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ORIGINAL ARTICLE

New sesquiterpenes, JBIR-27 and -28, isolated from a tunicate-derived fungus, *Penicillium* sp. SS080624SCf1

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In the course of our screening program for novel metabolites from tunicate-derived fungi, novel sesquiterpenoids, named JBIR-27 (1) and -28 (2), together with known sporogen-AO1 and phomenone, were isolated from the culture broth of *Penicillium* sp. SS080624SCf1. The structures of 1 and 2 were determined to be eremophilane analogs on the basis of extensive NMR and MS analyses. Sporogen-AO1, phomenone and 2 showed cytotoxicity against human cervical carcinoma cell line HeLa at IC_{50} values of 8.3, 19 and 92 μM, respectively, whereas 1 was inactive at a concentration of 80 μM.
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INTRODUCTION

Many bioactive substances have been isolated from marine organisms, such as marine microorganisms, phytoplankton, algae, sponges and tunicates. Moreover, marine microorganisms have been studied as the important resources for new biologically active metabolites.[1] Especially, marine-derived fungi are emerging as an attractive source for discovering new bioactive compounds.[2] Indeed, cytotoxic metabolites, such as diketopiperazine alkaloids,[3] trichodermatides[4] and carbonarones,[5] have been isolated from metabolites of marine-derived fungi. Tunicates are a rich source of unique and biologically active metabolites.[1] However, there have been few reports of compounds isolated from tunicate-derived fungi.[6,7] Therefore, we attempted to isolate fungi from a tunicate, *Didemnum molle,* and obtain secondary metabolites from the fungal culture broths.

In the course of chemical screening for novel compounds from the metabolites of tunicate-derived fungi, we isolated two novel sesquiterpenoid compounds, designated as JBIR-27 (**1**) and -28 (**2**), from the culture broth of *Penicillium* sp. SS080624SCf1 (Figure 1). In addition, we also isolated known derivatives, sporogen-AO1[8] and phomenone[9] (Figure 1). This paper describes the fermentation, isolation and brief biological activity of **1**, **2**, sporogen-AO1 and phomenone, in addition to the taxonomy of the producing microorganism. The structure elucidation of **1** and **2** is also reported.

RESULTS AND DISCUSSION

Taxonomy

The sequence analysis of ribosomal DNA and ITS region of the producing fungus showed high sequence similarities with *Penicillium* sp. strain NRRL 32575 (DQ123664, 99.6%) and *Penicillium roseopurpureum* strain NRRL 2064 (AF033415, 98.2%). Moreover, this strain showed morphological features typical to the genus *Penicillium,* such as penicillate conidiophore, verticillate phialides and phialides forming basipetal chains of dry conidia. On the basis of the characteristics described above, the strain SS080624SCf1 was identified as a member of the genus *Penicillium*.

Fermentation

Penicillium sp. SS080624SCf1 was cultivated in 50-ml test tubes containing 15 ml of the seed medium. The test tubes were shaken on a reciprocal shaker (355 r.p.m) at 27 °C for 3 days. Aliquots (5 ml) of the seed culture were inoculated to 500-ml Erlenmeyer flasks containing the production medium and incubated in static culture at 27 °C for 14 days.

Isolation

The culture broth (10 flasks) was extracted with 80% aqueous Me_2CO. After concentration *in vacuo*, the aqueous concentrate was extracted with EtOAc. After drying over Na_2SO_4, the EtOAc layer was evapo-

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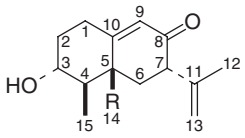
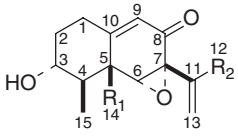


R = CH_2OH JBIR-27 (**1**)
R = CH_3 petasol



$R_1 = CH_2OH$ $R_2 = CH_3$ JBIR-28 (**2**)
$R_1 = R_2 = CH_3$ sporogen-AO1
$R_1 = CH_3$ $R_2 = CH_2OH$ phomenone

Figure 1 Structures of JBIR-27 (**1**), -28 (**2**), petasol, sporogen-AO1 and phomenone.

rated to dryness. The dried residue (1.012 g) was chromatographed on normal-phase medium-pressure liquid chromatography developed with a $CHCl_3$-MeOH gradient system, and fractions, including major metabolites, were collected by liquid chromatography-MS monitoring. As a result, sporogen-AO1 was obtained from 2% MeOH eluates. The 5% MeOH eluate (61.6 mg) was purified by preparative reversed-phase HPLC on an L-column2 ODS column developed with 35% MeOH-H_2O containing 0.1% formic acid (flow rate: 10 ml min^{-1}) to yield **1** (12.1 mg, Rt 14 min), **2** (1.4 mg, Rt 11 min) and phomenone (1.7 mg, Rt 9 min). Compound **1** (4.8 mg, Rt 14 min) was further purified by preparative reversed-phase HPLC on an X-bridge column developed with 35% MeOH-H_2O containing 0.1% diethylamine (flow rate: 10 ml min^{-1}).

Table 1 Physicochemical properties of 1 and 2

	1	*2*
Appearance	Colorless amorphous powder	Colorless amorphous powder
Melting point	135 °C	120 °C
$[\alpha]_D^{25}$ (MeOH)	+161.1 (*c* 0.5)	+188.7 (*c* 0.2)
HR-ESI-MS (*m/z*) found	251.1616 $(M+H)^+$	265.1424 $(M+H)^+$
Calcd	251.1647 (for $C_{15}H_{23}O_3$)	265.1440 (for $C_{15}H_{21}O_4$)
UV λ_{max} (MeOH) (nm) (ε)	238 (8700)	237 (11 700)
IR ν_{max} (KBr) (cm^{-1})	3320, 1660	3330, 1670

Table 2 ^{13}C- and ^{1}H-NMR data of 1 and 2 in CD_3OD

	1		*2*	
Position	^{13}C	^{1}H (*J* in Hz)	^{13}C	^{1}H (*J* in Hz)
1	31.7	2.60, ddd (14.7, 9.6, 5.0) 2.39, ddd (14.7, 4.1, 4.0)	30.7	2.60, ddd (14.4, 9.7, 4.9) 2.37, dt (14.4, 3.8)
2	35.1	2.14, dddd (14.5, 5.0, 4.6, 4.0) 1.41, dddd (14.5, 10.9, 9.6, 4.1)	35.1	2.12, dddd (14.3, 4.9, 4.3, 3.8) 1.38, dddd (14.3 10.9, 9.7, 3.8)
3	70.5	3.69, dt (10.9, 4.6)	70.2	3.59, dt (10.9, 4.3)
4	50.7	1.34, dq (10.9, 7.0)	44.3	1.78, dq (10.9, 7.0)
5	45.3		47.8	
6	40.4	2.31, dd (13.5, 5.2) 1.91, dd (14.3, 13.5)	67.6	3.45, s
7	51.6	3.63, dd (14.3, 5.2)	63.4	
8	201.2		194.5	
9	126.1	5.89, d (1.6)	123.8	5.78, d (1.7)
10	167.5		160.8	
11	144.0		140.4	
12	18.6	1.67, br s	18.5	1.83, br s
13	113.7	4.87[a], 4.80, m	112.5	5.20, 5.03, m
14	64.7	3.90, d (11.2), 3.85, d (11.2)	61.0	3.93, d (11.3), 3.82, d (11.3)
15	10.0	1.10, d (7.0)	10.6	1.24, d (7.0)

[a]Overlapped.

Structure elucidation

The physicochemical properties of **1** and **2** are summarized in Table 1. Compound **1** was obtained as a colorless amorphous powder. The HR-MS spectrum of **1** established its molecular formula as $C_{15}H_{22}O_3$ by HR-ESI-MS data [*m/z* 251.1616 $(M+H)^+$]. The IR spectrum showed absorbance for a hydroxyl ($\nu_{max,}$ 3320 cm^{-1}) and an α,β-unsaturated ketone ($\nu_{max,}$ 1660 cm^{-1}) group, respectively. Their structures were determined by detailed analyses of a series of NMR spectra. The tabulated ^{1}H- and ^{13}C-NMR spectral data obtained from heteronuclear single-quantum coherence spectrum are summarized in Table 2. Three partial structures were established by double-quantum-filtered-COSY and constant-time heteronuclear multiple-bond correlation spectra as follows (Figure 2).

The sequence from methylene protons H-1 (δ_H 2.60 and 2.39) to a doublet methyl proton H-15 (δ_H 1.10) through methylene protons H-2 (δ_H 2.14 and 1.41), an oxymethine proton H-3 (δ_H 3.69) and a methine proton H-4 (δ_H 1.34) was observed in double-quantum-filtered-COSY spectrum. In addition, H-15 showed ^{1}H–^{13}C long-range couplings to an oxymethine carbon C-3 (δ_C 70.5), a methine carbon C-4 (δ_C 50.7) and a quaternary carbon C-5 (δ_C 45.3) in the constant-time heteronuclear multiple-bond correlation spectrum. The constant-time heteronuclear multiple-bond correlations from doublet hydroxymethyl protons H-14 (δ_H 3.90 and 3.85) to a quaternary sp^2 carbon C-10 (δ_C 167.5), a methylene carbon C-6 (δ_C 40.4) and C-5, from H-1 to an olefinic carbon C-9 (δ_C 126.1) and C-10 deduced that these carbons constructed a six-membered ring system. A spin coupling system was observed between methylene protons H-6 (δ_H 2.31 and 1.91) and a methine proton H-7 (δ_H 3.63). The long-range couplings from H-6 to C-5, a methine carbon C-7 (δ_C 51.6), a ketone carbon C-8 (δ_C 201.2), C-10 and C-14, from H-7 to C-6 and C-8, from an olefinic proton H-9 (δ_H 5.89) to C-1, C-5 and C-7 established an octalone ring structure involving of an α,β-unsaturated carbonyl group. Finally, the long-range couplings from a singlet allylic methyl proton H-12 (δ_H 1.67) to a quaternary sp^2 carbon C-11 (δ_C 144.0), an exomethylene carbon C-13 (δ_C 113.7) and C-7, from exomethylene protons H-13 (δ_H 4.87 and 4.80) to a methyl carbon C-12 (δ_C 18.6) and C-7, from H-7 to C-11, C-12 and C-13 revealed that an isopropenyl group was substituted at the position of C-7. The relative configuration of **1** was determined by NOESY spectrum. NOESY correlations were observed between H-3/H-15, H-14/H-15, H-7/H-14 and H-4/[H-6ax (δ_H 1.91)]. Thus, the structure, including relative stereochemistry of **1,** was determined as shown in Figure 1.

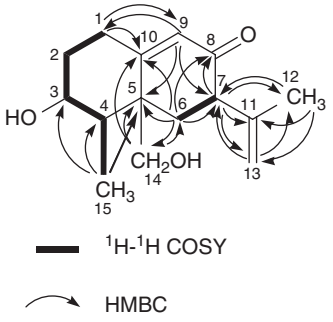


Figure 2 CT-HMBC and COSY correlations observed for **1**.

Compound **2** was obtained as a colorless amorphous powder. The molecular formula of **2** was determined as $C_{15}H_{20}O_4$, based on HR-ESI-MS data [*m/z* 265.1424 $(M+H)^+$]. The IR and UV spectra were similar to those of **1**. Furthermore, the NMR spectral data were almost similar to those of **1** (Table 2), except for chemical shifts at C-6 and C-7. The typical ^{13}C chemical shifts, C-6 (δ_C 67.6) and C-7 (δ_C 63.4), suggest an epoxy functional group. Thus, these data proved **2** to be 6,7-epoxyl JBIR-27 (Figure 1). The relative configuration of **2** was also determined by NOESY spectrum. NOESY correlations of **2** were observed between H-3/H-15, H-14/H-15, H-6/H-15 and H-6/H-14. Biosynthetically, the absolute configurations of **1** and **2** were assumed to be similar to those of petasol[10] and sporogen-AO1,[8] respectively, as these compounds were derivatives of petasol and/or sporogen-AO1 by oxygenation at C-14 in the skeleton (Figure 1).

We herein isolated two new eremophilane analogs, **1** and **2,** from the culture broth of a tunicate-derived *Penicillium* sp. SS080624SCf1. Although eremophilane analogs are known to be isolated from many fungal metabolites,[11–15] this paper is the first report that a tunicate-derived *Penicillium* sp. produces terpenoid compounds.

Biological activity

To evaluate the cytotoxicity of **1**, **2**, sporogen-AO1 and phomenone against mammalian cells, we used human cervical carcinoma cell line HeLa. Sporogen-AO1, phomenone and **2** showed cytotoxicity against HeLa cells at IC_{50} values of 8.3, 19 and 92 μM, respectively. In contrast, **1** had no cytotoxic activity at the concentration of 80 μM. Thus, these results suggest that the epoxy group and the methyl group at C-14 in sporogen-AO1 and phomenone might play important roles for the cytotoxic effects against HeLa cells. Sporogen-AO1 recently has been reported as an inhibitor of human inducible nitric-oxide synthase expression.[16] Therefore, studies on detailed biological activities are now underway.

METHODS

General experimental procedures

Melting points were determined with a Yanagimoto micro melting point apparatus (Yanagimoto, Kyoto, Japan). Optical rotations were assigned on a SEPA-300 polarimeter (Horiba, Kyoto, Japan). HR-ESI (electrospray ionization)-MS data were recorded on an LCT-Premier XE mass spectrometer (Waters, Milford, MA, USA). UV and IR spectra were measured on a DU730 UV/Vis spectrophotometer (Beckman, Fullerton, CA, USA) and an FT-720 spectrophotometer (Horiba), respectively. NMR spectra were taken on an NMR System 500 NB CL (Varian, Palo Alto, CA, USA) in CD_3OD with the residual solvent peak as an internal standard (δ_C 49.0, δ_H 3.30 p.p.m.). Normal-phase medium-pressure liquid chromatography was performed on a Purif-Pack SI-60 column (Moritex, Tokyo, Japan). Analytical reversed-phase HPLC was carried out using an L-column2 ODS column (4.6 i.d.×150 mm; Chemical Evaluation and Research Institute, Tokyo, Japan) with a 2996 photodiode array detector (Waters) and a 3100 mass detector (Waters). Preparative reversed-phase HPLC was carried out using an L-column2 ODS (20 i.d.×150 mm) or an X-bridge column (20 i.d.×150 mm; Waters) with an L-2455 photodiode array detector (Hitachi High Technologies, Tokyo, Japan). Reagents and solvents were of the highest grade available.

Microorganism

The producing fungus, designated as SS080624SCf1, was isolated from a tunicate, *D. molle*, collected from Ishigaki Island, Okinawa Prefecture, Japan. The SS080624SCf1 strain was identified based on its ribosomal RNA ITS region and morphology observed under a Zeiss Axio Plan 2 imaging system (Carl Zeiss, Oberkochen, Germany).

Medium

The seed medium, potato dextrose was composed of 2.4 g l^{-1} Potato Dextrose Broth (BD Biosciences, San Jose, CA, USA). The production medium consisted of 15 g brown rice (Hitomebore, Miyagi, Japan) and 45 ml solution containing 0.67 g l^{-1} Bacto-Yeast Extract (BD Biosciences), 0.33 g l^{-1} sodium tartarate dihydrate (Kanto Chemical, Tokyo, Japan) and 0.33 g l^{-1} K_2HPO_4 (Wako Pure Chemical, Tokyo, Japan) in a 500-ml Erlenmeyer flask.

Cytotoxic assay

Human cervical carcinoma cell line HeLa was used in this study. The cells were cultured in Dulbecco's modified Eagle's medium (Wako Pure Chemical) supplemented with 10% fetal bovine serum (Gibco, Carlsbad, CA, USA), penicillin (100 U ml^{-1}) and streptomycin (100 μg ml^{-1}) at 37 °C in a humidified incubator with 5% CO_2. The cytotoxic activity was estimated by WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] colorimetric assay. HeLa cells were incubated on 96-well plates at a density of 5.0×10^3 cells per well in 100 μl of medium for overnight, and then treated with compounds at various concentrations for 48 h. Next, 10 μl of WST-8 reagent solution (Cell Counting Kit; Dojindo, Kumamoto, Japan) was added and incubated for an hour at 37 °C in a humidified incubator with 5% CO_2. The absorbance of the formazan dye formed was measured at 450 nm.

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