FR227244, a Novel Antifungal Antibiotic from *Myrothecium cinctum* No. 002

II. Biological Properties and Mode of Action

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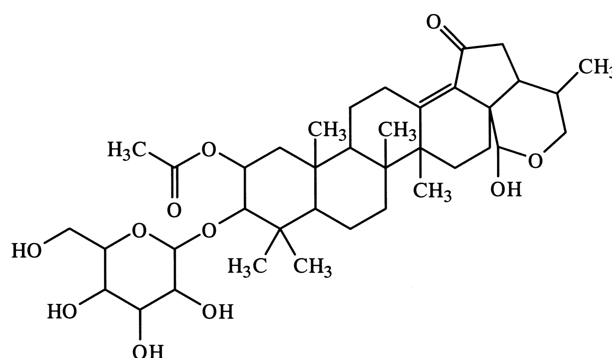
(Received for publication September 15, 2004)

FR227244 is a novel triterpene glycoside that exhibits *in vitro* antifungal activity against filamentous fungi such as *Aspergillus* sp. and *Trichophyton* sp. and yeast such as *Candida utilis* and *Candida parapsilosis* but shows low activity against *Candida albicans*, *Candida krusei* and *Candida tropicalis*. Specifically, FR227244 exhibits *in vitro* and *in vivo* antifungal activity against *Aspergillus fumigatus*. The minimum effective concentration (MEC) of FR227244 against *A. fumigatus* FP1305 in a micro-broth dilution test was 0.031 μg/ml. FR227244 showed good efficacy by subcutaneous injection and oral administration against *A. fumigatus* in a murine systemic infection model, with ED_{50}s of 1.9 and 18 mg/kg, respectively. FR227244 inhibited glucan synthesis in a 1,3-β-glucan synthase assay weakly and in whole cells strongly, but did not effect other macromolecule synthesis, including protein, nucleic acids, mannan and chitin. These results and the effect on hyphal morphology of *A. fumigatus* suggested that FR227244 showed antifungal activity based on inhibition of glucan synthesis.

In the previous paper[1)], we described a novel antifungal antibiotic FR227244 as a fungal metabolite that showed characteristic changes in hyphal morphology of *Aspergillus fumigatus*, similar to FR901379, which inhibits cell wall synthesis based on inhibition of 1,3-β-glucan synthase[2~4)].

The fungal cell wall, which is composed of glucan, chitin and mannan, is an essential structure for fungi and is not present in mammalian cells. It is expected to be a suitable target for antifungal agents and to fulfill the criteria for a safe drug. Therefore, we have been focused on the search for cell wall synthesis inhibitors from natural products, and discovered FR901379, FR901469[5~8)] and FR227244. The effect of FR227244 against *A. fumigatus* was similar to those of FR901379 and FR901469. ONISHI *et al.* reported that some acidic terpenoids, arundifungin[9)] and ergokonin A[10)], and some triterpene glycosides, ascosteroside[11,12)] and enfumafungin[13,14)], are 1,3-β-glucan synthase inhibitors[15)]. However, it is not clear whether FR227244 inhibits fungal cell wall component synthesis in the same way.

Fig. 1. Structure of FR227244.



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In this work, we carried out biochemical studies on the mode of action and *in vitro* and *in vivo* antifungal activity of FR227244. Based on these studies, the potential of FR227244 as a candidate lead compound for new antifungal therapeutic agents was evaluated.

Materials and Methods

Compounds

FR901379[2], FR901469[6] and nikkomycin X were purified in Fujisawa Pharmaceutical Co., Ltd. Nikkomycin Z was obtained from Calbiochem®. Amphotericin B and 5-fluorocytosine (5-FC) were purchased from Sigma. Cycloheximide was from Nacalai tesque Inc., while terbinafine was from Tokyo Kasei.

In Vitro Antifungal Activity

Antifungal activity was measured by microbroth dilution method assay using 96-well titer plates. Each inoculum was prepared as follows. The *Candida* cultures were incubated in yeast-maltose (YM) broth medium for 20 hours at 37°C in standing conditions. The culture of *Cryptococcus neoformans* 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. The filamentous fungi were cultured on YM agar slant for 7 days. The spores were harvested in sterile saline, and filtered through gauze. Finally, fungal cells or spores were resuspended in yeast nitrogen base-dextrose (YNBD) medium for inoculation. Test compounds were dissolved in methanol and diluted serially two-fold with YNBD. The test microorganisms were inoculated to each well to yield 1×10^4 cfu/well in 100 μl. The plates were incubated for 16 hours at 37°C (*Candida* sp. and *Aspergillus* sp.), 48 hours at 37°C (*C. neoformans*, *Aspergillus terreus* and *Malassezia furfur*), 48 hours at 32°C (*Candida parapsilosis*), 72 hours at 37°C (*Trichophyton* sp.) or 24 hours at 30°C (*Mucor racemousus*). Minimum effective concentration (MEC) was determined by microscopic observation.

In Vivo Antifungal Activities of FR227244 against *A. fumigatus* in Murine Infection Models

The *in vivo* anti-*Aspergillus* activities were evaluated in murine models of systemic infection. *A. fumigatus* FP1305, which is a clinical isolate in the Fujisawa culture collection, was used in these studies. For anti-*Aspergillus* activity, the inoculum was prepared from a seven-day old culture of YM agar slant. ICR mice (female, four weeks old), were administered cyclophosphamide (200 mg/kg) intraperitoneally for 4 days before challenge, and then were intravenously injected with 1×10^6 spores of the fungus. Five mice were used in each group. Test compounds were dissolved in 10% HCO-60/saline, saline, or sterile water in each experiment, and administered orally or subcutaneously one hour after challenge and once a day for three consecutive days. The ED_{50} was determined on the day when all control mice (vehicle only) died.

Membrane Fraction Preparations of *Candida albicans* and *A. fumigatus*

Membrane fraction preparations of *C. albicans* were conducted according to the method described by SAWISTOWSKA-SCHRÖDER *et al.*[16] with some modifications. Briefly, yeast cells of *C. albicans* FP633 were grown to logarithmic phase (absorbance at 660 nm; 0.42) in yeast nitrogen base-dextrose (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 β-mercaptoethanol, 1 M sucrose and 25 μM GTP). The cells were sonicated with 0.4 mm i.d. glass beads by a sonicator. 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 $100,000\times g$ 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.

Membrane fraction preparation of *A. fumigatus* was carried out as follows. *A. fumigatus* FP1305 was cultured on potato dextrose agar slant for 7 days at 30°C. The spores were harvested gently in sterile saline containing 0.1% Tween80, and filtered through gauze. Fungal spores were inoculated into 1 liter of YM medium to be find concentration of 2×10^8 spores. The culture broth was incubated under shaking conditions (100 rpm) for 16 hours at 30°C. The cells were filtered by Miracloth® (Calbiochem) and washed and suspended in ice-cold extraction buffer (50 mM HEPES (pH 7.5), 1 mM EDTA, 1 mM DTT, 10% glycerol, 1 μg/ml aprotinin, 1 mM phenylmethylsulfonylfluoride, 5 μg/ml leupeptin and 10 μM GTP-γ-S). The cells were sonicated with 0.4 mm i.d. glass beads by a sonicator. The glass beads and the cell debris were removed by centrifugation. The supernatant fluids were centrifuged at $100,000\times g$ for 60 minutes at 4°C. The pellet was resuspended in storage buffer (50 mM HEPES (pH 7.5), 1 mM EDTA, 1 mM DTT and 20% glycerol) and homogenized with a Dounce homogenizer. The homogenized sample was stored at -80°C as a source of

enzyme.

Glucan Synthase Assay

Glucan synthase assay procedures for *C. albicans* were conducted according to the method described by SAWISTOWSKA-SCHRÖDER *et al.*[16] 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 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.

Glucan synthase assay for *A. fumigatus* was carried out as follows. Five μl of test compound solution or vehicle was incubated with 50 μl of reaction buffer (50 mM Tris-HCl (pH 7.5), 10% glycerol, 1.5% BSA, 25 mM KF, 1 mM EDTA and 10 μM GTP-γ-S) and 35 μl of particulate enzyme solution (20 μg protein) for 15 minutes at room temperature. After incubation, 10 μl of substrate solution (UDP-[U-^{14}C]glucose (0.35 μCi/ml, 1 mM), 0.8 mM UDP-glucose and 1 U α-amylase) 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 30% TCA and allowed to stand on ice. The resultant precipitate was washed with ice-cold 30% TCA and dissolved with 1 N NaOH. After neutralization, radioactivity was counted with a toluene scintillator.

Chitin Synthase Assay

The chitin synthase assays for *C. albicans* and *A. fumigatus* were carried out according to the method described by SELITRENNIKOFF *et al.*[17] with some modifications. Membrane fractions of *C. albicans* or *A. fumigatus* were incubated with 1/100 protein amount of trypsine in 0.1 M Tris-HCl buffer (pH 7.5) for 10 minutes at room temperature. After incubation, 1/50 protein amount of trypsine inhibitor was added, and incubated on ice for 5 minutes. Trypsine-treated membrane fraction was used as the enzyme in the chitin synthase assay.

Two and a half μl of test compound solution or vehicle was incubated with 10 μl of enzyme solution and reaction buffer (100 mM MES (pH 6.5) - 20 mM $MgCl_2 \cdot 6H_2O$ for chitin synthase I (*CHS I*) or 100 mM Tris-HCl (pH 8.0) - 10 mM $Co(OAc)_2 \cdot 4H_2O$ for chitin synthase II (*CHS II*) assay) for 10 minutes at room temperature. After incubation, 25 μl of substrate solution (*N*-acetyl-[1-^{14}C] glucosamine (0.35 μCi/ml, 1 mM), 0.6 mg/ml UDP-*N*-acetylglucosamine and 177 mg/ml *N*-acetylglucosamine) 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% TCA. The resultant precipitation was washed with 10% ice-cold 10% TCA twice and dissolved with 1 N NaOH. After neutralization, radioactivity was counted with a toluene scintillator.

Whole Cell Macromolecular Synthesis Assay of *C. albicans* and *Candida utilis*

The effects of FR227244 on macromolecular synthesis were evaluated by pulse-labeling the cells with radioactive precursors of specific macromolecules. Whole cell macromolecular synthesis assay procedures for *C. albicans* and *C. utilis* were conducted according to the method described by YAMAGUCHI *et al.*[18] with some modifications. Exponentially growing cells of *C. albicans* FP633 and *C. utilis* YC123 were harvested by centrifugation and resuspended in a medium containing 0.4% glucose and 0.2% yeast extract supplemented with 0.8 M sorbitol as osmotic stabilizer (GYS medium). An experimental cell suspension was prepared to give approximately 0.7 of absorbance at 660 nm. Methods used for the incorporation of specific precursors were as follows:

(a) Leucine (protein synthesis): The reaction mixture contained per ml: 0.85 ml cell suspension, 20 μl antibiotic solution and 20 μl methanol or sterile water. After incubation for 20 minutes at 37°C, 100 μl of 4 μCi/ml L-[4,5-^{3}H]leucine and 10 μl of 1.31 mg/ml cold L-leucine were added. The reaction tubes were incubated at 37°C with shaking for 60 minutes. Reaction was terminated by addition of 1 volume of 10% TCA and heated at 90°C for 15 minutes. The insoluble material was collected on glass-fiber filters (Whatman, GF/C), washed with 5% TCA and dried. The dried filters were counted for radioactivity with a toluene scintillator.

(b) Adenine (nucleic acid synthesis): The reaction mixture contained per ml: 0.91 ml cell suspension, 20 μl antibiotic solution and 20 μl methanol or sterile water. After incubation for 20 minutes at 37°C, 40 μl of 4 μCi/ml [8-^{3}H]adenine and 10 μl of 2 mg/ml cold adenine sulfate were added. The reaction tubes were incubated at 37°C with shaking for 60 minutes. Reaction was terminated by addition of 1 volume of ice-cold 10% TCA. The cold TCA-insoluble material were collected on glass-fiber filters (Whatman, GF/C), washed with 5% TCA and dried. The dried filters were counted for radioactivity with a toluene

scintillator.

(c) Glucose (glucan, chitin and mannan synthesis): The reaction mixture contained per ml: 0.92 ml cell suspension, 20 μl antibiotic solution and 20 μl methanol or sterile water. After incubation for 20 minutes at 37°C, 40 μl of 4 μCi/ml D-[U-^{14}C]glucose or 40 μl of 4 μCi/ml *N*-acetyl-[1-^{14}C] glucosamine were added. The reaction tubes were incubated at 37°C with shaking for 90 minutes. Reaction was terminated by centrifugation of the reaction mixture, removed supernatant, addition of 1 volume of ice-cold 10% TCA and standing at 5°C for 10 minutes. The cold-TCA insoluble material were extracted with 30% KOH at 100°C for 30 minutes. The alkali-insoluble and alkali-soluble extracts were separated by centrifugation. A copper sulfate Fehling's solution was added to the alkaline soluble extract to precipitate the mannan[12)]. These precipitates were collected on glass-fiber filters (Whatman, GF/C), washed with 2 N acetic acid and dried. The dried filters were counted for radioactivity with a toluene scintillator. The alkaline insoluble materials were extracted with 2 N acetic acid at 100°C for 30 minutes. The acid insoluble materials, which contained glucan and chitin[19)], were collected on glass-fiber filters (Whatman, GF/C), washed with 2 N acetic acid and dried. The dried filters were counted for radioactivity with a toluene scintillator.

Whole Cell Macromolecular Synthesis Assay of *A. fumigatus*

A. fumigatus FP1305 was cultured on YM agar slant for 7 days. The spores were harvested in sterile saline, and filtered through gauze. The fungal spores were resuspended in GYS medium to 1×10^4/ml in the reaction tubes. Reaction tubes were incubated at 33°C for 13.5 hours without shaking. Methods used for the incorporation of specific precursors were as follows:

(a) Leucine (protein synthesis): The reaction mixture contained per ml: 940 μl cell suspension, 20 μl antibiotic solution and 20 μl methanol or sterile water. After incubation for 20 minutes at 33°C, 10 μl of 40 μCi/ml L-[4,5-^{3}H]leucine and 10 μl of 1.31 mg/ml cold L-leucine were added. The reaction tubes were incubated at 33°C without shaking for 4 hours. Reaction was terminated by addition of 1 ml of 10% TCA and heated at 90°C for 15 minutes. The subsequent procedures were the same as for *Candida*.

(b) Glucose (glucan synthesis): The reaction mixture contained per ml: 922.5 μl cell suspension, 20 μl antibiotic solution and 20 μl methanol or sterile water. After incubation for 20 minutes at 33°C, 37.5 μl of 40 μCi/ml D-[U-^{14}C]glucose were added. The reaction tubes were incubated at 33°C with shaking for 4 hours. Reaction was terminated by centrifugation of the reaction mixture, removed supernatant, addition of 1 ml of ice-cold 10% TCA and left at 5°C for 10 minutes. The subsequent procedures were the same as for *Candida*.

(c) Glucose (chitin synthesis): The reaction mixture contained per ml: 952.5 μl cell suspension, 20 μl antibiotic solution and 20 μl methanol or sterile water. After incubation for 20 minutes at 33°C, 7.5 μl of 4 μCi/ml *N*-acetyl-[1-^{14}C]glucosamine were added. The reaction tubes were incubated at 33°C with shaking for 4 hours. Reaction was terminated by centrifugation of the reaction mixture, removed supernatant, addition of 1 ml of ice-cold 10% TCA and left at 5°C for 10 minutes. The subsequent procedures were the same as for *Candida*.

Results

In Vitro Antifungal Activity

Table 1 shows the antifungal spectra of FR227244 and other antifungal agents against various yeast-like and filamentous fungi. FR227244 has potent activity against a variety of fungal species, especially *Aspergillus* species and *Trichophyton* species. FR227244 has weak activity against *Candida* species, except for *C. utilis* and *C. parapsilosis*. However, FR227244 was inactive against *C. albicans* FP633, *C. neoformans* YC203, *M. furfur* 12001 and IFO0657, *M. racemoses* F588, *Fusarium graminearum* F0497 and *Fusarium merismoides* F0501.

In Vivo Antifungal Activities of FR227244 against *A. fumigatus* in Murine Infection Models

The protective efficacy of FR227244 administered subcutaneously and orally against murine systemic infection with *A. fumigatus* was examined. The ED_{50} values for FR227244 in experiment 1 (subcutaneous injection) and experiment 2 (oral administration) on the day when all control mice died were 1.9 mg/kg and 18 mg/kg, respectively. As shown in Table 2, FR227244 was inferior to FR901469, but FR227244 significantly prolonged the survival of infected mice by oral administration. FR901469 and FR901379, which is an echinocandin-like lipopeptide, were inactive by oral administration in this model. Survival curves for the *in vivo* aspergillosis model are shown in Fig. 2.

Table 1. *In vitro* antifungal activities of FR227244 and other compounds by the microbroth dilution method.

Test organism	MEC (μg/ml)		
	FR227244	Amphotericin B	Terbinafine
Candida albicans FP633	>50	0.20	3.1
Candida albicans FP629	3.1	0.39	3.1
Candida albicans FP1830	6.3	0.39	3.1
Candida albicans 6406	6.3	0.20	3.1
Candida krusei YC109	25	3.1	25
Candida utilis YC123	0.39	0.20	0.39
Candida tropicalis IFO0006	16	0.39	1.6
Candida prapsilosis OUT6016	0.78	0.78	6.3
Cryptococcus neoformans YC203	>50	1.6	6.3
Aspergillus fumigatus FP1305	0.031	0.63	0.31
Aspergillus fumigatus 8004	0.031	0.16	0.31
Aspergillus fumigatus F163	0.039	0.16	0.31
Aspergillus fumigatus FD-050	0.063	0.31	0.31
Aspergillus fumigatus S-43	0.039	0.16	0.31
Aspergillus nidurans F50	0.13	0.16	0.039
Aspergillus flavuss ATCC9643	0.13	1.3	0.039
Aspergillus terreus IFO6123	0.039	1.3	0.078
Aspergillus niger ATCC9642	13	0.20	0.10
Trichophyton mentagrophytes TIMM1189	0.039	0.078	0.0050
Trichophyton mentagrophytes FP602	0.063	0.010	0.0050
Trichophyton rubrum FP596	0.063	0.039	0.0050
Trichophyton interdigitate FP595	0.063	0.039	0.0050
Malassezia furfur 12001	>50	25	3.1
Malassezia furfur IFO0657	>50	25	0.10
Mucor racemoses F588	>50	0.78	25
Fusarium graminearum F0497	>50	0.050	13
Fusarium merismoides F0501	>50	0.050	6.3

Table 2. *In vivo* antifungal activities of FR227244 and FR901469 against *A. fumigatus* in a murine infection model.

Compound	administration route	ED_{50} (mg/kg)[c]
FR227244	SC[a]	1.9
FR227244	PO[b]	18
FR901469	SC[a]	0.13

[a] subcutaneous injection

[b] oral administration

[c] ED_{50} was calculated based on the survival rate on the day when all control mice died.

Glucan Synthase and Chitin Synthase Inhibitory Activity

FR227244 inhibited 1,3-β-glucan synthase prepared from *A. fumigatus* FP1305 with an IC_{50} value of 4.04 μg/ml, but did not inhibit the synthase prepared from *C. albicans* FP633 (Table 3). FR901469, which is 1,3-β-glucan synthase inhibitor, inhibits 1,3-β-glucan synthase from *A. fumigatus* FP1305 and *C. albicans* FP633 with IC_{50} values of 0.22 μg/ml and 0.63 μg/ml, respectively (Table 3).

FR227244 did not inhibit chitin synthase I and II prepared from *A. fumigatus* FP1305 and *C. albicans* FP633 (Table 3). Nikkomycin, which is potent chitin synthase I inhibitor, inhibit chitin synthase I from *A. fumigatus* FP1305 and *C. albicans* FP633 with an IC_{50} value of 3.88 μg/ml and 0.039 μg/ml, respectively (Table 3).

Fig. 2. Antifungal activities of FR227244 against *A. fumigatus* in a murine infection model.

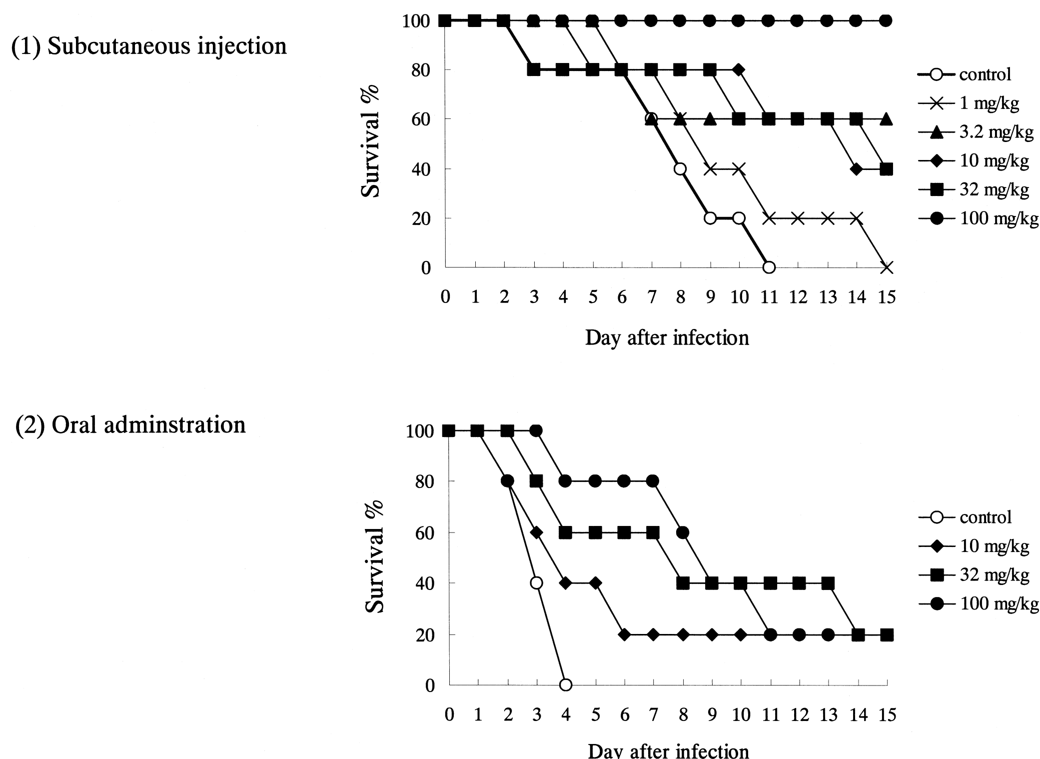


Table 3. Inhibitory activity of FR227244 and other compounds on 1,3-β-glucan synthase, chitin synthase and mannan synthase prepared from *C. albicans* and *A. fumigatus*.

(a) *Candida albicans* FP633

	IC50 (μg/ml)				MEC (μg/ml)
	glucan synthase	chitin synthase I	chitin synthase II	mannan synthase	antifungal activity (YNBD medium)
FR227244	>50	>50	>50	>50	>50
FR901469	0.63	NT	NT	NT	0.63
Nikkomycin Z	NT	0.039	27.9	NT	0.20

NT; not tested

(b) *Aspergillus fumigatus* FP1305

	IC50 (μg/ml)				MEC (μg/ml)
	glucan synthase	chitin synthase I	chitin synthase II	mannan synthase	antifungal activity (YNBD medium)
FR227244	4.04	>250	>250	NT	0.078
FR901469	0.22	NT	NT	NT	0.156
Nikkomycin Z	NT	3.88	9.13	NT	25

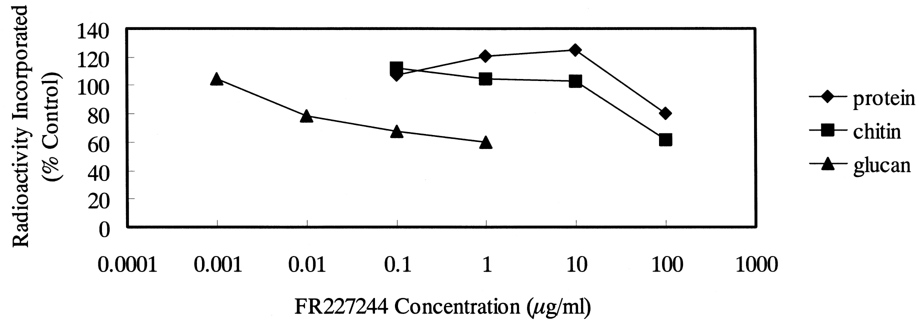
NT; not tested

Effect on Incorporation of Several Specific Radioactive Precursors into Major Cellular Composition

Effects of FR227244 on the synthesis of macromolecules and cell wall polysaccharides in growing cells of *A. fumigatus*, *C. albicans* and *C. utilis* in GYS medium containing 0.8 M sorbitol to protect cells from lysis, were explored by determination of the counts taken up of specific radioactive precursors. FR227244 inhibited incorporation of D-[U-^{14}C]glucose into glucan of *A. fumigatus* and

Fig. 3. Effect of FR227244 on incorporation of radioactive precursors into macromolecules in growing cells of *C. albicans* and *A. fumigatus*.

(1) *Aspergillus fumigatus* FP1305



(2) *Candida albicans* FP633

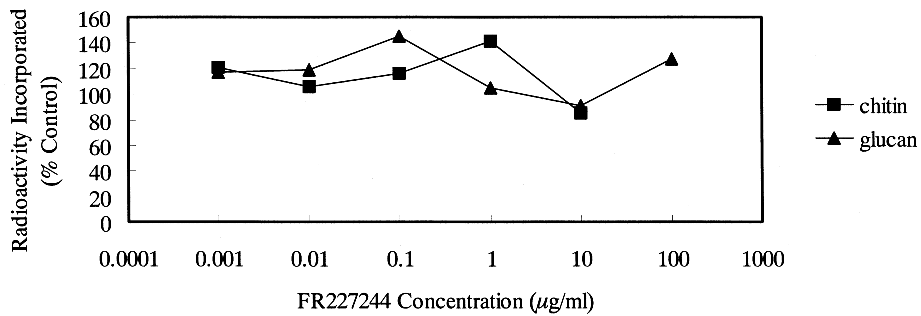
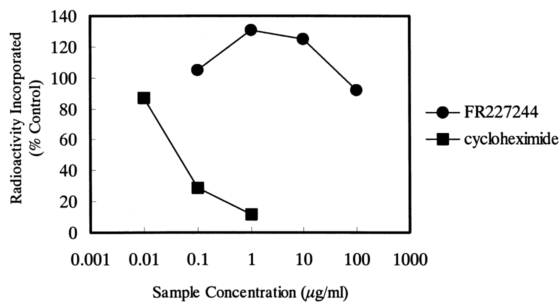
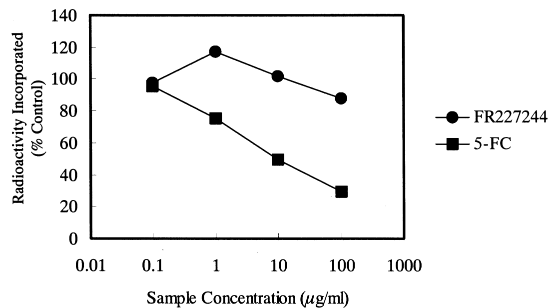


Fig. 4. Effect of FR227244 on incorporation of radioactive precursors into macromolecules in growing cells of *C. utilis*.

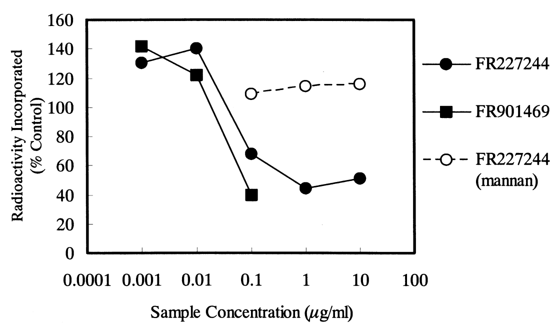
(1) Protein



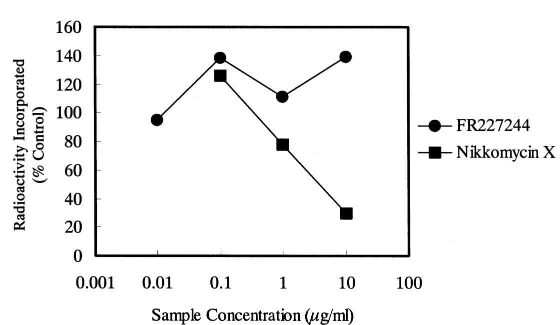
(2) Nucleic acid



(3) Glucan and Mannan



(4) Chitin



C. utilis (Figs. 3 and 4), but did not inhibit incorporation into *C. albicans* glucan (Fig. 3). Incorporation of other radioactive precursors was not inhibited (Figs. 3 and 4).

Discussion

In this study, the *in vitro* and *in vivo* antifungal activities and mode of action of FR227244 were evaluated. The results of *in vitro* studies indicated that FR227244 had potent anti-*Aspergillus* and anti-*Trichophyton* activity (Table 1). These results suggest that FR227244 may be a drug for chemotherapy of not only disseminated mycoses but also superficial mycoses.

FR227244 showed good efficacy in systemic infection models in mice infected with clinically relevant *A. fumigatus* (Fig. 2). However, the ED_{50} value of FR227244 was 10 times higher than that of FR901469 in the mouse model of systemic aspergillosis administered subcutaneously (Table 2), FR227244 showed efficacy when administered orally (ED_{50} value: 18 mg/kg). These results suggest that FR227244 may have advantages over echinocandin-like lipopeptides, which are not oral drugs, and fluconazole having weak activity against *A. fumigatus* in the therapy of aspergillosis in immunocompromised patients.

ONISHI *et al.* showed that acidic terpenoids (arundifungin[9] and ergokonin A[10]) and triterpene glycoside (enfumafungin[11,12] and ascosteroside[13,14]) were 1,3-β-glucan synthase inhibitor[15]. We also isolated ergokonin A, and its *in vivo* antifungal activities were evaluated in a murine model of systemic infection, however, ergokonin A did not show protective efficacy when administered subcutaneously at 32 mg/kg (data not shown). It has also been reported that arundifungin is not effective in a model of disseminated candidiasis when administered daily at up to 50 mg/kg (IP, t.i.d.×1 day)[9], and enfumafungin yielded an ED_{90} of 90 mg/kg[10]. Our experiments indicate that FR227244 inhibits the glucan synthesis and selectively inhibited fungal cell wall glucan assembly at their MECs (Figs. 3 and 4). FR227244 did not inhibit cell wall glucan assembly in *C. albicans* FP633 whose growth was not effected by FR227244 (Fig. 3). FR227244 also inhibited glucan synthase of *A. fumigatus* selectively, but the IC_{50} value for glucan synthase inhibition (4.04 μg/ml) is higher than MEC (0.031 μg/ml) against *A. fumigatus* (Table 3). Theoretically, the IC_{50} value for an inhibitor with a single mode of action for the target enzyme should be lower or the same as the concentration needed to inhibit growth (MEC). In nature, there are many kinds of terpenoid compounds, and these compounds have diverse biological activities. Therefore, we cannot conclude that FR227244 is a specific inhibitor of 1,3-β-glucan synthase.

In conclusion, further evaluation of FR227244 suggests that it may be a candidate lead compound for new antifungal agents which are orally absorbed and which inhibit fungal cell wall synthesis. We plan to explore FR227244-derived compounds as more effective and safer antifungal agents in subsequent work.

Acknowledgment

We are grateful to Dr. DAVID BARRETT, Medicinal Chemistry Research Laboratories, Fujisawa Pharmaceutical Co., Ltd., Osaka, Japan for assistance in the preparation of this manuscript.

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