INHIBITION OF SERINE PALMITOYL-TRANSFERASE ACTIVITY BY LIPOXAMYCIN

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(Received for publication September 20, 1993)

Serine palmitoyltransferase condenses serine with a fatty acyl-CoA to form ketodihydrosphingosine in the first committed step of sphingolipid biosynthesis. A family of novel compounds, called the sphingofungins, have recently been discovered to be the first specific inhibitors of this enzyme[1~3]. Initially identified as antifungal agents, the sphingofungins resemble the long chain base intermediates in the sphingolipid pathway and inhibit the serine palmitoyltransferase at the nanomolar level. Thus far, the sphingofungins have been isolated from two species of thermotolerant fungi: *Aspergillus fumigatus* which produces sphingofungins A, B, C and D[1] and *Paecilomyces variotii* which produces sphingofungins E and F[3]. Two other, previously identified antifungal compounds, myriocin[4,5] and fumifungin[6], are likely to be serine palmitoyltransferase inhibitors based on their strong structural resemblance to the sphingofungins; they are also produced by thermotolerant fungi. In this paper, we report on the identification of the first *Streptomyces* products that inhibit the enzyme. Lipoxamycin was discovered 22 years ago as an antifungal compound with an unknown mechanism of action[7,8]. We have now found that lipoxamycin and hydroxylipoxamycin, an analog coproduced in the fermentation, are very potent inhibitors of serine palmitoyltransferase, and inhibition of this enzyme accounts for their antifungal activity.

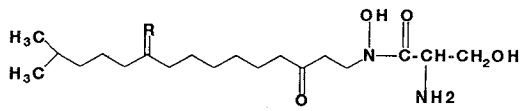
A standardized inoculum of the actinomycete Merck Culture Collection Number MA 6975 was prepared by growing the culture in 250-ml Erlenmeyer flasks containing 54 ml of the seed medium A. Medium A contained: 1 g of glucose, 10 g of dextrin, 3 g of beef extract, 5 g of Ardamine PH, 50 mg of $MgSO_4 \cdot 7H_2O$, 0.5 g of $CaCO_3$ and 2 ml of phosphate buffer in 1 liter of distilled water, final pH 7.3 with NaOH. Phosphate buffer contained 95 g of Na_2HPO_4 and 91 g of KH_2PO_4 per liter of distilled water. Medium A was distributed at 54 ml per baffled 250-ml Erlenmeyer flask. All fermentation media were steam sterilized at 15 psi for 20 minutes. After growth at 28°C for 3 days with agitation (220 rpm) the resulting culture was 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 B to prepare an inoculum for the production medium. Seed medium B contained: 22 g of lard water, 15 g of whey, 10 g of Ardamine PH, 75 g of cerelose, 1 g of $MgSO_4 \cdot 7H_2O$, 0.15 mg of $CoCl_2 \cdot 6H_2O$ and 2 g $CaCl_2$ in 1 liter of distilled water. Seed medium B was distributed at 54 ml per baffled 250-ml Erlenmeyer flask. Seed cultures were incubated at 28°C for 3 days with shaking at 220 rpm.

Liquid production medium C consisted of 10 g of beta-cyclodextrin, 40 g of dextrin, 7 g of distillers solubles and 5 g of yeast extract per liter of distilled water. Forty four ml of this medium was distributed into 250-ml unbaffled Erlenmeyer flasks. Production of antifungal activity was accomplished by adding 2 ml of the seed culture described above to each production flask. Production cultures were incubated at 28°C for 4 days. Antifungal activity was produced in the culture filtrate and could conveniently be detected using the formation of zones of inhibition against *Candida* and *Penicillium*.

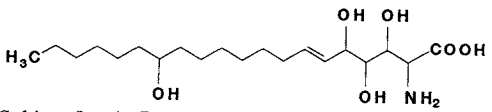
Two liters of the fermentation described above were centrifuged for 1 hour at 8,000 rpm. The supernatant was adsorbed onto a column packed with 150 ml of Dowex 50 (sodium form). The lipoxamycins were eluted from the column with 0.2 N ammonia. Active fractions were adsorbed onto a column packed with 150 ml of Amberlite XAD-2 (ROHM and HAAS), the column was rinsed with water and the lipoxamycins were eluted with methanol.

The active XAD-2 eluate was concentrated to dryness under vacuum and reconstituted in 60:40 methanol-0.01 M phosphate buffer (pH 7). Final purification was achieved by repetitive preparative HPLC on a Zorbax RX C-8 (21.2×250 mm) column eluted with 60:40 methanol-0.01 M phosphate buffer (pH 7), 10 ml/minute, monitored by UV absorption at 205 nm. Fractions were analyzed on an analytical Zorbax RX C-8 column (4.6×250 mm), eluted with 60:40 methanol-0.01 M phosphate buffer (pH 7), 1 ml/minute, monitored by UV absorption at 205 nm. Fractions containing lipoxamycin (Rt 21 minutes) were pooled and

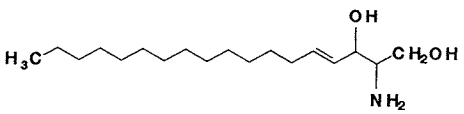
Fig. 1. Structures of lipoxamycins, sphingofungin B, and sphingosine.



Lipoxamycin R=O
Hydroxylipoxamycin R=OH, H



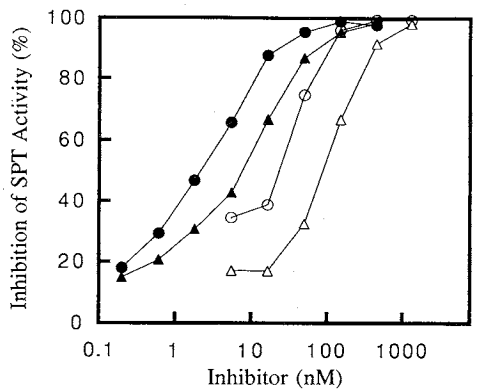
Sphingofungin B



Sphingosine

Fig. 2. Inhibition of serine palmitoyltransferase (SPT) activity.

Lipoxamycin (●, ○) and hydroxylipoxamycin (▲, △) were tested in the SPT assay[3] using microsomal membranes prepared from *Saccharomyces cerevisiae* (open symbols) and HeLa cells (closed symbols).



desalted by adsorption onto XAD-2 and elution with methanol yielding 1.5 mg. Fractions containing hydroxylipoxamycin (Rt 25 minutes) were similarly pooled and desalted on XAD-2 yielding 1.9 mg. The compounds were found to be unstable, most probably oxidizing over time[7].

UV spectra were recorded in methanol on a Beckman DU-70 spectrophotometer. IR spectra were recorded as neat samples on a ZnSe multiple internal reflectance (MIR) crystal using a Perkin-Elmer 1750 FT-IR spectrometer. Mass spectral data were obtained on a Finningan-MAT MAT-90, MAT-212 and JEOL HX110A instruments.

The structures of the lipoxamycins are shown in Fig. 1. The UV spectrum of lipoxamycin indicated an absorption maximum at 210 nm (methanol, ε 5,952). The IR spectrum of lipoxamycin showed absorbances for NH/OH at 3150~3400 cm^{-1} and C=O at 1709 and 1637 cm^{-1}. These UV and IR absorbances were in agreement with the published values[8]. The UV spectrum of hydroxylipoxamycin indicated a UV maximum at 208 nm (methanol ε 3,334). Hydroylipoxamycin exhibited NH/OH at 3150~3450 cm^{-1}, with a very weak absorbance at 1709 cm^{-1} which indicated that this compound had reduced carbonyl character compared to lipoxamycin.

The molecular formula for lipoxamycin, $C_{19}H_{36}N_2O_5$, was determined by HRFAB-MS (calculated for $(M+H)^+$ 373.2702, found 373.2723). Similarly HRFAB-MS afforded $C_{19}H_{38}N_2O_5$ for hydroxylipoxamycin (calculated for $(M+H)^+$ 375.2859, found 375.2842). Comparison of the trimethylsilyl derivatives of the lipoxamycins disclosed a unique ion at *m/z* 187 which corresponded to $[C_6H_{13}CH=OSi(CH_3)_3]^+$ in hydroxylipoxamycin. This finding, taken together with the IR data presented above allowed assignment of the structure as the C13 hydroxy analog of lipoxamycin. It is possible that the coproduced antibiotic found in the original isolation of lipoxamycin[8] was hydroxylipoxamycin.

Proton NMR data for lipoxamycin was obtained on a Varian 300 XL instrument in CD_3OD. A methyl doublet at δ 0.9 corresponding to the isopropyl methyls, aliphatic methylene multiplets at 1.3 and 1.6 ppm and a downfield methylene shift at 2.4 ppm representing methylenes adjacent to C=O were evident in the spectrum for lipoxamycin and are in agreement with the partial ^1H NMR data that has been reported for lipoxamycin[8].

Lipoxamycin and hydroxylipoxamycin have a long alkyl chain and an amino-containing polar head group, but otherwise do not resemble the sphingoid bases as closely as the sphingofungins do, as shown in Fig. 1. They are, however, potent inhibitors of the same enzyme. Inhibition of the serine palmitoyltransferase was measured *in vitro* using membranes prepared from *Saccharomyces cerevisiae* and cultured HeLa cells (Fig. 2). The estimated IC_{50} values against the fungal enzyme were 21 nM for lipoxamycin and 88 nM for hydroxylipoxamycin. Approximately 10-fold lower IC_{50} values were obtained against the HeLa enzyme. Thus, the lipoxamycins are on the same order of potency as the sphingofungins[2] and also have potent

Table 1. Antifungal activity of lipoxamycins and sphingofungin B.

Pathogen	MIC (μg/ml)		
	Sphingofungin B	Lipoxamycin	Hydroxylipoxamycin
Candida albicans (MY1028)	16	1	2
C. albicans (MY1750)	32	4	4
C. guillermondii (MY1019)	>32	16	16
C. parapsilosis (MY1010)	32	2	4
C. pseudotropicalis (MY2099)	1	4	4
C. tropicalis (MY1012)	16	0.5	0.5
Cryptococcus neoformans (MY1051)	0.25	0.25	0.5
C. neoformans (MY1146)	0.5	0.5	2
C. neoformans (MY2061)	0.25	0.5	1
C. neoformans (MY2062)	0.5	0.5	1
Saccharomyces cerevisiae (MY1976)	>32	16	32
Aspergillus fumigatus (MF4839)	>32	>32	>32
A. fumigatus (5668)	>32	>32	>32
A. fumigatus (5669)	>32	>32	>32

Minimum inhibitory concentrations (MIC) were determined by microtiter broth dilution assay in yeast nitrogen base glucose medium with fungi inoculated at $OD_{600}=7\times10^{-4}$ (*ca.* 1×10^4 yeast cells or conidia/ml). Inhibitors were tested at 32 μg/ml and serial 2-fold dilutions down to 0.015 μg/ml; the MIC value was the lowest concentration of inhibitor which prevented visible growth after 24 hours at 30°C.

Table 2. Reversal of antifungal activity with sphingolipid intermediates.

Inhibitor	Control	Zone size (mm)			
		PHS	DHS	SS	STA
Lipoxamycin (10 μg)	21	0	0	15 h	17
Sphingofungin B (20 μg)	17	0	0	14 h	18
Cerulenin (2 μg)	15	19	17.5	16	15
Amphotericin B (10 μg)	15	14	15	15	15

Inhibitors were spotted onto 7-mm paper discs and placed onto seeded agar plates consisting of yeast nitrogen base glucose, 1.5% agar, 2.5% tergitol, 1×10^4 cells/ml *C. albicans* (MY1055), and 4 μg/ml phytosphingosine (PHS), dihydrosphingosine (DHS), sphingosine (SS), or stearoylamine (STA). Zones of inhibition were measured after 24 hours growth; 'h' refers to weak, hazy growth visible within the zone of inhibition.

activity against the mammalian enzyme. Previous studies by WHALEY *et al.*[7] showed that lipoxamycin was highly toxic in mice when applied subcutaneously or topically. Toxicity may be mechanism based, since studies with a chinese hamster ovary cell mutant have shown that the serine palmitoyltransferase is an essential enzyme in mammalian cells[9].

Lipoxamycin and hydroxylipoxamycin have antifungal activity against a panel of human pathogenic fungi with better potency against some of the *Candida* species, but otherwise, a similar spectrum of inhibition as measured for sphingofungin B (Table 1). *Cryptococcus neoformans* was the most sensitive organism, followed by various species of *Candida*. *Aspergillus fumigatus* was not inhibited in the broth dilution assay, but other filamentous fungi were sensitive to the lipoxamycins in disk diffusion assays.

The antifungal activity of lipoxamycin was reversed by the addition of sphingolipid intermediates, indicating that the mechanism of antifungal activity is inhibition of the serine palmitoyltransferase. Table 2 shows that dihydrosphingosine and phytosphingosine reversed the zones of inhibition obtained with lipoxamycin and sphingofungin B, but not the zones seen with the fatty acid synthesis inhibitor, cerulenin, or with amphotericin

B, which binds to ergosterol. Sphingosine is the primary mammalian sphingoid base, but is not commonly found in fungi; lipoxamycin and sphingofungin B were inhibitory in the presence of sphingosine although partial reversal was evident by hazy growth within the zone. An amino-containing fatty acid, stearoylamine, was not effective in reversing antifungal activity. Thus, only intermediates in the fungal sphingolipid pathway are fully effective in rescuing the growth of *Candida albicans* in the presence of inhibitors of the serine palmitoyltransferase.

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