Synthesis and Evaluation of Oxodioxolenylmethyl Carbamate Prodrugs of Pseudomycins

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With the aim of increasing therapeutic indexes of novel cyclic depsinonapeptide pseudomycins, we synthesized and evaluated a series of mono-, di-, and trioxodioxolenylmethyl carbamate prodrugs (**2** and **4**) of pseudomycin B **1** and pseudomycin C′ **3**. It is rather encouraging to note that several members of the newly synthesized prodrugs described herein (e.g., **2a**, **2e**, and **4e**) exhibited comparable in vivo efficacy to that achieved by the parent compounds, yet free of tail vein irritation and histamine induced toxicity in vivo.

Introduction

The incidence of developing serious systemic fungal infections continues to grow and is generally associated with host immunodeficiecy. Statistically, *Candida albicans*, *Cryptoccus neoformans*, and *Aspergillus fumigatus* account for greater than 90% of the life-threatening systemic fungal infections (SFI).[1] The current available drugs for the treatment of such SFI are limited to amphotericin B (AMB) and several triazole based drugs (e.g., fluconazole, itraconazole). Unfortunately, the clinical utility of these drugs is further restricted either by its severe toxicity or by its narrow spectrum of activity and rapid development of drug resistance.[2] The combination of the information discussed herein clearly suggests the urgent need for the discovery and development of safe and broad spectrum antifungal agents. In view of this situation, several approaches have been taken either to further improve the biological and toxicology profiles of existing drugs (e.g., amphotericin B[3] and itraconazole[4]) or to develop novel structure based antifungal agents (such as the cyclic lipopeptides, echinocandin[5] and pseudomycins[6]). Within the pseudomycin family, PSB **1** and PSC′ **3** were identified as the most potent members (see Figure 1 for structures), both of which showed improved in vitro and in vivo activities against *Candida* and *Cryptococcus* in comparison to amphotericin B.[6d] Besides the excellent in vivo activity obtained with pseudomycins, recent reports indicated that the pseudomycins exert their antifungal effects through a unique mode of action that is different from existing antifungal agents or those presently in development. Despite the promising antifungal activities demonstrated by pseudomycins, the potential clinical utility of these natural products is compromised by the undesirable irritation occurring at the injection site. We speculated, on the basis of literature precedent on amphotericin B,[7] that the irritation potential found with pseudomycins may be caused, at least in part, by the presence of the amino or acid functional groups within the polar pseudomycin core structures. To circumvent the side effect mentioned above, we decided to prepare various analogues[8] (with 3- or 8-acid modified) and prodrug forms of pseudomycins[9] (with amino groups modified). In this report, we discuss the synthesis and biological and toxicology profiles of another novel class of pseudomycin prodrugs, namely, the N-acylated oxodioxolenylmethyl containing prodrugs **2a**,**d**,**e** and **4a**,**d**,**e**. It should be pointed out that although the oxodioxolenyl linker has been used frequently in the literature for the purpose of improving oral absorption of various drugs (e.g., Ampicillin,[10] Methyldopa,[11] Norfloxacin,[12] etc.), the use of this prodrug group for modifying therapeutic indexes of the parent drugs has not yet been reported.

Chemistry

The synthesis of PSB prodrugs **2a**−**e** and PSC′ prodrugs **4a**−**e** were accomplished via direct N-acylation of their corresponding parents **1** or **3** with the requisite prodrug synthon **5** (see Figure 1 for structures). The mix-carbonate **5** was prepared in turn from the known 4-hydroxymethyl-5-methyl-1,3-dioxol-2-one[13] via simple acylation. Under the general experimental conditions described for PSC′ prodrugs, the simple nonselective N-acylation reaction (mole ratio of PSC′**3** vs linker **5** = 1:1.5) produced all seven possible products (three mono, three di, and one tri) eluted within five peaks numbered **4a** through **4e**. The retention times recorded (on reverse-phase HPLC system)[14] for these five peaks increased according to the following order: **4a** < **4b** < **4c** < **4d** < **4e**. The combined total yield of PSC′ prodrugs **4(a**−**e)** was 49%. In a separate experiment, treatment of PSC′ **3** with three equiv of **5** afforded the desired tri-N-acylated prodrug **4e** in 60%. Among them, one pure monoprodrug **4a**, one pure diprodrug **4d**, and the 2,4,5-triprodrug **4e** were fully characterized by their respective proton NMR spectra obtained on a Bruker spectrometer at 500 MHz. Judging from their distinctive ^{1}H NMR spectra, compounds **4b** and **4c** were proven to be the mix-mono- and mix-diprodrugs of PSC′ (see Table S1, Supporting Information, for detailed proton assignments). Therefore, the detailed NMR assignments for **4b** and **4c** were not pursued. After more extensive analyses of their respective COSY spectra, **4a** was identified as the N_5-monoacylated prodrug, whereas **4d** was confirmed to be the N_2,N_4-bisacylated prodrug. The detailed proton NMR assignments for **4a**, **4d**, and **4e**, along with three closely related PSB prodrugs, **2a**,**d**,**e**,

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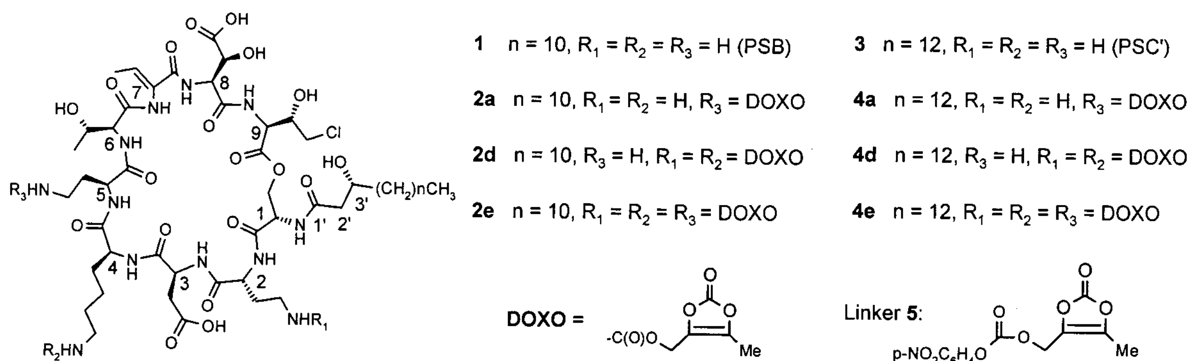

Figure 1. Structures of pseudomycin B and C′ and their prodrugs.

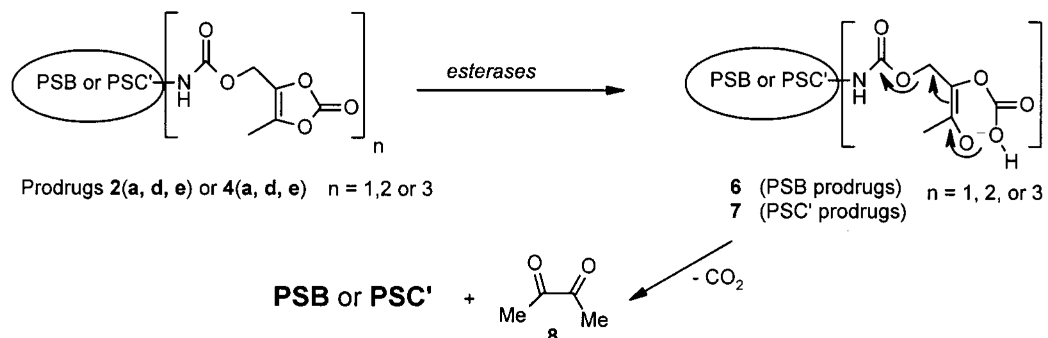

Figure 2. Bioactivation pathway of pseudomycin prodrugs **2** and **4**.

generated under identical reaction conditions from **1**, are listed in Table S1 (Supporting Information). As expected, downfield shifts (~0.15−0.20 ppm) for proton(s) at 2γ, 4ϵ, and 5γ positions were observed with various prodrugs examined. The detailed proton NMR assignments and copies of proton NMR and COSY spectra of **2a**, **2d**, **2e**, **4a**, **4d**, and **4e** are provided as Supporting Information.

Prodrug Bioactivation Study

The in vitro esterase mediated prodrug activation pathway for both **2** and **4** should proceed sequentially in a fashion similar to that proposed in the literature.[11a,11b] As briefly outlined in Figure 2, initial esterase attack on the cyclic carbonate moiety in **2** or **4** should lead to the corresponding negatively charged intermediates **6** or **7**. This should be followed by intramolecular fragmentation cascade, leading to the desired parent PSB **1** or PSC′ **3**, with concomitant release of CO_2 and 2,3-butanedione **8**. It should be pointed out that serum or tissue esterases mediated bioactivation of oxodioxolenylmethyl carbamate prodrug lenampicillin (KBT-1585) produced the corresponding parent drug along with nontoxic acetoin and 2,3-butanediol (instead of 2,3-butanedione) as the byproducts.[11c,11d] To further confirm this general mechanism, we studied the mouse plasma mediated bioactivation of PSB triprodrug **2e** in vitro (detailed protocol described in the Experimental Section). Indeed, upon incubation with freshly prepared mouse plasma in vitro for 1 h, **2e** 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 identified at this time point. In light of these results, it is evident that compound **2e** was qualified as the prodrug of PSB. With this information in hand, we were assured that the remaining pseudomycin derivatives discussed (**2a**, **2d**, **4a**, **4d**, and **4e**) should also function as prodrugs of the corresponding parent drugs.

Results and Discussion

In Vitro Evaluation. We evaluated the in vitro potencies of four prodrugs (**2a**, **2d**, **2e**, and **4e**) against *C. albican, C. neoformans,* and *A. fumigatus* according to the protocol described in the Experimental Section. As expected, the two triprodrugs **2e** and **4e** were totally devoid of antifungal activity. Similarly, 5-monoacylated and 2,4-bisacylated PSB prodrugs (**2a** and **2d)** were found to be 4- to 16-fold less potent than their parent against *Candida* and *Cryptococcus*. It is interesting to note that prodrugs **2a** and **2d** still exhibited some activity toward *Cryptococcus* in vitro. All four prodrugs evaluated showed poor activity against *Aspergillus* with MIC values of >20 μg/mL. In summary, the in vitro antifungal activities observed with all prodrugs were considerably weaker than those displayed by PSB **1** or PSC′ **3**.[15]

In Vivo Evaluation. To identify compounds that retain good in vivo antifungal efficacy while free of tail vein irritation observed with the parents, we routinely used the mouse disseminated *Candidiasis* model and tail vein irritation model to profile all of the oxodioxolenyl bearing pseudomycin prodrugs. On the basis of the results listed in Table 1, it is evident that all three PSB prodrugs (**2a**, **2d**, and **2e**) possessed slightly reduced (~3-fold) in vivo activity in comparison to the parent. More significantly, in contrast to the parent, prodrugs **2a** and **2e** were completely devoid of tail vein irritation. Improvement in tail vein irritation potential was also observed with **2d**. Within pseudomycin C′ series, two prodrugs **4a** and **4e** exhibited similar in vivo efficacy to

Table 1. Biological Evaluations of Pseudomycin Derivatives **1**−**4**

compd	in vitro MIC[a] (μg/mL)			ED50[a] (mg/kg × 4)		tail vein assay
	C. albicans	*C. neoformans*	*A. fumigatus*	1st expt	2nd expt	
1 (PSB)	0.625	0.01−0.078	>20	1.77	3.8	positive
2a (mono)	5.0	1.3	>20	5.95		negative
2d (di)	5.0	0.3	>20	7.07		partial positive
2e (tri)[c]	>20	10	>20	6.85	7.8	negative
3 (PSC′)	0.312	<0.02	10	7.8	12.4	positive
4a (mono)	NT	NT	NT	9.0		partial positive
4d (di)	NT	NT	NT	>20		partial positive
4e (tri)[d]	>20	5.0	>20	12.4	10.8	negative

[a] MIC: lowest drug concentration required to inhibit 90−100% of visible fungal growth compared to controls. [b] ED_{50}: drug concentration required to achieve 50% survival of fungal infection compared with untreated animals. [c] Dose elevation study in mice appeared normal at 25, 50, and 75 mg/kg. [d] Dose elevation study in rat appeared normal at 25 mg/kg.

that obtained with PSC′ **3** without evidence of tail vein irritation being observed for **4e**. Improvement in tail vein irritation potential was also demonstrated by **4a**. It is surprising to see that prodrug **4d** was devoid of in vivo efficacy. In summary, it is exciting to note that three of the prodrugs evaluated—**2a**, **2e**, and **4e**—exhibited good in vivo efficacy without inherent tail vein irritation.

Encouraged by these results, we selected two lead prodrugs—**2e** and **4e**—for a dose elevation study in mice and rats. These experiments were designed to assess the toxicity profiles of these prodrugs. In these experiments, healthy mice or rats were injected with a single dose of the testing prodrug at 25, 50, or 75 mg/kg for mice or at 25 mg/kg for rats. It is encouraging to note that the mice receiving **2e** (up to 75 mg/kg) as well as the rats injected with **4e** (at 25 mg/kg) were found to be normal. In contrast, the control groups receiving the same dose of PSB **1** showed symptoms of histamine induced pathology.

In conclusion, we described herein the preparation of a series of N-acylated oxodioxolenylmethyl bearing prodrugs of pseudomycin B and C′.[18] Among all six compounds tested, with the exception of **4d**, all of the prodrugs exhibited good in vivo efficacy (against *Candidiasis*) along with greatly reduced tail vein irritation potential. Furthermore, in sharp contrast to their respective parents, the two triprodrugs **2e** and **4e** did not produce histamine induced toxicity in the dose elevation study in mice (for **2e**) or rats (for **4e**). In view of these results, we are confident that it is possible to prepare novel pseudomycin prodrugs that are endowed with improved safety margins.

Experimental Section

Chemistry. All solvents and reagents were purchased from Aldrich, Fluka, or TCI (Japan) and used without further purification. Nuclear magnetic resonance (NMR) data were obtained on a Bruker AC-300 spectrometer (at 300 MHz for ^{1}H NMR). The detailed proton spectra assignments for the final products listed in Table S1 (Supporting Information) were made with the aid of COSY experiments on Bruker spectrometer at 500 MHz. Accurate mass measurements were obtained with Micromass Flatform LCZ. Preparative silica gel chromatography was carried out according to Still.[16]

The requisite prodrug linker **5** was prepared according to Alexander et al.[17] The proton NMR spectrum obtained with **5** was consistent with that reported in the patent literature.

Prodrugs **2a**−**e** were obtained in a fashion similar to that described for **4a**−**e**. The combined total yield of **2a**−**e** was 51%.

General Procedure for Preparing Pseudomycin Prodrugs. A DMF solution of PSC′ **3** (1.50 g, 1.20 mmol) was treated with **5** (1.80 mmol, 1.5 equiv). The reaction mixture was stirred at room temperature for 3 days. At this point, the solvent was partially removed, and that remaining was subjected to reverse phase HPLC purification (gradient 40−70% B in 40 min; buffer A = 0.1% TFA/H_2O and buffer B = 0.1% TFA/CH_3CN) to provide the pure monoprodrug **4a** (86 mg, 5.7%), the mix-monoprodrug **4b** (87 mg, 5.7%), the mix-diprodrug **4c** (177 mg, 11.8%), the pure-diprodrug **4d** (132 mg, 8.8%), and the triprodrug **4e** (248 mg, 16.5%). The retention times of all five products in the analytic HPLC system[14] are listed as follows: 10.11 min for **4a**, 10.34 min for **4b**, 11.10 min for **4c**, 11.33 min for **4d**, and 12.26 min for **4e**. Please note that the detailed proton NMR data for **4b** and **4c** were not available due to fact that these two compounds were mixtures.

General Protocol for the In Vitro Assay. All pseudomycin analogues and two positive controls, pseudomycin B and C′, were screened against the following three major fungi responsible for systemic fungal infections: *C. albicans*, *C. neoformans*, and *A. fumigatus*. The MIC value was defined as the lowest drug concentration required to inhibit 90−100% of visible growth compared to controls.

Sample Preparation. Pseudomycin analogues were dissolved in 100% DMSO. Compound solutions were diluted in sabourauds broth to yield a starting concentration of 20 μg/mL in the first wells following the addition of inoculum. Serial 2-fold dilutions in 100 μL aliquots were made in 96-well microtiter plates using a QuadFlex automatic pipeting liquid delivery instrument (Titertek Instruments Inc., Huntville, AL). The fungal yeast isolated and used for in vitro assays were grown on Sabouraud dextrose agar slants at 35 °C for 24 h. *C. albicans* and *C. neoformans* were suspended in saline and adjusted to 2×10^5 conidia/mL in Sabouraud dextrose broth (DIFCO, Detroit, MI). *A. fumigatus* spores were gently teased from mycelia grown on potato dextrose agar (DIFCO, Detroit, MI) and suspended in 0.1% Tween 80 and saline.

General Procedure for the Evaluation of Pseudomycin Analogues in a Disseminated Candidiasis Mouse Model: Animal. Outbred, male ICR mice (average weight, 18−20 g; harlan Sprangue Dawley, Indianapolis, IN) were used in a disseminated candidiasis ED_{50} survival study.

Organism and Culture Conditions. *C. albicans* A26 was cultured on Sabouraud dextrose agar (SDA; DIFCO Laboratories, Detroit, MI) slants at 35 °C overnight. Blastoconidia were washed from the surface of the slant in sterile saline and quantitated using a hemacytometer.

Immunosuppression. Mice were X-irradiated with 400 r 24 h prior to infection with a Gamacell 40 (Atomatic Energy of Canada Limited Commercial Products, Ottawa, Canada).

Survival Study. Mice were infected by an intravenous (iv) injection of 0.1 mL (containing 2×10^6 blastoconidia per mouse) in the lateral tail vein. Untreated controls were moribund within 3−4 days post-infection. Mice were dosed four times at 0, 4, 24, and 48 h post-infection with 0.2 mL of testing 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.

General Procedure for Performing the Tail Vein Toxicity Assay. Animal. Outbred, male ICR mice (average weight, 18−20 g; harlan Sprangue Dawley, Indianapolis, IN) were used in a tail vein irritation/toxicity assay.

Procedure. Mice were treated intravenously (iv) through the lateral tail vein with 0.1 mL of testing 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, tail loss, etc. Mice were also observed for any other signs of adverse effects indicating toxicity.

General Procedure for Dose Elevation Toxicity (Histamine Release Induced) Evaluation in Mice. Animal. Outbred, male ICR mice (average weight, 18−20 g; harlan Sprangue Dawley, Indianapolis, IN) were used in a dose elevation toxicity study.

Procedure. Mice were treated with a single intravenous (iv) injection of 0.1 mL of the testing 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.

General Procedure for the Prodrug Bioconversion Study. Pseudomycin analogues (e.g., **2e**) were incubated in mouse plasma obtained just prior to the experiments. Incubation occurred at 37 °C over a period of 4 h. Next, 200 μL aliquots were taken for extraction at $t = 0$, 1, and 4 h. The samples were extracted using Emnpore SDB-XC (7 mm × 3 mL) by the same procedure developed for plasma, except no ISTD was added. Following a 1 mL wash with 5/95 acetonitrile/H_2O, elution occurred with 1 mL of acetonitrile/HOAc (99/1). The extracts were dried under N_2 and reconstituted as indicated above.

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Supporting Information Available: Additional detailed ^{1}H NMR assignments and mass and chemical purity data for all prodrugs discussed herein (**2a**, **2d**, **2e**, **4a**, **4d**, and **4e**) are provided in Tables S1 and S2. This material is available free of charge via the Internet at http://pubs.acs.org.

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