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Chemical diversity in lipopeptide antifungal antibiotics

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In the course of screening for antifungal antibiotics, we have discovered a novel series of lipopeptide compounds structurally related to, but highly superior to, echinocandin B in terms of their water solubility due to the presence of a sulfate residue. These compounds, WF11899s, WF738s, WF14573s, WF16616 and WF22210, and their derivatives have diversity in their nuclear structures and acyl side chains. The producing strains were classified into two groups, the *Coleomycetes* group and the *Hyphomycetes* group. Compound FK463, a derivative of WF11899A, is currently in Phase 3 clinical development as a novel antifungal antibiotic. *Journal of Industrial Microbiology & Biotechnology* (2001) **27,** 157–162.

Keywords: antifungal compounds; lipopeptide; sulfate; *Coleomycetes*

Introduction

There is a growing need for new antifungal drugs because (a) increasing use of antitumor and broad-spectrum antibacterial agents can lead to serious fungal infections and (b) the growing population of AIDS patients and the prevalence of transplantation have increased the number of fungal opportunistic infections. A number of therapies are available to treat such fungal infections, but they are associated with major problems. Amphotericin B is broad-spectrum and fungicidal, but is relatively toxic. The broad-spectrum azole antifungal antibiotics are safer than amphotericin B, but are only fungistatic, leading to clinical failures in certain cases.

We require a fungi-selective target to discover safer and more effective antifungal agents. Fungal cells have a cell wall in their structure that is absent in mammalian cells. The cell wall is mainly composed of three macromolecules, β-1,3-glucan, chitin and mannan. Furthermore, recent studies have revealed that β-1,3-glucan and chitin are essential components for fungal cell growth. Therefore, inhibitors of such macromolecule biosyntheses are expected to be selective antifungal agents.

Echinocandin B [5], pneumocandin [7], aculeacin [4] and related compounds are known as selective inhibitors of β-1,3-glucan synthase, a key enzyme in β-1,3-glucan biosynthesis. Echinocandin-type compounds are lipopeptides that are structurally characterized by a cyclic hexapeptide nuclear structure acylated with a long fatty chain (Figure 1). They are sparingly soluble in water due to this lipophilic structure. Although echinocandins show potent *in vitro* and *in vivo* antifungal activity, their poor water solubility is a major barrier for progression to the clinical stage. In the course of screening for new antifungal antibiotics, we have concentrated our efforts on finding water-soluble β-1,3-glucan synthase inhibitors and have discovered compounds WF11899s [1,2], WF738s, WF14573, WF16616 and WF22210.

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Materials and methods

Compounds

Echinocandin B was isolated from culture broth of *Aspergillus nidulans var. roseus* A 42355 NRRL-11440. Pneumocandin B0 was isolated from culture broth of *Glarea lozoyensis*. Aculeacin A was isolated from culture broth of *A. japonicus var. aculeatus*.

In vitro antifungal activity

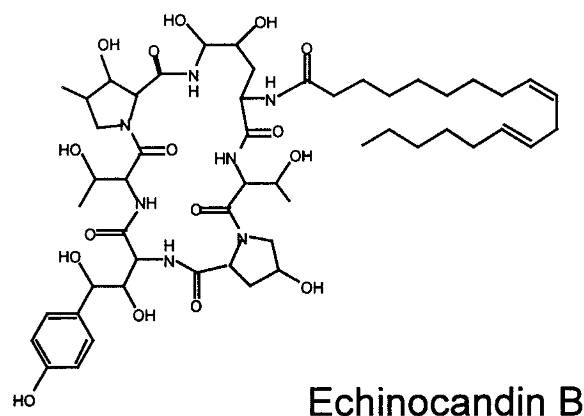
Microbroth dilution assay: Each inoculum was prepared as follows. The *Candida* cultures were incubated in yeast–maltose (YM) broth for 20 h at 37°C without shaking. The cell suspension was prepared by washing the cells with sterile saline. Antifungal activity was measured in yeast nitrogen base (YNB) medium containing 0.5% glucose by microbroth dilution assay using 96-well microtiter plates. The compounds were dissolved in methanol and diluted serially twofold with YNB medium in microplates. *Candida albicans* was inoculated to each well to yield 1×10^4 cfu/well in 100 μl. The plates were incubated for 22 h at 37°C. IC_{50} was determined by measuring turbidity at 600 nm on a microplate reader.

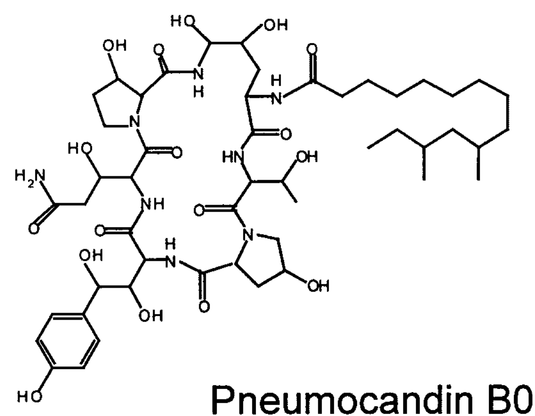
In vivo antifungal activity

The *in vivo* anti-*Candida* activity was evaluated using a murine systemic infection model. *C. albicans* FP-633, a clinical isolate in the Fujisawa culture collection, was used. The inoculum was prepared from a 3-day-old culture from a YM agar slant. ICR mice (female, 4 weeks old) were injected intravenously with 2×10^6 cells. All compounds were dissolved in saline or 10% HCO-60 saline and administered subcutaneously 1 h after challenge and once a day for three consecutive days.

Glucan synthase assay

Enzyme (membrane fraction) preparation and assay procedures for β-1,3-glucan synthase were conducted according to the method described by Sawistowska-Schroder *et al.* [6] with modifications. *C. albicans* 6406 was kindly supplied by Dr. Kerridge (University of Cambridge). Briefly, the yeast cells





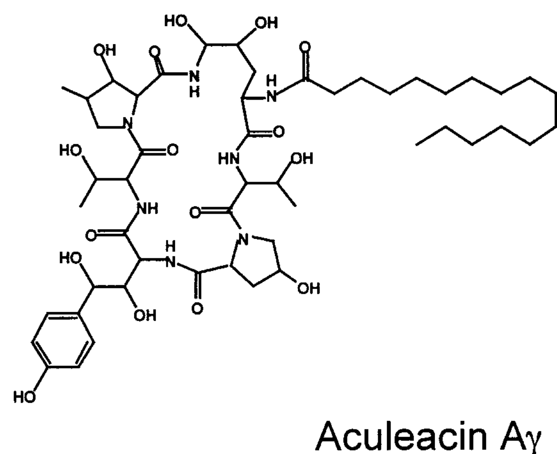


Figure 1 Structures of lipopeptide antifungal antibiotics.

were grown to logarithmic phase (absorbance at 660 nm, 0.42) in YNB medium supplemented with 2% glucose at 30°C with shaking. Cells were harvested by centrifugation, washed and suspended in ice-cold buffer A [50 mM Tris–HCl buffer (pH 7.5), 1 mM EDTA, 1 mM β-mercaptoethanol, 1 M sucrose and 25 μM GTP]. Cells were broken by mixing them with 0.4-mm glass beads on a vortex mixer and the beads were washed with ice-cold buffer B (buffer A minus 1 M sucrose), and the cell debris was removed by centrifugation. The supernatant fluids were centrifuged at 100,000×g for 45 min at 4°C. The pellet was washed by ultracentrifugation in buffer B, resuspended in buffer C (buffer B–glycerol, 2:1) at 10 mg protein/ml and stored at −80°C as a source of enzyme. Measurement of inhibitory activity on glucan synthase was carried out as follows: 2.5 μl of the test compound solution was incubated with 25 μl of reaction mixtures [50 mM Tris–HCl buffer (pH 8.0), 0.8% BSA, 0.1 mM GTP, 0.1% CHAPS, 0.05% Tween 80)] and the particulate enzyme (40 μg protein) for 15 min at room temperature. After the incubation, 25 μl of UDP-[U-^{14}C] glucose (0.35 μCi/ml, 1 mM) was added to the reaction vessel to react for 60 min at room temperature. The reaction was terminated by addition of 100 μl of ice-cold 5% trichloroacetic acid (TCA), which was then allowed to stand on the ice. The resultant precipitate was collected on a GF/C glass filter and counted for radioactivity in toluene scintillator.

Taxonomy of producing strains

The morphological observations of WF16616- and WF22210-producing strains were made after incubation at 25°C for 14 days on Miura's LCA plate [3]. In the case of WF11899-, WF738- and WF14573-producing strains, the morphological characteristics were determined on the basis of the cultures on sterilized azalea leaf affixed to a Miura's LCA plate because these strains produced conidial structures only on the leaf segment.

Results

Discovery of WF11899A, B and C

Using the cell-free assay, we screened for new water-soluble inhibitors of β-1,3-glucan synthase. Potent inhibitory activity was detected in the water-soluble fraction from a broth sample of fungal strain no. 11899. The producing strain, no. 11899, could

		R1	R2	R3	R4	R5	R6	MW	relative activity *Candida albicans*
WF11899	A	OH	CH_3	OH	CH_3	OSO_3H	OH	1174	1
	B	OH	CH_3	H	CH_3	OSO_3H	OH	1158	0.5
	C	H	CH_3	H	CH_3	OSO_3H	OH	1142	1
WF14573	A	OH	H	H	CH_3	OH	OSO_3H	1144	16
	B	OH	CH_3	H	CH_3	OH	OSO_3H	1158	2
WF738	A	OH	CH_3	H	H	OSO_3H	OH	1144	0.5
	B	OH	H	H	H	OSO_3H	OH	1130	0.5
	C	H	CH_3	H	H	OSO_3H	OH	1128	0.5

Figure 2 Chemical diversity in cyclic peptide nuclear structures.

form conidiomata only on the leaf segment. The producing strain was identified as *Coleophoma empetri*. Three active components were isolated from this fermentation broth using reverse-phase column chromatography. The new compounds, WF11899A, B and C, were found to be members of the echinocandin family [1,2]. While these compounds contained a sulfate residue in their structure, other echinocandins did not. The sulfate residue was the specific, unique feature of WF11899s (Figure 2) and WF11899A, B and C which were highly soluble in water even at a concentration of 50 mg/ml. Other echinocandin family lipopeptide compounds such as echinocandin B are hardly soluble in water.

WF11899A was treated with arylsulfatase derived from *Aerobacter aerogenes* to analyze the effect of the sulfate residue on its excellent water solubility. As a result, arylsulfatase treatment of WF11899A greatly diminished its water solubility, indicating that the excellent water solubility is attributed to the sulfate residue in its structure.

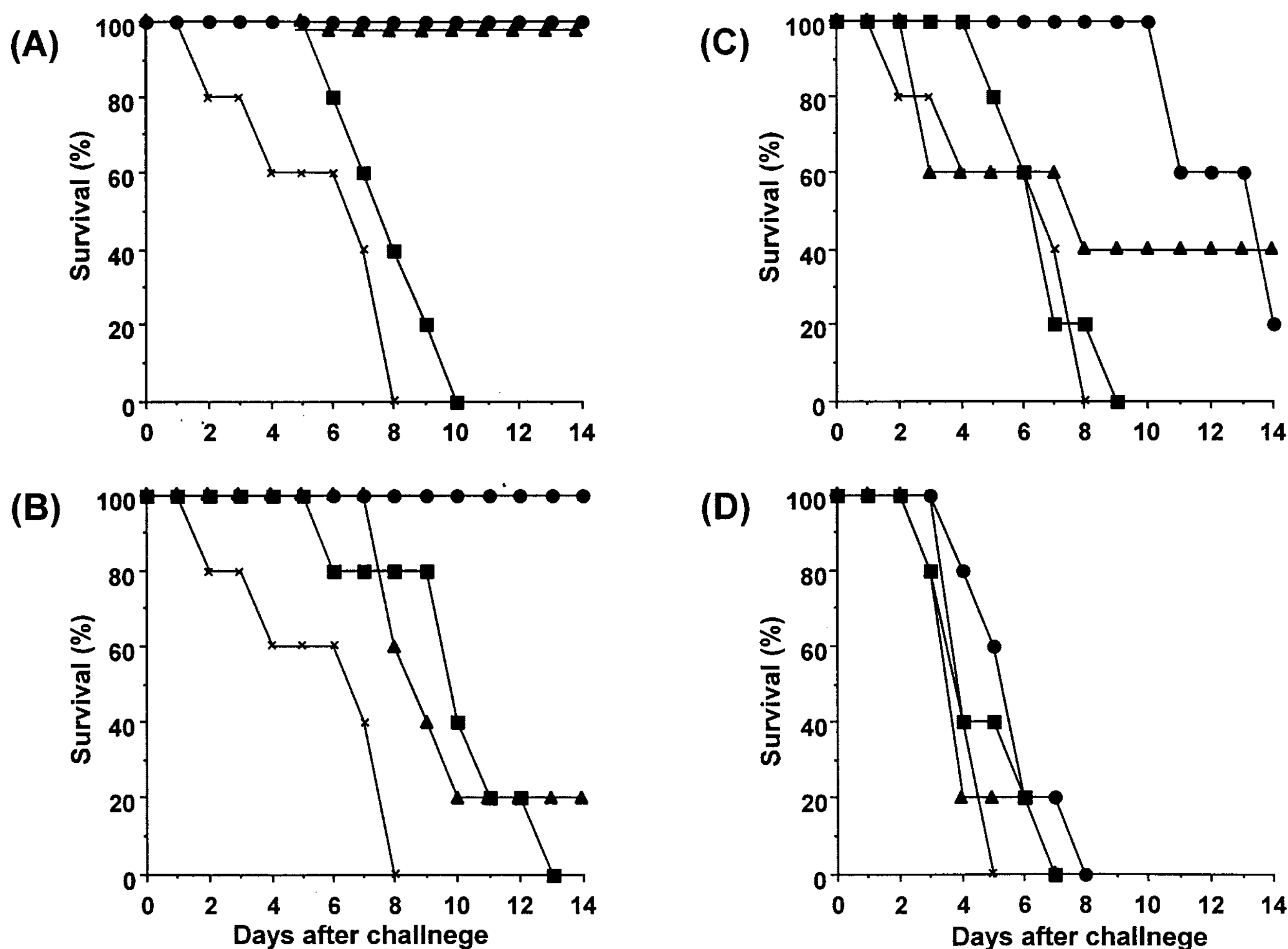


Figure 3 Protective effect of WF11899A, B, C and echinocandin B against systemic infection of *C. albicans* FP633. (A) WF11899A; (B) WF1189B; (C) WF11899C: (●) 10 mg/kg; (▲) 3.2 mg/kg; (■) 1.0 mg/kg; (×) saline; (D) echinocandin B: (●) 32 mg/kg; (▲) 10 mg/kg; (■) 3.2 mg/kg; (×) 10% HCO-60/saline.

WF11899A, B and C strongly inhibited β-1,3-glucan synthase activity and fungal cell growth. These compounds were active against *C. albicans* and *A. fumigatus*, but not against *C. neoformans*. The enzyme inhibitory activity of WF11899A was fourfold more potent than echinocandin B. The anti-*Candida* activities of WF11899A, B and C were 18-, 36- and 18-fold more potent than echinocandin B, respectively.

The *in vivo* anti-*Candida* activity was evaluated in a murine *C. albicans* infection model. Subcutaneous administration of WF11899A greatly enhanced the survival rate with an ED_{50} of 1.2 mg/kg. On the other hand, echinocandin B showed only a small effect *in vivo* and its ED_{50} was more than 32 mg/kg (Figure 3). The potency of WF11899A was clearly superior to that of echinocandin B.

Screening of back-up compounds

WF11899A showed strong antifungal activities against *C. albicans* and *A. fumigatus*, not only *in vitro* but also *in vivo*. However, we could not develop it as an antifungal drug because of its hemolytic activity. We needed to get chemical diversity to improve this point for further development.

The acyl side chain was readily replaced by semi-synthesis; however, modification of the cyclic peptide portion by chemical synthesis was not easy. Thus, we attempted to obtain diversity in the cyclic peptide nuclear structure by screening of fungal products. As a result, we found several new series of sulfate-containing lipopeptides, WF738s and WF14573s (Figure 2).

WF738A, B and C contain cyclic peptide nuclear structures with the same palmitoyl acyl side chain. WF738A showed the strongest inhibitory activity against β-1,3-glucan synthase. However, the *in vitro* anti-*Candida* activities of these three compounds were almost the same and twofold stronger than that of WF11899A (Figure 2). Interestingly, the producing strain also belonged to *Coleophoma* of the same genus as the WF11899-producing strain. This strain was identified as *Co. crateriformis* no. 738.

WF14573A and B also contained a sulfate residue in their structure, but the position was different from that of WF11899s and WF738s. Interestingly, the WF14573-producing strain was identified as *Co. empetri*, the same species as WF11899s.

The anti-*Candida* activity of WF14573A was 16-fold weaker than that of WF11899s, but WF14573B showed almost the same activity as WF11899A. WF11899B and WF14573B had the same cyclic peptide nuclear structure and acyl side chain except for the position of the sulfate residue. Therefore, we compared their activities to analyze the effect of the position of the sulfate residue on the antifungal activity. As shown in Figure 2, the anti-*Candida* activity of WF11899B was fourfold stronger than that of

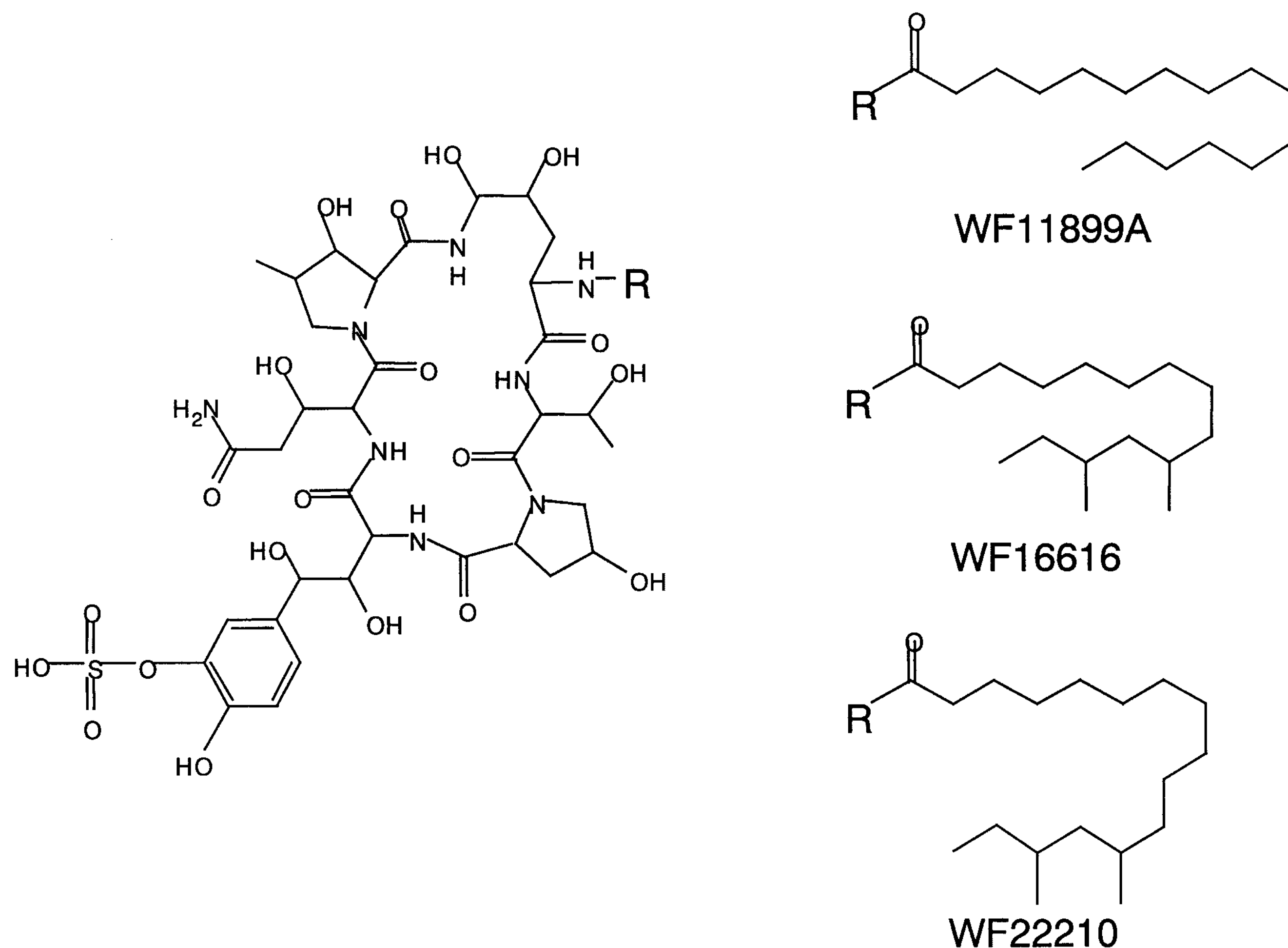


Figure 4 Chemical diversity in acyl side chains.

Table 1 The classification of lipopeptide antifungal antibiotics producing strains

Compound	Class	Producing strain
WF11899	Type-IA	*Coleophoma empetri*
WF738	(*Coelomycetes*)	*Coleophoma crateriformis*
WF14573		*Coleophoma empetri*
WF16616	Type-IB	*Tolypocladium parasiticum*
WF22210	(*Hyphomycetes*)	*Chalara* sp
Pneumocandin	Type-II	*Glarea lozoyensis*
Echinocandin		*Aspergillus nidulans var. echinulatus*
Aculeacin		*Aspergillus japonicus var. aculeatus*

WF14573B. We conclude that the presence of the sulfate residue at R4 is important for antifungal activity.

Although these three series of sulfate-containing lipopeptide antifungal antibiotics were isolated from *Coleophoma* strains, two compounds were further found in non-*Coleophoma* strains; WF16616 produced by *Tolypocladium parasiticum* and WF22210 produced by *Chalara* sp. contained different branched acyl side chains as shown in Figure 4.

Chemical diversity

Figure 2 shows a summary of the chemical diversity in the cyclic peptide nuclear structures of the three series of lipopeptides produced by *Coleophoma* and structure–activity relationships. Structural difference from WF11899A is shown by a bold letter. Structural diversity in their cyclic peptide nuclear structures was seen among the metabolites of the three strains belonging to the same genus, *Coleophoma*.

We also isolated WF16616 and WF22210 having the same cyclic peptide nuclear structure as WF1189A, but with a different side chain. The chemical diversity in the acyl side chain is shown in Figure 4. The two producing strains were different from *Coleophoma* species.

Producing strains

The producing strains of the lipopeptide antifungal antibiotics are classified in two groups: sulfate-containing lipopeptide, type I and echinocandin-like lipopeptide, type II; and type I classified into two sub-groups, as shown in Table 1. The type IA, class *Coleomycetes* produce the lipopeptides WF11899s, WF738s and WF14573s, which are sulfate-containing cyclic peptide nuclear structures with a palmitoyl chain. These strains could form the conidial structure only on the leaf segment. Type IB, class *Hyphomycetes* produce the lipopeptides WF16616 and WF22210, which are composed of the same peptide nuclear structure as WF11899A with various branched acyl side chains. These strains could form hyphal conidia in a general medium. Type II consists of other lipopeptide-producing strains, such as echinocandins, pneumocandins and aculeacins. The type I strains may have a specific enzyme, aryl sulfotransferase, to sulfonate the cyclic peptide structure.

Medicinal chemistry

To find a more potent antifungal agent, we focused on the lipophilic acyl side chain and synthesized various compounds with novel side chains. As a result, we discovered the novel sulfate-containing lipopeptide, FK463 [8] (Figure 5). The cyclic peptide nucleus, obtained by enzymatic cleavage of WF11899A, was acylated with activated ester to give FK463. FK463 exhibited broad-spectrum activity against clinically important phasogens including *Candida* species and *Aspergillus* species, but displayed no activity against *Cryptococcus neoformans*. FK463 was also effective against azole-resistant *C. albicans* (data not shown).

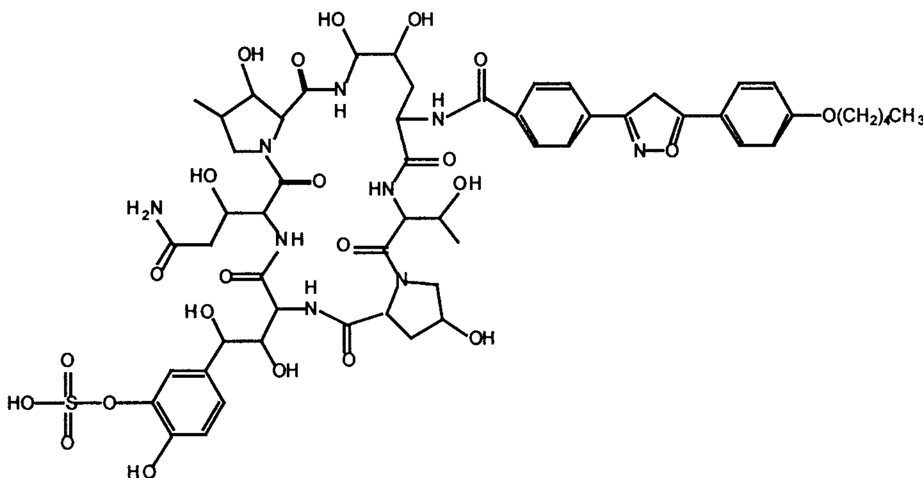


Figure 5 Structure of FK463.

Discussion

"Chemical diversity" in screening sources is one of the key factors for successful drug discovery. In the case of microbial screening, diversity can be created through suitable selection of producing strains and culture conditions. In the course of screening for antifungal antibiotics, we discovered a novel series of sulfate-containing lipopeptide compounds. These compounds were highly soluble in water, even at a concentration of 10 mg/ml. They were active against *C. albicans* and *A. fumigatus*, not only *in vitro*, but also *in vivo*. Chemical diversity in our compounds was observed not only in the cyclic peptide nuclear structures, but also in the acyl side chains. Interestingly, the producing strains of WF11899s, WF738s and WF14573s, which consist of different peptide nuclear structures with the palmitoyl chain, belong to the genus *Coleophoma*. We showed that a taxonomic method is useful to obtain chemical diversity. In the near future, we may be able to increase the chemical diversity in lipopeptide compounds using genetic engineering of biosynthetic genes.

We choose WF11899A, WF738B and WF14573B as lead compounds for optimization. FK463, a semi-synthetic derivative of WF11899A, is in Phase 3 clinical trials as a potent antifungal agent for disseminated fungal infections.

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