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Review Article

Antifungal lipopeptides: a tale of pseudomycin prodrugs and analogues

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CONTENTS

Abstract

Systemic fungal infections (SFI) can cause serious life-threatening diseases in normal healthy humans. *Candida albicans*, *Cryptococcus neoformans* and *Aspergillus fumigatus* are the major opportunistic pathogens responsible for SFI. The rising incidence of SFI, especially in immunocompromised patients, attests to the need for more effective therapies. Present treatment options are limited to three classes of compounds, the polyenes, the azoles and the recently approved lipopeptide caspofungin acetate. Amphotericin B and azole-based antifungal agents have an inadequate spectrum of activity and rapid emergence of fungal resistance, limited dosage forms and a narrow therapeutic window. Several approaches have been taken to address these deficiencies, such as improving the biological and/or toxicology profiles of existing drugs or the search for novel antifungal lipopeptides. As a result of this search, several cyclic peptides endowed with promising antifungal activities have been identified, including aureobasidins, echinocandins, papulacandin B and the recently disclosed pseudomycins. The most significant progress on this front was the 2001 launch of caspofungin acetate in the U.S. for the parenteral treatment of invasive aspergillosis in patients refractory to or intolerant of other antifungal therapies. This review provides a brief update on a number of recently discovered antifungal lipopeptides, as well as structural modifications and evaluation of pseudomycin analogues and prodrugs.

Introduction

Fungal infections range from superficial conditions of the skin (*e.g.*, ringworm and athlete's foot) and nails (onychomycoses) to life-threatening systemic fungal infections (SFI). SFI are those infections involving deep viscera such as liver and lung. Clinically, it has been shown that SFI can cause serious life-threatening diseases in normal healthy humans. *Candida albicans*, *Cryptococcus neoformans* and *Aspergillus fumigatus* are the major opportunistic pathogens responsible for SFI. The rising incidence of serious fungal infections such as SFI, especially in immunocompromised patients, attests to the need for more effective therapies (1-4). Present treatment options for SFI are limited to compounds in three classes, the polyenes (*e.g.*, amphotericin B), azoles (*e.g.*, fluconazole) and the recently approved lipopeptide caspofungin acetate (MK-0991, L-743872). Amphotericin B and azole-based antifungal agents have some serious liabilities, such as an inadequate spectrum of activity and rapid emergence of fungal resistance (*e.g.*, fluconazole) (5, 6), limited dosage forms and a narrow therapeutic window (*e.g.*, amphotericin B) (7, 8). To address these deficiencies, several approaches have been taken including to further improve the biological and/or toxicology profiles of existing drugs (*e.g.*, amphotericin B and itraconazole) (9, 10). These efforts led to the discovery of less toxic amphotericin derivatives as well as several new azole-based antifungal agents, including voriconazole (11), posaconazole (12), BMS-207147, *etc.* (13).

Parallel with these pursuits, the search for novel antifungal lipopeptides continues to attract even more attention from the medical community. As a result, several cyclic peptides endowed with promising antifungal activities have been identified. These include aureobasidins (14), echinocandins (15), papulacandin B (16, 17) and the recently disclosed pseudomycins (18-22). The most significant progress on this front was the 2001 launch in the U.S. of caspofungin acetate, the first semisynthetic analogue from the echinocandin/pneumocandin class, for the parenteral treatment of invasive aspergillosis in patients refractory to or intolerant of other antifungal therapies such as amphothericin B or its lipid formulation or itraconazole. This review will provide a brief update on a

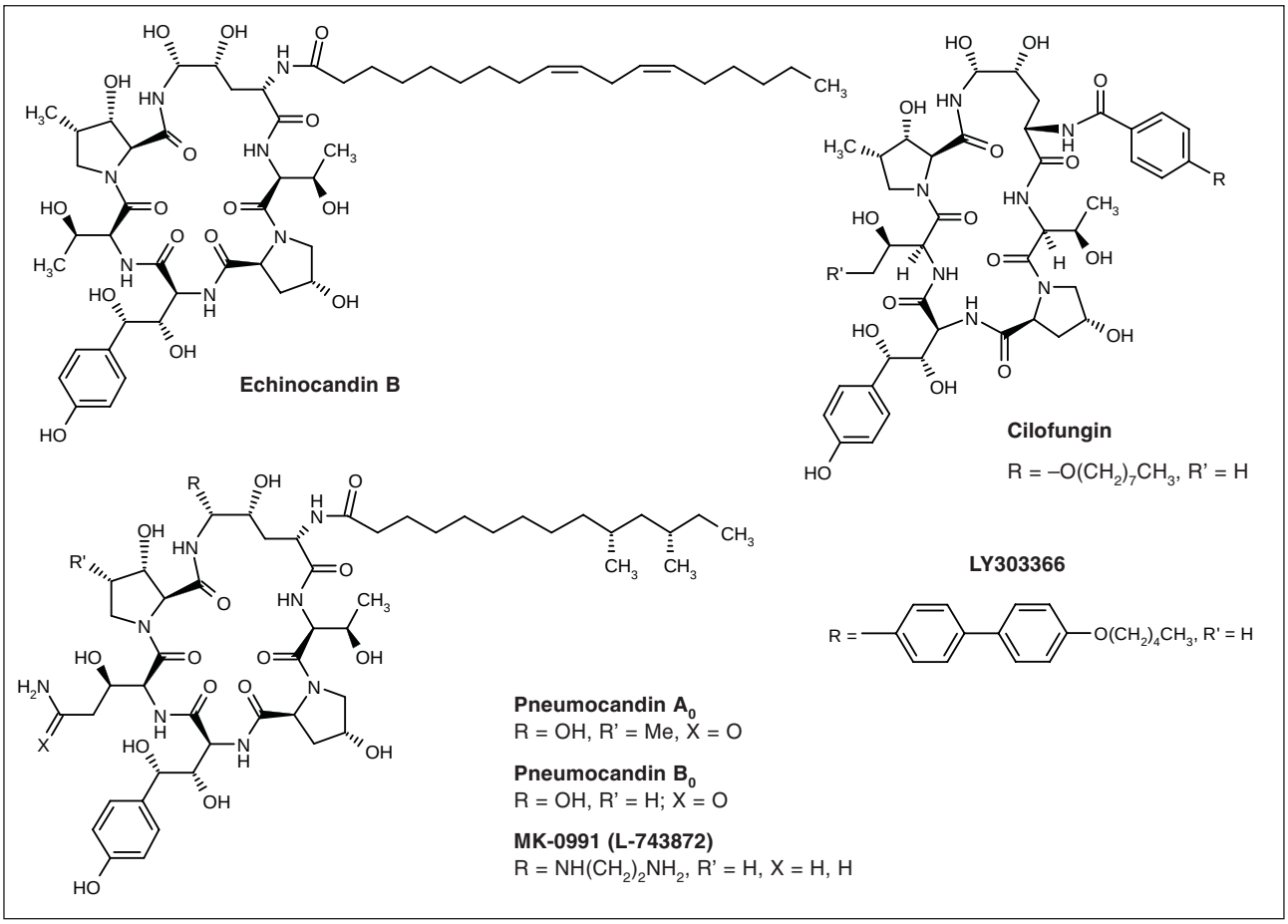


Fig. 1. Glucan synthesis inhibitors.

number of recently discovered antifungal lipopeptides (23, 24) and focus on structural modifications and evaluation of pseudomycin analogues and prodrugs. Our interest in this class of novel antifungals stemmed from the fact that pseudomycin B, the most promising member within the pseudomycin family, demonstrated improved *in vitro* and *in vivo* activity against *Candida albicans* and *Cryptococcus neoformans* when compared with amphotericin B.

Lipopeptides inhibiting glucan synthesis

Echinocandin B and analogues

Echinocandins and the related pneumocandins (15, 25-27) are natural products discovered in the 1970s that act as noncompeptitive inhibitors of (1,3)-β-D-glucan synthase, an enzyme complex that forms glucan polymers in the fungal cell wall (Fig. 1). Echinocandin B (ECB) is a potent anticandidal agent but was never used clinically due to toxicity, primarily associated with hemolysis. As a result of rather extensive side-chain SAR efforts, scientists at Lilly discovered a number of ECB side-chain analogues – for example, cilofungin (28, 29) and LY303366 (30, 31) – possessing improved anticandidal activity yet endowed with greatly reduced toxicity relative to ECB. Careful inspection of the antifungal activity shown below reveals that LY303366 demonstrated the most potent anticandidal activity both *in vitro* and *in vivo* in comparison to ECB and cilofungin (Table I).

Cilofungin (28, 29) has demonstrated promising activity for the treatment of *Candida* esophagitis and disseminated candidiasis in clinical trials. Unfortunately, nephrotoxicity, caused by polyethylene glycol in the i.v. formulation, led to discontinuation of clinical trials.

LY303366 (30, 31) exhibited potent activity against a broad range of *Candida* isolates studied to date and *Aspergillus fumigatus* as well as *Blastomyces dermatitidis* but was less potent against *C. parapsilosis* and inactive towards *C. neoformans*. LY303366 is also active against fluconazole-resistant *Candida* spp. and clinical isolates of *C. albicans* that are resistant to azole antifungals. The potent *in vitro* antifungal activity demonstrated by LY303366 translated well into good efficacy in stringent models of disseminated candidiasis when administered

Table I: Antifungal activity of echinocandin B and its derivatives.

Compound	MIC (μg/ml)	ED_{50} (mg/kg) i.p.	ED_{50} (mg/kg) oral
Echinocandin B	0.625	28.0	>100
Cilofungin	0.156	7.6	>100
LY303366	0.010	0.3	7.8

MIC: minimal inhibitory concentration against *C. albicans* A26; ED_{50}: calculated dose for 50% survival of mice infected with *C. albicans* A26.

i.p. or orally. Phase I data generated with LY303366 for both i.v. and oral formulations showed that it was well tolerated with a long half-life and was 3-5% oral bioavailable. In addition, a series of phosphate and phosphonate prodrugs of LY303366 and its analogue (linked at the phenolic hydroxyl moiety) have been reported (26, 27). A few such prodrugs retained *in vivo* antifungal activity while enhancing water solubility.

Also shown in Figure 1 are the structures of a series of pneumocandins. Pneumocandin A_0 was found to be less hemolytic than other members of the naturally occurring echinocandins (32). The anti-*Candida* activity of pneumocandin A_0 is similar to that of cilofungin. Pneumocandin B_0 (33, 34) isolated from the fermentation of the fungus *Glarea lozoyensis*, appears to be the most potent glucan synthase inhibitor within this series; however, the *in vitro* and *in vivo* antifungal activity of pneumocandin B_0 is comparable to that obtained with pneumocandin A_0. Continued core SAR work at Merck led to the discovery of MK-0991 (L-743872, caspofungin acetate) (34-37), a derivative of pneumocandin B_0 containing modifications at both the hydroxy aminal (R) and the β-Gln (X) sites (Fig. 1). MK-0991 has shown impressive *in vitro* and *in vivo* activity against various *Candida* species and *Aspergillus* spp. (MIC ≤ 0.09 μg/ml) but not active against *C. neoformans* (MIC = 32 μg/ml). Recent reports showed that MK-0991 was highly effective against fluconazole-susceptible and -resistant *Candida* spp., with MICs ranging from ≤ 0.20-0.80 μg/ml. In addition, this compound was effective against clinically important fungal isolates and well tolerated by rodents. On the basis of its favorable antifungal activity and toxicity profiles, caspofungin acetate was launched in the U.S. in 2001 for the treatment of certain invasive aspergillosis infections.

Another important echinocandin-like cyclic lipopeptide is FK-463 (micafungin sodium), which is a side-chain analogue of FR-901379 (38-40). The latter compound was isolated from the culture broth of *Colephoma empredi* strain F11899. FK-463 exhibited broad-spectrum activity against clinically important fungi such as *Candida* species (MIC range: ≤ 0.0031-0.125 μg/ml) and *Aspergillus* species (MIC range: 0.0078-0.0156 μg/ml), but displayed no activity towards *Cryptococcus neoformans* (MIC > 64 μg/ml). FK-463 was also effective against fluconazole-resistant *C. albicans* as well as azole-susceptible strains (MIC range: 0.0156-0.0313 μg/ml). FK-463 was launched in Japan in December 2002 for the prevention and treatment of fungal infections caused by *Aspergillus* and *Candida*, and is preregistered in Canada, the U.S. and the European Union for the same indication.

A recent report from Fujie *et al.* disclosed the structure and antifungal activity of FR-131535 (41) (structure not shown), another side-chain analogue of FK-901379 bearing cilofungin side-chain as shown in Figure 1. FR-131535 demonstrated potent *in vitro* and *in vivo* anticandidal activity yet was devoid of hemolytic activity.

Papulacandin B and analogues

The papulacandins, a class of unusual spirocyclic natural products originally isolated from *Papularia sphaerosperma* (16, 17), were documented as inhibitors of enzyme (1,3)-β-glucan synthase, a key component in the biosynthesis of the fungal cell wall (42). The papulacandins displayed potent *in vitro* activity against yeasts, particularly *Candida albicans* as well as modest efficacy *in vivo* via s.c. administration (*e.g.*, papulacandin B). Oral activity against SFI has remained elusive. A recent review regarding total synthesis efforts towards papulacandins has appeared (43). Since the discovery of papulacandins, other structurally related natural products have been reported, including chaetiacandin (44), Mer-WF3010 (45), saricandin (46), furanocandin (47), corynecandin (48) and fusacandin A (49).

FR-901469 and analogues

FR-901469 belongs to a new family of natural products inhibiting fungal (1,3)-β-glucan synthase activity (50-52). FR-901469 has shown potent antifungal activity in the murine systemic candidiasis model with an ED_{50} value of 0.22 mg/kg. However, hepatotoxicity was observed with this compound after multiple dosing (30 mg/kg i.v. q.d. x 14) in mice. In light of this drawback, chemical modifications were conducted on FR-901469 with the aim of improving the *in vivo* antifungal activity and reducing hepatotoxicity, thereby identifying analogues of FR-901469 with better therapeutic indexes. To this end, a D-ornithine bearing analogue of FR-901469 (53) was discovered by scientists at Nippon Roche Research Center. This compound exhibited 2-fold improved *in vivo* efficacy (ED_{50} = 0.1 mg/kg) and 3-fold lower hepatotoxicity in comparison to the parent compound FR-901469. Independent of Roche's effort, scientists from Fujisawa Pharmaceutical Company discovered another analogue of FR-901469 containing double modifications on the tyrosine residue, as shown in Figure 2 (54). This compound was found to be twice as potent as the parent compound in a disseminated candidiasis model. In a separate experiment, the Fujisawa analogue was shown to be 4-fold less hemolytic than the parent.

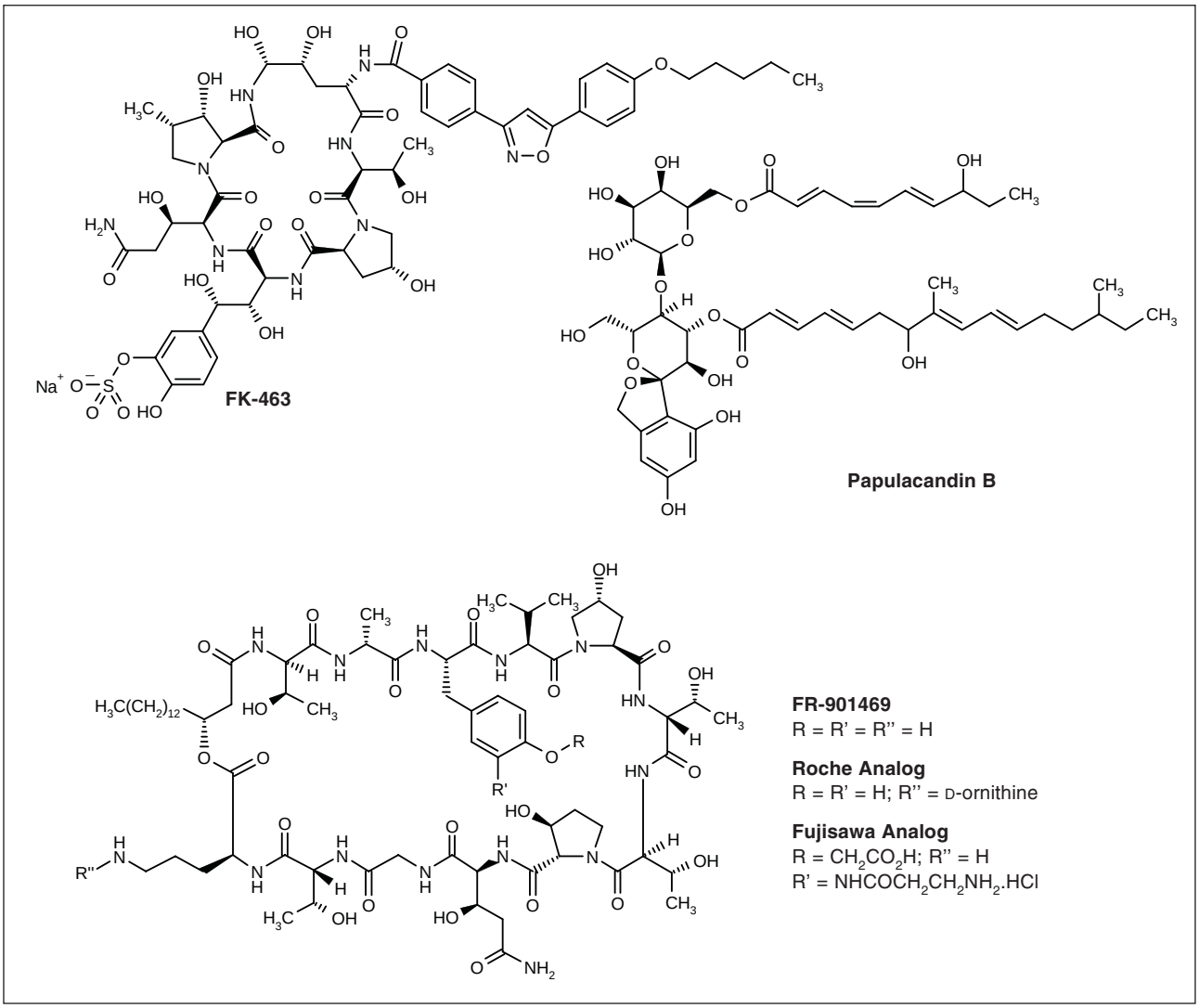


Fig. 2. Glucan synthesis inhibitors II.

Agents affecting chitin synthesis

Polyoxins, nikkomycins and FR-900403

Polyoxins (55), nikkomycins (56, 57) and FR-900403 (58) represent a series of natural products capable of inhibiting chitin synthase, an enzyme that catalyzes the polymerization of *N*-acetylglucosamine to form a major component of the fungal cell wall. As shown in Figure 3, all of the agents discussed herein are termed nucleoside peptides. Polyoxin D, produced by *Streptomyces cacaoi*, displayed inhibitory activity against *C. albicans*, with MIC values ranging from 0.12-2.0 μg/ml. Likewise, FR-900403, produced by *Kernia* spp., was active against *C. albicans* with an MIC value of 0.4 μg/ml. Nikkomycin Z, produced by *Streptomyces tendae*, is the most advanced compound within this series with an MIC value of 0.77 μg/ml. This agent has demonstrated additive and synergistic effects with both fluconazole and itraconazole against *C. albicans* and *C. neoformans in vitro* and *in vivo*. Marked synergism between nikkomycin Z and itraconazole was also observed against *A. fumigatus*. Single doses of up to 2 g of nikkomycin Z were well tolerated in phase I clinical trials.

Aureobasidins

The aureobasidins (14, 59-63), produced by *Aureobasidium pullulans*, exhibited potent antifungal activity against *Candida* species (MIC: < 0.04-0.16 μg/ml) and *C. neoformans* (MIC: 0.31-0.63 μg/ml), but not against *A. fumigatus* (MIC: 20 μg/ml). Compared with fluconazole and amphothericin B, aureobasidin A demonstrated superior activity. Aureobasidins are believed to be

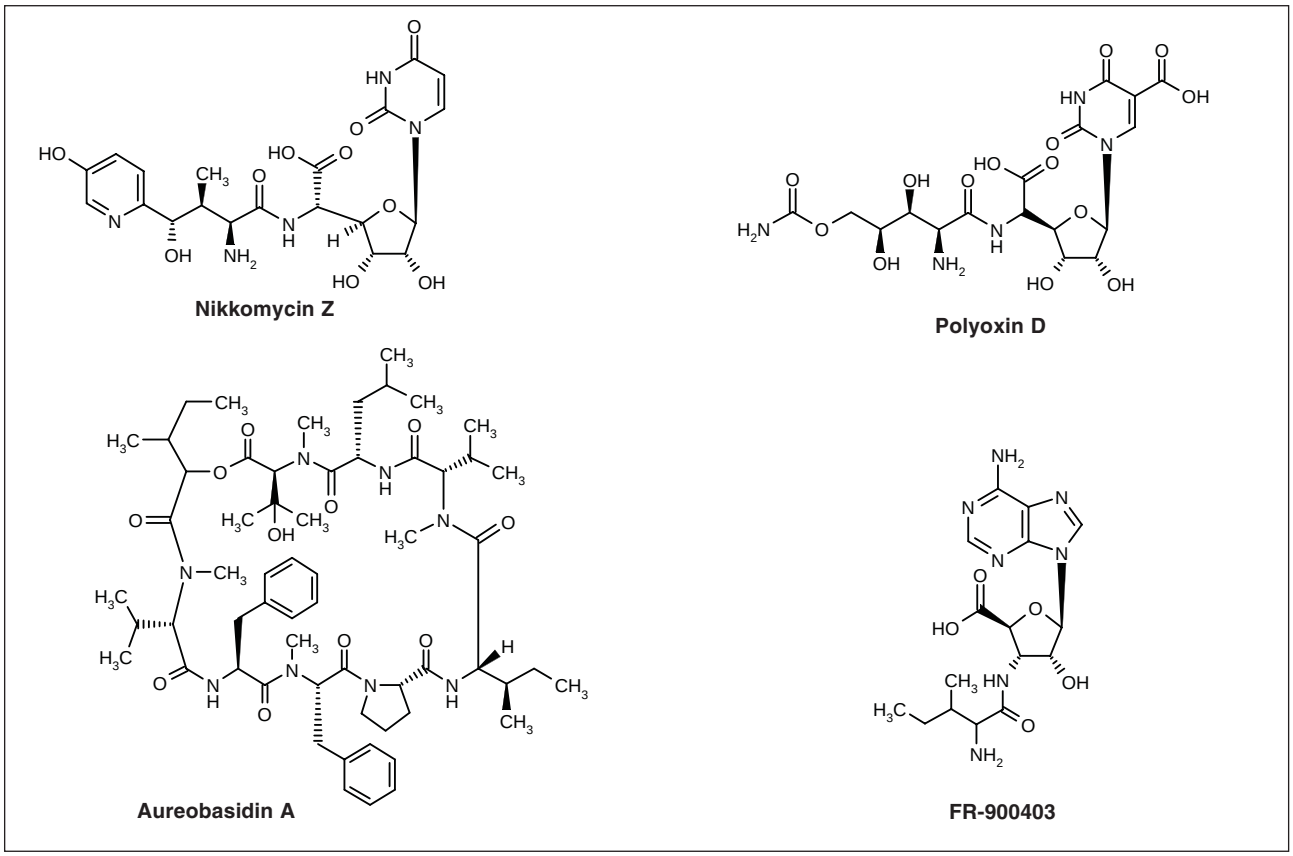


Fig. 3. Chitin synthesis inhibitors.

capable of altering actin assembly and delocalizing chitin in cell walls, thereby disrupting cell membranes.

Lipopeptides exerting antifungal activities via lysis

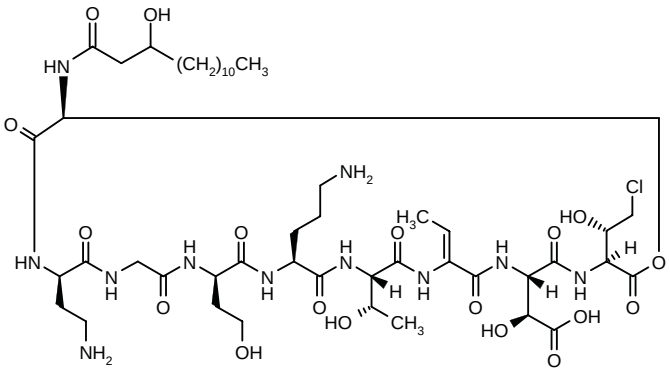
Syringotoxins, syringomycins and syrinostatins

All three of these cyclic lipodepsinonapeptides (CLPs) are produced by the plant bacterium *Pseudomonas syringae* pv. *syringae* (62-66). Individual strains within *P. syringae* pv. *syringae* produce one of such cyclic lipopeptide (Fig. 4). For example, the syringomycins are produced by *P. syringae* pv. *syringae* B301D, SCI and M1. It has been shown that the CLPs target the fungal plasma membrane (67, 68). Syringomycins alter several membrane functions such as membrane potential, protein phosphorylation, H^+-ATPase activity and cation transport fluxes. All three CLPs showed broad-spectrum antifungal activity against *Candida* species (MIC: 2.5-12.5 μg/ml) and *C. neoformans* (MIC: 0.8-10 μg/ml), but less activity against *A. fumigatus* (MIC: 6.25-25 μg/ml) (67, 68). All three CLPs caused lysis of sheep erythrocytes. Syringotoxin was the least toxic of the three CLPs tested.

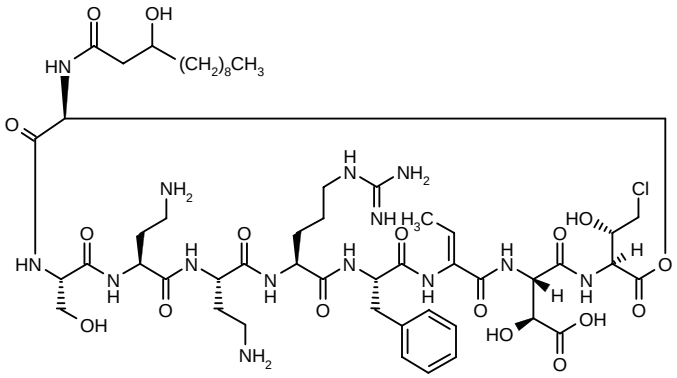
Pseudomycins

The emergence of fungal diseases resistant to current therapies has prompted the search for novel agents. Isolates of *Pseudomonas syringae*, which were first associated with plant diseases, are part of a large family of plant bacteria that have been the source of several interesting bioactive metabolites with potent antifungal activity against human pathogens. Research by Strobel *et al.* recognized that some of these isolates were actually plant symbionts that produced antifungal agents to protect the plant from fungal diseases (18, 19). The pseudomycins A-C'(PSA-C') are part of a new class of fungicidal agents derived from MSU 16H, a transposon-generated mutant of a wild-type strain of *Pseudomonas syringae* found on field grasses. In these particular isolates, the pseudomycins were identified as the natural products responsible for the observed broad spectrum *in vitro* activity against several of the common and medicinally important human pathogens: *Candida, Cryptococcus* and *Aspergillus* (69, 70).

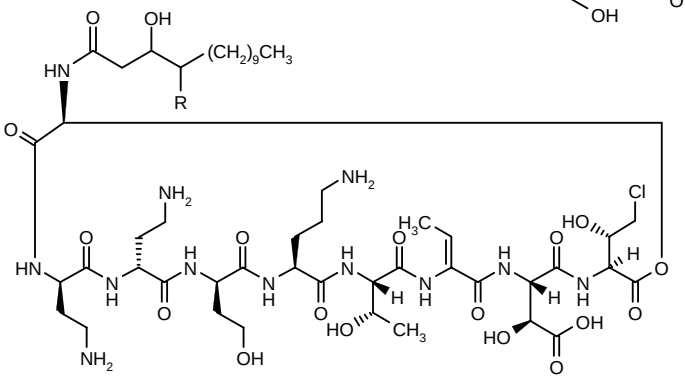
Pseudomycins represent a novel class of compounds having a unique mode of action over the existing antifungal agents currently on the market or in clinical trials. Pseudomycins are nonadepsipeptides, a cyclic peptide



Syringotoxin B



Syringomycin



Syrinostatins

Pseudomycins

Factors	n	R
A	9	OH
A'	10	OH
B	9	H
B'	7	H
C	11	OH
C'	11	H

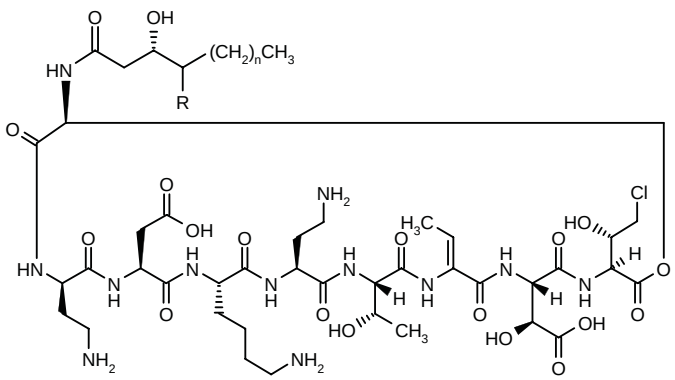
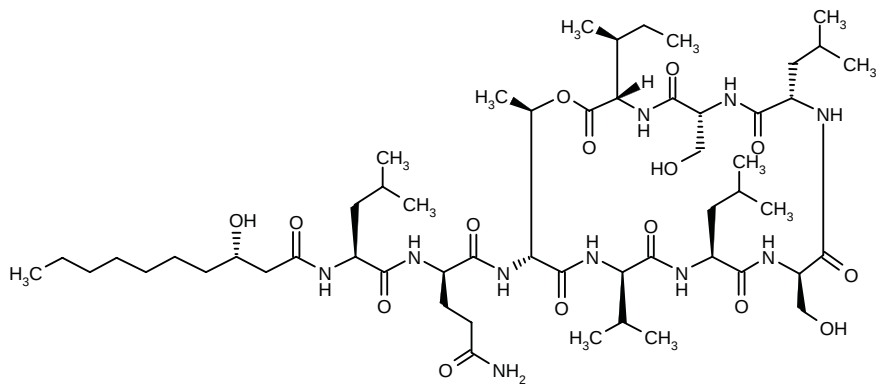


Fig. 4. Inhibitors via lysis mechanism.

Table II: MIC (μg/ml) of natural product pseudomycin (PSA-C') and echinocandin B (ECB).

Pathogen	PSA	PSA'	PSB	PSB'	PSC	PSC'	ECB
C. albicans	2.5	2.5	0.3	10	0.6	0.2	0.3
C. neoformans	1.3	1.3	0.3	1.3	0.04	0.08	>20
A. fumigatus	20	>20	10	>20	5.0	0.6	>20
H. capsulatum	2.5	5.0	0.6	2.5	1.3	0.3	2.5
C. parapsilosis	2.5	5.0	0.6	10	0.6	0.2	2.5



Viscosinamide

including one or more uncommon amino acids with a long, hydroxylated, aliphatic side chain. The amino acid residues on the cyclic peptide are identical for each factor. The significant structural difference among the factors resides only on the length and degree of the hydroxylation state of the side chain. With the factors readily available, it was encouraging to observe a mini SAR on the side chain. As the side-chain lipophilicity increases (PSB' to PSC'), the best overall *in vitro* spectrum was observed (71). In general, the *in vitro* antifungal activity for the six factors compared reasonably well against ECB, another well documented antifungal lipopeptide natural product. Additional *in vivo* and safety studies provided a means to help distinguish among the factors. From this preliminary research, the pseudomycin B (PSB) factor was selected for advanced studies. It was determined that PSB administered i.v. and i.p. was as effective as amphotericin B in mouse models of disseminated *C. albicans* and *C. neoformans*. However, for *A. fumigatus* infections in mouse models, only modest *in vivo* activity was observed with pseudomycin B (Table II).

Despite the encouraging *in vitro* and *in vivo* activity, and the fact that these compounds contained highly water soluble functional groups making them suitable for i.v. administration, the potential clinical utility of these agents was compromised by an undesirable irritation occurring at the site of injection presumably due to the charged nature and structural features of the natural product. To ameliorate the side effects at the site of injection and to improve the overall antifungal properties, research was initiated to identify semisynthetic analogues using analoguing and prodrug strategies detailed below (21, 22).

Antifungals with unknown mode of action

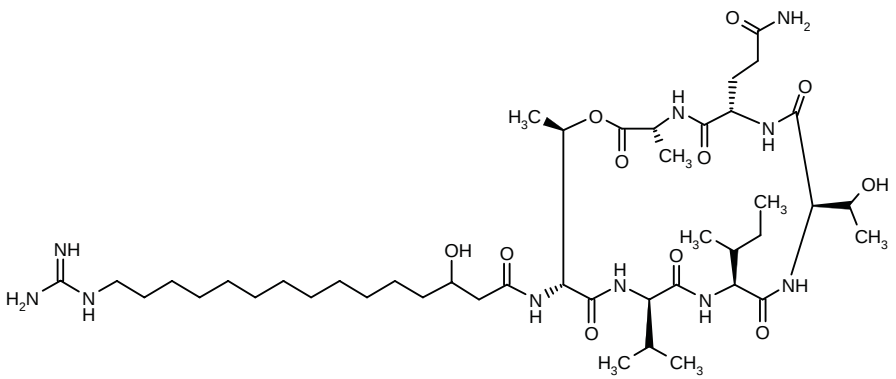
The structures and antifungal activities of a few recently reported cyclic lipopeptides are briefly discussed below. These include viscosinamide, LI-F antifungal antibiotics, glomosporin, jaspamides, cyclolithistide A, halolitoralins and lobocyclamides.

Viscosinamide

Viscosinamide was isolated from an extract of *Pseudomonas fluorescens* DR54 culture and is a depsipeptide containing nine common amino acids (72). This compound displays antifungal and surfactant properties. It demonstrated activity against the plant pathogen *Pythium ultimum* and *Rhizotonia*. Viscosinamide is capable of forming channels in membranes.

LI-F antifungal antibiotics

These compounds, produced by a strain of *Bacillus polymyxa* named L-1129, exhibited inhibitory activity against Gram-positive bacteria, mycobacteria and a wide range of fungi and yeasts, but were not active against



LI-F05b

Gram-negative bacteria. In general, LI-F antibiotics showed broad-spectrum activity against *Candida*, *Cryptococcus* and *Penicillum* strains with MIC values ranging from 1-8 μg/ml. Only weak activity against *Aspergillus* was observed with these compounds. Furthermore, this type of antibiotics produced synergistic effects with a number of important azole-based antifungal agents (73).

Glomosporin

Glomosporin is a novel antifungal agent produced by a culture of *Glomospora* spp. BAUA 2825. Structurally, glomosporin is a depsicyclic lipopeptide containing five L-amino acids and two D-amino acids as well as a side chain. Glomosporin showed broad, yet modest activity against most clinically important fungi with MIC values around 16-32 μg/ml (74). The acute toxicity of this compound was estimated to be 25 mg/kg i.v. in male CD-1 mice.

Jaspamides

Jaspamide, a novel cyclodepsipeptide isolated from a soft-bodied sponge, *Jaspis* spp., contains 2-bromoabrine with D-configuration and the rare amino acid (*R*)-β-tyrosine. Jaspamide was found to be fungicidal against *C. albicans*, *C. parapsilosis*, *C. glabrata* and *C. pseudotropicalis* with MIC values ranging from ≤ 0.3-3 μg/ml. In addition, jaspamide also exhibited potent insecticidal and anthelminthic properties as well as potent cytotoxicity against several prostate cancer cell lines (75-77).

A recent report from D'Auria *et al*. documented the isolation of jaspamide B and C (as minor products) along with jaspamide (as major product) from ethanolic extract of the sponge *Jaspis splendans*. Structurally, in contrast to the *endo*-cyclic double bond seen in jaspamide, jaspamide B or C contains an *exo*-cyclic double bond. Jaspamide B and C displayed cytotoxicity against the human NSCLC-N6 cell line with IC_{50} values of 3.3 and 1.1 μg/ml, respectively (78).

Cyclolithistide A

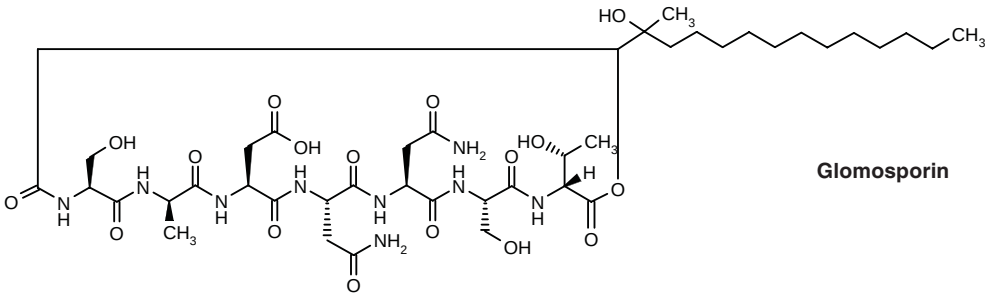
Cyclolithistide A is a novel cyclic depsipeptide that was isolated from a marine sponge, *Theonella swinhoei*. As can be seen from its structure, cyclolithistide A contains a few unique amino acids such as formyl-leucine and chloroisoleucine. Cyclolithistide A exhibited potent antifungal activity against *Candida albicans* (ATCC 24433) in the agar disk diffusion assay. When tested at a dose of 20 μg/disk, cyclolithistide A produced a zone of inhibition which was equal to 90-100 μg/disk standard nystatin (79).

Halolitoralins

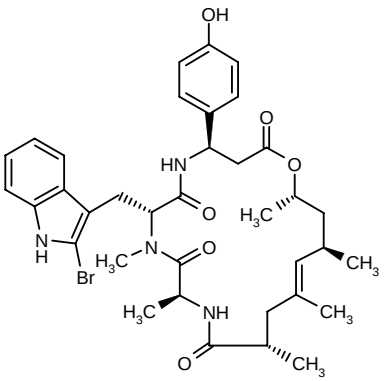
Halolitoralin A (hexapeptide), along with halolitoralin B and C (tetrapeptide), were isolated from the fermentation broth of a marine sediment-derived *Halobacillus litoralis* YS3106. These cyclopeptides contain surprisingly simple architectures with highly repeated hydrophobic residues (*e.g.*, Leu, Ile, Ala). Halolitoralin A, the most potent member within the class, showed moderate activity against *Candida albicans* with an MIC value of 20 μg/ml (80).

Lobocyclamides

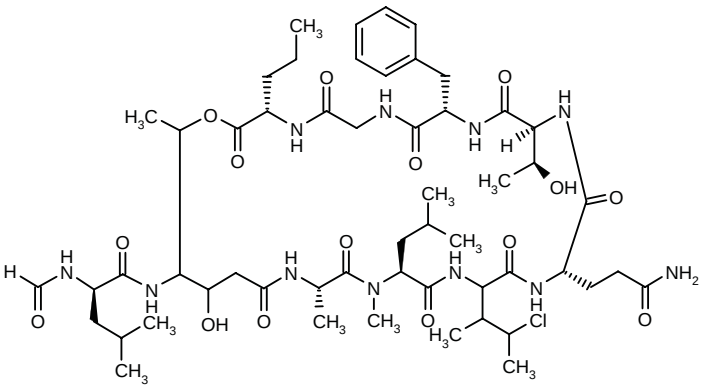
Lobocyclamide B, together with lobocyclamide C (two carbons shorter at the β-Ada residue site) and lobocyclamide A, were obtained from the methanol extracts of a benthic sample of *Lyngbya confervoides* collected at Cay Lobos, Bahamas (81). Lobocyclamides A-C exhibited modest antifungal activity against fluconazole-resistant *Candida albicans* with MIC values ranging from 30-100 μg/ml when tested separately. However, when tested together, lobocyclamides A and B (1:1 mixture) produced



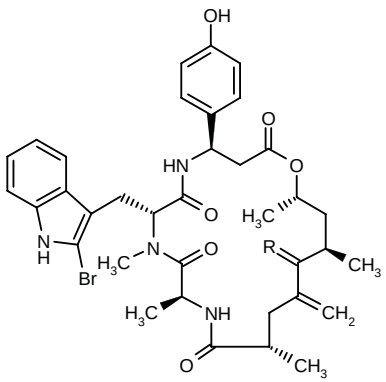
Glomosporin



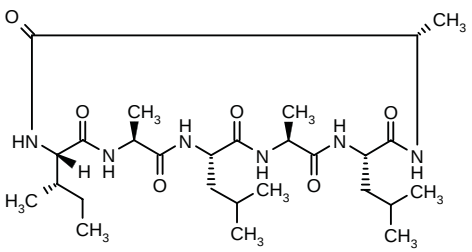
Jaspamide



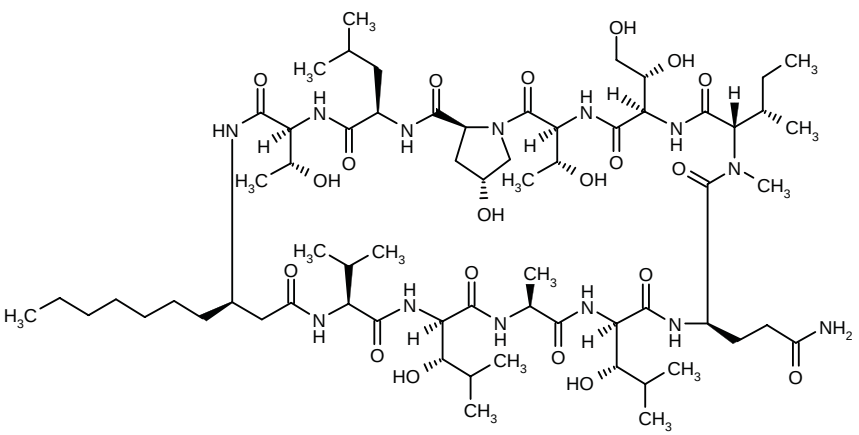
Cyclolithistide A



Jaspamide B R = O
Jaspamide C R = H, OH



Halolitoralin A



Lobocyclamide B

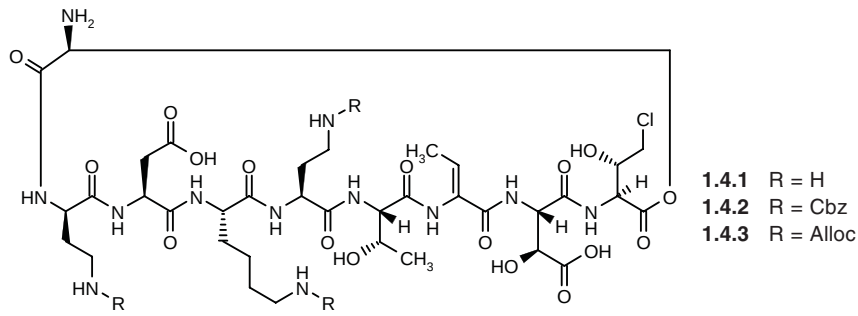
significant synergism with superior activity (MIC: 10-30 μg/ml).

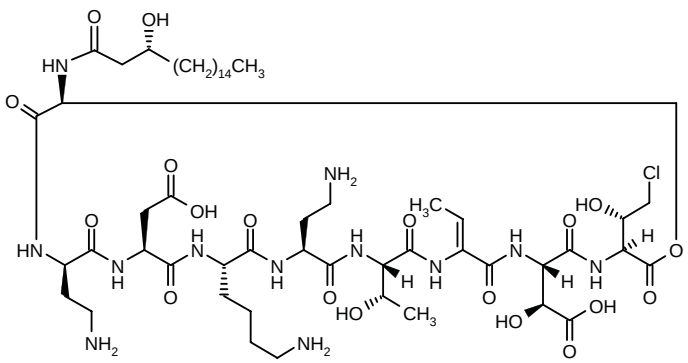
Pseudomycin analogues

Side-chain analogues

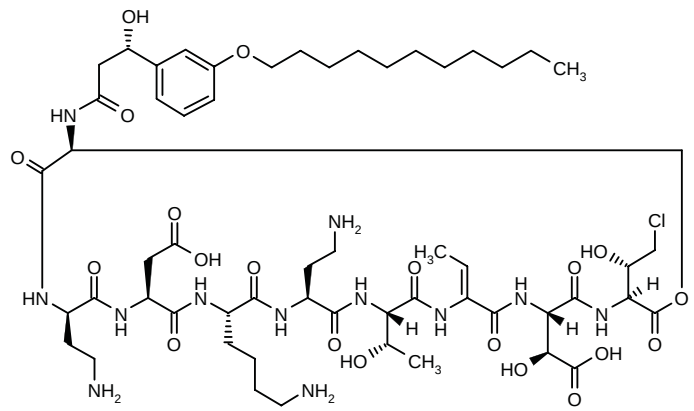
As can be seen from Table II, the length and the hydroxylation state of the side chain has a significant effect on the antifungal activity displayed by various pseudomycins. To take advantage of these findings, we decided to synthesize side-chain analogues in hopes of discovering novel pseudomycins possessing improved antifungal activity and toxicity profile. In accordance with the general semisynthetic route published from this institution (71), all side-chain analogues were prepared via *N*-acylation of ZPSN **1.4.2** or AllocPSN **1.4.3** with various side-chain acids.

As a result of our systematic side-chain modifications, we discovered two promising pseudomycin side-chain analogues, as shown in Figure 5. A further extended side-chain analogue **2.1.1** exhibited excellent activity against *C. albicans* (MIC = 0.625 μg/ml) and *C. neoformans*





2.1.1



2.1.2

Fig. 5. Representative pseudomycin side-chain analogues.

(MIC = 0.01 μg/ml) (83). In addition, compound **2.1.1** displayed 8-fold improved activity against *A. fumigatus* in comparison to PSB. When evaluated in the tail vein irritation assay, **2.1.1** did not induce irritation at a dose < 10 mpk. Although slight discoloration and swelling were observed at the highest dose (20 mpk), the level of tail vein irritation detected with **2.1.1** was better than that found with pseudomycin B (83).

In parallel with chain length modification, we also synthesized several series of rigidified side-chain analogues including ones incorporating an aromatic ring as exemplified by **2.1.2**, which was identified as the most potent analogue within these structural types (84). The *in vitro* antifungal activity and tail vein irritation potential of **2.1.2** were very similar to that found with pseudomycin B.

Prompted by the finding that certain amido linkage (instead of acid functionality) bearing amphotericin B (AMB) analogues demonstrated reduced toxicity relative to the parent AMB while retaining similar impressive antifungal activity (9), we decided to prepare corresponding amido pseudomycin analogues in hopes of discovering novel amido analogues of pseudomycins possessing overall improved biological and toxicological profiles (85-87).

Synthesis

The 3- and 8-bisamido pseudomycin analogues were prepared easily by treatment of ZPSB with excess amine (RNH_2) in the presence of HOBt/EDCI, followed by removal of Cbz protective groups via catalytic hydrogenation. When tested *in vitro* against various fungi responsible for SFI, none of the bisamido PSB analogues prepared exhibited significant activity. In light of this finding, we decided to synthesize 3- or 8-monoamido PSB analogues. Attempted regioselective synthesis of monoamido PSB analogues using HOBt/EDCI as the coupling reagents produced a mixture of the corresponding 3-amide, 8-amide and 3-,8-bisamide. After careful survey of various peptide coupling reagents, we discovered that the use of PyBOP led to selective 8-amidation, whereas treating ZPSB with TBTU (*O*-benzotriazol-1-yl-*N,N,N',N'*-tetramethyluronium tetrafluoroborate) in the presence of $EtPr_2N$ resulted in selective 3-amidation (Scheme 1) (88). The regioselectivity obtained with PyBOP and TBTU/$EtPr_2N$, in conjunction with basic, neutral and polar termini bearing amines, is summarized in Table III. Excellent selectivity (> 20:1) was achieved in most cases. The isolated yields (after preparative HPLC purification) ranged from 12-90%. The structures of 3- and 8-amides were confirmed upon detailed analyses of their proton NMR spectra (89).

Antifungal activity of 8-amides

The *in vitro* and *in vivo* (against *Candida*) antifungal activities and tail vein irritation potentials of eleven 8-amido PSB analogues are shown in Table IV (85, 90-93). With the exception of the simple amide derivative of PSB **2.2.1**, all other 8-amides exhibited reduced *in vitro* activity in comparison to PSB **1.2.1**. Nevertheless, all of the 8-amides still retained excellent activity against *C. neoformans*, with MIC values in the range of < 0.01-1.25 μg/ml. Upon careful examination of the data generated for *Candida albicans*, we observed the following trends: (i) the *in vitro* potency decreases according to the following order: **2.2.1** (R = H) > **2.2.2** (R = Me) > **2.2.3** (R = Et) > **2.2.4** (R = *n*-Pr); (ii) cycloalkyl bearing amides **2.2.5** and **2.2.7** displayed better activity than their corresponding *n*-alkyl containing counterparts **2.2.4** and **2.2.6**; and (ii) polar termini bearing analogues were found to be less potent than the parent PSB. Despite the promising *in vitro* activity seen with **2.2.1**, this analogue, along with other 8-amides, did not produce *in vivo* efficacy. The reason for the lack of correlation between *in vitro* potency and *in vivo* efficacy is currently unclear. When evaluated in the subsequent tail vein irritation assay *in vivo*, in sharp contrast to the findings observed with pseudomycin B and C', all of the 8-amides tested failed to induce tail vein irritation at the highest dose tested (20 mpk). These findings are shown in Table IV.

Antifungal activity of 3-amides

Three types of 3-amides were prepared during the course of core modifications. These include neutral alkyl, basic termini and amino acid termini bearing analogues. All 3-amides synthesized were first evaluated using an *in vitro* assay against three major fungi responsible for SFI, an *in vivo* efficacy model against murine systemic candidiasis (i.p.) and an *in vivo* tail vein irritation assay (i.v.) (86, 90-93). Analogues with good *in vivo* efficacy against *Candida* and clean results from the tail vein irritation assay were selected for further *in vivo* testing against *Cryptococcus* (i.p.). The results obtained from these evaluations are summarized in Table V. After careful review of the data in Table V, we made the following observations.

1) *In vitro* activity

All straight alkyl chain bearing 3-amides **2.3.1-2.3.6** exhibited excellent activity against *Cryptococcus* with MIC values ranging from < 0.01-5.0 μg/ml. They also displayed weak activity against *Aspergillus* with MIC values around 20 μg/ml. Their activity against *Candida* varied according to the nature of alkyl groups. Generally speaking, better activity against *Candida* was obtained with analogues bearing smaller alkyl groups. All of the *N',N'*-diMe(Et)alkyl-3-amides **2.3.7-2.3.10** showed excellent activity against *Cryptococcus*. Importantly, several such 3-amides (*e.g.*, **2.3.7** and **2.3.10**) demonstrated improved potency (~ 4-fold) against *Aspergillus* in comparison to the parent PSB. Compared with PSB, three amino acid termini containing 3-amides **2.3.11-2.3.13** showed

Scheme 1: Regioselective Synthesis of 3- and 8-Amido PSB Analogs

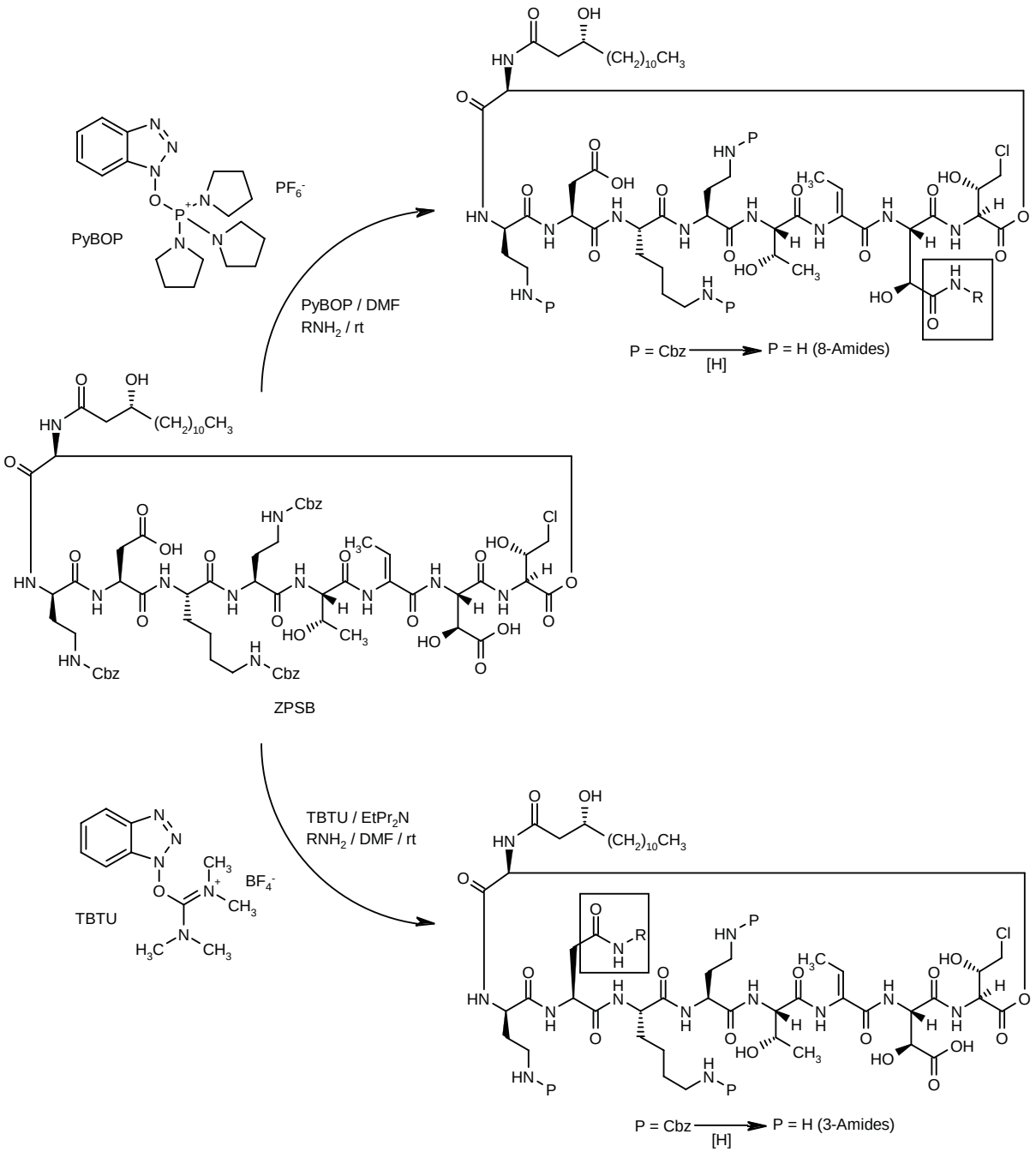


Table III: Selectivity for 8- and 3-amido pseudomycin analogues.

R=	PyBOP 8-/3-Amides	Yield (%) 8-Amide	TBTU 3-/8-Amides	Yield (%) 3-Amide
$CH_2CH_2NMe_2$	>20 : 1	90 (**2.2.9**)	>20 : 1	53 (**2.3.7**)
NHPhe	>20 : 1	12	>20 : 1	37
NH-*n*-Pr	>7 : 1	34	>20 : 1	52
CH_2CH_2OH	>20 : 1	71	>20 : 1	21

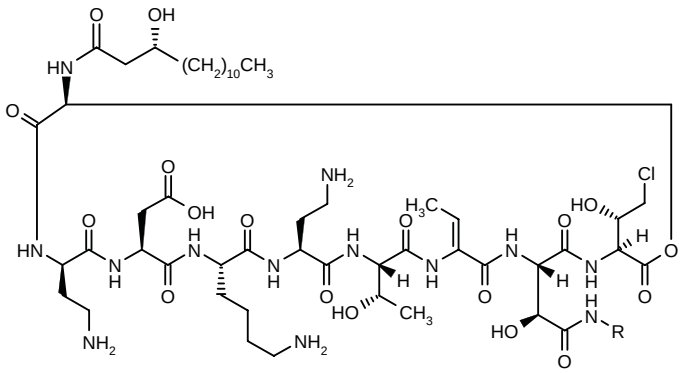


Table IV: Antifungal activity of 8-amido pseudomycin analogs.

Comp.	R	MIC (μg/ml) *C. albicans*	*C. neoformans*	*A. fumigatus*	IP ED_{50} (mg/kg x 4)	Tail Vein Assay (i.v.)
2.2.1	H	0.312	<0.01	>20	>20	Clean
2.2.2	Me	1.25	0.156	>20	>20	Clean
2.2.3	Et	10	1.25	>20	N.T.	N.T.
2.2.4	*n*-Pr	20	5.0	>20	14	Clean
2.2.5	*c*-Pr	5.0	0.312	>20	>20	Clean
2.2.6	*n*-Bu	20	1.25	>20	>20	Clean
2.2.7	*c*-Bu	5.0	1.25	>20	>20	Clean
2.2.8	CH_2CH_2OH	5.0	0.625	>20	—	Clean
2.2.9	$CH_2CH_2NMe_2$	5.0	1.25	>20	>20	Clean
2.2.10	GlyOMe	2.5	0.08	>20	>20	Clean
2.2.11	PheOMe	10	1.25	>20	>20	Clean
PSB 1.2.1	—	0.625	0.078	>20	2.4-7.2	Positive

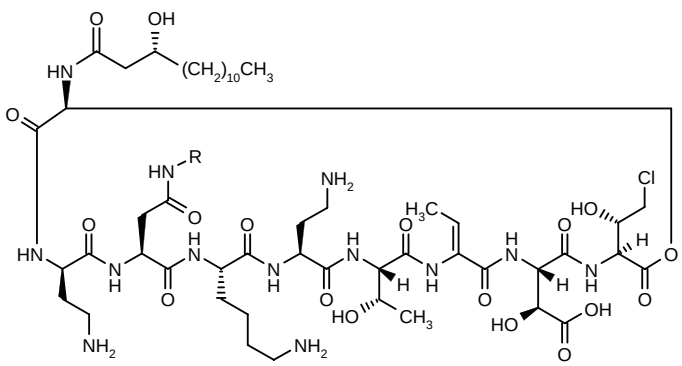


Table V: Antifungal activity of 3-amido pseudomycin analogues.

Comp.	3-amide R =	MIC (μg/ml) *C. albicans*	*C. neoformans*	*A. fumigatus*	Tail Vein 20 mg/kg x 4 (i.v.)	*In vivo* Candidiasis (i.p.)	ED_{50}(mg/kg) Cryptococcosis (i.p.)
PSB	—	0.625	0.01	>20	Positive	3.2-8.4	1.8
2.3.1	H	2.5	<0.01	10	Positive	<4.8	N.T.
2.3.2	Me	5.0	0.312	>20	Pat. Posit.	7.4 (4.5)	N.T.
2.3.3	Et	0.625	0.156	20	Positive	<5.0 (4.2)	N.T.
2.3.4	*n*-Pr	1.25	0.078	>20	Negative	6.0 (8.4)	N.T.
2.3.5	*c*-Pr	0.156	<0.01	20	Negative	<5.0 (7.2)	2.9
2.3.6	*n*-Bu	5.0	<0.01	20	Negative	5.9 (3.8)	N.T.
2.3.7	$(CH_2)_2NMe_2$	0.156	<0.01	5.0	Negative	<5.0 (4.5)	2.5
2.3.8	$(CH_2)_2NEt_2$	10	<0.01	10	Positive	4.9 (3.5)	N.T.
2.3.9	$(CH_2)_3NMe_2$	10	<0.01	10	Negative	<5.0 (3.9)	N.T.
2.3.10	$(CH_2)_4NMe_2$	20	<0.01	5.0	Negative	<5.0 (7.1)	N.T.
2.3.11	GlyOMe	1.0	0.01	20	Negative	<5.0 (3.8)	2.9
2.3.12	PheOMe	1.25	1.25	>20	Pat. Posit.	>15 (9.0)	N.T.
2.3.13	LysOMe	1.25	<0.01	>20	Negative	<5.0 (8.4)	N.T.

slightly reduced activities against *Candida* and *Crypotococcus*, respectively. When tested against *Aspergillus*, these analogues exhibited similar potency to that observed with the parent PSB.

2) *In vivo* activity

Five straight chain alkyl bearing 3-amides **2.3.2-2.3.6** (Table V) exhibited comparable *in vivo* efficacy (against murine candidiasis) to that obtained with PSB. All four basic termini bearing 3-amides (**2.3.7-2.3.10**), regardless of the length of the alkyl linker, demonstrated impressive *in vivo* efficacy against candidiasis (ED_{50} values <5.0 mg/kg). Within the amino acid series, two such analogues (**2.3.11** and **2.3.13**) exhibited good efficacy against candidiasis (ED_{50} values < 5.0 mg/kg). The bulky PheOMe bearing amide **2.3.12** displayed poor activity.

In light of the excellent *in vivo* activity achieved in the murine candidiasis model, compounds **2.3.5** and **2.3.7** were further evaluated *in vivo* against *Cryptococcus*. As can be seen in Table V, both compounds demonstrated good *in vivo* efficacy against *Cryptococcus* with ED_{50} values ranging from 2.5-2.9 mg/kg (the ED_{50} observed with PSB was 1.8 mpk in the same experiment).

3) Tail vein irritation

With the exception of small alkyl bearing 3-amides (**2.3.1-2.3.3**, wherein R = H, Me, Et), the remaining alkyl chain bearing 3-amides listed in Table V (**2.3.4-2.3.6**) were found to be clean in this assay. With respect to the dialkylamino termini amide series, except for the di-Et bearing amide **2.3.8**, all three di-Me 3-amides (**2.3.7, 2.3.9** and **2.3.10**) were found to be negative in this assay. It is encouraging to note that all of the amino acid termini containing 3-amides (**2.3.11-2.3.13**) demonstrated reduced irritation potential relative to the parent pseudomycin B **1.2.1** in this assay.

4) Dose elevation study

To further assess the safety profiles of the 3-amides, one member of each subset of 3-amides (**2.3.5, 2.3.7** and **2.3.13**) were selected, on the basis of their excellent *in vivo* efficacy and minimal tail vein irritation potential, for additional evaluation in the dose elevation study in mice. The positive control, PSB was found to be safe only at the dose of 25 mg/kg. When PSB was dosed at 50 mg/kg and higher, severe toxicity resulted. To our satisfaction, all three 3-amides evaluated failed to induce tail vein irritation at the highest dose tested (75 mg/kg). In light of the encouraging results obtained with **2.3.5** in mice, this analogue was selected for the 2-week toxicity study in rats. In this study, amide **2.3.5** was given to rats at doses of 50 and 75 mg/kg for 14 consecutive days. The results from the study showed that all drug-treated animals (at highest dose) were found to be normal at the end of the experiment. No clinical observations were recorded. Careful analysis of blood samples collected from drug-treated rats indicated that blood chemistry was also normal (Table VI).

Table VI: Toxicity profiles of selected 3-amido pseudomycin analogues.

Comp.	Mice Dose Elevation	Two-Week Rat Tox.
PSB 1.2.1	25 mpk	25 mpk
2.3.5	75 mpk	75 mpk
2.3.7	75 mpk	N.T.
2.3.13	75 mpk	N.T.

5) Summary of activity of 3-amides

To summarize the data presented thus far concerning 3-amido pseudomycin analogues, it appears that there is no clear correlation between *in vitro* potency and *in vivo* efficacy. It is also evident that many newly prepared 3-amides passed the primary *in vivo* efficacy screening without inherent tail vein toxicity. These include **2.3.4-2.3.7, 2.3.11** and **2.3.13**. More significantly, the 3-cyclopropyl amide **2.3.5** demonstrated superior *in vitro* and *in vivo* activity (against *C. albicans* and candidiasis) to that determined for PSB, without inherent tail vein irritation and long-term toxicity being observed in mice or rats.

N-Acyl prodrugs of pseudomycin analogues

In parallel with pseudomycin analoguing work, we were also interested in the design of novel pseudomycin prodrugs. The rationale behind prodrug design is built upon the general notion that, in some cases, the C_{max} related acute toxicity of a drug may be reduced via controlled release of the parent drug. Many esterase labile prodrugs were documented in the literature for this purpose, including the *N*-oxodioxolenylmethyl carbamate and *N*-acyloxymethyl carbamate pseudomycin prodrugs shown in Figure 6. It should be pointed out that although the oxodioxolenyl linker has been used frequently in the literature for improving oral absorption of various drugs, including ampicillin (94), methyldopa (95, 96), norfloxacin (97), *etc.*, the use of this prodrug group for modifying therapeutic indexes of the parent drugs has not yet been reported. Similar comments can be made about the acyloxymethyl prodrug linker (98). It should be mentioned that the acyloxymethyl linker was used recently for the purpose of improving the toxicity profile of the ribonucleotide reductase inhibitor 3-AP (99).

Chemical synthesis

The general synthetic routes for two types of pseudomycin prodrugs (100, 101) are depicted in

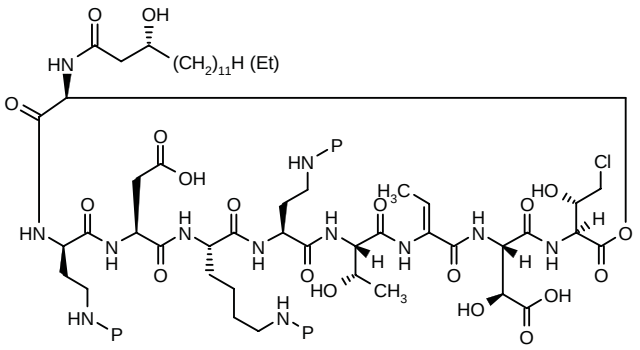


Fig. 6. Pseudomycin *N*-acyl prodrugs.

Scheme 2. Due to the fact that three amino functions located at residues 2, 4 and 5 reacted almost equally well with the acylating agents (*e.g.*, **3.2.3** and **3.1.6**), regioselective synthesis of any specific mono- or di-prodrugs could not be achieved. Alternatively, treatment of a DMF solution of PSB with three equivalents of the linker **3.1.6** provided, after semipreparative HPLC purification, the triprodrug **3.1.4** in 70% yield (Scheme 2). The preparation of the *N*-oxodioxolenyl triprodrug **3.2.2** was accomplished via *N*-acylation of PSC' with the mix-carbonate linker **3.2.3** (60%). Following the identical procedure shown in Scheme 2, all other prodrugs listed in Table VII were prepared in a similar fashion. Satisfactory mass spectra were obtained for all pseudomycin prodrugs synthesized (102).

Prodrug bioactivation

According to the literature precedents (95, 96, 98, 103), pseudomycin prodrugs should be converted to the corresponding parent compounds (PSB or PSC') via successive esterase-mediated prodrug linkers cleavage *in vivo*. Since three amino functional groups within the target prodrugs (at N^2, N^4 and N^5) were protected by various *transient* acyl linkers, it is expected that these prodrugs should retain good *in vivo* efficacy without tail vein irritation. Furthermore, it seems to be possible to modulate the rate of parent drug release (from its prodrugs) by varying the nature of the linker attached, such as the terminal esters used in Type 1 prodrug series. To further confirm the prodrug bioactivation mechanism, we studied mouse and human plasma mediated bioactivation of various pseudomycin prodrugs.

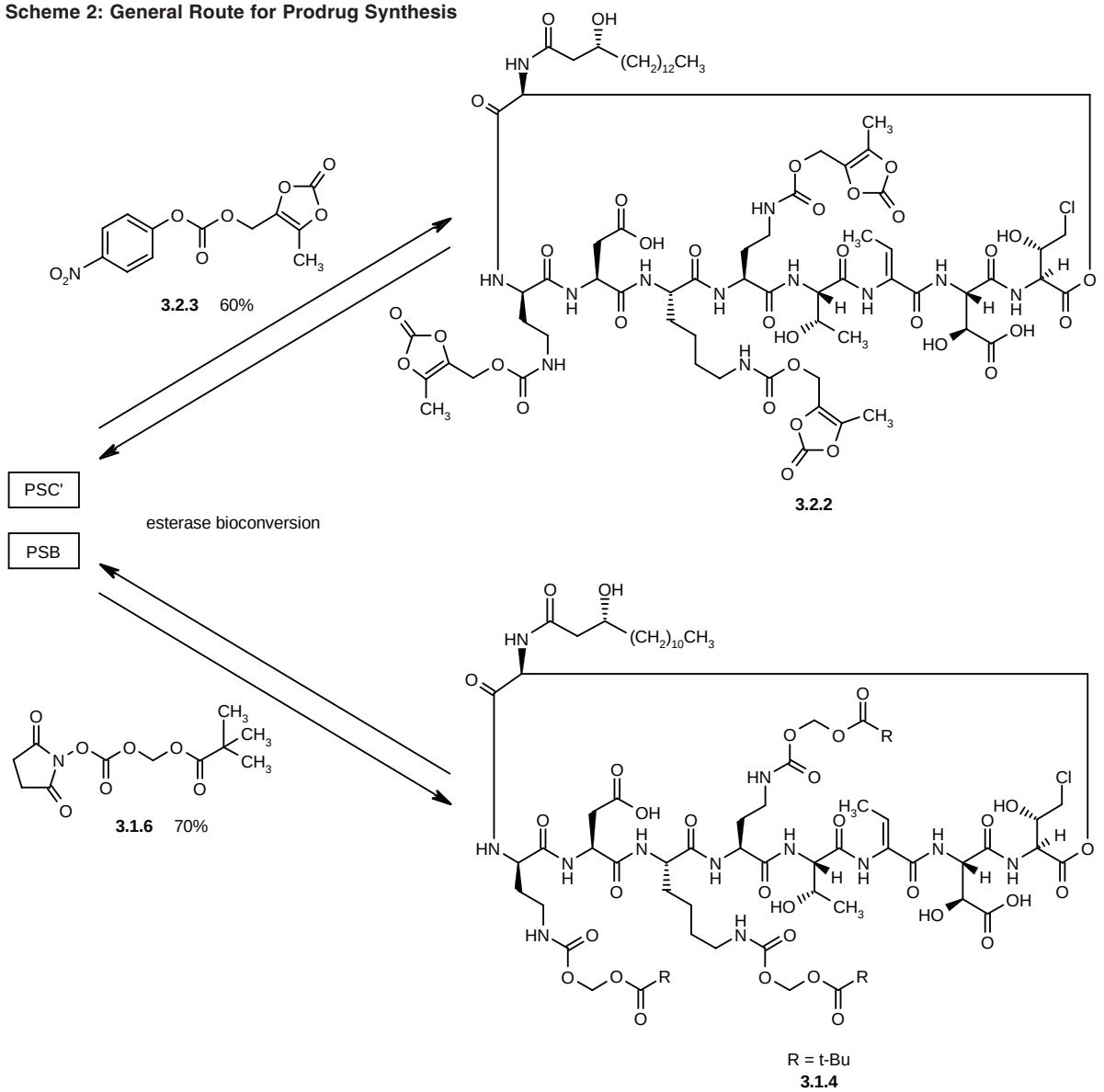
In the initial study using mouse plasma, we selected the most labile *O*-acetyl termini-bearing prodrug **3.1.1** and the least labile *O*-pivaloyl containing prodrug **3.1.4** as our candidates from Type 1 prodrug series. After incubation with mouse plasma for 1 h, no triprodrug **3.1.1** was detected. Analysis of the reaction mixture indicated the presence of di-, and monoprodrug along with the parent PSB. At the 4-h time point, the only pseudomycin-like compound detected was the parent drug. As expected, the mouse plasma mediated bioactivation of **3.1.4** was considerably slower than that found with **3.1.1**. Incubation of **3.1.4** with mouse plasma for 1 h led to only partial mono *N*-deacylation. The major product identified at this time point was the corresponding diprodrug. At the 4-h time point, the desired parent drug PSB, along with some monoprodrug (the major component) were identified on the basis of LC-MS analyses. In view of these results, it is evident that both **3.1.1** and **3.1.4** indeed served as the prodrug forms of PSB. Moreover, PSB was released much more readily from **3.1.1** than **3.1.4**.

Bioactivation of the *N*-oxodioxlenylmethyl prodrug **3.2.1** was also performed with freshly prepared mouse plasma. At the 1-h time point, **3.2.1** rapidly degraded into a mixture containing parent PSB and its corresponding mono- and diprodrugs. After incubation for 4 h, none of the *N*-acylated species were detected. Pseudomycin B was the only product detected at that time point. Given these results, it is evident that compound **3.2.1** qualifies as the prodrug of PSB.

Antifungal activity and tail vein toxicity

By virtue of being prodrugs, all seven triprodrugs listed in Table VII were devoid of *in vitro* antifungal activity (104). To identify pseudomycin prodrugs that retain good *in vivo* efficacy yet are free of tail vein irritation, we evaluated all seven prodrugs (**3.1.1-3.1.5, 3.2.1-3.2.2**) *in vivo* against the disseminated candidiasis mouse model (i.p.) and the tail vein irritation model (i.v.). PSB **1.2.1** or PSC' **1.3.1** were also included as positive controls in these studies. The ED_{50} values were determined using the method of Reed and Muench (105). It should be pointed out that the reason for using disseminated candidiasis model as our primary *in vivo* assay is because *Candida albicans* is the most important pathogen responsible for approx. 80% of SFI in hospitalized patients. As can be seen in Table VI, three *N*-acyloxylmethyl prodrugs (**3.1.1**,

Scheme 2: General Route for Prodrug Synthesis



3.1.3, **3.1.4**) and one *N*-oxodioxolenylmethyl PSB prodrugs **3.2.1** exhibited good *in vivo* activities with ED_{50} values ranging from 6.4-14.1 mg/kg (~2x weaker than that found with PSB). PSC' prodrugs **3.1.5** and **3.2.2** also showed similar *in vivo* efficacy to that found with PSC' with ED_{50} values ranging from < 5.0-12.4 mg/kg. Disappointingly, however, prodrug **3.1.2** failed to show measurable efficacy (ED_{50} > 20 mpk). The reason for this "unexpected" result will be discussed later.

In a subsequent experiment, all prodrugs listed in Table VII were evaluated in the tail vein toxicity assay. All test compounds, along with positive control PSB, were administered i.v. to mice at 20 mpk. Despite its favorable *in vivo* efficacy, prodrug **3.1.1** was found to be equally irritable as PSB. To our satisfaction, the remaining six prodrugs were found to be nontoxic in this assay. Thus, judging from the data shown in Table VII, it was evident that we had successfully discovered three *N*-acyloxymethyl prodrugs (**3.1.3**-**3.1.5**) as well as two *N*-oxodioxolenylmethyl prodrugs (**3.2.1**-**3.2.2**) that retained favorable *in vivo* antifungal activities and tail vein irritation/toxicity profiles.

Because of their promising overall profiles, **3.1.4** and **3.2.2** were evaluated next in a dose elevation study in mice. Administration of a single dose of PSB at 75 mpk to mice (i.v.) resulted in immediate death of the animal. In sharp contrast to this observation, all mice receiving **3.1.4**

Table VII: Antifungal activity and toxicity profiles of pseudomycin prodrugs.

Comp.	Parent Drug	Prodrug Linker	ED_{50}(mg/kg) Candidiasis	Tail Vein @ 20 mpk	Dose Elevat. mpk (mice)	Two-Week rat tox.(mpk)
1.2.1	PSB	—	3.2-8.4	Positive	< 25	< 25
3.1.1	PSB	OCH_2OCOMe	13	Positive	—	—
3.1.2	PSB	OCHMeOCOMe	>20	Negative	—	—
3.1.3	PSB	OCH_2OCO-*i*-Pr	11.4	Negative	—	~ 75
3.1.4	PSB	OCH_2OCO-*t*-Bu	6.4/14.1	Negative	> 75	~ 75
3.2.1	PSB	Dioxolenyl methyl	10.8/12.4	Negative	—	—
1.3.1	PSC'	—	7.8/12.4	Positive	< 25	< 25
3.1.5	PSC'	OCH_2OCO-*t*-Bu	9.0	Negative	—	—
3.2.2	PSC'	Dioxolenyl methyl	<5.0/6.8/7.8	Negative	> 75	~ 75

and **3.2.2** at 75 mpk were found to be normal. To further assess the long-term toxicity profiles of selected pseudomycin prodrugs, **3.1.3**, **3.1.4** and **3.2.2** along with PSB were dosed to rats daily (i.v.) for 2 weeks. The maximum tolerated dose found with PSB **1.2.1** was ≤ 25 mg/kg. To our satisfaction, the rats injected with all three prodrugs (**3.1.3**, **3.1.4** and **3.2.2**) were found to be normal. No symptoms of histamine-induced pathology were detected (Table VII).

With the *in vivo* efficacy and toxicity data (Table VII) and the plasma stability data in hand, we conducted a detailed data analysis and discovered the following trends: (i) a prodrug capable of delivering parent drug very rapidly upon incubation with plasma (*e.g.*, **3.1.1**) displayed favorable *in vivo* efficacy along with undesirable tail vein toxicity; (ii) a prodrug capable of generating only a minimal amount of parent drug after incubation with plasma was devoid of *in vivo* efficacy as well as tail vein toxicity (*e.g.*, **3.1.2**); and (iii) prodrugs capable of releasing an adequate (C_{max} and AUC profiles) amount of their corresponding parent drugs (*e.g.*, **3.1.4**, **3.1.5, 3.2.1** and **3.2.2**) were endowed with good *in vivo* efficacy yet devoid of tail vein irritation.

3-Amido prodrugs of pseudomycin analogues

As documented in Schemes 1 and 2 and Tables V-VII, we synthesized and evaluated two series of *N*-acylated prodrugs (100, 101) and 3-amido PSB analogues (86) possessing impressive antifungal activity yet devoid of inherent tail vein irritation potential and long-term toxicity. Encouraged by these findings, we designed and synthesized *N*-acylated prodrugs of 3-amides (termed combinations) as novel antifungal agents (106). It was expected that these amide-prodrug combinations would possess even greater safety profiles than their corresponding 3-amides and *N*-acylated prodrugs.

Synthesis

Following our recently developed regioselective methodology for 3-amidation, the combinations listed in Table VIII were synthesized in one step from the PSB prodrugs **3.1.3** or **3.1.4**. Generally speaking, TBTU and $EtPr_2N$ mediated 3-amidation of **3.1.3** and **3.1.4** provided the desired products (combinations) in low to modest yields along with various amounts of recovered starting prodrugs. Consistent with the selectivity obtained with 3-amidation reactions shown in Table III, the desired combinations (**4.2.1-4.2.6**) were always obtained as the predominant products with 3-amide/8-amide ratio exceeding 20:1.

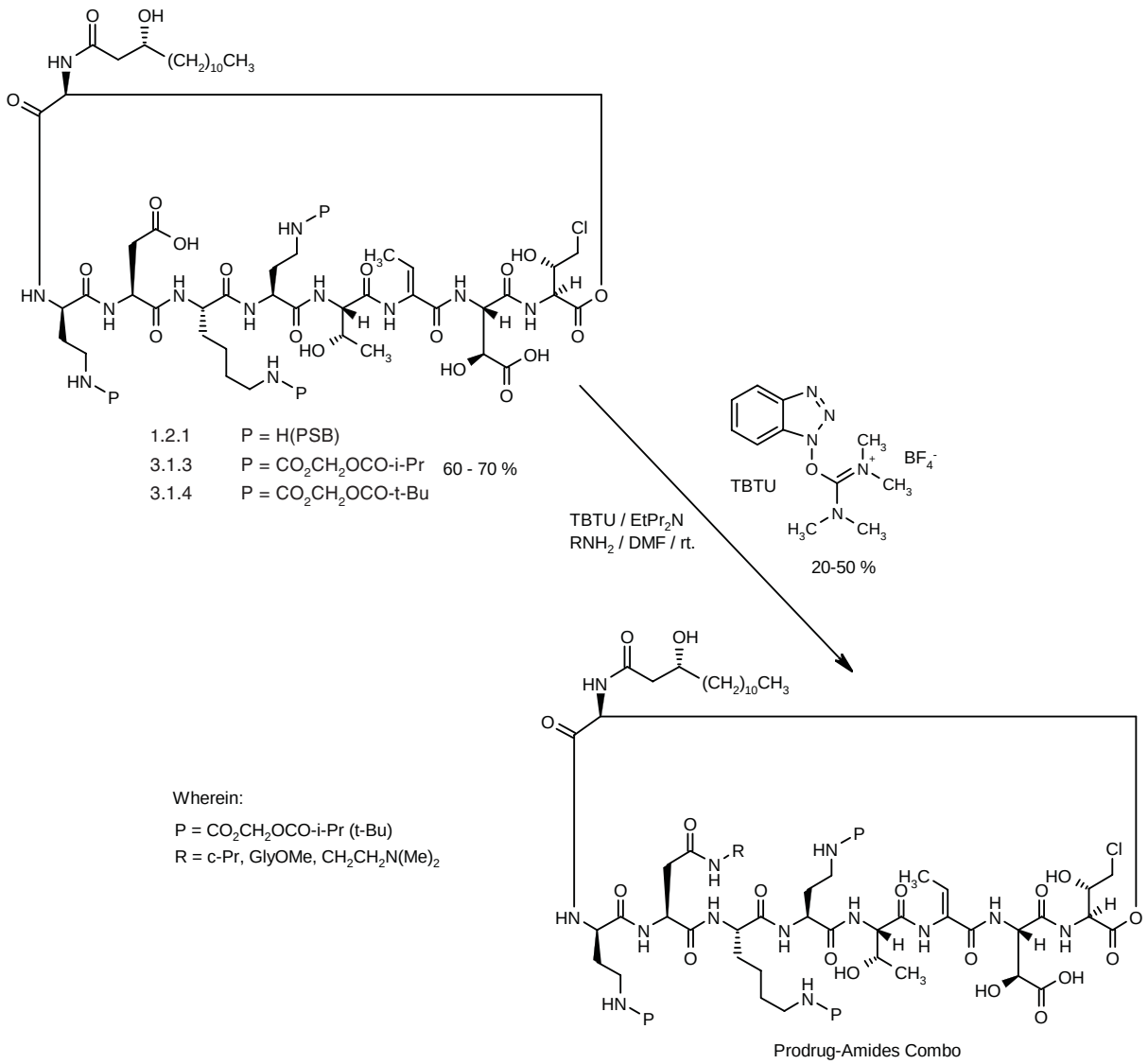
Biological evaluation

The *in vivo* efficacy and toxicity profiles of 3-cyclopropylamide **2.3.5**, 3-dimethylaminoethylamide **2.3.7**, 3-GlyOMe **2.3.11** and their corresponding *N*-acylated prodrugs (**4.2.1-4.2.6**) are summarized in Table VIII. As predicted, all combinations tested displayed relatively poor *in vitro* potencies (data not shown). When evaluated *in vivo*, all six combinations (**4.2.1-4.2.6**) exhibited excellent efficacy against murine candidiasis with ED_{50} values ranging between < 5.0-6.6 mg/kg. Three such combinations (**4.2.1**, **4.2.4** and **4.2.6**) were further tested in the murine cryptococcosis model. As shown in Table VIII, the *in vivo* activity demonstrated by **4.2.1** and **4.2.4** was 5-fold greater than that achieved by their corresponding 3-amides **2.3.5** and **2.3.7**. In view of the exciting *in vivo* activity observed with these combinations, we selected four of them (**4.2.1**, **4.2.3**, **4.2.5** and **4.2.6**) for further evaluation in the dose elevation study in mice (i.v.) at doses of 50, 75, 100 and 125 mg/kg. The test results showed that all four combinations were well tolerated at the highest dose tested (125 mpk). It was evident that the newly synthesized prodrug-amide combinations exhibited even greater safety profiles than their corresponding 3-amides (MTD ≤ 50 mg/kg).

Conclusions

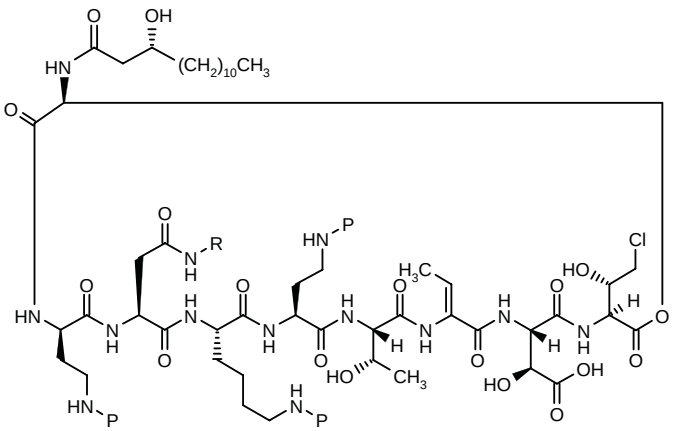
Due to the growing population of immunocompromised patients, SFI are becoming a major medical concern. Statistically, *Candida albicans*, *Cryptococcus*

Scheme 3: General Synthetic Route for Prodrug-Amide Combinations



neoformans and *Aspergillus fumigatus* account for more than 90% of SFI. The emergence of fungal pathogens resistant to current therapies further compounds the need for effective antifungal agents. Currently available antifungal drugs for the treatment of SFI are limited. Furthermore, the utility of these drugs is restricted either by severe host toxicity or by lack of broad-spectrum activity against all three major fungi mentioned above. Because of the urgent need for the development of safe and effective drugs for the treatment of SFI, we became interested in the SAR modifications of pseudomycins, a novel class of lipopeptides with significant *in vitro* and *in vivo* activity against *C. albicans* and *C. neoformans*. Unfortunately, despite its promising antifungal activity, the development of pseudomycin B as a therapeutic agent was rapidly discontinued due mainly to the irritation potential found at the injection site and long-term, end-organ toxicity. In the search for novel pseudomycin derivatives with overall desirable efficacy and safety profiles suitable for clinical use, we designed, synthesized and evaluated several series of pseudomycin analogues and *N*-acylated prodrugs.

Although side-chain modifications did not yield analogues possessing significantly improved safety profiles, core modifications such as 3-amidation provided many analogues that were endowed with excellent *in vitro* potency and *in vivo* efficacy against candiadiasis and cryptococcosis, without tail vein irritation being observed



3-Amides

Wherein R = GlyOMe, *c*-Pr, $CH_2CH_2NMe_2$

and P = CO_2CH_2OCO-*i*-Pr (*t*-Bu)

Table VIII: Efficacy and toxicity profiles of N-acylated prodrugs of 3-amides.

Comp.	Prodrug Linker	R=	Candidiasis ED_{50} (mpk)	Cryptococosis ED_{50} (mpk)	TailVein Toxicity	Max. safe dose Mice (mpk)
4.2.1	*i*-Pr	*c*-Pr	<5.0	0.56	-	125
4.2.2	*t*-Bu	*c*-Pr	<5.0	N.T.	-	N.T.
2.3.5	—	*c*-Pr	<5.0	2.9	-	50
4.2.3	*i*-Pr	$CH_2CH_2NMe_2$	6.6	N.T.	-	125
4.2.4	*t*-Bu	$CH_2CH_2NMe_2$	<5.0	0.44	-	N.T.
2.3.7	—	$CH_2CH_2NMe_2$	<5.0	2.5	-	50
4.2.5	*i*-Pr	GlyOMe	<5.0	N.T.	-	125
4.2.6	*t*-Bu	GlyOMe	<5.0	>5.0	-	125
2.3.11	—	GlyOMe	<5.0	2.9	-	<50
PSB 1.2.1	—	—	2.8 - 8.4	1.4 - 1.8	+	20

(*e.g.*, 3-cyclopropyl amide **2.3.5** and *N,N*-dimethylethyl amide **2.3.7**). In contrast to PSB, when evaluated in the dose elevation experiment in mice (at 75 mpk) and 2-week rat toxicology study (at 75 mpk), **2.3.5** treated animals were found to be normal.

Parallel with novel analogue design, we also investigated two types of *N*-acylated prodrugs, namely *N*-acyloxymethyl and *N*-oxodioxlenylmethyl carbamate prodrugs of PSB and PSC'. Most of the prodrugs exhibited comparable *in vivo* efficacy to that achieved by their parent drugs, without inherent tail vein irritation being detected (*e.g.*, **3.1.3**, **3.1.4**, **3.1.5**, **3.2.1** and **3.2.2**). Two such prodrugs (**3.1.4** and **3.2.2**) were further evaluated in the mice dose elevation study and 2-week rat toxicology study (dosed at 75 mpk), the results generated from these investigations showed that both prodrugs were well tolerated. No tail vein irritation or long-term toxicity was observed in these studies.

Lastly, to take advantage of the success achieved with 3-amidation and *N*-acylated prodrug approaches, we synthesized and evaluated *N*-acylated prodrugs of 3-amides (termed combination). As expected, all of the combinations demonstrated excellent *in vivo* efficacy against candidiasis. Furthermore, many such combinations exhibited even greater safety profiles than their corresponding 3-amides or *N*-acylated prodrugs.

Thus, based on the data presented in this chapter, we are convinced that it is possible to prepare safe and effective pseudomycin derivatives as useful therapeutic agents for the treatment of SFI via prodruging and analoguing approaches either alone or in combination.

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Special thanks to X. Sun, Y-Z. Zhang and M. Zweifel for the syntheses of various novel pseudomycin derivatives, R. Boyer and J. Paschal for structure determinations, and D. Zeckner and R. Sachs for *in vitro* and *in vivo* evaluations of pseudomycin analogues and prodrugs. In addition, we would like to thank N. Yumibe, C. McMillian and V. Vasudevan for ADME support, and D. Laska, J. Gidda and T. Jones for toxicology evaluation. We are also indebted to J. Munroe, B. Laguzza and J. McDonald for their advice, support and encouragement.

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88. We found that both low temperature and other bulky amines such as dicyclohexylethylamine favored the formation of 3-amido pseudomycin analogs.

89. An upfield shift (approx. 0.10-0.15 ppm) of 3beta and 3beta' protons relative to PSB was observed with the 3-amide **2.3.7** (Table V). In the case of the 8-amide **2.2.9**, a similar upfield shift (approx. 0.11 ppm) relative to PSB was observed for 8beta proton (Table IV).

90. General protocol for in vitro assay. All pseudomycin analogs and two positive controls, pseudomycin B and C' were screened against the following three major fungi responsible for systemic fungal infections: *Candida albicans, Cryptococcus neoformans, Aspergillus fumigatus.* MIC value was defined as the lowest drug concentration required to inhibit 90-100% of visible growth compared to controls.

91. General procedure for evaluation of pseudomycin analogs in a disseminated candidiasis mouse model. Mice were infected by an intravenous (IV) injection of 0.1 ml (containing 2 x 10^6 blastoconidia per mouse) in the lateral tail vein. Untreated controls were moribund within 3-4 days postinfection. Mice were dosed four times at 0, 4, 24 and 48 h postinfection with 0.2 ml of test compounds which were given at 20, 10 and 5 mg/kg. Compounds were formulated in 4.0% hydroxypropyl cyclodextrin and sodium acetate, pH 7.0 buffer and 1.75% dextrose. Infected sham-treated mice (10 animals) were dosed with vehicle alone. Morbidity and mortality were recorded for 7 days. The 50% effective doses (ED_{50}) were determined using the method of Reed and Muench. Statistical differences in treated groups compared to untreated infection controls were determined using the Student's *t* test.

92. General procedure for performing tail vein toxicity assay. Mice were treated i.v. through the lateral tail vein with 0.1 ml of test compounds (20 mg/kg) at 0, 24, 48 and 72 h. Two mice were included in each group. Compounds were formulated in 5.0% dextrose and sterile water for injection. Mice were monitored for 7 days following first treatment. Mice were observed closely for signs of irritation including erythema, swelling, discoloration, necrosis and tail loss, *etc*. Mice were also observed for any other signs of adverse effects indicating toxicity.

93. General procedure for dose elevation toxicity (histamine release induced) evaluation in mice. Mice were treated with a single intravenous (IV) injection of 0.1 ml of the test compounds at 75, 50 and 25 mg/kg. Two mice were included in each group. Compounds were formulated in 5.0% dextrose and sterile water for injection. Following dosing, mice were observed closely for clinical signs of histamine-induced pathology. These signs include dyspnea, agitation, convulsions and death. Mice were observed for 7 days.

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