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Funicone-related Compounds, Potentiators of Antifungal Miconazole Activity, Produced by *Talaromyces flavus* FKI-0076

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Talaromyces flavus FKI-0076, a soil isolate, was found to produce compounds which reinforce the anti-*Candida albicans* activity of miconazole. Four structurally related compounds, a novel one, designated actofunicone, and the knowns deoxyfunicone, vermistatin and NG-012, were isolated from the culture broth by solvent extraction, ODS column chromatography and HPLC. The structure of actofunicone was elucidated as benzoic acid, 3,5-dimethoxy-2-[[4-oxo-6-(2-acetyloxy propyl)-4*H*-pyran-3-yl]carbonyl]-, methyl ester by various spectroscopic analyses including NMR experiments. These compounds potentiated the anti-*C. albicans* activity of miconazole, decreasing the IC_{50} value of miconazole from 19 μM to 1.6~3.7 μM in the presence of the funicones.

Opportunistic infections caused by certain fungi, especially problematic *Candida albicans*, have increased recently and become a public concern. 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]. Azole derivatives, which inhibit fungal ergosterol biosynthesis by blockade of the cytochrome P-450 reaction involved in 14-α demethylation, are the most commonly used agents. However, new antifungal agents of a different mechanism of action have been sought extensively.

A concept of "anti-infective drugs"[2] includes not only compounds which inhibit the growth of pathogenic microorganisms statically or kill such microorganisms (so called chemotherapeutics) and vaccines but also compounds which control microbial adaptation/survival or pathogenecity, potentiate the activities of known antibiotics, or enhance the host immune system against microbial infection. For example, β-lactamase inhibitors such as clavulanic acid, sulbactam and tazobactam themselves showed very weak or no antimicrobial activity, but these compounds dramatically potentiated the antimicrobial activity of β-lactam antibiotics against β-lactamase producing bacteria[3].

HAZEL *et al.*[4] reported that two homologous genes PDR16 and PDR17 of yeast involved in multiple drug resistance affected lipid biosynthesis and that deletion of PDR16 resulted in hypersensitivity of yeast to azole derivatives. Other papers described that resistance to azole derivatives in *C. albicans* can be mediated by multidrug efflux transporters[5,6]. On the basis of these findings, an assay system was conducted to screen for compounds, which reinforce the antifungal activity of an azole compound, as a potential approach leading to development of anti-infective drugs. With the convenient assay described below, four funicone-related compounds (Fig. 1) were isolated from the culture broth of fungal strain FKI-0076. One was found to be new, designated actofunicone, and the other three were identified as deoxyfunicone[7], vermistatin[8] and NG-012 (BK-223A)[9~11], which were previously isolated from *Penicillium* spp. as a plant growth stimulator, an antibiotic and a potentiator of nerve growth factor, respectively.

In this paper, we describe the taxonomy of the producing fungus, fermentation, isolation, structure elucidation and

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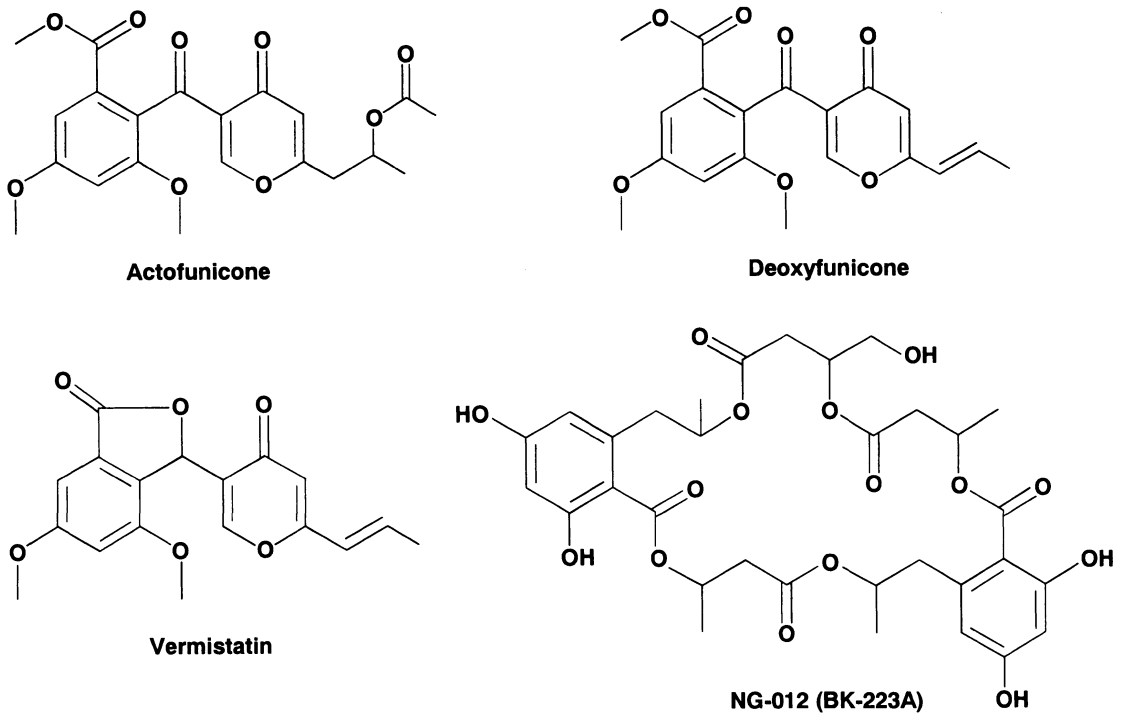
Fig. 1. Structures of actofunicone, deoxyfunicone, vermistatin and NG-012 (BK-223A).



miconazole-reinforcing activity of these funicones.

Materials and Methods

General Experimental Procedures

Strain FKI-0076 was isolated from a soil sample collected at Hiroo, Shibuya-ku, Tokyo, JAPAN and was used for production of four funicone-related compounds. *Candida albicans* KF-1 was purchased from ATCC. HPLC was carried out using a Gulliver system (JASCO). For determination of the amounts of compounds in culture broths, samples (ethyl acetate extracts) were dissolved in methanol and analyzed by HPLC under the following conditions; Symmetry C18/3.5 μm column (2.1×150 mm, Waters), a 20-minute linear gradient from 30% CH_3CN/0.05% H_3PO_4 to 70% CH_3CN/0.05% H_3PO_4, 0.2 ml/minute, and UV at 254 nm. Active compounds, actofunicone, deoxyfunicone, vermistatin and NG-012, were eluted as peaks with retention times of 9.0, 10.1, 10.4 and 10.9 minutes, respectively.

UV spectra were recorded on a Shimadzu UV-200S spectrophotometer. IR spectra were recorded on a Horiba FT-210 infrared spectrometer. Optical rotations were obtained with a JASCO DIP-370 digital polarimeter. EI-MS spectra were recorded on a JEOL JMS-D 100 mass spectrometer at 20 eV. FAB-MS spectra were recorded on a JMS-DX300 mass spectrometer. The various NMR spectra were obtained on a Varian XL-400 spectrometer.

Taxonomic Studies of the Producing Organism

Taxonomic studies were conducted according to the procedures described by PITT.[12] Morphological observations were done under a microscope (Olympus Vanox-S AH-2) and a scanning electron microscope (JEOL JSM-5600). Color Harmony Manual, 4th Ed., 1958 (Container Corporation of America, Chicago) was used for color names and hue numbers[13].

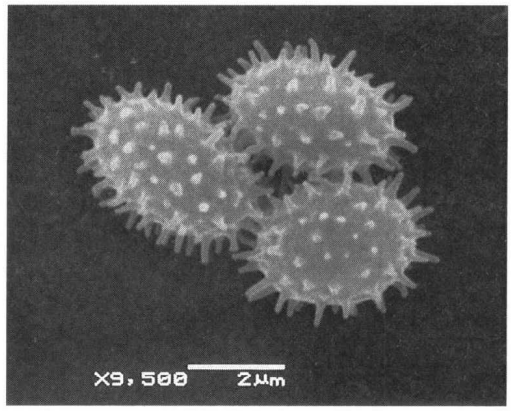
Assay for Miconazole-reinforcing Activity

C. albicans KF-1 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 (0.24 μM) (Plate B). The concentration (0.24 μM) of

miconazole showed no effect on the growth of *C. albicans*. Paper disks (8 mm, ADVANTEC) containing a sample were put on Plates A and B, which were incubated at 27°C for 24 hours. A sample which showed inhibition zone on Plate B and no inhibition on Plate A was selected as a potentiator of anti-*C. albicans* activity of miconazole.

In Method B, *C. albicans* was grown in 96-well microplates (Corning). The seed culture of *C. albicans* (0.1%, v/v) was added to GY agar containing serial concentrations of miconazole (0~2.4 mM) in the absence or presence of a sample (50 μM) in a total volume of 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). The IC_{50} values of miconazole against *C. albicans* in the absence or presence of a sample (50 μM) were calculated.

Fig. 2. Scanning electron micrograph of *Talaromyces flavus* FKI-0076.



Bar represents 2 μm.

Results

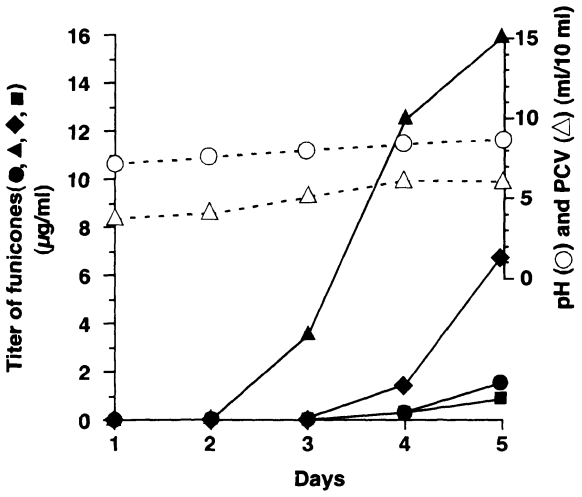
Taxonomy of the Producing Organism

For the taxonomic studies of the fungus, Czapek yeast extract agar (CYA), malt extract agar (MEA), 25% glycerol nitrate agar (G25N) and potato dextrose agar (PDA) were used. Colonies on CYA after 7 days at 25°C were 40 μm in size, floccose to velutinous and light yellow (1 1/2 ea) in color. Reverse of the colonies was brite yellow (1 1/2 la) to topaz (3ne). Ascomata developed abundantly but conidiogenesis was sparse. Colonies on MEA after 7 days at 25°C were 25 mm in diameter, floccose to velutinous and pale yellow (1 ca) to light lemon yellow (1 ga) in color. Reverse of the colonies was light yellow to orange brown. Ascomata were similar to those on CYA. Colonies grown on G25N were very restricted with conidiogenesis and dusty green (24 ge) in color, but without ascomata production. Colonies on CYA after 7 days at 37°C were 40 μm in diameter. Ascomata were not produced and conidiogenesis was sparse. No colonies were formed at 5°C on CYA. Ascomata were globose and 300~800 μm in diameter. Ascocarpic initials consisted of long vermiform ascogonia, 100~220 μm in length and 4.5~6.5 μm in diameter, with slender entwining antheridia. Asci were 8-spored, evanescent, subglobose, 7.5~11 μm in diameter. Ascospores were yellowish, ellipsoidial, spinulose, 3.5~4.5×2.5~3.5 μm in size as shown in Fig. 2. Conidiophores born from the substratum (180~250 μm in length) and aerial hyphae (50~120 μm in length), which were smooth-walled. Penicilli were mono- or bi-verticillate; metulae were 12.5~17.5 μm in length, in verticils of 2~4; phialides were 3~6 per metula, acerose, 11~15×1.8~3 μm in size; conidia were subglobose to ellipsoidal and 2~3×1.5~2.5 μm in size with smooth walls. From the above characteristics, the strain FKI-0076 was identified as *Talaromyces flavus*[12,14]. This strain was deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology Japan, as FERM BP-7037.

Fermentation

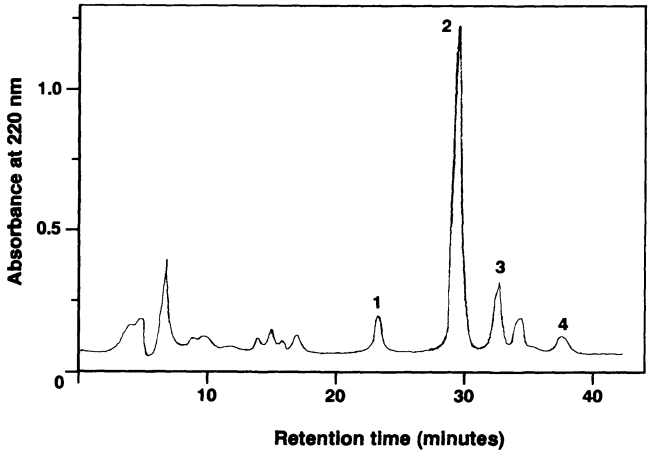
A slant culture of the strain FKI-0076 grown on LcA medium (glycerol 0.1%, KH_2PO_4 0.08%, K_2HPO_4 0.02%, $MgSO_4 \cdot 7H_2O$ 0.02%, KCl 0.02%, $NaNO_3$ 0.2%, yeast extract 0.02% and agar 1.5%, pH 6.0) was used to inoculate a 500-ml Erlenmeyer flask containing 100 ml of the seed medium (glucose 2.0%, 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 (200 ml) was transferred into a 30-liter jar fermenter (Mitsuwa Biosystem) containing 20 liters of the production medium (potato dextrose broth 2.4%, malt extract 0.5%, $Mg_3(PO_4)_2 \cdot 8H_2O$ 0.5% and agar 0.1%, pH 6.0). The fermentation was carried out at 27°C for 5 days with an aeration of 5 liters/minute and an agitation of 200 rpm. A typical time course of the fermentation is shown in Fig. 3. Deoxyfunicone was detected in the culture broth from day 2 after inoculation, and others were produced from day 3. The concentrations of actofunicone, deoxyfunicone, vermistatin and NG-012 at day 5 reached

Fig. 3. A typical time course of production of actofunicone and related compounds by *T. flavus* FKI-0076.



The amounts of actofunicone (●), deoxyfunicone (▲), vermistatin (◆) and NG-012 (■) in culture broths were determined by HPLC as described in Materials and Methods. PCV, packed cell volume (ml) from 10 ml of the whole culture broth after centrifugation at 3000 rpm.

Fig. 4. A chromatographic profile of purification of funicone-related compounds by preparative HPLC.



Sample, 2.0 mg of active materials (obtained through MPLC) dissolved in 500 μl of MeOH. Peak 1, actofunicone; 2, deoxyfunicone; 3, vermistatin; 4, NG-012.

levels of 1.5, 15.9, 6.7 and 1.0 μg/ml, respectively.

Isolation

The 5-day old culture broth (20 liters) was centrifuged to separate mycelium and supernatant. The mycelium was extracted with 10 liters of acetone. After the acetone extracts were filtered and concentrated, the resulting aqueous solution was extracted with 10 liters of ethyl acetate. The extract was dried over Na_2SO_4 and concentrated *in vacuo* to dryness to yield 10.2 g of oily material. The material was dissolved in a small volume of MeOH and subjected to a medium pressure liquid chromatography (MPLC) as follows; ODS column (30×300 mm, Pegasil, Senshu Sci. Co.), a linear gradient from 30% CH_3CN to 80% CH_3CN, 10 ml/minute and 13 ml/fraction×130 fractions. The reinforcing activity was observed in the fractions from 40 to 70 (1.77 g). Furthermore, the active principles from MPLC were purified by HPLC; ODS column (20×250 mm, Pegasil, Senshu Sci. Co.), 40% acetonitrile, 8.0 ml/minute, and UV at 220 nm. Under the conditions, actofunicone, deoxyfunicone, vermistatin and NG-012 were eluted as peaks with retention times of 24, 30, 34 and 38 minutes, respectively (Fig. 4). Each peak was collected and concentrated to yield actofunicone (17 mg), deoxyfunicone (265.5 mg), vermistatin (33.8 mg) and NG-012 (10.0 mg).

Structure Elucidation of Actofunicone

Physico-chemical properties of actofunicone are summarized in Table 1, where those of other funicones isolated from fungus FKI-0076 are also shown for comparative purpose. Similarity in their data indicates that they are structurally related. Absorption at 1735, 1724, 1683 and 1658 cm^{-1} in the IR spectrum suggested the presence of carbonyl groups[15] in the structure.

The molecular formula of actofunicone was determined as $C_{21}H_{22}O_9$ on the basis of HRFAB-MS measurement. The ^{13}C NMR spectrum (CD_3OD) of actofunicone showed 21 resolved peaks (Table 2), which were classified into two methyl, three *O*-methyl, one methylene, one *O*-methine, four sp^2 methine, six sp^2 quaternary and four carbonyl carbons by analysis of the DEPT spectra. The ^1H NMR spectrum (data not shown) displayed 22 proton signals. The connectivity of proton and carbon atoms (Table 2) was established by the HMQC spectrum. From the ^1H-^1H

Table 1. Physico-chemical properties of actofunicone, deoxyfunicone, vermistatin and NG-012.

	Actofunicone	Deoxyfunicone	Vermistatin	NG-012
Appearance	Pale yellow oil	Pale yellow powder	White powder	White powder
Molecular formula	$C_{21}H_{22}O_9$	$C_{19}H_{18}O_7$	$C_{18}H_{16}O_6$	$C_{32}H_{38}O_{15}$
Molecular weight	418	358	328	662
FAB-MS (*m/z*)				
Positive	419 $[M+H]^+$	359 $[M+H]^+$	329 $[M+H]^+$	663 $[M+H]^+$
	441 $[M+Na]^+$	381 $[M+Na]^+$	351 $[M+Na]^+$	685 $[M+Na]^+$
Negative		357 $[M-H]^-$	327 [M-H]	661 $[M-H]^-$
HRFAB-MS (*m/z*)				
	$C_{21}H_{22}O_9Na$ $[M+Na]^+$	$C_{19}H_{18}O_7Na$ $[M+Na]^+$	$C_{18}H_{16}O_6Na$ $[M+Na]^+$	$C_{32}H_{38}O_{15}Na$ $[M+Na]^+$
Calcd:	441.1162	381.0950	351.0845	685.2108
Found:	441.1173	381.0951	351.0853	685.2114
$[\alpha]_D^{24}$	+5.2 °	-5.6 °	-92.0 °	-17.0 °
	(*c* 0.23, MeOH)	(*c* 0.54, MeOH)	(*c* 0.1, MeOH)	(*c* 0.51, MeOH)
UV λ_{max}^{MeOH} nm (e)	208 (34,900)	206 (33,800)	210 (46,000)	216 (44,400)
	246 (11,000)	218 (sh)	262 (18,500)	264 (22,400)
	316 (3,950)	248 (29,600)	302 (10,100)	
		315 (sh)		
		361 (sh)		
IR ν_{max}^{KBr} (cm^{-1})	3410, 1735,	3430, 1716,	1768, 1653,	3410, 1732,
	1724, 1683,	1682, 1645,	1614, 1504,	1649, 1620
	1658, 1602	1604, 1556	1591	
	1579			
Melting point		127.5-128.5 °C	210.5-211.5 °C	120.0-121.0 °C
Solubility				
Soluble:	CH_3OH, $CHCl_3$,	CH_3OH, $CHCl_3$,	CH_3OH, $CHCl_3$,	CH_3OH, $CHCl_3$,
	Acetone, EtOH	Acetone,	Acetone, DMSO	Acetone,
	Ethyl acetate	Ethyl acetate	Ethyl acetate	Ethyl acetate
Insoluble:	H_2O, *n*-Hexane	H_2O, *n*-Hexane	H_2O, *n*-Hexane	H_2O, *n*-Hexane

COSY spectrum, one partial structure was determined (Fig. 5). ^{13}C-^1H long range couplings of ^2J and ^3J observed in the HMBC experiment (Fig. 6) gave evidence as follows; 1) The long range couplings from H-4 (δ 6.80) to C-2 (δ 125.64), C-3 (δ 159.50), C-5 (δ 163.09) and C-6 (δ 107.24), from H-6 (δ 7.06) to C-1 (δ 133.27), C-2, C-4 (δ 103.48), C-5 and C-18 (δ 168.01), from H_3-19 (δ 3.76) to C-18, from H_3-20 (δ 3.75) to C-3, and from H_3-21 (δ 3.88) to C-5 showed the presence of a benzoic acid 3,5-dimethoxy methyl ester moiety. 2) The cross peaks from H-8 (δ 8.52) to C-7 (δ 192.84), C-9 (δ 127.68), C-10 (δ 177.69) and C-12 (δ 167.77), and from H-11 (δ 6.29) to C-9, C-10 and C-12 showed the presence of the γ-pyran moiety. 3) The long-range couplings from H-11 to C-13 (δ 40.44), from H_2-13 (δ 2.84, 2.89) to C-11 (δ 118.62), C-12, C-14 (δ 69.13) and C-15 (δ 20.09), from H-14 (δ 5.25) to C-12, and C-16 (δ 171.95), from H_3-15 (δ 1.33) to C-13 and C-14, and from H_3-17 (δ 1.99) to C-16 indicated that the 14-*O*-acetoxy propyl residue is attached to the γ-pyran moiety. 4) The long-range couplings of ^4J from H-4 and H-11 to C-7 showed that the benzoic acid 3,5-dimethoxy methyl ester moiety is attached to the γ-pyran moiety.

Taken together, the structure of actofunicone was elucidated to be benzoic acid, 3,5-dimethoxy-2-[[4-oxo-6-(2-acetyloxy propyl)-4*H*-pyran-3-yl]carbonyl]-, methyl ester as shown in Fig. 1. The NOE experiments (Fig. 6) also supported the structure.

Table 2. NMR chemical shifts of actofunicone.

Carbon No.	^{13}C chemical shifts ppm[a)]	^{1}H chemical shifts ppm[b)]
C-1	133.27	
C-2	125.64	
C-3	159.50	
C-4	103.48	6.80 (1H, d, J =2.0 Hz)
C-5	163.09	
C-6	107.24	7.06 (1H., d, J =2.0 Hz)
C-7	192.84	
C-8	163.49	8.52 (1H, s)
C-9	127.68	
C-10	177.69	
C-11	118.62	6.29 (1H, s)
C-12	167.77	
C-13	40.44	2.84 (1H, dd, J =15.0, 8.0 Hz)
		2.89 (1H, dd, J =15.0, 5.0 Hz)
C-14	69.13	5.25 (1H, ddd, J =8.0, 6.0, 5.0 Hz)
C-15	20.09	1.33 (3H, d, J =6.0 Hz)
C-16	171.95	
C-17	20.91	1.99 (3H, s)
C-18	168.01	
C-19	52.91	3.76 (3H, s)
C-20	56.69	3.75 (3H, s)
C-21	56.26	3.88 (3H, s)

[a)] A sample was dissolved in CD_3OD. Chemical shifts are shown with reference to CD_3OD as 49.8 ppm. [b)] Chemical shifts are shown with reference to CD_3OD as 3.30 ppm.

Fig. 5. A partial structure of actofunicone deduced by COSY experiments.

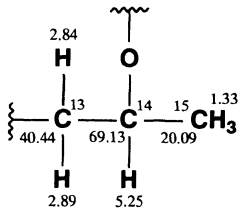


Fig. 6. Structure elucidation of actofunicone by HMBC and NOE experiments.

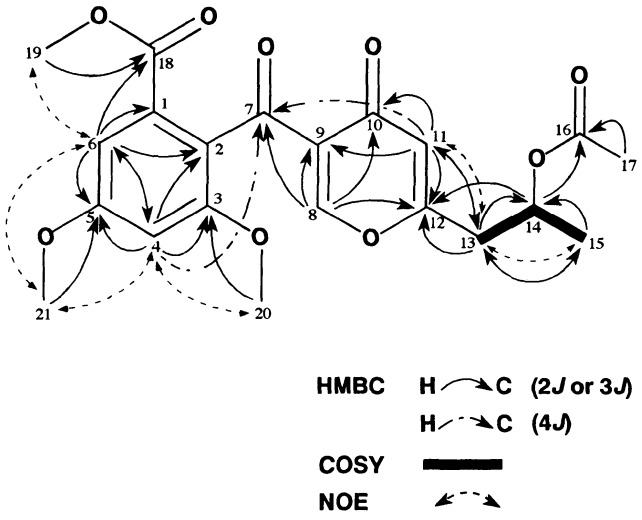
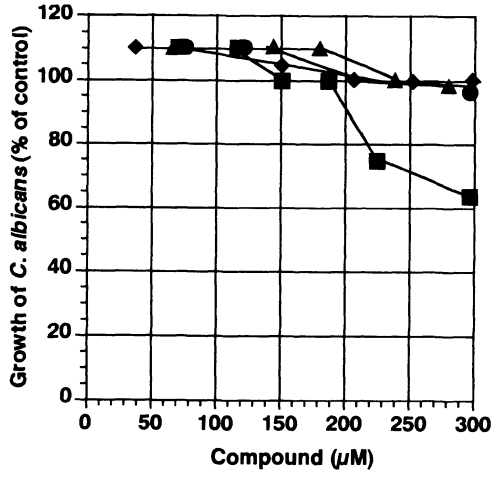


Fig. 7. Effect of funicones on the growth of *C. albicans*.



C. albicans was grown at 27°C for 24 hours in microplates containing GY agar supplemented with various concentrations of actofunicone (●), deoxyfunicone (▲), vermistatin (◆) and NG-012 (■). The growth was measured with a microplate reader as described in Materials and Methods.

Miconazole-reinforcing Activity by Funicones

First, the anti-*C. albicans* activity of the four funicones was investigated. The three compounds, actofunicone, deoxyfunicone and vermistatin showed no effect on the growth of *C. albicans* up to 300 μM, and a slight inhibition (35%) was observed at that concentration for NG-012 (Fig. 7). Then, their miconazole-reinforcing activity was tested by Method A. Actofunicone showed no inhibition against *C. albicans* up to 100 μg/disk on Plate A (Fig. 8 and Table 3), which was comparable to the result of Fig. 7. However, the compound showed a clear inhibition zone (8~15 mm

i.d.) at 10~100 μg/disk on Plate B containing 0.24 μM miconazole, indicating that actofunicone reinforces the anti-*C. albicans* activity of miconazole. Similarly, the other funicones potentiated the miconazole activity against *C. albicans* dose-dependently by Method A (Table 3).

To confirm their activity, the funicones were investigated by Method B. In the absence of the funicones, the IC_{50} value of miconazole against *C. albicans* was calculated to be 19 μM. However, in combination with the funicones (50 μM), the IC_{50} values were decreased to 1.6~3.7 μM (Table 4), demonstrating that they reinforce the anti-*C. albicans* activity of miconazole.

The funicones showed no cytotoxic effect on P388 cells up to 100 μM (data not shown).

Fig. 8. Miconazole-reinforcing activity by actofunicone (Method A).

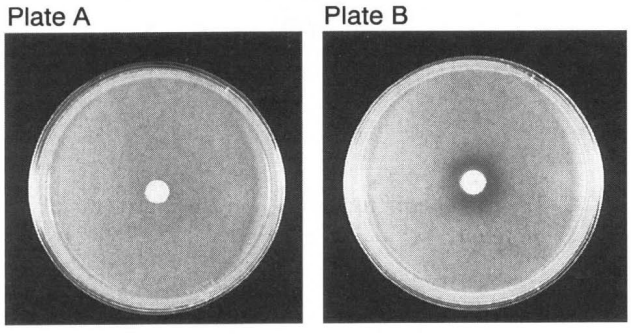


Plate A, GY agar; Plate B, GY agar containing 0.24 μM miconazole. Sample, 100 μg actofunicone/8 mm i.d. disk.

Discussion

As a new approach for development of anti-infective drugs, compounds which reinforce antifungal azole activity were screened from microbial metabolites. The compounds are expected to show no or very weak antifungal activity, and exhibit synergic antifungal activity in the presence of an azole. By establishing a convenient assay system using *C. albicans* and miconazole, four funicone-related compounds including new actofunicone were isolated from the culture broth of *T. flavus* FKI-0076. As we expected, the funicones did not inhibit the growth of *C. albicans* even at 200 μM (Fig. 7) and at 100 μg/disk (Table 3), and if any, it was reported that they showed very weak antifungal activity[7,8,11]. However, the funicones showed dose-dependent anti-*C. albicans* activity in combination with 0.24 μM miconazole in Method A (Fig. 8 and Table 3), although miconazole alone gave no effect on the fungal growth at the concentration. The miconazole-reinforcing activity by the funicones was also comfirmed by Method B, reducing the IC_{50} value of 19.2 μM for miconazole to 1.6~3.7 μM in the presence of the funicones (Table 4). Among them, deoxyfunicone has the most potent activity. The miconazole-reinforcing activity by the funicones was

Table 3. Reinforcement of miconazole anti-*Candida albicans* activity by funicone-related compounds.

Compound	μg /disk	Inhibition zone (mm)	
		Plate A	Plate B
Actofunicone	1	-	-
	10	-	8
	100	-	15
Deoxyfunicone	1	-	9
	10	-	14
	100	-	23
Vermistatin	1	-	-
	10	-	10
	100	-	17
NG-012	1	-	-
	10	-	9
	100	-	15

After incubation of paper disks (8mm i.d.) containing three concentrations of a compound on Plate A (*C. albicans* in GY agar) and Plate B (*C. albicans* in GY agar + 0.24μM miconazole) at 27 °C for 24 h, the diameters of inhibition zones were measured (Method A).

Table 4. Reinforcement of miconazole anti-*Candida albicans* activity by funicone-related compounds.

Addition	IC_{50} of miconazole (μM)	Ratio (control/+ drug)
No (control)	19.2	1.0
+ Actofunicone (50 μM)	3.7	5.2
+ Deoxyfunicone (50 μM)	1.6	11.8
+ Vermistatin (50 μM)	2.1	9.1
+ NG-012 (50 μM)	2.5	7.7

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

observed against *Saccharomyces cerevisiae* (data not shown). As previously reported[16], nikkomycin Z, an inhibitor of fungal chitin synthase, possessed synergistic interaction with fluconazole against *C. albicans*. However, the compound itself showed potent anti-*C. albicans* activity with MIC of 0.5 μg/ml (1.0 μM), whereas funicones showed no anti-*C. albicans* activity up to 200 μM, indicating that the funicones are a different type of potentiators.

Although the mechanism of action of the funicones remains unclear, several possibilities are speculated, namely, they might affect lipid composition of fungal membranes to change the drug permeability[4] or inhibit the azoles efflux transporters[17]. Furthermore, it may be interesting to investigate whether or not the funicones can reinforce the miconazole activity against azole-resistant *C. albicans*.

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