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Microbacterins A and B, New Peptaibols from the Deep Sea Actinomycete *Microbacterium sediminis* sp. nov. YLB-01(T)

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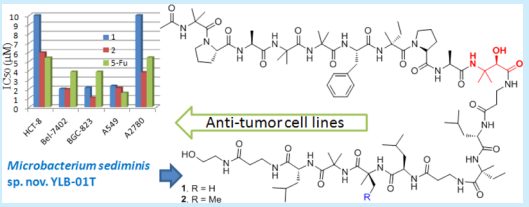
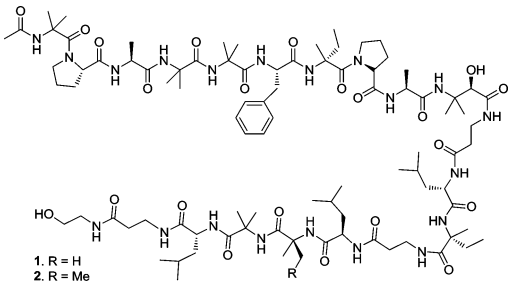
Supporting Information

ABSTRACT: Two new peptaibols, namely microbacterins A (**1**) and B (**2**), were isolated from the deep sea inhabited actinomycete *Microbacterium sediminis* spp. nov. YLB-01(T). The sequences of the amino acid residues were determined on the basis of intensive NMR and ESI-MS/MS spectroscopic analysis, in addition to the Marfey's method and CD and optical rotation data for the configurational assignment. Both **1** and **2** exhibited significant cytotoxic activities against a panel of human tumor cell lines.



Peptaibols are an irregular class of nonribosomally biosynthesized linear or cyclic peptides with different biological properties. Naturally occurring peptaibols are structurally characterized by the presence of a high proportion of α-aminoisobutyric acid (Aib) and/or isovaline (Iva) with an N-terminus that is usually modified with an acetyl group and a C-terminus modified with an amino alcohol.[1−10] The unusual Aib and Iva residues as the marker amino acids of peptaibols were depicted to be formed from abiotic and biotic origin, while the possible formation of abiotic Aib and Iva is suggested to be induced by aqueous or enzymatic hydrolysis of 5,5-dimethyl- and 5-ethyl-5-methylhydantoins which may be derived from volcanic action, while the carbonaceous meteorites may supply a new source of abiotic Aib and Iva in peptaibiotic-producing fungi.[11−13] Peptaibols exert antimicrobial activities against a range of Gram-positive/-negative bacterial and fungal phytopathogens. Their antibiotic function in the biological control of plant diseases may result from the formation of voltage-dependent ion channels in cell membranes due to the amphipathic property and the helical structures of peptaibols in nature. This specific mechanism is assumed to be related to their cytotoxic,[14,15] anti-HIV,[16] anti-inflammatory,[17] and antimycoplasmic[18] activities. In the course of our investigation of the bioactive metabolites from extremophile microorganisms, a chemically unknown actinomycete *Microbacterium sediminis* sp. nov. (YLB-01-T) isolated from the deep sea[19] showing antitumor activity was selected for chemical examination. Chromatographic separation of the EtOAc fraction of the solid fermentation broth resulted in the isolation of two new peptaibols, namely microbacterins A (**1**) and B (**2**).

The molecular formula of microbacterin A (**1**) was determined to be $C_{91}H_{152}N_{20}O_{22}$ on the basis of the HRESIMS data at m/z 1878.1463 $[M + H]^+$, 1900.1659 $[M + Na]^+$, and 1916.1394 $[M + K]^+$ in association with the NMR data. The ^1H



NMR spectrum exhibited 18 D_2O changeable protons ranging δ_H 7−9 ppm for amide protons, eight α-H ranging δ_H 4−5 ppm, and numerous protons in the alkyl region involving 14 methyl singlets, in addition to the ^{13}C NMR data for 20 carbonyl carbons and the resonances among δ_C 50−70 ppm and δ_C 5−40 ppm (Table S1, Supporting Information), featuring a peptaibol derivative.[20] Intensive interpretation of the COSY and HMBC spectra established 19 amino acid residues, including a Phe (phenylalanine), two Ala (alanine), two Pro (proline), three Leu (leucine), two Iva (isovaline), five Aib (aminoisobutyric acid), three β-Ala (β-alanine), as well as an 3-amino-2-hydroxyvaline. A moiety of 2-aminoethanol was recognized by the COSY correlations between NH (δ_H 7.80, t, J = 5.7 Hz)/β-H_2 (δ_H 3.10, m), β-H_2/α-H_2 (δ_H 3.38, m), and α-H_2/OH (δ_H 4.61, t, J = 4.8 Hz), while this moiety was considered to form a C-terminal end. The NMR resonances of two Iva residues were characterized by the presence of methyl triplets (δ_H 0.69, t, J = 7.2 Hz; δ_H 0.81, t, J = 7.2 Hz) for ethyl groups and the upfield shifts of methyl carbons at δ_C 8.3 and 7.9 ppm, along with the HMBC interactions from the methyl

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protons to the quaternary carbons at δ_C 59.8 and 59.3, respectively. The unusual residue of 3-amino-2-hydroxyvaline (AHV) was recognized by the hydroxymethine resonance at δ_C 75.3 and the methine proton at δ_H 4.19 (1H, d, J = 5.7 Hz) correlating to OH (δ_H 5.74, d, J = 5.7 Hz) in the COSY spectrum and the HMBC interactions of the OH proton with a carbonyl carbon at δ_C 171.9 and a quaternary carbon at δ_C 56.5, in addition to the HMBC relationships of two methyl singlets at δ_H 1.26 (3H, s) and 1.29 (3H, s) with the carbons at δ_C 56.5 and 75.3. In addition, an acetyl group (δ_H 1.96; δ_C 22.8 and 170.7) was identified by the NMR spectra. The sequential assignment was carried out by the NOE interactions from NH and α-H of one residue to those of adjacent residue, in association with the HMBC correlation which helped to assign the carbonyl carbon of each amino acid. The acetyl group was assumed to be in one terminal end as the cases found in known peptaibols. The NOE interpretation was initialized from the acetyl terminus, whose protons showed the NOE interaction with NH-1 (δ_H 8.73, s, Aib-1), indicating the acetyl group positioned at NH-1 of the first Aib residue. Subsequent NOE interactions were observed between NH-1/δ-H_2 (δ_H 3.22, 3.79, Pro-2), α-H (δ_H 4.26, Pro-2)/NH-3 (δ_H 7.91, Ala-3), α-H (δ_H 4.10, Ala-3)/NH-4 (δ_H 7.90, Aib-4), α-H (δ_H 4.30, Pro-8)/NH-9 (δ_H 7.78, Ala-9), NH-9/NH-10 (δ_H 7.09, AHMBA-10), α-H (δ_H 4.19, AHMBA-10)/NH-11 (δ_H 7.60, β-Ala-11), NH-11/NH-12 (δ_H 8.14, Leu-12), α-H (δ_H 4.13, Leu-12)/NH-13 (δ_H 7.83, Iva-13), NH-13/NH-14 (δ_H 7.60, β-Ala-14), and NH-19 (δ_H 7.42, β-Ala-19)/α-H (δ_H 4.10, Leu-18), as well as the NOE correlations from NH-5 (δ_H 7.32) to NH-4 and NH-6 (δ_H 7.60, Phe-6), NH-7 (δ_H 7.68, Iva-7) to NH-6 and δ-H_2 (δ_H 3.45, 3.65, Pro-8), NH-15 (δ_H 7.42, Leu-15) to NH-14 and HN-16 (δ_H 7.63, Aib-16), and NH-17 (δ_H 8.43, Aib-17) to NH-16 and NH-18 (δ_H 8.09, Leu-18). These findings enabled to establish a partial structure for 19 amino acid residues sequencing Ac-Aib-Pro-Ala-Aib-Aib-Phe-Iva-Pro-Ala-AHV-β-Ala-Leu-Iva-β-Ala-Leu-Aib-Aib-Leu-β-Ala. The remaining 2-aminoethanol unit was substituted at β-Ala-19 to form a C-terminus. These assignments were further clarified by the HMBC relationships from NH and α-H to the respective carbonyl carbon.

The sequence order of amino acid residues was also supported by the ESI-MS/MS data. Primary fragmentation of the molecular ion at m/z 1878.1463 $[M + H]^+$ afforded two main daughter ions at m/z 1166.7329 ($C_{55}H_{100}N_{13}O_{14}$) and m/z 712.4034 ($C_{36}H_{54}N_7O_8$), which were derived by α-cleavage between Iva-7 and Pro-8 to form y_{13} and b_7 segments. Further ESI-MS/MS ionization of the segment y_{13} at m/z 1166 resulted in a series of b-type fragments (m/z 1034, 921, 836, 751, 638, 567, 468, 355, 284, and 169) (Figure 1), which were in accordance with the sequential loss of amino acid residues from C-terminus. In addition, the ESI-MS/MS fragmentation of the second daughter ion b_7 at m/z 712 produced the ion peaks (Figure 2) which were characterized by the b-type in association with a-type cleavage, while these MS data were in agreement with the partial sequence from N-terminus.

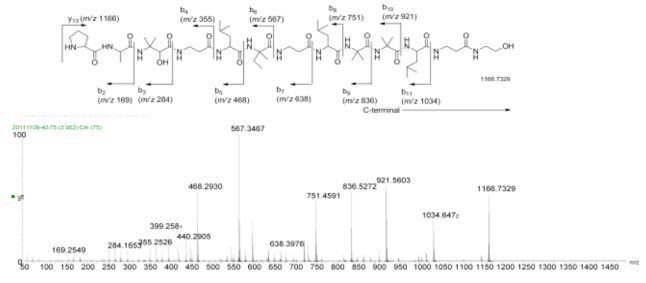

Figure 1. ESI-MS/MS ionization of fragment A of **1**.

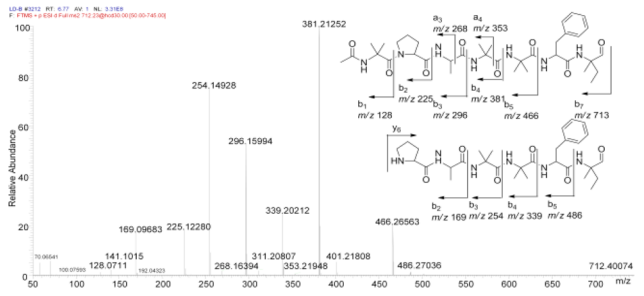

Figure 2. ESI-MS/MS ionization of fragment B of **1**.

The absolute configuration of the amino acids in **1** were determined by the acidic hydrolysis of **1** and subsequent derivertization according to the advanced Marfey's method.[21] Comparison of the resulting derivatives such as 5-fluoro-2,4-dinitrophenyl-L-alanine amide with those of appropriate standard amino acids using UPLC-MS techniques indicated L-form for Ala, Phe, Pro, and Leu. The absolute configuration of Iva residue can be determined on the basis of the chemical shift values of its ethyl group if the conformation of the structure is assigned. The occurrence of two negative maxima at 210 and 225 nm and a positive maximum near 195 nm in the CD curve (Figure 3) reflected the right-handed helical conformation of

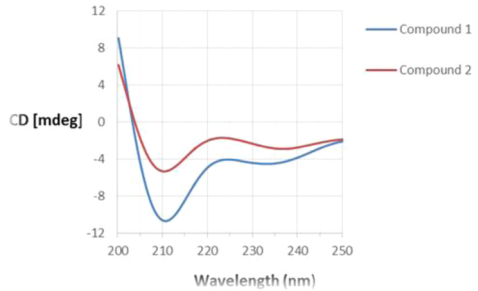

Figure 3. CD spectrum of compounds **1**-**2**.

1.[22] Thus, the chemical shifts of γ-H_3 (δ_H 0.81 for Iva-7, δ_H 0.69 for Iva-13, <0.89 ppm), $\Delta\delta_{\beta Hb\text{-}\beta Ha}$ (0.34 ppm for Iva-7, 0.23 ppm for Iva-13, >0.20 ppm), and βC (δ_C 28.7 for Iva-7 and δ_C 29.7 for Iva-13, <30 ppm) (Table 1) were in agreement with the R-configuration for the Iva residue.[23] Acidic hydrolysis of compound **1** and semipreparative HPLC separation resulted in the isolation of 3-amino-2-hydroxyvaline (AHV). On the basis of the density functional theory (DFT) methods,[24] the positive

Table 1. ^{1}H NMR Parameters of Iva of 1 and 2

	1		2		
NMR parameter	Iva-7	Iva-13	Iva-7	Iva-13	Iva-16
δ_H data of the γ-methl protons	0.69	0.81	0.69	0.81	0.74
δ_C data of the β-methylene carbon	29.7	28.7	29.6	28.7	27.5
$\Delta\delta$ of the two β-methylene protons	0.23	0.34	0.23	0.34	0.28

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sign (+12.5) of the optical rotation (OR) of *R*-isomer of AHV was calculated at the B3LYP/6-311++G (2d,p) level, whereas the computed OR sign for *S*-isomer of AHV was negative (−12.5). Thus, the measured specific rotation of AHV ($[\alpha]_D$ +10.6, *c* 0.04, MeOH) was in agreement with the *R* configuration.

The molecular formula of **2** was determined to be $C_{92}H_{154}N_{20}O_{22}$ on the basis of HRESIMS data, containing CH_2 unit more than that of **1**. Analysis of 1D and 2D NMR data revealed most amino acid residues and the order of their connection in **2** to be the same as those of **1**. The distinction was attributed to the absence of Aib-16 which was replaced by an Iva residue, as evident from the presence of an additional ethyl group (δ_C 7.9, 27.0), whose methyl protons (δ_H 0.70, t, *J* = 7.0 Hz) correlated to the quaternary carbon at δ_C 59.8 (α-carbon). The location of this Iva residue was supported by the ESI-MS/MS data. Primary fragmentation of the molecular ion at *m/z* 1892 $[M + H]^+$ produced two major daughter ions at *m/z* 712 (A) and *m/z* 1180 (B), while the composition of the ion peak of A segment and its ESI-MS/MS fragments were the same as those of **1**, indicating the same partial sequence. The ESI-MS/MS ionization of *m/z* 1180 resulted in the fragments at *m/z* 1048 (B − $C_5H_{11}N_2O$), 935 (B − $C_5H_{11}N_2O$ − Leu), 850 (B − $C_5H_{11}N_2O$ − Leu − Aib), 751 (B − $C_5H_{11}N_2O$ − Leu − Aib − Iva), and 638 (B − $C_5H_{11}N_2O$ − Leu − Aib − Iva − Leu), in addition to the remaining fragments to be the same as those of **1**, supported the sequential position of the additional Iva residue. The absolute configurations of the amino acid residues and AHV unit of **2** were the same as those of **1** based on the similar results provided by Marfey's method, CD effects, chemical shifts, and the specific rotation.

The antitumor bioassay revealed that compound **2** exhibited significant inhibitory effects against a panel of human tumor cell lines including HCT-8, Bel-7402, BGC-823, A549, and A2780 (Table 2) with IC_{50} values ranging 1.03−5.93 μM, which were compatible to those of positive control 5-fluorouracil. In addition, compound **1** showed selectively inhibition against Bel-7402, BGC-823, and A549. These findings informed that the antitumor effects of peptaibols are closely relied on the type of amino acid residues.

Table 2. Activities for Anti-tumor Cells of 1 and 2[a]

	IC_{50}(μM)				
	HCT-8	Bel-7402	BGC-823	A549	A2780
1	>10	1.98	2.11	2.30	>10
2	5.93	1.94	1.03	2.08	3.79
5-fluorouracil	5.38	3.85	3.84	1.54	5.40

[a]HCT-8 (human intestinal adenocarcinoma cell), Bel-7402 (human hepatoma cell), BGC-823 (human gastric cancer cell), A549 (human lung cancer cell), A2780 (human ovarian cancer cell). 5-Fluorouracil: positive control.

The documented peptaibols are exclusively derived from fungal origin, whereas the present work first reported marine-derived actinomycete to be a new source for the production of peptaibols. The unusual 3-amino-2-hydroxyvaline (AHV) was first isolated from a mushroom *Pleurocybella porrigens*,[25] while this is the first report for peptaibols containing this moiety and the second discovery of the naturally occurring AHV. The C-terminus linked by an aminoethanol is rarely found in the peptaibol family. The primary genomic analysis resulted in a nonribosomal peptide synthetase modules gene which contained an adenylate-forming domain, phosphopantetheine attachment domain, and a nonribosomal peptide synthetase terminal domain, whereas the biosynthetic study of the unusual peptaibols is in progress. The unusual peptaibols with significant antitumor effects suggested the deep sea derived microorganisms could be a potential source for lead compound discovery.

■ ASSOCIATED CONTENT

Supporting Information

Tables for NMR assignments of **1** and **2**, experimental section, spectroscopic copies of **1** and **2**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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