Two New Chymotrypsin Inhibitors Isolated from the Cyanobacterium *Microcystis aeruginosa* NIES-88

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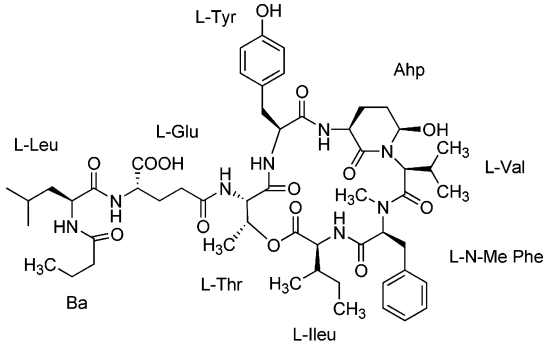
Micropeptins 88-N (**1**) and 88-Y (**2**), new 3-amino-6-hydroxy-2-piperidone (Ahp)-containing cyclic depsipeptides, were isolated from *Microcystis aeruginosa* NIES-88. The structures were elucidated by analyses of HRFABMS, 1D and 2D NMR spectra, and chemical degradation. Micropeptins 88-N and 88-Y inhibited chymotrypsin. The inhibitory activities were closely related to the amino acid residue that was attached to the amino group of Ahp.

Cyanobacteria produce many kinds of bioactive secondary metabolites including toxins and enzyme inhibitors.[1−3] 3-Amino-6-hydroxy-2-piperidone (Ahp)-containing cyclic depsipeptides are widely distributed in cyanobacteria such as *Microcystis*, *Oscillatoria*, *Nostoc*, and *Anabaena*.[4−23] These depsipeptides, such as micropeptin 90[13] and oscillapeptin G,[17] showed inhibitory activity to trypsin, chymotrypsin, tyrosinase, plasmin, and thrombin.

In *Microcystis aeruginosa*, we found two new Ahp-containing cyclic depsipeptides, **1** and **2**, during our investigation on peptides. In this paper, we report the structure elucidation of **1** and **2** and their structure−activity relationship as chymotrypsin inhibitors.



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Results and Discussion

Two new chymotrypsin inhibitors were obtained from *M. aeruginosa* NIES-88. These inhibitors were further purified by normal-phase TLC to give 6.5 mg (0.02% yield from dry algal cells) of micropeptin 88-N (**1**) and 7.5 mg (0.025% yield from dry algal cells) of micropeptin 88-Y (**2**), respectively. Using glycerol as the matrix for the positive HRFABMS of micropeptin 88-N, the $[M + H]^+$ ion was observed at *m/z* 1063.5605, and the dehydrated ion was observed at *m/z* 1045.5511 $[M - H_2O + H]^+$. From these results, the molecular formula of micropeptin 88-N was established to be $C_{54}H_{78}N_8O_{14}$ (calcd for [M + H]: 1063.5637, Δ 3.2 mmu). The ^1H and ^{13}C NMR spectral data (Table 1) suggested that **1** was a peptide. Amino acid analysis of the hydrolysate (6 N HCl, 110 °C, 20 h) indicated the presence of 1 mol each of threonine (Thr), valine (Val), isoleucine (Ile), tyrosine (Tyr), glutamic acid (Glu), *N*-methylphenylalanine (N-MePhe), and leucine (Leu). All seven of these residues were shown to have the L-configuration by LC/MS analysis of derivatives from the acid hydrolysate. Butyric acid was also identified from the hydrolysate by LC/MS analysis using methanol (50%) containing 0.01% formic acid aqueous solution.

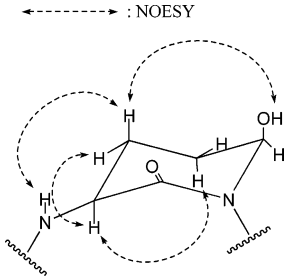

Figure 1. NOESY correlations of Ahp in cyclic depsipeptides **1** and **2** in DMSO-d_6.

In the ^1H NMR spectrum of **1**, a broad singlet was observed at 5.06 ppm. In the HSQC spectrum, this broad singlet connected with a carbon at 76.18 ppm and suggested it was both O- and N-substituted. After extensive two-dimensional NMR analyses, this broad singlet was elucidated as the H-6 signal in an Ahp unit. The ^1H and ^{13}C NMR data of the Ahp unit in **1** closely resembled those of micropeptins 88-B[20] and -C[20] and A90720A.[5] To confirm the relative stereochemistry of the Ahp in **1**, NOESY correlations were observed between Ahp-NH and H-4, H-4 and OH on C-6, and H-3 and H-5 (Figure 1). Furthermore, after the PCC oxidation of **1** followed by hydrolysis with HCl, L-Glu was identified in the hydrolysate by HPLC analysis of the L-Marfey's derivative.[24] Therefore, the configuration of the Ahp unit in **1** was determined to be (3*S*,6*R*)-3-amino-6-hydroxy-2-piperidone (L-Ahp).

The signal of H-3 in Thr was observed at 5.59 ppm and suggested that the hydroxy group of Thr was acylated. The sequence of **1** was mostly deduced by HMBC correlations from α-H's to carbonyl carbons (Table 1). The HMBC correlation from H-2 of Thr to C=O at the γ-position of Glu confirmed the iso-linkage between Thr and Glu. The methyl protons of N-MePhe showed HMBC correlations with the C=O of Val. Furthermore, Val and Ahp were connected as

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Table 1. NMR Spectral Data for Micropeptin 88-N at 500 MHz (^{1}H) and 125 MHz (^{13}C) in DMSO-d_6

unit	position	δ_H (J, Hz)	δ_C	HMBC (^{1}H)
Ba	1		175.86 (qC)	BA-2, BA-3, Leu-NH
	2	2.16, t, (7.0)	38.94 (CH_2)	
	3	1.53, m	20.52 (CH_2)	
	4	0.82, t (5.5)	13.99 (CH_3)	
Leu	1		170.51 (qC)	Leu-2, Ley-3, Glu-2
	2	4.50 (overlapped)	53.63 (CH)	Leu-NH
	3a	1.58 (dd) 14.5, 3.5)	40.80 (CH_2)	
	3b	1.95 (dd (14.5, 12.0)		
	4	1.67, m	26.03 (CH)	Leu-3a, Leu-3b, Leu-5, Leu-6
	5	0.87, d (6.0)	21.42 (CH_3)	Leu-3a, Leu-3b, Leu-6
	6	0.96, d (5.5)	23.78 (CH_3)	Leu-3a, Leu-3b, Leu-6
Glu	1		172.70 (qC)	Glu-3a, Glu-3b
	2	4.42, br d (4.5)	52.87 (CH)	
	3a	1.97 (overlapped)	29.52 (CH_2)	
	3b	2.26 (overlapped)	33.10 (CH_2)	
	4	2.48, m		
	5		175.86 (qC)	Glu-3a, Glu-3b, Thr-NH
Thr	1		171.67 (qC)	Thr-2, Thr-3, Tyr-NH
	2	4.73, d (9.0)	57.01 (CH)	Thr-4
	3	5.59, m	57.02 (CH)	Thr-4
	4	1.37, d (7)	18.80 (CH_3)	Thr-2, Thr-3
	NH	7.48, d (9.0)		
Tyr	1		173.84 (qC)	Tyr-3b, Ahp-NH
	2	4.65 (overlapped)	58.18 (CH)	Tyr-3a, Tyr-3b
	3a	2.29 (overlapped)	38.13 (CH_2)	Tyr-5, Tyr-9
	3b	3.15 (overlapped)		
	4		127.84 (qC)	Tyr-3a, Tyr-3b
	5, 9	7.07, d (7.0)	131.46 (CH)	
	6, 8	6.68, d (7.0)	116.22 (CH)	Tyr-5/Tyr-9
	7		157.27 (qC)	Tyr-5/Tyr-9, Tyr-6/Tyr-8
	NH	8.43, d (9.0)		
Ahp	2		171.3 (qC)	Ahp-2, Ahp-3, Ahp-5, Val-2
	3	4.60, m	48.79 (CH)	Ahp-NH
	4a	1.89, m	33.34 (CH_2)	Ahp-2, Ahp-5
	4b	2.79, m		
	5	1.89, m	31.34 (CH_2)	
	6	5.06, br s	76.18 (CH)	
	NH	7.27 (overlapped)		
	OH	5.99, br s		
Val	1		172.78 (qC)	Val-2, N-Me Phe-2, N-Me Phe-Me
	2	4.46 (overlapped)	58.18 (CH)	Val-3, Val-4, Val-5
	3	2.04 (overlapped)	28.93 (CH)	Val-2, Val-4, Val-5
	4	0.56, d (5.0)	17.84 (CH_3)	Val-2, Val-5
	5	−0.052, d (6.0)	17.84 (CH_3)	Val-2, Val-4
N-Me Phe	1		172.00 (qC)	N-Me Phr-2, Ileu-2
	2	5.25, br d (11.5)	62.98 (CH)	N-Me Phe-Me, N-Me Phe-3
	3a	2.85 (overlapped)	35.54 (CH_2)	
	3b	3.23 (overlapped)		
	4		138.94 (qC)	N-Me Phe-3a,3b, N-Me Phe-5/9
	5/9	7.29 (overlapped)	131.42 (CH)	N-Me Phe-3a,3b, N-Me Phe-7
	6/8	7.27 (overlapped)	129.98 (CH)	N-Me Phe-5/9
	7	7.19, t (6.5)	128.05 (CH)	N-Me Phe-5/9
	N-Me	2.83, s	31.34 (CH_3)	,N-Me Phe-2
Ileu	1		175.34 (qC)	Thr-3, Ileu-2
	2	4.50, d (4.5)	58.18 (CH)	Ileu-6
	3	1.89, m	38.13 (CH)	Ileu-4a,4b, Ileu-6
	4a	1.42, m	26.54 (CH_2)	Ileu-2
	4b	1.68, m		
	5	0.86, t (overlapped)	10.88 (CH_3)	
	6	0.93, d (7.5)	16.32 (CH_3)	Ileu-2

a hemiaminal as mentioned above. From the results, the structure of micropeptin 88-N was established as **1**.

Micropeptin 88-Y (**2**) was a colorless amorphous solid. In the positive HRFABMS using glycerol as the matrix, the $[M + H]^+$ ion was observed at *m/z* 1085.5057, and the dehydrated ion at *m/z* 1067.4955 $[M - H_2O + H]^+$ was also observed. From these results, the molecular formula of 88-Y was established to be $C_{55}H_{72}N_8O_{15}$ (calcd for $[M + H]$: 1085.5116, Δ −5.9 mmu). The ^{1}H and ^{13}C NMR spectral data of **2** (Table 2) suggested that it was also an Ahp-containing cyclic depsipeptide. Amino acid analysis of the hydrolysate (6 N HCl, 110 °C, 20 h) indicated the presence of 1 mol each of Thr, Val, Ile, Glu, and N-MePhe and 2 mol of Tyr. All of the amino acids were shown to have the L-configuration. Acetic acid was also identified from the hydrolysate by LC/MS analysis using methanol (50%) containing 0.01% formic acid aqueous solution as the mobile phase. The sequence of residues in **2** was mostly

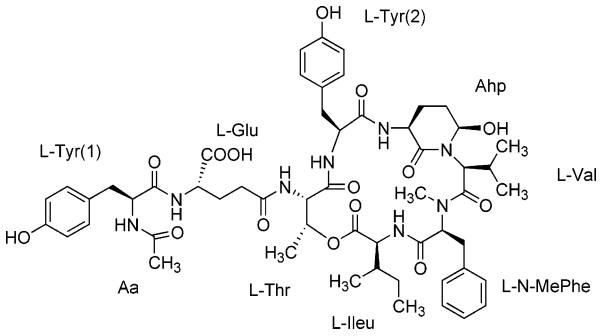
Table 2. NMR Spectral Data for Micropeptin 88-Y at 500 MHz (^{1}H) and 125 MHz (^{13}C) in DMSO-d_6

unit	position	δ_H (J, Hz)	$^1\delta_C$	HMBC (^{1}H)
Aa	1		173.14 (qC)	AA-2, Tyr(1)-NH,
	2	1.90, s (7.0)	23.59 (CH_3)	
Tyr(1)	1		173.77 (qC)	Tyr(1)-2, Glu-2
	2	4.47 (overlapped)	56.74 (CH)	Tyr(1)-NH
	3a	2.73, dd (14.5, 3.5)	36.46 (CH_2)	Tyr(1)-5/9
	3b	3.43, dd (14.5, 12.0)		
	4		129.98 (qC)	Tyr(1)-3a,3b, Tyr(1)-6/8
	5/9	7.01, d (8.5)	131.36 (CH)	Tyr(1)-3a,3b
	6/8	6.73, d (8.5)	116.43 (CH)	Tyr(1)-5/9, Tyr(1)-7
	7		155.20 (qC)	Tyr(1)-5/9
	NH	7.93, d (8.5)		
Glu	1		177.11 (qC)	Glu-3a,3b
	2	4.29, br d (4.5)	52.28 (CH)	Glu-3a, Glu-3b
	3a	2.31 (overlapped)	33.29 (CH_2)	
	3b	2.28 (overlapped)		
	4a	2.31, m	33.29 (CH_2)	
	4b	2.38, m		
	5		175.65 (qC)	Glu-3a, Glu-3b, Thr-2
Thr	1		171.67 (qC)	Thr-2, Thr-3, Thr-NH
	2	4.64, d (9.0)	56.82 (CH)	Thr-4
	3	5.54, m	73.71 (CH)	Thr-4
	4	1.30, d (7)	18.46 (CH_3)	Thr-2, Thr-3
	NH	7.48, d (9.0)		
Tyr(2)	1		173.31 (qC)	Tyr(2)-3b, Ahp-NH
	2	4.60 (overlapped)	56.60 (CH)	Tyr(2)-3a,3b
	3a	2.80 (overlapped)	38.18 (CH_2)	Tyr(2)-5/9
	3b	3.15 (overlapped)		
	4		129.98 (qC)	Tyr(2)-3a,3b
	5,9	7.07, d (7.0)	131.36 (CH)	Tyr(2)-6/8
	6,8	6.69, d (7.0)	116.18 (CH)	Tyr(2)-5/9
	7		157.20 (qC)	Tyr(2)-5/9, Tyr(2)-6/8
	NH	8.43, d (9.0)		
Ahp	2		171.50 (qC)	Ahp-2, Ahp-3, Ahp-5, Val-2
	3	4.61, m	51.09 (CH)	Ahp-NH
	4a	1.89, m	31.25 (CH_2)	Ahp-2, Ahp-5
	4b	2.80, m		
	5	1.87, m	31.34 (CH_2)	
	6	5.07, br s	76.16 (CH)	
	NH	7.27 (overlapped)		
Val	1		172.73 (qC)	Val-2, N-Me Phe-2, N-Me Phe-Me
	2	4.49 (overlapped)	56.74 (CH)	Val-3, Val-4, Val-5
	3	2.05 (overlapped)	28.90 (CH)	Val-2, Val-4, Val-5
	4	−0.04, d (5.0)	19.53 (CH_3)	Val-2, Val-5
	5	0.58, d (6.0)	18.46 (CH_3)	Val-2, Val-4
N-Me Phe	1		171.90 (qC)	N-Me Phe-2, Ileu-2
	2	5.23, br d (11.5)	63.00 (CH)	N-Me Phe-Me, N-Me Phe-3a,3b
	3a	2.83 (overlapped)	35.55 (CH_2)	N-Me Phe-2, N-Me Phe-5/9
	3b	3.23 (overlapped)		
	4		138.95 (qC)	N-Me Phe-3a,3b, N-Me Phe-5/9
	5/9	7.29 (overlapped)	129.39 (CH)	N-Me Phe-3a,3b, N-Me Phe-6/8
	6/8	7.26 (overlapped)	128.36 (CH)	N-Me Phe-5/9
	7	7.18, t (6.5)	130.96 (CH)	N-Me Phe-5/9
	N-Me	2.83, s	31.58 (CH_3)	N-Me Phe-2
Ileu	1		175.32 (qC)	Thr-3, Ileu-3
	2	4.58, d (4.5)	56.60 (CH)	Ileu-6
	3	1.90, m	38.37 (CH)	Ileu-4a,4b, Ileu-6
	4a	1.14, m	26.52 (CH_2)	Ileu-2
	4b	1.41, m		
	5	0.85, t (overlapped)	11.11 (CH_3)	Ileu-3
	6	0.94, d (7.5)	16.43 (CH_3)	Ileu-2

deduced by HMBC correlations from α-H's to carbonyl carbons (Table 2) and closely resembled those of **1**. The only difference between **2** and **1** was that the N-terminus of **2** contained an acetyl Tyr instead of an N-butyl Leu residue. From these results, the structure of micropeptin 88-Y was established as **2**.

A trypsin inhibitor was isolated from *M. aeruginosa* GU-1, and its structure was elucidated in a manner similar to that followed for **1** and **2**. On the basis of the molecular formula, amino acid composition, and 1D and 2D NMR spectra, the structure of **3** was suggested to be identical to micropeptin 90.[13]

In this study, the inhibitory activities of these cyclic peptides (**1**−**3**) against chymotrypsin and trypsin were assayed. The Ahp-containing cyclic peptides **1** and **2** showed inhibitory activities against chymotrypsin, and not against trypsin, whereas peptide **3** showed inhibitory activity against trypsin, and not against chymotrypsin. The



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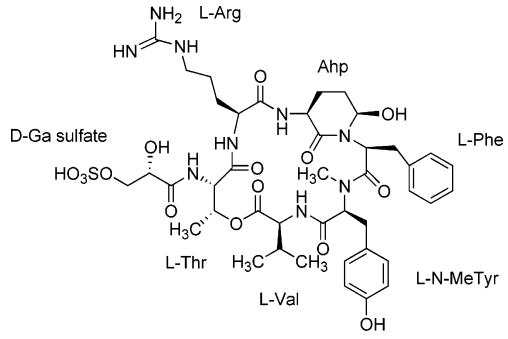
specificity of inhibitory activities of these Ahp-containing cyclic depsipeptides against chymotrypsin and trypsin appears to be due to the variations in the amino acid sequences of Tyr-Ahp in micropeptins 88-N and 88-Y and of Arg-Ahp in micropeptin 90, because it is known that chymotrypsin hydrolyzes the carboxy group side of Tyr, Phe, or hydrophobic amino acids, and trypsin hydrolyzes the carboxy group side of Arg or Lys in peptides.



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Many Ahp-containing cyclic depsipeptides have been obtained from cyanobacteria. Figure 2 presents the general structure of the peptides. In this general structure, A is an amino acid, esterified glyceric acid, or fatty acid-containing oligopeptides. B, C, and X are amino acids. In Ahp-containing cyclic depsipeptides, which show inhibitory activity against chymotrypsin and trypsin, amino acids occurring at B and C are usually Ile, Leu, Val, or Phe. In rare cases, Thr is located at C as seen in the molecule of oscillapeptin G.[17] Interestingly, Phe (micropeptin T-20[14]), Tyr (micropeptins 88-C,[21] 88-F,[21] SD979,[22] and SD1002[22]), HomoTyr (oscillapeptin[16]), or Leu (oscillapeptin G[17]) occur at X in the X-Aph sequence of chymotrypsin inhibitors. In peptides **1** and **2**, the new chymotrypsin inhibitors, Tyr also occurs at X. As presented in Table 3, the Tyr-Ahp sequence-containing peptides showed almost the same inhibitory activities. On the other hand, Arg (micropeptins SD999[22] and 90,[13] cyanopeptorin S,[9] A90720A[5]) or Lys (micropeptins SD944[22] and A,[14] oscillapeptin D[18]) occurs at X in the trypsin inhibitors. When a hydrophobic amino acid occurs at X, the Ahp-containing cyclic depsipeptide strongly inhibits chymotrypsin; however it does not inhibit trypsin. When basic amino acids such as Arg or Lys occur at X, the depsipeptides inhibit trypsin and not chymotyrypsin. This correlation can be extended to Ahp-containing cyclic depsipeptide analogues. On the basis of the correlation, aeruginopeptins[6,7] must be chymotrypsin inhibitors since the amino acid at X in the sequence of Thr-X-Ahp is Tyr or tetrahydroTyr. Furthermore, oscillapeptin J[19] must be a trypsin inhibitor since the amino acid at X is Arg. When

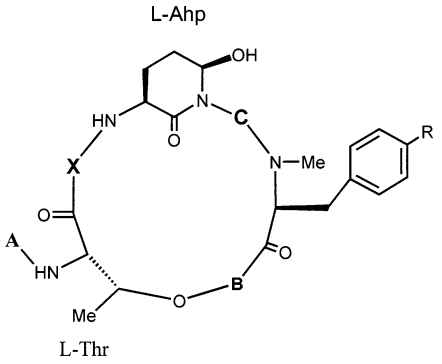

Figure 2. General structure of Ahp-containing cyclic depsipeptides. A: Glyceric acid sulfate (phosphate) ester or acylated di- or tripeptide. B: Leu, Ile, or Val. C: Phe, Val, Ile, Leu, or Thr. X: Lys, Arg, Tyr, Phe, Leu, or Glu. R: H or OH.

Table 3. Correlation between X in the General Structure of Ahp-Containing Cyclic Depsipeptides Shown in Figure 2 and Inhibitory Activities against Chymotrypsin and Trypsin

		inhibitory activity (IC_{50})	
Aph-containing cyclic depsipeptide [ref]	X	chymotrypsin (μM)	trypsin (μM)
micropeptin 88-N	Tyr	15	ni[a]
micropeptin 88-Y	Tyr	1.3	ni
micropeptin T-20 [14]	Phe	2.5×10^{-3}	ni
micropeptin 88-C [20]	Tyr	4.6	ni
micropeptin 88-D [20]	HcAla[b]	9	ni
micropeptin 88-E [20]	Leu	5	ni
micropeptin 88-F [20]	Tyr	3.4	ni
oscillapeptin [16]	HomoTyr	1.9	ni
oscillapeptin G [17]	Leu	4	ni
micropeptin SD979 [22]	Tyr	2.5	ni
micropeptin SD1002 [22]	Tyr	3.2	ni
micropeptin 88-B [20]	Glu	ni	ni
nostcyclin [15]	HomoSer	ni	ni
micropeptin 90 [13]	Arg	ni	2×10^{-2}
micropeptin A [12]	Lys	ni	7×10^{-2}
cyanopeptorin S [9]	Arg	ni	0.2
oscillapeptin D [18]	Lys	ni	1.3×10^{-2}
A90720A [5]	Arg	ni	1×10^{-3}
micropeptin SD944 [22]	Lys	ni	8.5
micropeptin SD999 [22]	Arg	ni	4

[a] ni, not inhibited ($>10^{-4}$ M). [b] HcAla, 3-(4′-hydroxy-2′-cyclohexyl)alanine.

HomoSer (nostocyclin[15]) or Glu (micropeptin 88-B[20]) occurs at X, a noninhibitory activity is observed against both trypsin and chymotrypsin.

By Lineweaver–Burk plots[26] of the proteases in the presence and absence of the inhibitors showed the competitive inhibition of the inhibitors against chymotrypsin and trypsin. These results suggest that these inhibitors attach to the active sites of the enzymes and do not undergo hydrolysis. As a result, the inhibitors block the active centers for chymotrypsin and trypsin. Probably, the three-dimensional structure of the peptide bond formed between X and Ahp in the inhibitors is different from that of peptide bonds formed between normal amino acids such as those occurring between digestible linear peptides.

The structures of peptides **1** and **2** and micropeptin 88-C precisely agree with each other except at the N-terminus. The residues butylLeu, acetylTyr, and butylTyr are located at N-termini of peptides **1** and **2** and micropeptin 88-C, and their chymotrypsin inhibitory concentrations (IC_{50}) are 1.5×10^{-2}, 1.3×10^{-3}, and 4.6×10^{-3} μM, respectively. The presence of butylLeu at the N-terminus of peptide **1** appears to weaken the inhibitory activity in comparison with those of micropeptins 88-Y and 88-C.

Experimental Section

General Experimental Procedures. NMR spectra were recorded on a JEOL JNM A-500 spectrometer (500 MHz). Chemical shifts of ^{1}H and ^{13}C NMR were referenced to TMS. Homonuclear ^{1}H connectivities were determined by COSY and HOHAHA experiments, and heteronuclear ^{1}H−^{13}C connectivities were determined by HSQC and HMBC experiments. Using glycerol as a matrix, fast-atom bombardment mass spectra (FABMS) were obtained on a JEOL JMS-700 mass spectrometer.

Chemicals. D- and L-amino acids were obtained from Sigma Chemical Company (St. Louis, MO). Solvents for NMR were purchased from E. Merck (Darmstadt, Germany). All other chemicals and solvents were of analytical grade. An Ahp-containing cyclic depsipeptide, nostcyclin,[14] was purified previously and was stored at −20 °C. D- and L-*N*-Methyltyrosine[14] were derived from D- and L-*N*-methylphenylalanine, respectively, which were purchased from Sigma.

Culture Conditions. *Microcystis aeruginosa* strain NIES-88 was obtained from the Microbial Culture Collection, National Institute for Environmental Studies, Japan. *M. aeruginosa* NIES-88 was grown axenically in a batch culture in 10 L volumes of MA medium of the following composition: 5 mg of $Ca(NO_3)_2 \cdot H_2O$, 10 mg of KNO_3, 5 mg of $NaNO_3$, 4 mg of Na_2SO_4, 5 mg of $MgCl_2 \cdot 6H_2O$, 10 mg of β-Na_2glycerophosphate$\cdot 5H_2O$, 0.5 mg of Na_2EDTA, 0.05 mg of $FeCl_3 \cdot 6H_2O$, 0.5 mg of $MnCl_2 \cdot 4H_2O$, 0.05 mg of $ZnCl_2$, 0.5 mg of $CoCl_2 \cdot 6H_2O$, 0.08 mg of $Na_2MoO_4 \cdot 2H_2O$, 2 mg of H_2BO_3, 50 mg of Bicine, and 100 mL of distilled water, pH 8.6. The cells were isothermally grown at 25 °C. Cultures were sparged with air at an approximate rate of 1.5 L/min. Light was supplied using white fluorescent tubes: an irradiance of approximately 250 μmol photon$\cdot m^{-2} \cdot s^{-1}$ was incident on the surface of the vessels. Cells of each strain were harvested from the stationary phase by centrifugation at 10 000*g* for 20 min to yield a pellet that was freeze-dried. *M. aeruginosa* GU-1 was also mass cultured in MA medium to obtain micropeptin 90.

Extraction and Isolation. Methanol extracts from 30 g of freeze-dried cells of *M. aruginosa* NIES-88 were evaporated under reduced pressure. The remaining residue was suspended in 5% (v/v) aqueous acetic acid solution. The suspension was centrifuged at 2000 rpm for 20 min, and the supernatant was retained. The extract was fractionated using Sep-pak C18 cartridges with 20% and 80% aqueous MeOH. The inhibitors present in the 80% MeOH fraction were isolated by reverse-phase HPLC (Mightysil RP-18, 20 mm i.d. × 25 cm) with methanol (60%) containing 0.05 M phosphate buffer (pH 3.0) at 10 mL/min. The isolated inhibitors were further purified by HPTLC (E. Merck, silica gel 60 containing fluorescent indicator) using chloroform/methanol/water (60/40/10, v/v) as the solvent. The yields of the inhibitors from *M. aeruginosa* NIES-88, micropeptins 88-N (**1**, t_R 15.2 min, R_f 0.60) and 88-Y (**2**, t_R 15.7 min, R_f 0.68), were 6.5 and 7.5 mg, respectively.

Micropeptin 90 was isolated from 17 g of lyophilized cells of *M. aeruginosa* GU-1 by the same method described above. The yield of micropetin 90 (**3**) was 16 mg.

Hydrolysis and Amino Acid Analysis. Each of the cyclic depsipeptides (100 μg) in 6 M HCl was heated at 110 °C for 20 h. The amino acids in the hydrolysate were derivatized with Marfey reagent[24] (1-fluoro-2,4-dinitrophenyl-5-L-alanine amide) and analyzed by LC/MS with a reverse-phase column (ZORBAX Eclipse, 2.1 mm × 100 mm). A linear gradient of 30%−80% acetonitrile in 10 mM ammonium acetate buffered at pH 3.0 (flow rate, 0.2 mL/min; MS detection, SIM mode) was used. The absolute stereochemistry for each amino acid was determined by comparing the retention time with those for the authentic D- and L-amino acid derivatives.

Identification of Butyric Acid and Acetic Acid. Butyric acid and acetic acid were analyzed by LC/MS with a reverse-phase column (ZORBAX Eclipse, 2.1 mm × 100 mm) using methanol (50%) containing 0.01% formic acid aqueous solution at 0.2 mL/min.

Chymotrypsin and Trypsin Inhibition Assay. Inhibitory activities against chymotrypsin and trypsin were assayed using α-chymotrypsin type II (Sigma C-4129) and trypsin (Sigma T-0134), with *N*-benzoyl-L-tyrosine ethyl ester as a substrate for chymotrypsin[25] and *N*-benzoyl-L-arginine ethyl ester for trypsin.[18]

Micropeptin 88-N (1): colorless amorphous solid (6.5 mg, 0.02% dry wt); $[\alpha]^{22}_D$ −57 (*c* 0.05, MeOH); UV (MeOH) λ_{max} (log ϵ) 278 (3.08); ^{1}H and ^{13}C NMR, see Table 1; FABMS (pos. glycerol) *m/z* 1063 $[M + H]^+$; HRFABMS (pos. glycerol) *m/z* 1063.5605 $[M + H]^+$ (calcd for $C_{54}H_{78}N_8O_{14}$, 1063.5637).

Micropeptin 88-Y (2): colorless amorphous solid (7.5 mg, 0.025% dry wt); $[\alpha]^{22}_D$ −52 (*c* 0.05, MeOH); UV (MeOH) λ_{max} (log ϵ) 278 (3.35); ^{1}H and ^{13}CNMR, see Table 2; FABMS (pos. glycerol) *m/z* 1085 $[M + H]^+$; HRFABMS (pos. glycerol) *m/z* 1085.5057 $[M + H]^+$ (calcd for $C_{55}H_{72}N_8O_{15}$, 1085.5116).

Micropeptin 90 (3): colorless amorphous solid (16 mg, 0.094% dry wt); $[\alpha]^{22}_D$ −52 (*c* 0.05, MeOH); UV(MeOH) λ_{max} (log ϵ) 278.6 nm (3.16); FABMS (pos. glycerol) *m/z* 962 $[M + H]^+$; HRFABMS (pos. glycerol) *m/z* 962.3987 $[M + H]^+$ (calcd for $C_{42}H_{60}N_9O_{15}S$, 962.3929).

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