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Cyclic Ether-Containing Macrolactins, Antimicrobial 24-Membered Isomeric Macrolactones from a Marine *Bacillus* sp.

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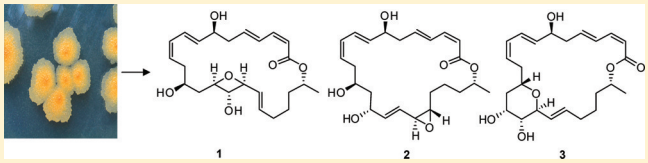
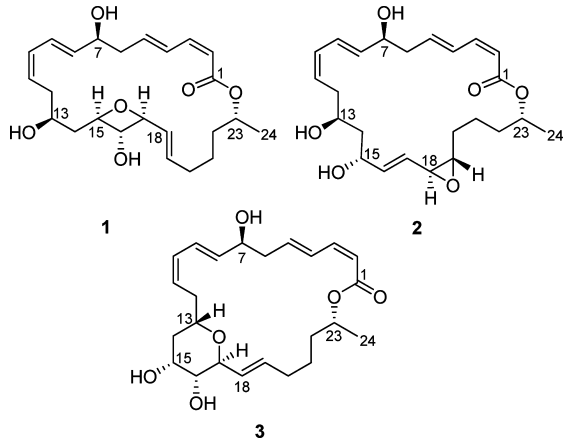
Supporting Information

ABSTRACT: Bioassay-guided isolation of bioactive metabolites from the ethyl acetate extract of a marine *Bacillus* sp. fermentation broth has led to the discovery of three new 24-membered macrolactones, macrolactins **1**−**3**, which contain an oxetane, an epoxide, and a tetrahydropyran ring, respectively. The configurations of **1**−**3** were assigned by a combination of coupling constants, ROESY data analysis, and application of the modified Mosher's method. Compounds **1**−**3** showed *in vitro* antimicrobial activity.



Currently, discovery of new drugs from terrestrial microbial sources is limited because of re-isolation of known natural products. Because new multidrug-resistant pathogens are emerging with regularity, discovery of novel antimicrobials has become a pressing need. In response to this, rational strategies to maximize the chemical diversity obtained from each microbial species during fermentation and subsequent biological screening are important to discover new bioactive metabolites. Marine microorganisms, which are taxonomically diverse and genetically distinct, have a high potential for producing novel bioactive substances.[1] Macrolactins, 24-membered macrolactones, are mainly produced by *Bacillus* sp. and show antimicrobial activity because of their unique chemical architecture.[2] Physiological processes of marine microorganisms are altered when cultured in low salinity to maintain osmotic balance.[3] Based on this phenomenon, large-scale fermentation of a marine *Bacillus* sp. 09ID194 and subsequent bioassay-guided fractionation and repeated chromatographic purifications of the EtOAc extract obtained from the fermentation broth has led to the discovery of three new bioactive macrolactones, macrolactins **1**−**3**. This paper reports the isolation, structure elucidation, and antimicrobial activity of **1**−**3**.

Compound **1** was obtained as an amorphous solid, and its molecular formula was determined as $C_{24}H_{34}O_6$ with eight degrees of unsaturation on the basis of the HRESIMS (*m*/*z* 441.2242 $[M + Na]^+$) and ^{13}C NMR data (Table 1). The IR spectrum of **1** contained bands at 3318 and 1681 cm^{-1}, consistent with hydroxy and carbonyl functionalities, respectively. The UV absorptions at 232 and 261 nm were suggestive of an extended conjugation system. This supposition was supported by the 10 sp^2 carbon signals in the ^{13}C NMR spectrum. In total 24 carbon resonances were observed in the ^{13}C NMR spectrum, which were ascribed to one carbonyl, six oxymethine, 10 olefinic, six methylene, and one methyl carbon with the help of an HSQC spectrum. The ^{13}C NMR spectrum indicated that **1** was composed of one ester and five double bonds, which accounted for six out of eight degrees of unsaturation. The remaining two degrees of unsaturation could be ascribed to the presence of two ring systems in **1**. The ^1H and ^{13}C NMR signals clearly indicated that **1** belongs to the macrolactin family[4] with the macrolactone accounting for one of the rings.



The planar structure of **1** (Figure 1) was established by analysis of the COSY, HSQC, and HMBC spectroscopic data. COSY correlations revealed one contiguous coupling sequence from H-2 at δ_H 5.51 (d, J = 11.5) to H_3-24 at δ_H 1.25 (d, J = 6.0). The point of cyclization of the ester in the macrolactone

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Table 1. NMR Data for Compounds 1 and 2 in CD_3OD

	1			2		
C	δ_C[a]	δ_H, mult. (J in Hz)[b]	HMBC[c]	δ_C[a]	δ_H, mult. (J in Hz)[b]	HMBC[c]
1	168.3			168.3		
2	118.3	5.51, d (11.5)	1, 4	118.3	5.53, d (11.3)	1
3	144.8	6.65, dd (11.5, 11.5)	1, 5	144.9	6.60, dd (11.3, 11.3)	1
4	130.7	7.23, dd (15.5, 11.5)	3, 6	130.6	7.23, dd (15.5, 11.3)	2
5	141.6	6.25, dt (15.5, 7.0)	3, 6, 7	141.6	6.12, m	3
6	42.9	2.40, m	4, 5, 7, 8	41.9	2.43, m	4, 5, 7, 8
		2.51, m			2.48, m	
7	73.2	4.25, m	5, 8, 9	72.9	4.24, m	5
8	136.4	5.64 (overlapped)	6, 7, 10	137.1	5.68 (overlapped)	9
		[5.53, dd (15.0, 7.5)][e]			[5.65, dd (15.5, 6.5)][d]	
9	127.9	6.55, dd (15.0, 11.0)	7, 10, 11	127.7	5.68 (overlapped)	8
					[6.42, dd (15.5, 11.0)][d]	
10	131.5	6.04, dd (11.0, 11.0)	8, 9, 12	131.5	6.09, dd (10.5, 10.5)	8, 9
11	129.7	5.44, dt (11.0, 6.0)	9, 12	129.5	5.45, dt (10.5, 6.0)	
12	35.3	2.12, m	10, 13	36.3	2.32, m	11, 13
		2.55, m			2.70, m	
13	76.1	3.45, m		78.6	4.05, m	
		[3.07, m][e]				
14	40.9	1.37, q (12.6)	12, 13, 15, 16	40.2	1.66, m	13, 15, 16
		1.95, ddd (12.6, 5.0, 1.5)			2.22, m	
15	74.0	3.52, ddd (11.3, 8.8, 5.0)		83.9	4.26, m	16, 17
		[3.36, ddd (11.3, 9.0, 5.0)][e]				
16	77.8	2.91, dd (9.0. 9.0)	15, 17, 18	136.8	5.68 (overlapped)	18
		[2.97, dd (9.0, 9.0)][e]				
17	80.3	3.45, m	16, 18, 19	129.0	5.68 (overlapped)	
		[3.46, dd (9.0, 5.0)][e]				
18	129.2	5.63 (overlapped)	17, 19, 20	73.4	4.08, dd (6.5, 2.5)	17
		[5.64, dd (15.1, 5.0)][e]				
19	132.3	5.67 (overlapped)	17, 18, 20	74.4	4.26, m	20
		[5.78, dt (15.1, 6.5)][e]				
20	34.6	2.04, m	18, 19, 21	38.0	1.48, m	
		2.08, m			1.62, m	
21	26.8	1.48, m	20, 22	22.5	1.48, m	
		1.51, m				
22	37.0	1.64, m	20, 23, 24	37.1	1.64, m	
23	73.1	4.95, m	1, 21, 24	72.7	4.96, m	1, 24
24	20.0	1.25, d (6.0)	22, 23	20.4	1.25, d (6.5)	22, 23

[a]Recored at 125 MHz. [b]Recorded at 500 MHz. [c]HMBC correlations are from proton(s) stated to the indicated carbon(s). [d]Chemical shifts and coupling constants were determined in DMSO-d_6. [e]Chemical shifts and coupling constants were determined in C_6D_6.

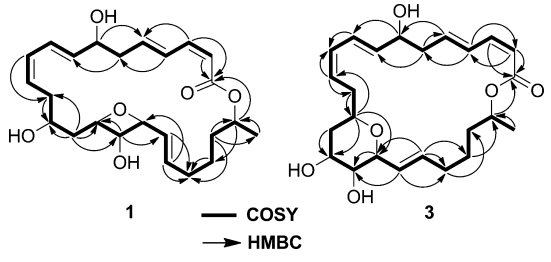

Figure 1. COSY and HMBC correlations for **1** and **3**.

ring of **1** was indicated by the chemical shift of H-23 at δ_H 4.95, which was clearly coupled to the H_3-24 methyl group and supported by an HMBC correlation (Figure 1) of H-23 with a carbonyl carbon at δ_C 168.3 (C-1). On the basis of the molecular formula and the requirement for another ring system, there must be a cyclic ether functionality in **1**. The location of the cyclic ether was determined by careful investigation of the COSY data recorded in different NMR solvents and coupling constants analysis. Analysis of the COSY spectra in DMSO-d_6 and C_6D_6 revealed a coupling sequence of three contiguous oxymethine protons (H-15 to H-17) (Supporting Information). In the COSY spectrum in DMSO-d_6, oxymethine protons H-7, H-13, and H-16 showed COSY correlations with OH protons attached to C-7, C-13, and C-16, respectively. As the oxymethine carbon C-23 was involved in the ester linkage with carbonyl carbon C-1, an oxygen bridge must be formed between C-15 and C-17 to create an oxetane ring to satisfy the molecular formula. This finding indicated that there was a 15,17-epoxy-16-hydroxy moiety in the molecule where H-16 coupled with H-15 and H-17 with a large coupling constant value (J = 9.0), which rules out an epoxide in this sequence. Compound **1** had an oxetan-3-ol moiety, which is found in several natural products.[5−7] The large coupling constants between H-15 and H-16, and between H-16 and H-17, and ROESY correlation between H-15 and H-17 indicated that H-15 and H-17 were *cis* oriented[8] (Table 1 and Supporting Information). The geometries of the double bonds at C-2, C-4,

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C-8, C-10, and C-18 were assigned as *Z*, *E*, *E*, *Z*, and *E*, respectively, on the basis of their respective ^{1}H NMR coupling constants: 11.5, 15.5, 15.0, 11.0, and 15.1 Hz.

The absolute configuration of **1** was addressed by the modified Mosher's method.[9−11] 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. All proton signals of the two triester derivatives were assigned by a COSY experiment, and ^{1}H NMR chemical shift values of the tris-(*R*)-MTPA esters (**1b**) were subtracted from the corresponding values of the tris-(*S*)-MTPA esters (**1a**). The $\Delta\delta_H$ ($\Delta\delta_H = \delta_S - \delta_R$) values are shown in Figure 3

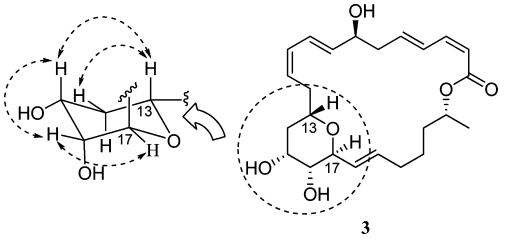

Figure 2. Key ROESY correlations for **3**.

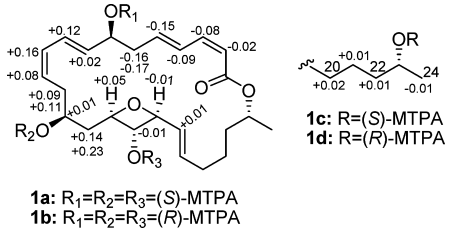

Figure 3. $\Delta\delta_H$ values ($\Delta\delta_H = \delta_S - \delta_R$) obtained for (*S*)- and (*R*)-MTPA esters of **1**.

(selected region). In the ^{1}H NMR spectra of the tris-Mosher's ester derivatives, negative $\Delta\delta_H$ values were observed for H-2−H_2-6, whereas positive values were found for H-8−H_2-12. These data allowed the assignment of the absolute configuration of C-7 as *S*. $\Delta\delta_H$ values from H-13 to H-16 corresponding to a typical $\Delta\delta_H$ pattern for diesters of *anti*-1,4-diols reported by Riguera[12] indicated that the absolute configurations of C-13 and C-16 of **1** were *S* and *R*, respectively. To establish the absolute configuration of C-23, **1** was first converted to the linear methyl ester by treating with NaOMe in MeOH. (*S*)- and (*R*)-Mosher's esters (**1c** and **1d**) of the linear methyl ester were prepared by the same methods as for **1a** and **1b**, respectively. Analysis of the ^{1}H NMR chemical shifts ($\Delta\delta_H = \delta_S - \delta_R$) of **1c** and **1d** in proximity of the esterified carbon (C-23) (Figure 3) allowed the assignment of the absolute configuration of C-23 as *R*. The absolute configurations of **1** for shared stereocenters were the same as those for macrolactin A.[13] Therefore, **1** was assigned as a new derivative, 15,17-epoxy-16-hydroxy macrolactin A.

Compound **2** also had a molecular formula of $C_{24}H_{34}O_6$ established by HRESIMS. The UV, IR, and ^{1}H and ^{13}C NMR spectra of **2** were almost identical to those of **1**. The IR absorption spectrum contained a strong hydroxy band at 3341 cm^{-1} and bands at 1685 and 1259 cm^{-1}, which were attributed to ester carbonyl and epoxide functionalities, respectively, suggesting that **2** was a positional isomer of **1**. UV absorbances at 234 and 261 nm were assigned to an extended conjugation system. The planar structure of **2** was established on the basis of extensive spectroscopic data analysis (COSY, HSQC, and HMBC). Analysis of the COSY spectrum revealed a single spin system from H-2 to H_3-24. The HMBC correlation from H-23 to C-1 (δ_C 168.3) indicated that C-23 was involved in an ester linkage. An epoxide group at C-18 and C-19 in **2** was established as **1**.

The geometries of the double bonds at C-2, C-4, C-8, and C-10 in **2** were found to be same as those of **1** on the basis of coupling constants (Table 1). The resonances of H-16 and H-17 were overlapped in both CD_3OD and DMSO-d_6, preventing direct measurement of their coupling constants. ROEs between H-15 and H-17 and between H-16 and H-18 indicated that the olefin configuration was *E*. The coupling constant of H-18 with H-19 (J = 2.5 Hz) indicated a *trans* orientation of the epoxide group. The weak COSY correlation between H-18 and H-19 also supported a *trans* orientation of the epoxide group. The *trans* orientation was also supported by ROESY correlations between H-16 and H-18 and between H-17 and H-19. Although a *trans* epoxide was established, the flexibility of the macrocyclic ring and the lack of ROESY correlations prevented the assignment of the configuration of the epoxide relative to the remaining stereocenters. Analysis of the corresponding ^{1}H NMR chemical shift differences between tris-(*S*)- and (*R*)-MTPA esters (Supporting Information) of **2** indicated that the absolute configurations of C-7, C-13, and C-15 stereogenic centers were *S*, *S*, and *R*, respectively. The ^{13}C and ^{1}H resonances of C-23 (δ_C 72.7) and H-23 (δ_H 4.96, m) were similar to those of **1**, and **1** and **2** were likely produced by a common biosynthetic pathway.[14] So it can therefore be assumed that the absolute configuration of C-23 in **2** was *R*. The structure of **2** was established to be an isomer of **1**, 18,19-epoxy macrolactin A.

Compound **3** was isolated as an optically active, amorphous solid, which gave a $[M + Na]^+$ ion at m/z 441.2244 in the HRESIMS spectrum, consistent with a molecular formula of $C_{24}H_{34}O_6$, isomeric with **1** and **2**. The planar structure of **3** was determined in a similar fashion to **1** and **2** (Figure 1). The continuity of protons from C-2 to C-24 positions was observed by a COSY spectral data analysis. The chemical shift of H-23 at δ_H 4.97 indicated that **3** was a cyclic ester, and the linkage was confirmed by an HMBC correlation of H-23 with the ester carbonyl carbon C-1 (δ_C 168.1). Six oxymethine carbon resonances were observed in the ^{13}C NMR spectrum. Of these, three oxymethines (H-7, H-15, and H-16) showed COSY correlations recorded in DMSO-d_6 with OH protons, indicating there were OH groups attached to C-7, C-15, and C-16. However, oxymethines H-13 and H-17 did not show COSY correlations with the OH group proton, suggesting that an oxygen bridge must be formed between C-13 and C-17 to create a tetrahydropyran ring. This was confirmed by an HMBC correlation of H-13 with C-17 (Figure 1).

Analysis of coupling constants indicated that the relative configurations of all disubstituted double bonds of **3** were the same as those of **1** and **2** (Tables 1 and 2). The ROESY correlations between H-13ax and H-14eq, H-13ax and H-15ax, H-15ax and H-16eq (J = 3.8 Hz), and H-16eq and H-17eq (J = 3.8 Hz) implied that the tetrahydropyran ring adopts a chair conformation (Figure 2). The difference in ^{1}H NMR chemical shift values $\Delta\delta_H$ ($\delta_S - \delta_R$) for the tris Mosher's esters indicated the 7*R* absolute configuration. Analysis of the $\Delta\delta_H$ values clearly established the absolute configurations of C-15 and C-16 of **3** as *R* on the basis of the modified Mosher's method for

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Table 2. NMR Data for Compound 3 in CD_3OD

C	δ_C[a]	δ_H, mult. (J in Hz)[b]	HMBC[c]
1	168.1		
2	118.2	5.52, d (11.5)	1, 4
3	144.9	6.63, dd (11.5, 11.5)	1, 5
4	130.8	7.24, dd (15.3, 11.5)	2, 3, 6
5	141.6	6.19, dt (15.3, 7.5)	3, 4, 7
6	42.7	2.50, dd (7.0, 7.0)	4, 5, 7, 8
7	72.2	4.29, m	5, 6, 8, 9
8	136.9	5.70 (partially overlapped) [5.66, dd (15.5, 6.5)][d]	6, 10
9	127.0	6.55, dd (15.2, 10.5)	7, 10
10	131.1	6.05, dd (10.5, 10.5)	8, 9, 12
11	129.5	5.54 (partially overlapped) [5.45, dt (10.5, 6.0)][d]	9, 12
12	34.4	2.80, m 2.10, m	10, 11, 13
13	71.9	3.73, m	11, 12, 15, 17
14	35.6	1.70, m 1.73, m	12, 15, 16
15	67.8	3.84, ddd (8.3, 5.6, 3.8)	13
16	72.1	3.60, dd (3.8, 3.8)	
17	77.5	4.33, dd (3.8, 3.8)	16
18	128.4	5.43 dd (15.5, 3.8)	16, 17, 20
19	135.6	5.68, dt (15.5, 7.0)	17, 20, 21
20	33.7	2.08, m 2.13, m	19, 21, 22
21	25.9	1.50, m 2.11, m	19, 20, 22, 23
22	36.4	1.60, m 1.70, m	21
23	72.5	4.97, m	1, 21
24	20.0	1.25, d (6.5)	22, 23

[a]Recorded at 125 MHz. [b]Recorded at 500 MHz. [c]HMBC correlations are from proton(s) stated to the indicated carbon(s). [d]Chemical shifts and coupling constant were determined in DMSO-d_6.

syn-1,2-diols described by Riguera[12] (Supporting Information). ROESY correlations (Figure 2) supported the *R* configuration of C-13. Thus, the structure of **3** was established to be 13,17-epoxy-16-hydroxy macrolactin A, a new derivative of macrolactin A containing a tetrahydropyran ring.

Compounds **1**−**3** each showed a minimum inhibitory concentration (MIC) of 0.16 μM against *Bacillus subtilis* and *Escherichia coli* in a standard *in vitro* broth dilution assay. Their MICs against *Saccharomyces cerevisiae* were 0.16, 0.02, and 0.16 μM, respectively. Macrolactin A,[4] which was isolated from an unclassified marine bacterium, inhibited *S. aureus* and *B. subtilis* in a standard disk diffusion assay and also showed significant antiviral and antitumor activities. A hydroxy group at C-15 was essential for the antibacterial activity, whereas the presence of a hydroxy group at C-7 or C-9 or the number of ring members did not affect the antibacterial activity.[15] Macrolactins mainly have shown antibacterial and moderate or weak antifungal activities.[16−18] The new macrolactins containing oxetane (**1**), epoxide (**2**), and tetrahydropyran (**3**) moieties exhibited antibacterial activities similar to the literature values,[2] and **2** showed very good activity against *S. cerevisiae*.

In conclusion, three new isomeric 24-membered macrolactones, macrolactins **1**−**3**, were isolated from the EtOAc extract of the culture broth of a marine *Bacillus* sp. and showed antimicrobial activities against both Gram-positive and Gram-negative pathogens. This is the first report on the isolation of 24-membered macrolactones containing oxetane (**1**), epoxide (**2**), and tetrahydropyran (**3**) moieties. The incorporation of an epoxide within the macrolactone ring appears to play an important role in the anti-*S. cerevisiae* activity. Interestingly, compounds **1**−**3** were produced by this *Bacillus* sp. only in low-salinity (12 g/L), but not in high-salinity (32 g/L) culture medium.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured on a JASCO (DIP-1000) digital polarimeter. UV spectra were obtained on a Shimadzu UV-1650PC spectrophotometer. IR spectra were recorded on a JASCO FT/IR-4100 spectrophotometer. 1D and 2D NMR spectra were acquired on a Varian Unity 500 spectrometer. High-resolution mass spectra were recorded on a hybrid ion-trap time-of-flight mass spectrometer (Shimadzu LC/MS-IT-TOF). Analytical HPLC was conducted with a PrimeLine binary pump with RI-101(Shodex) and variable UV detector (M 525). Continuous centrifugation was done on a centrifugal separator (Kansai Centrifugal Separator Manufacturing Co. Ltd.). 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). Natural seawater (NSW) was collected from the East Sea (Sea of Japan), Republic of Korea. All solvents used were either spectral grade or distilled prior to use.

Isolation and Taxonomy of Strain 09ID194. Strain 09ID194 was isolated from a sediment sample collected from Ieodo, Republic of Korea's southern reef, during an expedition in 2009. In brief, one gram of the sediment sample was diluted in sterilized seawater (10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4}) under aseptic conditions, and 100 μL from each dilution was spread onto modified Bennett's agar (0.1% yeast extract, 0.1% beef extract, 0.2% tryptone, 1% dextrose, 100% NSW, 1.8% agar, pH adjusted to 7.2 before sterilization). The plates were incubated for 14 days at 30 °C, and the resulting colony of strain 09ID194 was isolated and maintained on modified Bennett's agar. Strain 09ID194 formed well-developed yellowish colonies on modified Bennett's agar. The strain was identified as *Bacillus* sp. on the basis of 16S rDNA sequence analysis. The sequence was deposited in GenBank under accession number JN048684. This strain is currently preserved in the Microbial Culture Collection, KORDI, with the name of *Bacillus* sp. 09ID194 under the curatorship of H.J.S.

Seed and Large-Scale Cultures of the Strain 09ID194. The seed and large-scale cultures were carried out in modified Bennett's medium [0.1% yeast extract, 0.1% beef extract, 0.2% tryptone, 1% dextrose, diluted NSW (salinity 12 g/L), pH 7.6]. The medium (1 L) was dispensed in a 2 L conical flask and sterilized. A single colony of 09ID194 strain from the agar plate was inoculated aseptically into the flask and incubated at 24 °C for 2 days on a rotary shaker at 120 rpm. An aliquot (0.2% v/v) from the seed culture was inoculated aseptically into 2 L flasks (total 300 flasks) containing 1 L of sterilized culture medium. The production culture was incubated under the same conditions as the seed culture for 7 days and then harvested.

Extraction and Isolation. The production culture broth (300 L) was centrifuged, and the supernatant was extracted with EtOAc (2 × 300 L). The EtOAc layer was concentrated to dryness using rotary evaporators at 40 °C. The residual suspension (200 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 eluent. The fraction eluted with MeOH−H_2O (3:2, v/v) was again subjected to silica open column chromatography followed by gradient elution with *n*-hexane−EtOAc (v/v) (100:0, 4:1, 3:2, 2:3, 1:4, 0:100) and EtOAc−MeOH (v/v) (4:1, 3:2, 2:3, 1:4, 0:100). The fraction eluted with *n*-hexane−EtOAc (v/v) (0:100) was subjected to further fractionations by semipreparative silica HPLC (10% *n*-hexane in EtOAc; flow rate: 1.5 mL/min; detector: UV) to obtain 13 fractions (F-1−13). Compounds **1**−**3** were purified on analytical ODS HPLC (flow rate: 0.6 mL/min; detector: UV) from F-8, F-11, and F-9 using

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the following isocratic programs: 55% MeOH in H_2O; 60% MeOH in H_2O; and 58% MeOH in H_2O to yield pure compounds **1** (6.0 mg), **2** (3.1 mg), and **3** (3.5 mg), respectively.

15,17-Epoxy-16-hydroxy macrolactin A (1): amorphous solid; $[\alpha]^{23}_D$ −220 (*c* 0.05, MeOH); UV (MeOH) λ_{max} (log ε) 200 (4.08), 232 (4.23), and 261 (4.05) nm; IR (MeOH) ν_{max} 3318 (br), 2922, 1681, 1250, 1029 cm^{-1}; ^1H and ^{13}C NMR data (CD_3OD), see Table 1; HRESIMS *m*/*z* 441.2242 $[M + Na]^+$ (calcd for $C_{24}H_{34}O_6Na$, 441.2253).

18,19-Epoxy macrolactin A (2): amorphous solid; $[\alpha]^{23}_D$ −100 (*c* 0.05, MeOH); UV (MeOH) λ_{max} (log ε) 234 (3.95) and 261 (3.76) nm; IR (MeOH) ν_{max} 3341 (br), 2922, 1685, 1259, 1029, 1037 cm^{-1}; ^1H and ^{13}C NMR data (CD_3OD), see Table 1; HRESIMS *m*/*z* 441.2248 $[M + Na]^+$ (calcd for $C_{24}H_{34}O_6Na$, 441.2253).

13,17-Epoxy-16-hydroxy macrolactin A (3): amorphous solid; $[\alpha]^{23}_D$ −250 (*c* 0.05, MeOH); UV (MeOH) λ_{max} (log ε) 200 (4.11), 234 (4.42), and 261 (4.22) nm; IR (MeOH) ν_{max} 3373 (br), 2929, 1689, 1192 cm^{-1}; ^1H and ^{13}C NMR data (CD_3OD), see Table 2; HRESIMS *m*/*z* 441.2244 $[M + Na]^+$ (calcd for $C_{24}H_{34}O_6Na$, 441.2253).

Tris-(*S*)-MTPA Ester (1a) of 1. Compound **1** (1.0 mg) was dissolved in 150 μL of pyridine and stirred at room temperature (rt) for 10 min. For preparation of the tris-(*S*)-MTPA ester (**1a**) of **1**, 20 μL of (*R*)-(−)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride (MTPA-Cl) was added to the reaction vial, and the mixture was stirred at rt for 16 h. Completion of the reaction was monitored by LC/MS. The reaction mixture was dried *in vacuo* and redissolved in EtOAc, washed with H_2O, and purified on a silica HPLC using 5% MeOH in $CHCl_3$ as eluent to obtain **1a** (0.8 mg). All proton signals of the triester derivative (**1a**) were assigned by a COSY experiment. **1a**: white, amorphous solid; ^1H NMR (CD_3OD) (selected regions) δ_H 5.51 (d, *J* = 11.3, H-2), 6.50 (t, *J* = 11.3, H-3), 7.17 (dd, *J* = 15.3, 11.3, H-4), 5.88 (dt, *J* = 15.3, 7.0, H-5), 2.52 (m, H-6b), 2.65 (m, H-6a), 5.70 (dd, *J* = 14.8, 7.5, H-8), 6.78 (dd, *J* = 14.8, 11.0, H-9), 6.20 (t, *J* = 11.0, H-10), 5.61 (m, H-11), 2.23 (m, H-12b), 2.58 (m, H-12a), 3.57 (m, H-13), 1.61 (m, H-14b), 2.20 (m, H-14a), 5.15 (m, H-15), 3.15 (dd, *J* = 9.0, 9.0, H-16), 3.55 (dd, *J* = 9.0, 5.0, H-17), 5.64 (dd, *J* = 14.5, 5.0, H-18), 3.45 (OCH_3, s), 3.54 (2 × OCH_3, s) 7.29−7.63 (15H, m); ESIMS *m*/*z* 1089.81 $[M + Na]^+$.

Tris-(*R*)-MTPA Ester (1b) of 1. In an entirely analogous way, the tris-(*R*)-MTPA ester (**1b**) was obtained using (*S*)-(+)-MTPA-Cl. **1b**: white, amorphous solid (0.75 mg); ^1H NMR (CD_3OD) (assignment was done by a COSY experiment) (selected regions) δ_H 5.53 (d, *J* = 11.3, H-2), 6.58 (t, *J* = 11.3, H-3), 7.26 (dd, *J* = 15.5, 11.3, H-4), 6.03 (dt, *J* = 15.5, 7.0, H-5), 2.56 (m, H-6b), 2.72 (m, H-6a), 5.65 (dd, *J* = 14.8, 7.0, H-8), 6.66 (dd, *J* = 14.8, 11.0, H-9), 6.04 (t, *J* = 11.0, H-10), 5.53 (m, H-11), 2.14 (m, H-12b), 2.47 (m, H-12a), 3.56 (m, H-13), 1.38 (m, H-14b), 2.06 (m, H-14a), 5.10 (m, H-15), 3.16 (dd, *J* = 9.0, 9.0, H-16), 3.56 (dd, *J* = 9.0, 3.5, H-17), 5.63 (dd, *J* = 14.5, 3.5, H-18), (OCH_3, s), 3.53 (2 × OCH_3, s) 7.30−7.58 (15H, m); ESIMS *m*/*z* 1089.84 $[M + Na]^+$.

Methanolysis and Preparation of (*S*)- and (*R*)-MTPA Esters (1c and 1d) of 1. Compound **1** (1.2 mg) was dissolved in 0.5 N NaOMe solution (1.5 mL of MeOH) and refluxed at 90 °C for 1 h with stirring. The reaction mixture was neutralized by adding 1 N HCl and dried *in vacuo*. The residual material was dissolved in EtOAc (3 mL) and washed with H_2O (3 × 10 mL) to remove excess NaOMe. The methanolysis product (0.9 mg) was confirmed by ESIMS (*m*/*z* 449.23 $[M - H]^-$). Then the methanolysis product (0.45 mg in each case) (linear methyl ester of **1**) was subjected to esterification with (*R*)-(−)/(*S*)-(+)-MTPA-Cl according to the procedure described above, and purification on a semipreparative silica HPLC using 8% MeOH in $CHCl_3$ provided (*S*)-MTPA (**1c**) (0.30 mg) and (*R*)-MTPA (**1d**) (0.31 mg) esters of linear methyl ester of **1**, respectively. ^1H NMR of both of the Mosher's ester derivatives were analyzed in selected regions. **1c**: white, amorphous solid; selected ^1H NMR (CD_3OD) δ_H 2.04 (m, H_2-20), 1.38 (m, H_2-21), 1.60 (m, H_2-22), 1.23 (d, *J* = 6.5, H_3-24); ESIMS *m*/*z* 1314.56 $[M - H]^-$. **1d**: white, amorphous solid; selected ^1H NMR (CD_3OD) δ_H 2.02 (m, H_2-20), 1.37 (m, H_2-21), 1.59 (m, H_2-22), 1.24 (d, *J* = 6.0, H_3-24); ESIMS *m*/*z* 1314.56 $[M - H]^-$.

Antimicrobial Assays. The minimum inhibitory concentrations of compounds **1**−**3** were determined by using a standard broth dilution assay.[19] Compounds **1**−**3** were tested against three microbial strains: *Bacillus subtilis* (KCTC 1021), *Escherichia coli* (KCTC 1923), and *Saccharomyces cerevisiae* (KCTC 7913). Antibacterial and antiyeast tests were performed in nutrient broth and yeast maltose broth, respectively. A serial double dilution of each compound was prepared in 96-microtiter plates over the range 0.5−256 μg/mL. An overnight broth culture of each strain was prepared, and the final concentration of organisms in each culture was adjusted to 1.5 × 10^8 cfu/mL by comparing the culture turbidity with the 0.5 McFarland standard. Culture broth (30 μL) was added to each dilution of compounds **1**−**3**, and the final volume of each well was adjusted to 200 μL using the respective culture medium; the plates were incubated 24 h at 37 °C for bacteria and 48 h at 30 °C for yeast.[20,21] The MIC is the lowest concentration of a sample at which the microorganism did not demonstrate visible growth, as indicated by the presence of turbidity.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

HRESIMS and 1D and 2D NMR spectra of **1**−**3** and Mosher's ester data for **2** and **3**. This material is available free of charge via the Internet at http://pubs.acs.org.

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