A New Antifungal Cyclic Lipopeptide from *Bacillus marinus* B-9987

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A new cyclic lipopeptide, marihysin A (**1**), along with the three known cyclodipeptides cyclo(Ala-Ile) (**2**), cyclo(Ala-Leu) (**3**), and cyclo(Ala-Tyr) (**4**), was isolated from the fermentation broth of the marine microorganism *Bacillus marinus* B-9987 isolated from the tissues of rhizophere of *Suaeda salsa* in the intertidal zone of the Bohai Bay of P. R. China. Marihysin A (**1**) was established to be cyclo(Pro-Gln-Asn[1]-Ser-Asn[2]-Tyr-Asn[3]-β-aminotetradecanoic acid) by spectroscopic analysis, and it exhibits broad-spectrum but low activity against plant pathogens as determined by antifungal bioassay.

Introduction. – Marine microorganisms are being proven to be an extremely rich source of novel bioactive secondary metabolites [1] that have the potential of becoming new agricultural antibiotics [2][3]. The strain *Bacillus marinus* B-9987 isolated from the tissues of rhizophere of *Suaeda salsa* in the intertidal zone of the Bohai Bay of P. R. China shows excellent activity against plant pathogens both *in vitro* and *in vivo* as reported in [4][5], and some antimicrobial macrolactin constituents [6] have been identified from this strain. To search for further antimicrobial constituents from this strain, we finally obtained a new cyclic lipopeptide marihysin A (**1**; cyclo(Pro-Gln-Asn[1]-Ser-Asn[2]-Tyr-Asn[3]-β-aminotetradecanoic acid)) and three known cyclodipeptides, cyclo(Ala-Ile) (**2**), cyclo(Ala-Leu) (**3**), and cyclo(Ala-Tyr) (**4**; *Fig. 1*) determined based on the chemical analysis and all kinds of spectroscopic methods. Cyclic lipopeptides exhibit biological activities such as antifungal [7], antitumor [8], and anti-inflammatory [9] properties. Here, we describe the isolation, structural elucidation, and antifungal bioassay of the new cyclic lipopeptide and three known cyclodipeptides.

Results and Discussion. – Compound **1** was obtained as a white amorphous powder by a multi-step chromatography from the fermentation broth of the marine microorganism *B. marinus* B-9987. It gave an $[M+Na]^+$ peak in the HR-ESI-MS at m/z 1065.6316, which corresponds to the molecular formula $C_{48}H_{74}N_{12}O_{14}$ (18 degrees of unsaturation). The intense absorptions between 1600 and 1700 cm^{-1}, and between 3100 and 3400 cm^{-1} in the IR spectrum suggested the presence of the amide C=O and NH groups, respectively. Compound **1** was negative to ninhydrin reagent but positive after hydrolysis with 6M HCl [10]. Hence, **1** could be assigned as a cyclic lipopeptide.

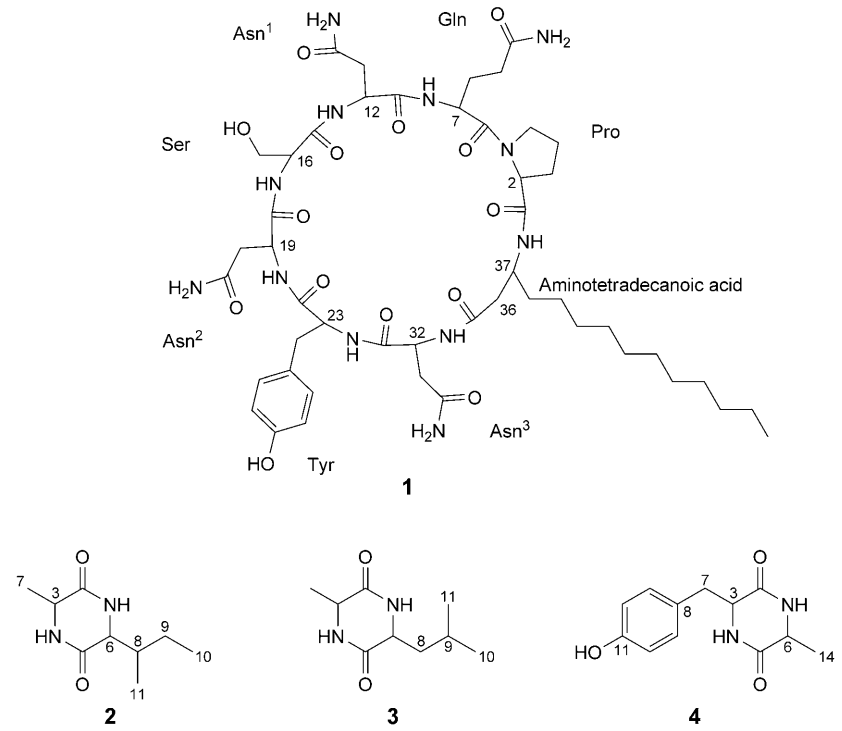

Fig. 1. *Structures of compounds* **1**–**4**

The ^{1}H- and ^{13}C-NMR spectra of **1** showed typical signals for a lipopeptide. In the ^{13}C-NMR spectrum of **1** (*Table 1*), there were signals corresponding to one ester C=O C-atom (δ(C) 171.24) and eleven amide C=O C-atoms (δ(C) 174.1, 173.3, 172.7, 171.9, 171.24, 171.17, 171.0, 170.9, 170.8, 170.6, and 170.3). The DEPT spectrum of **1** further indicated the presence of 14 quaternary C-atoms including 12 C=O groups (δ(C) 174.1 to 170.3) and two quaternary C-atoms of Tyr residue (δ(C) 155.8 and 127.9), twelve CH C-atoms including eight CH groups (δ(C) 45.4 to 60.8), 21 CH_2 C-atoms, and one Me C-atom. The ^{1}H-NMR spectrum of **1** in (D_6)DMSO revealed the presence of 8 H–C(α) atoms (δ(H) 4.51–4.52, 4.45–4.46, 4.44–4.45, 4.43–4.44, 4.17, 4.15–4.16, 4.01–4.03, and 3.98–4.00) and eleven NH/NH_2 groups (8.73, 8.08, 7.74, 7.40, 7.35, 7.24, 7.15, 6.99, 6.94, 6.90 and 6.88; partly overlapped). The ^{1}H-NMR signals at 7.03 (*d*, J = 8.1, 2 H), 6.67 (*d*, J = 8.1, 2 H) and the ^{13}C-NMR signals at 155.8, 129.7, 127.9 and 115.1 indicated a *p*-disubstituted aromatic ring, which was identified as Tyr by HMQC, HMBC, and ^{1}H,^{1}H-COSY data. Similarly, combining the NMR data of **1** (*Table 1*) with HMQC, HMBC, ^{1}H,^{1}H-COSY, and especially TOCSY (*Fig. 2*) experiments, we deduced that **1** was a lipopeptide composed of one Tyr, three Asn, one Pro, one Gln, and one Ser residues, and a long-chain fatty acid (*Table 1*). These amino acid residues were also confirmed by amino acid analysis following hydrolysis of **1** at 110°.

After subtraction of all seven amino acid residues determined, a molecular formula of $C_{14}H_{27}NO$ remained for the fatty acid portion of the molecule. The H-atom signals at

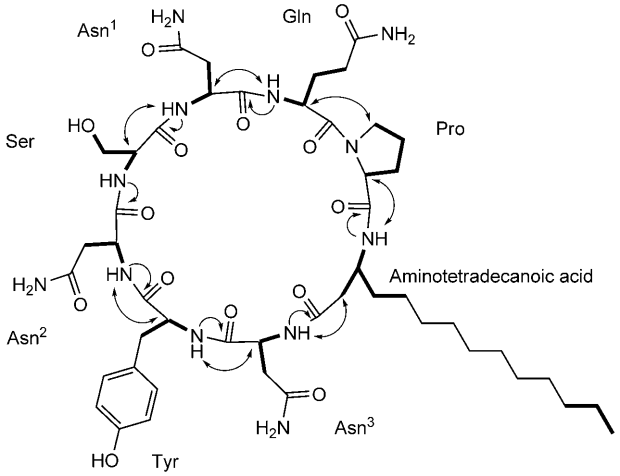

Fig. 2. *Key TOCSY* (—), *HMBC* (H → C), *and ROESY* (H ↔ H) *correlations of compound* **1**

δ(H) 1.24 (br. *s*, 16 H) and 0.85 (*t*, $J = 7.0$), and the C-atom signals at δ(C) 28.6 to 29.1, and 13.9 indicated that it was a normal-type long-chain amino fatty acid [11]. Analysis of HMBC and ^{1}H,^{1}H-COSY spectra showed that the amino group was at β-position (*Table 1*). Thus, the fatty acid residue should be the β-aminotetradecanoic acid residue.

The total sequence of residues in **1** was determined by analysis of HMBC and ROESY spectra (*Fig. 2*), and multiple stages of collisionally activated decompositions (CAD) technique in MS/MS (*Fig. 3*). Key correlations between the NH (H–C(4) of Pro residue) and H–C(α) of neighboring residues in ROESY, and NH and C=O of neighboring residues in HMBC (*Fig. 2*) finally allowed us to establish the structure of **1** as cyclo(Pro-Gln-Asn^1-Ser-Asn^2-Tyr-Asn^3-β-aminotetradecanoic acid).

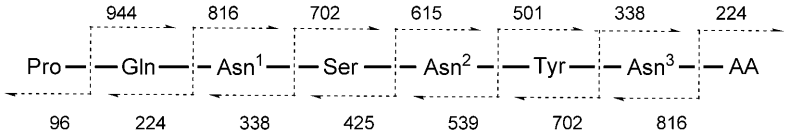

Fig. 3. *ESI-MS/MS* (neg.) *Fragmentation of compound* **1**

Antifungal activities of **1** are compiled in *Table 2*. Compound **1** exhibited a broad-spectrum but low activity against plant pathogens such as *Alternaria solani*, *Fusarium oxysporum*, *Verticillium alboatrum*, *F. graminearum*, *Sclerotium* sp., *Penicillium* sp., *Rhizoctonia solani*, and *Colletotrichum* sp. with the minimum inhibitory concentration (*MIC*) values of 100–200 μg/ml. In general, the antifungal activity of **1** is weaker than that of other cyclopeptides such as iturin D (*MIC* values against *F. oxysporum* and *Botrytis cinerea* are both 30 μg/ml) [12], bacillomycin Fa (*MIC* value against *B. cinerea* is 20 μg/ml) [13], and fengycin (*MIC* value against *R. solani* is 3.16 μg/ml) [14], but its antifungal activity against *F. oxysporum* is stronger than that of bacillomycin Fa (*MIC* value against *F. oxysporum* is > 320 μg/ml) [13].

Table 1. *NMR Data of Compound* **1** (recorded in (D_6)DMSO; δ in ppm, J in Hz).

Position		^{13}C	^{1}H	^{1}H,^{1}H-COSY	HMBC
Pro	1	173.3 (C=O)			
	2	60.8 (CH)	4.15–4.16 (*m*)	H–C(3)	C(1), C(3), C(4)
	3	28.7 (CH_2)	2.13–2.15 (*m*), 1.76–1.78 (*m*)	H–C(2)	C(2), C(4), C(5)
	4	24.6 (CH_2)	1.99–2.04 (*m*), 1.88–1.89 (*m*)	H–C(5)	C(2), C(3)
	5	47.2 (CH_2)	3.76–3.78 (*m*)	H–C(4)	C(2)
Gln	6	170.9 (C=O)			
	7	49.7 (CH)	4.51–4.52 (*m*)	H–C(8), 7-NH	C(6), C(8), C(9)
	8	26.5 (CH_2)	1.99–2.04 (*m*), 1.76–1.78 (*m*)	H–C(7), H–C(9)	C(7), C(9), C(10)
	9	30.6 (CH_2)	2.10–2.13 (*m*)	H–C(8)	C(7), C(8), C(10)
	10	174.1 (C=O)			
	10-NH_2		7.15 (*s*), 6.88 (*s*)		C(10)
	7-NH		6.99 (*d*, J = 7.5)	H–C(7)	C(7), C(11)
Asn[1]	11	171.0 (C=O)			
	12	49.7 (CH)	4.43–4.44 (*m*)	H–C(13), 12-NH	C(11), C(13)
	13	35.2 (CH_2)	2.70–2.74 (*m*), 2.45–2.51 (*m*)	H–C(12)	C(11), C(12), C(14)
	14	171.9 (C=O)			
	14-NH_2		7.40 (*s*), 6.88 (*s*)		C(13), C(14)
	12-NH		8.73 (*d*, J = 6.1)	H–C(12)	C(11), C(15)
Ser	15	170.3 (C=O)			
	16	56.2 (CH)	4.17 (*s*)	H–C(17), 16-NH	C(15), C(17)
	17	61.4 (CH_2)	3.67 (*s*)	H–C(16)	C(15), C(16)
	17-OH		4.89 (*t*, J = 5.5)		C(16), C(17)
	16-NH		7.35 (*s*)	H–C(16)	C(18)
Asn[2]	18	170.8 (C=O)			
	19	50.8 (CH)	4.45–4.46 (*m*)	H–C(20), 19-NH	C(18), C(20)
	20	36.0 (CH_2)	2.54–2.59 (*m*), 2.45–2.51 (*m*)	H–C(19)	C(18), C(21)
	21	171.24 (C=O)			
	21-NH_2		7.24 (*s*), 6.90 (*s*)		C(20), C(21)
	19-NH		8.08 (*d*, J = 7.1)	H–C(19)	C(19), C(22)
Tyr	22	171.17 (C=O)			
	23	56.3 (CH)	4.01–4.03 (*m*)	H–C(24), 23-NH	C(22)
	24	34.9 (CH_2)	2.97 (*d*, J = 11.3), 2.70–2.74 (*m*)	H–C(23)	C(23), C(25), C(26), C(30)
	25	127.9 (C)			
	26, 30	129.7 (2 CH)	7.03 (*d*, J = 8.1)	H–C(27), H–C(29)	C(24), C(25), C(27), C(28), C(29)
	27, 29	115.1 (2 CH)	6.67 (*d*, J = 8.1)	H–C(26), H–C(30)	C(25), C(26), C(28), C(30)
	28	155.8 (C)			
	28-OH		9.21 (*s*)		C(27), C(28), C(29)
	23-NH		8.73 (*d*, J = 6.1)	H–C(23)	C(23), C(31)
Asn[3]	31	172.7 (C=O)			
	32	50.8 (CH)	4.44–4.45 (*m*)	H–C(33), 32-NH	C(31), C(33)
	33	36.3 (CH_2)	2.28–2.32 (*m*), 2.16–2.17 (*m*)	H–C(32)	C(32), C(34)

Table 1 (cont.)

Position		^{13}C	^{1}H	$^{1}H,^{1}H$-COSY	HMBC
	34	170.6 (C=O)			
	34-NH_2		7.35 (*s*), 6.94 (*s*)		C(33), C(34)
	32-NH		7.74 (*d*, *J* = 3.1)	H–C(32)	C(35)
β-Aminotetra-decanoic acid	35	171.24 (C=O)			
	36	41.7 (CH_2)	2.34–2.35 (*m*)	H–C(37), 37-NH	C(35), C(37), C(38)
	37	45.4 (CH)	3.98–4.00 (*m*)	H–C(36), H–C(38)	C(38)
	38	34.6 (CH_2)	1.41 (*s*)	H–C(37), H–C(39)	C(39)
	39	28.6 (CH_2)	1.22–1.26 (*m*), 1.14–1.15 (*m*)	H–C(38), H–C(40)	C(40)
	40–44	29.1 (CH_2)	1.24 (br. *s*)	H–C(39)–H–C(48)	C(39)–C(48)
	45	31.3 (CH_2)	1.24 (br. *s*)	H–C(39)–H–C(48)	C(39)–C(48)
	46	25.4 (CH_2)	1.24 (br. *s*)	H–C(39)–H–C(48)	C(39)–C(48)
	47	22.1 (CH_2)	1.24 (br. *s*)	H–C(39)–H–C(48)	C(39)–C(48)
	48	13.9 (Me)	0.85 (*t*, *J* = 7.0)	H–C(47)	C(45), C(46), C(47)
	37-NH		7.15 (*s*)	H–C(1), H–C(36)	C(1), C(37)

Table 2. *The* MIC *Values of Compound* **1** *against Plant Pathogens*

Plant pathogens	*MIC*[a]) [μg/ml]
Alternaria solani	100
Fusarium oxysporum	200
Verticillium alboatrum	200
Fusarium graminearum	200
Sclerotium sp.	200
Penicillium sp.	200
Botrytis cinerea	> 200
Drechslera turcica	> 200
Rhizoctonia solani	200
Colletotrichum sp.	100
Fusarium oxysporum f. sp. *cuberse*	200

[a]) *MIC* = Minimum inhibitory concentration.

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Experimental Part

General. TLC: precoated silica-gel *H* plates (*Qingdao Haiyang Chemical Group Co.*). Column chromatography (CC): 200–300 mesh silica gel (*Qingdao Haiyang Chemical Group Co.*) and *Sephadex LH-20* (*American Pharmacia Fine Chemical Co., Ltd.*). HPLC: *ODS* column (*Sunfire*, 10 × 250 mm, 5 μm). UV Spectra: *Shimadzu-UV-1700* spectrophotometer; in MeOH; λ_{max} (ε) in nm. IR Spectra: *Magna-IR 550* spectrometer (*American Nicolet Co.*). ^{1}H-, ^{13}C-, and 2D-NMR Spectra: *Bruker AV-500* instrument; recorded in (D_6)DMSO at 500 and 125 MHz (^{1}H and ^{13}C, resp.); chemical shifts δ in ppm rel. to Me_4Si as internal standard, *J* in Hz. ESI-MS: *Q-Tof micro* instrument (*American Waters Co.*) at cap.

3 kV, sample cone 80 V, extraction cone 4 V, source temp. 80°, desolvation temp. 150°, ion energy 1 V, MCP detector 2200 V, and collision energy 10 V (MS) or 40 V (MS/MS); in *m/z* (rel. %). Amino acids analysis: *L-8900 High-speed amino acid analyzer* (*Japanese Hitachi Co.*).

Bacterial Material. The marine microorganism B-9987 was isolated from the tissues of rhizophere of *S. salsa* in the intertidal zone of the Bohai Bay, P. R. China. The strain was identified as *B. marinus* according to its morphological and biochemical characters, and the partial sequence of its 16S rDNA [4]. The strain was deposited with the Laboratory of Marine Bioprocess Engineering, State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, and preserved by China General Microbiological Culture Collection Center (CGMCC 2095).

Fermentation, Extraction, and Purification. The marine microorganism *B. marinus* B-9987 was incubated at 30° for 24 h in a 50-l tank containing 30 l of culture medium. The 30-l culture broth of *B. marinus* B-9987 was centrifuged at 4000 rpm for 10 min. The supernatant was then extracted three times with AcOEt and BuOH, resp. The AcOEt and BuOH extracts were then concentrated *in vacuo*. The AcOEt extract (24 g) was subjected to CC (silica gel; $CHCl_3$/MeOH from 10:1 to 1:1), and the fraction of $CHCl_3$/MeOH 6:1 was then applied to a *Sephadex LH-20* column with $CHCl_3$/MeOH 1:1 to afford **2** (6 mg), **3** (8 mg), and **4** (5 mg). The BuOH extract was subjected to CC (silica gel; $CHCl_3$/MeOH from 20:1 to 1:1), and the active fraction of $CHCl_3$/MeOH 1:1 was then purified by CC (*Sephadex LH-20*; MeOH) and finally purified by HPLC (70% MeOH; 7.0 ml/min) to yield **1** (20 mg).

Marihysin A (=*Cyclo[(3*R*)-3-aminotetradecanoyl-asparaginyl-tyrosyl-asparaginyl-seryl-asparaginyl-glutaminyl-prolyl]*; **1**). White amorphous powder ($CHCl_3$/MeOH). M.p. >250°. UV (MeOH): 210. IR (KBr): 3333, 2929, 2864, 1659, 1545, 1449, 1423, 1247, 1128. ^{1}H- and ^{13}C-NMR, ^{1}H,^{1}H-COSY, and HMBC: see *Table 1*. HR-ESI-MS: 1065.6316 ($[M+Na]^+$, $C_{48}H_{74}N_{12}NaO_{14}^+$; calc. 1065.6308).

Cyclo(Ala-Ile) (=*(3*S*,6*S*)-3-Methyl-6-(1-methylpropyl)piperazine-2,5-dione*; **2**). Colorless amorphous powder ($CHCl_3$/MeOH). ^{1}H-NMR: 7.98 (*s*, NH); 7.85(*s*, NH); 3.85–3.87 (*m*, H–C(3)); 3.75–3.77 (*m*, H–C(6)); 1.84–1.86 (*m*, H–C(8)); 1.39–1.41 (*m*, 1 H, CH_2(9)); 1.29 (*d*, *J* = 7.0, Me(7)); 1.18–1.20 (*m*, 1 H, CH_2(9)); 0.91 (*d*, *J* = 7.2, Me(11)); 0.85 (*d*, *J* = 7.4, Me(10)). ^{13}C-NMR: 168.5 (C(2)); 166.6 (C(5)); 58.7 (C(6)); 49.6 (C(3)); 37.9 (C(8)); 24.1 (C(9)); 19.8 (C(7)); 15.0 (C(11)); 11.8 (C(10)). ESI-MS: 185 (20, $[M+H]^+$), 86 (100).

Cyclo(Ala-Leu) (=*(3*S*,6*S*)-3-Methyl-6-(2-methylpropyl)piperazine-2,5-dione*; **3**). Colorless amorphous powder (MeOH). ^{1}H-NMR: 8.12 (*s*, NH); 8.10 (*s*, NH); 3.85–3.87 (*m*, H–C(3)); 3.75–3.77 (*m*, H–C(6)); 1.82–1.84 (*m*, H–C(9)); 1.60–1.62 (*m*, 1 H, CH_2(8)); 1.46–1.48 (*m*, 1 H, CH_2(8)); 1.27 (*d*, *J* = 7.0, Me(7)); 0.88 (*d*, *J* = 6.6, Me(10/11)); 0.86 (*d*, *J* = 6.6, Me(10/11)). ^{13}C-NMR: 168.8 (C(2)); 168.3 (C(5)); 52.6 (C(6)); 49.9 (C(3)); 42.5 (C(8)); 23.6 (C(9)); 22.9 (C(10/11)); 21.8 (C(10/11)); 19.5 (C(7)). ESI-MS:185 (20, $[M+H]^+$), 86 (100).

Cyclo(Ala-Tyr) (=*(3*S*,6*S*)-3-[(4-Hydroxyphenyl)methyl]-6-methylpiperazine-2,5-dione*; **4**). ^{1}H-NMR: 8.02 (*s*, NH(4)); 7.97 (*s*, NH(1)); 6.93 (*d*, *J* = 8.5, H–C(9), H–C(13)); 6.65 (*d*, *J* = 8.5, H–C(10), H–C(12)); 4.08–4.06 (*m*, H–C(3)); 3.61–3.59 (*m*, H–C(6)); 3.01 (*dd*, *J* = 3.6, 13.6, H_b–C(7)); 2.73 (*dd*, *J* = 4.8, 13.6, H_a–C(7)); 0.54 (*d*, *J* = 7.1, Me(14)). ^{13}C-NMR: 167.9 (C(5)); 166.1 (C(2)); 156.3 (C(9), C(13)); 131.5 (C(11)); 126.1 (C(8)); 115.0 (C(10), C(12)); 55.8 (C(3)); 49.9 (C(6)); 37.8 (C(7)); 20.0 (C(14)). ESI-MS: 235 (30, $[M+H]^+$), 107 (100).

Bioactivity Tests. The antifungal activities of **1** were determined by a two-fold serial agar dilution method using potato dextrose agar media for plant pathogens after incubation for 48 h at 25° (see *Table 2*).

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