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Gageopeptins A and B, new inhibitors of zoospore motility of the phytopathogen *Phytophthora capsici* from a marine-derived bacterium *Bacillus* sp. 109GGC020

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ABSTRACT

The motility of zoospores is critical in the disease cycles of the peronosporomycetes that cause devastating diseases in plants, fishes, vertebrates, and microbes. In the course of screening for secondary metabolites regulating the motility of zoospores of *Phytophthora capsici*, we discovered two new inhibitors from the ethyl acetate extract of the fermentation broth of a marine-derived strain *Bacillus* sp. 109GGC020. The structures of these novel metabolites were elucidated as new cyclic lipopeptides and named gageopeptins A (**1**) and B (**2**) by spectroscopic analyses including high resolution MS and extensive 1D and 2D NMR. The stereoconfigurations of **1** and **2** were assigned based on the chemical derivatization studies and reviews of the literature data. Although compounds **1** and **2** impaired the motility of zoospores of *P. capsici* in dose- and time-dependent manners, compound **1** (IC_{50} = 1 μg/ml) was an approximately 400-fold stronger motility inhibitor than **2** (IC_{50} = 400 μg/ml). Interestingly, the zoospores halted by compound **1** were subsequently lysed at higher concentrations (IC_{50} = 50 μg/ml). Compounds **1** and **2** were also tested against some bacteria and fungi by broth dilution assay, and exhibited moderate antibacterial and good antifungal activities.

Phytophthora capsici is a peronosporomycete plant pathogen that causes blight and fruit rot diseases in peppers and other important commercial crops worldwide.[1] This fungus-like pathogen has been reported to cause severe epidemics in the Central and South America, Europe, Asia, and many states of the United States, where vegetables are grown. The host range of *P. capsici* is wide including bell pepper, cacao, cantaloupe, chayote, cucumber, eggplant, honeydew melon, marigold, macadamia nut, papaya, pumpkin, some bean types, squash, tomato, and watermelon.[2]

Different types of chemical and synthetic compounds have been used as antimicrobial agents to inhibit plant pathogenic peronosporomycete and fungi for many years. Antimicrobials such as benzimidazoles, aromatic hydrocarbons, and sterol biosynthesis inhibitors are often used to control plant disease in agriculture. However, development of resistance against fungicide is a serious problem in efficacy of these chemicals.[3] Famers often use higher concentrations of these chemicals to overcome this problem, but this increases the risk of high-level toxic residues in the products and environment. Therefore, there has been a growing interest in research for the possible use of natural products, which can be safe for the products and environment, and relatively specific for the pest.[4]

As part of our ongoing research for the discovery of new inhibitory compounds against the notorious phytopathogen *P. capsici*, we isolated two new cyclic lipopeptides **1** and **2** from a marine-derived bacterial strain 109GGC020. Here, we report the isolation and characterization of **1** and **2** and their growth inhibitory activity against *P. capsici* and some bacteria and fungi.

The producing strain 109GGC020 was isolated from a marine sediment sample as described before[5] and identified as *Bacillus subtilis* based on the analysis of 16S rRNA gene sequencing data. The sequence was deposited in GenBank under the accession number JQ927413. This strain is currently preserved in the Microbial Culture Collection, KIOST, with the name *Bacillus subtilis*

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Table 1
^{1}H and ^{13}C NMR data of compounds **1** and **2**

Units	No.	**1**		**2**	
		$\delta_{H,mult.}$ (J in Hz)	δ_C	$\delta_{H,mult.}$ (J in Hz)	δ_C
Glu-1	1		174.5		174.3
	2	4.04 m	61.8	4.03 m	61.9
	3	2.01 m	28.2	2.00 m	28.1
		2.11 m		2.11 m	
	4	2.25 m	30.8	2.25 m	30.7
	COOH		176.4		176.7
Val-2	1		173.8		173.9
	2	4.42 m	53.5	4.40 m	53.5
	3	1.45 m	23.6	1.46 m	23.3
	4	0.89 m	21.7	0.89 m	21.9
	5	0.89 m	23.6	0.89 m	23.7
Leu-3	1		174.2		174.0
	2	4.44 m	53.3	4.42 m	53.1
	3	1.70 m	40.3	1.70 m	40.2
	4	1.70 m	26.3	1.70 m	26.3
	5	0.89 m	22.6	0.89 m	22.7
	6	0.89 m	23.8	0.89 m	23.8
Asp-4	1		173.6		173.9
	2	4.15 m	53.2	4.14 m	53.1
	3	2.85 dd (17.0, 9.0)	35.8	2.85 dd (17.0, 9.5)	35.9
		2.98 dd (17.0, 4.0)		2.98 dd (17.0, 4.0)	
	COOH		175.0		174.3
Ala-5	1		175.4		175.5
	2	4.79 dd (9.5, 4.5)	51.8	4.79 dd (9.5, 4.5)	51.7
	4	1.77 m	19.7	1.77 m	19.8
Leu-6	1		174.1		174.0
	2	4.33 dd (11.5, 6.5)	54.3	4.32 m	54.2
	3	1.70 m	41.1	1.70 m	41.0
	4	1.70 m	26.3	1.70 m	26.3
	5	0.93 m	23.3	0.93 m	23.2
	6	0.93 m	23.7	0.93 m	23.7
Leu-7	1		174.1		174.2
	2	4.22 dd (8.0, 6.0)	55.5	4.21 m	55.5
	3	1.70 m	40.5	1.70 m	40.5
	4	1.70 m	26.3	1.70 m	26.3
	5	0.93 m	22.2	0.93 m	22.0
	6	0.93 m	23.8	0.93 m	23.8
3-OH acid	1		176.7		176.7
	2	2.44 dd (14.0, 8.0)	43.3	2.43 dd (6.0, 3.0)	43.3
		2.64 dd (14.0, 4.0)		2.64 dd (15.0, 2.0)	
	3	5.30 m	73.6	5.29 m	73.4
	4	1.37 m	26.3	1.37 m	26.3
		1.50 m		1.50 m	
	5	1.55 m	42.9	1.55 m	42.8
	6–10	1.32 br s	30.4–31.4	1.32 br s	30.4–31.4
	11	1.17 m	40.5	—	—
	12	1.54 m	29.2	—	—
	13	1.32 br s	31.1	1.17 m	40.4
	14	1.32 br s	35.8	1.51 m	29.3
	15	1.12 m	37.9	1.32 m	38.5
		1.32 m			
	16	0.88 m	11.9	0.88 m	14.6
	17	0.88 m	18.4	0.88 m	19.8
	18	0.88 m	19.8		

109GGC020 under the curatorship of Shin. The bacterium was fermented for 7 days and the culture broth was extracted with EtOAc. The crude extract (11.8 g) was subjected to column chromatography and HPLCs to yield pure compounds **1** (8.9 mg) and **2** (4.5 mg) (Fig. 1).[6]

Gageopeptin A (**1**) was isolated as an amorphous solid and its molecular formula was determined to be $C_{53}H_{93}N_7O_{13}$ based on the HRESI mass at m/z 1034.6758 $[M-H]^-$. Preliminary, the ^{1}H NMR and ^{13}C NMR resonances (Table 1) of **1** were found quite similar to the previously reported cyclic lipopeptides.[7] Later, the detailed analysis of COSY and HMBC correlations enabled us to determine the sequence of amino acids in **1**, Glu-Val-Leu-Ala-Asp-Leu-Leu-3-hydroxy fatty acid, which was same as the published compounds (Fig. 2).[7] However, in the ^{13}C NMR spectra of **1** few more ^{13}C resonances were observed which guided us to carefully investigate the structure of **1**. It was found that among the 12 methyl carbons in **1**, 9 were attributed to five amino acids and therefore, the remaining 3 methyl carbons must be present in the fatty acid chain. Based on the HMBC correlations, the methyl carbon resonated at δ_C 11.9 was assigned as a terminal methyl,

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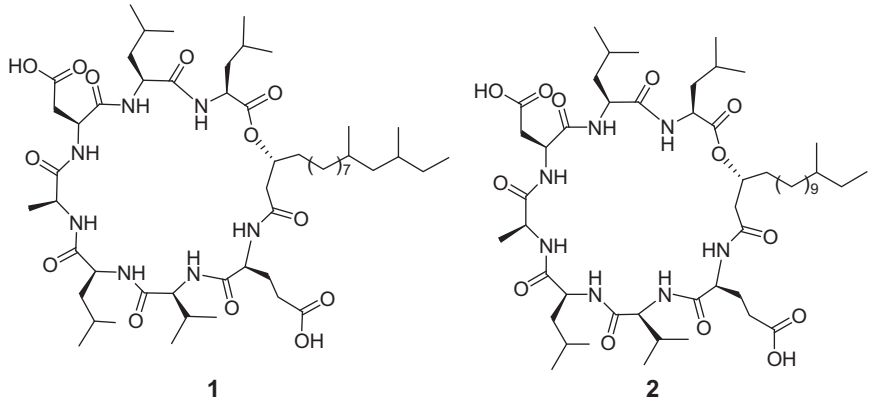

Figure 1. Structures of gageopeptins A (**1**) and B (**2**).

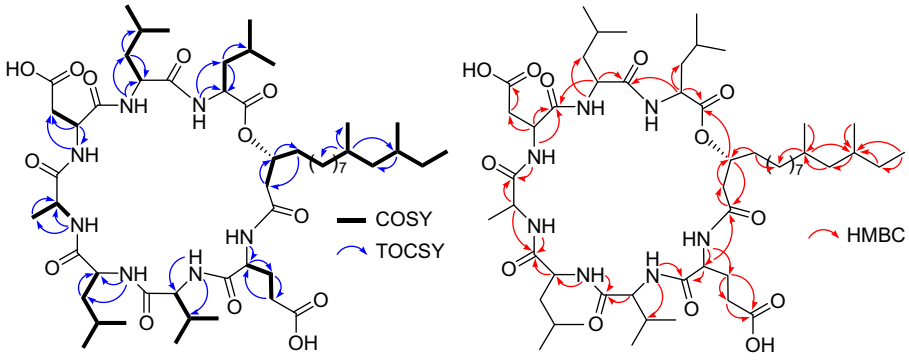

Figure 2. Key COSY, TOCSY and HMBC correlations in gageopeptin A (**1**).

whereas the methyl carbons resonated at δ_C 18.4 and δ_C 19.8 were located at C-12 and C-14 of the fatty acid, respectively. Primarily, the fatty acid chain was determined to be a C_{18} fatty acid based on the composition of the peptide moiety and the molecular weight of the molecule amidated to the N-terminal amine of the peptide. The fatty acid was then confirmed as a 3-hydroxy-12,14-dimethylhexadecanoic acid from the acid hydrolysis of **1** followed by APCI-MS analysis (m/z at 300.26 $[M+H]^+$) with a molecular rotation value $[\alpha]_D^{25}$ −22 (0.5, MeOH). Collectively, the data obtained allowed determination of the chemical structure of **1**.

Gageopeptin B (**2**) was isolated as an amorphous solid and its molecular formula was determined to be $C_{52}H_{91}N_7O_{13}$ based on the high-resolution ESIMS peak at m/z 1020.6595 $[M-H]^-$. The ^1H and ^{13}C NMR data of **2** showed a close similarity with **1**, indicating a lipopeptide nature of **2**. A careful investigation of the ^{13}C NMR data of **2** revealed the absence of a methine and a methyl carbon resonated at δ_C 11.9 and 29.2, which were observed in **1**. This observation lead to further detailed investigation of the structure of compound **2** by running a series of 1D and 2D NMR experiments. The detailed NMR data analysis revealed that **1** and **2** had the same amino acids sequence, indicating that **2** had a different type of fatty acid than that of **1**. Based on HMBC correlations, a methyl carbon resonated at δ_C 14.6 was found to be a terminal methyl and another methyl carbon resonated at δ_C 19.8 constituted side chain locating at C-14 in the fatty acid. The LC-MS analysis (m/z at 286.2508 $[M+H]^+$) of the hexane phase of the acid hydrolysate of **2** confirmed the chain length of the fatty acid. These extensive NMR data along with LC-MS results confirmed the presence of a 3-hydroxy-14-methylhexadecanoic acid in **2** with a molecular rotation value $[\alpha]_D^{25}$ −30 (0.2, MeOH). Finally, the complete chemical structure of compound **2** was constructed by the detailed analysis of COSY, TOCSY, and HMBC correlations.

The absolute configuration of the amino acid residues and the stereocenter at C-3 of the fatty acid moiety in **1** and **2** was determined by acid hydrolysis[8] followed by Mosher's MTPA and Marfey's methods sequentially.[9,10] Mosher's method lead to the assignment of absolute configuration at C-3 of the fatty acid in **1** as *R*-form (Fig. 4, SI-1).[11] Additionally, Marfey's method unambiguously established the configuration of each of the amino acids to be L-form (Fig. 5, SI-1). This result was consistent with the absolute stereochemistry of the previously reported (*R*)-3-hydroxy fatty acid and also supported by the literature reviews, as analogues of this fatty acid display negative specific rotation in MeOH.[12,13] The absolute stereochemistry at the 3-hydroxy position of the fatty acid in **2** was determined to possess *R* configuration by the comparison of optical rotation value with that of the fatty acid in **1**.

To infect host plants, pathogens like *P. capsici* asexually produce characteristic biflagellate (heterokont) motile spores, called zoospores.[14] Several lines of evidence indicate that these wall-less zoospores locate their hosts guided by the specific chemical signals secreted from the hosts.[15] When zoospores find potential infection sites of the hosts, they stop motility, attach to the host surface using posterior flagella, and rapidly transform to round-shaped cystospores after shedding flagella.[15] The cystospores then germinate to form germ tubes within 30–40 min to invade plant tissues and initiate infection. The successful infection of this obligate notorious phytopathogen can be attributed in part to the speed of asexual differentiation to generate biflagellate motile zoospores that target potential infection site of the plant through water films. Any disruption of zoospore release from sporangia

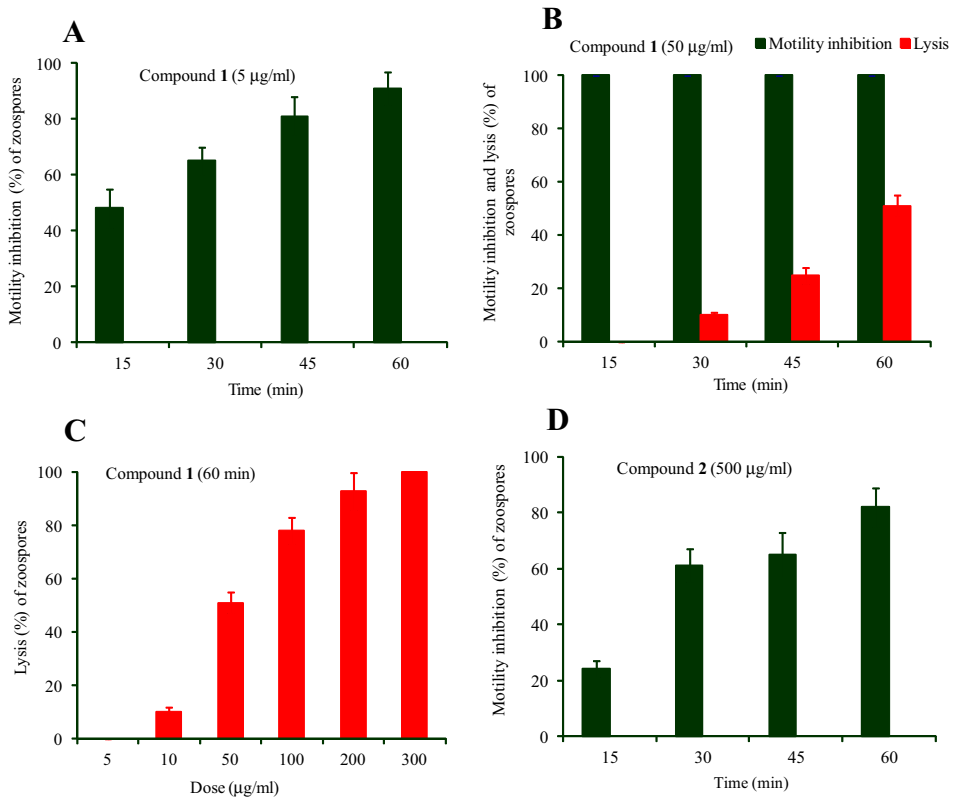

Figure 3. Time-course and dose-dependent motility inhibitory and lytic activities of compounds **1** and **2** against *P. capsici* zoospores. (A) Motility inhibitory activity of compound **1** at 5 μg/ml; (B) Motility inhibitory and lytic activity of compound **1** at 50 μg/ml; (C) Lytic activity of compound **1** at 5 μg/ml; (D) Motility inhibitory activity of compound **2** at 500 μg/ml.

Table 2
Minimum inhibitory concentrations (MICs) of **1** and **2**

Microorganisms	MICs (μg/ml)		
	1	**2**	P.C.
Fungi			
R. solani	4	8	1
C. acutatum	8	8	1
B. cinera	4	8	1
Gram positive bacteria			
S. aureus	16	16	2
B. subtilis	16	32	2
Gram negative bacteria			
S. Typhi	16	32	2
P. aeruginosa	16	16	2

P.C.: Positive control (Azithromycin for Bacteria and Amphotericin B for Fungi).

(zoosporogenesis) or zoospore motility remarkably decreases the potential for pathogenesis.[16] We tested the activity of compounds **1** and **2** on the motility and viability of *P. capsici* zoospores using homogeneous solution method.[5] Interestingly, compounds **1** and **2** significantly inhibited the motility of zoospores (Fig. 3). Moreover, in the presence of compounds **1** and **2**, zoospores moved very slowly in their axis or spun in tight circles instead of displaying straight swimming in a helical fashion. Compound **1** was very potent to arrest the motility of zoospores at the concentrations starting from 1 μg/ml (Table 3, SI-2). Nevertheless, compound **1** displayed lytic activity after increasing the concentration at 50 μg/ml. It might be noted that compound **1** showed lytic activity after 30 min. However, this compound displayed strong lytic activity after 1 h at 10 μg/ml. On the other hand, compound **2** was found to display motility inhibition at 500 μg/ml but did not show lytic activity at this concentration. These results suggested that the methyl group at C-12 of the fatty acid in **1** is critical for activity. Furthermore, minimum growth inhibitory activity of compounds **1** and **2** was evaluated against both Gram positive and Gram negative bacteria and fungi by broth dilution assay.[17] Different growth conditions of bacteria and fungi were maintained while culturing these microorganisms.[18,19] Compounds **1** and **2** showed good inhibitory activity against fungi compared to bacteria (Table 2). These results suggested the new compounds **1** and **2** could be good bioprobes for antimicrobial agents. In particular, compound **1** can be used to develop fungicides targeting the economically important pathogenic peronosporomycete *P. capsici*.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2015.05.070.

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6. *Strain culture, extraction and isolation of compounds*. The seed culture of the strain 109GGC020 was carried out into 100 ml flasks (total 3 flasks) containing 50 ml modified Bennett's culture medium (for 1 L, the medium composition was 1% dextrose, 0.1% yeast extract, 0.1% beef extract, 0.2% tryptone, 1.8% agar, 100% natural sea water, and pH 7.1). The seed culture (0.2% v/v) was inoculated aseptically into 2 L flasks (total 50 flasks) containing 1.2 L sterilized culture medium (same composition as above). The culture was incubated at 28 °C for 7 days. The culture was harvested (cells were separated from the broth by centrifugation) and the broth was extracted with EtOAc (2 times). The solvent was evaporated to dryness under reduced pressure at 40 °C. The obtained crude extract (11.8 g) was subjected to an ODS open column chromatography followed by stepwise gradient elution with MeOH–H2O (v/v) (1:4, 2:3, 3:2, 4:1 and 100:0) as eluent. The 100% MeOH fraction was fractionated again and purified by C_{18} semi-preparative and analytical HPLCs using a solvent system (95% MeOH in H_2O) to yield pure compounds **1** and **2**. Gageopeptin A (**1**): amorphous solid; $[\alpha]_D^{27}$ −152 (*c* 0.05, MeOH); IR (MeOH) ν_{max} 3290, 2930, 1718–1635 cm^{-1}; ^1H and ^{13}C NMR data (CD_3OD), Table 1; HRESIMS *m*/*z* 1034.6758 $[M-H]^-$. Gageopeptin B (**2**)**:** amorphous solid; $[\alpha]_D^{27}$ −123 (*c* 0.05, MeOH); IR (MeOH) ν_{max} 3310, 2945, 1742–1656 cm^{-1}; ^1H and ^{13}C NMR data (CD_3OD), Table 1; HRESIMS *m*/*z* 1020.6595 $[M-H]^-$.
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8. *Acid hydrolysis*. Compound **1** (2.5 mg) was dissolved in 6 N HCl (0.60 ml) and stirred at 125 °C for 23 h. The solution was cooled, diluted with water, and extracted with chloroform. The chloroform extract was concentrated by a stream of N_2 and the aqueous part was evaporated to dryness under reduced pressure. The fatty acid obtained from the chloroform extract was derivatized with Mosher's reagent to determine the absolute configuration at the stereocenter C-3 of the 3-hydroxy fatty acid. The aqueous part was subjected for the assignment of absolute stereochemistry of amino acids by Marfey's method. In an analogous way, compound **2** (1.8 mg) was hydrolyzed and analyzed. Compound **1a** (1.5 mg): colorless oil; ^1H NMR data (CD_3OD) δ_H 2.38 (H-2a, dd, *J* = 15.0, 8.5), 2.54 (H-2b, dd, *J* = 15.5, 5.0), 3.98 (H-3, m), 1.35 (H-4a, m, overlapped), 1.47 (H-4b, m, overlapped), 1.49 (H_2-5, m, overlapped), 1.29 (H_2-6-H_2-11, brds), 1.16 (H-12, m), 1.29 (H-13, m, overlapped), 1.49 (H-14, m, overlapped), 1.29 (H-15, m, overlapped), 0.86 (H_3-16, m, overlapped), 0.87 ($2H_3$-17,18, m, overlapped); APCI-MS *m*/*z* 300.26 $[M+H]^+$; molecular rotation $[\alpha]_D^{27}$ −22 (*c* 0.5, MeOH).
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