Prenylagaramides A and B, New Cyclic Peptides from Two Strains of *Oscillatoria agardhii*

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Prenylagaramides A (**1**) and B (**2**), two new cyclic peptides, were isolated from the cultured cyanobacteria *Oscillatoria agardhii* (NIES-205) and *O. agardhii* (NIES-596), respectively. The structures of **1** and **2**, which both contain a rare *O*-prenyltyrosine (Ptyr) unit, were established as *cyclo*(-Ptyr^1-Gly^2-Thr^3-Gly^4-Glu^5-Phe^6-Phe^7-Asn^8-Pro^9-) and *cyclo*(-Ptyr^1-Leu^2-Tyr^3-Pro^4-Ile^5-Asn^6-Pro^7-), respectively, by spectroscopic analysis and chemical degradation.

Cyanobacteria have been shown to be a rich source of unique secondary metabolites including cyclic peptides and depsipeptides,[1] such as cryptophycins[2] from *Nostoc* spp., laxaphycins[3] from *Anabaena laxa*, and microcystins,[4] which are highly effective inhibitors of protein phosphatases 1 and 2A, from several freshwater cyanobacterial species.

As part of a screening program for enzyme inhibitors from freshwater cyanobacteria, we have described previously some bioactive peptides from two strains of *Oscillatoria agardhii* Gomont (NIES-204 and 205, Hormogonales, Cyanophyceae): oscillapeptin;[5] agardhipeptins A and B;[6] microviridins D−F;[7] anabaenopeptins B,[8] E, and F;[9] and aeruginosins 205A and B,[10] which inhibit serine proteases such as trypsin and thrombin. Further investigation of the extract of *O. agardhii* (NIES-205) resulted in the isolation of a new cyclic nonapeptide, prenylagaramide A (**1**), which contains a rare *O*-prenyltyrosine (Ptyr) unit as a major peptide. In addition, we have isolated a novel new cyclic heptapeptide, prenylagaramide B (**2**), also containing a Ptyr unit, from *O. agardhii* (NIES-596). We report here the isolation and structure elucidation of **1** and **2**.

Oscillatoria agardhii (NIES-205) was isolated from Lake Kasumigaura, Japan, and mass cultured in our laboratory.[10] The 80% MeOH and 100% MeOH extracts of the freeze-dried alga were partitioned between *n*-BuOH and H_2O. The *n*-BuOH layer was subjected to ODS flash chromatography and eluted with aqueous MeOH followed by 100% MeOH and CH_2Cl_2. The 60% MeOH fraction was purified by reversed-phase HPLC on an ODS column (linear gradient of MeCN in H_2O containing 0.05% TFA, 20−60%) to yield prenylagaramide A (**1,** 71.2 mg). *O. agardhii* (NIES-596) was isolated from Veluwemeer in Holland and also mass cultured in our laboratory. The extraction and partitioning of this freeze-dried alga gave the *n*-BuOH-soluble material, which was fractionated by ODS flash chromatography with aqueous MeOH, 100% MeOH, and CH_2Cl_2. The 80% MeOH was purified by HPLC on an ODS column to yield prenylagaramide B (**2,** 1.7 mg).

Prenylagaramide A (**1**) was isolated as a colorless amorphous powder. The molecular formula of **1** was deduced as $C_{54}H_{68}N_{10}O_{14}$ by HRFABMS and NMR spectral data. The ^{1}H and ^{13}C NMR spectra of **1** (Table 1) contained resonances that were characteristic of peptide. Amino acid analysis of the acid hydrolysate indicated the presence of Asp, Thr, Glu, Pro, Tyr, Gly, and Phe. Extensive 2D NMR analysis of **1**, including the ^{1}H−^{1}H COSY, HMQC,[11] and HMBC[12] spectra, led to assignment of one of Asn, Thr, Glu, Pro, Tyr and two of Gly and Phe (Figure 1). The prenyl unit was identified by the above-mentioned 2D NMR experiments and a fragment ion peak in the positive FABMS [*m*/*z* 1013 (M − prenyl + H)$^+$]. Its methylene signal (δ_H 4.46, δ_C 64.2), which was unusually downfield, suggested that this prenyl unit might be attached to one amino acid residue through an ether linkage, which was confirmed by the HMBC correlation between the prenyl methylene protons and Tyr C-7 (δ_C 157.0) (Figure 1). Thus, the structure of a rare Ptyr unit was determined unambiguously. The cyclic nonapeptide moiety of **1** was determined as *cyclo*[-Ptyr-Gly(1)-Thr-Gly(2)-Glu-Phe(1)-Phe(2)-Asn-Pro-] by interresidue correlations in the HMBC spectrum [Pro CO/Ptyr NH, Ptyr CO/Gly (1) NH, Gly (1) CO/Thr NH, Thr CO/Gly (2) NH, Gly (2) CO/Glu NH, Glu CO/Phe (1) NH, Phe (1) CO/Phe (2) NH, Phe (2) CO/Asn NH] and the NOESY cross peak between Pro γ-H and Asn α-H (Figure 1).

Prenylagaramide B (**2**) was isolated as a colorless, amorphous powder. The molecular formula of **2** was deduced as $C_{49}H_{68}N_8O_{10}$ by HRFABMS and NMR spectral data. Amino acid analysis of the acid hydrolysate of **2** revealed the presence of Asp, Ile (not allo-Ile), Leu, Tyr, and Pro. Detailed analysis of the ^{1}H−^{1}H COSY, HMQC, HMBC, and NOESY spectra and a fragment ion peak in the positive FABMS [*m*/*z* 861, (M − prenyl + H)$^+$] indicated the presence of one residue each of Asn, Ile, Leu, Tyr, Ptyr and two of Pro (Figure 1). The two partial sequences, -Pro (1)-Ile-Asn-(**A**) and -Pro (2)-Ptyr-Leu-Tyr-(**B**), were determined by the HMBC correlations [Pro (1) CO/Ile NH, Ile CO/Asn NH] and [Pro (2) CO/Ptyr NH, Ptyr CO/Leu NH, Leu CO/Tyr NH], respectively. The partial sequence **A** was connected to **B** by the NOESY correlation between Pro (2) γ-H and Asn α-H. Although a Pro (1) γ-H/Tyr α-H correlation was not observed in the NOESY spectrum, Pro (1) α-H/Tyr α-H and Pro (1) α-H/Tyr 5-H correlations were observed. This suggested that Pro (1) in **A** was connected to Tyr in **B** (Figure 2). In support of this, the resonances in Pro (1) were shifted upfield, consistent with the anisotropic effect of an aromatic ring in Tyr. Thus, the amino acid sequence of **2** was determined as *cyclo*[-Ptyr-Leu-Tyr-Pro (1)-Ile-Asn-Pro (2)-] (Figure 1).

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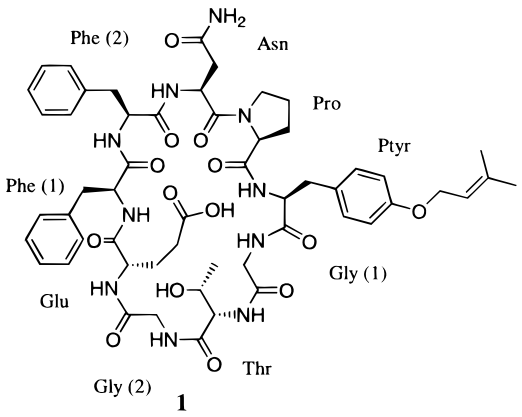

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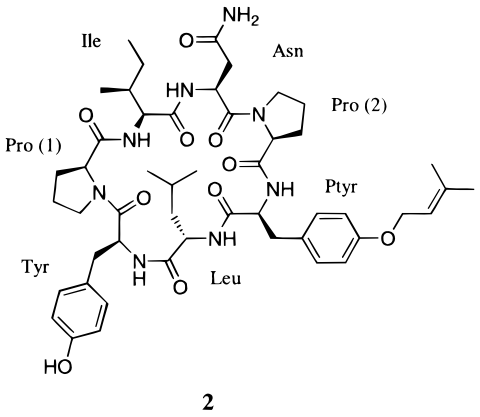

2

The absolute stereochemistry of the amino acid residues in **1** and **2** was determined by HPLC analysis of the acid hydrolysates derivatized with Marfey's reagent.[13] All the amino acids present in prenylagaramides had the L configuration.

Prenylagaramides A (**1**) and B (**2**) are unique cyclic peptides having a rare Ptyr unit. Some compounds with prenyl-linked unusual amino acids have been reported previously, such as oscillatorin[14] from *O. agardhii* (NIES-610) and kawaguchipeptin A[15] from *Microcystis aeruginosa* (NIES-88). However, to the best of our knowledge, compounds having a Ptyr unit like the prenylagaramides have not been described in any naturally occurring molecule until now. Compounds **1** and **2** showed no inhibitory activity against proteases (trypsin, thrombin, plasmin, chymotrypsin, elastase, leucine amino peptidase, and papain), no cytotoxicity against P-388 leukemia cells, and no antimicrobial activity against several Gram-positive and Gram-negative bacteria.

Experimental Section

General Experimental Procedures. Optical rotations were determined on a JASCO DIP-1000 digital polarimeter in CH_3OH. UV spectra were measured on a Hitachi 330 spectrometer. NMR spectra were recorded on a JEOL JNM-A600 or -A500 NMR spectrometer using DMSO-d_6 as solvent at 27 °C. ^1H and ^{13}C NMR chemical shifts were referenced to residual solvent peaks of DMSO-d_6 at δ_H 2.49 and δ_C 39.5.

Table 1. ^1H and ^{13}C NMR Data of Prenylagaramide A (**1**) in DMSO-d_6

	^1H (mult, *J* Hz)	^{13}C (mult)		^1H (mult, *J* Hz)	^{13}C (mult)
Ptyr			Phe (1)		
1		171.4 (s)	1		171.3 (s)
2	4.23 (ddd, 7.7, 3.9, 3.8)	56.0 (d)	2	3.98 (ddd, 8.5, 6.4, 4.3)	57.0 (d)
3	2.88 (dd, 13.7, 3.9)	35.1 (t)	3	2.65 (dd, 14.1, 6.4)	36.2 (t)
	2.97 (dd, 13.7, 3.8)			2.77 (dd, 14.1,8.5)	
4		130.0 (s)	4		136.8 (s)
5, 9	7.15 (d, 8.6)	129.7 (d)	5,9	6.95 (d, 6.8)	128.8 (d)
6, 8	6.82 (d, 8.6)	114.3 (d)	6,8	7.19 (dd, 8.5, 6.8)	128.2 (d)
7		157.0 (s)	7	7.17 (t, 8.5)	126.6 (d)
10	4.46 (d, 6.8)	64.2 (t)	NH	8.36 (d, 4.3)	
11	5.39 (d, 6.8)	120.1 (d)	Phe (2)		
12		136.9 (s)	1		170.4 (s)
13	1.67 (s)	18.0 (q)	2	4.22 (ddd, 13.9, 4.7, 3.9)	55.2 (d)
14	1.72 (s)	25.4 (q)	3	2.90 (dd, 14.0, 3.9)	36.2 (t)
NH	8.14 (d, 7.7)			3.06 (dd, 14.0, 4.7)	
Gly (1)			4		137.9 (s)
1		169.0 (s)	5,9	7.22 (d, 6.8)	128.8 (d)
2	3.67 (dd, 16.3, 5.6)	43.2 (t)	6,8	7.34 (dd, 7.3, 6.8)	128.4 (d)
	3.83 (dd, 16.3, 6.8)		7	7.25 (t, 7.3)	126.6 (d)
NH	7.67 (br)		NH	7.33 (m)	
Thr			Asn		
1		171.5 (s)	1		170.3 (s)
2	4.44 (m)	55.8 (d)	2	4.72 (ddd, 10.7, 8.5, 3.4)	47.5 (d)
3	4.40 (m)	67.8 (d)	3	2.40 (dd, 15.8, 3.4)	36.0 (t)
4	0.98 (d, 6.0)	19.2 (q)		2.74 (dd, 15.8, 10.7)	
NH	6.95 (d, 8.9)		4		173.3 (s)
Gly (2)			NH	7.10 (d, 8.5)	
1		169.7 (s)	NH_2	7.43 (br)	
2	3.71 (dd, 15.8, 5.6)	43.1 (t)		7.66 (br)	
	3.91 (dd, 15.8, 7.4)		Pro		
NH	7.50 (dd, 5.6, 7.4)		1		173.3 (s)
Glu			2	4.25 (dd, 8.6, 6.0)	62.4 (d)
1		171.6 (s)	3	1.36 (m)	29.0 (t)
2	4.42 (m)	51.6 (d)		1.93 (m)	
3	1.80 (m)	28.3 (t)	4	1.70 (m)	24.1 (t)
	2.15 (m)			1.82 (m)	
4	2.16 (m)	29.7 (t)	5	3.42 (m)	46.9 (t)
	2.32 (m)			3.62 (ddd, 12.8, 6.4, 3.4)	
5		173.8 (s)			
NH	7.33 (m)				

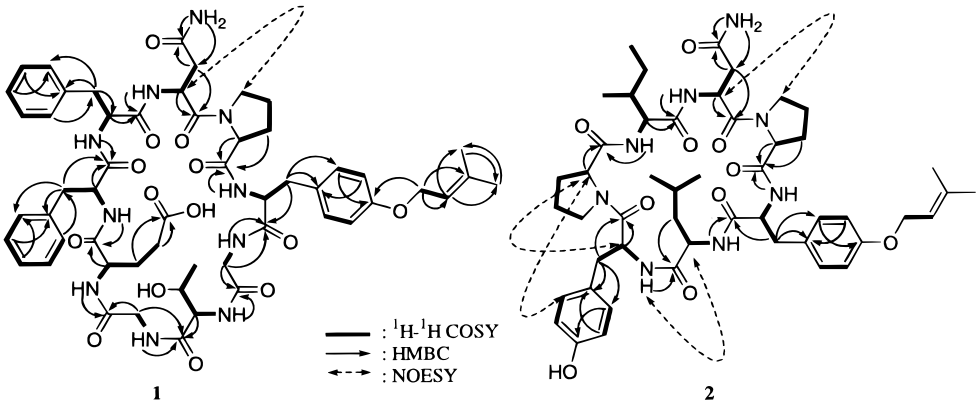

Figure 1. Selected ^{1}H-^{1}H COSY, HMBC, and NOESY correlations for **1** and **2**.

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

	^{1}H (mult, *J* Hz)	^{13}C (mult)		^{1}H (mult, *J* Hz)	^{13}C (mult)
Ptyr			Pro (1)		
1		170.35 (s)	1		169.9 (s)
2	4.24 (ddd, 9.4, 9.0, 3.4)	54.7 (d)	2	3.73 (m)	60.9 (d)
3	2.77 (m)	36.4 (t)	3	0.82 (m)	25.3 (t)
	3.05 (dd, 13.9. 3.4)			1.95 (m)	
4		129.9 (s)	4	1.27 (m)	21.3 (t)
5, 9	7.02 (d, 8.6)	130.3 (d)		1.61 (m)	
6, 8	6.79 (d, 8.6)	114.6 (d)	5	3.09 (m)	45.7 (t)
7		156.9 (s)		3.37 (m)	
10	4.45 (d, 6.4)	64.5 (t)	Ile		
11	5.38 (d, 6.4)	120.1 (d)	1		170.15 (s)
12		136.3 (s)	2	3.82 (t, 8.1)	60.5 (d)
13	1.67 (s)	18.2 (q)	3	1.94 (m)	36.2 (d)
14	1.72 (s)	25.7 (q)	4	1.03 (m)	24.8 (t)
NH	8.16 (d, 9.4)			1.45 (m)	
Leu			5	0.77 (t, 7.3)	11.0 (q)
1		171.10 (s)	6	0.85 (d, 6.8)	16.2 (q)
2	4.41 (ddd, 7.3, 4.7, 3.8)	51.2 (d)	NH	8.26 (d, 8.1)	
3	1.26 (m)	40.4 (t)	Asn		
	1.60 (ddd, 12.8, 10.3, 4.7)		1		170.25 (s)
4	1.42 (m)	24.8 (d)	2	4.65 (m)	49.7 (d)
5	0.91 (d, 6.8)	22.3 (q)	3	2.90 (m)	35.7 (t)
6	0.80 (d, 6.8)	24.9 (q)		2.97 (m)	
NH	7.41 (d, 7.3)		4		172.6 (s)
Tyr			NH	7.89 (d, 5.6)	
1		170.30 (s)	NH_2	7.33 (br)	
2	4.33 (ddd, 9.6, 6.4, 4.3)	53.8 (d)		7.88 (br)	
3	2.76 (m)	36.0 (t)	Pro (2)		
	2.90 (m)		1		170.20 (s)
4		126.0 (s)	2	4.06 (dd, 9.4, 7.2)	62.8 (d)
5, 9	6.97 (d, 9.0)	130.4 (d)	3	1.07 (m)	29.5 (t)
6, 8	6.68 (d, 9.0)	115.6 (d)		2.08 (m)	
7		156.1 (s)	4	1.73 (m)	25.4 (t)
OH	9.28 (s)			1.76 (m)	
NH	9.17 (d, 4.3)		5	3.40 (m)	48.2 (t)
				3.78 (m)	

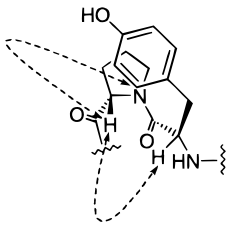

Figure 2. NOESY correlations between Tyr and Pro (1) in **2**.

FABMS were measured using glycerol as matrix on a JEOL JMS SX-102 mass spectrometer. Amino acid analysis was carried out with a Hitachi L-8500A amino acid analyzer.

Culture Conditions. Culture conditions of *Oscillatoria agardhii* (NIES-205) were the same as previously described.[10] *O. agardhii* (NIES-596) was obtained from the NIES-collection (Microbial Culture Collection, the National Institute for Environmental Studies, Japan) and cultured in 10-L glass bottles containing CT medium[16] with aeration (filtered, 0.3 mL/min, without CO_2). Cultures were illuminated with fluorescent lights on a 12L:12D cycle at an intensity of 250 μ/m^2·s 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. The freeze-dried cyanobacterium *O. agardhii* (NIES-205) (118.7 g from 350 L of culture) was extracted three times with 80% MeOH and once with 100% MeOH. The extracts were combined and concentrated to give a crude extract (28.9 g). The 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 (10.1 g) was subjected to ODS flash chromatography and eluted with aqueous MeOH, 100% MeOH, and CH_2Cl_2. The 60% MeOH fraction (617 mg) was purified by reversed-phase HPLC on a Cosmosil C_{18} MS column (linear gradient of MeCN

in H_2O containing 0.05% TFA, 20% to 60% in 50 min; flow rate 2.0 mL/min; UV detection at 210 nm) to yield prenylagaramide A (**1**, 71.2 mg, 0.059% yield, t_R 43 min).

The freeze-dried cyanobacterium *O.agardhii* (NIES-596) (135.9 g from 400 L of culture) was extracted twice with 80% MeOH and once with 100% MeOH. The extracts were combined and concentrated to give a crude extract (27.2 g). The 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 (4.41 g) was subjected to ODS flash chromatography and eluted with aqueous MeOH, 100% MeOH, and CH_2Cl_2. The 80% MeOH fraction (160 mg) was purified by reversed-phase HPLC on a CAPCELL PAK C_{18} UG column (linear gradient of MeCN in H_2O containing 0.05% TFA, 20% to 80% in 60 min; flow rate 2.0 mL/min; UV detection at 210 nm) to yield prenylagaramide B (**2**, 1.7 mg, 0.0012% yield, t_R 36 min).

Prenylagaramide A (1): colorless amorphous powder; $[\alpha]_D$ −22.1° (*c* 0.1, MeOH); UV (MeOH) λ_{max} 278 nm (ϵ 2800); ^1H and ^{13}C data, see Table 1; FABMS *m/z* 1081 $(M + H)^+$, 1079 $(M - H)^-$, 1013 $(M - \text{prenyl} + H)^+$; HRFABMS *m/z* 1081.4950 $(M + H)^+$ calcd for $C_{54}H_{69}N_{10}O_{14}$ (Δ −4.5 mmu).

Prenylagaramide B (2): colorless amorphous powder; $[\alpha]_D$ −43.7° (*c* 0.1, MeOH); UV (MeOH) λ_{max} 276 nm (ϵ 2400); ^1H and ^{13}C data, see Table 2; FABMS *m/z* 929 $(M + H)^+$, 927 $(M - H)^-$, 861 $(M - \text{prenyl} + H)^+$; HRFABMS *m/z* 929.5164 $(M + H)^+$ calcd for $C_{49}H_{69}N_8O_{10}$ (Δ +2.8 mmu).

Amino Acid Analysis. Compounds **1** and **2** (100 μg each) were dissolved in 6N 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. Retention times (min) in the amino acid analysis of **1**: Asp (10.40), Thr (15.06), Glu (20.42), Gly (33.41), Tyr (50.08), Phe (53.52), and Pro (31.84). Retention times (min) of **2**: Asp (10.66), Ile (45.76), Leu (47.46), Tyr (49.86), and Pro (32.08). Under this condition, Ile and allo-Ile were separated. Retention times (min): allo-Ile (43.68) and Ile (45.70).

HPLC Analysis of the Marfey Derivatives. To the acid hydrolysates of **1** and **2** (100 μg each), a 10% Me_2CO solution of 1-fluoro-2,4-bis(nitrophenyl)-5-L-alanine amide (L-FDAA, Marfey's reagent) in 1 M $NaHCO_3$ was added. The mixture was kept at 80 °C for 3 min followed by neutralization with 50 μL of 2N HCl. The reaction mixtures were dissolved in 50% MeCN and subjected to reversed-phase HPLC on a Cosmosil C_{18} MS (4.6 × 250 mm) (linear gradient of MeCN in H_2O containing 0.1% TFA, 0% to 60% in 60 min; flow rate 1.0 mL/min; UV detection at 340 nm). Retention times (min) of standard amino acids: L-Thr (36.5), L-allo-Thr (36.8), L-Asp (37.2), D-Asp (38.0), D-allo-Thr (38.2), L-Glu (38.8), D-Thr (39.2), D-Glu (40.0), L-Pro (41.6), D-Pro (42.8), L-Ile (50.2), L-Leu (50.8), L-Phe (52.6), D-Ile (54.4), D-Leu (54.8), D-Phe (55.2), L-Tyr (56.8), and D-Tyr (60.0). Retention times (min) of the amino acids of **1**: Thr (36.5), Asp (37.2), Glu (38.8), Pro (41.6), Phe (52.6), and Tyr (56.8). Retention times (min) of **2**: Asp (37.2), Pro (41.6), Ile (50.2), Leu (50.8), and Tyr (56.8).

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