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FR901469, a Novel Antifungal Antibiotic from an Unidentified Fungus No.11243

I. Taxonomy, Fermentation, Isolation, Physico-chemical Properties and Biological Properties

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FR901469 is a novel antifungal antibiotic produced by an unidentified fungus No.11243. This compound was isolated from the culture broth by solvent extraction, HP-20 and YMC ODS gel column chromatography, and lyophilization. FR901469 is a white powder which melts at 182~187°C and possesses the molecular formula $C_{71}H_{116}N_{14}O_{23}$. This compound has good water solubility. FR901469 inhibited the activity of 1,3-β-glucan synthase from *Candida albicans* with an IC_{50} value of 0.05 μg/ml, and displayed greater inhibitory activity than other 1,3-β-glucan synthase inhibitors such as, WF11899A, echinocandin B, aculeacin A, and papulacandin B.

β-Glucans are essential cell wall components in clinically important pathogenic fungi, including *Candida* and *Aspergillus* species. Enzymes necessary for the synthesis of β-glucans are considered to be effective targets for antifungal drugs because of the absence of similar mammalian enzymes[1]. A number of antifungal agents are well known as 1,3-β-glucan synthase inhibitors, for example, echinocandin B[2], aculeacin A[3], pneumocandins[4~6] and the papulacandins[7]. However, these compounds have various problems preventing clinical use, such as poor water-solubility and/or low *in vivo* efficacy against pathogenic fungal infection.

Recently, we isolated WF11899A and related compounds as 1,3-β-glucan synthase inhibitors[8~10]. These compounds are novel water-soluble echinocandin-like lipopeptides with a sulfonate moiety. WF11899A was subsequently enzymatically converted to the deacyl-derivative and then combined with novel acyl side chains to give more active and less toxic compounds. One of these compounds, FK463 is now under clinical study[11~14].

In the course of our continuous screening for new 1,3-β-glucan synthase inhibitors, we isolated a novel antifungal antibiotic, FR901469 (Fig. 1) from the culture broth of an unidentified fungus No.11243. FR901469 is a water-soluble 40-membered macrocyclic lipopeptidolactone consisting of 12 amino acids and a 3-hydroxypalmitoyl moiety. In this paper, we describe taxonomic studies on the producing strain, fermentation, isolation, physico-chemical properties, and biological properties of FR901469.

Materials and Methods

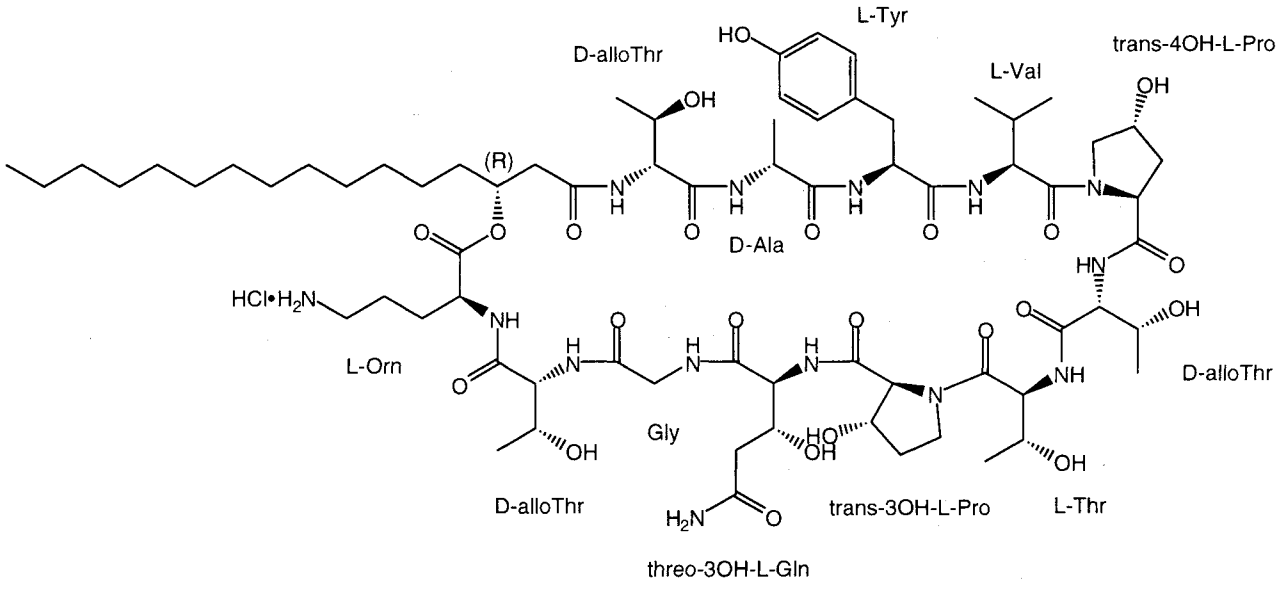
Compounds

Aculeacin A was a generous gift from Asahi Chemical Industry Co., Ltd., Tokyo, Japan. Echinocandin B was isolated from the culture broth of *Aspergillus nidulans* var. *roseus* A42355 NRRL-11440. Cilofungin was generously provided by Eli Lilly and Company, Indianapolis, Indiana, U.S.A. Papulacandin B and nikkomycin X were isolated from the culture broth of *Papularia* sp. and *Streptomyces* sp., respectively, both of which are strains in the Fujisawa culture collection.

General Procedures

Melting points were recorded on a Yanagimoto micro

Fig. 1. Structure of FR901469.



melting point apparatus. IR spectra were measured on a Shimazu IR-420 3S-48 spectrometer. Optical rotation was determined on a Jasco DIP-140 polarimeter, using a 10 cm-micro cell. Low-resolution and high-resolution FAB-MS spectra were obtained on a VG ZAB-SE mass spectrometer.

Taxonomy

The observations were made after two-week incubation at 25°C. Compositions of malt extract agar, Czapek's solution agar and MY20 agar were based on JCM Catalogue of Strains[15]. The color names used in this study were taken from the Methuen Handbook of Colour[16]. The temperature range of growth was determined on potato dextrose agar. The morphological characteristics were determined principally from the cultures on LCA plate by the method of MIURA and KUDO[17].

Glucan Synthase Assay

Inhibitory activity against 1,3-β-glucan synthase was measured according to the method described by SAWISTOSKA-SCHRÖDER *et al.*[18] with some modifications. Briefly, yeast cells of *Candida albicans* 6406 kindly supplied by Dr. D. KERRIDGE, University of Cambridge, were grown to logarithmic phase (absorbance at 660 nm; 0.42) in yeast nitrogen base-glucose (YNBD) medium at 30°C with shaking. The cells were harvested by centrifugation, washed and suspended in ice-cold buffer A (50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM β-mercaptoethanol, 1 M sucrose and 25 μM GTP). The cells were disrupted by mixing with 0.4 mm i.d. glass beads in a vortex mixer. The glass beads were then washed with ice-cold buffer B (buffer A without sucrose), and the cell debris was removed by centrifugation. The supernatant fluids were centrifuged at 100,000×g for 45 minutes at 4°C. The pellet was washed with 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. Two and a half μl of test compound solution or vehicle was incubated with 25 μl of reaction mixture (50 mM Tris-HCl (pH 8.0), 0.8% BSA, 0.1 mM GTP, 0.1% CHAPS, 0.05% Tween80 and the particulate enzyme (40 μg protein)) for 15 minutes 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 minutes at room temperature. The reaction was terminated by addition of 100 μl ice-cold 5% trichloroacetic acid (TCA) and allowed to stand on ice. The resultant precipitate was collected on a GF/C glass filter paper (Whatman International Ltd.) and counted for radioactivity in toluene scintillator.

Antifungal Activity

Antifungal activity was determined by a conventional paper disc diffusion assay on agar plate using YNBD medium against *Candida albicans*-7, a strain from the

Fujisawa culture collection.

HPLC Analysis

The quantitative and qualitative analysis of FR901469 was carried out by reverse phase HPLC (column: YMC Packed column AM-303, S-5, 120A ODS, 250×4.6 mm i.d., made by YMC Co.,) using a solvent system of 45% aqueous acetonitrile containing 0.5% $NH_4H_2PO_4$ (UV detection; 210 nm and flow rate: 1 ml/minute). The retention time was 10.9 minutes.

Acute Toxicity

The acute toxicity was determined with an intravenous injection to female ICR mice (four weeks old). Two mice were used in each group.

Results

Characteristics of Producing Strain

The fungus strain No.11243 was originally isolated from a decayed leaf sample collected at Ayabe, Kyoto Prefecture, Japan.

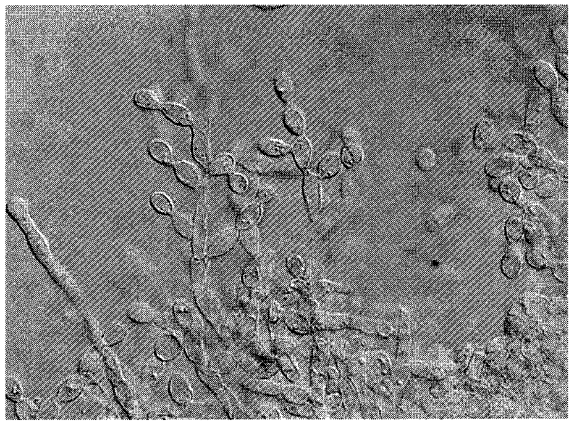
Cultural characteristics in various culture media are summarized in Table 1. This organism grew very restrictedly on agar media and formed pale orange colonies.

Table 1. Cultural characteristics of strain No. 11243.

Media	Cultural characteristics
Malt extract agar	G: Very restrictedly, 0.5-1.0 cm S: Circular, raised, produced hyaline mucous exudation, pale orange (5A3) R: Grayish orange (5B4)
Potato dextrose agar (Difco 0013)	G: Very restrictedly, 0.5-1.0 cm S: Circular, raised, radially sulcate, produced hyaline mucous exudation, pale orange (5A3) R: Pale yellow (4A3)
Czapek's solution agar	G: Very restrictedly, -0.5 cm S: Circular, plane, thin, orange white (5A2) R: Yellowish gray (4B2)
Sabouraud dextrose agar (Difco 0190)	G: Very restrictedly, 0.5-1.0 cm S: Circular, raised, radially sulcate, produced hyaline mucous exudation, grayish orange (5B3) R: Brown (6E7)
Emerson Yp Ss agar (Difco 0739)	G: Very restrictedly, 0.5-1.0 cm S: Circular, raised, radially sulcate, produced hyaline mucous exudation, light orange (5A4) R: Pale yellow (4A3)
Corn meal agar (Difco 0386)	G: Very restrictedly, 0.5-1.0 cm S: Circular to irregular, plane, radially sulcate, produced hyaline mucous exudation, orange white (5A2), and dark brown (6F8) at the center R: Dark gray (1F1)
Oatmeal agar (Difco 0552)	G: Very restrictedly, 0.5-1.0 cm S: Circular to irregular, plane, radially sulcate, orange white (5A2), and black at the center R: Pale yellow (4A3)
MY20 agar	G: Very restrictedly, -0.5 cm S: Irregular, raised, wrinkly, produced hyaline mucous exudation, light orange (5A4) R: Light orange (5A4)

Abbreviation G: growth, measuring colony size in diameter, S: colony surface, R: reverse.

Fig. 2. Micrograph of hyphal cells of strain No. 11243, grown on LCA plate after 30-day incubation at 25°C.



The colony surface was circular with an entire edge, raised, sometimes radially sulcate, and produced hyaline mucous exudation. The reverse side color was pale yellow to grayish orange. This strain was able to grow in the temperature range from 7 to 29°C, with the growth optimum at 22 to 26°C.

Neither reproductive organs nor typical conidial structures were observed on agar plates. Vegetative hyphae were smooth, septate, hyaline and branched. The hyphal cells were cylindrical and 1~4 μm in width. In old cultures (over one month), the colony was conspicuously raised, wrinkly, darkened, and sometimes produced dark brown soluble pigments. Aerial hyphae aggregated fascicularly and moistened by large amounts of mucilaginous exudations. The hyphal cells were swollen, oval or broadly ellipsoidal, attenuate at septa, 6~11 μm long, 4~6 μm thick, and formed in a chain- or tree-like fashion (Fig. 2). Attempts to separate each cell were difficult and/or unsuccessful. Chlamydospores were sometimes observed at the terminal or intercalary cells. They were hyaline, smooth, one-celled, subglobose, and 6~9 μm in diameter.

The above morphological characteristics indicate that the strain No.11243 is classified as an asporogenous fungi, which is a polyphyletic and artificial miscellaneous group. Because of its taxonomical uncertainty, we referred to this isolate simply as "Fungus strain No.11243". The strain has been deposited to the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Japan, as FERM BP-3373.

Fermentation

A seed medium (160 ml), consisting of 4% sucrose, 2% cotton seed meal, 1% dried yeast, 1% peptone, 0.2% KH_2PO_4, 0.2% $CaCO_3$ and 0.1% Tween80 was poured into each of two 500-ml Erlenmeyer flasks and sterilized at 121°C for 30 minutes. A loopful of the slant culture of fungus strain No.11243 was inoculated to each medium and cultured under shaking condition (250 rpm) at 25°C for 4 days.

A production medium (20 liters), consisting of 2% soluble starch, 0.5% glucose, 1% cotton seed meal, 1% gluten meal, 0.2% KH_2PO_4, 1.5% $Na_2HPO_4 \cdot 12H_2O$, 0.01% $ZnSO_4 \cdot 7H_2O$, 0.025% Adekanol LG-109, and 0.025% Silicone KM-70 was poured into a 30-liter jar fermentor and sterilized at 121°C for 30 minutes.

The resultant seed culture broth (320 ml) was transferred to the production medium, and the fermentation was carried out at 25°C for 4 days with aeration at 20 liters per minutes during culture. The agitation was carried out at 200 rpm. The amount of the FR901469 substance in the culture broth was determined by HPLC.

Isolation

The isolation scheme is shown in Fig. 3. The culture broth (75 liters) was extracted with an equal volume of acetone by stirring for 12 hours at room temperature. The extract was filtered through diatomaceous earth and to the filtrate was added an equal volume of water followed by evaporation *in vacuo* to remove acetone. The resultant aqueous solution was applied to a column (6.5 liters) of Diaion HP-20 (Mitsubishi Kasei. Corp.). After the column was washed with water and 40% aqueous acetone, the active fraction was eluted with 80% aqueous acetone containing 0.002 N HCl. The eluate was concentrated to 1.9 liters *in vacuo*, and adjusted to pH 6.0. After the solution was washed with ethyl acetate (one liter), it was extracted with *n*-BuOH (one liter). The *n*-BuOH extract was washed with 1% sodium bicarbonate (one liter) and then with aqueous hydrochloric acid (one liter) adjusted to pH 4.0. The organic solvent layer obtained was concentrated *in vacuo*. The residue was dissolved in 50% aqueous acetonitrile and applied to a column (1.3 liter) of HP-20. The column was washed with water, 50% aqueous methanol, 80% aqueous methanol and methanol. The active fraction was eluted with 80% aqueous acetone containing 0.002 N HCl. After the resultant eluate was concentrated *in vacuo*, the residue was dissolved in 20% aqueous methanol. The solution was subjected to ODS-AM (YMC GEL,

Fig. 3. Isolation and purification procedure of FR901469.

Culture broth (75 liters)
- Extracted with acetone

Acetone extracts
- Added water

Diaion HP-20 column chromatography
- Eluted with 80% acetone-0.002N HCl
- Concentrated to aqueous solution
- Washed with ethyl acetate
- Extracted with *n*-BuOH

n-BuOH extracts
- Concentrated to aqueous solution

Diaion HP-20 column chromatography
- Eluted with MeOH
- Concentrated

YMC-ODS column chromatography
- Eluted with 45% acetonitrile-0.5%$NH_4H_2PO_4$

YMC-ODS column chromatography
- Eluted with 43% acetonitrile-0.5%$NH_4H_2PO_4$

Diaion HP-20 column chromatography
- Eluted with 80% acetone-0.002N HCl
- Concentrated
- Lyophilized

White powder of FR901469 (72 mg)

ODS-AM, 120-S50, YMC Co., Ltd. 500 ml) column chromatography. The column was washed with 20~40% aqueous acetonitrile containing 0.5% $NH_4H_2PO_4$. The active fraction, eluted with 45% aqueous acetonitrile containing 0.5% $NH_4H_2PO_4$, was diluted with the same amount of water and then applied to a column (180 ml) of ODS-AM. After the column was washed with 30~40% aqueous acetonitrile containing 0.5% $NH_4H_2PO_4$, the column was eluated with 43% aqueous acetonitrile containing 0.5% $NH_4H_2PO_4$. This active fraction was diluted with the same amount of the water and then applied to a column (40 ml) of HP-20. The column was washed with water (180 ml), and eluted with 80% aqueous acetone containing 0.002 N HCl. After the eluate was concentrated *in vacuo* to remove acetone, the resultant aqueous solution was lyophilized to obtain 72 mg of the hydrochloride salt of FR901469 as a white powder.

Physico-chemical Properties

Physico-chemical properties of FR901469 are shown in Table 2. FR901469 is a white powder which is soluble in MeOH and water, slightly soluble in acetone and insoluble in *n*-hexane. It showed positive color reactions to ninhydrin,

Table 2. Physico-chemical properties of FR901469.

Appearance		White powder
MP		182~187 °C
$[\alpha]_D^{23}$		+29 ° (*c* 1.5, MeOH)
Molecular formula		$C_{71}H_{116}N_{14}O_{23}\cdot HCl$
HRFAB-MS (m/z)		Found: 1555.8240 $(M-HCl+Na)^+$
		Calcd: 1555.8235
UV $\lambda_{max}^{H_2O}$	nm (ε)	225(sh, 11200), 273(1260), 282 (sh, 1050)
UV $\lambda_{max}^{0.01NHCl}$	nm (ε)	225(sh, 11200), 273(1260), 282 (sh, 1050)
UV $\lambda_{max}^{0.01NNaOH}$	nm (ε)	240(95500), 291(1950)
IR ν_{max} (KBr) cm^{-1}		3400, 2920, 1730, 1660, 1635, 1540, 1520, 1460, 1250, 1090 cm^{-1}
TLC Rf value	System I[A)]	0.42
	System II[b)]	0.18
Solubility	Soluble	H_2O, MeOH
	Slightly	Acetone
	Insoluble	*n*-Hexane
Color reaction	Positive	Ninhydrin, I_2, $Ce(SO_4)_2$-H_2SO_4
	Negative	Molish, Ehrlich
Amino acid analysis (conventional)		Thr(4), Gly(1), Ala(1), Val(1), Tyr(1),Orn(1)

a) Silica gel 60 F_{254} (E. Merck): *n*-BuOH-acetic acid-H_2O (4:1:2)

b) RP-18 WF_{254} (E. Merck): 45% aqueous acetonitrile-0.5%$NH_4H_2PO_4$

Fig. 4. UV spectrum of FR901469.

—— H_2O, 0.01 N HCl, ------ 0.01 N NaOH

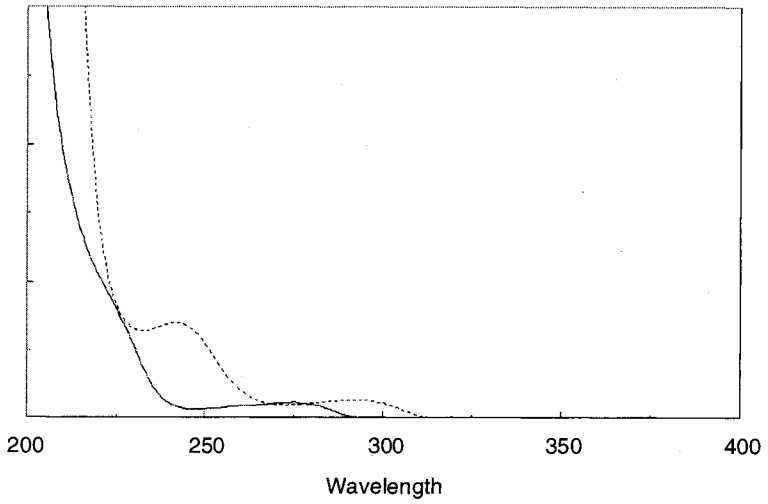
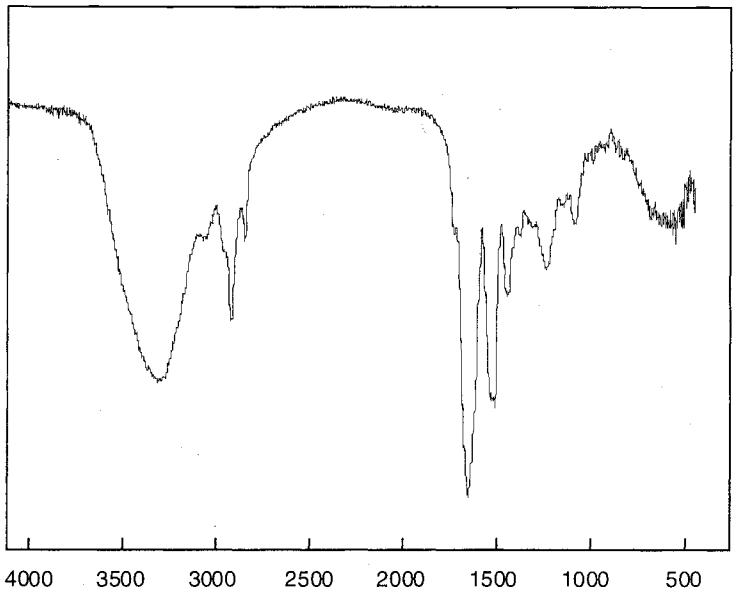


Fig. 5. IR spectrum of FR901469.



iodine vapor and ceric sulfate, and was negative to Molish and Ehrlich. The molecular formula was determined as $C_{71}H_{116}N_{14}O_{23}$·HCl based on the elemental analysis and fast atom bombardment (FAB)-MS. FR901469 exhibited absorption maxima at 225 nm (sh, ε 11200), 273 (ε 1260), 282 (sh, ε 1050) in water in the UV spectrum shown in Fig. 4. The IR spectrum is shown in Fig. 5.

Analysis of conventional amino acids was carried out as follows. One mg of FR901469 substance was hydrolyzed with one ml of 6 N HCl in a sealed tube at 110°C for 20 hours. After hydrolysis, the reaction mixture was evaporated to dryness, and the resulting solid was analysed with an amino acid analyser (Hitachi 835 Automatic Amino-Acid Analyser). The result indicated that the product comprised Thr (4), Gly (1), Ala (1), Val (1), Tyr (1), and Orn (1), as shown in Table 2. The details of the structural elucidation will be described in a separate paper (in preparation).

Table 3. Inhibitory activity of FR901469 on 1,3-β-glucan synthase from *Candida albicans*.

Compound	IC_{50} (μg/ml)
FR901469	0.05
WF11899A	0.8
Aculeacin Aγ	1.3
Echinocandin B	2.6
Cilofungin	2.9
Papulacandin B	2.5
Nikkomycin X	>100

Table 4. Acute toxicity of FR901469.

FR901469 (mg/kg)	Survivors 4days later
0	2/2
63	2/2
125	2/2
250	2/2
500	0/2
1000	0/2

Mouse: ICR, female, 4w, n = 2
Vehicle: saline
Dose: iv one shot

Biological Properties

FR901469 inhibited 1,3-β-glucan synthase prepared from *Candida albicans* 6406 with an IC_{50} value of 0.05 μg/ml (Table 3). The acute toxicity of FR901469 was determined with an intravenous injection to ICR mice (female, 4 weeks old). No lethal toxicity was observed at the dose of 250 mg/kg (Table 4). The body weight increase for all of the test mice was the same as that for the mice of a control group to which the substance was not injected. FR901469 was shown to have potent antifungal activity against *Candida* sp., *Aspergillus* sp. and *Pneumocystis carinii*, but less activity against *Cryptococcus* sp. Studies on the *in vitro* and *in vivo* antifungal activities of FR901469 are reported in the following paper.

Discussion

FR901469 was discovered from the culture broth of fungus strain No.11243. The producing strain No.11243 was not identifiable by traditional methods, because of its asporogenesis. Therefore, we are presently investigating the phylogenetic position of this strain based on 18S and internal transcribed spacers of the ribosomal DNA.

1,3-β-glucan synthase, one of the major cell wall synthesis enzymes in pathogenic fungi, is considered to be the target of antifungal agents, such as the echinocandin-like lipopeptides and papulacandins. However, these compounds have a number of problems, such as poor water-solubility and/or low *in vivo* efficacy against pathogenic fungal infection that prevents clinical evaluation. We have therefore carried out screening to find more potent 1,3-β-glucan synthase inhibitors from microbial products to overcome these obstacles. As a result, we discovered WF11899A and its related compounds, as novel water-soluble echinocandin-like lipopeptides with a sulfonate moiety. Enzymic removal of the palmitoyl side chain of WF11899A, followed by re-acylation with various acyl side chains gave more active and less toxic compounds. FK463[11~14] was synthesized in the course of this study and is now under clinical study.

FR901469 is another new compound discovered from continuous screening for new 1,3-β-glucan synthase inhibitors. The IC_{50} value of FR901469 against 1,3-β-glucan synthase from *Candida albicans* was determined to be 0.05 μg/ml, while those for echinocandin B, aculeacin A, WF11899A, and papulacandin B were over 1 μg/ml (Table 3). The inhibitory activity of FR901469 against 1,3-β-glucan synthase appears to be the most potent among the products produced by microorganisms. We also detected FR901469 with conventional paper disc diffusion assay on agar plate (YNBD medium) against *Candida albicans*-7. Microscopic examination of the inhibitory zone revealed extensive formation of protoplasts, suggesting the inhibitory activity of FR901469 against cell wall formation in *Candida albicans*. Concerning acute toxicity, the LD_{50} of FR901469 in mice after intraperitoneal injection was estimated to be 250~500 mg/kg. This data shows that FR901469 may be quite safe.

FR901469 has excellent solubility in water, being comparable to WF11899A (data not shown). This is a major advantage from the point of view of development of FR901469 for clinical use. While the peptide core portion of echinocandin-like lipopeptides, such as WF11899 A, aculeacin A, mulundocandin[19], echinocandin B, and pneumocandins is composed of 6 amino acids. According

to amino acid analysis, FR901469 has at least 8 common amino acids in its structure. This indicates that the structure of FR901469 is quite different from those of echinocandin-like lipopeptides and the papulacandins which are glycolipids. Actually, as shown in Fig. 1, FR901469 is a novel chemical entity. To date, 1,3-β-glucan synthase inhibitors have been classified into two broad chemical groups, echinocandin-like lipopeptides and papulacandins. Accordingly, with the discovery of FR901469, we propose that a new chemical group of 1,3-β-glucan synthase inhibitors, the non-echinocandin type lipopeptide group, can be created.

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