The Discovery of Australifungin, a Novel Inhibitor of Sphinganine *N*-Acyltransferase from *Sporormiella australis*

Producing Organism, Fermentation, Isolation, and Biological Activity

SUZANNE M. MANDALA, ROSEMARY A. THORNTON, BETH R. FROMMER, JAMES E. CUROTTO, WALTER ROZDILSKY, MYRA B. KURTZ, ROBERT A. GIACOBBE, GERALD F. BILLS, M. ANGELES CABELLO†, ISABEL MARTÍN†, FERNANDO PELÁEZ† and GUY H. HARRIS

Merck Research Laboratories,
R80Y-230, P.O. 2000, Rahway, NJ 07065, U.S.A.
†Merck Sharp & Dohme de España, S.A.,
Josefa Valcárcel 38, 28027, Madrid, Spain

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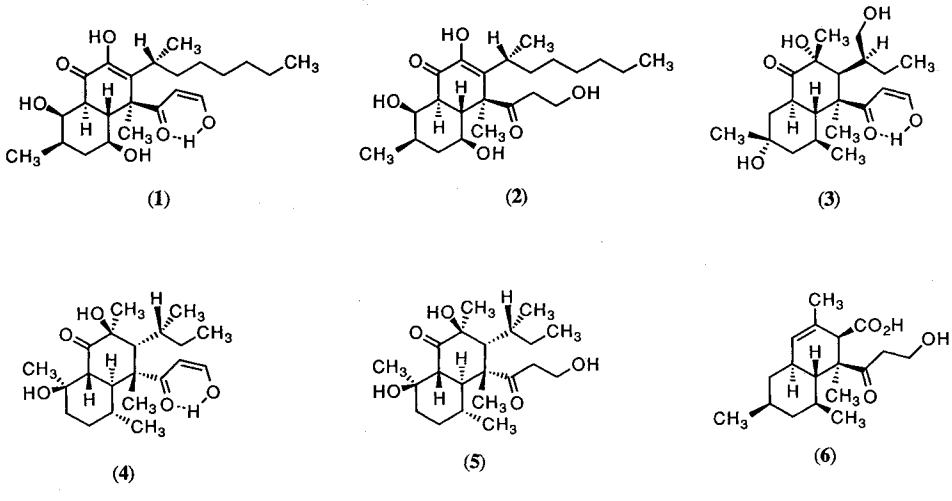
Potent antifungal activity was detected in fermentation extracts of *Sporormiella australis* and two related components were isolated from solid fermentations using silica gel and high speed countercurrent chromatography. The most active antifungal component, australifungin, contained a unique combination of α-diketone and β-ketoaldehyde functional groups. Australifungin exhibited broad spectrum antifungal activity against human pathogenic fungi with MICs against *Candida* spp., *Cryptococcus neoformans*, and *Aspergillus* spp. between 0.015 and 1.0 μg/ml. Mode of action studies revealed that australifungin interfered with fungal lipid metabolism by specifically inhibiting sphingolipid synthesis at the step converting sphinganine to ceramide.

The increasing incidence of life-threatening fungal infections coupled with emerging resistance to fluconazole and the toxicity of amphotericin B emphasizes the need for new fungicidal drugs with different modes of inhibition. Our natural products screening program has focused on fermentation products of a wide phylogenetic array of filamentous fungi as sources of antifungal agents with therapeutic potential. One of most easily accessible groups of chemically uninvestigated fungi are the coprophilous fungi that inhabit dung of herbivorous mammals. A high proportion of coprophilous fungi thus far investigated have yielded a surprisingly diverse array of novel and moderately potent antifungal compounds[1,2]. Below we report on the discovery of australifungin (**1**), a highly potent antifungal compound from a coprophilous fungus identified as *Sporormiella australis*. Australifungin inhibits sphingolipid synthesis at the sphinganine *N*-acyltransferase, a mechanism that is common to the mycotoxin, fumonisin B1[3]. The producing organism, fermentation, isolation and biological activity of australifungin are described; details of the structure elucidation are provided elsewhere[4].

Fig. 1. Structures of australifungin (**1**), australifunginol (**2**), stemphyloxin (**3**), betaenone C (**4**), betaenone B (**5**), and diplodiatoxin (**6**).



Materials and Methods

Fermentation

Vegetative mycelia of *S. australis* (MF5672=ATCC 74157) were prepared by inoculating mycelium, that had been frozen and stored in 10% glycerol (−80°C), into a 250-ml unbaffled Erlenmeyer flask containing 54 ml of the seed medium described previously[5]. Seed cultures were incubated for 3 days on a rotary shaker at 25°C and 50% relative humidity. The rotary shaker was operated at 220 rpm with a 5-cm throw in a room with continuous fluorescent light. Two ml of this culture were then inoculated into fresh seed medium to produce a second stage seed culture that was incubated similarly. Two ml of the resulting culture growth were used to inoculate either a solid corn-based or liquid production medium (Tables 1 and 2). The corn-based production medium A was incubated in 250-ml unbaffled Erlenmeyer flasks under static conditions at 25°C and 85% relative humidity for all the fermentation studies and initial isolation of australifungin. Fifty ml portions of the liquid production medium B were distributed into 250-ml unbaffled Erlenmeyer flasks. These flasks were incubated at 25°C on a rotary shaker in a room at 85% relative humidity for up to 21 days.

Media Components

The following nutrients were obtained from the sources indicated: yellow cracked corn, Bay-mor; Ardamine PH, Champlain Industries Inc.; oat flour, Quaker Oat Co. and Fidco yeast extract, Fidco division of Nestle Co., Inc. Carbon sources, amino acids, buffers and salts were reagent- or research-grade commercial products.

Isolation

Solid fermentations grown for 11 days were extracted by adding 50 ml of ethyl acetate to each flask. The material from 80 flasks was pooled, stirred with an overhead stirrer for 2 hours and filtered through a celite pad to yield an ethyl acetate extract (3,000 ml) containing crude australifungin and australifunginol. The extract was then concentrated to an oil *in vacuo* and partitioned between 650 ml of hexanes and 200 ml of methanol. The methanol layer was concentrated *in vacuo* (3.54 g) and dissolved in 17.8 ml of ethyl acetate. A 15.3 ml portion of the ethyl acetate solution was applied to a 377 g column, 5 cm × 45 cm, of silica gel 60 (0.040 ~ 0.063 mm, 230 ~ 400 mesh, E. Merck) which had been equilibrated with a solution of 3 parts hexanes: 2 parts ethyl acetate containing 1% glacial acetic acid. The column was eluted with the same solution at 15 ml/min collecting 24 ml fractions. Fractions 61 to 80 contained crude **1** as determined by analytical HPLC (Phenomenex Ultracarb 5 ODS 30, 15 cm × 4.6 mm, eluted with a mobile phase consisting of 55% acetonitrile - 45% aqueous 25 mM K_2HPO_4 adjusted to pH 6.9 with conc. H_3PO_4, flow rate 1 ml/minute at 55°C, detection at 275 nm). The silica gel column was then eluted with ethyl acetate containing 1% glacial acetic acid to give crude **2**.

The crude **1** fraction pool was concentrated *in vacuo* to a yellow oil (410 mg). The oil was further purified by high speed countercurrent chromatography (P.C. Inc., 11805 Kim Place, Potomac Maryland, U.S.A.) The sample was dissolved in 2 ml of the upper phase and 2 ml of the lower phase of a solvent system consisting of 7 parts hexanes: 3 parts ethyl acetate: 5 parts methanol: 5 parts aqueous 25 mM K_2HPO_4. It was then applied to the tail of a No. 14 analytical multilayer coil (P.C. Inc.) which had been filled completely with the lower phase of the above solvent system. The coil was then eluted with the upper phase of the solvent system at 3ml/minute from the tail to the head of the column, at a rotation speed of 800 rpm in the forward direction collecting 7.5 ml fractions. Australifungin eluted after 435 ml and the solvent was removed *in vacuo* to yield pure **1** (185 mg).

The crude **2** was purified by chromatography in multiple runs on Whatman Partisil 10 ODS3 (22 × 250 mm) eluted with CH_3CN - 25 mM aqueous KH_2PO_4, pH 6.8 (45 : 55) at 20 ml/minute. **2** was recovered by removal of CH_3CN *in vacuo* and then extraction of the remaining aqueous solution with ethyl acetate. The ethyl acetate solution was washed sequentially with H_2O, brine, dried over anhydrous Na_2SO_4, filtered and concentrated to yield pure **2** (785 mg).

Determination of Physico-chemical Properties

Mass spectral data were acquired on Finnigan-MAT models MAT212 and TSQ70B mass spectrometers. MAT212 spectra were obtained in the electron impact mode (EIMS) at 90 eV. Exact mass measurements were performed at high resolution (HR-EIMS) using perfluorokerosene (PFK) as an internal standard. TSQ70B spectra were acquired in the EI mode at 70 eV or by negative-ion fast atom bombardment (FAB-MS) employing ethanolamine as the matrix. Optical rotations were measured on a Perkin Elmer Model 241 polarimeter. Infrared spectra were recorded on a Perkin Elmer Model 1750 Fourier Transform Spectrometer by multiple internal reflectance as a thin film on a ZnSe crystal. Ultraviolet spectra were recorded on a Beckman Model DU70 spectrophotometer.

Antifungal Activity

Minimum Inhibitory Concentrations (MIC) were determined by microtiter broth dilution assay in Difco Yeast Nitrogen Base medium containing 2% glucose (YNBD) with fungi inoculated at $OD_{600} = 7 \times 10^{-4}$ (*ca.* 1×10^4 yeast cells or conidia/ml). Serial 2-fold dilutions of inhibitors were made from 32 μg/ml (**1**) or 128 μg/ml (**2**); the MIC value was the lowest concentration of inhibitor which prevented visible growth after 24 hours at 37°C.

Sphingolipid Synthesis

Five-ml cultures of logarithmic phase *C. albicans* (MY1055) cells in YNBD were pretreated with **1** or **2** at

0.25 μg/ml or 25 μg/ml, respectively, for 10 minutes at 30°C, and then 1 μCi/ml ^{3}H-inositol or 1 μCi/ml ^{3}H-palmitate was added. After a 2 hour labeling period at 30°C, the cultures were chilled on ice with an additional 0.5 ml of stationary phase cells, and subjected to centrifugation at 1,750 × *g* for 10 minutes at 4°C. The cells were washed with 5 ml cold H_2O and the lipids were extracted twice in 1 ml ethanol - water - diethylether - pyridine - NH_4OH (15 : 15 : 5 : 1 : 0.018) at 60°C for 15 minutes as described[6]. Half of the lipid extract was subjected to mild alkaline methanolysis by two treatments of 0.5 ml monomethylamine reagent as prepared by CLARKE and DAWSON[7], for 30 minutes at 52°C. Alkaline-stable lipids and total lipid extracts were dried under N_2, resuspended in 0.2 ml chloroform - methanol - H_2O and 25 μl was applied to silica gel TLC plates (Whatman Linear K5D) and resolved in chloroform - methanol - 4.2 N NH_4OH (9 : 7 : 2). Radioactive bands were visualized by X-ray film (Kodax XAR5) after spraying the plates with EN^{3}HANCE (DuPont).

Macromolecular Synthesis

Logarithmic phase *C. albicans* (MY1055) cells grown in Difco Yeast Carbon Base medium (YCB) containing 2 mM proline, 0.23 mM leucine, and 0.1 mM adenine were dispensed (100 μl) into 96 well plates containing different concentrations of **1**, **2**, and the protein synthesis inhibitor, verrucarrin, dissolved in MeOH. There were 4 replicates for each treatment and the final concentration of MeOH in each sample was 5%. After a 10 minute pretreatment with inhibitor, 0.5 μCi of ^{3}H-leucine was added to measure protein synthesis, or 0.05 μCi of ^{14}C-adenine was added to measure DNA and RNA synthesis over a 30 minute period at 30°C. An equal volume of 18% TCA was added and precipitates were harvested onto filtermats and radioactivity quantitated in a BetaPlate scintillation counter (Wallac). The same cultures were used to measure sphingolipid synthesis except duplicate samples of 0.4 ml of cells were labeled with 0.5 μCi of ^{3}H-sphinganine (10 μM) for 30 minutes at 30°C. Lipids were extracted and the entire sample was subjected to mild alkaline methanolysis and TLC as described above. The amount of incorporation of ^{3}H-sphinganine into ^{3}H-inositol phosphoceramide was quantitated using a BioScan counter.

In Vitro Enzyme Assays

Sphinganine *N*-acyltransferase activity was assayed using microsomal membranes from *Candida albicans* MY1055. To prepare membrane fractions, the cells were grown to OD_{600} of 1.2 in Difco Yeast Extract Peptone Dextrose media, collected by centrifugation, and resuspended in buffer containing 50 mM HEPES, pH 7.5, 5 mM DTT, 1 mM PMSF, and 1 μg/ml each chymostatin, aprotinin, pepstatin. Cells were disrupted with glass beads (0.5 mm) in a Mini Bead Beater (Biospec Products, Bartlesville OK) and homogenates were cleared by centrifugations at 3,000 × *g* for 10 minutes, and 9,500 × *g* for 10 minutes. Microsomal membranes were collected by ultracentrifugation (100,000 × *g*, 1 hour) and resuspended in disruption buffer containing 20% glycerol. Sphinganine *N*-acyltransferase assays were performed in 100 μl reactions containing 50 mM HEPES, pH 7.5, 0.02% tergitol NP-40, 10 mM ATP, 1 mM $MgCl_2$, 0.5 mM NADPH, 2 μM 4,5-^{3}H-sphinganine (0.05 μCi/ml), 10 μM lignoceroyl-CoA, and 50 μg membrane protein. After 30 minutes at 30°C, the reaction was terminated with 0.64 ml of chloroform - methanol (1 : 1) and the samples were centrifuged at 12,000 × *g* for 3 minutes. The supernatants were dried, resuspended, and applied to TLC plates as described above. Two forms of ceramide were resolved in chloroform - methanol - acetic acid (90 : 2 : 8) and radioactivity was quantitated using a BioScan counter.

Results

Description of the Producing Organism

Our strain of *Sporormiella australis* was isolated from dung of *Alces alces* (moose), collected in Cook Co., Minnesota, U.S.A. In the following description colonies were grown on media from Difco for 14 days at 20°C, 95% relative humidity, 12 hour photoperiod, under fluorescent lights. Capitalized color names are from RIDGWAY[8].

Colonies on oatmeal agar attaining 34～37 mm appressed to felty or sparsely floccose at the center, with margin even and submerged, dry, dull, pale gray to dark olivaceous gray, Pale Smoke Gray, Deep Grayish Olive, Iron Gray, Dark Olive-Black, often developing conspicuous lightly pigmented sectors that originate from inoculum source, with sectors pale olivaceous yellow to pinkish olive, Avellaneous, Deep Olive Buff, reverse dull olivaceous gray to gray, pinkish gray or yellow in the sectors. Exudates and odors absent.

Colonies on malt yeast extract agar, attaining 26～28 mm in 14 days appressed to felty, becoming sparsely floccose, dry, dull, with margin even, submerged, white, pale gray to dull olivaceous gray, Pale Smoke Gray, Smoke Gray, Grayish Olive, developing unpigmented sectors, reverse dark gray to nearly black, with yellow to grayish sectors. Exudates and odors absent.

Colonies on cornmeal agar, attaining 8～12 mm in 14 days, similar in color and appearance to colonies on malt yeast extract agar, but more translucent.

Colonies of this strain, are similar to those of other *Sporormiella* spp. and have a tendency to develop aberrant and attenuated sectors, especially after repeated transfer. The sectors are generally paler in color and have lost or have reduced their ability to differentiate stromata

and/or pseudothecia.

Pseudothecia evident in 10~21 days, maturing in 4~5 weeks on oatmeal agar. Pseudothecia single to densely gregarious, or confluent, embedded, with upper 10~60% protruding above the surface, 100~400 μm in diameter, globose to subglobose, with a minute apical papilla, non-ostiolate, glabrous, dull, black. In culture, pseudothecia often become moribund and fail to fully mature within 4~8 weeks. Often development is arrested with only the formation of asci initials and paraphyses. Peridium thin, 1~3 cells thick, a textura angularis. Peridial cells isodiametric, 3~8 μm in diameter, gray to dark olivaceous gray in KOH. Asci abundant, arising from the base of the pseudothecial cavity, bitunicate, 8-spored, cylindrical, straight to slightly curved, with broad rounded apex, 110~160×15~21 μm, tapering abruptly at the base into a short stalk, with basal stalk 6~10 μm long. Paraphyses abundant, interspersed among asci, filamentous, 1~3 μm wide, septate, approximately equal in length with asci. Ascospores biseriate within the ascus, 32~42×6~9 μm, 4-celled, constricted at the septa, with terminal cells with rounded apices, central cells cylindrical to doliform, each cell with a thin, faint lateral germ slit, with entire ascospore surrounded by a thin, refractive, hyaline sheath, with cells often separating when removed from ascus, dark olivaceous or brownish gray in KOH.

This strain can be assigned to the genus *Sporormiella* (Ascomycotina, Pleosporales) based on a combination of its saprobic coprophilous habitat, cleistothecioid or perithecioid pseudothecia, bitunicate asci, and transversely septate ascospores, with lateral germ slits surrounded by a gelatinous sheath. MF5672 can be identified as *S. australis* because the asci are cylindrical with a short stipe, the ascospores are 4-celled, biseriately arranged in the ascus, and mostly between 35~40 μm long. The morphological characteristics of this strain agree well with descriptions published by AHMED and CAIN[9] and by ELLIS and ELLIS[10].

Fermentation

Antifungal activity was initially detected and isolated from extracts of *S. australis* grown on the solid corn-based medium A shown in Table 1. Production of **1** in medium A started after day 7 and peaked at day 14 (67.1 μg/ml), and that of **2** started after day 4 and peaked at day 18 (501 μg/ml) (Fig. 2). Several liquid agitated media were screened, and liquid medium B (Table 2) was found to produce a 4.6- to 5-fold increase in the titer of **1** at day 14 using either mannitol or fructose as the soluble carbon source.

Isolation and Physico-chemical Properties

The isolation of **1** and **2** is summarized in Fig. 3. Solid fermentations were extracted with ethyl acetate and liquid fermentations were acidified to pH 3 before extraction with an equal volume of ethyl acetate. Compound **1** and **2** were easily separable by column chromatography on silica gel and **2** readily purified by preparative reverse phase HPLC. Compound **1**, however, required elevated temperatures to obtain satisfactory peak shape for reverse phase HPLC, making preparative HPLC impractical. This poor chromatographic behavior was probably due to extensive keto-enol tautomerism[4] and the chelating

Table 1. Components of the solid cracked corn-based fermentation medium A.

Component	Amount (per 250-ml flask)
Cracked corn	10.0 g
Ardamine PH	2.0 mg
KH_2PO_4	1.0 mg
$MgSO_4 \cdot 7H_2O$	1.0 mg
Na Tartrate	1.0 mg
$FeSO_4 \cdot 7H_2O$	0.1 mg
$ZnSO_4 \cdot 7H_20$	0.1 mg
Distilled water	10.0 ml

pH was not adjusted prior to autoclaving for 20 minutes. Immediately before use, the medium was moistened with 15 ml of water and autoclaved again for 20 minutes.

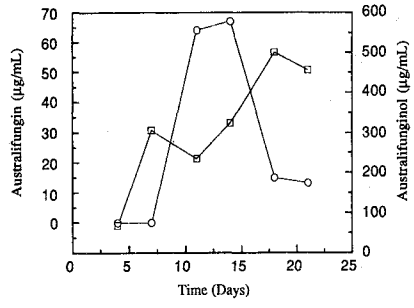
Table 2. Components of liquid production medium B.

Components	g/liter
Carbon source	75
Oat flour	15
Fibco yeast extract	5
L-Glutamic acid	4
MES	16.2

Carbon source was either mannitol or fructose. The medium was adjusted to pH 6.0 with sodium hydroxide before sterilization. [2-(*N*-morpholino)-ethanesulfonic acid], monohydrate (MES).

Fig. 2. Australifungin (**1**) and australifunginol (**2**) production by *S. australis* in solid corn-based medium A.

○ Australifungin, □ australifunginol.



Each point is a mean titer of three flasks analyzed by HPLC.

Fig. 3. Isolation scheme for australifungin (**1**) and australifunginol (**2**) from fermentations of *Sporormiella australis*.

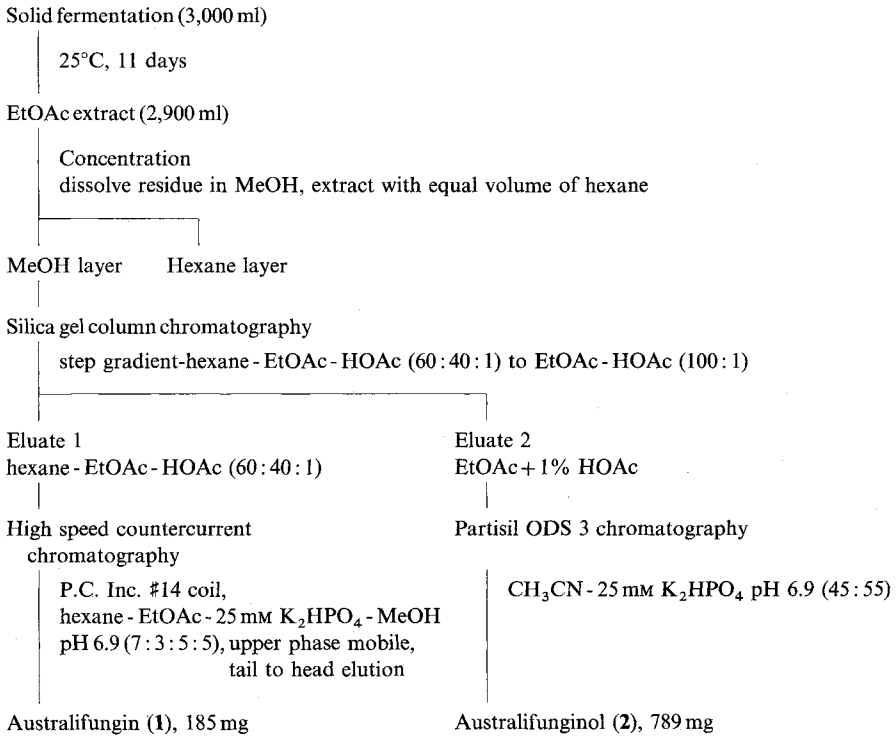


Table 3. Physico-chemical characteristics of australifungin (**1**) and australifunginol (**2**).

	Australifungin (**1**)	Australifunginol (**2**)
Appearance	White amorphous solid	White amorphous solid
Molecular formula	$C_{23}H_{36}O_6$	$C_{23}H_{38}O_6$
HREI-MS		
Calcd:	408.2512	410.2668
Found:	408.2515	410.2613
$[\alpha]_D^{20}$ (MeOH)	+98° (*c* 0.59)	+98° (*c* 0.99)
UV λ_{max}^{MeOH} nm (ε)	275 (13,100)	275 (11,600)
$\lambda_{max}^{MeOH-NaOH}$ nm (ε)	207, 287 (17,300), 306 (19,000)	275 (10,800)
IR (film on ZnSe) cm^{-1}	2929, 2797,1723 (C=O), 1666 (C=O), 1630, 1460, 1398, 1176, 1037	2958, 2932, 1698 (C=O), 1666 (C=O), 1635, 1397, 1041

property of the β-ketoaldehyde functionality. Final purification of **1** was accomplished using high speed countercurrent chromatography. A solvent system was developed by measuring the partition coefficient for 100 mg of **1** in a series of hexane-ethyl acetate-methanol-buffer systems. A solvent system (7 : 3 : 5 : 5) yielding a partition coefficient of 0.69 (organic/aqueous) was selected for chromatography.

The physico-chemical properties of **1** and **2** are summarized in Table 3 and the structure is shown in Fig. 1. ^1H and ^{13}C NMR data as well as details of the structure elucidation will be published elsewhere[4].

Biological Activity

Antifungal Activity

Antifungal activity against human pathogenic yeasts and filamentous fungi for **1** and **2** was evaluated in a microbroth dilution assay as shown in Table 4. Australifungin had MICs of 1 μg/ml or less against all of the species tested, with particularly good activity against *Candida pseudotropicalis*, *C. tropicalis*, and *Cryptococcus neoformans*. Much weaker activity was detected for australifunginol with MICs between 8 and 64 μg/ml. Both compounds were fungicidal in this assay (data not shown).

Sphingolipid Synthesis

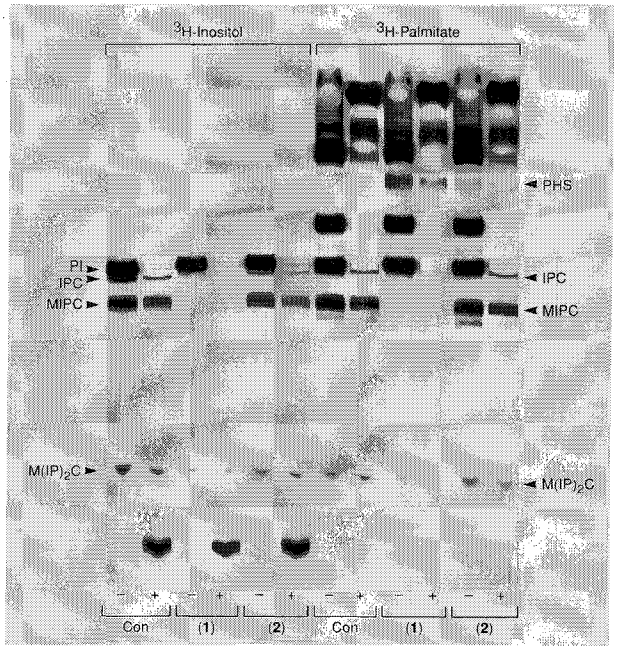
Investigations into the mechanism of antifungal activity for **1** revealed changes in lipid biosynthesis, with specific inhibition of the inositol-containing sphingolipids. Fig. 4 shows that **1**, at 0.25 μg/ml, almost completely inhibited ^{3}H-inositol incorporation into the deacylation resistant sphingolipids in *C. albicans*, with relatively little effect on phosphatidylinositol synthesis. When the cells were labeled with ^{3}H-palmitate, a precursor to the sphingolipid pathway, **1** inhibited incorporation into the mature sphingolipids but not the deacylation sensitive phospholipids. A new ^{3}H-palmitate-labeled lipid that comigrated with phytosphingosine accumulated with australifungin treatment (Fig. 4). A similar pattern of inhibition of the mature sphingolipids and accumulation of phytosphingosine was also found in ^{3}H-sphinganine and ^{3}H-serine labeled lipids (not shown). At 25 μg/ml, **2** had minimal effects on sphingolipid or phospholipid synthesis, although a small amount of phytosphingosine was visible in ^{3}H-palmitate labeled cells (Fig. 4) and ^{3}H-sphinganine labeled cells.

Table 4. Antifungal activity by microbroth dilution assay.

Pathogen	MIC (μg/ml)	
	Australifungin (**1**)	Australifunginol (**2**)
Candida albicans (MY1028)	0.25	32
C. albicans (MY1055)	0.5	ND
C. albicans (MY1750)	0.5	64
C. guillermondii (MY1019)	0.5	16
C. parapsilosis (MY1010)	0.5	64
C. pseudotropicalis (MY2099)	<0.015	16
C. tropicalis (MY1012)	0.031	32
Cryptococcus neoformans (MY1051)	0.062	8
C. neoformans (MY1146)	0.5	32
C. neoformans (MY2061)	<0.015	16
C. neoformans (MY2062)	0.125	16
Saccharomyces cerevisiae (MY1976)	0.25	16
Aspergillus fumigatus (MF4839)	1	32
A. fumigatus (5668)	0.125	32
A. fumigatus (5669)	1	32

Fig. 4. ^{3}H-Inositol and ^{3}H-Palmitate labeling of *C. albicans* lipids.

Absence of inhibitor (Con) or the presence of 0.25 μg/ml australifungin (**1**) or 25 μg/ml australifunginol (**2**).



Total lipid extracts (−) and lipids treated with mild alkali (+) were identified by co-migration with standards: phosphatidylinositol (PI), inositolphosphoceramide (IPC), mannosylinositol phosphoceramide (MIPC), mannosyldiinositoldiphosphorylceramide (M(IP)$_2$C), and phytosphingosine (PHS).

Table 5. Effects of australifungin (**1**) and australifunginol (**2**) on macromolecular synthesis.

Compound	Concentration (μg/ml)	Protein synthesis (% Control)	RNA & DNA synthesis (% Control)	Sphingolipid synthesis (% Control)
Australifungin (**1**)	0.008	NT	NT	61
	0.04	98	76.8	50
	0.2	111	79.2	0
	1.0	103	78.5	0
	5.0	109	61	NT
	25.0	2.8	6.5	NT
Australifunginol (**2**)	0.04	83.6	78.1	NT
	0.2	80.3	75.4	NT
	1.0	84.5	75.8	126
	5.0	88.4	89.5	93
	25.0	106	83.3	69
Verrucarin	1.0	68.1	63.2	NT
	5.0	7.9	69.3	152

NT = not tested.

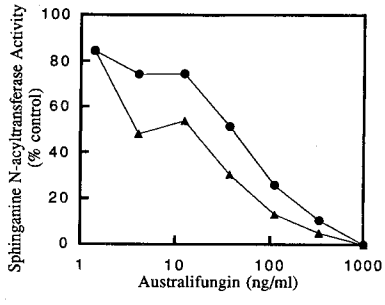
Dose Response on Macromolecular Synthesis

The specificity of inhibition of sphingolipid synthesis by **1** was tested by comparing its dose response on macromolecular synthesis as measured by pulse labeling with ^{3}H-leucine for protein synthesis, ^{14}C-adenine for RNA and DNA synthesis, and ^{3}H-sphinganine for sphingolipid synthesis (Table 5). Compound **1** did not significantly affect protein or nucleic acid synthesis at concentrations of 5 μg/ml or less, but was inhibitory at 25 μg/ml, a concentration that is well above the MIC. Sphingolipid synthesis was inhibited at much lower concentrations of **1** with 50% inhibition measured at 0.04 μg/ml. Compound **2** was not inhibitory to protein or nuclei acid synthesis at 25 μg/ml, but weakly inhibited sphingolipid synthesis.

In Vitro Enzyme Activity

The accumulation of phytosphingosine with australifungin treatment suggested a block at ceramide synthesis; this was confirmed in an *in vitro* enzyme assay for sphinganine *N*-acyltransferase. The assay, which employs ^{3}H-sphinganine and lignoceroyl-CoA as substrates with microsomal membranes prepared from *C. albicans*, results in the *in vitro* synthesis of two products, one of which comigrates with lignoceroyl sphinganine and releases ^{3}H-sphinganine upon acid hydrolysis, and the other which releases ^{3}H-phytosphingosine upon acid hydrolysis. Australifungin inhibited the synthesis of both products, as shown in Fig. 5. The phytosphingosine-containing ceramide was slightly more sensitive to **1** with an IC_{50} of 15 ng/ml (37 nM); the IC_{50} for the sphinganine-containing form was 38 ng/ml (93 nM). As expected from the whole cell labeling results, inhibition by **1** was specific for the sphinganine *N*-acyltransferase. The first enzyme in the sphingolipid pathway, the serine palmitoyltransferase, was not sensitive to 10 μg/ml of **1**. The less active analog, **2**, also inhibited the sphinganine *N*-acyltransferase *in vitro*, but was at least 50-fold less potent.

Fig. 5. *In vitro* inhibition of sphinganine-ceramide (●) and phytosphingosine-ceramide (▲) by australifungin (**1**).



Discussion

Australifungin is the first non-sphingosine-based inhibitor described for the sphingolipid biosynthetic pathway. Several natural product inhibitors have previously been discovered that have structural features resembling the sphingoid base intermediates. These compounds include the sphingofungins[11~13] and lipoxamycins[14] that inhibit the first enzyme in the pathway, as well as inhibitors of sphinganine *N*-acyltransferase, the fumonisins[3,15] and AAL toxins[16].

The fumonisins were initially isolated as tumor promoting agents found in contaminated corn feed[15] and are associated with severe toxicological effects such as equine leucoencephalomalacia and porcine pulmonary edema[17]. These compounds are the subject of intense examination to ascertain their natural occurrence and determine whether toxicity is mechanism based. Ceramide is the precursor to sphingomyelin, gangliosides, and glycosphingolipids, and is also a central intermediate in the metabolic pathway that may function as a lipid signal transduction pathway[18]. Ceramide and its derivatives, have been implicated as second messengers in cell differentiation, cell death by apoptosis, and cell proliferation[19]. Thus, disruption of ceramide synthesis by fumonisin, and the accumulation of the bioactive sphingoid bases has been suggested as the likely mechanism of toxicity and carcinogenicity[20]. Australifungin, which is structurally unrelated to sphingolipids and lacks a free amine group that in fumonisin, is critical for its toxic activity, should prove useful for dissecting the complex role of sphingolipids in cell regulation and evaluating the mechanism of fumonisin toxicity.

Australifungin is structurally related to several other phytotoxins. The β-ketoaldehyde functional group, which is relatively rare in a natural product, and the basic ring structure is shared by stemphyloxin (**3**)[21] and betaenone C (**4**)[22]. Reduction of the β-ketoaldehyde to the alcohol in betaenone B (**5**) reduces potency, as we have found for australifunginol (**2**). The mechanism of phytotoxic activity of the betaenones has not been reported, while stemphyloxin has been proposed to interfere with iron metabolism as it is a chelating agent[21]. Although lacking the β-ketoaldehyde group, structural similarity to the mycotoxin, diplodiatoxin[23], is also evident. Diplodiatoxin has been isolated from *Fusarium moniliforme*[24], the producer of the fumonisins[15], and like the fumonisins, is associated with severe toxicity in animals that consume contaminated corn. Inhibition of ceramide synthesis may be a mechanism common to all of these toxins.

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