Anabaenopeptins E and F, Two New Cyclic Peptides from the Cyanobacterium *Oscillatoria agardhii* (NIES-204)

Hee Jae Shin, Hisashi Matsuda, Masahiro Murakami,* and Katsumi Yamaguchi

Laboratory of Marine Biochemistry, Graduate School of Agricultural Life Sciences, The University of Tokyo, Bunkyo-ku, Tokyo 113, Japan

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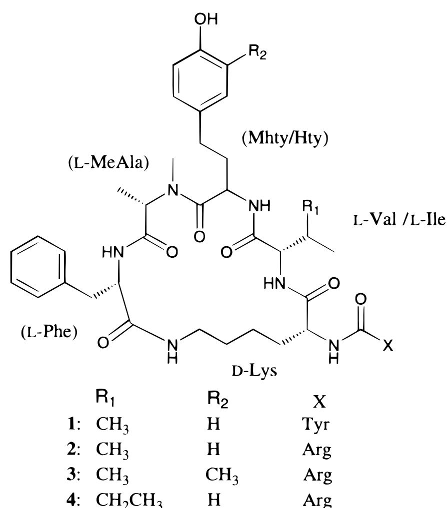
Anabaenopeptins E and F, two new ureido bond-containing cyclic peptides, were isolated from the cultured cyanobacterium *Oscillatoria agardhii* (NIES-204). The gross structures of anabaenopeptins E (**3**) and F (**4**) were elucidated by extensive 2D NMR techniques and chemical degradation. The absolute stereochemistry of usual amino acids in **3** and **4** was determined by GC and HPLC analyses.

Anabaenopeptins A (**1**) and B (**2**) were first isolated as a minor group of bioactive compounds from the cyanobacterium *Anabaena flos-aquae* NRC 525-17, and produced concentration-dependent relaxations in rat aortic preparations with endothelium precontracted with 0.1 μM norepinephrine.[1] In our continuing search for protease inhibitors from cyanobacteria,[2] we also isolated anabaenopeptin B (**2**) from the cultured *Oscillatoria agardhii* Gomont (NIES-204, Oscillatoriales, Oscillatoriaceae).[3] Further investigation of the extract of *O. agardhii* resulted in the isolation of two new congeners, anabaenopeptins E (**3**) and F (**4**). We report here their isolation and structure elucidation.



The freeze-dried cyanobacterial cells were extracted with 80% MeOH, and the extract was partitioned between *n*-BuOH and H_2O. The *n*-BuOH layer was subjected to ODS flash chromatography and eluted with aqueous MeOH and CH_2Cl_2. The 70% MeOH-eluted fraction was purified by reversed-phase HPLC on an ODS column (linear gradient of CH_3CN in H_2O containing 0.05% TFA, 30−80%) to yield anabaenopeptins E (**3**) and F (**4**) along with the known compound, anabaenopeptin B (**2**).

Anabaenopeptin E (**3**) was isolated as a colorless, amorphous powder from the cultured cyanobacterium *O. agardhii* in the yield of 0.018%: $[\alpha]^{20}_D$ −52.7° (*c* 0.2, MeOH); UV (MeOH) λ max (log ϵ) 280 nm (3.25). The molecular formula of **3** was deduced as $C_{42}H_{63}N_{10}O_9$ by HRFABMS [*m/z* 851.4763 (M + H)$^+$ Δ −1.8 mmu] and NMR spectral data. Amino acid analysis of the acid hydrolysate of **3** revealed the presence of one residue each of Phe, Val, Lys, Arg, and two unknown amino acids. Extensive NMR analyses of **3** including ^{1}H−^{1}H COSY, HMQC,[4] HMBC,[5] and HOHAHA[6] spectra indicated the presence of the above-mentioned amino acid residues together with *N*-methylalanine (MeAla) and 7-methylhomotyrosine (Mhty) structural units. A fragment peak in the negative ion FABMS (*m/z* 675, [M − Arg − H_2O − H]$^-$) also indicated the presence of Arg. The peptidic nature of **3** was suggested by its ^{1}H- and ^{13}C-NMR spectra, showing seven amide protons, six amide carbonyl groups, one ureido carbonyl group, one guanidine group, and four non-protonated signals, as shown in Table 1. The cyclic pentapeptide moiety of **3** was determined as cyclo-(Phe-MeAla-Mhty-Val-Lys) by interresidue correlations in the HMBC spectrum (Phe NH/MeAla CO, MeAla *N*-Me/Mhty CO, Mhty NH/Val CO, Val NH/Lys CO, Lys ϵ-NH/Phe CO) and NOESY spectrum (Phe NH/MeAla α-H, MeAla α-H/Mhty α-H, Mhty NH/Val α-H, Val NH/Lys α-H, Lys ϵ-NH/Phe α-H). The remaining Arg residue was attached to Lys through an unusual ureido linkage, which was confirmed by the NOESY correlations (Arg α-NH/Lys α-NH, Arg α-NH/Lys α-H) as well as HMBC cross peaks of Arg α-H/ureido CO (δC 157.3) and Lys α-H/ureido CO. It was also established by HMBC that the free carboxy group was present in the Arg group (Figure 1).

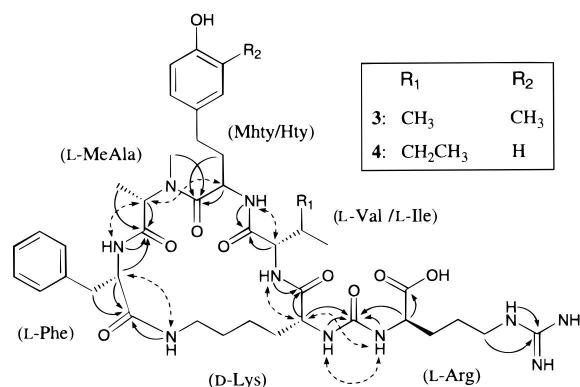


Figure 1. Important HMBC (half arrows) and NOESY (dashed arrows) correlations for anabaenopeptins E (**3**) and F (**4**) in DMSO-d_6.

* To whom correspondence should be addressed. Phone: 81-3-3812-2111 ext. 5298. FAX: 81-3-5684-0622. E-mail: amura@hongo.ecc.u-tokyo.ac.jp.

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Table 1. ^{1}H- and ^{13}C-NMR Assignments for Anabaenopeptins E (**3**) and F (**4**) in DMSO-d_6

anabaenopeptin E (**3**)			
units	no.	^{13}C (mult[a])	^{1}H (mult, J = Hz)
Phe	1	170.8 (s)	
	2	54.9 (d)	4.39 (ddd, 12.7, 8.8, 3.4)
	3	37.5 (t)	2.77 (dd, 13.9, 12.7)
			3.32 (dd, 13.9, 3.4)
	4	138.3 (s)	
	5, 9	128.9 (d)	7.06 (d, 7.0)
	6, 8	128.3 (d)	7.18 (m)
	7	126.1 (d)	7.13 (m)
	NH		8.69 (d, 8.8)
MeAla	1	169.9 (s)	
	2	54.3 (d)	4.83 (q, 6.7)
	3	13.8 (q)	1.07 (d, 6.7)
	N-Me	27.0 (q)	1.78 (s)
Mhty	1	170.9 (s)	
	2	48.7 (d)	4.73 (ddd, 7.9, 5.4, 5.4)
	3	33.2 (t)	1.71 (m)
			1.87 (m)
	4	30.5 (d)	2.38 (ddd, 13.7, 11.2, 6.2)
			2.59 (ddd, 13.7, 11.2, 4.3)
	5	131.0 (s)	
	6	130.3 (d)	6.89 (d, 2.4)
	7	123.7 (s)	
	8	153.6 (s)	
	9	114.4 (d)	6.66 (d, 8.1)
	10	126.2 (d)	6.80 (d, 8.1)
	7-Me	16.0 (q)	2.08 (s)
	NH		8.89 (d, 4.8)
	OH		9.18 (br s)
Val	1	172.2 (s)	
	2	58.0 (d)	3.91 (dd, 8.7, 7.1)
	3	30.3 (d)	1.97 (m)
	4	19.2 (q)	0.92 (d, 6.6)
	5	18.9 (q)	1.02 (d, 6.7)
	NH		6.97 (d, 7.1)
Lys	1	172.2 (s)	
	2	54.7 (d)	3.94 (ddd, 6.7, 6.7, 4.4)
	3	31.7 (t)	1.62 (m)
	4	20.3 (t)	1.15 (m)
			1.30 (m)
	5	28.1 (t)	1.43 (m)
	6	38.3 (t)	2.80 (m)
			3.58 (dddd, 13.3, 8.6, 8.6, 4.1)
	α-NH		6.49 (d, 7.1)
	ε-NH		7.20 (m)
Arg	1	174.2 (s)	
	2	52.0 (d)	4.09 (ddd, 8.1, 8.1, 4.9)
	3	29.2 (t)	1.53 (m)
			1.70 (m)
	4	25.0 (t)	1.48 (m)
	5	40.3 (t)	3.09 (m)
	6	156.8 (s)	
	α-NH		6.43 (d, 8.3)
	δ-NH		7.61 (t, 5.8)
	CO[b]	157.3 (s)	

anabaenopeptin F (**4**)			
units	no.	^{13}C (mult[a])	^{1}H (mult, J = Hz)
Phe	1	170.8 (s)	
	2	54.9 (d)	4.38 (ddd, 12.7, 8.8, 3.4)
	3	37.5 (t)	2.77 (dd, 13.9, 12.7)
			3.32 (dd, 13.9, 3.4)
	4	138.2 (s)	
	5, 9	128.8 (d)	7.05 (d, 7.4)
	6, 8	128.3 (d)	7.18 (m)
	7	126.1 (d)	7.14 (m)
	NH		8.67 (d, 8.8)
MeAla	1	169.8 (s)	
	2	54.2 (d)	4.78 (q, 6.7)
	3	13.8 (q)	1.05 (d, 6.7)
	N-Me	27.0 (q)	1.76 (s)
Hty	1	170.9 (s)	
	2	48.7 (d)	4.72 (ddd, 7.9, 5.4, 5.4)
	3	33.2 (t)	1.70 (m)
			1.87 (m)
	4	30.5 (t)	2.42 (ddd, 13.7, 11.0, 6.4)
			2.62 (ddd, 13.7, 11.0, 4.3)
	5	131.0 (s)	
	6, 10	129.0 (d)	6.98 (d, 8.2)
	7, 9	115.1 (d)	6.66 (d, 8.2)
	8	155.5 (s)	
	NH		8.93 (d, 4.6)
	OH		9.18 (br s)
Ile	1	172.7 (s)	
	2	56.6 (d)	3.97 (dd, 8.5, 7.3)
	3	35.7 (d)	1.78 (m)
	4	24.7 (t)	1.14 (m)
	5	10.3 (q)	0.82 (t, 7.3)
	6	14.9 (q)	0.88 (d, 6.4)
	NH		6.97 (d, 7.3)
Lys	1	172.1 (s)	
	2	54.7 (d)	3.94 (ddd, 6.6, 6.6, 4.3)
	3	31.7 (t)	1.61 (m)
	4	20.3 (t)	1.15 (m)
			1.29 (m)
	5	28.1 (t)	1.43 (m)
	6	38.2 (t)	2.80 (m)
			3.57 (dddd, 13.5, 8.7, 8.7, 4.2)
	α-NH		6.47 (d, 7.3)
	ε-NH		7.14 (m)
Arg	1	174.1 (s)	
	2	51.8 (d)	4.10 (ddd, 7.6, 7.6, 5.1)
	3	29.4 (t)	1.52 (m)
			1.69 (m)
	4	24.9 (t)	1.46 (m)
	5	40.3 (t)	3.08 (m)
	6	156.7 (s)	
	α-NH		6.43 (d, 8.2)
	δ-NH		7.57 (t, 5.9)
	CO[b]	157.1 (s)	

[a] Multiplicity and assignment are from HMQC experiment. [b] Ureido carbonyl group.

Anabaenopeptin F (**4**) was also isolated as a colorless, amorphous powder: $[\alpha]^{20}_{D}$ −41.4° (c 0.1, MeOH); UV (MeOH) λ max (log ϵ) 279 nm (3.19). The molecular formula of **4** was deduced as $C_{42}H_{63}N_{10}O_9$, which was identical with **3**, by HRFABMS [m/z 851.4810 $(M + H)^+$ Δ +3.1 mmu] and NMR spectral data. Amino acid analysis of the acid hydrolysate of **4** revealed the presence of one residue each of Phe, Ile, Lys, Arg, and two unknown amino acids. A detailed analyses of ^{1}H−^{1}H COSY, HMQC, and HOHAHA spectra revealed the presence of the MeAla and homotyrosine (Hty) and led to the complete assignment of the ^{1}H- and ^{13}C-NMR resonances of the six amino acid residues of **4** (Table 1). The amino acid sequence of **4** was determined to be cyclo-(Phe-MeAla-Hty-Ile-Lys) by the interpretation of interresidue HMBC and NOESY correlations (Figure 1). The HMBC and NOESY correlations observed between Lys and Arg established the whole structure of **4**. The difference between **4** and **3** was that **4** had Hty and Ile instead of Mhty and Val found in **3**.

The absolute stereochemistries of Phe, Val, and Ile in **3** and/or **4** were determined to be L and that of Lys to be D by chiral GC analysis of *N*-trifluoroacetyl isopropyl ester derivatives of the acid hydrolysate. MeAla and Arg were determined to be L by Marfey's method.[7] The absolute configurations of the Mhty and Hty residues remain to be determined.

Anabaenopeptins E and F are unique cyclic peptides having an unusual ureido bond and 7-Mhty or Hty residues. Cyclic peptides with a ureido linkage have been isolated from the cyanobacteria *Anabaena flos-aquae*[1] and *O. agardhii*.[8] Keramamide A[9] and konbamide,[10] which also contain a ureido group, were isolated from the Okinawan marine sponge *Theonella* sp., and

the authors speculated that these unique peptides might be produced by symbiotic microorganisms such as microalgae, bacteria, or fungi. The fact that *O. agardhii* produces anabaenopeptins E and F, closely related to keramamide A and konbamide, provides additional evidence that cyanobacteria might be the source of these compounds.

Experimental Section

General Experimental Procedures. NMR spectra were recorded on either a JEOL JNM-A600 or JEOL JNM-A500 NMR spectrometer 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 spectra were measured by using glycerol or polyethyleneglycol as matrix on a JEOL JMS SX-102 mass spectrometer. Amino acid analysis was carried out with a Hitachi L-8500A amino acid analyzer. HPLC was performed on a Shimadzu LC-6A liquid chromatograph with ODS L-column (10 × 250 mm, Chemicals Inspection and Testing Institute). Chiral GC experiments were performed on a Shimadzu GC-9A gas chromatograph fitted with an Alltech Chirasil-Val capillary column (25 m × 0.25 mm) with a flame ionization detector (FID). The oven temperature was increased from 80 to 200 °C at a rate of 4°/min. Optical rotations were measured on a JASCO DIP-1000 digital polarimeter. UV spectrum was measured on a Hitachi 330 spectrometer.

Culture Conditions. *O. agardhii* (NIES-204) 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.[11] 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 10−14 days. 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 (138 g from 400 L of culture) was extracted three times with 80% MeOH and concentrated to give a crude extract. This extract was partitioned between Et_2O and H_2O. The H_2O-soluble fraction was further partitioned between *n*-BuOH and H_2O. The *n*-BuOH layer was subjected to ODS flash chromatography and eluted with aqueous MeOH and CH_2Cl_2; 60% MeOH fraction (617 mg) was resubjected to ODS flash chromatography and eluted with 50, 60, 70, 100% MeOH, and CH_2Cl_2; 70% MeOH fraction was concentrated under reduced pressure to give bright yellow solid (312 mg). Final purification was achieved by reversed-phase HPLC on a ODS L-column (linear gradient of CH_3CN in H_2O containing 0.05% TFA, 30% to 80%; flow rate 2.5 mL/min; UV detection at 210 nm) to yield anabaenopeptins E (**3**, 24.5 mg, 0.018% yield, dry wt) and F (**4**, 8.9 mg, 0.006%) along with the known compound, anabaenopeptin B (**2**, 212.6 mg, 0.15%).

Anabaenopeptin E (3): colorless powder; $[\alpha]^{20}_D$ −52.7° (*c* 0.2, MeOH); UV (MeOH) λ max (log ϵ) 280 nm (3.25); FABMS *m/z* 851 (M + H)$^+$, 849 (M − H)$^-$, 675 (M − Arg − H_2O − H)$^-$; HRFABMS *m/z* 851.4763 (M + H)$^+$ calcd for $C_{42}H_{63}N_{10}O_9$ (Δ −1.8 mmu); For ^{1}H and ^{13}C data, see Table 1.

Anabaenopeptin F (4): colorless powder; $[\alpha]^{20}_D$ −41.4° (*c* 0.1, MeOH); UV (MeOH) λ max (log ϵ) 279 nm (3.19); FABMS *m/z* 851 (M + H)$^+$, 849 (M − H)$^-$, 675 (M − Arg − H_2O − H)$^-$; HRFABMS *m/z* 851.4810 (M + H)$^+$ calcd for $C_{42}H_{63}N_{10}O_9$ (Δ +3.1 mmu); For ^{1}H and ^{13}C data, see Table 1.

Amino Acid Analysis. Compounds **3** and **4** (100 μg each) were dissolved in 6 N HCl (500 μL) and sealed in separate reaction vials. The vials were heated at 110 °C for 16 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.

Chiral GC Analysis of Amino Acids. A solution of 10% HCl in isopropyl alcohol was added to each of the hydrolysates of **3** and **4** in reaction vials and heated at 100 °C for 30 min. The solvent was removed in a stream of dry nitrogen. Trifluoroacetic anhydride (300 μL) in CH_2Cl_2 (300 μL) was added to the residues, the vials were capped, and the solution was heated at 100 °C for 5 min and evaporated in a stream of dry nitrogen. The residues were dissolved in CH_2Cl_2 (500 μL) and immediately analyzed by chiral GC using a Chirasil-Val capillary column with a FID. Column temperature was kept at 80 °C for 3 min and then increased at a rate of 4°/min to 200 °C. Retention times (min) of standard amino acids were found as follows: D-Val (7.92), L-Val (8.68), D-Ile (9.97), L-Ile (11.02), D-Phe (21.75), L-Phe (22.63), D-Lys (32.66), L-Lys (33.21).

Marfey Analysis of Amino Acids. A 100-μg portion of **3** and **4** was dissolved in 6 N HCl (500 μL) and heated at 110 °C for 16 h. After removal of HCl in a stream of dry nitrogen, the residues were treated with 10% Me_2CO solution of 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (L-FDAA, Marfey's reagent) in 1 M $NaHCO_3$ at 80−90 °C for 3 min followed by neutralization with 50 μL of 2 N HCl. The reaction mixtures were dissolved in 50% MeCN and subjected to reversed-phase HPLC: column, Cosmosil MS (Nacalai Tesque Co., 4.6 × 250 mm), gradient elution from H_2O/TFA (100:0.1) to MeCN/H_2O/TFA (50:50:0.1) in 50 min, UV (340 nm). Retention times (min) of derivatized amino acids were found as follows: L-MeAla (31.6), D-MeAla (34.0), D-Arg (44.4), L-Arg (46.5).

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