Aeruginosin 103-A, a Thrombin Inhibitor from the Cyanobacterium *Microcystis viridis*

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Aeruginosin 103-A was isolated from the cultured freshwater cyanobacterium *Microcystis viridis* (NIES-103). Its structure was elucidated to be **1** on the basis of 2D NMR data. This linear peptide inhibited thrombin, with an IC_{50} of 9.0 μg/mL.

The cyanobacteria *Microcystis viridis* and *Microcystis aeruginosa* are well known to produce the hepatotoxic cyclic peptides, the microcystins.[1] Recently, we have reported that thrombin inhibitors, aeruginosins 102-A and -B, were obtained from *M. viridis* (NIES-102),[2] and a chymotrypsin inhibitor, micropeptin 103, was isolated from *M. viridis* (NIES-103).[3] In on-going studies of protease inhibitors from cyanobacteria, we have isolated a new thrombin inhibitor, aeruginosin 103-A (**1**), from the cultured freshwater cyanobacterium *M. viridis* (NIES-103).[4,5] Here we describe the isolation and structure elucidation of the new thrombin inhibitor, aeruginosin 103-A (**1**).

The 80% MeOH extract of freeze-dried alga (119 g) was partitioned between H_2O and Et_2O. The Et_2O layer was further separated by the Kupchan procedure.[6] The aqueous MeOH fraction, which inhibited thrombin, was subjected to ODS flash chromatography followed by reversed-phase HPLC with MeCN (20−50%) containing 0.05% TFA to yield aeruginosin 103-A (**1**, 4.6 mg) as a colorless amorphous powder.

The peptidic nature of **1** was suggested by its ^{1}H and ^{13}C NMR spectra (Table 1), but only Tyr was identified by amino acid analysis of the hydrolysate. Interpretation of NMR data, including ^{1}H−^{1}H COSY, HMQC,[7] and HMBC[8] spectra, revealed the partial structures, Hpla (*p*-hydroxyphenyllactic acid), Tyr, Choi (2-carboxy-6-hydroxyoctahydroindole), and Aeap (1-amidino-2-ethoxy-3-aminopiperidine) (Figure 1). Correlation between H-3a (δ 1.55) and H-4 (δ 1.23) in the Choi unit was not observed, but the similar chemical shifts of protons and carbons supported the presence of Choi (Figure 1 and Table 1) by comparison with other related peptides.[2] The linkage between Choi and Tyr was decided by the NOESY correlation between Choi H-7a (δ 3.62) and Tyr H-3 (δ 2.60, 2.72). The HMBC correlation from Aeap H-2 (δ 4.89) to C-6 (δ 40.0) indicated the presence of the ethoxy residue. The HMBC correlations from Aeap H-2 to C-5 (δ 19.1) and from H-2 to C=N (δ 158.0, indicating the presence of one guanidino group) established the structure of Aeap and its cyclic hemiaminal nature. All these data led to the planar structure of aeruginosin 103-A.

Table 1. ^{1}H and ^{13}C NMR Data for Aeruginosin 103-A in DMSO-d_6 (δ, ppm)

Unit		^{1}H	*J* (Hz)	^{13}C	
Hpla	1			173.0	(s)
	2	3.99	(br)	72.0	(d)
	3	2.52	(dd, 14.1, 8.1)	39.5	(t)
		2.80	(dd, 14.1, 3.4)		
	4			128.0	(s)
	5,9	6.99	(d, 8.6)	130.2	(d)
	6,8	6.62	(d, 8.6)	115.0	(d)
	7			155.9	(s)
	2-OH	5.65	(br d, 5.6)		
	7-OH	9.15	(s)		
Tyr	1			169.5	(s)
	2	4.60	(ddd, 8.1, 7.3, 7.3)	51.6	(d)
	3	2.60	(dd, 13.3, 8.1)	38.0	(t)
		2.72	(dd, 13.3, 7.3)		
	4			126.0	(s)
	5,9	6.90	(d, 8.1)	130.1	(d)
	6,8	6.63	(d, 8.1)	115.0	(d)
	7			156.0	(s)
	OH	9.30	(s)		
	NH	7.53	(d, 7.3)		
Choi	1			172.0	(s)
	2	4.09	(dd, 9.7, 8.1)	59.9	(d)
	3	1.67	(ddd, 12.8, 12.8, 9.7)	30.0	(t)
		1.82	(ddd, 12.8, 8.1, 5.6)		
	3a	1.55	(m)	36.0	(d)
	4	1.23	(m)	18.5	(t)
		1.75	(m)		
	5	1.31	(m)	25.9	(t)
		1.32	(m)		
	6	3.81	(m)	63.7	(d)
	7	1.42	(m)	30.3	(t)
		1.99	(m)		
	7a	3.62	(ddd, 11.7, 5.9, 5.9)	54.0	(d)
Aeap	2	4.89	(br)	83.5	(d)
	3	3.90	(m)	46.0	(d)
	4	1.41	(m)	22.0	(t)
		1.92	(m)		
	5	1.48	(m)	19.1	(t)
		1.91	(m)		
	6	3.10	(ddd, 13.6, 12.8, 3.0)	40.0	(t)
		3.52	(ddd, 13.6, 4.7, 3.0)		
	7	3.35	(m)	62.0	(q)
	8	1.10	(t, 7.1)	14.5	(t)
	C=N			158.0	(s)
	NH	7.79	(d, 7.7)		

The absolute stereochemistry of Hpla was determined to be the D-form by HPLC analysis of a menthyl ester derivative of acid hydrolysate of **1**.[2] Tyr was determined to be the D-form by Marfey's analysis of the acid hydrolysate of **1**.[9] The relative stereochemistry of Aeap was determined by the NOESY spectrum and coupling

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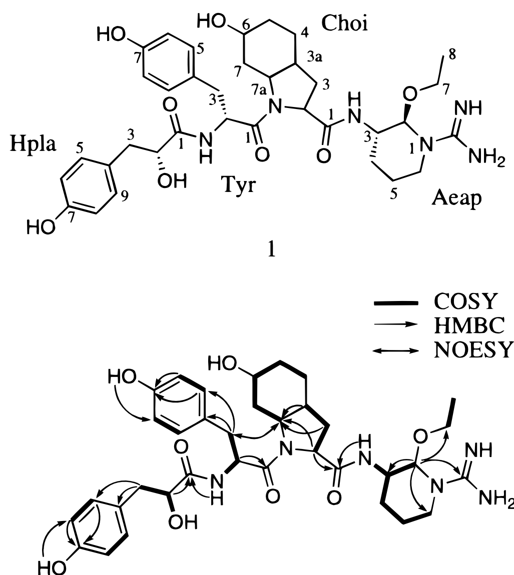

Figure 1. COSY, HMBC, and NOESY correlations of aeruginosin 103-A.

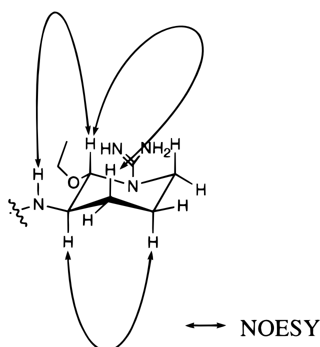

Figure 2. The relative stereochemistry of Aeap.

constants (Figure 2).[10] To decide the absolute stereochemistry of Aeap, **1** was oxidized with CrO_3. After hydrolysis with 6 N HCl, the reaction mixture afforded Arg, which was proved to be the L-form from HPLC analysis by Marfey's method. Therefore, the absolute stereochemistry of Aeap was assigned as (2*R*,3*S*)-1-amidino-2-ethoxy-3-aminopiperidine. The relative stereochemistry of Choi was determined by NOESY and ROESY spectra,[11] which was coincident with the relative stereochemistry of Choi in aeruginosins 102-A and -B.[2] A study of the absolute stereochemistry of Choi is now in progress.

Aeruginosin 103-A inhibited thrombin, trypsin, and plasmin, with an IC_{50} of 9.0, 51.0, and 68.0 μg/mL, respectively. The X-ray crystallographic structures of the trypsin−aeruginosin 98-B complex[12] and the thrombin−aeruginosin 298-A complex[13] have been recently reported. In the case of aeruginosin 98-B, the guanidium of agmatine was buried deeply in trypsin's specificity pocket. It is probable that the bulky argininal ethyl aminal structure of the Aeap unit in aeruginosin 103-A, compared with the linear structure of agmatine in aeruginosin 98-B, negatively affects its trypsin inhibitory activity.

Experimental Section

General Methods. The UV spectrum was recorded on a Hitachi 330 spectrophotometer. ^1H and ^{13}C NMR spectra were obtained with a JEOL JNM-A500 or 600 in DMSO-d_6 at 27.0 °C. The resonances of residual DMSO-d_6 at δ_H 2.49 and δ_C 39.5 were used as internal references for ^1H and ^{13}C NMR spectra, respectively. FABMS were recorded by a JEOL JMS SX-102 mass spectrometer. Amino acid analyses were carried out with a Hitachi L-8500A amino acid analyzer.

Culture Conditions. *Microcystis viridis* (NIES-103)[5] was obtained from the NIES collection (Microbial Culture Collection, the National Institute for Environmental Studies, Japan). The alga was cultured in 10-L glass bottles containing MA medium [$Ca(NO_3)_2 \cdot 4H_2O$ 5 mg, KNO_3 10 mg, $NaNO_3$ 5 mg, Na_2SO_4 4 mg, $MgCl_2 \cdot 6H_2O$ 5 mg, β-Na_2glycerophosphate 10 mg, $Na_2 \cdot$EDTA 2 H_2O 0.5 mg, $FeCl_3 \cdot 6H_2O$ 0.05 mg, $MnCl_2 \cdot 4H_2O$ 0.5 mg, $ZnCl_2$ 0.05 mg, $CoCl_2 \cdot 6H_2O$ 0.5 mg, $Na_2MoO_4 \cdot 2H_2O$ 0.08 mg, H_3BO_3 2 mg, BICINE 50 mg, distilled H_2O 100 mL, pH 8.6] under illumination of 250 $\mu E/m^2s$ on a 12L:12D cycle. After 4−8 weeks, the algal cells were harvested by continuous centrifugation at 10 000 rpm. The yield of the lyophilized cells was 119 g from 450 L of culture. The harvested cells were stored at −20 °C until extraction.

Isolation. Freeze-dried alga (119 g from 450 L of culture) was extracted with 80% MeOH (2 L × 3) and MeOH (2 L × 3). The combined 80% MeOH and MeOH extracts were concentrated to an aqueous suspension, and the suspension was extracted with Et_2O. The Et_2O fraction was partitioned between *n*-hexane and MeOH−H_2O (9:1), and the 90% MeOH layer was subsequently partitioned between CCl_4 and MeOH−H_2O (8:2).[6] The 80% MeOH fraction was subjected to ODS flash chromatography (YMC-GEL, 5 × 10 cm) with aqueous MeOH followed by CH_2Cl_2. The 20% MeOH fraction was subjected to reversed-phase HPLC (Cosmosil C18MS, 10 × 250 mm; 20% MeCN containing 0.05% TFA; flow rate, 2.0 mL/min; UV detection at 210 nm) to yield aeruginosin 103-A (**1**, 4.5 mg): $[\alpha]_D$ −7.6° (*c* 0.1, MeOH); UV absorption in H_2O at λ_{max} 224 nm (ϵ 11 600), 274 nm (2100); HRFABMS *m/z* 681.3633 $[M + H]^+$ calcd for $C_{35}H_{48}N_6O_8$ (Δ +2.2 mmu).

HPLC Analysis of the Menthyl Ester Derivatives. L-Menthol (100 mg) and MeCN (50 μL) were added to the acid hydrolysates (6 N HCl, 16 h) of **1**. The mixture was heated at 100 °C for 10 min, then concentrated in vacuo, diluted with MeCN, and analyzed by reversed-phase ODS−HPLC: Cosmosil 5C18AR (4.6 × 250 mm); gradient elution from MeCN−H_2O−TFA (50:50:0.1) to MeCN−H_2O−TFA (70:30:0.1) in 30 min; UV detection 210 nm; flow rate 1.0 mL/min. Retention times of standards (min): L-Hpla (20.4), D-Hpla (21.6). Retention time of Hpla of aeruginosin 103-A (min): 21.6.

HPLC Analysis of the Marfey Derivatives of Tyr. To the acid hydrolysate (6 N HCl, 16 h) of a 100 μg portion of **1**, 50 μL of 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (L-FDAA) in MeCO (10 mg/mL) and 100 μL of 1 M $NaHCO_3$ were added, and the reaction 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 the reaction mixture was analyzed by reversed-phase ODS−HPLC: Cosmosil 5C18MS (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 of the

standard amino acids (min): L-Tyr (58.4), D-Tyr (61.6). Retention time of Tyr of aeruginosin 103-A: 61.6.

Oxidation by CrO_3. Aeruginosin 103-A was dissolved in HOAc acid (500 μL) and combined with 2 mg of CrO_3. After being stirred at room temperature for 2 h, the reaction mixture was subjected to ODS chromatography (YMC Dispo SPE, 0−60% MeOH). Arg was detected in the hydrolysate of the 60% MeOH fraction by amino acid analysis.

HPLC Analysis of Arg. The 60% MeOH fraction was hydrolyzed with 6 N HCl for 16 h and the hydrolysate was derivatized with L-FDAA as described above. The reaction mixture was analyzed by reversed-phase ODS−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 of the standard amino acids (min): L-Arg (36.0), D-Arg (37.2). Retention time of Arg of oxydized aeruginosin 103-A (min): 36.0.

Serine Protease Inhibitory Assay. Serine protease inhibitory activities were determined by the method described in a previous paper.[14]

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(10) H-6 (3.10 ppm) was indicated to be axial position by the coupling constants (ddd, 13.6, 12.8, 3.0); H-6 (3.52 ppm) was indicated to be equatorial position by the coupling constants (ddd, 12.8, 4.7, 3.0).
(11) NOESY correlations: H-2 (4.09 ppm) to H-3 (1.82 ppm), H-3a (1.55 ppm) to (3.62 ppm), H-7a (3.62 ppm) to H-7 (1.99 ppm), H-3 (1.67 ppm) to H-4 (1.23 ppm), H-4 (1.23 ppm) to H-6 (3.81 ppm). ROESY correlation: H-3 (1.82 ppm) to H-3a (1.55 ppm).
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