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NOTE

MBJ-0110, a novel cyclopeptide isolated from the fungus *Penicillium* sp. f25267

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We have constructed an isolated natural compound library designed to facilitate extensive biological screenings.[1] The library consists of over 1000 isolates, including over 140 'JBIR compounds' that were discovered in our laboratory. To enrich this library, we recently initiated a screening program for rare microbial products using the advanced compound-identification system designated as 'MBJ's special selection'.[2,3] As a result, our program yielded novel compounds named 'MBJ compounds', such as a cytotoxic hydroxamate MBJ-0003 from *Micromonospora* sp. 29867;[4] cytotoxic eremophilane derivatives MBJ-0009 and MBJ-0010 from *Nectria* sp. f26111;[5] MBJ-0011, MBJ-0012 and MBJ-0013 from *Apiognomonia* sp. f24023;[2] cytotoxic chaetoglobosin derivatives MBJ-0038, MBJ-0039 and MBJ-0040 from *Chaetomium* sp. f24230;[3] bicyclic depsipeptides MBJ-0086 and MBJ-0087 from *Sphaerisporangium* sp. 33226;[6] and aziridine-containing peptide MBJ-0035 from *Streptosporangium* sp. 32552.[7] Further screening for novel compounds led to the identification of MBJ-0110 (**1**) from the culture of *Penicillium* sp. f25267. Herein we report the fermentation, isolation, structure elucidation and preliminary biological activity data.

Penicillium sp. f25267 was isolated from a soil sample collected in the Shiga Prefecture, Japan. The strain was cultured 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% $MgSO_4{\cdot}7H_2O$ (pH 7.4 before sterilization). The flasks were incubated 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 of the same composition, which were then cultured on a rotary shaker (220 r.p.m.) at 25 °C for 4 days.

The whole culture broth (2 l) was extracted with an equal volume of *n*-BuOH. After concentration *in vacuo*, the extract was successively partitioned between EtOAc (350 ml × 3) and H_2O (300 ml). The aqueous layer was evaporated to dryness and the residue (1.4 g) was fractionated by reversed-phase medium-pressure liquid chromatography (Purif-Pack ODS-30, Shoko Scientific, Yokohama, Japan; 40–100% aq. MeOH with 10% stepwise increments in the MeOH concentration). The fractions were monitored using an ultra performance liquid chromatography-diode array detection-evaporative light scattering-mass spectrometry system and **1** was isolated based on peak-guided fractionation. The 50% MeOH eluate (30.9 mg) was subjected to preparative reversed-phase HPLC using a Capcell Pak C_{18} MG II column (20 mm inside diameter (i.d.) × 150 mm; Shiseido, Tokyo, Japan) with a solvent system of 20% CH_3CN/H_2O containing 0.1% formic acid (flow rate: 10 ml min^{-1}), to yield semi-purified **1** (6.9 mg, retention time (Rt) = 15.3 min). Final purification was carried out by preparative HPLC using an X-Bridge C_{18} column (19 mm i.d. × 150-mm; Waters, Milford, MA, USA) with a solvent system of 20% CH_3CN/H_2O containing 0.1% formic acid (flow rate: 10 ml min^{-1}) to afford **1** (3.5 mg, Rt = 13.3 min).

MBJ-0110 (**1**) was obtained as a colorless amorphous powder: $[\alpha]^{24}_D$ –186 (MeOH; *c* 0.18); UV end; IR (attenuated total reflectance) ν_{max}: 3400 (hydroxy) and 1683 (carbonyl) cm^{-1}. The molecular formula of **1** was established as $C_{27}H_{41}N_5O_8$ by high-resolution (HR)-ESI–MS (*m/z* 564.3018 $[M+H]^+$, calcd for $C_{27}H_{42}N_5O_8$ *m/z* 564.3033). Its peptidic nature was evident from the resonances corresponding to α-methine protons (δ_H 4.00–5.08) and the resonances corresponding to the carbonyl carbons (δ_C 169.0–175.9) in the ^1H and ^{13}C NMR spectra of **1**, respectively. The direct connectivity between protons and carbons was established by a HSQC spectrum; Table 1 summarizes the ^{13}C and ^1H NMR spectroscopic data for **1.** The ^1H sequences and ^1H–^{13}C long-range couplings from α-methine protons to the corresponding amide carbonyl carbons, which were elucidated by double quantum-filtered COSY and constant time-HMBC[8] spectra, respectively, revealed the involvement of an isoleucine (Ile), a pipecolic acid (Pip), a proline (Pro) and an aspartic acid (Asp) residue, as shown in Figure 1b. In addition to the above-mentioned amino-acid moieties, the presence of a 4-hydroxypipecolic acid (C-22 to C-27) moiety was proved based on a ^1H sequence from an α-methine proton H-23 (δ_H 4.00) to nitrogen-bearing methylene protons H_2-27 (δ_H 3.39 and 2.98) via aliphatic methylene protons

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Table 1 ^{13}C and ^{1}H NMR spectroscopic data for 1

Position	δ_C	δ_H, *multiplicity (J in Hz)*
1	169.7	
2	59.2	4.36, d (7.8)
3	39.7	1.68, ovl[a]
4	26.7	1.52, m; 1.19, m
5	11.4	0.95, t (7.2)
6	15.9	0.93, d (6.6)
7	173.0	
8	54.8	5.08, br s
9	25.0	2.20, ovl[a]; 1.62, ovl[a]
10	20.9	1.78, ovl[a]; 1.70, ovl[a]
11	25.5	1.73, ovl[a]; 1.73, ovl[a]
12	45.0	3.97, br d (15.6); 3.00, ovl[a]
13	175.9	
14	59.7	4.95, dd (4.8, 8.4)
15	30.0	2.47, m; 1.80, ovl[a]
16	26.3	2.08, ovl[a]; 1.96, ovl[a]
17	50.2	3.76, ovl[a]; 3.70, ovl[a]
18	171.3	
19	37.0	3.10, dd (7.2, 12.0); 2.96, ovl[a]
20	51.7	4.82, m
21	175.7	
22	169.0	
23	55.5	4.00, d (7.2)
24	32.8	2.62, br d (13.8); 2.07, ovl[a]
25	68.9	5.17, br s
26	26.8	2.19, ovl[a]; 2.02, ovl[a]
27	40.6	3.39, br d (9.6); 2.98, ovl[a]

[a]Overlapped with other signals. NMR spectra were taken on a 600 NB CL NMR system (Varian, Palo Alto, CA, USA) in CD_3OD with the residual solvent peak as an internal standard (δ_C 49.0, δ_H 3.31 p.p.m.)

H_2-24 (δ_H 2.62 and 2.07), an oxymethine proton H-25 (δ_H 5.17, δ_C 68.9) and aliphatic protons H_2-26 (δ_H 2.19, 2.02), and ^{1}H–^{13}C long-range couplings from H-23 and H-24 (δ_H 2.07) to an amide carbonyl carbon C-22 (δ_C 169.0), and from H_2-27 to an α-methine carbon C-23 (δ_C 55.5).

The amino-acid sequence in **1** was determined by the HMBC correlations from an α-methine proton H-2 (δ_H 4.36) to a carbonyl carbon C-7 (δ_C 173.0), from an α-methine proton H-8 (δ_H 5.08) and ε-methylene protons H_2-12 (δ_H 3.97 and 3.00) to a carbonyl carbon C-13 (δ_C 175.9), from an α-methine proton H-14 (δ_H 4.95) to a carbonyl carbon C-18 (δ_C 171.3), from an α-methine proton H-20 (δ_H 4.82) to C-22 and from H-25 to an ester carbonyl carbon C-1 (δ_C 169.7). Although only ^{1}H–^{13}C HMBC information suggested two structural possibilities, α- and β-aspartyl amide linkages, we concluded that the aspartyl acid moiety is linked to adjacent Pro by β-amino bond because of the existence of a ROESY correlation between H_b-17 (δ_H 3.70) and H_a-19 (δ_H 3.10) and ^{1}H–^{15}N HMBC correlations from H_2-16 (δ_H 2.08, 1.96), H_b-15 (δ_H 1.80) and H_b-19 (δ_H 2.96) to a nitrogen atom of Pro (δ_N 140). Therefore, the structure of **1** was determined as shown in Figure 1b.

To verify the proposed structure, **1** was treated with 0.1 N NaOH overnight at room temperature, followed by ESI–MS/MS analysis of the alkaline hydrolysate (molecular formula: $C_{27}H_{43}N_5O_9$; HR-ESI–MS: $[M+H]^+$ *m/z* 582.3159, $C_{27}H_{44}N_5O_9$ 582.3139). The ESI–MS/MS data showed major fragment ions (*m/z* 185.0945, 243.0976, 324.1554, 340.1477 and 451.2165) that supported the proposed structure (Figure 1c).

The multiplicity and a large ^{1}H spin coupling constant value of H-23 (doublet, $J_{H-H} = 7.2$ Hz) and ROESY correlations between H-23/H_{ax}-27 (δ_H 2.98) and H-23/H_{eq}-24 (δ_H 2.62) implied that the piperidine ring is in the chair conformation and the H-23 is axially orientated. In addition, the broad singlet signal of H-25 proved its

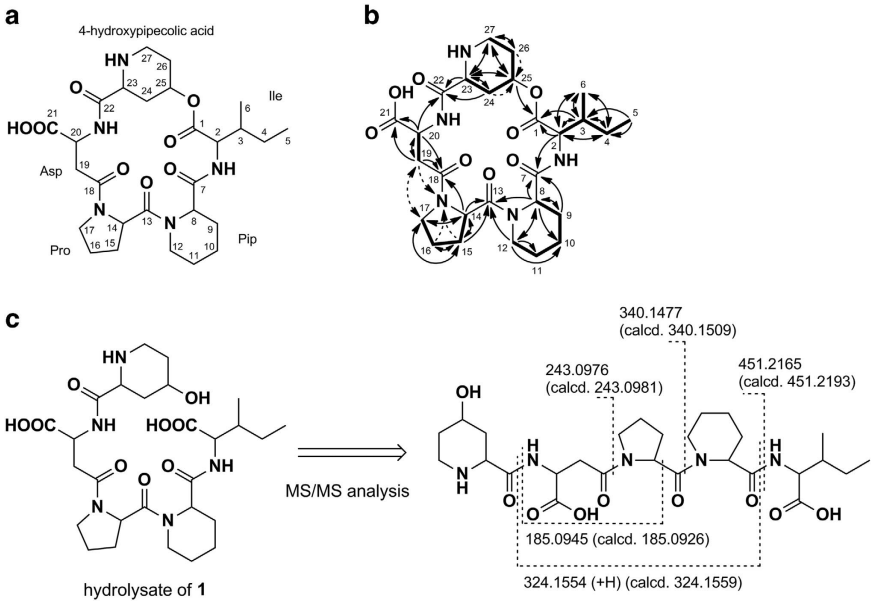

Figure 1 (**a**) Structure of **1**. (**b**) NMR analysis of **1**. COSY, bold line; ^{1}H–^{13}C HMBC, solid arrow; ^{1}H–^{15}N HMBC, dashed arrow; ROESY, bidirectional dashed arrow. (**c**) ESI–MS/MS fragmentation ions of **1**.

equatorial orientation. Taken together, the relative configurations of C-23 and C-25 were determined as 23*S** and 25*S**, respectively.

The absolute configurations of the amino-acid residues were determined to be L-Pro, L-Pip and L-Asp by using Marfey's method.[9] A portion of **1** (0.4 mg) was hydrolyzed in 6 N HCl at 110 °C for 12 h and then dried under air flow. The resulting hydrolysate was treated with 0.1 M $NaHCO_3$ (200 µl) and 1% *N*-(5-fluoro-2,4-dinitrophenyl)-L-alaninamide (L-FDAA) in Me_2CO (100 µl) at 40 °C for 30 min. Amino-acid standards were derivatized with L-FDAA in a similar manner. The Marfey's derivatives were analyzed using a HPLC–MS system as follows: a Capcell Pak C_{18} MG II column (4.6 mm i.d.× 150 mm) was developed with a linear gradient system of water/MeCN with 0.1% formic acid (20–50% MeCN, 15 min; flow rate, 1.0 ml min^{-1}). FDAA derivatives were detected by absorption at 340 nm, and assignment was secured by ion-selective monitoring. The retention times of the standard FDAA derivatives were as follows: L-Asp, 7.9 min; D-Asp, 8.2 min; L-Pip, 13.6 min; D-Pip, 12.8 min; L-Pro, 10.1 min; D-Pro, 10.7 min; L-Ile, 14.7 min; D-Ile, 16.8 min; L-*allo*-Ile, 14.7 min; and D-*allo*-Ile, 16.7 min. The retention times of the FDAA derivatives of **1** were as follows: Asp, 7.9 min; Pip, 13.6 min; Pro, 10.1 min; and Ile, 14.7 min.

The absolute configuration of the Ile residue in **1** was established by HPLC comparison of the 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate (GITC) derivative of hydrolysate of **1** with standard samples.[10] Triethylamine (50 µl) and a GITC solution (250 µl, prepared at 3.9 mg ml^{-1} in CH_3CN) were added to the acid hydrolysate of **1** or an authentic amino-acid standard. The reaction mixture was kept at room temperature for 30 min and the reaction was then quenched by adding 40 µl of MeCN–5% AcOH in H_2O (1:1). Analysis of the GITC derivatives was performed on a Capcell Pak ADME column (4.6 mm i.d. × 150 mm; Shiseido) employing an isocratic elution of 40% CH_3CN containing 0.1% formic acid (1.0 ml min^{-1}). GITC derivatives were detected by absorption at 248 nm, and assigned by ion-selective monitoring. The retention times of the GITC derivatives were as follows: L-Ile, 11.6 min and L-*allo*-Ile, 11.3 min. The retention time (11.6 min) of the GITC derivative of **1** implied that the Ile residue in **1** is L-Ile.

We evaluated the cytotoxic and antimicrobial activities of **1**, but it showed neither cytotoxicity to human ovarian adenocarcinoma SKOV-3 cell lines ($IC_{50} > 100$ µM) or human malignant pleural mesothelioma ACC-MESO-1 cell lines ($IC_{50} > 100$ µM), nor antimicrobial activity against *Micrococcus luteus* and *Bacillus subtilis*.

The obtained structure of **1** is very rare in nature; only petrosifungins A and B,[11] and JBIR-113, -114 and -115[12] have been isolated as pipecolic acid-containing peptides of fungal origin. To the best of our knowledge, there are no reports in the literature of peptide compounds possessing the 4-hydroxypipecolic acid moiety.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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