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Antifungal Activity of Bifunctional Sphingolipids. Intramolecular Synergism within Long-Chain α,ω-bis-Aminoalcohols

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Abstract—The in vitro antifungal activity of a series of α,ω-bifunctionalized aminoalcohols against *Candida glabrata* was measured. The dimeric bi-functionalized lipids exhibited activity about ~10-fold higher higher than D-sphingosine, which is a larger factor than expected from the simple additive effects of vicinal aminoalcohols groups. © 2002 Elsevier Science Ltd. All rights reserved.

We recently reported[1] the structure of a novel sphingolipid, the α,ω-*bis*-amino alcohol oceanapiside (**1**), which was isolated from the temperate marine sponge *Oceanapia phillipensis*. The structure of **1**—a C_{28} lipid chain terminated at each end by a vicinal amino alcohol—is differentially functionalized. The glycosidic terminus of **1** bears a conventional (2*S*,3*R*)-D-*erythro*-2-amino-1,3-diol, identical to that of D-sphingosine (**3**), while the opposing terminus lacks the primary OH group and displays an enantiomeric configuration 2*R*,3*R*, of opposite relative stereochemistry (*threo*). Compound **1** and its aglycone **2** exhibited potent selective antifungal activity against the fluconazole-resistant strain *Candida glabrata* (MIC 10 and 3 μg/mL, respectively), but not against other tested strains (*Candida albicans* ATCC 14503, *Candida krusei* or *Candida albicans* 96–489). Related compounds also exhibit biological activity.[2] For example, the isomeric calyxoside (**4**), from an undescribed *Calyx* sp.,[3] exhibited modest activity against two yeast indicator strains for DNA-damaging agents (RS321 and RS322, 62 and 36 μg/mL, respectively) while rhizochalin (**5**) from *Rhizochalia incrustata* showed modest antibacterial activity.[4]

In humans, the sphingolipid pool of the *stratum corneum* forms an important barrier to cutaneous infection by fungi and bacteria.[5] Sphingosine is known to inhibit the growth of *C. albicans*,[6] but the antifungal properties of non-natural dimeric aminoalcohols have not been described previously. We report here that dimeric sphingolipids have ~10-fold improved antifungal activity against *C. glabrata* compared to **3** or sphinganine.

C. glabrata is a human pathogen with recognized clinical importance due its association with fungemia caused by fluconazole-resistant yeasts.[7] The widespread use of fluconazole and the rise of resistant strains has lead to a shifting pathology of clinical patient isolates from *C. albicans* to fluconazole-resistant, non-albicans species.[7c] The selective activity of **1** against *C. glabrata* prompted us to investigate the structure–activity relationship of **1**, **2**, and several synthetic analogues with the goal of defining the contributions to activity as from the lipid-chain length and the bipolar sphingolipid-like head groups. We found a significant intramolecular synergism with long-chain α,ω-bis-aminoalcohols that leads to a higher than expected antifungal activity compared with simple mono-functionalized vicinal aminoalcohols such as **3**. These observations may have broader implications for the mode of action of bifunctional sphingolipids and their potential use in antifungal therapy.

Compounds tested in this study (Scheme 1) were obtained as follows. Oceanapiside (**1**) was isolated from extracts of *O. phillipensis,* as previously described.[1a] The algycone **2** was obtained by methanolysis of **1** followed by separation on silica chromatography. The dimeric bis-aminoalcohol **6** was synthesised from the terminal olefin **7**.[1b] Olefin metathesis of **7** (Grubbs' catalyst) gave

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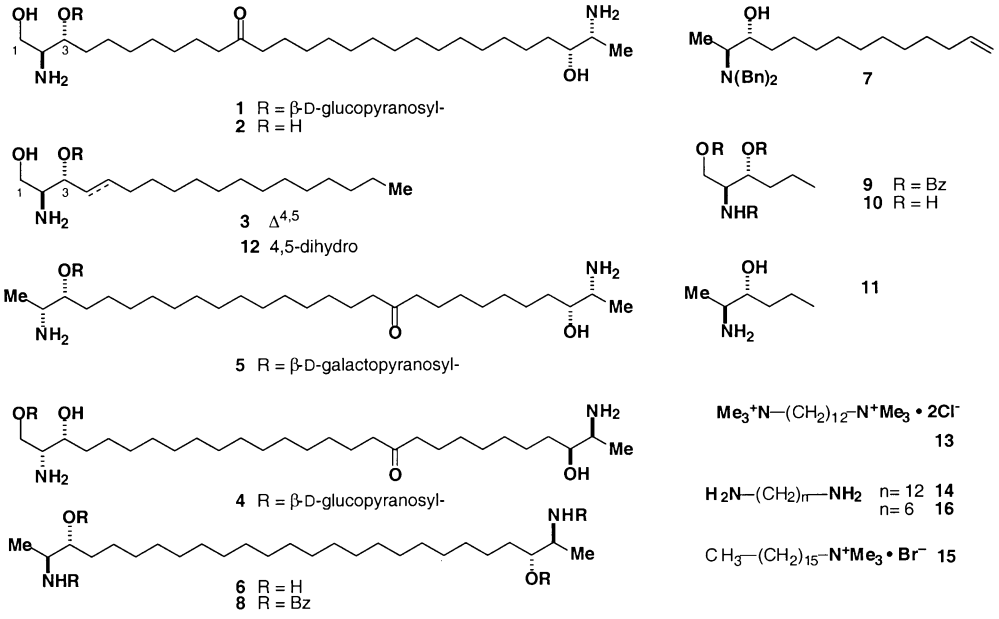

Scheme 1. Structures of naturally occurring α,ω-aminoalcohol lipids and synthetic analogues.

a tetrabenzyl dimer which was subjected to hydrogenation–hydrogenolysis to provide **6** and, subsequently, **8** (BzCl, pyr).[1b] Short-chain analogues **9**, **10**, and **11** were synthesized as described previously.[1b] D-Sphinganine (**12**) was obtained by hydrogenation of D-sphingosine (**3**).[8] Finally, the bis-quaternary ammounium compound **13** was obtained by exhaustive methylation of 1,12-diaminododecane (**14**).[9]

Bioassay of each compound for antifungal activity against *C. glabrata* was carried out using modifications of a published microbroth dilution assay.[10] The results are shown in Table 1. The most potent of the compounds tested was the algycone, oceanin (**2**). Removal of the *O*-glucosyl group enhanced activity, possibly by rendering the compound more membrane-permeable.

Table 1. Antifungal minimum inhibitory concentrations (MIC's) for aminoalcohols **1**, **2** and synthetic analogues against *C. glabrata*[a,b]

Compd	MIC (μg/mL)	MIC (μM)
1	10	15
2	3	6.2
3	30	100
6	10	24
8	>100	—
9	>100	—
10	>100	—
11	>100	—
12	30	100
13	>100	—
14	>100	—
15	5	14
16	>100	—

[a]Minimum inhibitory concentrations (MIC's) are from duplicate experiments.
[b]Dimeric compounds exhibited no significant activity against *C. albicans* ATCC 14503, the fluconazole-resistant strains *C. albicans* 96–489 (a patient isolate, MIC fluconazole >64 μg/mL) and *C. krusei* (up to 100 μg/mL).

The long lipid chain was found to be a requirement for activity—all the short chain analogues **9**–**12** were inactive (MIC >100 μg/mL). D-Sphingosine (**3**) and dihydrosphingosine (**12**) both inhibited *C. glabrata*, albeit with modest activity (MIC 30 μg/mL). Long-chain bis-analogue **6** also exhibited activity comparable to the natural product **1**. The corresponding per-benzoyl derivative **8**, and also short-chain analogue **9** were essentially inactive, which showed that antifungal activity required free primary NH_2 and OH groups.

Significant differences in activity were observed between the simple symmetrical C_{26} bipolar lipid **6** (MIC 10 μg/mL) and the C_{28} aglycone **2** (3 μg/mL). A difference of two CH_2 groups in the carbon chains seems unlikely to account for the differential activity, however, the presence of the 11-keto group in **1** and **2** is expected to predispose a 'hairpin' bend into the long alkyl chains. Consequently, the molecule suffers a reduction in the degrees of freedom of chain movement and closer average distance between the two polar head groups. Alternatively, the presence of the extra primary OH may play a role in the activity of **2**.

We tested the possibility that the antifungal activity against *C. glabrata* was manifested through nonspecific amphiphilic properties of long-chain aminolipids. The The MIC of the antiseptic cetyltrimethylammonium bromide (**15**) was 5 μg/mL, however, 1,6-diaminohexane (**16**), 1,12-diaminododecane (**13**), the corresponding *N*,*N*'-hexamethyl-bis-ammonium dichloride salt, **13**, were all inactive at up to 100 μg/mL. From these data we can draw the conclusion that the minimal requirements for antifungal activity in this series are the presence of a long lipid chain and a terminal vicinal 2,3-aminoalcohol. The minimum chain length required for activity in monofunctionalized aminoalcohols, as judged from antifungal activity of **3** and **9**–**12**, is between C6 and C18. No activity (MIC >100 μg/mL) is

Table 2. Fungicidal versus fungistatic activity of bifunctional sphingolipids against *C. glabrata*[a]

Compd	Compd concentration before dilution-replating (μg/mL)							
	0.3	1.0	3.0	5.0	6.0	10	12	30
Amphotericin B[c]	**FC** –	**FC** –	–	–	–	–	–	[d]
Fluconazole[c]	+	**FS** –	**FS** –	–	–	–	–	[d]
1	+	+	+	+	**FS** –	**FC** –	–	[d]
2	+	+	+	**FS** –	**FS** –	**FC** –	–	[d]
3[b]	+	+	+	+	+	+	+	–[b]
6	+	+	+	+	+	**FS** –	**FS** –	–

[a]*C. glabrata* was grown in the presence of compound and Saboraub media overnight (37 °C) in 96-well plates. Innocula from those wells which were near the MIC (10 μL) were then diluted and replated onto Saboraub agar plates before overnight reincubation. Codes: +, growth in media; –, no growth in media; FS, fungistatic (growth on plate); FC, fungicidal (no growth on plate). Compounds **11** and *threo*-**11** (structure not shown) were inactive in this assay (>100 μg/mL).
[b]Indistinct fungistatic activity.
[c]Amphotericin B and fluconazole were used as positive controls for fungicidal and fungistatic activity, respectively.
[d]Not tested.

seen in **10**–**11** where the correct functionality is present but the chain length is truncated (C6).

We briefly examined the fungistatic or fungicidal properties of the bifunctional sphingolipids in comparison to fungistatic fluconazole and fungicidal amphotericin B (Table 2). Fungistatic activity was estimated as that concentration which inhibited growth in liquid media, but produced viable colonies after dilution-replating. Oceanin (**2**) was fungistatic as low as 5.0 μg/mL, but at 10 μg/mL became fungicidal. Oceanapiside (**1**) was also fungicidal at 10 μg/mL, however in constrast, the synthetic dimer **6** was only fungistatic at the highest concentration tested (12 μg/mL). Under these conditions, sphingosine (**3**) exhibited only indistinct fungistatic activity at high concentration (30 μg/mL). It thus appears that the bipolarity of α,ω-bis-aminoalcohols and the presence of a keto group within the long chain lipid promotes fungicidal activity of sphingolipids, although additional analogues must be tested before we may draw more firm conclusions.

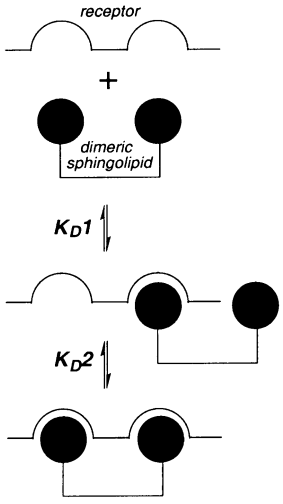

Figure 1. Hypothetical binding of dimeric sphingolipid to dimeric receptor or two units of monomeric receptor.

The most remarkable feature of the antifungal profile was the potent activity exhibited by the natural product **1** and its algycone **2** compared to D-sphingosine **3**. When compared on a molar basis, the MIC's for **1** and **2** appear to be an order of magnitude lower than those of **3**. The increase in activity (~10-fold) is much larger than expected from simple molar additivity of two vicinal aminoalcohol groups in each of the bifunctional compounds and is consistent with a synergistic effect.

The synergism may involve binding and inhibition of multiple receptor sites by **2** in a manner observed with multivalent drug-receptor interactions (Fig. 1). An obvious entropic advantage for binding is gained through dimerism as illustrated in a hypothetical ligand–receptor Scheme (Fig. 1). Binding of the first sphingolipid polar group (K_D1) promotes cooperativity in the second binding event (K_D2). For example, Whitesides et al. showed a synthetic dimer of the antibiotic vancomycin exhibited a 1000-fold increase in binding to a receptor peptide analogue (diacetyl-L-lysD-ala-D-ala) compared to the monomeric parent.[11] Improved binding to biological receptors may, in turn, translate to improved efficacy in antifungal drug–receptor interactions.

Oceanapiside (**1**) and related compounds exhibit pronounced synergism in their antifungal activity against *C. glabrata*. The activity of **1** and analogues appear to be specific to the bis-aminoalcohol functionality and not a simple function of the amphiphilic nature of these long chain polar lipids. While other natural product aminoalcohols have been reported with modest activity against *C. albicans*,[12] the dimeric functionality confers upon compounds **1** and **2** a unique selectivity and particularly high potency as a result of intramolecular synergism. Sphingofungin[13] and related compounds bear structural similarity to **1** and **3** and are known inhibitors of palmitoyl transferase, a key enzyme involved in the committed step of D-sphingosine biosynthesis. We are currently exploring the possibility that the natural products are specific inhibitors of one or more key steps in the biosynthesis of yeast sphingosine or sphingolipid metabolism. These results will be reported in due course.

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