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

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

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A novel antifungal antibiotic, FR227244, was isolated from the culture broth of a fungal strain No. 002. The strain was identified as *Myrothecium cinctum* from morphological and physiological characteristics. This compound was isolated from the culture broth by solvent extraction, HP-20 and YMC ODS gel column chromatographies, and *n*-hexane precipitation. FR227244 is a white powder which melts at 210~211°C and possesses the molecular formula $C_{38}H_{58}O_{11}$.

FR227244 is a novel triterpene glycoside with antifungal activity against *Aspergillus fumigatus*. The effects of FR227244 on the morphology of *A. fumigatus* were shown to be similar to those of FR901379 which is a known 1,3-β-glucan synthase inhibitor.

Fungal infections, particularly deep-seated mycoses, have dramatically increased in frequency in recent decades due to a growing number of immunocompromised and neutropenic patients[1]. Antifungal therapies are currently limited to a small number of compounds for the treatment of a rather diverse array of pathogenic fungi. 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].

In recent years, a new antifungal drug, micafungin which is a novel echinocandin-like lipopeptide, has been launched, and a number of clinical problems have been solved. However, micafungin has not completely satisfied unmet medical needs due to its poor oral absorption. We are therefore making efforts to discover new oral antifungal drugs. Micafungin is a synthetic analog of FR901379[3], and both are 1,3-β-glucan synthase inhibitors. A number of antifungal agents are well known as 1,3-β-glucan synthase inhibitors, for example, echinocandin B[4], aculeacin A[5], pneumocandins[6~8] and papulacandins[9]. Recently, new antifungal agents as inhibitors of glucan synthesis were reported, for example, arundifungin[10], enfumafungin[11]. The effect of these compounds on the morphology of *Aspergillus fumigatus* is characteristic based on the inhibition of cell wall synthesis. Examination of *A. fumigatus* cells after treatment with FR901379 revealed significant and characteristic changes in hyphal morphology (Fig. 1b).

In the course of our screening for new antifungal antibiotics focused these changes in hyphal morphology, we discovered a novel antifungal antibiotic, FR227244 (Fig. 2), which is produced by the strain *Myrothecium cinctum* No. 002. In this paper, we describe the taxonomy, fermentation, isolation and physico-chemical properties.

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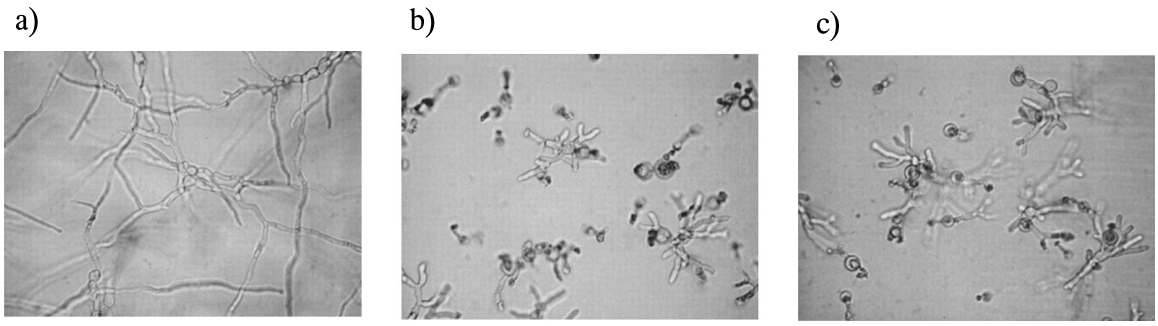
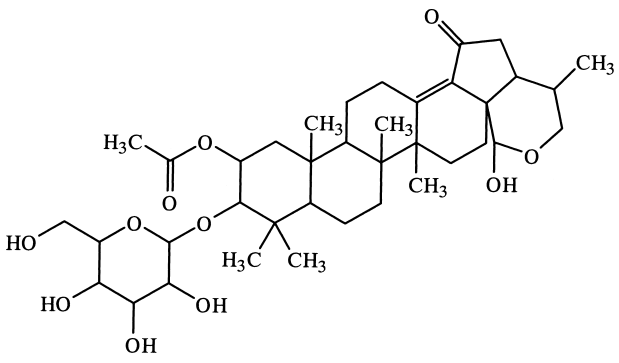
Fig. 1. Effect of FR901379 and FR227244 on the morphology of *A. fumigatus*.



a) Control (no drug); b) FR901379 (2.5 μl/ml); c) FR227244 (2.5 μl/ml).

Fig. 2. Structure of FR227244.



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[12].

General Procedures

Melting points were recorded on a Yanagimoto micro melting point apparatus. IR spectra were measured on a Perkin-Elmer 16PC FT-IR. Optical rotation was determined on a Jasco DIP-470 polarimeter, using a 10 cm-micro cell. ^{1}H and ^{13}C NMR were measured on a Bruker DRX500 NMR spectrometer.

Taxonomic Studies

The producing strain No. 002 was originally isolated from a soil sample collected in Japan. Cultural characteristics were determined after 14 days of incubation at 25°C using agar media as follows: malt extract agar, potato dextrose agar (PDA, Difco 0013), Czapek's solution agar, Sabouraud dextrose agar (SDA, Difco 0109), Emerson YpSs agar (Difco 0739), corn meal agar (CMA, 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[13]. The color names used in this study were used according to the Methuen Handbook of Colour[14]. The temperature range of growth was determined on potato dextrose agar (Nissui). Morphological characteristics were determined principally from the cultures on Miura's LCA[15].

Fermentation

An aqueous seed medium (160 ml) containing glucose 1%, sucrose 4%, soluble starch 2%, Pharmamedia 3%, soybean flour 1.5%, KH_2PO_4 1%, $CaCO_3$ 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 500-ml Erlenmeyer flasks and was sterilized at 120°C for 30 minutes. A loopful of the slant culture of *M. cinctum* No. 002 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 4 days, and 480 ml (three flasks) of the seed culture were inoculated to 20 liters of sterile production medium consisting of modified starch 6%,

glucose 0.5%, glycerol 8.5%, potato protein 2.5%, corn steep liquor 0.8%, $(NH_4)_2SO_4$ 0.05%, β-cyclodextrin 1%, 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 8 days under aeration of 20 liters/minute and agitation of 200 rpm.

HPLC Analysis

Detection of FR227244 in the fermentation broth and fractions during purification was performed by HPLC using a reverse phase column YMC Pack ODS-AM 303, S-5 120A (250×.6 mm i.d., YMC Co., Ltd.). The mobile phase was 45% aqueous acetonitrile containing 0.5% $NaH_2PO_4 \cdot 2H_2O$. The flow rate was 1.0 ml/minute. The detection wavelength was set at 210 nm.

Antifungal Activity against *A. fumigatus*

Antifungal activity was measured by the micro-broth dilution method in 96-well culture plates employing yeast nitrogen base-dextrose (YNBD) medium. *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 spores were resuspended in yeast nitrogen base-dextrose 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. Antifungal activity was determined by microscopic observation.

Results

Identification of the Producing Strain

The strain No. 002 grew rather rapidly and formed orange white to yellowish white colonies on various agar media (Table 1). The strain produced anamorph (conidiomata) on the media, while it did not form teleomorph. Colonies on PDA grew fairly rapidly, attaining 3.5～4.5 cm in diameter after two weeks at 25°C. The colony surface was plane to raised, velvety to cottony, sulcate or wrinkly, producing exudate, sometimes sectoring, orange white to pale orange at the center and the margin, and gray in the middle. Abundant conidia were produced on the media (Fig. 3). The reverse was pale orange to light orange. Colonies on CMA grew restrictedly, attaining 1.5～2.5 cm in diameter under the same conditions. The surface was plane, thin, sometimes sectoring, white to orange white at the center and the margin, and brownish gray to dark gray in the middle. Many conidial structures were formed. The reverse was white to orange white. Strain No. 002 was able to grow at the temperature range from 6 to 33°C, with the growth optimum at 19 to 22°C.

Conidiomata consisted of conidiophores and phialides, and dark green to dark gray conidial masses. The conidiophores were erect, semi-macronematous, hyaline, smooth to roughened, repeatedly branched, and formed a whorl of 2～4 phialides at the tips. The phialides were discrete, hyaline, roughened to granulate, cylindrical, with differentiated collarettes, and (9～/16～28(～34)×(1.5～)2～3 μm. Conidia were accumulated in a slimy drop, subhyaline to dark green, with oblique or longitudinal striae, one-celled, fusiform to lentiform, and 8.5～12×2.5～3.5(～4.5) μm. Chlamydospores were not observed. This strain formed sometimes sporodochial conidiomata on SDA. The sporodochia were composed of loosely aggregations of hyphae and conidiophores, and 100～300 μm in diameter.

On the basis of the morphological characteristics with fungal taxonomic criteria by VON ARX[16] and by BARRON[17], strain No. 002 was considered to belong to the hyphomycete genus *Myrothecium*. Moreover, above characteristics were corresponded the species description of *Myrothecium cinctum* (Corda) Sacc. 1886 by TULLOCH[18] and DOMSCH *et al.*[19] with few exceptions.

Thus, we identified the isolate No. 002 as *M. cinctum.* The strain has been deposited to the International Patent Organism Depositary in the National Institute of Advanced Industrial Science and Technology, Japan, as FERM BP-6380.

Fermentation

Figure 4 shows the time course of FR227244 production by strain No.002 in a 30-liter jar fermentor, along with the pH and the packed cell volume. The maximal production (131.8 mg/liter) was observed after 8 days of cultivation.

Isolation and Purification

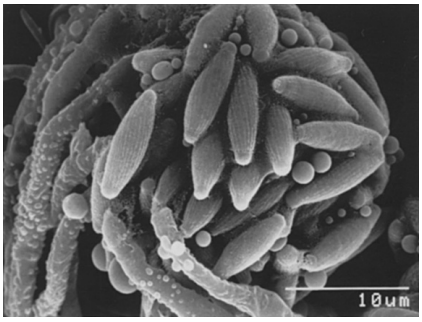
The culture broth (100 liters) was extracted with an equal volume of acetone by stirring for 2 hours at room temperature and filtered with the aid of diatomaceous earth. The filtrate was diluted with an equal volume of water and passed through a column (7 liters) of DIAION HP-20 (Mitsubishi Chemical Co., Ltd.) packed with 25% aqueous acetone. The column was washed with 50% aqueous methanol (22 liters) and then eluted with methanol (43

Table 1. Cultural characteristics of strain No. 002.

Medium	Cultural characteristics
Malt extract agar	G: Rather restrictedly, 2.5-3.5 cm S: Irregular, plane, felty, formed some conidial structures, yellowish white (2A2) at the center, white to orange white (5A2) at the margin, and olive gray (1F2) at the middle R: Pale yellow (4A3) to grayish yellow (4B3), and olive brown (4E4) at the middle
Potato dextrose agar (Difco 0013)	G: Fairly rapidly, 3.5-4.5 cm S: Circular, plane to raised, felty to cottony, sulcate or wrinkly, exudate, sometimes sectoring, formed many conidial structures, orange white (6A2) to pale orange (6A3) at the center and the margin, and gray (1E1) at the middle R: Pale orange (5A3-6A3) to light orange (5A4-6A4)
Czapeck's solution agar	G: Rather restrictedly, 2.5-3.5 cm S: Circular, centrally raised to umbonate, zonate, felty, radiately sulcate, exudate, formed few conidial structures, and yellowish white (3A2) to orange white (6A2) R: Pale yellow (4A3) to pale orange (6A3)
Sabouraud dextrose agar (Difco 0109)	G: Rather rapidly, 3.0-4.0 cm S: Circular to irregular, centrally raised to umbonate, felty, radiately sulcate or wrinkly, exudate, grayish yellow (4B3) to orange white (6A2), and formed some grayish dots of sporodochia R: Grayish orange (5B5) to brownish orange (5C4)
Oatmeal agar (Difco 0552)	G: Spreading broadly, 4.5-5.5 cm S: Circular, plane, felty, radiately sulcate, exudate, hygroscopic, sometimes sectoring, abundantly formed conidial structures, dark gray (1F1) to dark green (27F4), and pale orange (6A3) at the margin
Emerson Yp Ss Agar (Difco 0739)	G: Rather rapidly, 3.0-4.0 cm S: Circular, plane, felty, hygroscopic, formed many conidial structures, dark green (27F4-27F5), and orange white (6A2) at the margin R: Light orange (6A4-6A5)
Corn meal agar (Difco 0386)	G: Restrictedly, 1.5-2.5 cm S: Circular, plane, thin, sometimes sectoring, formed many conidial structures, white to orange white (5A2) at the center and the margin, and brownish gray (5F2) to dark gray (1F1) at the middle R: White to orange white (5A2)
MY 20 agar	G: Fairly rapidly, 3.5-4.5 cm S: Circular, plane to centrally raised, felty to cottony, exudate, hygroscopic, abundantly formed conidial structures, greenish white (28A2) to greenish gray (28B2) at the center, white to orange white (6A2) at the margin, and dull green (28D4) to dark green (28F4) at the middle R: Olive brown (4E4-4F4), and yellowish white (4A2) at the margin

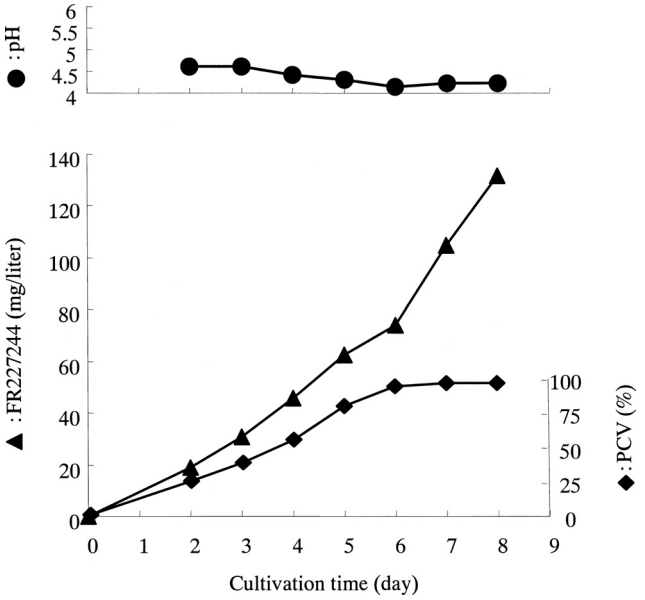
Abbreviation G: growth, measuring colony size in diameter, S: colony surface, R: reverse.

Fig. 3. Electron micrograph of strain No. 002.



Scale: 10 μm.

Fig. 4. Time course of FR227244 production in a 30-liter jar fermentor.



liters). The active fraction (10～25 liters) was diluted to 30 liters with water and passed through a column (4 liters) of YMC-GEL (ODS-AM 120-S50, YMC Co., Ltd.) packed with 50% aqueous methanol. The column was washed with 60% aqueous methanol (14 liters) and then eluted with 70% (18 liters) and 80% aqueous methanol (9 liters). The active fraction was diluted with an equal volume of water and passed through a column (16 liters) of YMC-GEL packed with 50% aqueous methanol. The column was eluted with 40% aqueous acetonitrile containing 0.5% $NaH_2PO_4 \cdot 2H_2O$ (87 liters). The active fraction (58～71 liters) was diluted with an equal volume of water and passed through a column (2 liters) of YMC-GEL packed with 20% aqueous acetonitrile containing 0.25% $NaH_2PO_4 \cdot 2H_2O$. The column was washed with 50% aqueous methanol (7 liters) and eluted with 75% aqueous methanol (8 liters) and methanol (3 liters). The active fraction was diluted with an equal volume of water and passed through a column (16 liters) of YMC-GEL packed with 37.5% aqueous methanol. The column was eluted with 75% aqueous methanol (66 liters). The active fraction (30～58 liters) was diluted with an equal volume of water and passed through a column (2 liters) of YMC-GEL packed with 37.5% aqueous methanol. The column was eluted with 95% aqueous methanol (3.5 liters). The active fraction (1～2.5 liters) was concentrated *in vacuo* to give white precipitates. The residue was dissolved in a small volume of ethyl acetate and added with a large amount of *n*-hexane, and then was dried up to give 6.1 g of FR227244 as white powder.

Physico-chemical Properties

As shown in Table 2, FR227244 was soluble in methanol, ethyl acetate and dimethyl sulfoxide, but insoluble in *n*-hexane and water. It displayed positive color reactions to iodine vapor and Molish, though it was negative against Dragendorff, Ehrlich, $FeCl_3$ and Ninhydrin. It showed a UV absorption at 260 nm. The ESI-MS spectrum showed a molecular ion peak at *m*/*z* 713 ($M+Na^+$) and 689 ($M-H^-$). The ^1H and ^{13}C NMR spectra of FR227244 are shown in Fig. 5 and Fig. 6, respectively.

Determinations of the structure were accomplished primarily by a series of 2-D NMR techniques. Detailed of the structure elucidation studies will be described elsewhere.

Antifungal Activity against *A. fumigatus*

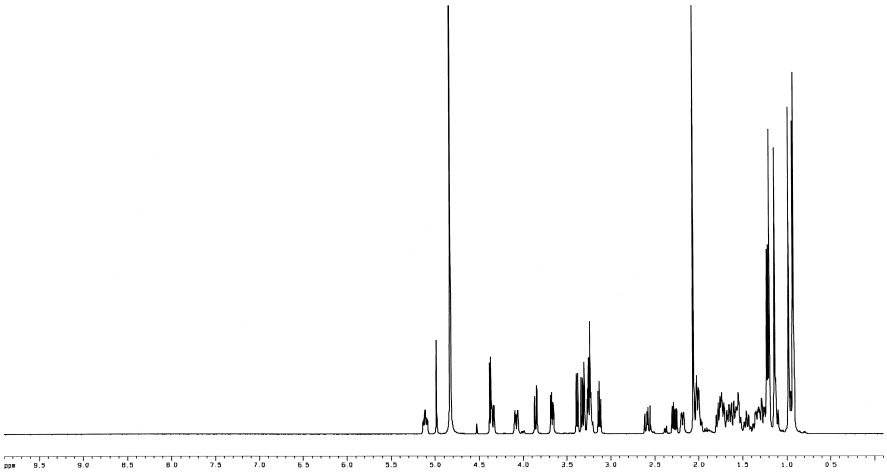
Examination of *A. fumigatus* cells after treatment with FR227244 revealed some relevant changes in hyphal morphology, in comparison with the control without antifungal agents (Fig. 1a). FR227244 (Fig. 1c) produced basically the same set of morphological alternations as FR901379 (Fig. 1b), *i.e.* hyphae abnormally grown, shortened, stunted and highly branched with bipolar or vesicular tips, swollen germ tubes and frequent balloon-like cells.

Table 2. Physico-chemical properties of FR227244.

Appearance	white powder
Melting point	210 - 211℃
$[\alpha]_D^{23}$	+58° (*c* 0.5, methanol)
ES-MS (m/z)	713 $(M + Na)^+$, 689 $(M - H)^-$
Molecular formula	$C_{38}H_{58}O_{11}$
Elemantal analysis	
Calcd for $C_{38}H_{62}O_{10} \cdot 3H_2O$:	C 61.27, H 8.66
Found:	C 61.08, H 8.83
UV $\lambda_{max}^{methanol}$ nm (e)	260 (13000)
Color test	
Positive	I_2, $Ce(SO_4)_2$-H_2SO_4, Molish
Negative	$FeCl_3$, Dragendorff, Ninhydrin, Ehrlich
Solubility	
Soluble	DMSO, methanol, ethyl acetate
Insoluble	water, n-hexane
IR v max (KBr) cm^{-1}	3430, 2950, 2880, 1710, 1620, 1450, 1370, 1260, 1100, 1080, 1040
TLC (Rf value)[a]	0.21

[a] Plate: Silica gel 60 F_{254} (E. Merck Co.), $CHCl_3$:CH_3OH = 8:1

Fig. 5. ^{1}H NMR spectrum of FR227244 (500 MHz, DMSO-d_6).

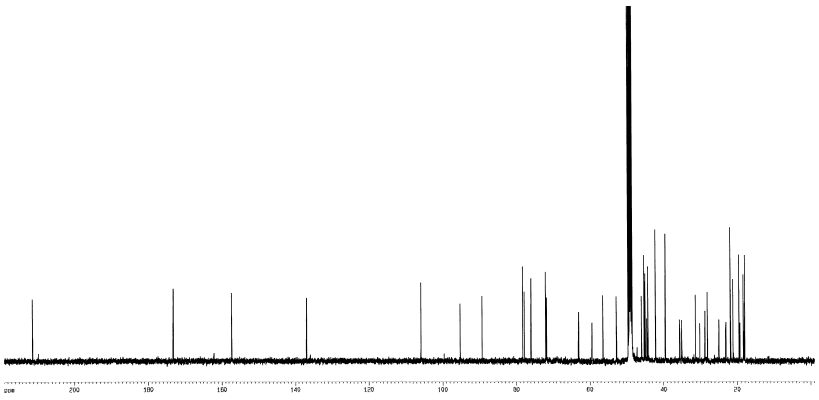


Discussion

In this paper, we have presented a novel antifungal antibiotic, FR227244, isolated from the fermentation broth of *M. cinctum* No. 002. This compound was discovered during a screening program for antifungal antibiotics with activity against *A. fumigatus* focused on characteristic changes in hyphal morphology (Fig. 1). Based on physico-chemical data and 2D-NMR spectra, this compound has a novel structure (Fig. 2). FR227244 is a triterpene glycoside, and its structure resembles enfumafungin and ascosteroside[11,20,21].

The effects of FR227244 on the morphology of *A. fumigatus* were similar to the effects of FR901379 (Fig. 1). According to the correlation that has been established between the pattern of morphological alternation and the mode of action of antifungal agents, these results suggest that FR227244 is acting on the fungal cell wall. The fungal

Fig. 6. ^{13}C NMR spectrum of FR227244 (125 MHz, DMSO-d_6).



cell wall is an essential structure to fungi and is not present in mammalian cells. As such, it is expected to be a suitable antifungal target and to fulfill the criteria for a safe drug.

The studies of *in vitro* and *in vivo* antifungal activities and the mode of action of this compound are described in the following paper[22]. The structural analysis will be described elsewhere.

Acknowledgment

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