SPHINGOFUNGINS A, B, C, AND D; A NEW FAMILY OF ANTIFUNGAL AGENTS

I. FERMENTATION, ISOLATION, AND BIOLOGICAL ACTIVITY

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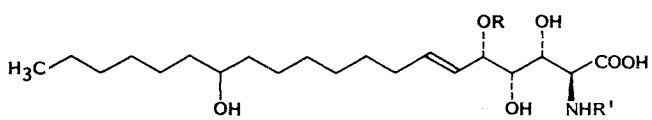
In screening for antifungal inhibitors from fungi, four related antifungal agents have been isolated from the cultivation of *Aspergillus fumigatus* ATCC 20857. These agents were initially produced by the microorganism growing on a solid millet-based medium. A liquid medium containing both glucose and glycerol has also been developed in which these antibiotics are produced in two phases. These novel compounds, sphingofungins A, B, C, and D, show a limited spectrum of antifungal activity but were especially effective against *Cryptococcus* species.

The recent increase in patients suffering from opportunistic infections[1] has further emphasized the need for safe and effective antifungal medications. In our efforts to identify new antifungal agents exhibiting novel modes of action, we found that a strain of *Aspergillus fumigatus* (ATCC 20857) produces four related antifungal agents which we have named sphingofungins A (**1**), B (**2**), C (**3**), and D (**4**). This paper describes the taxonomy, fermentation, isolation, and some biological properties of these sphingofungins. The structure elucidation of these compounds[2,3] as well as their novel mode of action[4] will be detailed elsewhere.

Identification of Producing Microorganism

The strain of *A. fumigatus* Fres. (ATCC 20857=MF 5038) was isolated from soil collected from a pasture in Young, Departmento Rio Negro, Uruguay. *A. fumigatus*, cosmopolitan in distribution, is a thermotolerant fungus, representing one of the most common microorganisms isolated from decomposing organic matter at elevated temperatures. Published descriptions of *A. fumigatus*[5~7] agree well with the key characters of the strain examined here. These characteristics include a uniseriate arrangement of the conidiogenous cells, columnar conidial heads, blue-green conidial masses and globose conidia. We observed



Sphingofungin A (**1**) R=H R'=C(NH)NH_2
Sphingofungin B (**2**) R=H R'=H
Sphingofungin C (**3**) R=Ac R'=H
Sphingofungin D (**4**) R=H R'=Ac

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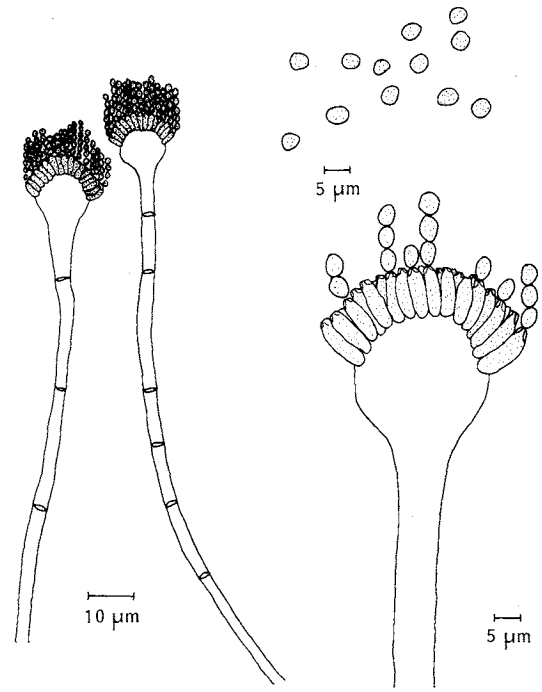
that the conidia are only finely roughened whereas *A. fumigatus* conidia are typically described with echinulate ornamentation. However, RAPER and FENNELL[7] reported many variations among strains of *A. fumigatus*. In the following description of colony morphology, capitalized color names are from RIDGEWAY[8]. The microscopic features are illustrated in Fig. 1.

Colonies on potato glucose agar at 25°C fast growing, attaining a diameter of 35~40 mm in 5 days. Mycelium varying from typically velvety to moderately floccose, white. Conidiogenesis confined to center of colony, dusky dull bluish green, dark bluish grey green, dark delft blue. Margin wide, up to 5 mm, hyaline, appressed, reverse light vinaceous lilac. Exudate and soluble pigment absent. At 37°C, colonies growing extremely fast, attaining a diameter of greater than 60 mm in 5 days, extremely floccose to cottony, white, slightly sulcate in center (as seen in reverse); conidiophores sparse, white; reverse pale vinaceous to hydrangea pink. Exudate and soluble pigment absent.

Fig. 1. *Aspergillus fumigatus* ATCC 20857.



Conidiophores and conidia on potato glucose agar.

Mycelium septate, 2~3 μm wide, hyaline in KOH. Conidial heads columnar, compact, usually 250~350 × 40 μm, usually colored. Conidiophores erect, very long, typically exceeding 5 mm in length by 4~5 μm in width, smooth, septate, arising from aerial hyphae and gradually enlarging upward into a vesicle. Vesicles globose to clavate, 15~20 μm in diameter. Conidiogenous cells strictly uniseriate, restricted to upper half of vesicle, 8~11 × 2~3 μm, with axes roughly parallel to the axis of the conidiophore. Conidia globose to subglobose, finely roughened, 3~3.5 μm. Sclerotia or cleistothecia absent.

Fermentation

Our initial experience in growing the culture for production of sphingofungins was on the solid millet-based medium A (Table 1) which supported a maximum titer of 23 units†/g of millet at 19 days.

Although this medium gave a satisfactory titer, a liquid medium was needed for production of these metabolites in fermenters. Past experience[9,10] has shown that high levels of a soluble carbohydrate along with one or more complex nitrogen sources are important factors in successfully converting a solid fermentation process to a liquid process for fungi. Based on this information liquid media were formulated with high levels of glycerol and various complex nitrogen sources. Our best liquid fermentation medium for production of sphingofungins is medium C (Table 2) which supports a titer of 406 units/ml at 15 days. Fig. 2 shows the fermentation time course, the utilization of glucose and glycerol, and broth pH in medium C over a 21-day period.

Antifungal activity initially is produced after glucose exhaustion at day 1 and increases as the glycerol

† See bioassay procedure in Experimental section for the definition of a unit.

Table 1. Components of the solid millet based fermentation medium (Medium A).

Component	Amount (per 250-ml flask)
Millet	15.0 g
Yeast extract	2.0 mg
KH_2PO_4	1.0 mg
$MgSO_4 \cdot 7H_2O$	1.0 mg
Sodium tartrate	1.0 mg
$FeSO_4 \cdot 7H_2O$	0.1 mg
$ZnSO_4 \cdot 7H_2O$	0.1 mg
Distilled water	10 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.

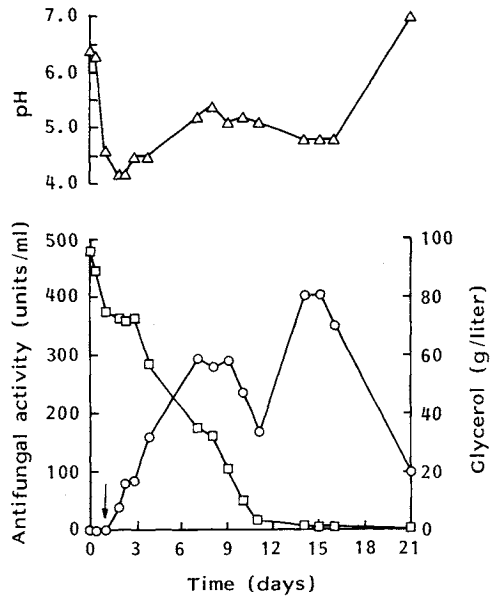
Table 2. Components of the liquid fermentation media.

Component	Medium B	Medium C
Glycerol	66.0 g	90.0 g
Glucose	10.0 g	10.0 g
Ardamine pH	5.0 g	5.0 g
$(NH_4)_2SO_4$	2.0 g	2.0 g
Soybean meal	5.0 g	5.0 g
PPG 2000	2.0 ml	2.0 ml
Tomato paste	5.0 g	5.0 g
Sodium citrate	2.0 g	2.0 g

Ingredients are given per liter of distilled water. In each case, pH of medium was adjusted to 7.0 before being distributed as 45 ml aliquots into 250 ml flasks and autoclaved. Post sterilization pH of medium C was 6.4.

Fig. 2. Time course for the production of sphingofungin activity during fermentation of *Aspergillis fumigatus* in medium C.

(Circles) antibiotic activity; (squares) residual glycerol concentrations; (triangles) pH of the culture. Each time point is the average determination from three flasks. An arrow marks the point at which glucose was exhausted.



concentration decreases. The antifungal activity curve appears to be biphasic and a second major increase in antifungal activity was observed after glycerol exhaustion at day 11. During the fermentation, the broth pH remained between 4.2 and 5.2 and increased only at the end of the fermentation when the antifungal activity decreased.

Isolation

Fermentations of this organism proved to be rich sources of biologically active natural products. Besides the four sphingofungins described herein, other compounds isolated from various regrowths of this organism were determined to be the known neurotoxin tryptoquivaline[11], linoleic acid, and a novel phospholipid[12].

The most abundant of the sphingofungins from this fermentation is sphingofungin C. Sphingofungins A, B, and D, although initially isolated and identified as natural products, were later prepared by synthetic transformations[3] of sphingofungin C in order to obtain sufficient quantities for biological evaluation. In fact, sphingofungin D is readily formed from sphingofungin C by a base catalyzed transacylation which occurs measurably under mildly basic conditions above pH 7.5.

Since the sphingofungins are partially mycelial bound, and this species is a known opportunistic pathogen, the whole broth was diluted with MeOH and extracted before separation of the solids. For the initial small scale isolations, the filtered extract was concentrated and purified by use of various chromatographic conditions including Amberlite XAD-2, silica gel, and Sephadex LH-20. The final separation

Table 3. Antifungal activity of sphingofungins A, B, C, and D compared to amphotericin B (minimum inhibitory concentrations in μg/ml).

Organism	Sphingofungin A	Sphingofungin B	Sphingofungin C	Sphingofungin D	Amphotericin B
Cryptococcus neoformans MY 1051	2	0.12	0.25	8	0.5
C. neoformans MY 1146	4	1	1	32	0.5
C. neoformans MY 2061	4	0.5	1	8	0.25
C. neoformans MY 2062	8	1	1	16	0.25
Candida albicans MY 1028	>128	>128	32	128	1
C. albicans MY 1055	>128	>128	32	128	1
C. albicans MY 1750	>128	>128	32	128	0.5
C. guilliermondii MY 1019	>128	>128	>128	>128	>128
C. parapsilosis MY 1010	32	8	16	128	1
C. pseudotropicalis MY 2099	2	0.25	2	16	0.5
C. tropicalis MY 1012	64	8	32	128	>128
Saccharomyces cerevisiae MY 1976	32	1	32	128	0.5
Aspergillus flavus MF 383	128	8	8	64	2
A. fumigatus MF 4839	>128	>128	>128	>128	2
A. fumigatus 10AF	128	>128	>128	>128	2
A. fumigatus ATCC 13073	128	>128	>128	>128	1

of the four related components was accomplished empolying reverse phase C-18 HPLC chromatography.

For larger scale liquid fermentations which produced a mixture of sphingofungins B and C at concentrations up to 10 mg/liter and 50 mg/liter, respectively, a five step isolation procedure was developed. After extraction with MeOH and removal of the solids by centrifugation, the aqueous filtrate was adsorbed onto an SP207 column. After elution of sphingofungins B and C with MeOH, the eluate was diluted with water and the pH of the solution was adjusted to 6.0. The solution was passed through a column of Dowex 1 (Cl^-) resin to remove contaminating carboxylic acids. The Dowex 1 eluant, containing sphingofungins B and C, was then adsorbed to Diaion HP-20 and eluted with a stepwise gradient of aqueous MeOH, which afforded both a concentration and a chromatographic cleanup. Polypropylene glycol 2000 (PPG), used as an antifoaming agent in the fermentation, and lipophilic contaminants were then removed by washing the aqueous MeOH solution with hexane-EtOAc. The MeOH and water were then removed to obtain a mixture of sphingofungins B and C with a combined purity of approximately 75%. Sphingofungins B and C could then be obtained separately by HPLC chromatography.

Antifungal Activity

As shown in Table 3, sphingofungins A, B, and C are potent antifungal agents especially against *Cryptococcus neoformans*. These three compounds also show selective activity against various *Candida* species and are essentially inactive against filamentous fungi and bacteria (data not shown). In general, sphingofungin D is much less potent than the other three compounds.

The sphingofungins show some similarities in structure to myriocin produced by an ascomycete[13], thermozymocidin produced by a thermophilic mold[14,15], fumifungin produced by *Aspergillus fumigatus*[16], and the fumonisins produced by *Fusarium moniliforme*[17].

Experimental

Fermentation

Vegetative mycelia of the culture were prepared by inoculating a 54-ml portion of seed medium[18], in

a 250-ml unbaffled Erlenmeyer flask with a lyophilized pellet of ATCC 20857. The resulting culture was incubated for 3 days on a rotary shaker at 28°C and 75% relative humidity. All cultures grown on a rotary shaker were agitated with a 5-cm throw at 220 rpm. A 2-ml portion of the 3-day culture was used to inoculate either a millet-based solid production medium A or liquid production media B and C (Tables 1 and 2). Production cultures were incubated at 25°C and 50% relative humidity for up to 21 days; all liquid production cultures were agitated on a rotary shaker and cultures on the solid medium were incubated under static conditions. Media A and B were used to provide material for the isolation of sphingofungins A, B, C, and D described below. To extract the sphingofungins, the cultures were shaken at 25°C with MeOH for 1 hour. Solid cultures received 45 ml of 70% MeOH while liquid cultures were diluted with equal volumes of MeOH.

Analysis of Sphingofungins

Sphingofungins A, B, C, and D can be quantitated by HPLC with detection at 205 nm, however, the presence of only a weak UV chromophore makes HPLC insensitive and requires a labor intensive concentration step prior to analysis. Due to this complication, a bioassay was the primary method of analysis for fermentation studies as well as for monitoring purification steps. The production of all four sphingofungins of different potencies (see Table 3), made interpretation of the analyses by bioassay ambiguous.

Bioassay Procedure

Disc diffusion assays on plates seeded with *Candida pseudotropicalis* (MY 2099, Merck Culture Collection) were used to measure antibiotic production. The indicator organism was maintained on Sabouraud dextrose agar (Difco) at 4°C with bimonthly transfers. Seeded agar plates were prepared from this culture as follows. Sabouraud dextrose broth (Difco) was inoculated with the MY 2099 stock, and the culture was incubated overnight (approx 18 hours) at 28°C, with agitation at 220 rpm. The turbidity of the culture was then adjusted with sterile water to a transmittance of 40% at 620 nm (Bausch and Lomb Spectronic 20 Spectrophotometer). The diluted culture was used as a 3.3% inoculum to seed potato glucose agar (300 ml, cooled to 52°C) which was then poured into glass assay plates (26 × 33 cm), allowed to harden, and refrigerated (4°C) until use. Assay samples and standards (10, 20, 40 μg/ml of sphingofungin C) (20 μl) were applied to 6.35 mm filter discs (Schleicher and Schuell) and air dried at room temperature prior to placement on seeded assay plates. Following incubation of the assay plates at 28°C for 18 hours, zones of inhibition were measured with a Bausch and Lomb Omnicon 2000 image analyzer; sample concentrations were calculated by comparison to the regression line computed from the zone diameters of the standards. The average standard concentration variability was 5%, and the assay sensitivity was 2 μg/ml for sphingofungin C. Sphingofungin B is 10 times more potent than either sphingofungin A or sphingofungin C, which in turn, are 100 times more potent than sphingofungin D against the bioassay organism MY 2099.

One unit of antifungal activity is defined as the activity equivalent to that produced by 1 μg of sphingofungin C in this bioassay. A test sample was considered to have an activity of 1 unit/ml if it produced a zone of inhibition equal to that produced by sphingofungin C at 1 μg/ml in this bioassay.

Carbohydrate Analysis

HPLC analysis of glucose and glycerol on a Bio Rad Aminex HPX-87H column (300 × 7.8 mm) was used to monitor carbohydrate utilization in the fermentation broths. The column was operated isocratically at 60°C with 0.009 N sulfuric acid at a flow rate of 0.6 ml/minute, and the effluent was monitored using an RI detector. Retention times for glucose and glycerol were 9.6 and 14.2 minutes, respectively.

Isolation

Isolation of Sphingofungin A

After 14 days growth, 90 ml of MeOH were added to each of fourteen 250-ml flasks, containing fermentations grown in millet-based medium A. The solids were mechanically broken up and the mixtures stirred for 1 hour. The mixtures were combined and filtered to obtain 1.8 liters of filtrate comprised of 65% MeOH - 35% water. The filtrate was concentrated *in vacuo* to a volume of 225 ml. A portion (18 ml) of the

concentrate was applied to an HPLC chromatography column (Dupont Zorbax ODS, 9.2 mm i.d. × 25 cm) and eluted with 75% MeOH - 25% water with a flow rate of 4 ml/minute. The material eluting from 11.0 to 11.9 minutes was combined and concentrated to dryness. The residue was redissolved in MeOH (1.5 ml), and chromatographed on a silica gel prep TLC plate (GF Analtech Uniplate) using a developing solvent of 90% CH_2Cl_2 - 10% MeOH. The band at the origin was recovered and eluted with MeOH. The MeOH eluate was concentrated to 0.5 ml, centrifuged, and the supernatant separated. Evaporation of the solvent resulted in a crude yellow oily residue (3.4 mg) identified by MS and NMR as sphingofungin A.

Isolation of Sphingofungins B and C

Two 1,000-liter fermentations grown in medium B were added to 2,000 liters of MeOH and the mixture was agitated for 24 hours. The mycelia were then separated using a Sharples centrifuge and the 3,900-liter of centrifugate was applied directly to an SP207 column (120 liters bed volume). The SP207 column was washed with 65% aqueous MeOH (5 × 60 liters), and the sphingofungins were eluted with 100% MeOH (fractions 1 ~ 5, 30 liters each, fractions 6 ~ 13, 60 liters each). The material showing antifungal disc diffusion activity *vs. Candida pseudotropicalis* MF 2099 (first 6 of the 100% MeOH fractions) was combined and diluted with water to 70% MeOH. This solution (410 liters) was buffered at pH 6.0 by adding 4 liters of 1.0 M potassium phosphate pH 6.0. Contaminating carboxylic acids were removed by passage of this solution through a Dowex 1 (Cl^-) column (40 liters bed volume), and washing the resin with 70% aqueous MeOH. The active eluant (500 liters) was then diluted with water to 43% MeOH (950 liters total volume) and applied to a Diaion HP-20 column (110 liters bed volume) equilibrated with 50% aqueous MeOH. The column was then washed with 50% aqueous MeOH (500 liters) and 60% aqueous MeOH (500 liters). The eluent was then changed to 100% MeOH and sixteen 20-liter fractions were collected. The antifungal activity eluted in fractions 2 ~ 8 and these were combined to give material that was approximately 50% pure, with the major contaminant being polypropylene glycol 2000. The methanolic solution was subjected to partitioning with hexane - EtOAc and the MeOH - water layer was concentrated *in vacuo* to obtain 65 g of 75% pure sphingofungin C.

A portion of this material (50 mg) was dissolved in 75% MeOH - 25% aqueous 0.1% H_3PO_4 (2 ml) and applied to an HPLC column (Vydac RP-C18, 20 mm i.d. × 25 cm) eluting with an isocratic mobile phase of 55% MeOH - 45% aqueous 0.1% H_3PO_4, at a flow rate of 9 ml/minute. The fractions eluting between 18 and 19 minutes were combined and the solvents were removed. The residue was dissolved in MeOH (2 ml), diluted with water (10 ml) and applied to an open RPC-18 column (2 ml). The column was washed with water and the sphingofungin B was eluted with MeOH. The solvents were removed to yield sphingofungin B (3 mg) as a cream colored solid identified by MS, ^1H NMR, and ^{13}C NMR.

The HPLC fractions eluting between 23 and 25 minutes were combined and desalted as above to yield sphingofungin C (28 mg) as a cream colored solid identified by MS, ^1H NMR, and ^{13}C NMR.

Preparation of Sphingofungin D from Sphingofungin C

Sphingofungin C (20 mg, 0.046 mmol) was dissolved in MeOH (4 ml) and triethyl amine (1 ml) was added. After stirring 20 minutes at room temperature, the solvents were removed *in vacuo*. The residue was dissolved in 5 ml MeOH and water (5 ml) was added with some clouding. The suspension was applied to an open RP-C18 column (2 ml) and eluted with 50% aqueous MeOH. The 50% MeOH fractions were combined and the solvents removed to yield sphingofungin D (15 mg) as a slightly yellowed glass. Sphingofungin D was identified by MS, ^1H NMR, and ^{13}C NMR.

Broth Microdilution Assay for MIC Determinations

Media: Yeast Nitrogen Base (Difco) with 1% glucose (YNBD) and Sabouraud dextrose agar (SDA) were used for MIC determinations.

Compounds: Test compounds were solubilized in 10% DMSO at 2,560 μg/ml. Amphotericin B was prepared according to the manufacturer's instructions and diluted to 2,560 μg/ml in distilled water. The compounds were then diluted to 256 μg/ml in YNBD and were serially diluted 2-fold yielding final drug concentrations ranging from 128 ~ 0.06 μg/ml. All tests were performed in duplicate.

Organisms: Yeasts included in the panel (see Table 3) were selected because of their resistance/susceptibility to known antifungal agents, virulence, source and clinical importance. Long-term

stock cultures were maintained in water and working stocks were maintained on SDA and transferred monthly.

Inoculum: Four-hour broth cultures from stocks were diluted to a turbidity equivalent to a 0.5 McFarland standard measured using a spectrophotometer set at 530 nm. This yielded a cell concentration of $1 \sim 5 \times 10^6$ colony forming units (cfu)/ml. Microplates (96-well) containing test compounds were inoculated using an MIC-2000 (Dynatech), which delivers 1.5 μl per well yielding a final inoculum per well of $1.5 \sim 7.5 \times 10^3$ cells. Drug-free growth control wells were included. Microplates were incubated at 35°C.

MIC Interpretation: Results were recorded as growth or no growth after 24 hours for *Candida* spp. and 48 hours for *Cryptococcus* spp. The MIC was defined as the lowest concentration of drug showing no visible growth.

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