New Anabaenopeptins, Potent Carboxypeptidase-A Inhibitors from the Cyanobacterium *Aphanizomenon flos-aquae*

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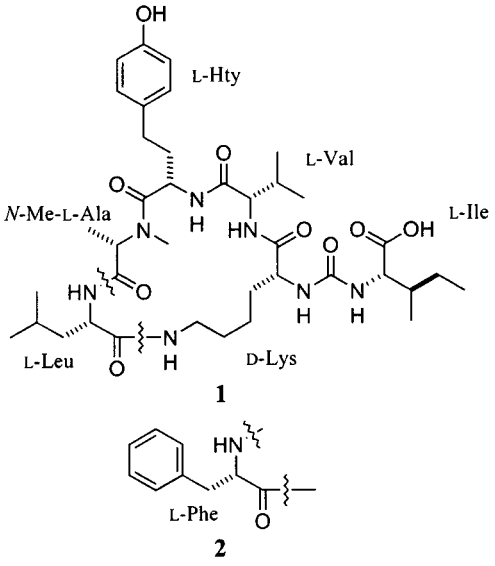
Anabaenopeptins I (**1**) and J (**2**), two new ureido bond-containing cyclic peptides, were isolated from the cultured cyanobacterium *Aphanizomenon flos-aquae* (NIES-81) as potent carboxypeptidase-A (CPA) inhibitors. The gross structures of **1** and **2** were established by spectroscopic analysis, including the 2D NMR techniques. The absolute configurations of **1** and **2** were determined by spectral and chemical methods. Anabaenopeptins I and J inhibited CPA with IC_{50} values of 5.2 and 7.6 ng/mL, respectively.

The search for new enzyme inhibitors from natural sources has led to the discovery of structurally diverse and biologically operative compounds for structure-based drug design. In our screening of inhibitory agents against carboxypeptidase-A (CPA) from microalgae, we isolated anabaenopeptins G and H from the cultured *Oscillatoria agardhii* Gomont (NIES-595, Hormogonales, Cyanophyceae)[1] and anabaenopeptin T from cyanobacterial water-bloom materials of lake Teganuma (Japan), collected in 1994.[2] Furthermore, as part of our ongoing screening, we found that extracts of the cyanobacterium *Aphanizomenon flos-aquae* Lemmermann (NIES-81, Hormogonales, Cyanophyceae) showed strong CPA-inhibitory activity. We report here the isolation and structure elucidation of anabaenopeptins I (**1**) and J (**2**) as the active principals.



1 **2**

A. flos-aquae (NIES-81) was obtained from the NIES-collection and cultured in 10-L glass bottles containing CB medium[3] under illumination of 250 μE/m^2•s on a 12L:12D cycle. The freeze-dried alga (61.0 g) was extracted with the 80% and 100% aqueous MeOH. After evaporation of the solvent, the residue was partitioned between Et_2O and H_2O. The active H_2O layer was extracted with *n*-BuOH and subjected to ODS flash chromatography with aqueous MeOH. The active 40% aqueous MeOH fraction was purified by reversed-phase HPLC on an ODS column (linear gradient of CH_3CN in H_2O containing 0.05% TFA, 30−60%) to yield anabaenopeptins I (**1**, 21.9 mg) and J (**2**, 97.5 mg).

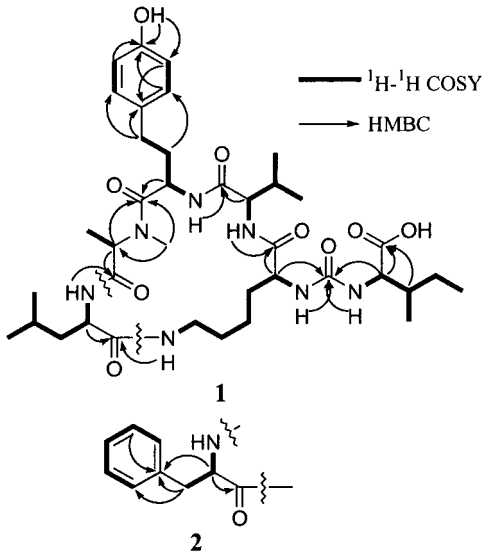


Figure 1.

Anabaenopeptin I (**1**) is a white powder with the molecular formula $C_{38}H_{61}N_7O_9$ as established by HRFABMS [*m/z* 760.4618 $(M + H)^+$ Δ +0.9 mmu]. The ^{1}H NMR spectra suggested the peptidic nature of **1** (Table 1). Amino acid analysis of the acid hydrolyzate of **1** (6 N HCl, 110 °C, 36 h) indicated the presence of one residue each of Leu, Lys, and Val. Extensive 2D NMR experiments, including ^{1}H−^{1}H COSY, HMQC, and HMBC, constructed four usual amino acids (Ile, Leu, Lys, and Val) and two unusual amino acids [homotyrosine (Hty) and *N*-Me-Ala] as shown in Figure 1. HMBC correlations (Leu NH/*N*-Me-Ala CO, *N*-Me-Ala *N*-Me/Hty CO, Hty NH/Val CO, Val NH/Lys CO, and Lys 6-NH/Leu CO) allowed us to establish the cyclic pentapeptide moiety of **1** as *cyclo*(−Leu−*N*-Me-Ala−Hty−Val−Lys−). In addition, both the Ile 2-NH and the Lys 2-NH showed correlations with an unassigned carbon signal (δ_C 157.38), which indicated that the Ile 2-NH was joined to the Lys 2-NH through a ureido moiety (Figure 1). The negative FABMS fragment ion peak (*m/z* 627 [M − Ile − 2H]$^-$) also supported it.

Anabaenopeptin J (**2**) is a white powder with the molecular formula $C_{41}H_{59}N_7O_9$ as established by HRFABMS

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Table 1. ^{1}H and ^{13}C NMR Data of Anabaenopeptins I (**1**) and J (**2**) in DMSO-d_6

anabaenopeptin I (**1**)			anabaenopeptin J (**2**)		
position	^{1}H (mult., *J* Hz)	^{13}C (mult.)	position	^{1}H (mult., *J* Hz)	^{13}C (mult.)
Leu			Phe		
1		171.8 (s)	1		170.8 (s)
2	4.17 (m)	51.3 (d)	2	4.38 (ddd, 12.2, 8.9, 3.4)	55.0 (d)
3	1.58 (m)	40.5 (t)	3	2.76 (m)	37.6 (t)
4	1.33 (m)	24.0 (d)		3.31 (br)	
5	0.73 (d, 6.4)	20.3 (q)	4		138.3 (s)
6	0.77 (d, 6.8)	23.2 (q)	5,9	7.05 (d)	128.8 (d)
NH	8.56 (d, 9.0)		6,8	7.18 (m)	128.3 (d)
N-Me-Ala			7	7.13 (m)	126.0 (d)
1		169.8 (s)	NH	8.65 (d, 8.9)	
2	4.88 (q, 6.4)	54.6 (d)	*N*-Me-Ala		
3	1.20 (d, 6.4)	14.0 (q)	1		170.0 (s)
N-Me	2.53 (s)	28.1 (q)	2	4.78 (d, 6.8)	54.2 (d)
Hty			3	1.06 (d, 6.8)	13.8 (q)
1		171.5 (s)	*N*-Me	1.77 (s)	27.0 (q)
2	4.77 (br)	48.4 (d)	Hty		
3	1.76 (m)	33.4 (t)	1		171.2 (s)
	1.91 (m)		2	4.72 (br)	45.9 (d)
4	2.44 (ddd, 14.3, 10.7, 6.4)	30.6 (t)	3	1.70 (m)	33.3 (t)
	2.64 (ddd, 14.3, 10.0, 4.3)			1.87 (m)	
5		130.9 (s)	4	2.43 (ddd, 14.3, 10.7, 6.4)	30.6 (t)
6,10	7.01 (d, 8.6)	129.0 (d)		2.63 (ddd, 14.3, 10.0, 4.3)	
7,9	6.67 (d, 8.6)	115.1 (d)	5		131.1 (s)
8		155.6 (s)	6,10	6.99 (d, 8.3)	129.0 (d)
NH	8.88 (d, 5.1)		7,9	6.66 (d, 8.3)	115.1 (d)
OH	9.20 (s)		8		155.6 (s)
Val			NH	8.92 (d, 4.3)	
1		172.4 (s)	OH	9.18 (s)	
2	3.90 (m)	57.5 (d)	Val		
3	1.85 (m)	30.6 (d)	1		172.8 (s)
4	0.80 (m)	18.5 (q)	2	3.90 (m)	59.0 (d)
5	0.87 (m)	18.9 (q)	3	1.96 (m)	30.1 (d)
NH	6.75 (d, 6.8)		4	0.92 (d, 6.4)	19.3 (q)
Lys			5	1.02 (d, 6.8)	19.0 (q)
1		172.2 (s)	NH	6.92 (d, 7.3)	
2	3.88 (m)	54.6 (d)	Lys		
3	1.61 (m)	31.3 (t)	1		172.3 (s)
4	1.13 (m)	20.3 (t)	2	3.93 (m)	54.8 (d)
	1.32 (m)		3	1.63 (m)	31.6 (t)
5	1.40 (m)	28.1 (t)	4	1.15 (m)	20.5 (t)
6	2.77 (m)	38.1 (t)		1.32 (m)	
	3.50 (m)		5	1.44 (m)	28.2 (t)
6-NH	7.05 (m)		6	2.80 (m)	38.3 (t)
2-NH	6.52 (d, 6.83)			3.57 (m)	
ureido		157.4 (s)	6-NH	7.15 (m)	
Ile			2-NH	6.49 (d, 6.8)	
1		173.9 (s)	ureido		157.5 (s)
2	4.04 (dd, 8.8, 5.1)	56.7 (d)	Ile		
3	1.73 (m)	37.0 (d)	1		173.9 (s)
4	1.12 (m)	24.6 (t)	2	4.05 (dd, 8.8, 5.1)	56.8 (d)
	1.37 (m)		3	1.72 (m)	36.8 (d)
5	0.84 (m)	11.5 (q)	4	1.10 (m)	24.6 (t)
6	0.84 (m)	15.8 (q)		1.35 (m)	
NH	6.33 (d, 8.8)		5	0.83 (m)	11.4 (q)
			6	0.84 (m)	15.7 (q)
			NH	6.33 (d, 8.8)	

Table 2. Inhibition of CPA by Anabaenopeptins

anabaenopeptin							inhibition of CPA (μg/mL)
B	L-Phe	*N*-Me-L-Ala	L-Hty	L-Val	D-Lys	L-Arg	no activity
E	L-Phe	*N*-Me-L-Ala	L-MeHty	L-Val	D-Lys	L-Arg	no activity
F	L-Phe	*N*-Me-L-Ala	L-Hty	L-Ile	D-Lys	L-Arg	no activity
G	L-Ile	*N*-Me-L-Hty	L-Hty	L-Ile	D-Lys	L-Tyr	$IC_{50} = 0.0070$
H	L-Ile	*N*-Me-L-Hty	L-Hty	L-Ile	D-Lys	L-Arg	$IC_{50} = 3.4$
I (**1**)	L-Leu	*N*-Me-L-Ala	L-Hty	L-Val	D-Lys	L-Ile	$IC_{50} = 0.0052$
J (**2**)	L-Phe	*N*-Me-L-Ala	L-Hty	L-Val	D-Lys	L-Ile	$IC_{50} = 0.0076$
T	L-Ile	*N*-Me-L-Hty	L-Hty	L-Val	D-Lys	L-Ile	$IC_{50} = 0.022$

[*m*/*z* 794.4462 $(M + H)^+$ Δ +0.9 mmu]. The ^{1}H NMR spectra suggested the peptidic nature of **2** (Table 1). Amino acid analysis of the acid hydrolyzate of **2** (6 N HCl, 110 °C, 36 h) indicated the presence of one residue each of Lys, Phe, and Val. Extensive 2D NMR experiments, including ^{1}H−^{1}H COSY, HMQC, and HMBC, constructed four usual amino acids (Ile, Lys, Phe, and Val) and two unusual amino acids (Hty and *N*-Me-Ala) as shown in Figure 1. The HMBC correlations established the cyclic pentapeptide moiety of **2** as *cyclo*(−Phe−*N*-Me-Ala−Hty−Val−Lys−). The remain-

ing Ile residue was attached to Lys through an unusual ureido linkage, which was confirmed by the HMBC correlations (Figure 1). The difference between **1** and **2** was that **1** had Leu instead of Phe as found in **2**.

Marfey's analysis[4] of the acid hydrolyzate of **1** and **2** revealed that Hty, Leu, *N*-Me-Ala, Phe, and Val have the L configuration, and Lys has D. The ureido bond could not be cleaved by the normal acid hydrolysis conditions, but treatment of **1** and **2** with anhydrous hydrazine produced Ile residues,[5] each of which was derivatized and identified as L-Ile.

The inhibitory activity of **1** and **2** against CPA was studied on the rate of CPA-catalyzed hydrolysis of hippuryl-L-phenylalanine, as monitored by absorbance changes at 254 nm.[6] Compounds **1** and **2** inhibited CPA with IC_{50} values of 5.2 and 7.6 ng/mL, respectively. Anabaenopeptins were first isolated from *Anabaena flos-aquae* NRC-525-17 by Harada and co-workers,[7] and we also previously reported five anabaenopeptins from *O. agardhii* (NIES-204 and 595)[1,8,9] and anabaenopeptin T from cyanobacterial water-bloom materials of lake Teganuma (Japan), collected in 1994.[2] Anabaenopeptins G and T inhibited CPA, with IC_{50} values of 7.0 and 22.0 ng/mL, respectively. Although it would be expected that anabaenopeptins B,[7,8] E, and F[9] were also CPA inhibitors, these peptides showed no activity at a concentration of 10 μg/mL. From this result and substrate specificity of CPA, it was suggested that the amino acid residue attached to the ureido group was essential to interact with CPA. However, inasmuch as anabaenopeptin H showed weak inhibitory activity (IC_{50} = 3.4 μg/mL), it was supposed that the cyclic pentapeptide moiety of anabaenopeptins might also be important for the inhibitory mechanism. It would be of interest to extend the structural study of these compounds to design new inhibitors against CPA.

Experimental Section

General Experimental Procedures. NMR spectra were recorded on a JEOL AM600 NMR spectrometer operating at 600 MHz for ^{1}H and 150 MHz for ^{13}C using DMSO as solvent at 27 °C. ^{1}H and ^{13}C NMR chemical shifts were referenced to solvent peaks: δ_H 2.49 and δ_C 39.5 for DMSO-d_6. FABMS were measured by using glycerol as matrix on a JEOL JMS SX-102 mass spectrometer. Amino acid analyses were carried out with a Hitachi L-8500A amino acid analyzer. HPLC was performed on a Shimadzu LC-10AS liquid chromatograph with COSMOSIL C_{18}-MS column (10 mm × 250 mm i.d.). Optical rotations were measured on a JASCO DIP-1000 digital polarimeter. UV spectra were measured on a Hitachi 330 spectrometer.

Culture Conditions. *Aphanizomenon flos-aquae* (NIES-81) was obtained from the NIES collection (Microbial Culture Collection, the National Institute for Environmental Studies, Japan) and cultured in 10-L glass bottles containing CB medium. Cultures were illuminated on a 12 L:12 D cycle at an intensity of 250 μE/m^2•s from fluorescent tubes and aerated with filtered air (0.3 L/min, without added CO_2) at 25 °C for 6−8 weeks. After incubation, cells were harvested by continuous flow centrifugation at 10 000 rpm. Harvested cells were lyophilized and kept in a freezer at −20 °C until extraction.

Extraction and Isolation. Freeze-dried cyanobacterium (61.0 g from 520 L of culture) was extracted with the 80% and 100% aqueous MeOH. After evaporation of the solvent, the residue was partitioned between Et_2O and H_2O. The H_2O-soluble fraction was further partitioned between *n*-BuOH and H_2O. The active *n*-BuOH layer was subjected to ODS flash column chromatography and eluted with 20, 60, 100% aqueous MeOH, and CH_2Cl_2. The 60% aqueous MeOH fraction was further subjected to ODS open column chromatography and eluted with 20, 40, 60, 80, 100% aqueous MeOH, and CH_2Cl_2. The 40% aqueous MeOH fraction showed CPA-inhibitory activity and was purified by ODS HPLC (30−60% MeCN containing 0.05% TFA in 30 min; flow rate 2.0 mL/min; UV detection at 210 nm) to yield anabaenopeptins I (**1**, 21.9 mg) and J (**2**, 97.5 mg).

Anabaenopeptin I[1]: white powder; $[\alpha]_D$ −30.0° (*c* 0.1, MeOH); UV (MeOH) λ_{max} 278 nm (ϵ 1300); ^{1}H and ^{13}C NMR, see Table 1; FABMS *m/z* 760 $(M + H)^+$, 758 $(M - H)^-$, 627 $(M - Ile - 2H)^-$; HRFABMS *m/z* 760.4618 $(M + H)^+$ calculated for $C_{38}H_{61}N_7O_9$ (Δ +0.9 mmu).

Anabaenopeptin J[2]: white powder; $[\alpha]_D$ −51.5° (*c* 0.1, MeOH); UV (MeOH) λ_{max} 278 nm (ϵ 1400); ^{1}H and ^{13}C NMR, see Table 1; FABMS *m/z* 794 $(M + H)^+$, 792 $(M - H)^-$, 661 $(M - Ile - 2H)^-$; HRFABMS *m/z* 794.4462 $(M + H)^+$ calculated for $C_{41}H_{59}N_7O_9$ (Δ +0.9 mmu).

Amino Acid Analyses. Compounds **1** and **2** (1.0 mg each) were dissolved in 6 N HCl (500 μL) and sealed in separate vials. The vials were heated at 110 °C for 36 h. The solution was evaporated in a stream of dry nitrogen with heating and redissolved in 0.1 N HCl in preparation for amino acid analysis.

Marfey Analyses of Amino Acids. To the acid hydrolyzate of a 1.0-mg portion of **1** and **2**, 1-fluoro-2,4-dinitrophenyl-5-L-alanineamide (L-FDAA) in acetone (50 μL) and 1 M $NaHCO_3$ (100 μL) were added. The mixtures were heated at 80 °C for 3 min followed by neutralization with 2 N HCl (50 μL). The ureido bond could not be cleaved by normal acid hydrolysis conditions, but treatment of a 1.0- mg portion of **1** and **2** with anhydrous hydrazine produced Ile residues, which were derivatized in the same manner as described above. The reaction mixtures were dissolved in 50% aqueous MeCN and subjected to reversed-phase HPLC: column, Cosmosil C_{18} MS (4.6 mm × 250 mm i.d.), gradient elution from H_2O/TFA (100:0.1) to MeCN/H_2O/TFA (60:40:0.1) in 60 min, flow rate 1 mL/min, UV detection at 340 nm. The identity of each peak was confirmed by co-injection with a solution of a standard that had been derivatized in the same manner, but the L-Hty−D-FDAA derivative was substituted for the D-Hty−L-FDAA derivative. Retention times (min): D-Lys (33.0), L-Lys (34.6), L-Val (47.0), L-Ile (51.0), L-Leu (51.2), D-Val (51.4), L-Phe (51.8), D-Phe (52.8), D-Ile (55.2), D-Leu (55.4), L-Hty (58.6), D-Hty (61.2). To separate *N*-Me-D,L-Ala clearly, a different gradient elution from H_2O/TFA (100:0.1) to MeOH/H_2O/TFA (60:40:0.1) in 60 min, was used. Retention time (min): *N*-Me-D-Ala (59.8), *N*-Me-L-Ala (60.4).

Enzyme Assay. CPA inhibitory activities were measured according to the method of Cannel et al.[8]

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