Aeruginosins 205A and -B, Serine Protease Inhibitory Glycopeptides from the Cyanobacterium *Oscillatoria agardhii* (NIES-205)

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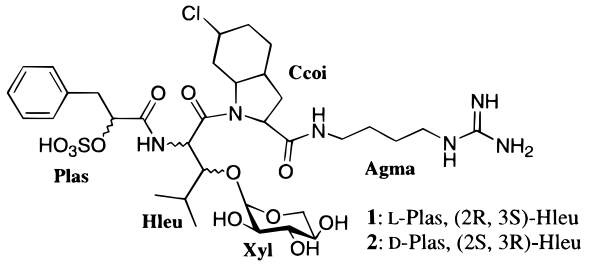
Serine protease inhibitory glycopeptides, aeruginosins 205 A (**1**) and -B (**2**), were isolated from the cyanobacterium *Oscillatoria agardhii* (NIES-205). The gross structures of these glycopeptides were elucidated by mass and 2D NMR spectral data. The planar structure of **2** was the same as that of **1** but differed in the stereochemistries of 3-hydroxyleucine (Hleu) and phenyllactic acid 2-*O*-sulfate (Plas). These glycopeptides are potent inhibitors of trypsin and thrombin.

Introduction

Cyanobacteria have received considerable attention as a source of novel natural products with unique structures and biological activities.[1] In this regard, we have reported on the structure elucidation of aeruginosins 298A[2] and 98A and -B,[3] protease inhibitors from *Microcystis aeruginosa*. Aeruginosins showed potent inhibitory activity against trypsin and thrombin. In the course of our ongoing screening program for enzyme inhibitors from cyanobacteria,[4] we isolated aeruginosins 205A and -B, new glycopeptides with potent serine protease inhibitory activity and unique structure from *Oscillatoria agardhii* (NIES-205). We report here the isolation and structure elucidation of unusual linear glycopeptides, aeruginosins 205A (**1**) and -B (**2**).



Results and Discussion

Isolation. *O. agardhii* (NIES-205) was isolated from Kasumigaura Lake, Japan,[5] and mass cultured in our laboratory. The freeze-dried cyanobacterium (119 g) was successively extracted with 80% MeOH and MeOH, and the resulting extracts were combined and concentrated to give a crude extract (28.9 g). The extract was partitioned between ether and water. The water-soluble fraction was further partitioned between *n*-BuOH and water. The *n*-BuOH layer (10.1 g) was subjected to ODS flash chromatography and eluted with 20−60% MeOH followed by MeOH and CH_2Cl_2. The active 50% MeOH fraction (327 mg) exhibiting considerable trypsin and thrombin inhibitory activity was purified by reversed-phase HPLC on a Cosmosil C-18 column to yield aeruginosins 205A (24.5 mg, 0.021% yield) and -B (7 mg, 0.006% yield).

Gross Structure Determination. Aeruginosin 205A (**1**) was isolated as a colorless microcrystalline solid, $[\alpha]^{20}_D = +17.7°$ (*c* 0.1, MeOH). The molecular formula of **1** was determined to be $C_{34}H_{53}N_6O_{12}ClS$ by HRFAB-MS and NMR data. The presence of chloride was supported by 3:1 isotope ion peaks at *m*/*z* 725/727 and 805/807 in the positive FAB-MS. The ^1H and ^{13}C NMR spectra of **1** (Table 1) contained resonances that were characteristic of peptide. The ^{13}C NMR spectrum contained 32 signals, including two methyl, 10 methylene, 17 methine, and five nonprotonated carbons (of which three were carbonyl carbons). Amino acid analysis indicated the presence of an unknown imino acid and the absence of usual amino acids. The extensive 2D NMR spectra employing $^1H-^1H$ COSY, HMQC,[6] HMBC,[7] NOESY, and HOHAHA experiments led to five partial structures (Figure 1), viz., Plas (phenyllactic acid 2-*O*-sulfate), Hleu (3-hydroxyleucine), Ccoi (2-carboxy-6-chlorooctahydroindole), Agma (agmatine), and Xyl (xylopyranose).

Plas. The $^1H-^1H$ COSY and HMBC spectra as well as MS data revealed the presence of the Plas unit. In the COSY spectrum, the H-2 oxygenated methine proton was correlated to H-3 methylene protons (δ_H, 2.66, 2.93), which sequentially correlated to C-1, C-4, and C-5,9 in the HMBC spectrum, indicating the presence of phenyllactic acid moiety. In the positive FAB-MS, the fragment ion peak at *m*/*z* 725 $(M - \text{sulfate} + H)^+$ indicated the presence of sulfate. The position of sulfate, whether it would be at C-2 of Plas or C-6 of Ccoi, could not be clearly determined by NMR data. But, the fragment ion peak at *m*/*z* 575 $(C_{25}H_{44}N_6O_7Cl, M - \text{Plas} + H)^+$ in the mass spectrum defined the placement of sulfate at C-2 of Plas (Figure 2).

Hleu. The 3-Hydroxyleucine unit was assigned from the COSY spectrum. The COSY experiment gave straightforward connectivities from amide proton at δ_H 8.02 to

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(2) Murakami, M.; Okita, Y.; Matsuda, H.; Okino, T.; Yamaguchi, K. *Tetrahedron Lett.* **1994**, *35*, 3129−3132.

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Table 1. ^{1}H and ^{13}C NMR Data of Aeruginosin 205A (1) in DMSO-d_6

units		^{1}H (mult, J (Hz))	^{13}C (mult)	HMBC[a] correlations
Plas	1		173.0 (s)	Plas 2, 3, Hleu NH
	2	4.15 (dd, 8.6, 4.6)	72.0 (d)	Plas 3
	3	2.66 (dd, 13.7, 8.6)	40.4 (t)	Plas 5, 9
		2.93 (dd, 13.7, 4.6)		
	4		138.1 (s)	Plas 3, 6, 8
	5, 9	7.22 (d, 7.3)	129.4 (d)	Plas 3, 7
	6, 8	7.25 (dd, 7.3, 7.0)	128.0 (d)	
	7	7.16 (t, 7.0)	126.0 (d)	Plas 5, 9
Hleu	1		167.3 (s)	Hleu 2
	2	4.93 (dd, 9.5, 2.1)	51.5 (d)	Hleu 3
	3	4.07 (dd, 10.7, 2.1)	68.7 (d)	Hleu 2, 5, 5′, Xyl 1
	4	1.83 (m)	27.7 (d)	Hleu 5, 5′
	5	0.86 (d, 6.7)	15.4 (q)	Hleu 3, 4, 5′
	5′	0.89 (d, 6.4)	20.4 (q)	Hleu 3, 4, 5
	NH	8.02 (d, 9.5)		
Ccoi[b]	1		171.2 (s)	Ccoi 2, 3, Agma 2, 1-NH
	2	4.21 (dd, 9.7, 8.0)	59.8 (d)	Ccoi 7′
	3a	1.85 (ddd, 13.1,13.1, 9.7)	30.7 (t)	Agma 1-NH
	3b	2.02 (ddd, 13.1, 8.0, 6.8)		
	3′	2.24 (dddd, 13.1, 6.8, 6.4, 5.8)	35.9 (d)	
	4a	1.48 (m)	19.4 (t)	
	4b	2.13 (m)		
	5	1.53 (2H, m)	24.7 (t)	
	6	3.83 (dddd, 2.4, 2.4, 2.4, 2.4)	68.7 (d)	
	7a	1.59 (ddd, 14.0, 11.9, 2.4)	28.6 (t)	
	7b	2.28 (ddd, 14.0, 6.4, 2.4)		
	7′	4.33 (ddd, 11.9, 6.4, 6.4)	54.3 (d)	Ccoi 2, 3
Agma	1	3.01 (m)	37.9 (t)	
		3.11 (m)		
	2	1.41 (2H, m)	26.3 (t)	Agma 1, 3
	3	1.44 (2H, m)	25.8 (t)	Agma 1, 2, 4
	4	3.09 (2H, m)	40.4 (t)	
	1-NH	7.92 (t, 5.2)		
	4-NH	7.48 (t, 5.1)		
	C=N		156.7 (s)	Agma 4
Xyl	l	4.95 (d, 3.5)	95.1 (d)	Xyl 5, Hleu 3
	2	3.28 (dd, 9.0, 3.5)	72.1 (d)	Xyl 3, 5
	3	3.58 (t, 9.0)	71.8 (d)	
	4	3.93 (ddd, 11.0, 9.0, 5.8)	75.0 (d)	Xyl 3, 5
	5	3.37 (t, 11.0)	59.5 (t)	Xyl 1
		3.67 (dd, 11.0, 5.8)		

[a] Optimized for $^{2,3}J_{CH}$ = 8.3 Hz. [b] Assignments of coupling constants of Ccoi were aided by spin-decoupling experiments.

H-5 and H-5′ methyl protons at δ_H 0.86 and 0.89, respectively.

Ccoi. In the COSY spectrum, the connectivities from H-2, which was correlated to the carbonyl carbon at δ_C 171.2 in the HMBC, to H-7′ were readily determined in a straightforward manner. The chemical shifts of C-2 (δ_H 4.21, δ_C 59.8) and C-7′ (δ_H 4.33, δ_C 54.3) indicated that both carbons were adjacent to the nitrogen atom, and the correlation between C-2 and H-7′ was observed in the HMBC spectrum. There were two possibilities for the position of chloride: one was at C-2 of Plas and the other was at C-6 of Ccoi. The former, however, was excluded by the location of sulfate. Therefore, the placement of chloride was determined to be at C-6 of Ccoi. These data fully supported the presence of the Ccoi residue.

Agma. The connectivities from the C-1 amide proton at δ 7.92 to the C-4 guanidine proton (δ 7.48) were inferred from the COSY and HOHAHA experiments. Furthermore, the ^{13}C NMR spectrum contained a signal for the guanidine group at δ 156.7, which was correlated to the H-4 methylene proton in the HMBC to complete the Agma unit.

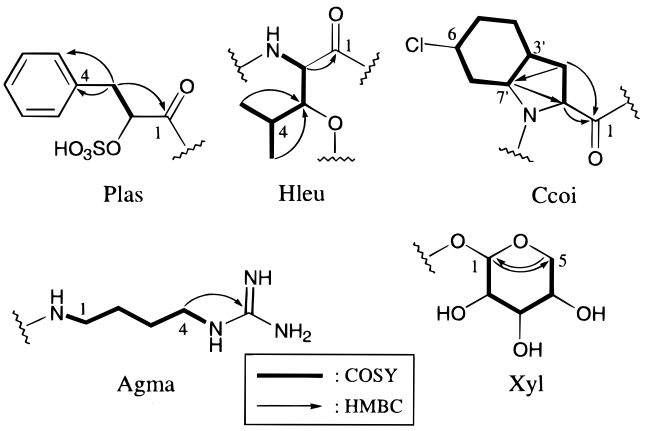


Figure 1. Partial structures of **1** and **2** derived from 2D NMR experiments.

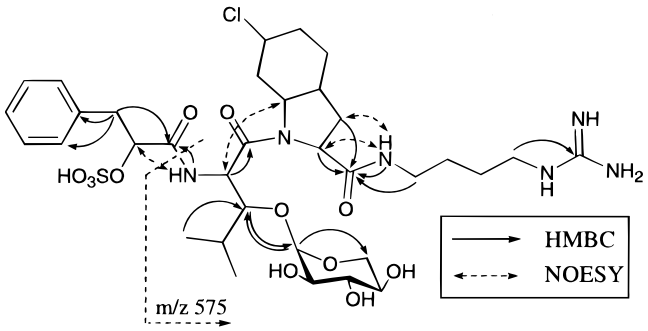


Figure 2. Important HMBC and NOESY correlations and a fragment ion peak for **1** and **2**.

Xyl. The COSY and HMBC spectra indicated the presence of a C5 pyranose unit. The unit was identified as xylopyranose on the basis of its ^{1}H and ^{13}C chemical shift values[8] and coupling constants (Table 1). The configuration of Xyl was considered to be α on the basis of the coupling constant of the anomeric proton signal at δ 4.95 ($J_{1,2}$ = 3.5 Hz) and the carbon signal at δ 95.1. The large coupling constants observed between the C2, C3, and C4 methine protons suggested that all protons were axial and all hydroxyl groups were equatorial. All of these results demonstrated that the pentose portion of **1** was α-xylopyranose.

Assembly of the Partial Structures. The HMBC (Table 1) and NOESY data (Figure 2) allowed us to connect the partial structures into gross structure of **1**. There were HMBC cross peaks between the NH protons and the carbonyl carbons of the adjacent amino acids, viz., Hleu NH/Plas CO and Agma 1-NH, H-1/Ccoi CO, that established the partial sequences Plas-Hleu and Ccoi-Agma. The connectivity between Hleu and Ccoi was determined by the NOESY correlation between Hleu H-2 and Ccoi H-7′, though no HMBC correlation was observed between these residues. The remaining xylose residue could be placed on the 3-hydroxy group of the Hleu as a result of the three-bond correlations between the anomeric proton at δ 4.95 and the C-3 carbon signal of the Hleu at δ 68.7 and between the oxymethine proton of Hleu at δ 4.07 and the anomeric carbon at δ 95.1. Thus, the gross structure of aeruginosin 205-A (**1**) was completed.

(8) (a) Kalinowski, H.-O.; Beerger, S. *Carbon-13 NMR Spectroscopy*; John Wiley and Sons: New York, 1988; p 441. (b) Bock, K.; Pedersen, C. *Adv. Carbohyd. Chem. Biochem.* **1983**, *41*, 27−66.

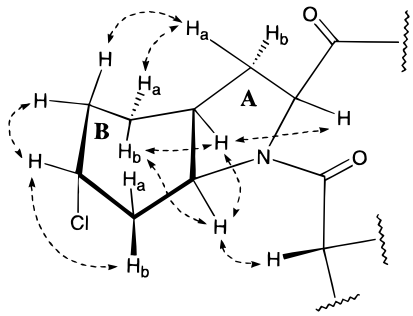
Table 2. ^{1}H and ^{13}C NMR data of Aeruginosin 205-B (2) in DMSO-d_6

unit		^{1}H (mult, *J* (Hz))	^{13}C (mult)	HMBC correlations
Plas	1		172.4 (s)	Plas 2, 3, Hleu NH
	2	4.19 (dd, 9.6, 7.3)	71.9 (d)	Plas 3
	3	2.78 (dd, 11.8, 7.3)	40.0 (t)	Plas 5, 9
		2.93 (dd, 11.8, 9.6)		
	4		137.9 (s)	Plas 3, 6, 8
	5, 9	7.23 (d, 7.3)	129.6 (d)	Plas 3, 7
	6, 8	7.25 (dd, 7.3, 7.0)	127.9(d)	
	7	7.18 (t, 7.0)	126.0 (d)	Plas 5, 9
Hleu	1		167.3 (s)	Hleu 2
	2	4.90 (dd, 9.6, 1.7)	51.1 (d)	Hleu 3
	3	3.98 (dd, 10.7, 1.7)	68.9 (d)	Hleu 2, 5, 5′, Xyl 1
	4	1.69 (m)	27.5 (d)	Hleu 5, 5′
	5	0.85 (d, 6.6)	20.8 (q)	Hleu 3, 4, 5′
	5′	0.88 (d, 6.4)	15.4 (q)	Hleu 3, 4, 5
	NH	7.62 (d, 9.6)		
Ccoi	1		171.2 (s)	Ccoi 2, 3, Agma 2, 1-NH
	2	4.19 (dd, 9.8, 8.0)	59.8 (d)	Ccoi 7′
	3a	1.84 (ddd, 13.0,13.0, 9.8)	30.8 (t)	Agma 1-NH
	3b	2.02 (ddd, 13.0, 8.0, 6.8)		
	3′	2.24 (dddd, 13.0, 6.8, 6.4, 5.8)	35.8 (d)	
	4a	1.48 (m)	19.4 (t)	
	4b	2.13 (m)		
	5	1.54 (2H, m)	24.7 (t)	
	6	3.83 (dddd, 2.4, 2.4, 2.4, 2.4)	68.7 (d)	
	7a	1.59 (ddd, 14.0, 11.9, 2.4)	28.7 (t)	
	7b	2.23 (ddd, 14.0, 6.4, 2.4)		
	7′	4.32 (ddd, 11.9, 6.4, 6.4)	54.2 (d)	Ccoi 2, 3
Agma	1	2.97 (m)	37.9 (t)	
		3.11 (m)		
	2	1.40 (2H, m)	26.2 (t)	Agma 1, 3
	3	1.44 (2H, m)	25.8 (t)	Agma 1, 2, 4
	4	3.09 (2H, m)	40.4 (t)	
	1-NH	7.92 (t, 5.8)		
	4-NH	7.44 (t, 5.2)		
	C=N		156.6 (s)	Agma 4
Xyl	1	4.93 (d, 3.7)	95.2 (d)	Xyl 5, Hleu 3
	2	3.28 (dd, 9.3, 3.7)	72.1 (d)	Xyl 3, 5
	3	3.58 (t, 9.3)	71.7 (d)	
	4	3.93 (ddd, 11.0, 9.3, 5.8)	74.9 (d)	Xyl 3, 5
	5	3.37 (t, 11.0)	59.6 (t)	Xyl 1
		3.67 (dd, 11.0, 5.8)		

Aeruginosin 205-B (**2**) was also isolated as a colorless microcrystalline solid, $[\alpha]^{20}_D = +40.3°$ (*c* 0.1, MeOH). The molecular formula of **2** was also determined to be $C_{34}H_{53}N_6O_{12}ClS$, which was consistent with that of **1**. The detailed analysis of the NMR data revealed that the planar structure of **2** was the same as that of aeruginosin 205-A (**1**). The key difference between **1** and **2** in the NMR data was the chemical shifts of Plas and Hleu (Tables 1 and 2), strongly suggesting that the absolute stereochemistry of the residues in **2** might be different from that of **1**.

Relative Stereochemistry. The relative stereochemistry of the Ccoi of **1** was established by vicinal ^{1}H−^{1}H coupling constants ($^3J_{vic}$) and NOEs from phase sensitive NOESY experiments (Chart 1). Since the NMR spectral data of Ccoi in **1** and **2** were essentially identical, they were assumed to have the same stereochemistry. An intense NOESY cross peak H3′/H7′ revealed a *cis* junction. H2 was assigned to an axial position on the basis of the large coupling ($J_{2,\ 3a} = 9.7$ Hz) with the adjacent axial proton at C3. A cross peak H2/H3′ indicated their *cis* relationship. H3′ exhibited a large coupling ($J = 13.1$ Hz) to H3a and showed a strong NOESY correlation to H3b, indicating an *anti* arrangement of H3′ and H3a. NOESY cross peaks of both H3a/H4a and H3a/H5 confirmed the *cis* ring junction between rings A and B.

Chart 1



In the decoupling experiments, the coupling between H3′ and H4a was very small, implying that the dihedral angle between H3′ and H4a was about 90°. A large coupling ($J = 11.9$ Hz) between H7′ and H7a indicated a diaxial arrangement for these protons. The *cis* relationship between H7′ and H4b was also revealed by the NOESY cross peak H7′/H4b. H6 showed only a small coupling ($J = 2.4$ Hz) to H5 and H7a,b and was assigned to an equatorial position.

Absolute Stereochemistry. The absolute stereochemistry of Plas in **1** and **2** was determined by HPLC analysis of the menthyl ester derivatives of the acid hydrolysates. The Plas in **1** had the L-configuration, while that in **2** had the D-form. Absolute stereochemistries of Hleu residues in **1** and **2** were determined to be (2R,3S) and (2S,3R), respectively, by HPLC analyses[9] of the acid hydrolysates derivatized with Marfey's reagent.[10] The stereochemistry of xylose in both **1** and **2** was determined as the D-form by chiral GC analyses of the acid hydrolysates.[11] The absolute stereochemistry of Ccoi is still under investigation.

Protease Inhibitory Activity. Both aeruginosins 205A (**1**) and -B (**2**) inhibited trypsin with an IC_{50} of 0.07 μg/mL. These compounds also inhibited thrombin with IC_{50} values of 1.5 and 0.17 μg/mL for **1** and **2**, respectively.

Conclusion. Aeruginosins 205A and -B contained new structural units; namely, 2-carboxy-6-chlorooctahydroindole and phenyllactic acid 2-*O*-sulfate. Aeruginosins 205A and -B had unique structures consisting of unusual amino acids or amino acid derivatives, sugar, and organic acid. Though a number of sugar-containing natural products are known, to the best of our knowledge, aeruginosins 205A and -B are only the third glycosides that have been isolated from cyanobacteria.[12] Aeruginosins 205A and -B also contained 3-hydroxyleucine,

(9) Professor Satoshi Omura (Director of the Kitasato Institute, Research Center of Biological Function, Japan) kindly provided us with three stereoisomers (2*S*,3*S*; 2*S*,3*R*; 2*R*,3*R*) of Hleu out of four possible stereoisomers (*Tetrahedron Lett.* **1993**, *34*, 4447−4448). In the HPLC analyses of the samples and the standards derivatized with L-Marfey's reagent, Hleu in **2** was determined to be (2*S*,3*R*), but the retention time of that in **1** was not consistent with either of the above three stereoisomers, suggesting that the absolute stereochemistry of Hleu in **1** might be (2*R*,3*S*). To clarify this fact, the (2*S*,3*R*) isomer was derivatized with D-Marfey's reagent, which is enantiomeric to or chromatographically equivalent to the (2*R*,3*S*) isomer derivatized with L-Marfey's reagent. HPLC analysis of the (2*S*,3*R*) isomer derivatized with D-Marfey's reagent allowed us to determine the stereochemistry of Hleu in **1** as (2*R*,3*S*) unambiguously.

(10) Marfey, P. *Carlsberg Res. Commun.* **1984**, *49*, 591−596.

(11) After methanolysis (10% HCl in MeOH, 100 °C, 1 h) of **1** and **2**, followed by treatment with TFAA/CH_2Cl_2 at 100 °C for 10 min, chiral GC analyses were carried out.

(12) (a) Stewart, J. B.; Bornemann, V.; Chen, J. L.; Moore, R. E.; Caplan, F. R.; Karuso, H.; Larsen, L. K.; Patterson, G. M. L. *J. Antibiot.* **1988**, *41*, 1048−1056. (b) Chen, J. L.; Moore, R. E.; Patterson, G. M. L. *J. Org. Chem.* **1991**, *56*, 4360−4364.

which is rare in naturally-occurring molecules. 3-Hydroxyleucine has been reported as a constituent of peptide antibiotics such as telomycin,[13] lysobactin,[14] and lactacystin.[15] Aeruginosins 205A and -B had the same planar structure, but interestingly, the stereochemistries of the Hleu and Plas in aeruginosin 205B were the opposite of those found in aeruginosin 205A.

Aeruginosins 205A and -B are unusual glycopeptides, and their remarkable activity may provide a useful model for protease inhibitors.

Experimental Section

General Methods. NMR spectra were measured at either 500 or 600 MHz for ^{1}H and 125 or 150 MHz for ^{13}C. ^{1}H and ^{13}C NMR chemical shifts were referenced to DMSO-d_6 solvent peaks at 2.49 and 39.5 ppm, respectively. Phase-sensitive NOESY spectra were measured with a mixing time of 800 ms. The HMBC spectra were acquired with an evolution time of 60 ms. FAB mass spectra were measured by using poly(ethylene glycol) sulfate or glycerol as matrices.

Strain and Culture Conditions. *O. agardhii* (NIES-205) was obtained from the NIES-collection (Microbial Culture Collection, the National Institute for Environmental Studies, Japan) and cultured in 10 L glass bottles containing CB medium.[5] Cultures were illuminated with fluorescent lights on a 12L:12D cycle at an intensity of 250 μE/m^2•s and aerated with filtered air (0.3 L/min, without added CO_2) at 25 °C for 10−14 days. After incubation, cells were harvested by continuous flow centrifugation at 10 000 rpm. Harvested cells were lyophilized and kept in a freezer at −20 °C until extraction.

Extraction and Isolation. Freeze-dried cyanobacterium (119 g from 350 L of culture) was extracted three times with 80% MeOH and once with MeOH. The extracts were combined and concentrated to give a crude extract (28.9 g). The extract was partitioned between ether and water. The water-soluble fraction was further partitioned between *n*-BuOH and water. The *n*-BuOH layer (10.1 g) was subjected to ODS flash chromatography (12 × 10.5 cm) and eluted with aqueous MeOH, 100% MeOH, and CH_2Cl_2. The active 50% MeOH fraction (327 mg), which showed trypsin and thrombin inhibitory activity, was purified by reversed-phase HPLC on a Cosmosil C_{18} MS column (linear gradient of CH_3CN in H_2O containing 0.05% TFA, 20 to 60% in 55 min; flow rate 2.0 mL/min; UV detection at 210 nm) to yield aeruginosins 205A (**1**, 24.5 mg, 0.021% yield, t_R 20.5 min) and -B (**2**, 7 mg, 0.006% yield, t_R 24.5 min).

Aeruginosin 205A (1): $[\alpha]^{20}_D$ +17.7° (*c* 0.1, MeOH); UV (MeOH) λ_{max} 258 nm (ϵ 520); FAB-MS *m/z* 805 $(M + H)^+$, 725 $(M - SO_3 + H)^+$, 575 $(M - Plas + H)^+$; HRFAB-MS *m/z* 803.3094 $(M - H)^-$ calcd for $C_{34}H_{52}N_6O_{12}ClS$ (Δ +4.1 mmu); for ^{1}H and ^{13}C data, see Table 1.

Aeruginosin 205B (2): $[\alpha]^{20}_D$ +40.3° (*c* 0.1, MeOH); UV (MeOH) λ_{max} 258 nm (ϵ 570); FAB-MS *m/z* 805 $(M + H)^+$, 725 $(M - SO_3 + H)^+$, 575 $(M - Plas + H)^+$; HRFAB-MS *m/z* 803.3088 $(M - H)^-$ calcd for $C_{34}H_{52}N_6O_{12}ClS$ (Δ +3.5 mmu); for ^{1}H and ^{13}C data, see Table 2.

Amino Acid Analysis. Each 100 μg of **1** and **2** was dissolved in 6 N HCl (500 μL) and sealed in reaction vials. The vials were heated at 110 °C for 16 h. The solution was evaporated in a stream of dry nitrogen with heating and redissolved in 0.1 N HCl to prepare for amino acid analysis.

HPLC Analysis of Marfey Derivatives. To the acid hydrolysates of **1** and **2** was added a 10% acetone solution of 1-fluoro-2,4-bis(nitrophenyl)-5-L-alanine amide (L-FDAA, Marfey's reagent) in 1 M $NaHCO_3$. The mixtures were kept at 80−90 °C for 3 min followed by neutralization with 50 μL of 2 N HCl. The reaction mixtures were dissolved in 50% MeCN and subjected to reversed-phase HPLC: column, Cosmosil C_{18} MS (4.6 × 250 mm); gradient elution from H_2O/TFA (100:0.1) to MeCN/H_2O/TFA (50:50:0.1) in 50 min, flow rate 1 mL/min, UV (340 nm). The derivatization of amino acids with D-FDAA[16] was done as in the case of L-FDAA. Retention times (min) of standard amino acids were found to be as follows: (2*S*,3*S*)-Hleu (51.2), (2*S*,3*R*)-Hleu (52.9), (2*R*,3*S*)-Hleu (53.4),[17] (2*R*,3*R*)-Hleu (53.9).

Methanolysis and Chiral GC Analysis. A solution of 10% HCl in MeOH was added to each 100 μg portion of **1** and **2** in reaction vials and heated at 100 °C for 1 h. The solvent was removed in a stream of dry nitrogen. Trifluoroacetic anhydride (300 μL) in CH_2Cl_2 (300 μL) was added to the residues, the vials were capped and heated at 100 °C for 5 min, and the solution was evaporated in a stream of dry nitrogen. The residues were dissolved in CH_2Cl_2 (500 μL) and immediately analyzed by chiral GC using Chirasil-Val capillary column. Column temperature was kept at 80 °C for 3 min and then increased at a rate of 4 °C/min to 200 °C. Retention times (min) were as follows: D-Xyl (20.3), L-Xyl (20.9).

Menthyl Esterification and HPLC Analysis. L-Menthol (10 mg) and MeCN (50 μL) were added to each of the hydrolysates of **1** and **2** and cooled to 0 °C. The mixtures were further added to trimethylsilyl chloride (20 μL) and heated at 100 °C for 10 min. Then the mixtures were concentrated *in vacuo*, diluted with MeCN, and analyzed by reversed-phase HPLC: column; Cosmosil C_{18} MS, isocratic elution, MeCN/H_2O/TFA (65/35/0.05), flow rate 1 mL/min, UV (210 nm). Retention times (min) were as follows: D-Plas (21.6), L-Plas (22.4).

Protease Inhibitory Activity Assay. Serine protease inhibitory activities were determined by the method described in a previous paper.[18]

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Supporting Information Available: ^{1}H, ^{13}C, and 2D NMR data (7 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

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