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Leucine aminopeptidase M inhibitors, cyanostatin A and B, isolated from cyanobacterial water blooms in Scotland

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Abstract

Two leucine aminopeptidase M inhibitors, cyanostatin A and B, were isolated from cyanobacterial water blooms at Loch Rescobie in Scotland, and specifically from a *Microcystis* species. Both inhibitors were lipopeptides containing 3-amino-2-hydroxydecanoic acid and weak inhibitors of protein phosphatase (PP2A). Both strongly inhibited the activity of leucine aminopeptidase M with IC_{50} values of 40 and 12 ng/ml, respectively.

Keywords: Cyanobacteria; Microcystis sp., Lipopeptide; Leucine aminopeptidase M inhibitor; Water bloom; Microginin; 3-Amino-2-hydroxydecanoic acid

1. Introduction

Some genera and species of cyanobacteria (blue-green algae), which form water blooms in eutrophic lakes and reservoirs, produce cyclic peptide hepatotoxins, named microcystins (Rinehart et al., 1994; Codd et al., 1999). Recently, cyanobacteria have been reported to produce lipopeptides that have various kinds of biological activities such as protease inhibitory, neurotoxic and ichthyotoxic properties (Ishida et al., 2000; Sano and Kayo, 1997; Kaya et al., 2002; Nogle et al., 2001). During investigations into toxins from cyanobacterial blooms of Loch Rescobie in Scotland, we have found two novel 3-amino-2-hydroxy-decanoic acid (Ahda)-containing lipopeptides (cyanostatin A **1** and B **2**) in microcystin and microcystin-containing extracts of the fractionated cyanobacteria. Here, we report on their structural elucidation and some bioactivities of the purified cyanostatins.

2. Results and discussion

The bloom material, initially collected in 2002, from Loch Rescobie consisted of a mixture of the cyanobacteria *Microcystis* and *Anabaena* species in approximately equal abundance, as visualized by light microscopy. When extracts of the lyophilised bloom were fractionated by reversed-phase HPLC (Mightysil RP-18, 20×250 mm, Kanto Chemicals, Japan; solvent; 60% MeOH in 50 mM phosphate buffer, pH 3.0), two peaks were obtained which showed inhibitory activity against leucine aminopeptidase M. Two inhibitors, named cyanostatin A (**1**) and B (**2**), were further purified by

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HPTLC with a normal phase system. Cyanostatins A (**1**) and B (**2**) were obtained at yields of 79.4 and 19.4 mg, respectively, from 40 g of the lyophilized material.

Additionally, while the 2002 bloom material consisted of a mixture of *Microcystis* and *Anabaena* species in approximately equal abundance, a more recent analysis of a November 2004 bloom sample from the same location, but consisting solely of *Microcystis*, also contained Cyanostatin A (**1**) and B (**2**), indicating *Microcystis* to have been a source of both lipopeptides in the 2002 material. However, at present, we cannot rule out the possibility that *Anabaena* from Loch Rescobie may also have been an additional cyanostatin-producer in 2002.

From the HR-FABMS spectrum of cyanostatin A (**1**), the molecular formula was deduced to be $C_{38}H_{57}N_5O_9$ ($[M + H]^+$ *m/z* 728.4196. Calc. for $C_{38}H_{58}N_5O_9$: Δ −3.8 mmu). The ^1H NMR spectrum of cyanostatin A (**1**) (Table 1) suggested that the compound was a lipopeptide. Analyses of 2D NMR spectra revealed that cyanostatin A (**1**) contained 4 amino acid units namely Ala, Val, *N*-Me-Tyr, and homotyrosine (Hty). The absolute configuration of these amino acid units were all determined to be of L configuration by Marfey's method (Marfey, 1984) and by chiral GC/MS analysis. The spectral data suggested the presence of an *N*- and *O*-substituted acyl side chain. From GC/MS spectra after acid hydrolysis following esterification and trifluoroacetylation, the structure of the unknown moiety was deduced to be 3-amino-2-hydroxydecanoic acid (Ahda). Other Ahda-containing peptides have been isolated from cyanobacteria *Microcystis* and *Planktothrix* (Okino et al., 1993; Sano and Kayo, 1997; Ishida et al., 2000). The Ahda was isolated after hydrolysis of

Table 1
^1H and ^{13}C NMR spectroscopic data for cyanostatin A (**1**) in CD_3OD

Position		^1H	*J* (Hz)	^{13}C	HMBC (C ⇒ H)
Ahda	1			173.4	Ahda-2,3, Ala-2
	2	4.20	(*d*, 3.4)	70.6	Ahda-3,4
	3	3.39	(*m*)	55.3	Ahda-4,5
	4	1.8	(*m*)	30.8	Ahda-6
		1.6	(*m*)		
	5	1.4	(*m*)	26.5	
	6	1.3	(*m*)	26.4	
	7	1.3	(*m*)	32.8	
	8	1.3	(*m*)	30.1	Ahda-6,9
	9	1.3	(*m*)	23.6	Ahda-7,8,10
	10	0.88	(*t*, 7.3)	14.4	Ahda-8,9
Ala	1			174.8	Ala-2,3, Val-2
	2	4.44	(*q*, 7.0)	49.8	Ala-3
	3	1.31	(*d*, 7.0)	18.2	Ala-2
Val	1			174.4	Val-2, *N*-Me
	2	4.38	(*d*, 6.7)	56.1	Val-4,4′
	3	1.3	(*m*)	30.4	Val-2,4,4′
	4	0.56	(*d*, 6.7)	19.9	Val-4′
	4′	0.73	(*d*, 6.7)	17.9	Val-4
N-Me-Tyr	1			170.6	*N*-Me-Tyr-2,3, Hty-2
	2	5.04	(*dd*, 4.9, 9.8)	64.1	*N*-Me, *N*-Me-Tyr-3
	3	3.24	(*dd*, 4.9, 14.3)	34.9	*N*-Me-Tyr-2,5,9
		2.87	(*dd*. 9.8, 14.3)		
	4			129.6	*N*-Me-Tyr-2,6,8
	5, 9	7.05	(*d*, 8.5)	131.5	
	6, 8	6.72	(*d*, 8.5)	116.6	
	7			157.6	*N*-Me-Tyr-5,9
	N-Me	2.92	(*s*)	30.4	*N*-Me-Tyr-2
Hty	1			178.5	Hty-2,3
	2	4.31	(*dd*, 4.6, 7.6)	56.4	Hty-4
	3	2.1	(*m*)	36.2	Hty-2
		1.9	(*m*)		
	4	2.5	(*m*)	32.5	Hty-2,3
	5			134.0	Hty-3,7,9
	6, 10	6.96	(*d*, 8.5)	130.3	
	7, 9	6.66	(*d*, 8.5)	116.2	
	8			156.4	Hty-6,10

cyanostatin A (**1**) according to the procedure of Ishida et al. (2000). The CD spectrum of the isolated Ahda from **1** showed a positive Cotton effect at 210 nm in 1 M HCl. The specific rotation of the isolated Ahda from **1** was a small negative value (−3°, *c* 0.033). In the ^{1}H NMR spectrum of the Ahda unit, the signal of H-3 appeared at 3.1 ppm. Matsuura synthesized 4 stereoisomers of Ahda, and reported the spectral data of these isomers (Matsuura et al., 1994). In comparison with the CD, ORD and NMR spectroscopic data, the absolute configuration of the Ahda unit in **1** was deduced to be 2*S*, 3*R*. The configuration of Ahda from **1** was the same as the Ahda moiety in microginin (Okino et al., 1993), an inhibitor of angiotensin-converting enzyme.

Cyanostatin B (**2**) was also a lipopeptide as suggested from analysis of the ^{1}H NMR spectrum. The molecular formula of cyanostatin B (**2**) was elucidated to be $C_{40}H_{59}N_5O_9$ ($[M + H]^+$ *m/z* 754.4364. Calc. for $C_{40}H_{60}N_5O_9$: Δ +2.6 mmu) from the HR-FABMS spectrum. Analyses of the 2D NMR spectra deduced the amino acid units namely *N*-Me-Ile, Pro, and two Tyr. The configurations of these amino acid units were determined to be L by Marfey's method and a chiral GC/MS method. Cyanostatin B (**2**) also contained an Ahda moiety, as in cyanostatin A, and its absolute configuration was also assigned to be 2*S*,3*R* from its ^{1}H NMR spectrum, CD and ORD values. The connectivities of these amino acids and the Ahda moiety in cyanostatins A (**1**) and B (**2**) were elucidated from 2D NMR spectra (HMBC) between carbonyl carbons and α-protons of amino acid units (Tables 1 and 2). The structures of cyanostatin A (**1**) and B (**2**) are shown in Fig. 1.

Table 2
^{1}H and ^{13}C NMR spectroscopic data for cyanostatin B (**2**) in CD_3OD

Position		^{1}H	*J* (Hz)	^{13}C	HMBC (C ⇒ H)
Ahda	1			172.9	Ahda-2,3, Tyr1-2
	2	4.10	(*d*, 4.0)	71.3	Ahda-3
	3	3.3	(*m*)	55.1	
	4	1.7	(*m*)	30.7	Ahda-2,3
		1.5	(*m*)		
	5	1.4	(*m*)	26.5	Ahda-3,4
	6	1.3	(*m*)	30.1	Ahda-5
	7	1.3	(*m*)	30.4	Ahda-6
	8	1.3	(*m*)	32.9	Ahda-6,7,10
	9	1.3	(*m*)	23.6	Ahda-8,10
	10	0.86	(*t*, 7.0)	14.4	Ahda-8,9
Tyr1	1			173.9	Tyr1-2,3, *N*-Me
	2	5.04	(*dd*, 6.4, 6.7)	52.1	Tyr1-3
	3	3.0	(*m*)	38.3	Tyr1-2,5,9
	4			127.9	Tyr1-3,6,8
	5, 9	6.97	(*d*, 8.5)	131.3	Tyr1-3
	6, 8	6.64	(*d*, 8.5)	116.4	
	7			157.8	Tyr1-5,9
N-Me-Ile	1			170.5	*N*-Me-Ile-2, Pro-2
	2	5.02	(*d*, 11.0)	59.8	*N*-Me-Ile-6
	3	2.0	(*m*)	34.4	*N*-Me-Ile-2,5
	4	1.8	(*m*)	25.4	*N*-Me-Ile-2,5,6
	5	0.78	(*t*, 7.3)	11.0	
	6	0.81	(*d*, 6.4)	15.9	
	N-Me	2.86	(*s*)	31.1	*N*-Me-Ile-2
Pro	1			173.0	Pro-2,3, Tyr2-2
	2	4.29	(*dd*, 3.7, 8.5)	62.0	Pro-3,4,5
	3	2.0	(*m*)	30.4	Pro-2,4,5
	4	1.8	(*m*)	25.4	Pro-2,5
	5	3.6	(*m*)	48.7	Pro-2
		3.4	(*m*)		
Tyr2	1			177.3	Tyr2-2,3
	2	4.33	(*dd*, 5.5, 5.8)	57.3	Tyr2-3
	3	3.04	(*dd*, 5.5, 13.7)	38.1	Tyr2-2,5,9
		3.0	(*m*)		
	4			129.9	Tyr2-2
	5, 9	7.02	(*d*, 8.5)	131.8	Try2-3
	6, 8	6.97	(*d*, 8.5)	115.9	
	7			156.9	Tyr2-5,9

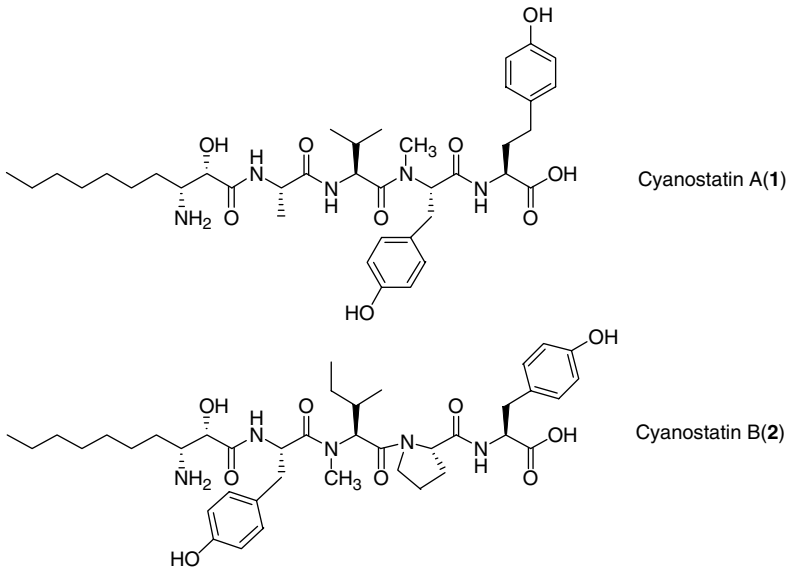

Fig. 1. Chemical strucures of cyanostatin A (**1**) and cyanostatin B (**2**) isolated from cyanobacterial bloom material.

When the purified cyanostatins were assayed for toxicity to juveniles (nauplii) of the brine shrimp (*Artemia salina*) in 24-h exposures, no toxicity was apparent at the highest concentrations tested (cyanostatin A, 73 μg/ml; cyanostatin B, 75 μg/ml), in comparison to an IC_{50} of 10 μg/ml for purified microcystin-LR (Metcalf et al., 2002).

Cyanostatins A (**1**) and B (**2**) strongly inhibited the activity of leucine aminopeptidase M with IC_{50} values at 40 and 12 ng/ml, respectively.

Cyanostatins A (**1**) and B (**2**) also inhibited the activity of angiotensin-converting enzyme (ACE) with IC_{50} values at 110 and 130 μg/ml, respectively. According to Ishida's report (Ishida et al., 2000), the 2*S* configuration of the Ahda unit is necessary for the inhibitory activity of cyanobacterial microginins (structurally related to the cyanostatins) against aminopeptidase M. The configurations of the Ahda unit in cyanostatins A (**1**) and B (**2**) were also 2*S*. The cyanostatins (**1** and **2**) inhibited the activity of aminopeptidase M with a potency between 50 and 100 times stronger than that of the microginins (see Ishida et al., 2000). However, the comparison of these inhibitory activities should be viewed cautiously, since the activities may be influenced by differences in the assay methods.

According to Ishida et al. (2000) the C-terminal structure (*N*-Me-Tyr-Tyr) of microginin plays an important role for the inhibition of ACE activity. Cyanostatin A (**1**) also inhibited ACE activity, but at approximately one-tenth of the potency of microginin. This difference in inhibitory activity was probably due to the difference in the amino acids around the C-terminals of the inhibitors when complexed to ACE. The amino acid sequence at the around C-terminal of microginin was *N*-Me-Tyr-Tyr, whereas that of cyanostatin A (**1**) was *N*-Me-Tyr-Hty. The difference in the amino acid sequences suggested that only one extra methylene unit of the C-terminal amino acid of cyanostatin A may weaken inhibitory activity against angiotensin-converting enzyme.

3. Experimental

3.1. General experimental procedures

NMR spectra were recorded on a JEOL JNM A-500 spectrometer (500 MHz). ^{1}H and ^{13}C chemical shifts were referenced to solvent ($CD^{3}OD$) peak (^{1}H, δ 3.30 ppm; ^{13}C, δ 49.0 ppm). Homonuclear ^{1}H connectivities were determined with COSY and HOHAHA experiments and heteronuclear ^{1}H–^{13}C connectivities were determined by HMQC and HMBC experiments. Low and high resolution FABMS spectra were performed with a JEOL JMS-700 spectrometer using glycerol as a matrix. Specific rotations were obtained on a Horiba SEPA-300 polarimeter. CD spectra were recorded on a JASCO J720 spectropolarimeter.

3.2. Cyanobacterial bloom samples

Cyanobacterial blooms were collected in September, 2002 from the surface water of Loch Rescobie, near Forfar in Scotland. The collected material was frozen at −20 °C immediately, lyophilized and the resulting cell powder (Sample ID: 120902_108) stored in the University of Dundee cyanobacterial archive at −20 °C.

3.3. Extraction and isolation

Lyophilized cyanobacterial cells (40 g dry wt) were extracted with 5% HOAc in H_2O, then MeOH. The extracts were combined and evaporated to dryness. The residue was resuspended in HOAc–H_2O (5:95) and passed through solid phase extraction cartridges (Sep-Pak C18, Waters, USA.) The cartridges were washed with MeOH–H_2O (1:4), then eluted with MeOH–H_2O (9:1). The eluate was evaporated in vacuo, and applied onto a semi-preparative HPLC column (Mightysil RP-18, 20 × 250 mm, Kanto Chemicals, Japan). Cyanostatins A (**1**) and B (**2**) were purified with a 4:6 solution of MeOH in 50 mM sodium phosphate buffer (pH 3.0), following HPTLC (Merck, silica gel $60F_{254}$; solvent, $CHCl_3$–MeOH–H_2O (6:4:1)).

Cyanostatin A (**1**): 79.4 mg (0.2% yield); colourless amorphous solid; λ_{max}^{MeOH} nm (log ε): 279 (3.5); $[\alpha]_D^{26\,°C}$ −70.5° (MeOH, *c* 0.67); FABMS (pos. glycerol) *m/z* 728 $[M + H]^+$; HRFABMS (pos. glycerol) *m/z* 728.4196 $[M + H]^+$ (Calc. for $C_{38}H_{58}N_5O_9$; Δ −3.8 mmu); for ^1H and ^{13}C NMR spectra, see Table 1.

Cyanostatin B (**2**): 19.4 mg (0.05% yield); colourless amorphous solid; λ_{max}^{MeOH} nm (log ε): 279 (3.4); $[\alpha]_D^{26\,°C}$ −65.6° (MeOH, *c* 0.17); FABMS (pos. glycerol) *m/z* 754 $[M + H]^+$; HRFABMS (pos. glycerol) *m/z* 754.4364 $[M + H]^+$ (Calc. for $C_{40}H_{60}N_5O_9$; Δ +2.6 mmu); for ^1H and ^{13}C NMR: spectra, see Table 2.

3.4. Hydrolysis and amino acid analysis

Cyanostatins A (**1**) and B (**2**) (100 μg) in 6 M HCl were heated at 110 °C for 20 h, then the amino acid hydrolysates were treated with 6 M HCl (0.2 ml) and iPrOH (0.2 ml) at 110 °C for 1 h. The mixtures were evaporated to dryness under a gentle stream of N_2. The residues were then treated with trifluoroacetic anhydride (100 μl) and CH_2Cl_2 (100 μl) at 100 °C for 5 min and evaporated under a N_2 stream. The CH_2Cl_2 solutions were then analyzed by GC/MS using an Chirasil-L-Val capillary column (25 m × 0.25 mm i.d.) under the following conditions: column temperature 40 to 200 °C at 8 °C/min.

3.5. Isolation of Ahda from cyanostatins

Cyanostatins A (**1**) and B (**2**) (10 and 5 mg, respectively), were hydrolyzed with 6 M HCl at 110 °C for 20 h. After dilution to about 10 ml with H_2O, the mixtures were passed through OASIS HLB cartridges (Waters, 60 mg). The cartridges were washed with H_2O, then eluted with MeOH–H_2O (9:1). The eluates were concentrated and applied on a HPLC (column: Zorbax Eclipse XDB C-18, 2.1 × 150 mm; solvent: 50% MeOH in 50 mM phosphate buffer, pH 3.0; flow rate: 0.2 ml/min). The peaks around 7 min were collected, and the fractions were passed through OASIS HLB cartridges, washed with 5% HOAc, then eluted with 90% MeOH. Ahda yields of 1.5 and 0.7 mg were obtained from 10 and 5 mg of cyanostatins A (**1**) and B (**2**), respectively.

Ahda from cyanostatin A (**1**): Colourless amorphous solid; $[\alpha]_D^{23\,°C}$ −3.0 (MeOH, *c* 0.033); $[\theta]_{210}$ 2×10^3 in 1 M HCl; ^1H NMR spectral data (500 MHz, DMSO-*d*6) δ 3.55 (1H, *d*, *J* = 3.3 Hz, H-2), 3.1 (1H, *m*, H-3), 1.6 (1H, *m*, H-4a), 1.4 (1H, *m*, H-4b), 1.2–1.3 (10H, *m*, H-5,6,7,8,9), 0.85 (3H, *t*, *J* = 6.9 Hz).

Ahda from cyanostatin B (**2**): Colourless amorphous solid; $[\alpha]_D^{23\,°C}$ −1.9 (MeOH, *c* 0.028); $[\theta]_{210}$ 2×10^3 in 1 M HCl; ^1H NMR spectral data (500 MHz, DMSO-*d*6) δ 3.55 (1H, *d*, *J* = 3.7 Hz, H-2), 3.1 (1H, *m*, H-3), 1.6 (1H, *m*, H-4a), 1.4 (1H, *m*, H-4b), 1.2–1.3 (10H, *m*, H-5,6,7,8,9), 0.85 (3H, *t*, *J* = 6.9 Hz).

3.6. Brine shrimp (*Artemia salina*) bioassay

This was performed according to Metcalf et al. (2002) with mortalities recorded after exposure for 24 h.

3.7. Leucine aminopepidase M inhibition assay

Inhibitory activities of cyanostatins were performed by a modification of the method of Ishida et al. (2000). The reaction mixture contained 0.1 M Tris–HCl buffer (pH 7.0), 1.4 mU leucine aminopeptidase (Sigma, porcine kidney microsomal), test compounds, and 0.4 mM L-leucine *p*-nitroanilide in 100 μl. The reaction was monitored at 410 nm for 30 min at 37 °C using a microplate reader.

3.8. Angiotensin-converting enzyme (ACE) inhibition assay

Inhibition assays were determined by a modification of the method of Matsas et al. (1984). The reaction mixture contained 0.1 M Tris–HCl buffer (pH 8.3), 300 mM NaCl, 10 μM $ZnCl_2$, 1 mU Angiotensin-converting enzyme (Sigma, porcine kidney), test compounds and 1 mM Hip-His-Leu in 100 μl. The reaction mixture was incubated for 30 min at 37 °C. After 30 min, 30 μl of HOAc was added to the reaction mixture and the mixture was centrifuged for 5 min at 13 000 rpm. The resulting hippuric acid was measured by HPLC (column: Agilent, Zorbax Eclipse XDB C-18, 2.1 × 150 mm, detect at 230 nm).

3.9. Protein phosphatase (PP2A) inhibition assay

Protein phosphatase inhibition assays were performed according to Mountfort et al. (1999) with modifications using purified protein phosphatase 2A (Upstate Biotechnology, NY, USA). Briefly, 2 μl of enzyme was diluted in 1 ml enzyme dilution buffer

according to the supplier's instructions. Cyanostatins A (**1**) and B (**2**) were diluted with Milli Q water and 10 μl of sample was added to 10 μl diluted enzyme in a 96-well microtitre plate, followed by 50 μl reaction buffer (50 mM Tris, pH 7 and 0.1 mM $CaCl_2$). Plates were incubated at room temperature for 2 min followed by the addition of 120 μl substrate buffer (50 mM Tris, pH 7, 1.67 mM $NiCl_2$, 41.67 μg BSA and 20 mg *p*-nitrophenylphosphate pre-warmed to 37 °C) and then absorbance read at 2 min intervals for 1 h at 37 °C in a VERSAMAX tuneable microplate reader at 405 nm. Results were compared to microcystin-LR standards in Milli Q water (0.1–50 μg/l) prepared and analysed in the same way as the samples.

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