NOTE

Cytotoxic sesquiterpenoids MBJ-0009 and MBJ-0010 from a saprobic fungus *Nectria* sp. f26111

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During our screening program for novel microbial metabolites using the advanced compound-identification system designated as ‘MBJ’s special selection’, we succeeded in the discovery of new natural compounds.^{1,2} Among them, ester-substituted sesquiterpenoids related to the eremophilane class MBJ-0009 (**1**) and MBJ-0010 (**2**) were isolated from a fungus *Nectria* sp. f26111 (Figure 1). These type of compounds isolated from fungal cultures have been reported as cytotoxic,^{3–5} antimalarial⁵ agents and cell-adhesion inhibitors.⁶ Here, we report the fermentation, isolation, structure elucidation and brief biological activity of **1** and **2**.

The fungus *Nectria* sp. f26111 was isolated from a soil sample collected in Kochi Prefecture, Japan. The strain was cultivated in 250-ml Erlenmeyer flasks, each containing 25 ml of a seed medium consisting of 2% potato starch (Tobu Tokachi Nosan Kako Agricultural Cooperative Assoc., Hokkaido, Japan), 1% glucose (Junsei Chemical, Tokyo, Japan), 2% soybean powder (Honen SoyPro, J-Oil Mills, Tokyo, Japan), 0.1% KH₂PO₄ and 0.05% MgSO₄·7H₂O. 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 2% potato starch (Tobu Tokachi Nosan Kako Agricultural Cooperative Assoc.), 1% glucose (Junsei Chemical), 2% soybean powder (Honen SoyPro, J-Oil Mills), 0.1% KH₂PO₄ and 0.05% MgSO₄·7H₂O, and cultured on a rotary shaker (220 r.p.m.) at 25 °C for 4 days. As our routine step, the fermentation broth (2 l) was directly extracted with an equal volume of *n*-BuOH.

The crude extract was then partitioned between EtOAc (300 ml × 3) and H₂O (300 ml) to yield EtOAc extract (730 mg). The obtained extract was applied to a silica gel medium-pressure liquid chromatography column (Purif-Pack SI-30; Shoko Scientific Co., Ltd, Yokohama, Japan) and eluted with a gradient system of hexane–EtOAc (0–25% EtOAc) followed by the stepwise solvent system of CHCl₃–MeOH (0, 1, 2, 5, 10, 20, 30 and 100% MeOH). The 2% MeOH eluate (200 mg) was subjected to octadecylsilane-medium-pressure liquid chromatography (Purif-Pack octadecylsilane-

30; Shoko Scientific Co., Ltd) by using the stepwise solvent system (50, 60, 70, 80, 90 and 100% aqueous MeOH). The 90% MeOH fraction (127 mg) was purified by reversed-phase HPLC using a CAPCELL PAK C18 MGII column (5.0 μm, 20 i.d. × 150 mm; Shiseido, Tokyo, Japan) with 70% aqueous CH₃CN containing 0.1% formic acid (flow rate: 10 ml min^{−1}) to yield **1** (57.4 mg, retention time (Rt) = 21.7 min) and crude **2** (8.5 mg, Rt = 19.8 min). The repetitive HPLC purification of **2** (3.0 mg, Rt = 19.8 min) from the crude material containing **2** was carried out using the same procedure as **1**.

MBJ-0009 (**1**) was isolated as a pale yellow amorphous powder: [α]_D²⁴ −10 (c 0.70, MeOH); UV λ_{max} nm (ε): 340 (15 000) in MeOH; IR (attenuated total reflectance) ν_{max} cm^{−1}: 3400 (hydroxy), 1747 (aliphatic ester carbonyl) and 1720 (unsaturated ester carbonyl). Its molecular formula was determined as C₃₁H₄₂O₈ by negative ion high-resolution ESI MS (*m/z* 541.2767 [M−H][−], calculated for C₃₁H₄₁O₈: *m/z* 541.2801). The planar structure was determined by the series of 2D NMR analyses, including DQF-COSY, HSQC, and constant-time HMBC⁷ (CT-HMBC) spectra (Figure 1a). The ¹³C and ¹H NMR data for **1** are listed in Table 1.

The DQF-COSY spectrum of **1** allowed ¹H coupling networks from an oxygenated methine proton H-1 (δ_H 4.54) through an oxygenated methine proton H-2 (δ_H 5.10) to methylene protons H₂-3 (δ_H 2.40, 2.03), and between methylene protons H₂-6 (δ_H 2.06 and 1.36) and a methine proton H-7 (δ_H 2.61). In the CT-HMBC spectrum, ¹H–¹³C long-range correlations from a methyl proton H₃-15 (δ_H 1.18) to a methylene carbon C-3 (δ_C 34.9) and quaternary carbons C-4 (δ_C 74.6) and C-5 (δ_C 39.8), and from H-1 and H-2 to an oxygenated quaternary carbon C-10 (δ_C 60.7) were observed. The presence of a 1,2,3,5-tetraoxygenated cyclohexane ring moiety was revealed by long-range couplings from a methyl proton H₃-14 (δ_H 1.27) to C-4, C-5 and C-10. The additional couplings from H₃-14 to a methylene carbon C-6 (δ_C 30.9) and from an oxygenated methine proton H-9 (δ_H 3.30) to C-1, C-10, C-5, a methine carbon C-7 (δ_C 44.3) and a ketal carbon C-8 (δ_C 102.1) indicated the

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Figure 1 (a) Structures of **1** and **2**. (b) Key COSY and HMBC correlations of **1**. COSY: bold line; HMBC (^1H to ^{13}C): arrow. (c) Key NOESY correlations of **1**.

presence of an additional 1,1,2,3-tetraoxygenated cyclohexane ring moiety (C-5 to C-10). HMBC correlations from exomethylene protons H_2 -13 (δ_{H} 4.96 and 4.94, δ_{C} 105.2) to C-7, an olefinic quaternary carbon C-11 (δ_{C} 150.2) and an oxygenated methylene carbon C-12 (δ_{C} 69.9) and from H_2 -12 (δ_{H} 4.57 and 4.50) to C-8 indicated the presence of a 3-methylenetetrahydrofuran moiety. The high-fielded chemical shifts of C-9 (δ_{C} 58.7) and C-10 are considered to form an epoxide ring at these carbons, and consequently the C-8 is a hemiketal carbon. A methyl proton H_3 -17 (δ_{H} 2.08) and H-2 were ^1H - ^{13}C long-range coupled to a carbonyl ketone carbon C-16 (δ_{C} 169.2), which suggested the presence of an acetoxy group at the C-2 position. Thus, the structure of the sesquiterpenoid substructure of **1** was determined as shown in Figure 1b. The other substructure was determined as follows. In the DQF-COSY spectrum, the presence of an antiepentane moiety was revealed by ^1H - ^1H couplings from a terminal methyl proton H_3 -12' (δ_{H} 0.82) through aliphatic methylene protons H_2 -11' (δ_{H} 1.38 and 1.26) to an allylic methine proton H-10' (δ_{H} 2.41), which was also spin coupled to both a doublet methyl proton H_3 -14' (δ_{H} 0.95) and an olefinic methine proton H-9' (δ_{H} 5.42). The sequence from an olefinic proton H-2' (δ_{H} 5.80) to an olefinic proton H-7' (δ_{H} 6.44) through olefinic protons H-3' (δ_{H} 7.32), H-4' (δ_{H} 6.29), H-5' (δ_{H} 6.64) and H-6' (δ_{H} 6.21) suggested the presence of a hexatriene moiety. In addition to these two proton sequences, HMBC correlations from a methyl proton

Table 1 ^{13}C and ^1H NMR spectroscopic data for MBJ-0009 (**1**) and MBJ-0010 (**2**)

Position	1^a			2^b		
	δ_{C}	δ_{H} , mult (J in Hz)		δ_{C}	δ_{H} , mult (J in Hz)	
1	72.4	4.54, dd (2.0, 2.6)		72.5	4.58, br s	
2	70.6	5.10, ddd (2.6, 2.6, 3.7)		70.5	5.11, ddd (2.5, 3.5, 3.5)	
3	34.9	2.40, dd (3.7, 15.7)		34.9	2.40, dd (3.5, 16.0)	
4	74.6			74.6		
5	39.8			39.8		
6	30.9	2.06, dd (13.6, 13.6)		30.9	2.08, ov ^c	
7	44.3	1.36, dd (6.3, 13.6)			1.38, ov ^c	
8	102.1	2.61, dddd (1.8, 1.8, 6.3, 13.6)		44.3	2.63, dd (6.0, 13.5)	
9	58.7			102.1		
10	60.7			58.2	3.24, s	
11	150.2			60.8		
12	69.9	4.57, ddd (2.3, 4.1, 12.6)		150.0		
13	105.2	4.50, ddd (2.1, 4.1, 12.6)		70.1	4.58, ov ^c	
14	21.00	4.96, dd (2.0, 4.1)			4.52, ov ^c	
15	23.3	4.94, dd (2.3, 4.1)		105.4	4.99, dd (2.0, 4.0)	
16	169.2				4.97, dd (2.0, 4.0)	
17	21.12	1.27, s		21.1	1.30, s	
1'	165.2	1.18, s		23.3	1.18, s	
2'	117.9	2.08, s		169.2		
3'	146.7	2.08, s		21.2	2.09, s	
4'	128.1	5.80, d (15.0)		165.3		
5'	143.2	5.80, d (15.0)		118.7	5.84, d (15.5)	
6'	125.2	7.32, dd (11.4, 15.0)		141.5	7.85, dd (11.0, 15.5)	
7'	143.7	6.29, dd (11.4, 14.7)		124.4	6.04, dd (11.0, 11.0)	
8'	132.7	6.64, dd (10.9, 14.7) h		139.7	6.42, dd (11.0, 11.0)	
9'	144.0	6.21, dd (10.9, 15.2)		120.6	6.65, dd (11.0, 14.5)	
10'	34.7	6.44, d (15.2)		143.8	6.43, d (14.5)	
11'	30.2	132.7		132.7		
12'	11.9	144.0		144.3	5.44, br d (9.5)	
13'	12.5	5.42, d (9.7)		34.8	2.43, ov ^c	
14'	20.4	2.41, ov ^c		30.2	1.39, ov ^c	
		1.38, ov ^c			1.26, ov ^c	
		1.26, ov ^c			1.26, ov ^c	
		0.82, t (7.2)		12.0	0.84, t (7.5)	
		1.77, s		12.7	1.82, d (1.0)	
		0.95, d (6.7)		20.4	0.95, d (8.5)	

^a600 MHz.

^b500 MHz. NMR spectra were taken on a Varian NMR System 500 NB CL (**1**) or 600 NB CL (**2**) in CDCl_3 with the residual solvent peak as an internal standard (δ_{C} 77.0, δ_{H} 7.25 p.p.m.).

^cOverlapped with other signals.

H_3 -13' (δ_{H} 1.77) to an olefinic methine carbon C-7' (δ_{C} 143.7), an olefinic quaternary carbon C-8' (δ_{C} 132.7) and C-9' (δ_{C} 144.0) indicated the connectivity between two partial structures through the olefinic quaternary carbon C-8'. Finally, the planar structure of **1** was determined by HMBC correlations from H-1, H-2' and H-3' to a carbonyl ketone carbon C-1' (δ_{C} 165.2), which suggested an ester linkage between C-1 and C-2', as shown in Figure 1b. The large vicinal J values between H-2' and H-3', H-4' and H-5', and H-6' and H-7' (15.0, 14.7 and 15.2 Hz, respectively) suggested that the geometries of the position at C-2', C-4' and C-6' were in all *E* geometries. The chemical shift of C-13' (δ_{C} 12.5) and a NOESY correlation between H-7' and H-9' also suggested an *E* geometry of the olefinic bond at C-8'.

The relative configuration of the sesquiterpenoid moiety was established from the NOESY spectrum and the corresponding coupling constants. A large coupling constant (13.6 Hz) between H-6ax (δ_{H} 2.06) and H-7 (δ_{H} 2.61) established that these protons are in diaxial orientation. In the NOESY spectrum, NOEs among H-7, H₃-14 and H-3ax (δ_{H} 2.40) allowed that H-7, H-3ax and C-14 are located on the same direction of the molecule. Small coupling constants between H-1 and H-2 (2.6 Hz), and between H-2 and both protons of H₂-3 (2.6 and 3.7 Hz, respectively) indicated that H-2 is in an equatorial location. As an NOE between H-1 and H-3ax was not observed, H-1 is concluded to be in an equatorial location. The existence of NOE between H₃-15 and H-6eq (δ_{H} 1.36) suggested that a hydroxy group at C-4 is in an axial orientation. Furthermore, an axial orientation of a hydroxy residue at C-8 was revealed by NOESY correlations among H-13 (δ_{H} 4.94), H-7 and H-6eq. Although an NOESY correlation between H-1 and H-9 was observed, the relative configuration at C-10 could not be determined. Thus, the partial relative configuration of **1** was elucidated as shown in Figure 1c.

MBJ-0010 (**2**) was isolated as a pale yellow amorphous powder: $[\alpha]_{\text{D}}^{24} +18$ (c 0.15, MeOH); UV λ_{max} nm (ϵ): 340 (17,000) in MeOH; IR (attenuated total reflectance) ν_{max} cm⁻¹: 3400 (hydroxy), 1747 (aliphatic ester carbonyl) and 1716 (unsaturated ester carbonyl). The molecular formula of **2** was led to be C₃₁H₄₂O₈ from the negative HRESIMS ion peak at m/z 541.2791 calculated for C₃₁H₄₁O₈. The UV and IR spectra of **2** were very similar to those of **1**. Furthermore, the ¹H and ¹³C NMR data and obtained planar structure of **2** were the same as **1**. A difference was found in the coupling constant between H-4' and H-5' (11.0 Hz) revealing the geometry of 4'Z, and hence, the structure of **2** was determined as shown in Figure 1a.

The obtained structures of **1** and **2** were structurally related to cytotoxic compounds berkleasmins isolated from saprophytic fungus

Berkleasium nigroapicale.⁵ The cytotoxic activities of **1** and **2** against human ovarian adenocarcinoma SKOV-3 cells were examined by using the WST-8 [(2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) colorimetric assay (Cell Counting Kit; Dojindo, Kumamoto, Japan). After 72 h of treatment, **1** and **2** exhibited moderate cytotoxicity with the IC₅₀ values of 24.7 and 11.2 μM , respectively.

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