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Discovery of micafungin (FK463): A novel antifungal drug derived from a natural product lead*

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Abstract: FR901379, which is a novel, water-soluble, echinocandin-like lipopeptide, was isolated from a microbial culture broth. It showed potent in vivo antifungal activity against *Candida albicans* and inhibits the synthesis of 1,3-β-glucan. However, this compound had hemolytic activity and was also less active against *Aspergillus fumigatus*. To overcome these shortcomings, we synthesized FR131535 bearing an octyloxybenzoyl acyl side chain instead of a fatty acid. FR131535 retained the original activity displayed by FR901379, acquired potent anti-*Aspergillus* activity, and its hemolytic activity was significantly reduced. Further extensive chemical modification of FR901379 has led to the discovery of micafungin (FK463), which is effective against *Candida* and *Aspergillus* spp. Micafungin has been marketed in Japan and the United States as a candin-class parenteral antifungal agent for life-threatening mycoses.

Keywords: micafungin; FK463; antifungal drugs; FR901379; echinocandin.

INTRODUCTION

Fungal infections are an important problem, particularly in immunocompromised patients, resulting from AIDS infections, aggressive cancer treatment, the growing use of organ transplants, and other nosocomial situations. Advanced medical therapies have created a critical need for new safe fungicidal agents that can be used to treat disseminated infections. Systemic mycoses are not easily diagnosed, and the patient has usually been infected for quite some time before symptoms appear. Thus, empiric therapy needs to begin immediately, but currently available treatments have problems with toxicity or resistance.

The clinically available drugs were limited to amphotericin B, azoles, and flucytosine when we started our antifungal drug development program, although voriconazole and caspofungin have since been marketed. However, these drugs have problems regarding antifungal spectra of activity, toxicity, or resistance. Furthermore, the emergence of multi-azole-resistant strains of *Candida* is leading to many serious fungal infections. Much interest has been focused on developing safer and more effective antifungal agents.

In the course of our screening program for new therapeutic leads, we isolated FR901379 and related compounds (Fig. 1), which are novel, water-soluble, echinocandin-like lipopeptides from a microbial culture broth [1]. FR901379 is a cyclic antifungal lipopeptide that has a hexapeptide nucleus with

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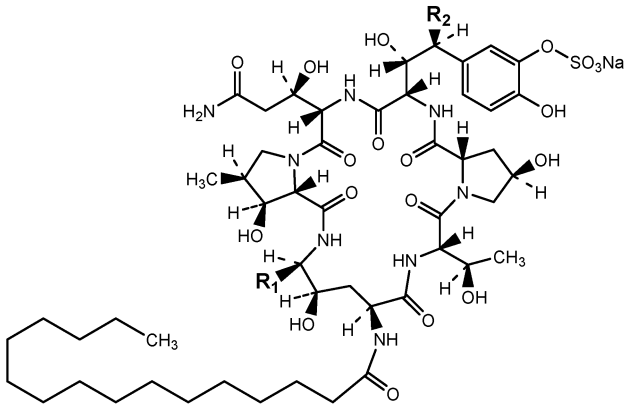
sulfonate, and bears a fatty acid acyl group attached to the N-terminus. FR901379 showed potent in vivo antifungal activity against *Candida albicans* and inhibited the synthesis of 1,3-β-glucan, a key component of the fungal cell wall [2]. Since the cell wall is a feature unique to fungi and is not present in eukaryotic cells, inhibitors of the synthesis of fungal cell wall components have potential for selective toxicity to fungi and not to the host [3].



	R1	R2
FR901379(WF11899A)	OH	OH
WF11899B	OH	H
WF11899C	H	H

Fig. 1 Structures of FR901379 and related compounds.

The acyl side chain in the FR901379 molecule is a palmitoyl group with a straight chain of 15 carbon atoms. FR901379 showed rather strong hemolytic activity in vitro, which is also a feature of other echinocandins. Studies on echinocandin-like lipopeptides have demonstrated that the different fatty acid acyl side chains of these otherwise identical cyclic peptide antibiotics were important determinants of their antifungal activity and toxicity [4]. We thus aimed to reduce the hemolytic activity of FR901379 by replacing the acyl side chain. As a result, we obtained the novel echinocandin-like lipopeptide FR131535 bearing an octyloxybenzoyl acyl side chain instead of a fatty acid for the trial study [5]. FR131535 retained the original activity of FR901379, acquired potent anti-*Aspergillus* activity, and its hemolytic activity was significantly reduced.

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 of extensive chemical modification of FR901379, we have discovered the novel echinocandin-like lipopeptide micafungin bearing an isoxazole ring which is effective against *Candida* and *Aspergillus* spp. [6,7]. In animal studies, micafungin is as efficacious as amphotericin B with respect to improvement of survival rate. Studies to date have shown that micafungin exhibits extremely potent antifungal activity against clinically important fungi, including *Aspergillus* and azole-resistant strains of *Candida* [8]. Micafungin has been marketed in Japan and the United States as a candin-class parenteral antifungal agent for life-threatening mycoses. In this paper, we report the synthesis and antifungal activity of micafungin.

DISCOVERY OF FR901379

The lead compounds from which micafungin was derived, FR901379, and several related compounds were discovered from amongst about 6000 broth samples, and were detected by antifungal activity

against *C. albicans* and *Aspergillus fumigatus*. These new compounds were found to be members of the echinocandin-like class of lipopeptides. Echinocandin B, pneumocandin B0, and other echinocandin-like lipopeptides are characterized structurally by a cyclic hexapeptide acylated with a long side chain, and have excellent anti-*Candida* activity attributed to selective inhibition of 1,3-β-glucan synthesis, although their intrinsic water insolubility was a major problem [9–11].

However, FR901379 and related compounds showed high water solubility, and also demonstrated strong antifungal effects on *Candida* species. The key structural difference between FR901379 and the other echinocandins is that FR901379 has a sulfate moiety in its molecule (Fig. 2). We thought that this portion might be involved in water solubility as FR901379 is highly soluble in water even at a concentration of 50 mg/ml, whereas the other compounds are hardly soluble in water (Table 1). To prove this hypothesis, we treated the FR901379 molecule with an arylsulfatase from *Aerobacter aerogenes.* The water solubility of the desulfated molecule was decreased to 1 mg/ml even though the inhibitory activity toward 1,3-β-glucan synthase did not drop dramatically. This result suggests that the excellent water solubility of FR901379 can be attributed to its sulfate functionality.

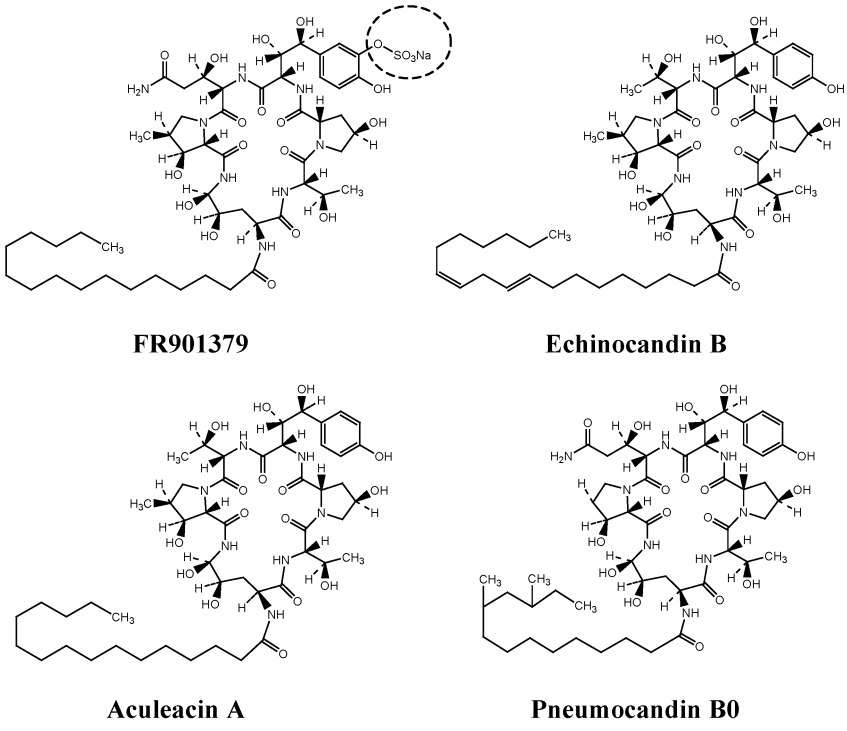
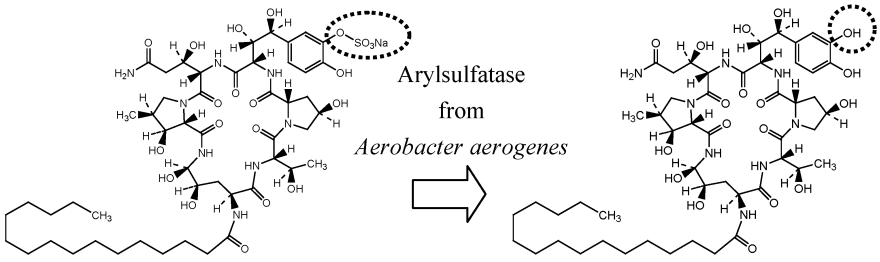


Fig. 2 Structures of the echinocandin-like class of lipopeptides.

Table 1 Effect of sulfate moiety on solubility in water.

Compound	Solubility in water (mg/ml)	Inhibition of 1,3-β-glucan synthase IC_{50} (ug/ml)
FR901379	>50	0.7
FR133302	1	1.3



FR901379 **FR133302**

The IC_{50} values of FR901379 and related compounds for 1,3-β-glucan synthase are 0.7, 0.7, and 1.8 μg/ml, respectively. These compounds inhibited 1,3-β-glucan synthase more strongly than echinocandin B (Table 2). The in vitro antifungal activity of FR901379 and related compounds against both *C. albicans* and *A. fumigatus* shows higher potency than that of aculeacin A (Table 3). They were, however, only weakly active against *A. fumigatus*. None of these compounds showed antifungal activity against *Cryptococcus neoformans*. Table 4 shows the therapeutic effect of FR901379 in a mouse *C. albicans* infection model. Drugs were administered subcutaneously for four consecutive days. FR901379 and related compounds significantly prolonged the survival of infected mice. FR901379 was the most potent compound with an ED_{50} value of 2.7 mg/kg at day 14. This value was almost comparable to that of fluconazole. In spite of its potent activity toward fungi and its good water solubility, FR901379 lysed red blood cells at low concentrations (Table 5). The lytic activity of FR901379 was weaker than that of Amphotericin B, but there was room for improvement.

Table 2 Inhibitory activity of FR901379 and related compounds on 1,3-β-glucan synthase.

Compound	IC_{50} (μg/ml)
FR901379 (WF11899A)	0.7
WF11899B	0.7
WF11899C	1.8
Aculeacin A	1.3
Echinocandin B	2.6

Table 3 In vitro antifungal activity of FR901379 and related compounds.

Test organism	IC_{50} (μg/ml) FR901379 (WF11899A)	WF11899B	WF11899C	Aculeacin A
C. albicans FR578	0.008	0.008	0.008	0.008
C. albicans FP582	0.025	0.015	0.03	0.06
C. albicans FP629	0.008	0.004	0.008	0.015
C .albicans FP633	0.025	0.025	0.03	0.06
C. tropicalis YC118	0.025	0.05	0.015	0.31
C. krusei YC109	0.16	0.16	0.16	0.62
C. utilis YC123	0.03	0.003	0.003	0.06
A. fumigatus FD050	1.9	1.6	0.62	2.5
A. niger ATCC9642	0.03	0.03	0.03	2.5
C. neoformans YC203	>2.5	>2.5	>2.5	>2.5

Microbroth dilution assay

Table 4 In vivo efficacy: neutropenic mouse model of disseminated Candidiasis.

Compound	ED_{50} (mg/kg)
FR901379 (WF11899A)	2.7
WF11899B	4.6
WF11899C	>10.0
Aculeacin A	6.4
Fluconazole	4.5

Infection: *C. albicans* FP633

Table 5 Hemolytic activity of FR901379 and related compounds.

Compound	MLC[a] (μg/ml)
FR901379 (WF11899A)	62
WF11899B	62
WF11899C	62
Aculeacin A	31
Echinocandin B	125
Amphotericin B	8

[a]Minimum lytic concentration.

The producer strain of FR901379 was originally isolated from a soil sample collected at Iwaki-City, Fukushima Prefecture, Japan. The morphological characteristics were determined on the basis of the cultures on sterilized azalea leaf affixed to a Miura's LCA plate because the strain produced conidial structures on the leaf segment alone. It was identified as *Coleophoma empetri* F-11899 (Fig. 3).

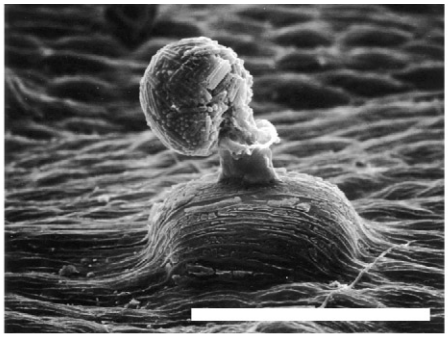


Fig. 3 Electron micrograph of *Coleophoma empetri* F-11899.

SYNTHESIS OF FR131535 FROM FR901379

FR901379 is a highly selective antifungal agent and an inhibitor of 1,3-β-glucan synthase. When we began our work, Merck and Lilly were already involved in this area [10,11]. However, FR901379 has some advantages compared with other analogs. One is good water solubility. We also knew that the hemolytic activity of FR901379 might be eliminated by substituting the acyl chain. While other compounds in the class suffered from poor solubility, the good aqueous solubility of FR901379 encouraged us to focus on transformation of the palmitoyl side chain, keeping the sodium sulfate group intact. Considering that this hemolytic activity may be due to its long alkyl side chain, we tried to reduce the hemolytic activity of FR901379 by substituting the side chain as previously achieved by scientists at Lilly [12].

Thus, an initial acyl side-chain modification study of FR901379 was conducted. The synthesis of this novel echinocandin-like lipopeptide is outlined in Fig. 4. The palmitoyl group of FR901379 was deacylated to give FR179642 by the acylase from *Actinoplanes utahensis*. The new acyl side chain was prepared from 1-bromooctane and 4-hydroxybenzoic acid. 2,4,5-Trichlorophenyl 4-(*n*-octyloxy) benzoate was then obtained from 4-(*n*-octyloxy) benzoic acid and 2,4,5-trichlorophenol using DCC in ether. We then reacylated at the free amino group of FR179642 with this chemically synthesized acyl group to yield FR131535. The water solubility of FR131535 was as high as that of FR901379 even after replacement of the acyl side chain. Echinocandin B and cilofungin did not dissolve in water under the same conditions. FR131535 inhibited 1,3-β-glucan synthase prepared from *C. albicans* 6406 with an IC_{50} value of 2.8 μg/ml, and the inhibition was noncompetitive (Ki 4.0 mM). This compound showed broad spectrum and potent activity against a variety of fungal species by the microbroth dilution method. FR131535 was active against most *Candida* and *Aspergillus* species. The protective efficacy of FR131535 administered subcutaneously against murine systemic infection with *C. albicans* was examined. As shown in Table 6, the ED_{50} of FR131535 was 3.7 mg/kg. This compound was superior to echinocandin B and cilofungin in the above model. Furthermore, the in vivo efficacy of FR131535 was almost as potent as fluconazole. Fluconazole is fungistatic against fungal pathogens, while FR131535 is an inhibitor of cell wall biosynthesis and fungicidal against *Candida* species. Furthermore, FR131535 also showed potent in vivo activity for *A. fumigatus* with a much improved ED_{50} of 4.3 mg/kg compared to >70 mg/kg for FR901379. This result encouraged us since there were no reports of candin agents having good anti-*Aspergillus* activity at the time of the study. The hemolytic activity of FR131535 was also greatly reduced compared to that of FR901379, thus meeting the objective of this trial study (Table 6). Our chemists were especially encouraged by these results. Therefore, they concentrated their work on synthesis and evaluation of novel benzoyl-type acyl side chains.

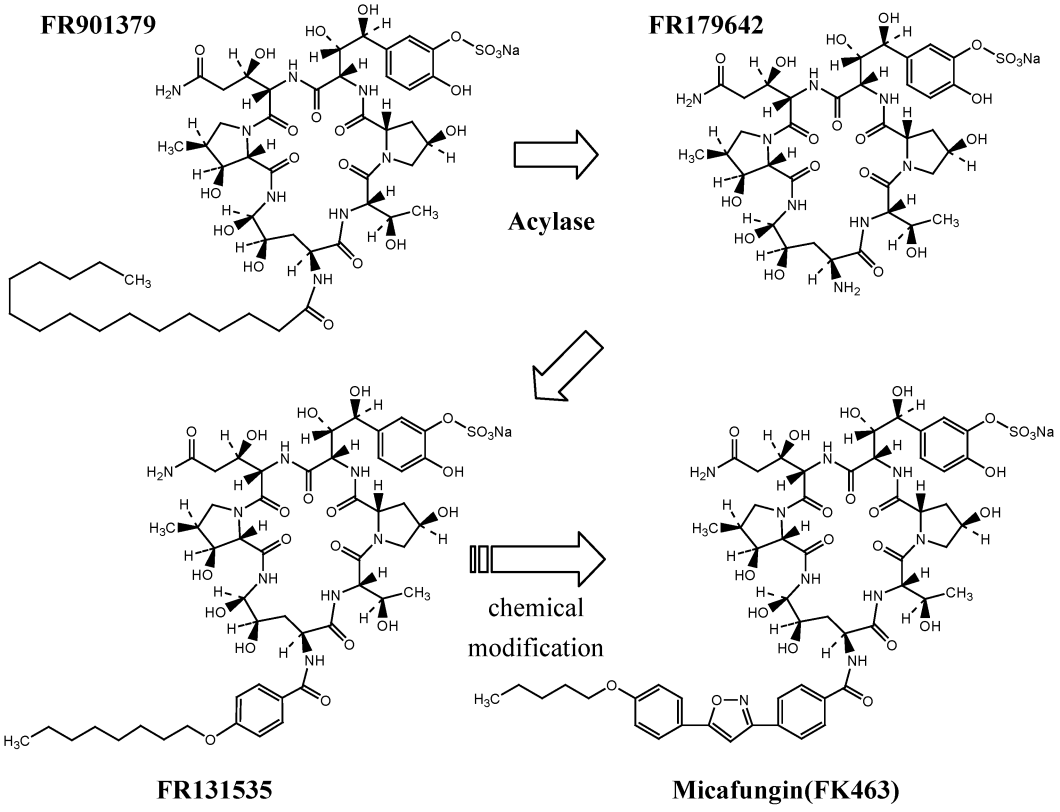
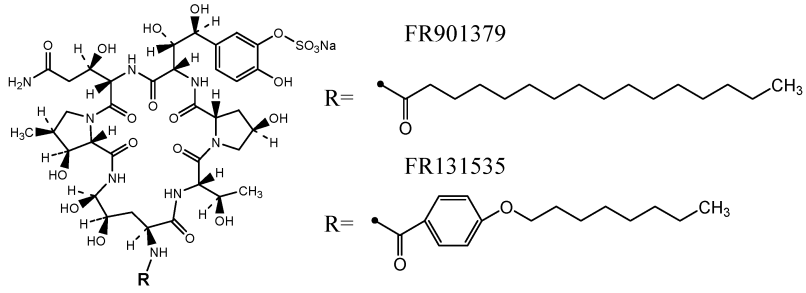


Fig. 4 Synthesis of micafungin (FK463).

Table 6 Influence of acyl side-chain group.

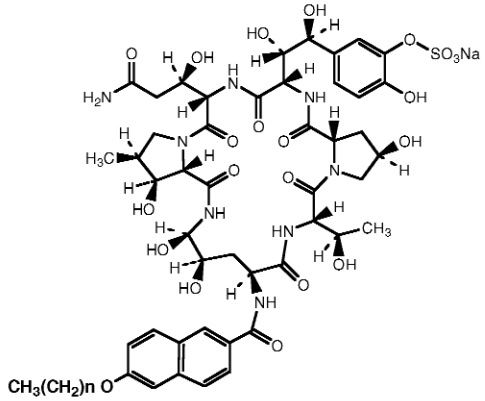


Compound	*C. albicans* FP633		*A. fumigatus* FP1305	Hemolysis
	MIC (μg/ml)	ED_{50} (mg/kg)	ED_{50} (mg/kg)	LC_{30}[a] (mg/ml)
FR901379	0.2	1.8	70	0.456
FR131535	0.78	3.7	4.3	>8

[a]Lytic concentration 30 %.

OPTIMIZATION FROM FR131535 TO FK463

Conversion of the acyl side chain led to expansion of the antifungal spectrum to *Aspergillus*. The relationship between the lipophilicity of the side chain and antifungal activity was then examined. Naphthalene side chains, which were compact and amenable for the modulation of lipophilicity, were chosen as the initial acyl side chains. The relationship between antifungal and hemolytic activities was examined by adjusting lipophilicity by varying the length of the alkyl chains. As shown in Fig. 5, an increase of lipophilicity resulted in improved anti-*Candida* activity, which was the most potent with an octyloxy group (n = 7). Furthermore, in vivo studies in mice reflected in vitro antifungal activities. As a tool to aid analog design, the ClogP value [octanol–water partition coefficient (calculated value)], which is a measure of the lipophilicity of the side chains, showed an excellent correlation with anti-*Candida* activity. The strongest in vivo effect was obtained when the ClogP value was set at approximately 6. However, the longer the alkyl chains were, the greater hemolytic activity was. As a result of this correlation, we were able to synthesize novel side chains with predicted strong activity by adjusting the lipophilicity, measured by ClogP, to approximately 6 by precalculation. Conversion of the benzene ring of the side-chain aromatic moiety of FR131535 into a naphthalene ring improved anti-*Candida* activity, and further introduction of aromatic rings into the side chain was also effective (Table 7).



n	*C. albicans* FP633 MIC(mg/ml)	Hemolysis (%, 2mg/ml)
3	12.5	1
5	0.78	5
6	0.39	-
7	0.1	34
9	0.2	100
11	0.78	100

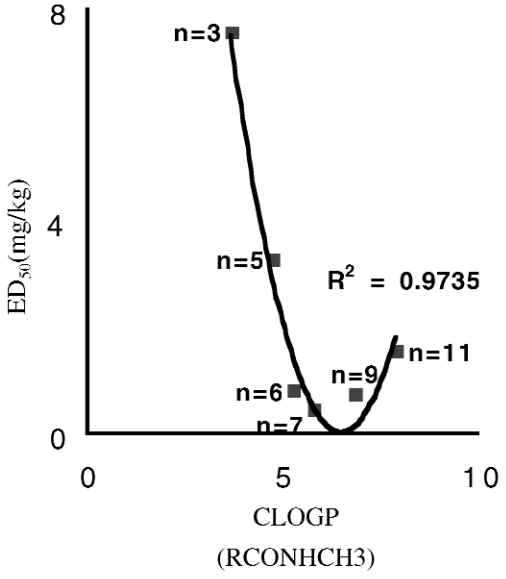
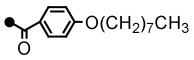
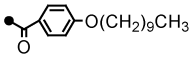
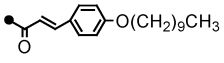
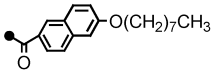
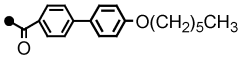
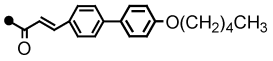
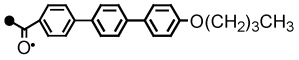


Fig. 5 Hydrophobicity and efficacy of side-chain lipophilicity.

Table 7 Side-chain modification—part 1 (introduction of heterocycles).

Compound No.	Acyl side-chain group	CLOGP	*C. albicans* FP633 MIC (μg/ml)[a)]	Serum MIC (μg/ml)[a)]	ED_{50} (mg/kg)[a)]	*A. fumigatus* FP1305 ED_{50} (mg/kg)	Hemolysis LC_{30} (mg/ml)
1		4.77	0.78 (1)	25 (1)	1.5-4.3 (1)	4.31	>10
2		5.80	0.2 (0.26)	-	1 (0.7)	22.9	>10
3		5.38	0.78 (1)	-	4.3 (1)	-	>10
4		5.80	0.1 (0.13)	6.25 (0.25)	0.742 (0.23)	0.788	10
5		5.37	0.2 (0.26)	6.25 (0.25)	0.658 (0.2)	-	1.74
6		5.68	0.05 (0.06)	3.13 (0.13)	0.563 (0.3)	0.894	3.95
7		6.14	0.0125 (0.02)	1.56 (0.06)	0.447 (0.14)	-	0.37

[a)] Figures in parentheses indicate the ratio of MIC(ED_{50})(drug)/MIC(ED_{50})(FR131535)

[b)] Represents the range of values of ED_{50} for FR131535 over a number of experiments

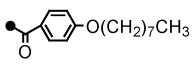
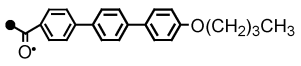
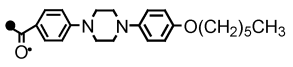
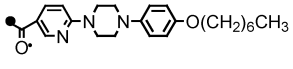
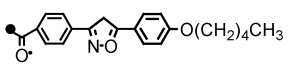
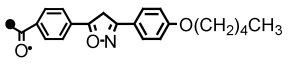
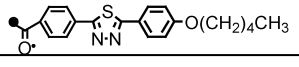
[c)] Lytic Concentration 30%

Anti-*Candida* activity tended to improve as the number of benzene rings increased, and compound 7 showed the strongest MIC (minimum inhibitory concentration) (0.0125 μg/ml). Furthermore, compound 4 [MIC ratio (0.13) and ED_{50} ratio (0.23)], which has a naphthalene ring, showed a large improvement in anti-*Aspergillus* activity (0.788 mg/kg) as compared with FR131535 (4.31 mg/kg). And the activities of compound 5 [MIC ratio (0.26) and ED_{50} ratio (0.23)] bearing acyl side chain with two benzene rings was similar to compound 4. Another problem was, however, encountered. Even though compound 7 showed the strongest MIC [MIC ratio (0.02)], its in vivo effect [ED_{50} ratio (0.14)] was only slightly better as compared with compound 5 [ED_{50} ratio (0.2)]: a 10-fold worse MIC. To improve the in vivo activity, we focused on the synthesis of compounds with superior MICs. We found a correlation between the in vivo effect (ED_{50} ratio) and the in vitro activity measured in the presence of mouse serum (serum MIC ratio). The addition of serum to the assay medium generally increased MICs as a result of decreased concentration of free compounds due to strong binding to serum protein. Addition of serum decreased MIC as a result of decreased concentration of free form because these compounds bound strongly to serum protein. After this discovery, prediction of the in vivo effect became feasible by measuring the serum MIC of synthetic derivatives, and this allowed rapid development of structure–activity relationships. Consequently, the structure–activity correlation with the serum MIC revealed that compound 7 type derivatives, with three linearly linked aromatic rings, have strong anti-*Candida* and anti-*Aspergillus* activities. However, hemolysis with these analogs was still evident.

We solved this issue by converting the central benzene ring of compound 7 into various heterocycles (Table 8). Initially, reducing the hemolytic activity was difficult because FR901379 derivatives

have an amphiphilic structure and surfactant activity. However, we found there were crucial differences between erythroid cell membranes and those of eukaryotic cells with respect to their branched fatty acid content. We hypothesized that hemolytic potential could be reduced by decreasing the linearity of the acyl side chains. As expected, the introduction of a heterocycle into the acyl side chain resulted in decreased hemolytic activity without losing the antifungal potency of compound 7. Finally, the cyclic peptide nucleus FR179642, obtained by enzymatic cleavage of the natural product FR901379, was acylated with an isoxazole ring in the acyl side chain (Fig. 4).

Table 8 Side-chain modification—part 2 (introduction of heterocycles).

Compound No.	Acyl side-chain group	CLOGP	*C. albicans* FP633 Serum MIC (μg/ml)[a)]	*C. albicans* FP633 ED_{50} (mg/kg)[a)]	*A. fumigatus* FP1305 ED_{50} (mg/kg)	Hemolysis (%, 1mg/ml)
1		4.77	25 (1)	1.5-4.3 (1)	4.31	<20
7		6.14	1.56 (0.06)	0.447 (0.14)	-	79
8		6.16	0.78 (1)	4.3 (1)	0.53 (0.15)	<20
9		6.29	0.1 (0.13)	0.742 (0.23)	-	<20
FK463		5.31	0.2 (0.26)	0.658 (0.2)	0.228 (0.06)	<20
10		5.31	0.05 (0.06)	0.563 (0.3)	-	38
11		6.24	0.0125 (0.02)	0.447 (0.14)	-	82

a) Figures in parentheses indicate the ratio of MIC(ED_{50})(drug)/MIC(ED_{50})(FR131535)

b) Represents the range of values of ED_{50} for FR131535 over a number of experiments

In the course of the optimization study of FR901379, we screened for new acylases for the production of peptide core FR179642 [15]. We discovered an acylase from *Streptomyces* sp. No. 6907 instead of *A. utahensis*. This new acylase from *Streptomyces* sp. No. 6907 was 10 times more efficient than that produced by *A. utahensis*. As a result of these efforts, FK463 was synthesized and we selected this compound as the clinical candidate.

Among all the candidate compounds prepared, FK463 had the strongest in vivo effects against *Candida* and *Aspergillus* spp. The efficacy of FK463 was evaluated in neutropenic mouse models of disseminated candidiasis and aspergillosis, and was compared with those of amphotericin B and fluconazole [9]. Table 9 shows the ED_{50}s calculated on the basis of the survival rate at 15 days after infection. The ED_{50}s of FK463 against disseminated infections with *C. albicans*, *C. glabrata*, *C. tropicalis*, and *C. krusei* ranged from 0.14 to 0.77 mg/kg. Although the efficacies of FK463 were 1.4 to 3.1 times inferior to those of amphotericin B (0.09 to 0.26 mg/kg), they were 9.6 to >77 times superior to those of FLCZ. The ED_{50} of FK463 against disseminated *C. parapsilosis* infection was 1.0 mg/kg, which was 11 times superior to that of FLCZ (10.9 mg/kg) and 18 times inferior to that of amphotericin

B (0.06 mg/kg). FK463 showed good activity against disseminated *A. fumigatus* infection, with ED_{50}s in the range of 0.25 to 0.50 mg/kg. The efficacies of FK463 were 1.7 to 2.3 times inferior to those of amphotericin B (0.11 to 0.29 mg/kg) and >80 times superior to those of fluconazole. These results indicate that FK463 is a potent parenteral therapeutic agent for disseminated candidiasis and aspergillosis in the neutropenic mouse model.

Table 9 In vivo efficacy of micafungin (FK463) in neutropenic mouse model of disseminated Candidiasis and Aspergillosis.

Organisms	ED_{50} (mg/kg)[a]		
	FK463	Fluconazole	Amphotericin B
C. albicans FP633	0.14	2.15	0.08
C. albicans 16010	0.21	4.51	0.12
C. albicans FP1839[b]	0.26	>20.0	0.18
C. glabrata 13002	0.30	6.27	0.11
C. tropicalis 16009	0.28	3.71	0.09
C. krusei FP1866	0.77	9.52	0.26
C. parapsilosis FP1946	1.00	10.9	0.06
A. fumigatus TIMM0063	0.25	>20.0	0.11
A. fumigatus IFM41209	0.50	>20.0	0.29

[a]Once-daily treatment for 4 days, starting at 1 h after infection.
[b]Fluconazole-resistant.

CONCLUSIONS

In this paper, we have reported the discovery of micafungin (FK463) starting from FR901379, which is a microbial product. Micafungin showed potent activities against *C. albicans* and *A. fumigatus*, which were superior to the parent natural product, FR901379. Furthermore, micafungin maintained the excellent water solubility of the original natural product while its hemolytic activity was greatly reduced.

Micafungin has been marketed in Japan and the United States as a candin-class parenteral antifungal agent for life-threatening mycoses and will soon be available in Europe and Canada.

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