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Lyngbyacyclamides A and B, novel cytotoxic peptides from marine cyanobacteria *Lyngbya* sp.

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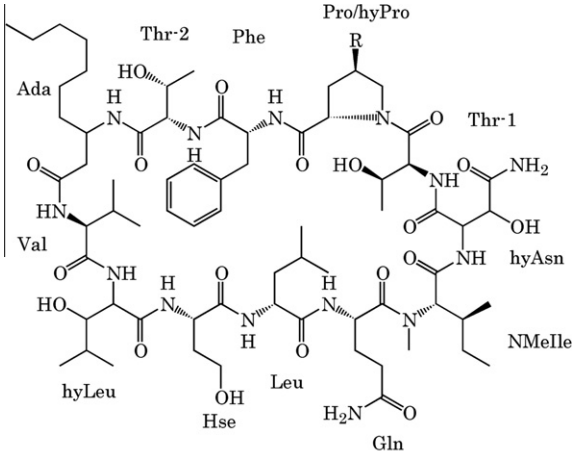
ABSTRACT

Lyngbyacyclamides A (**1**) and B (**2**), novel cyclic peptides, were isolated from marine cyanobacteria *Lyngbya* sp. collected in Okinawa, Japan. Their structures were determined by spectroscopic analyses and degradation studies. They moderately inhibited the growth of B16 mouse melanoma cells.

Cyanobacteria are photosynthetic prokaryotes that are widely distributed throughout marine and terrestrial environments. Members of the marine cyanobacteria genus *Lyngbya* are known to produce structurally interesting and biologically active secondary metabolites. Typically, linear/cyclic peptides and depsipeptides that include various nonproteinogenic amino acids are the major groups of these metabolites, which can exhibit potent cytotoxicity as represented by apratoxins,[1] bisebromoamide,[2] and laxaphycins.[3] We report here the isolation, structural determination, and biological activities of the novel cyclic peptide lyngbyacyclamides A (**1**) and B (**2**) (Fig. 1) from marine cyanobacteria *Lyngbya* sp.

Lyngbya sp. was collected at the Ishigaki Island, Okinawa Prefecture, Japan. The collected organism (500 g wet wt) was extracted with 80% aqueous ethanol (1 L) for 30 days. The extract was filtered, concentrated, and partitioned between EtOAc and water. The EtOAc-soluble material was further partitioned between 90% aqueous MeOH and hexane. The material obtained from the aqueous MeOH portion was subjected to fractionation using silica gel column chromatography (MeOH/$CHCl_3$), ODS column chromatography (MeOH/water), preparative TLC (silica gel, 15% MeOH/$CHCl_3$), and reversed-phase HPLC (Develosil ODS-HG-5, 40% aqueous acetonitrile) to give lyngbyacyclamides A (**1**) (44 mg) and B (**2**) (39 mg).

Lyngbyacyclamide A[4] was isolated as a colorless amorphous solid with a molecular formula of $C_{69}H_{114}N_{14}O_{19}$ on the basis of HRFABMS at *m/z* 1443.8462 $[M+H]^+$ (calcd for $C_{69}H_{115}N_{14}O_{19}$, 1443.8463). The ^1H and ^{13}C NMR spectra (Table 1) displayed characteristic peptide signals including α-protons of amino acid residues (δ_H 4.0–5.0), amide protons (δ_H 6.8–8.2), doublet methyl protons (δ_H 0.7–1.1), and 14 carbonyl carbons (δ_C 168–182). Automated amino acid analysis of **1** suggested the presence of the proteinogenic amino acids Phe, Leu, Val, Thr (×2), Pro, and Gln/Glu, as well as some nonproteinogenic amino acids. The structures of these amino acid residues were identified by 2D NMR



Lyngbyacyclamide A (**1**) R = H
Lyngbyacyclamide B (**2**) R = OH

Figure 1. Structures of lyngbyacyclamides A (**1**) and B (**2**).

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Table 1
^{1}H (500 MHz) and ^{13}C NMR (125 MHz) data for lyngbyacyclamides A (**1**) and B (**2**) in DMSO-d_6

Entry	Position	**1**		**2**	
		^{13}C	^{1}H multi (*J*, Hz)	^{13}C	^{1}H multi (*J*, Hz)
Phe	1	171.0 s[a]		171.0 s	
	2	53.9 d	4.67 m	53.9 d	4.66 m
	3	38.5 t	2.74 dd (9.2, 13.5), 3.01 dd (5.8, 13.5)	38.6 t	2.72 dd (9.8, 13.2), 2.97 m
	4	137.6 s		137.6 s	
	5, 9	128.1 d	7.21 m	128.1 d	7.21 m
	6, 8	129.5 d	7.21 m	129.5 d	7.21 m
	7	126.4 d	7.16 m	126.4 d	7.17 m
	NH		8.04 d (7.7)		8.12 d (7.4)
Pro/hyPro	1	171.4 s		171.4 s	
	2	59.5 d	4.29 m	58.7 d	4.35 m
	3	29.4 t	1.36 m, 1.82 m	37.8 t	1.41 m, 1.79 m
	4	24.2 t	1.63 m, 1.70 m	68.7 d	4.13 m
	5	47.4 t	3.53 m, 3.65 td (7.5, 9.4)	55.6 t	3.53 dd (4.0, 10.9), 3.61 dd (4.6, 10.9)
	OH				5.01 d (2.9)
Thr-1	1	168.6 s		168.8 s	
	2	56.1 d	4.35 m	56.2 d	4.39 m
	3	66.6 d	3.86 m	66.7 d	3.82 m
	4	19.1 q	1.04 d (6.0)	19.1 q	1.02 d (6.2)
	OH		4.94 d (4.3)		4.83 d (4.0)
	NH		7.07 d (6.9)		7.05 d (7.4)
hyAsn	1	169.5 s		169.4 s	
	2	55.9 d	4.58 dd (1.8, 8.3)	56.1 d	4.59 dd (1.7, 8.0)
	3	70.7 d	4.64 d (1.8)	70.7 d	4.34 m
	$CONH_2$	173.8 s	7.32 s	173.8 s	7.32 s
	OH		5.89 br		5.89 br
	NH		7.64 d (8.3)		7.72 d (7.5)
NMeIle	1	170.6 s		170.7 s	
	2	60.0 d	4.68 m	60.1 d	4.70 m
	3	31.7 d	1.90 m	31.9 d	1.89 m
	4	24.1 t	1.25 m	24.2 t	1.25 m
	5	10.6 q	0.75 t (7.2)	10.6 q	0.75 t (7.5)
	6	15.3 q	0.74 d (6.6)	15.3 q	0.74 d (7.4)
	N-Me	30.3 q	2.95 s	30.5 q	2.99 s
Gln	1	172.7 s		172.7 s	
	2	49.1 d	4.54 m	49.0 d	4.56 m
	3	26.2 t	1.71 m, 1.88 m	26.2 t	1.72 m, 1.91 m
	4	31.0 t	2.09 m	30.9 t	2.12 m
	$CONH_2$	175.1 s	6.85 br s, 7.25 br s	175.1 s	6.85 br s, 7.25 br s
	NH		7.95 d (6.6)		7.93 d (6.9)
Leu	1	172.0 s		172.0 s	
	2	51.8 d	4.18 m	51.7 d	4.22 m
	3	40.9 t	1.33 m, 1.47 m	41.1 t	1.36 m, 1.46 m
	4	24.1 d	1.58 m	24.2 d	1.55 m
	5	21.5 q	0.79 d (6.6)	21.5 q	0.79 d (6.3)
	6	23.2 q	0.84 d (6.9)	23.2 q	0.84 d (6.9)
	NH		7.84 d (7.8)		7.84 d (6.3)
Hse	1	171.5 s		171.5 s	
	2	50.7 d	4.30 m	50.7 d	4.32 m
	3	34.9 t	1.70 m, 1.85 m	34.9 t	1.73 m, 1.85 m
	4	57.6 t	3.30 m, 3.42 m	57.7 t	3.32 m, 3.42 m
	OH		4.47 t (5.2)		4.46 t (5.2)
	NH		7.80 d (7.2)		7.84 d (6.3)
hyLeu	1	171.2 s		171.3 s	
	2	55.7 d	4.33 m	55.7 d	4.33 m
	3	76.9 d	3.47 t (8.6)	76.8 d	3.48 t (7.5)
	4	30.8 d	1.57 m	30.7 d	1.59 m
	5	19.1 q	0.77 d (6.6)	19.1 q	0.77 d (6.9)
	6	19.4 q	0.91 d (6.6)	19.4 q	0.92 d (6.3)
	OH		4.68 s		4.68 s
	NH		7.96 d (7.2)		7.99 d (8.0)
Val	1	171.7 s		171.7 s	
	2	59.3 d	4.03 t (7.4)	59.3 d	4.04 t (7.4)
	3	29.8 d	1.96 m	29.8 d	1.97 m
	4	18.8 q	0.90 d (6.9)	18.8 q	0.90 d (6.9)
	5	19.3 q	0.85 d (6.6)	19.3 q	0.86 d (6.3)
	NH		8.10 d (7.2)		8.12 d (7.4)
Ada	1	171.2 s		171.4 s	
	2	40.5 t	2.25 dd (7.2, 14.0), 2.45 dd (6.0, 14.0)	40.3 t	2.26 dd (6.9, 13.8), 2.46 dd (5.7, 13.8)
	3	46.4 d	4.05 m	46.4 d	4.04 m
	4	34.1 t	1.30 m, 1.40 m	34.0 t	1.30 m, 1.43 m
	5	31.4 t	1.20 m	31.4 t	1.20 m
	6	29.0 t	1.20 m	29.0 t	1.20 m
	7	28.8 t	1.20 m	28.8 t	1.20 m

(*continued on next page*)

Table 1 (*continued*)

Entry	Position	1		2	
		^{13}C	^{1}H multi (*J*, Hz)	^{13}C	^{1}H multi (*J*, Hz)
	8	25.4 t	1.20 m	25.4 t	1.20 m
	9	22.3 t	1.20 m	22.3 t	1.20 m
	10	14.1 q	0.83 t (6.9)	14.1 q	0.83 t (6.9)
	NH		7.56 d (8.9)		7.62 d (8.6)
Thr-2	1	168.8 s		168.8 s	
	2	57.9 d	4.12 dd (3.7, 8.1)	58.0 d	4.15 m
	3	66.6 d	3.88 m	66.7 d	3.86 m
	4	14.1 q	0.82 d (6.9)	19.4 q	0.86 d (6.8)
	OH		4.84 d (4.9)		4.87 d (4.6)
	NH		7.92 d (8.1)		7.95 d (7.5)

[a] Multiplicity was based on the DEPT and HMQC spectra.

spectroscopic analyses. TOCSY and HMBC spectra enabled the construction of proteinogenic amino acids with Gln, and four partial structures of nonproteinogenic amino acids were assigned as *N*-methyl Ile (NMeIle), β-hydroxy leucine (hyLeu), β-hydroxy asparagine (hyAsn), and homoserine (Hse). The presence of a β-amino decanoic acid (Ada) was determined from TOCSY and HSQC-TOCSY data that showed a series of spin systems with α-CH_2 (δ_H 2.25, 2.45), β-CH (δ_H 4.05), β-NH (δ_H 7.56), γ-CH_2 (δ_H 1.30, 1.40), five internal methylenes (δ_H 1.20, δ_C 22.3, 25.4, 28.8, 29.0, 31.4), and a terminal methyl group (δ_H 0.83). The NMR assignments were supported by the LC/MS analysis for degradation products of **1** by HCl hydrolysis, which showed 11 $[M+H]^+$ ion peaks at *m/z* 116.0 (Pro), 118.1 (Val), 120.2 (Hse), 120.2 (Thr), 132.1 (Leu), 146.1 (NMeIle), 148.2 (hyLeu), 148.3 (Glu), 150.2 (hyAsp), 166.1 (Phe), and 188.2 (Ada). The HMBC correlations from amide protons, *N*-methyl protons, and α-protons to carbonyl carbons allowed us to connect each amino acid residue in two fragments, Leu-Gln-NMeIle-hyAsn-Thr-1 and Phe-Thr-2-Ada-Val-hyLeu-Hse (Fig. 2). These fragments were connected via Hse-Leu amide bond suggested by the cross-peak observed in the NOESY spectrum between the α-proton of Hse (δ_H 4.30) and the NH proton of Leu (δ_H 7.84). Finally, the NOESY cross-peaks between the α-proton of Pro (δ_H 4.30) and the amide proton of Phe (δ_H 8.04), and between the δ-protons of Pro (δ_H 3.53, 3.65) and the α-proton of Thr-1 (δ_H 4.35) allowed us to elucidate the Phe-Pro-Thr-1 connection and that the structure of **1** as a cyclic peptide consists of 12 amino acid residues.

Lyngbyacyclamide B[5] was isolated as a colorless amorphous solid with a molecular formula of $C_{69}H_{114}N_{14}O_{20}$ on the basis of HRFABMS *m/z* 1459.8407 $[M+H]^+$ (calcd for $C_{69}H_{115}N_{14}O_{20}$, 1459.8412). The ^{1}H and ^{13}C NMR spectra of **2** were similar to those of **1**. Significant differences were found with regard to the proline residue with a downfield shift at γ-CH (δ_H 4.13, δ_C 68.7). The COSY correlation of the γ-proton and hydroxy proton (δ_H 5.01) revealed the presence of 4-hydroxy proline (hyPro) in **2** instead of proline. The NOESY correlation of a hydroxy proton and an α-proton of hyPro revealed a *trans* relationship between the hydroxy group and the carbonyl group. TOCSY, HMBC, HSQC-TOCSY, and NOESY analyses led us to construct the same planar structures as those for **1** except for the proline.

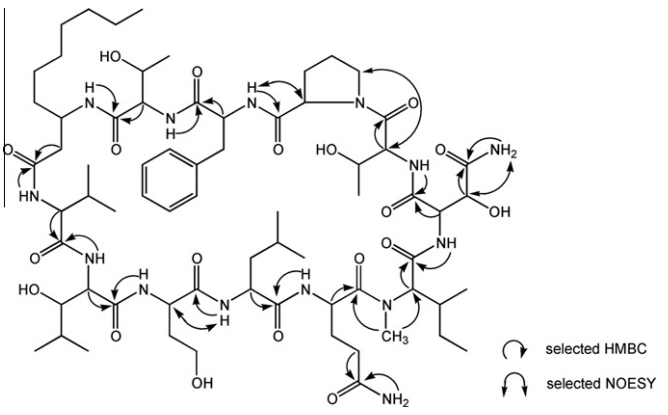

Figure 2. Partial structures of **1**, based on 2D NMR correlations.

The absolute configurations of the amino acid residues were determined by Marfey's analysis.[6] Hydrolysis (105 °C, 6 N HCl, 12 h) of **1** followed by the Marfey's derivatization and HPLC analyses[7,8] revealed that the configurations were L-Val, L-Thr, L-Pro, L-Gln, L-Hse, L-NMeIle, D-Phe, and D-Leu. The stereochemistries of **2** were determined according to the same protocol as that used for **1**. The HPLC analyses[9] showed the presence of L-hyPro and the same configurations for L-Val, L-Thr, L-Gln, L-Hse, L-NMeIle, D-Phe, and D-Leu as for **1**. The absolute configurations of hyLeu, hyAsn, and Ada residues are still being elucidated.

The biological activities of **1** and **2** were examined with regard to cytotoxicity against B16 mouse melanoma cells and toxicity against brine shrimp (genus *Artemia*). After incubation, **1** and **2** showed potency with an IC_{50} of 0.7 μM against the B16 cells. Meanwhile, they did not show definite toxicity at 70 μM against brine shrimp.

In summary, we isolated lyngbyacyclamides A (**1**) and B (**2**) from marine cyanobacteria *Lyngbya* sp. With the use of spectroscopic analyses and degradation reactions, **1** and **2** were determined to be novel cyclic peptides. Their structures resemble those of the natural products laxaphycin B[3] and lobocyclamide C.[10] The biological activities of **1** and **2** are quite interesting, since they showed significant cytotoxicity toward B16 cells but no toxicity toward brine shrimp.

Acknowledgments

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4. $[\alpha]_D^{20}$ −15.3 (*c* 0.38, MeOH); IR (neat): 3313, 1660, 1536, 1454 cm^{-1}; UV (MeOH): λ_{max} 214 nm; ^{1}H NMR (DMSO-d_6, 500 MHz), see Table 1; ^{13}C NMR (DMSO-d_6, 125 MHz), see Table 1; HRFABMS: [*m/z* (M+H)$^+$] found 1443.8462, calcd for $C_{69}H_{115}N_{14}O_{19}$ (Δ −0.1 mmu).
5. $[\alpha]_D^{20}$ −11.2 (*c* 0.23, MeOH); IR (neat): 3314, 1659, 1524, 1455 cm^{-1}; UV (MeOH): λ_{max} 209 nm; ^{1}H NMR (DMSO-d_6, 500 MHz), see Table 1; ^{13}C NMR (DMSO-d_6, 125 MHz), see Table 1; HRFABMS: [*m/z* (M+H)$^+$] found 1459.8407, calcd for $C_{69}H_{115}N_{14}O_{20}$ (Δ −0.5 mmu).

6. Marfey, P. *Carlsberg. Res. Commun.* **1984**, *49*, 591–596.
7. The retention times for the Marfey's derivatives of standards were: L-hyPro, 10.6 min; D-hyPro, 11.2 min; L-Glu, 35.8 min; L-Pro, 37.2 min; D-Pro, 41.5 min; D-Glu, 42.3 min; L-Val, 43.2 min; L-Phe, 44.1 min; D-Val, 48.4 min; L-Leu, 48.9 min; L-NMelle, 49.3 min; D-Phe, 50.8 min; D-Leu, 52.5 min, and D-NMelle, 56.6 min [Develosil ODS-MG-5, 4.6 × 250 mm, 1.0 mL/min, 340 nm, gradient of 20% MeOH/20 mM acetate buffer (pH 4.0) to MeOH over 60 min], and L-Hse, 33.8 min; L-Thr, 34.0 min; D-Hse, 35.1 min, and D-Thr, 37.9 min [Develosil ODS-MG-5, 4.6 × 250 mm, 0.8 mL/min, 340 nm, 30% MeOH/0.1% aqueous TFA].
8. The retention times for the Marfey's derivatives of **1** were: L-Glu, 36.0 min; L-Pro, 37.3 min; L-Val, 43.4 min; L-NMelle, 49.5 min; D-Phe, 51.0 min, and D-Leu, 52.7 min [Develosil ODS-MG-5, 4.6 × 250 mm, 1.0 mL/min, 340 nm, gradient of 20% MeOH/20 mM acetate buffer (pH 4.0) to MeOH over 60 min], and L-Hse, 33.7 min and L-Thr, 34.0 min [Develosil ODS-MG-5, 4.6 × 250 mm, 0.8 mL/min, 340 nm, 30% MeOH/0.1% aqueous TFA].
9. The retention times for the Marfey's derivatives of **2** were: L-hyPro, 10.6 min; L-Glu, 35.9 min; L-Val, 43.3 min; L-NMelle, 49.4 min; D-Phe, 50.9 min, and D-Leu, 52.6 min [Develosil ODS-MG-5, 4.6 × 250 mm, 1.0 mL/min, 340 nm, gradient of 20% MeOH/20 mM acetate buffer (pH 4.0) to MeOH over 60 min], and L-Hse, 33.7 min and L-Thr, 34.0 min [Develosil ODS-MG-5, 4.6 × 250 mm, 0.8 mL/min, 340 nm, 30% MeOH/0.1% aqueous TFA].
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