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LIPOPEPTIDE ANTIFUNGAL AGENTS: AMINE CONJUGATES OF THE SEMI-SYNTHETIC PNEUMOCANDINS L-731,373 AND L-733,560

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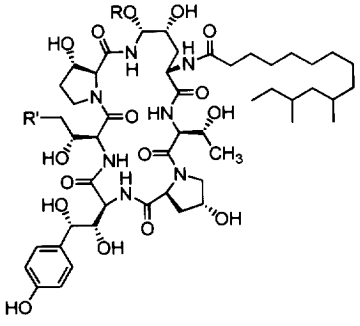
Abstract: Amine conjugates of the semi-synthetic 1,3-β-(D)-glucan synthesis inhibitors L-731,373 (**3**) and L-733,560 (**4**) were prepared and evaluated for in vitro and in vivo antifungal activity. Tricationic analogs were more potent than the dicationic which were more potent than the monocationic. The L-ornithine conjugate of **4** possessed excellent pharmacokinetic parameters but lacked sufficient antifungal spectrum for development.

Introduction

Due to an increasing number of mycotic infections and more frequent accounts of resistance there has been considerable interest in developing new antifungal agents with novel modes of action.[1] Our group has previously described potent cationic derivatives of the fungicidal lipopeptide, pneumocandin B_0 (**1**). L-705,589 (**2**), L-731,373 (**3**), and L-733,560 (**4**) are nanomolar inhibitors of 1,3-β-(D)-glucan synthesis with excellent in vitro activity and efficacy in rodent models of disseminated candidiasis and *P. carinii* pneumonia and, for **2** and **4**, excellent activity in mouse models of disseminated aspergillosis.[2,3] Compounds **3** and **4** possess a modified 3-hydroxyglutamine residue (3-OH *gln*). Further modifications of the carboxamide of the 3-OH *gln* revealed that charge type and lipophilicity at this position could greatly influence antifungal activity. Anionic groups such as carboxylate reduced the activity, small neutral substituents had little effect while cationic groups greatly increased potency.[4] In this report, we wish to describe amine conjugates at the reduced 3-OH *gln* position of compounds **3** and **4**.[5]



	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

Biological Assays

The biological assays have been described in detail elsewhere. Briefly, the 1,3-β-(D)-glucan synthase inhibition assay (GS)[6] employed a microsomal membrane preparation derived from *C. albicans* (MY 1208). An IC_{50} (nM) was determined (in duplicate) and refers to the concentration of drug required to inhibit the production of 50% of insoluble glucan compared to the controls.

Fungicidal activity was determined against a panel of clinical yeast isolates (in duplicate).[7] The MFC or minimum fungicidal concentration was defined as the concentration of drug (μg/mL) that inhibited regrowth of the organism.

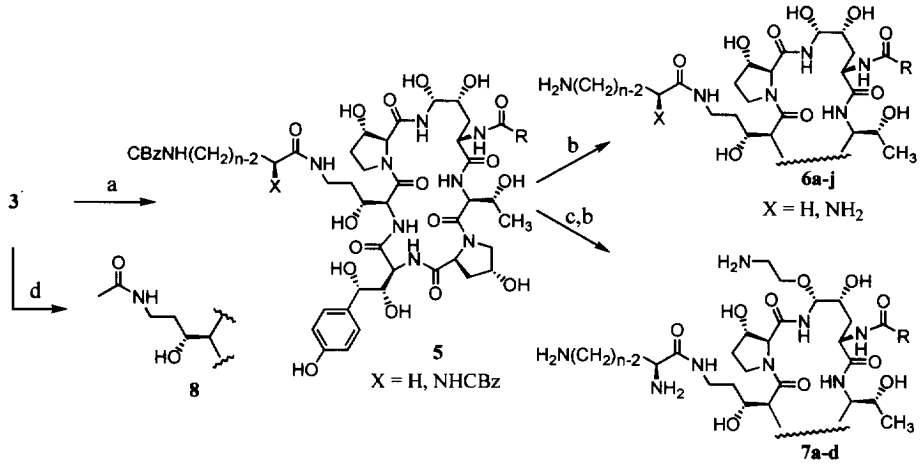
The in vivo anti-*Candida* activity was determined in a mouse model of disseminated candidiasis (TOKA).[8] Mice (n = 5) were infected i.v. with a 50% lethal dose of *C. albicans* (MY 1055) and dosed i.p., twice daily for 4 days with drug (fourfold serial dilutions). On day 7 post-infection, the kidney 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 ($ED_{99.9}$). The percentage of animals with no detectable *C. albicans* infection (% sterilization) was also recorded.

The in vivo anti-*Aspergillus* activity was determined in a mouse model of disseminated aspergillosis.[9] Mice (n = 10) were infected i.v. with a lethal dose of *A. fumigatus* (MF 5668) then dosed i.p., twice daily for 5 days with drug. Survival was followed out to study day 28 and an effective dose (mg/kg/dose) for 50% survival was calculated (ED_{50}).

Chemistry

The conversion of pneumocandin B_0 to **3** has previously been described.[2] The hydrochloride salt of **3** was used to synthesize amine conjugates of **3** and **4** as shown in Scheme 1. Compound **3** was N-acylated with an N-carbobenzyloxy (CBz)-protected aminoacid utilizing 1-hydroxybenzotriazole and 1-(3-dimethylamino-propyl)-3-ethylcarbodiimide hydrochloride in DMF to give 54–77% yields of the desired products **5**. Hydrogenolysis of the CBz group(s) under 1 atmosphere of hydrogen and 10% Pd-C in methanol gave the desired adducts **6a–j** in 20–73% yield. Derivative **8** was prepared in 85% yield by treating **3** with acetic anhydride. For the synthesis of **7a–d**, the aminoethyl ether was introduced by reacting **5** with ethanolamine hydrochloride in the presence of HCl/dioxane in dry DMSO for 4–7 days. The epimer at the C5-orn position was obtained as a minor product. The desired products were obtained in 23–50% yields. Subsequent hydrogenolysis gave the final adducts in 33–58% yields. All compounds were purified by preparative HPLC (acetonitrile/water/0.1% TFA, C18 DeltaPak, 220 nm) to >92% purity by analytical HPLC (acetonitrile/water/0.1% TFA, C18 ZORBAX, 210 nm). Satisfactory 400 MHz ^{1}H NMR and mass spectroscopic (FAB) data were obtained for all compounds.[10]

Scheme 1. Generalized scheme for the synthesis of amine conjugates **6a–j** and **7a–d** and acetamide **8**



(a) $HO_2CCH(X)(CH_2)_{n-2}NHCBz$ (where X = H or CBzNH and n = 2–7), DIPEA, HOBt, EDC, DMF; (b) H_2, 10% Pd-C, MeOH; (c) $H_2N(CH_2)_2OH$, HCl, DMSO; (d) Ac_2O, DIPEA, DMAP, DMF

Results and Discussion

Table 1 summarizes the in vitro and in vivo anti-*Candida* activity of parent compounds **3** and **4** and their conjugated analogs **6a–j**, **7a–d** and **8**. Overall, there was a poor correlation of glucan synthesis IC_{50}, *C. albicans* MFC and TOKA activity, however, there were several notable observations. N-Acetylation of **3**, which abolishes the cationic charge, led to compound **8** which had substantially lower in vitro and in vivo activity compared to **3**. Introduction of a cationic amine into the analog (**6a**), returned potent in vitro and in vivo potency. In the monocationic series, as the tether length was increased (**6a–6f**), a trend toward diminished antifungal activity was seen. It is interesting to note that the GS IC_{50} for the five carbon tether analog (**6d**) was an anomaly. A "local minimum" of activity was observed in the di- and tricationic analogs as well. In the monocationic series, the in vivo TOKA activity steadily decreased as the chain became longer. In both the di- and tricationic series, analogs with a chain length of n = 3 were less potent than analogs with longer tethers. This may be attributable to the lower basicity of the terminal amine in these compounds. Analogs lacking the C5-*orn* aminoethyl ether substituent have previously been shown to have poor anti-*Aspergillus* activity. Both **3** and diaminopropanoyl conjugate **6g** demonstrate this lower activity when compared to **4** and **7a**, respectively. The ornithine and lysine conjugates **7c** and **7d** possessed excellent activity in the disseminated aspergillosis model. The acute toxicity in mice was substantially lower in the conjugates vis-à-vis their respective parent compounds. One compound (**7c**), the L-ornithine conjugate of **4**, was selected for further evaluation since it possessed potent in vitro and in vivo activity along with improved acute toxicity.

Table 1. Effect of tether length and charge on antifungal activity and mouse acute toxicity

Classification	Compound	n	GS IC_{50} (nM)	*C. albicans* MFC[a] (μg/mL)	TOKA $ED_{99.9}$ (mg/kg)[b]	TOKA %mice sterilized[c]	Aspergillosis ED_{50} (mg/kg)[d]	Acute Toxicity[e] (mg/kg)
Parent	**3**	NA	10	≤0.06	0.375	70%	>20	100
Uncharged	**8**	NA	300	2	12	0%	---	>250
Monocations	**6a**	2	15	0.5	0.375	20%	---	250
	6b	3	25	0.125	1.5	100%	---	250
	6c	4	50	≤0.06	1.5	40%	---	>100
X = H	**6d**	5	20	0.5	1.5	0%	---	250
	6e	6	90	0.5	>1.5	0%	---	250
	6f	7	---	2	>1.5	0%	---	200
Dications	**6g**	3	30	1	1.5	100%	>1.25	>250
	6h	4	60	0.25	0.375	60%	---	>250
X = NH_2	**6i**	5	3	≤0.06	0.375	20%	---	>200
	6j	6	10	≤0.06	0.375	20%	---	150
Parent	**4**	NA	1	0.125	0.09	80%	0.03	30
Trications	**7a**	3	4	1	0.375	100%	0.07	>100
	7b	4	6	0.5	0.09	0%	---	100
	7c	5	1.5	≤0.06	0.09	70%	0.03	80
	7d	6	3	0.125	0.09	0%	<0.02	60

[a]Minimum fungicidal concentration against MY 1055. [b]Minimum dose to achieve at least a 3 log reduction over sham treated controls. [c]Percentage of mice with complete eradication of *Candida* in kidneys. [d]Minimum i.p. dose to achieve at least 50% survival. [e]Minimum i.v. dose administered to three mice that resulted in at least one death within 24 h.

Evaluation of L-Ornithine Conjugate (7c)

Conjugate **7c** showed substantially improved acute toxicity compared to the parent compound L-733,560 (**4**) while retaining potent antifungal activity. To assess this analog as a potential drug development candidate, additional in vitro and in vivo studies were performed. MFC_{90}s were determined in RPMI liquid medium according to the NCCLS protocol (M-27T), against a panel of clinical yeast isolates from the Merck collection. The antifungal spectrum of **7c** and **4** compared to amphotericin B is shown in Table 2. The data

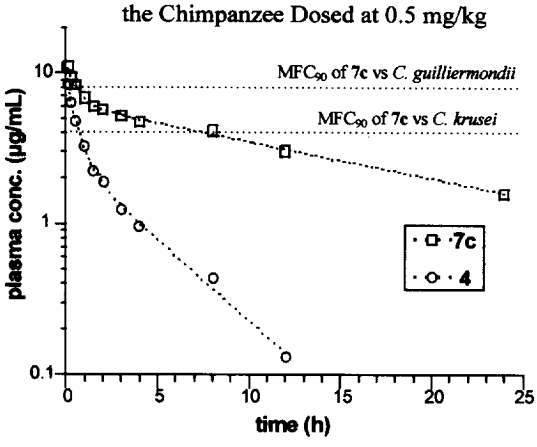
Table 2. Comparison of in vitro Range and MFC_{90} values (μg/mL) against a panel of clinical isolates[a]

Isolate	(# isolates)	Amphotericin B		4		7c	
		Range	MFC_{90}[b]	Range	MFC_{90}	Range	MFC_{90}
C. albicans	(40)	0.06-0.5	0.25	0.03-0.5	0.5	0.25-1	1
C. tropicalis	(20)	0.25-0.5	0.5	0.06-1	0.5	0.5-1	1
C. pseudotropicalis	(20)	0.125-0.5	0.5	0.06-0.5	0.5	0.25-1	1
C. parapsilosis	(20)	0.5-1	1	0.25-1	0.5	0.5-1	1
C. krusei	(20)	0.25-0.5	0.5	1-2	1	2-4	4
C. lusitaniae	(20)	0.5-2	2	0.06-0.5	0.5	0.125-1	0.5
C. glabrata	(20)	0.125-0.5	0.5	0.5-1	1	1-4	2
C. guilliermondii	(20)	0.125-0.5	0.5	0.5-4	4	1-16	8
C. neoformans	(20)	0.125-0.5	0.5	8-32	32	8-32	32

[a]Determined using NCCLS M-27T standard protocol. [b]Minimum fungicidal concentration effective against at least 90% of indicated clinical isolates

showed that **7c** had higher MFC_{90} values than either amphotericin B or **4** against all *Candida* species tested except *C. lusitaniae*. The relatively high range and MFC_{90} values against *C. krusei* and *C. guilliermondii* indicated that relatively high plasma levels of **7c** would be necessary to achieve the desired in vivo antifungal spectrum. The pharmacokinetic parameters of **7c** were determined in a chimpanzee which is an excellent model for human pharmacokinetics. A chimpanzee anesthetized with ketamine was administered an i.v. bolus of **7c** in water at a dose of 0.5 mg/kg. Plasma samples were taken out to 24 h post-injection and analyzed by HPLC to determine drug levels. A similar experiment was conducted in the same chimpanzee with **4** and the results are shown in Figure 1. Compound **7c** displayed excellent pharmacokinetics especially compared to **4**. The plasma clearance rate for **7c** was 0.074 mL/min/kg and for **4** was 0.58 mL/min/kg. The half-lives were 12.4 h and 2.8 h, respectively. The improved kinetics may be attributable to the presence of an additional cationic charge in **7c** versus **4**.

Figure 1. Pharmacokinetic Profiles of **7c** and **4** in the Chimpanzee Dosed at 0.5 mg/kg



During the course of our evaluation of other compounds of this class, we have noted a general correlation of drug plasma concentrations and efficacy. Attaining drug levels above the MFC for a particular pathogen for about two-thirds of the dosing interval gave full efficacy in our models. This analysis suggested that **7c** would be effective against most *Candida* spp., however, multiple daily dosing or doses significantly higher than 0.5 mg/kg would be required for efficacy against *C. krusei* and *C. guilliermondii* (see Figure 1). The weak activity against these organisms precluded the development of **7c** as a drug candidate.

In summary, potent amine conjugates of **4** at the reduced 3-hydroxyglutamine position were identified that possessed significantly improved acute toxicity. Tricationic analogs were more potent than dications which were more potent than monocations. The L-ornithine conjugate **7c** was further evaluated and found to possess excellent pharmacokinetic parameters in a chimpanzee compared to **4** but lacked sufficient in vitro potency against several *Candida* spp. to warrant further development.

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References and Notes

1. (a) Turner, W. W.; Rodriguez, M. J. *Curr. Pharmaceut. Des.* **1996**, *2*, 209. (b) Walsh, T. J.; Hiemenz, J. W.; Anaissie, E. *Infect. Dis. Clin. North Am.* **1996**, *10*, 365. (c) Graybill, J. R. *Clin. Infect. Dis.* **1996**, *22*, S166.

(d) Hiemenz, J. W.; Walsh, T. J. *Clin. Infect. Dis.* **1996**, *22*, S133. (e) Balkovec, J. M. *Expert Opin. Invest. Drugs* **1994**, *3*, 65.

2. Bouffard, F. A.; Zambias, R. A.; Dropinski, J. F.; Balkovec, J. M.; Hammond, M. L.; Abruzzo, G. K.; Bartizal, K. F.; Marrinan, J. A.; Kurtz, M. B.; McFadden, D. C.; Nollstadt, K. H.; Powles, M. A.; Schmatz, D. M. *J. Med. Chem.* **1994**, *37*, 222.
3. (a) Bartizal, K.; Scott, T.; Abruzzo, G. K.; Gill, C. J.; Pacholok, C.; Lynch, L.; Kropp, H. *Antimicrob. Agents Chemother.* **1995**, *39*, 1070. (b) Abruzzo, G. K.; Flattery, A. M.; Gill, C. J.; Kong, L.; Smith, J. G.; Krupa, D.; Pikounis, V. B.; Kropp, H.; Bartizal, K. *Antimicrob. Agents Chemother.* **1995**, *39*, 1077. (c) Schmatz, D.; Powles, M. A.; McFadden, D.; Nollstadt, K.; Bouffard, F. A.; Dropinski, J. F.; Liberator, P.; Andersen, J. *Antimicrob. Agents Chemother.* **1995**, *37*, 1320.
4. Zambias, R. A.; James, C.; Hammond, M. L.; Abruzzo, G. K.; Bartizal, K. F.; Nollstadt, K. H.; Douglas, C.; Marrinan, J.; Balkovec, J. M. *Bioorg. Med. Chem. Lett.* **1995**, *5*, 2357.
5. This work was presented in part: Balkovec, J. M.; Zambias, R. A.; James, C. A.; Nollstadt, K. H.; Douglas, C. M.; Marrinan, J. A.; Abruzzo, G. K. and Bartizal, K. F. "Antifungal Lipopeptides: SAR Of Novel Amino Acid Conjugates Of Pneumocandin B_0 Analogs L-731,373 and L-733,560" presented at the National ACS Meeting, Chicago, IL: Abstract No. MEDI 213, **August 23-28, 1995**.
6. Schmatz, D. M.; Abruzzo, G.; Powles, M. A.; McFadden, D. C.; Balkovec, J. M.; Black, R. M.; Nollstadt, K.; Bartizal, K. *J. Antibiot.* **1992**, *45*, 1886.
7. Bartizal, K.; Scott, T.; Abruzzo, G. K.; Gill, C. J.; Pacholok, C.; Lynch, L.; Kropp, H. *Antimicrob. Agents Chemother.* **1995**, *39*, 1070.
8. Bartizal, K.; Abruzzo, G.; Trainor, C.; Krupa, D.; Nollstadt, K.; Schmatz, D.; Hammond, M.; Balkovec, J.; Van Middlesworth, F. *Antimicrob. Agents Chemother.* **1992**, *36*, 1648.
9. Abruzzo, G. K.; Flattery, A. M.; Gill, C. J.; Kong, L.; Smith, J. G.; Krupa, D.; Pikounis, V. B.; Kropp, H.; Bartizal, K. *Antimicrob. Agents Chemother.* **1995**, *39*, 1077.
10. The preparation of **7c** is a representative experimental procedure: The hydrochloride salt of **3** (8.02 g, 7.64 mmol) and bis-carbobenzyloxy-L-ornithine (7.65 g, 19.1 mmol) were dissolved in 73 mL of dry, dimethylamine-free, *N,N*-dimethylformamide. Diisopropylethylamine (1.33 mL, 7.64 mmol), 1-hydroxybenzotriazole (2.58 g, 19.1 mmol) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (3.66 g, 19.1 mmol) were added. The reaction was stirred under a nitrogen atmosphere at room temperature for 18 h. The reaction mixture was purified directly in two equal batches by preparative HPLC (50 X 250 mm Deltapak C18, 60 mL/min, water/acetonitrile/0.1% TFA: step gradient 70/30 [until DMF has eluted] to 35/65 [as product begins to elute], 48 mL fractions, 220 and 277 nm) to give 7.54 g (69%) of **5** (n = 5, X = NHCBz) as a white amorphous solid after lyophilization.

    Compound **5** (n = 5, X = NHCBz) (7.54 g, 5.26 mmol) and ethanolamine hydrochloride (20.10 g, 210 mmol) were dissolved in 42 mL of anhydrous DMSO. 4M HCl in dioxane (1.3 mL, 5.2 mmol) was added and the reaction was stirred under a nitrogen atmosphere for 4 days. The mixture was diluted with 100 mL of 50% aqueous acetonitrile and purified by preparative HPLC (50 X 250 mm Deltapak C18, 60 mL/min, water/acetonitrile/0.1% TFA: step gradient 70/30 [until DMSO has eluted] to 50/50 [as product begins to elute], 48 mL fractions, 220 and 277 nm) to give 4.19 g (50%) of white amorphous solid after lyophilization. The product was a 2:1 mixture of aminoethyl ether epimers which was used in next reaction.

    The mixture of α- and β-epimers from the above reaction (4.19 g, 2.63 mmol) was dissolved in 80 mL of methanol. 10% Pd-C (2.51 g) was added and the vessel was flushed with nitrogen and then hydrogen gas. The mixture was stirred vigorously under one atmosphere of hydrogen for 18 h, filtered through celite and most of the methanol removed *in vacuo*. The solution was diluted with water and purified by preparative HPLC (50 X 250 mm Deltapak C18, 60 mL/min, water/acetonitrile/0.1% TFA: step gradient 75/25 to 70/30 [as product begins to elute], 48 mL fractions, 220 and 277 nm) to give 1.56 g (38%, >99% pure) of the desired compound **7c** and 0.80 g (20%, 97.9% pure) of the β-epimer.

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