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Macrolactin S, a New Antibacterial Agent with FabG-inhibitory Activity from *Bacillus* sp. AT28

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Abstract In the course of screening for FabG inhibitors from microbial sources, a new 24-membered ring lactone named macrolactin S, along with the known compound macrolactin B, has been isolated from the mycelium of liquid fermentation cultures of *Bacillus* sp. AT28. The structure of macrolactin S was determined on the basis of MS and NMR data. Macrolactin S showed a dose-dependent inhibition of *Staphylococcus aureus* FabG, not inhibiting *S. aureus* FabI. Also macrolactin S inhibited the growth of *S. aureus*, *Bacillus subtilis*, and *Escherichia coli*.

Keywords macrolactin, *Bacillus*, FabG, antibacterial, *Staphylococcus aureus*

Bacterial fatty acid synthase (FAS), which is essential for bacterial growth, consists of multiple individual enzymes, each encoded by a separate gene, in contrast to the mammalian fatty-acid synthase, which is a homodimer of single multifunctional polypeptide derived from a single gene. It is important that there is low protein sequence homology and that there are different arrangements of enzymatic-active sites between bacterial and mammalian FAS. These differences have been exploited to establish bacterial FAS as a target for antibacterial drug discovery [1～3]. β-Ketoacyl-acyl carrier protein (ACP) reductase (FabG) is an essential enzyme which catalyzes the reduction of β-ketoacyl-ACP to β-hydroxyacyl-ACP in the bacterial fatty acid synthesis pathway. Since the FabG gene is well conserved in most pathogenic bacteria, it is considered to be a new potential target for broad-spectrum antibacterial agents. Epigallocatechin gallate and related polyphenol compounds have been reported to inhibit *E. coli* FabG [4]. Some inhibitors of other bacterial FAS are known. Triclosan and isoniazid, which are currently used antibacterial agents, have been demonstrated to act by inhibiting FabI [5, 6]. Cephalochromin and unsaturated fatty acids acting as FabI inhibitors [7, 8], atromentin acting as a FabK inhibitor [9], cerulenin and thiolactomycin acting as FabH/B inhibitors [3], and platensimycin and phomallenic acid acting as FabF inhibitors [10, 11] were isolated from microorganisms. However, a FabG inhibitor of microbial origin has not been reported.

In the course of our screening for FabG inhibitors from microbial metabolites, we have isolated a new 24-membered lactone compound named macrolactin S (**1**) together with the known compound, macrolactin B (**2**) [12], from *Bacillus* sp. AT28 (Fig. 1). In this paper, we report the producing strain, fermentation, isolation, structure determination, selective inhibitory activity against FabG, and antibacterial activity of **1**.

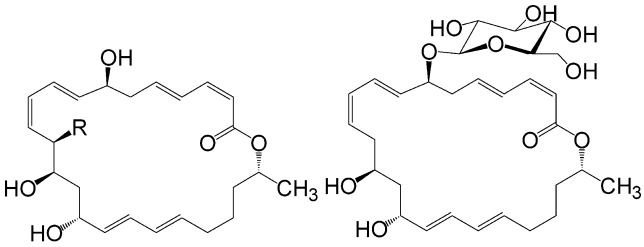

Fig. 1 Chemical structures of macrolactin S (**1**) and macrolactin B (**2**).

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Materials

Humic acid was purchased from Tokyo Kasei (Tokyo, Japan). Soluble starch, glucose, and inorganic salts were purchased from Junsei (Tokyo, Japan). Cycloheximide, nalidixic acid, *p*-aminobenzoic acid, biotin, Ca-pantothenate, inositol, and thiamine-HCl were purchased from Sigma (St. Louis, MO, USA). Beef extract and yeast extract were purchased from Difco (Sparks, MD, USA). Soybean meal was purchased from Seolim Food Co. Ltd. (Namyangjoo, Korea).

The Producing Strain

The producing strain AT28 was isolated from a soil sample that was collected in 2004 near Daejeon-City, Chungcheongnam-Do, Korea. The air-dried soil (1.0 g) was suspended in 10 ml of saline and after properly diluted the suspension was spread on the humic acid agar plate (humic acid 0.1%, Na_2HPO_4 0.05%, KCl 0.17%, $MgSO_4 \cdot 7H_2O$ 0.005%, $FeSO_4 \cdot 7H_2O$ 0.001%, $CaCO_3$ 0.002%, and agar 2.0% (adjusted to pH 7.2 before sterilization) containing antibiotics (cycloheximide 0.005%, nalidixic acid 0.002%) and vitamins (*p*-aminobenzoic acid 0.05×10^{-3}%, biotin 0.025×10^{-3}%, Ca-pantothenate 0.05×10^{-3}%, inositol 0.05×10^{-3}%, thiamine-HCl 0.05×10^{-3}%). Among colonies that appeared after incubation at 28°C for 2 weeks, the strain AT28 was isolated. The strain was identified as a *Bacillus* species on the basis of 16S rDNA sequence and deposited in the Korean Collection for Type Cultures, Daejeon, with accession number KCTC 13317.

Fermentation and Isolation

Fermentation was carried out in 500-ml Erlenmeyer flasks containing soluble starch 1.0%, glucose 2.0%, soybean meal 2.5%, beef extract 0.1%, yeast extract 0.4%, NaCl 0.2%, K_2HPO_4 0.025%, and $CaCO_3$ 0.2% (adjusted to pH 7.2 before sterilization). A piece of strain AT28 from a mature plate culture was inoculated into a 500-ml Erlenmeyer flask containing 80 ml of sterile seed liquid medium with the above composition and cultured on a rotary shaker (150 rpm) at 28°C for 3 days. For the production of **1** and **2**, 5-ml portions of the seed culture were transferred into 500-ml Erlenmeyer flasks containing 100 ml of the above medium and cultivated for 7 days using the same conditions. The culture broth (3 liters) was mixed with an equal volume of Me_2CO and then the filtrate was concentrated *in vacuo*. The Me_2CO extract was extracted with EtOAc three times and the EtOAc layer was concentrated *in vacuo* (Fig. 2). The resultant residue was subjected to silica gel (56×200 mm, Merck Art No. 7734.9025) column chromatography, eluting with $CHCl_3$-MeOH. Each fraction was tested for FabG-inhibitory activity. The first active fractions (1.5 liters) eluted with $CHCl_3$-MeOH (25 : 1) were pooled and concentrated *in vacuo*. The residue was applied to a Sephadex LH-20 (Amersham Biosciences, Uppsala, Sweden) column and eluted with $CHCl_3$-MeOH (1 : 1). After the active effluent (30 ml) was pooled and concentrated *in vacuo*, the resultant residue was purified by preparative silica gel TLC (Merck Art No. 1.05715.0001) developed with $CHCl_3$-MeOH (5 : 1). The active band was further purified by HPLC equipped with Waters 510 HPLC pump and Waters 996 Photodiode Array Detector. The reverse phase HPLC column (20×250 mm, YMC C_{18}) was eluted with CH_3OH-H_2O (70 : 30) at a flow rate of 5.0 ml/minute to afford 3.2 mg of **1** as a white powder (retention time of 20.5 minutes). The second active fraction from the silica gel column was eluted with $CHCl_3$-MeOH (10 : 1). The active effluent (450 ml) was concentrated *in vacuo* and applied again to a Sephadex LH-20 column, eluting with $CHCl_3$-MeOH (1 : 1). The active effluent was further purified by preparative silica gel TLC developed with CH_3OH-H_2O (5 : 1) to afford 13 mg of **2** as a white powder.

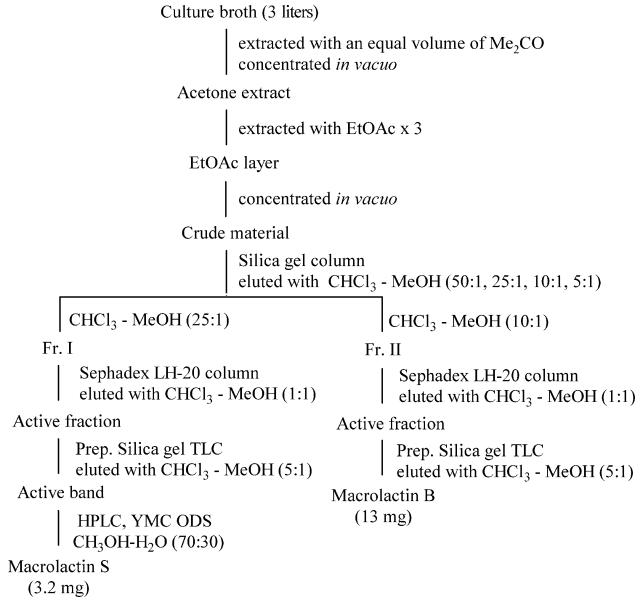

Fig. 2 Purification scheme of **1** and **2**.

Structure Elucidation

Compound **2** was identified as macrolactin B by direct HPLC comparison with an authentic sample that we previously isolated [14], together with comparison of MS and ^{1}H-NMR spectral data. The $[\alpha]_D$ value [−27.5 (*c* 0.38, MeOH)] of **2** was almost identical to that of macrolactin B [−28.3 (*c* 0.38, MeOH)]. Good agreement for coupling constants and chemical shifts in the ^{1}H-NMR spectrum compared with those of macrolactin B in the same solvent

Table 1 Physico-chemical properties of macrolactin S (**1**)

Appearance	White powder
Melting point	125~127°C
$[\alpha]_D$	−42.4 (*c* 0.1, MeOH)
ESI-MS (*m/z*)	441 $(M+Na)^+$, 417 $(M-H)^-$
HRESI-MS (*m/z*)	
found.	441.2250 $(M+Na)^+$
calcd.	441.2253
Molecular formula	$C_{24}H_{34}O_6$
UV λ_{max} nm (log ε) (MeOH)	232 (4.56), 262 (4.13)
IR (KBr) γ cm^{-1}	3434, 2923, 1640, 1455, 1122

indicates that **2** has the same structure and absolute configuration as macrolactin B [15].

The molecular formula of **1** was determined to be $C_{24}H_{34}O_6$ on the basis of high resolution ESI-MS [$(M+Na)^+$, 441.2250 *m/z* (−0.3 mmu error)] in combination with ^{1}H- and ^{13}C-NMR data. The IR data suggested the presence of a carbonyl (1640 cm^{-1}) and a hydroxyl (3434 cm^{-1}) moiety (Table 1). The ^{1}H- and ^{13}C-NMR data (Table 2) with DEPT and HMQC data suggested the presence of twelve olefinic methines, five oxygenated methines, five methylenes, a methyl, and a lactone carbonyl carbon. The ^{1}H-^{1}H COSY spectrum indicated the presence of $-^2CH=^3CH-^4CH=^5CH-^6CH_2-^7CH(OH)-^8CH=^9CH-^{10}CH=^{11}CH-^{12}CH(OH)-^{13}CH(OH)-^{14}CH_2-CH(OH)-^{16}CH=^{17}CH-^{18}CH=^{19}CH-^{20}CH_2-^{21}CH_2-^{22}CH_2-^{23}CH(O-)-^{24}CH_3$. The presence of this long structure and the hydroxylated methine at C-12 was confirmed by the HMBC spectrum summarized in Fig. 3. The hydroxylated methine proton at δ 4.45 (H-12)) showed HMBC correlations with the olefinic methine carbons at δ 129.6 (C-10) and δ 129.8 (C-11) and the methylene carbon at δ 38.6 (C-14). The geometries of C-2, C-4, C-8, C-10, and C-18 were assigned as *Z*, *E*, *E*, *Z*, and *E*, respectively, by their respective ^{1}H coupling constants of 11.5, 15.0, 15.0, 10.5, and 15.0 Hz. The geometry of H-16 and H-17 was assigned through NOESY because chemical shift overlap of H-10 and H-17 prevented direct measurement of their coupling constant. NOEs between H-15 and H-17, H-16 and H-18, and H-17 and H-19 were observed, while an NOE between H-15 and H-18 was not observed, indicating the geometry of C-16 to be *E* (Fig. 3). The stereochemistry of C-12 was also determined by NOESY data. H-12 showed strong NOEs with H-9 and H-13. Since the stereochemistry at C-13 is *S*, C-12 should be *S* (Fig. 3). Thus, the structure of **1** was determined as shown in Fig. 1.

Compound **1** is a derivative of macrolactin A [12] in which C-12 is hydroxylated. Macrolactin S was reported by another group earlier this year [13]. After our manuscript had been submitted to this journal, we learned about the previous publication in Natural Products Research reporting macrolactin S. It seems that the stereochemistry of **1** was the same as the previously reported macrolactin S even though there is a little discrepancy in optical rotation.

Table 2 ^{1}H- and ^{13}C-NMR spectral data of **1**

Position	δ_H (*J*, Hz)	δ_C
1		166.8 C
2	5.54 (1H, d, 11.5)	116.6 CH
3	6.57 (1H, t, 11.5)	144.0 CH
4	7.17 (1H, dd, 15.0, 11.5)	128.6 CH
5	6.19 (1H, m)	140.5 CH
6	2.35 (1H, m)	41.0 CH_2
	2.44 (1H, m)	
7	4.13 (1H, m)	72.2 CH
8	5.72 (1H, dd, 15.0, 8.4)	137.5 CH
9	6.38 (1H, dd, 15.0, 10.5)	125.7 CH
10	6.11 (1H, m)	129.6 CH
11	5.47 (1H, t, 10.5)	129.8 CH
12	4.45 (1H, dd, 10.5, 1.2)	70.8 CH
13	3.99 (1H, m)	71.4 CH
14	1.42 (1H, m)	38.6 CH_2
	1.51 (1H, m)	
15	4.32 (1H, m)	67.6 CH
16	5.55 (1H, m)	133.4 CH
17	6.11 (1H, m)	129.2 CH
18	6.01 (1H, dd, 15.0, 10.5)	130.2 CH
19	5.57 (1H, m)	133.5 CH
20	2.05 (1H, m)	31.5 CH_2
	2.17 (1H, m)	
21	1.42 (1H, m)	24.0 CH_2
	1.51 (1H, m)	
22	1.61 (2H, m)	34.5 CH_2
23	5.01 (1H, m)	70.6 CH
24	1.23 (3H, d, 6.0)	19.2 CH_3

^{1}H- and ^{13}C-NMR spectra were measured at 500 MHz and 125 MHz, respectively, in $CDCl_3+CD_3OD$.
The assignments were aided by ^{1}H-^{1}H COSY, DEPT, HMQC, and HMBC

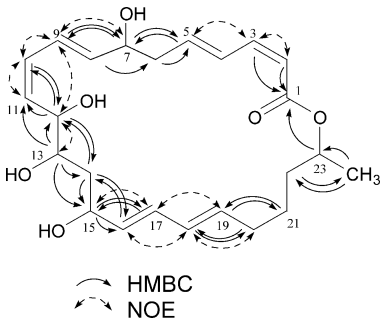

Fig. 3 Key HMBC and NOE correlations of **1**.

Appearance of compound **1** is quite different from the reported one: a white powder *vs.* an yellow oil.

Twenty macrolactins, a class of 24-membered lactones, such as macrolactins A∼R, 7-*O*-succinoylmacrolactin A, and 7-*O*-succinoylmacrolactin F, have been reported so far [12, 14∼20]. Macrolactins have been isolated from an unclassifiable deep-sea bacterium, *Actinomadura* sp., or *Bacillus* sp. Macrolactin A shows antibacterial activity, inhibits B16∼F10 murine melanoma cancer cells *in vitro*, shows significant inhibition of mammalian herpes simplex viruses (types I and II) [12], and prevents glutamate neurotoxicity in N18-RE-105 cells [16]. In addition, macrolactins F∼R, 7-*O*-succinoylmacrolactin A, 7-*O*-succinoylmacrolactin F, and 7-*O*-malonylmacrolactin A have been reported to exhibit antibacterial activity against *S. aureus* and *B. subtilis* [14, 17∼20].

The inhibitory activity of **1** against *S. aureus* FabG was evaluated with a previously described method [21] with some modifications as follows: assays contained 100 mM sodium phosphate (pH 7.4), 35 mM NaCl, 1.0% dimethyl sulfoxide, 50 μM acetoacetyl-CoA, 100 μM NADPH, and 0.39 nM *S. aureus* FabG in half-area, 96-well microtiter plates. Compound dissolved in dimethyl sulfoxide was added to each well. The rate of increase in the amount of NADPH in each reaction well was measured at 340 nm and 30°C by a microtiter ELISA reader using SOFTmax PRO software (Molecular Devices, California, USA). The inhibitory activity was calculated by the following formula: % of inhibition=100×[1−(rate in the presence of compound/rate in the untreated control)].

Compound **1** inhibited *S. aureus* FabG in a dose dependent manner with an IC_{50} of 0.13 mM (Table 3). Compound **2** also exhibited inhibitory effects on *S. aureus* FabG with similar potency. However, neither **1** nor **2** inhibited *S. aureus* enoyl-ACP reductase (FabI), the other reductase of bacterial FAS catalyzing the reduction of enoyl-ACP to acyl-ACP, even at 1.0 mM. This suggests that **1** and **2** selectively inhibit FabG, even though their activities are weak. The antibacterial activity of **1** against *S. aureus* (RN4220), *B. subtilis* (KCTC 1021), and *Escherichia coli* (KCTC 1924) was examined using microdilution broth method [14]. Compound **1** inhibited growth of *E. coli* and *B. subtilis* with an MIC of 64 μg/ml and showed weaker antibacterial activity against *S. aureus* with an MIC of 128 μg/ml. Compound **2** showed similar antibacterial activity. Compounds **1** and **2** showed quite different antibacterial activity from the previously reported macrolactin S which showed antibacterial activity against *E. coli* and *S. aureus*, not *B. subtilis*.

In summary, macrolactin S is a new 24-membered lactone isolated from a strain of *Bacillus* sp. AT28.

Table 3 Selective inhibitory activity of **1** and macrolactin B (**2**) against FabG and their antibacterial activity

	IC_{50} (mM)		MIC (μg/ml)		
	FabG	FabI	*E. coli*	*B. subtilis*	*S. aureus*
1	0.13	>1	64	64	128
2	0.10	>1	64	64	128

Macrolactin S selectively inhibited *S. aureus* FabG and also showed antibacterial activity against *E. coli*, *B. subtilis* and *S. aureus*.

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