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NOTE

Novel arginine-containing peptides MBJ-0173 and MBJ-0174 from *Mortierella alpina* f28740

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We have constructed a library of isolated natural products obtained from microbial sources in order to perform efficient screenings.[1] During the investigation of rare microbial products with promising biological and pharmacological properties, we have developed an advanced system for compound identification based on accumulated HPLC-MS profiling data and strain information designated as 'MBJ's special selection'. Using this screening method, we have already succeeded in discovering novel eremophilane derivatives MBJ-0009 and MBJ-0010 from *Nectria* sp. f26111,[2] MBJ-0011, MBJ-0012 and MBJ-0013 from *Apiognomonia* sp. f24023,[3] cytotoxic chaetoglobosin derivatives MBJ-0038, MBJ-0039 and MBJ-0040 from *Chaetomium* sp. f24230,[4] and a cytotoxic hydroxamate MBJ-0003 from *Micromonospora* sp. 29867.[5] During our continuous search for new substances, two metabolites named MBJ-0173 (**1**) and MBJ-0174 (**2**) were isolated together with plactin B,[6] from the culture broth of *Mortierella alpina* f28740 (Figure 1a). In this paper, the fermentation, isolation, structure elucidation and preliminarily biological activities of **1** and **2** are described.

The producing fungus *Mortierella alpina* f28740 was isolated from a soil sample collected in Ise, 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% $MgSO_4{\cdot}7H_2O$ (pH 7.4 before sterilization). 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 of the same composition and 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 the *n*-BuOH layer was evaporated *in vacuo*, the resulting residue was suspended in brine (350 ml) and then extracted with EtOAc (350 ml × 3) and *n*-BuOH (300 ml × 2), successively. The *n*-BuOH extract (3.4 g) was subjected to reversed-phase medium-pressure liquid chromatography (Purif-Pack ODS-100, size: 60 (39 g), Shoko Scientific Co., Ltd., Yokohama, Japan) by using an H_2O-MeOH stepwise solvent system (20%, 40%, 60%, 80% and 100% MeOH). The 40% and 60% MeOH fractions were combined (1.5 g) and chromatographed by preparative HPLC on an XSelect CSH C18 column (20 i.d. × 150 mm; Waters, Milford, MA, USA) with a linear gradient from 20 to 60% aqueous CH_3CN containing 0.1% formic acid over 20 min (flow rate: 10 ml min^{-1}) to afford crude **2** (59.3 mg). Further purification was achieved by preparative HPLC on the CSH column with an aqueous CH_3CN containing 0.1% formic acid linear gradient system (20–30% CH_3CN, 20 min; flow rate: 10 ml min^{-1}) to give **2** (4.0 mg, retention time 14.7 min). On the other hand, the 80% and 100% MeOH fractions were combined (0.54 g) and subjected to preparative HPLC on the CSH column eluted with a 20-min linear gradient from 20% to 60% aqueous CH_3CN containing 0.1% formic acid (flow rate: 10 ml min^{-1}) to obtain semi-purified **1** (41.2 mg, retention time: 12.3 min). Final purification was performed using HPLC (linear gradient, 20–50% aqueous CH_3CN containing 0.1% formic acid, 20 min, flow rate: 10 ml min^{-1}) to afford pure **1** (23.5 mg, retention time: 12.9 min).

MBJ-0173 (**1**) was isolated as a colorless amorphous solid: $[\alpha]^{23}_{D}$ −32 (*c* 0.04, MeOH); UV λ_{max} nm (log ε): 274 (4.5), 281 (4.5) and 289 (4.4) in MeOH; IR (ATR) ν_{max} 1635 cm^{-1} (carbonyl). The molecular formula of **1** was established as $C_{44}H_{62}N_{10}O_8$ by HR-ESIMS (*m/z* 859.4852 $[M+H]^+$, calcd for $C_{44}H_{63}N_{10}O_8$: 859.4830). The planar structure of **1** was determined by a series of 2D NMR analyses, including double quantum filtered COSY (DQF-COSY), heteronuclear single quantum coherence (HSQC) and constant-time heteronuclear multiple bond correlation[7] (CT-HMBC). The ^{13}C and ^{1}H NMR data of **1** is listed in Table 1. The ^{1}H and ^{13}C NMR data suggested that **1** was a peptidic compound, with five deshielded α-methine protons ($\delta_H \approx 4$), seven NH protons (δ_H 9–7) and seven carbonyl carbons (δ_C 175–162). Further analyses of 2D NMR data revealed that **1** was composed of an *N*-terminal acetic acid endcapped peptides consinting of 5 amino acid residues: arginine, phenylalanine, tryptophan and two leucine residues (Figure 1b). Additionally, the presence of a dehydrobutyrine (DHB) moiety was revealed by HMBC correlations from an olefinic methine proton H-DHB-β (δ_H 6.52), which was also ^{1}H spin coupled to a doublet methyl proton H_3-DHB-

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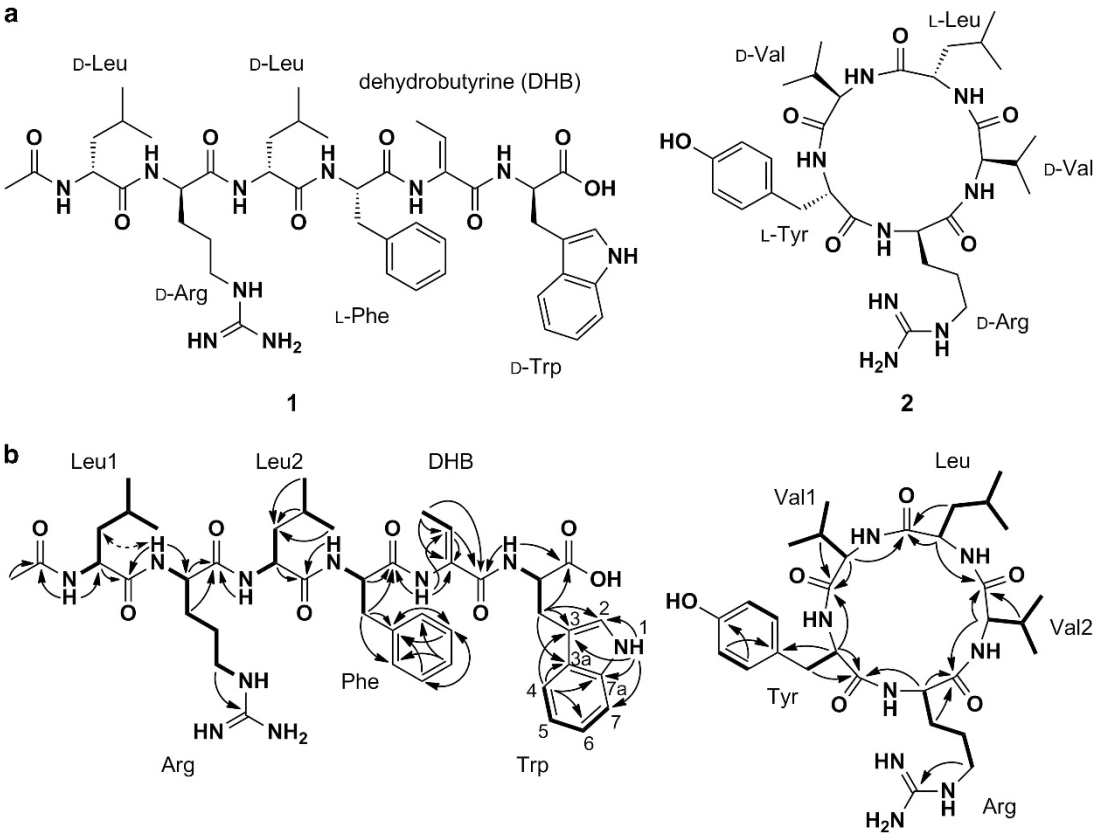

Figure 1 (**a**) Structures of **1** and **2**. (**b**, left) Structure determination of **1**. COSY, HMBC (^{1}H to ^{13}C) and ROESY correlations are shown as bold lines, arrows and dashed arrows, respectively. (**b**, right) Structure determination of **2**. COSY and HMBC (^{1}H to ^{13}C) correlations are shown as bold lines and arrows, respectively.

γ (δ_H 1.53), to an olefinic quaternary carbon C-DHB-α (δ_C 130.7) and an amide carbonyl carbon of DHB (δ_C 162.8). The connectivity among the amino acid units was determined by ^{1}H-^{13}C long-range couplings from NH-Arg (δ_H 7.95), NH-Leu2 (δ_H 8.04), NH-Phe (δ_H 8.51), NH-DHB (δ_H 9.13) and NH-Trp (δ_H 7.10) to carbonyl carbons of Leu1 (δ_C 172.13), Arg (δ_C 171.5), Leu2 (δ_C 172.09), Phe (δ_C 170.5) and DHB, respectively. Furthermore, HMBC correlations from an amide proton of Leu1 (δ_H 8.00) to an acetic carbonyl carbon (δ_C 169.3) indicated that *N*-terminus of **1** was acetylated. Thus, the planar structure of **1** was established as shown in Figure 1b. The stereochemistry of the trisubstituted double bond was established as *Z* by means of a *J*-resolved HMBC-2 spectrum.[8] The smaller ^{1}H-^{13}C long-range coupling value of 4.8 Hz between H-DHB-β and the carbonyl carbon of DHB indicated that these two nuclei were *cis* to each other, thus concluding the *Z* geometry for the double bond.[9–11]

The absolute configurations of the amino acid residues in **1** were determined using Marfey's method.[12] A portion of **1** (0.2 mg) was hydrolyzed in 6 N HCl (0.5 ml) at 110 °C for 12 h. After drying the reaction solution under an air flow, 0.1 M $NaHCO_3$ (0.2 ml) and 10 mM *N*-(5-fluoro-2,4-dinitrophenyl)-L-alaninamide (FDAA) in Me_2CO (0.1 ml) were added. The mixture was then reacted at 40 °C for 30 min. The resultant products were analyzed by HPLC-MS (Capcell Pak C_{18} MGII column (4.6 i.d. × 150 mm; Shiseido, Tokyo, Japan)) developed with aqueous CH_3CN containing 0.1% formic acid linear gradient systems (A: 10–30% CH_3CN in 15 min; B: 30–60% CH_3CN in 15 min, flow rate 1.0 ml min^{-1}). FDAA derivatives of target amino acids were detected by absorption at 340 nm and MS analyses Retention times of authentic FDAA-amino acids (min, solvent system): L-Arg (12.1, A), D-Arg (11.7, A), L-Trp (9.8, B), D-Trp (10.6, B), L-Phe (10.2, B), D-Phe (11.6, B), L-Leu (10.3, B) and D-Leu (12.1, B). The hydrolysate of **1** contained D-Arg (11.7, A), D-Trp (10.6, B), L-Phe (10.2, B) and D-Leu (12.1, B). Hence, the structure of **1** including the absolute configuration was determined as shown in Figure 1a.

MBJ-0174 (**2**) was obtained as a colorless amorphous powder: $[\alpha]_D^{24}$ +32 (*c* 0.04, MeOH); UV λ_{max} nm (log ε): 277 (3.9) in MeOH; IR (ATR) ν_{max} 3300 and 1633 cm^{-1} (hydroxy and carbonyl, respectively). The molecular formula of **2** was determined to be $C_{31}H_{50}N_8O_6$ on the basis of the HR-ESIMS data (*m/z* 631.3959 $[M+H]^+$, calcd for $C_{31}H_{51}N_8O_6$: 631.3932) in conjunction with the HSQC data. Analyses of a series of 2D NMR spectra revealed the presence of arginine, leucine, tyrosine and two valine moieties in **2** (Figure 1b). The amino acid sequence of **2** was determined by HMBC correlations from α-methine protons of Leu (δ_H 4.69), Val1 (δ_H 4.52), Arg (δ_H 4.30), Tyr (δ_H 4.99) and Val2 (δ_H 4.38) to carbonyl carbons of Val1 (δ_C 171.1), Arg (δ_C 172.6), Tyr (δ_C 172.4), Val2 (δ_C 171.5) and Leu (δ_C 172.1), respectively. Therefore, **2** is a cyclic pentapeptide as shown in Figure 1b.

The absolute configuration of **2** was determined using the Marfey's method by the same procedure as that of **1**. The obtained FDAA derivatives of **2** were analyzed using the same column and HPLC system as above with following aqueous CH_3CN (0.1% formic acid) linear gradient systems: (A) 10–30% CH_3CN in 15 min, (B) 30–60% CH_3CN in 15 min and (C) 20–50% CH_3CN in 15 min, flow rate 1.0 ml min^{-1}. Retention times of the standard FDAA derivatives were

Table 1 The ^{13}C (125 MHz) and ^{1}H (500 MHz) NMR spectroscopic data for MBJ-0173 (1) and MBJ-0174 (2)

	1			***2***	
Position	δ_C	δ_H, *mult (J in Hz)*	*Position*	δ_C	δ_H, *mult (J in Hz)*
Leu1			*Val1*		
Carbonyl	172.13		Carbonyl	171.1	
α	51.2	4.21, ovl[a]	α	57.6	4.52, d (8.4)
β	41.0	1.36, ovl[a]; 1.36, m	β	29.9	2.22, m
γ	24.3	1.54, ovl[a]	γ	18.12	0.87[b], d (6.0)
δ	23.3	0.83, d (6.5)	δ	17.4	0.90, d (6.0)
ε	21.6	0.78, d (6.5)	*Arg*		
N*H*		8.00, d (8.0)	Carbonyl	172.6	
Arg			α	54.7	4.30, dd (4.8, 9.6)
Carbonyl	171.5		β	27.4	2.02, n; 1.81, m
α	52.0	4.22, ovl[a]	γ	24.3	1.51, m; 1.45, m
β	29.1	1.66, ovl[a]; 1.49, ovl[a]	δ	39.6	3.15, m; 3.15, m
γ	25.0	1.35, ovl[a]; 1.35, ovl[a]	ε	156.2	
δ	40.5	2.99, ovl[a]; 2.90, m	*Tyr*		
ε	157.4		Carbonyl	172.4	
N*H*		7.95, d (7.5)	α	54.5	4.99, dd (7.8, 7.8)
N*H*		9.79, br s	β	35.8	3.10, m; 3.01, m
Leu2			1	126.6	
Carbonyl	172.09		2/6	129.8	7.13, ovl[a]
α	51.6	4.15, m	3/5	114.8	6.97, d (7.8)
β	40.7	1.16, m; 1.08, m	4	155.4	
γ	24.0	1.17, ovl[a]	*Val2*		
δ	22.9	0.73, d (5.5)	Carbonyl	171.5	
ε	22.2	0.70, d (5.5)	α	58.8	4.38, d (9.6)
N*H*		8.04, d (6.0)	β	28.4	2.07, m
Phe			γ	18.07	0.78, d (6.0)
Carbonyl	170.5		δ	17.7	0.87[b], d (6.0)
α	54.3	4.66, m	*Leu*		
β	37.8	3.16, ovl[a]; 2.77, dd (12.0, 12.0)	Carbonyl	172.1	
1	138.0		α	51.0	4.69, dd (7.8, 7.8)
2/6	129.5	7.25, ovl[a]	β	36.7	1.70, ovl[a]; 1.70, ovl[a]
3/5	128.2	7.23, ovl[a]	γ	23.7	1.61, m
4	126.4	7.17, dd (7.0, 7.0)	δ	21.3	0.75, d (6.0)
N*H*		8.51, d (8.0)	ε	20.9	0.69, d (6.0)
DHB					
Carbonyl	162.8				
α	130.7				
β	129.8	6.52, q (7.0)			
γ	13.2	1.53, d (7.0)			
N*H*		9.13, s			
Trp					
Carbonyl	175.8				
α	55.2	4.23, ovl[a]			
β	28.2	3.20, ovl[a]; 3.01, ovl[a]			
1-N*H*		10.66, s			
2	123.5	7.07, s			
3	111.5				
3a	128.1				
4	118.7	7.51, d (8.0)			
5	118.0	6.90, dd (8.0, 8.0)			
6	120.6	7.00, dd (8.0, 8.0)			
7	111.2	7.27, ovl[a]			
7a	136.1				
N*H*		7.10, d (8.0)			
Acetic acid					
1	169.3				
2	22.6	1.79, s			

Abbreviation: DHB, dehydrobutyrine.
NMR spectra were obtained using a Varian NMR System 500 NB CL (Palo Alto, CA, USA) in DMSO-d_6 (**1**) and pyridine-d_5: deuterium oxide = 5:1 (**2**) with the residual solvent peak as an internal standard (**1**: δ_C 39.7, δ_H 2.49 p.p.m., **2**: δ_C 135.5, δ_H 7.55 p.p.m.).
[a]Overlapped with other signals.
[b]Exchangeable.

as follows (min, solvent system): L-Val (12.9, C), D-Val (14.8, C), L-Tyr (11.3, C) and D-Tyr (12.0, C). The hydrolysate of **2** contained D-Arg (11.7, A), D-Val (14.8, C), L-Tyr (11.3, C) and L-Leu (10.3, B). Therefore, the absolute configurations of **2** were established as shown in Figure 1a.

The obtained structure of MBJ-0173 (**1**) shows significant similarity to EM-f2368 isolated as a fungal opportunistic pathogen inhibitor.[13] MBJ-0174 (**2**) is structurally related to plactins, isolated as stimulators of cellular fibrinolytic activity.[6] The cytotoxic activity and antibacterial activity of **1** and **2** were tested. Compounds **1** and **2** did not display any significant cytotoxicity against human ovarian adenocarcinoma SKOV-3 cells, human malignant pleural mesothelioma ACC-MESO-1 cells and T-lymphoma Jurkat cells up to a compound concentration of 100 μM, respectively. Compounds **1** and **2** did not exhibit antibacterial activity against *Micrococcus luteus* ($IC_{50} > 100$ μM) and *Escherichia coli* (10 μg per disk), respectively.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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1 Kawahara, T., Nagai, A., Takagi, M. & Shin-ya, K. JBIR-137 and JBIR-138, new secondary metabolites from Aspergillus sp. fA75. *J. Antibiot. (Tokyo)* **65**, 535–538 (2012).
2 Kawahara, T. *et al.* Cytotoxic sesquiterpenoids MBJ-0009 and MBJ-0010 from a saprobic fungus Nectria sp. f26111. *J. Antibiot. (Tokyo)* **66**, 567–569 (2013).
3 Kawahara, T. *et al.* Three eremophilane derivatives, MBJ-0011, MBJ-0012 and MBJ-0013, from an endophytic fungus Apiognomonia sp. f24023. *J. Antibiot. (Tokyo)* **66**, 299–302 (2013).
4 Kawahara, T. *et al.* New chaetoglobosin derivatives, MBJ-0038, MBJ-0039 and MBJ-0040, isolated from the fungus Chaetomium sp. f24230. *J. Antibiot. (Tokyo)* **66**, 727–730 (2013).
5 Kawahara, T. *et al.* New hydroxamate metabolite, MBJ-0003, from Micromonospora sp. 29867. *J. Antibiot. (Tokyo)* **67**, 261–263 (2014).
6 Inoue, T., Hasumi, K., Kuniyasu, T. & Endo, A. Isolation of plactins A, B, C and D, novel cyclic pentapeptides that stimulate cellular fibrinolytic activity. *J. Antibiot. (Tokyo)* **49**, 45–49 (1996).
7 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).
8 Furihata, K. & Seto, H. J-Resolved HMBC, a new NMR technique for measuring heteronuclear long-range coupling constants. *Tetrahedron Lett.* **40**, 6271–6275 (1999).
9 Kawahara, T. *et al.* MBJ-0086 and MBJ-0087, new bicyclic depsipeptides, from Sphaerisporangium sp. 33226. *J. Antibiot. (Tokyo)* **68**, 67–70 (2015).
10 Palermo, J. A., Rodríguez Brasco, M. F., Cabezas, E., Balzaretti, V. & Seldes, A. M. Celenamide E, a tripeptide alkaloid from the patagonian sponge Cliona chilensis. *J. Nat. Prod.* **61**, 488–490 (1998).
11 Shigematsu, N. *et al.* Structure of WS9326A, a novel tachykinin antagonist from a Streptomyces. *J. Org. Chem.* **58**, 170–175 (1993).
12 Marfey, P. Determination of D-amino acids. II. Use of a bifunctional reagent, 1,5-difluoro-2,4-dinitrobenzene. *Carlsberg Res. Commun* **49**, 591–596 (1984).
13 Hata, K., Fujita, M., Sakata, N. & Dobashi, K. EM-f2368 manufacture with Mortierella alpina. *Jpn Kokai Tokkyo Koho JP* 2001–354695 (2001).