New Diketopiperazine Derivatives Isolated from Sea Urchin-Derived *Bacillus* sp.

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Two new diketopiperazine derivatives, bacillusamides A (1) and B (2), have been isolated from the EtOAc extract of the sea urchin-derived *Bacillus* sp. along with the known cyclo(-L-pro-L-val-) (3), cyclo(-L-pro-L-tyr-) (4), cyclo(-L-pro-L-phe-) (5). These structures were elucidated by extensive spectroscopic methods. Furthermore, the absolute configurations of the amino acid residues were determined using Marfey's method. Compound 1 displayed weak antifungal activity against *Aspergillus niger*.

Key words *Bacillus* sp.; diketopiperazine derivative; antifungal activity

Marine microorganisms have attracted considerable attention as some of the most important resources for new biologically active metabolites.[1)] Recently, a number of diketopiperazine derivatives were obtained from various microorganisms.[2)] Many of these constituents exhibit interesting biological activities, *e.g.*, antitumor,[3)] antiviral,[4)] antifungal,[5)] antibacterial[6)] activities.

We are studying the bioactive compounds of marine microorganisms that live in the Nagasaki coast. Recently, as part of our research, it was observed that the extract of the culture of *Bacillus* sp., derived from digestive tract of sea urchin (*Anthocidaris crassispina*), showed antimicrobial activity against *Aspergillus niger*. So, we have researched the active constitutes of the bacterium. As a result, two new diketopiperazine derivatives bacillusamides A (**1**) and B (**2**) have been isolated along with known compounds **3**,[7)] **4**,[8)] and **5**.[9)] In this paper, we will describe the isolation, structure elucidation and biological activities of these compounds.

The marine bacterium *Bacillus* sp. was obtained from the digestive tract of the sea urchin, *Anthocidaris crassispina*, collected in the Nagasaki shitsu coast of Japan in 2007. The strain was cultured at 24 °C on a rotary shaker using a seawater-based medium. The fermentation broth (30 l) was successively partitioned with EtOAc. The broth extracted with 30 l of EtOAc in three times to yield 15.2 g of brown oily extract. The EtOAc extract was then subjected to Sephadex LH-20, silica gel column chromatography and octa decyl silyl (ODS) column chromatography, followed by reverse phase (RP)-HPLC to yield diketopiperazine derivatives, bacillusamides A (**1**) and B (**2**), along with known compounds **3**—**5**. Structures of these compounds were elucidated by using extensive spectroscopic methods.

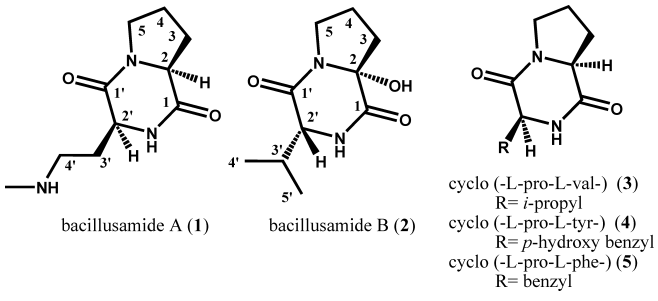

Fig. 1. Structures of Compounds **1**—**5**

Bacillusamide A (**1**) was obtained as a white amorphous powder, and its molecular formula was assigned as $C_{10}H_{18}N_3O_2$ by high resolution (HR)-FAB-MS (*m*/*z* 212.1420 $[M+H]^+$, Calcd for 212.1399, Δ +2.1 mmu), indicating 4 degrees of unsaturation. The IR absorptions of 3470, 1701, and 1630 cm^{-1} showed the presence of the amide group. In the ^{1}H-NMR spectrum, singlet methyl proton [δ_H 2.42 (3H, s)] and two amino protons [δ_H 5.15 (1H, br s), 9.40 (1H, br s)] were observed and these data suggested that compound **1** has the amino methyl group (Table 1). The ^{13}C-NMR spectrum showed 10 carbon signals, attributable to one methyl carbon (δ_C 38.5), five methylene carbons (δ_C 22.8, 23.5, 28.5, 45.7, 49.9), two methine carbons (δ_C 54.5, 59.4), and two carbonyl carbons (δ_C 166.8, 170.8) (Table 1). These data together with the degree of unsaturation revealed that **1** contain two rings in the molecule. A detailed analysis of the ^{1}H–^{1}H correlation spectroscopy (COSY) spectrum showed connectivity for three proton spin systems, H_2–H_3–H_4–H_5, NH–$H_{2'}$–$H_{3'}$–$H_{4'}$, and NH-Me. The heteronuclear multiple bond correlations (HMBC) of H-5 to C-2, H-2 to C-1, H-3 to C-1, H-4′ to N-Me, and N-Me to C-4′ were observed. Moreover HMBC correlation of H-3′ to C-1′ was also observed. These data defined the presence of proline moiety (**a**) and 2-amino-4-(methylamino)-butanoic acid moiety (**b**) in **1** (Fig. 2). The connectivity of these two moieties were revealed by the HMBC correlation of H-5 to C-1′ and NH to C-2. The relative stereochemistry of **1** was determined by the nuclear Overhauser enhancement spectroscopy (NOESY) spectrum. Namely, a nuclear Overhauser effect (NOE) correlation between H-2 with H-2′ suggested that the two protons had same orientation as the diketopiperazine ring system, thereby forming a boat conformation (Fig. 3). The absolute configuration of **1** was defined by acid hydrolysis and Marfey's method,[10)] using standard amino acids (Table 2). The absolute configurations of the proline residue was determined as L-form. Thus, the absolute configuration was defined as 2*S*, 2′*S*.

Bacillusamide B (**2**) was obtained as a light yellow amorphous powder. The molecular formula of **2** was assigned as $C_{10}H_{16}N_2O_3$ by HR-FAB-MS (*m*/*z* 213.1244 $[M+H]^+$, Calcd for 213.1239, Δ +0.5 mmu), indicating 4 degrees of unsaturation. The IR spectrum of **2** suggested the presence of the

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Table 1. ^{1}H- (500 MHz) and ^{13}C-NMR (125 MHz) Data of **1** and **2** in Pyridine-d_5[a)]

No.	**1**		**2**	
	δ_H	δ_C	δ_H	δ_C
1	—	170.8	—	169.5
2	4.18 (1H, t, J=8 Hz)	59.4	—	87.3
3	2.10 (2H, m)	28.5	2.27 (1H, m) 2.56 (1H, m)	37.9
4	1.62 (2H, m)	22.8	1.74 (1H, m) 2.23 (1H, m)	19.8
5	3.43 (1H, m) 3.56 (1H, m)	45.7	3.82 (2H, t, J=9.2 Hz)	45.8
1′	—	166.8	—	167.5
2′	4.39 (1H, t, J=4.8 Hz)	54.5	3.87 (1H, dd, J=4.0, 6.8 Hz)	64.3
3′	2.66 (2H, m)	23.5	2.69 (1H, m)	33.8
4′	3.16 (2H, m)	49.9	1.18 (3H, d, J=6.8 Hz)	19.9
5′			1.12 (3H, d, J=6.8 Hz)	19.5
NH	9.40 (1H, br s)	—	9.58 (1H, m)	—
NH-Me	2.42 (3H, s) 5.15 (1H, br s)	38.5		

a) Spectra were aquired at 23 °C. Chemical shifts were given in δ (ppm) and referenced to internal solvent for pyridine-d_5 at 7.19 (δ_H) and 123.5 ppm (δ_C).

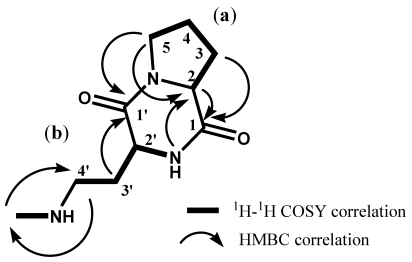

Fig. 2. Partial Structures (**a**) and (**b**) of Bacillusamide A (**1**)

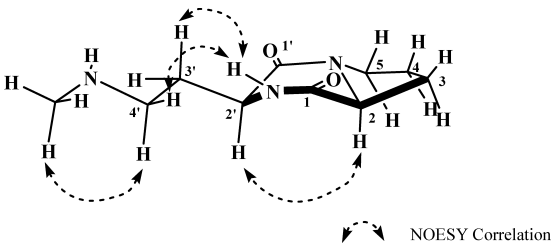

Fig. 3. NOESY Correlations of Bacillusamide A (**1**)

Table 2. Retention Times for Amino Acids Obtained from **1** and **2** as Their N^{α}-(5-Fluoro-2,4-dinitrophenyl)-L-leucinamide (FDLA) Derivatives

Residue	Standards retention time (min)	Acid hydrolysate of **1** retention time (min)	Acid hydrolysate of **2** retention time (min)
L-Pro	8.24	8.09	
D-Pro	12.68		
L-Val	19.99		
D-Val	21.10		21.08

Retention times were determined by HPLC analyses [Mightysil RP-18 (250×4.6 mm i.d., Kanto Chemical Co., Inc.), mobile phase; 40% MeCN–H_2O, flow rate; 1 ml/min, detection; UV 340 nm].

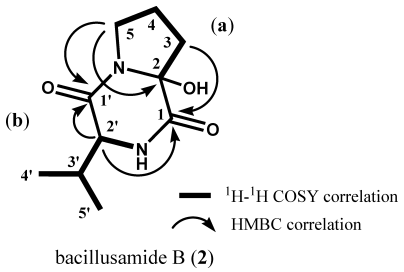

Fig. 4. Partial Structures (**a**) and (**b**) of Bacillusamide B (**2**)

hydroxyl group (3224 cm^{-1}) and amide carbonyl group (1644 cm^{-1}). The ^{1}H-NMR spectrum showed signals for the isopropyl group [δ_H 1.12 (3H, d, J=6.8 Hz), 1.18 (3H, d, J=6.8 Hz), 2.69 (1H, m)] and amide proton [δ_H 9.58 (1H, m)] (Table 1). The ^{13}C-NMR spectrum displayed 10 carbon signals, including two methyl carbons (δ_C 19.5, 19.9), two methylene carbons (δ_C 19.8, 37.9), one methylene carbon bearing nitrogen (δ_C 45.8), two methine carbons (δ_C 33.8, 64.3), one quaternary carbon bearing oxygen (δ_C 87.3), and two carbonyl carbons (δ_C 167.5, 169.5) (Table 1). These data showed the presence of two amide groups, and thus, **2** was found to have a bicyclic skeleton in the same manner as **1**. Detailed analyses of the 2D NMR spectral data such as heteronuclear single quantum coherence (HSQC), COSY and HMBC spectra showed the presence of two partial structures including 2-hydroxy-proline moiety (**a**) and valine moiety (**b**) (Fig. 4). Furthermore, the connectivity of these partial structure were revealed by the HMBC correlations from H-5 to C-1′ and from H-2′ to C-1. The relative stereochemistry of C-2 and C-2′ of compound **2** was confirmed on the basis of comparing the ^{13}C-NMR data of **2** with those of the known compound, notoamide M.[11—13)] Because the chemical shifts of C-1, C-2, C-3, C-4, C5, C-1′ and C-2′ of **2** showed the same data with those of notoamide M, the relative stereochemistry of C-2 and C-2′ on **2** was determined as shown in Fig. 1.

The absolute configuration of **2** was also, in the same manner as **1**, elucidated based on acid hydrolysis and Marfey's method, using standard amino acids (Table 2). The absolute configurations of the valine residue was determined as D-form. Thus, the absolute configuration was defined as 2*R*,

2′*R*.

The anti-microbial activities of compounds **1**—**5** were tested for the growth inhibition of 8 microbes with the paper disk method.[14] The growth inhibition was studied in a concentration of 125 μg/disk. As a result, compound **1** exhibited weak inhibition activity against *Aspergillus niger*, and compounds **4** and **5** exhibited moderate inhibition activity against *Aspergillus niger*. Compounds **2** and **3** did not show anti-microbial activity.

Experimental

General IR spectra were obtained with JASCO FT/IR-410 spectrophotometers. Optical rotations were measured with a JASCO DIP-370 digital polarimeter. ^{1}H- and ^{13}C-NMR, ^{1}H–^{1}H COSY, NOESY, HSQC and HMBC spectra were recorded with a Unity plus 500 spectrometer (Varian Inc., U.S.A.) operating at 500 MHz for ^{1}H, and 125 MHz for ^{13}C, respectively. FAB-MS were recorded on a JMS DX-303 spectrometer (JEOL Ltd., Japan), and *m*-nitrobenzyl alcohol or Magic bullet used as a matrix. Preparative HPLC was performed on a Develosil C-30-UG-5 (250×4.6 mm i.d., Nomura Chemical Co., Aichi, Japan), at a flow rate of 1.0 ml/min, equipped with a JASCO RID-300 detector and a JASCO BIP-I HPLC pump.

Bacterial Material and Fermentation The marine *Bacillus* sp. (strain number p-0707-517) was isolated from the digestive tract of sea urchin, *Anthocidaris crassispina*, collected in the Nagasaki Shitsu coast of Japan in 2007. The subcultures of the bacterium are deposited at the Garden for Medicinal Plants, Graduate School of Biomedical Sciences Nagasaki University. The bacterium was grown in a seawater medium (D-glucose 1%; polypeptone 0.5%; yeast extract 0.3%; KH_2PO_4 0.3%; $MgSO_4$ 0.1%; pH 7.5) rotary-shaking at 120 rpm for 21 d at 24 °C . The culture broth (30 l) was sonicated followed by filtration.

Extraction and Isolation The filtered broth was extracted with EtOAc (10 l×3). EtOAc extract concentrated under reduced pressure to dryness. The dried residue (15.2 g) was subjected to Sephadex LH-20 ($CHCl_3$–MeOH=5 : 5) to yield 4 fractions (fractions 1—4). The third fraction was chromatographed on silica gel using a *n*-hexane– acetone (from 8 : 2 to 5 : 5) followed by $CHCl_3$–MeOH–H_2O (from 95 : 5 : 0 to 5 : 5 : 1) to yield 8 fractions. Fraction 4 of the eight fractions (145.3 mg) was subjected to reversed phase HPLC (5% MeOH–H_2O) to give **3** (t_R=21.2 min, 1.3 mg). Fraction 6 of the eight fractions (612.7 mg) was chromatographed on ODS using 20% MeOH–H_2O as the eluent to give followed by a subject on a reversed phase HPLC (40% MeOH–H_2O) to give the mixture of **1** and **2**, **4** (t_R=4.1 min, 37.0 mg), and **5** (t_R=4.9 min, 9.9 mg). The mixture of **1** and **2** was subjected to reversed phase HPLC (5% MeOH–H_2O) to give **1** (t_R=19.6 min, 1.3 mg) and **2** (t_R=21.2 min, 4.0 mg).

Bacillusamide A (**1**): White amorphous powder; $[\alpha]_D^{30}$ −64.0° (*c*=0.04, Py); IR ν_{max} (dry film) 3470, 2962, 1701, 1630, 1447, 1133 cm^{-1}, ^{1}H- and ^{13}C-NMR data (see Table 1); (+)FAB-MS *m/z*: 212[M+H]$^+$, 197 [M−CH$_3$]$^+$; (+)HR-FAB-MS *m/z*: 212.1420 [M+H]$^+$ (Calcd for $C_{10}H_{18}N_3O_2$, 212.1399, Δ +2.1 mmu).

Basillusamide B (**2**): Light yellow amorphous powder; $[\alpha]_D^{30}$ +44.0° (*c*=0.04, MeOH) ; IR ν_{max} (dry film) 3224, 2974, 1644, 1442 cm^{-1}; ^{1}H- and ^{13}C-NMR data (see Table 1); (+)FAB-MS *m/z*: 213 [M+H]$^+$; (+)HR-FAB-MS *m/z*: 213.1244 [M+H]$^+$ (Calcd for $C_{10}H_{17}N_2O_3$, 213.1239, Δ +0.5 mmu).

Determination of Amino Acids Configuration A sample of **1** or **2** (100 μg) was hydrolyzed in 6 N HCl (100 μl) at 110 °C for 12 h. After concentration to dryness, the residue was dissolved in 25 μl of H_2O and 20 μg of N^{α}-(5-fluoro-2,4-dinitrophenyl)-L-leucinamide (FDLA) in acetone (50 μl) and 10 μl of 1 M $NaHCO_3$ aq. were added. The mixture was heated at 37 °C for 1 h. and 10 μl of 1 N HCl was added. The solution was diluted ten times with CH_3CN followed by analyses by HPLC. The conditions for HPLC analyses and the retention times for standard and hydrolysate FDLA derivatives are provided in Table 2.

Antibiotic Activity Assay Activities of the Compounds **1**—**5** were tested by the paper disk method against *Aspergillus niger*, *Penicillium crustosum*, *Schizophyllum commune*, *Trichophyton concentricum*, *Saccharomyces cerevisae*, *Bacillus subtilis* subsp. *subtilis*, *Serratia marcescens* subsp. *marcescens*, *Staphylococcus aureus* subsp. *aureus* with 125 μg/disk. As a result, compound **1** slightly showed an inhibition circle against *Aspergillus niger*, and compounds **4** and **5** exhibited a weak inhibition circle against *Aspergillus niger*.

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