Radiosumin, a Trypsin Inhibitor from the Blue-Green Alga *Plectonema radiosum*

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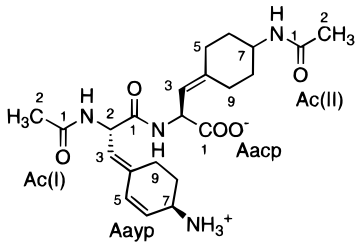
Radiosumin, a novel potent trypsin inhibitory dipeptide, was isolated from the freshwater blue-green alga *Plectonema radiosum* (NIES-515). Its structure was elucidated to be **1** on the basis of 2D NMR data and chemical degradation. The IC_{50} of **1** against trypsin was 0.14 μg/mL.

In recent years, blue-green algae have been demonstrated to be valuable sources of unique peptidic compounds, such as microviridin,[1] hormothamnin A,[2] calophycin,[3] microginin,[4] micropeptins,[5] and aeruginosins.[6] In the course of our screening program of protease inhibitors from microalgae, we have found that the aqueous layer from cultures of the freshwater blue-green alga *Plectonema radiosum* (NIES-515) strongly inhibited trypsin. We isolated radiosumin (**1**), a novel potent trypsin inhibitory dipeptide, and elucidated the structure to be **1** on the basis of 2D NMR data and chemical degradation. The IC_{50} of **1** against trypsin was 0.14 μg/mL. We herein report the isolation and structural determination of **1**.



1

P. radiosum was obtained from the NIES-collection and cultured in 10 L glass bottles containing CSi medium under illumination. The 80% MeOH extract of freeze-dried algal cells (110 g) was partitioned between water and diethyl ether, and the aqueous layer was further washed with BuOH. The aqueous layer was subjected to ODS column chromatography. The active fractions were combined and further subjected to ODS column chromatography followed by reversed phase HPLC to yield 120 mg of **1**.

The UV absorption in H_2O at λ_{max} 239 nm (ϵ 15000) indicated the presence of a conjugated diene, and the IR spectrum showed the presence of amide carbonyl groups (1660 cm^{-1}). The molecular formula was determined to be $C_{22}H_{32}N_4O_5$ by HRFABMS (*m/z* 433.2466 $[M + H]^+$ Δ1.5 mmu) and NMR. ^{13}C NMR, DEPT, and HMQC[7] spectral data in DMSO-d_6 indicated the presence of two methyl, six methylene, eight methine, and six nonprotonated carbons. The sharp one-proton doublets at δ_H 7.75, 8.24, and 8.40 were thought to be amide protons based on the HMQC spectrum and the chemical shifts. The broad ^1H doublet (3H) at 8.08 ppm was considered to be a primary ammonium signal from the chemical shift and the integration (Table 1).

Extensive analyses of $^1H-^1H$ COSY, HMQC, and HMBC[8] spectra revealed the existence of two new amino acid residues, Aayp (2-amino-3-(4-amino-2-cyclohexen-1-ylidene)propionic acid) and Aacp (2-amino-3-(4-amino-2-cyclohexylidene)propionic acid) as follows.

Aayp. The $^1H-^1H$ COSY spectrum revealed correlations of 2-NH(δ 8.24) to H-2(δ 5.15), H-2 to H-3(δ 5.33), H-5(δ 6.23) to H-6(δ 5.70), 7-NH_3^+(δ 8.08) to H-7(δ 3.85), H-7 to H_2-8(δ 1.52, 2.00), and H_2-8 to H_2-9(δ 2.31, 2.71) but not the correlation of H-6 to H-7 due to the small coupling constant between H-6 and H-7. HMBC correlations from the nonprotonated sp^2 carbon C-4 to H-2, H-5, H-6, H_2-8, and H_2-9 revealed the presence of a conjugated diene system. Correlations observed in the HMBC spectrum of H-5/C-7, H-6/C-8, 7-NH_3^+/C-6, and H-8b/C-6 clarified the connectivity between C-6 and C-7 which was not detected from the $^1H-^1H$ COSY spectrum. Furthermore, the correlations observed in the HMBC spectrum between H-2 and C-1 indicated the connectivity between C-1 and C-2. From these data, we elucidated the planar structure of one of the two new amino acids as Aayp, which contains a cyclohexylidene ring.

Aacp. The $^1H-^1H$ COSY spectrum revealed the correlations of 2-NH(δ 8.40) to H-2(δ 4.86), H-2 to the olefinic proton H-3(δ 5.13), and 7-NH(δ 7.75) to H-7(δ 3.70). The correlations of the two chemical shift-equivalent methylene protons at δ 1.19 and 1.77 (H_2-6, H_2-8) to H-7, H_2-5(δ 2.02, 2.16), and H_2-9(δ 1.85, 2.53) observed in the $^1H-^1H$ COSY spectrum suggested the connectivities from C-5 to C-9 via C-7, which was supported by HMBC correlations of H_2-5 with C-7, and H-9b with C-7. The ^{13}C NMR signals of C-6 and C-8 were unambiguously assigned as δ 33.3 and δ 32.4, respectively, from the correlations H_2-5/C-6 and H_2-9/C-8 (Table 1). The HMBC correlations

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(1) Ishitsuka, M. O.; Kusumi, T.; Kakisawa, H.; Kaya, K.; Watanabe, M. M. *J. Am. Chem. Soc.* **1990**, *112*, 8180−8182.
(2) Gerwick, W. H.; Jiang, Z. D.; Agarwal, S. K.; Farmer, B. T. *Tetrahedron* **1992**, *48*, 2313−2324.
(3) Moon, S. S.; Chen, J. L.; Moore, R. E.; Patterson, G. M. L. *J. Org. Chem.* **1992**, *57*, 1097−1103.
(4) Okino, T.; Matsuda, H.; Murakami, M.; Yamaguchi, K. *Tetrahedron Lett.* **1993**, *34*, 501−504.
(5) Okino, T.; Murakami, M.; Haraguchi, R.; Munekata, H.; Matsuda, H.; Yamaguchi, K. *Tetrahedron Lett.* **1993**, *34*, 8131−8134.
(6) (a) Murakami, M.; Okita, Y.; Matsuda, H.; Okino, T.; Yamaguchi, K. *Tetrahedron Lett.* **1994**, *35*, 3129−3132. (b) Murakami, M.; Ishida, K.; Okino, T.; Okita, Y.; Matsuda, H.; Yamaguchi, K. *Tetrahedron Lett.* **1995**, *36*, 2785−2788.
(7) Bax, A.; Subramanian, S. *J. Magn. Reson.* **1986**, *67*, 565−569.
(8) Bax, A.; Summers, M. F. *J. Am. Chem. Soc.* **1986**, *108*, 2093−2094.

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Table 1. ^{1}H and ^{13}C NMR Spectral Data of Radiosumin in DMSO-d_6

position		δ_C	δ_H	HMBC correlations (^{13}C)
Ac(I)	1	168.6 (s)		
	2	22.5 (q)	1.82 (s)	Ac(I) 1
Aayp	1	169.6 (s)		
	2	50.6 (d)	5.15 (dd, 9.3, 7.9)	Ac(I) 1, Aayp 1, 3, 4
	2-NH		8.24 (d, 7.9)	Ac(I) 1, Aayp 2
	3	126.5 (d)	5.33 (d, 9.3)	Aayp 1, 2, 5, 9
	4	135.4 (s)		
	5	133.7 (d)	6.23 (d, 9.7)	Aayp 3, 4, 7, 9
	6	126.1 (d)	5.70 (brdd, 9.7, 2.8)	Aayp 4, 8
	7	46.3 (d)	3.85 (brm)	
	7-NH_3^+		8.08 (brd, 5.2)	Aayp 6, 7, 8
	8a	26.4 (t)	1.52 (m)	Aayp 4, 7, 9
	8b		2.00 (m)	Aayp 4, 6, 7, 9
	9a	22.0 (t)	2.31 (m)	Aayp 3, 4, 7, 8
	9b		2.71 (m)	Aayp 3, 4, 5, 7, 8
Aacp	1	172.4 (s)		
	2	50.6 (d)	4.86 (dd, 9.1, 6.6)	Aayp 1, Aacp 1, 3, 4
	2-NH		8.40 (d, 6.6)	Aayp 1, Aacp and/or Aayp 2
	3	117.0 (d)	5.13 (d, 9.1)	Aacp 2, 5, 9
	4	143.2 (s)		
	5a	33.8 (t)	2.02 (m)	Aacp 3, 4, 6, 7
	5b		2.16 (m)	Aacp 3, 4, 6, 7, 9
	6a	33.3 (t)	1.19 (m)	Aacp 7,[a] 9[a]
	6b		1.77 (m)	
	7	46.9 (d)	3.70 (m)	
	7-NH		7.75 (d, 7.6)	Aacp 7, Ac(II) 1
	8a	32.4 (t)	1.19 (m)	Aacp 7,[a] 9[a]
	8b		1.77 (m)	
	9a	26.7 (t)	1.85 (m)	Aacp 8
	9b		2.53 (m)	Aacp 4, 7, 8
Ac(II)	1	168.4 (s)		
	2	22.7 (q)	1.76 (s)	Ac(II) 1

[a] Assignment is ambiguous due to overlapping of the ^{1}H signals.

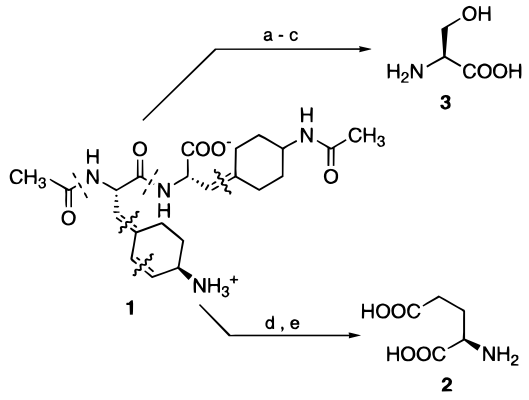
observed between H-2/C-4, H-3/C-5 and -9, H_2-5/C-3 and -4, H-5b/C-9, and H-9b/C-4 clarified the connectivities from C-2 to C-9. The connectivity between C-1 and C-2 was indicated by an HMBC correlation of α-proton H-2 to carbonyl carbon C-1. Therefore, the structure of the second unusual amino acid was deduced as Aacp, which is 5,6-dihydro Aayp.

Two methyl proton signals at δ 1.82 (Ac(I) H-2) and δ 1.76 (Ac(II) H-2) were confirmed to be acetyl groups, since long range couplings were observed between the methyl protons and carbonyl carbons at δ 168.6 (Ac(I) C-1) and δ 168.4 (Ac(II) C-1), respectively.

Connectivities of the two amino acid residues and the two acetyl groups were also decided by analysis of HMBC spectrum. One of the acetyl groups, Ac(I) was concluded to attach to Aayp 2-N, since the couplings were observed between Aayp H-2/Ac(I) C-1, and Aayp 2-NH/Ac(I) C-1. Similarly another acetyl group, Ac(II) was concluded to attach to Aacp 7-N from the observation of the coupling between Aacp 7-NH/Ac(I) C-1. The coupling of Aacp H-2/Aayp C-1 indicated the connectivity between Aayp and Aacp, which was supported by NOESY data.[9]

The *E*-geometry of the $\Delta^{3,4}$ double bond of Aayp was established based on NOESY correlations from H-2 to H-9 and H-3 to H-5. Other stereochemical assignments were elucidated by chemical degradation (Scheme 1). Ozonolysis of **1** followed by H_2O_2 oxidation afforded glutamic acid (**2**)[10] which proved to be the D-form from chiral GC analysis of the trifluoroacetyl isopropyl ester derivative. Thus, the stereochemistry at position 7 of Aayp was assigned as *R*. Cleavage of the double bonds of **1** with OsO_4−$NaIO_4$ followed by $NaBH_4$ reduction and

Scheme 1[a]



[a] (a) OsO_4, $NaIO_4$, H_2O, 2 h; (b) $NaBH_4$; (c) 6 N HCl, 110 °C, 16 h; (d) O_3, H_2O/AcOH, 0 °C, 30 min; (e) H_2O_2, H_2O/AcOH/HCl, 16 h.

hydrolysis with HCl afforded serine[10] (**3**). Only L-Ser was detected by chiral GC analysis, suggesting that of both Aayp and Aacp have the *S* configuration at the α-position.

As described above, the structure of radiosumin except for the stereochemistry of Aacp-7 was established as **1**, that is a novel dipeptide composed of two novel amino acid residues and two acetyl groups. Further study on the stereochemistry on Aacp-7 is in progress.

Plectonema radiosum is known to produce the cytotoxic nucleosides, tubercidin and tubercidin 5′-α-D-glucopyranose.[11] However, this is the first report of isolation of a protease inhibitory peptide from this species. Radiosumin inhibited trypsin with an IC_{50} of 0.14 μg/mL,

(9) NOESY correlations: Aayp H-2/Aacp 2-NH.

(10) Formation of these amino acids was confirmed by amino acid analysis.

(11) 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.

plasmin with an IC_{50} of 6.2 μg/mL, and thrombin with IC_{50} of 88 μg/mL, but **1** did not inhibit chymotrypsin, elastase, or papain at 200 μg/mL.

Experimental Section

General Methods. FAB mass spectra were measured by using diethanolamine or glycerol as matrices. NMR measurements were carried out at 500 or 600 MHz for ^{1}H and 150.9 or 75.5 MHz for ^{13}C. All NMR spectra were measured in DMSO-d_6 at 27 °C. The NOESY spectrum was acquired with a mixing time of 600 ms. The HMBC spectrum was acquired with an evolution time of 60 ms.

Culture Conditions. *Plectonema radiosum* (NIES-515) 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 CSi medium [$Ca(NO_3)\cdot 4H_2O$ 15 mg, KNO_3 10 mg, disodium β-glycerophosphate 5 mg, $MgSO_4\cdot 7H_2O$ 4 mg, vitamin B_{12} 0.01 μg, biotin 0.01 μg, thiamine HCl 1 μg, PIV metals 0.3 mL, $Na_2SiO_3\cdot 9H_2O$ 10 mg, HEPES 50 mg, distilled water 99.7 mL, pH 7.0][12] under illumination of 80 $\mu E/m^2$ s on a 12L:12D cycle. After 4−8 weeks, the algal cells were harvested by filtration. The yield of the lyophilized cells was 110 g from 350 L of culture. The cells were stored at −20 °C until extraction.

Isolation. The freeze-dried algal cells (110 g) were extracted with 80% MeOH (3 × 2 L). After removal of organic solvent, the extract was partitioned between water and diethyl ether. The aqueous layer was further washed with BuOH and then subjected to ODS column chromatography (YMC ODS 120 Å 70/230, 0−50% MeOH). The active 10 and 20% MeOH fractions were combined and further subjected to ODS column chromatography followed by reversed phase HPLC (CAPCELL PAK C_{18}, 8% MeCN 0.05% TFA) to yield 120 mg of radiosumin (**1**) as colorless amorphous powder: $[\alpha]^{20}_D$ +96° (*c* 0.77, H_2O); UV absorption in H_2O at λ_{max} 239 nm (ϵ 15000); IR 1660 cm^{-1}; HRFABMS *m*/*z* 433.2466 $(M + H)^+$, ($C_{22}H_{33}N_4O_5$).

Ozonolysis of Radiosumin. Radiosumin (5 mg) was treated with O_3 in H_2O/AcOH (1:1 4 mL) at 0 °C for 30 min. After removal of O_3 by N_2, 2 mL of H_2O_2, and 1 drop of concd HCl were added to the reaction mixture. After incubation at room temperature for 16 h, the solution was lyophilized to yield Glu.[10]

Oxidation by OsO_4 and Hydrolysis of Radiosumin. Radiosumin (5 mg) was dissolved in H_2O (1 mL) and then combined with 1 mg of OsO_4. $NaIO_4$ (18 mg) was carefully added to the solution with stirring at room temperature. After being stirred for further 80 min, an excess of $NaBH_4$ was added. The reaction mixture was then sealed in a test tube with 1 mL of concd HCl and heated at 110 °C for 16 h to yield Ser.[10]

Chiral GC Analysis of Amino Acids. Amino acids were heated in 10% HCl in *i*-PrOH (0.5 mL) at 100 °C for 1 h. After evaporation of the solvent, the residue was treated with trifluoroacetic anhydride/CH_2Cl_2 (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 120 °C at a rate of 4 °C/min. Helium was used as carrier gas. Retention times (min): D-Ser (12.5), L-Ser (13.3), D-Glu (19.8), L-Glu (20.9).

Trypsin Inhibitory Assay. Trypsin inhibitory activities were determined by the modified method of Cannel *et al.*[13] Trypsin was purchased from Sigma Chemical Co. It was dissolved in 50 mM Tris-HCl (pH 7.6) to prepare a 150 units/mL solution. In 1 mL of DMSO, 43.3 mg of *N*-benzoyl-D,L-arginine-*p*-nitroanilide (BAPNA) was dissolved, diluted with 100 mL of 50 mM Tris-HCl (pH 7.6), and used as substrate solution. A 30 μL volume of 0.4 M Tris-HCl (pH 7.6), 50 μL of trypsin solution, and 20 μL of test solution were added to each microtiter plate well and preincubated at 37 °C for 5 min. Then 100 μL of substrate solution was added to begin the reaction. The absorbance of the well was immediately read at 405 nm. The developed color was measured after incubation at 37 °C for 30 min.

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Supporting Information Available: Copies of NMR data of **1** (10 pages). This material is contained in the libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from ACS; see any current masthead page for ordering information.

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(12) Watanabe, M. M.; Satake, K. N. *NIES-Collection List of Strains Fourth Edition*; Microbial Culture Collection, Natl. Inst. Env. Stud., Tsukuba, 1994; pp 30−38.

(13) Cannell, R. J. P.; Kellam, S. J.; Owsianka, A. M.; Walker, J. M. *Planta Med.* **1988**, *54*, 10−14.