NOTE

Three eremophilane derivatives, MBJ-0011, MBJ-0012 and MBJ-0013, from an endophytic fungus *Apiognomonia* sp. f24023

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Eremophilane sesquiterpenoids are well-known cytotoxic, phytotoxic, mycotoxic and phytohormonic secondary metabolites that are obtained from fungi and higher plants.^{1–9} During this half decade, we have constructed the library of isolated natural products (CB library), mostly of microbial origin, with the aim of efficiently implementing several biological screenings.¹⁰ In the course of our screening for novel natural compounds, which will provide new scaffolds for drug development, we have developed the advanced compound-identification system designated as 'MBJ's special selection' and have succeeded in discovering many new natural compounds. Among them, eremophilane derivatives MBJ-0011 (**1**), MBJ-0012 (**2**) and MBJ-0013 (**3**) were discovered as cytotoxic compounds from an endophytic fungus *Apiognomonia* sp. f24023 (Figure 1). The fermentation, isolation, structural elucidation and preliminary biological activities of **1–3** are described herein.

The producing microorganism, *Apiognomonia* sp. f24023, classified based on partial sequence of the D1/D2 region of the 28S ribosomal RNA gene (data not shown), was isolated from a plant growing in Iwata, Shizuoka Prefecture, Japan. The strain was cultivated in 250-ml Erlenmeyer flasks, each containing 25 ml of a seed medium consisting of potato starch (Tobu Tokachi Nosan Kako Agricultural Cooperative Assoc., Hokkaido, Japan) 2%, glucose (Junsei Chemical, Tokyo, Japan) 2%, soybean powder (Honen SoyPro, J-Oil Mills, Tokyo, Japan) 2%, KH₂PO₄ 0.1%, and MgSO₄ · 7H₂O 0.05%. The flasks were shaken on a rotary shaker (220 r.p.m.) at 25 °C for 3 days. Aliquots (0.5 ml) of the broth were transferred to 500-ml Erlenmeyer flasks containing 50 ml of a production medium consisting of fructose (Junsei Chemical) 1.5%, xylitol (Junsei Chemical) 1.5%, maltose monohydrate (Junsei Chemical) 1.5%, galactose (Wako Pure Chemical Industries, Osaka, Japan) 1.5%, soybean powder 2%, gluten meal (Nihon Shokuhin Kako, Tokyo, Japan) 1%, yeast extract powder (Oriental Yeast, Tokyo, Japan) 0.5%, ZnSO₄ · 7H₂O 0.02%,

MgSO₄ · 7H₂O 0.02%, FeSO₄ · 7H₂O 0.02%, and 3-indoleacetic acid 0.002%, pH 6.0 (adjusted before sterilization), and were cultured on a rotary shaker (220 r.p.m.) at 25 °C for 4 days. After fermentation, the whole culture broth (2 l) was extracted with an equal volume of *n*-BuOH. The *n*-BuOH extract was partitioned between CHCl₃ (350 ml × 3) and brine (350 ml). The organic layer was evaporated and the concentrate (868 mg) was fractionated using medium-pressure silica gel chromatography (Purif-Pack SI-30, Shoko Scientific, Yokohama, Japan) with the gradient system of *n*-hexane–EtOAc (0–25% EtOAc) followed by the stepwise solvent system of CHCl₃–MeOH (0, 2, 5, 10, 20, 30 and 100% MeOH). Compound **1** (2.5 mg) was obtained after purification of the 2% MeOH fraction (21 mg) by reversed-phase HPLC using a CAPCELL PAK C18 MGII column (5.0 μm, 20 i.d. × 150 mm; Shiseido, Tokyo, Japan) with 60% aqueous MeOH containing 0.1% formic acid (flow rate: 10 ml min^{–1}, retention time (Rt) = 19.5 min). The 5% MeOH fraction (102 mg) was applied to reversed-phase medium-pressure column (Purif-Pack ODS-30, Shoko Scientific, Yokohama, Japan) and developed with the stepwise H₂O–MeOH solvent system (70, 80 and 100% MeOH). The 80% MeOH eluate (25.6 mg) was subjected to HPLC (CAPCELL PAK C18 MGII column, 70% aqueous MeOH containing 0.1% formic acid, flow rate: 10 ml min^{–1}) to yield **2** (17.2 mg, Rt = 14.6 min) and crude **3** (8.3 mg, Rt = 17.0 min). The crude material containing **3** was then re-chromatographed with the same system to give pure **3** (1.2 mg, Rt = 17.0 min).

MBJ-0011 (**1**) was isolated as a colorless amorphous solid: [α]_D²⁷ + 20 (c 0.13, MeOH); UV λ_{max} nm (log ε): 276 (4.2) in MeOH; IR (ATR) ν_{max} 1650 cm^{–1} (unsaturated ketone). Its molecular formula was determined as C₂₂H₂₈O₅ by HR electrospray ionization MS (HR-ESI-MS) (*m/z* 373.1994 [M + H]⁺, calcd for C₂₂H₂₉O₅: 373.2015). The planar structure was clarified by the series of 2D NMR analyses, including double quantum filtered COSY (DQF-COSY),

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Figure 1 Structures of 1–3.

heteronuclear single quantum coherence and constant-time heteronuclear multiple-bond correlation¹¹ (CT-HMBC; Figures 2a and b). The ¹³C and ¹H NMR data for 1 are listed in Table 1.

The structure determination of 1 was carried out by dividing it into two partial moieties on the basis of DQF-COSY and CT-HMBC as follows. The sequence from an oxymethine proton H-3 (δ_{H} 5.38) to a methyl proton H₃-14 (δ_{H} 0.99) through a methine proton H-4 (δ_{H} 1.88) was observed in the DQF-COSY. Long-range ¹H–¹³C couplings from the methyl proton H₃-14 to an oxymethine carbon C-3 (δ_{C} 74.0), a methine carbon C-4 (δ_{C} 43.7) and a quaternary carbon C-5 (δ_{C} 37.9), and from a methyl proton H₃-15 (δ_{H} 1.21) to C-4, C-5, a methylene carbon C-6 (δ_{C} 39.6) and an olefinic quaternary carbon C-10 (δ_{C} 160.1) revealed the sequence from C-3 to C-6 as shown in Figure 2a. HMBC correlations from an olefinic methine proton H-2 (δ_{H} 6.01), which was spin coupled to an olefinic methine proton H-1 (δ_{H} 6.22), to C-4 and the olefinic quaternary carbon C-10, and from the olefinic proton H-1 to C-3, C-5 and C-10 established the six-membered cyclic moiety. Long-range ¹H–¹³C coupling from methylene protons H₂-6 (δ_{H} 2.01 and 1.93) to an α,β -unsaturated ketone carbonyl carbon C-8 (δ_{C} 198.5), which in turn long-range coupled to a methine proton H-7 (δ_{H} 3.25), was observed. Together with these HMBC correlations, long-range couplings from an olefinic proton H-9 (δ_{H} 5.80) to C-5, a methine carbon C-7 (δ_{C} 50.6) and an olefinic carbon C-1 (δ_{C} 129.6) revealed an 2-decalone moiety. Furthermore, long-range couplings from a methyl proton H₃-13 (δ_{H} 1.72) to C-7, an olefinic quaternary carbon C-11 (δ_{C} 143.3) and an exomethylene carbon C-12 (δ_{C} 115.0) established a 6-oxygenated 4,4a,5,6-tetrahydro-4a,5-dimethyl-3-(1-methylethenyl)-2(3H)-naphthalenone moiety (an eremophilane skeleton) as shown in Figure 2a.

The other substructure of 1 was elucidated as follows. A sequence from doublet methyl protons H₃-7' (δ_{H} 1.25) to methylene protons H₂-5' (δ_{H} 2.57 and 2.56) through a methine proton H-2' (δ_{H} 2.77), an oxygenated methine proton H-3' (δ_{H} 4.71) and methylene protons H₂-4' (δ_{H} 2.38 and 1.96) was established by the analysis of the DQF-COSY. Long-range ¹H–¹³C couplings from H-3' and H-5' to an ester carbonyl carbon C-6' (δ_{C} 176.1) revealed a γ -lactone moiety

Figure 2 (a) Partial structure of 1 (sesquiterpenoid moiety). (b) Partial structure of 1 (tetrahydro- α -methyl-5-oxo-2-furanacetic acid moiety). (c) Key NOESY correlations of 1. (d) Partial structure of 2. (e) Partial structure of 3. (f) Key NOESY correlations of 3. COSY and HMBC (¹H to ¹³C) correlations are shown as bold lines and solid arrows, respectively.

(Figure 2b). In addition to these correlations, a long-range coupling from the methyl proton H₃-7' to an ester carbonyl carbon C-1' (δ_{C} 172.9) determined the presence of a tetrahydro- α -methyl-5-oxo-2-furanacetic acid moiety.

An ester linkage between the two partial structures was proved by an HMBC correlation from the low-field-shifted methine proton H-3 by acylation to the ester carbonyl carbon C-1'. Therefore, the gross structure of 1 was determined as shown in Figure 1. The relative configuration of the sesquiterpenoid moiety of 1 was clarified by vicinal ³J_{H,H} values and NOESY correlations (Figure 2c). The coupling constants of H-7 ($J = 5.0$ and 14.0 Hz) indicated that this proton had a pseudoaxial orientation. Furthermore, NOESY correlations between H-7 and H₃-14 and between H₃-14 and H-3 revealed that C-14 and H-3 are also pseudoaxial orientations. Furthermore, C-15 was determined to have pseudoequatorial orientation on the basis of a NOESY correlation between H₃-14 and H₃-15. Hence, the partial relative configuration of 1 was determined as shown in Figure 1.

MBJ-0012 (2) and MBI-0013 (3) were isolated as colorless amorphous solids with optical rotations of $[\alpha]_{\text{D}}^{27} -26$ (c 0.43, MeOH) for 2 and $[\alpha]_{\text{D}}^{27} -54$ (c 0.06, MeOH) for 3. They showed UV absorption maxima at 230 ($\log \epsilon$ 4.5) and 275 ($\log \epsilon$ 4.4) nm for 2, and at 229 ($\log \epsilon$ 4.4), 275 ($\log \epsilon$ 4.3) and 310 (sh) nm for 3, suggesting the presence of similar unsaturated systems, and their IR spectra indicated the presence of unsaturated ketone (1650 cm^{-1}) and hydroxy (3300 cm^{-1}) functional groups. Their HR-ESI-MS spectra gave the same molecular formula of C₂₆H₃₆O₅ (obsd: m/z 429.2649 $[M+H]^+$ for 2 and m/z 429.2666 $[M+H]^+$ for 3, calcd for C₂₆H₃₇O₅: 429.2641).

The NMR data for 2 indicated that it had the same sesquiterpenoid moiety as 1, including the stereochemistry. The determination of the remaining substructure was carried out as follows (Figure 2d). Based on the analysis of the DQF-COSY, the ¹H sequence from methyl protons H₃-11' (δ_{H} 1.18) to methyl protons H₃-10' (δ_{H} 1.19) through a methine proton H-2' (δ_{H} 2.62), an oxygenated methine proton H-3' (δ_{H} 4.25), olefinic methine protons H-4' (δ_{H} 5.59), H-5' (δ_{H} 6.26), H-6' (δ_{H} 6.11) and H-7' (δ_{H} 5.73), methylene protons H₂-8'

Table 1 ^{13}C and ^1H NMR spectroscopic data for MBJ-0011 (1), MBJ-0012 (2) and MBJ-0013 (3)

1			2			3		
Position	δ_{C}	δ_{H} , multiplicity (<i>J</i> in Hz)	δ_{C}	δ_{H} , multiplicity (<i>J</i> in Hz)		δ_{C}	δ_{H} , multiplicity (<i>J</i> in Hz)	
1	129.6	6.22, dd (2.0, 10.0)	129.4	6.19, dd (1.5, 10.0)		129.1	6.19, br d (10.0)	
2	135.3	6.01, dd (2.0, 10.0)	135.7	5.98, dd (1.5, 10.0)		135.0	5.95, br d (10.0)	
3	74.0	5.38, ddd (2.0, 2.0, 10.0)	73.5	5.37, br d (10.0)		73.6	5.34, br d (9.0)	
4	43.7	1.88, m	43.7	1.87, m		43.2	1.89, ovl ^a	
5	37.9		37.9			39.3		
6	39.6	2.01, ovl ^a ; 1.93, ovl ^a	39.6	2.00, dd (5.0, 13.0); 1.93, dd (13.0, 13.0)		39.5	2.93, d (13.0); 2.18, ovl ^a	
7	50.6	3.25, dd (5.0, 14.0)	50.6	3.24, dd (5.0, 13.0)		126.8		
8	198.5		198.6			191.0		
9	125.3	5.80, s	125.2	5.78, s		127.6	5.79, s	
10	160.1		160.2			158.0		
11	143.3		143.3			146.3		
12	115.0	4.99, br s; 4.86, br s	114.9	4.99, br s; 4.85, br s		23.3	2.16, s	
13	19.8	1.72, s	19.8	1.71, s		22.9	1.88, s	
14	10.3	0.99, d (7.0)	10.2	0.98, d (7.0)		10.3	1.02, d (7.0)	
15	16.7	1.21, s	16.7	1.20, s		16.4	1.00, s	
1	172.9		175.2			175.2		
2	44.8	2.77, dq (7.0, 7.0)	45.9	2.62, dq (7.0, 7.0)		45.8	2.62	
3	80.6	4.71, ddd (7.0, 7.0, 8.5)	74.5	4.25, dd (7.0, 7.0)		74.5	4.23, dd (7.0, 7.0)	
4	25.4	2.38, dddd (7.0, 7.0, 14.0, 14.0); 1.96, m	131.2	5.59, dd (7.0, 15.0)		131.3	5.60, dd (7.0, 15.5)	
5	28.5	2.57, ovl ^a ; 2.56, ovl ^a	132.6	6.26, dd (11.0, 15.0)		132.6	6.27, dd (10.0, 15.5)	
6	176.1		132.2	6.11, dd (11.0, 15.0)		132.3	6.12, dd (10.0, 15.0)	
7	12.6	1.25, d (7.0)	131.6	5.73, ddd (8.0, 8.0, 15.0)		131.6	5.73, ddd (7.0, 7.0, 15.0)	
8			42.5	2.27, ovl ^a ; 2.22, ovl ^a		42.5	2.28, ovl ^a ; 2.22, ovl ^a	
9			67.3	3.84, m		67.3	3.85, ddq (1.0, 6.0, 7.0)	
10			22.9	1.19, d (7.0)		23.0	1.20, d (7.0)	
11			14.1	1.18, d (7.0)		14.1	1.19, d (7.0)	

Measured on a 500 NB CL NMR spectrometer (Varian, Palo Alto, CA, USA) at 500 MHz for ^1H and 125 MHz for ^{13}C with the residual solvent peak as the internal standard (7.25 p.p.m. for ^1H and 77.0 p.p.m. for ^{13}C in CDCl_3).

^aOverlapped with other signals.

(δ_{H} 2.27, 2.22) and an oxygenated methine proton H-9' (δ_{H} 3.84) was established. HMBC correlations from H-2', H-3', H-11' and H-3 to an ester carbonyl carbon C-1' (δ_{C} 175.2) were indicative of the direct connectivity between C-1' and C-2' and the presence of an ester linkage between C-3 and C-1'. Therefore, the gross structure of **2** was established as shown in Figure 1. The geometries of the double bonds (C-4' and C-6') were deduced as 4'*E* and 6'*E* based on the large vicinal coupling constants between H-4' and H-5', and H-6' and H-7' (15.0 Hz and 15.0 Hz, respectively).

The 1D and 2D NMR spectra of **3** were quite similar to those of **2**, except for the signals corresponding to the C-7, C-11, C-12 and C-13 positions. In the CT-HMBC spectrum of **3**, allylic methyl protons H₃-12 (δ_{H} 2.16) and H₃-13 (δ_{H} 1.88) were long-range coupled to each other and both were also coupled to olefinic carbons C-7 (δ_{C} 126.8) and C-11 (δ_{C} 146.3) (Figure 2e). The assignments of the *gem*-methyl groups C-12 and C-13 were determined by NOESY correlations among H-13, H-14 and Ha-6 (Figure 2f). Given these, the planar structure was determined as shown in Figure 1. The relative configuration of the sesquiterpenoid moiety of **3** was established in the same manner as that of **1**. The large $^3J_{\text{H-3,H-4}}$ (9.0 Hz) showed the pseudodiaxial relationship between H-3 and H-4. NOESY correlations between H-4 and Hb-6 (δ_{H} 2.18) and between H-3 (δ_{H} 5.34) and H-14 suggested that the Hb-6 and C-14 are in pseudoaxial orientation and are located on the opposite side. Therefore, the partial relative configuration was determined as shown in Figure 2f.

The cytotoxic activities of **1–3** against human ovarian adenocarcinoma SKOV-3 cells were examined by using the WST-8 5-(2,4-

disulphophenyl)-3-(2-methoxy-4-nitrophenyl)-2-(4-nitrophenyl)-2*H*-tetrazolium, monosodium salt] colorimetric assay (Cell Counting Kit; Dojindo, Kumamoto, Japan). After 72 h of treatment, **1** exhibited moderate cytotoxic activity against SKOV-3 cells with the IC₅₀ of 3.4 μM . On the contrary, **2** and **3** showed weak cytotoxicity (IC₅₀ = 63 μM and 54 μM , respectively).

Although eremophilane derivatives have been isolated from many fungal metabolites,^{2–9} to the best of our knowledge, **1** is the first example of an eremophilane derivative possessing a tetrahydro- α -methyl-5-oxo-2-furanacetic acid moiety.

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