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

Takashi Fukuda, Yoko Hasegawa, Yasunari Sakabe, Hiroshi Tomoda, Satoshi Ōmura

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Abstract Two new aromatic alkaloids, designated citrinamides A and B, were isolated from the culture broth of *Penicillium* sp. FKI-1938 by solvent extraction, silica gel column chromatography and HPLC. Their structures were elucidated by spectroscopic analysis, including NMR and amino acid analysis. Citrinamides A and B showed moderate potentiation of miconazole activity against *Candida albicans*.

Keywords citrinamide, miconazole potentiator, *Penicillium* sp., fungal metabolite, *Candida albicans*

Introduction

Opportunistic infections caused by certain fungi, in particular *Candida albicans*, have increased to the point of public concern. Patients with compromised immune systems, *e.g.*, patients receiving organ transplants, cancer chemotherapy or those infected by human immunodeficiency virus, are particularly prone to such infections [1]. Recently, resistance to azole antifungals has become a significant problem. Several mechanisms of resistance have been proposed including 1) overexpression of transporters such as ATP binding cassette (ABC) transporter [2], 2) mutation of cytochrome P-450 14-α demethylase (P-450 14DM) [3] and 3) overexpression of P-450 14DM [4]. The mechanisms of resistance will indicate new targets for overcoming infections by azole-resistant *C. albicans*. That is, inhibiting the resistance mechanisms leads to enhance the activity of azoles against not only azole-resistant but also wild *C. albicans*. On the basis of this new concept of "anti-infective drugs" [5], we have screened microbial metabolites, which didn't show the antifungal activity by themselves but showed potentiation of miconazole activity against *C. albicans*. As a result, we discovered various new compounds: actofunicone [6], beauvericins [7], citridones [8, 9] and tensidoles [10] from fungi and phenatic acids [11] from an actinomycete. During detailed analysis of the metabolites of citridone-producing *Penicillium* sp. FKI-1938, two new aromatic alkaloids designated citrinamides A and B (Fig. 1) with core structures that differs from citridones, were discovered.

In this study, the structural elucidation, including the absolute stereochemistry and miconazole-potentiating activity of those citrinamides are described.

Materials and Methods

General Experimental Procedures

The strain FKI-1938 was isolated from soil collected on Ishigakijima, Okinawa, Japan and was used for production of citrinamides. *C. albicans* ATCC64548 was purchased from ATCC.

Optical rotations were recorded with a DIP-370 digital polarimeter (Jasco). FAB-MS spectrometry was conducted using a JMS-AX505H spectrometer (JEOL). UV and IR spectra were measured with a DU640 spectrophotometer (Beckman) and an FT-210 Fourier transform infrared

T. Fukuda, Y. Hasegawa, Y. Sakabe, S. Ōmura: Kitasato Institute for Life Sciences & Graduate School of Infection Control Sciences, Kitasato University, 5-9-1 Shirokane, Minato-ku, Tokyo 108-8641, Japan

H. Tomoda (Corresponding author)**:** School of Pharmacy, Kitasato University, 5-9-1 Shirokane, Minato-ku, Tokyo 108-8641, Japan, E-mail: tomodah@pharm.kitasato-u.ac.jp

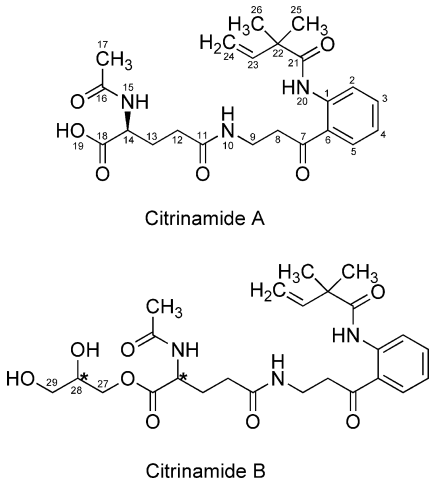

Fig. 1 Structures of citrinamides A and B.

spectrometer (Horiba), respectively. The various NMR spectra were measured with a MERCURY plus 300 MHz spectrometer (Varian).

Acid Hydrolysis

Citrinamide A (100 μg) was degraded in a gas phase of 6.0 N HCl (200 μl) at 108°C for 22 hours using a PICO·TAG work station (Waters). The degradation products were dissolved in H_2O (20 μl), and used for determining amino acid constituents.

Analysis of the Stereochemistry of the Amino Acid by HPLC

To determine the stereochemistry of the amino acid contents, a part of the H_2O (10 μl) was analyzed under the following conditions: pump, SSC-3210 (Senshu Scientific Co., ltd.), UV detector, SSC-5200 (Senshu Scientific Co., Ltd.), column, SUMICHIRAL OA-5000 (Sumika Chemical Analysis Service, Ltd.), 4.6 i.d.×150 mm; flow rate, 1.0 ml/minute; detection, UV at 254 nm; Solvent, 5.0% MeOH in 2.0 mM aq $CuSO_4$. L- and D-glutamic acids were purchased from Sigma.

Assay for Miconazole-potentiating Activity

The broth microdilution test using 96-well microplates (Corning) 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. A 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) was prepared in the absence or presence of a sample (50 μg/ml) 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 580 nm with a microplate reader (model Elx 808, Bio-Tek Instruments). The IC_{50} values of miconazole against *C. albicans* in the absence or presence of a sample were calculated.

Results

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 30-liter jar fermentor (Mitsuwa, Japan) containing 20 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 10.0 liters/minute and an agitation of 250 rpm.

Isolation

To 7-day-old culture broth (20 liters) of *Penicillium* sp. FKI-1938, Me_2CO (20 liters) was added. After the acetone extracts were filtered and concentrated, the aqueous solution (pH 7.1) was extracted with EtOAc (20 liters) to remove citridones A, B, B′ and C. The pH of the resulting aqueous solution was adjusted to 3.0 with HCl, and extracted with EtOAc again. The organic layer was dried over Na_2SO_4 and concentrated *in vacuo* to dryness to yield an oily material (1.4 g). The material was dissolved in a small volume of $CHCl_3$, applied to a silica gel column (80 g, 4.0×18 cm, 70~230 mesh, Merck), and eluted with $CHCl_3$ - CH_3OH solutions (500 ml each). Citrinamides A and B were recovered in the fraction eluted with the 5 : 1 solvent mixture, which was concentrated to give a brown material (149.1 mg). The material was purified by HPLC; ODS column (20×250 mm, Pegasil, Senshu Sci. Co.), a 30-minute linear gradient from 20 to 50% CH_3CN in 0.05% CF_3COOH, 8.0 ml/minute, and UV at 270 nm. Under these conditions, citrinamides A and B were eluted as peaks with a retention time of 27.6 and 26.0 minutes, respectively (Fig.

2). The peaks were pooled and concentrated to yield pure citrinamide A (13.4 mg) and citrinamide B (15.7 mg).

Structural Elucidation

Physico-chemical Properties of Citrinamides A and B

Physico-chemical properties of citrinamides A and B are summarized in Table 1. Citrinamides A and B showed similar peaks of absorption at 230, 260 and 322～324 nm. The IR spectrum of cirtinamide A showed the characteristic absorption at 3500～3000, 1733 and 1718 cm^{-1}, suggesting the presence of carboxylic acid and amide groups. Citrinamide B showed the absorption at 3600～3000 and 1739 cm^{-1}, suggesting the presence of hydroxyl and amide groups. Similarity of their spectral data indicated that they were close structural relationships.

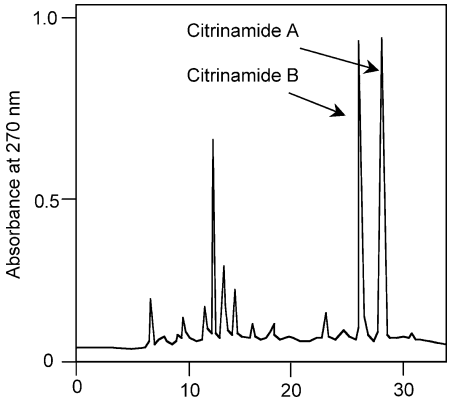

Fig. 2 A chromatographic profile of purification of citrinamides A and B by preparative HPLC.

Column, Senshu Pak PEGASIL ODS (20×250 mm); solvent, 30-minute linear gradient from 20 to 50% CH_3CN in 0.05% CF_3COOH; detection, UV at 270 nm; flow rate, 8.0 ml/minute; sample, 100 μg of active materials dissolved in 100 μl of MeOH.

Citrinamide A

The molecular formula of citrinamide A was determined to be $C_{22}H_{29}N_3O_6$ on the basis of HRFAB-MS measurement. The ^{13}C-NMR spectrum (in $CDCl_3$) showed 22 resolved signals (Table 2). They were classified as derived from three methyl carbons, five methylene carbons including one sp^2 methylene carbon, six methine carbons including five sp^2 methine carbons, and eight quaternary carbons including seven sp^2 quaternary carbons, by analysis of the DEPT spectra. The ^{1}H-NMR spectrum (in $CDCl_3$) showed three methyl signals, five methylene signals, two methine signals, four aromatic proton signals, three amino proton signals, and one hydroxyl signal (Table 2). The connectivity of proton and carbon atoms was established by the HMQC spectrum. Analysis of the ^{1}H-^{1}H COSY spectra gave four partial structures: position C-2 to C-5, C-8 to N-10, C-12 to N-15 and C-23 to C-24. Analysis of HMBC spectra gave the following information. The cross peaks from 2-H (δ 8.72) to C-1 (δ 140.9), C-4 (δ 122.5) and C-6 (δ 121.7), from 3-H (δ 7.53) to C-1 and C-5 (δ 130.7) from 4-H (δ 7.10) to C-2 and C-6, from 5-H (δ 7.86) to C-1, C-3 and C-7 (δ 202.7), from 8-H_2 (δ 3.29) to C-7 and C-9 (δ 35.2) 9-H_2 (δ 3.65) to C-7 and C-8 (δ 39.1) and from 10-H (δ 6.99) to C-9 supported the partial structure I. The cross peaks from 12-H_2 (δ 2.42, 2.36) to C-11 (δ 173.8), C-13 (δ 27.8) and C-14 (δ 52.4), from 13-H_2 (δ 2.12, 2.02) to C-11, C-12 (δ 32.5), C-14 and C-18 (δ 173.2), from 14-H (δ 4.40) to C-12, C-13, C-16 (δ 171.6) and C-18, from 15-H (δ 7.38) to C-14, C-16 and C-17 and from 17-H_3 (δ 2.02) to C-16 supported the partial structure II. The cross peaks from 20-H (δ 11.60) to C-21 (δ 176.4), from 23-H (δ 6.11) to C-21, C-22 (δ 46.7), C-25 (δ 24.7) and C-26 (δ 24.7) from 24-H_2 (δ 5.34, 5.29) to C-22 and C-23 (δ 142.4), from 25-H_3 (δ 1.41) to C-21, C-22, C-23 and C-26 and

Table 1 Physico-chemical properties of citrinamides A and B

	Citrinamide A	Citrinamide B
Appearance	Brown solid	Brown solid
$[\alpha]_D^{25}$	−1.9 (*c* 0.1, CH_3OH)	−7.7 (*c* 0.1, CH_3OH)
Molecular formula	$C_{22}H_{29}N_3O_6$	$C_{25}H_{35}N_3O_8$
Molecular weight	431	505
HR-FAB-MS *m/z* $(M+H)^+$		
Calcd	432.2135 (for $C_{22}H_{30}N_3O_6$)	506.2504 (for $C_{25}H_{36}N_3O_8$)
Found	432.2118	506.2502
UV $\lambda_{max}^{CH_3OH}$ nm (ε)	230 (39,000), 260 (9,300), 322 (3,200)	230 (20,400), 260 (8,000) 324 (3,300)
IR ν_{max}^{KBr} cm^{-1}	3299, 3093,1733, 1718, 1654	3434, 3299, 3087, 1739, 1656
Solubility		
Soluble	CH_3OH, $CHCl_3$, EtOAc	CH_3OH, $CHCl_3$, EtOAc
Insoluble	H_2O	H_2O

Table 2 ^{1}H- and ^{13}C-NMR chemical shifts of citrinamides A and B

	Citrinamide A		Citrinamide B	
	^{13}C chemical shifts (m)[a]	^{1}H chemical shifts (ppm)[b]	^{13}C chemical shifts (ppm)[a]	^{1}H chemical shifts (ppm)[b]
C-1	140.9		141.0	
C-2	121.0	8.72 (1H, d, J =8.5)	121.1	8.72 (1H, d, J =8.5)
C-3	135.2	7.53 (1H, dd, J =8.5, 7.5)	135.3	7.53 (1H, t, J =8.5)
C-4	122.5	7.10 (1H, dd, J =8.5, 7.5)	122.5	7.10 (1H, t, J =8.5)
C-5	130.7	7.86 (1H, d, J =8.5)	130.7	7.86 (1H, d, J =8.5)
C-6	121.7		121.7	
C-7	202.7		202.9	
C-8	39.1	3.29 (2H, t, J =6.5)	39.2	3.29 (2H, br t, J =6.0)
C-9	35.2	3.65 (2H, dd, J =6.5, 6.0)	34.8	3.65 (2H, m)
N-10		6.99 (1H, br t, J =6.0)		6.64 (1H, br s)
C-11	173.8		172.8	
C-12	32.5	2.42 (1H, m)	32.0	2.40 (2H, br m)
		2.36 (1H, m)		
C-13	27.8	2.12 (1H, m)	27.1	2.05 (2H, br m)
		2.02 (1H, m)		
C-14	52.4	4.40 (1H, q, J =7.0)	52.5	4.43 (1H, br m)
N-15		7.38 (1H, d, J =7.0)		7.16 (1H, br s)
C-16	171.6		170.9	
C-17	22.8	2.02 (3H, s)	22.9	2.02 (3H, s)
C-18	173.2		171.7	
O-19		5.80 (1H, br s)		
N-20		11.60 (1H, s)		11.59 (1H, s)
C-21	176.4		176.0	
C-22	46.7		46.8	
C-23	142.4	6.11 (1H, dd, J =18.0, 11.0)	142.5	6.11 (1H, dd, J =18.0, 11.0)
C-24	114.7	5.34 (1H, d, J =18.0)	114.7	5.36 (1H, d, J =18.0)
		5.29 (1H, d, J =11.0)		5.31 (1H, d, J =11.0)
C-25	24.7	1.41 (3H, s)	24.7	1.42 (3H, s)
C-26	24.7	1.41 (3H, s)	24.7	1.42 (3H, s)
C-27			66.4	4.23 (2H, br m)
C-28			69.6	3.94 (1H, br m)
C-29			63.1	3.68 (2H, br m)
OH				3.00 (2H, br s)

[a] Chemical shifts are shown with reference to $CDCl_3$ as 77.0 ppm.
[b] Chemical shifts are shown with reference to $CDCl_3$ as 7.26 ppm.

from 26-H_3 (δ 1.41) to C-21, C-22, C-23 and C-25 supported the partial structure III.

The cross peaks from 9-H and 10-H to C-11 and from 20-H to C-2 and C-6 indicated that the partial structures I, II and III are linked as shown in Fig. 3. The carboxylic acid at C-18 was determined from the chemical shifts and supported by the IR spectrum. The structure satisfied the molecular formula and the degree of unsaturation.

The absolute stereochemistry of the glutamic acid residue in citrinamide A was studied. The authentic amino acids, L-glutamic acid, D-glutamic acid and the acid hydrolysate of citrinamide A, were analyzed by HPLC using a chiral column. By comparing the retention times with L-glutamic acid (18.8 minutes) and D-glutamic acid (20.8 minutes), hydrolysate citrinamide A (18.8 minutes) was found to contain L-glutamic acid. Taken together, the structure of citrinamide A including the absolute stereochemistry was elucidated as shown in Fig. 1.

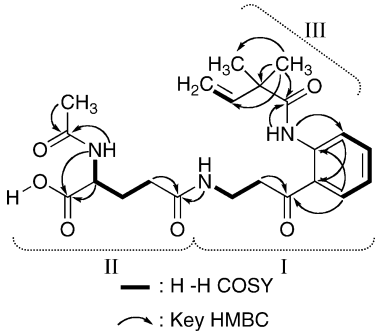

Fig. 3 Partial structures I, II and III and key cross peaks observed in ^{1}H-^{1}H COSY and HMBC experiments of citrinamide A.

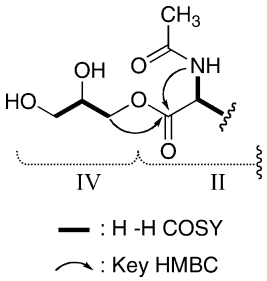

Fig. 4 Partial structure IV and key cross peaks observed in ^{1}H-^{1}H COSY and HMBC experiments of citrinamide B.

Citrinamide B

The molecular formula of citrinamide B ($C_{25}H_{35}N_3O_8$) is larger by $C_3H_6O_2$ compared with that of citrinamide A. The difference between citrinamides A and B is the presence of the partial structure IV for citrinamide B (Fig. 4), which was elucidated from the ^{1}H-^{1}H COSY, HMQC and HMBC spectra. The cross peaks from 27-H_2 (δ 4.23) to C-18 (δ 171.7) and 15-H (δ 7.16) to C-18 indicated that the partial structures IV and II are linked as shown in Fig. 4. Two hydroxyl moieties at C-28 (δ 69.6) and C-29 (δ 63.1) of citrinamide B were determined from the chemical shifts and supported by the IR spectrum. The structure satisfied the molecular formula and the degree of unsaturation. Thus, the structure of citrinamide B was elucidated as shown in Fig. 1. Determining the structural stereochemistry of citrinamide B will require further investigation.

Biological Properties

Miconazole-potentiating Activity

Citrinamides A and B showed no antifungal activity against wild *C. albicans* even at 250 μg/ml. However, citrinamides A and B (50 μg/ml) decreased the IC_{50} of miconazole (9.1 nM) to 5.0 nM and 4.4 nM, respectively.

Discussion

We isolated two new compounds, citrinamides A and B, from the culture broth of *Penicillium* sp. FKI-1938 as potentiators of miconazole activity against *C. albicans*. They moderately potentiated miconazole activity. We have already reported several miconazole-potentiators such as actofunicone, beauvericins and citridones. When compared with their potency, citrinamides are rather weak potentiators of antifungal miconazole activity. We speculated the potentiation mechanisms: 1) inhibiting the transporters such as ABC transporter 2) inhibiting P450 14DM 3) activation of $\Delta^{5,6}$ sterol desaturase [13]. It might be plausible that citrinamides inhibit one or some of these enzymes or proteins as a potential target of citrinamides in potentiating miconazole activity, but we need more investigation to define this point, such as testing using *Trichospron cutaneum* and azole-resistant *C. albicans*.

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