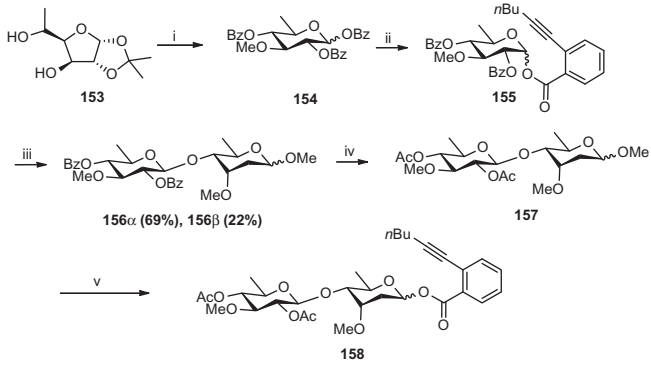


SCHEME 16 i: (a) Ac_2O, Py, 80 °C (90%); (b) H_2SO_4, MeOH/H_2O, reflux (91%); (c) TBDPSCl, imidazole, DMF, rt (99%); ii: (a) O_3, CH_2Cl_2, −60 °C; (b) Zn, AcOH, 70 °C (71%); (c) ethylene glycol, $HC(OEt)_3$, TsOH, C_6H_6, rt (97%); iii: (a) TBAF, THF, reflux; (b) Dess–Martin, CH_2Cl_2, rt (95%); iv: (a) LiHMDS, TMSCl, Et_3N, THF, −78 °C; (b) $Pd(OAC)_2$, CH_3CN, rt (compound **148**: 8% and compound **147**: 79%); v: (a) *t*BuOK, DMSO, rt; (b) $LiAlH_4$, THF, rt (56–71%); (c) TIPSCl, imidazole, DMF, rt (70%); vi: (a) tiglic acid, Yamaguchi reagent, Et_3N, DMAP, toluene, 60 °C (80%); (b) TBAF, THF, 0 °C, 90%; (c) PPTS, acetone, reflux 90%.

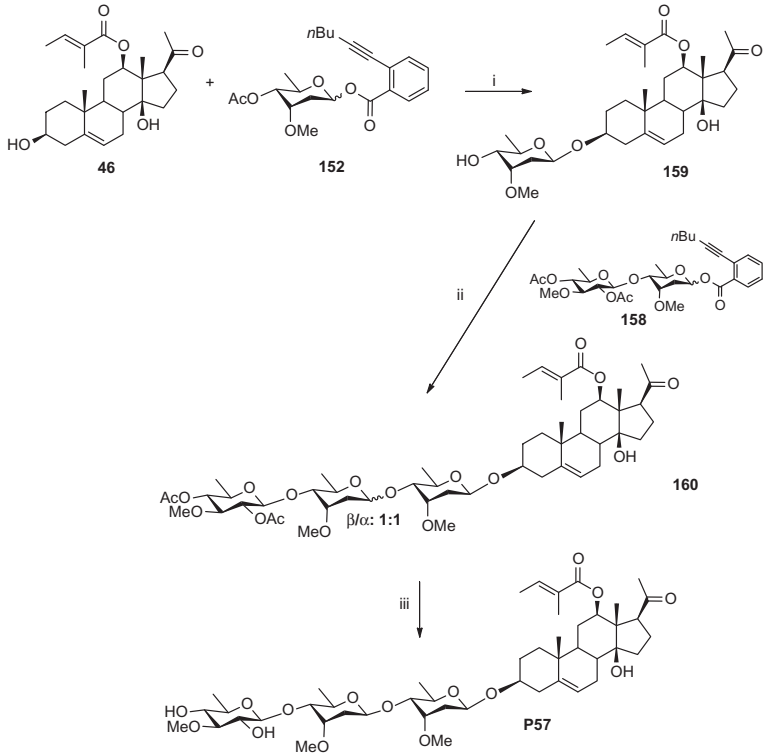


SCHEME 17 i: (a) MeI, NaH, THF, rt (quant.); (b) NBS, AIBN, $BaCO_3$, CCl_4, reflux; (c) $LiAlH_4$, THF, 80 °C (b+c: 88%); ii: (a) Ac_2O, Py, rt (87%); (b) 80% AcOH, 100 °C; (c) *o*-hexynylbenzoic acid, EDCl, DIPEA, DMAP, CH_2Cl_2, rt (70%).

To obtain P57, Hoodigogenin A **46** was first of all submitted to a coupling reaction with cymarosyl *o*-hexynylbenzoate **152** in the presence of PPh_3AuOTf as a catalyst affording the corresponding glycoside from which was obtained a 3.5:1 mixture of β and α isomers. Selective removal of the acetyl group on the β isomer delivered cymaroside **159**. The coupling of **159** with disaccharide **160** was realized in the presence of PPh_3AuOTf, affording the desired trisaccharide **28** as a 1:1 mixture of α and β isomers. Finally, starting from the **160β** isomer, P57 was obtained by final deprotection of the acetyl groups. Thus, according to Yu *et al.*, the synthesis of P57 was achieved in 20 steps in 2.4% overall yield (Scheme 19).



SCHEME 18 i: (a) 80% AcOH, TFA, 70 °C; (b) BzCl, DMAP, NEt_3, CH_2Cl_2, rt (a+b: 81%); (c) NH_3, MeOH, 0 °C (60%); ii: CCl_3CN, DBU, CH_2Cl_2, rt, 98%; iii: PPh_3AuOTf, 4 A MS, rt (**156α**: 69%; **156β**: 22%); iv: (a) $LiAlH_4$, THF, rt; (b) Ac_2O, Pyr, rt (90%); v: (a) 80% AcOH, 50 °C; (b) *o*-hexynylbenzoic acid, DMAP, EDCl, DIPEA, CH_2Cl_2, rt (85%).



SCHEME 19 i: (a) PPh_3AuOTf (0.2 eq), 4 A MS, CH_2Cl_2, rt (75%); (b) Na_2CO_3, MeOH, rt (90%); ii: **3**, PPh_3AuOTf (0.5 eq), 5 A MS, toluene, −40 °C; iii: **160α**+**160β**, KOH, C_6H_6, MeOH, rt 90%.

CONCLUSION

Plants have been used for medicinal purposes for thousands of years. The ethnopharmacological knowledge about applications of medicinal plants for achieving health benefits has laid the foundation for development of many modern drugs. Natural products such as strychnine, aspirin, morphine, colchicine, and artemisinin are some of the early examples of success of pharmacognostic evaluation of medicinal plants toward new drug discovery. During the first half of twentieth century, the central idea behind new drug discovery from medicinal plants was to explore the ethnopharmacological evidence about the medicinal properties of a plant and to purify the pharmacologically significant constituents. Although the paucity of advanced bioassay methods was evident, drug discovery efforts were upheld by the basic organic chemistry and chromatographic techniques. The advent of modern scientific tools, availability of advanced *in vitro* screening methods, and advancements in chromatographic and spectroscopic techniques have been helpful in speeding up the drug discovery process from medicinal plants. This has led to the development of several new chemical entities (NCEs) from the medicinal plants. Moreover, with modern synthetic methods, it has become possible for the researchers to synthetically mimic the chemical constituents obtained from natural sources. This has enabled large-scale production of such NCEs and in turn helped the diminishing natural resources. In past 50 years, there are many successful stories, in which, medicinal plants, especially those with evidence of traditional uses for medicinal purposes, have become the sources of new drug candidates. Thus, it can be reasonably argued that medicinal plants have contributed a lot to the modern drug discovery. Several reviews recognize the role of traditionally used plants for the development of clinically used drugs [92–95]. Of 520 new drugs approved for commercial use between 1983 and 1994, 30 were new natural products and 127 were chemically modified natural products. Furthermore, it was estimated by Newman *et al*. in 2003 that about 25% of the single drug molecules that are currently under clinical use are obtained from natural sources, that is, from medicinally important plants, directly or are synthetic derivatives of the natural products [96]. As a parallel paradigm, application of medicinal plants as botanical supplements for achieving health benefits has seen global resurgence in the past 3 decades. It should be noted that the purported pharmacological benefits from such supplements are due solely to the presence of unique phytochemical compounds in these medicinal plants. As supported by numerous pharmacological studies, these compounds can produce significant physiological effects even at low concentrations. In such scenario, the knowledge about chemical composition of a medicinal plant is crucial for establishing the identity, purity, efficacy, and safety of a herbal dietary supplement. In this regard, isolation of natural compounds from the medicinal plants of commercial interest is particularly important. In the current context, *H. gordonii* and *C. fimbriata* are two

very good examples, wherein traditional knowledge of possible pharmacological effects of these plants led to the discovery of new natural compounds comprising 14β-hydroxypregnane glycosides. Spectroscopic studies of the 14β-hydroxypregnanes from these two plants have generated fundamental information about their chemical structures and physicochemical properties. These compounds served as markers for the development of several quality control methods for the botanical products in market, which claim to contain *Hoodia* and *Caralluma*. Furthermore, these phytochemical studies of *Hoodia* and *Caralluma* were vital toward the total synthesis of Hoodigogenin A and P57. These pregnane derivatives have been popular for their purported appetite-suppressant and antiobesity potential and therefore may serve as models for developing future antiobesity drugs. Our synthetic studies of these compounds and related 14β-hydroxypregnane derivatives should provide opportunities for development of novel, more potent, and selective molecules with therapeutic potential.

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Chapter 10

Chemico-Biological Aspects of Plant Lectins with a Preference to Legume Lectins

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INTRODUCTION

Lectins were originally termed phytohemagglutinins because they were first detected in plant extracts. They are proteins that possess at least one non-catalytic domain that binds reversibly to a specific carbohydrate, which is normally termed carbohydrate recognition domain (CRD) or carbohydrate-binding domain (CBD) [1]. The carbohydrate specificity of lectins plays a role in important biological recognition phenomena involving cells and proteins, and lectins are distributed in all classes of living organisms, ranging from viruses to vertebrates. In the microbial world, they are called by other names, such as hemagglutinins, adhesins, and toxins. In animals, lectins serve different biological roles including cell adhesion, pathogen recognition, and glycoprotein synthesis [2,3]. In plants, they interact with microbes in a dual role, as defense molecules in one case and as molecules of recognition in another [4,5]. When consumed as part of the diet, plant lectins, in some cases, are known to cause allergies, trigger autoimmune diseases, or even interfere with the absorption of nutrients, thereby acting as antinutrition molecules [6,7]. Their role as defense molecules has received much attention in recent years with the creation of transgenic plants expressing genes for insecticidal lectins for self-defense. Examples of such transgenic crops include tobacco, rice, potato, sugarcane, and cotton, mostly expressing mannose (Man)-binding insecticidal lectins or legume lectins [8,9].

Despite the hype regarding some of the antinutrition effects that plant lectins have received, many lectins studied to date have numerous biological activities that have potential for application in the biomedical industry. Their roles in crop protection and immunomodulation are being explored to their fullest capacity. Recent advancements that have surfaced in the areas of computational biology and bioinformatics, coupled with efficient screening mechanisms, have led the pharmaceutical industries to pick the most efficient lectins and turn them into drugs. This is, indeed, a renewed and challenging activity of lectins in drug discovery, today. Studies on lectins since the 1990s have generated a wealth of data that address many aspects, such as isolation, structure, posttranslational process, and carbohydrate-binding ability of lectins. Therefore, among the other topics discussed in this chapter, the practical aspects of isolation, structure elucidation, and structure–activity relationship (SAR) of legume lectins have been included. For clarity, certain aspects related to nonlegume lectins have also been incorporated. Toward the end of the chapter, various biomedical applications of lectins have also been highlighted.

CLASSIFICATION OF PLANT LECTINS

Lectin classification has been a topic of debate and has not been settled for a long time, because of the changes that take place continuously in the parameters. Because of the diversity of lectins, a general agreement on their

classification into families has been difficult to achieve and lectin classification has seen many changes. A summary of the different classifications and the advantages/disadvantages associated with them are discussed here.

Based on Domain Architecture

Based on the overall domain architecture of plant lectins, the latter are classified into four major groups: (i) *merolectins*, for example, hevein, which comprise only one CBD and therefore cannot agglutinate cells; (ii) *hololectins*, composed of two or more identical or very homologous CBDs, which allows them to agglutinate cells and/or precipitate glycoconjugates, for example, *Phaseolus vulgaris* agglutinin (PHA) specific for galactose; (iii) *superlectins*, composed of two or more inequivalent CBDs to recognize structurally unrelated carbohydrates, for example, lectin from tulip bulbs with two distinctly different CBDs that bind mannose in one CBD and *N*-acetylgalactosamine (GalNAc) in another; and (iv) *chimerolectins,* composed of one or more CBDs in addition to a domain that exhibits biological activity that is independent of the CBD, for example, Type 2 ribosome-inactivating protein [10,11]. Most of the plant lectins isolated and characterized to date are hololectins. Although not many chimerolectins have been isolated and studied, the sequence analysis of complete plant genomes reveals that they are very abundant in plants [12]. Moreover, recent genome/transcriptome analyses of plants have provided evidence for the occurrence of many proteins containing one or more lectin domains embedded in a complex multidomain architecture [13].

Based on Carbohydrate Specificity and 3D Structure

For a long time, the classification of plant lectins based on their carbohydrate-binding specificities offered a good way to categorize and study them. These families included (i) mannose- and mannose/glucose-, (ii) mannose/maltose-, (iii) Gal/GalNAc, (iv) *N*-acetylglucosamine (GlcNAc)/$(GlcNAc)_n$-, (v) fucose-, and (vi) sialic acid-binding lectins. However, structurally unrelated proteins could also get included under these classes [11,14]. To overcome this, a system of classifying lectins based on their three-dimensional structures was created and six major groups were identified: α-D-mannose-specific monocot lectins, agglutinins with a hevein domain, β-prism plant lectins, β-trefoil lectins, cyanovirin-N homologs, and legume lectins [14,15].

Based on Sequence Similarity

A seven-family classification based on serological relatedness and sequence similarities was proposed by Van Damme *et al*. [13], which was later updated to a 12-family classification by further considering sequence and structural homology [16]. These lectin families are represented in Fig. 1 and are as

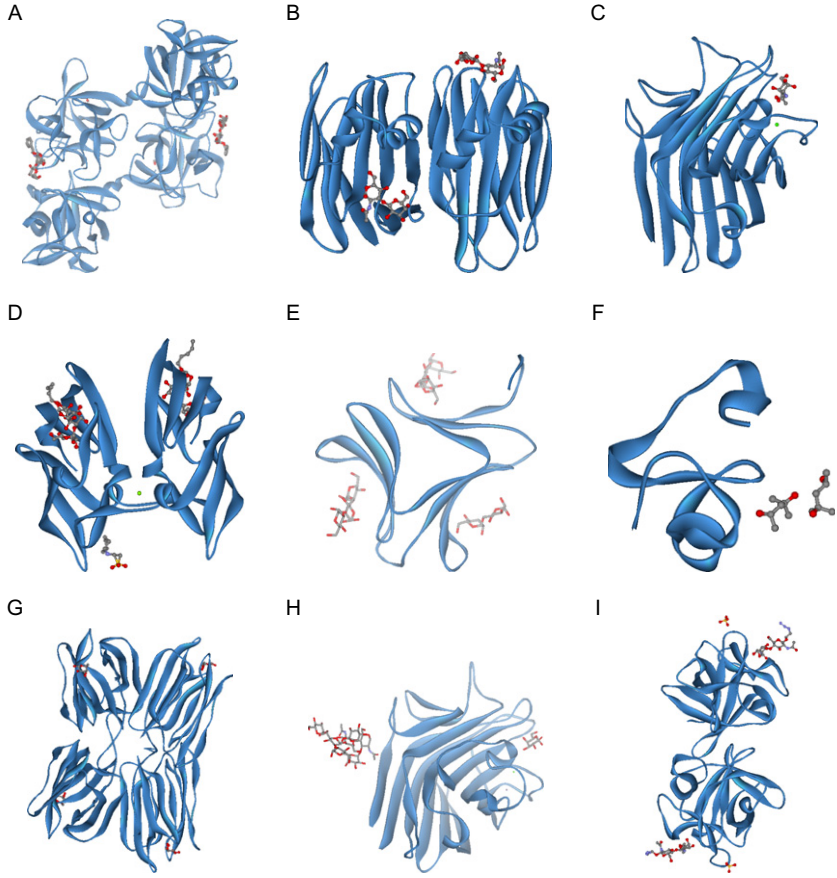

FIGURE 1 Representative proteins for 9 of the 12 lectin classes, based on the classification by Van Damme *et al*. [13], represented in ribbon form as complexes with their corresponding sugar ligands (ball and stick). Their PDB codes are given in parentheses. (A) *Agaricus bisporus* agglutinin in complex with lacto-*N*-biose (1Y2U); (B) *Amaranthus caudatus* agglutinin with bound T-antigen disaccharide (1JLX); (C) *Robinia pseudoacacia* chitinase-related agglutinin in complex with *N*-acetylgalactosamine (1FNZ); (D) Cyanovirin-N with high-mannose oligosaccharides (3GXY); (E) *Galanthus nivalis* agglutinin with mannose (1JPC); (F) Hevein–methyl pentanediol complex (1Q9B); (G) Jacalin–galactose complex (1UGW); (H) *Erythrina corallodendron* legume lectin (chain A) in complex with galactose (1AXZ); (I) *Clitocybe nebularis* ricin B-like lectin in complex with *N*,*N*′-diacetyllactosediamine (3NBE).

follows: (i) *Agaricus bisporus* agglutinin homologs, (ii) amaranthins, (iii) Class V chitinase homologs with lectin activity or chitinase-related agglutinins, (iv) cyanovirin family, (v) *Euonymus europaeus* agglutinin family, (vi) *Galanthus nivalis* agglutinin family, (vii) proteins with hevein domain (s), (viii) jacalins (JACs), (ix) proteins with legume lectin domains, and (x) lysin motif, (xi) nictaba, and (xii) ricin_B families.

Based on Phylogeny

The homology-based 12-family system of classification surpasses the traditional method of lectin classification based on carbohydrate monomer specificity. Within a given lectin family, most, but not necessarily all, of the members are made up of protomers with a similar primary structure and an overall 3D fold. The overall structure of the native lectins is not only determined by the structure of the protomers but is also dependent on the degree of oligomerization and, in some cases, on the posttranslational processing of the lectin precursors.

The classification systems designed so far have not accounted for lectin-like members in plant genomes from animal lectin sequences. The latest classification of the plant lectin gene superfamily is based on domain structures and phylogenetic analyses of plant genomes after the recent release of genome sequences of *Arabidopsis*, rice, and soybean [15]. The classification is identical to the structure- and homology-based classification of Van Damme *et al.* [11] and has 12 families, 4 of them consisting of recently identified plant lectin members. With the continual discovery of new lectins, even the newer systems of classification are bound to see changes for better accommodating these lectins with their unique properties.

ISOLATION OF PLANT LECTINS

Lectins are present in many plant families and are most abundantly seen in the leguminosae family. The location and relative abundance of lectins can vary between different families and also within the same family. Lectins are distributed in various tissues of the plant, including seeds, roots, stems, leaves, and fruits. To a lesser extent, they are also present in the bark, flower, ovaries, and nectar. In many leguminous plants, the seed contains a considerable amount of lectins, up to even 10–15% of the total protein content [1]. Quantitatively, the nonseed legume lectins are present in lesser amounts and are sometimes found in different tissues of the same plant [17].

General Methodology

Plant lectins are relatively soluble and can be easily extracted. In typical cases, a buffered saline extract is prepared by blending with the plant tissue, filtering, and spinning down at high speed to remove insoluble material. The lectin is then purified on an immobilized carbohydrate matrix followed by elution with the appropriate hapten. The molecular weight characterization and purity of the lectin can be studied by gel filtration chromatography, ion-exchange chromatography, and HPLC [18].

Purification and Assay

Since agglutinating lectins possess at least two carbohydrate binding sites, it becomes easy to follow the purification steps with a simple

agglutination/precipitation assay that uses erythrocytes, leucocytes, or certain glycoconjugates. For establishing the identity of lectins with only one CBD, other biological assays, such as toxicity or enzyme activity, may be helpful. For example, *ricin*, a highly toxic, nonagglutinating lectin can be studied for lectin activity by measuring its toxicity [19,20]. Lectins of different specificities have been purified occasionally on a single affinity adsorbent. For example, a Sepharose-fetuin affinity column was used in a single-step procedure to isolate the lectins, concanavalin A (Con A), favin, PHA, wheat germ agglutinin (WGA), and limulus hemagglutinin, all with different specificities. Lectins with unknown binding specificities can also be purified by the same procedure as demonstrated in the case of the extracts of small California white beans, Idaho red beans, and white pea beans [21].

Immobilized Lectins for the Separation of Glycoconjugates

The specificity of glycoconjugates toward lectins makes them important tools for preparing immobilized columns for the affinity purification of glycoproteins, glycopeptides, free glycans, and even glycolipids. Con A-Sepharose, WGA-agarose, and other lectin-immobilized columns have often been used for purifying glycoproteins from nonglycosylated proteins. The basic principle behind such purification involves binding of the glycoconjugate, via the specific sugar chain, washing unbound/nonspecifically bound compounds, and finally eluting the lectin-bound glycoconjugates by affinity chromatography combined with HPLC [22–24]. Multilectin affinity columns using the combined specificities of three different lectins, Con A, WGA, and JAC, have been prepared to make use of their combined specificities for fast affinity separations (10 min per run) of glycoproteins from human plasma [25]. Approximately 50% of all the proteins are now considered to be glycosylated, and many of these are potential biomarkers for certain diseases such as cancer. Lectin affinity columns are useful in enrichment of these glycoproteins, which are present in minute quantities in complex biological samples [26,27].

LEGUME LECTINS

Legume lectins are the largest and best characterized family of plant lectins and are present in abundance in the seeds of leguminous plants. They are synthesized during seed development and accumulate in the cotyledons and embryonic axes of seeds. Even the seed coat is often known to be rich in lectins, for example, *Datura stramonium* [28]. Currently, well over 190 three-dimensional structures of more than 40 legume lectins have been elucidated, either in free form or as complexes with ligands [29,30]. During the decade 1990–2000, extensive studies were carried out resulting in the enrichment of the literature with information on the structure, thermodynamics, sugar-binding activity, and metal-binding sites of these proteins. Some of these important aspects of legume lectins are discussed in the following sections.

General Features

The sequences of well over 210 legume lectins are known, and the proteins of this class display high sequence and tertiary structure homology, possessing diverse forms of quaternary associations and a wide range of binding specificities [15]. About 50% of their secondary structure comprises loops and β-bends. However, they lack any α-helix, with exceptions as seen in case of the seed agglutinin, *Dolichos biflorus* lectin (DBL), and the leaf and stem lectin, DB58, from the same plant [31]. The tertiary structure is a characteristic "jelly-roll" motif that is common to all legume lectins despite their widely varying sugar specificities. Legume lectins also display quaternary associations that consist of two or four identical or different subunits (or protomers), each with a relative molecular mass of about 25–30 kDa. Each monomer exhibits a dome shape and consists of three sets of antiparallel sheets, characteristic of the jelly-roll motif [32].

The Jelly-Roll Motif

The jelly-roll fold is common to many other proteins besides legume lectins, including the mammalian galectins, and this fold is associated with carbohydrate-binding activity. The fold typically consists of three sets of β-sheets, six strands forming a flat "back" sheet, seven strands forming a curved "front" sheet, and a small five-stranded "top" sheet that plays a major role in bringing the two sets of strands together, forming a hydrophobic core between them. The three strands are interconnected by several loops, with about 50% of the residues being present in it. The Ω-loop curls over the front sheet forming a second hydrophobic core [33–35]. The CBD and the metal-binding region are located mostly in the loops contiguous to the front sheet. More detailed descriptions of these regions are given later.

Metal Ion Binding

The metal-binding sites of legume lectins were first described for Con A and are well conserved in all lectins of this class. Metal ions confer a high degree of structural stability to legume lectins, endowing them with heat stability and proteolytic resistance. The CBD of legume lectins requires the simultaneous presence of one tightly bound ion each of Ca^{2+} and Mn^{2+} per subunit to exhibit sugar-binding activity. Manganese ions can be replaced by other transition metal ions without the loss of biological activity as demonstrated with Con A [36,37]. X-ray crystallography studies carried out at low temperature (Cryo-crystallography) for the saccharide-free Con A at a resolution of 0.94 A° show the Mn^{2+} in the S1 site bound to Asp10 (Mn–O: 2.15 A°), Glu8 (Mn–O: 2.17 A°), Asp19 (Mn–O: 2.19 A°), His24 (Mn–N: 2.23 A°), and two water molecules, with Mn–O being 2.18 A° and 2.26 A°, thus exhibiting a distorted octahedral geometry. Ca^{2+} ion in S2 sites binds to Asp10 in a bidentate manner with a

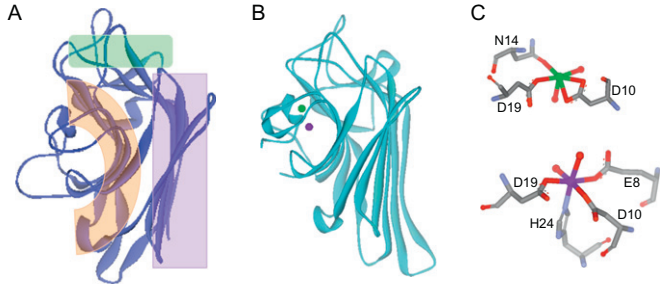

FIGURE 2 Jelly-roll motif and metal-binding sites of legume lectins: (A) Typical jelly-roll motif-with the three sets of antiparallel β-sheets ("front", "back," and "top" sheets) highlighted in orange, purple, and green, respectively, in the crystal structure of a monomer of phytohemmaglutinin-L (PDB code: 1FAT). (B) Crystal structure of a monomer of saccharide-free Con A (PDB code: 1NLS) showing metal ions, Mn^{2+} (purple) and Ca^{2+} (green) in the S1 and S2 sites, respectively. (C) S1 (purple Mn^{2+} center) and S2 sites (green Ca^{2+} center) of Con A in a ball-and-stick model, showing amino acid residues involved in the binding of metal ions, along with water moieties. The "C, H" backbone is shown in gray, "N" in purple, and "O" in red.

long, Ca–O: 2.47 Å distance (Fig. 2) [38]. The insecticidal legume proteins, arcelins, from *Phaseolus vulgaris* are considered lectin-like proteins as they are similar in sequence and tertiary structure to those of the legume lectins. Arcelins, however, lack sugar-binding activity as they do not possess the crucial metal-binding residues [39], suggesting the importance of the metal ion in imparting the activity to the protein.

Con A, and about half of the known legume lectins display *cis*/*trans* isomerization of the peptide bond between Asp 207 and 208. The *cis* to *trans* isomerization of this peptide bond is dependent on the binding of Ca^{2+} and Mn^{2+} and drastically affects the CBD of Con A. In such cases, the demetallation abolishes the sugar-binding activity. However, in the case of *Pterocarpus angolensis* lectin (PAL) and *Griffonia simplicifolia* isolectin I-B4 (GS-IB4), and their likes, demetallation neither induces a similar *cis* to *trans* isomerization nor does it have any major change in the carbohydrate-binding activity. The difference in the *cis*/*trans* isomerization of this nonproline *cis* peptide bond lies in the sequence of the C-terminal β-strand of the lectin monomer. The presence of Asn104 in the sequence of Con A and its likes, stabilizes the *cis* to *trans* isomerization, whereas the presence of a more bulky residue such as His226 in PAL or a hydrophobic residue in GS-IB4 prevents this isomerization [37,40].

Carbohydrate-Binding Domain

Since the metal-binding site and the CBD are in close proximity, some of the residues are common to both, for example, Asn14, as seen in Fig. 3. A comparison of the saccharide-bound and the saccharide-free forms of Con A and other legume lectins suggests that the binding site is substantially preformed, with water molecules replacing the key carbohydrate hydroxyls in the free form [38].

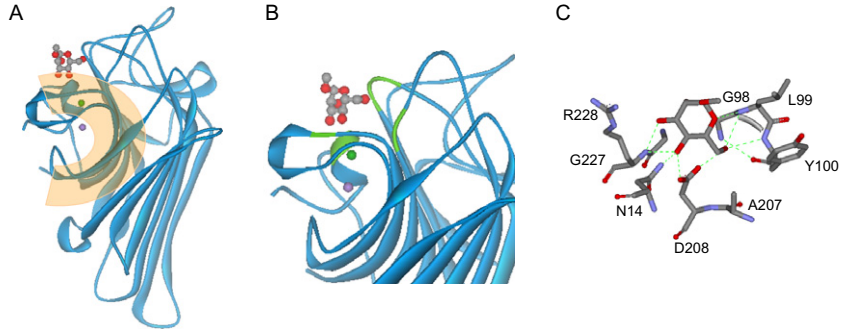

FIGURE 3 Carbohydrate-binding site of legume lectins: (A) Crystal structure of a monomer of Con A (PDB code: 5CNA) showing methyl-α-D-mannopyranoside (gray/red ball-and-stick representation) binding to the concave side of the front sheet (represented in the orange region), along with metal ions, Mn^{2+} (purple) and Ca^{2+} (green). (B) Enlarged portion of the CBD of Con A subunit showing four different loops (highlighted in green) involved in the binding to methyl mannoside. (C) Ball-and-stick representation of residues involved in the binding to methyl mannoside in the CBD. Hydrogen bonds involved are shown as green dotted lines.

Comparative analyses of 15 different families containing the legume lectin fold, but lacking in sequence similarity, were made to identify a minimal determining region in the fold. The minimal determining region is flexible and adaptable to recognize diverse carbohydrates [35]. Legume lectins bind carbohydrates at the top of the concave side of the front sheet of the jelly-roll motif. The carbohydrates interact with four loops, three of which (loop A: 91–106, loop B: 125–135, and loop C: 75–83) are largely common to all legume lectins and the fourth (loop D: 211–216) varies in size and conformation and hence determines the specificity. Thus, the legume lectin fold is linked to the recognition of carbohydrate, in general, and it is the variations in the CBD and also the loop lengths that determine the binding specificity [35,41].

Ligand Binding

Legume lectins bind a wider variety of carbohydrates compared to any other plant lectin family. Single crystal X-ray diffraction studies showed that despite the differences in the primary sequences, folding of the polypeptide chains in the CBD region is similar among many legume lectins. About ~20% of the primary sequences are invariant and another 20% comprises similar types of amino acids. Most of these conserved amino acids are present in the CBD and participate in hydrogen bonding and/or hydrophobic interactions with the sugar. Each monomer has a single CBD with low K_a for monosaccharides, in the range 10^3–10^6 M^{-1}. However, because very often they are multivalent, they possess a high binding avidity [42]. The CBD region of legume lectins, similar to that of other lectins, has shallow depressions on

the surface of the protein [43,44]. Carbohydrates bind at the top of the concave side of the front sheet and interact with residues from four loops, namely, A, B, C, and D. The first three loops are largely common to all legume lectins and interact with all monosaccharides irrespective of specificity. They contain a constellation of four invariant combining residues in legume lectins, wherein loop A has Asp, loop B has Gly (except in the case of Con A), and loop C has Asn along with a hydrophobic residue, namely, Phe, Tyr, Trp, or Leu. As loop D varies in size and conformation, it determines the binding specificity. Asp and Asn play key roles in forming hydrogen bonds with the carbohydrate and are also involved in coordination with calcium ions, explaining the need for metal ions in sugar-binding activity. The hydrophobic residue or Leu further stabilizes the binding by stacking interactions with the sugar. Weaker van der Waals interactions are also found in large numbers and contribute significantly to binding [41]. Different lectins combine with the same ligand by different hydrogen bonding side chains. Mannose, for example, binds to Con A (Asn, Asp), *Galanthus nivalus* agglutinin (Asn, Asp, Gln, Tyr), and mannose-binding protein A (Asn, Gln) by different side-chain residues [45].

Peptide Mimicry

The early 1990s witnessed the interesting discovery of peptide mimicry of carbohydrate binding in lectins [46,47]. Peptides containing the consensus sequence motif, namely, Tyr-Pro-Tyr, bind to Con A with affinities comparable to that of specific sugar ligands, for example, methyl-α-D-mannopyranoside (Fig. 4). Binding of such peptides to Con A could be competitively inhibited by α-D-mannopyranoside in a dose-dependent manner. Cross-recognition of peptide ligands of Con A by antisugar polyclonal antibodies demonstrates that both of them share topological equivalence [48]. However, despite the functional mimicry of the two different types of ligands, crystal structure studies of the respective complexes showed that they do not necessarily share the same binding sites [49,50].

Carbohydrate-mimetic peptides can be used as functional surrogates to generate carbohydrate-specific immune responses. Hence, they can be used in the preparation of veterinarian and human vaccines against certain pathogens and tumor cells for greater efficacy of the vaccine because carbohydrate antigens make weaker immunogens than peptides [51–53].

STRUCTURE–ACTIVITY RELATIONSHIP IN LEGUME LECTINS

Lectins are involved in a multitude of cellular functions ranging from cell adhesion to pathogen recognition. In plants, although there is much debate as to their exact function, lectins are mostly seen to act as molecules of defense. The basis for all this is the interaction that occurs between the sugar and the

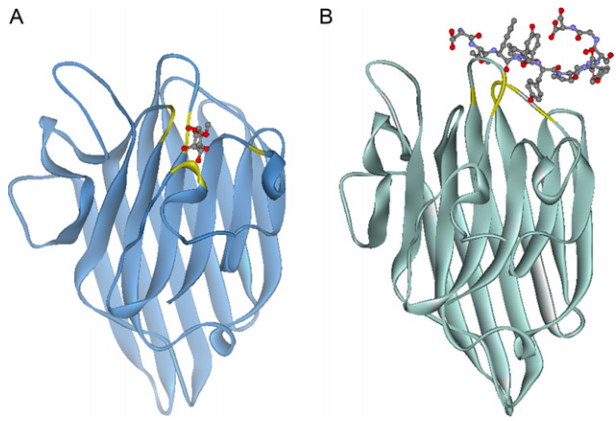

FIGURE 4 A comparison of the binding domains for 12-mer peptide and methyl mannoside of Con A: Ribbon representation of a subunit of Con A showing the binding regions for (A) 12-mer peptide DVFYPYPYASGS (PDB code: 1JYI) and (B) methyl-α-D-mannopyranoside (PDB code: 5CNA). The ligands are represented as balls and sticks. Different regions of the loops (highlighted in yellow) are involved in binding to the two ligands, despite the functional mimicry displayed by them.

protein. Elucidating the SAR of lectins provides a strong basis for understanding these processes and such understanding in turn helps to identify and/or improve the value of lectins in therapy. In addition, advancements in biophysical techniques and molecular biology, availability of synthetic glycoconjugates, and characterization of newer lectins with unique carbohydrate-binding properties have been helpful in understanding the SAR of plant lectins.

Glycoconjugates are made up of building blocks that mostly resemble each other in that they possess small patches of aliphatic portions, and a large number of —OH groups, with occasional *N*-acetyl groups. They are also known to possess carboxylate or sulfate groups to a lesser extent. Glycoconjugates thus contribute least to the selective recognition by proteins due to the lack of complexity of their building blocks, as compared to the peptides formed from 20 different amino acids plus the conformation flexibility built into them. Even oligosaccharide ligands, though they may seem more complex, lack the shape suitable for selective recognition because of their flexibility. Despite this, the specificity of protein–carbohydrate interactions is noteworthy.

Most legume lectins, though structurally similar to Con A, show significant differences in their activity. Lectins bind to a relatively restricted number of sugars; however, their binding specificities can vary, for example, *Datura stramonium* agglutinin recognizes oligosaccharides better than the monosaccharides, while Man/Glc-binding lectins show a high preference for the α-anomeric form. Others, such as Con A, bind to the internal rather than the terminal α-1,2 Glc or Man residues in glycoproteins and glycolipids [12].

The SAR of legume lectins has been discussed based on certain key aspects, such as the nature of the glycan moiety, configurational preferences of the lectin toward the sugar ligand, and its binding specificity.

Binding Specificity

Legume lectins are a large family of homologous proteins that display wide variety in binding specificities and hence are suited for the study of SAR of proteins. Site-directed mutagenesis and X-ray crystallography studies reported in 1990s helped to understand the basis of SAR in legume lectins, very often using Con A as the model lectin. In legume lectins, the carbohydrate-binding residues are distributed over several loops. For monosaccharides, a conserved core of residues (Asp208, Asn14, and Arg228 in Con A or Gly228 in the case of other legume lectins), which forms hydrogen bonds with Glc/Man, determines the exact shape of the monosaccharide binding site. In mono- and oligosaccharides containing a hydrophobic aglycon, a few distinct subsites next to the monosaccharide binding site are involved in the binding. The presence of metal ions, namely, Ca^{2+} and Mn^{2+}, is sometimes necessary for the sugar-binding activity of legume lectins as they stabilize the three-dimensional conformation of the CBD and its vicinity. Very often, the quaternary structure of the protein also plays an important role in contributing to a higher level of specificity, especially in the recognition of multivalent carbohydrates and in high affinity interactions of certain lectins, such as the tetrameric DBL with adenine and adenine-derived plant hormones [44,54,55].

Configurational Preference

Legume lectins are also known to display anomeric and configurational preferences. In the following examples, three highly conserved residues in the CBD of the lectins are able to distinguish between Gal and its 4-epimer Man (or Glc). *Lathyrus ochrus* lectin and lentil lectin recognize and bind to Glc/Man, and the sugars are oriented such that the hydrogen bonds are formed between the oxygens of the Asp side chain and the 4- and 6-OH groups of the sugar, the amide of Asn with 4-OH, and the main chain amide of Gly with 3-OH of the sugar. On the other hand, in the case of Gal-binding lectins, such as soybean agglutinin (SBA) and peanut agglutinin (PNA), oxygens of Asp form hydrogen bonds with 4-OH and 3-OH of the sugar and the amide of Asn and Gly are both bonded to 3-OH (Fig. 5). The correct positioning of these amino acids is directed via the two metal ions or via water molecules that provide the framework required for binding different sugars [45]. Figure 5 illustrates this more clearly. In a similar case of configurational specificity, the derivatives of galactose were unable to induce dimerization in blocked ricin owing to the lack of a C2-OH group. Blocked ricin is formed

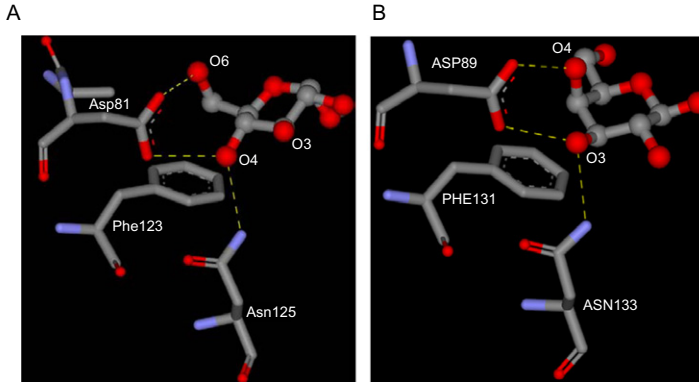

FIGURE 5 Schematic representation of the CBD with conserved residues, Asp and Asn, bound to (A) β-galactose in the combining site of *Erythrina corallodendron* lectin (PDB code: 1AXZ) and (B) α-glucose in the combining site of *Lathyrus ochrus* lectin-I (PDB code: 1LOA). The carbohydrate is shown in a ball-and-stick model.

when the two galactose-binding sites of the ricin B-chain are modified. At neutral pH, it is known to form dimers in the presence of sugars that have a similar relative orientation of the C2- and C4-OH groups as D-galactose. The orientation of the C3- and C6-OH groups has no influence on this phenomenon [56].

Oligosaccharide Affinity

The affinity of lectins for oligosaccharides is higher than for monosaccharides due to additional interactions with parts of the glycoconjugate occurring outside the CBD. This is seen in the higher (twice) affinity of galactose-specific *Erythrina corallodendron* lectin (ECorL) for lactose, which is attributed to the H-bond formed between the amide of Gln219 present in the variable region and the 3-OH of the glucose moiety of the disaccharide, but not its interaction with galactose. In addition, the H-bond between the same Gln219 and carbonyl of the acetyl of *N*-acetyl-lactosamine accounts for the four to eight times higher affinity of binding with ECorL as compared to lactose [45,57].

In general, in legume lectins, a constellation of three invariant residues, Asp, Asn, and an aromatic residue, are essential for sugar binding, irrespective of the sugar specificity of the lectin, and the binding involves H-bonds and hydrophobic interactions. In the case of nonleguminous lectins, the interactions are the same; however, the amino acids involved may differ even if the specificity of the lectins is the same [45].

SYNTHETIC GLYCOCONJUGATES AND INTERACTIONS WITH LECTINS

Lectin–carbohydrate interactions are highly specific. Since a single interaction between the CBD and the ligand is weak (mM to μM range), multiple interactions between them are necessary to achieve strong binding [58]. Lectins are useful tools in studying their interactions with glycoconjugates. These interactions depend on a number of factors, such as (a) the number of sugar moieties present, (b) the molecular weight and architecture of the glycoconjugate, and (c) the protein environment in and around the CBD [59]. Glycoconjugates of different architectures that mimic natural monosaccharide or oligosaccharide ligands (glycomimetics) have been synthesized to gain new information on lectin–carbohydrate interactions, and these may even modify the properties of lectins. Interactions with chemically modified glycoconjugates also contribute to the structure elucidation of lectins in solution via spectroscopic techniques, apart from the conventional crystallography studies.

Aromatic-Imino-Glycoconjugates

Inhibition of lectin activity and alteration of lectin properties by chemically modified glycoconjugates is of profound importance to glycobiology. In order to understand the lectin–carbohydrate interactions, experimental as well as computational studies of glycoconjugates have been carried out with various lectins, as reported recently. A comparative study of the agglutination and binding properties of eight different lectins with glyco-imino-aromatic conjugates clearly demonstrates the differential recognition of conjugates toward lectins. The lectins studied included those with Glc/Man specificity (*Dolichos lablab* lectin (DLL)-I, pea lectin, lentil lectin), Gal specificity (DLL-II, PNA, SBA, *Moringa oleifera* lectin), and Lac specificity (*Unio* lectin). Several C1-imino conjugates of D-galactose, D-lactose, and C2-imino conjugates of D-glucose, where the nitrogen center was substituted by salicylidene or naphthylidene, were synthesized and studied for their binding to these eight lectins. The results indicate that the agglutination inhibitions exhibited by the glycoconjugates are in line with the lectin specificity [60]. The presence of the hydrophobic naphthyl- and salicylyl- moieties makes the glycoconjugates more effective inhibitors (by 100–200 times), and is better than the corresponding simple sugars or sugar-amino derivatives [61].

In another study, the binding of JAC, a 66-kDa Gal-specific tetrameric lectin present in the seeds of the jackfruit, with the aromatic-imino glycoconjugates was examined. The relatively large size of the CBD of JAC and its flexibility enables the protein to interact with sugars having different hydroxyl orientations (epimers), including mannose, glucose, *N*-acetylneuraminic acid, and *N*-acetylmuramic acid, besides galactose [62]. Since the immediate vicinity of the CBD possesses hydrophobic amino acids, such as Trp, Tyr, and Phe, the

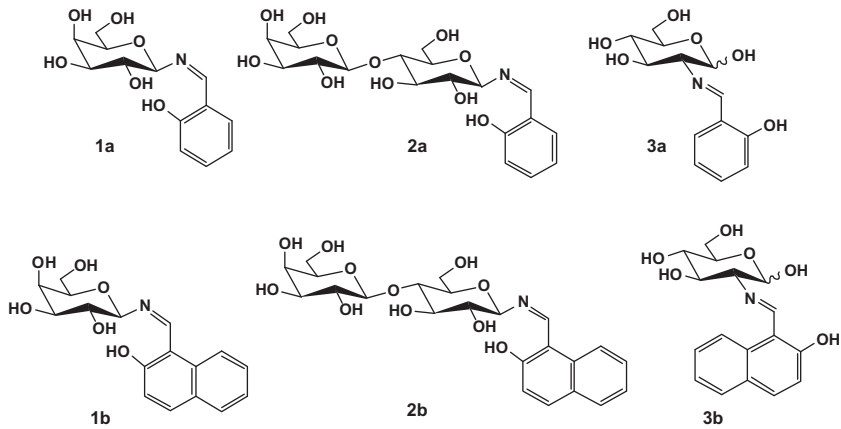

FIGURE 6 Schematic structures of the aromatic-imino-glycoconjugates studied as inhibitors of hemagglutination by jacalin: **1a**, **2a**, and **3a** are for the salicylidene-imino derivatives of galactose, lactose, and glucose, respectively. **1b**, **2b**, and **3b** are for the same derivatives but with a 2-hydroxynaphthalidene base.

synthesized glycoconjugates were designed to have hydrophobic moieties. In order to achieve this, the glycoportions were connected to the aromatic ones through imine linkage to result in a number of aromatic-imino-glycoconjugates. Salicylidene and 2-hydroxynaphthalidene imino conjugates of galactose, lactose, and glucose were synthesized and tested for their hemagglutination and for other studies (Fig. 6).

Out of the 10 glycoconjugates studied with JAC, galactosyl-naphthyl imine (**1b**) inhibited the agglutination effectively (viz., 260 times better than galactose) and showed better binding than galactose based on the IC_{50} values. Among the different glycoconjugates studied, the conjugates of galactose interacted better with JAC than the other glycol-derivatives. Among the galactose derivatives, 2-hydroxynaphthalidene-, salicylidene-, and amino- derivatives exhibited interaction with JAC in decreasing order. Docking experiments demonstrated the molecular interactions present between the glycoconjugate and JAC and the same result has also been derived based on the experimental data. The specific binding of galactose conjugates and the nonspecific binding of other conjugates were demonstrated based on ITC, wherein **1b** showed specific binding through exothermic isotherms with JAC. Both the far- and near- UV CD spectra show changes in the secondary structures of the JAC. The near-UV CD studies support the interaction of salicilyl and naphthyl conjugates with the aromatic amino acids, namely, Trp, Tyr, and Phe. Computational studies show that the galactose moiety interacts mainly through hydrogen bonding at the CBD region ensuring specificity, whereas the hydrophobic moiety is stabilized mainly by π...π interactions as well as by C—H...π interactions, leading to cumulative changes that alter the properties of protein. Conjugates of other sugars interact primarily through hydrophobic ones (Fig. 7) [61].

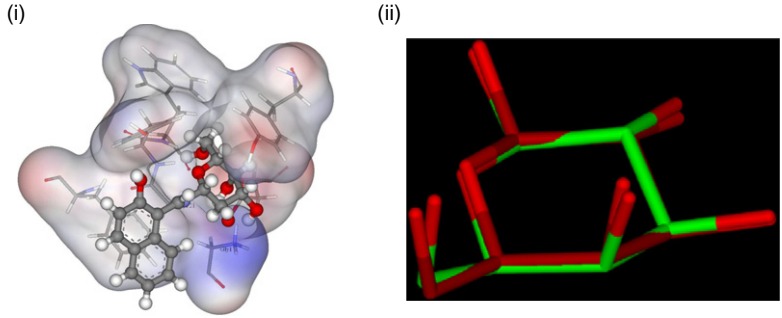

FIGURE 7 Docking of **1b** with jacalin: (i) van der Waals surface representations (colored with electrostatic potential) showing the binding of glycoconjugates with jacalin at CRD for **1b**. (ii) Overlap of the structures of galactose as obtained from crystal structures (red color) and that from docking studies (green).

The interactions between the glycoconjugate and the protein residues, as obtained from computational docking studies, were analyzed from the viewpoint of both the amino acid residues and the glycoconjugates, and the results are shown in the histogram given in Fig. 8A. The binding energies (Fig. 8B) were found to follow a trend, Gal < GC1A <<< GSI < GNI, suggesting that the imino-aromatic conjugates interact better with the CBD of JAC owing to the presence of their imine and aromatic moieties as compared to simple galactose or its C1-amine, which lacks such moieties [61].

Lactosylated Glycoclusters

In a similar study, Cecioni and coworkers synthesized lactosylated glycoclusters around a core scaffold of porphyrin and calix[4]arenes and studied their hemagglutination inhibition abilities in the presence of two lectins, *Erythrina cristagalli* (ECA), a legume lectin, and recombinant human galectin-1, both being specific for galactosides. The glycoconjugates were able to inhibit the hemagglutination displayed by both the lectins by interacting with the nonglycon unit, namely, porphyrin–calixarene, weakly in the case of ECA and preferentially in the case of galectin-1 [63].

Polymeric Glycoconjugates

Other studies on synthetic glycoconjugates include the design of oligocsaccharide-based glycopolymer ligands. Binding of WGA to the synthetic chitobiose polymer, poly ((*p*-vinylbenzamido)-β-diacetylchitobiose) was increased 1000-fold as compared to the oligosaccharide itself. The increased binding is attributed to the interactions of the lectin with the hydrophobic

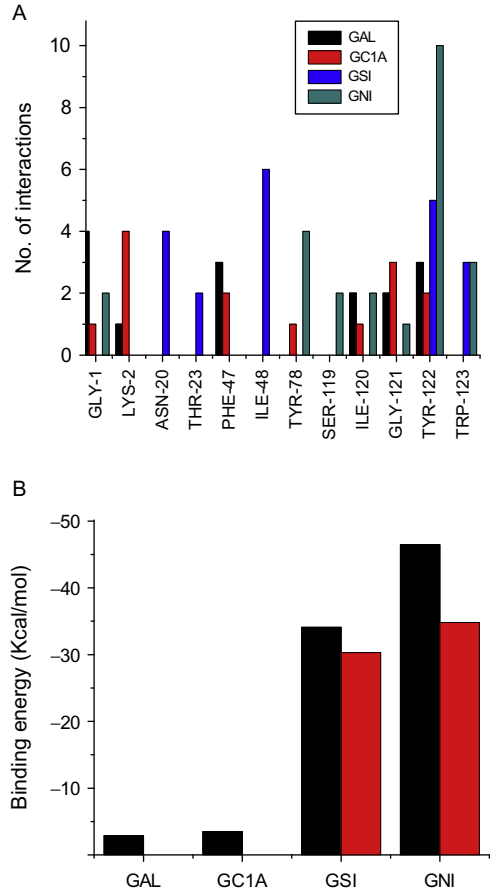

FIGURE 8 Interactions present between the glycoconjugate and the amino acid residues of jacalin in CBD: (A) based on the amino acid residues; (B) the corresponding binding energies. The red columns show the binding energy contribution from the aromatic portion.

phenyl aglycon and the multiantennary oligosaccharide chains [64]. Similar binding was observed with *Ricinus communis* agglutinin in the presence of synthetic glycoconjugates of styrene derivatives substituted by oligosaccharides due to the presence of the phenyl moiety [65]. In all these studies, the presence of the aglycon aromatic portion in the synthetic glycoconjugates contributed to more favorable binding interactions. Therefore, the introduction of aromatic moieties into drug molecules may influence the overall drug–protein interactions, thereby altering the original properties of the proteins they bind. These nonspecific and/or hydrophobic interactions of the drug moiety cannot be ignored completely though they bind the proteins through specific interactions.

LEGERE TO LECTINOMICS

Lectins are present ubiquitously in all classes of organisms. The term "lectin" was taken from the Latin word "legere," which means "to select," after it was noticed that extracts of castor bean had a component that selectively agglutinated red blood cells, as described by Stillmark for the first time in his doctoral thesis in 1888. Since the purification of various lectins by Sharon and Lis in 1972, the information available on lectins has grown enormously. Today, it is not surprising to find terms such as "lectinology" to describe a separate branch of lectin chemistry and "lectinomics" to forecast trends in lectin-biorecognition technology, as part of the neologisms in science [66,67]. Apart from the known biochemical and biophysical aspects of these proteins, several lectin genes have now been sequenced, cloned, and expressed in heterologous systems. Lectinology is a growing field with tremendous impending applications, from deciphering the proteins' native functions in plants to conferring new properties in unrelated systems, especially in the biomedical field. Some such uses of plant lectins are listed later.

Mitogenic, Immunomodulatory, and Antiproliferating Activity

Legume lectins such as PHA, Con A, SBA, and PNA have been extensively used as mitogenic stimulants of lymphocytes. Research on newer lectins has proved that they have tremendous applications in the field of biomedical research. For example, polymers coated with lectins such as SBA and *Ulex europaeus* agglutinin-I show promising potential as vaccine carriers [68,69]. Another lectin from *Bauhinia variegata* is known to reconstruct skin layers and heal surgical wounds in mice [70]. A galactose-binding legume lectin, *Astragalus membranaceus* lectin, displays antiproliferative properties toward human leukemia cells *in vitro* [71]. Such properties of lectins could easily be exploited in disease diagnosis and prophylaxis, and in the treatment of disease states. Plant lectins interact with animal lymphocytes in a manner that mimics microbial antigens. They mediate such functions by interacting with glycoproteins on the surface of lymphocytes, often turning on or turning off certain pathways involved in the initiation and regulation of these activities. Mitogenic lectins are presumed to bind to the T-cell receptor complex and initiate such a response [72]. Mistletoe lectin, which is used as an adjuvant in cancer therapy, is known to activate caspases, enzymes involved in the self-destruction of cells, through the tumor necrosis factor receptor I signaling pathway [73].

Tools of Segregation

The ability of lectins to differentiate carbohydrate ligands has been exploited in blood typing, bacterial typing, and cell and glycoconjugate separation. Because the blood groups are determined by carbohydrate antigens, blood

types H, N, and A1, other than the ABO types, can be distinguished by lectins such as those from *Ulex europaeus*, *Vicia graminea*, and *Dolichos biflorus*, respectively [74]. Based on the carbohydrates expressed on the surface of *Campylobacter concisus*, 45 strains of the pathogenic bacteria were grouped into 13 lectin-reaction types [75]. As seen already, lectin-immobilized columns can be used in the enrichment of glycoconjugates from complex biological samples [26] and in the isolation of certain cell types based on the expression of surface glycans. Lectins serve as tools in the identification of cell lineage-specific glycan markers and in the isolation of homogenous populations of cells as was seen in the isolation of human neuronal progenitor cells from a mixed population of embryonic stem cells by the lectin *Vicia villus* agglutinin [76].

Tools of Cytocharacterization

Studies on the uses of lectins in cytochemical characterization of cells and mapping of neuronal pathways have been reported. Histochemical characterization of the different cell types present in the equine mucosa of guttural pouches was performed with various lectins of different specificities. In such studies, lectins are often labeled with a dye that makes visualization of the cells or cellular compartments possible upon its binding to the lectin [77]. *Phaseolus vulgaris* leucoagglutinin (PHA-L) has been used as an anterograde label in tracing the path of efferent axons. In this technique, PHA-L is injected into a region of the brain and is taken up by the dendrites and cell bodies and transported by the axons, which can be visualized by microscopy and thus the path of the axon traced [78].

Design of Novel Lectins

Research on legume lectins has seen new developments in the designing of novel lectins with unique carbohydrate-binding specificities. For example, a chimeric lectin from leguminous lectins *Bauhinia purpurea* agglutinin (BPA), specific for lactose and galactose, and *Lens culinaris* agglutinin (LCA), specific for mannose, have been cloned and expressed in *Escherichia coli*. A nine amino acid residue sequence corresponding to the metal-binding site of BPA was replaced with the corresponding amino acid sequence from LCA to generate a chimeric lectin with binding properties different from those of either BPA or LCA. The chimeric lectin had affinity toward hybrid-type as well as high-mannose type glycoconjugates. Similarly, random mutations introduced into the cDNA of the CBD of BPA produced a lectin with specificity for mannose or GalNAc, rather than galactose or lactose. This opens up the possibility of designing artificial lectins (cyborg lectins) with desired carbohydrate-binding specificities [79,80].

Tangible Applications of Lectins

In the wake of the vast amount of data on plant lectins that has been generated, interesting aspects and potential uses of lectins are being revealed. Although most of them are limited to animal and *in vitro* cell studies, some of them are already being successfully used in more tangible applications. Some of these include insect-resistant crops expressing lectin genes of another species as an alternative to chemical pesticides [81], and the use of mistletoe extract, Iscador®, containing lectin as the active ingredient, in complementary cancer therapy [82]. Further studies on these bioactive plant products along with developments in technology can help these applications reach society at a much faster rate.

ABBREVIATIONS

BPA	*Bauhinia purpurea* agglutinin
CBD	carbohydrate-binding domain
CRD	Carbohydrate recognition domain
Con A	concanavalin A
DBL	*Dolichos biflorus* lectin
DLL	*Dolichos lablab* lectin
ECA	*Erythrina cristagalli*
ECorL	*Erythrina corallodendron* lectin
GalNAc	*N*-acetylgalactosamine
GlcNAc	*N*-acetylglucosamine
JAC	jacalin
LCA	*Lens culinaris* agglutinin
Man	mannose
PHA	*Phaseolus vulgaris* agglutinin
PNA	peanut agglutinin
SAR	structure–activity relationship
SBA	soybean agglutinin
WGA	wheat-germ agglutinin

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Chapter 11

Lycopene: A Review of Chemical and Biological Activity Related to Beneficial Health Effects

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Chapter Outline

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INTRODUCTION

Carotenoids were first discovered in biological tissues during the nineteenth century, when W.H. Wackenroder isolated carotene for the first time in 1831 [1], from carrot roots. According to a review by Davies *et al.* [2] and Isler [3], in 1837, J.J. Berzelius obtained yellow pigments from leaves, called xanthophylls. Officially, the class of carotenoids received its name in 1911 from M.S. Tswett, who was able to isolate and purify xanthophylls and carotenes using chromatography. It was during the period 1873–1927 that lycopene was separated from other carotenoids by Harsten and R. M. Willstätter, who isolated 11 g of lycopene from 75 Kg of tomatoes. Later, research conducted by P. Karrer, R. Kuhn, L. Zechmeister, and I.M. Heilbron led to the elucidation of the chemical structures of lycopene and other carotenoids, establishing relationships between the presence of conjugated double bonds, their color, and spectroscopic properties, as well as their similarities to retinol molecule. Following these studies, P. Karrer received the Nobel Prize in 1937 for the elucidation of the chemical structure of β-carotene [2,3]. From these first findings regarding the structure and properties of lycopene, substantial knowledge has been accumulated about its functions in plant and animal tissues, especially related to its role in the maintenance or improvement of human health.

Different biochemical functions of lycopene in preventing chronic diseases have been proposed: lycopene may act as an antioxidant, trapping reactive oxygen species (ROS), increasing the overall antioxidant potential, or reducing the oxidative damage to lipids, proteins, and deoxyribonucleic acid (DNA), thereby lowering oxidative stress, which may lead to a reduced risk for cancer and cardiovascular disease (CVD) with an anticarcinogenic and antiatherogenic effect. Alternatively, the increased lycopene status in the body may regulate gene functions and metabolism, improve intercellular communication, and modulate hormone and immune response, thus lowering the risk for different chronic diseases [4]. These mechanisms may also be interrelated and may act simultaneously to provide health benefits. However, the exact mechanisms of action of lycopene are still not clearly understood.

In this sense, critical reviews by experts, several epidemiological studies, cell culture, and animal and human intervention studies provide scientific evidence to support the role of lycopene in human health [5,6] and its protective effects against CVD (one of the main causes of mortality in developed countries), as well as hypertension, atherosclerosis, diabetes, certain types of cancer (prostate, lung, cervix, breast, pancreas, stomach), eye diseases, and skin damage among others [7–12].

On the other hand, the developments in lycopene analysis by spectroscopic and chromatographic techniques together with mathematical modeling have opened up new possibilities to evaluate and determine the concentration of this carotenoid in natural products. Methods for efficient lycopene extraction from food samples are reported, taking into account the importance of sample

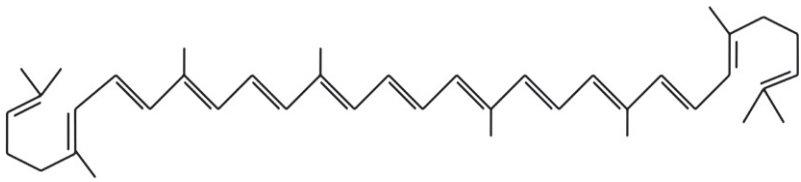
preparation, the selection of the extraction solvent, and the extraction procedure. The identification and quantification of lycopene using different advanced techniques and methods are also discussed. A very large number of reviews and chapters dealing with lycopene analysis exist in the literature. A personal selection has been made in this review, which covers the developments in the analysis of lycopene during the last decades. The review is the result of scanning through a large number of references. Nevertheless, many more exist and those listed reflect the personal choice of the authors.

LYCOPENE: CHEMISTRY, METABOLISM, AND BIOAVAILABILITY

Lycopene is a lipophilic red-colored carotenoid pigment, composed of eight isoprene units (octaprene) joined by regular head to tail bindings, except in the middle of the molecule where the binding is tail to tail, giving rise to a symmetric structure (Fig. 1). Lycopene is the prototype of other carotenoids, which may be formed through cyclizations, oxidations, or reductions. The presence of a system of 11 conjugated double bonds confers on lycopene its ability to absorb light in the UV–visible region.

Lycopene is insoluble in water, almost insoluble in methanol and ethanol, and soluble in organic solvents such as carbon disulphide, ethyl ether, petroleum ether, chloroform, and benzene.

More than 72 lycopene isomers have been identified, the most important being all-*trans*-lycopene, neolycopene A (6-*cis*-lycopene), 5-*cis*-lycopene, prolycopene (1-, 3-, 5-, 7-, 9-, 11-*cis*-lycopene), and *cis*-lycopene (1-, 3-, 5-, 6-, 7-, 9-, 11-*cis*-lycopene) [13]. Naturally, lycopene is found mainly in the all-*trans* form and can be isomerized to mono-*cis* or poly-*cis* through exposure to high temperatures, light, oxygen, acids, catalysts, and metal ions, with the *cis* form being less thermostable. Previous studies have shown a better stability of lycopene from natural matrices, such as tomatoes, than in its isolated form [14,15].



Lycopene

Molecular weight: 536.89

Molecular formula. $C_{40}H_{56}$

Molecular composition: C: 9.49%; H: 10.51%

FIGURE 1 Chemical structure of lycopene.

After ingestion, lycopene is quickly absorbed in the intestine, at a rate of 7–10%, together with dietary fat, and distributed to corporal tissues. The existence of protein-mediated transport mechanisms, which are saturable at low doses of lycopene (<30 mg), have been suggested. In an intervention study in humans, the maximum plasma concentrations of 4–11 μg/dL were reached after 15–32 h with an intake of 10–30 mg/day; higher intakes of up to 120 mg/day did not give rise to higher lycopene plasma concentrations [16,17].

Although the major form in food products is all-*trans*-lycopene, the bioavailability is higher for *cis*-lycopene isomers; this fact has been attributed to the lower length of the molecules, a lower tendency to precipitate in the gastrointestinal tract, and/or higher solubility in bile micelles, which make it easy for them to be incorporated into chylomicrons. Some authors have also indicated intense isomerization during or after absorption, which explains the higher levels of *cis*-lycopene found in plasma [18,19].

The food matrix, food processing, and the presence of different substances in the diet could also have an influence on lycopene availability. Some studies have shown equal bioavailability from different tomato products, such as lycopene-rich tomato juice, tomato oleoresin, and lycopene supplements, as well as from a synthetic lycopene source [20–22]. However, lycopene absorption can be improved by low heating as well as by reducing particle size, as is the case in many tomato products due to the technological processes applied [23,24]. The superior availability of lycopene from processed tomatoes than from raw tomatoes is probably due to the release of lycopene from the cellular matrix and the isomerization of all-*trans*-lycopene into *cis*-isomers induced by heating processes [25–27].

The presence of fat in tomato products can also improve the intestinal absorption of lycopene, as well as the simultaneous ingestion of β-carotene. On the other hand, any factor that decreases fat absorption will also decrease lycopene absorption, as is the case with the synthetic oil Olestra in food or drug formulations. Koonsvitsky *et al*. [28] reported a reduction of nearly 30% over 16 weeks in patients consuming Olestra together with lycopene, and the presence of very high levels of phytosterols in some functional foods, which have been demonstrated to reduce carotenoid absorption. Thus, consumption of lycopene and this kind of products should be separated by several hours [29,30].

In the serum, lycopene is one of the most abundant carotenoids, together with lutein, β-carotene, and γ-carotene, with normal levels in the range of 220–1168 nmol/L in healthy subjects (585 nmol/L on average). Variations may be influenced by factors such as ingestion of alcohol or smoking [29,31,32]. The maximum levels of lycopene in serum are found to be at 6 h after its ingestion with an average half-life of 12–20 h [24,33].

Lycopene is stored mainly in the liver, where it can be mobilized when serum levels decrease. Significant amounts have also been found in other tissues, especially those rich in lipids, such as adipose tissue, adrenal glands,

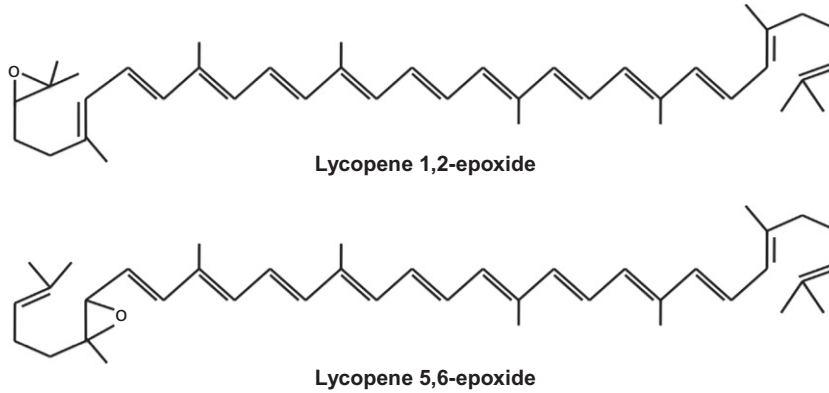


FIGURE 2 Chemical structure of the main lycopene metabolites.

prostate, kidney, and ovaries [14,30,34]. Although most of the ingested lycopene is in the all-*trans* form, between 50% and 88% of lycopene is found as *cis*-isomers [14]. During its metabolism, the oxidation of lycopene first produces lycopene 1,2-epoxide and then lycopene 5,6-epoxide as the main metabolites (Fig. 2), together with other minor metabolites such as lycopene 1,2;5,6-diepoxide; lycopene 1,2;5′,6′-diepoxide; lycopene 5,6;5′,6′-diepoxide; and lycopene 1,2;1′,2′-diepoxide, followed by ring opening and rearrangement to cyclic structures. [35].

Excretion of lycopene takes place through the feces and in much lower amounts through urine; some lycopene can also be excreted via sebaceous glands. Due to its lipidic nature, breast milk is a good vehicle for lycopene, solubilized in the lipid fraction, providing for lycopene intake for breastfed children. In general, lycopene is found in human milk at approximately 3.8 μg/100 g, or 10% of the serum concentrations [29,36], although a low correlation has been found between the amount of lycopene in the diet of tested mothers and concentrations in breast milk.

Lycopene from either natural sources or supplements is generally well tolerated by the population. Only one manifestation of excessive lycopene intake (about 2 L of tomato juice per day) has been described as lycopenemia, consisting of a high concentration of lycopene in the blood that produces a yellowish-orange pigmentation of the skin, with no health alterations, and reversibility after administration of a lycopene-poor diet [37]. Although there is no scientific evidence available for the metabolism of lycopene during pregnancy, animal studies have revealed the absence of adverse effects associated with the consumption of lycopene-rich foods during pregnancy, taking into account acute, subacute, and chronic toxicity studies, as well as reproductive and geno-toxicity studies [17].

Based on the available toxicology studies, a no-observed-adverse-effect level (NOAEL) of 3 g/kg/day is assumed, which is much higher than the

estimated lycopene intake among the general population. Because of the lack of adverse-effect data for lycopene in animals or healthy humans, the Institute of Medicine has not set a tolerable upper intake level for lycopene. Synthetic lycopene, tomato lycopene extracts, and crystallized lycopene extracts are generally recognized as safe (GRAS) for use as ingredients in a variety of foods at levels of 0.5–7% [17].

SOURCES OF LYCOPENE

Substances included in the denomination of carotenoids are natural pigments that can be synthesized by plants, algae, and bacteria, and reach animal tissues only through the feeding process. Thus, humans are unable to synthesize carotenoids and the level of these compounds in plasma depends on the consumption of different carotenoid sources in the diet [38].

Lycopene is not as widespread in nature as other carotenoids, contributing to the attractive colors of some animals (e.g., flamingo feathers) or plant tissues (flowers and specially fruits). Lycopene is naturally located in the chromoplasts of plant cells, and it is found primarily in tomato fruits (about 80% of their total carotenoid content) and transformed tomato products. Some other edible fruits may also be sources of lycopene for the diet, mainly watermelon, guava, and pink grapefruit (Table 1); it is found in minor amounts in other food products such as apricot, cloudberry, cranberry, eggplant, grape, papaya, and peach [39–41,45,46].

TABLE 1 Main Dietary Sources of Lycopene and Their Range of Contents

Fruit	Range of Lycopene Content (mg/100 g of Edible Portion)
Tomato (*Solanum pimpinellifolium* L.)	0.594–43.09
Tomato (*Solanum lycopersicon* L.)	1.60–18.46
Pink guava (*Psidium guajava* L.)	5.43–7.02
Watermelon (*Citrullus lanatus* (Thumb.) Mansf.)	2.45–7.30
Pink grapefruit (*Citrus paradisi*)	3.32–3.36
Papaya (*Carica papaya* L.)	2.54–3.72
Persimmon (*Diospyros kaki* L.)	0.158–0.359
Mango (*Mangifera indica* L.)	0.072–0.082
Wild cherry (*Prunus avium* L.)	0.010

Data obtained from Refs. [38–44].

Tomato is one of the vegetables that are in great demand, and tomato products have a great economical relevance worldwide. The importance of tomato lies in its highly appreciated sensory properties, its being able to integrate into diverse food preparations, either cooked or raw, and also in its nutritional properties, as an important source of bioactive compounds with antioxidant activity, lycopene being one of the more interesting ones. These features contribute to significantly increasing the value of these tomato products from the consumer point of view [42,47,48]. Furthermore, there is a special interest in tomato as it is a major component in the "Mediterranean diet," which has recently been associated with a healthier lifestyle.

Fresh tomato and its processed products are the main contributors to the total lycopene intake in the diet, present mainly in the all-*trans* configuration in variable amounts between 3.1 and 43.1 mg/100 g depending on the species, variety, and ripening stage. These contents may be different in varieties with different colors, the very red varieties having as much as 44 mg/100 g, and the yellow varieties as little as 5 mg/100 g [29,49]. The major part of the lycopene content in tomato is associated with the water-insoluble fraction, the exocarp or skin of the fruit being the part with higher contents, ranging from 35.4 to 53.56 mg/100 g.

Lycopene degradation in commercial tomato-based products not only affects the attractive color, but also the nutritional and functional value as an antioxidant in the final product. In fact, the stability of lycopene is critical for health benefits; therefore, it is essential to preserve its content in food products. Most commercial tomato products have quite a long shelf life, which makes them susceptible to lycopene losses [14,50,51].

There are some studies related to the effect of processing on lycopene stability that show that lycopene instability phenomena in biological tissues occur through *cis*-isomerization or oxidation. Lycopene autoxidation may occur at ambient temperature, in the presence of O_2, and can be accelerated with light, heat, oxygen, moisture, extreme pH, or the catalytic action of some metals. Oxidation may also take place by the action of lipoxygenase, with the formation of hydroperoxides, and the presence of some antioxidants may inhibit this reaction. These processes may be reduced by using techniques that avoid contact with oxygen in air, such as freeze-drying, microwave, or others [25,26,52–58].

The final concentration of lycopene in processed tomato products depends on the kind of technological treatment and the original raw material, and usually ranges between 0.85 and 94.0 mg/100 g [59–61]. Tomato juices may contain between 5.95 and 20.10 mg/100 g of lycopene, while ketchups usually contain a significantly higher content of lycopene, between 15.37 and 24.60 mg/100 g [62] (Table 2).

Consumption of lycopene through the diet and supplements can contribute to half of the carotenoids in the human serum [63]. Mackinnon *et al*. [64] studied the frequency of consumption of lycopene (from various sources) in a week among people aged between 25 and 79 years. The results showed that

TABLE 2 Lycopene Content (Average ± Standard Deviation) Found in Different Brands of Common Tomato-Based Foods During 2 Years' Storage Time [62]

	Tomato Products									
	Juices				*Sauces*			*Ketchups*		
Code of tomato brands	J1	J2	J3	J4	S1	S2	S3	K1	K2	K3
Lycopene (mg/100 g)	13.16 a ± 0.568	16.72 b ± 0.114	20.10 c ± 1.019	12.56 a ± 0.479	20.25 a ± 0.710	20.67 a ± 0.288	19.75 a ± 0.593	15.37 a ± 0.177	21.95 b ± 0.266	24.60 b ± 0.825

For each type of product, different letters mean significant statistical differences (ANOVA Tukey test, $p < 0.05$).

the consumption of lycopene is higher in the form of fresh tomato, followed by sauces, pizza, and ketchup. It was also shown that subjects between 25 and 49 years were significantly the greatest consumers of ketchup and pizza sauce.

Tucker *et al*. [65] used the Willett 126-item food-frequency questionnaire to determine lycopene intake. The reliability of this method was tested in elderly patients, showing that this questionnaire does not provide a reasonable evaluation of lycopene status in such patients since the effects of smoking, age, energy intake, and body mass index may alter the absorption and subsequent plasma levels of lycopene.

To date, no international standard recommendation for daily lycopene intake has been made, although different studies have been carried out with this purpose. Rao [12] proposed a lycopene intake of 7–8 mg/day as the recommended amount to achieve antioxidant properties in the human body. Considering this, the ingestion of 100 g of most of the varieties of tomato and tomato-based products would guarantee the intake of this recommended lycopene amount: 100 g of tomato sauce provides approximately 6.20 mg of lycopene, and 100 g of pizza sauce may contain about 12.71 mg of lycopene. Also, the daily ingestion of a glass of tomato juice (200–250 mL); 5 portions (10 mL) of ketchup; 120 g of tomato sauce; or 60 g pizza sauce would provide for 7–8 mg/day of lycopene, as indicated by Rao [12].

Regarding dietary lycopene supplements, lycopene is commercially available in many formulations, mainly used for prostate treatment, but it is also used by the general population. Its content is highly dependent on a variety of factors such as quality of the raw materials, manufacturing process, and packaging. Since no official standards have been established to regulate the production of lycopene dietary supplements, dosage ranges must be employed as guidelines. Generally, a daily dose of 5–10 mg is taken for general recommendations. In adults with hypercholesterolemia, 60 mg of daily intake may cause a 14% reduction in plasma low-density lipoprotein (LDL) cholesterol concentrations [66].

LYCOPENE MECHANISMS OF ACTION

Although different physiological mechanisms of action have been reported for lycopene in the scientific literature, some of the most studied have been those related to protection against oxidative damage, effects on cardiovascular health, and antineoplastic activity.

Antioxidant Activity

Oxidation processes involve electron transference reactions, with the participation of molecules, atoms, or ions. These processes usually occur enzymatically, as in the case of catalase or dehydrogenase reactions in mitochondria, acting in several stages of the respiratory chain. During the breathing process, oxygen is

consumed and adenosine triphosphate (ATP) is generated, with the production of carbon dioxide and water. However, this process is not perfect, since other pollutant molecules also take part, and ROS are produced. The very reactive free radicals formed normally as a result of the cellular aerobic activity have an unpaired electron with the potential to cause damage to a great number of biological molecules. Between 1% and 3% of the oxygen that our cells consume is transformed into ROS, which are free radicals or take part in their formation. The permanent production of free radicals is opposed by the organism through the production of antioxidants. When the balance between free radicals and antioxidants is lost, harmful processes associated with the development of several diseases are triggered.

Human antioxidant systems are grouped into two principal classes, enzymatic and nonenzymatic endogenous antioxidants. The endogenous system is quite efficient; however, it does not suffice and the human body depends on various types of antioxidants present in the diet to maintain free radical concentration at low levels.

The most important antioxidants present in the diet are carotenoids (such as lycopene), phenolic compounds, vitamin C (ascorbic acid), and vitamin E (α-tocoferol) [67,68]. Lycopene is one of the most powerful antioxidants among dietary carotenoids, two times more efficient than β-carotene [69]. Lycopene has the highest singlet oxygen-quenching capacity *in vitro*, which results in excited lycopene molecules that dissipate the newly acquired energy through a series of rotational and vibrational interactions with the solvent, thus returning to the unexcited state and allowing them to quench more radical species. This is the reason why its presence in the diet is considered of great significance [12,60,61,70]. Due to the presence of conjugated double bonds in the chemical structure of lycopene, it can capture ROS, and work as an antioxidant at low oxygen pressure [35,71].

Lycopene's antioxidant action is beneficial in the prevention and improvement of certain pathologies, since most of them start with a cellular oxidation process. Results obtained by Wenli *et al*. [72] showed the greater antioxidant capacity of lycopene (and all the isomeric forms of lycopene) in human tissues in comparison with other antioxidant substances. Böhm *et al*. [69] also reported higher trolox equivalent antioxidant capacity (TEAC) values for lycopene than for other carotenoids.

Modulation of Lipid Metabolism

Lipoprotein metabolism plays an important role in the development of several human diseases, including coronary artery disease and the metabolic syndrome. A good comprehension of the factors that regulate the metabolism of the various lipoproteins is therefore a key factor for a better understanding of the variables associated with the development of these diseases.

Related to CVD, elevated blood LDL-cholesterol concentrations are associated with increased risk of coronary heart disease (CHD); there is strong evidence for the biological basis through which it can contribute to the development of atherosclerosis (one pathway to CHD). Therefore, a decrease in blood LDL-cholesterol concentration may be considered beneficial in the context of reduction of disease risk for CHD. Similarly, reduction in systolic blood pressure may be considered beneficial in the reduction of disease risk for CHD or stroke.

It is now well recognized that atherosclerosis is an inflammatory disease that begins with the development of fatty streaks, formed when macrophages that have invaded the artery wall scavenge lipid from plasma lipoproteins in the subendothelial space, and eventually become so engorged that they can form foam cells. LDL plays a major role in macrophage foam cell formation; however, uptake of native LDL via the LDL receptor (LDLr) is downregulated when intracellular cholesterol levels begin to rise, and thus does not lead to foam cell formation. The modified LDL, however, is taken up mainly by unregulated scavenger receptors, allowing large amounts of lipid to accumulate intracellularly [73].

Lycopene as an isoprenoid is synthesized in plant and animal cells from mevalonate via the 3-hydroxy-3-methyl glutaryl-CoA reductase (or HMG-CoA reductase) pathway, an enzyme that produces cholesterol and other isoprenoids, regulated by an end-product repression. Competitive inhibitors of reductase induce the expression of LDL receptors in the liver, which in turn increase the catabolism of plasma LDL, lowering the plasma concentration of cholesterol.

Lycopene has anticholesterol effects as it is able to reduce the expression of HMG-CoA reductase in a dose- and time-dependent manner. The inhibition of HMG-CoA reductase by lycopene is also accompanied by a reduction in intracellular cholesterol levels [74], both in cultured macrophages [66] and in human subjects [75]. In agreement with these *in vitro* observations, dietary supplementation of lycopene in human subjects resulted in a significant reduction in plasma LDL-cholesterol concentrations [76].

Other variables in lipoprotein metabolism are regulators such as caveolins and caveolae. Caveolae are small plasma membrane invaginations that are observed in terminally differentiated cells. Their most important protein marker, caveolin-1, has been shown to play a key role in the regulation of several cellular signaling pathways and in the regulation of plasma lipoprotein metabolism by controlling plasma levels as well as their composition. Thus, caveolin 1 (cav-1) plays an important role in the development of atherosclerosis [77]. Palozza *et al*. [74] observed that lycopene induced the expression of both ATP-binding cassette, subfamily A member 1 (ABCA 1) and cav-1 in THP-1 macrophages in a dose-dependent manner, favoring cholesterol efflux through a potential mechanism involving Ras homolog gene family member

A (RhoA) inactivation and a subsequent increase in peroxisome proliferator-activated receptor-c (PPAR-c) and liver X receptor-a (LXRa) activity.

Other mechanisms that explain lycopene's effect in atherosclerosis prevention include prevention of endothelial injury; modulation of lipid metabolism through cholesterol synthesis control and oxysterol toxic activities; reduction of inflammatory response through changes in cytokine production; and inhibition of smooth muscle cell proliferation through regulation of molecular pathways involved in cell proliferation and apoptosis [74].

There is renewed interest in studying the possible beneficial effects of cytokines on the heart. The induction of most cytokine genes requires activation of the transcription factor, nuclear factor κβ (NF-κβ). In cardiac myocytes, the stimulation of p38 mitogen-activated protein kinase (MAPK) by the mitogen-activated protein kinase kinase (MAPKK), mitogen-activated protein kinase kinase 6 (MKK6), activates the transcription factor, NF-κβ, and protects cells from apoptosis [78]. Palozza *et al*. [74] reported the effect of lycopene in reducing macrophage foam cell formation in response to modified LDL, by decreasing lipid synthesis in the cells and down-regulating the activity and expression of scavenger receptors. The mechanism by which lycopene was able to inhibit oxysterol-induced pro-inflammatory cytokine production, at both protein and messenger ribonucleic acid (mRNA) levels, was a redox mechanism involving an inhibition of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, a decrease in ROS production and in MAPK, and NF-κβ activation.

Lycopene is able to modulate the redox status, since it has been reported to chemically interact with ROS and undergo oxidation; to modulate ROS-producing enzymes, such as NADPH oxidase, inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2) and 5-lipoxygenase (5-LO), and to control redox-sensitive molecular pathways. It has also been found that lycopene has a role in the inhibition of the pro-inflammatory cascade generated by macrophages since tumor necrosis factor alpha (TNF-α)-induced intercellular adhesion molecule 1 (ICAM-1) expression in human umbilical vein endothelial cells (HUVECs) was inhibited by lycopene, whereas COX-2 and platelet endothelial cell adhesion molecule (PECAM-1) expression was not affected [79].

These findings suggest that lycopene may act as an antiatherogenic agent through a mechanism involving, at least in part, an antioxidant mechanism.

Antineoplastic Activity

According to Clinton [29], much of lycopene's antineoplastic activity may be attributed to its antioxidant properties. However, other mechanisms underlying the inhibitory effects of lycopene on carcinogenesis have been described, such as upregulation of detoxification systems, interference with cell proliferation, induction of gap junctional communication (GJC), inhibition of cell cycle progression, and modulation of signal transduction pathways [80].

The antineoplastic activity of lycopene may be due to its inhibition of DNA synthesis [33]. Lycopene strongly inhibited proliferation of endometrial (Ishikawa), mammary (MCF-7), and lung (NCI-H226) human cancer cells with the half-maximal inhibitory concentration of 1–2 μM; lycopene also suppressed insulin-like growth factor-I-stimulated growth. Inhibition of cell proliferation by lycopene may involve a modulation of protein kinase C (PKC) activity, which is important in the signal transduction pathway leading to cell proliferation [81]. Thus, inhibition of proliferation might also be linked to lycopene's antioxidant effect.

Modulation of intercellular communication, which has been demonstrated in cell cultures, may be another mechanism for the antiproliferative effect of lycopene. The scientific findings show that lycopene differentially modulates gap-junctional intercellular communication (GJIC) depending on the dose, with beneficial effects on cell communication [82,83]. Lycopene may stimulate GJC through stabilization of connexin43 mRNA [84].

Another postulated mechanism for the antiproliferative effect of lycopene is inducing differentiation of cancer cells. This induction of differentiation has been observed in leukemic cell cultures exposed to a combination of both lycopene and 1,25 dihydroxyvitamin D3 [85].

In breast and endometrial cancer, lycopene's mechanism of action is based on the inhibition of cell cycle progression associated with reduction in cyclin D levels and retention of p27Kip1 in the cyclin E binds to G1 phase—cyclin-dependent kinase 2 (E-cdk2) complexes. In prostate cancer, different mechanisms are proposed for the inhibition of cancerous cell proliferation at the G0–G1 cell cycle transition and protection of DNA [86].

BENEFICIAL HEALTH EFFECTS OF LYCOPENE

A beneficial effect may relate to maintenance or improvement of a function. This implies the reduction (or beneficial alteration) of a risk factor for the development of a human disease (not reduction of the risk of disease). In this sense, a risk factor is a factor associated with the risk of a disease that may serve as a predictor of the development of that disease.

The protection of body cells and molecules such as DNA, proteins, and lipids from oxidative damage, including photo-oxidative (UV-induced) damage, may be a beneficial physiological effect, assuming that any significant oxidative modification of the target molecule is potentially harmful [87]. For the purpose of classifying human diseases, we have used the World Health Organization (WHO) International Statistical Classification of Diseases and Related Health [88].

The physiological effects of lycopene have been the subject of study in a total of 1969 articles, 1067 of them published in the last 5 years as scientific publications of international scope [89]. Among these publications, 1680 studies refer to its antioxidant activity in relation to the reduction in the incidence

of different chronic diseases. Considering the different types of study, 186 are review articles and 189 correspond to clinical trials. The rest of the studies are chemical, epidemiological, *in vitro*, etc. In this review, those studies have been selected that provide the highest level of evidence for the health relationship with respect to the role of lycopene.

Hypercholesterolemia and CVD

In the scientific literature, different epidemiological studies and interventional trials have evaluated the association between lycopene (or tomato products) supplementation and CVD and/or progression of the risk factors. In the last 10 years (2002–2012), 1 meta-analysis [90] and 34 published clinical trials in humans (various randomized controlled trials and multicenter studies) have assessed the association between consumption of products rich in lycopene and a lower incidence of CVD [89]. Several reports have appeared supporting the role of lycopene in the prevention of CVD, based mostly on epidemiological studies showing a dose–response relationship between lycopene and CVD. However, a more complex situation emerges from the interventional trials, where several works have reported conflicting results.

In some studies, *in vitro* lipid oxidation was subjected to inhibition by lycopene, while endothelial cell-mediated oxidation of LDLs was not inhibited [91,92]. Furthermore, *in vitro* studies in J-774A cell line macrophages have been used to measure cellular cholesterol synthesis, showing its reduction (73%) by 10 μmol of lycopene [66].

In model animal studies, reduced atherosclerosis risk was found in mice treated with a tomato supplement for 4 months, attributed to reduced lipid oxidation [93].

There is scientific evidence showing that lycopene reduces cholesterol levels in human studies. A 14% reduction in plasma LDL-cholesterol concentrations was noted when six males were given a tomato lycopene supplementation of 60 mg daily for 3 months [66]. However, no significant change in lipids was detected with a daily lycopene supplementation of 5 mg in 22 female adults for 6 weeks, and the antioxidant activity of the subject's plasma was not altered [94].

Some epidemiological studies found an association between intake of lycopene from fresh tomatoes and tomato products and the prevention of CVD. As discussed before, an epidemiological study from 10 European countries [8] showed a correlation between lycopene lipid levels and a reduced risk of myocardial infarction. A multicenter case-control study was performed evaluating the content of lycopene in human tissue taken after myocardial infarction. Analyses using conditional logistic regression models controlling for age, body mass index, socioeconomic status, smoking, hypertension, and maternal and paternal history of disease showed lycopene to be protective. The effect of lycopene was the strongest in nonsmokers. The protective effect of lycopene increased at each increasing level of polyunsaturated fat and was significant in individuals whose fat tissue contained more

than 16.1% polyunsaturates. Levels of lycopene in the fatty tissue provide a better measure of long-term lycopene intake than do serum levels or dietary records.

Oxidation of LDL cholesterol was significantly reduced by the administration of lycopene in the diet of 19 healthy subjects not taking other drugs, when it was administered as tomato juice, spaghetti sauce, and tomato oleoresin [95]. The different sources of lycopene did not produce significantly different reductions. The study was randomized and crossover in design. A tendency toward lower oxidation in these groups was noted but the results were not statistically significant. Sutherland *et al*. [96] reported that the susceptibility of LDLs to oxidation was not changed by increased lycopene plasma concentrations caused by supplementation with tomato juice.

Vivekananthan *et al*. [97] performed a meta-analysis of randomized trials of the use of antioxidant vitamins for the prevention of CVD. Researchers at Kobe University in Japan tested tomato extracts for antithrombotic effects and identified varieties that have this effect. Tests were performed *in vitro* and evaluated *in vivo,* by testing laser-induced thrombosis in mice. One of the tomato varieties tested (KG99-4) showed a significant antithrombotic activity both *in vitro* and *in vivo*, inhibiting not only thrombus formation but also showing a thrombolytic effect [98]. The intake of tomato products is effective in preventing lipid peroxidation, a risk factor for atherosclerosis and CVD. Researchers at the University of Milan (Italy) evaluated the effects of tomato consumption on parameters of lipid oxidation in normal volunteers [99]. The oxidizability of LDL significantly decreased after tomato consumption, which is correlated with serum lycopene concentration. These results support the role of tomato products in the prevention of lipid peroxidation, a risk factor for atherosclerosis and CVD. The intake of tomato products has a beneficial effect by lowering LDL-cholesterol levels and increases resistance to oxidation, a preventive factor against the formation of atherosclerotic plaques. This study conducted by researchers at the University of Oulu (Finland) studied the effects of increased dietary intake of tomato products on plasma lipids and LDL oxidation [100]. Dietary intervention included two groups, one with a reference period of 3 weeks on a diet without tomato and the other on a diet of 3 weeks with a high intake of tomatoes (400 mL of tomato juice and tomato sauce 30 mg of tomato sauce daily). The study involved 21 healthy individuals. The results showed a significant decrease of 5.9% in total cholesterol and LDL cholesterol decreased by 12.9% in individuals who were on a diet with a high intake of tomato products. These changes were significantly correlated with serum levels of lycopene, β-carotene and γ-carotene. As a conclusion, it was found that a high intake of tomatoes and tomato products produces atheroprotective effects by significantly reducing LDL-cholesterol levels and increasing resistance to LDL oxidation in healthy normotensive cholesterolemics. These atheroprotective features are associated with changes in serum lycopene, β-carotene, and γ-carotene levels [100].

The oxidation of LDL in the vascular endothelium is considered to be important in the development of early atherosclerosis [101]. These authors performed a study with the aim of investigating the influence of fat-soluble vitamins and carotenoids in the concentration of conjugated dienes in LDL as an indicator of lipid peroxidation. The study sample included 124 women and 225 men. The levels of plasma lycopene and lutein were the major determinants of the inhibition of LDL-conjugated diene formation in women, while the β-carotene was the most important factor in men. The results suggested that lycopene, lutein, and β-carotene are potent antioxidants that may explain the inhibition of LDL oxidation *in vivo*.

The results reported by Thies *et al*. [102] indicated that a relatively high daily consumption of tomato-based products (equivalent to 32–50 mg lycopene/day) or lycopene supplements (10 mg/day) was ineffective in reducing conventional CVD risk markers in moderately overweight, healthy, middle-aged individuals. However, Burton-Freeman *et al*. [103] and Xaplanteris *et al*. [104] showed that consuming tomato products with a meal attenuated postprandial lipemia-induced oxidative stress and the associated inflammatory response. The relevance of oxidized LDL and inflammation to vascular injury suggests a potentially important protective role of tomato in reducing CVD risk. Furthermore, in a Ried and Fakler meta-analysis [90], it has been suggested that lycopene taken in doses of $\geq$25 mg daily is effective in reducing LDL cholesterol by about 10%, which is comparable to the effect of low doses of statins in patients with slightly elevated cholesterol levels.

Although many aspects of lycopene are yet to be clarified, supplementation of low doses of lycopene has already been suggested as a preventive measure, for contrasting and ameliorating many aspects of CVD, which is important for patients and clinicians [105].

Cancer

There are several human studies that find a statistically significant association between intake of lycopene from fresh tomatoes and tomato products and the prevention of cancers due to the antioxidant effect itself. Epidemiological studies demonstrated some benefits of lycopene in the treatment of cancer, and a few showed no effect. Several small uncontrolled trials have examined the efficacy of oral lycopene supplementation as cancer therapy with mixed results. However, observations of the well-being of the patients showed some improvement with a lycopene-rich diet. Although several mechanisms for the antineoplastic actions of lycopene have been studied, it is thought that the antioxidant properties of lycopene protect against molecular damage that is associated with carcinogenesis and/or that lycopene may modulate signal transduction in gene expression.

Lycopene's antineoplastic effect may also be related to the enhancement of immune cell activity, but some studies show no significant effect [106].

In another study, lymphocyte oxidative DNA damage was reduced in 10 healthy women who consumed a lycopene-containing tomato puree [107].

The half-maximal inhibitory concentration of lycopene (IC50) for proliferation of endometrial (Ishikawa), mammary (MCF-7), and lung (NCI-H226) human cancer cell lines ranged from 1 to 2 μmol. When lycopene was added to these cell cultures, an inhibitory effect was noted within 24 h, which persisted for 2–3 days. In these test models, lycopene also suppressed insulin-like growth factor (IGF-I), which is a major autocrine/paracrine mammary and endometrial cancer cell growth regulator [33]. These results agree with those of Sharoni *et al*. [108] related to the influence of lycopene on IGF-I and the risk of prostate and breast cancers.

When tested for antioxidant properties in a multilamellar liposome system, lycopene was found to have the greatest antioxidant activity among carotenoids; ranked from greatest to least, they were lycopene, α-tocopherol, α-carotene, β-cryptoxanthin, zeaxanthin, β-carotene, and lutein [82]. Lycopene showed a dose-responsive ability to inhibit the mutagenicity of 2-amino-1-methyl-6-phenylimidazol(4-,5-b) pyridine (PhIP) with a 50% inhibitory dose (ID50) of 271 μg of lycopene [109].

Several epidemiological studies support the role of lycopene in prostate cancer prevention [110–112]. The World Cancer Research Fund (WCRF) reported that a high fruit and vegetable intake may be beneficial in reducing the risk of cancer, including a positive role of lycopene in prostate cancer prevention [113]. Specifically, in the last 10 years (2002–2012), 65 clinical trials in humans have assessed the impact of consumption of lycopene-rich products, showing a decrease in the incidence of different cancers, mostly prostate and breast cancer [89]. A statistically significant trend toward reduced prostate cancer risk was associated with self-reported intake of various fruits and vegetables containing lycopene [114]. The reduced risk of prostate cancer is associated only with lycopene in plasma and was related to a lowered risk of aggressive prostate cancers in 578 men who developed the disease over a 13-year period and a set of 1294 matched controls (age, marital status, and smoking) [115]. Other studies confirm the reduced risk of prostate cancer due to lycopene supplementation [116]. Some *in vitro* studies reveal the antiproliferative effect on prostate cancer cell lines with the combination of lycopene and α-tocopherol while this effect was not seen with lycopene alone [117]. In this line, a study reported by Zhang *et al*. [118] revealed that lycopene as an antioxidant factor in the diet could significantly inhibit DNA synthesis following a dose-dependent pattern, as it could inhibit the activity and expression of the androgen receptor gene.

A meta-analysis of observational studies (including cohort, case-control, and nested-case control) indicates that tomato products may play a role in preventing the risk of prostate cancer [119]. Edinger and Koff [120] studied the effect of a daily dietary intake of 50 g of tomato paste for 10 weeks, and their results reflect a significant reduction in the mean plasma prostate-specific antigen (PSA) levels in patients with benign prostatic hyperplasia, probably

linked to the high lycopene content in tomato paste. However, these analyses examined only the relationship between diet and cancer risk and could not distinguish if the relationship was specific to dietary lycopene or related to other components in lycopene-rich food. Basu and Imrhan [9] summarized the findings from 20 clinical studies reviewing the effects of tomato product supplementation containing lycopene on biomarkers of oxidative stress and carcinogenesis in human clinical trials. Most of the clinical trials with tomato products suggest a synergistic action of lycopene with other nutrients in lowering biomarkers of oxidative stress and carcinogenesis. The authors concluded from their review of clinical trials that the consumption of processed tomato products containing lycopene provides significant health benefits and can be attributed to various naturally occurring nutrients in tomatoes; the specific role of lycopene *per se* remains to be investigated.

On the other hand, a recent Cochrane systematic review of three randomized controlled trials, investigating the role of lycopene in preventing prostate cancer in a total of 154 participants, concluded that there is insufficient evidence to either support or refute the use of lycopene for the prevention of benign prostate hyperplasia or prostate cancer [121]. Given the lack of randomized control trials on this topic, clinicians and consumers may refer to the 2004 meta-analysis observational studies that identified a 1% relative reduction in the risk of prostate cancer diagnosis in men consuming lycopene. So a well-designed, high-methodological-quality, randomized controlled trial to investigate the effectiveness of lycopene in the prevention of prostate cancer is required [86,122].

Various cohort epidemiological [123,124] and case-control studies [125] evaluated the relative risk of developing breast cancer. Sato *et al.* [126] performed a prospective study of carotenoids and risk of breast cancer. A study of women with a history of breast cancer concluded that a diet with increased vegetable and fruit intake was linked with a significantly reduced risk of cancer recurrence [127]. Results suggested by Aune *et al.* [128] indicated that blood concentrations of carotenoids are more strongly associated with reduced breast cancer risk, and recommended the use of certain biomarkers to clarify the inconsistent and weak association between dietary intake and breast cancer risk.

There are different human observational case-control studies addressing the protective effect of lycopene against lung or pleural cancer [129–133]. Low plasma lycopene concentration is associated with increased mortality in a cohort of patients with a history of oral, pharynx, or larynx cancers [134].

Studies related to lycopene and cancer have been conducted in animal models. Narisawa *et al.* [135] reported that tomato juice containing lycopene protects against the development of induced colon cancer. A meta-analysis of observational studies indicates the potential role of lycopene in the prevention of colorectal cancer [136]. A case-control epidemiological study of 728 Italian rectal cancer subjects and 1225 colon cancer patients confirmed a 20% reduction in the risk of colorectal cancer by the addition of one daily serving of vegetable (in the form of tomato) [137].

Other studies also suggest different effects of lycopene on several cancer types. Lycopene in combination with the drug piroxicam, or lycopene with β-carotene, produced a preventive effect against induced urinary bladder cancers in rats [138]. Leal *et al*. [139] showed that lycopene administration to chicks protected them against some of the hepatotoxic effects of T-2 mycotoxin. Some studies suggest a synergic relation between lycopene and vitamin D3 regarding antiproliferative effects, and an additive effect regarding cellular cycle progression. For this reason, the inclusion of lycopene in the diet has been recommended as a cancer prevention strategy [85].

The heterogeneity in the literature may be related to different additional factors as studies are based on intake (limited by the assessment of intake, food composition databases, and differences in bioavailability). Future studies should explore new markers of cancer risk and clarify the specific role and mechanisms of lycopene in cancer prevention.

Skin Damage

Ultraviolet radiation causes the accumulation of free radicals, which causes lipid oxidation at skin level and the formation of wrinkles and aging. Due to the liposoluble nature of carotenoids, they may accumulate preferentially on the stratum spinosum of the epidermis. Lycopene appears to be carried to the skin via sebaceous secretion, and it has a tendency to accumulate in specific regions rich in sweat glands such as the forehead, nose, chin, hand, and palms. In these areas, lycopene can provide an *in situ* prevention effect against skin oxidative damage.

Basavaraj *et al*. [140] reviewed various skin diseases caused by nutritional deficiencies, the role of diet in skin immunity, and the role of antioxidants and other supplements in maintaining skin health. In this review, the role of food in the prevention of various skin disorders and the photo-protective potential of antioxidants are evaluated. Several studies suggest that the intake of foods rich in carotenoids is a useful strategy to protect the skin from photoaging. Of the various carotenoids, lycopene may play a role in preventing oxidative damage to the skin caused by exposure to ultraviolet light. Epidemiological studies link lycopene with a protective effect against UV radiation and, therefore, the induced erythema. Thus, a study conducted on the volar forearm of 16 healthy Caucasian women exposed to three times their minimal erythema dose (determined previously) of ultraviolet light reported a 31–46% reduction in skin concentrations of lycopene [141].

According to the results obtained in a previous study, lycopene naturally present in tomatoes and tomato paste protects against skin damage caused by solar radiation [142]. These are also the conclusions of the recently published study by Terao *et al*. [143]. There is much evidence that lycopene protects the skin against sunburn (solar erythema) by increasing the basal defense against UV light-mediated damage [144].

LYCOPENE ANALYSIS

Lycopene differs from other carotenoids in its physicochemical properties such as solubility and instability. Therefore, it is necessary to carefully handle lycopene during its purification and food analysis stages to avoid its degradation and isomerization. Various methods for an efficient extraction of lycopene from food samples are reported in this review taking into account the importance of sample preparation, the selection of the extraction solvent, and the extraction procedure (with special emphasis on saponification as a possible way to improve the extraction).

The identification and quantification of lycopene can be done using different methods: high-performance liquid chromatography (HPLC) with an ultraviolet (UV) or photodiode array detector (HPLC–PDA), liquid chromatography–tandem nuclear magnetic resonance (LC–NMR), liquid chromatography tandem mass spectrometry (LC–MS/MS), UV/visible spectroscopy, Fourier transform infrared spectroscopy (FTIR), and Raman resonance spectroscopy, including the separation and identification of *cis*/*trans* isomers of lycopene.

Mathematical models that have been used as useful tools to interpret spectroscopic and chromatographic data for the quantification of lycopene in complex mixtures are also considered.

Sampling and Sample Preparation

Lycopene is highly susceptible to degradation by heat, light, oxygen, and metal ions [57,145–149], which makes its preservation and analysis in complex samples such as foods difficult. It may undergo degradation, formation of stereoisomers, structural rearrangements, and other chemical reactions. The main mechanisms of lycopene losses are oxidation and isomerization of the all-*trans*-form to *cis*-isomers [150–153].

The solubility and instability of lycopene necessitates a very careful handling process and short analysis time to avoid degradation and isomerization. For this reason, the methods for lycopene analysis in vegetables are limited and the need for a reliable and rapid analytical method for this purpose is recognized [43,154]. Therefore, samples should be handled under dim or red light, the solvent evaporated with gas (nitrogen, argon, or helium), and stored in the dark at temperatures below −20 °C [155].

Knowledge of the sample matrix is critical for accurate quantification, as many factors regarding the food matrix must be considered for efficient carotenoid extraction (relative content of lipid to carotenoids, types and forms of the carotenoid present, etc.).

The sampling protocol must consider the variations among different fruits and the distribution of lycopene in each fruit. In order to ensure a complete extraction, samples should be ground or cut into small pieces.

Extraction Procedure

Solvent Extraction

As *carotenoids are lipophilic compounds*, they are usually extracted using a mixture of organic solvents [156]. Commonly used solvents for lycopene extraction are ethanol, hexane, acetone, ethyl acetate, chloroform dichloromethane, isopropanol, and petroleum ether [157–159]. As lycopene is insoluble in methanol (a solvent widely used for carotenoid analysis), it requires several extraction steps, which involves higher volumes and a more complex treatment of the extracts.

The use of *low-moisture samples* (<10%) usually simplifies the extraction process, and for this reason, freeze-drying or desiccation in a vacuum-oven is often carried out to reduce the water content in the samples with little damage to the carotenoids. According to Desobry *et al*. [160] and Gonzalez Castro *et al*. [161], freeze-drying does not cause significant changes in carotenoids, since it excludes O_2. Long-time storage of freeze-dried samples can cause carotenoid alterations in carotenoids [162]. However, some authors do not recommend a complete dehydration before extraction, since a small amount of water is often useful when a low-polarity solvent mixture is used in the extraction [163,164].

In the case of fruit and vegetables with a *high water content*, water-miscible solvents, such as acetone and tetrahydrofuran (THF), are needed for complete penetration of the sample [165].

Acetone, hexane, methanol, ethanol, ethyl ether, THF, dichloroethane, and their combinations have been used to extract carotenoids from foods [44,166–172]. After extraction, the pigment is transferred from the water-containing extract to the water-immiscible solvent by adding enough water or a saturated NaCl solution until the two layers separate [52,173].

The use of *nonpolar extraction solvents* such as hexane or THF enables a more efficient extraction and reduces the time and number of extractions. Partitioning, washing, and phase separation are sometimes applied to the extract, in order to change the solvent or eliminate undesirable compounds in the extracts. For example, partitioning with petroleum ether and methanol has been used to separate chlorophylls and carotenes in the organic phase and xanthophylls in the alcoholic phase from tomato seeds [151,163,174,175].

Chlorinated solvents could induce the formation of 9-*cis* and 13-*cis* isomers of carotenoids; however, THF or acetonitrile (ACN) does not have this effect [176]. THF rapidly dissolves carotenoid compounds and denatures proteins, avoiding the formation of emulsions, but it induces peroxide formation. To avoid this oxidation process, antioxidant agents, such as butyl-hydroxide-toluene (BHT), vitamin E, rosemary extract, or pyrogallol, is often added to THF [44,177,178]. Diethyl ether (Et_2O) must be freshly prepared (peroxide-free) before use [52]. Magnesium carbonate [159,166] or calcium carbonate

[168] is often added during the extraction process to neutralize organic acids that might be present in the sample [175,179,180].

The *influence of temperature* during the extraction procedure must be considered taking into account the stability of lycopene. Some authors recommend refrigeration conditions for carotenoid extraction. Konings *et al*. [179] and Tonucci *et al*. [181] performed the extraction of carotenoids from tomato products at 0 °C under gold fluorescent light.

On the other hand, due to the interference in carotenoid determination caused by the high sugar content, solubilization in a 100 g/L NaCl solution prior to the carotenoid extraction has been recommended [182]. In the method recommended by Craft, [180] methanol/THF (50:50, v/v) is applied until the extract is colorless (which usually can take more than 3 or 4 extraction steps in lycopene-rich samples); other authors have used combinations of hexane/acetone/ethanol (50/25/25) with very good efficiency of lycopene extraction from foods, involving only one extraction step [44,173].

The extraction should be repeated several times, until all the lycopene has been removed from the sample. Some authors [180,183] stop the extraction process when the sample is colorless and other authors [173] repeat the extraction process until no lycopene is found in the extracted sample.

Matrix solid-phase dispersion (*MSPD*) has been employed as a novel alternative to traditional solvent extraction methods. This technique combines sample preparation, extraction, and fractionation into one process, limiting the handling and transfer steps [184]. The sample is mechanically ground with a bounded-phase solid support material in a mortar and then packed into a chromatographic column to isolate the carotenoids of interest. In addition, MSPD has been found to require 95% less solvent and 90% less time than traditional liquid–liquid extraction methods [184]. While carotenoids are prone to oxidative degradation, several groups have suggested that MSPD precludes the formation of artifacts [185,186].

Saponification

Lycopene, as a hydrocarbon compound, does not form ester linkages and can be directly extracted by homogenization in the presence of lipophilic solvents, especially in samples with a low content of chlorophylls [180], but sometimes a saponification step has been reported to improve the accuracy of lycopene analysis [187]. Although saponification may be carried out directly with the homogenized matrix, it is frequently performed after organic extraction. In the determination of some carotenoids in plant foods (especially xanthophylls in chlorophyll-rich samples), saponification is an optimal method for removal of chlorophylls (which could otherwise sensitize photo-isomerization of carotenoids), unwanted lipids (as triglycerides in lipid-rich samples), and other interfering compounds, as well as the best way to hydrolyze carotenoid esters [187,188].

Different conditions have been used for the saponification: hydrolysis time, temperature, KOH concentration, and number and volume for partition and washing. Most of them use an alkaline treatment with 10–40% KOH/methanol over 5–60 min at 56–100 °C or 3–16 h at ambient temperature [167,179,180,182,189].

The disadvantage of this treatment is that certain saponification conditions may cause the degradation of carotenoids, and for this reason, the concentration of KOH, time, and temperature must be carefully assessed for the particular type of materials to avoid *trans–cis* isomerization and epoxidation [164,190]. These authors recommended saponification at room temperature in petroleum ether with 10% methanolic KOH, either under an N_2 atmosphere or in the presence of an antioxidant. For the determination of carotenoids in tomato and kale, this procedure was considered unnecessary.

Greater losses of lycopene (about 25%), as compared to other carotenoids in saponified extracts of vegetables and fruits, have been reported by Müller [189]. He found that the xanthophylls were the most resistant. On the contrary, Khachik *et al.* [191] and Scott [150] found greater losses of xanthophylls rather than carotenes after 3 h of saponification at ambient temperature. Shorter saponification times are thus desirable to minimize these losses. Wilberg and Rodriguez-Amaya [182] applied a 5–10 min saponification with 10% KOH and Granado *et al.* [187] developed a rapid saponification protocol (40% KOH/methanol, 5 min), which considerably improved the accuracy of lycopene HPLC determination in vegetables, with a negligible degree of lycopene isomerization due to shorter exposure of the sample to alkaline conditions.

For these reasons, saponification is recommended for chlorophyll-rich samples, and the need for this procedure should be assayed depending on the food matrix, the analytical method, and the particular aim of the analysis [188]. In the case of saponification, when alkaline and heat conditions are used, BHT should be avoided in the solvents because in these conditions, it can form polymers that absorb light in the visible range and may coelute with some carotenoids in HPLC analysis [180].

In order to know the magnitude of the possible loss of carotenoids during the analytical procedures, internal standards, such as ethyl-β-apo-8′-carotenoate, echinenone, or retinyl palmitate, are sometimes added to the samples [163,179,192].

Supercritical Fluid Extraction

Supercritical fluid extraction (SFE) has been used as an alternative method to extract carotenoids from food samples. This is an advanced separation technique based on the enhanced solvating power of gasses above their critical point. The preferred gas is CO_2, because of its lower critical temperature and its nontoxic, nonflammable nature, its low cost, and its high purity. It can be used at temperatures of 40–80 °C and pressures of 35–70 MPa [193].

SFE performs selective isolation of carotenoids in one step, avoiding elevated processing temperatures. This makes it favorable for the extraction of heat-sensitive carotenoids. Furthermore, the elimination of organic solvents offers the advantage that the extracts obtained are free of chemical residues [194]. SFE is compatible with supercritical fluid chromatography since both techniques can share the same mobile phase and devices, favoring the development of extraction and separation methodologies. This technique has been applied to successfully separate lycopene from other carotenoids in tomato fruits [43,195–199].

The extraction efficiency of carotenoids with CO_2 has been shown to increase with temperature and pressure. Among the various parameters tested, temperature has been observed to be the most important factor affecting lycopene yield in SFE extraction. Increasing the temperature increases the solubility of the carotenoids, which results in higher yields, but could also contribute to greater carotenoid degradation. The highest temperature used for the extraction gave the maximal extraction yield [200]. However, accounting for lycopene losses, lycopene yields at 60 °C extraction temperature were 14% greater than those obtained at 70 °C [201]. The extraction yield of lycopene from freeze-dried tomato pomace (peels and seeds) with CO_2 was determined at different technical parameters, such as temperature (40, 50, 60 °C) and pressure (35, 40, 45 MPa), using statistical analysis. The highest concentration of lycopene was obtained at over 35 MPa, while that of β-carotene and lutein was obtained at below 30 MPa. The product obtained by supercritical CO_2 extraction at 40 MPa and 60 °C contains the highest lycopene concentration of 32.52 ± 1.02 g/100 g dry material [202]. In another study carried out at almost similar conditions, the maximum recovery of lycopene (54%) and β-carotene (50%) was found at 300 bar with 5% ethanol (2 h with a 4 kg/h flow rate) [200], while 61% of lycopene was recovered at 86 °C, 34.47 MPa, and 500 mL of CO_2 at a flow rate of 2.5 mL/min [197].

Isomer separation of 9-*cis* and all-*trans* β-carotene extracted from algae using SFE at different dissolution rates in CO_2 has been reported [203]. The effect of CO_2 density on extraction of all-*trans*-lycopene from tomatoes was studied and a greater yield was found at the highest density (0.90 g/mL) [198].

Extraction of pigments (chlorophylls and carotenoids) from marjoram (*Origanum majorana* L.) with supercritical CO_2 was investigated and compared with Soxhlet extraction. The results showed that the amounts of lutein and β-carotene recovered using SFE were similar to those obtained from a traditional Soxhlet extraction [193].

Lycopene Extraction Without Solvent

Recently, advances have been made to devise new methods for the extraction of lycopene from food samples. These methods are based on the *action of enzymes* on lysed cells, using a combination of pH changes and hydrolytic

treatments. The lycopene-containing chromoplasts are then precipitated by lowering the pH and isolated through a centrifugation step. At this stage, the lycopene content of the isolated chromoplasts showed a 10-fold increase (3–5%, w/w, dry basis) with respect to untreated tomato peels [204]. A further improvement in lycopene concentration was obtained by a second enzymatic treatment using a protease cocktail as shown in Fig. 3. This catalytic step eliminated unwanted proteins bound to the chromoplasts, but not essential for their stability. The final extract showed a lycopene content of around 8–10% (w/w, dry basis), which represented a 30-fold increase with respect to the lycopene concentration of the untreated peels [204].

A coupling of ultrasonication and biocatalysis has been reported to improve the recovery of lycopene from tomato peel. Only cellulase treatment resulted in an increment of about 225% of lycopene (260 μg/g) over the untreated sample. 5% (w/w) of the biocatalyst and an incubation period of 20 min were found optimal for maximal lycopene extraction from the samples [205].

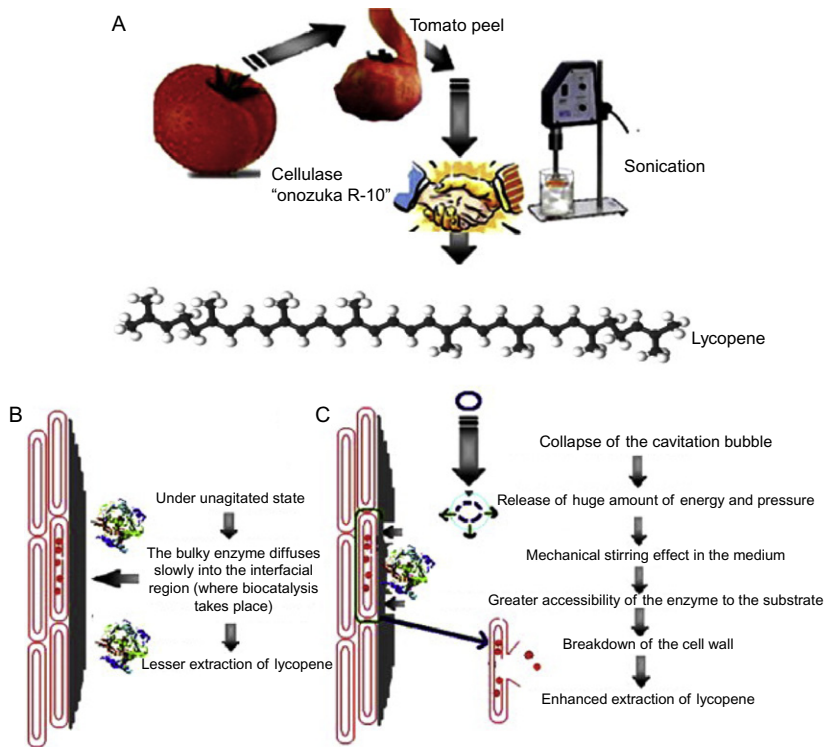


FIGURE 3 (A) Schematic representation of the coupled action of sonication and biocatalysis in the extraction of lycopene, (B) and (C) the proposed mechanism of enhanced extraction of lycopene under the concomitant exploitation of ultrasonication and biocatalysis. *Reprinted with permission from Ref. [205], published by Elsevier.*

This method works in accordance with green chemistry as it does not involve the use of organic solvents for the extraction of lycopene. Such methodology is expected to represent an economic alternative to the CO_2 extraction of lycopene to be used especially for food supplement analysis.

An extensive table summarizing the different extraction and analytical techniques can be found in the review by Rao [12].

Identification and Quantification

Chromatographic Methods

Chromatographic methods are specific and allow the separation of different compounds. However, gas chromatography is not a suitable technique for carotenoid analysis, since these compounds decompose when exposed to the high temperatures used in this technique.

The first method applied for separating carotenoids and chlorophylls by *open column chromatography* (*OCC*) using solid-phase $CaCO_3$ and petroleum ether as liquid phase was developed as early as 1906 [206]. OCC has been used especially for preparative purposes, with adsorbents, such as alumina or silica gel, MgO, $CaCO_3$, sugar or cellulose, and eluents of increasing polarity (starting with hexane) to obtain different fractions of pigments depending on its polarity [207]. These fractions can then be analyzed by spectrophotometry [175]. OCC has the advantage of being more economical to perform but is more time consuming.

Thin layer chromatography (*TLC*) has been successfully applied in carotenoid separation and purification from tomato, either alone or in combination with OCC. By OCC, the carotenoids can first be separated into fractions of different polarity, which are then further separated into individual compounds by TLC, using MgO, diatomaceous earth, and cellulose as the absorbent, and with solvent systems consisting of hexane, isopropanol, and methanol [52]. A chromato-scanner can be used as the detection system or a high-performance TLC (HPTLC) system can be applied to accurately quantify the compounds detected [208,209].

Numerous papers have been published on *HPLC* analysis of carotenoids including lycopene from food samples [210]. For the last few decades, HPLC has been the preferred method to separate, identify, and quantify carotenoids in food and biological samples, allowing us to distinguish between the geometrical structures of carotenoids, including some mono- and di-*cis*-isomers [211,212].

Stewart and Wheaton [213] developed the first HPLC method applied to carotenoid analysis, which was normal-phase using a laboratory-packed MgO column with a linear gradient of hexane/acetone containing 10% benzene. This method could separate several carotenoids in tomato samples, including lycopene. After this, several methods have been developed to analyze lycopene in food matrices by HPLC, especially reverse-phase (RP) ones.

Niizu *et al*. [214] used a monomeric C18 column to achieve carotenoid separation from salad vegetables; however, Jinno and Lin [215] recommended the use of a polymeric octadecylsilica (ODS) stationary phase for its better selectivity than monomeric ODS columns, taking into account their molecular shape and size recognition, as well as the better separation obtained using them. Most authors use C18 RP-columns for lycopene analysis [178,187,189] as they can provide a good resolution for lycopene and other carotenoids. The use of metal-free columns (such as titanium ones) is desirable, so as to avoid damages to the carotenoids during the analysis [150].

C30 RP-columns were specifically developed for the separation of carotenoids [216]. This stationary phase, with a very high efficiency, is preferred when the interest is focused on the separation of different isomers of carotenoids, including the positional and geometrical ones. Yeum *et al*. [217] separated five isomers of lycopene by using a C30 column and a gradient solvent system of methanol/methyl-ter-butyl-ether (MTBE)/water. Lee and Chen [211] compared two types of columns (C18 and C30) and various solvent systems for separation. All-*trans*-lycopene and 9-*cis* isomers (5-*cis*, 9-*cis*, 13-*cis*, 15-*cis*, and 4-di-*cis*-lycopene) were resolved by employing a C30 column with a mobile phase of n-butanol-ACN-dichloromethane (30:70:10, v/v/v) within 35 min [211]. Fröhlich *et al*. [218] also used a C30 column to distinguish between lycopene isomers. Extensive reviews of the development of C30 stationary phases for carotenoid analysis can be found elsewhere [219].

A *guard column* with a stationary phase similar to the column is sometimes used to increase the life of the column and improve the resolution of the peaks [167,187,220]. The control of *column temperature* is an additionally important factor in reducing analytical variation of the results, especially with C30 stationary phases [221], and it can also be useful to speed up the analysis. Temperatures should not be higher than 30 °C (to avoid isomerization); however, the effect of temperature should be assessed for each system, and conditions should be optimized for each case [215,222].

Regarding the *mobile phases*, they may include solvents such as ACN, methanol, 1-butanol, 2-propanol, ethyl acetate, THF, water, MTBE, or halogenated hydrocarbons (as dichloromethane or chloroform) [12]. Despite the poor solubility of lycopene in methanol, this solvent can be included in some proportion in the mobile phase, taking care that all the solutions injected do not have a high concentration of lycopene to avoid precipitation in the column [180]. The use of gradients is sometimes applied to achieve a better separation of the compounds.

Some extraction solvents can interfere with the HPLC mobile phases used for the separation of carotenoids. They can produce chromatographic artifacts, that is, broaden, or deform the chromatographic peaks. This interaction is usually produced by nonpolar extraction solvents (hexane or THF) and more polar HPLC solvents (methanol or ACN) [44]. For this reason, evaporation (if possible under N_2) is sometimes necessary, followed by a redissolution

in the mobile phase or in another noninterfering solvent. These practices usually involve a compromise between the solvent's compatibility with the mobile phase and the good solubility of lycopene (which can, in these cases, usually be restricted to 3–6 μg/mL maximum) [44,179].

The addition of a solvent modifier such as *n*-decanol, *N*,*N*-diisopropylethylamine, or triethylamine (TEA) to the mobile phase, in quantities ranging between 0.05% and 0.1%, has been shown to prevent nonspecific adsorption and oxidation and, thus, improve recovery of carotenoids; it may also reduce retention times with no compromise in resolution [167,180]. A mobile phase consisting of 90/10 methanol/ACN and 0.1% TEA has shown good results in lycopene separation from other carotenoids in several food samples [44,180].

The simplest *HPLC detection systems* for lycopene are UV–visible; usually λ of 475 nm is used, because it allows the quantification of other carotenoids present in the sample. The photodiode array (PDA) is one of the most commonly used detectors for HPLC carotenoid analysis, although other detectors, such as electrochemical detectors (ECDs), fluorescence, mass spectrometers, and nuclear magnetic resonance (NMR) can be used. PDA is useful to identify and quantify lycopene and other carotenoids in food samples, following the simultaneous elution in the full UV–visible range, which guarantees the identification of each pigment by its spectrum and its quantification at its absorbance maximum.

Olives Barba *et al*. [44] optimized and compared an HPLC method with the spectrophotometric standard method mentioned earlier for the determination of lycopene and β-carotene in vegetables. They used extraction of different fruits and vegetables with hexane/acetone/ethanol (50:25:25, v/v/v), evaporation of the hexane layer, redissolution in THF/ACN/methanol (15:30:55, v/v/v), and injection in a C18 column with methanol/ACN (90:10, v/v) + TEA 9 μM as mobile phase (a flow rate of 0.9 mL/min) and detection at 475 nm. The HPLC method was comparable to the standard spectrophotometric method in precision, accuracy, and sensitivity, involving a simple preparation of the samples (one-step direct extraction) and short run times (10 min) for the quantification of lycopene in fruit and vegetable samples.

Other detection systems include a coulometric ECD which is recommended when very low levels of carotenoids need to be quantified [168,180], or mass spectrometry, which should be performed under 100 °C because of the heat instability of carotenoids. Mass spectrometry allows the identification of lycopene and other carotenoids on the basis of the structural information obtained from the fragmentation of the molecules provided by classical ionization methods (such as electron impact and chemical ionization), or by soft ionization techniques (such as fast atom bombardment, matrix-assisted laser desorption/ionization, electrospray ionization, and atmospheric pressure chemical ionization), which have facilitated the molecular weight determination of carotenoids by minimizing fragmentation. The differentiation of structural

isomers (such as carotene and lycopene) can be carried out with the aid of collision-induced dissociation and tandem mass spectrometry, which augment fragmentation and obtain structurally significant fragment ions. For example, the ion of $[M\text{-}69]^{+}$*, indicating the presence of a terminal acyclic isoprene unit, is observed in the tandem mass spectra of lycopene, neurosporene and γ-carotene, but not of α-carotene, β-carotene, lutein, or zeaxanthin [223].

However, the structural elucidation of carotenoid stereoisomers can be accomplished only by the use of NMR spectroscopy. HPLC–NMR on-line coupling has been shown to be particularly advantageous as it allows the direct identification of carotenoid stereoisomers in food as well as in physiological samples [185].

Ultra high-performance liquid chromatography (UHPLC) has been used to monitor carotenoids, but mostly in conjunction with other fat-soluble vitamins [224,225]. UHPLC methods for the quantification of a wide range of carotenoids are uncommon. The column temperature should be controlled and maintained above 20 °C to promote a consistent separation and to prevent the carotenoids from crystallizing out of the solution [221,222].

Calibration of HPLC equipment with pure standards is often necessary, although standards for many carotenoids are not commercially available. The accuracy of the results would depend on the purity of these standards. In the case of lycopene, commercial standards of all-*trans*-lycopene can be used for identification and quantification purposes. *Cis*-lycopenes can be tentatively identified based on their spectral properties: they have smaller extinction coefficients than the all-*trans* forms. The mono-*cis* isomers result in a hypsochromic shift of about 4 nm when compared to the all-*trans* form. The central *cis*-isomers have a strong peak at about 340 nm and the di-*cis* isomers may be shifted to shorter wavelengths than the mono-*cis* isomers [211,226–228].

Commercial standards have the disadvantage of being expensive and unstable. For this reason, they should be verified using a spectrophotometer to avoid errors in quantitation due to possible impurities and to the fact that carotenoids dissolve slowly in many solvents. Accuracy of the analysis can be improved by calculating the real concentration of the standard solution from the absorbance and the extinction coefficient. The value assigned can be further refined by correcting for peak purity, which is obtained from the spectrum of each standard. Once the standard solution concentrations have been established, the individual standard solutions can be mixed to form the final calibration solutions [180].

All the standards should be prepared daily and used immediately. The use of stock solutions is not desirable because of the risk of degradation. Although lycopene solutions can be stable over one week at −20 °C if protected from light and oxygen [180], if standard stock solutions are to be used, they should be evaluated daily for purity by measurements of absorbance or chromatographic peak area [179]. The precipitation of lycopene can also take place in a standard solution when it is placed at freeze temperatures; in this

case, the standard must be redissolved by agitation in an ultrasonic bath, or filtered and its concentration reassigned.

Due to the problems of availability and instability of commercial standards of lycopene, some authors prefer to prepare their own standards by means of extraction from tomato samples, purification, and evaluation of the concentration. For this purpose, the usual organic solvent for carotenoid extraction can be used, followed by concentration in a rotatory evaporator. OCC has also been used for purification purposes in an MgO:Hyflosupercel column [214]. Rodriguez [164] also recommended the purification of carotenoids using crystallization of fractions derived from a preparatory chromatographic technique such as TLC or OCC.

Interlaboratory studies on HPLC procedures for carotenoid analysis on foods have shown relative uncertainties, attributed to the effect of the chromatographic system and standardization of the carotenoid extract, while the preparation of the carotenoid extract (protocol and efficiency) may account for more than half of the total variance [229,230]. To evaluate the magnitude of these uncertainties, some certified standard reference materials (CRM), available from different organisms, can be used [187], which are essential to the development and harmonization of methods. Scott *et al.* [229] developed a CRM consisting of a mixture of freeze-dried vegetables, including tomato, which maintained a 97% retention after 36 months of being stored at −18 °C under nitrogen and out of the incidence of light. There are some commercially available CRM for carotenoid analysis, consisting of vegetable mixtures and baby food composites; however, the lycopene content in these materials is usually given not as a certified value but as a reference one, because of the instability of this pigment.

UV/Visible Spectroscopy

Spectrophotometric methods are simple, but exhibit a lack of specificity for carotenoid analysis. However, they have been shown to be reliable methods to identify lycopene as the major pigment present in a mixture. A compilation of all spectra of the known carotenoids can be found in the *Carotenoids Handbook* [155]. As an example, a UV/visible spectrum of lycopene is shown in Fig. 4. The differences in the absorption spectrum between lycopene and other major carotenoids in foods such as α-carotene, β-carotene, or lutein make the quantification of lycopene easy at its characteristic wavelength maximum of 503 nm, with no interference of other compounds, in lycopene-rich samples such as tomato or watermelon. Fish *et al.* [231] stated that in samples where lycopene is at least 70% of the constituent carotenoids, the contribution of carotenoids other than lycopene to absorbance at 503 nm is less than 2% for watermelon, 4% for tomato, and 6% for pink grapefruit.

A large number of lycopene studies have been published that report the use of this method [173,232–234] as it allows for a quick routine analysis in the previously mentioned samples. Fish *et al.* [231] have also reported a

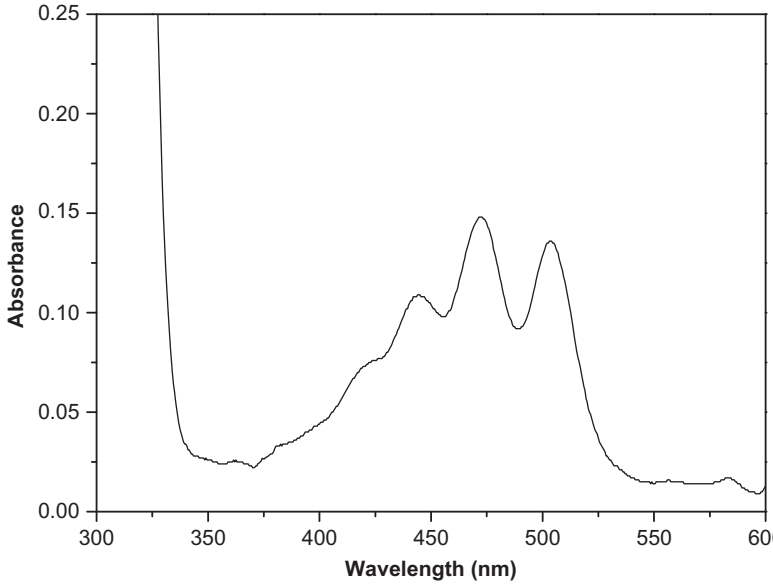


FIGURE 4 UV/visible spectrum of lycopene.

spectrophotometric method providing the advantage of using reduced volumes of organic solvents. For quantification purposes, the molar extinction coefficient of lycopene can be used [13], avoiding the problems of instability and availability of commercial standards.

Biehler *et al.* [235] have compared different spectrophotometric and HPLC methods for the rapid quantification of carotenoids in fruits and vegetables (with and without chlorophylls). A significant correlation was found between the results obtained by the newly developed method (based on the average molar absorption coefficient and wavelength) and standard HPLC.

New computerized approaches and linear models (LMs) to solve the UV/visible spectroscopy interference effects of β-carotene with lycopene analysis by neural networks (NNs) have been reported in recent years [236,237]. The data (absorbance values) obtained by UV/visible spectrophotometry were transferred to an NN-trained computer for modeling and prediction of output. Such an integrated NN/UV/visible spectroscopy approach is capable of determining β-carotene and lycopene concentrations with a mean prediction error 50 times lower than that calculated by LM/UV/visible spectroscopy.

Raman and Infrared Spectroscopy

The spectra in Et_2O solutions of α-carotene, β-carotene, leaf xanthophyll, lycopene, and β-ionone were determined as early as 1932 [238]. The nondestructive character of *Raman spectroscopy* in the analysis of organic pigments and dyes was also reported by B. Guineau [239]. Other studies have shown

that due to the strong electron–phonon coupling that occurs in carotenoids, two bands in the 1100–1200 and 1400–1600 cm^{-1} regions of the resonance Raman spectrum are strongly enhanced. This indicates that resonance Raman spectroscopy can be usefully applied to the investigation of carotenoids for analytical applications [240].

The use of spatially offset Raman spectroscopy (SORS) for nondestructive evaluation of the internal maturity of tomatoes has been assessed [241]. A Raman system using a 785-nm laser was developed to collect spatially offset spectra in the wavenumber range of 200–2500 cm^{-1}. Two peaks appeared consistently at 1001 and 1151 cm^{-1}, and the third peak was gradually shifted from 1525 cm^{-1} (lutein at the mature green stage) to 1513 cm^{-1} (lycopene at the red stage) owing to the loss of lutein and β-carotene and the accumulation of lycopene during tomato ripening. The Raman peak changes were evaluated by spectral information divergence (SID) with pure lycopene as the reference. The SID values decreased as the tomatoes ripened and so, these values can be used to evaluate the internal ripeness of tomatoes.

Near-infrared (*NIR*) excited Fourier transform (FT) Raman spectroscopy has been applied for *in situ* analysis of carotenoids in living plant samples. The *Pelargonium x hortorum* leaf has been mapped using a Raman mapping technique to illustrate the heterogeneous distribution of carotenoids (Fig. 5)

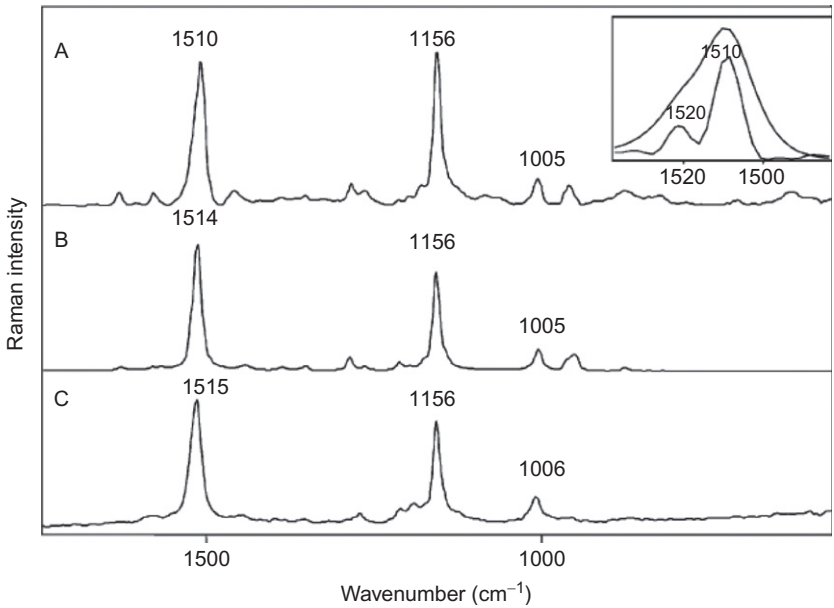


FIGURE 5 FT-Raman spectra of tomato puree (A), lycopene (B), and α-carotene (C). The inset presents a deconvoluted band of tomato puree. *Reprinted with permission from Ref. [243], published by American Chemical Society.*

[242]. NIR-FT-Raman spectroscopy has been shown to be a very useful technique for nondestructive analysis of carotenoids in various living plant tissues.

Nondestructive calibration methods have been established using FT-Raman, attenuated total reflection (ATR)-IR, and NIR spectroscopy for quantification of lycopene and β-carotene content in tomato fruits and related products (Fig. 6) [242,243]. It has been found that *FT-Raman spectroscopy* can be successfully applied for the identification of carotenoids directly in the plant tissue and food products without any preliminary sample preparation. Compared with the very intense carotenoid signals, the spectral impact of the surrounding biological matrix is weak and, therefore, it does not contribute significantly to the results obtained. On the other hand, ATR/FT-IR and NIR measurements mainly show strong water signals, and no characteristic bands for carotenoids are observed. However, calibration models for determination of lycopene and β-carotene contents in tomato samples obtained on the basis of the three applied vibrational methods show the best statistics when IR spectroscopy is used.

Calibration models were developed for the quantitative measurement of lycopene with the use of mathematical models. These methods simplified the use of the IR technique for the measurement of carotenoids in tomatoes [244–246].

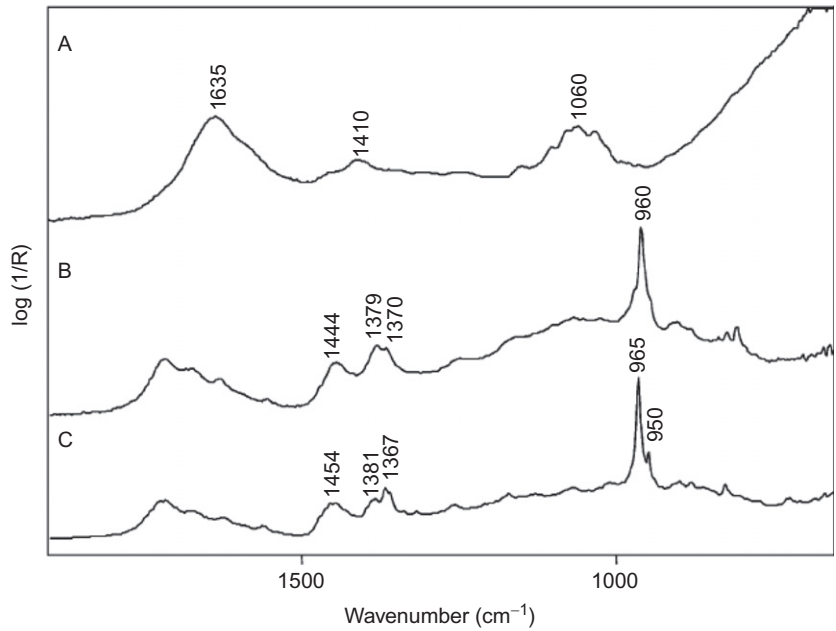


FIGURE 6 ATR-IR spectra of tomato puree (A), lycopene (B), and α-carotene (C). *Reprinted with permission from Ref. [243], published by American Chemical Society.*

CONCLUSIONS AND FUTURE PROSPECTS

The direction of an arrow is only known by its previous path, so the trajectory in research on lycopene shows a clear direction, and a significant growth in this field is anticipated in the coming years.

Scientific evidence strongly supports an association between a healthy diet and the prevention of chronic diseases. In recent years, there has been increasing interest in the role of lycopene, an antioxidant carotenoid present in many fruits and vegetables, in human health. Research activities on lycopene have proliferated exponentially in the past decade. The increasing number of scientific reports on the role of lycopene in the prevention of human diseases has led the food, pharmaceutical, and cosmetic industries to innovate, by including lycopene in their products, such as functional foods, supplements, and cosmetic products. However, the dosing of dietary supplements is highly dependent on a variety of factors such as quality of raw materials, manufacturing process, and packaging. To date, no official standards have been established in Europe to regulate the production of dietary supplements, so dosage ranges must be employed as guidelines only. There is an increasing involvement of consumers in health care, resulting in lifestyle modification and incorporation of complementary and alternative medicines into their dietary routine to maintain their health and prevent disease. In this regard, lycopene may help support prostate function and it is often used as an adjunctive therapy in treating prostate cancer.

To have a better understanding of the role of lycopene *in vivo*, well-controlled clinical and dietary intervention studies investigating its role in the different phases of chronic diseases should be conducted, focused on specific and standardized outcome measures of each specific disease.

Progress in the analytical techniques has brought about an improvement in the analysis of carotenoids in the last years. The accuracy of parent carotenoid as well as carotenoid metabolite identification and quantitation can be achieved by using sample preparation methods that allow a quick and efficient separation of the carotenoid content from its matrix, without any loss or degradation of the carotenoids.

Production of tomato varieties with an increased level of lycopene is desired, which requires the analysis of a greater number of samples. Therefore, analytical techniques that offer fast, simple, and accurate analysis are required. The studies conducted have shown that techniques such as UHPLC and various other spectroscopic methods such as IR and Raman spectroscopy provide a simple and fast protocol for the accurate, reproducible, and sensitive quantification of lycopene in tomatoes and tomato-based products. Raman chemical imaging has proved to be a promising spectroscopic technique for mapping constituents of interest in complex food matrices. The progress in UHPLC by the development of C30 columns, which can resist ultra-high pressures, would also increase the usefulness of this technique in performing such analyses.

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ABBREVIATIONS

ACN	acetonitrile
ATR	attenuated total reflection
BHT	butyl-hydroxide-toluene
cav-1	caveolin 1
CHD	coronary heart disease
COX-2	cyclooxygenase-2
CRM	certified reference materials
CVD	cardiovascular disease
DNA	deoxyribonucleic acid
ECDs	electrochemical detectors
Et_2O	diethyl ether
GJC	gap junction communication
HMG-CoA	3-hydroxy-3-methyl glutaryl-coenzyme A
HPLC	high-performance liquid chromatography
IGF-I	insulin-like growth factor
IR	infrared
LDL	low-density lipoprotein
LMs	linear models
MAPK	mitogen-activated protein kinase
mRNA	messenger ribonucleic acid
MS	mass spectrometers
MSPD	matrix solid-phase dispersion
MTBE	methyl-ter-butyl-ether
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
NF-κβ	nuclear factor κβ
NIR	near-infrared
NMR	nuclear magnetic resonance
NNs	neural networks
OCC	open column chromatography
ODS	octadecylsilica
PDA	photodiode array
RNA	ribonucleic acid
ROS	reactive oxygen species
RP	reverse-phase

SFE	supercritical fluid extraction
SID	spectral information divergence
TEA	triethylamine
THF	tetrahydrofuran
TLC	thin layer chromatography
UHPLC	ultra high-performance liquid chromatography

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Chapter 12

Oleuropeic and Menthiafolic Acid Glucose Esters from Plants: Shared Structural Relationships and Biological Activities

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Chapter Outline

INTRODUCTION

Many functionally important plant metabolites occur as glycosides of small lipophilic molecules. The most common of these are *O*-glycosides, which generally form when a glucosyltransferase (GT) joins the hydroxyl group of an aglycone to the anomeric center of glucopyranose in glucosides or di-, tri-, and higher carbohydrate moieties in glycosides [1]. The transfer of a sugar onto a lipophilic acceptor via the action of glycosyltransferases changes chemical properties of the aglycone such as solubility and stability, which in turn alters bioactivity and enables access to membrane transporter systems [2]. As a consequence, the resultant glycosides have diverse activities and functions in plants and play key roles in abiotic and biotic response processes including free radical scavenging in oxidative stress tolerance and antimicrobial and antiherbivore defense.

Glucose esters of small lipophilic molecules are also widely distributed in plants and are characterized by esterification of aromatic or aliphatic acid aglycones to a carbohydrate such as glucopyranose, typically at the anomeric

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or primary hydroxyl positions [3]. Similar to glycosides, glucose esters also fulfill many biologically important functions in plants. Indeed, it has been suggested that neutralizing the acid group via esterification may be more advantageous than glycosylation for plants as it improves water solubility for transport, particularly in phloem sap [4]. Common examples of aromatic acid aglycones of glucose esters are the hydroxycinnamic acids caffeic, cinnamic and ferulic acids, and the trihydroxybenzoic acid gallic acid. Aliphatic acid aglycones of glucose esters can be fatty acids, iridoids, carotenoids, or terpene acids such as triterpenes (e.g., oleanolic acid), diterpenes (e.g., retinoic acid), sesquiterpenes (e.g., artemisinic acid), or monoterpenes (e.g., perillic acid; [5]). This chapter focuses on the glucose esters of particular aliphatic monoterpene acids that have received increased attention in recent times.

Only a relatively small number of monoterpene acid glucose esters have been isolated from any sources to date. This is in contrast to monoterpene glycosides which are relatively common metabolites in plants with diverse functional roles and numerous human uses based on their flavor and fragrance properties (see reviews by Vasserot et al. [6–9]). Despite the apparent rarity of monoterpene acid glucose esters in general, a variety of glucose esters based on two isomeric monoterpene acids, oleuropeic acid (*S*)-4-(1-hydroxy-1-methylethyl)-1-cyclohexene-1-carboxylic acid (**1**) and menthiafolic acid (linalool-1-oic acid; 6-hydroxy-2,6-dimethyl-2,7-octadienoic acid; **5**), have been found with increasing prevalence in plants in recent years and have been shown to exhibit numerous biological properties [10]. The two aliphatic acids share a number of structural similarities: they both have α,β-unsaturated carbonyls, a nonoxidizable tertiary hydroxyl at C-8, and a molecular formula of $C_{10}H_{16}O_3$. They differ, however, in oleuropeic acid being cyclic and menthiafolic acid acyclic. Interestingly, both acids have also been found esterified to glucose in the same compound—froggattiside A (**8**; [11]). This chapter focuses on the glucose esters that contain oleuropeic and menthiafolic acids identified to date. We examine their biological activities and possible functional roles in plants and discuss how their chemical properties give them the potential for commercial application as pharmaceuticals.

OLEUROPEYL AND MENTHIAFOLOYL GLUCOSE ESTERS

Oleuropeic and menthiafolic acids have been found in their free form in plants, but their glucose esters appear to be more widely distributed (see Table 1). The apparent prevalence of glucose esters of these acids may reflect their potential functional roles (described in Functional Roles in Plants section), or it may in part be artifactual due to an experimental bias toward elucidating the structures of the glucose esters. Free oleuropeic acid has been found in olive oil extracted from the fruit of *Olea europaea* [49,50], in Sacha Inchi oil extracted from the seeds of *Plukenetia volubilis* [51], and in leaves or aerial parts of *Abies chensiensis* [52], *Eucalyptus maideni* [16,31], *Ledum*

TABLE 1 Thirty-Seven Compounds Containing Glucose Esterified to the Monoterpenoids Oleuropeic and/or Menthiafolic Acids have been Found in a Range of Plant Sources, but are Particularly Prevalent Within the Genus *Eucalyptus* (Myrtaceae)

Compound	C6 Primary Aglycone	C1 Anomeric Aglycone	Species	Family	Plant Tissue	References
Oleuropeic acid glucose esters						
Cuniloside A (**26**) (cuniloside)	Oleuropeic acid	Oleuropeic acid	*Cunila spicata*	Lamiaceae	Leaves	[12]
			E. globulus	Myrtaceae	Leaves	[13]
Cuniloside B (**27**) (eucalmaidin E)	Oleuropeic acid	Oleuropeic acid	*E. cypellocarpa*	Myrtaceae	Leaves	[11]
			E. froggattii	Myrtaceae	Leaves‡	[11,14]
			E. globulus	Myrtaceae	Leaves‡	[11]
			E. polybractea	Myrtaceae	Leaves‡	[11]
			E. behriana	Myrtaceae	Leaves	[11]
			E. loxophleba ssp. *lissophloia*	Myrtaceae	Leaves	[15]
			E. maidenii	Myrtaceae	Leaves	[16]
					Fruit	[17]
			23 Species of *Eucalyptus*	Myrtaceae	Leaves	[18]
			19 Species of *Eucalyptus*	Myrtaceae	Leaves‡	[19]

Continued

TABLE 1 Thirty-Seven Compounds Containing Glucose Esterified to the Monoterpenoids Oleuropeic and/or Menthiafolic Acids have been Found in a Range of Plant Sources, but are Particularly Prevalent Within the Genus *Eucalyptus* (Myrtaceae)—Cont'd

Compound	C6 Primary Aglycone	C1 Anomeric Aglycone	Species	Family	Plant Tissue	References
Cypellocarpin A (**11**)	Oleuropeic acid	Gallic acid	*E. cypellocarpa*	Myrtaceae	Leaves	[20]
			E. globulus	Myrtaceae	Leaves	[13]
			E. maidenii	Myrtaceae	Leaves	[16]
					Fruit	[17]
Cypellocarpin B (**20**)	Oleuropeic acid	Chromanone	*E. cypellocarpa*	Myrtaceae	Leaves	[20]
			E. maidenii	Myrtaceae	Leaves	[16]
			E. gomphocephala	Myrtaceae	Leaves	[21]
Cypellocarpin C (**21**) (camaldulenside)	Oleuropeic acid	Noreugenin	*E.camaldulensis*	Myrtaceae	Leaves	[22,23]
			E. cypellocarpa	Myrtaceae	Leaves	[20]
			E. globulus	Myrtaceae	Fruit	[24–27]
			E. maidenii	Myrtaceae	Leaves	[16]
			23 Species of *Eucalyptus*	Myrtaceae	Leaves	[18]
			15 Species of *Eucalyptus*	Myrtaceae	Leaves[‡]	[19]

Cypellogin A (**34**)	Oleuropeic acid	Isoquercetin	*E. cypellocarpa*	Myrtaceae	Leaves	[28]
			E. maidenii	Myrtaceae	Fruit	[17]
Cypellogin B (**35**)	Oleuropeic acid	Isoquercetin	*E. cypellocarpa*	Myrtaceae	Leaves	[28]
			E. maidenii	Myrtaceae	Leaves	[16]
					Fruit	[17]
Cypellogin C (**36**)	Di-hydro oleuropeic acid	Isoquercetin	*E. cypellocarpa*	Myrtaceae	Leaves	[28]
Eucaglobulin A (**9**)	Oleuropeic acid	Gallic acid	*E. globulus*	Myrtaceae	Leaves	[13,29,30]
					Fruit	[27]
			E. maidenii	Myrtaceae	Fruit	[17]
Eucaglobulin B (**10**)	Oleuropeic acid	Gallic acid	*E. dielsii*	Myrtaceae	Leaves‡	[19]
			E. platypus	Myrtaceae	Leaves‡	[19]
			E. spathulata	Myrtaceae	Leaves‡	[19]
Eucalmaidin A (**3**)	Oleuropeic acid	Not present	*E. maidenii*	Myrtaceae	Leaves	[16]
Eucalmaidin B (**12**)	Oleuropeic acid	Gallic acid	*E. maidenii*	Myrtaceae	Leaves	[16]
					Fruit	[17]
Eucalmaidin C (**13**)	Oleuropeic acid	Gallic acid	*E. maidenii*	Myrtaceae	Leaves	[16]
Eucalmaidin D (**31**)	Oleuropeic acid	Quercetin	*E. maidenii*	Myrtaceae	Leaves	[16]

Continued

TABLE 1 Thirty-Seven Compounds Containing Glucose Esterified to the Monoterpenoids Oleuropeic and/or Menthiafolic Acids have been Found in a Range of Plant Sources, but are Particularly Prevalent Within the Genus *Eucalyptus* (Myrtaceae)—Cont'd

Compound	C6 Primary Aglycone	C1 Anomeric Aglycone	Species	Family	Plant Tissue	References
Eucalmaidin F (**14**)	Oleuropeic acid	Gallic acid	*E. maidenii*	Myrtaceae	Leaves*	[31]
Eucalmalduside B (**22**)	Oleuropeic acid	Chromanone	*E. camaldulensis*	Myrtaceae	Leaves	[22]
Eucalmalduside C (**23**)	Oleuropeic acid	Chromanone	*E. camaldulensis*	Myrtaceae	Leaves	[22]
Froggattiside A (**8**)	Oleuropeic acid	Menthiafolic acid	*E. cypellocarpa*	Myrtaceae	Leaves	[11]
			E. froggattii	Myrtaceae	Leaves‡	[11,14]
			E. globulus	Myrtaceae	Leaves‡	[11]
			E. polybractea	Myrtaceae	Leaves‡	[11]
			E. behriana	Myrtaceae	Leaves	[11]
			15 Species of *Eucalyptus*	Myrtaceae	Leaves‡	[19]
			Melaleuca armillaris	Myrtaceae	Leaves‡	[19]

Galloyl cypellocarpin B (putative)	Oleuropeic acid	Chromanone	*E. gomphocephala*	Myrtaceae	Leaves	[21]
Globulusin B (**25**)	Gallic acid	Oleuropeic acid	*E. globulus*	Myrtaceae	Leaves	[13]
					Fruit	[27]
Resinoside A (**32**)	Oleuropeic acid	Kaempferol	*E. resinifera*	Myrtaceae	Leaves	[32]
Resinoside B (**33**)	Oleuropeic acid	Kaempferol	*E. resinifera*	Myrtaceae	Leaves	[32]
1-*O*-[(*S*)-oleuropeyl]-β-D-glucopyranose (**6**)	Not present	Oleuropeic acid	*C. spicata*	Lamiaceae	Leaves	[12]
6-*O*-[(*S*)-oleuropeyl]-D-glucose (**2**)	Oleuropeic acid	Not present	*C. spicata*	Lamiaceae	Leaves	[12]
6-*O*-oleuropeyl sucrose (**38**)	Oleuropeic acid	(Fructose)	*Olea europaea*	Oleaceae	Root bark	[33,34]
Menthiafolic acid glucose esters						
Eucalmalduside A (**24**)	Menthiafolic acid	Noreugenin	*E. camaldulensis*	Myrtaceae	Leaves	[22]
Froggattiside A (**8**)	Oleuropeic acid	Menthiafolic acid	*E. cypellocarpa*	Myrtaceae	Leaves	[11]
			E. froggattii	Myrtaceae	Leaves‡	[11]
			E. globulus	Myrtaceae	Leaves‡	[11]
			E. polybractea	Myrtaceae	Leaves‡	[11]
			E. behriana	Myrtaceae	Leaves	[11]
			15 Species of *Eucalyptus*	Myrtaceae	Leaves‡	[19]
			M. armillaris	Myrtaceae	Leaves‡	[19]

Continued

TABLE 1 Thirty-Seven Compounds Containing Glucose Esterified to the Monoterpenoids Oleuropeic and/or Menthiafolic Acids have been Found in a Range of Plant Sources, but are Particularly Prevalent Within the Genus *Eucalyptus* (Myrtaceae)—Cont'd

Compound	C6 Primary Aglycone	C1 Anomeric Aglycone	Species	Family	Plant Tissue	References
Jashemsloside B (**28**)	Menthiafolic acid	Iridoid	*Jasminum hemsleyi*	Oleaceae	Leaves	[35]
Phlebotrichin (**15**)	Menthiafolic acid	Phenol	*Viburnum phlebotrichum*	Adoxaceae	Leaves	[36]
			Breynia fruticosa	Euphorbiaceae	Leaves*	[37]
			B. rostrata	Euphorbiaceae	Leaves*	[37]
			Portucala oleracea	Portulacaceae	Leaves*	[38]
Phlebotricoside (**16**)	Menthiafolic acid	Phenol	*V. phlebotrichum*	Adoxaceae	Leaves	[39]
Rotundifoline A (**37**)	Menthiafolic acid	Vomifoliol	*Rotala rotundifolia*	Lythraceae	Leaves*	[40]
Syrveoside B (**30**)	Menthiafolic acid	Seciridoid	*Syringa velutina*	Oleaceae	Leaves	[41]
Tetrapteroside A (**39**)	Menthiafolic acid	Triterpene	*Tetrapleura tetraptera*	Fabaceae	Stem bark	[42]

6-*O*-[(2*E*,6*S*)-2,6-dimethyl-6-hydroxy-2,7-octadienoyl]-(α-β)-D-glucopyranose (**4**)	Menthiafolic acid	Not present	*P. oleracea*	Portulacaceae	Leaves*	[38]
6′-*O*-menthiafoloyl mussaenosidic acid (**29**)	Menthiafolic acid	Iridoid	*Veronica bellidioides*	Plantaginaceae	Leaves*	[43]
					Flowers	[44]
6′-*O*-menthiafoloyl verbascoside(**17**) (galypumoside C)	Menthiafolic acid	Phenylethyl glycoside	*J. subtriplinerve*	Oleaceae	Leaves*	[45]
			Globularia alypum	Plantaginaceae	Leaves	[46]
9-(6-*O*-[(2*E*,6*S*)-2,6-dimethyl-6-hydroxy-2,7-octadienoyl]-β-D-glucopyranosyloxy)-guaiacylglycerol (**18**)	Menthiafolic acid	Guaiacylglycerol	*P. oleracea*	Portulacaceae	Leaves*	[38]
9-(6-*O*-[(2*E*,6*S*)-2,6-dimethyl-6-hydroxy-2,7-octadienoyl]-β-D-glucopyranosyloxy)-syringoylglycerol (**19**)	Menthiafolic acid	Syringoylglycerol	*P. oleracea*	Portulacaceae	Leaves*	[38]
β-D-glucopyranosyl menthiafolate (**7**)	Not present	Menthiafolic acid	*Ligustrum robustum*	Oleaceae	Leaves	[47]
			Lantano lilacia	Verbenaceae	Leaves	[48]

‡*Compound localized specifically to the lumen of foliar essential oil secretory cavities.*
**Leaves and stems pooled for extractions, i.e. method describes 'aerial parts' or 'branches'.*

palustre [53], and *Schisandra chinensis* [54]. Free menthiafolic acid has been detected in more plant species than free oleuropeic acid, including flowers of *Albizia julibrissin* [55] and *Narcissus trevithian* [56]; fruits of *Euterpe oleracea* [57]; roots of *Cynanchum amplexicaule* [58]; and aerial parts of *Anethum graveolens* [59], *Artemisia sieberi* [60], *Exochorda racemosa* [61], and *Malva silvestris* [62].

The identification of oleuropeic and menthiafolic acid glucose esters in plant extracts is advantaged by the use of reversed-phase high-performance liquid chromatography (HPLC) coupled with electron spray ionization-mass spectrometry. The glucose esters can be readily separated using gradients of acetonitrile and water and importantly show unique tandem mass spectrometry (MS–MS) fragmentation patterns. As illustrated in Fig. 1, the anomeric moieties are readily cleaved from the glucose esters of both acids producing a key protonated fragment with *m*/*z* 347 and subsequent diagnostic fragments with the loss of one (*m*/*z* 329) or two (*m*/*z* 311) water molecules. This diagnostic fragmentation pattern was recently used to relatively rapidly screen extracts from 20 different species and identify numerous known monoterpenoid glucose esters and to detect the presence of many other unknowns that share this pattern and warrant further structural elucidation [19].

The first oleuropeic acid glucose ester reported was 6-*O*-[(*S*)-oleuropeyl] sucrose (**38**), isolated from the root bark of *Olea europea* [33]. Since then, a further 22 oleuropeic acid-containing compounds have been elucidated from plants, none of which contain sucrose, instead almost all contain the beta form of glucopyranose as the carbohydrate moiety (Fig. 2). The exceptions are resinoside B (**33**) and cypellogin B (**35**) containing β-D-galactopyranose, the diastereoisomer of glucopyranose, as the carbohydrate moiety. Similarly, β-D-galactopyranose is also found in cypellogin C (**36**) from *Eucalyptus cypellocarpa*, a compound identical to **35** except for the monoterpenoid acid aglycone being a di-hydro-derivative of oleuropeic acid [28]. It is noteworthy that the endocyclic double bond of oleuropeic acid has been shown to readily reduce to di-hydro oleuropeic acid in the biotransformation of α-terpineol in

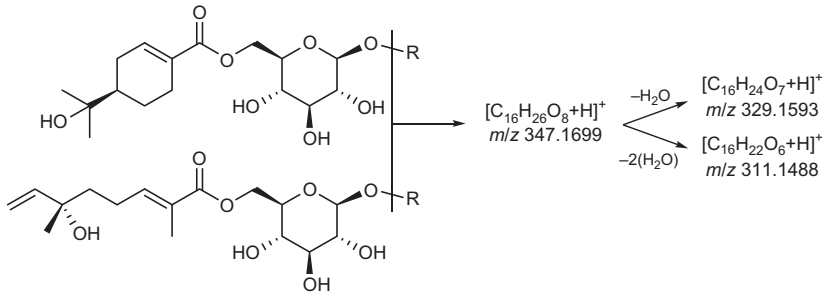

FIGURE 1 Diagnostic MS–MS fragmentation of glucose esters of oleuropeic and menthiafolic acids.

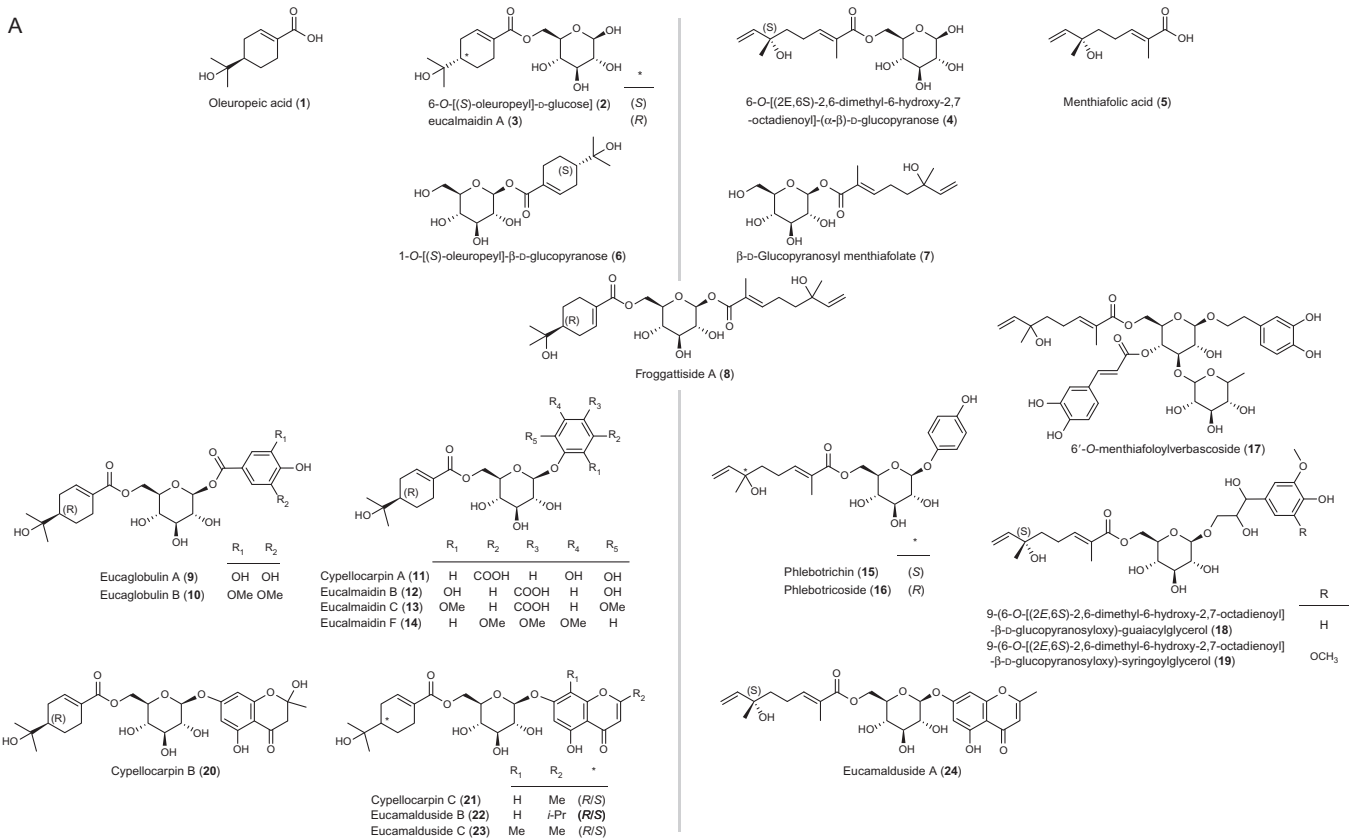

FIGURE 2—Cont'd

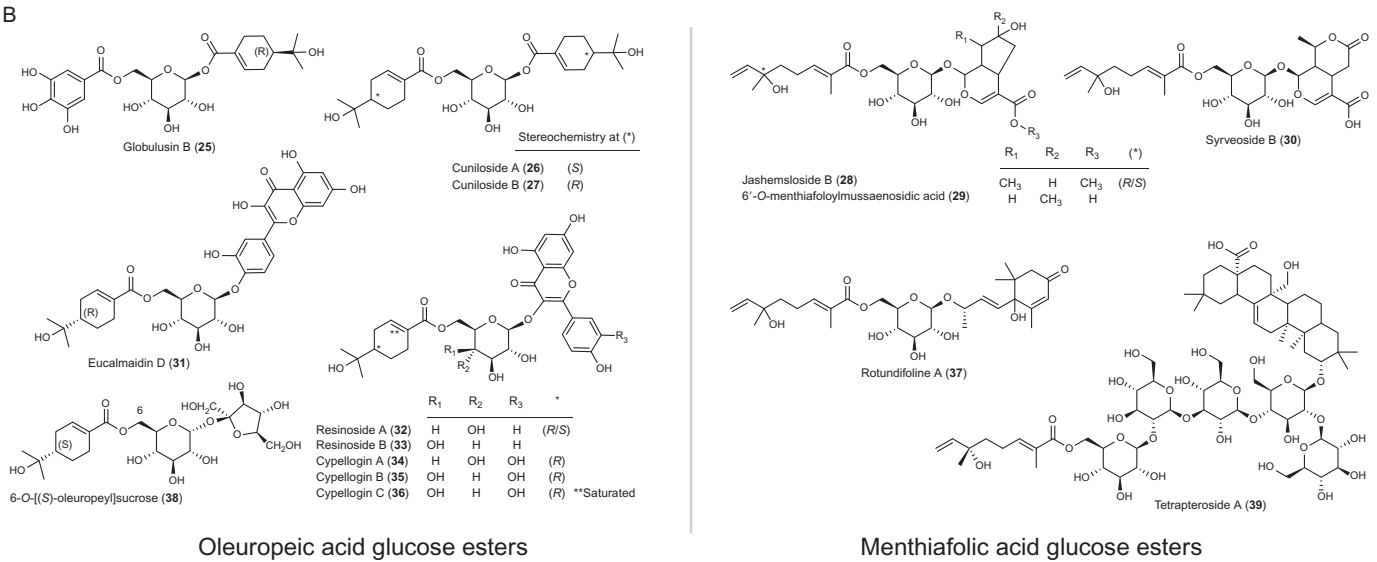

FIGURE 2 Structures of 37 different glucose esters of the monoterpenoids oleuropeic and menthiafolic acids. All compounds have been isolated from plant sources and some share structurally similar aglycones (A), whereas others do not (B).

rats, suggesting that the di-hydro-derivative of oleuropeic acid in **36** may simply be an enzymatic modification of oleuropeic acid prior to esterification to glucose [63]. Interestingly, 20 of the 23 oleuropeic acid-containing glucose esters have been found in the leaves and fruit of trees in the genus *Eucalyptus* (Table 1).

The first menthiafolic acid glucose ester reported was phlebotricoside (**16**), isolated from the leaves of *Viburnum phlebotrichum* [39]. Subsequently, 13 more menthiafolic acid glucose esters have been isolated from plants (Fig. 2). In contrast to the oleuropeic acid glucose esters, only two of the menthiafolic acid-containing compounds (**8** and **24**) have been isolated from the genus *Eucalyptus* (Table 1), although **8** has been detected in 18 different species within that genus [11,19].

There are some noteworthy structural similarities between the glucose esters of oleuropeic and menthiafolic acids (Fig. 2A). For example, both can contain phenolic moieties attached at the anomeric position of glucopyranose such as gallic acid derivatives in compounds **9**–**14** and **15**–**19**, and derivatives of the chromenone noreugenin in compounds **20**–**24**. Nonetheless, there are also some structural differences between the glucose esters of each acid (Fig. 2B). For example, some of the oleuropeic acid glucose esters contain phenolic flavonol aglycones glycosylated at the anomeric position of the glucopyranose moiety such as quercetin (**31**), isoquercetin (**34** and **35**), and kaempferol (**32** and **33**). Recently, a mass spectral analysis of an extract from *Eucalyptus gomphocephala* leaves tentatively identified another oleuropeic acid-containing compound based on cypellocarpin B (**20**), which has a chromenone derivative as its anomeric moiety, but this new compound also appears to have a gallic acid moiety attached at a position yet to be determined [21]. This suggests that the new compound may have a flavonol moiety structurally similar to those in eucalmaidin D (**31**), resinoside A (**32**), or cypellogin A (**34**). Gallic acid has also been found attached at the primary hydroxyl of glucopyranose in globulusin B (**25**), which is one of only four compounds with oleuropeic acid attached at the anomeric position, the others being the monoester 1-*O*-[(*S*)-oleuropeyl]-β-D-glucopyranose (**6**) and the diastereoisomeric diesters cuniloside A (**26**) and B (**27**). In contrast to the common phenolic aglycones of the oleuropeic acid glucose esters, a number of the menthiafolic acid glucose esters contain terpenoid aglycones glycosylated at the anomeric position of glucose including vomifoliol (**37**), a secoiridoid (**30**), iridoids (**28** and **29**), and a triterpenoid (**39**).

The stereochemistry of the monoterpenoid acid moieties has not been determined for all of the glucose esters presented in Fig. 1, often due to insufficient yields of compounds for accurate determinations. Nevertheless, it appears that both *R* and *S* forms of oleuropeic acid can be produced and even co-occur in some plants. For example, compounds **21**–**23** were isolated in 1:1 epimeric ratios from the same extract of *Eucalyptus camaldulensis var. obtusa* leaves [22], and **32** has also been found as a 1:1 epimeric mixture in an extract

of *Eucalyptus resinifera* leaves [32]. Although the stereochemistry of menthiafolic acid has less often been determined, an epimeric mixture of 18:82 (*R*:*S*) was determined for jashemsloside B (**28**), and both the enantiomers (*S*)-phlebotrichin (**15**) [36] and (*R*)-phlebotrichoside (**16**) [39] have been isolated from *V. phlebotrichum*, albeit in different studies. Interestingly, the *E. camaldulensis* extract that contained 1:1 epimeric ratios of (*R*)- and (*S*)-oleuropeic acid in compounds **21**–**23** also contained the menthiafolic acid glucose ester eucalmalduside A (**24**), but this occurred exclusively as the (*S*) enantiomer [22].

There is only one example of an *O*-glycoside of oleuropeic acid isolated from plants to date. That compound was isolated from *Juniperus communis* var. *depressa* and is glycosylated through the tertiary hydroxyl of (−)-(*S*)-oleuropeic acid to the anomeric position of glucopyranose with no other aglycone attached to the carbohydrate [64]. In contrast, a number of menthiafolic acid *O*-glycosides have been isolated from plants. These include the monoterpenoid mono-, di-, and tri-glycosides, anatoliosides A–E isolated from *Viburnum orientale* [65,66], the related chevangins A–D from *Jasminum subtriplinerve* [67], the diastereomeric triterpenoid saponins julibroside J_1 and J_9 isolated from *A. julibrissin* [68], and the phenolic diglycoside seguinoside I isolated from *Myrsine seguinii* [69].

BIOLOGICAL ACTIVITIES

The biological activities of free oleuropeic and menthiafolic acids have not been tested extensively, but in those studies that have assessed their properties, no significant activity has been observed. For example, the inhibitory activity of oleuropeic acid isolated from *Abies chensiensis* was tested against lipopolysaccharide-induced nitric oxide production in a murine marcrophage cell line but was found to have no detectable activity when compared to the positive control aminoguanidine [52]. Oleuropeic acid isolated from *Eucalyptus maidenii* was tested for cytotoxicity against five human cancer cell lines (human myeloid heukemia HL-60, hepatocellular carcinoma SMMC-7721, breast cancer MCH-7, colon cancer SW480, and lung cancer A-549) using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide method with cisplatin as a positive control, but at a concentration of 40 μm, it did not exhibit activity against any of the cell lines [31]. Furthermore, free oleuropeic acid isolated from leaves of the same species was evaluated for *in vitro* antiherpes simplex virus type 1 (HSV-1) activity using a cytopathic effect assay, but it displayed no HSV-1 inhibition [16]. Similarly, menthiafolic acid isolated from *Euterpe oleracea* fruit showed no activity in an *in vitro* hydroxyl radical scavenging assay [57], and when isolated from *Malva silvestris*, it was found to have no significant effect on germination, root growth, or shoot growth of *Lactuca sativa* [62]. Finally, in a recent study on antiobesity effects of compounds isolated from *A. julibrissin* flowers, free menthiafolic

acid at a concentration of 30 μm showed 31% inhibition of triglyceride accumulation in cultured 3T3-L1 adipocytes, but this was less than the positive control, and importantly no inhibition of 2-deoxyglucose uptake was observed in the menthiafolic acid-treated cells [55].

In contrast to the free acids, their glucose esters display many biological activities. The majority of the compounds presented in Fig. 2 contain α,β-unsaturated carbonyls in both aglycones [10]. These carbonyl groups draw electrons away from the olefinic bond rendering it electrophilic and thus able to act as a Michael reaction acceptor, which generates a number of important biological reactivities (see [70] for review). In particular, they can interact with electron-rich biological macromolecules, resulting in a wide range of adverse effects, including toxicity, allergenic reactions, mutagenicity, and carcinogenicity [71]. Despite these deleterious effects, Michael acceptors can also act as anticarcinogens by inducing chemoprotective enzymes in animal cells that then inactivate reactive, electrophilic forms of carcinogens [72]. Moreover, many of the compounds presented in Fig. 2 contain both Michael acceptor functionalities and phenolic hydroxyl groups, and as such are characterized as bi-functional antioxidants that can play dual protective roles by scavenging hazardous oxidants directly and instantaneously, and inducing cytoprotective enzymes to ensure long-term protection [73]. The fact that most of the glucose esters described here are also double Michael acceptors may enhance their activities as has previously been shown for curcumin from *Curcuma longa* [74] and for particular synthetic antiinflammatory triterpenoids [75].

Many of the oleuropeic acid glucose esters have been assessed for their antitumor, antiinflammatory, antioxidant, antiviral, antimicrobial, cytotoxic, and repellent properties and found to exhibit varying levels of activity. In particular, cypellocarpins A–C and eucaglobulin A have received the most attention. Cypellocarpins A (**11**), B (**20**), and C (**21**) isolated from *E. cypellocarpa* leaves have been evaluated for their tumor inhibiting activities [20]. First, they were assessed for their inhibitory effects on Epstein-Barr virus early antigen (EBV-EA) activation induced by 12-*O*-tetradecanoyl-13-*O*-acetylphorbol (TPA), using an *in vitro* short-term bioassay with Raji cells derived from Burkitt's lymphoma. Compounds **11**, **20,** and **21** inhibited EBV-EA activation at 100 mol ratio/TPA, and exhibited more significant inhibitory effects (68%, 67%, and 63% inhibition, respectively) at 500 mol ratio/TPA. Similar potencies at concentrations preserving high viability of Raji cells were observed for the positive control, (−)-epigallocatechin gallate (EGCG). Second, **20** and **21** were evaluated for their effects on *in vivo* two-stage carcinogenesis in mouse skin using nitric oxide as an initiator and TPA as a promoter. At a concentration of 50-mol ratio/TPA, both cypellocarpins reduced the percentage of tumor-carrying mice to 30%, 10 weeks after promotion. This was compared to control animals which showed a 100% incidence of papillomas at that time. Although papillomas were observed in all cypellocarpin-treated

mice by week 20, the average number of papillomas per mouse was reduced to 78% of the control in the **21** treatment and only 58% of control in the **20** treatment. The potencies of **20** and **21** were slightly lower than that of the EGCG positive control [20], but the result still suggests that these cypellocarpins have therapeutic potential. The therapeutic potential of **21** was further investigated in a study on its intestinal permeability using human Caco-2 cell monolayers as a model system for intestinal absorption [24]. The results from the model system estimated **21** to be a moderately well-absorbed drug, suggesting it is orally bioavailable if a drug therapy was to be developed.

Compounds **11**, **20**, **21**, eucalmaidins A (**3**), B (**12**), C (**13**), and cuniloside B (**27**), isolated from *E. maidenii* leaves [16], as well as eucaglobulin A (**9**), **11**, **12**, and **27** isolated from fruits of that species [17] were all evaluated for their *in vitro* anti-HSV-1 activity but none displayed any inhibition. In both studies, the maximal noncytotoxic concentration (MNCC) of each compound was also assessed using African green monkey kidney cells (Vero cells). Compounds **11** and **20** showed the strongest cytotoxicity with MNCC values of 0.03 and 0.02 mM, respectively [16]. Interestingly, free oleuropeic acid (**1**) was the least cytotoxic to vero cells (MNCC 1.09 mM), the monoester of oleuropeic acid (**3**) was intermediately cytotoxic (MNCC 0.58 mM), and the diester of oleuropeic acid (**27**) was the most cytotoxic (MNCC 0.20 mM; [16]).

Compounds **9**, **11**, globulusin B (**25**), and cuniloside A (**26**) isolated from *Eucalyptus globulus* leaves have been examined for antioxidant activity by their ability to suppress 1,1-diphenyl-2-pycrylhydrazyl (DPPH) free radical formation [13]. Compounds **9**, **11,** and **25** were able to scavenge DPPH free radicals, with half maximal inhibitory concentration (IC_{50}) values of 21, 4, and 122 μM, respectively, whereas **26** showed no activity. Indeed the DPPH radical scavenging activity of **25** was 10 times greater than that of the positive control, ascorbic acid ($IC_{50} = 12$ μM). In the same study, **9** was tested as an antiinflammatory agent via its ability to suppress production of the cytokines interleukin-1β and tumor necrosis factor-α in cultured human myeloma THP-1 cells, in which inflammation was induced with phorbol myristate acetate. Compound **9** quantitatively suppressed inflammatory production of both cytokines at doses of 2, 20, and 40 μM, but also showed cytotoxic activity against the THP-1 cells at 40 μM (29% mortality). Although cytokine suppression was significantly greater than the negative control at all doses, even at a concentration of 40 μM, **9** was incapable of as much suppression as the positive control—the antiinflammatory agent dexamethasone at a dose of only 2 μM [13]. Compound **9**, again isolated from *E. globulus* leaves, has also been tested for its antimicrobial activity. It was found to moderately inhibit proliferation in the yeast *Candida albicans* and the bacterium *Escherichia coli* with minimum inhibitory concentrations (MIC) of 0.5 mM and 1 mM, respectively [29]. Nevertheless, it showed little or no inhibitory activity against the bacteria *Staphylococcus aureus* (MIC = 2 mM), *Staphylococcus epidermidis*

(MIC = 4 mM), *Streptococcus sanguis* (MIC = 2 mM), and *Enterobacter gergoviae* (MIC = 4 mM) [29].

Cuniloside B (**27**) was recently tested for *in vitro* antileishmanial activity, but was found to be inactive [15]. In the same study, an Alamar blue bioassay at double the IC_{50} (μM) was used to show that **27** also has no cytotoxicity toward peripheral blood mononuclear cells separated from heparinized blood of a normal healthy individual. Finally, the repellent activity of resinoside A (**32**) isolated from *E. resinifera* leaves has been tested against the blue mussel *Myrtilus edulis* [32]. Resinoside A exhibits repellent activity at 0.78 μmol cm^{-2} which equates to 64% of the repellent activity of the marine antifouling control $CuSO_4$ applied at the same rate. Interestingly, **32** was much more repellent toward the blue mussel than any of its components: oleuropeic acid (8% of $CuSO_4$), the flavonol kaempferol (8% of $CuSO_4$) or the flavonol glycoside astragalin (5% of $CuSO_4$). This result is similar to the aforementioned vero cells cytotoxicity study [16] where the oleuropeic acid diester was more toxic than its components.

Only a small number of the menthiafolic acid glucose esters have been tested for biological activity, and these tests have generally been relatively less extensive. Despite this, some notable results have been reported. In particular, a recent study of *Portucala oleraceae* leaves and roots elucidated three new menthiafolic acid glucose esters (**4**, **18**, **19**) and the known glucose ester phlebotrichin (**15**). The antioxidant activities of these compounds were tested using the DPPH radical assay with tocopherol (vitamin E) as the positive control [38] and it was found that the antioxidative activity of **18** ($IC_{50} = 11.6 \pm 0.6$ μM) was greater than that of tocopherol ($IC_{50} = 14.8 \pm 0.8$ μM). Moreover, both **18** and **19** ($IC_{50} = 36.7 \pm 5.7$ μM) were stronger antioxidants than their precursor monoester **4** ($IC_{50} = 57.2 \pm 4.2$ μM), their constituent monolignan moiety guaiacylglycerol ($IC_{50} = 75.4 \pm 3.7$ μM) isolated in its free form, or menthiafolic acid ($IC_{50} = 122.8 \pm 5.2$ μM) produced via hydrolysis of **18** with NaOH. Both compounds **18** and **19** were also stronger antioxidants than **15** ($IC_{50} = 92.7 \pm 7.8$ μM) [38]. The only other study noting a chemical property of a menthiafolic acid glucose ester is the description of phlebotricoside (**16**) from *V. phlebotrichum* leaves as being bitter to taste [39], a property commonly related to plant deterrence of mammalian herbivores.

FUNCTIONAL ROLES IN PLANTS

There has been little research specifically addressing the biological or ecological roles of the monoterpene acid glucose esters presented in Fig. 2. The most detailed work comes from the aforementioned study that found four menthiafolic acid glucose esters in *P. oleraceae* [38]. That study also examined if the compounds could be induced when leaves were sprayed with a 2% aqueous solution of $CuCl_2$. After 48 h, leaves were harvested and in those from unsprayed control plants only the monoester **4** and guaiacylglycerol

(the monolignan precursor of **18**) were detected together with **15**, whereas in leaves from treated plants, lower amounts of **4** and guaiacylglycerol were detected along with **15**, **18**, and **19**. This result suggests that compounds **18** and **19** are stress metabolites induced in response to copper toxicity in *P. oleraceae*, although **15** evidently is not. Phenolic-containing compounds are known to chelate metals such as copper and consequently they are thought to be induced in plants as part of metal detoxification mechanisms [76,77].

Based on the predicted chemical properties of their chemical constituents, a number of other biological roles are possible for the oleuropeic and menthiafolic acid glucose esters. First, the potentially adverse biological activities of both the Michael acceptors and the phenol groups they contain may form part of biotic stress avoidance mechanisms such as plant chemical defense against herbivores and pathogens. The purported cytotoxicity, antimicrobial activity, bitterness, and repellency of some of these compounds are supportive of such a role, as are the well-characterized antiherbivorous properties of many of their constituent phenolic glycosides (see [78]). Nevertheless, no studies have yet been undertaken to clearly demonstrate the effectiveness of the glucose esters in plant defense.

Second, the glucopyranose moiety in the glucose esters is a strong indicator of a number of other potential roles in plants. Glycosylation has been shown to increase the water solubility of terpenes [8] and phenolics [79], and esterification to glucose is likely to impart similar properties. Enhanced solubility is an important property enabling mobility in phloem sap and partitioning into various other plant tissues and cell compartments [1,80]. In an ostensibly contradictory manner, glycosylation can also act to reduce the mobility of lipophilic aglycones under certain circumstances. For example, the addition of a carbohydrate onto a lipophilic acceptor can make compounds more polar and prevent any further free diffusion across lipid bilayers or between intracellular compartments [2]. Therefore, esterification to glucose, such as in the compounds presented here, may relate to a plants need to either transport or alternatively contain these potentially autotoxic metabolites to various tissues, extracellular spaces, or cellular compartments. No studies have yet been undertaken to explore these possibilities.

Glycosylation or esterification to glucose can also impart other potentially important properties to the aglycones. For example, it has been suggested that glycosylation of polyphenols at their less reactive groups has evolved to protect these molecules from deleterious oxidation by the plants' own enzymes [79]. In this way, certain important phenolic glycosides have a relatively extended half-life in plant cells, maintaining their beneficial antioxidant activities and biological properties, as well as increasing their solubility in the cell cytoplasm [81]. Glycosyl conjugation can also reduce the chemical reactivity of aglycones and has long been argued as a protective mechanism against the autotoxicity of compounds like phenolics [79] and terpenes [82] in plants. It is noteworthy that a recent attempt to genetically engineer overexpression of the monoterpenoid

geranic acid as an antifungal metabolite in *Zea mays* did not result in increased free acid in leaves but instead resulted in accumulation of an abundant geranic acid glucose ester [83]. This result suggests that the free geranic acid produced by transformed plants was rapidly conjugated with glucose, perhaps as a protective mechanism against deleterious effects of the acid.

Another possible functional role for the monoterpene glucose esters again relates to the potential autotoxicity of plant terpenes. Plant terpenes are commonly sequestered into specialized cells or larger extracellular spaces to avoid deleterious effects on tissues [84,85]. Many of the monoterpene acid glucose esters isolated from *Eucalyptus* have also been found to be localized exclusively to foliar secretory cavities that house terpenes [10,19], and can comprise over 50% of cavity lumen volume in adult leaves of some species [86]. We recently discussed the possibility that the relatively hydrophilic monoterpene acid glucose esters, with their polar glucopyranose moieties and relatively nonpolar terpenoid groups, may act in an amphipathic manner to form a protective "buffer zone" between the terpenes housed in the lumen interior and the epithelial cells lining the cavity lumen [10]. This idea was originally proposed as an explanation for unidentified, hydrophilic "mucilage material" observed to occur between terpenes and cap cells in glandular secreting trichomes of *Mentha* × *piperita* [87]. Some support for this proposed protective function has recently been found. In a microscopic study on *Eucalyptus* secretory cavities, Heskes and coworkers localized an autofluorescent domain to a zone between the central terpene pool and the secretory cells bounding the lumena [88]. Importantly, they found that one of the main constituents of the autofluorescent domain was the ester eucaglobulin B (**10**).

BIOSYNTHESIS

Despite great advances in understanding plant mono- and sesquiterpene biosynthesis in the last few decades (see [89]), surprisingly little is known about the biosynthesis of monoterpene acids or the process by which they are esterified to glucose. Based largely on structural similarities, it has been suggested that oleuropeic acid is likely derived from the cyclic monoterpene α-terpineol [25], whereas menthiafolic acid is likely derived from the acyclic monoterpene linalool [90]. Studies on biotransformation of plant products, particularly microbial degradation of terpenes [91,92], can be indicative of the processes and enzyme groups involved in plant biosynthesis of secondary metabolites. A number of such studies using a variety of organisms have provided supportive evidence for the biosynthesis of the monoterpene acids from linalool and α-terpineol, respectively. The likely pathways reported in these studies are summarized in Fig. 3.

First, studies have shown that the biotransformation of linalool can result in menthiafolic acid as one of the products. For example, menthiafolic acid was detected in urine after feeding linalool to rats (*Rattus norvegicus*) and it

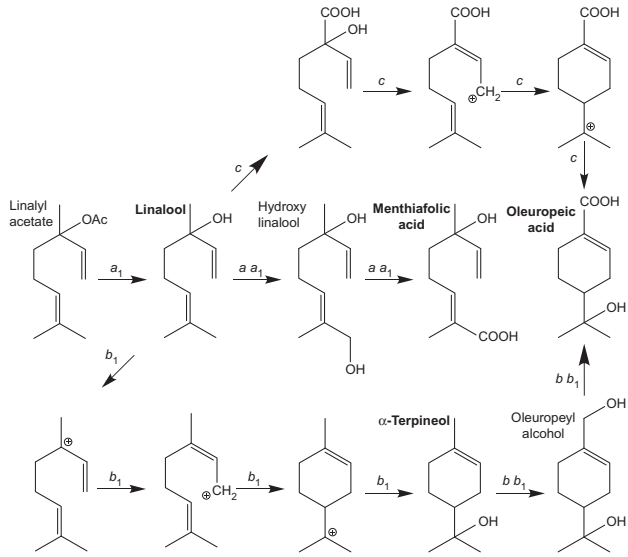

FIGURE 3 Possible relatedness between biosynthetic pathways for oleuropeic and menthiafolic acids based on the evidence from biotranformation work in *a* [93,94], a_1 [95], *b* [63,96–98], b_1 [94,95], and *c* [94].

was proposed that the acid was formed via a hydroxyl-linalool intermediate (pathway *a* in Fig. 3; [93]), which in roots of *C. amplexicaule* has been observed to co-occur with free menthiafolic acid [58]. Similarly, the conversion of linalool to wine lactone via menthiafolic acid as an intermediate has been observed in *Vitis vinifera* Muscat grapes [99] and Riesling wine [100]. Interestingly, after three days of dosing linalool to rats in the study by Chadha and Madyastha [93], significant increases in liver microsomes were observed, with a corresponding increase in both cytochrome P-450 (~50%) and cytochrome b_5 (~20%) enzyme concentrations, a result that is suggestive of the involvement of these enzymes in the redox steps [93].

Second, oleuropeic acid has been produced via the biotransformation of α-terpineol in a number of studies (Fig. 3 *b*). For example, when α-terpineol was fed as part of an artificial diet to larvae of the common cutworm (*Spodoptera litura*) numerous metabolites were detected in frass, including oleuropeic acid [96]. Similarly, when α-terpineol was fed to rats, oleuropeic acid was detected in urine along with its reduced derivative dihydrooleuropeic acid [63]. The administering of α-terpineol in the latter study coincided with a significant increase in the amount of rat liver microsomal cytochrome P-450 enzymes, again suggesting that P-450s are involved in degradation. There is some preliminary evidence for the involvement of P-450s in the biosynthesis of monoterpene acids in plants. For example, a cytochrome P-450 dependent monooxygenase has been isolated from the plant *Vinca rosea* which converts the acyclic monoterpenes geraniol and nerol to their respective hydroxy derivatives [101].

Further support for α-terpineol being a precursor for oleuropeic acid comes from studies on *Pseudomonas aeruginosa* [97] and *P. incognita* [98] where degradation of the monoterpene resulted in numerous neutral and acidic metabolites, including oleuropeic acid. Evidence from both studies indicated that this formation occurred via oxidation of the allylic methyl of α-terpineol to an hydroxyl terpineol (oleuropeyl alcohol) intermediate which is then further oxidized to the acid (Fig. 3 *b*). It should be noted that biotransformation of α-terpineol has not been shown to produce oleuropeic acid in all systems. For example, feeding of the monoterpene to *Nicotiana tabacum* suspension cells gave rise to hydroxylated products at different positions of α-terpineol, without the formation of oleuropeic acid [102]. Similarly, fungal degradation by *Armillariella mellea* resulted in the hydroxylation products *trans*-sobrerol and 1,2,8-*p*-menthantriol, but not the acid [103].

Oleuropeic acid can also be formed via microbial (Pseudomonad) degradation of α- and β-pinene [104], and interestingly, it can result from microbial fermentation of linalool, the purported precursor of menthiafolic acid. For example, a study on *P. incognita* isolated from soil by an enrichment culture technique and using 1% linalool as the sole carbon source produced *inter alia* hydroxylinalool, menthiafolic acid, and oleuropeic acid [94]. The authors suggested that linalool was metabolized by at least three different pathways of biodegradation. The first is initiated by specific oxygenation of linalool leading to hydroxylinalool, which is further oxidized to menthiafolic acid (Fig. 3 *a*). These reactions are likely catalyzed by cytochrome P-450 monooxygenases. The second pathway involves the degradation of linalool to oleuropeic acid via anchimerically assisted nucleophilic attack of the 2,3-double bond of linalool giving rise to α-terpineol (Fig. 3 b_1). The monocyclic cation produced during the elimination of water from linalool is likely the precursor of all monoterpenes with *p*-menthenoid skeletons [94]. It appears that α-terpineol progressively undergoes oxidation to yield oleuropeic acid as already noted. In support of this pathway as a potential biosynthetic route in plants, the application of ^{14}C-labeled linalool to *Citrus jambhiri* and *C. reticulate* resulted in labeled α-terpineol being detected in leaves and fruit [105]. A third possible degradation pathway in *P. incognita* was proposed by Madyastha et al. [94] which may involve oxidation of linalool before cyclization to yield oleuropeic acid (Fig. 3 *c*). This latter pathway, known as "prototropic cyclisation" [92], was not found in a subsequent study using linalyl acetate as the sole carbon source for *P. incognita* [95]. Instead, linalyl acetate was degraded to linalool (Fig. 3 a_1), which in turn was transformed to either menthiafolic acid via hydroxyl-linalool (Fig. 3 *a*) or oleuropeic acid via α-terpineol (Fig. 3 b_1).

There is a great deal of information on the biosynthesis of the phenolic aglycones present in many of the glucose esters presented here (see [106]). Furthermore, there is much known of the GTs that transfer glucose to phenolics in such compounds as flavonol glycosides (e.g., [107,108]). Nevertheless,

there is little knowledge of the specific GTs involved in esterifying glucose to monoterpene acids. A study on glucosylation of hydroxybenzoic and phenylpropanoic acids in *Arabidopsis* found regioselective glucosylation at the carboxylic acid position was restricted to eight GTs of a single phylogenetic group (group L; [109]). The ability of group L enzymes to recognize carboxyl groups was also reported for terpenoids (e.g., artemisinic and retinoic acids; [110]), but it is not known if they are involved in the biosynthesis of monoterpene acid glucose esters.

CONCLUDING REMARKS

There have been increasing reports of oleuropeic and menthiafolic acid glucose esters in recent years, particularly from plants in the genus *Eucalyptus*. Indeed, over two thirds of known glucose esters of oleuropeic and menthiafolic acids have been described from, or detected in, the genus. Many of the compounds contain phenolic aglycones and all contain one, and often two, α,β-unsaturated carbonyls—chemical constituents that afford a number of important potential therapeutic and biological reactivities. The therapeutic potential of some of the compounds has been examined and it typically relates to functions such as antitumor promotion, carcinogenesis suppression, and also antioxidant, antiinflammatory, and antimicrobial activities. From a biological perspective, the reported cytotoxicity, bitterness, repellency, and antimicrobial properties are suggestive of a role in plant defense against herbivores and pathogens, and recent work suggests they may also be involved in plant metal detoxification mechanisms. In addition, the localization of some of the compounds to the exterior of secretory cavity lumena in *Eucalyptus* suggests they may play a role in protecting secretory cells from the potentially toxic terpenes housed within these structures. Finally, one of the main challenges now is to elucidate the biosynthetic pathways in plants for production and storage of monoterpene acid glucose esters. Biotransformation experiments have provided many clues regarding the intermediate metabolites and the classes of enzymes that catalyze the individual steps. What is now required is to use the abundant genomic, transcriptomic and proteomic data for many species to match individual enzymes, substrates, and products.

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ABBREVIATIONS

DPPH	1,1-diphenyl-2-pycrylhydrazyl
EBV-EA	Epstein-Barr virus early antigen
EGCG	(−)-epigallocatechin gallate

GT	glucosyltransferase
HPLC	high-performance liquid chromatography
IC_{50}	half maximal inhibitory concentration
MIC	minimum inhibitory concentration
MNCC	maximal noncytotoxic concentration
MS–MS	tandem mass spectrometry
TPA	12-*O*-tetradecanoyl-13-*O*-acetylphorbol

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Chapter 13

Pharmacological Effects of *Cordyceps* and Its Bioactive Compounds

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Chapter Outline

INTRODUCTION

The *Cordyceps* genus includes more than 400 species, including *Cordyceps sinensis* and *Cordyceps militaris*. Among them, *C. sinensis* has long been used in traditional Chinese medicine as a tonic food and herbal medicine for various health-related issues, for example, dysfunctions of lung and kidney, impotence, and fatigue [1,2]. The term *Cordyceps* normally refers to the species of *C. sinensis* [2], which is a commonly known as "caterpillar fungi" in English (Chinese-"DongChongXiaCao") and is the result of parasitic relationship of the fungus (*Cordyceps*) and the larvae of a moth caterpillar. As the mycelium extends its growth, the fungal fruiting body grows out of the top of the caterpillar. The fruiting body is dark brown to black in color. The fruiting body and the host caterpillar of *C. sinensis* show similarity in terms of chemical composition, and both are used for medication [3]. There is a dispute whether the

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relation between fungus and the larvae of a caterpillar is parasitic or symbiotic. Some investigators have regarded the relationship as symbiotic with the host, and the connection is mutually beneficial [4]. *C. militaris* is a common substitute for *C. sinensis* and widely distributed species; therefore, proper identification is critical in assigning the biological activity of *C. sinensis* and its products [2,5].

C. sinensis (Berk) Sacc. (Family, Clavicipitaceae) grows at the high elevations, especially in the pasture above 3800 m in the Himalayan region of Nepal, Indian, the Tibetan plateau of China, and the Chinese provinces of Sichuan, Gansu, Hubei, Zhejiang, Shanxi, Guizhou, Qinghai, and Yunnan [6]. Because of centuries of popular use in the traditional Chinese medicines, *C. sinensis* has been extensively investigated for its pharmacological actions, such as antioxidative [7,8], antitumor [9,10], immunomodulatory [11], and antihyperglycemic agent [12,13]. Because of its rarity and increasing demand in world's market due to health beneficial claims, the price of *Cordyceps* skyrocketed to over $75,000/kg in San Francisco and other cities in 2008 [4]. However, there have been concerns over the possibility that the *Cordyceps* products being sold are adulterated/contaminated with heavy metals [14]. To meet the increasing demand for high-quality *Cordyceps*, many companies are now cultivating *Cordyceps* in the laboratory at large scale [15], and several *Cordyceps*-based products are now sold as over-the-counter treatments and/or dietary supplements to protect against aging and cancer and as aphrodisiacs, and immune boosting agents; however, there are little clinical evidences to support the efficacy. Due to the large variation in the quality of cultivated *Cordyceps*, many consumers believe that the medicinal quality of the wild product is better than that of the cultivated type [4]. However, with the new advancements in cultivation technology as well as in quality assessment, cultivated *Cordyceps* can be a realistic substitute for the wild *Cordyceps*.

PHARMACOLOGICAL STUDIES

While a range of traditional medicinal uses of *C. sinensis* have been reported, many of the claims regarding the efficacy and safety lack scientific evaluation. There are a number of comprehensive reviews describing historical and pharmacological functions of *Cordyceps* [2,4,16,17]. Proteins, peptides, amino acids, polyamines, nucleosides, polysaccharides, sterols, and fatty acids have been reported to be present in *Cordyceps*. Natural *C. sinensis* contains more than 7.99% free mannitol and a small amount of glucose [18]. Nucleosides cordycepin, polysaccharides, and sterols are the major active components in *C. sinensis*, which are associated with multiple pharmacological effects, for example, antitumor, antimetastatic, immunomodulatory, antioxidant, anti-inflammatory, hypoglycemic, hyperlipidemic, and antiaging [19].

Aphrodisiac Effects

As mentioned earlier, *C. sinensis* is believed by many to enhance libido and fertility in both sexes. Studies have shown that *C. sinensis* has ability to increase 17 beta estradiol (E2) and testosterone [20,21]. Studies by Chang et al. [22] demonstrated that *Cordyceps* supplemented diets enhance both sperm numbers and sperm motility in rats and that the increased levels of sex hormones may underlie these effects; however, precise mechanism(s) involved in aphrodisiac effects of *Cordyceps* is still unclear.

Anticancer Effects

Cancer is a leading cause of death in both developing and developed countries. Although conventional cancer therapies such as chemotherapy and surgery have been increasingly used to treat several different cancers, the side effects associated with these treatments have been the major concern. In quest of finding natural products with potent anticancer activity, *C. sinensis* has shown great promise. Buenz et al. [5] provide an excellent review on the ability of *C. sinensis* to alter apoptotic homeostasis (ability by which a biological system maintains stability while adjusting to changing condition). A series of studies indicated that water and organic soluble fractions of *C. sinensis* are cytotoxic to cancer cells. For example, fractions obtained from super-critical carbon dioxide extraction of *C. sinensis* displayed strong free radical scavenging activity and inhibition of the growth of colorectal and hepatocellular cancer cells [9].

Rin et al. [23] reported that the hot-water extract of cultured *C. sinensis* mycelium inhibits the metastasis of B16 melanoma cells in mice. These studies also indicated that oral administration (250 mg/kg administration) of the hot-water extract of *C. sinensis* inhibits lung tumor colonies in the models of the experimental metastasis and in spontaneous metastasis.

Extensive investigations have been conducted to define its potential anticancer compound(s).Nucleoside cordycepin is a well-known cytotoxic compound extracted from the mycelium of *C. sinensis* and has significant antitumor effects [24,25]. It enters cells that are undergoing deoxyriboneculeic acid (DNA) replication and damages the DNA, leading to apoptosis, by interruption of nucleolar ribonucleic acid (RNA) synthesis, at least in HeLa cells [26]. Other studies show that cordycepin accumulates in multiple myeloma cells as a triphosphate metabolite 3′-dATP (3′-deoxyadenosine-5′-triphosphate) and subsequently inhibits RNA synthesis and cell growth [27]. Thus, it acts as a nucleoside antimetabolite. The inhibition of nucleic acid methylation may also underlie its cytotoxic effects [28]. The inhibitory effects of cordycepin on RNA synthesis are further supported by other reports [29,30].

It has been suggested that *in vivo* cordycepin effects may be the result of the depletion of cellular ATP pools and the altered ability of 3′-dATP to

substitute for ATP-dependent reactions [31]. Lee and coworkers [32] elucidated the molecular mechanisms for the antitumor effects of cordycepin in bladder cancer cell lines, by demonstrating that cordycepin at a dose of 200 μM (IC50) significantly inhibited the growth of bladder cancer cell lines, 5637 and T-24 due to G2/M-phase arrest involving JNK (c-Jun N-terminal kinases) activation. The anticancer effect of cordycepin has also been studied in oral squamous cancer (OEC-M1) and testicular cancer [24,33]. These reports indicate that cordycepin induces apoptosis in MA-10 mouse Leydig tumor cells through a caspase-9 3, and 7-dependent pathway. Cordycepin has been shown to inhibit the proliferation of human neuroblastoma SK-N-BE(2)-C and human melanoma SK-MEL-2 cells with IC_{50} values of 120 and 80 μM, respectively, causing induction of apoptotic cell death [34]. Taken together, the mechanisms involved in anticancer effects of *C. sinensis* likely include inhibition of RNA synthesis and nucleic acid methylation, induction of tumor cell apoptosis, and regulation of signal pathways.

Glucose Homeostasis

Diabetes is characterized by chronic hyperglycemia that can lead to complications related to cardiovascular disease, renal failure, blindness, and neurological disorders. It is related to either a lack of insulin production by the pancreatic β-cells or the inability of the body to efficiently use the insulin that is produced. In recent years, there has been a growing interest in antidiabetic agents from natural products, in part, because plant sources with ethnomedicinal use appear to have less toxicity and other side effects than synthetic medicines.

In vitro and *in vivo* studies demonstrate that *Cordyceps* can reduce the concentration of sugar in the blood (hypoglycemic). Extract obtained from the fruiting body of *Cordyceps* has been shown to exhibit antihyperglycemic activity in streptozotocin- and nicotinamide-induced diabetic rats [12], and fermented mycelia and broth of *C. sinensis* have antihyperglycemic properties similar to the fruiting bodies (Hsu et al., 2006). It is interesting to note that metals like vanadium have the ability to mimic the action of insulin, and recent studies have shown that the complex enriched of *Cordyceps* vanadium complex is beneficial in the treatment of both diabetes and depression [35].

Polysaccharides extracted from cultured mycelium of *Cordyceps* have been shown to have potent hypoglycemic activity when given intraperitoneally in genetically diabetic mice [12]. A similar effect was observed when the neutral polysaccharide (CS-F30) extracted from mycelium of *C. sinensis*, which significantly decreased the plasma glucose in normal mice after oral administration [36]. In addition to polysaccharides, other metabolites of *Cordyceps* such as adenosine, cordycepin, and amino acids may modify glucose metabolism. While *Cordyceps* polysaccharides and other components have shown to have a great potential in lowering blood glucose levels in diabetes,

Cordyceps supplementation does not appear to significantly lower the circulating lipids (hypolipidemic) [12].

Immunomodulatory Effects

The immune system of the body provides defense against infectious microorganisms. The immunostimulatory effects of various extracts (petroleum ether, ethyl acetate, and ethanol and glycoprotein) of *C. sinensis* have been studied in mice [37,38]. A polysaccharide from *C. sinensis* with the average molecular weight of 83 kDa significantly enhanced the serum IgG, IgG1, and IgG2 levels [37,38]. Therefore, polysaccharides from *Cordyceps* have attracted significant interest for their potential benefits in several health complications, including cancers. With regard to immune activity, different fractions of *C. sinensis* polysaccharides enhance the phagocytic function of monocytes and macrophages [39] and increase the mass of thymus and the spleen in mice that are dexamethasone inhibited. Reports from Kuo et al. [40] indicate that immunostimulatory components of submerged cultured *C. sinensis* reside in the culture filtrate. Cordysinocan, a novel polysaccharide from cultured *C. sinensis*, stimulates cultured T-lymphocytes and macrophages [41].

Similarly, a polysaccharide of molecular weight ~210 kDa isolated from cultured *Cordyceps* mycelia is a strong antioxidative [42]. Pretreatment with this polysaccharide on the cultured rat pheochromocytoma PC12 cells led to significant protection against hydrogen peroxide (H_2O_2)-induced insult. Another line of evidence indicates that the soluble polysaccharide from *C. sinensis* fraction (CS-Ps) greatly enhances TNF-α production [43]. Structurally, CS-Pp appears to be 1,3-beta-D-glucan containing some 1,6 branched chains. Taken together, these findings suggest that *Cordyceps* polysaccharides play active role in immunomodulatory activity.

In addition to polysaccharides, small molecule cordycepin and adenosine regulate the phenotypic switch on macrophages and they may potentially be used as immunomodulatory agents in the treatment of inflammatory disease [44]. Since nucleosides are basic building blocks of nucleic acids RNA and DNA well known for their effects in immune and central nervous systems, cordycepin and its analogs would be expected to affect immunomodulation. Studies have shown that cordycepin inhibits the production of nitric oxide by downregulating inducible nitric oxide synthase and cyclooxygenase-2 gene expression via the suppression of NF-κB activation, and Akt and p38 phosphorylation [45].

Type 1 diabetes is an autoimmune disease (the condition when antibodies are produced against tissues of our own body) that is caused by the destruction of insulin-producing beta-cells. Beta-cell interleukin 10 (IL-10) is an immunoregulatory cytokine that plays an important role in both inflammatory and immune reactions [46]. There is a great deal of interest in identifying natural products which upregulate IL-10 and cordycepin, which, in turn, appears to

upregulate IL-10 production and IL-10 mRNA expression in human peripheral blood mononuclear cells [47].

Nephroprotective Effects

Chronic kidney diseases are increasingly recognized as a major public health concern that affects the quality of life. *Cordyceps* has been considered to be beneficial for innate vigor or reducing symptoms of aging in traditional Chinese medicine. A clinical study of *C. sinensis* on T-lymphocyte subsets in chronic kidney failure patients suggests that administration of *C. sinensis* improves renal and immune functions in chronic renal failure patients [48]. A polysaccharide (CPS-2) from *C. sinensis* was tested for its effects on a model of chronic renal failure, and it protected from renal injuries, improving blood urea nitrogen and serum creatinine levels [49].

The mouse model (MRL−1pr/1pr) develops a severe autoimmune lupus syndrome characterized by strong autoantibody production and severe lymphoproliferation. This model was used to evaluate the autoimmune inhibitory effects of a pure compound (H1-A) from *C. sinensis* and showed that H1-A (40 μg/day orally) for 8 weeks could improve renal function [50]. A number of studies have demonstrated that *C. sinensis* is beneficial in ameliorating aminoglycoside-induced nephrotoxicity [51,52]. Aminoglycosides are used to treat bacterial infection; however, they may cause kidney damage. Studies on nephroprotective effects of *C. sinensis* on aminoglycosides in elderly patients suggested that the patients receiving *C. sinensis* display less nephrotoxicity [51]. Also, a clinical study showed that *Cordyceps* treatment is useful for renal transplantation recipients, especially in immunoregulation after organ transplantation [53]. Another clinical study reported that *Cordyceps* could be used as adjunctive therapy in clinical renal transplantation [54].

ACTIVE COMPONENTS

Nucleosides

Nucleosides are glycosylamine consisting of a nitrogenous base (a purine or pyrimidine) bound to a ribose or deoxysugar. Cordycepin ($C_{10}H_{13}N_5O_3$; molecular weight 251 Da), structurally very similar to adenosine (except for the absence of 3′-OH of its ribose moiety; Fig. 1), is one of the major bioactive nucleosides from *Cordyceps* (Fig. 2). It is now available commercially. Several studies have used high-performance liquid chromatography (HPLC) coupled with diode array detection and liquid chromatography–mass spectrometry (LC–MS) for quantitative and quantitative analyses of nucleosides in natural and cultured *C. sinensis* [1,49,55–58]. Quantitative analysis of 13 nucleosides and bases (cytosine, uracil, cytidine, hypoxanthine, guanine, uridine, thymine, adenosine, inosine, guanosine, thymidine, adenosine, and cordycepin; Fig. 2)

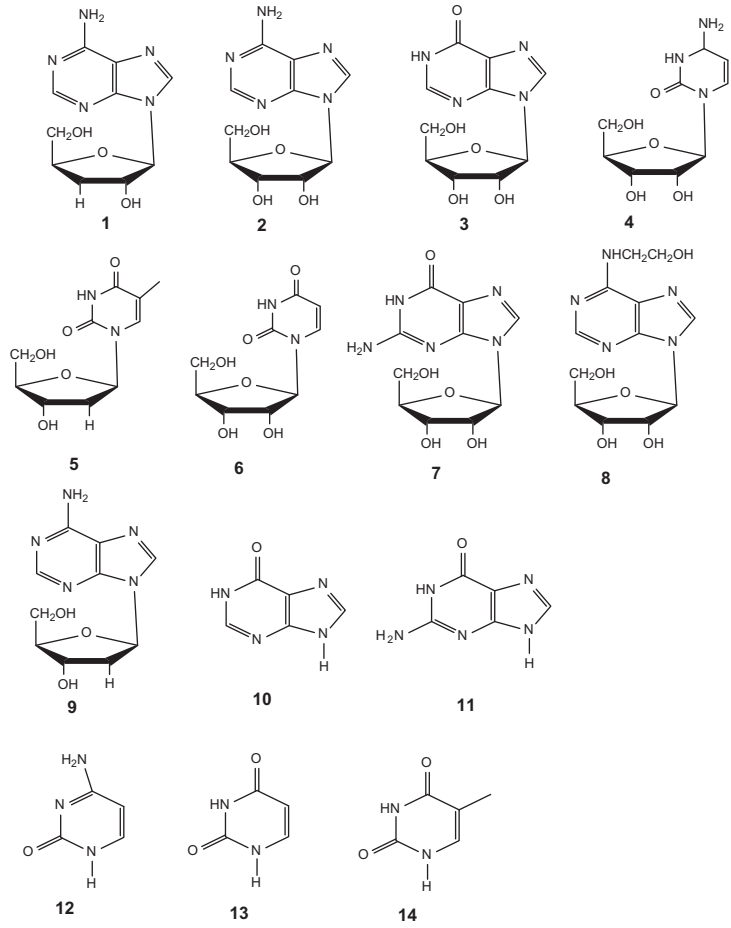

FIGURE 1 Nucleosides and their bases from Cordyceps. Cordycepin (**1**), adenosine (**2**), inosine (**3**), uridine (**4**), thymidine (**5**), cytidine (**6**), guanosine (**7**), 6-hydroxyethyladenosine (**8**), 2′-deoxyadenosine (**9**), hypoxanthine (**10**), guanine (**11**), cytosine (**12**), uracil (**13**), thymine (**14**).

from *C. sinensis* (natural and cultured) and cultured *C. militaris* indicates that cordycepin readily available (36.3–57.1 μg/g) in naturally grown *C. sinensis* [55]; however, cultured *C. militaris* contains a much higher level of cordycepin (1629–12,761 μg/g) than cultured *C. sinensis* (2.8 μg/g).

Results from these studies indicate that the amounts of nucleosides, for example, uridine, adenosine, and guanosine, in cultured *C. sinensis* are higher than those of naturally grown *C. sinensis*. However, cordycepin and inosine are higher in natural (compared to cultured) *C. sinensis* (Fig. 2). Extensive chemical investigation for anti-inflammatory principles of the mycelium of cultivated *C. sinensis* identified 50 compounds, including 5 new compounds, cordysinins A–E [59].

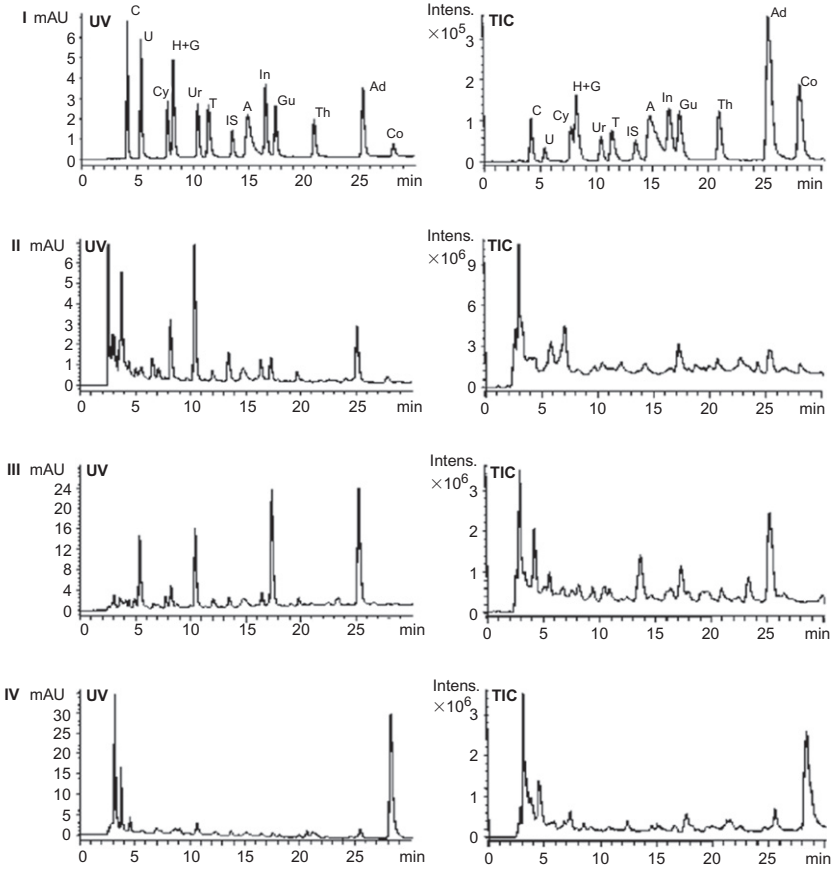

FIGURE 2 HPLC-UV MS chromatogram of (**I**) mixture of authentic standards, (**II**) natural, *C. sinensis*, (**III**) cultured *C. sinensis*, and (**IV**) cultured *C. militaris*. The representative samples of natural and cultured *C. sinensis* and cultured *C. militaris* were Tobet 2, wanfeng and HKUST, respectively. C. cytosine; U, uracil; Cy, cytidine; H, hypoxanthine; G, guanine; Ur, uridine; T, thymine; IS, 2′-deoxyuridine (internal standard); A, adenine; In, inosine; Gu, guanosine; Th, thymidine; Ad, adenosine; Co, cordycepin. *Reprinted with permission from Ref. [55], published by Elsevier.*

Mass spectrometry and NMR analyses of cordysinin A indicate that it is cyclo (L-leucine-L-hydroxyproline) (Fig. 3). Cordysinin B is an adenosine derivative, only differing from adenosine at 2′ position with methoxyl substitution. Cordysinins C and D are enantiomeric mixture and structurally are 1-(9H-β-carbolin-1-yl) ethanol. Cordysinin E is an analog of cordysinins C and D with the presence of a —$CH_2CHOHCH_2OH$ at C-1 position ([59]; Fig. 3). Other interesting adenosine analogs such as 2′-deoxyadenosine and 6-hydroxyethyladenosine have also been identified from *C. sinensis* (Fig. 1; [55]). Cordycepin of *Cordyceps* has a large spectrum of biological activities, including antiproliferative, proapoptotic, and anti-inflammatory effects [30].

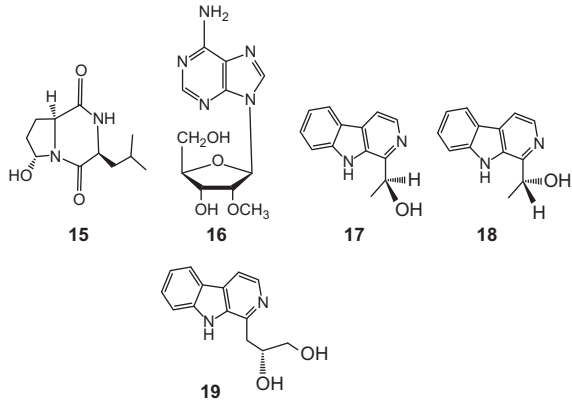

FIGURE 3 Structures of cordysinins A–E (**15–19**) identified from *C. sinensis* [59].

Polysaccharides

Polysaccharides constitute major bioactive compounds of *C. sinensis*. A series of polysaccharides have been isolated and characterized from the fruiting body and mycelium of *Cordyceps*. Zhong et al. [39] provides a comprehensive review on production, isolation, and purification of *Cordyceps* polysaccharides.

Cordyceps polysaccharides have been investigated for their hypoglycemic, hypolipidemic, and anticancer properties. The anticancer properties of the polysaccharide are associated with their role in immunomodulatory activity. The following are polysaccharides that are from *C. sinensis*.

CS-F10 is a polysaccharide isolated from a hot-water extract of *C. sinensis* and composed of galactose, glucose, and mannose in a molar ration of 43:33:24 with molecular weight about 15,000 Da [60]. It has alpha-D-glucopyranosyl residue on the terminal of the side-chain and 1,5-linked beta-D-galactofuranosyl residue. *CS-F30* is another polysaccharide ([alpha] D + 21 degrees in water) composed of galactose, glucose, and mannose (molar percent, 62:28:10), and its molecular weight is about 45,000 Da [36].

CPS-2 is a water-soluble polysaccharide obtained from the cultured *C. sinensis* extract and consists of a-(1–4)-D-glucose and a-(1–3)-D-mannose branched with molecular weight of 4.39×10^4 Da mostly consisting of α-(1→4)-D-glucose and α-(1→3)-D-mannose, branched with α-(1→4,6)-D-glucose every 12 residues on average [61].

CME-1 is also a water-soluble polysaccharide from mycelia of *C. sinensis* with molecular weight 27.6 Da containing of mannose and galactose in a respective ratio of 4:6 [62].

CS-PS was isolated by Zhang and coworkers [63], who characterized this polysaccharide from cultured *C. sinensis*. It is composed of mannose, rhamnose, arabinose, xylose, glucose, and galactose. The average molecular weight of CS-PS is 12 kDa.

Cordysinocan is a novel polysaccharide of molecular weight approximately 82 kDa that was isolated from the conditioned medium of cultured *Cordyceps*. It contains glucose, mannose, and galactose in a ratio of 2.4:2:1 and induces the cell proliferation and the secretion of interleukin-2, interleukin-6, and interleukin-8 [41].

SCP-1 was isolated from the mycelium of *C. sinensis* [37,38]. Based on NMR and IR spectra, it consists of a backbone composed of (1→4)-D-glucosyl residues and carries a single (1→6)-linked D-glucosyl residue, α-D-glucosidic linkages.

An acid polysaccharide was isolated by ion-exchange and sizing chromatography, from cultured *Cordyceps* mycelia. This polysaccharide was composed of mannose, glucose, and galactose in an approximate molar ratio of 3.3:2.3:1 and showed protective effects on hydrogen peroxide-induced damage in PC12 cells [64].

PS is a polysaccharide fraction from the edible mycelia of *C. sinensis* that has been isolated by Sephadex G-100 chromatography, an ion-exchange resin eluted with 0.2 M NaCl resulted *PS* with the average molecular weight 8.3×10^4 Da [37,38]. It displays d-Glc, d-Man, l-Ara, and d-Gal in a molar ratio of 8:90:1:1.

CS-Pp is a 1,3-beta-D-glucan isolated from cultured mycelia of *C. sinensis* [43].

EPS is a polysaccharide from cultivated *C. sinensis*, consisting of mannose, glucose, and galactose in a ratio of 23:1:2.6. Its molecular weight is about 1.04×10^5 Da [65]. In addition to these, a polysaccharide of molecular weight ~210 kDa with strong antioxidative activity was isolated from cultured *Cordyceps* mycelia by ion-exchange and sizing chromatography [42].

Cyclopeptides has been considered in only a few reports. A cyclopeptide–cordyheptapeptide A with antimalarial and cytotoxic activities was isolated from the insect pathogenic fungus *cordyceps* sp. BCC 1788 and characterized by HPLC, high-resolution mass spectrometry, NMR, and X-ray diffraction analyses [66].

Similarly, a phytochemical investigation of the crude extract of the fungus *Isaria farinosa* that colonizes *C. sinensis* led to the isolation of cycloaspeptides F and G, two new cyclic pentapeptides, the known cycloaspeptides A and C, and bisdethiodi(methylthio)hyalodendrin [67].

Steroids

A number of bioactive steroids have been isolated from *C. sinensis*. Two antitumor sterols 5α,8α-epidioxy-24(R)-methylcholesta-6,22-dien-3β-D-glucopyranoside (**20**) and 5,6-epoxy-24(R)-methylcholesta-7,22-dien-3β-ol (**21**) together with previously known ergosteryl-3-*O*-β-D-glucopyranoside (**22**) and 22-dihydroergosteryl-3-*O*-β-D-glucopyranoside (**23**) (Fig. 4) were isolated and characterized from the methanol extract of *C. sinensis* [68,69]. Similarly, Matsuda and coworkers

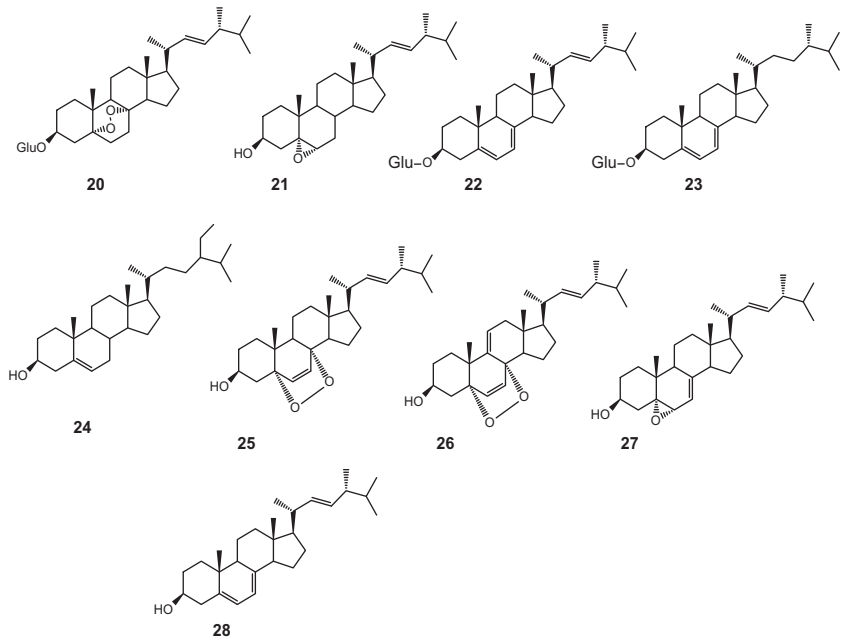

FIGURE 4 Structures of sterols isolated from *C. sinensis*. 5a,8a-epidioxy-24(R)-methylcholesta-6,22-dien-3-D-glucopyranoside (**20**), 5a,6a-epoxy-24(R)-methylcholesta-7,22-dien-3-ol (**21**), ergosteryl-3-*O*-D-glucopyranoside (**22**), 22-dihydroergosteryl-3-*O*-β-D-glucopyranoside (**23**), sitosterol (**24**), 5a,8a-epidioxy-22E-ergosta-6,22-dien-3-ol (**25**), 5a,8a-epidioxy-22E-ergosta-6,9,22-trien-3-ol (**26**), 5a,6a-epoxy-5-ergosta-7,22-dien-3-ol (**27**), ergosterol (**28**). Glu, glucose.

identified five apoptosis-inducing sterols, namely, sitosterol (**24**), 5α,8α-epidioxy-22E-ergosta-6,22-dien-3β-ol (**25**), 5α,8α-epidioxy-22E-ergosta-6,9,22-trien-3β-ol (**26**), 5α,6α-epoxy-5α-ergosta-7,22-dien-3β-ol (**27**), and ergosterol (**28**) from the ethyl acetate fraction of *C. sinensis* [70].

Ten Fatty acids, namely, lauric acid, myristic acid, pentadecanoic acid, palmitoleic acid, palmitic acid, linoleic acid, oleic acid, stearic acid, docosanoic acid, and lignoceric acid and four free sterols including ergosterol, cholesterol, campesterol, and beta-sitosterol in natural (wild) have been identified from *C. sinensis* using gas chromatography–mass spectrometry [71]. The unsaturated fatty acids constitute 57.84%; linoleic acid being the highest (38.44%) [2].

Although a large number of bioactive secondary metabolites have been isolated and characterized from *Cordyceps*, this fungus may contain unidentified bioactive metabolites.

Cordycepin Pharmacokinetics

Being adenosine analogue, its metabolism and pharmacokinetic profile is similar to that of adenosine [72]. It is quickly deaminated by adenosine deaminase

and rapidly metabolized to an inactive metabolite, 3′-deoxyhypoxanthinosine, *in vivo* [73]. Pharmacokinetic studies of adenosine and cordycepin indicated that in rats they have short elimination half-lives (10.4 ± 0.9 and 1.6 ± 0.0 min, respectively) at a dose of 10 mg/kg i.v. [72]. In this study, the area under the curve, maximum concentration observed, and clearance of cordycepin were determined to be 38.5 ± 10.3 min μg/mL, 3.1 ± 0.9 μg/mL, and 2.1 ± 1.2 L/min/kg, respectively. An unidentified HPLC peak was observed in the blood and liver samples after 20–30 min cordycepin administration, but not in the case of adenosine treatment, indicating it could be a metabolite of cordycepin [72].

QUALITY CONTROL

Since there are several mycelial strains of *Cordyceps*, proper identification of *Cordyceps* species is the first important step in its quality control. It is also critical to confirm that biological activities of extracts and pure compounds are from the *Cordyceps* of interest, not from other fugal contaminant [16]. As alluded to above, *Cordyceps* is a rare natural product with very high-market price, and thus the possibility of selling adulterated *C. sinensis* products is very high. Two cases of lead poisoning caused by contaminated *Cordyceps* have been reported, and the lead content in the *Cordyceps* powder has been found to be as high as 20,000 ppm [74] in commercial products. In addition, variation in concentrations of major active components of *Cordyceps* collected from different places is also an important issue. Reports have shown that nucleosides concentrations in wild versus cultivated *C. sinensis* are different [55]. These factors contribute to poor quantitative data of *C. sinensis*-based products, and consequently, there exist a large variability observed for oral intake level of *C. sinensis*, and its safety and efficacy. This underscores the need for qualitative and quantitative determination of the active compounds in *C. sinensis*. For this, development of sensitive and specific analytical methods is needed. HPLC and liquid chromatography tandem mass spectrometry (LC–MS/MS) have been extensively used for quality control of herbal products [75–77]. HPLC is very sensitive and used for identification, authentication, and quantification of compounds of interest. HPLC with diode array detection had been used for the quality evaluation of *Cordyceps* [49,78,79]. For greater sensitivity and specificity, LC–MS has been used for the analysis of main nucleosides in *Cordyceps* that obtained from different sources [57,58,80]. In LC–MS, selected ion monitoring, and LC–MS/MS, selected reaction monitoring (SRM) can be used for quantitative analysis. LC–MS/MS operated in SRM offers tremendous sensitive to quantify as low as 4.64 ng/mL of cordycepin in *Cordyceps* [55]. Since the difference in nucleoside levels has been used to distinguish between *C. sinensis* and *C. militaris*, precise quantitative analysis of nucleosides would be useful for the study and quality control of *C. sinensis* versus other species [57]. Furthermore, Yu and coworkers reported

hierarchical clustering analysis based on the typical peaks of adenosine, cordycepin, and inosine in HPLC profiles from the 11 tested samples and concluded that natural and cultured *Cordyceps* were in different clusters, which could provide a means of discriminating between *Cordyceps* of different origins [78]. Results also showed that adenosine, cordycepin, and inosine could be used as markers for quality control of *Cordyceps* [78]. Other investigations on nucleoside analysis also indicate that cultured *C. militaris* contains a much higher level of cordycepin than *C. sinensis* [55].

Information on chemical composition of small molecules other than nucleosides can also be utilized in the quality control of *Cordyceps* being traded. For example, palmitic acid, linoleic acid, oleic acid, stearic acid, and ergosterol are main components in both natural and cultured *Cordyceps*, but the contents of palmitic acid and oleic acid in natural *Cordyceps* are significantly higher than those in the cultured ones [71]. Taken together, the metabolite profile (nucleosides, nitrogen bases, steroids, and free fatty acids) may be useful for the quality control of *C. sinensis* products.

CONCLUSIONS

Throughout the past two decades, research on health effects of *Cordyceps* and its compounds has been conducted at a brisk pace. An increasing number of publications on *Cordyceps* and its active compounds clearly reflect the broad attention that this medicinal product has attracted, primarily due to its health benefits, based on results obtained from *in vitro* and animal experiments, and a few clinical trials [4]. There are many challenges associated with the use and health claims of *Cordyceps*, for example, proper identification of *Cordyceps* and many conflicting reports of pharmacological function in the literature [5]. Generally, *Cordyceps* is considered safe; however, critical toxicological studies have not been reported. *Cordyceps* may be useful for aging, renal, and hepatic functions. It has a potential to be used as adjunctive therapy in diabetes, cancer, and renal failure, but there is a lack of double blind, randomized placebo-control clinical studies to demonstrate its safety and efficacy [17]. Further well-defined clinical trials are needed to test *Cordyceps*'s health benefits. Just as importantly for the safety of users, the therapeutic value of this fungal product must rest on properly characterized products with defined chemical fingerprinting.

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Chapter 14

Silymarin Flavonolignans: Structure–Activity Relationship and Biosynthesis

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Chapter Outline

INTRODUCTION

Silybum marianum (L.) Gaertn (Asteraceae) is an annual herb native to the Mediterranean and North African regions [1]. It grows wild throughout Europe, North Africa, the Americas, and Australia [2]. The plant has many common names such as milk thistle, Marian Thistle, Mary Thistle, blessed milk thistle, and mariendistel. The fruits of this plant (Fig. 1) contain a mixture of flavonolignans collectively known as silymarin. Most research and applications on silymarin is directed toward disorders of the liver [3–5]. Silymarin is one of the most well-studied plant extracts with known mechanism of action for treatment of liver diseases. The annual consumption of silymarin for treatment of liver diseases in the Egyptian market is 40–50 tons. This quantity is imported to an annual rate of 75 million Egyptian pounds [6].

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FIGURE 1 *Silybum marianum* fruit head containing the medicinally active fruits.

FIGURE 2 *Silybum marianum* fruits at different stages of maturity.

In the early 1990s, several reports showed that milk thistle is a potential cancer chemopreventive agent. Silymarin has shown efficacy in arresting human prostate carcinoma proliferation in cancer cell cultures [7] and in human cancer models [8].

S. marianum fruits vary from milky white to nearly black as they develop toward maturity (Fig. 2). There are about 42 fruits/g. A fruit head can produce from 100 to 190 fruits. Silymarin content in the fruits varies between 1.5–3%. Fruits containing 3–6% are considered of high quality. Extract prepared from the fruits is standardized, so the silymarin concentration is 70–80% of the extract weight.

CHEMISTRY AND COMPOSITION OF MILK THISTLE EXTRACT

Most plant extracts contain several bioactive compounds yet are usually only standardized for one compound or a few compounds with similar spectroscopic characteristics. Milk thistle ingredients are described using different terms in the

literature [9]. Milk thistle extract is the early extract of crushed milk thistle fruits. It contains 65–80% silymarin and 20–35% fatty acids such as linoleic acid. The term *silymarin* was introduced in 1968 by the German phytochemist Hildebert Wagner [10] who described the mixture of flavonolignans that had been characterized at the time (silybin, isosilybin, silydianin, and silychristin). This mixture was resolved into seven flavonolignans and one flavonoid (Fig. 3) that comprise 65–80% of milk thistle extract. What was first termed silybin can be resolved into two diastereoisomers, silybin A and silybin B.

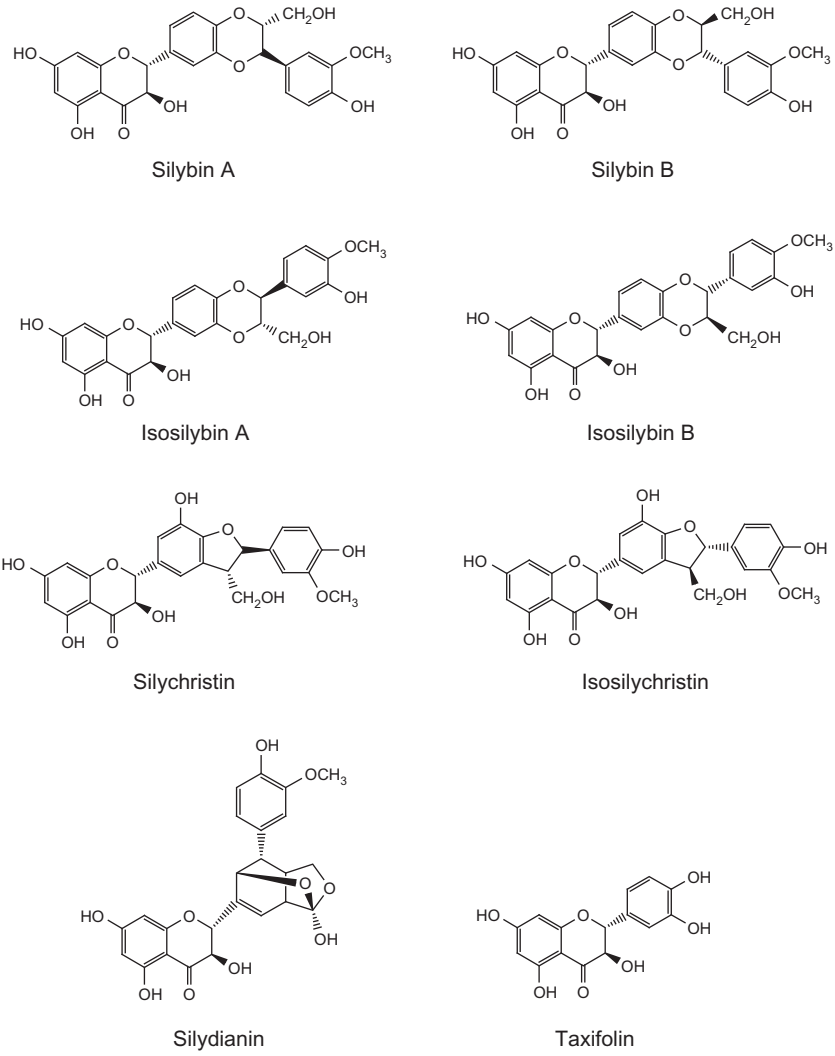


FIGURE 3 Chemical structures of flavonolignans and flavonoid comprising silymarin.

A similar diastereoisomeric mixture exists, yielding isosilybin A and isosilybin B. These are regioisomers of silybin A and silybin B. The three other flavonolignans, which are structural isomers of the earlier mentioned compounds, are silychristin, isosilychristin, and silydianin. Silibinin is a semipurified commercially available fraction of silymarin. It is a roughly 1:1 mixture of the two diastereoisomeric compounds, silybin A and silybin B. Isosilybinin, a semipurified fraction of silymarin, contains a roughly 1:1 mixture of the two diastereoisomeric compounds, isosilybin A and isosilybin B. Milk thistle extract proved useful in preclinical studies. This required complete and precise chemical characterization of the extract for progression to the clinical studies. Milligram quantities of all milk thistle flavonolignan compounds were purified for biological studies [11].

STEREOCHEMISTRY OF FLAVONOLIGNANS

Silybin A, silybin B, isosilybin A, and isosilybin B have similar ^{1}H and ^{13}C NMR spectra and no characteristic signals for easy identification of individual isomers. Reversed-phase HPLC showed significant difference in retention times for these isomers. These diastereoisomeric pairs were separated by sequential silica gel column chromatography, preparative reversed-phase HPLC, and recrystallization from CH_2Cl_2—MeOH [12]. This allowed isolation of pure diastereoisomers. The ^{1}H NMR spectrum of silybin A confirmed the *trans* conformation of H-2, H-3, and H-7′–H-8′ from the observed coupling constants (11.4 and 8.1 Hz). The stereochemistry at 2*R* and 3*R* was determined by dehydrogenation of silybin [13]. The stereochemistry of C-7′ and C-8′ was deduced as 7′*R* and 8′*R* by comparison of the optical rotation with that of isosilybin A, which was determined by X-ray crystallography. The ^{1}H NMR spectrum of isosilybin B is very similar to that of silybin A with less than 0.01 ppm differences in chemical shifts. The ^{1}H NMR spectrum of a mixture of silybin A and silybin B looks like that of one compound. The difference in chemical shifts in ^{13}C NMR spectra of silybin A and silybin B is not greater than 0.06 ppm. The 7′*S* and 8′*S* configurations were assigned based on the differences in optical rotation between silybin A ($[\alpha]_D + 20°$) and silybin B ($[\alpha]_D - 1.07°$). The stereochemistry of isosilybin A was assigned by X-ray crystallography in combination with optical rotation data as 2*R*, 3*R*, 7′*R*, and 8′*R*. The significant differences in optical rotation between isosilybin A ($[\alpha]_D + 48.15°$) and isosilybin B ($[\alpha]_D - 23.55°$) confirmed the configuration 7′*S* and 8′*S*.

Isosilybin C and isosilybin D (Fig. 4) were isolated from milk thistle extract as minor compounds. These two compounds have regiochemistry similar to that of isosilybin A and isosilybin B. The major structural difference in these compounds from other flavonolignans is 1,3,5-substitution pattern in the lignan-derived aromatic ring.

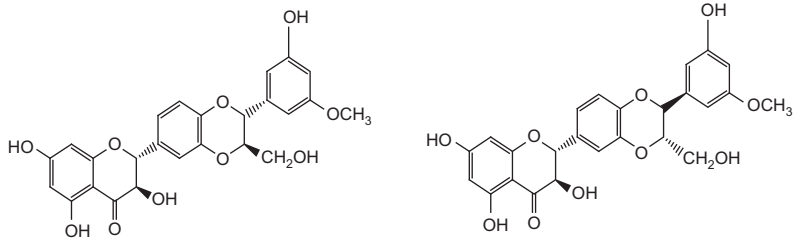


FIGURE 4 Chemical structures of minor flavonolignans in silymarin.

BIOSYNTHESIS OF SILYMARIN FLAVONOLIGNANS

Flavonolignans are produced by oxidative coupling between a flavonoid (taxifolin) and a phenylpropanoid, usually coniferyl alcohol (Fig. 5) [14]. Flavonoids are produced by the enzyme chalcone synthase that catalyzes the addition of a cinnamoyl-CoA unit to three malonyl-CoA units and subsequent cyclization giving chalcones. Chalcones are precursors for a wide range of flavonoid derivatives found in plants. The resulting compound contains a six-membered heterocyclic ring, formed by Michael-type nucleophilic attack of a phenyl group on the unsaturated ketone giving a flavanone. Hydroxylation in the two aromatic rings occurs at the flavanone or dihydroflavonol stage. Coniferyl alcohol is derived by reduction of cinnamic acid by NADPH-dependent reaction using coenzyme A. This is followed by aromatic hydroxylation and methylation.

The oxidative coupling reaction between taxifolin and coniferyl alcohol is mediated by free radical formation and catalyzed by peroxidase enzyme that is known to be radical generator (Fig. 6). Unfortunately, the enzyme catalyzing the oxidative coupling of flavonolignans has not yet been characterized. Mechanistically, the reaction proceeds by one-electron oxidation of the dihydroflavonol (+)-taxifolin to provide phenoxy radical [15]. This free radical couples with quinone methide radical generated from coniferyl alcohol to produce an adduct in a reaction that is neither regio- nor enantioselective. The final step of biosynthesis is an intramolecular nucleophilic attack of the hydroxyl group on the quinone methide ring to produce silybins and isosilybins (Fig. 6). Silychristin and silydianin are derived from a mesomer of the taxifolin-derived free radical.

Silandrin and isosilandrin (Fig. 7) were isolated from white-flowered *S. marianum*, in which the flavonolignan constituents markedly differ from purple-flowered type [16–18]. HPLC analysis of the methanol extract of the white-flowered *S. marianum* has allowed separation of diastereoisomers of silandrin and isosilandrin. Besides, *cis*-isomers of silandrin A and silandrin B were isolated and named *cis*-silandrin and isocissilandrin (Fig. 7). The presence of *cis*-stereoisomers clearly showed that a quinine methide intermediate

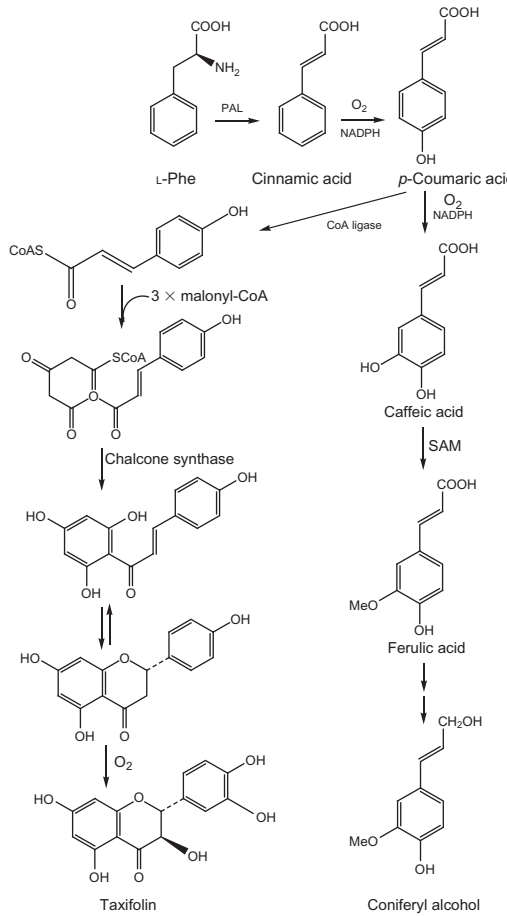


FIGURE 5 Formation of flavonolignan precursors. PAL, phenylalanine ammonia lyase.

is formed in the biosynthetic pathway, leading to flavonolignan formation in *S. marianum*. These results clearly showed that the *O*-β coupling step of flavonolignan biosynthesis in *S. marianum* is neither regio- nor enantioselective.

REGULATION OF FLAVONOLIGNAN METABOLIC PATHWAY

Silymarin flavonolignans are constitutive compounds that accumulate in the pericarp of the fruits of *S. marianum*. Production of these compounds is reduced in cell, tissue, and organ cultures of this species [19,20]. Many approaches have been used in increased yield of flavonolignans *in vitro* such as change in media composition [21], precursor feeding [22], and elicitation [23]. Elicitation has proven to be an effective approach for enhancement of accumulation of secondary metabolites and for a better understanding of their

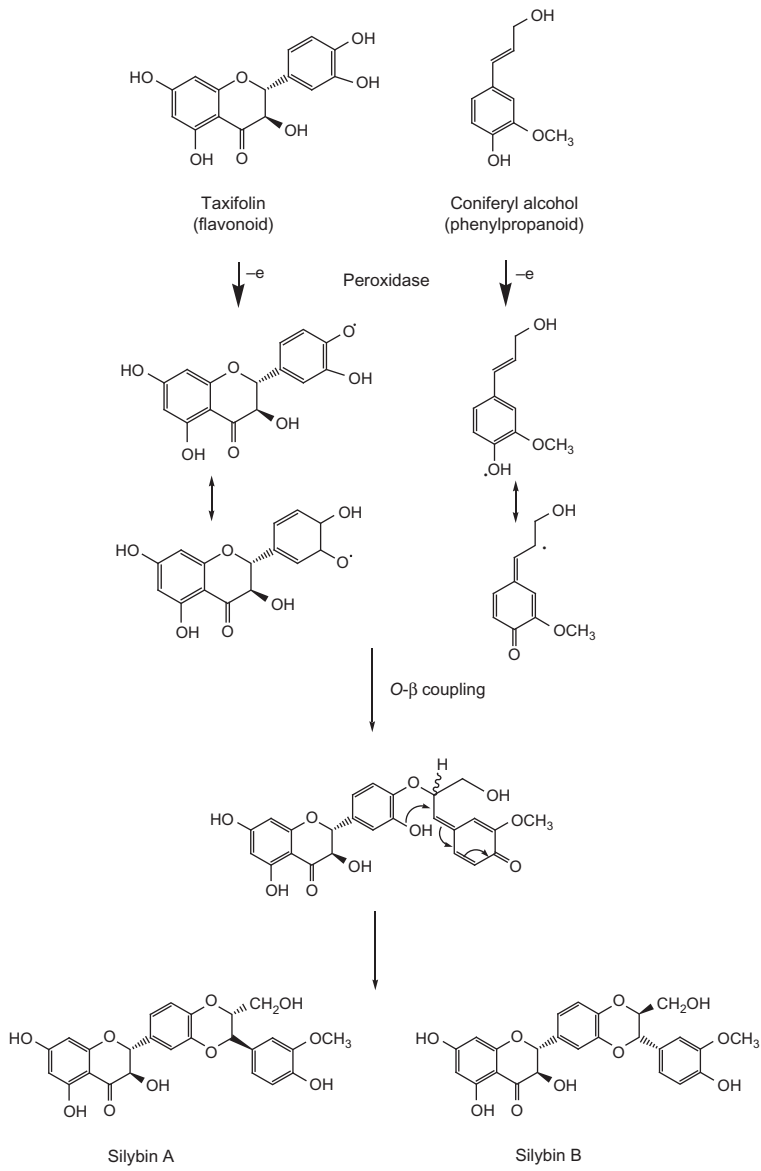


FIGURE 6 Biosynthesis of silybins from taxifolin and coniferyl alcohol.

biosynthesis. Elicitors can either be physical or chemical, biotic or abiotic agents. For example, Ag^{+} in low concentrations can increase silymarin production in cell suspension and hairy root cultures of *S. marianum* [24,25]. Yeast extract and methyl jasmonate promoted the accumulation of silymarin in cell and root and hairy root cultures [26–29]. Time course for production

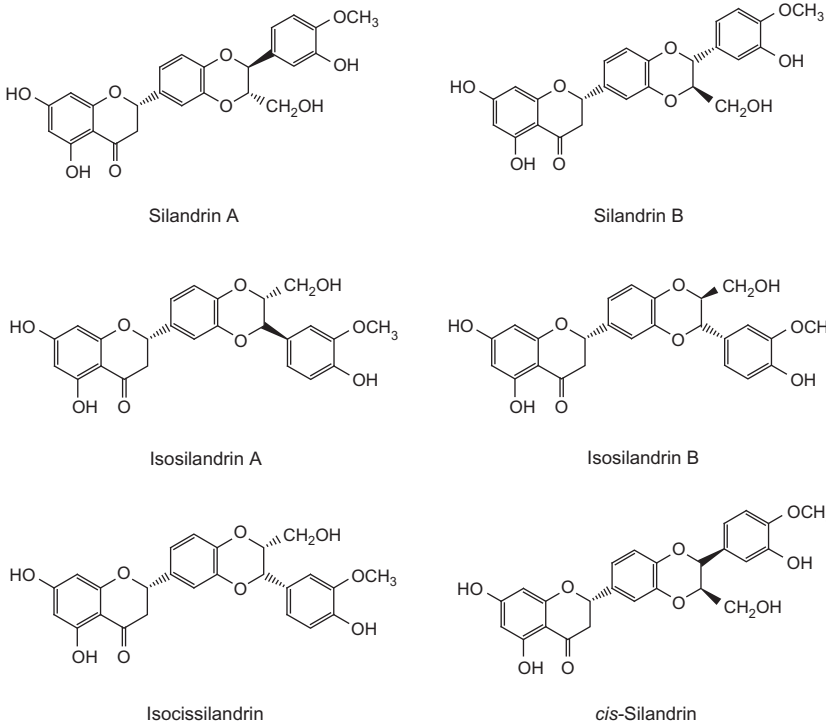
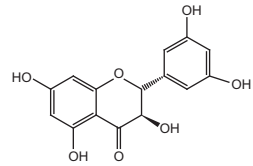


FIGURE 7 Chemical structures of flavonolignans isolated from white-flowered *Silybum marianum*.

of silymarin in cell cultures of *S. marianum* treated with 50 μg/ml medium of yeast extract was studied [26]. Signal transduction pathway involved in elicitor-induced silymarin accumulation was investigated. Elicitation with 100 μM methyl jasmonate resulted in a significant and strong accumulation of silymarin in cells (10-fold compared to elicitation with yeast extract). Methyl jasmonate was nontoxic in a wide range of concentrations, and its elicitor effect lasted for a long time. Methyl jasmonate induced synthesis of silymarin by acting in the early steps in the pathway. Activation of synthesis of cinnamoyl CoA esters contributed to the elicitation. Chalcone synthase, a key enzyme in pathway leading to flavonoid biosynthesis, is not crucial in the silymarin elicitation. This was concluded when the increase in activity of chalcone synthase by methyl jasmonate was not dependent on *de novo* protein synthesis.

S. marianum root culture was used as an experimental system to study methyl jasmonate elicitation effects [28]. Production of silychristin, silydianin, and taxifolin was enhanced by methyl jasmonate addition. No silybin or isosilybin could be detected after elicitation of the cultured roots with methyl jasmonate. 3,3′,5,5′,7-Pentahydroxyflavanone (Fig. 8) was identified among the compounds enhanced by methyl jasmonate addition. This suggests



3,3′,5,5′,7-pentahydroxyflavanone

FIGURE 8 Chemical structure of flavonoid elicited by methyl jasmonate in root culture of *Silybum marianum*.

that flavonoid biosynthesis, not coniferyl alcohol biosynthesis, may be the candidate component of the signaling pathway involved in enhancement of flavonolignan production with methyl jasmonate.

Elicitor stimulation for silymarin production was studied in hairy roots of *S. marianum* [30]. Yeast extract has increased production of silymarin to a level about two fold higher than that of the control in hairy roots. Jasmonate signaling pathway was hypothesized to be involved in the yeast extract-induced production of silymarin. This occurs by increase of lipoxygenase activity and linoleic acid content [31]. In the referred study, it was also suggested that yeast extract induces the production of hydrogen peroxides and other reactive oxygen species.

One- and two-dimensional nuclear magnetic resonance spectroscopy was used for metabolic profiling of *S. marianum* cell cultures elicited with yeast extract or methyl jasmonate [32]. This revealed changes in the phenylpropanoid production after elicitation with methyl jasmonate or yeast extract. The profile of phenylpropanoids was different in methyl jasmonate- and yeast extract-treated cultures, showing that phenylpropanoid metabolism was altered by elicitation but different phenylpropanoid is produced according to the elicitor used. Though this result clearly shows the activation of phenylpropanoid metabolic pathways at the level of the enzymes and the genes, no study is available about the effect of elicitors on the enzymes involved in the biosynthesis of coniferyl alcohol. On the other hand, flavonoids biosynthesis, which also involves phenylpropanoid precursor, is affected by the elicitors. More detailed studies on the enzymatic and genetic levels are required for better understanding of biosynthesis and metabolic regulation of flavonolignan production in *S. marianum*.

BIOLOGICAL EFFECTS OF SILYMARIN FLAVONOLIGNANS

Many biological effects have been attributed to silymarin [33–39]. This review focuses on the most recent research on flavonolignans structure–activity relationship for the followings:

1. Hepatoprotective activity
2. Antiproliferative effects against human prostate carcinoma cell lines
3. Radical-scavenging activity

STRUCTURE–ACTIVITY RELATIONSHIP

Hepatoprotective Activity

Silymarin exerts its hepatoprotective activity through antiviral, anti-inflammatory, antioxidant, and immunomodulatory actions in liver and immune cells [40–43]. These actions are mediated by blocking HCV cell culture infection of human hepatoma cultures, inhibition of TNF-α and TCR-induced NF-κB-dependent transcription, and suppression of TCR-mediated proliferation and inflammatory cytokine production from T-cells. The hepatoprotective actions of the major flavonolignans and the flavonoid taxifolin that comprise silymarin were evaluated for the inhibition of HCV cell culture infection, NS5B polymerase activity, TNF-α-induced NF-κB transcription, virus-induced oxidative stress, and T-cell proliferation [44].

Cytotoxicity

All the major components comprising silymarin except isosilybin B were well tolerated by Huh 7.5.1. human hepatoma cells up to 80 μM. Silydianin, silychristin, isosilychristin, and taxifolin did not show cytotoxicity at 100 μM. Isosilybin B, which showed the highest potential as an antiproliferative agent against human prostate carcinoma cells, was toxic to cells above 10 μM.

Antiviral Effect

Silymarin blocks HCV entry and fusion to liver cells as well as virus production [43]. Taxifolin, isosilybin A, and silybin A were the most effective HCV protein expression inhibitors. These compounds were more potent than silymarin as antiviral agents. Isosilychristin, silydianin, and isosilybin B did not inhibit HCV protein expression. Taxifolin was the most potent HCV RNA replication blocker. This was followed by isosilybin A and silybin B.

Anti-Inflammatory Effect

Induction of inflammatory response is blocked by silymarin through inhibition of NF-κB transcription [40]. Silybin A and silybin B induced dose-dependent inhibition of NF-κB transcription. Taxifolin induced inhibition of NF-κB transcription at lower doses, but as a plateau blockade.

Antioxidant Effect

Silymarin induces antioxidant effect in the context of HCV infection by inhibiting the induced oxidative stress. This occurs by direct antioxidant action and antiviral action which reduces HCV replication and oxidative stress [44]. Direct antioxidant action of silymarin is responsible for its hepatoprotective effects in liver diseases of nonviral origin [45,46].

Silibinin, taxifolin, and isosilybin A were found to have antioxidant activity equal to silymarin.

Immunomodulatory Effect

Silymarin, silibinin, silybin A, and isosilybin A were the most potent suppressor for T-cell proliferation. Silymarin had higher potency in T-cell proliferation. Isosilybin B showed the highest potency in T-cell proliferation inhibition because of toxicity rather than specific function.

Table 1 shows the most potent silymarin flavonolignan components in each of the aforementioned effects. It is noteworthy to mention that most of these effects have been observed *in vitro* using high silymarin concentrations. Further studies are needed to assess toxicity of standardized silymarin on human at the used concentrations.

Prostate Cancer Prevention

Milk thistle extracts have shown efficacy in suppressing prostate cancer cell growth *in vitro* and tumor formation *in vivo* [47,48]. These studies have used crude extract, silymarin, or silibinin. No toxicity was found to normal prostate epithelium at antitumor concentration. Silymarin was reported to be more effective than silibinin against cell proliferation [48].

Purification of silymarin flavonolignan components in milligram quantities has allowed assessment of antiproliferative activities of pure compounds against human prostate carcinoma cell lines [49]. These activities included prostate cancer cell growth suppression, cell cycle distribution, suppression of prostate-specific antigen secretion, and DNA topoisomerase IIα promoter

TABLE 1 Silymarin Components Hepatoprotective Activity [44]

Activity	Active Component
HCV infection	
HCV protein expression inhibition	Taxifolin/isosilybin A
HCV protein RNA replication blocking	Taxifolin
Anti-inflammatory effect	
NF-κB transcription blockade	Silybin A/silybin B
Antioxidant effect	
Inhibition of oxidative stress	Silibinin
Immunomodulatory effect	
Suppression of T-cell proliferation	Silymarin

activity. Isosilybin B was most potent suppressor of cell growth. Silybin B was the most effective in causing G_1 cell cycle accumulation. Isosilybin A and isosilybin B were the most effective suppressor of prostate-specific antigen secretion. Isosilybin B was the most effective suppressor for DNA topoisomerase IIα gene promoter activity. Silybin A was the least potent of the four major silymarin flavonolignans. Isosilybin A was of similar potency as isosilybin B in suppressing prostate-specific antigen secretion and of intermediate potency in other activities. Table 2 shows the most potent silymarin flavonolignan components in assays related to prostate cancer prevention.

Gram-scale isolation of the major flavonolignans from milk thistle allowed analysis of structure–activity relationship toward prostate cancer [50]. Prostate cancer chemopreventive, antiproliferative, and proapoptotic properties were evaluated for these compounds. Isosilybin B and isosilybin A were the most potent in the used assays. Isosilybin C and isosilybin D had decreased activity relative to the other flavonolignans. This shows the importance of *ortho* relationship of the hydroxy and methoxy substituents that is more favorable than the *meta* relationship for the same substituents in isosilybin C and isosilybin D.

Safety profile of silymarin suggests that high doses can be given to produce plasma concentrations consistent with the previously mentioned effects. Though, as mentioned under hepatoprotective effects of silymarin, concentrations used *in vitro* have to be reassessed *in vivo*. Bioavailability studies on human using pure silymarin components to follow their concentrations are needed.

Radical-Scavenging Activity

The molecular mechanism of the radical-scavenging activity of silybin and 2,3-dehydrosilybin was studied [51]. The latter compound was isolated as a minor component from seeds extract of *S. marianum* subsp. anatolicum [52]. 2,3-dehydrosilybin was reported to have a higher antioxidant activity than silybin based on structure-activity relationship studies [53]. In these

TABLE 2 Silymarin Components Chemopreventive Activity [49]

Activity	Active Component
Prostate cancer cell growth suppression	Isosilybin B
Cell cycle effects	Silybin B
Topoisomerase IIα promoter suppression	Isosilybin B
Prostate antigen secretion suppression	Isosilybin B

studies it was shown that in flavonoids 3-OH group connected to 2,3-double bond conjugated with the C-4 carbonyl group is important for a high radical scavenging activity. A detailed structure–activity relationship study was performed on selectively methylated derivatives of silybin and 2,3-dehydrosilybin to determine sites for scavenging free radicals. The activity was evaluated using three different assays (diphenyl picrylhydrazyl radical scavenging, inhibition of lipid peroxidation, and inhibition of superoxide anion production). The mechanism of the antiradical activity of silybin was proposed based on laccase-mediated radical dimerization of its 7-*O*-methyl derivatives (Fig. 9) [54].

The 2-OH group of silybin was proposed to be the most important radical-scavenging moiety. The 7-OH group was proposed to possess pro-oxidant activity. The 3-OH group in 2,3-dehydrosilybin is the most important radical-scavenging group. This can be explained by the resonance stabilization which can lead to a stable resonance structure containing *o*-diphenol moiety (Fig. 10). The presence of this motif is an important prerequisite to ease O—H bond dissociation and facilitate H abstraction by the free radical.

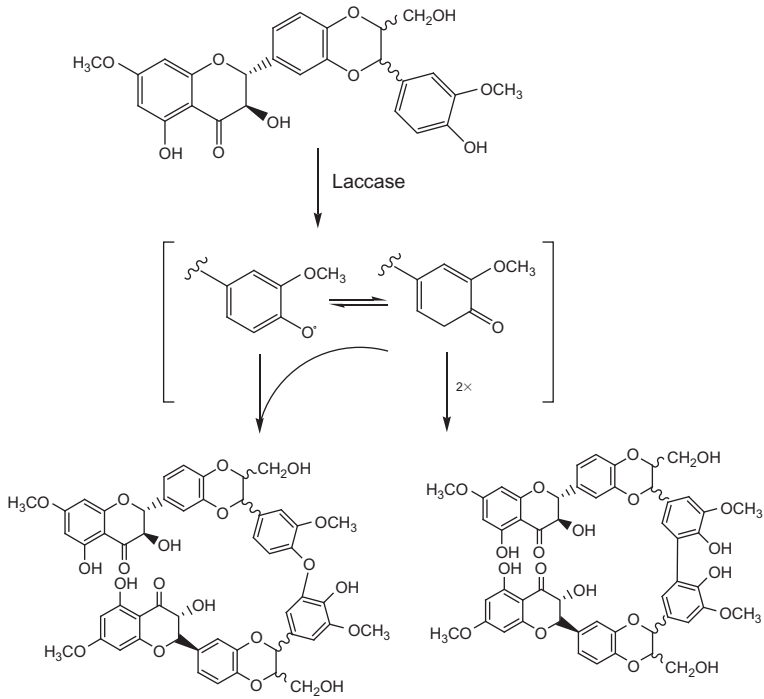


FIGURE 9 Dimers formed from reaction of 7-methyl silybin with laccase.

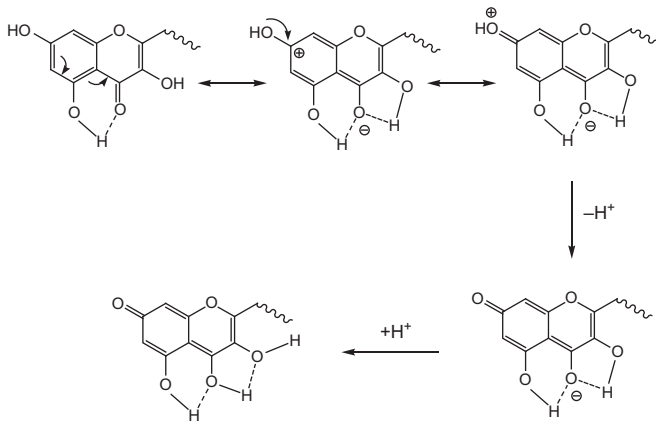


FIGURE 10 Resonance stabilization of dehydrosilybin.

CONCLUSION

Silymarin is a valuable natural product that has many biological activities. Silymarin flavonolignan and flavonoid components have been isolated in quantities sufficient for biological studies. This allowed assessment of hepatoprotective, antiproliferative, and antioxidant activities of pure compounds. Results suggested promise for the treatment of hepatitis C viral infection and prostate carcinoma. Plant tissue culture studies have allowed better understanding of biosynthesis and regulation of silymarin in *S. marianum*. More studies are needed for the identification of candidate components of the signaling pathway involved in silymarin production.

ABBREVIATIONS

HCV	hepatitis C virus
HPLC	high performance liquid chromatography
NF	necrosis factor
RNA	ribonucleic acid
TCR	T-cell receptor
TNF-α	tumor necrosis factor-α

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Chapter 15

Natural Antioxidants in Cosmetics

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Chapter Outline

INTRODUCTION

Cosmetics are pharmaceutical products that are used for improving skin appearance and body odor. These products are available in various forms, ranging from lotions, creams, powders, and so forth. Cosmetics are used for cleansing, protecting, and moisturizing the skin. Generally, consumers prefer to choose cosmetics that have less harmful effects on their skin. "Cosmeceutical" products have now been developed by many pharmaceutical industries. According to Duroja *et al*., a cosmeceutical is the combination of a topical cosmetic and a pharmaceutical that is used for enhancing beauty through ingredients that have biological functions related to the skin [1]. The topical cosmetic, which contains antioxidants, is used to protect human skin against the damage caused by ultraviolet radiation (UVR) and by free radicals [2].

Antioxidant compounds are very interesting for pharmaceutical industries. Antioxidants can be added to cosmetic preparations because of their activities against free radicals. Unfortunately, most antioxidants are not stable, and they can cause many problems in the formulation of cosmetics. The selection of antioxidants and their concentrations in cosmetic formulations must be optimized. Nowadays, the use of plant-derived natural antioxidants in cosmetics is

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preferred over synthetic antioxidants [3]. Extracts of plant-derived antioxidants generally contain a mixture of natural compounds, which could have synergetic effects; therefore, they can have better effects and less toxicity [3].

Due to their instability, keeping constant the activities of antioxidants in formulations during their claimed shelf life is often problematic. For this reason, the concentration of each antioxidant should be determined and controlled for its chemical stability to ensure that the consumer gets a product that has the claimed activity. Activities assays of plant derived antioxidant are not simple because the compounds are usually very complex.

The aim of this chapter is to review all aspects of the use of (natural) antioxidants in cosmetics. Free radicals, human skin defense systems, the use of antioxidants in cosmetics, determination of antioxidants' activity/capacity, and important natural antioxidants and their mechanisms are summarized and discussed.

FREE RADICALS AND REACTIVE OXYGEN SPECIES

Most of the biological molecules are nonradicals, which are characterized by two outer orbital electrons (pair-electron). When a chemical reaction breaks the bonding of these electrons, they become unpaired and are called free radicals. Free radicals are very unstable and reactive due to their capability to lose or gain electrons [4–6]. They can be formed by hemolytic bond fission or via electron transfer [7]. Free radicals can be continuously formed by cellular metabolism and by the induction of external factors such as UVR, chemicals, air pollutants and cigarette smoke, drugs, pesticides, anesthetics, and industrial solvents [5,8–10].

Due to their instability, free radicals will always be transformed into their stable forms, making their lifetimes very short. Free radicals can initiate continuous chain reactions that could become very dangerous because of their damaging effects. Reactions of free radicals with important components of cells or the cell membrane can be very hazardous because it can affect the cell functions or even kill the cells [5]. Free radicals can cause metabolism disorder by forming covalent bonds with enzymes, receptors, and membrane components, as well as by disturbing transport processes [7].

Cell components that are very sensitive to oxidation are lipids, proteins, DNA, and carbohydrates. The interaction of free radicals with the target molecule can cause irreversible damage(s) to it or alter its function(s). Chain reactions take place when free radicals interact with lipids to form lipid peroxidation, which results in an increase in cell permeability and eventually causes cellular damage [5,7,11]. Interactions of free radicals with protein residues affect the functions of essential proteins such as immunoglobulin, albumin, and hemoglobin [6]. Reactions of free radicals with thiol groups affect the activities of the enzymes [7], while the reaction with DNA can lead to irreparable damage or inaccurate repairs [5], which can trigger cytotoxicity or mutation [7].

Reactive oxygen species (ROS), reactive nitrogen species, atomic hydrogen, many heavy transition metals, chlorine, certain drugs, ionizing radiation, and environmental wastes are different types of free radicals [5]. Generally, free radicals are formed dominantly by ROS [12,13]. ROS can be formed via the by-products of various metabolic pathways, which are located in different cellular compartments [14,15].

The toxicity of ROS depends on its half-life. ROS needs some time to reach its target from the site of formation. The toxicity of ROS is determined more by the time needed to reach the target than its reactivity. The interaction of ROS and the target molecules can be inhibited by the antioxidants that are available at the site of ROS formation [15,16]. Hydroxyl radicals are very reactive, and these radicals directly react at the site of formation [17]. Tocopheroxyl-, ascorbyl-, and certain semiquinones-type radicals are intermediate radicals that will cause damage only under suitable conditions [17]. One of the persistent radicals that are present in the skin and have physiological effects is the melanin radical [17].

ROS can affect the structure and function of skin endogenously and exogenously [12,13]. Almost 80% of ROS that affect the skin are produced by UVR, which comprises 95–98% of UVA and 2–5% UVB [18]. The main target of UVR is O_2 that occurs in the mid-lower epidermis. Donation of one electron can transform O_2 into superoxide anion (O_2^{*-}), which is very unstable and reactive. For transformation into stable molecules, it initiates reactions that yield other free radical *OH. Carbon-centered radicals are formed in the process of lipid peroxidation, while sulfur-centered radicals are formed in the oxidation of thiol compounds [17].

Interaction of UVR and skin surface can result in free radicals or ROS and biochemical transformation in collagen, elastin, and connective tissues. They can affect skin firmness and elasticity, making the skin dry and appear wrinkled. The UVB can cause erythema and sunburn. In comparison to UVB, UVA can penetrate the skin deeper; indirectly, it can penetrate 50% of the exposure through the skin even in the shade, and UVA cannot be filtered by glass. UVA is also more potent in inducing pigment darkening and tanning than UVB. UVA causes lipid peroxidation 10 times more potent than UVB. UVA is more cytotoxic than UVB [9,18–22].

SKIN DEFENSE MECHANISM

It is known that skin is the outermost human organ, so it is often influenced by endogenous and exogenous free radicals [12,23]. To deal with the adverse effects of the free radicals, skin has its own defense mechanism, which derives from endogenous and exogenous antioxidants. Endogenous antioxidants originate from melanin, while exogenous antioxidants derive from antioxidants that are administrated orally and topically. This defense mechanism is related to the structure and function of constituent skin layers [23].

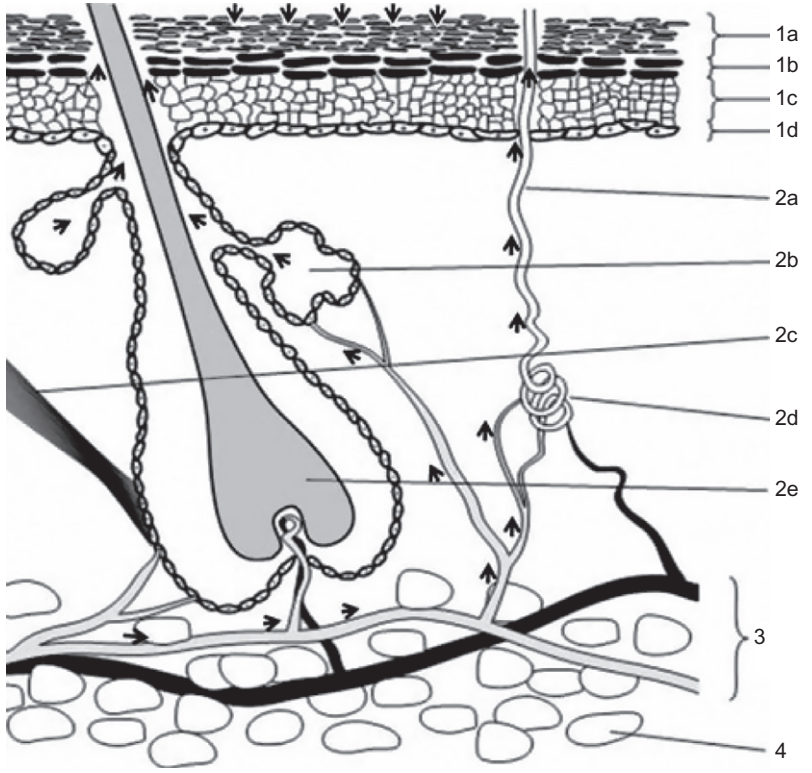

FIGURE 1 Diffusion and accumulation of antioxidants in human skin. (1a) Stratum corneum, (1b) stratum granulosum, (1c) stratum spinosum, (1d) stratum basale, (2a) sweat duct, (2b) Sebaceous gland, (2c) arrector pili muscle, (2d) sweat gland, (2e) hair follicle, (3) blood vessels, (4) fat lobules. Arrows show the diffusion of oral antioxidants and accumulation of topical antioxidants in the epidermis. *Modified from Ref. [12].*

Skin comprises three layers (Fig. 1). The uppermost layer is the epidermis, which contains corneocytes, keratinocytes, and melanocytes; they are cells responsible for melanin synthesis. The epidermis, which comprises the stratum corneum, stratum granulosum, stratum spinosum, and stratum germinativum/basale, has no blood vessels. The stratum basale is composed of keratinocyte and melanocyte cells. Melanocytes synthesize melanin, which can absorb UVR. The stratum corneum is a layer surrounded by the epidermis, which is composed of nondividing cells or dead cells named corneocytes or horny cells, and has *ceramide* as its main component. Ceramide has an important role in water retention and keeping the skin moisturized; it plays an important role in maintaining the integrity of the skin defense. Ceramide is the primary lipid, while the other lipids are fatty acid and cholesterol. These lipid bilayers have amphiphilic characteristics. Intrinsic and extrinsic proteins such as enzymes are also tucked inside these bilayers. A healthy stratum

corneum is the best protection for the epidermis, and a determinant of healthy skin appearance [10,24,25].

The next layer is the dermis, which keeps the characteristics of the skin and serves as a water reservoir for the skin. This layer contains fibroblasts, collagen, elastin, and hyaluronic acid. The hypodermis is the deepest tissue layer that contains collagen and extracellular matrix (as adipocytes and used as fat storage) [10,24,25]. Skin defense systems against ROS comprise enzymatic and nonenzymatic mechanisms, and both of these could deactivate ROS. Enzymatic defense systems are done by superoxide dismutase, catalase, and glutathione (GSD) peroxide [26], while nonenzymatic mechanisms are done by low-molecular weight antioxidants [27]. However, antioxidant defense mechanisms are taking place in a comprehensive way between enzymatic and nonenzymatic antioxidants [28,29]. Concentrations of enzymatic and nonenzymatic antioxidants vary in the different skin cells. Fibroblasts have a higher concentration of antioxidant enzymatic antioxidants than keratinocytes, while melanocytes have no enzymatic antioxidants. Cells with high levels of antioxidants are more resistant to oxidative stress [30].

First, the enzyme defense system converts ROS into hydrogen peroxide and oxygen; subsequently, hydrogen peroxide is transformed into water and oxygen. Nonenzymatic reactions are performed by vitamin C, vitamin E, vitamin B, vitamin A, and other antioxidants. These molecules scavenge the free radicals and delay the oxidation process [31]. Very complex interactions between antioxidants occur; therefore, changes in the redox status or concentration of the antioxidant will affect other antioxidants in the system [28,29].

Nonenzymatic defense systems cannot be provided by the human body; they can be supplied through oral or topical preparations that contain vitamins, carotenes, or other antioxidants. These antioxidant molecules are generally accumulated in the epidermis through diffusion processes from adipose glands, blood and lymph, and via secretion by sweat glands or sebaceous glands to the epidermis, as presented in Fig. 1 [12].

The *stratum corneum* contains hydrophilic and hydrophobic antioxidants. The concentration of antioxidants in the epidermis is higher than in the dermis [23]. The activity and capacity of the antioxidants in the epidermal layer are higher than in the dermis, although the thickness of the epidermis is only 10% of the skin. This is due to the early defense system of the epidermal layer. The skin antioxidant capacity is also higher than that of other organs [23,28,29]. Consumption of rich antioxidant-containing foods or application of topical antioxidants can overcome the occurrences of oxidative stress caused by the inequilibrium between ROS and the skin defense system [12,32]. Skin can naturally defend itself against ROS by the use of antioxidants [21].

Two mechanisms of antioxidants are stabilizing the free radicals by scavenging and slowing the oxidation; by these mechanisms, the formation of further free radicals can be suppressed [33,34]. The main defense systems of the skin dealing with the UV inductions are melanogenesis and enhancement of

DNA repair [23]. ROS can stimulate lipid peroxide, which can generate hyperpigmentation and melanogenesis, and this could develop into skin darkening. Enzyme tyrosinase can convert tyrosine into melanin; melanin, a skin pigment, can absorb UVR [35]. Melanogenesis, which stimulates the abnormal production of melanin, can be overcome by increasing the antioxidant defense capacity of the skin. A correlation is observed between the defense system of the epidermis and skin pigmentation. The addition or use of antioxidants in cosmetics is one way to maintain the amount of antioxidant pool in the skin [21,26].

The major target of UVR that penetrates into the skin is mitochondrial DNA. Oxidation of DNA can cause various damages to it, such as DNA strand break. Self-cellular defense systems can carry out DNA repair. Antioxidants might increase the capability of the repair enzyme systems by posttranscriptional gene regulation of the transcriptional factor [23].

Healthy skin has intrinsic antioxidants, which can protect it against exposure to free radicals. On the contrary, the defense system of old skin is impaired, so an external supply of both oral and topical antioxidants is needed. Antioxidants have the ability to donate a hydrogen atom for stabilizing the radical electron, so it can delay or minimize the propagation or initiation of chain reactions and ultimately prevent skin damage [36].

For preventing skin from the damages of oxidation reactions, the amount of endogenous antioxidants must be balanced with the amount of ROS. Under certain conditions, when there is very high exposure to ROS, the body's antioxidant defense systems are not sufficient to maintain the balance. That is why exogenous antioxidants should be administrated either orally or topically [12,21,32,37].

Exogenous antioxidants include vitamins, trace elements, and phyto-antioxidants [32]. GSD and vitamin C are hydrophilic antioxidants, while vitamin E and ubiquinol-10 are lipophilic antioxidants [21,32]. Vitamin E (tocopherol) can inhibit lipid peroxidation to form tocopheryl stable compounds, which can stop the chain reaction of membrane lipid peroxidation [32]. Vitamin C can react with tocopheryl radicals for restoring and regenerating vitamin E. Trace elements such as selenium, copper, zinc, manganese, and iron are a cofactor of the enzymatic antioxidants [38]. Phyto-antioxidants are compounds of plant extract mixtures usually containing terpenes and polyphenols [32]. Antioxidants from plant extracts, which contain a mixture of compounds, are now used in cosmetic preparations [39]. The use of mixtures of compounds as antioxidants is preferable on account of its advantages, that is, the combined effects of various compounds contained therein either as biologically active compounds or as protectors of other ingredients [32].

NATURAL ANTIOXIDANTS

Based on their function, antioxidants can be divided into primary or natural and secondary or synthetic antioxidants. Primary antioxidants comprise

mineral antioxidants (such as selenium, copper, iron, zinc, and manganese), vitamins (C and E), and phyto-antioxidants. Generally, a mineral antioxidant is a cofactor of enzymatic antioxidants. The functions of secondary or synthetic antioxidants are capturing free radicals and terminating the chain reaction. Some examples of secondary antioxidants are butylated hydroxy anisole (BHA), butylated hydroxytoluene (BHT), propyl gallate and metal chelating agent, tertiary butylhydroquinone, and nordihydroguaiaretic acid [38,40].

The increasing application of plant antioxidants could replace the application of synthetic antioxidants [32,41,42]. Natural antioxidants can be a single pure compound/isolate, a mixture of compounds, or plant extracts; these antioxidants are now widely used in cosmetic products [39]. Natural antioxidants function as singlet and triplet oxygen quenchers, free radical scavengers, peroxide decomposers, and enzyme inhibitors [32,41,42].

Phyto-antioxidants consist mostly of polyphenols and terpenes; this differentiation is based on their molecular weight, polarity, and their solubility. Polyphenols have —OH groups attached to the benzene ring. Their activity as antioxidants is determined by the number and the position of —OH groups on the benzene ring. Phenolic groups modulate protein phosphorylation by their ability to inhibit lipid peroxidation (as chain-breaking peroxyl radical scavengers). Flavonoids and stilbenes are the largest group of polyphenols, while the largest group of terpenes are carotenoids that serve as singlet oxygen quenchers [32]. A summary of some natural antioxidants generally used in cosmetic preparations and their mechanisms of action are presented in Table 1. The chemical structures of the pure natural antioxidants are presented in Fig. 2.

APPLICATION OF ANTIOXIDANTS IN COSMETICS

Antioxidants are responsible for the chain-breaking of radical scavengers and for inhibiting the oxidation reaction; by these mechanisms, antioxidants can prevent oxidative damage [56,57]. In cosmetic preparations, antioxidants have two functions, that is, as the active ingredients and as protectors of other ingredients against oxidation [38].

Currently, the application of antioxidants in cosmetics is increasing; however, to obtain the desired activities, some strategies should be considered. The short life of ROS can be overcome by using antioxidants that have high reactivity and capacity. Antioxidants must not be transformed into their radicals such as ascorbyl- or tocopheryl radicals; this will trigger the chain reaction. Antioxidants should remain stable in the product; they must not react with the other ingredients and should be protected from oxygen radicals. The selection of antioxidants that can be used in cosmetics depends on their hydrophobic or lipophillic characteristics. Unfortunately, sometimes the selection of antioxidant(s) (by pharmaceutical industries) used in cosmetic products is not based on scientific judgment, but rather on their price.

TABLE 1 Summary of Natural Antioxidants Which Have Been Used in Cosmetics

Antioxidants	Sources	Use In Cosmetics as	Mechanism of Action of the Antioxidants	Products (™/®)	References
α-Tocopherol	Wheat germ, sunflower	Pure compound or isolate, plant oils	Via glutathione pathway, and chain breaking lipid peroxidation	Estee Lauder Re-Nutriv Ultimate Youth Creme Reviews, Body Lotion, Cosmetics Bakery, Singapore	[18,43]
Aloesin (low-molecular weight glycoprotein)	*Aloe vera*	Plant extracts, pure compound	*Tyrosinase* inhibitor (at DOPA-oxidation site)	Constance Cosmetic, Aloe Vera Jelly (aloe therapy and oily skin moisturizer), The Intensive Whitening Cream	[44,45]
Anthocyanin, proanthocyanin	Berry species (*Vaccinium* sp.)	Plant extracts, pure compound	Radical scavenger, Inhibition of low density lipoprotein oxidation	Revale Skin (day cream, night cream), DDF Doctor's Dermatologic Formula Protect	[46–49]
Arbutin	*Vaccinium vitisidaea*	Pure compound or isolate	Inhibition of tyrosinase and melanosome, but without any cytotoxicity effects on melanocytes	Buhna W Quinone Cream 15 g (Arbutin cosmetics)	[44]
Ascorbic acid	Rosehip oil	Pure compound or isolate, plant oils	Quenching UV-induced free radicals, and regenerating vitamin E	100% Pure Argan Oil Hydration Facial Moisturizer SPF 30	[18,19]
Azelaic acid	*Pityrosporum ovale*	Pure compound or isolate	Inhibition of thioredoxin reductase, so the toxicity effects of melanocytes are hampered	Ampleur Luxury White Cream AO	[44]

Carotenoid (lycopene, β-carotene, lutein)	*Lycopersicon esculentum, Daucus carota, Hippophae rhamnoides*	Carotenoid, plant extracts, pure compound	Breaking chain in lipid peroxidation, decreasing UV-induced erythema, and sunburn cell formation	Ilike Rich Carrot Moisturizer, Dharpin's Products	[19,43,50]
Carnosic acid	*Rosmarinus officinalis*	Plant oil	Scavenging lipid free radicals	Natural skin facial toner, wildcrafted herbal product	[32,51]
Curcumin	*Curcuma longa*	Pure compound or isolate	Radical scavenging	EWG's Skin Deep, Gentle Earth Products Virgin Coconut Rose Face Cream	[19]
Epigallocatechin-3-gallate (EGCG)	Green tea, black tea (*Camellia sinensis*)	Plant extracts, pure compound	Radical scavenging, sunscreen	Olay, Regenerist Perfecting Cream, Sunday Riley's products	[19,52]
Licorice, flavonoid liquertin, isoliquertin.	*Glycyrrhiza glabra*	Plant extracts	Inhibiting tyrosinase without interfering with DNA synthesis	YUKIMIZU Natural Skin Cream	[44]
Kojic acid	*Aspergillus* spp., *Penicillium* spp.	Pure compound or isolate	Inhibiting copper binding to tyrosinase, so it can be used as a lightening agent	100% Pure Skin Brightening Facial Cleanser	[44]
Pycnogenol (proanthocyanidin)	*Pinus pinaster*	Bark extract	Converting vitamin C radical into its active form, and increasing the levels of glutathione and other radical scavengers	Dermae Pycnogenol Cream	[44,53]
Quercetin (flavonol)	Contained in various fruits, vegetables,	Pure compound or isolate	Iron chelator, which maintains and protects the	EltaMD UV Physical Broad-Spectrum, SPF 41	[19,32,54,55]

Continued

TABLE 1 Summary of Natural Antioxidants Which Have Been Used in Cosmetics—Cont'd

Antioxidants	Sources	Use In Cosmetics as	Mechanism of Action of the Antioxidants	Products (™/®)	References
	beverages, and herbs, Leaf of *Psidium guajava*		enzymatic antioxidant activity, sunscreen	EltaMD by Swiss-American Products	
Resveratrol (stilben)	Contained in many grapes and berries	Plant extracts, pure compound	Lowering generation of H_2O_2 by UVB, inhibiting the increase in lipid peroxidation, it can ward off free radical damage (caused by UV rays and pollution), and encourages cell renewal, so it can reduce wrinkles and dark spots	Moisture Reservoir Hand Cream, Love Life Skin, 46 Graphic Place Moonachie, NJ Estee Lauder Re-Nutriv Ultimate Youth Creme Reviews	[19,43]

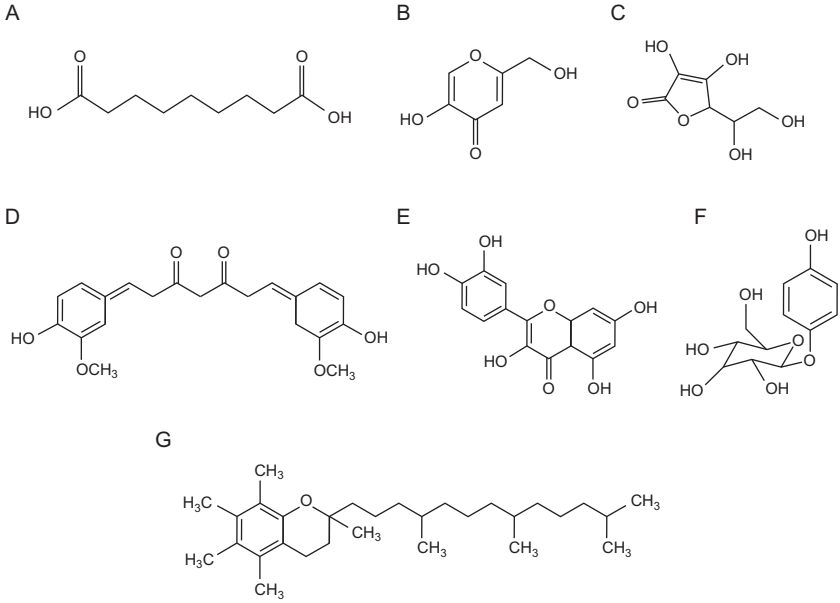

FIGURE 2 Chemical structures of pure natural antioxidants. (A) Azalaic acid, (B) kojic acid, (C) ascorbic acid, (D) curcumin, (E) quercetin, (F) arbutin, (G) α-tocopherol.

Generally, antioxidant by nature are unstable, deeply colored, and susceptible to hydrolysis and photodegradation in the presence of oxygen; that is why it is very difficult to have good cosmetic formulations and to maintain their aesthetic validity and acceptability. Modifying the chemical structure of the antioxidant such as substitution wiht its esters (e.g., tocopheryl acetate, ascorbyl palmitate), or by shortening the lipophilic chain of CoQ10, may be able to improve its stability, but unfortunately, it reduces its activity.

For being active, a stable antioxidant is needed, but unfortunately, antioxidants are generally unstable compounds. This instability can cause many problems. In a cosmetic formulation, the concentration of antioxidants must be stable for achieving the desired activity. Their color should not change in the production processes and storage, so that their antioxidant activities remain constant and the product retains an aesthetic appearance. All this raises many problems in the formulation of cosmetic products [12,21,32]. That is why a valid method is needed for determining the antioxidant's capacity to evaluate its activity [32].

Application of the relatively new "lipid-based delivery system" technology could protect and maintain the stability of the antioxidants. This technology also has protective effects against skin dehydration. Lipid carriers can increase the skin penetration of the antioxidant, so its desired activity can be guaranteed. Various lipid carriers such as nanoparticle emulsion, various vesicular systems (liposomes, phytosomes, transfersomes, etosomes, niosomes, and

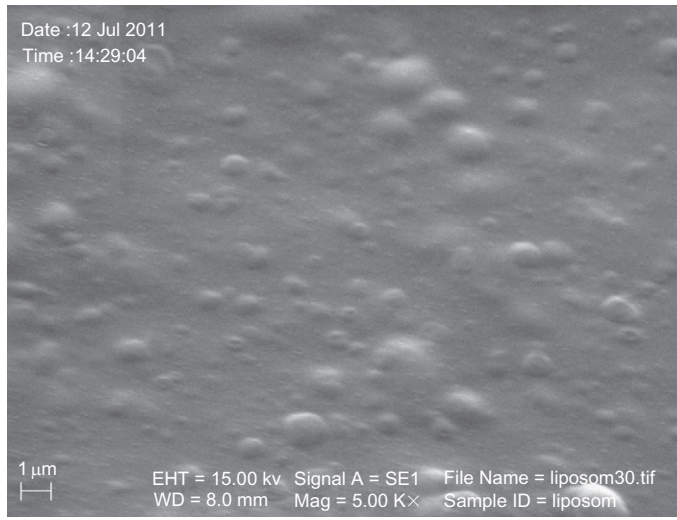

FIGURE 3 SEM of the liposomes of quercetin (5000×). *Cited from Ref. [55].*

nanotopes), and particulate systems (lipid microparticles and lipid nanoparticles) have been developed and are being used. The stability of ascorbyl palmitate and vitamins K and A in cosmetics can be enhanced by using lipid nanocarriers. Phytosomes of green tea and grape seed can improve their free radical scavenging and UV protection activity. Skin penetration of vitamin E acetate was increased by using Nanotop^{TM}. The antiaging effect of vitamin E acetate and CoQ10 was improved by the application of nanoemulsions [36,56–60].

We have now developed various lipid carrier systems, such as liposomes, phytosomes, and lipid nanoparticles for the natural antioxidants pycnogenol, quercetin, squalene, and *p*-metoxycinnamic acid, which will be used in UV-protector preparations. Figure 3 shows liposomes of quercetin, which were prepared in our laboratory; the liposomes were viewed by using SEM. These liposomes can increase the permeation of quercetin into human skin, so it will have the desired photoprotective activity [55]. This part of our work is still in progress.

ANTIOXIDANT CAPACITY; METHODS OF DETERMINATION AND QUALITY CONTROL

The determination of the antioxidant capacity of a sample can be used for getting information regarding its resistance to oxidation, the quantitative contribution of its antioxidant substances, and its antioxidant activity. For example, the antioxidant capacity of food depends on the colloidal properties, the condition and stages of oxidation, localization of antioxidants in different

phases, and the free radical generator or oxidant used [61]. Jung used the other term "antioxidant power"; this term is similar in meaning to the term antioxidant capacity [36].

In determining an antioxidant's capacity, several constraints have to be faced, particularly for phytoantioxidants, which may contain thousands of different compounds that often work synergistically. It is known that cosmetic products usually have a mixture of ingredients, which may have different characteristics (hydrophilic and hydrophobic), so they need to have a mixture of antioxidants that have the same characteristics. This gives rise to problems in the determination of the total activities of the antioxidants [38].

Several methods can be used for measuring antioxidant capacity (activity). Generally, spectrometric methods are used for the determination of *in vitro* antioxidant activity; these methods are based on the reaction of antioxidants with free radicals. Antioxidants donate their hydrogen atom to the free radicals. Electrochemical methods are also widely used.

Generally, antioxidant capacity (activity) evaluations can be classified according to the chemical reactions:

1. Based on electron transfer (ET-based) that involves a redox reaction [32] and a single electron transfer mechanism [38]. This assay is based on measurement of the capacity of an antioxidant in the reduction of an oxidant. The oxidant compound will change its color when it is reduced, and the degree of the color change depends on its concentration in the sample; examples of this assay are [61] as follows:
   - **a.** DPPH (2,2′-diphenyl-1, 1′-picrylhydrazyl) assay:
     DPPH is a free radical that is unstable at room temperature; it can react by reducing the hydroxyl group(s) of the antioxidant molecules. The percentage of the scavenging activity of DPPH generally depends on the concentrations of the antioxidants [42]. This method is suitable for antioxidants that are soluble in organic solvents, especially alcohol. Light, oxygen, pH, and solvent type could lead to biased interpretations of the results. The disadvantages of this method are the narrow linear range and steric hindrance [38]. Recently, Olech *et al.* reported the application of an HPTLC–DPPH method; this method, combined with imaging processing software, can be used to evaluate the antioxidant activities of the chemical constituents of plant materials [62]. A schematic picture of the DPPH reaction is shown (Fig. 4).
   - **b.** TEAC (trolox equivalent antioxidant capacity) assay:
     A TEAC assay is based on the activation of metmyoglobin with hydrogen peroxide in the presence of ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) [42,65]. Reaction between ATBS with peroxidase and H_2O_2 will yield the colored ABTS radicals. The occurrence of antioxidants in the sample will inhibit the formation of colored ABTS radicals. This method can be used for evaluating both hydrophilic and

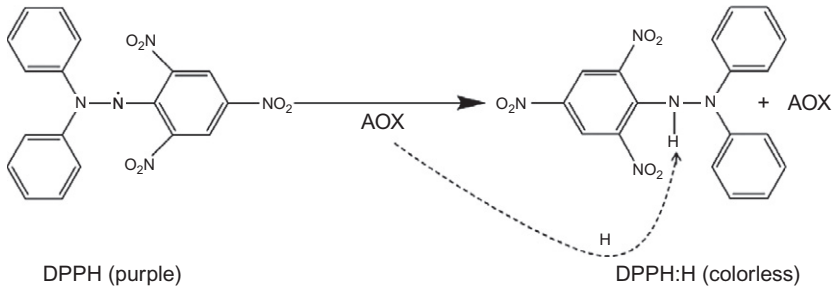

FIGURE 4 Schematic of DPPH assay: Donation of hydrogen from AOX to DPPH molecule can lead to a decrease in the absorbance (decolorization). DPPH radical scavenging activity can be measured spectrophotometrically at 517 nm against methanol. AOX, antioxidant; ●, odd electron. *Modified from Refs. [63,64].*

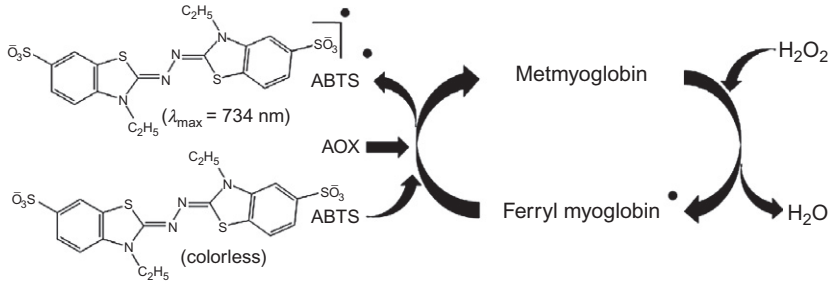

FIGURE 5 Schematic of TEAC reaction (for details see text). AOX, antioxidant; ●, odd electron. *Modified from Ref. [65].*

lipophilic antioxidant samples [38,66]. The TEAC reaction might be too fast, so it could contribute to the reduction of the ferryl myoglobin radical, and the addition of hydrogen peroxide could oxidize the antioxidants in the sample before it is measured. ABTS radicals are not ROS, so the method does not show the actual antioxidant activity. This method only indicates specific oxidant-reducing power [67]. Figure 5 shows the schematic reaction of a TEAC assay.

2. Based on hydrogen atom transfer (HAT-based). The method involves antioxidants' ability to donate hydrogen atoms [32]; this can measure the neutralization capacity of the free radicals [38]. In this case, antioxidant and substrate compete for thermally generated peroxyl radicals via decomposition of azo compounds; examples of this assay are described in brief [61].
   a. ORAC (oxygen radical absorbance capacity) assay:
      ORAC measures antioxidant scavenging activity against peroxyl radicals induced by AAPH (2,2-azobis-(2-amidino-propane)-dihydrochloride) at 37 °C (15 min). It measures the ability of the antioxidant to inhibit the declining of the fluorescence of β-phycoerythrin (B-PE) or FL (fluorescein or 3′,6′-dihydroxy-spiro[isobenzofuran-1[3H],9′[9H]-xanthen]-3-one).

The loss of fluorescence of B-PE or FL is a sign of the extent of damage caused by its reaction with peroxyl radicals. The protective effect can be measured by comparing an area of the sample under fluorescent decay with antioxidants and without antioxidants (blank sample). The limitation of B-PE was its variability in different lots. It seems that FL is a better probe for this assay. Trolox, a water-soluble analog of vitamin E, is used as a control antioxidant standard. ORAC can be used for biological samples, hydrophilic and liphophylic emulsions, and commercial products. Detailed methods have been described [37,40,47,67–70]. The schematic of this assay is presented in Fig. 6.

b. TRAP (total radical trapping antioxidant parameter) assay:
According to Sies, TRAP can be defined as "a quantitative measure of the total secondary antioxidant content of a biological fluid" [70]. This method is based on luminol (3-amino phthal hydrazide)-enhanced chemiluminescence (CL) for determining the antioxidant capacity of substances. Alkyl peroxyl radicals from the decomposition of AAPH in the presence of luminol will produce luminescence, which is quenched by the addition of antioxidants. The TRAP value is determined by the duration of the time period (T_{sample}) in which the sample quenches the CL signal in the presence of antioxidants. Generally, Trolox (8 nM) is used as reference inhibitor (T_{trolox}):

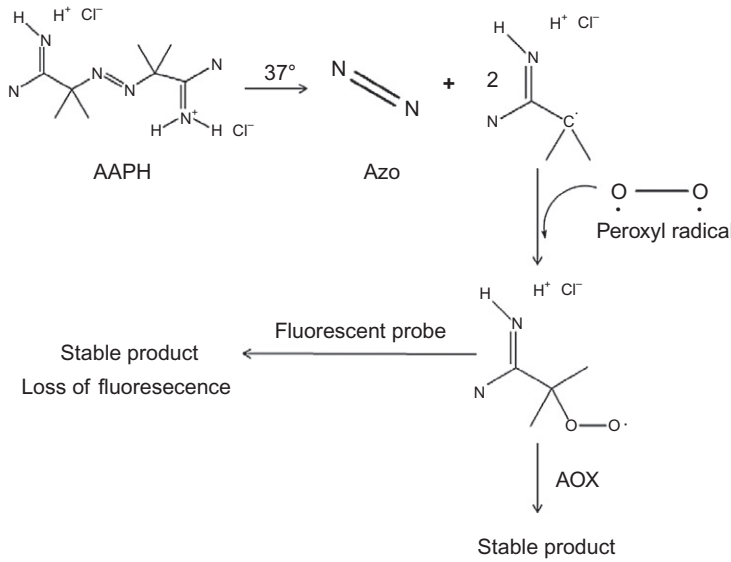

FIGURE 6 Schematic of ORAC assay: Peroxyl radical is more reactive to antioxidant (AOX) compared to the fluorescence probe (e.g., B-PE), so the decline in the fluorescence of B-PE can be inhibited. *Modified from Ref. [61].*

$$\text{TRAP value} = 2.0[\text{Trolox}]T_{\text{sample}}/fT_{\text{trolox}}$$

where f is the dilution of the sample and 2.0 is the stoichiometric factor of trolox (the number of peroxyl radicals trapped per molecule of trolox). This method is suitable only for nonenzymatic antioxidants [69]. It can measure antioxidant capacity in hydrophilic and lipophilic compartments of plasma and tissue homogenate [71]. The detailed method can be referred to in previous publications [47,70,72–75]. This assay is presented schematically in Fig. 7.

3. Based on electrochemical characteristics.
This method can be used to measure either a single compound or a mixture. It is based on the determination of the redox capacity, which requires neither reagents nor dependent absorbance. Cyclic voltammetry methods have been adapted to the quantification of the overall reducing capacity of antioxidants in different biological fluids [32,38,76]. Validation procedures for measuring global antioxidants in the stratum corneum by cyclic voltammetry have been described in detail by Ruffien-Ciszak *et al*. [77].

It seems very difficult to choose an appropriate method for the determination of the antioxidant capacity in samples because each different antioxidant has its own type of reaction mechanisms. No single method can be used to

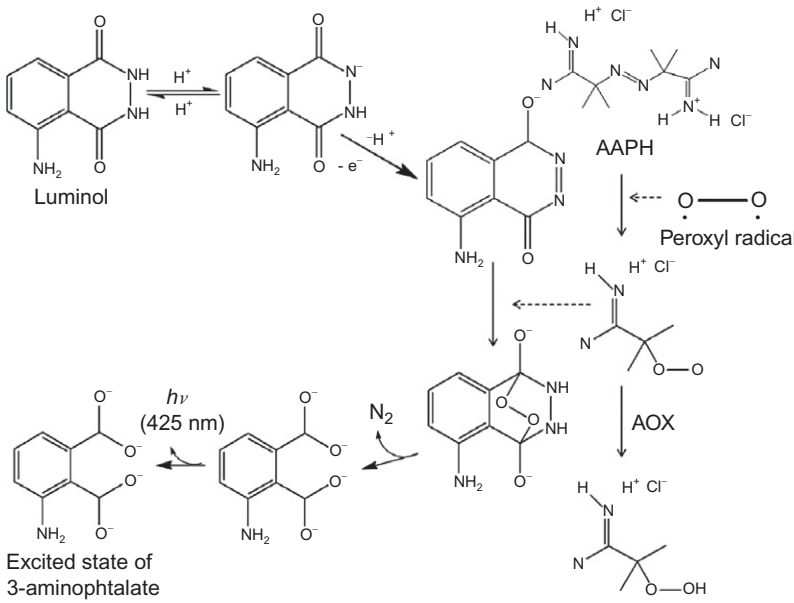

FIGURE 7 Schematic of TRAP assay: Luminol is oxidized by peroxyl radicals; in neutral or alkaline conditions, luminol is oxidized to anion form, and further oxidation by H_2O_2 yields the excited state of 3-aminophtalate; then, it returns to its ground state by emission of characteristic luminescence (CL) at 425 nm. Antioxidant (AOX) can quench CL. *Modified from Refs. [61,74].*

describe the different modes of action of the different antioxidants. An *in vitro* assay only shows the activity for a given reaction system, and unfortunately, its correlation with *in vivo* systems is uncertain. It is recommended to perform more than one type of *in vitro* antioxidant assay, and it will be better if the assay could be correlated with the *in vivo* system. Comprehensive discussions on these aspects have been published by Badarinath *et al*. [69].

The selected method of antioxidant assay must be validated first before being routinely used; this is because the ingredients in cosmetics are very complex. The method should have the capability to determine all types of antioxidants. The activities of each antioxidant must be determined before evaluating the activity of the finished products [66]. Due to the complexity of the composition of plant extracts, food, and cosmetics, both the antioxidant capacity and the concentrations of each of its active ingredients including the antioxidants need to be determined [42].

Unfortunately, for quality control purposes in the pharmaceutical industry in Indonesia, only the content of the active ingredients is determined quantitatively, and bioactivity evaluations are generally not performed. For determination of the concentrations of each active ingredient including the antioxidant compounds in samples, it is recommended to evaluate the accuracy profiles of each of the active ingredients including the antioxidants, instead of performing the validation method using classical methods [78].

Due to the possibility of variations in the content of secondary metabolites, including antioxidants caused by different conditions at the sites of cultivation, metabolite profiling studies of the plant extracts are recommended. It is well known that the conditions at the site of cultivation, the method of harvesting and drying, etc. can influence the content of the secondary metabolites in plants both qualitatively and quantitatively.

In addition, in cosmetics that have plant extracts as one of their active ingredients (see Table 1), the possibility of contamination with some toxic materials such as heavy metals, toxic microorganisms, microbial toxins, pesticides, and residual solvents must be evaluated; the content of all these toxic components must be lower than their maximum permitted levels [79].

CONCLUDING REMARKS

At present, the market development of cosmeceuticals is growing very rapidly worldwide. This might be due to the increasing demands of consumers for safe and effective cosmetics.

For relatively good, stable, acceptable cosmeceutical formulations, the selection of the antioxidants, other active ingredients, and the excipients must be optimized. A total quality control of the cosmetics must be performed. This is not being done as of now.

The routine quality control procedures should be able to determine both the bioactivities of all the active ingredients in products (including antioxidant

capacity) and their concentrations before the products are released to the market. This is very important on account of the instability characteristics of known antioxidants.

In conclusion, to provide the consumer with an effective and safe product, it is important that metabolite profiling studies of plant extracts are performed. For effectivity, it is of extreme importance to ensure the stability of the plant extracts or pure compounds to be able to support clinical results, and for safety reasons, it is important to investigate the concentrations of certain toxic components such as heavy metals. The cosmetic industry has undertaken several measures to date to increase the stability of active ingredients using conjugates to change the molecular characteristics as well as the addition of synthetic antioxidants such as BHT/BHA. The issue with this development is that the antioxidant capacity is often reduced and the additive synthetic compounds could have irritancy and sensitizing properties [80].

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ABBREVIATIONS

AAPH	2,2-azobis-(2-amidino-propane)-dihydrochloride
ABTS	2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
AP	antioxidant power
BHA	butylated hydroxyl anisole
BHT	butylated hydroxytoluene
B-PE	β-phycoerythrin
CL	chemiluminescence
CoQ10	coenzyme Q10
DNA	deoxyribonucleic acid
DPPH	2,2′-diphenyl-1,1′-picrylhydrazyl
EDTA	ethylenediaminetetraacetate
ET	electron transfer
FL	fluorescein
GSD	glutathione synthetase deficiency
HAT	hydrogen atoms transfer
HPTLC	high-performance thin layer chromatography
NDGA	nordihydroguaiaretic acid
ORAC	oxygen radical absorbance capacity
PG	propyl gallate
RNS	reactive nitrogen species
ROS	reactive oxygen species

SEM	scanning electron microscope
SOD	super oxide dismutase
TBHQ	tertiary butylhydroquinone
TEAC	trolox equivalent antioxidant capacity
TRAP	total radical trapping antioxidant parameter
UVR	ultraviolet radiation

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Index

Note: Page numbers followed by "*f*" indicate figures, and "*t*" indicate tables.

D

E

F

G

H

I

J

K

L

M

N

O

P

Q

R

S

T

U