Micropeptins from the Freshwater Cyanobacterium *Microcystis aeruginosa* (NIES-100)

Takaya Kisugi and Tatsufumi Okino*

Graduate School of Environmental Earth Science, Hokkaido University, Sapporo 060-0810, Japan

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Micropeptins C (**1**), D (**2**), E (**3**), and F (**4**) have been isolated from the freshwater cyanobacterium *Microcystis aeruginosa* (NIES-100). The structures were elucidated by analyses of MS, NMR spectra, and chemical degradation. Micropeptins C, D, E, and F inhibited chymotrypsin with IC_{50}'s of 1.1, 1.2, 1.0, and 1.5 μg/mL, respectively.

Cyanobacteria are well-known as producers of a variety of bioactive peptides.[1] The genus *Microcystis* produces six classes of peptides. The classes consist of microcystins,[2] aeruginosins,[3] microginins,[4] anabaenopeptins,[5,6] micropeptins[7] (aeruginopeptins,[8] cyanopeptolins,[9] nostopeptins,[10] and oscillapeptins[11]), and microviridins,[12] all of which are known protease inhibitors. Micropeptins have a characteristic unit, 3-amino-6-hydroxy-2-piperidone (Ahp), and a cyclic structure with six amino acid residues. This unique unit (Ahp) is also distributed in marine cyanobacterial depsipeptides such as largamides,[13] somamides,[14] and tasipeptins.[15] Most of these depsipeptides show inhibitory activity against serine proteases, and the structure−activity relationship has been explained.[16] Microginin and micropeptins A and B isolated from *Microcystis aeruginosa* (NIES-100) were the first examples of cyanobacterial protease inhibitors. In the course of our screening program for protease inhibitors, we reinvestigated the strain NIES-100 to find chymotrypsin inhibitors. Here, we describe the isolation and structural elucidation of novel Ahp-containing depsipeptide inhibitors.

The 80% MeOH and MeOH extract of freeze-dried cyanobacterial cells was partitioned between water and diethyl ether. The aqueous layer was further extracted with *n*-BuOH, and the *n*-BuOH layer was subjected to ODS flash column chromatography. By bioassay-guided fractionation and LC-MS analyses, fractions eluted with 40% and 60% MeOH were found to contain new active compounds. These were purified with reversed-phase HPLC to yield micropeptins C (**1**), D (**2**), E (**3**), and F (**4**) as colorless, amorphous solids.

The molecular formula of **1** was established to be $C_{53}H_{69}N_7O_{14}$ by the ESI-TOFMS and NMR spectroscopic data. The ^{1}H NMR in DMSO-d_6 revealed five doublet NH proton signals between δ 7.0 and 8.5 ppm and one *N*-Me proton signal at 2.75 ppm. The aromatic protons between 6.56 and 7.18 ppm indicated the presence of two *p*-substituted and one monosubstituted benzene ring. A broad singlet at 6.00 ppm correlated to a carbon at 73.7 ppm in the HMBC spectrum and was a characteristic proton of the hydroxy group in Ahp. The structure of the Ahp unit was confirmed by analyses of ^{1}H−^{1}H COSY and TOCSY. In addition, the ^{1}H−^{1}H COSY, TOCSY, and HMBC spectra determined the presence of Glu, Thr, Tyr, Phe, *N*-Me-Tyr, and Val residues. However, the Phe residue was suggested to be an *N*,*N*-disubstituted derivative because of no correlation to an amide proton (Figure 1). The last unit was determined as hexanoic acid (HA).

The sequence of **1** was deduced by HMBC correlations from α-methines and amide protons to carbonyl carbons of amino acid residues, but the HMBC correlations from Ahp to Tyr, and from *N*-Me-Tyr to Val, could not be observed. Their connections were confirmed by the NOESY spectrum (Figure 1). Furthermore, the absolute configurations of the amino acid residues, except for Ahp,

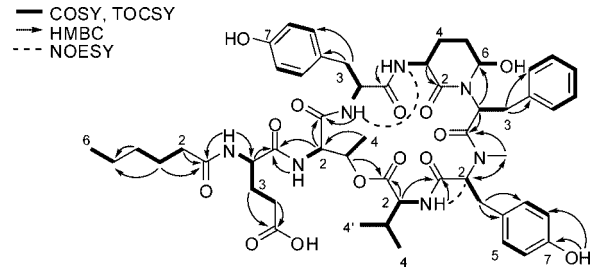


Figure 1. ^{1}H−^{1}H COSY, TOCSY, HMBC, and NOESY correlations of **1**.

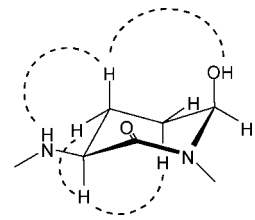
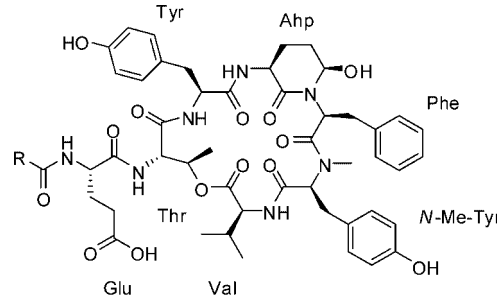


Figure 2. NOESY correlations of the Ahp unit.

were determined to be the L-form by chiral HPLC analysis of the hydrolysate of **1**. The relative configuration of Ahp was determined as shown in Figure 2. The absolute configuration of Ahp was deduced by the modified method of Ishida et al.[17] Pentahomoserine was obtained from the hydrolysate followed by the reduction of **1** with $NaBH_4$ and clarified to be the L-form by chiral HPLC analysis. Therefore, the absolute configuration of Ahp could be assigned as (3*S*,6*R*)-3-amino-6-hydroxy-2-piperidone.



1: R = C_5H_{11}, **2**: R = C_7H_{15}

The molecular formula of **2** was established to be $C_{55}H_{73}N_7O_{14}$ by the ESI-TOFMS and NMR spectroscopic data. ^{1}H and ^{13}C NMR spectra of **2** were similar to those of **1**, but the molecular weight of **2** was 28 mass units larger than **1**. The differences in ^{13}C NMR were an additional two methylene carbons (28.4, 28.5 ppm). Therefore, micropeptin D (**2**) was suggested to be substituted by

* To whom correspondence should be addressed. Tel: +81-11-706-4519. Fax: +81-11-706-4867. E-mail: okino@ees.hokudai.ac.jp.

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Table 1. NMR Spectroscopic Data (600 MHz, DMSO-d_6) for Micropeptin C (**1**)

unit	C/H no.	δ_C, mult.	δ_H (J in Hz)	HMBC[a]	NOESY[b]
HA	1	172.4, qC			
	2	35.0, CH_2	2.10, m	1,3,4	Glu-NH
	3	24.9, CH_2	1.49, m	1,2,4,5	2
	4	30.8, CH_2	1.21, m	2,3,5	
	5	21.8, CH_2	1.27, m	3,4,6	
	6	13.8, CH_3	0.84 t, (7.0)	4,5	
Glu	1	171.9, qC			
	2	51.7, CH	4.35, m	1,3	3,3′,4,NH, Thr-NH
	3	27.0, CH_2	1.69, m	2	NH
	3′		1.84, m	3	Phe-2, Thr-NH
	4	30.3, CH_2	2.20, m	2,3,5	2,NH
	5	174.0, qC			
	NH		7.97, d (7.7)	2, HA-1	2,3,3′,4, HA-2
Thr	1	168.8, qC			
	2	54.2, CH	4.51, d (9.4)	1, Glu-1	3,4,NH, Tyr-NH
	3	72.0, CH	5.37, q (6.3)	Glu-4, Val-1	2,4,NH, Ahp-NH, Tyr-NH
	4	17.5, CH_3	1.10, d (6.3)	2,3	2,3,NH, Tyr-NH
	NH		7.70, brd	Glu-1	2,3,4, Glu-2,3,3′,
Tyr	1	169.5, qC			
	2	53.3, CH	4.36, m		3,3′,5,NH, Ahp-NH
	3	34.9, CH_2	2.53, dd (9.7, 14.2)	2,4,5	2,3′,5,NH
	3′		3.10, brdd (3.4, 14.2)	1,2,4,5	2,5,NH
	4	128.0, qC			
	5,5′	129.5, CH	6.88, d (8.0)	3,7	2,3,3′,6,NH
	6,6′	114.9, CH	6.56, d (8.0)	4,7	
	7	155.6, qC			
	NH		8.42, d (8.6)	1	2,3,3′,5,5′, Thr-2,3,4, Ahp-NH
Ahp	2	168.8, qC			
	3	48.8, CH	3.60, m	2	3,3′,4,4′,NH
	4	21.6, CH_2	1.61, m	4	2,3′,NH
	4′		2.40, m		2,3,4′,NH,OH
	5	29.3, CH_2	1.54, m		2,3′,5,OH, Phe-5
	5′		1.68, m		3′,5,OH
	6	73.7, CH	5.04, brs		3′,4,4′,OH, Phe-3,3′,5, Val-NH
	NH		7.08, d (8.8)		2,3,3′, Tyr-2,NH, Thr-3
	OH		6.00, brs		3′,4,4′,5, Phe-3′, *N*-Me-Tyr-Me, Val-4
Phe	1	170.3, qC			
	2	50.2, CH	4.74, dd (3.6, 11.4)	1,3, Ahp-5	3,3′,5, *N*-Me-Tyr-3,3′,5, Val-NH
	3	35.3, CH_2	1.78, brdd (3.6, 14.0)	5	2,3′,5, Ahp-6
	3′		2.85, brdd (11.4, 14.0)	2,4,5	2,3,5, Ahp-6,OH
	4	136.7, qC			
	5,5′	129.4, CH	6.83, d (7.2)	3,7	2,3,3′,6, Ahp-5,5′,6
	6,6′	127.7, CH	7.18, dd (7.2, 7.2)	4,5	5
	7	126.2, qC	7.13, t (7.2)	5	
N-Me-Tyr	1	169.1, qC			
	2	60.8, CH	4.88, brd (11.2)		3,3′,5, Phe-3, Val-NH
	3	32.8, CH_2	2.70, brdd[c]	4,5	2,5
	3′		3.08, brd[c]	4,5	2,5, Phe-2
	4	127.4, qC			
	5,5′	130.4, CH	6.99, d (8.0)	3,7	2,3,3′,6,OH, Phe-2,3
	6,6′	115.3, CH	6.76, d (8.0)	4,7	5
	7	156.2, qC			
	N-Me	30.2, CH_3	2.75, s	2, Phe-1	5, Val-4,4′,NH, Ahp-OH
	OH		9.32, s	6,7	6,6′
Val	1	171.9, qC			
	2	55.8, CH	4.67, dd (4.4, 9.4)	1,3,4,4′, *N*-Me-Tyr-1	3,4,4′,NH, Thr-NH
	3	30.8, CH_2	2.03, m	4,4′	2,4,4′
	4	17.2, CH_3	0.70, d (6.7)	2,4′	2,3,NH, *N*-Me-Tyr-Me, Ahp-OH
	4′	19.2, CH_3	0.84, d (6.7)	2,4	2,3,NH, *N*-Me-Tyr-Me
	NH		7.39, d (9.4)	*N*-Me-Tyr-1	2,3,4,4′, Ahp-4′,6,OH, *N*-Me-Tyr-2,*N*-Me, Phe-2

[a] HMBC correlations are from proton(s) stated to the indicated carbon. [b] NOESY correlations are from proton(s) stated to the indicated proton(s). [c] Signal partially obscured.

octanoic acid (OA) in place of HA in **1**. The structure of **2** including the configurations was supported by extensive NMR analyses including $^1H-^1H$ COSY, TOCSY, HSQC, HMBC, and NOESY spectra and chiral HPLC analysis of its hydrolysate.

The molecular formula of **3** was established to be $C_{50}H_{71}N_7O_{14}$ by the ESI-TOFMS and NMR spectroscopic data. In the ^1H NMR spectrum, the isobutyl protons at 0.40, 0.49, 0.69, 0.97, and 1.55 ppm were observed instead of aromatic protons of Phe in **1**. These protons were assigned as a part of Leu by analyses of the $^1H-^1H$ COSY and TOCSY spectra. The sequence and absolute configuration of **3** were determined by the same method for **1**.

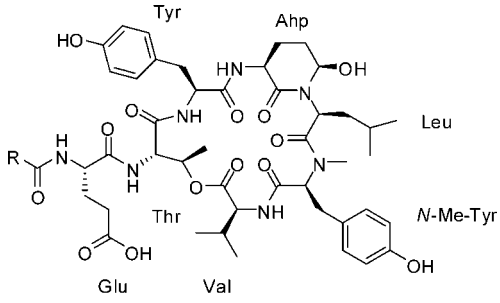
The molecular formula of **4** was established to be $C_{52}H_{75}N_7O_{14}$ by the ESI-TOFMS and NMR spectroscopic data. ^1H and ^{13}C NMR spectra of **4** were similar to those of **3**. The molecular weight of **4** was 28 mass units larger than **3**. In conclusion, micropeptin F (**4**) was substituted by OA in place of HA in **3**, which was supported by extensive 2D NMR analyses and chiral HPLC analysis of its hydrolysate. The amino acid sequences of **3** and **4** were reported by Czarnecki et al. as cyanopeptoline 993 and 1021 based on mass spectrometry.[18] However, they were not fully characterized. Due to the lack of detailed information, we could not conclude that they are identical with **3** and **4**.

Table 2. NMR Spectroscopic Data (600 MHz, DMSO-d_6) for Micropeptins D (**2**), E (**3**), and F (**4**)

		micropeptin D (**2**)				micropeptin E (**3**)				micropeptin F (**4**)	
unit	C/H no.	δ_C	δ_H (J in Hz)	unit	C/H no.	δ_C	δ_H (J in Hz)	unit	C/H no.	δ_C	δ_H (J in Hz)
OA	1	172.4		HA	1	172.4		OA	1	172.4	
	2	35.1	2.11, m		2	35.0	2.13, m		2	35.1	2.11, m
	3	25.2	1.48, m		3	24.9	1.49, m		3	25.2	1.48, m
	4	28.5	1.23, m		4	30.8	1.21, m		4	28.4	1.23, m
	5	28.4	1.23, m		5	21.8	1.25, m		5	28.5	1.23, m
	6	31.2	1.21, m		6	13.8	0.84, t (7.2)		6	31.2	1.22, m
	7	22.0	1.24, m	Glu	1	171.9			7	22.0	1.24, m
	8	13.9	0.83, t (6.8)		2	51.7	4.36, m		8	13.9	0.84, t (6.8)
Glu	1	171.9			3	27.0	1.71, m	Glu	1	171.9	
	2	51.7	4.34, m		3′		1.85, m		2	51.7	4.36, m
	3	27.0	1.69, m		4	30.3	2.20, m		3	27.0	1.71, m
	3′		1.83, m		5	174.1			3′		1.85, m
	4	30.3	2.19, m		NH		7.99, d (7.8)		4	30.3	2.22, m
	5	174.1		Thr	1	168.9			5	174.1	
	NH		7.98, d (7.7)		2	54.3	4.55, d (9.5)		NH		7.99, d (7.7)
Thr	1	168.8			3	72.0	5.46, q (6.4)	Thr	1	168.9	
	2	54.2	4.50, d (9.5)		4	17.5	1.13, d (6.4)		2	54.3	4.55, d (9.5)
	3	72.0	5.37, q (6.4)		NH		7.72, d (9.5)		3	72.0	5.46, q (6.4)
	4	17.5	1.10, d (6.4)	Tyr	1	169.9			4	17.5	1.13, d (6.4)
	NH		7.70, d (9.5)		2	53.5	4.45, m		NH		7.71, d (9.5)
Tyr	1	169.5			3	35.1	2.61, dd (9.8, 14.6)	Tyr	1	169.9	
	2	53.3	4.35, m		3′		3.22, dd (3.9, 14.6)		2	53.6	4.45, m
	3	34.9	2.53, dd (9.8, 14.3)		4	128.1			3	35.1	2.62, dd
	3′		3.10, dd (3.8, 14.3)		5,5′	129.6	6.93, d (8.4)				(10.0, 14.5)
	4	128.0			6,6′	115.0	6.57, d (8.4)		3′		3.21, dd (3.6, 14.5)
	5,5′	129.6	6.88, d (8.3)		7	155.6			4	128.1	
	6,6′	114.9	6.56, d (8.3)		NH		8.48, d (8.6)		5,5′	129.6	6.93, d (8.3)
	7	155.6		Ahp	2	169.1			6,6′	115.0	6.59, d (8.3)
	NH		8.42, d (8.7)		3	49.1	4.38, m		7	155.6	
Ahp	2	168.8			4	21.7	1.73, m		NH		8.48, d (8.6)
	3	48.8	3.61, m		4′		2.52, m	Ahp	2	169.1	
	4	21.5	1.61, m		5	29.8	1.71, m		3	49.2	4.37, m
	4′		2.40, m		6	73.4	4.88, br		4	21.7	1.72, m
	5	29.2	1.55, m		NH		7.32, d (9.1)		4′		2.53, m
	5′		1.68, m		OH		5.99, brd (3.0)		5	29.7	1.71, m
	6	73.7	5.04, brs	Leu	1	170.8			6	73.4	4.88, br
	NH		7.09, d (8.9)		2	47.7	4.58, dd (3.8, 10.7)		NH		7.32, d (9.0)
	OH		6.00, brd (2.7)		3	38.4	0.40, m		OH		6.00, brs
Phe	1	170.3			3′		1.55, m	Leu	1	170.8	
	2	50.3	4.74, dd (3.8, 11.3)		4	23.6	0.97, m		2	47.7	4.58, dd (3.5, 10.7)
	3	35.3	1.78, brdd		5	22.1	0.49, d (6.6)		3	38.4	0.41, m
			(3.8, 14.3)		5′	23.9	0.69, d (6.6)		3′		1.54, m
	3′		2.86, brdd	*N*-Me-	1	169.3			4	23.6	0.97, m
			(11.3, 14.3)	Tyr	2	60.8	4.90, brdd[a]		5	22.1	0.49, d (6.5)
	4	136.7			3	32.8	2.66, brdd[a]		5′	23.8	0.69, d (6.5)
	5,5′	129.4	6.83, d (7.3)		3′		3.07, brdd[a]	*N*-Me-	1	169.3	
	6,6′	127.7	7.18, dd (7.3, 7.3)		4	127.2		Tyr	2	60.8	4.90, brdd[a]
	7	126.2	7.13, t (7.3)		5,5′	130.0	6.89, d (8.4)		3	32.8	2.66, brdd[a]
N-Me-	1	169.1			6,6′	115.3	6.60, d (8.4)		3′		3.07, brdd[a]
Tyr	2	60.8	4.88, brd (11.3)		7	156.0			4	127.2	
	3	32.8	2.70, brdd[a]		*N*-Me	30.4	2.69, s		5,5′	130.0	6.89, d (8.2)
	3′		3.08, brd[a]		OH		9.20, s		6,6′	115.3	6.62, d (8.2)
	4	127.5		Val	1	172.1			7	156.0	
	5,5′	130.4	6.99, d (8.3)		2	55.8	4.68, dd (4.7, 9.4)		*N*-Me	30.4	2.69, s
	6,6′	115.3	6.76, d (8.3)		3	30.8	2.03, m		OH		9.21, s
	7	156.2			4	17.3	0.71, d (6.8)	Val	1	172.1	
	N-Me	30.3	2.74, s		4′	19.2	0.85, d (6.0)		2	55.9	4.67, dd (4.6, 9.4)
	OH		9.33, s		NH		7.47, d (9.4)		3	30.8	2.03, m
Val	1	171.9							4	17.3	0.71, d (6.8)
	2	55.8	4.67, dd (4.5, 9..5)						4′	19.2	0.84, d (6.1)
	3	30.8	2.03, m						NH		7.48, d (9.4)
	4	17.2	0.70, d (6.8)								
	4′	19.2	0.84, d (6.3)								
	NH		7.39, d (9.5)								

[a] Signal partially obscured.

Micropeptins C, D, E, and F inhibited chymotrypsin with IC_{50}'s of 1.1, 1.2, 1.0, and 1.5 μg/mL, respectively. These peptides did not inhibit trypsin and thrombin at 20 μg/mL, although micropeptins A and B isolated from the same strain were reported as trypsin inhibitors.[7] Specificity to serine proteases of Ahp-containing depsipeptides was related to the amino acid residue at the N-terminal side of Ahp.[15,19] When a hydrophobic residue such as Tyr or Phe connects to Ahp, peptides are known to inhibit chymotrypsin. When a basic residue such as Lys or Arg connects to Ahp, peptides are trypsin inhibitors. Micropeptin T-1, which has *N*-Me-L-Trp instead of *N*-Me-L-Tyr in micropeptin C, inhibited chymotrypsin with an IC_{50} of 3.0 μg/mL, similar to that of micropeptin C.[20] Micropeptin B, containing L-Lys instead of L-Tyr as in micropeptin C, inhibited trypsin with an IC_{50} of 0.25 μg/mL. Therefore, our results were in



3: R = C_5H_{11}, **4**: R = C_7H_{15}

a good agreement with the reported specificity to serine proteases of Ahp-containing depsipeptides.

Experimental Section

General Experimental Procedures. IR spectra were recorded on a Jasco FT/IR-460 Plus infrared spectrometer. Optical rotations were measured on a Horiba SEPA-300 high sensitive polarimeter. NMR spectra were obtained with a JEOL JMN-ECA-600 spectrometer in DMSO-d_6. The resonances of residual DMSO-d_6 at δ_H 2.49 and δ_C 39.5 ppm were used as internal references for ^{1}H and ^{13}C NMR spectra, respectively. High-resolution mass spectra were obtained with a Bruker Daltonics microTOF mass spectrometer. LC-MS analyses were carried out under the following conditions: YMC Pack Pro C18 column and Develosil ODS HG-5 column applying a MeCN/0.01% HCOOH (in H_2O) gradient with a flow rate of 0.2 mL/min.

Biological Materials. *Microcystis aeruginosa* (NIES-100) was obtained from the NIES collection (Microbial Culture Collection, the National Institute for Environmental Studies, Japan) and cultured in 5 L bottles containing MA medium with aeration at 25 °C for 2 weeks under a 12 h/12 h light−dark cycle. Cells were harvested by centrifugation, lyophilized, and kept in a freezer at −20 °C until extraction.

Isolation Procedure. The freeze-dried cells (64.5 g) were extracted with 80% MeOH and MeOH. The combined extract (12.5 g) was partitioned with diethyl ether and water. The aqueous layer was further extracted with *n*-BuOH, and the *n*-BuOH layer was subjected to ODS (YMC-GEL, ODS-A, 22 × 295 mm) flash column chromatography with aqueous MeOH followed by $CHCl_3$ to obtain fractions 1−6.

Fraction 2 (4:6 MeOH−H_2O) was subjected to reversed-phase HPLC (Develosil ODS HG-5, 10.0 × 250 mm) with a gradient of aqueous MeCN (30−45%) containing 0.05% TFA to yield crude peptide fractions I and II. Fraction I was subjected to reversed-phase HPLC (Develosil CN-UG-5, 10.0 × 250 mm) with 33% MeCN containing 0.05% TFA to yield 3.4 mg of micropeptin E (**3**). Fraction II was subjected to reversed-phase HPLC (Inertsil ODS-3, 10.0 × 250 mm) with 45% MeCN containing 0.05% TFA to yield 2.4 mg of micropeptin C (**1**).

Fraction 3 (6:4 MeOH−H_2O) was subjected to reversed-phase HPLC (Develosil ODS HG-5, 10.0 × 250 mm) with a gradient of aqueous MeCN (35−55%) containing 0.05% TFA. Final purification was achieved by reversed-phase HPLC (Inertsil ODS-3, 10.0 × 250 mm) with 50% MeCN containing 0.05% TFA to yield 5.4 mg of micropeptin F (**4**) and 3.1 mg of D (**2**).

Micropeptin C (1): colorless, amorphous solid; $[\alpha]^{25}_D$ −14 (*c* 0.05, MeOH); IR ν_{max} 3367, 3299, 2958, 2929, 2894, 2852, 1734, 1641 cm^{-1}; ^{1}H NMR and ^{13}C NMR data, see Table 1; HRMS (ESI-TOF) *m/z* 1026.4786 [M − H]$^-$ (calcd for $C_{53}H_{68}N_7O_{14}$, 1026.4830).

Micropeptin D (2): colorless, amorphous solid; $[\alpha]^{25}_D$ −6.5 (*c* 0.2, MeOH); IR ν_{max} 3369, 3298, 2958, 2929, 2891, 2852, 1732, 1641 cm^{-1}; ^{1}H NMR and ^{13}C NMR data, see Table 2; HRMS (ESI-TOF) *m/z* 1054.5161 [M − H]$^-$ (calcd for $C_{55}H_{72}N_7O_{14}$, 1054.5143).

Micropeptin E (3): colorless, amorphous solid; $[\alpha]^{25}_D$ −46 (*c* 0.1, MeOH); IR ν_{max} 3363, 3290, 2958, 2927, 2894, 2852, 1734, 1641 cm^{-1}; ^{1}H NMR and ^{13}C NMR data, see Table 2; HRMS (ESI-TOF) *m/z* 992.4993 [M − H]$^-$ (calcd for $C_{50}H_{70}N_7O_{14}$, 992.4981).

Micropeptin F (4): colorless, amorphous solid; $[\alpha]^{25}_D$ −39.5 (*c* 0.05, MeOH); IR ν_{max} 3365, 3292, 2958, 2929, 2894, 2852, 1732, 1641 cm^{-1}; ^{1}H NMR and ^{13}C NMR data, see Table 2; HRMS (ESI-TOF) *m/z* 1020.5287 [M − H]$^-$ (calcd for $C_{52}H_{74}N_7O_{14}$, 1020.5294).

Determination of the Absolute Configurations of the Amino Acids. Each compound was dissolved in 6 M HCl (500 μL). The reaction mixture was then placed in a sealed glass tube at 110 °C for 22 h. After evaporation *in vacuo*, the residue was dissolved in H_2O (100 μL), and chiral HPLC analyses were carried out using a SUMICHIRAL OA-5000 column (Sumitomo Chemical Industry, 4.6 × 150 mm, eluent: MeOH−H_2O (5:95) containing 2.0 mM $CuSO_4$; flow rate: 1.0 mL/min; UV detection: 254 nm; oven temperature: 40 °C). Retention times (min) of authentic amino acids were as follows: L-Thr (4.0), D-Thr (4.5), L-allo-Thr (5.4), D-allo-Thr (5.8), L-Val (9.4), D-Val (15.7), L-Tyr (24.2), D-Tyr (39.8), *N*-Me-L-Tyr (28.8), *N*-Me-D-Tyr (31.3), L-Glu (37.1), D-Glu (43.9), L-Leu (27.5), D-Leu (45.7), L-Phe (79.3), and D-Phe (113.5). Retention times of the hydrolysate of **1** were as follows: L-Thr (4.0), L-Val (9.4), L-Tyr (24.2), *N*-Me-L-Tyr (28.8), L-Glu (37.1), and L-Phe (79.3). Retention times of the hydrolysate of **3** were as follows: L-Thr (4.0), L-Val (9.4), L-Tyr (24.3), L-Leu (27.6), *N*-Me-L-Tyr (28.8), and L-Glu (37.1). Retention times of hydrolysates of **2** and **4** were identical to those of **1** and **3**, respectively.

Synthesis of DL-Pentahomoserines. Each of Boc-D- and Boc-L-Glu(OBzl) (200 mg) was dissolved in anhydrous THF (1.0 mL), and then $LiBH_4$ (31.5 mg) was added to the solution with stirring at room temperature under argon for a further 16 h. EtOAc (1.0 mL) was added, and the solution was stirred at room temperature for 3 h. After evaporation, the reaction mixture was subjected to Si gel (silica gel 60, 70−230 mesh, Merck Ltd., 15 × 235 mm) column chromatography with $CHCl_3$ to $CHCl_3$−MeOH (1:1). The fraction containing Boc-pentahomoserine was evaporated and dissolved in HCOOH (1.0 mL). After stirred at room temperature for 4 h, the reaction mixture was evaporated and purified using RP-HPLC (Develosil C30 UG-5, 10.0 × 250 mm; H_2O; 3.0 mL/min: UV detection 210 nm) to obtain D- and L-pentahomoserine.

Reduction of Micropeptin C (1) to F (4). Each of **1** to **4** (300 μg) was dissolved in anhydrous MeOH (1.0 mL), and then an excess amount of $NaBH_4$ was added to the solution with stirring at room temperature. After stirring for 3 h, H_2O was added and evaporated. The reaction mixture was passed through a disposable ODS column (YMC Dispo SPE C18; H_2O−MeOH) and evaporated. The MeOH extract was dissolved in 6 N HCl (500 μL) and placed in a sealed glass tube at 110 °C for 16 h. After evaporation *in vacuo*, the residue was dissolved in H_2O (300 μL), and chiral HPLC analyses were carried out using a SUMICHIRAL OA-5000 column (Sumitomo Chemical Industry, 4.6 × 150 mm, eluent: H_2O containing 2.0 mM $CuSO_4$; flow rate: 1.0 mL/min; UV detection: 254 nm; oven temperature: 40 °C). Retention times (min) of standard amino acids were as follows: L-pentahomoserine (5.4), D-pentahomoserine (8.2). Retention times of the reductive hydrolysates of **1** to **4**: L-pentahomoserine (5.4).

Protease Inhibition Assay. All the enzymes and substrates were purchased from Sigma Chemical Co. The enzyme (α-chymotrypsin type II) was dissolved in 50 mM Tris-HCl (pH 7.6) to prepare a 15 U/mL solution. A 1 mg/mL solution of *N*-succinyl-L-phenylalanyl-*p*-nitroanilide in the buffer was used for the substrate solution. A 30 μL buffer solution, 50 μL enzyme solution, and 20 μL of test solution were added to each microtiter plate well and preincubated at 37 °C for 5 min. Then, 100 μL of substrate solution was added to start the reaction. The absorbance of the well was immediately measured at 405 nm. The developed color was measured after incubation at 37 °C for 30 min.

Thrombin inhibitory activity was determined by the modified method of Svendsen et al.[21] The following stock solutions were prepared for Tris-HCl buffer: (I) a mixture of equal volumes of 0.1 M imidazole-HCl and 0.1 M Tris-HCl; (II) a mixture of equal volumes of 0.1 M imidazole and 0.1 M Tris, both in 0.1 M NaCl. These two stock solutions were then mixed to adjust to pH 8.2 and diluted with an equal volume of 0.2 M NaCl. Thrombin was dissolved in Tris-imidazole buffer to prepare a 1.3 U/mL solution. A 0.25 mg/mL solution of *N*-benzoyl-Phe-Val-Arg-*p*-nitroanilide in buffer was used for the substrate solution. A 90 μL enzyme solution and 20 μL test solution were added to each mictotiter plate well and preincubated at 37 °C for 5 min. Then 90 μL of substrate solution was added to start the reaction. The absorbance of the well was immediately measured at 405 nm. The developed color was measured after incubation at 37 °C for 30 min.

Trypsin inhibitory activity was determined by the method of Yamaguchi et al.[22] except that the enzyme solution was 300 U/mL.

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Supporting Information Available: 1D and 2D NMR spectra for micropeptins C (**1**), D (**2**), E (**3**), and F (**4**). This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

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