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# A New 24-membered Lactone and a New Polyene δ-Lactone from the Marine Bacterium *Bacillus marinus*

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**Abstract** A new 24-membered macrolide macrolactin T (**1**), and a new polyene δ-lactone macrolactin U (**2**), along with macrolactins A, B, D, O, and S, were isolated from the cultured broth of the bacterium *Bacillus marinus*, which was isolated from *Suaeda salsa* collected in the coastline of Bohai Sea of China. The structures of **1** and **2** were elucidated on the basis of extensive spectroscopic data analyses. The inhibitory activity of macrolactins T, B and D against fungi *Pyricularia oryzae* and *Alternaria solani*, and bacteria *Staphylococcus aureus* is reported.

**Keywords** *Bacillus marinus*, macrolactin T, macrolactin U, structural elucidation, antibiotic activity

## Introduction

The macrolactins represent a group of unique structural patterns containing a 24-membered ring lactone, which were mainly isolated from the bacterial strains *Bacillus* and *Actinomadura* [1, 2]. Thus far nineteen macrolactins (macrolactins A~S) have been reported, of which macrolactins A~F were found from unidentified deep sea bacterium [3]. Macrolactins possess a wide range pharmacological activities, *e.g.* macrolactin A showed significant antiviral activity toward *Herpes simplex* virus (types I and II), potential utility in controlling human HIV replication, and inhibition against B16~F10 murine melanoma cell replication [3~6]. In the continuation of our interest in the bioactive metabolites from marine microorganisms, the bacterium *Bacillus marinus* was isolated from *Suaeda salsa* collected in the coastline of Bohai Sea of Eastern China. A chemical investigation of the fermented broth resulted in the isolation of seven secondary metabolites including two new compounds, macrolactin T (**1**) and macrolactin U (**2**). This paper reports the structural determination of the new metabolites and their primary antibiotic results.

## Results and Discussion

On the basis of a bioassay guided fermentation of *B. marinus*, the cultured broth possessed inhibitory activities against fungus *Alternaria solani* and bacterium *Staphylococcus aureus*. Analyses of $^{1}$H-NMR spectroscopic data and HPLC chromatographic spectra implied that the EtOAc extract of the cultured broth contained a group of polyene components. Repeated column chromatography of the EtOAc fraction led to the isolation of six 24-membered macrolactins and a polyene lipid. The known compounds were determined as macrolactins A, B, D, O, and S, based on their spectroscopic data analysis and comparison with those

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**Table 1** Comparison of $^{1}$H- and $^{13}$C-NMR data between **1** and macrolactin A in DMSO-$d_6$

| No. | **1** | | Macrolactin A | |
|---|---|---|---|---|
| | $\delta_H$ | $\delta_C$ | $\delta_H$ | $\delta_C$ |
| 1 | | 166.8 (s) | | 166.3 (s) |
| 2 | 5.51 (d, *J*=11.5) | 117.9 (d) | 5.55 (d, *J*=11.5) | 117.4 (d) |
| 3 | 6.65 (dd, *J*=11.5, 11.5) | 144.4 (d) | 6.66 (dd, *J*=11.5, 11.5) | 144.2 (d) |
| 4 | 7.00 (dd, *J*=15.0, 11.5) | 129.3 (d) | 7.04 (dd, *J*=15.5, 11.5) | 129.1 (d) |
| 5 | 6.17 (ddd, *J*=15.0, 7.5, 7.5) | 143.4 (d) | 6.17 (ddd, *J*=15.5, 7.5, 7.5) | 143.1 (d) |
| 6 | 2.25 (m) | 42.8 (t) | 2.27 (m) | 42.5 (t) |
| | 2.33 (m) | | 2.33 (m) | |
| 7 | 4.18 (ddd, *J*=8.5, 5.0, 3.0) | 70.5 (d) | 4.18 (ddd, *J*=8.5, 5.0, 3.0) | 70.2 (d) |
| 8 | 5.72 (dd, *J*=15.0, 5.0) | 138.5 (d) | 5.73 (dd, *J*=15.0, 5.5) | 138.1 (d) |
| 9 | 6.48 (dd, *J*=15.0, 11.5) | 124.7 (d) | 6.47 (dd, *J*=15.0, 11.5) | 124.3 (d) |
| 10 | 6.02 (dd, *J*=10.0, 11.5) | 130.6 (d) | 6.03 (dd, *J*=10.0, 11.5) | 130.3 (d) |
| 11 | 5.47 (dd, *J*=10.0, 8.0) | 128.8 (d) | 5.49 (dd, *J*=10.0, 8.0) | 128.5 (d) |
| 12 | 2.12 (m) | 36.5 (t) | 2.14 (m) | 36.1 (t) |
| | 2.40 (m) | | 2.37 (m) | |
| 13 | 3.57 (m) | 67.9 (d) | 3.62 (m) | 67.4 (d) |
| 14 | 1.41 (m) | 44.3 (t) | 1.39 (m) | 44.0 (t) |
| 15 | 4.11 (dt, *J*=6.5, 7.0) | 68.5 (d) | 4.12 (dt, *J*=6.5, 7.0) | 67.9 (d) |
| 16 | 5.56 (dd, *J*=14.5, 6.5) | 137.9 (d) | 5.55 (dd, *J*=14.5, 6.5) | 136.6 (d) |
| 17 | 6.05 (dd, *J*=10.5, 14.5) | 129.3 (d) | 6.01 (dd, *J*=10.5, 14.5) | 129.1 (d) |
| 18 | 6.07 (dd, *J*=14.5, 10.5) | 129.8 (d) | 6.07 (dd, *J*=14.5, 10.5) | 130.9 (d) |
| 19 | 5.59 (dd, *J*=14.5, 6.0) | 138.3 (d) | 5.60 (dt, *J*=14.5, 7.5) | 134.0 (d) |
| 20 | 3.98 (dt, *J*=6.0, 5.5) | 71.6 (d) | 2.06 (m) | 32.1 (t) |
| 21 | 1.47 (m) | 34.2 (t) | 1.41 (m) | 24.8 (t) |
| 22 | 1.48 (m) | 32.6 (t) | 1.51 (m) | 35.1 (t) |
| | 1.57 (m) | | | |
| 23 | 4.83 (m) | 72.3 (d) | 4.91 (m) | 71.0 (d) |
| 24 | 1.19 (d, *J*=6.0) | 20.9 (q) | 1.20 (d, *J*=6.0) | 20.3 (q) |

reported in literature [3, 6, 7].

Macrolactin T (**1**) had a molecular formula of $C_{24}H_{34}O_6$ as determined by HRFAB-MS (*m/z* 419.2419 $[M+H]^+$, calcd. 419.2428), implying eight degrees of unsaturation. The UV bands at 236, 262 nm, and IR absorptions at 3378, 1693, 1638, and 1581 $cm^{-1}$ suggested the presence of hydroxyl, carbonyl and olefinic groups. The $^{1}$H- and $^{13}$C-NMR data (Table 1) of **1** were closely related to those of macrolactin A [3], indicating a congener of macrolactins. The $^{13}$C-NMR spectrum exhibited totally 24 carbon resonances including 12 vinyl carbons for six double bonds and a carbonyl carbon (Table 1), which accounted to seven sets of unsaturation. Thus, the structural pattern of **1** was composed with a monocyclic ring. The DQFCOSY correlations enabled to establish a lipid fragment starting from H-2 ($\delta$ 5.51, d, *J*=11.5 Hz) to the end of $H_3$-24 *via* a long chain as the case of macrolactin A. The difference between **1** and macrolactin A was found by the presence of an additional hydroxymethine at $\delta$ 3.98 (1H, dt, *J*=6.0, 5.5 Hz), which was assigned to C-20 ($\delta$ 71.6, d) based on the COSY and HMQC relationship. The position of lactone cyclization was determined by the downfield shift of H-23 ($\delta$ 4.83, m) which showed HMBC coupling to C-1 ($\delta$ 166.8, s) and COSY coupling to a methyl protons ($\delta$ 1.19, d, *J*=6.0 Hz). The geometric configurations of double bonds could be determined by the *J* values. The coupling constants of H-2 ($J_{2,3}$=11.5 Hz) and H-10 ($J_{10,11}$=10.0 Hz) were ascribed to 2*Z* and 10*Z*, while the *J* values of H-4 ($J_{4,5}$=15.0 Hz), H-8 ($J_{8,9}$=15.0 Hz), H-16 ($J_{16,17}$=14.5 Hz), and H-18 ($J_{18,19}$=14.5 Hz) were indicative of 4*E*, 8*E*, 16*E*, and 18*E*. Although NOE effects can be controversial in the assignment of the relative configuration of conformationally flexible macro-ring, the correlations observed for compound **1** could provide the evidence to support the relative configuration as depicted in Fig. 1. The ROESY correlations between H-7/H-9, H-7/H-5, H-20/H-

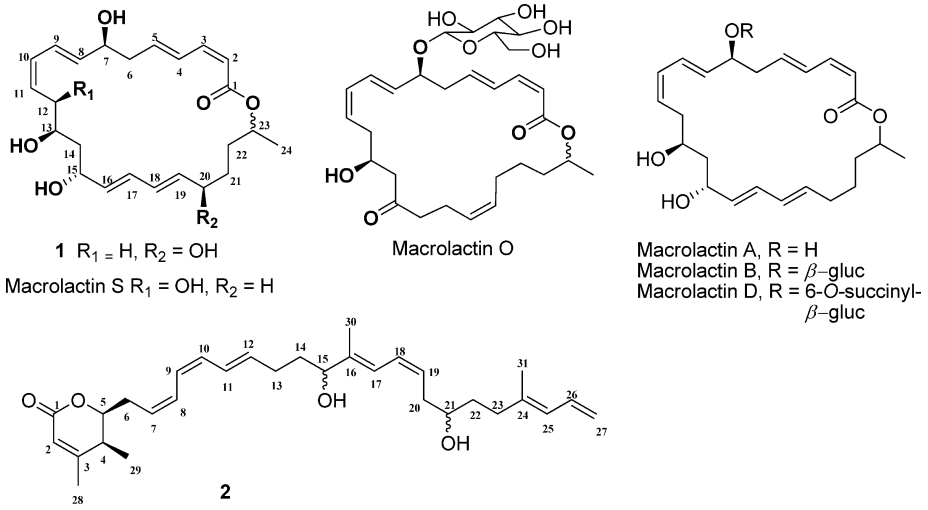



**Fig. 1** Structures of compounds **1**∼**2** and macrolactins A, B, D, O, and S.

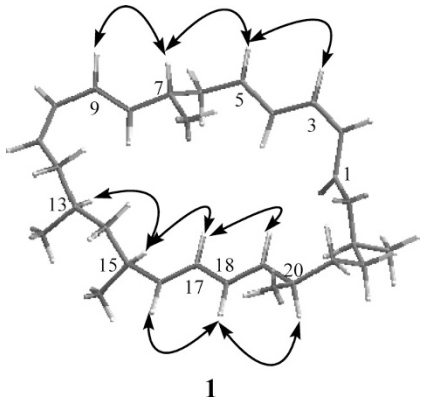



**Fig. 2** Key NOE correlations of **1**.

18, and a weak NOE between H-13/H-15 (Fig. 2), suggested OH-7$\beta$, OH-13$\beta$, OH-15$\alpha$, and OH-20$\beta$, which were in agreement with the $MM_2$ optimized conformation of macrocyclic nucleus. Since the stereogenic centers of macrolactin A and its C-7 glucosed derivative macrolactin B had been determined as 7*S*, 13*S*, 15*R*, and 23*R* by a combination of $^{13}$C-acetonide analysis, oxidative degradation, and chemical correlation [4], the close similarity of $^{1}$H- and $^{13}$C-NMR data (Table 1), *J* values, along with the NOE correlation and optical rotation of **1** which were compatible to those of macrolactin A indicated that **1** shared the same stereochemistry as the latter compound biogenetically. Accordingly, C-20 of **1** was suggested to be *R*. The structure of **1** was thus supposed to be 20*R*-hydroxymacrolactin A.

Compound **2** had a molecular formula of $C_{31}H_{44}O_4$ as determined by HREI-MS and NMR data, implying ten degrees of unsaturation. The IR absorptions at 3421, 1711, and 1643 cm$^{-1}$ suggested the presence of hydroxyl, carbonyl, and olefinic groups. The $^{13}$C-NMR and DEPT spectra exhibited totally 31 carbon resonances including 16 olefinic carbons (Table 2), a carbonyl carbon ($\delta$ 167.7, s), and four methyl resonances. The HMQC spectrum assigned all protons and their associated carbons. The $^{1}$H-$^{1}$H COSY correlations established three partial structures from C-4 to C-15, C-17 to C-23, and C-25 to C-27. The connection of each subunit was achieved by HMBC relationships. The HMBC cross peaks observed from the methyl protons at $\delta$ 1.69 (3H, s, Me-31) to C-23 ($\delta$ 36.1, t), C-24 (140.3, s), and C-25 ($\delta$ 125.8, d), along with the HMBC relationship of methyl protons at $\delta$ 1.72 (3H, s, Me-30) with C-15 ($\delta$ 67.7, d), C-16 ($\delta$ 141.5, s), and C-17 ($\delta$ 121.6, d), leading to the establishment of a full side chain, which possessed seven double bonds and two hydroxyl groups being positioned at C-15 and C-21, respectively. The remaining NMR resonances were attributed to an unsaturated $\delta$-lactone, which was evident from the HMBC cross peaks between H-2 ($\delta$ 5.74, s) and C-1 ($\delta$ 163.6), C-4 ($\delta$ 36.3, d), and C-28 ($\delta$ 21.7, q), in association with the correlations between H-5 ($\delta$ 4.26, m) and C-1, C-3 ($\delta$ 162.9, s), C-4, and C-7 ($\delta$ 127.1, d). Accordingly, the gross structure was established as a conjugated $\delta$-lactone, which was partially related to an antitumor agent kazusamycin A [8] with the difference at the side chain. The geometric configurations of the double bonds were determined on the basis of coupling constants and NOE relationships. The *J* values of $J_{7,8}$=11.0 Hz, and $J_{9,10}$=11.0 Hz, $J_{18,19}$=11.5 Hz were assigned to 7*Z*, 9*Z*, and 18*Z*, while the coupling constant $J_{11,12}$=15.0 Hz and the NOESY correlations between $H_3$-31 ($\delta$ 1.69, s)/H-26 ($\delta$ 6.54), $H_3$-30 ($\delta$ 1.72, s)/H-18 ($\delta$ 6.26) resulted in 11*E*, 16*E*, and 24*E*. The methyl group $H_3$-29 ($\delta$

**Table 2** $^{1}$H- and $^{13}$C-NMR data, HMBC, and NOESY correlations of **2** in DMSO-$d_6$

| No. | $\delta_H$ | $\delta_C$ | HMBC (H→C) | NOESY |
|---|---|---|---|---|
| 1 | | 163.6 (s) | | |
| 2 | 5.74 (s) | 115.6 (d) | C-1, C-28, C-4 | H-28 |
| 3 | | 162.9 (s) | | |
| 4 | 2.32 (m) | 36.3 (d) | C-2, C-28, C-3, C-29, C-5 | H-5 |
| 5 | 4.26 (m) | 82.1 (d) | C-1, C-3, C-29, C-6, C-7 | H-4 |
| 6 | 2.56 (m) | 31.4 (t) | C-5, C-7, C-8 | |
| 7 | 5.49 (dt, *J*=11.0, 7.5) | 127.1 (d) | C-5, C-6, C-9 | H-8 |
| 8 | 6.58 (dd, *J*=11.0, 12.5) | 126.9 (d) | C-6, C-10, C-9 | H-7, H-11 |
| 9 | 6.05 (dd, *J*=11.0, 12.5) | 122.5 (d) | C-7, C-11 | H-10 |
| 10 | 6.01 (dd, *J*=11.0, 11.0) | 131.1 (d) | C-8, C-12 | H-9, H-12 |
| 11 | 6.51 (dd, *J*=15.0, 11.0) | 126.4 (d) | C-9, C-13 | H-8, H-13 |
| 12 | 5.78 (dt, *J*=15.0, 7.5) | 137.4 (d) | C-10, C-13, C-14 | H-10 |
| 13 | 2.08 (m) | 29.4 (t) | C-11, C-12, C-14, C-15 | |
| 14 | 1.41 (m) | 35.2 (t) | C-12, C-15, C-16 | |
| | 1.60 (m) | | | |
| 15 | 4.53 (t, *J*=7.0) | 67.7 (d) | C-14, C-16, C-17, C-30 | |
| 16 | | 141.5 (s) | | |
| 17 | 6.07 (d, *J*=11.0) | 121.6 (d) | C-15, C-30, C-19 | H-15 |
| 18 | 6.26 (dd, *J*=11.0, 11.5) | 125.1 (d) | C-16, C-20 | H-30, H-19 |
| 19 | 5.39 (dt, *J*=11.5, 7.5) | 127.7 (d) | C-17, C-20, C-21 | H-18 |
| 20 | 2.24 (m) | 35.5 (t) | C-18, C-19, C-21 | |
| 21 | 3.43 (m) | 70.1 (d) | C-19, C-20, C-22 | |
| 22 | 1.41 (m) | 35.2 (t) | C-20, C-21, C-24 | |
| | 1.49 (m) | | | |
| 23 | 2.02 (m) | 36.1 (t) | C-21, C-31, C-24 | |
| | 2.14 (m) | | | |
| 24 | | 140.3 (s) | | |
| 25 | 5.81 (d, *J*=10.5) | 125.8 (d) | C-23, C-31, C-27 | H-23, H-27b |
| 26 | 6.54 (ddd, *J*=10.5, 10.0, 16.5) | 134.1 (d) | C-24, C-25 | H-31, H-27a |
| 27 | 4.96 (dd, *J*=2.0, 10.0) | 115.6 (t) | C-25 | H-26 |
| | 5.06 (dd, *J*=2.0,16.5) | | | H-25 |
| 28 | 1.92 (s) | 21.7 (q) | C-2, C-3, C-4 | H-2, H-29 |
| 29 | 1.10 (d, *J*=7.0) | 16.6 (q) | C-3, C-4, C-5 | H-28, H-6 |
| 30 | 1.72 (s) | 18.9 (q) | C-15, C-16, C-17 | H-18 |
| 31 | 1.69 (s) | 17.2 (q) | C-23, C-24, C-25 | H-26 |

1.10, d) and H-5 were defined as *trans* due to the NOE relationship between $H_3$-29 and $H_2$-6. The configurations of both hydroxyl groups were not determined due to the minor amount of the unstable sample.

Macrolactins B, D, and T (**1**) were tested against fungi *Alternaria solani* and *Pyricularia oryzae* and bacterium *Staphylococcus aureus*. Macrolactins T and B exhibited inhibitory activity toward *Alternaria solani*, *Pyricularia oryzae*, and *Staphylococcus aureus*, whereas macrolactin D showed no activity (Table 3). This fact implied that the succinyl group at C-6′ of the latter reduced the antibiotic activity. Besides, the vesicles occurred at sporule top of the

**Table 3** The inhibitory activity of **1**, and macrolactins B and D

| Strain | MIC ($\mu$g/ml) | | |
|---|---|---|---|
| | **1** | Macrolactin B | Macrolactin D |
| *P. oryzae* | 0.8 | 7.5 | >100 |
| *A. solani* | 2.8 | 20.1 | >100 |
| *S. aureus* | 5.5 | 4.5 | >100 |

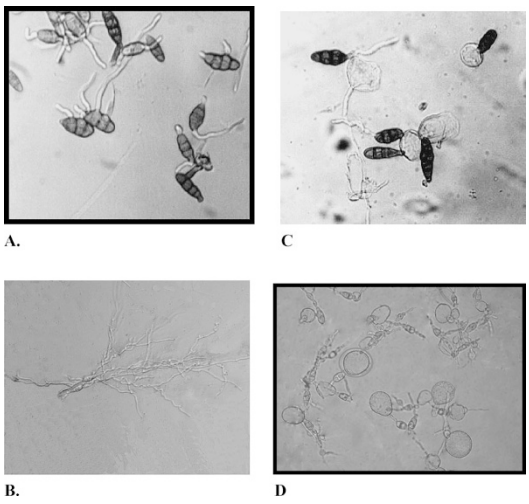



**Fig. 3** The shapes of two fungi under optical microscope.

A. the control of *Alternaria solani*; B. the control of *Pyricularia oryzae*; C. the vesicle formed on top of *Alternaria solani* spora in MIC of macrolactin B; D. the vesicle produced by spora of *Pyricularia oryzae* in MIC of macrolactin B.

fungus *Alternaria solani* after adding macrolactin B, and the same phenomenon was also observed in fungus *Pyricularia oryzae* (Fig. 3). This finding suggested that the fungal inhibition of macrolactin B was probably caused by destroying the fungal cell wall.

## Experimental

### General

Optical rotations were measured on a Perkin-Elmer 243B polarimeter. The IR spectra were recorded on a Thermo Nicolet Nexus 470 FT-IR spectrometer. The UV spectra were measured on a UV-VIS spectrophotometer 756 MC. The $^{1}$H- and $^{13}$C-NMR spectra were measured on a Varian-500 FT NMR and a Bruker Avance-500 FT NMR spectrometers (500 MHz for $^{1}$H, 125 MHz for $^{13}$C) using TMS as an internal standard. HRFAB-MS were measured on a Bruker Daltonics Apex II mass spectrometer (FAB mode), and EIS-MS were performed on MDS-SCIEX-QSTAR mass spectrometer. Column chromatography was carried with silica gel (200～300 mesh), and H-silica gel, and $GF_{254}$ silica gel for TLC was obtained from Qingdao Marine Chemistry Co. Ltd., Qingdao, People's Republic of China. Sephadex LH-20 (18～110 $\mu$m) was obtained from Pharmacia Co., while ODS (chromatorex, 100～200 mesh) was purchased from Fuji Silysia Co.

### Bacterial Strain

The strain *Bacillus marinus* was isolated from the *Suaeda salsa* (Linn) located in sediment of intertidal Bohai Sea of China, in 1999. The strain was identified by Dr. Li Tian of the Qingdao Institute of Marine Bioactive Substance, and deposited at the State Key laboratory of Natural and Biomimetic Drug, Peking University. The strain was preserved in China General Microbiological Culture Collection Center (CGMCC). The species was determined by 16S rDNA genes and sequencing analysis and was aligned with published sequences from the GenBank database using the NCBI BLASTN comparison software.

The strain was identified by 16S rDNA genes and sequencing analysis PCR amplification of the 16S rDNA genes, and the sequencing Genomic DNA was prepared by using a method as described by Sambrook [9]. The 16S rDNA genes were amplified from genomic DNA using the universal primer set 27f 5′-AGAGTTTGATCCTGGCT-CAG-3′ and 1492r 5′-TACCTTGTTACGACTT-3′. The thermal cycling parameters selected starting a 5 minutes hot at 95°C, followed by 32 cycles of denaturation for 1 minute at 94°C, annealing at 55°C for 1 minute, and extension for 1.5 minutes at 72°C, with a final extension of 20 minutes at 72°C. The PCR products were purified by an Omega Cycle-Pure PCR purification kit and sequences were determined directly using conserved bacterial 16S rDNA sequencing primers provided by Shanghai Invitrogen. The 16S rDNA sequences were aligned with published sequences from the GenBank database using the NCBI BLASTN comparison software. Phylogenetic trees were constructed by the neighbor-joining method using the DNAMAN software (version 5.1, Lynnon Biosoft, Quebec, Canada). The closest relatives of the remaining sequences were obtained from the GenBank database using BLAST program and sequence identity was 99% between strain B-9987 and *Bacillus marinus* AJ237708, AB021190. For phylogenetic analysis, reference strains were chosen from the BLAST results. Phylogenetic analysis showed that strain B9987 is closely related to *B. marinus* DSM 1297 (Fig. 4) [10].

### Culture Condition

The strain was cultured in a seawater-based medium (40 liters) (100×1 liter Erlenmeyer flasks) containing peptone (1.0 g/liter), yeast extract (0.5 g/liter), glucose (5.0 g/liter), NaCl (20 g/liter), $MgCl_2 \cdot 6H_2O$ (1.3 g/liter), KCl (0.1 g/liter), $FePO_4$ (0.01 g/liter), and pH of the medium (PYG media) was adjusted to 7.0. The flasks were cultured on a rotary shaker (150 rpm) for 9 days at 20°C.

**Extraction and Isolation**

The fermentation broth (40 liters) of *Bacillus marinus* was extracted three times with EtOAc (10 liters for each). The extracts were concentrated under vacuum to afford a brown residue (5.0 g), which was subjected to a silica gel column (4.5×30 cm) chromatography with a gradient eluant of petroleum ether - EtOAc (4 : 1, 2 : 1, 1 : 1, 1 : 2) to collect 5 fractions (FA-FE, 500 ml for each). The fraction FC (700 mg) was chromatographied on a Sephadex LH-20 column (3.5×100 cm) eluting with MeOH, to obtain **1** (3.1 mg, 200 ml) and macrolactin S (5.0 mg, 200 ml). The fraction FD (600 mg) was subjected to Sephadex LH-20 column (3.5×100 cm) with MeOH as an eluant to afford **2** (2.6 mg, 160 ml), macrolactin O (6.2 mg, 120 ml), macrolactin A (3.3 mg, 120 ml), macrolactin B (48.0 mg, 160 ml). and macrolactin D (30.5 mg, 120 ml) (Scheme 1).

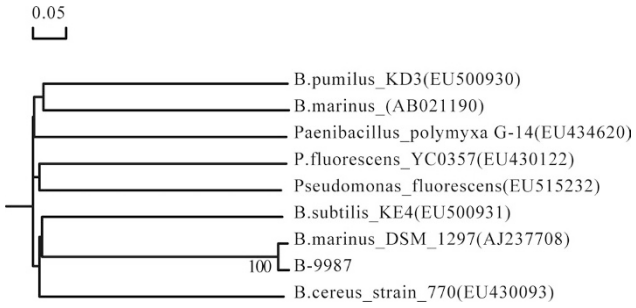


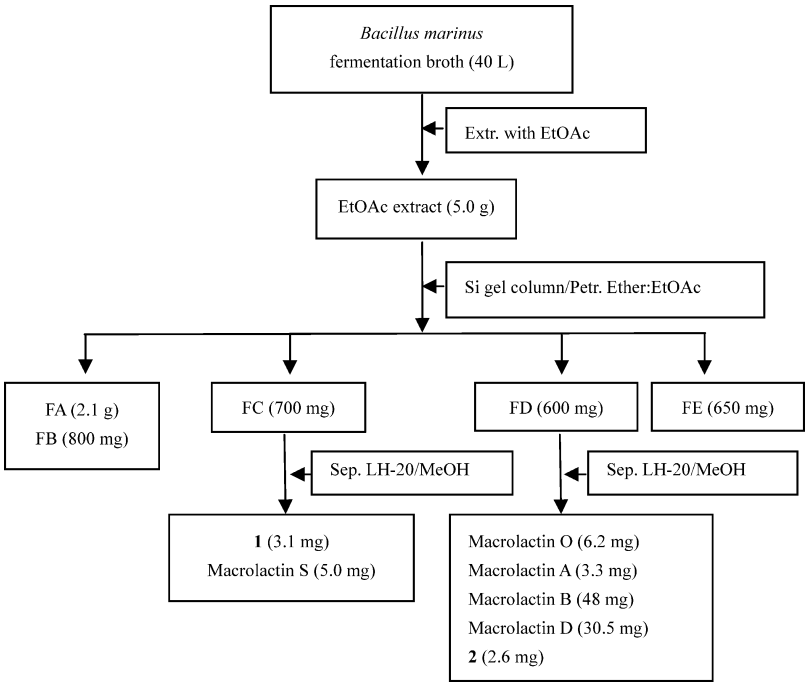

**Fig. 4** 16SrDNA phylogenetic tree of strain B9987 and related strains.

**Bioassay**

Pathogenic fungi *Pyricularia oryzae* and *Alternaria solani* and bacterium *Staphylococcus aureus* were incubated at 27°C for 12 to 14 days. The spores were then collected in 10 ml sterile water. Macrolactins B, D, and T were dissolved in DMSO/$H_2O$ (1.0%) and followed by the addition of 300 $\mu$l sabourand's liquid medium. Each well of the 96-well microtiter plate contained 50 $\mu$l spores liquid. Serial 2-fold dilution of macrolactins B, D, and T was made from 200 $\mu$g/ml, respectively. The 96-well microtiter was cultured at 27°C for 16 hours. The zone of growth inhibition was measured using a hand-held digital calipers. The MIC was determined by the microbroth dilution method [11]. The MIC was defined as the lowest concentration of antimicrobial agent that completely inhibited visible growth of the organism.

Macrolactin T (**1**)

**1** was obtained as a colorless oil; $[\alpha]_D^{25}$ −2.6 (*c* 0.3 in MeOH). UV $\lambda_{max}$ (MeOH) (log $\varepsilon$): 236 (4.52), 262 nm (4.24). IR (KBr) $\nu_{max}$ $cm^{-1}$: 3378, 2925, 1693, 1638,1581, 1422, 1281, 1190, 1025. $^1$H- and $^{13}$C-NMR data, see Table 1. HRFAB-MS *m/z*: 419.2419 $[M+H]^+$ (calcd for $C_{24}H_{35}O_6$, 419.2428).




**Scheme 1** The isolation procedure for macrolactins.

Macrolactin U (**2**)

**2** was obtained as colorless oil; $[\alpha]_D^{25}$ −32.1 (*c* 0.4 in MeOH). IR (KBr) $\nu_{max}$ $cm^{-1}$: 3421, 2933, 1711, 1643, 1440, 1382, 1152. $^1H$- and $^{13}C$-NMR data, see Table 2. HRFAB-MS *m/z*: 481.3315 $[M+H]^+$ (calcd for $C_{31}H_{45}O_4$, 481.3318).

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