

Tensidols, New Potentiators of Antifungal Miconazole Activity, Produced by *Aspergillus niger* FKI-2342

Takashi Fukuda, Yoko Hasegawa, Keiichi Hagimori, Yuichi Yamaguchi, Rokuro Masuma, Hiroshi Tomoda, Satoshi Ōmura

Dedicated to the memory of Professor Kenneth L. Rinehart.

Received: June 2, 2006 / Accepted: August 7, 2006
© Japan Antibiotics Research Association

Abstract Two new fuopyrrols, designated tensidols A and B, were isolated from the culture broth of *Aspergillus niger* FKI-2342 by solvent extraction, silica gel column chromatography and HPLC. Their structures were elucidated and shown to have the common skeleton of 6-benzyl-6*H*-furo[2,3-*b*]pyrrole. Tensidols A and B potentiated miconazole activity against *Candida albicans*. Tensidols also showed moderate antimicrobial activity only against *Pyricularia oryzae*.

Keywords tensidol, miconazole potentiator, *Aspergillus niger*, fuopyrrol, fungal metabolite, *Candida albicans*

Introduction

Opportunistic infections caused by certain fungi, in particular problematic *Candida albicans*, have increased to the point of public concern. Patients with compromised immune systems, e.g. patients receiving organ transplants and cancer chemotherapy, or those infected by human immunodeficiency virus, are particularly prone to such infections [1]. We reported actofunicone [2], beauvericins [3], phenatic acids [4] and citridones [5], produced by fungal or actinomycete strains, as potentiators of miconazole activity against *C. albicans*. During the course

of our screening program, new compounds, designated tensidols A and B (Fig. 1), were isolated from the culture broth of *Aspergillus niger* FKI-2342. Both compounds were found to potentiate miconazole activity against *C. albicans*.

In this study, we describe the taxonomy of the producing fungus, fermentation, isolation, structure elucidation and biological properties of tensidols.

Materials and Methods

General Experimental Procedures

The strain FKI-2342 was isolated from soil collected at Ooura Tensyudou, Nagasaki, Japan, and was used for production of tensidols. *C. albicans* ATCC64548 was purchased from ATCC (Virginia, USA). Packed cell volume (PCV) was measured after the whole culture broth (10 ml) was centrifuged at 3000 rpm for 10 minutes. The pH of the culture broth was measured by using a compact pH meter (Horiba, Kyoto, Japan).

Optical rotations were recorded with a DIP-370 digital polarimeter (Jasco, Tokyo, Japan). FAB-MS spectrometry was conducted on a JMS-AX505H spectrometer (Jeol, Tokyo, Japan). UV and IR spectra were measured with a DU640 spectrophotometer (Beckman, California, USA)

H. Tomoda (Corresponding author): School of Pharmacy, Kitasato University, 5-9-1 Shirokane, Minato-ku, Tokyo 108-8641, Japan, E-mail: tomudah@pharm.kitasato-u.ac.jp
Y. Yamaguchi, S. Ōmura: The Kitasato Institute, 5-9-1 Shirokane, Minato-ku, Tokyo 108-8642, Japan

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

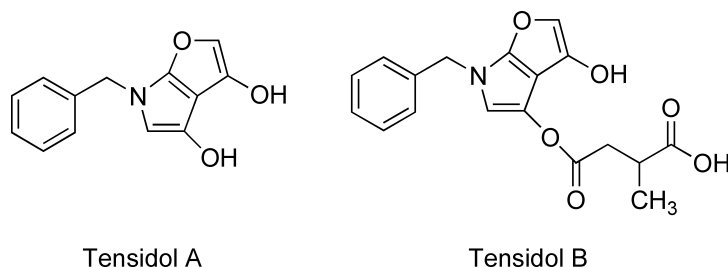


Fig. 1 Structures of tensidols A and B.

and an FT-210 Fourier transform infrared spectrometer (Horiba, Kyoto, Japan), respectively. The various NMR spectra were measured with a MERCURY plus 300 MHz spectrometer (Varian, California, USA).

Taxonomic Studies of the Producing Organism

Taxonomic studies and identification were conducted according to the procedures described by Klich [6]. For the taxonomic studies of fungus, Czapeck yeast extract agar (CYA), malt extract agar (MEA) and Czapeck yeast extract agar with 20% sucrose (CY20S) were used. Morphology was observed 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 [7].

Assay for Miconazole-potentiating Activity

The miconazole-potentiating activity of tensidols was tested by paper disk method previously reported by Arai *et al.* [2]. *C. albicans* ATCC 64548 was used as a test organism.

Antimicrobial Activity

Antimicrobial activity against 14 species of microorganisms was measured by our established method [4] using *Bacillus subtilis* PCI 219, *Staphylococcus aureus* FDA 209P, *Micrococcus luteus* PCI 1001, *Mycobacterium smegmatis* ATCC 607, *Escherichia coli* NIHJ, *Pseudomonas aeruginosa* P-3, *Xanthomonas campestris* pv. *Oryzae* KB 88, *Bacteroides fragilis* ATCC 23745, *Acholeplasma laidlawii* PG 8, *Pyricularia oryzae* KF 180, *Aspergillus niger* ATCC 6275, *Mucor racemosus* IFO 4581, *C. albicans* ATCC 64548 and *Saccharomyces cerevisiae*. The following media were employed: GAM agar (Nissui Seiyaku Co., Tokyo, Japan) for *Bacteroides fragilis*; Bacto PPLO agar (Sanko Junyaku Co. Ltd., Tokyo, Japan) supplemented with 15% horse serum, 0.1% glucose, 0.25% phenol red (5 mg/ml) and 1.5% agar for *A. laidlawii*; Taiyo agar (Shimizu Syokuhin Kaisya Ltd., Shizuoka, Japan) for the other bacteria; a medium composed of 1.0% glucose, 0.5% yeast extract and 0.8% agar for fungi and yeasts. A paper disk (i.d. 6 mm,

Toyo Roshi Kaisha Ltd., Tokyo, Japan) containing 10 μ g of a sample was placed on an agar plate. Bacteria, with the exception of *X. oryzae*, were incubated at 37°C for 24 hours. Yeasts and *X. oryzae* were incubated at 27°C for 24 hours. Fungi were incubated at 27°C for 48 hours. Antimicrobial activity was expressed as diameter (mm) of the inhibitory zone.

Results

Taxonomy of the Producing Strain FKI-2342

Colonies on CYA were 62~65 mm diameter after 7 days at 25°C, velutinous to farinaceous, ebony (2 po) to dark brown (3 pn) in color. The reverse side was pale yellow (1 ca) to pale lemon yellow (2 ea). Colonies on MEA were 42~45 mm diameter, velutinous to farinaceous, ebony (2 po) to dark brown (3 pn) in color. The reverse side was pale yellow (1 ca) to olive gray (1 ig). Colonies on CY20S were 70~72 mm diameter, velutinous to farinaceous, ebony (2 po to 3 po) in color. The reverse side was pale yellow (1 ca) to pale lemon yellow (2 ea). Conidiogenesis on each medium was abundant. This strain was able to grow on CYA at 37°C, 65 mm diameter after 7 days. At 5°C, no colonies were formed on CYA. Conidial structures were consisted of conidiophores, vesicles with biseriate aspergilla. Conidiophores were arised from foot cells and 1000~2000 μ m long. They were smooth-walled and hyaline to slightly yellowish brown. Vesicles were globose and 32~50 μ m in diameter. Biseriate aspergilla were formed metulae and phialides; metulae were covering the entire surface of the vesicle, cylindrical and 7~9 \times 3.5~5 μ m in size with 2 to 4 phialides, phialides were ampulliformed and 5.5~8 \times 3~4 μ m in size, producing long chains of conida. Conidia were globose to subglobose with finely roughened-wall and 3~4 \times 3~4 (*av.* 3.4 \times 3.7) μ m in size (Fig. 2). This strain can grow at the temperature range from 14 to 43.5°C and has the optimum growth range at 24 to 41°C.

From the above characteristics, strain FKI-2342 was

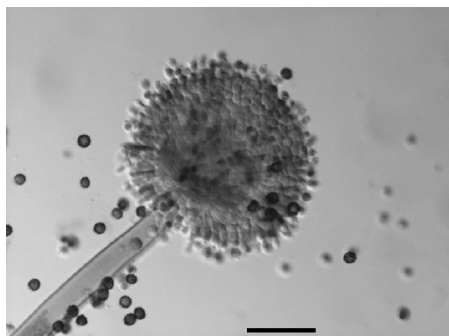


Fig. 2 *Aspergillus niger* FKI-2342 on CYA.
Scale bar: 20 μ m.

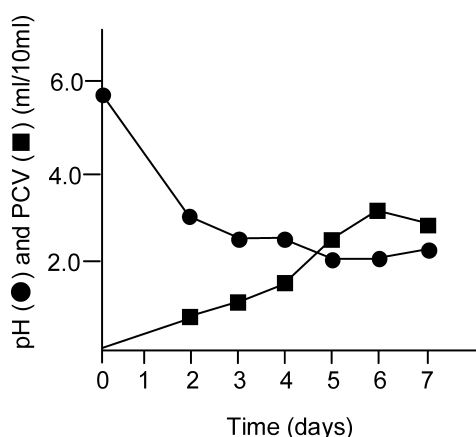


Fig. 3 A typical time course of PCV (■) and pH (●) in culture broths by *A. niger* FKI-2342.

identified as the genus *Aspergillus niger*.

Fermentation

A slant culture of the strain FKI-2342 grown on LcA medium (glycerol 0.1%, KH_2PO_4 0.08%, K_2HPO_4 0.02%, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.02%, KCl 0.02%, NaNO_3 0.2%, yeast extract 0.02% and agar 1.5%, pH 6.0) was used to inoculate 100 ml of the seed medium (glucose 2.0%, yeast extract 0.2%, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.05%, polypepton 0.5%, KH_2PO_4 0.1% and agar 0.1%, pH 6.0) contained in a 500-ml Erlenmeyer flask. The flask was shaken on a rotary shaker at 27°C for 3 days. One ml of the seed culture was incubated with 100 ml of the production medium (glycerol 3.0%, oat meal 2.0%, dry yeast 1.0%, KH_2PO_4 1.0%, Na_2PO_4 1.0%, $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ 0.5%) contained in a 500-ml Erlenmeyer flask. The fermentation was carried out at 27°C for 7 days. A typical time course of the fermentation is shown in Fig. 3. The pH value changed from 5.8 to 2.0 on day 5 and PCV was increased gradually up to day 6.

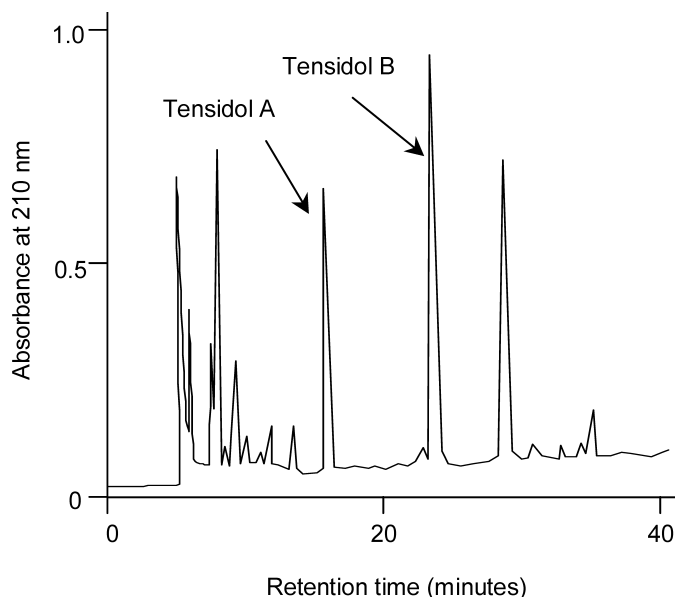


Fig. 4 A chromatographic profile of purification of tensidols A and B by preparative HPLC.

Column, Senshu Pak PEGASIL ODS (20 \times 250 mm); solvent, 40-minute linear gradient from 30 to 50% CH_3CN in 0.05% H_3PO_4 ; detection, UV at 210 nm; flow rate, 8.0 ml/minute; sample, 10 μ g of active materials dissolved in 10 μ l of MeOH.

Isolation

To the 7-day old culture broth (1.4 liter) was added acetone (1.4 liter). After the acetone extracts were filtered and concentrated, the resulting aqueous solution was extracted with ethyl acetate (1.4 liter). The ethyl acetate layer was dried over Na_2SO_4 and concentrated *in vacuo* to dryness to yield an oily material (1.33 g). The material was dissolved in a small volume of CHCl_3 , applied on a silica gel column (50 g, 3.0 \times 15 cm, 70~230 mesh, Merck), and eluted stepwise with 100:0, 100:1, 50:1, 10:1 and 0:100 (v/v) of CHCl_3 - CH_3OH (500 ml each). The miconazole-potentiating activity was observed in the fraction, eluted with the 50:1 solvent mixture, which was concentrated to give a brown material (79.1 mg). The material was purified by HPLC; ODS column (20 \times 250 mm, Pegasil, Senshu Sci. Co. Tokyo, Japan), a 40-minutes linear gradient from 30 to 50% CH_3CN in 0.05% H_3PO_4 , 8.0 ml/minute, and UV at 210 nm. Under the conditions, tensidols A and B were eluted as peaks with retention times of 16.0 and 23.5 minutes, respectively (Fig. 4). Each fraction was collected and extracted with ethyl acetate to yield tensidols A (1.2 mg) and B (2.6 mg) as yellow powder.

Table 1 Physico-chemical properties of tensidols A and B

	Tensidol A	Tensidol B
Appearance	yellow powder	yellow powder
$[\alpha]_D^{25}$	+27.6 (c 0.1, CH ₃ OH)	+3.7 (c 0.1, CH ₃ OH)
Molecular formula	C ₁₃ H ₁₁ NO ₃	C ₁₈ H ₁₇ NO ₆
Molecular weight	229	343
HR-FAB-MS m/z (M+H) ⁺		
Calcd	230.0817 (for C ₁₃ H ₁₂ NO ₃)	344.1134 (for C ₁₈ H ₁₈ NO ₆)
Found	230.0813	344.1056
UV $\lambda_{\max}^{\text{CH}_3\text{OH}}$ nm (ϵ)	206 (13,700), 250 (4,100)	206 (25,000), 243 (11,000)
IR ν_{\max}^{KBr} cm ⁻¹	3360, 1683, 1668	3530, 3360, 1697, 1683, 1670
Solubility		
Soluble	CH ₃ OH, CHCl ₃ , EtOAc	CH ₃ OH, CHCl ₃ , EtOAc
Insoluble	H ₂ O	H ₂ O

Structure Elucidation

Physico-chemical Properties of Tensidols

Physico-chemical properties of tensidols A and B are summarized in Table 1. Tensidols A and B showed similar absorption at 206 and 243~250 nm. Tensidol A showed absorption at 3360 cm⁻¹, suggesting the presence of hydroxyl groups. Tensidol B showed the absorption at 3530 and 3360 cm⁻¹, suggesting the presence of carboxylic acid and hydroxyl groups. Similarity of their spectral data indicated close structural relationships.

Tensidol A

The molecular formula of tensidol A was determined to be C₁₃H₁₁NO₃ on the basis of HRFAB-MS measurement (Table 1). The ¹³C NMR spectrum (in CDCl₃) showed 13 resolved signals, which were classified into one methylene carbon, seven *sp*² methine carbons and three quaternary carbons by analysis of DEPT spectra. The ¹H NMR spectrum (in CDCl₃) showed one methylene signal, two methine signals and five aromatic signals. The connectivity of proton and carbon atoms was established by the ¹³C-¹H HMQC spectrum as shown in Table 2. Analysis of the ¹H-¹H COSY and ¹³C-¹H HMBC spectra revealed the two partial structures I and II (Fig. 5). The ¹³C-¹H long range couplings of ²J and ³J observed in the HMBC experiments (Fig. 5) gave the following information. The cross peaks from 7-H₂ (δ 3.90) to C-8 (δ 133.7) and C-9 (δ 129.1) and from 9-H (δ 7.25) to C-7 (δ 39.6) supported the partial structure I. The cross peaks from 2-H (δ 8.75) to C-3 (δ 178.0), C-3a (δ 119.1) and C-6a (δ 168.8) and from 5-H (δ 6.25) to C-3a, C-4 (δ 164.3) and C-6a supported the partial structure II. The cross peaks from 5-H to C-7 and

from 7-H to C-6a and C-5 indicated that the partial structures I and II are linked at a nitrogen as shown in Fig. 5. The structure satisfied the molecular formula and degree of unsaturated.

Taken together, the structure of tensidol A was elucidated as shown in Fig. 1.

Tensidol B

The molecular formula of tensidol B (C₁₈H₁₇NO₆) is larger by C₅H₆O₃ when compared with tensidol A. The difference between tensidols A and B is the presence of the partial structure III for tensidol B (Fig. 3), which was elucidated by the ¹H-¹H COSY and HMBC spectra. The cross peaks from 2'-H₂ (δ 2.92, 3.28) to C-1' (δ 172.8), C-3' (δ 34.4), C-4' (δ 177.7) and C-5' (δ 17.3), from 3'-H (δ 3.07) to C-1', C-2', C-4' and C-5' and from 5'-H (δ 1.30) to C-2', C-3' and C-4' were observed in the ¹³C-¹H HMBC experiments to confirm the partial structure III (Fig. 6). Because the chemical shift of C-4 (δ 161.2) moved to the higher field in comparison with that of tensidol A (δ 164.3), the partial structure III is linked to C-4 *via* an oxygen as shown in Fig. 1. The structure satisfied the molecular formula and the unsaturated degree (Table 1). Thus, the structure of tensidol B was elucidated as shown in Fig. 1.

Biological Properties

Miconazole-potentiating Activity

The miconazole-potentiating activity of tensidols was tested. Tensidols A and B showed no inhibition zone against wild *C. albicans* even at 50 μ g/disk on Plate A (GY agar). However, tensidols A and B gave dose-dependent

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

	Tensidol A		Tensidol B	
	^{13}C chemical shifts (ppm) ^a	^1H chemical shifts (ppm) ^b	^{13}C chemical shifts (ppm) ^a	^1H chemical shifts (ppm) ^b
C-2	162.0	8.75 (1H, s)	163.1	8.74 (1H, s)
C-3	178.0		177.7	
C-3a	119.1		119.8	
C-4	164.3		161.2	
C-5	116.0	6.25 (1H, s)	116.2	6.30 (1H, s)
C-6a	168.8		169.3	
C-7	39.6	3.90 (2H, s)	39.6	3.90 (2H, s)
C-8	133.7		133.6	
C-9	129.1	7.29 (2H, m)	129.1	7.36 (2H, m)
C-10	127.8	7.25 (2H, m)	128.0	7.24 (2H, m)
C-11	129.1	7.29 (1H, m)	129.1	7.36 (1H, m)
C-1'			172.8	
C-2'			41.8	2.92 (1H, dd, $J=8.5, 5.0$) 3.28 (1H, dd, $J=8.5, 5.0$)
C-3'			34.4	3.07 (1H, m)
C-4'			177.7	
C-5'			17.3	1.30 (3H, d, $J=7.5$)
3-OH		5.90 (1H, br s) ^c		11.79 (1H, s)
4-OH		9.05 (1H, br s) ^c		

^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.

^c The assignments may be exchangeable.

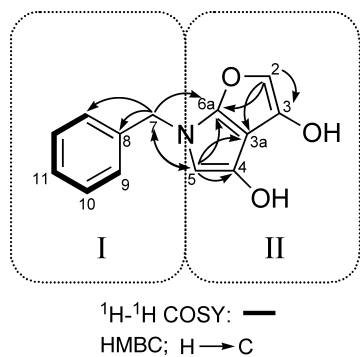


Fig. 5 Partial structures I and II and key cross peaks observed in ^1H - ^1H COSY and HMBC experiments of tensidol A.

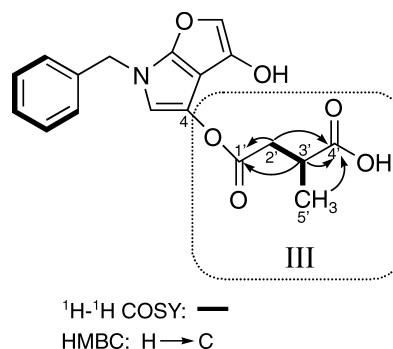


Fig. 6 Partial structure III and key cross peaks observed in ^1H - ^1H COSY and HMBC experiments of tensidol B.

inhibition zones on Plate B (Plate A containing miconazole ($0.06 \mu\text{M}$)) (Table 3). These results indicated that tensidols A and B potentiate miconazole activity against *C. albicans*.

Antimicrobial Activity

Tensidols A and B showed weak antifungal activity against *P. oryzae* (inhibition zone at a concentration $10 \mu\text{g}/6 \text{ mm}$ disk: 13 and 13 mm, respectively), but no activity against the other microorganisms: *Bacillus subtilis*, *S. aureus*, *M.*

Table 3 Potentiation of miconazole activity against *C. albicans* by tensidols

Tensidol	Concentration	Inhibition zone (mm)	
	$\mu\text{g}/\text{disk}$	Plate A	Plate B
A	50	—	21
	25	—	19
	10	—	14
B	50	—	15
	25	—	12
	10	—	—

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. The concentration (0.06 μM) of miconazole is one fourth of the MIC value against *C. albicans* that showed no effect on the growth of *C. albicans*.

luteus, *M. smegmatis*, *E. coli*, *P. aeruginosa*, *X. campestris*, *Bacteroides fragilis*, *A. laidlawii*, *A. niger*, *M. racemosus*, *C. albicans* and *S. cerevisiae*.

Discussion

As described in this study, tensidols A and B are new potentiators of miconazole activity against *C. albicans*. The potentiating activity of tensidol A is more potent than that of tensidol B, suggesting that the 2-methyl butanoic acid moiety at the hydroxy group interferes with the activity. Tensidols A and B did not potentiate miconazole activity against fluconazole-resistant *C. albicans* (data not shown). It suggests that tensidols act on the potentiating mechanism other than the efflux pumps, but we need more investigation to define this point. We have reported several miconazole-potentiators such as actofunicone, beauvericins, phenatic acids and citridones. When compared with their potency, tensidols are rather weak potentiators of antifungal miconazole activity.

Tensidols have the unique 6a-dihydro-3aH-furo[2,3-b]pyrrole skeleton in common, which has not been reported so far. The chemical shifts of C-2 (162.0 and 163.1 ppm) and C-3 (178.0 and 177.7 ppm) of furan ring in tensidols A and B are much lower than those of usual furan rings (about 110~140 ppm), concluding the presence of a 3-hydroxyl

furan ring. Regarding the structure of tensidol B, there are four possible ester forms; 3-OH or 4-OH of the furopyrrol ring is bound to 1' or 4' carboxylic acid of methylsuccinic acid. We concluded that 4-OH is linked to 1'-carboxylic acid because the chemical shift of C-4 (161.2 ppm) moved to a higher field in comparison with that (164.3 ppm) of tensidol A, and the chemical shift of an ester carbonyl carbon (C-1', 172.8 ppm) should be in a higher field than that of a hydroxyl carbonyl carbon (C-4', 177.7 ppm). Further experiments, e.g., X-ray crystallography or total synthesis, are needed to corroborate this assessment.

Acknowledgments We wish to thank Ms. Noriko Sato, Ms. Akiko Nakagawa and Ms. Chikako Sakabe, School of Pharmacy, Kitasato University, for measurements of NMR and mass spectra. This study was supported by the 21st Century COE Program and KAKENHI 16073215, Ministry of Education, Culture, Sports, Science and Technology, Japan.

References

- Nishiyama Y, Yamaguchi H. Mode of action of antifungal agents for treatment of systematic fungal infection. *Antibiot Chemother* 16: 19–26 (2000)
- Arai M, Tomoda H, Okuda T, Wang H, Tabata N, Masuma R, Yamaguchi Y, Ōmura S. Funicone-related compounds, potentiators of antifungal miconazole activity, produced by *Talaromyces flavus* FKI-0067. *J Antibiot* 55: 172–180 (2002)
- Fukuda T, Arai M, Yamaguchi Y, Masuma R, Tomoda H, Ōmura S. New beauvericins, potentiators of antifungal miconazole activity, produced by *Beauveria* sp. FKI-1366. I. Taxonomy, fermentation, isolation, and biological properties. *J Antibiot* 57: 110–116 (2004)
- Fukuda T, Mathumoto A, Takahashi Y, Tomoda H, Ōmura S. Phenatic acids A and B, new potentiators of antifungal miconazole activity produced by *Streptomyces* sp. K03-0132. *J Antibiot* 58: 252–259 (2005)
- Fukuda T, Yamaguchi Y, Masuma R, Tomoda H, Ōmura S. Citridones, potentiators of antifungal miconazole activity, produced by *Penicillium* sp. FKI-1938 I. Taxonomy, fermentation, isolation, and biological properties. *J Antibiot* 58: 309–314 (2005)
- Klich AM. Identification of Common *Aspergillus* Species. Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands (2002)
- Taylor HD, Knoche L, Granville WC. Color Harmony Manual 4th Ed. Container of America, Chicago (1958)