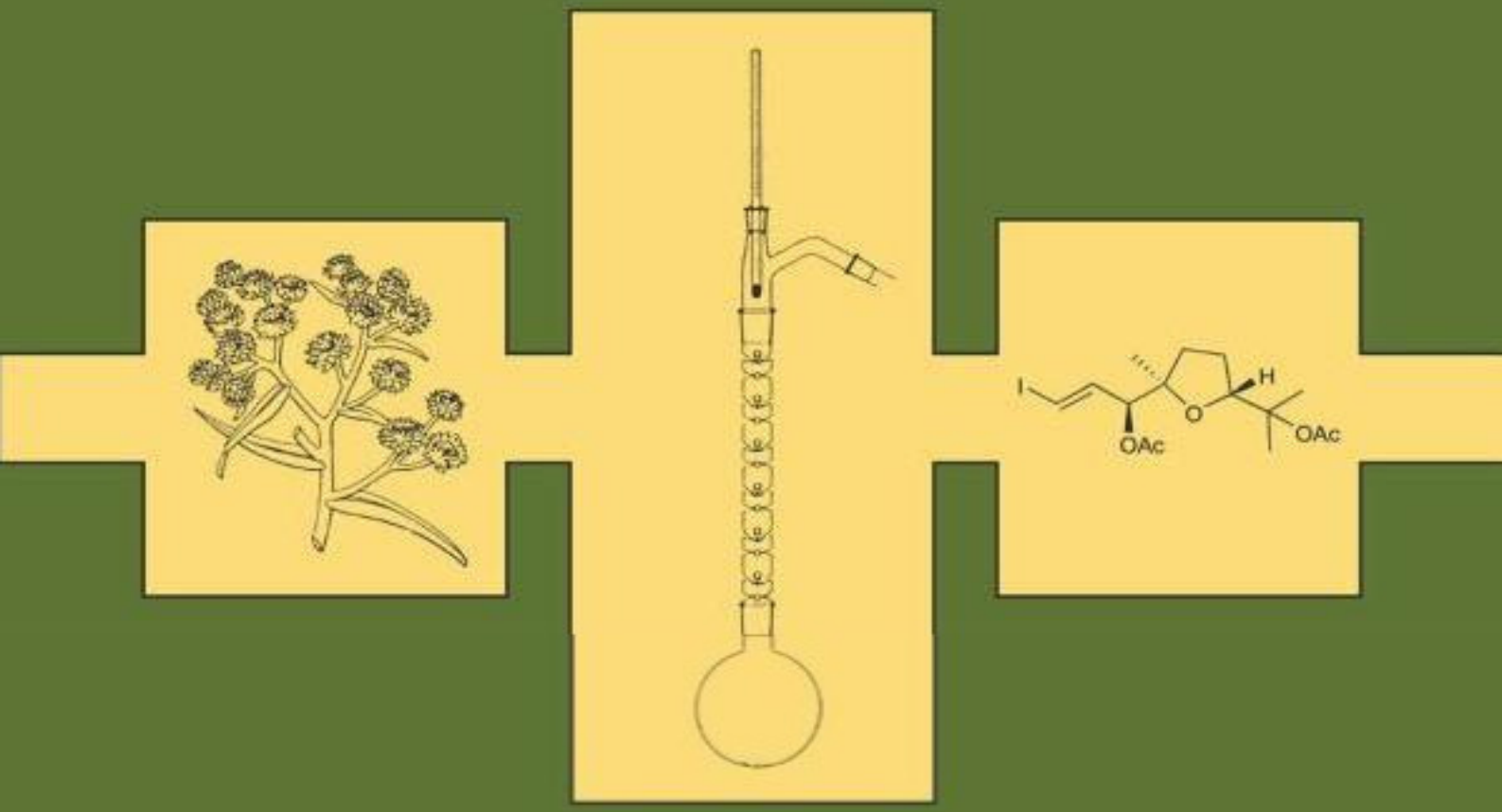
Studies in Natural Products Chemistry

Atta-ur-Rahman, FRS
Editor



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Preface

This volume represents the 38th volume of this series and contains a number of interesting chapters by leading scientists in their respective fields on bioactive natural products.

Chapter 1 by Cvejić and co-workers reviews the developments in the field of phytoestrogens, also called "dietary estrogens." These can bind to estrogen receptors, thereby activating or blocking them. They therefore exhibit estrogenic or anti-estrogenic activities. They also have other potentially beneficially effects on cancer, osteoporosis, vascular diseases, and menopausal systems. The discovery of brevisamide, a key biosynthetic precursor to the ladder-frame polyethers isolated from the red tide dinoflagellate *Karenia brevis*, led to many synthetic approaches that are presented by Lindsley and Fadeyi in Chapter 2. Isoquinoline alkaloids exhibit a wide diversity of biological activities including anti-leukemic, anti-tumor, anti-inflammatory and anti-microbial activities. Chapter 3 by Georghiou and co-workers describes the recent asymmetric synthetic approaches to benzyltetrahydroisoquiniline-based alkaloids.

Chapter 4 by Peduto and co-workers presents the synthesis and biological activities of 1-methoxy-canthin-6-one and related β-carbolines. The reviews by Socolsky, Brown, Wada, Ho and co-workers on fern acylphloroglucinols, mitogen-activated protein kinase and natural phenolic compounds in cardiovascular remodeling, biological activity of diterpenoid alkaloids, respectively, present the current frontiers of research in these fields (Chapters 5–7 and 9). Another interesting chapter on novel plant-derived biological agents and their biosynthetic origin has been contributed by Ata et al. (Chapter 8).

This volume also contains interesting chapters on histone deacetylases as cancer chemoprevention targets, natural products as strategic tools for modulation of biofilm formulation, medicinal herb and plant extracts for their anti-influenza effects and for modulation of immune responses by Ho, Řezanka, and Chon and their co-workers, respectively (Chapters 9–11). Chapter 12 on bioactive compounds from marine gorgonian corals by Qi describes the compounds obtained worldwide from gorgonians including their structures, biological activities, source organisms and structure–activity relationships. An interesting chapter has been written by Flamini on natural herbicides that offer a safer and more environmental friendly approach to weed control (Chapter 13). Chapter 14 by Orhan and co-authors is on selected Lamiaceae plants for their use in neuroprotection. The last chapter by Veluthoor and co-workers describes the potential and use of phytochemicals as anti-tubercular drugs (Chapter 15).

I hope that this volume will be yet another useful addition to the series *Studies in Natural Products Chemistry* which I initiated in 1988. It should be of considerable interest to a large number of scientists working in the fields of phytochemistry, pharmacognosy, medicinal chemistry, and organic chemistry who are interested in natural products for their potential use in medicine.

I would like to express my gratitude to Ms. Taqdees Malik and Mr. Wasim Ahmed 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

Phytoestrogens: "Estrogene-Like" Phytochemicals

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INTRODUCTION

It is widely recognized that many plant-derived bioactive nonnutrients called "phytochemicals" may provide significant health benefits if incorporated into diet either naturally as an integral part of food or as a food supplement [1]. Epidemiological data have shown a strong interdependence between the incidence of the major diseases of Western populations (hormone-dependent diseases and cardiovascular diseases) and diet. Numerous scientific studies are focused on identifying the individual or multiple components of a diet with health beneficial properties. The number of such compounds is rapidly expanding as epidemiologic studies continue to indicate possible associations between diet and various diseases. Among these phytochemicals is the broad class of polyphenolic compounds called phytoestrogens, derived from plants, with "estrogen-like" biological properties.

The majority of phytoestrogens belong to a large group of substituted phenolic compounds known as flavonoids. Because of their biological activities, even being a nonsteroidal phytochemicals, phytoestrogens are also called "plant-derived estrogens" or "dietary estrogens." Regarding the chemical structure, this group of compounds consists of following subgroups: isoflavones, lignans, coumestans, stilbens, and prenylated flavonoids (Fig. 1). Nonflavonoid group of phytoestrogens includes lignans, coumestans, and stilbens.

Due to their structural similarity to female hormone estradiol (17-β-estradiol) phytoestrogens have a potential of exerting "estrogen-like" effects (Fig. 2). Some of these benefits include effects on cancer, cardiovascular diseases, osteoporosis, and menopausal symptoms. Considering purported beneficial effects

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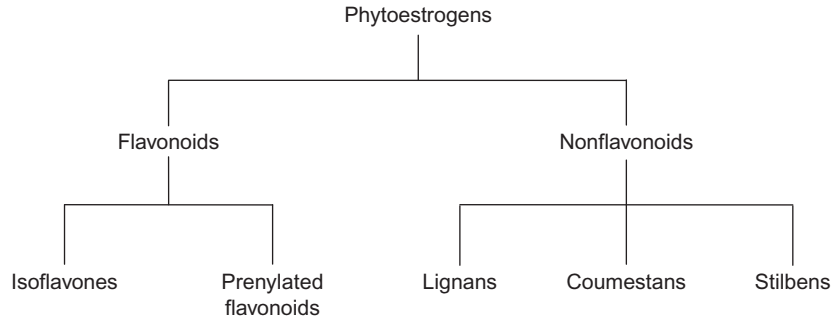

FIGURE 1 Examples of compounds belonging to different groups of phytoestrogens.

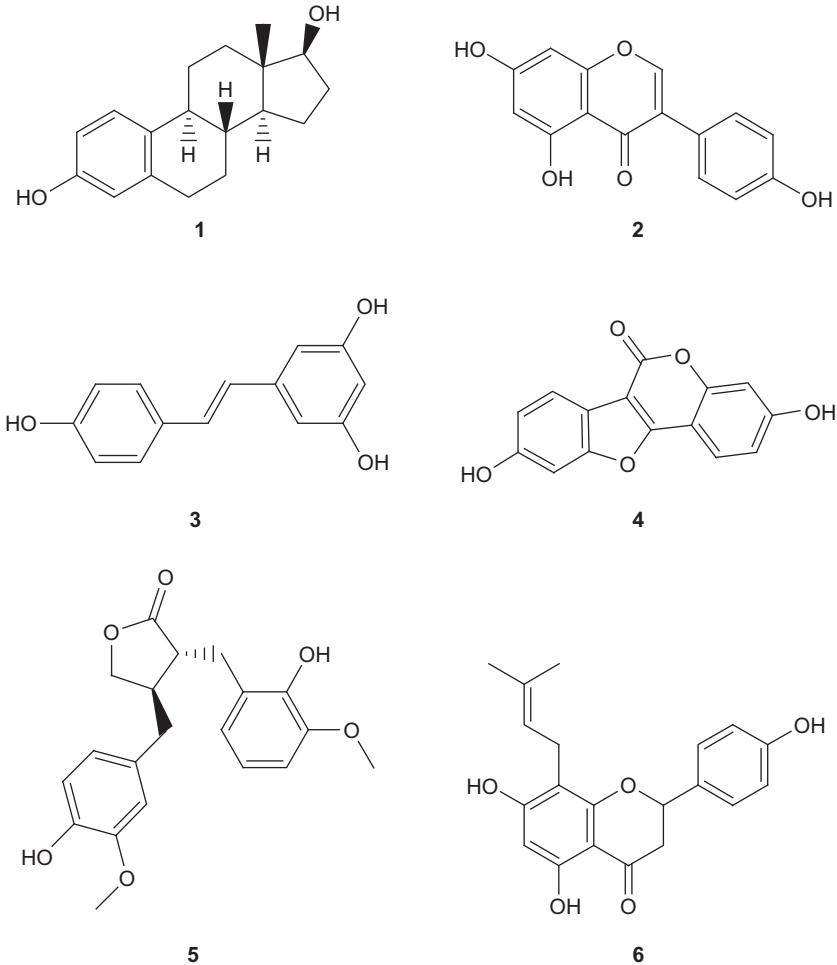

FIGURE 2 Structural similarities of phytoestrogens and estradiol: (**1**) 17β-estradiol, (**2**) genistein (isoflavone), (**3**) *trans*-resveratrol (stilbene), (**4**) coumestrol (coumestan), (**5**) matairesinol (lignan), and (**6**) 8-prenyl naringenin.

and the fact that hormone replacement therapy is not as safe or effective as previously thought [2], the interest in phytoestrogens and use of dietary products, as well as supplements containing phytoestrogens, has significantly increased.

The high content of isoflavones in the diet of some populations has been used as an argument in favor of protective effects of these compounds. Food containing important amounts of phytoestrogens is extensively consumed in the Asian diets [3]. Epidemiological studies have shown a low incidence of several hormone-dependent diseases, such as breast and prostate cancer and postmenopausal symptoms, in Asian countries, which is assumed to be due to high consumption of soy and soy-based foods [4]. Many authors confirm that cardiovascular problems as well as osteoporosis and menopausal symptoms have lower incidence in Asian than in Western countries [5–7].

PHYTOESTROGENS

In the 1940s, it was first realized that some plant-derived compounds could cause an estrogenic effect [8].

It was observed that sheep fed with red clover had multiple fertility problems. Later, it was shown that the clover had high amounts of the isoflavones, formononetin and biochanin A [9], which were among the first phytoestrogens discovered. Biochanin A was isolated in the 1950s [10], from red clover extracts, and later Schultz [11] showed that biochanin A and formononetin are present in red clover as glycosides.

Most phytoestrogens are present in plants and food as glycosides (bound to carbohydrate moiety) and usually just a small fraction appears to be in the aglycone form (unbound phytoestrogens). The glycosides can also occur in acetylated or malonylated form. Today, the most popular phytoestrogens used for human consumption as food or dietary products (supplements) are isoflavones and resveratrol.

Isoflavones

From nutritional and health perspective, isoflavones are the most important class of phytoestrogens of current interest. From all known plant estrogens, this group of compounds has been studied most extensively.

Soybeans (*Glycine max*) and soy products are the most important sources of these compounds in human diet. Soy contains 12 different phytoestrogens including daidzein, genistein, and glycitein (Fig. 3). They are the aglycons which can form three glucoside forms [12], a β-glucoside, a 6″-*O*-malonyl-glucoside and a 6″-*O*-acetyl-glycoside (Fig. 4).

Red clover (*Trifolium pratense*), commonly used as the source of phytoestrogens for dietary supplements, mainly includes biochanin A and formononetin (Fig. 3), while genistein and daidzen are also present. Red clover also contains small amounts of coumestans [13], which do not contribute to a large

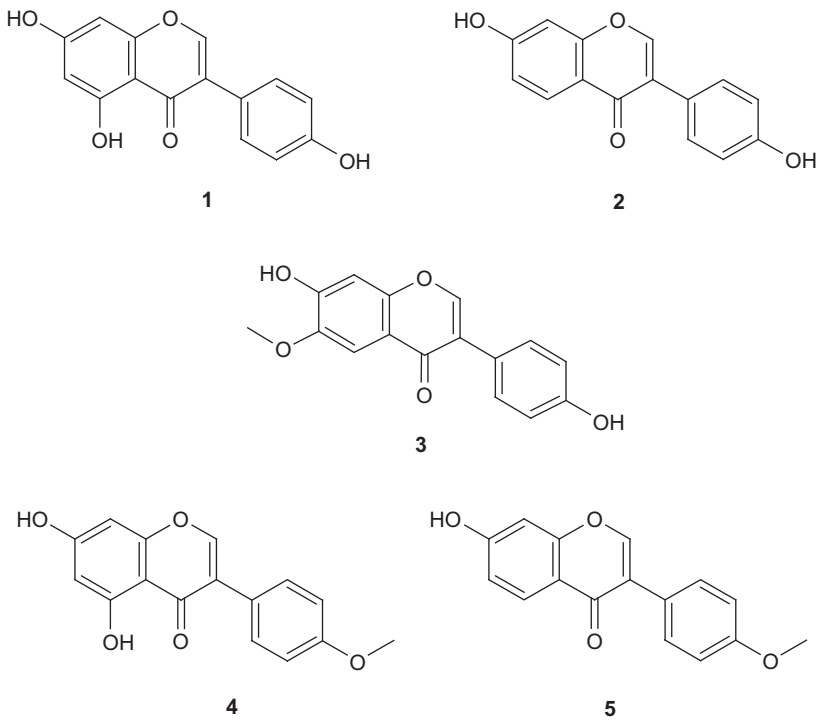

FIGURE 3 Isoflavones: (**1**) Daidzein, (**2**) genistein, (**3**) glycitein, (**4**) biochanin A, and (**5**) formononetin.

extent to its estrogenic effects. Isoflavones are present in red clover as aglycones, glycosides, and malonyl glycosides [14].

Different phytoestrogens do not have the same biological activity. Genistein is the most active of the isoflavones contained in red clover and soybeans [15–17].

The content of isoflavones in soybean is found to be about 0.1–0.4% of dry weight, and the amount and composition of isoflavones varied according to the year, growing area, and environment as well as genotype [18,19].

Depending on the origin and cultivar, the content of isoflavones varies in different red clover samples. Red clover cultivars with high isoflavone content are selected and grown as raw material for pharmaceutical use and preparation of dietary supplements. The concentrations of biochanin A and formononetin in red clover are generally 10 times higher than those of genistein and daidzein and are usually present in amount between 0.025% and 0.3%.

Sources of Isoflavones in Human Diet

Soybean is the most important source of phytoestrogens in human diet. Traditionally, soybeans consumed in Asia are usually divided into two groups:

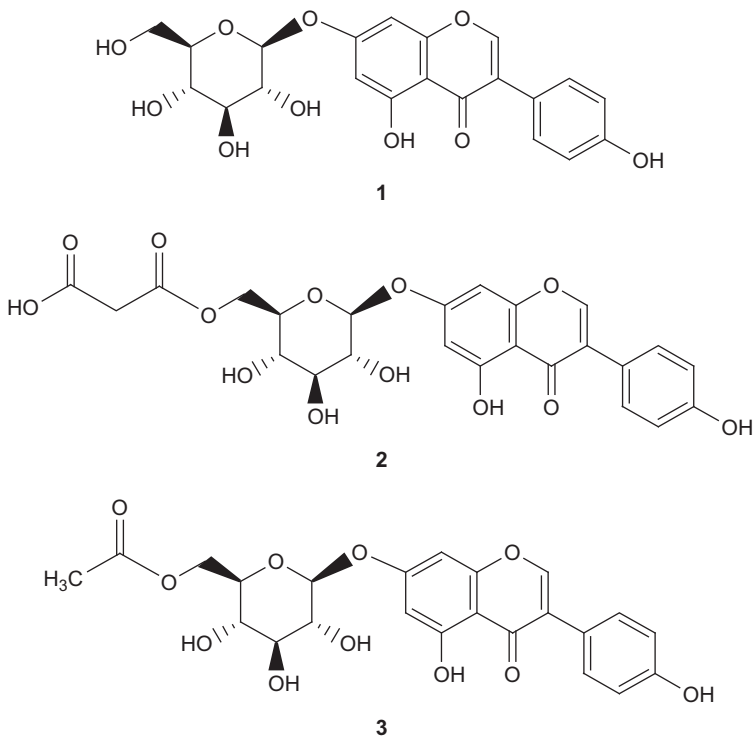

FIGURE 4 Examples of isoflavone glycosidased forms: (**1**) Genistein-β-glucoside, (**2**) genistein-6″-*O*-malonyl-glucoside, and (**3**) genistein-6″-*O*-acetyl-glycoside.

fermented and nonfermented foods. The nonfermented soy foods include fresh soybeans, soybean sprouts, soymilk, tofu, toasted soy protein flour, while miso, natto, soy sauce, and tempeh are considered as fermented soybean products [20]. There are also numerous products, which are soy-added second generation of soy foods (soy bacon, soy hot dog, tofu yogurt, etc.,).

Traditional nonfermented soyfoods, roasted soybeans (1.625 mg/g), and instant soy beverage powder (1.001–1.183 mg/g) have 2–3 times the total amount of isoflavone as compared with fermented soy foods, tempeh (0.625 mg/g), bean paste (0.593 mg/g), miso (0.294 mg/g), and fermented bean curd (0.390 mg/g). The second generation of soy foods contains total isoflavones from 0.034 to 0.289 mg/g. It is observed that fermented soy foods have aglycons as major form present, probably due to hydrolysis during fermentation [21]. Analysis of soy-based infant formulas marketed in the United States showed that content of total isoflavones varied from 214 to 267 μg/g of dry formula or 25–30 μg/ml of reconstituted formula [22].

Red clover is not usually a part of human diet, but it has become very important raw material for production of dietary supplements used for treatment of menopausal symptoms. There are numerous natural supplements

based on soy on the market, and the number of supplements containing red clover extracts is still increasing.

Influence of Various Factors on Isoflavone Content in Soy and Red Clover

It has been shown that different factors influence the content of isoflavones in plant sources. Various studies are focused on determination of these factors in order to produce and/or select a material for further processing into food ingredients or dietary supplements which, due to their favorable characteristics, may enhance health benefits of these products.

Temperature is one of the factors that can influence isoflavone concentration in soybeans. In a study by Tsukamoto *et al.* [23], the isoflavone content was significantly lower in seeds that developed in higher temperatures than in seeds exposed to low temperatures. Different temperature regimes influenced isoflavone concentration in cotyledons, while isoflavone content remained relatively constant in hypocotyls.

Effect of the crop season was researched in Taiwan. Tsai *et al.* [24] compared isoflavone composition in spring (February to June) and autumn (August to December) crops of soybean. Isoflavone contents were significantly higher in the autumn crops than those in the spring crops. The observed result was mainly related to temperature differences between spring and autumn.

Low temperatures and high soil moisture conditions influence the production of seeds with highest isoflavone concentrations [25]. Decrease in temperature significantly increased daidzein, genistein, and therefore the total isoflavone content. On the other hand, glycitein content was less influenced by temperature. In general, it was demonstrated that the magnitude of changes in seed isoflavone content as a response to changes in temperature and soil moisture is cultivar dependent.

Lee *et al.* [26] analyzed isoflavone content in 15 Korean soybean cultivars for 3 years at three different locations and reported significant effect of environmental factors on the isoflavone content.

Seeds of six soybean cultivars grown in four different locations in Ohio in 2002 were analyzed for isoflavone content [27]. It was confirmed that planting location and cultivar influenced total isoflavone content. The only environmental variable that correlated with total isoflavones was rainfall during seed fill.

It is clear that environmental factors can have large effect on isoflavone content, but it is considered that the potential for this trait is mainly under genetic control. Hoeck *et al.* [28] evaluated importance of genotype, year, location, and their interactions with isoflavone content in soybean cultivars. Despite the significant genotype/environment interactions, the performance of two genotypes with the highest and the lowest mean total isoflavone

concentration was quite consistent among the 16 different environments. These results also suggest that genotype is an important factor which potentially determines the total isoflavone content.

It was [21] reported that the total isoflavone contents vary from 1.176 to 3.309 mg/g across years and from 1.176 to 1.749 mg/g across sites within the same year for single soybean cultivar. It was suggested that content and composition of isoflavones could be derived from parent genotype to its hybrids and therefore could be utilized for breeding of soybean cultivars with favorable characteristics [18].

Some recent studies examine the interdependence between soybean seed coat color and isoflavone concentration. Lee *et al*. [29] analyzed 268 soybean samples categorized into five groups according to their seed coat color (black, brown, green, mottled, and yellow). It was concluded that seed coat and cotyledon color differences are not strongly associated to differences in isoflavone concentration. The only consistent difference observed between soybeans of different color was the percentage of aglycones, and the highest concentration of glycitein present in brown seeds compared with other seed colors. Similar results were obtained in the study of Kumar *et al*. [30], where isoflavone levels were not significantly different between yellow, black, and green soybean seeds.

Isoflavone concentration and profile in red clover are also affected by many factors. It is considered that both genetic and environmental factors are important. Sivesind and Seguin [31] analyzed effects of environment, cultivar, plant maturity, plant part, and storage method on the content of two dominant isoflavones in red clover—formononetin and biochanin A. It was established that cultivars had the greatest impact on isoflavone concentrations. At all stages of maturity and in all cultivars, average isoflavone concentrations were the highest in leaves (11.97 mg/g), medium in stems (4.90 mg/g), and the lowest in inflorescence (3.30 mg/g). Also as plant maturity progresses, isoflavone content varies depending on the plant part. The total isoflavone content was 22% higher in fresh material than in either silage or hay. No significant difference in total isoflavone content between the diploid and tetraploid red clover cultivars was established.

Study of Vetter [32] showed different results concerning red clover plant parts. The highest concentrations of isoflavones were found in flowers and the lowest in stems. Tsao *et al*. [33] reported the highest concentration in leaves, medium in stems, and the lowest in petioles. These information suggest that it is not yet clear which plant part is the richest in phytoestrogens.

In general, it can be concluded that different plant parts and same parts harvested at different growth periods show different content of phytoestrogens.

Management can also affect isoflavone content in red clover. It was noticed that harvest time has influence on isoflavone profile. For example, when the first harvest was delayed for 21 days, the formononetin

concentration decreased by 39% [34]. Composition of isoflavones in red clover may vary significantly. Although formononetin and biochanin A are often reported as isoflavones most present in red clover, there are some reports that imply that this is not the general rule [14].

Stilbenes—Resveratrol

Resveratrol (*trans*-3,5,4′-trihydroxystilbene) (Fig. 3) is a phenolic stilbene phytoalexin, which is produced by grapevines as a defense response to fungal infection or other exogenous stimuli (UV radiation, chemical stressors, etc.,) [35]. This phytoestrogen is mainly known as implicated in beneficial cardioprotective actions of moderate regular red wine consumption, so-called French paradox [36]. Observed effect is connected to the ability of resveratrol to act as an antioxidant and an inhibitor of platelet aggregation [37,38]. Moreover, as phytoestrogen, resveratrol may favorably influence several physiological processes [39]. The carcinopreventive activity of resveratrol has been proved [40]. It has been shown that resveratrol has cancer chemopreventive activity in assays representing three major stages of carcinogenesis (initiation, promotion, and progression) and that it has great potential both in prevention and in therapy of a wide variety of tumors [41–43].

Although there are two isomers of resveratrol, *cis* and *trans*, only the *trans* form has been reported to be estrogenic [44]. Physiological activity of *cis*-resveratrol has only been described once and was shown to have potential anticancer activity, as does the *trans*-isomer, by inhibiting kinase activity, a cancer-related factor [45]. As *cis*-resveratrol has not been extensively studied, it has not been clarified yet whether biological activity of this form could be comparable to that of *trans* isoform.

Red grapes and wines usually contain concentrations from 0.2 to 8 mg/l of resveratrol, sometimes even up to 15 mg/l [46,47]. Resveratrol can be present in wine also as glucoside form, called piceid (Fig. 5), which is biologically inactive. After ingestion, piceid is transformed in corresponding aglycone.

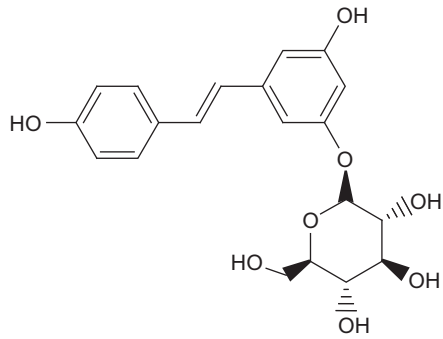

FIGURE 5 Structure of resveratrol glycoside—piceid.

Some authors have reported that resveratrol has high bioavailability, estimated to be over 50% in humans, and that physiological levels can be obtained through drinking red wine [48–50]. Later studies point out to relatively low bioavailability of this compound [51–54]. The oral bioavailability of resveratrol is almost zero due to rapid and extensive metabolism and the consequent formation of various metabolites, for example, resveratrol glucuronides [51]. Resveratrol has low water solubility. Some of the proposed methods for improvement of low solubility and limited biological availability include liposomal [55] and micellar solubilization [56].

Sources of Resveratrol

In the early 1980s, it was reported that dried roots of *Polygonum cuspidatum* (Japanese Knotweed or Mexican Bamboo), contain resveratrol as primary active compound. It has been used in traditional Japanese and Chinese medicine to treat a wide range of ailments, including diseases of heart, liver, and blood vessels [57]. The resveratrol levels in this plant can be as high as 377 mg/100 g of dry root [58]. *P. cuspidatum* is one of the richest sources of resveratrol, and it is also present in grapes, wine, peanuts, and peanut products. In addition to these food products, resveratrol has been isolated from several grass species [59], pine bark [60], ivy, and lilies [61].

Resveratrol is not found in grape flesh, only in the skin. In the process of red vinification, maceration with skin and seed during fermentation leads to a higher concentration of resveratrol in red wines in comparison to white wines [47,62]. The *trans*-isomer occurs in the berry skins of most grape cultivars, and its synthesis is stimulated by UV light, injury, and fungal infection [35,63].

Influence of Various Factors on Resveratrol in Wine

Resveratrol content in wine depends on many different factors including variety, harvest year, climatic conditions, UV light, wine-making technology applied, and presence of *Botrytis cinerea* fungus [37,39,61,64,65]. Also, grapes exposed to influence of *Uncinula necator* give extracts with higher resveratrol content [66,67]. In addition, it has been shown that skin contact time could affect resveratrol extraction, but it has been observed that the maximum extraction time is not the same for all grape varieties [68].

Lignans

Lignans are group of compounds that contain dibenzylbutane skeleton and they have a role in building plant cell wall. These compounds are widely spread in various sources, from which the most included in human diet are cereals, fruit, and vegetables [69]. The most studied phytoestrogenic lignans are secoisolariciresinol (Fig. 6) and matairesinol (Fig. 3) which are transformed under

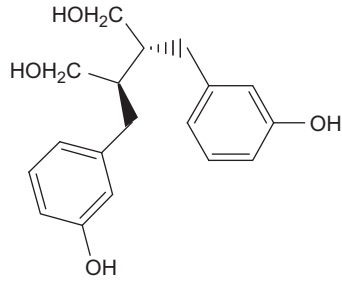

FIGURE 6 Structure of secoisolariciresinol.

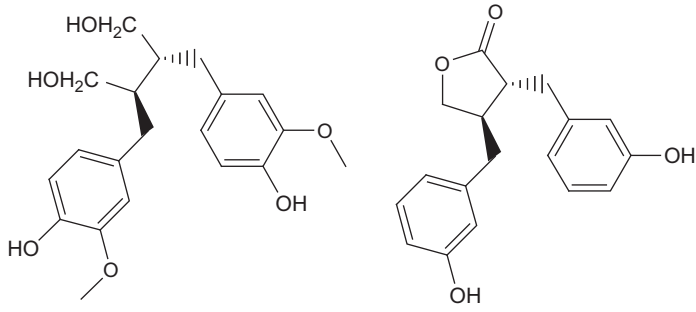

FIGURE 7 Examples of mammalian lignanes: (**1**) Enterodiol and (**2**) Enterolactone.

the influence of gut bacterial flora into enterodiole and enterolactone, respectively [70]. Additionally, enterodiole can be metabolized to enterolactone (Fig. 7). These compounds (enterodiole and enterolactone) are metabolic products and are not normally present in plants. Secoisolariciresinol and matairesinol do not possess estrogenic activity, but their metabolic products (enterodiole and enterolactone) have phytoestrogen properties, and they are often referred to as mammalian lignans. Recently, more dietary precursors of enterolactone, such as arctigenin, lariciresinol, and syringaresinol have been identified [71,72].

Many food products have been analyzed for plant lignan content or the ability to produce enterodiole and enterolactone. Although lignans are widely spread in different food products, their biological properties have not been so extensively studied as isoflavones [69]. Foodstuff rich in lignans include: rye bread, oil seeds, brewed, black tee, grains, cereals, pumpkin seed, etc., [73]. Flaxseed is one the richest sources of lignans, with about 0.8 mg of secoisolariciresinol per g of dry weight [74]. Secoisolariciresinol is widely present in diet, while matairesinol is found in smaller amounts or traces [73].

Concerning daily intake of secoisolariciresinol and matairesinol, substantial differences have been observed across populations. For example,

postmenopausal non-Asian women from California consume an average of 139 and 36 μg/day of secoisolariciresinol and matairesinol, respectively, mainly from orange juice, coffee, bread, and fruit [75]. On the other hand, high consumption of grain and rye bread, contributes significantly to high levels of daily intake of secoisolariciresinol and matairesinol in postmenopausal Dutch women (130 and 80 μg/day, respectively) [76].

Estrogens are mitogenic factors necessary for the growth and differentiation of certain tissues, for example, breast and prostate [77], and have been implicated in the initiation and promotion of several cancers [77,78]. Because of its structural similarity to 17β-estradiol, lignans can influence cancer through estrogen-mediated pathways. Also, lignans can act through mediation of other growth hormones such as insulin-like growth factor and vascular endothelial growth factor.

Because of its structural similarity to endogenous estrogens, enterolactone may act as a weak estrogen agonist or antiestrogen. Numerous studies have demonstrated the biphasic nature of enterolactone *in vitro* and *in vivo*. Studies conducted by Thompson [79] demonstrated the ability of purified lignans to compete with estradiol, stimulate sex hormone binding globulin production, and inhibit steroid binding. Also, it has been observed that lignans are able to inhibit enzymes involved in steroid synthesis [77].

Another study confirmed that flaxseed supplementation significantly increases the ratio of urinary biomarkers used to assess breast cancer risk (an increase in the ratio is considered protective) in both postmenopausal [80] and premenopausal women [81]. Still, because of the fact that flaxseed consists of different potentially active components, the effects observed cannot be attributed exclusively to lignans.

Coumestans

The main coumestans with phytoestrogenic activity are coumestrol (Fig. 2) and 4-methoxycoumestrol (Fig. 8). Coumestans are less common in human diet than isoflavones [69]. They are also found in legumes, especially food plants such as sprouts of alfalfa and mung bean (*Vigna radiata* (L.) Fabaceae) [73,82,83] and they are abundant in clover [84]. Soy sprouts also show high levels of coumestrol (71.1 μg/g wet weight) [69]. This compound is

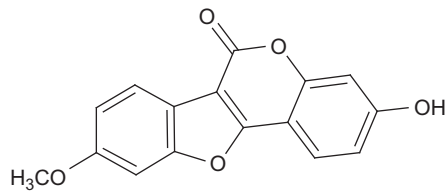

FIGURE 8 Structure of 4-methoxycoumestrol.

often considered to be phytoalexin, a compound formed in a plant as a result of injury or disruption, which can also protect the plant from insects or bacteria [69].

Some studies showed that coumestrol competes with estradiol for cytoplasmic receptors in mammary tumors and breast cancer cells [85,86]. Also, it is important to emphasize that coumestrol and genistein have similar binding affinities to estrogenic receptors [87]. *In vitro* coumestrol has been reported to inhibit bone resorption and to stimulate bone mineralization [88].

ABSORPTION AND BIOAVAILABILITY OF PHYTOESTROGENS

For the full performance of the biological activities of phytoestrogens it is important that compounds are present in a chemical form that is biologically available to the organism.

Conversion of Phytoestrogens in Organism

The main part of isoflavones in the plant is conjugated to sugar residues and is abound as β-glycoside. To these glycosides malonyl or acetyl residues may be attached. For their absorption in an organism, the hydrolysis of the glycosides is necessary [89].

The evidence in the literature suggests that biological activity of isoflavones does not depend upon the type of glycoside form. However, some results show that there is a certain difference in absorption rate between aglycone and conjugated forms in favor of aglycones [90]. Biochanin A and formononetin are the methylated precursors of genistein and daidzein, respectively. After ingestion, in the gut, intestinal bacteria convert conjugated isoflavones to their respective aglycones (malonyl and acetyl derivatives are converted into glycosides) and methylated isoflavones to their demethylated forms (biochanin A and formononetin to genistein and daidzein, respectively). The glycoside forms of isoflavones are further hydrolyzed by β-glucosidases to the aglycone forms in the jejunum [91]. Aglycones are either absorbed by the enterocytes or in the case of daidzein partly converted into equol (Fig. 9) and/or methylangelolensin which are also absorbed [92–94]. It has been shown that the ability to metabolize daidzein into equol is not the same for all persons [95,96]. Most probably, the bacterial environment is essential

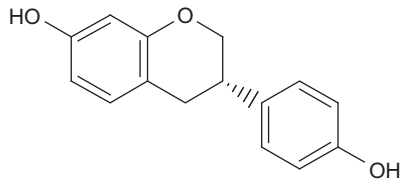

FIGURE 9 Structure of equol.

for the conversion of isoflavones into aglycones and equol [97]. Not all persons seem to absorb isoflavones to the same extent. This may be due to individual differences in the status of the flora of the large intestine.

Bioavailability of Isoflavones

The bioavailability is not significantly different when the isoflavones are consumed as either aglycone or glucoside [98]. On the contrary, estrogenic activity of the isoflavones depends on the type of aglycone. It has been found that genistein has higher affinity for estrogen receptors in comparison to daidzein and glycitein [99].

As isoflavones from red clover are methylated derivatives (formononetin and biochanin A), soy phytoestrogens are bioavailable after less metabolizing steps than those from red clover. Due to their low binding affinity to estrogen receptor, formononetin and biochanin A can be considered as precursors of daidzein and genistein, their demethylated derivatives [100]. It was shown that when present in the aglycone forms, isoflavones are absorbed faster than in the conjugated forms. This could be explained by the fact that conversion of glycosides to aglycones by glucosidases may be a rate determining step [90].

The great variety in results concerning the benefits of consuming an isoflavone-rich diet could be due to difference in equol-production. One of the examples of interindividual variation of isoflavone metabolism is difference of the metabolization of daizdein to equol among individuals [101]. As equol has a higher estrogenic activity than its precursor daizdein, "equol producers," with higher rate of daizdein metabolization might benefit more from isoflavones than "nonequol" producers.

In the manufacturing process of food and infant formula, soy protein isolate is generally used. It was shown that it can efficiently deliver isoflavone and is capable of providing significant portions of the total dosage into circulation in the active aglycone form [102].

PHYTOESTROGENS "ESTROGEN-LIKE" ACTIVITIES

Biological "estrogen-like" activities of phytoestrogens derive from their structural similarity to human estrogen, as well as from consequent binding ability to the estrogen receptors. These compounds could act as estrogen agonists and antagonists, and are thus able to function as selective estrogen receptor modulators.

Phytoestrogens Structural Similarity to Estrogens

Regarding the structure, phytoestrogens have similar polarity and molecular weight as estradiol. A significant feature of the chemical structure of

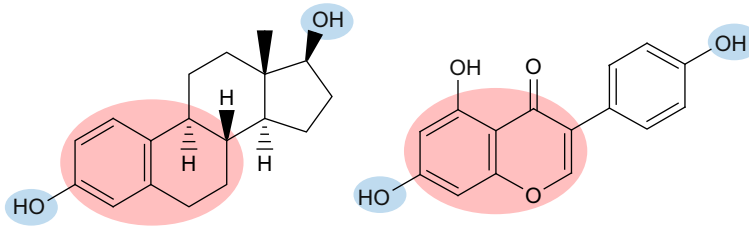

FIGURE 10 Structural similarity of estradiol and genistein.

phytoestrogens is the presence of a phenolic ring that, generally, is a prerequisite for binding to the estrogen receptor [103].

In isoflavone structure, the A and C rings are similar to the A and B rings in estradiol. Also, comparing isoflavone genistein to estradiol it is obvious that the actual distance between the two hydroxyl groups on both molecules is similar (Fig. 10). These groups are critically located to enable binding to the estrogen receptor protein. The flavonoid core structure with two to four hydroxyl groups in certain positions is fundamental for the estrogenicity, but it was observed that methylation of hydroxyl groups diminishes the estrogenic effect. Isoflavones have a greater estrogenic activity than other flavonoids, due to the two hydroxyl groups positioned in the benzyl ring [104].

As bioactive compounds structurally similar to estrogen, phytoestrogens have the ability to bind to estrogen receptors either activating or blocking them, depending on the type of estrogen receptors, and consequently, exhibit estrogenic and/or antiestrogenic effects [105–109]. These compounds preferentially bind to β-estrogen receptors which are found in the central nervous system, bones, vascular walls, and the urogenital tract. Unlike estrogens, isoflavones have low affinity to α-receptors of breast and uterine tissues. These effects are also shown to be tissue specific.

Mechanism of Action

Estrogens are hormones which are produced in the ovaries and testicles. Besides in the reproductive system, these compounds have many biological effects in other parts of the organism.

Agonist and Antagonistic Behavior of Phytoestrogens

When estrogen binds to estrogen receptors, localized in the nucleus, dimers are formed. These dimers then interact with the estrogen response element, which regulates transcription of estrogen-responsive genes. The dominant form of estrogen in the body is 17β-estradiol, although any compound that induces receptor dimerization and subsequent binding to the estrogen response element can be considered an estrogen.

Antagonistic effects can occur when a compound is able to bind to the receptor, but dimer formation either does not occur or the correct configuration for activation is not attained. Compounds having the ability to act as estrogen agonists and antagonists are referred to as Selective Estrogen Receptor Modulators. These agonist/antagonistic effects are believed to be responsible for the different effects of phytoestrogens compared to estradiol.

There are two known estrogen receptors, estrogen receptor α (ERα) and estrogen receptor β (ERβ) which can be localized within the same cell. However, they vary in tissue distributions and can have different effects on mixed agonists and antagonists [110]. Their actions at the cellular and molecular level are influenced by many factors, including but not limited to concentration dependency, receptor status, presence or absence of endogenous estrogens, and the type of target organ or cell. Human estrogen has a high binding affinity for the α receptor, while isoflavones have a high affinity for the β receptor.

Depending on the concentrations of estradiol, phytoestrogens exert a selective action—in some tissues they display proestrogenic responses, whereas in others they inhibit estogenic action [111–113]. When the amount of circulating endogenous estrogen is low, like in postmenopausal women, the binding of natural plant estrogens to cells will exhibit an agonistic effect and increase the overall estrogen effect. This could be beneficial in all conditions where low estrogen levels have negative effects on cell metabolism and organ function, as in case of osteoporosis or postmenopausal symptoms (e.g., hot flushes). For example, estrogen activity of phytoestrogens that occurs in nonreproductive tissues has positive influence on prevention of osteoporosis and cardiovascular conditions. On the other hand, in case when normal estrogen levels are present, they may demonstrate an antiestrogenic action. This may be an important feature in prevention of hormone-dependent cancers like breast cancer.

Isoflavones Binding to Estrogen Receptors

Isoflavones have receptor binding affinity for ERα, ERβ, progesterone receptor (PR) and androgen receptor (AR). The higher binding affinity to ERβ compared to ERα can explain why isoflavones present in food supplements could be used to treat menopausal disorders and may reduce risk of breast cancer. The preferential binding of nonsteroidal estrogens to the ERβ receptor suggests that they may exert their actions through distinct and separate pathways from those of classical steroidal estrogens. The binding affinity of the plant estrogens is, however, much weaker than that of the human estrogen. Dietary estrogens are weakly estrogenic (10^{-2} to 10^{-3} fold, depending on the system examined) when compared to estradiol or estrone, the principal circulating estrogens of most mammals [114–116]. Isoflavones, as the female hormone estrogen, can bind to cellular estrogen receptors of different organs

in human body. The relative binding affinity of genistein has been shown to be only 0.0125 compared to 1.0 for estradiol [116], and the relative estrogenic potency has been calculated to be 0.0008 versus 1.0 for estradiol [117]. In general, genistein and daidzein have 100 and 1000 times lower affinity for binding to estrogen receptor than estradiol [116,117].

Genistein has one-third the potency of estradiol when it interacts with ERβ, and one thousandth the potency of estradiol when it interacts with ERα as determined by expression of luciferase reporter gene construct in kidney cells that had been cotransfected with ERα and ERβ [117].

HEALTH AND PHYTOESTROGENS

Many potential health benefits of these compounds have been investigated, including their effect on cancer, vascular disease, osteoporosis, and menopausal symptoms. Apart the estrogenic and antiestrogenic activity, phytoestrogens have also effects that are unrelated to estrogen activity. As well as other compounds present in plants, phytoestrogens may act as natural antioxidants [118,119].

Epidemiological evidence and experimental data obtained from animal studies are highly suggestive of beneficial effects of isoflavones on health, but the clinical data supportive of such effects remain to be definitively established.

Although there are numerous studies concerning isoflavones, experimental animal data on their long-term effects are not clearly in accordance with results of epidemiological studies. Some of the reported studies show significant positive effects on human health.

Menopausal Symptoms

Estrogen hormones play a vital role in different aspects of women's health. These compounds improve maintenance of bone strength, reduce incidence of cardiovascular diseases, and also influence emotional conditions. Many women suffering from menopausal disorders, such as hot flushes, are seeking an alternative to conventional hormone replacement therapy, mainly because of the risk of serious adverse effects such as breast cancer, heart disease, etc.

As women in menopause often have low estrogen blood level, phytoestrogens can act as hormonal substitution by reducing some symptoms connected with estrogen deficiency. This theory corroborates with the fact that women in Oriental countries, generally having higher phytoestrogen intake by diet than women in other regions, have less frequent hot flashes and other menopausal symptoms. Many scientific studies were, and still are, focused on the potential of plant estrogens to reduce menopausal disorders.

It was shown that under certain conditions plant isoflavones have the same efficiency in reduction of these symptoms as oral estrogens [120]. However,

scientific evidence in this area are contradictory, considering the fact that some studies reported reduction of hot flashes and other menopausal symptoms [121,122], while others did not give similar results [123–125]. Reduction of hot flashes was observed just in three out of eight studies performed in duration longer than 6 weeks [126], while other studies have not shown positive effects of isoflavones within 24 weeks [127] or 2 years [128]. In 11 studies, statistically significant decrease in hot flushes is obtained only with more than 15 mg of genistein, except for one case [129].

Studies of red clover supplements for relief of menopausal hot flashes have been generally disappointing, although their interpretation is unclear because of lacks in design, such as short duration or low dosage. There are still ongoing studies that aim at defining whether the estrogen activity of phytoestrogens is sufficient for a significant effect on estrogen deficiency. Numerous different commercial preparations containing phytoestrogens are available without medical prescription, and they are recommended for treating menopausal disorders. In order to confirm that isoflavones are solely responsible for purported biological activities, intensive monitoring of effects and products is necessary.

Osteoporosis

It has been observed that in female populations consuming food rich in phytoestrogens (the Asian population) frequency of bone fracture is generally lower than among the Western populations. Many studies have been carried out in order to reveal whether the increased consumption of soy isoflavones in Western populations could assist in prevention of osteoporosis. Some indicators, such as population studies and animal models support the hypothesis that isoflavones may affect the preservation of bone mass, which could have a potential application for reducing the risk of osteoporosis in postmenopausal women.

However, performed clinical studies are usually of insufficient scale and duration, often resulting in incoherent results which disable clear conclusions. Short-term clinical trials showed different results concerning the influence of increased phytoestrogens intake on biochemical markers of bone formation and bone resorption.

While some controlled studies showed that increased intake of phytoestrogens (by consuming soy foods, soy protein or soy isoflavones) improves markers of bone formation and resorption in postmenopausal women [130–132], in other cases this effect is missing [7,133,134]. Also, it was shown that studies measuring bone marker levels comparing with those measuring actual bone mineral density or bone mineral content may yield different results. Thus, some studies showed an improvement in mineral content, but not in mineral density of bones [7,135]. On the other hand, two of the three clinical studies that used soybean isoflavone extracts are suggestive of a positive effect on bone mineral density [136].

During two clinical studies, it has been observed that reduction of mineral density after 6 months was lower in menopausal women who consumed soy protein with isoflavones than in women who consumed the same amount of milk proteins [7,99]. Experiments on animals in which genistein or estradiol were given simultaneously showed that the tested substances have similar properties related to the increase in bone mass [137]. Two-year clinical study demonstrated that daily consumption of soy milk, which contains isoflavones, reduces bone mineral density loss in the lumbar region of the spinal cord, compared to the same consumption of soy milk without isoflavones [130].

It has been shown that chronic dietary exposure to soy isoflavones may have beneficial impact on bone health and clearly is different from results obtained after acute therapeutic application studies of treating already existing symptoms [138].

Studies using dietary supplements containing phytoestrogens for preservation of bone health show some initial promise, but follow-up studies are needed to determine the specific types and locations of bone(s) that receive benefit from isoflavones.

There is evidence that a diet rich in isoflavones has moderate effects on bones, but for a definitive conclusion whether increased intake of soy isoflavones markedly reduces the risk of osteoporosis or fractures due to osteoporosis, wider studies with monitoring of various parameters need to be performed.

Cancer

Mild estrogen activity of phytoestrogens suggests that these compounds could extenuate the negative effect of more potent endogenious estrogens on breast and endometrial cancers. It was observed that incidence of hormon-depending cancers is lower in countries where the intake of phytoestrogens is higher (Asian countries) [139]. It was also reported that incidence of prostate cancer is relatively low. Apart from the diet, some other factors, for example, quality of life or hereditary factors, could affect this difference.

Numerous epidemiological studies indicate that correlation between intake of soy foods and phytoestrogens with incidence of breast cancer varies among different studies, some showed protective effect, while others showed none [139–141]. Positive effect of isoflavones was demonstrated on animal models of breast and endometrial cancers [77,142].

Genistein shows anticancerogenic effects as antiangiogenesis and apoptosis in both, *in vivo* and *in vitro* studies. Study of genistein influence on breast cancer cells indicate that this molecule leads to partial blockage of estrogen receptors, but its inhibitory activity on cancer cells is very complex [135]. In the research conducted so far, red clover isoflavones do not seem to influence the endometrium or breast.

Prevention of Cardiovascular Diseases

Cardiovascular diseases are the worldwide leading cause of mortality, in both, men and women. The main factors that increase risk of cardiovascular diseases are high cholesterol and blood pressure, improper diet and lifestyle, overweight and physical inactivity, diabetes, gender, and family history of disease.

Low-density lipoprotein (LDL) cholesterol has the central role in the process of atherosclerosis. It passes through the walls of blood vessels, where it becomes oxidized by free radicals and accumulated, which may cause blockage of blood vessels lumen, as well as a blood vessels thrombosis. On the other hand, high-density lipoprotein (HDL) cholesterol demonstrates protective effects by preventing the oxidation of LDL cholesterol and reducing the accumulation of cholesterol in blood.

After epidemiological studies indicated a connection between consumption of soy and its products with reducing risk of cardiovascular disease, many researchers have focused their interest on the role of isoflavones in these effects. Cardiovascular prevention achieved through consuming phytoestrogen-rich diet is affected by several mechanisms, some of which include: reduction of LDL oxidation by antioxidants present in soy, improving vascular reactivity, modulation of lipoprotein metabolism [3,143].

Earlier studies pointed out the possible influence of phytoestrogens on some important parameters for atherosclerosis development. Exposure to dietary soy during fetal development and early life may reduce susceptibility to cardiovascular diseases and obesity in adulthood [144].

Controlled clinical studies performed before 1995 suggested that consuming 25–50 g/day of soy proteins instead of animal proteins reduces serum LDL cholesterol by 13% [143]. However, recent researches show that this percentage is much lower.

In a meta-analysis of 17 randomized trials [145], it has been found that isoflavone extracts have no effect on serum lipids. Data from 13 studies suggest that soybean food consummation may have a beneficial effect on lipoprotein status, while there are limited data to support a lipid-lowering effect of isoflavone extracts [136].

Recent evaluation of 22 controlled studies pointed out that the inclusion of 50 g/day of soy protein in diet reduces LDL cholesterol by around 3% [146]. Some data show that consummation of soy protein containing isoflavones is more effective in lowering LDL cholesterol than consumption of soy protein without isoflavones [147,148], although the consumption of isolated soy isoflavones (as supplements or extracts) showed no significant effects on serum lipid profile [149].

Enriched isoflavone supplements do not have consistent, clear effects on LDL or HDL levels in most menopausal women, but biochanin A—rich supplements may selectively lower LDL in men.

Several studies have been focused on detailed examination of the effects of isoflavones on the cardiovascular system [150,151]. Also, meta-analysis shows that isoflavones do not affect the concentration of blood lipids [152]. Given that isolated isoflavones have no effect on reducing cholesterol level in humans, probably other active substances, which could have synergistic effects with isoflavones, contribute to cardioprotective effects of consumption of soy and its products [3].

A normal artery function plays an important role in preventing cardiovascular diseases. The ability of artery dilatation and elasticity of blood vessels are compromised in people with high risk of cardiovascular disease. The majority of studies have not found significant improvement in vasodilatation in postmenopausal women consuming isoflavones [153–155].

A strong correlation between arterial rigidity and atherosclerosis has been demonstrated. Soy isoflavones and protein supplementation in periods of 5 weeks and 3 months leads to a significant reduction in arterial rigidity [156,157]. Isoflavone supplements seem to significantly improve arterial elasticity, which is expected to decrease atherosclerosis risk. Preliminary studies therefore suggest that supplementation with isoflavones may cause the reduction of arterial rigidity, and thus could play a role in preventing cardiovascular disease. These observations require further confirmation.

Additional Remarks

In the majority of studies, analysis of biological effects of consuming products and supplements rich in phytoestrogens were observed after administration of certain dosages of isoflavones, supplements, or food. It is possible that in some cases, similar studies have contradictory results because of different content of biologically active components in the administered products. In addition, isoflavones are not equally absorbed by all individuals, which probably depends on individual differences in gastrointestinal flora status [158,159] and contributes to the final effect.

The observed discrepancies in health benefits of isoflavone supplementation in clinical trials may occur due to: differences in intestinal bacterial flora which could influence bioavailability of soy isoflavone metabolites [136,160], differences in dosage–response effects [161], length of isoflavone supplementation, limited number of subjects, and the preexisting metabolic status of subjects included in supplementation trials [162].

Given that a large number of epidemiological studies show numerous positive effects of products containing phytoestrogens on human health, it is very important to accurately determine all active components as well as mechanism of their action. For this purpose, it is necessary to determine and to correlate the concentrations of individual potentially active components with the observed biological effects, which could contribute to disease prevention and improve health status and quality of life of population.

DIETARY SUPPLEMENTS

A dietary supplement is a product taken orally that contains a "dietary ingredient" intended at supplementing diet. The "dietary ingredients" in these products may include: vitamins, minerals, herbs or other botanicals, amino acids, and substances such as enzymes, organ tissues, glandulars, and metabolites. Dietary supplements can also be extracts or concentrates, and may be found in many forms such as tablets, capsules, softgels, gelcaps, liquids, or powders. They can also be in other forms, such as a bar, but if they are, information on their label must not represent the product as a conventional food or a sole item of a meal or diet [163].

Phytoestrogen Supplementation

Due to the purported beneficial effects, use of products and supplements containing isoflavones has significantly increased. They are in everyday use for prevention or reduction of different health problems. Number of commercially available products containing phytoestrogens is significantly increasing [3,164]. Dietary supplements containing phytoestrogens are commonly used for treatment of menopausal symptoms in women as an alternative to hormonal substitution therapy [151,165]. These products are widely commercialized in many countries as alternative therapy for alleviating menopausal discomforts and the possible prevention of diseases such as osteoporosis and breast cancer. The most common reason for supplementation with phytoestrogens is treatment of vasomotor symptoms (menopausal hot flashes) [166].

Actually, regulation does not clearly define criteria regarding the quality of dietary products. Focus is mainly on health safety and not on efficiency of these products. Widely present on market, products based on soy or red clover extracts are more often used as natural alternative of hormonal therapy. These preparations, sold as nutritional supplements, are often blend of extracts from different plants and usually contain extracts from soy (*G. max*) and/or red clover (*T. pratense*).

Isoflavone Content in Supplements

The obtained results confirm that in large percentage of the analyzed supplements, the determined content of total isoflavones does not comply with data given by the manufacturer. Usually, only the total isoflavone content or a ratio of summed isoflavone content for commercial isoflavone products is declared, rather than specific chemical content of isoflavones. Because of that, it could be difficult to determine clinical doses of specific isoflavones, as well as to correlate the obtained biological effects and an individual compound.

Results obtained by analysis of phytoestrogens content in dietary supplements showed no uniformity in the profile and concentration of these

compounds among the products of a particular category. The phytoestrogen levels also vary from batch to batch and from product to product, causing more difficulty in interpretation of the results. Since isoflavones in aglycone forms are absorbed faster than in conjugated forms it could be concluded that the aglycone-rich isoflavone diet or supplements are more effective than glycoside-rich products. Accordingly, isoflavone supplements should contain mainly aglycones, which can be more easily absorbed [17]. Also, products are different in composition of isoflavones, which can influence their quality, because all phytoestrogens do not have the same biological activity. It was implied that supplement labeling is unclear about isoflavone concentration, and also that very different information are given by manufacturer about recommended daily intake.

In a study performed by Boniglia *et al*. [167], only the concentrations of free, aglycone forms were determined. In all analyzed samples genistein or daidzein were dominant, while the glicitein content was generally low. From the analyzed samples, 64% had lower isoflavones content than declared which corroborates with results obtained by Nurmi *et al*. [168].

Results reported by Stürtz *et al*. [169] showed that in the most of the dietary supplements determined phytoestrogen content does not match the content specified by the manufacturer on the package. It has been shown that in dietary supplements isoflavones are predominantly present in glycoside forms (glycitin, genistin, and daizdin) [169]. In the same study, no malonyl glycoside has been detected in the analyzed supplement samples. Values of total amount of phytoestrogens, obtained as the sum of all glycosides greatly exceeded the declared values for corresponding product. Even expressed as aglycon equivalent, these values were, in more than 73% of the analyzed samples, different from those declared for isoflavone content (Table 1). The declared values were mostly in a range between 17.5 and 50 mg, in some cases even up to 100 mg of phytoestrogens per tablet/capsule [169].

In another study, it has been observed that the analyzed supplements contained from 23% up to 68% less isoflavones than declared (Table 1) [168]. Total amount of isoflavones, calculated as aglycon equivalents, was from 0.12 to 201 mg/g. As manufacturers did not indicate in which way the content was expressed, as aglycone equivalents or conjugate forms, authors assumed that the declared amount of isoflavones refers to total isoflavones in conjugated forms [168].

In general, it has been observed that manufacturers reported only the total content of isoflavones, with no clear indication on how the concentrations were calculated and whether the stated amount of isoflavones is given as free or conjugated forms [167–169]. As it is not possible to asses with which isoflavone form the declared mass is related and given that the molecular weight of glucose unit makes up to 50% by weight of glycoside [168], this approach significantly complicates the interpretation of the results. As individual isoflavones do not have the same biological activities, it is important that

TABLE 1 Review of Study Results for Dietary Supplements Containing Phytoestrogens

Reference	Number of Samples	Supplement Type	Results
[168]	15	Supplements based on soy	– in 10 supplements—content lower than declared (from −23 to −68%) – in 1 supplement—complies with labeled content – in 4 supplements—do not contain isoflavones
[170]	6	Supplements based on soy, red clover, and kudzu	– isoflavone content—7.3–80.4 mg/g of sample – obtained values are not compared to declared content
[171]	13	Supplements based on soy	– isoflavone content—0.4–57.6 mg/g of product – obtained values are not compared to declared content
[167]	14	Supplements based on soy	– in 9 supplements—isoflavone content lower than labeled (from −9% to −91%) – in 3 supplements—higher content (from +18 to +34%) – in 1 supplement—complies with labeled content – in 1 supplement—does not contain isoflavones
[169]	11	Supplements based on soy	– in 2 supplements—content higher than declared (from +39.5% to +66%) – in 6 supplements—content lower than declared (from −11% to −49%) – in 3 supplements—comply with labeled content

composition of isoflavones and amounts of individual compounds are specified on the supplement labeling.

The qualitative differences of individual compounds were observed, which is explained by differences in raw material origin as well as processing method applied [167–169,171].

Delmonte and collaborators examined isoflavone content in dietary supplements containing extracts of soybeans, red clover or kudzu (*Pueraria*

lobata), before and after acid hydrolysis [170]. The hydrolysis was performed in order to convert isoflavone conjugates into aglycone forms and simplify the analysis because of complexity of supplements composition [170].

As in most previous studies, in a study performed by Prabhakaran, the total isoflavone content was expresses as aglycone equivalents. Significant differences were observed in the recommendations concerning the daily intake of supplements among different producers. The corresponding values obtained ranged from 30 to even 186 mg for daily dosage of phytoestrogens. Also, in several preparations with the same declared contents of isoflavones, large differences in the total content of these compounds were found. Additionally, profile of individual isoflavone content among various supplements was found to be different. However, in the majority of the analyzed samples genistin was the dominant component while in others it was daidzin [171].

In soybean, glycoside forms of isoflavones are present (mostly malonyl glycosides) [19,172,173], and variation in later treatment could cause a different aglycone/glycoside ratio in the final product [174]. Numerous studies showed that the treatment of soybean extracts used for dietary supplement preparations includes heating and/or use of acid which results in changes of isoflavone forms. Namely, malonyl and acetyl glycosides convert to β-glycosides or aglycones [175]. Data concerning the bioavailability of different isoflavone forms are often ambiguous [176], so it is still difficult to assess the most advantageous ratio of these components in supplements.

SAFETY ASPECTS

Some studies indicate the possible side effects of isoflavone consumption, which include substantial impacts on female and male fertility [177]. Considering the ability of phytoestrogens to compete with estradiol in binding to estrogen receptors as well as their mild estrogenic effects, it is hypothetically possible that these compounds could have detrimental effects in cancer patients taking tamoxifen or other selective estrogen receptor modulator-like drugs [178].

Studies in experimental animal models showed some evidence for potential adverse effects such as stimulation of estrogen-dependent mammary tumors and aberrant perinatal development [179–182].

Bioavailability of phytoestrogens from appropriate food matrices and exposure to these compounds during different life stages are critical determinants of phytoestrogens biological effects. For that reason and in order to reach more complete understanding of potential susceptibility issues, it is important to compare isoflavone bioavailability in adults to that in fetal and neonatal animals. Plasma measurements were performed in rats treated with different levels of dietary genistein exposure. It has been shown that the dietary test dosages produced circulating levels of genistein that are directly comparable to population groups such as ones with negligible exposures to genistein (adults

consuming typical Western diets), group with modest exposures (adults consuming traditional Asian foods) and group with much higher exposures (Western infants who consume all their nutritional input from soy infant formula) [180–184]. Measuring of total genistein level in serum indicates that serum concentrations in rats fed with the control and low dose (5 μg/g) diet are similar to those in human adults consuming a typical Western diet (0.1 μmol/l); in rats fed the 100-μg/g genistein diet they are similar to human adults consuming a typical Asian diet (0.1–1.2 μmol/l) or soy nutritional supplements (0.5–0.9 μmol/l) [185]; and in rats fed the 500-μg/g genistein diet they are similar to infants consuming soy formulas (2–7 μmol/L) [186].

It was reported that exposure of rats to genistein throughout the entire lifespan has produced a number of effects on reproductive tissues, immune function, neuroendocrine function, and behavior [180–182]. It was observed that an early onset of aberrant estrous cycles suggesting early reproductive senescence occurs in females receiving 500 μg/g diet of genistein. In the same study, a significant trend for increased incidences of mammary adenoma and adenocarcinomas has been shown, which corroborates with previously reported results on the effect of early life estrogen exposures on mammary carcinogenesis [187]. A significant positive trend in the incidences of mammary gland adenoma or adenocarcinoma (combined) was reported, and the incidence in the 500-μg/g diet group was significantly greater than that in the control group.

Significant dosage-dependent increases in total genistein concentration in endocrine-responsive tissues such as brain, liver, mammary, ovary, prostate, testis, thyroid, and uterus has been shown [183]. The physiologically active, aglycone form, was present in tissues at fractions up to 100%, and the concentration was greater than that obtained in serum. Contrary to the results observed in tissues, genistein conjugated forms are dominant in serum (95–99%). These results for measured amounts of genistein, present as aglycone and conjugates in putative target tissues, provide a connection with studies where correlation of blood concentrations and physiologic effects of genistein is targeted.

Placental and lactational transfer could be a route of developmental exposure to phytoestrogens. Examination of the placental transfer of genistein in rats showed that fetal or neonatal serum concentrations of total genistein were approximately 20 times lower than maternal serum concentrations [188]. However, the biologically active genistein aglycone concentration was only fivefold lower than in maternal serum. On the other hand, genistein aglycone concentration in fetal brain is similar to that in the maternal brain. These results suggest that genistein aglycone is present in maternal serum in levels that are relevant to those observed in humans consuming foods and supplements with phytoestrogens, crosses the rat placenta and can reach fetal brain.

It was observed that limited lactational transfer of genistein in rats occurs. However, the internal exposures to the aglycone form of genistein are

generally lower than those obtained for the fetal period [189]. Developmental effects which are previously attributed to genistein exposure are probably more likely to be due to fetal exposures because of the higher levels of the active estrogenic aglycone form of genistein in utero, although the possibility of neonatal responses is also present.

The very low margin of exposure between dosages of genistein that produce demonstrably adverse effects in experimental animal models and those in 20–25% of all formula-fed children in the United States suggests that the possibility of adverse effects should be considered.

Reported toxicology and exposure assessments of rats treated with dietary genistein demonstrate clearly adverse effects as increased incidences of mammary adenoma or carcinoma in females from chronic exposures, accelerated reproductive senescence in females and increased incidences of mammary adenoma from developmental exposures.

To our knowledge, no studies have been conducted to follow the long-term survival or tumor recurrence rate of cancer patients who use red clover isoflavone supplements. Red clover isoflavones can inhibit certain metabolic liver enzymes [190,191] which may cause increased plasma levels of some drugs. However, there has been no evidence of clinically significant interactions of red clover with drugs until now.

Red clover is considered a class 2b herb ("Herbs for which the following use restrictions apply, unless otherwise directed by an expert qualified in the use of the described substance. Not to be used during pregnancy") by the American Herbal Products Association and as such is contraindicated during pregnancy [192].

More trials involving chronic exposure of large patient populations to products containing phytoestrogens are needed to assess long-term risks and safety.

CONCLUSION

Diet and nutrition are well-known essential factors for preserving healthy and quality life. Thus, biologically active nutrients are still main focus of extensive research. In this decade, there has been a rising scientific interest in isoflavones, which are considered to be a group of phytochemicals with crucial benefit to human health.

Because of their structural similarity to human estrogen, these compounds may exhibit "estrogen-like" activities. Having weak estrogen activity, phytoestrogens have been attributed to have many potential health benefits including effects on cancer, vascular disease, osteoporosis, and menopausal symptoms. The obtained experimental data from animal studies are highly suggestive of beneficial effects of isoflavones on health, but the clinical data supportive of such effects remain to be definitively established. Performed studies show that commercially available isoflavone preparations have

multiple beneficial effects on different disorders or syndromes. This effect of phytoestrogens, could also be additionally supported by synergistic effect with other phytochemicals.

It has been shown that phytoestrogens may have different affinity to the estrogen receptors from human estrogens. Moreover, they preferentially bond to ERβ comparing to ERα. Having the ability to act as estrogen agonists and antagonists phytoestrogens can function as selective estrogen receptor modulators. These properties provide the basis for separating the undesirable effects of estrogenic compounds on cell proliferation from their beneficial diseases preventive functions. For that reason, these molecules have the ability to improve some estrogen-dependent conditions and might be a promising alternative to conventional method of hormone replacement therapy. More specifically, they seem to ease or to prevent menopausal disorders and related diseases, such as hot flushes or osteoporosis, without exhibiting severe side effects.

Due to the promising results from different epidemiological studies, as well as development of numerous commercially available preparations, isoflavone supplements are increasingly consumed among population. However, performed studies have shown that in most of the dietary supplements available on the market, the actually determined phytoestrogen content does not match the content specified by the manufacturer. Generally, the analytically obtained amount of isoflavones in the preparations was smaller, and only for a few samples it was equal to the declared content. Also, it has been observed that only the total content of isoflavones is specified on the product. Bearing in mind that all isoflavones do not show the same biological activity, it is recommended that more detailed information concerning their individual composition is stated in specification. All the above stated facts point out to the fact that quality control standards for isoflavone preparations, concerning the content and the composition of active substances, should be created.

Variations in the isoflavone composition observed in food and supplements may be due to different conditions of cultivation of plant used as phytoestrogens source as well as the natural variability of the composition of isoflavones. It was shown that the processing method applied could also be a factor of influence. In order to improve the use of available phytoestrogen sources as well as the content and profile of containing phytochemicals and consequently obtain favorable raw material for supplement production, additional scientific contribution is needed.

Supplements containing phytoestrogens are widely used by women suffering from menopausal disorders, often without medical advice. As the molecular mechanisms of action of plant extracts have not been fully elucidated yet, additional studies are necessary to evaluate the risks of a long-term use of phytoestrogens. Also, noticeably wide range in recommended dosage by manufacturer points out to the importance of urgent determination of the optimal phytoestrogen dose. Patients can benefit from supplementation, and

at the same time remain protected to the greatest extent possible only if amount of phytoestrogen daily intake through supplements as well as treatment duration are clearly specified.

Described contradictions in effects of dietary isoflavones as well as phytoestrogen supplementation may be due to the differences in the composition and concentration of these compounds in applied extracts. Also, some other factors, including the duration of the period of administration and age could have an important impact on the obtained effects.

Obviously, more research is necessary in order to entirely define the pharmacological effect of dietary phytoestrogens. It is essential that future studies should be fully monitored and conducted with standardized and structurally characterized mixtures of compounds.

An exciting prospect of the future phytoestrogen research lies in comprehension of their effects and complete determination of benefit/risk ratio. These results could provide new information as well as additional explanation of already obtained results and thus elucidate how to efficiently and safely use these promising natural compounds in improving human health.

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ABBREVIATIONS

AR androgen receptor
ERα estrogen receptor α
ERβ estrogen receptor β
HDL high-density lipoprotein
LDL low-density lipoprotein
PR progesterone receptor

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Chapter 2

The Discovery and Synthesis of Brevisamide

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INTRODUCTION

In recent years, natural marine toxins are becoming more prevalent around the world, affecting an estimated 500,000 individuals annually, and having deleterious impacts on health, resulting in a global mortality rate of 1.5% [1]. These toxins (dinoflagellates), which poison wildlife as well as humans, are known to be produced by a very large and diverse group of eukaryotic algae in the marine ecosystem, during the course of harmful algal blooms (red tides) [2]. Interestingly, many marine toxins are known to have fascinating, complex structures. In particular, the dinoflagellate toxins are structurally and functionally diverse, usually possessing multiple cyclic ether rings which are often aligned in a ladder frame, with a long carbon chain backbone bearing many hydroxyl groups [3–5]. These polycyclic ether marine natural products have shown unique and extremely potent biological activities such as neurotoxicity, anticancer, and antifungal properties [3–7].

Karenia brevis is a marine dinoflagellate known for producing complex fused polyethers such as brevetoxin A (**1**), B (**2**), and hemibrevetoxin B (**3**) (Fig. 1) [8–10]. Complex ladder polyethers possess many structural and stereochemical similarities. Structurally, a carbon–carbon–oxygen unit is present through the length of the polycyclic ether ladder. As seen in the structure of brevetoxin A (**1**) and other polyether toxins, the repeating C–C–O units are independent of the substitution, ladder length, and ether ring size. The relative stereochemistry of the ladder ring function possesses a *trans–syn–trans* relationship except for maitotoxin, which contains a *trans–anti–trans* relationship [8–16].

In 1985, after the discovery and structural determination of brevetoxin B (**2**), Nakanishi proposed that the structural and stereochemical features of the ladder-frame polyethers could arise through a cascade of successive

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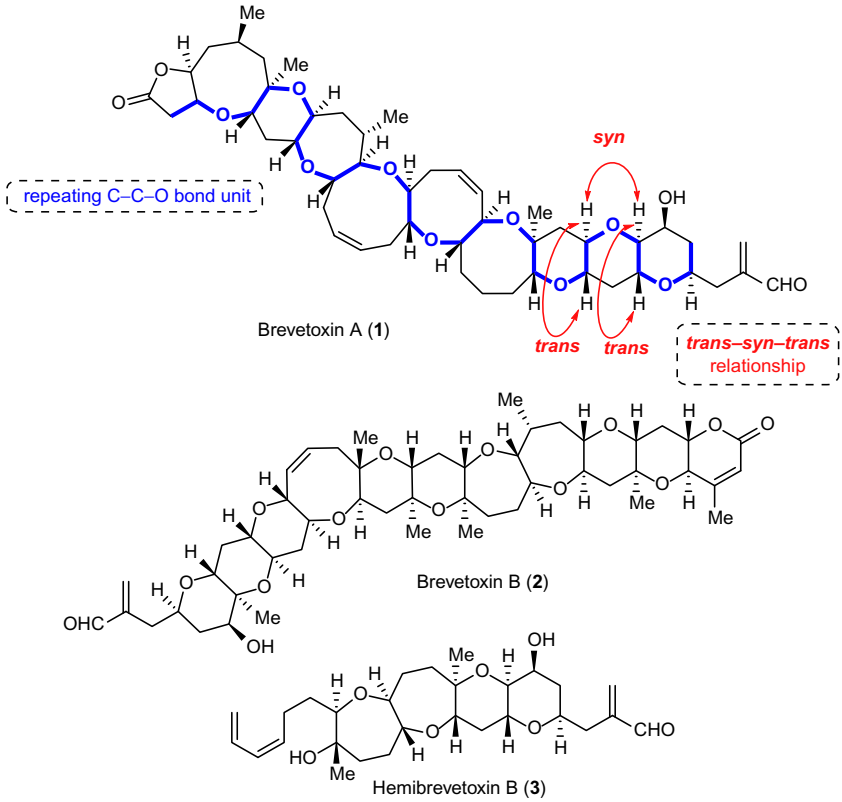


FIGURE 1 Structures of brevetoxin A (**1**), brevetoxin B (**2**), and hemibrevetoxin B (**3**).

endo-tet epoxide openings of a polyepoxide precursor [7]. Therefore, each epoxide opening must proceed stereospecifically with complete inversion of stereochemistry. A similar proposal has been suggested independently by Shimizu [17] and Nicolaou [18,19]. Specifically, Nakanishi [7] proposed that brevetoxin B (**2**) is assembled from a polyepoxide precursor (**5**) via a cascade of S_N2 epoxide openings and further proposed that the polyepoxide precursors could arise from epoxidation of polyene (**4**) (Fig. 2). Recently, Rein and coworkers reported the first evidence of resident polyketide synthase (PKS) genes in *K. brevis* and other dinoflagellates [20,21]. This work corroborates the proposal of Nakanishi that polyketides are the origin of the carbon skeleton in ladder polyethers.

In 2006, Gallimore and Spencer showed that contiguous rings in any single polyether can be derived from stereochemically identical *trans* epoxides [22]. Therefore, there is a stereochemical regularity at the ether ring junctures which supports the previous hypothesis by Nakanishi. Furthermore, they suggested that a single epoxidase is likely responsible for the selective and

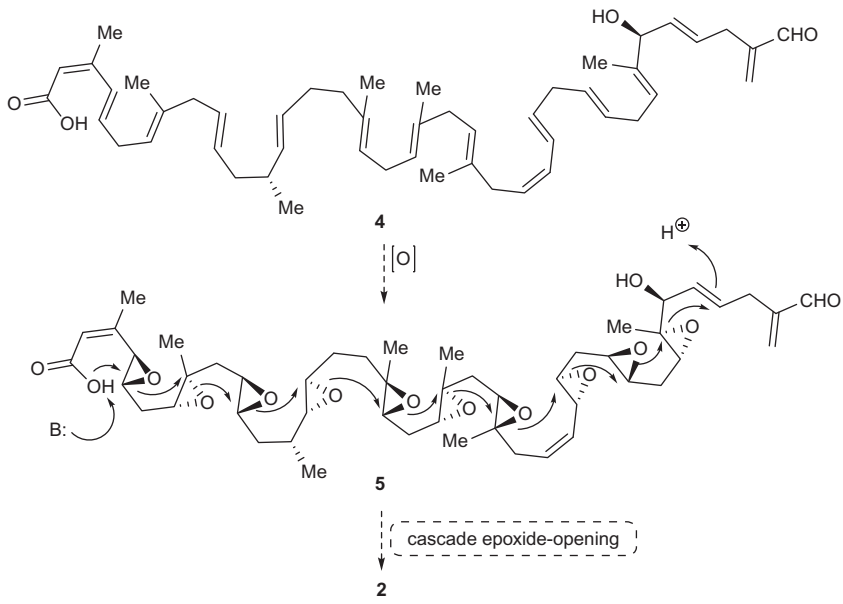


FIGURE 2 Nakanishi's proposed biosynthesis of brevetoxin B (**2**).

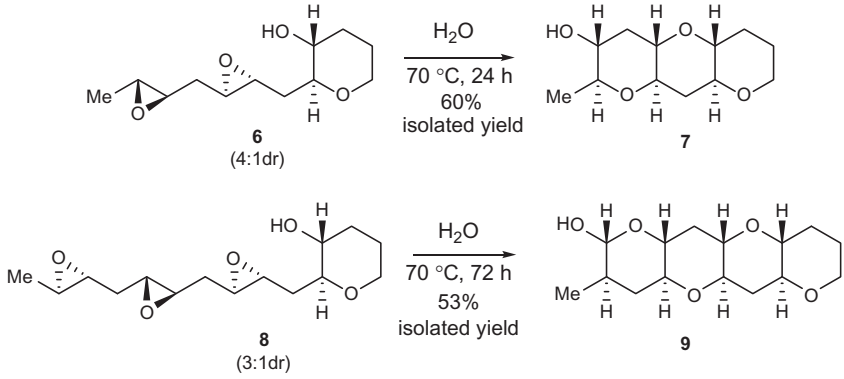


FIGURE 3 Water-promoted epoxide-opening cascade with a template 3-hydroxypyran.

uniform epoxidation of the *trans* polyene precursor [22]. Recently, an important breakthrough in understanding the formation of ladder polyether marine natural products was reported by Jamison's group [23]. Their work demonstrated the importance of the first ring toward the cascade formation of multiple ether rings present in the ladder-frame polyethers. It was reported that the endo-tet cyclization of a polyepoxide precursor for the formation of ladder-frame polyethers can only proceed in aqueous media of neutral pH, and an initial 3-hydroxy-tetrahydropyranyl ring moiety must be built into the polyepoxide intermediate (Fig. 3). Jamison's work suggests that after enzyme-

catalyzed formation of the first ether ring, the cascade polyepoxide opening should proceed spontaneously, relying on the spatial and configurational properties of the epoxide intermediate [23,24].

In subsequent years, *K. brevis* also afforded brevenal (**10**) and brevisin (**11**), smaller ladder-frame polyethers with a unique 3,4-dimethyl-2,4-dienal side chain (Fig. 4) [25,26]. In 2008, Satake, Tachibana, Wright, and coworkers reported the isolation and characterization of brevisamide (**12**), an unprecedented monocyclic ether alkaloid, from the dinoflagellate *K. brevis* [27]. The extraction of 400 L of cultured cells led to 0.2 mg of brevisamide (**12**) as an amorphous solid. Brevisamide (**12**), which displayed similar UV data to brevenal (**10**), had very distinctive ^{1}H NMR spectra compared to other known brevetoxins. The structural assignment was elucidated by 500 MHz 2D-NMR experiments including ^{1}H–^{1}H COSY, ^{1}H–^{13}C HMBC, TOSCY, HSQC, and NOE experiments [27]. Brevisamide (**12**) contains the same conjugated 3,4-dimethyl-2,4-dienal side chain as the more complex polycyclic ether brevenal (**10**) and brevisin (**11**) (Fig. 4). Thus, brevisamide (**12**) is believed to be a biosynthetic precursor for these complex polyether natural products **10** and **11**. Interestingly, the brevisamide (**12**) skeleton matches well with Jamison's template ring system in the formation of ladder polyethers

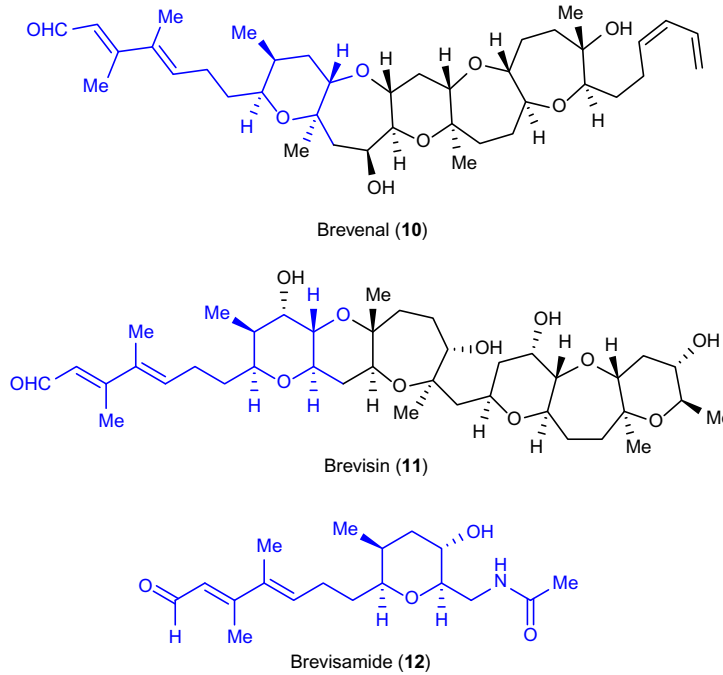


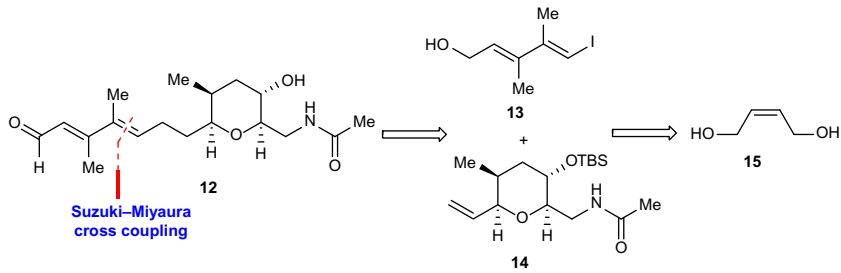
FIGURE 4 Structures of brevenal (**10**), brevisin (**11**), and brevisamide (**12**).

(Fig. 3) with the ether ring oxygen *anti* to the hydroxyl oxygen and a carbon–carbon–oxygen unit [23,24]. These features are consistent with the structural and stereochemical trend found in complex ladder polyethers such as brevetoxins. Brevisamide (**12**) might prove the existence of the tetrahydropyran template in nature [27]. Wright and coworkers suggested that based on the established biosynthesis pathway of other dinoflagellate metabolites, glycine could be the source of the amide nitrogen of brevisamide (**12**) and acts as a starter unit in a NRPS/PKS hybrid pathway [27]. Due to the unique role brevisamide (**12**) could play in further understanding the biogenetic origin of fused, ladder-frame polyether marine natural products, it has garnered a great deal of interest among the synthetic community, with five total syntheses, two formal syntheses, and one unnatural stereoisomer total synthesis reported [28].

TOTAL SYNTHESES OF BREVISAMIDE

Satake and Tachibana [29]

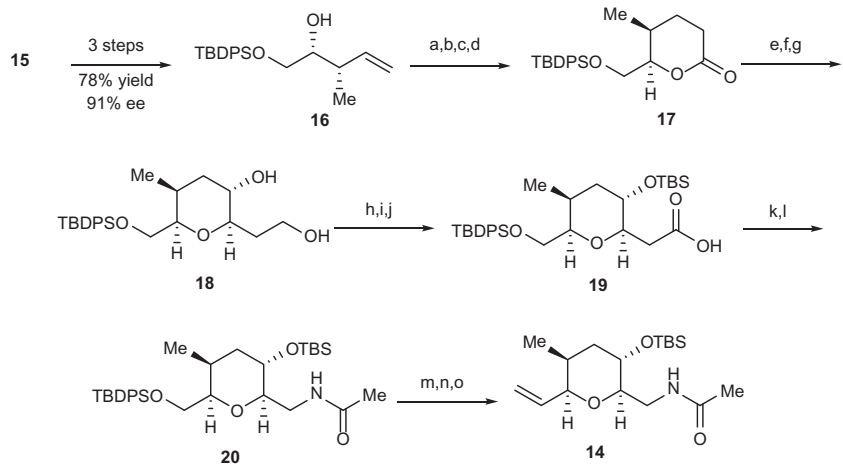
Within months of the isolation and characterization of **12**, the same group reported the first total synthesis of brevisamide (**12**) [29]. Importantly, this first synthesis confirmed the structure of **12** and the stereochemistry as the (−)-enantiomer. Satake's strategy involves Suzuki–Miyaura coupling of iodide **13** and amino cyclic ether fragment **14** (Scheme 1). Both fragments can be obtained from a commercially available starting material, *cis*-but-2-ene-1,4-diol **15**. The synthesis of amino cyclic ether **14** began from optically active homoallylic alcohol **16**, which was prepared in three steps (silyl-monoprotection, ozonolysis, and Brown crotylation) from diol **15** (78% yield, 91% ee). Ozonolysis of the homoallylic alcohol **16**, Wittig reaction of the resulting aldehyde, hydrogenation, and transesterification of the enoate gave lactone **17** in 71% after four steps. Ketene acetal triflate generation from **17**, followed by Stille coupling, generated the dienol ether which was further subjected to hydroboration conditions to give the pyran ring **18**. TBS protection of the diol, followed by selective deprotection of the primary



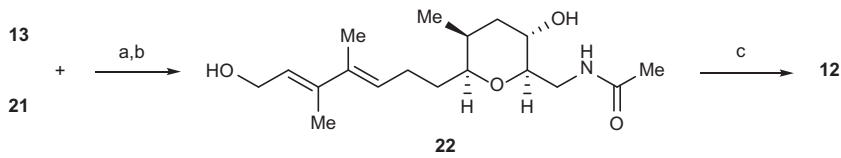
SCHEME 1 Satake and Tachibana's retrosynthesis of brevisamide (**12**).

silyl alcohol and 2,2,6,6-tetramethylpiperidine *N*-oxide (TEMPO) oxidation, gave carboxylic acid **19** in 80% yield for three steps. Curtius rearrangement of pyran acid **19** generated the terminal amino group of the pyran ring, and subsequent acetylation delivered the desired amide **20**. Selective deprotection of the silylether, Parikh–Doering oxidation, and Wittig reaction of the resulting aldehyde furnish the desired key ether ring fragment **14** (Scheme 2) [29].

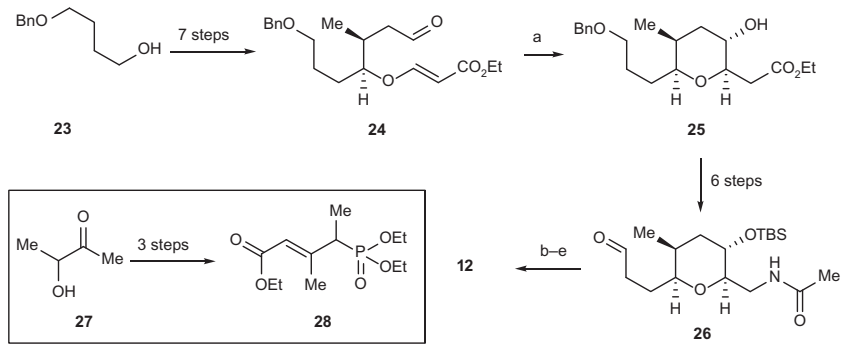
The key vinyl iodide (**13**) is accessed in seven steps from commercial diol **15**. Hydroboration of the terminal olefin in amino cyclic ether fragment **14** afforded the alkylborane **21**, which was coupled with iodide side chain fragment **13** (Cs_2CO_3, cat. $PdCl_2$ (dppf), DMF, 45 °C) to give the cross-coupled product. TBAF deprotection of the crude product gave diol **22** in 40% yield for two steps. Finally, selective allylic oxidation with MnO_2 provided the first synthetic brevisamide (**12**) in 55% yield (Scheme 3). This synthesis required 28 total steps, 21 steps in the longest linear sequence, with an overall yield of 2.6%. Subsequent syntheses of **12** employed alternative strategies to construct the pyran moiety and to install the dienyl side chain [29] and the preceding reviews will capture the most significant steps en route to **12**.



SCHEME 2 Synthesis of Suzuki–Miyaura coupling fragment amino cyclic ether (**14**). *Reagents and conditions*: (a) O_3, CH_2Cl_2, −78 °C; PPh_3, rt; (b) $Ph_3P{=}CHCO_2Me$, THF, rt; (c) H_2, Pd/C, EtOAc, rt; (d) PPTS, benzene, reflux (71% yield for four steps); (e) KHMDS, Tf_2NPh, DMPU, THF, −78 °C; (f) $CH_2{=}CHSn$*n*-Bu_3, $Pd(PPh_3)_4$, LiCl, THF, reflux (85% yield for two steps); (g) thexylborane, THF, 0 °C, 30% H_2O_2, sat. $NaHCO_3$ aq., rt; (h) TBSCl, imidazole, DMF, rt; (i) CSA, MeOH—CH_2Cl_2, 0 °C; (j) TEMPO, NaOCl, KBr, TBAC, NaCl, $NaHCO_3$, CH_2Cl_2—H_2O, 0 °C (80% yield for three steps). (k) DPPA, Et_3N, toluene, 80 °C, 4 N LiOH, THF, rt, 1 h, 85%; (l) Ac_2O, pyridine, quant; (m) TBAF, AcOH, THF, 0 °C to rt, 83%; (n) SO_3–pyridine, Et_3N, CH_2Cl_2—DMSO, 0 °C; (o) $Ph_3P^+CH_3Br^-$, NaHMDS, THF, −78 °C to rt (56% yield for two steps).



SCHEME 3 Satake and Tachibana's synthesis of brevisamide (**12**). *Reagents and conditions*: (a) 9-BBN, THF, rt; 3 M, Cs_2CO_3, $PdCl_2$(dppf), DMF, 45 °C; (b) TBAF, THF, 0 °C (40% yield for 2 steps); (c) MnO_2, CH_2Cl_2, rt 55%.



SCHEME 4 Fadeyi and Lindsley's synthesis of brevisamide (**12**). *Reagents and conditions*: (a) SmI_2, MeOH, THF, 0 °C, 73%; (b) **28**, *n*-BuLi, THF, −78 °C to rt then **26**, 78%; (c) DIBAL, CH_2Cl_2, −78 °C; (d) TBAF, THF, 0 °C to rt (71% for over two steps); (e) MnO_2, CH_2Cl_2, rt, 74%.

Fadeyi and Lindsley [30]

The second synthesis of natural (−)-brevisamide (**12**) was performed by Fadeyi and Lindsley, utilizing a key SmI_2-mediated reductive cyclization reaction to construct the pyran (Scheme 4) [30]. Another point of divergence was the application of a Horner–Wadsworth–Emmons reaction between aldehyde **26** and phosphonate ester **28** to install the dienyl moiety. Starting from monobenzyl diol **23**, the key α,β-unsaturated ester **24** was prepared in 5 steps, setting the stage for the critical SmI_2-mediated reductive cyclization reaction, which afforded pyran **25** in 73% yield and 14 steps from **23**. A series of functional group conversions over six steps, including a Curtius rearrangment, delivered aldehyde **26**. Phosphonate ester **28** was prepared in three steps from 3-hydroxybutan-2-one **27**, enabling a Horner–Wadsworth–Emmons reaction to install the dienyl side chain in 78% yield. Adjustment in oxidation state then delivered (−)-brevisamide (**12**) in 5.2% overall yield over 21 total synthetic steps from commercial materials with 18 steps longest linear sequence [30].

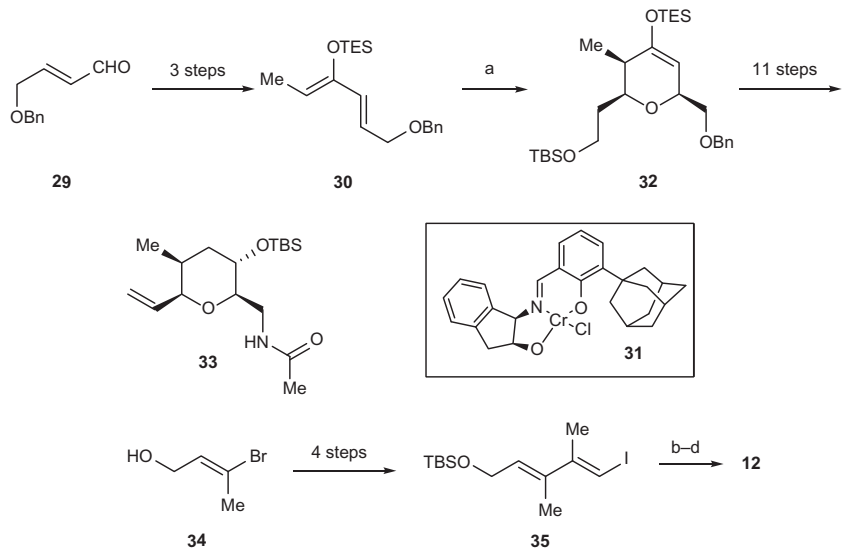
Ghosh and Li [31]

Ghosh and Li reported the third total synthesis of (−)-brevisamide (**12**) based on the same coupling strategy reported by Satake and coworkers [31]. The

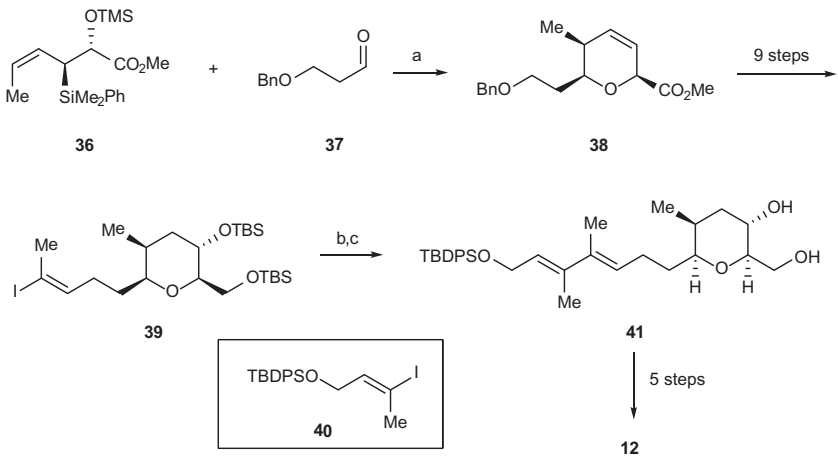
synthesis (Scheme 5) features a Suzuki–Miyaura coupling of pyran **33** and iodide **35**. The pyran **33** was prepared via Jacobsen's asymmetric Diels–Alder reaction with catalyst **31**. Starting from aldehyde **29**, a three-step protocol provided silyoxydiene **30** that underwent a Diels–Alder reaction, catalyzed by **31**, to deliver pyran **32** in 37% overall yield and excellent diasteroselectivity (dr = 95%, ee = 96%). An 11-step sequence of transformations led to the required alkene **33**. The iodide fragment (**35**) was derived from the previously known *E*-bromocrotyl alcohol **34** in four steps. Then, completion of **12** followed Satake's approach, save for employing TEMPO for the final chemoselective oxidation, and proceeded in 1.7% overall yield. This route featured 24 total synthetic steps with 18 steps longest linear sequence [31].

Lee and Panek [32]

The fourth synthesis of (−)-brevisamide (**12**) was reported by Lee and Panek and utilized Panek's silicon-directed [4 + 2]-annulation reaction to form the pyran and a Negishi coupling to install the dienyl unit (Scheme 6) [32]. Here, reaction of the known crotyl silane **36** with 3-benzyloxypropanal **37** afforded pyran **38** in 10:1 dr and 70% yield. Nine synthetic steps then provided vinyl iodide **39**. Vinyl iodide **39** was converted to the vinyl zinc reagent, via the organolithium, and subjected to a modified Negishi coupling with fragment **40** in the presence of 10 mol% $Pd(PPh_3)_4$. Selective desilylation afforded



SCHEME 5 Gosh and Li's synthesis of brevisamide (**12**). *Reagents and conditions*: (a) 3-TBSOCH$_2$CH$_2$CHO, 10 mol% **31**, 52%; (b) 9-BBN, then aq. Cs_2CO_3, $PdCl_2$(dppf)–CH_2Cl_2; (c) TBAF, THF (40% yield for two steps); (d) TEMPO, $PhI(OAc)_2$, 87%.



SCHEME 6 Panek and Lee's synthesis of brevisamide (**12**). *Reagents and conditions*: (a) TMSOTf, CH_2Cl_2, PhH, −50 °C, 70% yield, 10:1 dr; (b) *t*-BuLi, $ZnCl_2$, THF, −78 °C to 0 °C, $Pd(PPh_3)_4$; (c) CSA, MeOH, CH_2Cl_2 (58% yield for 2 steps).

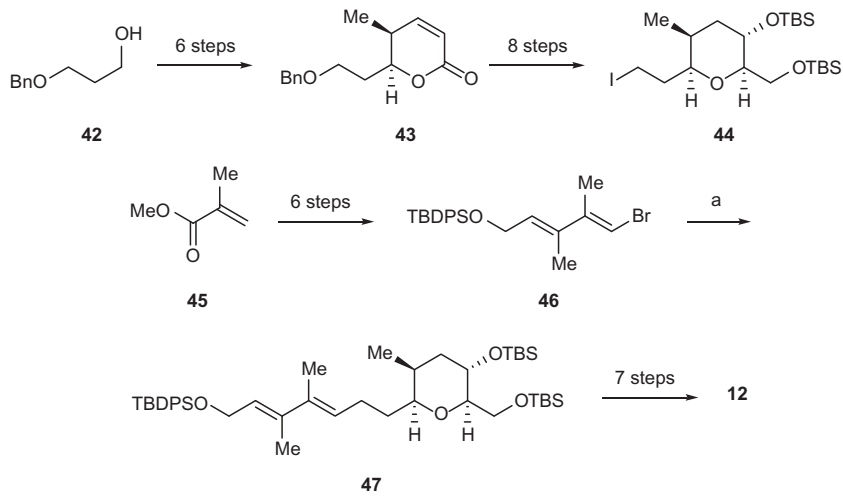
diene **41** in 58% yield for two steps. Completion of the synthesis was achieved through five additional synthetic steps and proceeded in 6.4% overall yield. This route featured 29 total synthetic steps with 17 steps longest linear sequence [32].

Satake and Tachibana [33]

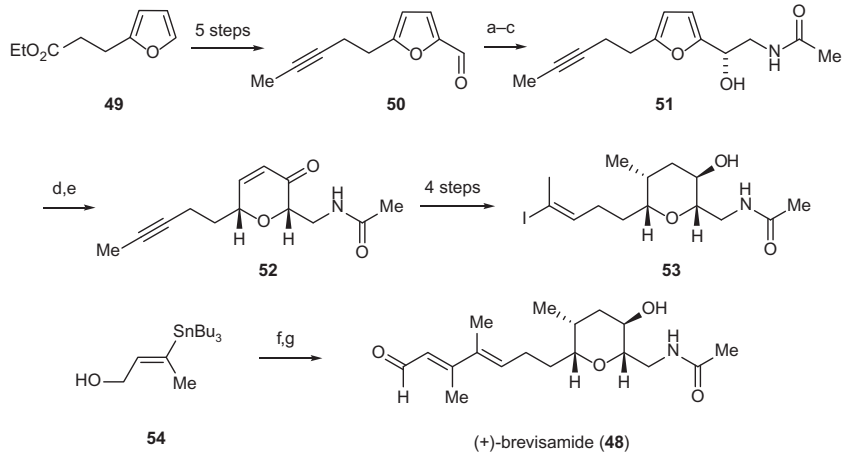
In 2010, Satake and Tachibana published an improved synthesis of (−)-brevisamide (**12**) [33]. Once again, a Suzuki–Miyaura coupling is employed to install the dienyl unit, but fundamentally different chemistry was employed to prepare the pyran (Scheme 7). Starting from 3-benzyloxypropanol **42**, a series of six reactions including an Evans Aldol and one-pot Horner–Emmons–Wadsworth/lactonization sequence delivered lactone **43**. Eight straightforward reactions then produced the key iodide coupling partner **44**. The dienyl bromide **46** was prepared in six steps from commercial methyl methacrylate **45**. Iodide **44** was then converted into the alkyl bromide, via Li–I exchange and trapping with *B*-OMe-9-BBN, to facilitate the Suzuki–Miyaura coupling with **46** to produce **47**. Seven additional steps completed the fifth total synthesis of (−)-brevisamide (**12**) in an improved 8.6% overall yield (compared with 0.23% overall yield from their first synthesis) in 30 total steps and 21 steps longest linear sequence [33].

Herrmann and Zakarian [34]

In 2011, Zakarian and coworkers reported on the first total synthesis of unnatural (+)-brevisamide (**48**), utilizing a key asymmetric Henry reaction and an



SCHEME 7 Satake and Tachibana's improved, second-generation synthesis of brevisamide (**12**). *Reagents and conditions*: (a) *B*-OMe-9-BBN, *t*-BuLi, Et_2O/THF, −78 °C to rt, then **46** and 3 M, Cs_2CO_3, $PdCl_2$(dppf), aq. DMF, 64%.



SCHEME 8 Herrmann and Zakarian's synthesis of (+)-brevisamide (**48**). *Reagents and conditions*: (a) $Cu(OAc)_2$, chiral amine ligand, $MeNO_2$, EtOH, rt, 69%, 99% ee; (b) $LiAlH_4$, THF, −15 °C to reflux; (c) Ac_2O, EtOAc:MeOH (4:1), rt, 67% for two steps; (d) NBS, $NaHCO_3$, NaOAc, THF:H_2O (10:1), 0 °C; (e) Et_3SiH, $BF_3{\cdot}OEt_2$, CH_2Cl_2, 0 °C, 54% for two steps; (f) $Pd_2(dba)_3$, Ph_3As, CuBr·DMS, THF:DMSO (1:1), rt, 78%; (g) TEMPO, $PhI(OAc)_2$, CH_2Cl_2, rt, 90%.

Achmatowicz rearrangement to generate the pyran ring, and a Stille coupling to install the dienyl side chain (Scheme 8) [34]. Importantly, this route was protecting group free. Commercial ethyl 3-(furan-2-yl)-propionate **49** is converted in five steps to aldehyde **50**. An asymmetric Henry reaction provides

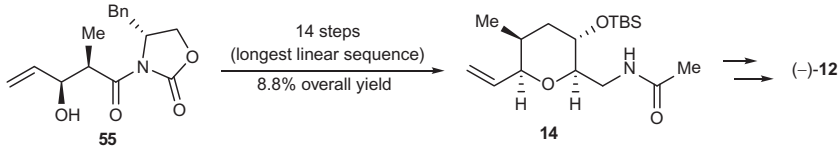
99% ee, followed by reduction and acylation of the nitro moiety to afford β-amino alcohol **51**. A two-step Achmatowicz oxidative ring expansion delivered pyran **52**, which was converted in four steps to the vinyl iodide **53**. Stille coupling with known stannane **54** and TEMPO oxidation afforded (+)-brevisamide (**48**) in 2.5% overall yield and 16 total steps without protecting group manipulations [34].

Interestingly, the stereochemistry could have been established in the asymmetric Henry reaction to ultimately provide natural (−)-brevisamide (**12**); however, the authors did not comment on the rationale for the synthesis of **48** instead of **12** [34].

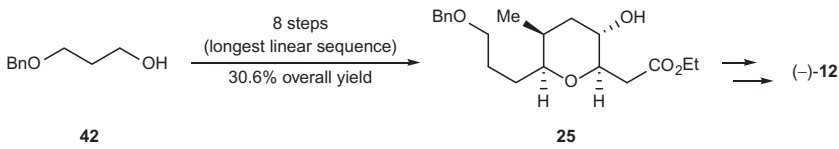
FORMAL TOTAL SYNTHESES OF BREVISAMIDE

In 2011, two formal total syntheses of (−)-brevisamide (**12**) appeared from the Smith [35] and Sabitha groups [36]. Smith and coworkers accessed tetrasubstituted pyran **14**, a key intermediate in the first total synthesis of (−)-**12** by Satake and Tachibana, from the known starting material **55** [35]. The formal total synthesis proceeded in 14 steps (longest linear sequence) with an overall yield of 8.8% (Scheme 9). Their route featured a chemoselective asymmetric dihydroxylation and a one-pot Fraser-Reid epoxidation/*p*-methoxybenzyl (PMB) protection sequence [35].

Sabitha and coworkers reported on a palladium hydroxide catalyzed isomerization of primary alcohols to aldehydes and completed a formal total synthesis of (−)-brevisamide (**12**) by preparing advanced Fadeyi/Lindsley pyran **25** [36]. Starting from alcohol **42**, pyran **25** was synthesized in eight steps (longest linear sequence) with an overall yield of 30.6% (Scheme 10) [36].



SCHEME 9 Smith's formal total synthesis of (−)-brevisamide (**12**).



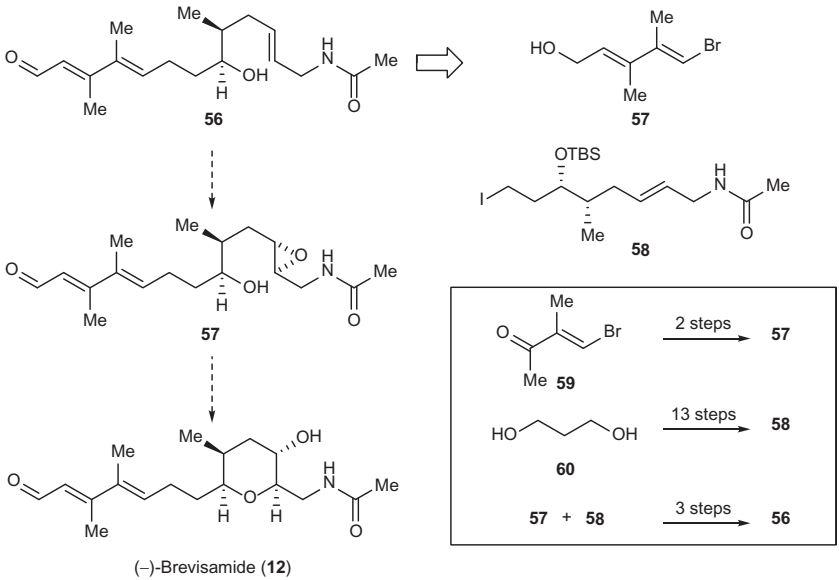
SCHEME 10 Sabitha's formal total synthesis of (−)-brevisamide (**12**).

BIOSYNTHESIS OF BREVISAMIDE

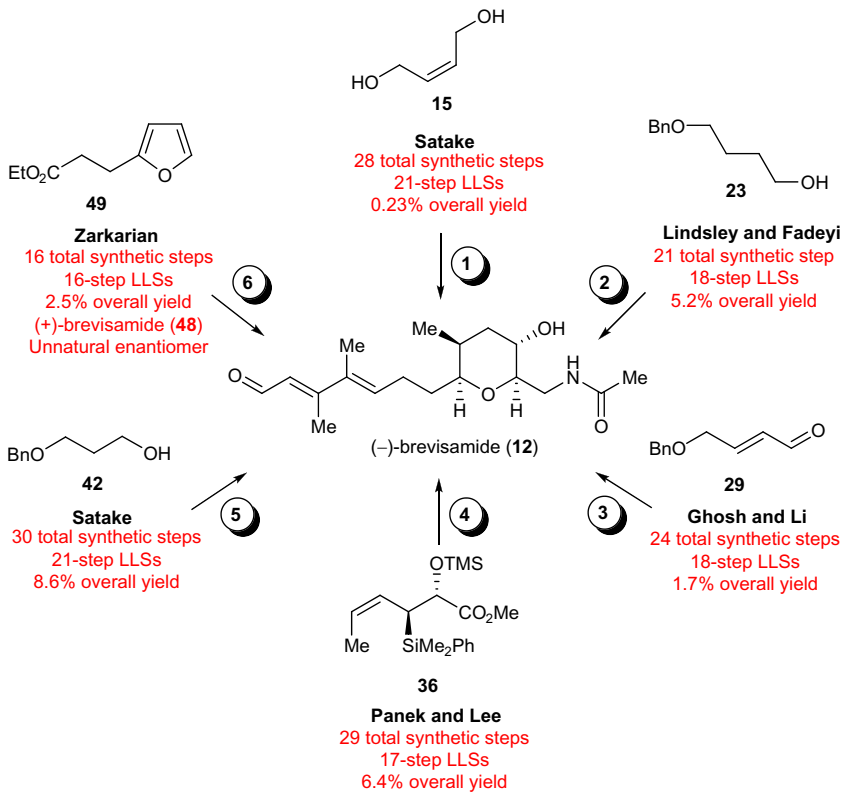
In the original isolation paper of **12**, the authors posed a possible biosynthetic mechanism for the formation of the pyran ring (Scheme 11) [27]. Here, acyclic polyketide **56** is enantioselectively epoxidized to form **57**, followed by an S_N2 attack of the hydroxyl moiety to form the pyran in **12**. In 2010, Satake and Tachibana synthesized the proposed biosynthetic intermediate **56**; however, the first communication did not describe any attempts to convert **56** into **12** [37]. Retrosynthetic analysis led to fragment targets **57** and **58**. The dienyl bromide **57** was prepared in 2 steps from commercial **59**, and iodide **58** was prepared in 13 steps from propane diol **60**. Coupling of the fragment **57** and **58** via a Suzuki–Miyaura coupling and two additional steps afforded the proposed biosynthetic precursor **56**. The synthesis proceeded in 18 steps (13 steps longest linear sequence) with an overall yield of 3.4% [37].

SUMMARY

In 2008, Satake, Tachibana, Wright, and coworkers reported the isolation and characterization of brevisamide (**12**), an unprecedented monocyclic ether alkaloid, from the dinoflagellate *K. brevis* and believed to be a biosynthetic precursor to all of the complex polyether natural products. Due to the unique role, brevisamide (**12**) could play in further understanding the biogenetic origin of fused, ladder-frame polyether marine natural products, it has garnered a great deal of interest among the synthetic community, with five total



SCHEME 11 Satake and Tachibana's proposed biosynthesis of (**12**) and synthesis of the biosynthetic precursor (**56**).



SCHEME 12 Overview of the synthetic efforts toward (−)-brevisamide (**12**) and the unnatural enantiomer (+)-brevisamide (**48**).

syntheses, two formal syntheses, and one unnatural stereoisomer total synthesis reported. Here, we reviewed the synthetic efforts to date, which are summarized in Scheme 12.

ACKNOWLEDGMENTS

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ABBREVIATIONS

CSA	camphor sulfonic acid
DPPA	diphenylphosphoryl azide
PPTS	*para*-toluene sulfonic acid
TEMPO	2,2,6,6-tetramethylpiperidine *N*-oxide
THF	tetrahydrofuran

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Chapter 3

Recent Asymmetric Syntheses of Tetrahydroisoquinolines Using "Named" and Some Other Newer Methods

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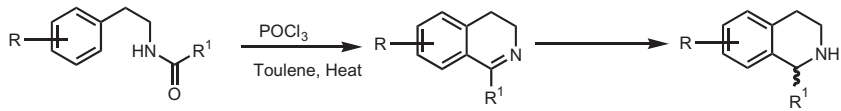
Department of Chemistry, Memorial University of Newfoundland, St. John's, Newfoundland and Labrador, Canada

ASYMMETRIC BISCHLER–NAPIERALSKI CYCLIZATION–REDUCTION SYNTHESES

The Bischler–Napieralski reaction (Scheme 1) is notably one of the most widely used reactions for the synthesis of 3,4-tetrahydroisoquinolines from β-ethylamides of electron-rich arenes, using condensation reagents such as P_2O_5, $POCl_3$, or $ZnCl_2$. The number and the position of the electron-donating groups on the aryl ring of the β-arylethylamides influence the regioselectivity of the reaction [3]. The choice, however, of the reducing agent in the next step to produce the tetrahydro ring is crucial since it generates a new stereogenic center at C-1 [4]. Several approaches toward the stereoselective reduction at this center will be addressed in this review.

Using Asymmetric Catalytic Hydrogen-Transfer Hydrogenation

Hydrogen-transfer reactions are those in which double or triple bonds are reduced using a hydrogen donor in the presence of a catalyst. In most cases, the hydrogen donors used are alcohols, including chiral ones, and formic acid.



SCHEME 1 The Bischler–Napieralski cyclization–reduction reaction.

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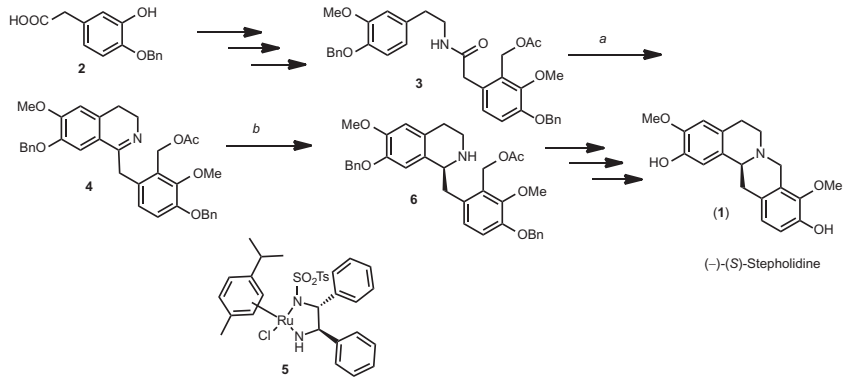
For asymmetric hydrogen-transfer reactions, chiral phosphines are the most popular ligands used for the asymmetric catalysts. Recently, however, for enantioselective hydrogen-transfer reactions, the most commonly used are chiral auxiliaries containing nitrogen and not phosphorus, as the donor atom [5]. Asymmetric transfer hydrogenation catalyzed by suitably designed chiral Ru(II) complexes as developed by Noyori *et al.* [6] has been shown to be an excellent method for the enantioselective reduction of cyclic imines with formic acid/triethylamine. Since its development, it has become the method of choice for the enantioselective hydrogen-transfer reduction of cyclic imines. Asymmetric transfer hydrogenations have been used to produce important chiral enantio-enriched compounds and many different tetrahydroisoquinolines have been prepared in high yields with ee values ranging from 90% to 97% using chiral Noyori's catalyst and formic acid/triethylamine as the hydrogen source. Some recent examples are illustrative of this methodology.

Cheng and Yang's Synthesis of (−)-(S)-Stepholidine

(−)-(*S*)-Stepholidine (**1**) is a tetrahydroprotoberberine (a class of naturally occurring tetracyclic alkaloids that also contain isoquinoline core) and is one of the protoberberine alkaloids extracted from *Stephania intermedia*. (−)-(*S*)-Stepholidine plays a unique pharmacological activity toward dopamine receptors and also plays a major role in the treatment of drug abuse [7]. Yang's synthesis [8] of **1** proceeded via the chiral tetrahydroisoquinoline **6** which, in turn, was synthesized as outlined in Scheme 2.

Yang used a Noyori asymmetric hydrogen-transfer hydrogenation catalyzed by the chiral Ru(II) complex **5** to reduce the imine **4** to **6** using formic acid/triethylamine. Imine **4** was prepared from the corresponding amide **3**, which was synthesized from the phenylacetic acid **2**, via a Bischler–Napieralski cyclization reaction. The Bischler–Napieralski reaction was the key step used to



SCHEME 2 Yang's synthesis of (−)-(*S*)-Stepholidine (**1**). *a*: $POCl_3$, CH_3CN. *b*: **5**, HCO_2H, TEA, DMF, 84% for two steps.

produce **4** in excellent yield using $POCl_3$ in CH_3CN. Since this imine was found to be unstable at room temperature, it was reduced directly when freshly prepared, without further purification.

Opatz's Synthesis of (−)-Norlaudanosine

Benzyltetrahydroisoquinolines containing a B ring that is reduced at the C1–C2 and C3–C4 positions are known to be key biosynthetic precursors to many naturally occurring alkaloids. These include morphine and codeine which are found in, or are derived from, the opium poppy *Papaver somniferum* [9]. The structures of some of these constituents which are of interest are shown in Fig. 1 and include laudanosine (**8**), reticuline (**9**), codamine (**10**), laudanine (**11**), and the enantiomers of *N*-norlaudanidine **12** and **12a**.

Opatz and coworkers [10] synthesized the tetrahydroisoquinolines (−)-(*S*)-norlaudanosine (**13**), (+)-(*R*)-*O*,*O*-dimethylcoclaurine (**14**), and (+)-(*R*)-salsolidine (**15**) (Fig. 2) as outlined in Scheme 3. The syntheses were accomplished via the intermediate α-aminonitrile **18**. Functionalization of **16** with formic acid and a subsequent classic $POCl_3$-mediated Bischler–Napieralski reaction produced the imine **17** which, with potassium cyanide, afforded the α-aminonitrile **18**. Alkylation of **18**, spontaneous elimination of HCN, and then asymmetric hydrogen-transfer reduction using the Noyori catalyst (**21**) with formic acid/triethylamine afforded the respective target compounds. The configuration of the catalyst controlled the configuration of the newly formed C-1 stereogenic center: With the (*S*,*S*)-enantiomer of the catalyst, the (*R*)-configured *O*,*O*-dimethylcoclaurine (**14**) and salsolidine (**15**)

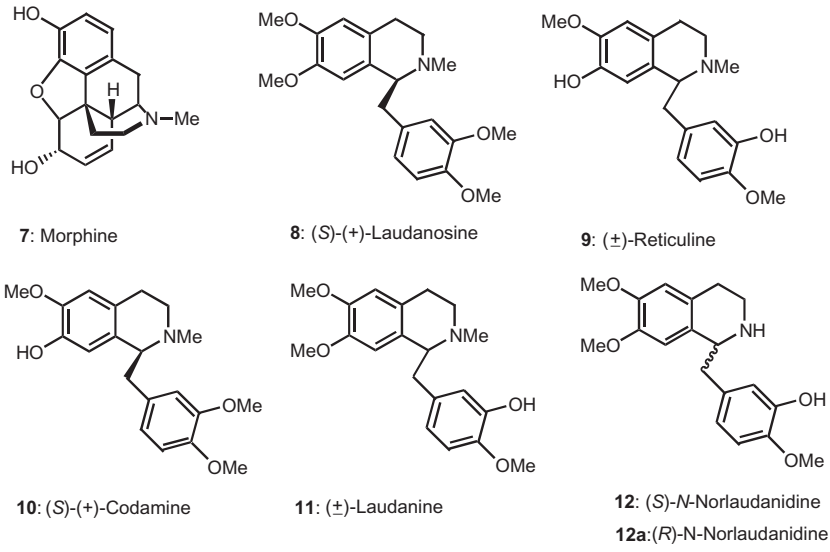
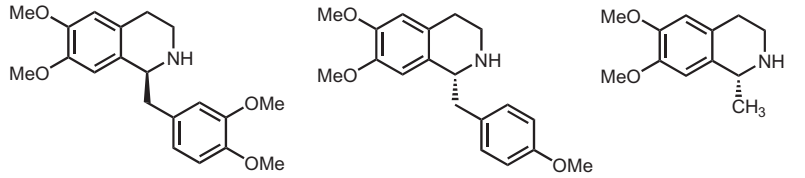


FIGURE 1 Structures of some benzyltetrahydroisoquinoline alkaloids related to opium.

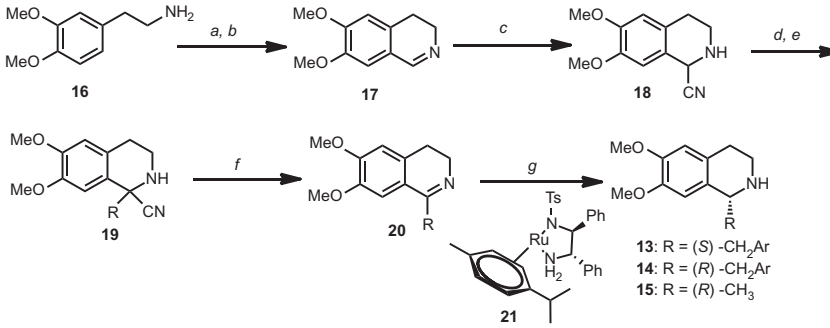


13: (–)-Norlaudanosine
78% from 17 using (*R*,*R*)-20, 93% ee

14: (+)-*O*,*O*-Dimethylcoclaurine
55% from 17, 96% ee

15: (+)-Salsolidine
51% from 17, 91% ee

FIGURE 2 Tetrahydroisoquinolines synthesized by Opatz *et al*.



SCHEME 3 Opatz's synthesis of tetrahydroisoquinolines **13**–**15**. *a*: HCO_2H, heat. *b*: $POCl_3$, heat. *c*: KCN, $HCl_{(aq)}$, 60%. *d*: KHMDS, THF, −78 °C. *e*: RX, THF, −78 °C. *f*: spontaneous —HCN. *g*: HCO_2H, Et_3N.

were obtained with 96% and 91% ee, respectively. However, using the (*R*,*R*)-enantiomer of the catalyst, the (*S*)-enantiomer of norlaudanosine (**13**) was obtained in 93% ee (Fig. 2).

Using Chiral-Auxiliary-Modified Amines

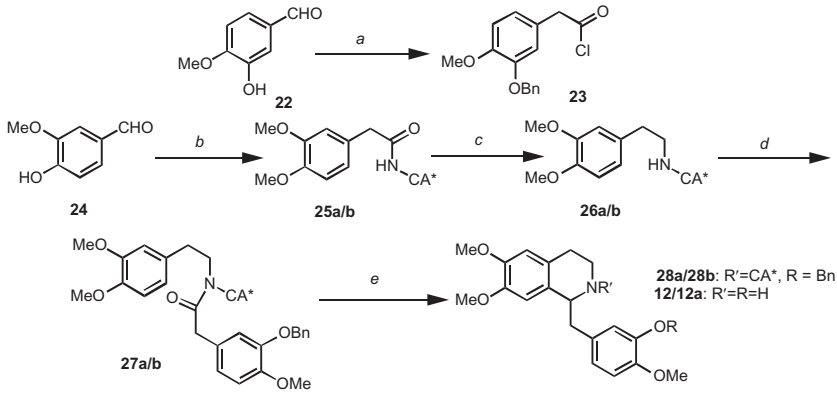
Chiral benzyltetrahydroisoquinoline alkaloids can also be asymmetrically synthesized via Bischler–Napieralski cyclization followed by stereoselective hydride reduction of the 3,4-dihydroisoquinolinium derived from the amine functionalized with a chiral auxiliary. Many different chiral auxiliaries have been used in such reactions, and the conditions and solvents used are very crucial in the reduction step to produce the C-1 stereogenic center.

Georghiou's Synthesis of (+)- and (−)-N-Norlaudanidine

The enantioselective synthesis of each of the enantiomers (**12** and **12a**) of *N*-norlaudanidine, a minor *P. somniferum* opium benzyltetrahydroisoquinoline alkaloid was achieved using a chiral auxiliary-mediated Bischler–Napieralski cyclization–sodium borohydride reduction strategy by the Georghiou group [11].

Laudanine (**11**), which is also known as "racemic laudanidine," and reticuline (**9**) occur in both enantiomeric forms in opium [12]. (−)-*N*-norlaudanine (**12a**) is a component part of the pathway for the biosynthesis of the benzylisoquinoline alkaloids in *P. somniferum*. It has been found to be incorporated into palaudine, another minor benzylisoquinoline constituent found in opium [13]. The fact that radiolabelled *N*-norlaudanine, whose absolute configuration was not defined by Brochmann–Hannssen and coworkers, was incorporated into palaudine was evidence that complete methylation was not necessary for dehydrogenation to take place in *P. somniferum*.

The syntheses of both **12** and **12a** were accomplished as outlined in Scheme 4 with the only difference being the choice of the chiral auxiliary used: **12** was obtained using (*S*)-α-methylbenzylamine and **12a** with (*R*)-α-methylbenzylamine. The "benzyl" component was synthesized via the intermediate **23** which was obtained in 78% overall yields from five steps, starting from the commercially available 3-hydroxy-4-methoxybenzaldehyde (isovanillin) (**22**) which was elongated using the method of Kim *et al*. [14]. Using (*S*)-α-methylbenzylamine as the chiral auxiliary, intermediate **25a** was obtained in 70% overall yields from six steps from vanillin (**24**). The corresponding enantiomer, **25b**, was obtained in the same manner, with (*R*)-α-methylbenzylamine as the chiral auxiliary. Reduction of **25a** (or **25b**) to the secondary chiral auxiliary-functionalized amines **26a** (or **26b**) was accomplished in 86% yields via BF_3·etherate-mediated reactions with B_2H_6 in THF. Schotten–Baumann amidation between **26a** (or **26b**) and **23** formed the amides **27a** (or **27b**) in 72% yields. Bischler–Napieralski cyclization–$NaBH_4$ reduction of **27a** and



SCHEME 4 Synthesis of (+)- and (−)-*N*-norlaudanidine (**12**). *a*: 1. BnBr, DMSO, 98%; 2. CBr_4, PPh_3, CH_2Cl_2, 93%; 3. Pyrrolidine, H_2O, rt, 91%; 4. 1.0 M $HCl_{(aq)}$/dioxane; 5. $(COCl)_2$, benzene, 94%. *b*: 1. $(CH_3)_2SO_4$, K_2CO_3, acetone, 95%; 2. CBr_4, PPh_3, CH_2Cl_2, 92%; 3. Pyrrolidine, H_2O, rt, 91%; 4. 1.0 M $HCl_{(aq)}$/dioxane; 5. $(COCl)_2$, benzene, 96%; 6. **CA*** = (*S*)- or (*R*)-α-Methylbenzylamine, 5% $NaOH_{(aq)}$, CH_2Cl_2, 92%. *c*: B_2H_6, THF, BF_3. Et_2O, 88%. *d*: **23**, 5% $NaOH_{(aq)}$, CH_2Cl_2, 72%. *e*: 1. $POCl_3$, benzene; 2. $NaBH_4$, MeOH, 89%, (95%ee); 3. H_2, 10% Pd/C, EtOH, 10% $HCl_{(aq)}$: **12** or **12a** ~72%.

27b afforded **28a** and **28b**, respectively, with 95% de. The same group reported the enantioselective total syntheses and X-ray structures of both (*S*)-tetrahydropalmatrubine (**29**) and (*S*)-corytenchine (**30**) [15], a class of naturally occurring tetracyclic alkaloids that also contain isoquinoline cores, and are a subclass of the protoberberine alkaloids. These compounds are found in at least eight plant families and possess a variety of biological activities including, for example, anti-inflammatory, antimicrobial, antifungal, and antitumor properties [16]. The most common of these tetrahydroprotoberberine derivatives, such as (*S*)-tetrahydropalmatrubine (**29**), have oxygen functionalities at the C-2, C-3 and C-9, C-10 positions on the A and D aromatic rings, respectively.

Less commonly found is the class of "pseudotetrahydroprotoberberines" such as (*S*)-corytenchine (**30**) and (*S*)-xylopinine (**31**), for which oxygen functionalities are on the C-2, C-3 and C-10, C-11 positions (Fig. 3). Both (*S*)-tetrahydropalmatrubine (**29**) and (*S*)-corytenchine (**30**) were derived from (*S*)-*N*-norlaudanidine, a benzyltetrahydroisoquinoline that was synthesized with high (>95% ee) enantioselectivity using a chiral auxiliary-assisted Bischler–Napieralski cyclization/reduction approach. Conversion of **12** into (S)-corytenchine (**30**) was then effected by reaction at 0 °C with formaldehyde (37% formalin) in acetonitrile, followed by the addition of $NaBH_3CN$ and acetic acid. The reaction afforded almost quantitative formation of **30**. Using formalin in ethanol "without acid" showed approximately 70% of **30** and 30% of the regioisomeric product **29** (Scheme 5).

Lipshutz's Synthesis of (+)-Korupensamine

Michellamine B (**32b**), a naphthylisoquinoline alkaloid isolated from Cameroonian liana *Ancistrocladus korupensis*, has exhibited significant *in vitro* activity as a potent anti-HIV-1 and anti-HIV-2 agent (Fig. 4). All of these naturally occurring dimeric naphthylisoquinolines have in common a central binaphthalene part and the restricted rotation around the C-5/C-8′ and C-8″/C-5‴ bonds produce different atropisomers of michellamine [17]. Isomerization does not take place thermally, but under basic conditions, they do isomerize. The structure of a michellamine shows it to be a heterodimeric product of tropdiastereomeric korupensamine B (**34**) and contains two stereogenic centers in each of their two tetrahydroisoquinoline rings (at C-1, C-3, C-1‴, and C-3‴) and two stereogenic biaryl bonds between these rings and the

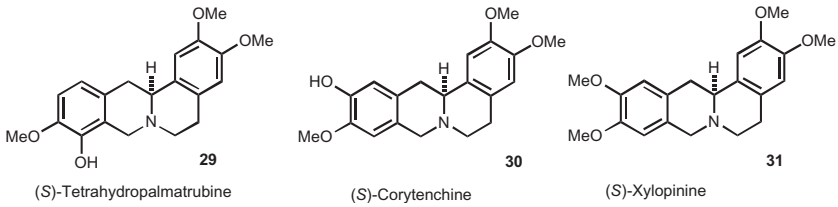
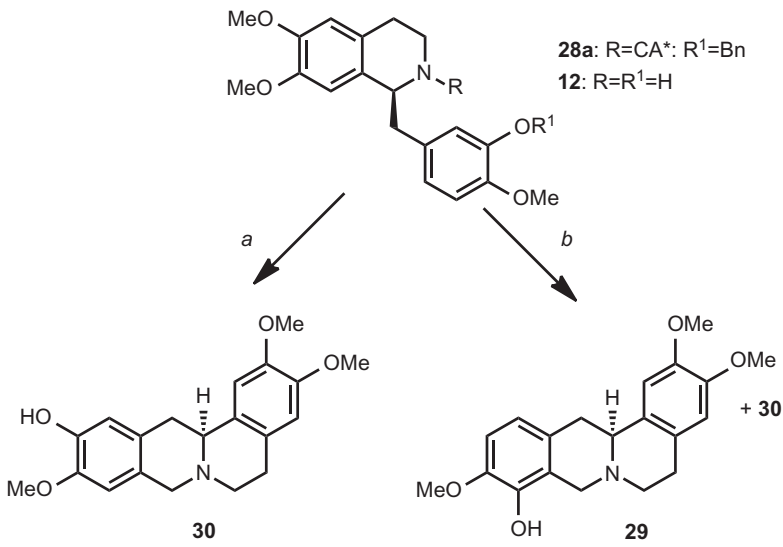
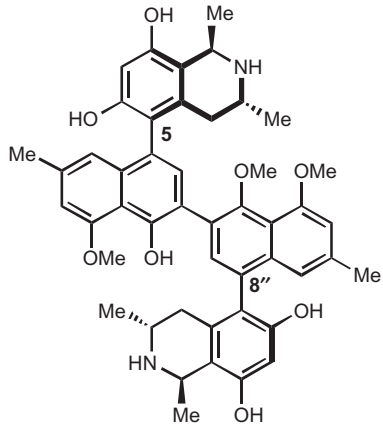


FIGURE 3 Structures of some common tetrahydroprotoberberine alkaloids.



SCHEME 5 Syntheses of (*S*)-tetrahydropalmatrubine (**29**) and (*S*)-corytenchine (**30**). *a*: 1. HCHO, CH_3CN; 2. $NaBH_3CN$; 3. CH_3CO_2H, 95%. *b*: 1. HCHO, EtOH; 2. $NaBH_4$, ~70% **30** and ~30% **29**.



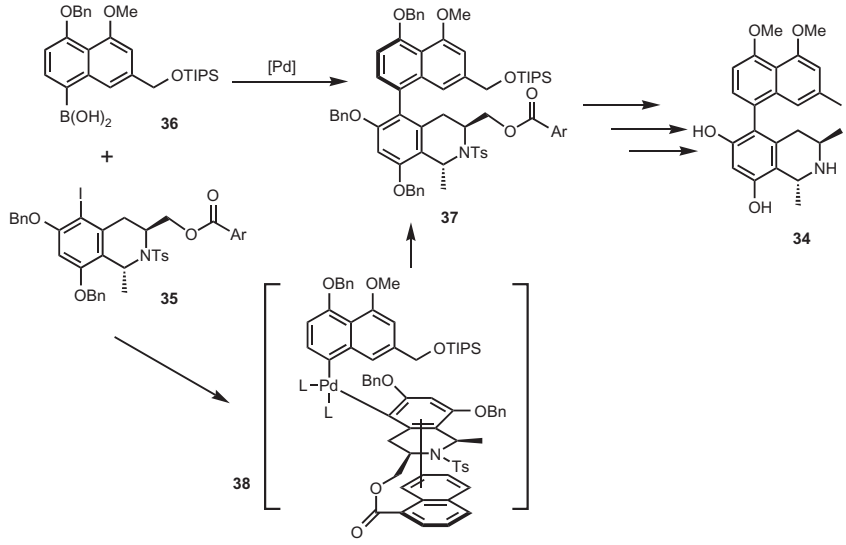
Configuration of stereogenic 5/8″ biaryl axis:

32a Michellamine A *S*/*S*
32b Michellamine B *R*/*S*
33 Michellamine C *R*/*R*

FIGURE 4 Structures of michellamines A–C.

central binaphthalene ring system. Lipshutz *et al.* [18] synthesized the nonracemic korupensamines B (**34**) via a tropselective intermolecular Suzuki–Miyaura biaryl coupling for the construction of the fully fashioned naphthylisoquinoline framework that invokes π-stacking as a possible source of the stereocontrol (Scheme 6).

The strategy for the atropselective synthesis of korupensamines B (**34**), which are biosynthetic precursors to the michellamines, is based on the



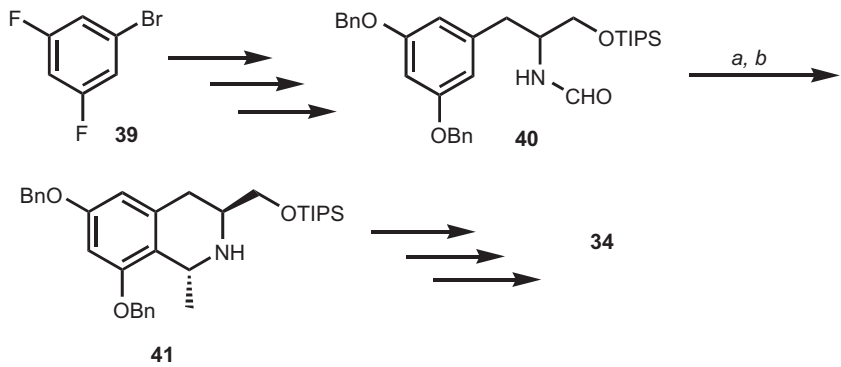
SCHEME 6 Lipshutz's synthesis of korupensamine B (**34**).

intramolecular π-stacking interactions between the electron-rich tetrahydroisoquinoline ring and its electron-deficient naphthyl ester part in the intermediate **35** which would orient one face of the tetrahydroisoquinoline ring as shown in structure **38**. The bulky triisopropylsilyl ether on the second component after metal-exchange with the palladium catalyst is thereby positioned to avoid steric interactions with the ligands in a square-planar array around the metal in **38**.

The tetrahydroisoquinoline **41** which has stereogenic centers at C-1 and C-3 was prepared as the main diastereomer in a Bischler–Napieralski cyclization step using $POCl_3$ in combination with 2-methylpyrazine, to afford the corresponding imine in 73% yield. The crucial intermediate, formamide **40**, in turn, was prepared from the chiral primary amine obtained from the commercially available 1-bromo-3,5-difluorobenzene (**39**) via several steps (Scheme 7). The imine was then treated with MeMgCl in Et_2O at low temperature to produce korupensamine B (**34**) in 85% isolated yield with excellent *trans* diastereoselectivity (>20:1 de).

Gurjar's Synthesis of Schulzeines B and C

Three novel tetrahydroisoquinoline alkaloids (Fig. 5) designated as schulzeines A–C (**42–44**), which were isolated from the Japanese marine sponge, *Penares schulzei*, were reported by Fusetani and coworkers in 2004. This group of natural products strongly inhibits yeast α-glucosidase at a concentration as low as 48 nM [19].



SCHEME 7 Synthesis of the precursors in Lipshutz's synthesis of korupensamine B. *a*: $POCl_3$, 2-methylpyrazine, CH_2Cl_2, 0^0 C to rt, 73% benzene. *b*: MeMgCl·Et_2O, −78 °C to rt, 85% (*trans*:*cis* > 20:1).

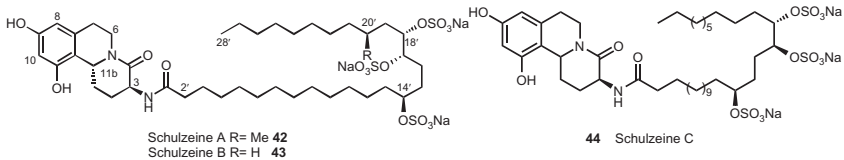
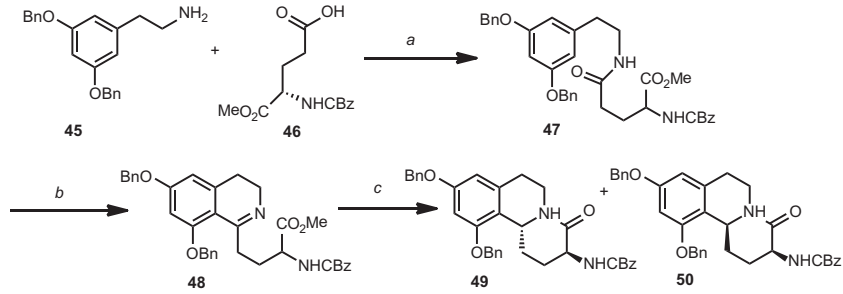


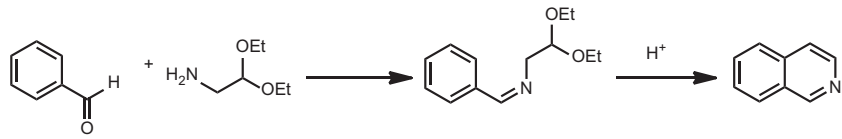
FIGURE 5 Structures of schulzeines A–C.

The structures of these schulzeines each have tricyclic units consisting of a tetrahydroisoquinoline with a fused γ-lactam and a 28-carbon-sulfated fatty acid side chain linked together by an amide bond. The tricyclic unit has two stereogenic centers at C-11b and C-3. In all members of the group, the C-3 stereogenic centers have the same *S*-configuration. The C-11b stereogenic center in schulzeines A (**42**) and C (**44**), however, have the *R*-configuration, whereas schulzeine B (**43**) has the *S*-configuration at this position. The C-28 fatty acid side chain contains three stereogenic centers at C-14, C-17, and C-18, each having *S*-configurations. Schulzeine A (**42**), however, has an extra C-20 *S*-stereogenic center which has a methyl substituent.

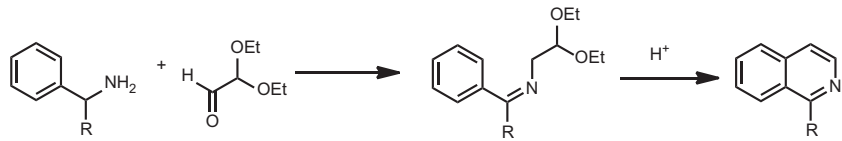
A short synthetic route (Scheme 8) toward the tricyclic core of schulzeines has been reported by Kuntiyong *et al*. [20], but the first total syntheses of schulzeines B (**43**) and C (**44**) was reported by Gurjar and coworkers, in 2007 [21]. A nonstereoselective Bischler–Napieralski cyclization–reduction reaction was the key step to prepare the tetrahydroisoquinoline moiety of these molecules starting from the amide derivative **47** using $POCl_3$ in $CHCl_3$ at 70 °C to afford **48**. Reduction of the resulting imine with $NaCNBH_3$ and subsequent stirring with $NaHCO_3$ resulted in cyclization to form the tricyclic derivatives **49** and **50** in a 2:3 ratio. The isomers could be separated by simple-column chromatography and were characterized by 2D-NMR spectral studies.



SCHEME 8 Gurjar's synthesis of Schulzeines B and C. *a*: EDC, HOBt, CH_2Cl_2, 0 °C-rt, 15 h, 84%. *b*: $POCl_3$, $CHCl_3$, 70 °C, 3 h. *c*: $NaBH_3CN$, AcOH, CH_2Cl_2, 0 °C, 1 h, aq. $NaHCO_3$, rt, 3 h, 65% for the two steps.



SCHEME 9 The Pomeranz–Fritsch reaction.



SCHEME 10 The Schlittler–Müller modification.

ASYMMETRIC POMERANZ–FRITSCH AND RELATED REACTIONS

Acid-catalyzed cyclization of a benzalaminoacetal results in the formation of the isoquinoline nucleus. This reaction was first reported by Pomeranz [22a] and Fritsch [22b] and, with some of the modifications described below, has been used in the synthesis of a variety of isoquinoline and other isoquinoline-ring-based compounds. The basic reaction is carried out in two stages. In the first step, condensation of an aromatic aldehyde and an aminoacetal leads to the formation of a benzalaminoacetal (a Schiff base). In the second step, an acid-catalyzed ring closure leads to the isoquinoline (Scheme 9).

An alternative route is the Schlittler–Müller modification [23], which involves the condensation of a benzyl amine with glyoxal semiacetal (Scheme 10).

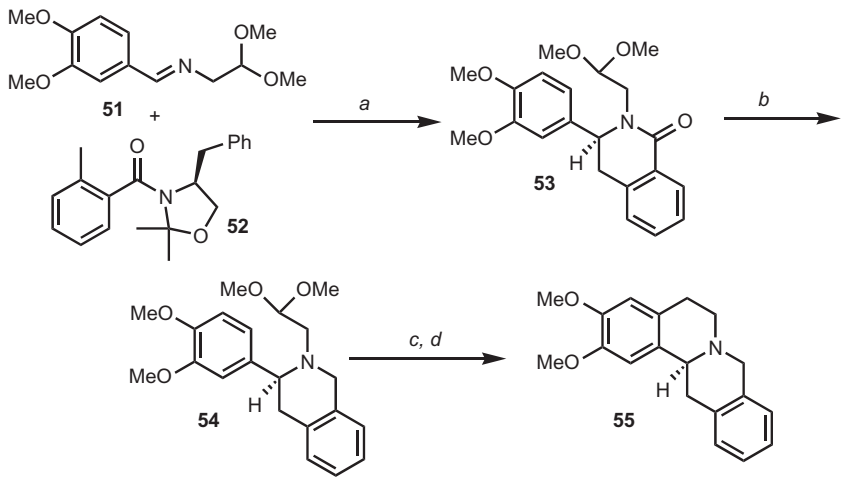
The Bobbitt modification of the Pomeranz–Fritsch methodology involves the reductive cyclization of an *N*-benzylaminoacetaldehyde to afford the 1,2,3,4-tetrahydroisoquinoline core [24]. The following section reviews some recently reported asymmetric syntheses of benzyltetrahydroisoquinoline alkaloids which have employed the Pomeranz–Fritsch and its modified reactions.

Synthesis of (S)-(−)- and (R)-(+)-O-Methylbharatamine Using Chiral o-Toluamide

The protoberberines are a large class of naturally occurring alkaloids and possess antitumor, antimicrobial, and other biological activities [25]. *O*-methylbharatamine has served for many years as a model compound for designing new methods for the synthesis of the protoberberine skeleton [26]. Chrzanowska *et al*. [27] synthesized (*S*)-(−)- and (*R*)-(+)-*O*-methylbharatamines in high enantiomeric purity using a Pomeranz–Fritsch–Bobbitt methodology (Scheme 11). In this synthetic approach, a new stereogenic center was created by addition of the Pomeranz–Fritsch imine **51** to the benzylic carbanion generated from an enantiopure *o*-toluamide. Treatment of the (*S*)-(−)-*o*-toluamide **52**, or its (*R*)-(+)-enantiomer with *n*-butyllithium, followed by addition of the prochiral imine **51** at −72 °C, leads to the formation of compound **53**. Further transformation, involving LAH-reduction of **53** to give compound **54**, followed by cyclization–hydrogenolysis, resulted in the formation of (*S*)-(−)-methylbharatamine (**55**) in 88% ee, or its enantiomer in 73% ee.

Synthesis of (S)-(−)-O-Methylbharatamine Using (S)-N-tert-Butanesulfinimine

A diastereoselective Pomeranz–Fritsch–Bobbitt methodology was used in the synthesis of (*S*)-(−)-*O*-methylbharatamine using (*S*)-*N*-*tert*-butylsulfinimine as a substrate, as has been reported by Rozwadowska *et al*. [28]. In their synthetic approach, the readily available (*S*)-sulfinaldimine (**56**) and the *o*-toluamide **57** were used as the starting materials. The key step of the



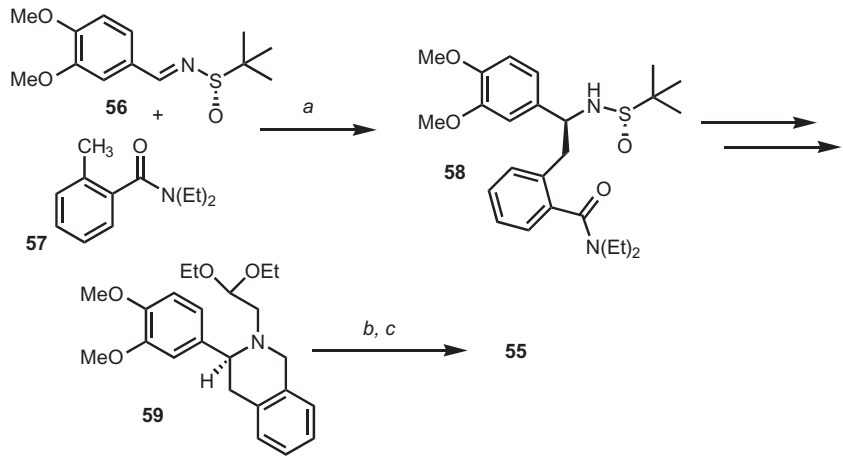
SCHEME 11 Chrzanowska's synthesis of (*S*)-(−)- and (*R*)-(+)-*O*-methylbharatamines. *a*: *n*-BuLi, THF. *b*: $LiAlH_4$, THF. *c*: 5 M $HCl_{(aq)}$. *d*: $NaBH_4$/TFA.

process is one in which the generation of a new stereogenic center is formed by the addition of the laterally lithiated *N*,*N*-diethyl-*o*-toluamide (**57**) to the imine C=N (Scheme 12). The amide carbanion was generated with *tert*-BuLi at −72 °C and the addition with **56** forms the product **58** which, with further transformation, leads to the cyclized tetrahydroisoquinoline compound **59**. Treatment of **59** with hydrochloric acid followed by reduction with sodium borohydride/TFA yields (*S*)-(−)-*O*-methylbharatamine **55** in 88% ee.

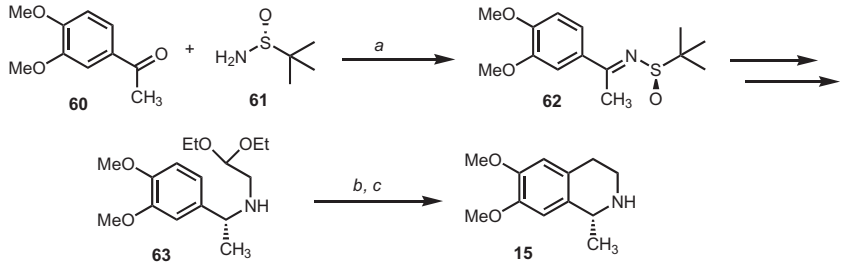
Synthesis of (R)-(+)- and (S)-(−)-Salsolidine

(*R*)-(+)-Salsolidine (**15**) was synthesized by Grajewska and Rozwadowska [29] in high enantiomeric purity, using the Pomeranz–Fritsch–Bobbitt methodology (Scheme 13). In this approach, 3,4-dimethoxyacetophenone (**60**) was condensed with (*R*)-*tert*-butanesulfinylamide (**61**) using $Ti(OEt)_4$, to afford the ketimine **62**, which was used for the key step of the synthesis. The sulfanilamide effectively served as a chiral auxiliary. Hydride addition to **62** using diisobutylaluminium hydride (DIBAL-H), followed by further transformations led to **63**. Cyclization to the tetrahydroisoquinoline ring was effected by a two-step, one-pot procedure involving the treatment of aminoacetal **63** with 6 M hydrochloric acid followed by $NaBH_4$/TFA reduction to afford (*R*)-(+)-salsolidine **15** in 58% yield with 95.5% ee.

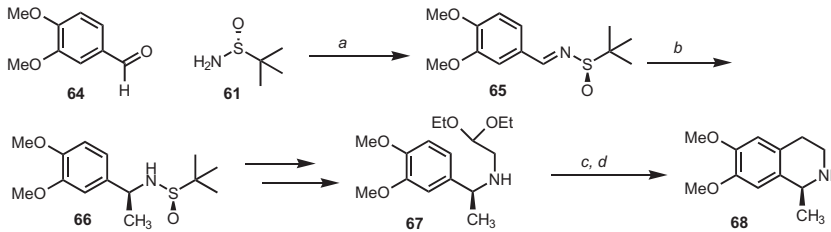
The enantiomeric form of **15**, namely, (−)-salsolidine (**68**), was synthesized via a diastereoselective Pomeranz–Fritsch–Bobbitt synthesis by using chiral **61** as a chiral auxiliary, attached to the nitrogen atom of ketimine **65** derived from 3,4-dimethoxybenzaldehyde (**64**) [30]. The chiral auxiliary permitted the diastereoselective addition of methylmagnesium bromide to



SCHEME 12 Rozwadowska's synthesis of (*S*)-(−)-*O*-methylbharatamine (**55**). *a*: *tert*-BuLi, −72 °C. *b*: 5 M $HCl_{(aq)}$. *c*: $NaBH_4$/TFA.



SCHEME 13 Grajewska and Rozwadowska's synthesis of (*R*)-(+)-salsolidine (**15**). *a*: $Ti(OEt)_4$, THF. *b*: 6 M $HCl_{(aq)}$. *c*: $NaBH_4$/TFA (trifluoroacetic acid).



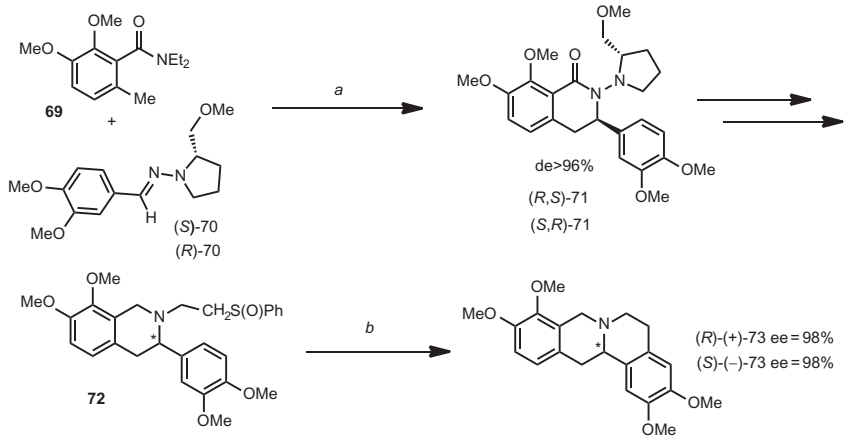
SCHEME 14 Kosciolowicz and Rozwadowska's enantioselective synthesis of (−)-salsolidine (**68**). *a*: $Ti(O^iPr)_4$, THF, 65 °C. *b*: CH_3MgBr, THF, −27 °C. *c*: 6 M $HCl_{(aq)}$. *d*: $NaBH_4$/TFA, DCM.

the imine C═N to afford **66**. Removal of the chiral auxiliary and the usual transformations afforded the Pomeranz–Fritsch amine **67**. The synthesis was completed in a one-pot procedure in which **67** was treated with 6 M hydrochloric acid followed by reduction with $NaBH_4$/trifluoroacetic acid to afford (*S*)-(−)-salsolidine **68** in 98% ee (Scheme 14).

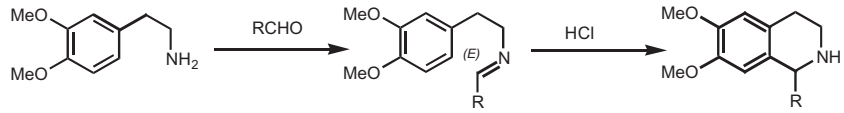
Asymmetric Synthesis of Tetrahydropalmatine

Tetrahydropalmatine (**73**), which belongs to the tetrahydroprotoberberine family, racemic form of tetrahydropalmatine, was shown to possess insecticidal activity against the larvae and adults of *Drosophila melanogaster* [31], whereas its (*S*)-(−)-enantiomer showed an inhibitory effect on the *Epstein–Barr* virus [32].

An asymmetric synthesis of tetrahydropalmatine via a tandem 1,2-addition/cyclization methodology has been reported by Boudou and Enders [33]. In their synthetic approach (Scheme 15), the diethylbenzamide **69** and the SAMP or RAMP [(*S*)- or (*R*)-1-amino-2-methoxymethylpyrrolidine] hydrazone of 3,4-dimethoxybenzaldehyde (**70**) gave the corresponding dihydroisoquinolones **71** in 96% de and 54–55% yields. Removal of the chiral auxiliaries and further transformation lead to the *N*-functionalized-3-substituted tetrahydroisoqinolines



SCHEME 15 Boudou and Enders' synthesis of tetrahydropalmatine (**73**). *a*: *N*,*N*,*N*′,*N*′-Tetramethylethylenediamine (TMEDA), (7.0 eq)/*s*-BuLi (6.5 eq), Et_2O/THF (5/1), −78 °C. *b*: Conc. $HCl_{(aq)}$.



SCHEME 16 A typical Pictet–Spengler reaction.

72 which, upon ring closure by a typical Pomeranz–Fritsch reaction, produced (*R*)-(+)-**73** in 9% and overall yield over seven steps and (*S*)-(−)-**73** in 17% overall yield with high enantioselectivity (98% ee).

ASYMMETRIC PICTET–SPENGLER SYNTHESES

The Pictet–Spengler reaction which was discovered in 1911 by Amé Pictet and Theodor Spengler [34] has remained an important reaction in alkaloid and pharmaceuticals synthesis. The reaction is an important acid-catalyzed transformation for the synthesis of tetrahydroisoquinolines from carbonyl compounds and β-arylethylamines (Scheme 16). The reactions are usually carried out in an aprotic solvent in the presence of an acid catalyst and afford high yields when the number of electron-donating groups on the phenylethylamine aromatic ring is increased.

The regioselectivity for isoquinoline synthesis in a Pictet–Spengler cyclization depends on the electron-donating group in the aromatic ring of phenylethylamine and that the least sterically hindered *ortho* position is the predominant cyclization site. Some other catalysts and dehydrating agents such as trifluoromethanesulfonic acid, acetic acid, and trifluoroacetic acid can also be used to increase the yield and regioselectivity. No relationship

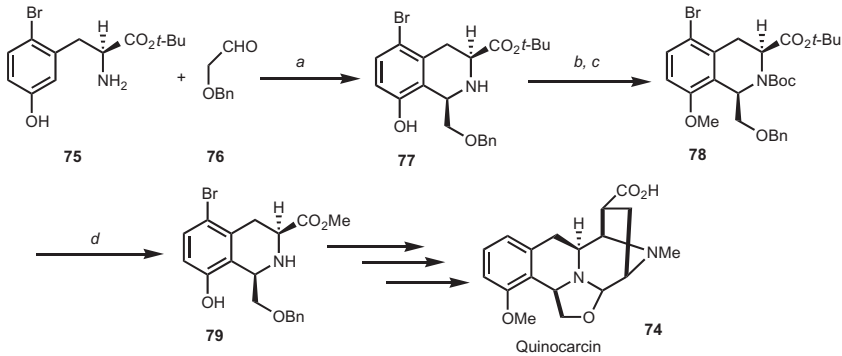
has been established between this reaction's pH and yield or regioselectivity [35]. When an aldehyde other than formaldehyde is used, a new chiral center at C-1 is generated. The chirality of this new center can be controlled by using a chiral auxiliary introduced to either the β-arylethylamine or the aldehyde component, thereby involving a diastereoselective synthesis.

Using Chiral Amines

The use of a chiral amine or carbonyl component in an intermolecular Pictet–Spengler condensation is a valuable strategy for obtaining chiral iminium intermediates and achieving the chirality to the newly generated C-1 stereocenter. The following highlights some recent syntheses using chiral amines.

Zhu's Synthesis of (−)-Quinocarcin

(−)-Quinocarcin (**74**) is a pentacyclic antitumor and antibiotic tetrahydroisoquinoline alkaloid isolated from *Streptomyces melanovinaceus* nov. sp. by Takahashi and Tomita in 1983 [36]. It is structurally similar to the saframycins and ecteinascidins (ETs) in the A-, B-, and C-rings but differs in the D-ring, which is a five- rather than a six-membered ring. Three asymmetric synthesis of (−)-quinocarcin have been reported in the literature [37–39]. In 2008, Zhu *et al*. published an asymmetric total synthesis of (−)-quinocarine [40] in which the key step to establish the A- and B-rings was a Pictet–Spengler reaction, as shown in Scheme 17. Condensation of L-*tert*-butyl-2-bromo-5-hydroxyphenylalanate (**75**) with benzyloxyacetaldehyde (**76**) under mild acidic conditions afforded the 1,3-*cis* tetrahydroisoquinoline **77** as single diastereomer in 91% yield. The bromine atom on the phenyl ring not only led to an increase in the diseteroselectivity during the cyclization step but also determined the regioselectivity. Protecting the secondary amine as its



SCHEME 17 Zhu's synthesis of (−)-quinocarcin (**74**). *a*: AcOH, CH_2Cl_2, 4 Å MS, rt, 36 h, 91%. *b*: Boc_2O, DIPEA, MeCN, rt, 6 h, 85%. *c*: Me_2SO_4, acetone, Cs_2CO_3, rt, 4 h, 92%. *d*: $SOCl_2$, MeOH, reflux, 4 h, 95%.

N-tert-butoxycarbamate (*N-Boc*), followed by methylation of the phenol, provided the key intermediate **78** in 78% overall yield.

Zhu's Syntheses of (−)-Ecteinascidin597 and (−)-Ecteinascidin583

ETs, whose structures incorporate a piperazine-bridged bis(tetrahydroisoquinoline) framework similar to that of the saframycin class of antitumor antibiotics, were first isolated by Reinhart *et al*. in 1990 from the Caribbean tunicate *Ecteinascidia turbinate* [41]. They include ETs 729, 743, 745, 759A, 759B, and 770 and have a wide range of antitumor and antimicrobial activities. Four putative biosynthetic precursors (ETs 594, 597, 583, and 596) were isolated in 1996 [42]. Although ET597 has less of a cytotoxic effect than that of ET743 against P388, A549, HT29, and the CV-1 cell lines, it has a large antiproliferative activity (Fig. 6).

Zhu *et al*. [42] completed the asymmetric total syntheses of ET597 and ET583 which contain two tetrahydroisoquinoline units using a Pictet–Spengler reaction for the key intermediate(s) in the synthesis (Scheme 18). Reaction of the chiral amine **82** with $TrocOCH_2CHO$ in acetic acid and dichloromethane in the presence of molecular sieves produced a single diastereomer **83** in 90% yields, thus forming the A- and B-rings. Zhu determined the high diastereoselectivity in this Pictet–Spengler reaction as being due to the iminium intermediate which had a *trans* and the pseudoequatorial orientation of the substituents at C-3 and C-4 as shown in **84** (Fig. 7). The presence of the phenolic free hydroxyl group in the A ring also was found to be essential for the reaction to occur. The C-ring cyclization step formed the important intermediate **85**. A second, one-pot, condensation–cyclization step formed the

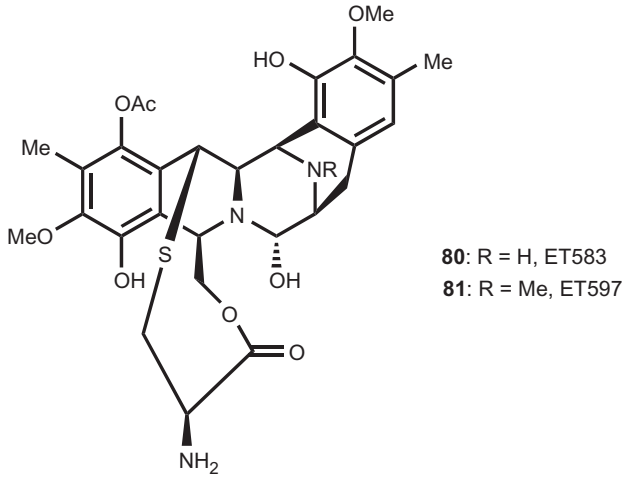
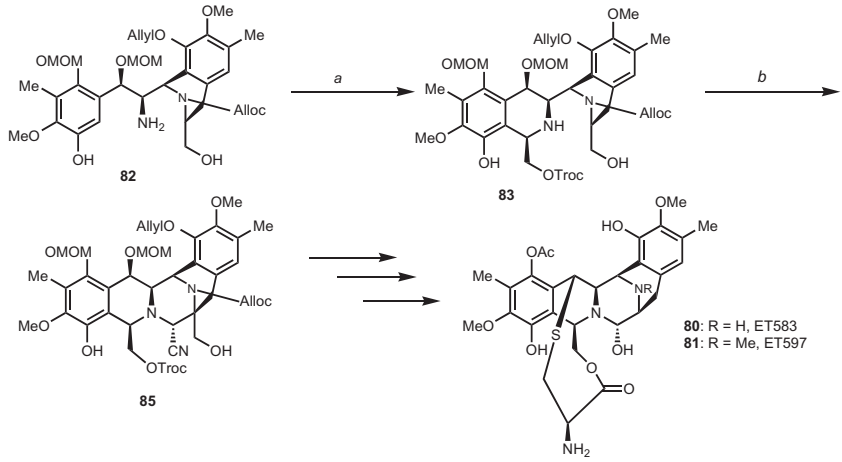


FIGURE 6 Structures of ecteinascidins ET583 (**80**) and ET597 (**81**).



SCHEME 18 Zhu's syntheses of (−)-ecteinascidin583 (**80**) and (−)-ecteinascidin597 (**81**). *a*: AcOH, $TrocOCH_2CHO$, 3 Å MS, CH_2Cl_2, rt, 90%. *b*: $(COCl)_2$, DMSO, CH_2Cl_2, −60 °C, then TMSCN, $ZnCl_2$, CH_2Cl_2, rt, 87%.

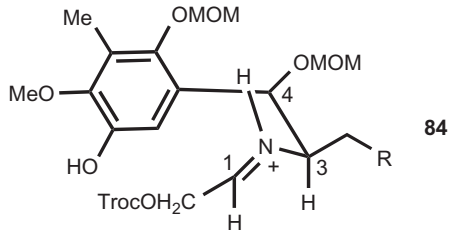


FIGURE 7 Zhu's proposed iminium intermediate structure **84**.

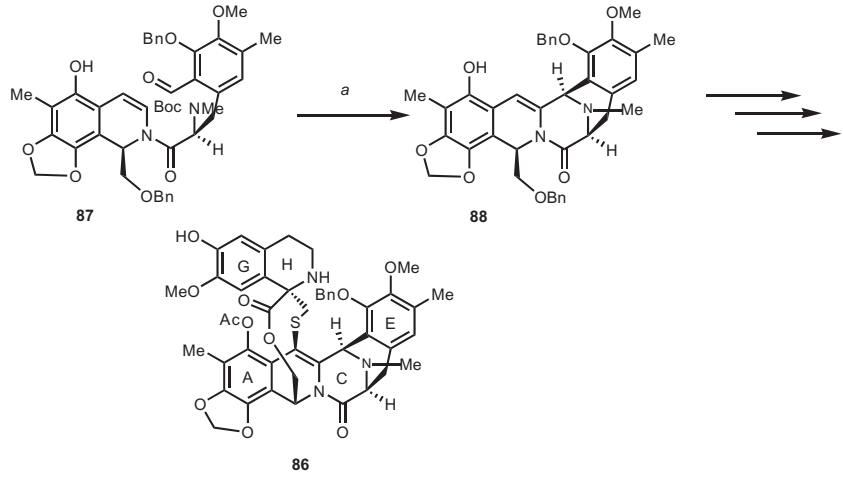
key intermediate **85** which was subsequently transformed into ET597 or ET583 in good overall yield.

Danishefsky's Synthesis of ET743

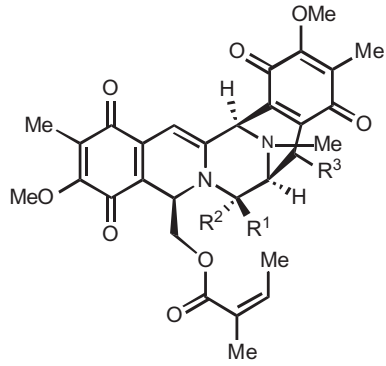
Danishefsky [43] used an intramolecular Pictet–Spengler cyclization in his stereospecific total synthesis of ET743 (**86**). This natural product was isolated from a marine tunicate, *E. turbinate*, and is one of the most highly cytotoxic natural products found to be an antitumor agent. The stereoselective formation of the C-11 stereocenter in **88** was achieved from the intermediate **87** using difluoroacetic acid and heating in benzene solution. Although the yield was only 42–58% yield, the desired stereocenter at C-11 was obtained (Scheme 19).

Williams' Synthesis of (−)-Cribrostatin 4 (Renieramycin H)

The renieramycins are a group of pentacyclic alkaloids isolated from different sponge species. Renieramycins A–D were isolated from *Reniera* sp. by Frincke



SCHEME 19 Danishefsky's synthesis of ET743 (**86**). *a*: CHF_2CO_2H, $MgSO_4$, benzene, 100 °C, 45 min, 42–58%.



Renieramycins

A: $R^1 = R^2 = R^3 = OH$ (**89**)
B: $R^1 = R^2 = H$, $R^3 = OMe$ (**90**)
C: $R^1 = R^2 = O$, $R^3 = OH$ (**91**)
D: $R^1 = R^2 = H$, $R^3 = OEt$ (**92**)
E: $R^1 = H$, $R^2 = O$, $R^3 = H$ (**93**)
F: $R^1 = H$, $R^2 = OH$, $R^3 = OMe$ (**94**)
G: $R^1 = R^2 = O$, $R^3 = H$ (**95**)

FIGURE 8 Structures of renieramycins A–G.

and Faulkner; [44] renieramycins E–G were isolated from the Fijian sponge *Xestospongia caycedoi* [45], and two renieramycins H and I were isolated by Parameswaran *et al*. from the sponge *Haliclona cribricutis* [46] (Fig. 8).

The structure of renieramycin H was later revised to that of **96** by Kubo and coworkers who isolated the compound from *Cribrochalina* sp. and named it cribrostatin 4 [47]. Renieramycin H has moderate antimicrobial activities (Fig. 9).

Williams *et al*. [48] used two different strategies to form the two tetrahydroisoquinoline units in the renieramycin pentacycle. The key step for installing the D and E-rings was to use a reductive opening/elimination of the C-3, C-4 β-lactam in **100** followed by the formation of the iminium ion and

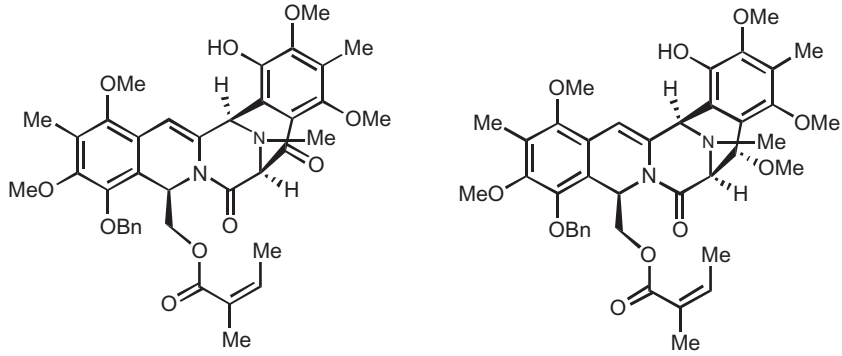
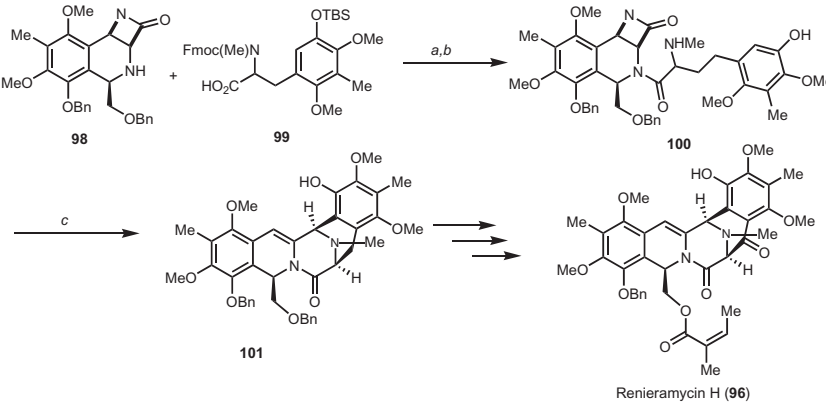


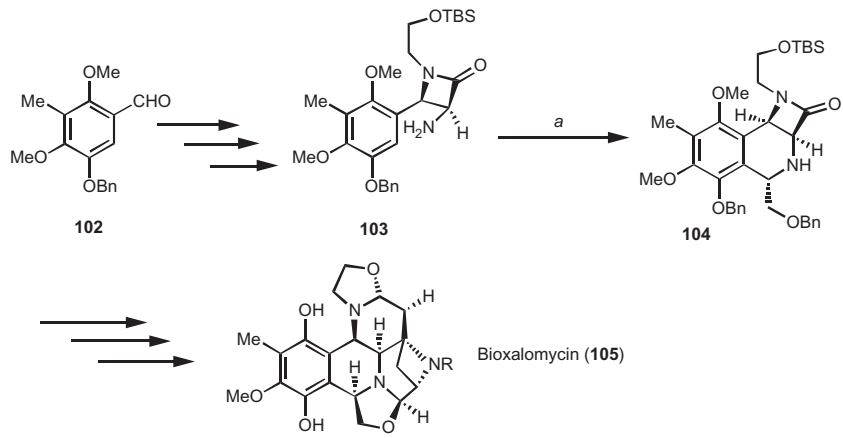
FIGURE 9 Structures of renieramycin H (cribrostatin 4) and renieramycin I.



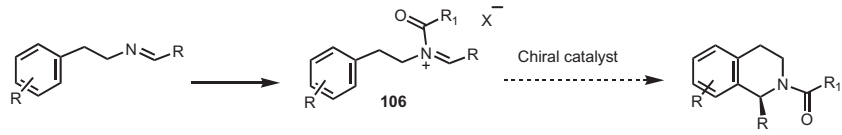
SCHEME 20 Williams' synthesis of (−)-cribrostatin 4 (renieramycin H). *a*: $(COCl)_2$, DMF, CH_2Cl_2, 2,6-lutidine, rt, 79%. *b*: TBAF, THF, rt, 92%. *c*: $LiEt_3BH$, THF, 30 min, 0 °C then aq NH_4Cl, 62%.

subsequent Pictet–Spengler cyclization to afford the pentacyclic intermediate **101** which could be converted to the desired product cribrostatin 4 (**96**). Scheme 20 outlines the synthesis including that of **100** from the condensation reaction of **98** and **99**.

The asymmetric syntheses of the tetrahydroisoquinoline **104** which is related to **100** were used to prepare highly functionalized tetrahydroisoquinolines relevant to the bioxalomycin (**105**) and ecteinascidin families of antitumor alkaloids. Williams *et al*. also produced a single diastereomer of **104** in 85% yield when the amine **103** was condensed with benzyloxyacetaldehyde in MeOH at 50 °C (Scheme 21).



SCHEME 21 Williams' synthesis of intermediate **104** toward bioxalomycin (**105**). *a*: $BnOCH_2$-CHO, MeOH, 50 °C, 86%.



SCHEME 22 Chiral catalyst-mediated Pictet–Spengler reaction.

Using Chiral Aldehydes or Chiral Catalysts

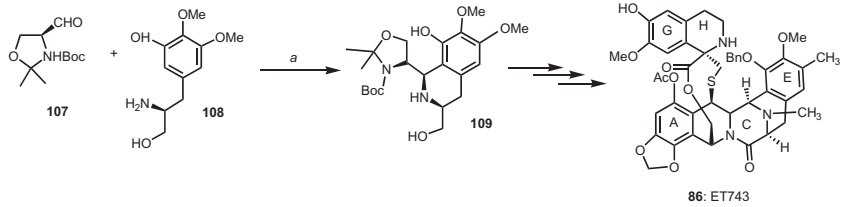
Many natural and biologically important alkaloids have been synthesized using optically active carbonyl components in a Pictet–Spengler reaction as one of the key steps. These include the use of sulfur chirality (**106**), cyclohexyl derivatives such as menthol, bicyclics such as camphor, and amino acid-derived aldehydes [49] (Scheme 22).

Zhu's Synthesis of ET743

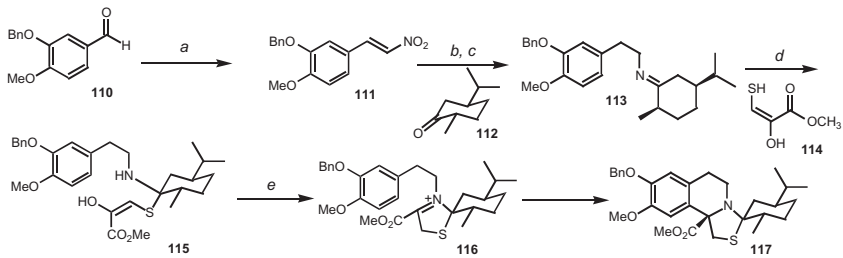
Zhu [50] has also completed the total synthesis of ET743 (**86**) in 31 steps with 1.7% overall yield using a highly distereoselective Pictet–Spengler condensation reaction to establish the D-E fragment **109** using Garner's (*S*)-chiral aldehyde [51] (**107**) to control the C-11 stereogenic center. Condensation of **107** with L-3-hydroxy-4-methoxy-5-methylphenylalanol (**108**) under acidic conditions provided the desired tetrahydroisoquinoline **109** diastereoselectively as the only isolable product, in 84% yield (Scheme 23).

Corey's Synthesis of ET743

Corey's synthesis of ET743 used (+)-tetrahydrocarvone as a chiral auxiliary for the stereoselective preparation of the intermediate **117**, the



SCHEME 23 Zhu's synthesis of ET743 (**86**). *a*: AcOH, CH_2Cl_2/CF_3CH_2OH (7/1), 3 Å MS, rt, 20 h, 88%.



SCHEME 24 Corey's synthesis of ET743 via chiral auxiliary-functionalized intermediate **117**. *a*: CH_3NO_2, piperidine, AcOH. *b*: $LiAlH_4$. *c*: 112, 3 Å MS. *d*: AcOH, 114. *e*: CH_3SO_2H, 3 Å MS.

spirotetrahydroisoquinoline unit which forms rings G and H of ET743 (Scheme 24) [52]. 3-Benzyloxy-4-methoxybenzaldehyde **110** was subjected to nitroaldol condensation to afford nitrostyrene **111**, which, upon reduction and condensation with (+)-tetrahydrocarvone, formed the Schiff base, **113**. The crude imine was added to methyl 3-mercaptopyruvate to diastereoselectively form the *N*,*S*-ketal **115** which, when treated under acidic conditions, formed **117** via the putative intermediate **116**.

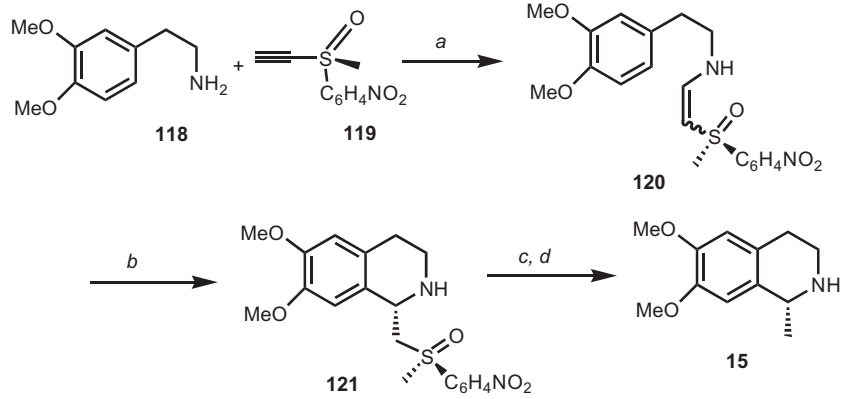
Lee's Synthesis Using Chiral Acetylenic Sulfoxides

Lee *et al*. [53] prepared chiral (*R*)-(+)-ethynyl-*o*-nitrophenylsulfoxides (**119**) which were submitted to a Michael addition with the primary amine 3,4-dimethoxyphenethylamine **118**, producing vinyl sulfoxides **120**, which were cyclized using TFA catalysis to obtain single diastereomers of tetrahydroisoquinoline **121** as the only isolated product (Scheme 25).

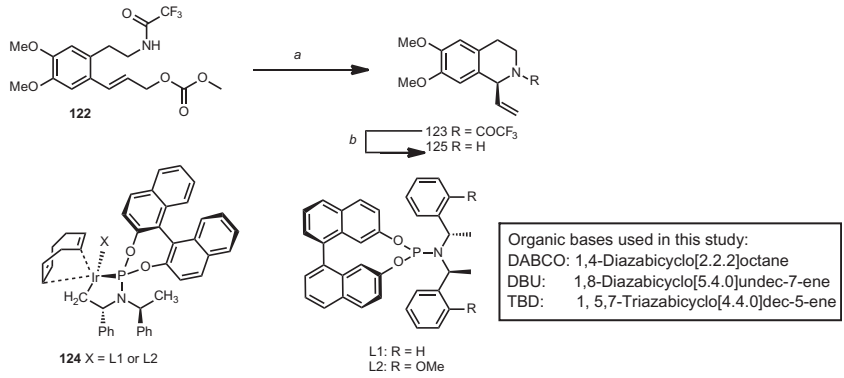
METAL-CATALYZED CYCLIZATIONS

Iridium-Catalyzed Asymmetric Synthesis of Tetrahydroisoquinolines

Feringa *et al*. reported an enantioselective synthesis of tetrahydroisoquinolines by an intramolecular iridium-catalyzed asymmetric intramolecular allylic amidation [54]. This synthetic approach formed tetrahydroisoquinoline



SCHEME 25 Lee's use of chiral (*R*)-(+)-ethynyl-*o*-nitrophenylsulfoxide (**119**). *a*: $CHCl_3$, rt, 2 h. *b*: TFA, 0 °C, 1 h. *c*: $CH_2O/NaBH_3CN$. *d*: Raney Ni.



SCHEME 26 Feringa's iridium-catalyzed asymmetric synthesis of tetrahydroisoquinolines. *a*: Ir catalyst **128** (5 mol%), base (1.0 equiv), THF. *b*: K_2CO_3, $MeOH/H_2O$, rt.

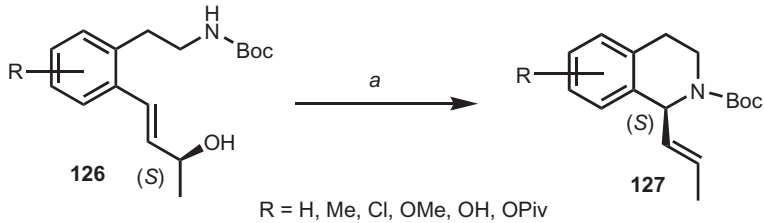
moieties in high yields and appreciable enantioselectivity. The conversion of allylic carbonate **122** to the corresponding protected chiral tetrahydroisoquinoline **123** was facilitated by the presence of iridium catalyst **124** and DBU (Scheme 26). The reaction shown was performed with different bases such as DBU, TBD, K_3PO_4, Cs_2CO_3, and DABCO, but high enantioselectivity (81% ee) was reported only with DBU. The reaction was carried out in the presence of the base and iridium catalysts containing different ligands, such as phosphoramidite ligand (**L1**) and methoxy-substituted phosphoramidite (**L2**) (Scheme 26). The intramolecular asymmetric allylic amidation with the highest yields (90%) and enantioselectivity (95% ee) was reported for the iridium catalyst containing ligand **L2**. Using this methodology, various tetrahydroisoquinolines containing donor substituents such as methoxy, dioxo, and methyl groups were reportedly obtained with high yields and

enantioselectivities (91–95% ee) [53]. Deprotection of the trifluoroacetamide **123** with K_2CO_3 in MeOH/H_2O (7:1) gave the corresponding chiral tetrahydroisoquinoline **125**.

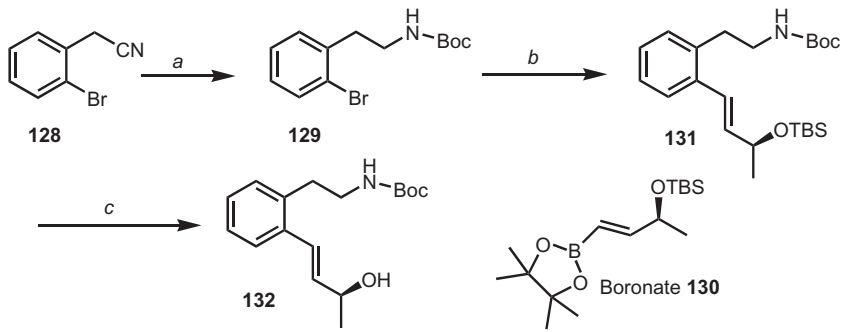
Bi(OTf)$_3$-Catalyzed Chiral Synthesis of C-1-Substituted Tetrahydroisoquinolines

Other C-1-substituted tetrahydroisoquinoline compounds are also known to have diverse biological and pharmacological properties [55]. Kawai *et al.* reported the synthesis of chiral C-1-substituted tetrahydroisoquinolines by an intramolecular 1,3-chirality transfer reaction catalyzed by $Bi(OTf)_3$ [56]. Thus, the reaction of chiral amino alcohols **126**, in the presence of $Bi(OTf)_3$ catalyst, leads to the formation of 1-substituted tetrahydroisoquinolines **127** (Scheme 27).

The stereochemistry at the newly formed chiral center is due to a *syn* S_N2'-type approach [55]. In this reaction, the substituent on the benzene ring of **126** affects the reactivity and selectivity. Scheme 28 shows the general synthesis of the cyclization precursor **132** (and **126**). Reduction of 2-(*o*-bromophenyl) acetonitrile (**128**) with $NaBH_4$ and a catalytic amount of $NiCl_2$ in methanol,



SCHEME 27 Kawai's $Bi(OTf)_3$-catalyzed synthesis of C-1-substituted tetrahydroisoquinolines. *a*: $Bi(OTf)_3$ (10 mol%), 4 Å MS, DCM.



SCHEME 28 General synthesis of Kawai's cyclization precursor **132**. *a*: $NiCl_2$, $NaBH_4$, Boc_2O, MeOH, 0 °C, 1 h. *b*: Boronate **130**, $PdCl_2$dppf, $NaHCO_3$, dioxane-H_2O, 80 °C, 1 h. *c*: TBAF (tetrabutylammonium fluoride), THF, rt.

followed by protection with Boc_2O, yields compound **129**. The cross-coupling reaction of **129** and boronate **130** in the presence of $PdCl_2$(dppf) as catalyst, and $NaHCO_3$, affords compound **131**; further deprotection with TBAF in THF affords compound **132**.

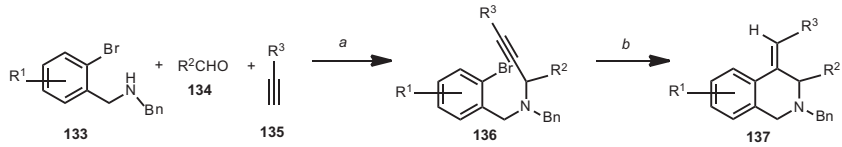
The effect of the counterion was examined for the Bi-catalyzed cyclization of **126**, which included chloride, bromide, and triflate; high yields and enantioselectivity were reported with $Bi(OTf)_3$. Various *N*-protecting substituents on the amino group were found to affect the reactivity and enantioselectivity of the cyclization reaction, but the highest yields and enantioselectivities being found with the *tert*-butyloxycarbonyl (Boc) protected amine.

Palladium-Catalyzed Synthesis of Functionalized Tetrahydroisoquinolines

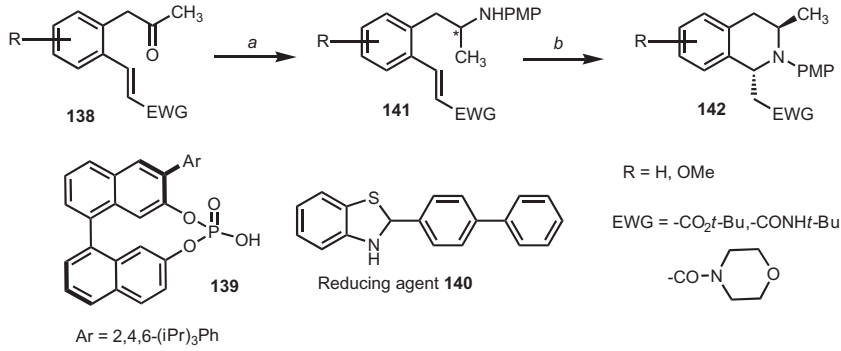
The synthesis of functionalized tetrahydroisoquinolines via palladium-catalyzed 6-*exo-dig* carbocyclization of propargylamine has been reported by Perumal *et al*. [57]. In this synthetic approach, the first step involves a CuI-catalyzed three-component coupling reaction of a terminal alkyne, an aldehyde, and an amine to give a propargylamine, as shown in Scheme 29. The second step involves the regio- and stereoselective palladium-catalyzed 6-*exo-dig* carbocyclization of the propargylamine leading to the formation of the substituted tetrahydroisoquinolines (Scheme 29). The reaction was reported to proceed with various substrates of R^1, R^2, and R^3 in the presence of CuI catalyst, leading to the formation of corresponding propargylamines. High yields were reported with $R^1 = H$, $R^2 = 4\text{-}ClC_6H_4$, $R^3 = Ph$ (87%) and $R^1 = H$, $R^2 =$ isopropyl, $R^3 = Ph$ (90%). The intramolecular cyclization of propargylamines **136** was achieved using 3 mol% of $Pd(PPh_3)_4$ catalyst and sodium formate as a reducing agent in DMF/H_2O (3:1).

Asymmetric Synthesis of Trans*-1,3-Disubstituted Tetrahydroisoquinolines*

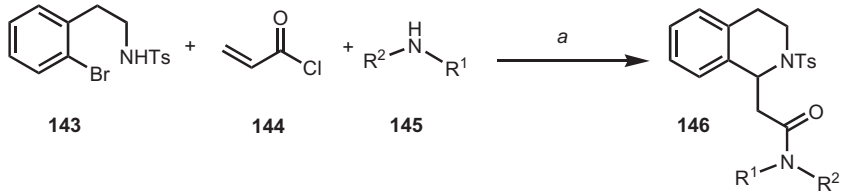
Enders *et al*. reported the organocatalytic asymmetric synthesis of *trans*-1,3-disubstituted tetrahydroisoquinolines via a reductive amination/aza-Michael sequence shown in Scheme 30 [58].



SCHEME 29 Palladium-catalyzed synthesis of functionalized tetrahydroisoquinolines. *a*: CuI (15 mol%), toluene, 100 °C, 3 h. *b*: $Pd(PPh_3)_4$ 3.0 mol%, HCO_2Na, 1.5 equiv., DMF/H_2O, 100 °C.



SCHEME 30 Enders' organocatalytic asymmetric syntheses. *a*: 10 mol% of **139**, reducing agent **140** (140 mol%), *p*-anisidine, mesitylene, 45 °C. *b*: *t*-BuOK, THF, rt.



SCHEME 31 Stewart's one-pot, three-component synthesis of tetrahydroisoquinolines. *a*: Pd $(OAc)_2/PPh_3$, K_2CO_3, PhMe, 120 °C, 16 h.

In this synthetic approach, a chiral Bronsted acid-catalyzed [59] reductive amination of three components followed by an aza-Michael cyclization leads to the formation of the tetrahydroisoquinolines. Amination of various methyl ketones **138** with *p*-anisidine in the presence of the chiral Bronsted acid catalyst **139** and the reducing agent **140** leads to the formation of **141** (Scheme 30). The aza-Michael cyclization of **141** in the presence of *t*-BuOK afforded the *trans*-1,3-disubstituted tetrahydroisoquinolines **142** with high enantioselectivity (93–98% ee) and yields ranging from 81% to 97%.

Synthesis of Tetrahydroisoquinolines Using Domino Heck–aza-Michael Reactions

Stewart *et al*. reported a one-pot, three-component approach [60] to the synthesis of functionalized tetrahydroisoquinolines using a domino Heck–aza-Michael reaction [61]. In this synthetic approach, nucleophilic addition of a primary or secondary amine **145** to acryloyl chloride **144** in the presence of K_2CO_3 forms an acrylamide which when followed by the addition of Pd $(OAc)_2$, PPh_3, and the 2-bromophenethylsulfonamide substrate **143** undergoes a domino Heck–aza-Michael reaction leading to the formation of a series of C-1-acetamido-tetrahydroisoquinolines **146** (Scheme 31). Several tetrahydroisoquinolines were synthesized with yields are ranging from 28% to 97%.

CONCLUSIONS

This review has highlighted some very recent creative applications of classical or "named" reactions for the syntheses of tetrahydroisoquinoline motifs within and toward the total asymmetric syntheses of challenging synthetic targets. These targets are compounds which have demonstrated significant biological activities which may eventually find their way into the arsenal of therapeutic agents which will be of increasing importance. In many cases, the classical methodologies with minor modifications have been successfully employed to afford highly enantioselective products.

ACKNOWLEDGMENTS

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Chapter 4

1-Methoxy-Canthin-6-One and Related β-Carbolines: From Natural Compound to Synthesis and Biological Activities

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INTRODUCTION

Tree of heaven, *Ailanthus altissima* (Mill.) Swingle (Simaroubaceae), is a medicinal tree used in traditional medicines in many parts of Asia, including China: its bark and leaves have been used for their bitter-tonic, astringent, vermifuge, and antitumoral properties. Extracts of this plant have been employed in case of leucorrhoea, as a vulnerary and an antidiarrhoeal, to treat cold, dysentery, endoparasites, and gastric diseases [1,2]. A number of phytochemical studies demonstrated the presence in the plant of several classes of chemical compounds, among which quassinoids [1–8], lipids and fatty acids [1,7–12], volatile compounds [1,13–15], phenolic compounds [1,16], flavonoids [1,17,18], and coumarines [1,19].

Alkaloids are the most representative class of secondary metabolites in *A. altissima*. Among them, canthin-6-ones are a subclass of β-carboline-based natural alkaloids in which an additional six-membered ring (D) has been fused to the β-carboline nucleus. In the 1970s, canthin-6-one (**1**), canthin-6-one-3*N*-oxide (**5**), and 1-methoxy-canthin-6-one (**2**) (Fig. 1) were isolated and identified from the wood of the plant [20]. Canthin-6-one [21,22] and canthin-6-one 3*N*-oxide [22] were isolated also from root bark; the first was present also in the leaves [23]: 1-hydroxycanthin-6-one (**3**) [24], 5-hydroxymethylcanthin-6-one (**4**) [25], and 1-methoxy-canthin-6-one-3*N*-oxide (**6**) (Fig. 1) [22] were

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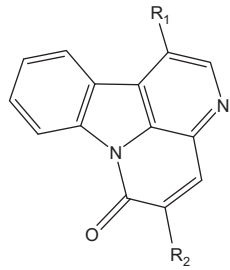
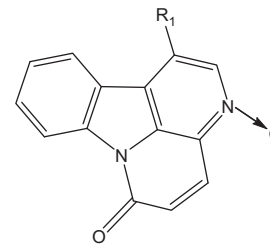
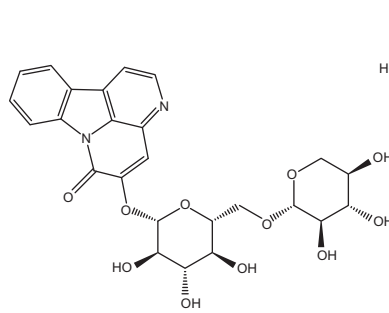




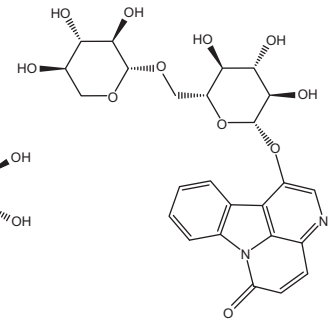
Canthin-6-one (**1**): $R_1 = R_2 = H$
1-Methoxy-canthin-6-one (**2**): $R_1 = OMe$, $R_2 = H$
1-Hydroxy-canthin-6-one (**3**): $R_1 = OH$, $R_2 = H$
5-Hydroxymethyl-canthin-6-one (**4**): $R_1 = H$, $R_2 = CH_2OH$

Canthin-6-one-3N-oxide (**5**): $R_1 = H$
1-Methoxy-canthin-6-one-3N-oxide (**6**): $R_1 = OMe$

FIGURE 1 Structures of canthinones isolated from *Ailanthus altissima*.



Ailantcanthinoside A (**7**)

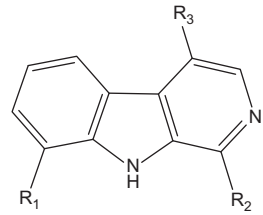


Ailantcanthinoside B (**8**)

FIGURE 2 Alkaloid glycosides from *Ailanthus altissima*.

subsequently isolated (Fig. 1). The alkaloid 1-methoxy-canthin-6-one is the most studied compound, found in *A. altissima* [21,22,26–28]. In 2007, Zhang and coworkers [29] isolated two new alkaloid glycosides from the root bark of *A. altissima*, canthin-6-one-5-O-β-D-xylopyranosyl-(1 → 6)-β-D-glucopyranoside (**7**) and canthin-6-one-1-O-β-D-xylopyranosyl-(1 → 6)-β-D-glucopyranoside (**8**), named ailantcanthinoside A and B, respectively (Fig. 2).

Several carboline alkaloids (Fig. 3) were also found in the plant: 1-acetyl-4-methoxy-β-carboline (**9**) [22,30], 1-ethyl-4-methoxy-β-carboline (**10**), 1-(2′-hydroxyethyl)-4-methoxy-β-carboline (**11a**) and 1-(1′,2′-dihydroxyethyl)-4-methoxy-β-carboline (**11b**) [22,24], 1-(1-Hydroxy-2-methoxy)-ethyl-4-methoxy-β-carboline (**12**), β-carboline-1-propionic acid (**14**), methyl-β-carboline carboxylate (**15a**), 1-carbamoyl-β-carboline (**16**) [25], 4-methoxy-1-vinyl-β-carboline (**13**), methyl-4-methoxy-β-carboline carboxylate (**15b**) [21] and methyl-4,8-dimethoxy-β-carboline carboxylate (**15c**) [31].



1-Acetyl-4-methoxy-β-carboline (**9**)	R_1 = H, R_2 = $OCOCH_3$, R_3 = OCH_3
1-Ethyl-4-methoxy-β-carboline (**10**)	R_1 = H, R_2 = CH_2CH_3, R_3 = OCH_3
1-(2′-Hydroxyethyl)-4-methoxy-β-carboline (**11a**)	R_1 = H, R_2 = CH_2CH_2OH, R_3 = OCH_3
1-(1′,2′-Dihydroxyethyl)-4-methoxy-β-carboline (**11b**)	R_1 = H, R_2 = $CH(OH)CH_2OH$, R_3 = OCH_3
1-(1-Hydroxy-2-methoxy)-ethyl-4-methoxy-β-carboline (**12**)	R_1 = H, R_2 = $CH(OCH_3)CH_2OH$, R_3 = OCH_3
4-Methoxy-1-vinyl-β-carboline (**13**)	R_1 = H, R_2 = CH = CH_2, R_3 = OCH_3
β-Carboline-1-propionic acid (**14**)	R_1 = H, R_2 = CH_2CH_2COOH, R_3 = H
Methyl-β-carboline carboxylate (**15a**)	R_1 = H, R_2 = $COOCH_3$, R_3 = H
Methyl-4-methoxy-β-carboline carboxylate (**15b**)	R_1 = H, R_2 = $COOCH_3$, R_3 = OCH_3
Methyl-4,8-dimethoxy-β-carboline carboxylate (**15c**)	R_1 = OCH_3, R_2 = $COOCH_3$, R_3 = OCH_3
1-Carbamoyl-β-carboline (**16**)	R_1 = H, R_2 = $CONH_2$, R_3 = H

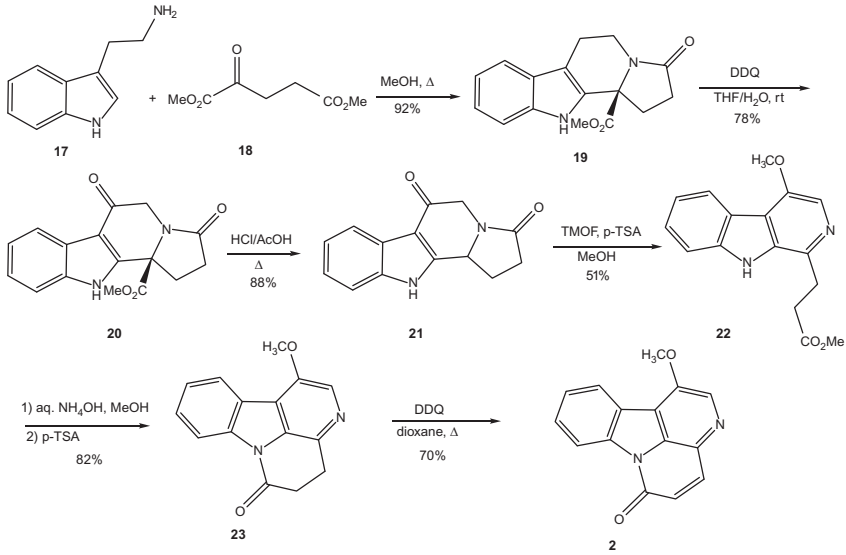
FIGURE 3 β-Carboline alkaloids from *Ailanthus altissima*.

1-METHOXY-CANTHIN-6-ONE: EXTRACTION FROM NATURAL SOURCE

In 2005, our research group isolated 1-methoxy-canthin-6-one from *A. altissima*. In particular, powder of air-dried roots of the plant was successively extracted at room temperature with solvents of increasing polarity [petroleum ether, chloroform, chloroform–methanol (9:1), methanol, and water]. Chloroform extract, the most active in biological assays, was fractionated on a silica-gel column, eluting with chloroform and increasing polarity mixtures of chloroform and methanol. A total of 762 fractions of 10 mL were obtained and pooled in 13 major fractions on the basis of their TLC similarity. These major fractions, dissolved in DMSO, were assayed on HeLa cell proliferation at a dose of 10 μg/mL. Fraction XXIII–XXVII (100% of cytotoxic activity) was further purified on a silica-gel column, collecting 127 fractions of 5 mL, pooled in nine major subfractions (A–I). Subfraction C, showing a strong antiproliferative activity, was demonstrated to contain, in TLC assays, a single substance, positive to Dragendorff reagent [27]. The identification of this active substance, recognized as 1-methoxy-canthin-6-one, was performed by analyses of its ^{1}H NMR, ^{13}C NMR, and ^{13}C NMR DEPT data and by comparison with literature data [32].

1-METHOXY-CANTHIN-6-ONE: SYNTHETIC PROCEDURES

The first synthesis of 1-methoxy-canthin-6-one **2** was reported by Cook and coworkers [33,34]. It began with a Pictet–Spengler reaction between tryptamine hydrochloride (**17**) and dimethyl α-ketoglutarate (**18**) (scheme 1), to

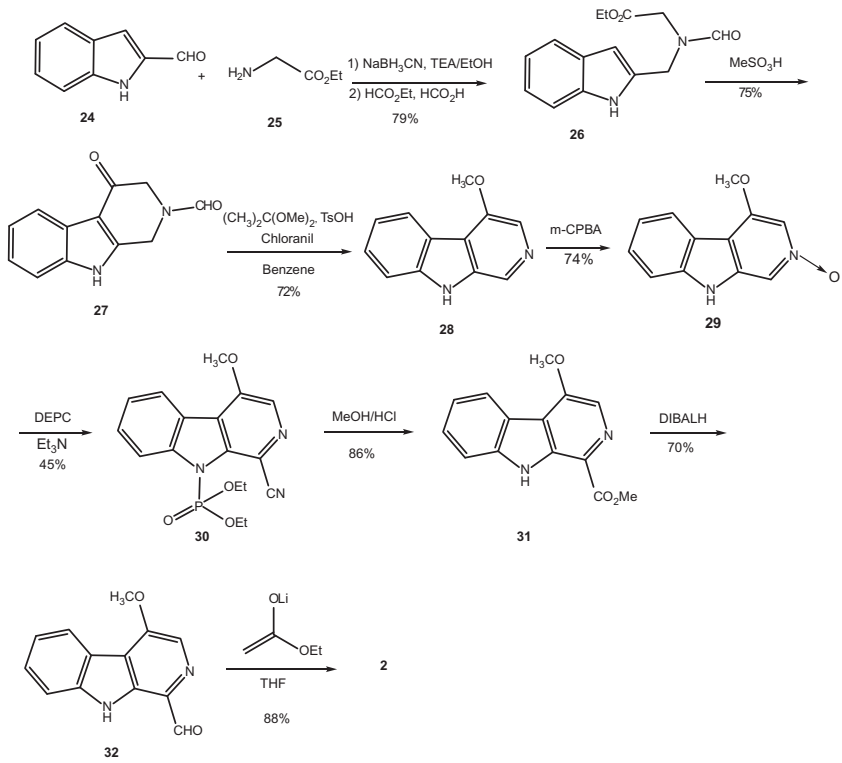


SCHEME 1 Synthesis of 1-methoxy-canthin-6-one by Cook and Hagen.

furnish the desired γ-lactam **19** in 92% yield, in a one-pot reaction. Treatment of **19** with DDQ (2,3-dichloro-5,6-dicyanobenzoquinone) at room temperature for 3 days, in a mixture of THF:H_2O 9:1, resulted in selective oxidation at C-1 (**20**) in 78% yield. Hydrolysis and decarboxylation were carried out, heating in HCl/AcOH, according to the procedure of Hobson [35], giving the keto lactam **21** in 88% yield. Heating **21** in methanol with trimethyl orthoformate, in presence of *p*-toluenesulfonic acid, resulted in concomitant enol ether formation, oxidative aromatization, and methanolysis of the lactam to give intermediate **22** in 51% yield. Hydrolysis of the ester with ammonium hydroxide followed by acid-catalyzed lactamization gave 4,5-dihydro-1-methoxy-canthin-6-one (**23**) in 82% yield. Dehydrogenation with DDQ in refluxing dioxane provided the final product **2** in 60% yield.

Recently, Suzuki and coworkers [36] have reported another synthesis of 1-methoxy-canthin-6-one (**2**) (Scheme 2).

Starting from indole-2-carboxaldehyde (**24**), reductive amination with ethyl glycinate (**25**) and sodium cyanoborohydride, followed by formylation with ethyl formate and formic acid gave amide ester **26** in 79% overall yield. Acid-catalyzed ring closure could be effected using methanesulfonic acid, to furnish tetrahydro-β-carboline-4-one (**27**). Oxidative ketalization to 4-methoxy-β-carboline (**28**) was accomplished in one-pot and in 72% yield by treatment with 2,2-dimethoxy-propane and chloranil, as oxidizing agent, in the presence of *p*-toluenesulfonic acid. Functionalization at C-1 of β-carboline was accomplished by a modified Reissert–Henze reaction [37]. *N*-oxidation with *m*-chloroperbenzoic acid (74%), followed by the reaction with diethyl phosphorocyanidate and triethylamine in



SCHEME 2 Synthesis of 1-methoxy-canthin-6-one by Suzuki.

an aprotic solvent gave **30** in 61% yield. Acid-catalyzed methanolysis easily gave the ester **31**, which was reduced to aldehyde **32** in 70% yield, by treatment with diisobutylaluminum hydride. The addition of ethyl acetate to LiHMDS generated the corresponding enolate, to which was added β-carboline aldehyde **32**. Aldol condensation and intramolecular acylation provided 1-methoxy-canthin-6-one (**2**) in 88% yield after appropriate work-up conditions and purification by silica-gel column chromatography.

BIOLOGICAL PROPERTIES OF 1-METHOXY-CANTHIN-6-ONE AND RELATED β-CARBOLINES

1-Methoxy-canthin-6-one was reported to possess several biological activities, such as antiviral [38], chemopreventive [39], and antitumoral [27,28,38,40,41].

Antiviral Activity

In an *in vitro* anti-HIV assay, 1-methoxy-canthin-6-one suppressed significantly HIV-infected H9 cell growth and had an impressive therapeutic index

(TI:IC_{50}/EC_{50}) > 391. The corresponding IC_{50} (concentration inhibiting uninfected H9 cell growth by 50%) and EC_{50} (concentration that inhibited viral replication by 50%) values were >100 and 0.256 μg/mL, respectively [38].

Chemopreventive Activity

Murakami and coworkers [39] reported inhibitory effects of 1-methoxycanthin-6-one (**2**) and analogs, isolated from Simaroubaceous plants, on 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced Epstein–Barr virus early antigen (EBV-EA) activation, in Raji cells. Compounds demonstrated 100% inhibition at 1000 mol ratio/TPA and 100–73.3% inhibition at 500 mol ratio/TPA (Table 1).

Antitumoral Activity

The cytotoxicity of canthin-6-one (**1**), 1-methoxy-canthin-6-one (**2**), 5-methoxycanthin-6-one, and canthin-6-one-3-*N*-oxide (**5**) to guinea pig ear keratinocytes was compared, and the IC_{50} values ranged from 1.11 to 5.76 μg/mL. There is no significant difference in activity among these four cytotoxic alkaloids (Table 2) [40].

Recently, Xu and coworkers [38] also found that 1-methoxy-canthin-6-one (**2**), isolated from *Leitneria floridana* Chapman, suppressed the growth of several human tumor cell lines, including epidermoid carcinoma of the nasopharynx (KB), lung carcinoma (A 549), ileocecal carcinoma (HCT-8), renal

TABLE 1 Inhibitory Effects of Canthinones on TPA-Induced EBV-EA Activation

Cpd	% EBV-EA Positive Cells[a]			
	Compound Concentration (mol/ratio/32 pmol TPA)[b]			
	1000	***500***	***100***	***10***
Canthin-6-one (**1**)	0(70)[c]	26.7	84.9	100
1-Methoxy-canthin-6-one (**2**)	0(70)	19.3	85.0	100
1-Hydroxy-canthin-6-one (**3**)	0(70)	0	36.3	82.2
1,10-Dimethoxy-canthin-6-one	0(70)	21.5	80.8	100

[a] *Positive control (100%).*
[b] *mol/ratio/TPA (32pmol = 20 ng/mL), 1000 mol ratio = 32 nmol; 500 mmol ratio = 16 nmol; 100 nmol ratio = 3.2 nmol; 10 nmol = 0.32 nmol.*
[c] *Values in parentheses are viability of Raji cells of the test compounds relative to the positive control (100%).*

TABLE 2 Cytotoxicity (GPK Epithelial Cells) of Some Canthin-6-One Alkaloids

Alkaloid	IC_{50} (μg/mL)
Canthin-6-one (**1**)	1.11
1-Methoxy-canthin-6-one (**2**)	5.76
5-Methoxy-canthin-6-one	5.44
Canthin-6-one-3*N*-oxide (**5**)	4.21

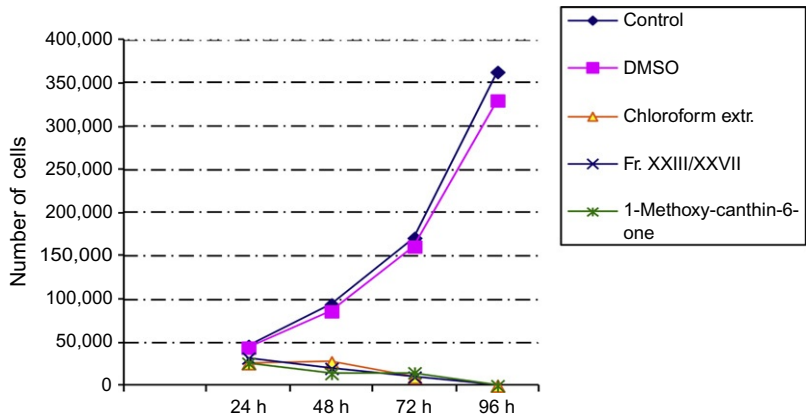


FIGURE 4 Cytotoxic assay. Growth inhibition of Hela cells after treatment with $CHCl_3$ root extract, its chromatographic fraction XXIII–XXVII, and 1-methoxy-canthin-6-one.

cancer (CAK-1), breast cancer (MCF-7), and melanoma (SK-MEL-2), with IC_{50} value in the range of 2.5–20 μg/mL.

Our research group reported that 1-methoxy-canthin-6-one showed a good antiproliferative and proapoptotic effectiveness on several tumoral cell lines [27].

The effects of the extracts, chromatographic fractions of root chloroform extract, and 1-methoxy-canthin-6 one on cytotoxicity were studied *in vitro* on HeLa (human cervical carcinoma cell line). Cell vitality was evaluated after different times of continuous exposure at doses of 10 μg/mL (Fig. 4). These results clearly indicate that chloroform root extract (10 μg/mL), its active fraction (10 μg/mL), and the pure alkaloid (4×10^{-5} M) are highly cytotoxic in HeLa cells, in a time-dependent way.

In order to investigate whether the cell growth inhibition reported above was caused by apoptosis or other mechanism(s), the potential apoptotic effects of *A. altissima* preparations on several tumoral cell lines were studied. SAOS (human osteosarcoma cell line), HeLa, U87MG (human glioma cell line), and

U937 (human monocytic leukemia cell line) were treated with a dose of 10 μg/mL of the active extract, its active fraction, and the alkaloid (final concentration 4×10^{-5} M) for 24 h. In SAOS cells (Fig. 5), spontaneous cell apoptosis was less than 4% and this percentage was similar in culture of DMSO alone. However, in SAOS treated with crude chloroform extract, apoptosis increased to more than 45% while cells incubated with pure 1-methoxycanthin-6-one (4×10^{-5} M) showed an apoptosis of about 20%. Staurosporine, a protein kinase inhibitor used as a strong inducer of apoptosis [42], provoked apoptosis in 69% of the treated cells. In HeLa cells, apoptosis induced by crude chloroform extract increased more than 40% and about 30% in culture treated with pure alkaloid (Table 3). It appears remarkable that HeLa cells, treated with chloroform extract, presented apoptosis levels very

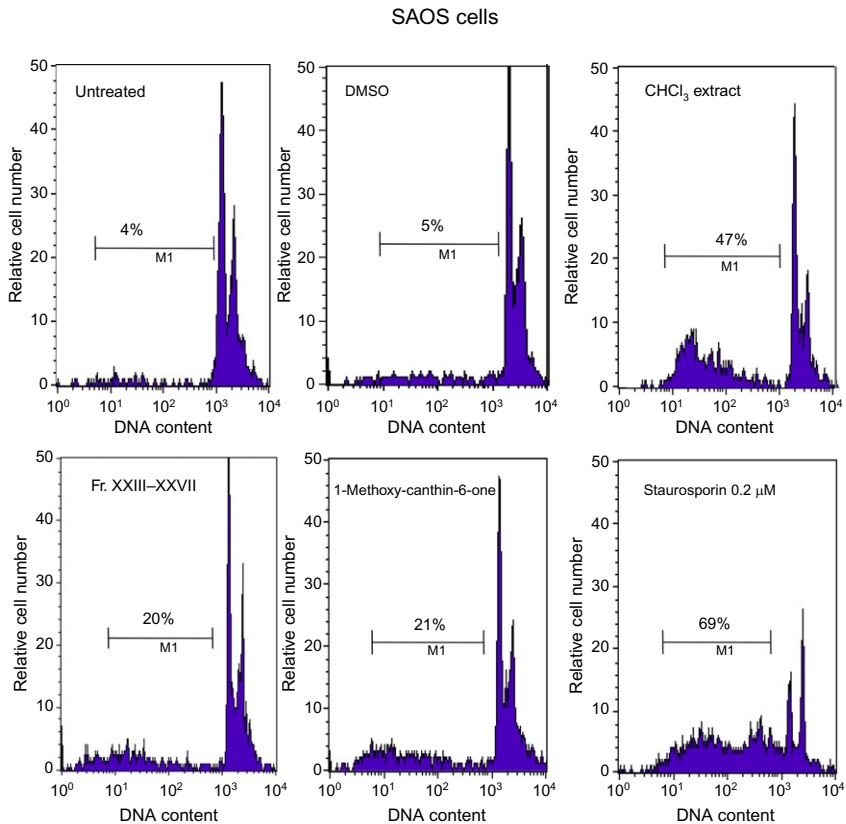


FIGURE 5 Effects of *Ailanthus altissima* on cytotoxicity-induced apoptosis in SAOS cells. The percentage of the apoptotic elements in the hypodiploid region was calculated in permeabilized SAOS cells. Cells were untreated or incubated for 24 h with DMSO or $CHCl_3$ root extract, its chromatographic fraction XXIII–XXVII and 1-methoxy-canthin-6-one and then stained with propidium iodide.

TABLE 3 Effects of *Ailanthus altissima* on Cytotoxicity-Induced Apoptosis in HeLA, U87MG, and U937 cells

Percentage of the Apoptotic Elements in the Hypodiploid Region			
	HeLa cells	*U87MG*	*U937*
Untreated	10	3	3
DMSO	10	3	3
$CHCl_3$ extract	41	20	19
Fraction XXIII–XXVII	28	8	11
1-Methoxy-canthin-6-one	27	9	11
Staurosporine 0.2 μM	46	11	9

The percentage of the apoptotic elements in the hypodiploid region was calculated in permeabilized cells. Cells were untreated or incubated for 24 h with DMSO or $CHCl_3$ root extract, its chromatographic fraction XXIII–XXVII and 1-methoxy-canthin-6-one and then stained with propidium iodide.

similar to staurosporine-treated cells (41% *vs.* 46%). Apoptosis was also confirmed in U87MG and U937 (Table 3) cell lines upon 24-h treatment with the crude extract and its active fraction and with a concentration of 4×10^{-5} M of the alkaloid. Spontaneous apoptosis in U87MG was about 3%, and after treatment with crude chloroform extract, it increased to 20% (staurosporine apoptosis 11%). At 24 h, the chloroform extract induced 19% of apoptosis in U937 cell lines (staurosporine apoptosis 9%). In U87MG and U937 cells, biological activity of both fraction XXIII–XXVII of root chloroform extract and pure 1-methoxy-canthin-6-one was minor in comparison to the crude chloroform extract, in which other compounds could present additive effects.

The results clearly showed an induced cytotoxicity in HeLa cells and apoptotic death in human U87MG, U937, HeLa, and SAOS cells. SAOS cells are p53 deficient; therefore, the mechanism(s) underlying cytotoxicity and apoptosis do not involve p53 activity. The resistance of cancer cells to classical chemotherapy may be probably due in part to the high frequency of mutation in p53 that impairs p53-dependent apoptosis. Most of the common anticancer agents, such as doxorubicin, vincristine, or 5-fluorouracil, induce apoptosis via a p53-dependent pathway [43].

The requirement of the p53 tumor suppressor gene for activation of apoptosis by these agents provides an attractive explanation for their poor efficacy on p53 mutant tumor. Thus, chemotherapeutic agents that induce apoptosis independently from the p53 apoptotic pathway are of major interest.

To verify and characterize the proapoptotic effect of 1-methoxy-canthin-6-one in human cells of diverse origins, our research group analyzed the dose-dependent induction of apoptosis in leukemia (Jurkat), thyroid carcinoma

(NPA, ARO), or HuH7 (Hepatocarcinoma) cell lines. Following a 24 h incubation with 1-methoxy-canthin-6-one, the four tested lines showed percentages of apoptotic (sub-G1) cells ranging from 50% to >70%. The apoptosis-inducing activity was evident at <10 μM 1-methoxy-canthin-6-one and half maximal at a compound concentration of about 40 μM (Fig. 6A–D). The compound did not show any proapoptotic or toxic activity in PBMCs from two different donors (Fig. 6E).

The appearance of sub-G1 elements was evident in cells incubated with 1-methoxy-canthin-6-one for <15 h (Fig. 7A).

To investigate whether mitochondrial events were involved in the induction of apoptosis, we analyzed mitochondrial membrane depolarization by

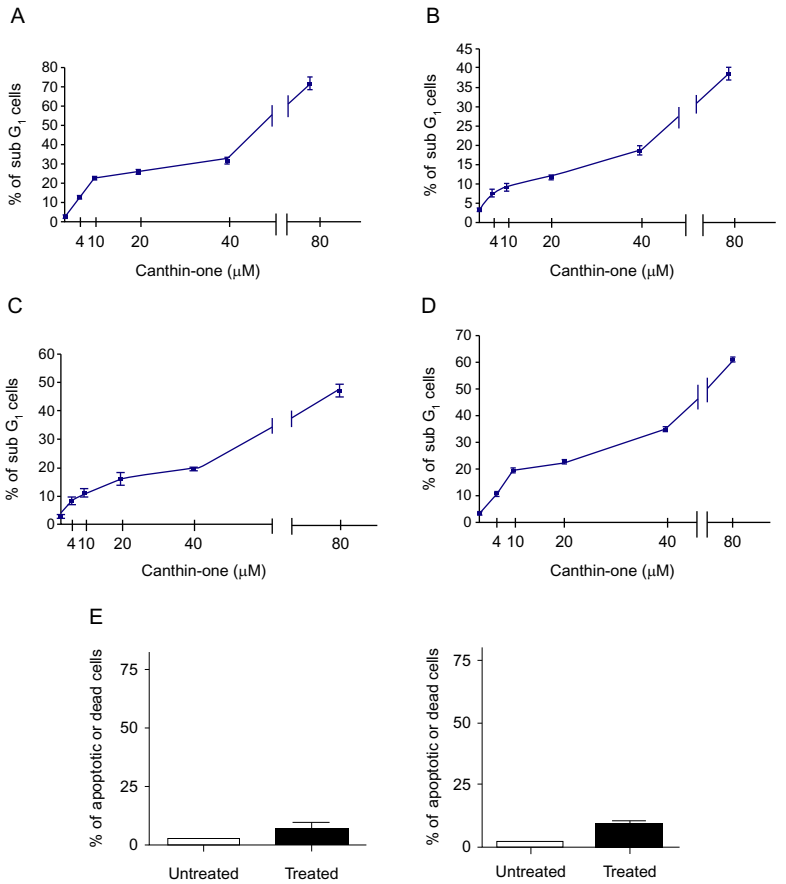


FIGURE 6 Apoptosis induction by 1-methoxy-canthin-6-one. Jurkat (A), NPA (B), ARO (C), and HuH7 (D). After 24 h, apoptosis was measured as percentage of sub-G1 nuclei by flow cytometry (**10**). (E) PBMCs from two (A and B) healthy donor. Following 24 h, cell death and percentages of sub-G1 nuclei were analyzed by trypan blue exclusion and flow cytometry, respectively.

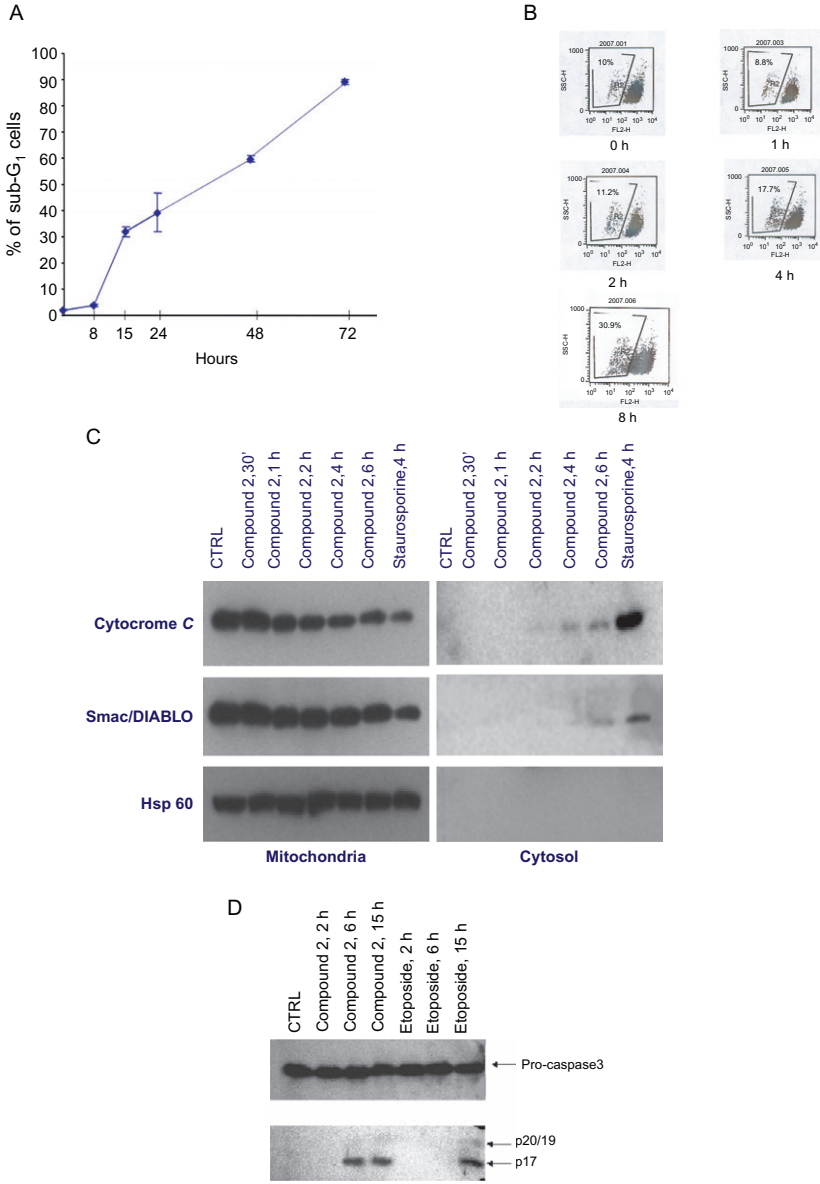


FIGURE 7 Effect of 1-methoxy-canthin-6-one on mitochondrial membrane depolarization, cytochrome *c*, and Smac/DIABLO release and caspase-3 cleavage. (A) Jurkat cells, in the absence or presence of 1-methoxy-canthin-6-one. Apoptosis was measured at the indicated time intervals as percentage of sub-G1 nuclei by flow cytometry. (B) Jurkat cells, in the absence or presence of 1-methoxy-canthin-6-one. At the indicated time intervals, mitochondrial membrane depolarization was evacuate by cell staining with TMRE by flow cytometric analysis. (C) Jurkat cells, in the absence or presence of 1-methoxy-canthin-6-one, by Western blotting. (D) Jurkat cells, in the absence or presence of 1-methoxy-canthin-6-one. Cell incubation with etoposide was used as a positive control for caspase-3 activation and appearance of the caspase-3 active cleaved forms p20/19 and/or p17, by Western blotting.

flow cytometry and the release of cytochrome *c* and Smac/DIABLO by Western blotting in cells incubated with the compound. The cells clearly showed a loss of mitochondrial membrane polarity from four to eight hours following cell incubation with 1-methoxy-canthin-6-one (Fig. 7B); in parallel, we detected a decrease in the intramitochondrial and an increase in the cytosolic amounts of cytochrome *c* and Smac/DIABLO proteins (Fig. 7C). These findings indicated that the mitochondrial pathway of apoptosis was triggered by this molecule. As expected, procaspase-3 was accordingly cleaved, leading to the appearance of a faint p20/19 and an intense p17 signal (13), in cells exposed to the drug (Fig. 7D).

To further explore the potential use of 1-methoxy-canthin-6-one as antineoplastic agent, we investigated its proapoptotic effect in combination with TRAIL, a molecule that induces apoptosis in a vast series of different neoplasias [28]. To this purpose, we analyzed apoptosis by flow cytometry in cells treated with suboptimal doses of both compounds. When different cell lines were incubated with 10 μmol/L 1-methoxy-canthin-6-one and/or suboptimal TRAIL, the single agents induced $<10\%$ of apoptotic elements, whereas their combination elicited apoptotic responses ranging from 45% to $>80\%$. Such effect was not detectable in normal PBMCs (Fig. 8A). The synergistic effect in neoplastic cells was evident at the levels of caspase-3 activation (Fig. 8C) and mitochondrial depolarization (Fig. 8B), indicating that its mechanism was to be investigated in earlier step(s) of the apoptotic program. Therefore, we verified whether incubation with 1-methoxy-canthin-6-one modulated the expression of TRAIL receptors.

As shown in Fig. 9, Jurkat cells incubated with the molecule (10 μmol/L) showed an enhanced binding to an anti-TRAIL-R1 antibody, as detected by immunofluorescence: indeed, the mean intensity of fluorescence increased from 24.1 (Fig. 9) (TRAIL-R1, green) to 44.8 after 15 h of cell incubation with the drug (Fig. 9) (TRAIL-R1, orange). This effect is specific because we could not detect any increase in the expression of other TRAIL receptors or unrelated proteins (i.e., integrins, Fig. 9).

The activity of JNK influences cell apoptosis and mediates the proapoptotic effects of some antineoplastic compounds [28]. We, therefore, investigated the role of the kinase in 1-methoxy-canthin-6-one-induced apoptosis and synergism with hrTRAIL. Incubation of Jurkat cells with 1-methoxy-canthin-6-one induced JNK activation, as revealed by the appearance of the phosphorylated forms of JNK and its substrate c-Jun, analyzed by Western blot (Fig. 10A). To verify whether JNK activation was required for apoptosis induction, we used the JNK inhibitor (JNKI), SP600125 [44]. The addition of this molecule to cell cultures inhibited apoptosis by $>80\%$ (Fig. 10C) indicating that JNK activity mediated the effect of the drug. Furthermore, the inhibitor reduced TRAIL-R1 upregulation (Fig. 11A) and the synergistic activity of 1-methoxy-canthin-6-one with hrTRAIL (Fig. 11B). Indeed, whereas apoptosis induced by the two drugs together in cell culture was $>45\%$, it was reduced to 21% in the presence of JNKI. Because the

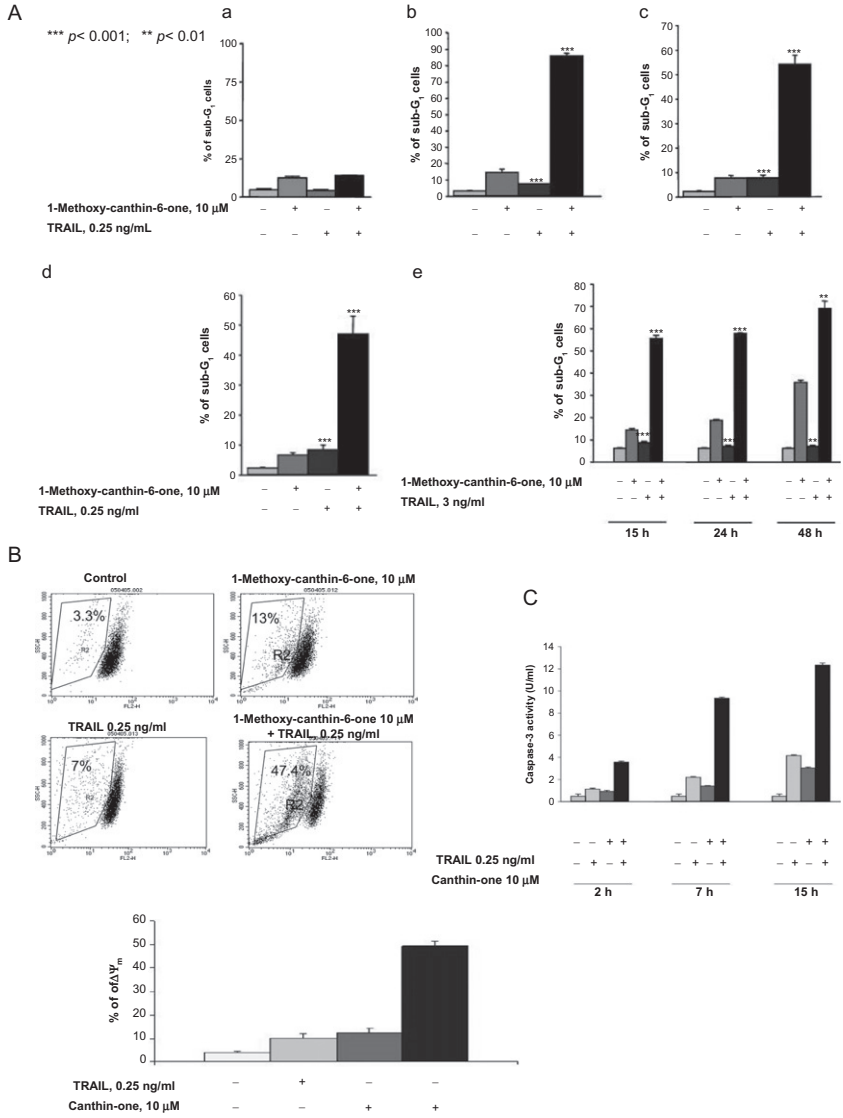


FIGURE 8 Effect of 1-methoxy-canthin-6-one on TRAIL-induced cell apoptosis. (A) Normal PBMC (a), HuH7 (b), NPA (c), Jurkat (d), and ARO (e) cells were incubated with 1-methoxy-canthin-6-one at a concentration, with or without TRAIL at the indicated suboptimal concentrations. After 24 h (a–d) or the indicated times (e), apoptosis was measured as percentage of sub-G1 nuclei by flow cytometry. (B) Jurkat cells were incubated with 1-methoxy-canthin-6-one, without or with TRAIL, for 4 h. Then cells were harvested, incubated with TMRE, and analyzed by flow cytometry. (C) Jurkat cells were incubated with 1-methoxy-canthin-6-one, without or with TRAIL, for the indicated time intervals. Then caspase-3 activity was measured by fluorometric assay.

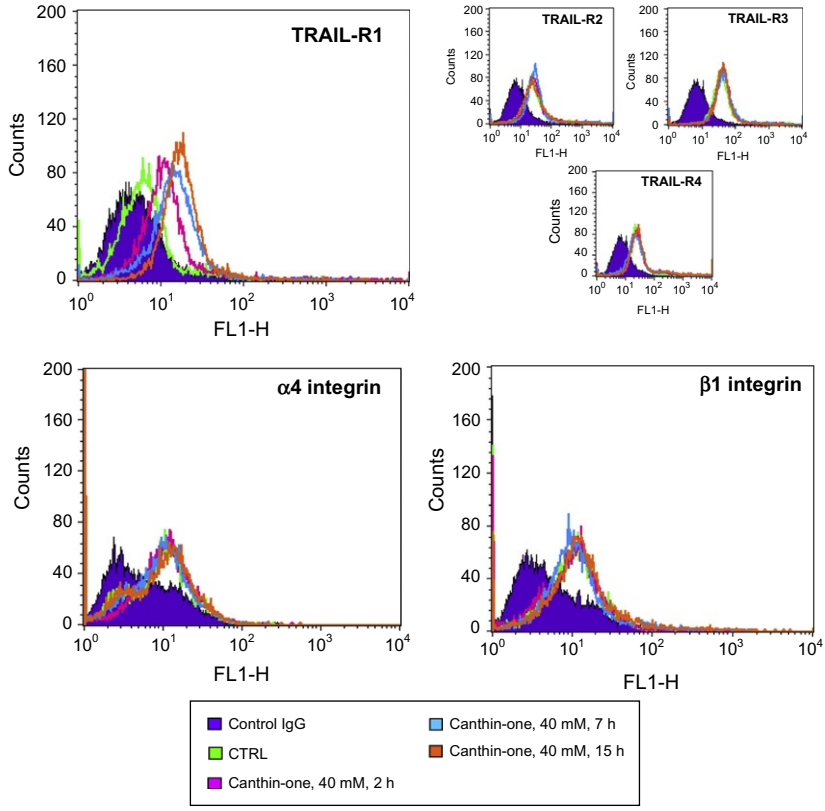


FIGURE 9 Effect of 1-methoxy-canthin-6-one on the expression of TRAIL receptors.

synergism was not completely abrogated by the inhibitor, other element(s), in addition to JNK activation, apparently contributed to the enhancement of TRAIL-induced apoptosis by 1-methoxy-canthin-6-one. Understanding of such an effect of this molecule supports its potential therapeutic application. The mechanism of 1-methoxy-canthin-6-one activity seemed to entirely rely on JNK activation; indeed, the inhibition of the kinase was able to completely prevent apoptosis induced by the compound. In this respect, these findings provide one of the clearest evidence attesting that JNK activation can exert a major role in the activity of antineoplastic molecules. Various antineoplastic agents, including topoisomerase inhibitors, histone deacetylase inhibitors, rapamycin, have been shown to activate JNK. Inhibition of the kinase mostly reduced the proapoptotic activity of these agents, whereas completely abrogated that displayed by 1-methoxy-canthin-6-one. In this case, therefore, JNK activation did not simply participate in promoting or amplifying the proapoptotic signal, but was decisive for subverting the cell survival/death balance and might instead support the potential utility of strategies aimed at

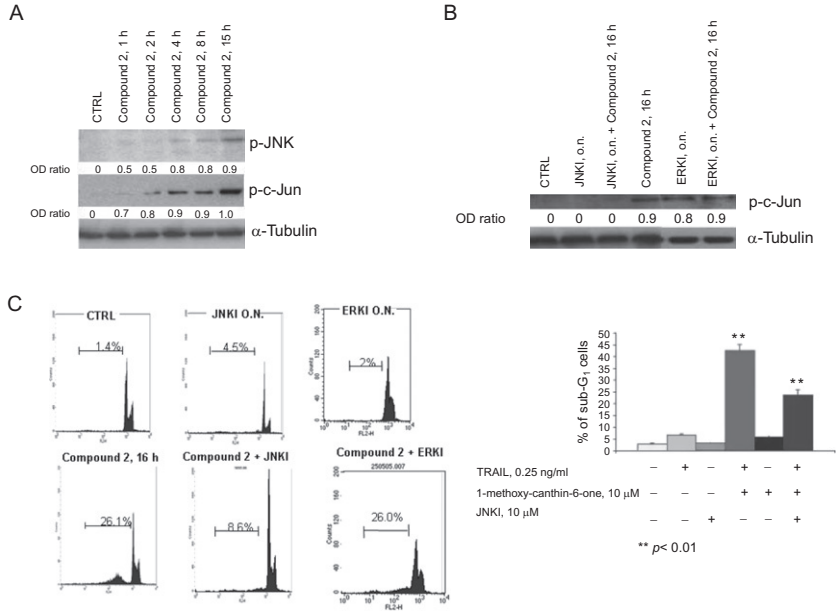


FIGURE 10 Effect of 1-methoxy-canthin-6-one on JNK activation. (A) Cells were incubated with 1-methoxy-canthin-6-one for the indicated times, in Western blot. (B) Cells were incubated with 1-methoxy-canthin-6-one for the indicated times, with or without overnight pre-treatment with JNKI or ERKI, in Western blot. (C) Cells, preincubated with medium alone or JNKI or ERKI overnight, were incubated with 1-methoxy-canthin-6-one for 16 h. Then apoptosis was measured as percentage of sub-G1 nuclei.

specifically activating the kinase, to trigger apoptosis in neoplastic cells. JNK activity has been shown to influence the levels of TRAIL-R2 [28]. We found that it can also upregulate TRAIL-R1 because the levels of this receptor were enhanced by 1-methoxy-canthin-6-one in a JNK-dependent manner: indeed, JNK inhibition completely prevented the effect. However, the inhibition of the kinase only partially reduced the synergism of 1-methoxy-canthin-6-one with TRAIL. Therefore, 1-methoxy-canthin-6-one apparently regulated, in addition to JNK and TRAIL receptor levels, other factor(s) that participated in TRAIL-induced apoptosis. A more extensive analysis of such factors (DISC components, IAPs, etc.) could contribute to deeply evaluating the proapoptotic activity of 1-methoxy-canthin-6-one and its synergism with agents that induce tumor cell death.

This finding reveals the promising antitumor properties of 1-methoxy-canthin-6-one that can represent a candidate for *in vivo* studies of monotherapies or combined antineoplastic therapies.

Unfortunately, as often happens in extraction procedure of active compounds from plants, only small quantities of pure substance can be achieved, even starting from large amounts of plant material. For this reason, in order to carry out further investigations about the antiproliferative properties of

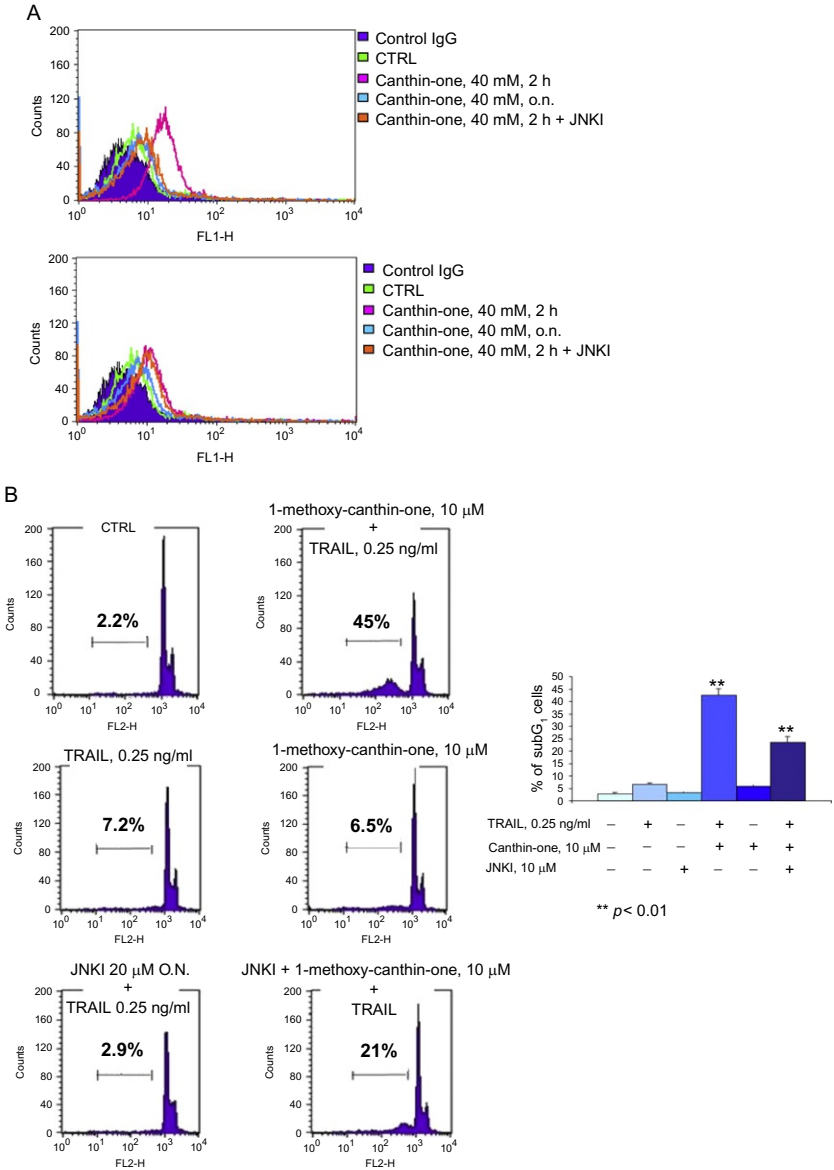


FIGURE 11 Effect of JNK inhibition on the enhancing of TRAIL-induced apoptosis by 1-methoxy-canthin-6-one. (A) Jurkat cells were incubated overnight, in the absence or presence of JNKI or ERKI, and treated with 1-methoxy-canthin-6-one for additional 2 h. Then cell expression of TRAIL-R1 was analyzed by flow cytometry. (B) Jurkat cells were treated with JNKI overnight and then incubated with 1-methoxy-canthin-6-one with or without TRAIL. After 24 h, apoptosis was measured as percentage of sub-G1 nuclei.

1-methoxy-canthin-6-one, we have decided to obtain the active alkaloid via a synthetic method, following the procedure reported by Suzuki [36] (Scheme 2). Moreover, in order to acquire important informations about the structural requirements responsible of the cytotoxic activity of this compound and to elucidate the structure–activity relationships (SARs) between substituent properties and antitumor activity, we have synthesized and tested a series of novel 1,4-disubstituted and 1,4,9-trisubstituted β-carbolines and a tetracyclic derivative related to 1-methoxy-canthin-6-one (Fig. 12) [45].

With regard to β-carboline compounds, we have chosen to synthesize new compounds showing an oxygen function in the same position of methoxy group of 1-methoxy-canthin-6-one. In particular, compound **33** can be considered the regioisomer of harmine (**38**) (Fig. 13).

Harmine is the most representative β-carboline alkaloid endowed with antitumor properties. It was first isolated from seeds of *Peganum harmala* L. (Zygophyllaceae) [46]. Several studies are reported about its biological properties. Ishida and coworkers [47] reported that harmine and β-carboline analogues exhibited significant activities against several human tumor cell lines, including three drug-resistant KB (epidermoid carcinoma of nasopharynx cell line) sublines, with various resistance mechanisms. Harmine had a broad cytotoxicity spectrum against 1A9 (ovarian cancer cell line), U87MG, KB, SAOS-2, A549 (lung carcinoma cell line), SK-MEL-2 (melanoma cancer cell line), and MCF-7 (breast cancer cell line), with ED_{50} values ranging from 1.9 to 18.5 μg/mL. Cao and coworkers [48] demonstrated that harmine and its nine substituted derivatives, containing planar tricyclic systems, had

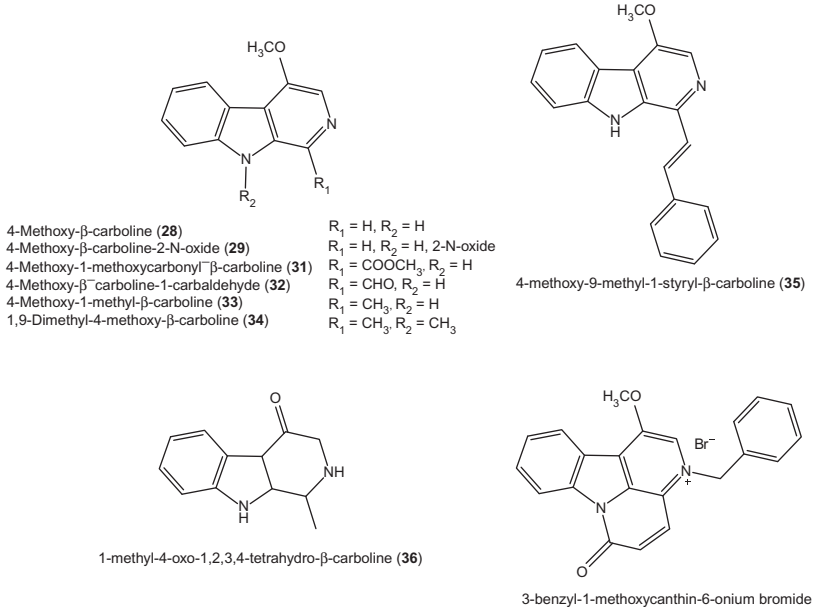
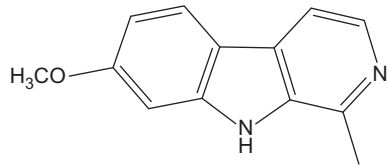


FIGURE 12 Structures of synthesized β-carboline and canthinone derivatives.



Harmine (**38**)

FIGURE 13 Chemical structure of harmine.

remarkable DNA intercalation capacity, this stabilization of DNA double helix probably contributing to the inhibition of DNA replication, thus leading to antitumor activity. Moreover, they showed the capability of this alkaloid to inhibit the DNA relaxation activity of Topoisomerase I, while no effect on Topoisomerase II was observed. Recently, harmine and numerous related β-carboline derivatives were found as potent and specific inhibitors of cyclin-dependent kinases (CDKs). It is worthy noted that harmine specifically inhibited CDK1, CDK2, and CDK5. The SARs analysis demonstrated that the degree of aromaticity of the tricyclic ring and the positioning of substituents were crucial for inhibitory activity [49,50]. Ma and Wink [51] reported that harmine can inhibit the BCRP (breast cancer resistance protein) and increase the cytotoxicity of anticancer drugs mitoxantrone and camptothecin in a BCRP overexpressing breast cancer cell line MDA-MB-231. The most relevant side effect of harmine is the neurotoxicity *in vivo*, probably due to the presence of a methoxy group in position 7 [52]. For this reason, our design has provided for the synthesis of compounds that maintained the methoxy group, but it was shifted from position 7 to position 4 (compound **33**). In order to expand SAR data, we have investigated also the role of the substituents in position 1 (compounds **28**, **31**, **32**, **35**) and 9 (compound **34**), the loss of aromaticity of pyridine ring (compound **36**), and the insertion of a N_2-oxide function on β-carboline ring (**29**). At the same time, in order to improve the biological profile of 1-methoxy-canthin-6-one, we have inserted a benzyl group on N_3 position of canthin-6-one nucleus. In literature is well described that N_2-benzylated β-carbolinium bromides are endowed with excellent antiproliferative properties [53]. This is probably due to the fact that β-carbolines, in order to intercalate into DNA and inhibit Topo I, must pass through cell membrane and penetrate into nucleus. N_2-benzylated β-carbolinium ions can penetrate into plasma membrane and nuclear envelope more easily than those molecules without substituents at position 2.

The cytotoxic potential of all synthesized compounds has been evaluated *in vitro* against a panel of human tumor cell lines, comparing 1-methoxy-canthin-6-one and its derivatives to harmine activity.

Our experiments have confirmed the cytotoxic effect of 1 methoxy-canthin-6-one and, for the first time, we have demonstrated the potential cytotoxic activity of compounds **28**, **29**, **31**, **32**, **33**, **34**, **35**, **36**, and **37** in different cancer cell lines (Table 4).

TABLE 4 *In Vitro* Antitumor Activity of Derivatives

Compounds	IC50 (μM)[a]							
	M14	*MCF-7*	*HT-29*	*A549*	*PC-3*	*Jurkat*	*ARO*	*T98G*
Harmine	n.s.[b]	n.s.[b]	79.4±0.9	n.s.[b]	22±0.8	n.s.[b]	26.5±0.7	35±1.1
33	n.s.[b]	n.s.[b]	50±1.5	80±0.5	20±1.2	n.s.[b]	50±0.8	n.s.[b]
34	36±0.6	32±0.1	27±0.2	50±0.4	22±1.2	65±0.8	30±0.5	40±1.2
28	n.s.[b]	n.s.[b]	n.s.[b]	n.s.[b]	n.s.[b]	n.s.[b]	64±1.2	n.s.[b]
31	n.s.[b]	n.s.[b]	n.s.[b]	n.s.[b]	50±0.6	n.s.[b]	n.s.[b]	n.s.[b]
32	38.9±0.1	35±0.45	90±0.3	45±1.2	25±0.2	28.8±0.9	39±0.4	n.s.[b]
35	n.s.[b]	n.s.[b]	n.s.[b]	n.s.[b]	60±0.9	n.s.[b]	n.s.[b]	n.s.[b]
36	n.s.[b]	n.s.[b]	n.s.[b]	n.s.[b]	24±0.8	n.s.[b]	18±0.6.[b]	n.s.[b]
29	n.s.[b]	n.s.[b]	n.s.[b]	n.s.[b]	n.s.[b]	n.s.[b]	n.s.[b]	n.s.[b]
2	50±1.1	50±0.3	31±0.1	n.s.[b]	26±0.1	50±0.2	15±0.6	90±0.7
37	37±0.3	46±0.75	72±0.4	n.s.[b]	8±0.9	n.s.[b]	28±1.0	n.s.[b]

[a] *IC_{50}, compound concentration required to inhibit tumor cell proliferation by 50%.*
[b] *n.s., not significant. Data represent the mean values of three independent determinations performed in triplicate.*

On the basis of biological effects, it was possible to perform a SAR analysis. In particular, the shift of methoxy group from position 7 to 4 (harmine *vs*. **33**) leads to an increase of activity against HT-29 (Human colon adenocarcinoma grade II) and A549 cell lines, while no significant activity is detected in other cell lines. The introduction of a methyl group into position 9 of compound **33** leads to an improvement of activity to all tumor cell lines (**34**). When methyl group in position 1 is replaced with benzylidine substituent (**35**), there is a loss of activity. Compounds **28** and **29**, with no substituent at position 1, are inactive against all tumor cell lines. Interestingly, the compound bearing a carboxaldehyde substituent in position 1 (**32**) of β-carboline ring system displays a strong antiproliferative effect against almost all tumor cell lines. In addition, the compound with a carboxylate (**31**) substituent is inactive to all tumor cell lines except to PC-3. Tetrahydro-β-carboline derivative **36** shows good activity only against PC-3 and ARO cells. As a whole, the present results show that all the compounds studied, except **28** and **29**, are very selective against prostate cancer cells PC-3 at IC_{50} values nearly to 20 μM. In particular, **37** demonstrates an important antiproliferative effect at low concentration (IC_{50} 8 μM).

Selected compounds **2**, **32**, **33**, **34**, **36**, and **37** have been subjected to further studies to investigate their mechanism of antiproliferative activity. Especially, they have been evaluated for their ability to inhibit Topoisomerase I. They have been tested for their intercalation, inhibition of relaxation activity, and poisoning at the concentration of 312 μM. Compounds **33**, **34**, and **37** demonstrate intercalation and inhibition of relaxation activity (IC_{50} see Table 5). Other three compounds do not show any activity. Compound **37** exhibits strong Topoisomerase I inhibition, and **33** and **34** show, respectively, moderate and weak activity.

TABLE 5 Inhibition Activity Against DNA Topoisomerases I of Compounds in Comparison with Harmine

Compound	Topoisomerase I Activity (IC_{50}) (μM)
2	(–)
33	61.44 ± 9.63
34	117.75 ± 21.41
32	(–)
36	(–)
37	17.77 ± 2.75
Harmine	65.25 ± 14.09

Note: (–) indicates that the compound is inactive (negative at 312 μM or with $IC_{50} > 250$ μM).

It appears of interest that (i) the regioisomer of harmine (compound **33**) maintains the same activity of the lead harmine (61.44 μM *vs.* 65 μM); (ii) the substitution of methyl group in position 1 with a carboxaldehyde (**32**) is not effective; (iii) N_9-methylation of β-carboline nucleus (compound **34**) is detrimental for the activity; (iv) the loss of aromaticity of pyridine ring (**36**) leads to an inactive compound; (v) 1-methoxy-canthin-6-one (**2**) is unable to inhibit topo I, while its N_2 benzylated analog (**37**) shows to be the most potent inhibitor of the series, better than the harmine.

CONCLUSION

It is today accepted that the plant kingdom is a vast, extremely diverse source of chemicals having bioactive properties, particularly compounds with pharmaceutical value. Biologically active plant-derived chemicals provide the basis and inspiration for the semisynthesis or total synthesis of effective new drugs. On the basis of natural compounds, provided of strong biological activity, and considering semisynthetic and synthetic substances related, it is possible to study, in a lot of cases, the mechanism of their biological actions and the SAR. Data presented in this chapter confirm that the study of medicinal plants represents a privileged approach for the achievement of bioactive compounds and for new models of drugs. Moreover, the plant kingdom is "the prolific and promising treasure-trove of the ethnopharmacological knowledge" [54].

ABBREVIATIONS

TLC	Thin Layer Chromatography
DMSO	Dimethyl sulfoxide
HeLa	human cervical carcinoma cell line
NMR	Nuclear Magnetic Risonance
NMR DEPT	Nuclear Magnetic Risonance Distorsionless Enhancement by Polarization Transfer
DDQ	2,3-dichloro-5,6-dicyanobenzoquinone
THF:H2O	tetrahydrofuran:water
HCl/AcOH	chloridric acid/acetic acid
LiHMDS	Lithium bis(trimethylsilyl)amide
HIV	Human immunodeficiency virus
H9	lymphoblastoid CD4+
TI:IC_{50}/EC_{50}	therapeutic index: half maximal inhibitory concentration/half maximal effective concentration
TPA	12-O-tetradecanoylphorbol-13-acetate
EBV-EA	Epstein–Barr virus early antigen
KB	epidermoid carcinoma of the nasopharynx
A 549	lung carcinoma

HCT-8	ileocecal carcinoma
CAK-1	renal cancer
MCF-7	breast cancer
SK-MEL-2	melanoma
SAOS	human osteosarcoma cell line
U87MG	human glioma cell line
U937	human monocytic leukemia cell line
Jurkat	CD4(+) T-cell leukemia cell line
NPA	thyroid papillary carcinoma
ARO	Anaplastic thyroid carcinoma
HuH7	Hepatocarcinoma
PBMC	Peripheral blood mononuclear cell
TRAIL	tumor necrosis factor-related apoptosis-inducing ligand
i.e.	*id est*
JNK	c-Jun N-terminal kinase
hrTRAIL	human recombinant TRAIL
SARs	structure–activity relationships
KB	epidermoid carcinoma of nasopharynx cell line
1A9	ovarian cancer cell line
SK-MEL-2	melanoma cancer cell line
CDKs	cyclin-dependent kinases
BCRP	breast cancer resistance protein
MDA-MB-231	human breast cancer cells
HT-29	human colon adenocarcinoma grade II
PC-3	human prostatic carcinoma cell line
TMRE	tetramethylrhodamine ethyl ester

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Chapter 5

Fern Acylphloroglucinols: Structure, Location, and Biological Effects

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INTRODUCTION

Acylphloroglucinols are derivatives of the compound known as phloroglucinol (benzene-1,3,5-triol) and are usually composed of two or more rings bound together through methylene bridges. Fern acylphloroglucinols attracted the attention of chemists for their anthelmintic properties. They are produced by various species from the genera *Dryopteris*, *Arachniodes*, and *Elaphoglossum*. Powdered rhizomes and extracts of *Dryopteris* ferns, especially those of *D. filix-mas*, were used since antiquity due to their vermifuge properties, which have been compiled in several pharmacopeias [1,2]. Although their use has been frequently focused on human health, they have also been employed in ethnoveterinary [1].

Extensive investigations on the bioactive constituents of *Dryopteris* ferns and their chemical structures were performed by Böhm in 1896–1903 and resulted in the isolation of several acylphloroglucinols and structure elucidation of some of them [3–7]. Zwimpfer and Büchi reviewed phytochemical aspects of fern acylphloroglucinols in 1962 [8], while their biogenesis, distribution in the genus *Dryopteris*, and biological evaluation were published in 1967 [2]. Further reviews by Penttila and Sundman in 1970 [9], Soeder in 1985 [10], and Murakami and Tanaka in 1988 [11] summarized the chemistry, detection, and identification of this type of fern constituents. The distribution of acylphloroglucinols in Japanese *Dryopteris* fern species was studied in 1961 by Hisada and Noro, who developed a paper

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electrophoretic method for their detection in rhizome extracts [12]. The location and distribution of acylphloroglucinols in different fern families and genera was reported in 1983 [13]. Further surveys for phloroglucinol derivatives in *Arachniodes* [14–16], *Dryopteris* [17–20], and related fern genera [19] with the aim of drawing out chemotaxonomic conclusions were performed by Widén *et al*. Although many of the aforementioned articles referred to the anthelmintic effects of acylphloroglucinols, none of them included biological evaluation of the isolated constituents. A review on bioactive phloroglucinol derivatives of natural origin (plants, marine, and microorganisms) was published in 2006 and included the structures of some compounds isolated from ferns [21].

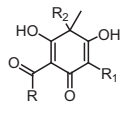
The presence of acylphloroglucinols in ferns has been associated with the presence of external or internal secreting glands located on their rhizomes and stipe bases [13–16,19]. Even though phenolic compounds were detected in two *Elaphoglossum* species analyzed in 1983 [13], neither internal nor external glands were described or even mentioned in the literature for *Elaphoglossum* ferns so far. Our studies of six Argentine *Elaphoglossum* species indicated the presence of acylphloroglucinols in all of them, presumably located in the fern scales. Analysis of herbarium and fresh plant material (rhizomes and scales) of six of the eight Argentine *Elaphoglossum* species indicated the presence of external secreting structures (glands) producing a yellow-orange oleoresin. External secreting glands in *Elaphoglossum* ferns are reported here for the first time.

The purpose of this review is to present the sources, chemistry, location in the plant, distribution, and biological activities of fern acylphloroglucinols, a rapidly growing class of natural compounds.

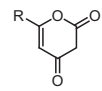
STRUCTURAL FEATURES OF FERN ACYLPHLOROGLUCINOLS

Even in structurally complex fern acylphloroglucinols, one or more of the molecular building blocks presented in Scheme 1 may be recognized. In oligomeric fern acylphloroglucinols, two or more of these building blocks are linked via methylene bridges.

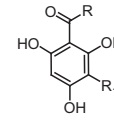
The natural origin of some monocyclic acylphloroglucinols, like aspidinol and fraginol, has always been under discussion. They have been considered, by many authors, to be artifacts formed during the isolation procedures by alkaline cleavage or by heating [2,9,22]. In order to detect the presence of the above-mentioned compounds or their analogs, we analyzed the crude diethyl ether extracts of *Elaphoglossum piloselloides* (C. Presl) T. Moore, and *Elaphoglossum gayanum* (Fée) T. Moore by Gas Chromatography–Mass Spectrometry (GC–MS) and Nuclear Magnetic Resonance (NMR). GC–MS analysis showed the presence of aspidinol



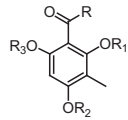
1-Acylfilicinic acid—R_1 = H, R_2 = CH_3
2-Fraginol—R_1 = R_2 = CH_3
3-Stenolepin—R_1 = CH_3, R_2 = OH



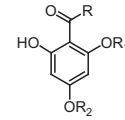
4- 6-Alkyl-2,3-dihydropyran-2,4-dione



5-Acylphloroglucinol—R_1 = H
6-Methylacylphloroglucinol—R_1 = CH_3



7-Pseudoaspidinol—R_1 = CH_3, R_2 = R_3 = H
8-Aspidinol—R_1 = R_3 = H, R_2 = CH_3
9-iso-Aspidinol—R_1 = R_2 = H, R_3 = CH_3



10-Desaspidinol—R_1 = H, R_2 = CH_3
11-*ortho*-Desaspidinol—R_1 = CH_3, R_2 = H

For all structures, R = alkyl residue (1–5 carbon atoms). The accepted nomenclatures for acyl groups are as follows: A, P, B, iB, V, C, accounting for acetyl (R = Me), propionyl (R = Et), butyryl (R = *n*-Pr), isobutyryl (R = *i*-Pr), *n*-valeryl (R = *n*-C_4H_9), or *n*-caproyl (R = *n*-C_5H_{11}), respectively.

SCHEME 1 Building blocks of fern acylphloroglucinols.

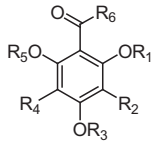
and desaspidinol; however, no signals arising from any of these two compounds could be detected in the ^{1}H NMR spectra of the crude extracts, suggesting that aspidinol and desaspidinol are artifacts produced during the gas chromatographic process.

ACYLPHLOROGLUCINOLS FROM *DRYOPTERIS* AND RELATED GENERA

As ancient remedies effective against helminthiasis, *Dryopteris* ferns have been the subject of chemical investigation since the end of the nineteenth century. These studies lead to the isolation and characterization of many acylphloroglucinols made of two to six rings.

MONOMERS

A few glycosilated, terpenylated, and methoxylated monocyclic acylphloroglucinols have been isolated from ferns. *Dryopteris fragrans* (L.) Schott contains the glycoside **12** and the terpenylated derivative of butyrylfilicinic acid, dryofragin (**18**) [21,23,24]. The glucosilated desaspidinol A, pleoside (**13**), together with compounds **14** and **15**, was isolated from *Arachniodes standishii* (T. Moore) Ohwi [21,25], whereas **16** and **17** came from *Arachniodes festina* (Hance) Ching and *Arachniodes nigrospinosa* (Ching) Ching [26].



12—R_1 = H, R_2 = R_4 = R_5 = R_6 = CH_3, R_3 = β-D-glucopyranosyl

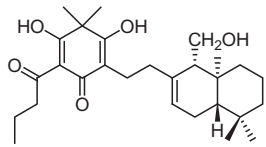
13—R_1 = β-D-glucopyranosyl, R_2 = R_4 = R_5 = H, R_3 = R_6 = CH_3

14—R_1 = R_3 = R_5 = CH_3, R_2 = R_4 = H, R_6 =

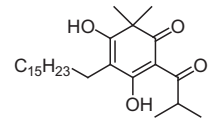
15—R_1 = R_2 = R_4 = H, R_3 = R_5 = CH_3, R_6 = *O*-β-D-allopyranosyl

16—R_1 = R_3 = R_5 = CH_3, R_2 = H, R_4 = OCH_3, R_6 =

17—R_1 = R_3 = R_5 = CH_3, R_2 = H, R_4 = OCH_3, R_6 =



18



19

The structure of subtriangularin-iB (**19**) could only be solved in part [27]. Nevertheless, this compound seems to be a sesquiterpenylated fraginol iB (methylisobutyrylfilicinic acid).

DIMERS AND POLYMERS

Nomenclature

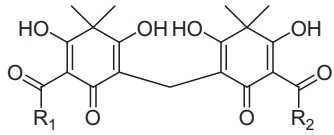
In 1970, Penttila and Sundman summarized the nomenclature for *Dryopteris* bi- and polycyclic acylphloroglucinols [9]. Compounds within a group differ from one another in the acyl residue attached to each ring. The acyl moieties found in phloroglucinol derivatives from *Dryopteris* and related fern genera are acetyl (A), propionyl (P), butyryl (B), isobutyryl (iB), and *n*-valeryl (V). Nevertheless, the butyryl group is the most common. We consider that a systematization of the acylphloroglucinol nomenclature is necessary. In the following schemes, the main structural types of phloroglucinol derivatives are described.

Dimers

As shown in Scheme 2, albaspidins are constituted by two acylfilicinic acid rings combined via a methylene bridge. For example, albaspidin AP has an acetyl group attached to one of the filicinic acid moieties and a propionyl residue linked to the other.

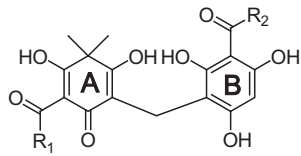
Norflavaspidic acids are made of an acylphloroglucinol ring linked through a methylene bridge to an acylfilicinic acid ring, whereas in flavaspidic acids, the B ring is replaced by a methylacylphloroglucinol residue (Scheme 3).

Aspidins are dimers composed of an acylfilicinic acid ring and a pseudoaspidinol ring, whereas in para-aspidins, the B ring is replaced by an aspidinol ring. In iso-aspidins, the B ring is iso-aspidinol (Scheme 4).

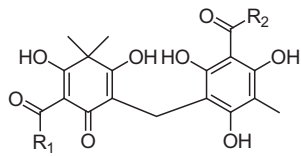


20-Albaspidin AA—$R_1 = R_2 = CH_3$
21-Albaspidin AB—$R_1 = CH_3$, $R_2 = n$-Pr
22-Albaspidin BB—$R_1 = R_2 = n$-Pr
23-Albaspidin PB—R_1 = Et, $R_2 = n$-Pr
24-Albaspidin PP—$R_1 = R_2$ = Et
25-Albaspidin AP—$R_1 = CH_3$, R_2 = Et
26-Albaspidin iBiB—$R_1 = R_2 = i$-Pr
27-Albaspidin BV—$R_1 = n$-Pr, $R_2 = n$-C_4H_9
28-Albaspidin iBV—$R_1 = i$-Pr, $R_2 = n$-C_4H_9
29-Albaspidin VV—$R_1 = R_2 = n$-C_4H_9

SCHEME 2 Structure of albaspidins.



30-Norflavaspidic acid AB—$R_1 = CH_3$, $R_2 = n$-Pr
31-Norflavaspidic acid BB—$R_1 = R_2 = n$-Pr
32-Norflavaspidic acid AP—$R_1 = CH_3$, R_2 = Et
33-Norflavaspidic acid PB—R_1 = Et, $R_2 = n$-Pr



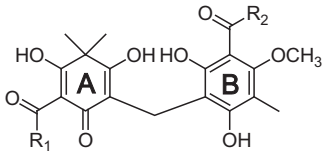
34-Flavaspidic acid AB—$R_1 = CH_3$, $R_2 = n$-Pr
35-Flavaspidic acid BB—$R_1 = R_2 = n$-Pr
36-Flavaspidic acid AP—$R_1 = CH_3$, R_2 = Et
37-Flavaspidic acid PA—R_1 = Et, $R_2 = CH_3$
38-Flavaspidic acid PB—R_1 = Et, $R_2 = n$-Pr
39-Flavaspidic acid BP—$R_1 = n$-Pr, R_2 = Et
40-Flavaspidic acid PP—$R_1 = R_2$ = Et
41-Flavaspidic acid VV—$R_1 = R_2 = n$-C_4H_9
42-Flavaspidic acid VB—$R_1 = n$-C_4H_9, $R_2 = n$-Pr
43-Flavaspidic acid BV—$R_1 = n$-Pr, $R_2 = n$-C_4H_9

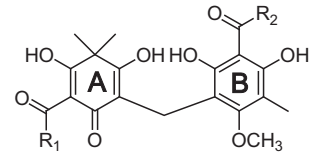
SCHEME 3 Structures of norflavaspidic and flavaspidic acids.

An acylfilicinic acid bound to a desaspidinol ring by a methylene bridge gives a desaspidin, whereas in ortho-desaspidin, the desaspidinol ring is replaced by ortho-desaspidinol (Scheme 5).

As seen in Scheme 6, when the acylfilicinic acid ring of a desaspidin, a para-aspidin, or an aspidin is substituted by methylacylphloroglucinol, phloraspins, margaspidins, or aemulins are obtained, respectively.

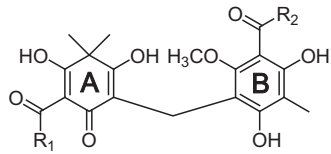
Phloraspidinols are obtained by replacing the acylfilicinic acid ring of desaspidin by an aspidinol ring. Methylene-bis-aspidinols and methylene-bis-desaspidinols, as indicated by their names, are dimers of aspidinol and desaspidinol, respectively (Scheme 7).





44-Aspidin AA—$R_1 = R_2 = CH_3$
45-Aspidin AB—$R_1 = CH_3$, $R_2 = n$-Pr
46-Aspidin BB—$R_1 = R_2 = n$-Pr
47-Aspidin AP—$R_1 = CH_3$, R_2 = Et
48-Aspidin PA—R_1 = Et, $R_2 = CH_3$
49-Aspidin PB—R_1 = Et, $R_2 = n$-Pr
50-Aspidin BP—$R_1 = n$-Pr, R_2 = Et
51-Aspidin PP—$R_1 = R_2$ = Et
52-Aspidin iBiB—$R_1 = R_2 = i$-Pr
53-Aspidin VB—$R_1 = n$-C_4H_9, $R_2 = n$-Pr
54-Aspidin iBB—$R_1 = i$-Pr, $R_2 = n$-Pr

55-Para-aspidin AA—$R_1 = R_2 = CH_3$
56- Para-aspidin AB—$R_1 = CH_3$, $R_2 = n$-Pr
57-Para-aspidin BB—$R_1 = R_2 = n$-Pr
58- Para-aspidin AP—$R_1 = CH_3$, R_2 = Et
59- Para-aspidin PA—R_1 = Et, $R_2 = CH_3$
60- Para-aspidin BP—$R_1 = n$-Pr, R_2 = Et
61- Para-aspidin PB—R_1 = Et, $R_2 = n$-Pr
62-Para-aspidin PP—$R_1 = R_2$ = Et



63-Iso-aspidin AB—$R_1 = CH_3$, $R_2 = n$-Pr
64-Iso-aspidin BB—$R_1 = R_2 = n$-Pr
65-Iso-aspidin PB—R_1 = Et, $R_2 = n$-Pr

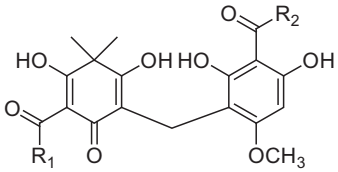
SCHEME 4 Structures of aspidins, para-aspidins, and iso-aspidins.

When an acylfilicinic acid ring is linked through a methylene bridge to a pyrendionic ring, a phloropyrone is formed, while in phloraspyrone, the pyrendionic ring is bound to a desaspidinol ring (Scheme 8).

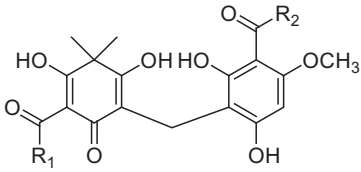
Abbreviatins and Araspidins

As seen in Scheme 9, abbreviatins are dimers of methylacylphloroglucinol linked via a methylene bridge, whereas in araspidins, the B ring is replaced by an acylphloroglucinol moiety. So far, only one araspidin (**87**) was isolated from a methanol extract of the rhizomes of *Arachniodes exilis* (Hance) Ching [28].

In pulvinuliferins, the A ring is stenolepin, and it is linked through a methylene bridge to an aspidinol moiety (Scheme 10). Only one pulvinuliferin (**88**) was isolated from *Dryopteris pulvinulifera* (Bedd.) Kuntze [27].

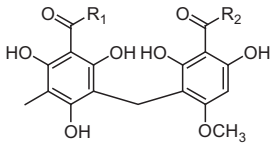


66-Desaspidin AB—R_1 = CH_3, R_2 = *n*-Pr
67-Desaspidin BA—R_1 = *n*-Pr, R_2 = CH_3
68-Desaspidin BB—R_1 = R_2 = *n*-Pr
69-Desaspidin BP—R_1 = *n*-Pr, R_2 = Et
70-Desaspidin PB—R_1 = Et, R_2 = *n*-Pr
71-Desaspidin AP—R_1 = CH_3, R_2 = Et
72-Desaspidin PA—R_1 = Et, R_2 = CH_3
73-Desaspidin PP—R_1 = R_2 = Et
74-Desaspidin VV—R_1 = R_2 = *n*-C_4H_9
75-Desaspidin VB—R_1 = *n*-C_4H_9 R_2 = *n*-Pr
76-Desaspidin BV—R_1 = *n*-Pr R_2 = *n*-C_4H_9

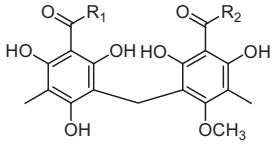


77-Ortho-desaspidin BB—R_1 = R_2 = *n*-Pr

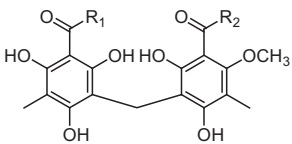
SCHEME 5 Structures of desaspidins and ortho-desaspidin.



78-Phloraspin BB—R_1 = R_2 = *n*-Pr
79-Phloraspin PB—R_1 = Et, R_2 = *n*-Pr
80-Phloraspin BP—R_1 = *n*-Pr, R_2 = Et
81-Phloraspin PP—R_1 = R_2 = Et
82-Phloraspin VB—R_1 = *n*-C_4H_9, R_2 = *n*-Pr
83-Phloraspin BV—R_1 = *n*-Pr, R_2 = *n*-C_4H_9
84-Phloraspin VV—R_1 = R_2 = *n*-C_4H_9

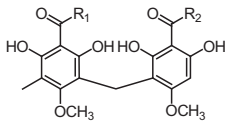


85-Margaspidin AA—R_1 = R_2 = CH_3
86-Margaspidin AB—R_1 = CH_3, R_2 = *n*-Pr
87-Margaspidin BB—R_1 = R_2 = *n*-Pr
88-Margaspidin BP—R_1 = *n*-Pr, R_2 = Et
89-Margaspidin PB—R_1 = Et, R_2 = *n*-Pr
90-Margaspidin AP—R_1 = CH_3, R_2 = Et
91-Margaspidin PA—R_1 = Et, R_2 = CH_3
92-Margaspidin PP—R_1 = R_2 = Et
93-Margaspidin VB—R_1 = *n*-C_4H_9, R_2 = *n*-Pr
94-Margaspidin BV—R_1 = *n*-Pr, R_2 = *n*-C_4H_9
95-Margaspidin VV—R_1 = R_2 = *n*-C_4H_9

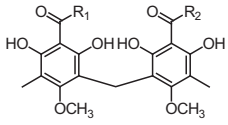


96-Aemulin BB—R_1 = R_2 = *n*-Pr

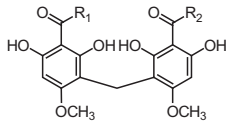
SCHEME 6 Structures of phloraspins, margaspidins, and aemulin.



97-Phloraspidinol BB—$R_1 = R_2 = n$-Pr
98-Phloraspidinol VB—$R_1 = n$-C_4H_9, $R_2 = n$-Pr
99-Phloraspidinol BV—$R_1 = n$-Pr, $R_2 = n$-C_4H_9

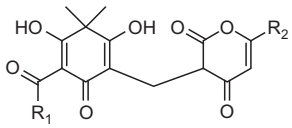


100-Methylene-bis-aspidinol BB—$R_1 = R_2 = n$-Pr
101-Methylene-bis-aspidinol PB—R_1 = Et, $R_2 = n$-Pr
102-Methylene-bis-aspidinol PP—$R_1 = R_2$ = Et
103-Methylene-bis-aspidinol VB—$R_1 = n$-C_4H_9, $R_2 = n$-Pr
104-Methylene-bis-aspidinol VV—$R_1 = R_2 = n$-C_4H_9

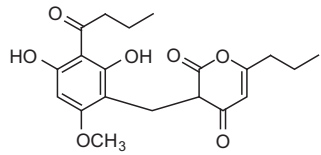


105-Methylene-bis-desaspidinol BB—$R_1 = R_2 = n$-Pr

SCHEME 7 Structures of phloraspidinols, methylene-bis-aspidinols, and methylene-bis-desaspidinol.

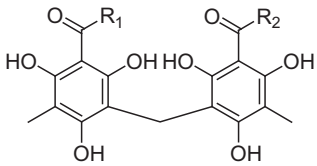


106-Phloropyrone BB—$R_1 = R_2 = n$-Pr
107-Phloropyrone PB—R_1 = Et, $R_2 = n$-Pr
108-Phloropyrone BP—$R_1 = n$-Pr, R_2 = Et
109-Phloropyrone VB—$R_1 = n$-C_4H_9, $R_2 = n$-Pr
110-Phloropyrone BV—$R_1 = n$-Pr, $R_2 = n$-C_4H_9
111-Phloropyrone PP—$R_1 = R_2$ = Et

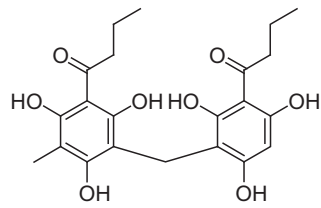


112-Phloraspyrone BB

SCHEME 8 Structures of phloropyrones and phloraspyrone.

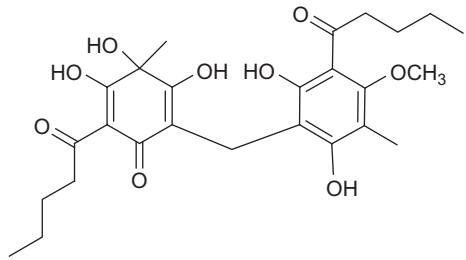


113-Abbreviatin AB—$R_1 = CH_3$, $R_2 = n$-Pr
114-Abbreviatin BB—$R_1 = R_2 = n$-Pr
115-Abbreviatin PB—R_1 = Et, $R_2 = n$-Pr
116-Abbreviatin PP—$R_1 = R_2$ = Et



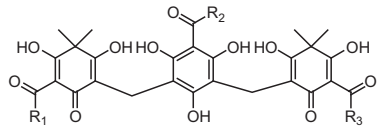
117-Araspidin BB

SCHEME 9 Structures of abbreviatins and araspidin.



118-Pulvinuliferin VV

SCHEME 10 Structure of pulvinuliferin.



119-Filixic acid AAA—$R_1 = R_2 = R_3 = CH_3$
120-Filixic acid ABA—$R_1 = R_3 = CH_3$, $R_2 = n$-Pr
121-Filixic acid ABB—$R_1 = CH_3$, $R_2 = R_3 = n$-Pr
122-Filixic acid BBB—$R_1 = R_2 = R_3 = n$-Pr
123-Filixic acid ABP—$R_1 = CH_3$, $R_2 = n$-Pr, R_3 = Et
124-Filixic acid APA—$R_1 = R_3 = CH_3$, R_2 = Et
125-Filixic acid PBB—R_1 = Et, $R_2 = R_3 = n$-Pr
126-Filixic acid PBP—$R_1 = R_3$ = Et, $R_2 = n$-Pr
127-Filixic acid VBB—$R_1 = n$-C_4H_9, $R_2 = R_3 = n$-Pr
128-Filixic acid VBV—$R_1 = R_3 = n$-C_4H_9, $R_2 = n$-Pr
129-Filixic acid VVV—$R_1 = R_2 = R_3 = n$-C_4H_9

SCHEME 11 Structure of filixic acids.

Trimers

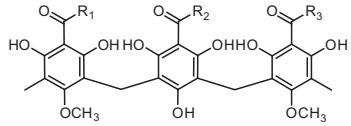
All the known trimers have an acylphloroglucinol residue intercalated between the rings of any of the previously listed two-ring compounds. Usually, the middle ring is a phloroglucinol B. Except for filixic acids, their name alludes to the related dimer.

In filixic acids, an acylphloroglucinol ring is placed between the two rings of an albaspidin (Scheme 11).

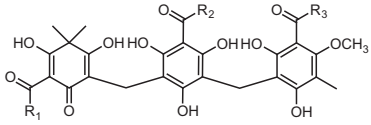
Trisaspidins are derived from aspidins, trispara-aspidins from para-aspidins, trisdesaspidins from desaspidins, trisflavaspidic acids from flavaspidic acid, and so on, by intercalating an acylphloroglucinol moiety between their two rings (Scheme 12).

Tetra-, Penta-, and Hexamers

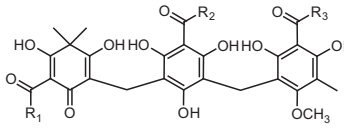
Tetra-, penta-, and hexamers (and higher members of the series) can be pictured as sandwiches of two or more acylphloroglucinol rings, the breads being the two rings of any of the previously mentioned dimers. For example, a hexa-albaspidin is formed by intercalating four acylphloroglucinol rings between the rings of albaspidin (Scheme 13).



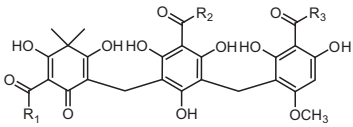
130-Trisaspidinol BBB—$R_1 = R_2 = R_3 = n$-Pr
131-Trisaspidinol PBB—R_1 = Et, $R_2 = R_3 = n$-Pr
132-Trisaspidinol PBP—$R_1 = R_3$ = Et, $R_2 = n$-Pr
133-Trisaspidinol VBB—$R_1 = n$-C_4H_9, $R_2 = R_3 = n$-Pr



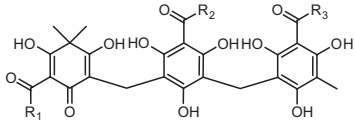
134-Trisaspidin BBB—$R_1 = R_2 = R_3 = n$-Pr



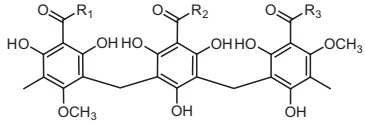
135-Trispara-aspidin ABB—$R_1 = CH_3$, $R_2 = R_3 = n$-Pr
136-Trispara-aspidin BBB—$R_1 = R_2 = R_3 = n$-Pr
137-Trispara-aspidin VBB—$R_1 = n$-C_4H_9, $R_2 = R_3 = n$-Pr
138-Trispara-aspidin BBV—$R_1 = R_2 = n$-Pr, $R_3 = n$-C_4H_9
139-Trispara-aspidin VVB—$R_1 = R_2 = n$-C_4H_9, $R_3 = n$-Pr
140-Trispara-aspidin BVV—$R_1 = n$-Pr, $R_2 = R_3 = n$-C_4H_9
141-Trispara-aspidin PBB—R_1 = Et, $R_2 = R_3 = n$-Pr
142-Trispara-aspidin BBP—$R_1 = R_2 = n$-Pr, R_3 = Et
143-Trispara-aspidin PBP—$R_1 = R_3$ = Et, $R_2 = n$-Pr
144-Trispara-aspidin PPB—$R_1 = R_2$ = Et, $R_3 = n$-Pr
145-Trispara-aspidin BPP—$R_1 = n$-Pr, $R_2 = R_3$ = Et



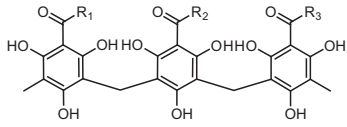
146-Trisdesaspidin ABB—$R_1 = CH_3$, $R_2 = R_3 = n$-Pr
147-Trisdesaspidin PBP—$R_1 = R_3$ = Et, $R_2 = n$-Pr
148-Trisdesaspidin BBB—$R_1 = R_2 = R_3 = n$-Pr
149-Trisdesaspidin ABP—$R_1 = CH_3$, $R_2 = n$-Pr, R_3 = Et
150-Trisdesaspidin PBA—R_1 = Et, $R_2 = n$-Pr, R_3 = Et



151-Trisflavaspidic acid ABB—$R_1 = CH_3$, $R_2 = R_3 = n$-Pr
152-Trisflavaspidic acid BBB—$R_1 = R_2 = R_3 = n$-Pr
153-Trisflavaspidic acid PBB—R_1 = Et, $R_2 = R_3 = n$-Pr

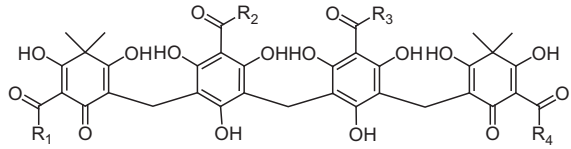


154-Trisaemulin BAB—$R_1 = R_3 = n$-Pr, $R_2 = CH_3$
155-Trisaemulin BBB—$R_1 = R_2 = R_3 = n$-Pr

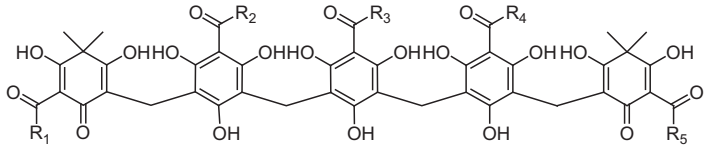


156-Trisabbreviatin BBB—$R_1 = R_2 = R_3 = n$-Pr

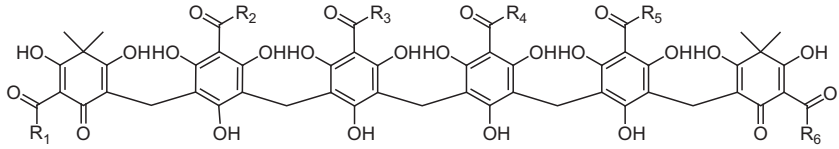
SCHEME 12 Structures of trisaspidinols, trisaspidin, trispara-aspidins, trisdesaspidins, trisflavaspidic acids, trisaemulins, and trisabbreviatin.



157-Tetra-albaspidin ABBA—$R_1 = R_4 = CH_3$, $R_2 = R_3 = n$-Pr
158-Tetra-albaspidin BBBB—$R_1 = R_2 = R_3 = R_4 = n$-Pr



161-Penta-albaspidin BBBBB—$R_1 = R_2 = R_3 = R_4 = R_5 = n$-Pr



162-Hexa-albaspidin BBBBBB—$R_1 = R_2 = R_3 = R_4 = R_5 = R_6 = n$-Pr

SCHEME 13 Structure of some tetra-, penta-, and hexamers.

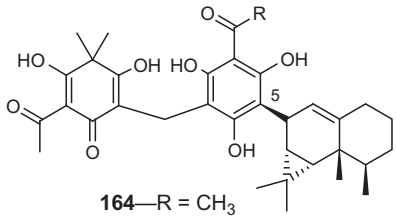
The most common tetra-, penta-, and hexamers are those whose parent dimer is albaspidin or flavaspidic acid.

TERPENYLATED ACYLPHLOROGLUCINOLS

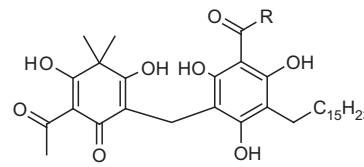
Some terpenylated flavaspidic and norflavaspidic acids have been isolated from *Dryopteris* ferns. The terpenyl moiety is usually attached to C-5 of the acylphloroglucinol ring or to the methyl group of the methylacylphloroglucinol residue. From *D. atrata* (Wall. ex Kunze) Ching, two sesquiterpenylated acylphloroglucinols called atrata-phloroglucinols A (**164**) and B (**165**) were obtained [29]. Interestingly, atrata-phloroglucinols are of the few *Dryopteris* acylphloroglucinols that were isolated from an extract of the fronds instead of that of the rhizomes [29]. The absolute configuration of these rare phloroglucinol derivatives was established by comparison of their specific rotation with that of (+)-aristol-9-ene, which is the terpenyl moiety of these acylphloroglucinols.

The first terpenylated derivative of flavaspidic acid named pentherin-I (**166**) was isolated from *Dryopteris pentheri* (Krasser) C. Chr. in 1973, though its structure could not be established at that moment and was called "brown unknown" by the authors [30]. The same authors proposed that pentherin-I (**166**) was also present in the diethyl ether extracts of the rhizomes of

Dryopteris ardechensis [31]. Additional sesquiterpenylated flavaspidic acids were obtained from *Dryopteris juxtaposita* H. Christ [20,31], *Dryopteris lepidopoda* Hayata [20,31], *Dryopteris sledgei* Fraser-Jenk. [17], and *Dryopteris nigropaleacea* (Fraser-Jenk.) Fraser-Jenk [31] and assigned the codes Ju-1 (**167**), Ju-2 (**168**), Ju-3 (**169**), Le-1 (**170**), Le-2 (**171**), Le-3 (**172**), Sl-1 (**173**), and Sl-2 (**174**). Their structures could not be established, but the authors suggested that these compounds were closely related to pentherin-I (**166**). They possibly differ from one another in their sesquiterpenyl moieties. It seems that a unique terpenyl moiety is attached to flavaspidic acid within a particular species.



164—R = CH_3
165—R = *n*-Pr

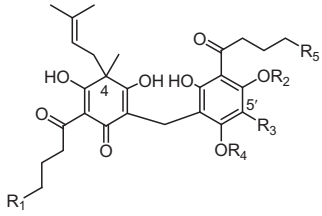


166-Pentherin-I

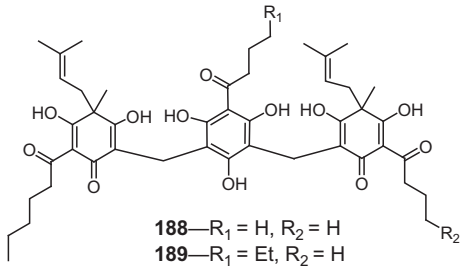
ELAPHOGLOSSUM ACYLPHLOROGLUCINOLS

The presence of acylphloroglucinols in herbarium specimens of ferns belonging to the genus *Elaphoglossum* (*E. erinaceum* (Fée) T. Moore and *E. paleaceum* (Hook. & Grev.) Sledge) was detected more than 25 years ago [13]. The authors described them as "unknown phenolics" that were "clearly not identical with those occurring in *Dryopteris* and some other related genera." But it was not until 2009 that the first *Elaphoglossum* phloroglucinol derivatives from *E. piloselloides* were isolated and structurally characterized [32]. Moreover, preliminary chemical analyses of the Ethyl ether (Et_2O) extracts of six of the eight Argentine *Elaphoglossum* species indicated the presence of acylphloroglucinols in all of them. In previous publications, we reported on the isolation and characterization of 18 new acylphloroglucinols (**180**–**197**) from *E. piloselloides*, *E. gayanum*, *E. yungense* de la Sota, and *E. lindbergii* (Mett. ex Kuhn) Rosenst [32–35]. A characteristic structural feature of *Elaphoglossum* acylphloroglucinols is that they carry prenyl side chains attached to C-4 of the acylfilicinic acid residue, and, in some cases, to C-5′ of the aromatic ring as well. Sometimes, the latter cyclizes to form a chromene-, chromane-, or chromanol-type ring. Another distinctive feature is that acyl groups present in *Elaphoglossum* phloroglucinol derivatives contain an even number of carbons (e.g., acetyl, butyryl, caproyl).

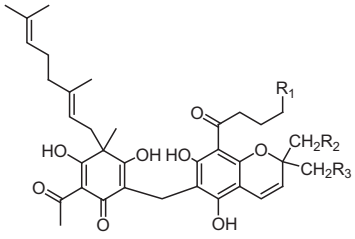
In fact, *Elaphoglossum* acylphloroglucinols structurally resemble those of *Hypericum*'s [36–38] rather than *Dryopteris*' [17,20].



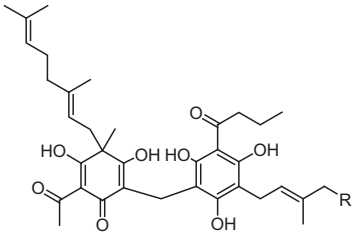
180-R_1 = H, R_2 = H, R_3 = H, R_4 = CH_3, R_5 = H
181-R_1 = H, R_2 = H, R_3 = H, R_4 = CH_3, R_5 = Et
182-R_1 = H, R_2 = CH_3, R_3 = H, R_4 = H, R_5 = H
183-R_1 = H, R_2 = CH_3, R_3 = H, R_4 = H, R_5 = Et
184-R_1 = H, R_2 = H, R_3 = CH_3, R_4 = CH_3, R_5 = H
185-R_1 = Et, R_2 = H, R_3 = H, R_4 = CH_3, R_5 = H
186-R_1 = Et, R_2 = H, R_3 = H, R_4 = CH_3, R_5 = Et
187-R_1 = Et, R_2 = CH_3, R_3 = H, R_4 = H, R_5 = Et



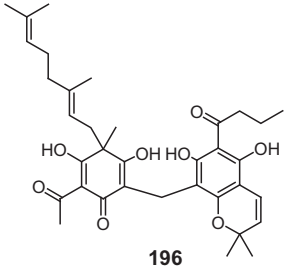
188—R_1 = H, R_2 = H
189—R_1 = Et, R_2 = H
190—R_1 = Et, R_2 = Et



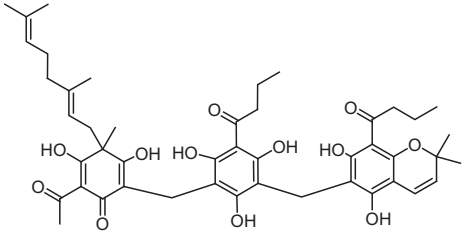
191—R_1 = R_2 = R_3 = H
192—R_1 = Et, R_2 = R_3 = H
193—R_1 = R_2 = H, R_3 = 



194—R = H
195—R = 



196



197

So far, only four species of *Elaphoglossum* were chemically analyzed for their phloroglucinol derivatives. Each one contained characteristic acylphloroglucinols not found in any other species analyzed up to the present day (Table 1).

PHYTOCHEMICAL AND BOTANICAL CONSIDERATIONS

Acylphloroglucinols occur mainly in ferns of the genera *Dryopteris* [17,18,20], *Arachniodes* [14–16,19], and *Elaphoglossum* [11–15]. The presence of these compounds has also been reported from a few species belonging to the genera *Stigmatopteris*, *Dryopsis*, *Polystichum*, *Rumohra*, *Nothoperanema*, *Lastreopsis*, *Polybotrya*, *Acrophorus*, *Ctenitis*, *Pleocnemia*, and *Peranema* [13,19]. According to the latest fern classification, based on a compromise of morphological and molecular features [72], acylphloroglucinol-containing ferns are included in the family Dryopteridaceae with the exceptions of *Lastreopsis marginans*, *Pleocnemia conjugata*, and *P. irregularis* (Tectariaceae). In this new classification, several groups were moved to the family Dryopteridaceae including genera from the family Lomaropsidaceae, such as *Bolbitis* and *Elaphoglossum*. In addition, a few genera originally placed in Dryopteridaceae remained in the families Athyriaceae, Tectariaceae, Onocleaceae, and Lomaropsidaceae [73].

Dryopteridaceae Herter is a family of worldwide distribution, with over 45 genera and about 1700 species, 70% of which are grouped in four genera: *Ctenitis*, *Dryopteris*, *Elaphoglossum*, and *Polystichum* [72]. This family is pantropical, with many species in the neotropical region, but the east region of Asia has the greatest diversity. Dryopteridaceae family is part of the most derived indusiate ferns, along with Aspleniaceae, Blechnaceae, Davalliaceae, Grammitidaceae, Lomaropsidaceae, Nephrolepis, Oleandraceae, Polypodiaceae, and Thelypteridaceae [74,75]. The rhizome of the fern species belonging to Dryopteridaceae is erect, very rarely arborescent, decumbent, long creeping or long ascending, stout to slender, and scaly; the stipe base is also scaly. *Arachniodes* Blume is a pantropical genus of about 50 species [16] distributed in the subtropical and tropical forest regions of the world, mainly in China and southern and southeastern Asia [76]. According to Widén *et al.* [14,16], most of the species of *Arachniodes* have secreting glandular trichomes formed by a round head and one-celled foot situated on the stipe base. These trichomes are located on the epidermis of the rhizome and are long to short stalked (38–70 μm). These authors also found two- to three-celled internal secretory glands in the rhizome cortex. Regarding the distribution of scales in the sporophyte, *Arachniodes* has a creeping or erect rhizome from 1 to 3 cm, scaly with shiny, lanceolate, and entire scales; the petiole being yellowish to brown and scaly at the base. *Dryopteris* Adans is a genus of about 150 species, 25 of which are American. It has worldwide distribution with the greatest diversity in Asia [77].

TABLE 1 Distribution of Phloroglucinol Derivatives in Ferns

#	Compounds	Species in Which the Phloroglucinol Derivative Was Detected
3	Stenolepin B	***Dryopteris stenolepis*** (Baker) C. Chr. [39], ***D. nigropaleacea*** [20], ***D. marginata*** (C. B. Clarke) H. Christ [20]
12	3,5-Dimethyl-6-hydroxy-2-methoxy-4-*o*-β-D-glucopyranosyloxy-acetophenone	***Dryopteris fragrans*** [23]
13	Pleoside	***Arachniodes standishii*** [25]
14	(*E*)-1-(2,4,6-Trimethoxyphenyl)but-2-en-1-one	***Arachniodes standishii*** [25]
15	3-β-D-Allopyranosyloxy-1-(2-hydroxy-4,6-dimethoxyphenyl)butan-1-one	***Arachniodes standishii*** [21,25]
16	(*E*)-1-(2,3,4,6-Tetramethoxyphenyl)but-2-en-1-one	***Arachinodes nigrospinosa*** [26], ***A. festina*** [26]
17	(*E*)-1-(2,3,4,6-Tetramethoxyphenyl)pent-2-en-1-one	***Arachniodes nigrospinosa*** [26], ***A. festina*** [26]
18	Dryofragin	***Dryopteris fragrans*** [21,24]
19	Subtriangularin-iB	***Dryopteris subtriangularis*** (C. Hope) C. Chr. [19,27]
20	Albaspidin AA	*Arachniodes hasseltti* (Blume) Ching (=***Acrorumohra hasseltii*** (Blume) Ching) [16], ***A. nipponica*** (Rosenst.) Ohwi [16], *A. simplicior* (Makino) Ohwi var. *major* Ohwi [16], ***Ctenitis mannii*** (C. Hope) Ching [15], *D. affinis* (Lowe) Fraser-Jenk. ssp. *affinis* Fraser-Jenk. var. *affinis* [31], *D. affinis* ssp. *affinis* var. *splendens* (Ehrler ex Becherer) Fraser-Jenk. [17], *D. affinis* ssp. *borreri* (Newman) Fraser-Jenk. [31], *D. affinis* ssp. *cambrensis* Fraser-Jenk. var. *insubrica* Oberholzer & Tavel ex Fraser-Jenk. [17], *D. affinis* ssp. *cambrensis*

Continued

TABLE 1 Distribution of Phloroglucinol Derivatives in Ferns—Cont'd

#	Compounds	Species in Which the Phloroglucinol Derivative Was Detected
		var. *paleaceo-crispa* (T. Moore) Fraser-Jenk. [17], *D. affinis* ssp. *pontica* Fraser-Jenk. [17], *D. affinis* ssp. *pseudo-disjuncta* (Fraser-Jenk.) Fraser-Jenk. [17], *D. ardechensis* Fraser-Jenk. [31], ***D. chinensis*** (Baker) Koidz. [16,40], ***D. cochleata*** (D. Don) C. Chr. [14], ***D. crassirhizoma*** Nakai [41], ***D. cristata*** (L.) A. Gray [42], ***D. dickinsii*** (Franch & Sav.) C. Chr. [39,40], ***D. dilatata*** (Hoffm.) A. Gray [43], ***D. hawaiiensis*** (Hillebr.) W. J. Rob. [44], *D. hayatae* Tagawa (=***D. subexaltata*** (H. Christ) C. Chr.) [16], *D. hirtipes* (Blume) Kuntze ssp. *atrata* (Kunze) Fraser-Jenk. (=***D. atrata*** (Wall. ex Kunze) Ching) [17], ***D. juxtaposita*** [20], *D. khullarii* Fraser-Jenk. [17], ***D. lepidopoda*** Hayata [17], *D. maderensis* Alston [43], ***D. nigropaleacea*** [20], *D. odontoloma* (Bedd.) C. Chr. (=***D. juxtaposita***) [18], ***D. patula*** (Sw.) Underw. [45], ***D. redactopinnata*** S. K. Basu & Paniraghi [17], ***D. sledgei*** Fraser-Jenk. [17], ***D. sparsa*** (D. Don) Kuntze [16,19], ***D. stenolepis*** (Baker) C. Chr. [39], *D. stewartii* Fraser-Jenk. [20], *D. wallichiana* (Spreng.) Hyl. [46], ***D. wallichiana*** ssp. ***coriacea*** (Fraser-Jenk.) Fraser-Jenk. [17], ***D. wallichiana*** ssp. ***madrasensis*** (Fraser-Jenk.) Fraser-Jenk. [17], *D. wallichiana* ssp. *reichsteinii* Fraser-Jenk. [17], *Nothoperanema squamiseta* (Hook.) Ching (=***Nothoperanema squamisetum*** (Hook.) Ching) [19], ***Pleocnemia conjugata*** (Blume) C. Presl [15]
21	Albaspidin AB	***Arachniodes amabilis*** (Blume) Tindale [16], ***A. aristata*** (G. Forst.) Tindale [14], *A. cavalerii* (H. Christ) Ching [14], ***A. dimorphophyllum*** (Hayata) Ching [16], *A. hasseltii* [16], *A. maximowiczii* (Baker) Ohwi [16], ***A. nipponica*** [16], *A. simplicior* var. *major* [16], ***Ctenitis mannii*** [15], *D. affinis* ssp. *affinis* var. *affinis* [31], *D. affinis* ssp. *affinis* var. *splendens* [17], *D. affinis* ssp. *borreri* [31], *D. affinis* ssp. *cambrensis* var. *insubrica* [17,31], *D. affinis* ssp. *cambrensis* var. *paleaceo-crispa* [17], *D. affinis* ssp. *pontica* [17], *D. affinis* ssp. *pseudo-disjuncta* [17], *D. aitoniana* Pic. Serm. [47], *D. antarctica* (Baker) C. Chr. [18], *D. ardechensis* [31], *D. assimilis* S. Walker (=***Dryopteris expansa*** (C. Presl) Fraser-Jenk. & Jermy) [42], ***D. athamantica*** (Kunze) Kuntze [30], ***D. austriaca*** (Jacq.)

		Woyn. ex Schinz & Thell. [40], ***D. azorica*** (H. Christ) Alston [43], ***D. barbigera*** (T. Moore ex Hook.) Kuntze [20], ***D. callolepis*** C. Chr. [30], ***D. campyloptera*** (Kuntze) Clarkson [42], ***D. celsa*** (Palmer) Knowlton, Palmer, & Pollard [18], ***D. championii*** (Benth.) C. Chr. ex Ching [19,40], ***D. chinensis*** [16,40], ***D. cochleata*** [14], ***D. cristata*** [42], ***D. dilatata*** [43], ***D. erythrosora*** (D. C. Eaton) Kuntze [19], *D. hayatae* [16], *D. hirtipes* ssp. *atrata* [17], ***D. intermedia*** (Muhl. ex Willd.) A. Gray [42], ***D. juxtaposita*** [20], *D. khullarii* [17], ***D. lepidopoda*** [17], *D. maderensis* [43], ***D. marginata*** [20], ***D. nigropaleacea*** [20], *D. odontoloma* [20], *D. pallida* (Bory) Fomin ssp. *balearica* (Litard.) Fraser-Jenk. [20], ***D. pallida*** ssp. ***libanotica*** (Rosenst.) G. Nardi [20], *D. pallida* ssp. *pallida* [20], *D. pallida* ssp. *raddeana* (Fomin) Nardi (=***D. raddeana*** Fomin) [20], *D. para-chrysocoma* Ching & Z. R. Wang (=***D. chrysocoma*** (H. Christ) C. Chr.) [20], ***D. ramosa*** (C. Hope) C. Chr. [20], ***D. redactopinnata*** [17], ***D. remota*** (A. Braun) Hayek [48], ***D. sledgei*** [17], ***D. sparsa*** [16,19], *D. spinulosa* (Muell.) Watt (=***D. carthusiana*** (Vill.) H. P. Fuchs) [42], ***D. stenolepis*** [39], *D. stewartii* [20], *D. submontana* (Fraser-Jenk. & Jermy) Fraser-Jenk. (=***D. submontana*** Fraser-Jenk.) [20], ***D. subtriangularis*** [19], *D. tyrrhena* Fraser-Jenk. & Reichstein [31], *D. undulata* (Bedd.) Kuntze [19], *D. villarii* (Bell.) Woynar [49], *D. villarii* ssp. *pallida* var. *balearica* [47], *D. villarii* ssp. *pallida* (Bory) Heywood [47], ***D. wallichiana*** ssp. ***coriacea*** [17], ***D. wallichiana*** ssp. ***madrasensis*** [17], *D. wallichiana* ssp. *reichsteinii* [17], ***D. woodsiisora*** Hayata [20], ***D. yigongensis*** Ching [17], *Nothoperanema rubiginosa* (Brack) A. R. Sm. & D. R. Palmer (=***Nothoperanema rubiginosum*** (Brack.) A.R. Sm. & D.R. Palmer) [19], *N. squamiseta* (Hook.) Ching (=***N. squamisetum*** (Hook.) Ching) [19], ***Polystichum tsus-simense*** (Hook.) J. Sm. [14]
22	Albaspidin BB	***Arachniodes amabilis*** [16], ***A. aristata*** [14], *A. cavalerii* [14], ***A. dimorphophyllum*** [16], *A. hasseltii* [16], *A. maximowiczii* [16], ***A. nipponica*** [16], *A. simplicior* var. *major* [16], ***A. tripinnata*** (Goldm.) Sledge [14], ***Ctenitis mannii*** [15], *Diacalpe aspidioides* Blume (=***Peranema aspidioides*** (Blume) Mett.) [19], ***Dryopsis apiciflora*** (Wall. ex Mett.) Holttum & P. J. Edwards [19], ***D. clarkei*** (Baker) Holttum & P. J. Edwards [19], ***D. nidus*** (Baker) Holttum & P. J. Edwards [19], *Dryopteris aitoniana* [47,50], ***D. acutodentata*** Ching [17], *D. affinis* ssp. *affinis* var. *affinis* [31], *D. affinis* ssp. *affinis* var. *splendens* [17], *D. affinis* ssp. *borreri* [31], *D. affinis* ssp. *cambrensis* var. *insubrica* [17,31], *D. affinis* ssp.

Continued

TABLE 1 Distribution of Phloroglucinol Derivatives in Ferns—Cont'd

#	Compounds	Species in Which the Phloroglucinol Derivative Was Detected
		pontica [17], *D. affinis* ssp. *pseudo-disjuncta* [17], *D. antarctica* [18], *D. ardechensis* [31], *D. assimilis* [42,51], ***D. athamantica*** [30,47], ***D. austriaca*** [12,40], ***D. azorica*** [43], ***D. barbigera*** [20], *D. bissetiana* (Baker) C. Chr. (=***D. setosa*** (Thunb.) Akas.) [40], ***D. callolepis*** [30], ***D. campyloptera*** [42], ***D. celsa*** [18], ***D. championii*** [12,40], ***D. chinensis*** [12,16,40], ***D. clintoniana*** (Eaton) Dowell. [18], ***D. cochleata*** [14], ***D. crassirhizoma*** [12], ***D. cristata*** [18,42], ***D. decipiens*** (Hook.) Kuntze [12], ***D. dickinsii*** [12], ***D. dilatata*** [30,43], ***D. erythrosora*** (D. C. Eaton) Kuntze [12,40], ***D. filix-mas*** (L.) Schott [50,52], *D. filix-mas* var. *rigidiformis* Rouy [47], ***D. fragrans*** [20], ***D. fuscipes*** C. Chr. [12], ***D. gymnophylla*** (Baker) C. Chr. [12], *D. hayatae* [16], *D. hirtipes* ssp. *atrata* [17], ***D. hondoensis*** Koidz. [12], ***D. inaequalis*** (Schltdl.) Kuntze [30], ***D. indusiata*** (Makino) Makino & Yamam. [12], ***D. intermedia*** [42], ***D. juxtaposita*** [20], ***D. kinkiensis*** Koidz. ex Tagawa [12], *D. khullarii* [17], ***D. lacera*** (Thunb.) Kuntze [12], ***D. lepidopoda*** [17], ***D. ludoviciana*** (Kunze) Small [18], *D. maderensis* [43], ***D. manniana*** (Hook.) C. Chr. [30], ***D. marginata*** [20], ***D. marginalis*** (L.) A. Gray [18], ***D. nigropaleacea*** [17,20], *D. nipponensis* Koidz. [12], *D. odontoloma* [20], *D. pallida* ssp. *balearica* [20], ***D. pallida*** ssp. ***libanotica*** [20], *D. pallida* ssp. *pallida* [20], *D. pallida* ssp. *raddeana* [20], ***D. patula*** [45], *D. para-chrysocoma* [20], ***D. pentheri*** [30], ***D. polylepis*** (Franch. & Sav.) C. Chr. [12,40,53], ***D. pulvinulifera*** [14,27], ***D. ramosa*** [20], ***D. redactopinnata*** [17], ***D. remota*** [48], ***D. sacrosancta*** Koidz [40], *D. schimperana* (A. Braun) C. Chr. (=*D. schimperiana* C. Chr.) [30], ***D. sordidipes*** Tagawa [12], ***D. sparsa*** [16,19], *D. spinulosa* [42,51], ***D. splendens*** (Wall. ex Hook.) Kuntze [18], ***D. stenolepis*** [39], *D. stewartii* [20], *D. submontana* [20], ***D. subtriangularis*** [19,27], ***D. tokyoensis*** (Matsum. ex Makino) C. Chr. [12], *D. tyrrhena* [31], *D. undulata* [19], ***D. uniformis*** (Makino) Makino [12], ***D. varia*** (L.) Kuntze [12], *D. villarii* [49], *D. villarii* ssp. *pallida* [47], *D. villarii* ssp. *pallida* var. *balearica* [47], *D. villarii* ssp. *villarii* [20,47], ***D. wallichiana*** (Spreng.) Alston & Bonner

		[12], *D. wallichiana* ssp. *reichsteinii* [17], ***D. wallichiana*** ssp. ***wallichiana*** [17], ***D. woodsiisora*** [20], ***D. yigongensis*** [17], *Nothoperanema rubiginosa* [19], *N. squamiseta* [19], ***Polybotrya caudata*** Kunze [13], ***Polystichum rigens*** Tagawa [14], ***P. tsus-simense*** [14]
23	Albaspidin PB	***Arachniodes amabilis*** [16], ***A. aristata*** [14], *A. cavalerii* [14], *A. dimorphophylla* (Hayata) Ching (=***A. dimorphophyllum*** (Hayata) Ching) [16], *A. hasseltii* [16], *A. maximowiczii* [16], ***A. nipponica*** [16], *A. simplicior* var. *major* [16], ***A. tripinnata*** [14], ***Ctenitis mannii*** [15], ***Dryopsis nidus*** [19], *Dryopteris antarctica* [18], ***D. athamantica*** [30,47], ***D. celsa*** [18], ***D. chinensis*** [16,40], ***D. clintoniana*** [18], ***D. cochleata*** [14], ***D. crassirhizoma*** [54], ***D. cristata*** [18,42], ***D. dilatata*** [30], ***D. erythrosora*** [40], *D. filix-mas* var. *rigidiformis* [47], *D. hayatae* [16], ***D. inaequalis*** [30], ***D. juxtaposita*** [20], ***D. ludoviciana*** [18], ***D. manniana*** [30], ***D. marginata*** [20], ***D. nigropaleacea*** [17,20], *D. odontoloma* [20], *D. pallida* ssp. *balearica* [20], ***D. pallida*** ssp. ***libanotica*** [20], *D. pallida* ssp. *pallida* [20], *D. pallida* ssp. *raddeana* [20], *D. para-chrysocoma* [20], ***D. polylepis*** [17,40], ***D. pulvinulifera*** [14], ***D. remota*** [48], ***D. sparsa*** [16], *D. spinulosa* [42,51], *D. villarii* [49], *D. villarii* ssp. *pallida* [47], ***Polystichum rigens*** [14], ***P. tsus-simense*** [14]
24	Albaspidin PP	***Arachniodes amabilis*** [16], ***A. aristata*** [14], *A. cavalerii* [14], *A. dimorphophylla* [16], *A. hasseltii* [16], *A. maximowiczii* [16], ***A. nipponica*** [16], *A. simplicior* var. *major* [16], ***A. tripinnata*** [14], ***Ctenitis mannii*** [15], ***Dryopsis nidus*** [19], *Dryopteris antarctica* [18], ***D. athamantica*** [30,47], ***D. celsa*** [18], ***D. chinensis*** [16,40], ***D. clintoniana*** [18], ***D. cochleata*** [14], ***D. crassirhizoma*** [54], ***D. cristata*** [18,42], ***D. dilatata*** [30], *D. filix-mas* var. *rigidiformis* [47], *D. hayatae* [16], ***D. juxtaposita*** [20], ***D. ludoviciana*** [18], ***D. manniana*** [30], ***D. nigropaleacea*** [17,20], *D. odontoloma* [20], *D. pallida* ssp. *balearica* [47], ***D. pallida*** ssp. ***libanotica*** [20], *D. pallida* ssp. *pallida* [20,47], *D. pallida* ssp. *raddeana* [20], ***D. polylepis*** [17,40], ***D. pulvinulifera*** [14], ***D. remota*** [48], ***D. sparsa*** [16], *D. spinulosa* [42,51], *D. villarii* [49], ***Polystichum rigens*** [14], ***P. tsus-simense*** [14]

Continued

TABLE 1 Distribution of Phloroglucinol Derivatives in Ferns—Cont'd

#	Compounds	Species in Which the Phloroglucinol Derivative Was Detected
25	Albaspidin AP	***Arachniodes amabilis*** [16], ***A. aristata*** [14], *A. cavalerii* [14], *A. hasseltii* [16], *A. maximowiczii* [16], ***A. nipponica*** [16], *A. simplicior* var. *major* [16], ***Ctenitis mannii*** [15], ***Dryopsis nidus*** [19], ***Dryopteris celsa*** [18], ***D. chinensis*** [16,40], ***D. cochleata*** [14], ***D. crassirhizoma*** [54], ***D. cristata*** [42], ***D. dilatata*** [43], ***D. goldiana*** (Hook. ex Goldie) A. Gray [18], ***D. hawaiiensis*** [44], *D. hayatae* [16], *D. maderensis* [43], ***D. nigropaleacea*** [17,20], *D. odontoloma* [20], *D. pallida* ssp. *balearica* [47], *D. pallida* ssp. *pallida* [20,47], ***D. remota*** [48], ***D. sparsa*** [16], *D. spinulosa* [42], *D. villarii* [49], ***Polystichum tsus-simense*** [14]
26	Albaspidin iBiB = Japonicine A	***Dryopteris erythrosora*** [40], ***D. subtriangularis*** [19,27]
27/28	Albaspidin BV/iBV	***Dryopteris erythrosora*** [40], ***D. subtriangularis*** [27]
29	Albaspidin VV	***Dryopteris subtriangularis*** [27]
30	Norflavaspidic acid AB	*Dryopteris affinis* ssp. *affinis* var. *affinis* [31], *D. affinis* ssp. *affinis* var. *jessenii* [17], *D. affinis* ssp. *affinis* var. *splendens* [17], *D. affinis* ssp. *cambrensis* var. *insubrica* [17], *D. affinis* ssp. *cambrensis* var. *paleaceo-crispa* [17,31], *D. affinis* ssp. *pontica* [17], ***D. conjugata*** Ching [17], ***D. crassirhizoma*** [54], ***D. dickinsii*** [21,39], *D. fusco-atra* (Hillebr.) W. J. Rob. (= ***D. fusco-atra*** W. J. Rob.) [44], *D. fusco-atra* var. *lamoureuxii* Fraser-Jenk. [17], *D. hirtipes* ssp. *atrata* [17], *D. hirtipes* ssp. *hirtipes* [17], ***D. juxtaposita*** [20], *D. khullarii* [17], *D. odontoloma* [20], ***D. oreades*** Fomin [17], *D. sledgei* [17], ***D. stenolepis*** [39], ***D. wallichiana*** ssp. ***wallichiana*** [17]
31	Norflavaspidic acid BB	*Dryopteris affinis* ssp. *affinis* var. *affinis* [31], *D. affinis* ssp. *affinis* var. *jessenii* [17], *D. affinis* ssp. *affinis* var. *splendens* [17], *D. affinis* ssp. *cambrensis* var. *insubrica* [17], *D. affinis* ssp. *cambrensis* var. *paleaceo-crispa* [17,31], *D. affinis* ssp. *pontica* [17], ***D. conjugata*** [17], *D. fusco-atra* var. *fusco-atra* [17], *D. fusco-atra* var. *lamoureuxii* [17], *D. hirtipes* ssp. *atrata* [17], *D. hirtipes* ssp. *hirtipes* [17], ***D. juxtaposita*** [20], *D. khullarii* [17], *D. odontoloma* [20], ***D. oreades*** [17], *D. sledgei* [17], *D. tyrrhena* [31], ***Stigmatopteris longicaudata*** (Liebm.) C. Chr. [19]

32	Norflavaspidic acid AP	***Dryopteris stenolepis*** [39]
33	Norflavaspidic acid PB	*Dryopteris fusco-atra* [44]
34	Flavaspidic acid AB	*Arachniodes rhomboidea* (Wall. ex Mett.) Ching [19], *Dryopteris abbreviata* Newman (=***D. pseudomas*** Holub & Pouzar) [47,55], ***D. acutodentata*** [17], *D. aemula* (Aiton) Kuntze [31], *D. affinis* ssp. *affinis* var. *affinis* [31], *D. affinis* ssp. *affinis* var. *jessenii* [17], *D. affinis* ssp. *affinis* var. *paleaceo-lobata* [17], *D. affinis* ssp. *affinis* var. *splendens* [17], *D. affinis* ssp. *borreri* [31], *D. affinis* ssp. *cambrensis* var. *insubrica* [17,31], *D. affinis* ssp. *cambrensis* var. *paleaceo-crispa* [17,31], *D. affinis* ssp. *persica* [17], *D. affinis* ssp. *pontica* [17], *D. affinis* ssp. *pseudo-disjuncta* [17], *D. ardechensis* [31], *D. arguta* (Kaulf.) Watt [20], ***D. barbigera*** [20], ***D. blanfordii*** (C. Hope) C. Chr. [20], ***D. celsa*** [18], ***D. chinensis*** [40], *D. corleyi* Fraser-Jenk. [31], ***D. crassirhizoma*** [41,54,56,57], ***D. dickinsii*** [39], ***D. filix-mas*** [17], ***D. fragrans*** [20], *D. fusco-atra* [44], *D. fusco-atra* var. *lamoureuxii* [17], ***D. juxtaposita*** [20], *D. khullarii* [17], ***D. lacera*** [40], ***D. lepidopoda*** [17], ***D. manniana*** [30], ***D. neorosthornii*** Ching [17], ***D. nigropaleacea*** [20], *D. odontoloma* [20], ***D. oreades*** [17,31], *D. pallida* ssp. *pallida* [20], *D. pallida* ssp. *raddeana* [20], *D. parachrysocoma* [20], *D. parallelogramma* (Kunze) Alston (=*D. wallichiana* (Spreng.) Hyl.) [45], *D. parrisiae* Fraser-Jenk. [17], ***D. polylepis*** [40,53], ***D. pseudo-filix-mas*** (Fée) Rothm. [17], ***D. ramosa*** [20], ***D. redactopinnata*** [17], *D. sledgei* [17], ***D. stenolepis*** [39], *D. stewartii* [20], ***D. subbipinnata*** W. H. Wagner & Hobbi [17], *D. submontana* [20], *D. tyrrhena* [31], ***D. wallichiana*** ssp. ***coriacea*** [17], ***D. wallichiana*** ssp. ***madrasensis*** [17], *D. wallichiana* ssp. *reichsteinii* [17], ***D. wallichiana*** ssp. ***wallichiana*** [17], *D. villarii* ssp. *villarii* [20], ***D. woodsiisora*** [20], *D. xanthomelas* (H. Christ) C. Chr. (=***D. rosthornii*** (Diels) C. Chr.) [17], ***D. yigongensis*** [17], *D. zayuensis* Ching & S. K. Wu (=***D. squamifera*** (Ching & S. K. Wu) [17], ***Peranema cyatheoides*** D. Don [19], ***Pleocnemia conjugata*** [15]

Continued

TABLE 1 Distribution of Phloroglucinol Derivatives in Ferns—Cont'd

#	Compounds	Species in Which the Phloroglucinol Derivative Was Detected
35	Flavaspidic acid BB	***Arachniodes amabilis*** [14,16], ***A. aristata*** [14,15], ***A. assamica*** (Kuhn) Ohwi [14,19], *A. cantilenae* Sa. Kurata [15], *A. cavalerii* [14], *A. dimorphophylla* [16], ***A. exilis*** [16], *A. hasseltii* [16], ***A. hekiana*** Sa. Kurata [15], ***A. japonica*** (Sa. Kurata) Nakaike [15], *A. maximowiczii* [16,19], *A. miqueliana* (Maxim. ex Franch. & Sav.) Ohwi (=***Leptorumohra miqueliana*** (Maxim. ex Franch. & Sav.) H. Itô) [16,19], *A. mutica* (Franch. & Sav.) Ohwi (=***Leptorumohra mutica*** Czer. comb. nova) [16], ***A. nipponica*** [16], *A. okinawensis* Nakaike [16], *A. rhomboidea* [19], ***A. simplicior*** [16], *A. simplicior* var. *major* [16], ***A. tripinnata*** [14,15], *A. yasu-inouei* Sa. Kurata [14], ***Ctenitis mannii*** [15], ***Ctenitis setosa*** (C. Presl) Holttum [19], *Diacalpe aspidioides* [19], ***Dryopsis apiciflora*** [19], ***D. clarkei*** [19], ***D. nidus*** [19], *Dryopteris abbreviata* [47], ***D. acutodentata*** [17], *D. aemula* [43], *D. affinis* ssp. *affinis* var. *affinis* [31], *D. affinis* ssp. *affinis* var. *jessenii* [17], *D. affinis* ssp. *affinis* var. *paleaceo-lobata* [17], *D. affinis* ssp. *affinis* var. *splendens* [17], *D. affinis* ssp. *borreri* [17], *D. affinis* ssp. *cambrensis* var. *insubrica* [17,31], *D. affinis* ssp. *cambrensis* var. *paleaceo-crispa* [17,31], *D. affinis* ssp. *persica* [17], *D. affinis* ssp. *pontica* [17], *D. affinis* ssp. *pseudo-disjuncta* [17], *D. aitoniana* [47,50], *D. ardechensis* [31], *D. arguta* [20], ***D. athamantica*** [30,47], *D. assimilis* [42,58], ***D. atrata*** [12], ***D. austriaca*** [12,40], ***D. azorica*** [43], ***D. barbigera*** [20], ***D. blanfordii*** [20], *D. bissetiana* [40], *D. borreri* var. *disjuncta* [47], *D. borreri* var. *pseudo-disjuncta* [47], ***D. callolepis*** [30], ***D. campyloptera*** [42], ***D. caucasica*** Fraser-Jenk. & Corley [17], ***D. celsa*** [18], ***D. championii*** [12,40], ***D. chinensis*** [12,16,40], ***D. cochleata*** [14], *D. corleyi* [31], ***D. crassirhizoma*** [12,40], *D. crispifolia* Rasbach, Reichst., & G. Vida [43], ***D. cristata*** [18,42], ***D. decipiens*** [12], ***D. dickinsii*** [12,40], ***D. dilatata*** [30,43], ***D. erythrosora*** [40], ***D. filix-mas*** [17,18,42,47,50,52], *D. filix-mas* var. *rigidiformis* [47], ***D. formosana*** (H. Christ) C. Chr. [12], ***D. fragrans*** [20], ***D. fuscipes*** [12], *D. fusco-atra* [44], *D. fusco-atra* var. *lamoureuxii* [17], ***D. gymnophylla*** [12], *D. hayatae* [16], *D. hirtipes* ssp. *atrata* [39], ***D. hondoensis*** [12], ***D. inaequalis*** [30], ***D. intermedia*** [42], ***D. juxtaposita*** [20], ***D. kinkiensis*** [12], *D. khullarii* [17], ***D. lacera***

		[12,40], ***D. lepidopoda*** [17], ***D. ludoviciana*** [18], *D. maderensis* [43], ***D. manniana*** [30], ***D. marginalis*** [42], ***D. marginata*** [20], ***D. monticola*** (Makino) C. Chr. [12], ***D. neorosthornii*** [17], ***D. nigropaleacea*** [20], *D. odontoloma* [20], *D. oligodonta* (Desv.) Pic. Serm. [30], ***D. oreades*** [17,18,31], *D. pallida* ssp. *pallida* [20,47], *D. pallida* ssp. *balearica* [20], *D. pallida* ssp. *raddeana* [20], *D. para-chrysocoma* [20], *D. parallelogramma* [45], *D. parrisiae* [17], ***D. patula*** [45], ***D. pentheri*** [30], ***D. polylepis*** [12,40], ***D. pseudo-filix-mas*** [17], ***D. pulvinulifera*** [14], ***D. ramosa*** [20], ***D. redactopinnata*** [17], *D. schimperana* [30], ***D. remota*** [48], ***D. sacrosancta*** [12,40], *D. sieboldii* (van Houtte) Kuntze [12], *D. sledgei* [17], ***D. sordidipes*** [12], ***D. sparsa*** [16], *D. spinulosa* [42,51], ***D. splendens*** [18], ***D. stenolepis*** [39], *D. stewartii* [20], ***D. subbipinnata*** [17], *D. submontana* [20], ***D. tokyoensis*** [12], *D. tyrrhena* [31], ***D. uniformis*** [12], ***D. varia*** [12], *D. villarii* ssp. *villarii* [20,47], ***D. wallichiana*** ssp. ***coriacea*** [17], ***D. pallida*** ssp. ***libanotica*** [20], ***D. wallichiana*** [12], ***D. wallichiana*** ssp. ***madrasensis*** [17], *D. wallichiana* ssp. *reichsteinii* [17], ***D. wallichiana*** ssp. ***wallichiana*** [17] ***D. woodsiisora*** [20], *D. xanthomelas* [17], ***D. yigongensis*** [17], *D. zayuensis* [17], *Nothoperanema squamiseta* [19], ***Peranema cyatheoides*** [19], ***Polybotrya caudata*** [13,19], ***Polystichum rigens*** [14], ***P. tsus-simense*** [14,16], ***Stigmatopteris longicaudata*** [19]
36	Flavaspidic acid AP	*Dryopteris ardechensis* [31], ***D. goldiana*** [18], ***D. lacera*** [40]
36/37	Flavaspidic acid AP/PA	***Dryopteris subimpressa*** Loyal [59]
38	Flavaspidic acid BP	***Dryopteris celsa*** [18]
30	Flavaspidic acid PB	*Dryopteris abbreviata* [47,55], *D. affinis* ssp. *affinis* var. *affinis* [17], *D. affinis* ssp. *borreri* [17], *D. ardechensis* [31], *D. arguta* [20], ***D. blanfordii*** [20], *D. borreri* var. *disjuncta* [47], *D. borreri* var. *pseudo-disjuncta* [47], ***D. celsa*** [18], ***D. crassirhizoma*** [54,56,57], ***D. cristata*** [18], ***D. filix-mas*** [18], ***D. lacera*** [40], ***D. manniana*** [30], *D. odontoloma* [20], ***D. polylepis*** [40], ***D. ramosa*** [20], ***D. splendens*** [18], *D. stewartii* [20], *D. xanthomelas* [17]

Continued

TABLE 1 Distribution of Phloroglucinol Derivatives in Ferns—Cont'd

#	Compounds	Species in Which the Phloroglucinol Derivative Was Detected
40	Flavaspidic acid PP	***Dryopteris celsa*** [18], ***D. chinensis*** [40], ***D. cristata*** [18], *D. fusco-atra* [44], ***D. polylepis*** [40], ***D. subimpressa*** [59]
41	Flavaspidic acid VV	*Dryopteris schimperana* [30]
42	Flavaspidic acid VB	*Dryopteris schimperana* [30]
43	Flavaspidic acid BV	*Dryopteris schimperana* [30]
44	Aspidin AA	*Arachniodes cavalerii* [14], *A. mutica* [16], ***Dryopteris gymnosora*** (Makino) C. Chr. [19], ***D. subimpressa*** [59]
45	Aspidin AB	***Arachniodes amabilis*** [16], ***A. aristata*** [14,15], *A. cavalerii* [14], *A. dimorphophylla* [16], ***A. exilis*** [16], ***A. japonica*** [15], ***A. nipponica*** [16], *A. rhomboidea* [19], ***A. simplicior*** [16], *A. simplicior* var. *major* [16], *A. yasu-inouei* [14], *D. aemula* [43], *D. assimilis* [58], ***D. azorica*** [43], ***D. callolepis*** [30], ***D. championii*** [40], *D. corleyi* [31], *D. crispifolia* [43], ***D. dilatata*** [30,43], ***D. erythrosora*** [40], ***D. fragrans*** [20], *D. guanchica* Gibby & Jermy [18], ***D. intermedia*** [42], *D. intermedia* ssp. *intermedia* [18], *D. maderensis* [43], ***D. pallida*** ssp. ***libanotica*** [20], *D. pallida* ssp. *raddeana* [20], ***D. patula*** [45], ***D. pulvinulifera*** [14,27], ***D. remota*** [48], *D. spinulosa* [42,51], ***D. subimpressa*** [59], ***Polystichum tsus-simense*** [16]
46	Aspidin BB	*Acrophorus nodosus* C. Presl (=***Peranema nodosa*** (C. Presl) Fraser-Jenk.) [14], ***Arachniodes amabilis*** [14], ***A. aristata*** [14,15], *A. cantilenae* [15], *A. cavalerii* [14], *A. dimorphophylla* [16], ***A. exilis*** [16], ***A. hekiana*** [15], ***A. japonica*** [15], *A. miqueliana* [16], *A. mutica* [16], ***A. nipponica*** [16], *A. rhomboidea* [19], ***A. simplicior*** [16], *A. simplicior* var. *major* [16], ***A. tripinnata*** [14,15], *A. yasu-inouei* [14], *Dryopteris aemula* [43], *D. aitoniana* [47], *D. ardechensis* [31], *D. assimilis* [42,58], ***D. azorica*** [43], ***D.***

		callolepis [30], ***D. campyloptera*** [42], ***D. championii*** [12,40], *D. crispifolia* [43], *D. corleyi* [31], ***D. crassirhizoma*** [54], ***D. decipiens*** [12], ***D. dilatata*** [30,43], ***D. erythrosora*** [12,40], ***D. formosana*** [12], ***D. fragrans*** [20], ***D. fuscipes*** [12], *D. guanchica* [18], ***D. gymnosora*** [12], ***D. hondoensis*** [12], ***D. indusiata*** [12], ***D. intermedia*** [42], *D. intermedia* ssp. *intermedia* [18], *D. intermedia* ssp. *maderensis* (Alston) Fraser-Jenk. [18], ***D. kinkiensis*** [12], *D. maderensis* [43], *D. nipponensis* [12], *D. pallida* ssp. *balearica* [20,47], ***D. pallida*** ssp. ***libanotica*** [20], *D. pallida* ssp. *pallida* [20], *D. pallida* ssp. *raddeana* [20], ***D. patula*** [45], ***D. pulvinulifera*** [14,27], ***D. remota*** [48], ***D. sparsa*** [16], *D. spinulosa* [42,51], *D. submontana* [20], *D. unidentata* (Hook. & Arn.) C. Chr. var. *paleacea* (Hillebr.) Fraser-Jenk. [18], ***Polybotrya caudata*** [13], ***Polystichum tsus-simense*** [16]
47/48	Aspidin AP/PA	***Dryopteris subimpressa*** [59]
49	Aspidin PB	*Acrophorus nodosus* [14], ***Arachniodes amabilis*** [14], *A. cavalerii* [14], *A. dimorphophylla* [16], ***A. exilis*** [16], *A. miqueliana* [16], *A. mutica* [16], ***A. nipponica*** [16], ***A. simplicior*** [16], *A. simplicior* var. *major* [16], ***A. tripinnata*** [14], *A. yasu-inouei* [14], ***Dryopteris championii*** [40], *D. corleyi* [31], *D. guanchica* [18], ***D. sparsa*** [16], *D. spinulosa* [51], ***D. pulvinulifera*** [14], ***Polystichum tsus-simense*** [16]
49/50	Aspidin PB/BP	***Dryopteris subimpressa*** [59]
51	Aspidin PP	***Dryopteris subimpressa*** [59]
52	Aspidin VB	***Dryopteris erythrosora*** [40]
53/54	Aspidin iBiB/iBB	***Dryopteris erythrosora*** [19]
55	Para-aspidin AA	*Dryopteris arguta* [49]
56	Para-aspidin AB	*Arachniodes maximowiczii* [16], ***A. tripinnata*** [14,15], ***Dryopteris acutodentata*** [17], *D. aemula* [43], *D. affinis* ssp. *borreri* [31], *D. affinis* ssp. *pontica* [17], ***D. blanfordii*** [20], ***D. celsa*** [18], *D. corleyi* [31], *D. crispifolia* [43], ***D. hawaiiensis*** [44], ***D. ramosa*** [20], *D. stewartii* [20], ***D. wallichiana*** ssp. ***wallichiana*** [17]

Continued

TABLE 1 Distribution of Phloroglucinol Derivatives in Ferns—Cont'd

#	Compounds	Species in Which the Phloroglucinol Derivative Was Detected
57	Para-aspidin BB	***Arachniodes amabilis*** [14], *A. maximowiczii* [16,19], *A. okinawensis* [16], ***A. tripinnata*** [14], ***Dryopsis apiciflora*** [19], ***D. clarkei*** [19], ***D. nidus*** [19], ***Dryopteris acutodentata*** [17], *D. aemula* [43], *D. affinis* ssp. *borreri* [17,31], *D. affinis* ssp. *pontica* [17], *D. aitoniana* [47,50], *D. arguta* [20], *D. assimilis* [42,58], ***D. athamantica*** [30,47], ***D. austriaca*** [40,60], ***D. barbigera*** [20], ***D. blanfordii*** [20], *D. borreri* var. *pseudo-disjuncta* [47], ***D. callolepis*** [30], ***D. campyloptera*** [42], ***D. carthusiana*** [18], ***D. caucasica*** [17,18,22], ***D. cochleata*** [14], *D. corleyi* [31], *D. crispifolia* [43], ***D. cristata*** [18,42], ***D. dilatata*** [43], ***D. filix-mas*** [17,18,42,47,50,52], *D. filix-mas* var. *rigidiformis* [47], ***D. fragrans*** [20], ***D. hawaiiensis*** [44], ***D. inaequalis*** [30], ***D. ludoviciana*** [18], ***D. marginalis*** [42], *D. oligodonta* [30], ***D. pallida*** ssp. ***libanotica*** [20], *D. pallida* ssp. *pallida* [20,47], *D. pallida* ssp. *raddeana* [20], ***D. panda*** (C. B. Clarke) H. Christ [18], ***D. ramosa*** [20], ***D. remota*** [48], ***D. sparsa*** [19], *D. spinulosa* [42], *D. stewartii* [20], *D. submontana* [20], *D. tyrrhena* [31], *D. villarii* ssp. *villarii* [20,47], ***D. wallichiana*** ssp. ***wallichiana*** [17], ***Polystichum rigens*** [14]
58	Para-aspidin BP	***Dryopteris athamantica*** [30], ***D. caucasica*** [18], ***D. celsa*** [18], ***D. inaequalis*** [30]
59	Para-aspidin PB	*Arachniodes maximowiczii* [16], *A. okinawensis* [16], ***A. tripinnata*** [14], ***Dryopteris athamantica*** [30], ***D. caucasica*** [18], ***D. celsa*** [18], ***D. cochleata*** [14], ***D. cristata*** [18,42], ***D. filix-mas*** [18,42], ***D. inaequalis*** [30], ***D. marginalis*** [42], *D. pallida* ssp. *pallida* [20], ***Polystichum rigens*** [14]
60	Para-aspidin PP	***Dryopteris caucasica*** [18], ***D. celsa*** [18], ***D. cristata*** [18], ***D. inaequalis*** [30]
61/62	Para-aspidin AP/PA	***Dryopteris hawaiiensis*** [44]

63	Iso-aspidin AB	*Arachniodes dimorphophylla* [16], ***A. nipponica*** [16]
64	Iso-aspidin BB	*Arachniodes cantilenae* [15], *A. dimorphophylla* [16], ***A. hekiana*** [15], ***A. nipponica*** [16]
65	Iso-aspidin PB	*Arachniodes dimorphophylla* [16], ***A. nipponica*** [16]
66	Desaspidin AB	***Arachniodes aristata*** [14], *A. dimorphophylla* [16], *A. miqueliana* [16], *Dryopteris arguta* [49], ***D. cochleata*** [14], ***D. cristata*** [42], ***D. intermedia*** [42], ***D. patula*** [45], *D. spinulosa* [51]
66/67	Desaspidin AB/BA	***Dryopteris subimpressa*** [59]
68	Desaspidin BB	***Arachniodes amabilis*** [14,16]**,** ***A. aristata*** [14], ***A. assamica*** [14,19], *A. dimorphophylla* [16], ***A. japonica*** [15], *A. miqueliana* [16,19], *A. rhomboidea* [19], ***A. simplicior*** [16], *A. simplicior* var. *major* [16], ***A. tripinnata*** [15], ***Dryopsis clarkei*** [19], *Dryopteris aemula* [43], *D. assimilis* [42,58], ***D. austriaca*** [12,40], *D. borreri* var. *pseudo-disjuncta* [47], ***D. callolepis*** [30], ***D. caucasica*** [17,18,22], ***D. championii*** [12,40], ***D. clintoniana*** [18], ***D. cochleata*** [14], *D. crispifolia* [43], ***D. cristata*** [18,42], ***D. dilatata*** [30,43], ***D. filix-mas*** [17,18,42,47,50,52], ***D. fragrans*** [20], ***D. gymnophylla*** [12], ***D. intermedia*** [42], ***D. kinkiensis*** [12], ***D. manniana*** [30], ***D. marginalis*** [18,42], *D. pallida* ssp. *balearica* [20,47], ***D. pallida*** ssp. ***libanotica*** [20], *D. pallida* ssp. *pallida* [20,47], ***D. patula*** [45], ***D. remota*** [48], *D. spinulosa* [42,51], ***D. subimpressa*** [59], *D. submontana* [20], ***D. tokyoensis*** [12,18], *D. villarii* ssp. *villarii* [20,47], ***Polybotrya caudata*** [13], ***Polystichum tsus-simense*** [14,16]
69	Desaspidin PB	***A. amabilis*** [14,16]**,** ***A. aristata*** [14], ***A. assamica*** [14], *A. dimorphophylla* [16], *A. miqueliana* [16], ***A. simplicior*** [16], *A. simplicior* var. *major* [16], *D. arguta* [49], ***D. caucasica*** [18], ***D. clintoniana*** [18], ***D. cochleata*** [14], ***D. cristata*** [18,42], ***D. filix-mas*** [18,42], ***D. marginalis*** [42], *D. spinulosa* [42,51], ***P. tsus-simense*** [14,16]
69/70	Desaspidin PB/BP	***Dryopteris subimpressa*** [59]
70	Desaspidin BP	***Dryopteris caucasica*** [18]

Continued

TABLE 1 Distribution of Phloroglucinol Derivatives in Ferns—Cont'd

#	Compounds	Species in Which the Phloroglucinol Derivative Was Detected
71	Desaspidin PP	*Dryopteris arguta* [49], ***D. cristata*** [18], ***D. subimpressa*** [59]
72	Desaspidin AP	*Dryopteris arguta* [49]
72/73	Desaspidin AP/PA	***Dryopteris subimpressa*** [59]
74	Desaspidin VV	*Arachniodes rhomboidea* [16]
75/76	Desaspidin VB/BV	*Arachniodes rhomboidea* [16]
77	Ortho-desaspidin BB	***Dryopteris austriaca*** [61], ***D. dilatata*** [30]
78	Phloraspin BB	*Ctenitis apiciflora* (Wall. ex Mett.) Ching (=***Dryopsis apiciflora***) [21], *C. crinita* (Poir.) Ching var. *hispida* (Kuhn) Tardieu (=***C. crinita*** (Poir.) Tardieu var. ***hispida*** (Kuhn) Tardieu) [19], ***C. setosa*** [19], ***C. subglandulosa*** (Hance) Ching [19], ***Dryopsis apiciflora*** [19], ***D. clarkei*** [19], ***Dryopteris athamantica*** [47], *D. corleyi* [31], *D. filix-mas* var. *rigidiformis* [47], ***D. inaequalis*** [30], ***D. marginalis*** [18,42]
79/80	Phloraspin VB/BV	*Ctenitis apiciflora* [21], ***Dryopsis apiciflora*** [19], ***D. clarkei*** [19]
81	Phloraspin VV	***Dryopsis clarkei*** [19]
82/83	Phloraspin PB/BP	***Dryopsis apiciflora*** [19], ***D. clarkei*** [19]
84	Phloraspin PP	***Dryopsis clarkei*** [19]
85	Margaspidin AA	***Dryopteris hawaiiensis*** [44]
86	Margaspidin AB	***Dryopteris hawaiiensis*** [44]

87	Margaspidin BB	*Ctenitis crinita* var. *hispida* [19], ***Dryopsis apiciflora*** [19], ***D. clarkei*** [19], ***D. nidus*** [19], *Dryopteris aemula* [21,43], *D. bissetiana* [40,62], *D. corleyi* [31], ***D. fragrans*** [20], ***D. hawaiiensis*** [44], ***D. pacifica*** (Nakai) Tagawa [62], ***D. inaequalis*** [30], ***D. marginalis*** [18,42], ***D. panda*** [18], ***D. sacrosancta*** [40,62], ***D. saxifraga*** H. Itô [62]
88/89	Margaspidin BP/PB	***Dryopsis apiciflora*** [19], ***D. clarkei*** [19], ***D. nidus*** [19], *Dryopteris bissetiana* [40], ***D. hawaiiensis*** [44], ***D. panda*** [18], ***D. sacrosancta*** [40]
90/91	Margaspidin AP/PA	***Dryopteris hawaiiensis*** [44]
92	Margaspidin PP	***Dryopsis apiciflora*** [19], ***D. clarkei*** [19], ***D. nidus*** [19], *Dryopteris bissetiana* [40], ***D. sacrosancta*** [40]
93/94	Margaspidin VB/BV	***Dryopsis apiciflora*** [19], ***D. clarkei*** [19], ***D. nidus*** [19], *Dryopteris bissetiana* [40], ***D. sacrosancta*** [40]
95	Margaspidin VV	***Dryopsis apiciflora*** [19], ***D. nidus*** [19]
96	Aemulin BB	*Dryopteris aemula* [21,43]
97	Phloraspidinol BB	*Ctenitis crinita* var. *hispida* [19], ***Dryopsis apiciflora*** [19], ***D. clarkei*** [19], ***D. nidus*** [19], *Dryopteris aemula* [43], ***D. athamantica*** [47], ***D. austriaca*** [63], ***D. hawaiiensis*** [44], ***D. inaequalis*** [30], ***D. marginalis*** [42], ***D. sparsa*** [19]
98/99	Phloraspidinol VB/BV	***Dryopsis clarkei*** [19]
100	Methylene-bis-aspidinol BB	***Ctenitis setosa*** [19], ***C. subglandulosa*** [19], ***Dryopsis apiciflora*** [19], ***D. clarkei*** [19], ***D. nidus*** [19], *Dryopteris aemula* [43], *D. bissetiana* [40], *D. crispifolia* [43], ***D. inaequalis*** [30], ***D. marginalis*** [18,42], ***D. panda*** [18], ***D. sacrosancta*** [40]
101	Methylene-bis-aspidinol PB	***Dryopteris inaequalis*** [30], ***D. sacrosancta*** [40]
102	Methylene-bis-aspidinol PP	***Dryopteris inaequalis*** [30], ***D. sacrosancta*** [40]
103	Methylene-bis-aspidinol VB	***Dryopsis nidus*** [19]

Continued

TABLE 1 Distribution of Phloroglucinol Derivatives in Ferns—Cont'd

#	Compounds	Species in Which the Phloroglucinol Derivative Was Detected
104	Methylene-bis-aspidinol VV	***Dryopsis nidus*** [19]
105	Methylene-bis-desaspidinol BB	***Dryopteris austriaca*** [63], ***D. inaequalis*** [30], ***D. marginalis*** [42]
106	Phloropyrone BB	*Ctenitis apiciflora* [21], *C. nidus* (C. B. Clarke) Ching (=***Dryopsis nidus***) [21], ***C. setosa*** [19], ***C. subglandulosa*** [19], *Diacalpe aspidioides* [19], ***Dryopsis apiciflora*** [19], ***D. clarkei*** [19], ***D. nidus*** [19], *Dryopteris aemula* [43], *D. assimilis* [42,58], ***D. callolepis*** [30], ***D. campyloptera*** [42], *D. crispifolia* [43], ***D. dilatata*** [30,43], *D. spinulosa* [42], ***Polystichum rigens*** [14], ***Rumohra adiantiformis*** (G. Forst.) Ching [19]
107/ 108	Phloropyrone PB/BP	***Ctenitis apiciflora*** [21], ***C. nidus*** [21], ***Dryopsis nidus*** [19]
109/ 110	Phloropyrone VB/BV	***Dryopsis nidus*** [19]
111	Phloropyrone PP	***Dryopsis nidus*** [19]
112	Phloraspyrone BB	***Dryopsis clarkei*** [19], ***D. nidus*** [19], ***Dryopteris austriaca*** [63],
113	Abbreviatin AB	*Dryopteris pseudosikkimensis* Ching & S. K. Wu (=***D. sikkimensis*** (Bedd.) Kuntze) [18]
114	Abbreviatin BB = methylene-bis-methylphlorobutyrophenone	*Dryopteris abbreviata* [21,55], ***D. crassirhizoma*** [54], ***D. juxtaposita*** [20], ***D. marginata*** [20], *D. para-chrysocoma* [20], *D. pseudosikkimensis* [18], ***D. ramosa*** [20], ***D. stenolepis*** [39], *D. stewartii* [20], ***D. woodsiisora*** [20]
115	Abbreviatin PB	*Dryopteris abbreviata* [21,55]
116	Abbreviatin PP	*Dryopteris pseudosikkimensis* [18]

117	Araspidin BB	***Arachniodes exilis*** [28]
118	Pulvinuliferin VV	***Dryopteris pulvinulifera*** [27]
119	Filixic acid AAA	***Dryopteris stenolepis*** [39]
120	Filixic acid ABA	***Dryopteris acutodentata*** [17], *D. affinis* ssp. *affinis* var. *affinis* [17,31], *D. affinis* ssp. *affinis* var. *jessenii* [17], *D. affinis* ssp. *affinis* var. *paleaceo-lobata* [17], *D. affinis* ssp. *affinis* var. *splendens* [17], *D. affinis* ssp. *borreri* [17], *D. affinis* ssp. *cambrensis* var. *insubrica* [17,31], *D. affinis* ssp. *cambrensis* var. *paleaceo-crispa* [17,31], *D. affinis* ssp. *persica* [17], *D. affinis* ssp. *pontica* [17], *D. affinis* ssp. *pseudo-disjuncta* [17], *D. ardechensis* [31], *D. arguta* [20], ***D. barbigera*** [20], ***D. blanfordii*** [20], *D. borreri* var. *disjuncta* [47], *D. borreri* var. *pseudo-disjuncta* [47], ***D. conjugata*** [17], ***D. crassirhizoma*** [17,41,64], ***D. dickinsii*** [39,40], ***D. filix-mas*** [18], *D. filix-mas* var. *rigidiformis* [47], *D. fusco-atra* [44], *D. fusco-atra* var. *lamoureuxii* [17], *D. gamblei* (C. Hope) C. Chr. (=***D. stenolepis***) [18], *D. hirtipes* ssp. *atrata* [17], *D. hirtipes* ssp. *hirtipes* [17], ***D. juxtaposita*** [20], *D. khullarii* [17], ***D. lepidopoda*** [17], ***D. manniana*** [30], ***D. neorosthornii*** [17], ***D. nigropaleacea*** [20], *D. odontoloma* [20], ***D. oreades*** [17,18,31], *D. para-chrysocoma* [20], *D. parallelogramma* [45], *D. parrisiae* [17], ***D. polylepis*** [17], ***D. pseudo-filix-mas*** [17], ***D. ramosa*** [20], ***D. redactopinnata*** [17], *D. sledgei* [17], *D. stewartii* [20], *D. tyrrhena* [31], *D. wallichiana* [46], ***D. wallichiana*** ssp. ***coriacea*** [17], ***D. wallichiana*** ssp. ***madrasensis*** [17], *D. wallichiana* ssp. *reichsteinii* [17], ***D. wallichiana*** ssp. ***wallichiana*** [17], ***D. woodsiisora*** [20], *D. xanthomelas* [17], ***D. yigongensis*** [17], *D. zayuensis* [17], ***Peranema cyatheoides*** [19]
121	Filixic acid ABB	***Dryopteris acutodentata*** [17], *D. affinis* ssp. *affinis* var. *affinis* [17,31], *D. affinis* ssp. *affinis* var. *jessenii* [17], *D. affinis* ssp. *affinis* var. *paleaceo-lobata* [17], *D. affinis* ssp. *affinis* var. *splendens* [17], *D. affinis* ssp. *borreri* [17], *D. affinis* ssp. *cambrensis* var. *insubrica* [17,31], *D. affinis* ssp. *cambrensis* var. *paleaceo-crispa* [17,31], *D. affinis* ssp. *persica* [17], *D. affinis* ssp. *pontica* [17], *D. affinis* ssp. *pseudo-disjuncta* [17], *D. ardechensis* [31], *D. arguta* [20], ***D. barbigera*** [20], ***D. blanfordii*** [20], *D. borreri* var. *disjuncta* [47], *D. borreri* var. *pseudo-disjuncta* [47], ***D. caucasica*** [18], ***D. conjugata***

Continued

TABLE 1 Distribution of Phloroglucinol Derivatives in Ferns—Cont'd

#	Compounds	Species in Which the Phloroglucinol Derivative Was Detected
		[17], ***D. dickinsii*** [39,40], ***D. filix-mas*** [18], *D. filix-mas* var. *rigidiformis* [47], *D. fusco-atra* [44], *D. fusco-atra* var. *lamoureuxii* [17], *D. gamblei* [18], *D. hirtipes* ssp. *atrata* [17], *D. hirtipes* ssp. *hirtipes* [17], ***D. juxtaposita*** [20], *D. khullarii* [17], ***D. lacera*** [40], ***D. lepidopoda*** [17], ***D. manniana*** [30], ***D. marginata*** [20], ***D. neorosthornii*** [17], ***D. nigropaleacea*** [20], *D. odontoloma* [20], ***D. oreades*** [17,18,31], *D. pallida* ssp. *pallida* [20], ***D. panda*** [18], *D. para-chrysocoma* [20], *D. parallelogramma* [45], *D. parrisiae* [17], ***D. polylepis*** [17], ***D. pseudo-filix-mas*** [17], ***D. ramosa*** [20], ***D. redactopinnata*** [17], ***D. sacrosancta*** [40], ***D. serratodentata*** (Bedd.) Hayata [18], *D. sledgei* [17], ***D. sparsa*** [19], *D. stewartii* [20], *D. submontana* [20], *D. tyrrhena* [31], *D. villarii* ssp. *villarii* [20], *D. wallichiana* [46], ***D. wallichiana*** ssp. ***coriacea*** [17], ***D. wallichiana*** ssp. ***madrasensis*** [17], *D. wallichiana* ssp. *reichsteinii* [17], ***D. wallichiana*** ssp. ***wallichiana*** [17], ***D. woodsiisora*** [20], *D. xanthomelas* [17], ***D. yigongensis*** [17], *D. zayuensis* [17], *Nothoperanema squamiseta* [19], ***Peranema cyatheoides*** [19]
122	Filixic acid BBB	*Dryopteris abbreviata* [47,55], ***D. acutodentata*** [17], *D. affinis* ssp. *affinis* var. *affinis* [17,31], *D. affinis* ssp. *affinis* var. *jessenii* [17], *D. affinis* ssp. *affinis* var. *paleaceo-lobata* [17], *D. affinis* ssp. *affinis* var. *splendens* [17], *D. affinis* ssp. *borreri* [17], *D. affinis* ssp. *cambrensis* var. *insubrica* [17,31], *D. affinis* ssp. *cambrensis* var. *paleaceo-crispa* [17,31], *D. affinis* ssp. *persica* [17], *D. affinis* ssp. *pontica* [17], *D. affinis* ssp. *pseudo-disjuncta* [17], *D. ardechensis* [31], *D. arguta* [20], ***D. atrata*** [12], ***D. austriaca*** [65], ***D. barbigera*** [20], *D. bissetiana* [40], ***D. blanfordii*** [20], *D. borreri* var. *disjuncta* [47], *D. borreri* var. *pseudo-disjuncta* [47], ***D. caucasica*** [17,18,22], ***D. conjugata*** [17], ***D. crassirhizoma*** [12], ***D. dickinsii*** [12,39,66], ***D. filix-mas*** [17,18,42,47,50,52], *D. filix-mas* var. *rigidiformis* [47], *D. fusco-atra* [44], *D. fusco-atra* var. *lamoureuxii* [17], *D. gamblei* [18], *D. hirtipes* ssp. *atrata* [17,39], *D. hirtipes* ssp. *hirtipes* [18], ***D. inaequalis*** [30], ***D. juxtaposita*** [20], *D. khullarii* [17], ***D. lacera*** [12,40], ***D. lepidopoda*** [17], ***D.***

		manniana [30], ***D. marginata*** [20], ***D. monticola*** [12], ***D. neorosthornii*** [17], ***D. nigropaleacea*** [20], *D. odontoloma* [20], *D. oligodonta* [30], ***D. oreades*** [17,18,31], *D. pallida* ssp. *pallida* [20], *D. pallida* ssp. *raddeana* [20], ***D. panda*** [18], *D. parrisiae* [17], *D. para-chrysocoma* [20], ***D. polylepis*** [12,40,53], ***D. pseudo-filix-mas*** [17], ***D. ramosa*** [20], ***D. sacrosancta*** [40], *D. sieboldii* [12], *D. sledgei* [17], ***D. sparsa*** [19], ***D. stenolepis*** [39], *D. stewartii* [20], *D. submontana* [20], ***D. tokyoensis*** [12], *D. tyrrhena* [31], ***D. uniformis*** [12], *D. villarii* ssp. *villarii* [20,47], ***D. wallichiana*** [12], ***D. wallichiana*** ssp. ***madrasensis*** [17], ***D. wallichiana*** ssp. ***wallichiana*** [17], ***D. woodsiisora*** [20], *D. xanthomelas* [17], ***D. yigongensis*** [17], *D. zayuensis* [17], *Nothoperanema squamiseta* [19], ***Peranema cyatheoides*** [19]
123	Filixic acid ABP	***Dryopteris acutodentata*** [17], *D. affinis* ssp. *affinis* var. *affinis* [17], *D. affinis* ssp. *borreri* [17], *D. arguta* [20], ***D. blanfordii*** [20], *D. borreri* var. *disjuncta* [47], *D. borreri* var. *pseudo-disjuncta* [47], ***D. crassirhizoma*** [54], ***D. filix-mas*** [18], *D. filix-mas* var. *rigidiformis* [47], *D. fusco-atra* var. *fusco-atra* [18,22], ***D. juxtaposita*** [20], ***D. lacera*** [40], ***D. manniana*** [30], ***D. oreades*** [18], ***D. panda*** [18], ***D. sacrosancta*** [40], ***D. serratodentata*** [18], *D. sledgei* [17], *D. stewartii* [20]
124	Filixic acid APA	***Dryopteris stenolepis*** [39]
125	Filixic acid PBB	*Dryopteris abbreviata* [47], ***D. acutodentata*** [17], *D. affinis* ssp. *affinis* var. *affinis* [17], *D. affinis* ssp. *borreri* [17], *D. arguta* [20], ***D. barbigera*** [20], ***D. blanfordii*** [20], *D. borreri* var. *disjuncta* [47], *D. borreri* var. *pseudo-disjuncta* [47], ***D. caucasica*** [18], ***D. filix-mas*** [18], *D. filix-mas* var. *rigidiformis* [47], *D. fusco-atra* [44], ***D. inaequalis*** [30], ***D. juxtaposita*** [20], ***D. lacera*** [40], ***D. manniana*** [30], ***D. marginata*** [20], *D. odontoloma* [20], *D. oligodonta* [30], ***D. oreades*** [18,47], *D. para-chrysocoma* [20], ***D. ramosa*** [20], ***D. sacrosancta*** [40], ***D. serratodentata*** [18], *D. sledgei* [17], *D. stewartii* [20]
126	Filixic acid PBP	*Dryopteris abbreviata* [47], ***D. acutodentata*** [17], *D. affinis* ssp. *affinis* var. *affinis* [17], *D. affinis* ssp. *borreri* [17], *D. arguta* [20], ***D. barbigera*** [20], ***D. blanfordii*** [20], *D. borreri* var. *disjuncta* [47], *D. borreri* var. *pseudo-disjuncta* [47], ***D. caucasica*** [18], ***D. filix-mas*** [18], *D. filix-mas* var. *rigidiformis* [47], *D. fusco-atra* [44], ***D. inaequalis*** [30],

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TABLE 1 Distribution of Phloroglucinol Derivatives in Ferns—Cont'd

#	Compounds	Species in Which the Phloroglucinol Derivative Was Detected
		D. juxtaposita [20], ***D. lacera*** [40], ***D. manniana*** [30], ***D. marginata*** [20], *D. odontoloma* [20], *D. oligodonta* [30], ***D. oreades*** [18,47], ***D. panda*** [18], *D. para-chrysocoma* [20], ***D. ramosa*** [20], ***D. sacrosancta*** [40], ***D. serratodentata*** [18], *D. sledgei* [17], *D. stewartii* [20], ***D. wallichiana*** ssp. ***madrasensis*** [17], *D. xanthomelas* [17]
127	Filixic acid VBB	*Dryopteris schimperana* [30]
128	Filixic acid VBV	*Dryopteris schimperana* [30]
129	Filixic acid VVV	*Dryopteris schimperana* [30]
130	Trisaspidinol BBB	*Dryopteris bissetiana* [40], ***D. inaequalis*** [21,30], ***D. sacrosancta*** [40]
131	Trisaspidinol PBB	***Dryopteris inaequalis*** [30], ***D. sacrosancta*** [40]
132	Trisaspidinol PBP	***Dryopteris inaequalis*** [30], ***D. sacrosancta*** [40]
133	Trisaspidinol VBB	***Dryopteris sacrosancta*** [40]
134	Trisaspidin BBB	***Dryopteris austriaca*** [21,65]
135	Trispara-aspidin ABB	***Dryopteris remota*** [48]
136	Trispara-aspidin BBB	***Dryopsis apiciflora*** [19], ***Dryopteris acutodentata*** [17], *D. affinis* ssp. *borreri* [31], *D. aitoniana* [47,50], ***D. athamantica*** [47], ***D. barbigera*** [20], ***D. filix-mas*** [17,52], *D. filix-mas* var. *rigidiformis* [47], *D. oligodonta* [30], ***D. pallida*** ssp. ***libanotica*** [20], *D. pallida* ssp. *pallida* [20,47], *D. pallida* ssp. *raddeana* [20], ***D. remota*** [48], ***D. sacrosancta*** [19], *D. stewartii* [20], *D. submontana* [20], *D. tyrrhena* [31], ***D. wallichiana*** ssp. ***wallichiana*** [17]

137/ 138	Trispara-aspidin VBB/BBV	***Dryopsis apiciflora*** [19]
139/ 140	Trispara-aspidin VVB/BVV	***Dryopsis apiciflora*** [19]
141	Trispara-aspidin PBB	*Dryopteris pallida* ssp. *raddeana* [20]
141/ 142	Trispara-aspidin PBB/BBP	***Dryopsis apiciflora*** [19]
143/ 144	Trispara-aspidin PPB/BPP	***Dryopsis apiciflora*** [19]
145	Trispara-aspidin PBP	*Dryopteris pallida* ssp. *raddeana* [20]
146/ 147	Trisdesaspidin ABB/PBP	***Dryopteris subimpressa*** [21,59]
148	Trisdesaspidin BBB	***Arachniodes assamica*** [19], ***A. japonica*** [15], *A. miqueliana* [19], ***A. simplicior*** [16], *A. rhomboidea* [16], ***Dryopteris assimilis*** [58], ***D. austriaca*** [21,40,65], ***D. caucasica*** [17,21,22], ***D. championii*** [40], ***D. cristata*** [18,42], ***D. filix-mas*** [17,52], ***D. manniana*** [30], *D. pallida* ssp. *balearica* [20,47], ***D. pallida*** ssp. ***libanotica*** [20], *D. pallida* ssp. *pallida* [20,47], ***D. remota*** [48], *D. spinulosa* [42,51], *D. submontana* [20], ***D. wallichiana*** ssp. ***wallichiana*** [17], ***Polystichum tsus-simense*** [14]
149/ 150	Trisdesaspidin ABP/PBA	***Dryopteris subimpressa*** [59]
151	Trisflavaspidic acid ABB	*Arachniodes rhomboidea* [19], *Dryopteris affinis* ssp. *affinis* var. *affinis* [31], *D. affinis* ssp. *affinis* var. *jessenii* [17], *D. affinis* ssp. *affinis* var. *paleaceo-lobata* [17], *D. affinis* ssp. *affinis* var. *splendens* [17], *D. affinis* ssp. *borreri* [31], *D. affinis* ssp. *cambrensis* var. *insubrica* [17,31], *D. affinis* ssp. *cambrensis* var. *paleaceo-crispa* [17,31], *D. affinis* ssp. *persica* [17], *D. affinis* ssp. *pontica* [17], *D. affinis* ssp. *pseudo-disjuncta* [17], ***D.***

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TABLE 1 Distribution of Phloroglucinol Derivatives in Ferns—Cont'd

#	Compounds	Species in Which the Phloroglucinol Derivative Was Detected
		barbigera [20], ***D. blanfordii*** [20], ***D. crassirhizoma*** [41], ***D. filix-mas*** [17], *D. gamblei* [18], ***D. juxtaposita*** [20], *D. khullarii* [17], ***D. lepidopoda*** [17], ***D. neorosthornii*** [17], ***D. nigropaleacea*** [17,20], *D. odontoloma* [20], ***D. oreades*** [17], *D. parrisiae* [17], ***D. pseudo-filix-mas*** [17], ***D. ramosa*** [20], ***D. redactopinnata*** [17], *D. tyrrhena* [31], ***D. wallichiana*** ssp. ***coriacea*** [17], ***D. wallichiana*** ssp. ***madrasensis*** [17], *D. wallichiana* ssp. *reichsteinii* [17], ***D. wallichiana*** ssp. ***wallichiana*** [17], *D. xanthomelas* [17], ***D. yigongensis*** [17], *D. zayuensis* [17]
152	Trisflavaspidic acid BBB	*Arachniodes cantilenae* [15], ***A. hekiana*** [15], ***A. nipponica*** [16], *A. rhomboidea* [19], ***Ctenitis mannii*** [15], ***Dryopsis apiciflora*** [19], ***Dryopteris acutodentata*** [17], *D. affinis* ssp. *affinis* var. *affinis* [31], *D. affinis* ssp. *affinis* var. *jessenii* [17], *D. affinis* ssp. *borreri* [31], *D. affinis* ssp. *cambrensis* var. *insubrica* [17,31], *D. affinis* ssp. *cambrensis* var. *paleaceo-crispa* [17,31], *D. affinis* ssp. *affinis* var. *paleaceo-lobata* [17], *D. affinis* ssp. *affinis* var. *splendens* [17], *D. affinis* ssp. *persica* [17], *D. affinis* ssp. *pontica* [17], *D. affinis* ssp. *pseudo-disjuncta* [17], *D. aitoniana* [21,47,50], *D. arguta* [20], ***D. athamantica*** [30], ***D. austriaca*** [21,40,65], ***D. barbigera*** [20], ***D. blanfordii*** [20], ***D. championii*** [40], ***D. chinensis*** [16,40], *D. corleyi* [31], ***D. filix-mas*** [17,50,52], *D. filix-mas* var. *rigidiformis* [47], ***D. juxtaposita*** [20], ***D. lepidopoda*** [17], ***D. marginata*** [20], ***D. neorosthornii*** [17], ***D. nigropaleacea*** [20], *D. odontoloma* [20], ***D. oreades*** [17,31], ***D. pallida*** ssp. ***libanotica*** [20], *D. pallida* ssp. *pallida* [20], *D. pallida* ssp. *raddeana* [20], *D. parrisiae* [17], *D. para-chrysocoma* [20], ***D. pentheri*** [30], ***D. ramosa*** [20], ***D. redactopinnata*** [17], ***D. stenolepis*** [39], *D. submontana* [20], *D. tyrrhena* [31], *D. villarii* ssp. *pallida* [47], *D. villari* ssp. *pallida* var. *balearica* [47], ***D. wallichiana*** ssp. ***madrasensis*** [17], *D. wallichiana* ssp. *reichsteinii* [17], ***D. wallichiana*** ssp. ***wallichiana*** [17], ***D. woodsiisora*** [20], *D. xanthomelas* [17], ***D. yigongensis*** [17], *D. zayuensis* [17], ***P conjugata*** [15]

153	Trisflavaspidic acid PBB	***Dryopteris barbigera*** [20,67]
154	Trisaemulin BAB	*Dryopteris aemula* [21,43]
155	Trisaemulin BBB	*Dryopteris aemula* [21,43]
156	Trisabbreviatin BBB	*Dryopteris abbreviata* [21,55]
157	Tetraalbaspidin ABBA=dryocrassin	***Dryopteris acutodentata*** [17], *D. affinis* ssp. *affinis* var. *affinis* [17], *D. affinis* ssp. *affinis* var. *jessenii* [17], *D. affinis* ssp. *affinis* var. *splendens* [17], *D. affinis* ssp. *cambrensis* var. *insubrica* [17], *D. affinis* ssp. *cambrensis* var. *paleaceo-crispa* [17], *D. affinis* ssp. *persica* [17], *D. affinis* ssp. *pontica* [17], ***D. blanfordii*** [20], ***D. chinensis*** [16], ***D. conjugata*** [17], ***D. crassirhizoma*** [17,21,40,41,64], *D. fusco-atra* [44], *D. fusco-atra* var. *lamoureuxii* [17], ***D. juxtaposita*** [20], *D. khullarii* [17], ***D. lacera*** [40], ***D. lepidopoda*** [17], ***D. neorosthornii*** [17], ***D. nigropaleacea*** [20], *Dryopteris parrisiae* [17], ***D. polylepis*** [40,53], ***D. pseudo-filix-mas*** [17], ***D. redactopinnata*** [17], *D. sledgei* [17], *D. stewartii* [20], ***D. wallichiana*** ssp. ***coriacea*** [17], ***D. wallichiana*** ssp. ***madrasensis*** [17], *D. wallichiana* ssp. *reichsteinii* [17], ***D. wallichiana*** ssp. ***wallichiana*** [17], *D. xanthomelas* [17], ***D. yigongensis*** [17], *D. zayuensis* [17]
158	Tetraalbaspidin BBBB	***Dryopteris acutodentata*** [17], *D. affinis* ssp. *affinis* var. *affinis* [17], *D. affinis* ssp. *affinis* var. *splendens* [17], *D. affinis* ssp. *cambrensis* var. *insubrica* [17], *D. affinis* ssp. *pontica* [17], *D. aitoniana* [21,50], ***D. austriaca*** [21,68], ***D. blanfordii*** [20], ***D. chinensis*** [16], ***D. lepidopoda*** [17], ***D. nigropaleacea*** [20], *D. sledgei* [17], ***D. wallichiana*** ssp. ***madrasensis*** [17], *D. wallichiana* ssp. *reichsteinii* [17], ***D. wallichiana*** ssp. ***wallichiana*** [17]
159	Tetraflavaspidic acid ABBB	***Dryopteris lepidopoda*** [17], ***D. wallichiana*** ssp. ***wallichiana*** [17]
160	Tetraflavaspidic acid BBBB	***Dryopteris acutodentata*** [17], *D. aitoniana* [21,50], *D. affinis* ssp. *affinis* var. *affinis* [17], *D. affinis* ssp. *cambrensis* var. *paleace-ocrispa* [17], *D. affinis* ssp. *pontica* [17], ***D. barbigera*** [20], ***D. blanfordii*** [20], ***D. filix-mas*** [17,50,52], ***D. juxtaposita*** [20], ***D. lepidopoda*** [17], ***D. neorosthornii*** [17], ***D. nigropaleacea*** [20], *D. odontoloma* [20], ***D. oreades*** [17], ***D. redactopinnata*** [17], ***D. stenolepis*** [39], ***D. wallichiana*** ssp.

Continued

TABLE 1 Distribution of Phloroglucinol Derivatives in Ferns—Cont'd

#	Compounds	Species in Which the Phloroglucinol Derivative Was Detected
		madrasensis [17], *D. wallichiana* ssp. *reichsteinii* [17], ***D. wallichiana*** ssp. ***wallichiana*** [17], ***D. woodsiisora*** [20], *D. xanthomelas* [17], ***D. yigongensis*** [17], *D. zayuensis* [17]
161	Penta-albaspidin BBBBB	*Dryopteris aitoniana* [21,50], ***D. nigropaleacea*** [20]
162	Hexa-albaspidin BBBBBB	*Dryopteris aitoniana* [21,50], ***D. nigropaleacea*** [20]
163	Hexaflavaspidic acid BBBBBB	*Dryopteris aitoniana* [21,50], ***D. nigropaleacea*** [20], ***D. juxtaposita*** [20], *D. odontoloma* [20]
164	Atrata-phloroglucinol A	***Dryopteris atrata*** [21,29]
165	Atrata-phloroglucinol B	***Dryopteris atrata*** [21,29]
166	Pentherin-I	*Dryopteris ardechensis* [31], ***D. pentheri*** [30]
167	Ju-1	***Dryopteris juxtaposita*** [20], ***D. nigropaleacea*** [20]
168	Ju-2	***Dryopteris juxtaposita*** [20], ***D. nigropaleacea*** [20]
169	Ju-3	***Dryopteris juxtaposita*** [20], ***D. nigropaleacea*** [20]
170	Le-1	***Dryopteris lepidopoda*** [17]
171	Le-2	***Dryopteris lepidopoda*** [17], ***D. wallichiana*** ssp. ***wallichiana*** [17]
172	Le-3	***Dryopteris lepidopoda*** [17], ***D. wallichiana*** ssp. ***wallichiana*** [17]
173	Si-1	*Dryopteris sledgei* [17]
174	Si-2	*Dryopteris sledgei* [17]

175	Fu_1	*Dryopteris fusco-atra* [44]
176	Fu_2	*Dryopteris fusco-atra* [44]
177	Fu_3	*Dryopteris fusco-atra* [44]
178	Wa-1	***Dryopteris wallichiana*** var. ***wallichiana*** [17]
179	Wa-2	***Dryopteris wallichiana*** var. ***wallichiana*** [17]
180	Elaphopilosin A	***Elaphoglossum piloselloides*** [32]
181	Elaphopilosin B	***Elaphoglossum piloselloides*** [32]
182	Elaphopilosin C	***Elaphoglossum piloselloides*** [33]
183	Elaphopilosin D	***Elaphoglossum piloselloides*** [33]
184	Elaphopilosin E	***Elaphoglossum piloselloides*** [33]
185	Elaphogayanin A	***Elaphoglossum gayanum*** [33]
186	Elaphogayanin B	***Elaphoglossum gayanum*** [33]
187	Lindbergin A	***Elaphoglossum lindbergii*** [34]
188	Lindbergin B	***Elaphoglossum lindbergii*** [34]
189	Lindbergin C	***Elaphoglossum lindbergii*** [34]
190	Lindbergin D	***Elaphoglossum lindbergii*** [34]
191	Yungensin A	***Elaphoglossum yungense*** [35]
192	Yungensin B	***Elaphoglossum yungense*** [35]
193	Yungensin C	***Elaphoglossum yungense*** [35]

Continued

TABLE 1 Distribution of Phloroglucinol Derivatives in Ferns—Cont'd

#	Compounds	Species in Which the Phloroglucinol Derivative Was Detected
194	Yungensin D	***Elaphoglossum yungense*** [35]
195	Yungensin E	***Elaphoglossum yungense*** [35]
196	Yungensin F	***Elaphoglossum yungense*** [35]
197	Yungensin G	***Elaphoglossum yungense*** [35]

The distribution of acylphloroglucinols in ferns, as cited in previous publications, is compiled in this table. The compound number (#), as presented, may be found in Schemes 1–13 and in previous paragraphs. It is important to point out that, except for compounds isolated and reported from species of the genus *Elaphoglossum*, data mostly come from a TLC analysis (R_f and color after spraying with "fast blue salt B" in comparison with authentic samples) and/or EI-MS analysis of mixtures of acylphloroglucinols. Species' names in bold are accepted according to "The Plant List," "Tropicos," and/or "Catalogue of Life 2010" databases [69–71].

There is a positive correlation between the presence of phloroglucinols and secretory structures. Many species of this genus have secretory glandular-headed trichomes, long or short stalked (30–53 μm) and clavate trichomes (85–105 μm), located on the epidermis of the rhizome and stipe base [14]. Internal two- to three-celled secretory glands can also be found in different species. *Dryopteris villari* and *D. arguta* have acylphloroglucinols secreted from glandular trichomes located on the leaf blade [49]. Widén *et al.* [19] found that glands present in rachis and costa were negative for phloroglucinols in *D. sparsa* and pointed out that internal clavate glands (85–105 μm) and long cylindrical secretory glands (150–280 μm) with or without resinous cells were found in the rhizome. The presence of glands with secretory function in the rhizome scales of *D. wallichiana* and *D. patula* is reported here for the first time (Fig. 1). Regarding the distribution of scales in the sporophyte, it has been reported that the rhizome of *Dryopteris* is short, stout, erect to suberect, and densely scaly, while the petioles are scaly especially at the base [78]. *Elaphoglossum* Schott is a genus of about 600 species and ranks as one of the world's largest and taxonomically most difficult pantropical fern genus [79]. The neotropic is the most diverse region, where 80% of the species grow [80]. *Elaphoglossum* species can be found in North Mexico, Central America, the Antilles, and South America (from Venezuela to Argentina and Chile). In the paleotropics, it has been reported for Africa, Madagascar, India, Sri Lanka, Malaysia, South China, NE of Japan, New Guinea, Australia, and Hawaii [81]. The six chemically analyzed *Elaphoglossum* species from Northwestern Argentina contain phloroglucinols. A detailed anatomical research on secretory glands of these species shows that the rhizome scales bear glands in the apex as well as on the sides and, in the particular case of *E. lindbergii*, all over the surface of the scale. These glands have secretory function and are reported for the first time for *E. gayanum*, *E. piloselloides*, *E. lorentzii*, *E. yungense*, *E. lindbergii*, and *E. crassipes* (Fig. 1). These structures were reported neither for the Argentine species by Lavalle and Rodriguez [82] nor by any of the morphological descriptions of *Elaphoglossum* species made so far [13,82]. Rohuan *et al.* [80], in their work about the species of *Elaphoglossum* from the French Polynesia, refer to the scales as having entire to subentire margins with few short teeth, occasionally glandular. Detailed observations indicate that the sources of phloroglucinol secretion are the glands present in the rhizome scales. The glands have different shapes and locations in the scale. The secretions deposit on the rhizome epidermis and between the scales (Fig. 2). Regarding the distribution of the scales in the sporophyte, morphologically the genus is characterized by scaly rhizomes, simple glabrous to densely scaly fronds, acrostichoid sorus, and frond dimorphism [83]. Glands, situated in the scales, play an important role in the secretion of phloroglucinols in the *Elaphoglossum* species studied.

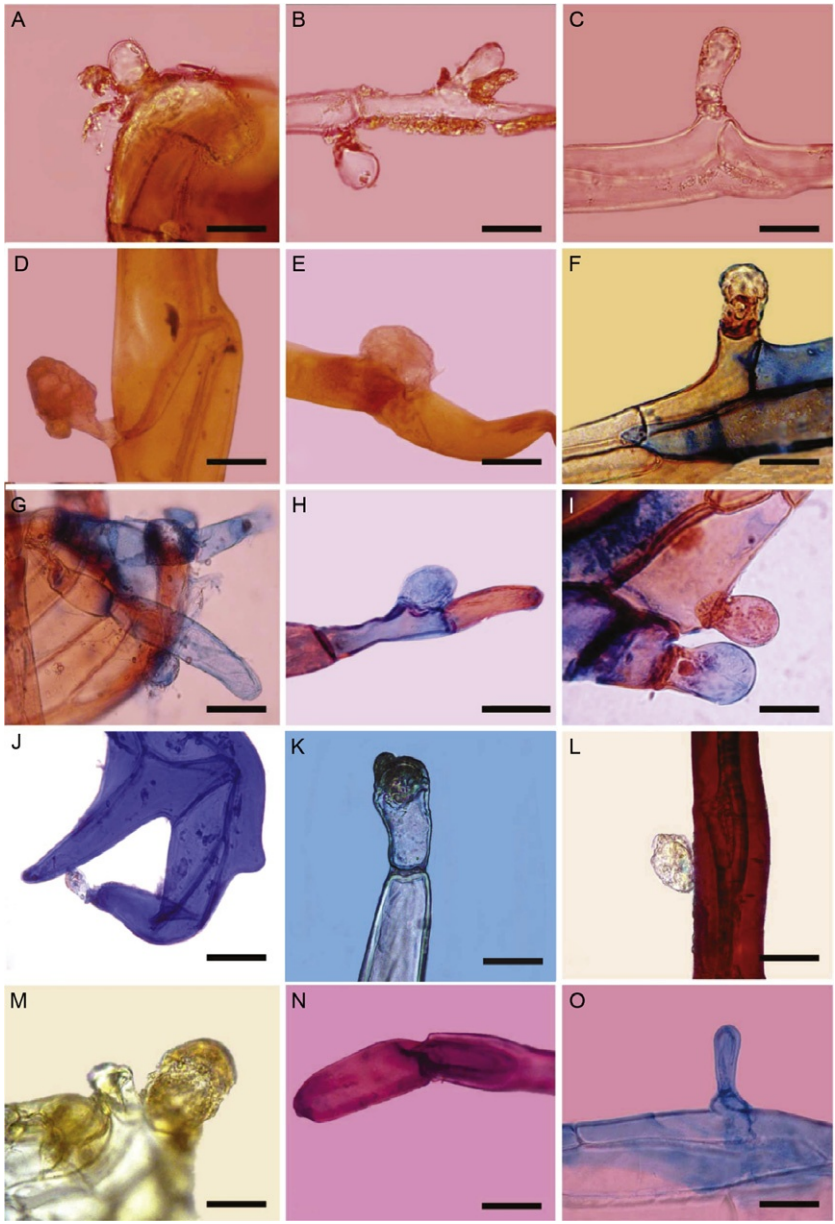

FIGURE 1 Secretory glands. (A–C) *Elaphoglossum lindbergii*. (A) Lateral gland at the base. (B–C) Lateral gland near the scale apex. (D–E) *Elaphoglossum crassipes*. Lateral glands. (F–G) *Elaphoglossum lorentzii*. (F) Lateral glands. (G) Glands at the scale base. (H–K) *Elaphoglossum pilloseloides*. (H–I) Lateral glands. (J) Gland at the scale base. (K) Apical gland. (L) *Elaphoglossum yungense*. Lateral gland. (M) *Elaphoglossum gayanum.* Gland on the scale base. (N) *Dryopteris walliciana.* Apical gland. (O) *Dryopteris patula* lateral gland. Bar: 20 μm (K, M, N); 30 μm (C–I, L, O); 40 μm (A, B); 50 μm (J).

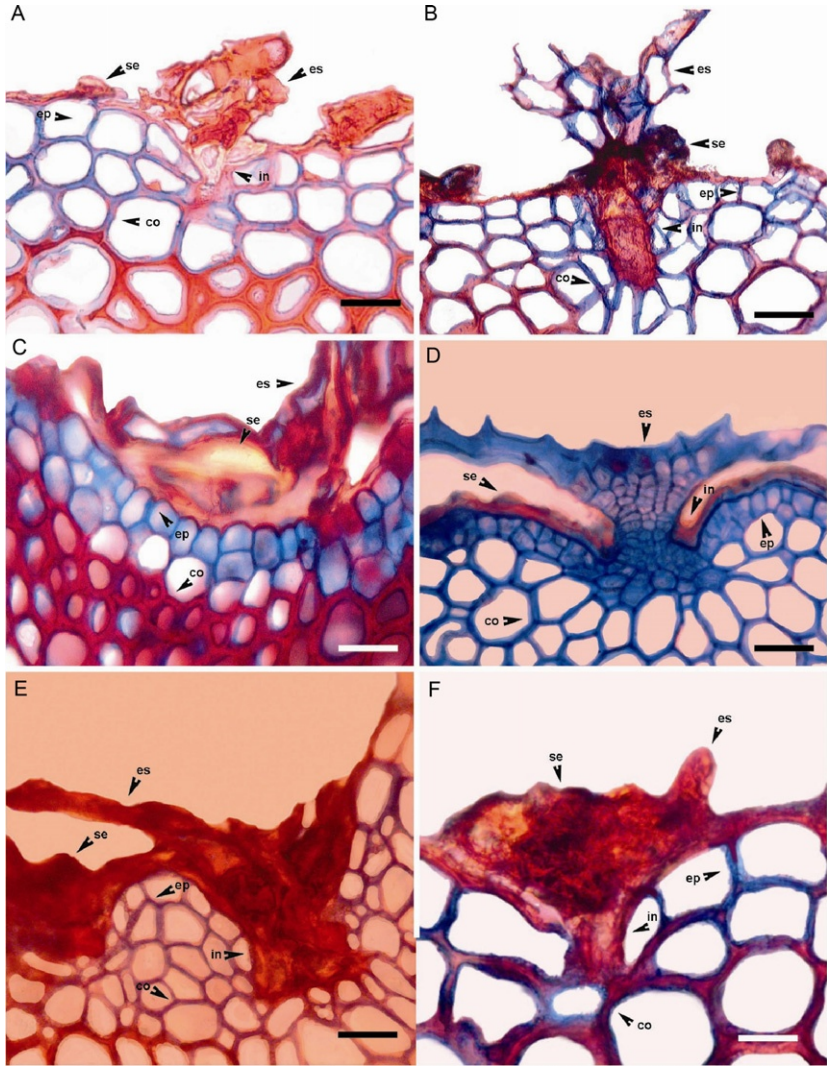

FIGURE 2 Rhizome cross-sections. (A, B, F) *Elaphoglossum lindbergii*. Scale insertion zone with secreting material; (C) *Elaphoglossum yungense*. Rhizome epidermis, scale remains, and secreting material; (D) *Elaphoglossum gayanum*. Scale insertion with secretion on the rhizome surface; (E) *Elaphoglossum crassipes*. Secretion in the scale insertion zone. The whole rhizome surface is covered by secretions. Bar: 80 μm (A, C, D, and E); 50 μm (B); 60 μm (F). se, secretion; ep: epidermis; co: cortex; es: scales; in: insertion zone.

The presence of secretory glands situated in the scales of *Dryopteris wallichiana*, *D. patula*, *Elaphoglossum crassipes*, *E. gayanum*, *E. lindbergii*, *E. lorentzii*, *E. piloselloides*, and *E. yungense* is reported for the first time in this review.

IMPROVEMENT OF DETECTION AND ISOLATION PROCEDURES

Previous Techniques. The so-called standard procedure [52] for the isolation or concentration of phloroglucinol derivatives for their further identification by Thin Layer Chromatography (TLC) comprises the following steps:

1. Plant material consisting of rhizomes or stipe bases is air dried (air at 20–45 °C for several days), powdered, and extracted with peroxide-free diethyl ether. The solvent is then evaporated to give a viscous, honey-like extract.
2. The resulting "crude ether extract" is then treated with MgO and water to transform fatty acids into insoluble magnesium salts and phenolics into water-soluble magnesium salts.
3. Filtration eliminates the excess of MgO, insoluble salts, and neutral material known as "MgO cake."
4. Phenolics are then precipitated from the water solution by adding acid (HCl). The material obtained by this procedure is often called "MgO filicin."
5. Additional amounts of phenolics can be obtained from the "MgO cake" by adding aqueous $Ba(OH)_2$.
6. Phenolics are precipitated from this solution by adding acid to obtain the so-called crude "$Ba(OH)_2$-filicin" which contains the less acidic phloroglucinols such as phloraspin and phloraspidinol.

Most of the phloroglucinol derivatives were detected as constituents of the crude extract or filicins on paper chromatography or TLC by comparison with authentic samples. Different solvent systems were employed and "fast blue salt B" (*o*-dianisidine bis(diazotized) zinc double salt in H_2O) was used to visualize the spots.

Some acylphloroglucinols were isolated by column chromatography, usually as a mixture of homologues that were identified by analysis of the peaks observed on their mass spectra.

Usually, Na_2SO_3 was added in steps 2 and 6 to avoid oxidation. It is known that acylphloroglucinols are stable to acids but sensitive to alkaline conditions and, thus, some degradation and alteration of phenolics to give artifacts by alkaline cleavage can take place in the aforementioned steps. For this reason, an "improved method" that avoided the use of MgO and $Ba(OH)_2$ was developed in 1985 that allowed the isolation and identification of compounds with up to six rings [50]. In this method, the preparation of the plant material and extraction procedure (step 1 in the previous procedure) remains unaltered and is followed by the steps described in the next paragraphs.

1. Before evaporation of the solvent, the extract is washed with aqueous HCl and water to eliminate organic and inorganic cations. After evaporation, the "cation-free crude ether extract" is obtained.
2. Partition of the extract between hexane and aqueous MeOH (95%) is then performed. The material recovered from the MeOH phases contains the more polar acylphloroglucinols (e.g., flavaspidic acids), while the less polar phenolics, such as albaspidins, remain in the hexane phase.
3. Column chromatography on microcrystalline cellulose (for the most polar compounds), polyamide (useful for the least polar compounds), or buffered SiO_2 (pH 4, 6, or 7).

Current Techniques. High-resolution chromatographic technique (HPLC) is the selected tool for the isolation of natural products. For prenylated acylphloroglucinols, normal phase HPLC has proved to be a useful technique, especially for those samples that are insoluble in MeOH [34,84]. A mixture of *n*-hexane–Ethyl acetate (EtOAc) is usually enough to accomplish resolution of a mixture, but sometimes the addition of Acetic acid (HOAc) is necessary to avoid tailing or broadening of the peaks. When the separation is carried out on reverse phase, the addition of HOAc to the mobile phase is mandatory. We recommend the use of HOAc instead of other acids because it can be easily removed *in vacuo*.

TLC detection can be achieved by the observation of the plate under Ultraviolet (UV) light (254 nm) and further spraying with Godin's reagent [85]. Prenylated acylphloroglucinols give orange, brown, red, or burgundy spots after spraying with the reagent and further heating on a hot plate.

The presence of acylphloroglucinols in extracts can be easily detected by a routine ^{1}H NMR spectrum in $CDCl_3$. Acylphloroglucinols show singlets that appear over 12 ppm (and up to 19 ppm) and can be easily identified in complex mixtures, for no other signals appear in that range (Fig. 3).

Biological Activity

Due to their known anthelmintic effect, *Dryopteris* ferns have been chemically studied since the nineteenth century in search of their bioactive constituents. Much has been done in terms of isolation and identification of acylphloroglucinols, but only a few reports describe their biological effects. Only in the past three decades, some articles were published in which their piscicidal, anthelmintic, antitumor, and other effects were investigated (Table 2).

Although there were few records of the use of any *Elaphoglossum* fern in ethnomedicine, the presence of acylphloroglucinols in their rhizomes and roots Et_2O extracts encouraged us to evaluate the biological active compounds. We tested the phloroglucinol derivatives isolated and identified in our laboratory for their molluscicidal and antibacterial effects (Table 2).

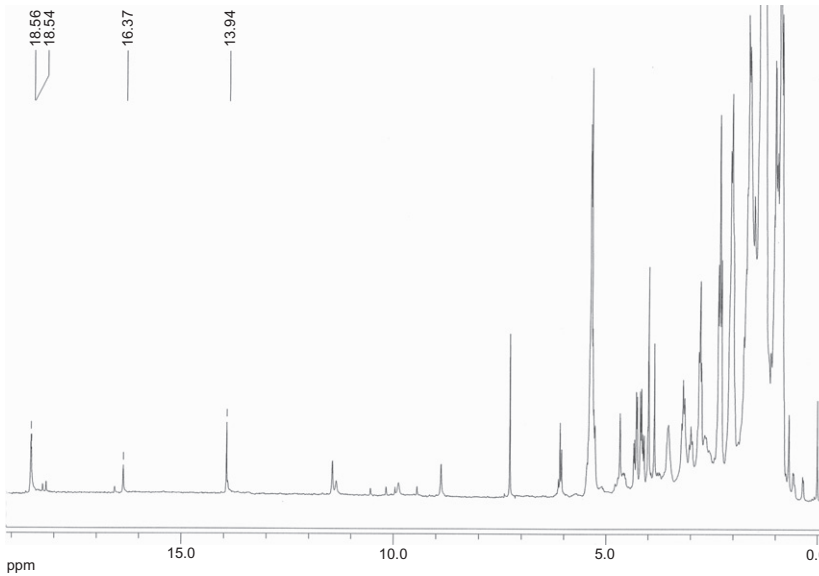

FIGURE 3 ^{1}H NMR spectrum of the diethyl ether extract of the fern *Elaphoglossum piloselloides* ($CDCl_3$, 200 MHz).

TABLE 2 Reported Effects of Fern Acylphloroglucinols

Acylphloroglucinol	Reported Biological Activity
Aspidinol B (**8**)	– Fatty acid synthase inhibition (IC_{50} = 49.1 μM) [54] – Piscicidal against *Oryzias latipes* (TLm = 1.5 μg/mL) [21,86] – *In vitro* tumor inhibition [87] – *In vitro* anthelmintic against *Hymenolepis nana* (Minimum lethal concentration: 10 ppm) [88]
Dryofragin (**18**)	– Piscicidal against *Oryzias latipes* (TLm = 1.6 μg/mL) [21,86]
Albaspidin AA (**20**)	– Molluscicidal against *Biomphalaria peregrina* (LD_{50} = 14.0 ppm) [46] – *In vitro* tumor inhibition [87]
Albaspidin BB (**22**)	– *In vitro* tumor inhibition [87]
Albaspidin PB (**23**)	– Fatty acid synthase inhibition (IC_{50} = 56.1 μM) [54]
Albaspidin PP (**24**)	– Fatty acid synthase inhibition (IC_{50} = 60.2 μM) [54]

TABLE 2 Reported Effects of Fern Acylphloroglucinols—Cont'd

Acylphloroglucinol	Reported Biological Activity
Albaspidin AP (**25**)	– Fatty acid synthase inhibition (IC_{50} = 71.7 μM) [54]
Norflavaspidic acid AB (**30**)	– Fatty acid synthase inhibition (IC_{50} = 29.7 μM) [54]
Flavaspidic acid AB (**34**)	– Fatty acid synthase inhibition (IC_{50} = 28.7 μM) [54] – Antibacterial against Gram (+) bacteria (*Staphylococcus aureus*, *Bacillus subtilis*, and *Streptococcus mutans*) [56] – Antioxidant activity on lipid peroxidation inhibitory test (IC_{50} = 13.1 μM), DPPH radical scavenging (IC_{50} = 76.3 μM), and superoxide radical scavenging (IC_{50} = 64.4 μM) [57] – *In vitro* tumor inhibition [87]
Flavaspidic acid BB (**35**)	– Schistosomicidal against *Schistosoma mansoni* adult worms ($LD_{100} \leq 50$ μM) [89] – *In vitro* tumor inhibition [87] – *In vitro* and *in vivo* anthelmintic against *Hymenolepis nana* (Minimum lethal concentration: 2 ppm) [88]
Flavaspidic acid PB (**38**)	– Fatty acid synthase inhibition (IC_{50} = 23.1 μM) [54] – Antibacterial against Gram (+) bacteria (*Staphylococcus aureus*, *Bacillus subtilis*, and *Streptococcus mutans*) [56] – Antioxidant activity on lipid peroxidation inhibitory test (IC_{50} = 12.9 μM), DPPH radical scavenging (IC_{50} = 71.7 μM), and superoxide radical scagenting (IC_{50} = 58.6 μM) [57] – *In vitro* tumor inhibition [87]
Aspidin AB (**45**)	– Piscicidal against *Oryzias latipes* (TLm = 2.4 μg/mL) [86]
Aspidin BB (**46**)	– Fatty acid synthase inhibition (IC_{50} = 32.6 μM) [54] – Piscicidal against *Oryzias latipes* (TLm = 1.2 μg/mL) [86] – Schistosomicidal against *Schistosoma mansoni* adult worms ($LD_{100} \leq 25$ μM) [89] – *In vivo* tumor inhibition [87] – *In vitro* and *in vivo* anthelmintic against *Hymenolepis nana* (Minimum lethal concentration: 0.2 ppm) [88]

Continued

TABLE 2 Reported Effects of Fern Acylphloroglucinols—Cont'd

Acylphloroglucinol	Reported Biological Activity
Aspidin PB (**47**)	– Piscicidal against *Oryzias latipes* (TLm = 1.4 μg/mL) [86]
Para-aspidin AB (**56**)	– *In vitro* tumor inhibition [87]
Para-aspidin BB (**57**)	– *In vitro* tumor inhibition [87]
Desaspidin BB (**68**)	– Schistosomicidal against *Schistosoma mansoni* adult worms ($LD_{100} \leq 25$ μM) [89] – *In vivo* tumor inhibition [87] – *In vivo* anthelmintic against *Diphyllobothrium latum* (clinical trial) [90] – *In vitro* and *in vivo* anthelmintic against *Hymenolepis nana* (minimum lethal concentration: 2–100 ppm) [88]
Phloraspin BB (**78**)	– *In vitro* tumor inhibition [87]
Margaspidin AB (**86**)	– *In vitro* tumor inhibition [87]
Margaspidin BB (**87**)	– *In vitro* tumor inhibition [87]
Phloraspidinol BB (**97**)	– *In vitro* tumor inhibition [87]
Methylene-bis-aspidinol BB (**100**)	– Schistosomicidal against *Schistosoma mansoni* adult worms ($LD_{100} \leq 100$ μM) [89] – *In vitro* tumor inhibition [87]
Phloropyrone BB (**106**)	– *In vitro* tumor inhibition [87] – *In vitro* anthelmintic against *Hymenolepis nana* (minimum lethal concentration: 10 ppm) [88]
Abbreviatin BB = Methylene-bis-methylphlorobutyrophenone (**114**)	– Fatty acid synthase inhibition ($IC_{50} = 25.4$ μM) [54]
Filixic acid ABA (**120**)	– Molluscicidal against *Biomphalaria peregrina* ($LD_{50} = 8.4$ ppm) [46] – *In vitro* tumor inhibition [87]
Filixic acid ABB (**121**)	– Molluscicidal against *Biomphalaria peregrina* ($LD_{50} = 19.6$ ppm) [46] – *In vitro* tumor inhibition [87]
Filixic acid BBB (**122**)	– *In vitro* tumor inhibition [87]
Filixic acid ABP (**123**)	– Fatty acid synthase inhibition ($IC_{50} = 31.0$ μM) [54]
Filixic acid PBB (**125**)	– *In vitro* tumor inhibition [87]
Filixic acid PBP (**126**)	– *In vitro* tumor inhibition [87]
Tetraalbaspidin ABBA = dryocrassin (**157**)	– *In vitro* tumor inhibition [87]

TABLE 2 Reported Effects of Fern Acylphloroglucinols—Cont'd

Acylphloroglucinol	Reported Biological Activity
Elaphopilosin A (**180**)	– Molluscicidal against *Biomphalaria peregrina* (LD_{50} = 2.9 ppm) [32]
Elaphopilosin B (**181**)	– Molluscicidal against *Biomphalaria peregrina* (LD_{50} = 0.94 ppm) [32]
Elaphopilosin C (**182**)	– Molluscicidal against *Biomphalaria peregrina* (LD_{50} = 2.15 ppm) [46]
Elaphopilosin D (**183**)	– Molluscicidal against *Biomphalaria peregrina* (LD_{50} = 7.2 ppm) [33]
Elaphopilosin E (**184**)	– Molluscicidal against *Biomphalaria peregrina* (LD_{50} = 11.7 ppm) [33]
Elaphogayanin A (**185**)	– Molluscicidal against *Biomphalaria peregrina* (LD_{50} = 5.9 ppm) [46]
Elaphogayanin B (**186**)	– Molluscicidal against *Biomphalaria peregrina* (LD_{50} = 1.90 ppm) [46]
Lindbergin A (**187**)	– Mild antibacterial activity against *Staphylococcus aureus* (45% inhibition at 100 μg/mL) [34]
Lindbergin B (**188**)	– Considerably reduces biofilm production by *Staphylococcus aureus* (82% at 100 μg/mL) [34] – Mild antibacterial activity against *S. aureus* (86% inhibition at 100 μg/mL) [34]
Lindbergin C (**189**)	– Mild antibacterial activity against *Staphylococcus aureus* (41% inhibition at 100 μg/mL) [34]
Lindbergin D (**190**)	– Mild antibacterial activity against *Staphylococcus aureus* (35% inhibition at 100 μg/mL) [34]
Yungensin A (**191**)	– Antibacterial against *Staphylococcus aureus* and *Pseudomonas aeruginosa* (MIC = 10 μg/mL) [35] – Stimulates biofilm production by *Staphylococcus aureus* and *Pseudomonas aeruginosa* [35]
Yungensin B (**192**)	– Antibacterial against *Staphylococcus aureus* and *Pseudomonas aeruginosa* (MIC = 10 μg/mL) [35] – Stimulates biofilm production by *Staphylococcus aureus* and *Pseudomonas aeruginosa* [35]

Continued

TABLE 2 Reported Effects of Fern Acylphloroglucinols—Cont'd

Acylphloroglucinol	Reported Biological Activity
Yungensin C (**193**)	– Antibacterial against *Staphylococcus aureus* and *Pseudomonas aeruginosa* (MIC = 50 μg/mL) [35]
Yungensin D (**194**)	– Antibacterial against *Staphylococcus aureus* and *Pseudomonas aeruginosa* (MIC = 100 and 50 μg/mL, respectively) [35] – Molluscicidal against *Biomphalaria peregrina* (LD_{50} = 5.1 ppm) [46]
Yungensin E (**195**)	– Antibacterial against *Staphylococcus aureus* and *Pseudomonas aeruginosa* (MIC = 100 μg/mL) [35] – Stimulates biofilm production by *Staphylococcus aureus* and *Pseudomonas aeruginosa* [35] – Molluscicidal against *Biomphalaria peregrina* (LD_{50} = 17.0 ppm) [46]
Yungensin F (**196**)	– Antibacterial against *Staphylococcus aureus* and *Pseudomonas aeruginosa* (MIC = 50 μg/mL) [35] – Stimulates biofilm production by *Staphylococcus aureus* and *Pseudomonas aeruginosa* [35] – Molluscicidal against *Biomphalaria peregrina* (LD_{50} = 4.0 ppm) [46]

FINAL REMARKS

Among fern acylphloroglucinols, a large number comes from European species of the genus *Dryopteris*, the one that deserved the most extensive phytochemical studies through the years. These derivatives have been incorporated to the present review together with acylphloroglucinols isolated from a few other genera. In addition, we presented in a tabular way, an important group of phloroglucinol derivatives that have been isolated from Argentine species of the genus *Elaphoglossum*. These compounds, isolated in our laboratories in the past 8 years, have been identified employing modern spectroscopic techniques, particularly 600 MHz NMR and High Resolution–Mass Spectrometry after exhaustive isolation and purification employing HPLC. Evaluation of their bioactivity revealed that their molluscicidal effects are the most significant and that they are comparable, in potency, to the synthetic molluscicides employed in the control of snails of the genus *Biomphalaria*, the known schistosomiasis vectors. Finally, a recent evaluation of the antidepressant-like activity in mice of a

phloroglucinol-containing fraction of *E. crassipes* [84] opens new horizons in the pharmacological applications of fern acylphloroglucinols.

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Chapter 6

Mitogen-Activated Protein Kinase and Natural Phenolic Compounds in Cardiovascular Remodeling

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INTRODUCTION

Mitogen-activated protein kinases (MAPKs) include many serine/threonine kinases involved in regulating cellular processes such as proliferation, differentiation, stress adaptation, and apoptosis. The MAPKs are activated in response to free radical-induced injury in cells and contribute to the processes of cellular survival or death [1]. Free radicals are electron-rich molecules with the ability to react with lipids, proteins, DNA, and other macromolecules [2]; their presence in biological systems was first described in 1954 [3]. This was followed by the hypothesis in 1956 that free radicals were generated through the enzymatic processes of the biological system and were responsible for initiation of disease processes [4]. Oxidative stress is defined as the cellular damage resulting from excessive formation of highly reactive molecules such as reactive oxygen species (ROS) including superoxide anions and hydrogen peroxide and reactive nitrogen species such as nitric oxide together with insufficient removal of these species by enzymes such as superoxide dismutase (SOD), catalase, and peroxidase [5,6]. ROS activate a wide variety of serine/threonine and tyrosine kinases (TKs), which are key regulatory proteins of signal transduction pathways important in mediating cellular growth, apoptosis, survival, migration, and aging. ROS have been proposed to exert their effects through targeting the cysteine regions of the active sites of tyrosine phosphatases, which in turn activate TKs.

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Strategies targeting the MAPK pathway as a common signaling step are predicted to provide new therapeutic options for chronic diseases including diabetes, cardiovascular diseases, and cancer [7] as these diseases may be initiated and promoted by oxidative stress [8]. The potential of this strategy in cardiovascular diseases is shown by the regulation of oxidative stress by antagonists at the angiotensin AT_1 receptor, thus decreasing cardiovascular remodeling including hypertension, hypertrophy, and fibrosis. The peptide hormone, angiotensin II, activates NADPH oxidase and stimulates ROS generation in the cardiovascular system by activation of AT_1 receptors [9]. AT_1 receptor activation triggers Ras and Raf-1-dependent phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2) and c-Jun N-terminal kinase (JNK) [10,11]. Angiotensin II stimulates tyrosine phosphorylation of the Janus kinases JAK2 and Tyk2, resulting in an activation of the latent transcription factors STAT1, STAT2, and STAT5 in neonatal cardiomyocytes [12]. Increased ROS concentrations also activate multiple redox signaling pathways including NF-κB that in turn enhance other angiotensin II-mediated inflammatory responses by upregulating inflammatory mediators such as TNFα, MCP-1, and CRP. MAPKs are also involved in TNFα-induced vascular smooth muscle cell (VSMC) migration and mitogenic signaling by growth factors in VSMCs.

Naturally occurring phenolic compounds are antioxidants that may alter MAPK-induced signaling pathways as these compounds decrease inflammation, platelet aggregation, atherosclerosis, and hypertension thus protecting the heart [13]. Phenolic compounds such as apocyanin may prevent oxidative stress by inhibiting NADPH oxidase-mediated free radical generation or by directly scavenging free radicals which induce mitochondrial transition pore complex opening by decreasing production of Bax and Bad proteins, favoring an increase in Bcl2–BclXL/Bax–Bak ratio [14]. Thus, phenolic compounds may exert protective effects by selectively inhibiting or stimulating key proteins in the cell signaling cascades.

MAPKs are an increasing area of interest. A PubMed search with the keyword "MAPKs" retrieved 4420 references and the keywords "MAPKs in Heart" retrieved 262 references. Searching the Web of Sciences for "MAPKs" produced 4487 references and "MAPKs in Heart" produced 214 items. For this review, we selected articles having more detailed information relevant to the topic of this review, emphasizing the actions on the cardiovascular system. Thus, the primary purpose of this review is to describe the MAPK pathways in the cardiovascular system that are activated by oxidative stress, ischemia/reperfusion, and hypertrophy signals and then discuss the potential role of natural phenolic compounds for protection.

CARDIAC REMODELING

Remodeling is usually an adaptive process that occurs in response to long-term changes in hemodynamic conditions, but it may subsequently contribute

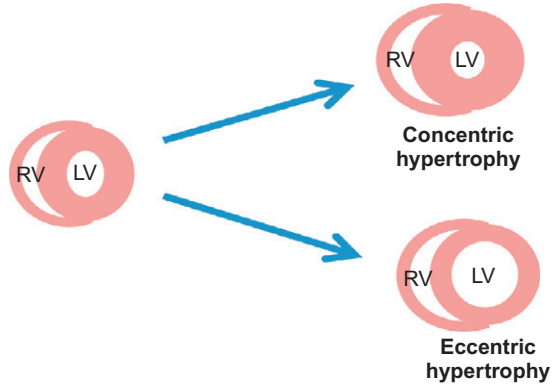

FIGURE 1 Hypertrophy of heart.

to the pathophysiology of vascular diseases and circulatory disorders. Cardiac remodeling is best described as molecular, cellular, interstitial, and genomic changes in the heart, which are shown clinically as alterations in size, shape, and function of the heart following cardiomyocyte injury [15]. Cardiac remodeling involves changes in cardiomyocytes, proliferation of cardiac fibroblasts, alteration in collagen formation and degradation, and apoptosis [16]. Important initiators of cardiac hypertrophy include hemodynamic load, increased wall stress, neurohormonal activation (sympathetic nervous system, renin–angiotensin–aldosterone system, and endothelin), cytokines, oxidative stress, and ischemia. Myocardial cells cannot divide to increase their numbers, but they can increase in length or in size. Following an insult, cardiac myocytes may undergo different changes. Surviving myocytes may elongate with thinning of the wall (Fig. 1). Hypertrophy, usually at sites distant from an infarct, may temporarily improve cardiac output and diminish wall stress [17].

VASCULAR REMODELING

Vascular remodeling is an active process of structural alteration that involves changes in at least four cellular processes: cell growth, cell death, cell migration, and production or degradation of extracellular matrix. Most of these events are dependent on a dynamic interaction between locally generated growth factors, vasoactive substances, and hemodynamic stimuli [18]. The biologic process of vascular remodeling may be divided into the following components: the detection of signals due to changes in hemodynamic conditions and humoral factors (sensors); the relay of signals within the cell and to adjacent cells (transducers); the synthesis and release or activation of substances that influence cell growth, death, or migration or the composition of the extracellular matrix (mediators); and the resultant structural changes in the vessel wall (both cellular and noncellular components) [18]. The classical

feature of vascular remodeling is narrowing of the luminal diameter of blood vessels. Normal arterial walls contain three distinct layers: tunica intima, tunica media, and tunica externa. The innermost tunica intima consists of endothelial cells (ECs) facing the lumen and residing on a basement membrane that overlies a thin extracellular matrix substrate. Degradation of extracellular matrix enables VSMCs to migrate and proliferate and inflammatory cells to infiltrate the arterial wall during the remodeling process. Increased MMP activity has been reported in various inflammatory, malignant, and degenerative disorders. Also, loss of control of MMP activity could result in pathological vascular remodeling and vascular disease [19]. Since MMP activity is a redox-sensitive process, free radical generation via NADPH oxidase may increase the MMP activity in experimental animals [20,21]. The wall thickness was increased in the aorta of hypertensive rats; antioxidant supplementation normalized the blood pressure and prevented thickening of aorta by reducing the oxidative stress [22].

MAPK PATHWAYS

The first important molecular event involved in cellular responses is the activation of intracellular signal transduction pathways, particularly the activation of protein kinases. One common pathway of G-protein-coupled receptor (GPCR) signaling convergence is the MAPK pathway, which ultimately activates downstream transcription factors and gene expression related to cardiac growth. The MAPK pathway is a TK-dependent pathway normally stimulated by growth factors and cellular stress or inflammatory cytokines [23]. The MAPK cascade consists of three kinase modules: MAP kinase kinase kinase (MKKK, MAPKKK, or MEKK), MAP kinase kinase (MKK, MAPKK, or MEK), and MAPK [24]. MKKK activates the downstream MKK, which in turn activates MAPK by catalyzing the phosphorylation of threonine and tyrosine residues within the catalytic domain [24]. The three major MAPK cascades identified in the myocardium are the ERKs and two stress-activated MAPK subfamilies, SAPK/JNK and p38 MAPKs (Fig. 2) which are believed to be activated by dual phosphorylation on both threonine and tyrosine residues within a Thr-Xaa-Tyr motif with the identity of Xaa assisting in the classification of subgroup membership [25]. Activation of the MAPK by MKK requires phosphorylation of threonine and tyrosine residues [26]. There is increasing evidence that MAPK pathway is involved in various cardiovascular disorders such as cardiac hypertrophy and atherosclerosis [27]. The effects of MAPKs producing cardiac hypertrophy to heart failure are summarized in Table 1.

ERK SUBFAMILY

ERKs, the most studied kinases among the MAPKs, are activated in response to mitogenic and growth factors acting through receptor protein tyrosine

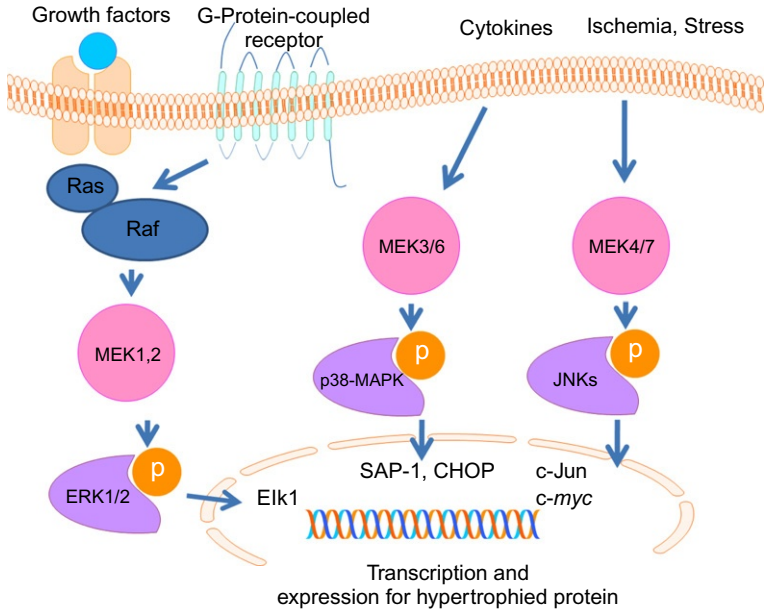

FIGURE 2 Mitogen-activated protein kinase cascades showing their upstream activators and downstream targets involved in cardiac hypertrophy.

kinases (PTKs) or GPCR [40]. ERK1/2 is a 42-kDa protein first identified in 1991 [41]. ERKs are characteristically activated by many hormones and growth factors and are associated with cell growth and hypertrophy. The mammalian ERK1/2 module, also known as the classical mitogen kinase cascade, consists of the MAPKKKs A-Raf, B-Raf, and Raf-1, the MAPKKs MEK1 and MEK2, and the MAPKs ERK1 and ERK2. Typically, cell surface receptors such as tyrosine kinases (RTK) and GPCRs transmit activating signals to the Raf/MEK/ERK cascade through different isoforms of the small GTP-binding protein Ras [42]. Studies also have demonstrated evidence for Ras/Raf-independent activation of MEK1,2 by both p21 kinase (PAK) and MEKK1-3 kinases [43,44]. In cardiac myocytes, members of the ERK MAPK family have been implicated in survival signaling in response to ischemia-reperfusion, oxidative stress, hypoxia, and β-adrenoceptor stimulation. Cardiomyocyte stretching and aortic bending-induced pressure overload activate the ERK and provide evidence about the regulatory role of hypertrophic response [45]. Hydrogen peroxide-induced apoptosis in cultured cardiomyocytes and selective inhibition of ERKs increases the number of apoptotic cells [13]. Oxidative stress and hydrogen peroxide activate ERKs in cultured cardiomyocytes through the downstream activator in the Ras/Raf-1/MEK pathway [13]. Development of transgenic mice gives us a good insight into the regulatory role of MAPKs *in vivo*. MEK1 transgenic mice demonstrate

TABLE 1 Effect of MAPK Subfamilies on Cardiac Hypertrophy to Failure

MAPKs	Intervention	Outcome	Reference
ERKs	MEK1 transgenic mice and MEK1 adenovirus-infected cultured cardiomyocytes	MEK1–ERK1/2 signaling pathway stimulated a physiologic hypertrophy, augmented cardiac function, and partial resistance to apoptosis	[28]
	Aortic banding in adult male Charles River guinea pigs	ERK1/2, p38, Src, and BMK1 were activated by chronic pressure overload leading to cardiac hypertrophy	[29]
p38 MAPK	Infection of cardiomyocytes with adenoviral vectors expressing upstream activators for the p38 kinases	The hypertrophic response was enhanced by coinfection of an adenoviral vector expressing wild-type p38β and was suppressed by the p38β dominant-negative mutant	[30]
	Spontaneously hypertensive stroke-prone (SHRSP) rats receiving a high-salt/high-fat diet	Myocardial p38 MAPK was activated persistently during the development of cardiac hypertrophy and inactivated during decompensation	[31]
	Cardiac-specific transgenic mice expressing dominant-negative mutants of p38α, MKK3, or MKK6	Dominant-negative p38α, MKK3, and MKK6 transgenic mice each showed enhanced cardiac hypertrophy following aortic banding, Ang II infusion, isoproterenol infusion, or phenylephrine infusion for 14 days	[32]
	Left anterior descending (LAD) coronary artery ligation produced anterior MI	Treatments with SB203580 (a p38 MAPK inhibitor) suppressed myocardial fibrosis and LV remodeling, as well as attenuated the expressions of p-p38-MAPK, TNFα, α-SMA, and collagen I	[33]
	Cardiac remodeling was induced in male albino rats by chronic inhibition of nitric oxide (NO) synthesis by *N*-nitro-L-arginine methyl ester (L-NAME)	An elevation in cardiac phosphorylated p38 MAPK concentration, tumor necrosis factor alpha concentration, and in cardiac caspase-3 activity was also observed which was ameliorated by statins and retinoic acid administration	[34]
	Ligation of the LAD coronary artery in outbred Sprague–Dawley rats induces myocardial infarction	Long-term but not short-term p38 mitogen-activated protein kinase (MAPK) inhibition by RWJ 67657 improved cardiac function and reduced cardiac remodeling postmyocardial infarction	[35]

JNKs	Neonatal rat ventricular myocytes	The alpha1-AdrR effect on JNK occurred through a pathway requiring Ras and MEK kinase (MEKK)-activated JNK-induced hypertrophied signal	[36]
	Isolated perfused rat heart treated for global ischemia	JNK activation was also associated with ischemia/reperfusion	[37]
	Cardiac hypertrophy in stroke-prone spontaneously hypertensive rats (SHRSP)	AT_1 receptor was involved in the enhanced cardiac JNK activity in both the onset and development of cardiac hypertrophy of hypertensive rats	[38]
	Adenovirus solution containing approximately 1010 plaque-forming units of either AdLacZ or AdSEK-1(KR) injected from the apex of the left ventricle to the aortic root with supravalvular aortic banding in heart of male Sprague–Dawley rats	Adenovirus-mediated gene transfer of SEK-1(KR), a dominant inhibitory mutant of the immediate upstream activator of the stress-activated protein kinases (SAPKs), inhibited pressure-overload-induced cardiac hypertrophy	[39]

concentric hypertrophy without signs of cardiomyopathy or lethality up to 12 months of age and show resistance to ischemia/reperfusion-induced apoptosis [28]. Overexpression of constitutively active Ras mutant in Ras mutant mouse heart induces cardiac hypertrophy with a myopathic phenotype [46]. Ras is a direct upstream activator of Raf-1, which in turn leads directly to MEK1 and ERK1/2 activation in the heart. How ERKs provide signals for cellular survival is yet not fully understood but several pathways have been proposed. Of these, ERK1/2 forms a complex with PKC_{ε} in the mitochondria facilitating the phosphorylation and inactivation of the proapoptotic Bcl-2 family member Bad [47]. Moreover, ERK1/2 activities can suppress Fas-mediated apoptosis by inhibiting the formation of the death-inducing signaling complex [48].

The big mitogen-activated kinase 1 (BMK1 or ERK5; 80 kDa) has recently been characterized. It can be activated by oxidative stress and plays a role in early gene expression triggered by serum [49]. GPCRs can potently stimulate ERK5 through a mechanism that involves Gαq and Gα13, independently of Rho, Rac1, and Cdc42 [50]. ERK5 appears to play an important role in the regulation of early gene expression through the phosphorylation of the transcription factor myocyte enhancer factor 2 [51].

p38 SUBFAMILY

p38 MAPK was cloned in 1994 by two independent research groups [52,53]. The p38 module consists of several MAPKKKs, including MEKKs1 to 4 (MEKK1–4), MLK2 and 3, DLK, ASK1, Tpl2 (also termed Cot), and Tak1, and the MAPKKs, MEK3 and MEK6 (also termed MKK3 and MKK6, respectively). Four p38 kinases, namely p38α, p38β, p38γ, and p38δ, were identified [54]; p38α and p38β exist in the myocardium [54]. p38 activity is critical for normal immune and inflammatory responses. p38 is activated in macrophages, neutrophils, and T cells. MEK3 and MEK6 are activated by MAPKKKs in response to physical and chemical stresses, such as oxidative stress, UV irradiation, hypoxia, ischemia, and cytokines including interleukin-1 (IL-1) and TNFα [55]. One substrate of p38-MAPKα and p38-MAPKβ is MAPK-activated protein (MAPKAP) kinase 2, an enzyme that phosphorylates the small heat-shock protein 25/27 (HSP25/27) [53]. In unstressed cells, HSP25/27 exists predominantly in an unphosphorylated state as high molecular mass aggregates [56].

The association between p38 MAPKs and the regulation of cardiac hypertrophy has primarily been investigated in cultured neonatal rat cardiomyocytes. Overexpression of activated MKK3 or MKK6 in cultured neonatal cardiomyocytes induces hypertrophy and atrial-natriuretic factor expression *in vitro*, further implicating p38 as a positive regulator of cardiomyocyte growth [30]. Also, reduced signaling of p38 in the heart promotes myocyte differentiation via a mechanism involving calcineurin–NFAT signaling [57].

In cardiac-specific p38α-conditional knockout mice, p38α is not an essential regulator for cardiac hypertrophy under pressure overload but rather surprisingly plays a protective role against cardiac myocyte apoptosis and myocardial remodeling [58]. Transgenic animal studies also show the potential role of p-38MAPKs in developing cardiac complications. MKK3 and MKK6 transgenic mice rapidly develop heart failure characterized by reduced functional performance, interstitial fibrosis, and thinned ventricular walls [59]. p38 MAPK activity is also elevated by pressure overload hypertrophy in aortic-banded mice [24,27,60]. In spontaneously hypertensive stroke-prone (SHRSP) rats receiving a high-salt/high-fat diet, myocardial p38 MAPK is activated persistently during the development of cardiac hypertrophy [31].

c-JUN KINASE

The JNKs are strongly activated in response to cytokines, UV irradiation, growth factor deprivation, DNA-damaging agents, and, to a lesser extent, some GPCR, serum, and growth factors [61]. The JNK family consists of at least 10 isoforms, derived from three genes: JNK1, JNK2, and JNK3 [62]. Like ERK1/2 and p38, JNK activation also requires dual phosphorylation on tyrosine and threonine residues within a conserved Thr-Pro-Tyr motif. Regulation of the JNK pathway is extremely complex and is influenced by many MKKKs. Phosphorylation of c-Jun on Ser63 and Ser73 by JNK leads to increased c-Jun-dependent transcription [63]. c-Jun is also a component of the AP-1 transcription complex, which is an important regulator of gene expression. JNK1 and 2 are critical regulators of cardiac hypertrophy *in vitro* and *in vivo*. In cultured cardiomyocytes, JNK isoforms become phosphorylated in response to stress stimuli (stretching) or GPCR activation [64,65]. JNK activation is also associated with load-induced cardiac hypertrophy in the rat [39]. JNK activation results in marked induction of TGF-β, and the selective induction of fibronectin suggests that TGF-β-mediated extracellular matrix remodeling is a likely factor contributing to the increased myocardial stiffness observed in JNK-activated hearts [66].

Pharmacological Inhibitors of MAPKs

The pharmacological responses of inhibitors of MAPKs (Table 2) have attracted attention, supporting their ever-increasing role in the development of cardiovascular complications, from ischemia to hypertrophy. As examples, inhibition of p38 during prolonged ischemia slows the rate of infarction and death [75] and inhibits the production of inflammatory cytokines, such as TNFα, IL-1, and IL-8 which aggravate the ischemic injury [75]. SB203580 and SKF-86002 show potent p38 inhibitory activity and inhibit cytokine

TABLE 2 Pharmacological Inhibitors of MAPKs Under Development

MAPKs	Name of Inhibitor	References
MEK1–2/ERKs	1. PD98059 and U0126	[67]
p38 MAPKs	2. Vertex 745 (VX745) for rheumatoid arthritis 3. RPR200765A for rheumatoid arthritis 4. SCIO469 for rheumatoid arthritis 5. SB235699 (HEP689) for psoriasis 6. SB203580, SB216995, SB218655, and SB220025	[60,67,68]
	7. RWJ 67657 [69] {4-[4-(4-fluorophenyl)-1-(3-phenylpropyl)-5-(4-pyridinyl)-1-Himidazol-2-yl]-3-butyn-1-ol} inhibits the release of TNFα 8. BIRB 796 BS [70] {1-(5-*tert*-butyl-2-*p*-tolyl-2H-pyrazol-3-yl)-3-[4-(2-morpholin-4-yl-ethoxy)-naphtalen-1-yl]-urea} 9. R-130823 [71] {2-(4-fluorophenyl)-4-(1-phenethyl-1,2,3,6-tetrahydropyridin-4-yl)-3-(pyridin-4-yl)-1Hpyrrole} 10. 230963 [72] [*trans*-1-(4-hydroxycyclohexyl)-4-(4-fluorophenyl)-5-(2-methoxypyridimidin-4-yl) imidazole]	[69–72]
JNKs	11. SP600125 12. CEP1347 (KT7515)	[67,73]
Raf-1	13. Bay439006—Phase I trials in locally advanced or metastatic cancer	[67,74]

production [68]. Later on, SB242235, RWJ 67657, VX-745, BIRB-796, and RO3201195 were developed and have advanced to clinical trials [76]. SB242235 is well tolerated in clinical trials and suppresses production of TNFα, IL-1β, IL-6, and IL-8 [76]. RWJ 67657 improves left ventricular (LV) dysfunction in rats [77].

The first report of neuronal protection *in vivo* with a kinase inhibitor used direct injection into the cerebral ventricles of PD98059, a MEK1–2 inhibitor that activates ERKs and may be useful in preventing stroke [78]. SP600125 is a JNK inhibitor that inhibits the phosphorylation of c-Jun, the expression of inflammatory genes COX-2, IL-2, IFN-gamma, and TNFα, and prevents the activation and differentiation of primary human CD4 cell cultures [79]. Treatment with SP600125 in LmnaH222P/H222P mice (mutations in LMNA, which encodes A-type nuclear lamins, causing disorders of striated muscle that have a common feature of dilated cardiomyopathy) delays the development of LV dilation and prevents decreases in cardiac ejection fraction and fibrosis [80].

UPSTREAM ACTIVATORS OF MAPK FAMILIES, GPCR AND SMALL G-PROTEINS

GPCRs play a critical role in mediating the responses of hormones and neurotransmitters. GPCRs comprise an extracellular amino terminus, seven-transmembrane-spanning R-helices (TM1–TM7) connected by alternating extracellular and intracellular loops, and a cytoplasmic carboxyl terminal region [81]. Dysregulation of GPCR generates a wide array of pathologies, promoting GPCR as a powerful drug discovery research area [82,83] that accounts for 30% of marketed drugs [83]. G-proteins transmit a wide variety of extracellular signals to effector molecules within cells. G-proteins consist of two functional units, a guanine nucleotide-binding α-subunit and a β-subunit/γ-subunit dimer, and are classified according to their α-subunits into four subfamilies: Gs, Gi, Gq, and G12. Further, the emerging concept of oligomerization is giving direction for new signaling GPCR responses [84–86]. G-proteins and TK receptors modulate MAPK signaling independently. Further, G-proteins activate MAPKs by two distinct mechanisms. Both Gαq and Gαo stimulate MAPK via a PKC-dependent pathway [87,88]. In contrast, Gβγ subunits activate MAPK by a mechanism that is independent of PKC but involves both phosphoinositide 3-kinase (PI3K) and PTKs as mediators [89]. The G12 subfamily, which is comprised of the α-subunits Gα12 and Gα13, has been implicated in such cellular processes as Rho-dependent cytoskeletal shape changes, activation of JNK, and stimulation of Na^+/H^+ exchange [90].

The small G-proteins (also called small GTP-binding proteins) are monomeric proteins with a low molecular weight of 20–40 kDa with intrinsic GTP-hydrolyzing activity. Therefore, they are also called GTPases [91]. More than 100 small G-proteins have been identified and comprise a superfamily. They are structurally divided into at least five subfamilies: Ras (Ras, Rap, Rad, Ral, Rin, and Rit), Rho (Rho, Rac, Cdc42, and Rnd), Rab, Sar1/ADP ribosylation factor (Arf, Arl, Ard, and Sarl), and Ran [91]. The Ras and Rho small G-proteins are the most investigated of these molecules in the cardiovascular system, and multiple downstream effector protein kinases of small G-proteins have been identified [91]. The Rho family small G-proteins consisting of Rho, Rac, and Cdc42 subfamilies play pivotal roles in many aspects of cellular functions including cytoskeletal reorganization, growth and transformation [92], cell migration [93], adhesion and differentiation of neuronal cells [94], and also in programmed cell death during limb development [95].

The membrane-associated small G-protein Ras is an essential component of all growth factor signaling pathways and is also involved in differentiation and survival. Ras exists in an inactive, GDP-bound and an active, GTP-bound state. In mammals, the Ras superfamily includes three highly homologous proteins, namely H-, N-, and K-Ras [96]. H-Ras is expressed throughout development, with most predominant expression in the adult brain [97].

The Ras superfamily is implicated in both mitogenic and apoptotic pathways and mediates the upregulation of vascular endothelial growth factor by triggering the MAPK pathway [96]. Mutations in ras genes can produce permanent activation, being found in 20–25% of all human tumors and in up to 90% of specific tumor types [98]. The Ras signaling pathway initiates its interaction with its first effector protein, Raf-1, which is a threonine/serine kinase. The Ras/Raf complex is involved in the signaling steps leading to the induction of intracellular oxidative stress [99]. Mechanical stress stimulates the secretion of angiotensin II from cardiac myocytes, and angiotensin II activates Src family TKs and Ras in cardiac myocytes [40]. c-Src is a member of a family of cytoplasmic TKs that are involved in many biological processes such as cell proliferation, migration, and differentiation [100,101]. Stimulation of receptor tyrosine kinases activates the Raf-1-MAPK/ERK kinase (MEK)–ERK cascade through Ras in many cell types [102]. Activation of the Src family of TKs and Ras is required for angiotensin II-induced activation of ERKs in smooth muscle cells [103].

Rho family members including RhoA, Rac1, and Cdc42 regulate the activity of MAPK subfamilies such as JNK and p38 MAPK in HeLa and COS-7 cells [104,105]. Rho family proteins are critically involved in regulation of the cytoskeleton, such as formation of actin microfilaments and focal adhesions [106]. Rho proteins are involved in the integrin-dependent activation of ERKs [107], and they activate ERKs through activation of p21-activated kinase [108]. Integrins activate ERKs when bound to the extracellular matrix [109,110]. PI3K regulates the activation of Rac, which, in turn, activates p21-activated kinase resulting in the induction of p38 MAPK and JNK [108].

Rac proteins are involved in the development of cardiac hypertrophy [111,112]. Rac proteins are essential for the assembly of the plasma membrane NADPH oxidase and lead to the production of ROS in phagocytic cells [113]. The regulators of G-protein signaling 14 (RGS14) is an integrator of G-protein and MAPK signaling pathways [114].

ANGIOTENSIN RECEPTORS

Angiotensin II is a vasoconstrictor with proliferative effects involved in the regulation of salt and water homeostasis and pathological remodeling of the heart and vessels usually through AT_1-subtype receptors [115]. AT_1 receptors belong to the seven-membrane-domain superfamily of GPCR and are expressed in VSMCs, heart, lung, brain, liver, kidney, and adrenal glands [116]. AT_1 receptors are coupled to several intracellular signaling molecules, including phospholipases A_2, C, and D, adenylate cyclase, voltage-dependent Ca^{2+} channels, and kinases involved in phosphorylation cascades [9,117]. Stimulation of the AT_2-subtype receptor leads to vasodilation and inhibition of vascular smooth muscle growth [118]. AT_2 receptors are expressed in developing arteries during embryogenesis and seem to play a role in curbing vascular smooth muscle

growth to determine the ultimate thickness of the vascular wall [118,119]. Although interesting and important roles of the AT_2 receptor continue to be defined, most of the known functions of angiotensin II are related to AT_1 receptor activation. Circulating or locally generated angiotensin II may also be able to alter gene expression via activation of second messenger systems [10]. In experimental studies, both angiotensin II receptor density and mRNA for the AT_1 receptor subtype are increased [120]. This receptor is also implicated in cardiac hypertrophy in rats [120,121]. Collagen synthesis is increased by angiotensin II in cardiac fibroblasts by activation of AT_1 receptors [122]. Angiotensin II is also directly responsible for lesions that increase coronary artery permeability [123], allowing diffusion of growth factors into the myocardial interstitium, and has a cytotoxic effect on cardiac myocytes, causing cellular necrosis and fibrosis [124]. Angiotensin II administration in rats causes a substantial increase in vascular superoxide formation and impairs endothelium-dependent vasodilation [125]. Treatment with liposome-encapsulated SOD or the membrane-permeable SOD mimetic tempol blunts the increase in blood pressure caused by angiotensin II and preserves the bioavailability of nitric oxide, suggesting that the increase in vascular superoxide formation is critical for the response to the octapeptide [126]. Increased vascular NADPH oxidase activity and increased expression of its subunit p22phox are observed in angiotensin II-treated rats *in vivo* [127].

AT_1 receptor activation leads to the production of ROS in the vessel wall, in part because the AT_1 receptor is linked to activation of an NADPH oxidase in vascular cells [9]. This oxidase system, which has similarities to the neutrophil oxidase, is a major source of ROS in ECs, VSMCs, and adventitial fibroblasts [128]. ECs contain all of the subunits of the neutrophil oxidase, including gp91phox, p47phox, p67phox, p22phox, and the small GTPase Rac1 [129,130]. In contrast, VSMCs contain NOX (nonphagocytic oxidase), which seems to replace gp91phox as one of the membrane components [114]. Angiotensin II- and endothelin-1-mediated transactivation of the EGF receptor is mediated by several intermediary signaling molecules including Ca^{2+}, ROS, metalloproteases that generate EGF-like ligands, and TKs such as c-Src [131,132]. c-Src is one of the first kinases to be activated by angiotensin II and it plays a key role in VSMC signaling events [133,134]. Consistent with the *in vivo* observations, *in vitro* data supports the idea that angiotensin II stimulation increases superoxide and hydrogen peroxide generation and has been demonstrated in cultured VSMCs, ECs fibroblasts, cardiac myocytes, and fibroblasts [135]. Furthermore, angiotensin II-induced superoxide inactivates nitric oxide and promotes the formation of peroxynitrite, leading to endothelial dysfunction [136]. Further, NOX is essential for angiotensin II-induced ROS generation in the cardiovascular system [137,138]. ROS are involved in angiotensin II-induced proliferation and endothelin-1 gene expression, and combined AT_1 and ET_A receptor antagonism plus antioxidants may prevent excessive cardiac fibrosis [139].

EPIDERMAL GROWTH FACTOR RECEPTOR

Many of the proliferative and mitogenic effects of GPCRs are mediated through transactivation of the epidermal growth factor receptor (EGFR) [140]. EGFR (HER1 or erbB1) is a 170-kDa cell-surface-bound glycoprotein that belongs to the erbB family of receptor TKs, the RTKs (other members are erbB2, 3, and 4) [141]. Epidermal growth factor (EGF) or heparin binding changes EGF receptor to homo- or hetero-dimers that in turn results in autophosphorylation of the intracellular TK activity [142]. The autophosphorylated dimer recruits adaptor proteins to lead to activation of multiple signaling pathways, including the classical cytosolic ras/raf/MAPK pathway [143]. Binding of EGF to its cognate receptors leads to the tyrosine phosphorylation of several substrates including EGFRs. Such phosphorylation sites serve as docking sites for the binding of adapter proteins that contain structural motifs involved in protein–protein interactions. These adapter proteins include Grb2 and SEM5 [144], which possess an Src homology 2 (SH2) domain and two SH3 domains, and Shc (an adapter protein in the Ras pathway), which possesses a phosphotyrosine binding (PTB) domain, an SH2 domain, and an SH3 domain [145]. Both PTB and SH2 domains bind phosphotyrosine-containing polypeptides [145]. Shc is also a substrate for the EGFR and, upon tyrosine phosphorylation, binds to the SH2 domain of Grb2 [146]. Thus, activation of EGFRs results in the association of Grb2 to the EGFR, which also results in the recruitment of Son of Sevenless (SOS) (SOS refers to a set of genes encoding guanine nucleotide exchange factors that act on the Ras subfamily of small GTPases), which binds to the SH3 domains of Grb2 [147]. SOS stimulates the exchange of GDP bound to Ras for GTP [147] and initiates a protein kinase cascade. β-Arrestin-dependent, G-protein-independent activation of the EGFR by the β_1-adrenoceptor confers cardioprotection in mice chronically stimulated with catecholamines [148]. Heparin-binding epidermal growth factor (HBEGF) knockout mice develop severe heart failure caused by diminished cardiac function, enlarged ventricular chambers, and enlarged cardiac valves. Interestingly, mice lacking EGFR and cardiac-specific deletion of ErbB2 have similar cardiac defects. These results imply that HBEGF-mediated signaling by ErB2 and EGFR is essential for heart development [149–151]. In addition, other EGF receptors (ErbB3 and ErbB4) are crucial for cardiac morphogenesis since mice deficient in ErbB3 demonstrate heart valve malformation and ErbB4 null mice die early *in utero* due to abnormal ventricular trabecularization [152,153].

ENDOTHELIN SYSTEM

The endothelin family consists of three structurally related peptides, ET-1, ET-2, and ET-3 [154]. In the vasculature, proendothelin may be released from the nonluminal surface of the ECs and converted to mature endothelin

extracellularly by membrane-bound endothelin-converting enzymes, which are neutral metalloproteinases. Two separate receptors for endothelin have been identified: ET_A receptors, which are most commonly found on VSMCs, induce vasoconstriction by increasing intracellular calcium, and ET_B receptors located on ECs, which stimulate the release of vasodilating agents such as nitric oxide and prostacyclin. However, ET_B receptors also appear on VSMCs where they stimulate vasoconstriction. Theoretically, either selective blockade of ET_A receptors or nonselective blockade of both ET_A and ET_B receptors could dilate local vessels. Endothelins are potent vasoconstrictor peptides through coupling to ET_A receptors. Endothelin does not appear to be stored in ECs but is synthesized *de novo* in response to several substances (thrombin, angiotensin II, and cytokines) or physical stimuli (shear stress, hypoxia). ET_A receptor activation leads to myocyte hypertrophy, mediated by Gαq stimulation. In addition to its vasoconstrictor actions, endothelin may stimulate release of renin and aldosterone and enhance the conversion of angiotensin I to angiotensin II in ECs [155]. A further important action is derived from its mitogenic potential. It stimulates proliferation of VSMCs and fibroblasts and increases the expression of certain proto-oncogenes [156]. Angiotensin II and endothelin-1 regulate p38 MAPK, JNK, and ERK5 in human VSMCs in a ROS-dependent mechanism [157].

MITOCHONDRIAL ROS PRODUCTION AND MAPK PATHWAY

Mitochondria are energy-producing cellular organelles responsible for regulation of cytosolic calcium levels and tissue oxygen gradients, hydrogen peroxide signaling, and the modulation of apoptosis. Maintaining the cellular respiration is the most important task mediated by the mitochondrial electron cycle chain to produce ATP as a source of cellular energy. Mitochondria utilize more than 90% of cellular oxygen and most of it is transformed to water at complex IV of the mitochondrial electron transport chain. Approximately 1–2% of the oxygen atoms receive electrons directly from complexes I and III to form superoxide. The electron transport chain is a sequence of electron transport carriers (complexes I, II, III, and IV) that transport electrons gradually to molecular oxygen. Coenzyme Q shuttles the electrons between complex I or II and III and further between III and IV. The energy for ATP synthesis is provided by the oxidation of NADH and $FADH_2$ by the electron transport chain. Physiologically, more than 98% of electrons are tightly coupled with the production of ATP and only 1–2% "leak" to form superoxide and are scavenged primarily by mitochondrial MnSOD. Other pathways scavenging ROS include catalase, peroxidase, and low molecular weight antioxidants including ascorbate, glutathione, and phenolic compounds. However, when these enzymes cannot convert ROS such as the superoxide radical to water fast enough, oxidative damage occurs and accumulates in the mitochondria. A schematic diagram for antioxidant defense mechanism is presented in

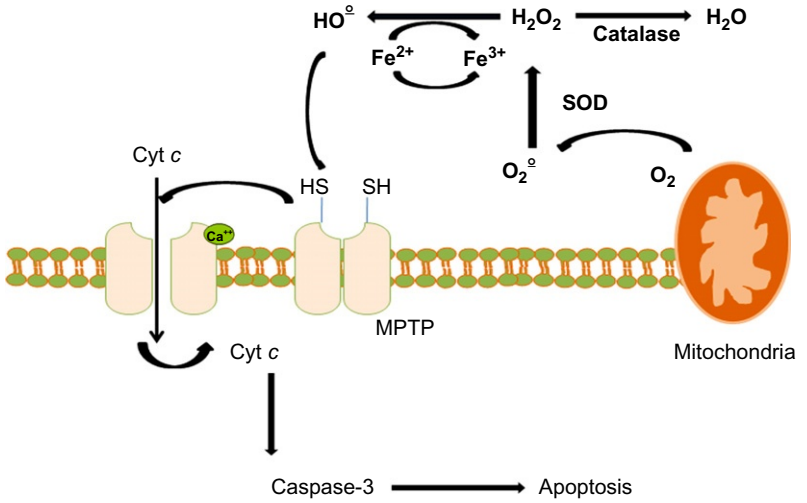

FIGURE 3 Schematic process of mitochondrial ROS generation and MPTP opening for cytochrome *c* release and triggering apoptosis via caspase activation.

Fig. 3. High glucose concentrations result in increased metabolic input into mitochondria, which overwhelms the electron transport chain causing mitochondrial hyperpolarization, leading to electron backup within the electron transport chain and ROS overproduction [158,159]. In the intermembrane space, the steady-state concentration of superoxide is controlled by the copper and zinc-containing SOD (Cu, Zn-SOD) [160].

Mitochondria and MAPK signaling process are closely related as both are responsible for the cell survival and proliferation and growth. The mitochondria express MAPK proteins. MAPK activation is a common target of exogenous or endogenously generated ROS [161]. Mitogen-induced ERK2 activation is redox sensitive in T cells [162]. Mitochondria-derived ROS generation may play a regulatory role in cell function and signaling and affect activation of a proapoptotic MAPK, JNK1 [163]. ROS from mitochondria is the upstream activator of ERK2 in EC hypoxia [164]. Moreover, the opening of mitoK_{ATP} channels by diazoxide increases superoxide generation in the heart indicating the important role of these K_{ATP} channels in the activation of p38 and JNK MAPK in response to angiotensin II stimulation [165].

NADPH AND MAPK PATHWAY

Superoxide anion can be dismutated enzymatically or nonenzymatically to hydrogen peroxide, which is then scavenged by catalase or peroxidase to

prevent oxidative stress damage to macromolecules, membranes, and DNA [166]. Almost all cell types in the cardiovascular system including cardiomyocytes, endothelial, and VSMCs, fibroblasts, and infiltrating inflammatory cells generate ROS [167]. Potential sources of ROS in these cell types include the mitochondrial electron transport chain, xanthine oxidases, "uncoupled" nitric oxide synthases, cytochrome P450, and NADPH oxidases [167]. Among these sources, the NADPH oxidases may be considered unique in that they generate ROS in a highly regulated manner, whereas ROS are generated as a by-product of enzymatic activity for all the other sources [167]. Furthermore, NADPH oxidases can stimulate further ROS production from one or more of the above enzymes, thereby being able to act as initiating sources of ROS. Five NADPH oxidase isoforms each encoded by a separate gene and with distinct tissue distribution have been identified [129,168]. These isoforms are distinguished by the presence of distinct catalytic subunits, Nox1–Nox5, which mediate the electron transfer process. In the cardiovascular system, Nox1 is expressed mainly in VSMCs. Nox2 is expressed in ECs, cardiomyocytes, fibroblasts, and some VSMCs [167]. Nox4 is expressed in ECs, VSMCs, cardiomyocytes, and fibroblasts [167]. Nox3 is not expressed in cardiovascular cells while Nox5 has been reported in human ECs and smooth muscle cells but is not found in rodents [167]. The cytosolic components of the classical NADPH oxidase, p47phox, p67phox, p40phox, and Rac1, have been detected at both mRNA and protein levels in most cardiovascular cells except for p67phox which could not be detected in cultured VSMCs [169,170]. A schematic diagram for GPCR (AT_1)-stimulated NADPH oxidase through small GTPase Rac and redox signal generated for MAPK activation is presented in Fig. 4.

In ECs, hydrogen peroxide activates p38 MAPK and its downstream target, MAPKAP kinase 2/3, leading to phosphorylation of heat-shock protein 27 [170]. ERK1/2 activation also seems to be redox sensitive in this cell type, based on the observation that shear stress-induced ERK1/2 phosphorylation is inhibited by antioxidants and dominant-negative Rac-1 [171]. In neonatal rat ventricular myocytes, all three MAPKs (ERK1/2, p38 MAPK, and JNK) are activated by hydrogen peroxide [172]. NADPH oxidase activity can be regulated in a growth-dependent manner [173]. A role for NADPH oxidase activity has been reported in TNFα- and fibroblast growth factor-induced c-Fos expression and TNFα-induced JNK1 activation in chondrocytes [173].

PHENOLIC COMPOUNDS AS NEW THERAPEUTIC AGENTS FOR TARGETING MAPKs

Dietary polyphenols represent a group of secondary metabolites found abundantly in nature. Polyphenolic compounds produce relevant biological functions, such as protection against oxidative stress, cellular signaling, and

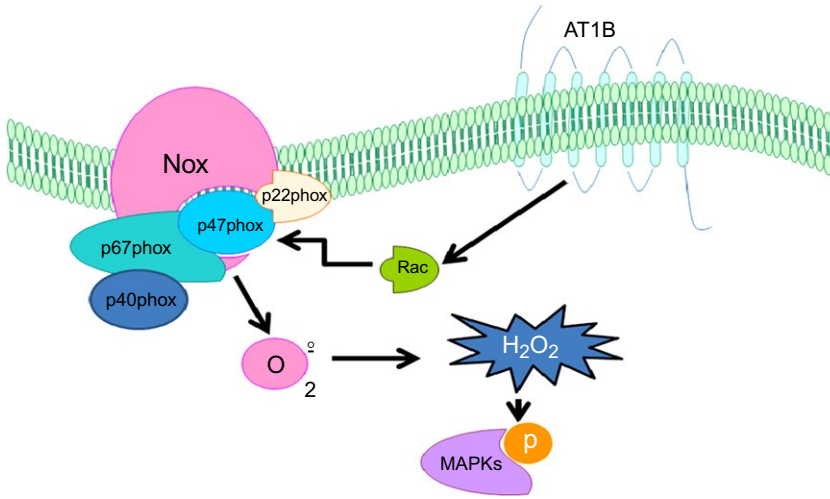

FIGURE 4 G-protein-coupled receptor (AT_1) stimulates NADPH oxidase through small GTPase Rac and redox signal generated for MAPK activation.

apoptosis. Most of these biological functions are attributed to their intrinsic antioxidant capabilities. The structure of natural polyphenols varies from simple molecules, such as phenolic acids, to highly polymerized compounds, such as condensed tannins (Fig. 5). Flavonoids are a group of more than 4000 polyphenolic compounds that occur naturally in foods of plant origin. These compounds share a common phenylbenzopyrone structure (C6–C3–C6). Possible effects of phenolic acids on the cardiovascular system are presented in Fig. 6 and individual effects are summarized in Table 3. Apigenin is reported to be the most potent inhibitor of COX-2 and iNOS expression in LPS-stimulated mouse macrophages [188] and suppresses inflammatory responses through inactivation of NF-κB [188,189]. Kaempferol and quercetin reduce the activity of iNOS and COX-2 by suppressing the signaling of STAT1, NF-κB, and AP-1 in LPS- or cytokine-stimulated macrophages and HUVECs [190,191]. Flavonoids such as (−)-epigallocatechin gallate (EGCG) and quercetin attenuate oxidative stress-induced apoptosis, nuclear condensation and fragmentation, and the expression of Bax-2 and caspase-3 cleavage but restored the expression of Bcl-2 protein in ECs [192]. Angiotensin II stimulation activates TKs such as focal adhesion kinases, Janus kinases, the receptor kinases, EGF, and platelet-derived growth factor. Several polyphenolic compounds have potent angiotensin-converting enzyme inhibitory activity [193]. Ferulic acid reduces angiotensin-converting enzyme activity in hypertensive obese rats [194] and inhibits VSMC proliferation due to angiotensin II

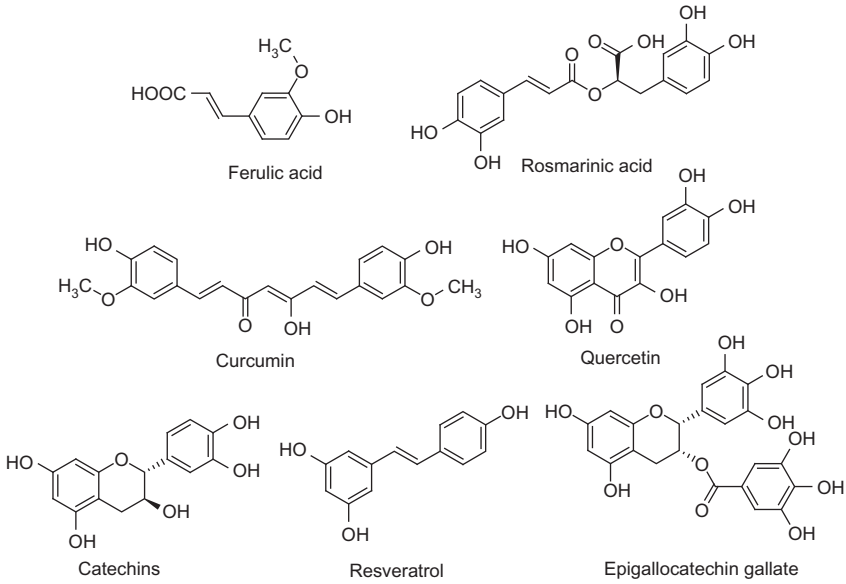

FIGURE 5 Phenolic and polyphenolic compounds.

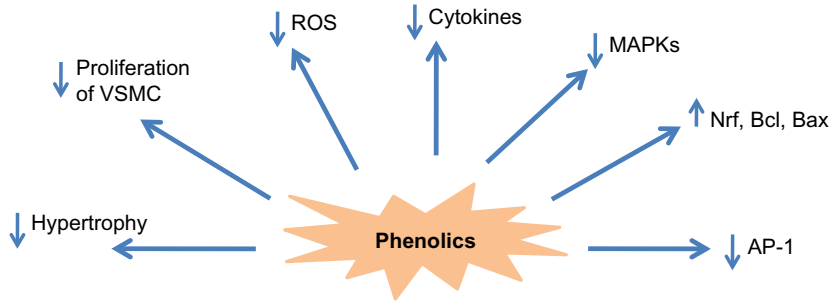

FIGURE 6 Schematic effects of phenolic acid on cardiovascular system.

stimulation [174]. Phenolic compounds can exert modulatory actions in cells by interacting with a wide spectrum of molecular targets central to the cell signaling machinery. These include MAPKs, PKC, serine/threonine protein kinase Akt/PKB, phase II antioxidant detoxifying enzymes, downregulation of proinflammatory enzymes (COX-2 and iNOS) through the activation of peroxisome proliferator-activated receptor gamma (PPARγ), regulation of calcium homeostasis, inhibition of PI3K, TKs, NF-κB, and c-Jun [195].

TABLE 3 Beneficial Effects of Known Phenolic Compounds in Different Models of Cellular Hypertrophy and Proliferation Affecting the MAPK Signal Transduction Pathways

Antioxidant	Experimental Model	Activity	Reference
Ferulic acid	Vascular smooth muscle cell (VSMC)	ANG-II-induced VSMC proliferation markedly inactivated the ERK1/2 and JNKs, while no effect on p38	[174]
Rosmarinic acid	H9C2 cardiac muscle cells	Rosmarinic acid can inhibit ADR-induced apoptosis in H9C2 cardiac muscle cells by inhibiting ROS generation and JNK and ERK activation	[175]
Curcumin	HUVECs	Curcumin reduced intracellular ROS, phosphorylation of JNKs, p38, and STAT-3 in TNFα-stimulated HUVECs	[176]
Quercetin	Rats	The activities of ERK1/2, p38 MAP kinase, Akt, and GSK-3β were increased with pressure overload and attenuated by quercetin treatment	[177]
Quercetin	HUVEC	Hydrogen peroxide-induced endothelial cell apoptosis was abolished by quercetin, via blocking the phosphorylation of p38 MAPK, JNK, and c-Jun by hydrogen peroxide	[178]
Catechins such as EGCG	VSMCs	Ang II increased the phosphorylation of the ERK1/2, JNK1/2, or p38 MAPKs and mRNA expression of c-Jun and c-fos. The EGCG pretreatment inhibited the Ang II-induced phosphorylation of ERK1/2, JNK1/2, or p38 MAPK, and the expression of c-Jun or c-fos mRNA	[179]
EGCG	Neonatal rat cardiac myocyte	Ang II-induced ERK1/2, p38, and JNK1 activation was completely impaired by EGCG	[180]

TABLE 3 Beneficial Effects of Known Phenolic Compounds in Different Models of Cellular Hypertrophy and Proliferation Affecting the MAPK Signal Transduction Pathways—Cont'd

Antioxidant	Experimental Model	Activity	Reference
EGCG	Male Sprague–Dawley rats, pressure overload model	EGCG attenuated Ang II- and pressure-overload-mediated cardiac hypertrophy	[180]
(−)-EGCG	HUVEC	The phosphorylation of JNK, c-Jun, and p38 MAPK was modestly inhibited in HUVEC treated with (−)-EGCG	[178]
EGCG	Aortic VSMCs from apoE$^{-/-}$ mice	Antiproliferative effects exhibited by EGCG included inhibition of the nuclear translocation of c-Jun and AP-1-binding activity and reduced expression of iNOS	[181]
Resveratrol	Rat aortic smooth muscle cells	Resveratrol suppressed angiotensin II-induced activation of PI3K/AKT, ERK1/2, and p70 S6 kinase and subsequent hypertrophy	[182]
Resveratrol	Bovine aortic smooth muscle cells	Blocking oxLDL-induced activation of ERK1/2 in bovine aortic smooth muscle cells and proliferation	[183]
Resveratrol	Coronary artery smooth muscle cell	Attenuated endothelin-1-evoked protein tyrosine phosphorylation of ERK1/2, JNK1, and p38	[184]
Resveratrol	Cardiac fibroblasts	Resveratrol targeted MEK, an ERK pathway kinase, and ERK activation in the inhibition of cardiac fibroblast mitogenic signaling, proliferation, and differentiation into myofibroblasts	[185]
Resveratrol	Rat aortic smooth muscle cells	Inhibited angiotensin II-induced endothelin-1 gene expression and subsequent proliferation	[186]
Resveratrol	Ischemic heart	Through MAPK/SAPK/cAMP response element-binding protein	[187]

Phenolic Antioxidants Prevent Ischemic Reperfusion Injury by Inhibiting MAPKs

Ischemic heart disease is a major cause of morbidity and mortality. Myocardial ischemia can result in LV remodeling, characterized by thinning of the infarcted myocardium, LV chamber dilation, fibrosis, and hypertrophy of viable myocytes [77]. Early remodeling may be adaptive to maintain LV function, but long-term remodeling contributes to functional breakdown and eventual pump failure [196]. The MAPK family is activated due to ischemic reperfusion in heart [197,198]. Reperfusion injury promotes release of inflammatory cytokines, such as interleukins and TNFα [199,200]. Polyphenolic compounds show antioxidant and antiinflammatory activity in numerous cell lines and *in vivo*. Phenolic antioxidants such as ferulic acid, resveratrol, and EGCG inhibit production of macrophage cytokines such as TNFα and the interleukins [201]. They are also potential inhibitors of NF-κB. Resveratrol prevents damage to the myocardium due to ischemic reperfusion injury in isolated hearts [202]. Caffeic acid phenethyl ester (CAPE) protection appears to be through attenuation of cardiac myocyte death during ischemia-reperfusion injury. CAPE cardiac protection is complemented by direct inhibition of cell death pathways in cardiac myocytes by preventing p38 MAPK phosphorylation and caspase-3 activation, both of which play important roles in ischemia-reperfusion-induced cell death [203]. Curcumin reduces damage to rat heart myoblasts (H9c2 cells) due to ischemia-reperfusion injury by scavenging ROS and involving the NF-κB and JNK pathways [204].

Phenolic Antioxidants Prevent Hypertrophy Signals by Inhibiting MAPKs

We have discussed the effects of the MAPKs on cardiac hypertrophy and remodeling. Natural antioxidants prevent the pathologic hypertrophic response and remodeling. Resveratrol prevents angiotensin II-mediated hypertrophy of cardiomyocytes and aortic smooth muscle cells [205]. Cardiac fibroblast proliferation is vital for ventricular remodeling. Resveratrol inhibits proliferation of cardiac fibroblast by attenuation of ERK1/2 signaling [185]. A resveratrol analog, isorhapontigenin, attenuates cardiac hypertrophy via blocking MAPK signaling transduction pathways in angiotensin II-induced cardiac hypertrophy [206]. Ferulic acid inhibits angiotensin II-induced VSMC proliferation and markedly inactivates ERK1/2 and JNK, while no effect is observed on p38 MAPK [174]. EGCG inhibits angiotensin II-induced VSMC hypertrophy through blockade of JNKs [207]. EGCG also attenuates angiotensin II- and pressure-overload-mediated cardiac hypertrophy by inhibiting NF-κB and AP-1 activation [180]. AP-1 is another transcription factor that plays an important role in the induction of cardiac hypertrophy [208]. ERKs and JNKs activate AP-1 transcriptional factor. Activation of ERK results in

an increase in AP-1 activity via c-fos induction, whereas JNK activation leads to c-Jun induction [208]. Inhibition of the hypertrophic response in transaortic abdominal aortic constriction rats by EGCG is mediated via modulating MAPK signals [209].

CONCLUSION

The role of MAPKs has been well established in many physiological and pathophysiological conditions. The signaling pathway is of prime importance in cardiovascular disease. MAPK inhibitors have shown potential in clinical trials. Further studies on naturally occurring phenolic compounds could lead to potent and selective analogs.

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ABBREVIATIONS

ADR	Adriamycin
AP-1	Activator protein 1
AT1	Angiotensin receptor-1
BMK-1	Big mitogen activated protein kinase-1
COX-2	Cyclooxygenase 2
CRP	C-reactive protein
ECs	Endothelial cells
EGF	Endothelial growth factor
EGCG	Epigallocatechin gallate
ERKs	Extracellular signal-regulated kinases
ETA	Endothelin receptor A
ETB	Endothelin receptor B
Fos, c-Fos	A cellular proto-oncogene belonging to the immediate early gene family of transcription factors
GDP	Guanosine diphosphate
GPCR	G-protein coupled receptor
GRB	Growth factor receptor-bound protein
GTP	Guanosine triphosphate
HSP	Heat shock protein
HUVEC	Human umbilical vein endothelial cells
iNOS	Inducible nitric oxide synthase
JNK	C-Jun N-terminal kinase
LPS	Lipopolysaccharide
MAPK	Mitogen activated protein kinase

MCP-1	Monocyte chemotactic protein-1
MEK-1	Mitogen-activated protein kinase kinase 1
MMP	Matrix metalloproteinase
NADPH oxidase	Nicotinamide adenine dinucleotide phosphate-oxidase
NF-κB	Nuclear factor κB
NOS	Nitric oxide synthase
NOX	NADPH oxidase or Nonphagocytic oxidase
PI3K	Phosphoinositide 3-kinase
PKC	Protein kinase C
PPAR-γ	Peroxisome proliferator-activated receptor gamma
PTK	Protein tyrosine kinase
ROS	Reactive oxygen species
RTK	Receptor tyrosine kinase
SAPK	Stress activated kinase
SH	Src homology
SOD	Superoxide dismutase
SOS	Son of Sevenless
STAT	Signal Transducers and Activators of Transcription family
TNF-α	Tumor necrosis factor alpha
VSMC	Vascular smooth muscle cell

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Chapter 7

Chemistry and Biological Activity of Diterpenoid Alkaloids

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INTRODUCTION

Numerous diterpenoid alkaloids have been isolated from various species of *Aconitum*, *Consolida,* and *Delphinium* (Ranunculaceae) and are classified according to their chemical structure as C_{18}-, C_{19}-, and C_{20}-diterpenoid alkaloids (Fig. 1) [1,2]. The second group includes aconitine (**1**), mesaconitine (**2**), hypaconitine (**3**), and jesaconitine (**4**), which all exhibit extremely high toxicity, whereas the third group includes lucidusculine (**5**), kobusine (**6**), pseudokobusine (**7**), and atisine (**8**), which are far less toxic. The roots of *Aconitum* plants have been used as "bushi," an herbal drug in some prescriptions of traditional Chinese medicine for the treatment of hypometabolism, dysuria, cardiac weakness, chills, neuralgia, gout, and certain rheumatic diseases [3–5].

The pharmacological properties of the C_{19}-diterpenoid alkaloids have been studied extensively and reviewed [5,6]. Alkaloids **1** and **2** are representative toxins that exhibit activity both centrally and peripherally by preventing the normal closing of sodium channels, with their predominant effects on the

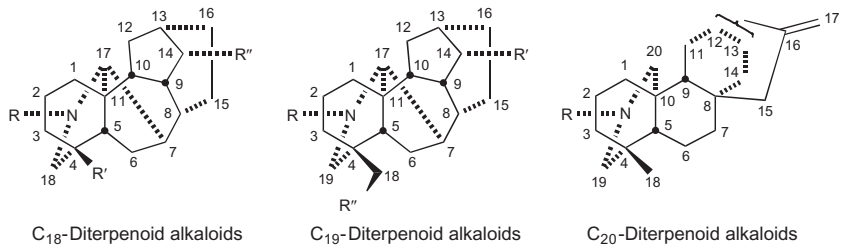

FIGURE 1 Types of diterpenoid alkaloids.

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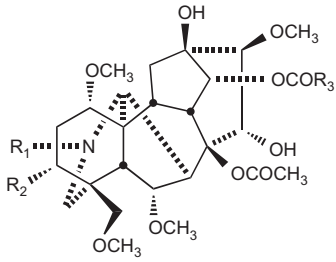
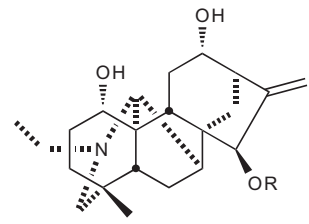
cardiovascular and respiratory systems [7,8]. Although less information has been reported about the pharmacological properties of the C_{20}-diterpenoid alkaloids and their chemically transformed products, certain activities have been reported. Alkaloids **6**, **7**, and 6,11,15-tribenzoylpseudokobusine (**9**) show analgesic activity [9,10]. Alkaloid **5** can cause peripheral and coronary vasodilation [11,12] and reduction of blood pressure [13]. Luciculine (**10**) displays an antagonistic effect on aconitine-induced arrhythmia [7].

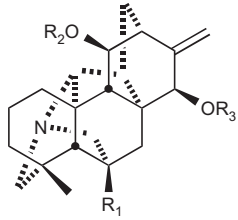
The majority of drugs used in cancer chemotherapy can be categorized as plant alkaloids, alkylating agents, antimetabolites, antibiotics, topoisomerase inhibitors, monoclonal antibodies, and other antitumor agents [14–22]. However, little information on the cytotoxic properties of *Aconitum* alkaloids has been reported despite their extreme toxicities. Two reports on the effects of C_{19}-diterpenoid alkaloids on cancer cells appeared in 2005 and 2006. 8-*O*-Azeloyl-14-benzoylaconine, an aconitine-type C_{19}-diterpenoid alkaloid, exhibited antiproliferative activity [23], and the cytotoxic effects of various C_{19}-diterpenoid alkaloids against tumor cell lines were reported [24]. Since 2007, many C_{19}- and C_{20}-diterpenoids as well as semisynthetic derivatives were evaluated for cytotoxicity by using cell growth, clonogenic, cell cycle distribution, and cell cycle-related assays against four different human tumor cell lines, A172, A549, HeLa, and Raji [25–28] (Fig. 2).



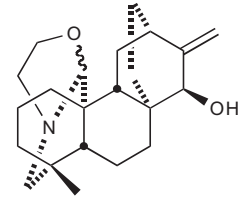
1 : $R_1 = CH_2CH_3$, $R_2 = OH$, $R_3 = C_6H_5$
2 : $R_1 = CH_3$, $R_2 = OH$, $R_3 = C_6H_5$
3 : $R_1 = CH_3$, $R_2 = H$, $R_3 = C_6H_5$
4 : $R_1 = CH_2CH_3$, $R_2 = OH$, $R_3 = C_6H_4OCH_3$ (*p*)



5 : $R = COCH_3$
10 : R = H



6 : $R_1 = R_2 = R_3 = H$
7 : $R_1 = OH$, $R_2 = R_3 = H$
9 : $R_1 = OCOC_6H_5$, $R_2 = R_3 = COC_6H_5$



8 : $C_{22}H_{33}NO_2$

FIGURE 2 Structure of diterpenoid alkaloids **1–10**.

RESULTS AND DISCUSSION

The Peripheral Microcirculation

C_{20}-Diterpenoid alkaloids were examined for peripheral vasoactivities by continuously measuring blood flow in the hind foot of anesthetized mice with a Doppler-type laser blood flowmeter after intravenous (*i.v.*) administration [29–32]. The laser blood flowmeter used in the study was designed subsequent to the finding that there is a linear relationship between the integrated intensity of the power spectrum of scattered light from a given tissue and the volume of red blood cells moving at a low density in the microcirculation [33,34].

Effects of Reference Drugs

Laser flowmetric monitoring of cutaneous blood flow revealed the characteristic effects of reference vasodilators on the peripheral microcirculation in mice (Table 1) [29]. Prazosin produced a continual rise in blood flow during the observation time of 20 min, while nifedipine showed a biphasic pattern, with an immediate increase in blood flow followed by a second rise, which was larger than the initial rise. Hydralazine increased blood flow sharply reaching a peak at 3 min, which was nearly equal to the second peak of nifedipine. The increase in blood flow with hydralazine was followed by a decrease to lower than the basal flow level. Papaverine caused a transitory fall followed by a rise in blood flow. Prazosin is known as a selective α_1-adrenergic antagonist as well as a relatively potent inhibitor of cyclic nucleotide phosphodiesterases and thus is capable of reducing peripheral vascular resistance without secondary reflex tachychardia [35]. In contrast, nifedipine relaxes arterial resistance vessels rather selectively by blocking the entry of Ca^{2+} into smooth muscle cells with little effect on venous pooling, resulting in a reduction in afterload and compensatory increases in heart rate and ejection fraction [36]. Hydralazine causes direct relaxation predominantly of the arteriolar smooth muscle but does not relax venous smooth muscle; the action is believed to be associated with powerful stimulation of the sympathetic nerve endings to release catecholamines [37]. Papaverine directly dilates vascular smooth muscle in combination with an inhibition of cellular cyclic nucleotide phosphodiesterases [38].

Effects of Aconitum Extracts

Aconitum yesoense var. *macroyesoense* (Nakai) Tamura is a plant that grows naturally in the Jozankei district of Hokkaido in Japan, and extracts from the roots were found to contain the C_{19}-diterpenoid alkaloids delcosine (**11**) and 14-acetyldelcosine (**12**) and the C_{20}-diterpenoid alkaloids **5**, **6**, and **7**, as major alkaloids, together with 31 minor alkaloids [39–44]. A methanol extract of the root was shown to increase cutaneous blood flow in the hind foot of mice after peroral (*p.o.*) administration (Table 2). The increased blood flow reached a peak

TABLE 1 Time Courses of Cutaneous Blood Flow in Anesthetized Mice After Intravenous Administration of Reference Drugs with Vasoactivity

	Blood Flow (ml/min/100 g)								
Time (min)	*1*	*2*	*3*	*4*	*5*	*6*	*10*	*15*	*20*
Drugs									
Prazosine (0.2 mg/kg)	0.43	0.18	0.27	0.85	1.42	2.00	2.68*	4.18*	6.07*
Hydralazine (20 mg/kg)	1.00	4.08	6.58**	5.67*	5.42**	3.50*	−0.17	−2.08	−2.58
Papaverine (10 mg/kg)	−1.75	−1.42	−0.67	0.17	1.92	4.00	3.25	2.25	1.58
Nifedipine (0.05 mg/kg)	5.20*	4.07	2.67	3.47	6.84*	6.13	3.23	2.97	2.80
Control	−0.78	0.33	0.43	0.20	0.40	0.25	−0.33	−0.83	−0.45

Blood flow was measured by laser blood flowmeter. Each point is presented as a mean of three to five experiments.
*$p<0.05$, **$p<0.01$: Significantly different from the basal value determined before administration (Student's paired *t*-test).

TABLE 2 Time Courses of Cutaneous Blood Flow in Anesthetized Mice After Peroral Administration of Methanol Extract and Solvent Fractions with Vasoactivity

	Blood Flow (ml/min/100 g)								
Time (min)	*15*	*25*	*35*	*45*	*55*	*65*	*75*	*85*	*95*
Extracts									
MeOH extr. (333 mg/kg)	0	2.15	6.80*	6.05*	4.40	4.40	5.50	3.60	1.15
Water extr. (333 mg/kg)	0	−0.35	−2.15	−2.15	−2.55	−2.80	−2.80	−3.55*	−4.65*
Hexane extr. (50 mg/kg)	0	1.25	1.45*	1.00*	0.35	0.30	0.55	0.00	−0.20
$CHCl_3$ (50 mg/kg)	0	1.14	1.67	2.36	2.69*	2.61*	0.39	0.28	−1.28
Water extr. (50 mg/kg)	0	0.15	−0.60	−0.45	−0.35	−0.60	−1.15	−1.40	−1.80*
Control	0	−0.20	−1.45	−2.25	−2.45	−2.70	−2.90	−3.30	−2.85

Blood flow was measured by laser blood flowmeter. Each point is presented as a mean of five experiments.
*$p < 0.05$: Significantly different from the basal value determined before administration (Student's paired *t*-test).

at 40 min and the blood flow then decreased to 4–5 ml/min/100 g during 50–80 min after administration. The methanol extract was partitioned into hexane, chloroform, and water fractions [32]. The chloroform extract contained diterpenoid alkaloids and caused increased blood flow reaching a peak at 60 min in the hind foot of mice after *p.o.* administration (Table 2).

Effects of Diterpenoid Alkaloids

Among the five major alkaloids from the *A. yesoense* root, alkaloids **6** and **7** were fairly comparable to hydralazine regarding the intensity of increased cutaneous blood flow in the murine hind foot and the peak time of the increase after *i.v.* administration (Table 3) [29]. However, with **6**, the increase in blood flow was then followed by a sharp decrease to less than basal level lasting until the end of observation, whereas with **7**, a more gradual decline occurred after the peaked flow, as was also observed with nifedipine. Compared to **6** and **7**, alkaloids **5**, **11**, and **12** had less effect on blood flow, with **12** producing an early increase followed by a quick return to the basal level and a subsequent decrease to less than basal level. 1-Acetyldelcosine (**13**) did not show significantly different activity from the parent alkaloid (**11**). Of the lucidusculine-related compounds (Fig. 3), **10** and dehydroluciculine (**14**), as well as their 12-acetyl derivatives [12-acetyllucliculine (**15**), 12-acetyldehydroluciculine (**16**)], were approximately two times more active than **5** in increasing blood flow immediately after their administration, but this early effect was transitory and they differed as to whether blood flow was thereafter increased or decreased. On the other hand, 1-acetyllucliculine (**17**) was unique; it slowly increased blood flow to a single peak of nearly equal magnitude to the peaks of **10** and **14**–**16**.

In contrast, dehydrolucidusculine (**18**) showed a papaverine-like action producing a transient fall of blood flow before a gradual increase. In summary, alkaloids, especially of the C_{20}-diterpenoid type, from the root of *A. yesoense* are postulated to have varying degrees of peripheral vasodilating activity in mice, probably due to their direct action on the cutaneous microvasculature, as also shown by hydralazine.

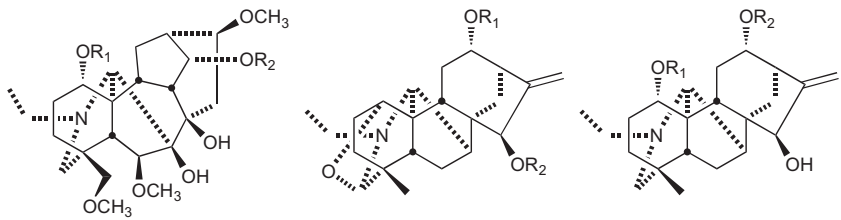
Effects of Alkaloids and Derivatives

Alkaloid **6**, 15-acetylkobusine (**19**), and 15-benzoylkobusine (**20**) were injected intravenously in mice at doses of 1 and 5 mg/kg (Table 4) [30]. Alkaloid **6** was barely effective at 1 mg/kg, resulting in a very slight but not significant increase in blood flow immediately after the injection. At the same dosage, the two derivatives **19** and **20** significantly increased blood flow to nearly the same extent and eventually gave an almost identical pattern of blood flow over the time course. At the higher dosage of 5 mg/kg, **6** produced a gradual increase in blood flow to reach a maximum that was twofold larger than that produced by **6** at 1 mg/kg. Alkaloids **19** at 5 mg/kg and **20**

TABLE 3 Time Courses of Cutaneous Blood Flow in Anesthetized Mice After Intravenous Administration (20 mg/kg) of Diterpenoid Alkaloids

	Blood Flow (ml/min/100 g)									
Time (min)	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*10*	*15*	*20*
Alkaloids										
5	4.69**	3.19*	1.63	0.50	0.06	0.38	0.63	−0.19	−0.56	−0.75
6	−0.20	8.70**	8.30*	3.76	−0.02	−1.64	−2.81	−4.01*	−5.31**	−4.15*
7	6.85	9.20**	8.05**	6.35*	5.95*	5.95	5.40	2.70	1.05	0.90
10	9.67**	7.75	2.50	1.50	1.58	2.17	3.00	3.42	1.92	0.83
11	2.69	1.01	0.45	−0.01	−0.23	0.35	−0.30	−0.06	−1.23	−0.84
12	5.08*	0.89	−0.86	−1.39**	−1.44*	−1.43*	−1.46	0.29	−1.31	−1.63
13	2.67	−0.33	−0.92	−1.00	−0.92	−0.92	−0.83	−0.92	−0.25	0.33
14	9.42*	0.20	0.53	0.75	0.67	0.70	1.08	1.60**	0.87	1.68
15	3.13	9.38*	5.00*	2.13	0.13	−0.44	−0.25	−0.94	−2.44	−1.81
16	8.42*	1.33	0.17	−0.58	2.75	3.92	4.58	3.83	0.50	−2.00
17	4.42	5.50	6.67	7.42*	8.33*	8.08*	7.42	3.25	2.25	2.67
18	−5.03	0.97	2.57	3.33	3.90	2.60	6.27	3.00	3.57	4.10
Control	−0.83	1.42	1.58	1.75	2.00	1.58	1.08	−0.13	−0.25	−0.38

Blood flow was measured by laser blood flowmeter. Each point is presented as a mean of three to five experiments.
*$p<0.05$, **$p<0.01$: Significantly different from the basal value determined before administration (Student's paired *t*-test).



11 : $R_1 = R_2 = H$
12 : $R_1 = H$, $R_2 = COCH_3$
13 : $R_1 = COCH_3$, $R_2 = H$

14 : $R_1 = R_2 = H$
16 : $R_1 = COCH_3$, $R_2 = H$
18 : $R_1 = H$, $R_2 = COCH_3$

15 : $R_1 = H$, $R_2 = COCH_3$
17 : $R_1 = COCH_3$, $R_2 = H$

FIGURE 3 Structure of diterpenoid alkaloids **11–18**.

at 2.5 mg/kg both increased blood flow to a greater extent than the parent compound **6**, each reaching a peak of almost the same magnitude at the respective peak times (3 min for **19**, 6 min for **20**). The benzoate had a longer delayed peak time compared with the acetate, probably because of an accompanying rapid onset of a decrease in blood flow immediately after the start of the injection. As also depicted in Table 4, pseudokobusine (**7**) at 1 mg/kg increased blood flow to the same extent as **6** at 1 mg/kg and the magnitude of the increase rose as the dose increased to 5 mg/kg. Alkaloids **6** and **7** showed similar patterns of blood flow over the time course after the injection. 15-Acetylpseudokobusine (**21**) at 1 mg/kg reached a prominent plateau of increased blood flow, which was larger in magnitude than that caused by the parent alkaloid **7** or 15-benzoylpseudokobusine (**22**) at the same dosage. In addition, with the low dose of **21**, the blood flow decreased late in the time course. At 5 mg/kg, **21** caused a sharp increase in blood flow that was rapidly replaced by a significant decrease to below the basal level. On the other hand, **22** at 5 mg/kg was unique in that blood flow continued to increase steadily, reaching a level of the same magnitude as the relatively sharp peak produced by the same dose of either the parent **7** or **21** at 4 and 2 min, respectively. The increase caused by **22** at 1 mg/kg, however, was mild, and a very low level of increased blood flow persisted throughout the time course (Fig. 4).

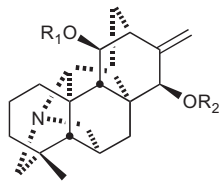
Effects of Substituents on Activity of Kobusine

To compare the effects of substituents on the blood-flow enhancing activity of **6**, the maxima of increased blood flow for **6** and **18** ester derivatives are summarized in Table 5 [30,31]. Alkaloid **6** and the 15-acetate (**19**), 11-acetate (**23**), 11,15-diacetate (**24**), 11-benzoate (**25**), 11-propionate (**26**), 15-propionate (**27**), 11,15-dipropionate (**28**), 11-cinnamate (**29**), 15-cinnamate (**30**), and 11-nicotinoate (**35**) were dosed at 1 and 5 mg/kg; the 15-benzoate (**20**) and 15-nicotinoate (**36**) were dosed at 1 and 2.5 mg/kg; the 11-anisoate (**31**), 15-anisoate (**32**), 11-veratroate (**33**), 11-pivaloate (**37**), and 15-pivaloate (**38**) were dosed at 0.5 and 1 mg/kg; and finally, the 15-veratroate (**34**) was dosed at 0.05 and 0.1 mg/kg. At their highest doses, the acetyl (**19**, **23**, **24**), 11-

TABLE 4 Time Courses of Cutaneous Blood Flow in Anesthetized Mice After Intravenous Administration of Diterpenoid Alkaloids

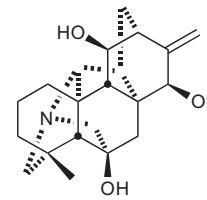
	Blood Flow (ml/min/100 g)									
Time (min)	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*10*	*15*	*20*
Alkaloids										
6 (1 mg/kg)	2.47	−0.43	0.07	0.20	0.43*	0.57*	0.53*	0.07	0.10	0.63
6 (5 mg/kg)	0.87	3.50	4.13	3.60	3.20	5.30	5.43	3.40	1.80	1.23
7 (1 mg/kg)	−0.40	1.38	1.73	1.98*	3.30**	4.20**	3.60**	0.18	−0.43	−0.03
7 (5 mg/kg)	0.38	5.60	5.48	6.73	5.90	4.93	3.00	1.70	0.68	0.63
19 (1 mg/kg)	0.27	0.67	3.20*	4.77**	4.90	2.80	1.77	0.57	−0.20	0.83
19 (5 mg/kg)	2.67	9.73	9.93	6.90	5.93	4.80	3.90	2.43	0.17	3.60
20 (1 mg/kg)	0.77	1.03	4.53*	6.00*	4.40	3.47	2.70	1.57	0.93	1.43
20 (2.5 mg/kg)	−3.95	0.70	6.00*	11.50**	9.45**	9.50**	8.35**	3.15	1.80	1.80
21 (1 mg/kg)	−0.88	5.84	5.44*	4.84*	5.84**	5.34**	4.30*	−0.88	−1.60	−1.64
21 (5 mg/kg)	2.80	7.53*	3.78	1.03	0.28	−1.33	2.08	−2.60	−3.53	−2.73
22 (1 mg/kg)	2.13	3.00	1.93	1.57	1.47*	1.57*	1.53*	1.63*	0.67	−1.00
22 (5 mg/kg)	−0.90	0.87	1.47	2.80	3.07	3.23	4.13*	5.50**	6.93**	5.63*
Control	−0.83	1.42	1.58	1.75	2.00	1.58	1.08	−0.13	−0.25	−0.38

Blood flow was measured by laser blood flowmeter. Each point is presented as a mean of three to five experiments.
*$p<0.05$, **$p<0.01$: Significantly different from the basal value determined before administration (Student's paired *t*-test).



19 : R_1 = H, R_2 = $COCH_3$
20 : R_1 = H, R_2 = COC_6H_5
23 : R_1 = $COCH_3$, R_2 = H
24 : R_1 = R_2 = $COCH_3$

25 : R_1 = COC_6H_5, R_2 = H
26 : R_1 = $COCH_2CH_3$, R_2 = H
27 : R_1 = H, R_2 = $COCH_2CH_3$
28 : R_1 = R_2 = $COCH_2CH_3$
29 : R_1 = $COCH{=}CHC_6H_5$, R_2 = H
30 : R_1 = H, R_2 = $COCH{=}CHC_6H_5$
31 : R_1 = $COC_6H_4OCH_3$ (*p*), R_2 = H
32 : R_1 = H, R_2 = $COC_6H_4OCH_3$ (*p*)
33 : R_1 = $COC_6H_3(OCH_3)_2$ (3,4), R_2 = H
34 : R_1 = H, R_2 = $COC_6H_3(OCH_3)_2$ (3,4)
35 : R_1 = COC_5H_4N, R_2 = H
36 : R_1 = H, R_2 = COC_5H_4N
37 : R_1 = $COC(CH_3)_3$, R_2 = H
38 : R_1 = H, R_2 = $COC(CH_3)_3$



21 : R = $COCH_3$
22 : R = COC_6H_5

FIGURE 4 Structure of diterpenoid alkaloids **19–38**.

benzoyl (**25**) [5 mg/kg], and 15-benzoyl (**20**) [2.5 mg/kg] derivatives showed significant effects. The 15-acetyl (**19**) and 15-benzoyl (**20**) derivatives were remarkably active and produced higher blood flow maxima than **6**. The propionoyl (**26–28**) and cinnamoyl (**29** and **30**) derivatives showed varying degrees of activity at 5 mg/kg. The 15-cinnamate (**30**) at 5 mg/kg resulted in a maximal increased blood flow equal to that produced by **6** at 5 mg/kg, while the remaining four compounds were less active. Derivatives **35** (11-nicotinoate) and **36** (15-nicotinoate) produced extremely significant and comparable maxima in blood flow, but the latter compound (2.5 mg/kg dose) was more potent than the former (5 mg/kg). At the lower dose of 1 mg/kg, derivatives **19**, **20**, **35**, and **36** were the most active compounds. All derivatives (**31**−**38**) tested at 0.1−1 mg/kg showed significant effects. At a dose of 1 mg/kg, derivatives **31**−**33**, **37**, and **38** caused blood flow maxima that were twofold greater than that produced by the parent alkaloid (**6**) at the same dose. Remarkably, the 15-veratroate (**34**) at only 0.1 mg/kg caused a gradual increase in blood flow to a maximum equal to that produced by **6** at 5 mg/kg.

Effects of Substituents on Activity of Pseudokobusine

The results for pseudokobusine compounds are shown in Table 6 [30,31]. The acetates (**21**, **39–41**) and benzoates (**22**, **42**, **43**), as well as the parent pseudokobusine (**7**), produced significant increases in blood flow at 5 mg/kg, and the 11-acetate (**40**) produced the highest maximum blood flow. The effects of **7**, **21** (15-acetate), and **40** were also statistically significant at 1 mg/kg. Compound **21** had essentially the same maximum values at the doses of 1 and 5 mg/kg. Among the propionates (**44–46**) and cinnamates (**47–49**), only the compounds esterified at the C-15 hydroxy group were statistically effective at the low dose. The rank order of potency at both doses was C-15 esters (**46**, **49**) > C-11 esters (**45**, **48**) > C-6 esters (**44**, **47**). The anisoates (**50**−**52**), veratroates (**53**−**55**), *p*-nitrobenzoates (**56**−**58**), nicotinoates (**59**, **60**), and pivaloates (**61**−**63**) produced significant increases in blood flow at doses of 5, 1, or 0.1 mg/kg. The effects of **50**−**60**, **62**, and **63** were also statistically

TABLE 5 Maxima of Increased Cutaneous Blood Flow in Anesthetized Mice After Intravenous Administration of Kobusine and Its Derivatives

	Blood Flow (ml/min/100 g)			Blood Flow (ml/min/100 g)	
Dose (mg/kg)	*1*	*5*	*Dose (mg/kg)*	*1*	*2.5*
Alkaloids			*Alkaloids*		
6	4.50 ± 1.08	9.23 ± 2.00*	**20**	6.00 ± 0.62**	11.50 ± 1.29**
19	5.50 ± 1.23*	11.73 ± 3.43*	**36**	6.90 ± 0.91**	10.60 ± 1.21**
23	3.50 ± 0.70	6.73 ± 0.20**			
24	2.80 ± 0.26	6.77 ± 0.67**	**Dose (mg/kg)**	**0.5**	**1**
25	4.43 ± 0.67*	8.63 ± 0.50**	**31**	4.33 ± 0.87*	9.75 ± 1.02**
26	2.31 ± 0.56	4.75 ± 0.96*	**32**	7.60 ± 1.04**	11.30 ± 1.93**
27	3.00 ± 0.75	6.33 ± 2.14*	**33**	5.17 ± 0.60**	11.00 ± 0.68**
28	4.25 ± 1.52	5.25 ± 1.75	**37**	6.10 ± 1.20*	9.75 ± 2.86*
29	2.81 ± 0.78	6.10 ± 0.89*	**38**	6.85 ± 0.62**	10.55 ± 2.69*
30	5.08 ± 1.75	9.42 ± 2.03*			
35	5.80 ± 0.88*	11.00 ± 1.13**	**Dose (mg/kg)**	**0.05**	**0.1**
Control	2.27 ± 0.58	–	**34**	4.75 ± 0.69*	9.50 ± 1.08**

The data obtained were expressed as the mean ± S.E. of three to five mice of a group.
*$p < 0.05$, **$p < 0.01$: Significantly different from the basal value determined before administration (Student's paired *t*-test).

TABLE 6 Maxima of Increased Cutaneous Blood Flow in Anesthetized Mice After Intravenous Administration of Pseudokobusine and Its Derivatives

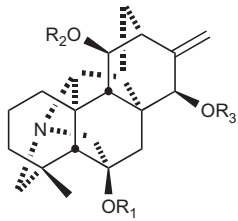
	Blood Flow (ml/min/100 g)			Blood Flow (ml/min/100 g)	
Dose (mg/kg)	*1*	*5*	*Dose (mg/kg)*	*1*	*5*
Alkaloids			*Alkaloids*		
7	4.88 ± 0.28**	9.80 ± 0.73**	**56**	5.85 ± 0.68**	9.45 ± 2.18*
21	7.84 ± 1.28*	8.65 ± 0.42**	**59**	7.15 ± 1.24*	12.00 ± 1.77**
22	4.47 ± 0.95	8.20 ± 0.62**	**61**	6.44 ± 1.67	9.50 ± 1.01**
39	3.93 ± 0.80	7.80 ± 0.46**			
40	5.80 ± 0.93*	13.20 ± 2.03**	**Dose (mg/kg)**	**0.5**	**1**
41	3.60 ± 0.78	7.53 ± 0.91**	**51**	5.56 ± 1.28*	10.10 ± 1.16**
42	3.85 ± 1.03	7.78 ± 0.89**	**54**	8.05 ± 0.56**	12.55 ± 2.41*
43	4.33 ± 1.13	8.02 ± 1.10**	**57**	6.25 ± 1.18*	10.90 ± 1.89*
44	2.65 ± 0.90	3.75 ± 0.66	**60**	6.20 ± 1.12*	11.20 ± 2.30*
45	3.63 ± 0.58	7.38 ± 1.01**	**62**	6.81 ± 0.84**	11.60 ± 2.31*
46	4.25 ± 0.47*	10.31 ± 1.48**	**63**	8.85 ± 1.15**	12.45 ± 1.32*
47	3.40 ± 0.58	4.55 ± 1.12			
48	4.75 ± 1.57	7.69 ± 1.00**	**Dose (mg/kg)**	**0.05**	**0.1**
49	5.50 ± 0.44**	11.00 ± 1.42**	**52**	4.69 ± 0.77*	9.75 ± 1.50**
50	5.20 ± 0.78*	9.19 ± 2.25*	**55**	6.40 ± 0.77**	11.05 ± 1.15**
53	6.75 ± 0.94**	9.63 ± 2.71*	**58**	6.15 ± 0.93*	10.30 ± 1.18**
Control	2.27 ± 0.58	–			

The data obtained were expressed as the mean ± S.E. of three to five mice of a group.
*$p < 0.05$, **$p < 0.01$: Significantly different from the basal value determined before administration (Student's paired *t*-test).

significant at their lowest dose of 1, 0.5, or 0.05 mg/kg. The effects of the C-15 ester derivatives **52**, **55**, and **58** were particularly remarkable. At 0.1 mg/kg, these three most potent derivatives produced a maximum blood flow that was twofold larger than that produced by **7** at 1 mg/kg. Derivative **55** (15-veratroate) produced the highest blood flow maximum, followed closely by **58** (15-*p*-nitrobenzoate) and then **52** (15-anisoate) at the same dose. The maximal blood flow values for C-11 ester derivatives **51**, **54**, **57**, **60**, **62**, and **63** at 1 mg/kg were twofold larger than that produced by **7** at 1 mg/kg and still greater than that produced at 5 mg/kg. Among the C-6 esters, only **59** at 5 mg/kg increased blood flow more than the parent alkaloid at the same dose. Finally, the same general rank order of potency found with **44–49** applied to these ester derivatives, C-15 > C-11 > C-6 (Fig. 5).

Effects of the Hydroxy Group and Double Bond on Activity of Kobusine and Pseudokobusine

The results are shown in Table 7 [31]. Yesoline (**64**) produced statistically significant increases in blood flow at the doses of 1 and 2.5 mg/kg and was more potent than yesonine (**65**), *N*-acetyl-*N*,6-seco-6-dehydropseudokobusine (**66**), and *N*,11,15-triacetyl-*N*,6-seco-6-dehydropseudokobusine (**67**). The 6-dehydro derivatives **65–67**, but not **64**, were also less active than pseudokobusine (**7**). The effects of 15-dehydro-11-veratroylkobusine (**68**) and 11-dehydro-15-veratroylkobusine (**69**) were statistically significant at 1, 0.5, and 0.1 mg/kg, and the latter compound was at least twofold more potent than the former compound at the identical dose (0.5 mg/kg). Compared with the respective parent alkaloids **6** and **7**, isokobusine (**70**) and isopseudokobusine (**71**) had lower effects on blood flow, while dihydrokobusine (**72**) and dihydropseudokobusine (**73**) had greater effects. Thus, changing the exocyclic methylene to



39 : $R_1 = COCH_3$, $R_2 = R_3 = H$
40 : $R_1 = R_3 = H$, $R_2 = COCH_3$
41 : $R_1 = R_2 = R_3 = COCH_3$
42 : $R_1 = COC_6H_5$, $R_2 = R_3 = H$
43 : $R_1 = R_3 = H$, $R_2 = COC_6H_5$
44 : $R_1 = COCH_2CH_3$, $R_2 = R_3 = H$
45 : $R_1 = R_3 = H$, $R_2 = COCH_2CH_3$
46 : $R_1 = R_2 = H$, $R_3 = COCH_2CH_3$

47 : $R_1 = COCH{=}CHC_6H_5$, $R_2 = R_3 = H$
48 : $R_1 = R_3 = H$, $R_2 = COCH{=}CHC_6H_5$
49 : $R_1 = R_2 = H$, $R_3 = COCH{=}CHC_6H_5$
50 : $R_1 = COC_6H_4OCH_3$ (*p*), $R_2 = R_3 = H$
51 : $R_1 = R_3 = H$, $R_2 = COC_6H_4OCH_3$ (*p*)
52 : $R_1 = R_2 = H$, $R_3 = COC_6H_4OCH_3$ (*p*)
53 : $R_1 = COC_6H_3(OCH_3)_2$ (3,4), $R_2 = R_3 = H$
54 : $R_1 = R_3 = H$, $R_2 = COC_6H_3(OCH_3)_2$ (3,4)
55 : $R_1 = R_2 = H$, $R_3 = COC_6H_3(OCH_3)_2$ (3,4)
56 : $R_1 = COC_6H_4NO_2$ (*p*), $R_2 = R_3 = H$
57 : $R_1 = R_3 = H$, $R_2 = COC_6H_4NO_2$ (*p*)
58 : $R_1 = R_2 = H$, $R_3 = COC_6H_4NO_2$ (*p*)
59 : $R_1 = R_3 = H$, $R_2 = COC_5H_4N$
60 : $R_1 = R_2 = H$, $R_3 = COC_5H_4N$
61 : $R_1 = COC(CH_3)_3$, $R_2 = R_3 = H$
62 : $R_1 = R_3 = H$, $R_2 = COC(CH_3)_3$
63 : $R_1 = R_2 = H$, $R_3 = COC(CH_3)_3$

FIGURE 5 Structure of diterpenoid alkaloids **39–63**.

TABLE 7 Maxima of Increased Cutaneous Blood Flow in Anesthetized Mice After Intravenous Administration of Derivatives

	Blood Flow (ml/min/100 g)			Blood Flow (ml/min/100 g)	
Dose (mg/kg)	*1*	*5*	*Dose (mg/kg)*	*1*	*5*
Alkaloids			*Alkaloids*		
7	$4.88 \pm 0.28^{**}$	$9.80 \pm 0.73^{**}$	**6**	4.50 ± 1.08	$9.23 \pm 2.00^{*}$
65	2.83 ± 1.20	5.42 ± 1.42	**70**	$4.00 \pm 0.57^{*}$	$7.80 \pm 0.89^{**}$
66	1.63 ± 0.39	$3.42 \pm 0.36^{*}$	**72**	$5.20 \pm 0.82^{*}$	$11.00 \pm 1.68^{**}$
67	$2.85 \pm 0.27^{*}$	4.92 ± 1.42			
71	$4.75 \pm 0.47^{**}$	$8.00 \pm 1.48^{*}$	**Dose (mg/kg)**	**0.5**	**1**
73	$6.45 \pm 0.93^{**}$	$11.60 \pm 2.14^{**}$	**68**	$4.65 \pm 0.77^{*}$	$10.60 \pm 2.25^{*}$
Dose (mg/kg)	**1**	**2.5**	**Dose (mg/kg)**	**0.1**	**0.5**
64	$6.33 \pm 0.68^{**}$	$11.42 \pm 0.98^{**}$	**69**	$6.25 \pm 1.16^{*}$	$12.00 \pm 2.10^{**}$

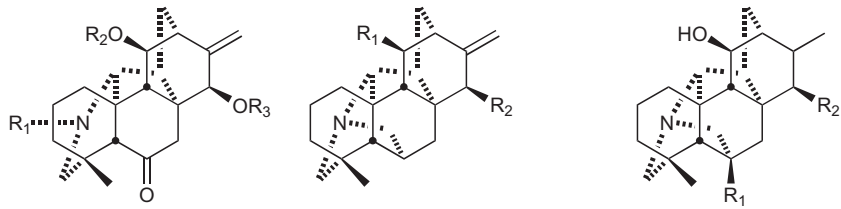
The data obtained were expressed as the mean ± S.E. of three to five mice of a group.
$^{*}p < 0.05$, $^{**}p < 0.01$: significantly different from the basal value determined before administration (Student's paired *t*-test).

a methyl group increased potency, while a subsequent change of the C-15 hydroxy group to a C-15 ketone carbonyl decreased potency (Fig. 6).

Summary of Effects on Peripheral Microcirculation

Both alkaloids **6** and **7** share an identical dose–response line for the ability to increase peripheral blood flow within the same dose range in mice [30]. Therefore, the two alkaloids may have similar modes of action. However, regarding the time course of blood flow after *i.v.* injection, while administration of **6** at a high dose of 20 mg/kg resulted in a rapid reversal of the initial blood flow increase to a level below the basal flow (Table 3), **7** produced a large monophasic increase followed by a gradual decrease to the basal flow at the same dose. It is assumed that such a counter effect on peripheral blood flow, described as hydralazine-like, may be commonly produced by both **6** and **7** at a high dose and that the presence of the additional hydroxy group at C-6 in the pseudokobusine molecule may contribute to diminishing such counter activity, while enhancing the ability to increase blood flow.

With the exception of the hydroxy groups at C-11 and C-15 in **6** and at C-6, C-11, and C-15 in **7**, alkaloids **6** and **7** lack other reactive substituents. Generally, the dehydro derivatives (**64**–**71**) with C═O rather than OH at C-6, C-11, or C-15 had lower effects on blood flow compared with the parent alkaloids **6** or **7** or corresponding derivatives (e.g., compare **69** and **34**). An OH group at C-6 could augment the effect on blood flow as seen by the comparison of **55** with **34**. The results of this study suggest that the hydroxy groups are essential for action on the peripheral vasculature leading to dilation. While esterification of the C-6 OH group of pseudokobusine (**7**) did not have much effect on activity, esterification of the C-15 OH could enhance activity relative to the parent alkaloids more than esterification of the C-11 OH group. Acetyl, benzoyl, propionyl, cinnamoyl, anisoyl, veratroyl, *p*-nitrobenzoyl, nicotinoyl, and pivaloyl substitutions of alkaloids **6** and **7** were advantageous, and the effects of kobusine 15-veratroate (**34**), pseudokobusine 15-anisoate (**52**), 15-veratroate (**55**), and 15-*p*-nitrobenzoate (**58**) on blood flow were



64 : $R_1 = CH_3$, $R_2 = H$, $R_3 = COC_6H_4OCH_3$ (*p*)
65 : $R_1 = CH_3$, $R_2 = R_3 = H$
66 : $R_1 = COCH_3$, $R_2 = R_3 = H$
67 : $R_1 = R_2 = R_3 = COCH_3$

68 : $R_1 = OCOC_6H_3(OCH_3)_2$ (3,4), $R_2 = O$
69 : $R_1 = O$, $R_2 = OCOC_6H_3(OCH_3)_2$ (3,4)

70 : $R_1 = H$, $R_2 = O$
71 : $R_1 = OH$, $R_2 = O$
72 : $R_1 = H$, $R_2 = OH$
73 : $R_1 = R_2 = OH$

FIGURE 6 Structure of diterpenoid alkaloids **64–73**.

remarkable. The maximal blood flow reached with these four compounds at 0.1 mg/kg was equivalent or better than that produced by **7** at 5 mg/kg. Alkaloids **72** and **73** with a methyl group at C-16 increased blood flow to a greater extent than did the parent alkaloids **6** and **7**, respectively, which have an exocyclic methylene at this position.

The Cytotoxicity Against Human Tumor Cells

In order to investigate the pharmacological properties of natural diterpenoid alkaloids and their novel derivatives, compounds were tested against the growth of various tumor cell lines, including A172 human malignant glioma, A549 human lung cancer, HeLa human epitheloid cervix carcinomae, and Raji non-Hodgkin's lymphoma. In order to obtain diterpenoid alkaloids for rapid testing of the structure–activity relationships among substituted diterpenoid alkaloids, the readily available kobusine (**6**), pseudokobusine (**7**), and luciculine (**10**) were used as templates for functional group transformations. The natural alkaloids **6**, **7**, 14-acetyldelcosine (**12**), dehydrolucidusculine (**18**), 15-veratroylpseudokobusine (**55**), 14-acetylbrowniine (**74**), yesoxine (**75**), and 12-acetyllucidusculine (**76**) were isolated and purified from the roots of *A. yesoense* var. *macroyesoense* according to procedures described in the literature [39–43]. Five alkaloids from *A. japonicum*, aljesaconitine A (**77**), deoxyjesaconitine (**78**), hokbusine A (**79**), hypaconitine (**80**), and deoxyaconitine (**81**), were purified from the roots as described previously [45–49]. Delpheline (**82**) was purified from the seeds of *Delphinium elatum* cv. Pacific Giant by a previously described procedure [50,51]. Other novel derivatives were prepared from the parent alkaloids [25–28].

Effects of C_{19}-Diterpenoid Alkaloids

Table 8 summarizes the inhibitory effects of several C_{19}-diterpenoid alkaloids on the growth of A172, A549, HeLa, and Raji cells [25–28]. In a test at a single concentration (1 μg/ml/well, or 3 μg/ml/well for Raji cells) against a human cell line, an alkaloid is considered active when it reduces the cell growth rate relative to untreated controls. None of the five aconitine-type alkaloids (**77**–**81**) showed much inhibition of cell growth. Aljesaconitine A (**77**) caused a slight growth reduction of A172 and Hela cells. Deoxyjesaconitine (**78**) and hokbusine A (**79**) also slightly reduced the growth of Hela cells. These three compounds differ structurally in their *N*-substituent (*N*-ethyl in **77** and **78**, *N*-methyl in **79**), C-3 substituent (OH in **77** and **79**, H in **78**), C-8 substituent (*O*-methyl in **77** and **79**, *O*-acetyl in **78**), and C-14 substituent (*O*-anisoyl in **77** and **78**, *O*-benzoyl in **79**). Deoxyaconitine (**81**) had a slight effect on the growth of Raji cells. Chodoeva and coworkers reported that 8-*O*-azeloyl-14-benzoylaconine, an aconitine-type C_{19}-diterpenoid alkaloid, exhibited antiproliferative activity [23]. Thus, the identity of the C-8

TABLE 8 Inhibitory Effects of C_{19}-Diterpenoid Alkaloids on Human Tumor Cell Growth

	Mean ± SD			
Compound	*A172 (1 μg/ml)*	*A549 (1 μg/ml)*	*HeLa (1 μg/ml)*	*Raji (3 μg/ml)*
Control	1.00	1.00	1.00	1.00
12	1.11 ± 0.32	1.16 ± 0.11	1.09 ± 0.05	0.95 ± 0.01*
74	0.99 ± 0.18	1.01 ± 0.03	1.04 ± 0.04	1.00 ± 0.02
77	0.93 ± 0.08	0.97 ± 0.06	0.91 ± 0.03*	1.00 ± 0.03
78	0.97 ± 0.02	0.98 ± 0.06	0.93 ± 0.04	0.99 ± 0.01
79	1.03 ± 0.20	1.01 ± 0.03	0.92 ± 0.05	0.98 ± 0.08
80	1.08 ± 0.13	1.13 ± 0.12	1.05 ± 0.17	1.01 ± 0.02
81	0.99 ± 0.02	1.01 ± 0.04	0.95 ± 0.07	0.90 ± 0.06
82	0.90 ± 0.25	1.13 ± 0.16	1.07 ± 0.11	1.06 ± 0.04
83	0.93 ± 0.22	1.18 ± 0.19	0.95 ± 0.09	1.04 ± 0.05
84	1.04 ± 0.07	1.15 ± 0.12	1.04 ± 0.11	1.05 ± 0.04
85	1.14 ± 0.13	1.17 ± 0.21	1.03 ± 0.07	0.99 ± 0.12
86	1.10 ± 0.05	1.08 ± 0.02	1.07 ± 0.02	1.00 ± 0.08

*$p < 0.05$: Significantly different from control value (Student's paired *t*-test).

group may contribute to enhanced cytotoxic activity. Among the seven lycoctonine-type alkaloids (**12**, **74**, **82–86**) tested, *N*-deethyldelcosine (**82**) and *N*-deethyldelsoline (**83**) showed slight growth inhibition of A172 cells, while *N*-deethylanhydrohydroxydelcosine (**84**) and *N*-deethylanhydrohydroxydelsoline (**85**) did not. 14-Acetyldelcosine (**12**), which has an OH group at C-1 compared with the OMe found in 14-acetylbrowniine (**74**), slightly reduced the growth of Raji cells. Delpheline (**86**) was inactive (Fig. 7).

Effects of Veatchine-Type C_{20}-Diterpenoid Alkaloids

Table 9 shows the inhibitory effects of C_{20}-diterpenoid alkaloids on the growth of A172, A549, HeLa, and Raji cells [25–28]. The alkaloid yesoxine (**75**), which has an exocyclic epoxy group, was inactive. Dehydrolucidusculine (**18**), a natural alkaloid, was also inactive. Among the C-12 aryl ester derivatives (**87–89**) of luciculine, 12-benzoylluciculine (**87**) showed a modest growth inhibitory effect against A172, A549, and Raji cells. Addition of a *p*-methoxy group on the benzoyl group [12-anisoylluciculine (**88**)] led to the

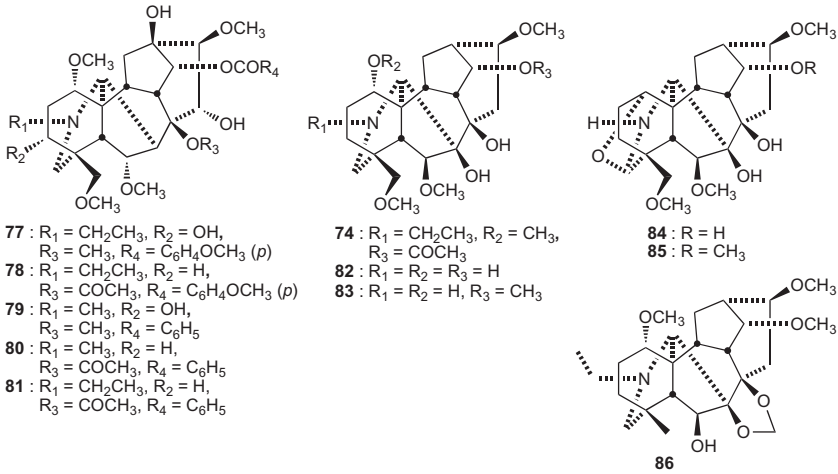


FIGURE 7 Structure of diterpenoid alkaloids **74–86**.

TABLE 9 Inhibitory Effects of Veatchine-Type C_{20}-Diterpenoid Alkaloids on Human Tumor Cell Growth

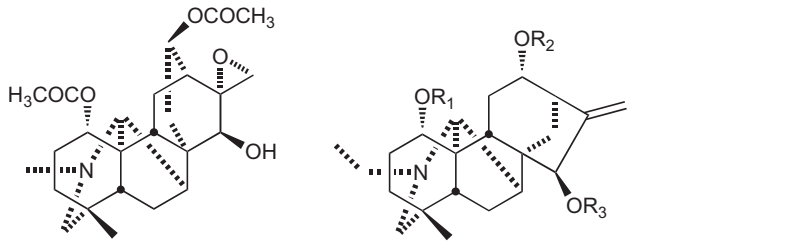
	Mean ± SD		Mean ± SD		
Compound	*A172 (1 μg/ml)*	*IC_{50} (μg/ml)*[a]	*A549 (1 μg/ml)*	*HeLa (1 μg/ml)*	*Raji (3 μg/ml)*
Control	1.00	–	1.00	1.00	1.00
15	0.66 ± 0.11*	5.6 ± 0.15	0.88 ± 0.06*	0.92 ± 0.07	0.98 ± 0.01
18	1.18 ± 0.09	> 5.0	1.12 ± 0.16	0.96 ± 0.05	1.03 ± 0.03
75	1.01 ± 0.22	> 5.0	1.11 ± 0.12	0.97 ± 0.06	1.08 ± 0.04
76	1.03 ± 0.22	ND	1.07 ± 0.02	0.95 ± 0.06	1.00 ± 0.03
87	0.82 ± 0.06*	ND	0.89 ± 0.02*	0.96 ± 0.06	0.90 ± 0.01
88	0.84 ± 0.38	ND	0.95 ± 0.01	0.91 ± 0.14	0.96 ± 0.07
89	0.97 ± 0.23	ND	0.95 ± 0.11	0.99 ± 0.04	0.99 ± 0.07
90	1.02 ± 0.28	> 5.0	0.97 ± 0.01	0.95 ± 0.07	0.95 ± 0.11

ND, Not determined.
*$p < 0.05$: Significantly different from control value (Student's paired *t*-test).
[a]IC_{50} is the compound concentration required to inhibit tumor cell growth by 50%. Data are expressed as means ± SD from the dose–response curves of at least three independent experiments.

same potency against A172, but lower potency against A549 and Raji cell lines. The inclusion of two methoxy substituents [12-veratroylluciculine (**89**)] led to a loss of inhibitory effect. Among the acetyl derivatives (**15**, **76**, and **90**) of luciculine, 12,15-diacetylluciculine (**76**, 12-acetyllucidusculine) and 1,12,15-triacetylluciculine (**90**) were inactive. In contrast, 12-acetylluciculine (**15**) had a potent growth inhibitory effect against A172, a modest effect against A549, and a small effect against HeLa cells. Therefore, the acetyl group had a position-dependent contribution on the cytotoxic activity. Among these veatchine-type alkaloids, hydroxy rather than acetoxy groups at the C-1 and C-15 positions were preferred for A172 cell growth inhibition, and certain esterifications of the C-12 hydroxy group contributed to enhanced activity (Fig. 8).

Effects of Atisine-Type C_{20}-Diterpenoid Alkaloids

The inhibitory effects of various atisine-type C_{20}-diterpenoid alkaloids (**6**, **7**, **42**, **47**, **48**, **53–58**, **91–104**) against the growth of A172, HeLa, and Raji cells were also examined (Table 10) [25–28]. The alkaloids kobusine (**6**) and pseudokobusine (**7**) inhibited the growth of A172 cells to the same extent as the veatchine-type alkaloids 12-benzoylluciculine (**87**) and 12-anisoylluciculine (**88**) (Table 9). Alkaloids **6** and **7** contain two and three hydroxy groups, respectively, at the C-11, C-15, and C-6 (**7** only) positions of their shared atisine skeleton. The effects of modifications of these groups in semisynthetic derivatives were examined. *N*-Benzyl-*N*,6-seco-6-dehydropseudokobusine (**91**) and *N*,15-dibenzyl-*N*,6-seco-6-dehydropseudokobusine (**92**) were inactive against A172 and HeLa cells and showed modest growth inhibitory activity against Raji cells. Among the benzoyl derivatives (**42**, **94**) of **7**, 6-benzoylpseudokobusine (**42**) had moderate inhibitory effects against the growth of Raji cells, while 6,11-dibenzoylpseudokobusine (**94**) had a similar or more potent inhibitory effect against Raji and A172 cells, respectively, as well as a slight inhibitory effect against Hela cells. 15-Benzoyl-6,11-di-*p*-nitrobenzoylpseudokobusine (**93**),



75 : $C_{25}H_{35}NO_6$

76 : $R_1 = H, R_2 = R_3 = COCH_3$
87 : $R_1 = R_3 = H, R_2 = COC_6H_5$
88 : $R_1 = R_3 = H, R_2 = COC_6H_4OCH_3$ (*p*)
89 : $R_1 = R_3 = H, R_2 = COC_6H_3(OCH_3)_2$ (3,4)
90 : $R_1 = R_2 = R_3 = COCH_3$

FIGURE 8 Structure of diterpenoid alkaloids **75–90**.

TABLE 10 Inhibitory Effects of Atisine-Type C_{20}-Diterpenoid Alkaloids on Human Tumor Cell Growth

	Mean ± SD	IC_{50} (μg/ml)[a]	Mean ± SD	Mean ± SD	IC_{50} (μg/ml)[a]
Compound	*A172*		*HeLa*	*Raji*	
Control	1.00	–	1.00	1.00	–
6	0.76 ± 0.28	> 5.0	0.96 ± 0.06	1.04 ± 0.04	ND
7	0.84 ± 0.23	> 5.0	0.94 ± 0.05	1.01 ± 0.02	ND
42	0.93 ± 0.11	ND	0.97 ± 0.05	0.84 ± 0.04*	ND
47	0.97 ± 0.16	ND	1.00 ± 0.05	0.97 ± 0.04	ND
48	0.49 ± 0.18**	0.89 ± 0.16	0.86 ± 0.01*	0.83 ± 0.04*	ND
50	0.99 ± 0.13	ND	0.95 ± 0.03	0.97 ± 0.05	ND
51	0.23 ± 0.04**	1.3 ± 0.11	0.54 ± 0.07*	0.62 ± 0.13*	2.2 ± 0.40
52[b]	0.95 ± 0.04	ND	ND	ND	ND
53	0.84 ± 0.19	3.7 ± 0.57	1.01 ± 0.93	0.93 ± 0.04	ND
54	0.47 ± 0.07**	1.2 ± 0.10	0.62 ± 0.17*	0.93 ± 0.15	ND
55	0.97 ± 0.09	2.9 ± 0.09	0.89 ± 0.06	0.97 ± 0.04	ND
56	0.96 ± 0.12	ND	0.94 ± 0.03*	0.86 ± 0.04*	ND
57	0.22 ± 0.11**	1.5 ± 0.01	0.66 ± 0.12*	0.52 ± 0.16*	ND
58[b]	0.95 ± 0.04	ND	ND	ND	ND
91	1.01 ± 0.14	ND	0.98 ± 0.03	0.84 ± 0.04*	ND
92	1.04 ± 0.10	> 5.0	0.99 ± 0.01	0.79 ± 0.06*	ND
93	0.97 ± 0.09	ND	0.92 ± 0.04	0.90 ± 0.02*	ND
94	0.76 ± 0.14*	1.3 ± 0.72	0.95 ± 0.06	0.85 ± 0.02*	ND
95	0.58 ± 0.16**	ND	0.83 ± 0.02*	0.79 ± 0.10*	2.4 ± 0.42
96	1.04 ± 0.12	ND	0.93 ± 0.01	0.82 ± 0.04*	ND
97	0.60 ± 0.09**	ND	0.89 ± 0.04*	0.87 ± 0.07*	ND
98	0.89 ± 0.04	ND	ND	ND	ND
99[b]	1.06 ± 0.08	ND	ND	ND	ND
100[b]	0.83 ± 0.02	ND	ND	ND	ND
101[b]	0.71 ± 0.11	ND	ND	ND	ND

Continued

TABLE 10 Inhibitory Effects of Atisine-Type C_{20}-Diterpenoid Alkaloids on Human Tumor Cell Growth—Cont'd

	Mean ± SD	IC_{50} (μg/ml)[a]	Mean ± SD	Mean ± SD	IC_{50} (μg/ml)[a]
Compound	*A172*		*HeLa*	*Raji*	
102[b]	1.14 ± 0.17	ND	ND	ND	ND
103[b]	1.04 ± 0.22	ND	ND	ND	ND
104[b]	1.24 ± 0.18	ND	ND	ND	ND

ND, Not determined.
*$p < 0.05$, **$p < 0.01$: Significantly different from control value (Student's paired *t*-test).
[a]IC_{50} is the compound concentration required to inhibit tumor cell growth by 50%. Data are expressed as means ± SD from the dose–response curves of at least three independent experiments.
[b]No published data.

which has a third benzoyl group at C-15 together with nitrated benzoyl groups at C-6 and C-11, exhibited mild inhibitory effects against the growth of HeLa and Raji cells. Among the veratroyl derivatives (**53**–**55**) of **7**, 6-veratroylpseudokobusine (**53**) and 15-veratroylpseudokobusine (**55**) displayed modest inhibitory effects, while 11-veratroylpseudokobusine (**54**) had significantly more potent inhibitory effects against A172 and HeLa cells. Thus, the contribution of an acyl group to activity was position dependent. Placing an acyl group at C-6 (**47**, **50**, **56**, **96**), C-15 (**52** and **58**), C-6, C-11 (**98** and **101**), C-6, C-15 (**99** and **102**), C-11, C-15 (**100** and **103**), and C-6, C-11, C-15 (**104**) resulted in less active compounds. In contrast, like compound **54**, other 11-acyl derivatives (**48**, **51**, **57**, **95**) generally exhibited potent inhibitory effects, to varying degrees against different cell lines depending on the identity of the C-11 ester substituent. Against A172 cells, the inhibitory effect of 11-cinnamoylpseudokobusine (**48**) was equivalent to that of **54**. Against the same cell line, 11-(*m*-trifluoromethylbenzoyl)pseudokobusine (**95**) and 11-(*m*-trifluoromethylbenzoyl)kobusine (**97**) showed comparable cytotoxic effects, but both were less potent relative to **54**. At the tested concentration, 11-anisoylpseudokobusine (**51**) and 11-*p*-nitrobenzoylpseudokobusine (**57**) exhibited the greatest reduction of the growth of A172 as well as Raji cells, while compound **48** exhibited the lowest IC_{50} value (0.89 μg/ml) against the A172 cell line. Compounds **51**, **54**, and **57** displayed the most potent inhibitory effects on HeLa cells. Compounds **51** and **95** showed significant suppressive effects, and their IC_{50} values were 2.2 μg/ml and 2.4 μg/ml against Raji cells, respectively [27] (Fig. 9).

Because antitumor drugs usually affect cell cycle distribution [52], the cell cycle distribution in Raji cells treated with compounds **51** and **95** was analyzed by flow cytometry [27]. Compound **95** induced a significant increase

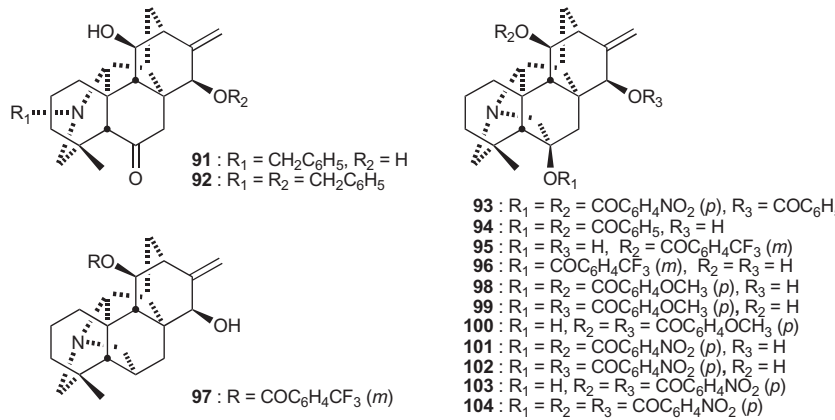


FIGURE 9 Structure of diterpenoid alkaloids **91–104**.

of G1 and/or a partial increase of sub-G1 phase population, which was dependent on both dose and time. To evaluate the mechanisms of the G1 arrest induction by compound **95**, the effects of **95** on proliferating pathways were investigated. Erk is generally activated during cell proliferation, and CDK2 activation is indispensable for the transition from G1 phase into S phase [53–55]. In the control group, Erk was gradually activated for up to 6 h. On the other hand, compound **95** significantly inhibited Erk activation. However, there were no observable CDK2 alterations under the indicated conditions. Compound **95** clearly induced the accumulation of G1 and sub-G1 phase cells, thereby showing a growth inhibitory effect in a time-dependent manner against Raji cells. These results were supported by a Western blotting analysis, which showed inhibition of Erk activation, but no modulation of CDK2 in comparison with the control. However, down regulation of PI3K phosphorylation and expression of CDK2 were observed in the combined treatment. Compound **95** clearly inhibited the phosphorylation of extracellular signal-regulated kinase, induced enhanced phosphoinositide 3 kinase phosphorylation, and led to the subsequent accumulation of G1 and/or sub-G1 phase cell populations in Raji cells [27].

Effects of Atisine-Type C_{20}-Diterpenoid Alkaloids on A549 Cells

Table 11 shows the inhibitory effects of various atisine-type C_{20}-diterpenoid alkaloids (**6**, **7**, **42**, **47**, **48**, **50**, **51**, **53–57**, **91–110**) against the growth of A549 cells [26,28]. Among the pseudokobusine derivatives, **42**, **91**, **92**, and **93** were inactive, while **94** had a weak cytotoxic effect; these results were similar to those in the A172 cell line. Among the veratroyl ester derivatives (**53–55**) of **7**, the C-6 ester (**53**) was inactive and the C-15 ester (**55**) displayed only a small cytotoxic effect, while the C-11 ester (**54**) had a significant cytotoxic effect against the A549 cell line. Therefore, the highest suppressive

TABLE 11 Inhibitory Effects of Atisine-Type C_{20}-Diterpenoid Alkaloids on A549 Cell Growth

	Mean ± SD		
Compound	*1 μg/ml*	*5 μg/ml*	*IC_{50} (μM)*[a]
Control	1.00	–	–
6	1.07 ± 0.16	1.00 ± 0.05	ND
7	1.12 ± 0.16	0.93 ± 0.04	ND
25	1.06 ± 0.19	0.64 ± 0.11*	ND
29	0.93 ± 0.19	0.44 ± 0.11*	ND
31	0.98 ± 0.14	0.35 ± 0.04*	11.42 ± 0.71
33	1.04 ± 0.25	0.50 ± 0.06*	ND
42	1.02 ± 0.01	ND	ND
47	0.95 ± 0.04	0.90 ± 0.02	ND
48	0.59 ± 0.10*	ND	4.24 ± 0.00
50	1.02 ± 0.01	1.05 ± 0.04	ND
51	0.65 ± 0.08*	ND	2.20 ± 0.11
52	0.89 ± .03	0.56 ± 0.04*	ND
53	0.98 ± 0.06	0.86 ± 0.03	ND
54	0.65 ± 0.06*	ND	4.07 ± 0.00
55	0.94 ± 0.01	ND	ND
56	0.93 ± 0.04	0.83 ± 0.03	ND
57	0.75 ± 0.08*	ND	5.08 ± 0.15
58	0.93 ± 0.05	0.47 ± 0.04*	ND
72	1.09 ± 0.06	1.04 ± 0.05	ND
91	1.01 ± 0.03	ND	ND
92	0.98 ± 0.02	ND	ND
93	0.96 ± 0.06	ND	ND
94	0.88 ± 0.06*	ND	ND
95	0.69 ± 0.09*	ND	4.67 ± 0.08
96	1.05 ± 0.06	ND	ND
97	0.64 ± 0.06*	ND	3.75 ± 0.14

Continued

TABLE 11 Inhibitory Effects of Atisine-Type C_{20}-Diterpenoid Alkaloids on A549 Cell Growth—Cont'd

	Mean ± SD		
Compound	*1 μg/ml*	*5 μg/ml*	*IC_{50} (μM)*[a]
98	0.66 ± 0.04*	0.15 ± 0.04*	3.68 ± 0.30
99	1.04 ± 0.14	0.25 ± 0.06*	ND
100	0.54 ± 0.14*	0.03 ± 0.01*	1.72 ± 0.03
101	0.86 ± 0.11	0.11 ± 0.08*	ND
102	0.98 ± 0.11	0.42 ± 0.10*	ND
103	0.74 ± 0.14*	0.03 ± 0.01*	2.66 ± 0.21
104	1.05 ± 0.06	0.91 ± 0.05	ND
105	1.03 ± 0.05	1.17 ± 0.31	ND
106	1.09 ± 0.19	0.72 ± 0.07*	ND
107	0.72 ± 0.11*	0.21 ± 0.04*	5.44 ± 0.41
108	0.91 ± 0.19	0.37 ± 0.10*	ND
109	0.92 ± 0.23	0.89 ± 0.04*	ND
110	0.75 ± 0.22	0.27 ± 0.24*	3.02 ± 0.47

ND, Not determined.
*$p < 0.05$: Significantly different from control value (Student's paired *t*-test).
[a]IC_{50} is the compound concentration required to inhibit tumor cell growth by 50%. Data are expressed as means ± SD from the dose–response curves of at least three independent experiments.

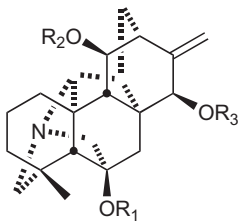
effects were elicited by the presence of an acyl substituent at C-11. Among the anisoyl (**50**–**52**, **98**–**100**) and *p*-nitrobenzoyl (**56**–**58**, **101**–**104**) derivatives of **7**, pseudokobusine 6-anisoate (**50**), 6,15-dianisoate (**99**), 6,15-di-*p*-nitrobenzoate (**102**), and 6,11,15-tri-*p*-nitrobenzoate (**104**) were inactive. Pseudokobusine 6-*p*-nitrobenzoate (**56**) and 15-*p*-nitrobenzoate (**58**) displayed small cytotoxic effects, and pseudokobusine 15-anisoate (**52**) and 6,11-di-*p*-nitrobenzoate (**101**) showed only weak cytotoxic effects. Pseudokobusine 11-anisoate (**51**), 11-*p*-nitrobenzoate (**57**), 6,11-dianisoate (**98**), 11,15-dianisoate (**100**), and 11,15-di-*p*-nitrobenzoate (**103**) had significant cytotoxic effects. Accordingly, the 6-esters (**50**, **56**) exhibited weaker cytotoxic effects than the 6,11-esters (**98**, **101**), and 11-esters (**51**, **57**) had more potent cytotoxic effects than 6,11-esters (**98**, **101**). In fact, 11-acyl derivatives (e.g., **48**, **95**) generally exhibited more potent cytotoxic effects than corresponding 6-acyl derivatives (e.g., **47**, **96**), but the C-6 and C-11 *p*-trifluoromethylbenzoyl (**105**, **106**) derivatives were inactive. In addition, pseudokobusine 11,15-dianisoate (**100**) and

11,15-di-*p*-nitrobenzoate (**103**) were found to be about 1.3- and 2-fold more potent than pseudokobusine 11-anisoate (**51**) and 11-*p*-nitrobenzoate (**57**), respectively. Based on the identity of the acyl group at C-11 of **7**, an unsubstituted benzoate (**94**) and *p*-trifluoromethylbenzoate (**106**) led to weaker or no cytotoxic activity, while an anisoate (**51**, $IC_{50} = 2.20\ \mu M$), veratroate (**54**, $IC_{50} = 4.07\ \mu M$), cinnamate (**48**, $IC_{50} = 4.24\ \mu M$), *m*-trifluoromethylbenzoate (**95**, $IC_{50} = 4.67\ \mu M$), and *p*-nitrobenzoate (**57**, $IC_{50} = 5.08\ \mu M$) resulted in significant cytotoxic effects. Thus, the rank order of potency regarding the substituent on the benzoyl aromatic ring was 4-OMe, 3,4-OMe, 3-CF_3, 4-NO_2, H, 4-CF_3.

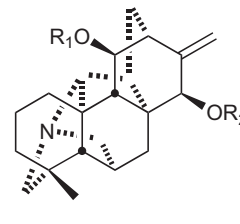
Similarly, the suppressive effects of kobusine derivatives (**25**, **29**, **31**, **33**, **72**, **97**, **107–110**) were examined against the growth of A549 cells (Table 11). At 1 μg/ml, kobusine 11-benzoate (**25**), 11-veratroate (**33**), and dihydrokobusine (**72**) were inactive. Kobusine 11-cinnamate (**29**) and 11-anisoate (**31**) displayed very weak cytotoxic effects at this same concentration. Both kobusine 11-*m*-trifluoromethylbenzoate (**97**) and 11-*p*-trifluoromethylbenzoate (**107**) had significant cytotoxic effects. Among the *p*-nitrobenzoyl derivatives (**108–110**) of **6**, kobusine 11-*p*-nitrobenzoate (**108**) and 15-*p*-nitrobenzoate (**109**) exhibited only slight cytotoxic effects at 1 μg/ml; however, 11,15-di-*p*-nitrobenzoylkobusine (**110**) exhibited a significant cytotoxic effect at 1 μg/ml and had an IC_{50} value against A549 cells of 3.02 μM. Except for the trifluoromethyl benzoates (**97**, **107**) of **6**, the cytotoxic effects of derivatives of kobusine (**6**) were weaker compared with the cytotoxic effects of derivatives of pseudokobusine (**7**). Hence, the presence of a hydroxy group at the C-6 position enhanced the suppressive effects against the growth of A549 cells.

Twenty-six alkaloids (**6**, **7**, **25**, **29**, **31**, **33**, **47**, **50**, **52**, **53**, **56**, **58**, **72**, **98–110**) were also assayed at 5 μg/ml against the growth of A549 cells. Pseudokobusine (**7**)-derived alkaloids **47**, **53**, **56**, **104**, and **109** displayed weak cytotoxic effects. Compounds **25**, **29**, **33**, **52**, **58**, **102**, and **106** had significant cytotoxic effects and were found to be about 1.4- to 2.4-fold more potent at the higher concentration. However, compounds **31**, **98**, **99**, **101**, **107**, **108**, and **110** were significantly more potent (three- to eightfold) at the higher concentration. Compounds **100** (11,15-dianisoate of **7**) and **103** (11,15-di-*p*-nitrobenzoate of **7**) showed the greatest cytotoxic activity against A549 cells (Fig. 10).

The cell cycle distribution of A549 cells at 24 and 48 h after treatment was analyzed by a fluorescence cell analyzer. Compounds **98**, **100**, and **107** showed remarkable G1 enhancement at doses of up to twice their IC_{50} values at 24 h, increasing the G1 phase population of A549 cells in a time-dependent manner [28]. These results suggest that cytotoxic derivatives can disturb the G1 to S phase transition [56–58]. It is well established that cell cycle progression is highly dependent on cyclins, Cdks (cyclin-dependent kinases) and Cdk inhibitors [59]. Raf-MEK-Erk signaling and/or PTEN-PI3K-AKT signaling contributes to the G1 → S transition *via* subsequent cyclin production, and



105 : $R_1 = COC_6H_4CF_3$ (*p*), $R_2 = R_3 = H$
106 : $R_1 = R_3 = H$, $R_2 = COC_6H_4CF_3$ (*p*)



107 : $R_1 = COC_6H_4CF_3$ (*p*), $R_2 = H$
108 : $R_1 = COC_6H_4NO_2$ (*p*), $R_2 = H$
109 : $R_1 = H$, $R_2 = COC_6H_4NO_2$ (*p*)
110 : $R_1 = R_2 = COC_6H_4NO_2$ (*p*)

FIGURE 10 Structure of diterpenoid alkaloids **105–110**.

inhibition of MEK and PI3K activity induces complete G1 phase arrest [60]. Moreover, in a previous study, a C-11 acyl derivative showed an inhibitory effect on growth of A549 cells without enhancement of apoptosis or DNA damage [28]. Thus, it appears that these diterpenoid alkaloid derivatives do not induce genotoxic stress, but inhibit cell growth through G1 arrest.

Because antitumor drugs also usually act as radiosensitizers, compound **51** was tested for its ability to induce radiation sensitization [26]. In the preliminary experiments, compound **51** did not show radiosensitizing effects on HeLa and Raji cells, but did show a radiosensitizing effect before or after X-ray irradiation on A549 cells. This function may be due to a slight interference of the cell cycle, but no effect was observed on the expression of cell cycle-related molecules such as p21, cyclin B, cyclin D, and cyclin E. In addition, a combination of X-ray irradiation with **51** did not result in a synergistic effect on the recovery of DNA double-strand break repair, thus suggesting that the compound's effects may not be related to a disruption of the DNA repair mechanism [61,62]. Based on the above findings, there is a possibility that the cytotoxic activity of alkaloids, including compound **51**, depends on the cell type.

Structure–Activity Relationships of Diterpenoid Alkaloids

Diterpenoid alkaloids **6** and **7** have the same atisine skeleton with two and three hydroxy groups, respectively, at the C-11, C-15 and, for **7**, the C-6 positions, which are important for cytotoxic effects. Placement of an acyl group at C-11 generally resulted in enhanced activity compared with the parent alkaloids, which have a hydroxy group at this position. Moreover, the presence by an acyl group at both C-11 and C-15 [e.g., pseudokobusine 11,15-dianisoate (**100**), 11,15-di-*p*-nitrobenzoate (**103**), and 11,15-di-*p*-nitrobenzoylkobusine (**110**)] could result in further enhancement of the cytotoxic effect. These three compounds incorporated all of the favorable modifications identified to date. They possess a novel structure and show remarkable IC_{50} values in the submicromolar range. The identity of the acyl group had varying effects. Simple benzoyl ester derivatives were generally less active. Cinnamoyl,

p-nitrobenzoyl, *m*-trifluoromethylbenzoyl, and veratroyl substitutions were effective. Anisoate esters were generally found to be more potent than the other esters tested. These discoveries may lead to the development of new specific antitumor compounds with an optimal balance between the antitumor effects and myelosuppression from atisine-type structures.

MATERIAL AND METHODS

Chemicals

The reference drugs, prazosin hydrochloride, hydralazine hydrochloride, papaverine hydrochloride, and nifedipine were obtained from Wako Pure Chemical Ind., Ltd. (Osaka, Japan).

The *A. yesoense* var. *macroyesoense* samples were obtained from roots found in Jozankei, the city of Sapporo, Hokkaido, Japan. The root samples were freeze-dried and then ground. The root powders (10 g) were stirred with solvents (water or methanol, 150 ml) at room temperature for 1 day. The liquid phase was filtered and the residue was further extracted in the same manner with the same solvent (150 ml each) for 3 days. The water phase was filtered and then freeze-dried. The methanol phase was filtered and then evaporated. The root powders (50 g) were stirred with methanol (750 ml) at room temperature for 1 day. The liquid phase was filtered, and the residue was further extracted in the same manner with the same solvent (500 ml) for 3 days. The water phase was filtered and then freeze-dried. The methanol phase was filtered and then evaporated. The methanol extract was dissolved with 5% (v/v) HCl and was partitioned in hexane (50 ml × 3), and then the organic phase was evaporated to dryness. A 28% NH_4OH solution was added to the aqueous phase until the pH reached 10. The aqueous phase was then extracted with chloroform (100 ml × 7), and the organic phase was evaporated to dryness. The aqueous phase was freeze-dried.

Lucidusculine (**5**), kobusine (**6**), pseudokobusine (**7**), luciculine (**10**), delcosine (**11**), 14-acetyldelcosine (**12**), 1-acetylluciculine (**17**), dehydroluciculine (**14**), dehydrolucidusculine (**18**), 15-benzoylpseudokobusine (**22**), 15-veratroylpseudokobusine (**55**), yesoline (**64**), yesonine (**65**), 14-acetylbrowniine (**74**), yesoxine (**75**), and 12-acetyllucidusculine (**76**) were used after extraction from the root of *A. yesoense* var. *macroyesoense*, followed by purification and identification by the methods described previously [39–42]. Five alkaloids from *A. japonicum*, aljesaconitine A (**77**), deoxyjesaconitine (**78**), hokbusine A (**79**), hypaconitine (**80**), and deoxyaconitine (**81**), were purified from the roots as described previously [45–49]. Delpheline (**86**) was purified from the seeds of *D. elatum* cv. Pacific Giant by a previously described procedure [50,51].

1-Acetyldelcosine (**13**) was prepared from delcosine (**11**) [29]. Luciculine 12-acetate (**15**), 12-benzoate (**87**), 12-anisoate (**88**), 12-veratroate (**89**), and 1,12,15-triacetate (**90**) were prepared from luciculine (**10**) [25,29,40,63].

12-Acetyldehydroluciculine (**16**) was prepared from dehydroluciculine (**14**) [29]. Kobusine15-acetate (**19**), 15-benzoate (**20**), 11-acetate (**23**), 11,15-diacetate (**24**), 11-benzoate (**25**), 11-propionate (**26**), 15-propionate (**27**), 11,15-dipropionate (**28**), 11-cinnamate (**29**), 15-cinnamate (**30**), 11-anisoate (**31**), 15-anisoate (**32**), 11-veratroate (**33**), 15-veratroate (**34**), 11-nicotinoate (**35**), 15-nicotinoate (**36**), 11-pivaloate (**37**), 15-pivaloate (**38**), 11-*p*-trifluoromethylbenzoate (**107**), 11-*p*-nitrobenzoate (**108**), 15-*p*-nitrobenzoate (**109**), 11,15-di-*p*-nitrobenzoate (**110**), 15-dehydro-11-veratroylkobusine (**68**), 11-dehydro-15-veratroylkobusine (**69**), isokobusine (**70**), and dihydrokobusine (**72**) were prepared from kobusine (**6**) [28,30,31]. Pseudokobusine 15-acetate (**21**), 6-acetate (**39**), 11-acetate (**40**), 6,11,15-triacetate (**41**), 6-benzoate (**42**), 11-benzoate (**43**), 6-propionate (**44**), 11-propionate (**45**), 15-propionate (**46**), 6-cinnamates (**47**), 11-cinnamates (**48**), 15-cinnamate (**49**), 6-anisoate (**50**), 11-anisoate (**51**), 15-anisoate (**52**), 6-veratroate (**53**), 11-veratroate (**54**), 6-*p*-nitrobenzoate (**56**), 11-*p*-nitrobenzoate (**57**), 15-*p*-nitrobenzoate (**58**), 11-nicotinoate (**59**), 15-nicotinoate (**60**), 6-pivaloate (**61**), 11-pivaloate (**62**), 15-pivaloate (**63**), 15-benzoyl-6,11-di-*p*-nitrobenzoate (**93**), 6,11-dibenzoate (**94**), 11-*m*-trifluoromethylbenzoate (**95**), 6-*m*-trifluoromethylbenzoate (**96**), 6,11-dianisoate (**98**), 6,15-dianisoate (**99**), 11,15-dianisoate (**100**), 6,11-di-*p*-nitrobenzoate (**101**), 6,15-di-*p*-nitrobenzoate (**102**), 11,15-di-*p*-nitrobenzoate (**103**), 6,11,15-tri-*p*-nitrobenzoate (**104**), 6-*p*-trifluoromethylbenzoate (**105**), 11-*p*-trifluoromethylbenzoate (**106**), *N*-acetyl-*N*,6-seco-6-dehydropseudokobusine (**66**), *N*,11,15-triacetyl-*N*,6-seco-6-dehydropseudokobusine (**67**), isopseudokobusine (**71**), dihydropseudokobusine (**73**), *N*-benzyl-*N*,6-seco-6-dehydropseudokobusine (**91**), and *N*,15-dibenzyl-*N*,6-seco-6-dehydropseudokobusine (**92**) were prepared from pseudokobusine (**7**) [10,25,28,30,31,40,43]. *N*-Deethyldelcosine (**82**) and *N*-deethylanhydrohydroxydelcosine (**84**) were prepared from delcosine (**11**) [25]. *N*-Deethyldelsoline (**83**) and *N*-deethylanhydrohydroxydelsoline (**85**) were prepared from delsoline [25].

Animals

Male ICR SPF mice at 5 weeks of age were purchased from Japan SLC Inc. (Shizuoka, Japan) and used after acclimatization for 7 days. They were housed five in a polycarbonate cage, with free access to food and water. The animal room was maintained at a constant temperature of $23 \pm 1\,^{\circ}C$, in a relative humidity of $50 \pm 5\%$, and with a 12-h light and 12-h dark cycle.

Measurement of Cutaneous Blood Flow

A Doppler-type laser blood flowmeter (Type ALF21R, Advance Co., Ltd., Tokyo, Japan) was used for measurement of cutaneous blood flow in the hind foot of mice under anesthesia with an intraperitoneal dose of 70 mg/kg of sodium pentobarbital (Nembutal®, Abbott Labs., North Chicago, IL, USA) followed by *p.o.* or *i.v.* administration of each test extract or compound,

respectively. Test compounds were dissolved in 50% ethanol and injected intravenously to mice in a constant volume of 0.02 ml/10 g of body weight. The probe was fixed in place on the center of the dorsal skin surface of the left hind foot by wrapping it with elastic adhesive tape. The flow signals generated in the cutaneous region of skin 2 mm in diameter and 1 mm in depth were detected continuously for 20 min. Blood flow was digitally recorded as ml/min/100 g of tissue with a three channel pen recorder (EB 22005, Chino Co., Tokyo), and the differences before and after administration were determined. The vehicle served as the negative control.

Statistical Analysis

The data obtained were expressed as the mean ± S.E. of three to five mice of a group, and the differences from the values determined before administration were analyzed statistically by Student's paired *t*-test at the significant level of 0.05 or less.

Cell Lines

The human tumor cell line A172 (glioblastoma cells) was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). A549 (lung cancer), HeLa (epithelioid cervix carcinoma), and Raji (Burkitt EBV-infected lymphoma cells) cell lines were purchased from the RIKEN Bio-Resource Center (Tsukuba, Japan). The A172 cells were maintained in a continuous culture in Dulbecco's MEM medium (DMEM, Sigma, St. Louis, MO, USA) supplemented with 10% heat-inactivated fetal bovine serum (Bioserum, UBC, Japan); A549 and HeLa cells in DMEM medium (Sigma); Raji cells in RPMI 1640 (Gibco® Invitrogen, Carlsbad, CA, USA) in a humid atmosphere at 37 °C and 5% CO_2.

Cell Culture

Each cell line was seeded in 24-well tissue culture plate (Falcon, Becton Dickinson Biosciences, Franklin Lakes, NJ, USA) with 1 ml of culture medium at a concentration of $4–5 \times 10^3$ cells/well. After 24 h incubation, media were discarded by aspiration, and then each diterpenoid alkaloid sample (1 μg/ml) was added to the culture. After culturing (A172 cells for 6 days, A549 cells and HeLa cells for 3 days), cells were harvested by 0.1% trypsin-EDTA (Gibco® Invitrogen), and total cell numbers were analyzed by a Z™ Series Particle counter (Coulter Electronics, Hialeah, FL, USA). In the case of Raji cells, the culture was performed at 1×10^5 cells/well, and diterpenoid alkaloid samples at 3 μg/ml were added to the culture. Four days later, the total cell numbers harvested were analyzed in the same way.

Statistical Analysis

The data are expressed as the mean ± SD of three cultures of a group, and the significance of differences between the control and experimental groups was determined using either Student's *t*-test or Mann–Whitney's U-test, depending on the data distribution. Statistical analysis was performed using the Excel 2003 software package (Microsoft, Redmond, WA, USA) with the add-in software Statcel 2 (OMS, Saitama, Japan).

Cell Cycle Analysis by Flow Cytometry

A549 cells were treated with each compound at two doses (IC_{50} value and twice IC_{50} value) and incubated for 24 h. The harvested cells were treated with PBS containing 0.1% Triton X-100 (Wako, Osaka, Japan) and were stained with propidium iodide (50 μg/ml, Sigma). Analysis of cell cycle distribution was performed using a flow cytometer (Beckman–Coulter, Cell Lab Quanta^{TM} SC MPL, Fullerton, CA, USA).

In Vitro Irradiation of Cells

A549 cells were seeded in a 60 mm tissue culture dish (Iwaki, Chiba, Japan). After 2 days of culture, 11-anisoylpseudokobusine (**51**) (0.5 μg/ml) was added within 1 h either before or after X-ray irradiation. The cells were irradiated with X-rays (150 kV, 20 mA) by using 0.5-mm aluminum and 0.3-mm copper filters at a distance of 45 cm from the focus at a dose of 92.3–94.4 cGy/min (MBR-1520R, Hitachi Medical Corporation, Tokyo, Japan) in the range of 1–8 Gy. During X-ray exposure, the dose intensity was evaluated by the probe with an ionization chamber. The exposed cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2. On day 7, the cells were fixed in methanol for 5 min at room temperature, air dried, and stained by Giemsa stain solution for 30 min. The colonies consisting of more than 50 cells were counted using an inversion microscope.

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ABBREVIATIONS

i.v. intravenous
p.o. peroral

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Chapter 8

Novel Plant-Derived Biomedical Agents and Their Biosynthetic Origin

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INTRODUCTION

Natural product chemistry is considered as one of the major contributors of lead compounds to the drug discovery program. Recent studies indicate that 50% of the prescribed pharmaceuticals are of natural product origin [1,2]. The structural diversity provided by natural product chemistry is enormous compared to combinatorial chemistry and genomic approaches [3]. An extensive research in combinatorial chemistry has yielded only one anticancer drug, sorafenib, compared to natural products which have provided 49% of 877 small molecules introduced as pharmaceuticals during 1981–2002 [4,5].

One of the aspects of drug discovery process is the identification of small molecules with enzyme-inhibiting activities. Enzymes are essential to human life, mediating biochemical processes including metabolism, cellular signal transduction, cell cycling, and development. Malfunction in these biochemical systems often leads to disease that can be caused by the dysfunction, overexpression, or hyperactivation of the enzymes involved [6]. An understanding of diseases at the molecular level has provided several enzyme inhibitors in clinics. For instance, galanthamine, a potent acetylcholinesterase (AChE) inhibitor, is used to treat Alzheimer's disease (AD) [7]. *In vitro* enzyme inhibition assays can easily be performed in any natural product chemistry lab that help to discover lead compounds against various biological targets. For instance, glutathione *S*-transferase (GST) inhibitors have applications in overcoming the drug resistance problems in cancer and parasitic chemotherapy. Fatty acid synthase inhibitors are used to discover antimalarial, antiparasitic, anti-TB, and antifungal compounds. For discovering antifungal agents, natural

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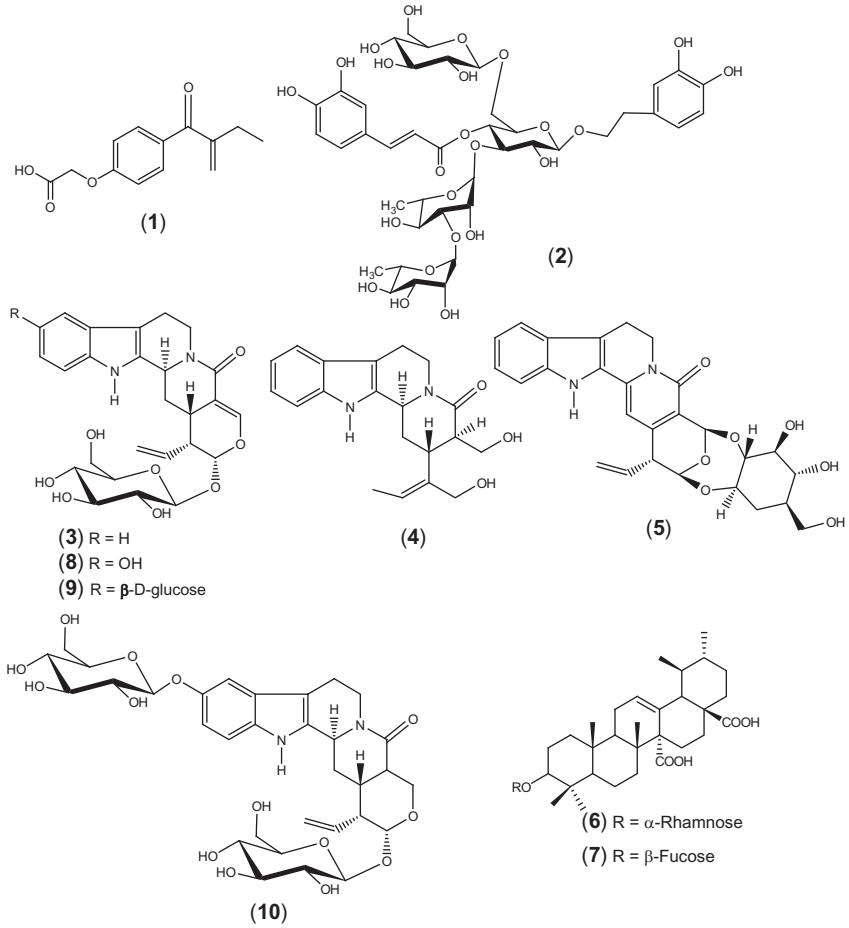
products should be screened against a yeast *Candida albicans*. Any compound active against this yeast is assumed to be capable of entering cells of *Saccharomyces cerevisiae*, a yeast used in haploinsufficiency determination of drug cellular targets, and provides a detailed functional information on bioactive compounds through chemical genetic profiling for hypersensitivity against an array of about 4800 viable haploid deletion mutant strains of *S. cerevisiae* [8]. This gene-deleting process rendering cells hypersensitive to a specific compound identify pathways that protect the cell against the toxic effects of the drug and provide information about its mode of action and cellular target. These days, it would be worthwhile for natural product chemists to expand the range of bioassays in their labs as the collection and preparation of plant extracts are very costly and time consuming. It has been reported that the cost of individual plant extract is nearly over US $ 200 [9]. This expansion of screening targets would help to overcome this problem and also this suit of bioassays will be useful to determine the biomedical applications of pure natural products. In our lab, we are involved in discovering new natural products exhibiting inhibitory activities against GST, AChE, and α-glucosidase. In this chapter, the structures of different classes of natural products exhibiting aforementioned enzyme inhibition activities and their structure–activity relationships are described.

GST Inhibitors

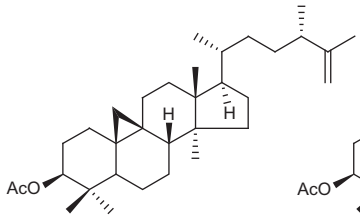
Drug resistance is one of the major clinical obstacles to the currently used anticancer and antiparasitic pharmaceuticals. There is an active research going on to understand the mechanism of acquired drug resistance during anticancer and antiparasitic chemotherapy. The isozymes, GSTs (EC 2.1.5.18), have been reported to play an active role during these chemotherapies. GSTs are phase II detoxification isozymes that catalyze reactions of exogenous or endogenous electrophilic substrates with glutathione to make water soluble adduct. This adduct can easily be excreted from the body [10]. Anticancer drugs containing electrophilic centers make glutathione adduct and are excreted from the body, thus lowering the effectiveness of anticancer pharmaceuticals. In humans, GSTs exist in various dimerized isoenzyme classes: α (A), μ (M), ω, π (P), θ (T), ζ (Z), and σ classes. Their existence in different forms provides broad substrate specificities to improve detoxification of many toxic substances. Overexpression of GSTs in various cancer cells was observed compared to normal tissues. A twofold increase in GST activity is reported in the literature in lymphocytes obtained from chronic lymphocytic leukemia (CLL) patients, resistant to chlorambucil when compared with untreated CLL patients [11]. This drug resistance problem of cancer chemotherapeutic agents might be overcome by the use of GST inhibitors as adjuvant during cancer chemotherapy. Ethacrynic acid (**1**), first generation of GST inhibitor, exhibits significant *in vitro* potentiating activity but has diuretic side effects and lack of isoenzyme specificity. These drawbacks restricted its use in drug resistance obstacles [12]. Toward this end, we

screened several medicinally important plants in GST inhibition assay and discovered that the crude methanolic extracts of *Barleria prionitis*, *Nauclea latifolia*, *Artocarpus nobilis*, and *Caesalpinia bonduc* exhibited anti-GST activity with IC_{50} (concentration required to inhibit 50% activity of enzyme) values of 160.0, 10.5, 125, and 83.0 μg/ml, respectively. Due to the colorimetric nature of enzyme inhibition assays, polyphenols, present in crude plants extracts, often give false-positive results. In order to rule out the possibility of false-positive results, it is worthwhile to remove polyphenols by filtering plant extracts through MN-polyamide SC6 resin before screening for enzyme inhibition activities [13]. Our recent phytochemical studies on the crude methanolic extract of *B. prionitis*, collected from Sri Lanka, yielded barlerinoside (**2**) exhibiting GST inhibitory activity with an IC_{50} value of 12.4 μM. The potency of compound **2** was more or less comparable with the GST inhibitory activity of a positive control, ethacrynic acid (**1**), a substrate GST inhibitor ($IC_{50} = 16.5$ μM). Compound **2** also showed free radical scavenging activity with an IC_{50} value of 0.42 μg/ml [14].

Our detailed chemical studies on the crude ethanolic extract of *Nauclea latifolia* of Nigerian origin yielded five known GST-inhibiting compounds: strictosamide **3** ($IC_{50} = 20.3$ μM), naucleamides A **4** ($IC_{50} = 37.2$ μM), naucleamide F **5** ($IC_{50} = 23.6$ μM), quinovic acid-3-*O*-β-rhamnosylpyranoside **6** ($IC_{50} = 143.8$ μM), and quinovic acid 3-*O*-β-fucosylpyranoside **7** ($IC_{50} = 53.5$ μM). Compound **3** showed significant anti-GST property and was isolated in a large quantity. It was, therefore, decided to prepare analogues by using microbial reactions and to evaluate them for GST inhibitory activity in order to study their structure–activity relationships. Microbial reactions of compound **3** using fungi, *Cunninghamella blakesleeana* and *Rhizopus circinans*, afforded three metabolites: 10-hydroxystrictosamide (**8**), 10-β-glucosyloxyvincoside lactam (**9**), and 16,17-dihydro-10-β-glucosyloxyvincoside lactam (**10**). *C. blakesleeana* is reported to perform hydroxylation reactions on aromatic compounds, while *R. circinans* was discovered for the first time to perform this reaction [15]. In order to determine the sequence for the formation of metabolites **8–10**, the time-dependent biotransformation experiments were performed. These experiments were carried out by incubating compound **3** in the liquid culture of *R. circinans*; this afforded compounds **8** and **9**. Incubation of compound **9** in the liquid culture of this fungus yielded compound **10**. These studies suggested that *R. circinans* initially performed microbial hydroxylation at C-10 of compound **3** to yield compound **8**. This metabolite further underwent glycosylation, followed by reduction of the Δ^{16-17} double bond to give compounds **9** and **10**, respectively. Compounds **8–10** also exhibited GST inhibitory activity with IC_{50} values of 18.6, 12.3, and 46.6 μM, respectively. The bioactivity of **9** was approximately two-fold increased compared to the parent compound (**3**) [16]. This higher potency might be due to the presence of a sugar moiety at C-10 that might have increased its solubility in water to have better interactions with GST.



GST inhibition-directed fractionations on the ethanolic extract of *A. nobilis* of Sri Lankan origin yielded four known triterpenoids, cyclolaudenyl acetate (**11**), lupeol acetate (**12**), β-amyrin acetate (**13**), and zizphursolic acid (**14**), along with five known flavonoids, artonins E (**15**), artobiloxanthone (**16**), artoindonesianin U (**17**), cyclocommunol (**18**), and multiflorins A (**19**). Compounds **11–19** showed anti-GST properties with IC_{50} values of 195.1, 146.1, 251.0, 68.5, 2.0, 1.0, 6.0, 3.0, and 14.0 μM, respectively. The bioactivity data suggest that compounds (**15–18**) exhibited more potent GST inhibitory activity compared to ethacrynic acid (**1**) and rest of the isolates of this plant. It has been reported in the literature that flavonoids usually exhibit GST inhibitory activity with the IC_{50} values in the range of 15–30 μM [17]. Compounds **15–18** are also members of flavonoid class of natural products and their higher potency might be due to the presence of prenyl group incorporated in their structures [18].



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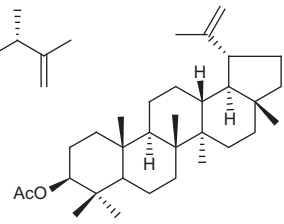

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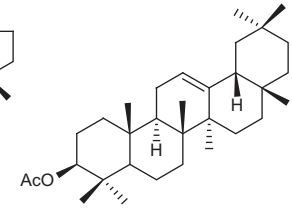

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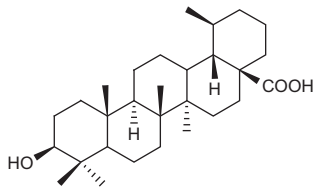

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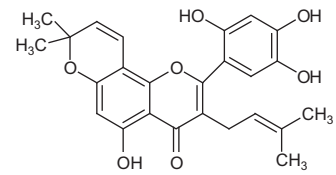

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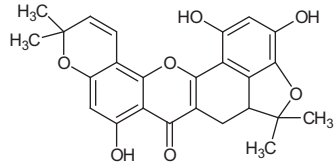

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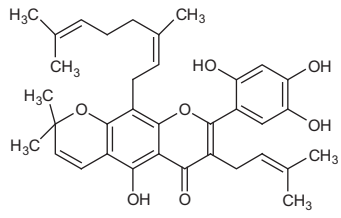

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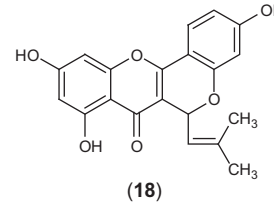

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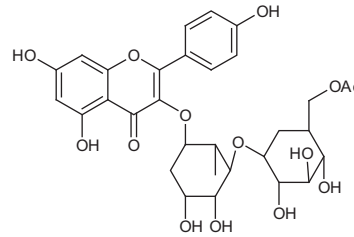
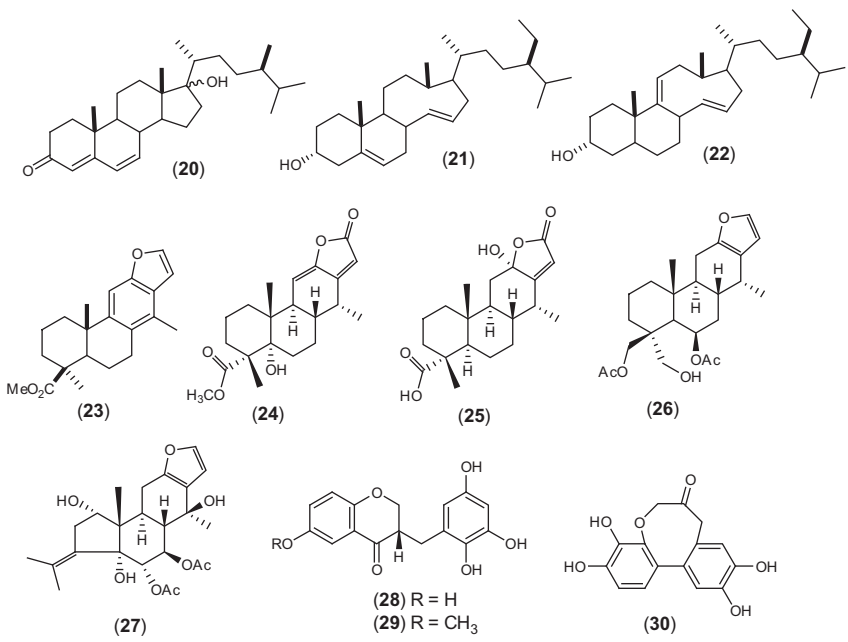


(**19**)

From the bioactive fractions of *C. bonduc* of Sri Lankan origin, 17-hydroxycompesta-4,6-dien-3-one (**20**), 13,14-*seco*-stigmasta-5,14-dien-3α-ol (**21**), 13,14-*seco*-stigmasta-9(11),14-dien-3*a*-ol (**22**), caesaldekarin J (**23**), neocaesalpin P (**24**), neocaesalpin H (**25**), cordylane A (**26**), caesalpinin B (**27**), caesalpinianone (**28**), 6-*O*-methylcaesalpinianone (**29**), and hematoxylol (**30**) were isolated [19–21]. Compounds **20–30** exhibited anti-GST activity with IC_{50} values of 380, 230, 248, 259, 200, 218, 250, 350, 16.5, 17.1, and 23.6 μM, respectively. The bioactivity data of all of these compounds indicated that homoisoflavonoids, **28** and **29,** have shown their potential as GST inhibitor [21]. Compound **28** was isolated in large quantity by us, and we decided to modify its structure by using whole cell cultures of *Curvularia lunata*. These studies yielded compounds **31** and **32**. The time-dependent microbial transformation experiments revealed that *C. lunata* carried out microbial hydroxylation at C-2 to afford compound **31**. The latter underwent dehydration reaction to yield metabolite **32** as shown in Fig. 1. Compounds **31** and **32** exhibited anti-GST activity with IC_{50} values of 15.0 and 0.8 μM, respectively. The higher potency of **32** might be due to the presence of a double bond adjacent to C-4 carbonyl group in its structure [22]. We are in the process of incorporating prenyl groups in compounds **28–32** in order to further improve their anti-GST activity, as we have observed the higher potency of compounds **15–18** that was due to the presence of prenyl groups in them compared to nonprenylated flavonoids.



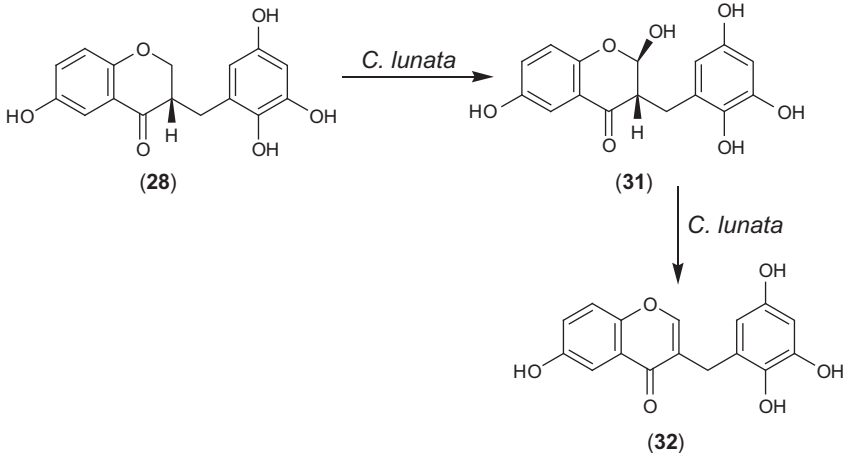


FIGURE 1 Biotransformation products of caesalpinianone (**28**).

A very careful comparison of structures **1–3**, **8–9**, **15–19**, and **32** reveals the presence of α,β-unsaturated carbonyl group in all of these compounds. All of these compounds exhibit significant GST inhibitory activity which suggests that this may constitute a pharmacophore for the expression of this bioactivity. This was further confirmed by a significant decrease in the bioactivity of compound **10** ($IC_{50} = 46.6$ μM), in which a double bond adjacent to the carbonyl group was reduced by microbial reaction. The α,β-unsaturated carbonyl group would lead to the formation of a glutathione adduct of these compounds through Michael addition to inhibit the activity of GST [23].

One of the major problems in drug discovery process is the supply of lead bioactive natural products for *in vivo* testing or clinical trials as most of these compounds are present in minor quantities and their synthesis on large scales seems to be difficult due to the presence of chiral centers in their structures. Currently, most of the bioactive compounds are mainly supplied for detailed *in vivo* and clinical trials by extracting plants. This method of obtaining lead bioactive compounds from plants will damage our rainforests and cause environmental problems. To overcome all these problems, it is therefore required to develop new biotechnological methods to produce lead compounds. In order to develop biotech method for the production of lead bioactive natural products, it is necessary to elucidate their biosynthetic origin. The biosynthetic studies provide information regarding enzymology involved in the synthesis of these compounds in nature. These biosynthesis experiments can be used as an assay to purify key enzymes involved in the synthesis of bioactive natural products. These enzymes can then be cloned in a suitable vector, commonly used *Escherichia coli*, to overexpress them in order to develop biotechnological methods to produce natural products on a large scale. Toward this end, we recently discovered that homoisoflavonoids, **28** and **29,** are biosynthesized using phenylalanine, acetate, and methionine to

give 2′-methoxychalcone (**33**) [24–26]. The latter compound undergoes cyclization to afford 3-benzylchroman-4-one (**34**). Our biosynthetic studies reveal that compound **28** is produced in nature by the hydroxylation of **34**. Methionine has methylated the C-6 hydroxyl group to afford **29**. The biosynthesis of **28** and **29** is outlined in Fig. 2.

Currently, we are in the process of purifying enzymes involved in the biosynthesis of intermediates **33** and **34** and to clone them in *E. coli* in order to develop biotech method to produce compounds **28** and **29**. Incubation of phenylalanine, acetate, and methionine with this particular *E. coli* strain would provide the homoisoflavonoids skeleton. This skeleton can then be subjected to microbial hydroxylation reaction to produce compounds **28** and **29** in the lab.

AChE Inhibitors

AD, a neurodegenerative disorder, causes severe health problems in elderly people [27]. One of the major causes of memory loss in AD patients is due to the deficiency of acetylcholine in the brain [28,29]. Accordingly, enhancement of acetylcholine levels in the brain is one of the most effective approaches to treat AD [30,31]. This task can be accomplished by using

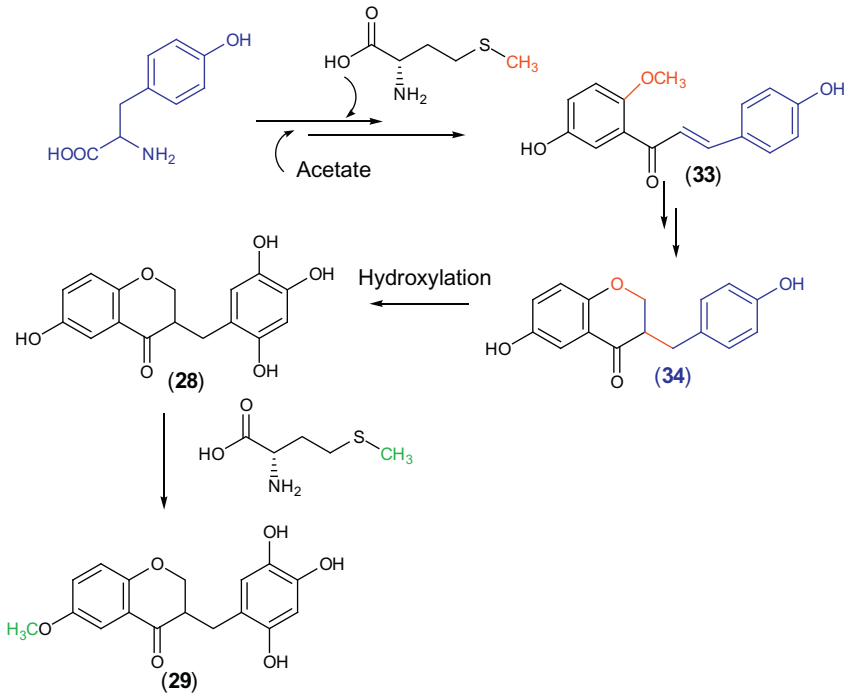
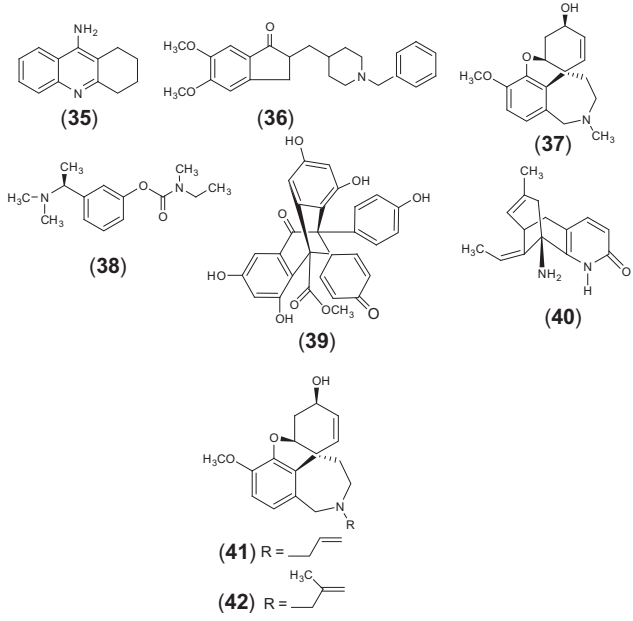


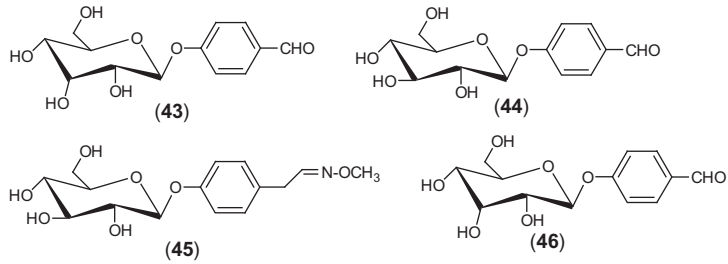
FIGURE 2 Biosynthesis of compounds **28** and **29**.

potent AChE inhibitors. AChE inhibitors also play a role in preventing proaggregating activity of AChE, leading to the deposition of β-amyloid, another cause of AD [32]. AChE inhibitors also have applications in treating senile dementia, ataxia, myasthenia gravis, and Parkinson's disease [33]. Two types of AChE inhibition assays (microplate reader and thin layer chromatography (TLC) based assays) are used to screen plant extracts and pure natural products [34]. Microplate reader bioassay is more accurate and reliable in order to determine the accurate potency of pure natural products while TLC-based bioassay can be used to guide the bioactivity and to determine chromatographic properties of bioactive fractions. Four AChE inhibitors, tacrine (**35**), donepezil (**36**), galanthamine (**37**), and rivastigmine (**38**) are approved by FDA to be used in clinics [35]. These compounds have limited effectiveness and a number of side effects [35]. For example, tacrine exhibits hepatotoxic lability and rivastigmine has a short half-life.

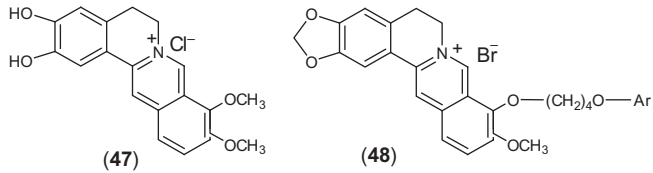
In this area, during the past decade, a few natural products or their analogues have been reported as potent AChE inhibitors. Hopeahainol (**39**), isolated from *Hopea hainanensis*, showed a potent AChE inhibitory activity with an IC_{50} value of 4.33 μM [36]. This bioactivity is more closely related to huperzine A (**40**), a prescribed drug used to treat AD and is active in this bioassay with an IC_{50} value of 1.6 μM. *N*-alkylated galanthamine derivatives, *N*-allylnorgalanthamine (**41**) and *N*(14-methylallyl)norgalanthamine (**42**), exhibit *in vitro* anti-AChE activity with IC_{50} values of 0.18 and 0.17 μM, respectively [37]. Compounds **41** and **42** are found to be more potent than galanthamine (**37**) that exhibit AChE inhibition activity with an IC_{50} value of 1.82 μM.



The aglycone of helicid (**43**), purified from *Helicia nilagirica*, exhibits potent GABA transaminase activity with an IC_{50} value of 4.1 μg/ml. Studies on the structure–activity relationships indicate that this bioactivity was due to aldehyde and hydroxyl groups on benzene ring [38]. Song *et al*. report the weak AChE inhibition activity ($IC_{50} = 37.8$ mM) of this aglycone. These authors synthesize compounds **44–46** using compound **43** as a template. Compounds **44–46** exhibit anti-AChE activity with IC_{50} values of 0.45, 0.49, and 0.20 μM, respectively [39].

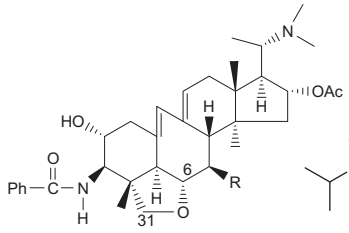


Similarly, compound **48**, a synthetic analogue of berberine (**47**), exhibits significant AChE inhibitory activity with an IC_{50} value of 0.097 μM. This compound is more potent than galanthamine (**37**) [40]. In this compound, berberine (**47**) is linked with phenol by 4-carbon spacers. Initial structure–activity relationship studies indicate that AChE inhibition is mainly due to the functional group present at the end of the chain and the length of the connecting tether.

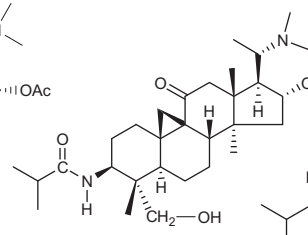


Buxus alkaloids isolated from *B. hyrcana* have shown their potential as AChE inhibitors. These alkaloids are O^6-buxafurandiene (**49**) and 7-deoxy-O^6-buxafurandiene (**50**), exhibiting this bioactivity with IC_{50} values 17.0 and 13.0 μM, respectively [41]. A number of *Buxus* alkaloids are reported in the literature with moderate AChE inhibition activity [42–46]. These moderately active alkaloids can be used as a template to synthesize potent AChE inhibitor. For instance, using *Buxus* alkaloid, *N*-3-isobutyrylcyclobuxidine-F (**51**), alkaloids (**52–56**) are synthesized by Guillou and collaborators [47]. These synthetic alkaloids (**52–56**) exhibit potent AChE inhibitory activity with IC_{50} values of 31, 13, 27, 18, and 14 nM, respectively [47]. These compounds

represent the first example of triterpenoidal alkaloids exhibiting potent anti-AChE activity.



(**49**) R = OH
(**50**) R = H



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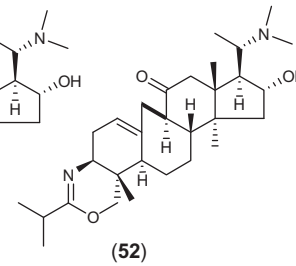
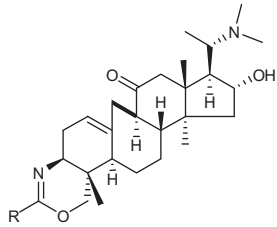
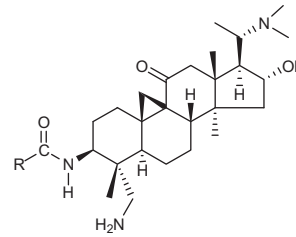


(**52**)

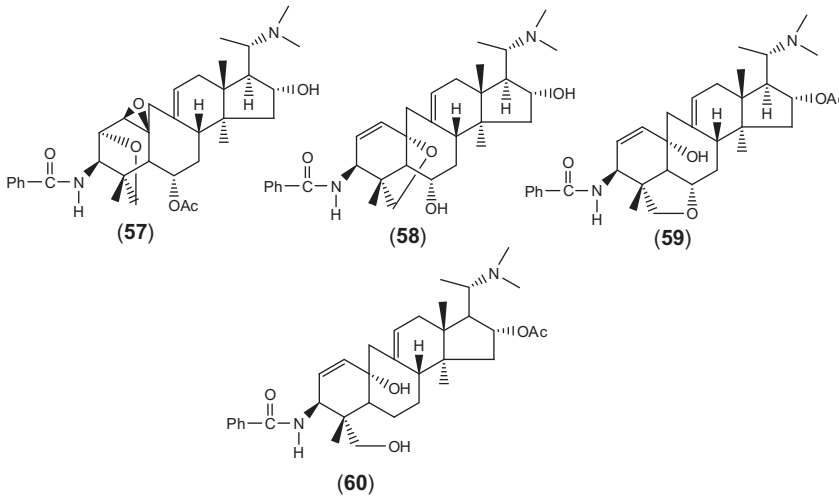


(**53**) R = CH_3
(**54**) R = $CH_3CH—CH_2—CH_3$



(**55**) R = iPr
(**56**) R = (*S*)—$(CH_3)CH(C_2H_5)_2$

These studies prompted us to collect *B. natalesis* from South Africa based on the ethnomedicinal use of this plant to enhance memory in elderly people by the local traditional healers [48]. The crude methanolic extract of this plant exhibited anti-AChE activity with an IC_{50} value of 28 μg/ml in our bioassay. AChE-inhibition-directed phytochemical studies on this extract yielded two new natural products, O^2-natafuranamine **57** ($IC_{50} = 3.0$ μM) and O^{10}-natafuranamine **58** ($IC_{50} = 8.5$ μM), along with two known alkaloids, buxafuranamide **59** ($IC_{50} = 14.0$ μM) and buxalongifolamidine **60** ($IC_{50} = 30.2$ μM), displaying different levels of anti-AChE activity [49]. The bioactivity of compound **57** was almost identical to huperzine A. Compounds **49**, **50**, **58**, and **59** were equally potent in AChE inhibition assay, suggesting the bioactivity of these compounds might be due to the presence of a tetrahydrofuran ring incorporated in their structures. The structural analysis of these compounds further indicated that the location of an ether linkage in these compounds does not play any role in enzyme inhibition activity, as **49**, **50**, and **59** contain ether linkage between C-31 and C-6, while **58** has an ether linkage between C-31 and C-10. Compound **57** has an ether linkage between C-31 and C-2 and an epoxy functionality at C-1/C-10. The higher potency of this compound was assumed to be due to the presence of these two functionalities.



Buxus alkaloids are plausibly reported to be produced in nature from cycloartenol type triterpenoids as no experimental report on biosynthesis of these alkaloids is present in the literature [50,51]. During our phytochemical studies on *Artocarpus* species, we have identified 4,4,14-trimethyl-9β, 19-cyclopregnane-3,20-dione (**61**) as a new natural product which is thought to be a putative intermediate involved in the biosynthesis of *Buxus* alkaloids. To confirm this hypothesis, it was decided to incorporate tritium at C-21 methyl group by using chemo-enzymatic approach. In order to achieve this goal, compound **61** was fermented using the liquid culture of *Cunninghamella echinulata* to afford C-21 alcohol (**62**). Intermediate **62** was reacted with TMSI using a mixture of methanol and $^{3}H_2O$ as solvent to afford compound **63**, as shown in Fig. 3 [52].

The compound **63** was fed to the fresh leaves of *B. natalaensis*, and investigation of these leaves, after 2 weeks, afforded C-21 tritium-labeled compounds **57–60**. During these studies, we have also identified compound (**64**) as a putative intermediate involved in the biosynthesis of compound **57** as shown in Fig. 4. Further studies on the biosynthesis of these compounds in order to elucidate the mechanism for the incorporation of amino group at C-3 and C-20, opening of 9β,10β-cyclopropane ring to afford 9(10→19) *abeo* diene system, and substitution of other functional group are currently underway in our lab. These biosynthetic studies will provide us the knowledge of key enzymes involved in the synthesis of these secondary metabolites in nature. We will then use this information to purify key enzymes and clone them into a suitable vector, *E. coli*, in order to devise a biotechnological method to produce these compounds in the lab as an alternative method of their supply for *in vivo* and clinical trials.

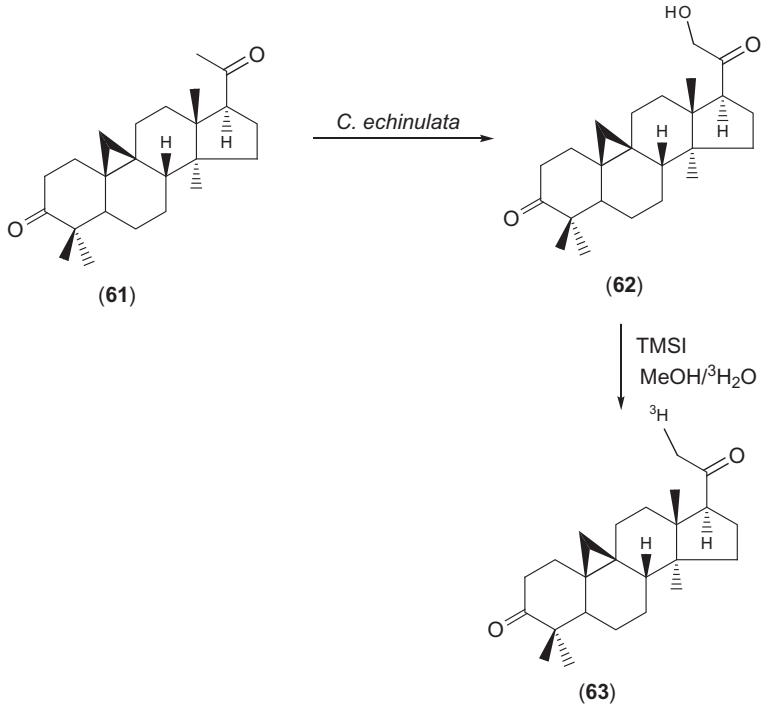


FIGURE 3 Chemo-enzymatic synthesis of compound **63**.

α-Glucosidase Inhibitors

α-Glucosidase is a membrane-bound enzyme and lies at intestinal cells. This enzyme catalyzes the final step of carbohydrates digestion by hydrolyzing the glycosidic bonds in carbohydrates to liberate free glucose and causing postprandial hyperglycemia. This results in type 2 diabetes mellitus that affects approximately 311 million people worldwide [53]. Its treatment mainly relies on suppressing hyperglycemia that includes reduction of glucose absorption in gut. This task can be accomplished by using potent α-glucosidase inhibitors [54]. These inhibitors can also be used to overcome obesity problems [55]. Recent studies indicate that natural products adopt a novel mode of action in inhibiting the activity of α-glucosidase. For instance, aegeline (**65**), a hydroxyl amide alkaloid, is reported to suppress both blood glucose and plasma triglycerides levels [56]. Tabopda *et al*. identified two new ellagic acid derivatives, 3,4′-di-*O*-methylellagic acid 3′-*O*-β-D-xylopyranoside (**66**) and 4′-*O*-galloy-3,3′-methylellagic acid 4-*O*-β-D-xylopyranoside (**67**) from *Terminalia superba*. Compounds **66** and **67** exhibit α-glucosidase inhibition activity with IC_{50} values of 7.95 and 21.21 μM, respectively [57]. Both of these compounds also show significant immunoinhibitory activities with no cytotoxic effects. Chebulagic acid (**68**), identified from *T. chebula*, exhibits

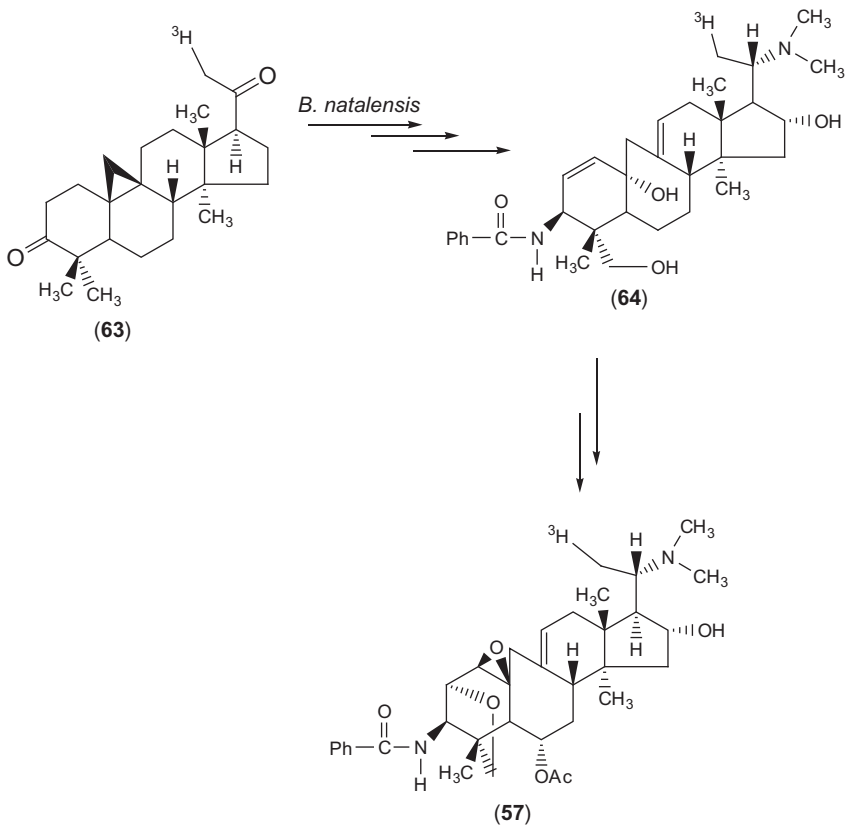
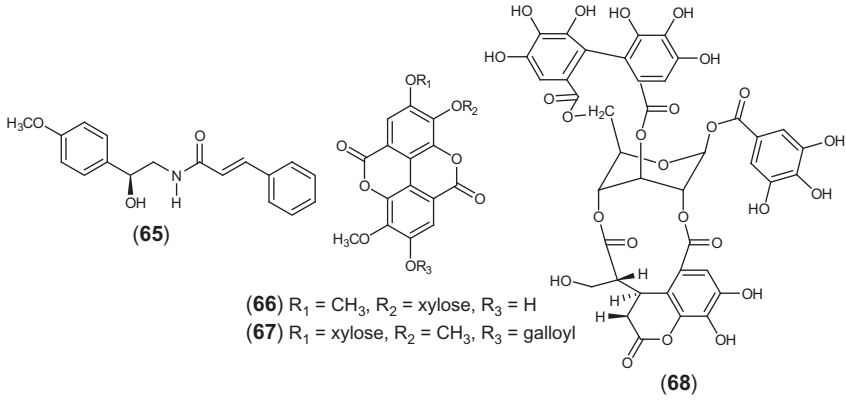
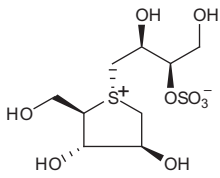


FIGURE 4 Biosynthesis of compound **57**.

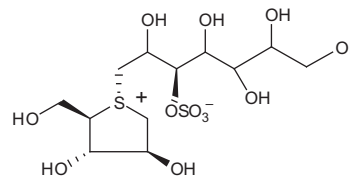
anti-α-glucosidase activity with an IC_{50} value of 6.6 μM. Kinetic studies on this inhibition activity suggest that this compound is a reversible and non-competitive inhibitor of maltase [58].



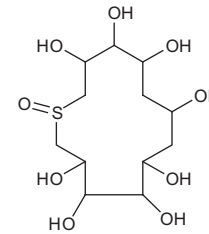
Salacia reticulate (Kothala-himbutu), a medicinal plant, is used to treat diabetes mellitus in Sri Lanka [59]. α-Glucosidase inhibition-directed investigation of this plant afforded salacinal (**69**), kotalanol (**70**), and a polyhydroxylated cyclic 13-membered sulfoxide (**71**) as active principles [60–62]. All of these compounds exhibit potent α-glucosidase inhibitory activity: **69** (IC_{50}: maltase 9.58 μM, sucrase 2.51 μM, isomaltase 1.77 μM); **70** (IC_{50}: maltase 6.60 μM, sucrase, 1.37 μM, isomaltase 4.48 μM); **71** (IC_{50}: maltase 0.227 μM, sucrase 0.186 μM, isomaltase 0.099 μM). The bioactivity data of **71** indicate that it is 42-, 14-, and 18-fold more potent against maltase, sucrase, and isomaltase compared to compounds **69** and **70** [60–62]. The high potency of compound **71** is hypothesized due to the orientation of hydroxyl, ring structure, and sulfoxide groups [62]. This assumption is only made by looking at the structure, but it is absolutely necessary to study the structure–activity relationships of compound (**71**) to confirm this hypothesis. These studies will also help to determine the active pharmacophore present in **71** that will provide a rational to design new α-glucosidase inhibitors.



(**69**)

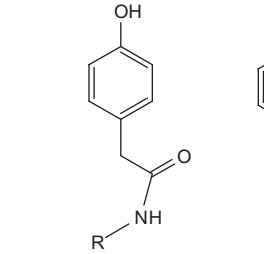


(**70**)

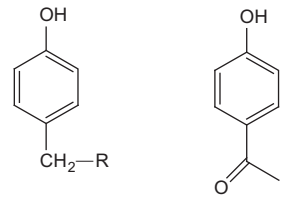


(**71**)

Our recent phytochemical studies on the methanolic extract of *Drypetes gossweileri*, collected from South Africa, afforded *N*-β-D-glucopyranosyl-*p*-hydroxyphenylacetamide (**72**), *p*-hydroxyphenylacetic acid (**73**), *p*-hydroxyphenylacetonitrile (**74**), *p*-hydroxyacetophenone (**75**), 3,4,5-trimethoxyphenol (7**6**), dolichandroside A (**77**), and β-amyrone (**78**). Compounds **72–78** exhibited α-glucosidase inhibitory activity with IC_{50} values of 12, 50, 48, 50, 56, 20, and 25 μM, respectively [63]. Compound **72** was found to be more potent compared to the rest of the isolates and represented the first example of the plant natural product containing *N*-glucose moiety incorporated in its structure. The acidic hydrolysis of compound **72** afforded compound (**79**), which exhibited α-glucosidase inhibition activity with an IC_{50} value of 60.0 μM, suggesting the higher potency of the parent compound (**72**) was due to the presence of *N*-glucose moiety.

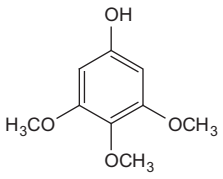


(**72**) R = β-D-glucose
(**79**) R = H



(**73**) R = COOH
(**74**) R = CN

(**75**)



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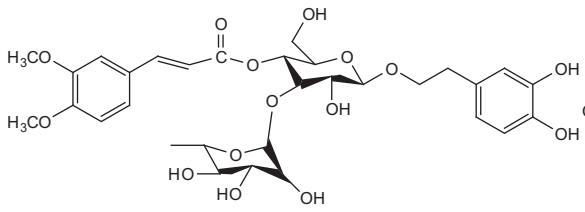

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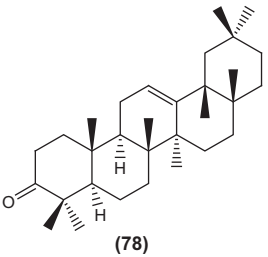
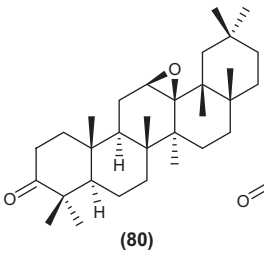
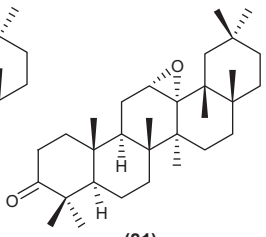


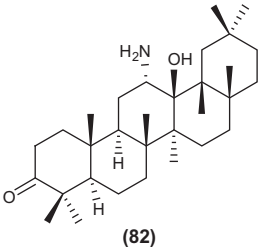
(**78**)



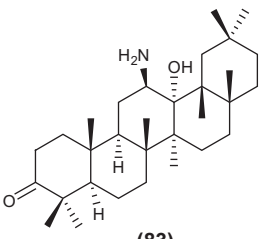
(**80**)



(**81**)



(**82**)



(**83**)

Compounds **72** and **78** also exhibited antifungal activity against *C. albicans* with minimum inhibitory concentrations (MIC) of 8.0 and 16 μg/ml, respectively. Compound **79** was weakly active in this bioassay with MIC value of 32 μg/ml, again indicating the higher potency of

compound **72** was due to the presence of *N*-glucose moiety in it. Compound **78** was reacted with *m*-chloroperbenzoic acid to afford compounds **80** and **81**. Both of these compounds underwent further reaction with 20% ammonium hydroxide solution to afford compound **82** and **83** [64]. Compounds **80–83** exhibited antifungal activity with MIC values of 4.0 and 8.0, 8.0 and $\leq$2.0 μg/ml. Compounds **80–83** also showed anti-α-glucosidase activity with IC_{50} values of 4.0, 10.0, 1.0, and 10.0 μM, respectively. The bioactivity data of compounds **80–83** suggest that the introduction of a β-oriented C-12/C-13 epoxy functionality and an amino group at C-12 in these compounds improve their bioactivity. It was also discovered that α-orientation of the C-12 amino group and β-orientation of the C-13/OH group play a significant role in these aforementioned bioactivities. These studies suggest that it is worthwhile to study the structure–activity relationships on moderately bioactive natural products to improve their bioactivities.

Investigation of *Epilobium angustifolium* of Manitoban origin afforded new compounds **84–85** displaying potent anti-α-glucosidase (yeast) activity with IC_{50} values of 120 and 122 nM, respectively. Compound **84** was a minor secondary metabolite of *E. angustifolium*. We, therefore, have developed a chemo-enzymatic method for the synthesis of this minor bioactive metabolite from commercially available betulin (**86**). Compound **86** was reacted with TMSCl in the presence DMAP to afford compound **87**. Microbial hydroxylation at C-21 in **87**, using the whole cell culture of *Cunninghamella blakesleeana*, afforded 21-hydroxybetulin (**88**). The reaction of **88** with $Hg(CF_3COO)_2$ and $NaBH_4$ followed by treatment with HF yielded compound **84** [65,66]. The chemo-enzymatic synthesis of **84** is outlined in Fig. 5. This novel approach using a combination of chemical and microbial reaction helps to prepare minor lead bioactive compounds from commercially available compounds or major secondary metabolites, isolated during bioassay-directed fractionation method. This novel green chemistry approach needs to be applied in the synthesis of other bioactive compounds that are difficult to synthesize using traditional synthetic chemistry. This chemo-enzymatic method will be a useful method to scale up the minor lead bioactive natural products for structure–activity relationships studies and detailed *in vivo* and clinical testing.

In summary, we have successfully identified natural products exhibiting *in vitro* anti-enzymatic activities against GST, AChE, and α-glucosidase. These potent lead enzyme inhibitors need to be evaluated for *in vivo* enzyme inhibition assays in order to determine their biomedical applications. Currently, our lab is in the process of devising biotech/chemo-enzymatic methods to produce these newly discovered potent lead-enzyme-inhibiting natural products for their detailed *in vivo* screenings.

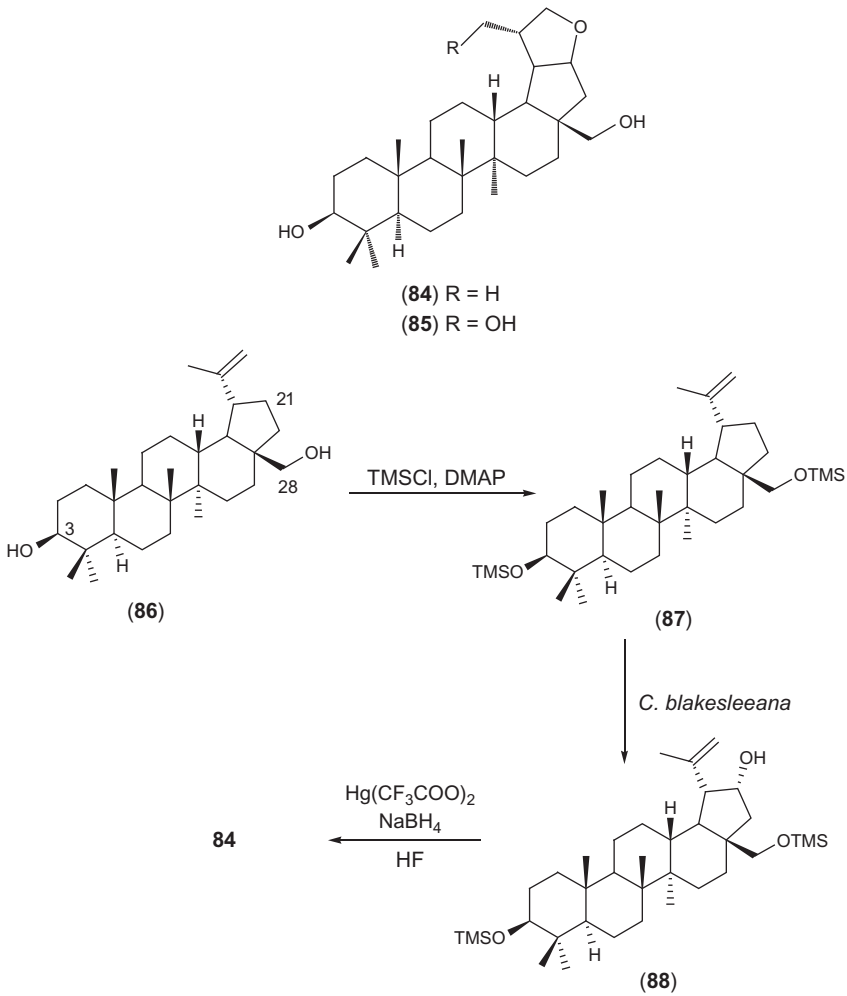


FIGURE 5 Chemo-enzymatic synthesis of compound **84**.

ACKNOWLEDGMENTS

I would like to thank all of my undergraduate and graduate students who were involved in discovering GST, AChE, and α-glucosidase inhibitors and their names are cited in the references. Funding provided by Natural Sciences and Engineering Research Council of Canada, Manitoba Health Research Council, and Research Corporation, USA, is gratefully acknowledged. I am also grateful to the Department of Chemistry, The University of Manitoba, for my appointment as adjunct professor. I am also thankful to my beloved mother, Sharifan Bibi, for all her moral support, kind prayers, and love throughout my entire life and I lost her on September 10, 2011.

ABBREVIATIONS

AChE acetylcholinesterase
AD Alzheimer's disease
CLL chronic lymphocytic leukemia
GST glutathione *S*-transferase
IC_{50} concentration required to inhibit 50% activity of enzyme
TLC thin layer chromatography

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Chapter 9

Histone Deacetylases as Cancer Chemoprevention Targets for Natural Products

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INTRODUCTION

Cancer is considered to be the result of a wide variety of genetic and genomic alterations. It has been established that most human cancers are induced by environmental factors, including chemicals, radiation, and biological agents [1]. Given the high probability of developing cancer over the period of a normal lifespan, cancer chemoprevention provides an attractive therapeutic strategy for the delay or reversal of this process [2–4].

Recent evidence shows that cancer is associated with identifiable epigenetic changes and dysfunctional transcriptional regulatory mechanisms [5]. Both the acetylation and the deacetylation of histones play a fundamental role in the remodeling of chromatin and epigenetic regulation of gene expression. Transcriptionally active genes are associated with hyperacetylated chromatin, whereas transcriptionally silent genes are associated with hypoacetylated chromatin. The steady-state histone acetylation level arises from the opposing action of histone acetyltransferase (HAT) and histone deacetylase (HDAC) enzymes [6,7].

HDACs are enzymes that cleave acetyl groups from acetyl-lysine residues in histones and various nonhistone proteins. HDACs are grouped into four classes based on the structure of their accessory domains. Classes I, II, and IV belong to the Rpd3/Hda1 family [8]. Class I has nuclear localization and includes the constitutively expressed HDACs 1–3 [9] and HDAC8. HDAC8 is the only isotype that is not strictly Zn^{2+} dependent [10]. Class II is subdivided into classes IIa (HDAC4, -5, -7, and -9) and IIb (HDAC6 and -10).

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Enzymes from class IIa have a weak deacetylase activity [11] and shuttle between the cytosol and nucleus. Class IIb is mostly found in the cytosol where HDAC6 deacetylates proteins with a preference for nonhistone proteins [12]. HDAC11 is the sole member of the class IV enzymes but shares features of both class I and II. The inhibition of HDAC activity has been explored as a therapeutic strategy in cancer [13]. Thus, HDAC inhibitors (HDACi) represent a new class of prominent anticancer agents and have been shown to exhibit antimetastatic and antiangiogenic activities toward malignantly transformed cells *in vitro* and *in vivo* [14,15]. Over the past several years, the number of HDAC enzyme subtypes has expanded considerably, offering opportunities for the development of HDACi with improved specificity [16]. Unlike the other three out of four classes of HDACs that have been identified in humans, which are zinc-dependent amidohydrolases, class III HDACs depend on nicotinamide adenine dinucleotide (NAD^+) for their catalytic activity. The seven members of this class are named sirtuins for their homology to Sir2p, a yeast HDAC. Sirtuin inhibitors have been critical for the linkage of sirtuin activity to many physiological and pathological processes, and sirtuin activity has been associated with the pathogenesis of cancer, HIV, and metabolic and neurological diseases [17].

Epigenetic alterations are often involved in the earliest stages of tumor progression, usually preceding neoplastic transformation, and have been shown to affect tumor formation in genetic mouse models of carcinogenesis [18]. Therefore, the real clinical interest of cancer epigenetics may be in prevention, which is where epigenetics might have its greatest impact. The most suitable interventions for preventing early epigenetic changes would be lifelong, nontoxic dietary approaches [19]. Thus, there is a growing interest in identifying pharmacologically safe dietary compounds with HDAC modulating activities [20].

This review presents an overview of the most important natural products known for their cancer chemopreventive potential by modulating HDAC activity. In the latter part, available enzyme- and cell-based assays are presented with a special focus on the screening of plant extracts and natural products.

THE ROLE OF HDACs IN CARCINOGENESIS

HDACs are well known for their contribution to epigenetic control; besides DNA methylation [21], histone acetylation regulates transcription with a subsequent condensation of chromatin sections [22]. In consequence, histone deacetylation silences gene expression, for example, p21 by HDAC2 [23], but also induces transcriptional upregulation of an important number of proteins [8], such as 5-lipoxygenase [24]. Some studies suggest that selective class I inhibition is sufficient for chemotherapy [25] because selective inhibition of

this class abrogates cell proliferation and survival. Moreover, unselective inhibitors may lead to adverse effects, such as interference with MEF2 [26].

Nonhistone substrates, recognized by distinct HDAC classes, not only contribute to transcriptional regulation but also contribute to other effects that are relevant to cancer development, such as posttranslational modifications. These targets may also offer additional benefits in cancer prevention and/or therapy. Nonhistone substrates comprise tumor relevant proteins, such as transcription factors, inflammation and signal transduction mediators, hormone receptors, and structural elements [6,8]. For example, HDAC6 is well known to deacetylate HSP90 and α-tubulin [12]. Although not stringently associated with acetylation or deacetylation, several tested HDACi functionally alter some of the cancer-related pathways, at least *in vitro*, including the following pathways: growth arrest by downregulation of cyclines and upregulation of p21, antiangiogenic effects by interference with VEGF and HIF-1α, activation of and enhanced susceptibility to the intrinsic and extrinsic apoptosis pathways, and increased reactive oxygen species production [6,8]. Mainly inflammation-related proteins, such as cytokines [27], cyclooxygenase-2, or matrix metalloproteinases, are influenced by HDACs through interference with nuclear factors, such as NF-κB and STATs or signal transduction mediators, such as ERK and PI3K [28]. A unique finding regards HDAC11, which suppresses the anti-inflammatory cytokine IL-10, thereby modulating the global T-cell balance [29]. The alteration of all these carcinogenesis-related effects by HDACi is, therefore, regarded as beneficial for cancer chemoprevention [25,30,31].

HDACi FROM NATURAL SOURCES

The first HDACi to be identified was the naturally occurring butyrate, shown in Fig. 1, which is present in butter and vegetable oils and is also available *in vivo* after fiber intake and digestion [32]. Since then, the presence of HDACi in products of natural origin, mainly dietary products, has been associated with cancer chemoprevention. The contribution of dietary natural compounds as chemopreventive agents will be herein discussed for isothiocyanates (cruciferous vegetables), short-chain fatty acids (fiber, butter, and oil), organosulfur compounds (garlic, onion, and broccoli), organoselenium compounds (seleniferous vegetables), and polyphenols (edible fruits). Some of these products are HDACi as such, while others provide *in situ* active metabolites. They are usually weak ligands for HDAC and are mostly associated with cancer chemoprevention [33]. It has been postulated that HDAC inhibition by dietary agents enables acetyltransferase–coactivator complexes to acetylate histone tails, thereby loosening DNA–chromatin interactions and allowing access of transcription factors to the promoters of such genes as *CDKN1A* (*p21*) and *Bax*. The expression of these genes facilitates cell cycle arrest and apoptosis in the context of chemoprevention [20].

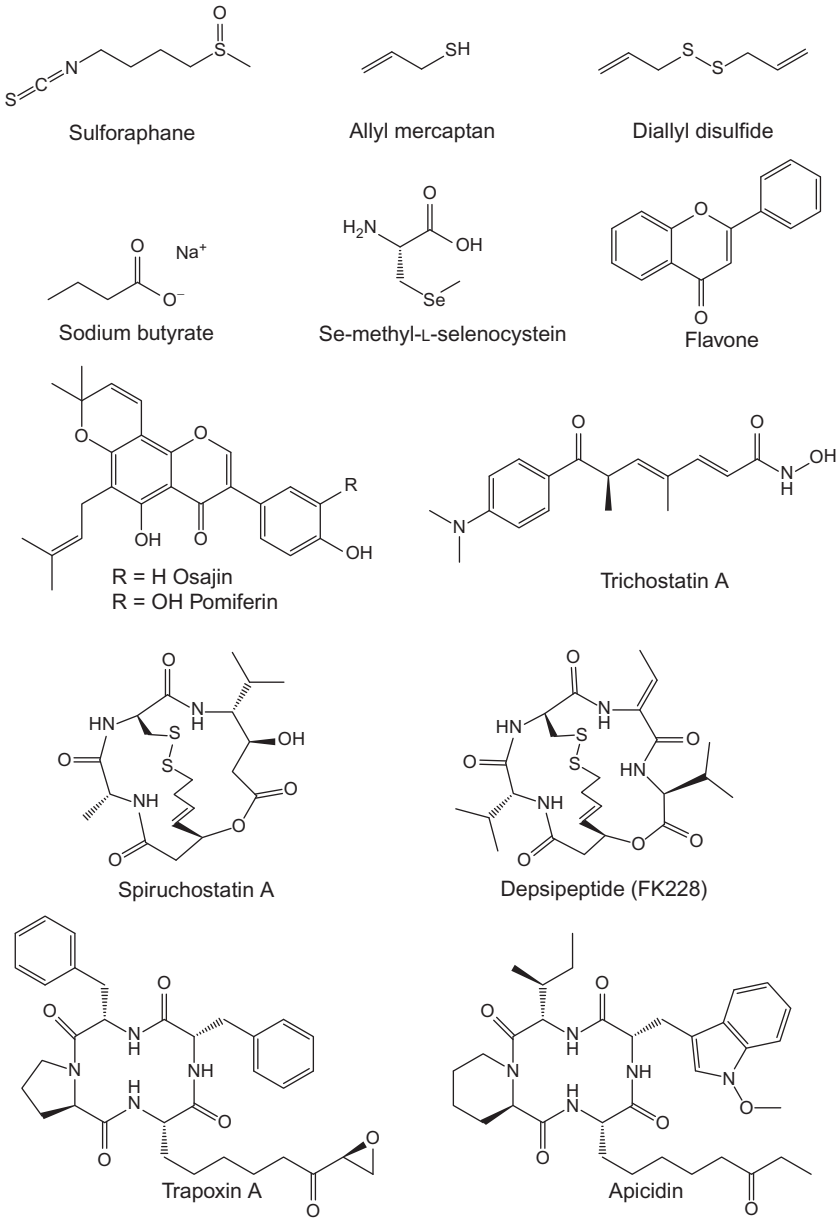

FIGURE 1 Chemical structures of selected natural HDAC inhibitors.

Natural HDACi have also been identified in bacteria (depsipeptide) and fungi (trichostatin A (TSA) and apicidin, see Fig. 1) as well as in marine organisms (psammaplin and largazole). These potent natural HDACi have been studied in the context of cancer therapy and will be briefly mentioned in this review.

Isothiocyanates

Brassica vegetables are known for containing glucosinolates, which are hydrolyzed by myrosinase, an enzyme activated in plants upon damage, and are rearranged to provide isothiocyanates. One example of isothiocyanate is sulforaphane (SFN, see Fig. 1), which is derived from broccoli. This compound has been considered to be a chemopreventive agent according to results obtained from several animal models [34,35]. The first mechanisms associated with its chemopreventive effect were the induction of phase II detoxification enzymes as well as the inhibition of phase I enzymes responsible for the activation of chemical carcinogens [34–36]. The HDAC inhibitory activity of SFN was first reported on nuclear extracts of HEK293 cells previously treated with this compound. Interestingly, no HDAC inhibition could be observed when SFN was incubated with HeLa nuclear extracts. However, when the culture medium of HEK293 cells treated with SFN was incubated with HeLa nuclear extracts, HDAC inhibition was found (Table 1) [37]. This result suggests that SFN cell metabolites may be responsible for the activity. SFN is metabolized via the mercapturic acid pathway with the formation of SFN-GSH and with subsequent metabolization to SFN-Cys and SFN-NAC [38]. Individual metabolites have been tested on HeLa nuclear extracts showing that SFN-Cys and SFN-NAC were able to inhibit HDAC activity, while SFN and SFN-GSH had little inhibitory activity [37]. It has been shown that other dietary isothiocyanates metabolized via the mercapturic acid pathway, such as SFN-4, -6, -9, erucin, phenylbuthylisothiocyanate, and phenethylisothiocyanate, display similar HDAC inhibition after incubation with HTC116 colon cancer cells followed by an HDAC *in vitro* assay using the cell lysate [39].

SFN is especially capable of reaching millimolar concentrations in mammalian cells [35]. The parent compound and its metabolites have been observed at micromolar concentrations in the plasma and urine of humans consuming broccoli [40].

During preclinical and clinical evaluation for chemoprevention in the breast, a SFN dithiocarbamate metabolite was found in the mammary gland of rats (18.8 pmol/mg tissue 1 h after oral administration of 150 μmol SFN) and humans (1.45–2.00 pmol/mg tissue 1 h after oral administration of 200 μmol SFN) [41].

TABLE 1 Inhibitory Activity of the Main Natural HDAC Inhibitors, According to the Literature

Compound	Inhibition Assay	Activity	References
Sulforaphane	HeLa nuclear extract	NA	[37]
	HEK293 cell incubation (transfected with HDAC1)	Significantly dose-dependent inhibition at 3, 9, and 15 μM	[37]
Butyrate	HeLa Mad38	$IC_{50}=0.09$ mM	[50]
	HT29 nuclear extract	$IC_{50}=0.01$ mM	[50]
	HeLa Mad38 cell incubation	$IC_{50}=2$ mM	[50]
Diallyl disulphide	Caco-2 nuclear extract	29% inhibition at 200 μM	[51]
Allylmercaptan	Caco-2 nuclear extract	92% inhibition at 200 μM	[51]
Se-methyl-L-selenocysteine	LNCaP, C4-2, PC-3, DU145 nuclear extracts	NA at 2.5 mM	[52]
Flavone	Recombinant HDAC1 incubated in U937 cell lysate	>50% inhibition at 270 μM	[53]
Osajin	HDAC (not specified)	$IC_{50}=6.5$ μM	[54]
Pomiferin	HDAC (not specified)	$IC_{50}=1.1$ μM	[54]
Trichostatin A	Breast cancer cell lines, crude extracts	$IC_{50}=2.4$ nM	[55]
	HDAC1[a]	$IC_{50}=15.3$ nM	[56]
	HDAC6[a]	$IC_{50}=61.2$ nM	[56]
Spiruchostatin A (reduced form)	HDAC1[a]	$IC_{50}=0.6$ nM	[56]
	HDAC6[a]	$IC_{50}=360$ nM	[56]
Depsipeptide (FK228)	Purified enzyme fraction[a]	$IC_{50}=1.1$ nM	[57]
Trapoxin A	Purified enzyme fraction[a]	$IC_{50}=0.3$ nM	[57]
Apicidin	*Pf*HDAC purified from parasitic nuclear extract	$IC_{50}=0.7$ nM	[58]

NA, not active; *Pf*, *Plasmodium falciparum*.
[a]*Source not specified.*

Short-Chain Fatty Acids

Butyrate, depicted in Fig. 1, is a product of the fermentation of dietary fibers by endosymbiotic bacteria during the digestive process, and it can also be found in butter and vegetable oils. This short-chain fatty acid was among the first compounds discovered to increase histone acetylation in cells (Table 1) [32]. Butyrate has potent activity as a differentiating agent and inducer of cell cycle arrest, apoptosis, inhibition of invasion, and metastasis in cell culture assays [42–44]. Butyrate inhibits HDAC *in vitro* with a millimolar IC_{50} [45], and its synthetic prodrug derivative tributyrin was shown to be more potent at differentiating leukemia cells [46]. A number of clinical trials have been conducted using sodium butyrate and synthetic derivatives, mainly tributyrin, phenylacetate, phenylbutyrate, and pivaloyloxymethyl butyrate, usually showing dose-limiting toxicity and the need for administration of large volumes of infusion to evaluate the effects on cancer chemotherapy [47]. Moreover, no changes were detected in histone acetylation in peripheral blood mononuclear cells before and after oral administration of tributyrin. Therefore, the role of HDAC inhibition on the clinical effects observed for tributyrin is not well established and needs further investigation [48]. Despite the fact that the clinical use of butyrate-like compounds for cancer chemotherapy seems challenging, the hypothesis that dietary short-chain fatty acids contribute to chemoprevention of several cancer types is supported by epidemiological data on fiber intake. As a matter of fact, the chemopreventive effects of less potent short-chain fatty acid HDACi (millimolar IC_{50} ranges and weak HDAC ligands) from the diet is under investigation in studies that attempt to correlate dietary fiber intake and fermentation, colon cell turnover, global protein acetylation, and early carcinogenesis [49].

Organosulfur Compounds

The anticarcinogenic activity of the garlic organosulfur compound diallyl disulphide (DADS, see Fig. 1) has been reported in various *in vivo* models, indicating inhibition of the initiation and promotion phases of colon, stomach, and renal carcinogenesis [59,60]. Corroborating these observations, epidemiological studies point out the protective effect of garlic consumption on gastrointestinal cancers [61]. The first mechanisms associated with this protection were the modulation of phase I and II drug-metabolizing enzymes [62] as well as changes in gene expression [63]. The ability of DADS to inhibit HDAC was determined on the basis of the *in vitro* assay on nuclear extracts from HeLa and Caco-2 cells (Table 1). One of the metabolites of DADS, allylmercaptan, shown in Fig. 1, was even more efficient [64].

Another interesting *in vivo* metabolite of DADS is *S*-allylmercaptocysteine, which presents structural characteristics that are similar to those of butyrate and SNF-Cys [65]. This metabolite showed an induction of histone

acetylation after incubation with DS19 cells. However, it is a less effective HDACi than allylmercaptan *in vitro* on nuclear extracts. The increase of cellular histone acetylation may be due to the metabolization of *S*-allylmercaptocystein into allylmercaptan [66].

Organoselenium Compounds

Dietary seleniferous plants, such as garlic, onions, and broccoli, have been considered to be of great interest for cancer chemoprevention. All of these plants contain the natural organoselenium compound Se-methyl-L-selenocysteine (MSC, see Fig. 1), which has been associated with chemoprevention through *in situ* generation of methylselenol by β-lyases [67]. Selenium compounds have been shown to have antitumorigenic activities in animal models [68]. Several hypotheses have been proposed to elucidate the inhibition of tumor development by selenium supplementation, including protection from oxidative damage, influences in carcinogen metabolism, production of cytotoxic selenium metabolites, inhibition of protein synthesis, inhibition of specific enzymes, and stimulation of apoptosis [69].

Recently, a metabolization hypothesis for MSC and L-selenomethionine has been formulated, suggesting that these compounds provide methylselenol and α-ketoacid metabolites by elimination (β- or γ-liase) and transamination (aminotransferase, e.g., GTK), respectively. When tested for *in vitro* HDAC inhibition, the α-ketoacid metabolites presented significant activity rather than methylselenol and MSC (Table 1) [52]. Clinical trials demonstrated a protective role of selenium supplementation against carcinogenesis [69]. Despite all of this evidence, the results provided by a large clinical prevention trial on selenium and vitamin E supplementation for the prevention of prostate cancer (SELECT) failed to show a positive outcome [70]. However, such an outcome is still controversial, and the chemopreventive potential of selenium is supported by successful clinical trials on other types of cancer [69,71].

Polyphenols

Although polyphenols are often associated with the activation of human SIRT1, some phenolic compounds, mainly isoflavones and flavones, are known for causing HDAC inhibition *in vitro*. One such example is flavone, shown in Fig. 1, which is isolated from acetonic extracts of the edible fruits of *Feijoa sellowiana* (Myrtaceae). The total extract was able to exert antiproliferative activity along with modulation of the cell cycle and apoptosis. Thus, the inhibition of HDAC by the isolated flavone may contribute to the observed effect (Table 1) [53]. In addition, the prenylated isoflavonesosajin and pomiferin, shown in Fig. 1 and isolated from *Maclura pomifera* (Moraceae), were able to inhibit HDAC activity as well as the growth of various human cancer cell lines at micromolar concentrations (Table 1) [54].

HDACi from Fungi, Bacteria, and Marine Organisms

TSA, shown in Fig. 1, is a hydroxamic acid of bacterial origin that was first isolated from *Streptomyces hygroscopicus* together with trichostatin C (TSC) [72]. Following the observation that TSA was able to promote cell cycle arrest and cell differentiation [73], TSA appeared to be a potent HDACi and is now extensively used as a reference compound in the search for new HDACi. The full characterization of TSA, as well as its cocrystalization with the human enzyme HDAC8, has widely contributed to the design of new and potent HDACi [74,75]. Other trichostatins have been isolated from *Streptomyces* spp. such as trichostatic acid (TSacid), TSC, trichostatin RK, and FL657C [76]. To our knowledge, only TSacid and TSC have been tested for HDAC inhibition. While the former was considered inactive, the latter showed some HDAC inhibitory activity ($IC_{50} = 10.0\ \mu M$), but was less potent than TSA, which is active at the nanomolar (nM) level (Table 1) [77].

From a structural point of view, the inhibitors of deacetylase based on a cyclopeptidic scaffold include the most structurally complex molecules. Some of the natural cyclic peptides known for HDAC inhibition are apicidinas shown in Fig. 1, which is isolated from the filamentous fungi *Fusarium* spp. [58,78]; trapoxin A, shown in Fig. 1, and B, isolated from the fungus *Helicoma ambiens*; depsipeptide of bacterial origin (FK228, see Fig. 1) isolated from *Chromobacterium violaceum* [57]; spiruchostatin A, shown in Fig. 1, and B, which are depsipeptides from *Pseudomonas* spp. [56,79]; azumamides, from the marine sponge *Mycale izuensis* [80]; and largazole from the cyanobacterium *Symploca* sp. (Table 1) [81].

A few other non-peptidic HDACi of natural origin have been studied, such as psammaplin A from the sponges *Pseudoceratina purpurea* and *Psammaplysilla* sp. as well as the two-sponge association *Jaspis* sp. and *Poecillastrawondoensis* [82]; depudecin, isolated from the fungal pathogen *Alternaria brassicicola* [83]; and bispyridinium derivatives such as the macrocyclic cyclostellettamines isolated from the marine sponges *Haliclona* sp. and *Xestospongia* sp. [84,85].

THE ROLE OF SIRTUINS IN CARCINOGENESIS

When compared to the class of small molecule inhibitors of classical HDACs (class I, II, and IV enzymes), a small number of sirtuin inhibitors have been described to date [17]. Much less is known about the biological consequences of sirtuin inhibition [86], which were mostly focused on the inhibition of human SIRT1 and/or SIRT2. One reason for the optimism that SIRT1 is a druggable target is the known beneficial effect of classical activators of SIRT1, such as resveratrol [87]. Nevertheless, the interaction between sirtuins and cancer development is especially confusing. Whether sirtuins serve as tumor suppressors or tumor promoters is a hot debate [88]. On the one hand,

as promoters of cell survival, one might predict that some sirtuins, such as SIRT1 and SIRT3, would possess an oncogenic function. For example, they are able to inhibit apoptosis by deacetylating p53, Ku70, PARP, Smad7, and HSF1 [89], which may engender cancer in tissues with dividing cells. In particular contexts, the activation of SIRT1 may be undesirable, and inhibitors of SIRT1 were shown to be beneficial [90]. On the other hand, as promoters of the survival of various organisms, one might predict a tumor suppressor function for sirtuins, which are involved in DNA damage responses. It is also important to consider the complicated and sometimes competing effects of individual sirtuins on cellular processes that influence cancer development, suppression, and progression [91]. Of all the sirtuins, SIRT1 has received the largest amount of research attention and its impact on other proteins is best understood. There are several reviews that claim either oncogenic or tumor suppressor functions for sirtuins [92,93]. This scenario suggests that much more research is required.

Moreover, it would be prudent to bear in mind the following points when considering sirtuin inducers. The potential problems in the coactivation of multiple sirtuins require special attention. Because SIRT1 modulates energy metabolism, the development of any SIRT1-based therapeutics to be administered systemically should consider possible secondary effects on the basal metabolic rate of the subjects [94]. Finally, existing evidence suggests that, while there is some overlap in the proteins that the various sirtuin deacetylates, each of the proteins plays a distinct and different role in protein deacetylation depending on their subcellular location, tissue distribution, and protein affinities [95].

MODULATORS OF SIRTUINS FROM NATURAL SOURCES

A broad range of human health benefits have been reported for plant polyphenols, including cardioprotection, neuroprotection, and cancer suppression [96,97]. Interestingly, similar beneficial effects are observed in calorie-restricted rodents [98]. It is possible that many of the effects of polyphenols might be the result of a caloric restriction-mimetic defense response mediated by sirtuins [99]. Polyphenols are members of a large and diverse group of

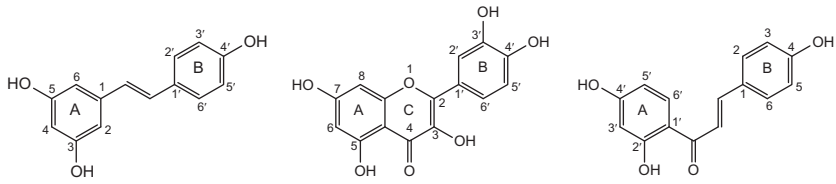

FIGURE 2 Chemical structures of selected natural sirtuin inducers.

plant secondary metabolites that includes flavones, flavanones, isoflavones, catechins (flavan-3-ols), chalcones, stilbenes, tannins, and anthocyanidins.

Many but not all of the most active sirtuin activators include hydroxyls in the two meta-positions of the A ring and *trans* to the B ring with a 4′- or 3′,4′-hydroxyl pattern, shown in Fig. 2. A potentially coplanar orientation and *trans*-positioning of the hydroxylated rings also appears to be important for activity. Among them, resveratrol was shown to be the most active [99].

Resveratrol

Resveratrol, which is a natural phytoalexin produced in such food plants as grapes, peanuts, and berries as a defensive response against fungal infections and other environmental stressors [100], has exceptional potential as a treatment modality due to its cardioprotective, anti-inflammatory, chemopreventive, and antiangiogenic properties [101]. A great number of *in vitro* studies and preclinical research strongly support the wide-ranging anticancer effects of resveratrol, including the induction of apoptosis and the inhibition of cell growth and angiogenesis [102].

The effect of resveratrol on SIRT1 has been the focus of several reports in which both upstream and downstream molecular mechanisms have been extensively studied. In human fibrosarcoma cells, the activation of SIRT1 in the presence of resveratrol inhibited the expression of MMP-9 through the inhibition of ROS and the activation of NF-κB and AP-1 [103]. In breast cancer cell lines, a mechanism through which resveratrol could inhibit protein translation by activating the AMPK-mTOR pathway was proposed. These data showed that resveratrol-induced AMPK activation decreased tumor cell proliferation. This mechanism depends on the expression of SIRT1 [104].

The caloric restriction-like beneficial effect of resveratrol has now been proven in many animal studies [87]. However, it did not extend the lifespan of mammals [105]. The majority of studies on the interaction of resveratrol with sirtuins have used stressed, impaired, or knockout models [106]; few studies have analyzed this interaction in healthy experimental animals. In one of these studies, an increase was observed in the levels of the SIRT1 transcript and protein in rats fed with a low-protein, high-carbohydrate diet plus resveratrol, but not in rats fed with a normal diet [107]. In healthy volunteers, no correlation with the modulation of sirtuins was found [108,109]. The regulation of SIRT1 by resveratrol may be quite complex. Whether SIRT1 is regulated at the transcriptional, translational, or posttranslational level has not been thoroughly elucidated. Additionally, such factors as target tissue, differences in the tissue distribution of resveratrol, compound administration, and food interference need to be considered [110]. Also, its specificity (in terms of SIRT1 activation) as well as bioavailability has been in doubt [94]. Some results indicate that *in vitro* SIRT1 is not a direct target of

resveratrol [111–113]; nonetheless, it is possible that the beneficial effects attributed to resveratrol are partly mediated by SIRT1 *in vivo* [99].

Combinations between HDACi and resveratrol have also been studied. Combined suberoylanilide hydroxamic acid (SAHA) and resveratrol treatment resulted in a substantial increase in p53 acetylation [114]. This result opens new avenues for combination therapies between resveratrol (or its analogs) and chemotherapeutic HDACi. This concept may evolve as a new anticancer strategy because SAHA is an FDA-approved anticancer agent [115] and resveratrol is a dietary compound.

Lately, small compounds with *in vitro* SIRT1 activation efficacies up to 3 magnitudes greater than that of resveratrol have been synthesized [116]. A number of these compounds are already in phase II clinical trials, and new compounds are still being identified and developed [94].

Other Polyphenols

Besides resveratrol, other polyphenols are sirtuin inducers. Treatment of fibroblasts with oligomeric proanthocyanidins isolated from persimmon (*Diospyros kaki*) peels led to a decrease in 8-OHdG, which is one of the most abundant oxidative DNA adducts, and an elevation in SIRT1 expression [117]. In a mouse model, an extended life span was observed after oral administration of oligomeric proanthocyanidins [118]. These studies suggest that these compounds might have a beneficial effect on DNA damage and carcinogenesis.

Quercetin, a natural polyphenolic flavonoid present in a wide variety of food plants, including red onions, apples, and berries [119], also showed interesting activity. Seven days of quercetin feeding increased SIRT1 expression in both the skeletal muscle and brain tissue of mice [120]. With some of these polyphenols, large differences were seen between their effects on intracellular SIRT1 and on the isolated SIRT1 protein. This difference might be due to the metabolism and stability of polyphenols, which affect the stimulation of SIRT1. Therefore, extrapolation of *in vitro* SIRT1 stimulation results to physiological effects should be done with caution [121].

BIOLOGICAL ASSAYS

The emerging interest in HDACi as new targets, mainly in cancer therapy, motivates the development of assay platforms that are suitable for fast, easy to perform, and cost-effective screening of large chemical and natural compound libraries. The moderate substrate specificity of HDAC enzymes, which certainly promoted the development of a broad set of screening strategies during the past dozen years, simultaneously hampers subtype specificity of many HDAC ligands [122]. In addition, the activity of many HDACs is tightly regulated by protein complexes [123–125]. To date, only class-specific and, with the exception of sirtuins [126,127], substrate-competitive inhibitors are

known. As a result, subtype specific assays are usually bound to costly purified enzyme preparations.

Early assays for monitoring histone acetylation and, later, for inhibitor screening were based on radioactive substrates. Usually, [^{13}C]- or [^{3}H]-acetyl labeled core histones from biologically labeled erythrocyte nuclei [128] or from cell lines [129] are used today. In better-defined assays, synthetic [^{3}H]-acetylated peptides based on histone sequences are employed [130]. Both strategies involve subsequent extraction steps with organic solvents followed by the quantification of released radiolabeled acetate by liquid scintillation count. A more recent approach is based on the scintillation proximity principle by means of a biotinylated octapeptide [131]. Although it does not require additional workup, this latter strategy still uses radioactive material.

Only recently, nonradioactive assays have been developed. Most of the commercially available kits have their origin in the cell membrane-permeable and weakly fluorescent Boc-Lys(Ac)-7-amino-4-methylcoumarin ($\lambda_{ex} = \sim 330$ nm and $\lambda_{em} = \sim 390$ nm) (shown in Fig. 3) [132,133]. The original protocol, which involved an isolation step, HPLC separation and UV fluorescence detection, has since been adapted to high-throughput and whole-cell suitability by coupling the initial deacetylation reaction to an enzymatic liberation of the highly fluorescent 7-amino-4-methylcoumarin ($\lambda_{ex} = 365$–380 nm and $\lambda_{em} = 430$–460 nm) by *in situ* incubation with trypsin [134,135] or the lysyl endopeptidase Lys-C

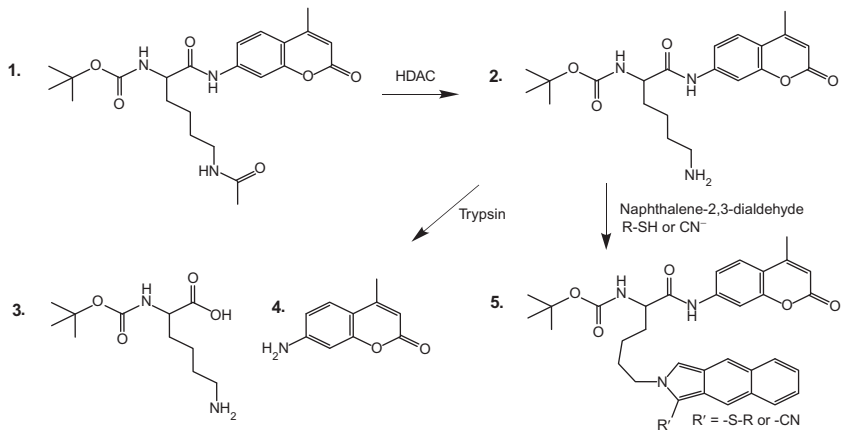

FIGURE 3 Two-step fluorescence-based HDAC assay principles. The first published profluorophore Boc-Lys(Ac)-7-amino-4-methylcoumarin (**1**) undergoes HDAC-catalyzed hydrolysis of the ε-acetate, leading to compound (**2**). This compound subsequently serves as a substrate for enzymatic digestion or chemical derivatization. Trypsin is the commonly used enzyme, and the reaction results in a nonfluorescent product (**3**) and the strongly fluorescent 7-amino-4-methylcoumarin (**4**). Intermediate **2** can also be condensed with an aromatic dialdehyde, such as naphthalene-2,3-dicarboxaldehyde, in the presence of free thiols or cyanide ions to form a fluorescent benzisoindole moiety. The latter, together with the conjugated coumarin, gives a FRET couple (**5**) with an annihilated emission at 390 nm.

[136] (commonly termed "development step" or "developer"), as shown in Fig. 3. A variety of analogs containing short peptide sequences instead of the Boc protecting group have been tested, among others, by Wegener et al. [137]. Other coupled enzyme assays use similar substrates, which bear, instead of a coumarin derivative, *p*-nitroanilide for colorimetric detection [138,139] or aminoluciferin for luminescence measurements [140]. Some assays are based on classical colorimetric or fluorescence detection [141] of the liberated ε-amine from short peptides with, for example, fluorescamine [136] or naphthalene-2, 3-dicarboxaldehyde, which is likely to be used in certain commercial assay kits. The latter detection principle has also been used in conjunction with the 7-amino-4-methylcoumarin derivative or other nonfluorescent quenchers, thereby resulting in intramolecular fluorescence quenching [142,143] and reducing eventual interactions of the detection reaction with test compounds (see Fig. 3).

Almost all of these strategies involve end-point detections and often lack subtype specificity but are well standardized and suitable for high-throughput inhibitor screenings and IC_{50} measurements. Although elegant continuous assays, for example, those based on fluorescence polarization [144,145] or FRET analysis in which HDAC tryptophans serve as acceptors [146], do exist, convenient and cost-saving assays are unavailable for most applications.

In addition to the radioactive and spectrophotometric approaches, the common western blot and ELISA techniques as well as standard chromatin immunoprecipitation using acetyl-specific antibodies allow for determination of the acetylation state of several HDAC substrates. Additionally, the immunoprecipitation of histone–complexes can be used to measure transcriptionally active chromatin by subsequent qPCR or Southern blot [125,147]. Modified histones are commercially available for a broad set of protein–protein interaction studies, and even surface plasmon resonance measurements have been performed for protein–small molecule interactions [113,148,149]. The latest published strategies use flow cytometry [150] or HPLC/MS [151] to monitor the acetylation state of diverse HDAC substrates. Because HDACs are heavily involved in epigenetic control [152] and because they influence diverse intracellular signaling cascades [153] and directly regulate transcription factors, such as NF-κB [154], a wide range of assay principles for transcriptional activities have been employed. These assay principles involve common PCR techniques, siRNA knockout experiments [155], transfection with fusion proteins [156], transgenic overexpression in mice [157], isothermal titration calorimetry [111,113], and standard protein quantification of a broad set of HDAC-related gene sequences and proteins. However, all of these approaches are less suitable for high-throughput drug discovery and inhibitor screenings but offer additional advantages, such as HDAC subtype specificity, complex functional insights, and substrate uncompetitive ligand discovery, and are suitable for the analysis of living cells or even for *in vivo* experiments.

Nevertheless, a high-throughput assay that combines a sensitive, selective, and continuous one-step reaction, ease of experimentation by using automated

pipetting systems, and cost-effective commercially available materials is needed. At best, this method should be applicable for measurements in solution and in living cells as well as for *in vivo* experiments.

Precaution is indicated especially when screening preparations of natural origin are used in conjunction with spectrophotometric two-step assays or when sirtuin specific commercial assays are used. It is well known that many plants contain UV-active or colored constituents, such as flavonoids or anthraquinones, respectively, which could positively or negatively interfere with the spectrophotometric detection. Other plants contain amides, such as alkaloids, amino acids, and derivatives thereof or thiols; therefore, it is recommendable to test for undesired reactions with the "developer" (*vide supra*), which is seldom specified, using a deacetylated substrate as control. For the latter case, it has been demonstrated that resveratrol and related compounds interfere with fluorophore bearing peptide sequences, such as the Fluor de Lys®-SIRT1 substrate (Enzo Life Sciences), by altering substrate binding and subsequently increasing catalysis in an artificial manner, leading to false positive read-outs [111,113,158,159]. In fact, results for resveratrol obtained in such a way lead to a lively controversy, and it is still unclear whether and how this compound, best known as the "healthy" constituent of red wine, contributes to the French paradox [160].

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ABBREVIATIONS

DADS	diallyl disulphide
HAT	histone acetyltransferase
HDAC	histone deacetylase
HDACi	histone deacetylase inhibitors
MSC	Se-methyl-L-selenocysteine
NAD^+	nicotinamide adenine dinucleotide
SAHA	suberoylanilide hydroxamic acid
SFN	sulforaphane
TSacid	trichostatic acid
TSA	trichostatin A
TSC	trichostatin C

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Chapter 10

Natural Products: Strategic Tools for Modulation of Biofilm Formation

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INTRODUCTION

Microorganisms tend to adhere to biotic and abiotic surfaces and to form a structured community—biofilm. Growth in such systems is accompanied by a change of cell phenotype manifested by different metabolic pathways, increased virulence, resistance to antibiotics, etc. [1]. Biofilm formation and its maturation and stability are influenced by physical, chemical, and biological factors. The course of colonization of the surfaces can be divided into several interconnected phases.

The phase prior to cell adhesion is called conditioning of the surface and includes an adsorption of inorganic and organic molecules presented in the bulk flow leading to changes of the physicochemical properties of the surface [2]. The conditioning film can affect biofilm formation both positively and negatively [3]. Initial phase of cell adhesion is often described by DLVO theory as an interaction of colloidal particles with a surface [4].

Similarity of microbial cells with a colloidal particle is, however, only partial. The process of microbial adhesion is considerably more complex.

A microbial cell interacting with the conditioned surface exhibits a high heterogeneity of envelope layers. The individual chemical substances in cell envelopes provide local areas with different surface hydrophobicity, charge, etc.; structures such as pili and flagella also come into play. Presence of extracellular polymeric substances (EPS), produced by biofilm microorganisms, is of considerable importance for the construction of biofilm architecture [5].

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Active participation of microbial cells leading to dynamic changes of interacting structures is essential for the adhesion process.

Regulatory mechanisms of microorganisms that are involved in biofilm formation and the development of a new phenotype are often controlled by chemical signals in the environment of cells. Such signaling molecules are produced specifically by the microorganism itself and then are included in the quorum-sensing (QS) regulatory system, or they are products of other microorganisms and can interfere with this regulatory system [6].

Natural products can affect biofilm formation in all its phases, and they can interact with the mature biofilm. They are able to change physicochemical properties of the environment (e.g., surface tension) or interfere with the structures of cell envelope layers, which are involved in adhesion. Natural products are able to modulate very effectively biofilm formation at the level of regulatory mechanisms [7].

Special and often unique properties of microbial cells growing in biofilm are preferably used in many technological processes; on the other hand, they cause important economic losses (e.g., biofouling) and serious health problems.

For these reasons, considerable attention is devoted to tools that can specifically affect biofilm formation, its stability, and structure. Research aimed at using natural products in the role of such tools occupies one of the leading positions.

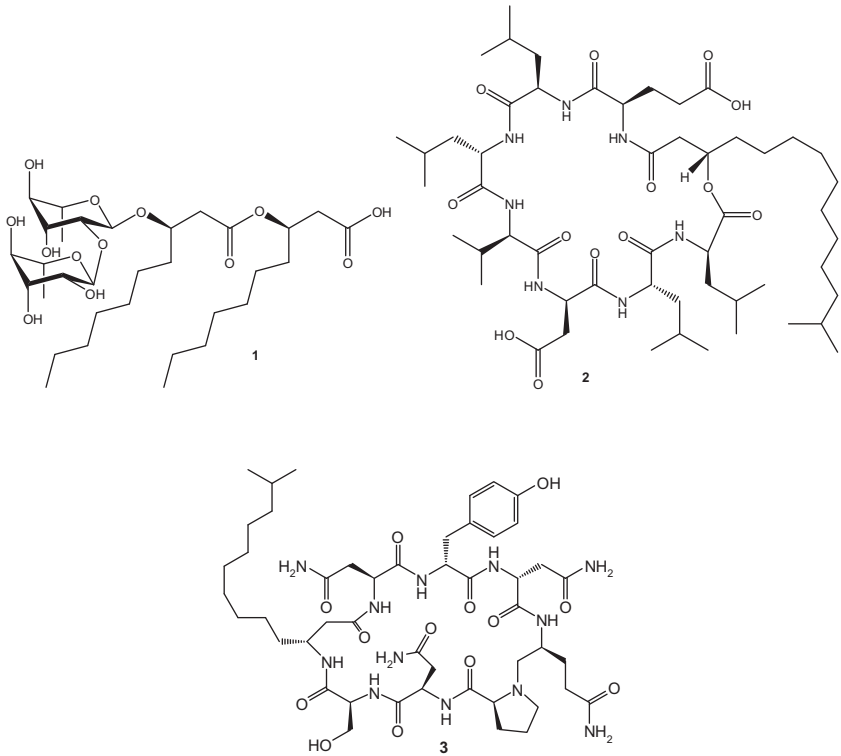
The following sections are devoted to such natural substances, produced by unicellular and higher organisms, which affect the physicochemical properties of the surfaces (surface activity) or interfere with the microbial regulatory mechanisms that are important for biofilm formation. The greatest attention is given to compounds that primarily affect adhesion of microbial cells or the stability of the biofilm structure but not the compounds which primarily kill microbial cells and thereby also cause biofilm breakdown.

SURFACE ACTIVE NATURAL PRODUCTS

Adhesion of microbial cells and the subsequent biofilm formation depends on physicochemical properties of surfaces and interfacial tension. The surface-active compounds produced by living organisms (biosurfactants) are, apart from antimicrobial activity, able to significantly influence the microbial adhesion or biofilm formation. Many structurally different biosurfactants are currently known, including glycolipids, lipoproteins, polysaccharides, or proteins [8]. Fungi and bacteria are the most common producers.

It appears that surfactants are necessary for their producers to initiate biofilm formation, as well as for migration of subpopulations within the biofilm. The participation of *Pseudomonas aeruginosa* surfactants in maintaining channels between multicellular structures was also confirmed [9]. Al-Tahhan *et al*. [10] found that very low concentrations of rhamnolipids (**1**)

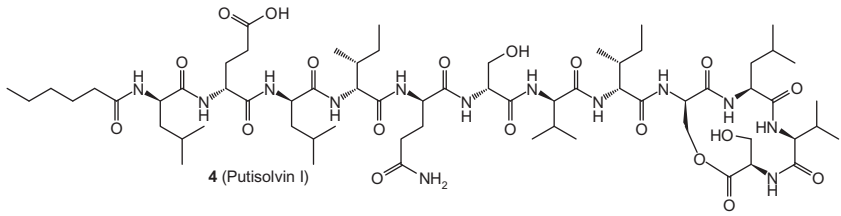
(amphipathic anionic glycolipids composed of rhamnose and β-hydroxy-fatty acids) in the environment increase cell surface hydrophobicity due to the release of lipopolysaccharide from the outer membrane and thus promote cell adhesion. Direct interaction (adsorption) of surfactant molecules with cell envelopes can change their hydrophobicity as well, depending on orientation of adsorbed molecules [11]. Zhong *et al*. [12] indicate that rhamnolipids (mono- or di-) at low concentration occupy adsorption sites on the cell surface of *P. aeruginosa* with hydrophobic tail extending to the environment and increase the cell surface hydrophobicity. A higher surfactant concentration saturates the adsorption sites and multilayer adsorption or accumulation of hemimicelles occurs. Under such conditions, the cell surface hydrophobicity can fall again. Likewise, surfactin (**2**) and iturin A (**3**), lipopeptides produced by *Bacillus subtilis*, increase cell surface hydrophobicity and are responsible for enhanced ability to colonize surfaces [13]. Lopez *et al*. [14], however, showed that surfactin has also the function of a signal molecule that triggers cannibalism and biofilm formation in *B. subtilis*.



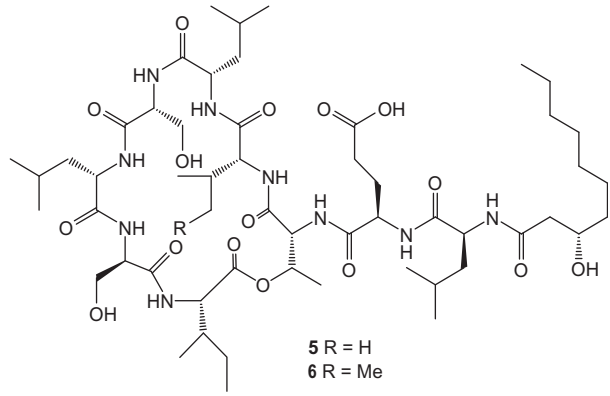
Because they possess the ability to reduce the surface and interfacial tension, biosurfactants are very often referred to as the "antibiofilm" active compounds toward other microorganisms. A lot of works using rhamnolipids as

antiadhesive substances were published in this context [9,11,15–17]. Rhamnolipids caused an effective decomposition of the biofilm of marine bacteria *Bacillus pumilus* [18], as well as the respiratory pathogen *Bordetella bronchiseptica* [19]. Interest in commercial applications of rhamnolipids led to the development of biotechnological processes, allowing their industrial production.

Antibiofilm activity was also demonstrated in the case of lipopeptides. The above-mentioned surfactin prevents biofilm formation of *Listeria monocytogenes* and *Enterobacter sakazakii*, important pathogens in food [20], as well as *Salmonella enterica* sv. *typhimurium* [21]. Inhibition of biofilm formation by putisolvins (**4**), cyclic lipopeptides, produced by *Pseudomonas putida* was described by Kuiper *et al*. [22]. Putisolvins inhibited *P. aeruginosa* PA14 and *Pseudomonas fluorescens* WCS365 biofilms.

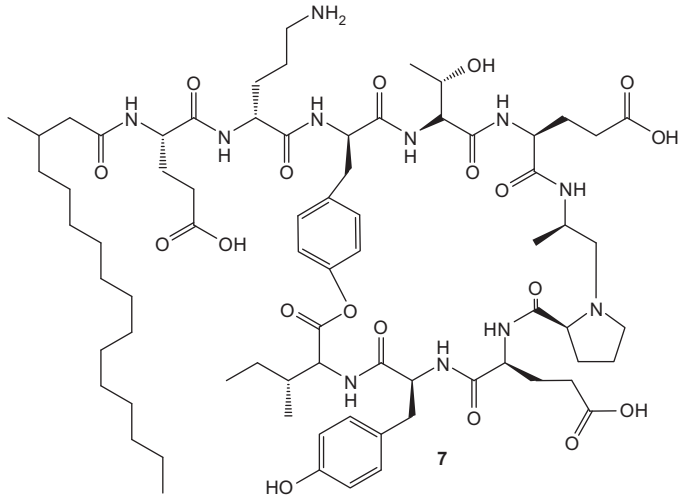


Other cyclic lipopeptides acting as biosurfactants/antibiotics, viscosin (**5**) and massetolide A (**6**), from *P. fluorescens* prevent the formation of *P. aeruginosa* PAO1 microcolonies [23].



Rivardo *et al*. [24] found in *B. subtilis* and *Bacillus licheniformis* biosurfactants containing two separate fractions, each containing the surfactin-like and the fengycin-like (**7**) biosurfactant families in varying proportion. The reactions showed selective antiadhesive activity in biofilm inhibition of pathogenic strains G^- *Escherichia coli* CFT073 and G^+ *Staphylococcus aureus* ATCC 29213. The V9T14 biosurfactant, active against the G^- strain, was ineffective against the G^+ one. The V19T21 fraction acted in an opposite

manner. Subsequent studies carried out by these authors [25] demonstrated synergistic activity of biosurfactant V9T14 with some antibiotics on the destruction of mature biofilm of *E. coli* CFT073. The authors hypothesize that the action of V9T14 biosurfactant lies in its interaction with the bacterial membrane, increasing the activity of antimicrobial agents by forming pores in the outer membrane and thus facilitating the entrance of antibiotics into biofilm cells.



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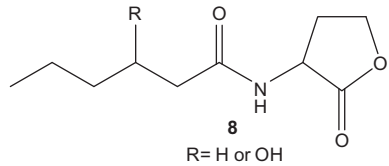
A lipopeptide biosurfactant isolated from marine bacterium *Bacillus circulans*, which was not exactly specified, suppressed adhesion and caused destruction of mature biofilm of several potentially pathogenic strains. Applications of 10 g/l had a nearly 90% efficiency [26]. Rodrigues *et al*. [27] isolated a biosurfactant from probiotic lactic acid bacteria *Lactococcus lactis* 53, which after adsorption on the surface of silicone rubber reduced its hydrophobicity and very effectively suppressed the deposition of several strains of staphylococci and yeasts C*andida albicans* and *C. tropicalis* on this carrier. Also other lactic acid bacteria such as *Lactobacillus acidophilus* [28,29] and *L. fermentum* RC-14 [30] produce surfactants whose importance is associated with an effective ability to suppress the adhesion of uropathogenic bacteria in the urogenital tract.

NATURAL PRODUCTS IN BIOFILM REGULATION MECHANISMS

Current knowledge of the regulatory processes controlling microbial cell adhesion, biofilm formation and maintenance, or its dispersal was very well summarized by Landi *et al*. [31]. A relatively universal regulatory process that occurs in the development of a biofilm phenotype is QS, a process based on

the ability of cells to identify the cell population density by certain threshold concentration of an autoinducer in the environment. There are a lot of different extracellular signal molecules in G^+ and G^- bacteria and their number continuously grows.

N-Acyl homoserine lactones (**8**) (AHLs) are the common signaling molecules (autoinducers) of G^- bacteria. The side chain lengths vary from 4 to 18 carbon atoms.



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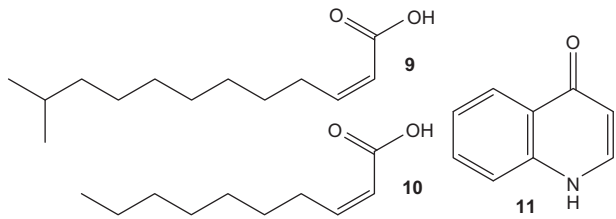
R= H or OH

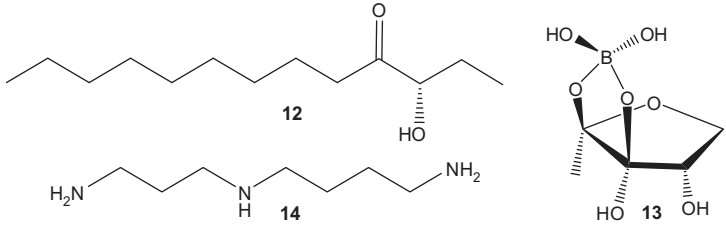
They regulate the production of EPS, one of the most important components in the biofilm [32]. AHLs are further involved in regulatory mechanisms of both virulence factors and synthesis of rhamnolipids (pathogens) and pili and flagella, that is, substances (structures) that directly affect the biofilm formation [33,34]. The autoinducers have a variety of functions not only in intraspecies communication but also they are also involved in mutual interactions (relationships) at the level of interspecies and interkingdoms. Autoinducers can act in such cases as agonists or antagonists. In connection with our topic, they are able to stimulate or suppress biofilm formation [35].

AHLs are not the only autoinducers in G^- bacteria. For example, *Xanthomonas campestris* uses substituted fatty acid messenger, *cis*-11-methyl-2-dodecanoic acid (**9**), and called diffusible signal factor [36]. Another small messenger fatty acid, *cis*-2-decenoic acid (**10**), is produced by *P. aeruginosa* [37]. This compound induces biofilm dispersion and inhibits its formation in a number of G^+ and G^- bacteria and yeast, as well as in the producers themselves. *P. aeruginosa* produces 4-quinolones (**11**) that serve as another type of QS signals. Some of these compounds play an important role in the formation and maintenance of the biofilm by a number of different mechanisms [38–40].



Vibrio fischeri is a bioluminescent bacterium, in which regulation by QS was observed in 1970 for the first time [41]. The genus *Vibrio* is being constantly studied in this context. *Vibrio cholerae* uses two autoinducers that control pathogenicity and biofilm formation. Cholerae autoinducer-1

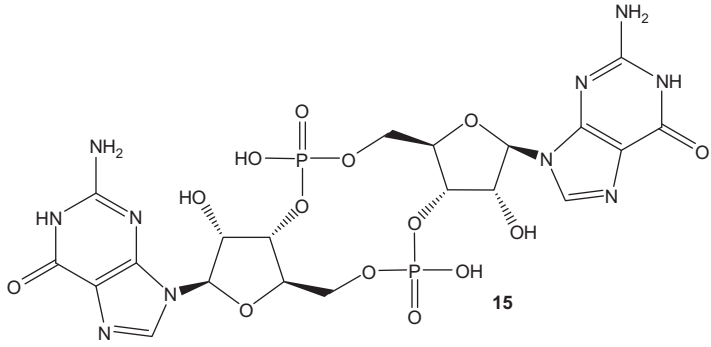
(CAI-1)—(*S*)-3-hydroxytridecan-4-one (**12**) and autoinducer-2 (AI-2)—furanosyl borate diester (**13**) function synergistically to control gene regulation [42]. Polyamines are required for normal growth of both eukaryotes and prokaryotes. They are involved in numerous and diverse cellular processes including the regulation of biofilm formation. McGinis *et al*. [43] confirmed the regulatory effects of spermidine (**14**) on the formation of *V*. *cholerae* biofilms.



Indole is a common natural product. Furthermore, it is important interspecies signal molecule produced by a variety of both G^+ and G^- bacteria. Indole affects many regulatory mechanisms and is also involved in biofilm formation/inhibition [44]. Extracellular indole suppresses *E*. *coli* adhesion but induces pseudomonad adhesion in dual-species biofilm [45].

Ribosomally synthesized short peptides are common signal molecules in QS in G^+ bacteria. Posttranslational modification of the peptides is often of great importance [46].

The regulatory cascade involving the cyclic-di-guanosine monophosphate (**15**) (c-di-GMP) as a second messenger is another important regulation process associated with the multicellular (biofilm) behavior of microbial populations. The GGDEF and EAL domain proteins involved in c-di-GMP biosynthesis and degradation are widespread in bacterial genomes [47]. Intracellular level of c-di-GMP plays a central role in regulation of production of extracellular polysaccharides (EPS), adhesines, pili and flagella, and in this connection influences biofilm formation or destruction [48].



The complexity of natural regulatory processes provides a large number of targets that can interfere with chemical compounds of various origins, which

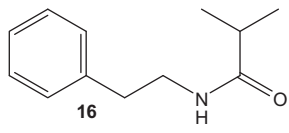
in turn leads to positive or negative changes in cell adhesion and the ability of microorganisms to form biofilm. Moreover, a great importance is attributed to enzymes that are able to degrade AHLs and other signal molecules, which have been found in some bacteria and also in plant and animal tissues [49–51].

A more detailed overview of natural products affecting mechanisms of microbial adhesion in the following sections is subdivided according to the producers of these substances.

MICROBIAL NATURAL PRODUCTS

Microorganisms are undoubtedly one of the most important groups of organisms producing natural products. In the early stages of research, the most attention has been devoted to compounds with antibiotic activity or to toxins. Recent years saw a marked increase in the research of the regulatory functions of natural products. Study of interferences of these substances with mechanisms that are directly or indirectly involved in the adhesion and/or formation of a biofilm cell phenotype is an integral part of this upswing.

The considerable versatility of signaling molecules directly involved in regulation of microbial adhesion renders their wide impact across the microbial world quite logical. However, signaling by AHLs may be diminished (eliminated) by competitive antagonists, which do not have their own signaling activity, but bind to the same receptor site as the native agonists. Metabolites of marine G^+ bacterium *Halobacillus salinus* can be one of many examples. *H. salinus* produces two compounds, phenylethylamides (*N*-(2-phenylethyl)-isobutyramide (**16**) and 3-methyl-*N*-(2-phenylethyl)-butyramide) (**17**), which resemble in their structure AHLs, that is, they possess a ring system with a side chain connected via an amide bond [52].



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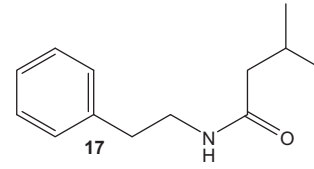
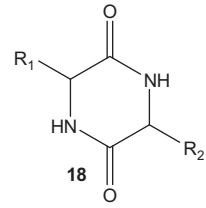


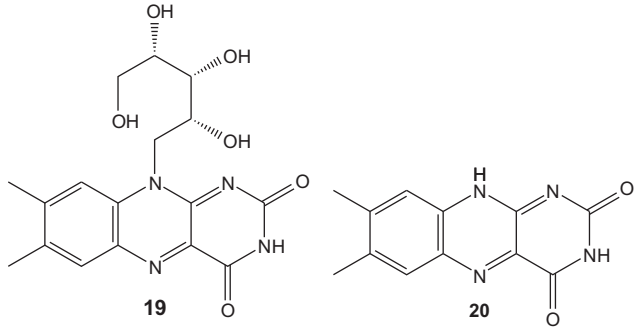
17

Some pseudomonads produce substances that activate or antagonize LuxR-based QS systems in other G^- bacteria. However, the structure of these substances greatly differs from the AHL molecules. They are diketopiperazines (**18**), the smallest possible cyclic peptides (cyclo(D-Ala-L-Val), cyclo (L-Pro-L-Tyr), and cyclo(L-Phe-L-Pro)), which are capable of mimicking the action of AHLs [53].



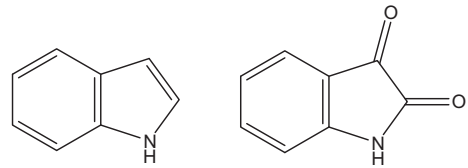
18

Examples of other compounds that can mimic AHLs and thereby manipulate QS in bacteria by interacting with the Las-R receptors are riboflavin (**19**) and lumichrome (**20**). It should be noted that these substances are commonly secreted by bacteria, algae, and plants, and so their activity can reach across the kingdoms.



19 20

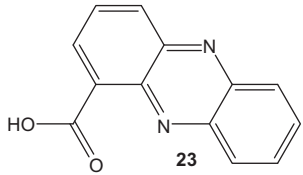
Indole (**21**) is a substance produced by many microorganisms, whose activities associated with the induction of virulence, cell cycle regulation, resistance to acid and biofilm formation were summarized by Hu *et al*. [54]. The ability of indole to stimulate/inhibit biofilm formation is not entirely clear, and one explanation could be strain specificity [55,56]. Many nonindole-producing bacteria have oxygenases which can oxidize indole and the resulting products have antibiofilm activity. In this context, it is interesting to note the role of isatin (**22**) (indole-2,3-dione), a stress-related biological substance in animals. The presence of isatin represses indole biosynthesis in pathogenic *E. coli* O157:H7 and thus enhances biofilm formation [57].



21 22

Phenazines represent a very large group of nitrogen-containing secondary metabolites of bacteria. Some affect the regulation of biofilm, while the specific effect is determined by the molecular structure [58]. Pierson and Pierson [59] found that *Pseudomonas chlororaphis* produces phenazine-1-carboxylic

acid (**23**) which is partly converted into 2-hydroxy-phenazine-1-carboxylic acid (**24**). The former may facilitate growth within the biofilm while the latter may facilitate cell adhesion.



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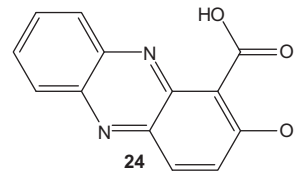
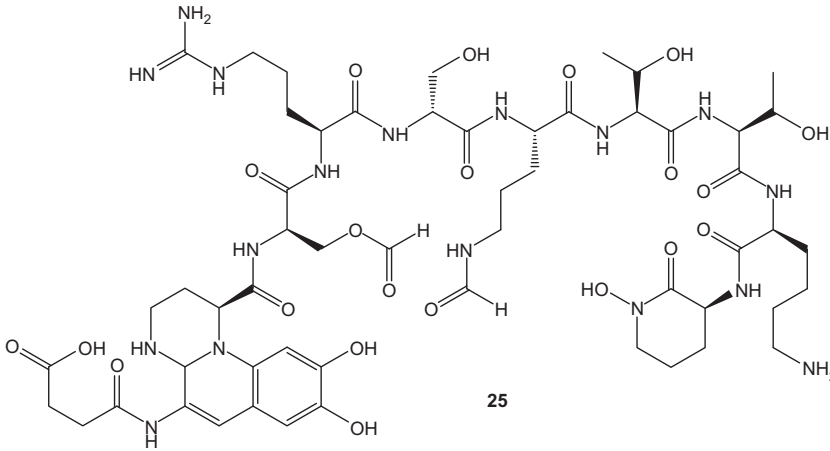


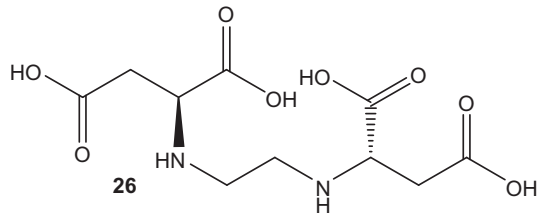
24

Siderophores as iron chelators are also involved in *P. aeruginosa* biofilm formation, albeit indirectly. Iron serves as a signal in *P. aeruginosa* and, for example, pyoverdine (**25**) can ensure its sufficiently high intracellular level in an iron-poor environment [60].



25

Aminopolycarboxylic acid ethylenediaminedisuccinate (**26**) was isolated from culture filtrate of the actinomycete *Amycolatopsis orientalis* [61]. Wen *et al*. [62] have shown that this natural chelator was able to enhance the treatment of sulfate-reducing bacteria biofilms by glutaraldehyde.

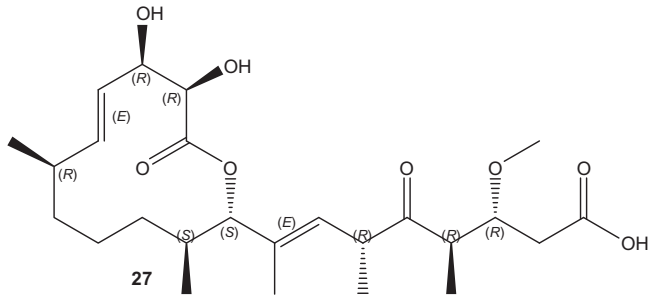


26

Bacteria produce D-amino acids in the stationary phase of growth [63]. Some of them may contribute to the destruction of the formed biofilm. Kolodkin-Gal *et al*. [64] demonstrated that D-tyrosine, D-leucine, D-tryptophan, and D-methionine

are able to inhibit biofilm formation by *B. subtilis*, while a combination of these acids showed synergistic effect. The authors also found that mixtures of these D-amino acids or D-tyrosine itself can inhibit the biofilm of pathogenic bacteria such as *S. aureus* and *P. aeruginosa*.

Carolacton (**27**) is a macrolide metabolite of the myxobacterium *Sorangium cellulosum*. This compound exhibits significant inhibitory activity against *Streptococcus mutans*, a major pathogen in human dental caries. An important characteristic of this compound is its ability to damage *S. mutans* biofilm at nanomolar concentrations under anaerobic conditions [65].



27

In addition to studies on individual products of microorganisms, a number of papers deal with the effects of variant types of extracts of microorganisms on biofilm formation.

Actinomycetes are known for extensive production of secondary metabolites. You *et al.* [66] screened 88 marine actinomycetes and found that extracts from 33 of them dispersed the mature biofilm. Strain A66, identified as *Streptomyces albus*, was rated as the most effective with regard to the biofilm attenuation and AHLs inhibition.

The antibiofilm activity against *P. aeruginosa* PAO1 and *Vibrio* spp. was also demonstrated in extracts of culturable bacteria isolated from sediments in the Palk Bay [67,68]. None of the extracts showed any antibacterial activity. Some extracts have shown QS inhibition, others showed biofilm dispersion and disruption of the biofilm architecture by reducing the EPS production and hydrophobicity index.

Similarly, numbers of bacteria isolated from the marine environment produce substances that inhibit QS [69]. Marine bacterial extracts appear as valuable sources for developing biofilm inhibitors against pathogens and biofouling. Although the participation of bacterial biofilms in biofouling is important, microorganisms with antibiofilm effects can be found even in this complex system. So epiphytic bacterium *Pseudoalteromonas tunicata* growing on green algae *Ulva lactuca* synthesizes yellow pigmented substances which block biofilm formation [70].

Released exopolysaccharides (r-EPS) produced by probiotic *L. acidophilus* A4 have shown antibiofilm activity against a number of G^+ and G^- pathogens [71]. Biofilm formation of certain bacteria is stimulated by the presence of highly adhesive curli fibers on the cell surface [72]. As found in the case of

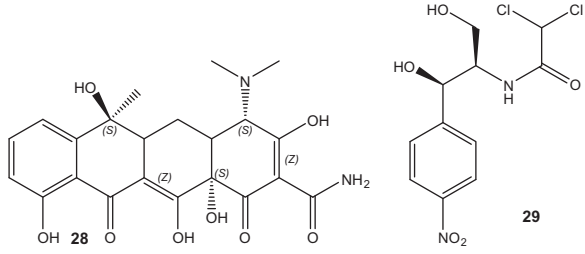
E. coli O157: H7, r-EPS significantly repressed genes responsible for production of curli [71].

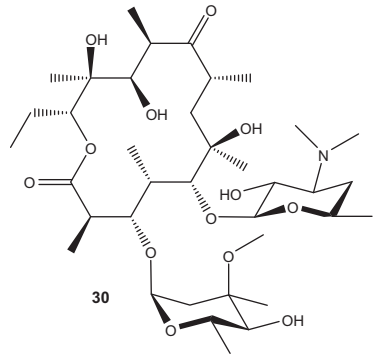
In addition to bacteria, imperfect fungi are important producers of secondary metabolites. Screening of a wide set of *Penicillium* species yielded two compounds with a significant effect on *P. aeruginosa* QS [73]. Both penicillic acid and patulin affect QS-controlled gene expression. Expectedly, patulin was found to enhance biofilm susceptibility of *P. aeruginosa* to tobramycin treatment.

Antibiotics

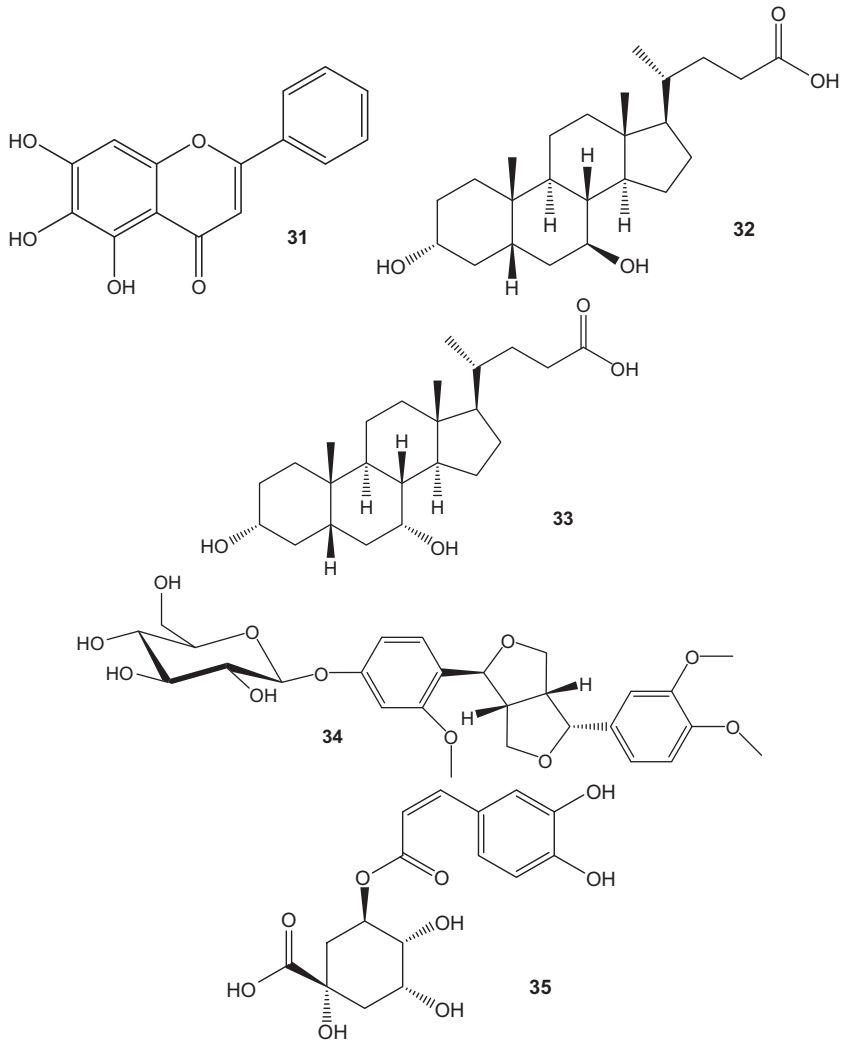
Antibiotics are an inherent group of microbial natural products that are essential for man. Accurate separation of their activities suppressing microbial growth and activities associated with cell adhesion is rather difficult. Nevertheless, it was shown that some substances, originally isolated as antibiotics, can exhibit signal activity based on an interference with the microbe communication.

The number of microorganisms resistant to the "old" antibiotics has been growing over the years. The importance of these substances may, however, lie in the suppression of microbial colonization. Tetracycline (**28**) and chloramphenicol (**29**) demonstrated the ability to effectively inhibit the biofilm formation of such pathogens as *Achromobacter* sp. *P. aeruginosa*, *K. pneumoniae*, and *B. pumilis* [74]. Heerden *et al.* [75] found that chloramphenicol and the steroid antibiotic fusidic acid used for coating the surface of silicone implants were able to inhibit the adhesion of pathogenic bacterium *Staphylococcus epidermidis in vitro*. Erythromycin A (**30**) as a natural product and some other semisynthetic macrolides inhibit EPS production in *P. aeruginosa* even at subinhibitory concentrations [76].

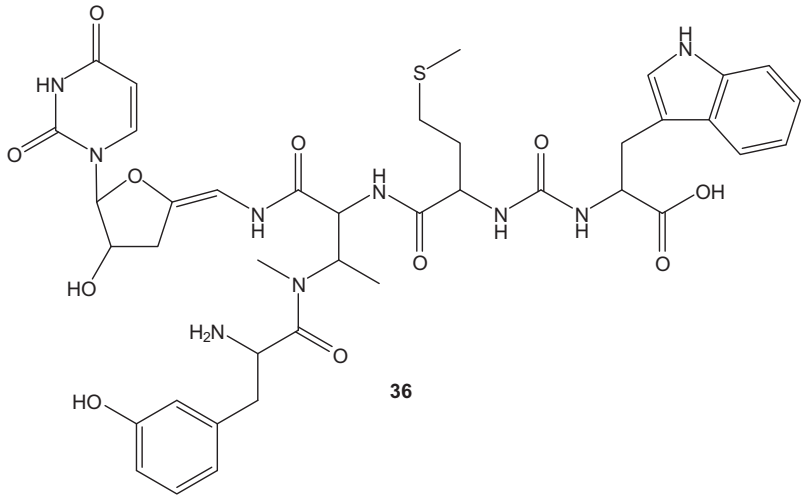




Traditional Chinese medicine uses TanReQuin (TRQ) as an antibacterial agent to treat infections of upper airways. Baicalein (**31**), ursodeoxycholic acid (**32**), chenodeoxycholic acid (**33**), forsythin (**34**), and chlorogenic acid (**35**) are the main active compounds in the formulation. Good efficiency of TRQ to infections caused by *S. aureus*, for example, lies in its ability to inhibit biofilm formation [77]. Baicalein is also able to regulate biofilm formation in the pathogenic yeast *C. albicans*. Yeast has *CSH1* gene, which encodes the synthesis of protein responsible for the rise of cell surface hydrophobicity. Baicalein reduces the *CSH1* expression and thus can reduce the cell surface hydrophobicity and, consequently, reduce cell adhesion [78].

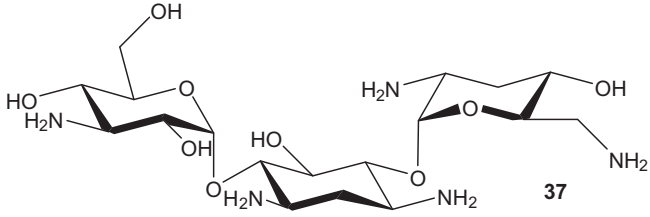


Sansanmycin (**36**), a novel narrow-specific antibiotic, was isolated from not otherwise specified *Streptomyces* sp. SS (CGMCC No. 1764). Chemically, it is classified as a nucleosidyl-peptide antibiotic [79]. Sansanmycin, especially in combination with macrolides, inhibits *P. aeruginosa* biofilms [80].



36

Interactions of tobramycin (**37**) (product of *Streptomyces tenebrarius*) with signal systems regulated by AHLs are not fully understood. Linares *et al.* [81] proved that subinhibitory concentration of tobramycin induces swarming and biofilm formation in a clinical isolate of *P. aeruginosa* PAO1. On the other hand, this antibiotic suppresses formation of C4-HSL in environmental *P. aeruginosa* strain PUPa [82].

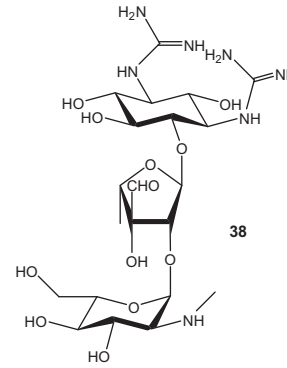


37

Positive effect on biofilm formation was also identified during the testing of other antibiotics. In this context, the hypothesis was expressed that in nature antibiotic-producing microorganisms could serve as an organizing force in integrated microbial communities, helping, for instance, nonantibiotic members of the community to colonize surfaces and form biofilm [81].

Subinhibitory concentrations of aminoglycoside antibiotics are able to induce biofilm formation in pathogenic strains *P. aeruginosa* and *E. coli*. The aminoglycoside streptomycin (**38**) interferes with the regulation of c-di-GMP (second messenger) biosynthesis whose intracellular level is

essential for cell surface adhesiveness [83]. It was shown that the loss of potassium from intracellular space of *B. subtilis* stimulates the activity of protein kinase KinC, which governs the expression of genes involved in biofilm formation. The polyene antibiotic nystatin A1 (**39**) or the nonribosomally encoded peptide gramicidin S (**40**) are causing cation leakage and it is therefore not surprising that both substances enhance the biofilm formation [14].



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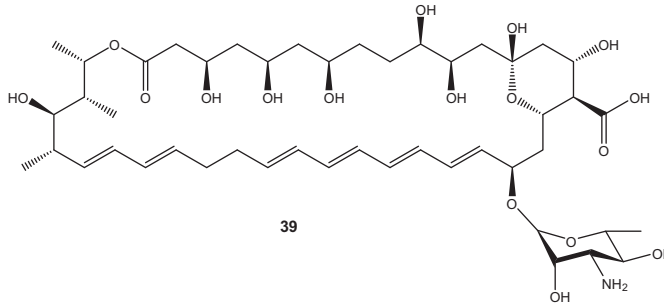

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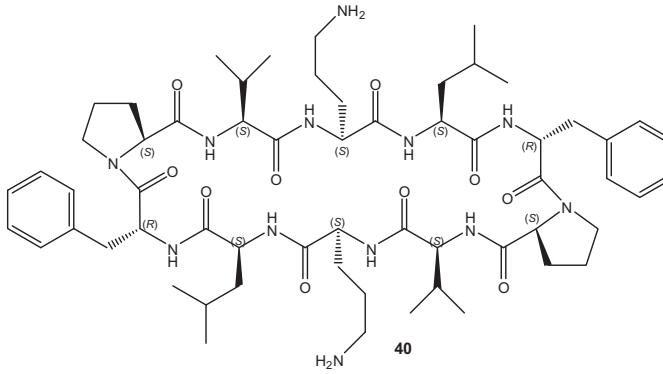
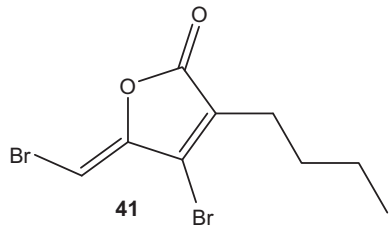


40

MARINE ORGANISMS

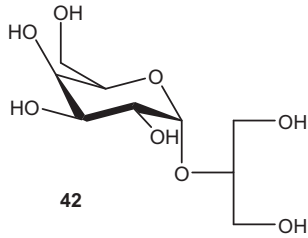
Marine ecosystems are in many aspects little explored regions, which is also confirmed by numerous new discoveries concerning the metabolism of and metabolites produced by their inhabitants. Marine microorganisms producing antiadhesive substances were described above.

Natrah *et al*. [84] published a comprehensive study devoted to the negative effects of marine organisms on bacterial cell-to-cell communication, which often led to suppression of adhesion ability and bacterial biofilm formation. A very important group of these compounds, halogenated furanones, was identified in marine red algae *Delisea pulchra* by Manfield *et al*. [85] in 1999. The compounds are structurally similar to AHLs and can specifically interfere with AHL-dependent gene transcription at the level of the LuxR-like regulatory protein (they displace the AHL signal from its receptor protein). Further studies then showed that one of the *D. pulchra* furanones ((5*Z*)-4-bromo-5-(bromomethylene)-3-butyl-2-(5*H*)-furanone) (**41**) significantly inhibited *E. coli* and *B. subtilis* biofilms [86–88]. Zang *et al*. [89] found that a naturally occurring brominated furanone covalently modifies and inactivates LuxS (*S*-ribosylhomocysteine lyase, EC 4.4.1.21), the enzyme which produces AI-2. The activity of natural furanones has led to the preparation of synthetic analogues with a higher ability to suppress QS. 5-(Bromomethylene)-4-ethyl-3-methyl-2-pyrrolinone, a strong inhibitor of bacterial swarming and biofilm formation of *P. aeruginosa*, can be mentioned as an example [90].



41

The Korean red alga *Ahnfeltiopsis flabelliformis* produces three structurally different compounds, which, though different from the AHLs structure, interfere with *N*-octanoyl-DL-homoserine lactone (OHL) [91]. A mixture of floridoside (**42**) and isethionic acid (**43**) exhibited a dose-dependent inhibitory effect on the function of the OHL. Conversely, the third compound, betonicine (**44**), exhibited dose-dependent stimulatory effect [92].



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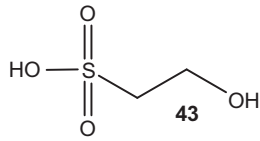

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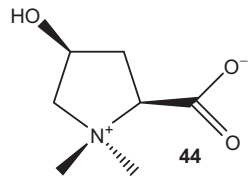
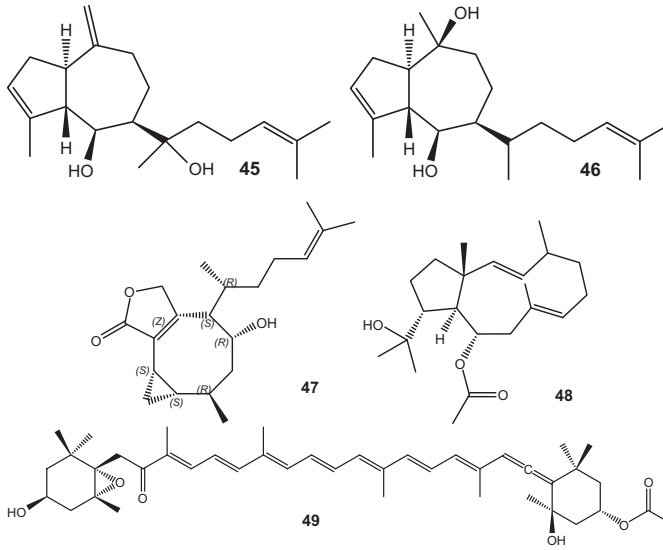
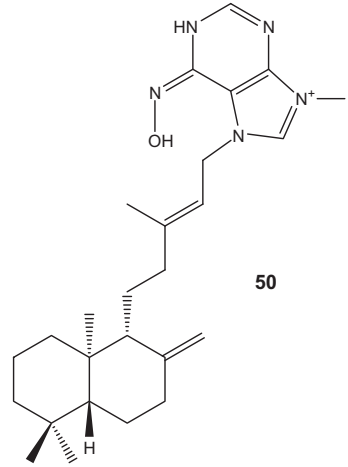


44

Species of Mediterranean brown alga *Dictyota* are important producers of secondary metabolites. Significant portions are diterpenoids. Viano *et al*. [93] isolated 11 compounds from the algae of which 4 were new diterpenes. Dictyols E (**45**) and C (**46**), hydroxycrenulide (**47**), 9-acetoxy-15-hydroxy-1, 6-dollabelladiene (**48**), fucoxanthin (**49**), and two of the new diterpens (dolabellanes) showed antibiofilm activity, while dictyol C and fucoxanthin were the most efficient.

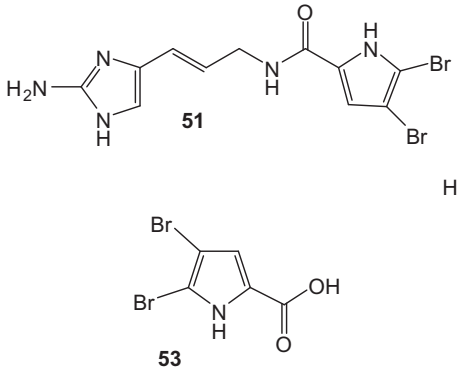


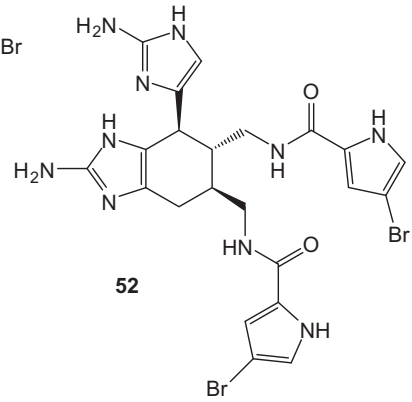
Agelas nakamurai also produces diterpenes; the diterpenoid alkaloid ageloxime D (**50**) prevents bacteria *S. epidermidis* from forming the biofilm [94].

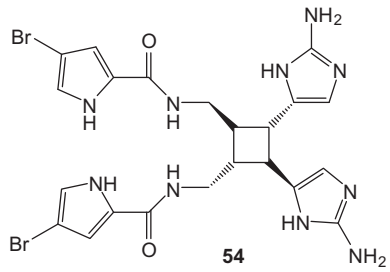


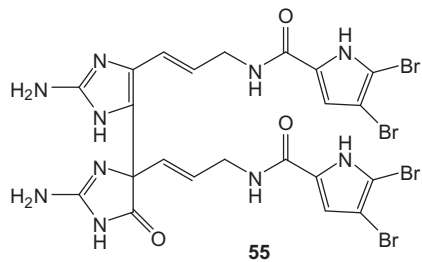
Similarly, other bromoalkaloids such as oroidin (**51**), ageliferin (**52**) and 4,5-dibromopyrrole-2-carboxylic acid (**53**) isolated from *Agelas clathrodes*, and sceptrin (**54**) isolated from *A. conifera* inhibited bacterial attachment at natural concentrations. Tsukamoto *et al*. [95] found an oroidin dimer

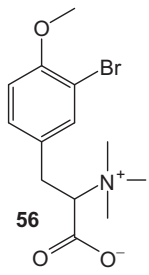
mauritiamine (**55**) in *A. mauritinia* with the same antibiofilm property. Marine sponge *Ailochroia crassa* synthesizes bromotyrosine-derived natural products—for instance (**56**). They are often able to influence the growth and motility of bacteria. Ianthellin (**57**) was identified as the metabolite that inhibited bacterial attachment [96].

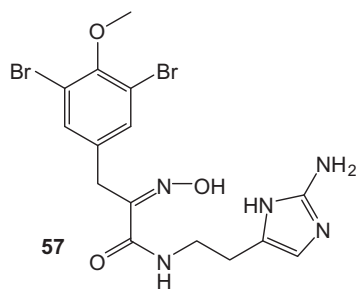




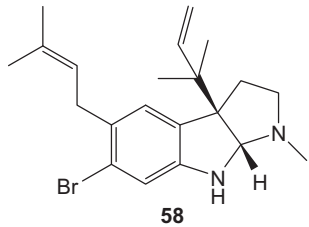








The North Sea bryozoan *Flustra foliacea* produces several secondary metabolites and thus prevents the colonization of their surfaces. Two of these compounds have been identified as bromoalkaloids, that is, flustramine D (**58**) and dihydroflustramine C (**59**) and are effective antagonists of AHL-dependent QS signaling systems [97].



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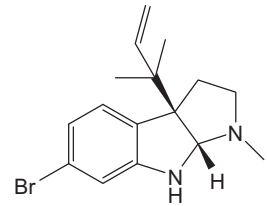
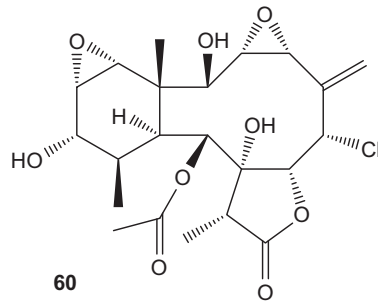


59

Yamada *et al*. [98] isolated from the marine sponge *Psammaplysilla purpurea* the chlorinated polycyclic metabolite bis (deacetyl) solenolide D (**60**) possessing antibiofilm activity, expressed as a decrease of the acidic EPS produced by biofilm-forming marine bacterium *Rhodospirillum salexigenes* SCRS 113.



60

PLANTS

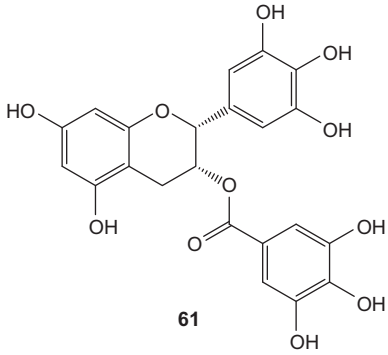
Plants produce an enormous number of natural products. Many of them have been used by people in the form of whole plants, their parts or extracts since ancient times. Despite the long-term research, plants seem to be an inexhaustible treasure of new compounds in the present.

Extracts of many plants contain components (phenol derivatives, terpenes, etc.) that have an ability to suppress microbial cell attachment and a biofilm growth [99].

Natural polyphenols are a group of substances exhibiting extensive biological activities. In particular, plants are rich sources of these compounds. It was shown [100] that epigallocatechin-3-gallate (**61**) (EGCG), a rich source of which is green tea, and ellagic acid (**62**), which is contained in various fruits (blackberries, raspberries, strawberries, cranberries), act as antagonists of AHLs-dependent microorganisms. The latter compound had a high antibiofilm activity.

Antibiofilm activity of green tea polyphenols was also demonstrated on attached pathogenic yeast *C. albicans* [101], EGCG being more effective than epigallocatechin (**63**) or epicatechin-3-gallate (**64**). This study [101] suggests that the metabolic instability produced by the catechin-induced proteasome

inactivation was a contributor to the decrease in the growth rate constant as well as biofilm formation and maintenance.



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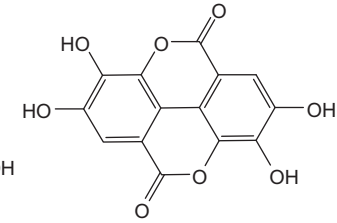

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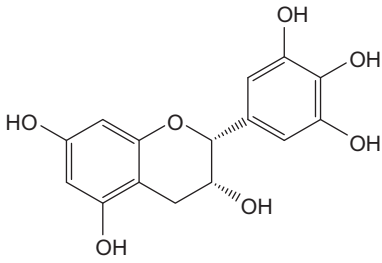

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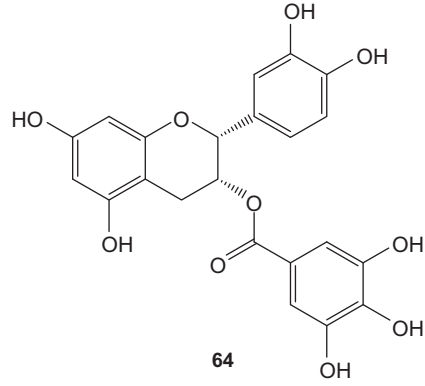
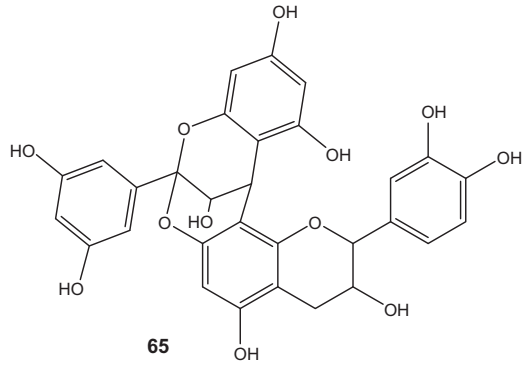


64

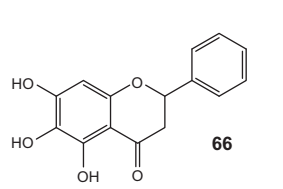
Daglia *et al*. [102] found that dealcoholised red wine is able to inhibit *S*. *mutans* biofilm formation on human teeth. Proanthocyanidins, for example, proanthocyanidin A1 (**65**), were the components most involved in the antiadhesion and antibiofilm activity. Cranberry (*Vaccinium macrocarpon*) contains less frequent A-type linked proanthocyanidins. In contrast to the more frequent proanthocyanidins containing B-type linkages, the A-type showed far greater antiadhesive activity to uropathogenic *E*. *coli* [103]. It was proven that the cranberry juice cocktail significantly decreases nanoscale adhesion forces between P-fimbriated *E*. *coli* and uroepithelial cells [104].



65

Zeng *et al*. [105] carried out an analysis of 51 active compounds used in traditional Chinese medicine. Five of them had a proven ability to inhibit biofilm formation, the flavonoid baicalein (**66**) being the most effective. This substance is contained, for example, in *Oroxylum indicum* or in the roots of *Scutellaria baicalensis*. Baicalin (**67**), the glucuronide of baicalein (**31**), has significant antibiofilm activity against *Burkholderia cenocepacia* or *B. multivorans* [106]. Many flavonoids are prominent secondary metabolites present in citrus species. So naringenin (**68**), kaempferol (**69**), quercetin (**70**), and apigenin (**71**) have shown the ability to inhibit biofilm formation in *Vibrio harvey* and *E. coli* O157:H7 [107]. *Combretum albiflorum* (Tul.) Jongkind, which is endemic in Madagascar, contains flavonoids in bark. The flavan-3-ol catechin (**72**) has quorum-quenching activity and partly suppresses biofilm formation [108].

Hamamelitannin (**73**) (2′,5-di-*O*-galloyl-D-hamamelose) is a natural product found in the bark of *Hamamelis virginiana* (witch hazel). Kiran *et al*. [109] have been found that this compound acted as nonpeptide analog of the QS inhibitor RNAIII-inhibiting peptide, and that it effectively prevented an attachment of *S. aureus* and *S. epidermidis*.



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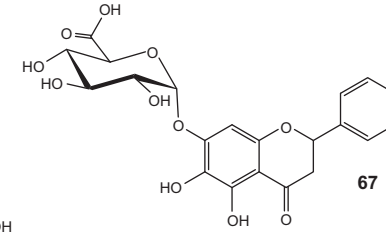

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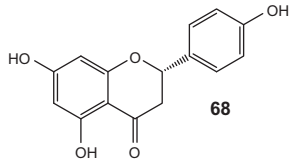

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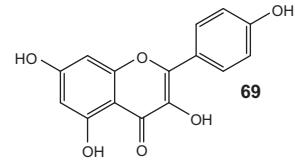

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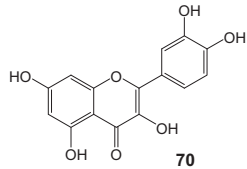

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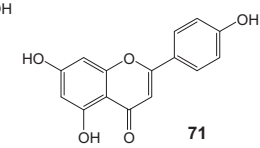

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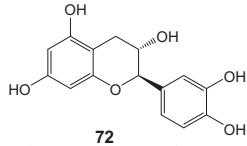

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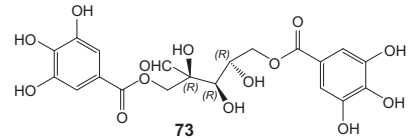
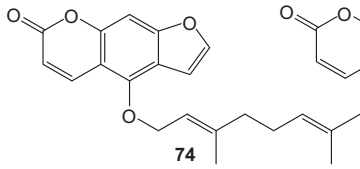


73

Plant furanocoumarines are substances known as mediators of the activities of certain drugs [110]. In this context, grapefruit juice was mainly studied because of the content of bergamottin (**74**) and dihydroxybergamottin (**75**). Both of these substances are also potent inhibitors of G^- bacteria autoinducers and suppress the formation of biofilms of *E. coli* O157: H7, *S. typhimurium*, and *P. aeruginosa* [111].

6,7-Dihydroxycoumarin (**76**) (aesculetin) present in horse chestnut, *Aesculus hippocastanum*, was proved to be efficient in preventing biofilm formation by *S. aureus* [112].



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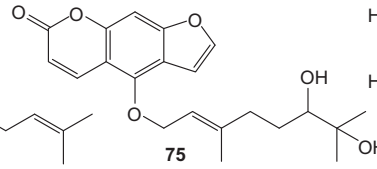

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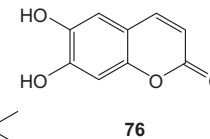
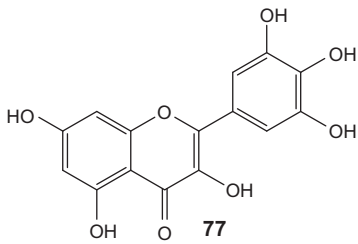


76

Jeon *et al.* [113] studied antibiofilm activity of myricetin (**77**) (flavonol) and *tt*-farnesol (**78**), compounds ubiquitously found in fruits (cranberries and red wine grapes), and propolis (a resinous mixture collected from tree buds, sap flows, or other botanical sources by honey bees), against *S. mutans* causing dental caries. They showed that the mixture of the natural products in combination with fluoride disrupted the accumulation and structural organization of EPS and bacterial cells in the matrix, which affected the biochemical and physiological properties of the biofilms.



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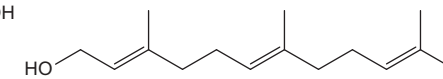
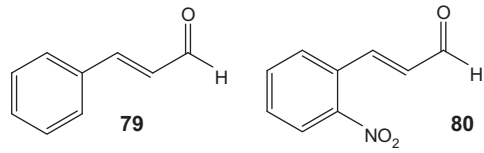


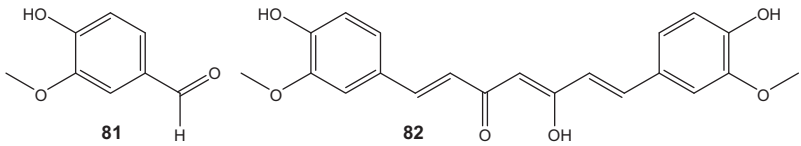
78

Certain compounds contained in the commonly used spices can affect the adhesion of microorganisms. The flavor essence in the bark of cinnamon trees,

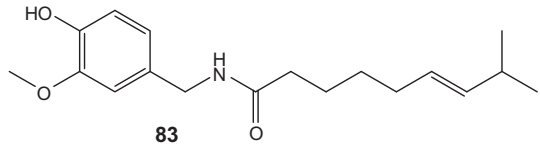
cinnamaldehyde (**79**), has an inhibitory effect on target protein LuxR in *V. harvey* [114]. Some semisynthetic derivatives such as 2-nitrocinnamaldehyde have the same effect (**80**). The authors also showed that these compounds affect the production of EPS and thereby suppress biofilm formation.



Similarly, vanilla, a spice popular for its distinctive aroma, can inhibit bacterial QS [115]. It has long been known that aldehyde vanillin (**81**), the primary flavoring component of the vanilla, inhibits many bacterial strains [116]. Later, it was found that vanillin is an important inhibitor of C4-HSL and 3-Oxo-C8-HSL in *Aeromonas hydrophila*. A significant effect of vanillin in suppression of the bacterial biofilm was also confirmed [117]. Kannan *et al*. [118] found that the interaction efficiency of vanillin with AHLs is dependent on the molecular structure of AHL, mainly on the length of the side chain. Weak antibiofilm activity has been also found in peppermint (*Mentha piperita*) extract against biofilms of *P. aeruginosa* and *C. albicans* [119]. Turmeric (*Curcuma longa*), commonly used as spice, contains the active substance curcumin (**82**). Rudrappa and Bais [120] found that this substance downregulates genes associated among others with the QS and biofilm initiation in *P. aeruginosa* strain PAO1.

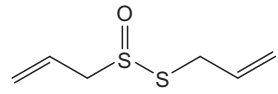


Capsaicin (**83**) (8-methyl-*N*-vanilly-6-nonenamide), the natural product from chili pepper responsible for the "hotness" of the pepper, also effectively prevents bacterial attachment and biofilm formation [121,122].



The prophylactic role of garlic (*Allium sativum*) was evaluated as an alternative to conventional antibiotics in the treatment of infections caused by biofilm-forming bacteria [123]. Garlic contains a large amount of highly biologically active compounds. Persson *et al*. [124] found six sulfur compounds in a garlic extract, which were not toxic to bacteria, but had high activity

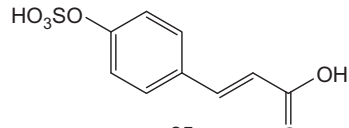
toward the LuxR QS system. Interestingly, these inhibitors were structurally different from the AHLs. Allicin (**84**), applied at subinhibitory concentration, is involved in specific enzymatic inhibition of polysaccharide intracellular adhesin (PIA) synthesis. Suppression of PIA production, the main substance in *S. epidermidis* agglutination, leads to prevention of biofilm formation by this pathogen [125].



84

Simultaneous application of the garlic extract and the antibiotic tobramicin enable effective destruction of *P. aeruginosa* biofilm [126,127].

Eelgrass *Zostera marina* produces another sulfur compound, zosteric acid (**85**), which was studied in the light of its ability to act as a nontoxic antifoulant [121,122,128].

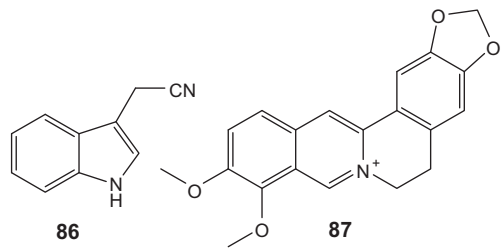


85

Nitrogenous natural substances produced by plants did not remain ignored in the context of microbial adhesion, either.

Auxin 3-indolylacetonitrile (**86**) produced by cruciferous vegetables is a potent inhibitor of pathogenic *E. coli* O157:H7 biofilm formation. As mentioned above, *E. coli* produces in the stationary growth phase indole, which suppresses biofilm formation. The 3-indolylacetonitrile deregulates synthesis of the indole and thus increases its concentration [129].

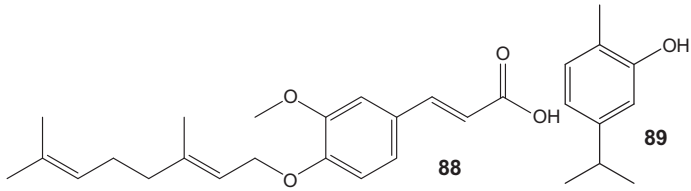
Herbal isoquinoline-type alkaloid, berberine (**87**), has a number of medically usable properties. Very important is the finding of Wang *et al.* [130] that subinhibitory concentration of berberine can effectively block biofilm formation of *S. epidermidis*.



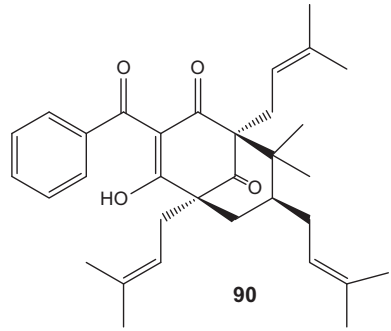
86 **87**

Plants produce a variety of substituted terpene compounds. Also this group includes compounds with significant antibiofilm activity. The bark of *Acronychia baueri* Schott (an Australian small tree belonging to the family of

Rutaceae) contains 3-(4′-geranyloxy-3′-methoxyphenyl)-2-*trans* propenoic acid (**88**) in which geranyl chain is attached to phenolic group [131].This compound, as well as some of its synthetic derivatives, effectively inhibits biofilm formation, which was documented on two oral pathogens *Porphyromonas gingivalis* and *S. mutans* [132]. Essential oil of Summer Savory (*Satureja hortensis*) has limited antibiofilm effect in a subinhibitory concentration against *Prevotella nigrescens*, commonly isolated from endodontic infections [133]. *Satureja khuzistanica* Jamzad (Lamiaceae) is an endemic plant widely distributed in the southern parts of Iran. Iranians have been using it as an analgesic and antiseptic. Essential oil from this plant (called "Dentol"), whose main component is similar to *S. hortensis*, carvacrol (**89**), has anti-inflammatory, antinociceptive, antidiabetic, and antioxidant effects [134]. This essential oil also caused changes in the structure of *P. aeruginosa* biofilm, causing lowered levels of jelly appearance. This phenomenon indicates that Dentol could block polysaccharide production [135].

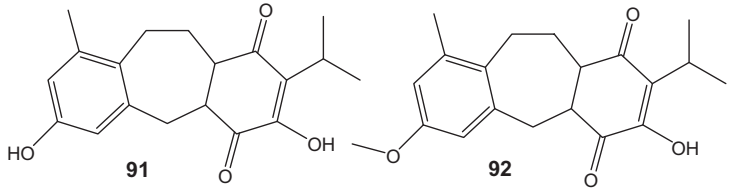


Almeida *et al.* [136] evaluated the antimicrobial activity of extracts obtained from *Rheedia brasiliensis* fruit. They characterized the polyprenylated benzophenone 7-*epi*-clusianone (**90**) as a new agent to control *S. mutans* biofilms.

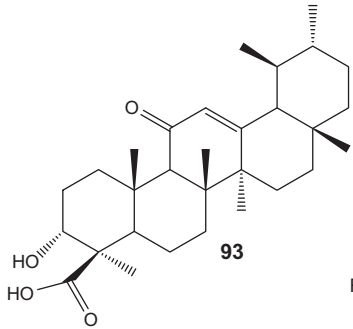


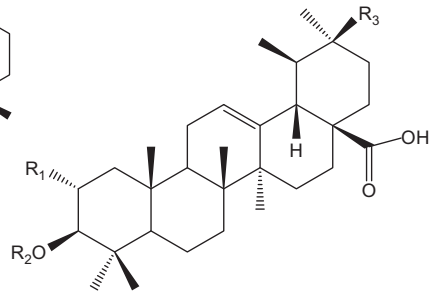
Exudate from the aerial parts of *Salvia corrugata* contains, among others, two diterpenoid compounds, quinones demethylfruticuline A (**91**) and fruticuline A (**92**). Both compounds exhibit different levels of antibiofilm activity on pathogenic bacteria such as *S. aureus*, *S. epidermidis*, and *Enterococcus faecalis*. The authors [137] assume that this effect could depend not only on

inhibition of EPS synthesis but also on their chelating activity and on changes in the microorganism's surface, including cell hydrophobicity.



Boswellia serrata, also known as Indian frankincense, has been used in traditional Ajurvedic medicine for the treatment of inflammatory diseases. The gum resin from this plant contains among others pentacyclic triterpenes called boswellic acids [138]. The 11-keto-β-boswellic acid (**93**) is a biologically very active substance, which can also inhibit formation and cause eradication of biofilm of pathogens such as *S. aureus* or *S. epidermis* [139].





	R_1	R_2	R_3
94	H	*cis-p*-coumaroyl	OH
95	OH	*trans-p*-coumaroyl	H

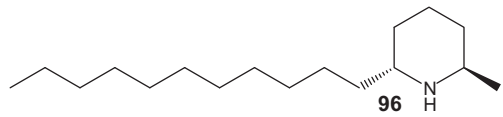
This genus *Diospyros* is known to yield triterpenes. Ursene triterpenes, for example, 3β-*O*-*cis*-*p*-coumaroyl-20β-hydroxy-12-ursen-28-oic acid (**94**) and 2α-hydroxy-3β-*O*-*trans*-*p*-coumaroyl-12-ursen-28-oic acid—jacoumaric acid (**95**) from *D. dendo* inhibited the formation of bacterial biofilm *P. aeruginosa* PAO1 [140].

Extracts from various plants are the subject of growing interest to find compounds with anti-QS or antibiofilm activity [141,142]. Adonizio *et al.* [143] studied the anti-QS activity of aqueous extracts of several terrestrial plants from Florida. Suppression of QS in *P. aeruginosa* PAO1 by extracts of *Callistemon viminalis* (Myrtaceae), *Quercus virginiana* (Fagaceae), and *Tetrazygia bicolor* (Melastomataceae) resulted in more than 80% reduction in biofilm formation.

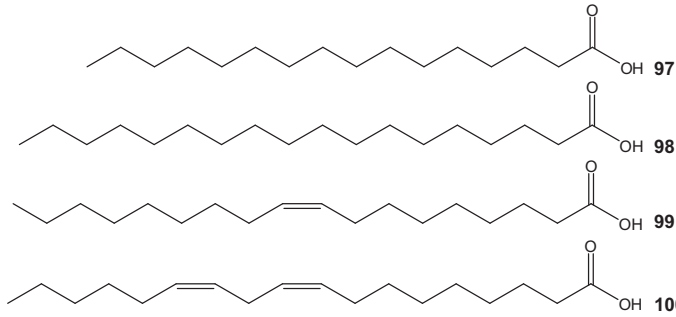
ANIMALS

Animal kingdom does not belong to the priority groups synthesizing natural products. Still, compounds are described that have the ability to influence microbial QS.

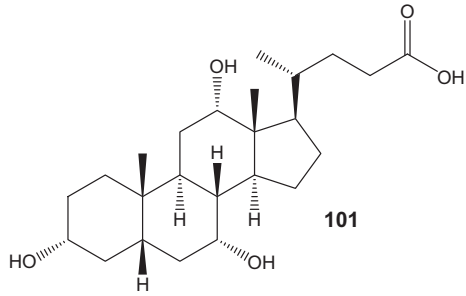
Solenopsin A (**96**), a venom alkaloid from the fire ant *Solenopsis invicta*, efficiently disrupted *P. aeruginosa* QS signaling [144]. Biofilm formation in *P. aeruginosa* was gradually reduced in the presence of solenopsin A in a dose-dependent manner.



Ground beef extract contains several fatty acids such as palmitic acid (**97**), stearic acid (**98**), oleic acid (**99**), and linoleic acid (**100**) that were able to inhibit AI-2 activity. A mixture of these fatty acids, prepared at concentrations equivalent to those present in the ground beef extract, negatively influenced *E. coli* K-12 biofilm formation. Similarly, fatty acids isolated from poultry meat added in the appropriate ratio had concentration-dependent effect on the AI-2 inhibition of *V. harvey* BB 170 [145].

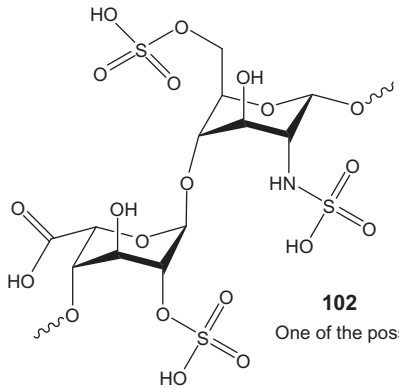


Bile acids (cholic acid (**101**), etc.) promote *V. cholerae* biofilm formation. The reason is probably an effort to reduce bacteria toxicity of this substance, which is quite significant against planktonic cells. Bile acid induction of biofilms was found to be dependent on the *vps* genes, which are responsible for the synthesis of EPS [146].



101

S. aureus is a versatile human and animal pathogen, commonly associated with catheter-related bloodstream infections. Biofilm formation by several *S. aureus* strains is stimulated by heparin (**102**), highly sulfated glycosaminoglycan. The exact mechanism of its effect on *S. aureus* adhesion is unknown [147].



102

One of the possible disaccharide units

Traditional Chinese medicine extensively uses extracts from *Nidus Vespae* (the honeycomb of *Polistes olivaceous*, *P. japonicus*, and others). The chloroform/methanol fraction of these materials showed the highest antibiofilm activities against *S. mutans*, the principal etiological agent of dental caries [148].

Application of honey in traditional medicine is also widely known. Alnaqdy *et al*. [149] studied its effect on adhesion of *Salmonella interitidis* to isolated intestinal epithelial cells *in vitro*. Bacteria pretreated with dilute solutions of honey up to a ratio of 1:8 had a significantly reduced ability of adhesion to epithelial cells. Chestnut honey and especially its aqueous extract have the ability to both degrade AHLs and inhibit the AHLs production of a number of bacterial strains. The result of such exposure was a significant suppression of biofilm formation by *Erwinia carotovora*, *Yersinia enterocolitica*, and *Aeromonas hydrophyla* [150].

CONCLUSION

The ability to control biofilm growth is becoming increasingly important for several reasons. First, biofilms cause considerable economic losses associated with, for example, biofouling; second, they give rise to health problems caused by increasing drug resistance of populations colonizing the tissue, implants, and medical instruments. On the other hand, biofilms are increasingly applied as biocatalysts in biotechnological processes, where their stability is one of the key factors. From the previous text, it is evident that natural compounds are very promising tools for coping with these situations. Substances, whose mechanism of action on the biofilm formation was identified, form a basis for creation of modified structures with higher activity or selectivity. It is also clear that the ongoing screening of natural materials brings many other structures which are able to interact with the regulatory mechanisms involved in adhesion of microbial cells and biofilm formation. New drugs based on a combination of substances with antibiofilm and antibiotic activity, paints, or covering films containing antiadhesion natural substances are just some examples of the expected practical applications.

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ABBREVIATIONS

AHL	N-Acyl homoserine lactone
AI-2	autoinducer 2
c-di-GMP	cyclic di-guanosine monophosphate
DLVO	Derjaguin, Landau, Verwey and Overbeek theory
DNA	deoxyribonucleic acid
EGCG	epigallocatechin-3-gallate
EPS	extracellular polymeric substances
G−	Gram negative bacteria
G+	Gram positive bacteria
HSL	homoserine lacton
m-RNA	messenger ribonucleic acid
OHL	N-octanoyl-DL-homoserine lactone
PIA	polysaccharide intracellular adhesin
QS	quorum-sensing
r-EPS	released exopolysaccharides
TRQ	TanReQuin

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Chapter 11

Medicinal Herbs and Plant Extracts for Influenza: Bioactivity, Mechanism of Anti-influenza Effects, and Modulation of Immune Responses

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INTRODUCTION

Influenza, commonly referred to as the flu, is an acute respiratory infectious disease caused by the influenza virus. These viruses are enveloped RNA viruses that belong to the family *Orthomyxoviridae*, which can cause significant morbidity and mortality in humans [1,2]. Influenza viruses can result in periodic epidemics, and sometimes pandemics, due to their antigenic drift or antigenic shift characteristics [3]. The pandemic virus is still a threat. Influenza viruses, including currently circulating H1N1 and H3N2, cause 3–5 million cases of severe illness and up to 500,000 deaths worldwide per year [4]. A new pandemic (H1N1, swine flu) virus has been recently reported in Mexico and the United States in April 2009, and the World Health Organization (WHO) announced the emergence of a novel influenza A virus for this new human transmissible H1N1 virus.

These viruses are single-stranded RNA viruses with eight gene segments, namely, PB1, PB2, PA, HA, NP, NA, M, and NS. Their subtype is determined by the serological reactivity of two surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA). The viral envelope glycoprotein HA contains the receptor binding site for initial attachment to the sialylated cellular receptors and is critical for binding to cellular receptors and fusion of the viral and

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endosomal membranes. NA is a surface glycoprotein that possesses enzymatic activity essential for viral replication in influenza virus. It is responsible for catalyzing the cleavage of the α-ketosidic linkage that exists between a terminal sialic acid and an adjacent sugar residue [5]. The breaking of this bond has several important effects including (1) allowing for the release of virus from infected cells, (2) preventing the formation of viral aggregates after release from host cells, and (3) preventing viral inactivation and promoting viral penetration into respiratory epithelial cells [6–8]. Thus, effective HA, NA inhibitors, or inhibitors of M2 and other protein targets can be used for preventing and curing influenza infections.

The number of antiviral agents against influenza virus has increased in the past few years, and many chemical and biological antiviral agents have been investigated. There are now four U.S. Food and Drug Administration (FDA)-approved drugs, including amantadine, rimantadine, oseltamivir, and zanamivir, on the market [2]. Antiviral synthetic drugs such as amantadine and rimantadine (M2 ion channel protein inhibitors) were the first drugs available for the treatment of influenza and have been used for decades, but these chemical drugs also have side effects and limitations [9]. Oseltamivir (NA inhibitor) is the first orally active drug commercially developed and widely used for the treatment of influenza. Especially, the acquisition of resistance to NA inhibitors such as oseltamivir and zanamivir is a potential problem.

An alternative approach is the use of broad-spectrum and chemically standardized antiviral herbal extracts or their compounds with demonstrated efficacy against influenza virus *in vitro*. Traditional medicinal plants or herbs employ complex formulae which contain multiple ingredients to prevent or treat various symptoms related to influenza virus infection. These formulae are effective both as antiviral agents and in immunomodulation and are believed to mitigate adverse side effects linked to the toxicity of individual drugs. This review describes several active components and their medicinal herbs with antiviral activity and focuses on elucidating antiviral mechanisms against influenza virus produced by direct virucidal effects or indirect immune responses.

BIOACTIVITY

Active Compounds of Anti-influenza Herbs

Numerous studies have identified bioactive components or compounds in medicinal herbs and plant extracts that may be useful for treating influenza or for use in prophylactic treatment of influenza. A variety of polyphenols, flavonoids, alkaloids, essential oils, and aromatic compounds isolated from medicinal plants as well as plant extracts have been extensively studied and tested for anti-influenza activity using various experimental methods. Table 1 lists some natural extracts or their active constituents demonstrating antiviral effects against influenza.

TABLE 1 Active Compounds Exerting Antiviral Effects Against Influenza from Plant or Its Extracts or Its Compounds

Active Component or Compound	Plant	Common Name	Part Used	Cited Literature (Year)
Polyphenol or polyphenolic complex	*Abies sibirica*	Siberian fir	NS	[10] (2005)
	Agrimonia pilosa	Agrimony	Roots	[11] (2010)
	Caesalpinia sappan	Sappanwood	NS	[12] (2009)
	Camellia sinensis	Green tea	Leaves	[13] (1993); [14] (2005)
	Chaenomeles sinensis	Chinese Quince, Mugua	Fruits	[15] (2008)
	Chaenomeles speciosa	Flowering quince, Japanese quince	Fruits	[16] (2010)
	Cistus incanus	Soft-hairy rockrose	NS	[17] (2007); [18] (2007); [19] (2009)
	Citrus junos Tanaka (Rutaceae)	Yuzu	Fruits	[20] (2001)
	Cleistocalyx operculatus	Water banyan	NS	[21] (2010)
	Cudrania tricuspidata	Storehousebush	Roots	[22] (2009)
	Cydonia oblonga Mill.	Quince	Fruits	[23] (2005)
	Echinacea purpurea	Cone flower	Herb and roots	[24] (2009); [25] (2010)
	Elsholtzia rugulosa	Ye ba zi	NS	[26] (2008)
	Ephedrae herba	Ma huang	Whole parts	[27] (1999)
	Folium Isatidis	Woad	Leaves	[28] (2010)
	Geranium sanguineum L.	Bloody cranesbill	Aerial roots	[29] (1993); [30] (1997);

Continued

TABLE 1 Active Compounds Exerting Antiviral Effects Against Influenza from Plant or Its Extracts or Its Compounds—Cont'd

Active Component or Compound	Plant	Common Name	Part Used	Cited Literature (Year)
				[31] (1998); [32] (2005); [33] (2005); [34] (2006)
	Ginkgo biloba	Ginkgo	Leaves	[35] (2007)
	Glycyrrhiza uralensis	Liquorice	Roots	[36] (2010)
	Gnetum pendulum		Heartwood	[21] (2010)
	Houttuynia cordata	Chameleon plant	Whole parts	[37] (2009)
	Mosla scabra	*Elsholtzia* weeds	NS	[38] (2010)
	Muehlenbeckia hastulata		Whole parts	[39] (2010)
	Pseudocydonia sinensis	Chinese quince	Fruits	[40] (2007)
	Punica granatum	Pomegranate	NS	[41] (2009)
	Reynoutria elliptica		Roots	[42] (2003)
	Rhodiola rosea	Roseroot	Roots	[43] (2009)
	Scutellaria baicalensis	Skullcap	Leaves	[44] (1992)
			Roots	[45] (1990); [46] (1995); [47] (2010)
Alkaloids	*Castanospermum australe*	Moreton Bay chestnut	Seeds	[48] (1983)
	Commelina communis	Asiatic dayflower	NS	[49] (2009)
	Mahonia bealei	Beale's barberry	Roots	[50] (2006)
	Thalictrum simplex L.	Meadow-rue	NS	[51] (2003); [14] (2003)

TABLE 1 Active Compounds Exerting Antiviral Effects Against Influenza from Plant or Its Extracts or Its Compounds—Cont'd

Active Component or Compound	Plant	Common Name	Part Used	Cited Literature (Year)
Organic or aromatic compounds	*Alpinia officinarum*	Galangal	NS	[52] (2010)
	Chaenomeles speciosa	Sweet Nakai	Fruits	[16] (2010)
	Cynanchum stauntonii	Bai Qian	Roots	[53] (2005)
	Echinacea purpurea	Eastern purple coneflower	Roots	[54] (2009)
	Erythrina addisoniae		Roots	[55] (2010)
	Ferula assa-foetida	Asafoetida	Roots	[56] (2009)
	Glycyrrhiza glabra	Licorice	Roots	[57] (1997); [58] (2009)
	Glycyrrhiza inflata	Chinese licorice	Roots	[59,60] (2011)
	Houttuynia cordata	Chameleon plant	Whole parts	[61] (1995)
Protein or sugars	*Clematis montana*	Bergwaldreben	Stem	[59] (2009)
	Codiaeum variegatum	Garden croton	Leaves	[62] (2008)
Unspecified extracts	*Alpinia katsumadai*	Katsumadai, Cao dou kou	Seeds	[63] (2010)
	Clinacanthus siamensis		Leaves	[64] (2009)
	Forsythia suspensa	Weeping golden-bell	Fruits	[65] (2005); [66] (2006)
	Myrica rubra	Yumberry	Leaves	[67] (2008)
	Ribes nigrum L.	Blackcurrant	Fruits	[68] (2003)
	Sanicula europaea L.	Wood sanicle	Leaves	[69] (1996)
	Toddalia asiatica Lam.	Orange climber	Roots	[24] (2005)

NS, not specified.

Polyphenolic Complexes or Polyphenols

Polyphenol compounds have been shown to have diverse antiviral mechanisms, including inhibition of NA activity, membrane fusion, the viral protein, or RNA synthesis, and their actions are probably directed against an early stage of infection. Such compounds also inhibit viral adsorption. Polyphenols from *Reynoutria elliptica* exhibited high inhibitory activity for NA [42], as determined by a simple NA assay system developed by Myers [70]. Polyphenols from *Folium lsatidis* leaves reduced the pulmonary index and significantly lessen the mortality rate together with prolonging life time in a mouse model [28]. In addition, polyphenols from the fruits of *Chaenomeles sinensis* showed anti-influenza virus activity by inhibiting hemagglutination activity and by suppressing NS2 protein synthesis [15].

Flavonoids are a large class of polyphenolic compounds and are also abundantly found in vegetables, fruits, and plant-derived beverages. Flavonoid compounds inhibit both NA activity and membrane fusion. As early as 1990, 5,7,4′-trihydroxy-8-methoxyflavone, from the root of *Scutellaria baicalensis*, was found to be an influenza virus sialidase inhibitory compound [45]. Later, 5,7,8,4′-tetrahydroxyflavone (isoscutellarein) from the leaf of *S. baicalensis* was also found to have potent influenza virus sialidase inhibitory activity when administered intranasally or orally to mice [44]. In a study of anti-influenza virus activity from compounds extracted from *Citrus junos*, a new flavanone hesperetin, 7-O-(2″, 6″-di-*O*-α-rhamnopyranosyl)-β-glucopyranoside, was reported as being effective for inhibition of the influenza A virus [20].

Recently, flavonoids have received attention due to their various antiviral activities against the influenza virus [71]. In particular, quercetin 3-rhamnoside, which is present in *Houttuynia cordata*, has been reported to have inhibitory effects on influenza A virus replication [37]. *Agrimonia pilosa*, especially the juice of the root, has been used for treating coughs and colds [26]. The extract of this plant was later shown to contain abundant flavonoids such as catechin, hyperoside, quercitrin, quercetin, and rutin [72], and human influenza viruses including H1N1 and H3N2 were inhibited by this extract in embryonated chicken eggs and Madin–Darby canine kidney (MDCK) cells [11]. The flavonoids which are used in the treatment of cold and fever have also been studied in medicinal plants [35,71].

Polyphenol-rich extracts or polyphenolic complexes isolated from aerial roots of the Bulgarian medicinal plant *Geranium sanguineum* L. have shown strong antiviral activity against influenza virus. Their selective anti-influenza activity was demonstrated *in vitro* and *in ovo*, as well as in mice and chickens [29,31,34]. A polyphenol-rich extract from the Mediterranean plant *Cistus incanus* exerted a potent antiviral activity in A549 and MDCK cell cultures infected with avian and human influenza strains of different subtypes [18]. Punicalagin, which is one of the major polyphenols in the pomegranate juice

from *Punica granatum*, demonstrated virucidal effects against influenza H1H1 and H3N2 *in vitro* [41].

Recent studies have also shown antioxidant abilities, such as radical scavenging capabilities, from extracts of *G. sanguineum* in addition to antiviral activity [33]. Polyphenols in fruits also showed antiviral activity against influenza virus together with antioxidant activity. Chinese quince (*Cydonia oblonga* Mill.) contains large amounts of phenolics, consisting mainly of high polymeric procyanidins, and the antioxidant activities were superior to those of ascorbic acid evaluated in both the linoleic acid peroxidation and the 1,1-diphenyl-2-picrylhydrazyl radical scavenging systems [73].

Concerning the active components in *Ephedrae herba* extract responsible for antiviral activity on the growth of influenza A/PR/8 in MDCK cells, tannin is strongly suggested to be one of the candidate components, based on the complete or partial reversal of the inhibitory effect after $FeCl_3$ treatment of the extract [27]. It has been reported that *E. herba* extract contains many types of condensed tannins and this condensed tannin component is composed of flavan units, mostly consisting of (+)-catechin, (−)-epicatechin (EC), or their analogs [23]. Also, it is well known that these tannins are contained in green tea. Tea-condensed tannins, such as (−)-epigallocatechin gallate (EGCG) and theaflavin digallate, have been reported to inhibit the growth of influenza A and B viruses [13]. Green tea is produced from the leaves of the evergreen plant *Camellia sinensis*, and its major active ingredients are polyphenolic compounds, known as catechins. Catechins include EGCG, (−)-epigallocatechin, (−)-epicatechin gallate, and (−)-EC, and EGCG comprise approximately 50% of the total amount of catechins in green tea [74]. Among these components, EGCG was found to be a potent inhibitor of influenza viruses, including H1N1 and H3N2, in an MDCK cell culture, and the 3-galloyl group of catechins performs an important role for observing antiviral activity [75].

Alkaloids

Alkaloids widely exist in medicinal plants and natural products. Some alkaloids, such as (−)-thalimonine, isolated from the Mongolian plant *Thalictrum simplex*, have shown antiviral activity against H7N7 and H7N1 in cell cultures of chicken embryo fibroblasts. This isoquinoline alkaloid markedly inhibited the influenza virus reproduction *in vitro* by reducing the expression of viral glycoproteins HA and NA on the surface of infected cells, virus-induced cytopathic effects (CPEs), infectious virus yields, and virus-specific protein synthesis. However, the synthetic analogues of (−)-thalimonine were not active [14]. The total alkaloid extract from *Commelina communis* showed antiviral activity against influenza virus H1N1 *in vitro* and *in vivo*. This inhibitory action was exhibited when administered prior to or after infection of mice

with influenza virus and suggests that this extract has a protective effect against influenza A virus infection [49].

An initial study using castanospermine, which is an indolizin alkaloid first isolated from the seeds of *Castanospermum australe*, native to the east coast of Australia and the Pacific islands, showed this plant alkaloid to be a potent inhibitor of glycoprotein processing because it inhibited glucosidase I, and 80–90% of the viral glycopeptides of influenza virus were found to be susceptible to the action of endoglucosaminidase H [48].

Organic or Aromatic Compounds

The volatile oils of the roots of *Cynanchum stauntonii* exhibited antiviral activity against the influenza virus *in vitro* and prevented influenza virus-induced deaths in a dose-dependent manner *in vivo*. The major constituents were identified as (E,E)-2,4-decadienal, 3-ethyl-4-methypentanol, 5-pentyl-3H-furan-2-one, (E,Z)-2,4-decadienal, 2(3H)-furanone, and dihydro-5-pentyl [53]. Recently, two new sesquiterpene coumarins and one new diterpene were isolated from the roots of *Ferula assafoetida*, and these compounds showed greater potency against influenza A virus (H1N1) than amantadine [56].

Glycyrrhiza glabra is a perennial herb and native to central and south-western Asia and cultivated in temperate and subtropical regions of the world. The root, which is called licorice, is dried and processed and has a characteristic odor and sweet taste [76]. The triterpene saponin, glycyrrhizin, is the main active component of licorice roots and is found to protect cells from infection with influenza A virus, H3N2. Glycyrrhizin treatment produced a clear reduction in the number of influenza virus-infected human lung cells [58]. In an experimental mouse model study, when mice were treated intraperitoneally with glycyrrhizin 1 day before exposure to virus and 1 and 4 days after infection with influenza virus H2N2, all of the animals survived and the pulmonary consolidation and the virus titers in the lung tissues of infected mice treated with glycyrrhizin were significantly lower than those in the lung tissues of infected mice [57]. More recently, one new licochalcone and several chalcones have been isolated as active principles from *Glycyrrhiza inflat*e for influenza viral strains, H1N1, H9N2, novel H1N1, and oseltamivir-resistant novel H1N1 [60].

Proteins or Sugars and Derivatives

A novel mannose-binding lectin was isolated from the stem of *Clematis montana* and showed marked antiviral activity against various viruses including influenza A subtype H1N1 and H3N2 in cell culture [59].

A number of natural and synthetic sugar analogues have been tested for antiviral activity against influenza virus and found to disturb glycosylation by inhibiting the trimming enzymes needed to complete the synthesis of sugar chains. Higher concentrations of 2-deoxy-D-glucose and D-glucosamine were

found to impair glycosylation of viral glycoproteins. Only the sugar to which a benzyl group introduced has antiviral activity against influenza virus [77]. A bioactive cyanoglucoside from *Codiaeum variegatum* leaves showed anti-influenza activity, and this was the first report indicating antiviral activity of a cyanoglucoside [62]. In addition, platyphyllone, from the bark of *Alnus japonica*, was strongly active against H9N2 avian influenza virus [78].

Multiple Bioactive Components

Antiviral herbal extracts frequently exhibit multiple bioactivities, possibly by acting in synergy, while also providing a relatively safe drug with few side effects. For example, some *Echinacea*-derived preparations are known to contain potentially bioactive compounds, such as phenolic caffeic acid derivatives, alkylamides, and polysaccharides, and also possess selective antibacterial and immune modulation activities that might also contribute to their beneficial properties [79,80]. *Echinacea purpurea* shows the markers for standard to have its bioactive characteristics. As a commercially standardized extract of the herb *E. purpurea*, this remedy has shown potent antiviral activity against human H1N1 and H3N2, avian H5N1 and H7N7, and the pandemic H1N1. In addition, the lack of emergence of *E. purpurea*-resistant viruses during sequential passage is a significant advantage over Tamiflu®, which under similar culture conditions readily allows the development of resistant virus strains [54].

Some Extracts from Plants with Anti-influenza Activity

Compared with the previously mentioned isolated constituents or components, additional work has been conducted to determine the antiviral effects of crude extracts, although the main components contributing to the antiviral effects are still unclear. The influence of a water-soluble extract of *Sanicula europaea* L. on influenza (A/PR/8/34) virus growth has been investigated in MDCK cells. Fractions from Sanicula were found to be nontoxic for MDCK cells, but the growth of influenza virus was completely inhibited by these fractions [69]. More recently, more than 200 Chinese medicinal herb extracts were screened for antiviral activities against influenza (A/PR/8/34) virus, and *Toddalia asiatica* showed potent antiviral activities against H1N1 virus. In addition, antiviral activity was best observed using cotreatment with influenza virus infection, and the effects were observed even when extracts were administrated 24 h before or after the initiation of infection [24].

MECHANISMS OF ANTIVIRAL EFFECTS AGAINST INFLUENZA

Intensive studies on the mode of the protective effects of the plant extracts or medicinal herbal compounds indicate that the protection may possibly be attributed to a combination of biological activities. The presence of a variety

of biologically active compounds as well as the possible synergistic interactions between them seems to be decisive for the overall antiviral effect. The inhibition of virus infectivity might be attributed to multiple mechanisms of action, for example, specific inhibition of an early stage in viral intracellular multiplication or nonspecific interference with virus and cell interactions. The methods to illuminate the mechanism of antiviral activity can be carried out by *in vitro* tests including tests for viral replication, cell survival rate, membrane fusion, and assays of hemagglutination or sialidase activity. *In vivo* tests including animal challenge experiments and embryo culture studies are also applied to evaluate either direct or indirect activity against virus.

Inhibiting Acidification of Viral Membrane or Fusion of the Virus with Endosome

The tannin components of *E. herba*, traditional Chinese medicines for influenza, inhibit the acidification of intracellular compartments such as endosomes and lysosomes, thereby inhibiting the growth of influenza A virus *in vitro* [27]. It has been also shown that acidic conditions in endosomes and lysosomes are essential for the uncoating process of influenza virus by triggering the viral envelop fusion activity [81].

The antiviral mechanism involved with catechins from green tea leaves can have two modes of action: interference of viral membrane fusion by inhibition of acidification of endosome, and/or inhibition of virus adsorption onto the host cells by binding itself to the HA of influenza virus [13,82]. The dual actions for antiviral activity were also found in the plant flavone from the roots of *S. baicalensis*, and this flavone reduced the replication of mouse-adapted influenza virus by inhibiting the fusion of the virus with the endosome/lysosome membrane, which occurs at early stage of the virus infection cycle. The antiviral activity of *S. baicalensis* extract against influenza virus was also indicated by its ability to inhibit the budding of progeny virus from the cell surface, when it was added at budding stage of the virus infection cycle [46].

Cell entry of enveloped viruses, such as influenza virus or human immunodeficiency virus, requires a wide-fusion-pore mechanism, involving clustering of fusion-activated proteins and fluidization of the plasma membrane and the viral envelope. When cells treated with glycyrrhizin were replaced in glycyrrhizin-free medium, the fluidity of the membrane increased with time and the cells showed enhanced susceptibility to infection and fusion. Factors regulating the formation of a wide fusion pore include the number of receptors and gp120 molecules and the degree of fluidity [83]. In a recent report, glycyrrhizin reduced H3N2 titers in lung-derived cells. However, the observed antiviral activity of glycyrrhizin was assumed to be mediated by an interaction with the cell membrane possibly resulting in reduced endocytotic activity and virus uptake; in other words, cell entry, rather than absorption of the virus by

interfering with the cellular membrane, eventually led to a reduction in endocytosis [58]. These factors could afford dual antiviral actions of one component, by interfering with one or more essential stages in viral replication or dissemination.

Inhibiting Viral Entry

Hemagglutination assays show whether a compound inhibits the receptor binding activity of a virus by interfering with the viral entry into cells. *E. purpurea* extracts showed potent antiviral activity against human H1N1 and H3N2 and avian strains H5N1 and H7N7. Also, direct contact between virus and this extract was found to be required for the inhibitory effect because pretreatment of cells before virus infection led to substantially less inhibition. This was confirmed by the use of hemagglutination assays, which clearly showed that this extract inhibited HA activity and consequently would block entry of treated virus into the cells [54].

The extract of the Mediterranean plant *C. incanus*, which has been used in traditional medicine for centuries in southern Europe without any reported complications, exerted a potent antiviral activity in cell culture experiments. On a molecular basis, the protective effect of this extract appears to be mainly due to binding of the polymeric polyphenol components of the extract to the virus surface, thereby inhibiting binding of the HA to host's cellular receptors [18]. This active component may not be taken up by the cell because of its higher molecular weight; therefore, this antiviral agent had to be applied locally as an aerosol formulation in a mouse infection model. Mice treated with extract of *C. incanus* did not develop disease and exhibited no histological alterations of the bronchiolus epithelial cells against a highly pathogenic avian influenza A virus (H7N7) [17]. High molecular weight polyphenols of *Ch. sinensis* also showed anti-influenza virus activity by inhibiting hemagglutination activity [15]. In addition, a polyphenolic complex isolated from *G. sanguineum* L. was shown to have selective anti-influenza activity, possibly due to nonspecific binding to viral membrane components [31].

Interfering Virus Replication or Suppressing Viral RNA Synthesis

Flavonoids derived from *H. cordata* were evaluated for antiviral activity against influenza virus using a CPE reduction method, and quercetin was found to possess a strong antiviral effect with the reduction of the formation of a visible CPE. This compound inhibited virus replication in the initial stage of virus infection by indirect interaction with the viral particles [37]. In contrast, the antiviral components of the steam distillate derived from fresh plants of *H. cordata* were found to be different. They included essential oils, and their major components were methyl *n*-nonyl ketone, lauryl aldehyde, and caprylic aldehyde. The antiviral activity against influenza virus demonstrated

that the essential oils provide virucidal activity by interfering with the function of the viral envelope [61].

Pomegranate polyphenol extract purified from *P. granatum* was found to have antiviral properties against influenza virus and to suppress replication of influenza A virus in MDCK cells. Its virucidal effects were considered to result from blocking replication of the virus RNA and inhibiting agglutination of red blood cells by the virus [41]. Quantitative RT-PCR analysis data showed that the extract of *A. pilosa*, to some degree, suppressed viral RNA synthesis of human influenza A virus in MDCK cells, and the antiviral effect of the extract was stronger than that of the ion channel blocker, amantadine [11]. When diarylheptanoids isolated from *Alpinia officinarum* were orally administered to mice infected with influenza A virus, these aromatic compounds significantly reduced body weight and prolonged survival times of infected mice and also reduced viral titers in bronchoalveolar lavage fluids of the lungs. These compounds demonstrated dose-dependent suppression of viral messenger RNA [52].

Inhibiting Neuraminidase/Sialidase Inhibitory Activity

In the course of screening NA inhibitors as herbal medicines, extracts of *R. elliptica* exhibited high inhibitory activity, and the anthraquinones were initially as a possible NA inhibitors [72]. Flavonoids from 103 species were tested for inhibitory activity against influenza virus sialidase, and flavones from the root of *S. baicalensis* showed the most potent activity [45]. Flavones from the roots of *Cudrania tricuspidata* [84] and *Rhodiola rosea* [43] were also found to have NA inhibitory activities.

In a recent study, the structure–activity relationships of 25 flavonoids were studied regarding NA activity of influenza virus, and typical influenza virus strains of H1N1 and H3N2 were used as the source of NA. The analyses of effects of flavonoids on influenza virus NA revealed that the 4′-OH, 7-OH, C4O, and C2C3 functionalities were essential for a strong inhibitory effect, and the presence of a glycosylation group greatly reduced NA inhibition [22]. The structure–activity relationship of the flavonoids with different chemical structures and their anti-influenza virus activities were also addressed in a study of *Elsholtzia rugulosa* [71]. In addition, polyphenol compounds isolated from the roots of *Glycyrrhiza uralensis* demonstrated NA inhibition, and the scaffolds of these compounds showed a resveratrol-like motif that is commonly found in traditional medicinal compounds [36].

MODULATION OF IMMUNE RESPONSES

Previously, the antiviral mechanisms of medicinal plants have been considered to be different from those of existing antiviral agents, such as the viral NA inhibitors or ion channel blockers. In addition to the direct virucidal

effect, the involvement of host immune responses, including cytokine production, has been suggested to play an important role in the pathogenesis of influenza virus infection. Herbal drugs act as anti-influenza agents in another way by inhibiting the virus indirectly, by inducing interferon (INF) or other regulating immune functions. Simply stated, they promote phagocytosis to enhance the immune function of the cells or enhance macrophage activation through inducing INFs or producing proinflammatory cytokines. It is also known that several medicinal plants alleviate flu-like symptoms by suppressing local and systemic proinflammatory cytokines, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1α, IL-6, IL-8, and Th1 cytokines such as IL-12 and IFN-γ, in the host rather than through direct effects on viral replication.

Alternatively, overproduction of cytokines in the host has been shown to correlate with elevated levels of local and systemic proinflammatory cytokines, including TNF-α and IL-6 [85,86]. Cytokines are produced locally and systemically in animals during influenza infection [87–89], and the occurrence of the cytokine storm "hypercytokinemia" has been proposed to contribute to the increased severity of diseases caused by the highly pathogenic viruses [90–92]. In addition, antagonism of TNF-α, IL-1α, and IL-6 has been found to partly prevent the decrease in feeding and loss of body weight of mice induced by influenza virus infection [93]. It has also been reported that the reduction of IFN-γ production contributes to the alleviation of influenza infection by reducing the number of infiltrated cells in the lungs and the levels of nitric oxide metabolites [94].

Immune-Enhancing Activities

Modern interest in Echinacea has focused on its immunomodulatory effects, particularly in the prevention and treatment of upper respiratory tract infections [79]. A variety of studies on Echinacea have been published. They found Echinacea to contain a number of bioactive compounds such as cichoric acids, caffeic acids, alkylamides, and polysaccharides, many of which have been implicated in various models of immune modulation against upper respiratory infections such as influenza virus, which can also induce proinflammatory cytokine secretion in epithelial cells [95]. Among them, *E. purpurea* is widely used for prevention or treatment of the common cold, with the proposed mechanism related to stimulate the immune system.

Daily dietary supplements of the soluble root extracts of this American coneflower revealed that natural killer (NK) cells and monocytes were numerically and significantly increased in both the bone marrow and the spleen, and all other hemopoietic and immune cell populations in these two organs remained at control levels, even after 2 weeks administration of the dietary herb. The specific nature of Echinacea-derived phytochemicals as stimulants of NK cells and monocytes may be responsible for nonspecific immunity as

the first line of defense against virus-infected cells, especially in treatment of acute upper respiratory infections such as the common cold and influenza [96,97]. In contrast, the ethanol extracts, which were prepared from dormant roots of *E. purpurea* grown in different locations throughout North Carolina, displayed a variety of effects ranging from suppression to stimulation of mediator production; therefore, it has been suggested that *E. purpurea* extracts have a potential for use in alleviating the symptoms and pathology associated with infections due to influenza A virus [98].

The mode of anti-influenza activity of polyprenols from Siberian silver fir (*Abies sibirica*) has been based on nonspecific stimulation of immune cell activities [10], and the anti-influenza effect of *Zingiber officinale* was suggested to result from macrophage activation leading to production of TNF-α, and not an inhibitory effect on the growth of influenza virus [25]. The antiviral effect of glycyrrhizin, which is extracted from *G. radix*, was investigated in mice infected with influenza virus. Mice were protected from lethal H2N2 infection by glycyrrhizin, although no influence on viral replication was detected. When splenic T cells from glycyrrhizin-treated mice were adoptively transferred to mice exposed to influenza virus, all of the recipients survived, compared to 0% survival for recipient mice inoculated with naïve T cells or splenic B cells and macrophages from glycyrrhizin-treated mice. The protective mechanism was therefore suggested to be the induction of IFN-γ in T cells [57]. In a more recent study, the immune responses induced by glycyrrhizin may result in the loss of control of viral replication by cytotoxic immune cells, including NK cells and $CD8^+$ T lymphocytes [92].

Immunomodulatory Activities

Influenza A virus causes infection of the upper respiratory tract, and the virus-infected epithelial cells and macrophages have been shown to produce various chemotactic cytokines. Among them, regulated on activation, normal T-cell expressed and secreted (RANTES) is a potent chemoattractant for monocyte/macrophages, basophils, and T cells. Macrophage inflammatory protein-1 (MCP-1) recruits and activates monocyte/macrophages in inflammatory sites in pediatric patients with acute upper respiratory tract infections caused by adenovirus, influenza, and parainfluenza virus [99]. The human airway epithelium infected with influenza virus A, H3N2, has also been studied both *in vitro* and *in vivo*, and significant levels of RANTES protein were detected [100]. In addition, significant amounts of IL-6 and IL-8 as well as RANTES were released when bronchial epithelial cells were infected with influenza virus A, H3N2 [101]. There have been numerous reports of immunomodulatory properties linked with respiratory viral infections in virus-infected respiratory cells, epithelial cells, or nasal wash samples obtained from patients [99,102–104].

A plant extract of *Forsythia suspense* exhibited a consistent inhibitory effect on influenza virus-stimulated RANTES secretion and displayed both

negative and positive regulatory effects on virus-stimulated MCP-1 production, dependent on the concentrations used [65,66]. Especially, results from *in vitro* immune assays using human peripheral blood mononuclear cells showed that *E. purpurea* stimulated production of anti-inflammatory IL-10, together with TNF-α and IL-12 [105,106]. More recently, isolated alkylamides from *E. purpurea* suppressed the production of TNF-α and PGE2 from infected cells and also strongly inhibited the production of granulocyte colony-stimulating factor, chemokine (C–C motif) ligand 2 (CCL2)/MCP-1, CCL3/macrophage inflammatory protein-1α, and CCL5/RANTES [98]. In addition, glycyrrhizin, which is the active component of licorice roots, has been found to impair H5N1-induced production of C–X–C motif chemokine 10 , IL-6, and CCL5 and inhibits H5N1-induced apoptosis in human monocyte-derived macrophages [92].

Influenza virus infection is an example of a viral infection associated with immunosuppression. However, immune responses to the virus accurately reflect immune responses of host defensive capabilities: the depression on nonspecific responses and the development of virus-specific systemic, local, and recruited responses [107]. A polyphenol-rich extract isolated from the medicinal plant *G. sanguineum* L. was found to restore the suppressed functions of phagocytes in mice infected with influenza virus H3N2 by increasing the numbers of peritoneal and alveolar macrophages [32].

CONCLUSION

Traditional medicinal herbs have long been used as remedies against influenza virus in Asian, Eastern European, North American, and some South American countries and include *E. purpurea*, *C. incanus*, *G. sanguineum* L., *G. uralensis* F., *E. herba*, etc. These herbs contain polyphenols, polyphenolic complexes, and aromatic compounds, which can be potential HA inhibitors or NA inhibitors, and may be responsible for reducing flu symptoms, according to their antiviral actions. Some flavonoids have direct antiviral effects through inactivating the influenza virus by inhibiting acidification of intracellular endosome compartments required for fusion of viral and cellular membranes or reducing membrane fluidity, which would inhibit fusion of the viral membrane.

A single small molecule isolated from one herb may exhibit certain antiviral activity *in vitro*, but are often less clinically effective. In fact, whole plants work very differently in the human body than do the isolates, and they are thought to contain synergistic elements that interact with the virus in different ways. We believe that the interaction between the compounds from plants and the state of the host might be far more complex than merely the result of a direct antiviral activity exerted by a single chemical entity. Therefore, a formulation containing a combination of herbal plants holds the potential to become the therapeutic of choice in the future, due to the synergistic

effect achieved by the multiple ingredients that inhibit the virus at different stages, simulating the impaired immune system and improving the overall symptoms.

In conclusion, the antiviral mechanisms of medicinal plants are considered to be different from those of existing FDA-approved viral NA inhibitors or ion channel blockers. Herbal drugs, as anti-flu agents, comprise multiple compounds regulating multiple targets, such as intracellular endosomes, and viral surface HA or NA, etc. In addition, other protein targets or characteristics for alleviating flu-like symptoms or producing antioxidant or anti-inflammatory effects can be used for preventing and curing influenza infections. By further investigating and elucidating the mechanisms and compounds as reviewed in this study, a better drug discovery system may be made available to our generation. Although the published evidence to date and long history of using these medicinal herbs in some countries support the safety and the effectiveness of these herbs for treatment for influenza virus infection, their use as antiviral drugs against influenza virus infection is limited, and additional research is needed before definitive recommendations can be made.

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ABBREVIATIONS

CCL	chemokine (C–C motif) ligand
CPE	cytopathic effect
EC	epicatechin
EGCG	epigallocatechin gallate
HA	hemagglutinin
IL	interleukin
INF	interferon
MCP	macrophage inflammatory protein
MDCK	Madin–Darby canine kidney
NA	neuraminidase
NK	natural killer
RANTES	regulated on activation, normal T-cell expressed and secreted
TNF	tumor necrosis factor

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Chapter 12

Bioactive Compounds from Marine Gorgonian Corals

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INTRODUCTION

Gorgonian corals (also known as sea fans, sea whips, or sea plumes) are conspicuous members of most tropical and subtropical marine habitats and grow in ocean from tideland to about 4000 m deep. There are 13 families, over 6100 species gorgonian corals in all over the world. The tropical Western Atlantic (West Indian) and the Indo-Pacific regions are two main areas of the globe gorgonian corals. Gorgonian corals are a rich source of novel, bioactive secondary metabolites, and they have attracted a great deal of attention from scientists in the fields of chemistry, pharmacology, and ecology. Studies of the natural products chemistry of this interesting group of marine invertebrates began in the late 1950s. These marine invertebrates are recognized to mainly produce novel acetogenins, sesquiterpenoids, diterpenoids, prostanoids, and, in some cases, highly functionalized steroids that are largely unknown from terrestrial sources. Coil *et al*. reviewed the chemistry and chemical ecology of octocorals in 1992 [1], and Rodríguez *et al*. summarized the natural products chemistry of West Indian gorgonian octocorals in 1995 [2]. Until now, totally over 1000 new compounds have been obtained from approximately 1% (12 families, 38 genera) of the worldwide gorgonian corals. These metabolites are responsible for various bioactivities such as antitumor, antituberculosis, anti-inflammation, antiplasmodial, antiviral, and antioxidant activities and have been shown to play a very important role in the survival of gorgonian corals in their natural environment, such as feeding deterrence, coevolved predator, and antifouling.

This review focuses on the compounds with significant bioactivities from the worldwide gorgonians with emphasis on their structures, relevant biological

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activities, and structure–activity relationships, covering the literatures from 1993 up to now. Its aim is to give the reader a brief view of the bioactive compounds from the marine invertebrates and provide models on which to base extensive synthetic programs leading to still more efficacious pharmaceuticals.

BIOACTIVE COMPOUNDS FROM MARINE GORGONIANS

The compounds with significant bioactivities isolated from gorgonians since 1993 up to now mainly covered diterpenes, sesquiterpenes, nitrogen-containing compounds, and steroids.

Diterpenoids

The principal terpenoids elaborated by gorgonians are diterpenes and sesquiterpenes that account for about 65% and 10% of all the reported metabolites from gorgonians, respectively. There was chemotaxonomic value associated with the distribution of terpenoid types across gorgonian corals as Gerhart reported [3]. The representative structures of diterpenoids by carbon skeleton class from gorgonians included briarane type, cembrane type, eunicellan type, xenicane type, pseudopterosin type, dilophol type, etc. These diterpenoids show a variety of biological activities, such as cytotoxicity, antifungal, antiplasmodial, antituberculosis, antiparasitic, anti-inflammatory, antimycobacterial, hemolytic, and antiviral activities.

Bioactive Briarane-Type Diterpenes

Since the discovery of the first briarane-type metabolite (briarein A) in 1977 by Burks *et al*. from a West Indian gorgonian *Briareum asbestinum* [4], over 300 compounds of this type have been isolated from gorgonian *Briareum* and *Junceella* genera. In 2001, Sung *et al*. reviewed the survey of briarane-type diterpenoids of marine origin [5]. Briarane-type diterpenoids have attracted the attentions of investigations because of the structural complexity and interesting biological activities (e.g., cytotoxicity, anti-inflammatory, antiviral, immunomodulatory activity, insect control, antifouling) associated with numerous compounds of this type [6–16]. Here, we selectively introduce the representative briarane-type diterpenoids with significant bioactivities.

According to the literature, over 21 briarane-type diterpenoids from gorgonians showed significant cytotoxicity (Table 1), such as stecholide L (**1**); excavatolides C–F (**2**–**5**); excavatolides J, K, M, O, Q, Z (**6**–**11**) [6–9]; briaexcavatolides B, F, L, P (**12**–**15**) [10–12]; stecholide A (**16**); briarane (**17**) [13]; 2β-acetoxy-2-debutyryloxystecholide E (**18**) [14]; 9-deacetylstylatulide lactone (**19**) [14]; 2β-acetoxy-2-debutyryloxystecholide E acetate (**20**) [14]; and 9-deacetylbriareolide H (**21**) [15].

In addition, juncins R–ZI (**22**–**31**) had strong antifouling activities toward barnacle *Balanus amphitrite* larvae with EC_{50} (50% effective concentration) values of 0.004, 0.34, 2.65, 1.61, 3.77, 21.06, 0.004, 0.14, 1.47, and 0.51 μg/mL [16]. Comparison of the antifouling activities of compounds **22**–**27**, **29**, and **30** suggested that the potency of briarane-type diterpenoids

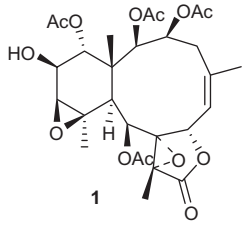
TABLE 1 *In Vitro* Cytotoxicity of Compounds 1–21

Compounds	Cytotoxicity
1	CC_{50} (P-388, A-549, HT-29, MEL28) = 10, 2.5, 5, 5 μg/mL
2	CC_{50} (P-388, KB, A-549, HT-29) = 0.3, 1.9, 1.9, 1.9 μg/mL
3	CC_{50} (P-388, KB, A-549, HT-29) = 1.8, 4.2, >50, 1.3 μg/mL
4	CC_{50} (P-388, KB, A-549, HT-29) = 1.6, 0.8, 1.2, 1.6 μg/mL
5	CC_{50} (P-388, KB, A-549, HT-29) = 6.2, 7.0, 5.2, 5.5 μg/mL
6	CC_{50} (P-388, KB, A-549, HT-29) = 3.8, 6.5, 5.2, 5.2 μg/mL
7	CC_{50} (P-388, KB, A-549, HT-29) = 0.9, 3.3, 3.0, 1.3 μg/mL
8	CC_{50} (P-388, KB, A-549, HT-29) = 0.001, 1.0, 0.1, 2.2 μg/mL
9	CC_{50} (P-388, A-549, HT-29, MEL28) = 5, 5, 5, 10 μg/mL
10	CC_{50} (P-388, A-549, HT-29, MEL28) = 5, 10, 10, 10 μg/mL
11	CC_{50} (P-388, KB, A-549, HT-29) = 1.3, 6.5, 11.2, 2.8 μg/mL
12	CC_{50} (P-388, KB) = 1.3, 1.5 μg/mL
13	CC_{50} (A-549) = 1.3 μg/mL
14	CC_{50} (P-388) = 0.5 μg/mL
15	CC_{50} (P-388, A-549, HT-29) = 0.9, 4.8, 3.1 μg/mL
16	CC_{50} (P-388) = 4.5 μg/mL
17	CC_{50} (P-388, HT-29) = 0.4, 1.1 μg/mL
18	CC_{50} (P-388, KB, A-549, HT-29) = 0.61, >50, >50, 6.96 μg/mL
19	CC_{50} (P-388, KB, A-549, HT-29) = 1.12, >50, >50, 1.79 μg/mL
20	CC_{50} (P-388, KB, A-549, HT-29) = 1.59, 24.45, 17.39, 10.07 μg/
21	CC_{50} (P-388, KB, A-549, HT-29) = 0.28, 0.27, 10.35, 8.27 μg/mL

CC_{50}: 50% cytotoxic concentration; P-388 (mouse lymphoid neoplasm), KB (human mouth epidermal carcinoma), A-549 (human lung adenocarcinoma), HT-29 (human colon adenocarcinoma) tumor cells.

inhibiting larval settlement could be increased as the exocyclic oxymethylene C-16 (such as —CH_2OH and —CH_2OCH_3 in **24**, **25**, and **26**) was substituted by a methylene-bearing chlorine (—CH_2Cl in **22** and **23**) and decreased as the exocyclic oxymethylene C-16 was esterified (—CH_2OAc in **27**) or the acetoxymethylene C-16 (—CH_2OAc in **29**) was oxygenated to be an esterified carboxyl group (—COOMe in **30**). Further comparison of the antifouling activities of compounds **22**–**27** also indicated that the chain lengths of esters at C-2, C-12, C-13, and C-14 could affect the potency of briarane-type diterpenoids. Moreover, compound **28** was more potent than compounds **29** and **30**,

which suggested that the exocyclic 11,20-epoxy group was important for the antifouling activities of briarane-type diterpenoids. The relationship between their structures and biofouling activity showed a certain consistency to that between their structures and cytotoxicity.



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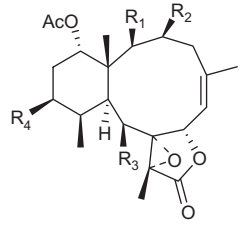
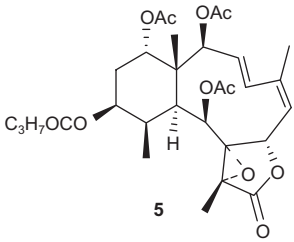


2. $R_1 = R_2 = R_3 = OAc$, $R_4 = OH$
3. $R_1 = R_4 = OH$, $R_2 = R_3 = OAc$
4. $R_1 = OAc$, $R_2 = H$, $R_3 = R_4 = OH$
6. $R_1 = R_2 = R_3 = OAc$, $R_4 = OCOC_3H_7$
7. $R_1 = R_2 = R_3 = R_4 = OAc$
8. $R_1 = R_3 = R_4 = OH$, $R_2 = OAc$



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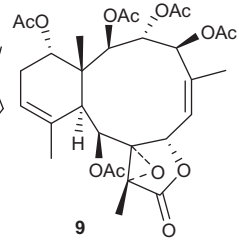

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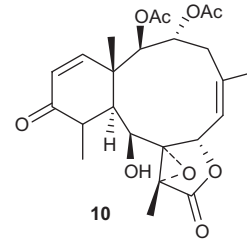

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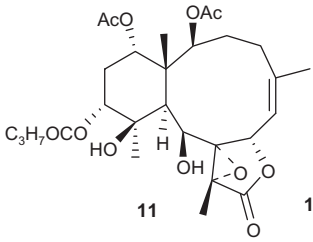

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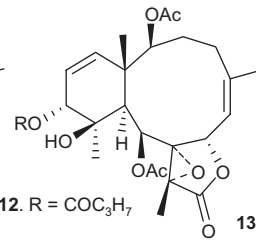
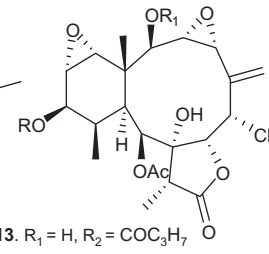
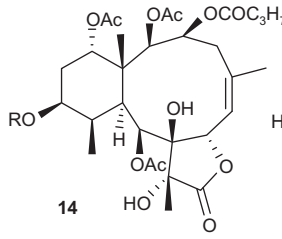


12. $R = COC_3H_7$



13. $R_1 = H$, $R_2 = COC_3H_7$



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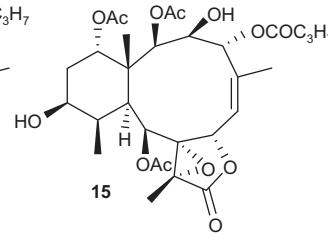

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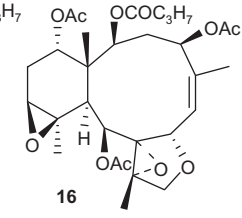

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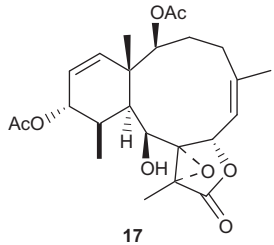

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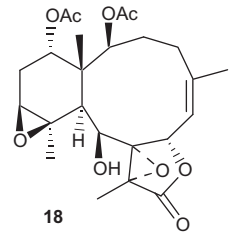

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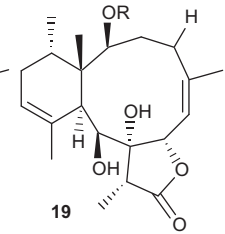

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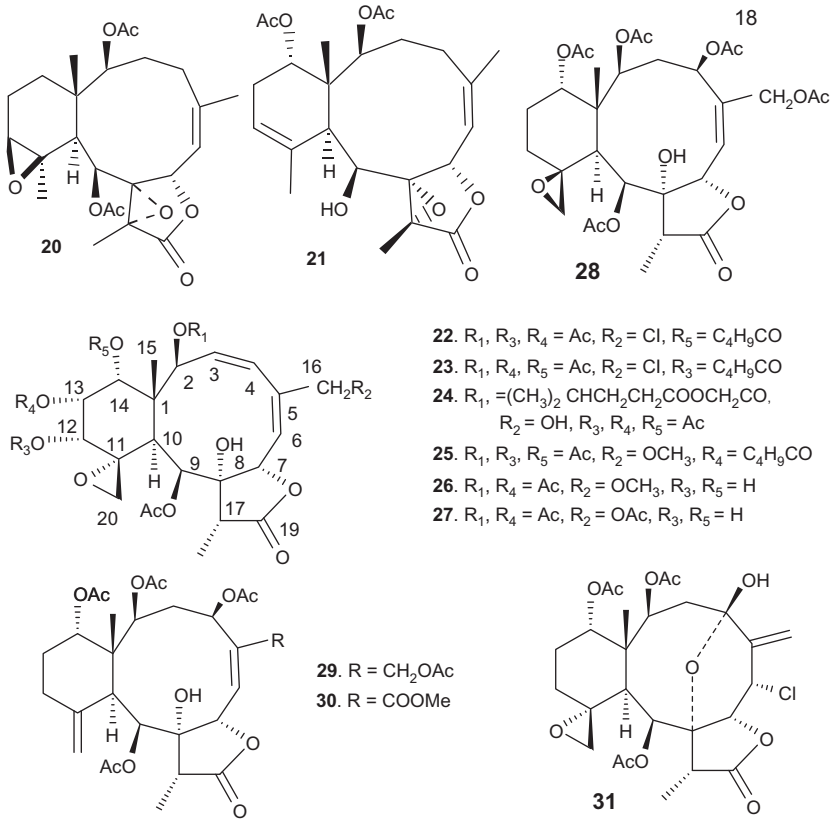


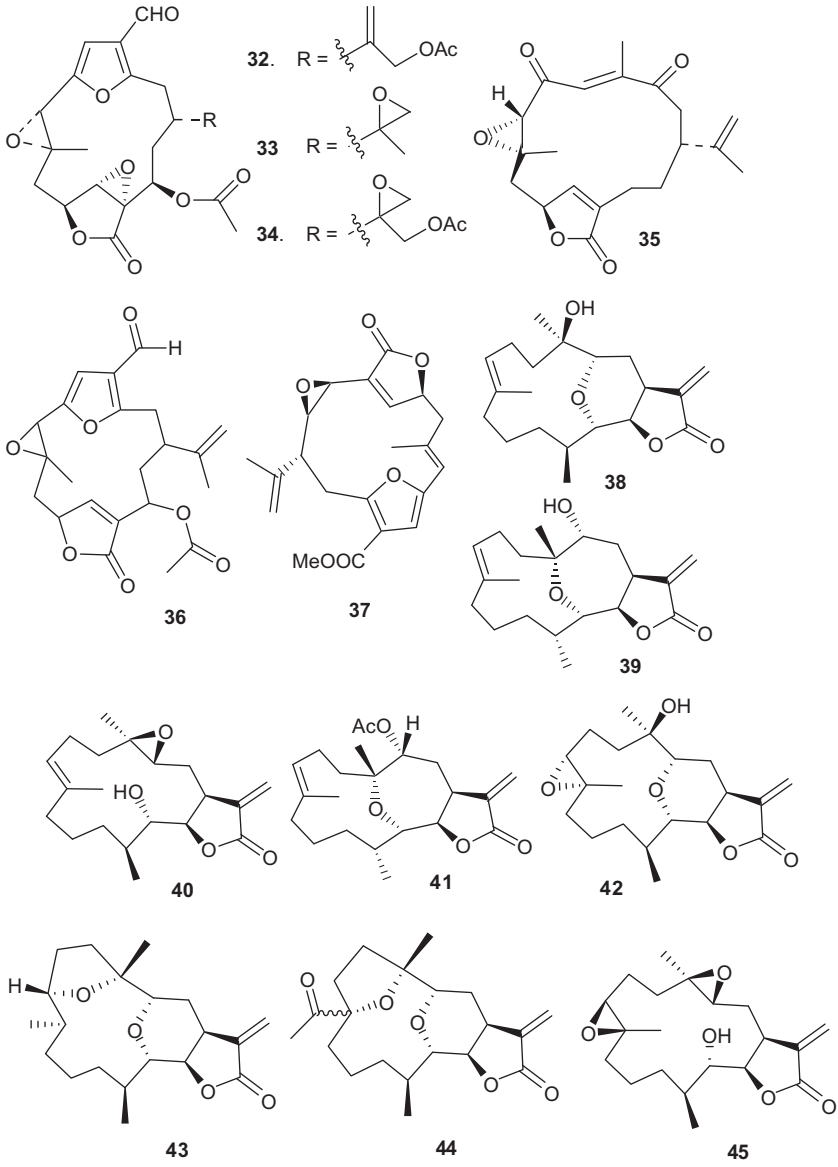
Structures of compounds **1–31**

Bioactive Cembrane-Type Diterpenes

Cembrane-type diterpenes and their cyclized derivatives are the most abundant and characteristic metabolites of gorgonian *Leptogorgia*, *Plexaura*, *Pseudoplexaura*, and especially *Eunicea* and *Pseudopterogorgia* genera. About 200 cembrane-type diterpenoids have been isolated from the 5 genera. Although these compounds seem to play an important role in chemical defense against other corals, fishes, or microorganisms, some cembrane derivatives also possess significant biomedical activities, including anti-inflammatory, Ca-antagonistic, antiparasitic, and, more frequently, cytotoxic properties. Because of their structural complexity and interesting biological activities, this type of compounds has attracted a great deal of attention from chemistry to synthesize their analogs [17].

Many cembrane-type diterpenoids showed significant cytotoxicity. 17-Acetoxylophotoxin (**32**), 15,16-epoxylophotoxin (**33**), 17-acetoxy-

15,16-epoxylophotoxin (**34**), isoepoxylophodione (**35**), and deoxylophotoxin (**36**) were isolated from *Leptogorgia peruana* [18]. Their *in vitro* cytotoxicity has been tested against three tumor cell lines MDA-MB-231 (human breast carcinoma), A-549, and HT-29. Among these compounds, **33–36** showed a significant activity against the three tumor cell lines with most of the CC_{50} [half maximal (50%) cytotoxicity concentration] values lower than 5.0 μM, and their LC_{50} (concentration that causes 50% cell killing) values were lower than 10 μM. Compound **35** was the most active with CC_{50} values of 2.7, 2.9, and 4.1 μM against MDA-MB-231, A-549, and HT-29, respectively. Compound **32** showed moderate cytotoxicity (usually CC_{50} value ranging from 10 to 50 μM means moderate cytotoxicity) against the three tumor cell lines with CC_{50} values of 14.5–17.9 μM. Acerolide (**37**) isolated from *Pseudopterogorgia acerosa* showed strong cytotoxicity against a panel of 14 tumor cell lines [DU-145 (prostate carcinoma), LN-caP (prostate carcinoma), IGROV (ovarian adenocarcinoma), SK-BR3 (breast adenocarcinoma), SK-MEL-28 (melanoma), A-549, K-562 (chronic myelogenous leukemia), PANC1 (pancreas carcinoma), HT-29, LOVO (colon adenocarcinoma), LOVO-DOX (colon adenocarcinoma resistant to doxorubicin), and HeLa (cervix epithelial adenocarcinoma)] with CC_{50} values lower than 10 μM [19]. 12-*epi*-Eunicin (**38**), 4-*epi*-jeunicin (**39**), and 13-*epi*-eupalmerin (**40**) isolated from *Eunicea mammosa* and their synthetic analogs (**41–45**) were studied *in vitro* with A-549, H116 (human colon carcinoma), PSN1 (human pancreatic adrenocarcinoma), and T98G (human Caucasian glioblastoma) tumor cells [20]. The results showed that **38–41**, **43**, and **44** had moderate cytotoxicity with CC_{50} values of 15.0–30.0 μM, and diepoxide **45** was the most active and selective against A-549, H116, and PSN1 with CC_{50} value of 1.3 μM. It was noteworthy that the synthetic analogs (e.g., **43** and **45**) displayed greater potency than the parent natural products. The introduction of cyclic ether linkages across the cembrane skeleton resulted in an enhancement of cytotoxic activity. Thus, compound **43** was 10 times more active against H116 and PSN1 tumor cells than **38** ($CC_{50} = 30.0$ μM in **38** to $CC_{50} = 3.0$ μM in **43**). Further, the cytotoxic activity present in **40** was significantly enhanced by the introduction of an extra epoxide functionality in **45** against H116 and PSN1 ($CC_{50} = 15.0$ μM in **40** to $CC_{50} = 1.3$ μM in **45**). These results corroborated the assessment that analogs of this series appeared to be attractive targets for the development of antitumor agents. Incubation of cell-free extracts of *E. mammosa* with ^{3}H-GGPP afforded radioactive labeling of all three metabolites, with significantly higher specific activity observed in **38**, indicating a possible central role of **38** in the biosynthesis of **40**.

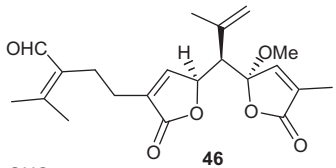


Structures of compounds **32–45**

In addition, some cembrane-type diterpenoids showed significant antiplasmodial and antituberculosis activities [21–24]. Caucanolide A (**46**) and caucanolide D (**47**) from *Pseudopterogorgia bipinnata* demonstrated significant

in vitro antiplasmodial activity against chloroquine-resistant *Plasmodium falciparum* W2 with IC_{50} (concentration that causes 50% inhibitory) values of 17 and 15 μg/mL, respectively. On the other hand, caucanolide A inhibited growth of human cancer cells T-47D (human breast epithelial tumor cell), CCRF-CEM (human leukemic lymphoblast cell), NCIH460 (human large-cell lung carcinoma line), and MCF-7 (human breast carcinoma) with IC_{50} values of 25.8, 25.5, 12.5, and 7.6 μg/mL, respectively, and when tested for *in vitro* antituberculosis activity against *Mycobacterium tuberculosis* H37Rv, caucanolide A marginally inhibited mycobacterial growth by 21% at a concentration of 6.25 μg/mL [21]. Bipinnapterolide B (**48**) isolated from *P. bipinnata* could cause 66% inhibition against *M. tuberculosis* H37Rv at 128 μg/mL [22]. Kallolide D (**49**), kallolide C acetate (**50**), and kallolides E–I (**51**–**55**) from *P. bipinnata* and *Pseudopterogorgia kallos* showed antimycobacterial active against *M. tuberculosis* at the two concentrations of 128 and 64 μg/mL with 6.6–49.2% inhibition [23]. Kallolides A, D, F–I also showed mild antiplasmodial activity toward *P. falciparum* with IC_{50} values of 30.6–83.3 μM. The significant difference in biological activity of compounds **49**–**55** indicated that neither the furan ring, the C-2 hydroxy, nor the γ-butenolide moiety alone appeared to play a pivotal role in the antiparasitic activity. Nevertheless, these results provided valuable data to suggest that the conformational nature and size of the carbocyclic ring was essential for antiplasmodial activity.

Bielschowskysin (**56**); bipinnatin E (**57**); and bipinnatins K, M, N, O, and Q (**58**–**62**) were isolated from *P. kallos* [24,25]. Bielschowskysin (**56**) exhibited antiplasmodial activity ($IC_{50} = 10$ μg/mL) when tested against *P. falciparum* and was found to display strong and specific *in vitro* cytotoxicity against the EKVX non-small-cell lung cancer ($CC_{50} < 0.01$ μM) and CAKI-1 renal cancer ($CC_{50} = 0.51$ μM) [24]. When tested against the National Cancer Institute's (NCI) 60-cell line cancer panel, bipinnatin Q exhibited CC_{50} values of 5.8, 4.2, 4.1, and 2.6 μM against the leukemia cell lines CCRF-CEM, MOLT-4 (human lymphocytic leukemia line), RPMI-8226 (human myeloma cell), and SR, respectively [25]. Bipinnatins E, K, M, N, O, and Q were evaluated as inhibitors of the acetylcholine-binding protein from *Aplysia californica* with IC_{50} values ranging from 0.23 to 0.83 μM. The most potent inhibitory diterpenoid in this series was bipinnatin E that had an IC_{50} value of 0.23 μM at the *A. californica* nicotinic acetylcholine receptor. It lacked a C-2 acetate group and possessed both the furanyl epoxide array and the α,β-unsaturated aldehyde moiety in the C-1 position. The present lophotoxin analogs could be useful probes for understanding the structure and function of neuronal and muscle nicotinic acetylcholine receptors, and their strong activity may also prove to be useful in a variety of clinical applications.



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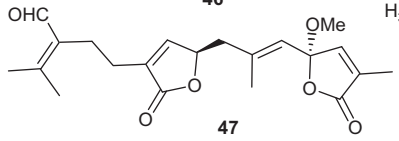

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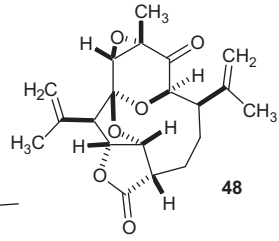

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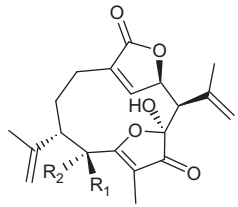
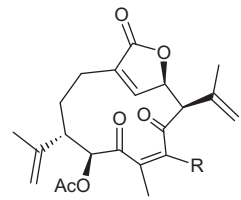
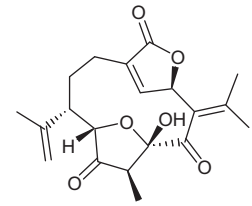


49. R_1 = OH, R_2 = H
51. R_1 = H, R_2 = OH



50. R = OH
54. R = H



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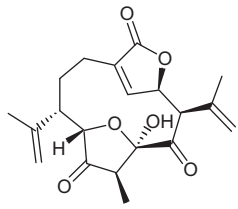

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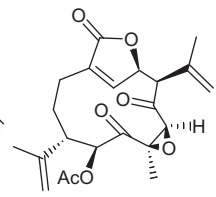

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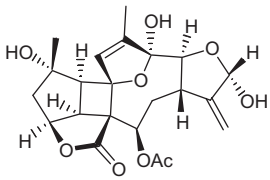

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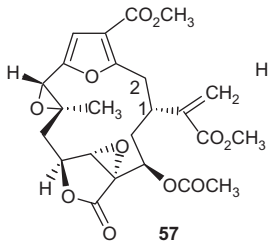

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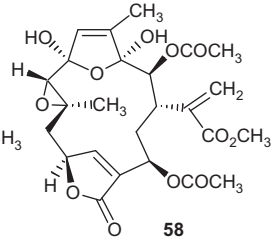

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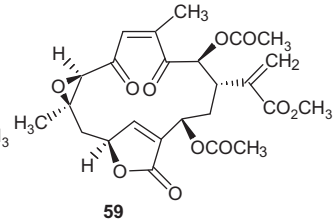

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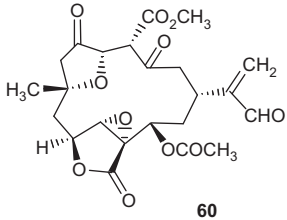

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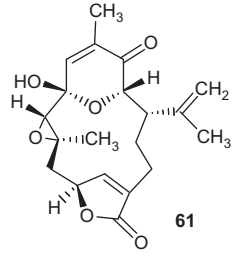

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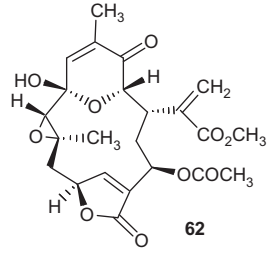


62

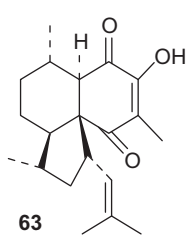
Structures of compounds **46–62**

Bioactive Pseudopterosin-Type Diterpenoids

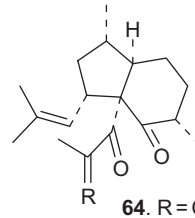
Pseudopterosin-type diterpenoid is the characteristic chemical constituent of gorgonian *Pseudopterogorgia* genus. About 90 pseudopterosin-type diterpenoids have been obtained from the genus, some of which showed cytotoxicity, antituberculosis, anti-inflammatory, antiviral, and antimalarial activities [26–30].

Elisabethins A–C (**63–65**), elisabanolide (**66**), pseudopteroxazole (**67**), secopseudopteroxazole (**68**), erogorgiaene (**69**), and 7-hydroxyerogorgiaene (**70**) were isolated from *Pseudopterogorgia elisabethae* [26–28]. Biological screening of **64** and **67** in the NCI 60-cell line tumor panel indicated that **64** had significant cytotoxicity with concentrations of 10^{-5} M, eliciting significant differential responses at the CC_{50} level from all the renal, CNS, and leukemia cancer cell lines, and **67** had no insignificant cytotoxicity. In an *in vitro* antituberculosis screen (against *M. tuberculosis* H37Rv), **65–69** caused 42%, 39%, 97%, 66%, and 96% inhibition at a concentration of 12.5 μg/mL, and **70** inhibited 77% of mycobacterial growth at a concentration of 6.25 μg/mL, which indicates that C-7 hydroxylation did not reduce the activity, and the benzoxazole moiety was not essential for the activity. In addition, **63** has been synthesized utilizing intramolecular [4 + 2] cyclization under biomimetic conditions [27].

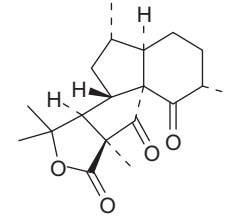
Elisabethadione (**71**); pseudopterosin N (**72**); secopseudopterosin E (**73**); and pseudopterosins P (**74**), Q (**75**), U–V (**76**, **77**) were also isolated from *P. elisabethae* [29–31]. Compounds **71**, **72**, and **73** exhibited 83%, 88%, and 88% inhibition in a mouse ear anti-inflammatory assay, respectively, and they exhibited potencies equal to or somewhat greater than pseudopterosin A and were significantly more potent when compared to pseudopterosin E computed [27]. Pseudopterosin P (**74**) exhibited the strongest inhibitory activity (76%) toward the growth of *M. tuberculosis* H37Rv at a concentration of 6.25 μg/mL [31] and also showed antiviral activity against herpes simplex viruses (HSV-1, HSV-2), human cytomegalovirus (HCMV), and varicella-zoster virus (VZV) with EC_{50} values of 2.9, 2.9, 2.9, and 2.6 μM [selectivity index (SI) values <2.4, <2.4, <3.6, and <3.2, respectively] [SI = the ratio of CC_{50} (50% cytotoxicity concentration) to IC_{50}], which exhibited pseudopterosin P to be very toxic against each virus. Compounds **74–77** also showed antimalarial activity against the *P. falciparum* W2 (chloroquine resistant) strain with IC_{50} values of 12, 15, 19, and 1 μg/mL, respectively [30].



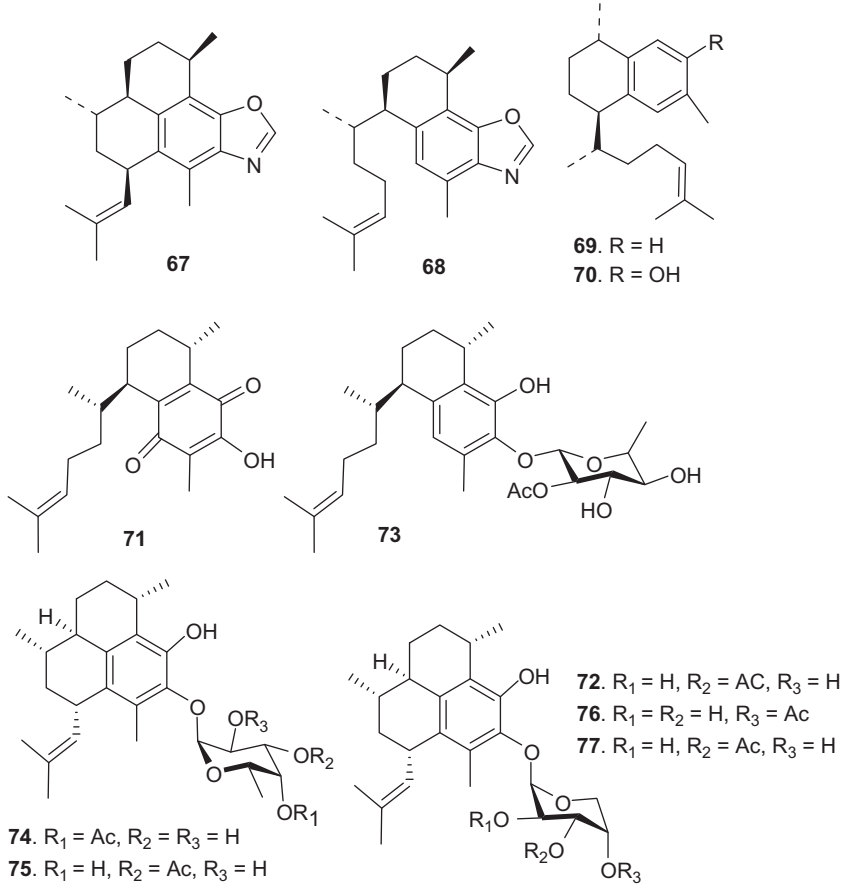
63



64. R = CH_3
65. R = H_2



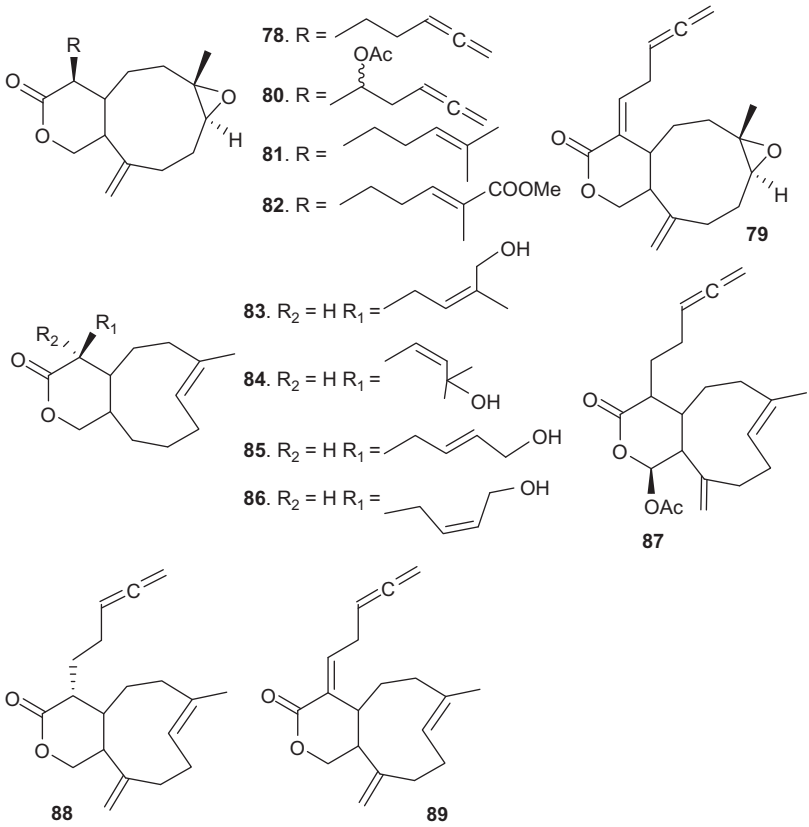
66



Structures of compounds **63–77**

Bioactive Other Diterpenes

Totally, over 20 xenicane-type diterpenes have been isolated from gorgonian *Acalycigorgia* and *Corallium* genera. Acalycixeniolides C–F (**78**–**81**) and acalycixeniolides H–L (**82**–**86**) isolated from *Acalycigorgia inermis* showed cytotoxicity toward lung cancer cell K-562 with CC_{50} values of 1.6, 5.2, 4.7, 0.2, 3.9, 1.2, 2.0, 1.8, and 1.5 μg/mL, respectively [32]. Ginamallene (**87**) and acalycixeniolides B′ and C′ (**88**, **89**) isolated from *Acalycigorgia* sp. were not only cytotoxic against P-388 leukemia cells with CC_{50} of 0.27, <2.5, and 2.5 μg/mL, respectively, but also active in the sea urchin *Hemicentrotus pulcherrimus* egg assay with IC_{50} values of 1.0, 15.0, and 50.0 μg/mL, respectively [33,34].

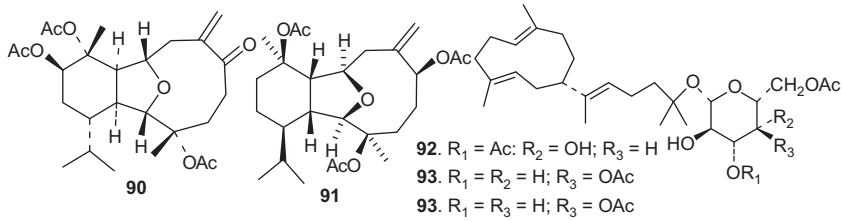


Structures of compounds **78–89**

Over 40 eunicellan-type diterpenes have been isolated from gorgonian *Acalycigorgia*, *Muricella*, *Briareum*, *Erythropodium*, and *Eunicella* genera; however, only two of which showed significant cytotoxicity. Labiatin B (**90**) from *Eunicella labiata* was active in inhibiting the growth of HCT-116 human colon tumor cells with CC_{50} of 0.85 μg/mL [35], and palmonine B (**91**) from *Eunicella verrucosa* was active against P-388 mice lymphoma and MEL28 human melanoma with CC_{50} value of 5 μg/mL [36].

Three dilophol-type diterpene glycosides calyculaglycosides A–C (**92–94**) were isolated from the Caribbean gorgonian *Eunicea* sp. [37]. Calyculaglycoside B was an effective topical anti-inflammatory agent stronger in potency than the industrial standard indomethacin. Calyculaglycoside B reduced arachidonic acid-induced mouse ear inflammation by 92% at a testing dose of 125 μg/ear and also inhibited edema induced by croton oil (a mixture of phorbol esters) at a level comparable with indomethacin (77% and 43% inhibition at 125 and

60 μg calyculaglycoside B/ear, respectively). Calyculaglycoside B inhibited the synthesis of both prostaglandin PGE2 and leukotriene LTB4, suggesting it was a nonselective inhibitor of the 5-lipoxygenase and cyclooxygenase pathways. At concentrations of 10^{-4}–10^{-5} M, calyculaglycoside B produced LC_{50}-level differential responses against a majority of the NCI ovarian cancer lines and several of the renal, prostate, and colon tumor lines. Calyculaglycoside C gave a similar pattern but required higher concentrations. Thus, the calyculaglycosides and related metabolites may be of interest for *in vivo* evaluation in appropriate xenograft tumor models.



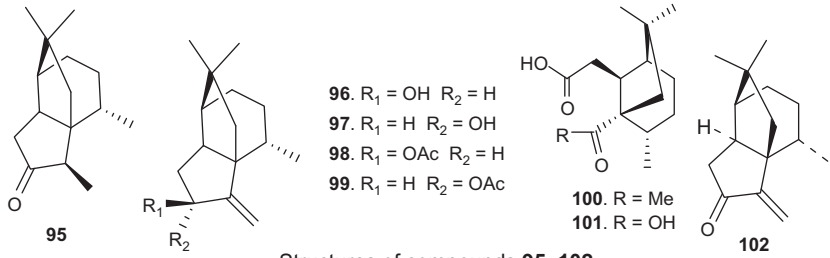
Structures of compounds **90–94**

Sesquiterpenoids

The representative structures of sesquiterpenoids by carbon skeleton class from gorgonians included sesquiterpene hydrocarbon, aromatic sesquiterpene, suberosane type, guaiane-furano type, caryophyllane type, acyclic sesquiterpenes, subergane type, etc. These sesquiterpenes exhibited many kinds of bioactivities, such as cytotoxicity, antifungi, antiplasmodial, antituberculosis, antiparasitic, anti-inflammatory, antimycobacterial, and antiviral activities [38–50].

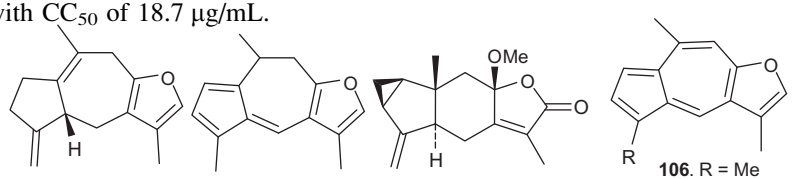
Suberosane-type sesquiterpene was the characteristic metabolites of gorgonian *Isis* genus. Suberosanone (**95**), suberosenols A and B (**96**, **97**), suberosenol A acetate (**98**), suberosenol B acetate (**99**), and two 4,5-secosuberosane sesquiterpenoids, isishippuric acids A and B (**100**, **101**) were isolated as cytotoxic components of *Isis hippuris* [38,39]. Compounds **95**–**99** and **101** were significantly cytotoxic against the proliferation of P-388, A-549, and HT-29 cancer cell lines with CC_{50} values $< 10^{-1}$–10^{-6} μg/mL, and the most active compound was **96** that contained a β-hydroxyl group at the allylic position of the 5,6-double bond, which suggested that the molecular skeleton, not the functionalities, was the main factor for the potent cytotoxicity of these suberosane terpenoids. Further, compound **98**, which was the acetyl derivative of **96**, was found to be less cytotoxic than **96**, revealing that the substitution might also influence the cytotoxicity of these compounds. The above results indicate that the suberosane-related compounds, both natural and synthetic [40], may warrant further biological studies for the discovery of the useful anticancer drugs in the future. The first enantioselective total synthesis of isishippuric acid B (**101**) bearing a novel 4,5-seco-6-norquadrane skeleton was accomplished from

(*R*)-citronellal with use of a Diels–Alder cycloaddition and an intramolecular Michael addition as the ring-forming steps [41]. Suberosenone (**102**), which was a relatively potent cytotoxic sesquiterpene from *Subergorgia suberosa* with particularly sensitive cytotoxicity toward ovarian, renal, and melanoma lines [42], has been synthesized as the corresponding racemate [43].



Structures of compounds **95–102**

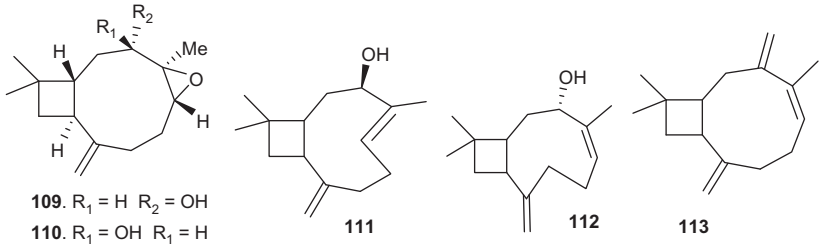
Three guaiane-furano-type sesquiterpenes, iso-echinofuran (**103**), 8,9-dihydro-linderazulene (**104**), and echinofuran isolated from *Echinogorgia complexa*, displayed a moderate activity for the inhibition of the integrated electron transfer chain (nicotinamide adenine dinucleotide phosphate-oxidase (NADH) activity) in beef heart submitochondrial particles with IC_{50} values of 4.3 ± 0.15, 2.5 ± 0.04, and 2.2 ± 0.31 μM, respectively, whereas full inhibition of rotenone-sensitive NADH oxidase activity was achieved at approximately 2 μM [44]. Heterogorgiolide (**105**) from *Heterogorgia uatumani*, at natural volumetric concentrations (0.8 mg/cm^3), significantly inhibited feeding relative to controls with high statistical significance. [45]. Linderazulene (**106**) and two congeners (**107** and **108**) were isolated as mildly cytotoxic constituents of a deep sea collection of the gorgonian *Paramuricea* sp. [46]. Compounds **106–108** show moderate *in vitro* cytotoxicity against the P-388 murine leukemia cell line with CC_{50} of 18.8, 2.7, and 15.6 μg/mL, and **107** also showed moderate activity against the PANC1 pancreatic tumor cell line with CC_{50} of 18.7 μg/mL.



Structures of compounds **103–108**

Over 10 caryophyllane-type sesquiterpenes have been isolated from gorgonian *Rumphella* and *Subergorgia* genera. Among them, suberosols A–D (**109–112**) and buddledin C (**113**) were isolated from *S. suberosa* [47]. Suberosols A–D exhibited significant cytotoxicity against P-388 cancer cells with

CC_{50} values of 3.8, 7.4, 2.1, and 3.3 μg/mL, respectively. Suberosol D and buddledin C exhibited significant cytotoxicity toward A-549 cancer cells with CC_{50} values of 4.2 and 3.8 μg/mL, respectively. Suberosols C and D, and buddledin C were found to exhibit significant activity against the growth of HT-29 cells with CC_{50} values of 2.3, 3.8, and 3.6 μg/mL, respectively.

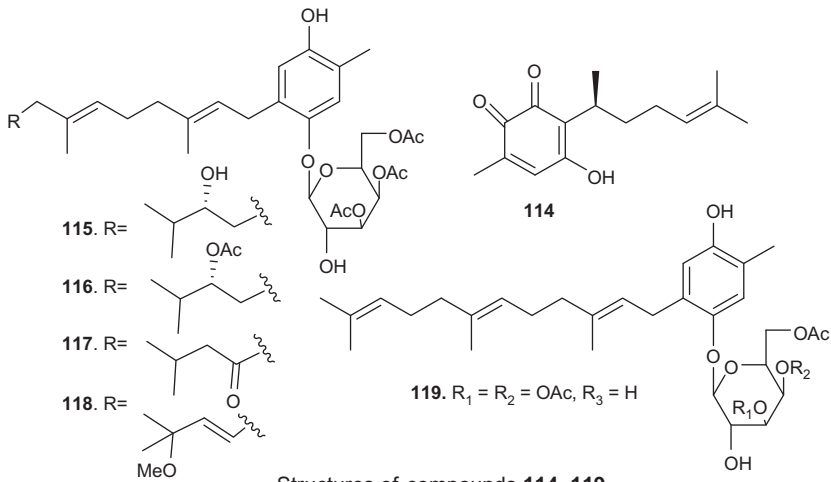


109. R_1 = H R_2 = OH
110. R_1 = OH R_1 = H

111 **112** **113**

Structures of compounds **109–113**

An aromatic sesquiterpene, rigidone (**114**), was isolated from *Pseudopterogorgia rigida* as an inhibitor of macrophage scavenger receptors with IC_{50} value of 5.6 μM [48]. Eight farnesylhydroquinone derivatives euplexides A–E (**115**–**119**) isolated from *Euplexaura anastomosans* showed significant cytotoxicity against the human leukemia cell line K-462 with CC_{50} values of 2.6, 3.1, 5.2, 8.1, and 9.4 μg/mL, respectively [49,50]. At the concentration of 10 μg/300 μL, euplexides A–C and E also displayed antioxidizing activity of 3.42, 3.56, 3.45, and 3.13 times, respectively, higher than that of superoxide dismutase. In addition, euplexides A and B exhibited 52% and 71%, respectively, inhibition of phospholipase A2 (PLA_2) at the concentration of 50 μg/mL.



115. R=
116. R=
117. R=
118. R=

114

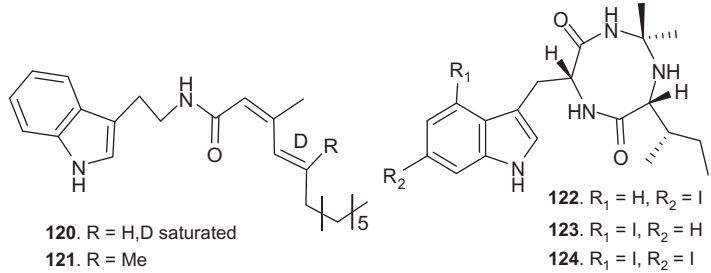
119. R_1 = R_2 = OAc, R_3 = H

Structures of compounds **114–119**

Nitrogen-Containing Compounds

Nitrogen-containing compounds contain only about 2.2% of the metabolites from gorgonians, of which the bioactive compounds included tryptamine derivatives, purine alkaloids, and amide derivatives [51–59].

Granulatamides A and B (**120**, **121**) isolated from *Eunicella granulate* showed strong or moderate cytotoxicity against a panel of 16 human tumor cell lines [for **120**, CC_{50} (DU-145, LN-caP, SK-OV-3, IGROV, IGROV-ET, SK-BR3, SK-MEL-28, HMEC1, A-549, K-562, PANC1, HT-29, LOVO, and LOVO-DOX) = 1.7, 4.7, 11.6, 6.7, 12.7, 2.7, 3.9, 6.2, 6.7, 6.8, 10.4, 2.2, 10.5, and 12.0 μM, respectively; for **121**, CC_{50} (DU-145, LN-caP, IGROV, IGROV-ET, SK-BR3, SK-MEL-28, A-549, K-562, PANC1, HT-29, LOVO, LOVO-DOX, HeLa, and HeLa-APL) = 7.7, 3.5, 8.2, 6.7, 6.0, 10.6, 8.9, 4.3, 6.5, 13.8, 8.6, 10.0, 9.7, and 9.0 μM, respectively] [49]. Novel eight-membered heterocycles, named hicksoanes A–C (**122–124**) isolated from *Subergorgia hicksoni*, showed antifeeding activity against goldfish at natural concentration (≈10 μg/mL) [52].

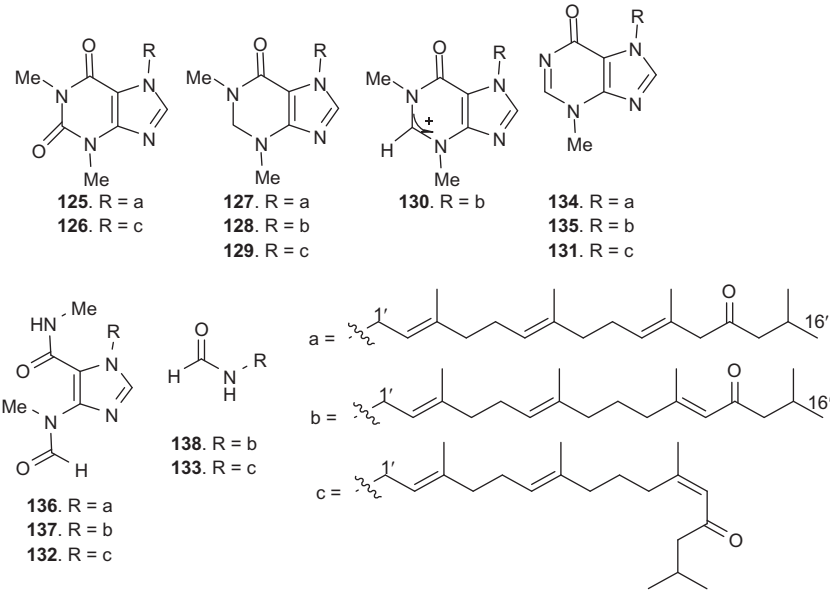


120. R = H,D saturated
121. R = Me

122. R_1 = H, R_2 = I
123. R_1 = I, R_2 = H
124. R_1 = I, R_2 = I

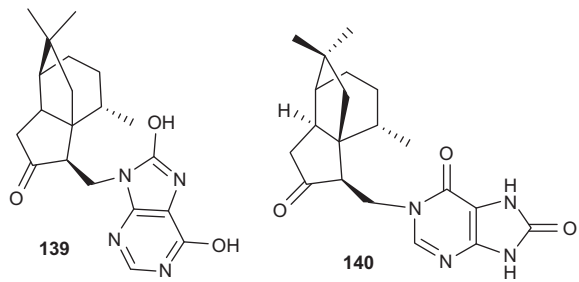
Structures of compounds **120–124**

Six tetraprenylated purine alkaloids, nuttingins A–F (**125–130**), and eight malonganenones A–H (**131–138**) were isolated from *Euplexaura nuttingi* [53]. Compounds **131–138** were mildly cytotoxic against six esophageal cancer cell lines (WHCO1, WHCO5, WHCO6, KYSE70, KYSE180, and KYSE520) and the nonmalignant breast epithelial cell line MCF-12 with $CC_{50} > 17.0$ μM [54]. Nuttingins A–E (**125–129**) and malonganenones D–G (**134–137**) could inhibit growth of K-562 and UT723 cells. The tests were done on mixtures of compounds **125–126**, **127–130**, **134–136**, and **137–138**, as there was no real difference between the activities of compounds differing only in the side chain. At 0.4 μg/mL, compounds **127–130** induced 50% inhibition of cell growth in UT723 cells and 30% in K-562 cells, after 48 h of exposure to the compounds. Compounds **125–126**, **134–136**, and **136–137** also displayed inhibitory activity on proliferation of both cell lines, although they were approximately threefold less potent than compounds **127–130** [53].



Structures of compounds **125–138**

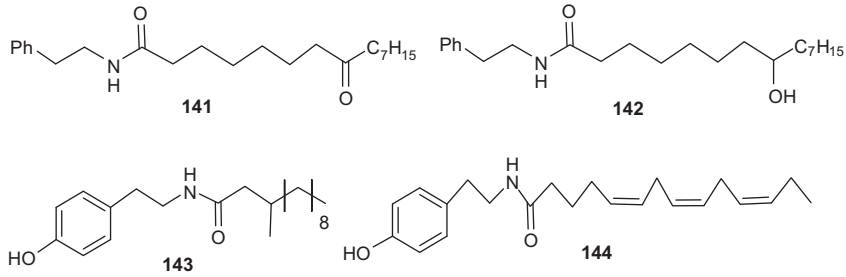
Two isomeric purine alkaloids, 6-(9′-purine-6′,8′-diolyl)-2β-suberosanone (**139**) [55] and 6-(1′-purine-6′,8′-dionyl)-suberosanone (**140**) [56], were obtained from *S. suberosa*. Compound **139** showed moderate cytotoxicity against human breast carcinoma MDA-MB-231 cell line with an CC_{50} of 8.87 μg/mL [55], while **140** showed weak cytotoxicity [56], which suggested that the location of the connection between the 6′,8′-purinedione moiety and the suberosanone moiety in the isomers could significantly affect their cytotoxic activity.



Structures of compounds **139–140**

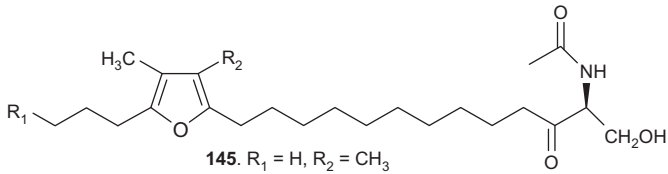
Amides **141** and **142** isolated from *Telesto riisei* showed cytotoxic toward P-388 with CC_{50} values of 2.1 and 2.2 μg/mL, respectively [57]. Two new tyramines (**143** and **144**) isolated from *Muricea austere* and a series of synthetic tyramine derivatives were evaluated *in vitro* against a drug-resistant

P. falciparum and intracellular form of *Trypanosoma cruzi* [58]. Compounds **143–144** showed moderate antiplasmodial activity against chloroquine-resistant *P. falciparum*, with IC_{50} values between 11 and 38 μg/mL. Variations in the structure and activity of the natural and synthetic tyramides suggested that increasing the number of carbons of the fatty acid chain produced an increase in potency, while the presence of polar groups on the fatty acid chain decreased the potency. On the other hand, the introduction of bromine atoms on the tyramine aromatic ring produced an increase in the antiplasmodial activity, and the change in the position of the amide bond also produced an increase in antimalarial activity.



Structures of compounds **141–144**

Calicogorgins A, B, and C (**145–147**), three novel sphinganine derivatives possessing lethal and repellent activities against the muricid gastropod *Drupella fragum* were isolated from *Calicogorgia* sp. [59]. They exhibited 100% mortality in the snail at 30 ppm within 24 h and showed a marked activity to the snail at 45 μg/cm^2 for repellent activity.



145. R_1 = H, R_2 = CH_3
146. R_1 = CH_3CH_2, R_2 = CH_3
147. R_1 = CH_3CH_2, R_2 = H

Structures of compounds **145–147**

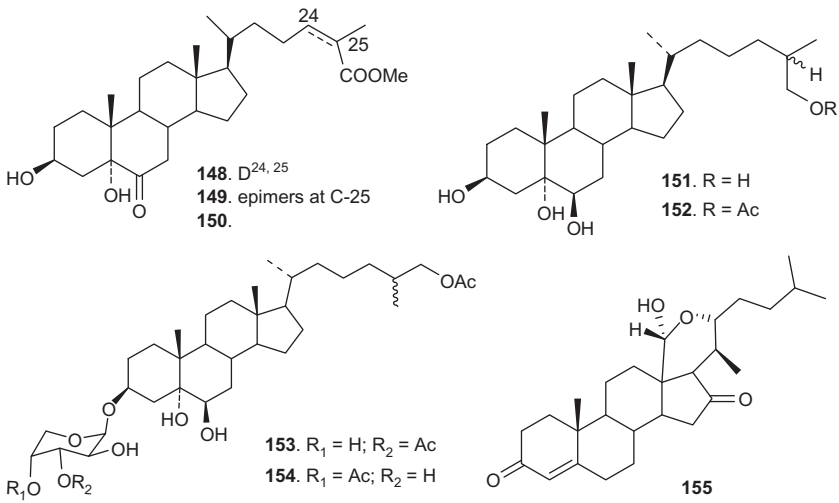
Steroids

Gorgonians have proven to be a natural source of polyoxygenated sterols with unprecedented structures that contain about 16% of the metabolites from gorgonians. In particular, the gorgonian *Isis* genus is unique in its steroidal constituents: structures having both polyoxygenation and the side chain variation as hippurin or hippuristanol type having a spiroketal, gorgosterol type possessing a cyclopropane, and hippuristerone type containing a 3-keto function.

These types also differ in the side chain alkylation pattern. Some of these steroids have been reported to have significant antitumor activity [60,57,61–73].

Bioactive Mono- and Polyhydroxylated Steroids

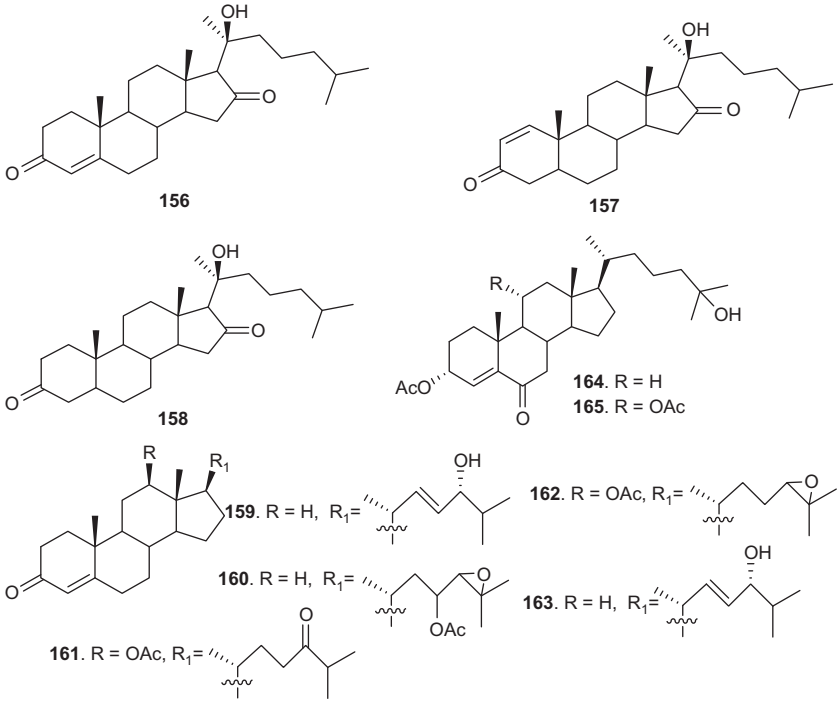
Sterols (**148–150**) isolated from *A. inermis* displayed moderate cytotoxicity against the human leukemia cell line K-562 with CC_{50} values of 1.1, 0.9, and 9.7 μg/mL, respectively [60]. Cholestane-3β,5β,6β,26-tetrol (**151**) and the corresponding 26-acetate **152** isolated from *T. riisei* were cytotoxic to P-388 cells with CC_{50} values of 2.4 and 1.3 μg/mL, respectively [57]. Riiseins A **153** and B **154** were isolated from *T. riisei* with cytotoxicity toward HCT-116 human colon adenocarcinoma with CC_{50} values of 2.0 μg/mL [61]. A mixture of isomeric hemiacetals (**155**) (22-hydroxy-3,16-dioxocholest-5-en-18-ol) isolated from *Ctenocella* sp. showed cancer cell antiproliferative properties ($CC_{50} = 0.23$ μg/mL on KB cells, $CC_{50} = 2.9$ μg/mL on NSCLCN6 cells) [62].



Structures of compounds **148–155**

Three cytotoxic steroids, (20*S*)-20-hydroxycholest-4-ene-3,16-dione (**156**), (20*S*)-20-hydroxycholest-1-ene-3,16-dione (**157**), and (20*S*)-20-hydroxycholestane-3,16-dione (**158**), were obtained from *Leptogorgia sarmentosa* [63]. Compounds **156–158** showed significant, although nonselective, cytotoxicity against P-388 suspension culture of mouse lymphoid neoplasm and the monolayer cultures of A-549 human lung carcinoma, HT-29 human colon carcinoma, and MEL28 human melanoma exhibiting CC_{50} values of 1 μg/mL. Using tigogenin as starting material, **156–158** were synthesized in four steps [64]. Seven polyoxygenated steroids (**159–165**) from *Dasystenella acanthina* showed significant activities as growth inhibitors of several human tumor cell

lines (DU-145, LN-caP, IGROV, SK-MEL-28, K-562, PANC1, HT-29, LOVO). The higher levels of activity were observed against the cell lines LN-caP and K-562, with most of the compounds displaying CC_{50} values lower than 2 μg/mL [65]. In addition, **159–165** showed the cytostatic effects with all the compounds being active against the cell lines LN-caP and K-562 with TGI_{50} values of 3.4–9.9 μg/mL. Compounds **164** and **165** presented the broader range of activity with significant cytostatic effects on most of the cell lines. Especially, **165** was active against DU-145, LN-caP, IGROV, SK-MEL-28, K-562, PANC1, and LOVO cell lines with LC_{50} values of 7.3–9.9 μg/mL.



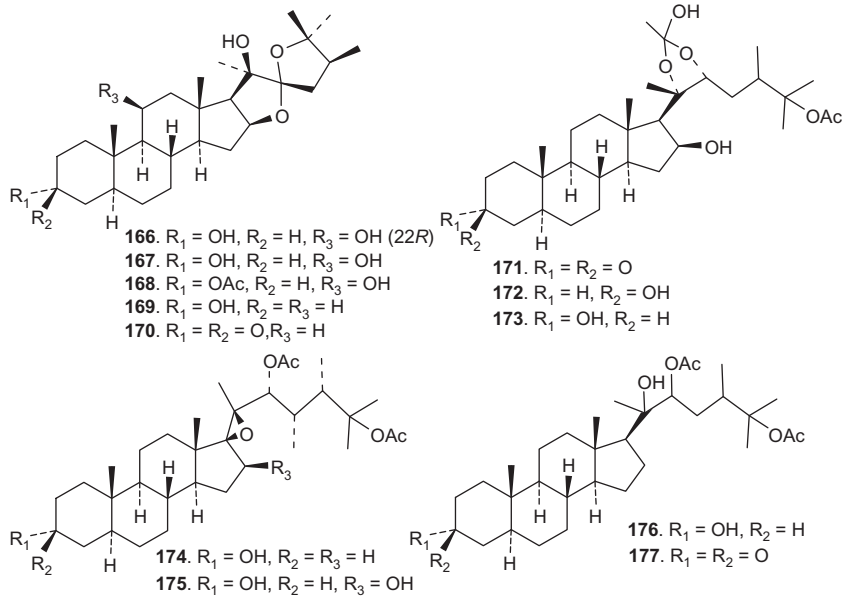
Structures of compounds **156–165**

Twelve polyoxygenated sterols, hippuristanol (**166**), 22-*epi*-hippuristanol (**167**), 3-acetyl-22-*epi*-hippuristanol (**168**), 11-dehydroxy-22-*epi*-hippuristanol (**169**), 11-dehydroxy-22-*epi*-hippuristan-3-one (**170**), orthohippurinsterone A (**171**), orthohippurinsterol A (**172**), orthohippurinsterol B (**173**), hippurinsterol B (**174**), hippurinsterol A (**175**), hippurinsterol D (**176**), and hippurinsterone D (**177**), were isolated from *I. hippuris* [66]. These compounds have a range of cytotoxic activities against P-388, A-549, HT-29, and MEL28 cell lines with CC_{50} values from 5 to 0.1 μg/mL (Table 2). The steroids bearing a spiroketal ring were more active than those without this feature, with

TABLE 2 Cytotoxicity of Compounds 166–177

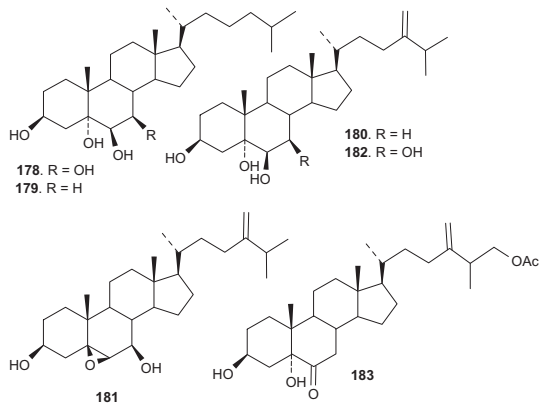
Compounds	Cytotoxicity
166	CC_{50} (P-388, A-549, HT-29, MEL28) = 0.1, 0.1, 0.1, 0.1 μg/mL
167	CC_{50} (P-388, A-549, HT-29, MEL28) = 0.1, 0.1, 0.1, 0.1 μg/mL
168	CC_{50} (P-388, A-549, HT-29, MEL28) = 1, 0.125, 0.5, 0.125 μg/mL
169	CC_{50} (P-388, A-549, HT-29, MEL28) = 5, 5, 5, 5 μg/mL
170	CC_{50} (P-388, A-549, HT-29, MEL28) = 5, 5, 5, 5 μg/mL
171	CC_{50} (P-388, A-549, HT-29, MEL28) = 2.5, 5, 5, 5 μg/mL
172	CC_{50} (P-388, A-549, HT-29, MEL28) = 2.5, 5, 5, 5 μg/mL
173	CC_{50} (P-388, A-549, HT-29, MEL28) = >10, 5, 1, >10 μg/mL
174	CC_{50} (P-388, A-549, HT-29, MEL28) = 1.25, 1.25, 1.25, 1.25 μg/mL
175	CC_{50} (P-388, A-549, HT-29, MEL28) = 1, 1, 1, 1 μg/mL
176	CC_{50} (P-388, A-549, HT-29, MEL28) = 1, 1, 1, 1 μg/mL
177	CC_{50} (P-388, A-549, HT-29, MEL28) = 1, 1, 1, 1 μg/mL

hippuristanol and 22-*epi*-hippuristanol, being the most active. Among the spiroketal steroids, several conclusions can be reached. First, these compounds can be divided into rings A and C: (i) class A contains hippuristanol, 22-*epi*-hippuristanol, 3-acetyl-22-*epi*-hippuristanol (**168**), and 3,11-diacetyl-22-*epi*-hippuristanol with two oxygenated positions at C-3 and C-11; (ii) class B consists of 3-acetyl-2α-hydroxy-22-*epi*-hippuristanol and 3,2-diacetyl-22-*epi*-hippuristanol with three oxygenated positions at C-2, C-3, and C-11; and (iii) class C covers 11-dihydroxy-22-*epi*-hippuristanol (**169**) and 11-dihydroxy-22-*epi*-hippuristan-3-one (**170**) with one oxygenated position at C-3. In class A, the C-22 epimerization does not influence the cytotoxic activity (epimers hippuristanol and 22-*epi*-hippuristanol have the same level of activity). Acetylation of the OH at C-3 (**168**) decreases the activity but increases the cytotoxic selectivity and, finally, diacetylation leads to complete loss of activity. Compounds belonging to class B are inactive as either the monoacetylated (3-acetyl-2α-hydroxy-22-*epi*-hippuristanol) or the diacetylated (3,2-diacetyl-22-*epi*-hippuristanol) derivatives. On the other hand, for compounds in class C, the oxidation level at C-3, that is, the hydroxyl group (**169**) or ketone (**170**), does not influence the activity. It is worth noting that *epi*-hippuristanol is being patented as a cytotoxic compound. The data on the structure–biological activity relationships in these compounds could be very valuable in the future synthetic studies [66].



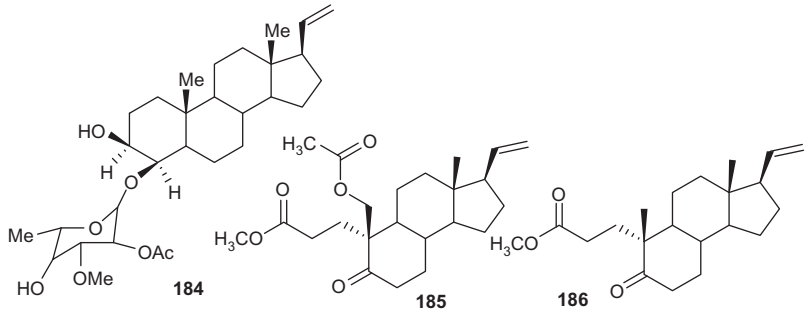
Structures of compounds **166–177**

Six cytotoxic steroids, cholestane-3β,5α,6β,7β-tetrol (**178**), cholestane-3β, 5α,6β-triol (**179**), ergost-24(28)-ene-3β,5α,6β-triol (**180**), 5β,6β-epoxyergost-24(28)-ene-3β,7β-diol (**181**), ergost-24(28)-ene-3β,5α,6β,7β-tetrol (**182**), and an unseparable 1:1 mixture of the epimers (25*R*) and (25*S*)-26-acetoxy-3β,5α-dihydroxyergost-24(28)-en-6-one (**183**), were isolated from *Plexaurella grisea* [67]. Compounds **178** and **179** were active against the A-549 and HT-29 cell lines with CC_{50} values of 1 μg/mL, and **180**–**183** exhibited strong and selective cytotoxicity against the HT-29 cell line with CC_{50} values of 0.1, 0.25, and 0.1 μg/mL, respectively.



Structures of compounds **178–183**

Verrucoside, a cytotoxic pregnane glycoside 4β-*O*-[2-*O*-acetyl-α-L-digitalopyranosyl]-5β-pregn-20-en-3β-ol (**184**) isolated from *E. verrucosa*, had cytotoxicity toward P-388, A-549, and HT-29 with IC_{50} values of 5.9, 7.2, and 6.3 μg/mL, respectively [68]. Two degraded pregnanes muricenones A and B (**185**, **186**) from *Muricea* sp. show a significant and selective activity as inhibitors of the growth of A-549 cells with CC_{50} values of 2.0 and 3.0 μg/mL, respectively [69].



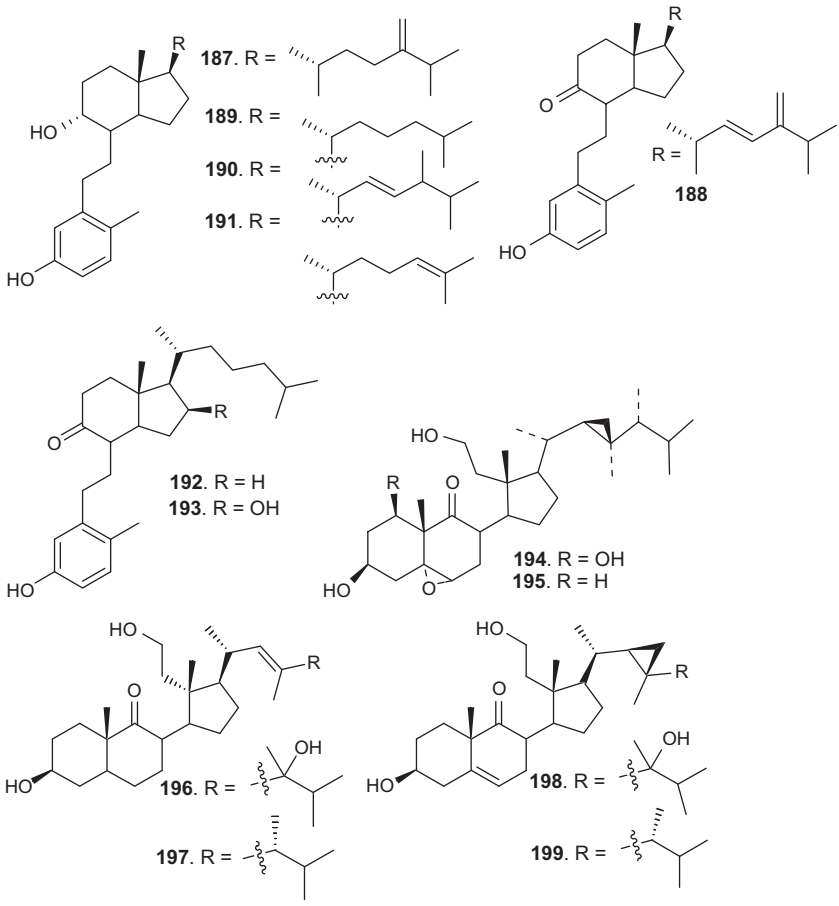
Structures of compounds **184–186**

Bioactive Secosterols

Calicoferols C–I (**187–193**) were isolated from *Muricella* sp., and compounds 190, 191, and 193 had obvious cytotoxicity toward A-562 with CC_{50} values of 3.2, 2.1, and 9.6 μg/mL, and calicoferols G and H showed media inhibition toward PLA_2 [70,71]. Two secosterols, 1β,3β-dihydroxy-5α,6α-epoxy-9-oxo-9,11-secogorgostan-11-ol (**194**) and 3β-hydroxy-5α,6α-epoxy-9-oxo-9,11-secogorgostan-11-ol (**195**) isolated from *Pseudopterogorgia americana*, exhibited significant cytotoxic activity against prostate cancer (LN-caP, $CC_{50} = 15.49$, and 11.0 μg/mL, respectively) and lung cancer cell lines (Calu-3, $CC_{50} = 18.43$ and 12.0 μg/mL, respectively), which suggested that the epoxide ring in their structures was partly responsible for the observed activity [72].

Protein kinase C (PKC) assay-guided study of the extracts of *Pseudopterogorgia* sp. led to the isolation of four 9,11-secosterols **196–199** [73]. Secosterols **196–198** exhibited moderate inhibitory activity against human PKC enzymes α, BI, BII, γ, δ, ε, η, and ζ with IC_{50} values in the range of 12 to >50 μM. Since PKC has been implicated in both inflammatory and proliferative processes, secosterols **195–198** were tested in cells related to these processes. All the compounds inhibited MCF-7 human breast carcinoma proliferation as measured by tritiated thymidine (3H-T) incorporation in the range of 3–13 μM and were even more potent against proliferation of normal human epidermal keratinocytes in the 0.6–5.0 μM range. Direct cytolethality as measured by MTT formazan dye reduction was 10- to 20-fold higher than antiproliferative activity for all compounds, suggesting cellular activity may

be due to a block in cell cycle progression rather than due to a directly cytolethal event.



Structures of compounds **187–199**

CONCLUSION

Gorgonian metabolites possess novel structures that are largely unknown from terrestrial sources. Totally, over 1000 new compounds have been obtained from the worldwide gorgonian corals. Among these compounds, 199 bioactive compounds were reviewed in the chapter with emphasis on their structures, relevant biological activities, and structure–activity relationships, covering diterpenes, sesquiterpenes, nitrogen-containing compounds, and steroids. Over half of these bioactive compounds showed potent cytotoxicity toward a series of tumor cells, specially, diterpenes (briarane type, eunicellan type, dilophol type, and xenicane type) and sesquiterpenes (guaiane-furano type

and suberosane type) appeared to be attractive targets for the development of antitumor agents. In addition, some diterpenoids (cembrane type, dilophol type, and pseudopterosin type) exhibited significant anti-inflammatory, antiplasmodial, antituberculosis, or antiviral activities. A selected few of them have already found application as probes for new mechanisms of action and may be found under development as pharmaceutical agents (i.e., the pseudopterosins are currently in clinical application for topical skin diseases). These natural products isolated from the marine gorgonian corals provide a rich source of biologically active compounds of medicinal importance; even more importantly, they provide models on which to base extensive synthetic programs leading to still more efficacious pharmaceuticals.

ACKNOWLEDGMENTS

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ABBREVIATIONS

CC_{50}	half maximal (50%) cytotoxicity concentration
EC_{50}	50% effective concentration
IC_{50}	concentration that causes 50% inhibitory
LC_{50}	concentration that causes 50% cell killing
SI	selectivity index, the ratio of CC_{50} to IC_{50}

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Chapter 13

Natural Herbicides as a Safer and More Environmentally Friendly Approach to Weed Control: A Review of the Literature Since 2000

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INTRODUCTION

Before starting a review about natural weed control efforts, it is important to first address the definition of a "weed." There is no universal definition that it is accepted by all scientists[1]. Generally speaking, a weed is an undesirable plant growing where it is not wanted. This is the same definition used in 1967 by the Weed Science Society of America: "a plant growing where it is not desired." In 1989, this definition was changed to "any plant that is objectionable or interferes with the activities or welfare of man." The European Weed Research Society used the following definition: "any plant or vegetation, excluding fungi, interfering with the objectives or requirements of people"[1]. All of these definitions clearly express the same idea, but unfortunately, they have a major drawback in that man must interpret what is truly harmful or unwanted. The same plant may not be considered a weed by different people, that is, many plants used as fodder are considered useful by breeders, but they are weeds when they invade cultivated fields. Dandelion (*Taraxacum officinale* L., Asteraceae) may be an annoying weed for the farmer, but at the same time, a useful medicinal plant for the herbalist[2,3].

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AGRICULTURE AND WEEDS

Plants are universally considered weeds when they interfere with a crop plant in cultivation. Both weeds and cultivated plants are well adapted to grow in disturbed habitats such as tilled fields, but the former tend to compete with the latter causing various problems for their cultivation. Most of the problems arise from characteristics of weeds, in particular their very rapid growth and maturation, as well as ease of pollination and reproduction. Furthermore, many of them are capable of easy adaptation and resistance to adverse climatic and edaphic conditions, have seeds resistant to decay, and can remain dormant for long periods in soil. These characteristics enable weeds to efficiently compete with crops for nutrients, light, and water[1]. No recent accurate estimate is available for each country of the costs due to weed control and reduction in crop yields. However, their impact can be globally quantified in the range of hundreds of millions of dollars per year[4–7]. It has been calculated that when plant pests and weeds are not systematically controlled, about 40% of a typical crop is lost preharvest and an additionally 20% is lost postharvest[8].

Before the introduction of herbicides (or organic farming), weeds were controlled by mechanical or cultural means, that is, harrows, hoes, weeders, tillage, crop rotation, etc. However, these serve as effective control methods for a limited number of weeds, usually annual ones. Perennial weeds are quite tolerant to traditional mechanical control methods and even to the most modern ones. In some cases, mechanical methods may lead to the spread of some pest species[9,10]. Even if these methods have not been abandoned after the discovery of substances capable of interfering with the growth of weedy plants, when these molecules has become widely available, the chemical control of weeds has become more and more dependent on herbicides. The chemical control of weeds has as a main advantage in both its simplicity and rapidity with respect to the other weeding methods. At present, the control of weeds is mainly based on the use of effective herbicides; for this reason, the lack of effective molecules sometimes prevents their control.

The use of commercial active principles poses many problems; in addition to the resistance shown by some plant species, the environmental damage and the toxicity of many synthetic herbicides represent the main problems and strongly limit their use. The emergence of resistance to herbicides is a serious challenge faced by modern farmers[11–14]. Perhaps it is the simplicity of treating weeds with very effective substances on a routine basis and the proven cost-effective gains in productivity that will accrue in the short term that has led to the predominance of synthetic herbicides. Broadly speaking, resistance is the ability of the weed to survive and reproduce when exposed to doses of herbicide that would normally kill plants of the same species and growth stage. A dramatic increase in the number of species showing resistance has been observed during the past 20 years. More than 300 species have

evolved resistance to one or more of all the major synthetic groups of herbicides, among which resistance to glyphosate is the greatest concern[14]. Resistance is inherited, so survivors of the treatment pass genes for resistance on to their offspring[15–17]. These genes are initially rare in the population, arising as rare mutations in genes, but as selection continues, the proportion of resistance genes in the population increases and so does the proportion of resistant individuals. Furthermore, detoxification and translocation processes within the plant organs can contribute to herbicide effectiveness and/or resistance (nontarget site). Herbicide susceptibility is a resource that needs to be preserved using appropriate methods of weed management. In some cases, susceptibility of some plant species to certain herbicides has been lost forever. One approach to this problem is to develop new susceptibilities by using novel molecules.

The application of synthetic chemicals is still the most common method to control weeds. In general, the toxicity of herbicides is due to the inhibition of enzyme functions essential to plant survival. The enzyme involved is defined as the herbicide target site. Unfortunately, many herbicides have nonspecific properties, affecting other organisms (crops, nonvertebrates, and vertebrates) to varying degrees. The damage to these nontarget organisms may be reflected by different effects, such as direct mortality, long-term population reductions, and bioaccumulation within the food web. The indiscriminate dispersion of these substances must be regarded as potentially hazardous to man and the environment. Among the examples on this subject, we can mention the toxicity to useful arthropods such as the predatory mite *Phytoseiulus persimilis* [18], aquatic invertebrates[19], and fishes[20]. Because of their mechanism of action, herbicides are less toxic to mammals[21], but the risk of exposure cannot be neglected, even considering the additional impact that may result from farmers switching to more toxic herbicides[22].

Plants are the richest source of organic compounds on Earth. The greatest part of them are not directly involved in the normal growth and development of the plant (secondary metabolites), but they are important in many other functions, most of which have not yet been completely clarified. However, there is no doubt that some of these chemicals are important as defense compounds against microorganisms, animals, and other plants. Since ancient times, farmers have observed that some plants inhibited the growth of other species, both weeds and crops. This phenomenon is known as allelopathy, from the Greek words αλλήλων (allelon = mutual) and πάθος (pathos = suffering), that is, the reciprocal sufferings of two organisms. The term was proposed in 1937 by the Czech scientist Hans Molisch. At first, he did not give the term a negative connotation, but he simply meant the effect of a plant on another. Later, the meaning was limited to those interactions that inhibit the growth of nearby plants[23]. One of the first records of allelopathy was reported by the Latin scholar Marcus Terentius Varro in his *Rerum rusticarum* about oak and walnut: “ut quercus sic iugulandes magnae et crebrae finitimae

fundi oram faciunt sterilem" (like oak, large nearby walnut trees make the margins of the farm sterile).

Given the large amount of material on the subject, this review is restricted to publications on pure characterized substances, and it will not take into account the activity of crude extracts. It is also restricted to publications written in Western languages.

AGRICULTURAL USES

Because of their effects, allelopathic compounds could be used to control weeds in crops. Apart from their direct use as control agents, allelochemicals can also be used in association with synthetic herbicides to reduce their doses while maintaining the same effectiveness[24–27].

Allelochemicals belong to many different chemical classes and are always secondary metabolites. Often they are water-soluble substances, so they can be easily washed away by rain from plant surfaces and carried into the soil, where they exert their action. Sometimes they are produced by roots as exudates or released into the environment as volatiles.

Phenols

Among allelochemicals, many phenolic compounds have been identified (Fig. 1). Phenols are small molecules containing one or more phenolic groups and can be further classified into different subgroups, starting from the simplest C-6 derivatives, such as phenol, pyrogallol, catechol, and benzoquinone, to the most complex structures of polyphenols such as tannins, through a series of molecules of intermediate complexity, such as salicylic derivatives, acetophenones, hydroxycinnamic acids, coumarins, naphthoquinones, anthraquinones, flavonoids, and lignans.

Among the intermediate complexity molecules, seven derivatives were identified in the roots and in the shoots of 17 days old wheat (*Triticum aestivum* L., Poaceae) seedlings and in their associated water-agar growth medium: *p*-hydroxybenzoic, vanillic, *trans*- and *cis*-coumaric, syringic, *trans*- and *cis*-ferulic acids[28]. They were tested for growth inhibition of annual ryegrass (*Lolium rigidum* Gaudin, Poaceae). Only *p*-hydroxybenzoic, vanillic, and *trans*-ferulic acids were significantly associated with the inhibition of root elongation of the weed. Interestingly, it was observed that those individual compounds that did not show any activity individually, became effective when examined in association. Authors suggested a possible additive or synergistic effect among allelochemicals.

Some rice (*Oryza sativa* L., Poaceae) cultivars are known for their high allelopathic potential. Studying the phenols released by rice straw, decomposed straw, and hulls, Chung *et al.*[29] identified benzoic, ferulic, *o*-hydroxyphenylacetic, *p*-hydroxybenzoic, salicylic, syringic, *m*-, *o*-, *p*-coumaric acids.

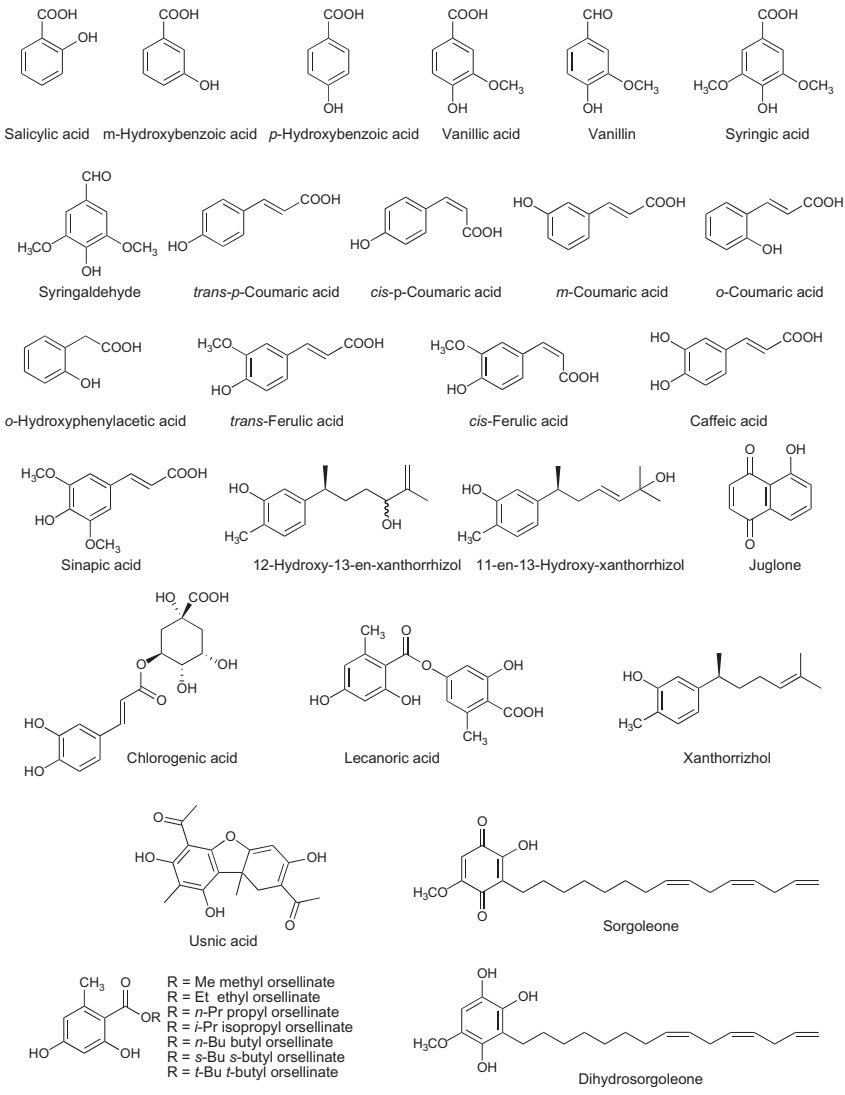


FIGURE 1 Phenols.

They were tested for their effects on seed germination and seedlings growth of barnyardgrass (*Echinochloa crus-galli* var. *orzycola* Ohwy, Poaceae), both as individual compounds and as an equimolar mixture at 10^{-3}–10^{-5} M concentrations. All the chemicals showed allelopathic activity and most had inhibitory effects on seed germination and early growth of seedling, irrespective of their concentration. Among the tested compounds, ferulic, *p*-hydroxybenzoic acid, *p*- and *m*-coumaric acids showed the highest activity. Mixtures of phenols were

less inhibitory than individual compounds, in contrast with the observations of Wu *et al*.[28] and Einhellig[30].

Aqueous and methanol extracts of 16 wild Asteraceae plants showed variable allelopathic activity on lucerne (*Medicago sativa* cv. Vernal, Fabaceae) and *E. crus-galli* (Poaceae) depending on plant species[31]. Among the main possible allelochemicals quantified in the extracts, C6–C3 phenols such as *trans*-cinnamic, chlorogenic, *o*- and *p*-coumaric acids and coumarin were identified. The same authors[32] tested the water extract of four crop plants in the Poaceae family: barley (*Hordeum vulgare* L.), oats (*Avena sativa* L.), rice (*O. sativa* L.) and wheat (*T. aestivum* L.), for allelopathic activity on alfalfa (*M. sativa* L.), barnyardgrass (*E. crus-galli* (L.) Beauv. var. *orzycola* Ohwi.) and eclipta (*Eclipta prostrata* L., Asteraceae) seeds. The substances responsible for the activity, identified by HPLC, were *trans*-cinnamic, hydrocinnamic, salicylic, ferulic, caffeic, *m*-, *o*-, *p*-coumaric acids. Furthermore, coumarin, which was detected in very low amounts, showed the highest phytotoxicity on plant root growth.

Similar phenolic compounds were identified in the methanol extract of the underground parts of *Ophiopogon japonicus* (L. f.) Ker Gawl., Asparagaceae [33]. Tests against two major weeds of rice fields in Japan, *E. crus-galli* (L.) Beauv. and *Monochoria vaginalis* (Burm. f.) C. Presl, Pontederiaceae, verified the significant activity of salicylic and sinapic acids on the germination of the seeds of *E. crus-galli* at the lowest doses (250–500 ppm). All compounds inhibited root elongation dose-dependently, with the exception of syringic acid and syringaldehyde. A comparable effect was noted on shoot growth, but this time sinapic and *p*-hydroxybenzoic acids were the ineffective compounds. All the phenols, except sinapic acid, inhibited seed germination and growth of roots and shoots in the case of *M. vaginalis*. A further study on this plant species also confirmed the phytotoxicity of phenolic acids on lettuce (*Lactuca sativa* L., Asteraceae)[34]. The most active compound was salicylic acid. When compared with its *meta* and *para* analogues, the effectiveness scale was *ortho* > *para* > *meta*. Furthermore, methylation of the phenolic group considerably reduced the activity.

Again, salicylic acid and vanillin resulted as the most phytotoxic components against the paddy weeds *E. crus-galli* and *M. vaginalis* isolated from *Stylosanthes guianensis* (Aubl.) Sw. var. *guianensis*, Fabaceae. This species and its chemicals gave promising results both *in vitro* assays and in greenhouse or field trials, reducing shoots and roots length or the weed biomass, respectively.

Four bisabolane-type sesquiterpene phenols have been characterized in the aerial parts of *Lagascea mollis* Cav., Asteraceae[35]. Among these chemicals, 12-hydroxy-13-en-xanthorrhizol and 11-en-13-hydroxy-xanthorrhizol showed significant inhibitory activity on the germination of *Sorghum halepense* (L.) Pers. at 100 and 1000 ppm. Xanthorrhizol was considerably less effective, while no data have been reported about the efficacy of 12,13-epoxy-xanthorrhizol.

Dodder (*Cuscuta hygrophilae* H. Pearson, Convolvulaceae) showed strong inhibition against the growth of noxious paddy weeds both in bioassays and in greenhouse trials[36]. The most active phenol was found to be vanillin, together with the related compounds cinnamic acid and methyl cinnamate. The unusual aspect of this discovery is that dodder is a parasitic weed and as such, it grows only by penetrating tissues of host plants to obtain water and preformed nutrients. Hence, its allelopathic potential would seem a contradiction in terms. However, it is still unclear whether these allelochemicals are able to suppress the growth of the hosts or whether they can contribute to the strong invasiveness of dodder. The same hypothesis was suggested by Qasem *et al*.[37].

Besides higher plants, also lichens may exert allelopathy due to the production of phenols[38]. *Parmotrema tinctorum* (Nyl.) Hale, Parmeliaceae, is very rich in lecanoric acid, a phenolic acid that significantly inhibited the growth of *L. sativa* L. hypocotils. However, this component was ineffective on seed germination. Authors prepared some esters (orsellinates), observing that an increase in the number of carbon atoms in the ester chain led to higher activity.

To verify if the paddy weed *E. crus-galli* may have an allelopathic potential on rice, Khanh *et al*.[39] studied the soil on which the plant was grown, isolating some constituents among which cinnamic acid methyl ester showed the strongest herbicidal activity. Another plant that releases phenols into the soil is *Allium ursinum* L., Amaryllidaceae[40]. Among these compounds, *p*-coumaric, ferulic, *p*-hydroxybenzoic and vanillic acids were identified. They inhibited the growth of seedlings of *L. sativa* L., *Amaranthus caudatus* L., and *T. aestivum* L. transplanted in this soil by 37–44%. Furthermore, the phenolic fraction obtained by solvent extraction of the soil caused strong inhibition of seed germination.

As mentioned above, phenolic acids have been shown to be strongly implicated in allelopathy and are therefore of interest as a basis of new natural herbicides. However, apart from their chemical structure, their effectiveness is directly related to the amounts that are bioavailable to the root system. Phenols occur in the soil in three forms: free, reversibly bound and bound. Free phenols are bioavailable, those reversibly bound are potentially bioavailable, whereas bound phenols are considered to not be bioavailable. Bioavailability of phenols obviously depends on the soil properties, even if the sorption-desorption processes are not fully elucidated. It has been observed that sorption dynamics are complicated and cannot be generalized using a single soil variable, such as organic matter content, pH, metal oxide content, clay content, etc., as reported in many studies carried out so far[41]. In addition, soil microbes can degrade phenolic allelochemicals, further reducing their bioavailability[42].

Quinones are other simple phenols that show allelopathic properties. Among them, juglone is one of the most famous derivatives to be found in

nature, and is responsible for walnut allelopathy[43]. It was tested against four common weeds in wheat and barley fields: wild mustard (*Sinapis arvensis* L., Brassicaceae), creeping thistle (*Cirsium arvense* (L.) Scop., Asteraceae), field poppy (*Papaver rhoeas* L., Papaveraceae), and henbit (*Lamium amplexicaule* L., Lamiaceae)[44]. Tests were conducted by spraying water solutions of juglone on the leaves of the potted weeds to examine growth inhibition. Interestingly, it was also found that juglone was harmful to all of the weeds without causing any significant decrease in the growth of wheat and barley. From these results, authors hypothesized that juglone affects dicots and monocots differently. Hejl and Koster[45] observed that juglone disrupts root plasma membrane H^+-ATPase activity and impairs water uptake, root respiration and growth in soybean (*Glycine max* (L.) Merr., Fabaceae) and corn (*Zea mays* L., Poaceae). However, when released by the source plant into the soil, juglone can be irreversibly absorbed or degraded. Only the portion that accumulates on soil particles in dynamic equilibrium with the solution phase can reach the target organism. It has been observed that beneath a 19-year-old walnut plantation, the concentrations of juglone into the soil (particularly acidic with low organic carbon and poor fertility) can reach the inhibition threshold for the crops commonly used for intercropping, such as maize and soybean[46]. In addition, the process of bacterial degradation does not seem to completely remove juglone from the soil[47].

Species of *Sorghum* constitute a crop with a high allelopathic potential. It was observed that the cultivation of *Sorghum* inhibits weed growth. The negative impact of accumulated compounds in the soil remains during the following months. The allelopathic properties of this species are related to the presence of hydrophilic constituents such as phenolic acids, as well as hydrophobic derivatives such as sorgoleone and dihydrosorgoleone[48], produced and secreted by specialized glandular root hairs[49]. It was noted that sorgoleone inhibits photosynthesis, in particular photosystem II[50,51], and mitochondrial respiration[52] in target plants. Sorgoleone is one of the most effective allelochemicals so far isolated. It was demonstrated that it can inhibit the growth of many weeds at the concentration of only 10 μM[53]. For this reason, its structure has been used as a model for the preparation of new effective herbicides[54]. Furthermore, because of its high hydrophobicity, it is strongly adsorbed by soil and detectable levels were still measurable 7 weeks after incorporation[55]. Even if this is a desirable feature for a herbicide, it is suspected to be the main responsible factor for the soil sickness in fields that were planted with sorghum and to negatively affect the growth of other crops in rotation systems[56,57].

Coumarins

Coumarins are derivatives of 2H-chromen-2-one. Their name comes from the French word “coumarou,” the common name of the tonka bean (*Dipteryx*

odorata (Aubl.) Willd., Fabaceae). Some plants, mainly Rutaceae and Apiaceae members, produce furano- and pyranocoumarins in which an additional furan or pyran ring is fused to the main skeleton (Fig. 2).

Coumarin, the simplest derivative of this chemical class, has been reported in many studies as an allelopathic compound. It was observed that coumarin has the ability to inhibit the growth of *Daucus carota* L. cv. Saint Valery, Apiaceae[58], or differentially affect the morphology of *Z. mays* L. cv. Cecilia seedlings[59]. It has been suggested that this compound, together with simple phenols, may play a significant role in the phytotoxicity of *S. guianensis* (Aubl.) Sw., Fabaceae[60]. Coumarin may act as an inhibitor of germination of the seeds of *E. crus-galli*[61] as well as the growth of its roots and those of *M. sativa* L., Fabaceae[32]. The root exudate of sweet vernal grass (*Anthoxanthum odoratum* L., Poaceae) affected the growth and phosphorus uptake of nearby plants because of the presence of coumarin. This chemical is contained in all plant parts and can reach the soil both as root exudate and as leachate. Studies on the dynamics of coumarin in the soil showed that its levels tend to be halved in about 7 days; however, if the soil was disinfected coumarin maintains constant levels for more than 7 days. Consequently, it was supposed that microorganisms are involved in the degradation of coumarin in soil[62].

Two more complex coumarins isolated from *Zosima absinthifolia* (Vent.) Link., Apiaceae, 7-prenyloxy coumarin, and auraptene, completely inhibited germination of seeds and growth of roots and shoots of lettuce at 0.1 mg/ml[63].

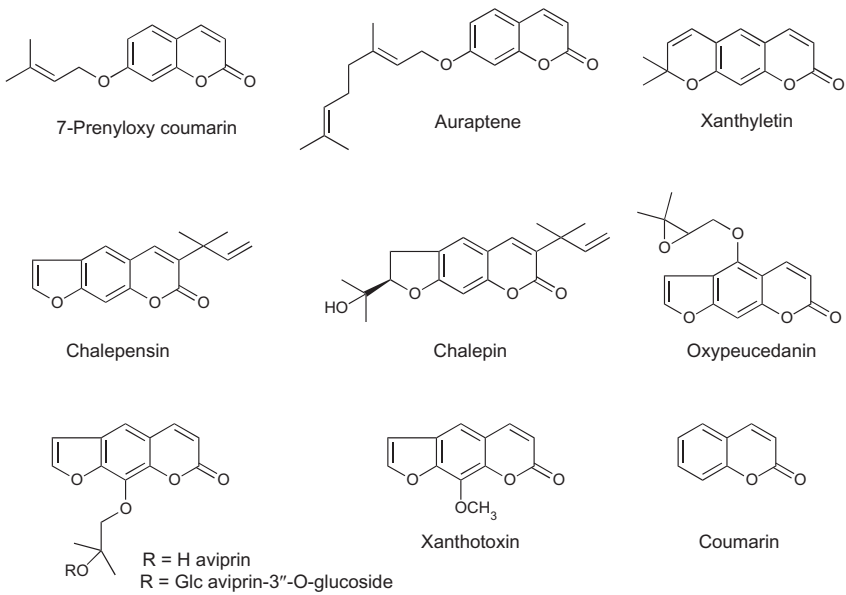


FIGURE 2 Coumarins.

In a structure–activity correlation study, furanocoumarins appeared to be more active than pyrano- and simple coumarins, particularly those whose molecules lack an oxygenated function at C-8[64]. One furanocumarin, xanthotoxin, was identified in the seed epicuticular waxes of *Ammi majus* L., Apiaceae and in the leachates obtained from them. It has been observed that rain can completely leach this coumarin from the epicuticular waxes, permitting the germination of the seeds of *A. majus*. At the same time, the liberation of this compound into the soil reduces or prevents germination and growth of competing plants. The general accepted mechanism of furanocoumarin toxicity is based upon the electrophilic addition of the photoactivated furan ring to DNA. However, since allelopathy occurs underground, in absence of light, another mechanism of action should be hypothesized[65]. Given their phenylpropanoid origin, it is likely that coumarins may share similar activity and mechanisms of action with cinnamic acids.

From the extracts of the Mexican tree *Stauranthus perforatus* Liebm., Rutaceae, the pyranocoumarin xanthyletin and the furanocoumarins chaleensin and chalepin were isolated and found very effective in the reduction of the root growth and seed germination of the weed *Amaranthus hypochondriacus* L., while *E. crus-galli* was found to be less sensitive to these compounds [66]. Another furanocoumarin, oxypeucedanin, showed considerable phytotoxic activity inhibiting seed germination as well as shoot and root growth of lettuce at quite low concentrations. The IC_{50} values of oxypeucedanin were calculated as 0.21, 0.59, and 0.62 mg/ml, respectively[67]. Phytotoxic assays showed that aviprin, a linear furanocoumarin significantly reduced seed germination of lettuce seeds in a dose-dependent manner. The IC_{50} value was 0.27 mg/ml. On the contrary, its 3″-*O*-glucopyranoside resulted less effective, having an IC_{50} value of 0.575 mg/ml[68].

Lignans

Lignans are widely distributed within the plant kingdom. They are phenylpropanoids dimers that are receiving widespread interest because of their many biologically important activities (Fig. 3). Lignans with an aryltetralin skeleton are particularly abundant in plants of the genus *Podophyllum*, both as aglycones and as *O*-β-glucosides. Compounds obtained from mayapple, *Podophyllum peltatum* L., Berberidaceae, were tested for their phytotoxic activity against *L. sativa* L. cv. Iceberg, *Lolium multiflorum* L. cv. Gulf, and *Allium cepa* L. cv. Evergreen[69]. All the lignans were more active against monocotyledonous plants than lettuce. Podophyllotoxin was the most phytotoxic lignan, inhibiting 75–94% of *L. multiflorum* root growth, while its glucoside resulted less active. Even less active were β-peltatin and its glucoside, while the activity of α-peltatin and its glucoside were marginal. Observations on onion root tips showed a reduction in the number of actively dividing cells and an abnormal mitotic organization. Despite their effectiveness and

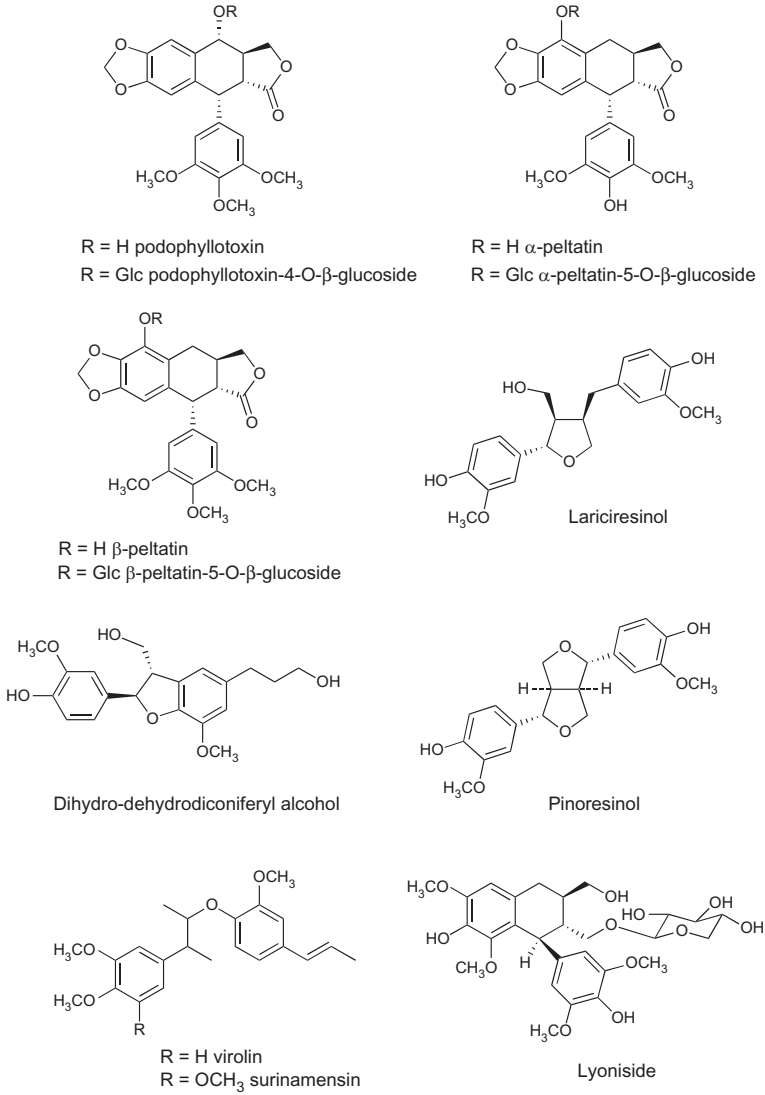


FIGURE 3 Lignans.

selectivity against monocots, the antimitotic effect of aryltetralin lignans on mammalian systems will most likely preclude their use in agriculture.

The lignans contained in a water extract of sunflower (*Helianthus annuus* L., Asteraceae) leaves were tested using the wheat coleoptiles bioassay and their phytotoxicity was also tested against seeds of tomato, lettuce, cress, and onion[70]. Authors concluded that lignans showed high levels of activity in the former bioassay. This activity was influenced by the lipophilicity of

the molecule: a tetrahydrofuran ring in the structure increased the bioactivity, whereas a second tetrahydrofuran ring did not modify the activity. The most active compounds in coleoptile bioassay, pinoresinol and lariciresinol, showed low levels of phytotoxicity, whereas the most phytotoxic compound was dihydro-dehydrodiconiferyl alcohol.

Two neolignans, surinamensin and virolin, have been isolated from the leaves of the Brazilian species *Virola surinamensis* (Rol. ex Rottb.) Warb., Myristicaceae[71]. Tests on seed germination as well as root and hypocotyl elongation of the Fabaceae species *Mimosa pudica* L., *Senna obtusifolia* (L.) H.S. Irwin & Barneby, and *Senna occidentalis* (L.) Link showed a dose-dependent activity of both compounds, with maximum inhibition at 8.0 mg/l.

Recently, from the rhizomes and stems of bilberry, *Vaccinium myrtillus* L., Ericaceae, a new phenyltetralin lignan named lyoniside, was characterized. Its levels were higher during the winter and decreased in the spring, suggesting that the accumulated compound may be exuded into the surrounding soil, similarly to many other phenolic allelochemicals. Indeed, lyoniside was detected in samples of soil obtained from the natural habitat of bilberry. The allelopathic potential was estimated on seed germination and subsequent seedling growth. It moderately inhibited the germination of dicotyledonous plants, but it strongly suppressed the growth of the radicle. It showed phytotoxicity also on conifers, in particular larch (*Larix decidua* Mill., Pinaceae) and, with minor effectiveness, on pine and spruce (*Pinus sylvestris* L. and *Picea abies* (L.) H. Karst., Pinaceae). The association of lyoniside with two triterpenes, oleanolic, and ursolic acids, which also occur in bilberry, exerted a much stronger effect on germination and growth of the seedlings[72].

Flavonoids

Flavonoids are another class of phenolic compounds widely studied for phytotoxic activity. They are a large group of pigments characterized by a C6–C3–C6 skeleton and are widely distributed in bryophytes and vascular plants. Among their many physiological roles and biological activities, flavonoids are also endowed with allelopathic properties (Fig. 4).

Extracts from *Castanea sativa* Mill., Fagaceae leaves, as well as the purified flavonoids quercetin and rutin, inhibited seed germination, root elongation, and epicotyl growth of *Raphanus sativus* L., Brassicaceae. Of these flavonoids, quercetin was the most active one. A third flavonoid, apigenin, was only able to inhibit the germination of seeds[73].

Allelopathy is often the result of shed and decaying fallen plant material. To test this aspect, Tseng *et al*.[74] purified two prenylflavanones from the fallen leaves of *Macaranga tanarius* (L.) Müll. Arg., Euphorbiaceae. They showed a weak inhibitory effect on the growth of the radicle of germinating lettuce seeds: at 200 ppm tanariflavanone B reduced by 30% root elongation, while tanariflavanone A caused 11% inhibition.

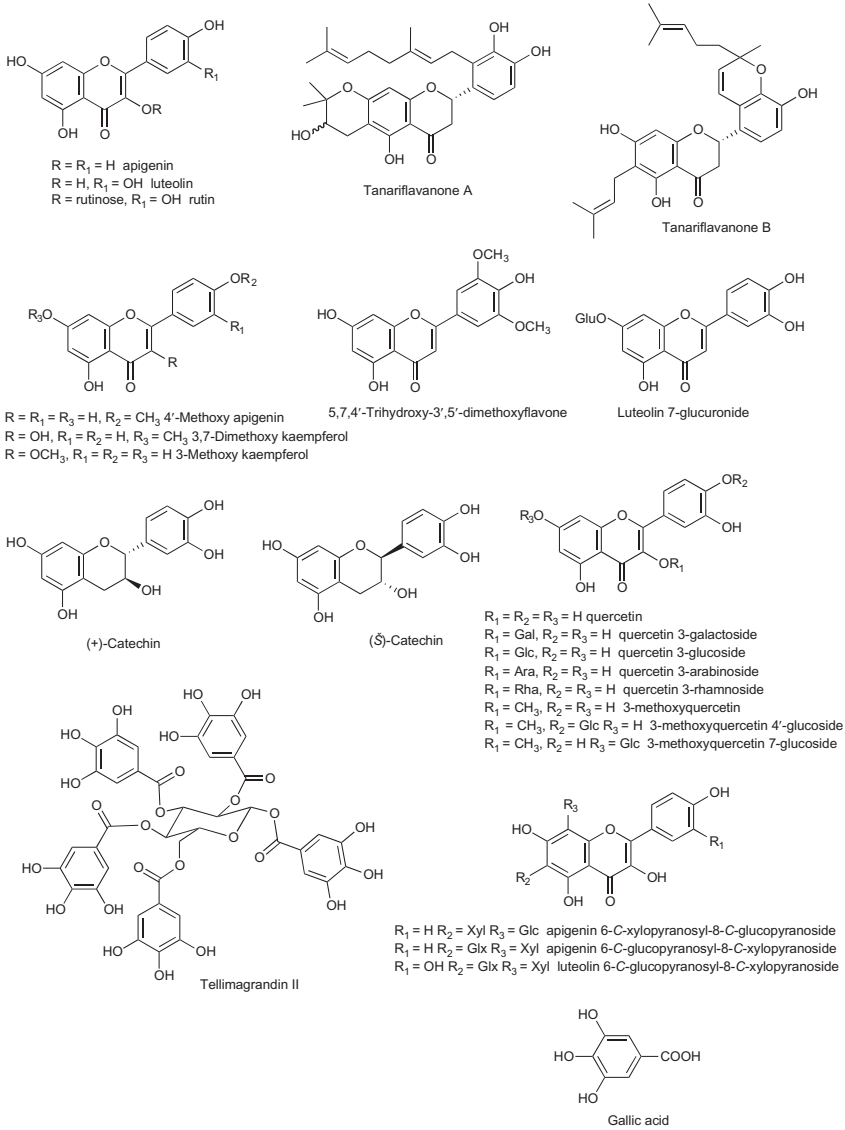


FIGURE 4 Flavonoids and tannins.

Cistus ladanifer L., Cistaceae, is a species that secretes an exudate rich in phenolics. Its flavonoid content has marked qualitative and quantitative seasonal variation. Given the remarkable phytotoxicity of this species, it was examined to check whether the flavonoid aglycones extracted from the leaf exudate may be responsible for this activity, using curled dock, *Rumex crispus* L.,

Polygonaceae, as target species[75]. The germination and cotyledon emergence percentages were not affected by any of the compounds. However, root length was significantly inhibited by pure 4′-methoxy apigenin and 3,7-dimethoxy kaempferol and by a mixture of these compounds. None of the pure flavonoid affected the cotyledon size, but they were smaller when treated with a mixture of 3-methoxy kaempferol and and 3,7-dimethoxy kaempferol. This latter mixture, as well as pure 4′-methoxy apigenin, had a clear effect on prolonging the time required for germination and cotyledon emergence. All the flavonoids were also found in the soils where the plants grew naturally, even though their amounts were not constant throughout the year. The long persistence into the soil of *C. ladanifer* flavonoids was further confirmed by a recent study[76].

One of the active principles of an allelopathic rice accession (PI312777) was found to be a flavone, 5,7,4′-trihydroxy-3′,5′-dimethoxyflavone[77]. This aglycone had inhibitory effects on growth of common weeds associated with rice in South China, *E. crus-galli* (L.) P. Beauv., Poaceae, *Cyperus difformis* L., and *Cyperus iria* L., Cyperaceae, with IC_{50} values of 200, 100, and 150 μg/g, respectively. Interestingly, it showed no effect on the growth of rice itself. This flavone was also found in the soil containing the rice accession starting from 15 days after seedling emergence, and its amounts continued to increase during the following days. In the presence of *E. crus-galli*, rice released higher amounts of the flavonoid into the soil. It must be pointed out that it was not present in the weed, and thus it was actually released from rice, substantiating the hypothesis of an allelopathic interaction. In a similar study, two flavone glycosides, 5,4′-dihydroxy-3′,5′-dimethoxy-7-*O*-β-glucopyranosylflavone and 7,4′-dihydroxy-3′-5′-dimethoxy-5-*O*-β-glucopyranosylflavone, were identified in rice seedlings[78]. Subsequent bioassays showed that both glycosides were highly active against *E. crus-galli* and *C. difformis*. In laboratory conditions, it was observed that these compounds were primarily synthesized in the shoots of rice seedlings and then exuded from living rice into the environment. However, the glycosides could not be detected in soil surrounding rice, and only their aglycone could be found in the soil. This aglycone, 5,7,4′-trihydroxy-3′,5′-dimethoxyflavone, is the same active compound tested by Kong *et al.*[77].

In a structure–activity study, quercetin and seven of its derivatives were tested on the growth of *Arabidopsis thaliana* (L.) Heynh., Brassicaceae seedlings. The flavonoid 3-methoxyquercetin and its 4′-*O*-glucoside and 7-*O*-glucoside completely inhibited the growth of the seedlings at 100 ppm. However, all the flavonoids (quercetin, quercetin-3-*O*-galactoside, quercetin-3-*O*-glucoside, quercetin-3-*O*-arabinofuranoside, quercetin-3-*O*-rhamnoside) inhibited at least in part the growth of *A. thaliana*. These results may be indicative that the presence of a methyl group has an important role for activity[79].

Chrysanthemum species are known to be a rich source of secondary metabolites with a variety of biological activities. In a study on *C. morifolium* Ramat., Asteraceae, two flavonoids, luteolin 7-*O*-β-glucuronide and diosmetin

7-*O*-β-glucuronide, were purified and tested for their allelopathic properties [80]. Only the glucuronide of luteolin was found to be effective, inhibiting the growth and the chlorophyll content of *Lemna gibba* L., Araceae.

The brassicaceous species *Lobularia maritima* (L.) Desv. produces kaempferol derivatives. To investigate their potential allelopathic role, the aglycone and its glycosides were tested against three coexisting herbaceous plants, *Phleum subulatum* (Savi) Asch. & Graebn., Poaceae, *Petrorhagia velutina* (Guss.) P.W. Ball & Heyw., Caryophyllaceae, and *Dactylis hispanica* Roth, Poaceae[81]. The following glycosides were identified: kaempferol 3-*O*-β-glucopyranosyl-(1→2)-*O*-α-xylopyranoside, kaempferol 3-*O*-α-rhamnopyranosyl-(1→2)-*O*-α-arabinopyranoside, kaempferol 3-*O*-β-glucopyranosyl-(1→2)-*O*-β-glucopyranosyl-7-*O*-α-rhamnopyranoside, and kaempferol 3-*O*-β-glucopyranosyl-(1→2)-*O*-β-galactopyranosyl-7-*O*-α-rhamnopyranoside. The aglycone had different levels of phytotoxicity depending mainly on concentrations and in the case of root elongation, also on the test species, with *P. subulatum* and *P. velutina* more sensitive than *D. hispanica*. The author hypothesized that in the glycosides the sugar moiety could modulate the biological response on the basis of the number of sugar units and the nature of carbohydrates present in the saccharide moiety, indicating a structure–activity relationship.

Among flavonoid glycosides, also *C*-glycosides showed allelopathic properties. This activity was verified for apigenin-6-*C*-β-xylopyranosyl-8-*C*-β-glucopyranoside, apigenin-6-*C*-β-glucopyranosyl-8-*C*-β-xylopyranoside, and luteolin-6-*C*-β-glucopyranosyl-8-*C*-β-xylopyranoside, isolated from *Carex distachya* Desf., Cyperaceae, against *P. subulatum* (Savi) Asch. & Graebn., Poaceae, *D. hispanica* Roth, Poaceae, and *P. velutina* (Guss.) P.W. Ball & Heyw., Caryophyllaceae[82], as well as isoorientin-2″-*O*-glucoside and isovitexin-2″-*O*-arabinoside from *A. sativa* L. against lettuce seeds[83].

The case of catechin deserves separate treatment. Catechin is a flavan-3-ol that occurs in many plants, often as a racemic mixture. Over the past decade, a lively scientific debate has tried to clarify whether this substance was indeed a powerful allelochemical. The debate started when it was claimed that the high phytotoxicity of (–)-catechin could explain the invasive success of spotted knapweed, *Centaurea maculosa* Lam., Asteraceae, in the USA[84,85]. *C. maculosa* and other knapweeds constitute a severe problem for American grasslands, where they are reducing native biodiversity and quality of forage [86,87]. A racemic mixture of catechin was isolated from the root exudates of the plant[88]. Since then many studies about the susceptibility of plants to catechin, both *in vitro* and in the field, reported contrasting results (see the review by Li *et al.* and references therein cited[89]). Of the four possible isomers, it was assumed that only (–)-catechin was the real allelopathic compound that imparts *C. maculosa* its invasive success against non-adapted native species. However, other researchers reported conflicting results on the concentrations of catechin in the soil, which often were too low to exert

phytotoxic effects[90–92]. Furthermore, catechin degrades rapidly in the soil [93,94]. Consequently, the debate is still ongoing.

Tannins

Tannins are polyphenols contained in various plant parts that can be classified in two broad groups: hydrolyzable tannins and condensed (or nonhydrolizable) tannins (Fig. 4). The former are composed by sugar molecules, usually glucose, whose hydroxyl groups are partially or totally esterified by phenolic acids, such as gallic or ellagic acids (gallotannins and ellagitannins, respectively). Condensed tannins are flavonoid polymers, generally catechin polymers. They make up a significant portion of forest carbon pools: foliage and bark may contain up to 40% of these chemicals. They were believed to function primarily as herbivore deterrents, but recent evidence casts doubts on their universal effectiveness against herbivores. An alternative hypothesis, which, however, does not entirely exclude the previous one, suggests that they may play an important role in plant–plant and plant–litter–soil interactions. Tannins can affect nutrient cycling by hindering decomposition rates, complexing proteins, inducing toxicity to soil microbial populations, and inhibiting enzymes[95].

Spike watermilfoil, *Myriophyllum spicatum* L., Haloragaceae, contains large amounts of hydrolyzable tannins that can account for up to 10% of its dry weight. Among them, tellimagrandin II has been identified as the main allelochemical because of its ability to induce 40% inhibition of photosystem II[96]. At first glance, 40% inhibition may be considered weak, but even a moderate reduction of photosynthetic activity may impair normal growth of co-occurring species.

Phragmites australis (Cav.) Trin ex. Steud. Poaceae, is a common North American plant that during the past 200 years has expanded rapidly and it is now considered one of the most aggressive invasive species in marsh communities[97]. From chloroplast DNA analysis, it was noted that invasive plants have a different haplotype, similar to that of species widespread in Europe and Asia. Previous studies showed an allelopathic effect of the root exudate of this species because of the presence of the phytotoxin gallic acid. However, in exotic invasive *P. australis* populations, authors detected increased levels of polymeric gallotannin that serve as source of gallic acid production via the tannase activities of native microbes and plants.

Benzoxazolinones

These chemicals are produced by a limited number of plant families, such as Acanthaceae, Poaceae, Ranunculaceae, and Scrophulariaceae[98] (Fig. 5). Containing a cyclic hydroxamic acid moiety, they have attracted attention since the early 1960s, when 2,4-dihydroxy-(2H)-1,4-benzoxazin-3(4H)-one (DIBOA) and 2,4-dihydroxy-7-methoxy-(2H)-1,4-benzoxazin-3(4H)-one (DIMBOA)

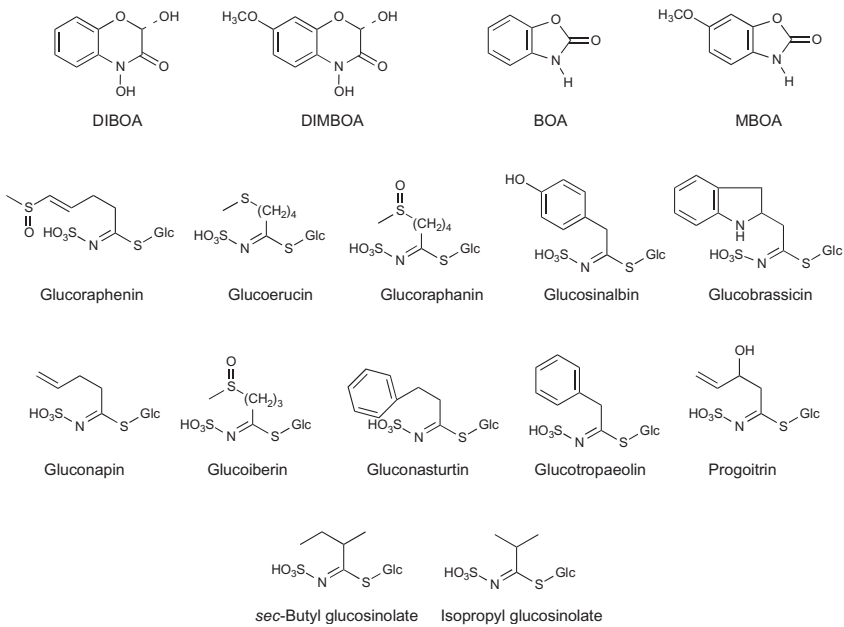


FIGURE 5 Benzoxazolinones and glucosinolates.

where characterized for the first time[99,100]. Usually, they are present in plants as glucosides, which are enzymatically converted into agluconic forms by action of β-glucosidases after plant injury. However, some authors reported the presence of agluconic forms in intact plants. Most agluconic forms are unstable and undergo a transformation in ring-contracted active compounds, such as benzoxazolin-2-one (BOA) and 6-methoxy-benzoxazolin-2-one (MBOA)[101].

Almost all studies concerned agroecosystems, in particular those involved in the production by cereals such as rice, wheat, rye, maize, and dynamics in the soils of these products. In the case of maize, a synergistic effect with phenolic acids has been observed[102].

Within the same species, the content of benzoxazinones can vary greatly depending on the cultivar[103]. The activity is directed against a variety of target plants, both weeds and crops, that is, *A. cepa* L. and *Phaseolus aureus* Roxb., Fabaceae[104], or *E. crus-galli*, *Chenopodium album* L., Amaranthaceae, *Setaria viridis* (L.) P. Beauv., Poaceae, *Abutilon theophrasti* Medik., Malvaceae, *Amaranthus retroflexus* L., Amaranthaceae[105], and *L. rigidum* Gaudin, Poaceae[28]. Studies on the structure–activity relationships of benzoxazinones and of their degradation products[106] have led to them being proposed as useful leads for herbicide models development[107]. The knowledge of the dynamics of soil degradation may further contribute to assess the real effectiveness and sustainability of these allelochemicals[108,109].

Glucosinolates

Glucosinolates are β-thioglucoside *N*-hydroxysulfates, mainly distributed in the Brassicaceae family. However, at least 500 species of non-cruciferous dicots have been reported to contain one or more of the over 120 known glucosinolates. These relatively nonreactive molecules are converted to isothiocyanates upon wounding of plant tissues that causes the release of myrosinase, an enzyme that coexists with, but is physically segregated from, its glucosinolate substrates[110] (Fig. 5).

It has been observed that changes in the mean level of glucosinolates in a species can lead to changes in the plant community structure that in turn, with a potential feedback mechanism, may affect selection on the allelochemical [111]. The allelopathic potential of glucosinolates has been discussed as a major factor influencing plant–plant interactions, in particular between exotic invasive species and neighbors native to the invaded range. However, results were highly controversial, ranging from no to high activity, probably because of the different methods used to test their inhibitory properties [112]. Furthermore, the real chemicals responsible for the allelopathic effect are not the glucosinolates themselves, but their hydrolysis products, as demonstrated by Siemens *et al*.[113] in their tests of allylglucosinolate alone and in combination with myrosinase: the root length of *Lolium perenne* L., Poaceae, was only reduced when the enzyme was present.

In a field experiment to reduce the use of synthetic herbicides in sweet corn cultivation, Malik *et al*.[114] reported the isolation and identification of 10 potential allelopathic glucosinoloates from wild radish (*Raphanus raphanistrum* L., Brassicaceae): glucoiberin, progoitrin, glucoraphanin, glucoraphenin, glucosinalbin, gluconapin, glucotropaeolin, glucoerucin, glucobrassicin, and gluconasturtin. Besides direct phytotoxicity, exotic weeds can also interfere with neighboring species by disrupting their interaction with soil organisms, such as arbuscolar mycorrhizal fungi. Both mechanisms have been proposed for two allelochemicals isolated from tall hedge mustard (*Sisymbrium loeselii* L., Brassicaceae), isopropyl glucosinolate and *sec*-butyl glucosinolate[115].

For some plants, such as *Alliaria petiolata* (Bieb.) Cavara and Grande, Brassicaceae, the allelopathic potential has been well established in laboratory conditions, but the importance of these processes in natural settings has not yet been fully elucidated. In an attempt to recover glucosinolates from the soil where the plant was grown, no results were obtained, both in sterile and non-sterile soils. Hence, authors concluded that the observed allelopathic effects are most likely either due to degradation derivatives of the compounds produced by the plant, or to other unknown mechanisms[116]. In further studies, the same authors examined whether the allelopathic effects of *A. petiolata* could be due to direct competition through interference (i.e., allelopathy), or due to indirect competition for access to a limited resource. In the latter case,

the "winner" will be the species most efficient at acquiring that resource[117]. The exposure of *Impatiens pallida* Nutt., Balsaminaceae, to glucosinolates from *A. petiolata* gave no appreciable results, whereas plant density affected the growth of the target species, suggesting that resource competition was the dominant factor.

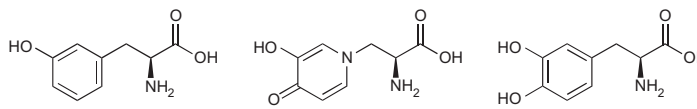
Another use of glucosinolates in agriculture is as biofumigation agents which may serve as a useful technique for suppression of soil-borne pathogens and weed seed germination. In principle, it uses the volatile active hydrolysis products, namely, isothiocyanates, released in the soil after incorporation of glucosinolate-containing plant tissues. It can be achieved by incorporating fresh plant material (green manure), seed meals (a by-product of seed crushing for oil), or dried plant material treated to preserve isothiocyanate activity[118–120].

Amino Acids

Of the hundreds of amino acids occurring in nature, only 20 are used for the synthesis of proteins. All the other ones are intermediate or end-products of metabolism in both plants and animals (Fig. 6). Most of these nonmetabolic amino acids isolated from plants are often very toxic to animals: a well-known example is the Fabaceae genus *Lathyrus*. The seeds or foods made from the seeds of *L. sativus* L., *L. cicera* L., and *L. clymenum* L. have been implicated as causes of neurolathyrism, a neurological disorder of both man and animals[121].

Since 1963, it has been observed that some plants are susceptible to these substances[122]. Some species of the tropical legumes *Leucaena* and *Mimosa* produce a toxic nonprotein amino acid, β-(3-hydroxy-4-pyridon-1-yl)-L-alanine, known as mimosine, considered an allelopathic agent, inhibiting germination and growth of several horticultural and forestry species. This chemical is contained in dormant seeds and it is also produced by germinating plants, especially if grown in the presence of light[123]. Mimosine was detected in all plant parts of *Leucaena leucocephala* (Lam.) de Wit, Fabaceae. In general, with the exception of seeds, the young plant parts contain a larger amount of this amino acid. Mimosine was found to be exuded and adsorbed in the soil where it can exert a potent allelopathic effect[124].

An unusual amino acid in plants, L-DOPA, was found to play an important role as allelochemical in velvet bean (*Mucuna pruriens* (L.) DC., Fabaceae)



m-Tyrosine Mimosine L-DOPA

FIGURE 6 Nonprotein aminoacids.

and hairy vetch (*Vicia villosa* Roth., Fabaceae) for weed control in orchard, vegetable and rice production in Japan[125]. Plants exuded L-DOPA from their roots into the soil, where it reached levels high enough to exert its activity[126].

Fine fescue (*Festuca rubra* L. ssp. *commutata* Gaud., Poaceae) displaces neighboring plants by depositing large amounts of an aqueous phytotoxic root exudate in the soil rhizosphere. By means of bioassay-guided fractionation efforts based on inhibition of lettuce radicle elongation, 3-hydroxyphenylalanine, a nonprotein amino acid known as *m*-tyrosine, was identified as the main component of the active fraction. Its absolute configuration was determined to be the L form. However, in assays using enantiomerically pure samples of *m*-tyrosine, the D and L enantiomers proved equally effective in inhibiting lettuce root growth, with IC_{50} being 17 and 21 μM, respectively. In contrast to the inhibition exerted by *m*-tyrosine, its isomers *p*-tyrosine and *o*-tyrosine actually stimulated root growth at concentrations as low as 50 μM[127].

The same research team also evaluated *m*-tyrosine activity in a series of laboratory assays performed on field soil or growth media as a basis for future trials on field soils containing living plant roots. Unfortunately, persistence of *m*-tyrosine in the soil was limited and its rapid degradation requires the development of formulations that can stabilize the product before it can be effectively used as an herbicide[128].

Alkaloids

Alkaloids constitute one of the most diverse classes of plant secondary metabolites. Some of them are also produced or sequestered by animals. They can be biosynthesized through different metabolic pathways and have many ecological and pharmacological activities. Despite their large distribution in nature, little is known about their effects as allelopathic agents because most studies have focused on the pharmacological aspects (Fig. 7).

Fagopyrum esculentum Moench, Polygonaceae was known for its allelopathic activity observed in field and laboratory studies[129]. Iqbal *et al.* [130], by means of bioassay-guided fractionation of the hydroalcoholic extract of the aerial parts of this species, isolated three allelochemicals involved in this activity: fagomine, 4-piperidone, and 2-piperidinemethanol. All compounds inhibited the elongation of roots of lettuce seedlings, with fagomine and 2-piperidinemethanol as the most effective ones, but showed different behavior against weeds species, such as *Digitaria ciliaris* (Retz.) Koeler, Poaceae, *E. crus-galli*, *Trifolium repens* L., Fabaceae, *Brassica juncea* (L.) Czern., Brassicaceae, and *Amaranthus palmeri* S. Watson, Amaranthaceae. However, when comparing the activity of pure substances with the crude extract, authors found that alkaloids alone could not account for the allelopathic activity of the plant and probably other allelochemicals are present in the extract.

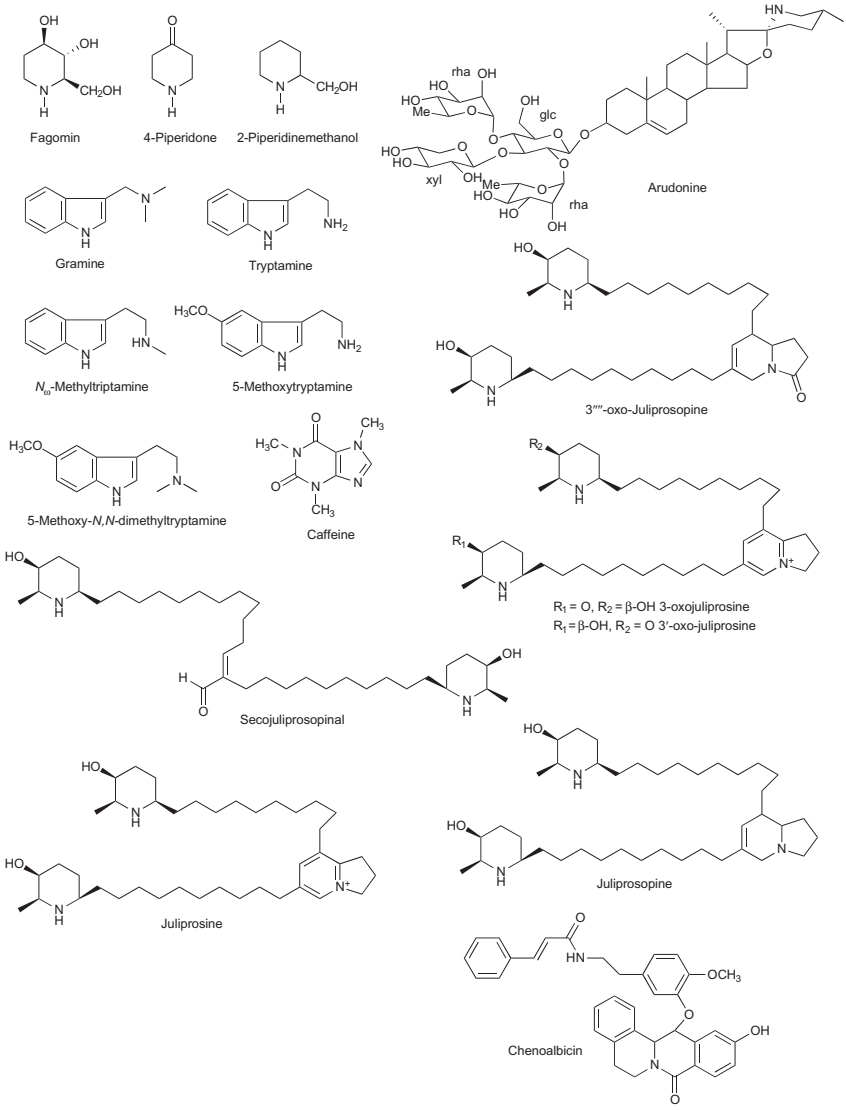


FIGURE 7 Alkaloids.

Fukuhara *et al*.[131] observed that other plants were rarely found in the proximity of *Solanum arundo* Mattei, Solanaceae, and hypothesized an allelopathic effect. Bioassay-guided fractionation of the methanol extract of the root bark of the plant led to the isolation of a new steroidal glycoalkaloid, named arudonine, having a solasodine skeleton as aglycone and four sugar moieties. This compound completely inhibited the growth of the roots of lettuce seedlings at 1000 ppm. The roots lacked hairs were brown in color. It should be

noted that the inhibitory activity of solasodine was less than 30% at the same dose. Therefore, the sugar moiety of arudonine could play an important role in the activity.

A well-known purine alkaloid, caffeine, has been found in the soil of coffee and tea plantations, where it causes autotoxicity to young plants, probably via an inhibition of mitosis and/or fragmoplast and cell plate formation[132]. The role of caffeine as an allelopathic agent seems to be supported by the fact that it degrades slowly, even in aged leaves, and it appears not to act as a nitrogen reserve since considerable amounts remain in leaves after abscission[133].

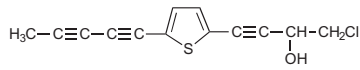
Besides phenolics, coumarins, and hydroxamic acids derivatives, alkaloids were also found among allelochemicals produced by cereals. In particular, these plants produce indole alkaloids, whose allelopathic potential is not yet fully understood. To gain a deeper comprehension of the phytotoxicity of these metabolites, Bravo *et al*.[134] tested gramine, tryptamine, 5-methoxytryptamine, 5-methoxy-*N*,*N*-dimethyltryptamine, and N_{ω}-methyltryptamine against *Chlorella vulgaris*. All the tested compounds displayed toxic effects at doses comprised between 30 and 1000 ppm. Gramine showed the highest activity and for this reason was selected to evaluate its phytotoxicity on the crops *H. vulgare* L., *Secale cereale* L. cv. *tetra*, *Triticum durum* Desf., *A. sativa* L., *L. sativa* L., and the weed *L. rigidum* Gaudin. It was observed that gramine did not cause autotoxicity on barley and did not inhibited rye. The highest activity was exerted against wheat and oat.

The structure–activity relationships of five alkaloids isolated from mesquite, *Prosopis juliflora* (Sw.) DC, Fabaceae, were evaluated by growth inhibition of shoot and root of monocots (*E. crus-galli* L., *O. sativa* L., and *Phleum pratense* L.) and dicots (*Amaranthus viridis* L., *L. sativa* L., and *Lepidium sativum* L.)[135]. The alkaloids were identified as 3″-oxo-juliprosopine, secojuliprosopinal, a 1:1 mixture of 3-oxo- and 3′-oxo-juliprosine, juliprosine, and juliprosopine. They generally showed growth inhibitory activity against both monocots and dicots plants, in particular against the growth of root. The most active compounds were juliprosine and juliprosopine. Author suggested that the active sites in the chemical structure of alkaloids from mesquite were the functional groups at C3 and C3′ of piperidine and the indolizine skeleton.

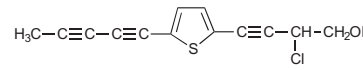
Finally, chenoalbicin, a new natural compound with an alkaloid moiety linked to cinnamic acid amide, isolated from *C. album* L., Amaranthaceae, showed a weak inhibitory activity on the growth of seedlings of lettuce[136].

Polyacetylenes

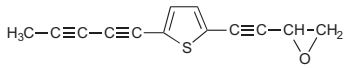
Polyacetylenes are an unusual group of naturally occurring hydrocarbons that have one or more acetylenic groups in their structures (Fig. 8). They have a limited distribution in higher plants and occur regularly in only



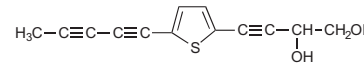
4′-Chloro-1′-(5-penta-1,3-diyn-1-yl-2-thienyl)-but-2′-yn-3′-ol



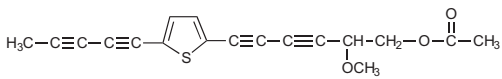
3′-Chloro-1′-(5-penta-1,3-diyn-1-yl-2-thienyl)-but-2′-yn-4′-ol



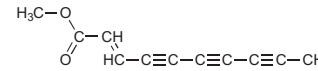
3′-(5-Penta-1,3-diynyl-thiophen-2-ylethynyl)-oxirane



1′-(5-Penta-1,3-diyn-1-yl-2-thienyl)-but-2′-yne-3′,4′-diol



5′-Methoxy-1′-(5-prop-1-yn-1-yl-2-thienyl)-hexa-2′,4′-diyn-6′-yl acetate



Dehydromatricaria ester

FIGURE 8 Polyacetylenes.

five families: Campanulaceae, Asteraceae, Araliaceae, Pittosporaceae, and Apiaceae[137].

Often they are produced by invasive plants, such as Russian knapweed, *Acroptilon repens* (L.) DC, Asteraceae (formerly *Centaurea repens*). Using bioassay-guided fractionation, five polyacetylenes were isolated from the roots of the plant, 4′-chloro-1′-(5-penta-1,3-diyn-1-yl-2-thienyl)-but-2′-yn-3′-ol, 3′-chloro-1′-(5-penta-1,3-diyn-1-yl-2-thienyl)-but-2′-yn-4′-ol, 5′-methoxy-1′-(5-prop-1-yn-1-yl-2-thienyl)-hexa-2′,4′-diyn-6′-yl acetate, 3′-(5-penta-1,3-diynyl-thiophen-2-ylethynyl)-oxirane, and 1′-(5-penta-1,3-diyn-1-yl-2-thienyl)-but-2′-yne-3′4′-diol[138]. All the compounds, with the exception of 3′-chloro-1′-(5-penta-1,3-diyn-1-yl-2-thienyl)-but-2′-yn-4′-ol, caused phytotoxicity on *A. thaliana* (L.) Heynh., Brassicaceae seedlings. Interestingly, the other chlorinated derivative presented the strongest phytotoxic activity. Thus, the different position of the OH and Cl may play an important role in the ability of these compounds to inhibit growth. Some of these derivatives were also found in the root exudate and in the soil surrounding the plants, indicating a probable role as allelopathic agents for these chemicals.

Dehydromatricaria ester is a well-known allelopathic polyacetylene isolated from Asteraceae species[139,140]. Recently, it was found that concentrations of 16 ppm effectively reduced the germination success of *Asclepias syriaca* L., Apocynaceae a common perennial competitor of *Solidago altissima* L., Asteraceae in laboratory trials[141]. In another study, the adsorption, downward mobility, and degradation of this compound released by *S. altissima* in different soils were determined. It was observed that the phytotoxicity is dependent on the soil type, but more importantly, it depends on the amount of water contained in the soil itself and its ability to dissolve the chemical[142].

Terpenes

Terpenes are probably the most widespread group of plant natural products. They are based on a definite number of isoprene units (i.e., 2 in monoterpenes, 3 in sesquiterpenes, etc.). Mono- and sesquiterpenes are the main components of essential oils (Fig. 9), even if sometimes other volatiles may be present in large amounts. Higher terpenes are usually nonvolatile and constitute resins, waxes, and rubber (Fig. 10). Phytoalexines, gibberellins, and carotenes are also terpene compounds involved in other functions.

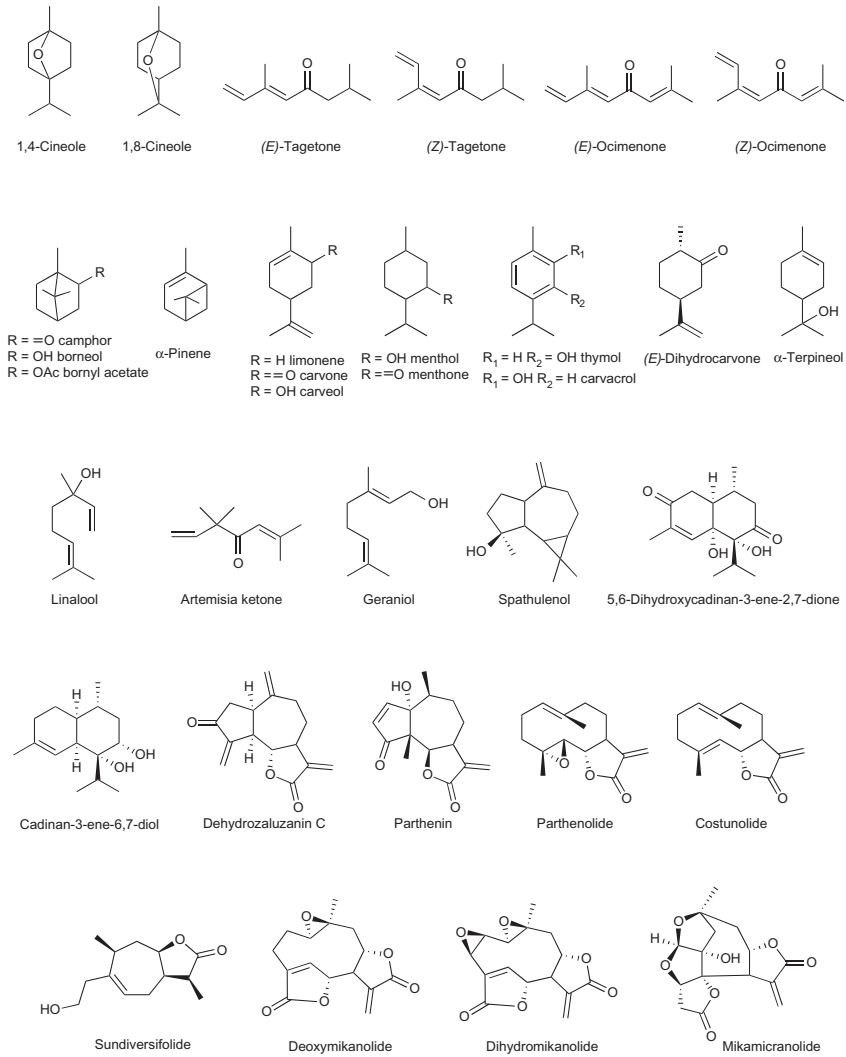


FIGURE 9 Monoterpenes, sesquiterpenes, and sesquiterpene lactones.

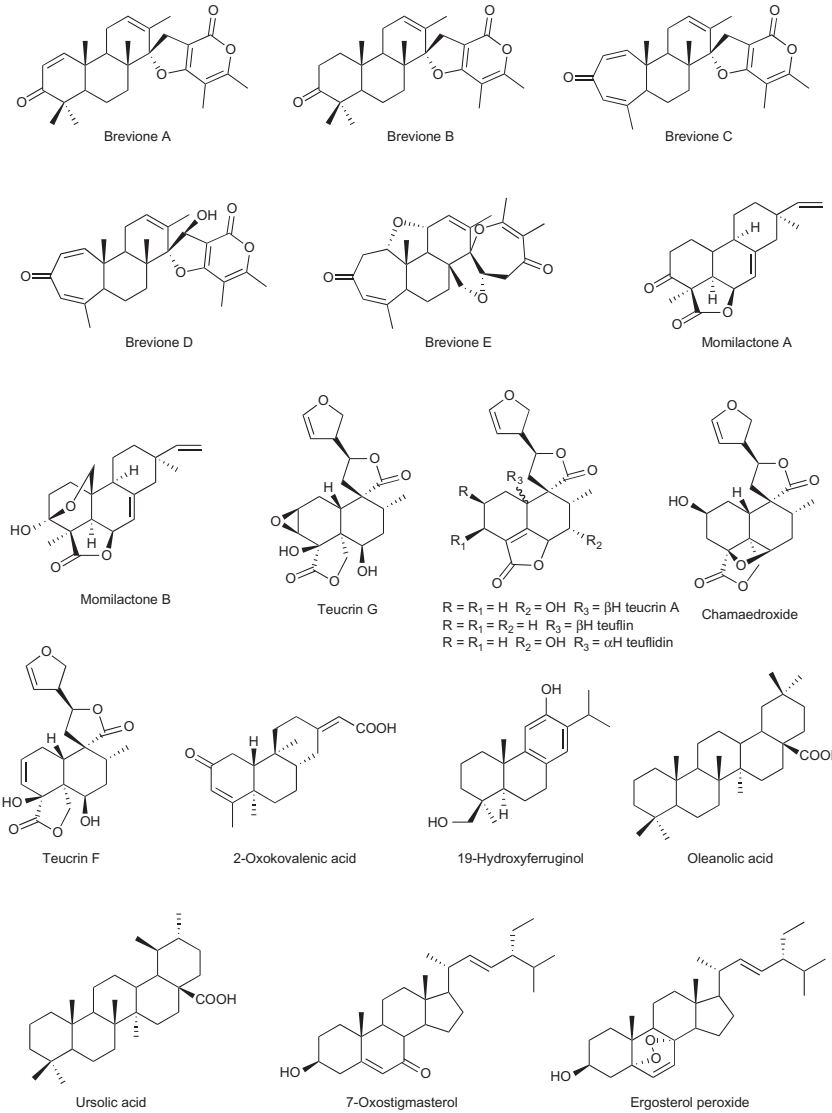


FIGURE 10 Diterpenes, triterpenes, and sterols.

Volatile Terpenes and Essential Oil

One of the most common monoterpenes found as component of essential oils is 1,8-cineole, or eucalyptol. Its isomer, 1,4-cineole, often occurs in the same plant species although normally in much lower concentrations than 1,8-cineole. The phytotoxicity of these molecules seems to be due to their epoxide moiety[143]. Romagni *et al.*[144] compared the phytotoxicity of 1,4- and

1,8-cineole on two weeds, the monocot *E. crus-galli* and the dicot *Cassia obtusifolia* L., Fabaceae. The two monoterpenes inhibited growth of both plant species, with the greatest effect observed on the monocot weed. Both compounds had similar effects on the growth of roots, but 1,4-cineole had a greater effect on shoots. Inhibitory effects of either cineole on the dicot weed were not as strong as on the monocot. Furthermore, 1,4-cineole showed inhibitory effect at lower concentrations, suggesting that it is a more active analog. Using assays on the mitotic index of onion roots, it was observed that both cineole isomers inhibited prophase of mitosis. However, 1,4-cineole did not inhibited any other phase, whereas 1,8-cineole inhibited all stages of mitosis.

Some essential oils have as their main constituents compounds other than terpenes, such as that of *Ruta graveolens* L., Rutaceae, which is characterized by the great prevalence of 2-ketones. In a sample from Italy, undecan-2-one (46.8%) and nonan-2-one (18.8%) were found to be the main volatiles[145]. This essential oil inhibited both germination and radicle elongation of radish seeds in a dose-dependent manner, particularly when tested in the dark. However, when tested as pure compounds, 2-ketones were completely ineffective, while the active principles were found to be monoterpenes, with 1,8-cineole and α-pinene as the most phytotoxic ones. Within the Lamiaceae family, many species release phytotoxic monoterpenes that hinder the development of herbaceous species. Angelini *et al.*[146] evaluated the allelopathic potential of the essential oils obtained from thyme (*Thymus vulgaris* L.), savory (*Satureja montana* L.), and two accessions of rosemary (*Rosmarinus officinalis* L.) obtained from plants cultvated in Pisa (Italy). Rosemary type A mainly affected the germination of seeds of radish, pepper, lettuce, *E. crus-galli*, and *C. album*, while rosemary B showed phytotoxic activity on the same species, but it was more effective than type A. The essential oil of thyme was effective against all the species, suggesting that its allelopathic activity was high, but devoid of selectivity in relation to weeds. A similar result was observed for the essential oil of savory. Among the main constituents of these oils, carvacrol and thymol inhibited germination in all species except radish; 1,8-cineole increased the mean germination time, while borneol completely impaired germination in all weed species and only in lettuce and pepper among the crop ones. Furthermore, authors were able to detect terpene compounds in samples of soil.

Bioassays conducted on *Tagetes minuta* L., Asteraceae, and *Schinus areira* L., Anacardiaceae, oils and their pure principal constituents revealed strong inhibitory activity on the root growth of *Z. mays* L., Poaceae seedlings[147]. The former oil was more phytotoxic than the latter. Previous studies reported that plants exposed to monoterpene vapors underwent structural breakdown and decomposition of cell membrane. To verify a possible oxidative damage, the authors verified the oxidant potential of the essential oil on membrane lipids with positive results. Among the main constituents of the oil, limonene and ocimenone were found to be the most active, followed by α-pinene.

The stomata constitute the interface between plant interiors and the surrounding atmosphere. It is conceivable that stomata will respond to volatiles released by neighboring plants and that the consequences of altered stomatal movements could interfere with plant growth. To verify this hypothesis, leaf epidermal sheets were exposed to vapors of the essential oil obtained from *Prinsepia utilis* Royle, Rosaceae leaves, and were found to cause inhibition of stomatal opening. The proposed mechanism was the inhibition of K^+ influx into the guard cells[148].

The fate of exogenous monoterpenes applied to germinating seeds is still not completely understood. In an attempt to delineate this issue, Dudai *et al.* [149] exposed the seeds of *T. aestivum* L. "Dariel" to various monoterpenes in the gaseous phase. A correlation was observed between the inhibition of germination caused by compounds and the amount of total metabolites quantified in the seeds. The ketone monoterpene carvone was reduced to (*E*)+(*Z*)-carveol, (*E*)-dihydrocarvone to *neo*-dihydrocarveol and dihydrocarveol. Similarly, artemisia ketone was reduced to artemisia alcohol. The monoterpene alcohol carveol was converted to dihydrocarveol, which was either further reduced to carvomenthol or oxygenated to carvenone. Oxygenation also occurred in the cases of linalool (to 8-acetoxylinalool) and α-terpineol (to sobrerol). The number of structurally different substances, which were either reduced or oxidized, might indicate that nonspecific enzyme systems are involved.

Suppression of understory vegetation has also been noted beneath *Juniperus* species, both for grass and forb. The allelopathic potential of the essential oil and aqueous leachate of *J. ashei* Buchh. on germination and growth of *Bouteloua curtipendula* (Michx.) Torr., Poaceae was therefore investigated [150]. Three constituents of the essential oil, camphor, bornyl acetate, and limonene were detected in the leachate obtained from fresh leaves (but not in the leachate derived from degraded litter). The leachate significantly reduced germination and growth of the target species.

Normally, the composition of an essential oil changes during the year and the phenological stages of a plant species and the climatic conditions. These temporal variations may have ecological implications related to alterations in the allelopathic potential. To verify this issue, the variability of the essential oil composition and its phytotoxicity was studied during the life cycle of *T. minuta* L., Asteraceae[151]. The main components of the essential oil were tagetones [(*E*)- + (*Z*)-2,6-dimethyl-5,7-octadien-4-one], ocimenones [(*E*)- + (*Z*)-2,6-dimethyl-2,5,7-octatrien-4-one] and spathulenol. The content of these chemicals varied significantly according to the month and the plant part. The phytotoxic effect was positively correlated with the concentration of ocimenones, with broad-leaved species more sensitive than grasses.

Terpenoids are considered sparingly soluble in water, given their nonpolar character. Yet, some researchers have reported that plant volatiles appear to be sufficiently soluble in water to cause strong inhibitory effects of aqueous

solutions. A saturated water solution of the volatile oil of *Descurainia sophia* (L.) Webb. ex Prantl., Brassicaceae possessed strong phytotoxic potential to cause germination reduction and seedling growth inhibition of eight different wheat cultivars[152]. In a similar study, a saturated solution of the essential oil of *Aster lanceolatus* Willd., Asteraceae in water showed a strong inhibition of *L. sativa* germination[153].

Other effective essential oils were obtained from *Lantana camara* L., *Eucalyptus camaldulensis* Dehnh., *Eriocephalus africanus* L.[154], *Anethum graveolens* L., *Foeniculum vulgare* Mill., *Ocimum basilicum* L., *Coriandrum sativum* L., *Petroselinum crispum* (Mill.) Nyman ex Hill, *Phacelia tanacetifolia* Benth., *Pimpinella anisum* L.[155], *Schinus molle* L.[156], and *Pituranthos tortuosus* (Coss.) Maire[157]. Among pure constituents of essential oils, 5,6-dihydroxycadinan-3-ene-2,7-dione and cadinan-3-ene-6,7-diol, obtained from *Eupatorium adenophorum* Spreng. showed promising activity[158].

He *et al*.[159] calculated the optimum combination of five oxygenated terpenes to obtain the highest phytotoxic effect on *E. crus-galli*. Based on the optimized regression equation, it was found to be composed by 0.033 mM carveol, 0.03 mM carvone, 0.08 mM menthone, 0.02 mM carvyl acetate, and 0.001 mM cedrol.

For some volatile terpenes, the mechanism of action has been ascertained. It was observed that some monoterpenes strongly affect the respiratory activity of primary root mitochondria. Although the effects observed for camphor, 1,8-cineole, limonene, and α-pinene were somewhat distinct, all monoterpenes shared, at different concentration ranges, a common effect, leading to a complete suppression of respiratory control. Their potency was found in the following order: α-pinene > limonene > 1,8-cineole > camphor[160]. Another proposed mechanism of action is due to the lipophilic nature of most monoterpenes that could alter the packing, fluidity, and/or physical arrangement of phospholipids in the membrane. It has been observed that treatment of maize roots with geraniol, 1,8-cineole, camphor, menthol, and thymol induces a modification of the sterol proportion, mainly in the free sterols fraction, a change in the unsaturation of the fatty acids and an increased percentage of unsaturated phospholipids[161].

Sesquiterpene Lactones

Sesquiterpene lactones constitute a large and diverse group of biologically active plant chemicals that have been identified in several plant families, but they are mainly produced by Asteraceae. They are classified on the basis of their carbocyclic skeletons and the suffix "olide" refers to the lactone function (Fig. 9). They have been shown to exhibit a wide range of biological activities. Most of them are cytotoxic and have been proposed as anticancer agents. However, also anti-inflammatory, antiviral, antibacterial, and antifungal properties have been verified. A particular derivative, artemisinin,

a sesquiterpene lactone containing an endoperoxide linkage, was found to be superior plasmocidal and blood schizontocidal agent to conventional antimalaria drugs[162].

Dehydrozaluzanin C is a sesquiterpene lactone with a guaianolide skeleton obtained from different weeds of the Asteraceae family. Its phytotoxicity was evaluated on plant species selected as representatives of main monocotyledon and dicotyledon weeds and important commercial crops[163]. Among dicots, lettuce (*L. sativa* L. cv. nigra and cv. roman), tomato (*Lycopersicon esculentum* L. cv. Tres Cantos), carrot (*D. carota* L. cv. Coral), and cress (*L. sativum* L.) were selected, while onion (*A. cepa* L. cv. Valenciana), wheat (*T. aestivum* L. cv. Cortex), barley (*H. vulgare* L. cv. Wellam), and maize (*Z. mays* cv. Oropesa) were chosen as representatives of monocots. Dehydrozaluzanin C was active starting from 100 μM but only cress growth was inhibited. A similar behavior was observed for monocots, with barley as the most sensitive species. In most cases dehydrozaluzanin C showed a phytotoxic activity higher than Logran®, the reference synthetic herbicide. Authors concluded that dehydrozaluzanin C can be considered as a potent growth regulator, specially for dicotyledon species and proposed it as a lead compound for the development of new herbicides.

Sunflower (*H. annuus* L.) seedlings are well known for their strong allelopathic properties. From the exudates of the roots, a dinorxanthanolide was obtained. It was identified as sundiversifolide and showed a species-selective activity on the shoot and root growth of the tested plants. Among the six species examined, tomato growth was inhibited most. Other sensitive species were crabgrass (*D. ciliaris* (Retz.) Koeler) and barnyard grass (*E. crus-galli*), both being serious weeds the world over[164].

Billygoat weed, *Ageratum conyzoides* L., is a weed commonly associated with wheat in hilly tracts of North India. The possibility of using parthenin to control its growth has been considered. This allelochemical was obtained from the dried leaves of *Parthenium hysterophorus* L. Within the 50–200 μM range, germination and growth was significantly reduced and at 400 μM it was completely inhibited. Spray treatment with 200 μM parthenin solution on plants grown in pots caused reduction of chlorophyll content and plant respiration. The treatment adversely affected the amounts of some macromolecules, such as water-soluble proteins and carbohydrates and the specific activities of the enzymes (protease, amylase, etc.). Parthenin possesses an α-methylen-γ-lactone and an α,β-unsubstituted cyclopentenone ring, around a seven-membered ring, which makes it chemically reactive toward a wide variety of biological nucleophiles. The presence of the unsubstituted ring provides potential for chemical modification, making it an ideal molecule for development as a natural herbicide[165].

The inhibitory effects of two sesquiterpene lactones, costunolide and parthenolide, isolated from the leaves of *Magnolia grandiflora* L., were tested on seed germination and seedling growth of wheat (*T. aestivum* L.), lettuce

(*L. sativa* L.), radish (*R. sativus* L.), and onion (*A. cepa* L.). Both compounds inhibited seed germination of the four species at 500 μg/ml. In general, parthenolide was more active than costunolide. A similar result was observed for growth inhibition, particularly in the case of root[166]. Another study considered the same sesquiterpene lactones, costunolide and parthenolide, together with the derivative 1,10-epoxyparthenolide on germination and seedling growth of wild oat, *Avena fatua* L. All the compounds reduced seed germination, with costunolide as the most active one. Furthermore, the three sesquiterpene lactones strongly inhibited root and shoot growth of the weed. Root was more sensitive than shoot. Parthenolide was also tested for its effect on acetolactate synthase activity. The observed inhibition of the enzyme indicated that the herbicidal activity of this lactone is mediated by this mechanism[167].

Three sesquiterpene lactones, deoxymikanolide, dihydromikanolide, and mikamicranolide, obtained from *Mikania micrantha* H.B.K. showed inhibitory effects on seedling growth of *Brassica parachinensis* Bail. All the compounds exhibited marked root growth inhibition that was concentration dependent. Deoxymikanolide was the most effective compound. A similar result was observed for inhibition of shoot growth[168].

Diterpenes

Diterpenes are fungal and plant secondary metabolites formed from four isoprene units. Most diterpenes are nonvolatile but sometimes they are found in very small amounts in essential oils (Fig. 10).

The genus *Penicillium* is well known for producing a variety of bioactive metabolites, possessing many biological properties, including plant growth regulators. After semisolid fermentation of a strain of *P. brevicompactum* from leaf litter, a spiroditerpene, brevione A, was isolated by means of bioassay-guided fractionation. This compound inhibited wheat coleoptile growth starting from 1 mM concentration; it was of particular interest being the first member of bioactive diterpenes with a new skeleton[169]. Further studies afforded four new related compounds, breviones B–E. The most active metabolite was brevione E, followed by brevione C, brevione A, and brevione B. The fourth derivative, brevione D, was not available in sufficient amounts to carry out bioassays. Authors proposed that the expansion of the A ring observed in breviones C and E may account for the increased activity. Their levels of activity suggest that they may be lead compounds for new agrochemicals[170].

As mentioned above, some rice cultivars are endowed with high allelopathic properties due to the production of a variety of phytotoxins. Diterpenes have been identified among these allelochemicals. From the root exudates of *O. sativa* L. cv. Koshihikari, momilactone B was isolated and found to inhibit the root and hypocotyl growth of cress and lettuce. Observations during field trials that rice seedlings were able to inhibit the growth of neighboring plants after the exclusion of competitive interference, led to the hypothesis that rice

seedlings produce growth-inhibiting substances that are released into the soil [171]. Further investigations verified this hypothesis and confirmed that momilactone B may play an important role in rice allelopathy[172]. Besides momilactone B, momilactone A was also isolated from rice root exudates. However, it was less phytotoxic than momilactone B, at least on the growth of *L. sativa* L. cv. Santanasu and *Brassica rapa* L. cv. Harumaki-ichigou[173].

With the aim to verify if *Teucrium chamaedrys* L. produces secondary metabolites with selected modulated effects on nearby plants, Fiorentino *et al.*[174] characterized nine neoclerodane diterpenes that were assayed for their effects on seed germination and seedling growth of four stenomediterranean coexisting species (*D. hispanica* Roth, *P. velutina* (Guss.) Ball et Heyw., *Petrorhagia saxifraga* (L.) Link, and *P. subulatum* (Savi) Asch. & Graebn.) and on two weeds (*A. retroflexus* L. and *A. fatua* L.). In general, teucrins A and G were the most phytotoxic derivatives, followed by teucrin F and chamaedroxide. The least active ones were teuflin and teuflidin. However, each species showed different behavior in relation to the type of compound and its concentration. Authors concluded that these results confirmed the hypothesis that *T. chamaedrys* produces a wide range of metabolites with different chemical properties, responsible for the observed modulating effects.

Exotic plants may represent the core of agricultural production in many countries. This is the case of teak (*Tectona grandis* L.) in Cuba. This species showed a very high allelopathic activity due at least in part, to diterpenes. The most phytotoxic compounds against both monocots and dicots species were found to be 2-oxokovalenic acid and 19-hydroxyferruginol[175].

Triterpenes and Sterols

Both triterpene and sterols derive from a common intermediate, squalene, formed by six isoprene units (Fig. 10).

Vigna unguicolata (L.) Walp, cowpea, is probably the most consumed legume, and in many countries its production occupies 90% of the working population. Unfortunately, in South Benin, this economically important crop is endangered by the presence of *Justicia anselliana* (Nees) Anders which was found to produce the triterpene lupeol and the sterols stigmasterol and β-sitosterol. These compounds showed a good inhibitory activity on the growth of cowpea[176].

It has been observed that several *Vaccinium* species have a capacity to strongly restrict the growth of other plants, including seedlings of trees. Therefore, Szakiel and Mroczek[177] investigated the profile of triterpenoids in the soil obtained from native cowberry (*Vaccinium vitis-idaea* L.) habitat and their influence on germination and growth of cress (*L. sativum* L.), lettuce (*L. sativa* L.), wheat (*T. aestivum* L.), and pine (*P. sylvestris* L.). Free oleanolic and ursolic acids, as well as traces of their derivatives, were detected in the soil samples. The ratio of these two triterpenes was identical in the soil and in

rhizomes, suggesting a direct exudation or simple decay of the underground plant parts. Oleanolic and ursolic acids slightly inhibited cress and lettuce, while wheat was more sensitive. However, the most phytotoxic effect was exerted on the germination of pine seeds, a frequently co-occurring species.

Among the many allelochemical produced by rice cultivars, sterol derivatives also seem to play an important role. Two out of eight sterols, 7-oxostigmasterol and ergosterol peroxide, isolated from *O. sativa* cv. Puntal showed high inhibition properties on *E. crus-galli*, with IC_{50} values of about 500 μM. Comparison with other inactive sterols, permitted the inference of some structure–activity relationships. In particular, a higher oxidation of ring B and a double bond in the side chain seem to enhance the phytotoxic activity. The α,β-unsatured carbonyl moiety in 7-oxostigmasterol can act as a Michael acceptor with nucleophilic residues located in biomolecules, while the endoperoxide moiety of ergosterol epoxide can be transformed by peroxidases, which are very common enzymes in plant tissues. Moreover, endoperoxides could provoke the appearance of free radicals that could be responsible for cellular damages[178].

Triterpenes and sterols may occur in plants as glycosides where they are more specifically known as saponins. The genus *Solanum* is known to produce allelopathic spirosolane glycoalkaloids. However, Ye *et al*.[179] demonstrated that steroidal glycosides are also involved in this phenomenon. Both desgalactotigonin and soladulcine 3-*O*-β-glucopyranosyl-(1→2)-[β-xylopyranosyl-(1→3)]-β-glucopyranosyl-(1→4)-β-galactopyranoside exhibited inhibitory effects on the radicle growth of *Medicago hyspida* Gaertn. and *Agrostemma githago* L. at 100 ppm.

To investigate structure–activity relationships between the sugar chain of triterpenoid saponins and their germination and growth regulation effects on alfalfa (*M. sativa* L.) seeds, several monodesmosidic saponins with betulin as an aglycone were assayed. Betulin glycosides showed stronger effects than betulin; furthermore, betulin glycosides with two to four glucose residues had the most inhibitory activity. Therefore, the inhibitory effects of triterpenoids saponins are thought to be attributed not to the properties of their aglycons but to the molecule in its entirety. Betulinic acid had a higher effect than betulin, suggesting the importance of the carboxyl group at the 28 position[180].

Several polygalacic acid and bayogenin saponins were isolated from two cucurbitaceous climbing plants, *Microsechium helleri* (Peyr.) Cogn. and *Sicyos bulbosus* sp. Nov. These compounds had variable effects on root growth of *L. sativa*, *Lycopersicum esculentum*, and *L. perenne*, with *L. sativa* being the most sensitive species[181].

Apocarotenoids

Carotenoids are vital not only in their intact form but also constitute important precursors for the biosynthesis of bioactive compounds. The multifaceted

carotenoid cleavage products of plants include pigments and aroma compounds, as well as regulatory molecules. Specific tailoring of carotenoids to apocarotenoids is performed by regiospecific oxidative enzymes targeting different double bonds of the carotenoid polyene chain. These carotenoid cleavage oxygenases can act via a single cleavage of the substrate or via several sequential cleavage events, leading to a multitude of products[182] (Fig. 11).

The analysis of an aqueous infusion of fresh aerial parts of *C. album* L. led to the identification of 18 apocarotenoids that were tested on germination and growth of *L. sativa* cv. Napoli. Seven of them showed a variable response, whereas for some compounds no dose-dependence effects were observed. The active compounds were (*3R,6R,7E,9E,11E*)-3-hydroxy-apo-α-caroten-13-one, (*6S, 7E,9E,11E*)-3-oxo-13-apo-α-caroten-13-one, a grasshopper ketone isomer, (*3R,6R,7E*)-3-hydroxy-4,7-megastigmadien-9-one, (*6R,7E*)-4,7-megastigmadien-3,9-dione, blumenol A, and (+)-dehydrovomifoliol [183]. A further 13 derivatives were obtained by the same research group from the leaves of *Cestrum parqui* L'Her. Only one of them, (*3R,6R,7E*)-3-hydroxy-4,7-megastigmadien-9-one, inhibited germination of lettuce seeds [184]. A derivative of blumenol A, 4,5-dehydroblumenol A, was found to be responsible for the allelopathic activity of *Tachigali myrmecophyla*

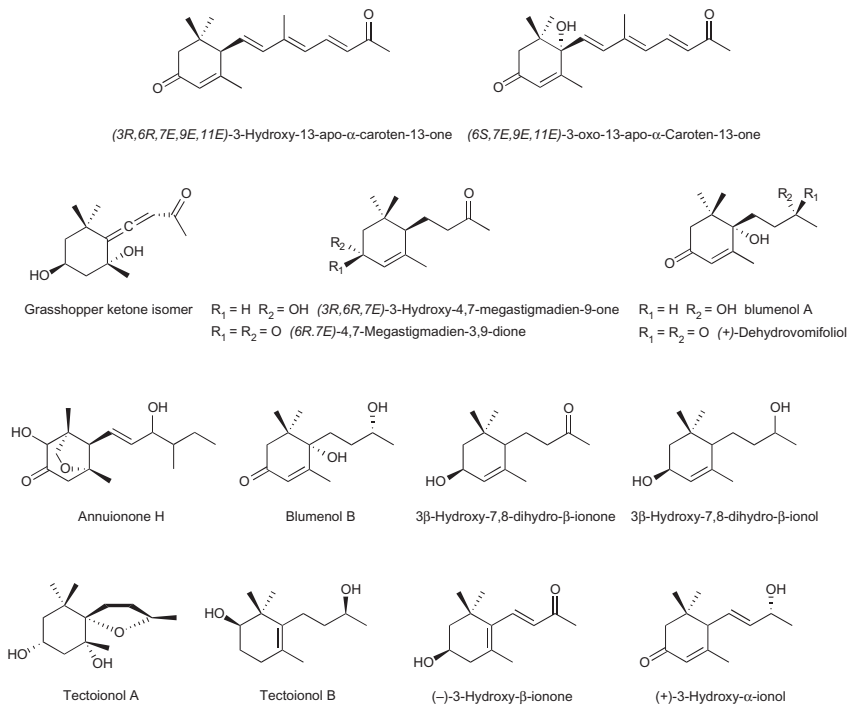


FIGURE 11 Apocarotenoids.

(Leg. -Pap.). This metabolite showed a dose-dependent activity on germination and growth of *M. pudica* L. and *S. obtusifolia* L.[185].

Apocarotenoids were found to be among the various allelochemicals produced by sunflower, *H. annuus* L. From the aqueous extract of the leaves of this species, the bioactive annuionone H was obtained. Among the five weeds tested, *Phalaris minor* Retz. showed the highest tolerance. Broadleaved weeds, especially *Rumex dentatus* L. and *C. album* L., were the most affected[186].

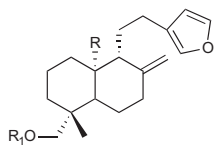
Besides the above-cited diterpenes, *T. grandis* produces apocarotenoids. Seven derivatives have been isolated from the aqueous extract of dried leaves and assayed on the coleoptiles of *T. aestivum* L. cv. duro. Five of them were obtained in sufficient amounts and their IC_{50} values were calculated. This permitted to establish their order of increasing activity: tectoionol B > 3β-hydroxy-7,8-dihydro-β-ionone > 3β-hydroxy-7,8-dihydro-β-ionol > tectoionol A > blumenol B. The three most active compounds were characterized by a double bond between C-5 and C-6, a missing feature in the least active ones[187].

Two potent growth inhibitors were isolated in rattail fescue, *Vulpia myuros* (L.) Gmel. (syn. *Festuca myuros* L.), and identified as (–)-3-hydroxy-β-ionone and (+)-3-oxo-α-ionol. Both substances were active on various test species at concentrations greater than 0.3 μM[188]. 3-Hydroxy-β-ionone is also produced by the moss *Rhynchostegium pallidifolium* (Mitt.) Jaeg. At concentrations greater than 1 and 3 μM, it inhibited the growth of cress shoots and roots, respectively. This may explain why several higher plants did not grow in places where these bryophytes live[189].

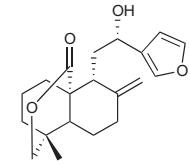
NONAGRICULTURAL USES

The management of weeds in agriculture is of course the most studied application of allelopathy and phytotoxic agents. However and especially in recent years, a number of nonagricultural applications have been reported or proposed.

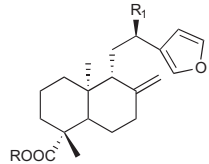
One of the most promising uses is to prevent and/or control harmful algal blooms (HAB) (Fig. 12). In recent years, eutrophication in waterbodies worldwide has become much more serious. It causes explosive growth of phytoplankton accompanied by several negative impacts, such as decreased water quality and release of algal toxins harmful for animals and even human. Moreover, floating algal mats block off light and depletes dissolved oxygen in water, resulting in suffocation of aquatic animals and plants, and consequent reduction of biodiversity[190]. It is estimated that the economic loss from serious algal bloom events in the United States is usually over several billion U.S. dollars annually[191]. Although some methods have been proposed to control HAB, most of them, such as application of copper sulfate by airplanes, treatment with flocculants, or algicidal bacterial strains, are not



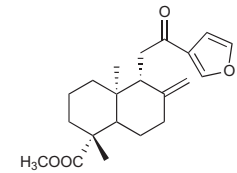
R = CHO R_1 = Ac 19-Acetoxy-15,16-epoxy-8(17),13(16),14-*ent*-labdatrien-20-al
R = OH, R_1 = H 10α-Dihydroxy-15,16-epoxy-8(17),13(16),14-*nor*-*ent*-labdatriene



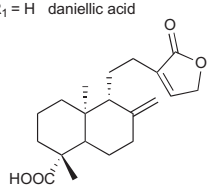
12(S)-Hydroxy-15,16-epoxy-8(17),13(16),14-*ent*-Labdatrien-20,19-olide



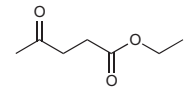
R = CH_3 R_1 = OAc methyl 15,16-epoxy-12(R)-acetoxy-8(17),13(16),14-*ent*-labdatrien-19-oate
R = H R_1 = H daniellic acid



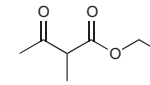
methyl 15,16-epoxy-12oxo-8(17),13(16),14-*ent*-labdatrien-19-oate



8(17),13-*ent*-Labdadien-15Ŝ>16-lactone-19-oic acid



ethyl levulinate



ethyl 2-methylacetoacetate

FIGURE 12 Algicidal allelochemicals.

easily applicable due to high cost, secondary pollution, or impracticability [191,192].

Application of crop straw in particular barley and rice straw was an economical method that showed good results in the control of HAB[193–195]. Analysis performed on crop straw evidenced the presence of algicidal phenols[194]. Aquatic macrophytes and algae are known to have an antagonistic relationship in aquatic ecosystems because of allelopathic effects that could be used for an environmental-friendly control of blooms. *Potamogeton natans* is a freshwater plant, distributed throughout the Mediterranean basin, which produces algicidal diterpenes. During a chemical investigation, six new furanoid *ent*-labdanes were characterized. Three of them showed good toxic effects on the unicellular green alga *Raphidocelis subcapitata* (formerly *Selenastrum capricornutum*). The most active compounds were 12(*S*)-hydroxy-15,16-epoxy-8(17),13(16),14-*ent*-labdatrien-20,19-olide and 10α,19-dihydroxy-15,16-epoxy-8(17),13(16),14-*nor*-*ent*-labdatriene, with IC_{50} values of 4.40 and 2.84 μM/l, respectively, while 19-acethoxy-15,16-epoxy-8(17),13(16),14-ent-labdatrien-20-al was less active (IC_{50} = 58.27)[196]. Further six *ent*-labdanes were isolated from another species of the same genus, *P. pectinatus*, and tested against the alga *R. subcapitata*. Methyl 15,16-epoxy-12-oxo-8

(17),13(16),14-*ent*-labdatrien-19-olide was the most toxic derivative (IC_{50} = 1.45 μM/l), followed by daniellic acid and methyl 15,16-epoxy-12-acetoxy-8(17),13(16),14-*ent*-labdatrien-19-oate (IC_{50} = 17.2 and 18.2 μM/l), while the effect of 8(17),13-*ent*-labdadien-15→16-lactone-19-oic acid was more moderate[197].

Being many polyphenols quite soluble in water, some plants release them in the aquatic environment as allelopathic agents. This is the case of *M. spicatum* that produces pyrogallic acid, gallic acid, catechin, and ellagic acid among its phenolic allelochemicals. The first two mentioned compounds were the most active against one of the most undesirable blue-green alga in Japan, *Mycrocystis aeruginosa*. When used in mixture, these polyphenols showed synergistic action[198].

Experiments against the broadly distributed green algae *Chlorella pyrenoidosa* and *C. vulgaris* and the blue-green alga *M. aeruginosa* in Japan, permitted to verify the activity of two isomeric metabolites, ethyl 2-methylacetoacetate and ethyl levulinate, isolated from *Phragmitis communis*. Only the former was endowed with antialgal activity, probably via an oxidative stress due to an increased production of ROS that, in turn, may cause lipid peroxidation[191]. A species-specific algicidal effect was noted for ethyl 2-methylacetoacetate during a test on six species of algae. Among these species, the inhibited chlorophytes all have a pyrenoid (an organelle within the chloroplast), while the uninhibited one has no pyrenoid. This suggests that pyrenoid might be one of the most sensitive parts of the algal cell[199]. A method to provide allelochemicals in lakes and ponds is to use artificial floating islands prepared with allelopathic species[200,201].

Among the problems caused by HAB, damages to the fisheries and aquaculture industries have been reported. The metabolites of a macroalga, *Ulva pertusa* Kjellman (Ulvaceae) showed a good effectiveness against the growth of the microalgae *Heterosigma akashiwo* Y. Hada (Raphidophyceae) and *Alexandrium tamarense* (Lebour) Balech (Gonyaulacaceae), known for their production of ichthyotoxins. The simple coexistence in the same environment reduced the growth of both microalgae. The effectiveness of the macroalga culture medium filtrate indicated the involvement of allelopathic metabolites [202]. Further studies permitted the characterization of some algicidal polyunsatured fatty acids, hexadeca-4,7,10,13-tetraenoic, octadeca-6,9,12,15-tetraenoic, α-linolenic, and linoleic acids[203].

Besides toxic effects, also organoleptic problems may be caused by HAB to aquaculture industries. The second largest cause of economic losses to channel catfish producers in the United States is off-flavor problems, mainly musty and earthy off-flavors, caused by the absorption of 2-methylisoborneol and geosmin into the flesh of catfish, thereby rendering them unpalatable and unmarketable. These compounds are produced by blue-green algae that can form blooms in the catfish ponds. Among natural products, quinones (anthraquinone in particular) have been found to be the most promising and selective

allelochemicals[204]. For further information about control of algal bloom by allelopathy, a recent review can be consulted[190].

Weeds can have substantial negative impacts on the quantity and quality of outdoor recreational activities such as fishing, hunting, hiking, and water-based recreation[205]. The lawn care industry is expanding and has annual associated revenues in excess of $1.5 billion in the United States. Currently, there are more than 12 millions hectares of maintained turfgrass, including lawns, parks, and right-of-ways in the United States. In addition, sport turf market includes golf courses and athletic fields[206]. Weeds detract from the beauty of lawns and, when they invade a sport field, they may affect its optimal use. In addition, weeds compete with the desired grass plants for available water and nutrients, usually resulting in thinning of the desirable plant cover. To avoid all the problems associated with the use of synthetic herbicides, the development of allelopathic turfgrass represents an interesting possibility. Fine leaf fescues (*Festuca* sp.) are the main turfgrass species currently used in golf course fairways, athletic fields, and private and public lawns throughout the temperate regions of North and South America and Europe. Fescue plants are known for reducing growth of co-occuring species because of the production of phytotoxic root exudates[207,208]. Among the main allelochemicals produced by fescues, *m*-tyrosin[127], (−)-3-hydroxy-β-ionone, and (+)-3-oxo-α-ionol[188] should be cited.

CONCLUSIONS

The control of weeds is mainly based on the use of effective herbicides, mostly in agriculture but also for invasive weeds in ecologically sensitive areas and outdoor recreational activities. For this reason, the lack of effective molecules often prevents the control of some species. However, the use of synthetic herbicides involves many problems that strongly limit their use: foremost the resistance problem showed by some weeds, the environmental damage, and the toxicity of many of these substances. In addition, herbicide residues in plant and animal food products are important reasons for considerable economic losses. The European Community's law (EC n. 834/2007) regarding organic farming prohibits the use of synthetic herbicides. All these problems are stimulating the search for new alternative control methods, including effective compounds characterized by smaller environmental impacts in terms of residues and toxicity. Since plant-derived compounds are generally more easily degradable and could show reduced environmental damage with respect to synthetic chemicals, the evaluation of natural substances of plant origin is being increasingly investigated. Most of the compounds cited in this review have shown promising activity and many of them may represent useful leads for the development of new herbicides. However, only a few natural products are at present commercially available, such as bilanofos, a tripeptide produced by some actinomycete strains, and some

essential oils (i.e., clove and pine essential oils) or their pure constituents (i.e., 2-phenethyl propionate)[209]. Most of these molecules have the drawback that their main useful feature, that is their biodegradability, is also their weakness. Often, many products are not able to persist in the environment for a period of time sufficient for effective weed control. Further studies are necessary to prepare better formulations that permit a solution to this problem. Other important future research topics should concentrate on the fate of these compounds and their degradation derivatives in the environment and on the evaluation of their possible toxicity, which are unknown features for many natural compounds.

ABBREVIATIONS

HPLC	High Performance Liquid ChromatographyHAB
ATP	Adenosine Triphosphate
DNA	Deoxyribonucleic Acid
IC_{50}	Median Inhibition Concentration
DOPA	Dihydroxyphenylalanine
HAB	Harmful Algal Bloom
ROS	Reactive Oxygen Species

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Chapter 14

Recent Approaches Towards Selected Lamiaceae Plants for Their Prospective Use in Neuroprotection

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INTRODUCTION

Neurodegeneration is characterized by functional loss in nerves, finally leading to neuronal death. The diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), and multiple sclerosis are strongly associated with neurodegeneration and have life-threatening effects. Degenerative nerve diseases may cause deterioration in body functions and abnormalities in mental and behavioral status. Although neurodegenerative diseases are strongly linked to genetic structure and polymorphism [1,2], multifactorial reasons including lifestyle, nutritional habits, environmental factors generating oxidative stress, inflammation, and metal accumulation also contribute to occurrence of neurodegeneration [3–6]. Neurodegenerative diseases create a major problem especially in elderly population as their incidence increase over age of 65. Since neuropathology of these diseases is quite complex and not fully elucidated, yet, only symptomatic treatment is available at the moment with the current drugs. In this regard, the most prescribed drug classes for AD are cholinesterase (ChE) inhibitors (tacrine, rivastigmine, donepezil, galanthamine), *N*-methyl-D-aspartate (NMDA) receptor antagonists (memantine), and cerebrovascular circulation-increasing drugs (*Ginkgo biloba*) (Picture 1) [7]. Among them, ChE family has special importance, comprising two sister enzymes: acetylcholinesterase (AChE) and butyrylcholinesterase (BChE). As deficiency in

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PICTURE 1 *Ginkgo biloba* (by Ilkay Erdogan Orhan)

acetylcholine (ACh) is evident in AD, it is an important strategy to inhibit AChE, whose function is to hydrolyze ACh [8].

Lamiaceae, also known as "mint family," is one of the largest flowering plant families in the world. The family consists of approximately 240 genera and 7000 species, which have generally aromatic property used as spice and tea as well as in cosmetic industry [9]. However, the plants in Lamiaceae have been reported to possess a wide range of biological activities as well as a rich diversity in phytochemistry with many chemical classes such as terpenes, flavonoids, and phenolic acids.

Up-to-date, a remarkable number of the Lamiaceae species have been investigated for their neuroprotective activity by *in vitro* and *in vivo* experimental models. Some of these species including *Salvia* sp., *Rosmarinus officinalis*, *Scutellaria baicalensis*, etc., were recorded to be used for memory-enhancing purpose in traditional medicine in Europe and China. In this review, some selected species belonging to Lamiaceae will be covered by evaluating the relevant literature regarding their neuroprotective effects.

ROSMARINUS OFFICINALIS L.

R. officinalis L. (Picture 2), known as "rosemary" in English, is a woody evergreen shrub with small blue flowers. The plant has an attractive smell and has been recorded to be a symbol of remembrance in Europe. Although *R. officinalis* is native to the Mediterranean region, it has been naturalized throughout Europe and temperate parts of America.

R. officinalis, which has been recorded for treating memory loss in Danish folk medicine, was investigated for its AChE inhibitory effect, and its aqueous and methanol extracts inhibited the enzyme at moderate level, which was defined as over 15% at 100 $\mu g\ mL^{-1}$ [10]. In a similar study by our group [11], the petroleum ether, ethyl acetate, chloroform, methanol extracts, rosmarinic acid (Fig. 1), and the essential oil obtained from *R. officinalis* sample

PICTURE 2 *Rosmarinus officinalis* (by Ilkay Erdogan Orhan)

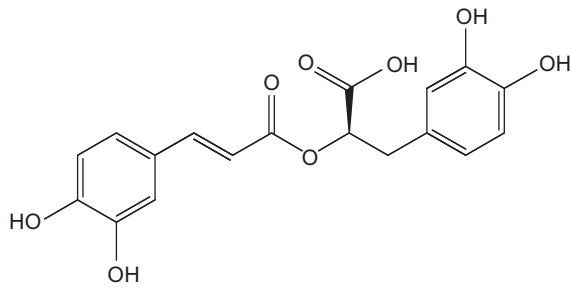

FIGURE 1 Rosmarinic acid.

growing in Turkey were tested for its inhibitory potential against AChE and BChE by spectrophotometric method of Ellman (Table 1). Rosmarinic acid was found to cause 85.8% of inhibition against AChE at 100 μg mL^{-1}, while the methanol extract was the most active in these assays. Since our high-performance liquid chromatography (HPLC) showed that rosmarinic acid constituted a foremost amount ($12.21 \pm 0.95\%$) of the methanol extract, it was concluded to be the major contributor to the inhibitory activity of the plant.

Later on, Falé *et al*. [12] also reported that rosmarinic acid had a remarkable anticholinesterase effect, in accordance with our finding [11]. Muscarinic acetylcholine receptor (mAChR), similar to the NMDA glutamate subtype receptor, is involved in memory and learning. Some rosemary components have been found to prevent free radical-dependant damage to mAChR [13]. Through *in vitro* results, rosmarinic acid was also suggested to be effective inhibitor of amyloid plaques, which frequently occur in the brains of AD patients [14]. According to this finding, the dense and symmetric structure of rosmarinic acid was proposed to be suitable for specific binding of free β-amyloid and inhibition of its polymerization into the fibrillar form. However, rosmarinic acid has been

TABLE 1 AChE and BChE Inhibitory Activities of *Rosmarinus officinalis* Essential Oil, Extracts, and Rosmarinic Acid [11]

	Percentage of Inhibition (100 μg mL^{-1})	
	AChE	*BChE*
Petroleum ether	8.5 ± 0.56[a]	54.2 ± 1.55***
Ethyl acetate	–[b]	34.2 ± 0.85
Chloroform	–	–
Methanol	–	83.9 ± 0.97***
Essential oil	63.7 ± 1.23***	74.0 ± 0.79***
Rosmarinic acid	47.3 ± 1.05	85.8 ± 1.31***
Galanthamine[c]	99.8 ± 0.31	80.3 ± 1.14

[a]*Values were expressed as mean ± S.E.M. (n = 3), p > 0.05; *p < 0.05, ** p < 0.01, ***p < 0.001.*
[b]*No inhibition.*
[c]*Reference drug.*

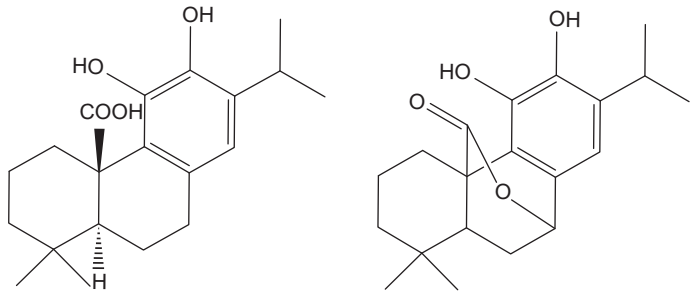

FIGURE 2 Carnosic acid (left) and carnosol (right).

reported to be associated with complement-inhibitory action, another mechanism involved in neuroprotection, by inhibiting classical and alternative pathways of complementary system [15,16].

Glutamate has been proven to play a major role in learning and memory in part *via* NMDA receptor-mediated pathways. High concentrations of glutamate induce a novel type of neuronal, named as "oxidative glutamate toxicity." Consequently, antioxidant molecules have been shown to protect neurons against excitotoxicity and oxidative glutamate toxicity [17]. Relevantly, carnosic acid and carnosol (Fig. 2), typical components of rosemary, were reported to protect cortical neurons by inducing phase 2 enzymes, which are involved in the redox regulation of cells. Induction of these enzymes often affords resistance to oxidative stress, which was initiated by activation of the Keap1/Nrf2 pathway [18,19].

PD is the most common movement disorder of neurodegenerative nature, affecting more than 6 million people worldwide [20]. Carnosol has been shown to protect nigral dopaminergic neurons [21]. The mechanism of the neuroprotective effect of carnosol and carnosic acid related to their antioxidant potential has also been proposed as follows by Tamaki *et al.* [22]: *S*-alkylation → activation of the Keap1/Nrf2 pathway → transcriptional activation → induction of phase 2 enzymes → activation of glutathione metabolism → neuroprotection. In a recent study [23], the methanol extract of *R. officinalis* was tested on H_2O_2-induced apoptosis in human dopaminergic cells (SH-SY5Y). The results indicated that the extract was quite successful in attenuating the distraction of mitochondrial membrane potential and H_2O_2-induced apoptotic cell death. Besides, pretreatment with the methanol extract of *R. officinalis* in this study significantly attenuated the downregulation of tyrosine hydroxylase and aromatic amino acid decarboxylase gene in SH-SY5Y cells. Therefore, it was concluded by the authors that *R. officinalis* might be used as a promising agent for prevention of several human neurodegenerative diseases caused by oxidative stress and apoptosis. Seham *et al.* [24] studied on effects of rosemary extract on monoamines, Na^+ ion, γ-aminobutyric acid (GABA) contents, and AChE activity in male albino rats, which were administered with a dose of 582.4 mg kg^{-1} body weight (0.5 mL solution rat^{-1}) for 4 weeks. According to the outcomes of this study, the extract decreased monoamine reuptake or induce their turnover. It also possessed anticonvulsant and antinociceptive properties as it can prevent Na^+ currents in neurons. Another finding obtained from this study was that the reduction in AChE activity by *R. officinalis* might be due to anti-AChE activity of the plant, which improves memory.

Effects of carnosic acid were examined on dieldrin-induced neurotoxicity model, which is an organochlorine pesticide implicated in sporadic type of PD, using cultured dopaminergic cells [25]. Carnosic acid attenuated significantly dieldrin-induced downregulation of brain-derived neurotrophic factor production. These results underlined that carnosic acid seems to be able to protect dopaminergic neurons against environmental neurotoxins. However, carnosol (100 μM) was found to attenuate sodium nitroprusside-induced cytotoxicity in C6 glial cells through modulation of apoptotic events as well as nitric oxide (NO) production [26]. Oxidative stress has been consistently linked to aging-related neurodegenerative diseases, and both of these compounds have been confirmed with a high antioxidant activity shown by many mechanisms [27].

SCUTELLARIA BAICALENSIS GEORGI

The genus *Scutellaria*, known as "skullcap," consists of approximately 300 species. Some of its species are used as herbal medicine. Among them, a special importance is attributed to *S. baicalensis* Georgi (Picture 3), ("Huang Qin" in

PICTURE 3 *Scutellaria baicalensis* (taken from http://www.botanicalgarden.ubc.ca/potd/scutellaria_baicalensis2.jpg, Accessed date: June 30, 2011)

Chinese), for its memory-enhancing, immunostimulant, anti-inflammatory, antiepileptic, hepatoprotective properties in Chinese and Korean traditional medicine. This particular species grows abundantly in the regions of Baikal Lake, eastern Siberia, and northern China, and chemical composition of *S. baicalensis* comprises flavonoids, steroidal saponins, alkaloids, glycosides, volatile oils, iridoids, and tannins.

In a study published in 2000 [28], the flavonoids from *S. baicalensis* were shown to protect the neuronal cells against H_2O_2-induced injury. Neuroprotective effect of the methanol extract of the root of *S. baicalensis* was evaluated in transient global ischemia using rat four-vessel occlusion model [29]. The data revealed that the microglia may be activated by this extract during brain damage including ischemia, inflammation, and infection, and the extract inhibited microglial tumor necrosis factor-alpha (TNF-α) and NO production and protected pheochromocytoma (PC12) cells from H_2O_2-induced toxicity *in vitro*. In another study [30], the extract of the plant was shown to possess improving effect on learning and memory by passive avoidance and Y-maze tests in rats. The ethanol extract of *S. baicalensis* was examined on spinal cord injury (SCI) by enhancing functional recovery *via* inhibiting inflammation and oxidative stress after SCI [31]. In a study by our group [32], we screened the ethyl acetate and ethanol extracts of the 33 *Scutellaria* taxa growing naturally in Turkey for their neuroprotective effect against the enzymes; AChE and BChE, related to AD, as well as tyrosinase, linked to PD, using enzyme-linked immunosorbent assay (ELISA) microplate reader along with several antioxidant activity assays. The extracts generally showed weak inhibition against AChE and BChE, while the best tyrosinase inhibition ($51.58 \pm 1.20\%$ at 1000 mg mL^{-1}) was caused by the methanol extract of *Scutellaria brevibracteata* subsp. *subvelutina*. The extracts had a very high 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging effect and moderate antioxidant activity in ferrous ion-chelating and ferric-reducing antioxidant power (FRAP) tests.

Wogonin (Fig. 3), an O-methylated flavone found in *S. baicalensis*, was reported to possess neuroprotection action by protecting neuronal cells damaged by oxygen and glucose deprivation in rat hippocampal slices in culture [33] and preventing the overactivation of microglial cells, possibly by inactivating nuclear factor-(NF)-κβ signaling pathway [34].

Cerebral hypoxia is known to be involved in many neurodegenerative diseases such as AD and cerebrovascular dementia. The total flavonoid fraction obtained from the stems and leaves of *S. baicalensis* improved learning and memory abilities, reduced the neuronal pathological alteration induced by D-galactose in mice, and showed protective effect against cerebral hypoxia in mice [35]. Neuroprotective action of wogonin was studied in neural precursor cells (NPCs), which exist in the hippocampus and subventricular zone of adult brains [36]. The outcome of this work revealed that wogonin induced differentiation of NPCs both in culture and *in vivo* by which wogonin protects neurons in damaged brain.

On the other hand, baicalein (Fig. 4), another flavonoid abundantly found in *S. baicalensis*, was reported to exert neuroprotective effect by assorted mechanisms. For instance, baicalein was tested on dopaminergic neurons, primary midbrain neuron-glia cultures from E-14 rat embryos stimulated by lipopolysaccharide (LPS) and baicalein (5 μM) completely obstructed LPS-induced activation of microglia [37]. Besides, it exerted protection from 6-hydroxydopamine (6-OHDA)-induced damage by the attenuation of reactive oxygen species (ROS) in SH-SY5Y cells, a dopaminergic neuronal cell line [38]. Its positive effect was also reported on hypoxic–ischemic brain damage

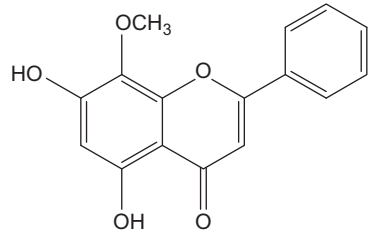

FIGURE 3 Wogonin.

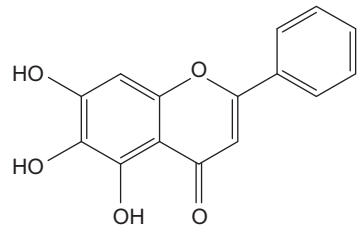

FIGURE 4 Baicalein.

in neonatal rats [39] and baicalein reversed methamphetamine- [40], 6-OHDA- [41], methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced [42] dopaminergic neurotoxicity.

Baicalin (Fig. 5), another flavonoid derivative from *S. baicalensis*, was also reported to display protection toward ischemic-like or excitotoxic injury in rat hippocampal slices by inhibiting protein kinase Cα (PKCα) translocation [43], while baicalein and baicalin were shown to provide prevention of cellular damage by the Aβ-induced ROS, which is known to increase free radical production and lipid peroxidation in PC12 nerve cells [44].

Oroxylin A (Fig. 6), another O-methylated flavone in *S. baicalensis*, was tested for its neuroprotective property on memory impairment induced by transient bilateral common carotid artery occlusion in mice as well as passive avoidance task, the Y-maze task, and the Morris water maze task models in mice, and the results suggested that oroxylin A dramatically attenuates the memory impairment [45]. Oroxylin A was investigated on the memory impairments and pathological changes induced by amyloid-β peptide$_{25–35}$ (A$\beta_{25–35}$) in mice, and subchronic studies revealed that oroxylin A (1 or 5 mg kg^{-1} per day) for 7 days ameliorated the memory impairment induced by A$\beta_{25–35}$ peptide [46]. Moreover, LPS-induced by A$\beta_{25–35}$ was also reduced

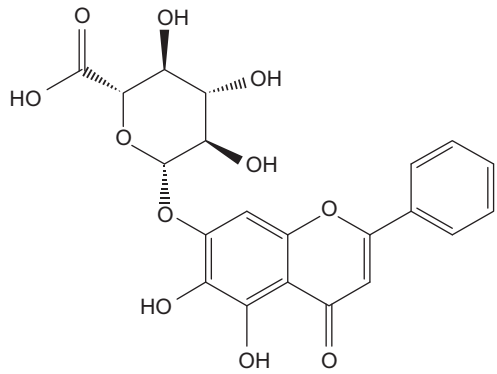

FIGURE 5 Baicalin.

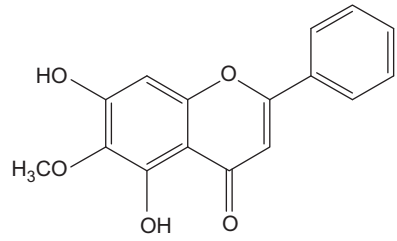

FIGURE 6 Oroxylin A.

by oroxylin A. In another study, baicalein, baicalin, and oroxylin A were tested for their anticonvulsant activity and 5,7-dihydroxyl group present in the structure of these three flavones was stated to be the key moiety for the mentioned activity [47].

SALVIA L.

The *Salvia* L., referred to "sage" in English, is the largest genus of Lamiaceae with approximately 900 species worldwide. Many members of this genus have utilization as culinary herb, spice, tea, and in perfume and cosmetic industries. Most of *Salvia* species have been used in folk medicines for different purposes and have a wide range of biological activities.

Salvia officinalis L. and *Salvia lavandulaefolia* L.

Among them, *S. officinalis* L. (Picture 4) was recorded to be used for memory enhancement in European folk medicine [48]. After successive studies on neuroprotective effect of *S. officinalis* (garden sage) and *S. lavandulaefolia* (Spanish sage), it was confirmed that both of these species possessed properties related to the attenuation of the cognitive decline associated with the downregulation of the cholinergic system seen in natural aging and dementia, by inhibiting ChE enzyme family [49,50].

The essential oils and extracts of *S. officinalis* var. *purpurea* were demonstrated to inhibit AChE, while its essential oil inhibited BChE in a dose-dependent manner in human postmortem brain tissue or bovine erythrocytes [49,51–53]. In our previous study [54], we also screened the essential oils from *Salvia sclarea* cultivated using organic and chemical fertilizers and *S. officinalis* using organic fertilizer against AChE and BChE at 100 $\mu g\ mL^{-1}$. The essential oil of *S. officinalis* displayed AChE inhibition of $63.8 \pm 1.32\%$ and BChE inhibition of $66.3 \pm 1.45\%$, whereas the essential

PICTURE 4 *Salvia officinalis* (by Fatma Sezer Senol)

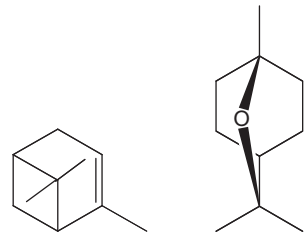

FIGURE 7 α-Pinene (left) and 1,8-cineole (right).

oils of *S. sclarea* did not inhibit AChE. Nevertheless, the essential oil of *S. sclarea* cultivated with organic fertilizer showed higher inhibition toward BChE (76.0 ± 0.76%) than that of the one cultivated with chemical fertilizer (45.1 ± 1.06%). However, α-pinene and 1,8-cineole (*syn.* eucalyptol) (Fig. 7), the monoterpene-type of components, were found to inhibit AChE in uncompetitive and reversible manner acting synergistically and, therefore, responsible for inhibitory effect of the essential oil of *Salvia* species [49,52]. As a matter of fact, since the anticholinesterase effect of 1,8-cineole was accounted for half the activity of the plant extract *per se*, it was considered as the main agent, which could show synergy with other constituents existing in the plant. In addition, a minor synergy was revealed in 1,8-cineole/α-pinene and 1,8-cineole/caryophyllene oxide combinations at its higher concentrations, whereas antagonism was apparent in 1,8-cineole/camphor combinations.

Furthermore, the essential oil of *S. lavandulaefolia* was clinically tested on 24 healthy young volunteers and proved to have capability in acute modulation and cognition [55]. In a randomized, placebo-controlled, double-blind, balanced, five-period crossover study, acute effects of a standardized extract of *S. officinalis* were investigated on cognitive performance in older adults and the extract administered at 333 mg of dose exerted a significant enhancement of secondary memory performance at all testing times [56].

Effect of a standardized extract prepared from the leaves of *S. officinalis* and its active ingredient rosmarinic acid was tested on Alzheimer Aβ peptide-induced toxicity in cultured rat PC12 cells and rosmarinic acid blocked ROS formation, LPS, DNA fragmentation, caspase-3 activation, and *tau* protein hyperphosphorylation as well as phosphorylated p38 mitogen-activated PK [57]. Consequently, it was suggested that rosmarinic acid could partially contribute to neuroprotective activity of sage.

Salvia fruticosa L. (*syn. Salvia triloba* L.)

S. fruticosa L. (Picture 5) has been recorded for its use against memory problems in Lebanese folk medicine [58]. Inhibitory effect of the ethanol and aqueous extracts of *S. triloba* of Lebanese origin was tested against AChE by Ellman method, and the tested extracts did not inhibit or showed a very

PICTURE 5 *Salvia fruticosa* (by Fatma Sezer Senol)

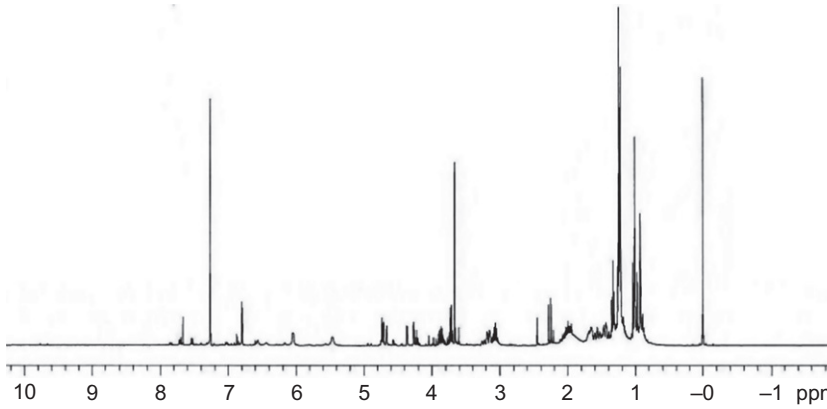

FIGURE 8 ^{1}H NMR spectrum (in $CDCl_3$) of the active subfraction of *S. fruticosa*.

low inhibition in this assay. However, the ethanol extract had a modest affinity to the $GABA_A$-benzodiazepine receptor site. According to the data obtained, the authors concluded that this species might be showing neuroprotection by some other mechanisms.

The leaves of *S. fruticosa* are consumed as tea (1–5% infusion) to treat simple disorders in Anatolian folk medicine [59]. In our screening study on 55 Turkish *Salvia* species [60], we found out that the dichloromethane extract of *S. fruticosa* was the most effective one in regard with ChE inhibition using spectrophotometric Ellman method. Therefore, we investigated the dichloromethane extract of this species cultivated in Turkey further in order to elucidate the active component(s) by activity-guided fractionation applying various chromatographic techniques [61]. ^{1}H NMR interpretation of the active subfraction underlined possible existence of terpenoid derivatives (Fig. 8).

The essential oil of the plant showed the best inhibitory activity against AChE, whose major components were elucidated as 1,8-cineol, camphor, and thujon by gas chromatography–mass spectrometry (GC–MS). In the light of these findings, we put forward that it is more advantageous for *S. fruticosa* to have both anticholinesterase and antioxidant activities and its standardized extract could be evaluated for further studies in treatment of AD. We also examined antiamnesic activity of the ethanol extract of *S. fruticosa* collected from the wild using step-through passive avoidance test in mice by scopolamine-induced amnesia model [62]. The extract was found to be the most effective in antiamnesic experiment at 100, 200, and 400 mg kg^{-1} doses having 22.7%, 57.1%, and 71.4% of relative effects, respectively.

Salvia miltiorrhiza Bunge

S. miltiorrhiza, known as "danshen" in Chinese, has been clinically prescribed in China for treatment of patients with stroke. The roots of the plant have a long history of use for cardiovascular and cerebrovascular diseases in China, Japan, Taiwan, and Korea. Since its roots are in red color, this species is also called "red sage" (Picture 6).

Neuroprotective effect of *S. miltiorrhiza* was examined in forebrain cerebral ischemia model in gerbils, and protection by this species was concluded to be caused by attenuating the dysfunctions of monoamine neurotransmitters [63]. Its protective effect was studied in cultured neurons damaged by lactate acid and found to possess positive effect possibly acting by the mechanism of which may be correlated to its Ca^{2+} antagonistic action [64]. The roots of the plant were found to inhibit superoxide generation by microglia in primary microglia cell cultures from rat brains [65]. The neuroprotection caused by *S. miltiorrhiza* was previously suggested to occur through the inhibition of leukocyte

PICTURE 6 The roots of *Salvia miltiorrhiza* (danshen) (taken from http://www.horizonherbs.com/images/products/SageChineseSalvia%20MiltiorrhizaDanShenRoot.jpg, Access date: June 30, 2011)

adherence to the endothelium, which is connected to neuronal death by some mechanisms [66]. Many neurotransmitters including norepinephrine may create brain damage by inducing excitotoxicity and by oxidizing to generate oxygen radicals or other mechanisms. *S. miltiorrhiza* has been shown to reduce the release of norepinephrine, dopamine, and serotonin during brain ischemia [63].

The main active ingredients in *S. miltiorrhiza* extract are the lipophilic diterpenes named as tanshinones (Tan), comprising of tanshinone I (Tan I), tanshinone IIA (Tan IIA), cryptotanshinone, and dihydrotanshinone as well as the water-soluble danshensu (3,4-dihydroxyphenyl lactic acid), salvianolic acid A (Sal A) and salvianolic acid B (Sal B), and isotanshinones [67].

Neuroprotective effects of Tan IIA and Tan IIB (Fig. 9) were investigated in adult mice with transient focal cerebral ischemia caused by middle cerebral artery occlusion, and the tested Tan reduced brain infarct volume in the company of a significant decrease in the observed neurological deficit [68]. Li *et al.* [69] revealed the protective effect of Tan on the neuropathological changes induced by $A\beta_{1-40}$ injection in hippocampus in rats. Tan IIB was appraised in regard with neuroprotective effects in experimentally stroked rats, which significantly reduced the focal infarct volume, cerebral histological damage, and apoptosis subjected to middle cerebral artery occlusion [70] and found to show its neuroprotective effect through inhibition of apoptosis in rat cortical neurons in another experiment by suppressing the elevated Bax protein and decreasing bcl-2 and caspase-3 proteins induced by staurosporine [71]. The neuroprotective effect of Tan IIA (10 mg/kg, i.p.) was examined against hypoxia–ischemia brain damage induced in neonatal rats and treatment with Tan IIA considerably reduced the severity of brain injury, as indicated by the increase in ipsilateral brain weight and neuron density [72]. The same compound was also tested in permanent focal cerebral ischemia in mice in order to resolve the probable mechanisms of its neuroprotective effect [73]. In this experiment, biochemical analyses for malondialdehyde (MDA) content and super oxide dismutase (SOD) activity in serum, and NO content and the inducible nitric oxide synthase (iNOS) activity in brain tissue were performed at 24 h after ischemia. The results illustrated that Tan IIA showed its protective effect from ischemic injury in the brain by suppressing the oxidative stress and the radical-mediated inflammatory affront.

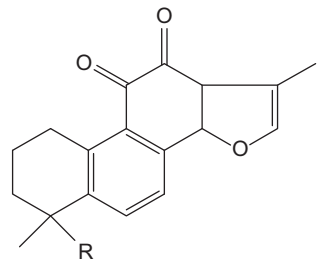

FIGURE 9 Tan IIA (R═CH_3) and Tan IIB (R═CH_2OH).

Salvianolic acid derivatives in *S. miltiorrhiza* appear to be synthesized from monoterpenoids [74]. Acetylsalvianolic acid A, a semisynthetic derivative of salvianolic acid from *S. miltiorrhiza* is neuroprotective in middle cerebral artery thrombosis. Sal B (Fig. 10), also named as "lithospermic acid B or tanshinoate B," was investigated against $A\beta_{25-35}$-induced neurotoxicity and the expression of brain-pancreas relative protein (BPRP), which is a new protein and mainly expressed in brain and pancreas in PC12 cells and the findings pointed out that Sal B, as an antioxidant, has ability to protect toward $A\beta_{25-35}$-induced reduction in expression of BPRP *via* its suppressing effect on the production of ROS, calcium flux, and apoptosis [75] as well as H_2O_2-induced injury in rat PC12 cells [76].

Pretreatment of the cells with Sal B (0.1–10 μM), prior to H_2O_2 exposure, blocked H_2O_2-induced cytotoxicity remarkably. Neuroprotective effect of Sal B was proved against 6-OHDA-induced cell death in human neuroblastoma SH-SY5Y cells [77]. Pretreatment of SH-SY5Y cells with Sal B significantly reduced 6-OHDA-induced generation of ROS and prevented 6-OHDA-induced increases in intracellular calcium. The obtained data demonstrated that 6-OHDA-induced apoptosis was reversed by Sal B treatment. Sal B was also tested for its inhibitory potential toward $A\beta_{1-40}$ fibril formation and destabilization of the preformed $A\beta_{1-40}$ fibrils using thioflavin T fluorescence assay and Aβ aggregating immunoassay [78]. The results revealed that Sal B prevented the formation of β-structured aggregates of $A\beta_{1-40}$ in dose-dependent manner and addition of preincubated Sal B with $A\beta_{1-42}$ significantly reduced its cytotoxic effects on human neuroblastoma SH-SY5Y cells.

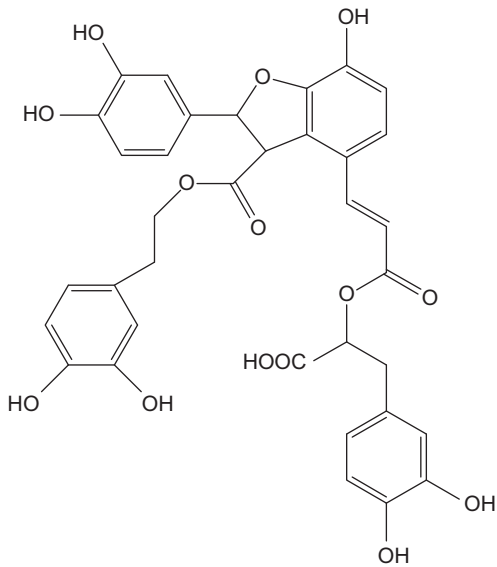

FIGURE 10 Salvianolic acid B (Sal B).

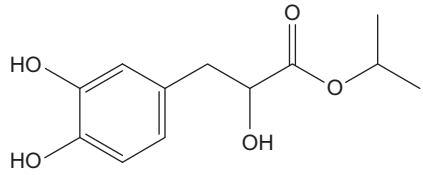

FIGURE 11 ND-309 [isopropyl-β-(3,4-dihydroxyphenyl)-α-hydroxypropanoate].

Magnesium lithospermate B was evaluated for its neuroprotective effect in ischemic stroke using a cortical brain slice-based assay model and found to inhibit Na^+, K^+-ATPase, which contributes to anti-ischemic neuroprotection by *S. miltiorrhiza* [79]. Sal A isolated from *S. miltiorrhiza* has been shown to exert protective effect on neurotoxicity induced by 1-methyl-4-phenylpyridinium ion (MPP^+), an inhibitor of mitochondrial complex I, in human neuroblastoma SH-SY5Y cells, related to PD [80].

Another compound coded as ND-309 [isopropyl-β-(3,4-dihydroxyphenyl)-α-hydroxypropanoate] (Fig. 11) has been found to be a new metabolite of danshen in rat brain [81]. In adult male rats, ND-309 considerably reduced neurological deficit scores, infarct volume, as well as the edema compared with the model group. In addition, ND-309 significantly improved mitochondrial energy metabolism and attenuated the elevation of MDA content, while it caused a decrease in SOD and generation of ROS in brain mitochondria.

CONCLUSION

Lamiaceae, one of the largest plant families in the world, contains a considerable number of medicinal and aromatic plants. Some of the members of this family have been investigated for their neuroprotective effects through various *in vitro* and *in vivo* methods. Since most of these species are consumed as culinary herbs, spices, tea, and folk medicine, it is important to establish their neuroprotective prospective. Intensive studies by our group as well as other researchers have shown that some Lamiaceae species such as *R. officinalis* (rosemary), *S. baicalensis* (skullcap), and several *Salvia* (sage) species have a great neuroprotective potential. Among them, especially *S. miltiorrhiza* (danshen), whose active components are almost established, is quite promising in terms of neuroprotection. The research on the same subject is in fast progress in the world to discover an effective plant-originated drug candidate from the Lamiaceae plants.

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ABBREVIATIONS

$A\beta_{25-35}$	amyloid-β $peptide_{25-35}$
ACh	acetylcholine
AChE	acetylcholinesterase
AD	Alzheimer's disease
BChE	butyrylcholinesterase
BPRP	brain-pancreas relative protein
ChE	cholinesterase
DPPH	2,2-diphenyl-1-picrylhydrazyl
ELISA	enzyme-linked immunosorbent assay
FRAP	ferric-reducing antioxidant power
GABA	γ-aminobutyric acid
GC–MS	gas chromatography–mass spectrometry
HD	Huntington's disease
HPLC	high-performance liquid chromatography
iNOS	inducible nitric oxide synthase
LPS	lipopolysaccharide
mAChR	muscarinic acetylcholine receptor
MDA	malondialdehyde
MPP^+	1-methyl-4-phenylpyridinium ion
MPTP	methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NF	nuclear factor
NMDA	*N*-methyl-D-aspartate
NMR	nuclear magnetic resonance
NO	nitric oxide
NPCs	neural precursor cells
OHDA	hydroxydopamine
PC12	pheochromocytoma
PD	Parkinson's disease
PK	protein kinase
ROS	reactive oxygen species
Sal A	salvianolic acid A
Sal B	salvianolic acid B
SCI	spinal cord injury
SOD	super oxide dismutase
Tan	tanshinones
Tan I	tanshinone I
Tan IIA	tanshinone IIA
Tan IIB	tanshinone IIB
TNF-α	tumor necrosis factor-alpha

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Chapter 15

Phytochemicals: In Pursuit of Antitubercular Drugs

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The person who takes medicine must recover twice, once from the disease and once from the medicine.

William Osler, M.D.

INTRODUCTION

From the times when man lived in harmony with nature to these present times, treatment methods have come full circle. The relationship between the healing power of nature and cure of diseases is being revisited, mainly because the mainstream methods of treatment have not been effective enough. Antibiotics that were once thought to be "the panacea" are failing to combat the ever-mutating microorganisms. The logical answer seems to be going back to nature. Natural products appear to be a promising source of cure as it is less risky, more economical, and reliable enough to alleviate the root cause with fewer side effects. For ages, plants have been a good source of food and they provide essential nutrition, valuable therapeutics, and notable physiological effect to life [1].

This review is complementary to earlier reviews and covers studies of naturally occurring compounds from medicinal plants, the rich source for biologically active compounds having antitubercular properties. The World Health Organization (WHO) has estimated that approximately 80% of the world's inhabitants rely mainly on traditional medicines for their health care [2].

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The complementary and alternative forms of medicines practiced worldwide have been relying mainly on the local flora for their medical needs. The use of plant drugs and derivatives in the allopathic system is also increasing. It is obvious that an increased use of natural product-based drugs will be followed in the future. The first written records on the medicinal uses of plants appeared in about 2600 BC from the Sumerians and Akkaidians [3]. Documentation of the Ayurvedic system, recorded by Susruta and Charaka, dates from about 1000 BC [4]. The first book on traditional Chinese medicine (TCM) was compiled around the first century BC [5]. An improvement of traditional health-care systems (such as TCM, Ayurveda, Naturopathy) and studies on the derivation of drugs through bioprospecting of the medicinal plants are thus of great importance [6]. A thorough survey of the literature yields several ethanobotanicals and phytomolecules that display promising activity against different species of mycobacteria [7].

AN ANCIENT SCOURGE: TUBERCULOSIS

Tuberculosis (TB) describes an ancient scourge, an infectious disease that has plagued humans since the Neolithic times and even today is responsible for more than a million deaths worldwide annually [8], easily making it one of the most deadly diseases. In 460 BC, the Greek physician Hippocrates described TB as an "almost always fatal disease of the lungs" [9]. Physicians in ancient Greece called this illness "*phthisis*" to reflect its wasting character [10]. As an airborne nightmare [11], it is highly contagious and spreads mainly through coughs and sneezes. The bacteria can attack any part of the body, but they usually stick to the lungs [12]. The ease with which TB infection spreads [13] has helped to sustain this scourge at current levels. In spite of half a century of anti-TB chemotherapy, one-third of the world's population still harbors a dormant or latent form of *Mycobacterium tuberculosis* (*Mtb*). This gives rise to a lifelong risk of disease reactivation. One in 10 latent infections eventually progress to active disease, which if left untreated, kills more than 50% of its victims.

THE NEXT CATASTROPHE: TB TSUNAMI

With each passing year, this epidemic is assuming the proportions of a tsunami. According to the WHO, there were 8.4 million new cases in 1999 up from 8 million the year before [9]. It is estimated that about 2 billion people, equaling one-third of the world's total population, are infected with *Mtb* [8,14] and new infections occur at a rate of about one per second. The years of neglect and mismanagement have created multidrug-resistant (MDR) strains of TB; this almost incurable form of TB is spreading quickly from Russia and other "TB hot spots" to the rest of the world [15].

THE CULPRIT: *MTB*

The initial studies on TB bacteria were conducted by Robert Koch in the year 1882. Systematically, he showed how TB was caused by a bacterium through a four-step procedure that is still referred to as Koch's postulates [16]. The genus *Mycobacterium* (Mycobacteriaceae) is highly diverse with 85 species, yet known. Some cause human and animal diseases and others are omnipresent in nature. The three most common human mycobacterial diseases are the primary pulmonary disease, TB, and two skin diseases, leprosy (*M. leprae*) and buruli ulcer (*M. ulcerans*). Once the causative agent was identified, the entire attention was focused on killing the bacteria. But the TB bacteria are very difficult to kill as they have a tough, waxy outer shell, and when attacked and isolated by the macrophages, they pile up and form tubercles. These tubercles have hard walls made of scar tissue and inside lie the live bacteria sealed up and away from the reach of the so-called drugs and antibiotics. These bacteria get reactivated when the immunity is compromised.

For example, in case of human immunodeficiency virus (HIV) infection, this weakens the immune system and makes people at least 800 times more susceptible to reactivation. Management of the disease in patients coinfected with HIV has the following challenges:

1. Pill burden: Strictly following the directly observed treatment short-course (DOTS) regimen for TB (about 10–12 pills/day) and the recommended regimen for acquired immune deficiency syndrome (AIDS) therapy ($\sim$20 pills/day), round the clock puts tremendous stress on the patients.
2. Drug interactions between the TB and AIDS medication can create problems in the prescribed drug regimen.
3. Functional monotherapy leads to MDR infections among AIDS patients infected with TB.

Similarly, diabetes mellitus is also a well-known risk factor for TB [17]. Diabetes is known to complicate the management of TB. Diabetes medication may interact with TB treatment (rifampicin in particular) with corresponding complications in glycemic control. Diabetic patients are not only more susceptible to infection like TB, but also when infections do occur, they are more severe as the diabetic is a compromised host.

CURRENT DRUGS

It is easier to kill the bacteria in the initial stages because the possibility of drug resistance is very low. As the disease progresses, lack of patient compliance to the prescribed combination therapy is mainly the reason for worldwide presence of MDR strains and extremely drug-resistant (XDR) strains [18,19]. Once created, these strains spread in the same way as ordinary TB, resulting in a human-made disease which few antibiotics can possibly cure [15].

The commonly followed treatment regimen usually includes streptomycin (introduced in 1946), isoniazid (INH) and pyrazinamide (PZA) (introduced in 1952), ethambutol (1963), and rifampin (1966). It is almost 50 years since a new drug has been introduced in the treatment of TB. MDR-TB describes strains of TB that are resistant to at least the two main first-line TB drugs—isoniazid and rifampin. Genetic and molecular analysis of drug resistance in *Mtb* suggests that resistance is usually acquired by the bacilli either by alteration of the drug target through mutation [20] or by titration of the drug through overproduction of the target [21]. XDR-TB or extensive drug-resistant TB (also referred to as extreme drug resistance) is MDR-TB that is resistant to three or more of the six classes of second-line drugs (SLDs). XDR-TB poses a grave public health threat, especially in populations with high rates of HIV and also economically challenged societies. In 2008, an estimated 440,000 cases of MDR-TB emerged globally [22]. Together, India and China comprise the largest population of MDR-TB, estimated to be about 50% of the world's total cases. It is of greater concern that almost three quarters of the estimated cases of MDR-TB occur in previously undiagnosed patients [23].

The regimen currently followed for the treatment of the MDR- and XDR-TB is as follows:

1. The first-line drugs are ethambutol, isoniazid, PZA, rifampicin, and streptomycin.
2. There are six classes of SLDs used for the treatment of TB. A drug may be classed as second line instead of first line for one of three possible reasons: (1) Effectiveness is lower than the first-line drugs (e.g., *para*-aminosalicylic acid (PAS/P)), (2) toxic side effects (e.g., cycloserine), and (3) unavailability in many developing countries (e.g., fluoroquinolones). Examples of SLDs are as follows:
   - Aminoglycosides, for example, amikacin (AMK), kanamycin
   - Polypeptides, for example, capreomycin, enviomycin
   - Fluoroquinolones, for example, ciprofloxacin, levofloxacin
   - Thioamides, for example, ethionamide, prothionamide
   - Cycloserine (the only antibiotic in its class)
   - PAS/P
3. The third-line drugs are not on the WHO list but are listed here either because they are not very effective (e.g., clarithromycin) or because their efficacy has not yet been proven (e.g., linezolid). Rifabutin is effective but comparatively very expensive. Some examples of third-line drugs are as follows:
   - Rifabutin
   - Macrolides, for example, clarithromycin (CLR)
   - Linezolid (LZD)
   - Thioacetazone

- Thioridazine
- Arginine

Unfortunately, the fact remains that all the above listed drugs (even the first-line drugs) have adverse side effects like thrombocytopenia caused by rifampin, neuropathy caused by isoniazid, rashes most frequently caused by PZA, and so on. Rifampin commonly causes itching without a rash in the first 2 weeks of treatment. The single biggest problem with TB treatment is drug-induced hepatitis, which has a mortality rate of around 5% [24]. Three drugs can induce hepatitis: PZA, isoniazid, and rifampin (in decreasing order of frequency) [25,26].

The WHO declared TB a global health emergency in 1993. The Stop TB Partnership developed a strategy to stop TB that aims to save 14 million lives worldwide between 2006 and 2015 [22]. DOTS that stands for "directly observed treatment, short course" is the strategy of TB treatment regimen in the WHO Global Plan to Stop TB. The DOTS strategy focuses on five main points of action which include government commitment to control TB, diagnosis based on sputum-smear microscopy tests done on patients who actively report TB symptoms, direct observation short-course chemotherapy treatments, a definite supply of drugs, and standardized reporting and recording of cases and treatment outcomes. [27]

Drugs are not used singly (except in latent TB or chemoprophylaxis) because of the simple reason that single drugs result in the rapid development of resistance and treatment failure [25,28]. Moreover, the different drugs in the combination therapy regimen have different modes of action. Isoniazid is bactericidal against replicating bacteria. Ethambutol is bacteriostatic at low doses but is used in TB treatment at higher bactericidal doses. Rifampicin is bactericidal and has a sterilizing effect. PZA is only weakly bactericidal but is very effective against bacteria located in acidic environments, inside macrophages, or in areas of acute inflammation.

NEED FOR ALTERNATIVE AND INTEGRATIVE FORMS OF TREATMENT

Although the existing drugs are of great value in controlling TB, they have several shortcomings. Drug resistance, adverse effects, failure to act on the latent bacilli, etc. are some of the few. Moreover, the current drug regimen is 40 years old and takes 6–9 months to administer. In addition, the emergence of drug-resistant strains and HIV coinfection mean that there is an urgent need for new anti-TB drugs [29]. With the rising incidence of drug resistance to existing TB drugs, as demonstrated by the occurrence of MDR strains of *Mtb* and the recent outbreak in South Africa of XDR-TB [30], the search for new anti-TB drug leads urgently needs to gain momentum.

DRUG TARGETS OF CURRENT DRUGS

Identification and validation of appropriate targets for designing drugs are critical steps in drug discovery, which are at present major bottlenecks. Still now, among 3999 proteins encoded by *Mtb* genome, only 9 have been pharmaceutically investigated as drug targets. The drug–target interaction network for all structurally characterized approved drugs bound to putative *Mtb* receptors is now referred to as the "TB-drugome." The TB-drugome reveals that approximately one-third of the drugs examined have the potential to be repositioned to treat TB and that many currently unexploited *Mtb* receptors may be chemically druggable and could serve as novel antitubercular targets [31].

The drug targets for the currently used drugs are as follows:

Isoniazid and ethionamide inhibit the mycolic acid synthesis [32], while cycloserine and ethambutol inhibit synthesis of peptidoglycan [33] and arabinogalactan, respectively, needed for building up the cell wall [34], thereby weakening the cell wall of the *Mycobacterium*. Rifampin and AMK exert their pharmacological action by inhibiting bacterial RNA (ribonucleic acid) or protein synthesis [35]. Summarized in Table 1 is an exhaustive account of the current and probable drug leads with their site of action.

This review highlights the structural variations in antitubercular phytochemicals since the past 4 years. The different classes of compounds discussed here are alkaloids, terpenes, phenolics, flavones, steroids, fatty acids, and other compounds from plants. Our intention is to generate sustained interest in this area by natural product chemists, synthetic chemists, biochemists, and computational chemists to construct analog classes of these compounds that can be further investigated for drug development.

Alkaloids

Natural product literature shows an ever-increasing number of publications based on plant-derived antimycobacterial alkaloids. Several alkaloids have reportedly displayed promising activity against different species of mycobacteria. In the review by Newton *et al.* [87], 12 pure alkaloids were among the metabolites reported to have antimycobacterial activities. Since then, many new and known naturally occurring alkaloids and analogs have been assayed. Identification of new targets for plant products, especially the alkaloids, may be crucial in the context of finding new drugs effective against MDR-TB. Table 2 and Fig. 1 represent a list of alkaloids with promising results against *Mtb*.

Phenols

Plant phenols, sometimes called phenolics, are a class of most common and widespread plant metabolites consisting of a hydroxyl functional group (—OH) attached to an aromatic hydrocarbon group. As a general rule, the

TABLE 1 Drugs, Drug Leads, and Known Targets

S. No.	Inhibitor/Drug	Enzyme Target	References
1.	Pyrazinamide	Cyclosporane fatty acyl phospholipid synthase 1	[36]
2.	Rifabutin	RpoB, RNA polymerase	[37]
3.	Rifampin	Probable DNA-directed RNA polymerase	[38,39]
4.	Ethambutol	Integral membrane indolyl acetyl inositol arabinosyl transferase	[40–42]
5.	Isoniazid	NADH-dependent enoyl reductase (INHA)	[43]
6.	Thiacetazone	Methoxy mycolic acid synthase (MMA1)	[44]
7.	Meropenem	Class A beta lactamase (BLAC)	[45,46]
8.	D-Cycloserine	D-Alanine ligase	[47]
9.	Streptomycin	Protein synthesis	[48]
10.	Fusidic acid	Protein synthesis	[49]
11.	AMK	Protein synthesis	[35,50]
12.	Levofloxacin	DNA gyrase	[51]
13.	Diarylquinoline R207910/TMC207	Probable ATP synthase (C chain ATPE)	[52]
14.	Isoxyl	Possible linoleoyl CoA desaturase	[53]
15.	Triclosan	NADH-dependent enoyl reductase (INHA)	[54]
16.	Gemfibrozil	NADH-dependent enoyl reductase (INHA)	[55]
17.	Comtan	NADH-dependent enoyl reductase (INHA)	[56]
18.	Fosmidomycin	Probable 1-deoxy D-xylulose 5-phosphate reducto isomerase (DXR)	[57]
19.	Myxopyronin	RNA polymerase	[38]
20.	BTZ043	Decaprenylphosphoryl beta D-ribose 2′-oxidase (DPRE1)	[45,58]

Continued

TABLE 1 Drugs, Drug Leads, and Known Targets—Cont'd

S. No.	Inhibitor/Drug	Enzyme Target	References
21.	Clavulanate	Class A beta lactamase (BLAC)	[45,46]
22.	Albendazole	Cell division protein (FtsZ)	[59,60]
23.	Thiabendazole	Cell division protein (FtsZ)	[59,60]
24.	Chelidamic acid	Probable dihydrodipicolinate synthase (DAPA)	[61]
25.	3-Bromopyruvate	Isocitrate lyase (ICL1)	[62,63]
26.	Trifluoperazine	Conserved hypothetical protein	[64]
27.	Platensimycin	3-Oxoacyl synthase 2 (KASB)	[65,66]
28.	Indisulam	Beta carbonic anhydrase (CANA)	[67]
29.	Acetazolamide	Probable transmembrane carbonic anhydrase	[67]
30.	Tetrahydrobenzothiophene AX20017	Serine/threonine protein kinase	[68]
31.	Double site isoxazole based compounds	Phosphotyrosine protein phosphatase (PTPB)	[69]
32.	Nitrobenzothiazole	Probable ATP phosphoribosyltransferase (HisG)	[70]
33.	Hexahydroisoindole-1,3-dione	Probable L-lysine ε-aminotransferase (LAT)	[71]
34.	Cyclohexylmethylamine derivatives	Probable L-lysine ε-aminotransferase (LAT)	[71]
35.	Ribitypurinetrione compounds	Probable bifunctional riboflavin biosynthesis protein (RIBG)	[72]
36.	*N*1,*Nn*-bis-(5-Deoxy-*a*-D-xylofuranosylated) diamines	Probable DNA ligase (LigA)	[73]
37.	Glycosyl ureides	Probable DNA ligase (LigA)	[74]
38.	Pyridochromanones	Probable DNA ligase (LigA)	[74]
39.	5′-*O*-[(*N*-Acyl)sulfamoyl] adenosines	Bifunctional enzyme (MBTA) salicyl AMP ligase	[75]

TABLE 1 Drugs, Drug Leads, and Known Targets—Cont'd

S. No.	Inhibitor/Drug	Enzyme Target	References
40.	Methyl 2-(2-bromoacetamido)-5-(3-chlorophenyl) thiazole-4-carboxylate	3-Oxoacyl synthase III (FABH)	[76]
41.	Butein	(3*R*)-Hydroxyacyl ACP dehydratase subunit (HADB)	[77]
42.	5-*tert*-Butyl-*N*-pyrazol-4-yl-4,5,6,7-tetrahydrobenzo[*d*] isoaxazole-3-carboxamide derivatives	Probable pantoate-beta alanine ligase (PANC)	[78]
43.	Tripeptide inhibitor WYY	Dihydrofolate reductase (DFRA)	[79]
44.	BB-3497	Probable polypeptide deformylase (PDF)	[80]
45.	Actinonin	Probable PDF	[80]
46.	1,10-Phenanthroline	PDF	[80]
47.	Hydroxylamine hydrochloride	PDF	[80]
48.	Galardin	PDF	[80]
49.	Deoxygenated α (1 → 5)-linked arabinofuranose disaccharides	Arabinosyltransferases	[81]
50.	Tetrahydrolipstatin	Probable conserved membrane protein	[82]
51.	Lipiarmycin	RNA polymerase	[39]
52.	Novobiocin	DNA gyrase (gyrA)	[83]
53.	Lauroyl sulfamate	Fatty acid AMP ligase (fadD28)	[84]
54.	1-(3-(5-ethyl-5H-[1,2,4] Triazino[5,6-β]indol-3-ylthio) propyl)-1H-benzo[*d*]imidazol-2(3H)-one	dTDP-4-dehydrarhamnose 3,5,epimerase (RMLC)	[85]
55.	E-600	Triacyl glycerol lipase (lipY)	[86]
56.	Isoliquiritigenin	(3*R*)-Hydroxyacyl ACP dehydratase subunit (HADB)	[77]

These data are partially from OSDD repository (courtesy OSDD-CSIR India).

TABLE 2 Alkaloids

S. No.	Phytomolecule	Plant/Plant Part Used	Bacterial Strain Tested	Assay Used	Activity MIC (μg/mL) or % Inhibition	References
1	Micromeline **1**	*Micromelum hirsutum* Merr.	Mtb H37Rv	Fluorometric microplate Alamar blue assay	31.5 ± 0.2	[88,89]
2	Lansine **2**	Stem bark			14.3 ± 0.9	
3	3-Formylcarbazole **3**				42.3 ± 0.5	
4	3-Formyl-6-methoxycarbazole **4**				15.6 ± 0.2	
5	Chabamide **5**	*Piper chaba Hunter* Stem bark	Mtb H37Ra	MABA	12.5	[89,90]
6	Piperolactam A **6**	*Piper sanctum* (Miq.) Schl. Leaves	Mtb H37Rv	MABA	8	[89,91]
7	Chelerythrine **7**	*Sanguinaria canadensis* L. Stem	*M. aurum*	Microdilution	7.3	[89,92]
8	Sanguinarine **8**				9.61	
9	Ambroxol **9**	*Adhatoda vasica* Nees. Leaves	*Mtb*	Broth dilution	64	[89,93]
10	Bromhexine **10**				128	
11	8-Hydroxy canthin-6-one **11**	*Allium neapolitanum* Cirillo Fresh bulbs	*M. phlei* (ATCC 11758)	Broth dilution	**11** **12**	[89,94]
12	Canthin-6-one **12**		*M. smegmatis* (ATCC $mc^2$2700)		2 8 8 8	

13	2-(Methyldithio)pyridine-*N*-oxide **13**	*Allium stipitatum* Regel	*M. bovis* BCG	Spot culture	**13**	**14**	[89,95]
14	2-[(Methylthiomethyl) dithio]pyridine-*N*-oxide **14**	Bulbs	Mtb H37Rv	Growth inhibition assays	0.1	0.1	
					0.1	0.1	
15	Cryptolepine hydrochloride **15**	*Cryptolepis sanguinolenta* (Lindl.) Schltr	*M. aurum*	MABA assay	2		[89,96]
			M. phlei		12.5		
		Roots	*M. smegmatis*		4		
			M. bovis		8		
16	2-Acetyl benzyl amine **16**	*Adhatoda vasica* Ness.		Agar dilution method	**16**	**17**	[97]
17	Vasicine acetate **17**	Leaves	Mtb		100	100	
			Resistant		200	200	
			(HR-Res)				
			Sensitive		50	50	
			(HR-Sen)				
18	(−)-Nordicentrine **18**	*Goniothalamus laoticus* (Finet & Gagnep.) Bân Flowers	Mtb	Microplate Almar blue assay	12.5		[98]

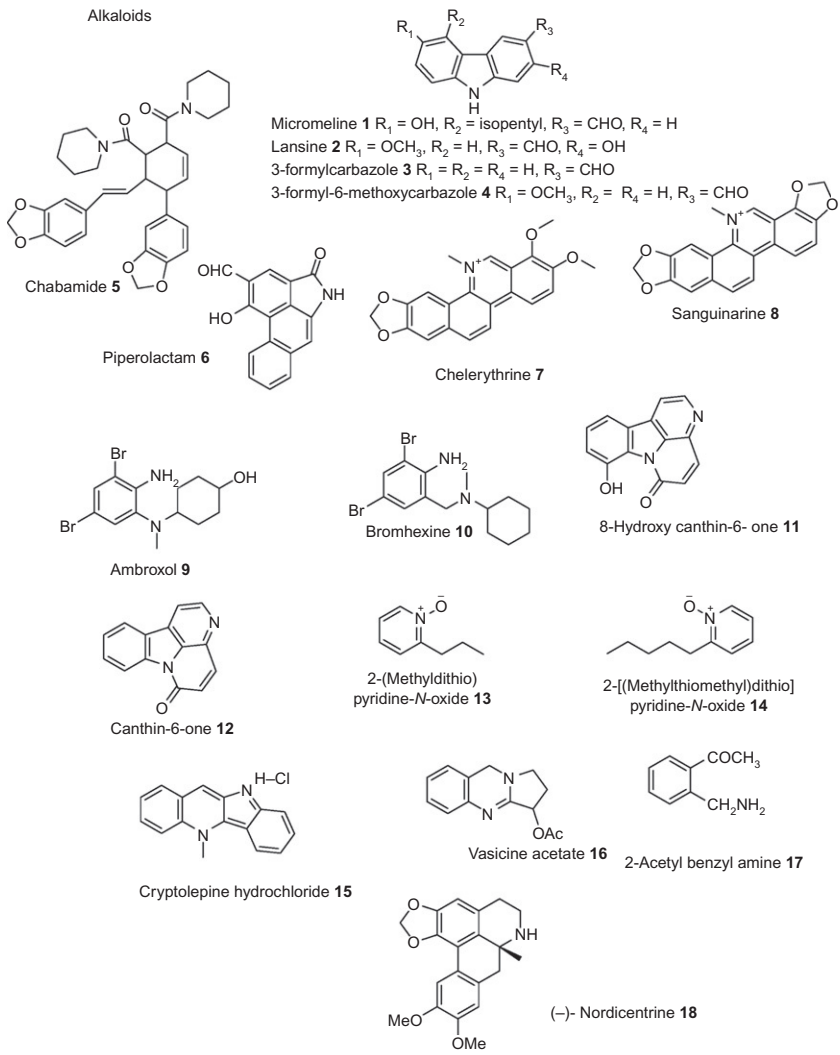


FIGURE 1 Alkaloids with antitubercular activity.

terms phenolics and polyphenols refer to all secondary natural metabolites arising biogenetically from the shikimate–phenylpropanoid–flavonoid pathways, producing monomeric and polymeric phenols and polyphenols. Tabulated in Table 3 and Figs. 2–4 are several phenolic metabolites, such as pterocarpans, isoflavones, flavanones, chalcones, etc. that have displayed significant antimicrobial activity and can be further pursued as potential drug leads.

TABLE 3 Phenols

	Phytomolecule	Plant/Plant Part Used	Bacterial Strain Tested	Assay	Activity MIC (μg/mL) or % inhibition	References
1	Indicanine B **19**	*Erythrina variegata* L. var. *orientalis* (L.) Merr.	*M. smegmatis*	Agar streak dilution	18.5	[99]
2	Indicanine C **20**	Root bark			>150	
3	5,4′-*O*- methyl-alpinumisoflavone **21**				>450	
4	Cajanine **22**				>400	
5	Diospyrin **23**	*Euclea undulata* Thunb.	*M. fortuitum*	Modified twofold	41.67	[100]
6	7-Methyljuglone **24**	Root	(ATCC 6841)	serial dilution assay	1.57	
10	*E*-ω-Benzoyloxyferulenol **25**	*Ferula communis* L.	*M. aurum* (Pasteur	Muller-Hinton	8	[89,101]
11	Ferulenol **26**	Root	Institute 104482)	broth	2	
12	Glabridin **27**	*Glycyrrhiza glabra* L. Root	Mtb (1) H37Rv (2) H37Ra	BACTEC 460 system	(1) 29.16 (2) 29.16	[89,102]
13	Licochalcone A **28**	*Glycyrrhiza inflata* Batalin Root	Mtb	BACTEC 460 system	7.1	[89,103]

Continued

TABLE 3 Phenols—Cont'd

	Phytomolecule	Plant/Plant Part Used	Bacterial Strain Tested	Assay	Activity MIC (μg/mL) or % inhibition	References
14	Ermanin **29**	*Haplopappus sonorensis* (A. Gray) S.F. Blake	Mtb H37Rv	BACTEC 460 system	100	[89,102,104]
15	Kumatakenin **30**	Whole plant			100	
16	5-Hydroxy-trimethoxy flavones 31				100	
17	Peucenin-7-methyl ether **32**	*Harrisonia perforata* (Blanco) Merr.	Mtb H37Ra	Microplate Alamar blue assay	50	[89,105]
18	*O*-Methylalloptaeroxylin **33**	Branches			100	
19	Perforamone A **34**				200	
20	Perforamone B **35**				25	
21	Perforamone C **36**				200	
22	Perforamone D **37**				25	
23	Eugenin **38**				100	
24	Greveichromenol **39**				100	

25	α-Mangostin **40**	*Garcinia mangostana* L.	Mtb H37Ra	MABA	12.5	[89,106]
26	β-Mangostin **41**	Green fruit hulls			6.24	
27	γ-Mangostin **42**				12.5	
29	Garcinone D **43**				6.24	
30	Mangostenol **44**				6.24	
31	Garcinone B **45**				25	
32	Prenylated xanthone derivative **46**				25	
33	Mangostatin **47**				100	
34	Mangostenone A **48**				12.5	
35	Mangostanol **49**				25	
36	Trapezifolixanthone **50**				200	
37	Tovophyllin B **51**				25	
38	Demethyl calabaxanthone **52**				25	
40	Mangostinone **53**				200	
41	7-Demethylartonol E **54**	*Artocarpus rigidus* Blume subsp. *rigidus*	Mtb H37Ra	MABA	50	[89,107]
42	Artorigidusin **55**	Not given			12.5	
43	Artonol B **56**				6.25	
44	Artoindonesianin C **57**				12.5	
45	Artonin F **58**				100	
46	Cycloartobiloxanthone **59**				25	

Continued

TABLE 3 Phenols—Cont'd

	Phytomolecule	Plant/Plant Part Used	Bacterial Strain Tested	Assay	Activity MIC (μg/mL) or % inhibition			References
47	(−)-Butin **60**	*Butea monosperma* (Lam.) Taub.	Mtb H37Ra	Microplate Alamar blue assay	25			[89,108]
48	Butein **61**	Flowers			12.5			
49	(−)-Butrin **62**				50			
50	(−)-Isomonospermoside **63**				25			
51	Dihydromonospermoside **64**				25			
52	(−)-Liquiritigenin **65**				50			
53	Monospermoside **66**				50			
54	Isoliquiritigenin **67**				25			
55	7,3′,4′-Trihydroxyflavone **68**				25			
56	Formononetin **69**				50			
57	Afrormosin **70**				25			
58	Fomononetinin-7-*O*-β-D-glucopyranoside **71**				100			
59	Licarin A **72**	*Aristolochia taliscana* Hook. & Arn.		MABA assay	**72**	**73**	**74**	[89,109]
60	Licarin B **73**	Roots	*M. non-chromogenicum*		12.5	25	ND	
61	Eupomatenoid-7 **74**		*M. smegmatis*		>200	25	6.25	
			M. fortuitum		>200	50	6.25	
			M. chelonae		>200	25	3.25	
			M. avium		>200	50	6.25	

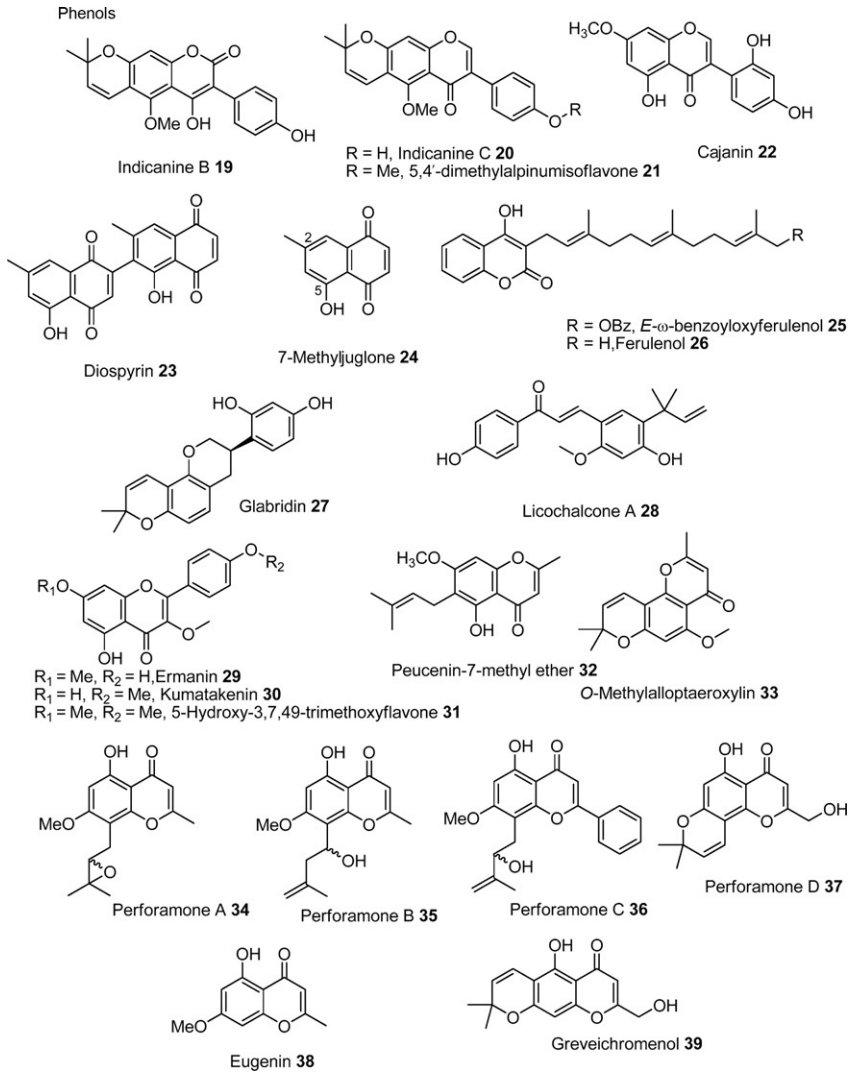
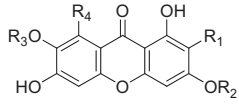
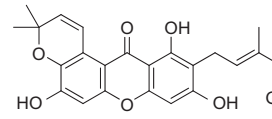


FIGURE 2 Phenols with antitubercular activity.

Flavones are a class of flavonoids based on the backbone of 2-phenylchromen-4-one (Table 4). In recent years, scientific and public interest in flavones has grown enormously due to their putative beneficial effects against atherosclerosis, osteoporosis, diabetes mellitus, and certain cancers [114]. Tabulated in Table 5 and Figs. 5 and 6 are a list of flavones derived from plants, showing activity against *Mycobacterium* species and which can be further modified and modeled to become potential drug leads.

Phenols II

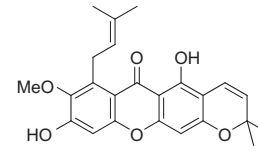




Garcinone B **45**

R1 = R4 = Prenyl, R2 = H, R3 = Me, α-Mangostin **40**
R1 = R4 = Prenyl, R2 = R3 = Me, β-Mangostin **41**
R1 = R4 = Prenyl, R2 = R3 = H, γ-mangostin **42**
R1 = Prenyl, R2 = H, R3 = Me, R4 =

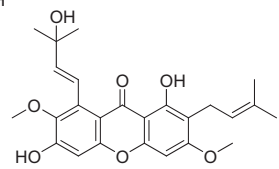
Garcinone D **43**



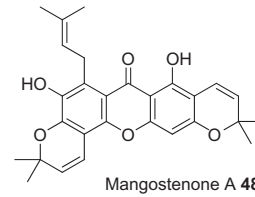
R1 =

R2 = H, R3 = Me, R4 = Prenyl, Mangostenol **44**

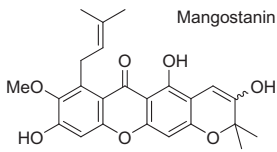
5,9-Dihydroxy-8-methoxy-2,2-dimethyl-7-(3-methylbut-2-enyl)-2H,6H-pyrano[3,2-β]xanthen-6-one. **46**



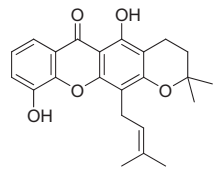
Mangostanin **47**



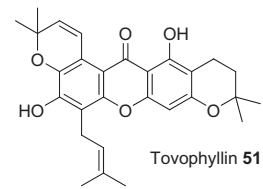
Mangostenone A **48**



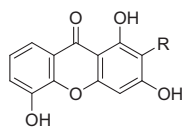
Mangostanol **49**



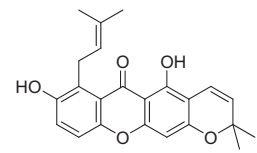
Trapezifolixanthone **50**



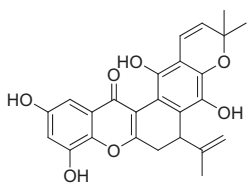
Tovophyllin **51**



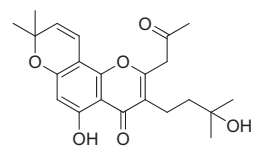
R = Geranyl,Mangostinone **53**



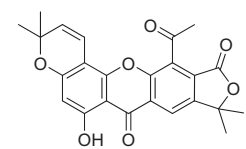
Demethylcalabaxanthone **52**



7-Demethylartonol E **54**



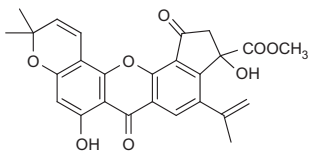
Artorigidusin **55**



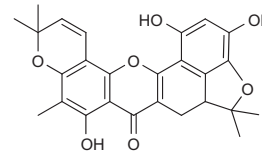
Artonol B **56**

FIGURE 3 Phenols with antitubercular activity.

Phenols III

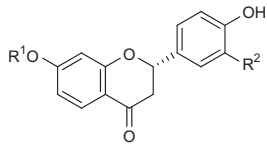


Artoindonesianin C **57**



R =  Artonin F **58**

R = H,Cycloartobiloxanthone **59**



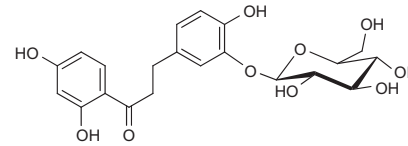
R^1 = H, R^2 = OH, Butin **60**

R^1 = Glu, R^2 = OGlu, Butrin **62**

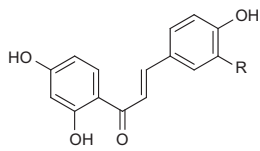
R^1 = H, R^2 = OGlu, Isomonospermoside **63**

R^1 = R^2 = H, Liquiritigenin **65**

R^1 = R^2 = OH, 7, 3′, 4′-Trihydroxyflavone **68**



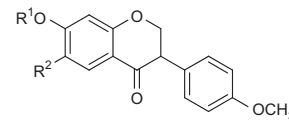
Dihydromonospermoside **64**



R = OH, Butein **61**

R = OGlu Monospermoside **66**

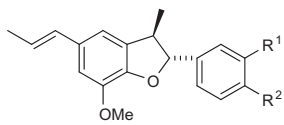
R = H Isoliquiritigenin **67**



R^1 = R^2 = H, Formononetin **69**

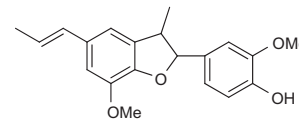
R^1 = H, R^2 = OCH_3, Afrormosin **70**

R^1 = Glu, R^2 = H, Formononetin-7-*O*-β-D-glucopyranoside **71**



R^1-R^2 = $–OCH_2O–$, Licarin A **72**

R^1 = OMe, R^2 = H, Licarin B **73**



Eupomatenoid-7 **74**

FIGURE 4 Phenols with antitubercular activity.

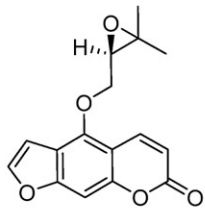
TABLE 4 Flavones

S. No.	Phytomolecule	Plant/Plant Part Used	Bacterial Strain Tested	Assay/Control Used	Activity MIC (μg/mL) or % Inhibition		References
1	Oxypeucedanin **75**	*Anethum graveolens* L. Whole plant	*M. fortuitum* (ATCC 6841)	Columbia Blood Agar	**75**	**76**	[110]
2	Oxypeucedanin hydrate **76**		*M. phlei* (ATCC 11758)		32	32	
			M. aurum (Pasteur Inst 104482)		128	128	
			M. smegmatis (ATCC 14468)		64	64	
					32	64	
3	3-Methoxy-(3″,4″-dihydro-3″,4″-diacetoxy)-2″,2″-dimethylpyrano-(7,8:5″,6″)-flavone **77**	*Derris indica* (Lam.) Bennet Stem	Mtb H37Ra	MABA			[111]
4	8,4″-Dimethoxy-7-y,y-dimethylallylisoflavone **78**				25		
5	3,4-Methylenedioxy-10-methoxy-7-oxo[2] benzopyrano[4,3-β]benzopyran **79**				100		
6	Desmethoxy kanugin **80**				6.25		
7	Lacheolatin B **81**				50		
8	Pongachromene **82**				50		
9	3,7-Dimethoxyflavone **83**				100		
10	Pachycarin D **84**				50		
11	Maackiain **85**				50		
12	Medicarpin **86**				100		
13	Karanjachromene **87**				12.5		
14	Pinnatin **88**				12.5		

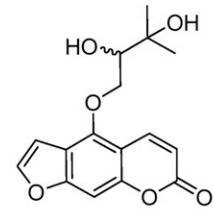
15	Khonklonginol A **89**	*Eriosema chinense* Vogel Roots	Mtb H37Ra	Microplate Alamar blue assay	25	[112]
16	Khonklonginol B **90**				50	
17	Khonklonginol F **91**				100	
18	Khonklonginol H **92**				25	
19	Lupinifolinol **93**				25	
20	Dehydrolupinifolinol **94**				12.5	
21	Flemichin D **95**				12.5	
22	Eriosemaone A **96**				12.5	
23	Lupinifolin **97**				12.5	
24	(2*S*)-5,7,2′-Trihydroxyflavanone **98**	*Galenia africana* Leaves	Mtb	BACTEC method	367.60 µM	[113]
25	(*E*)-3,2′,4′-Trihydroxychalcone **99**				416.60 µM	
26	(*E*)-2′,4′-Dihydroxychalcone **100**				195.30 µM	
27	(*E*)-3,2′,4′-Trihydroxy-3′-methoxychalcone **101**				174.80 µM	
28	8-Hydroxy-6-methoxy-3-pentylisocoumarin **102**	*Xylosma longifolia* Clos. Stem bark	Mtb H37Rv	MABA	40.2	

TABLE 5 Steroids and Fatty Acids

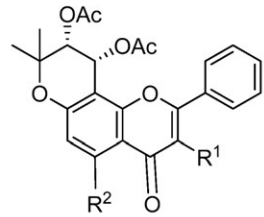
S. No.	Phytomolecule	Plant/Plant Part Used	Bacterial Strain Tested	ASSAY/Control Used	Activity MIC (μg/ml) or % Inhibition	References
1	6β-Benzoyl-7*β*-hydroxyvouacapen-5α-ol **103**	*Caesalpinia pulcherrima* (L.) Sw.	Mtb	Microplate Alamar blue assay	6.25	[115]
2	6β-Cinnamoyl-7*β*-hydroxyvouacapen-5α-ol **104**	Root			25	
3	Linoleic acid **105**	*Humulus lupulus* L.	*M. aurum* Pasteur Institute 104482	Nil	8	[89,116]
4	Oleic acid **106**	Whole plant		Nil	8	
5	Scleropyric acid **107**	*Scleropyrum wallichianum* (Wight & Arn.) Arn. Twigs	Mtb H37Ra	Microplate Alamar blue assay	25	[117]
6	Sitosteryl-3-*O*-6-palmitoyl-β-D-glucoside **108**	*Alpinia purpurata* (Vieill.) K. Schum	Mtb H37Rv	Microplate Alamar blue assay	64	[89,118]
7	β-Sitosteryl galactoside **109**	Leaves			64	
8	5(ζ)-Hydroxy-octadeca-6(*E*)-8(*Z*)-dienioc acid **110**	*Allium neapolitanum* Cirillo	*M. smegmatis* $mc^2$2700	Antibacterial assay	16	[94]
		Fresh bulbs	*M. phlei* ATCC 11758		16	
9	Campesta-6,22-dien-5α,8α-epidioxy-3β-ol **111**	*Morinda citrifolia* L.	Mtb H37Rv (ATCC 27294)	Radiorespirometric assay	2.5	[91,119]
10	β-Sitosterol **112**	Leaves			128	
11	β-Stigmasterol **113**				32	



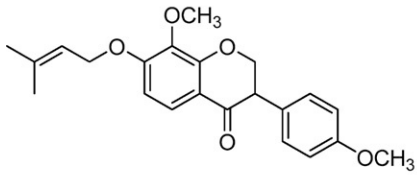
Oxypeucedanin **75**



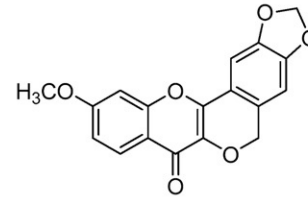
Oxypeucedanin hydrate **76**



3-Methoxy-(3″,4″-dihydro-3″,4″-diacetoxy)-2″,2″-dimethylpyrano-(7,8:5″,6″)-flavone **77**



8,4′-Dimethoxy-7-O-γ, γ-dimethylallylisoflavone **78**



3,4-Methylenedioxy-10-methoxy-7-oxo[2]benzopyrano[4,3-β]benzopyran **79**

FIGURE 5 Flavones with antitubercular activity.

Flavones

Few steroids and fatty acids have been shown to possess antimycobacterial activity as given in Table 5 and Fig. 7.

Terpenoids

Plant terpenoids also called as isoprenoids are the compounds which are derived from five-carbon isoprene units assembled and modified in infinite ways (reference). Table 6 and Figs. 8–11 list a large variety of terpenes and their corresponding activity against various mycobacterial strains.

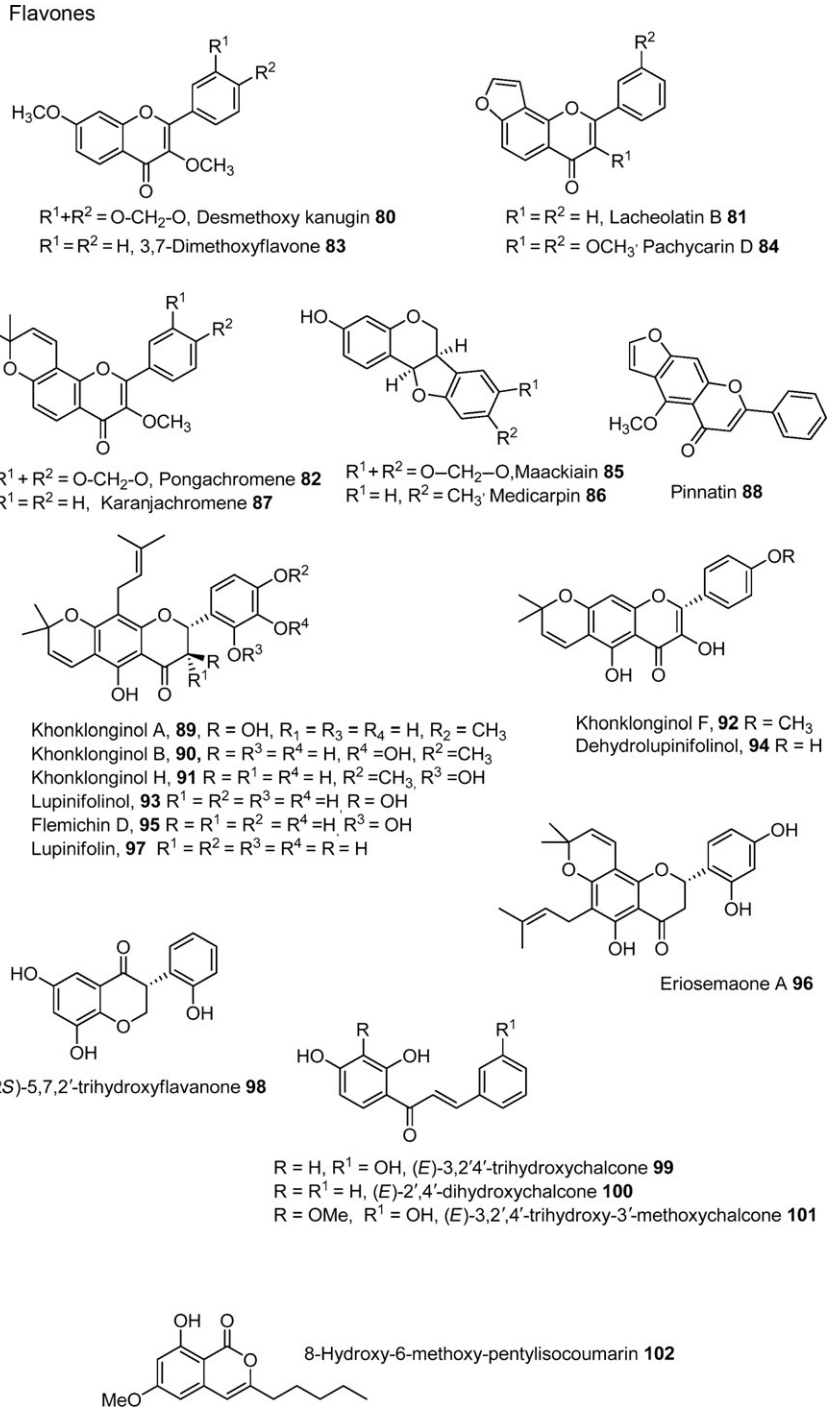


FIGURE 6 Flavones with antitubercular activity.

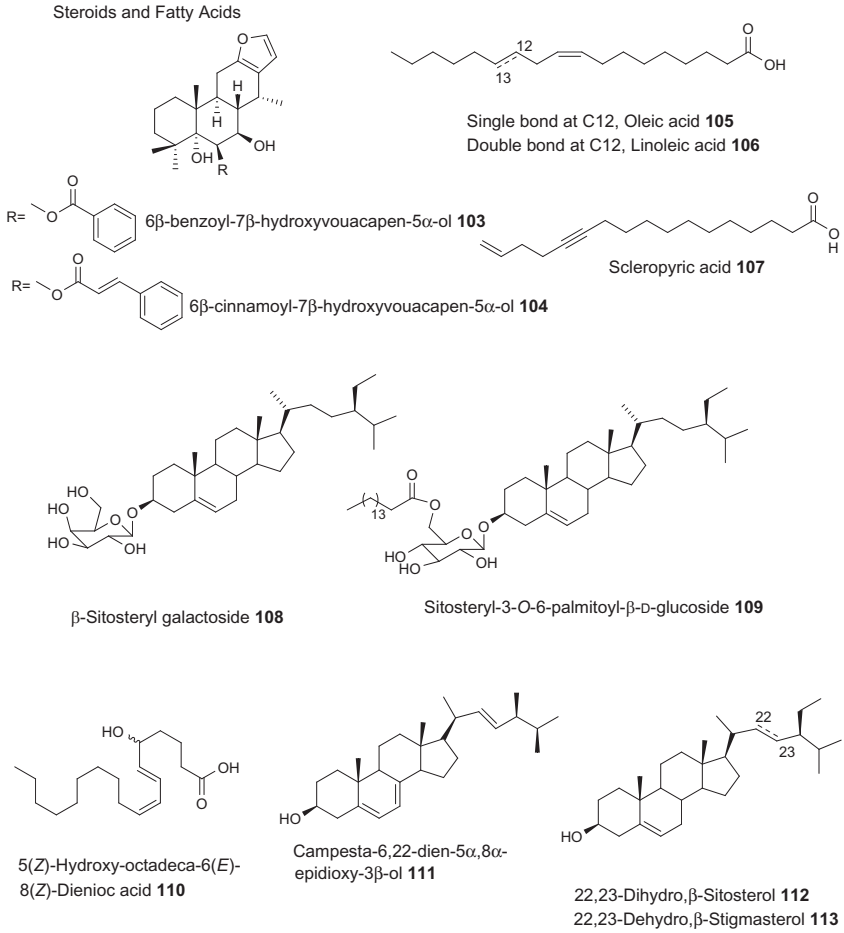


FIGURE 7 Steroids and fatty acids with antitubercular activity.

Miscellaneous

Table 7 and Figs. 12 and 13 summarize phytochemicals having proven antitubercular activity as obtained from microbial assays but cannot be categorized under a particular class because of differences in their basic scaffold.

STRUCTURE–ACTIVITY RELATIONSHIPS

A nice study involving the *N*-oxides has been conducted by Gibbons *et al*. Their experiments show a possible inhibition of fatty acid biosynthesis by these compounds. The RNA microarray analysis of 2-(methyldithio-)pyridine-*N*-oxide suggests that the mechanism of action could be the production of damaged proteins and other oxidative stress signals [95].

TABLE 6 Terpenes

S. No.	Phytomolecule	Plant/Plant Part Used	Bacterial strain tested	ASSAY/Control Used	Activity MIC (μg/mL) or % Inhibition	References
1	Alantolactone **114**	*Inula helenium* L.	Mtb H37Rv	Radiorespirometry assay using BACTEC system	32	[89,120]
2	Isoalantolactone **115**	Root			100	[102]
3	Dehydrocostuslactone **116**	*Laurus novocanariensis*	Mtb H37Rv	Fluorometric Alamar blue microassay	12.5	[89,121]
4	Costunolide **117**	Fixed oil			6.25	[122]
5	E-phytol **118**	*Leucas volkensii* Gurke	Mtb H37Rv	Radiorespirometric bioassay	2	[89,123]
6	Farnesol **119**	Arial part			8	
7	Ostruthin **120**	*Peucedanum osthruthin* Koch Root	*M. aurum* (Pasteur Institute 104482)	Muller-Hinton broth, Columbia	3.4	[89,124]
8	3-*O*-Vanillylceanothic acid **121**	*Ziziphus cambodiana* Pierre	Mtb H37Ra	Microplate Alamar blue assay	25 (IC50:3.7)	[125]
9	Betulinaldehyde **122**	Root bark			25 (IC50:6.5)	
10	Betulinic acid **123**				25	
11	2-*O*-*E*-*p*-Coumaroylalphitolic **124**				12.5 (IC50:0.9)	
12	Alphitolic acid **125**				50	
13	Zizyberanalic acid **126**				50	
14	Shinjulactone-K **127**	*Alianthus altissima* (Mill.) Swingle	Mtb		12.5	[89,126]
15	Alianthone **128**	Not given			12.5	
16	Shinjudilactone **129**				12.5	

17	ψ-Taraxasterol **130**	*Chrysanthemum morifolium* Ramat.	H37Rv (ATCC 27294)	Microplate Alamar blue assay	>64	[89,127]
18	Heliantriol **131**	Flowers			>64	
19	Faradiol **132**				16	
20	22α-Methoxyfaradiol **133**				32	
21	Faradiol α-epoxide **134**				32	
22	Taraxasterol **135**				64	
23	Arnidiol **136**				16	
24	β-Amyrin **137**				>64	
25	Maniladiol **138**				4	
26	Erythrodiol **139**				>64	
27	Longispinogenin **140**				32	
28	α-Amyrin **141**				32	
29	Brein **142**				>64	
30	Lupeol **143**				>64	
31	3-Epilupeol **144**				4	
32	Calenduladiol **145**				64	
33	Heliantriol B2 **146**				>64	
34	24-Methylenecycloartanol **147**				64	
35	(24*R*)-Cycloartane-3β,24,25-18 triol **148**				>64	
36	(24*S*)-Cycloartane-3β,24,25-triol **149**				32	
37	(24*S*)-25-Methoxycycloartane-3β,24-diol **150**				>64	
38	Helianol **151**				>64	
39	4,5α-Epoxyhelianol **152**				6	
40	D-7-Tirucallo **153**				>64	
41	Dammaradienol **154**				32	

Continued

TABLE 6 Terpenes—Cont'd

S. No.	Phytomolecule	Plant/Plant Part Used	Bacterial strain tested	ASSAY/Control Used	Activity MIC (μg/mL) or % Inhibition	References
42	Neroplomacrol **155**	*Oplopanax horridus* (Sm.) Miq.	Mtb H37R*a*	Microplate Alamar blue assay	>128	[128]
43	Neroplofurol **156**	Inner stem bark			>128	
					157 **158** **159**	[129]
44	*rel*-15(ζ),16-Epoxy-7*R*-hydroxypimar-8,14-ene **157**	*Plectranthus ernstii* Cood.	*M. fortuitum*	Microplate Alamar blue assay	16 128 128	
45	*rel*-15(ζ),16-Epoxy-7-oxopimar-8,14-ene **158**	Whole plant	*M. phlei*		8 64 128	
46	1*R*,11*S*-dihydroxy-8*R*,13*R*-epoxylabd-14-ene **159**		*M. smegmatis*		16 128 128	
47	Globiferin **160**	*Cordia globifera* W. W. Sm.	Mtb H37Ra	Microplate Alamar blue assay	6.2	[130]
48	Cordiachrome B **161**	Roots			12.5	
49	Cordiachrome C **162**				1.5	
50	Cordiaquinol C **163**				25	
51	Alliodorin **164**				12.5	
52	Eelaeagin **165**				12.5	
53	Cordiachromene **166**				12.5	
54	Kaurenoic acid **167**	*Pleurothyrium cinereum* van der Werff. Leaves	Mtb H37Rv	MABA	94.2% at 100 μg/mL	[131]
55	labda-8(17),12-Diene-15,16-dial **168**	*Curcuma amada* Roxb. Rhizome	Mtb H37Rv	BACTEC assay	500 μg/mL	[132]

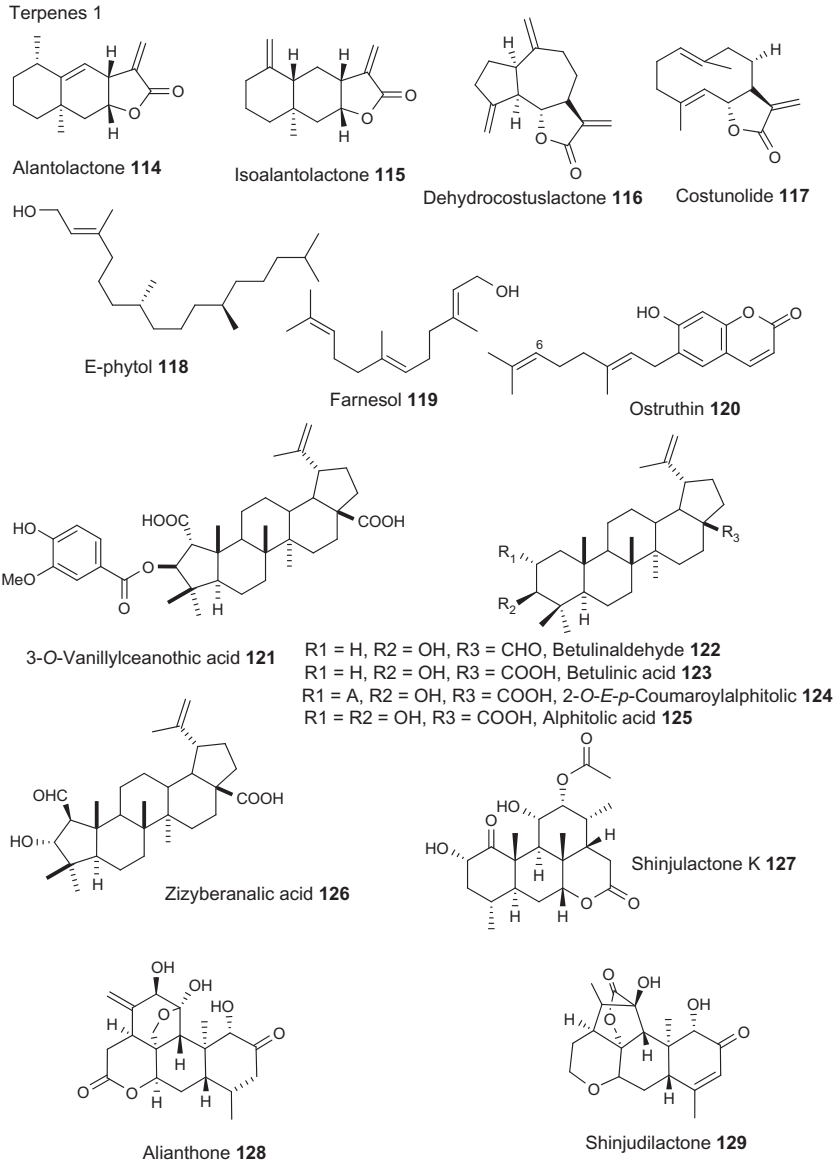


FIGURE 8 Terpenes with antitubercular activity.

Ma *et al*. has isolated seven carbazole alkaloids from *Micromelum hirsutum* Merr. They perceive that the presence of the aldehyde group enhances the mycobacterial activity compared to the ones lacking this functionality (compounds not shown here). Furthermore, methoxy group renders the compounds (**2** and **4**) twice as active as their free hydroxyl (phenolic) analog as in the case of **1** and **3**.

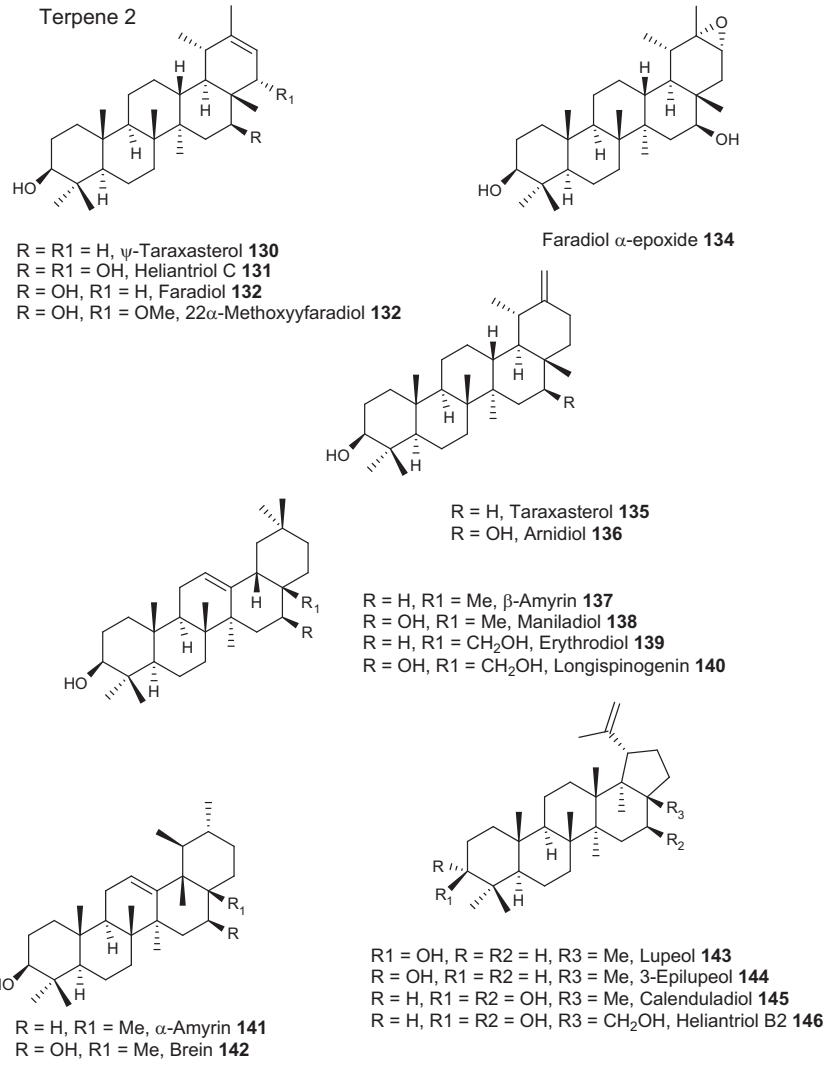


FIGURE 9 Terpenes with antitubercular activity.

Microarray screening of chelerythrine (**7**) on *Mtb* shows an effect on genes involved in many important pathways, urease genes, methyl mycolic acid synthase-related genes, genes involved in heat-shock response, protein synthesis, and mainly it inhibits the isocitrate lyase genes [136].

Many well-known anti-TB drugs are known to target the biosynthetic pathways that involve the production of macromolecules such as proteins, nucleic acids, or cell wall polymers. In selecting targets for antitubercular agents, it is advantageous to avoid targets that are close to the counterparts in mammalian cells [137].

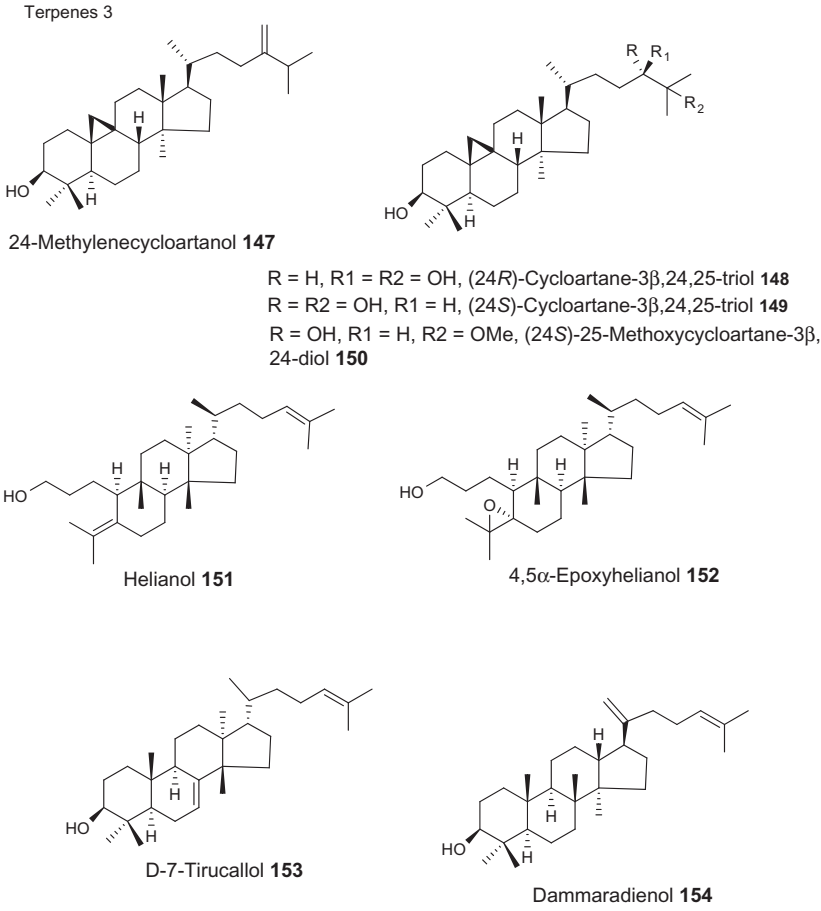


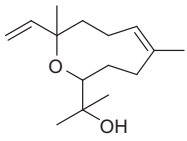
FIGURE 10 Terpenes with antitubercular activity.

The iminium salts of sanguinarine (**8**) and chelerythrine (**7**) demonstrate appreciable activity against *Mycobacterium avium*, *Mycobacterium bovis*, and *Mycobacterium smegmatis*. It appears as if the iminium ion improves the lipophilicity, thereby increasing the bioavailability of the alkaloids to the organisms [92].

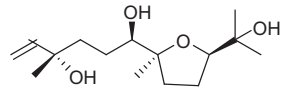
Structure–activity relationship (SAR)-based studies on the quinoline alkaloids point to the fact that the aromatic quinoline ring and a 4-methoxyl group are essential for exhibiting the alkaloid potency [138].

Berberine (reviewed earlier), an active constituent of many plants, has shown antimycobacterial activity against the pathogen *Mycobacterium intracellulare* with MIC of 0.78–1.56 μg/mL [139]. Moreover, at MIC of 25 μg/mL, this metabolite successfully inhibited the growth of *M. smegmatis* and *Mtb* [140,141]. SAR studies on the benzophenanthridine plant alkaloids

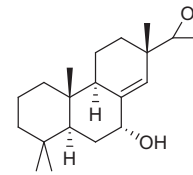
Terpenes 4



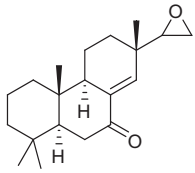
Neroplomacrol **155**



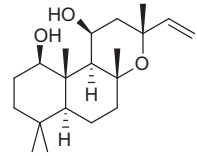
Neroplofurol **156**



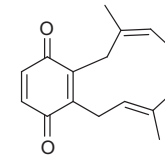
rel-15(*Z*),16-epoxy-7*R*-hydroxypimar-8,14-ene **157**



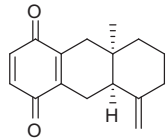
rel-15(*Z*),16-epoxy-7-oxopimar-8,14-ene **158**



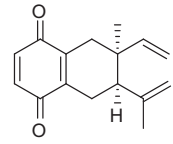
1*R*,11*S*-Dihydroxy-8*R*,13*R*-Epoxylabd-14-ene **159**



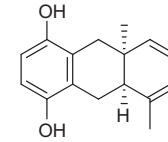
Globiferin **160**



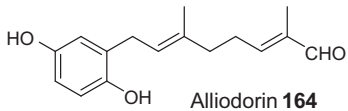
Cordiachrome B **161**



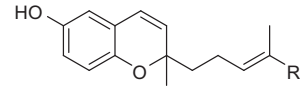
Cordiachrome C **162**



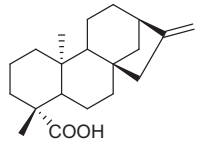
Cordiaquinol C **163**



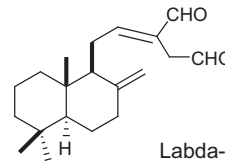
Alliodorin **164**



R = CHO, Elaeagin **165**
R = CH_3, Cordiachromene **166**



Kaurenoic acid **167**



Labda-8(17), 12-diene-15,16-dial **168**

FIGURE 11 Terpenes with antitubercular activity.

TABLE 7 Miscellaneous

S. No.	Phytomolecule	Plant/Plant Part Used	Bacterial Strain Tested	Assay/ Control Used	Activity MIC (μg/mL) or % Inhibition	References
1	Micromolide **169**	*Micromelum hirsutum* Merr. Stem bark	Mtb H37Rv	Fluorometric microplate Alamar blue assay	1.5 ± 0.4	[88,89]
2	2-Oxo-14-(3′,4′-methylenedioxyphenyl) tetradecane **170**	*Piper sanctum* (Miq.) Schl.	Mtb H37Rv	MABA	6.25	[89,91]
3	2-Oxo-16-(3′′,4′′-methylenedioxyphenyl)hexadecane **171**	Leaves and stem			6.25	
4	Falcarindiol **172**	Anethum graveolens L.	*M. abscessus*	Columbia Blood Agar	2	[89,110]
			M. aurum		4	
		Whole plant	*M. phlei*		2	
			M. smegmatis		4	
			Erdman strain (*Mtb*)		6	
			H37Rv		26.7	

Continued

TABLE 7 Miscellaneous—Cont'd

S. No.	Phytomolecule	Plant/Plant Part Used	Bacterial Strain Tested	Assay/ Control Used	Activity MIC (μg/mL) or % Inhibition				References
5	Oplopandiol **173**	*Oplopanax horridus* (Sm.) Miq.	Mtb and *M. avium*	MABA assay	100 μg/mL for all four compounds				[83]
					173	174	175	176	[34]
6	9*Z*,17-Octadecadiene-12,14-diyne-1,11,16-triol,1-acetate **174**	Inner stem bark			49.5	1.4	>60	>60	
7	8-Hydroxy-1-methoxy-(*Z*)-9-heptadecene-4,6-diyn-3-one **175**	*Angelica sinensis*	Erdman strain (*Mtb*)		50.2	25.3	>60	>60	
8	Heptadeca-1-ene-9,10-epoxy-4,6-diyne-3,8-diol **176**	Roots	H37Rv						
9	Debilisone B **177**	*Polyalthia debilis root*	Mtb H37Ra	Microplate Alamar blue assay (MABA)	25				[133]
10	Debilisone C **178**				12.5				
10	Debilisone E **179**				25				
11	Lakoochin A **180**	*Artocarpus lakoocha* Wall. ex Roxb.	Mtb H37Ra	MABA	12.5				[134]
12	Lakoochin B **181**	Root			50				
13	Plumbagin **182**	*Diospyros canaliculata* De Wild. and *Diospyros crassiflora* Hiern	Mtb H37Ra	MABA	183	182	181		[135]
14	Diospyrone **183**	Stem bark			4.88	2.44	4.88		
15	Crassiflorone **184**				4.88	2.44	>39.06		
					4.88	1.22	39.06		

16	Goniotriol **185**	*Goniothalamus laoticus* (Finet & Gagnep.) Bân	Mtb	Microplate Almar blue assay	100	[98]
17	(+)-Altholactone **186**	Flowers			6.25	
18	Howiinin A **187**				6.25	
19	Cinerin A **188**	*Pleurothyrium cinereum* van der Werff.	Mtb H37Rv	MABA	8.1	[131]
20	Cinerin B **189**	Leaves			19.5	
21	Cinerin C **190**				49.4	
22	Cinerin D **191**				7.3	

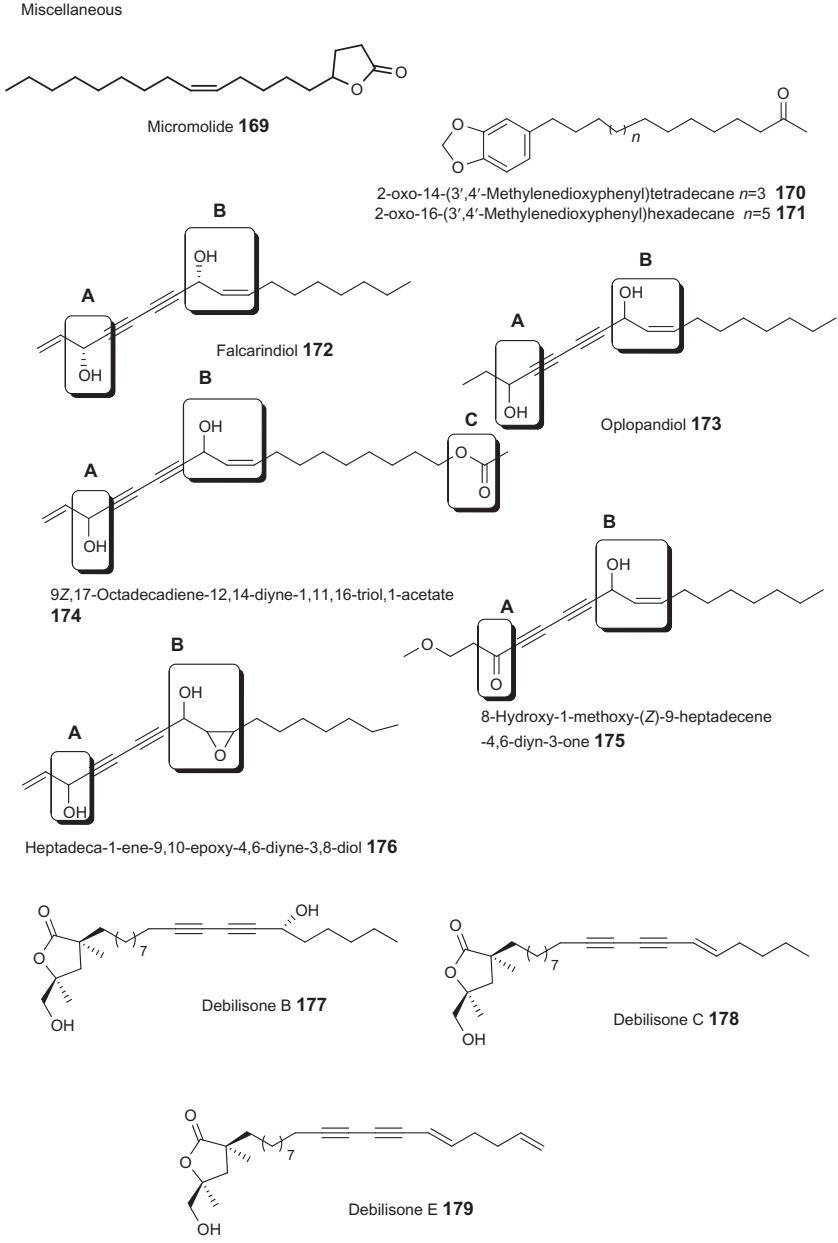


FIGURE 12 Miscellaneous phytochemicals with antitubercular activity.

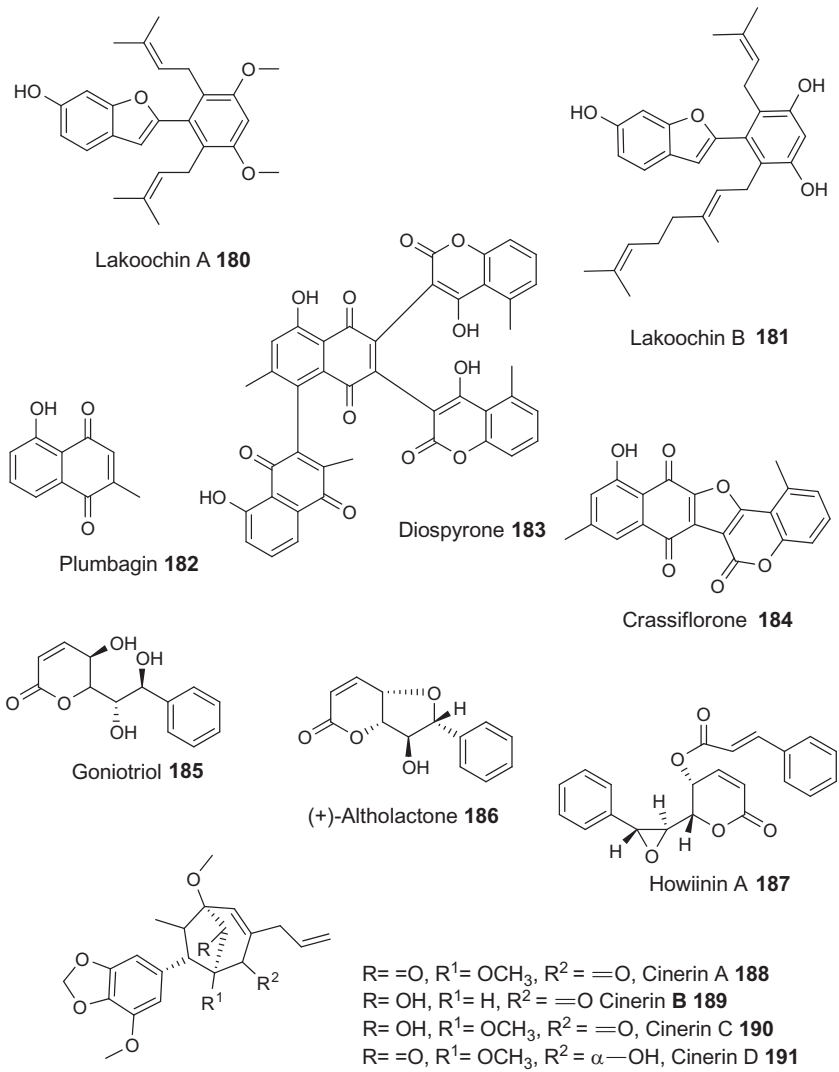


FIGURE 13 Miscellaneous phytochemicals with antitubercular activity.

like chelerythrine (**7**), sanguinarine (**8**), chelirubine, and macarpine (reviewed previously) showed remarkable inhibition on growth of *Mtb* H37Rv by $\geq$94% at 12.5 μg/mL [142]. This proves that the alkoxy substitution, in the form of methoxy or methylenedioxy functional groups, is needed for displaying the antitubercular potency of these alkaloids [138].

Many natural and synthetic nonprenylated xanthones have shown significant inhibitory effect [143] on the growth and multiplication of *Mtb* [144,145] like those obtained from *Garcinia mangostana* L. Quantitative

structure–activity relationships (QSARs) show correlations between ^{13}C NMR chemical shifts and lipophilicity of the various synthetic nonprenylated xanthones and the growth inhibition of TB [146,147]. The minimum inhibitory concentration (MIC) data of a group of xanthones suggested that, for a moderate to high antimycobacterial activity, there should be a presence of tri- or tetra-oxygen functions on the xanthone nucleus. Such xanthones exemplified by mangostin (**40**), the major constituent, β-mangostin (**41**), and garcinone B (**45**) exhibited the most potent activities with the same MIC value of 6.25 mg/mL [106].

QSARs reveal that the presence of aldehyde group at the C-3 position of the carbazole alkaloids is responsible for exhibiting greater activity against TB than those without the presence of this functional group. Similarly, the presence of a methoxy group in lansine (**2**) and 3-formyl-6-methoxycarbazole (**4**) is responsible for their improved antitubercular activity. But further biological studies need to be conducted on the carbazole alkaloids before laying down definitive structure–activity correlations [88]. Several studies conclusively prove that quinolones generally target the DNA gyrase of the *Mycobacterium* organisms [148]. Another set of study indicates that the drug target of diarylquinolines is the proton pump of adenosine triphosphate (ATP) synthase [52].

The naphthoquinone 7-methyljuglone (**24**) (5-hydroxy-7-methyl-1,4-naphthoquinone) obtained from the root extracts of *Euclea natalensis* shows good antitubercular activity. On analyzing the SAR of various structural derivatives of the basic juglone scaffold, it can be concluded that methylation of the C-2 position (menadione) or the 5-hydroxyl group is highly unfavorable with regard to substrate binding with the plasmid-encoding recombinant Mtr [149].

A variety of antitubercular compounds like flavonoids, coumarins, chromones, chalcones, etc. are isolated from *Haplopappus sonorensis* [104]. It was seen that the coumarins are active against *Mtb* most probably due to the presence of the prenyl group. Ostruthin (**120**) from *Peucedanum ostruthium* (L.) also has a prenyl group at C-6, the reason for the antimycobacterial activity. The interesting observation is that the MICs are similar to the control, isoniazid. Flavonoids are generally shown to inhibit the mycolic acid biosynthesis in mycobacteria [77].

The importance of epoxide ring for *in vitro* anti-TB activity was observed when the triterpene alcohols obtained from the nonsaponifiable lipid fraction of chrysanthemum extract (*Chrysanthemum morifolium* Ramat) showed potent antitubercular activity against *Mtb* [150,151].

Phytochemical research and subsequent studies led to the isolation of polyynes from the plant *Angelica sinensis* (Oliv.), namely, falcarindiol **172,** 9*Z*,17-octadecadiene-12,14-diyne-1,11,16-triol,1-acetate **174**, and oplopandiol **173**. The SAR studies reveal that both functional groups in area A (hydroxyl groups) and B (olefinic bond) are needed for the anti-TB activity of

polyynes [34]. This is supported by the results obtained in another study where there was a significant correlation between the antimicrobial activity and lipophilicity parameters [152]. Moreover, the presence of functional groups in area C (an acetate group attached to a saturated aliphatic terminal position) could be the key factor for the improved antitubercular activity [34].

Activity on MDR- and XDR-TB

MICs of (+)-calanolide A, licarin A, B, eupomatenoid-7, and the costunolides on drug-resistant, MDR, and XDR strains of *Mtb* are summarized in Table 8 [109,153].

SYNERGISM

In a bioassay-guided separation, the initial extract containing these two compounds was found to be active, but as single molecules, they were only moderately active. This observation is suggestive of the apparent synergism in *Oplopanax horridus* (Sm.) Miq. [128]. A study involving several PDF (peptide deformylase) inhibitors showed synergistic effect with rifampicin and isoniazid. Each of these molecules in combination with rifampicin and isoniazid illustrated an FICI (fractional inhibitory concentration index) of ≤ 0.5 [80]. Activities of isoniazid and rifampicin have been found to enhance in the presence of 7-methyljuglone, a naphthaquinone isolated from *E. natalensis* A. DC., as indicated by FICI values of 0.5 and 0.2, respectively [154].

Similarly, synergistic activity is indicated in madeira laurel oil by costunolide and dehydrocostus lactone. These compounds in combination (alone they show moderate activity against *Mtb)* are shown to exhibit activity against various drug-resistant and MDR clinical strains of mycobacteria (Table 8) [121].

CONCLUSION

In this overview, many compounds have shown great potential as antitubercular drug candidates. 8-Hydroxy canthin-6-one **11** shows high activity with *M. smegmatis* (mc22700) strain expressing the *Mtb* FASI gene. 2-(Methyldithio)pyridine-*N*-oxide **13** and 2-[(methylthiomethyl)dithio]pyridine-*N*-oxide **14** shows the highest activity in this review of 0.1 mg/mL, against *Mtb* H37Rv and *M. bovis Bacillus Calmette Guerin* (BCG). However, the cytotoxicity of these compounds against cancer cells is higher than against normal cells, and so are potential anticancer agents. If the cytotoxicity effect of cryptolepine hydrochloride **15** can somehow be neutralized by synthetic derivatization, then this compound stands a great chance for further evaluation. Ferulenol **26**, the outstanding activity, low toxicity, and the evidence for definite SAR make this prenylated 4-hydroxycoumarin an interesting antimycobacterial worth further investigation. (*E*)- and (*Z*)-Phytol (**118**) and the phytanol which were isolated

TABLE 8 MDR and XDR Activity

S. No.	Mycobaterial Strains	Drug Resistance Pattern	(+)-Calanolide A	73	74	72	117	116
1.	H37Rv	Susceptible	8	50	25	25	12.5	6.25
2.	INH-R (ATCC 35822)	INH resistant	8	25	25	3.12	12.5	12.5
3.	CSU 36	RIF resistant	8	–	–	–	–	–
4.	CSU 38	STR resistant	8	–	–	–	–	–
5.	EMB-R (ATCC 35837)	EMB resistant	8	25	25	6.25	12.5	6.25
6.	STR-R (35820-ATCC)	STR resistant	–	25	25	3.12	25	50
7.	RIF-R (ATCC-35838)	RIF resistant	–	50	12.5	6.25	6.25	12.5
8.	MMDO	INH, EMB	–	12.5	12.5	3.12	25	50
9.	MTY650	STR, INH	–	12.5	50	6.25	–	–
10.	MTY663	STR, INH, RIF, EMB, PZA	–	12.5	50	12.5	–	–
11.	MTY675	STR, INH, EMB	–	12.5	50	12.5	–	–
12.	MTY282	STR, INH, EMB, PZA	–	12.5	50	12.5	–	–
13.	HG8	EMB, CLR, ETH	–	25	25	3.12	–	–
14.	SIN3	STR, INH, RIF, EMB, RFB, CLR, ETH	–	25	25	6.25	–	–
15.	MTY234	STR, INH, RIF, PZA	–	25	50	12.5	–	–
16.	MTY112	STR, INH, RIF, EMB	–	25	50	12.5	–	–

17.	MTY559	STR, EMB	–	25	50	12.5	–	–
18.	SIN4	STR, INH, RIF, EMB, RFB, ETH, OFX	–	50	6.25	3.12	25	50
19.	MTY172	INH, PZA	–	50	50	12.5	–	–
20.	MTY 147	INH, RIF	–	–	–	–	50	25

All data in μg/mL.

from *Leucas volkensii* Gurke are very interesting to be pursued further for evaluation.

The highly anti-TB active compound micromolide **169** has been evaluated for its cytotoxicity and has been found to have great potential as an anti-TB agent. The polyyene **174** deserves more in-depth evaluation due to its high activity as well as its low cytotoxicity (selectivity index of 117 against Erdman strain).

It is also worth studying the synergistic relationships of the active molecules with the existing drugs to improve upon and develop better drug formulations for short treatment regimes. Furthermore, studies on compounds with anti-MDR- and XDR-TB activity such as (+)-calanolide A, licarin A, B, eupomatenoid-7, and the costunolides are of great value. Drug development against MDR- and XDR-TB is the need of the hour due to its high prevalence, globally. In conclusion, there are a lot of leads presented here for mining of new drug compounds using the global flora.

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ABBREVIATIONS

AIDS	acquired immune deficiency syndrome
AMK	amikacin
ATP	adenosine triphosphate
BCG	Bacillus Calmette Guerin
CLR	clarithromycin
CSIR	Council of Scientific and Industrial Research
DNA	deoxyribonucleic acid
DOTS	directly observed treatment short course
EMB	ethambutol
FICI	fractional inhibitory concentration index
HIV	human immunodeficiency virus
INH	isoniazid
IP	intellectual property
LZD	linezolid
MABA	microplate Alamar blue assay
MDR	multidrug resistant
MIC	minimum inhibitory concentration
Mtb	*Mycobacterium tuberculosis*
NMR	nuclear magnetic resonance
OFX	ofloxacin
OSDD	open source drug discovery

PAS/P	para-aminosalicylic acid
PDF	peptide deformylase
PZA	pyrazinamide
QSAR	quantitative structure activity relationship
RFB	rifabutin
RIF	rifampicin
RNA	ribonucleic acid
SAR	structure–activity relationship
SLDs	second-line drugs
STR	streptomycin
T	thioridazine
TB	tuberculosis
TCM	traditional Chinese medicine
WHO	World Health Organization
XDR	extremely drug resistant

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