

Antimicrobial abietane diterpenoids against resistant bacteria and biofilms

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Antimicrobial resistance (AMR) is a growing worldwide threat to public health due to the increase of resistant pathogens to the existing antimicrobial therapy. Common and nosocomial infections are beginning to require 2nd and 3rd line antibiotic therapy, increasing time and cost of treatment, and also contributing to the development of multidrug resistance (MDR). Natural products remain an important source of new bioactive compounds and drug prototypes that can lead to new and more effective antimicrobial agents. Abietane diterpenoids display a wide spectrum of biological activities, including antibacterial, antifungal, antiviral and anti-parasitic. These compounds were found to be effective against *Mycobacterium tuberculosis* and *Staphylococcus aureus*, including methicillin-resistant (MRSA) strains and biofilm infection of *S. aureus*. Structure-activity relationship analysis suggests that the antimicrobial activity of abietane diterpenoids is related to some characteristic features of the abietane skeleton and is dependent on the degree of substitution, the nature and position of the substituents, and the overall lipophilicity of the compound.

Keywords: abietane diterpenoids; antimicrobial activity; biofilms; natural products

1. Introduction

1.1 Antimicrobial resistance

Antimicrobial resistance (AMR) is a global problem that affects both developing and developed countries, as a result of the use and misuse of antimicrobial agents over the past decades [1]. Infection diseases are still among the main causes of morbidity and mortality in developing countries while healthcare-acquired infections (HAI) with resistant microorganisms are a major cause of death worldwide [2]. In fact, the emergence of methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *S. aureus* (VRSA), vancomycin-resistant *Enterococcus* (VRE) and multidrug-resistant (MDR) strains of these microorganisms have become a common occurrence in the hospital environment [3–5]. Moreover, one of the most potent class of antibiotics, the carbapenems, is being compromised by the growth, mainly in intensive care units, of MDR strains of *Klebsiella pneumoniae*, *Acinetobacter baumannii*, and *Pseudomonas aeruginosa* [6].

A combination of increasing rates of AMR and a decline in the development of new antibiotics mainly due to a low return on investment contributed to the current crisis in fighting against antibiotic-resistant microorganisms, raising the spectre that once treatable infections may soon become untreatable [6]. Not surprisingly, the World Health Organization (WHO) identified antimicrobial resistance as a public health threat just at the beginning of the millennium [7], a consequence of the rapidly emergence and spread of antimicrobial resistant pathogens that cannot be treated with currently available antibiotics.

AMR to conventional antimicrobial agents mainly results from enzymatic inactivation of the drug, alteration of its receptor or target site, preventing the drug to gain access to the target site through altered membrane permeability or over-expression of efflux pumps [8–10]. Furthermore, many antibiotics were originally developed to target microorganisms growing in planktonic cultures, but it is now clear that many bacteria live as complex communities known as biofilms. Biofilms are complex microbial consortia surrounded by an exopolysaccharide matrix that protects the microbial community from environmental stress, including bacteriophage, amoebae and biocide attack as well as against the host immune response(s) and antibiotic chemotherapy. Microbial biofilms are unequivocally responsible for the recalcitrance of many infections to conventional antimicrobial therapy, mostly due to the inability of the antimicrobial agents to penetrate the biofilm matrix [11–13]. Therefore, novel classes of antimicrobial agents with improved efficacy and new or combined mechanisms of action to reduce the likelihood of acquired resistance are an urgent requirement to contain the global threat of AMR.

1.2 Antimicrobial drug discovery from natural products

Natural products remain an important and viable source of new drug candidates and lead molecules despite increasing competition from combinatorial and classical compound libraries, molecular modelling, and advances in synthetic chemistry technologies [14–18]. Moreover, natural products with low bioactivity or bioavailability can be synthetically modified to improve their pharmacological profiles. Many of the drugs in the market are natural products or natural

product-derived, with approximately one-third of the world top-selling drugs being natural products or their synthetic derivatives [16].

Recently there has been a renewed interest in natural products research due to the failure of alternative drug discovery methods to deliver many lead compounds in key therapeutic areas, such as infectious diseases. The wide structural diversity of natural products aiming at providing the producer organism with a survival advantage in environments threatening its growth and/or survival often affords biologically and environmentally friendly lead molecules due to their co-evolution with the target sites in biological systems [19, 20]. Resistance-modifying properties of natural products have been observed, and synergism between natural products and antibiotics holds a promise in the fight against infectious diseases [21–23].

Medicinal plants are a valuable source of novel chemotypes and pharmacophores, with the plant-derived products still playing a major role in modern drug discovery and development efforts [21, 22, 24–33]. About 80% of the population in developing countries relies on traditional medicine, mainly based on medicinal plants, for their primary healthcare, while in developed countries the use of herbal drugs is escalating in the form of complementary and alternative medicine [16, 29, 30].

Diterpenes, a class of natural products widespread in several botanical families such as Asteraceae, Celastraceae, Hydrocharitaceae, and Lamiaceae, are promising sources of antimicrobial prototype compounds due to their structural diversity and wide range of oxidized patterns [20, 31–42]. This mini-review will focus on the antimicrobial abietane diterpenoids, namely the aromatic dehydroabietane derivatives, with an emphasis on their antibacterial activity, especially against resistant bacterial strains and biofilms. Synthetic dehydroabietane derivatives with enhanced antimicrobial activities will also be addressed as promising new antimicrobial drug candidates for efficient chemotherapy of infectious diseases.

2. Abietane diterpenoids

2.1 Chemical structure and occurrence

The abietanes are a family of natural occurring diterpenoids that have been isolated from a large variety of terrestrial plants [20, 31–43]. These compounds are known to possess a broad range of biological activities, including, antimicrobial, antiulcer, antioxidant, anti-inflammatory, cardiovascular and cytotoxic activities, which have attracted a considerable interest from both the pharmaceutical and medical communities [32, 34, 37–45]. During the last decades, many new members of the abietane diterpenoid family have been isolated and characterized, and research efforts have also been made regarding the chemical synthesis of these natural products and the development of synthetic derivatives with improved bioactivity and/or pharmacological profiles [43–48]. Abietanes have the characteristic carbon skeleton of 20 carbon atoms (**I**, abietane skeleton) exemplified by that of abietic acid (**1a**) (Fig. 1), which shows an equatorial carboxylic group (C-18) and two conjugated double bonds at positions 7 and 13.

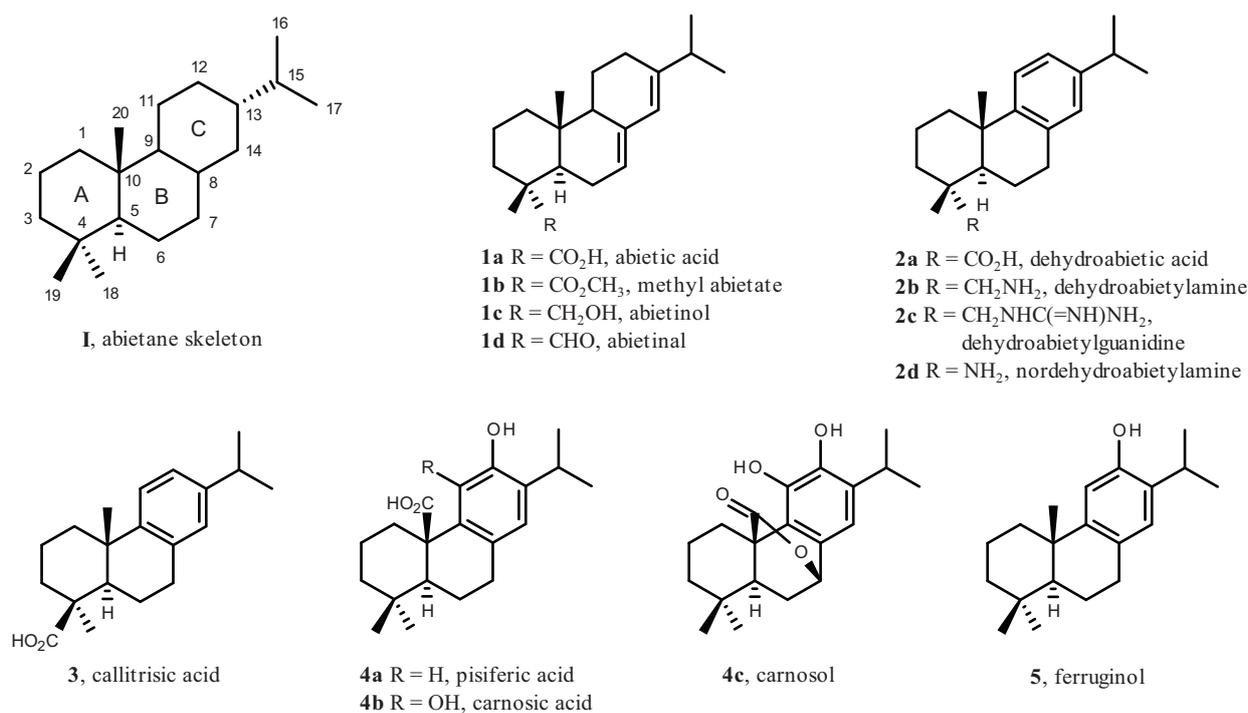


Fig. 1 Abietane numbering system and abietanes (1–5).

Aromatic abietanes, which comprise the largest group of naturally occurring abietanes, are characterized by an aromatic ring C, as exemplified by dehydroabietic acid (**2a**, DHA) (Fig. 1). There are about two hundred compounds up to now that belong to this group of natural products, being commonly known as dehydroabietic acid derivatives or dehydroabietanes [44, 45]. While DHA (**2a**) possesses an equatorial carboxylic group (C-18), in other natural congeners the carboxylic group adopts the axial configuration (C-19), namely in callitricic acid (**3**), or it represents C-20, such as in pisiferic (**4a**) and carnosic acids (**4b**) (Fig. 1). The carboxyl function is lacking in ferruginol (**5**) (Fig. 1), an aromatic abietane first isolated from the resin of the Miro tree (*Podocarpus ferrugineus*) endemic to New Zealand. Other functionalities are often present in dehydroabietanes, such as hydroxy and carbonyl groups, and in some compounds an endoperoxide moiety can also be found [44–46].

The main source of abietane diterpenoids is colophony, also known as rosin, which is the distillation residue of pine oleoresins [44]. Rosin is chiefly a mixture of resin acids mainly composed of abietic (7,13-abietadien-18-oic) acid (**1a**), its equilibrium isomers levopimaric (8(14),12-abietadien-18-oic), palustric (8,13-abietadien-18-oic) and neoabietic (8(14),13(15)-abietadien-18-oic) acids, and dehydroabietic (8,11,13-abietadien-18-oic) acid (**2a**), as well as some other non-abietane diterpenoids, such as pimaric and isopimaric acids [44, 45]. Dehydroabietylamine (**2b**, DHAA) (Fig. 1), an abietane diterpenic amine derived from abietic acid (**1a**), is the main component of disproportionated rosin amine [46]. There are three major types of colophony depending on whether the source of the oleoresin is gum, wood or tall oil. Gum rosin is recovered from the sap of living pine trees, wood rosin is extracted from pine stumps and tall oil is obtained as a by-product of paper pulp production. However, the abietanes can also be found in extracts or resins of other conifers belonging to different families such as Cupressaceae, Araucariaceae, Podocarpaceae, Phyllocladaceae, and Pinaceae, and they also occur in several Angiosperm species, particularly in the families Asteraceae, Celastraceae, Hydrocharitaceae, and Lamiaceae [44–46, 49].

The secretion of oleoresin by coniferous plants is an important defence mechanism against injury. Oleoresin is a complex mixture of terpenoids consisting of turpentine (volatile terpenes) and rosin (mainly resin acids). When the plant is wounded, oleoresin is exuded into the damaged site. Once at the surface, evaporation of the volatile turpentine leaves the diterpenoid resin acids that polymerize, forming a protective barrier that seals the wound while simultaneously trapping insect invaders and microbial pathogens [44–46, 49].

Pine resin, as whole or its derivatives, have been used since ancient times as expectorants, vesicants and rubefacients, and as antiseptics for the treatment of wounds, pyodermas and boils. Several of the rosin acids, including abietic (**1a**) and dehydroabietic (**2a**) acids, their salts and their amides with aminoacids are employed in cosmetics and dermatological preparations, mainly due to their surfactant and antimicrobial properties [28, 44, 45, 49].

Studies on rosin and the resin acids demonstrated their antibacterial effects, mainly against Gram-positive bacteria [49–51]. The abietic acids were stronger antibacterial agents than pimaric and labdane acids, and among the individual resin acids, DHA (**2a**) was generally the most potent. The growth inhibiting capacity of zinc oxide combined with Portuguese rosin, abietic acid (**1a**) or DHA (**2a**) led to synergistic antibacterial effects [52]. Recently, rosin proved to be an effective microbicidal against a wide range of microorganisms, including methacillin-sensitive *S. aureus* (MSSA), MRSA, *Bacillus subtilis*, *Escherichia coli*, *P. aeruginosa*, and *Candida albicans*, in the European Pharmacopoeia challenge test [53].

2.2 Antimicrobial activities of abietane derivatives

2.2.1 Abietic acid derivatives

Abietic acid (**1a**) occurs widely as the major constituent of commercial resins, prepared from the crude oleoresins of conifers by heat and mineral acid treatment. Under these conditions, several other resin acids, such as levopimaric, neoabietic, pimaric and isopimaric acids, undergo isomerisation to abietic acid (**1a**) [44, 51]. Despite abietic acid (**1a**) being one of the main compounds responsible for the antibacterial activity of resin acids, only a few reports have appeared concerning the biological study of its derivatives. Recently, González *et al.* [48] synthesized several abietic acid derivatives (**1b–d**, Fig. 1) and evaluated their antimycotic, antiviral and cytotoxic activities. Starting from commercial (–)-abietic acid (**1a**), methyl abietate (**1b**) was obtained by esterification reaction, while reduction of the carboxylic acid function with LiAlH_4 led to abietinol (**1c**) further oxidized to abietinal (**1d**) using pyridinium chlorochromate (PCC). Abietinol (**1c**) and abietinal (**1d**) have also been isolated as natural products. The compounds had no antimycotic activity *in vitro* against *C. albicans*, *C. parapsilosis*, *C. krusei*, *C. tropicalis*, *Aspergillus fumigatus*, *A. flavus*, *A. niger*, and *A. terreus*, at concentrations below 100 $\mu\text{g/mL}$, with the exception of abietanal (**1d**), which showed a minimum inhibitory concentration (MIC) value of 50 $\mu\text{g/mL}$ against *A. fumigatus* [48]. The antiviral activity of the abietanes (**1a–d**) against herpes simplex virus type 1 (HSV-1) was determined using a modified end-point titration technique, with abietinol (**1c**) showing the highest antiviral activity. This compound reduced the HSV-1 replication at a concentration of 6.25 $\mu\text{g/mL}$. Isomerisation of the double bonds present in abietic acid (**1a**) led to less active neoabietic acid derivatives [48].

2.2.2 Dehydroabietic acid derivatives

DHA (**2a**), which can be obtained by disproportionation of rosin or abietic acid (**1a**), is commercially available. In marked contrast with abietic acid (**1a**), a lot of research has been committed to the synthesis of DHA derivatives with improved antimicrobial, antitumor and antiulcer properties. Modifications in rings B and C as well as manipulation of the carboxyl group at C-18 of DHA (**2a**) have been studied in order to enhance its properties [44–47, 54].

The antibacterial effects of DHA (**2a**) are well documented, especially against *Staphylococcus* sp. and other Gram-positive microorganisms [28, 51–53]. DHA (**2a**) isolated from the oleoresin of *Pinus elliottii* showed bacteriostatic and bactericidal effects against Gram-positive bacteria, including MDR clinical isolates of *S. aureus*, *S. epidermidis*, *S. capitis*, *S. haemolyticus*, *Enterococcus faecium*, and *E. faecalis*, with MIC and minimum bactericidal concentration (MBC) values of 6.25–50 µg/mL and 6.25–100 µg/mL, respectively [55]. The compound had no activity against MDR clinical isolates of *K. pneumoniae* and had also been found inactive against other Gram-negative microorganisms, including *K. pneumoniae*, *E. coli*, *P. aeruginosa*, and *P. mirabilis*, in a previous study [51]. Fallarero *et al.* [56] reported on the activity of DHA (**2a**) against planktonic and bacterial biofilm infections of *S. aureus*. Minimum biofilm inhibitory concentration (MBIC) of 10.2 µg/mL and minimum biofilm eradication concentration (MBEC) of 24.4 µg/mL have been obtained, which demonstrated not only the ability of DHA (**2a**) to prevent bacterial colonisation, but also to inhibit of existing biofilms.

Recently, González *et al.* [47] demonstrated that DHA (**2a**) and hydroxylated DHA derivatives (**6a**) and (**6b**) possess anti-*Aspergillus* activity (Fig. 2), but no impact on *Candida* at concentrations below 100 µg/mL. DHA (**2a**) displayed a MIC value of 39.7 µg/mL against *A. terreus*, while for (**6a**) MIC values of 50 and 63 µg/mL were obtained against *A. fumigatus* and *A. niger*, respectively. The phenol derivative (**6b**) had MIC values of 25, 50 and 25 µg/mL against *A. fumigatus*, *A. niger* and *A. terreus*, respectively [49]. Introduction of a ketone function at C-7 (**6c**) led to less active compounds. Comparatively to the abietic acid analogues [48], aromatization of ring C in the dehydroabietane series did not improve anti-*Candida* activity but led instead to enhanced activity against *Aspergillus* [47, 49].

The isomeric compounds (**7a**) and (**7b**) (Fig. 2), which are C-13 deisopropylated derivatives of DHA (**2a**), were the most active inhibiting the growth of several filamentous fungi (*Actinomucor harzii*, *Cladosporium cucumerinum*, *Mucor racemosus*, *Rhizopus arrhizus*, *R. stolonifer*, and *Syncephalastrum racemosum*) and also Gram-positive bacteria (*S. aureus*) [57, 58]. The compounds alone did not inhibit the growth of Gram-negative bacteria, *E. coli* and *K. pneumoniae*, however the combination of both isomers was able to inhibit the growth of those organisms, suggesting a synergistic effect. Substitution of the ester group of (**7b**) by an aldehyde group gave compound (**7c**) (Fig. 2), with anti-yeast activity against *C. albicans*, *C. kruzei* and *C. parapsilosis* [59].

A series of dehydroabietyl-1,2,4-triazolo-thiazolidinones, including compounds (**8a-d**) (Fig. 2), have been synthesised and tested at 50 µg/mL for antifungal activity against *Fusarium oxysporum*, *Alternaria solani*, *Physalospora piricola*, *Cercospora arachidicola* and *Fusarium graminearum* [46, 60]. With the exception of (**8a**) bearing an unsubstituted phenyl ring, all compounds were most effective against *F. graminearum*, the most potent being the compound (**8b**) with the phenol substituent, showing an inhibition ratio of 70.9%. Compounds (**8a**) and (**8c**) exhibited the greatest inhibition ratios against *F. oxysporum* (51.9%) and *P. piricola* (56.8%), respectively. Other antimicrobial studies included the synthesis of some dehydroabietoyl thioureas (**9a-h**) and the corresponding 1,2,4-triazolo-aniline derivatives (**10a-f**) (Fig. 2) [46, 61]. Compounds (**9d**), (**9e**), (**9h**) and (**10b**) showed antibacterial activity against *B. subtilis* while compounds (**9b**), (**9f**), (**9g**) and (**10d**) were active against *E. coli*.

A series of novel dibenzo-carbazole derivatives of DHA, such as (**11a-f**) (Fig. 2), were synthesized through reaction of methyl 7-oxo-dehydroabietate with a variety of substituted phenylhydrazines and tested against bacteria (*B. subtilis*, *S. aureus*, *E. coli*, and *P. fluorescens*) and fungi (*Trichophyton rubrum*, *C. albicans* and *A. niger*) [62]. Compounds (**11c-e**) exhibited pronounced antibacterial activities, with (**11c**) exhibiting stronger antibacterial activity against *B. subtilis* with a MIC value of 1.9 µg/mL, comparable to that of 0.9 µg/mL for the positive control (amikacin). Compound (**11e**) also showed moderate antifungal activity. Later on, the same authors [63] reported the synthesis and antimicrobial evaluation of *N*-substituted dibenzo-carbazole derivatives of DHA, those bearing heterocyclic substituents (**12a-h**) (Fig. 2) displaying pronounced antimicrobial activity against *B. subtilis*, *S. aureus*, *E. coli*, and *P. fluorescens*, with low MIC values (0.9–15.6 µg/mL). Compounds (**12d**) and (**12g**) exhibited antibacterial activity comparable to reference drug amikacin. These authors [64] have also described the synthesis and antibacterial evaluation of new *N*-acylhydrazone derivatives (**13a-j**) (Fig. 2) obtained from DHA hydrazide by reaction with a variety of substituted arylaldehydes. Antibacterial activity was enhanced in compounds (**13e**), (**13g**) and (**13i**) bearing electron-withdrawing substituents (halogen and/or nitro) in the aromatic ring of the hydrazone moiety. The antibacterial activity of the nitrofuranyl derivative (**13i**) against *S. aureus* and *B. subtilis* was comparable to positive control amikacin. Compounds (**13e**), (**13g**) and (**13i**) also inhibited the growth of Gram-negative bacteria (*E. coli* and *P. fluorescens*) with MIC values of 3.9–7.8 µg/mL.

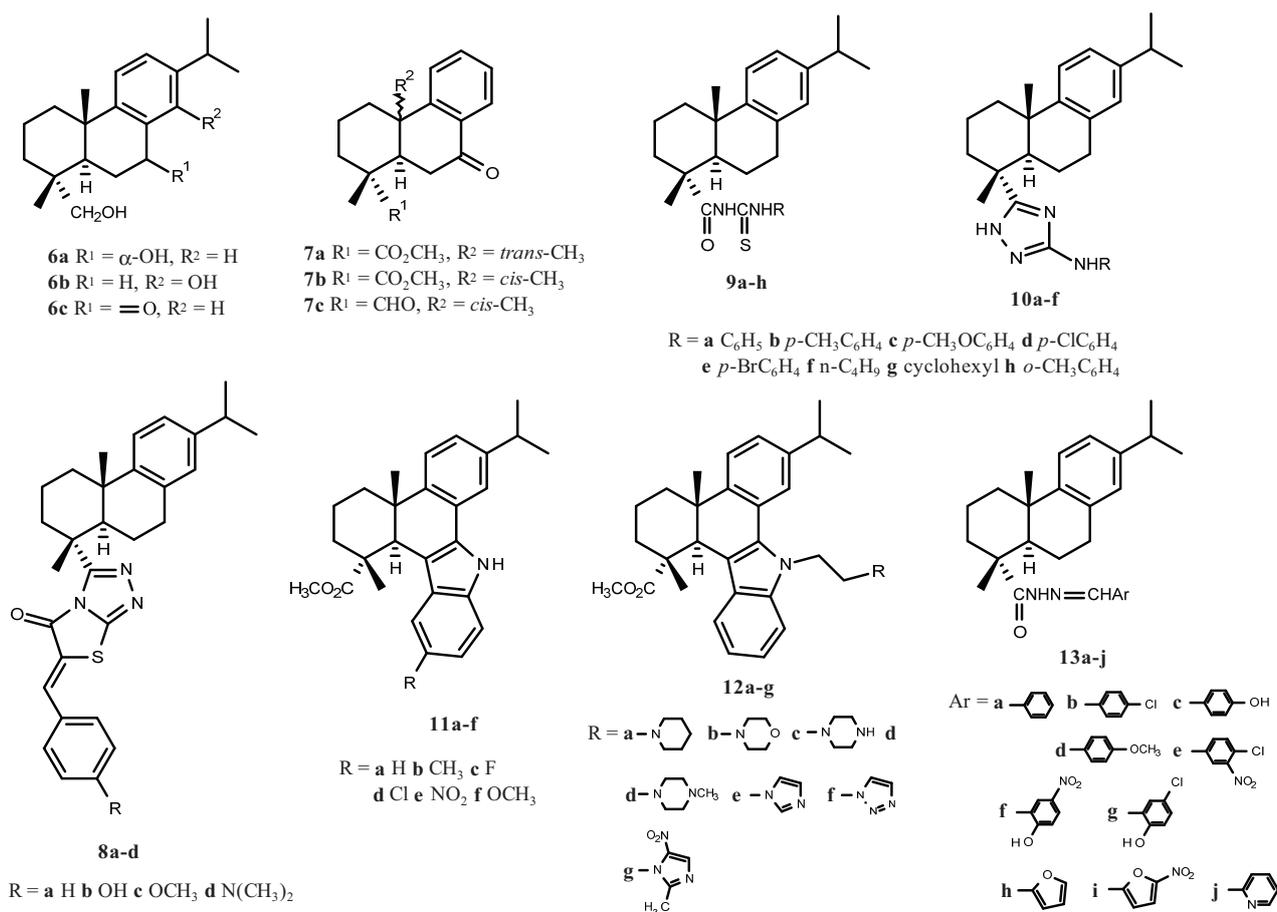


Fig. 2 Dehydroabietylamine derivatives (6-13) with antimicrobial properties.

2.2.3 Dehydroabietylamine derivatives

Dehydroabietylamine (**2b**, DHAA) (Fig. 1), also called leelamine, is a primary amine obtained as a part of a mixture of amines prepared from rosin. DHAA (**2b**) and its derivatives are widely used in the paper-making, coating and rubber industries due to their surfactant and dye properties, and as resolution agents in chiral separation technologies [44, 46]. DHAA (**2b**) also have pharmacological properties, and some of its derivatives are potent antibacterial and anticancer agents [44, 46, 65]. Among those, dehydroabietylguanidine (**2c**) (Fig.1), in the form of acetate salt, is a broad spectrum antibiotic effective against *Mycobacterium tuberculosis* (H₃₇Rv strain) with a MIC value as low as 1 μ g/mL [65]. The isopropyl substituent at C-13 was found to be essential for the tuberculostatic activity of dehydroabietylguanidine, but not for its antibacterial activity against Gram-positive (*S. aureus*) and Gram-negative (*E. coli*) bacteria [65]. Substitution in ring B of DHAA (**2b**), namely introduction of nitro substituents, originated potent antibacterial compounds, such as 12-nitro-dehydroabietylamine and 12,14-dinitro-dehydroabietylamine, with strong bactericidal activity against *E. coli*, *S. aureus* and *P. fluorescens* [46].

Several quaternary ammonium salts (**14a-f**) (Fig. 3) of DHAA displayed antibacterial activity against *S. aureus* and *E. coli* with MIC values of 7.81–31.25 μ g/mL for *S. aureus*, and 250–500 μ g/mL for *E. coli* [66, 67]. The cationic surfactants with *N,N*-dimethyl groups (**14a-c**) had biocidal activity comparable to that of commercially available benzalkonium bromide, being more effective than the corresponding analogues (**14d-f**) bearing diethyloxy groups.

Some peptoid derivatives of DHAA, including (**15**) and (**16**) (Fig. 3), are active at concentrations in the range 3–12 μ g/mL against a panel of Gram-positive and Gram-negative bacteria, including isolates resistant to known antibiotics [68]. The antimicrobial activity of these compounds is generally slightly more potent against Gram-positive than Gram-negative isolates, being bactericidal against *S. aureus*. Compound (**16**) was able to protect *S. aureus*-infected mice in a simple infection model.

Other DHAA derivatives, such as Schiff base derivatives (**17a-d**) (Fig. 3) obtained by reaction of the amino group with differently substituted benzaldehydes, exhibited bactericidal activity against *S. aureus*, *B. subtilis* and *E. coli* [69]. Compounds (**17c**) and (**17d**), derived from *p*-chlorobenzaldehyde and *p*-fluorobenzaldehyde, were the most active towards *B. subtilis* and *S. aureus*, respectively. The most active agent toward *E. coli* was compound (**17a**) derived from unsubstituted benzaldehyde.

Recently, a study on the activity of DHAA (**2b**) and nordehydroabietylamine (**2d**) (Fig. 1) against planktonic and biofilm infections of *S. aureus*, reported MIC values of 11.4 and 43.4 $\mu\text{g/mL}$, MBIC of 33.7 and 31.4 $\mu\text{g/mL}$ and MBEC of 21.2 and 7.7 $\mu\text{g/mL}$, respectively [56]. Besides preventing biofilm formation in the low micromolar range, only 2 to 4-fold higher concentrations of the amine compounds were needed to significantly reduce viability and biomass of existing biofilms, unlike typical antibiotics.

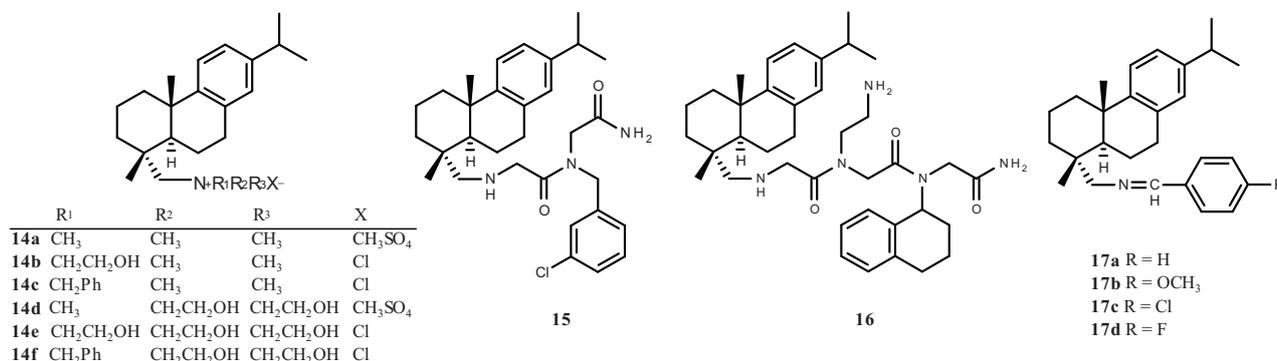


Fig. 3 Dehydroabietylamine derivatives (**14-17**) with antimicrobial properties.

2.2.4 Callitricic acid derivatives

Callitricic acid (**3**) (Fig. 1) can be found as a component in the resins of several *Callitris* species (Cupressaceae) and also in plants of the genus *Juniperus*, *Calceolaria* and *Illicium*. Recently, callitricic acid (**3**) and a series of aromatic abietane diterpenoids bearing a carboxylic acid function at C-19 have been isolated from the roots and stems of *Illicium jiadifengpi* (Illiciaceae) [70, 71], including 7α -hydroxycallitricic acid (**18a**), angustanoic acid F (**18b**) and its 7α -hydroxy derivative (**18c**), angustanoic acid E (**19a**), jiadifenoic acids B (**19b**), C (**19c**) and G (**19d**), angustanoic acid G (**19e**), jiadifenoic acid D (**20a**), and its C-15 epimer jiadifenoic acid E (**20b**) (Fig. 4). The compounds exhibited strong antiviral activity against Coxsackie virus (CV) B2, B3, B4 and B6, which was evaluated in African green monkey kidney cells (Vero cells) using a cytopathogenic effect assay. Compounds (**18a**), (**18c**), (**19b**) and (**19c**) were the most potent ones with IC₅₀ values of 2.7–23.2 $\mu\text{mol/mL}$. Additionally, (**19b**) had a SI value of 55.9 against CVB6, (**19c**) had selective index (SI) values of 52.8 and 49.9 against CVB2 and CVB6, respectively, while (**18a**) had SI values of 90.4, 46.5 and 69.0 against CVB2, CVB4 and CVB6 [70]. Compound (**18c**) had IC₅₀ of 21.9 $\mu\text{mol/L}$ and SI value of 45.6 against CVB3 [71].

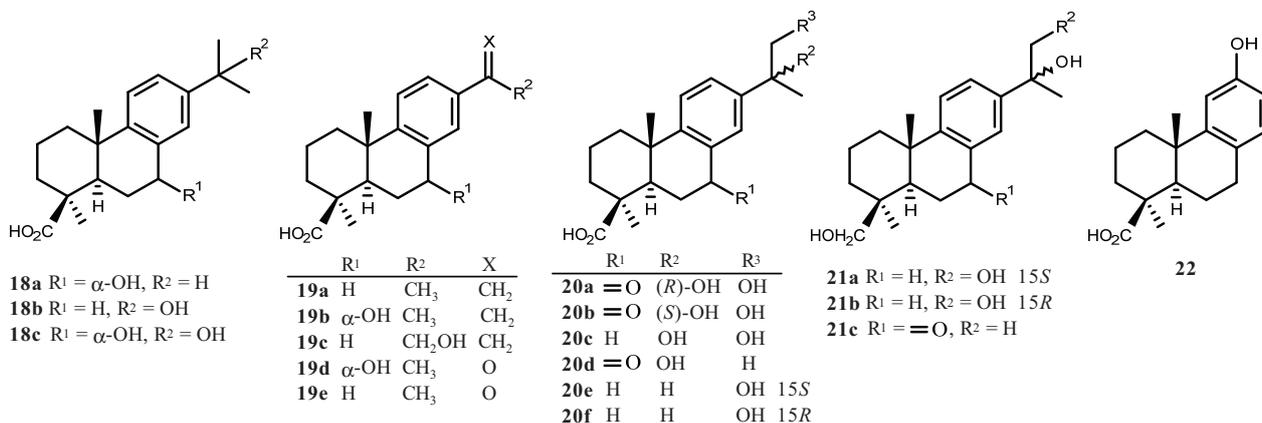


Fig. 4 Antiviral callitricic acid derivatives (**18-21**) and podocarpic acid (**22**).

Callitricic acid (**3**) has also been isolated from the roots of *Illicium majus*, along with majusanic acids B (**20c**), D (**20d**), E (**20e**) and F (**20f**) (Fig. 4), which also displayed antiviral activity against the Coxsackie B3 virus [72]. The majusanic acids showed IC₅₀ values of 3.3–51.7 $\mu\text{mol/mL}$ and SI values of 3.9–8.0, compared with IC₅₀ of 0.9 $\mu\text{mol/mL}$ and a SI value of 9.0 for the positive control ribavirin. The corresponding C-19 alcohol analogues, majusanins A-C (**21a-c**) (Fig. 4) were inactive, suggesting that the carboxylic acid group at C-19 plays a significant role in the antiviral activity [72]. Podocarpic acid (**22**) (Fig. 4), found in the resin of *Podocarpus cupressinum*, *P. dactyloides* and *Dacrydium cupressinum*, is a podocarpane diterpenoid with antiviral properties that also bears a C-19 carboxylic acid function, targeting the viral protein-mediated membrane fusion process [73].

2.2.5 Pisiferic acid and carnosic acid derivatives

Pisiferic acid (**4a**) (Fig. 1), first isolated from the methanol extract of leaves and twigs of *Chamaecyparis pisifera* Endle (Cupressaceae) and also found in some *Salvia* species, has antibacterial activity against Gram-negative bacteria (*P. aeruginosa*, *Proteus vulgaris* and *K. pneumoniae*), Gram-positive bacteria (*S. aureus* and *B. subtilis*) and antifungal activity against rice blast fungus *Pyricularia oryzae* [74, 75]. Methylation of the carboxylic acid group of pisiferic acid (**4a**) abolished its activity against Gram-negative bacteria *P. vulgaris* [74] while methylation of the phenol group abolished its antifungal activity [75]. Methylation of both the carboxyl and phenol groups almost eradicated antimicrobial activity [74, 75].

Carnosic acid (**4b**, CA) (Fig. 1) a derivative of pisiferic acid (**4a**), is found in the popular Lamiaceae herbs, sage (*Salvia officinalis*) and rosemary (*Rosmarinus officinalis*), along with its oxidation product carnosol (**4c**, CS), which exists predominantly in the form of lactone (**4c**) (Fig. 1). CA (**4b**) and CS (**4c**) have potent antioxidant activity due to the o-diphenol structure that undergoes oxidation easily. Several biological activities have been reported for CA (**4b**) and its derivatives, including antimicrobial, antitumor, anti-inflammatory, antiatherosclerosis, neuroprotective, antiangiogenic and antiadipogenic [44–46]. Antiviral properties have also been attributed to CA (**4b**), which has a strong inhibitory effect on HIV-1 protease (regarded as a potential target for the development of anti-HIV drugs) and HIV-1 virus replication, with IC₉₀ values of 0.08 µg/mL and 0.32 µg/mL, respectively [76].

CS (**4c**) and CA (**4b**) are the major components of the leaf extracts of *R. officinalis* and *S. officinalis*, which are known for their broad antimicrobial spectrum, being effective against a wide range of bacteria, yeasts and dermatophytes [67, 78]. CA (**4b**) has shown antimicrobial activity against aerobic (e.g. *Staphylococcus* sp., *Streptococcus* sp., *Enterococcus* sp., *Corynebacterium* sp.) and anaerobic (e.g. *Clostridium perfringens*, *Propionibacterium acnes*, *Fusobacterium nucleatum*, *Porphyromonas gengivalis*) bacteria, with MIC and MBC values in the range 16–128 µg/mL [78]. CS (**4c**) and CA (**4b**) are particularly effective in inhibiting the growth of oral pathogens responsible for dental caries, among them *S. mutans*, *S. sobrinus*, *S. mitis*, *S. sanguinis*, *S. salivarius* and *E. faecalis*, with MIC values in the range 15.0–100.0 µg/mL [77].

The antibacterial and resistance modifying activities of CA (**4b**) and CS (**4c**) have been described [79]. The compounds showed MIC values of 16–64 µg/mL against *S. aureus* strains possessing efflux mechanisms of resistance. CA (**4b**) and CS (**4c**) caused a 32- and 16-fold potentiation of the activity of erythromycin against an erythromycin effluxing strain, respectively. CA (**4b**) was shown to inhibit ethidium bromide efflux with an IC₅₀ of 50 µM in a strain of *S. aureus* over-expressing NorA. The antibacterial and efflux inhibitory activities of these natural products make them interesting templates for potential chemotherapeutic drugs with bacterial MDR modulation properties. Furthermore, CS (**4c**) was also found to greatly reduce the MICs of several aminoglycosides in vancomycin-resistant enterococci (VRE), thus potentiating antibiotic activity [80]. CA (**4b**) showed similar activity, and a synergistic effect was observed when CS (**4c**) or CA (**4b**) was combined with gentamicin [80].

O'Neill *et al.* [81] characterized several redox-active compounds with antistaphylococcal action, including CA (**4b**), as potential topical antibiofilm agents. CA (**4b**) displayed antistaphylococcal activity against planktonic cultures and was capable of eradicating established staphylococcal biofilms, with MIC and MBEC values of 8–32 µg/mL and 64–128 µg/mL, respectively. The authors also found that the antibacterial action was mediated by membrane perturbation, while the antibiofilm mode of action apparently involved disruption of the biofilm matrix.

The antimicrobial activity of carnosic acid-related compounds, such as 11-acetoxycarnosic acid, isolated from *Salvia* species, and 12-methoxycarnosic acid, isolated from *Dauphinea brevilabra* (Lamiaceae), has been reported [82]. The former showed considerable antibiotic activity against the Gram-positive microorganisms *S. aureus* and *B. subtilis* (MIC 10–25 µg/mL) while the latter also inhibited *Streptomyces scabies*, with MIC values in the range 1.0–20.0 µg/mL [45, 82]. Several semisynthetic derivatives from CA (**4b**) with cytotoxic and antimicrobial activities have been prepared [83]. Among those, the methyl O-12-methylcarnosate showed activity against *Mycobacterium tuberculosis*, with a MIC value of 28 µM.

2.2.6 Ferruginol derivatives

Ferruginol (**5**) (Fig. 1), the simplest phenolic abietane diterpenoid, occurs in plants belonging to the Podocarpaceae, Cupressaceae, Lamiaceae and Verbenaceae families, among others. This aromatic abietane has attracted considerable attention regarding its numerous biological activities, such as antioxidant, antimicrobial, antiplasmodial, antileishmanial, nematocidal, antitumor, anti-inflammatory and anti-ulcer [44–46]. Ferruginol (**5**) also showed antiviral properties, namely anti-severe acute respiratory syndrome associated coronavirus (SARS-CoV) activity, in a cell-based assay measuring SARS-CoV-induced cytopathogenic effect on Vero E6 cells [84]. Ferruginol (**5**) and other abietane-type diterpenoids were among the most potent compounds, with ferruginol (**5**) inhibiting 50% of viral replication at a concentration of 1.39 µM (EC₅₀) with a SI value of 58. The same study demonstrated that the antiviral activity of ferruginol (**5**) was due to specific inhibition of SARS-CoV protease, which plays a central role in processing viral proteins and controlling replicase complex activity [84].

The antimicrobial activity of ferruginol (**5**) and related common oxygenated derivatives, including hinokiol (**23a**) and sugiol (**24a**) (Fig. 5), has been evaluated against *S. aureus*, *S. epidermidis*, *E. faecalis*, *P. mirabilis*, *E. coli*, *P. aeruginosa*, *K. pneumoniae*, and *C. albicans* [85], as well as against antibiotic resistant bacteria [86]. 1-Oxo-ferruginol (**23b**) (Fig. 5) from the roots of *Salvia viridis* (Lamiaceae) showed antimicrobial activity against *S. aureus*, *S. epidermidis* and *B. subtilis* [85]. Sugiol (**24a**) and montbretol (**25a**) (Fig. 5) were active against MRSA, but not against VRE [86]. 2 β -Acetoxylferruginol (**23c**) (Fig. 5) isolated from the bark of *Prumnopitys andina* (Podocarpaceae) had an antibacterial activity of 8 $\mu\text{g/mL}$ against two effluxing strains of *S. aureus*, despite being inactive against a wild-type strain and a MRSA clinical isolate [87]. Since ferruginol (**5**) was active against the mentioned strains, the results indicate that the presence of the acetoxy group at C-2 in (**23c**) is detrimental for the antibacterial activity against certain *S. aureus* strains.

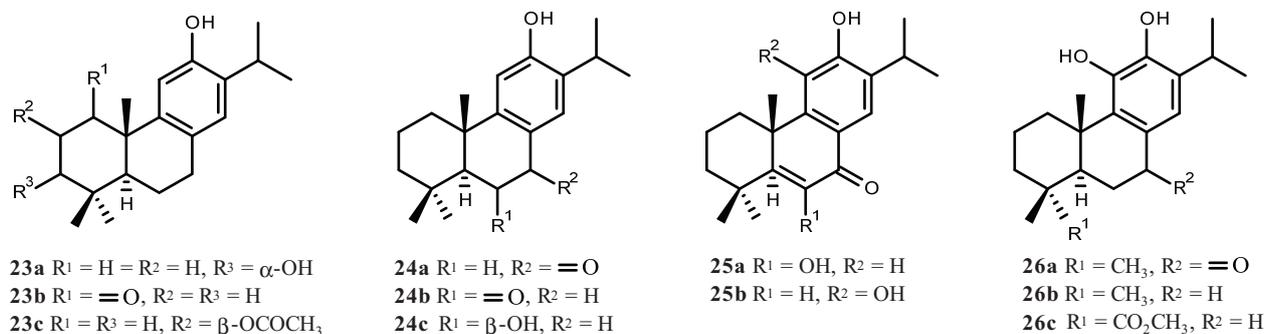


Fig. 5 Ferruginol derivatives (**25-28**) with antimicrobial activity.

Tada *et al.* [88] developed a synthetic route to (+)-ferruginol (**5**) and the corresponding enantiomer (–)-ferruginol, and used the same methodology to prepare several oxidized derivatives, including 6-oxoferruginol (**24b**), 6 β -hydroxyferruginol (**24c**) and the catechols salvinolone (**25b**) and demethyl cryptojaponol (**26a**) (Fig. 5). The compounds exhibited potent antibacterial activity with MIC values of 4–8 $\mu\text{g/mL}$ against MRSA and 4–16 $\mu\text{g/mL}$ against VRE strains. Non-natural (–)-ferruginol showed stronger activity than natural (+)-ferruginol (**5**) [88].

The synthetic catechol derivatives (**26b-c**) (Fig. 5) prepared by Gigante *et al.* [89] starting from abietic acid (**1a**) showed potent antifungal activity against dermatophytes *Microsporum canis*, *Trichophyton mentagrophytes* and *Epidermophyton floccosum*, with MIC values of 13.1–210.0 μM , below that obtained for the control drug fluconazole (MIC values of 52.2–208.9 μM). Compound (**26c**) also had antiherpetic activity and moderate anti-HIV activity [89].

3. Structure-activity relationship analysis

The activity of antimicrobial diterpenoids against certain microorganisms is associated to the presence in the molecules of functional groups such as carboxyl, hydroxyl, aldehyde or ketone, among other groups [57–59]. The ability of these polar functionalities to act as hydrogen donors or acceptors with the microbial targets and the importance of their position within the hydrocarbon skeleton led to the establishment of some important structure-activity relationships.

Urzúa *et al.* [90] have identified some structural requirements for the activity of several antimicrobial diterpenoids and their derivatives, which included a hydrophobic moiety, consisting of a substituted decalin skeleton, capable of insertion into a lipophilic region, and a hydrophilic region possessing a hydrogen bond donor group, able to interact with hydrogen bond acceptor groups of the target site. These structural features were responsible for an optimal insertion of the compounds into cell membranes, as suggested by the results of docking some of them into a model phospholipid bilayer [90].

The DHA skeleton has been extensively investigated to determine structural effects on its antibacterial and antifungal properties [59]. Substitution of the carboxylic acid group of DHA (**2a**) by an aldehyde or hydroxymethyl group improved antimicrobial activity, while removal of the isopropyl side chain from ring C could enhance activity depending on the microorganism [47, 57–59]. Antimicrobial activity could also be enhanced by the presence of a hydroxyl group at C-12, e.g. ferruginol (**5**), and an additional carbonyl at C-7, e.g. sugiol (**24a**). A study on the structure-activity relationships of aromatic diterpenoids from *Salvia* species revealed that the free catechol group, such as in CA (**4b**) and CS (**4c**), was essential for antimicrobial activity against Gram-positive bacteria, which was enhanced by oxidation of catechol group to quinone [91]. On the other hand, the stereochemistry of the A/B ring junction did not appear to display a significant role in antibacterial activity [57–59].

The activity of pisiferic acid (**4a**) against Gram-negative bacteria (*K. pneumoniae*, *P. vulgaris* and *P. aeruginosa*) and fungi can be almost completely neutralized by esterification of the carboxylic acid or methylation of the phenol group, respectively [74, 75]. These studies clearly demonstrated the need for a carboxyl group at C-10, such as in pisiferic acid (**4a**), for antimicrobial activity against Gram-negative bacteria [74], while antifungal activity seems to require a hydroxyl group at C-12 [75], which also provides activity against Gram-positive bacteria [74]. The hydrophobic nature

of pisiniferic acid derivatives has been correlated with their antimicrobial potencies, and it has been found that higher lipophilicity is associated with higher activity against Gram-positive bacteria (*S. aureus*, *B. subtilis*), whereas a lower lipophilicity increases activity against Gram-negative microorganisms [74].

Comparative studies have also been conducted on the antimicrobial activities of the aromatic diterpenoids DHA (**2a**), pisiniferic acid (**4a**), podocarpic acid (**22**) and ferruginol (**5**) [92]. Antimicrobial potency against Gram-positive bacteria was found to decrease in the order ferruginol (**5**) > pisiniferic acid (**4a**) > DHA (**2a**) > podocarpic acid (**22**). The results showed that the aromatic ring C and the isopropyl substituent at C-13 strongly contributed to the antimicrobial activity against Gram-positive bacteria, which was enhanced when the hydroxyl group was *ortho* to the isopropyl group. The change of the carboxyl group from C-4 (ring A) of DHA (**2a**) to C-10 in pisiniferic acid (**4a**) increased antimicrobial activity against Gram-positive bacteria while also conferring activity against Gram-negative microorganisms [92].

The diterpenoids with a carboxylic acid function at C-19, such as in callitricic (**3**) and podocarpic (**22**) acids, have potent antiviral activities [70–73]. In the callitricic acid series (Fig. 2), hydroxyl substitution at C-7 significantly enhanced antiviral activity against Cocksackie virus B (CVB) and decreased cytotoxicity compared to the corresponding non-hydroxylated derivatives [72]. Similarly, introduction of a hydroxyl group at C-17 improved antiviral activity, but hydroxyl substitution at C-12 or C-15 had no impact on either antiviral or cytotoxic properties. Moreover, acetyl substitution at C-13 promoted antiviral activity and reduced the cytotoxicity while isopropenyl substitution had no effect on both properties.

4. Mode of action

Although the huge amount of different new secondary metabolites that have been isolated and identified in the last decades, little is known about the mode of action for most of the natural antimicrobial agents. The mechanisms that might be involved in the antibacterial activity of abietane diterpenoids, and antibacterial natural products in general, are multifactorial and often specific for a certain microorganism or class of microorganisms.

Antimicrobial diterpenoids can act on multiple biochemical targets of the microorganisms and it has been suggested that the activity of these compounds results from their ability to cross or damage microbial cell membranes due to their amphiphilic nature [25, 90, 93]. The interaction of abietic acid (**1a**) with phospholipid membranes has been studied and its bacteriolytic action against *B. cereus* has been established [90, 94].

Disruption of the membrane topology leads to increased membrane fluidity and permeability, disturbance of membrane embedded proteins, inhibition of respiration, and alteration of ion transport processes in both Gram-positive and Gram-negative bacteria [93]. Oxygen consumption and respiratory-driven proton translocation in *P. aeruginosa* are inhibited by totarol, which also inhibits bacterial NADH-cytochrome c reductase, NADH-DPIP reductase and NADH-CoQ reductase [70–73]. The site of respiratory inhibition of this diterpene is thought to be near CoQ in the bacterial electron transport chain [95].

Once inside the cell, antimicrobial diterpenes can inhibit microbial proteins and enzymes, including MDR pumps and enzymes involved in cytoplasmic and cell wall metabolism, or interfere with microbial nucleic acid synthesis and protein synthesis [25]. Cell wall peptidoglycan synthesis was the primary target site for the inhibitory action of pisiniferic acid (**4a**) and some derivatives on *B. subtilis* [74]. Horminone, an abietane diterpene quinone, was found to bind to ribosomal RNA phosphate groups [96], thus inhibiting bacterial protein synthesis, whereas ferruginol (**5**) potentiates oxacillin activity by acting as an efflux pump inhibitor [97].

Moreover, plant-derived antiviral natural products are known to interfere with many viral targets, including inhibition of viral enzymes, such as integrase, protease, nucleic acid polymerase, or reverse transcriptase [72, 73, 76, 84]. Additionally, some of these compounds are able to block ligands necessary for viral attachment to host cells, thus preventing virus adsorption. This is the case of podocarpic acid (**22**) and related analogues that have been shown to inhibit (H1N1) influenza A virus by targeting the viral hemagglutinin-mediated membrane fusion [73].

5. Conclusion

Plants have developed effective defence strategies during the course of evolution to protect themselves from phytopathogenic microbes and herbivorous in their environment. Disease resistance in plants depends on the activation of coordinated, multicomponent defence mechanisms. Therefore a single mechanism or compound with a single target site is thought to be unsatisfactory due to fast development of resistant strains of pathogens to one selected substance. The multitude of antimicrobial secondary metabolites with different target sites and the multifactorial nature of the mechanisms underlying their biological activities make emergence of resistance unlikely and disclose the importance of the plant kingdom as a source of new antimicrobial compounds with diverse modes of action and resistance-modifying properties.

Bioactive plant-derived secondary metabolites are likely to be used as leads to synthetic new and more effective antimicrobial agents as well as compounds with new pharmacological effects by repeated structural modification. Coniferous plants, being rich in abietane type diterpenes, many of which with antibacterial, antifungal and antiviral

properties, represent an interesting source of antimicrobial leads. It is expected that the structurally modified natural products will exhibit increased potency, selectivity, duration of action and bioavailability with reduced toxicity.

Acknowledgements The authors dedicate this book chapter to M. F. Simões, former Professor of Pharmaceutical Chemistry at FFUL and now retired.

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