# Dehydroradiosumin, a Trypsin Inhibitor from the Cyanobacterium *Anabaena cylindrica*

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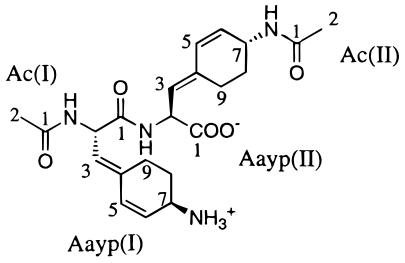
Dehydroradiosumin, a novel potent trypsin inhibitory dipeptide, was isolated from the freshwater cyanobacterium *Anabaena cylindrica* (NIES-19). Its structure was elucidated as **1** on the basis of 2D NMR data and chemical degradation. The $IC_{50}$ of **1** against trypsin was 0.1 $\mu$g/mL.

Recently, freshwater cyanobacteria have been demonstrated to be a rich source of unique and interesting bioactive peptides. The best known hepatotoxic cyclic peptides, the microcystins and congeners, have been isolated from various cyanobacteria (*Microcystis*, *Oscillatoria*, *Anabaena*, and *Nodularia*), and over 40 microcystin-type peptides have been reported to date.[1] In the course of our screening program for protease inhibitors of microalgae, we have found that many strains of freshwater cyanobacteria showed strong protease inhibitory activities and have isolated many peptidic compounds from *Microcystis*,[2] *Oscillatoria*,[3] and *Nostoc*.[4] Some strains of *Anabaena* also showed protease inhibitory activities. Circinamide,[5] which has a very similar structure to E-64 isolated *Aspergillus japonicus*,[6] has been previously isolated from *Anabaena circinalis* (NIES-41) and exhibited a potent papain inhibitory activity. Harada et al. isolated anabaenopeptins A and B from *A. flos-aquae* NRC 525-17, and these compounds produced concentration-dependent relaxations in rat aortic preparations with endothelium precontracted with 0.1 $\mu$M norepinephrine.[7] Related compounds were also isolated from *Oscillatoria agardhii* (NIES-204) by our group but did not show any protease inhibitory activities.[8]

In this paper, we report the isolation and structure elucidation of dehydroradiosumin (**1**) isolated from *Anabaena cylindrica* (NIES-19). Dehydroradiosumin is a congener to radiosumin,[9] which was isolated as a trypsin inhibitor from *Plectonema radiosum* (NIES-515).




**1**

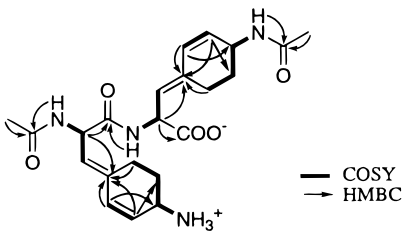



**Figure 1.** $^1H-^1H$ COSY, HMBC correlations of dehydroradiosumin.

*A. cylindrica* was obtained from the NIES collection and cultured in 10 L glass bottles containing CB medium under illumination. The freeze-dried alga was extracted with 80% MeOH and MeOH, and the extract was partitioned between $Et_2O$ and $H_2O$. The aqueous layer was further extracted with *n*-BuOH. The *n*-BuOH layer was subjected to ODS column chromatography followed by reversed-phase HPLC to yield dehydroradiosumin (**1**, 20.8 mg).

The UV absorption in $H_2O$ at $\lambda_{max}$ 239 nm ($\epsilon$ 26 000) indicated the presence of a conjugated diene. The molecular formula was determined to be $C_{22}H_{30}N_4O_5$ by HRFABMS and NMR spectra. Its peptidic nature was suggested by $^1H$ and $^{13}C$ NMR spectra (Table 1). The sharp one-proton doublets at $\delta_H$ 7.95, 8.25, and 8.47 were thought to be amide protons based on the HMQC spectrum and chemical shifts. The broad $^1H$ signal (3H) at 8.04 ppm was considered to be a primary ammonium signal from the chemical shift (Table 1).

Extensive analyses of $^1H-^1H$ COSY, HMQC,[10] and HMBC[11] spectra revealed that dehydroradiosumin was a dipeptide comprised of two units of the unusual amino acid Aayp (2-amino-3-(4-amino-2-cyclohexene-1-ylidene)-propionic acid) and two acetyl groups as in Figure 1. The existence of the Aayp units was confirmed by a comparison of $^1H$ and $^{13}C$ NMR chemical shifts with those of the Aayp unit of radiosumin.[9] Correlations observed in the HMBC spectrum of H-5/C-7 and H-6/C-8 in Aayp units clarified the connectivity between C-6 and C-7, which was not detected from the $^1H-^1H$ COSY spectrum (Figure 1).

Two methyl proton signals at $\delta$ 1.83 (Ac (I) H-2) and 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 $\delta$ 168.6 (Ac (I)

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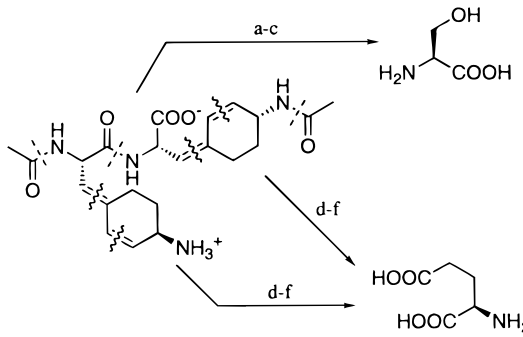
**Table 1.** $^1$H and $^{13}$C NMR Spectral Data of Dehydroradiosumin in DMSO-$d_6$

| position | | $^1$H ($J$, Hz) | $^{13}$C | HMBC correlations (from $^1$H to $^{13}$C) |
|---|---|---|---|---|
| Ac (I) | 1 | | 168.6 (s) | |
| | 2 | 1.83 (s) | 22.8 (q) | Ac (I) 1 |
| Aayp (I) | 1 | | 170.0 (s) | |
| | 2 | 5.15 (dd, 9.4, 8.1) | 50.5 (d) | Ac (I) 1, Aayp (I) 1, 3, 4 |
| | 2-NH | 8.25 (d, 8.1) | | Ac (I) 1, Aayp (I) 2 |
| | 3 | 5.34 (d, 9.4) | 126.5 (d) | Aayp (I) 5, 9 |
| | 4 | | 135.5 (s) | |
| | 5 | 6.24 (d, 9.8) | 133.8 (d) | Aayp (I) 3, 4, 7, 9 |
| | 6 | 5.70 (dd, 9.8, 2.8) | 125.9 (d) | Aayp (I) 4, 8 |
| | 7 | 3.85 (m) | 46.2 (d) | |
| | 7-NH$^{3+}$ | 8.04 (br) | | |
| | 8a | 1.52 (m) | 26.2 (t) | Aayp (I) 7 |
| | 8b | 2.00 (m) | | Aayp (I) 4, 6, 7, 9 |
| | 9a | 2.32 (m) | 22.0 (t) | Aayp (I) 4, 8 |
| | 9b | 2.71 (m) | | Aayp (I) 3, 4, 5, 7, 8 |
| Aayp (II) | 1 | | 172.0 (s) | |
| | 2 | 4.93 (dd, 9.0, 7.2) | 50.4 (d) | Aayp (I) 1, Aayp (II) 1, 3, 4 |
| | 2-NH | 8.47 (d, 7.2) | | Aayp (I) 1, Aayp (II) 2 |
| | 3 | 5.32 (d, 9.0) | 122.0 (d) | Aayp (II) 5, 9 |
| | 4 | | 137.5 (s) | |
| | 5 | 6.10 (d, 9.8) | 130.8 (d) | Aayp (II) 3, 4, 7, 9 |
| | 6 | 5.65 (dd, 9.8, 3.4) | 132.0 (d) | Aayp (II) 4, 8 |
| | 7 | 4.32 (m) | 44.5 (d) | |
| | 7-NH | 7.95 (d, 8.1) | | Ac (II) 1, Aayp (II) 7 |
| | 8a | 1.42 (m) | 28.0 (t) | Aayp (II) 7 |
| | 8b | 1.77 (m) | | Aayp (II) 4, 6, 7, 9 |
| | 9a | 2.21 (m) | 22.5 (t) | Aayp (II) 4, 7, 8 |
| | 9b | 2.51 (m) | | Aayp (II) 4 |
| Ac (II) | 1 | | 168.6 (s) | |
| | 2 | 1.79 (s) | 22.8 (q) | Ac (II) 1 |

**Scheme 1**[a]




[a] Key: (a) $OsO_4$, $NaIO_4$, $H_2O$, 40 min; (b) $NaBH_4$, 80 min; (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; (f) 6 N HCl, 110 °C, 16 h.

C-1) and 168.4 (Ac (II) C-1), respectively (Figure 1). Connectivities of the two amino acid residues and the two acetyl groups were also decided by the HMBC spectrum. One of the acetyl groups, Ac (I), was assigned to Aayp (I) 2-N, since correlations were observed between Aayp (I) H-2/Ac (I) C-1 and Aayp (I) 2-NH/Ac (I) C-1. Similarly, the other acetyl group, Ac (II), was assigned to attach to Aayp (II) 7-N from observation of the correlation between Aayp (II) 7-NH/Ac (II) C-1. The coupling of Aayp (II) H-2/Aayp (I) C-1 indicated the connectivity between Aayp (I) and Aayp (II), which was supported by the NOESY correlation of Aayp (I) H-2/Aayp (II) 2-NH.

The *E*-geometries of the $\Delta^{3,4}$ double bonds of Aayp (I) and Aayp (II) were established on the basis of NOESY correlations from H-2 to H-9 and H-3 to H-5.[9] Other stereochemical assignments were elucidated by chemical degradation (Scheme 1). Ozonolysis of **1** followed by $H_2O_2$ oxidation and hydrolysis with HCl afforded Glu (Scheme 1).[12] Only D-Glu was detected by the Marfey analysis,[13] suggesting that both Aayp (I) and Aayp (II) have the *R* configuration at position 7. Cleavage of the double bonds of **1** with $OsO_4$−$NaIO_4$ followed by $NaBH_4$ reduction and hydrolysis with HCl afforded Ser (Scheme 1).[12] Only L-Ser was detected by chiral GC analysis, suggesting that both Aayp (I) and Aayp (II) have the *S* configuration at the α-position. The structure of dehydroradiosumin was thus established as **1**.

The dipeptide radiosumin, containing Aayp and Aacp (2-amino-3-(4-amino-2-cyclohexylidene) propionic acid), has previously been obtained from *P. radiosum*[9] and was synthesized by Sioiri et al.[14] It is of interest that dehydroradiosumin, a congener to radiosumin, was obtained from *A. cylindrica*, which is taxonomically different from *P. radiosum*. Considering the identity of the stereochemistries of the Aayp units in radiosumin and dehydroradiosumin, they are probably formed by the same biogenetic pathway. Dehydroradiosumin inhibited trypsin with an $IC_{50}$ of 0.1 μg/mL and plasmin with an $IC_{50}$ of 90 μg/mL but not thrombin, chymotrypsin, elastase, or papain at 100 μg/mL.

## Experimental Section

**General Methods.** The UV spectrum was recorded on a Hitachi 330 spectrophotometer. $^1$H 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 $\delta_H$ 2.49 and $\delta_C$ 39.5 were used as internal references for $^1$H and $^{13}$C NMR spectra, respectively. FAB mass spectra 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.** *A. cylindrica* (NIES-19) 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 CB medium [$Ca(NO_3)_2 \cdot 4H_2O$ 15 mg,

$KNO_3$ 10 mg, $\beta$-$Na_2$ glycerophosphate 5 mg, $MgSO_4 \cdot 7H_2O$ 4 mg, vitamin $B_{12}$ 0.01 $\mu$g, biotin 0.01 $\mu$g, thiamine HCl 1 $\mu$g, PIV metals 0.3 mL, bicine 50 mg, and distilled $H_2O$ 99.7 mL, pH 9.0] under illumination of 250 $\mu$E $m^{-2}$ $s^{-1}$ on a 12 L/12 D cycle. After 4−8 weeks, the algal cells were harvested by continuous centrifugation at 10 000 rpm. The yield of the lyophilized cells was 68.2 g from 185 L of culture. The harvested cells were stored at −20 °C until extraction.

**Isolation.** Freeze-dried alga (68.2 g from 185 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 this was extracted with $Et_2O$. The aqueous layer was then extracted with *n*-BuOH. The *n*-BuOH layer 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; 12% MeCN containing 0.05% TFA; flow rate, 2.0 mL/min; UV detection at 210 nm) to yield 20.8 mg of dehydroradiosumin (**1**) as a colorless amorphous powder: $[\alpha]_D$ +17.4° (*c* 0.1, $H_2O$); UV absorption in $H_2O$ at $\lambda_{max}$ 239 nm ($\epsilon$ 26 000); HRFABMS *m/z* 431.2336 $(M + H)^+$, calcd for $C_{22}H_{31}N_4O_5$ 431.2295.

**Ozonolysis of Dehydroradiosumin.** Dehydroradiosumin (1.0 mg) was treated with $O_3$ in $H_2O$/AcOH (1:1, 1 mL) at 0 °C for 30 min. After removal of $O_3$ by $N_2$, 1 mL of $H_2O_2$ and 1 drop of concd HCl were added to the reaction mixture. The reaction mixture was concentrated by $N_2$, and the residue was then sealed in a test tube with 1 mL of 6 N HCl and heated at 110 °C for 16 h to yield Glu.

**Oxidation by $OsO_4$ and Hydrolysis of Dehydroradiosumin.** Dehydroradiosumin (1 mg) was dissolved in $H_2O$ (1 mL) and then combined with 1 mg of $OsO_4$. $NaIO_4$ (4 mg) was carefully added to the solution with stirring at room temperature for 40 min. An excess of $NaBH_4$ was added and stirred for 80 min. The reaction mixture was subjected to ODS flash column chromatography (YMC ODS-A 120−230/70, 1.7 × 10 cm, 0−100% MeOH). The 10% MeOH fraction was concentrated by $N_2$, and the residue was then sealed in a test tube with 1 mL of 6 N HCl and heated at 110 °C for 16 h to yield Ser.

**Marfey Analysis of Glu.** The Glu was treated with a 10% $Me_2CO$ solution of 1-fluoro-2,4-dinitrophenyl-5-L-alanineamide (L-FDAA, Marfey's reagent) in 1 M $NaHCO_3$ at 80−90 °C for 3 min followed by neutralization with 50 $\mu$L of 2 N HCl. The reaction mixture was dissolved in 50% MeCN and subjected to reversed-phase HPLC: column, 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 detector (340 nm). Retention times (min) of derivatized amino acids were found as follows: L-Glu (40.0), D-Glu (41.0).

**Chiral GC Analysis of Ser.** The Ser was heated in 10% HCl in MeOH (0.5 mL) at 100 °C for 30 min. 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 Chiral-L-Val capillary column (Chrompak, 25 m × 0.25 mm) with a flame-ionization detector (FID). Column temperature was 60 °C for 3 min and increased to 120 °C at rate of 4 °C/min. Helium was used as carrier gas. Retention times (min): D-Ser (13.6), L-Ser (14.5).

**Trypsin Inhibitory Assay.** Serine protease inhibitory activities were determined by the method described in a previous paper.[9]

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