SPHINGOFUNGINS E AND F: NOVEL SERINEPALMITOYL TRANSFERASE INHIBITORS FROM *Paecilomyces variotii*

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Over the years researchers have had little success in identifying fungicidal agents that are not toxic to mammalian cells. The lack of such antifungal therapeutics underlines the need to look for antifungal agents with new modes of action. While screening for antifungal agents, sphingofungins E and F were isolated from a fermentation of *Paecilomyces variotii* (ATCC 74097=MF 5537). Sphingofungins E (**1**) and F (**2**) are sphingosine-like compounds which inhibit serinepalmitoyl transferase, an enzyme essential in the biosynthesis of sphingolipids. These compounds are novel structures in the sphingofungin family of compounds. The first compounds in the sphingofungin family to be isolated[1~3] were also serinepalmitoyl transferase inhibitors[4]. These compounds bear a strong structural resemblance to myriocin (**3**), also referred to as thermozymocidin, an antifungal compound originally isolated approximately two decades ago[5,6] and to fumifungin[7].

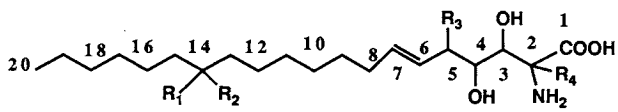
We report here on the producing organism, fermentation, isolation, structure determination and biological activity of these novel sphingofungins.

Paecilomyces variotii Bainier was isolated from the dung of a cottontail rabbit (*Sylvilagus floridanus*) collected in the Santa Catalina Mountains of Arizona. In the following description, the organism was observed on YpSs agar (Difco Laboratories), V8 juice agar (200 ml V8 juice from Campbell Soup Co., 0.3% $CaCO_3$, 2.0% agar in distilled H_2O), and CYA, Czapek yeast autolysate agar (yeast extract 0.5%, sucrose 3.0%, K_2HPO_4 0.1%, $NaNO_3$ 0.03%, KCl 0.005%, $MgSO_4 \cdot 7H_2O$ 0.005%, $FeSO_4 \cdot 7H_2O$ 0.0001%, 2.0% agar in distilled H_2O).

Colonies on YpSs, V8 juice, or CYA at 25°C, were slightly raised, densely velutinous to almost villose, with the margin minutely fimbriate, dull, dry, powdery in age, hyaline to white at the margin, soon developing a distinct pale yellow to yellow zone near the margin, becoming Straw Yellow (capitalized color names from RIDGWAY[8]), Naples Yellow, Deep Colonial Buff, finally light olivaceous brown to olivaceous brown, Olive-Ocher, Isabella Color, Ecru-Olive, Old Gold, Buffy Citrine, or Citrine-Drab. Colonies were similar in reverse, with slight aromatic odor. Exudates absent. Some sulcate medium buckling when colonies were grown at higher temperatures.

Conidiophores arising from surface and aerial mycelium, 80~450 μm tall, determinate, macronematous to semi-macronematous, mononematous, with 1 to 5 tiers of sparsely to densely arranged branches, forming either verticillate or penicillate groups of metulae and phialides, thin-walled, with walls smooth, with finely granular cytoplasm. Conidiogenous cells discrete, enteroblastic, phialidic, 8~35×2~4 μm, 1~2 μm wide at conidiogenous locus, single or in dense clusters on metulae. Conidia ellipsoidal, broadly ellipsoidal, to subglobose, 4~6×2.5~4 μm, smooth, adhering in long chains, with chains tangled and widely divergent, joined by faint connectives, hyaline in KOH, light olivaceous brown in mass. Hyphae septate, branched, smooth or occasionally incrusted in age, up to 7 μm in diameter. Chlamydospores abundant

Fig. 1. Comparison of sphingofungins E (**1**) and F (**2**), myriocin (**3**) and sphingofungin B (**4**).



1	R_1, R_2=O	R_3=OH	R_4=CH_2OH
2	R_1, R_2=O	R_3=OH	R_4=CH_3
3	R_1, R_2=O	R_3=H	R_4=CH_2OH
4	R_1, R_2=OH, H	R_3=OH	R_4=H

in older mycelium, arising directly from hyphae, pyriform to subglobose, smooth, with slightly thickened walls, 5~8 μm in diameter, with refractive cytoplasm. Cleistothecia and sclerotia absent.

Paecilomyces variotii is distinguished from other species in the genus by a combination of olivaceous brown to yellowish brown colony colors, absence of cleistothecia, variable conidia shape ranging from subglobose to ellipsoidal, and thermotolerant growth. The thermotolerant nature of the strain is observed by its relatively robust growth at 37 and 45°C. The radial growth rate (mm) of this organism was examined at 25, 37 and 45°C on YpSs, V8, and CYA agar over a one week period. While it grew well at all three temperatures, it was found to grow more rapidly on average at 37°C (46.5 mm i.d.) than at 25°C (28.5 mm i.d.) or 45°C (25.3 mm i.d.). This strain agrees with all essential anatomical characteristics as described by SAMSON[9]. *P. variotii* is a common and widely distributed organism. It has been reported as a saprobe from a variety of organic substrata, including soils, composts, decaying plants, as an agent of biodeterioration, as being associated with mycotoxicoses, and as an infectious agent causing paecilomycosis in man and animals.

A standardized inoculum of *P. variotii* ATCC 74097 was prepared by growing the fungus in 250-ml Erlenmeyer flasks containing 54 ml of the seed medium described previously[10]. The resulting culture was then mixed with an equal volume of 20% glycerol. The inoculum was stored in 2 ml portions at −80°C. This standard inoculum was diluted 28-fold into seed medium to prepare an inoculum for the production medium. Seed cultures were incubated at 28°C for 3 days with shaking at 220 rpm.

The solid production medium consisted of 10 g of brown rice, 20 mg of yeast extract (Difco), 10 mg of sodium tartrate, 10 mg of KH_2PO_4 and 20 ml of water in each 250-ml Erlenmeyer flask. This medium was steam sterilized at 1.05 kg/cm^2 for 20 minutes. The medium in each production flask was moistened with 10 ml of distilled water and subjected to a second steam sterilization immediately prior to inoculation. Production of the active compounds was accomplished by adding 2 ml of the seed culture described above to each production flask. Production cultures were incubated at 25°C for 14 days. At harvest, the antibiotics were extracted by the addition of 40 ml of methanol to each production flask. These flasks were shaken for 30 minutes at 25°C on a rotary shaker at 220 rpm.

Extracts from sixty 250-ml flasks of the solid fermentation described above were pooled. Following vacuum filtration through Celite the combined culture solids were extracted overnight in 1 liter of methanol then filtered through Celite. The filtered methanol extracts were combined, diluted to 20 : 80 methanol - water and adsorbed on a 4-liter column of Mitsubishi SP-207. The column was eluted with 40% aqueous acetone, 60% acetone and finally methanol. Sphingofungins E and F eluted with 60% acetone. Active fractions were concentrated by adsorption on a 75-ml SP-207 column and eluted with methanol. This methanol eluate was concentrated under vacuum and reconstituted in 250 ml of 10 : 90 methanol - water, adjusted to pH 4.4 and adsorbed on a 50-ml Dowex 50 (H^+) column. The sphingofungins eluted with 0.2 N aqueous pyridine. Final purification was achieved by repetitive semi-preparative HPLC on a Phenomenex ODS-30, 25 cm × 9.4 mm, column eluted with 70 : 30 methanol - 0.01 M phosphate buffer (pH 7), monitored by UV at 205 nm. Fractions were analyzed by analytical HPLC using the conditions described above. Fractions containing sphingofungin E (Rt = 10 minutes) were pooled and desalted by adsorption onto HP-20, and elution with methanol. Fractions containing sphingofungin F (Rt = 11 minutes) were similarly pooled and desalted on HP-20.

^{1}H, COSY, ^{13}C, APT and HETCOR data were recorded in CD_3OD on a Varian XL-300 NMR spectrometer. HMBC data was acquired on a Varian Unity 500 NMR spectrometer. UV spectra were taken on a Beckman DU-70 spectrophotometer and IR spectra on a Perkin-Elmer 1750 fourrier transfer spectrophotometer using ZnSe. Mass spectral data were obtained on a Finnigan MAT-212 mass spectrometer at 90 eV (EI-MS) and MAT-90 (FAB-MS).

The structure of sphingofungin E (**1**) is shown in Fig. 1. It was soluble in methanol and DMSO. The UV spectrum of this compound was found to have an absorption maximum at 205 nm (methanol, ε = 3,254) and an IR spectrum with absorbances at 3387, 2921, 1707, 1636, 1408, 1062 and 731 cm^{-1}. IR data suggested the presence of a carboxylic acid and hydroxyl groups.

^{1}H and COSY data allowed the structure to be assigned tentatively. The line assignments for the methine carbons bearing hydroxyls (C-3, C-4, C-5), the methylene and methyl groups were assigned using HETCOR data. The chemical shift of the hydroxy methyl group on C-2 was unambiguously assigned using HMBC data. See Table 1 for specific assignments.

Table 1. ^1H and ^{13}C NMR data for sphingofungin E (CD_3OD)[a].

	^{13}C	^1H
C-1	174.22	
CH_2OH	64.95	3.85 d (J=10.7 Hz)
		3.95 m
C-2	71.84	
C-3	70.30	3.95 m
C-4	76.21	3.65 d (J=7.0 Hz)
C-5	75.59	4.10 t (J=7.3 Hz)
C-6	130.18	5.45 dd (J=15.3, 7.3 Hz)
C-7	135.66	5.80 dt (J=15.3, 6.9 Hz)
C-8	33.39	2.10 br
C-9	30.17[d]	1.40 br
C-10	23.55	1.30 m
C-11	29.98	1.30 m
C-12	24.89[b]	1.55 br
C-13	43.49[c]	2.45 t (J=7.3 Hz)
C-14	214.63	
C-15	43.51[c]	2.45 t (J=7.3 Hz)
C-16	24.87[b]	1.55 br
C-17	30.10[d]	1.30 m
C-18	30.17[d]	1.30 m
C-19	32.78	1.30 m
C-20	14.33	0.9 t (J=6.7 Hz)

[a] Chemical shifts are given in ppm relative to the solvent peak at (^{13}C) and (^1H).
[b,c,d] Values are interchangeable.

Table 2. ^1H and ^{13}C NMR data for sphingofungin F (CD_3OD)[a].

	^{13}C	^1H
C-1	175.28	
CH_3	21.81	1.40 s
C-2	66.18	
C-3	72.45	3.87 br s
C-4	76.23	3.69 d (J=7.4 Hz)
C-5	75.71	4.11 t (J=7.4 Hz)
C-6	130.18	5.47 dd (J=15.2, 7.6 Hz)
C-7	135.67	5.76 dt (J=15.2, 6.6 Hz)
C-8	33.45	2.05 br
C-9	32.83	1.30 br
C-10	25.56	1.29 br
C-11	30.01	1.29 br
C-12	24.87[b]	1.55 br
C-13	43.48[c]	2.44 t (J=7.4 Hz)
C-14	214.63	
C-15	43.49[c]	2.44 t (J=7.6 Hz)
C-16	24.89[b]	1.55 br
C-17	30.04[d]	1.29 br
C-18	30.16[d]	1.29 br
C-19	30.18[d]	1.29 br
C-20	14.38	0.90 t (J=6.6 Hz)

[a] Chemical shifts are given in ppm relative to the solvent peak at (^{13}C) and (^1H).
[b,c,d] Values are interchangeable.

A molecular weight of 417 was assigned by FAB-MS and EI-MS. In the trimethylsilyl derivative, a strong ion corresponding to loss of water from the molecular ion was observed at m/z 615 (tris-trimethylsilyl derivative of 399). This corresponded to 399.2601 (calculated 399.2620) by HR-MS, which indicated $C_{21}H_{39}NO_7$ as a molecular formula. This was in agreement with the structure shown in Fig. 1. The *O*-methyl oxime was prepared by adding an excess of methoxyamine hydrochloride to the substrate in methanol and leaving the solution at room temperature for four hours. The methoxime was formed in order to confirm that the ketone group was located on C-14. Derivatizing the carbonyl to the methoxime and silylating gave a base peak at m/z 344 (HR-MS $C_{17}H_{32}NO_2$) (C-5 thru C-20 fragment) and m/z 142 (HR-MS $C_8H_{16}NO$) (C-14 thru C-20 fragment), confirming the proposed position of the carbonyl.

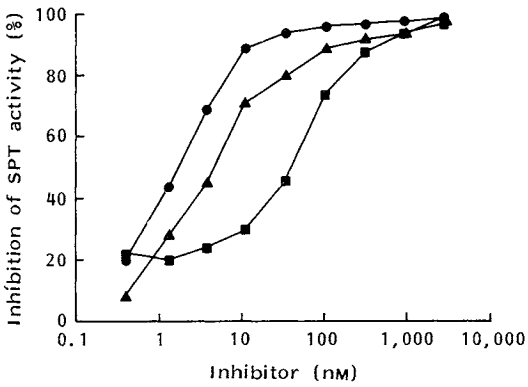
The structure of sphingofungin F (**2**) is shown in Fig. 1. It was soluble in methanol and DMSO. The UV spectrum of this compound was found to have a λ_{max} (methanol) = 205 nm (ε = 4,387). The structure was assigned based on ^1H, COSY, ^{13}C and APT NMR experiments in CD_3OD. Chemical shift data is presented in Table 2.

Mass spectral data were consistent with this structure. The molecular weight was determined to be 401 by FAB-MS and EI-MS. In the trimethylsilyl derivative an ion corresponding to loss of water from the molecular ion was observed at m/z 599 (trimethylsilyl derivative of 383). This corresponded to 383.2664 (calculated 383.2671) by HR-MS, which indicated $C_{21}H_{39}NO_6$ as the molecular formula. As the trimethylsilyl derivative, fragment ions indicated that the aliphatic chain was identical to that in sphingofungin E.

Sphingofungins E and F are clearly structurally similar to the sphingofungins which have been shown to be potent inhibitors of the first committed enzyme in the sphingolipid pathway, the serine-palmitoyl transferase[4]. The activity of sphingofungins E and F was compared to sphingofungin B in the *in vitro* enzyme assay for the serinepalmitoyl transferase using membranes prepared from *Saccharomyces cerevisiae*. Although these novel compounds were less potent than sphingofungin B, sphingofungins E and F inhibited the enzyme at nanomolar levels; the estimated IC_{50} values were 2.5 nM for sphingofungin B,. 7.2 nM for sphingofungin E, and 57 nM for sphingofungin F. These

Fig. 2. Inhibition of the serinepalmitoyl transferase of *Saccharomyces cerevisiae*.

Sphingofungin B (●), sphingofungin E (▲), and sphingofungin F (■).



Tested in the *in vitro* assay for the serinepalmitoyl transferase consisting of 100 mM HEPES pH 8.5, 2.5 mM EDTA, 0.1 mM palmitoylCoA, 0.1 mM pyridoxyl 5'-phosphate, 0.1 mM ^{3}H-serine, 5 mM DTT and 50 μg of microsomal membranes. After a 30 minutes reaction at 30°C, TCA precipitable cpm representing incorporation of label into ^{3}H-ketodihydrosphingosine were determined.

results are illustrated in Fig. 2.

From the limited set of natural product inhibitors of serinepalmitoyl transferase, a few conclusions can be drawn about structure activity relationships. All of these inhibitors resemble sphingolipid biosynthesis intermediates. Addition of either a hydroxyl or carbonyl functionality at the C-14 position, as well as addition of a carboxylic acid group at the C-2 position distinguish these inhibitors from the long chain base intermediates in the sphingolipid pathway. Changing the moiety at the C-2 position from a CH_2OH as in sphingofungin E to a methyl as in sphingofungin F adversely affects the potency of the inhibitor. Myriocin has all of the structural features of these compounds and is likely to be an inhibitor of the serinepalmitoyl transferase[6].

Bacteria do not make sphingolipids and as expected, this family of inhibitors does not have any antibacterial activity. The compounds do however, inhibit the mammalian serinepalmitoyl transferase; they are 5- to 20-fold more potent against the human placental enzyme than the enzyme from *Saccharomyces cerevisiae* (data not shown).

Sphingofungin E has antifungal activity against several human pathogenic fungi as shown in Table 3. The spectrum of inhibition is similar to that of the other sphingofungins[1] with good activity against

Table 3. Antifungal activity of sphingofungin E and sphingofungin B.

Test organism	Strain No.	MIC (μg/ml)	
		Sphingofungin E	Sphingofungin B
Candida albicans	MY 1028	32	32
C. albicans	MY 1055	>32	128
C. guillermondii	MY 1019	>32	>128
C. parapsilosis	MY 1010	32	2
C. pseudo-tropicalis	MY 2099	8	1
C. tropicalis	MY 1012	>32	16
Cryptococcus neoformans	MY 1051	1	0.25
Saccharomyces cerevisiae	MY 1976	8	1
Aspergillus flavus	MF 383	32	8
A. fumigatus	MF 4839	>32	128
A. fumigatus	10 AF	>32	>128

Cryptococcus neoformans and marginal activity against *Candida albicans*. Although *Aspergillus fumigatus* was not very sensitive, other filamentous fungi were strongly inhibited by sphingofungin E in a disk diffusion assay, including *Ceratocystis ulmi* and *Alternaria solani*.

Sphingofungin F was not active up to 32 μg/ml against any of the fungi shown in Table 3. Against a limited panel of yeast, weak activity was detected against *C. albicans* MY 1055, *C. neoformans* MY 1051, and *S. cerevisiae* MY 1976 when the dose was increased to 128 μg/ml (MIC values of 128, 64 and 128 μg/ml, respectively).

The reduced potency of sphingofungins E and F in antifungal activity relative to sphingofungin B is consistent with the higher IC_{50} values against the target enzyme. Inhibition of growth is a result of inhibiting the serinepalmitoyl transferase; the addition of the long chain base intermediates in the sphingolipid pathway abolished antifungal activity in a disk diffusion plate assay, as was previously demonstrated for the sphingofungins[4].

It is interesting to note that all of the sphingofungin-like compounds discovered to date have been isolated from a taxonomically diverse group of thermotolerant Ascomycetes. Sphingofungins E and F were produced by the thermotolerant fungus *P. variotii* while sphingofungins A, B, C, D and fumifungin were produced by another thermotolerant fungus, *Aspergillus fumigatus*. Myriocin was produced by the obligate thermophile *Myriococcum albomyces*. It is therefore tempting to speculate that

there may be an underlying ecological or environmental relationship between the thermotolerance of these fungi and their synthesis or use of these compounds.

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