6′-Hydroxy-3′-methoxy-mitorubrin, a New Potentiator of Antifungal Miconazole Activity, Produced by *Penicillium radicum* FKI-3765-2

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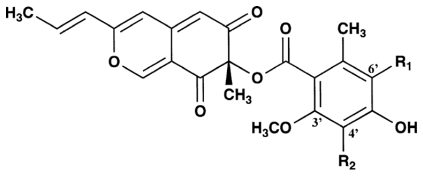
A new mitorubrin congener designated 6′-hydroxy-3′-methoxy-mitorubrin (1) was isolated along with structurally related 4′-hydroxy-3′-methoxy-(*S*)-mitorubrin (2) and monomethyl-(*S*)-mitorubrin (3) from the culture broth of *Penicillium radicum* FKI-3765-2 by solvent extraction, octadecyl silyl (ODS) column chromatography and HPLC. The structure of 1 was elucidated by various spectral analyses, including NMR experiments. They have a common isochromane-like ring with a similar hydrophobic side chain. These compounds moderately potentiated miconazole activity against *Candida albicans*.

Key words 6′-hydroxy-3′-methoxy-mitorubrin; miconazole potentiator; *Candida albicans*; *Penicillium radicum*; fungal metabolite

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-a demethylase (P-450 14DM),[3] and 3) overexpression of P450 14DM.[4] The mechanisms of resistance will indicate new targets of inhibition for overcoming infections by azole-resistant *C. albicans*. On the basis of the new concept of "antiinfective drugs,"[5] we have screened microbial metabolites for potentiators of miconazole activity against *C. albicans*. As a result, we discovered various new compounds: actofunicone,[6] beauvericins,[7] citridones,[8,9] tensidoles[10] and citrinamides[11] from fungi and phenatic acids[12] from an actinomycete. From precise analysis of the metabolites of xanthoradone-producing *Penicillium radicum* FKI-3765-2,[13] a new compound, designated 6′-hydroxy-3′-methoxy-mitorubrin (**1**, Fig. 1), with a core structure different from that of xanthoradones[14] was isolated along with known 4′-hydroxy-3′-methoxy-(*S*)-mitorubrin[15] (**2**) and monomethyl-(*S*)-mitorubrin[16] (**3**). In this study, fermentation, isolation, and structural elucidation, including the absolute stereochemistry and miconazole-potentiating activity, of **1** are described.



	R_1	R_2
6'-Hydroxy-3'-methoxy-mitorubrin (1)	-OH	-H
4'-Hydroxy-3'-methoxy-mitorubrin (2)	-H	-OH
Monomethyl-mitorubrin (3)	-H	-H

Fig. 1. Structures of **1** to **3**

Experimental

Microorganisms *P. radicum* FKI-3765-2, previously reported as a xanthoradone-producing fungus,[13,14] was used for production of **1**. The following seven microorganisms were used for antimicrobial tests: *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* FDA 209P, *Micrococcus luteus* PCI 1001, *Escherichia coli* NIHJ, *Xanthomonas campestris* pv. *Oryzae* KB 88, *Mucor racemosus* IFO 4581 and *C. albicans* KF-1.

General Experimental Procedures SSC-ODS-7515-12 (Senshu Scientific Co., Tokyo, Japan) was used for octadecyl silyl (ODS) column chromatography. HPLC was carried out using the L-6200 system (Hitachi, Ltd., Tokyo, Japan). To determine the amounts of **1** to **3** in the culture broths, samples (ethyl acetate extracts) were dissolved in methanol and analyzed by the HP1100 system (Hewlett-Packard Co., Palo Alto, CA, U.S.A.) under the following conditions: column, Symmetry (2.1×150 mm; Waters Corp., Milford, MA, U.S.A.); flow rate, 0.20 ml/min; mobile phase, a 20-min linear gradient from 30% CH_3CN to 70% CH_3CN containing 0.050% H_3PO_4; detection, UV at 210 nm. Under these conditions, **1** to **3** were eluted with a retention time of 9.94, 10.6 and 12.0 min, respectively.

UV spectra were recorded on a spectrophotometer (8453 UV–Visible spectrophotometer; Agilent Technologies Inc., Santa Clara, CA, U.S.A.). IR spectra were recorded on a Fourier transform infrared spectrometer (FT-710; Horiba, Ltd., Kyoto, Japan). Optical rotations were measured with a digital polarimeter (DIP-1000; JASCO, Tokyo, Japan). FAB-mass spectra were recorded on a mass spectrometer (JMS-DX300; JEOL, Tokyo, Japan), and HR-FAB-mass spectra were recorded on a mass spectrometer (JMS-AX505 HA; JEOL, Tokyo, Japan). Various NMR spectra were measured with a spectrometer (XL-400; Varian, Inc., Palo Alto, CA, U.S.A.).

Fermentation *P. radicum* FKI-3765-2 was fermented according to the method similar to xanthoradone production described previously.[13] Briefly, a slant culture of strain FKI-3765-2 grown on LCA (0.10% glycerol, 0.080% KH_2PO_4, 0.020% K_2HPO_4, 0.020% $MgSO_4 \cdot 7H_2O$, 0.020% KCl, 0.20% $NaNO_3$, 0.020% yeast extract and 1.5% agar, adjusted to pH 6.0 before sterilization) was inoculated into a 50-ml tube containing 10 ml of the seed medium (2.0% glucose, 0.50% polypeptone, 0.050% $MgSO_4 \cdot 7H_2O$, 0.20% yeast extract, 0.10% KH_2PO_4 and 0.10% agar, adjusted to pH 6.0 before sterilization). The tube was shaken reciprocally for 3 d at 27 °C. A 1 ml portion of the seed culture was then inoculated into a 500-ml Erlenmeyer flask (IWAKI, Tokyo, Japan) containing the production medium (50 g Italian rice; Japan Europe Trading Co., Ltd., Tokyo, Japan). The production medium was prepared as follows; Italian rice (50 g) was soaked with water for 2 h, and then collected through a colander. Soaked rice was placed in a 500-ml Elenmeyer flask and sterilized by autoclaving. Fermentation was carried out at 27 °C for 13 d under static conditions.

Isolation of Mitorubrin Congeners The 13-d-old whole culture (1000 g) was extracted with 2.0 l of acetone. After the acetone extracts were filtered and concentrated to remove acetone, the aqueous solution was extracted with ethyl acetate. The extracts were dried over Na_2SO_4 and concentrated *in vacuo* to dryness to yield a red brown material. The material (1.78 g) was dissolved in 30% CH_3CN, applied on an ODS column (100 g), and eluted stepwise with 30%, 50%, 70%, 100% CH_3CN containing 0.050% trifluoroacetic acid (TFA) [200 ml×5 fractions for each solvent]. Active

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fractions from the 3rd to 5th tube of 50% CH_3CN containing **1** to **3** were concentrated *in vacuo* to dryness to give red brown material (120.8 mg). This material was finally purified by preparative HPLC (column, PEGASIL ODS, 20×250 mm; Senshu Scientific Co.; solvent, 35% CH_3CN containing 0.050% TFA; detection, UV at 210 nm; flow rate, 8.0 ml/min). Under these conditions, **1** to **3** were eluted as peaks with retention times of 52.9, 62.5 and 70.4 min, respectively. The fractions were concentrated *in vacuo* to dryness to give pure **1** (5.2 mg), **2** (1.8 mg) and **3** (2.5 mg) as yellow material.

Assay for Miconazole-Potentiating Activity *C. albicans* ATCC64548 was inoculated into a 50-ml test tube containing 10 ml seed medium (potato extract containing 0.50% peptone and 1.0% glucose), and was grown for 24 h on a rotary shaker. The assay for potentiating miconazole activity against *C. albicans* in combination with a sample was carried out by paper disk methods.[6] Briefly, the seed culture of *C. albicans* (0.10%, v/v) was transferred to two different agar plates, GY agar (1.0% glucose, 0.50% yeast extract and 0.80% agar, Plate A) and GY agar containing 0.060 μM miconazole (Plate B). The concentration (0.060 μM) of miconzole had no effect on the growth of *C. albicans*. Paper disks (6 mm i.d.; Toyo Roshi Kaisha Ltd., Tokyo, Japan) containing various concentrations of a sample were placed on Plates A and B, and incubated at 37 °C for 24 h. A sample which showed an inhibition zone on Plate B and no inhibition on Plate A was selected as a potentiator of miconazole activity against *C. albicans*.

Antimicrobial Assays Antimicrobial activity against 6 species of microorganisms was measured by the paper disk method. Media for microorganisms were as follows: Nutrient agar (Sanko Junyaku Co., Ltd., Tokyo, Japan) for the bacteria; a medium composed of 1.0% glucose, 0.50% yeast extract and 0.80% agar for fungi and yeasts. A paper disk containing a sample (10 μg) was placed on an agar plate. Bacteria except *X. oryzae* were incubated at 37 °C for 24 h. Yeasts and *X. oryzae* were incubated at 27 °C for 24 h. Antimicrobial activity of a sample was expressed as the diameter (mm) of the inhibitory zone.

Results and Discussion

Physico-Chemical Properties The physico-chemical properties of **1** are summarized in Table 1. This compound had absorption maxima at 250 nm and 347 nm on UV spectra. The IR absorption at 1450 cm^{-1}, 1625—1712 cm^{-1} and 3426 cm^{-1} suggested the presence of phenyl, carbonyl and hydroxy groups in the structure. These data were similar to those of **2** and **3**,[15,16] suggesting that **1** also has a common isochromane-like ring with a similar hydrophobic side chain.

Structure Elucidation The molecular formula of **1** was determined to be $C_{22}H_{20}O_8$ on the basis of HR-FAB-MS measurement. The ^{13}C-NMR spectrum (in DMSO-d_6) showed 22 resolved signals, which were classified into three methyl carbons, one oxygenated methyl carbon, one oxy-

Table 1. Physico-Chemical Properties of **1** to **3**

	1	**2**	**3**
Appearance	Yellow powder	Yellow powder	Yellow powder
Molecular weight	412	412	396
Molecular formula	$C_{22}H_{20}O_8$	$C_{22}H_{20}O_8$	$C_{22}H_{20}O_7$
HR-FAB-MS (*m*/*z*)			
Calcd:	413.1236 $(M+H)^+$	413.1236 $(M+H)^+$	397.1287 $(M+H)^+$
Found:	413.1233 $(M+H)^+$	413.1243 $(M+H)^+$	397.1288 $(M+H)^+$
UV (MeOH) λ_{max} nm (ε)	250 (21292), 347 (22907)	253 (3459), 348 (2377)	253 (13692), 348 (12844)
$[\alpha]_D^{26}$	191.9° (*c*=0.1, CH_3OH)	203.4° (*c*=0.1, CH_3OH)	190.0° (*c*=0.1, CH_3OH)
IR (KBr) ν_{max} (cm^{-1})	3426, 2931, 1712, 1625, 1540, 1450	3419, 2929, 1708, 1627, 1542, 1448	3442, 2964, 1710, 1625, 1540, 1448

Table 2. ^1H- and ^{13}C-NMR Chemical Shifts of **1** to **3**

Position	**1**		**2**		**3**	
	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
1	154.7	8.25 s	153.5	8.26 s	153.5	8.24 s
2	114.4	—	115.1	—	115.1	—
3	192.6	—	193.1	—	192.9	—
4	84.4	—	85	—	84.4	—
5	191.4	—	192.4	—	193.2	—
6	106.9	5.57 d (*J*=1.0 Hz)	107.8	5.63 d (*J*=1.5 Hz)	107.9	5.64 d (*J*=1.5 Hz)
7	142.7	—	142.8	—	142.9	—
8	108.4	6.57 d (*J*=1.0 Hz)	108.6	6.12s	108.6	6.10 d (*J*=1.5 Hz)
9	154.9	—	155.4	—	155.4	—
10	122.6	6.24 dq (*J*=15.0, 1.5 Hz)	122.4	6.10 dq (*J*=15.0, 2.0 Hz)	122.4	6.01 dq (*J*=15.0, 1.5 Hz)
11	134.9	6.51 dq (*J*=15.0, 7.0 Hz)	135.5	6.51 m	135.4	6.57 m
12	18.3	1.88 dd (*J*=7.0, 1.5 Hz)	18.6	1.94 dd (J= 7.0, 2.0 Hz)	18.6	1.93 dd (*J*=7.0, 1.5 Hz)
13	22.2	1.42 s	22.5	1.63 s	22.4	1.60 s
1′	165.8	—	165.8	—	166.7	—
2′	112.1		115.1		113.2	
3′	150.9	—	146.6	—	160.1	—
4′	98.6	6.35 s	134.2	—	97.0	6.25 s
4′-OH	—	8.54 s				
5′	147.8	—	146.9	—	158.4	—
5′-OH	—	9.67 s	—	9.57 s	—	9.84 s
6′	136.9	—	113.4	6.56 s	109.5	6.25 s
6′-OH	—	7.98 s				
7′	124.4	—	131.5	—	113.2	—
8′	56.6	3.60 s	62.9	3.91 s	56.2	3.78 s
9′	13.0	2.17 s	20.2	2.42 s	20.2	2.44 s

genated sp^3 quaternary carbon, six sp^2 methine carbons, four sp^2 quaternary carbons, four oxygenated sp^2 quaternary carbons, and three carbonyl carbons by analysis of the distortionless enhancement by polarization transfer (DEPT) and heteronuclear single quantum coherence (HSQC) spectra. The ^{1}H-NMR spectrum (in DMSO-d_6) displayed 20 proton signals, six of which were suggested to be two hydroxy protons (δ 7.98, 9.67) and one aromatic oxygenated methyl proton (δ 3.60). These results supported the molecular formula. The connectivity of proton and carbon atoms was established by the ^{13}C–^{1}H HSQC spectrum (Table 2). Analyses of ^{1}H–^{1}H correlation spectroscopy (COSY) revealed the presence of one partial structure I, as shown in Fig. 2a. Furthermore, ^{13}C–^{1}H long-range couplings of ^2J and ^3J observed in the ^{13}C–^{1}H heteronuclear multiple bond correlation (HMBC) spectrum gave the following linkages (Fig. 2b): (1) Cross peaks from H-11 (δ 6.24) to C-9 (δ 154.9), from H-10 (δ 6.57) to C-8 (δ 108.4) and C-9, from H-8 (δ 8.25) to C-7 (δ 142.7), C-9 and C-10 (δ 122.6), from H-1 (δ 8.25) to C-2 (δ 114.4), C-7 and C-9 and chemical shifts of C-1 (δ 154.7) and C-9 (δ 154.9), which correspond to an oxygenated carbon, indicated that a pyran skeleton connects the partial structure I at C-9. Furthermore, a large coupling constant (J=15.0 Hz) between H-10 and H-11 indicated that the geometry of the double bond between C-10 and C-11 has *E* configuration. (2) Cross peaks from H-1 to C-3 (δ 192.6), from H-8 to C-6 (δ 106.9), from H-6 (δ 5.57) to C-4 (δ 84.4), C-7 and C-8, from H_3-13 (δ 1.42) to C-3, C-4 and C-5 (δ 191.4) indicated that **1** has a substructure of a *m*-quinone skeleton connecting the pyran ring. This substructure was also supported by the chemical shifts of two carbonyl carbons (C-3 (δ 192.6) and C-5 (δ 191.4)) deriving from α,β-unsaturated ketones. (3) Cross peaks from H-4′ (δ 6.35) to C-2′ (δ 112.1), C-3′ (δ 150.9), C-5′ (δ 147.8) and C-6′ (δ 136.9), from OH-5′ (δ 9.67) to C-4′ (δ 98.6), C-5′ and C-6′, from OH-6′ (δ 7.98) to C-5′, C-6′ and C-7′ (δ 124.4), from H_3-8′ (δ 3.60) to C-3′ and from H_3-9′ (δ 2.17) to C-6′, C-7′ and C-2′ indicated the presence of a 3′-methoxy-6′-hydroxy-7′-methyl-phenol moiety. Furthermore, the two substructures should be connected *via* an ester bond between C-4 and C-2′ because the chemical shift of C-4 (δ 84.4) corresponded to an oxygenated carbon and long-range coupling of ^4J was observed from H-4′ to C-1′ (δ 165.8) in ^{13}C–^{1}H HMBC experiments. Taken together, the structure of **1** was elucidated as shown in Fig. 1. The structure satisfied the degree of unsaturation and the molecular formula. All spectral data were reasonable when compared with those of **2**[15] and **3**[16] (Fig. 1). Furthermore, the optical rotational values of **1** ($[\alpha]_D$ +191.9° in CH_3OH) revealed that the absolute configuration of **1** has 4*S* from comparison with those of (*R*)-mitorubrin (4*R*, $[\alpha]_D$ −428.0° in CH_3OH),[15,17] **2** (4*S*, $[\alpha]_D$ +190.0° in CH_3OH)[15] and **3** (4*S*, $[\alpha]_D$ +203.4° in CH_3OH).[16]

Biological Properties Compound **2** was reported as a geranylgeranyltransferase I (GGTase I) inhibitor,[15] while the biological activity of **3** has not been reported. In this study, they were found to have miconazole-potentiating activity against *C. albicans*. Namely, **1** to **3** showed no inhibition zone against *C. albicans* even at 50 μg/6 mm disk on Plate A (GY agar); however, these compounds gave dose-dependent inhibition zones on Plate B (Plate A containing 0.060 μM miconazole) (Table 3). These results indicated that **1** to **3** potentiate miconazole activity against *C. albicans*. GGTase I was reported to be responsible for cell wall biosynthesis of *C. albicans*.[15] Therefore, it might be that mitorubrins inhibit GGTase I to potentiate miconazole activity, but it remains to be defined. Regarding antimicrobial activity against other microorganisms, as described in Experimental, these compounds showed very weak antibacterial activity only against *X. camperstris* with the same size (7 mm) of inhibition zone at 10 μg/6 mm disk, but no activity against the other five microorganisms.

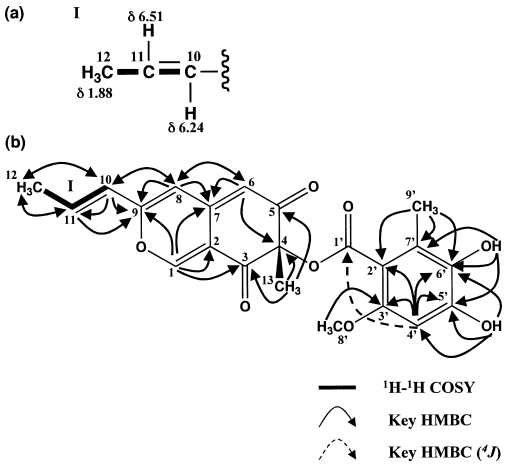

Fig. 2. Partial Structure (a) and ^{1}H–^{1}H COSY and ^{13}C–^{1}H HMBC Experiments (b) of **1**

Table 3. Potetiation of Micanazole Activity against *C. albicans* in Combination with **1** to **3**

Compound	Concentration	Inhibition zone (mm)	
	μg/disk	Plate A[a]	*Plate B[b]
1	0.1	—	—
	1	—	7
	20	—	10
	25	—	18
	50	—	23
2	0.1	—	—
	1	—	8
	20	—	10
	25	—	18
	50	—	20
3	0.1	—	—
	1	—	8
	20	—	16
	25	—	22
	50	—	30

a) Plate A; GY agar. *b*) Plate B; Plate A containing 0.060 μM miconazole.

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