Viridiofungins, Novel Inhibitors of Sphingolipid Synthesis

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Viridiofungins are broad spectrum antifungal agents that inhibit the squalene synthase *in vitro*, but do not specifically inhibit fungal ergosterol synthesis in whole cells, indicating a different mode of action for antifungal activity. In this report, we show that viridiofungins are potent *in vitro* inhibitors of serine palmitoyltransferase, the first committed enzyme in sphingolipid biosynthesis, and their antifungal activity is due to inhibition of sphingolipid synthesis. Additional related components with the same mode of action were isolated from the producing culture, *Trichoderma viride*, and inhibition of the serine palmitoyltransferase and antifungal activity is presented.

The isolation and structure of viridiofungins A, B, and C, members of a novel family of amino alkyl citrates from *Trichoderma viride* were presented previously[1]. The viridiofungins have potent, broad spectrum antifungal activity and were found to inhibit the yeast and rat squalene synthases at the micromolar level, but as described in the accompanying paper, antifungal activity was unrelated to inhibition of ergosterol synthesis[2]. Further characterization of their mode of action has revealed that the viridiofungins are nanomolar inhibitors of the first enzyme in the sphingolipid biosynthetic pathway, the serine palmitoyltransferase, and that inhibition of sphingolipid synthesis accounts for their antifungal activity. Related compounds were isolated as minor components from *T. viride*[3] and several semi-synthetic derivatives of viridiofungin A were made that inhibit the serine palmitoyltransferase. The viridiofungins are structurally distinct from previously described inhibitors of this enzyme, the sphingofungins[4~6], ISP-1/myriocin[7], or the lipoxamycins[8].

Materials and Methods

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 $OD_{600}=7\times10^{-4}$ (*ca.* 1×10^4 yeast cells or conidia/ml). Serial 2-fold dilutions of inhibitors were made from 32 μg/ml; the MIC value was the lowest concentration of inhibitor which prevented visible growth after 24 hours at 37°C.

Sphingolipid Synthesis

One-ml cultures of logarithmic phase *Candida albicans* (MY1055) cells in YNBD were pretreated with **A** at 1, 0.25, 0.062, or 0.015 μg/ml for 15 minutes at 37°C, and then 5 μCi/ml ^{3}H-palmitate was added. Labeling was terminated after a 1 hour incubation at 37°C by chilling the culture on ice. Stationary phase cells were added (0.5 ml) and a cell pellet was collected by centrifugation at $1,750\times g$ for 10 minutes. The cells were washed with 5 ml cold H_2O and fixed with 5% trichloroacetic acid for 15 minutes at 4°C. Following centrifugation, the lipids were extracted from the cell pellets and subjected to mild alkaline methanolysis and TLC analysis as described[9]. Radioactive bands were visualized by X-ray film (Kodak XAR5) after spraying the plates with EN^{3}HANCE (DuPont). The sphingolipids and phospholipids were identified by comigration with standards and quantitated by scraping the silica and counting in a Beckman liquid scintillation counter.

Phytosphingosine Reversal of Antifungal Activity

A culture of *C. albicans* (MY1055) was grown to 5×10^6 cells/ml and diluted 100-fold into Yeast Nitrogen Base-Glucose agar containing 0.025% tergitol, with or without 11.3 μM phytosphingosine or 15 μM stearylamine. After solidification, 5 to 10 μl of inhibitors suspended in DMSO were applied to the plates. Zone sizes were scored after 24 hours growth at 30°C.

In Vitro Enzyme Assays

Microsomal membranes from *C. albicans* (MY1055) and *Saccharomyces cerevisiae* (MY2141) were prepared from cells disrupted with glass beads (0.5 mm) in a Mini Bead Beater (Biospec Products, Bartlesville OK) as described[9]. HeLa cells were grown and a nuclear-free cytosolic extract was prepared as described by DIGNAM *et al.*[10]. Microsomal membranes were collected by ultracentrifugation ($100,000\times g$, 1 hour) and resuspended

in 50 mM HEPES pH 7.5, 1 mM DTT, 1 mM EDTA, 1 mM PMSF, 1 μg/ml each aprotinin, leupeptin, pepstatin, and 20% glycerol. Serine palmitoyltransferase activity was assayed in microtiter plates in 100 μl reactions containing 100 mM HEPES, pH 8.5, 2.5 mM EDTA, 0.1 mM pyridoxal 5'-phosphate, 0.1 mM ^{3}H-serine (1.0×10^6 cpm), 5 mM DTT, 0.2 mM palmitoyl-CoA, and 50 μg microsomal membranes. After a 2 hour incubation at 37°C the reaction was terminated with an equal volume of 18% trichloroacetic acid and the precipitated ^{3}H-ketodihydrosphingosine was collected on glass fiber filters (Wallach) using a plate harvester (Cambridge Technology, Inc.) and counted in a BetaPlate liquid scintillation counter (Wallach). Sphinganine *N*-acyltransferase activity was assayed as described[9].

Partial Esterification of Viridiofungin A

Acid catalyzed, partial esterification of viridiofungin A was observed upon storage at high concentration in CH_3OH. The products were purified using preparative reverse phase HPLC and characterized by ^{1}H, ^{13}C NMR, UV and MS.

Viridiofungin A, 1-Methyl Ester (**1**)

^{1}H NMR (8.6 mg in 0.7 ml CD_3OD, 300 MHz): δ 0.90 (t, 7.0, 3H), 1.29 (m, 14H), 1.53 (m, 4H), 1.90 (m, 2H), 2.44 (t, 8.3, 4H), 2.59 (d, 15.6, 1H), 2.88 (dd, 8.7, 14.3, 1H), 2.90 (d, 15.5, 2H), 3.13 (dd, 4.3, 14.3, 1H), 3.21 (d, 7.6, 1H), 3.64 (s, 3H, $-OCH_3$), 4.61 (dd, 4.2, 8.7, 1H), 5.54 (m, 2H), 6.67 (d, 8.2, 2H), 7.03 (d, 9.1, 2H). ^{13}C NMR (8.6 mg in 0.7 ml CD_3OD, 75 MHz): δ 14.4, 23.7, 24.8, 24.9, 29.8, 29.9, 30.1, 30.26, 30.29, 32.9, 33.5, 37.5, 43.1, 43.5, 52.2 ($-OCH_3$), 55.1, 57.7, 78.0, 116.2, 124.4, 128.7, 131.3, 137.6, 157.4, 172.0, 173.8, 174.5, 175.5, 214.6. UV (CH_3OH): 225, 277 nm. EI-MS: 605 *m/z* (M^+), silylation yielded a tetra-TMS derivative and EI-MS fragmentation was consistent with methylation at C-1.

Viridiofungin A, 1'-Methyl Ester (**2**)

^{1}H NMR (22 mg in 0.7 ml CD_3OD, 300 MHz): δ 0.90 (t, 7.0, 3H), 1.29 (m, 14H), 1.53 (m, 4H), 1.99 (m, 2H), 2.44 (t, 7.2, 4H), 2.61 (d, 16.2, 1H), 2.89 (dd, 8.7, 13.8, 1H), 2.92 (d, 16.2, 2H), 3.07 (dd, 5.4, 14.1, 1H), 3.22 (d, 8.4, 1H), 3.715 (s, 3H, $-OCH_3$), 4.62 (m, 1H), 5.54 (m, 2H), 6.68 (d, 8.7, 2H), 7.00 (d, 8.7, 2H), 8.23 (br d, 8.1, N*H*). UV (CH_3OH): 225, 277 nm. FAB-MS: 605 *m/z* (M^+); silylation yielded a tetra-TMS derivative and EI-MS fragmentation was consistent with methylation at C-1'.

Viridiofungin A, 1,1'-Dimethyl Ester (**3**)

^{1}H NMR (12 mg in 0.7 ml CD_3OD, 300 MHz): δ 0.90 (t, 7.0, 3H), 1.29 (m, 14H), 1.53 (m, 4H), 1.99 (m, 2H), 2.44 (t, 7.5, 4H), 2.59 (d, 16.2, 1H), 2.88 (dd, 9.0, 14.1, 1H), 2.92 (d, 16.2, 2H), 3.08 (dd, 5.1, 13.8, 1H), 3.22 (d, 7.5, 1H), 3.65 (s, 3H, $-OCH_3$), 3.717 (s, 3H, $-OCH_3$), 4.62 (m, 1H), 5.54 (m, 2H), 6.67 (d, 8.4, 2H), 7.00 (d, 8.4, 2H), 8.23 (br d, 7.8, N*H*). UV (CH_3OH): 225, 277 nm. FAB-MS: 619 *m/z* (M^+); silylation yielded a tri-TMS derivative and EI-MS fragmentation was consistent with methylation at C-1 and C-1'.

Preparation of 5,6-Dihydro-viridiofungin A (**4**)

Viridiofungin A (83 mg) was dissolved in EtOAc (3 ml) and stirred with 10% Pd on carbon (40 mg) under 1 atm hydrogen for 72 hours. ^{1}H NMR (7 mg in 0.7 ml CD_3OD, 300 MHz): δ 0.90 (t, 7.0, 3H, H-20), 1.30 (m, 20H), 1.54 (m, 2H, H-11, 15), 2.44 (t, 7.3, 2H, H-12*), 2.45 (t, 7.3, 2H, H-14*), 2.55 (dd, 3.4, 11.7, 1H, H-4), 2.63 (d, 16.1, 1H, H-2a), 2.83 (dd, 10.4, 14.3, 1H, H-3a'), 2.94 (d, 16.1, 1H, H-2b), 3.18 (dd, 4.4, 14.3, 1H, H-3b'), 4.68 (m, 1H, H-2'), 6.69 (d, 8.5, 2H, H-6', 8'), 7.08 (d, 8.5, 2H, H-5', 9'), 8.30 (d, 8.3, 1H, N*H*).

Results

Sphingolipid Inhibition

Viridiofungins A (**A**), B (**B**), and C (**C**) are alkyl citrate compounds amide linked to tyrosin, phenylalanine, or tryptophan, respectively[1]. Fig. 1 shows the structures of **A**, **B**, and **C**, and additional members of the viridiofungin family that were isolated as minor components from fermentations of *T. viride*[3]. The structures of semisynthetic derivatives of **A** are also shown.

Although the viridiofungins were initially isolated as inhibitors of squalene synthase, characterization of their mode of action as antifungal agents led to the discovery that **A**, **B**, and **C** did not specifically inhibit ergosterol synthesis[2], but did inhibit sphingolipid synthesis. Table 1 shows that sub-MIC concentrations of **A** (≤ 1.0 μg/ml), inhibited ^{3}H-palmitate incorporation into deacylation resistant sphingolipids in *C. albicans*, with relatively little effect on phospholipid synthesis. Sphingolipid synthesis was inhibited approximately 50% at 0.062 μg/ml **A**. There was no visible accumulation of early sphingolipid intermediates, unlike treatment with australifungin, an inhibitor of the sphinganine *N*-acyltransferase, that causes the accumulation of phytosphingosine and dihydrosphingosine[9]. These data are consistent with inhibition of the serine palmitoyltransferase and was confirmed by potent inhibition of this enzyme in an *in vitro* assay.

The serine palmitoyltransferase from *C. albicans* was inhibited by **A** with an IC_{50} of 0.013 μg/ml (22 nM), and similar activity was measured with **B** and **C** as shown in Table 2. The serine palmitoyltransferase from HeLa microsomes was somewhat more sensitive, but the enzyme from *S. cerevisiae* was surprisingly resistant to the viridiofungins. *S. cerevisiae* is less sensitive to the viridiofungins in MIC assays[2], and the level of inhibi-

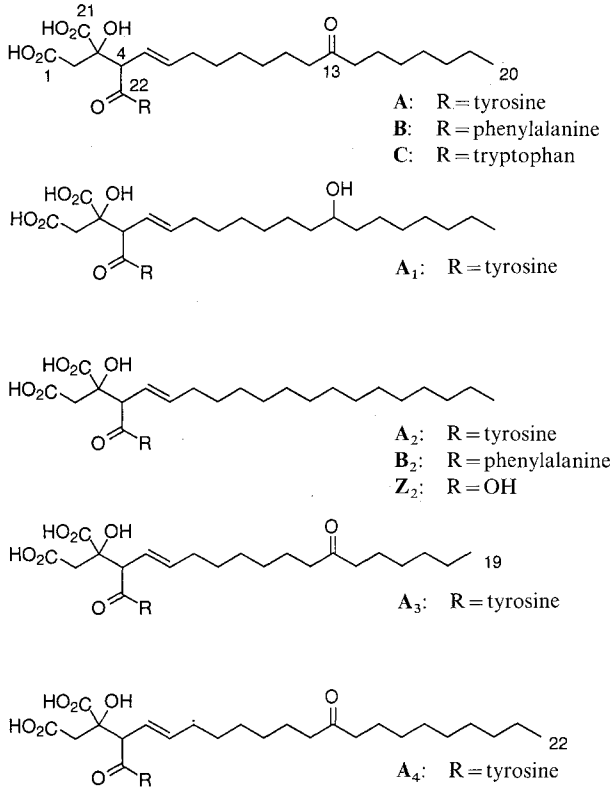
tion against the serine palmitoyltransferase is on the same order of magnitude as inhibition of the squalene synthase, suggesting a lack of specificity in this organism. The difference in sensitivity between *C. albicans* and *S. cerevisiae* is unique to this class of compound as sphingofungin B had similar potency against both fungal serine palmitoyltransferase enzymes (Table 2). The sphinganine *N*-acyltransferase was not inhibited by the viridiofungins.

The potent *in vitro* inhibition of the serine palmitoyltransferase and inhibition of whole cell sphingolipid synthesis at MIC levels pointed to inhibition of the serine palmitoyltransferase as the likely mechanism of antifungal activity for the viridiofungins. This mechanism was confirmed by reversal of antifungal activity with phytosphingosine. Phytosphingosine, a long chain base intermediate in the sphingolipid pathway, bypasses the block at the first enzyme, and as shown in Table 3, almost completely reversed the growth inhibitory activity against *C. albicans* of **A**, **B**, and **C**, and sphingofungin B. Stearylamine did not reverse antifungal activity indicating that this effect was specific for the sphingolipid intermediate. As expected, the antifungal activities of a sphinganine *N*-acyltransferase inhibitor, australifungin[9], and a fatty acid synthesis inhibitor, cerulenin, were not reversed by phytosphingosine. Phytosphingosine reversal of antifungal activity of viridiofungins against one of the most sensitive fungi, *Ustilago zeae*[2] was also detected (data not shown).

The minor components that were isolated from the *T. viride* producing culture primarily showed substitutions in the alkyl chain. Table 4 shows the potency of the natural product analogs at inhibiting the *C. albicans* serine palmitoyltransferase and in whole cell MIC assays.

Fig. 1. Structures of viridiofungins.

(A) Natural product viridiofungins



(B) Semisynthetic viridiofungins

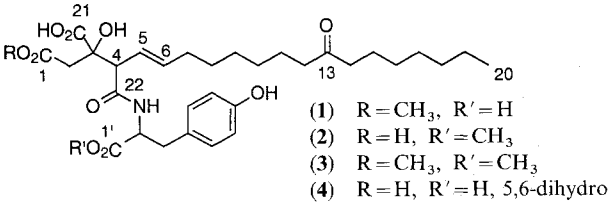


Table 1. Inhibition of ^{3}H-palmitate into lipids of *C. albicans* by viridiofungin A.

Concentration (μg/ml)	^{3}H-palmitate incorporation (% control)	
	Sphingolipids	Phospholipids
1.0	18	81
0.25	22	109
0.062	53	92
0.015	137	130

Table 2. Inhibition of serine palmitoyltransferase activity by viridiofungins.

Compound	Serine palmitoyltransferase IC_{50} (μg/ml)			Sphinganine *N*-acyltransferase IC_{50} (μg/ml)
	C. albicans	*S. cerevisiae*	HeLa	*C. albicans*
Viridiofungin A	0.013	2.8	0.003	>50
Viridiofungin B	0.010	1.1	0.003	>50
Viridiofungin C	0.015	2.7	0.003	>50
Sphingofungin B	0.019	0.020	0.001	>50

Table 3. Antifungal activity of viridiofungins with phytosphingosine (PHS) or stearylamine (STA).

Inhibitor	Zone size (mm)		
	Control	PHS	STA
Viridiofungin A (100 μg)	35	10	34
Viridiofungin B (100 μg)	32	10	18
Viridiofungin C (100 μg)	27	11	25
Sphingofungin B (10 μg)	21	0	20
Australifungin (10 μg)	30	26	25
Cerulenin (5 μg)	30	32	30

Table 4. IC_{50} of serine palmitoyltransferase activity and MIC against *C. albicans*.

Compound	IC_{50} (ng/ml)	MIC (μg/ml)
A	13.1	4.0
$\mathbf{A_1}$	14.2	8.0
$\mathbf{A_2}$	16.4	2.0
$\mathbf{A_3}$	30.8	>32
$\mathbf{A_4}$	3.2	2.0
B	10.3	>32
$\mathbf{B_2}$	62.5	>32
C	15.5	8
$\mathbf{Z_2}$	3817	16
1-C-1 methyl ester	412	>32
2-C-1′ methyl ester	16.7	16
3-C-1,1′ dimethyl ester	465	>32
4-5,6-dihydro	9.1	32

Structure activity relationships in the tyrosine series indicated that loss of the C-13 ketone ($\mathbf{A_2}$) or reduction to a hydroxyl group ($\mathbf{A_1}$) had minimal effects on *in vitro* or whole cell potency. Shortening the alkyl chain to C-19 ($\mathbf{A_3}$) was slightly deleterious to activity, while lengthening the chain to C-22 ($\mathbf{A_4}$) yielded the most potent compound. The phenylalanine (**B**) and tryptophan (**C**) containing analogs had similar *in vitro* potencies to the tyrosine compound (**A**), but lower whole cell antifungal activity. **B** gave a zone of inhibition in agar diffusion assays against *C. albicans* (Table 3), but did not completely prevent fungal growth in the liquid MIC assay. Loss of the amino acid ($\mathbf{Z_2}$) resulted in extremely poor inhibition of the serine palmitoyltransferase. Thus, the antifungal activity of $\mathbf{Z_2}$ may be due to a secondary mechanism. In addition to the natural products, structure activity relationships were also derived from semisynthetic analogs of **A** that were prepared: monomethyl ester derivatives at C-1 (**1**) and C-1′ (**2**), a C-1, C-1′ dimethyl ester (**3**), and a 5,6-dihydro (**4**) derivative. Table 4 shows that for inhibition of the serine palmitoyltransferase, a methyl ester at C-1′ was tolerated, but modification at the C-1 position in the mono- or dimethyl ester resulted in 30-fold lower potency. Hydrogenation of the alkyl chain had little effect on *in vitro* potency.

Discussion

Viridiofungins inhibit the serine palmitoyltransferase of *C. albicans* at nanomolar concentrations, specifically inhibit ^{3}H-palmitate incorporation into sphingolipids, and show phytosphingosine reversal of growth inhibition, thus indicating that the anti-*Candida* activity of viridiofungins is due to inhibition of the first enzyme in the sphingolipid biosynthetic pathway. In contrast, the mechanism of action against a less sensitive fungus, *S. cerevisiae*, is less clear, where the inhibition of the serine palmitoyltransferase and the squalene synthase is in the micromolar range. The antifungal activity in *S. cerevisiae* may be the result of multiple mechanisms. Structurally, the viridiofungins and other natural product inhibitors of the serine palmitoyltransferase, the sphingofungins[4~6], myriocin[7], and lipoxamycins[8], have a long alkyl chain in common that is unsaturated near the polar head group and modified by a carbonyl or hydroxy group at C-13 or C-14. Structure-activity-relationships derived from all of the natural-product viridiofungins and semisynthetic derivatives reveal that changes in the length of the alkyl chain, the C-13 functional group, or hydrogenation result in relatively modest differences in *in vitro* potency. Unlike the other inhibitors of the serine palmitoyltransferase, the viridiofungins are citric acid based and contain a hydrophobic amino acid. These functional groups are important for inhibition of the enzyme as the C-1 methyl ester (**1**) is 30-fold less potent, while loss of the amino acid ($\mathbf{Z_2}$) results in a 300-fold loss in potency.

Sphingolipids and their metabolic intermediates have recently been described as lipid second messengers involved in cell differentiation, apoptosis, and proliferation[11,12]. In addition to their antifungal properties, natural product inhibitors of the sphingolipid biosynthetic pathway have been isolated as modulators of cellular proliferation. Myriocin, an inhibitor of the serine palmitoyltransferase has immunosuppressive activity[7], and the fumonisins, inhibitors of the sphinganine *N*-acyltransferase[13], were isolated as tumor promoters[14]. The viridiofungins are an interesting new family of inhibitors of the sphingolipid pathway that should help to elucidate the role of these lipids in cell signaling.

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