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FR220897 and FR220899, Novel Antifungal Lipopeptides from *Coleophoma empetri* No. 14573

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Abstract Novel antifungal lipopeptides, FR220897 and FR220899, were isolated from the fermentation broth of a fungal strain No. 14573. This strain was identified as *Coleophoma empetri* No. 14573 from morphological and physiological characteristics. FR220897 and FR220899 showed antifungal activities against *Aspergillus fumigatus* and *Candida albicans* attributed to inhibition of 1,3-β-glucan synthesis. Furthermore, FR220897 was effective in a murine model of systemic candidiasis.

Keywords FR220897, FR220899, antifungal, 1,3-β-glucan synthesis

Introduction

Life-threatening infections caused by *Aspergillus fumigatus* and *Candida albicans* have been increasing in prevalence [1]. However, antifungal therapies in patients are currently limited to a small number of compounds. Toxicity is an issue with treatments based on amphotericin B and resistance is beginning to emerge as a problem with the safer, but fungistatic, azoles [2]. Therefore, new, safe and effective therapeutic agents are highly desirable for the treatment of infections caused by *A. fumigatus* and *Candida albicans*.

In the course of our continued screening program for new antifungals from microbial products, novel lipopeptides, FR220897 and FR220899, were isolated from the cultured broth of *Coleophoma empetri* No. 14573. These compounds have similar structures to FR901379 [3, 4], the points of difference having different amino acid constituents compared to FR901379 and, most strikingly, a sulphate residue presenting at a different position of the aryl ring. Other related antifungal lipopeptides of fungal origin are FR901379 [3, 4], echinocandin B [5] aculeacins [6], mulundocandin [7] and pneumocandins [8, 9], which are produced by *Coleophoma empetri* No. 11899, *A. nidulans*, *A. aculeatus*, *A. sydowi* and *Glarea lozoyensis*, respectively. These antibiotics have excellent anti-*Aspergillus* and anti-*Candida* activities attributed to inhibition of 1,3-β-glucan synthesis [10]. Recently, a new antifungal drug, Micafungin® which is a synthetic analog of FR901379, has been launched, and a number of clinical problems have been solved [11].

In this paper, we describe the taxonomy, fermentation, isolation and physico-chemical properties of FR220897 and FR220899.

Materials and Methods

Compound

FR901379 was isolated from the culture broth of *Coleophoma empetri* F-11899 which is a strain in the Fujisawa culture collection [3].

General Procedures

General procedures are described in the preceding paper.

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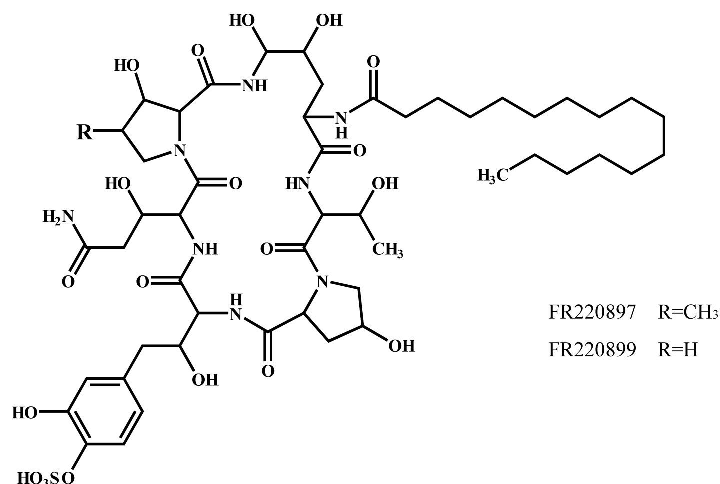

Fig. 1 Structures of FR220897 and FR220899.

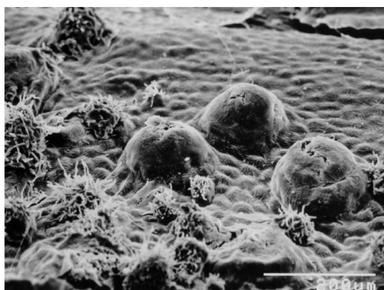

Fig. 2 Electron micrograph of strain No. 14573.

Taxonomic Studies

The producing fungus, strain No. 14573, was originally isolated from a decayed leaf sample collected at Tsushima Island, Nagasaki Prefecture, Japan. The cultural characteristics on various agar media were observed after 14 days of incubation at 25°C: malt extract agar, potato dextrose agar (Difco 0013), Czapek's solution agar, Sabouraud dextrose agar (Difco 0190), Emerson Yp Ss agar (Difco 0739), corn meal agar (Difco 0386), MY20 agar and oatmeal agar (Difco 0552). The compositions of malt extract agar, Czapek's solution agar and MY20 agar were based on the JCM Catalogue of Strains [12]. The color descriptions used in this study were taken from the Methuen Handbook of Colour [13]. The temperature range of growth was determined on potato dextrose agar (NISSUI). The morphological characteristics were examined from the cultures of sterile leaf segments affixed on a Miura's LCA plate [14].

Fermentation

Production of FR220897: An aqueous seed medium (30 ml) containing sucrose 4.0%, glucose 1.0%, soluble starch 2.0%, Pharmamedia 3.0%, soybean flour 1.5%, KH_2PO_4 1.0%, $CaCO_3$ 0.2%, Adekanol LG-109 (defoaming agent, Asahi Denka Co., Ltd.) 0.05% and Silicone KM-70 (defoaming agent, Shin-Etsu Chemical Co., Ltd.) 0.05% was placed in each of three 100-ml Erlenmeyer flasks and was sterilized at 120°C for 30 minutes. A loopful of *Coleophoma empetri* No. 14573, grown on YpSs agar at 25°C for 2 weeks, was inoculated in each of the seed flasks. The inoculated flasks were shaken on a rotary shaker (220 rpm, 5.1 cm throw) at 25°C for 5 days, and 8 ml of the seed culture was transferred to 160 ml of the same sterile seed medium in the 500-ml Erlenmeyer flask. The flasks were shaken on a rotary shaker (220 rpm, 5.1 cm throw) at 25°C for 2 days, and 640 ml (four flasks) of the second seed culture was inoculated to 20 liters of sterile production medium consisting of sucrose 8.0%, dried yeast 4.0%, $CaCO_3$ 0.5%, Adekanol LG-109 0.05% and Silicone KM-70 0.05% (pH 6.3 adjusted with 1 N NaOH) in a 30-liter jar fermentor. Fermentation was carried out at 25°C for 5 days under aeration of 20 liters/minute and agitation of 250 rpm.

Production of FR220899: The second seed culture (640 ml) was inoculated to 20 liters of sterile production medium consisting of modified starch 5.0%, Pharmamedia 2.0%, oat meal 0.5%, KH_2PO_4 3.5%, $Na_2HPO_4 \cdot 12H_2O$ 2.6%, $(NH_4)_2SO_4$ 0.6%, L-isoleucine 0.5%, L-proline 0.5%, Adekanol LG-109 0.05% and Silicone KM-70 0.05% in a 30-liter jar fermentor. Fermentation was carried out at 25°C for 5 days under aeration of 20 liters/minute and agitation of 250 rpm.

HPLC Analysis

Detection of FR220897 and FR220899 from the

fermentation broth and fractions under purification was performed by HPLC using a reverse phase column YMC Pack ODS-AM 303, S-5 120A (250×4.6 mm i.d., YMC Co., Ltd.). The mobile phase was 50% aqueous acetonitrile containing 0.1% TFA. The flow rate was 1.0 ml/minute. The detection wavelength was set at 210 nm.

In Vitro Antifungal Activity

Antifungal activity was measured by the micro-broth dilution method in 96-well culture plates employing yeast nitrogen base - dextrose (YNBD) medium. *Candida albicans* FP633, a clinical isolate in the Fujisawa culture collection, was incubated in yeast - maltose (YM) broth for 20 hours at 37°C at the standing condition. The cell suspension was prepared by washing the cultured cells with sterile saline. *A. fumigatus* FP1305, a strain from the Fujisawa culture collection, was cultured on YM agar slant for 7 days. The spores were harvested in sterile saline, and filtered through gauze. Finally, the fungal cells or spores were resuspended in YNBD medium for inoculation. Test compounds were diluted serially two fold with YNBD. The test microorganism was inoculated to each well to yield 1×10^4 cfu/well in 100 μl. The plates were incubated for 20 hours at 37°C. Minimum effective concentration (MEC) was determined by microscopic observation.

Glucan Synthase Assay

Candida albicans glucan synthase assays were conducted according to the method described by Sawistowska-Schröder *et al.* with some modifications [10]. Briefly, 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 10% trichloroacetic acid (TCA) and allowed to stand on ice. The resultant precipitate was dissolved with 1 N NaOH. After neutralization, radioactivity was counted with a toluene scintillator.

In Vivo Antifungal Activity of FR220897 against *Candida albicans* in a Murine Infection Model

The *in vivo* anti-*Candida* activity was evaluated in a murine model of systemic infection. *Candida albicans* FP633, a clinical isolate in the Fujisawa culture collection, was used in this study. The inoculum was prepared from a three-day old culture of YM agar slant. ICR mice (female, four weeks old) were intravenously injected with 1×10^6 cells of the yeast. Five mice were used in each group. Test compounds were dissolved in saline and administered subcutaneously one hour after challenge and once a day for three consecutive days. The ED_{50} value was determined on the day when all control mice (vehicle only) died.

Results

Characteristics of the Producing Strain

The cultural characteristics of strain No. 14753 are summarized in Table 1. The growth of the microorganism was rather restricted on various agar media and grayish colonies were fromed. The growth on potato dextrose agar was restricted, attaining 1.5~2.5 cm in diameter after 2 weeks at 25°C. The colony surface was flat to raised, velvety to cottony and light gray to dark gray. The margin was yellowish white and the reverse was olive. The spread of colonies on corn meal agar was rather restricted, attaining 2.5~3.5 cm in diameter under the same conditions. The surface was flat to centrally raised, dark gray and lustrous. The colony center was velvety to cottony and purplish gray to dark purple. Mycelium near the margin was subimmersed and white. The reverse was dark gray to dark green; its margin was yellowish white. Strain No. 14573 was able to grow at a temperature range of 3 to 30°C, with the growth optimum at 21~24°C.

Strain No.14573 produced pycnidial to stromatic conidiomata in the autoclaved leaf segments affixed on LCA media (Fig. 2), while it formed neither teleomorph nor anamorph on/in agar media. The conidiomata were superficial to semi-immersed, separate and dark brown to black. Their shape was convex to discoid, sometimes papillate, non-ostiolate or indistinctly ostiolate, unilocular, flattened at the base, thin-walled at the upper part, 70~170 μm in diameter and 40~90 μm high. The lower cells of inner pycnidial walls were thick, dark brown, of irregular shape, and composed of textura angularis. The inner cells produced directly conidiogenous cells, but they sometimes formed conidiophores. The conidiophores were hyaline, smooth, septate, simple to sparingly branched, and 10~17×3.5~4.5 μm. The conidiogenous cells were discrete, acrogenous or intercalary, hyaline, smooth, ampulliform to lageniform, sometimes cylindrical, and 5~11(~16)×2~4.5 μm. Conidia were hyaline, smooth, amerosporous, cylindrical, rounded at the apical end, with a small projection at the base, and (11~)13~20×2~3 μm. The conidiogenous cells and conidia were sometimes covered with large sheaths. Paraphysis-like structures often formed on or amongst conidiophores, and they were similar to sheaths. They were hyaline, thin-walled, campanulate to

Table 1 Cultural characteristics of strain No. 14573

Media	Cultural characteristics
Malt extract agar	G: Very restricted, 1.0∼2.0 cm
	S: Circular, flat, velvety, not formed reproductive structures, brownish gray (5F2) to grayish brown (6F3)
	R: Olive (3F4) to olive brown (4F4), and olive (3E3-3E4) at the center
Potato dextrose agar (Difco 0013)	G: restricted, 1.5∼2.5 cm
	S: Circular, flat to raised, velvety to cottony, not formed reproductive structures, light gray (1D1) to dark gray (1F1), yellowish white (3A2) at the margin
	R: Olive (3D3-3F3)
Czapek's solution agar	G: restricted, 1.5∼2.5 cm
	S: Circular, submerged, thin, flat, not formed reproductive structures, olive brown (4F4)
	R: Olive brown (4F4)
Sabouraud dextrose agar (Difco 0190)	G: restricted, 1.5∼2.5 cm
	S: Circular, flat to centrally raised, velvety, partly hygroscopic, sectoring, not formed reproductive structures, orange white (5A2) to grayish yellow (4C4), dark gray (1F1) at the center and sectors
	R: Grayish yellow (4B4-4C3), and brownish gray (5F2) at the center and sectors
Emerson Yp Ss agar (Difco 0739)	G: restricted, 2.0∼3.0 cm
	S: Circular, flat, velvety, not formed reproductive structures, gray (1F1) to olive gray (1F2), yellowish white (3A2) at the margin
	R: Greenish gray (25F2) to dark green (25F3), and yellowish white (3A2) at the margin
Corn meal agar (Difco 0386)	G: Rather restricted, 2.5∼3.5 cm
	S: Circular, flat to centrally raised, not formed reproductive structures, lustrous, dark gray (1F1); velvety to cottony, purplish gray (14F2) to dark purple (14F3) at the center; submerged and white (1A1) at the margin
	R: Dark gray (1F1) to dark green (25F3), yellowish white (3A2) at the margin
MY20 agar	G: Very restricted, 1.0∼2.0 cm
	S: Circular, flat, hygroscopic, lustrous, not formed reproductive structures, grayish yellow (4B3)
	R: Light yellow (4A4-4A5)
Oatmeal agar (Difco 0552)	G: Rather rapidly, 3.0∼4.0 cm
	S: Circular, flat to centrally raised, velvety to cottony, not formed reproductive structures, dark gray (1F1), and yellowish white (4A2) at the margin

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

cylindrical, collapsing at old culture, and 18∼30(∼35)× 2.5∼5 μm. Vegetative hyphae were smooth, septate, brown and branched. The hyphal cells were cylindrical and 2∼7 μm in width. Chlamydospores were not observed.

According to the fungal taxonomic criteria of von Arx [15], the strain No. 14573 was considered to belong to the Coelomycete genus *Coleophoma* Höhn. 1907. There were few differences between the above characteristics and descriptions of *Coleophoma empetri* (Rostr.) Petrak 1929 by Sutton [16] and by Wu *et al.* [17]: except superficial and indistinctly ostiolate conidiomata. In conclusion, we identified the isolate as a strain of *Coleophoma empetri*, and named it *Coleophoma empetri* No. 14573. This strain has been deposited to the International Patent Organism Depositary in the National Institute of Advanced Industrial Science and Technology, Japan, as FERM BP-6252.

Isolation and Purification of FR220897

An equal volume of acetone was added to the culture broth. The mixture was filtered with an aid of diatomaceous earth. The filtrate was diluted with an equal volume of water and passed through a column (3.0 liters) of DIAION HP-20 (Mitsubishi Chemical Co., Ltd.) packed with water. The column was washed with water (9.0 liters) and 50% aqueous methanol (10 liters) and then eluted with methanol (29 liters). The active fraction (0∼20 liters) was diluted with an equal volume of water and passed through a column (1.0 liter) of YMC-GEL (ODS-AM 120-S50, YMC

Co., Ltd.) packed with water. The column was washed with 60% (5.0 liters) and 70% (2.8 liters) aqueous methanol and then eluted with 80% aqueous methanol (2.8 liters). The active fraction (0.8∼2.8 liters) was diluted with an equal volume of water and passed through a column (1.0 liter) of YMC-GEL (ODS-AM 120-S50) packed with water. The column was washed with 40% aqueous methanol (1.0 liter) and eluted with 50% aqueous acetonitrile containing 0.5% $NH_4H_2PO_4$ (3.3 liters). The active fraction (2.0∼2.5 liters) was diluted with an equal volume of water and passed through a column (2.0 liters) of YMC-GEL (ODS-AM 120-S50) packed with water. The column was washed with 40% aqueous methanol (6.0 liters) and eluted with 80% aqueous methanol (4.4 liters). The active fraction (2.5∼4.4 liters) was concentrated *in vacuo* to an aqueous solution and lyophilized to give 411 mg of crude FR220897. A portion of this powder (120 mg) was dissolved in a small volume of water and further purified by preparative HPLC, using YMC-packed column (ODS-AM SH-343-5AM S-5, 250×20 mm i.d., YMC Co., Ltd.) with 50% aqueous acetonitrile containing 0.5% $NaH_2PO_4 \cdot 2H_2O$ as a mobile phase. A flow rate was 9.9 ml/minute. Fractions containing the FR220897 were collected. These active fractions were diluted with an equal volume of water and passed through YMC-packed column (ODS-AM SH-343-5AM S-5, 250×20 mm i.d.) equilibrated with 25% aqueous acetonitrile containing 0.25% $NaH_2PO_4 \cdot 2H_2O$. The column was washed with 30% aqueous methanol (240 ml) and then eluted with 80% aqueous methanol at a flow rate of 9.9 ml/minute. The eluate was concentrated *in vacuo* and lyophilized to give 70 mg of FR220897 as white powder.

Isolation and Purification of FR220899

An equal volume of acetone was added to the culture broth. The mixture was filtered with an aid of diatomaceous earth. The filtrate was diluted with an equal volume of water and passed through a column (6.0 liters) of DIAION HP-20 packed with water. The column was washed with 50% aqueous methanol (19 liters) and then eluted with methanol (34 liters). The active fraction (0∼20 liters) was diluted with an equal volume of water and passed through a column (4.0 liters) of YMC-GEL (ODS-AM 120-S50) packed with 50% aqueous methanol. The column was washed with 50% (6.5 liters) and 60% (12 liters) aqueous methanol and then eluted with 70% aqueous methanol (16 liters). The active fraction (5.8∼16 liters) was concentrated *in vacuo* to 5.3 liters. One liter of this solution was diluted with an equal volume of water and passed through a column (1.0 liters) of YMC-GEL (ODS-AM 120-S50, YMC Co., Ltd.) packed with 20% aqueous methanol. The column was washed with 40% aqueous acetonitrile containing 0.5% $NH_4H_2PO_4$ (3.0 liters) and eluted with 50% aqueous acetonitrile containing 0.5% $NH_4H_2PO_4$ (2.9 liters). The active fraction was diluted with an equal volume of water and passed through a column (2.0 liters) of YMC-GEL (ODS-AM 120-S50) packed with 25% aqueous acetonitrile. The column was washed with 40% aqueous methanol and eluted with 80% aqueous methanol. The active fraction was concentrated *in vacuo* to an aqueous solution and lyophilized to give 798 mg of crude FR220899. This powder was dissolved in a small volume of water and further purified by preparative HPLC, using a YMC-packed column (ODS-AM SH-343-5AM S-5, 250×20 mm i.d.) with 50% aqueous acetonitrile containing 0.5% $NaH_2PO_4 \cdot 2H_2O$ as a mobile phase. A flow rate was 9.9 ml/minute. Fractions containing the FR220899 were collected. These active fractions were diluted with an equal volume of water and passed through YMC-packed column (ODS-AM SH-343-5AM S-5, 250×20 mm i.d.) equilibrated with 25% aqueous acetonitrile containing 0.25% $NaH_2PO_4 \cdot 2H_2O$. The column was washed with 30% aqueous methanol and then eluted with 70% aqueous methanol at a flow rate of 9.9 ml/minute. The eluate was concentrated *in vacuo* and lyophilized to give 121 mg of FR220899 as white powder.

Physico-chemical Properties

The physico-chemical properties of FR220897 and FR220899 are summarized in Table 2. Both are soluble in water, methanol and dimethyl sulfoxide, but insoluble in *n*-hexane and chloroform. They displayed positive color reactions to iodine vapor and ceric sulfate though they were negative against Molish, Dragendorff and $FeCl_3$. They have UV absorption maxima at 276 nm. Their ESI-MS spectra showed molecular ion peaks at 1157 and 1143, respectively.

Structure Elucidation of FR220897

The molecular formula of FR220897 ($C_{51}H_{82}N_8O_{20}S$ as free acid) was derived from ESI-MS and elemental analysis (Table 2) and consistent with ^{13}C NMR data (Table 3). A ^{13}C NMR comparison with FR901379 [3, 18] showed that the difference of one oxygen atom between FR901379 and FR220897 was ascribable to the replacement of a hydroxymethine (75.5 ppm) in FR901379 with a methylene (41.1 ppm) at C-4 of the 3,4-dihydroxyhomotyrosine (DiOHTyr) residue in FR220897. Initial structural effort therefore was focused on the DiOHTyr moiety. A 1,3,4-trisubstituted benzene substitution pattern was evident from three benzene protons (δ_H 7.18 (d, J=8 Hz), 6.80 (d, J=2 Hz) and 6.67 (dd, J=8 and 2 Hz). By simple inspection of vicinal ^{13}C chemical shifts (δ_C 150.3 (C-3′) and δ_C 140.1 (C-4′)), the position of attachment of the

Table 2 Physico-chemical properties of FR220897 and FR220899

	FR220897	FR220899
Appearance	White powder	White powder
Melting point	220°C	230°C
$[\alpha]_D^{23}$	−15° (*c* 0.9 MeOH)	−12° (*c* 0.5 MeOH)
ESI-MS (*m/z*)	1157 $(M-H)^-$	1143 $(M-H)^-$
Molecular formula	$C_{51}H_{81}N_8O_{20}SNa$	$C_{50}H_{79}N_8O_{20}SNa$
	$C_{51}H_{82}N_8O_{20}S$ as free acid	$C_{50}H_{80}N_8O_{20}S$ as free acid
Elemental analysis		
Calcd as tetrahydrate	C 48.87, H 7.16, N 8.94, S 2.56, Na 1.83	C 48.46, H 7.08, N 9.04
Found:	C 48.79, H 7.34, N 8.96, S 2.80, Na 1.69	C 48.39, H 7.15, N 8.95
UV λ_{max}^{MeOH} nm (ε)	276 (3150)	276 (2880)
Color test		
Positive	I_2, $Ce(SO_4)_2$-H_2SO_4	I_2, $Ce(SO_4)_2$-H_2SO_4
Negative	Molish, Dragendorff, $FeCl_3$	Molish, Dragendorff, $FeCl_3$
Solubility		
Soluble	H_2O, methanol, DMSO	H_2O, methanol, DMSO
Insoluble	*n*-hexane, chloroform	*n*-hexane, chloroform
IR λ_{max} (KBr) cm^{-1}	3360, 2940, 2830,1670, 1630, 1530, 1440, 1270, 1240, 1050	3360, 2920, 2830, 1670, 1630, 1540, 1440, 1270, 1240, 1050
TLC (Rf value)*	0.45	0.39

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

sulfate could not be determined unambiguously. To define the positions of the sulfate and the phenolic OH, a full set of DMSO-d_6 NMR data was measured. Methylene protons (δ_H 2.45/δ_C 39.6) showed HMBC correlations to two hydrogen-bearing aromatic carbons at 118.3 ppm (δ_H 6.68 (d, J=2 Hz), C-2′) and at 120.4 ppm (δ_H 6.56 (dd, J=8 and 2 Hz), C-6′) and to an aromatic carbon at 135.6 ppm (C-1′). The 6′-H proton (6.56 ppm) gave a strong HMBC cross peak to an aromatic carbon at 139.2 ppm (s). The phenolic OH proton resonating at 8.74 ppm showed HMBC cross peaks to C-2′ and two aromatic carbons at 148.7 ppm (s) and 139.2 ppm (s). This NMR evidence indicated that the position of attachment of the phenolic OH was C-3′ and thus position of the sulfate was secured at C-4′. With the structure known, ^{13}C NMR data (CD_3OD) was analyzed by a combination of ^1H-^1H COSY, HSQC and HMBC and presented in Table 3 along with FR901379.

Structure Elucidation of FR220899

The negative mode of ESI-MS data indicated a molecular formula of $C_{50}H_{80}N_8O_{20}S$ which was consistent with the ^{13}C NMR data (Table 3). The absence of a doublet methyl at 1.06 ppm implied the loss of the methyl group present in the 3-hydroxy-4-methylproline residue of FR220897, consistent with the difference of a methylene group between FR220899 and FR220897. A ^{13}C NMR comparison with FR220897 indicated that the methine carbon (39.1 ppm) of FR220897 was replaced by a methylene (34.6 ppm) in FR220899. The modification of CH_3CH to CH_2 was further supported by an up-field shift of the methylene (47.0 ppm) in FR220899 as compared with the C-5 methylene (52.9 ppm) of OHMePro in FR220897. With the structure in hand, a complete ^{13}C NMR data analysis was achieved by extensive analysis of 2D NMR spectra (^1H-^1H COSY, HSQC and HMBC) and presented in Table 3.

In Vitro Activities of FR220897 and FR220899

The *in vitro* antifungal activities of FR220897 and FR220899 were compared with that of FR901379. The results are shown in Table 4. The antifungal activities of FR220897 and FR220899 were similar to that of FR901379 in that hyphal morphological changes were observed in *A. fumigatus*, *i.e.* hyphae abnormally grown, shortened, stunted and highly branched with bipolar or vesicular tips, swollen germ tubes and frequent balloon-like cells (data not shown). Although the antifungal activity of FR220897 was almost equivalent in potency to FR901379, inhibition of 1,3-β-glucan synthase by FR220897 was about 50-fold weaker than that of FR901379 (Tables 4 and 5).

Table 3 ^{13}C NMR data for FR220897, FR220899 and FR901379 (125 MHz, CD_3OD)

Position		FR220897	FR220899	FR901379
4,5-Dihydroxyornithine (DiOHOrn)				
	C-1	174.3 s	174.4 s	174.4 s
	C-2	51.3 d	51.2 d	51.5 d
	C-3	34.9 t	35.0 t	35.0 t
	C-4	70.7 d	70.7 d	70.7 d
	C-5	73.9 d	73.9 d	74.4 d
Threonine (Thr)				
	C-1	172.8 s	172.9 s	172.6 s
	C-2	58.6 d	58.5 d	58.4 d
	C-3	68.2 d	68.3 d	68.2 d
	C-4	19.5 q	19.5 q	19.8 q
4-Hydroxyproline (OHPro)				
	C-1	174.1 s	174.1 s	173.5 s
	C-2	62.4 d	62.4 d	62.4 d
	C-3	38.9 t	38.9 t	38.4 t
	C-4	71.3 d	71.3 d	71.3 d
	C-5	57.2 t	57.2 t	57.1 t
3,4-Dihydroxyhomotyrosine (DiOHTyr)				
	C-1	172.5 s	172.5 s	172.5 s
	C-2	58.0 d	58.0 d	57.1 d
	C-3	74.0 d	74.0 d	76.3 d
	C-4	41.1 t	41.1 t	75.5 d
	C-1′	137.6 s	137.6 s	134.6 s
	C-2′	119.4 d	119.4 d	123.2 d
	C-3′	150.3 s	150.3 s	141.1 s
	C-4′	140.1 s	140.1 s	150.3 s
	C-5′	124.0 d	124.0 d	118.3 d
	C-6′	122.0 d	121.9 d	125.5 d
3-Hydroxyglutamine (OHGln)				
	C-1	169.3 s	169.3 s	169.4 s
	C-2	55.5 d	55.4 d	55.5 d
	C-3	70.5 d	70.5 d	70.7 d
	C-4	39.8 t	39.7 t	39.7 t
	C-5	176.7 s	176.8 s	176.8 s
3-Hydroxy-4-methylproline (OHMePro)				
	C-1	172.5 s	172.7 s	172.7 s
	C-2	70.2 d	69.8 d	70.1 d
	C-3	75.9 d	74.2 d	75.7 d
	C-4	39.1 d	34.6 t	39.1 d
	C-5	52.9 t	47.0 t	53.0 t
	4-Me	11.1 q		11.1 q
Palmitoyl				
	C-1	176.0 s	175.9 s	175.8 s
	C-2	36.7 t	36.7 t	36.7 t
	C-3	27.0 t	27.0 t	26.9 t
	C-4	30.3 t	30.3 t	30.3 t
	C-5	30.4 t	30.5 t	30.4 t
	C-6	30.5 t	30.6 t	30.5 t
	C-7	30.7 t	30.7 t	30.7 t
	C-8	30.8 t	30.8 t	30.8 t
	C-9	30.8 t	30.8 t	30.8 t
	C-10	30.8 t	30.8 t	30.8 t
	C-11	30.8 t	30.8 t	30.8 t
	C-12	30.8 t	30.8 t	30.8 t
	C-13	30.7 t	30.7 t	30.7 t
	C-14	33.1 t	33.1 t	33.1 t
	C-15	23.7 t	23.7 t	23.7 t
	C-16	14.4 q	14.4 q	14.4 q

Table 4 *In vitro* antifungal activities (MECs) of FR220897, FR220899 and FR901379 by microbroth dilution method

Compound	*Candida albicans* FP633	*Aspergillus fumigatus* FP1305
FR220897	0.04	0.02
FR220899	0.31	0.08
FR901379	0.02	0.02

MEC (μg/ml).

Table 5 Inhibitory activities of FR220897, FR220899 and FR901379 against 1,3-*β*-glucan synthase prepared from *Candida albicans*

Compound	IC_{50} (μg/ml)
FR220897	36.2
FR220899	19.7
FR901379	0.77

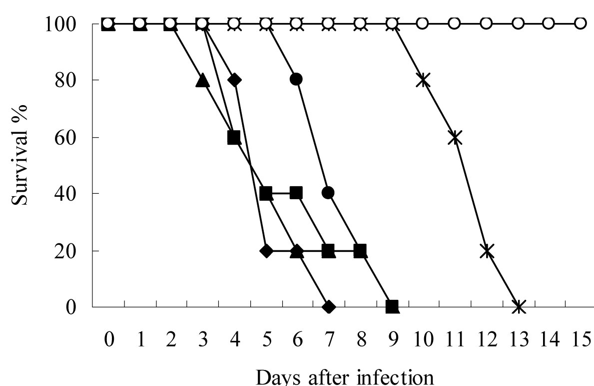

Fig. 3 Protective effect of FR220897 against systemic infection with *Candida albicans* FP633.

◆, Control; ■, 1.0 mg/kg; ▲, 3.2 mg/kg; ●, 10 mg/kg; *, 3.2 mg/kg; ○, 100 mg/kg

In Vivo Antifungal Activity of FR220897 against *Candida albicans* in a Murine Infection Model

The *in vivo* antifungal activity of FR220897, which showed more potent *in vitro* antifungal activity than FR220899, was evaluated. The protective efficacy of FR220897 administered subcutaneously against murine systemic infection with *Candida albicans* was examined. The ED_{50} value for FR220897 on the day when all control mice died was 10 mg/kg. Survival curves for the *in vivo* candidiasis model are shown in Fig. 3.

Discussion

In this paper, we have presented the novel antifungal lipopeptides, FR220897 and FR220899, isolated from the fermentation broth of *Coleophoma empetri* No. 14573. These compounds belong to the echinocandin-like class of lipopeptides consisting of a cyclic hexapeptide linked to a fatty acyl chain. A number of echinocandin analogs, which differ in the amino acid constituents of the cyclic peptide portion and in the fatty acyl chains, have been isolated from fungi. The acyl side chain is readily replaced by semi-synthesis; however, modification of the cyclic peptide portion by chemical synthesis is not easy. We thus attempted to obtain diversity in the cyclic peptide nuclear structure by screening for new, related, chemical products. FR220897 and FR220899 have different amino acid constituents compared to FR901379 and, most strikingly, a sulphate residue present at a different position of the aryl ring.

FR220897 showed almost equivalent antifungal activity to FR901379; however, the inhibitory activity of FR220897 on 1,3-*β*-glucan synthase was weaker than that of FR901379. This may suggest that FR220897 inhibits the fungal growth in a different manner than FR901379. Though further studies are required to elucidate this phenomenon, the mechanism of the antifungal activities of FR220897 and FR220899 is considered to be inhibition of 1,3-*β*-glucan synthesis from the observed morphological changes. It is known that echinocandins affect the function of the FKS genes which encode the 1,3-*β*-glucan synthases. Radding *et al.* [19] sought to define the echinocandin target within a *Candida albicans* membrane preparation by photoaffinity labeling with an echinocandin analog and identified two major proteins which have high similarity to *Saccharomyses cerevisiae* Pil1 and Lsp1, respectively. Subsequently, Edlind *et al.* [20] suggested the possibility that Pil1 and Lsp1 associate with 1,3-*β*-glucan synthase and , along with Rho1, play a role in its regulation.

FR220897 was effective in a *Candida albicans* murine infection model and could therefore constitute a lead compound providing an echinocandin-like lipopeptide scaffold with a novel cyclic peptide portion. We plan to synthesis and evaluate a series of chemically modified FR220897 derivatives in subsequent work.

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