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Citridones, New Potentiators of Antifungal Miconazole Activity, Produced by *Penicillium* sp. FKI-1938

I. Taxonomy, Fermentation, Isolation and Biological Properties

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Abstract New phenylfuropyridinones and related compounds, designated citridones A, B, B′ and C, were isolated along with known CJ-16,173, from the culture broth of *Penicillium* sp. FKI-1938 by solvent extraction, silica gel column chromatography and HPLC. Citridones (75 μM) potentiate the miconazole activity against *Candida albicans*, decreasing the IC_{50} value of miconazole from 14.5 nM to 3.5∼6.3 nM.

Keywords citridones, anti-infective, azole potentiator, *Penicillium*

Introduction

Opportunistic infections caused by certain fungi, in particular problematic *Candida albicans*, have increased and studied the therapy of these infections [1]. Patients with compromised immune systems, *e.g.* patients receiving organ transplants and cancer chemotherapy, or those infected by human immunodeficiency virus (HIV), are particularly prone to such infections [1]. Recently, resistance to azole antifungals became a significant problem. Several resistant mechanisms have been proposed; 1) overexpression of transporters like ATP binding cassette (ABC) transporter [2], 2) mutation of cytochrome P-450 14-α demethylase (P-450 14DM) [3], 3) overexpression of P450 14DM [4], and so on. Therefore, the resistant mechanisms will be a new target of inhibition for overcoming infections by azole-resistant *C. albicans*.

On the basis of new concept "antiinfective drugs" [5], we have discovered actofunicone [6] and beauvericins D, E and F [7, 8], all produced by fungal strains, as potentiators of antifungal miconazole activity. The compounds showed no antimicrobial activity themselves, but reinforced miconazole activity against *C. albicans*. During the course of our continuous screening program, new compounds, designated citridones A, B, B′ and C (Fig. 1), were isolated from the culture broth of *Penicillium* sp. FKI-1938 along with known CJ-16,173 [9]. All citridones potentiate miconazole activity against *C. albicans* (wild type).

In this paper, we describe the taxonomy of the producing fungus, fermentation, isolation and miconazole-potentiating activity of these citridones.

Materials and Methods

General Experimental Procedures

The strain FKI-1938 was isolated from soil collected on Ishigaki Island, Okinawa, Japan and was used for production of citridones and CJ-16,173. *C. albicans*

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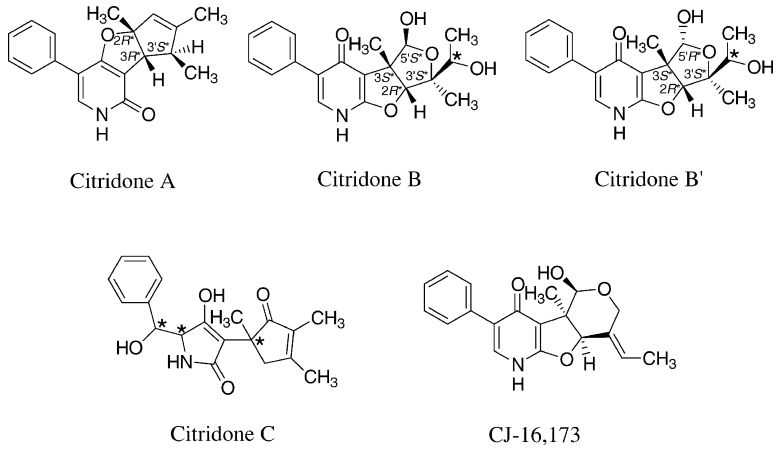

Fig. 1 Structures of citridones A, B, B′, C and CJ-16,173.

ATCC64548 (wild type) were purchased from ATCC (Virginia, USA). For determination of the amounts of citridones A, B, B′ and C in the culture broths, the samples, dissolved in methanol, were analyzed on an HP 1100 system (Hewlett Packard Inc., Germany) under the following conditions: column, Symmetry (2.1×150 mm, Waters Inc., Missouri, USA); flow rate, 0.2 ml/minute; mobile phase, a 20-minute linear gradient from 30% CH_3CN/0.05% H_3PO_4 to 70% CH_3CN/0.05% H_3PO_4; detection, UV at 210 nm. Citridones A and C were eluted as peaks with retention times of 13.6 and 7.1 minutes, respectively. Citoridones B and B′ were found to exist in an equilibrium, eluting as broad peaks with a retention time of 3.2∼3.9 minutes under the HPLC conditions. Packed cell volume (PCV) was measured after the whole culture broth at indicated times (10 ml) was centrifuged at 3,000 rpm for 10 minutes. The pH of the culture broth was measured by using compact pH meter (HORIBA).

Taxonomic Studies of the Producing Organism

Taxonomic studies and identification were conducted to the procedures described by Pitt [10]. Morphological observations were done under a light microscope (Olympus Vanox-S AH-2) and a scanning electron microscope (JEOL JSM-5600). Color names and hue numbers were determined according to the Color Harmony Manual [11].

Assay for Miconazole-potentiating Activity

Wild type of *C. albicans* was inoculated into a 50-ml test tube containing 10 ml of seed medium (potato extract containing peptone 0.5% and glucose 1%), and was grown for 24 hours on the rotary shaker. In Method A, the seed culture of *C. albicans* (0.1%, v/v) was transferred to the two different agar plates, GY agar (glucose 1%, yeast extract 0.5% and agar 0.8%) (Plate A) and GY agar plus miconazole (0.06 μM) (Plate B). The concentration (0.06 μM) of miconazole is one fourth of the MIC value against *C. albicans*, and showed no effect on the growth of *C. albicans*. Paper disks (8 mm, ADVANTEC, Tokyo, Japan) containing a sample were put on Plates A and B, which were incubated at 27°C for 24 hours. Samples showing inhibition zones selectively on Plate B were selected as potentiators of miconazole activity against *C. albicans*. In Method B, the broth microdilution test using 96-well microplates (Corning, New York, USA) was performed according to the guidelines of NCCLS document M27-A [12]. Five colonies of wild *C. albicans* with diameters of 1 mm were suspended in sterile 0.85% saline to adjust to a 0.5 McFarland standard by spectrophotometric measurement. The seed of *C. albicans* was diluted 1,000 times with medium A (165 mM morpholinopropanesulfonic acid buffer (pH 7.0)), and the diluted seed (100 μl) containing serial concentration of miconazole (0∼80 nM) in the absence or presence of a sample (75 μM) to make the total volume 200 μl/well. *C. albicans* in microplates was incubated at 27°C for 24 hours, and the growth of *C. albicans* was measured at 630 nm with a microplate reader (model Elx 808, BIO-TEK Instruments, Vermont, USA). The IC_{50} values of miconazole against *C. albicans* in the absence or presence of a sample were calculated.

Results

Taxonomy of the Producing Strain FKI-1938

Strain FKI-1938 was originally isolated from a soil sample collected at Ishigakijima Island, Okinawa Perfecture, Japan.

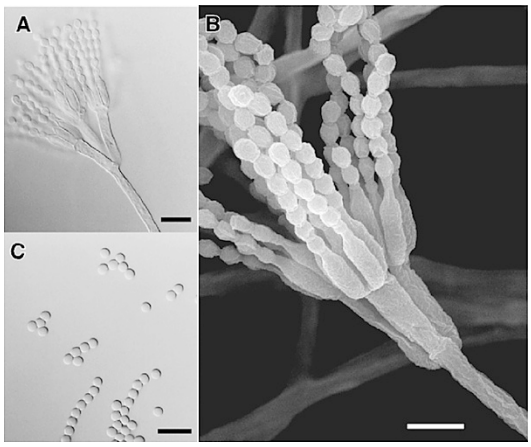

Fig. 2 *Penicillium* sp. FKI-1938 on CYA. (A) Biverticillate penicilli under a light microscope. (B) Biverticillate penicilli and conidia under a scanning electron microscope. (C) Conidia under a light microscope. Scale bar: A, C; 10 μm, B; 5 μm.

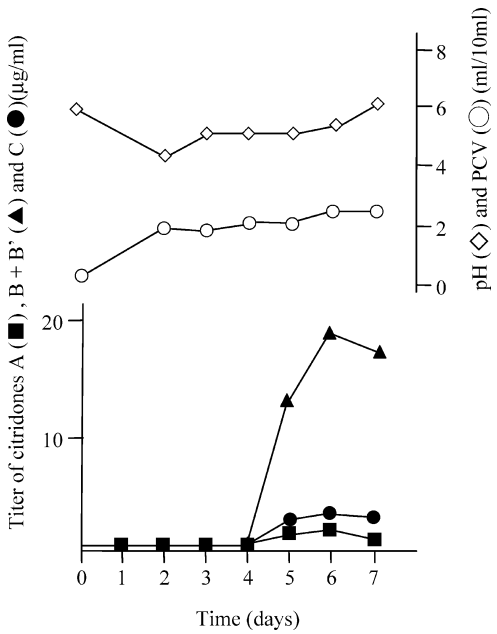

Fig. 3 A typical time course of citridone production by *Penicillium* sp. FKI-1938. The amounts of citridones A (■), B+B′ (▲) and C (●), PCV (○) and pH (◇) in culture broths were determined as described in Materials and Methods.

For the taxonomic studies of fungus, Czapek yeast extract agar (CYA), malt extract agar (MEA) and 25% glycerol nitrate agar (G25N) were used. Colonies on CYA were 30～36 mm diameter after 7 days at 25°C, floccose to velutinous, radially sulcate, sage gray (24 ih) to mistletoes gray (24 1/2 ih) in color. The reverse side was bamboo (2 gc) to cream (1 1/2 ca). Colonies on MEA were 20～22 mm diameter, velutinous, plane to light radially sulcate, dark olive green (24 1/2 nl) to mistletoes green (24 1/2 li) in color. The reverse side was bamboo (2 gc) to covert tan (1 1/2 ca). Colonies on G25N were 19～21 mm diameter, floccose to velutinous, radially sulcate, celadon gray (24 fe) to sage gray (24 ih) in color. The reverse side was pearl pink (3 ca) to light yellow (2 ea). At 5°C and 37°C, on CYA colonies were no growth. Soluble pigment was not produced on each medium. Conidiogenesis on each medium was moderate to abundant. Conidiophores on CYA were borne from subsurface or aerial hyphae, with 150～500 (～800) μm long, smooth-walled, and bearing biverticillate penicilli. Metulae were in (2～) 3～4 divergent clusters and 14～18×2.8～3.5 μm in size (Fig. 2A, 2B). Phialides were 5～8 per metula, ampulliform with 8～11×2～3 μm in size and smooth-walled (Fig. 2A). Conidia after 7 days incubation, were spherical to subsperical, 2.3～3.0 μm, smooth-walled, and in divergent long chains (Fig. 2B, 2C). From the above characteristics, strain FKI-1938 was considered to belong to the genus *Penicillium* and named it *Penicillium* sp. FKI-1938. Futhemore, the strain was considered to be classified in *Penicillium citrinum* series that are characterized by conidiophore are smooth walled and divergent whorls of metulae usually without further branches; conidia are globose to subglobose, smooth walled and not exceeding 3 μm diameter [13, 14]. The strain has been deposited to the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology Japan, as FERM ABP-10149.

Fermentation

A slant culture of the strain FKI-1938 grown on LcA medium (glycerol 0.1%, KH_2PO_4 0.08%, K_2HPO_4 0.02%, $MgSO_4 \cdot 7H_2O$ 0.02%, KCl 0.02%, $NaNO_3$ 0.2%, yeast extract 0.02% and agar 1.5%, pH 6.0) was used to inoculate a 500-ml Erlenmeyer flask containing 100 ml of the seed medium (glucose 2.0%, yeast extract 0.2%, $MgSO_4 \cdot 7H_2O$ 0.05%, polypepton 0.5%, KH_2PO_4 0.1% and agar 0.1%, pH 6.0). The flask was shaken on a rotary shaker at 27°C for 3 days. The seed culture (100 ml) was transferred into a 7.5-liter jar fermenter (B. E. Marubishi, Japan) containing 5 liters of the production medium (glycerol 2.0%, sucrose 1.0%, ammonium acetate 0.5%, K_2HPO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05%, KCl 0.05%, Cultivater #100 (Yazu Suisankagaku Industry CO., LTD.) 0.2% and agar 0.1%, pH 6.0). The fermentation was carried out at 27°C for 7 days with an aeration of 2.5 liters/minute and an agitation of 200 rpm.

A typical time course of the fermentation is shown in Fig. 3. Citridone A, the mixture of citridones B and B′, and

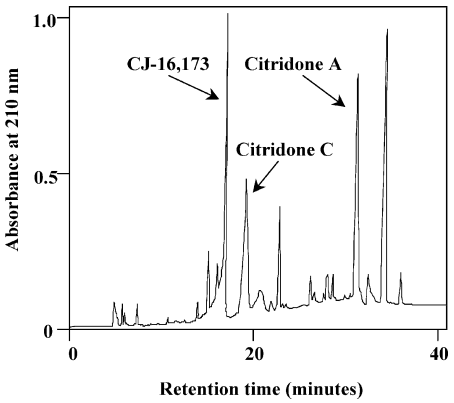

Fig. 4 A chromatographic profile of purification of citridones A and C by preparative HPLC. Column, Senshu Pak PEGASIL ODS (4.6×250 mm); solvent, 40-minute linear gradient from 30 to 70% CH_3CN in 0.05% H_3PO_4; detection, UV at 210 nm; flow rate, 1.0 ml/minute; sample, 10 μg of active materials dissolved in 10 μl of MeOH.

citridone C were detected in the culture broth from day 5 after inoculation, and their concentrations reached a maximum (1.3, 19.3 and 2.6 μg/ml, respectively) on day 6.

Isolation

To the 7-day old culture broth (5 liters) was added acetone (5 liters). After the acetone extracts were filtered and concentrated, the resulting aqueous solution was extracted with ethyl acetate (5 liters). The ethyl acetate layer was dried over Na_2SO_4 and concentrated *in vacuo* to dryness to yield an oily material (2.9 g). The material was dissolved in a small volume of $CHCl_3$, applied on a silica gel column (60 g, 3.4×28 cm, 70~230 mesh, Merck), and eluted stepwise with 100 : 0, 100 : 1, 75 : 1, 50 : 1, 40 : 1, 30 : 1, 10 : 1 and 0 : 100 (v/v) of $CHCl_3$ - CH_3OH solvents (1000 ml each). The miconazole-potentiating activity was observed in the 100 : 1 fraction, which was concentrated to give a brown material (237.5 mg). The material was purified by HPLC; ODS column (4.6×250 mm, Pegasil, Senshu Sci. Co. Tokyo, Japan), a 40-minute linear gradient from 30 to 70% CH_3CN in 0.05% H_3PO_4, 1.0 ml/minute, and UV at 210 nm. Under the conditions, citridones A and C were eluted as peaks with retention times of 32.0 and 18.8 minutes, respectively (Fig. 4). Each peak was collected and concentrated to yield citridone A (6.4 mg) as white needles and citridone C (11.7 mg) as pale yellow amorphous. The 50 : 1 fraction (white powder) was recrystallized from MeOH to give a mixture of citridones B and B′ (59.2 mg) as white needles. Analysis of the mixture by HPLC as described (HP 1100 system) showed broad two peaks with a retention time of 3.2~3.9 minutes. Each peak collected was analyzed under the same condition, showing the same broad two peaks. As described in the preceding paper [15], citridones B and B′ existed in equilibrium (about 3 : 2) of hemiacetal epimerization in a solution, and could not be separated.

Table 1 Potentiation of miconazole activity against *C. albicans* by citridones and CJ-16,173

Compound	Concentration	Inhibition zone (mm)	
	μg/disk	Plate A	Plate B
Citridone A	10	—	17
	25	—	19
	50	—	23
Citridones B+B′	10	—	—
	25	—	13
	50	—	15
Citridone C	10	—	—
	25	—	11
	50	—	17
CJ-16,173	10	—	—
	25	—	—
	50	—	—

Paper disks (8 mm i.d.) containing three concentrations of a compound were put on Plate A (*C. albicans* in GY agar) and Plate B (*C. albicans* in GY agar+0.06 μM miconazole), and incubated at 27°C. After 24 hours, the diameters of inhibition zones were measured (Method A). The concentration (0.06 μM) of miconazole is one fourth of the MIC value against *C. albicans*, and showed no effect on the growth of *C. albicans*.

Table 2. Reinforcement of miconazole activity against *C. albicans* by citridones.

Addition	IC_{50} of miconazole (nM)	Ratio (control/+drug)
None (control)	14.5	1
+Citridone A	5.0	2.9
+Citridones B+B′	6.0	2.4
+Citridone C	3.5	4.1
+CJ-16,173	6.3	2.3

The IC_{50} value of miconazole against the growth of *C. albicans* in the absence (control) or presence of a compound (75 μM) were measured using 96-well microplates (Method B).

Biological Properties

Miconazole-potentiating Activity by Citridones

The miconazole-potentiating activity of citridones was tested by Methods A and B. In Method A, all citridones

themselves showed no inhibition against wild *C. albicans* at up to 50 μg/disk on Plate A. However, all citridones gave dose-dependent inhibition zones on Plate B (Table 1). These results indicated that citridones potentiate miconazole activity against wild *C. albicans*. However, under the similar methods no potentiation of miconazole activity by citridones was observed against *Aspergillus* and *Mucor*.

Potentiation of miconazole activity by citridones was evaluated by Method B. The IC_{50} values of all citridones against *C. albicans* were over 400 μM (data was not shown). In the absence of citridones, the IC_{50} value of miconazole against *C. albicans* was calculated to be 14.5 nM. In combination with citridone A, a mixture of citridones B and B′, citridone C and CJ-16,173 (75 μM each), the respective IC_{50} values of miconazole were decreased to 5.0, 6.0, 3.5 and 6.3 nM (Table 2). Thus, it was confirmed that they potenitate miconazole activity against *C. albicans*.

Discussion

Several phenylfuropyridines or phenylfuropyridones such as CJ-15,696 (cladobotryal), CJ-16,170, CJ-16,171 and CJ-16,173, structurally related to citridones A, B and B′ (Fig. 1) were isolated from *Caldobotrium* varium [9, 16]. Such known ones have linked ring systems (6-6/5 or 6-6/5/6), which were different from the citridones' (6-6/5/5). But the citridones-producing *Penicillium* sp. FKI-1938 also produced CJ-16,173 as described in this paper. Among the CJ compounds, CJ-15,696 was reported to possess moderate activity against various Gram-positive bacteria including some drug-resistant strains [9]. Citridones and CJ-16,173 showed no activity even at 1 mg/ml against 15 microorganisms including Gram-positive bacteria (*Bacillus subtillis*, *Staphylococcus aureus*, *Mycobacterium smegmatis* and *Micrococcus luteus*) and Gram-negative bacteria (*Escherichia coli*, *Escherichia coli* CJ-2, *Xanthomonas oryzae*, *Pseudomonas aeruginosa*, *Bacteroides fragilis* and *Acholeplasma laidlawii*), fungi (*Mucor racemosus*, *Aspergillus niger* and *Pyricularia oryzae*) and yeast (*C. albicans* and *Saccharomyces cerevisiae*). However, we found that they potentiate antifungal miconazole activity against *C. albicans* (Table 2). Among them, citridone C having an isolated-ring system showed the most potentiating activity. It might be interesting whether or not CJ compounds show the miconazole potentiating activity to study the structure-activity relationship.

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