NOTE

New hydroxamate metabolite, MBJ-0003, from *Micromonospora* sp. 29867

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In the course of our screening program for new bioactive substances from microorganisms using the advanced compound-identification system designated as 'MBJ's special selection',^{1,2} with which we have selected over 50 strain culture samples and consequently have succeeded in discovering new cytotoxic eremophilane derivatives MBJ-0009, MBJ-0010 and MBJ-0011, MBJ-0012 and MBJ-0013 from *Nectria* sp. f26111³ and *Apiognomonina* sp. f24023,¹ respectively, and new chaetoglobosin derivatives MBJ-0038, MBJ-0039 and MBJ-0040 from *Chaetomium* sp. f24230.² Further screening resulted in the isolation of a new hydroxamate metabolite, designated as MBJ-0003 (**1**, Figure 1a), from the culture of *Micromonospora* sp. 29867. In this paper, we described the fermentation, isolation, structure elucidation and brief biological activities of **1**.

Micromonospora sp. 29867 was isolated from a shellfish collected in Suruga Bay, Shizuoka Prefecture, Japan. The strain was cultivated in 250 ml Erlenmeyer flasks, each containing 25 ml of a medium consisting of 2% potato starch (Tobu Tokachi Nosan Kako Agricultural Cooperative Association, Hokkaido, Japan), 2% glucose (Junsei Chemical, Tokyo, Japan), 2% soy bean powder (Honen SoyPro, J-Oil Mills, Tokyo, Japan), 0.5% yeast extract powder (Oriental Yeast, Tokyo, Japan), 0.25% NaCl (Junsei Chemical), 0.32% CaCO₃ (Wako Pure Chemical Industries, Osaka, Japan), 0.0005% CuSO₄·5H₂O (Wako Pure Chemical Industries), 0.0005% ZnSO₄·7H₂O (Wako Pure Chemical Industries) and 0.0005% MnCl₂·4H₂O (Junsei Chemical). The flasks were placed on a rotary shaker (220 r.p.m.) at 28 °C and incubated for 3 days. Aliquots (0.5 ml) of the broth were then transferred to 500 ml Erlenmeyer flasks containing 50 ml of the same medium and were cultured on a rotary shaker (220 r.p.m.) at 28 °C for 4 days. The whole culture broth (2 l) was extracted with an equal volume of *n*-BuOH. The *n*-BuOH layer was evaporated *in vacuo* to dryness and the residue was successively suspended in water (350 ml), then washed with EtOAc (350 ml × 3), followed by extraction with *n*-BuOH (300 ml × 2). The *n*-BuOH layer was evaporated and the extract (2.9 g) was subjected to normal-phase medium-pressure liquid

chromatography (Purif-Pack SI-30, size: 60 (29 g), Shoko Scientific, Yokohama, Japan) and developed with a gradient system of *n*-hexane–EtOAc (0–25% EtOAc over 12 min and was kept at 25% for 3 min, flow rate: 20 ml min^{−1}), followed by the stepwise solvent system of CHCl₃–MeOH (100 ml each of 0, 2, 5, 10, 20, 30 and 100% MeOH). The fractions were monitored by using an UPLC-DAD-ELS-MS system. The 100% MeOH-eluted fraction (608 mg) was then chromatographed by reversed-phase medium-pressure liquid chromatography (Purif-Pak ODS-30, size: 60 (30 g), Shoko Scientific) with the developing solvent of the H₂O–MeOH–CHCl₃ system (100 ml each of 60, 70, 80 and 100% aqueous MeOH and CHCl₃/MeOH = 1:1). The CHCl₃/MeOH eluate (214 mg) was subjected to preparative HPLC on a CAPCELL PAK C18 MGII column (5.0 μm, 20 i.d. × 150 mm; Shiseido, Tokyo, Japan) with 55% aqueous MeCN containing 0.1% formic acid (flow rate: 10 ml min^{−1}) to give semi-purified **1** (7.9 mg; retention time: 9.0 min). Final purification was carried out by preparative HPLC on an XSelect CSH C18 column (20 i.d. × 150 mm; Waters, Milford, MA, USA) with 40% aqueous MeCN containing 0.1% formic acid (flow rate: 10 ml min^{−1}) to afford **1** (1.7 mg; retention time: 16.3 min).

1 was found to be an optically inactive compound; a colorless amorphous solid with UV end and IR (ATR) $\nu_{\max}$ 3300 and 1650 cm^{−1} (hydroxy and carbonyl). The molecular formula was established as C₃₇H₇₂N₆O₈ by negative mode high-resolution electrospray ionization mass spectrometry (m/z 727.5353 [M–H][−], calcd for C₃₇H₇₁N₆O₈ 727.5333). The direct connectivity between protons and carbons was established by a heteronuclear single-quantum coherence spectrum; the tabulated ¹³C and ¹H NMR spectroscopic data for **1** are listed in Table 1. The structure determination of **1** was carried out through analysis of DQF-COSY and constant-time heteronuclear multiple bond correlation (CT-HMBC)⁴ data as described below.

DQF-COSY and CT-HMBC spectra revealed the presence of seven fragments (A to G) as follows (Figure 1b). A cadaverine moiety (fragment C) was revealed by the ¹H spin couplings between aliphatic

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Figure 1 (a) Structure of **1**. (b) Key correlations observed in 2D NMR spectra of **1** by DQF-COSY (bold line) and CT-HMBC (arrow, from ^1H to ^{13}C). (c) ESI-MS/MS fragment ions of **1**.

methylene protons H₂-16 (δ_{H} 1.64) and methylene protons H₂-15 (δ_{H} 3.60), which is considered to connect with a nitrogen atom from its ^{13}C chemical shift (δ_{C} 48.5), and between aliphatic methylene protons H₂-18 (δ_{H} 1.53) and another nitrogen-bearing methylene protons H₂-19 (δ_{H} 3.17, δ_{C} 40.3), in addition to HMBC correlations from the H₂-15 and H₂-19 to aliphatic methylene carbon C-17 (δ_{C} 24.9). By considering integration values of corresponding signals, additional two cadaverine moieties were also established in the same manner, as shown in Figure 1b (fragments E and G).

The presence of two succinyl moieties (fragments D and F) were revealed by proton spin couplings between allylic methylene protons H₂-22 (H₂-33) ($\delta_{\text{H2-22}}$ 2.47, $\delta_{\text{H2-33}}$ 2.47) and H₂-23 (H₂-34) ($\delta_{\text{H2-23}}$ 2.76, $\delta_{\text{H2-34}}$ 2.76), and by HMBC correlations from these protons to both amide- or ester-carbonyl ketone carbons C-21 (C-32) ($\delta_{\text{C-21}}$ 174.7, $\delta_{\text{C-32}}$ 174.9) and C-24 (C-35) ($\delta_{\text{C-24}}$ 174.5, $\delta_{\text{C-35}}$ 174.6), respectively.

A remaining alkyl side chain substructure was elucidated as follows. The ^1H sequence from equivalent doublet geminal methyl protons, H₃-1 and H₃-2-Me (δ_{H} 0.88, 6H) to methylene protons H₂-4

(δ_{H} 1.29) through a methine proton H-2 (δ_{H} 1.52) and methylene protons H₂-3 (δ_{H} 1.17), and ^1H - ^{13}C long-range correlations from the proton H₂-3 to a methylene carbon C-5 (δ_{C} 30.5–31.0) revealed the presence of a 2-methylpentanyl moiety (fragment A). A ^1H spin coupling between methylene protons H₂-11 (δ_{H} 1.60) and H₂-12 (δ_{H} 2.46), and HMBC correlations from the H₂-11 and H₂-12 to an aliphatic methylene carbon C-10 (δ_{C} 30.5–31.0) and an amide or ester carbonyl carbon C-13 (δ_{C} 176.1) were observed, as shown in Figure 1b (fragment B). According to the molecular formula, and ^{13}C - and ^1H -NMR chemical shifts of **1**, four methylene units should be assigned as an alkyl chain moiety. The molecular formula of **1** and the low-field-shifted ^1H NMR chemical shifts of the acylated nitrogen-substituted methylene protons (δ_{H} 3.17 vs δ_{H} 3.60–3.63) suggested the presence of three hydroxamic acid moieties at the position of N-14, N-25 and N-36, respectively. The connectivity among fragments B, C, D, E, F and G was determined by HMBC correlations from H₂-15 to C-13, H₂-19 to C-21, H₂-26 (δ_{H} 3.60) to C-24, H₂-30 (δ_{H} 3.17) to C-32 and H₂-37 (δ_{H} 3.63) to C-35, as shown in Figure 1b. Analysis of the ESI-MS/MS data also supports the

Table 1 ^{13}C and ^1H NMR spectroscopic data for MBJ-0003 (1)

Position	δ_{C}	δ_{H} (multiplicity, J in Hz)
1	23.1 ^a	0.88, d (7.2)
2-Me	23.1 ^a	0.88, d (7.2)
2	29.2	1.52, ovl ^b
3	40.3 ^a	1.17, m
4	28.6	1.29, ovl ^b
5-10	30.5–31.0	1.29–1.35, ovl ^b
11	26.0	1.60, ovl ^b
12	33.3	2.46, ovl ^b
13	176.1	
15	48.5 ^a	3.60, ovl ^b
16	27.4 ^a	1.64, ovl ^b
17	24.9 ^a	1.34, ovl ^b
18	30.0 ^a	1.53, ovl ^b
19	40.3 ^a	3.17, t (6.6)
21	174.7	
22	31.4 ^a	2.47, ovl ^b
23	29.0 ^a	2.76, t (6.6)
24	174.5	
26	48.5 ^a	3.60, ovl ^b
27	27.4 ^a	1.64, ovl ^b
28	24.9 ^a	1.34, ovl ^b
29	30.0 ^a	1.53, ovl ^b
30	40.3 ^a	3.17, t (6.6)
32	174.9	
33	31.4 ^a	2.47, ovl ^b
34	29.0 ^a	2.76, t (6.6)
35	174.6	
37	48.4 ^a	3.63, ovl ^b
38	27.0	1.68, ovl ^b
39	24.3	1.40, m
40	28.1	1.67, ovl ^b
41	40.7	2.92, t (6.6)

Measured on a 600 NB CL NMR spectrometer (Varian, Palo Alto, CA, USA) at 600 MHz for ^1H and 150 MHz for ^{13}C with the residual solvent peak as the internal standard (δ_{H} 3.31, δ_{C} 49.0 p.p.m.).

^aExchangeable.

^bOverlapped with other peak.

12-methyltridecanate side chain structure as shown in Figure 1c. Thus, the structure of **1** was established as shown in Figure 1a. The structure of **1** resembles those of promicroferrioxamine⁵ and tenacibactins⁶ that were reported as siderophores.

From the structural point of view, **1** was expected to exert cytotoxic activities. Therefore, we evaluated the cytotoxic and antimicrobial activities of **1**. The cytotoxic activity of **1** against human ovarian adenocarcinoma SKOV-3 cells, which was relatively sensitive to cytotoxic compounds and thus routinely used for evaluation of cytotoxicity and biological screening in our laboratory, was 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).⁷ After 72 h of treatment, **1** exhibited moderate cytotoxicity with the IC₅₀ values of 11 μM . Compound **1** did not show antimicrobial activity against *Micrococcus luteus* or *Bacillus subtilis*.

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