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Macrolactin W, a new antibacterial macrolide from a marine *Bacillus* sp.

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ABSTRACT

Bioactivity-guided isolation for the new/novel metabolites from the EtOAc extract obtained from the culture broth of a marine *Bacillus* sp. 09ID194 followed by chromatographic fractionations and subsequently HPLC purifications led to the isolation of two known macrolides, macrolactins A (**1**) and Q (**2**), together with a new glycosylated marcrolide, macrolactin W (**3**). The chemical structures of compounds **1–3** were assigned based on extensive MS and NMR spectral data analysis and literature review. Compound **3** showed potent antibacterial activity against both Gram-positive and Gram-negative pathogenic bacteria.

Terrestrial microorganisms have been extensively studied for the discovery of novel bioactive metabolites than marine microorganisms. Marine microbial metabolites exhibit a broad spectrum of biological activities because of living in quite different environment compared to terrestrial counterparts and may have unique metabolic pathway to produce metabolites which possess novel structures and activities.[1] 24-Membered macrolactins were generally produced by *Bacillus* sp. and exhibited antibacterial, anticancer and antiviral activities.[2] As a part of our ongoing research on isolation of bioactive secondary metabolites from marine microorganisms, we isolated a marine bacterium 09ID194 from a sediment sample collected from Ieodo, South Korea's southern reef, and later identified as *Bacillus* sp. based on 16S rDNA sequencing.[3] Macrolactins (**1–3**) (Fig. 1) were purified from the EtOAc extract of the culture broth of this strain by C-18 reversed-phase HPLC.[4,5] Here we report the isolation, structure determination and biological activities of compounds **1–3**.

The molecular formulas of compounds **1** and **2** were assigned to be $C_{24}H_{34}O_5$ and $C_{30}H_{44}O_{10}$ respectively, based on their MS, ^{1}H and ^{13}C NMR spectral data analysis. Compounds **1** and **2** gave molecular ion adduct peaks in ESIMS at *m*/*z* 425.34 ([M+Na]$^+$) and 587.38 ([M+Na]$^+$), respectively. The MS, ^{1}H and ^{13}C NMR spectral data and specific rotation values ($[\alpha]_D$) of compounds **1** and **2** were identical to those of known macrolactins A[6] and Q[7] respectively, hence the identities of **1** and **2** were established.

Compound **3** was isolated as an amorphous solid and analyzed for the molecular formula $C_{34}H_{48}O_{13}$ by its HRESIMS (*m*/*z* 687.2987 [M+Na]$^+$; Δ 0.0 mmu), ^{1}H and ^{13}C spectral data (Table 1), implying 11 degrees of unsaturation. The ^{1}H and ^{13}C NMR signals of **3** were characteristics of both macrolide and sugar moiety. The infrared spectrum of **3** (in MeOH) showed absorbance bands at 3372, 1742, 1700 and 1070 cm^{-1}, indicative of hydroxyl (OH), olefinic (C=C) and ester (C=O) functionalities. Ultraviolet absorbances at λ_{max} (log ε) 226 (4.74) and 258 (4.35) nm were assigned to a chromophore with extended conjugation. Analysis of ^{13}C NMR spectrum (Table 1) showed three ester carbonyl carbon resonances between δ_C 168.0 and 173.4, twelve methine carbons between δ_C 118.7 and 144.5 assigned to six double bonds, nine oxygenated methine carbons between δ_C 69.5 and 96.0, one oxygenated methylene carbon at δ_C 62.3, and nine aliphatic carbons between δ_C 20.3 and 44.3. In the macrolactin ring, six double bonds, one ester carbonyl carbon, and its ring accounted for a total of eight degrees of unsaturation and the remaining three degrees of unsaturation, of which two were attributed to two succinate ester carbonyl carbons (one for each) and leaving one degree of unsaturation for cyclic structure of glucose moiety, accounted for 11 degrees of unsaturation required by the molecular formula of **3**. The extensive COSY and HMBC (Fig. 2) spectroscopic data analysis revealed that **3** has three spin systems: H-2 to H-24, H-2′ to H-3′ and H-1″ to H-6″ and in these three spin systems, the coupling to each adjacent protonated center were determined sequentially in a stepwise fashion. The geometries (*Z* and *E* form) of the carbon-carbon double bonds in the α-, β-, γ-, δ-unsaturated ester (C-1 through C-5) and the two pairs of conjugated dienes (C-8 to C-11 and C-16 to C-19) were assigned on the basis of their ^{1}H coupling constants (Table 1) and ROESY correlations (Fig. 3). The coupling constant (J = 11.3 Hz) of H-2 with H-3 and ROESY correlation (Fig. 3) between H-2 and H-3 clearly established the

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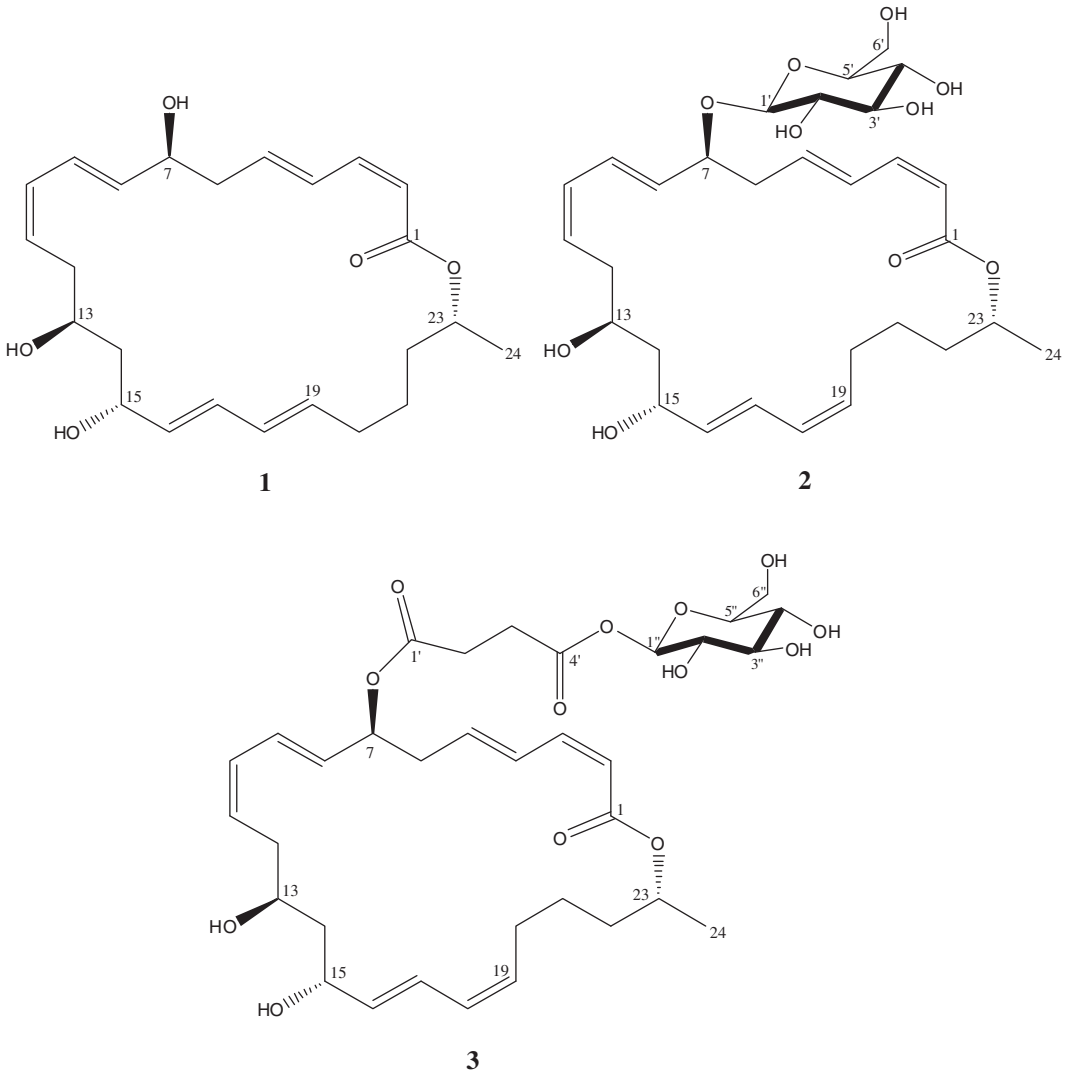

Figure 1. Chemical structures of macrolactins A (**1**), Q (**2**) and W (**3**).

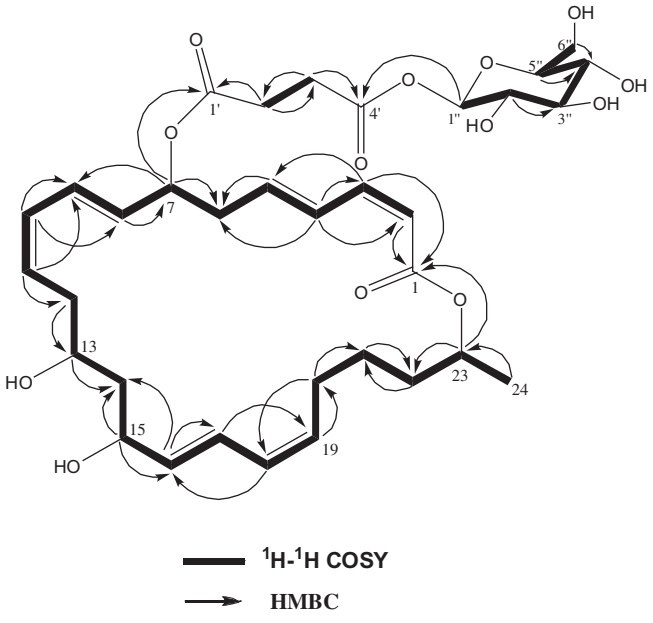

Figure 2. Key ^{1}H–^{1}H COSY and HMBC correlations of macrolactin W (**3**).

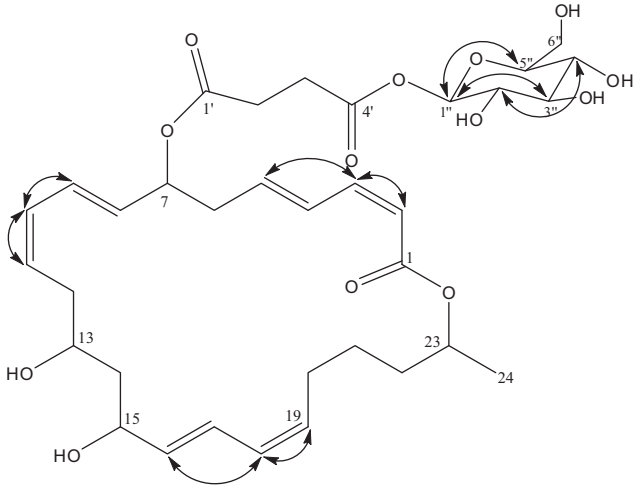

Figure 3. Key ROESY correlations of macrolactin W (**3**).

geometry of C-2/C-3 double bond to be *Z*. The *E* geometry of the C-4/C-5 olefin was supported by large vicinal proton–proton coupling constant (J = 14.8 Hz) of H-4 with H-5. The large scalar coupling

Table 1
^{1}H and ^{13}C NMR data of compound **3** in CD_3OD-d_4

Position	δ_C	δ_H, m (J in Hz)	HMBC
1	168.0		
2	118.7	5.55, d (1H, 11.3)	C-1
3	144.5	6.62, t (1H, 11.3)	C-1, C-5
4	131.1	7.21, dd (1H, 11.3, 14.8)	C-2, C-3, C-6
5	140.5	6.06, m (1H)	C-6, C-7
6	40.2	2.56, m (2H)	C-5, C-7, C-8
7	74.6	5.46, m (1H)	C-5, C-6, C-8, C-9, C-1′
8	132.2	5.71, dd (1H, 5.0, 15.3)	C-6, C-7
9	127.7	6.58, dd (1H, 11.3, 15.3)	C-7, C-11
10	130.8	6.09, t (1H, 11.3)	C-8, C-9, C-12
11	129.9	5.61, m (1H)	C-9, C-12
12	36.3	2.22, m (1H)	C-13, C-14
		2.59, m (1H)	
13	69.5	3.77, m (1H)	C-11, C-14, C-15
14	44.3	1.63, m (2H)	C-12, C-13
15	69.9	4.33, m (1H)	C-13, C-14, C-16, C-17
16	135.1	5.54, dd (1H, 7.0, 15.3)	C-14, C-17
17	131.5	6.19, dd (1H, 11.5, 15.3)	C-19
18	131.6	6.05, t (1H, 11.5)	C-16
19	135.5	5.69, m (1H)	C-20, C-21
20	33.26	2.12, m (1H)	C-18, C-19, C-21, C-22
		2.19, m (1H)	
21	26.0	1.50, m (2H)	C-22
22	36.25	1.58, m (1H)	C-20, C-21
		1.64, m (1H	
23	72.5	4.99, m (1H)	C-1, C-22, C-24
24	20.3	1.25, d (3H, 6.0)	C-23
1′	173.4		
2′	29.97	2.73, m (2H)	C-1′, C-3′
3′	29.98	2.64, m (1H)	C-2′, C-4′
		2.68, m (1H)	
4′	173.1		
1″	96.0	5.49, d (1H, 8.0)	C-4′
2″	74.1	3.33, dd (1H, 8.0, 8.4)	C-1″, C-3″
3″	77.9	3.42, dd (1H, 8.4, 8.8)	C-2″, C-5″
4″	78.9	3.35, m (1H)	
5″	71.0	3.36, m (1H)	C-4″
6″	62.3	3.66, dd (1H, 4.0, 12.2)	C-4″
		3.81, dd (1H, 2.0, 12.2)	

constant (J = 15.3 Hz) between H-8 and H-9, and small coupling constant (J = 11.3 Hz) of H-10 with H-11, and ROESY correlations (Fig. 3) between H-9 and H-10 and between H-10 and H-11, definitely certified the geometries of C-8/C-9 and C-10/C-11 double bonds as *E* and *Z*, respectively. The relative configurations of the disubstituted C-16/C-17 and C-18/C-19 conjugated olefins were deduced to be *E* and *Z*, respectively, in similar fashion simply by the analysis of their coupling constants ($J_{H\text{-}16/H\text{-}17}$ = 15.3 Hz, $J_{H\text{-}18/H\text{-}19}$ = 11.5 Hz) (Table 1) and ROESY correlations (H-16/H-18 and H-18/H-19) (Fig. 3).

The point of cyclization of the ester in macrolactone ring of **3** was indicated by the low-field shift of H-23 at δ_H 4.99, which was clearly coupled to the H-24 methyl group at δ_H 1.25 (d, J = 6.0 Hz) and firmly supported by showing long range HMBC correlation (Fig. 2) of H-23 with ester carbonyl carbon at δ_C 168.0 (C-1). The COSY correlation between H-2′ and H-3′ and the HBMC correlations of H-2′ with C-1′ (δ_C 173.4) and H-3′ with C-4′ (δ_C 173.1) indicated the presence of succinyl moiety. The down-field shift of H-7 (δ_H 5.46, m) and its HMBC connectivity with C-1′ and the HMBC correlation of the anomeric proton at δ_H 5.49 (d, J = 8.0 Hz, H-1″) with C-4′ corroborated that the succinic acid moiety was esterified to macrolactone ring at C-7 and sugar moiety at C-1″. A six carbons pyranose glycoside constituent was established by an acetal carbon resonance at δ_C 96.0, four methine carbon signals between δ_C 71.0 and 78.9 and a methylene carbon at δ_C 62.3. Appropriate ^{1}H NMR resonances for a β-pyranose sugar including the anomeric (axial) proton at δ_H 5.49, were also observed. Coupling constants analysis revealed diaxial couplings ranging from 8.0 to 8.8 Hz between all of the glycoside ring protons, thus defining the presence of β-glucopyranosyl moiety which was also supported by ROESY correlations (H-1″/H-5″, H-1″/H-3″, and H-2″/H-4″) (Fig. 3). Compound **3** was assigned to be a new derivative of 7-*O*-succinyl macrolactin A[8] and so named macrolactin W. In 24-membered macrolactins, compound **3** is first macrolactin where succinic acid moiety was fully esterified. Compound **3** showed optical rotation $[\alpha]_D^{23}$ −56.7° (*c* 0.10, MeOH) which is similar to macrolactin Q[7] and it can, therefore, be assumed that compound **3** has the same absolute stereochemistry as macrolactin Q.

The absolute configurations of the sugar and the aglycon part in **3** were determined by acid hydrolysis,[9] where the glycon portion was converted to glucose and the aglycon to macrolactin A, followed by TLC comparison with authentic sample and measurement of optical rotation after purification. The glucose isolated from the acid hydrolysis of **3** gave a positive specific rotation $[\alpha]_D^{23}$ +23° (*c* 0.15, H_2O), indicating that it was D-glucose. This fact was further confirmed by comparing its R_f value with authentic sample. Both the glucose isolated and authentic sample exhibited same R_f value (0.55) in same condition. The aglycon portion obtained from **3** showed optical rotation $[\alpha]_D^{23}$ −13.2° (*c* 1.5, MeOH) which is similar to macrolactin A.[6] Together with the agreement of the ^{1}H and ^{13}C NMR data with the literature values, this result suggested that the aglycon portion in **3** has the same absolute stereochemistry as macrolactin A.

The minimum inhibitory concentration (MIC) of compound **3** was determined by serial dilution technique.[10] The MIC of compound **3** against *Bacillus subtilis* (KCTC 1021), *Staphylococcus aureus* (KCTC 1916), *Escherichia coli* (KCTC 1923) and *Pseudomonas aeruginosa* (KCTC 2592) was 64 μg/mL. The cytoxicity[11] of compound **3** 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. The new compound **3** failed to register any cytotoxicity at a concentration of 10 μg/mL against cancer cell lines tested.

Gustafson and co-workers[6] reported that macrolactin A, which was isolated from unclassified marine bacterium, inhibited *S. aureus* and *B. subtilis* in standard 'disc diffusion assay' at a concentration of 5 and 20 μg/disc, respectively. Macrolactins F and K[12] in which C-15 was a ketone carbonyl group showed weak antibacterial activity. Macrolactins G–M[12] inhibited *S. aureus*, regardless of the positions of hydroxyl group at C-7 or C-9 or of the number of ring members. So the position of hydroxyl group at C-15 may play an important role in the antibacterial activity of macrolactins. Macrolactins O–R[7] inhibited *S. aureus* PDF in dose-dependent manners with IC_{50} (μM) values of 53.5, 57.7, 12.1 and 61.5, respectively. Macrolactin S[13] isolated from a marine *B. subtilis*, showed significant antimicrobial activity against *E. coli* but weak activity against *B. subtilis* and *S. aureus*. Macrolactin T,[14] and V[15] exhibited antifungal and significant antibacterial activity, respectively. Compound **3** and 7-*O*-succinyl macrolactin A[8] showed almost similar antibacterial activities. This result suggested that full esterification of succinic acid moiety in macrolactins does not affect antibacterial activity. The strain 09ID194 produced more amount of compounds **1–3** only in low salinity (12 psu) compare to high salinity (32 psu) and it may be due to maintaining osmoregulation in their physiological process.

In conclusion, macrolactin W (**3**) is a new 24-membered glycosylated lactone compound isolated from the culture broth of a marine *Bacillus* sp. 09ID194. Macrolactin W showed potent antibacterial activity against both Gram-positive and Gram-negative bacteria which may serve for the development of new antibacterial agent.

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3. The strain 09ID194 was isolated from a sediment sample collected from Ieodo, South Korea's southern reef, during expedition in 2009 by serial dilution technique. In brief, one gram of the sediment sample was diluted in sterilized sea water (10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}) in aseptic conditions and 100 μL from each dilution was spread on modified Bennett's agar medium (0.1% yeast extract, 0.1% beef extract, 0.2% tryptone, 1% dextrose, 100% natural sea water, 1.8% agar and pH adjusted to 7.2 before sterilization). The plates were incubated for 14 days at 30 °C, and the resulting colony of the strain 09ID194 was isolated and maintained on the modified Bennett's agar. The strain 09ID194 formed well-developed reddish substrate and aerial mycelium on modified Bennett's agar medium. The strain was identified as *Bacillus* sp. on the basis of 16S rDNA sequence analysis. This strain is currently deposited in the Microbial Culture Collection, KORDI, with the name of *Bacillus* sp. 09ID194 under the curatorship of H.J.S.
4. The seed and mass culture were carried out in modified Bennett's broth medium. The composition and pH of the seed culture medium (0.1% yeast extract, 0.1% beef extract, 0.2% tryptone, 1% dextrose, 1.2% psu, pH 7.6) were same as mass culture medium. 50 mL of the medium was dispensed in 250 mL conical flask. A single colony of 09ID194 strain from the agar plate was inoculated into the flask and incubated at 30 °C for 2 days on a rotary shaker at 120 rpm. An aliquot (0.2% v/v) from the seed culture was inoculated into a 20 L fermenter containing 15 L culture medium (15 L × 2). The culture was carried out at 30 °C, 120 rpm for 7 days and then harvested.
5. The fermentation broth (15 L × 2) was centrifuged by continuous centrifuge, and the supernatant was extracted with EtOAc (30 L × 2). The EtOAc layer was concentrated to dryness using rotary evaporator in vacuo at 40 °C. The residual suspension (3.0 g) was subjected to ODS open column chromatography followed by stepwise gradient elution with MeOH/H_2O (v/v) (1:4, 2:3, 3:2, 4:1 and 100:0) as eluant. The fraction eluted with MeOH/H_2O (4:1, v/v) was purified by reversed-phase HPLC (YMC ODS-A column, 250 × 10 mm i.d.: 70% MeOH in H_2O; flow rate: 1.5 mL/min; detector: RI) to yield pure compounds **1** (28 mg), **2** (5 mg) and **3** (17.5 mg).
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9. Compound **3** (3 mg) was refluxed in 1 N HCl (1 mL) at 90 °C for 2 h. The completion of hydrolysis was confirmed by LC/MS analysis (in ESIMS, sugar *m/z* 203.05 $[M+Na]^+$; macrolactin A *m/z* 425.16 $[M+Na]^+$). After cooling, the reaction mixture was extracted with EtOAc (2 mL × 2). The EtOAc extract was evaporated and the residue was purified through a silica column (YMC-Pack-SIL, 250 × 10 mm i.d.: 10% $CHCl_3$ in EtOAc; flow rate: 1.5 mL/min; detector: UV) to yield pure aglycon (macrolactin A, 0.8 mg). The aqueous phase was neutralized with 1 N NaOH and evaporated to dryness and purified through ODS analytical column (YMC-Pack-ODS-A, 250 × 4.6 mm i.d.: 55% MeOH in H_2O; flow rate: 0.6 mL/min; detector: RI) to obtain pure glycon (D-glucose, 0.7 mg). The glycon was analyzed by TLC (Kieselgel, eluting solvent $CHCl_3$/MeOH-1:1, sprayed with 1% H_2SO_4/vanillin and heated) to reveal the presence of D-glucose as its R_f value (0.55) was coincident with the authentic sample.
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