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Fungicidal activity of truncated analogues of dihydrosphingosine

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Abstract−We determined the minimal fungicidal concentration (MFC) of dihydrosphingosine (DHS), phytosphingosine (PHS) and 5 short-chain DHS derivatives for *Candida albicans* and *Candida glabrata*. We found that a C15- and a C17-homologue of DHS showed a 2- to 10-fold decreased MFC as compared to native DHS (i.e. C18-DHS). DHS derivatives that were active, i.e. comprising 12, 15, 17 or 18 carbon atoms, induced accumulation of reactive oxygen species (ROS) in *C. albicans*, whereas inactive DHS derivatives, i.e. C5- and C9-DHS, did not. The most active DHS derivatives, i.e. C15-DHS and C17-DHS, induced ROS production to the highest extent. Interestingly, the presence of 10 mM of the antioxidant ascorbic acid decreased the fungicidal activity of C12-, C15- and C17-DHS against *C. albicans*, whereas the presence of ascorbic acid had no effect on the fungicidal activity of native DHS and PHS. These data point to a link between the fungicidal activity and ROS induction capacity of selected truncated DHS derivatives in *C. albicans*.

Long-chain sphingoid bases, e.g. phytosphingosine (PHS), sphingosine and sphinganine (dihydrosphingosine, DHS) inhibit the growth of several yeast and fungal species *in vitro*, including *Candida albicans*[1], *Malassezia furfur*[1], *Aspergillus nidulans*[2], *Saccharomyces cerevisiae*[3], *Trichophyton mentagrophytes* and *T. tonsurans*.[4] Sphingosines also possess antimicrobial activity *in vitro*: they are effective against *Staphylococcus aureus*, *Streptococcus pyogenes*, *Micrococcus luteus*, *Propionibacterium acnes*, and *Brevibacterium epidermidis*.[5] Cheng and coworkers found that the antifungal activity of DHS and PHS against *A. nidulans* acts through the rapid induction of metacaspase-independent apoptosis, associated with the rapid accumulation of reactive oxygen species (ROS).[2] Regarding the *in vivo* antifungal activity of sphingoid bases, Bibel and coworkers demonstrated that DHS and sphingosine, when topically applied on human skin, are effective against *C. albicans* infections and also prove curative in experimental guinea-pig models for *C. albicans* and *T. mentagrophytes* infections.[6] Interestingly, no gross toxicity was observed among animals or human volunteers[6], which points to the therapeutic potential of sphingoid bases against fungal infections.

The aim of this study was to analyse the *in vitro* antifungal activity of PHS, DHS and truncated analogues of DHS. It has been previously demonstrated that the minimum chain length required for antifungal activity of sphingoid bases against *C. glabrata* lies in the C7-C18 range, based on the fact that 3 DHS analogues with C6 chain displayed no antifungal activity up to 100 µg/ml.[7] Therefore, we synthesized a series of truncated DHS analogues with C5 (C5-DHS), C9 (C9-DHS), C12 (C12-DHS) or C15 (C15-DHS) chain lengths and determined the minimal fungicidal concentration (MFC) of these derivatives along with C17-DHS, C18-DHS and C18-PHS against *Candida albicans* strain CAI4[8] and *Candida glabrata* strain BG2[9]. *C. glabrata* is a human pathogen with recognized clinical importance due to its association with fungemia caused by fluconazole-resistant yeasts.[10] Furthermore, we determined whether C18-PHS, C18-DHS and the DHS derivatives induce ROS accumulation in *C. albicans*.

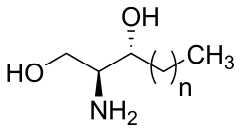
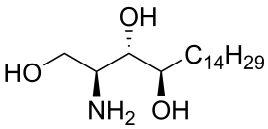
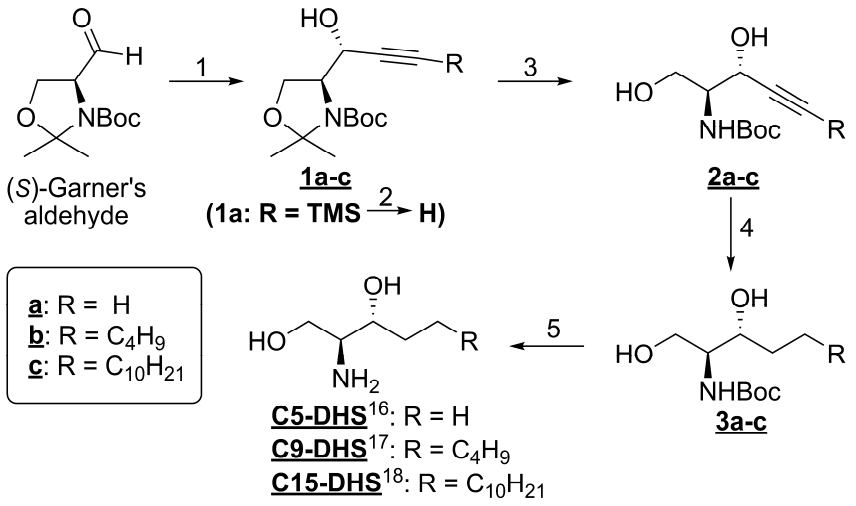
	**C5-DHS**: n = 1 **C9-DHS:** n = 5 **C12-DHS:** n = 8 **C15-DHS:** n = 11 **C17-DHS**: n = 13 **C18-DHS:** n = 14
	**C18-PHS**

Chart 1. Overview of tested compounds

Compounds tested in this study (Chart 1) were obtained as follows. C17-DHS, C18-PHS and C18-DHS were purchased from Avanti Polar Lipids (Alabaster, AL, US). C12-DHS was synthesized as previously described.[11] Compounds C5-DHS, C9-DHS and C15-DHS[12] were synthesized from Garner's aldehyde (Scheme 1). Treatment of *S*-enantiomer of Garner's aldehyde with an appropriate lithium alkyl acetylide in the presence of

HMPA to ensure *erythro*-selectivity afforded compounds **1a-c**[13] in reasonable yield and with excellent stereoselectivity (traces of *threo*-derivatives). In case of C5-DHS, deprotection of the intermediate TMS-protected acetylene was achieved using TBAF in THF.[13] Selective deprotection of the isopropylidene moiety[13] afforded synthons **2a-c.** Reduction of the alkyne functionality using Pd/C and subsequent deprotection of the *tert*-Boc protecting group under acidic conditions gave access to the envisioned compounds in good overall yield.



1) lithium alkyl acetylide, HMPA, THF, -78°C to rt, o.n. (**a**: 72%;[14] **b**: 64%;[15] **c**: 70%); 2) TBAF, THF, rt, 2h, 100%; 3) AcOH:H_2O (9:1), 5h, 60°C (**a**: 94%; **b**: 85%; c: 94%); 4) Pd/C (10%), H_2, EtOAc, o.n., rt (**a**: 92%; **b**: 96%; **c**: 95%); 5) 2M HCl:dioxane(1:1), 5h, 70°C (**a**: 100%; **b**: 99%; **c**: 72%).

Scheme 1. Synthesis of compounds C5-DHS, C9-DHS and C15-DHS.

The fungicidal activity of each compound against *C. albicans* and *C. glabrata* was determined in PBS[19] and the MFC for each compound was calculated as the minimal concentration resulting in less than 1% survival of the yeast strain relative to the DMSO control (table 1).

Table 1. Minimal fungicidal concentration (MFC) for C18-PHS, C18-DHS and its derivatives in the absence and presence of 10 mM ascorbic acid against *C. albicans* and *C. glabrata*

	MFC (µg/ml)		
	C. glabrata	*C. albicans*	
Compound	0 mM AA[a]	0 mM AA	10 mM AA
C5-DHS	> 100	> 100	ND[b]
C9-DHS	> 100	> 100	ND
C12-DHS	10	10	> 25
C15-DHS	0.5	0.5	10
C17-DHS	0.5	0.5	2
DHS	1	5	5
PHS	1	1	1

[a] ascorbic acid; [b] not determined

C15- and C17-DHS are the most active homologues against both yeast species: their MFC is 2- to 10-fold lower as compared to native DHS, and 20-fold lower as compared to C12-DHS. DHS derivatives with shorter chain length, i.e. C5-DHS and C9-DHS, are not active against the tested yeast species. Interestingly, native PHS is 5-fold more active as compared to native DHS against *C. albicans*, indicating that an additional hydroxyl group at position 4 can increase the antifungal activity against *C. albicans*. Moreover, we tested the fungicidal activity of C2- and C6-dihydroceramides, with C2 and C6 being the number of C atoms in the acyl residue (Avanti Polar Lipids, AL, US,) and found these ceramides to be completely inactive (MFC > 100 µg/ml) against both yeast species, indicating that a free amine at position C2 of the sphingoid base is necessary for fungicidal activity of sphingolipids. These data corroborate with the data of Chung and coworkers who demonstrated that C2-phytoceramide is not active against *Saccharomyces*

cerevisiae.[20] In conclusion, the optimal chain length for fungicidal activity of DHS derivatives against *C. albicans* and *C. glabrata* lies between C15 and C17.

In the literature, only two other studies report on derivatives of sphingoid bases with increased antifungal activity. One study describes a series of new PHS analogues with natural or altered stereochemistry at C3 and/or C4, and OH, NH_2 or N_3 substituents at C1, but without alteration of the sphingoid backbone length.[21] The 1-azido derivative, exhibiting the natural D-ribo stereochemistry, showed 10-fold improved antifungal activity against *C. albicans* as compared to PHS, based on determination of their minimal inhibitory concentration (MIC). However, antifungal activity of the compounds against *C. glabrata* was not reported.[21] Another study reports on the antifungal activity of dimeric aminoalcohols.[7] The most potent derivative was the dimeric aminoalcohol oceanin, which is characterized by 10-fold improved antifungal activity against *C. glabrata* as compared to DHS, based on their MIC. Oceanin is a C28 lipid chain with two polar head groups: one with a (2*S*,3*R*)-D-*erythro*-2-amino-1,3-diol moiety as in natural sphingosine, the other one with a (2*R*,3*R)*-2-aminopropan-3-ol group (*threo*).[7] The MFC of oceanin against *C. glabrata* is 10 μg/ml. Interestingly, oceanin is not active against *C. albicans*.

It should be noted that determination of MFC values is preferred over MIC values, since the former reflects fungicidal activity whereas the latter may account for both fungistatic as well as fungicidal activity. Since fungicidal activity pinpoints to inhibition of targets that are essential for fungal growth[22] or induction of an active cell death pathway (i.e. apoptosis), these values are more relevant for the design of antifungal drugs. Interestingly, it has previously been demonstrated that PHS and DHS induce apoptosis in *Aspergillus nidulans*, concomitant with an accumulation of reactive oxygen species (ROS).[2]

In search of the mode of action of PHS, DHS and its derivatives against *C. albicans*, we determined ROS accumulation upon incubation with various concentrations of the compounds using 2',7'-dichlorofluorescin diacetate staining as previously described.[23] As can be seen in Figure 1, the inactive C5-DHS and C9-DHS fail to induce ROS, even at 100 μg/ml, whereas C12-, C15- and C17-DHS, and native DHS and PHS induce ROS

accumulation in *C. albicans*. The most active DHS derivatives, i.e. C15-DHS and C17-DHS, induced ROS production to the highest extent. Interestingly, the presence of 10 mM of the antioxidant ascorbic acid decreased the fungicidal activity of C12-, C15- and C17-DHS, whereas the presence of ascorbic acid had no effect on the fungicidal activity of native DHS and PHS (Table 1). These data point to a link between the fungicidal activity and ROS induction capacity of short-chain DHS derivatives for *C. albicans*. In contrast, based on our data, there exists no causal link between ROS induction and cell death in yeast in case of native sphingoid bases. In this respect, Cheng and coworkers demonstrated that PHS and DHS induce an ROS-independent apoptotic cell death in *Aspergillus nidulans*.[2] Hence, our findings point to a ROS-dependent fungicidal activity of short-chain DHS derivatives on yeast, in contrast to the ROS-independent fungicidal activity of native sphingoid bases on yeast.

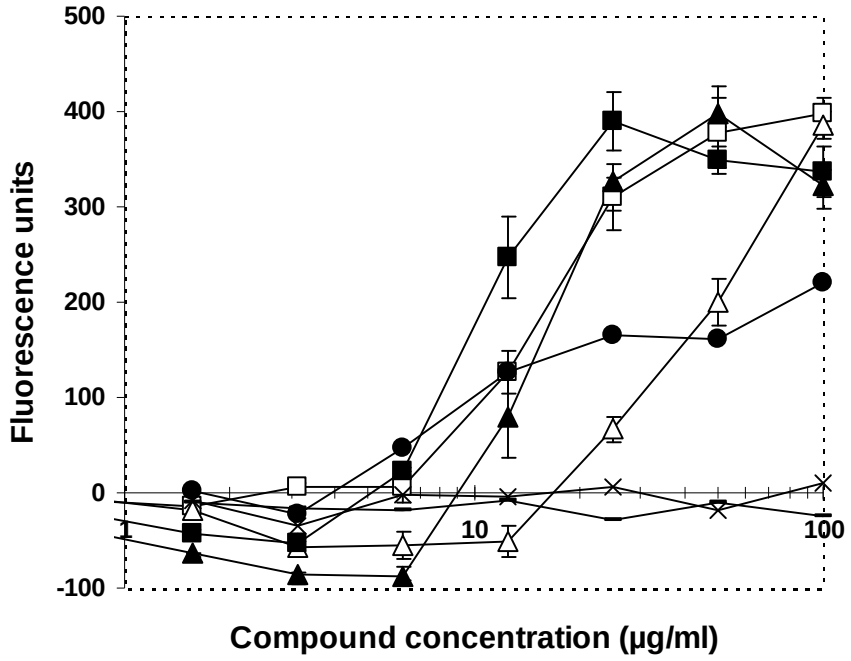


Figure 1. Accumulation of endogenous ROS in *C. albicans* upon treatment with antifungal compounds. Logarithmically growing *C. albicans* cells were suspended in PBS, pre-incubated with the compounds for 3 h at 37°C, washed with PBS and incubated with 2',7'-dichlorofluorescin diacetate for 3 h at 37°C. Compounds used are DHS (open

triangles), PHS (open squares), C5-DHS (crosses), C9-DHS (stripes), C12-DHS (black circles), C15-DHS (black squares) and C17-DHS (black triangles). Fluorescence emitted by the cells was measured using fluorescence spectrometer (λ_{ex} = 485 nm and λ_{em} = 525 nm). Experiments have been performed in triplicate.

In conclusion, we report on a series of synthetically easily accessible, truncated DHS-analogues. Based on MFC measurements, C15- and C17-DHS, DHS homologues consisting of 15 and 17 carbon atoms respectively, prove 10-fold more active against *C. albicans* and 2-fold more active against *C. glabrata* as compared to native DHS. Since PHS, bearing a hydroxyl group at position 4, has 5-fold increased fungicidal activity against *C. albicans* as compared to DHS, the question remains whether introduction of such hydroxyl group at position 4 of C15- and C17-DHS can likewise decrease their MFC for *C. albicans*. Since it has previously been demonstrated that DHS is non-toxic upon topical administration and is effective against *C. albicans* infections *in vivo* [6], C15- and C17-DHS hold promising therapeutic potential as novel antimycotics. Further studies addressing the mode of action of C15- and/or C17-DHS and their toxicity are underway.

Acknowledgments

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16. Spectroscopic data for C5-DHS: ^{1}H NMR (pyridine-*d5*) δ: 1.08 (t, 3H, *J* = 7.3 Hz), 1.71 – 1.86 (m, 2H), 3.60 (ddd, 1H, *J* = 4.4, 7.0 and 8.8 Hz), 4.14-4.36 (m, 3H); ^{13}C NMR (pyridine-*d5*) δ: 10.86, 26.91, 58.13, 61.68, 73.12; HRMS (ESI) calculated for $C_5H_{14}NO_2^+$: 120.1019 found: 120.1029.

17. Spectroscopic data for C9-DHS: ^{1}H NMR (CD_3OD-*d4*) δ: 0.91 (t, 3H, *J* = 6.7 Hz), 1.28 – 1.61 (m, 10H), 3.24 (app. dt, 1H, *J* = 4.1 and 8.2 Hz), 3.73 (dd, 1H, *J* = 8.2 Hz and 11.7 Hz), 3.80 – 3.86 (m, 1H), 3.86 (dd, 1H, *J* = 4.1 and 11.7 Hz); ^{13}C NMR (CD_3OD-*d4*) δ: 13.34, 22.52, 25.83, 29.08, 31.77, 33.05, 57.28, 57.77, 69.13; HRMS (ESI) calculated for $C_9H_{22}NO_2^+$: 176,1645, found: 176.1642.

18. Spectroscopic data for C15-DHS: ^{1}H NMR (CD_3OD-*d4*) δ: 0.89 (br. s, 3H), 1.12 – 1.81 (m, 22H), 3.12-3.36 (m, 1H), 3.63 - 3.94 (m, 3H); ^{13}C NMR (CD_3OD-*d4*) δ: 13.33, 22.58, 25.88, 29.32, 29.43, 29.46, 29.61, 31.92, 33.03, 57.28, 57.73, 69.12; HRMS (ESI) calculated for $C_{15}H_{34}NO_2^+$: 260,2584, found: 260,2584.

19. Overnight cultures of *C. albicans* and *C. glabrata* were 1/400 diluted in PBS and treated with the compounds or DMSO in the presence or absence of 10 mM ascorbic acid for 0h and 5h at 37°C, whereafter colony forming units were counted on YPD (1% yeast extract, 2% peptone, 2% glucose; 1% agar) plates after 2 days of incubation at 30°C. MFCs are means of at least three replicates with standard errors typically below 10%.

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