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FR209602 and Related Compounds, Novel Antifungal Lipopeptides from *Coleophoma crateriformis* No. 738

II. *In Vitro* and *In Vivo* Antifungal Activity

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Abstract The biological activities of the novel echinocandin-like lipopeptides, FR209602, FR209603 and FR209604, were evaluated. These compounds showed antifungal activity against *Candida albicans* and *Aspergillus fumigatus* attributed to inhibition of 1,3-β-glucan synthesis. The minimum effective concentrations of these compounds against *C. albicans* and *A. fumigatus* ranged from 0.02 to 0.04 μg/ml by microbroth dilution assay, and the IC_{50} values on *C. albicans* 1,3-β-glucan synthase were 0.49, 0.64 and 0.72 μg/ml, respectively. FR209602 and FR209603 showed good efficacy by subcutaneous injection against *C. albicans* in a murine systemic infection model, with ED_{50} values of 2.0 and 1.9 mg/kg, respectively.

Keywords FR209602, FR209603, FR209604, antifungal, 1,3-β-glucan synthesis

Introduction

Life-threatening infections caused by *Candida albicans* and *Aspergillus fumigatus* have been increasing in prevalence [1]. Antifungal therapies, however, are currently limited to a small number of compounds. Toxicity is an issue with treatments based on amphotericin B [2] and resistance is beginning to emerge as a problem with the safer but fungistatic azoles [3]. Therefore, new, safe and effective therapeutic agents are highly desirable for the treatment of infections caused by *Candida albicans* and *A. fumigatus*.

In the previous paper [4], we described the novel antifungal lipopeptides, FR209602, FR209603 and FR209604, produced by *Coleophoma crateriformis* No. 738. These compounds have potent antifungal activity against *Candida albicans* and *A. fumigatus* attributed to inhibition of 1,3-β-glucan synthesis. Fungal cell wall components, especially 1,3-β-glucan, are promising targets for antifungals, because this is essential to fungi and absent from mammalian cells [5]. In recent years, the new antifungal, micafungin [6~8] and caspofungin [9], which belong to the echinocandin-like family of lipopeptides, have been successfully launched as antifungal drugs.

In this paper, we describe the *in vitro* and *in vivo* antifungal activities of the newly isolated lipopeptides, FR209602, FR209603 and FR209604.

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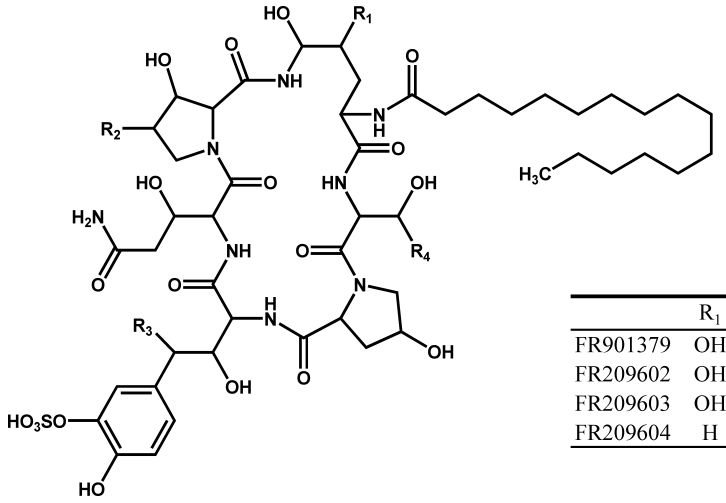
Materials and Methods

Compound

FR901379 was isolated from the culture broth of *Coleophoma empetri* F-11899 which is a strain in the Fujisawa culture collection [10, 11].

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*



	R_1	R_2	R_3	R_4
FR901379	OH	CH_3	OH	CH_3
FR209602	OH	CH_3	H	H
FR209603	OH	H	H	H
FR209604	H	CH_3	H	H

Fig. 1 Structures of FR901379, FR209602, FR209603 and FR209604.

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 culture of *Cryptococcus neoformans* YC203 was grown in YM broth medium for 20 hours at 30°C with shaking. 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 yeast nitrogen base - dextrose 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 (*Candida albicans* FP633 and *A. fumigatus* FP1305) or 48 hours at 37°C (*Cryptococcus neoformans* YC203). Minimum effective concentration (MEC) was determined by microscopic observation.

Membrane Fraction Preparation of *Candida albicans*

Membrane fraction preparation from *Candida albicans* was conducted according to the method described by Sawistowska-Schröder *et al.* [12] with some modifications. Briefly, yeast cells of *Candida albicans* FP633 were grown to logarithmic phase (absorbance at 660 nm; 0.42) in 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), 1.0 mM β-mercaptoethanol, 1.0 M sucrose and 25 μM GTP). The cells were sonicated with 0.4 mm i.d. glass beads using a sonicator. The glass beads were then washed with ice-cold buffer B (buffer A without sucrose), and the cell debris were 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 particulate enzyme for glucan synthase assay.

Glucan Synthase Assay

The assay procedure for *Candida albicans* glucan synthase was conducted according to the method described by Sawistowska-Schröder *et al.* [12] with some modifications. 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 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.

***In Vivo* Antifungal Activities of FR209602 and FR209603 against *Candida albicans* in a Murine Infection Model**

The *in vivo* anti-*Candida* activities were evaluated in a murine model of systemic infection using *Candida albicans* FP633. 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

Table 1 *In vitro* antifungal activities (MECs) of FR209602, FR209603, FR209604 and FR901379 by microbroth dilution method

	FR209602	FR209603	FR209604	FR901379
Candida albicans FP633	0.04	0.04	0.04	0.08
Aspergillus fumigatus FP1305	0.04	0.02	0.04	0.08
Cryptococcus neoformans YC203	>50	>50	>50	50

MEC (μg/ml).

Table 2 Inhibitory activities of FR209602 and related compounds on 1,3-β-glucan synthase prepared from *Candida albicans*

Compound	IC_{50} (μg/ml)
FR209602	0.49
FR209603	0.64
FR209604	0.72
FR901379	0.77

the day when all control mice (vehicle only) died.

Results

In Vitro Activities of FR209602, FR209603 and FR209604

Table 1 shows the antifungal activities of FR20902, FR209603 and FR209604. They showed almost equivalent activities to FR901379 against *Candida albicans* FP633 and *A. fumigatus* FP1305. These compounds were also inactive against *Cryptococcus neoformans* as was FR901379. With all of these compounds, 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). These morphological changes were characteristic of the inhibition of 1,3-β-glucan synthesis. Table 2 shows the inhibitory activities of these compounds on β-1,3-glucan synthase derived from *Candida albicans* FP633. Significant inhibition of β-1,3-glucan synthase was observed in all of these compounds, which was consistent with the *in vitro* antifungal activities of these compounds.

In Vivo Antifungal Activities of FR209602 and FR209603 against *Candida albicans* in a Murine Infection Model

In vivo antifungal activities of FR209602 and FR209603 against *Candida albicans* in a murine infection model were evaluated, and compared to that of FR901379. Both FR209602 and FR209603 significantly prolonged the survival of infected mice by subcutaneously administration with ED_{50} values of 2.0 mg/kg and 1.9 mg/kg, respectively (Table 3). These efficacies were almost equivalent to that of FR901379. Survival curves for the *in vivo* systemic infection model are shown in Fig. 2.

Table 3(a) *In vivo* antifungal activities of FR209602 and FR901379 against *Candida albicans* in a murine infection model

	FR209602	FR901379
ED_{50} (mg/kg)	2.0	1.2

Table 3(b) *In vivo* antifungal activities of FR209603 and FR901379 against *Candida albicans* in a murine infection model

	FR209603	FR901379
ED_{50} (mg/kg)	1.9	0.9

Discussion

In this paper, we have presented *in vitro* and *in vivo* activities of novel antifungal lipopeptides, FR209602, FR209603 and FR209604, isolated from the fermentation broth of *Coleophoma crateriformis* No. 738. These compounds have potent antifungal activities against *Candida albicans* and *A. fumigatus*. The effects of these compounds on the morphology of *A. fumigatus* were characteristic of inhibition of 1,3-β-glucan synthesis (data not shown). The IC_{50} values of these compounds on *Candida albicans* 1,3-β-glucan synthase were 0.49, 0.64 and 0.72 μg/ml, respectively. Moreover, FR209602 and FR209603 were effective in a *Candida albicans* murine

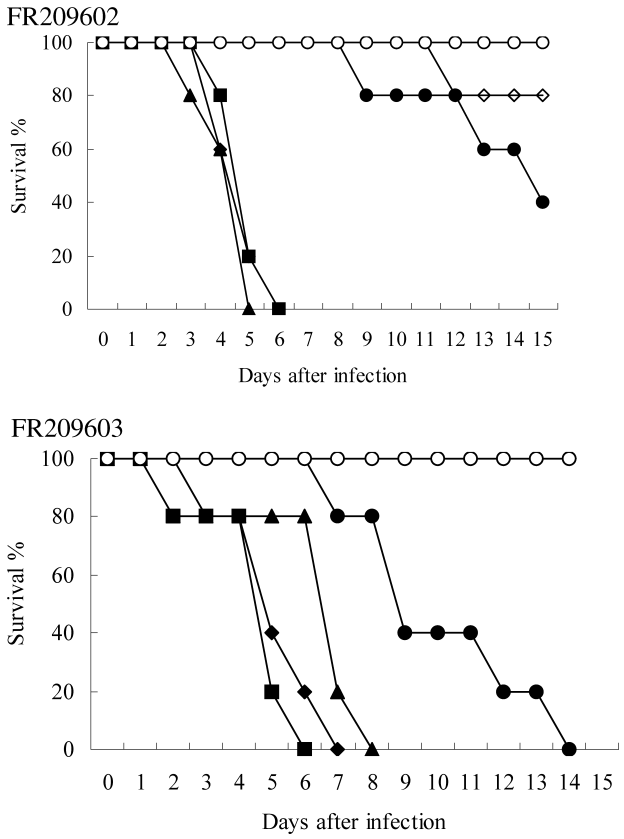


Fig. 2 Protective effect of FR209602 and FR209603 against systemic infection of *C. albicans* FP633.

◆, Control; ■, 0.32 mg/kg; ▲, 1.0 mg/kg; ●, 3.2 mg/kg; ◇, 10 mg/kg; ○, 32 mg/kg

infection model.

These compounds have similar structures to FR901379, the points of difference being the amino acid constituents of the cyclic peptide portion of their structures. It is known that amino acid composition affects both the antifungal spectrum as well as the chemical stability of lipopeptides. FR209602-4 could therefore provide a new direction for developing the next generation of antifungal agents.

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