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ANTIFUNGAL LIPOPEPTIDES: STRUCTURE-ACTIVITY RELATIONSHIPS OF 3-HYDROXYGLUTAMINE-MODIFIED PNEUMOCANDIN B_0 DERIVATIVES

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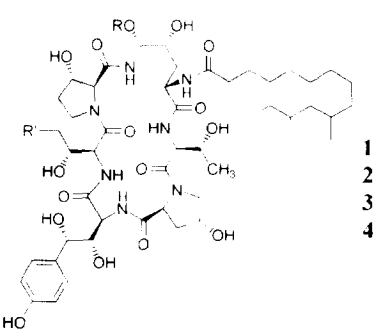
Abstract: Selective methanolysis or dehydration followed by reduction of the 3-hydroxyglutamine residue of pneumocandin B_0 **(1)** or its dideoxy analog **5** (L-692,289) gave the methyl 3-hydroxyglutamate and 3-hydroxy-ornithine analogs **6** and **9**, respectively. Further derivatization of these analogs allowed a study of the SAR at this position. In general, carboxylic acid-containing derivatives were poorer antifungal agents than neutral derivatives while amine-bearing analogs displayed the greatest potency.

Introduction

The incidence of serious fungal infection has steadily grown over the last two decades despite the introduction of a number of new agents. Immunosuppression from AIDS, anticancer therapy, the use of broad spectrum antibiotics and chemotherapy in organ transplantation accounts for this growing trend.[1] The majority of life-threatening fungal infections are caused by opportunistic pathogens such as *Candida* spp., *Aspergillus* spp., *Pneumocystis carinii* and *Cryptococcus neoformans*.[2] Currently available antifungal agents suffer drawbacks due to toxicity, static rather than cidal activity or inadequate spectrum. In addition, in some cases the selection of resistant organisms has been seen as the usage of these agents has increased.[3] Therefore, there is a considerable need for the development of new antifungal agents with improved properties.



	OR	R'
1 Pneumocandin B_0	OH	$CONH_2$
2 L-705,589	$O(CH_2)_2NH_2$	$CONH_2$
3 L-731,373	OH	CH_2NH_2
4 L-733,560	$O(CH_2)_2NH_2$	CH_2NH_2

The pneumocandins belong to a class of closely related fungicidal lipopeptides isolated from the fungus *Zalerion arboricola*.[4] Like the structurally-related echinocandins, these compounds inhibit the synthesis of β-1,3-glucan, an essential component of the fungal cell wall that is absent in mammalian cells. Thus, the inhibition of β-1,3-glucan synthesis represents a fungal-specific, potentially non-toxic target. Pneumocandin B_0 **(1)**, a cyclic hexapeptide possessing a 10,12-dimethylmyristoyl side chain, has provided an important platform for the synthesis of potent fungicidal derivatives. Recently, Bouffard, *et al.* have described several cationic derivatives of **1**.[5] L-705,589 **(2)**, L-731,373 **(3)**, and L-733,560 **(4)** are potent inhibitors of β-1,3-glucan synthase with excellent *in vitro* activity and efficacy in rodent models of disseminated candidiasis, aspergillosis and *P. carinii* pneumonia.[6] Compounds **3** and **4** possess a modified 3-hydroxyglutamine residue (gln→orn). In this report, we wish to expand on the structure-activity relationships at the 3-hydroxyglutamine (3-OH *gln*) position.

Biological Assays

The β-1,3-glucan synthase inhibition assay was conducted using a crude membrane system derived from *C. albicans* (MY 1208) as previously described.[7] An IC_{50} (μM) was determined and refers to the concentration of drug required to inhibit the production of 50% of the insoluble glucan compared to the control.

Fungicidal activity was determined against a panel of *Candida* spp., and *Cryptococcus neoformans* (in duplicate).[6a] The MFC or minimum fungicidal concentration is defined as the concentration of drug (μg/mL) that inhibits regrowth of the organism. Compounds showed weak to no activity (32 - >128 μg/mL) against *C. neoformans*. Data are presented for *C. albicans* and the inherently more resistant *C. parapsilosis*.

The *in vivo* anti-*Candida* activity was determined in a mouse model of disseminated candidiasis (TOKA).[8] Mice (n=5) were infected I.V. with a 50% lethal dose of *C. albicans* (MY 1055) and dosed I.P. BID for 4 days with drug. On day 7 post-infection, the kidney burden was quantitated and an effective dose (mg/kg/dose) for at least 99.9% reduction in colony forming units (CFUs) as compared to control animals was determined ($ED_{99.9}$).

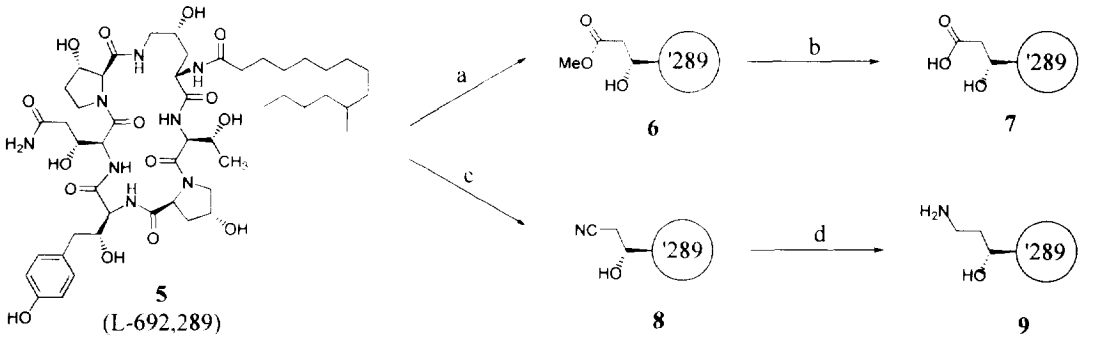
Chemistry

The 3-OH *gln* residue was envisioned to undergo selective hydrolysis to a 3-OH *glu* or selective reduction to a 3-OH *orn*. Since **1** is unstable at low and high pH,[9] we first investigated the chemistry of the stable dideoxy-analog, L-692,289 **(5)**.[10] Selective hydrolysis was accomplished by acid-catalyzed methanolysis to give **6**[11] followed by basic hydrolysis of the methyl ester to give **7**. The selective dehydration of the primary amide of **5** afforded nitrile **8** which was reduced to the 3-OH *orn* analog **9** using in situ-generated cobalt boride

and sodium borohydride in methanol[12] (Scheme 1). With these key intermediates available, the preparation of compounds **10-16** could be accomplished (see Table 1).

The hydroxamic acid **10** and hydrazide **11** were prepared by treatment of ester **6** with either hydroxylamine hydrochloride and aqueous sodium hydroxide in methanol or hydrazine in methanol in 35% and 78% yields, respectively. Carboxylic acid **7** was obtained as a by-product in the formation of **10** in 20% yield. The reduction of ester **6** to the carbinol **12** was accomplished with 4 molar equivalents of $LiBH_4$ in isopropanol in 20% yield. The relatively lipophilic thioamide **13** was obtained from nitrile **8** by treatment with hydrogen sulfide gas in a mixture of diethylamine/DMF (1:3) at 60 °C in 35% yield. Amides **14** and **15** were prepared from acid **7** and the corresponding amine employing 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride and 1-hydroxybenzotriazole in DMF in 44% and 69% yields, respectively. Hydrolysis of methyl ester **15** gave the carboxylic acid **16**. The cationic products were isolated as their TFA salts.

Scheme 1. Selective Hydrolysis or Reduction of the 3-Hydroxyglutamine Residue of **5**



(a) CSA, MeOH, 45 °C (20%); (b) 2N NaOH (aq), MeOH (66%); (c) cyanuric chloride, DMF, (65%); (d) $CoCl_2 \bullet 6H_2O$, $NaBH_4$, MeOH (50%)

Attempted methanolysis of **1** was unsuccessful leading to solvolysis of the C-5 ornithine and C-4 homotyrosine hydroxyl groups. The selective dehydration of the glutamine residue could be accomplished to give nitrile **17** (see Table 2) by carefully controlling the cyanuric chloride stoichiometry, reaction time and temperature as previously described.[5] The crude product was reduced with cobalt (II) chloride and sodium borohydride in methanol to give an overall 44% yield of the primary amine **3**. Compound **3** was acylated with acetic anhydride and diisopropylethylamine in DMF to give **18** in 85% yield. Alkylation of **3** with excess bromoacetonitrile gave the dialkylated adduct **19** in 44% yield with none of the quaternary analog detected. Synthesis of the methylamino analog **20** first required reductive alkylation to the N-benzyl adduct (Structure B, R = $-CH_2NHCH_2C_6H_5$) using benzaldehyde and sodium cyanoborohydride in DMF containing 1% acetic acid (49% yield). Next, methylation with 37% formaldehyde and sodium cyanoborohydride in aqueous acetonitrile gave the N-methyl-N-benzyl adduct in 72% yield. Hydrogenolysis of the benzyl group under 1 atm of H_2 with

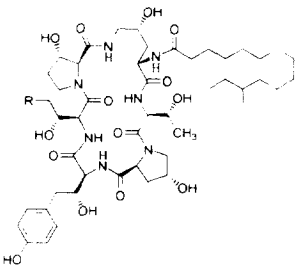
10% Pd-C as catalyst gave **20** in 84% yield. The N,N-dimethyl adduct **21** was obtained by treatment of **3** with 37% formaldehyde and sodium cyanoborohydride in aqueous acetonitrile. The quaternary ammonium analog **22** was obtained by treatment of **21** with excess MeI in DMF. The guanidine analog **23** was prepared from **3** by treatment with formamidinesulfonic acid[13] in the presence of Hunig's base in 46% yield. Satisfactory 400 MHz ^{1}H-NMR spectra (CD_3OD) and FAB-MS were obtained for all compounds. Final compounds were purified by preparative reverse phase HPLC (C8 or C18 ZORBAX, acetonitrile-water-0.1% TFA) and were >92% pure by analytical HPLC (λ=210 nm).

Results

The *in vitro* and *in vivo* anti-*Candida* activities of pneumocandin B_0 **(1)** and its dideoxy-analog **5** are quite similar[7] allowing a valid comparison between derivatives of either of these compounds. Indeed, nitrile analogs **8** and **17** and amine analogs **9** and **3** also display similar activities (see Tables 1 and 2). Thus, the SAR from series A can be assumed to parallel that from series B.

The β-1,3-glucan synthase enzyme assay is a crude membrane preparation where the cell wall has been digested and the disrupted plasma membrane and its components have been separated by centrifugation. Thus, it is not a pure enzyme and contains lipids and other materials that may influence the "activity" of a compound based on the compound's physicochemical properties. With this in mind, several general structure-activity relationships were apparent from the enzyme inhibition data. Neutral groups at the 3-OH *gln* position, whether polar (**1**, **5**, **10**, **11** and **12**) or lipophilic (**6, 8, 13, 15** and **17**), possessed similar activity. Compounds possessing a carboxylic acid substituent (**7** and **16**) were poorer inhibitors than the neutral analogs. With the amine analogs, a substantial increase in potency was noted that roughly correlated with the basicity of the amine. The basic analogs **3**, **9**, **14**, **20, 21, 22** and **23** had significantly lower IC_{50}s than **1** or **5** but the non-basic amine analog **19** was substantially less active especially when compared to **21**. Alkyl substitution of the amine had little influence on enzyme activity (**3**, **20**, **21** and **22**). The acetamide derivative **18** was a fourfold poorer inhibitor than **1** suggesting that a carbonyl group is unfavorable in this position. Nonetheless, the isosteric and basic guanidine analog **23** showed a tenfold increase in activity relative to **1** and at least a 28-fold increase compared to **18**, highlighting the positive influence of a basic substituent at that position.

The *in vitro* fungicidal activity (MFC) of the compounds against two different *Candida* species is shown in Tables 1 and 2. The *C. albicans* (MY 1055) is a clinical isolate and is the organism used in the *in vivo* TOKA model. The *C. parapsilosis* (MY 1010) is a species that is inherently more resistant to the lipopeptides. Although the MFCs did not correlate completely with glucan synthase inhibition, several of the amine analogs (**3, 22** and **23**) displayed potent activity against both *Candida* species. The monomethyl and dimethylamino analogs **20** and **21** were less potent against the whole organism even though they were potent enzyme inhibitors.



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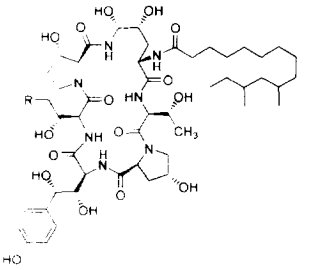

B

Table 1. Biological Data for Dideoxy-Pneumocandin B_0 Analogs (Structure A)

(Structure A) R		Glucan Synthase IC_{50} (μM)	*In Vitro* MFC (μg/mL) *C. albicans* (MY 1055)	*In Vitro* MFC (μg/mL) *C. parap.* (MY 1010)	*In Vivo* TOKA $ED_{99.9}$ (mg/kg)
(5)	$-CONH_2$	0.07	0.25	2	>6 (2.93)[a]
(6)	$-CO_2Me$	0.18	0.5	4	>6 (0)[a]
(7)	$-CO_2H$	0.4	0.25	8	>6 (0)[a]
(8)	-CN	0.1	1	4	---
(9)	$-CH_2NH_2$	0.01	0.125	---	1.5
(10)	-CONHOH	0.08	0.25	4	6
(11)	$-CONHNH_2$	0.11	4	8	---
(12)	$-CH_2OH$	0.2	1	8	---
(13)	$-CSNH_2$	0.12	0.25	4	>6 (0)[a]
(14)	$-CONH(CH_2)_6NH_2$	0.038	0.5	---	>6 (1.6)[a]
(15)	$-CONH(CH_2)_5CO_2Me$	0.25	4	>128	---
(16)	$-CONH(CH_2)_5CO_2H$	0.9	2	64	---

[a]log reduction in CFUs at indicated dose

Table 2. Biological Data for Pneumocandin B_0 Analogs (Structure B)

(Structure B) R		Glucan Synthase IC_{50} (μM)	*In Vitro* MFC (μg/mL) *C. albicans* (MY 1055)	*In Vitro* MFC (μg/mL) *C. parap.* (MY 1010)	*In Vivo* TOKA $ED_{99.9}$ (mg/kg)
(1)	$-CONH_2$	0.07	0.25	1	6
(3)	$-CH_2NH_2$	0.01	<0.06	0.5	0.375
(17)	-CN	0.1	2	2	---
(18)	$-CH_2NHAc$	0.3	4	8	12
(19)	$-CH_2N(CH_2CN)_2$	>0.2	4	8	>1.5 (0.94)[a]
(20)	$-CH_2NHMe$	0.007	2	2	0.375
(21)	$-CH_2NMe_2$	0.005	1	2	1.5
(22)	$-CH_2NMe_3^+$	0.009	0.125	0.5	0.375
(23)	$-CH_2NHC(=NH)NH_2$	0.007	<0.06	0.5	1.5

[a]log reduction in CFUs at indicated dose

The *in vivo* activity correlated well with the glucan synthase assay. The 3-OH *orn* analog of pneumocandin B_0 **3** was fourfold more potent than the corresponding dideoxy-analog **9**. Compound **3** and its trimethylammonium derivative **22** were the most potent compounds tested. Similar to the MFC assay, the monomethyl and dimethyl analogs were approximately two- to fourfold less potent.

In summary, cationic substituents at the 3-OH *gln* position of the pneumocandins significantly increased the enzyme, whole cell activity and *in vivo* potency of this class of compounds. Anionic groups, such as carboxylate, decreased the activity of analogs while neutral groups generally had little effect on activity.

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11. Preparation of **6**: p-Toluenesulfonic acid monohydrate (0.25 g, 1.3 mmol) was added to a solution of **5** (1.0 g, 0.97 mmol) in 40 mLs of methanol. The reaction vessel was heated to 49 °C and sealed. After stirring at 45-49 °C for 120 h, HPLC analysis showed a ratio of 1.6:1 for **6**:**5**. The mixture was concentrated *in vacuo* and purified by reverse phase HPLC (22.5 x 500 mm C8 ZORBAX, 57% acetonitrile in water). The appropriate fractions were lyophilized to give 200 mg (20%) of **6** as a white powder of 97% purity (λ=210 nm).
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