# Emerging novel antifungal agents

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Promising new compounds have recently been identified in an effort to supplement the relatively sparse portfolio of antifungal drugs. Many of these compounds have defined mechanisms of action against fungal cells and have, in some cases, aided the identification of new selective targets in fungi. For most of these compounds, however, factors such as a narrow spectrum of activity, susceptibility to efflux pumps, protein binding, serum inactivation and poor pharmaceutical properties prevent their use in the clinic. Even so, these compounds are novel substrates for synthetic modifications that could lead to the discovery of future antifungal drugs.

Discovery of new drugs for systemic opportunistic fungal infections is a major challenge in infectious disease research. The 'standards' of therapy have been the fungicidal (but toxic) polyene, amphotericin B, and the safer (but fungistatic) azoles, fluconazole and itraconazole. While there are several new azoles[1] in clinical and pre-clinical development, success in synthetically modifying the echinocandin class of fungal cell wall biosynthesis inhibitors has now enabled these latter compounds[2,3] to be the next class of antifungal drugs.

Despite these advances, the continuing increase in the incidence of fungal infections together with the gradual rise in azole resistance highlights the need to find novel compounds with divergent mechanisms of action. Hence, considerable research is being directed to screening for lead compounds with defined mechanisms of action that can serve as templates for further medicinal chemistry modifications. After finding these chemical templates, research will focus on obtaining analogs with a broad spectrum of activity against *Candida albicans*, azole-resistant *C. albicans*, non-albicans *Candida* (e.g. *C. glabrata, C. krusei* and *C. parapsilosis*), *Aspergillus fumigatus* and *Cryptococcus neoformans*. *C. albicans* and *A. fumigatus* cause most opportunistic mycoses and, hence, finding compounds with activity against these two organisms is considered to be crucial. A high efficacy of these compounds in murine mycoses models is generally thought to demonstrate potential clinical utility. This review covers a selection of new chemical templates that fulfil many of these objectives and that, with continued research, might emerge as novel classes of antifungal drugs.

## New inhibitors of cell wall biosynthesis

The fungal cell wall is a unique organelle, required for the growth and the maintenance of osmotic stability of the cell. As demonstrated for the echinocandins and the glycolipid papulacandin, inhibition of fungal cell wall glucan biosynthesis leads to cessation of growth and cell lysis. For these reasons, much effort has focused on the discovery of useful inhibitors of the cell wall glucan, chitin, and of mannoprotein biosynthesis[1].

In an attempt to find compounds with the desired potency, spectrum of activity and appropriate pharmaceutical properties, the peptidylnucleosides (such as nikkomycin) and the naphthacene quinones (such as pradimycin), which interfere with chitin synthesis and mannoprotein function, respectively, have been subjects of intensive research. The following is a description of some of the newer natural products and their derivatives that have promising *in vitro* cell wall biosynthesis inhibition and antifungal activities.

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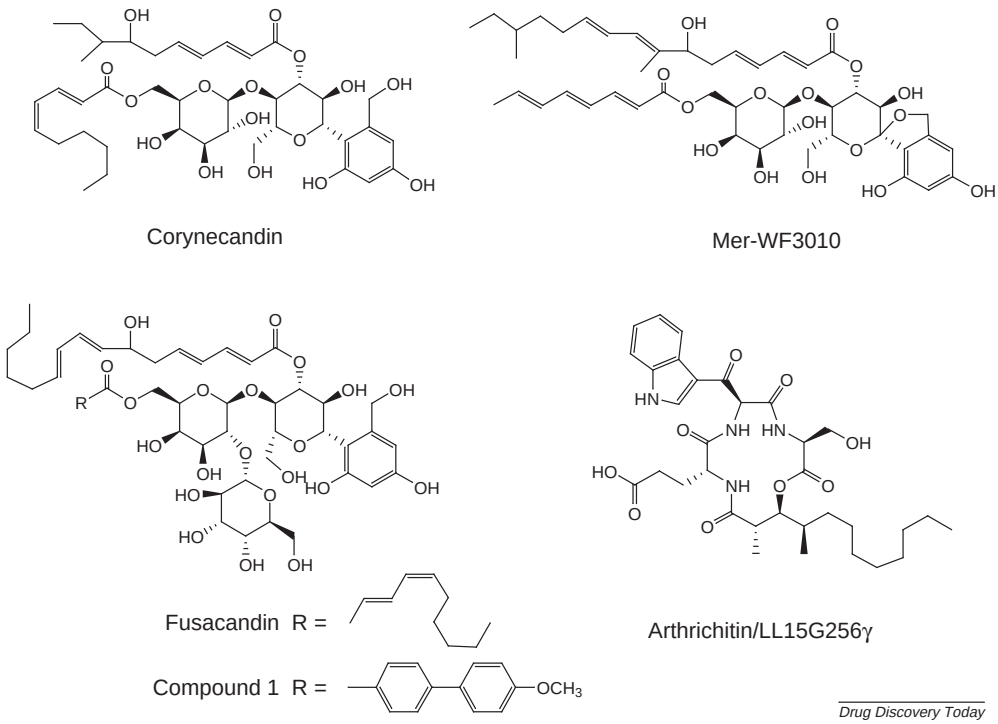


***Figure 1.*** *New inhibitors of cell wall biosynthesis.*

tered intraperitoneally at 1000 mg $kg^{-1}$.

### *Fusacandins*

Fusacandin, isolated from *Fusarium sambucinum*, is another structural variant of chaetiacandin (Fig. 1)[6]. This compound is unusual among this class of drugs in that it is a trisaccharide rather than a disaccharide. As for other papulacandin congeners, fusacandin demonstrated poor activity in animal models. In an attempt to find analogs with improved activity *in vivo*, the structure–activity relationships (SAR) of the fusacandins[7,8] have been extensively explored. These studies showed that the lack of *in vivo* potency of these compounds is caused by their inactivation through serum protein binding. The C-6′ fatty acyl substituent on fusacandin appeared to contribute to protein binding. Subsequent replacements of the fatty acid (see compound 1, Fig. 1) reduced protein binding and improved *in vivo* activity[8]. These results suggest that glycolipid analogs with clinically useful activities might be accessible through further synthetic alterations.

### *Corynecandin*

Corynecandin (Fig. 1)[4] is a novel glycolipid analogous to the structural variant of papulacandin, named chaetiacandin. Corynecandin was isolated from cultures of *Coryneum modonium* using a cell wall biosynthesis assay to monitor biological activity. Addition of the osmoprotectant sorbitol to the culture medium rescued cell growth, confirming that, analogous to other glycolipids, corynecandin inhibits cell wall biosynthesis. Corynecandin has an $IC_{50}$ of 12.9 μg $ml^{-1}$ against *C. albicans* glucan synthase, which is comparable to that of papulacandin B (1.9 μg $ml^{-1}$) and cilofungin (52.0 μg $ml^{-1}$)[4].

### *Mer-WF3010*

Another new member of the papulacandin class is Mer-WF3010, which was isolated from the culture broth of *Phialophora cyclaminis* (Fig. 1)[5]. Mer-WF3010 is similar to papulacandin B in that it inhibits the growth of *C. albicans* and *C. kefyr*, but not of *A. fumigatus* or *C. neoformans*[5]. Minimum inhibitory concentration (MIC) values against several *C. albicans* strains ranged from 0.31 to 1.25 μg $ml^{-1}$. As with other glycolipids, Mer-WF3010 is fungicidal to growing cells but inactive against resting cells. Furthermore, the compound is not toxic to mice when adminis-

### *Arthrichitin/LL15G256γ*

Arthrichitin[9] is a cyclic depsipeptide that was identified through screening for chitin synthesis inhibitors. It can be isolated from the culture broth of *Arthrinium phaeospermum* or as LL15G256γ from the marine fungus *Hypoxylon oceanicum* (Fig. 1)[10,11]. Arthrichitin has a broad-spectrum of activity against *Candida* spp., *Trychophyton* spp. and several phytopathogens. *In vitro* testing has shown that arthrichitin inhibits membrane preparations of fungal chitin and glucan synthases, with a greater potency against chitin synthase. Fungal cells exposed to arthrichitin undergo morphological changes[9] similar to the effects of chitin synthase inhibitor polyoxin B. The morphological effects of arthrichitin occur at concentrations that are tenfold below those required to inhibit chitin synthase. This suggests the existence of another target for arthrichitin, possibly an isozyme of chitin

synthase not present in the membrane preparation[12]. It is also possible that the compound alters regulatory processes in the cell cycle that affect the cell wall.

The *in vitro* potency of arthrichitin is too low for its use in the clinic. However, it has been suggested that the development of analogs, such as those based on the much larger cyclic peptides, the echinocandins, might yield congeners with improved activity[12].

## Selective inhibitors of fungal protein and amino acid synthesis

The activity of several clinically useful antibacterial agents (such as the macrolides, streptogramins, lincosamides, aminoglycosides, chloramphenicol and the tetracyclines) is caused by their selective inhibition of bacterial protein synthesis. By analogy, compounds that inhibit fungal protein synthesis are likely to be useful antifungal agents. The challenge is to identify aspects of the eukaryotic protein synthesis machinery that are unique to fungi.

Hence, previous studies[13] have identified elongation factor 3 (EF3), a unique fungal ribosomal factor essential for protein synthesis, as an obvious target for antifungal drug discovery. Recent work with the sordarins, a class of specific fungal protein synthesis inhibitors, has revealed that there are enough differences between other fungal ribosomal and ribosome-associated proteins and their mammalian cognates, such as EF2, to enable the development of selective antifungal protein synthesis inhibitors.

### *Sordarins*

Sordarin[14], an antifungal agent bearing the tetracyclic diterpene aglycone sordaricin, was isolated from *Sordaria araneosa* in 1971, but its mode of action was only elucidated[15,16] after the discovery of a closely related analog[17] GR135402 from *Graphium putredinis*. A further analog, BE31405, was isolated from *Penicillium minioluteum* (see Fig. 2)[18]. These three natural products differ only in the sugar portion of the molecule. Unlike that of sordarin, the sugar in GR135402 is acylated on the 3′-hydroxy group. Although the sugar in BE31405 has the same basic substitution pattern and configuration as sordarin, it is a keto sugar and is further oxidized at the C-5′ methyl group and elaborated into a tricyclic acetal.

Sordarins inhibit protein synthesis by stabilizing the complex formed between EF2 and the ribosome[19]. Furthermore, they are inactive against mammalian cells or subcellular fractions, indicating a specificity for fungal protein biosynthesis. Genetic studies in *Saccharomyces cerevisiae* have identified sites on EF2 that, when mutated, stimulate resistance to this class of compounds[15,20]. These studies have also enabled the putative binding site on EF2 to be defined, while the proposed amino acid side chain contact points have been identified by molecular docking experiments[20]. Further work has identified mutations in the eukaryotic ribosomal protein rpP0 that confer similar resistance to sordarins[21,22]. This protein corresponds to the prokaryotic ribosomal protein L10, which plays a crucial role in the ribosomal stalk architecture and in elongation. As the binding of the sordarins to EF2 is greatly enhanced by the addition of ribosomes, it is speculated that rpP0 is involved in the high-affinity binding and selectivity of these compounds.

Although sordarin is potent against *C. albicans*, *C. tropicalis* and *C. kefyr*, with an MIC for most isolates of $<0.01$ μg ml$^{-1}$, it is 100-fold less potent against *C. glabrata*, *C. parapsilosis* and *C. krusei*[19,23]. Ribosomes from *C. parapsilosis* do not bind to sordarin. However, addition of soluble factors from *C. albicans* enables sordarin to bind to

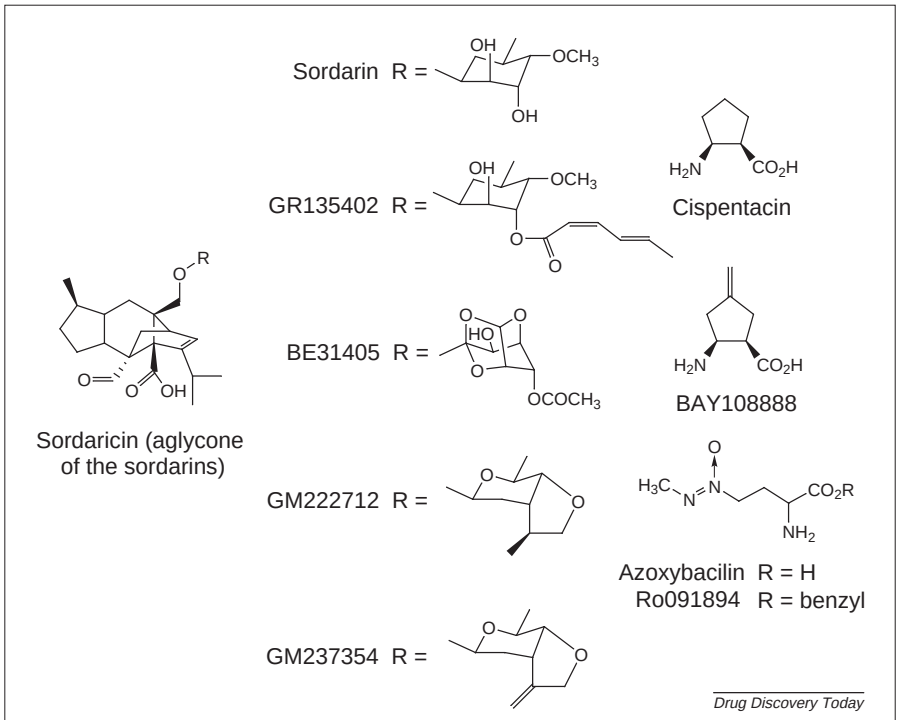


***Figure 2.*** *Selective inhibitors of fungal protein and amino acid biosynthesis.*

*C. parapsilosis* ribosomes, suggesting differences between *C. parapsilosis* and *C. albicans* EF2 proteins. Fungi from other genera also show selective sensitivity. For example, *A. flavus* is sensitive while *A. fumigatus* is not.

The SAR for binding to EF2 from different species lies in the nature of the sugar residue of the glycoside. BE31405, whose sugar is a rigid tricyclic ring system, has a broader spectrum of activity compared to sordarin. Synthetic modifications of the sugar in sordarin to provide more rigid ring systems has produced GM222712 and GM237354, which have a broader spectrum of activity compared with the parent compound (Fig. 2)[23].

Although the sordarins still lack activity against *A. fumigatus*, studies on the SAR of these compounds have only just begun. The comprehensive studies examining the mechanism of action, as well as the structural biology work to define their interaction with EF2 could lead to potent, broad spectrum, highly selective and clinically useful compounds.

### Cispentacin

Cispentacin[24] from *Bacillus cereus* and FR109615 (Ref. 25) from *Streptomyces setonii* are unnatural cyclic β-amino acids. These compounds and their analogs[26], such as BAY108888, appear to have a dual mode of action (Fig. 2). As cispentacin is rapidly accumulated in fungal cells by active transport[27], its mechanism of action was speculated to be by interfering with amino acid transport and cellular regulation of amino acid metabolism. This was confirmed by preincubation of cells with isoleucine or valine (these are transported by a specific branched chain amino acid carrier), which inhibited the uptake of this class of compounds into cells[26].

Cispentacin and its analogs are also low-affinity inhibitors of isoleucine-tRNA synthase and interfere with protein biosynthesis, BAY108888 having a $K_i$ of 1 mM against *C. albicans*. This mechanism is involved in the antifungal activity of these compounds, as strains of *S. cerevisiae* overexpressing isoleucine-tRNA synthase are resistant to BAY108888. Furthermore, the observed level of protein synthesis inhibition in whole cells is consistent with the $K_i$ against isoleucine-tRNA synthase and the extent (200-fold) of intracellular accumulation.

The SAR for these compounds indicates certain strict structural requirements. The carboxyl and amino groups must be in a 1(*R*),2(*S*) relationship on a cyclopentane ring[28]. Although more planar analogs (such as BAY108888, which bear an exocyclic methylene group) are more potent, introduction of a ring double bond leads to a loss of activity[28]. The only substituted analogs with activity comparable to that of cispentacin are aminoacyl derivatives of the 2-amino group, although it is unclear whether these have an intrinsic activity or are prodrugs of cispentacin.

The main barrier to the clinical use of these compounds is antagonism observed in the presence of several amino acids. Thus, cispentacin shows promising *in vivo* efficacy, but has no *in vitro* activity in rich media[29]. Cispentacin is also ineffective in aspergillosis, somewhat limiting its potential use. However, synthetic modifications of these compounds might yield a clinically useful derivative.

### Azoxybacilin

Azoxybacilin[30,31], an aliphatic amino acid with an azoxy side-chain, was isolated from *B. cereus* and has a broad spectrum of antifungal activity. MICs for azoxybacilin against *Candida* spp. (including azole-resistant *C. albicans*) and *Aspergillus* spp. are $<10$ μg ml$^{-1}$, while the *Cryptococcus* spp. are resistant. Although azoxybacilin does not directly alter protein biosynthesis, it affects enzymes in the sulfate assimilation (SA) pathway that are unique to fungi[31]. The SA pathway is essential for the *de novo* synthesis of sulfur-containing amino acids in fungi and involves the conversion of $SO_4^{2+}$ to $H_2S$ for use in the synthesis of cysteine and methionine. Azoxybacilin interferes with the induction of gene expression by low levels of methionine in the SA pathway. These genes include *MET3*, *MET10*, *MET14*, *MET16* and *MET25*, as well as the transcriptional regulator *MET4* (Ref. 32). Azoxybacilin is the first reported antifungal inhibitor of transcriptional control, and interferes with the induction of *MET4* in a dose-dependent manner, relying on the presence of promoters within 325 base pairs of the transcription start site.

The SA pathway is dispensable for fungal cells in the presence of exogenous sulfur-containing amino acids. However, the levels of these amino acids in the blood can be as low as 5 μg ml$^{-1}$, which is insufficient to support fungal growth when the *de novo* synthesis of cysteine or methionine is inhibited[32]. Furthermore, *C. albicans* strains that are auxotrophic for methionine are not pathogenic, suggesting that the SA pathway is a *bona fide* antifungal target. Despite this, azoxybacilin only has weak activity *in vivo*, perhaps because of a poor pharmacokinetic profile and antagonism by even the low *in vivo* concentrations of methionine. Synthetic manipulation focusing on identifying compounds that are not antagonized by methionine has led to the production of Ro091824, which is reported to be antagonized to a lesser extent[33]. Further work to improve the pharmacokinetic profile might impart the properties required to make these compounds clinically useful.

### Inhibitors of sphingolipid biosynthesis

The first committed step in sphingolipid biosynthesis is the condensation of serine and fatty acyl-Coenzyme A (acyl-CoA), usually palmitoyl-CoA, to form the long-chain base, ketodihydrosphingosine. This reaction is carried out by serine palmitoyltransferase. Ketodihydrosphingosine is subsequently converted to phytosphingosine in fungi and sphingosine in mammals. Condensation with fatty acyl-CoA produces ceramide. In fungi, phosphatidylinositol is transferred to ceramide by the unique fungal enzyme, inositol phosphoceramide (IPC) synthase, to yield inositol phosphoceramide. IPC synthase has now been shown to be an attractive fungicidal target[34] and is the target for the following compounds.

#### *Aureobasidin*

Aureobasidin A (AbA, LY295337)[35–37] is produced by *Aureobasidium pullulans* and belongs to a family of potent cyclic depsipeptide antifungals (Fig. 3). The L-β-hydroxy-*N*-methylvaline residue at position 9 of the compound was derivatized via a tandem retro-aldol–aldol reaction procedure to explore the stereoelectronic effects of that crucial residue on its activity[38]. These studies indicated that the preferred ring conformer was one that avoided intramolecular hydrogen-bonding by the tertiary hydroxy-group, suggesting a role for this group in binding to the target.

Recent studies have replaced the L-allo-isoleucine, *N*-methyl-L-valine and L-leucine at positions 6, 7 and 8, respectively, of aureobasidin A with amino acids bearing carboxyl or hydroxy side-chains, such as L-glutamic acid and δ-hydroxy-L-norvaline[39]. These new side-chains were acylated or esterified with long-chain fatty acids or alcohols with a view to increasing lipophilicity and potentially potency. However, these modifications led to decreased activity against *Candida* spp. and *C. neoformans*.

AbA is a substrate for ABC transporters, such as the MDR subgroup, in both yeasts and human tumor cells[40] and competitively inhibits the efflux of other substrates. The fact that AbA is an MDR substrate might account for its lower potency against filamentous fungi. The hydroxy-group at residue 9 was found to be important for both antifungal and MDR activity. However, the stereoelectronic requirements of that residue are different for the two activities, as the unnatural D-isomer at residue 9 reduced antifungal activity while increasing MDR pump inhibitory activity. By contrast, replacement of the phenylalanine at position 3 with tyrosine derivatives improved activity against *C. neoformans* while reducing MDR activity. The SAR for the two activities, therefore, appear to be different[41], suggesting that further synthetic modifications could produce antifungal analogs that are not substrates for the MDR pumps.

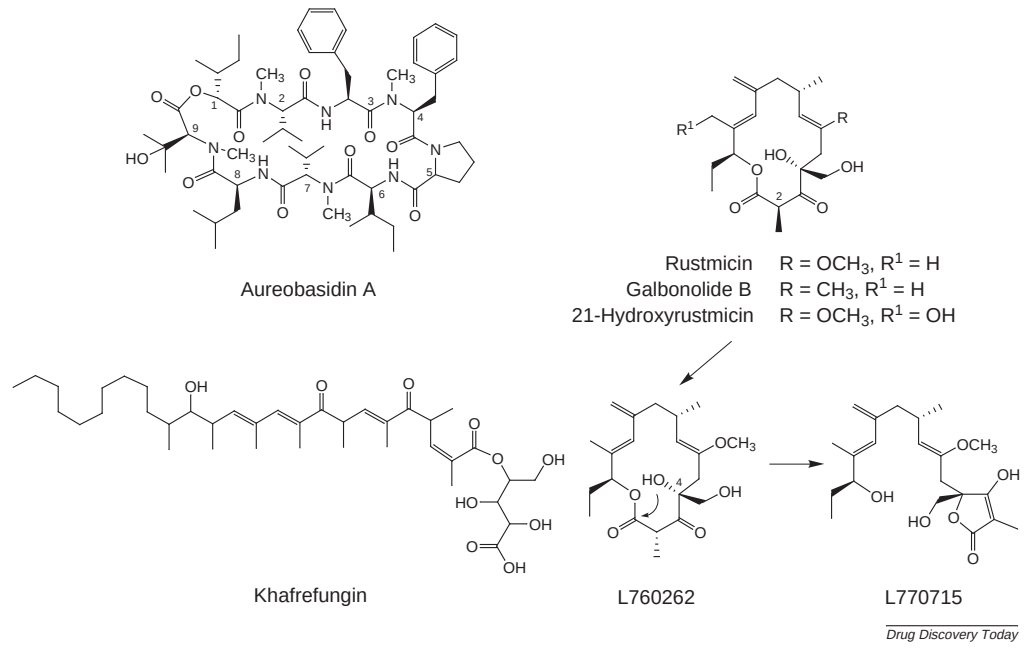


***Figure 3.*** *Inhibitors of sphingolipid biosynthesis.*

#### *Rustmicin*

Rustmicin (galbonolide A) and galbonolide B, as well as analogous[42–44] antifungal macrolides isolated from *Micromonospora* sp., are inhibitors of sphingolipid biosynthesis[45]. The order of potency with respect to inhibition of sphingolipid biosynthesis is rustmicin, followed by galbonolide B and 21-hydroxy-rustmicin, with 21-hydroxy-galbonolide B showing the least inhibition (Fig. 3). However, this trend in potency does not hold for antifungal activity, as the MIC is affected by chemical stability and susceptibility to multidrug efflux pumps[46].

Rustmicin is a reversible inhibitor of IPC synthase with $IC_{50}$ values of 70 pM and 3.8 nM against the *C. neoformans* and *C. albicans* enzymes, respectively[46]. Rustmicin is correspondingly more potent and fungicidal against *C. neoformans*. The compound is also efficacious in a mouse cryptococcosis model, although the efficacy was lower than that expected for the observed *in vitro* potency.

Rustmicin is unstable and undergoes epimerization at C-2 to produce L760262. This epimer then undergoes translactonization via the 4-hydroxy group to the inactive butenolide L770715 (Fig. 3). This degradation process is accelerated by the presence of serum and is likely to contribute to its lower efficacy *in vivo*. Rustmicin is also a substrate for fungal multidrug transporters, as *S. cerevisiae* mutants lacking Pdr5p are over 200-fold more sensitive to rustmicin than normal strains[46].

### *Khafrefungin*

Khafrefungin was isolated from an unidentified sterile fungus from a Costa Rican plant sample (Fig. 3)[47], and consists of aldonic acid derivatized at the 4-hydroxy group with a C-22 linear polyketide acid. Khafrefungin is active against *C. albicans* and *C. neoformans*, and cells treated with the compound accumulate hydroxyceramide. *In vitro* studies also show inhibition of inositol incorporation into sphingolipid but not into phosphatidylinositol, therefore confirming the target as IPC synthase.

Khafrefungin inhibits membrane preparations of IPC synthase with $IC_{50}$ values of 0.6 nM, 7 nM and 31 nM against *C. albicans*, *S. cerevisiae* and *C. neoformans* IPC synthases, respectively. The compound has no effect on incorporation of radiolabeled serine into mammalian lipids, indicating that khafrefungin is fungal-specific. Furthermore, khafrefungin does not inhibit the growth of *A. fumigatus*. However, at high concentrations, synthesis of alkali-stable inositol lipids is inhibited, suggesting that the target is present but is either less sensitive or less accessible to the drug. The specificity of khafrefungin against IPC synthase is speculated to be caused by the resemblance of the polar aldonic acid head group to phosphoinositol.

All these sphingolipid biosynthesis inhibitors have helped to define the value of IPC synthase as a selective antifungal target. They are also attractive starting points for further chemical optimization.

## Inhibitors of electron transport

A series of related antibiotics with a nine-membered dilactone ring, including UK2A and UK3A, has been isolated from *Streptomyces* sp. 517-02 (Fig. 4)[48–50]. This class of compounds has broad-spectrum antifungal activity including against *C. albicans* and *Aspergillus* spp. UK2A and UK3A also do not have antibacterial activity or significant cytotoxicity ($IC_{50}$ 18–100 μg $ml^{-1}$). This contrasts with the structurally related antibiotic, antimycin A, which has similar antifungal potency but 10- to 1000-fold increased cytotoxicity. All three compounds inhibit incorporation of nucleic acid precursors, amino acids, glucose and mannose into acid-insoluble material in a dose-dependent manner, making it difficult to identify the specific mode of antifungal action[51].

Antimycin A, UK2A and UK3A, however, are known to inhibit mitochondrial electron transport and interfere with *in vitro* respiration in both yeast and rat liver mitochondria. UK2A has $IC_{50}$ values comparable to antimycin A against yeast, while against rat liver mitochondria, UK2A is fivefold less active than antimycin A. Meanwhile, UK3A is approximately 50-fold less potent than antimycin A in both tests. Despite the relatively small difference in potency between UK2A and antimycin A against rat liver mitochondria, UK2A is significantly less cytotoxic against mammalian cell lines. It has now been determined that, in contrast to UK2A, antimycin A rapidly stimulates intracellular production of reactive oxygen species[52].

This suggests that while the primary mechanism of antifungal activity of the dilactones is inhibition of yeast mitochondrial respiration, the additional reactivity of antimycin A might account for its increased mammalian cytotoxicity. At any rate, characterization of the mechanism of action of the dilactones has led to the identification of respiration as an antifungal target.

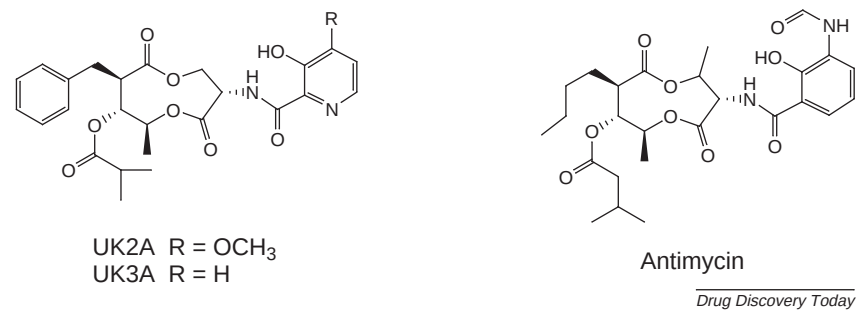


***Figure 4.*** *Inhibitors of electron transport.*

## Summary

The intensive research effort to increase the armamentarium of drugs against

**Table 1. Antifungal activities and challenges of emerging agents**

| Mechanistic class | Agent(s) | *In vitro* spectrum | | | | Major challenge |
|---|---|---|---|---|---|---|
| | | CA[a] | CG[b] | AF[c] | CN[d] | |
| Inhibitors of cell wall biosynthesis | Corynecandin | + | NR[e] | NR | NR | Poor *in vivo* efficacy |
| | Mer-WF3010 | + | NR | − | − | Poor *in vivo* efficacy |
| | Fusacandins | + | + | NR | NR | Poor *in vivo* efficacy |
| | Arthrichitin | + | + | NR | NR | Weak *in vitro* activity |
| Inhibitors of protein and amino acid synthesis | Sordarins | + | + | +/− | + | Weak activity versus *A. fumigatus* |
| | Cispentacin | + | NR | − | + | Lack of activity versus *A. fumigatus* |
| | Azoxybacillin | + | + | + | − | Inactive *in vivo* |
| Inhibitors of sphingolipid biosynthesis | Aureobacidin | + | + | − | + | Inactive versus *A. fumigatus* |
| | Rustmicin | + | + | − | + | Chemically unstable |
| | Khafrefungin | + | NR | − | + | Inactive versus *A. fumigatus* |
| Inhibitors of electron transport | UK2A | + | NR | + | NR | NR |
| | UK3A | + | NR | + | NR | NR |

[a] CA = *Candida albicans*; [b] CG = *Candida glabrata*; [c] AF = *Aspergillis fumigatus*; [d] CN = *Candida neoformans*; [e] NR = Not reported.

systemic fungal infections is yielding promising lead structures. The mode of action, spectrum of antifungal activity against key organisms and problems to be solved through synthetic modifications of these intriguing compounds are summarized in Table 1. The agents discussed in this review represent a fraction of the novel antifungal structures, mostly from natural sources, that have been isolated and characterized. With the advent of fungal genomics, combinatorial chemistry and other modern techniques, the stage is set for the development of an antifungal portfolio to rival the diversity of antibacterial drugs.

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