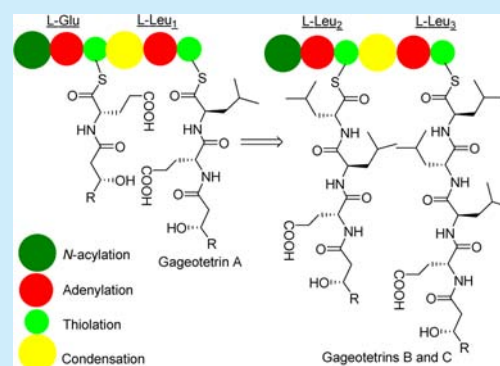


Gageotetrins A–C, Noncytotoxic Antimicrobial Linear Lipopeptides from a Marine Bacterium *Bacillus subtilis*Fakir Shahidullah Tareq,^{†,‡} Min Ah Lee,[‡] Hyi-Seung Lee,[‡] Yeon-Ju Lee,[‡] Jong Seok Lee,[‡] Choudhury M. Hasan,[§] Md. Tofazzal Islam,^{||} and Hee Jae Shin^{*,†,‡}[†]Department of Marine Biotechnology, University of Science and Technology, Republic of Korea[‡]Marine Natural Products Laboratory, Korea Institute of Ocean Science and Technology, Republic of Korea[§]Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Dhaka, Bangladesh^{||}Department of Biotechnology, Bangabandhu Sheikh Muzibur Rahman Agricultural University, Dhaka, Bangladesh

S Supporting Information

ABSTRACT: Gageotetrins A–C (1–3), a unique class of linear lipopeptides, consisting of di- and tetrapeptides and a new fatty acid were isolated from a Marine *Bacillus subtilis*. The structures of 1–3 were assigned by spectroscopic data and their absolute stereochemistries were ascertained by chemical derivatization. Compounds 1–3 displayed good antimicrobial activities with MIC values of 0.01–0.06 μ M. However, these compounds failed to register any cytotoxicity (GI_{50} > 30 μ g/ml) against human cancer cell lines.



Lipopeptides (LPs) have been reported to be produced by distinctively different groups of bacteria and fungi, and these compounds were found to exert roles in antagonistic interactions with other organisms, e.g., plant pathogenicity and antifungal, antibacterial, antiviral, and antitumor properties.¹ In particular, LPs of the surfactin, iturin, and fengycin families are important metabolites produced by the bacterial strain *Bacillus* sp., and their involvement in disease control has been widely reported.^{2,3} However, linear lipodipeptides, especially lipotetrapeptides possessing three Leu and Glu amino acids along with a new fatty acid residue, have been unprecedented from natural sources.

As part of our continuous exploration of bioactive secondary metabolites from marine-derived bacteria, we isolated the strain 109GGC020 from marine sediment samples collected from Gageocho, Republic of Korea, which revealed 100% 16S rRNA sequencing similarity to *Bacillus subtilis*. The unicellular bacterium was cultured, and the fermentation broth (100 L) was extracted with EtOAc. Thereafter, the chromatographic separation of the EtOAc extract using flash column chromatography in combination with semipreparative and analytical HPLC yielded a novel class of linear lipopeptides, gageotetrins A (3.2 mg), B (5.6 mg), and C (2.8 mg) (Figure 1).

Gageotetrin A (1) was isolated as an amorphous solid with a molecular formula of $C_{25}H_{46}N_2O_7$ as determined by HRESI-MS at m/z 509.3203 [$M + Na$]⁺. The IR absorbencies of 1 at 3285, 2923, and 1583 cm^{-1} were consistent with the presence

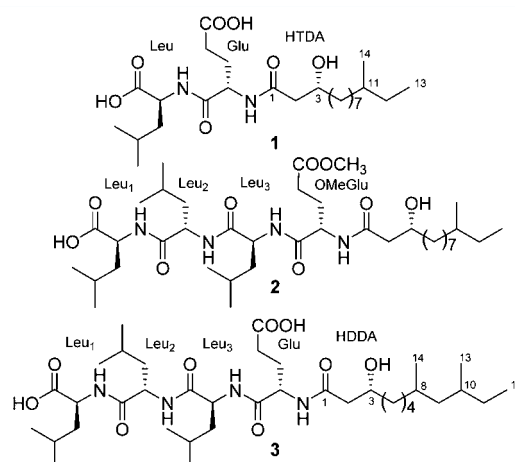


Figure 1. Structures of gageotetrins A–C (1–3).

of amides, a long aliphatic chain, and carbonyl groups, respectively. Two NH signals at δ_H 7.57 and δ_H 8.38, α -methines at δ_H 4.29 and δ_H 4.30 in the 1H NMR spectrum and the carbonyl signals at δ_C 173.6–181.7 together with α -carbons at δ_C 54.9 and δ_C 55.5 in the ^{13}C NMR spectrum revealed the peptidic nature of 1 (Table 1). In addition, a broad singlet at δ_H 1.29 and an oxygenated proton at δ_H 3.98 were observed in the

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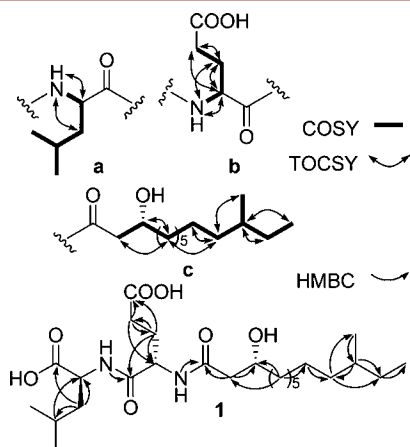
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Table 1. ^1H and ^{13}C NMR and ROESY data of **1** in CD_3OH

	position	δ_{H} , mult (J, Hz)	δ_{C}	ROESY
Leu	1		179.7	
	2	4.29, m (overlapped)	54.9	NH, 3, 4
	3	1.56, m	43.6	2
		1.63, m		
	4	1.67, m	26.1	2, 5, 6
	5	0.93, d (2.0)	22.6	3, 4
	6	0.93, d (2.0)	23.9	3, 4
	NH	7.57, d (8.0)		2
Glu	1		173.6	
	2	4.30, m (overlapped)	55.5	3, 4
	3	1.91, m	29.6	2, 4
		2.12, m		
	4	2.27, t (7.5)	35.4	2, 3
	NH	8.38, d (7.0)		
	COOH		181.7	
3-OH acid (HTDA)	1		174.7	
	2	2.32, dd (14.0, 8.5)	44.9	3, 4
		2.42, dd (13.5, 4.0)		
	3	3.98, m	69.9	2, 4, 5
	4	1.47, m	38.5	2, 3
	5	1.47, m	26.8	3
		1.30, m (overlapped)		
	6–9	1.28, brds.	30.7–30.8	
	10	1.29, m (overlapped)	33.1	14
		1.17, m		
	11	1.29, m (overlapped)	38.5	13
	12	1.29, m (overlapped)	30.8	
	13	0.89, t (7.5)	14.5	
	14	0.91, d (2.0)	23.8	

^1H NMR spectrum, revealing the presence of aliphatic chain in the molecule. These detailed IR and NMR results clearly indicated the lipopeptidic nature of **1**.

Analysis of 1D and 2D NMR spectra allowed construction of three partial structures that constituted **1** (Figure 2). Two amino acid residues were deduced as glutamic acid (Glu) and leucine (Leu), while a third, nonamino acid moiety, was identified as a 3-hydroxy fatty acid. The spin systems of the α -methines and NH resonances of Leu (δ_{H} 4.29/7.57) and Glu (δ_{H} 4.30/8.38) and the α -methines to the side chain of the

Figure 2. COSY, TOCSY, and HMBC correlations of **1** in CD_3OH .

individual amino acid residues were clearly observed in the COSY and TOCSY spectra (partial structures **a** and **b**).

Moreover, the NH signal at δ_{H} 8.23 showed TOCSY connectivities with α -methine and methylene protons at δ_{H} 4.30 and δ_{H} 1.91/2.12, and further HMBC connectivities of methylene signals at δ_{H} 1.91/2.12 and δ_{H} 2.27 with a carbonyl carbon at δ_{C} 181.7 confirmed the presence of the Glu residue in **1**. The oxygenated proton at δ_{H} 3.98 showed HMBC connectivities with the carbonyl carbon at δ_{C} 174.7 and also with the aliphatic chain. These HMBC correlations together with TOCSY correlations allowed the assignment of a 3-hydroxy fatty acid (partial structure **c**) in **1**.

Based on the composition of the peptide moiety and the molecular weight of the molecule, the fatty acid was primarily determined to be a C_{14} fatty acid, amidated to the N-terminal amine of the peptide. The molecular formula of the fatty acid was determined as $\text{C}_{14}\text{H}_{28}\text{O}_3$ by LC–MS analysis (m/z 243.02 $[\text{M} - \text{H}]^-$) of the acid hydrolysate of **1** (Supporting Information). In the ^{13}C spectrum, four methyl carbons were observed, among them two were attributed to a Leu residue and the remaining two were assigned to be present in the fatty acid chain. The methyl carbon at δ_{C} 14.5 was assigned as a terminal group and the second methyl (δ_{C} 23.8) was attached at C-11 of the fatty acid chain, corroborated by the analysis of TOCSY and HMBC correlations. Finally, the fatty acid was found as a 3-hydroxy-11-methyltridecanoic acid (HTDA) with an optical rotation value of $[\alpha]_{\text{D}}^{27} -22.0$ (0.1, MeOH). Collectively, the lipopeptide structure of **1** was then determined constructing the sequence of Leu-Glu-HTDA by ROESY and HMBC experiments (Figure 2).

Gageotetrin B (**2**) was isolated as an amorphous solid, and its molecular formula was determined to be $\text{C}_{38}\text{H}_{70}\text{N}_4\text{O}_9$ by HRESIMS at m/z 749.5020 $[\text{M} + \text{Na}]^+$. A series of ^{13}C resonances at δ_{C} 174.1–175.1 were assigned to amide or ester carbonyls. In addition, the ^1H NMR spectrum of **2** contained resonances of four amide signals at δ_{H} 7.79–8.24, α -methines at δ_{H} 4.26–4.44 and side-chain functionalities, suggesting **2** contained a peptide component (Table 2, Supporting Information). Consistent with this observation, detailed analysis of the COSY, TOCSY, and HMBC data led to the identification of three Leu and a methoxy-Glu (OMeGlu) residues in **2** (Figure 1). The methoxy signal at δ_{H} 3.67 (singlet) did not show COSY and TOCSY correlations but displayed an HMBC correlation to the carboxylic carbon at δ_{C} 175.1, corresponding to the presence of an OMeGlu residue in **2**. A 3-hydroxy fatty acid residue was also assigned by TOCSY and HMBC correlations from the observation of a distinct proton signal at δ_{H} 3.98 which showed HSQC cross peak with the carbon at δ_{C} 70.0. Subtraction of the atoms attributed to the four amino acid residues from the molecular formula of **1** ($\text{C}_{38}\text{H}_{70}\text{N}_4\text{O}_9$) showed that the remaining fragment, i.e., the fatty acid of the molecule had a molecular formula of $\text{C}_{14}\text{H}_{28}\text{O}_3$. The molecular formula of the fatty acid was further confirmed by the acid hydrolysis followed by LC–MS analysis (m/z 243.423 $[\text{M} - \text{H}]^-$) with an optical rotation value of $[\alpha]_{\text{D}}^{27} -27.8$ (0.1, MeOH). This result confirmed the presence of the same fatty acid residues in compounds **1** and **2**. The sequence of residues in **2** was then confirmed as Leu-Leu-Leu-OMeGlu-HTDA on the basis of the TOCSY, ROESY and HMBC experiments.

Gageotetrin C (**3**) was isolated as an amorphous solid, and its molecular formula was assigned to be $\text{C}_{37}\text{H}_{68}\text{N}_4\text{O}_9$ with the aid of HRESI-MS $[\text{M} + \text{Na}]^+$ at m/z 735.4883. Preliminary, the

NMR analysis showed a close similarity between the spectra of 2 and 3 (Table 2, Supporting Information), indicating a lipopeptide nature of 3. The difference between these compounds was identified from the lack of methoxy peak that was appeared as singlet at δ_{H} 3.67 in 2. Detailed analysis of NMR data of 3 revealed that it contained same amino acid residues as 2 except for the presence of Glu instead of OMeGlu. Similar to compounds 1 and 2, the presence of a 3-hydroxy fatty acid was identified and the LC–MS analysis of the hexane phase of the hydrolysate of 3 revealed that it had the same molecular formula of $\text{C}_{14}\text{H}_{28}\text{O}_3$ like in 1 and 2. But, interestingly the ^{13}C NMR spectrum of 3 displayed nine methyl carbons at δ_{C} 11.9–23.2; six of them were attributed to three Leu residues, and as the amino acids sequence of both 2 and 3 was found to be same by 2D data analysis the remaining three methyls must be located in the fatty acid chain. The methyl carbon at δ_{C} 11.9 was located as a terminal methyl and the carbons at δ_{C} 19.8 and 23.2 were located at C-8 and C-11 in the fatty acid moiety, respectively, by the TOCSY and HMBC correlations. Finally, the fatty acid was determined as a 3-hydroxy-8,10-dimethyldodecanoic acid (HDDA) with an optical rotation value of $[\alpha]_{\text{D}}^{27} -31$ (0.1, MeOH). The search results from natural products database confirmed this fatty acid as a new fatty acid derivative from natural sources. The complete structure of 3 was then elucidated on the basis of the 1D and 2D NMR data, constructing the sequence of Leu-Leu-Leu-Glu-HDDA.

The absolute configuration of the amino acids in 1–3 was determined by Marfey's method⁴ (Figure 3), and the

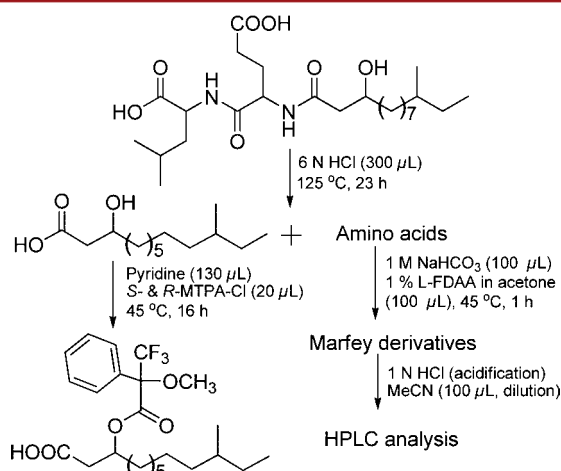


Figure 3. Acid hydrolysis and derivatization studies of 1.

configurations of all Leu and Glu residues were found to be the L-form (Supporting Information). In addition, the absolute configuration at C-3 of the fatty acid in 1 was determined by Mosher's method⁵ (Figure 3). A consistent distribution of positive and negative $\Delta\delta_{\text{H}}$ values ($\Delta\delta_{\text{H}} = \delta_{\text{S}} - \delta_{\text{R}}$) obtained from S-MTPA and R-MTPA esters around C-3 allowed the assignment of the R-configuration at the C-3 position (Figure 4). The absolute stereochemistry at C-3 of the fatty acids in 2 and 3 was also determined to be R-form by comparing optical rotation values of the fatty acids obtained from 1–3 and literature reviews.^{6,7}

Gageotetrins A–C might be biosynthetically produced in a ribosome-independent manner with megaenzymes called nonribosomal peptide synthetases via the function of the

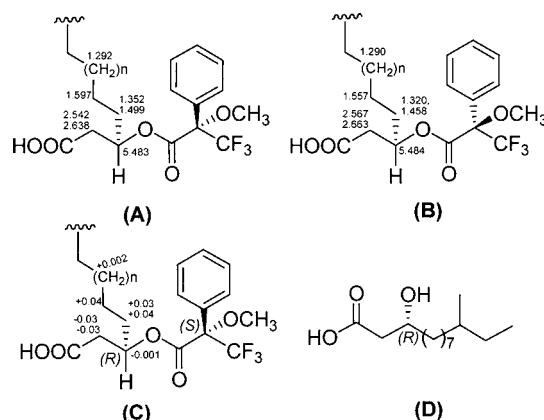


Figure 4. Absolute stereochemistry determination of 3-hydroxy fatty acid in 1. (A) ^1H NMR data of S-MTPA and (B) R-MTPA esters; (C) $\Delta\delta_{\text{H}}$ ($\delta_{\text{S}} - \delta_{\text{R}}$) values of derivatized products; (D) R-configuration of C-3 of fatty acid in 1.

catalytic unit, referred to as a module (Figure 5).⁸ Each module is composed of specific domains that are responsible for

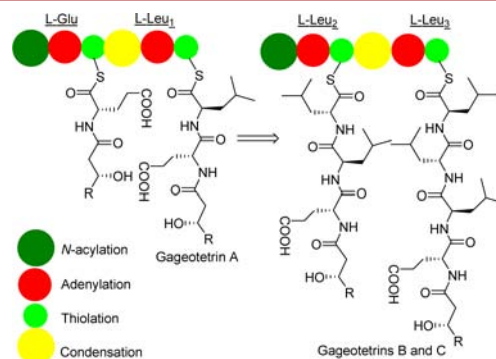


Figure 5. Hypothetical biosynthetic pathways of gageotetrins.

catalyzing different enzymatic activities.⁹ The adenylation (A) domain is responsible for amino acid recognition and adenylation at the expense of ATP to form an acyl adenylate intermediate. The intermediate covalently binds to a phosphopantetheine carrier of the adjacent thiolation (T) domain. Peptide bond formation of two consecutively bound amino acids is catalyzed by the condensation (C) domain. An additional C-domain, the N-acyl domain, is located at the N-terminal domain of the first module, suggesting that the first amino acid is initially N-acylated with a 3-hydroxy fatty acid in this domain.^{10,11} This proposed biosynthetic pathways are quite consistent with the chemical synthetic procedure of surfactin B.¹²

Antimicrobial activities of 1–3 were investigated against both bacteria and pathogenic fungi by broth dilution assay.¹³ Compounds 1–3 were found more active against fungi compared to bacteria with MIC values of 0.02–0.04 μM (Figure 6). Moreover, compounds 2 and 3 displayed good time course motility inhibition and lytic activity against the late blight pathogen *Phytophthora capsici*, which causes enormous economic damage in cucumber, pepper, tomato and beans, at 0.02 μM (Figure 7). *P. capsici* caused huge losses to numerous vegetable producers in Michigan.¹⁴ The pathogen is also important on a global scale and is potentially the most destructive organism of peppers in Spain.¹⁵ In addition, the

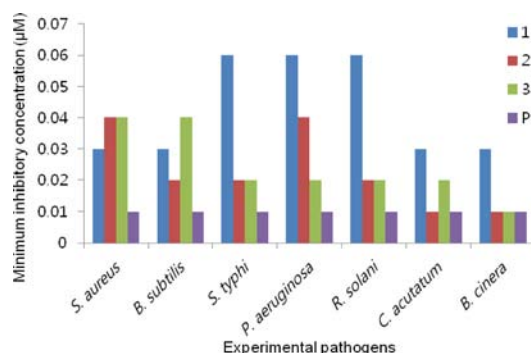


Figure 6. Antimicrobial activities of 1–3 and positive control P (azithromycin for bacteria and amphotericin B for fungi).

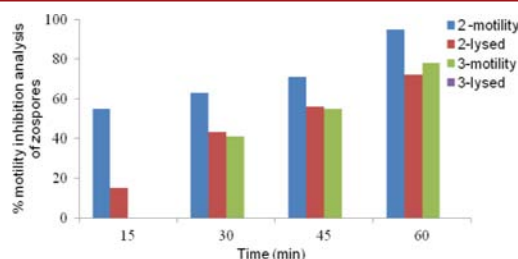


Figure 7. Time-course motility inhibitory and lytic activities of 2 and 3 against *P. capsici* at 0.02 μ M. Compound 3 failed to lyse zoospores at a concentration of 0.02 μ M but showed activity when the concentration was increased (Supporting Information, Table 1).

cytotoxicity of 1–3 was evaluated against six human cancer cell lines: ACHN renal cancer, HCT-15 colon cancer, MDA-MB-231 breast cancer, NCI-H23 lung cancer, NUGC-3 stomach cancer, and PC-3 prostate cancer cell lines by sulforhodamine B (SBR) assay¹⁶ but failed to register any cytotoxicity at a concentration of 30 μ g/mL. Nontoxic activity of 1–3 was further confirmed by WST-1 assay¹⁷ against the human leukemia cell line (K-562).

To the best of our knowledge, gageotetins A–C represent the first example of rare bioactive linear lipopeptides consisting of a Leu-rich peptide backbone and a new 3-hydroxy fatty acid (HDDA) from a marine-derived bacterium. Several lipopeptides containing a 3-hydroxy fatty acid have been reported including surfactins and biosurfactants and showed that the amino acid residue composition and the fatty acid play an important role for their activity.¹⁸ In fact, 3-hydroxy fatty acid itself displays good antifungal properties.¹⁹ Moreover, caspofungin, which possesses a fatty acid slightly resembling the fatty acid present in 3, has been approved as an antifungal agent exhibiting a unique mechanism of action relative to the other currently approved antifungal agents.²⁰ Consequently, antimicrobial activity of 1–3 resulting from this study could be promising for finding new frontiers to develop nontoxic antibiotics.

■ ASSOCIATED CONTENT

Supporting Information

Experimental and spectroscopic data for 2 and 3. 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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