Fungal Metabolites for the Control of Biofilm Infections

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Abstract: Many microbes attach to surfaces and produce a complex matrix of polymers surrounding their cells, forming a biofilm. In biofilms, microbes are much better protected against hostile environments, impairing the action of most antibiotics. A pressing demand exists for novel therapeutic strategies against biofilm infections, which are a grave health wise on mucosal surfaces and medical devices. From fungi, a large number of secondary metabolites with antimicrobial activity have been characterized. This review discusses natural compounds from fungi which are effective against fungal and bacterial biofilms. Some molecules are able to block the cell communication process essential for biofilm formation (known as quorum sensing), others can penetrate and kill cells within the structure. Several targets have been identified, ranging from the inhibition of quorum sensing receptors and virulence factors, to cell wall synthesizing enzymes. Only one group of these fungal metabolites has been optimized and made it to the market, but more preclinical studies are ongoing to expand the biofilm-fighting arsenal. The broad diversity of bioactive compounds from fungi, their activities against various pathogens, and the multi-target trait of some molecules are promising aspects of fungal secondary metabolites. Future screenings for biofilm-controlling compounds will contribute to several novel clinical applications.

Keywords: fungal metabolites; biofilm infections; quorum sensing; species interactions; antibiotic resistance; non-microbicidal drugs

1. Biofilm Infections Are a Therapeutic Challenge

In biofilms microbial cells are embedded in a complex matrix of macromolecules that adhere to various surfaces. While biofilms have several roles in the environment, like cleaning waste water in sewage plants, they can also be harmful, e.g., causing damage to buildings and monuments or clogging tubes and pipelines in industrial plants. In humans, they can play a protective role in the skin and mucosa, but often can represent an infectious threat. In this review we focus on infectious biofilms formed by pathogenic microorganisms, and we review approaches for their control using compounds produced by fungi. Biofilm infections are caused not only by bacteria, e.g., *Pseudomonas aeruginosa*, *Escherichia coli*, or *Staphylococcus aureus*, but also by fungi, e.g., *Candida albicans* or *Aspergillus fumigatus*. Biofilms can form on any implant and also on mucosal surfaces, e.g., in the cystic fibrosis lung [1], the middle ear [2], the urinary tract [3], or gastric mucosa [4]. Biofilm formation is a key factor for microbial survival in hostile environments, from where cells can disperse to colonize new habitats [5]. Infections caused by biofilms often result in severe complications and 12% to 25% of bloodstream infections are attributable to patient mortality [6].

To form a biofilm, microbes have to determine their cell density and to coordinate their population behavior. This is mediated by small molecules released into the environment and acting as autoinducers to start genetic programs [7]. If the autoinducer concentration reaches a certain threshold, the genetic

program of biofilm formation starts, followed by the production of virulence factors. This intercellular communication principle is used by many microbes and called quorum-sensing (QS) [8]. To date more than 100 different autoinducers are known for bacteria, archaea, and fungi. The best studied autoinducers are *N*-acyl-L-homoserine lactones, only known from Gram-negative bacteria. The acyl side chains are 4–16 carbons long, usually saturated, and can be oxidized at C-3 **1–3** [9] (Figure 1). Contrary to the acyl-homoserine lactones, autoinducer-2 (AI-2) is known both from Gram-positive and -negative bacteria, having the rather unusual cyclic boronic ester **4** [10]. There is also some species-specific autoinducers, e.g., the *Pseudomonas* quinolone signal (PQS) **5**. Gram-positive bacteria use small peptides as autoinducers which are derived by post-translational processing of larger precursor peptides [11].

1 R=H or alkyl   **2** R=H or alkyl   **3** R=H or alkyl

4 Autoinducer-2   **5** PQS   **6** Tyrosol

7 Farnesol   **8** 3R-Hydroxy-tetradecanoic acid
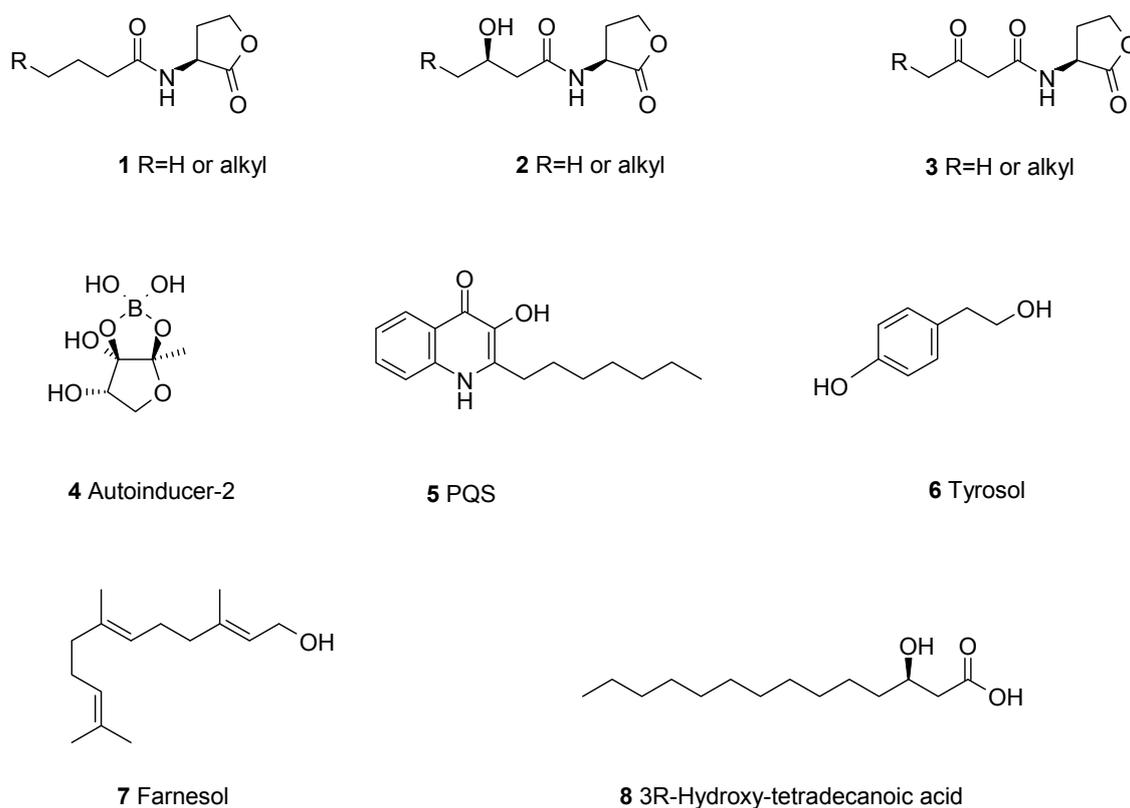
Figure 1. Molecules involved in quorum sensing in bacteria (**1**–**5**) and in fungi (**6**–**8**).

Quorum sensing is also known from fungi [12,13] and best studied in *Candida albicans* [14]. The known quorum sensing molecules from fungi are structurally very different from those found in bacteria. Tyrosol **6** [15], farnesol **7** [16,17] and 3R-hydroxy-tetradecanoic acid **8** [18], have been reported. These compounds are probably not very specific for *C. albicans*, since one third of all Ascomyotina strains tested could produce farnesol [19].

Most investigations on biofilm control have been done with pure strains; however, in nature, microbes seldom live alone and often form polymicrobial communities [20]. The individual species within these communities do not exist independently from each other, having complex and multifold interactions [21]. Any future drug for the control of biofilm infections has to deal with these interactions in order to avoid suppressing one pathogen while fostering the development of another one in the same biofilm community.

The eradication of biofilm infections is complicated because of the high protection of pathogens against host defenses (e.g., macrophages) and antibiotics [22]. For instance, 220-times higher antibiotic concentrations were required for killing *E. coli* in a biofilm than for the same strain living in planktonic

form in the serum [23]. Similar high resistances have been reported for fungal biofilms [24]. Because of these complications and under the background of increasing antibiotic resistances [25] novel antibiotics and novel targets are needed to control biofilm infections [26]. Several approaches are being developed in this context. One is the search for novel, biofilm-penetrating antibiotics [27]. Another one aims to disrupt communication of pathogens by blocking quorum sensing [28,29]. Most of these efforts are directed against bacteria, but also a number of techniques have been described for the screening of compounds against pathogenic fungi biofilms [30]. The search for biofilm-modulating secondary metabolites has been undertaken by several groups. The rationale behind this approach is that all organisms are confronted with the formation of pathogenic biofilms on their bodies, meaning they are driven to develop strategies to fight pathogenic biofilms. Compounds emerging from these searches are therefore already tailored for biofilm-control, and can serve as lead compounds for drug development. Bacteria and plants seem to be currently the main sources for such bioactive compounds; however fungi, mainly those of marine origin, have also been included in these screenings [31].

In this review we discuss the control of bacterial and fungal biofilm infections by novel antibiotics and biofilm-modulating compounds of fungal origin. The advantages and short-comings of the different classes of compounds are evaluated and an outlook for future developments is given.

2. Fungal Metabolites Reported to Modulate Biofilms of Pathogens

In the attempts to find novel natural products for the control of biofilm infections, fungal extracts have been the subject of several screenings. One approach is to look for sporocarps (fruiting bodies) of fungi which are exposed to a wet climate, favorable conditions for biofilm formation. In a small study of sporocarps from Basidiomycotina it was found that ectomycorhizal fungi had a higher diversity of bacterial biofilms on their fruiting bodies than saprophytic ones. Consequently, saprophytic Basidiomycotina had a higher percentage of biofilm-inhibiting extracts than their ectomycorrhizal cousins [32]. For obtaining the required novel isolates of fungi, sometimes unusual methods have been applied, for instance crowdsourcing, where samples from citizen scientists were included in the screening procedure [33]. Often these extracts showed activity, however sometimes the investigations ended in the screening, and no results on the nature of the bioactive compounds or their modes of action were reported. This leaves us only with the knowledge that there is some activity, but not what causes it. Many of these studies were directed against specific pathogenic biofilms, e.g., *Lentinula edodes* extract against the bacterial human pathogens *Actinomyces naeslundii*, *Prevotella intermedia*, *Neisseria subflava*, and *Streptococcus sanguinis* [34,35], sometimes using highly elaborated and specific methods [36]. Also lichens, the association between fungi and algae, have been investigated over decades for their spectrum of bioactive secondary metabolites; however, only relatively few reports appeared on biofilm-modulating compounds from this group of organisms [37].

2.1. Biofilm-Modulating Terpenes from Fungi

The finding that high concentrations of farnesol **7**, a quorum sensing compound in *C. albicans*, inhibits filamentation led to the application of this sesquiterpene for preventing biofilm formation by pathogenic *C. albicans*. When added at an early stage of biofilm formation, 300 µM farnesol could completely prevent the formation of a fungal biofilm [38]. Similar to the observation for many quorum sensing quenchers active against bacteria, the effect was less pronounced for mature biofilms. To find out whether the effect of farnesol is specific for *C. albicans*, Weber et al. investigated the production of eight different *Candida* species under different culture conditions. They found that only *C. albicans* and *C. dubliniensis* produced farnesol and from these two species, *C. albicans* produced more farnesol and stronger biofilms. For *C. kefyr*, *C. glabrata*, *C. krusei*, *C. parapsilosis*, *C. tropicalis*, and *C. guilliermondii* only low concentrations of farnesol were found in the media. The amount of farnesol production was not only species- but also strain-dependent [39].

When analyzing biofilm formation of a *Pneumocystis* species, Cushion et al. found that the ability of farnesol to prevent biofilm formation is not limited to *Candida* species. Species of the fungal genus

Pneumocystis can cause severe pneumonia in immune-compromised patients, but lack of experimental models still prevents a better understanding of these pathogens. The addition of farnesol to cultures of *P. carinii* from rat or *P. murina* from mouse could completely prevent the formation of biofilms, which is a probable virulence mechanism in the lung [40]. Farnesol has inhibiting effects not only on fungal but also on bacterial biofilms. Here it seems that the results vary a lot between the different species tested. When methicillin-resistant *Staphylococcus aureus* biofilms were treated with 150 μM farnesol the resistance against methicillin could be reversed. More importantly, the sensitivity against several other antibiotics, e.g., gentamycin, could be enhanced 100-fold for established biofilms [41]. In the closely related bacteria *S. epidermidis*, 200 μM farnesol were required to have an effect both on planctonic and biofilm-embedded cells, a result which was comparable to that of vancomycin [42]. Not surprisingly, a reduction in biofilm volume was also observed [43]. Such a reduction in biofilm volume was also found for *Streptococcus mutans* biofilms; however, here farnesol caused dispersion of the biofilm rather than significant killing of the cells [44].

Biofilms of *C. albicans* often develop resistance against the antifungal fluconazole. When these resistant biofilms were treated with farnesol at an early stage of biofilm development, the resistance could be reduced by more than three orders. Treatment at a later stage of biofilm development had a somewhat weaker but still very significant effect [45]. A similar observation was made also for the yeast *C. dubliniensis* [46], and it is noteworthy that these are the two farnesol-producing *Candida* species.

Downregulation of genes involved in hyphal formation, GTPase activation, mitosis and DNA replication in *C. albicans* when treated with 40 μM farnesol **7** shed some light on the mode of action of this compound. Interestingly, this could be reversed by the addition of 1-oleoyl-2-acetyl-sn-glycerol, a diacylglycerol analogue. In mammalian cells this diacylglycerol is an activator of protein kinase C; however, in *C. albicans* a PKC1 deletion mutant still showed the same response indicating that protein kinase C is not the target of 1-oleoyl-2-acetyl-sn-glycerol [47]. While farnesol showed no effect at all on the Gram-negative bacteria *Burkholderia multivorans* and *B. cenocepacia* [48] it affected the virulence of another Gram-negative pathogen, *Pseudomonas aeruginosa*. Here treatment with farnesol led to a decreased production of the *Pseudomonas* quinolone signal (PQS), and the virulence factor pyocyanin, which is controlled by PQS. Transcriptome analysis revealed that the transcription of the genes involved in PQS-synthesis was reduced [49], adding another mode of action of farnesol in biofilms. The authors also tested related compounds and found that farnesyl acetate **9** and geranyllinalool **10** were active too, but not the saturated and unbranched alcohol n-dodecanol **11** (Figure 2). Farnesol has also been shown to induce apoptosis in *C. albicans*, causing up-regulation of the caspase gene MCA1 and the intracellular presence of caspases [50]. Apoptosis is triggered by farnesol not only in *C. albicans*, but also in *Aspergillus nidulans* [51] and *Saccharomyces cerevisiae*, where reactive oxygen species participate in programmed cell death [52]. Finally, apoptosis caused by farnesol was also reported in human carcinoma cell lines [53].

Besides farnesol only a few other fungal terpenes have been reported possessing biofilm-modulating activities. One of the simplest ones, mevalonolactone **12**, is produced by an unidentified marine fungus isolated from the sponge *Mycale magnirhaphidifera*, and was found to prevent biofilm formation by *S. epidermidis* [54]. From a culture of a marine-derived strain of *Emericella variecolor*, the sesterterpenes ophiobolin K **13**, 6-epi-ophiobolin K **14**, and 6-epi-ophiobolin G **15** were isolated. All three ophiobolins inhibited biofilm formation of *Mycobacterium smegmatis* with biofilm inhibitory concentrations (BICs) of 4.1–65 μM, showing no antimicrobial activity at these concentrations. The most active compound was ophiobolin K, which was also active against *M. bovis* BCG [55]. The fungus *Neosatorya fischeri* KUTC 6344 was isolated from soil and shown to produce several secondary metabolites, including sartorypyrone A **16** and aszonapyrone A **17** and B **18** [56]. When applied at minimal inhibitory concentration (MIC)-levels, aszonapyrone A or sartorypyrone A almost completely prevented biofilm formation by *S. aureus*, *Bacillus subtilis* or *Enterococcus faecalis*, but were much less active in dispersing mature biofilms. Aszonapyrone B **18**, only lacking the acetoxy group of aszonapyrone A **17**, was inactive. Similar to most antibiotics, these compounds cause stress

to planctonic cells since at subtoxic concentrations they were not only unable to prevent biofilm formation but, on the contrary, fostered it [57]. An endophytic *Guignardia* species isolated from the plant *Euphorbia sieboldiana* produced several meroterpenes when grown as solid culture on rice grains. Two of these terpenes, guignardone N **19** and guignardic acid **20**, were synergistic with fluconazole in biofilm inhibition and killing of drug-resistant *C. albicans*, lowering the MIC from >1000 µg·mL^{-1} for fluconazole alone to 63 µg·mL^{-1} for the combination fluconazole–meroterpene [58].
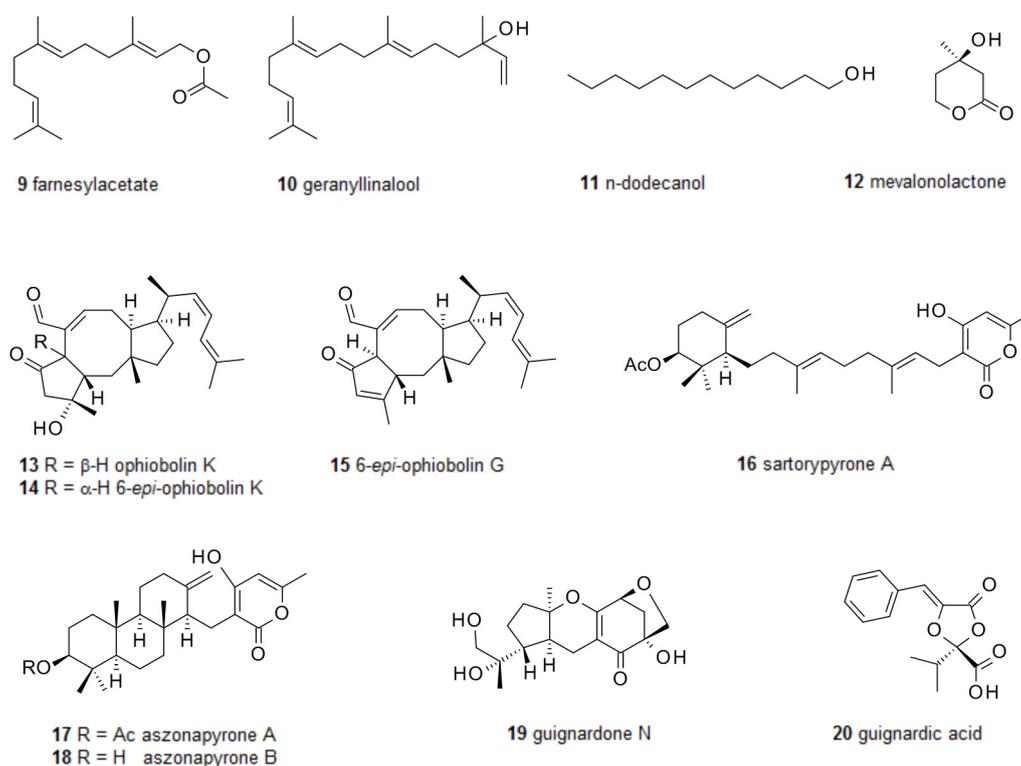
Figure 2. Terpene-derived secondary metabolites from fungi reported to inhibit biofilms of bacteria or fungi, and related compounds.

2.2. Fungal Metabolites of Polyketide Origin for Biofilm-Control

For more than a century lichens have been used against bacterial infections. One of the best known compounds in this respect is usnic acid **21**, produced by the fungal part in several lichens (Figure 3) [59]. It is commercially available and has been studied for its several bioactivities and its toxicity [60]. Usnic acid acts as antibiotic on group A *Streptococcus* [61] and *C. albicans* biofilms [62]. As a consequence it has been applied as surface coating against bacterial biofilm formation. Here it does not prevent biofilm formation but kills the cells when they settle at the surface [63]. Another lichen metabolite, protolichesterinic acid **22**, has been reported to inhibit biofilm formation by interfering with the quorum sensing systems of *P. aeruginosa* and *B. cenocepacia* [64]. Screening a number of lichen extracts further identified thamnolic acid **23** as active against *C. albicans* biofilms [65].

A number of rather diverse secondary metabolites from fungi have been characterized which are formed via polyketide precursors and are able to suppress biofilm development. Patulin **24** and penicillic acid **25** from *Penicillium* species were found displaying quorum quenching activity, i.e., suppression of communication by quorum sensing. As already observed for most quorum-quenchers, patulin enhanced *P. aeruginosa* biofilm susceptibility to tobramycin. Furthermore, patulin and penicillic acid activate polymorphonuclear neutrophils and break the blockage of the oxidative burst [66]. *Coprinus comatus* is an edible fungus which is also used for various medical applications. From this fungus the small gamma-lactone **26** has been isolated and shown to cause dispersion of *P. aeruginosa*

biofilms by interfering with its quorum sensing. It also reduces the production of pyocyanin and rhamnolipids, two virulence factors of *P. aeruginosa* [67]. From *Leucostoma persoonii*, isolated from red mangrove, a number of cytosporones have been isolated. One of them, cytosporone E **27**, could inhibit biofilm formation of methicillin-resistant *S. aureus* at 39 μM, about half of its MIC, and was also active against *Plasmodium flaciparum* [68]. Terreic acid **28**, long known from *Aspergillus terreus*, has been demonstrated to suppress biofilm growth of *E. coli* by inhibiting the bifunctional GlmU uridyltransferase, which catalyzes the acetylation and uridylation of glucosamine-1-phosphate [69]. Natural products often have several rather than only one target in the cell, and terreic acid has also been shown to covalently inhibit the enzyme MurA, which catalyzes the reaction of this substrate with phosphoenolpyruvate [70]. The soil derived fungal strain *Metarhizium anisopliae* produced a number of isocoumarin glycosides when growing in solid culture on rice. One of these metabolites, **29**, strongly inhibited the formation of biofilms by *P. aeruginosa*. 6-Acetylation of the sugar seems to be essential for activity because metabolite **30**, lacking the acetoxy group, did not show any activity [71]. Myriocin **31,** a strong antifungal agent, was first isolated from *Myriococcum albomyces* [72] and later also from *Isaria sinclairii* and *Mycelia sterilia*. Myriocin acts by strong inhibition of serine palmitoyltransferase, a key enzyme in the synthesis of sphingolipids [73]. It has been demonstrated that *C. albicans* biofilms possess a higher ratio of sphingolipids than planktonic cells, and accordingly, treatment with myriocin completely prevented the formation of *C. albicans* biofilms [74], as well as biofilms of *Aspergillus fumigatus* clinical isolates [75]. Although these reports are rather optimistic, one should keep in mind that myriocin is toxic for eukaryotes and has a small therapeutic window.
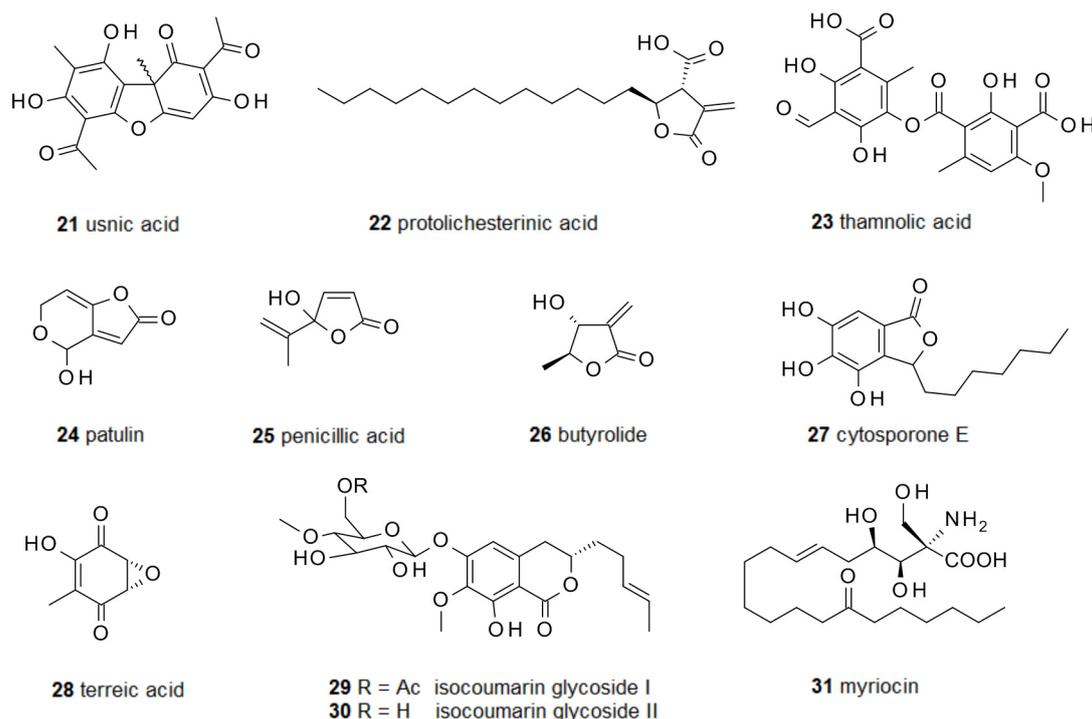
Figure 3. Biofilm-controlling metabolites from fungi synthesized via a polyketide biosynthetic pathway.

Besides these relatively simple compounds, a number of much more complex fungal secondary metabolites have been demonstrated to possess biofilm-modulating activities. From a *Penicillium* sp. several closely related alkaloids were isolated and two of them, shearinines D **33** and E **34**, inhibited the formation of *C. albicans* biofilms (Figure 4); however, they were unable to disperse already established ones. Interestingly, their parent compound, shearinine A **32**, not oxidized at C-22, did not show any activity [76]. *Bionectria ochroleuca* produced the polyketide glycosides bionectriol B-D **35–37** and TMC-151 C-F **38–41**, which could all prevent biofilm formation by *C. albicans* [77]. Waikialoid A **43**

and waikialide A **42** from *Aspergillus* sp. could do the same; remarkably, waikialoid A had an IC_{50} of 1.4 µM, one of the lowest so far reported for the inhibition of *C. albicans* biofilms [78].
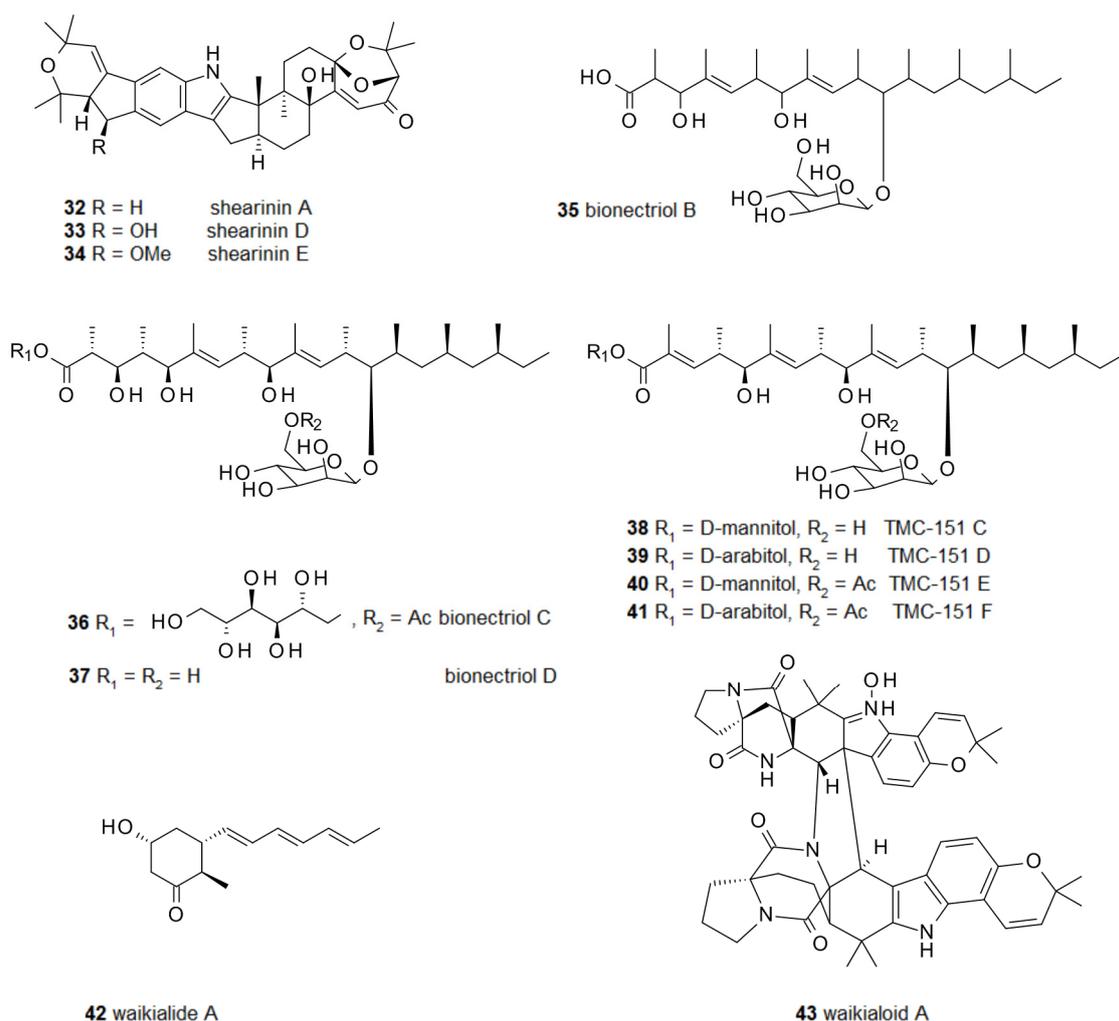
Figure 4. Relatively complex secondary metabolites from fungi directed against biofilms of *C. albicans*.

2.3. Amino Acids and Derivatives Controlling Biofilm Formation

Tyrosol **6** is derived from tyrosine and acts in *C. albicans*, together with farnesol, as a quorum sensing compound. At 200 mM, tyrosol reduced biofilm formation and metabolic activity of *C. albicans*, *C. glabrata*, *S. mutans* and their mixed biofilms, at a degree comparable to the effect of chlorhexidine gluconate [79].

Bioactive diketopiperazines have been reported from fungi including cyclo(L-Phe-L-Pro) **44** from *Rosellinia necatrix* [80] and cyclo(L-Tyr-L-Pro) **45** from *Alternaria alternata* [81]. These compounds interfere with the quorum sensing system of some bacteria by inhibiting the action of acyl-homoserine lactones [82], but there are contradicting reports. Cyclo(L-Tyr-L-Pro) **45** reduced colony expansion in *Serratia liquefaciens* and was able to inhibit the swarming behavior of a *S. liquefaciens* swarming motility mutant, when added together with autoinducer butyl-homoserine lactone. Diketopiperazines not only interfere with quorum sensing by competing with acyl-homoserine lactones in Gram-negative bacteria, but can also modulate biofilms of Gram-positive bacteria. Cyclo(L-Phe-L-Pro) **44** and cyclo(L-Tyr-L-Pro) **45** strongly inhibit the Ptst promoter of the accessory gene regulator (agr) quorum sensing system of *S. aureus*. This interaction seems to be not very specific since inhibition has been observed for all four known agr subgroups of *S. aureus*, despite differences in their autoinducer structures [83].

A marine *Penicillium* sp. isolated from the sponge *Axinella corrugata* produced cyclo(L-Tyr-L-Leu) **46** which inhibited biofilm formation by *Staphylococcus epidermidis* [84]. Cyclo(L-Leu-L-Pro) **47** from an isolate of *Penicillium commune* was active against *S. aureus* biofilms [85]. From the marine fungus *Cladosporium* sp., F14 cyclo-(Phe-Pro) **44** and cyclo-(Val-Pro) **48** were isolated. When tested against biofilm-forming bacteria species, both compounds were active against *Loktanella hongkongenis*, but only cyclo-(Phe-Pro) showed antimicrobial activity against *Micrococcus luteus* and *Ruegeria* sp. [86]. Flavipesin A **49** is an antibiotic from a marine isolate of *Aspergillus flavipes* which can penetrate mature biofilms of *S. aureus* and *B. subtilis* [87]. The activity of several diketopiperazines against biofilms is in line with the many activities displayed by this class of natural compounds [88] (Figure 5).
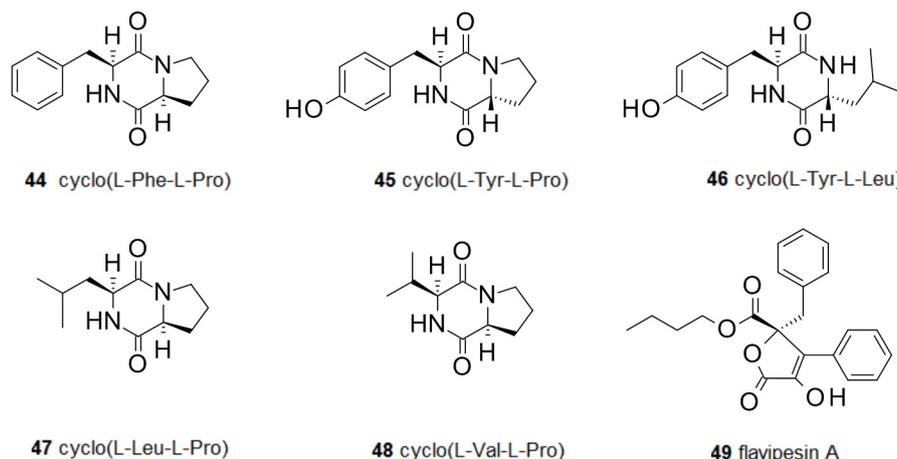
Figure 5. Biofilm-controlling metabolites from fungi derived from amino acids.

Campbell et al. synthesized several diketopiperazines and tested their library against a *Vibrio fischeri* reporter strain. The authors could not confirm that certain diketopiperazines can promote quorum sensing in bioreporter strains. They found that the diketopiperazines reported to be active showed no quorum sensing activity. In their screening they identified a few diketopiperazines which prevented biofilm formation of bacteria, but none of these compounds competed with acyl-homoserine lactones for their receptor. During the study a diketopiperazine macroarray, consisting of 400 different compounds, was constructed [89] which can form a basis for systematic structure-activity studies on quorum sensing control by diketopiperazines [90].

2.4. From Papulacandins and Echinocandins to Drugs against Fungal Biofilms

Infections caused by fungal pathogens are difficult to treat and fungal biofilm infections pose an even greater challenge. This dilemma caused a continuous search for novel secondary metabolites able to destroy fungal biofilms. In 1977, the first compounds from a series of double esterified disaccharides were isolated from the fungus *Papularia sphaerosperma.* They were named papulacandins **50–53** because of their high efficiency in inhibiting *Candida albicans* [91]. The mechanism of action interferes with the production of 1,3-β-glucan via inhibition of the enzyme 1,3-β-D-glucan synthase, which is required for fungal cell wall synthesis. Although the anti-biofilm activity of this class of compounds is still largely unexplored, the pivotal role played by 1,3-β-glucan in the extracellular matrix of *Candida albicans* biofilms [92] offers strong indication of the therapeutic potential of such inhibitors.

The discovery of the first papulacandins was followed by the findings of the closely related compounds L-687,781 **54** from *Dactyochaeta simplex* [93,94], Mer-WF3010 **54** from *Phialophora cyclaminis* [95,96], F-10748 A1-D1 **56–59** from a *Lophodermium* species [97], BU-4794F **60** from a *Gilmaniella* species [98], and PF-1042 **61** and saricandin **62** from a *Fusarium* species [99] (Figure 6).
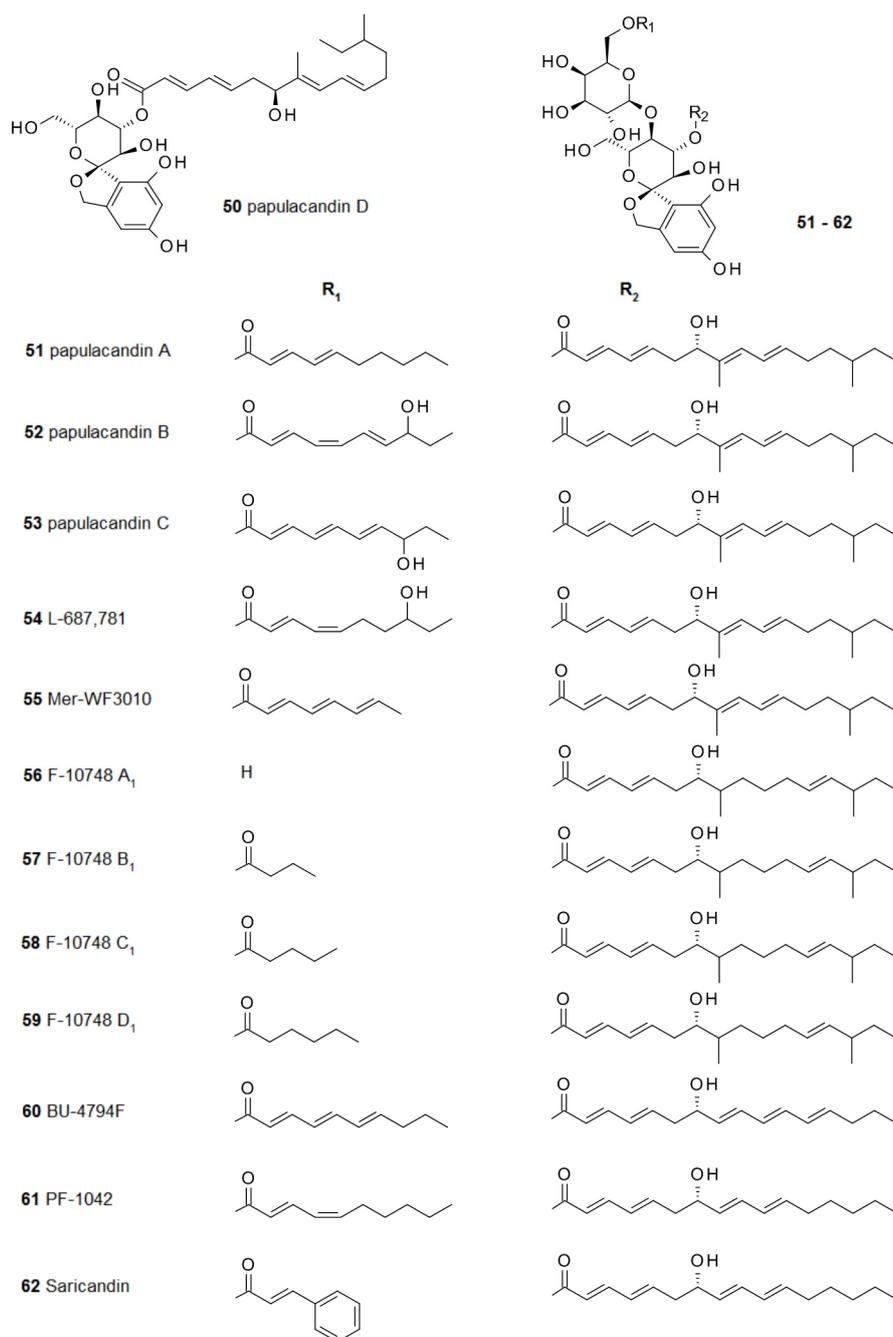
Figure 6. Papulacandins and related antifungal compounds possessing a spiroketal moiety.

The skeleton of these papulacandins bears a spiroketal moiety and its two esters are required for the activity against *Candida* sp. The long fatty acid at the spirocarbohydrate is essential for the bioactivity, and it has been assumed that it is required for the interaction with the fungal cell wall.

In a slightly smaller group of papulacandins the spiroketal moiety has been reduced, leading to a hydroxybenzyl-moiety. To this group of secondary metabolites belong F-10748 A2 **63**, B2 **64**, C2 **65**, and D2 **66**, also from *Papularia sphaerosperma*, chaetiacandin **67** from *Monochaetia dimorphospora* [100,101], and corynecandin **68** from *Coryneum modonium* [102]. Fusacandin A **69** and B **70** have also such a reduced spiroketal, but they are unique due to the attachment of an additional galactose to the galactose unit [103,104] (Figure 7). Chaetiacandin **67** and corynecandin **68** are the only papulacandins with a shortened fatty acid at the glucose unit.

Figure 7. Antifungal papulacandins and the galactosylated fusacandins lacking the spiroketal moiety.

Several papulacandins show high activities against several strains of *Candida* sp., but their pathogen spectrum is narrow and very strain-dependent. In order to improve the performance of papulacandins several synthetic derivatives were synthesized and tested [105]. Systematic variations of papulacandins revealed that the second sugar is not required for activity. This has been demonstrated by papulacandin D **50**, which lacks this sugar. Hydrogenation of the two fatty acids, or even removal of the shorter fatty acids in the galactose unit have only small effects on the inhibition of glucan biosynthesis, although it completely abolishes growth inhibition. The presence of the long fatty acid is essential for activity [106]. It seems that the configuration of the fatty acid is important for penetration into the cells, thus reaching the target site to block 1,3-glucan synthase.

In 1974 the first member of another group of antifungals also active against fungal biofilms was found [107,108]. This group of cyclopetides esterified with a long-chain fatty acid is named echinocandins after its first representative echinocandin B **78**, from *Aspergillus echinulatus* [109,110]. Later, a number of similar compounds were discovered and their biological activities were characterized [111]. These compounds include: echinocandins C **79** and D **80** from *Aspergillus rugulosus* [112]; aculeacin Aα **74**, Aγ **75**, Dα **76**, and Dγ **77** from *Aspergillus aculeatus* [113], in addition to aculeacins B, C, D, E, F and G of which the structures have not been reported [114]; FR190293 **81** and FR227673 **85** from a *Chalara* species and from *Tolypocladium parasiticum* (now identified as *Phialophora* sp.) [115]; FR209602 **82** from *Coleophoma crateriformis* [116]; FR901379 **86** from *Coleophoma empetri*; mulundocandin **89** and desoxy-mulundocandin **90** from *Aspergillus mulundensis* [117]; pneumocandins A0-A4 **91–95**, B0 **96**, B2 **97** and C0 **98** from *Zalerion arboricola, Glarea lozoyensis*, and some *Cryptosporiopsis* sp. [118]; and sporiofungins A-C **99–101** from a *Cryptosporiopsis* species [119] (Figure 8).

Echinocandins are efficient inhibitors of 1,3- and 1,6-D-glucan synthases which are required for fungal cell wall synthesis. They are active against *Candida* spp., especially *C. parapsilosis*, *C. lusitaniae*, and *C. guilliermondii*, *Aspergillus* spp., and *Pneumocystis carinii*, but not against Zygomycetes. However as for most antibiotics, their activity is also strain-dependent, indicating differences in cell permeability. As more and more genome sequences of echinocandin-producing fungi become available, a much clearer picture of the gene organization and evolution of echinocandins emerges. Echinocandins are found in Eurotiomycetes (*Aspergillus* spp.) and in Leotiomycetes (*Glarea*, *Coleophoma*, *Phialophora* species), which diverged about 350 million years ago. Gene organizations point to aculeacin **74** as having the most ancient biosynthesis pathway, whereas FR190293 **81** has the most evolved one. Since no sign of horizontal gene transfer from bacteria could be observed, an origin of echinocandin biosynthesis in the genus *Aspergillus* can be assumed [120,121].

In animal experiments it became evident that one complication of treatments with natural echinocandins was hemolysis. As further studies identified the amide side chain as the main factor for this problem, semi-synthetic derivatives were generated and tested [122]. To produce the cyclic peptide needed for derivatizations, the fatty acid side chain has to be selectively removed. In order to achieve this for FR901379 **86**, 3800 microbial isolates were tested for cyclic lipopeptide acylase activity. As a result, five strains (three *Streptomyces* spp. and two fungi) were identified with high levels of the enzyme [123]. Similar approaches were taken for a number of echinocandins, yielding the primary amine which was further on esterified with a number of different long chain fatty acids. A rigid linear aromatic chain and a flexible aliphatic tail were found to be favorable for these fatty acids, leading to highly active semi-synthetic echinocandins [124].

Starting from the sulfate bearing echinocandin FR901379 **86** several derivatives were synthesized, all varying in the ester side chain [125,126]. Their biological activity was evaluated [127]. From these compounds, caspofungin **71**, micafungin **72** [128], and anidulafungin **73** [129] finally made it to the market [130,131]. The long and challenging way from the discovery of the bioactive natural compound, over optimization of the producing strain, fermentation scale-up, optimization of the enzymatic cleavage of the side chain, formulation of the drug and, finally, to the introduction into the market has been described by Balkove et al. in the case of caspofungin **71** [132]. Comparisons of the characteristics, specificities, and pharmacokinetics of commercial echinocandins were given in a detailed review [133]. The latest addition to the semi-synthetic echinocandins is aminocandin, derived from desoxy-mulundocandin **90**, which is currently being assessed in clinical trials. Results so far demonstrated good efficacy of aminocandin against fluconazole-resistant *C. albicans* [134] and itraconazole-resistant *Aspergillus fumigatus* strains [135].
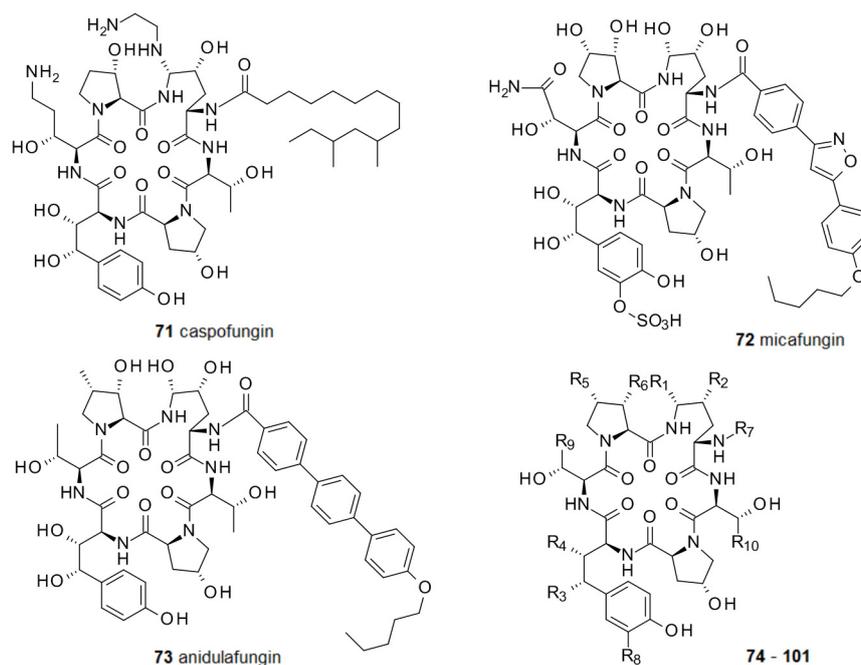
Metabolite	R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8	R_9	R_{10}
74 Aculeacin Aα	OH	OH	OH	OH	Me	OH	C14:0	H	Me	Me
75 Aculeacin Aγ	OH	OH	OH	OH	Me	OH	C16:0	H	Me	Me
76 Aculeacin Dα	OH	OH	H	OH	Me	OH	C14:0	H	Me	Me
77 Aculeacin Dγ	OH	OH	H	OH	Me	OH	C16:0	H	Me	Me
78 Echinocandin B	OH	OH	OH	OH	Me	OH	Linoleoyl	H	Me	Me
79 Echinocandin C	OH	OH	H	OH	Me	OH	Linoleoyl	H	Me	Me
80 Echinocandin D	H	H	H	OH	Me	OH	Linoleoyl	H	Me	Me
81 FR190293	OH	OH	OH	OH	Me	OH	Me-C14:0	H	CH_2CONH_2	Me
82 FR209602	OH	OH	H	OH	Me	OH	C16:0	SO_4H	CH_2CONH_2	H
83 FR209603	OH	OH	H	OH	H	OH	C16:0	SO_4H	CH_2CONH_2	H
84 FR209604	OH	H	H	OH	Me	OH	C16:0	SO_4H	CH_2CONH_2	H
85 FR227673	OH	OH	OH	OH	Me	OH	diMe-16:0	H	CH_2CONH_2	Me
86 FR901379	OH	OH	OH	OH	Me	OH	C16:0	SO_4H	CH_2CONH_2	Me
87 FR901381	OH	OH	H	OH	Me	OH	C16:0	SO_4H	CH_2CONH_2	Me
88 FR901382	OH	H	H	OH	Me	OH	C16:0	SO_4H	CH_2CONH_2	Me
89 Mulundocandin	OH	OH	OH	OH	Me	OH	Me-14:0	H	H	Me
90 Deoxy-mulundocandin	OH	OH	H	OH	Me	OH	Me-14:0	H	H	Me
91 PneumocandinA0	OH	OH	OH	OH	Me	OH	diMe-14:0	H	CH_2CONH_2	Me
92 PneumocandinA1	OH	OH	OH	H	Me	OH	diMe-14:0	H	CH_2CONH_2	Me
93 PneumocandinA2	H	H	OH	OH	Me	OH	diMe-14:0	H	CH_2CONH_2	Me
94 PneumocandinA3	OH	H	H	H	Me	OH	diMe-14:0	H	CH_2CONH_2	Me
95 PneumocandinA4	H	H	H	H	Me	OH	diMe-14:0	H	CH_2CONH_2	Me
96 PneumocandinB0	OH	OH	OH	OH	H	OH	diMe-14:0	H	CH_2CONH_2	Me
97 PneumocandinB2	H	H	OH	OH	H	OH	diMe-14:0	H	CH_2CONH_2	Me
98 PneumocandinC0	OH	OH	OH	OH	OH	H	diMe-14:0	H	CH_2CONH_2	Me
99 Sporiofungin A	OH	OH	H	OH	Me	OH	diMe-14:0	H	CH_2CONH_2	Me
100 Sporiofungin B	OH	OH	H	OH	H	OH	diMe-14:0	H	CH_2CONH_2	Me
101 Sporiofungin C	OMe	OH	H	OH	Me	OH	diMe-14:0	H	CH_2CONH_2	Me

Figure 8. Secondary metabolites of the echinocandin-type active against several fungal pathogens organized in biofilms. C14:0 = tetradecanoic acid, C16:0 = hexadecanoic acid, Me-14:0 = 12-methyl-tetradecanoic acid, diMe-14:0 = 10,12-dimethyl-tetradecanoic acid, diMe-16:0 = 12,14-dimethyl-hexadecanoic acid.

One of the most important characteristics of these semisynthetic echinocadins is their activity against biofilms. Micafungin showed antifungal activity against *Candida albicans* and *C. parapsilosis* biofilms, but has a better activity against biofilms with high metabolic activity [136,137]. Micafungin showed a considerably higher acitivity against *C. tropicalis* biofilms when compared to amphotericin [138]. Caspofungin could eradicate mature *C. lusitaniae* or *C. guilliermondii* biofilms at concentrations feasible for catheters [139]. Nevertheless, some biofilms of *C. albicans* are difficult to clear by echinocandins as an animal model of foreign-body infection demonstrated. Here, anidulafungin or caspofungin reached only a cure rate of 25%, with caspofungin being the most effective [140]. In an in vitro model, andulafungin was effective against nine *Candida* strains, four of them were *C. albicans*. However, when combined with the nonsteroidal anti-inflammatory drugs aspirin, diclofenac or ibuprofen, a clear synergism in biofilm clearance was observed [141]. The effect of caspofungin or micafungin alone or in combination with farnesol **7** against *C. parapsilosis* biofilms has been tested. Both echinocandins could clear the biofilm, but were up to 64-fold more effective when combined with farnesol. Interestingly, micafungin could not clear biofilms older than 24 h [142].

3. Conclusions

The present review brings an extensive, although not exhaustive, list of small metabolites from fungi which have anti-biofilm activity. Figure 9 summarizes examples of biofilm formation mechanisms which are targeted by fungal bioactive compounds. The pathway from finding interesting activities in fungal extracts to actual drug discovery, however, is a long and winding one. Among the hundred compounds discussed here, only three echinocandins (caspofungin, micafungin. and anidulafungin) were fully developed and are currently approved as commercial drugs. They are indicated mainly for the treatment of invasive or disseminated fungal infections, and despite being notorious for their ability to disrupt fungal biofilms, their clinical application against biofilm-related diseases and colonization of medical devices is still being continuously developed [107,133].
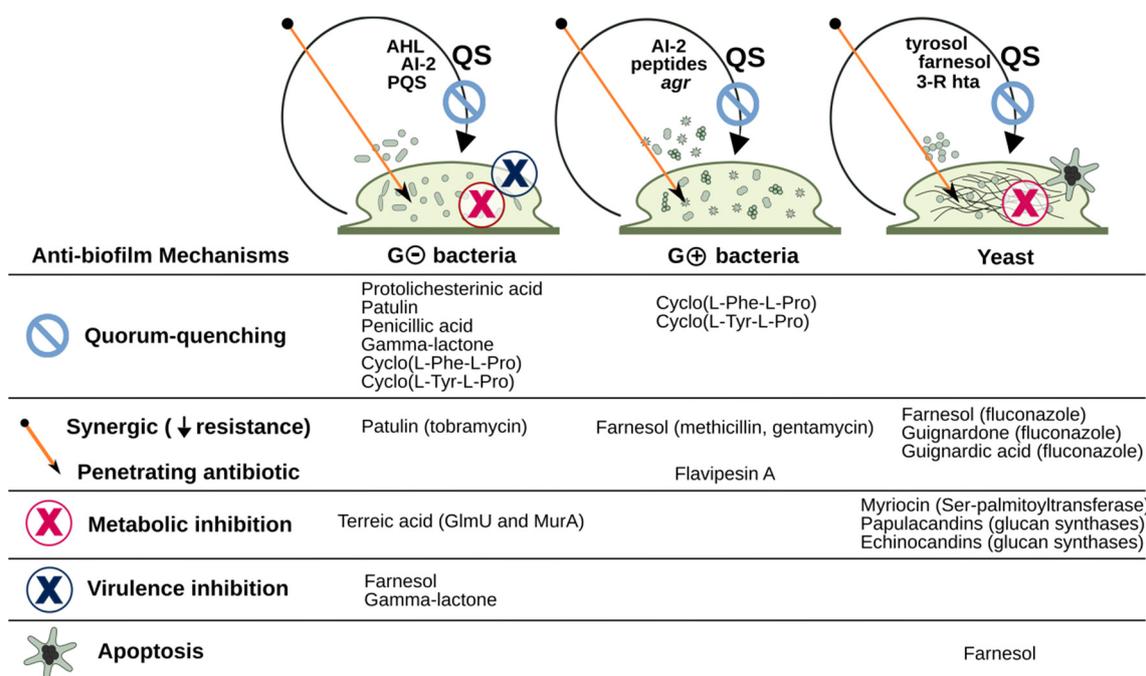
Figure 9. Anti-biofilm mechanisms of fungal molecules against Gram positive bacteria, Gram negative bacteria and yeasts, with examples of active compounds. QS: quorum sensing; AHL: acyl homoserine lactone; AI-2: auto-inducer-2; agr: accessory gene regulator system; PQS: Pseudomonas quinolone signal; 3R hta: 3R-hydroxy-tetradecanoic acid.

Two important issues are confronted when applying echinocandins in the clinics. One, which is inherent to virtually all antibiotics, is the increasing number of clinical strains displaying resistance [143]. Another concern, which is rather distinctive of echinocandins, is known as the paradoxical effect; it is characterized by an ability of the target organism to reverse growth inhibition at high concentrations, above the MIC drug concentrations [144]. Both phenomena jeopardize the efficacy of such drugs in the clinics, and deserve researchers' attention. Importantly, echinocandin resistance, as well as the paradoxical effect, is shown to be divergent between planktonic cells and biofilms of given *Candida* isolates [145]. The mechanism of action of echinocandins has the cell wall synthesis as a major target, and mutations in the beta-glucan synthase enzyme (*fks1* gene) are implicated in resistance-development [146]. The paradoxical effect, in turn, is also correlated to cell wall stress and general stress responses, including Hsp90. Additionally, the influence of paradoxical effect-related stress responses in the induction of resistance development has also been demonstrated [147]. Altogether, it highlights the need for a continuous effort from the research community, to maintain or enhance the effectiveness of currently available anti-fungal agents, as well as to open up new avenues from natural compounds with divergent modes of action.

Apart from glucan-synthesis inhibition by antifungal compounds, a number of the metabolites reviewed here act through different mechanisms, some even combining more than one target. This is the case for terreic acid, which has been shown to inhibit two different bacterial biosynthetic enzymes [69,70]. The multi-target trait is a highly desired one when trying to avoid resistance development. Also, some diketopiperazines are reported to be multi-functional in that they are able to inhibit Gram-negative biofilm formation by competing with acyl-homoserine lactone quorum-sensing signaling, as well as to interfere with Gram-positive *agr* quorum-sensing system [83]. Focusing on quorum-quenching compounds will likely expand the potential use of fungal metabolites in the control of bacterial biofilms. Indeed, several compounds of different classes can be highlighted here for displaying both anti-fungal and anti-bacterial activities (e.g., farnesol, tyrosol, usnic acid, cytosporone E and some echinocandins), and these should be considered of particular interest for drug development.

Noticeably, most studies on anti-biofilm compounds of fungal origin to date have demonstrated their ability to kill and/or disrupt fungal biofilms, and in particular the model organism and medically important pathogen genus *Candida*. Accordingly, biofilm-related fungal infections are the targets for a number of fungal metabolites tested in preclinical setups, i.e., using in vivo models, medical surfaces or isolates from patients. Also bacterial biofilm infections and mixed-species biofilms (as is often the reality in the clinics) are being addressed in some such studies. Table 1 gives a summary of preclinical results on selected fungal compounds, with focus on substantial advancements in their prospective use as anti-biofilm drugs, indicating they are in continuous progress towards clinical application. Further reading on in vivo models for anti-biofilm drug discovery, and translational research of echinocandins can be found in recent reviews [107,148,149].

Table 1. Examples of preclinical studies focusing on anti-biofilm properties of fungal compounds.

Experimental Setup for Anti-Biofilm Tests	Target Organisms	Fungal Compounds and Combinations Tested	References
in vitro biofilm formation by clinical isolates	*Aspergillus* spp.	anid, casp, casp + DNase	[150,151]
	Candida albicans	anid, casp, mica, farnesol, cas + voriconazole, cas + amb, shearinine, shearinine + amb	[137,150,152–163]
	Candida spp. (non-albicans)	anid, casp, mica, farnesol, casp + farnesol, mica + farnesol, tyrosol, tyrosol + amb, shearinine, shearinine + amb	[76,138,142,150,152,153, 156,157,159,161,163–166]
	Trichosporon asahii	casp, casp + voriconazole	[167]
	Pseudomonas aeruginosa	mica	[168]
	Group A *Streptococcus*	usnic acid	[61]
in vivo model of catheter biofilm in rabbit	*C. albicans*	casp, mica	[169,170]
in vivo model of subcutaneous device infection in rat	*C. albicans*	anid, casp, mica, casp + diclofenac	[171–173]
	C. glabrata	anid, casp, mica	[174]
mixed species oral biofilm, in vitro QS (quorum-sensing) interference in biofilm from dental plaque isolates	*C. albicans, C. glabrata, S. mutans*	tt-farnesol, tyrosol	[79,175]
	Gram-positive bacteria	usnic acid	[176]
in vivo model of biofilm infection by clinical isolates in *Galleria mellonella* larvae	*C. albicans*	anid	[177]
in vitro biofilms in catheters and biomaterials, simulated endocardial vegetation (SEV)	*Candida* spp.	casp, mica	[178–181]
	Staphylococcus aureus, P. aeruginosa	usnic acid	[63]
in vivo murine model of central venous catheter or subcutaneous catheter infection	*C. albicans*	casp	[182]
	S. epidermidis	tt-farnesol	[183]
in vivo model of foreign-body infection in guinea pig	*C. albicans*	casp, anid	[140]
in vivo murine model of cystic fibrosis	*Burkholderia cepacia, P. aeruginosa*	patulin	[184]
synthetic urine medium	*C. albicans*	casp	[185]

Notes: anid: anidulafungin; casp: caspofungin; mica: micafungin; amb: amphotericin B; tt-farnesol: *trans,trans*-farnesol.

4. Outlook

Additional alternative strategies to benefit from anti-biofilm compounds of fungal origin in the treatment of infectious diseases are under study, which may in the future offer promising results in the clinics. One such strategy is the combination of fungal metabolites with other classes of anti-fungal drugs, to enhance their performance, especially towards biofilms (some examples are presented in Table 1, and a recent review is available [186]). Another approach consists in immobilizing the drugs on medical surfaces. This has been described for caspofungin on propanal biosurfaces [187] and on titanium substrata which were therefore rendered less susceptible to *C. albicans* biofilm colonization, at the same time supporting osseointegration (integration of the implant into the bone) [188]. Osteocompatibility is an important trait to be taken into account, the lack of which can limit the clinical use of cytotoxic compounds (e.g., farnesol, [189]). Also, usnic acid has been successfully incorporated onto biodegradable polymer thin films [190], or cellulose-silica membranes [191] for the production of biocompatible, *S. aureus*-resistant surfaces. A third possibility still to be explored is the production of nanostructures for a more efficient delivery of anti-biofilm drugs. This has recently been tested with cefotaxime-impregnated chitosan particles [192] and farnesol-loaded cationic nanoparticles [193]. Polymer microparticles and magnetic nanoparticles also yielded positive results as carriers for usnic acid in vitro antimicrobial and anti-biofilm assays [194,195].

An astounding biodiversity found within the kingdom Fungi, which is estimated to include not less than five million species [196], associated to an unparalleled metabolic diversity inherent to a broad range of ecological strategies [197], draws attention to the biotechnological potential contained among fungal secondary metabolites. The number of different compounds summarized in this review highlights the usefulness of such sources in the much-needed search for novel antibiotic drugs which are effective against biofilm infections [198]. This urging issue is not a novelty in the context of the so-called "post-antibiotic era", neither is the fact surprising that natural compounds' research still consists of a promising approach. Not only the constant exposure of fungal tissues to other microorganisms in favorable environmental conditions, posing a natural demand for the production of anti-biofilm metabolites to avoid potentially harmful inter-species interactions, but also the biochemical machinery employed in intra-specific cell communication and self-regulation seem to offer us valuable leads for anti-biofilm drug development. We believe that a thorough exploration of natural compounds from fungi will broaden the possibilities for their use in the treatment of biofilm infections, offering novel ways to close the gaps left by current therapies.

Author Contributions: Both authors were involved in searching and evaluating the literature and writing the review. W.R.A. did the drawings of chemical formulae.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

The following abbreviations are used in this manuscript:

MIC	Minimal inhibitory concentration
IC$_{50}$	Half maximal inhibitory concentration

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