The Journal of Antibiotics (2014) 67, 843–845

www.nature.com/ja

NOTE

A highly oxygenated ergostane—MBJ-0005—from *Anthostomella eucalyptorum* f25427

Miho Izumikawa[1], Masashi Itoh[2], Teppei Kawahara[1], Noriaki Sakata[2], Toshio Tsuchida[2], Tamio Mizukami[3] and Kazuo Shin-ya[4]

The Journal of Antibiotics (2014) **67,** 843–845; doi:10.1038/ja.2014.82; published online 25 June 2014

Fungal secondary metabolites are considered to be good sources for the screening of lead compounds of clinical drugs. Among fungal species, ~6400 compounds are obtained from various filamentous fungi.[1] Among them ~950, 900 and 350 compounds are isolated from *Aspergillus*, *Penicillium* and *Fusarium* species, respectively. Moreover, several other members of filamentous and endophytic genus (*Trichoderma*, *Phoma*, *Alternaria*, *Acremonium* and *Stachybotrys*) also produce several hundreds of bioactive compounds. However, the rate of discovery of novel compounds from these fungi has decreased significantly. To increase the chances of discovering novel compounds, we chose to examine rare endophytic fungi. During our screening program, we isolated a novel cytotoxic compound, MBJ-0005 (**1**), consisting of a highly oxygenated ergostane-based skeleton, from the culture of *Anthostomella eucalyptorum* f25427. *Anthostomella* sp. are endophytic fungi, and notably only a few succinic acid derivatives from *Anthostomella* sp. have been reported.[2] Herein, we report the isolation, structure determination and brief biological activity of **1**.

The strain *A. eucalyptorum* f25427 was isolated from a plant 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 (SoyPro, J-Oil Mills, Tokyo, Japan), 0.1% KH_2PO_4 and 0.05% $MgSO_4 \cdot 7H_2O$. 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 containing 2% potato starch (Tobu Tokachi Nosan Kako Agricultural Cooperative Assoc.), 1% glucose (Junsei Chemical), 2% soybean powder (SoyPro, J-Oil Mills), 0.1% KH_2PO_4 and 0.05% $MgSO_4 \cdot 7H_2O$, and were cultured on a rotary shaker (220 r.p.m.) at 25 °C for 4 days.

The entire broth (2 l) was extracted using an equal volume of *n*-BuOH. After concentrating *in vacuo*, the residual aqueous concentrate was extracted using EtOAc (100 ml × 3). The separated organic layer was dried over Na_2SO_4 and concentrated *in vacuo*. The dried residue (320 mg) was applied on a normal-phase medium-pressure liquid column (Purif-Pack SI-30; Shoko Scientific, Yokohama, Japan), and the column was successively eluted using a hexane–EtOAc solvent system (0, 5, 10, 20 and 25% EtOAc) followed by a $CHCl_3$–MeOH solvent system (0, 2, 5, 10 and 20% MeOH). The target eluate (10% MeOH, 35 mg) was further purified by preparative reversed-phase HPLC using a CAPCELL PAK C_{18} MGII column (5.0 μm, 20 i.d. × 150 mm; Shiseido, Tokyo, Japan) with a 2996 photodiode array detector (Waters, Milford, MA, USA) and a 3100 mass detector (Waters) developed with 70% aqueous MeOH containing 0.1% formic acid (flow rate, 10 ml min^{-1}) to yield **1** (7.3 mg, retention time = 24.1 min).

Compound **1** was obtained as a colorless amorphous powder: $[\alpha]_D^{26}$ −5.8 (*c* 0.6, in MeOH); UV λ_{max} (ε) in MeOH: 247 (12 800) nm. The molecular formula was determined by high resolution-ESI-MS to be $C_{28}H_{42}O_7$ (found: 489.2856 $[M-H]^-$, calcd for $C_{28}H_{41}O_7$: 489.2852). The presence of hydroxy and α,β-unsaturated carbonyl functionalities was established from its IR spectrum (ν_{max} (attenuated total reflection) 3417, 1678 and 1619 cm^{-1}). The direct connectivity between the protons and carbons was established from the heteronuclear single-quantum coherence spectrum, and the ^{13}C and ^{1}H NMR spectroscopic data for **1** are listed in Table 1. The structure of **1** was established on the basis of its DQF-COSY, TOCSY and constant-time HMBC (CT-HMBC)[3] spectra as follows.

Three substructures were established through analyses of the DQF-COSY and TOCSY spectra, and the connectivity of those three units was established by the CT-HMBC spectrum. The sequence from methylene protons H_2-1 (δ_H 2.41, 1.75) to methylene protons H_2-4 (δ_H 1.99, 1.84) through two oxymethine protons H-2 (δ_H 4.08) and H-3 (δ_H 3.99) was revealed by the DQF-COSY spectrum. The ^{1}H–^{13}C long-range couplings from a singlet methyl proton H_3-19 (δ_H 1.15) to a methylene carbon C-1 (δ_C 32.9), oxygenated quaternary carbons C-5 (δ_C 80.7) and C-9 (δ_C 76.3), and a quaternary carbon C-10 (δ_C 42.5), and from oxymethine proton H-3 and methylene protons H_2-4

[1]Japan Biological Informatics Consortium (JBIC), Tokyo, Japan; [2]Bioresource Laboratories, MicroBiopharm Japan Co., Ltd. (MBJ), Shizuoka, Japan; [3]Nagahama Institute of Bio-Science and Technology, Shiga, Japan and [4]National Institute of Advanced Industrial Science and Technology (AIST), Tokyo, Japan
Correspondence: Dr K Shin-ya, Biomedicinal Information Research Center (BIRC), National Institute of Advanced Industrial Science and Technology (AIST), 2-4-7 Aomi, Koto-ku, Tokyo 135-0064, Japan.
E-mail: k-shinya@aist.go.jp
Received 12 January 2014; revised 21 April 2014; accepted 2 June 2014; published online 25 June 2014

Table 1 ^{13}C (150 MHz) and ^{1}H (600 MHz) NMR data for **1**

	1	
No.	δ_C	δ_H *(multiplicity, J, in Hz)*
1	32.9	2.41, dd (14.4, 2.3)
		1.75, dd (14.4, 2.9)
2	70.6	4.08, ddd (2.9, 2.7, 2.3)
3	69.2	3.99, dt dd (11.1, 2.7, 4.3)
4	31.4	1.99, dd (13.9, 4.3)
		1.84, dd (13.9, 11.1)
5	80.7	
6	199.9	
7	121.1	5.60, d (2.0)
8	164.3	
9	76.3	
10	42.5	
11	29.4	2.00, m
		1.73, m
12	36.2	1.92, ddd (10.3, 4.4, 2.1)
		1.72, m
13	46.4	
14	52.7	2.77, dd (11.7, 6.9)
15	23.5	1.67, m
		1.58, m
16	28.8	2.05, m
		1.47, m
17	57.4	1.49, m
18	12.3	0.68, s
19	23.4	1.15, s
20	37.8	1.62, m
21	19.7	1.00, d (6.6)
22	35.4	2.31, ddd (14.9, 3.2, 1.8)
		2.08, m
23	141.2	6.66, dd (8.8, 3.2)
24	139.9	
25	28.5	2.92, septet (7.1)
26	21.3	1.18, d (7.1)
27	21.2	1.17, d (7.1)
28	171.0	

The NMR spectra were obtained using a Varian NMR system 600 NB CL (Palo Alto, CA, USA) in CD_3OD, and the solvent peak was used as the internal standard (δ_H 3.31 p.p.m. and δ_C 49.0 p.p.m.).

to the oxygenated quaternary carbon C-5 observed in the CT-HMBC spectrum of **1** revealed a 5-methylcyclohexane-1,2,4-triol substructure (ring A, Figure 1b). The sequence from a methine proton H-14 (δ_H 2.77) to a doublet methyl proton H_3-21 (δ_H 1.00) through methylene protons H_2-15 (δ_H 1.67, 1.58) and H_2-16 (δ_H 2.05, 1.47), two methine protons H-17 (δ_H 1.49), and H-20 (δ_H 1.62), along with a sequence from H-20 to an olefinic proton H-23 (δ_H 6.66) through methylene protons H_2-22 (δ_H 2.31, 2.08), established a 3-methylhept-1,4,7-yl substructure (Figure 1b). The ^{1}H–^{13}C long-range couplings from a singlet methyl proton H_3-18 (δ_H 0.68) to a methine carbon C-14 (δ_C 52.7), quaternary carbon C-13 (δ_C 46.4) and methine carbon C-17 (δ_C 57.4), along with the HMBC correlations from methine proton H-14 to quaternary carbon C-13 and methine carbon C-17 established the cyclopentane moiety (ring D).

The ^{1}H–^{13}C long-range couplings from methylene protons H_2-11 (δ_H 2.00, 1.73), which were ^{1}H–^{1}H spin-coupled with methylene protons H_2-12 (δ_H 1.92, 1.72), to olefinic quaternary carbon C-8 (δ_C 164.3) and quaternary carbon C-13; from methylene protons H_2-12 to oxygenated quaternary carbon C-9; from methine proton H-14 to a methylene carbon C-12 (δ_C 36.2); from methylene protons H_2-15 to olefinic quaternary carbon C-8; from methylene protons H_2-16 to quaternary carbon C-13; and from singlet methyl proton H_3-18 to methylene carbon C-12 established the cyclohexane–cyclopentane moiety (rings C and D).

The ^{1}H–^{13}C long-range couplings from olefinic methine proton H-7 (δ_H 5.60) to two oxygenated quaternary carbons, C-5 and C-9, were observed. Moreover, the ^{1}H–^{13}C long-range couplings from methylene protons H_2-4 to carbonyl carbon C-6 (δ_C 199.9), from methine proton H-14 to olefinic methine carbon C-7 (δ_C 121.1), and from singlet methyl proton H_3-19 to oxygenated quaternary carbon C-9 established the 7-en-6-one system in the steroid nucleus.

As described previously, the sequence C21–C23 was established by DQF-COSY spectrum. The sequence from doublet methyl proton H_3-26 (δ_H 1.18) to another doublet methyl proton H_3-27 (δ_H 1.17) through methine proton H-25 (δ_H 2.92), which was in turn long-range coupled to olefinic quaternary carbon C-24 (δ_C 139.9) and carbonyl carbon C-28 (δ_C 171.0), was observed in the DQF-COSY spectrum. Finally, this sequence was determined to be attached to C-23 (δ_C 141.2) at C-25 (δ_C 28.5) through olefinic quaternary carbon C-24, as revealed by the ^{1}H–^{13}C long-range couplings from H-23 to C-24, C-25 and C-28. Moreover, carbonyl carbon C-28 was determined to be a carboxylic acid carbon based on the molecular formula. The NOESY between H-23 and H-25 established the geometry at C-23 as being *Z*. Thus, the planar structure including the stereochemistry at C-23 of **1** was determined to be a steroid derivative, as shown in Figure 1a.

The relative configuration was assigned on the basis of coupling constants and the analysis of the differential NOESY experiment. The large coupling constants for $J_{3H,4Hax}$ (11.1 Hz) (Figure 1c) established that H-3 is in axial orientation. The small coupling constant between H-3 and H-2 (2.7 Hz) proved that H-2 is in equatorial orientation. The NOESY correlations between H-3/H_{ax}-1 (δ_H 1.75) and between H_{ax}-4 (δ_H 1.84)/H_3-19 indicated that this cyclohexane ring (ring A) should be a chair conformation. Therefore, the methyl group (C-19) attributed to be in axial orientation, and the hydroxy groups at C-2 and C-3 should be in axial and equatorial orientations, respectively. The strong NOESY correlations between H_{ax}-11 (δ_H 1.73)/H_3-19 and between H_{ax}-11/H_3-18 revealed that H_{ax}-11, H_3-19 and H_3-18 are in the upper side of ring moieties. Furthermore, the NOE between H_{ax}-12 (δ_H 1.72)/H-14 proved that the cyclohexane ring (ring C) should form the chair conformation. Therefore, the 9-OH group is elucidated to be in the lower side of the ring C. Further, the NOESY correlations between H-14/H-17 and between H_3-18/H_3-21 revealed that the side chain was located in the upper side of the cyclopentane moiety (ring D). Thus, the structure of **1** was established as (23*Z*)-24-carboxy-2β,3β,5α9α-tetrahydroxyergosta-7,23-dien-6-one (Figure 1). Although many ergostane derivatives have been reported, a 7-en-6-one system[4,5] is rare in fungal secondary metabolites.[6,7] This is the first report of an ergostane isolated from *Anthostomella* sp.

The cytotoxic activities of **1** against human embryonic kidney (HEK) 293 cells and human cervical carcinoma HeLa cells were determined using a colorimetric assay with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich, St Louis, MO, USA) for 48 h. Compound **1** exhibited weak cytotoxic activities against HEK293 and HeLa cells with IC_{50} values of 51 and 26 μM, respectively.

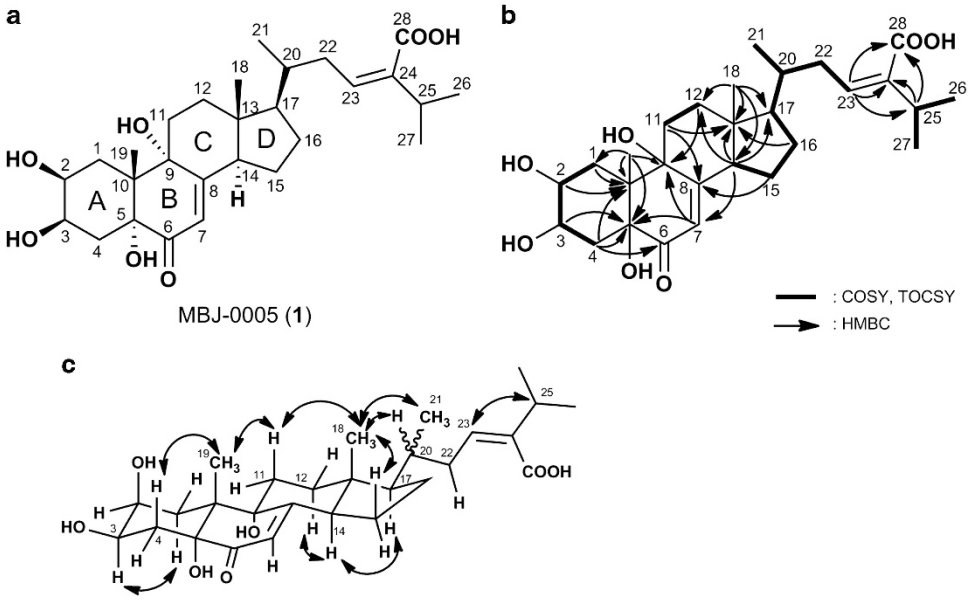

Figure 1 (**a**) Structure of MBJ-0005 (**1**). (**b**) Correlations in DQF-COSY, TOCSY (bold lines) and CT-HMBC (arrows) spectra of **1**. (**c**) Key NOESY correlations of **1**.

ACKNOWLEDGEMENTS

This work was supported by a grant from the New Energy and Industrial Technology Department Organization (NEDO) of Japan and a Grant-in-Aid for Scientific Research (23380067 to KS) from the Japan Society for the Promotion of Science (JSPS).

1 Berdy, J. Bioactive microbial metabolites–a personal view. *J. Antibiot.* **58,** 1–26 (2005).
2 Anderson, J. R., Edwards, R. L. & Whalley, A. J. S. Metabolites of the higher fungi. Part 22. 2-butyl-3-methylsuccinic acid and 2-hexylidene-3-methylsuccinic acid from xylariaceous fungi. *J. Chem. Soc. Perkin Trans* **1,** 1481–1485 (1985).
3 Furihata, K. & Seto, H. Constant time HMBC (CT-HMBC), a new HMBC technique useful for improving separation of cross peaks. *Tetrahedron Lett.* **39,** 7337–7340 (1998).
4 Takemoto, T., Nomoto, K., Hikino, Y. & Hikino, H. Structure of capitasterone, a novel C_{29} insect-moulting substance from *Cyathula capitata*. *Tetrahedron Lett.* **47,** 4929–4932 (1968).
5 Aiello, A., Fattorusso, E., Magno, S. & Menna, M. Isolation of five new 5α-hydroxy-6-keto-Δ^7 sterols from the marine sponge *Oscarella lobularis*. *Steroids* **56,** 337340–4932 (1991).
6 Yaoita, Y., Yoshihara, Y., Kakuda, R., Machida, K. & Kikuchi, M. New sterols form the edible mushrooms, *Pleurotus eryngii* and *Panellus serotinus*. *Chem. Pharm. Bull.* **50,** 551–553 (2002).
7 Yaoita, Y. *et al*. Sterol constituents from five edible mushrooms–Part III. *Chem. Pharm. Bull.* **46,** 944–950 (1998).