

PF1163A and B, New Antifungal Antibiotics Produced by *Penicillium* sp.

I. Taxonomy of Producing Strain, Fermentation, Isolation and Biological Activities

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Two novel antifungal antibiotics, PF1163A and B, were isolated from the fermentation broth of *Penicillium* sp. They were purified from the solid cultures of rice media using ethyl acetate extraction, silica gel and Sephadex LH-20 column chromatographies. PF1163A and B showed potent growth inhibitory activity against pathogenic fungal strain *Candida albicans* but did not show cytotoxic activity against mammalian cells. These compounds inhibited the ergosterol biosynthesis in *Candida albicans*.

The incidence of life-threatening systemic infections caused by opportunistic yeasts and fungi has increased in patients infected by HIV or received cancer chemotherapy¹⁾. The most prevalently used antifungal drugs include the polyenes and the azoles. All these agents, however, have problems in, for example, potency, toxicity and resistance development^{2,3)}. Therefore, the discovery and development of novel antifungal agents are required to resolve these problems.

As part of an antifungal screening program, we searched for compounds from the fermentation broths that inhibited the fungal ergosterol biosynthesis. In this paper, we describe the taxonomy of the producing strain, fermentation, isolation and biological activities of the novel compounds designated PF1163A and B isolated from the microbial broths of *Penicillium* sp.

Materials and Methods

Taxonomic Studies

The fungal strain PF1163 was deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Japan, under the accession number FERM P-15473. Taxonomic studies of the strain PF1163 were done according to the method of

PITT⁴⁾. The color guide of KORNERUP and WANSCHER⁵⁾ was used in the determination and standardization of colors. CYA (yeast extract 0.5%, glucose 3.0%, K₂HPO₄ 0.1%, NaNO₃ 0.2%, KCl 0.05%, MgSO₄·7H₂O 0.05%, FeSO₄·7H₂O 0.001%, agar 1.3%) and MEA media (malt extract 2.0%, peptone 0.1%, glucose 2.0%, agar 2.0%) were used for the identification of the fungus.

Fermentation

Strain PF1163 was inoculated from an agar slant into a 100-ml Erlenmeyer flask containing 20 ml of seed medium made up of 1.0% starch, 1.0% glucose, 0.6% wheat germ, 0.2% soybean meal, 0.3% yeast extract, 0.5% polypepton, 0.2% CaCO₃ and tap water (pH 7.0 before sterilization). The inoculated flask was shaken on a rotary shaker (200 rpm) at 25°C for 2 days. One milliliter of the first seed culture was transferred into 100 ml of the same medium in 500-ml Erlenmeyer flasks. After shaking at 25°C for 3 days, five milliliters of the second seed culture was added to a 500-ml Erlenmeyer flasks containing 100 g of production medium made up of rice containing water and 2.5% soybean meal (pH non-adjusted). The inoculated flasks were incubated as stationary phase cultures for 14 days at 28°C.

Antifungal Activity

Minimum inhibitory concentration (MIC) were determined by microbroth dilution assay in RPMI1640 medium (Nissui). Serial 2-fold dilutions of antibiotics were made from 256 $\mu\text{g/ml}$; the MIC value was the lowest concentration of antibiotic which prevented visible growth after 20 hours at 37°C.

Preparation of Cell-free Homogenate

Cell-free homogenate was prepared from *Candida albicans* TIMM1768 strain. Cells from an overnight culture (1 liter) were harvested by centrifugation, washed with 0.1 M $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ buffer (pH 7.4), and suspended in a minimum volume of ice-cold similar buffer. A cell suspension was passed twice through a French pressure cell (OHTAKE WORKS 5501-M) at an output pressure of 1,300~1,500 kg/cm^2 . The cell suspension was centrifuged at 3,000 g for 10 minutes and unbroken cells were removed. The supernatant (5~10 mg protein/ml) was stored at -80°C until use.

Assay of Ergosterol Synthesis

Ergosterol synthesis from [^{14}C]acetate was measured by the method of MARRIOTT *et al.*⁶⁾ with some modification. The reaction mixture consisted of cell-free extract. The assay mixture was incubated at 30°C with continual shaking for 120 minutes and stopped by the addition of 50 μl of freshly prepared 10% KOH/EtOH. The mixture was then saponified for 30 minutes at 80°C. After saponification, non-saponified lipid fraction was extracted two times with 50 μl of petroleum ether. The combined extracts were evaporated to dryness under reduced pressure and dissolved in 30 μl of petroleum ether. The solution was applied to silica gel TLC plate (Merck F254) and developed with hexane and ethyl acetate (5:1). Radioactive metabolic zones were located by imaging analyzer BAS3000 and the positions were based on relative mobilities of authentic standards of ergosterol, lanosterol and squalene purchased from sigma.

Results

Taxonomy of Strain PF1163

The mycological characteristics of strain PF1163 were as follows. Colonies growing on CYA at 25°C attained a diameter of 25~30 mm in 7 days, and were white to Brownish Orange (5C3), velvety, and radially sulcate. Conidiogenesis was sparse but was produced in sufficient quantities to influence colony appearance within 7 days.

Exudate and soluble pigment were absent. The reverse sides of the colonies were Cocoa Brown (6E6). Colonies growing on MEA at 25°C attained a diameter of 20~25 mm in 7 days, and were white to Greyish Orange (5B4), velvety, plane and slightly sulcate. Conidiogenesis was conspicuous. Exudate and soluble pigment were absent. The reverse side of the colonies were Dark Yellow (4C8). At 37°C, the growth was nil.

Penicilli were biverticillate. Stipes of conidiophores were smooth-walled, 150~300 \times 2~2.5 μm . Metulae were 3~5 per stipe, 8~13 \times 2~2.5 μm . Phialides were amplyform, smooth to slightly roughened, 4~7 per metula, 5~8 \times 1.8~2 μm . Conidia were globose to subglobose, smooth-walled, 2~2.5 μm in diameter, borne in short columus.

The above characteristics of strain PF1163 indicate that this fungus belongs to the genus *Penicillium*. We did not attempt the determination of species.

Isolation

The isolation scheme is shown in Fig. 1. The fermented rice medium (7.5 kg) was extracted with EtOAc (15 liters) and the extract was concentrated to dryness under reduced pressure. The oily residue (27 g) was washed three times with hexane. The residue (15 g) was applied to a column of silica gel (170 g). The column was washed with CHCl_3 and eluted with CHCl_3 -MeOH (100:1) and (100:2). The eluted fraction was evaporated to give the crude PF1163A (3.8 g) containing a little of PF1163B and crude PF1163B (600 mg). The former was applied to a column of silica gel (170 g). The column was washed with CHCl_3 and eluted with CHCl_3 -MeOH (100:0) and (100:1). The eluted fraction was evaporated to give the crude PF1163A (1.2 g) and crude PF1163B (100 mg). The former was applied to a column of silica gel (40 g). The column was washed with hexane-EtOAc (1:1) and eluted subsequently with hexane-EtOAc (1:1), (2:3) and (1:2). The eluted fraction containing PF1163A was evaporated to give pure PF1163A (830 mg) as an oily substance. The residue (700 mg) containing PF1163B was applied to a column of silica gel (65 g). The column was washed with hexane-EtOAc (7:3) and eluted subsequently with hexane-EtOAc (7:3), (1:1) and (1:2). The eluted fraction containing PF1163B was evaporated to give the residue containing PF1163B. It was applied to a column of Sephadex LH-20 (700 ml). The column was eluted with MeOH and the eluted fraction was evaporated to give pure PF1163B (300 mg) as a oily substance.

The structures of PF1163A and B are given in Fig. 2. The physico-chemical properties, structure elucidation⁷⁾

Fig. 1. Isolation procedure of PF1163A and B.

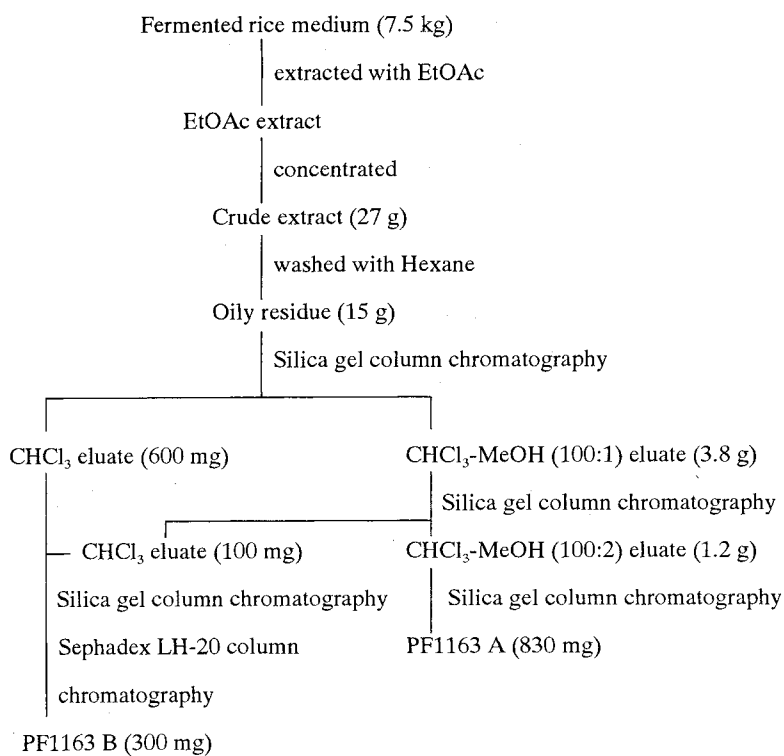
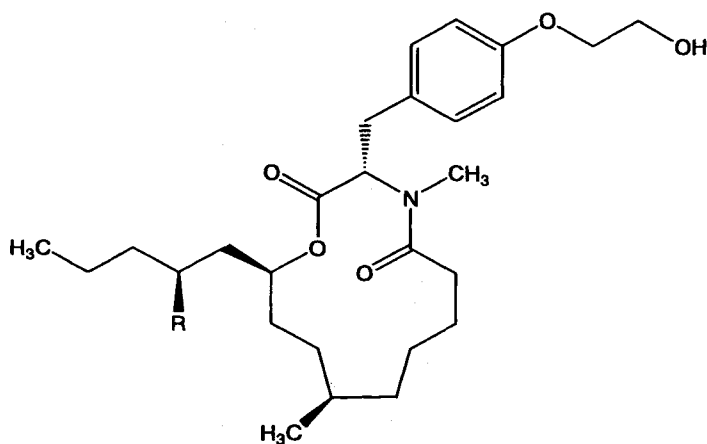


Fig. 2. Structures of PF1163A and B.



PF1163A R = OH

PF1163B R = H

Table 1. Antifungal activity by microbroth dilution assay.

Organisms (strain number)	MIC ($\mu\text{g/ml}$)	
	PF1163A	PF1163B
<i>C. albicans</i> TIMM1768	8	32
<i>C. glabrata</i> IFO-0005	≥ 256	256
<i>C. krusei</i> IFO-0584	≥ 256	256
<i>C. parapsilosis</i> IFO-0585	≥ 256	256
<i>A. fumigatus</i> TIMM1775	≥ 256	128

and total synthesis⁸⁾ of PF1163A and B will be described in the following papers.

Biological Activity

Antifungal and Cytotoxic Activity

Antifungal activities of PF1163A and B against the pathogenic yeasts and fungi were evaluated in a microbroth dilution assay as shown in Table 1. The MIC values of PF1163A and B were 8 and 32 $\mu\text{g/ml}$, respectively, against *Candida albicans* TIMM1768. These compounds were inactive against *Aspergillus fumigatus*.

The cytotoxicity of PF1163A and B against HepG2 human hepatoblastoma cell line was examined. Both compounds did not show 50% growth inhibition against HepG2 even at a concentration of 33.3 $\mu\text{g/ml}$.

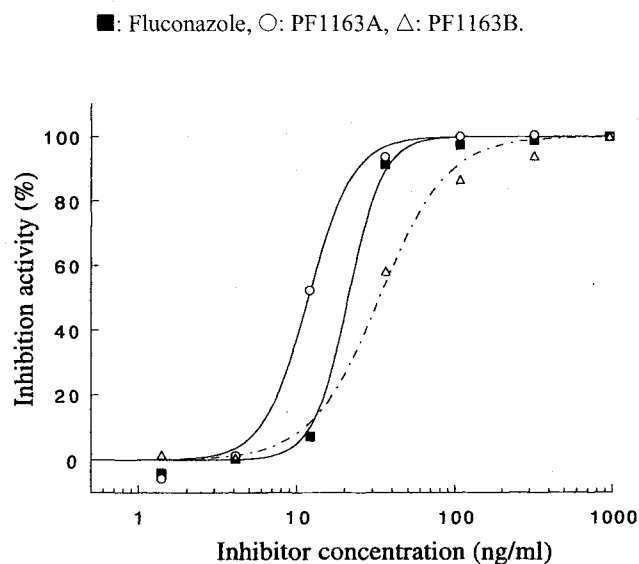
Inhibition of Ergosterol Synthesis

As shown in Fig. 3, PF1163A was as potent as fluconazole in inhibiting the synthesis of ergosterol from [¹⁴C]acetate. The inhibitory activity of PF1163B was a little weak. The IC₅₀ values of PF1163A and B were 12 ng/ml and 34 ng/ml, respectively.

Discussion

In the course of screening for antifungal agents, we discovered the novel compounds, PF1163A and B, from the fermentation broth of *Penicillium* sp. PF1163A and B were

Fig. 3. Inhibition of ergosterol synthesis by PF1163A and B.



active against *Candida albicans* and both compounds inhibited the ergosterol biosynthesis of *Candida albicans*. The target for antifungal agents, such as the azoles, is the fungal P450 lanosterol C-14 demethylase. Recently the inhibitors of ergosterol biosynthesis, isolated from microbial broth, have been reported by some groups^{9,10)}. These compounds were also known to inhibit the C-14 demethylation of lanosterol. PF1163A and B were proved to inhibit the biosynthetic pathway from lanosterol to ergosterol by the inhibitory assay of ergosterol synthesis.

Further experiments, however, will be needed to understand the mechanism of PF1163 compounds because PF1163A and B seemed to inhibit ergosterol biosynthesis at a step different to lanosterol C-14 demethylase. It is known that there are several genes responsible for sterol biosynthesis from lanosterol to ergosterol¹¹⁾. The detail mechanism of antifungal action of PF1163A and B are now under investigation.

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