

ORIGINAL ARTICLE

Fungal growth inhibitory properties of new phytosphingolipid analogues

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Abstract**Aims:** To study the growth inhibitory properties of a series of phytosphingosine (PHS) and phytoceramide (PHC) analogues.**Methods and Results:** A panel of two yeast (*Candida albicans* and *Saccharomyces cerevisiae*) and six moulds (*Aspergillus repens*, *Aspergillus niger*, *Penicillium chrysogenum*, *Cladosporium cladosporioides*, *Arthroderma uncinatum* and *Penicillium funiculosum*) has been used in this study. A series of new PHS and PHC analogues differing at the sphingoid backbone and the functional group at C1 position were synthesized.**Conclusions:** Among PHS analogues, 1-azido derivative 1c, bearing the natural *D-ribo* stereochemistry, showed a promising growth inhibitory profile. Among PHC analogues, compound 12, with a bulky *N*-pivaloyl group and a *Z* double bond at C3 position of the sphingoid chain, was the most active growth inhibitor. Minimal inhibitory concentration values were in the range of 23–48 $\mu\text{mol l}^{-1}$ for 1c and 44–87 $\mu\text{mol l}^{-1}$ for 12.**Significance and Impact of the Study:** Only scattered data on the antifungal activity of phytosphingolipids have been reported in the literature. This is the first time that a series of analogues of this kind are tested and compared to discern their structural requirements for antifungal activity.**Introduction**

Sphingolipids (SLs) are ubiquitous components of cellular membranes of eukaryotic cells. Until the late 1980s, they were believed to have a protecting role against harming environmental factors, forming a mechanically stable and chemically resistant outer leaflet of the plasma membrane lipid bilayer comprising a set of different glycolipids also known as glycocalix (Kolter and Sandhoff 1999). However, in addition to this structural role, SLs are now considered as biomolecules with important implications in cell recognition and signalling processes (Hakomori 1990), cell growth regulation (Chung *et al.* 2001; Fishman 2002) and other processes related with the pathogenesis of several diseases (Claus *et al.* 2000). Over the last years, much effort has been made in order to better understand the biosynthesis and regulation of SLs in different species.

In this context, important biosynthetic/metabolic and structural differences between mammal and fungal SLs have been observed (Sugimoto *et al.* 2004), which set the stage for the development of new and selective antifungal agents (Thevissen *et al.* 2005). Thus, the common precursor dihydrosphingosine (DHS, Fig. 1) is hydroxylated in fungi at C4 position to phytosphingosine (PHS) and further acylated or phosphorylated to phytoceramide (PHC) or PHS-1-phosphate, respectively. In addition, fungal glycosphingolipids (GSLs) are biosynthesized from inositolphosphorylceramide (IPC) by means of a specific synthase (IPC synthase), a common enzyme in fungi and plants (Sugimoto *et al.* 2004).

In recent years, the number of patients with serious fungal opportunistic infections has risen dramatically because of the increased number of immunocompromised patients, either by HIV infection (Ampel 1996;

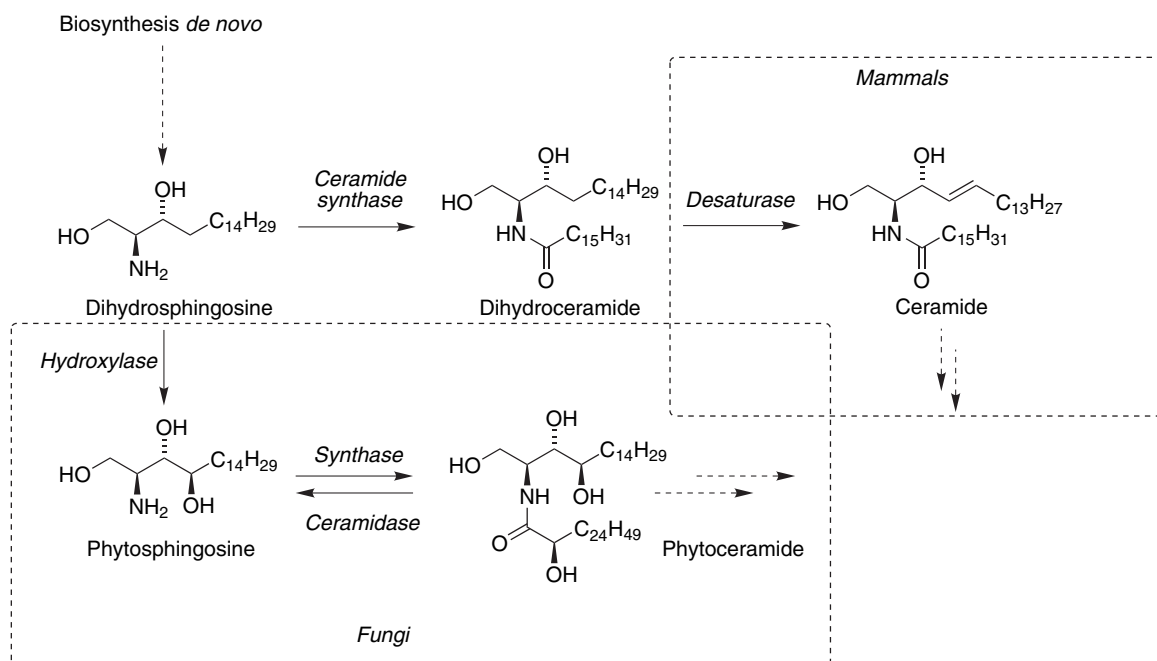


Figure 1 Metabolic routes in mammals and fungi.

Samaranayake *et al.* 2002), intense cancer chemotherapy (Bow 1998; Castagnola *et al.* 2006) or organ transplant (Marr and Bowden 1999; Fishman 2002; Brown 2004). However, the increasing tax of fungal resistance to standard treatments (Chamilos and Kontoyiannis 2005; Klepser 2006) has boosted the need of new therapeutic agents for specific pharmacological targets. Consequently, the use of SL analogues as potential and selective inhibitors of SL metabolism in fungi is an attractive approach.

Sphingoid long chain bases have been reported to have important functional roles in yeast cell signalling and heat stress responses (Liu *et al.* 2005b). In addition, DHS and PHS have been reported as fungicides *Aspergillus nidulans* (Cheng *et al.* 2003) and also as inhibitors of tryptophan import and yeast cell growth (Skrzypek *et al.* 1998). This effect is promoted by stimulation of ubiquitin-dependent proteolysis of the nutrient permeases (Chung *et al.* 2000). Furthermore, some alkaloids containing an alkyl amino alcohol skeleton, isolated from different marine invertebrate species, have also been described as antifungal agents (Clark *et al.* 2001; Nicholas *et al.* 2002; Searle and Molinski 1993).

In this study, we would like to report on the antifungal properties of a series of phytosphingolipid (PSL) analogues resulting from the systematic replacement of the C1–OH group present in natural SLs with an amino or an azido group. On the other side, all different stereochemistries at the C3–C4 moiety of the sphingoid chain have been explored, as well as its replacement

with a *Z* or *E* double bond. Finally, PHC analogues arising from acylation of the corresponding sphingoid bases with a bulky pivaloyl residue have been synthesized and tested. The *N*-pivaloyl group was chosen by analogy with other SL analogues previously synthesized in our group (unpublished data). From a structural standpoint, the new PSL analogues here reported can be viewed as: (i) PHS analogues (Fig. 2); (ii) PHC analogues (Fig. 3) and (iii) unsaturated PHS or PHC analogues resulting from replacement of the C3–C4 dihydroxy moiety with a double bond (Fig. 4).

Materials and methods

Micro-organisms

The micro-organisms used to evaluate the activity of the new compounds were yeast of the genera *Candida albicans* ATCC 10231 and *Saccharomyces cerevisiae* ATCC 9763 and the moulds *Aspergillus repens* IMI 0161144, *Aspergillus niger* ATCC 16404, *Penicillium chrysogenum* ATCC 9480, *Cladosporium cladosporioides* ATCC 16022, *Arthroderma uncinatum* ATCC 6082 and *Penicillium funiculosum* CECT 2914. Micro-organisms were maintained frozen at -80°C on cryobilles (EAS laboratories, Paypin, France). Prior to minimal inhibitory concentration (MIC) analysis, a cryobille of each micro-organism was removed from the freezer and cultivated on Sabouraud dextrose agar (ADSA Micro, Barcelona, Spain) at 25°C for 72–96 h and subcultured twice in the same conditions.

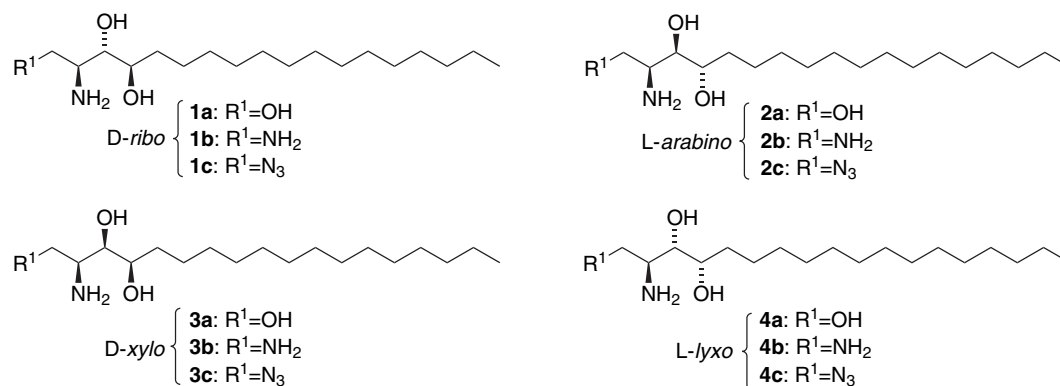


Figure 2 Phytosphingosine analogues tested in this study.

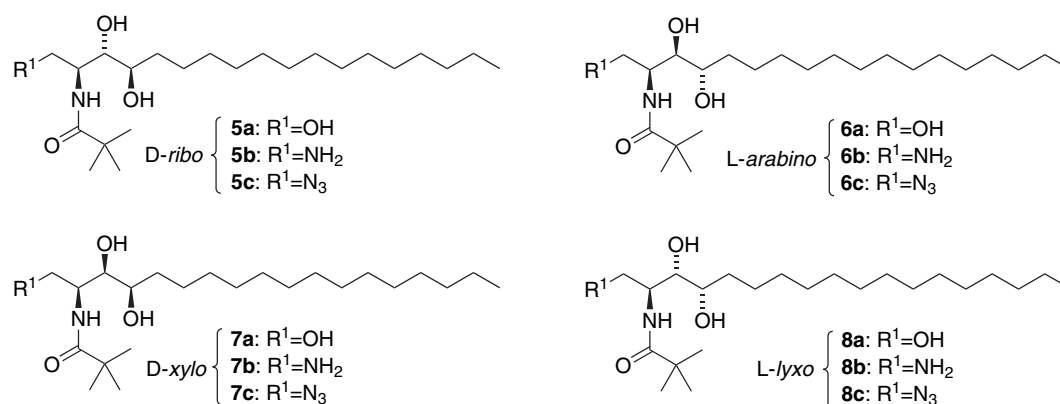


Figure 3 Phytoceramide analogues tested in this study.

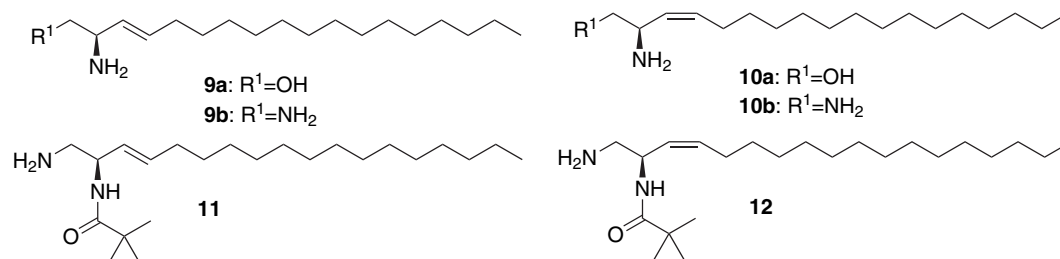


Figure 4 Unsaturated analogues tested in this study.

Antimicrobial agents

The compounds tested for antifungal properties were synthesized as described by Mormeneo *et al.* (2007). The antimicrobial solutions were prepared by a serial twofold dilutions of the product diluted in the assay medium to get the test concentration (256–0.25 mg l⁻¹). An aliquot of 200 μ l was dispensed in each well.

Minimal inhibitory concentration

The minimal inhibitory concentration was determined *in vitro* by using a broth microdilution assay following the guidelines of the Clinical and Laboratory Standard (NCCLS) described elsewhere (Espinel-Ingroff *et al.* 2003; Woods and Washington 1995). The test was performed by using sterile disposable 96-well polystyrene microtiter plate (Nunc, Roskilde, Denmark).

Preparation of inocula

In the case of yeast, four colonies were picked up from the Sabouraud dextrose agar media and suspended in sterile saline to match the turbidity produced by 0.5 McFarland barium sulfate standard. This produces a cell suspension of about 10^6 CFC ml⁻¹. In the case of the filamentous fungi, a saline solution with polysorbate (1% w/v) of spores harvested from Sabouraud dextrose agar medium was counted in a Petrof-Hausser chamber (Hauser Scientific, Horsman, PA) and adjusted by diluting 1 : 100 in Sabouraud dextrose broth to 10^6 spores ml⁻¹; 200 μ l of broth media containing the antimicrobial compounds were added to each well of a microtitre plate using a multichannel pipette. About 10 μ l of inoculum of each micro-organism was added to provide a final test inocula of about 10^4 UFC ml⁻¹. MIC was defined as the lowest concentration of antimicrobial agent that inhibits the development of visible growth after 24–96 h of incubation at 30°C. Experiments were conducted in duplicate.

Results

The activities of 1-octadecanol (C₁₈OH), 1-azidooctadecane (C₁₈N₃) and octadecylamine (C₁₈NH₂) were determined to evaluate any unspecific background activity, together

with that of the reference antifungal drug miconazole (Sud and Feingold 1981). Aliphatic octadecyl alcohol and azide were inactive (MIC > 474 μ mol l⁻¹ and 433 μ mol l⁻¹, respectively) in all the antimicrobial assays. Similarly, octadecylamine showed no significant growth inhibitory properties against the micro-organisms under study (Tables 1 and 2).

PHS analogues

1-Amino PHS analogues (1b–4b; Fig. 2), were inactive in all the assays, with MIC \geq 404 μ mol l⁻¹, a concentration higher than that required for octadecylamine for growth inhibition, whereas only 1-azido PHS analogue *D-ribo* 1c (Fig. 2) showed a significant activity, with MIC values ranging from 23 to 48 μ mol l⁻¹ in all the micro-organisms tested (Table 1).

Activity of 1-hydroxy PHS diastereomers (1a–4a; Fig. 2) against yeasts indicated that only *L-lyxo* 4a was active against both *C. albicans* and *S. cerevisiae* (MIC: 50 μ mol l⁻¹), while *L-arabino* 2a was active against *S. cerevisiae* and 1a and 3a were less active against *S. cerevisiae* (MIC: 101 μ mol l⁻¹) or inactive against *C. albicans* (Table 1). Similar growth inhibitory profile was observed for 1a–4a against *A. uncinatum* and *C. cladosporoides*,

Table 1 PHS analogues and unsaturated PHS analogues

Micro-organism	Compound MIC (μ mol l ⁻¹)													
	Miconazole	C ₁₈ NH ₂	1a	2a	3a	4a	1c	2c	3c	4c	9a	10a	9b	10b
<i>Candida albicans</i>	77	>238	>403	>403	>403	50	48	>374	>374	>374	>452	>452	>453	>453
<i>Saccharomyces cerevisiae</i>	77	>238	101	50	101	50	23	374	94	187	113	28	>453	>453
<i>Aspergillus repens</i>	0.6	238	50	0.8	101	50	48	374	187	187	226	113	>453	453
<i>Aspergillus niger</i>	9.6	>238	403	>403	>403	>403	48	>374	>374	>374	452	113	>453	>453
<i>Penicillium chrysogenum</i>	2.4	n.a.	50	101	1202	50	48	>374	374	>374	226	113	n.a.	n.a.
<i>Cladosporium cladosporioides</i>	9.6	238	50	25	25	50	23	374	187	187	226	226	227	453
<i>Arthroderma uncinatum</i>	2.4	119	50	25	50	25	23	374	187	187	113	56	113	227
<i>Penicillium funiculosum</i>	77	>238	403	50	403	202	48	374	374	374	113	113	>453	>453

n.a., not available; PHS, phytosphingosine; MIC, minimal inhibitory concentration.

Micro-organism	Compound MIC (μ mol l ⁻¹)										
	Miconazole	C ₁₈ NH ₂	5b	6b	7b	8b	11	12			
<i>Candida albicans</i>	77	>238	>320	>320	>320	320	349	44			
<i>Saccharomyces cerevisiae</i>	77	>238	>320	>320	320	160	44	44			
<i>Aspergillus repens</i>	0.6	238	160	>320	320	320	175	44			
<i>Aspergillus niger</i>	9.6	>238	80	160	>320	160	175	44			
<i>Penicillium chrysogenum</i>	2.4	n.a.	160	160	320	160	349	44			
<i>Cladosporium cladosporioides</i>	9.6	238	160	320	320	320	349	87			
<i>Arthroderma uncinatum</i>	2.4	119	160	160	320	160	44	44			
<i>Penicillium funiculosum</i>	77	>238	320	160	320	160	87	87			

n.a., not available; PHS, phytosphingosine; MIC, minimal inhibitory concentration.

Table 2 1-Amino-PHC and unsaturated 1-amino-PHC analogues

while *A. niger* was resistant in all cases (Table 1). It is worth noting the significant activity of *L-arabino* **2a** against *A. repens* (MIC: $0.8 \mu\text{mol l}^{-1}$) and *A. uncinatum* or *C. cladosporoides* (MIC: $25 \mu\text{mol l}^{-1}$), compared with the corresponding diastereomer *D-ribo* **1a**, which presented a moderate activity against *A. repens*, *P. crisogenum*, *C. cladosporoides* and *A. uncinatum* (MIC: $50 \mu\text{mol l}^{-1}$). Similar growth inhibition properties were found for **4a** (Table 1). Higher MIC values were observed for **3a**, with the exception of *C. cladosporoides* (MIC: $25 \mu\text{mol l}^{-1}$) and *A. uncinatum* (MIC: $50 \mu\text{mol l}^{-1}$).

Concerning unsaturated 1-hydroxy PHS analogues **9a** and **10a** (Table 1), the *Z* isomer **10a** was slightly more active against all the micro-organisms tested in the study. As the simple aliphatic octadecanol was inactive in the same microbial panel, it is reasonable to assume that the activity shown by **10a** must be due to specific interactions with cellular targets. On the other hand, the activities observed for alcohols **9a** and **10a** were not paralleled by those of the unsaturated diamines **9b** and **10b** (Table 1). In these cases, MICs were higher than that of simple octadecylamine, this suggesting the operation of nonspecific modes of action.

PHC analogues

The 1-hydroxy (**5a–8a**) and 1-azido (**5c–8c**) PHC analogues (Fig. 3) tested in this study were not active as growth inhibitors against the selected microbial panel, while some of the 1-amino derivatives **5b–8b** (Fig. 3) were moderately active (Table 2). Unsaturated PHC analogues **11** and **12** (Fig. 4) afforded interesting results. It is worth noting the good, even though unspecific, growth inhibitory activity shown by the unsaturated PHC analogue **12** and the lower activity shown by the isomeric *E* analogue **11** (Table 2). The influence of *N*-acylation on

the activity of these unsaturated analogues is also remarkable. Thus, *N*-pivaloyl derivatives **11** and **12** were more active than their corresponding lyso counterparts **9b** and **10b** (Table 1), respectively. This effect was even more pronounced on *Z* isomers, as evidenced by comparison of the growth inhibition patterns of **10b** and **12** (Tables 1 and 2).

Discussion

Design and synthesis of different PSL analogues, based on the modifications on the C1, C3 and C4 positions, were carried out as a new strategy on the search of antifungal compounds with the aim to interfere specifically on biochemical pathways of fungal SL biosynthesis. The above results (summarized in Fig. 5) show that the growth inhibitory properties of the tested analogues depend on a delicate balance between the functionality at C1 and C3–C4 positions, as well as *N*-acylation of the sphingoid chain. In some instances, the combination of two of the above requirements is essential to improve the activity, as in 1-amino-*N*-pivaloyl PHC analogues. To the best of our knowledge, no systematic studies of the antifungal activity of a series of PHC analogues have been reported so far and only a short chain ceramide (C2-Cer) analogue has been tested in *S. cerevisiae* (Fishbein *et al.* 1993).

Concerning PHS analogues, it has been reported that *C. albicans* growth was inhibited by natural *D-ribo* PHS (**1a**) at concentrations between 152 and $269 \mu\text{g ml}^{-1}$ (Nenoff and Haustein 2002), whereas different values have been found for *S. cerevisiae*, depending on the strain used. In this case, both natural *D-ribo* PHS (**1a**) and diastereomers **2a–4a** (Fig. 2) showed growth inhibitory properties, although with different selectivities and concentration ranks. It is accepted that effective incorporation of PHS into PSLs requires both phosphorylation and

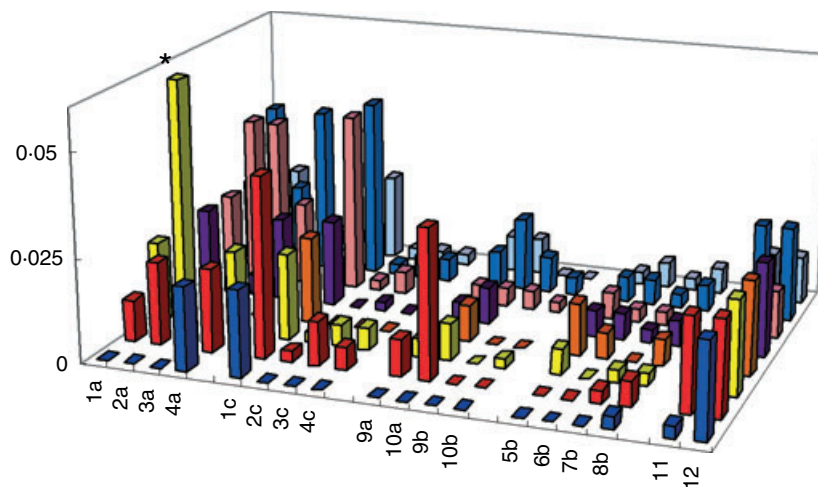


Figure 5 Activity of phytosphingolipid analogues tested in this study. The Z axis is expressed as $1/\mu\text{mol}^{-1}$, the reciprocal of the minimal inhibitory concentration. *Value: 1.25 ($0.8 \mu\text{mol l}^{-1}$). (■), *C. albicans*; (■), *S. cerevisiae*; (■), *A. repense*; (■), *A. niger*; (■), *P. chrysogenum*; (■), *C. cladosporides*; (■), *A. uncinatum*; (■), *P. funiculosum*.

subsequent dephosphorylation. These reactions serve to properly localize the sphingoid base and to allow for efficient action of downstream enzymes, because yeast with deleted phosphatase gene (*LCB3*) are more resistant to PHS treatment (Brace *et al.* 2007). Although additional experiments are required along this line, targets of natural PHS, such as AGC-type protein kinases (Liu *et al.* 2005a) and/or nutrient permeases (Chung *et al.* 2000), seem reasonable candidate targets for the above PHS analogues. Replacement of the 1-hydroxy functionality with an azide group was deleterious, except for the *D-ribo* isomer **1c**, one of the most potent analogues found in this study (Table 1). Azide **1c**, but not its diastereomers at C3–C4 positions, is a growth inhibitor of all the organisms tested at low μM concentration (Table 1 and Fig. 5). This stereoselectivity suggests the operation of specific interactions with yet undisclosed cellular targets.

Interestingly, simple unsaturated amino alcohols **9a** and **10a** behaved as modest fungal growth inhibitors of several of the tested species, specially the *Z* isomer **10a** (Table 1 and Fig. 5). Activity was lost in 1-amino analogues **9b** and **10b** (Table 1) but notably increased in 1-amino-*N*-pivaloyl analogues, in particular the *Z* isomer **12**, a good growth inhibitor of the entire microbial panel (Table 2 and Fig. 5). On the other hand, the corresponding *E* isomer **11** showed a higher selectivity, because it was as active as **12** against only three of the eight organisms tested in this study.

In conclusion, compounds **1c** and **12** have been discovered as two new PSL analogues with promising antifungal growth inhibitory properties. Further studies are currently underway to disclose its mode and/or mechanism of action, as well as their roles as modulators of SL biosynthesis.

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