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Four New Microginins, Linear Peptides from the Cyanobacterium *Microcystis aeruginosa*

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Abstract: Four new linear peptides, related to microginins 299-A (**1**) and 299-B (**2**), were isolated from the cyanobacterium *Microcystis aeruginosa*. Microginins 299-C (**3**) and 299-D (**4**), from *M. aeruginosa* (NIES-299), inhibited leucine aminopeptidase with IC_{50}'s of 2.0 and 6.4 μg/mL, respectively. Microginins 99-A (**5**) and 99-B (**6**), from *M. aeruginosa* (NIES-99), did not inhibit leucine aminopeptidase. These structures were elucidated to be **3**-**6** on the basis of 2D NMR data and chemical degradation.

Microcystis aeruginosa has been shown to be a rich source of unique and bioactive secondary metabolites, especially peptides.[1] In 1993, we reported isolation and structure of microginin, a linear pentapeptide, as an angiotensin-converting enzyme inhibitor from the cyanobacterium *M. aeruginosa* (NIES-100).[2] Recently, the total synthesis of microginin has been reported by Shioiri and co-workers,[3] and the asymmetric synthesis of the *N*-terminal unit, 3-amino-2-hydroxydecanoic acid (Ahda), of microginin has been also reported by Davies and co-workers.[4] The linear peptides of the microginin type have been also isolated from the cultured cyanobacterium *Oscillatoria agardhii* (NIES-610)[5] and a water bloom of *Microcystis* species in a German lake.[6] In the course of our screening program of protease inhibitors from microalgae, we previously isolated microginins 299-A (**1**) and 299-B (**2**),[7] leucine aminopeptidase inhibitors, from the cyanobacterium *M. aeruginosa* (NIES-299). Further investigation on the extract of this cyanobacterium led to isolation of two new congeners, microginins 299-C (**3**) and 299-D (**4**). Moreover, microginins 99-A (**5**) and 99-B (**6**),[8] two new congeners, were also isolated from the cyanobacterium *M. aeruginosa* (NIES-99). Here we describe the isolation and structure elucidation of these peptides.

M. aeruginosa (NIES-299)[9] was isolated from a bloom in Lake Kasumigaura and mass-cultured in our laboratory as previously described. The 80% methanol extract of freeze-dried alga was partitioned between water and diethyl ether. The aqueous layer 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 microginins 299-C (**3**, 7.0 mg) and 299-D (**4**, 5.0 mg) as colorless amorphous powders.

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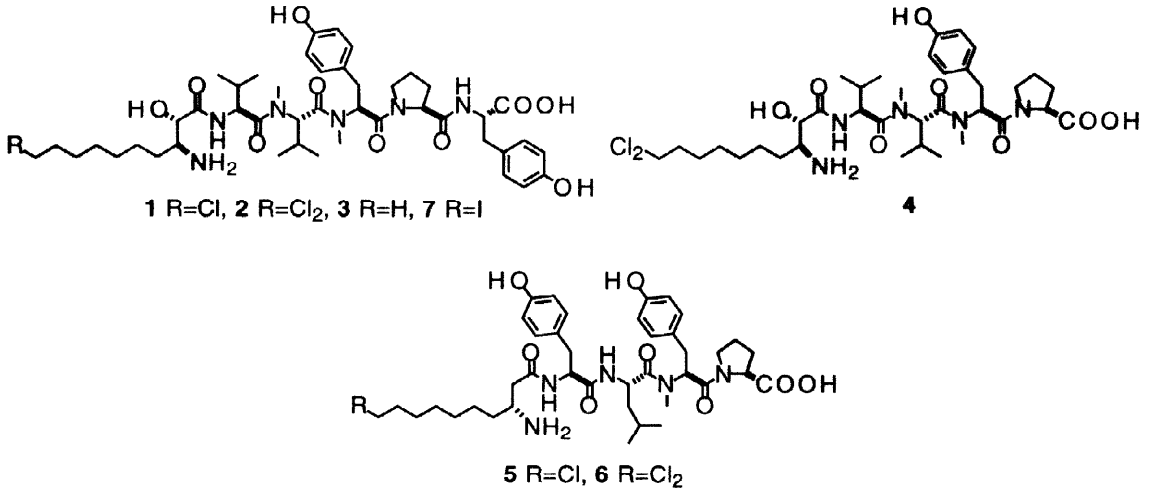

The molecular formula of microginin 299-C (**3**) was established as $C_{45}H_{68}N_6O_{10}$ by the high resolution FABMS and NMR spectral data (Table 1). The amino acid analysis of the hydrolysate and interpretation of the ^1H-^1H COSY, HMQC[10] and HMBC[11] spectra revealed the presence of one residue each of Val, *N*-Me Val, *N*-Me Tyr, Pro and Tyr (Fig. 1), which were also found in microginins 299-A (**1**) and 299-B (**2**). The structure of 3-amino-2-hydroxydecanoic acid (Ahda), which was also found in microginin,[2] was deduced as follows. In the ^1H-^1H COSY spectrum, the connectivities from H-2 (δ 4.20) to H-5 (δ 1.19 and 1.34) and from H-8 (δ 1.21) to H-10 (δ 0.84) were determined, and the HMBC correlations between H-4 and C-6 (δ 28.8), H-7 and C-5 (δ 24.8) and H-8 (δ 1.21) and C-6 connected H-5 to H-8 (Fig. 1).

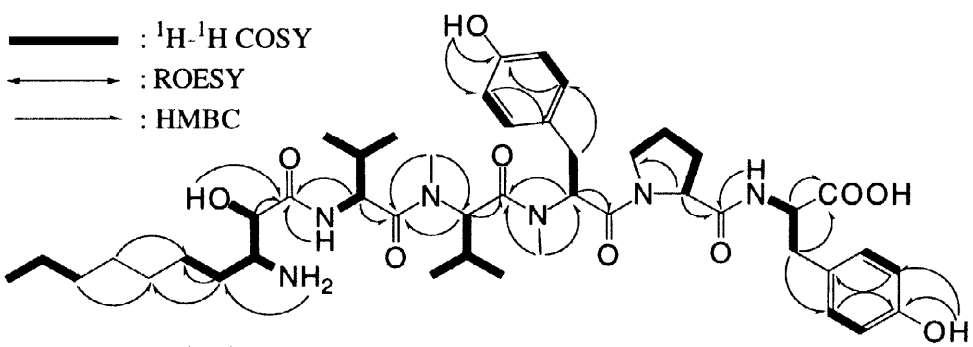

Fig. 1. ^1H-^1H COSY and HMBC correlations of microginin 299-C (**3**).

The sequence of **3** was mostly deduced by the HMBC correlations from α-H, β-H, NH and *N*-Me to C=O (Fig. 1), but the correlations from Pro α-H or δ-H to *N*-Me Tyr C=O could not be observed. The positive and negative FABMS of **3** using glycerol as a matrix revealed the sequence from Ahda to Tyr (Fig. 2).

The stereochemistries of the usual amino acids and *N*-methyl amino acids were determined as L by HPLC analysis of the derivatives of the acid hydrolysate with L- or D-Marfey's reagent.[12] The absolute stereochemistry of Ahda was deduced as follows. Halogen-halogen exchange reaction[13] product (**7**) of **1** was reduced with $LiBH_4$ to afford dechlorinated microginin 299-A. The correspondence of the ^1H and ^{13}C NMR spectra and an optical rotation value ($[\alpha]_D^{23}$ -89.8° in **3** and $[\alpha]_D^{23}$ -89.6° in dechlorinated microginin 299-A) between **3** and dechlorinated microginin 299-A indicated that the stereochemistry of Ahda was 2*S*, 3*S* configuration.

The high resolution FABMS spectrum and NMR data established that microginin 299-D (**4**) had a

molecular formula of $C_{36}H_{56}N_5O_8Cl_2$. The ^1H and ^{13}C NMR spectra of **4** resembled those of **2**, but Tyr was not observed by the amino acid analysis of the hydrolysate. The interpretation of the ^1H-^1H COSY, HMQC and HMBC spectra revealed the structure of microginin 299-D corresponded to that of deTyr microginin 299-B (**4**) (Table 1).

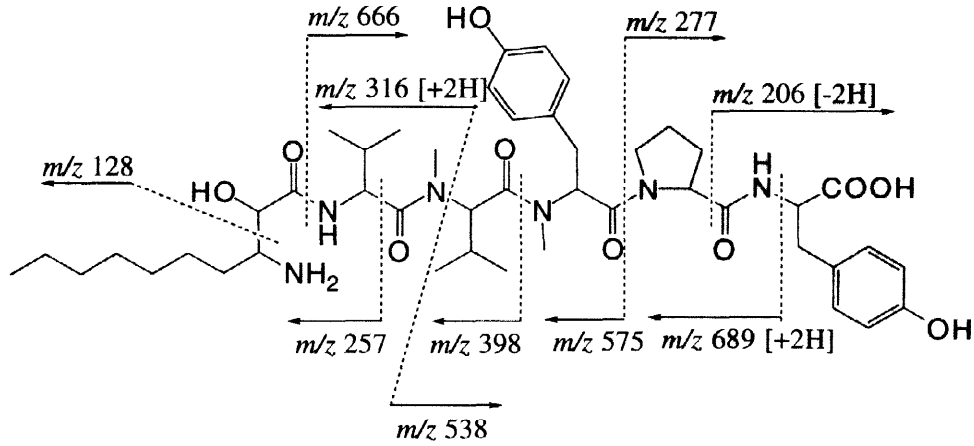

Fig. 2. Positive (left arrows) and negative (right arrows) FABMS fragmentations of microginin 299-C (**3**).

The stereochemistries of the ususal amino acids and *N*-methyl amino acids of **4** were also determined by the above-mentioned procedures. To determine the absolute stereochemistry of dichloro Ahda, microginin 299-B (**2**) was hydrolyzed with carboxypeptidase A[14] and resulting deTyr microginin 299-B was purified. The correspondence of the ^1H and ^{13}C NMR spectra and an optical rotation value ($[\alpha]_D^{23}$ -99.2° in **4** and $[\alpha]_D^{23}$ -98.2° in deTyr microginin 299-B) between **4** and deTyr microginin 299-B indicated that the stereochemistry of dichloro Ahda was 2*S*, 3*S* configuration.

M. aeruginosa (NIES-99)[15] was isolated from a bloom in Lake Suwa and mass-cultured in our laboratory as previously described. The 80% methanol extract of freeze-dried alga was partitioned between water and diethyl ether. The aqueous layer was further extracted with *n*-butanol. The diethyl ether layer was further separated by Kupchan procedure.[16] The 80% MeOH layer and *n*-butanol layer were combined 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 microginins 99-A (**5**, 9.3 mg) and 99-B (**6**, 7.5 mg) as colorless amorphous powders.

The pseudomolecular ions at *m/z* 772/774 $[M+H/M+H+2]^+$ of positive FABMS using glycerol as matrix of microginin 99-A (**5**) revealed the presence of one chlorine atom. The molecular formula of **5** was established to be $C_{40}H_{58}N_5O_8Cl$ by the HRFABMS and NMR spectral data (Table 2). Its peptidic nature was suggested by the ^1H and ^{13}C NMR spectra of **5**, and amino acid analysis of the hydrolysate gave Tyr, Leu and Pro. The extensive NMR analyses including ^1H-^1H COSY, HOHAHA, HMQC and HMBC spectra revealed the spin systems of Tyr, Leu and *N*-MeTyr (Fig. 3). Overlapping ^1H and ^{13}C NMR signals (δ_C 24.4, Pro C-4 and chloro Ada C-5, δ_C 28.5, Pro C-3 and chloro Ada C-6) prevented to deduce the structures of Pro and 10-chloro-3-amino-decanoic acid (Chloro Ada) unit. Therefore, we analyzed the HMQC-HOHAHA[17] spectrum, showing the correlations from Pro H-4 (δ 1.79 and 1.85) to Pro C-2 (δ 58.7), C-3 and C-5 (δ 46.1) and from chloro Ada H-6 (δ 1.18) to chloro Ada C-4 (δ 31.8), C-5, C-7 (δ 28.0) and C-8 (δ 26.1) (Fig. 3). These correlations and the correlations in the ^1H-^1H COSY and HMBC spectra revealed the spin systems of Pro and

chloro Ada (Fig. 3).

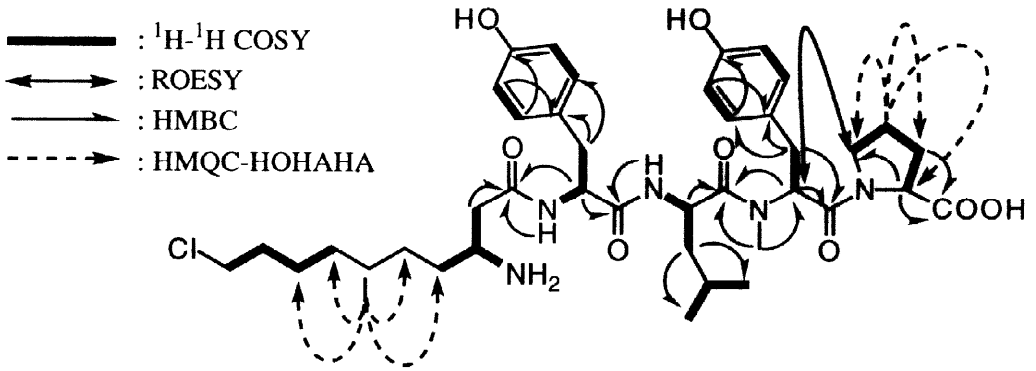

Fig. 3. ^{1}H-^{1}H COSY, ROESY, HMBC and HMQC-HOHAHA correlation of microginin 99-A (**5**).

The sequence of **5** was mostly deduced by the HMBC correlations from α-H, β-H, NH and *N*-Me to C=O (Fig. 3), but the correlations from Pro α-H or δ-H to *N*-Me Tyr C=O could not be observed. The ROESY correlation between *N*-Me Tyr α-H and Pro δ-H connected *N*-Me Tyr to Pro (Fig. 3).

The stereochemistries of the usual amino acids and *N*-Me Tyr were determined as L by HPLC analysis of the derivatives of the acid hydrolysate with L- or D-Marfey's reagent. The determination of the absolute stereochemistry at C-3 of chloro Ada was achieved by the modified Mosher's method.[18] The *N*-(*R*)- and -(*S*)-MTPA derivatives of chloro Ada methylester, obtained by acid hydrolysate of **5**, were prepared by treatment with (*R*) and (*S*)-MTPA chlorides in pyridine anhydride, and Δδ values (δ_S-δ_R) were determined at 600 MHz. Negative Δδ were found for the protons on C-2 and CO_2Me side of the MTPA plane, whereas positive values were found for protons on C-4 to C-10 side (Fig. 4). Therefore, the absolute stereochemistry at C-3 of chloro Ada was *R* configuration.

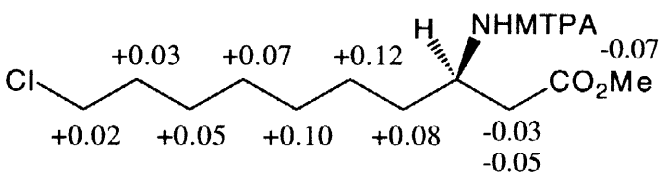

Fig. 4. Δδ (δ_S-δ_R) values in ppm obtained at 600 MHz for *N*-(*R*)- and -(*S*)-MTPA chloro Ada methylester.

The high resolution FABMS spectrum and NMR data established that microginin 99-B (**6**) had a molecular formula of $C_{40}H_{57}N_5O_8Cl_2$. The ^{1}H and ^{13}C NMR spectra of **6** resembled those of **5**, but an isotopic cluster at *m/z* 806/808/810 $[M+H/M+H+2/M+H+4]^+$, consistent with two chlorine atoms, was observed and the ^{1}H and ^{13}C chemical shifts shifted from chloro Ada H-10 (δ 3.61) and C-10 (δ 45.3) to dichloro Ada H-10 (δ 6.30) and C-10 (δ 74.8) (Table 2). Therefore, the Ada moiety of **6** was determined to be 10-dichloro-3-aminodecanoic acid (dichloro Ada). The sequence and the stereochemistries of the usual amino acids and *N*-Me Tyr of **6** were also determined by the above-mentioned procedures. The similarity of the ^{1}H and ^{13}C NMR spectra between dichloro Ada in **6** and chloro Ada in **5** indicated the 3*R* stereochemistry for dichloro Ada.

Microginins 299-C (**3**) and 299-D (**4**) inhibited leucine aminopeptidase with IC_{50}'s of 2.0 and 6.4 μg/mL, respectively, but microginins 99-A (**5**) and 99-B (**6**) did not inhibit at 100 μg/mL. These peptides did not inhibit angiotensin-converting enzyme, papain, trypsin, thrombin, plasmin, chymotrypsin and elastase at 100 μg/mL.

Table 1. ^{1}H and ^{13}C NMR Data for Microginins 299-C (**3**) and 299-D (**4**) in DMSO-d_6.

		microginin 299-C (**3**)					microginin 299-D (**4**)			
Position		^{1}H	*J* (Hz)	^{13}C		Position	^{1}H	*J* (Hz)	^{13}C	
Ahda	1			170.2	(s)	Dichloro			170.2	(s)
	2	4.20	(dd, 5.6, 3.0)	70.6	(d)	Ahda 2	4.21	(dd, 6.2, 2.8)	70.6	(d)
	3	3.37	(m)	53.0	(d)	3	3.36	(m)	52.9	(d)
	4	1.34	(m)	26.9	(t)	4	1.33	(m)	26.9	(t)
		1.49	(m)				1.49	(m)		
	5	1.19	(m)	24.8	(t)	5	1.19	(m)	24.6	(t)
		1.34	(m)				1.35	(m)		
	6	1.20	(m)	28.8	(t)	6	1.20	(m)	28.6	(t)
	7	1.22	(m)	28.2	(t)	7	1.26	(m)	27.5	(t)
	8	1.21	(m)	31.1	(t)	8	1.45	(m)	25.2	(t)
	9	1.23	(m)	22.0	(t)	9	2.12	(m)	42.8	(t)
	10	0.84	(t, 7.1)	13.9	(q)	10	6.28	(t, 5.8)	74.8	(t)
	2-OH	6.45	(brd, 5.6)			2-OH	6.30	(d, 6.0)		
	3-NH_2	7.88	(br)			3-NH_2	7.80	(br)		
Val	1			170.9	(s)	Val 1			170.9	(s)
	2	4.47	(dd, 9.0, 5.1)	53.2	(d)	2	4.48	(dd, 9.0, 5.6)	53.2	(d)
	3	1.75	(m)	29.7	(d)	3	1.75	(m)	29.6	(d)
	4	0.75	(d, 6.6)	16.7	(q)	4	0.75	(d, 6.8)	16.7	(q)
	4'	0.83	(d, 6.4)	19.9	(q)	4'	0.83	(d, 6.8)	19.9	(q)
	NH	7.81	(d, 9.0)			NH	7.78	(d, 9.0)		
N-Me Val	1			168.9	(s)	*N*-Me Val 1			168.9	(s)
	2	4.88	(d, 10.7)	57.9	(d)	2	4.89	(d, 10.7)	57.9	(d)
	3	2.11	(m)	26.3	(d)	3	2.10	(m)	26.3	(d)
	4	0.59	(d, 6.4)	17.8	(q)	4	0.59	(d, 6.8)	17.7	(q)
	4'	0.74	(d, 6.8)	19.6	(q)	4'	0.75	(d, 6.4)	19.5	(q)
	N-Me	2.37	(s)	29.2	(q)	*N*-Me	2.40	(s)	29.2	(q)
N-Me Tyr	1			168.3	(s)	*N*-Me Tyr 1			168.3	(s)
	2	5.58	(dd, 11.3, 4.9)	54.4	(d)	2	5.58	(dd, 10.5, 5.3)	54.4	(d)
	3	2.81	(dd, 14.3, 4.9)	33.0	(t)	3	2.76	(m)	33.0	(t)
		2.90	(dd, 14.3, 11.3)				2.93	(m)		
	4			126.9	(s)	4			126.9	(s)
	5, 9	6.98	(d, 8.6)	129.8	(d)	5, 9	6.98	(d, 8.4)	129.8	(d)
	6, 8	6.63	(d, 8.6)	114.9	(d)	6, 8	6.64	(d, 8.4)	114.9	(d)
	7			155.9	(s)	7			155.9	(s)
	N-Me	2.77	(s)	29.9	(q)	*N*-Me	2.79	(s)	29.9	(q)
	7-OH	9.21	(br)			7-OH	9.21	(s)		
Pro	1			171.3	(s)	Pro 1			173.1	(s)
	2	4.32	(dd, 8.4, 2.7)	59.4	(d)	2	4.22	(dd, 8.8, 3.6)	58.7	(d)
	3	1.77	(m)	28.8	(t)	3	1.83	(m)	28.7	(t)
		1.97	(m)				2.14	(m)		
	4	1.76	(m)	24.2	(t)	4	1.82	(m)	24.5	(t)
	5	3.40	(m)	46.8	(t)		1.89	(m)		
		3.52	(m)			5	3.45	(m)	46.8	(t)
Tyr	1			172.9	(s)		3.52	(ddd, 9.8, 7.7, 4.7)		
	2	4.31	(ddd, 8.5, 7.7, 5.6)	53.8	(d)					
	3	2.79	(dd, 14.0, 8.5)	35.9	(t)					
		2.91	(dd, 14.0, 5.6)							
	4			127.4	(s)					
	5, 9	7.01	(d, 8.6)	130.1	(d)					
	6, 8	6.64	(d, 8.6)	114.9	(d)					
	7			155.9	(s)					
	NH	7.96	(d, 7.7)							
	7-OH	9.21	(br)							

Table 2. ^{1}H and ^{13}C NMR Data for Microginin 99-A (**5**) and 99-B (**6**) in DMSO-d_6.

		microginin 99-A (**5**)					microginin 99-B (**6**)		
Position		^{1}H	*J* (Hz)	^{13}C	Position		^{1}H	*J* (Hz)	^{13}C
Chloro	1			169.1 (s)	Dichloro	1			169.1 (s)
Ada	2	2.28	(dd,15.4,5.0)	36.8 (t)	Ada	2	2.28	(dd,15.5,5.9)	36.8 (t)
		2.43	(dd,15.4,6.3)				2.43	(dd,15.5,6.3)	
	3	3.26	(brd,6.3)	48.1 (d)		3	3.26	(br)	48.1 (d)
	4	1.33	(m)	31.8 (t)		4	1.35	(m)	31.8 (t)
	5	1.19	(m)	24.4 (t)		5	1.20	(m)	24.3 (t)
	6	1.18	(m)	28.5 (t)		6	1.18	(m)	28.4 (t)
	7	1.22	(m)	28.0 (t)		7	1.25	(m)	27.8 (t)
	8	1.35	(m)	26.1 (t)		8	1.44	(m)	25.2 (t)
	9	1.68	(m)	32.0 (t)		9	2.13	(brd,5.9)	42.8 (t)
	10	3.61	(td,6.6,2.3)	45.3 (t)		10	6.30	(t,5.9)	74.8 (t)
	3-NH_2	7.72	(br)			3-NH_2	7.69	(d,5.0)	
Tyr	1			170.9 (s)	Tyr	1			170.9 (s)
	2	4.48	(ddd,10.4,8.4,4.1)	53.9 (d)		2	4.48	(ddd,10.2,8.5,4.2)	53.9 (d)
	3	2.57	(dd,14.0,8.4)	36.7 (t)		3	2.57	(dd,14.1,8.5)	36.7 (t)
		2.83	(dd,14.0,4.1)				2.83	(dd,14.1,4.2)	
	4			127.7 (s)		4			127.7 (s)
	5,9	7.03	(d,8.5)	130.0 (d)		5,9	7.03	(d,8.5)	130.0 (d)
	6,8	6.63	(d,8.5)	115.0 (d)		6,8	6.63	(d,8.5)	115.0 (d)
	7			155.8 (s)		7			155.8 (s)
	NH	8.32	(d,8.4)			NH	8.31	(d,8.5)	
	OH	9.13	(br)			OH	9.13	(br)	
Leu	1			171.6 (s)	Leu	1			171.6 (s)
	2	4.67	(ddd,9.7,8.4,4.7)	47.6 (d)		2	4.67	(ddd,9.5,8.4,4.7)	47.6 (d)
	3	1.37	(m)	40.5 (t)		3	1.37	(m)	40.5 (t)
		1.44	(m)				1.44	(m)	
	4	1.55	(m)	24.1 (d)		4	1.55	(m)	24.1 (d)
	5	0.84	(d,6.5)	21.5 (q)		5	0.84	(d,6.6)	21.5 (q)
	5'	0.86	(d,6.7)	23.0 (q)		5'	0.86	(d,6.7)	23.0 (q)
	NH	8.23	(d,8.4)			NH	8.23	(d,8.4)	
N-MeTyr	1			168.0 (s)	*N*-MeTyr	1			168.0 (s)
	2	5.17	(t,7.2)	56.4 (d)		2	5.17	(t,7.2)	56.4 (d)
	3	2.62	(dd,14.4,7.2)	33.2 (t)		3	2.62	(dd,14.4,7.2)	33.2 (t)
		3.05	(dd,14.4,7.2)				3.05	(dd,14.4,7.2)	
	4			127.7 (s)		4			127.7 (s)
	5,9	6.95	(d,8.5)	129.8 (d)		5,9	6.95	(d,8.5)	129.8 (d)
	6,8	6.60	(d,8.5)	114.8 (d)		6,8	6.60	(d,8.5)	114.8 (d)
	7			155.7 (s)		7			155.7 (s)
	N-Me	2.88	(s)	31.1 (q)		*N*-Me	2.88	(s)	31.1 (q)
	OH	9.10	(br)			OH	9.10	(br)	
Pro	1			173.1 (s)	Pro	1			173.1 (s)
	2	4.17	(dd,8.8,3.7)	58.7 (d)		2	4.17	(dd,8.9,3.7)	58.7 (d)
	3	1.80	(m)	28.6 (t)		3	1.80	(m)	28.6 (t)
		2.10	(m)				2.10	(m)	
	4	1.79	(m)	24.4 (t)		4	1.79	(m)	24.4 (t)
		1.85	(m)				1.85	(m)	
	5	3.35	(m)	46.1 (t)		5	3.35	(m)	46.1 (t)

At first the absolute configuration at the C-2 of the Ahda unit in microginin[2] was decided to be *R* by the CD spectrum, but the stereochemistry of the Ahda unit was revised to be 2*S*, 3*R* by the total synthesis.[3] Although the absolute configurations at the C-3 of the β-amino acid units in microginins 99-A (**5**) and 99-B (**6**) were *R*, those of the α-hydroxy-β-amino acid units in microginins 299-A[7] to 299-D (**1**-**4**) were *S*. The stereochemistry of α-hydroxy-β-amino acid in microginin 299-A (**1**) was determined by a combination of spectral and chemical studies. In this studies, we confirmed the stereochemistry of the Ahda unit in microginin to be 2*S*, 3*R* by the same procedure.[19] Recently, the absolute configuration at the C-3 of Ahda in microginin was also determined as *R* by the application of advanced Marfey's method.[20] It is interesting to differ with the absolute

configuration at the C-3 of the α-hydroxy-β-amino acid units by difference in strains of *M. aeruginosa*.

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 of microginins 299-C and 299-D were measured on JEOL JNM-A500 and 600 NMR spectrometers. Two-dimensional NMR spectra of these peptides were recorded on a JEOL JNM-A500 and 600 NMR spectrometers equipped with a VAXserver 4000-200 computer. ^{1}H and ^{13}C NMR spectra of microginins 99-A and 99-B were measured on Bruker AM600 spectrometer. Two-dimensional NMR spectra of these peptides were recorded on a Bruker AM600 NMR spectrometer equipped with an ASPECT 1000 computer. The HMBC spectra were acquired with an evolution time of 60 ms. The ROESY spectra of microginins 99-A and 99-B were recorded with mixing time of 200 ms. 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 of *M. aeruginosa* (NIES-299 and NIES-99) were the same as previously described.[7]

Isolation of Microginins 299-C and 299-D. Freeze-dried alga (91 g from 360 L of culture) was extracted with 80% MeOH (2 L × 3) and MeOH (2 L × 1). Combined 80% MeOH and MeOH extracts were concentrated to an aqueous suspension which was then extracted with Et_2O. The aqueous layer was extracted with *n*-BuOH. The *n*-BuOH layer was evaporated under reduced pressure to green dry solid (12.9 g), which was subjected to flash chromatography on ODS (YMC-GEL, 120Å, 10 cm × 12 cm) with aqueous MeOH followed by CH_2Cl_2. The 60% MeOH fraction was subjected to reversed-phase HPLC (Capcell pak C18 UG, 20 × 250 mm; UV-detection 210 nm; flow rate 6.0 mL/min) with 35% MeCN containing 0.05% TFA and reversed-phase HPLC (Cosmosil 5C18-MS, 10 × 250 mm; UV-detection 210 nm; flow rate 2.0 mL/min) with 34% MeCN containing 0.05% TFA to yield two fracitons containing **3** and **4**, respectively. Each of fractions containing **3** and **4** was subjected to reversed-phase HPLC (Cosmosil 5C18-MS, 10 × 250 mm; UV-detection 210 nm; flow rate 2.0 mL/min) with 34% MeCN containing 0.05% TFA to yield **3** (7.0 mg) and **4** (5.0 mg).

Isolation of Microginins 99-A (5) and 99-B (6). Freeze-dried alga (34 g from 100 L of culture) was extracted with 80% MeOH (1 L × 3) and MeOH (1 L × 1). Combined 80% MeOH and MeOH extracts were concentrated to an aqueous suspension which was then extracted with Et_2O. The aqueous layer was extracted with *n*-BuOH. Et_2O layer (6.2 g) was further separated by the Kupchan procedure,[16] employing gradient solvent systems of Hexane/90%MeOH and CCl_4/80%MeOH. The 80%MeOH layer (1.0 g) and *n*-BuOH layer (3.3 g) were combined, concentrated and subjected to flash chromatography on ODS (YMC-GEL, 120Å, 10 cm × 12 cm) with aqueous MeOH followed by CH_2Cl_2. The 70% MeOH (246.7 mg) fraction was subjected to reversed-phase HPLC (Cosmosil 5C18-MS, 10 × 250 mm; UV-detection 210 nm; flow rate 2.0 mL/min) with 30-50% MeCN containing 0.05% TFA to yield **5** (5.5 mg) and **6** (2.7 mg). The MeOH fraction (1.3 g) was subjected to reversed-phase HPLC (Cosmosil 5C18-MS, 10 × 250 mm; UV-detection 210 nm; flow rate 2.0 mL/min) with 30-50% MeCN containing 0.05% TFA to yield **5** (3.8 mg) and **6** (4.8 mg).

Microginin 299-C (3). $[\alpha]_D^{23}$ -89.8° (*c* 0.10, MeOH); UV (MeOH) λmax 279 nm (ε 3100); ^{1}H and ^{13}C NMR see Table 1; HRFABMS *m/z* 851.4878 $[M - H]^-$ ($C_{45}H_{67}N_6O_{10}$, Δ -4.1 mmu).

Microginin 299-D (4). $[\alpha]_D^{23}$ -99.2° (*c* 0.10, MeOH); UV (MeOH) λmax 271 nm (ε 3400); ^{1}H and ^{13}C NMR see Table 1; HRFABMS *m/z* 756.3549 $[M - H]^-$ ($C_{36}H_{56}N_5O_8Cl_2$, Δ +4.3 mmu).

Microginin 99-A (5). $[\alpha]_D^{23}$ -70.3° (*c* 0.10, MeOH); UV (MeOH) λmax 278 nm (ε 2200); ^1H and ^{13}C NMR see Table 2; HRFABMS *m/z* 772.4067 $[M - H]^-$ ($C_{40}H_{59}N_5O_8Cl$, Δ +1.5 mmu).

Microginin 99-B (6). $[\alpha]_D^{23}$ -72.0° (*c* 0.10, MeOH); UV (MeOH) λmax 278 nm (ε 2200); ^1H and ^{13}C NMR see Table 2; HRFABMS *m/z* 806.36472 $[M + H]^+$ ($C_{40}H_{58}N_5O_8Cl_2$, Δ +1.0 mmu).

Acid Hydrolysis. For amino acid analysis, 100 μg each of **3** - **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) in the amino acid analysis of **3** and **4**: Pro (31.7, 31.7), Val (39.8, 39.8), Tyr (49.3, -). Retention times (min) in the amino acid analysis of **5** and **6**: Pro (32.3, 32.3), *N*-MeTyr (44.1, 44.2), Leu (48.6, 48.7), Tyr (50.9, 51.0).

HPLC Analysis of the Marfey Derivatives. To the acid hydrolysate of a 100 μg portion of **3** - **6**, 50 μL of 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide in acetone (L-FDAA) (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 5C18-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-Pro (42.3), D-Pro (43.5), L-Val (47.6), D-Val (51.8), *N*-Me L-Val (50.8), *N*-Me D-Val (53.2). Retention times (min) of the amino acid derivatives of **3** and **4**: L-Pro (42.5, 42.2), L-Val (47.8, 47.7), *N*-Me L-Val (50.9, 51.0). Retention times (min) of the standard amino acids: L-Pro (40.2), D-Pro (41.2), L-Leu (49.6), D-Leu (53.4), L-Tyr (55.6), D-Tyr (58.4). Retention times (min) of the amino acid derivatives of **5** and **6**: Pro (40.0, 40.0), Leu (49.6, 49.6), Tyr (55.6, 55.6).

N-Me L-Tyr was derivatized with D- and L-FDAA as described above, respectively. The derivatives were analyzed by reversed-phase HPLC (Cosmosil 5C18-MS, 4.6 × 250 mm; MeCN/H_2O/TFA (40: 60: 0.1); UV-detection 340 nm; flow rate 1.0 mL/min). Retention times of standards of **3** and **4** (min): *N*-Me L-Tyr-(*N*, *O*)-L-FDAA (25.6), *N*-Me L-Tyr-(*N*, *O*)-D-FDAA (27.3). Retention times (min) of *N*-MeTyr-(*N*, *O*)-L-FDAA in the acid hydrolysate of **3** and **4**: 25.3 and 25.6. Retention times of standards of **5** and **6** (min): *N*-Me L-Tyr-(*N*, *O*)-L-FDAA (25.2), *N*-Me L-Tyr-(*N*, *O*)-D-FDAA (26.8). Retention times (min) of *N*-MeTyr-(*N*, *O*)-L-FDAA in the acid hydrolysate of **5** and **6**: 24.8 and 24.8.

Dechlorinated Microginin 299-A. Microginin 299-A (**1**; 10.0 mg) was dissolved in dried acetone (2.0 mL) and then NaI (10.0 mg) was added, and the reaction mixture was reflux under argon for 24 h. The solvent was evaporated and partitioned between H_2O and Et_2O. After the Et_2O layer was evaporated, the reaction mixture was subjected to reversed-phase HPLC (Cosmosil 5C18-MS, 10 × 250 mm; MeCN/H_2O/TFA (37:63:0.05); UV-detection 210 nm; flow rate 2.0 mL/min) to yield iodide Ahda-microginin 299-A (4.4 mg; 39.9%). Iodide Ahda-microginin 299-A was dissolved in dried THF and then $LiBH_4$ was added, and stirred under argon at room temperature for 5 h. The reaction mixture was quenched with 6 N HCl and saturated aqueous $NaHCO_3$. Following removal of solvent, the residue was partitioned between H_2O and Et_2O. After the Et_2O layer was evaporated, the reaction mixture ws subjected to reversed-phase HPLC (Cosmosil 5C18-MS, 10 × 250 mm; MeCN/H_2O/TFA (35:65:0.05); UV-detection 210 nm; flow rate 2.0 mL/min) to yield dechlorinated microginin 299-A (1.3 mg; 33%); $[\alpha]_D^{23}$ -89.6° (*c* 0.05, MeOH); HRFABMS *m/z* 853.5019 $[M+H]^+$ ($C_{45}H_{69}N_6O_{10}$, Δ -5.6 mmu); ^1H and ^{13}C NMR (DMSO-d_6), Ahda 1 (δ_C 170.2), 2 (δ_H 4.23, dd 6.0, 3.0, δ_C 71.0), 3 (δ_H 3.37, m, δ_C 53.0), 4 (δ_H 1.33, m and 1.47, m, δ_C 71.0), 5 (δ_H 1.19, m and 1.34, m, δ_C 24.8), 6 (δ_H 1.20, m, δ_C 28.8), 7 (δ_H 1.21, m, δ_C 28.2), 8 (δ_H 1.21, m, δ_C 31.1), 9 (δ_H 1.24, m, δ_C

22.0), 10 (δ_H 0.85, m, δ_C 13.9), 2-OH (δ_H 6.42, d 6.0), 3-NH_2 (δ_H 7.87, br), Val 1 (δ_C 170.9), 2 (δ_H 4.47, dd 8.8, 5.3, δ_C 53.3), 3 (δ_H 1.75, m , δ_C 29.7), 4 (δ_H 0.75, d 6.4, δ_C 16.7), 4' (δ_H 0.83, d 6.8, δ_C 19.9), NH (δ_H 7.78, d 8.6), *N*-Me Val 1 (δ_C 168.9), 2 (δ_H 4.88, d 10.7, δ_C 58.2), 3 (δ_H 2.11, m, δ_C 26.3), 4 (δ_H 0.59, d 6.8, δ_C 17.8), 4' (δ_H 0.74, d 6.4, δ_C 19.6), *N*-Me (δ_H 2.37, s, δ_C 29.2), *N*-Me Tyr 1 (δ_C 168.3), 2 (δ_H 5.59, dd 11.1, 5.1, δ_C 54.4), 3 (δ_H 2.80, dd 14.3, 4.9 and 2.89, dd 14.3, 11.3, δ_C 33.0), 4 (δ_C 126.9), 5, 9 (δ_H 6.97, d 8.6, δ_C 129.8), 6, 8 (δ_H 6.63, d 8.6, δ_C 114.9), 7 (δ_C 155.9), *N*-Me (δ_H 2.77, s, δ_C 29.9), 7-OH (δ_H 9.21, s), Pro 1 (δ_C 171.3), 2 (δ_H 4.33, dd 8.8, 2.8, δ_C 59.7), 3 (δ_H 1.77, m and 1.97, m, δ_C 28.8), 4 (δ_H 1.76, m, δ_C 24.2), 5 (δ_H 3.40, m and 3.52, m, δ_C 46.8), Tyr 1 (δ_C 172.9), 2 (δ_H 4.30, ddd 9.3, 8.1, 5.6, δ_C 53.6), 3 (δ_H 2.78, dd 14.0, 8.5 and 2.90, dd 14.0, 5.6, δ_C 35.9), 4 (δ_C 127.4), 5, 9 (δ_H 7.01, d 8.6, δ_C 130.1), 6., 8 (δ_H 6.64, d 8.6, δ_C 114.9), 7 (δ_C 155.9), NH (δ_H 7.94, d 8.1), 7-OH (δ_H 9.21, s).

DeTyr Microginin 299-B. To reaction vial was added: 4 mL of 1 M Tris HCl, 0.5 M NaCl, pH 7.5; 0.6 mL MeOH containing of microginin 299-B (**2**, 6.0 mg), 0.5 mL of 10% LiCl containing 200 units carboxypeptidase A. The vial was incubated at 37 °C for 2 h, and then, 2.0 mL of MeOH was added and inactivated at 60 °C. The reaction mixture was centrifugated (3000 rpm for 20 min), filtered, and subjected to reversed-phase HPLC (Cosmosil 5C18-MS, 10 × 250 mm; MeCN/H_2O/TFA (35:65:0.05); UV-detection 210 nm; flow rate 2.0 mL/min) to yield deTyr microginin 299-B (4.0 mg; 80%); $[\alpha]_D^{23}$ -98.2° (*c* 0.2, MeOH); HRFABMS *m/z* 758.3632 $[M+H]^+$ ($C_{36}H_{58}N_5O_8$, Δ -3.1 mmu); ^1H and ^{13}C NMR (DMSO-d_6), Dichloro Ahda 1 (δ_C 170.2), 2 (δ_H 4.21, dd 6.0, 3.0, δ_C 70.6), 3 (δ_H 3.36, m, δ_C 52.9), 4 (δ_H 1.33, m and 1.48, m, δ_C 26.9), 5 (δ_H 1.20, m and 1.35, m, δ_C 24.6), 6 (δ_H 1.20, m, δ_C 28.6), 7 (δ_H 1.26, m, δ_C 27.5), 8 (δ_H 1.45, m, δ_C 25.1), 9 (δ_H 2.13, m, δ_C 42.8), 10 (δ_H 6.29, t 6.0, δ_C 74.8), 2-OH (δ_H 6.43, d 6.0), 3-NH_2 (δ_H 7.85, br),Val 1 (δ_C 170.9), 2 (δ_H 4.48, dd 8.6, 5.6, δ_C 53.2), 3 (δ_H 1.75, m, δ_C 29.7), 4 (δ_H 0.75, d 6.8, δ_C 16.8), 4' (δ_H 0.83, d 6.8, δ_C 19.8), NH (δ_H 7.81, d, 8.6), *N*-Me Val 1 (δ_C 168.9), 2 (δ_H 4.89, d 10.7, δ_C 57.9), 3 (δ_H 2.11, m, δ_C 26.3), 4 (δ_H 0.59, d 6.8, δ_C 17.7), 4' (δ_H 0.75, d 6.4, δ_C 19.5), *N*-Me (δ_H 2.40, s, δ_C 29.2), *N*-Me Tyr 1 (δ_C 168.1), 2 (δ_H 5.58, dd 10.7, 5.1, δ_C 54.4), 3 (δ_H 2.77, dd 15.4, 10.7 and 2.92, dd 15.4, 5.1, δ_C 32.9), 4 (δ_C 126.9), 5, 9 (δ_H 6.98, d 8.6, δ_C 129.8), 6, 8 (δ_H 6.63, d 8.6, δ_C 114.8), 7 (δ_C 155.9), *N*-Me (δ_H 2.80, s, δ_C 29.9), 7-OH (δ_H 9.21), Pro 1 (δ_C 173.1), 2 (δ_H 4.22, dd 9.0, 3.4, δ_C 58.7), 3 (δ_H 1.83, m and 2.14, m, δ_C 28.6), 4 (δ_H 1.83, m, and 1.89, m, δ_C 24.4), 5 (δ_H 3.45, m and 3.52, ddd 9.8, 7.7, 4.7, δ_C 46.5).

Isolation of 10-Chloro-3-Aminodecanoic Acid (Chloro Ada). Methanol solution (3 mL) of **3** (15.0 mg) was dissolved in 3 mL of 6 N HCl and heated at 90 °C for 16 h. After the solvent was removed by evaporation, the reaction mixture was freeze-dried, and subjected to reversed-phase HPLC (Cosmosil 5C18-MS, 10 × 250 mm; 0-80% MeOH; UV-detection at 210 nm; flow rate 2.0 mL/min) to yield chloro Ada methylester (1.4 mg): HRFABMS, *m/z* 236.1383 $[M + H]^+$ ($C_{11}H_{23}NO_2Cl$, Δ -3.4 mmu); ^1H and ^{13}C NMR (600 MHz, $CDCl_3$), 2 (δ_H 2.83-2.88, m, δ_C 35.1), 3 (δ_H 3.58, m, δ_C 49.0), 4 (δ_H 1.57, m , δ_C 29.4), 5 (δ_H 1.27, m and 1.37, m, δ_C 24.6), 6 (δ_H 1.28, m, δ_C 28.9), 7 (δ_H 1.35, m, δ_C 27.9), 8 (δ_H 1.34, m and 1.38, m, δ_C 29.3), 9 (δ_H 1.70, m, δ_C 32.0), 10 (δ_H 3.61, t 6.4, δ_C 45.3).

***N*-(*S*,*R*)-MTPA-10-Chloro-3-Aminodecanoic Acid Methylester.** A solution of chloro Ada methylester (200 μg) in anhydrous pyridine was added with (*S*)-MTPA chloride (2.14 mg), and the mixture was allowed to stand at room temperature for 2 h under argon. *N*,*N*-Dimethyl-1,3-propanediamine was added to quench the excess chloride, and the pyridine was removed by freeze-drying. The residue was subjected to

reversed-phase HPLC (Cosmosil 5C18-MS, 10 × 250 mm; 30-100% MeOH; UV-detection at 210 nm; flow rate 2.0 mL/min) to yield *N*-(*S*)-MTPA-10-chloro-3-aminodecanoic acid methylester (300 μg; 78%); ^{1}H and ^{13}C NMR (600 MHz, $CDCl_3$), 2 (δ_H 2.502, dd 15.8, 5.6 and 2.545, dd 15.8, 5.1), 3 (δ_H 4.250, m), 4 (δ_H 1.580, m), 5 (δ_H 1.330, m), 6 (δ_H 1.290, m), 7 (δ_H 1.290, m), 8 (δ_H 1.400, m), 9 (δ_H 1.742, m), 10 (δ_H 3.506, m), NH (δ_H 7.187, d 9.4), COOMe (δ_H 3.600, s), OMe (δ_H 3.378, s), phenyl (δ_H 7.358-7.390, m). *N*-(*R*)-MTPA-10-chloro-3-aminodecanoic acid methyl ester was also derivatized as described above. *N*-(*R*)-MTPA-10-chloro-3-aminodecanoic acid methyl ester (300 μg; 78%); ^{1}H and ^{13}C NMR (600 MHz, $CDCl_3$), 2 (δ_H 2.535, dd 15.8, 5.6 and 2.593, dd 15.8, 5.1), 3 (δ_H 4.250, m), 4 (δ_H 1.505, m), 5 (δ_H 1.210, m), 6 (δ_H 1.190, m), 7 (δ_H 1.220, m), 8 (δ_H 1.350, m), 9 (δ_H 1.716, m), 10 (δ_H 3.488, m), NH (δ_H 7.140, d 9.4), COOMe (δ_H 3.670, s), OMe (δ_H 3.430, s), phenyl (δ_H 7.358-7.388, m).

Leucine Aminopeptidase Inhibitory Assay. Leucine aminopeptidase inhibitory activity was determined by the method described in a previous paper.[7]

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