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Aeruginosins, Protease Inhibitors from the Cyanobacterium *Microcystis aeruginosa*

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Abstract: Five new protease inhibitors, related to aeruginosins 298-A (**5**), 98-A (**1**) and 98-B (**2**), were isolated from the cyanobacterium *Microcystis aeruginosa*. Aeruginosins 98-C (**3**) and 101 (**4**) differed from **1** in the Hpla unit. Aeruginosin 298-B (**6**) lacked the argininol unit in **5**. Aeruginosins 89-A (**7**) and 89-B (**8**) were observed as the tautomers in HPLC because of the presence of the argininal units. Structures **3**, **4** and **6-8** were determined on the basis of spectral data and chromatographic analyses of degradation products. The absolute stereochemistry of **1-8** was deduced by a combination of spectral and chemical studies.

Microcystis aeruginosa has been shown to be a rich source of unique and bioactive secondary metabolites, especially peptides.[3] In 1994, we reported the isolation and structure elucidation of aeruginosin 298-A (**5**), a linear peptide, as a thrombin and trypsin inhibitor from the cyanobacterium *M. aeruginosa* (NIES-298).[4] This linear peptide was consisted of four units, *viz.*, Hpla (*p*-hydroxyphenyllactic acid), Leu, Choi (2-carboxy-6-hydroxyoctahydroindole) and argininol units. On and after this year, we have reported the isolations of the aeruginosin-type peptides, aeruginosins 98-A (**1**) and 98-B (**2**) from *M. aeruginosa* (NIES-98),[5] aeruginosins 102-A and 102-B from *M. viridis* (NIES-102),[6] and aeruginosins 205A and 205B from *Oscillatoria agardhii* (NIES-205).[7] Recently, the absolute stereochemistries and protease inhibitory modes of **2** and **5** were determined by the single crystal X-ray diffraction of the trypsin-aeruginosin 98-B (**2**) complex[8] and of the ternary complex of aeruginosin 298-A (**5**) bound to hirugen-thrombin,[9] respectively. These results indicated that aeruginosin-type peptides are useful as new protease inhibitory motifs. To research new aeruginosin-type peptides, further investigation on the extracts of *M. aeruginosa* (NIES-98 and -298)[10] led to the isolation of two new congeners, aeruginosins 98-C (**3**) and 298-B (**6**). Moreover, aeruginosins 101 (**4**), 89-A (**7**) and 89-B (**8**), three new congeners were also isolated from other strains *M. aeruginosa* (NIES-101 and -89).[10] Here we describe the isolation, structure and stereochemistry of these peptides.

Isolation and structure elucidation of **1** and **2** was previously reported,[5] but the stereochemistries of the chloro Hpla, Hpla and Choi sulfate units have not been determined. The stereochemistries of chloro Hpla and Hpla were determined to be D-form by ODS HPLC analyses of menthyl esters[6] of the acid hydrolysates. Standard optically active Hpla was synthesized from *p*-aminophenylalanine.[6] Chloro Hpla was synthesized from Hpla using SO_2Cl_2.[11] The absolute stereochemistry at C-2 of the Choi sulfate (2-carboxy-6-

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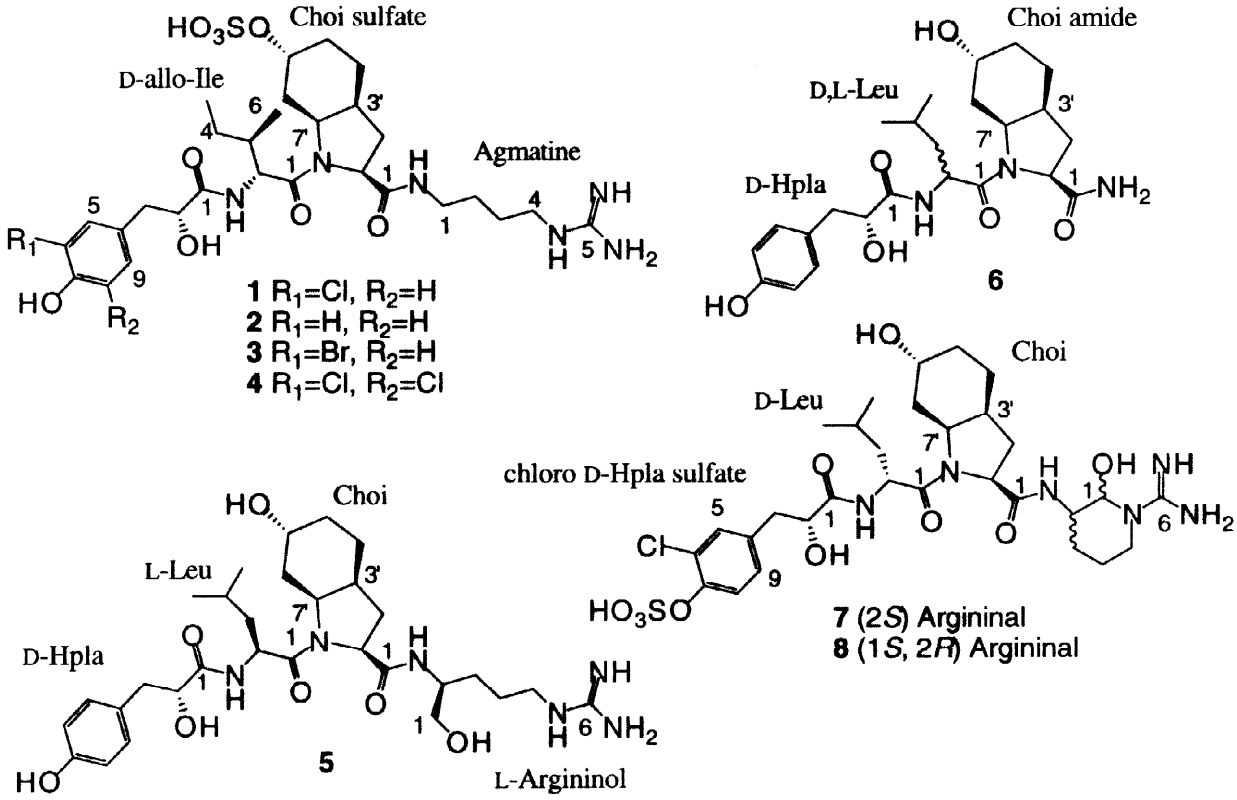
hydroxyoctahydroindole sulfate) unit was determined as follows. Choi diacetate was obtained as two conformers by acetylation on acid hydrolysate of **1**. The (*R*)- and (*S*)-PGME (phenylglycine methyl ester) amides of Choi diacetate were prepared by treatment with (*R*)- and (*S*)-PGME in DMF using the coupling and additive reagents (PyBOP, HOBt), and $\Delta\delta$ values (Δ_S-Δ_R) were determined with 600 MHz NMR.[12] Negative $\Delta\delta$ values were observed for the protons on C-7, C-7' and *N*-Ac side of the PGME plane, whereas positive values for the protons on C-2 to C-5 side (Fig. 1). Therefore, the absolute stereochemistry at C-2 of Choi was *S* configuration. The relative stereochemistry of the Choi sulfate unit in **1** was decided as shown in Fig. 2 by observing NOESY correlations (Choi sulfate H-2/H-3', H-2/H-3b, H-3a/H-5a, H-3a/H-7a, H-3'/H-7', H-5a/H-7a, H-7b/H-7'). Furthermore, H-6 showed small coupling constants (J = 2.8 Hz) to H-5a, 5b, 7a, 7b due to equatorial position. These results pointed that the stereochemistry of the Choi sulfate unit in **1** was 2*S*, 3'*R*, 6*S*, 7'*R*. The correspondence of ^{1}H and ^{13}C NMR signals of Choi sulfate of **1** and **2** and the NOESY correlations[13] indicated that the stereochemistry of the Choi sulfate unit of **2** was also 2*S*, 3'*R*, 6*S*, 7'*R*.



The HRFABMS spectrum and NMR data established that aeruginosin 98-C (**3**) had a molecular formula of $C_{29}H_{45}N_6O_9SBr$ (Table 1). The ^{1}H and ^{13}C NMR spectra of **3** resembled those of **1**, but an isotopic cluster at *m/z* 733/735/737 $[M+H/M+H+2/M+H+4]^+$ was observed and suggested the presence of a bromine in **3** instead of a chlorine in **1** by the ^{1}H-^{1}H COSY and HMBC spectra (Fig. 3). The stereochemisty of allo Ile was determined as D by the HPLC analysis of the derivatives of the acid hydrolysate with L-Marfey's reagent. The stereochemistries of bromo Hpla was determined by the above-mentioned procedures. Bromo Hpla was synthesized from Hpla using dioxane dibromide.[14,15] The correspondence of the ^{1}H and

^{13}C NMR spectra of Choi sulfate between **1** and **3** indicated that the stereochemistry of the Choi sulfate unit of **3** was also 2*S*, 3'*R*, 6*S*, 7'*R*.

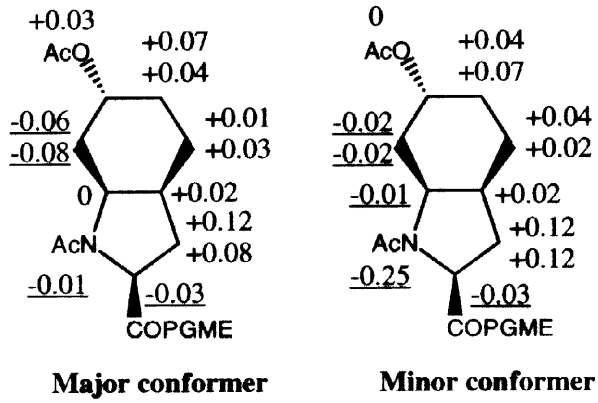


Fig. 1. $\Delta\delta$ (δ_S-δ_R) values in ppm obtained at 600 MHz for Choi (*S*)- and (*R*)-PGME amide diacetate.

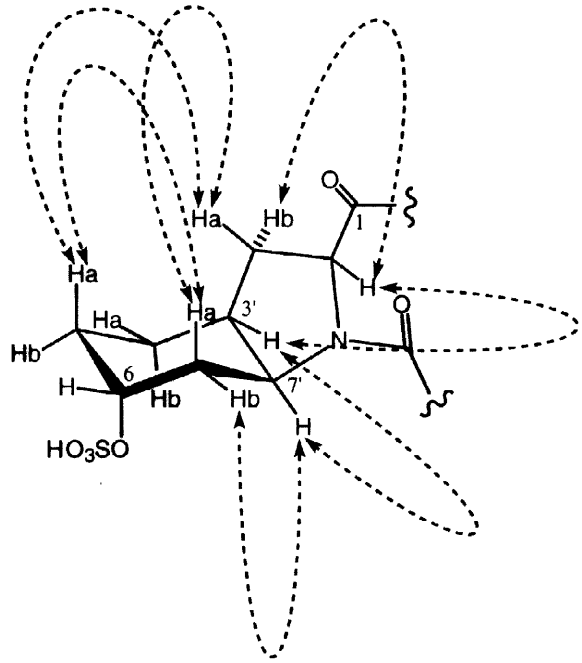


Fig. 2. Relative stereochemistry of the Choi sulfate unit in aeruginosin 98-A (**1**) (Dashed arrows: NOESY).

The molecular weight of aeruginosin 101 (**4**) was larger than **1** by one chlorine atom from the low and high resolution FABMS spectral data, and the signal of singlet methine proton (δ_H 7.19) was observed in the ^1H NMR spectrum, suggesting the presence of 3,5-dichloro-4-hydroxyphenyllactic acid (dichloro Hpla). Interpretation of the NMR data (Table 1) and the amino acid analysis of the hydrolysate allowed that the structure of **4** was almost the same as that of **1** except for the halogenated Hpla moiety. The ^{13}C NMR spectrum of the dichloro Hpla unit confirmed the presence of a tri-substituted phenol moiety by its typical chemical shifts (Fig. 3) and by the interpretation of the ^1H-^1H COSY, HMQC and HMBC spectra.

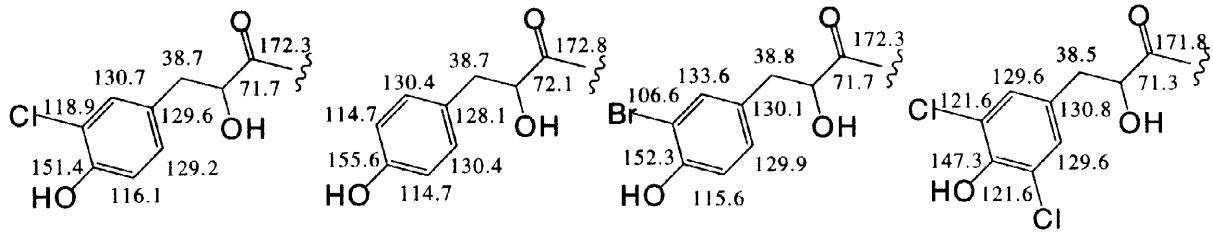


Fig. 3. ^{13}C NMR data of chloro Hpla (**1**), Hpla (**2**), bromo Hpla (**3**) and dichloro Hpla (**4**) in DMSO-d_6.

The stereochemistries of dichloro Hpla and allo-Ile of **4** was determined by above-mentioned procedure. The correspondence of the ^1H and ^{13}C NMR spectra of Choi sulfate between **1** and **4** and the NOESY correlations[16] indicated that the stereochemistry of the Choi sulfate unit was also 2*S*, 3'*R*, 6*S*, 7'*R*.

Isolation and structure elucidation of **5** was previously reported,[4] but the stereochemistries of the Hpla, Choi and argininol (Argol) units have not been determined. The stereochemistries of the Hpla unit of **5** was

determined by the above-mentioned procedure. The correspondence of the ^1H and ^{13}C NMR spectra of Choi sulfate between **1** and **5** and the NOESY correlations[17] indicated that the stereochemistry of the Choi sulfate unit was also 2*S*, 3'*R*, 6*S*, 7'*R*. The stereochemisty of Argol was determined as L by the HPLC analysis of the derivatives of the acid hydrolysate with L- and D-Marfey's reagent. L-Argol was prepared from Boc L-Arg (NO_2) as previously described by Hamada *et al.*[18]

Table 1. ^1H and ^{13}C NMR Data for Aeruginosins 98-C (**3**) and 101 (**4**) in DMSO-d_6

		Aeruginosin 98-C (**3**)				Aeruginosin 101 (**4**)		
Position		^1H *J* (Hz)	^{13}C	Position		^1H *J* (Hz)	^{13}C
bromo	1		172.3 (s)	dichloro	1		171.8 (s)
Hpla	2	4.08 (br)	71.7 (d)	Hpla	2	4.14 (dd, 7.2, 3.7)	71.3 (d)
	3	2.67 (dd, 14.0, 7.2)	38.8 (t)		3	2.73 (dd, 14.0, 7.2)	38.5 (t)
		2.85 (dd, 14.0, 3.6)				2.87 (dd, 14.0, 3.7)	
	4		130.1 (s)		4		130.8 (s)
	5	7.32 (s)	133.6 (d)		5,9	7.19 (s)	129.6 (d)
	6		106.6 (s)		6,8		121.6 (s)
	7		152.3 (s)		7		147.3 (s)
	8	6.83 (d, 8.3)	115.6 (d)		7-OH	9.81 (s)	
	9	7.00 (d, 8.3)	129.9 (d)	allo-Ile	1		168.8 (s)
	2-OH	5.82 (br)			2	4.46 (dd, 8.5, 4.2)	52.3 (d)
	7-OH	9.91 (s)			3	1.52 (m)	37.5 (d)
allo-Ile	1		169.0 (s)		4	0.89 (m)	26.1 (t)
	2	4.44 (dd, 8.5, 4.4)	52.6 (d)			1.10 (m)	
	3	1.53 (m)	37.4 (d)		5	0.84 (t, 7.1)	11.8 (q)
	4	0.94 (m)	25.8 (t)		6	0.63 (d, 6.7)	13.6 (q)
		1.15 (m)			NH	7.33 (d, 8.5)	
	5	0.84 (dd, 7.5, 7.4)	11.6 (q)	Choi	1		171.5 (s)
	6	0.66 (t, 6.7)	13.8 (q)	sulfate	2	4.18 (dd, 9.5, 8.3)	59.9 (d)
	NH	7.33 (d, 3.6)			3	1.75 (ddd, 13.0, 13.0, 9.5)	30.6 (t)
Choi	1		171.5 (s)			2.00 (m)	
sulfate	2	4.17 (dd, 9.0, 8.9)	59.9 (d)		3a	2.28 (m)	35.8 (d)
	3	1.76 (m)	30.6 (t)		4	1.43 (m)	19.3 (t)
		2.00 (m)				1.99 (m)	
	3a	2.27 (m)	35.6 (d)		5	1.31 (m)	23.3 (t)
	4	1.43 (m)	19.3 (t)			1.86 (m)	
		1.98 (m)			6	4.35 (m)	70.8 (d)
	5	1.31 (m)	23.4 (t)		7	1.67 (m)	31.7 (t)
		1.86 (m)				2.20 (m)	
	6	4.36 (br)	70.8 (d)		7a	4.01 (ddd, 11.1, 6.3, 6.3)	53.9 (d)
	7	1.66 (br)	31.6 (t)	agmatine	1	3.01 (m)	37.8 (t)
		2.26 (br)			2	1.40 (m)	25.9 (t)
	7a	4.03 (m)	54.0 (d)		3	1.44 (m)	26.2 (t)
agmatine	1	3.01 (m)	37.6 (t)		4	3.08 (m)	40.4 (t)
	2	1.41 (m)	25.8 (t)		1-NH	7.82 (t, 5.9)	
	3	1.43 (m)	26.2 (t)		4-NH	7.42 (t, 6.0)	
	4	3.08 (m)	40.4 (t)		5		156.6 (s)
	1-NH	7.78 (dd, 5.7, 5.9)					
	4-NH	7.42 (dd, 5.4, 5.9)					
	5		156.6 (s)				

Aeruginosin 298-B (**6**) was first isolated as a single peak by reversed-phase HPLC, but ^1H and ^{13}C NMR spectra in DMSO-d_6 at 300 K indicated the presence of two components in ratio of 3:1. Two components could not be separated by reversed-phase HPLC (ODS C18, CN and Ph). The amino acid analysis and the HPLC analysis of the derivatives with L-Marfey's reagent of the acid hydrolysate of these components gave D,L-Leu in a ratio of 3:1. Therefore, it was confirmed that **6** was consisted of epimers,

which were different in the stereochemistry of Leu, and the structure elucidation of **6** was carried out with the mixture. The molecular formula of **6** was established as $C_{24}H_{35}N_3O_6$ by the HRFABMS and NMR spectral data (Table 2). The extensive NMR analyses including ^{1}H-^{1}H COSY, HMQC and HMBC spectra revealed the same spin system of Hpla, Leu and Choi as in **5**. The HMBC correlation from the primary amide protons (δ 6.83, 7.22) to carbonyl carbon (δ 173.0) confirmed that the C-terminus was protected by the primary amide. The sequence of **6** was deduced by the HMBC correlations from α-H, β-H and NH to C=O (Fig. 4).

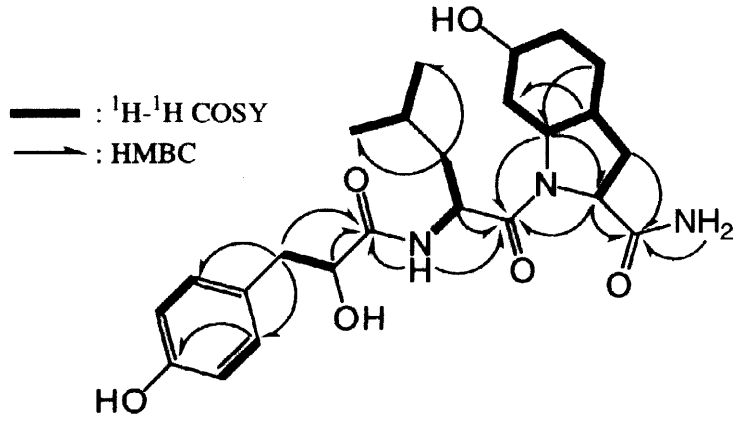


Fig. 4. ^{1}H-^{1}H COSY and HMBC correlations in aeruginosin 298-B (**6**).

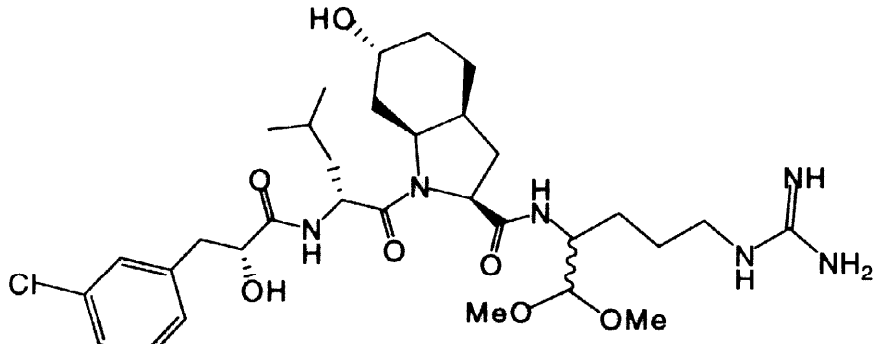
Table 2. ^{1}H and ^{13}C NMR Data for Aeruginosin 298-B (**6**) in DMSO-d_6

	Major component					Minor component				
Position		^{1}H	*J* (Hz)	^{13}C		Position	^{1}H	*J* (Hz)	^{13}C	
Hpla	1			172.3	(s)	Hpla 1			172.3	(s)
	2	4.01	(m)	71.8	(d)	2	4.00	(m)	72.0	(d)
	3a	2.63	(dd, 13.3, 7.7)	39.0	(t)	3a	2.61	(dd, 14.4, 7.3)	39.0	(t)
	3b	2.84	(dd, 14.1, 3.9)			3b	2.81	(dd, 13.7, 4.3)		
	4			127.9	(s)	4			125.2	(s)
	5,9	6.98	(dd, 8.6, 1.7)	130.00	(d)	5,9	6.95	(dd, 8.6, 1.7)	129.96	(d)
	6,8	6.62	(dd, 8.6, 1.7)	114.44	(s)	6,8	6.60	(dd, 8.6, 1.7)	114.39	(s)
	7			155.4	(s)	7			155.4	(s)
Leu	1			169.4	(s)	Leu 1			169.8	(s)
	2	4.53	(t, 9.0)	47.4	(d)	2	4.19	(ddd, 10.0, 9.0, 3.0)	46.9	(d)
	3a	1.20	(ddd, 13.3, 9.0, 4.7)	41.8	(t)	3a	1.24	(m)	41.7	(t)
	3b	1.33	(m)			3b	1.41	(m)		
	4	1.32	(m)	23.6	(d)	4	1.28	(m)	23.7	(d)
	5	0.80	(d, 6.4)	22.97	(q)	5	0.76	(d, 6.0)	23.00	(q)
	5'	0.87	(d, 6.4)	21.4	(q)	5'	0.71	(d, 6.0)	21.3	(q)
	NH	7.40	(d, 8.5)			NH	7.33	(d, 9.0)		
Choi	1			173.0	(s)	Choi 1			173.3	(s)
amide	2	4.12	(t, 8.0)	59.4	(d)	amide 2	4.64	(t, 8.6)	59.1	(d)
	3a	1.80	(ddd, 14.2, 13.3, 8.0)	30.1	(t)	3a	1.85	(m)	30.1	(t)
	3b	1.98	(m)			3b	2.22	(m)		
	3'	2.25	(m)	35.8	(d)	3'	2.22	(m)	35.9	(d)
	4a	1.41	(m)	18.8	(t)	4a	1.45	(m)	18.7	(t)
	4b	2.02	(m)			4b	2.02	(m)		
	5	1.42	(m)	25.9	(t)	5a	1.39	(m)	25.9	(t)
	6	3.92	(br)	63.7	(d)	5b	1.98	(m)		
	7a	1.65	(ddd, 14.2, 11.6, 1.9)	33.2	(t)	6	3.84	(br)	63.7	(d)
	7b	2.00	(m)			7a	1.43	(m)	33.4	(t)
	7'	4.02	(m)	53.6	(d)	7b	1.85	(m)		
	NH_2	6.83	(br)			7'	4.27	(ddd, 11.5, 6.0, 6.0)	53.9	(d)
		7.22	(br)			NH_2	7.10	(br)		
							7.60	(br)		

The stereochemistries of the Hpla and Leu units of **6** were determined by the above-mentioned procedure. The similarity of the ^1H and ^{13}C NMR spectra of Choi (sulfate) between **1** and **6** and the NOESY correlations[19] indicated that the stereochemistry of the Choi sulfate unit was also 2*S*, 3'*R*, 6*S*, 7'*R*.

The chromatogram of aeruginosins 89-A (**7**) and 89-B (**8**) on ODS HPLC showed a very complex pattern. After re-isolation, these individual peaks gave the same chromatographic pattern again. Furthermore, an aldehyde proton signal (δ_H 9.82) in ^1H NMR spectrum of a mixture of **7** and **8** in DMSO-d_6 was observed. From these results, both **7** and **8** were considered to be a mixture of tautomers as leupeptins,[20] aeruginosins 102-A and 102-B.[6] To determine the stereochemistries of these compounds, a mixture of **7** and **8** was treated with MeOH containing 10% HCl to obtain desulfated aeruginosins 89-A (**9**) and 89-B (**10**) dimethylacetal by HPLC, and these compounds were oxidized with $NaClO_2$ and hydrolyzed. The HPLC analysis of Marfey derivatives of Arg in the acid hydrolysates indicated that the stereochemistries of argininal dimethylacetal in **9** and **10** were L- and D-forms, respectively. Consequently, it is considered that **7** and **8** were epimers which differ in the stereochemistry of argininal.

Fortunately, in the each ^1H and ^{13}C NMR spectra of **7** and **8**, which were purified by ODS HPLC using aqueous MeCN containing 0.05% TFA, only one set of signals was observed in DMSO-d_6.



9 (*S*) Argal dimethylacetal
10 (*R*) Argal dimethylacetal

The molecular formula of aeruginosin 89-B (**8**) was decided to be $C_{30}H_{44}N_6O_{10}SCl$ by low and high resolution FABMS and NMR data (Table 3), but its 2 D NMR data were not clear for its broard spectra. The pseudomolecular ions at *m/z* 717/719 $[M+H/M+H+2]^+$ of the positive FABMS revealed the presence of a chlorine atom, and a desulfated ion at *m/z* 637 $[M-SO_3+H]^+$ was also observed. Its peptidic nature was suggested by the ^1H and ^{13}C NMR spectra and the amino acid analysis of acid hydrolysate gave Leu. Interpretation of NMR data including ^1H-^1H COSY, HMQC and HMBC spectra revealed the partial structures, chloro Hpla sulfate and Choi. The structures of the Leu and Argal (argininal) units were deduced by the interpretation of 2 D NMR spectra and comparison of the ^{13}C NMR data with those of other aeruginosins because the HMBC correlations between Leu H-2, H-3 and NH/Leu C-1 (δ 169.2) and Argal H-1 and H-5/Argal C-6 (δ 156.7) were not observed (Fig. 5). The sequence of these partial structures was determined by the HMBC and NOESY spectra. In the HMBC spectrum, Leu NH coupled to chloro Hpla sulfate C-1 and Argal NH coupled to Choi C-1. The NOESY spectrum showed the correlation between Choi H-7' and Leu H-2 (Fig. 5).

The stereochemistries of the chloro Hpla sulfate and Leu units of **8** were determined by the methods as above mentioned. The correspondence of the ^1H and ^{13}C NMR spectra of Choi sulfate between **1** and **8**, and the NOESY correlations[21] indicated that the stereochemistry of the Choi sulfate unit was also 2*S*, 3'*R*, 6*S*,

7'*R*. The absolute configuration on C-2 of the Argal unit was determined to be D-form by Marfey's analysis of acid hydrolysate of oxyproduct of **8**, in which Argal was oxydized to Arg. Moreover the Argal unit appeared to have a chair form by NOESY correlations shown in Fig. 6. These results indicated that the Argal unit was 1*S*, 2*R* argininal.

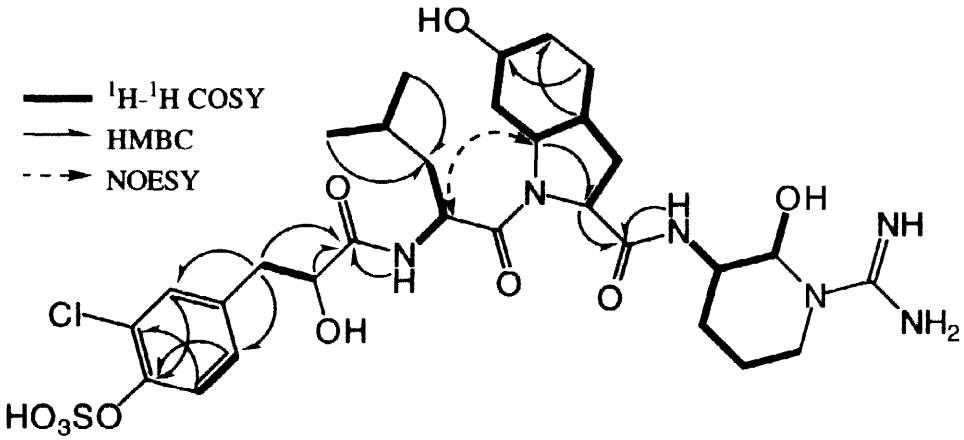


Fig. 5. ^{1}H-^{1}H COSY, HMBC and NOESY correlations of Aeruginosin 89-B (**8**).

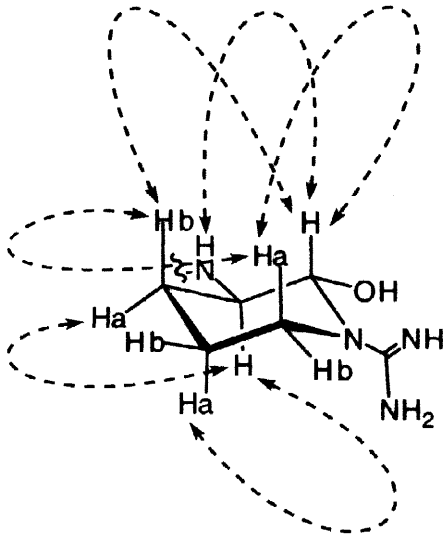


Fig. 6. Relative stereochemistry of argininal in aeruginosin 89-B (**8**) (Dashed arrows: NOESY).

The molecular formula of aeruginosin 89-A (**7**) was decided to be $C_{30}H_{44}N_6O_{10}SCl$, which was the same as that of **8**, by low and high resolution FABMS and NMR data (Table 3). The existence of a sulfate was also suggested by the fragment ion of positive FABMS. ^{1}H and ^{13}C NMR spectra were similar to those of **8**, but chemical shift data of the Argal and Choi units were different distinctly. Detailed analyses of 1 D and 2 D NMR spectra and comparison of the ^{13}C NMR data with those of other aeruginosins revealed that the planar structure of **7** was identical to that of **8**. The absolute stereochemistry of the Argal unit was L-form, which was different from **8**. However the relative stereochemistry of the Argal unit was not determined because of poor NOESY correlations. The absolute stereochemistries of the chloro Hpla sulfate, Leu and Choi[22] were identical to those of **7**.

Aeruginosin-type peptides are the linear peptides consisting of Hpla derivatives, usual amino acids, Choi derivatives and Arg derivatives. Among these peptides, aeruginosin 298-B might be considered to be a biosynthetic intermediate, because it lacks Arg derivative and is protected by a primary amide. On the other hand, Shin *et al.*[7] isolated aeruginosins 205-A and 205-B from *Oscillatoria agardhii* (NIES-205), which are the further modified aeruginosins with xylopyranose.

In the present study, the absolute configuration of the Choi unit of aeruginosin 98-A was decided to be 2*S*, 3'*R*, 6*S*, 7'*R* by chemical studies. Its absolute configuration was identical with the X-ray crystallographic structure of the trypsin-aeruginosin 98-B complex.[8] In 1987, the synthesis of 2-carboxy-6-oxooctahydroindole using Diels-Alder reaction of 1-methoxy-1,3-cyclohexadiene and dehydroalanine

derivative has been reported by Souchet *et al.*[23] Bonjoch *et al.* has also reported the synthesis of Choi using Birch reduction of *O*-Me Tyr and the relative configuration of Choi of aeruginosin 298-A was assigned to be 2*S*, 3'*R*, 6*S*, 7'*R* configuration.[24]

Table 3. ^{1}H and ^{13}C NMR Data for Aeruginosins 89-B (**8**) and 89-A (**7**) in DMSO-d_6

	Aeruginosin 89-B (**8**)				Aeruginosin 89-A (**7**)		
Position		^{1}H *J* (Hz)	^{13}C	Position		^{1}H *J* (Hz)	^{13}C
Chloro Hpla	1		172.0 (s)	Chloro Hpla	1		172.1 (s)
sulfate	2	4.08 (dd, 7.3, 3.9)	71.6 (d)	sulfate	2	4.10 (m)	71.6 (d)
	3a	2.70 (dd, 13.7, 7.3)	39.0 (t)		3a	2.70 (dd, 13.9, 7.5)	39.0 (t)
	3b	2.93 (dd, 13.7, 3.9)			3b	2.92 (dd, 13.9, 3.4)	
	4		133.8 (s)		4		133.9 (s)
	5	7.20 (d, 1.7)	130.3 (d)		5	7.20 (s)	130.3 (d)
	6		123.6 (s)		6		123.6 (s)
	7		147.8 (s)		7		147.8 (s)
	8	7.46 (d, 8.6)	121.2 (d)		8	7.46 (d, 8.5)	121.0 (d)
	9	7.05 (dd, 8.6, 1.7)	128.6 (d)		9	7.05 (d, 8.5)	128.5 (d)
Leu	1		169.2 (s)	Leu	1		169.5 (s)
	2	4.57 (br)	53.8 (d)		2	4.57 (m)	47.8 (d)
	3a	1.23 (m)	42.1 (t)		3a	1.23 (m)	42.0 (t)
	3b	1.38 (m)			3b	1.39 (m)	
	4	1.36 (m)	24.0 (d)		4	1.35 (m)	24.0 (d)
	5	0.82 (d, 6.4)	23.4 (q)		5	0.82 (d, 7.4)	23.3 (q)
	5'	0.88 (d, 6.4)	21.3 (q)		5'	0.88 (d, 7.4)	21.4 (q)
	NH	7.39 (d, 9.0)			NH	7.45 (d, m)	
Choi	1		170.9 (s)	Choi	1		170.8 (s)
	2	4.25 (t, 8.6)	59.6 (d)		2	4.28 (t, 9.0)	59.4 (d)
	3a	1.72 (m)	30.7 (t)		3a	1.87 (m)	30.2 (t)
	3b	2.05 (m)			3b	1.97 (m)	
	3'	2.26 (m)	36.0 (d)		3'	2.29 (m)	36.0 (d)
	4a	1.41 (m)	19.0 (t)		4a	1.43 (m)	19.0 (t)
	4b	2.03 (m)			4b	2.06 (m)	
	5	1.42 (m)	26.0 (t)		5	1.44 (m)	26.0 (t)
	6	3.90 (br)	63.8 (d)		6	3.91 (br)	63.8 (d)
	7a	1.68 (m)	33.5 (t)		7a	1.66 (t, 11.7)	33.5 (t)
	7b	2.02 (m)			7b	2.03 (m)	
	7'	4.02 m)	54.0 (d)		7'	4.06 (m)	53.9 (d)
Argal	1			Argal	1	5.25 (br)	76.2 (d)
	2	5.25 (br)	76.6 (d)		2	3.70 (m)	49.0 (d)
	3a	3.73 (m)	48.7 (d)		3a	1.57 (m)	23.3 (t)
	3b	1.50 (m)			3b	1.72 (m)	
	4	1.63 (m)	23.9 (t)		4	1.49 (m)	23.4 (t)
	5a	1.47 (m)			5a	3.11 (m)	39.5 (t)
	5b	1.68 (m)	23.3 (t)		5b	3.46 (m)	
	2-NH	7.56 (d, 8.1)			2-NH	7.70 (d, 11.3)	
	1-OH	6.50 (br)			1-OH	6.40 (br)	
	6		156.7 (s)		6		156.6 (s)

Leupeptin[25-27] and antipain[28,29] are well known protease inhibitors which possess Argal at C-terminus. In the case of leupeptin, Argal tautomerized as in Fig. 7. The tautomerization of aeruginosins 89-A and 89-B may be also due to the same manner. Umezawa's group has reported that the tautomerization of leupeptins is stopped by refluxing in butanol, reduction with sodium borohydride and oxidation with potassium permanganate.[25-27] However these treatments yielded various desulfate (refluxing in 10% HCl containing MeOH) and dechloro (sodium borohydride) derivatives of aeruginosins 89-A and 89-B. Consequently, the complete separation of aeruginosins 89-A and 89-B was very difficult.

Aeruginosin-type peptides inhibited serine proteases trypsin, thrombin and plasmin potently. Serine protease inhibitory activities of aeruginosins are summarized in Table 4.

The trypsin inhibitory mode of aeruginosin 98-B has closely resembled that of D-Phe-Pro-agmatine by the X-ray crystallographic structure of the trypsin-aeruginosin 98-B complex.[8] However aeruginosin 98-B binds exclusively to specificity and recognition elements in an unprecedented fashion for a natural product so that the nucleophilic oxygen of Ser195 makes no close contacts with any atom of aeruginosin 98-B. The thrombin inhibitory mode of aeruginosin 298-A has been also decided to resemble to that of D-Phe-Pro-Arg chloromethyl ketone by the X-ray crystallographic structure of the thrombin-aeruginosin 298-A complex. In this report, Steiner *et al.* has described that the new and unusual manner in which the L-Leu and Hpla groups of aeruginosin 298-A at the P3 and P4 positions interact with thrombin reveals that the binding properties are not always predicted by the sequence or stereochemistry alone.[9]

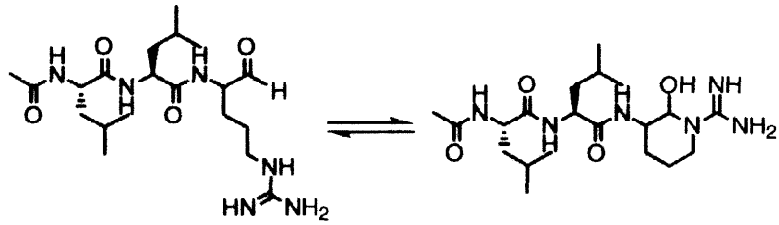


Fig. 7. Tautomerization of leupeptin.

Table 4. Serine and cysteine protease inhibitory activities of aeruginosins

IC_{50}=μg/mL	Trypsin	Thrombin	Plasmin	Papain	Other enzymes*
Aeruginosin 98-A[5]	0.6	7.0	6.0	100	>100
Aeruginosin 98-B[5]	0.6	10.0	7.0	100	>100
Aeruginosin 98-C	3.9	3.3	5.0	-	>100
Aeruginosin 101	3.0	3.2	3.3	-	>100
Aeruginosin 298-A[4]	1.0	0.3	>10	>10	>10
Aeruginosin 298-B	>100	>100	>100	>100	>100
Aeruginosin 89-A	0.4	0.03	0.02	>10	>10
Aeruginosin 89-B	6.6	0.05	0.46	>10	>10
Aeruginosin 102-A[6]	0.2	0.04	0.3	>10	>10
Aeruginosin 102-B[6]	1.0	0.1	0.8	>10	>10
Aeruginosin 205-A[7]	0.07	1.5	-	-	-
Aeruginosin 205-B[7]	0.07	0.17	-	-	-

*; elastase and chymotrypsin, -; unknown data

In the present study, it is found that aeruginosins having argininal at the C-terminus unit shows stronger protease inhibitory activity than those of having agmatine and argininol (Table 4). Especially, its tendency is more remarkable against thrombin (Table 4). Therefore, an aldehyde of argininal binding to S1 site may be very important to inhibit thrombin.

3. Experimental Section

General Methods. Ultraviolet spectrum was measured on a Hitachi 330 spectrophotometer. Optical rotation was measured on a JASCO DIP-1000 polarimeter. ^1H and ^{13}C NMR spectra were measured on either Bruker AM600, JEOL JNM-A500 or -A600 NMR spectrometers. Two-dimensional NMR spectra

were recorded on either a Bruker AM600 NMR spectrometer equipped with an ASPECT 1000 computer or a JEOL JNM-A600 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 either a Hitachi 835 or L-8500A amino acid analyzers.

Culture Conditions. Culture conditions of *M. aeruginosa* (NIES-98, NIES-101, NIES-298 and NIES-89) were the same as previously described.[4,5]

Isolation of Aeruginosins 98-A (1), 98-B (2) and 98-C (3). Freeze-dried alga (*M. aeruginosa* (NIES-98); 115.4 g from 420 L of culture) was extracted with 80% MeOH (2 L × 3) and MeOH (1.8 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 (11.4 g), which was subjected to flash chromatography on ODS (YMC-ODS AM120Å, 10 × 7 cm) with aqueous MeOH followed by CH_2Cl_2.

The 30% MeOH fraction (175 mg) was subjected to reversed-phase HPLC (J'sphere ODS H-80, 10 × 250 mm; 20-45% MeCN containing 0.05% TFA; UV-detection at 210 nm; flow rate 2.0 mL/min) to yield **1** (10.2 mg). The 40% (377 mg) and 50% MeOH (535 mg) fractions were combined, concentrated and subjected to flash chromatography on ODS (YMC-ODS AM120Å, 5 × 8 cm) with aqueous MeOH followed by CH_2Cl_2.

The 30%MeOH fraction (50 mg) was subjected to reversed-phase HPLC (CAPCELL PAK C18 AG120Å, 10 × 250 mm; 20-50% MeCN containing 0.05% TFA; UV-detection at 210 nm; flow rate 2.0 mL/min) to yield **1** (25.0 mg) and **2** (6.0 mg). The 40% MeOH fraction was subjected to reversed-phase HPLC (Cosmosil 5C18-MS, 10 × 250 mm; 20-45% MeCN containing 0.05% TFA; UV-detection at 210 nm; flow rate 2.0 mL/min) to yield **1** (45.2 mg) and **2** (11.0 mg). The 50% MeOH fraction (249 mg) was subjected to reversed-phase HPLC (YMC-Pack AM-324 120Å, 10 × 300 mm; 20-44% MeCN containing 0.05% TFA; UV-detection at 210 nm; flow rate 2.0 mL/min) to yield **1** (34.1 mg), **2** (14.4 mg) and fraction containing **3** (11.5 mg). The fraction containing **3** was subjected to reversed-phase HPLC (YMC-Pack AM-324 120Å, 10 × 300 mm; 26% MeCN containing 0.05% TFA; UV-detection at 210 nm; flow rate 2.0 mL/min) to yield **3** (5.0 mg).

Isolation of Aeruginosin 101 (4). Freeze-dried alga (*M. aeruginosa* (NIES-101); 120.0 g from 290 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 (8.0 g), which was subjected to flash chromatography on ODS (YMC-ODS AM120Å, 10 × 7 cm) with aqueous MeOH followed by CH_2Cl_2.

The 30%, 40% and 50%MeOH fractions were combined, concentrated and subjected to flash chromatography on ODS (YMC-ODS AM120Å, 8 × 7 cm) with aqueous MeOH followed by CH_2Cl_2. Each of the six fractions (20%, 30%, 40%, 50%, 60% and 100%MeOH fractions) was subjected to reversed-phase HPLC (J'sphere ODS H-80, 10 × 250 mm; 20-45% MeCN containing 0.05% TFA; UV-detection at 210 nm; flow rate 2.0 mL/min) to yield crude fraction containing **4** (60.3 mg). The fraction containing **4** was subjected to reversed-phase HPLC (YMC-Pack AM-324Å, 10 × 300 mm; 23% MeCN containing 0.05% TFA; UV-detection at 210 nm; flow rate 2.0 mL/min) to yield **4** (12.2 mg).

Isolation of Aeruginosins 298-A (5) and 298-B (6). Isolation of **5** was previously described.[4] Freeze-dried alga (*M. aeruginosa* (NIES-298); 130.0 g from 362 L of culture) was extracted with 80% MeOH (1.5 L × 3) and MeOH (1.5 L × 1). Combined 80% MeOH and MeOH extracts were concentrated to an aqueous suspension which was then extracted with diethyl ether. The aqueous layer was extracted with *n*-BuOH. The *n*-BuOH layer was evaporated under reduced pressure to green dry solid (5.0 g), which was subjected to flash chromatography on ODS (YMC-ODS AM120Å, 10 × 12 cm) with aqueous MeOH followed by CH_2Cl_2 to yield the 40-80%MeOH fraction containing **6** (0.92 g). The diethyl ether layer was further separated by the Kupchan procedure,[30] employing gradient solvent systems of hexane/90% MeOH and CCl_4/80% MeOH. The 80% MeOH layer (0.5 g) was subjected to flash chromatography on ODS (YMC-ODS AM120Å, 3 × 10 cm) with aqueous MeOH followed by CH_2Cl_2 to yield the 20% MeOH fraction containing **6** (255 mg). These fractions containing **6** were combined, concentrated and subjected to flash chromatography on ODS (YMC-ODS AM120Å, 10 × 12 cm) with aqueous MeOH followed by CH_2Cl_2. The 50% MeOH fraction (136 mg) was subjected to reversed-phase HPLC (Cosmosil 5C18-MS, 10 × 250 mm; 20-60% MeCN containing 0.05% TFA; UV-detection 210 nm; flow-rate 2.0 mL/min) to yield **6** (27.7 mg).

Isolation of Aeruginosins 89-A (7) and 89-B (8). Freeze-dried alga (*M. aeruginosa* (NIES-89); 105 g from 620 L of culture) was extracted with 80% MeOH (3 L × 3) 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 (2.6 g), which was subjected to flash chromatography on ODS (YMC-ODS AM120Å, 6 × 10 cm) with aqueous MeOH followed by CH_2Cl_2.

40% MeOH fraction was subjected to reversed-phase HPLC (YMC-Pack AM, 20 × 250 mm; 20-50% MeCN containing 0.05% TFA; UV-detection at 210 nm; flow rate 6.0 mL/min) to yield an active fraction (75.5 mg). This active fraction was subjected to reversed-phase HPLC (YMC-Pack AM324Å, 10 × 300 mm; 23% MeCN containing 0.05% TFA; UV-detection at 210 nm; flow rate 2.0 mL/min) to yield **7** (5.5 mg) and crude fraction containing **8** (65.0 mg). Fraction containing **8** was subjected to reversed-phase HPLC (Cosmosil 5C18-MS, 10 × 250 mm; 20-25% MeCN containing 0.05% TFA; UV-detection 210 nm; flow-rate 2.0 mL/min) to yield **8** (2.5 mg).

Aeruginosin 98-A (1). Properties of **1** were previously described.[5]

Aeruginosin 98-B (2). Properties of **2** were previously described.[5]

Aeruginosin 98-C (3). $[\alpha]_D$ -13.0° (*c* 0.25, H_2O); UV (H_2O) λmax 281 nm (ε 860); HRFABMS *m/z* 731.2077 $[M-H]^-$ ($C_{29}H_{44}N_6O_9SBr$, Δ +0.3 mmu); HMBC correlations: bromo Hpla C-1/allo Ile NH; bromo Hpla C-2/H-3; bromo Hpla C-3/H-5, H-9; bromo Hpla C-4/H-3, H-8; bromo Hpla C-5/H-3, H-9; bromo Hpla C-6/H-5, H-8, 7-OH; bromo Hpla C-7/H-5, H-8, H-9, 7-OH; bromo Hpla C-9/H-3, H-5; allo Ile C-1/NH; allo Ile C-2/H-6; allo Ile C-3/H-5, H-6; allo Ile C-4/H-5, H-6; allo Ile C-5/H-4; allo Ile C-6/H-4; Choi sulfate C-1/H-2, H-3, agmatine H-1, NH; Choi sulfate C-2/H-3; Choi sulfate C-3/H-2, H-7'; Choi sulfate C-7'/H-3, H-4; agmatine C-2/H-1, H-4; agmatine C-3/H-1, H-4; agmatine C-4/H-2, 4-NH; agmatine C-5/H-4, 4-NH.

Aeruginosin 101 (4). $[\alpha]_D$ -11.0° (*c* 0.50, 50% MeOH); UV (MeOH) λmax 290 nm (ε 1810); HRFABMS *m/z* 721.2149 $[M-H]^-$ ($C_{29}H_{44}N_6O_9SCl_2$, Δ -4.0 mmu); HMBC correlations: dichloro Hpla C-1/H-2, H-3, allo Ile NH; dichloro Hpla C-2/H-3; dichloro Hpla C-3/H-2, H-5, H-9; dichloro Hpla C-4/H-2,

H-3; dichloro Hpla C-5,9/H-3, H-5, H-9; dichloro Hpla C-6,8/H-5, H-9, 7-OH; dichloro Hpla C-7/H-5, H-9; allo Ile C-1/H-2; allo Ile C-2/H-4, H-6, NH; allo Ile C-3/H-2, H-4, H-5, H-6; allo Ile C-4/H-5, H-6; allo Ile C-5/H-4; allo Ile C-6/H-2, H-3, H-4; Choi sulfate C-1/H-2, H-3, agmatine H-1; Choi sulfate C-2/H-3, H-7'; Choi sulfate C-3/H-3', H-7'; Choi sulfate C-3'/H-3, H-7'; Choi sulfate C-5/H-3'; Choi sulfate C-7/H-3'; agmatine C-1/H-2; agmatine C-2/H-1, H-3, H-4; agmatine C-3/H-1, H-4; agmatine C-4/H-2, H-3, 4-NH; agmatine C-5/H-4.

Aeruginosin 298-A (5). Properties of **5** were previously described.[4]

Aeruginosin 298-B (6). UV (MeOH) λmax 277 nm (ε 2030); HRFABMS *m/z* 462.2617 $[M+H]^+$ ($C_{24}H_{36}N_3O_6$, Δ +1.3 mmu); HMBC correlations (Major component): Hpla C-1/H-2, H-3, Leu NH; Hpla C-2/H-3; Hpla C-3/H-2, H-5, H-9; Hpla C-4/H-2, H-5, H-6, H-8, H-9; Hpla C-5,9/H-5, H-9; Hpla C-6,8/H-5, H-6, H-8, H-9; Hpla C-7/H-5, H-6, H-8, H-9; Leu C-1/H-2, H-3, NH, Choi amide H-2, H-7'; Leu C-2/NH; Leu C-3/H-5, H-5'; Leu C-4/H-5, H-5'; Leu C-5/H-3, H-5'; Leu C-5'/H-3, H-5; Choi amide C-1/H-2, H-3, NH_2; Choi amide C-2/H-3, H-7', NH_2; Choi amide C-3/H-2, H-3', H-7'; Choi amide C-3'/H-3, H-4, H-7'; Choi amide C-5/H-3', H-7; Choi amide C-6/H-4; Choi amide C-7/H-3'; Choi amide C-7'/H-3', H-4, H-7.

Aeruginosin 89-A (7). HRFABMS *m/z* 715.2527 $[M-H]^-$ ($C_{30}H_{44}N_6O_{10}SCl$, Δ -0.7 mmu); HMBC correlations: chloro Hpla sulfate C-1/H-2, Leu NH; chloro Hpla sulfate C-2/H-3; chloro Hpla sulfate C-3/H-5, H-9; chloro Hpla sulfate C-4/H-2, H-3, H-8; chloro Hpla sulfate C-5/H-3, H-9; chloro Hpla sulfate C-6/H-8; chloro Hpla sulfate C-7/H-5, H-8, H-9; chloro Hpla sulfate C-9/H-3, H-5; Leu C-3/H-5, H-5'; Leu C-5/H-5'; Leu C-5'/H-5; Choi C-1/H-2, Argal 2-NH; Choi C-2/H-7'; Choi C-3/H-2, H-7'.

Aeruginosin 89-B (8). $[\alpha]_D^{23}$ +9.4° (*c* 0.1, MeOH); UV (MeOH) λmax 283 nm (ε 1660); HRFABMS *m/z* 715.2593 $[M-H]^-$ ($C_{30}H_{44}N_6O_{10}SCl$, Δ +6.5 mmu); HMBC correlations: chloro Hpla sulfate C-1/H-2, H-3, Leu NH; chloro Hpla sulfate C-2/H-3; chloro Hpla sulfate C-4/H-3, H-8; chloro Hpla sulfate C-5/H-3, H-9; chloro Hpla sulfate C-6/H-5, H-8; chloro Hpla sulfate C-7/H-5, H-8, H-9; chloro Hpla sulfate C-9/H-3, H-5; Leu C-3/H-5, H-5'; Leu C-4/H-3, H-5, H-5'; Leu C-5/H-5'; Leu C-5'/H-5; Choi C-1/H-2, Argal NH; Choi C-2/H-7'; Choi C-3/H-2; Choi C-3'/H-7; Choi C-5/H-3'; Choi C-6/H-4; Choi C-7/H-3'; Choi C-7'/H-4.

Acid Hydrolysis. For amino acid analysis, 100 μg each of **1** to **8** 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: allo Ile (90.9 in **1**, 43.8-44.9 in **2-4**), Ile (96.0 in **1**, 45.7-46.8 in **2-3**), Leu (98.1 in **5**, 47.0-47.6 in **6-8**); and acid hydrolysates of **1-4**: **1**, allo Ile (90.9); **2**, allo Ile (43.6); **3**, allo Ile (43.6); **4**, allo Ile (44.9); **5**, Leu (98.1); **6**, Leu (47.0); **7**, Leu (47.6); **8**, Leu (47.2).

HPLC Analyses of the Marfey Derivatives. To the acid hydrolyzate of a 100 μg portion of the peptides, 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 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-Leu (51.6-52.0 in **6-8**), D-Leu (55.6-55.8 in **6-8**), L-allo Ile (48.8 in **4**, 51.3 in **1-3**), D-allo Ile (52.8 in **4**, 55.4

in **1-3**); and amino acid derivatives from **1-4** and **6-8**: **1-3**, D-allo Ile (55.4); **4**: D-allo Ile (52.8); **6**, L-Leu (51.6)/D-Leu (55.6) 3:1; **7** and **8**, D-Leu (55.8).

***p*-Hydroxyphenyllactic Acid (Hpla). L-Hpla.** *p*-Amino-L-phenylalanine (100-300 mg) was dissolved in 1 N HCl (10-15 mL) and cooled to 0 °C. To this solution, $NaNO_2$ (100-200 mg) dissolved in water (5-10 mL) was added dropwise. After the solution was stirred at rt for 16 h, it was heated under reflux for 30 min. Then the solution was subjected to ODS column chromatography (Cosmosil 140C18, 3 × 11 cm) with aqueous MeOH and reversed-phase HPLC (Cosmosil 5C18-MS, 10 × 250 mm, 17% MeCN containing 0.05% TFA, UV-detection 210 nm, flow rate 2.0 mL/min) to yield L-Hpla (25-35%); HRFABMS *m/z* 181.0474 $[M-H]^-$ ($C_9H_9O_4$, Δ -2.7 mmu). **D-Hpla.** This was obtained from *p*-amino-D-phenylalanine (250 mg) in 31% yield.

3-Chloro-4-Hydroxyphenyllactic Acid (chloro Hpla). Chloro D-Hpla. The mixture of D-Hpla (70 mg), sulfuryl chloride (200 μL) and CH_2Cl_2 (2 mL) was warmed at 80 °C until gas no longer evolved. Then CH_2Cl_2 (2 mL) was added to the reaction flask and warmed at 80 °C until gas no longer evolved. The excess sufuryl chloride was removed by lyophilization. The reaction mixture was subjected to reversed-phase HPLC (Cosmosil 5C18-MS, 10 × 250 mm, 20-40% MeCN containing 0.05% TFA; UV-detection at 210 nm; flow rate 2.0 mL/min) to yield chloro D-Hpla (18.0 mg; 21%). Chloro D-Hpla; HRFABMS *m/z* 215.0128 $[M-H]^-$ ($C_9H_8O_4Cl$, Δ +1.7 mmu); $[\alpha]_D^{23}$ + 15.6° (*c* 0.5, H_2O); ^1H NMR (DMSO-d_6), δ 2.68 (H-3, dd, 13.7, 8.1), δ 2.84 (H-3, dd, 13.7, 4.7), δ 4.08 (H-2, dd, 8.1, 4.7), δ 6.85 (H-8, d, 8.1), δ 6.97 (H-9, dd, 8.1, 2.1), δ 7.17 (H-5, d, 2.1); ^{13}C NMR (DMSO-d_6), δ 37.8 (C-3), δ 70.4 (C-2), δ 115.6 (C-8), δ 118.9 (C-6), δ 129.5 (C-9), δ 129.7 (C-4), δ 130.0 (C-5), δ 151.3 (C-7), δ 174.9 (C-1). **Chloro D,L-Hpla.** This was obtained from D,L-Hpla (100 mg) in 23% yield.

3-Bromo-4-Hydroxyphenyllactic Acid (bromo Hpla). Bromo L-Hpla. To the mixture of L-Hpla (20 mg) and dried THF (1.0 mL), dioxane dibromide (27.3 mg; prepared from bromine and dried 1,4-dioxane) was added at -20 °C and stirred for 15 min. After stirring at room temperature for 1 h, the solvent was removed by evaporation. The reaction mixture was subjected to reversed-phase HPLC (Cosmosil 5C18-MS, 10 × 250 mm, 22-52% MeCN containing 0.05% TFA; UV-detection at 210 nm; flow rate 2.0 mL/min) to yield bromo L-Hpla (10.5 mg; 37%). Bromo L-Hpla; HRFABMS *m/z* 258.9611 $[M-H]^-$ ($C_9H_8O_4Br$, Δ +0.5 mmu); $[\alpha]_D^{23}$ - 13.5° (*c* 0.5, H_2O); ^1H NMR (DMSO-d_6), δ 2.76 (H-3, dd, 13.9, 8.1), 2.83 (H-3, dd, 13.9, 4.4), 4.06 (H-2, dd, 8.1, 4.4), 6.82 (H-8, d, 8.4), 7.01 (H-9, dd, 8.4, 2.2), 7.31 (H-5, d, 2.2), 9.97 (7-OH, br); ^{13}C NMR (DMSO-d_6), δ 38.6 (C-3), 70.9 (C-2), 108.6 (C-6), 115.8 (C-8), 129.6 (C-9), 130.2 (C-4), 133.4 (C-5), 152.3 (C-7), 174.9 (C-1). **Bromo D,L-Hpla.** This was obtained from D,L-Hpla (30 mg) in 69% yield.

3,5-Dichloro-4-Hydroxyphenyllactic Acid (Dichloro Hpla). Dichloro L-Hpla. The mixture of L-Hpla (70 mg) and sulfuryl chloride (900 μL) was warmed at 80 °C until gas no longer evolved. The excess sufuryl chloride was removed by lyophylization. The reaction mixture was subjected to reversed-phase HPLC (Cosmosil 5C18-MS, 10 × 250 mm, 22-52% MeCN containing 0.05% TFA; UV-detection at 210 nm; flow rate 2.0 mL/min) to yield dichloro L-Hpla (27.0 mg; 28%). Dichloro L-Hpla; HRFABMS *m/z* 248.9743 $[M-H]^-$ ($C_9H_7O_4Cl_2$, Δ +2.2 mmu); $[\alpha]_D^{23}$ - 10.9° (*c* 0.5, H_2O); ^1H NMR (DMSO-d_6), δ 2.71 (H-3, dd, 14.1, 8.1), δ 2.85 (H-3, dd, 14.1, 4.3), δ 4.10 (H-2, dd, 8.1, 4.3), d 5.6 (2-OH, d, 5.1), δ 7.02 (H-5,9, s), δ 9.90 (7-OH, br); ^{13}C NMR (DMSO-d_6), δ 38.3 (C-3), δ 70.5 (C-2), δ 121.7 (C-5,9), δ 129.4

(C-6,8), δ 131.1 (C-4), δ 147.3 (C-7), δ 175.0 (C-1). **Dichloro D,L-Hpla.** This was obtained from D,L-Hpla (100 mg) in 8% yield.

Derivatization and HPLC Analysis of Hpla and Halogenated Hpla. *l*-Menthol (10.0 mg) and MeCN (50 μL) were added to each of the acid hydrolysates of **1** to **8** (100 or 200 μg). After cooling, TMSCl (20 μL) was added, and the mixture was sealed in a test tube with screw cap, and heated at 100 °C for 10 min. Then the solvent was removed in a stream of nitrogen and diluted with MeCN (100 μL), and analyzed by reversed-phase HPLC (Cosmosil 5C18-MS, 10 × 250 mm, 50% (10 min)-70% (gradient elution of 1%/min) MeCN containing 0.05% TFA, UV-detection 210 nm, flow rate 1.0 mL/min). Retention times (min) of standards: D-Hpla (25.2 min), L-Hpla (26.4 min), chloro D-Hpla (30.0), chloro L-Hpla (31.2), bromo D-Hpla (33.0), bromo L-Hpla (34.4), dichloro D-Hpla (36.4), dichloro L-Hpla (37.6); and derivatives from **1**-**8**: D-Hpla (33.0) in **2**, **5** and **6**; chloro D-Hpla (30.0) in **1**, **7** and **8**; bromo D-Hpla (33.0) in **3**; dichloro D-Hpla (36.4) in **4**.

2-Carboxy-6-Hydroxyoctahydroindole diacetate (Choi diacetate). Aeruginosin 98-A (**1**; 30.0 mg) was disssolved in 6 N HCl (5.0 mL) and heated at 110 °C for 28 h. After the solvent was removed by evaporation, the reaction mixture was lyophylized and subjected to reversed-phase HPLC (Cosmosil 5C18-MS, 10 × 250 mm; 0-60% MeCN containing 0.05% TFA; UV-detection 210 nm, flow rate 2.0 mL/min) to yield the fraction containing Choi (4.0 mg). To this fraction, acetic anhydride (0.5 mL) and pyridine (0.5 mL) was added and stirred at room temperature for 16 h. After the solvent was removed by evaporation and lyophylization, the reaction mixture was subjected to reversed-phase HPLC (Cosmosil 5C18-MS, 10 × 250 mm; 10-60% MeCN containing 0.05% TFA; UV-detection 210 nm, flow rate 2.0 mL/min) to yield Choi diacetate (2.5 mg): ^{1}H NMR (CD_3OD; 600 MHz), **major conformer** δ 1.65 (H-4, m), 1.65 (H-5, m), 1.74 (H-5, m), 1.83 (H-7, ddd, 15.0, 11.5, 2.6), 2.04 (H-3, m), 2.04 (H-4, m), 2.06 (*O*Ac, s), 2.07 (*N*Ac, s), 2.20 (H-7, m), 2.26 (H-3, ddd, 12.4, 8.1, 7.3), 2.50 (H-3a, m), 4.14 (H-7a, ddd, 10.7, 6.4, 6.4), 4.35 (H-2, dd, 10.3, 8.6), 5.08 (H-6, br); **minor conformer** δ 1.60 (H-7, ddd, 14.1, 11.6, 2.6), 1.60 (H-5, m), 1.63 (H-4, m), 1.68 (H-5, m), 1.91 (*N*Ac, s), 2.02 (H-4, m), 2.05 (*O*Ac, s), 2.16 (H-3, m), 2.24 (H-7, m), 2.35 (H-3a, m), 2.43 (H-3, ddd, 11.5, 8.4, 7.0), 4.39 (H-7a, m), 4.53 (H-2, t, 8.6), 5.03 (H-6, br).

***N*-(*S*,*R*)-PGME-2-Carboxy-6-Hydroxyoctahydroindole diacetate.** A solution of Choi diacetate (1.0 mg) in DMF (0.5 mL) was added with (*R*)-PGME (1.0 mg), PyBOP (3.3 mg), HOBT (1.0 mg) and *N*-methylmorphorine (1.0 μL) at 0 °C, and the mixture was stirred at room temperature for 16 h. After the solvent was removed by lyophylization, the reaction mixture was subjected to reversed-phase HPLC (Cosmosil 5C18-MS, 10 × 250 mm; 20-100% MeCN containing 0.05% TFA; UV-detection 210 nm; flow rate 2.0 mL/min) to yield *N*-(*R*)-PGME-Choi diacetate (1.5 mg; 97%): ^{1}H NMR (CD_3OD; 600 MHz), **major conformer** δ 1.58 (H-5, m), 1.65 (H-4, m), 1.70 (H-5, m), 1.84 (H-7, m), 1.96 (H-3, m), 2.01 (H-4, m), 2.05 (*N*Ac, s), 2.08 (*O*Ac, s), 2.14 (H-3, ddd, 12.4, 7.7, 7.3), 2.15 (H-7, m), 2.46 (H-3a, m), 3.70 (CO_2Me, s), 4.52 (H-2, t 10.0), 5.04 (H-6, br), 5.50 (H-1', s), 7.30-7.40 (phenyl, m); **minor conformer**, δ 1.60 (H-5, m), 1.63 (H-4, m), 1.68 (H-5, m), 1.75 (H-7, m), 1.97 (*N*Ac, s), 2.02 (H-4, m), 2.04 (H-3, m), 2.04 (*O*Ac, s), 2.26 (H-3, m), 2.26 (H-7, m), 2.32 (H-3a, m), 3.70 (CO_2Me, s), 4.37 (H-7a, ddd, 12.4, 6.4, 5.1), 4.53 (H-2, t 8.1), 5.04 (H-6, br), 5.42 (H-1', s), 7.30-7.40 (phenyl, m). *N*-(*S*)-PGME-Choi diacetate was also derivatized as described above. *N*-(*S*)-PGME-Choi diacetate (1.5 mg; 97%): ^{1}H NMR (CD_3OD; 600MHz), **major conformer**, δ 1.65 (H-5, m), 1.66 (H-4, m), 1.74 (H-5, m),

1.90 (H-7, ddd, 14.5, 11.5, 2.6), 2.04 (H-4, m), 2.04 (*N*Ac, s), 2.06 (*O*Ac, s), 2.08 (H-3, m), 2.15 (H-7, m), 2.21 (H-3, ddd, 12.8, 7.7, 7.2), 2.48 (H-3a, m), 3.70 (CO_2Me, s), 4.12 (H-7a, ddd, 12.2, 6.4, 5.8), 4.49 (H-2, dd, 9.8, 8.1), 5.07 (H-6, br), 5.44 (H-1', s), 7.31-7.42 (phenyl, m); **minor conformer**, δ 1.64 (H-5, m), 1.67 (H-4, m), 1.72 (*N*Ac, s), 1.73 (H-7, m), 1.75 (H-5, m), 2.04 (H-4, m), 2.04 (*O*Ac, s), 2.16 (H-3, m), 2.24 (H-7, m), 2.34 (H-3a, m), 2.38 (H-3, m), 3.71 (CO_2Me, s), 4.36 (H-7a, ddd, 12.0, 6.4, 5.6), 4.51 (H-2, dd, 9.0, 8.1), 5.04 (H-6, br), 5.50 (H-1', s), 7.31-7.42 (phenyl, m).

L-Argininol. Boc L-Arg (NO_2) (200 mg) was dissolved in dried MeOH (2.0 mL) - benzene (7.65 mL) and then $TMSCHN_2$ (0.35 mL) was added and stirred at room temperature for 3 h under argon. After the solvent was removed by evaporation, the reaction mixture was subjected to silica gel column chromatography (Kieselgel 60, 3 × 7 cm) with $CHCl_3$, $CHCl_3$/MeOH (9:1) and $CHCl_3$/MeOH (4:1) to yield Boc L-Arg (NO_2) methylester (178.2 mg; 85%): FABMS (matrix: glycerol, positive) *m/z* 334 $[M+H]^+$; ^1H NMR ($CDCl_3$; 600 MHz), δ 1.40-1.46 (s, 9H), 1.50-1.78 (m, 3H), 1.84-1.91 (m, 1H), 3.23-3.31 (m, 1H), 3.52-3.60 (br, 1H), 3.75-3.79 (s, 3H), 4.30-4.36 (br, 1H), 5.32-5.37 (d 8.1, 1H), 7.24-7.44 (br, 1H).

Boc L-Arg (NO_2) methylester (150 mg) was dissolved in dried THF (5.0 mL) and then $LiBH_4$ (20 mg) was added to the solution with stirred at room temperature under argon. After being stirred for further 24 h, 10%citric acid was added and stirred at room temperature for 30 min. The reaction mixture was extracted with EtOAc, dried over Na_2SO_4, concentrated and subjected to silica gel column chromatography (Kieselgel 60, 3 × 7 cm) with $CHCl_3$, $CHCl_3$/MeOH (49:1), $CHCl_3$/MeOH (9:1) and $CHCl_3$/MeOH (4:1) to yield Boc L-Arg (NO_2) ol (111.5 mg; 81%): FABMS (matrix: glycerol, positive) *m/z* 306 $[M+H]^+$; ^1H NMR ($CDCl_3$; 600 MHz), δ 1.40-1.45 (s, 9H), 1.47-1.55 (m, 1H), 1.58-1.90 (m, 3H), 3.28-3.37 (br, 1H), 3.47-3.56 (br, 1H), 3.56-3.62 (dd, 11.1, 4.7, 1H), 3.66-3.73 (m, 1H), 4.94-5.00 (d, 6.8, 1H), 7.20-7.40 (br, 1H).

Boc L-Arg (NO_2) ol (100 mg) was dissolved in EtOH (4.0 mL) and Palladium black (20 mg) was added to the solution with stirred at room temperature for 24 h under hydrogen. The reaction mixture was filtered and evaporated to give a crude alcohol, which was dissolved in TFA (2.0 mL). After being stirred for 1 h, this solution was lyophylized, dissolved in EtOAc, washed with H_2O and dried over Na_2SO_4. The reaction mixture was evaporated and subjected to reversed-phase HPLC (Cosmosil 5C18-MS, 10 × 250 mm; H_2O-50% MeOH; UV-detection 210 nm; flow rate 2.0 mL/min) to yield L-argininol (27.0 mg; 51%): $[\alpha]_D^{26.5}$ +4.7° (*c* 1.0, MeOH); HRFABMS *m/z* 161.1403 $[M+H]^+$ ($C_6H_{17}N_4O$, Δ +0.1 mmu); ^1H NMR (D_2O; 600 MHz), δ 1.50-1.65 (m, 4H), 3.09-3.13 (t, 6.8, 2H), 3.18-3.25 (m, 1H), 3.50-3.55 (dd, 12.4, 4.7, 1H), 3.19-3.23 (dd, 12.4, 3.4, 1H).

Derivatization and HPLC Analysis of L-Argininol. The acid hydrolysate of **5** (100 μg) was derivatized with D- and L-FDAA as described above and analyzed by reversed-phase HPLC (Cosmosil 5C18-MS, 10 × 250 mm, 20% MeCN containing 0.05% TFA, UV-detection 210 nm, flow rate 1.0 mL/min). Retention times (min) of standards: L-argininol-D-FDAA (11.2), L-argininol-L-FDAA (13.4); and derivative from aeruginosin 298-A (**5**): L-argininol (13.4).

Desulfated Aeruginosins 89-A and 89-B dimethyl acetal (9 and 10). A solution of aeruginosins 89-A (**7**) and 89-B (**8**) mixture (50.0 mg) in MeOH containing 10% HCl (0.4 mL) was stirred at room temperature for 10 min. After the solvent was removed by lyophylization, the reaction mixture was subjected to ODS chromatography (YMC-ODS AM120Å, 1.5 × 7.0 cm) with aqueous MeOH and reversed-phase HPLC (Capcell Pak C18 UG, 10 × 250 mm, 25% MeCN containing 0.05% TFA, UV-detection 210 nm, flow rate 2.0 mL/min) to yield **9** (4.1 mg) and **10** (3.8 mg). Desulfate aeruginosin 89-A dimethyl acetal

(**9**): ^{1}H NMR (DMSO-d_6), chloro Hpla [δ 2.70 (H-3, dd, 14.0, 6.8), 2.84 (H-3, dd, 14.0, 3.7), 4.09 (H-2, ddd, 6.8, 4.8, 3.7), 5.80 (2-OH, d, 4.8), 6.83 (H-8, d, 8.5), 6.94 (H-9, dd, 8.5, 1.1), 7.13 (H-5, d, 1.1), 9.80 (7-OH, s)], Leu [δ 0.79 (H-5, d, 6.2), 0.86 (H-5', d, 6.2), 1.17 (H-3, m), 1.24 (H-4, m), 1.36 (H-3, m), 4.55 (H-2, ddd, 10.4, 8.8, 3.1), 7.38 (NH, d 8.8)], Choi [δ 1.42 (H-4, m), 1.43 (H-5, m), 1.64 (H-7, ddd, 11.0, 8.6, 2.4), 1.82 (H-3, ddd, 14.5, 9.5, 8.2), 1.97 (H-3, ddd, 14.5, 8.5, 7.7), 2.03 (H-7, m), 2.04 (H-4, m), 2.27 (H-3a, m), 3.93 (H-6, br), 4.05 (H-7a, ddd, 8.7, 8.6, 6.5), 4.20 (H-2, dd, 9.5, 8.5)], Argal dimethyl acetal [δ 1.30 (H-3, m), 1.43 (H-4, m), 1.55 (H-4, m), 3.05 (H-5, dd, 13.2, 6.6), 3.24 (*O*Me, s), 3.29 (*O*Me', s), 3.77 (H-2, m), 4.14 (H-1, d, 4.8), 7.38 (5-NH, t, 6.6), 7.74 (2-NH, d, 8.8)], ^{13}C NMR (DMSO-d_6), chloro Hpla [δ 38.8 (C-3), 70.8 (C-2), 116.0 (C-8), 118.9 (C-6), 129.1 (C-9), 129.6 (C-4), 130.6 (C-5), 151.4 (C-7), 172.0 (C-1)], Leu [δ 21.3 (C-5'), 23.2 (C-5), 23.9 (C-4), 42.2 (C-3), 47.8 (C-2), 169.7 (C-1)], Choi [δ 19.0 (C-4), 26.0 (C-5), 30.3 (C-3), 33.3 (C-7), 35.9 (C-3a), 54.0 (C-7a), 59.6 (C-2), 63.8 (C-6), 171.4 (C-1)], Argal dimethyl acetal [25.0 (C-4), 25.7 (C-3), 40.7 (C-5), 49.9 (C-2), 54.2 (*O*Me), 55.9 (*O*Me'), 105.3 (C-1), 156.6 (C-6)]. Desulfate aeruginosin 89-B dimethyl acetal (**10**): ^{1}H NMR (DMSO-d_6), chloro Hpla [δ 2.68 (H-3, dd, 14.1, 7.3), 2.84 (H-3, dd, 14.1, 3.9), 4.06 (H-2, dd, 7.3, 3.9), 6.83 (H-8, d, 8.1), 6.94 (H-9, dd, 8.1, 2.1), 7.13 (H-5, d, 2.1)], Leu [δ 0.80 (H-5, d, 6.8), 0.86 (H-5', d, 6.4), 1.21 (H-3, m), 1.29 (H-4, m), 1.37 (H-3, m), 4.48 (H-2, m), 7.45 (NH, d 8.1)], Choi [δ 1.42 (H-4, m), 1.43 (H-5, m), 1.64 (H-7, ddd, 13.4, 11.0, 3.0), 1.79 (H-3, ddd, 14.0, 9.8, 8.1), 2.03 (H-3, m), 2.03 (H-7, m), 2.04 (H-4, m), 2.27 (H-3a, m), 3.93 (H-6, br), 4.05 (m), 4.22 (H-2, dd, 9.8, 8.1)], Argal dimethyl acetal [δ 1.32 (H-3, m), 1.32 (H-4, m), 1.43 (H-4, m), 3.03 (H-5, m), 3.25 (*O*Me, s), 3.34 (*O*Me', s), 3.77 (H-2, m), 4.20 (H-1, d, 5.1), 7.46 (5-NH, t, 6.6), 7.50 (2-NH, d, 9.0)], ^{13}C NMR (DMSO-d_6), chloro Hpla [δ 38.8 (C-3), 71.6 (C-2), 116.0 (C-8), 118.9 (C-6), 129.1 (C-9), 129.6 (C-4), 130.6 (C-5), 151.4 (C-7), 172.3 (C-1)], Leu [δ 21.3 (C-5'), 23.4 (C-5), 23.9 (C-4), 41.6 (C-3), 48.2 (C-2), 169.7 (C-1)], Choi [δ 19.0 (C-4), 26.0 (C-5), 30.6 (C-3), 33.3 (C-7), 36.0 (C-3a), 54.0 (C-7a), 59.7 (C-2), 63.8 (C-6), 171.4 (C-1)], Argal dimethyl acetal [25.4 (C-4), 25.4 (C-3), 40.6 (C-5), 49.8 (C-2), 54.1 (*O*Me), 55.7 (*O*Me'), 105.3 (C-1), 156.6 (C-6)].

Oxidation and HPLC Analysis of 7 and 8. Each of compounds **7** and **8** (200 μg) was dissolved in *t*-BuOH (400 μL) and H_2O (100 μL). To this solution were added 2-methyl-2-butene (4.0 mg), NaH_2PO_4 (1.0 mg) and $NaClO_2$ (80% purity, 4.0 mg). After the mixture was stirred at room temperature for 1.5 h, was evaporated and passed through a disposable ODS column (YMC Dispo SPE C18; H_2O-80%MeOH) and evaporated, and the residue was dissolved in 6 N HCl (500 μL) and heated at 105 °C for 12 h. Each acid hydrolysate of the oxyproducts of **7** and **8** was derivatized with D- and L-FDAA as described above 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: D-Arg (33.2) and L-Arg (34.4); and amino acid derivatives from **7** and **8**: **7**; L-Arg (34.4), **8**; D-Arg (33.2).

Oxidation and HPLC Analysis of 9 and 10. Each of compounds **9** and **10** (500 μg) in 1.0 mL of 0.02 N HCl was heated at 60 °C for 3 h. After the solvent was removed by lyophylization, oxidation and HPLC analyses of the reaction mixtures were carried out by above mentioned method. Retention times (min) of the standard amino acids: D-Arg (37.6) and L-Arg (38.8); and amino acid derivatives from **9** and **10**: **9**; L-Arg (38.8), **10**; D-Arg (37.6).

Protease Inhibitory Assay. Serine and cysteine protease inhibitory activities were carried out by the methods previously described.[31]

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