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LETTER

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Natural Product-Based 6-Hydroxy-2,3,4,6-tetrahydropyrrolo[1,2-*a*]pyrimidinium Scaffold as a New Antifungal Template

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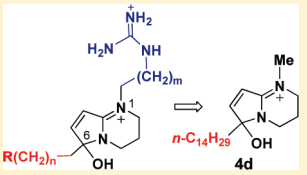
Supporting Information

ABSTRACT: Synthetic analogues of the marine-derived class of natural products phloeodictines have been prepared and exhibited potent in vitro fungicidal activities against a broad array of fungal pathogens including drug-resistant strains. The 6-hydroxy-2,3,4,6-tetrahydropyrrolo[1,2-*a*]pyrimidinium structural moiety with a C12 to C16 aliphatic side chain at C-6 has been shown to be the antifungal pharmacophore and may serve as a new antifungal template for further lead optimization.



KEYWORDS: Phloeodictines, fungicidal, antifungal template, mycosis

Since 2005, three new antifungal drugs, posoconazole, micafungin, and anidulafungin, have been added to the armamentarium against life-threatening disseminated mycosis.[1,2] In addition, isavuconazole and albaconazole representing the second generation triazole antifungals, the newer echinocandin class, aminocandin, and a polyene analogue, SPK-863, are currently in clinical trials.[3,4] These new and emerging treatments have improved antifungal spectra or pharmacokinetic and pharmacodynamic properties and may offer clinicians more effective and less toxic alternatives to conventional amphotericin B (AMB). However, because they incorporate slight structural modifications based on the pharmacophores of existing antifungal drugs, cross-resistance is an important concern, in particular for the azole class.[3] Thus, there remains an urgent need to discover novel prototype antifungal agents in light of the emerging resistance and increasing occurrence of inherently resistant species as human pathogens.

The phloeodictines,[5−7] originally isolated from marine sponges, are a small group of alkaloids featuring a novel bicyclic 6-hydroxy-2,3,4,6-tetrahydropyrrolo[1,2-*a*]pyrimidinium skeleton with two side chains: a hydrophobic C9−C16 alkyl/alkenyl chain at C-6 and a hydrophilic guanidino C4−C5 alkyl chain at N-1 (Figure 1, the positive charge at N-1 can be delocalized among N-1−C-9−N-5). They are generally reported as inseparable mixtures of two or more closely related compounds with similar side chain lengths[6,7] and have demonstrated varying degrees of antibacterial,[5,6] antimalarial,[7] and cytotoxic[5−7] activities. The total synthesis of (±)-phloeodictine A1 has been reported.[8]

As part of our ongoing effort to search for antifungal compounds from natural sources,[9−11] we identified the phloeodictines (as a mixture of phloeodictines A, A6, and A7 and phloeodictyne 5,9a in a ratio of approximately 2:2:2:1, Figure 1) as the antifungal constituents in the marine sponge *Pellina eusiphonia*.[12] As shown in Table 1, this mixture (designated PDT) shows potent antifungal activity (comparable to AMB) against the three major opportunistic fungal pathogens, *Candida albicans*, *Cryptococcus neoformans*, and *Aspergillus fumigatus*, which cause life-threatening infections in immunocompromised patients with AIDS, cancer, and organ transplants. In addition, PDT was determined to be slightly less cytotoxic than AMB against the mammalian Vero cell line using our published assay protocol[11] (IC_{50} of 12.2 vs 4.7 μg/mL, Table 1). In an unsuccessful effort to separate the mixtures, we acetylated PDT with Ac_2O/pyridine and obtained a mixture of tetra- and triacetylated products (designated acetylated PDT). We were surprised to observe that acetylated PDT retained antifungal activity and, more importantly, showed slightly less in vitro cytotoxicity than PDT (IC_{50} of 22.8 vs 12.2 μg/mL, Table 1). These results led us to conclude that the guanidino group at the N-1 side chain and possibly the C-6 hydroxy group may not be necessary for the antifungal activity of the phloeodictines.

The unique structures and potent in vitro antifungal activities of the phloeodictines prompted us to synthesize analogues without the guanidino group to (1) address the supply of single entity compounds for better biological studies and potential pharmaceutical development, (2) reduce the toxicity of the compounds while enhancing their antifungal potencies, and (3) explore the structure−activity relationships (SAR) of this class of compounds. We were convinced of the feasibility of the first goal, given the previous report of the total synthesis of (±)-phloeodictine A1.[8] We believed the second goal to also be achievable in light of previous reports that several guanidine-containing marine natural products are cytotoxic.[13−15] An added benefit was that placement of the guanidino side chain at N-1 is synthetically challenging and not likely to be cost-effective from the perspective of drug development. Thus, to confirm whether the guanidino group was necessary for antifungal activity, we synthesized a simple N-1-methyl phloeodictine analogue. Finally, to get a preliminary understanding of the effect of the C-6 side

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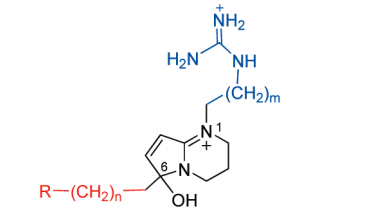
chain on activity, we prepared several analogues with C-6 side chains from C6 to C18 by the synthetic approach as shown in Scheme 1. Thus, the key intermediate amidine **1** was first synthesized by the reported procedure.[8] Then, addition of a 1:1 mixture of the Grignard reagent RMgBr and $CeCl_3$ to **1** in THF at 0 °C afforded amidium bromide **2**, which was treated with 1 M NaOH to regenerate base **3**. Alkylation of **3**, which is an unstable intermediate, with MeI in CH_3CN yielded the final products **4a**−**4f**. The six synthesized analogues are 6-hexyl-6-hydroxy-1-methyl-2,3,4,6-tetrahydropyrrolo[1,2-*a*]pyrimidin-1-ium iodide (**4a**), 6-decyl-6-hydroxy-1-methyl-2,3,4,6-tetrahydropyrrolo[1,2-*a*]pyrimidin-1-ium iodide (**4b**), 6-dodecyl-6-hydroxy-1-methyl-2,3,4,6-tetrahydropyrrolo[1,2-a]pyrimidin-1-ium iodide (**4c**), 6-hydroxy-1-methyl-6-tetradecyl-2,3,4,6-tetrahydropyrrolo[1,2-*a*]-pyrimidin-1-ium iodide (**4d**), 6-hexadecyl-6-hydroxy-1-methyl-2,3,4,6-tetrahydropyrrolo[1,2-*a*]pyrimidin-1-ium iodide (**4e**), and 6-hydroxy-1-methyl-6-octadecyl-2,3,4,6-tetrahydropyrrolo[1,2-*a*]pyrimidin-1-ium iodide (**4f**).

The highly purified synthetic phloeodictine analogues **4a**−**4f** (>95% assessed by HPLC and NMR) were evaluated for in vitro antifungal activities against *C. neoformans* ATCC 90113, *C. albicans* ATCC 90028, and *A. fumigatus* ATCC 90906. The results are shown in Table 1. Compound **4d** with a chain length of C14 was the most potent fungicidal compound against *C. neoformans*, with a minimum fungicidal concenctration (MFC) of 0.31 μg/mL as compared to 0.63 and 0.78 μg/mL for AMB and PDT, respectively. It also showed potent activities against *C. albicans* and *A. fumigatus*, with MFCs of 2.5 and 5.0 μg/mL, respectively. Compound **4a** with a C6 chain length, the shortest side chain in the series, was not active against the three fungal pathogens; compound **4b** with a C10 chain length was only active against *C. neoformans* and *C. albicans*, with MICs of 10 and 20 μg/mL, respectively. Compounds **4c** and **4e** with chain lengths of C12 and C16, respectively, also exhibited potent activities against the three fungal pathogens, for example, both showing an MFC of 0.63 and 1.25 μg/mL, respectively, against *C. neoformans*. It appears that the antifungal activity slightly diminishes with an increase of the side chain length from C14 to C18, such as in compound **4f** with a C18 chain length, which exhibited an MFC of 1.97 μg/mL against *C. neoformans*. Compounds **4a**−**4f** were also evaluated for in vitro cytotoxicity against mammalian Vero cells. Their IC_{50} values range from 12.3 to >25 μg/mL, in comparison to the IC_{50} of 12.2 μg/mL for the natural product PDT. However, all compounds were less cytotoxic than AMB (IC_{50} of 4.7 μg/mL). On the basis of the cytotoxicity to mammalian cells, the selectivity index of the compound was calculated in terms of the ratio of IC_{50} of cytotoxicity and MFC value. Compound **4d** showed the highest selectivity index followed by **4c**, indicating that the effective dose for fungicidal action of these compounds was much lower (about 50- and 30-fold) than the dose responsible for cytotoxicity. A selectivity index of 8 was observed for the standard drug AMB.

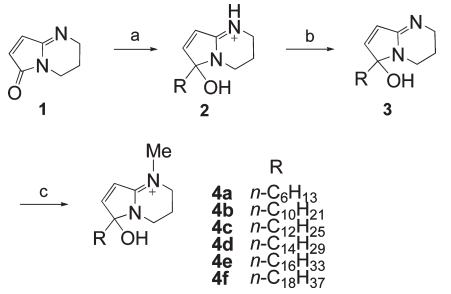
The above results led us to conclude that (1) we can prepare, through total synthesis, single entity synthetic phloeodictine



	R	m	n
Phloeodictine A	$-CH_2CH=CH_2$	3	10
Phloeodictine A6	$-CH(CH_3)_2$	4	9
Phloeodictine A7	$-CH(CH_3)_2$	3	9
Phloeodictyne 5,9a	$-CH_2CH=CH_2$	4	10

Figure 1. Structures of natural phloeodictines.

Scheme 1. Synthesis of Phloeodictine Analogues[a]



[a] Reagents and conditions: (a) $RMgBr/CeCl_3$ (1:1), THF, 0 °C, 30 min. (b) One molar NaOH, DCM, 25 °C, 5 min. (c) MeI, CH_3CN, 25 °C, 1 h.

Table 1. In Vitro Antifungal Activity and Cytotoxicity of Natural Phloeodictines and Synthetic Analogues

	antifungal activity [MIC[a] (MFC[b]), μg/mL]			cytotoxicity (IC_{50}[c], μg/mL)	SI[d]
	C. neoformans ATCC 90113	*C. albicans* ATCC 90028	*A. fumigatus* ATCC 90906	Vero[e]	Vero IC_{50}/fungal MFC[f]
PDT[g]	0.2 (0.78)	2.5 (2.5)	3.15 (12.5)	12.2	16
acetylated PDT[h]	0.94 (1.88)	5.0 (12.5)	5.0 (7.5)	22.8	12
4a	*i*	*i*	*i*	>25	N/A[j]
4b	10 (10)	20 (*i*)	*i*	21.3	2
4c	0.63 (0.63)	7.5 (10.0)	5.0 (10.0)	18.3	29
4d	0.31 (0.31)	1.88 (2.5)	1.88 (5.0)	16.5	53
4e	1.25 (1.25)	2.5 (2.5)	5.0 (5.0)	16.0	13
4f	1.61 (1.97)	2.5 (2.5)	10 (*i*)	12.3	6
AMB[k]	0.63 (0.63)	0.94 (1.25)	1.25 (1.25)	4.7	8

[a] Minimum inhibitory concentration (lowest concentration that allows no detectable growth). [b] Minimum fungicidal concentration (the lowest concentration that kills the fungus). [c] 50% inhibitory concentration. [d] Selective index. [e] African green monkey kidney cells. [f] Calculated by using the MFC against *C. neoformans*. [g] Phloeodictines A, A6, and A7 and phloeodictyne 5,9a in a ratio of approximately 2:2:2:1. [h] Acetylated products of the phloeodictine mixture. [i] Not active at the highest test concentration 20 μg/mL. [j] Not available. [k] Amphotericin B.

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analogues that have comparable or improved antifungal and cytotoxicity profiles, (2) the guanidino group is not required for antifungal activity, and (3) the bicyclic tetrahydropyrrolopyrimidinium structural moiety attached to the C-6 side chain is the antifungal pharmacophore, with C12 to C16 being optimal chain lengths.

The C-6 aliphatic side chain length appears to play an important role for antifungal activity of the phloeodictines. Previous studies suggest that the antifungal activity of some fatty acids is associated with their chain lengths and positional double or triple bonds.[16,17] It is also interesting to note that in the development of the very successful antifungal drug caspofungin from pneumocandin B_0 and of micafungin from FR901379, achieving the appropriate chain length of approximately 15 carbons in the aliphatic side chain was a determinant of the antifungal activity, and alteration of the aliphatic chain in FR901379 to a rigid, aromatic substituent containing side chain in micafungin greatly improves the hemolytic adverse effects.[18] In a recent study, we also demonstrated that among the seven acetylenic acids with chain lengths from C16 to C20, only 6-octadecynoic acid and 6-nonadecynoic acid possess potent antifungal activity against *C. albicans*.[11]

To further explore antifungal spectra of this class of compounds, synthetic analogues **4c**−**4f** and the natural product mixture PTD were tested against 23 strains of fungal pathogens including three *Cryptococcus*, seven *Candida albiacns*, four non-albicans *Candida*, five *Aspergillus*, and four *Trichophyton* strains, using our published protocols,[11] and the activities were compared with the two antifungal drugs AMB and fluconazole (FLU). The results show that compound **4d** is overall the most active among the tested compounds across various strains within each species of fungal pathogen, comparable to or more potent than the two control drugs (Table 2). Most notable is that **4d** is fungicidal to all of the pathogens tested, including FLU-resistant strains of *C. gattii* ATCC 32609 and *C. albicans* isolates 1 and 17, AMB-resistant strain of *C. albicans* ATCC 200955,[19] as well as *C. glabrata* ATCC 90030 and *C. krusei* ATCC 6258 that are intrinsically resistant to FLU. It must be pointed out that some *C. gattii* genotypes are highly virulent[20,21] and have recently drawn considerable public attention due to their causative role in the cryptococcosis outbreak throughout the Pacific Northwest.[22]

On the basis of these data, we believe that **4d** has broad spectrum antifungal activity and is a promising lead that warrants further study. Preliminary mechanistic studies using transcriptional profiling[23,24] have indicated that **4d** generates a gene expression profile indicative of targeting both cell membrane and cell wall in fungal cells. Fitness profiling using a whole-genome yeast deletion mutant collection[25] showed that mutants carrying deletions in genes required for mitochondrial function were hypersensitive to **4d**. Further examination of the transcriptional profile revealed that many mitochondrial genes (~10% of the total responding genes) were induced by **4d**, suggesting that **4d** also inhibits mitochondrial pathways in a manner similar to the amphipathic acetogenins, which are potent inhibitors of mitochondrial complex I.[26] Thus, the phloeodictine class of compounds, although containing detergent-like structural moieties,[27] appears to possess a novel antifungal mechanism.

Given that this novel pharmacophore differs from all existing drugs and no single synthetic analogue has been made for biological testing, there is great potential for the phloeodictines to be developed into new antifungal drugs. Through lead optimization, this class may be used for the treatment of disseminated mycosis, in particular for the life-threatening central nervous system cryptococcal infections for which there are few therapeutic options in a clinical setting.[28−30]

Our future work includes the synthesis of analogues with hydrophilic nitrogen-containing N-1 side chains to enhance pharmaceutical properties and modifications to the tetrahydropyrrolopyrimidium skeleton to improve antifungal selectivity.

Table 2. Comparison of the in Vitro Antifungal Activities of Compound 4d with Amphotericin B (AMB) and Fluconazole (FLU) against 23 Fungal Strains

	MIC[a] (MFC[b]) (μg/mL)		
	4d	AMB	FLU
	C. neoformans		
ATCC 90113	0.31 (0.31)	0.63 (0.63)	9.38 (25)
ATCC 66031	0.23 (0.31)	0.16 (0.31)	3.91 (12.5)
	C. gattii		
ATCC 32609	0.63 (0.63)	0.57 (0.57)	50 (50)
	C. albicans		
ATCC 14053	1.88 (2.5)	0.94 (1.25)	0.25 (*c*)
ATCC 60193	1.25 (1.25)	0.63(0.63)	0.2 (*c*)
ATCC 32354	1.25 (10)	0.63 (0.63)	0.94 (*c*)
ATCC 90028	1.88 (2.5)	0.94 (1.25)	0.51 (*c*)
ATCC 200955	1.25 (1.25)	1.88 (2.5)	0.35 (*c*)
isolate 1[d]	1.25 (1.25)	0.63 (0.63)	10.39 (*c*)
isolate 17[d]	2.5 (2.5)	0.94 (1.25)	*c*
	C. glabrata		
ATCC 90030	1.25 (1.25)	1.25 (1.25)	45 (*c*)
	C. krusei		
ATCC 6258	1.25 (1.25)	1.25 (1.25)	45 (80)
	C. parapsilosis		
ATCC 22019	1.25 (1.25)	1.25 (2.5)	2.8 (*c*)
	C. tropicalis		
ATCC 750	1.25 (1.25)	1.25 (2.5)	13.12 (*c*)
	A. fumigatus		
ATCC 204305	2.5 (10)	1.25 (2.5)	*c*
ATCC 90906	1.88 (5)	1.25 (2.5)	*c*
ATCC 13073	2.5 (2.5)	1.5 (10)	*c*
	A. flavus		
ATCC 204304	2.5 (2.5)	2.5 (5)	*c*
	A. niger		
ATCC 16404	2.5 (2.5)	1.25 (*e*)	*c*
	T. mentagrophytes		
ATCC MYA-4439	1.25 (1.25)	1.25 (1.25)	NT[f]
ATCC 9533	1.25 (1.25)	1.25 (1.25)	NT
	T. rubrum		
ATCC MYA-4438	1.25 (1.25)	0.63 (0.63)	NT
ATCC 10218	1.25 (1.25)	1.25 (1.25)	NT

[a] Minimum inhibitory concentration. [b] Minimum fungicidal concentration. [c] Not active at the highest test concentration 160 μg/mL. [d] AIDS patient isolates during fluconazole therapy: isolate 1 (first isolate); isolate 17 (last isolate), azole-resistant strain. [e] Not active at the highest test concentration 5 μg/mL. [f] Not tested.

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The most promising compounds (antifungal activity and selectivity index) will be subjected to mechanistic studies to uncover novel antifungal molecular targets toward further antifungal drug development.

■ ASSOCIATED CONTENT

Supporting Information. Experimental details and NMR spectra of key compounds **4a**−**4f**. This material is available free of charge via the Internet at http://pubs.acs.org.

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(27) A referee raised the question of whether the observed activity could be due to a detergent effect. This is unlikely to be the case because our compounds are much more potent than would be expected from a

detergent effect. For example, we have tested two detergent-like compounds, *tert*-butyltridecylaminium chloride and ethyltridecylaminium chloride. Neither compound is active against *C. albicans* ATCC 90028 or *C. glabrata* ATCC 90030 and are only moderately active against *C. neoformans* ATCC 90113 (MFC = 20 μg/mL) and *A. fumigatus* ATCC 90906 (MFC $\geq$ 20 μg/mL). To our knowledge, no detergents show antifungal potency comparable to the phloeodictines.

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NOTE ADDED AFTER ASAP PUBLICATION

This manuscript was originally published on the web March 18, 2011, with an error in one of the data entries in Table 1. The corrected version was reposted on March 23, 2011.