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Aeruginosin 298-A, A Thrombin and Trypsin Inhibitor from the Blue-green Alga *Microcystis aeruginosa* (NIES-298)

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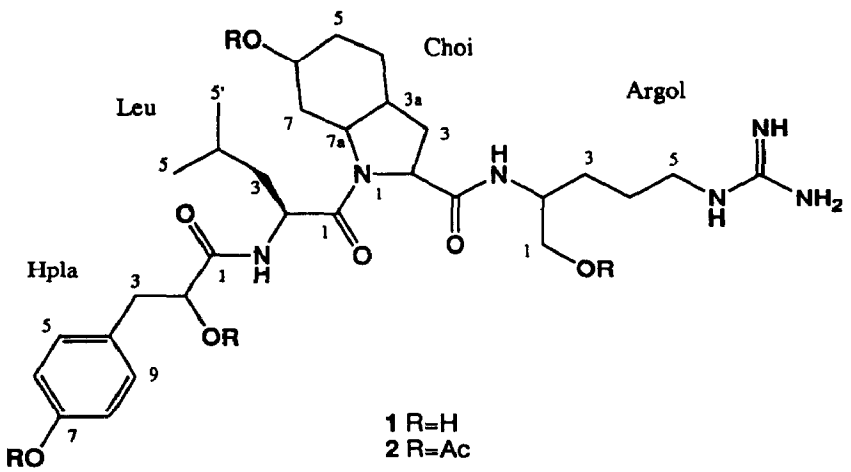
Abstract: Aeruginosin 298-A was isolated from the freshwater blue-green alga *Microcystis aeruginosa* (NIES-298). Its structure was elucidated to be **1** on the basis of 2D NMR data. This linear peptide inhibited thrombin and trypsin potently.

Blue-green algae have been shown to produce unique biologically active peptides such as microcystins[1] from *Microcystis aeruginosa*, microviridin[2] from *M. viridis*, nodularin[3] from *Nodularia spumigena*, and puwainaphycins[4] from *Anabaena* BQ-16-1. We have also reported the new protease inhibitory peptides from freshwater blue-green algae, microginin, micropeptins A and B, and radiosumin[5]. In the course of our screening program of new protease inhibitors, we found that *M. aeruginosa* (NIES-298) had potent inhibitory activities on thrombin and trypsin. In this paper, we report the isolation and structural elucidation of **1**.



M. aeruginosa (NIES-298) was obtained from the NIES-collection (Microbial Culture Collection, the National Institute for Environmental Studies, Japan) and 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_2EDTA·$2H_2O$ 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 water 100 mL, pH 8.6][6] under illumination of 250 $\mu E/m^2 \cdot s$ on a 12L:12D cycle.

Table 1. ^1H and ^{13}C NMR Spectra of Aeruginosin 298-A in DMSO-d_6

Position		H (mult, *J* Hz)	C (mult)	HMBC (^1H)
Hpla	1		172.8 (s)	Leu NH, Hpla 2, 3, 2-OH
	2	4.04 (m)	72.1 (d)	Hpla 3, 2-OH
	3	2.64 (dd, 14.0, 7.5)	39.3 (t)	Hpla 5, 9
		2.85 (dd, 14.0, 3.6)		
	4		128.1 (s)	Hpla 3, 6, 8
	5,9	6.94 (d, 8.5)	130.4 (d)	Hpla 6, 8
	6,8	6.60 (d, 8.5)	114.7 (d)	Hpla 5, 9, 7-OH
	7		155.7 (s)	Hpla 5, 6, 8, 9, 7-OH
	2-OH	5.69 (brd)		
	7-OH	9.12 (brd)		
Leu	1		169.8 (s)	Leu 2
	2	4.51 (ddd, 10.1, 8.3, 3.5)	48.1 (d)	Leu 3
	3	1.20 (t, 3.5)	41.8 (t)	Leu 2, 5, 5'
		1.40 (m)		
	4	1.35 (m)	23.9 (d)	Leu 3, 5, 5'
	5	0.82 (d, 6.4)	23.3 (q)	Leu 5'
	5'	0.88 (d, 6.5)	21.4 (q)	Leu 3, 5
	NH	7.45 (d, 8.3)		
Choi	2	4.16 (t, 8.9)	59.9 (d)	Choi 7a, 3
	3	1.80 (td, 12.7, 10.1)	30.7 (t)	Choi 3a, 7a, 2
		2.00 (m)		
	3a	2.27 (m)	36.0 (d)	Choi 4, 7a, 3
	4	1.43 (m)	26.0 (t)	Choi 3a, 5
	5	1.43 (m)	19.0 (t)	Choi 3a, 6
		2.02 (m)		
	6	3.92 (s)	63.9 (d)	Choi 5
	7	1.65 (t, 11.8)	33.4 (t)	Choi 3a
		2.02 (m)		
	7a	4.04 (m)	54.0 (d)	Choi 3a, 7, 3
	CO		171.3 (s)	Argol 2-NH, Choi 2, 3
Argol	1	3.21 (dd, 10.7, 6.2)	63.3 (t)	Argol 2
		3.31 (dd, 10.7, 5.1)		
	2	3.64 (m)	50.2 (d)	Argol 3, 2-NH
	3	1.30 (m)	28.0 (t)	Argol 1
		1.60 (m)		
	4	1.50 (m)	25.0 (t)	Argol 3, 5-NH
	5	3.07 (m)	40.8 (t)	Argol 4, 5-NH
	2-NH	7.54 (s)		
	5-NH	7.52 (s)		
	C=N		156.7 (s)	Argol 5

The 80% MeOH extract of freeze-dried algal cells (130 g from 362 L of culture) was partitioned between water and diethyl ether. Both layers showed potent thrombin and trypsin inhibitory activities. The aqueous layer was further extracted with *n*-butanol and subjected to ODS flash chromatography with increasing amounts of MeOH in water. The ether layer was partitioned between *n*-hexane and 90% MeOH and subsequently between CCl_4 and 80% MeOH. The active aqueous MeOH layer was fractionated by ODS flash chromatography with increasing amounts of MeOH in water. The active fractions from both layers by ODS chromatography were combined and resubjected to ODS flash chromatography. The 80% MeOH fraction was subjected to reversed-phase HPLC (SHISEIDO CAPCELL PAK C_{18}) with 32% MeCN containing 0.05% TFA followed by HPLC on Nomura Chemical Develosil CN-5 eluted with an MeCN gradient (20-50%) containing 0.05% TFA to yield pure aeruginosin 298-A (**1**, 12.1 mg).

Aeruginosin 298-A is colorless amorphous powder: $[\alpha]_D$ +22.3° (c = 0.36, H_2O); UV (H_2O) λmax 275 nm (ε 1160), 281 (sh). The molecular formula of **1** was determined to be $C_{30}H_{48}O_7N_6$ by HRFABMS [*m/z* 605.3657 $(M+H)^+$ Δ -0.6 mmu] and NMR data. The spectra of ^1H and ^{13}C NMR (Table 1) were characteristic of peptide. Amino acid analysis of the acid hydrolyzate of **1** (6N HCl, 16 hr) gave Leu and one unknown imino acid. The detailed analyses of ^1H-^1H COSY, HOHAHA[7], HMQC[8] and HMBC[9] spectra supported the presence of Leu (Fig. 1). The ^1H and ^{13}C NMR spectra suggested the presence of *p*-substituted phenol moiety that was easily identified as 4-hydroxyphenyllactic acid (Hpla) by COSY and HMBC spectra. Positive coloration with Sakaguchi reagent and ^{13}C NMR signal at δ 156.7 showed the existence of guanidine group. The COSY and HMBC experiments (Fig. 1) revealed that the residue containing a guanidine group had an Arg-like structure. The chemical shift of C-1 oxygenated methylene (δ_H 3.21, 3.31; δ_C 63.3), which was correlated to C-2 methine (δ_H 3.64; δ_C 50.2), suggested that this residue was argininol (Argol). The hydroxy group of Argol could not be assigned in ^1H NMR, but the C-1 methylene protons were shifted to lower field (δ_H 3.21 to 3.98, 3.31 to 4.08) after acetylation with acetic anhydride/pyridine (1:1)[10]. The structure of the last new imino acid (2-carboxy-6-hydroxyoctahydroindole; Choi) was deduced as follows. In the COSY and HOHAHA spectra, the connectivity from C-2, which was correlated to the carbonyl carbon (δ 171.3), to C-5 was easily determined. The C-3a angular methine proton (δ 2.27) was coupled to methine at δ4.04 (H-7a), which was in turn correlated to methylene at δ 1.65 and 2.02 (H_2-7). These methylene protons were also coupled to the oxygenated C-6 methine (δ_H 3.92; δ_C 63.9). In the COSY spectrum, the cross peaks between

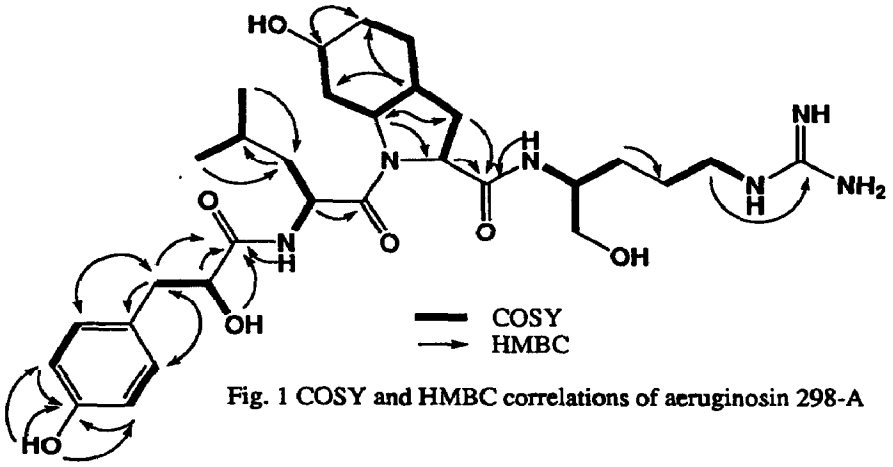


Fig. 1 COSY and HMBC correlations of aeruginosin 298-A

H_2-5 and H-6 were not observed, but this connectivity was unambiguously deduced by the HOHAHA and HMBC spectrum. Judging from the chemical shifts, C-2 and C-7a were adjacent to the nitrogen atom and the correlation between C-2 and H-7a was recognized in the HMBC spectra. Finally, the structure of this new imino acid was determined to be 2-carboxy-6-hydroxyoctahydroindole (Choi).

The sequence of aeruginosin 298-A was deduced by HMBC correlations (Hpla CO/Leu NH, Choi CO/Argol NH), but the correlation between Leu and Choi was not observed in HMBC spectrum. There were two possibilities of the connectivity between Leu and Choi. The chemical shift of the C-6 oxygenated methine proton (δ_H 3.92) was at high field for ester linkage and was shifted to lower field (δ_H 3.92 to 5.12) after acetylation[10]. These facts excluded the possibility of ester linkage between Leu and Choi.

The stereochemistry of Leu was determined to be L-form by the chiral GC analysis of *N*-trifluoroacetyl isopropyl ester derivative of the acid hydrolyzate. Study on the other stereochemistries is in progress.

Aeruginosin 298-A inhibits thrombin and trypsin with an IC_{50} of 0.3 μg/mL and 1.0 μg/mL respectively, but not papain, chymotrypsin, elastase and plasmin.

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10. **Aeruginosin 298-A tetraacetate (2)**: ^{1}H NMR (CD_3OD): **Hpla** δ 5.24 (H2), 3.05 (H3), 3.12 (H3), 7.25 (H5, 9), 7.02 (H6, 8); **Leu** 4.62 (H2), 1.33 (H3), 1.66 (H3), 1.57 (H4), 0.94 (H5), 0.96 (H5'); **Choi** 4.45 (H2), 2.06 (H3), 2.19 (H3), 2.50 (H3a), 2.08 (H4), 1.64 (H5), 1.77 (H5), 5.12 (H6), 1.95 (H7), 2.42 (H7), 4.12 (H7a); **Argol** 3.98 (H1), 4.08 (H1), 4.08 (H2), 1.57 (H3), 1.64 (H4), 3.13 (H5), 3.21 (H5). Four acetate-methyls are not assigned.

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