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Melleolides K, L and M, New Melleolides from *Armillariella mellea*

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Three new sesquiterpenoid aromatic esters designated melleolides K (**1**), L (**2**) and M (**3**) were isolated from the cultured mycelia of *Armillariella mellea* (Vahl. ex Fr.) Karst. Structures of these compounds were determined on the basis of various NMR spectral data, chemical transformations and X-ray analysis. Compounds **1**, **2** and **3** showed antimicrobial activities.

During our screening for new antibiotics, we isolated three new antibiotics from the cultured mycelia of Basidiomycetes, *Armillariella mellea*. (Vahl. ex. Fr.) Karst. *A. mellea* was well known to produce a range of sesquiterpenoid aromatic esters with the protoilludene skeleton including melleolide[1)], melleolides B~H[2,3)], melledonal[4)], melledonol[4)] and armillaric acid[5)]. Melleolide I and J[6)] had been isolated from *Armillaria novae-zelandiae*. Our three compounds were found to be new members of sesquiterpenoid aromatic esters, and named melleolides K (**1**), L (**2**) and M (**3**) (Fig. 1). These compounds showed antimicrobial activities.

We describe herein the isolation procedure, physico-chemical properties, structure determination and biological activities of **1**, **2** and **3**.

Materials and Methods

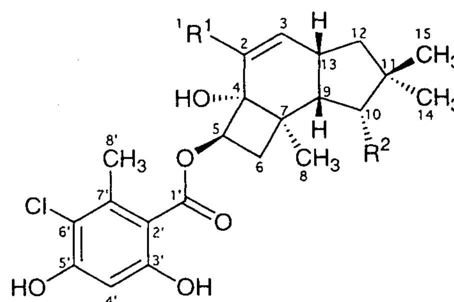
Microorganism

The melleolides K (**1**), L (**2**) and M (**3**) producing strain, K-1784 was isolated from a fruit body of *Armillariella mellea* (Vahl. ex Fr.) Karst collected in Akita prefecture, Japan. This strain has been deposited to the culture collection at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Ministry of International Trade and Industry, Tsukuba, Japan under the accession number FERM P-17059.

Production of Melleolides K (1), L (2) and M (3)

The K-1784 strain grown on a slant culture (potato dextrose agar, Difco) was inoculated into a 1-liter Erlenmeyer flask containing 500 ml of medium consisting of glucose 1.0%, peptone 0.5%, KH_2PO_4 0.3%, $MgSO_4 \cdot 7H_2O$ 0.1%. After cultured in a stationary mode for 7 days at 27°C, the flask was stirred by magnetic stirrer for additional 7 days. This culture (5 ml) was inoculated into 500-ml Sakaguchi flasks each containing 125 ml of the

Fig. 1. Structures of melleolides K (**1**), L (**2**) and M (**3**).



same medium. The flasks were incubated without shaking for 7 days at 27°C, and then shaken at 130 rpm on a reciprocal shaker for 7 days at 27°C.

Biological Activities

The MICs of **1**, **2** and **3** against bacteria were determined by the agar dilution method using Mueller-Hinton agar (Difco). The MICs of **1**, **2** and **3** against yesat and fungi were determined by the same method using synthetic amino acid medium for fungi agar.

Results and Discussion

Isolation of Melleolides K (1), L (2) and M (3)

The culture broth (5 liters) was filtrated to separate the supernatant and the mycelial cake. The mycelial cake was extracted twice with MeOH (1 liter). The MeOH extract was evaporated to dryness to give a brown oil (7.6 g). This oily substance was chromatographed on a silica gel column (Wakogel C-200) and eluted stepwise with mixtures of $CHCl_3$ - MeOH (100 : 0, 50 : 1 and 10 : 1). The active fractions containing **1**, **2** and **3** were eluted with the mixture of $CHCl_3$ - MeOH (100 : 0), (50 : 1) and (10 : 1), respectively and were collected and concentrated under reduced pressure to give pale yellow pastes in amounts of 1.7 g, 1.0 g and 1.8 g, respectively.

The material containing **1** was applied to a Sephadex LH-20 column and developed with a mixture of $CHCl_3$ - MeOH (1 : 1). The active fractions were concentrated to give yellow powder (548 mg). The crude **1** was further purified by reverse phase HPLC using Capcell Pak UG (Shiseido, 2.0×25 cm) with a solvent consisted of CH_3CN - H_2O (65 : 35) at flow rate of 10 ml/minute to give 149 mg of **1**.

The material containing **2** was purified by reverse phase HPLC as described above with a mixture of CH_3CN - H_2O (1 : 1) to give 226 mg of **2**.

The material containing **3** was subjected to a Sephadex LH-20 column chromatography with MeOH as the eluting solvent. Combined active fractions were concentrated *in vacuo* to dryness, and then the residue was chromatographed using reverse phase HPLC with a mixture of CH_3CN - H_2O (45 : 55). The active fractions were evaporated under reduced pressure to give a crude **3**. Further purification of **3** using a silica gel TLC (Merck Silica gel $60F_{254}$ Art. 105715, toluene - acetone - conc NH_4OH, 80 : 120 : 1) provided 70 mg of **3**.

Physico-chemical Properties of Melleolides K (1), L (2) and M (3)

Physico-chemical properties of **1**, **2** and **3** are shown in Table 1. The compounds are soluble in MeOH, $CHCl_3$, EtOAc or DMSO , but insoluble in hexane or H_2O. The UV spectra of **2** and **3** were similar to these of **1**. Compounds **1**, **2** and **3** gave positive color reactions with molybdophosphoric acid-sulfuric acid and $FeCl_3$. While **1** and **2** gave positive color reactions with 2,4-dinitrophenylhydrazine, **3** gave a negative reaction.

Structure Determination of Melleolide K (1)

The molecular formula of **1** was established as $C_{23}H_{27}ClO_6$ with HRFAB-MS. The ^1H and ^{13}C NMR data of **1** are shown in Table 2. The ^1H, ^{13}C NMR, DEPT and HMQC spectra of **1** revealed the presence of twelve sp^3 carbons and ten sp^2 carbons. The ^1H NMR spectrum indicated the presence of three aliphatic methyl groups (δ 1.00, 1.03 and 1.33), an aromatic methyl group (δ 2.43), an aldehyde group (δ 9.48), an olefinic proton (δ 6.82), an oxygenated methine (δ 5.64) and a hydrogen bonding hydroxyl group (δ 11.17).

The results of ^1H - ^1H COSY and HMBC experiments of **1** are summarized in Fig. 2. Two partial structures were established by analysis of the ^1H-^1H COSY spectrum. In the HMBC spectrum, two quaternary methyl groups C-14 and C-15 (δ 31.51 and 31.04) were coupled with each other. Both methyl groups C-14 and C-15 were coupled to a quaternary carbon C-11 (δ 37.93), and two methylene C-10 (δ 41.63) and C-12 (δ 46.54). These data were consistent with the presence of a cyclopentane ring with two methyl groups at C-11. An aldehyde proton at δ 9.48 (C-1) showed cross peaks to C-2 (δ 137.28), C-3 (δ 158.56) and C-4 (δ 74.91). These observations indicated the presence of an α,β-unsaturated aldehyde group. Both C-2 and C-4 were coupled to an oxygenated methine proton at δ 5.63 (5-H). A quaternary methyl protons at δ 1.33 (8-H) showed cross peaks to C-4, C-6 (δ 33.13), C-7 (δ 37.64) and C-8 (δ 21.15). Hence, the above evidence revealed the presence of a protoilludene moiety in the structure of **1**.

The UV spectrum of **1** showed the bands at λ_{max} nm (log ε) 219 (4.39), 262 (3.93) and 310 (3.66), which shifted to 240 (4.14), 290 (sh, 4.03) and 317 (4.17) in alkaline solution. The absorption bands and the bathochromic shift were similar to those of melleolide[1], suggesting the presence of *p*-hydroxybenzoyl moiety. In addition, another phenolic hydroxyl group at δ 11.17 (3′-OH) was deduced to be at C-3′, because its chemical shift indicated that this

Table 1. Physico-chemical properties of melleolides K (**1**), L (**2**) and M (**3**).

	1	**2**	**3**
Appearance	Colorless powder	Colorless powder	Colorless powder
MP	71 ~ 74 ℃	94 ~ 95 ℃	89 ~ 92 ℃
$[\alpha]_D^{24}$	+ 121.9 ° (*C* 1.0, MeOH)	+ 98.7 ° (*C* 1.0, MeOH)	+ 12.0 ° (*C* 1.0, MeOH)
Molecular formula	$C_{23}H_{27}ClO_6$	$C_{23}H_{27}ClO_7$	$C_{23}H_{29}ClO_7$
HRFAB-MS (*m/z*)			
Calcd:	435.1574 (as $C_{23}H_{28}ClO_6$)	451.1524 (as $C_{23}H_{28}ClO_7$)	453.1680 (as $C_{23}H_{30}ClO_7$)
Found:	435.1586 $(M+H)^+$	451.1537 $(M+H)^+$	453.1686 $(M+H)^+$
UV λ max nm, (log ε) in			
MeOH	219 (4.39), 262 (3.93), 310 (3.66)	219 (4.38), 261 (3.93), 311 (3.63)	212 (4.35), 264 (3.88), 310 (3.61)
MeOH-NaOH	240 (4.14), 290 (sh, 4.03), 317 (4.17)	241 (4.13), 290 (sh, 4.00), 317 (4.18)	245 (3.96), 289 (sh, 4.06), 316 (4.22)
MeOH-HCl	218 (4.36), 262 (3.92), 308 (3.54)	219 (4.35), 262 (3.92), 309 (3.54)	212 (4.35), 264 (3.89), 308 (3.53)
IR ν max (KBr cm^{-1})	3420, 2950, 2865, 1670, 1655, 1610, 1465, 1425, 1385, 1310	3435, 2920, 2875, 1655, 1655, 1610, 1465, 1425, 1385, 1310	3365, 2960, 1655, 1610,1465, 1445, 1430, 1380, 1310
Rf value*	0.51	0.39	0.17

* Silica gel TLC (Merck Art. 105715) : Toluene-Acetone (3:1)

Table 2. The ^{13}C and ^1H NMR assignments of **1**, **2** and **3** in $CDCl_3$.

Position	**1**		**2**		**3**	
	^{13}C ppm (mult.)	^1H ppm (mult., *J* (Hz))	^{13}C ppm (mult.)	^1H ppm (mult., *J* (Hz))	^{13}C ppm (mult.)	^1H ppm (mult., *J* (Hz))
1	195.97 (d)	9.48 (s)	195.68 (d)	9.44 (s)	65.68 (t)	4.29 (dd, 2.0, 12.4) 4.00 (d, 12.4)
2	137.28 (s)		137.28 (s)		132.54 (s)	
3	158.56 (d)	6.82 (d, 2.0)	158.74 (d)	6.95 (d, 2.4)	135.83 (d)	5.87 (d, 2.4)
4	74.91 (s)		74.19 (s)		76.23 (s)	
-OH		4.45 (br)		4.54 (br)		Not observed
5	77.84 (d)	5.63 (t, 8.6)	76.18 (d)	5.73 (t, 8.6)	77.23 (d)	5.64 (t, 8.6)
6α	33.13 (t)	2.10 (dd, 8.6, 11.6)	32.83 (t)	2.12 (dd, 8.6, 11.0)	32.99 (t)	2.04 (dd, 8.6, 10.8)
β		1.57 (dd, 8.6, 11.6)		1.58 (dd, 8.6, 11.6)		1.64 (dd, 8.6, 10.8)
7	37.64 (s)		35.47 (s)		36.59 (s)	
8	21.15 (q)	1.33 (s)	20.77 (q)	1.44 (s)	21.19 (q)	1.38 (s)
9	44.05 (d)	2.28 (ddd, 7.0, 7.0, 13.0)	47.3 (d)	2.40 (dd, 3.2, 9.6)	46.83 (d)	2.29 (dd, 3.9, 9.6)
10α	41.63 (t)	1.29 (dd, 12.6, 13.0)	80.59 (d)		82.5 (d)	
β		1.51 (dd, 7.0, 12.6)		3.65 (d, 3.2)		3.62 (d, 3.9)
-OH				2.23 (br)		Not observed
11	37.93 (s)		42.62 (s)		42.5 (s)	
12α	46.54 (t)	1.58 (dd, 2.6, 13.8)	43.09 (t)	1.65 (dd, 6.0, 13.0)	44.62 (t)	1.49 (dd, 5.0, 13.4)
β		2.03 (dd, 9.6, 13.8)		2.08 (dd, 10.8, 13.0)		1.99 (dd, 10.0, 13.4)
13	40.27 (d)	3.02 (m)	36.11 (d)	3.11 (m)	34.96 (d)	2.85 (m)
14	31.51 (q)	1.00 (s)	23.30 (q)	1.08 (s)	23.80 (q)	1.05 (s)
15	31.04 (q)	1.03 (s)	28.22 (q)	1.01 (s)	29.20 (q)	0.99 (s)
1'	169.91 (s)		169.80 (s)		170.14 (s)	
2'	106.99 (s)		106.87 (s)		107.10 (s)	
3'	162.72 (s)		162.67 (s)		162.49 (s)	
-OH		11.17 (br)		11.12 (br)		10.80 (br)
4'	102.09 (d)	6.49 (s)	102.08 (d)	6.47 (s)	102.10 (d)	6.50 (s)
5'	156.05 (s)		156.15 (s)		156.33 (s)	
6'	113.76 (s)		113.83 (s)		114.10 (s)	
7'	138.97 (s)		138.97 (s)		139.28 (s)	
8'	20.00 (q)	2.43 (s)	19.94 (q)	2.42 (s)	19.67 (q)	2.49 (s)

Fig. 2. Summary of ^{1}H-^{1}H COSY and HMBC experiments of **1**.

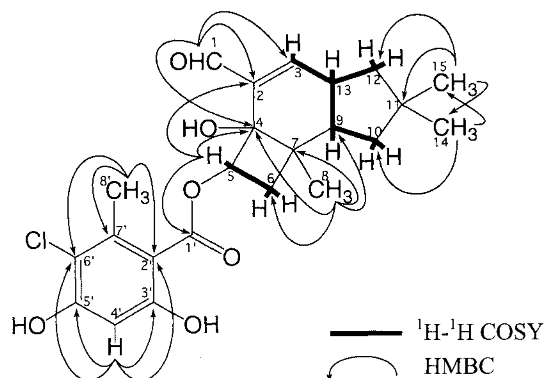
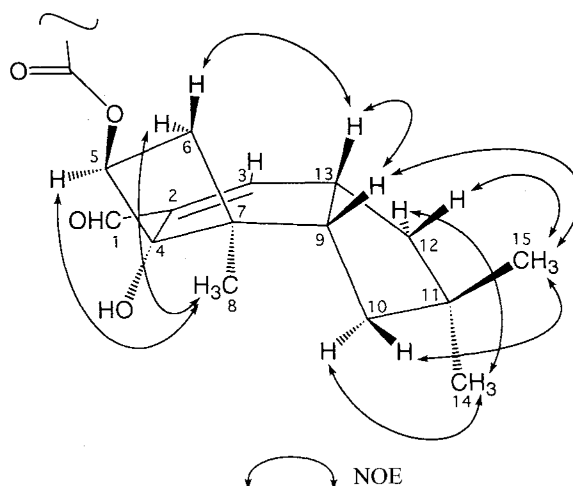


Fig. 3. Selected NOE correlations of **1**.



phenol group formed a hydrogen bond with a carbonyl group at δ 169.91 (C-1'). An aromatic methyl protons at δ 2.43 (8'-H) showed cross peaks to C-2' (δ 106.99), C-6' (δ 113.76) and C-7' (δ 138.97) in the HMBC spectrum. Higher field chemical shifts of two aromatic carbons C-2' and C-6' were attributed to mesomeric effects of two phenol groups at C-3' and C-5'. Therefore, the aromatic methyl group was placed on the C-7' position. Also a chlorine atom should be connected to C-6', considering the molecular formula. These results indicated that a 3-chloro-orsellinate moiety was present in the structure of **1**.

The connectivity between a protoilludene moiety and a 3-chloro-orsellinate moiety was demonstrated by the HMBC spectrum. The carbonyl carbon C-1' was coupled to a methine proton at C-5, indicating an ester bond between C-5 and C-1'.

Consequently, the compound **1** was found to be a new melleolide antibiotic as shown in Fig. 1.

The relative stereochemistry of **1** was elucidated on the basis of magnitude of the coupling constants and NOESY spectrum. The results of NOESY experiment of **1** are summarized in Fig. 3. The geometry of the protoilludene skeleton was assigned as *cis-anti-cis* relationship on the basis of J value 7.0 Hz between 9-H (δ 2.28) and 13-H (δ 3.02) and the NOE interactions between 9-H and 13-H, and 6β-H (δ 1.57) and 9-H, respectively.

Structure Determination of Melleolide L (2)

The molecular formula of **2** was established as $C_{23}H_{27}ClO_7$ with HRFAB-MS. The molecular formula of **2** indicated the presence of one more oxygen atom compared with that of **1**. The ^{1}H and ^{13}C NMR spectra of **2** were very similar to those of **1** (Table 2). However, the signals of methylene protons corresponding to 10α, β-H_2 (δ 1.29 and 1.51) of **1** were not observed in the ^{1}H NMR spectrum of **2**, but a methine proton signal bearing an oxygen atom and a hydroxyl group signal appeared at δ 3.65 and 2.23, respectively. Therefore, the structure of **2** was determined as a 10-hydroxy analogue of **1** (Fig. 1).

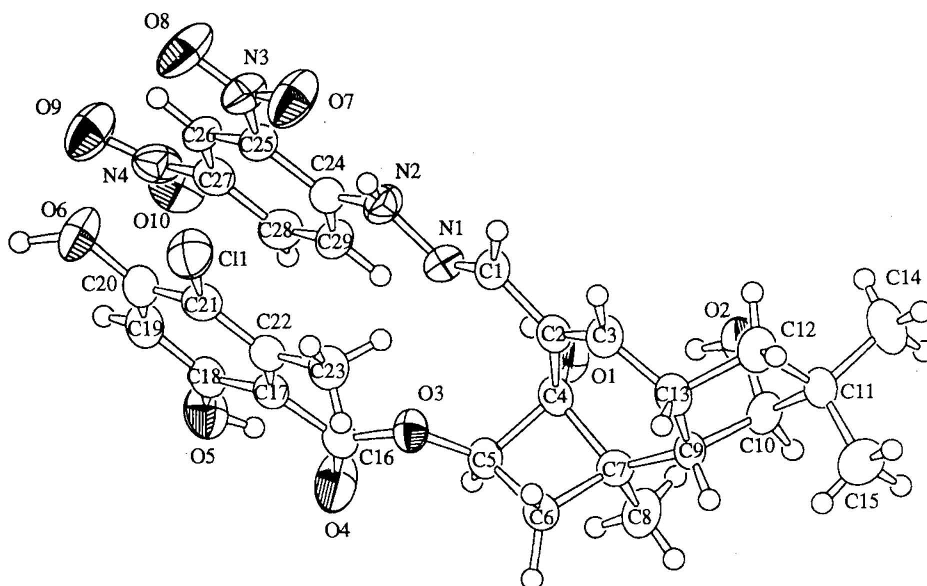
The absolute configuration of **2** was determined by X-ray crystallographic analysis of its 2,4-dinitrophenylhydrazone (**4**) using anomalous scattering of chlorine, oxygen and nitrogen atoms. The absolute configuration of **4** was established to be as shown in Fig. 4.

Structure Determination of Melleolide M (3).

The molecular formula of **3** was established as $C_{23}H_{29}ClO_7$ with HRFAB-MS. The molecular formula of **3** indicated the presence of two more hydrogen atoms than **2**. The ^{1}H and ^{13}C NMR spectra of **3** were very similar to those of **2** (Table 2). However, an aldehyde proton signal at δ 9.44 of **2** was not observed in ^{1}H NMR spectrum of **3**, but methylene proton signals bearing an oxygen atom appeared at δ 4.00 and 4.29. Therefore, the structure of **3** was determined as a 1-dihydro analogue of **2** (Fig. 1).

The absolute configuration of **3** was deduced by conversion of **2** into **3** as follows. The reduction of **2** was carried out with sodium borohydride ($NaBH_4$) for 30

Fig. 4. ORTEP drawing of **4**.



minutes at −5°C to yield 1-dihydro compound (**3**). The spectroscopic data and optical rotation of synthetic **3** were in full agreement with those of natural **3**. Therefore, the absolute configration of **3** was concluded to have the same stereochemistry as **2**.

Biological Activities

The antimicrobial activities of **1**, **2** and **3** are summarized in Table 3. Compound **1** inhibited growth of Gram-positive bacteria, yeast and fungi, but did not inhibit Gram-negative bacteria. Compounds **2** and **3** showed weak antimicrobial activities.

The acute toxicities (LD_{50}) of **1**, **2** and **3** in mice (i. p.) were estimated to be more than 100 mg/kg.

Experimental

General

NMR spectra were obtained on a JEOL JNM-A500 spectrometer at 500 MHz for ^{1}H NMR and at 125 MHz for ^{13}C NMR. Chemical shifts are given in ppm using TMS as an internal standard. UV absorption spectra were measured using a Hitachi U-3210 spectrophotometer. FAB-MS and HRFAB-MS spectra were measured using a JEOL JMS-SX 102 spectrometer. APCI-MS spectrum was measured using a Hitachi M-1200H spectrometer. Optical rotations were determined using a Perkin-Elmer 241 polarimeter.

2,4-Dinitrophenylhydrazone of 2

A suspension of 2,4-dinitrophenylhydrazine (20 mg) and acetic acid (0.1 ml) in MeOH (2 ml) was stirred for 5 minutes at room temperature, and filtered. To the filtrate was added **2** (4.2 mg) and stirred for 1 hour at room temperature. After evaporated the solvent, the residue was purified by a silica gel TLC (toluene - acetone, 3 : 1) to give **4** (5.8 mg) as yellow crystals. MP 253~255°C. HRFAB-MS *m/z* 631.1778 (calcd *m/z* 631.1807 for $C_{29}H_{32}O_{10}N_4Cl$). ^{1}H NMR ($CDCl_3$) δ 1.02 (3H,s), 1.09 (3H, s), 1.51 (3H, s), 1.61 (1H, dd, J=5.6, 13.0 Hz), 1.80 (1H, dd, J=8.8, 10.6 Hz), 2.03 (1H, dd, J=10.4, 13.0 Hz), 2.10 (1H, dd, J=8.8, 10.6 Hz), 2.23 (1H, br s), 2.43 (3H, s), 3.09 (1H, m), 3.69 (1H, d, J=3.2 Hz), 4.66 (1H, br s), 5.99 (1H, t, J=8.8 Hz), 6.11 (1H, br s), 6.14 (1H, s), 6.47 (1H, d, J=3.2 Hz), 7.53 (1H, d, J=9.6 Hz), 7.68 (1H, s), 8.28 (1H, dd, J=2.4, 9.6 Hz), 9.05 (1H, d, J=2.4 Hz), 11.71 (1H, br s), 11.12 (1H, s). ^{13}C NMR ($CDCl_3$) δ 20.04, 21.17, 23.41, 28.41, 32.31, 35.65, 36.02, 42.75, 43.88, 47.15, 75.64, 77.20, 81.09, 101.65, 106.24, 114.04, 116.03, 123.27, 129.22, 129.37, 130.09, 138.48, 139.19, 143.92, 147.49, 150.57, 156.33, 163.07, 169.95.

Table 3. The antimicrobial activities of **1**, **2** and **3**.

Test organism	MIC (µg/ml)		
	1	**2**	**3**
Staphylococcus aureus FDA209P	6.25	50	50
S. aureus Smith	12.5	100	100
S. aureus MS9610	12.5	100	100
S. aureus MS16526 (MRSA)	12.5	100	100
S. aureus TY-04282 (MRSA)	12.5	>100	100
Micrococcus luteus IFO3333	12.5	50	>100
Bacillus subtilis PCI219	6.25	50	>100
Corynebacterium bovis 1810	25	>100	>100
Escherichia coli K-12	>100	>100	>100
Shigella dysenteriae JS11910	>100	>100	>100
Salmonella typhi T-63	>100	>100	>100
Proteus vulgaris OX19	>100	>100	>100
Providencia rettgeri GN311	>100	>100	>100
Serratia marcescens	>100	>100	>100
Pseudomonas aeruginosa A3	>100	>100	>100
Klebsiella pneumoniae PCI602	>100	>100	50
Mycobacterium smegmatis ATCC607*	50	50	>100
Candida albicans 3147**	>100	>100	>100
*C. pseudotropicalis***	50	100	100
Cryptococcus neoformans v. neof. TIMM 1313 ← CDC551**	50	>100	>100
*Saccharomyces cerevisiae***	25	100	>100
Trichophyton rubrum IFO 9185**	25	>100	50
T. mentagrophytes 833**	50	>100	>100
*Aspergillus niger***	>100	>100	>100
A. fumigatus TIMM 2905**	50	>100	>100

Mueller Hinton agar (Difco) 37 ℃ 18 hours

*Mueller Hinton agar (Difco) 37 ℃ 42 hours

**Synthetic amino acid medium for fungi agar 27 ℃ 47 hours

Reduction of **2**

Compound **2** (8.0 mg) in MeOH (1 ml) was treated with $NaBH_4$ (8.0 mg) for 30 minutes at −5°C. To the reaction mixture, acetone (0.1 ml) and H_2O (20 ml) were added. The mixture was extracted with $CHCl_3$ (20 ml) and the $CHCl_3$ extract was dried over anhydrous sodium sulphate. After the solvent was evaporated, the residue was purified using a silica gel TLC (toluene - acetone, 3 : 1) to give **3** (8.0 mg) as colorless powder. The ^1H and ^{13}C NMR and APCI-MS data of synthetic **3** were identified with those of natural **3**. The optical rotations of synthetic and natural **3** were $[\alpha]_D^{24}$ +12.6° (*c* 0.23, MeOH) and $[\alpha]_D^{24}$ +12.0° (*c* 1.0, MeOH), respectively.

X-Ray Crystallography

Crystals of **4** were obtained from a CH_3CN solution. A yellow prism crystal having approximate dimensions of 0.03×0.04×0.35 mm was mounted on a glass fiber. All measurements were made on a Rigaku AFC7R diffractometer with graphite monochromated Cu-Kα radiation and a rotating anode generator. Crystal data are as follows. $C_{29}H_{31}N_4O_{10}Cl$, formula weight: 631.04, crystal system: monoclinic, space group: $P2_1$, a=12.7784(5) Å, b=7.3134(5) Å, c=15.4667(8) Å, β=93.186(4)° V=1443.2(1) Å^3, Z=2, D_{calc}=1.452 g/cm^3, μ(CuKα)=17.48 cm^{-1}. Of the 5613 reflections which were collected, 2687 were unique (R_{int}=0.043). No decay correction was

applied. The structure was solved by direct methods[7] and expanded using Fourier techniques[8]. The non-hydrogen atoms were refined anisotropically. Hydrogen atoms were included but not refined. The final cycle of full-matrix least-squares refinement was based on 1994 observed reflections ($I>1.5\sigma(I)$) and 396 variable parameters and converged with unweighted and weighted agreement factors of $R=0.044$ and $R_w=0.055$. The maximum and minimum peaks on the final difference Fourier map corresponded to 0.19 and $-0.22\,e^-/\text{Å}^3$, respectively. Comparing $|F_O(hkl)|/|F_O(\bar{h}\bar{k}\bar{l})|$ and $|F_C(hkl)|/|F_C(\bar{h}\bar{k}\bar{l})|$ for 180 Bijvoet mates for which the difference $||F_C(hkl)|-|F_C(\bar{h}\bar{k}\bar{l})||/(\sigma^2(F_O(hkl))+\sigma^2(F_O(\bar{h}\bar{k}\bar{l})))^{1/2}$ are larger than 1.0, 167 pairs showed consistently the absolute configuration in Fig. 3. All calculations were performed using the teXsan[9] crystallographic software package of Molecular Structure Corporation.

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