Pergamon

Tetrahedron 54 (1998) 5545–5556

TETRAHEDRON

Micropeptins 88-A to 88-F, Chymotrypsin Inhibitors from the Cyanobacterium *Microcystis aeruginosa* (NIES-88)

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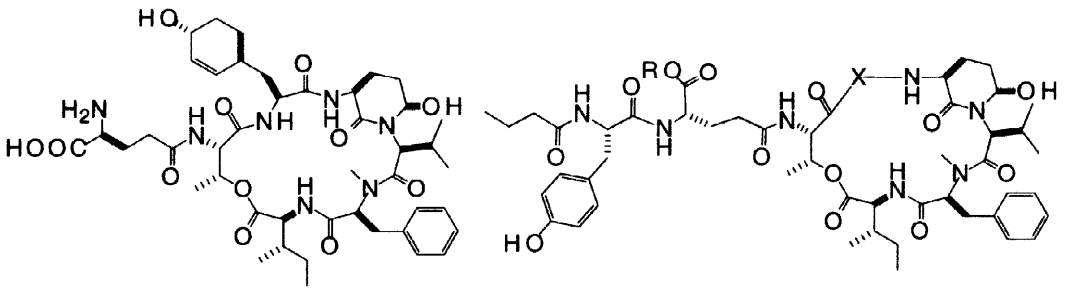
Received 9 February 1998; accepted 13 March 1998

Abstract: Micropeptins 88-A to 88-F (**1-6**) have been isolated from the cyanobacterium *Microcystis aeruginosa* (NIES-88). Their structures were determined by two-dimensional ^{1}H-^{1}H and ^{1}H-^{13}C NMR correlation experiments and confirmed by mass spectral and amino acid analyses. Their absolute stereochemistries were deduced by a combination of spectral and chemical studies. Micropeptins 88-A and 88-C to 88-F inhibited chymotrypsin potently with IC_{50} of 0.4-10.0 μg/mL.

The cyclic depsipeptides containing the Ahp (3-amino-6-hydroxy-2-piperidone) unit have been frequently found in the constituents of freshwater-blooming cyanobacteria.[1] In addition, these peptides are known to be protease inhibitors, such as micropeptins A and B,[1d] micropeptin 90[1e,1h] and micropeptins 478-A and 478-B[1i] from *Microcystis aeruginosa*, which inhibit plasmin and trypsin, and oscillapeptin[1f] from *Oscillatoria agardhii*, nostopeptins[2] from *Nostoc minutum* and micropeptin 103[1j] from *Microcystis viridis*, which inhibit elastase and chymotrypsin. In the course of our screening program of protease inhibitors from microalgae, we found that *M. aeruginosa* (NIES-88), which produces kawaguchipeptins,[3] had a potent inhibitory activity on chymotrypsin. Here we describe the isolation and structure elucidation of new chymotrypsin inhibitors, micropeptins 88-A to 88-F (**1-6**).



1

2: X=L-Glu, R=H
3: X=L-Tyr, R=H
4: X=HcAla, R=H
5: X=L-Leu, R=H
6: X=L-Tyr, R=Me

M. aeruginosa (NIES-88)[4] was isolated from a bloom in Lake Kawaguchi and mass-cultured in our laboratory as previously described.[5] The 80% methanol extract of freeze-dried alga was partitioned between

0040-4020/98/$19.00
PII: S0040-4020(98)00242-7

water and diethyl ether. The aqueous layer, which inhibited chymotrypsin, was further extracted with *n*-butanol and fractionated by ODS flash column chromatography (20-100% MeOH elution) followed by reversed-phase HPLC, using 0.05% TFA in aqueous MeCN, to yield micropeptins 88-A (**1**), 88-B (**2**), 88-C (**3**), 88-D (**4**), 88-E (**5**) and 88-F (**6**) as colorless amorphous powder.

The molecular formula of micropeptin 88-C (**3**) was established as $C_{57}H_{76}N_8O_{15}$ by the high resolution FABMS and NMR spectral data (Table 2). It's peptidic nature was suggested by the ^1H and ^{13}C NMR spectra of **3** (Table 2), and the amino acid analysis of the hydrolysate gave 1 mole each of Thr, Glu, Val, and Ile and 2 moles of Tyr. The ^1H-^1H COSY and HMQC[6] spectra also indicated the presence of these amino acids except for Val. The Val unit was suggested to be present as an *N*,*N*-disubstituted derivative, since its amide proton was not recognized (Fig. 1). The *N*-Me Phe residue was assigned from the ^1H-^1H COSY, HMQC and HMBC[7] spectra of **3**, which showed correlations from *N*-methyl protons (δ 2.72) to α-carbon (δ 60.4). The structure of Ahp was deduced as follows. In the ^1H-^1H COSY and HMQC spectra of **3**, the connectivity from NH (δ 7.42) to OH (δ 6.07) was determined. The correlation of C-2 (δ 169.3) with H-3 (δ 4.46), H-6 (δ 4.82) and H-2 (δ 4.33) of the Val derivative was observed in the HMBC spectrum of **3**. The unit containing the triplet methyl protons (δ 0.71) was identified as butyric acid (Ba) at the *N*-terminus by the ^1H-^1H COSY and HMBC spectra (Fig. 1).

The sequence of **3** was determined by the HMBC correlations from α-H, β-H and NH to C=O. The HMBC correlation from the downfield Thr H-3 (δ 5.47) to Ile C-1 (δ 172.4) confirmed the ester formation between Thr and Ile. The HMBC correlations (Thr H-2 and NH/Glu C-5, Glu H-3 and H-4/Glu C-5) and the NOESY correration (Thr NH/Glu H-4) connected Thr NH and Glu C-5 (Fig. 1).

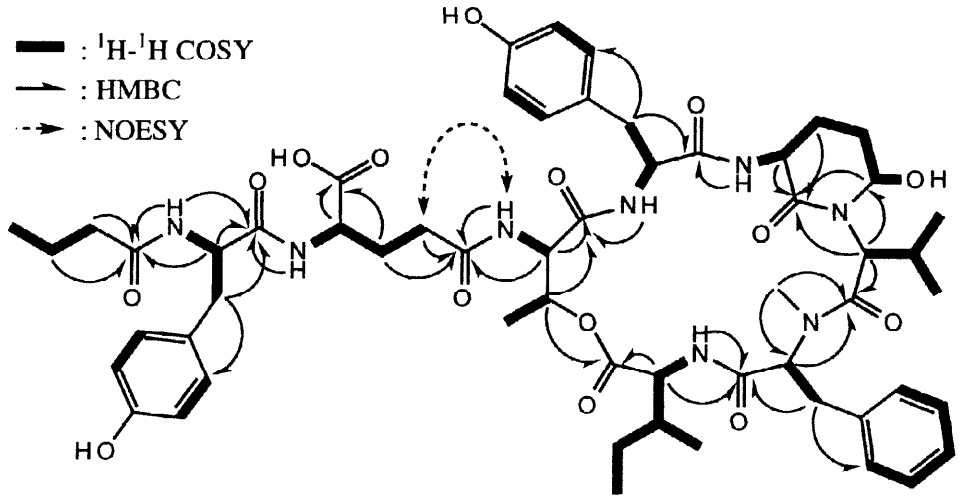


Fig. 1. ^1H-^1H COSY and HMBC correlations of micropeptin 88-C (**3**)

The stereochemistries of the usual amino acids and *N*-Me Phe were determined as all L by HPLC analysis of the derivatives of the acid hydrolysate with L- and D-Marfey's reagents.[8,9] The stereochemistry of the Ahp unit was deduced as follows. The reduction of **3** with $NaBH_4$ followed by hydrolysis with HCl afforded Pro and pentahomoserine,[10] which were proved to be both L-form from HPLC analysis of their derivatives with L- and D-Marfey's reagents (L-pentahomoserine was synthesized from Boc-L-Glu (OBzl) using $LiBH_4$). The relative stereochemistry of the Ahp unit in **3** was decided as shown in Fig. 2 by the NOESY correlations

between Ahp NH and H-4a, H-4a and OH, H-4b and H-3, and H-3 and H-5b. Therefore, the stereochemistry of Ahp was assigned as (*3S,6R*)-3-amino-6-hydroxy-2-piperidone.

The molecular formula of micropeptin 88-F (**6**) was larger than **3** by CH_2 from the HRFABMS data, and the signal of methyl protons (δ_H 3.61) was observed in the ^1H NMR spectrum, suggesting the presence of an extra methyl group. Interpretation of the NMR data and the amino acid analysis of the hydrolysate allowed that the structure of **6** was almost the same as that of **3** except for the presence of methyl protons. By the HMBC correlation from Glu methyl protons (δ_H 3.61) to Glu C-1 (δ_C 172.2), micropeptin 88-F was confirmed as methyl ester of micropeptin 88-C . The absolute stereochemistry of **6** was determined by the above-mentioned procedures.

Micropeptins 88-B (**2**) and 88-E (**5**) had the molecular formulas $C_{53}H_{73}N_8O_{16}$ and $C_{54}H_{78}N_8O_{14}$, respectively. The amino acid analyses of the acid hydrolysates suggested that these peptides were closely related to micropeptin 88-C (**3**) except for the presence of Glu and Leu in **2** and **5**, respectively, instead of one of two Tyr in **3**. Interpretations of the NMR data of **2** allowed that the structure of **2** was almost the same as that of **3** except for the presence of Glu (II) instead of Tyr (II) in **3**. Glu (II) was determined as the α-linkage by the HMBC correlations (Ahp NH/Glu (II) C-1 and Glu (II) H-4/Glu (II) C-5), the NOESY correlations (Ahp NH/Glu (II) H-2 and Glu (II) NH) and the chemical shift of Glu (II) C-5 (δ_C 173.6). Interpretations of the NMR data of **5** allowed that the structure of **5** was also almost the same as that of **3** except for the presence of the Leu unit instead of Tyr (II) in **3**. The absolute stereochemistries of **2** and **5** were determined by the above-mentioned procedures (The stereochemistry of the usual amino acids in **2** was determined as all L by the Chiral GC analysis of *N*-trifluoroacetyl isopropyl ester derivatives of the acid hydrolysate.).

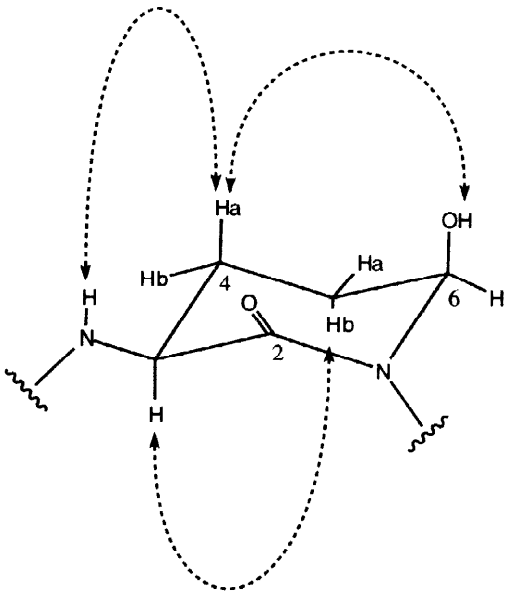


Fig. 2. Relative stereochemistry of the Ahp unit in micropeptin 88-C (**3**) (Dashed arrows: NOESY)

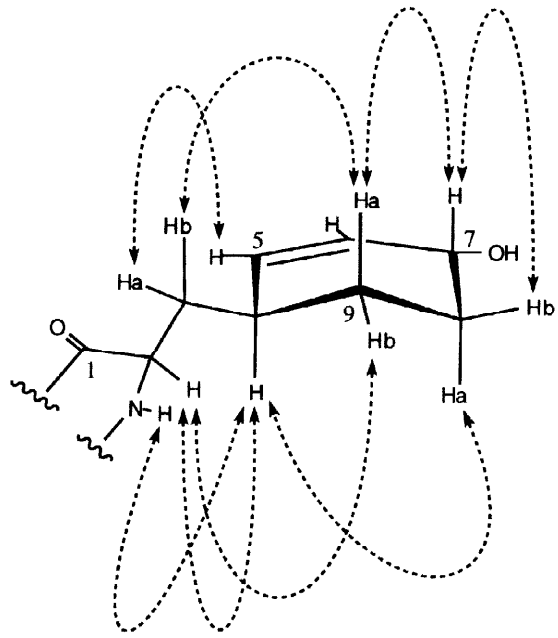
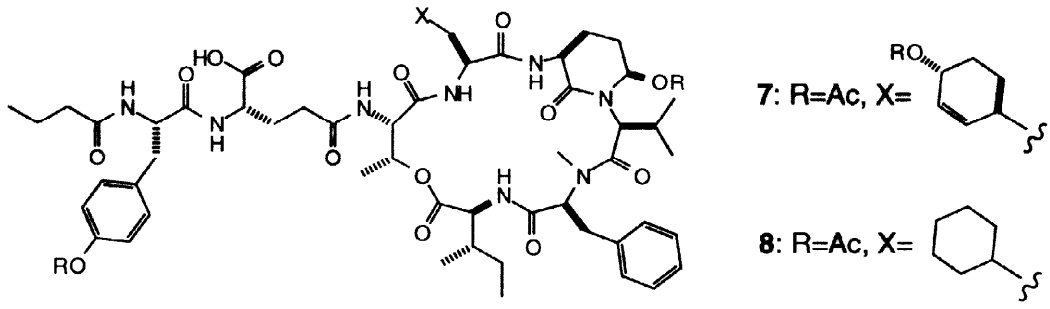


Fig. 3. Relative stereochemistry of the HcAla unit in micropeptin 88-D (**4**) (Dashed arrows: NOESY)

The molecular weight of micropeptin 88-D (**4**) was larger than **3** by four daltons. The amino acid analysis of the hydrolysate allowed the absence of 1 mole of Tyr in **3**, and one disubstituted E-geometric

double bond (δ_H 5.43, brd, J=10.0 Hz and δ_H 5.59, ddd, J=10.0, 2.4, 2.4 Hz) in the ^{1}H NMR spectrum suggested the presence of the 3-(4'-hydroxy-2'-cyclohexenyl)alanine (HcAla) unit,[11] which was decided by the ^{1}H-^{1}H COSY and HMBC spectra.[12] Interpretations of the NMR data of **4** allowed that the structure of **4** was almost the same as that of **3** except for the presence of the HcAla unit instead of the Tyr (II) unit in **3**. The absolute stereochemistry except the HcAla unit of **4** was determined by the above-mentioned procedures. The stereochemistry of HcAla was deduced as follows. The hydrogenated product (**8**) of micropeptin 88-D triacetate (**7**) was hydrolyzed with HCl to afford 2-amino-3-cyclohexylpropionic acid, which was proved to be *S* configuration from the HPLC analysis of its derivatives with L- and D-Marfey's reagents ((*S*)-2-amino-3-cyclohexylpropionic acid[13] was synthesized from L-Phe using PtO_2). The relative stereochemistry of HcAla unit in **4** was decided as shown in Fig. 3 by NOESY correlations (HcAla NH/H-4, H-2/H-4, H-2/H-9b, H-3a/H-5, H-3b/H-9a, H-4/H-8a, H-7/H-8b and H-7/H-9a). Therefore, the stereochemistry of HcAla was decided to be (2*S*, 1'*S*, 4'*R*)-3-(4'-hydroxy-2'-cyclohexenyl)alanine.



Micropeptin 88-A (**1**) had the molecular formula $C_{44}H_{65}N_7O_{12}$. The presence of two methine protons (δ_H 5.44 and 5.60) in the ^{1}H NMR spectrum suggested that **1** was closely related to **4**. The amino acid analysis of the hydrolysate of **1** did not allow the presence of Tyr. The methyl protons (δ_H 0.71) and two methylene protons (δ_H 1.38 and 1.99) due to the butyric acid unit detected in the ^{1}H NMR spectrum of **4** were not observed in that of **1**. These results and interpretation of the NMR data allowed that the structure of **1** was almost the same as that of **4** except for the absence of Glu and the butyric acid unit in **4**. The absolute stereochemistry except for the HcAla unit of **1** was also determined by the above-mentioned procedures, and the similarity of ^{1}H and ^{13}C NMR signals due to the HcAla unit in **1** and **4** (Tables 1 and 2, respectively) indicated that the stereochemistry of the HcAla unit in **1** was also (2*S*, 1'*S*, 4'*R*).

Micropeptin 88-A (**1**) inhibited chymotrypsin and elastase with IC_{50}'s of 0.4 and 3.5 μg/mL, respectively. Micropeptins 88-C to 88-F (**3**-**6**) also inhibited chymotrypsin with IC_{50}'s of 5.0, 10.0, 5.2 and 3.4 μg/mL, respectively, but did not inhibit elastase at 100 μg/mL. Micropeptin 88-B (**2**) did not inhibit chymotrypsin and elastase at 100 μg/mL. These peptides did not inhibit thrombin, trypsin, plasmin and papain at 100 μg/mL. These results suggest that the amino acid between Thr and Ahp is involved in the chymotrypsin inhibition-mechanism. Moreover, the side chain (buytric acid-Tyr-Glu-) may also relate to the mechanism.

Experimental Section

General Information. Ultraviolet spectra were measured on a Hitachi 330 spectrophotometer. Optical rotations were measured on a JASCO DIP-1000 polarimeter. ^{1}H and ^{13}C NMR spectra were measured on

Table 1. ^1H and ^{13}C NMR Data for Micropeptins 88-A (1) and 88-B (2) in DMSO-d_6

		micropeptin 88-A (1)				micropeptin 88-B (2)		
Position		^1H J (Hz)	^{13}C		Position	^1H J (Hz)	^{13}C	
Glu	1		172.1	(s)	Butyric	1	171.8	(s)
	2	3.68 (br)	52.5	(d)	acid	2	2.00 (t,7.3)	37.1 (t)
	3a	1.90 (m)	26.6	(t)		3	1.38 (m)	18.5 (t)
	3b	2.00 (m)				4	0.73 (t,7.5)	13.4 (t)
	4	2.45 (m)	31.0	(t)	Tyr	1		171.8 (s)
						2	4.48 (m)	53.8 (d)
	5		172.2	(s)		3a	2.61 (dd,13.9,10.4)	36.7 (t)
Thr	1		169.5	(s)		3b	2.92 (dd,13.9,3.7)	
	2	4.59 (d, 9.2)	55.3	(d)		4		126.6 (s)
	3	5.48 (q, 7.0)	71.5	(d)		5,9	7.03 (d,9.0)	130.0 (d)
	4	1.23 (d, 7.0)	17.8	(q)		6,8	6.62 (d,9.0)	114.7 (d)
	NH	8.22 (d, 9.2)				7		155.6 (s)
HcAla	1		170.6	(s)		NH	7.84 (d,8.8)	
	2	4.32 (br)	50.0	(d)	Glu(I)	1		172.4 (s)
	3a	1.49 (m)	36.4	(t)		2	4.22 (ddd,7.7,6.2,4.4)	51.4 (d)
	3b	1.84 (m)				3a	1.84 (m)	27.0 (t)
	4	2.03 (m)	31.6	(d)		3b	2.00 (m)	
	5	5.44 (d, 10.2)	132.0	(d)		4a	2.34 (m)	31.2 (t)
	6	5.60 (dd, 10.2, 2.6)	132.6	(d)		4b	2.40 (m)	
	7	3.99 (m)	65.1	(d)		5		173.1 (s)
	8a	1.25 (m)	31.3	(t)	Thr	1		169.5 (s)
	8b	1.83 (m)				2	4.58 (d,8.8)	55.5 (d)
	9a	1.00 (m)	25.8	(t)		3	5.48 (m)	71.5 (d)
	9b	1.74 (m)				4	1.23 (m)	17.7 (q)
	NH	8.45 (d, 8.4)				NH	8.13 (d,8.8)	
Ahp	2		169.4	(s)	Glu(II)	1		170.0 (s)
	3	4.45 (ddd, 12.1, 9.1, 6.6)	49.7	(d)		2	4.43 (m)	51.4 (d)
	4a	1.71 (m)	21.7	(t)		3	1.71 (m)	
	4b	2.52 (m)				4a	2.60 (m)	
	5a	1.71 (m)	29.7	(t)		4b	2.69 (m)	
	5b	1.80 (m)				5		173.6 (s)
	6	4.93 (d, 3.3)	74.0	(d)		NH	8.56 (d,8.5)	
	NH	7.40 (d, 9.1)			Ahp	2		169.1 (s)
	OH	6.11 (d, 3.3)				3	4.47 (m)	48.8 (d)
Val	1		169.6	(s)		4a	1.71 (m)	21.6 (t)
	2	4.31 (br)	55.7	(d)		4b	2.52 (m)	
	3	1.92 (m)	27.4	(d)		5a	1.72 (m)	29.6 (t)
	4	-0.20 (d, 6.4)	17.9	(q)		5b	1.80 (m)	
	4'	0.47 (d, 6.6)	18.0	(q)		6	4.93 (d,3.1)	73.9 (d)
N-Me Phe	1		169.1	(s)		NH	7.42 (d,8.6)	
	2	5.09 (dd, 11.4, 3.3)	60.4	(d)		OH	6.11 (d,3.1)	
	3a	2.81 (dd, 14.6, 11.4)	34.1	(t)	Val	1		169.7 (s)
	3b	3.25 (br)				2	4.32 (d,10.4)	55.7 (d)
	4		137.5	(s)		3	1.90 (m)	27.3 (d)
	5,9	7.24 (d, 8.4)	129.6	(d)		4	-0.20 (d,6.5)	17.8 (q)
	6,8	7.27 (dd, 8.4, 7.0)	128.5	(d)		4'	0.47 (d,6.9)	17.9 (q)
	7	7.19 (t, 7.0)	126.6	(d)	*N*-Me Phe	1		169.1 (s)
	N-Me	2.72 (s)	30.0	(q)		2	5.07 (dd,11.7,3.1)	60.4 (d)
Ile	1		172.6	(s)		3a	2.80 (dd,14.2,11.7)	34.1 (t)
	2	4.76 (dd, 9.5, 5.5)	55.4	(d)		3b	3.27 (dd,14.2,3.1)	
	3	1.78 (m)	37.2	(d)		4		137.5 (s)
	4a	1.00 (m)	24.5	(t)		5,9	7.25 (d,8.1)	129.6 (d)
	4b	1.25 (m)				6,8	7.28 (dd,8.1,7.8)	128.5 (d)
	5	0.80 (dd, 7.7, 7.3)	11.1	(q)		7	7.18 (t,7.8)	126.5 (d)
	6	0.84 (d, 6.6)	16.0	(q)		*N*-Me	2.72 (s)	30.0 (q)
	NH	7.71 (9.5)			Ile	1		172.5 (s)
						2	4.75 (m)	55.4 (d)
						3	1.78 (m)	37.2 (d)
						4a	1.00 (m)	24.5 (t)
						4b	1.25 (m)	
						5	0.79 (m)	11.1 (q)
						6	0.84 (m)	15.9 (q)
						NH	7.67 (m)	

Table 2. ^{1}H and ^{13}C NMR Data for Micropeptins 88-C (3) and 88- D (4) in DMSO-d_6

	micropeptin 88-C (3)				micropeptin 88-D (4)			
Position		^{1}H	J (Hz)	^{13}C	Position	^{1}H	J (Hz)	^{13}C
Butyric acid	1			171.83 (s)	Butyric acid 1			171.83 (s)
	2	2.00	(m)	37.16 (t)	2	1.99	(m)	37.3 (t)
	3	1.38	(m)	18.5 (t)	3	1.38	(m)	18.5 (t)
	4	0.71	(t, 7.3)	13.4 (q)	4	0.71	(t, 7.3)	13.5 (q)
Tyr(I)	1			171.83 (s)	Tyr 1			171.83 (s)
	2	4.48	(ddd, 10.2, 8.6, 3.9)	53.9 (d)	2	4.49	(ddd, 10.7, 8.6, 3.9)	53.8 (d)
	3a	2.61	(dd, 13.6, 10.2)	36.7 (t)	3a	2.61	(dd, 14.1, 10.7)	37.2 (t)
	3b	2.93	(dd, 13.6, 3.9)		3b	2.93	(dd, 14.1, 3.9)	
	4			128.3 (s)	4			128.3 (s)
	5, 9	7.03	(d, 8.1)	130.0 (d)	5, 9	7.03	(d, 8.6)	130.1 (d)
	6, 8	6.61	(d, 8.1)	114.7 (d)	6, 8	6.61	(d, 8.6)	114.7 (d)
	7			155.6 (s)	7			155.6 (s)
	NH	7.86	(d, 8.6)		NH	7.85	(d, 8.6)	
	7-OH	9.10	(s)		Glu 1			173.2 (s)
Glu	1			173.2 (s)	2	4.22	(ddd, 9.0, 8.1, 4.7)	51.5 (d)
	2	4.20	(ddd, 9.0, 7.7, 4.7)	52.4 (d)	3a	1.80	(m)	27.1 (t)
	3a	1.80	(m)	27.1 (t)	3b	2.01	(m)	
	3b	2.01	(m)		4	2.36	(m)	31.4 (t)
	4a	2.27	(ddd, 15.4, 10.3, 5.1)	31.3 (t)	5			171.76 (s)
	4b	2.36	(ddd, 15.4, 10.3, 5.1)		NH	8.21	(d, 8.1)	
	5			171.76 (s)	Thr 1			169.5 (s)
	NH	8.19	(d, 7.7)		2	4.62	(brd, 9.0)	55.1 (d)
Thr	1			169.1 (s)	3	5.47	(q, 6.4)	71.7 (d)
	2	4.64	(d, 8.6)	54.7 (d)	4	1.21	(d, 6.4)	17.6 (q)
	3	5.47	(q, 6.8)	72.0 (d)	NH	8.04	(d, 9.0)	
	4	1.15	(d, 6.8)	17.6 (q)	HcAla 1			170.6 (s)
	NH	7.87	(d, 8.6)		2	4.33	(br)	49.8 (d)
Tyr(II)	1			169.9 (s)	3a	1.50	(m)	36.8 (t)
	2	4.39	(ddd, 9.8, 8.6, 4.3)	54.4 (d)	3b	1.83	(m)	
	3a	2.57	(dd, 14.5, 9.8)	35.2 (t)	4	2.02	(m)	31.6 (d)
	3b	3.23	(dd, 14.5, 4.3)		5	5.43	(brd, 10.0)	132.1 (d)
	4			128.5 (s)	6	5.59	(ddd, 10.0, 2.4, 2.4)	132.7 (d)
	5, 9	6.97	(d, 8.1)	129.8 (d)	7	3.98	(m)	65.1 (d)
	6, 8	6.60	(d, 8.1)	114.9 (d)	8a	1.23	(m)	31.4 (t)
	7			155.6 (s)	8b	1.82	(m)	
	NH	8.51	(d, 8.6)		9a	0.99	(m)	25.9 (t)
Ahp	2			169.3 (s)	9b	1.72	(m)	
	3	4.46	(ddd, 11.5, 9.0, 6.8)	48.9 (d)	NH	8.40	(d, 8.6)	
	4a	1.77	(m)	21.6 (t)	Ahp 2			169.4 (s)
	4b	2.56	(m)		3	4.45	(ddd, 12.0, 9.4, 6.8)	48.7 (d)
	5a	1.72	(m)	29.6 (t)	4a	1.71	(m)	21.7 (t)
	5b	1.81	(m)		4b	2.56	(m)	
	6	4.92	(m)	73.9 (d)	5a	1.70	(m)	29.7 (t)
	NH	7.39	(d, 9.0)		5b	1.80	(m)	
	6-OH	6.07	(d, 3.4)		6	4.92	(br)	74.0 (d)
Val	1			169.6 (s)	NH	7.42	(d, 9.4)	
	2	4.33	(d, 10.3)	55.7 (d)	6-OH	6.08	(d, 3.0)	
	3	1.90	(m)	27.4 (d)	Val 1			169.6 (s)
	4	0.48	(d, 6.8)	18.0 (q)	2	4.31	(d, 10.3)	55.7 (d)
	4'	-0.20	(d, 6.4)	17.9 (q)	3	1.89	(m)	27.4 (d)
N-Me Phe	1			169.0 (s)	4	0.48	(d, 6.8)	18.0 (q)
	2	5.06	(dd, 11.5, 3.4)	60.4 (d)	4'	-0.20	(d, 6.4)	17.9 (q)
	3a	2.80	(dd, 14.2, 11.5)	34.2 (t)	*N*-Me Phe 1			169.1 (s)
	3b	3.24	(dd, 14.2, 3.4)		2	5.07	(dd, 11.5, 3.0)	60.4 (d)
	4			137.5 (s)	3a	2.81	(dd, 14.3, 11.5)	34.2 (t)
	5, 9	7.23	(d, 7.7)	129.6 (d)	3b	3.26	(dd, 14.3, 2.8)	
	6, 8	7.26	(dd, 7.7, 7.7)	128.5 (d)	4			137.6 (s)
	7	7.18	(dd, 7.7, 7.7)	126.6 (d)	5, 9	7.23	(d, 8.1)	129.6 (d)
	N-Me	2.72	(s)	30.0 (q)	6, 8	7.26	(dd, 8.1, 7.3)	128.5 (d)
Ile	1			172.4 (s)	7	7.18	(dd, 7.3, 7.3)	126.7 (d)
	2	4.74	(dd, 9.7, 5.3)	55.4 (d)	*N*-Me	2.72	(s)	30.0 (q)
	3	1.76	(m)	37.2 (d)	Ile 1			172.4 (s)
	4a	0.97	(m)	24.4 (t)	2	4.77	(dd, 9.6, 5.4)	55.4 (d)
	4b	1.20	(m)		3	1.79	(m)	37.2 (d)
	5	0.78	(t, 7.3)	11.2 (q)	4a	0.98	(m)	24.4 (t)
	6	0.80	(d, 6.8)	16.0 (q)	4b	1.21	(m)	
	NH	7.64	(d, 9.4)		5	0.79	(t, 7.3)	11.2 (q)
					6	0.82	(d, 6.8)	16.0 (q)
					NH	7.66	(d, 9.4)	

Table 3. ^{1}H and ^{13}C NMR Data for Micropeptins 88-E (5) and 88-F (6) in DMSO-d_6

Position		micropeptin 88-E (5) ^{1}H	J (Hz)	^{13}C		Position		micropeptin 88-F (6) ^{1}H	J (Hz)	^{13}C	
Butyric	1			171.83	(s)	Butyric	1			171.8	(s)
acid	2	1.99	(m)	37.2	(t)	acid	2	2.00	(m)	37.1	(t)
	3	1.38	(m)	18.5	(t)		3	1.38	(m)	18.5	(t)
	4	0.71	(t, 7.3)	13.4	(q)		4	0.71	(t, 7.3)	13.4	(q)
Tyr	1			171.83	(s)	Tyr(I)	1			172.0	(s)
	2	4.48	(ddd, 10.3, 8.6, 3.8)	53.9	(d)		2	4.48	(ddd, 10.3, 7.3, 4.1)	53.8	(d)
	3a	2.61	(dd, 14.1, 10.3)	36.7	(t)		3a	2.61	(dd, 13.7, 10.3)	36.8	(t)
	3b	2.93	(dd, 14.1, 3.8)				3b	2.90	(dd, 13.7, 4.1)		
	4			128.3	(s)		4			128.3	(s)
	5, 9	7.03	(d, 8.1)	130.0	(d)		5, 9	7.03	(d, 8.1)	130.0	(d)
	6, 8	6.61	(d, 8.1)	114.7	(d)		6, 8	6.61	(d, 8.1)	114.7	(d)
	7			155.6	(s)		7			155.64	(s)
	NH	7.84	(d, 8.6)				NH	7.86	(d, 8.6)		
Glu	1			173.2	(s)	Glu-*O*Me	1			172.2	(s)
	2	4.22	(ddd, 9.0, 8.1, 4.7)	52.4	(d)		2	4.20	(ddd, 8.6, 7.7, 4.7)	52.4	(d)
	3a	1.81	(m)	27.1	(t)		3a	1.80	(m)	27.1	(t)
	3b	2.01	(m)				3b	2.01	(m)		
	4a	2.35	(ddd, 15.0, 10.3, 6.0)	31.3	(t)		4a	2.27	(ddd, 15.0, 9.8, 5.6)	31.3	(t)
	4b	2.37	(ddd, 15.0, 10.0, 5.6)				4b	2.36	(ddd, 15.0, 9.8, 6.4)		
	5			172.1	(s)		5			171.7	(s)
	NH	8.20	(d, 7.7)				NH	8.25	(d, 7.7)		
Thr	1			169.4	(s)		OMe	3.61	(s)	51.8	(q)
	2	4.65	(d, 9.4)	54.7	(d)	Thr	1			169.0	(s)
	3	5.47	(q, 6.4)	72.0	(d)		2	4.64	(dd, 9.4, 1.3)	54.4	(d)
	4	1.19	(d, 6.4)	17.6	(q)		3	5.47	(qd, 6.0, 1.3)	72.1	(d)
	NH	8.02	(d, 9.4)				4	1.15	(d, 6.0)	17.6	(q)
Leu	1			170.7	(s)		NH	7.83	(d, 9.4)		
	2	4.30	(ddd, 12.4, 7.3, 3.4)	50.5	(d)	Tyr(II)	1			169.9	(s)
	3a	1.37	(m)	39.3	(t)		2	4.39	(ddd, 9.8, 9.0, 4.3)	54.2	(d)
	3b	1.83	(m)				3a	2.55	(dd, 14.3, 9.8)	35.2	(t)
	4	1.53	(m)	24.2	(d)		3b	3.24	(dd, 14.3, 4.3)		
	5	0.78	(d, 6.8)	21.1	(q)		4			128.5	(s)
	5'	0.88	(d, 6.8)	23.3	(q)		5, 9	6.97	(d, 8.1)	129.8	(d)
	NH	8.36	(d, 9.0)				6, 8	6.60	(d, 8.1)	114.9	(d)
Ahp	2			169.4	(s)		7			155.58	(s)
	3	4.45	(ddd, 12.4, 9.4, 6.8)	48.9	(d)		NH	8.50	(d, 9.0)		
	4a	1.71	(m)	21.8	(t)	Ahp	2			169.3	(s)
	4b	2.55	(m)				3	4.46	(ddd, 11.6, 9.4, 6.5)	49.0	(d)
	5a	1.71	(m)	29.6	(t)		4a	1.77	(m)	21.6	(t)
	5b	1.79	(m)				4b	2.57	(m)		
	6	4.92	(m)	74.0	(d)		5a	1.72	(m)	29.6	(t)
	NH	7.42	(d, 9.4)				5b	1.81	(m)		
	6-OH	6.07	(d, 3.0)				6	4.82	(m)	73.9	(d)
Val	1			169.6	(s)		NH	7.39	(d, 9.4)		
	2	4.33	(d, 10.3)	55.7	(d)		6-OH	6.07	(d, 3.0)		
	3	1.90	(m)	27.4	(d)	Val	1			169.6	(s)
	4	0.48	(d, 6.8)	18.0	(q)		2	4.33	(d, 10.7)	55.7	(d)
	4'	-0.20	(d, 6.4)	17.9	(q)		3	1.90	(m)	27.4	(d)
N-Me Phe	1			169.0	(s)		4	0.48	(d, 6.8)	18.0	(q)
	2	5.06	(dd, 11.5, 3.4)	60.4	(d)		4'	-0.20	(d, 6.8)	17.9	(q)
	3a	2.80	(dd, 14.5, 11.5)	34.2	(t)	*N*-Me Phe	1			169.0	(s)
	3b	3.24	(dd, 14.5, 3.4)				2	5.06	(dd, 11.8, 3.2)	60.4	(d)
	4			137.5	(s)		3a	2.80	(dd, 14.3, 11.8)	34.2	(t)
	5, 9	7.23	(d, 7.7)	129.6	(d)		3b	3.23	(dd, 14.3, 3.2)		
	6, 8	7.26	(dd, 8.1, 7.7)	128.5	(d)		4			137.5	(s)
	7	7.18	(dd, 8.1, 8.1)	126.6	(d)		5, 9	7.23	(d, 7.5)	129.6	(d)
	N-Me	2.72	(s)	30.0	(q)		6, 8	7.26	(dd, 7.7, 7.5)	128.5	(d)
Ile	1			172.5	(s)		7	7.18	(dd, 7.7)	126.6	(d)
	2	4.78	(dd, 9.6, 5.4)	55.4	(d)		*N*-Me	2.72	(s)	30.0	(q)
	3	1.76	(m)	37.2	(d)	Ile	1			172.4	(s)
	4a	0.97	(m)	24.4	(t)		2	4.74	(dd, 9.4, 5.1)	55.4	(d)
	4b	1.20	(m)				3	1.76	(m)	37.2	(d)
	5	0.80	(t, 7.3)	11.2	(q)		4a	0.97	(m)	24.4	(t)
	6	0.82	(d, 6.8)	16.0	(q)		4b	1.20	(m)		
	NH	7.64	(d, 9.8)				5	0.78	(t, 7.3)	11.2	(q)
							6	0.80	(d, 6.8)	16.0	(q)
							NH	7.64	(d, 9.4)		

either a JEOL JNM-A500, -A600 or Bruker AM600 NMR spectrometer. Two-dimensional NMR spectra of micropeptins 88-A and 88-B were recorded on a Bruker AM600 NMR spectrometer equipped with an ASPECT 1000 computer. Two-dimensional NMR spectra of micropeptins 88-C to 88-F were recorded on a JEOL JNM-A500 NMR spectrometer equipped with a VAXserver 4000-200 computer. FAB mass spectra, including high resolution mass measurements, were measured on a JEOL SX-102 mass spectrometer. Amino acid analyses were carried out with a Hitachi L-8500A amino acid analyzer.

Cultivation of Alga. Culture conditions were the same as previously described.[3a]

Isolation of Micropeptins 88-A to 88-F. Freeze-dried alga (121.7 g from 590 L of culture) was extracted with 80% MeOH (2 L × 2) and MeOH (2 L × 1). Combined 80% MeOH and MeOH extracts were concentrated to an aqueous suspension which was then extracted with ether. The aqueous layer was extracted with *n*-BuOH. The *n*-BuOH layer was evaporated under reduced pressure to green dry solid (7.0 g), which was subjected to flash chromatography on ODS (YMC-GEL, 120 Å, 10 × 12 cm) with aqueous MeOH followed by CH_2Cl_2.

The 50% MeOH fraction (230.8 mg) was subjected to reversed-phase HPLC (Cosmosil MS, 10 × 250 mm; 30-65% MeCN containing 0.05% TFA; UV-detection at 210 nm; flow rate 2.0 mL/min) to yield micropeptins 88-D (**4**, 17.8 mg) and 88-C (**3**, 22.6 mg).

The 60% MeOH fraction (527.5 mg) was subjected to reversed-phase HPLC (Capcell Pak C18 UG, 20 × 250 mm; 38-55% MeCN containing 0.05% TFA; UV-detection at 210 nm; flow rate 6.0 mL/min) to yield micropeptin 88-C (**3**, 19.8 mg) and crude peptide fractions **I**, **II**, **III** and **IV**. The fraction **I** was subjected to reversed-phase HPLC (Cosmosil MS, 10 × 250 mm; 35-50% MeCN containing 0.05% TFA; UV-detection at 210 nm; flow rate 2.0 mL/min) to yield micropeptin 88-B (**2**, 8.4 mg). The fraction **II** was subjected to reversed-phase HPLC (Deverosil ODS-5, 10 × 250 mm; 35-55% MeCN containing 0.05% TFA; UV-detection at 210 nm; flow rate 2.0 mL/min) to yield micropeptin 88-C (**3**, 13.6 mg). The fraction **III** was subjected to reversed-phase HPLC (Cosmosil MS, 10 × 250 mm; 40% MeCN containing 0.05% TFA; UV-detection at 210 nm; flow rate 2.0 mL/min) to yield micropeptin 88-D (**4**, 21.8 mg). The fraction **IV** was subjected to reversed-phase HPLC (Cosmosil MS, 10 × 250 mm; 40% MeCN containing 0.05% TFA; UV-detection at 210 nm; flow rate 2.0 mL/min) to yield micropeptins 88-D (**4**, 17.0 mg), 88-C (**3**, 84.1 mg), 88-E (**5**, 20.1 mg) and 88-F (**6**, 9.7 mg).

The 80% MeOH fraction (287.2 mg) was subjected to reversed-phase HPLC (Cosmosil MS, 10 × 250 mm; 30-60% MeCN containing 0.05% TFA; UV-detection at 210 nm; flow rate 2.0 mL/min) to yield micropeptin 88-D (**4**, 13.9 mg) and crude peptide fractions **V** and **VI**. The fraction **V** was subjected to reversed-phase HPLC (Cosmosil MS, 10 × 250 mm; 33% MeCN containing 0.05% TFA; UV-detection at 210 nm; flow rate 2.0 mL/min) followed by reversed-phase HPLC (Capcell Pak C18 UG, 10 × 250 mm; 33% MeCN containing 0.05% TFA; UV-detection at 210 nm; flow rate 2.0 mL/min) to yield micropeptin 88-A (**1**, 1.8 mg). The fraction **VI** was subjected to reversed-phase HPLC (Cosmosil MS, 10 × 250 mm; 40% MeCN containing 0.05% TFA; UV-detection at 210 nm; flow rate 2.0 mL/min) to yield micropeptins 88-C (**3**, 20.8 mg), 88-E (**5**, 12.1 mg) and 88-F (**6**, 5.3 mg).

Micropeptin 88-A (1). $[\alpha]_D^{23}$ -46.7° (*c* 0.10, MeOH); UV (MeOH) λmax 278 nm (ε 1170); ^1H and ^{13}C NMR see Table 1; HRFABMS *m/z* 884.4726 $[M + H]^+$ ($C_{44}H_{66}N_7O_{12}$, Δ -4.4 mmu).

Micropeptin 88-B (2). $[\alpha]_D^{23}$ -17.2° (*c* 0.50, MeOH); UV (MeOH) λmax 278 nm (ε 1100); ^1H and ^{13}C NMR see Table 1; HRFABMS *m/z* 1079.5208 $[M + H]^+$ ($C_{53}H_{74}N_8O_{16}$, Δ -9.4 mmu).

Micropeptin 88-C (3). $[\alpha]_D^{23}$ -47.4° (*c* 0.10, MeOH); UV (MeOH) λmax 278 nm (ε 3000); ^1H and ^{13}C NMR see Table 2; HRFABMS *m/z* 1095.5405 $[M - H_2O + H]^+$ ($C_{57}H_{75}N_8O_{14}$, Δ +0.3 mmu).

Micropeptin 88-D (4). $[\alpha]_D^{23}$ -29.2° (*c* 0.10, MeOH); UV (MeOH) λmax 278 nm (ε 1600); ^1H and ^{13}C NMR see Table 2; HRFABMS *m/z* 1117.5814 $[M + H]^+$ ($C_{57}H_{81}N_8O_{15}$, Δ -0.7 mmu), *m/z* 1099.5706 $[M - H_2O + H]^+$ ($C_{57}H_{79}N_8O_{14}$, Δ -1.0 mmu), *m/z* 1081.5596 $[M - 2H_2O + H]^+$ ($C_{57}H_{77}N_8O_{13}$, Δ -1.4 mmu).

Micropeptin 88-E (5). $[\alpha]_D^{23}$ -64.0° (*c* 0.10, MeOH); UV (MeOH) λmax 278 nm (ε 1830); ^1H and ^{13}C NMR see Table 3; HRFABMS *m/z* 1063.5716 $[M + H]^+$ ($C_{54}H_{79}N_8O_{14}$, Δ -6.0 mmu).

Micropeptin 88-F (6). $[\alpha]_D^{23}$ -27.2° (*c* 0.05, MeOH); UV (MeOH) λmax 278 nm (ε 2700); ^1H and ^{13}C NMR see Table 3; HRFABMS *m/z* 1109.5500 $[M - H_2O + H]^+$ ($C_{58}H_{77}N_8O_{14}$, Δ -5.9 mmu).

Acid Hydrolysis. For amino acid analysis, 100 μg each of **1** to **6** in 0.5 mL of 6 N HCl was heated at 110 °C for 16 h. The reaction mixture was dried, dissolved in 0.6 mL of 0.02 N HCl and subjected to amino acid analysis. Retention times (min) of the standard amino acids: Thr (15.04-15.33), Glu (20.42-20.80), Val (40.05-40.64), allo Ile (43.78), Ile (45.76-46.29), Leu (47.44) and Tyr (49.84-50.29). Retention times (min) in the amino acid analyses of **1** to **6**: **1**, Thr (15.06), Glu (20.48), Val (40.64) and Ile (46.32); **2**, Thr (15.01), 2×Glu (20.34), Val (40.58), Ile (46.16) and Tyr (50.08); **3**, Thr (15.33), Glu (20.93), Val (40.02), Ile (45.81) and 2×Tyr (49.81); **4**, Thr (15.30), Glu (20.77), Val (39.97), Ile (45.65) and Tyr (49.70); **5**, Thr (15.30), Glu (20.82), Val (40.10), Ile (45.84), Leu (47.49) and Tyr (49.84); **6**, Thr (15.33), Glu (20.82), Val (40.00), Ile (45.73) and 2×Tyr (49.73).

HPLC Analyses of the Marfey Derivatives. To each acid hydrolysate of a 100 μg portion of the peptides, 50 μL of 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (L-FDAA) in acetone (10 mg/mL) and 100 μL of 1 M $NaHCO_3$ were added, and the mixture was kept at 80 °C for 3 min. To the reaction mixture, 50 μL of 2 N HCl and 300 μL of 50% MeCN were added and analyzed by reversed-phase HPLC (Cosmosil MS, 4.6 × 250 mm; gradient elution from H_2O/TFA (100:0.1) to MeCN/H_2O/TFA (60:40:0.1) in 60 min; UV-detection 340 nm; flow rate 1.0 mL/min). Retention times (min) of the standard amino acids: L-Glu (39.6), D-Glu (40.6), L-Thr (37.0), L-allo-Thr (37.4), D-allo-Thr (38.5), D-Thr (40.2), L-Val (47.4), D-Val (51.4), L-Ile (50.8) and D-Ile (55.0); and amino acid derivatives from **1**: L-Glu (39.6), L-Thr (37.0), L-Val (47.4) and L-Ile (50.8). Retention times (min) of the standard amino acids: L-Glu (39.4), D-Glu (40.5), L-Thr (37.2), L-allo-Thr (37.6), D-allo-Thr (38.7), D-Thr (40.4), L-Val (47.6), D-Val (51.8), L-Ile (51.3), D-Ile (55.6), L-Leu (52.3), D-Leu (56.2), L-Tyr (57.9) and D-Tyr (61.1); and amino acid derivatives from **3-6**: L-Glu (39.5), L-Thr (37.2), L-Val (47.5), L-Ile (51.3), L-Leu (**5**; 52.2) and L-Tyr (57.8).

N-Me D- and L-Phe were also derivatized as above. The derivatives were analyzed by reversed-phase HPLC (Cosmosil MS, 4.6 × 250 mm; mobile phase MeCN/H_2O/TFA (35:65:0.1); UV-detection 340 nm; flow rate 1.0 mL/min). Retention times of standards (min): *N*-Me L-Phe (23.2) and *N*-Me D-Phe (24.6); and amino acid derivative from **1**: *N*-Me Phe (23.4). Retention times of standards (min): *N*-Me L-Phe (23.0) and *N*-Me D-Phe (24.4); and amino acid derivative from **2**: *N*-Me Phe (23.0). Retention times of standards (min): *N*-Me L-Phe (22.8) and *N*-Me D-Phe (24.2); and amino acid derivative from **3-6**: *N*-Me Phe (22.8).

Chiral GC Analyses of Micropeptin 88-B (2). To the acid hydrolysate of a 100 μg portion of **2**, 10% HCl in *i*-PrOH (400 μL) was added and heated at 100 °C for 30 min. The product was evaporated, dissolved in trifluoroacetic anhydride (200 μL) and CH_2Cl_2 (200 μL), reacted at 100 °C for 5 min and evaporated. The residue was dissolved in CH_2Cl_2 and analyzed by GC with a Chiral-L-Val capillary column (Chrompak, 0.25 mm × 25 m). The oven temperature was maintained for 5 min at 60 °C and raised to 200 °C at 4 °C/min and at

200 °C for 3 min. Retention times of the standard amino acids (min): D-Val (12.29), L-Val (13.47), D-Thr (13.13), L-Thr (14.57), D-Ile (15.11), L-Ile (16.25), D-Glu (28.12), L-Glu (28.86), D-Tyr (33.09) and L-Tyr (33.83). Retention times of the amino acid derivatives of **2** (min): L-Val (13.44), L-Thr (14.53), L-Ile (16.28), L-Glu (28.86) and L-Tyr (33.79).

Synthesis of L-Pentahomoserine. Boc L-Glu (OBzl) (1.0 g) was dissolved in dried THF (5.0 mL) and then 132 mg of $LiBH_4$ was added to the solution with stirring at room temperature under argon. After stirred for further 16 h, EtOAc (5.0 mL) was added and stirred at room temperature for 3 h. After evaporation, the reaction mixture was subjected to silica gel column chromatography (Wako gel C-300, 3 × 9 cm) with $CHCl_3$ to $CHCl_3$/MeOH (1:1). The fraction containing Boc L-pentahomoserine (863 mg) was evaporated and HCOOH (10 mL) was added and stirred at room temperature for 4 h. The reaction mixture was concentrated to yield an orange oil (722 mg). A 53.6 mg portion of the orange oil was subjected to reversed-phase HPLC (Cosmosil $5NH_2$, 10 × 250 mm; H_2O; flow rate 2.0 mL/min; UV detection 210 nm) to yield L-pentahomoserine (46.3 mg). L-Pentahomoserine: $[\alpha]_D^{18}$ +4.9° (*c* 0.5, H_2O) and +14.8° (*c* 0.5, fresh in 5N HCl); HRFABMS *m/z* 134.0820 ($C_5H_{12}O_3N$, Δ +0.3 mmu); ^1H and ^{13}C NMR (DMSO-d_6; 600 MHz), H-2 (δ_H 3.03, br, δ_C 54.8), H-3 (δ_H 1.50, m, 1.73, m, δ_C 29.9), H-4 (δ_H 1.50, m, δ_C 29.3) and H-5 (δ_H 3.36, t, 6.4, δ_C 60.7). [lit.[14] $[\alpha]_D^{25}$ +6.0° (*c* 2.0, H_2O) and +28.8° (*c* 2.0, fresh in 5N HCl).]

Reduction of Micropeptins 88-A (1) to 88-F (6). Each of **1** to **6** (500 μg) was dissolved in dried MeOH (1.0 mL), and then an excess amount of $NaBH_4$ was added to the solution with stirring at room temperature. After the mixture was stirred for further 3 h, H_2O was added and evaporated. The reaction mixture was passed through a disposable ODS column (YMC Dispo SPE C18; H_2O-50% MeCN) and evaporated, and the residue was dissolved in 6 N HCl (500 μL) and heated at 110 °C for 16 h. To each acid hydrolysate of the reduced products of **1** to **6** was added 50 μL of L-FDAA in acetone (10 mg/mL) and 10 μL of 1 M $NaHCO_3$, and the mixture was kept at 80 °C for 3 min, added 50 μL of 2 N HCl and 200 μL of 50% MeCN, and analyzed by ODS-HPLC: column Cosmosil MS (4.6 × 250 mm); gradient elution from H_2O/TFA (100:0.1) to MeCN/H_2O/TFA (60:40:0.1) in 60 min; UV detection at 340 nm. L-Pentahomoserine was derivatized with D- and L-FDAA as described above, respectively. Retention times of standards (min): L-pentahomoserine-L-FDAA (39.3), L-pentahomoserine-D-FDAA (39.8), L-Pro (42.4) and D-Pro (43.4). Retention times of the acid hydrolysates of reduced products of **1** to **6** (min): pentahomeserine (39.3) and Pro (42.4).

Synthesis of (2*S*)-2-Amino-3-Cyclohexylpropionic Acid. A solution of L-Phe (1 g) in H_2O (3.0 mL) and glacial acetic acid (6.0 mL) was treated with PtO_2 (50 mg) and 45 psi of hydrogen at 50 °C for 18 h on a Parr shaker apparatus. The reaction mixture was cooled to room temperature and filtered. The filtrate was evaporated in vacuo and the remaining solid triturated with Et_2O and filtered. The white solid was subjected to flash chromatography on ODS (YMC-GEL ODS-A 120-230/70, 3 × 8 cm) with H_2O and aqueous MeOH to yield (2*S*)-2-amino-3-cyclohexylpropionic acid (902 mg; 87%): $[\alpha]_D^{23}$ +13.9 ° (*c* 0.1, 6 N HCl); HRFABMS *m/z* 172.1358 ($C_9H_{18}O_2N$, Δ +2.1 mmu); ^1H NMR (D_2O; 600 MHz), δ 0.78-0.90 (m, 2H), 1.00-1.18 (m, 3H), 1.26 (m, 1H), 1.50-1.66 (m, 7H) and 3.62 (dd, 8.8, 5.5, 1H). [lit.[13] ^1H NMR (D_2O; 300 MHz), δ 0.80-1.00 (m, 2H), 1.05-1.46 (m, 4H), 1.50-1.80 (m, 7H) and 3.72 (dd, 1H)]

Acetylation and Hydrogenation of Micropeptin 88-D (4). Micropeptin 88-D (3.0 mg) was dissolved in pyridine (0.5 mL) and then anhydrous acetic acid (0.5 mL) was added to the solution and stirred at room temperature for 20 h. The reaction mixture was evaporated, lyophylized, and subjected to reversed-phase

HPLC (Cosmosil MS, 10 × 250 mm; 50-90% MeCN containing 0.05% TFA; flow rate 2.0 mL/min; UV detection 210 nm) to yield the triacetate (**7**; 3.9 mg): FABMS (matrix: glycerol, negative) *m/z* 1241 $[M-H]^-$; ^1H NMR (CD_3OD), butyric acid [δ 2.18(H-2, t, 7.7), 1.55 (H-3, tq, 7.7, 7.5), 0.85 (H-4, t, 7.5)], Tyr (OAc) [δ 4.72 (H-2, dd, 8.6, 6.8), 3.00 (H-3a, dd, 14.1, 8.6), 3.16 (H-3b, dd, 14.1, 6.8), 7.29 (H-5,9, d, 8.6), 7.01 (H-6,8, d, 8.6), 2.24 (Ac, s)], Glu [δ 4.39 (H-2, dd, 8.6, 4.7), 1.87 (H-3a, m), 2.25 (H-3b, m), 2.38 (H-4a, m), 2.46 (H-4b, m)], Thr [δ 4.68 (H-2, br), 5.60 (H-3, m), 1.37 (H-4, d, 6.4)], HcAla (OAc) [δ 4.56 (H-2, m), 1.67 (H-3a, m), 2.07 (H-3b, m), 2.26 (H-4, m), 5.65 (H-5, brd, 10.3), 5.72 (H-6, brd, 10.3), 5.23 (H-7, m), 1.55 (H-8a, m), 1.87 (H-8b, m), 1.27 (H-9a, m), 1.87 (H-9b, m), 2.00 (Ac, s)], Ahp (OAc) [δ 4.59 (H-2, ddd, 12.4, 9.0, 6.8), 1.86 (H-3a, m), 2.80 (H-3b, m), 1.90 (H-4, m), 5.07 (H-5, br), 2.02 (Ac, s)], Val [δ 4.46 (H-2, d, 10.7), 2.04 (H-3, m), -0.08 (H-4, d, 6.8), 0.57 (H-4', d, 6.8)], *N*-Me Phe [δ 5.25 (H-2, dd, 11.5, 2.7), 2.80 (H-3a, dd, 14.8, 11.5), 3.47 (H-3b, dd, 14.8, 2.7), 7.27 (H-5,9, d, 7.7), 7.25 (H-6,8, dd, 7.7, 6.8), 7.18 (H-7, t, 6.8), 2.83 (*N*-Me, s)], Ile [δ 4.53 (H-2, dd, 8.6, 7.7), 1.89 (H-3, m), 1.16 (H-4a, m), 1.40 (H-4b, m), 0.87 (H-5, t, 7.5), 0.93 (H-6, d, 6.8).

Compound **7** was dissolved in EtOH (1.0 mL) and then 5.0 mg of palladium black was added to the solution with stirred at room temperature for 5 h under hydrogen atmosphere. The reaction mixture was filtered and subjected to reversed-phase HPLC (Cosmosil MS, 10 × 250 mm; 50-90% MeCN containing 0.05% TFA; flow rate 2.0 mL/min; UV detection 210 nm) to yield **8** (200 μg): FABMS (matrix: glycerol, negative) *m/z* 1185 $[M-H]^-$. ^1H NMR spectrum of **8** allowed the absence of the signals due to the HcAla (OAc) unit observed in that of **7** [H-5 (δ 5.65), H-6 (δ 5.72) and Ac (δ 2.00)].

Compound **8** (100 μg) was dissolved in 6 N HCl (400 μL) and heated at 110 °C for 16 h. The acid hydrolysate of **8** was derivatized with L-FDAA and analyzed by reversed-phase HPLC as above. (2*S*)-2-Amino-3-cyclohexylpropionic acid was also derivatized with D- and L-FDAA as above, respectively. Retention times of standards (min): (2*S*)-2-amino-3-cyclohexylpropionic acid-L-FDAA (60.8) and (2*S*)-2-amino-3-cyclohexylpropionic acid-D-FDAA (65.2). Retention time of the acid hydrolysate of **8** (min): 60.8.

Protease Inhibitory Assay. Serine and cysteine protease inhibitory activities were determined by the method previously described.[15]

Acknowledgment. We thank Prof. K. Yamaguchi, Tokyo University of Agriculture & Technology, for reading this manuscript and valuable discussion. Thanks are also due to Dr. T. Watanabe, Mr. M. Horigome and Miss K. Uchida, The University of Tokyo, for advice and guidance on the synthesis of (2*S*)-2-amino-3-cyclohexylpropionic acid. This work was partly supported by a Grant-in-aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan. K. Ishida and H. Matsuda are financially supported by Research Fellowships of the Japan Society for the Promotion of Science for Young Scientists.

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