



## Aeruginosins 102-A and B, New Thrombin Inhibitors from the Cyanobacterium *Microcystis viridis* (NIES-102)

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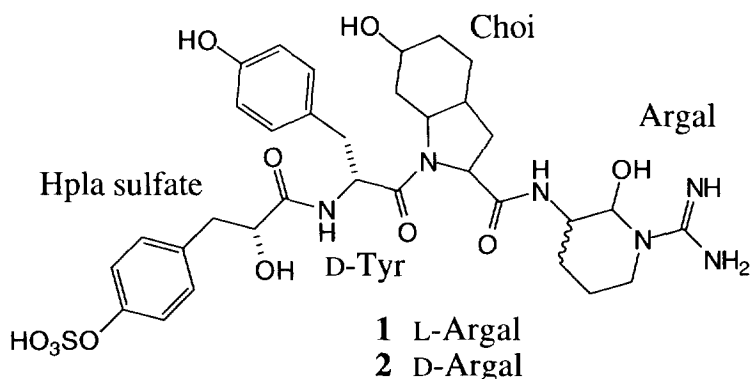
**Abstract:** Aeruginosins 102-A and B were isolated from the freshwater cyanobacterium *Microcystis aeruginosa* (NIES-102). Their structures were elucidated to be **1** and **2** on the basis of 2D NMR data and chemical degradation. These peptides inhibited thrombin potently.

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Recently, *Microcystis aeruginosa* and *M. viridis* have been shown to produce unique peptides. Microcystins<sup>3</sup> and microviridins<sup>4</sup> were isolated from both species. In addition, *M. aeruginosa* are known to produce many other peptides, such as microginin,<sup>5</sup> aeruginopeptins,<sup>6</sup> microcystilide A,<sup>7</sup> cyanopeptolins,<sup>8</sup> micropeptins,<sup>9</sup> kawaguchipeptin A,<sup>10</sup> and aeruginosins.<sup>11</sup> In the course of our study for bioactive compounds from blue-green algae, *M. viridis* was also found to contain aeruginosins. In this paper the isolation and structure elucidation of new thrombin inhibitors, aeruginosins 102-A and B, will be described.

*M. viridis* (NIES-102) obtained from the NIES-collection was cultured in our laboratory. The 80% methanol extract of freeze-dried cells was partitioned between water and diethyl ether. The aqueous layer, which inhibited thrombin, trypsin, and plasmin, was further extracted with *n*-butanol and subjected to ODS column chromatography. The active fraction was purified by ODS HPLC to obtain two compounds, named aeruginosins 102-A(**1**) and B(**2**).

Fig. 1 shows the chromatogram of aeruginosins 102-A and B on ODS HPLC. In this chromatogram, two sets of three peaks were observed and after isolation these individual peaks gave the same chromatographic pattern again. From these results, both aeruginosins 102-A and B were thought to be a mixture of



tautomers. Fortunately, in the  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra of **1** in  $\text{DMSO-}d_6$ , only one set of signals was observed in spite of the tautomeric nature. In the case of **2**, two sets of signals were observed.

The molecular formula of aeruginosin 102-A (**1**) was decided to be  $\text{C}_{33}\text{H}_{44}\text{N}_6\text{O}_{11}\text{S}$  by low and high resolution FABMS and NMR data. The existence of a sulfate was suggested by a fragment ion ( $m/z$  653,  $[\text{M-SO}_3+\text{H}]^+$ ) of positive FABMS. Its peptidic nature was suggested by the  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra and the amino acid analysis of acid hydrolyzate gave Tyr and an unknown amino acid.

Interpretation of NMR data including  $^1\text{H}$ - $^1\text{H}$  COSY, HMQC and HMBC spectra revealed the partial structures, Hpla sulfate (*p*-hydroxyphenyllactic acid sulfate), Tyr, Choi (2-carboxy-6-hydroxyoctahydroindole), Argal (argininal)(Fig. 2). The  $^1\text{H}$ - $^1\text{H}$  COSY correlations from an oxymethine proton at  $\delta$ 4.02 (Hpla sulfate H-2) to methylene protons at  $\delta$ 2.59 and  $\delta$ 2.90 (Hpla sulfate H<sub>2</sub>-3) were observed. The coupling between aromatic protons at  $\delta$ 7.06 and  $\delta$ 7.09 (Hpla sulfate H-5,9 and H-6,8) was also observed. The HMBC correlations from these aromatic protons (Hpla sulfate H-5,9/C-7; H-6,8/C-7; H-6,8/C-4) showed the presence of *p*-substituted phenol-like moiety of Hpla sulfate. The upfield shift of Hpla sulfate C-7 indicated that this carbon is sulfated.<sup>11</sup> In the HMBC spectrum, correlations from Hpla sulfate H<sub>2</sub>-3 to C-4 and C-5 and a carbonyl carbon at  $\delta$ 172.5 (Hpla sulfate C-1) were observed. These data established the presence of Hpla sulfate. The presence of Choi was suggested by comparison of the  $^{13}\text{C}$  NMR data with that of other aeruginosins.<sup>12</sup> Two spin systems observed in the  $^1\text{H}$ - $^1\text{H}$  COSY spectrum (Choi H-2,3,3a; H-4,5,6,7,7a) and HMBC correlations from the bridgehead methine protons (Choi H-3a/C-4; H-3a/C-7; H-3a/C-7a; H-7a/C-2), which connected these spin systems, revealed 6-hydroxyoctahydroindole moiety. The HMBC correlations from Choi H-2 ( $\delta$ 4.20) and H-3 ( $\delta$ 1.74) to a carbonyl carbon at  $\delta$ 170.8 established the structure of Choi. A hydroxyl proton at  $\delta$ 6.36 (Argal OH) coupled to a methine proton at  $\delta$ 5.23 (Argal H-1) which coupled to a methine proton at  $\delta$ 3.69 (Argal H-2). Argal H-2 coupled to both an amide proton at  $\delta$ 7.68 (Argal NH) and methylene protons at  $\delta$ 1.55 and  $\delta$ 1.70 (Argal H<sub>2</sub>-3). The  $^1\text{H}$ - $^1\text{H}$  COSY spectrum provided one more correlations between two pairs of methylene protons (Argal H<sub>2</sub>-4, H<sub>2</sub>-5). The chemical shift of Argal C-5 ( $\delta$ 39.4) and H<sub>2</sub>-5 ( $\delta$ 3.12, 3.44) suggested that this methylene carbon connected to nitrogen. The linkage between Argal C-3 and C-4 was decided by the HMBC correlation from one of the methylene protons at  $\delta$ 3.44 (Argal H-5) to C-3 ( $\delta$ 23.5), although  $^1\text{H}$ - $^1\text{H}$  COSY correlations were not observed due to overlap of the signals. A  $^{13}\text{C}$  signal at  $\delta$ 156.6 indicated that there is one guanidino group in **1**. The HMBC correlations from Argal H-1 to C-5 and guanidino carbon established the structure of argininal, which formed cyclic hemiaminal.

The sequence of these partial structures were determined by HMBC and NOESY spectrum (Fig. 2). In the HMBC spectrum, Tyr NH coupled to Hpla sulfate C-1 and Argal NH coupled to Choi CO. The NOESY spectrum showed the correlations between Choi H-7a/Tyr H-2, and Choi H-7a/Tyr H<sub>2</sub>-3. Although phenolic hydroxyl proton in Tyr and aliphatic hydroxyl protons in Hpla sulfate and Choi were not observed in NMR spectra, chemical shift data and molecular formula decided the presence of these hydroxyl groups. All these data led to the planar structure of aeruginosin 102-A.

The relative stereochemistry of Choi was determined by interpretation of NOESY data shown in Fig. 3. The absolute stereochemistry of Hpla sulfate was determined to be D-form by ODS HPLC analysis of menthyl ester of acid hydrolyzate of **1**.<sup>7</sup> Standard optically active Hpla was synthesized from *p*-aminophenylalanine. Tyr was decided to be D-form by chiral GC analysis of *N*-trifluoroacetyl methyl ester derivatives of the acid hydrolyzate. The absolute stereochemistry of Argal was determined to be L-form by Marfey's analysis of acid hydrolyzate of oxidation product of **1**, in which Argal was converted to Arg.

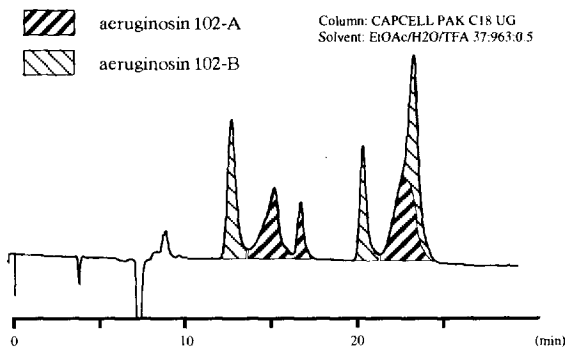


Fig. 1 HPLC chromatogram of aeruginosins 102-A, B

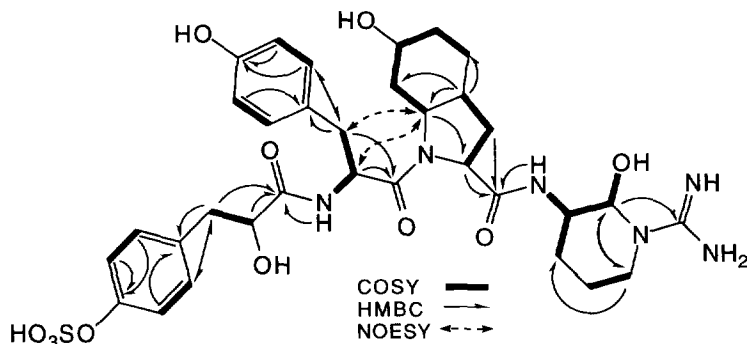


Fig.2 COSY, HMBC and NOESY correlations of Aeruginosin 102-A

Table 1.  $^{13}\text{C}$  and  $^1\text{H}$  NMR data for aeruginosins 102-A(1) and B(2) in  $\text{DMSO}-d_6$ 

Position	Aeruginosin 102-A		Aeruginosin 102-B (major)		Aeruginosin 102-B (minor)		
	$\delta_{\text{C}}$	$\delta_{\text{H}}$	$\delta_{\text{C}}$	$\delta_{\text{H}}$	$\delta_{\text{C}}$	$\delta_{\text{H}}$	
Hpla	1	172.5(s)	172.5(s)		172.5(s)		
sulfate	2	72.0(d)	4.02(dd,9.6,3.3)	72.1(d)	4.01(dd,8.8,3.3)	71.9(d)	4.05(dd,7.7,3.7)
	3	39.6(t)	2.59(dd,13.9,9.6)	39.7(t)	2.58(dd,13.6,8.8)	39.6(t)	2.65(dd,13.9,7.7)
			2.90(dd,13.9,3.3)		2.92(dd,13.6,3.3)		2.89(dd,13.9,3.7)
	4	133.1(s)		133.1(s)		132.8(s)	
	5,9	129.7(d)	7.09(d,8.4)	129.7(d)	7.08(d,8.7)	129.7(d)	7.08(d,8.7)
	6,8	120.2(d)	7.06(d,8.4)	120.3(d)	7.05(d,8.7)	120.3(d)	7.05(d,8.7)
	7	151.9(s)		151.9(s)		151.9(s)	
Tyr	1	168.7(s)		168.6(s)		169.2(s)	
	2	51.1(d)	4.67(ddd,8.0,8.0,5.9)	51.2(d)	4.65(ddd,8.4,7.7,6.1)	51.5(d)	4.60(ddd,8.8,7.3,6.1)
	3	38.2(t)	2.64(dd,13.1,8.0)	38.1(t)	2.63(dd,13.3,8.4)	38.1(t)	2.58(dd,13.6,8.8)
			2.74(dd,13.1,5.9)		2.76(dd,13.3,6.1)		2.78(dd,13.6,6.1)
	4	126.3(s)		126.3(s)		126.1(s)	
	5,9	130.3(d)	6.90(d,8.4)	130.3(d)	6.90(d,9.0)	130.3(d)	6.92(d,8.7)
	6,8	114.9(d)	6.65(d,8.4)	114.9(d)	6.65(d,9.0)	115.0(d)	6.67(d,8.7)
	7	156.1(s)		156.1(s)		156.2(s)	
NH		7.58(d,8.0)		7.51(d,7.7)		7.50(d,7.3)	
Choi	1	170.8(s)		170.9(s)		171.5(s)	
	2	59.2(d)	4.20(dd,9.0,9.0)	59.6(d)	4.14(dd,8.8,8.8)	59.9(d)	4.08(dd,9.9,8.0)
	3	30.0(t)	1.74(m)	30.5(t)	1.60(m)	30.1(t)	1.66(m)
			1.84(m)		1.90(m)		1.84(m)
	3a	35.7(d)	1.62(m)	35.7(d)	1.62(m)	35.8(d)	1.53(m)
	4	18.8(t)	1.28(m)	18.8(t)	1.27(m)	18.8(t)	1.27(m)
			1.82(m)		1.81(m)		1.79(m)
	5	25.8(t)	1.35(m)	25.8(t)	1.35(m)	25.8(t)	1.35(m)
	6	63.8(d)	3.84(m)	63.8(d)	3.83(m)	63.8(d)	3.83(m)
	7	33.1(t)	1.55(m)	33.2(t)	1.56(m)	32.9(t)	1.46(m)
		1.93(m)		1.94(m)		1.94(m)	
Argal	7a	54.1(d)	3.68(m)	54.1(d)	3.68(m)	54.3(d)	3.61(ddd,11.6,6.3,6.3)
	1	76.2(d)	5.23(br)	76.6(d)	5.23(br)	77.5(d)	5.07(br)
	2	49.0(d)	3.69(m)	48.7(d)	3.70(m)	47.0(d)	3.84(m)
	3	23.5(t)	1.55(m)	23.8(t)	1.46(m)	21.4(t)	1.40(m)
			1.70(m)		1.65(m)		1.96(m)
	4	23.3(t)	1.48(m)	23.3(t)	1.46(m)	23.3(t)	1.49(m)
		1.70(m)		1.68(m)		1.85(m)	
5	39.4(t)	3.12(ddd,13.0,13.0,2.8)	39.6(t)	3.12(ddd,12.5,12.5,2.8)	39.8(t)	3.25(ddd,13.0,13.0,3.6)	
		3.44(ddd,13.0,13.0,2.9)		3.42(m)		3.47(m)	
C=N	156.6(s)		156.8(s)		157.6(s)		
OH		6.36(br)		6.42(br)		6.23(br)	
NH		7.68(d,8.4)		7.49(d,7.3)		7.74(d,7.9)	

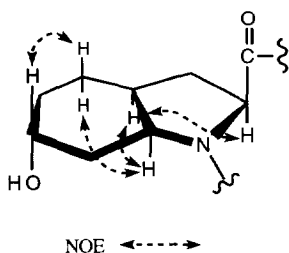


Fig. 3 Relative stereochemistry of Choi

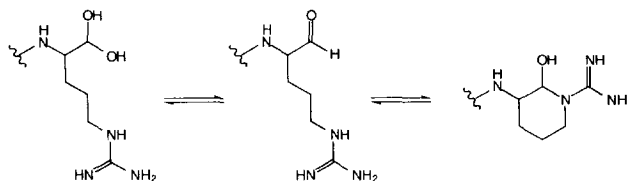


Fig. 4 Tautomerization of Argal

The molecular formula of aeruginosin 102-B (**2**) was decided to be  $C_{33}H_{44}N_6O_{11}S$ , which is the same as that of **1**, by low and high resolution FABMS and NMR data. The existence of a sulfate was also suggested by the fragment ion of positive FABMS. The amino acid analysis of acid hydrolyzate gave Tyr and an unknown amino acid.  $^1H$  and  $^{13}C$  NMR spectra were similar to those of **1**, but chemical shift data of Argal and Choi were different distinctly. The detailed analyses of 1D and 2D NMR spectra revealed that the planar structure of **2** is identical to that of **1**. The absolute stereochemistry of Argal was D-form, which was different from **1**. The absolute stereochemistry of Hpla sulfate and Tyr were identical to those of **1**.

Leupeptin<sup>13</sup> and antipain<sup>14</sup> are well-known protease inhibitors which possess Argal at C-terminus. In the case of leupeptin, it was presumed that Argal tautomerizes as in Fig. 4.<sup>15</sup> Separation of aeruginosins 102-A and B into three peaks each on HPLC may be also due to the tautomerization in the same manner.

Protease inhibitory activities of aeruginosins 102-A and B and other peptides are shown in Table 2. Aeruginosin 102-A inhibited thrombin with the  $IC_{50}$  of 0.04  $\mu g/mL$  and trypsin and plasmin with the  $IC_{50}$  of 0.2 and 0.3  $\mu g/mL$ , respectively. Aeruginosin 102-B inhibited thrombin, trypsin and plasmin with the  $IC_{50}$  of 0.1, 1.1 and 0.8  $\mu g/mL$ , respectively. Aeruginosins 102-A and B inhibited thrombin more potently than other peptides listed in Table 2. Aeruginosin 102-A, which possesses L-Argal, inhibited these three enzymes more potently than aeruginosin 102-B, which possesses D-Argal.

Table 2 Protease inhibitory activities of aeruginosins and other peptides containing arginal

	$IC_{50}$ ( $\mu g/ml$ )							
	Aeruginosins				Leupeptins <sup>14</sup>		Antipain <sup>15</sup>	
	102-A	102-B	98-A <sup>12</sup>	98-B <sup>12</sup>	298-A <sup>12</sup>	(L-L-L)	(L-L-D)	
Thrombin	0.04	0.1	7.0	10.0	0.3	10000*		-
Trypsin	0.2	1.1	0.6	0.6	1.0	1.0	22.0	0.26
Plasmin	0.3	0.8	6.0	7.0	>10	4.5	70.0	93.0

- : not determined \* : mixture of diastereomers

## EXPERIMENTAL

**General methods:** NMR spectra were recorded on a Bruker AM600 NMR spectrometer operating at 600 MHz for  $^1H$  and 150MHz for  $^{13}C$ .  $^1H$  and  $^{13}C$  NMR chemical shifts were referenced to solvent peaks:  $\delta_H$  2.49 and  $\delta_C$  39.5 for DMSO- $d_6$ . Ultraviolet spectra were measured on a Hitachi 330 spectrophotometer. FAB mass spectra were measured on a JEOL SX102 mass spectrometer. Amino acid analyses were carried out with Hitachi 835 and Hitachi L-8500A amino acid analyzer.

**Culture of *Microcystis viridis*:** *Microcystis viridis* (NIES-102) was obtained from the NIES-collection and cultured in 10L glass bottles containing MA medium [ $Ca(NO_3)_2 \cdot 4H_2O$  5 mg,  $KNO_3$  10 mg,  $NaNO_3$  5 mg,

Na<sub>2</sub>SO<sub>4</sub> 4 mg, MgCl<sub>2</sub>·6H<sub>2</sub>O 5 mg, β-Na<sub>2</sub>glycerophosphate 10 mg, Na<sub>2</sub>EDTA·2H<sub>2</sub>O 0.5 mg, FeCl<sub>3</sub>·6H<sub>2</sub>O 0.05 mg, MnCl<sub>2</sub>·4H<sub>2</sub>O 0.5 mg, ZnCl<sub>2</sub> 0.05 mg, CoCl<sub>2</sub>·6H<sub>2</sub>O 0.5 mg, Na<sub>2</sub>MoO<sub>4</sub>·2H<sub>2</sub>O 0.08 mg, H<sub>3</sub>BO<sub>3</sub> 2 mg, BICINE 50 mg, distilled water 100 mL, pH 8.6] with aeration at 25 °C under illumination of 250 μE/m<sup>2</sup>·s on a 12L:12D cycle. The algal cells were harvested by continuous flow centrifugation. The cells were yielded at 0.58 g/L on an average and kept in a freezer at -20 °C until extraction.

**Extraction and isolation:** Freeze-dried alga (207 g) was extracted with MeOH/H<sub>2</sub>O (8:2), concentrated and partitioned between Et<sub>2</sub>O and H<sub>2</sub>O. The H<sub>2</sub>O soluble fraction was partitioned between *n*-BuOH and H<sub>2</sub>O. The *n*-BuOH layer was subjected to ODS flash chromatography with aqueous MeOH. The fractions eluted with 40% and 70% MeOH were combined and subjected to ODS flash chromatography with aqueous MeOH again. The fraction eluted with 40% MeOH was subjected to ODS medium pressure column chromatography with MeOH/0.1 M NaClO<sub>4</sub>. The active fraction was separated by HPLC on CAPCELL PAK C18 UG (EtOAc/H<sub>2</sub>O/TFA 37:963:0.5) to yield aeruginosin 102-A (**1**, 20.3 mg) and B (**2**, 23.7 mg).

**Aeruginosin 102-A (1):** colorless amorphous powder; UV (MeOH) λ<sub>max</sub> 275 nm (ε 1500); positive FABMS *m/z* 733 (M+H)<sup>+</sup>, 653 (M-SO<sub>3</sub>+H)<sup>+</sup>; negative FABMS *m/z* 731 (M-H)<sup>-</sup>; HRFABMS *m/z* 731.2715 [M-H]<sup>-</sup> (Δ +0.4 mmu, σ 2.0 mmu); HMBC correlations: Hpla sulfate 1 / 2, 3, Tyr NH; Hpla sulfate 2 / 3; Hpla sulfate 3 / 5,9; Hpla sulfate 4 / 2, 3, 6,8; Hpla sulfate 5,9 / 2, 5,9; Hpla sulfate 6,8 / 6,8; Hpla sulfate 7 / 5,9, 6,8; Tyr 1 / 2, 3; Tyr 2 / 3; Tyr 3 / 2, 5,9; Tyr 4 / 3, 6,8; Tyr 5,9 / 3, 5,9; Tyr 6,8 / 6,8; Tyr 7 / 5,9, 6,8; Choi 1 / 2, 3; Argal NH; Choi 2 / 7a; Choi 3 / 3a, 4, 7a; Choi 4 / 6; Choi 5 / 3a; Choi 6 / 4; Choi 7a / 3, 4, 6; Argal 1 / 3, 5; Argal 2 / 1, 3; Argal 3 / 1, 5; Argal 4 / 5; Argal 5 / 1; Argal C=N / 1.

**Aeruginosin 102-B (2):** colorless amorphous powder; UV (MeOH) λ<sub>max</sub> 275 nm (ε 2300); positive FABMS *m/z* 733 (M+H)<sup>+</sup>, 653 (M-SO<sub>3</sub>+H)<sup>+</sup>; negative FABMS *m/z* 731 (M-H)<sup>-</sup>; HRFABMS *m/z* 731.2717 [M-H]<sup>-</sup> (Δ +0.6 mmu, σ 2.0 mmu). HMBC correlations of major tautomer: Hpla sulfate 1 / 2, 3, Tyr NH; Hpla sulfate 2 / 3; Hpla sulfate 3 / 5,9; Hpla sulfate 4 / 2, 3, 6,8; Hpla sulfate 5,9 / 2, 5,9; Hpla sulfate 6,8 / 6,8; Hpla sulfate 7 / 5,9, 6,8; Tyr 1 / 2, 3; Tyr 2 / 3; Tyr 3 / 2, 5,9; Tyr 4 / 2, 3, 6,8; Tyr 5,9 / 3, 5,9; Tyr 6,8 / 6,8; Tyr 7 / 5,9, 6; Choi 1 / 2, Argal NH; Choi 2 / 7a; Choi 3 / 7a; Choi 3a / 7a; Argal 2 / 1; Argal 3 / 1; Argal 5 / 1; Argal C=N / 1; HMBC correlations of minor tautomer: Hpla sulfate 1 / 2, 3, Tyr NH; Hpla sulfate 2 / 3; Hpla sulfate 3 / 5,9; Hpla sulfate 4 / 2, 3, 6,8; Hpla sulfate 5,9 / 2, 5,9; Hpla sulfate 6,8 / 6,8; Hpla sulfate 7 / 5,9, 6,8; Tyr 1 / 2, 3; Tyr 2 / 3; Tyr 3 / 2, 5,9; Tyr 4 / 2, 3, 6,8; Tyr 5,9 / 3, 5,9; Tyr 6,8 / 6,8; Tyr 7 / 5,9, 6; Choi 1 / 2, Argal NH; Choi 2 / 7a; Choi 3 / 7a; Argal 2 / 1; Argal 3 / 1; Argal 5 / 1; Argal C=N / 1.

**Amino acid analyses:** Each of compounds **1** and **2** (100 μg) was dissolved in 6 N HCl (1 mL) and sealed in reaction vials. The vials were heated at 110 °C for 16 h. The solution was evaporated and redissolved in 0.02 N HCl to subject on the amino acid analyzer.

**Chiral GC analyses:** Each acid hydrolyzates of **1** and **2** each was heated in the reaction vials with 10 % HCl in MeOH (0.5 mL) at 100 °C for 1 h and then treated with trifluoroacetic anhydride / CH<sub>2</sub>Cl<sub>2</sub> (1:1, 0.6 mL) at 100 °C for 5 min. Chiral GC was carried out by using a Chirasil Val III capillary column (0.32 mm × 25 m) with a flame ionization detector (FID). Column temperature was kept at 80 °C for 3 min and increased to 200 °C at a rate of 4 °C/min. Helium was used as carrier gas. Retention times (min): D-Tyr (30.5), L-Tyr (31.1).

***p*-Hydroxyphenyllactic acid:** (a) L-Isomer. *p*-Amino-L-phenylalanine (100 mg) was dissolved in 1N HCl (10 mL) and cooled to 0 °C. To this solution, NaNO<sub>2</sub> (100 mg) dissolved in water (5 mL) was added dropwise. After the solution was stirred overnight, it was heated under reflux for 15 min. Then the solution was subjected to ODS open column chromatography (YMC AM-ODS) and eluted with 20% MeOH to yield 38 mg of L-*p*-hydroxyphenyllactic acid. (b) D-Isomer. This was obtained from *p*-amino-D-phenylalanine (100 mg) in 25 % yield.

**Derivatization and HPLC analysis of *p*-hydroxyphenyllactic acid (Hpla):** L-Menthol (250 mg) and MeCN (50 μL) were added to each of the acid hydrolyzates of **1** and **2**. The mixture was sealed in a test tube with screw cap, and heated at 100 °C for 10 min. Then the mixture was concentrated *in vacuo* and diluted with MeCN, and analyzed by ODS-HPLC equipped with a photodiode array detector: column SHISEIDO

Supiorex ODS (4.6 × 150 mm); solvent 75% MeOH; flow rate 0.8 mL/min. Retention times (min): D-Hpla (10.4), L-Hpla (11.1).

**Oxidation, hydrolysis and Marfey's analysis:** Each of compounds **1** and **2** (100 µg) was dissolved in 31% H<sub>2</sub>O<sub>2</sub> and one drop of conc. HCl was added. The solution was left for 24 h at room temperature. Then the solution was lyophilized and hydrolyzed as described previously. After evaporation, 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (L-FDAA) in acetone (50 µL, 10 mg/mL) and 1 M NaHCO<sub>3</sub> (100 µL) were added, and the mixture was heated at 80 °C for 3 min. 2 N HCl (50 µL) and 50% MeCN (300 µL) were added to the mixture, and the mixture was analyzed by reversed phase HPLC: column Cosmosil MS (4.6 × 250 mm); gradient elution from H<sub>2</sub>O/TFA (100:0.1) to MeCN/H<sub>2</sub>O/TFA (50:50:0.1) in 50 min; UV detection 340 nm; flow rate 1.0 mL/min. Retention times (min): D-Arg (34.5) L-Arg (35.9).

**Protease inhibitory activity assay.** Thrombin, Trypsin and Plasmin inhibitory activities were determined by the method previously described.<sup>16</sup>

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