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Antimicrobial Gageomacrolactins Characterized from the Fermentation of the Marine-Derived Bacterium *Bacillus subtilis* under Optimum Growth Conditions

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Supporting Information

ABSTRACT: Marine bacteria are a potential source of structurally diversified bioactive secondary metabolites that are not found in terrestrial sources. In our continuous effort to search for new antimicrobial agents from marine-derived bacteria, we isolated bacterial strain 109GGC020 from a marine sediment sample collected from Gageocho, Republic of Korea. The strain was identified as *Bacillus subtilis* based on a 16s rRNA sequence analysis. After a 7-day fermentation of the *B. subtilis* strain under optimum growth conditions three new and four known secondary metabolites were discovered using chromatographic procedures, and their biological activities were evaluated against both bacteria and crop-devastating fungi. The discovered metabolites were confirmed by extensive 2D NMR and high-resolution ESI-MS data analyses to have the structures of new macrolactin derivatives gageomacrolactins **1**−**3** and known macrolactins A (**4**), B (**5**), F (**6**), and W (**7**). The stereoconfigurations of **1**−**3** were assigned based on coupling constant values, chemical derivatization studies, and a literature review. The coupling constants were very crucial to determine the relative geometries of olefins in **1**−**3** because of overlap of the ^{1}H NMR signals. The NMR data of these compounds were recorded in different solvents to overcome this problem and obtain accurate coupling constant values. The new macrolactin derivatives **1**−**3** displayed good antibiotic properties against both Gram-positive (*S. aureus*, *B. subtilis*, and *B. cereus*) and Gram-negative (*E. coli*, *S. typhi*, and *P. aeruginosa*) bacteria with minimum inhibitory concentration (MIC) values of 0.02−0.05 μM. Additionally, the antifungal activities of **1**−**7** were evaluated against pathogenic fungi and found to inhibit mycelial growth of *A. niger*, *B. cinerea*, *C. acutatum*, *C. albicans*, and *R. solani* with MIC values of 0.04−0.3 μM, demonstrating that these compounds were good fungicides.

KEYWORDS: *B. subtilis, marine sediment, antibacterial activity, fungicides, stereoconfigurations*

INTRODUCTION

The growing problem of antimicrobial resistance has become a significant public health concern worldwide, and it involves practically all types of pathogens, including bacteria, mycobacteria, viruses, fungi, and parasites.[1] The resistance problem is due to the excessive use of antibiotics and agrochemicals to treat pathogenic infections, which increases the chance of treatment failure. Moreover, application of agrochemicals/chemical fungicides to control pathogen-related damage to crops has become a serious threat to the environment, as the chemicals spread in the air, accumulate in the soil, and have negative effects on health.[3] A large number of synthetic pesticides have been banned because of their undesired attributes such as chronic and acute toxicity, long degradation periods, accumulation in the food chain, and extension of their power to destroy both useful and harmful pests.[4] Beside the synthetic productions, the development of most antimicrobial agents currently in use has relied largely on incremented modifications of natural products templates and semisynthetic variations or improved subclasses.[5] These approaches have produced therapeutic agents with sufficient degradability that narrowly, but temporarily evade existing mechanisms of resistance. Natural products of microbial origins have been proven to be effective and inherently biodegradable due the complexity of structures.[6,7] Especially, marine microorganisms possess unique metabolic pathways because of their diversified characteristics and capabilities of living in extreme conditions, thereby producing metabolites with interesting biological and pharmaceutical properties for their survival.[8] Thus, searching for additional new natural scaffolds from marine microbial origins that inhibit microbial targets can satisfy long-term concerns over microbial resistance and the environment.

Macrolactins are a group of 24-membered macrolides, possessing several bioactivities, most of which are obtained from marine microorganisms. Macrolactin A has been isolated from an unclassified marine bacterium and displays selective antibacterial activities, cytotoxicity against murine melanoma cancer cells (B16-F-10), and antiviral activities against Herpes simplex and HIV.[9] We also reported on three cyclic ether-containing antibacterial macrolactins from the marine sediment bacterium *Bacillus subtilis* in a preceding study.[10] As part of our

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Table 1. NMR Data of Gageomacrolactins 1−3 Recorded in CD_3OD[a]

	1		2		3	
carbon no.	δ_H'	δ_C'	δ_H'	δ_C'	δ_H'	δ_C'
1		167.9 C		167.9 C		168.2 C
2	5.55 d (11.5)	118.0 CH	5.56 d (11.5)	117.9 CH	5.55 d (11.2)	117.9 CH
3	6.61 t (11.5)	145.5 CH	6.64 t (11.5)	145.6 CH	6.63 t (11.2)	145.4 CH
4	7.28 m	130.4 CH	7.22 dd (14.8, 11.0)	130.1 CH	7.24 dd (15.0, 11.0)	130.4 CH
	7.15 dd (15.2, 11.0)‴					
5	6.10 m	141.8 CH	6.22 m	142.4 CH	6.21 m	141.9 CH
	6.05 dd (15.2, 10.5)‴					
6	2.30 m	43.1 CH_2	2.45 m	41.5 CH_2	2.45 m	41.3 CH_2
	2.47 m		2.57 m		2.56 m	
7	4.20 m	71.6 CH	4.49 q (6.5)	78.2 CH	4.51 q (7.0)	78.0 CH
	4.01 t (4.5)‴					
8	5.71 m	133.9 CH	5.62 dd (15.3, 7.5)	133.9 CH	5.58 m	133.9 CH
	5.62 dd (15.0, 6.5)″				5.40 dd (15.2, 9.0)‴	
9	5.72 m	129.0 CH	6.70 dd (15.3, 11.0)	129.8 CH	6.69 dd (15.2, 11.0)	129.6 CH
	5.64 dd (15.0, 7.0)‴					
10	4.21 m	85.7 CH	6.14 t (10.5)	131.5 CH	6.12 t (11.5)	131.2 CH
11	4.06 m	77.5 CH	5.58 m	129.2 CH	5.56 m	129.4 CH
	3.85 d (4.5)‴					
12	1.56 m	41.6 CH_2	2.41 m	36.5 CH_2	1.63 m	36.8 CH_2
	2.33 m				2.39 m	
13	4.04 m	76.6 CH	3.86 m	68.8 CH	3.91 m	69.1 CH
	3.80 t (6.5)‴					
14	1.92 m	44.9 CH_2	1.56 m	42.9 CH_2	1.54 m	44.6 CH_2
15	4.12 m	72.4 CH	3.83 m	80.2 CH	4.25 q (6.0)	70.3 CH
16	5.61 dd (15.5, 6.5)	134.7 CH	5.36 dd (15.3, 8.0)	132.5 CH	5.43 dd (15.3, 7.0)	135.0 CH
17	6.19 dd (15.5, 10.5)	132.1 CH	6.17 m	134.1 CH	5.61 dd (15.3, 7.5)	131.7 CH
	6.05 dd (15.0, 10.5)‴					
18	6.06 m	131.7 CH	6.07 dd (14.8, 10.0)	131.5 CH	2.04 m	33.0 CH_2
	5.95 dd (15.2, 10.5)‴					
19	5.71 m	135.4 CH	5.67 dd (14.8, 7.0)	135.6 CH	1.38 m	29.8 CH_2
20	2.06 m	33.3 CH_2	2.13 m	32.8 CH_2	1.33 m	29.4 CH_2
	2.21 m		2.20 m			
21	1.50 m	26.1 CH_2	1.51 m	25.2 CH_2	1.35 m	26.0 CH_2
22	1.67 m	36.6 CH_2	1.62 m	35.8 CH_2	2.44 m	36.8 CH_2
23	5.02 m	71.9 CH	5.05 m	71.8 CH	4.97 m	72.2 CH
24	1.25 d (6.0)	20.2 CH_3	1.25 d (6.5)	20.1 CH_3	1.24 d (6.0)	20.4 CH_3
1′			4.32 d (8.0)	101.3 CH	4.32 d (7.5)	101.4 CH
2′			3.23 m	75.3 CH	3.25 m	75.3 CH
3′			3.33 m	78.4 CH	3.33 m	78.3 CH
4′			3.27 m	71.9 CH	3.26 m	71.9 CH
5′			3.19 m	78.1 CH	3.21 m	78.1 CH
6′			3.65 dd (11.5, 6.0)	62.9 CH_2	3.65 dd (12.0, 6.0)	62.9 CH_2
			3.88 m		3.86 m	
OCH_3			3.26 s	56.6		

[a] $^1H'$ (δ in ppm and J in Hz) and $^{13}C'$ NMR data recorded at 500 and 125 MHz, respectively. Chemical shifts and coupling constants were determined in $CDCl_3$″ and DMSO-d_6‴.

continuing study on marine-derived bacteria, we discovered three new macrolactin derivatives, gageomacrolactins **1**−**3**, along with known macrolactins A (**4**), B (**5**), F (**6**), and W (**7**) from the fermentation broth of the bacterium *B. subtilis* under optimum growth conditions and tested them against both bacteria and fungi (harmful for human health and crops) to screen for potential biological activities. Accordingly, here we report the isolation, structural elucidation, and antimicrobial activities of **1**−**7**. It is noteworthy that we report here for the first time antifungal properties of **1**−**7** against the crop-devastating pathogens *A. niger*, *B. cinerea*, *C. acutatum*, and *R. solani*, which are well known for causing infections in several crops such as rice, strawberry, grapes, onions, peanuts, banana, beans, and cucumbers.

MATERIALS AND METHODS

Materials and Instrumentation. Dextrose and agar were supplied by Junsei Chemical Co. Ltd. (Tokyo, Japan). Tryptone, yeast extract, and beef extracts were purchased from Becton, Dickinson and Company (Sparks, USA), and organic solvents used were obtained from Duksan Pure Chemicals (Ansan, South Korea). Natural seawater was collected from the East Sea of South Korea at a depth of 20 m. Microorganisms were collected from Korean Collection for Type Cultures (KCTC), Korea Research Institute of Bioscience and Biotechnology (KRIBB), South Korea, for antimicrobial assay. All

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solvents used were either spectral grade or distilled prior to use. NMR spectroscopic data were acquired on a Varian Unity 500 spectrometer. IR spectra were recorded on a JASCO FT/IR-4100 spectrophotometer. Optical rotation values were measured on a JASCO (DIP-1000) digital polarimeter. UV spectra were obtained on a Shimadzu UV-1650PC spectrophotometer. High-resolution ESI-MS were recorded on a hybrid ion-trap time-of-flight mass spectrometer (Shimadzu). Analytical HPLC was conducted on an ODS column (YMC-Pack-ODS-A, 250 × 4.6 mm i.d., 5 μm) 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.

Microorganism Isolation and Taxonomy. Strain ID-109GGC020 was isolated by a standard serial microdilution technique from a marine sediment sample collected from Gageocho, Republic of Korea's southern reef, in 2010. The sediment sample was poured into a conical tube containing sterilized seawater (10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4}) under aseptic conditions and stirred, and 10 μL from each dilution was spread onto a modified Bennett's media agar plate (composition of culture media was 1% dextrose, 0.1% yeast extract, 0.1% beef extract, 0.2% tryptone, 1.8% agar for 1 L 100% natural seawater, and the pH was adjusted to 7.2). The plates were incubated for 4 days at 28 °C, and the resulting colonies of strain 109GGC020 were isolated and stocked in 40% glycerol in seawater. The strain was identified as *B. subtilis* based on a 16S rRNA sequence analysis (GenBank accession no. JQ927413). This strain is currently preserved in the Microbial Culture Collection, Korea Institute of Ocean Science and Technology (KIOST), under the curatorship of H.J.S. with the name *B. subtilis*, ID 109GGC020.

Fermentation and Extraction. The seed culture (50 mL) of the strain was carried out using modified Bennett's medium (composition as above) in 100 mL flasks (3 flasks) prior to mass culture. An aliquot (0.2% v/v) from the seed culture was inoculated aseptically into 2 L flasks (total of 38 flasks) containing 1.2 L of sterilized culture medium. The growth condition of the strain was optimized by culturing it in different combinations of salinity, pH, and temperature. The strain showed optimal growth at 24 °C, pH 7.0, and salinity of 18.5 g/L for the metabolite production. The culture was fermented under optimum conditions for 7 days and then harvested. The culture broth was extracted with EtOAc. The solvent was evaporated to dryness under reduced pressure at 40 °C to yield 8.2 g (dry) of crude extract.

Isolation of Compounds. The crude extract was subjected to ODS open column (350 mm × 50 mm i.d., C18) chromatography followed by stepwise gradient elution with MeOH–H_2O (v/v) (1:4, 2:3, 3:2, 4:1, and 5:0, 2 L each) as the eluent. The fraction eluted with MeOH–H_2O (3:2) was subjected to further fractionation by C18 MPLC [140 mm × 30 mm i.d., eluent: MeOH–H_2O (3:2–4:1), 1.5 L each] to yield 10 subfractions. Semipreparative silica HPLC (column: YMC-Pack-SIL, 250 mm × 10 mm i.d., 5 μm, eluent: 10% $CHCl_3$ in MeOH; flow rate: 1.5 mL/min; detection: 254 nm) was used to collect fractions from the subfractions. Compounds **1**–**7** were then purified on an analytical ODS HPLC (column: YMC-Pack-ODS-A, 250 mm × 4.6 mm i.d., 5 μm, flow rate: 0.6 mL/min; detection: 254 nm) from different subfractions using an isocratic program with MeOH–H_2O (v/v) as the eluent.

Gageomacrolactin ***1*** *(3.2 mg):* amorphous solid; $[\alpha]^{23}_D$ −222 (*c* 0.2, MeOH); UV (MeOH) λ_{max} (log ε) 232 (3.96) and 261 (3.85) nm; IR (MeOH) ν_{max} 3366, 1715, and 1251 cm^{-1}; ^1H and ^{13}C NMR data (Table 1); HR-ESI-MS *m*/*z* 441.2245 $[M + Na]^+$ (calcd *m*/*z* 441.2253 $[M + Na]^+$).

Gageomacrolactin ***2*** *(2.1 mg):* amorphous solid; $[\alpha]^{23}_D$ −61 (*c* 0.2, MeOH); UV (MeOH) λ_{max} (log ε) 234 (4.23) and 261 (4.05) nm; IR (MeOH) ν_{max} 3324 (br), 1600 cm^{-1}; ^1H and ^{13}C NMR data (Table 1); HR-ESI-MS *m*/*z* 601.2981 $[M + Na]^+$ (calcd *m*/*z* 601.2989 $[M + Na]^+$).

Gageomacrolactin ***3*** *(1.8 mg):* amorphous solid; $[\alpha]^{23}_D$ −62 (*c* 0.2, MeOH); UV (MeOH) λ_{max} (log ε) 231 (3.95) and 259 (3.86) nm; IR (MeOH) ν_{max} 3341 (br), 1623 cm^{-1}; ^1H and ^{13}C NMR data (Table 1); HR-ESI-MS *m*/*z* 589.2980 $[M + Na]^+$ (calcd *m*/*z* 589.2989 $[M + Na]^+$).

Determination of Absolute Stereochemistry. *Preparation of Tris-(S)-MTPA and Tris-(R)-MTPA Esters (**1a** and **1b**) of **1**.* The absolute configuration of **1** was assessed by the modified Mosher's method.[11−13] Compound **1** (1.4 mg) was dissolved in 150 μL of pyridine in a 4 mL vial and stirred at room temperature for 10 min. The tris-(*S*)-MTPA ester (**1a**) of **1** was prepared by adding 20 μL of (*R*)-(−)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride (MTPA-Cl) to the reaction vial, and the mixture was stirred at room temperature for 15 h. The reaction was monitored by ESI-MS. The reaction mixture was dried using a rotary evaporator under reduced pressure and again dissolved in EtOAc, washed with H_2O using a separating funnel, and purified on silica HPLC using 5% MeOH in CH_2Cl_2 as eluent to obtain **1a** (0.5 mg). In an entirely analogous way, the tris-(*R*)-MTPA ester **1b** (0.7 mg) was obtained using (*S*)-MTPA-Cl. All proton signals of the triester derivatives were assigned by ^1H–^1H COSY correlations. **1a**: amorphous solid; ^1H NMR (CD_3OD) δ_H 5.52 (d, *J* = 11.5, H-2), 6.40 (t, *J* = 11.5, H-3), 6.84 (dd, *J* = 15.5, 11.5, H-4), 5.76 (dt, *J* = 15.5, 7.5, H-5), 2.48 (m, H_2-6), 5.55 (m, H-7), 5.66 (dd, *J* = 15.5, 7.5, H-8), 6.44 (dd, *J* = 15.5, 11.0, H-9), 5.98 (t, *J* = 11.0, H-10), 5.40 (dt, *J* = 11.0, 5.0, H-11), 1.66 (m, H-12b), 2.14 (m, H-12a), 4.07 (m, H-13), 1.73 (m, H-14b), 2.58 (m, H-14a), 5.52 (m, H-15), 4.55 (t, *J* = 4.5, H-16), 5.82 (overlapped, H-17), 5.82 (overlapped, H-18), 4.60 (m, H-19), 1.52 (m, H-20), 1.27 (m, H-21b), 1.30 (m, H-21a), 1.55 (m, H_2-22), 4.50 (m, H-23), 1.20 (d, *J* = 6.0, H-24), 3.47 (OCH_3, s), 3.53 (OCH_3, s), 3.54 (OCH_3, s), 7.34–7.56 (15H, m); ESI-MS *m*/*z* 1089.90 $[M + Na]^+$. **1b** (0.7 mg): amorphous solid; ^1H NMR (CD_3OD): δ_H 5.54 (d, *J* = 11.5, H-2), 6.51 (t, *J* = 11.5, H-3), 6.97 (dd, *J* = 15.5, 11.5, H-4), 6.00 (dt, *J* = 15.5, 7.5, H-5), 2.54 (m, H_2-6), 5.66 (m, H-7), 5.50 (dd, *J* = 15.5, 7.5, H-8), 6.40 (dd, *J* = 15.5, 11.0, H-9), 5.92 (t, *J* = 11.0, H-10), 5.37 (dt, *J* = 11.0, 5.0, H-11), 1.85 (m, H-12b), 2.15 (m, H-12a), 4.07 (m, H-13), 1.68 (m, H-14b), 2.56 (m, H-14a), 5.50 (m, H-15), 4.53 (t, *J* = 4.5, H-16), 5.80 (overlapped, H-17), 5.80 (overlapped, H-18), 5.43 (m, H-19), 1.56 (m, H-20), 1.29 (m, H-21b), 1.43 (m, H-21a), 1.54 (m, H_2-22), 4.77 (m, H-23), 1.88 (d, *J* = 6.0, H-24), 3.49 (OCH_3, s), 3.50 (OCH_3, s), 3.54 (OCH_3, s), 7.36–7.60 (15H, m); ESI-MS *m*/*z* 1089.77 $[M + Na]^+$.

Antibacterial and Antifungal Activity. The antimicrobial activity of compounds **1**–**7** was determined by using a standard "broth dilution assay"[14] against Gram-positive and Gram-negative bacteria and fungi. A serial double dilution of each compound was prepared in 96-microtiter plates over the range 0.5–256 μg/mL. Culture broth of each strain was prepared, and the final concentration of microorganisms in each culture was adjusted to 1.5 × 10^8 cfu/mL by comparing the culture turbidity with 0.5 McFarland standards. The culture broth (30 μL) was added to each dilution of compounds, the final volume of each well was adjusted to 200 μL using the respective culture medium, and the plates were incubated 24 h at 37 °C for bacteria and 72 h at 30 °C for fungi.[15,16] The minimum inhibitory concentration (MIC, the lowest concentration of a sample at which the microorganism did not demonstrate visible growth, as indicated by the presence of turbidity) was then recorded visually.

RESULTS AND DISCUSSION

Culture Conditions Optimization and Fermentation. The producing strain ID 109GGC020 was isolated from a marine sediment sample and identified as *B. subtilis*. Because the growth of bacteria and their capability to produce the required metabolites for their survival largely depend on the surrounding living conditions and metabolic profiles can be changed with slight changes in condition,[17,18] the optimum growth conditions of the bacteria for maximum metabolite production were optimized by varying the salinity of the seawater. A comparison of ^1H NMR data for the culture extracts obtained using different salinity of the culture medium is shown in Figure 1. It was observed that when the producing

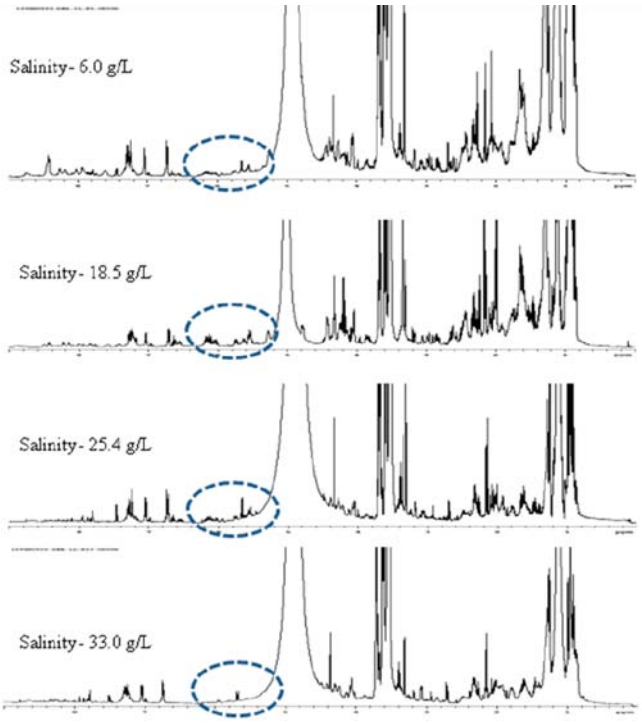

Figure 1. ^{1}H NMR data comparison of the culture extracts of *B. subtilis* grown in different salinity. On the basis of these NMR data analyses, the salinity of the growth medium of the strain 109GGC020 was fixed at 18.5 g/L for large-scale culture.

strain was cultured at different salinities of 6.0, 25.4, and 33.0 g/L, macrolactin production was not good. However, when it was cultured at a salinity of 18.5 g/L, more peaks corresponding to macrolactins were observed in the ^{1}H NMR spectrum. Accordingly, the salinity of the growth medium was fixed at 18.5 g/L for mass culture.

Isolation and Characterization of Compounds. The fermentation broth (50 L) of strain 109GGC020 was extracted with EtOAc; thereafter three new (**1**−**3**) along with four known 24-membered macrolactins (Figure 2) were isolated by sequential fractionation and purification employing flash column chromatography followed by reversed phase and normal phase HPLC.

Gageomacrolactin **1** was isolated as an amorphous solid, analyzed for the molecular formula $C_{24}H_{34}O_6$ based on the HR-ESI-MS in combination with the ^{1}H and ^{13}C data. The IR absorbance bands at 3366, 1715, and 1251 cm^{-1} in association with the ^{13}C NMR data suggested the presence of hydroxyl, carbonyl, and epoxide functionalities, respectively. Moreover, the ^{1}H and ^{13}C NMR data (Table 1) and HSQC spectrum suggested the presence of 10 olefinic methines, six oxygenated methines, six methylenes, and a methyl and carbonyl carbon. This observation clearly indicated that **1** belongs to the macrolactin family with epoxide functionality.[11] The existence of an epoxide group was determined by the close observation of the ^{1}H−^{1}H COSY, ROESY, and HMBC cross-peaks recorded in both CD_3OD and DMSO-d_6. These spectra revealed that, among the six oxygenated methines, three carbons at δ_C 71.6 (C-7), 76.6 (C-13), and 72.4 (C-15) were directly connected with OH groups, whereas the carbon resonating at δ_C 71.9 (C-23) formed an ester linkage with a carbonyl carbon at δ_C 167.9 (C-1). The remaining two carbons at δ_C 85.7 (C-10) and 77.5 (C-11) were assigned as an epoxide group in **1**. These

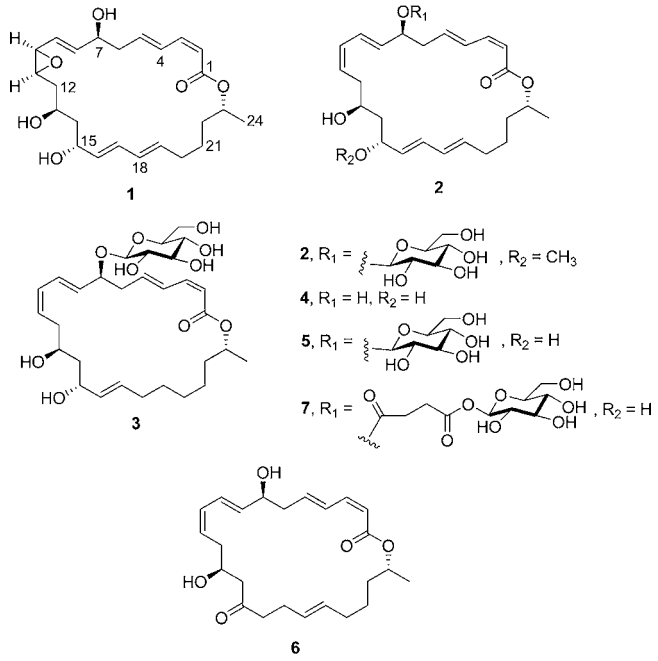

Figure 2. Structures of gageomacrolactins **1**−**3** and macrolactins A (**4**), B (**5**), F (**6**), and W (**7**).

observations established an additional ring system with the macrolactone ring. The ^{1}H−^{1}H COSY and HMBC correlations (Figure 3) also supported the position of the epoxide ring. The

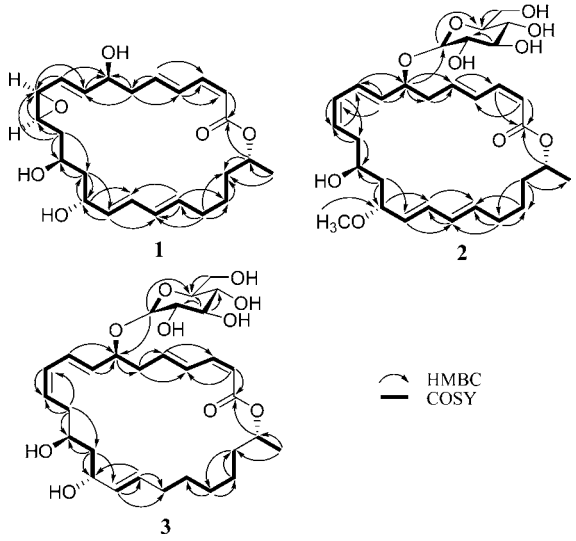

Figure 3. Key COSY and HMBC correlations of gageomacrolactins **1**−**3**.

coupling constant value (J = 4.5 Hz) between H-10 and H-11 indicated that H-10 and H-11 were *cis* oriented.[10,19] The *cis* orientation was also supported by ROESY correlations between H-8 and H-10 and between H-11 and H-12. The position of the hydroxy groups at C-7, C-13, and C-15 was also established by the ^{1}H−^{1}H COSY and HMBC correlations. The point of cyclization of the ester in the macrolactone ring of **1** was indicated by the chemical shift of H-23 at δ_H 5.02, which usually shows coupling to the H-24 methyl proton and the HMBC correlation to the carbonyl carbon at C-1.[9,10] Furthermore, the remaining structure of compound **1** was established on the

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basis of COSY and HMBC spectra. The geometries of the carbon−carbon double bonds at C-2, C-4, C-8, C-16, and C-18 in **1** were determined as *Z*, *E*, *E*, *E*, and *E*, respectively, based on coupling constants and ROESY correlations. The absolute configuration of **1** at the selected stereocenters C-7, C-13, and C-15 was confirmed by applying the modified Mosher's ester method. Compound **1** was treated with (*R*)-(−)- and (*S*)-(+)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride (MTPA-Cl) in dry pyridine separately to yield tris-(*S*)- and (*R*)-MTPA ester derivatives **1a** and **1b**, respectively. The ^{1}H−^{1}H COSY experiment was helpful to assign proton resonances of the two derivatives. The analysis of $\Delta\delta_H$ ($\Delta\delta_H = \delta_S - \delta_R$) values (Figure 4) suggested the absolute

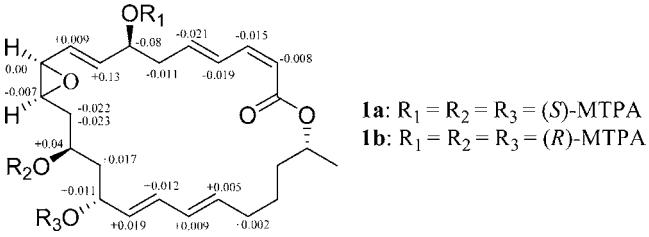

Figure 4. $\Delta\delta_H$ values ($\Delta\delta_H = \delta_S - \delta_R$) obtained for (*S*)- and (*R*)-MTPA esters of **1**.

configuration of the stereocenter at C-7 was *S*. The positive $\Delta\delta_H$ values for H-13 (+0.04), H_2-14 (+0.017), and H-15 (+0.011), which are consistent with the $\Delta\delta_H$ pattern for diesters of *anti*-1,3-diols reported by Freire et al.,[20] indicated that the configurations at C-13 and C-15 of **1** were *S* and *R*, respectively. Moreover, ^{1}H and ^{13}C resonances of **1** at C-23 were very close to those of macrolactin A, and the configuration at C-23 was assigned as *R*, as both compounds were suggested to be produced by the same biosynthetic pathways.[9,10] Thus, the structure of **1** was established to be a new macrolactin derivative, 10,11-epoxymacrolactin A, from the spectroscopic evidence.

Gageomacrolactin **2** was isolated as an amorphous solid, and its molecular formula was determined to be $C_{31}H_{46}O_{10}$ based on the HR-ESI-MS in combination with the ^{1}H NMR and ^{13}C NMR spectroscopic data. The IR absorption bands at 3324 cm^{-1} (br) and 1600 cm^{-1} suggested the presence of hydroxyl and carbonyl moieties, respectively. The ^{1}H and ^{13}C NMR resonances (Table 1) as well as ^{1}H−^{1}H COSY and HSQC suggested the presence of 12 olefinic methines, four oxygenated methines, six methylenes, a methyl, a methoxy, a lactone carbonyl carbon, and resonances attributable to a glucopyranoside moiety. These data revealed that compound **2** possessed structural similarities to macrolactin B[9] but differed from the molecular formula of macrolactin B by the addition of $-CH_3$. This methyl group was identified by a distinct singlet peak (methoxy peak) observed at δ_H 3.26 in the proton spectrum, which was supported by the associated carbon resonating at δ_C 56.6 in the HSQC spectrum. The attachment of the methoxy group at C-15 was confirmed by the long-range HMBC correlation between the methoxy proton and the methine carbon resonating at δ_C 80.2. A single contiguous spin system from H-2 to H_3-24 obtained from the analysis of the COSY spectrum was helpful to assign the gross structure of **2**. The carbon resonances at δ_C 62.9 (CH_2), 78.1 (CH), 71.9 (CH), 78.4 (CH), 75.3 (CH), and 101.3 (CH) in the ^{13}C NMR spectrum of **2** revealed the presence of a β-glucopyranoside. The anomeric proton at δ_H 4.32 (d, J = 8.0 Hz) correlated to the carbon signal at δ_C 101.3 in the HSQC spectrum, which also supported the β-configuration of the glucopyranoside moiety.[21−23] The anomeric proton was also used in the analysis of the COSY spectrum as a starting point for sequential assignment of the proton resonances in the glucopyranoside moiety. The intercorrelations among all carbons and protons in molecule **2** were also established with the help of HMBC connectivities (Figure 3). The ester linkage of the lactone ring was assigned from the HMBC correlation between H-23 (δ_H 5.05) and C-1 (δ_C 167.9). The connection between the β-glucopyranoside and the hydroxyl group at C-7 of the lactone ring was also determined by the long-range HMBC correlation between the oxygenated methine proton at δ_H 4.49 (H-7) and the anomeric carbon at δ_C 101.3. On the basis of the coupling constant values (Table 1) and ROESY correlations the geometries of the double bonds at C-2, C-4, C-8, C-10, C-16, and C-18 in **2** were *Z*, *E*, *E*, *Z*, *E*, and *E*, respectively. The overall stereoconfigurations of **2** were determined to be the same as macrolactin B, as the ^{1}H and ^{13}C NMR data of both compounds were very close and showed similar specific rotation values ($[\alpha]^{23}_D$ −61 (*c* 0.3, MeOH)) as well. Thus, the structure of compound **2** was a new macrolactin derivative, 15-methoxymacrolactin B, from the spectroscopic data analysis.

Gageomacrolactin **3** was isolated as an amorphous solid, and its molecular formula was determined to be $C_{30}H_{46}O_{10}$ based on the HR-ESI-MS in combination with the ^{1}H and ^{13}C data (Table 1). The IR absorption bands at 3341 (br) and 1623 cm^{-1} suggested the presence of hydroxyl and carbonyl groups, respectively. The NMR results were essentially identical to those of macrolactin B except the presence of two methylene carbons at δ_C 33.0 and 29.8 instead of two downfield methine carbons. The positions of these two methylenes of C-18 and C-19 were determined from ^{1}H−^{1}H COSY, ROESY, and HMBC correlations. The COSY cross-peaks confirmed the correlations of methylene protons between H-18 and H-19 and between H-19 and H-20. This assignment was further supported by the HMBC correlations of H-16 and H-17 with C-18 and between H-20 and C-19. The ^{1}H and ^{13}C NMR chemical shifts suggested the presence of a sugar moiety similar to that of gageomacrolactin **2** and macrolactin B. As the anomeric proton at δ_H 4.32 (d, J = 7.5 Hz) displayed a clear correlation with the methine carbon at δ_C 78.0 in the HMBC spectrum, the position of the sugar moiety was determined to be at C-7. In addition, the gross structure of **3** was determined by analyzing 1D and 2D spectroscopic data similar to compounds **1** and **2**. The relative geometries of the conjugated dienes of compound **3** at C-2, C-4, C-8, C-10, and C-16 were assigned on the basis of the coupling constant values and ROESY correlations and were the same as those of **2**. The ^{1}H and ^{13}C chemical shifts and optical rotation value suggested that the absolute configuration of **3** was in accord with that of macrolactin B. Thus, the structure of gageomacrolactin **3** was assigned as a new macrolactin derivative, 18,19-dihydromacrolactin B, from the spectroscopic data analysis.

The structures of known macrolactins A (**4**), B (**5**), F (**6**), and W (**7**) were confirmed by interpreting the ^{1}H and ^{13}C NMR chemical shifts together with ^{1}H−^{1}H COSY, HSQC, HMBC, and ESI-MS data.

Antimicrobial Assay. The antibacterial activities of new compounds **1**−**3** were evaluated against both Gram-positive and Gram-negative bacteria and exhibited good activity (Figure 5), but known macrolactins **4**−**7** were not tested in this study,

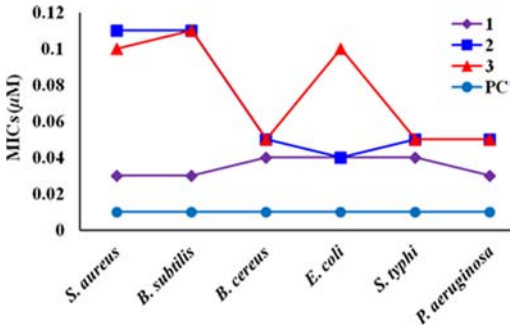

Figure 5. Antibacterial activity of gageomacrolactins **1**–**3** (PC: positive control, azithromycin).

as their antibacterial properties have been reported.[24] Compound **1** displayed similar activities to cyclic ether-containing macrolactin **2** reported by Mondol et al.[10] against *B. subtilis* and *E. coli* with MIC values of 0.03 and 0.04 μM, respectively. The only difference between these two compounds was the position of the epoxide group. Compound **2**, which possesses a similar structure to macrolactin B with an additional methoxy group attached at C-15, showed weak antimicrobial activity against bacteria and fungi compared to that of **1**. Consequently, the hydroxyl group at C-15 of the macrolactone ring was detected as an important factor for antimicrobial activity. It is also revealed that macrolactins F and K, which contain a ketonic group at C-15, exhibit less antibacterial activity than that of macrolactins possessing a hydroxyl group at C-15.[9,25] Compound **3** also displayed good activity against Gram-negative bacteria. Macrolactins have drawn the attention of scientists due to their broad-spectrum antibacterial properties. For example, macrolactin A has become an attractive target for asymmetric synthesis because of an unreliable cell culture supply as well as its structural uniqueness and broad therapeutic potential.[26] 7-*O*-Malonyl macrolactin A is a new antibiotic and is the most active compound against selected pathogens and clinical multidrug-resistant and Gram-negative pathogens compared to commercially available antibiotics. It also shows a strong growth-inhibiting effect against vancomycin-resistant enterococci and methicillin-resistant *S. aureus*.[27] Because the antifungal activity of macrolactins has not been reported yet, we were interested in evaluating such activities against crop-devastating pathogens. Among three new macrolactin derivatives (Figure 6), compound **1** displayed good inhibitory activity against the mycelial growth of *A. niger* and *S. cerevisiae* with MIC values of 0.07, 0.11, and 0.06 μM, respectively. Compound **2** showed

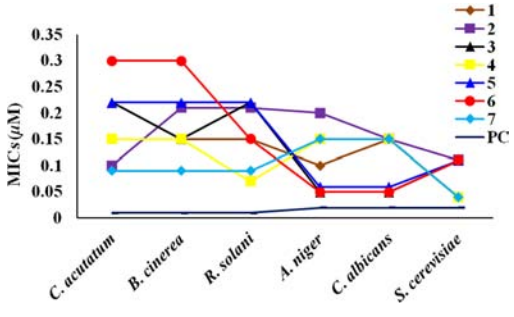

Figure 6. Antifungal activity of isolated macrolactins **1**–**7** (PC: positive control, amphotericin B).

good activity against *C. aculatum* and *S. cerevisiae* but less activity against *B. cinerea* and *R. solani*. Similarly, compound **3** exhibited good activity against *A. niger* and *C. albicans*. Known macrolactins **4**–**7** also displayed meaningful antifungal activity against all fungi with MIC values of 0.04–0.3 μM; in particular macrolactins A and W were more active than B and F. As these isolated compounds showed good antibacterial and antifungal properties, it was necessary to evaluate the toxic effects. Accordingly, the cytoxicity[28] of compounds **1**–**3** along with macrolactins B and F was evaluated against a panel of cancer cell lines: ACHN human renal cancer, HCT 15 human colon cancer, MDA-MB-231 human breast cancer, NCI-H23 human lung cancer, NUGC-3 human stomach cancer, and PC-3 human prostate cancer cell lines. These compounds failed to register any cytotoxicity (GI_{50} > 30) at a concentration of 30 μg/mL against the cancer cell lines tested. The cytotoxic and noncytotoxic effects of macrolactins A and W, respectively, have been reported[9,29] and were not investigated in this study.

In summary, the antimicrobial results suggested that macrolactins **1**–**7** or their modified forms might be useful bioactive precursors for further development of effective antibiotics and fungicides, which would be helpful to treat infections and to secure crop production.

■ ASSOCIATED CONTENT

Supporting Information

1D, 2D NMR (^{1}H–^{1}H COSY, HSQC, HMBC, and ROESY), and HRESIMS spectra of gageomacrolactins (**1**–**3**). This information 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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NOTE ADDED AFTER ASAP PUBLICATION

This paper was published on the Web on March 25, 2013, with an error to the Supporting Information. The corrected version was reposted April 2, 2013.