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Bacilotetrins A and B, Anti-Staphylococcal Cyclic-Lipotetrapeptides from a Marine-Derived *Bacillus subtilis*

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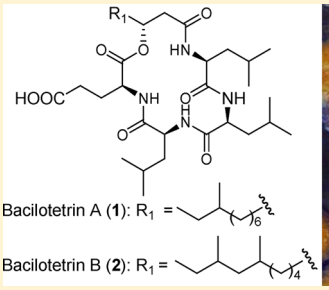
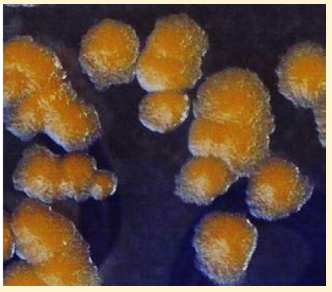
Supporting Information

ABSTRACT: LC-MS and NMR spectroscopy guided metabolic profiling and dereplication of a crude extract obtained from the fermentation of a marine-derived bacterium, *Bacillus subtilis*, followed by chromatographic isolation yielded two new cyclic-lipotetrapeptides, bacilotetrins A (**1**) and B (**2**). Based on extensive 1D and 2D NMR and high-resolution ESIMS data analysis, the structures of **1** and **2** were elucidated, revealing the unique structures of these lipopeptides consisting of three leucines and a glutamic acid residue cyclized with a lipophilic 3-hydroxy fatty acid. The absolute stereochemistries at selected stereocenters in **1** and **2** were assigned by chemical derivatization and comparison to literature data. Compounds **1** and **2** exhibited anti-MRSA activity with MIC values of 8 to 32 μg/mL. However, these compounds showed no cytotoxicity when tested against prostate and liver cancer cell lines using the standard SRB assay.





Marine-derived *Bacillus subtilis* isolates have been a good source of bioactive secondary metabolites.[1] Several bioactive compounds including lipopeptides, polypeptides, macrolactones, fatty acids, polyketides, and isocoumarins have been reported from this species.[2] The production of secondary metabolites is normally associated with the bacterium's response to a growth-limiting environment.[3] Faced with the depletion of essential nutrients, *B. subtilis* can adopt several responses, including motility, secretion of extracellular enzymes, competence for genetic transformation, antibiotic production, and finally sporulation. Depending on the environmental signal, one or more of these processes can be stimulated or inhibited.[4] This is evident from our studies reporting that upon alteration of growth conditions this species produces diverse classes of compounds.[5] On the basis of these observations, we have reported previously several classes of metabolites including macrolatins and lipopeptides having antibacterial and antifungal activities from the marine-derived *B. subtilis* 109GGC020.[5−7] Furthermore, our continuous chemical investigations on the ethyl acetate extract obtained from the culture of the same species upon alteration of growth conditions resulted in the isolation of two previously unknown cyclic-lipotetrapeptides, bacilotetrins A (**1**) and B (**2**). Here, we described their isolation, characterization, and anti-staphylococcal and cytotoxic activities.

The producing strain 109GGC020 was isolated from a marine sediment sample collected from the Gageocho reef, Republic of Korea, and identified as *B. subtilis* based on the 16S *r*RNA sequencing. The bacterium *B. subtilis* was cultured in large scale maintaining optimal growth conditions to obtain maximum metabolite production. The culture broth was filtered, and the cell-free supernatant was extracted with ethyl acetate. The crude extract was fractionated by open column chromatography and characterized by LC-MS and ^{1}H NMR data. The LC-MS and ^{1}H NMR metabolic profiling data guided the purification of compounds **1** and **2**.

Bacilotetrin A (**1**) possesses a molecular formula of $C_{37}H_{66}N_4O_8$ as determined from HR-(+)-ESIMS. The IR spectra of **1** gave a broad peak at 3361 cm^{-1} (NH) and 1694 (CO) cm^{-1} consistent with the presence of amide carbonyl groups, a smaller peak at 1743 cm^{-1}, which was indicative for an ester carbonyl, and a peak at 2931 cm^{-1} confirming the presence of an aliphatic chain. The ^{1}H NMR data (Table 1), recorded in CD_3OD and CD_3OH, of the chromatographically homogeneous material revealed the presence of a peptide backbone by four NH signals at δ_H 7.33 to 9.10 together with four α-protons at δ_H 3.74 to 4.60 and a long aliphatic chain at δ_H 1.29 (SI 1 and 5). A resonance at δ_H 5.16 indicated the presence of a further oxygenated proton, and CH_3 signals were observed at δ_H 0.89 to 0.98. The ^{13}C NMR data of **1** indicated the presence of six carbonyl carbons, four of which resonated at δ_C 173.3, 173.9, 174.6, and 175.9, attributed to amino acids, and the remaining two carbonyl carbons at δ_C 173.0 and 173.3

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Table 1. ^{1}H and ^{13}C NMR Data of 1 and 2 in CD_3OD

bacilotetrin A (1)		bacilotetrin B (2)	
δ_C, type	δ_H (J in Hz)	δ_C, type	δ_H (J in Hz)
Leu-1			
175.9, C		176.0, C	
NH	8.40, d (4.5)[a]	NH	8.42, d (4.0)[a]
55.5, CH	4.11, t (7.5)	55.7, CH	4.07, t (7.0)
40.7, CH_2	1.65, m	40.6, CH_2	1.65, m
26.4, CH	1.68, m	26.4, CH	1.68, m
21.8, CH_3	0.91, m	21.8, CH_3	0.91, m
24.1, CH_3	0.95, m	24.1, CH_3	0.95, m
Leu-2			
173.3, C		173.3, C	
NH	9.10, d (6.5)[a]	NH	9.10, d (6.5)[a]
54.9, CH	3.75, dd (11.0, 4.0)	54.9, CH	3.73, dd (11.0, 3.5)
38.6, CH_2	1.65, m	38.6, CH_2	2.02, m
26.4, CH	1.68, m	26.3, CH	1.68, m
21.3, CH_3	0.91, m	21.3, CH_3	0.91, m
23.9, CH_3	0.95, m	23.9, CH_3	0.95, m
Leu-3			
174.6, C		174.6, C	
NH	7.43, d (9.5)[b]	NH	7.74, d (8.5)[a]
53.4, CH	4.44, m	53.3, CH	4.41, m
40.5, CH_2	1.65, m	40.4, CH_2	1.71, m
25.8, CH	1.68, m	26.3, CH	1.68, m
21.3, CH_3	0.91, m	21.3, CH_3	0.91, m
23.9, CH_3	0.95, m	23.8, CH_3	0.95, m
Glu			
173.9, C		173.9, C	
NH	7.33, d (8.5)[b]	NH	7.77, d (10.0)[a]
51.6, CH	4.58, m	51.6, CH	4.56, m
35.5, CH_2	1.81, m	35.5, CH_2	1.96, m
31.5, CH_2	2.43, m	31.4, CH_2	2.43, m
173.3, C	COOH	173.3, C	COOH
***β*-OH acid**			
173.0, C		173.0, C	
41.6, CH_2	2.29, dd (14.0, 8.5)	41.5, CH_2	2.26, dd (14.0, 8.0)
	2.73, dd (13.5, 4.5)		2.70, dd (13.5, 4.5)
73.8, CH	5.16, m	73.9, CH	5.12, m
40.4, CH_2	1.58, m	40.4, CH_2	1.58, m
25.8–30.9, CH_2	1.29, brds	25.8–33.2, CH_2	1.29, brds
30.6, CH	1.50, m	29.3, CH	1.52, m
33.2, CH_2	1.29, brds	30.8, CH_2	1.29, brds
		35.8, CH	1.29, brds
14.6, CH_3	0.89, m	37.9, CH_2	1.12, m
			1.29, brds
23.9, CH_3	0.90, m	11.9, CH_3	0.87, m
		19.8, CH_3	0.86, m
		23.2, CH_3	0.87, m

[a,b]Chemical shifts and coupling constants of **1** and **2** were determined in CD_3OH and DMSO, respectively. The ^{13}C and ^{1}H NMR data of **1** and **2** were measured at 125 and 500 MHz, respectively.

(overlapped) were attributable to ester and carboxylic functionalities. These detailed IR absorbances together with the ^{1}H and ^{13}C NMR data analysis revealed the lipotetrapeptidic nature of **1**.

On the basis of COSY, TOCSY, and HMBC correlations, two partial substructures, **1a**, a tetrapeptide consisting of four amino acids, and **1b**, a lipophilic unit (3-hydroxy fatty acid), were elucidated for compound **1** (Figure 2). The substructure **1a** was deduced from the intra-COSY and TOCSY correlations

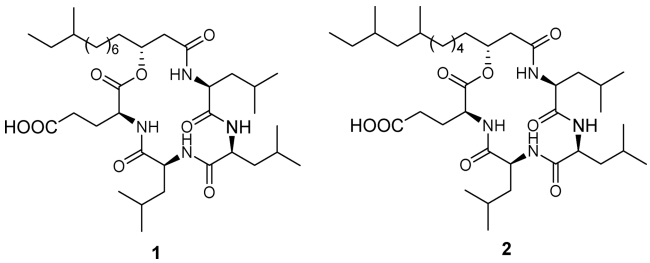

Figure 1. Structures of bacilotetrins A (**1**) and B (**2**).

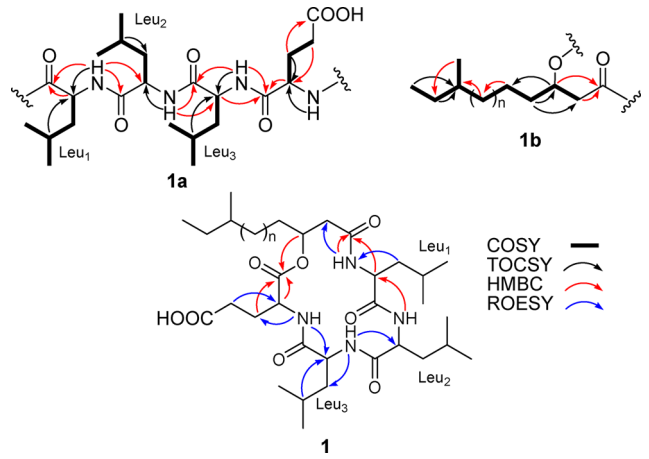

Figure 2. COSY, TOCSY, HMBC, and ROESY inter- and intraresidue correlations for **1**.

in each individual amino acid and inter-HMBC correlations among α-protons, NH protons, and carbonyl carbons of four amino acids (Figure 2). The substructure **1b** was deduced from the TOCSY and HMBC correlations of the oxygenated proton resonating at δ_H 5.16 with the carbonyl carbon at δ_C 173.0 and a long aliphatic chain at δ_H 1.29. The ^{13}C NMR spectrum of **1** contained eight methyl carbons; among them, six methyl carbons were attributed to three Leu residues, and the remaining two methyl groups were assumed to be present in the fatty acid chain. One methyl group at δ_C 14.6 was assigned as a terminal methyl, and another methyl group at δ_C 23.9 was located at C-11 in the fatty acid unit by the analysis of TOCSY and HMBC correlations. Based on the molecular weight of **1**, the fatty acid chain was determined to be a C_{14} fatty acid amidated to the N-terminal amine of the peptide. The chain length of the fatty acid was confirmed by the LC-(−)-APCIMS data of the acid hydrolysate of **1** (SI 22). Thus, the fatty acid was determined to be the same as the previously described fatty acid 3-hydroxy-11-methyltridecanoic acid.[8] The cyclic-lipotetrapeptide structure of **1** was then deduced by connecting the substructures **1a** and **1b** on the basis of ROESY and HMBC experiments showing long-range correlations of an NH proton at δ_H 8.40 and an α-proton at δ_H 4.11 of Leu_1 to the methylene signals at δ_H 2.29/2.73 and a carbonyl carbon at δ_C 173.0 of the fatty acid, resulting in the sequence Leu-Leu-Leu-Glu-fatty acid (Figure 2). The point of cyclization of **1** was deduced from the long-range HMBC correlation of an oxygenated proton at δ_H 5.16 of the fatty acid with a carbonyl carbon at δ_C 173.9 of the carboxyl group of the C-terminal Glu residue.

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Table 2. Anti-Staphylococcal Activity of Bacilotetrin A (1) and Bacilotetrin B (2)[a]

	MRSA strain (MIC, μg/mL)				
compound	ATCC25923	XU2120	SA1199B	RN4220	EMRSA15
bacilotetrin A	8	16	8	32	NA
bacilotetrin B	16	16	32	NA	NA
norfloxacin	16	16	64	2	4

[a]Norfloxacin: positive control; NA: not active.

Bacilotetrin B (**2**) possesses a molecular formula of $C_{38}H_{68}N_4O_8$ deduced by HR-(+)-ESIMS. Comparison of the ^{1}H NMR chemical shifts of **2** with those of **1** implied that they shared the same planar structure except for the presence of an additional methylene group as indicated by the molecular formula. The methylene group might be located either on the alkyl chain or on the peptide chain. To determine the exact location of the methylene group, a detailed investigation on the structure of **2** was undertaken by a series of 1D and 2D NMR experiments (SI 15 to 20). These experiments showed the presence of the same amino acid sequence Leu-Leu-Leu-Glu in **1** and **2**. Therefore, the additional methylene group must be located in the alkyl chain. This observation was confirmed by the LC-(−)-APCIMS peak at m/z 257.05 $[M - H]^-$ of the hexane phase of the acid hydrolysate of **2** (SI 23) and identified the lipophilic part as a 3-hydroxytetradecanoic acid. The careful interpretation of the ^{13}C NMR data of **2** revealed nine methyl carbons at δ_C 11.9 to 23.9 in **2**, six of them attributed to three Leu residues by 2D data analysis and the remaining three methyl groups located in the fatty acid chain based on the TOCSY and HMBC correlations. The methyl carbon resonating at δ_C 11.9 was located as a terminal methyl, and the remaining methyl carbons resonating at δ_C 19.8 and 23.2 were located at C-9 and C-11 in the fatty acid unit, respectively. The fatty acid was then confirmed as a previously reported 3-hydroxy-9,11-dimethyltridecanoic acid.[9] Finally, the structure of **2** was elucidated based on the same tetrapeptide chain Leu-Leu-Leu-Glu in **1**, cyclized at the C-terminal of Glu by the 3-hydroxy-9,11-dimethyltridecanoic acid.

The absolute stereochemistry of the amino acid residues (Leu_{1-3} and Glu) in **1** and **2** was determined to be L by acid hydrolysis and derivatization with Marfey's reagent followed by chiral HPLC analysis.[10] In addition, the 3*R*-hydroxy configuration of the fatty acids in **1** and **2** was assigned by direct comparison of their specific rotation values $[\alpha]^{27}_D$ −6.2 (*c* 0.05, MeOH) and $[\alpha]^{27}_D$ −29.0 (*c* 0.05, MeOH), respectively, with those of previously reported 3-hydroxy fatty acids.[7,8,11]

Anti-staphylococcal activity of compounds **1** and **2** was evaluated against clinically isolated MRSA strains (ATCC25923, XU212, SA1199B, RN4220, and EMRSA15) by the broth dilution assay according to the guidelines of the BSAC (Table 2).[12] Compounds **1** and **2** were found to be noncytotoxic when tested against two human cancer cell lines (prostate and liver cancer cell lines) by the sulforhodamine B (SRB) assay.[13]

EXPERIMENTAL SECTION

General Experimental Procedures. NMR spectroscopic data were acquired on a Varian Unity 500 spectrometer. UV spectra were obtained on a Shimadzu UV-1650PC spectrophotometer. IR spectra were recorded on a JASCO FT/IR-4100 spectrophotometer. Optical rotations were measured on a JASCO (DIP-1000) digital polarimeter. High-resolution ESIMS data were recorded on a hybrid ion-trap time-of-flight mass spectrometer (Shimadzu LC/MS-IT-TOF). HPLC was conducted with a PrimeLine binary pump with RI-101 (Shodex) and variable UV detector (M 525). Semipreparative HPLC was performed using ODS (YMC-Pack-ODS-A, 250 × 10 mm i.d. 5 μm) and silica (YMC-Pack-SIL, 250 × 10 mm i.d, 5 μm) columns. Analytical HPLC was conducted on an ODS column (YMC-Pack-ODS-A, 250 × 4.6 mm i.d. 5 μm). All solvents used were either spectral grade or distilled prior to use. Natural seawater was collected from the East Sea of South Korea at depths of 20 m.

Isolation and Taxonomy of the Strain 109GGC020. The strain 109GGC020 was isolated from a sediment sample collected from the Gageocho reef, Republic of Korea, in 2010 and cultured on a modified Bennett's media agar plate (media composition: 1 L of 100% natural seawater with a salt concentration of 18.5 g/L, 1% dextrose, 0.1% yeast extract, 0.1% beef extract, 0.2% tryptone, and 1.8% agar with the pH adjusted to 7.1) and identified as *Bacillus subtilis* on the basis of 16S rRNA sequence analysis. The sequence was deposited in GenBank under accession number JQ927413. This strain is currently preserved in the Microbial Culture Collection, KIOST, with the name *B. subtilis* 109GGC020 under the curatorship of Hee Jae Shin.

Culture Conditions. The seed culture of the strain 109GGC020 was performed in triplicate into 100 mL flasks, containing 50 mL of medium (composition as above). An aliquot (5 mL) from the seed culture was inoculated aseptically into 2 L flasks (total 50 flasks) containing 1.2 L of sterilized culture medium with the same composition. The growth conditions for the strain was optimized to obtain maximum production prior to large-scale culture by varying salt concentrations in the medium and monitoring the metabolite production by ^{1}H NMR analysis as described before.[5] The production culture was incubated with constant shaking at 28 °C for 7 days.

Extraction and Isolation of Compounds. The culture broth was filtered by high-speed centrifugation, and the cell-free filtrate was extracted with EtOAc (two times). The EtOAc layer was evaporated to dryness under reduced pressure using a chilled rotary evaporator and subjected to ODS (C18) open column chromatography followed by a stepwise gradient program of MeOH in H_2O (1:4, 2:3, 3:2, 4:1, and 100:0) as eluent. The 100% MeOH fraction, which showed interesting characteristic peaks of peptides, was subjected to further fractionations and purification by C18 semipreparative and analytical HPLC using isocratic elution with 90% MeOH in H_2O to yield pure compounds **1** and **2**.

Bacilotetrin A (1) (5.8 mg): amorphous solid; $[\alpha]^{27}_D$ −22.1 (*c* 0.05, MeOH); IR (MeOH) ν_{max} 3366 (NH), 1694 (CO), 1743, 2931 cm^{-1} (aliphatic chain); ^{1}H and ^{13}C NMR data (CD_3OD and CD_3OH), see Table 1; HR-(+)-ESIMS m/z 695.4953 $[M + H]^+$ (calcd for $C_{37}H_{66}N_4O_8$, 695.4959 $[M + H]^+$).

Bacilotetrin B (2) (3.6 mg): amorphous solid; $[\alpha]^{27}_D$ −18.6 (*c* 0.05, MeOH); IR (MeOH) ν_{max} 3260 (NH), 1575 (CO), 1734, 2925 cm^{-1} (aliphatic chain); ^{1}H and ^{13}C NMR data (CD_3OD and CD_3OH), Table 1; HR-(+)-ESIMS m/z 731.4935 $[M + Na]^+$ (calcd for $C_{38}H_{68}N_4O_8$, 731.4935 $[M + Na]^+$).

Acid Hydrolysis of 1 and 2. Compound **1** (1.5 mg) was heated at 123 °C for 23 h with 6 N HCl (500 μL) under constant stirring. The reaction was monitored from time to time by LC-MS analysis. The reaction mixture was cooled, mixed with water, and extracted with hexane (Hx). The Hx extract was concentrated under a stream of N_2, and the aqueous part was evaporated to dryness under reduced pressure. The Hx extract containing a fatty acid (**1a**) was subjected to recording specific rotation values to assign the stereochemistry at C-3 of the fatty acid, and the aqueous part was used for the determination of the stereochemistry of the amino acids by Marfey's method. In an

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analogous way, the absolute stereochemistry at C-3 of the fatty acid and amino acids in **2** was assigned.

Amino Acid Analysis by Marfey's Method in 1 and 2. The aqueous hydrolysate of **1** was resuspended in H_2O (100 μL) and treated with 0.1% 1-fluoro-2,4-dinitrophenyl-5-L-alaninamide solution in acetone (Marfey's reagent, 20 μL) and 1 N $NaHCO_3$ (10 μL) at 40 °C for 2 h. The solution was cooled to room temperature, neutralized with 1 N HCl (4 μL), and evaporated to dryness. The residue was dissolved again in 10% CH_3CN in H_2O (80 μL) and analyzed by reversed-phase HPLC (YMC-Pack ODS, 250 × 4.6 mm, 5 μm, flow rate of 0.5 mL/min) using a linear gradient of 30% CH_3CN in H_2O containing 0.1% acetic acid (AcOH) for 60 min. Similarly, standard amino acids (both L and D) were derivatized with Marfey's reagent and analyzed by HPLC. The derivatized Leu and Glu residues in the hydrolysate of **1** were obtained at the same retention time as the derivatized standard L-Leu (39.0 min) and L-Glu (29.6 min). In a similar manner, the hydrolysate of **2** was analyzed and had the same retention time as the derivatized standard L-Leu (39.0 min) and L-Glu (29.6 min).

Anti-Staphylococcal Activity Test of 1 and 2. The anti-MRSA activity of compounds **1** and **2** was investigated with ATCC 25923, SA-1199B, RN4220, XU212, EMRSA-15, and EMRSA-16 by the broth dilution assay. These strains were cultured on nutrient agar (Oxoid) plates and incubated for 24 h at 37 °C. An inoculum density of 5×10^5 colony-forming units of each bacterial strain was prepared in normal saline (9 g/L) by comparison with a 0.5 MacFarland turbidity standard. The inoculum (125 μL) was added to all wells, and the microtiter plate was incubated at 37 °C for the corresponding incubation time. For MIC determination, 20 μL of a 5 mg/mL methanolic solution of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was added to each of the wells and incubated for 20 min. Bacterial growth was indicated by a color change from yellow to dark blue. The MIC was recorded as the lowest concentration at which no growth was observed.[5]

Cytotoxicity Test by SRB Assay. Cancer cell growth inhibitory activity of compounds **1** and **2** was determined according to an SRB assay. In brief, a 96-well plate was loaded with selected cell lines (liver cancer and prostate cancer), and test samples (30, 10, and 3 μg/mL) were added. After incubation for 48 h, anchorage-dependent cells were fixed with 50% (wt/vol) trichloroacetic acid and stained for 60 min. Access dye was washed with SRB solution (0.4% sulforhodamine B in 1% acetic acid). The protein-bound dye was dissolved in 10 mM Tris base solution, and absorbance was measured at 510 nm using a microplate reader.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.7b00356.

1D and 2D NMR spectra of **1** and **2**, amino acid analysis data, and HRESIMS data (PDF)

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Notes

The authors declare no competing financial interest.

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