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FR227673 and FR190293, Novel Antifungal Lipopeptides from *Chalara* sp. No. 22210 and *Tolypocladium parasiticum* No. 16616

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Abstract Novel antifungal lipopeptides, FR227673 and FR190293, were isolated from the fermentation broths of fungal strains *Chalara* sp. No. 22210 and *Tolypocladium parasiticum* No. 16616, respectively. These compounds have the same cyclic peptide nuclear structure as FR901379, with different side chains, and showed antifungal activity against *Aspergillus fumigatus* and *Candida albicans* attributed to inhibition of 1,3-β-glucan synthesis.

Keywords FR227673, FR190293, antifungal, 1,3-β-glucan synthesis

Introduction

Serious systemic infections caused by fungi such as *Aspergillus fumigatus* and *Candida albicans* are an increasing problem in recipients of transplanted organs and in other immunosuppressed conditions. Side effects are an issue with treatments based on amphotericin B and drug resistance is beginning to emerge as a problem with the safer but fungistatic azoles [1]. Therefore, new, safe and effective therapeutic agents are highly desirable for the treatment of infections caused by fungi. Recently, the novel echinocandin-like lipopeptides, micafungin [2~4] and caspofungin [5], have been launched as antifungal antibiotics. They have excellent anti-*Candida* and anti-*Aspergillus* activities attributed to inhibition of 1,3-β-glucan synthesis. Fungal 1,3-β-glucan is an attractive target for antifungals due to the presence of a number of fungus-specific enzymes in its biosynthetic pathway, leading to the expectation of selective toxicity to the parasite and not to the host [6]. During our screening for fungal 1,3-β-glucan synthesis inhibitors, novel lipopeptides, FR227673 and FR190293, were isolated from the fermentation broths of fungal strains *Chalara* sp. No. 22210 and *Tolypocladium parasiticum* No. 16616, respectively. They have good water-solubility because it has a sulfate moiety. Other sulfate containing echinocandin-like lipopeptides were all produced by coelomycetes, which form conidial structures only on a leaf segment. In contrast, FR226673 and FR190293 were produced by hyphomycetes that can form hyphal conidia in a general medium.

In this paper, we describe the taxonomy, fermentation, isolation, physico-chemical properties and biological activities of FR227673 and FR190293.

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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 [7].

Taxonomic Studies

The producing fungi, strains No. 22210 and No. 16616, were isolated from soil samples collected from Ibaraki and Nagano prefectures, respectively. The cultural characteristics

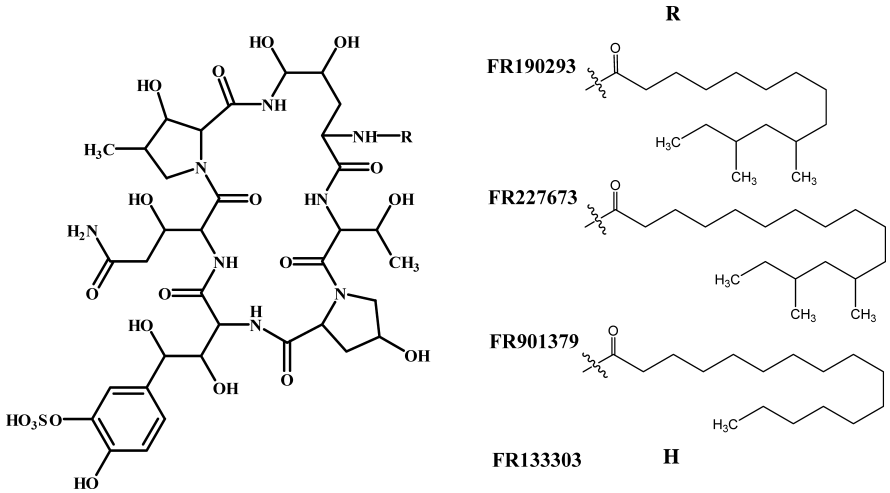

Fig. 1 Structures of FR190293, FR227673, FR901379 and FR133303.

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), and MY20 agar. The compositions of malt extract agar, Czapek's solution agar and MY20 agar were based on the JCM Catalogue of Strains [8]. The cultural characteristics of these strains are summarized in Table 1. The color descriptions used in this study were taken from the Methuen Handbook of Colour [9]. The temperature range of growth was determined on potato dextrose agar (NISSUI). The morphological characteristics were examined by the cultures on a Miura's LCA plate [10]. For the genus identification, fungal taxonomic criteria by von Arx [11] and Domsch *et al.* [12] were cited.

Fermentation

For FR227673 fermentation, 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 the slant culture of the strain No. 22210 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 flasks. 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 used to inoculate 20 liters of sterile production medium consisting of glucose 1%, mannitol 10%, Pharmamedia 3.5%, KH_2PO_4 0.9%, 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 10 days under aeration of 20 liters/minute and agitation of 250 rpm.

For FR190293 fermentation, an aqueous seed medium (160 ml) containing glycerol 2.0%, sucrose 2.0%, Pharmamedia 2.0%, dried yeast 1.0%, polypeptone 1.0%, KH_2PO_4 0.1% and Tween80 0.1% was placed in each of three 500 ml Erlenmeyer flasks and was sterilized at 120°C for 30 minutes. A loopful of *T. parasiticum* No. 16616, grown on YpSs agar at 25°C for 2 weeks, was inoculated in the seed flasks. The inoculated flasks were shaken on a rotary shaker (220 rpm, 5.1 cm throw) at 25°C for 7 days and were used to inoculate 20 liters of sterile production medium consisting of corn starch 2.0%, glucose 1.0%, Pharmamedia 1.0%, soybean meal 0.5%, dried yeast 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 6 days under aeration of 20 liters/minute and agitation of 200 rpm.

HPLC Analysis

Detection of FR227673 and FR190293 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 60% aqueous acetonitrile containing 0.5% $NaH_2PO \cdot 2H_2O$. The flow rate was 1.0 ml/minute. The detection wavelength was set at 210 nm.

Deacylation of FR190293

The cyclic peptide nucleus of FR190293 was obtained by enzymatic cleavage according to the method described by

Table 1 Cultural characteristics of strains No. 22210 and No. 16616

Media	Cultural characteristics	
	No. 22210	No. 16616
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	G : restricted, 1.5~2.0 cm S : Circular, flat, velvety, formed conidial structures abundantly, white to pale yellow (5A3) at the center and margin, olive brown (4F4) at their spaces R Olive (3F3) at the center, olive white (5A2) at the margin
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)	G: restricted, 1.5~2.0 cm S : Circular, flat, somewhat raised, velvety to cottony, formed conidial structures abundantly, white to pale yellow (4A3) at the center and margin, grayish brown (5F3) at their spaces R : Olive brown (4F3), yellowish white (4A2) at the margin
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)	G : restricted, 2.0~2.5 cm S : Circular, cottony, formed conidial structures abundantly, white R : White to yellowish white (3A2)
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	G : restricted, 1.0~2.0 cm S : Circular, cottony, formed conidial structures abundantly, grayish brown (7E3) at the center, yellowish gray (4B2) to pale orange (5A3) at the margin, producing pale orange soluble pigments R : Olive brown (4F4) or pale orange (5A3)
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	G : restricted, 2.0~2.5 cm S : Circular, flat, velvety to cottony, formed conidial structures abundantly, white to pale orange (4A3) R : Pale orange (4A3)
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	G : restricted, 1.5~2.0 cm S : Circular, flat, thin, velvety, formed conidial structures abundantly, olive gray (2F3) at the center, yellowish white (3A2) at the margin R : Olive gray (2F2), 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)	G : Very restricted, 0.5~1.0 cm S : Circular, raised, velvety, formed conidial structures R : Yellowish gray (5F4), light orange (5A4) at the margin

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

Debono *et al.* [13]. Briefly, FR190293 was incubated with the vegetative mycelium of *Actinoplanes utahensis* in 1 M sodium-phosphate buffer (pH 5.8) at 50°C with stirring for 1 hour. Decrease of FR190293 and production of deacyl-FR190293 were monitored by HPLC. Deacyl-FR190293 was purified by YMC GEL (ODS-AM 120-S50) column chromatography.

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. The *Candida albicans* cultures were incubated in yeast - maltose (YM) broth for 20 hours at 30°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 samples 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

The *Candida albicans* glucan synthase assay was conducted according to the method described by Sawistowska-Schröder *et al.* [14] with some modifications. 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, 1.0 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.

Results

Characteristics of strain No.22210 and strain No.16616

The growth of strain No. 22210 on various agar media was restricted. The colonies formed were yellowish-white. The culture 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, radiately sulcate, hygroscopic, lustrous, and yellowish white to grayish yellow or pale gray. The reverse color was grayish yellow to grayish brown, and pale orange soluble pigments were produced. This strain was able to grow at a temperature range from 2 to 28°C, with the growth optimum at 21～25°C. Strain No. 22210 produced conidial structures on Miura's LCA media, while it did not form a teleomorph. The anamorph consisted of phialidic conidiogenous cells and conidia (Fig. 2). Conidiophores were absent. The conidiogenous cells formed directly from the swelling hyphal cells, and they were discrete, acrogenous, pale brown, smooth, and ampulliform with long tube-like collarettes. The bases of the conidiogenous cells were 11～14×4～5.5 μm in size and the tips were 14～20(～22)×2～2.5 μm. Conidia were born endogenously within the upper half of conidiogenous cells and aggregated at the tips. The conidial masses were hyaline. The conidia were enteroblastic, phialidic, hyaline, smooth, amerosporous, oblong, truncate at both ends, and 6～9.5(～10.5)×1.5～2(～2.5) μm in size. Hyphal cells were cylindrical, 1.5～3.5 μm in width, and sometimes swelled to 3.5～9×3.5～5.5 μm. The abovementioned characteristics show that strain No. 22210 belongs to the Hyphomycete genus *Chalara* (Corda) Rabenh. 1844. Thus, we named it *Chalara* sp. No. 22210.

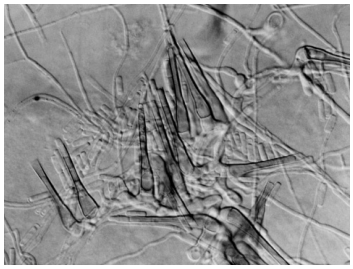

Fig. 2 Electron micrograph of strain No. 22210.

The growth of the second strain, No. 16616, was also restricted on agar media, forming powdery yellowish-white colonies. The culture on potato dextrose agar was restricted, attaining 1.5～2.0 cm in diameter after two weeks at 25°C. The colony surface was velvety to cottony, somewhat raised, and the color was white or yellowish white at center and margin, but their space was grayish brown. The reverse color was olive brown and yellowish white. The temperature range of strain No. 16616 was 3～30°C and the growth optimum was at 16～22°C. This strain abundantly produced conidial structures consisting of verticillate phialides and conidial masses on various agar media (Fig. 3), while a teleomorph was

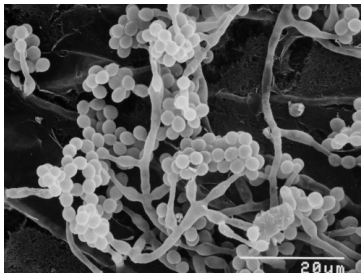

Fig. 3 Electron micrograph of strain No. 16616.

not observed. Conidiophores were macronematous or semi-macronematous, monomematous, hyaline to brown, smooth, directly erect from vegetative hyphae or branched from aerial mycelium as short laterals. They were simple or branched phialides verticillately or solitarily. The phialides were discrete, acrogenous, hyaline, smooth, ampulliform, and consisted of a swelling base and an elongated tip. The bases were 11～14×4～5.5 μm and the tips were 14～20(～22)×2～2.5 μm. Enteroblastic conidia were born from phialides and aggregated in slimy drops or dry chains. They were hyaline, smooth, one-celled, globose to subglobose, and 3～4×2.5～3 μm. Hyphal cells were cylindrical, 1～3 μm in width. Chlamydospores were absent, but sometimes a single conidium was born on top of the phialide and did not separate. After comparison of these characteristics with those described in the monograph by Bissett [15], strain No. 16616 was considered to belong to the Hyphomycete species *Tolypocladium parasiticum* Barron 1980. This species was originally described as a fungal parasite against rotifers in soils, however Barron [16] also reported it was culturable on artificial media. No. 16616 did not form typical chlamydospores like *T. parasiticum*, while other characteristics were identical. So, we named it *Tolypocladium parasiticum* No. 16616.

Isolation and Purification of FR227673

The culture broth (30 liters) was extracted with an equal volume of acetone by stirring for 2 hours at room temperature. The mixture was filtered with the aid of diatomaceous earth. The filtrate was diluted with an equal volume of water and passed through a column (4.0 liters) of DIAION HP-20 (Mitsubishi Chemical Co., Ltd.) packed with 25% aqueous acetone. The column was washed with 50% aqueous methanol (12 liters) and eluted with methanol (28 liters). The active fraction (0～15 liters) was concentrated *in vacuo* and lyophilized to give brown powder. This powder was dissolved in 2.0 liters of 50% aqueous methanol. This solution was passed through a column (2.0 liters) of YMC GEL (ODS-AM 120-S50, YMC Co., Ltd.) packed with 50% aqueous methanol. The column was washed with 60% (5.5 liters) and 70% (5.0 liters) aqueous methanol and eluted with 80% aqueous methanol (10 liters). The active fraction (0～6 liters) was diluted with an equal volume of water containing 1% $NaH_2PO_4 \cdot 2H_2O$ and passed through a column (2.0 liters) of YMC GEL (ODS-AM 120-S50) packed with 40% aqueous methanol containing 0.5% $NaH_2PO_4 \cdot 2H_2O$. The column was washed with 45% aqueous acetonitrile containing 0.5% $NaH_2PO_4 \cdot 2H_2O$ (5.7 liters) and eluted with 55% aqueous acetonitrile containing 0.5% $NaH_2PO_4 \cdot 2H_2O$ (3.9 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 27.5% aqueous acetonitrile containing 0.25% $NaH_2PO_4 \cdot 2H_2O$. The column was washed with 50% (7 liters) and 70% (6 liters) aqueous methanol and eluted with 90% aqueous methanol (5.7 liters). The active fraction (1.2～5.7 liters) was concentrated *in vacuo* to an aqueous solution and lyophilized to give 427.7 mg of crude FR227673 substance. This powder (97.7 mg) was dissolved in small volume of water and further purified by preparative HPLC, using a YMC-packed column (ODS-AM SH-343-5 AM S-5, 250×20 mm i.d., YMC Co., Ltd.) with 60% 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 FR227673 were collected. These active fractions were diluted with an equal volume of water and passed through YMC-packed column (ODS-AM SH-343-5 AM S-5, 250×20 mm i.d.) equilibrated with 30% aqueous acetonitrile containing 0.25% $NaH_2PO_4 \cdot 2H_2O$. The column was washed with 50% aqueous methanol and eluted with 95% aqueous methanol at a flow rate of 9.9 ml/minute. The eluate was concentrated *in vacuo* and lyophilized to give 62.2 mg of FR227673 as a white powder.

Isolation and Purification of FR190293

An equal volume of acetone was added to the culture broth. The mixture was filtered with the aid of diatomaceous earth. The filtrate was concentrated to 40 liters and pH was adjusted to 3.95. The aqueous solution was passed through a column (1.5 liters) of DIAION HP-20 packed with water. The column was washed with distilled water (5 liters) and 50% aqueous methanol (8 liters) and then eluted with methanol (6 liters) and acetone (4.5 liters). The active fraction (4.5～6 liters of methanol and 0～3 liters of acetone) was dried *in vacuo* and dissolved in small amount of methanol. The residue was applied to a column (300 ml) of silica gel. The column was washed with 0.9 liters of ethyl acetate, ethyl acetate/acetone (1 : 1) and acetone, and then eluted with 0.9 liters of acetone/methanol (10 : 1) and

Table 2 Physico-chemical properties of FR227673 and FR190293

	FR227673	FR190293
Appearance	White powder	White powder
Melting point	230～235°C	210～215°C
$[\alpha]_D^{23}$	−27° (*c* 0.5, MeOH)	−20° (*c* 1.0, MeOH)
ESI-MS (*m*/*z*)	1203 $(M+H)^+$	1175 $(M+H)^+$
HRESI-MS (*m*/*z*)		
Found	1203.5709	1175.5388
Calcd for M+H	1203.5706	1175.5393
Molecular formula	$C_{53}H_{86}N_8O_{21}S$	$C_{51}H_{82}N_8O_{21}S$
Elemental analysis		
Calcd as pentahydrate	C 48.39, H 7.28, N 8.52	Not determined
Found:	C 48.35, H 7.47, N 8.37	Not determined
UV λ_{max}^{MeOH} nm (ε)	277 (2020)	276 (1700)
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, $FeCl_3$
Solbility		
Soluble	H_2O, methanol, DMSO	H_2O, methanol, DMSO
Insoluble	*n*-hexane, chloroform	*n*-hexane, chloroform
IR λ_{max} (KBr) cm^{-1}	3300, 2930, 1670, 1630, 1540, 1520, 1460, 1440, 1250, 1050	3350, 2960, 2930, 2860, 1670, 1630, 1530, 1440, 1400, 1270, 1150, 1050,
TLC (Rf value)*	0.44	0.38

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

acetone/methanol (1 : 1). The active fraction was dried *in vacuo* and dissolved in 200 ml of 50% aqueous methanol, and then passed through a column (350 ml) of YMC-GEL (ODS-AM 120-S50) packed with 50% aqueous methanol. The column was washed with 50% (1 liter) and 60% (1 liter) aqueous methanol and then eluted with 70% aqueous methanol (1.5 liters). The active fraction (0.65～1.1 liters) was concentrated *in vacuo* and further purified by preparative HPLC, using a YMC-packed column (ODS-AM SH-343-5AM S-5, 250×20 mm i.d., YMC Co., Ltd.) with 45% 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 FR190293 were collected, diluted with equal volume of distilled water, and then passed through a YMC-packed column (ODS-AM SH-343-5AM) equilibrated with 50% aqueous methanol. The column was washed with 50% aqueous methanol and eluted with 60% aqueous methanol at a flow rate of 9.9 ml/minute. The eluate was concentrated *in vacuo* and lyophilized to give 47 mg of FR190293 as a white powder.

Physico-chemical Properties

Physico-chemical properties of FR227673 and FR190293 are summarized in Table 2. Both of them were 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 negative against Molish and Dragendorff. FR227673 and FR190293 showed UV absorption maxima at 277 nm and 276 nm, respectively.

Structure Elucidation of FR190293

The compound's high water solubility suggested the presence of a sulfate group. The molecular formula $C_{51}H_{82}N_8O_{21}S$ was derived from Electrospray Ionization (ESI) MS (*m*/*z* 1175 $(M+H)^+$), the ^{13}C NMR data (Table 3) and confirmed by HR-ESIMS (see Table 2). FR190293 possessed the same molecular formula of FR901379 [7, 17]. The ^1H NMR spectrum was quite similar to that of FR901379 apart from the signals relating to the acyl chains. The ^{13}C NMR chemical shifts due to six amino acid components matched well with those of relevant constituent of FR901379 and the difference of ^{13}C NMR spectrum lay in the acyl side chain. This fact revealed that FR190293 had the same nucleus and was acylated with a different fatty acid. The identity of the nucleus between FR190293 and FR901379 was established as follows. FR133303 is the deacylation product of FR901379 and a key intermediate

Table 3 ^{13}C NMR data for FR190293, FR227673 and FR901379

Position		FR190293[a]	FR227673[a]	FR901379[b]
4,5-Dihydroxyornithine (DiOHOrn)				
	C-1	174.4 s	174.4 s	174.4 s
	C-2	51.4 d	51.4 d	51.5 d
	C-3	34.9 t	34.9 t	35.0 t
	C-4	70.7 d	70.7 d	70.7 d
	C-5	74.2 d	74.3 d	74.4 d
Threonine (Thr)				
	C-1	172.7 s	172.7 s	172.6 s
	C-2	58.4 d	58.4 d	58.4 d
	C-3	68.2 d	68.2 d	68.2 d
	C-4	19.8 q	19.7 q	19.8 q
4-Hydroxyproline (OHPro)				
	C-1	173.5 s	173.5 s	173.5 s
	C-2	62.4 d	62.4 d	62.4 d
	C-3	38.4 t	38.3 t	38.4 t
	C-4	71.3 d	71.3 d	71.3 d
	C-5	57.1 t	57.1 t	57.1 t
3,4-Dihydroxyhomotyrosine (DiOHTyr)				
	C-1	172.5 s	172.6 s	172.5 s
	C-2	56.9 d	57.0 d	57.1 d
	C-3	76.4 d	76.3 d	76.3 d
	C-4	75.5 d	75.5 d	75.5 d
	C-1′	134.5 s	134.5 s	134.6 s
	C-2′	123.2 d	123.2 d	123.2 d
	C-3′	141.1 s	141.1 s	141.1 s
	C-4′	150.3 s	150.3 s	150.3 s
	C-5′	118.2 d	118.2 d	118.3 d
	C-6′	125.6 d	125.5 d	125.5 d
3-Hydroxyglutamine (OHGln)				
	C-1	169.3 s	169.3 s	169.4 s
	C-2	55.5 d	55.5 d	55.5 d
	C-3	70.6 d	70.6 d	70.7 d
	C-4	39.6 t	39.6 t	39.7 t
	C-5	176.9 s	176.9 s	176.8 s
3-Hydroxy-4-methylproline (OHMePro)				
	C-1	172.6 s	172.5 s	172.7 s
	C-2	70.1 d	70.1 d	70.1 d
	C-3	75.7 d	75.7 d	75.7 d
	C-4	39.1 d	39.1 d	39.1 d
	C-5	53.0 t	53.0 t	53.0 t
	4-Me	11.1 q	11.1 q	11.1 q
		10,12-Dimethylmyristoyl	Palmitoyl	Palmitoyl
	C-1	175.8 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	30.6 t	30.6 t	30.4 t
	C-6	30.7 t	30.7 t	30.5 t
	C-7	31.1 t	30.8 t	30.7 t
	C-8	28.0 t	30.8 t	30.8 t
	C-9	38.1 t	31.1 t	30.8 t

Table 3 Continued

Position		FR190293[a]	FR227673[a]	FR901379[b]
	C-10	31.2 d	28.0 t	30.8 t
	C-11	45.9 t	38.0 t	30.8 t
	C-12	32.9 d	31.2 d	30.8 t
	C-13	30.3 t	45.9 t	30.7 t
	C-14	11.6 q	32.9 d	33.1 t
	10-Me	20.7 q	30.3 t	23.7 t
	12-Me	20.2 q	11.6 q	14.4 q
			20.7 q	
			20.2 q	

a: 125 MHz in CD_3OD, b: 100 MHz in CD_3OD.

for the preparation of Micafungin [2, 18]. Deacylation of FR190293 was achieved in a similar manner. The nucleus obtained was identical to FR133303 in all respects (^1H, ^{13}C, $[\alpha]_D$).

In the ^{13}C NMR data of the acyl side chain, two chemical shifts (11.6 ppm (CH_3) and 45.9 ppm (CH_2)) were diagnostic. The primary methyl signal at 11.6 ppm was characteristic for the *anteiso* regioisomer. In the HMBC spectra, two secondary methyl (δ_H 0.85/δ_C 20.7 and 0.85/20.2) showed HMBC correlations to the down-field shifted methylene at 45.9 ppm. The presence of 10,12-dimethylmyristoyl group was indicated by the above information and confirmed by a comparison of the ^{13}C NMR data with that of pneumocandin [19]. With the structure of FR190293 in hand, a full NMR assignment was achieved by a combination of ^1H-^1H COSY, HSQC and HMBC and the ^{13}C data are presented in Table 3 alongside those of FR901379.

Structure Elucidation of FR227673

HR-ESIMS revealed the molecular formula to be $C_{53}H_{86}N_8O_{21}S$ (C_2H_4 more than FR190293) which was consistent with the elemental analysis and the ^{13}C NMR data (Table 3). The ^1H NMR spectrum was quite similar to that of FR190293 except for the intensity of overlapping acyl methylene signals (1.3 ppm). The ^{13}C NMR spectrum was almost superimposable to that of FR190293. Two additional methylene signals (30.8 and 30.8 ppm) appeared. The above information indicated that structure of acyl side chain was 12,14-dimethylpalmitoyl. A combined analysis of ^1H-^1H COSY, HSQC and HMBC confirmed the structure as depicted in Fig. 4.

***In Vitro* Activities of FR227673 and FR190293**

The antifungal activities of FR227673 and FR190293 were compared to that of FR901379 (Table 4). FR227673 showed almost equivalent antifungal activity to FR901379; however, the antifungal activity of FR199293 was 4-fold weaker than that of FR901379. On the other hand, the inhibitory activity of FR227673 on 1,3-β-glucan synthase was 2-fold weaker than that of FR190293 (Table 5).

Discussion

FR227673 and FR190293 belong to the echinocandin-like class of lipopeptides, consisting of a cyclic peptide nucleus and a lipophilic acyl side chain [20]. FR227673 and FR190293 had the same cyclic peptide nucleus as FR901379; however, acyl side chains were different from that of FR901379. The most prominent characteristic of FR227673, FR190293 and FR901379 is the presence of a sulfate ester moiety in the nucleus. Other sulfate containing echinocandin-like lipopeptides were reported by us [21, 22], all produced by coelomycetes, which form conidial structures only on a leaf segment. In contrast, FR226673 and FR190293 were produced by *Chalara* sp. No. 22210 and *T. parasiticum* No. 16616, respectively. These are hyphomycetes that can form hyphal conidia in a general medium.

FR227673 and FR190293 have branched side chains. The antifungal activity of FR227673 having a longer side chain was more potent than FR190293; however, inhibition of 1,3-β-glucan synthase by FR227673 was weaker than by FR190293. Further studies are required to elucidate this phenomenon. FR190293 has the same branched side chain as pneumocandin which was chemically modified to give Caspofungin. FR227673 with excellent water solubility could be a promising lead compound for a new antifungal drug.

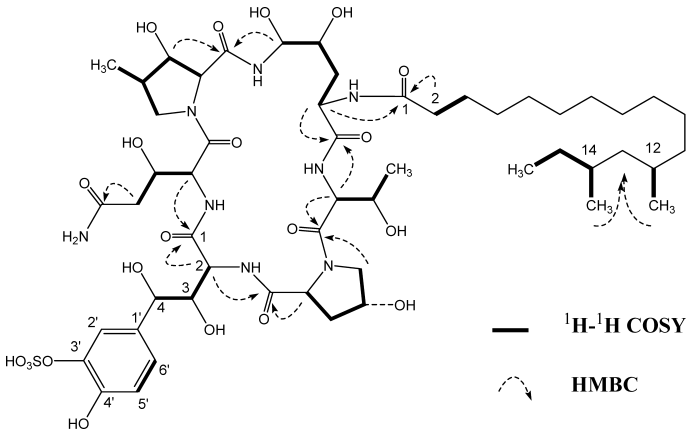

Fig. 4 ^{1}H-^{1}H COSY and selected HMBC correlations of FR227673.

Table 4 *In vitro* antifungal activities (MECs) of FR227673, FR190293 and FR901379 by microbroth dilution method

Compound	*Candida albicans* FP633	*Aspergillus fumigatus* FP1305
FR227673	0.02	0.01
FR190293	0.08	0.04
FR901379	0.02	0.02

MEC (μg/ml).

Table 5 Inhibitory activities of FR227673, FR190293 and FR901379 on 1,3-β-glucan synthase prepared from *Candida albicans*

Compound	IC_{50} (μg/ml)
FR227673	2.0
FR190293	0.87
FR901379	0.77

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