ORIGINAL ARTICLE

New antimicrobial compounds from a marine-derived *Bacillus* sp.

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In the course of our ongoing research for antimicrobial agents, we isolated five new compounds, three 24-membered macrolactones, macrolactins X–Z (1–3) and two hydroxy unsaturated fatty acids, lineiodolides A (5) and B (6), together with a known metabolite, macrolactinic acid (4), from the culture broth of a marine *Bacillus* sp. The structures of these metabolites were determined on the basis of their spectroscopic data interpretation. Their absolute configurations were addressed by modified Mosher's method and literature data review. Compounds 1–6 showed moderate to potent inhibitory activities (8–128 $\mu\text{g ml}^{-1}$) against selected pathogenic microorganisms.

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INTRODUCTION

Marine microorganisms, which are taxonomically diverse and genetically special, have powerful potential in producing novel bioactive compounds.¹ Lately, marine bacteria have drawn much interest owing to their unique secondary metabolites, which differ significantly from those of their terrestrial counterparts.^{2,3} Marine microorganisms are highly diversified and may have unique metabolic pathway because of extreme living conditions resulting in a potential for the discovery of novel drugs and remain mostly unexplored.⁴

Discovery of new antimicrobial drugs are decreasing steadily, whereas infectious diseases caused by drug-resistant pathogens are emerging day by day.^{5,6} Therefore, new antimicrobial drugs are urgently needed to treat these infectious diseases. We have also reported new antimicrobial compounds from a marine sediment *Bacillus* sp.^{7–9} During our continuous screening for new metabolites with antimicrobial activities, we could isolate five new (1–3, 5 and 6) and one known (4) antimicrobial compounds from the fermentation broth of a marine-derived *Bacillus* sp. 09ID194. Here, we report isolation, structure elucidation and antimicrobial activity of these compounds (1–6).

RESULTS

Structure elucidation of macrolactin X (1)

Macrolactin X (1) was isolated as an amorphous solid. Its molecular formula, $\text{C}_{24}\text{H}_{34}\text{O}_6$, was determined by high-resolution electrospray ionization mass spectrometry (HRESIMS) (m/z 441.2249 [$\text{M} + \text{Na}$]⁺), which suggested eight degrees of unsaturation. The IR

spectrum of 1 displayed characteristic absorption bands for hydroxy (3482 cm^{-1}), carbonyl (1691 cm^{-1}) and epoxide (1250 cm^{-1}) functionalities. UV absorptions at 232 and 261 nm indicated the presence of an extended conjugation system in the molecule. 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 quaternary carbonyl, 10 sp^2 , six oxymethine, six methylene and one methyl carbons by analysis of an HSQC spectrum. One carbonyl and five double bonds accounted for six degrees of unsaturation and the remaining two degrees of unsaturation were ascribed to the existence of two cyclic ring systems in 1. ^1H and ^{13}C NMR signals clearly indicated that 1 belongs to macrolactin family.¹⁰

The gross structure of 1 (Figure 1) was established by analysis of ^1H – ^1H COSY, HSQC and HMBC spectroscopic data. ^1H – ^1H COSY correlations revealed one coupling sequence from H-2 at δ_{H} 5.55 (d, $J = 11.5\text{ Hz}$) to H₃-24 at δ_{H} 1.25 (d, $J = 6.0\text{ Hz}$). The oxymethine proton H-23 (δ_{H} 4.93) showed an HMBC cross-peak with a carbonyl carbon (C-1, δ_{C} 167.9), confirming the presence of an ester linkage as suggested by its deshielded ^1H NMR chemical shift value.¹⁰ A 15,16-epoxide was established as H₂-14 and H-17 showed HMBC correlations with C-15 and C-16, respectively.

The relative configuration of 1 was addressed by a combination of coupling constants and ROESY data analysis. The geometries of the double bonds at C-2, C-4, C-8 and C-10 were assigned as *Z*, *E*, *E* and *Z*, respectively, based on their respective ^1H coupling constants: 11.5, 15.5, 15.5 and 11.0 Hz (Table 1) and ROESY correlations between H-2

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Figure 1 ^1H – ^1H COSY and HMBC correlations for **1–3**, **5** and **6**.

and H-3, H-3 and H-5, H-9 and H-10 and H-10 and H-11 (Figure 2). Resonances of H-17 and H-18 were overlapped in CD_3OD preventing direct measurement of their coupling constants. The ROESY correlations between H-16 and H-18, and between H-17 and H-19 indicated that the olefin configuration was *E*. The coupling constant of H-16 with H-15 ($J = 5.0\text{ Hz}$) indicated a *cis* orientation of the epoxide moiety.¹¹ The epoxide group lies α face, which is supported by the lack of ROESY correlation observed between H-13 and H-15. The absolute configurations of **1** at selected stereocenters were determined by the modified Mosher's method.^{12–14} **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 ^1H – ^1H COSY experiment and ^1H chemical shift values of the tris-(*R*)-MTPA esters (**1b**) were subtracted from the corresponding values of the tris-(*S*)-MTPA esters (**1a**). The $\Delta\delta_{\text{H}}$ ($\Delta\delta_{\text{H}} = \delta_{\text{S}} - \delta_{\text{R}}$) values are shown in Figure 3. Analysis of $\Delta\delta_{\text{H}}$ values indicated that the absolute configurations of stereocenters at C-7, C-13 and C-19 were *S*. ^1H and ^{13}C resonances of H-23 (δ_{H} 4.93, m) and C-23 (δ_{C} 72.1) of **1** were quite similar to those of macrolactin A^{10,15} and both **1** and macrolactin A might be produced by a common biosynthetic pathway.¹⁶ So, it might therefore be assumed that the absolute configuration of C-23 in **1** was *R*, as all the macrolactin family members are produced in same biogenesis and the shared stereocenters between macrolactin A and its derivatives had the same absolute configurations.

Structure elucidation of macrolactin Y (**2**)

Macrolactin Y (**2**) was isolated, by repeated chromatographic separations from the EtOAc extract, as an amorphous solid. The molecular formula of **2** was established as $\text{C}_{25}\text{H}_{38}\text{O}_7$ on the basis of HRESIMS measurements (m/z 473.2511 $[\text{M} + \text{Na}]^+$), indicating that **2** contained seven degrees of unsaturation. The degree of unsaturation was further illustrated by intense UV absorptions at 261 and 234 nm, which also suggested the presence of an extended conjugation system. The IR spectrum of **2** showed characteristic absorptions at 3311 and 1646 cm^{-1} , suggesting the presence of hydroxy and ester carbonyl groups, respectively. The ^{13}C NMR spectrum (in CD_3OD) showed 25 resolved signals, which were classified as derived from one methyl, one methoxy, 10 sp^2 methine, six methylene, six oxymethine and one ester carbonyl carbons (Table 1). The connectivities of all protons and carbon atoms, and carbon backbone of **2** were established by

interpretation of its ^1H – ^1H COSY, HSQC and HMBC NMR spectroscopic data (Tables 1 and 2, and Figure 1). Only one spin system was identified by analysis of a ^1H – ^1H COSY spectrum, which showed sequential connectivity from H-2 to H-24 (Figure 1). The downfield shift of H-23 indicated an ester linkage, which was further confirmed by an HMBC correlation between H-23 and an ester carbonyl carbon (C-1). The methoxy group (OCH_3) protons at δ_{H} 3.28 showed an HMBC cross-peak with a carbon (C-18) resonating at δ_{C} 86.6 indicating a methoxy group attached at C-18.

The *Z*, *E*, *E*, *Z* and *E* configurations of double bonds at C-2, C-4, C-8, C-10 and C-16 were evident from their respective vicinal coupling constants of 11.5, 15.0, 15.3, 11.3 and 15.3 Hz, respectively, and ROESY correlations (Table 1 and Figure 2). The H-18 and H-19 were shown to be in a *syn* relationship ($J = 3.8\text{ Hz}$), which was also supported by ROESY correlation (Figure 2). The absolute configurations of **2** at selected chiral centres were addressed by modified Mosher's method in a similar fashion as **1**. Analysis of $\Delta\delta_{\text{H}}$ values obtained from tetra-(*S*) and (*R*)-MTPA ester derivatives (**2a** and **2b**) around stereocenters at C-7 and C-19 indicated that the absolute configurations of C-7 and C-19 were *S*. Positive $\Delta\delta_{\text{H}}$ values for H-13 (+0.19), H-14 (+0.02/+0.15) and H-15 (+0.18), corresponding to a typical $\Delta\delta_{\text{H}}$ pattern for diesters of anti-1,3-diols reported by Freire *et al.*¹⁴ indicated that the absolute configurations of C-13 and C-15 of **2** were *S* and *R*, respectively. Macrolactins might be produced in common biogenesis,¹⁶ and ^1H and ^{13}C resonances of H-23 (δ_{H} 4.95, m) and C-23 (δ_{C} 72.2) of **2** were similar to those of macrolactin A.^{10,15} So, it might therefore be assumed that the absolute configuration of C-23 in **2** was *R*.

Structure elucidation of macrolactin Z (**3**)

The molecular formula of **3** (macrolactin Z) was established as $\text{C}_{29}\text{H}_{40}\text{O}_8$ from the HRESIMS measurements, which exhibited an $[\text{M} + \text{Na}]^+$ ion at m/z 539.2617. The UV spectrum of **3** showed absorption bands at λ_{max} 227 and 256 nm, which were assigned to an extended conjugation system. The IR absorptions at 3374 and 1733 cm^{-1} indicated the presence of hydroxy (OH) and ester carbonyl ($\text{C}=\text{O}$) groups, respectively. 1D and 2D NMR spectra of **3** were identical to those of 7-*O*-succinyl macrolactin A,¹⁷ except for the presence of an additional methoxy group, which was located at C-4' (Figure 1) by an HMBC correlation from the methoxy group protons (δ_{H} 3.66) to C-4' (δ_{C} 174.5). The optical rotation value $[\alpha]_{\text{D}}^{25} -11$ (c 0.05, MeOH) of **3** was similar to macrolactin A.¹⁵ Hence, **3** was identified as 4'-*O*-methyl-7-*O*-succinyl macrolactin A.

Table 1 ^1H NMR data [δ_{H} , mult. (J in Hz)] (500 MHz) of 1–3, 5 and 6 in CD_3OD

C#	1	2	3	5	6
2	5.55 d (11.5)	5.54 d (11.5)	5.56 d (11.5)	2.48 m	5.88 d (15.2)
3	6.61 t (11.5)	6.61 t (11.5)	6.61 t (11.5)	4.27 m	7.60 dd (15.2, 11.0)
4	7.25 dd (15.5, 11.5)	7.29 dd (15.0, 11.5)	7.22 dd (15.2, 11.5)	1.55 m	6.27 t (11.0)
5	6.14 dt (15.5, 7.5)	6.14 dt (15.0, 7.5)	6.05 dt (15.2, 7.0)	4.06 m	5.96 dt (11.0, 8.5)
6	2.46 m	2.68 m	2.52 m	1.58 m	2.47 m
7	4.24 m	4.28 m	5.46 m	4.33 m	3.90 m
8	5.65 dd (15.5, 7.5)	5.70 dd (15.3, 5.5)	5.68 d (15.3, 5.5)	5.60 dd (15.3, 6.0)	1.59 m
9	6.53 dd (15.5, 11.0)	6.56 dd (15.3, 11.3)	6.57 dd (15.3, 11.2)	6.20 dd (15.3, 10.2)	4.31 m
10	6.06 t (11.0)	6.08 t (11.3)	6.09 t (11.2)	6.05 dd (15.0, 10.2)	5.58 dd (14.8, 6.0)
11	5.46 dt (11.0, 5.0)	5.54 dt (11.3, 6.5)	5.61 dt (11.2, 7.0)	5.68 dt (15.0, 7.0)	6.20 dd (14.8, 11.5)
12	2.36 m	2.32 m	2.59 m	2.10 m	6.06 dd (14.7, 11.5)
13	2.66 dt (14.5, 9.5)	2.45 m	2.66 m		
	4.01 m	3.89 m	3.80 m	1.46 m	5.67 dt (14.7, 7.5)
14	1.62 m	1.59 m	1.63 m	1.50 m	
	2.31 m	1.62 m	2.18 m	1.55 m	2.08 m
15	4.32 q (5.0)	4.39 m	4.33 m	3.72 m	1.42 m
16	4.24 t (5.0)	5.77 dd (15.3, 4.5)	5.56 dd (15.5, 6.5)	1.15 d (6.0)	1.50 m
17	5.73 (overlapped)	5.64 dd (15.3, 8.2)	6.19 dd (15.5, 10.7)		1.42 m
18	5.73 (overlapped)	3.54 dd (8.2, 3.8)	6.05 dd (15.5, 10.7)		3.70 m
19	4.06 m	3.61 m	5.70 m		1.13 d (6.0)
20	1.55 m	1.43 m	2.12 m		
21	1.48 m	1.45 m	2.18 m		
	1.56 m	2.21 m	1.50 m		
22	1.65 m	1.34 m	1.53 m		
		1.39 m	1.61 m		
23	4.93 m	4.95 m	1.65 m		
24	1.25 d (6.0)	1.25 d (6.5)	5.00 m		
1'			1.25 d (6.0)		
2'			2.68 m		
3'			2.62 m		
4'					
OCH ₃		3.28 s	3.66 s	3.68 s

Figure 2 Key ROESY correlations for 1–3, 5 and 6.**Structure elucidation of macrolactinic acid (4)**

By the comprehensive analysis of 1D and 2D NMR (^1H , ^{13}C , ^1H – ^1H COSY, HSQC, HMBC), UV, IR, ESIMS data (m/z 419 [$\text{M} - \text{H}$] $^-$), and optical rotation value [$[\alpha]_{\text{D}}^{23} -13$ (c 0.05, MeOH)], compound 4 was identified as macrolactinic acid.^{10,15}

Structure elucidation of linieodolide A (5)

The molecular formula of 5, $\text{C}_{17}\text{H}_{30}\text{O}_6$ with three degrees of unsaturation, was deduced from its HRESIMS, which showed an $[\text{M} + \text{Na}]^+$ ion at m/z 353.1935. The IR spectrum of 5 showed absorption bands at 1650 and 3317 cm^{-1} , indicating the presence of

an ester carbonyl and hydroxy groups, respectively. A conjugated diene system was suggested by an absorption band at 230 nm in the UV spectrum. The ^{13}C NMR spectrum (in CD_3OD) showed 17 resolved signals, which were classified as derived from one ester carbonyl (C-1), two methyl including one oxygenated, 4 sp^2 methine, four oxymethine and six methylene carbons. The ^1H NMR spectrum of 5 in CD_3OD showed methyl, olefinic, methylene and methine signals. The connectivities of all protons and carbon atoms were established by interpretation of ^1H – ^1H COSY, HSQC and HMBC NMR spectroscopic data (Tables 1 and 2; Figure 1). One proton sequence from H_2 -2 (δ_{H} 2.48, m) to H_3 -16 (δ_{H} 1.15, d, $J = 6.0\text{ Hz}$)

Figure 3 $\Delta\delta_H$ values ($\Delta\delta_H = \delta_S - \delta_R$) obtained for (S)- and (R)-MTPA esters of **1**, **2** and **5**.

Table 2 ^{13}C NMR data (δ_C) (125 MHz) of **1–3**, **5** and **6** in CD_3OD

C#	1	2	3	5	6
1	167.9	168.0	167.9	173.8	171.1
2	118.0	118.0	118.6	43.7	123.6
3	144.9	145.0	144.3	66.4	140.6
4	130.6	130.4	131.0	45.7	129.3
5	141.0	141.8	140.2	65.9	138.0
6	42.0	42.2	40.0	46.2	37.5
7	72.8	71.9	74.3	69.9	68.7
8	136.8	137.1	132.1	135.37	45.1
9	127.9	126.3	127.7	131.1	69.8
10	131.0	131.0	130.7	131.34	135.2
11	129.7	128.8	129.4	135.3	131.2
12	36.3	36.8	36.3	33.6	131.3
13	78.5	69.2 ^a	69.4	26.6	135.4
14	40.6	44.5	44.2	39.6	33.6
15	74.1	69.2 ^a	69.8	68.4	26.6
16	83.4	140.1	135.1	23.5	39.6
17	129.1	126.5	131.4		68.4
18	137.0	86.6	131.6		23.5
19	73.4	74.0	135.3		
20	37.9	33.1	33.0		
21	22.4	22.4	25.8		
22	36.8	36.7	36.1		
23	72.1	72.2	72.4		
24	20.5	20.1	20.1		
1'			173.3		
2'			29.7		
3'			30.2		
4'			174.5		
OCH ₃		56.8	52.3	54.4	

^aOverlapping signals.

was established by analysis of a ^1H – ^1H COSY spectral data. An HMBC cross-peak from the OCH₃ protons (δ_H 3.68, s) to C-1 supported the connectivity of OCH₃ group to a carbonyl carbon, C-1.

The disubstituted C-8/C-9 and C-10/C-11 double bonds of **5** were elucidated to have *E* configuration by the large vicinal coupling

constant values of H-8/H-9 ($J = 15.3$ Hz) and H-10/H-11 ($J = 15.0$ Hz). Using a modified Mosher's method,^{12–14} the absolute configuration of C-15 was determined as *R*¹⁸ on the basis of the diagnostic proton chemical shift values [$\Delta\delta_H = \delta_S - \delta_R$] between (S)- and (R)-MTPA esters of **5** (**5a** and **5b**) (Figure 3). There is no effective method to determine the absolute configurations of 1,3,5-triols system. The relative configuration of 3,5,7-triols system of **5** was assigned by considering the 1,3,5-triols moiety using Kishi's Universal NMR Database (Database 2 in CD_3OD).¹⁹ The 1,3,5-triols system was assigned as *anti/anti* between C-3/C-5 and C-5/C-7 on the basis of comparison of the δ_C value at C-5 with the characteristic δ_C value of the central carbon of the 1,3,5-triols model system (Figure 4). On the basis of all spectral data (1D and 2D), the structure of **5** was confidently established as (3*R**,5*R**,7*S**,8*E*, 10*E*, 15*R*)-methyl 3,5,7,15-tetrahydroxyhexadeca-8,10-dienoate and named lineodolide A.

Structure elucidation of lineodolide B (**6**)

The ESIMS of **6** in the positive and negative modes showed quasimolecular ions at m/z 323 $[\text{M}-\text{H}]^-$ and 347 $[\text{M}+\text{Na}]^+$, respectively, consistent with the molecular formula, $\text{C}_{18}\text{H}_{28}\text{O}_5$, suggesting five degrees of unsaturation. This was confirmed by its HRESIMS, which showed an $[\text{M}+\text{Na}]^+$ ion at m/z 347.1826. Its UV and IR spectra were similar to **5** indicating the presence of conjugated olefin moiety, hydroxy and carbonyl groups. Correlations in a ^1H – ^1H COSY spectrum allowed the assignment of a single-spin system from H-2 to H₃-18 (Figure 1). The location of a carbonyl carbon was established at C-1 (δ_C 172.1) by the HMBC cross peaks between H-2 and C-1, and H-3 and C-1 (Figure 1). The relative configurations of double bonds at C-2, C-4, C-10 and C-12 corroborated as *E*, *Z*, *E* and *E*, respectively, based on their respective proton coupling constants: 15.2, 11.0, 14.8 and 14.7 Hz, and ROEs between H-2 and H-4, H-10 and H-12, and H-11 and H-13 (Table 1 and Figure 2). Data were not obtained to determine the absolute configurations of **6** due to the limited amount of sample. The relative configuration of C-7 and C-9 stereogenic centres of **6** was addressed by considering the 1,3-diols moiety using Kishi's Universal NMR Database (Database 5 in CD_3OD).¹⁹ The ^{13}C NMR chemical shift values of C-7/C-9 were in good agreement with *anti* arrangement of the 1,3-diols model system (Figure 4). The resonances of H-17 (δ_H 3.70, m) and C-17 (δ_C 68.4)

Figure 4 Assignment of the relative configuration of lineiodolides A (**5**) and B (**6**) based on Kishi's Universal NMR Database (Database 2 and 5 in CD₃OD). $\Delta\delta$ values between the model system and **5** and **6** are shown. The relative configuration shown is based on the best fit with the model system.

were identical to H-15 (δ_H 3.72, m) and C-15 (δ_C 68.4) of **5**, respectively, and both might be produced by the same biosynthetic pathway. Therefore, it might be assumed that the absolute configuration of C-17 was *R*. By the extensive analysis of spectroscopic data, the structure of **6** was established as (2*E*,4*Z*,7*S**,9*R**,10*E*,12*E*,17*R*)-7,9,17-trihydroxyoctadeca-2,4,10,12-tetraenoic acid (lineiodolide B).

Antimicrobial activity of 1–6

The minimal inhibitory concentrations (MICs) of compounds **1–6** against three pathogenic microorganisms are shown in Table 3. Compounds **1–3**, **5** and **6** showed a meaningful antimicrobial activity against tested pathogenic microbial strains. Moreover, macrolactinic acid (**4**) exhibited potent antibacterial activity.

DISCUSSION

Bacillus species are ubiquitous and diverse in marine ecosystems. Marine *Bacillus* species are well known for producing antimicrobial macrolactin A, a 24-membered macrolactone, and their derivatives.²⁰ Macrolactin A showed potent antibacterial activity against *Staphylococcus aureus* and *Bacillus subtilis* in standard 'disc diffusion assay' at a concentration of 5 and 20 μ g per disc,¹⁰ respectively. The position of hydroxy groups in macrolactone ring is important for antimicrobial activity of macrolactins. The hydroxy group at C-15 in macrolactone ring increases antimicrobial activity of macrolactins, whereas introduction of carbonyl group at C-15 decreases antimicrobial activity.²¹ The hydroxy group at C-7 and C-9, and the number of ring members have no effect on the antibacterial activity of macrolactins.²¹ Macrolactins exhibit antibacterial activity by inhibiting peptide deformylase in a dose-dependent manners.²² New macrolactins X–Z (**1–3**) showed antimicrobial activity

Table 3 MICs of **1–6**

Compounds	MICs (μ g ml ⁻¹)		
	<i>Bacillus subtilis</i>	<i>Escherichia coli</i>	<i>Saccharomyces cerevisiae</i>
1	16	16	64
2	16	32	64
3	16	32	64
4	8	8	32
5	64	64	128
6	64	32	128
Azithromycin (positive control)	4	4	—
Amphotericin B (positive control)	—	—	2

—, not tested.

comparable to the literature values, as there were no major structural differences among new macrolactins and published macrolactins.²⁰ By comparing the antibacterial activity among different fatty acids, it has been shown that unsaturated and hydroxy fatty acids (**4–6**) showed better antibacterial activity compared with saturated fatty acids.^{23,24} Although there are several reports regarding the mode of action of long-chain unsaturated fatty acids, the precise mechanism for the antimicrobial activity is not clearly understood. Lately, it was suggested that the antimicrobial activity of unsaturated fatty acids is related to the inhibition of bacterial fatty acid synthesis.²⁵ Interestingly, compounds **1–6** were produced by this *Bacillus* sp. only in low salinity (12 g l⁻¹), but not in high salinity (32 g l⁻¹) culture medium.

CONCLUSION

In conclusion, five new (**1–3**, **5** and **6**) and one known (**4**) antimicrobial compounds have been discovered through repeated chromatographic steps of the EtOAc extract obtained from the low-salinity mass culture broth of a marine *Bacillus* sp. It has been shown that the salinity of the culture medium had a profound effect on new antimicrobial compounds production by this *Bacillus* sp. All the compounds showed antimicrobial activity against tested pathogenic microorganisms. However, continued investigation of related compounds coupled with structural modification studies could be helpful to develop and optimize lead antimicrobial agents.

METHODS

General experiments, microorganism, fermentation and extraction

General experiments, isolation of producing strain, large-scale fermentation and extraction were done according to the previously described procedures.⁷

Purification

The residual suspension (200 g) was subjected to 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 fraction eluted with MeOH–H₂O (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 EtOAc–MeOH (v/v) (4:1) was subjected to further fractionations by semipreparative ODS HPLC (H₂O–MeOH–MeCN: 3:1:1, flow rate: 1.5 ml min⁻¹, Detector-UV) to obtain 11 fractions (Fr.3–Fr.11). Compounds **1–6** (Figure 5) were purified on silica HPLC from Fr.3, Fr.4, Fr.11, Fr.4, Fr.1 and Fr.1, respectively (Supplementary information).

Figure 5 Chemical structures of antimicrobial compounds **1–6**.

Physicochemical Properties

Macrolactin X (1). Amorphous solid; $[\alpha]_D^{25}$ -60 (c 0.05, MeOH); UV (MeOH) $\lambda_{\max}$ (log ϵ) 200 (4.12), 232 (4.30) and 261 (4.09) nm; IR (MeOH) $\nu_{\max}$ 3482 (br), 2922, 1691, 1250, 1195, 1038 cm^{-1} ; ^1H and ^{13}C NMR data (CD_3OD) (Tables 1 and 2); HRESIMS m/z 441.2249 $[\text{M} + \text{Na}]^+$ (calcd for $\text{C}_{24}\text{H}_{34}\text{O}_6\text{Na}$, 441.2253).

Macrolactin Y (2). Amorphous solid; $[\alpha]_D^{25}$ -84 (c 0.05, MeOH); UV (MeOH) $\lambda_{\max}$ (log ϵ) 200 (4.14), 234 (4.45) and 261 (4.25) nm; IR (MeOH) $\nu_{\max}$ 3311 (br), 2921, 1646, 1409, 1104, 1024 cm^{-1} ; ^1H and ^{13}C NMR data (CD_3OD) (Tables 1 and 2); HRESIMS m/z 473.2511 $[\text{M} + \text{Na}]^+$ (calcd for $\text{C}_{25}\text{H}_{38}\text{O}_7\text{Na}$, 473.2515).

Macrolactin Z (3). White, amorphous solid; $[\alpha]_D^{25}$ -11 (c 0.05, MeOH); UV (MeOH) $\lambda_{\max}$ (log ϵ) 200 (3.90), 227 (4.18) and 256 (3.83) nm; IR (MeOH) $\nu_{\max}$ 3374 (br), 2929, 1733, 1169, 990 cm^{-1} ; ^1H and ^{13}C NMR data (CD_3OD) (Tables 1 and 2); HRESIMS m/z 539.2617 $[\text{M} + \text{Na}]^+$ (calcd for $\text{C}_{29}\text{H}_{40}\text{O}_8\text{Na}$, 539.2621).

Linieodolide A (5). White, amorphous solid; $[\alpha]_D^{25}$ -34 (c 0.05, MeOH); UV (MeOH) $\lambda_{\max}$ (log ϵ) 201 (4.03), 230 (4.23) and 256 (3.28) nm; IR (MeOH) $\nu_{\max}$ 3317 (br), 2925, 1650, 1413, 1017 cm^{-1} ; ^1H and ^{13}C NMR data (CD_3OD) (Tables 1 and 2); HRESIMS m/z 353.1953 $[\text{M} + \text{Na}]^+$ (calcd for $\text{C}_{17}\text{H}_{30}\text{O}_6\text{Na}$, 353.1940).

Linieodolide B (6). White, amorphous solid; $[\alpha]_D^{25}$ -62 (c 0.05, MeOH); UV (MeOH) $\lambda_{\max}$ (log ϵ) 231 (4.26) and 259 (4.05) nm; IR (MeOH) $\nu_{\max}$ 3340 (br), 2928, 1623, 1456, 1039 cm^{-1} ; ^1H and ^{13}C NMR data (CD_3OD) (Tables 1 and 2); HRESIMS m/z 347.1826 $[\text{M} + \text{Na}]^+$ (calcd for $\text{C}_{18}\text{H}_{28}\text{O}_5\text{Na}$, 347.1834).

Tris-(S)-MTPA esters (1a) of 1. Compound **1** (0.8 mg) was dissolved in 150 μl pyridine and stirred at room temperature for 10 min. For preparation of the tris-(S)-MTPA esters (**1a**) of **1**, 20 μl (R)-(–)- α -methoxy- α -(trifluoromethyl)-phenylacetyl chloride (MTPA-Cl) was added to the reaction vial and the mixture was stirred at room temperature 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 CH_2Cl_2 as eluent to obtain **1a** (0.5 mg). All proton signals of the triesters derivative (**1a**) were assigned by a ^1H - ^1H COSY experiment. **1a**: Amorphous solid; ^1H NMR (CD_3OD) δ_{H} 5.52 (d, J = 11.5 Hz, H-2), 6.40 (t, J = 11.5 Hz, H-3), 6.84 (dd, J = 15.5, 11.5 Hz, H-4), 5.76 (dt, J = 15.5, 7.5 Hz, H-5), 2.48 (m, H-6), 5.55 (m, H-7), 5.66 (dd, J = 15.5, 7.5 Hz, H-8), 6.44 (dd, J = 15.5, 11.0 Hz, H-9), 5.98 (t, J = 11.0 Hz, H-10), 5.40 (dt, J = 11.0, 5.0 Hz, 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 Hz, 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-22), 4.50 (m, H-23), 1.20 (d, J = 6.0 Hz, H-24), 3.47 (OCH₃, s), 3.53 (OCH₃, s), 3.54 (OCH₃, s), 7.34~7.56 (15 H, m); ESIMS m/z 1089.90 $[\text{M} + \text{Na}]^+$.

Tris-(R)-MTPA esters (1b) of 1. In an entirely analogous way, the tris-(R)-MTPA esters (**1b**) was obtained using (S)-(+)- α -methoxy- α -(trifluoromethyl)-phenylacetyl chloride (MTPA-Cl). **1b** (0.4 mg): amorphous solid; ^1H NMR (CD_3OD) δ_{H} 5.54 (d, J = 11.5 Hz, H-2), 6.51 (t, J = 11.5 Hz, H-3), 6.97 (dd, J = 15.5, 11.5 Hz, H-4), 6.00 (dt, J = 15.5, 7.5 Hz, H-5), 2.54 (m, H-6), 5.66 (m, H-7), 5.50 (dd, J = 15.5, 7.5 Hz, H-8), 6.40 (dd, J = 15.5, 11.0 Hz, H-9), 5.92 (t, J = 11.0 Hz, H-10), 5.37 (dt, J = 11.0, 5.0 Hz, 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 = 5.0 Hz, 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-22), 4.77 (m, H-23), 1.88 (d, J = 6.0 Hz, H-24), 3.49 (OCH₃, s), 3.50 (OCH₃, s), 3.54 (OCH₃, s), 7.36~7.60 (15 H, m); ESIMS m/z 1089.82 $[\text{M} + \text{Na}]^+$.

Tetra-(S)- and (R)-MTPA esters (2a and 2b) of 2. Tetra-(S)- and (R)-MTPA ester derivatives of **2** were prepared from (R)-(–)- and (S)-(+)-MTPA-Cl, respectively, according to the procedure described above. All ^1H NMR signals were assigned by a ^1H - ^1H COSY experiment. **2a** (0.5 mg): white, amorphous solid; ^1H NMR (CD_3OD) δ_{H} 5.51 (d, J = 11.5 Hz, H-2), 6.46 (t, J = 11.5 Hz, H-3), 7.18 (dd, J = 15.0, 11.5 Hz, H-4), 5.82 (dt, J = 15.0, 7.5 Hz, H-5), 2.46 (m, H-6), 5.58 (m, H-7), 5.70 (dd, J = 15.3, 5.5 Hz, H-8), 6.46 (dd, J = 15.3, 11.3 Hz, H-9), 5.06 (t, J = 11.3 Hz, H-10), 5.25 (dt, J = 11.3, 6.5 Hz, H-11), 2.39 (m, H-12b), 2.48 (m, H-12a), 5.17 (m, H-13), 1.58 (m, H-14b), 1.83 (m, H-14a), 5.42 (m, H-15), 5.46 (dd, J = 15.3, 4.5 Hz, H-16), 5.49 (dd, J = 15.3, 4.5 Hz, H-17), 3.80 (dd, J = 8.2, 3.8 Hz, H-18), 5.18 (m, H-19), 1.48 (m, H-20b), 1.58 (m, H-20a), 2.23 (m, H-21), 1.35 (m, H-22b), 1.40 (m, H-22a), 5.00 (m, H-23), 1.16 (d, J = 6.5 Hz, H-24), 3.25 (OCH₃, s), 3.45 (OCH₃, s), 3.48 (OCH₃, s), 3.50 (OCH₃, s), 3.54 (OCH₃, s), 7.32~7.62 (20 H, m); ESIMS m/z 1338.51 $[\text{M} + \text{Na}]^+$. **2b** (0.45 mg): white, amorphous solid; ^1H NMR (CD_3OD) δ_{H} 5.56 (d, J = 11.5 Hz, H-2), 6.55 (t, J = 11.5 Hz, H-3), 7.30 (dd, J = 15.0, 11.5 Hz, H-4), 6.04 (dt, J = 15.0, 7.5 Hz, H-5), 2.64 (m, H-6), 5.68 (m, H-7), 5.68 (dd, J = 15.3, 5.5 Hz, H-8), 6.36 (dd, J = 15.3, 11.3 Hz, H-9), 6.05 (t, J = 11.3 Hz, H-10), 5.23 (dt, J = 11.3, 6.5 Hz, H-11), 2.30 (m, H-12b), 2.36 (m, H-12a), 4.98 (m, H-13), 1.56 (m, H-14b), 1.68 (m, H-14a), 5.23 (m, H-15), 5.58 (dd, J = 15.3, 4.5 Hz, H-16), 5.44 (dd, J = 15.3, 4.5 Hz, H-17), 3.71 (dd, J = 8.2, 3.8 Hz, H-18), 5.21 (m, H-19), 1.73 (m, H-20b), 1.60 (m, H-20a), 2.29 (m, H-21), 1.53 (m, H-22b), 1.63 (m, H-22a), 5.95 (m, H-23), 1.18 (d, J = 6.5 Hz, H-24), 3.20 (OCH₃, s), 3.47 (OCH₃, s), 3.52 (OCH₃, s), 3.54 (OCH₃, s), 3.55 (OCH₃, s), 7.35~7.60 (20 H, m); ESIMS m/z 1338.40 $[\text{M} + \text{Na}]^+$.

Tetra-(S)- and (R)-MTPA esters (5a and 5b) of 5. Tetra-(S)- and (R)-MTPA esters of **5** were prepared from (R)-(–)- and (S)-(+)-MTPA-Cl by an

analogous procedure described above. **5a** (0.2 mg): white, amorphous solid; ^1H NMR (CD_3OD) (key resonances) δ_{H} 2.25 (m, H_2 -12), 1.48 (m, H_2 -13), 1.58 (m, H_2 -14), 0.95 (d, $J = 6.5$ Hz, H_3 -16); ESIMS m/z 1217.34 $[\text{M} + \text{Na}]^+$. **5b** (0.2 mg): white, amorphous solid; ^1H NMR (CD_3OD) (key resonances) δ_{H} 2.23 (m, H_2 -12), 1.42 (m, H_2 -13), 1.28 (m, H_2 -14), 0.90 (d, $J = 6.5$ Hz, H_3 -16); ESIMS m/z 1217.35 $[\text{M} + \text{Na}]^+$.

Antimicrobial assays

The MICs of compounds **1–6** were determined by using a conventional broth dilution assay.²⁶ Compounds **1–6** 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 (beef extract 0.3%, peptone 0.5% and pH 7.2 before sterilization) and yeast maltose broth (dextrose 1%, beef extract 0.3%, peptone 0.5%, malt extract 0.3% and pH 7.2 before sterilization), respectively. A serial two-fold dilution of each compound was prepared in 96-well microtiter plates over the range of 0.5–256 $\mu\text{g ml}^{-1}$. An overnight broth culture of each strain was prepared, and the final concentration of microorganisms in each culture was adjusted to 1.5×10^8 c.f.u. ml^{-1} by comparing the culture turbidity with the 0.5 McFarland standard. Culture broth (30 μl) was added to each dilution of compounds **1–6**, the final volume of each well was adjusted to 200 μl using the respective culture medium, and the plates were incubated for 24 h at 37 °C for bacteria and 48 h at 30 °C for the yeast.^{27,28} 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.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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