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NOTE

Antibacterial and antilarval compounds from marine gorgonian-associated bacterium *Bacillus amyloliquefaciens* SCSIO 00856

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It is well understood that corals harbor diverse microbial communities. Recently, many coral-associated bacteria have been characterized as sources of marine natural products, especially as the coral surface is more nutrient rich than seawater or even sediments.[1,2] Colonization of coral surfaces by bacteria and other microorganisms is mostly nondestructive to corals. Owing to the close spatial vicinity of these biofilm-forming bacteria, it can be expected that the indigenous microbial population is adapted to competitive conditions.[3] The production of secondary metabolites is a common adaptation of these bacteria to compete in such microenvironments. There have been few reports documented on exploration of coral-associated bacteria in the South China Sea as a source of secondary metabolites.

In order to obtain biologically active natural products from coral-associated bacteria, we studied the marine bacterium *Bacillus amyloliquefaciens* SCSIO 00856 isolated from the South China Sea gorgonian *Junceella juncea*. In the preliminary experiments, we found that the culture broth of the strain had strong antibacterial activity towards three bacteria (*Escherichia coli*, *Bacillus subtilis*, and *Staphylococcus aureus*) and antilarval activity towards laboratory-reared bryozoan *Bugula neritina* larvae. In this study, a new 24-membered ring lactone, macrolactin V (**1**) (Figure 1), together with four known compounds, macrolactin S (**2**),[4] 4-butoxyphenol (**3**),[5] 4-propoxyphenol (**4**),[6] and 4-ethoxyphenol (**5**),[6] was isolated from a culture broth of the strain. Macrolactins are polyene macrolides containing a 24-membered lactone ring. About 20 macrolactins have been chemically characterized, including macrolactins A-U, 7-*O*-succinyl macrolactin A, and 7-*O*-succinyl macrolactin F, from marine *Bacillus* sp. sediment isolate, and 7-*O*-malonyl macrolactin A from *Bacillus subtilis* soil isolate.[4,7–13] Some of these compounds showed strong antibacterial, anti-tumor, and antivirus activities.[4,7,9–13] The antibacterial activity of **1–5** against four bacterial strains and the antilarval activity of **3–5** against larval settlement of *Bugula neritina* and *Balanus amphitrite* larvae were evaluated. This paper deals with the isolation, structural elucidation of **1** and biological activity of **1–5**.

The producing strain, *Bacillus amyloliquefaciens* SCSIO 00856, was isolated from the gorgonian coral *Junceella juncea* in Sanya, Hainan province, China. The strain culture (2 ml) was used to inoculate 50 ml seed medium consisting of 0.4% yeast extract, 0.4% glucose, 0.5% malt meal, and 1.8% sea salt (pH 7.3), in a 250-ml flask. A volume of 20 ml of the resulting culture was used to inoculate 400 ml of the seed medium, in a 1-l flask. The culture was incubated at 27 °C on a rotary shaker operating at 150 r.p.m. for 5 days. The resulting 400-ml culture was used to inoculate 120 l of production medium, in a 200-l fermenter, containing the following components per liter of water: yeast extract 4 g, malt meal 5 g, glucose 4 g, and sea salt 18 g. The production fermentation tanks were operated at 27 °C, a back-pressure of 0.1 MPa, and an agitation rate of 150 r.p.m. pH was controlled at 7.3 by addition of NaOH. The fermenter was operated for 10 days, at which time the culture was harvested for isolation of metabolites. The culture broth (120 l) was extracted with EtOAc. The EtOAc extract was concentrated *in vacuo* to afford 13.9 g of residue, which was subjected to column chromatography (CC) on silica gel, using $CHCl_3$-MeOH (from 10:0 to 0:10) as eluent. Elution of CC with 8% MeOH in $CHCl_3$ gave a fraction of 835 mg. The fraction was subsequently subjected to CC on silica gel, eluted with $CHCl_3$-MeOH, then purified with semi-preparative HPLC (Luna C18(2), 250×10 mm i.d., 5 ml min^{-1}), using MeOH-water (65:35) as eluent to yield **1** (2.6 mg) and **2** (19.7 mg). Elution of the first CC with 20% MeOH in $CHCl_3$ gave a 342-mg fraction, which was rechromatographed on a silica gel column using a gradient elution of $CHCl_3$-MeOH to yield **3** (60 mg). The fraction (163 mg) eluted with 35% MeOH in $CHCl_3$ at the first CC was repeatedly subjected to silica gel column using a gradient elution of $CHCl_3$-MeOH to yield **4** (7.5 mg) and **5** (7.0 mg). The structures

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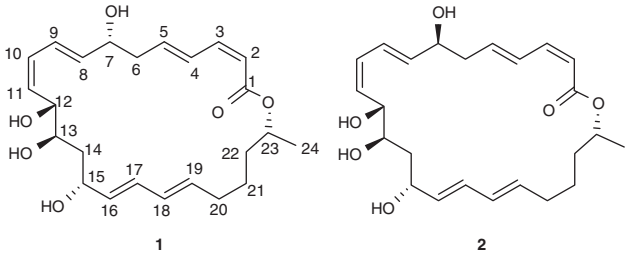

1 **2**

Figure 1 Structures of **1** and **2**.

Table 1 ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR data of 1 and 2 in CD_3OD

	1		**2**	
Position	δ_H, *mult (J in Hz)*	δ_C	δ_H, *mult (J in Hz)*	δ_C
1		167.8 C		167.8 C
2	5.58 d (12.0)	118.4 CH	5.58 d (12.0)	117.9 CH
3	6.66 dd (11.5, 11.5)	145.0 CH	6.66 dd (11.5, 11.5)	145.4 CH
4	7.25 dd (11.5, 15)	130.4CH	7.25 dd (11.5, 15)	129.9 CH
5	6.13 m	140.3 CH	6.13 m	142.1 CH
6	2.57 m	39.7 CH_2	2.47 m	42.6 CH_2
7	5.38 m	75.7 CH	4.18 m	73.6 CH
8	5.75 dd (7.5, 15.0)	138.5 CH	5.75 dd (7.5, 15.0)	138.8 CH
9	6.60 dd (11.5, 15.0)	130.0 CH	6.60 dd (11.5, 15.0)	127.4 CH
10	6.15 m	130.6 CH	6.15 m	130.6 CH
11	5.57 m	132.8 CH	5.57 m	131.5 CH
12	4.51 m	72.4 CH	4.51 m	72.4 CH
13	4.03 m	72.9 CH	4.03 m	73.0 CH
14	2.60 m	39.7 CH_2	2.60 m	39.7 CH_2
15	4.31 m	69.0 CH	4.31 m	69.0 CH
16	5.62 m	135.5 CH	5.62 m	135.5 CH
17	6.10 dd (10.5,15.0)	130.7 CH	6.10 dd (10.5, 15.0)	130.9 CH
18	5.57 dd (10.5,15.0)	131.8 CH	5.57 dd (10.5, 15.0)	131.8CH
19	5.56 m	134.8 CH	5.56 m	134.8 CH
20	2.09 m	32.7 CH_2	2.09 m	32.7 CH_2
	2.24 m		2.24 m	
21	1.53 m	25.5 CH_2	1.53 m	25.4 CH_2
22	1.56 m	36.0 CH_2	1.56 m	36.0 CH_2
	1.65 m		1.65 m	
23	5.08 m	71.7 CH	5.08 m	71.6 CH
24	1.25 (d, 6.0)	20.2 CH_3	1.25 (d, 6.0)	20.2 CH_3

of **3–5** were established by analyzing their 1D NMR spectral data and by comparison with the literature.[5,6]

Compound **1** had the molecular formula of $C_{24}H_{34}O_6$ as deduced from NMR spectra and HR-ESI-MS. The ^{1}H NMR spectrum showed signals for one methyl group at δ_H 1.25 (3H, d, J=6.0 Hz), 12 olefinic protons and five oxymethine protons at the chemical shift downfield of δ_H 4.03–7.25 (Table 1). Its ^{13}C NMR spectrum showed the presence of one methyl, five methylenes, six 1,2-disubstituted double bonds, five oxymethines, and a carbonyl group (δ_C 167.8) (Table 1). These ^{1}H and ^{13}C NMR data of **1** were very similar to those of macrolactin S (**2**)[4] (Table 1). Extensive analyses of the ^{1}H NMR, ^{13}C NMR, HSQC, HMBC (Figure 2), and ^{1}H–^{1}H COSY spectra of **1** inferred that **1** should have the same chemical planar structure as **2.** In the ^{1}H NMR spectrum of **1**, the ^{1}H coupling constants of H-2/H-3 (J=11.5 Hz), H-4/H-5 (J=15.0 Hz), H-8/H-9 (J=15.0 Hz), H-10/H-11 (J=10.0 Hz),

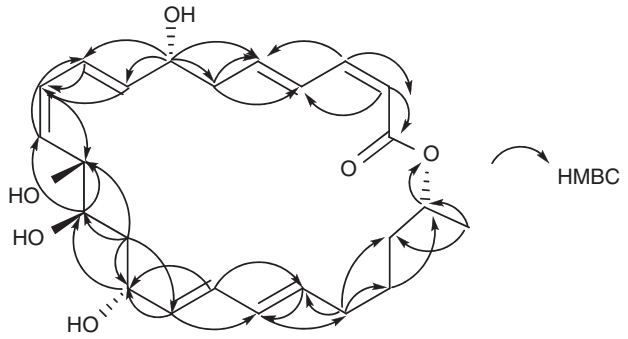

Figure 2 Key HMBC correlations of **1**.

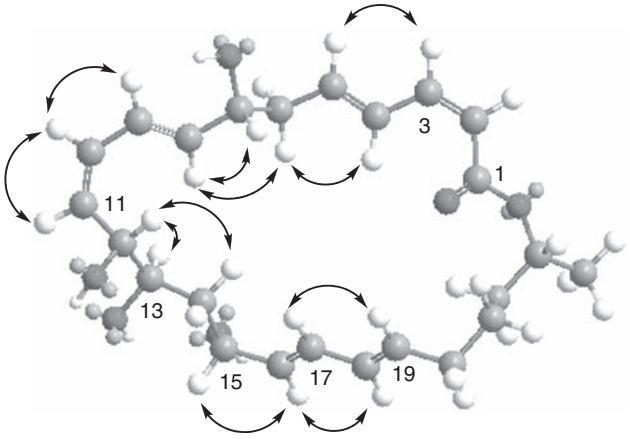

Figure 3 Key NOESY correlations of **1**.

H-16/H-17 (J=15.0 Hz), and H-18/H-19 (J=15.0 Hz) indicated that geometric configurations of **1** were 2*Z*,4*E*,8*E*,10*Z*,16*E*,18*E*. The ^{1}H and ^{13}C NMR data of H-12/C-12, H-13/C-13, H-15/C-15, and H-23/C-23 in **1** were almost the same as those in **2**, which suggested that the stereochemistry of chiral carbons C-12, C-13, C-15, and C-23 was the same as that in **2**.

However, compared with the ^{1}H and ^{13}C NMR data of **2**, the downfield shift of H-6 (from δ_H 2.47 in 2 to δ_H 2.57 in 1), H-7 (from δ_H 4.18 to δ_H 5.38), C-7 (from δ_C 73.6 to δ_C 75.7), and C-9 (from δ_C 127.4 to δ_C 130.0), and upfield shift of C-5 (from δ_C 142.1 to δ_C 140.3) and C-6 (from δ_C 42.6 to δ_C 39.7) were obviously observed in the NMR spectra of **1**, which should be caused by the change of the three-dimensional effect at C-7 position, and suggested that the configuration of −OH at C-7 in **1** should be α-configuration instead of β-configuration as it existed in **2** and other previously isolated macrolactins.[4,7–13] The suggestion was supported by the NOESY (Figure 3), in which NOE correlations of H-6β with H-8/H-4 and H-7 with H-8 were observed, whereas no NOE correlations between H-7 and H-9/H-5 were observed, which were in agreement with the MM2 optimized conformation of **1**. However, NOE correlations of H-7 with H-9/H-5 were obviously observed in the NOESY of **2**.[4] Thus, the structure of **1** was determined as shown and named macrolactin V. The optical rotation value of **1** is −0.08 (c 0.11, MeOH), which is different from that of **2** (−0.62 (c 0.15, MeOH)).[4]

The antibacterial activity of compounds **1–5** was measured against *E. coli*, *Bacillus thuringiensis*, *Bacillus subtilis*, and *S. aureus* using standard disc diffusion assay.[14] The MICs of compound **1–5** were

determined by a dilution method.[15] The results showed that macrolactin V (**1**) had strong antibacterial activity against *E. coli*, *Bacillus subtilis*, and *S. aureus*, with an MIC value of 0.1 μg ml^{-1}, and no activity against *Bacillus thuringiensis*, and macrolactin S (**2**) showed strong antibacterial activity against *E. coli* and *S. aureus* with MIC values of 0.3 and 0.1 μg ml^{-1}, respectively, but weak activity against *Bacillus subtilis* (MIC 100 μg ml^{-1}), which indicated that the configuration of OH-7 could affect the antibacterial activity of the epimers **1** and **2**. Compounds **3–5** showed no or weak inhibition towards all tested bacteria.

The antilarval activity of compounds **3–5** was evaluated in settlement inhibition assays with laboratory-reared *Bugula neritina* (Bryozoa) and *Balanus amphitrite* (Cirripedia) larvae. The procedures were the same as previously reported.[16] Larval settlement bioassays revealed that **3–5** showed potent antilarval activity towards *Bacillus amphitrite* larvae with EC_{50} values of 23.0, 22.9, and 24.1 μg ml^{-1}, respectively. However, only **3** could inhibit *Bacillus neritina* larvae settlement at the concentration of 50 μg ml^{-1}, but no activity at 25 μg ml^{-1}, and **4** and **5** were inactive even at 50 μg ml^{-1}. The EC_{50} values of **3–5** against *Bacillus amphitrite* larvae settlement were lower than the standard requirement of an EC_{50} of 25 μg ml^{-1} established by the US Navy program as an efficacy level for natural antifoulants, indicating that **3–5** are potential selective natural antifouling agents. This was the first time to report **3–5** from culture broths of bacteria and their antilarval activities against marine invertebrate larvae.

EXPERIMENTAL PROCEDURE

Taxonomy

Blast search results at EzTaxon.org Server showed that the 16S rRNA gene sequence of the strain SCSIO 00856 has the highest similarity (99.21%) with *Bacillus amyloliquefaciens* ATCC 23350^{T}. On the basis of the morphological features and the 16S rRNA gene sequence, the strain SCSIO 00856 was identified as *Bacillus amyloliquefaciens* and named *Bacillus amyloliquefaciens* SCSIO 00856. It was deposited at the Guangdong Key Laboratory of Marine Materia Medica, South China Sea Institute of Oceanology, The Chinese Academy of Sciences, China (Accession No. SCSIO 00856).

Physico-chemical properties

Macrolactin V: yellow oil; $[\alpha]_D^{20}$ −0.08° (c 0.11, MeOH), UV (MeOH) λ_{max} (log ε) 229 (4.57), 261 (4.18) nm; IR (KBr): 3295, 1700, 1667 cm^{-1}; for ^{1}H and ^{13}C NMR data, see Table 1; HR-ESI-MS *m/z*: $[M+H]^+$ 419.2357 (calcd. for $C_{24}H_{35}O_6$: 419.2355).

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