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Syntheses and Antifungal Activities of Novel 3-Amido Bearing Pseudomycin Analogues

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Abstract—As a result of our core SAR effort, we discovered a large number of 3-amido pseudomycin B (PSB) analogues (e.g., **4e** LY448212 and **5b** LY448731) that retain good in vitro and in vivo (IP) activities against *Candida* and *Cryptococcus* without inherent tail vein irritation. Several dimethylamino termini bearing 3-amides (e.g., **5b**) also exhibited improved potency against *Aspergillus* in vitro. When evaluated in a two-week rat toxicology study, it was found that all animals receiving **4e** (up to 75 mg/kg) were found to be normal. On the basis of these observations, we are convinced that it is possible to broaden the antifungal spectrum and improve the safety profile of pseudomycin analogues at the same time.

Due to the growing population of immunocompromised patients, fungal infections are becoming a major medical concern.[1] In particular, those systemic fungal infections (SFIs), defined as infections involving deep viscera such as lung and liver, can cause serious life-threatening diseases even in healthy humans. Statistically, *Candida albicans*, *Cryptococcus neoformans* and *Aspergillus fumigatus* account for more than 90% of SFIs. The emergence of fungal pathogens resistant to current therapies further compounds the dearth of antifungal agents.[2] Currently available antifungal drugs for treatment of SFIs are limited to amphotericin B (AMB) and a few azole based compounds (e.g., fluconazole). Unfortunately, the utility of these drugs is further restricted either by severe host toxicity or by lack of broad spectrum of activity against all three major fungi mentioned above. In light of the urgent need for the development of safe and effective drugs for the treatment of SFIs, we became interested in the SAR modifications of pseudomycins, a novel class of lipodepsipeptides reported recently.[3] In a series of recent publications from this institution, we have disclosed biological and toxicity profiles of pseudomycin B.[4] In comparison with AMB, PSB **1** exhibited better activity against *C. albicans* and *C. neoformans* both in vitro and in vivo.[3c] In addition, we have also reported on preliminary findings of pseudomycin side-chain[5] and core SAR efforts including *N*-acylated pseudomycin prodrugs[6,7] and 8-amido pseudomycin analogues **3**.[8] Our results showed that several PSB prodrugs indeed exhibited good in vivo efficacy without inherent tail vein toxicity and long term end organ toxicity. Encouraged by these promising results, we decided to expand our core SAR effort by synthesizing novel 3-amido PSB analogues **4**, **5**, and **6** as shown in Figure 1. Our interest in preparing analogue **5** stemmed from the observations that certain vancomycin related glycopeptides bearing basic dialkylamino termini exhibited improved antibacterial activity.[9] In this communication, we wish to report the synthesis, antifungal activity and safety data of these 3-amido pseudomycin analogues (**4**–**6**).

The syntheses of 3-amido PSB analogues (**4**–**6**) were accomplished using our recently developed chemoselective reactions. After many unsuccessful attempts, we discovered that treatment of a DMF solution of ZPSB **2** (1 equiv) with 1.3 equiv of TBTU (*O*-benzotriazol-1-yl-*N,N,N′,N′*-tetramethyluronium tetrafluoroborate) in the presence of 6 equiv of $EtPr_2N$ afforded mainly the desired Cbz protected 3-amide intermediates, which were converted next, upon standard hydrogenation, to the final 3-amido bearing PSB analogues. Following this three-step sequence, we synthesized a total of 13 straight alkyl chain bearing amides **4a**–**m**, 10 basic termini and five amino acid containing analogues (**5** and **6**). The yields of the TBTU mediated 3-amidation reaction and

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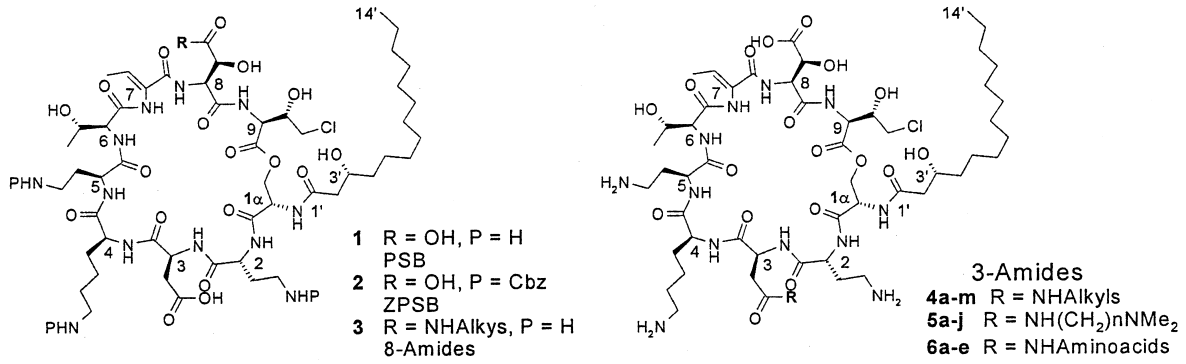

Figure 1. Structures of pseudomycin B analogues.

subsequent deprotection are listed in Tables 1 and 2. It should be mentioned that all of the 3-amides synthesized showed satisfactory mass spectra (see data below).

The structures of several newly synthesized 3-amides (**4**–**6**) were confirmed by detailed NMR analyses. All NMR spectra were obtained on a Bruker AMX550 spectrometer. The samples were dissolved in a 3:1 mixture of acetonitrile-d_3/deuterium oxide plus one drop of TFA-d_1. Careful inspection of the proton NMR spectra of the 3-amides (e.g., **5b**) indicated an upfield shift (~0.10–0.15 ppm) of the $3\beta_1$ and $3\beta_2$ protons for **5b** relative to the parent compound. The detailed proton assignments for **5b** (LY448731) and PSB are listed in Table 3. The structures of other 3-amido PSB analogues were assigned in a similar fashion.

All 3-amido pseudomycin B analogues were evaluated first in the following three assays: in vitro assay for MIC determinations; in vivo efficacy against murine systemic *Candidiasis* (ip) and in vivo tail vein irritation assay (iv). Analogues with good in vivo efficacy and clean results

Table 1. Yields and molecular weights of 3-amido pseudomycin B analogues **4a**–**m**

Compd	R	Coupling yield (%)	Deprotection yield (%)	Molecular formula	Molecular weight (salt free)
4a	H	5[a]	90	$C_{51}H_{88}ClN_{13}O_{18}$	1207
4b	Me	37	83	$C_{52}H_{90}ClN_{13}O_{18}$	1221
4c	Et	36	77	$C_{53}H_{92}ClN_{13}O_{18}$	1235
4d	*n*-Pr	52	44	$C_{54}H_{94}ClN_{13}O_{18}$	1249
4e	*c*-Pr	41	99	$C_{54}H_{92}ClN_{13}O_{18}$	1247
4f	*n*-Bu	[b]	[b]	$C_{55}H_{96}ClN_{13}O_{18}$	1263
4g	*n*-Amyl	50	100	$C_{56}H_{98}ClN_{13}O_{18}$	1277
4h	*i*-Amyl	22	98	$C_{56}H_{98}ClN_{13}O_{18}$	1277
4I	*n*-Hexyl	36	73	$C_{57}H_{100}ClN_{13}O_{18}$	1291
4j	*n*-Heptyl	35	92	$C_{58}H_{102}ClN_{13}O_{18}$	1305
4k	*n*-Octyl	32	42	$C_{59}H_{104}ClN_{13}O_{18}$	1319
4l	*n*-Nonanyl	36	84	$C_{60}H_{106}ClN_{13}O_{18}$	1333
4m	*n*-Decyl	35	84	$C_{61}H_{108}ClN_{13}O_{18}$	1347

[a]Compound **4a** was synthesized using Rink amide resin (purchased from Advanced ChemTech).
[b]Compound **4f** was provided initially by our external collaborators.

Table 2. Yields and molecular weights of 3-amido PSB analogues **5a**–**j** and **6a**–**e**

Compd	R	Coupling yield (%)	Deprotection yield (%)	Molecular formula	Molecular weight
5a	$NH(CH_2)_2NH_2$	47	27	$C_{53}H_{93}ClN_{14}O_{18}$	1250
5b	$NH(CH_2)_2NMe_2$	48	78	$C_{55}H_{97}ClN_{14}O_{18}$	1278
5c	$NH(CH_2)_2Net_2$	22	72	$C_{57}H_{101}ClN_{14}O_{18}$	1306
5d	$NH(CH_2)_3NMe_2$	24	95	$C_{56}H_{99}ClN_{14}O_{18}$	1292
5e	$NH(CH_2)_3Net_2$	41	93	$C_{58}H_{103}ClN_{14}O_{18}$	1320
5f	$NH(CH_2)_4NMe_2$	47	61	$C_{57}H_{101}ClN_{14}O_{18}$	1306
5g	$NH(CH_2)_6NMe_2$	57	94	$C_{59}H_{105}ClN_{14}O_{18}$	1334
5h	$NH(CH_2)_7NMe_2$	49	83	$C_{60}H_{107}ClN_{14}O_{18}$	1348
5I	NMe_2	27	77	$C_{53}H_{92}ClN_{13}O_{18}$	1235
5j	$NMe(CH_2)_2NMe_2$	63	85	$C_{56}H_{99}ClN_{14}O_{18}$	1292
6a	GlyOMe	—	—	$C_{54}H_{92}ClN_{13}O_{20}$	1277
6b	PheOMe	37	52	$C_{61}H_{98}ClN_{13}O_{20}$	1369
6c	HisOMe	25	80	$C_{58}H_{96}ClN_{15}O_{20}$	1359
6d	LysOMe	32	90	$C_{58}H_{96}ClN_{15}O_{20}$	1350
6e	ArgOMe	30	88	$C_{58}H_{101}ClN_{16}O_{20}$	1378

from the tail vein irritation assay were selected for further evaluation in the following testing: in vivo efficacy against murine systemic *Cryptococosis* (ip) and *Aspergillosis* (ip); dose elevation test in mice and two week long term toxicity in rats (iv).

Protocol for in vitro assay: all 3-amido analogues (**4**, **5**, and **6**) and PSB (positive control) were evaluated against the following three major fungi responsible for systemic fungal infections. These are *Candida albicans*, *Cryptococcus neoformans*, and *Aspergillus fumigatus*. The MIC values reported were defined as the lowest drug concentration required to inhibit 90–100% visible growth compared to controls.

Protocol for in vivo efficacy against murine *Candidiasis*: 3-Amides of interest along with PSB and amphotericin B (both used as positive control) were dosed four times at 0, 4, 24, and 48 post-infection with testing analogue given at 20, 10, and 5 mg/kg. The 50% effective doses (ED_{50}) were determined using the method of Reed and Muench.[10]

Protocol for tail vein irritation test: each testing compound was dosed four times to mice (Outbred, male ICR mice, ~18–20 g weight) at 0, 24, 48, and 72 h. Mice were observed closely for signs of irritation including erythema, swelling, discoloration, necrosis and tail loss.

Subsequently, a few selected 3-amides were also evaluated against murine *Cryptococosis* (analogue dosed with BID ×4 at the doses of 5.0, 1.25, 0.312, and 0.078 mg/kg) and *Aspergillosis* (analogue dosed with BID ×3 at 50, 10, and 1 mg/kg) in vivo.

Protocol for dose elevation study: mice were treated with a single injection (iv) of the testing compounds (dissolved in 5% dextrose and sterile water) at the dose of 50, 75, 100, and 125 mg/kg. Outbred, male ICR mice (~18–20 g, Harlan Sprague–Dawley, Indianapolis, IN) were used in this experiment. Two mice were included in each group. Following dose, mice were observed closely (for seven days) for clinical signs of histamine induced pathology, including dyspnea, agitation, convulsions and death.

After careful reviewing of the data shown in Tables 4 and 5, we made the following observations: In vitro Activity: (1) All straight alkyl chain bearing 3-amides **4a**–**m** exhibited excellent activity against *Cryptococcus* with MIC values ranging from < 0.01–5.0 μg/mL. They also displayed weak activities against *Aspergillus* with MIC values around 20 μg/mL. Their activity towards

Table 3. Proton NMR assignments for PSB and **5b** (in CD_3CN/D_2O/TFA-d_3)

Positions	PSB 1	448731	Positions	PSB 1	448731
Residue 1			Residue 5		
α	4.59	4.59	α	4.29	4.29
β1	4.38	4.42	β1	1.99	2.00
β2	4.53	4.50	β2	2.14	2.15
Residue 2			γ1	2.89	2.91
α	4.13	4.16	γ2	2.91	2.91
β1	2.01	2.01	Residue 6		
β2	2.07	2.07	α	4.27	4.27
γ1	2.90	2.91	β	3.92	3.92
γ2	2.97	2.98	γ	1.18	1.18
Residue 3			Residue 7		
α	4.55	4.56	β	6.53	6.53
β1	2.82	2.66	γ	1.70	1.71
β2	2.87	2.77			
1′α, 1′β	—	3.42/3.52			
2′	—	3.16			
4′,5′-Me	—	2.80/2.81			
Residue 4			Residue 8		
α	4.13	4.14	α	4.96	4.96
β1	1.75	1.73	β	4.75	4.75
β2	1.78	1.77	Residue 9		
γ1	1.26	1.27	α	4.87	4.87
γ2	1.32	1.33	β	4.31	4.32
δ1	1.53	1.56	γ1	3.44	3.46
δ2	1.56	1.56	γ2	3.50	3.51
ε	2.84	2.85			
Side chain			Side chain		
2′α	2.25	2.25	4′	1.38	1.39
2′β	2.36	2.38	5′-13′	~1.22	~1.23
3′	3.86	3.87	14′	0.83	0.83

Table 4. In vitro, in vivo and tail vein toxicity of 3-amides **4a**–**m**

Compd	R	MIC[a] (μg/mL)			Tail vein toxicity	ED_{50} (ED_{50}PSB)[b] (mg/kg×4, IP)
		C. albicans	*C. neoformans*	*A. fumigatus*		
PSB	—	0.625	0.01	>20	Yes	3.2–8.4
4a	H	2.5	< 0.01	10	Yes	< .8 (4.5)
4b	Me	5.0	0.312	>20	Pat. yes[c]	7.4 (4.5)
4c	Et	0.625	0.156	20	Not tested	< .0 (4.2)
4d	*n*-Pr	1.25	0.078	>20	No	6.0 (8.4)
4e	*c*-Pr	0.156	< 0.01	20	No	< 5.0 (7.2)
4f	*n*-Bu	5.0	< 0.01	20	No	5.9 (3.8)
4g	*n*-Amyl	0.312	< 0.01	>20	No	8.5 (6.6)
4h	*i*-Amyl	0.312	< 0.01	>20	Yes	10.9 (8.4)
4i	*n*-Hexyl	5.0	0.156	>20	No	>12 (3.2)
4j	*n*-Heptyl	20	0.312	>20	No	Toxic
4k	*n*-Octyl	20	0.625	>20	No	>20 (7.1)
4l	*n*-Nonanyl	20	5.0	>20	No	>20
4m	*n*-Decyl	>20	1.25	>20	No	>20 (7.1)

[a]MIC values were defined as the lowest drug concn required to inhibit 90–100% visible growth compared to controls.
[b]ED_{50}: drug concentration required to achieve 50% survival of fungal infection compared with untreated animals.
[c]Partial normal/partial yes meaning: one normal, one abnormal.

Candida varies according to the nature of alkyl groups. Generally speaking, better activities against *Candida* were obtained with analogues bearing smaller alkyl groups. (2) All of the *N*′,*N*′-diMe(Et)alkyl-3-amides **5** listed in Table 5 showed excellent activity against *Cryptococcus*. The activity of **5a**–**j** for *Candida* ranged from 0.16 to 20 μg/mL. Importantly, several such 3-amides (e.g., **5a**, **5b**, and **5f**) demonstrated improved potencies (~4-fold) against *Aspergillus* in comparison to the parent (see Table 5). (3) Five amino acid containing 3-amides **6a**–**e** showed modest to excellent activities against *Candida* and *Crypotococcus*, respectively. When tested against *Aspergillus*, these analogues exhibited similar potencies to that observed with the parent (see Table 5).

In vivo activity against *Candidiasis*: (1) Seven straight chain alkyl bearing 3-amides listed in Table 4 (**4b**–**h**) exhibited comparable in vivo activity against murine *Candidiasis*. It is also evident that further extension of alkyl chain beyond six carbons (e.g., **4j** and **4k**) was detrimental to in vivo efficacy. (2) In contrast to the trends observed with **4**, all nine basic termini bearing 3-amides (**5a**–**h** and **5j**), regardless of the length of the alkyl linker, demonstrated similar impressive in vivo efficacy against *Candidiasis* (ED_{50} values < 5.0 mg/kg). (3) Within the amino acid series, four out of five analogues (**6a** and **6c**–**e**) showed good efficacy against *Candidiasis* (ED_{50} values < 5.0 mg/kg). The bulky PheOMe bearing amide **6b** displayed poor activity. Comparison of the efficacy data obtained from **6b** and **6d** or **6e** seemed to indicate that better in vivo activities were associated with those analogues bearing basic termini.

Tail vein irritation: (1) With the exception of **4a**, **4b**, and **4h**, most of the straight chain 3-amides prepared were found to be clean in this assay (Table 4). (2) As shown in Table 5, only two analogues within the dialkylamino-termini amide series, **5a** and **5f**, were capable of inducing tail vein irritation. The remaining eight analogues passed this test. (3) It is encouraging to note that three out of five amino acids containing 3-amides (**6a**, **6d**, and **6e**) were proved to be clean in this experiment. The remaining two analogues (**6b** and **6c**) showed some improvement over the parent PSB with respect to tail vein irritation potential (see Table 5).

To summarize the data discussed so far, it is important to point out that no clear correlation existed between in vitro potency and in vivo efficacy. As shown in Table 5, compound **5b** (with excellent MIC against *Candida*) possessed equivalent in vivo efficacy to **5f** (with MIC value >20 μg/mL for *Candida*). It is also evident that many newly prepared 3-amides passed the primary in vivo efficacy screening without inherent tail vein toxicity. These include **4c**–**g**, **5b**–**d**, **5f**–**j**, **6a**, and **6d**–**f**.

Table 5. In vitro, in vivo (*Candidiasis*) and tail vein toxicity of 3-amides **5a**–**j** and **6a**–**e**

Compd	R	MIC[a] (μg/mL)			Tail vein toxicity	ED_{50} (ED_{50}PSB)[b] (mg/kg×4, IP)
		C. albicans	*C. neoformans*	*A. fumigatus*		
PSB	—	0.625	0.01	>20	Yes	3.2–9.0
5a	$(CH_2)_2NH_2$	10	<0.01	5.0	Yes	<5.0 (7.1)
5b	$(CH_2)_2NMe_2$	0.156	<0.01	5.0	No	<5.0 (4.5)
5c	$(CH_2)_2NEt_2$	10	<0.01	10	No	4.9 (3.5)
5d	$(CH_2)_3NMe_2$	10	<0.01	10	No	<5.0 (3.9)
5e	$(CH_2)_3NEt_2$	20	<0.01	>20	Yes	<4.0
5f	$(CH_2)_4NMe_2$	20	<0.01	5.0	No	<5.0 (7.1)
5g	$(CH_2)_6NMe_2$	5.0	<0.01	20	No	<5.0 (3.5)
5h	$(CH_2)_7NMe_2$	10	<0.01	>20	No	<4.0
5i	NMe_2	10	<0.01	10	No	<4.5 (5.4)
5j	$NMe(CH_2)_2NMe_2$	5.0	<0.01	20	No	4.0 (3.2)
6a	GlyOMe	1.0	0.01	20	No	<5.0 3.8)
6b	PheOMe	1.25	1.25	>20	Pat. yes[c]	>15 (9.0)
6c	HisOMe	0.312	<0.01	20	Pat. yes[c]	<5.0 (3.2)
6d	LysOMe	1.25	<0.01	>20	No	<5.0 (8.4)
6e	ArgOMe	5.0	0.01	>20	No	<5.0 (5.4)

[a]MIC values were defined as the lowest drug concd required to inhibit 90–100% visible growth compared to controls.
[b]ED_{50}: drug concentration required to achieve 50% survival of fungal infection compared with untreated animals.
[c]Partial normal/partial yes meaning: one normal, one abnormal.

Table 6. Additional efficacy and toxicity profiles of LY448212 (**4e**) and 448731(**5b**)

Compd	*Cryptococosis* ED_{50} (mg/kg)[a]	*Aspergillosis* survival extension		Dose elevation			
		1 mg/kg	20 mg/kg	50 mg/kg	75 mg/kg	100 mg/kg	125 mg/kg
4e	2.9	29%	Not tested	Normal	Pat. Norm[b]	Death	Death
5b	2.5	23%	Not tested	Normal	Death	Death	Death
4d	—	—	—	Normal	Normal	Death	Death
6d	—	—	—	Normal	Normal	Death	Death
PSB 1	1.8	Not tested	46%	Death	Death	Death	Death

[a]ED_{50}: drug concentration required to achieve 50% survival of fungal infection compared with untreated animals.
[b]Partial normal/partial yes meaning: one normal, one abnormal.

Consequently, **4e** and **5b** were further evaluated in vivo against *Cryptococosis* and *Aspergillosis*. As can be seen in Table 6, both analogues tested demonstrated good in vivo efficacy against *Cryptococosis* with ED_{50} values ranging from 2.5 to 2.9 mg/kg. Although no significant efficacy towards *Aspergillosis* was obtained with **4e** and **5b**, both analogues were found to be capable of extending survival time in animals infected with this fungus at doses as low as 1 mg/kg.

To further assess the safety profiles of 3-amides, a total of 3-amides (**4d**, **4e**, **5b**, and **6d**) were selected, on the basis of tail vein irritation testing, for additional evaluation in the dose elevation study (see Table 6). The positive control, PSB, was found to be safe only at the dose of 25 mg/kg (data not shown). When PSB was dosed at 50 mg/kg and higher, severe toxicity resulted. In comparison, 3-amides **4e** and **5b** were found to be safe at the dose of 50 mg/kg. More impressively, the other two amides **4d** and **6d** passed this test at the dose of 75 mg/kg.

In light of its good in vivo and tail vein toxicity profile, **4e** was selected for two-week toxicity study in rats. Towards this end, amide **4e** was given to rats at doses of 50 and 75 mg/kg for 14 days. To our satisfaction, all drug treated animals (at highest dose) were found to be normal at the end of this experiment. No clinical observations were recorded. Careful analysis of blood samples collected from those drug treated rats indicated that blood chemistry was found to be normal also.

In conclusion, we succeeded in the preparation of three kinds of 3-amido bearing pseudomycin analogues (**4**–**6**) via a three-step sequence in a regioselective fashion. In general, all of the newly synthesized 3-amides exhibited excellent in vitro activity against *Cryptococcus*. Their potencies towards *Candida* varied according to the nature of the amide linkages, with MIC values ranging from 0.156 to 20 μg/mL. Several members of dialkylamino termini bearing amides exhibited improved potencies against *Aspergillus*, with MIC values at ~5 μg/mL. In addition to in vitro testing, we also evaluated all of the 3-amides synthesized in the tail vein toxicity assay and in vivo efficacy assay against *Candidiasis*. On the basis of these testing results, we identified a number of 3-amides with good in vivo efficacy yet without tail vein toxicity, such as **4d**, **4e**, **5b**, and **6d**. When tested in the dose elevation study, all four 3-amides (**4d**, **4e**, **5b**, and **6d**) demonstrated improved safety profiles (Therapeutic Index value calculated for **4d**: 12.5; **4e**: >10; **5b**: >10; and **6d**: >15) than that observed with the parent. Furthermore, **4e** and **5b** also demonstrated good activity in the murine systemic *Cryptococosis* model. More importantly, 3-cyclopropylamide **4e** passed our long term toxicity test in rats (detailed results not shown). To take all of the findings presented herewith as a whole, it is evident that the desirable antifungal activity of pseudomycin B analogues can be preserved without inherent tail vein irritation and long term organ toxicity.

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