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Metabolites of the marine fungus *Humicola fuscoatra* KMM 4629

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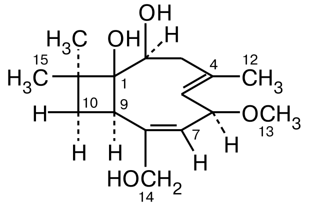
A new sesquiterpene of the caryophyllene series, fuscoatrol A (**1**), and known compounds, 11-epiterpestacin (**2**) and β-nitropropionic acid (**3**), were isolated from the marine fungus *Humicola fuscoatra (Traaen)* KMM 4629 associated with the Kuril colonial ascidium. The structure of **1** was established on the basis of X-ray diffraction data and 2D NMR spectroscopy. The antimicrobial and cytotoxic activities of compounds **1—3** were studied.

Key words: *Humicola fuscoatra,* marine fungi, sesquiterpenes, sesterterpenes, caryophyllenes.

As opposed to the terrestrial fungi, which have been studied for more than 60 years and have served as sources of diverse biologically active secondary metabolites,[1—4] marine fungi have been little studied. However, in recent years, the interest in them has enhanced and now they have been found[5—8] to be efficient producers of biologically active substances. The isolates of marine fungi are usually prepared from various substrates such as algae, sponges, echinoderms, sea water, and bottom sediments. When investigating the microbiota associated with the Kuril colonial ascidium, we found that the fungus *Humicola fuscoatra* produces compounds that exhibit antimicrobial activities. Here we report of the isolation and identification of a number of compounds from this fungus. These compounds include a new caryophyllenic sesquiterpene, fuscoatrole A (**1**), and two known compounds, bicyclic sesterpene 11-epiterpestacin (**2**) and β-nitropropionic acid (**3**). Previously,[4,9—12] caryophyllene type sesquiterpenes have been isolated from some plants and terrestrial fungi. This is the first case of detection of compounds **1—3** in the isolates of marine fungi.

The ethyl acetate extract of the fungus mycelium exhibiting an antimicrobial activity was subjected to column chromatography on silica gel. The subsequent chromatography of the active fraction on Sephadex LH 20 yielded pure compounds **1**, **2**, and **3**. Compound **1** was additionally purified by HPLC.

The molecular weight of the quasi-molecular ion $[M + Na]^+$ of compound **1** was determined by of high-resolution matrix-assisted laser desorption/ionization (MALDI) mass spectroscopy. The value (*m/z* 305.1692) corresponded to the molecular formula $C_{16}H_{26}O_4$. The



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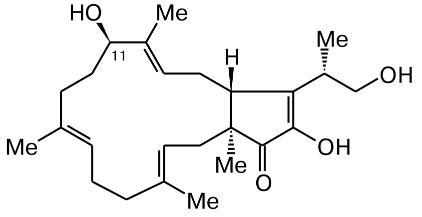

2

$O_2N—CH_2—CH_2—COOH$

3

absorption bands at 1603, 1462, and 3600—3250 cm^{-1} in the IR spectrum of **1** and the signals at δ 129.3, 133.8, 137.1, 139.7, 67.1, 73.5, 76.1, and 82.6 in the ^{13}C NMR spectrum (Table 1) indicated the presence of two double bonds and four sp^3-hybridized C—O fragments. X-Ray diffraction analysis of the obtained single crystal demonstrated that fuscoatrol A (**1**) has the structure of 8-hydroxymethyl-6-methoxy-4,11,11-trimethylbicyclo[7.2.0]undeca-4,7-diene-1,2-diol (Table 2, Fig. 1) and, thus, it is a sesquiterpene of the hydroxylated caryophyllene series.

The intensities of the sixteen major and sixteen minor ^{13}C NMR signals of **1** are related as 3 : 1, as well as two

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Table 1. ^{13}C and ^{1}H NMR spectra (δ, *J*/Hz) of compound **1**

Position	Major conformer		Minor conformer	
	^{13}C	^{1}H	^{13}C	^{1}H
1	82.6 (s)		84.6 (s)	
2	73.5 (d)	4.21 (dd, 1 H, C(2)H, J_1 = 4.6, J_2 = 11.1)	76.5 (d)	4.23 (dd, 1 H, C(2)H, J_1 = 7.6, J_2 = 10.4)
3	44.7 (t)	2.35 (dd, 1 H, C(3)H_a, J_1 = 4.7, J_2 = 11.1); 2.54 (t, 1 H, C(3)H_b, J = 11.1)	39.8 (t)	1.78 (dd, 1 H, C(3)H_b, J_1 = 10.4, J_2 = 14.2); 2.91 (dd, 1 H, C(3)H_a, J_1 = 7.0, J_2 = 14.2)
4	133.8 (s)		142.5 (s)	
5	129.3 (d)	5.14 (br.dd, 1 H, C(5)H, J_1 = 10.2, J_2 = 1.4)	120.6 (d)	5.64 (dt, 1 H, C(5)H, J_1 = 7.2, J_2 = 1.8)
6	76.1 (d)	4.46 (br.dt, 1 H, C(6)H, J_1 = 10.2, J_2 = 2.1)	76.1 (d)	4.30 (dt, C(6)H, J_1 = 7.1, J_2 = 2.1)
7	139.7 (d)	5.94 (br.d, 1 H, C(7)H, J = 2.1)	140.0 (d)	5.97 (br.s, C(7)H)
8	137.1 (s)		134.1 (s)	
9	40.3 (d)	3.10 (br.t, 1 H, C(9)H, J = 9.5)	45.4 (d)	3.21 (br.dd, 1 H, C(9)H, J_1 = 9.0, J_2 = 11, 2)
10	34.2 (t)	1.64 (dd, 1 H, C(10)H_a, J_1 = 10.3, J_2 = 12.3); 2.05 (dd, 1 H, C(10)H_b, J_1 = 8.4, J_2 = 12.3)	35.9 (t)	1.57 (dd, 1 H, C(10)H_a, J_1 = 8.6, J_2 = 10.8); 2.11 (t, 1 H, C(10)H_b, J = 11.0)
11	41.5 (s)		40.7 (s)	
12	17.8 (q)	1.89 (d, 3 H, C(12)H, J = 1.3)	24.9 (q)	1.76 (br.s, 3 H, C(12)H)
13	55.6 (q)	3.30 (s, 3 H, C(13)H)	56.1 (q)	3.31 (s, 3 H, C(13)H)
14	67.1 (t)	3.93 (d, 1 H, C(14)H, J = 11.1); 4.34 (br.dd, 1 H, C(14)H, J_1 = 1.5, J_2 = 11.0)	67.2 (t)	3.92 (d, 1 H, C(14)H_a, J = 10.8); 4.19 (br.dd, 1 H, C(14)H, J_1 = 3.0, J_2 = 9.7)
15	24.7 (q)	1.10 (s, 3 H, C(15)H)	24.1 (q)	1.07 (s, 3 H, C(15)H)
16	22.7 (q)	1.29 (s, 3 H, C(16)H)	25.7 (q)	1.33 (s, 3 H, C(16)H)

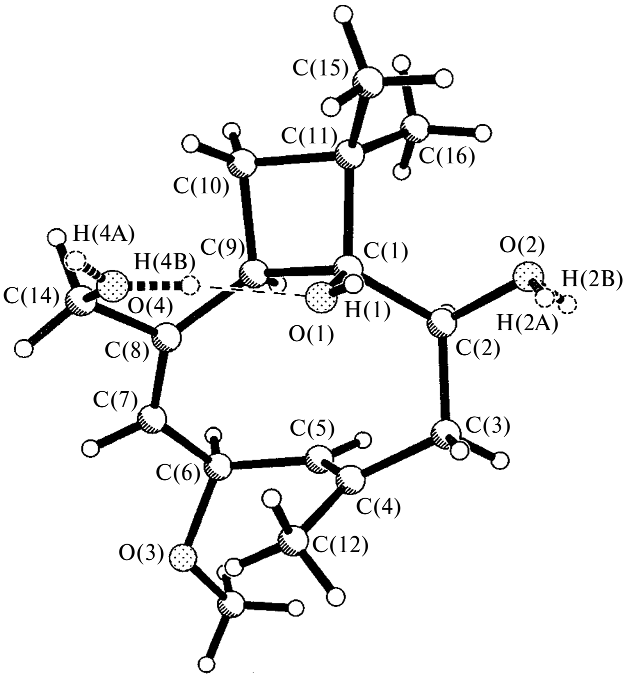

Fig. 1. Structure of compound **1**.

series of partially overlapped signals in the ^{1}H NMR spectra ($CDCl_3$, 22 °C). In a DMSO solution at 145 °C, the signal intensity ratio is 2.2 : 1. These data provide the conclusion that compound **1** occurs in solutions as two predominant conformers. A similar conformation equilibrium has been described for solutions of caryophyllene[13] and some its derivatives.[14,15]

The ^{1}H and ^{13}C NMR signals for both conformers were assigned using the ^{1}H—^{1}H-COSY, HSQC, and HMBC techniques (see Table 1).

Some stereochemical details were elucidated by measurement of the nuclear Overhauser effect (NOE). For the major conformer, NOE was detected between $H_3C(12)$ and the HC(2), HC(6), and HC(9) protons; the HC(5) and HC(7), HC(7) and $H_aC(14)$, as well as $H_aC(14)$ and $H_3C(15)$ protons. The NOE correlations between the HC(5) and HC(6), HC(2) and HC(13), as well as HC(10α) and HC(9) were determined for the minor conformer. These data indicate, in particular, different mutual arrangements of the $H_3C(12)$ group and the HC(9) proton in the conformers.

It is noteworthy that fuscoatrol A **1** has been unknown previously; however, its acetyl form, pestalotiopsin B, has already been isolated from the endophytic fungus associated with the bark and the leaves of *Taxus brevifolia*.[16]

The spectroscopic characteristics and the specific rotation of the 11-epiterpestacin **2** isolated by us almost coincided with those reported in the literature.[17] The last component of the extract, compound **3**, was identified as β-nitropropionic acid, according to X-ray diffraction data.[18]

Compound **1** possesses an antimicrobial activity against *Staphylococcus aureus* and *Bacillus subtilis* (MIC = 12.5 μg mL^{-1}) and exhibits a cytotoxic action on the developing eggs of sea urchin *Strongylocentrotus intermedius* (MIC_{50} = 40 μg mL^{-1}). Compound **2** has an antimicrobial activity against *S. aureus* and *B. subtilis* (MIC = 100 μg mL^{-1}), while **3** is active against *S. aureus*,

Table 2. Crystal data and X-ray experiment and structure refinement details of compound **1**

Parameter	Value
Molecular formula	$C_{16}H_{26}O_4$
Molecular weight	282.37
Temperature/K	173(1)
Wavelength/Å	Mo-Kα (0.71073)
System	Monoclinic
Space group	*C*2
a/Å	20.016(3)
b/Å	7.989(1)
c/Å	10.556(1)
ω/deg	112.502(2)
V/Å^3	1559.4(4)
Z	4
d_{calc}/g cm^{-3}	1.203
μ/mm^{-1}	0.085
F(000)	616
Crystal shape	Prism
Crystal size/mm	0.26×0.21×0.08
Data collection range for θ/deg	2.78—28.00
Range of reflection indices	$-26 \le h \le 25$ $-7 \le k \le 10$ $-13 \le l \le 13$
The number of measured reflections	5074
The number of independent reflections	2962
R_{int}	0.0347
Reflections with c $I > 2\sigma(I)$	2556
Refinement method	Full-matrix Least-squares for F^2
The number of refined parameters	201
GOOF	1.031
R-factors for $F^2 > 2\sigma(F^2)$	
R_1	0.0379
wR_2	0.0922
R-factors for all reflections	
R_1	0.0462
wR_2	0.0974
Extinction coefficients	0.0007(8)
Residual electron density (min/max)/e · Å^{-3}	−0.136/0.210

B. subtilis, *Candida albicans*, and *Escherichia coli* (MIC = 100 μg mL^{-1}).

Experimental

X-Ray diffraction analysis was performed for a faceted prismatic single crystal with the dimension 0.08×0.21×0.26 mm on a SMART 1000 CCD diffractometer (Mo-Kα-radiation, graphite monochromator) at 173(1) K. The data were collected in groups of 906, 660, and 345 frames for φ = 0, 90, and 180°, respectively, in the ω-scan mode with a step of 0.2° and a 20-s exposure per frame; the crystal—detector distance was 45 mm. The structure was solved by the direct method and refined by least squares in the anisotropic approximation for nonhydrogen atoms. The hydrogen atoms of the hydroxy groups were revealed from the difference Fourier synthesis, and their positions were refined with fixed thermal parameters. The hydrogen atoms at O(2) and O(4) were found to be statistically disordered over two positions (A and B) with equal occupancies (Table 3). The other hydrogen atoms were placed into geometrically calculated positions and included in the refinement in the "rider" model. The data collection and editing and the refinement of unit cell parameters were performed using the SMART SAINT Plus program package.[19] All calculations involved in structure solution and refinement were carried out using the SHELXTL/PC program package.[20]

Table 3. Geometric parameters in structure **1**

The O—H...O bond	Distance/Å			Angle O—H...O /deg
	O—H	H...O	O...O	
O(1)—H(1)...O(1)[a]	0.65(1)	2.15(1)	2.748(1)	153(2)
O(2)—H(2A)...O(2)[a]	0.88(2)	1.87(2)	2.730(2)	163(3)
O(2)—H(2B)...O(4)[b]	0.82(4)	1.93(4)	2.712(2)	161(3)
O(4)—H(4A)...O(2)[c]	0.70(4)	2.04(5)	2.712(2)	160(5)
O(4)—H(4B)...O(1)	0.98(4)	1.68(4)	2.662(2)	174(4)

Symmetric transformations: [a] $(-x + 2, y, -z + 1)$, [b] $(x, y - 1, z)$, [c] $(x, y + 1, z)$.

The crystal data for **3** are deposited at the Cambridge Crystallographic Center (CCDC 209830).

The ^{1}H and ^{13}C NMR spectra of solutions in $CDCl_3$ were recorded on Bruker DPX-300 (300.13 and 75.47 MHz) and Bruker DRX-500 (500.13 and 125 MHz) spectrometers with Me_4Si as the internal standard. The IR spectra of solutions in $CHCl_3$ were measured on a Specord M 82 instrument (Carl Zeiss, Jena). Melting points were determined on a Leica Galen III instrument. Optical rotation was measured on a Perkin—Elmer 141 polarimeter. MALDI mass spectra were run on a Bruker Biflex III mass spectrometer equipped with an N_2 laser (337 nm). Mass spectra (EI, 70 eV) were obtained on a LKB 9000s mass spectrometer.

Cultivation of the fungus. The KMM 4629 strain was isolated from a nonidentified colonial ascidium collected from a depth of 120 m near the Shikotan island (the Kuril isles). The fungus was cultivated for 21 day at 22 °C in twenty 500-mL flasks each containing the medium of the following composition: rice 20 g, yeast extract 20 mg, KH_2PO_4 10 mg, sea water 40 mL.

Isolation of compounds 1—3. The fungus mycelium with the medium was extracted twice with ethyl acetate. The extract was concentrated and the residue was dissolved in an ethanol—water mixture (70 : 30). The resulting solution was extracted with hexane. Ethanol was evaporated *in vacuo* and the remaining aqueous layer was extracted twice with *n*-butanol. The butanol extract was concentrated *in vacuo*. The dry residue (200 mg) was chromatographed on a column (25×2 cm) with SiO_2. Compounds **1** and **3** were eluted with a 85 : 15 hexane—ethyl acetate mixture, and compound **2** (30 mg) was eluted with a 80 : 20 hexane—ethyl acetate mixture. Separation of compounds **1** and **3** on Sephadex LH-20 (elution with chloroform and with a chloroform—ethanol mixture (2 : 1)) gave 30 mg of **1** and 50 mg of **3**.

Compound **1**, m.p. 144—145 °C (from acetonitrile), $[\alpha]_D^{23}$ −310 (*c* 0.15, $CHCl_3$). IR ($CHCl_3$), ν/cm^{-1}: 3600—3250, 2929, 2855, 1603, 1462, 1380, 1207, 1095. MS (EI, 70 eV), m/z ($I_{\rm rel}$ (%)): 282 $[M]^+$ (0.1), 232 $[M - H_2O - MeOH]^+$ (2), 207 (12.2), 176 $[M - H_2O - MeOH - C_4H_8]^+$ (4.7), 161 (7.9), 147 (12.5), 133 (12.8), 105 (26.5), 69 (36.0), 41 (100); (+)-HRMALDI/TOF-MS, m/z: 305.1692 $[M + Na]^+$; 305.1724 (Calculated for $C_{16}H_{26}O_4Na$).

Compound **2**, $[\alpha]_D^{23}$ −23.1 (c 0.12, MeOH) (*cf.* Ref. 17). IR ($CHCl_3$), ν/cm^{-1}: 3618, 3494, 1703, 1660, 1451, 1388, 1250, 1134, 1046. MS (EI, 70 eV), m/z ($I_{\rm rel}$ (%)): 402 $[M]^+$ (21), 384 (8), 279 (18.4), 248 (8.7), 221 (9.9), 215 (8.5), 189 (12), 187 (10.4), 147 (31), 137 (50), 95 (91), 93 (70), 81 (98.5), 57 (100), 55 (99.8). ^{13}C NMR (CD_3OD), δ: 10.5, 14.5, 15.4, 15.7, 16.3, 23.9, 28.9, 29.8, 35.0, 37.2, 39.4, 40.4, 49.0, 49.7, 66.2, 76.7, 121.6, 124.4, 129.0, 133.0, 136.6, 138.1, 146.6, 148,6, 207.8.

Compound **3**, m.p. 65—66 °C (from chloroform) (see Ref. 18). IR ($CHCl_3$), ν/cm^{-1}: 2800—3200, 1723, 1562, 1425, 1402, 1376, 1263, 1144. MS (EI, 70 eV), m/z ($I_{\rm rel}$ (%)): 102 $[M - 17]^+$ (10.0), 73 (99.1), 56 (39.0), 55 (100), 53 (27.6), 46 (93.2), 45 (99.8), 43 (99.0), 42 (99.0), 31 (99.1). ^{13}C NMR ($CDCl_3$), δ: 30.8, 69.3, 175.2. ^{1}H NMR ($CDCl_3$), δ: 2.99 (m), 4.59 (m).

The antimicrobial activities of compounds **1—3** were tested by a usual procedure.[21] The cytotoxicity was determined using a suspension of developing eggs of the sea urchin *Strongylocentrotus intermedius* by a procedure described previously.[22]

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