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N-Acyloxymethyl Carbamate Linked Prodrugs of Pseudomycins Are Novel Antifungal Agents

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Abstract—We describe herein the synthesis, bioconversion, antifungal activity, and preliminary toxicology evaluation of a series of *N*-acyloxymethyl carbamate linked triprodrugs of pseudomycins. The syntheses of these prodrugs (**3**–**6**) were achieved via simple *N*-acylation of PSB (**1**) or PSC′ (**2**) with various prodrug linkers (**7**–**9**). As expected, upon incubation with mouse and/or human plasma, many of these prodrugs (**3**, **5**, and **6**) were converted to the parent compound within a few hours. Of particular significance, two pseudomycin triprodrugs (**5** and **6**) showed excellent in vivo efficacy against systemic *Candidiasis* without tail vein irritation being observed.

Introduction

Fungal infections, especially systemic fungal infections, have attracted more and more attention from the medical community due to the increasing occurrence of such diseases in hospitalized patients. Clinically, *Candida albicans*, *Cryptococcus neoformans* and *Aspergillus fumigatus* are the major opportunistic fungi responsible for systemic infections.[1] Patients at risk of developing invasive fungal infections are those with AIDS and other immunocompromised conditions, or those receiving broad-spectrum antibiotics or cancer chemotherapy. Amphotericin B (AMB)[2] and several triazoles (e.g., fluconazole)[3] are the available treatments for these systemic fungal infections. Unfortunately, the clinical utility of these drugs is limited either by the severe side effects (as observed with AMB) or by the rapid development of drug-resistance and the lack of broad spectrum of activity (as observed with fluconazole). To meet the urgent medical needs, many efforts are being made in search of more efficacious and safe antifungal drugs. These include further modification of the existing drugs (e.g., amphotericin B and triazole series)[4] and the development of novel antifungal agents such as recently reported echinocandin B analogues.[5]

More recently, we and others disclosed the structures and preliminary biological evaluation of pseudomycins, a series of potent and broad spectrum antifungal agents.[6] Pseudomycins were initially isolated as metabolites produced by *Pseudomonas syringae* in plants.[7] Each member of the pseudomycins family is comprised of a polar core and a hydrophobic tail. Depending on the nature of the side chain, as shown in Figure 1, the pseudomycins are subdivided into PSB (bearing a 14-carbon side chain) and PSC′ (bearing a 16-carbon side chain). In a previous presentation from this institution, we demonstrated that PSB and PSC′ are potent antifungal agents with activities against a wide variety of fungi.[6b] When evaluated against *Candida* and *Cryptococcus* either in vitro or in vivo, both PSB and PSC′ showed better activity than that obtained with amphotericin B, the most commonly used antifungal agent in clinical use. When tested against *Aspergillus*, PSB and PSC′ displayed similar activity to that found with fluconazole, with MIC values in the range of 10–20 μg/mL.

Despite the promising antifungal activity demonstrated by pseudomycins, the development of this class of novel lipopeptides as new antifungal agents is slow. This is primarily due to the irritation potential observed with the naturally occurring pseudomycins. For example, when mice were dosed (iv) with PSB or PSC′ (25 mg/kg×4) through their tail veins, they quickly developed swollen and dark tails. This was followed by tail loss within the next few days. Thus, in order to maximize the

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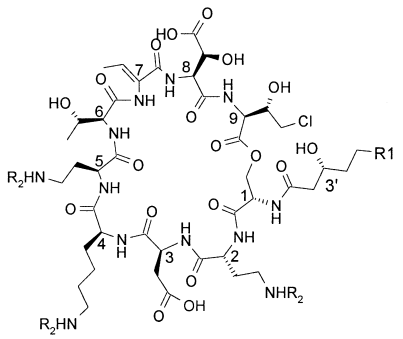
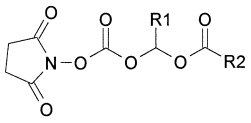


1 $R_1 = C_9H_{19\text{-}n}$, R_2 = H (Pseudomycin B)
2 $R_1 = C_{11}H_{23\text{-}n}$, R_2 = H (Pseudomycin C')
3 R1 = $C_9H_{19\text{-}n}$, R_2 = -C(O)OCH_2OC(O)Me
4 $R_1 = C_9H_{19\text{-}n}$, R_2 = -C(O)OCHMeOC(O)Me
5 $R_1 = C_9H_{19\text{-}n}$, R_2 = -C(O)OCH_2OC(O)Bu-t
6 $R_1 = C_{11}H_{23\text{-}n}$, R_2 = -C(O)OCH_2OC(O)Bu-t



7 R_1 = H, R_2 = Me
8 $R_1 = R_2$ = Me
9 R_1 = H, R_2 = t-Bu

Figure 1. Structures of pseudomycin derivatives (**1**–**6**) and prodrug linkers (**7**–**9**).

therapeutic utility of pseudomycins, we must identify pseudomycin derivatives that retain antifungal activity yet are devoid of the undesirable tail vein irritation potential. To achieve these goals, we have taken two markedly different approaches. The first approach relies on systematic SAR modifications of pseudomycins at both the core[8] and side chain,[9] whereas the second one takes advantage of novel prodrug design.[10] The rationale behind prodrug design is built upon the general notion that, in some cases, the C_{max} related drug acute toxicity may be reduce via controlled release of the parent drug. Many esterase labile prodrug linkers were documented in the literature for this purpose, including the recently reported *N*-oxodioxolenylmethyl carbamate linked pseudomycin prodrugs.[10,11] In this paper, we wish to discuss our efforts directed towards the synthesis and evaluation of a series of *N*-acyloxymethyl carbamate bearing pseudomycin prodrugs (**3**–**6**) designed to address the acute toxicity mentioned above (see Fig. 1 for structures).

Chemical Synthesis

The synthesis of *O*-acetyl bearing prodrug **3** was accomplished in 21% yield via coupling of one equivalent of PSB **1** with one equivalent of the mix-carbonate **7** (itself prepared via the reaction of its corresponding chloroformate and *N*-hydroxysuccinimide). In addition to the triprodrug **3**, all three possible monoprodrugs and three diprodrugs were also produced and isolated (via reverse-phase HPLC) in a combined yield of 15 and 23%, respectively. In general, the *N*-acylation reaction occurred at three residues (2, 4, and 5) was not regioselective. Alternatively, reacting PSB **1** (1 equiv) with three equivalents of the mix-carbonate **8** provided the triprodrug **4** as the major product in only 12% yield. The low yield in this case was attributed to the poor chemical stability of the linker used. Following the identical reaction conditions described for **3**, two *O*-pivaloyl ester bearing triprodrugs **5** and **6** were prepared, via *N*-acylation of PSB **1** or PSC′ **2** with linker **9** in 40%, respectively. In a separate experiment, triprodrug **5** was obtained in 80% yield via reacting PSB **1** with three equivalents of linker **9**. It should be mentioned that satisfactory mass spectra were obtained for all pseudomycin prodrugs synthesized.

Structure Determination

The structures of various pseudomycin prodrugs were determined on the basis of detailed proton NMR and COSY analyses. Careful examination of the NMR spectra of triprodrug **3** showed three pairs of additional singlets located around 5.58 ppm (consistent with the –O–CH_2–O– acetal moiety) and 2.02 ppm (assigned to the acetyl functionality on the linker). In addition, downfiled shifts of 2γ (~0.2 ppm), 4ε (~0.25 ppm), and 5γ (~0.2 ppm) protons relative to PSB were observed for **3**, indicating that *N*-acylations occurred at the residues 2, 4, and 5 (Table 1). The structures of two *O*-pivaloyl bearing prodrugs **5** and **6** were assigned in an analogous manner to that described for **3**. Since the prodrug linker **8** was racemic, the triprodrug **4** was composed of eight possible diastereomers. Although the gross structure of **4** was confirmed upon careful inspection of its ^{1}H NMR and COSY spectra, the more detailed peak assignments were found to be extremely difficult. Therefore, no proton NMR data was reported for **4**.

Antifungal Activity Evaluation

Prior to our systematic chemistry effort directed towards the preparation of *N*-acyloxymethyl carbamate linked pseudomycin prodrugs, we prepared several

Table 1. Key proton NMR assignments for PSB and its prodrugs (**3**, **5**, and **6**)

Sites	PSB	3	5	6
2α	*4.13*	4.09	4.07	4.07
2β	*2.01/2.07*	1.81/1.91	1.83/1.90	1.81/1.90
2γ	*2.90/2.97*	**~3.15**	**3.12/3.17**	**3.11/3.16**
4α	*4.13*	4.19	4.19	4.19
4β	*1.75/1.78*	1.73	1.72	1.71
4γ	*1.26/1.32*	1.27/1.33	1.23/1.30	1.24/1.29
4δ	*1.53/1.56*	1.39/1.43	1.37/1.43	1.36/1.42
4ε	*2.84*	**3.01**	**3.01**	**3.01**
5α	*4.29*	4.17	4.13	4.13
5β	*1.99/2.14*	1.80/1.98	1.78/1.98	1.76/1.98
5γ	*2.89/2.91*	**3.00/3.14**	**3.00/3.12**	**3.00/3.11**

Italic indicates the data generated for parent pseudomycin B; bold indicates significant chemical shift differences observed between pseudomycin analogues and the parent pseudomycin B.

stable *N*-acyl derivatives [e.g., R_2 = –C(O)Me] via simple *N*-acylation of PSB. Interestingly, such *stable* PSB analogue (towards mouse plasma mediated hydrolysis) was devoid of both the in vivo activity as well as tail vein irritation. In light of these findings, we decided to synthesize a series of *N*-acyloxymethyl carbamate bearing pseudomycin prodrugs **3**–**6**. In principle, these prodrugs should be converted to the corresponding parents (PSB or PSC′) via successive esterase-mediated prodrug linkers cleavage in vivo (see Fig. 2). 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 R_1 and R_2 depicted in **7**–**9**). To verify these hypotheses, three bioassays were carried out to screen all of the prodrugs prepared. These include (a) in vitro MIC determinations, (b) in vivo efficacy evaluation, and (c) tail vein toxicity assays. Three PSB prodrugs (**3**, **4**, and **5**) were also evaluated in the plasma stability assay with the intention to correlate the in vivo efficacy with the efficiency of prodrug bioactivation (if any).

In vitro evaluation

By virtue of being prodrugs, all of the newly synthesized PSB and PSC′ prodrugs were expected to be less active in vitro in comparison to their corresponding parent drugs. Therefore, only two *O*-pivaloyl ester containing prodrugs **5** and **6** were evaluated in the in vitro assay against the following three fungi: *C. albicans, C. neoformans*, and *A. fumigatus*. All three pathogens included here are the most important fungi responsible for systemic fungal infections. As expected, prodrugs (**5** and **6**) displayed much weaker activity against all three fungi tested relative to their respective parents. In view of these results, we felt that it was not necessary to test the remaining prodrugs (**3** and **4**) in the in vitro assay (see Table 2).

In vivo evaluation and tail irritation study

To identify pseudomycin prodrugs that retain good in vivo efficacy yet are free of tail vein irritation, we evaluated all four *N*-acylated prodrugs (**3**–**6**) in vivo against the disseminated *Candidiasis* mouse model (ip) and the tail vein irritation model (iv), respectively. PSB **1** or PSC′ **2** were also included as positive controls in these studies. The ED_{50} values were determined using the method of Reed and Muench.[13] It is worthwhile to point out that the reason to use a disseminated *Candidiasis* model as our primary in vivo assay is because *C. albicans* is the most important pathogen responsible for ~80% systemic fungal infections in hospitalized patients. As can be seen in Table 2, two PSB prodrugs **3** and **5** exhibited good in vivo activities with ED_{50} values ranging from 6.4 to 14.1 mg/kg. In comparison to PSB **1**, the level of in vivo efficacy demonstrated by **3** or **5** was about 2-fold lower. The *O*-pivaloyl bearing PSC′ prodrug **6** showed similar in vivo efficacy to that found with PSC′ **2**. Disappointingly however, prodrug **4** exhibited much weaker in vivo efficacy (ED_{50} > 20 mg/kg). The reason for this unexpected result will be discussed below.

In a subsequent experiment, four prodrugs (**3**–**6**) were evaluated in the tail vein toxicity assay (iv). In this case,

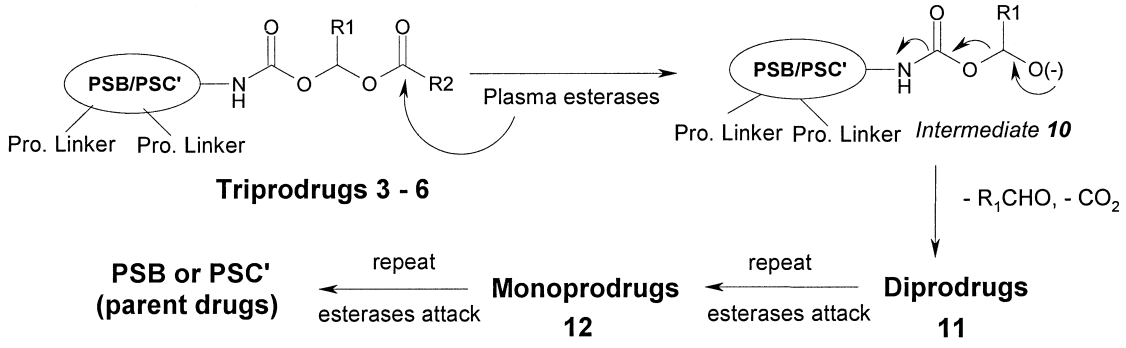


Figure 2. Prodrug bioconversion pathway.

Table 2. Antifungal activities of pseudomycin analogues (**1**–**6**)

Compound	MIC (μg/mL)			In vivo (ED_{50}) (mg/kg×4, ip)	Tail vein toxicology (20 mg/kg×4, iv)
	C. albicans	*C. neoformans*	*A. fumigatus*		
PSB (1)	0.625	0.039	20	2.5–7.2	Positive
PSC′ (2)	0.312	<0.02	5–10	7.8–12.4	Positive
3	N/A	N/A	N/A	13.0	Positive
4	N/A	N/A	N/A	>20	Negative
5	>20	>20	>20	6.4/14.1	Negative
6	>20	>20	>20	9.0	Negative

MIC, lowest drug concentration required to inhibit 90–100% of visible fungal growth compared to controls; N/A, not tested; ED_{50}, drug concentration required to achieve 50% survival of fungal infection compared with untreated animals.

mice were treated intravenously through the lateral tail vein with testing compounds (20 mg/kg) at 0, 24, 48, and 72 h. Mice were monitored (for 7 days) following first treatment for signs of irritation including erythema, swelling, discoloration, necrosis and tail loss. As mentioned in the introduction section, both PSB and PSC′ were capable of inducing tail vein irritation at the injection site. Despite its favorable in vivo efficacy, prodrug **3** was found to be equally irritable as pseudomycin B. To our satisfaction, compounds **4**, **5**, and **6** were found to be clean in the tail vein toxicity assay. Thus, judging from the data shown in Table 2, it is evident that we had successfully discovered two *O*-pivaloyl acetal linked prodrugs (**5** and **6**) that retained similar antifungal activities (in vivo) without inherent tail vein irritation.

Prodrug bioconversion study

With the aim to correlate in vivo efficacy with acute tail vein toxicity (if any) and to understand the lack of in vivo activity of **4**, we studied mouse or human plasma mediated bioactivation of various pseudomycin prodrugs. Mechanistically, prodrug bioactivation should occur via a two-step sequence as briefly outlined in Figure 2. Esterase mediated hydrolysis of terminal ester linkage on various triprodrug linkers (R_2 = Me, *t*-Bu) should lead, at different rate, to the negatively charged intermediate **10**, which should then undergo subsequent fragmentation to give the corresponding diprodrugs **11**, with the concomitant loss of CO_2 and R_1CHO (R_1 = H, Me). Following the same sequence, diprodrugs **11** would be further degraded to the respective monoprodugs **12**, and thereafter to the parent drugs (PSB **1** or PSC′ **2**). Furthermore, the parent drug release rate can be modulated by both R_1 and R_2 located on the prodrug linkers. In theory, the more bulky linker-bearing prodrug should generate the corresponding parent at a relatively slower rate than those bearing more esterase labile linkers.

In the initial prodrug activation study using mouse plasma, we selected the most labile *O*-acetyl terminibearing prodrug **3** and the least labile *O*-pivaloyl containing prodrug **5** as our candidates. After incubation of **3** with mouse plasma for 1 h, no triprodrug **3** was detected. Analysis of the reaction mixture indicated the presence of di- and monoprodrugs 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 **5** was considerably slower than that found with **3**. Incubation of **5** with mouse plasma for 1 h led to only partial mono *N*-deacylation. Based on the LC–MS result, the major product identified at this time-point was the corresponding diprodrug. At the 4-h time-point, the desired parent drug PSB **1**, along with some monoprodrug (the major component) were identified on the basis of LC–MS analyses. In light of these results, it is evident that both **3** and **5** indeed served as the prodrug forms of PSB **1**. Moreover, PSB was released much more readily from **3** than **5**. Since prodrug **6** was acylated with the same prodrug linker **9** as used in compound **5**, the outcome of bioactivation of **6** should be very similar to that observed with **5**.

In a separate experiment, prodrug **4** was incubated with human plasma for 4 h. Surprisingly, only minimal amounts of PSB was detected this time. The major products obtained in this case were the remaining **4** and its corresponding diprodrug.[14] In light of these results, it is evident that the poor in vivo efficacy observed with **4** may be attributed to its inability to generate sufficient levels of the parent drug in vivo (see Table 2).

With the in vivo efficacy data (Table 2) and the plasma stability data in hand, we conducted a detailed data analysis and found the following trends: (i) prodrugs capable of delivering parent drug very rapidly upon incubation with plasma (e.g., **3**) possessed favorable in vivo efficacy along with undesirable tail vein toxicity; (ii) prodrugs capable of generating only a minimal level of its parent drug after incubation in the presence of plasma was devoid of in vivo efficacy as well as tail vein toxicity (e.g., **4**); and (iii) prodrugs capable of releasing adequate (C_{max} and AUC profiles) amounts of their corresponding parent drugs (e.g., **5** and **6**) are endowed with good in vivo efficacy yet are devoid of tail vein irritation.

In view of their promising overall profiles, prodrugs **5** and **6** were further evaluated in a dose elevation study in mice.[10] When mice were injected with a single dose of PSB at 75 mg/kg, immediate animal death resulted. In sharp contrast to this observation, all mice receiving **5** or **6** at the same dose were found to be normal.

In conclusion, we disclosed herein the synthesis and biological and preliminary toxicity profiles of a series of *N*-acyloxymethyl carbamate linked triprodrugs of pseudomycins (**3**–**6**). Based on results obtained from in vitro bioactivation studies, prodrugs **3**, **5**, and **6** were capable of generating sufficient amounts of parent drugs, albeit at different rates. Consequently, all three prodrugs demonstrated good in vivo activities against systemic *Candidiasis*. Of these three compounds, prodrugs **5** and **6** were found to be clean in the tail vein irritation assay. In light of these promising results, further in vivo evaluation of prodrugs **5** and **6** against other fungi (e.g., *Cryptococcus* and *Aspergillus*) are clearly warranted. The results of these investigations will be published elsewhere.

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