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A Dhb-microcystin from the filamentous cyanobacterium *Planktothrix rubescens*

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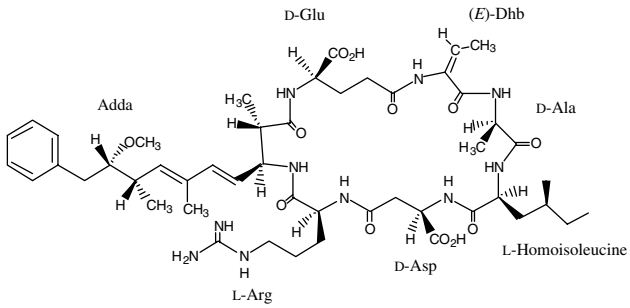
Abstract

A Dhb-microcystin variant was isolated from the filamentous cyanobacterium *Planktothrix rubescens*. Its structure was elucidated as (*E*)-Dhb-microcystin-HilR ([D-Asp[3], (*E*)-Dhb[7]]microcystin-HilR) on the basis of spectral data and amino acid analysis after acid hydrolysis.

Keywords: *Planktothrix rubescens*; Cyanobacterium; Microcystin; Dhb-microcystin; Hepatotoxin; Cyanotoxin

1. Introduction

Several genera and species of cyanobacteria (blue–green algae) which form waterblooms in eutrophic lakes and reservoirs can produce cyclic heptapeptide hepatotoxins, named microcystins (Rinehart et al., 1994; Codd et al., 1999). Their general structure is cyclo(-D-Ala-X-D-MeAsp-Z-Adda-D-Glu-Mdha-), where *X* and *Z* are variable L-amino acids, D-MeAsp is D-*erythro*-β-methyl-aspartic acid, Mdha is *N*-methyldehydroalanine, and Adda is (2*S*, 3*S*, 8*S*, 9*S*)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4(*E*),6(*E*)-dienoic acid. In previous studies on cyanobacterial hepatotoxins, eight Dhb-microcystins, which contained dehydrobutyrine (Dhb) unit instead of a Dha unit, were isolated from filamentous cyanobacteria *Planktothrix* and *Nostoc* (Sano and Kaya, 1995, 1998; Beattie et al., 1998; Sano et al., 1998). Recently, it was reported that there are considerable differences in the reactivity with sulfhydryl groups such as glutathione between Dhb-microcystins and Dha-containing microcystins (Kaya et al., 2001). This means that the in vivo metabolism of Dhb-microcystins may be different from that of Dha-containing microcystins. The Dhb-containing cyclic peptide toxin, nodularin, was reported as not only a tumor promoter but also a carcinogen. From the structural similarity of Dhb-microcystins with nodularin, Dhb-microcystins are possibly also carcinogenic. Herein we report the isolation and structural elucidation of a new (*E*)-Dhb-microcystin (**1**) from *Planktothrix rubescens* which contained L-homoisoleucine at position X.



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2. Results and discussion

HPLC analysis of the microcystin fraction of *P. rubescens* CCAP 1459/14 revealed one unknown

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microcystin peak which was eluted after (*E*)-Dhb-microcystin-LR using photodiode array detection. The unknown microcystin was purified by preparative reversed-phase HPLC (Mightysil RP-18, 25 cm × 10 mm i.d.; solvent: 60% MeOH in 50 mM phosphate buffer, pH 3.0; flow rate: 4 ml/min). After further purification with normal phase HPTLC, **1** was isolated as a colourless amorphous solid in 0.004% yield. The pseudomolecular ion ($[M+H]^+$) was observed at *m/z* 995 on FABMS spectrum using glycerol as matrix. From the high resolution FABMS spectrum, the molecular formula of **1** was elucidated as $C_{49}H_{74}N_{10}O_{12}$ ($[M+H]^+$ *m/z* 995.5553, calcd for $C_{49}H_{75}N_{10}O_{12}$, Δ −1.2 mmu). The pseudomolecular ion and the molecular formula of **1** are identical with those of microcystin-LR. It is difficult to distinguish each other with MS spectra even using MS/MS. The ^1H and ^{13}C NMR spectral data of **1** (Table 1) suggested that **1** was a peptide. Amino acid analysis (Sano and Kaya, 1998; Marfey, 1984) and extensive analysis of 2D NMR spectra of **1** revealed D-Ala, D-Asp, L-Arg, Adda, Glu, and Dhb units. In the ^1H NMR spectrum of **1**, the methine proton of Dhb unit appeared at 5.75 ppm as a quartet, and from its chemical shift, the geometrical configuration of the Dhb unit in **1** was assigned as *E* (Sano et al., 1998). The unknown amino acid unit in **1** contained two methyl protons (triplet at 0.88 ppm and doublet at 0.91 ppm), two methylene protons (2H at 1.81 ppm, and 1.41 and 1.10 ppm as geminal protons), and two methine protons (4.32 and 1.58 ppm). From 2D NMR spectral analysis, such as COSY and HOHAHA, this amino acid unit was deduced to be homoisoleucine (Hil). Its stereochemistry was determined to be (2*S*, 4*S*) by synthesis of four stereoisomers and chiral GC analysis according to Namikoshi et al. (1995). The amino acid sequence of **1** was determined from analysis of the HMBC spectrum. From these data, **1** was assigned as (*E*)-Dhb-microcystin-HilR ([D-Asp^3, (*E*)-Dhb^7]microcystin-HilR), and it was found that it strongly inhibited protein phosphatase 2A (PP2A) activity with an IC_{50} value of 0.3 ng/ml, this IC_{50} value was the same as that of microcystin-LR.

The molecular formula of **1** is identical with that of microcystin-LR, and it cannot be distinguished from microcystin-LR using MS spectral analysis even if with MS/MS or CID analyses, i.e. because the degraded fragments, which contain both Asp and Hil, show the same mass number as those of microcystin-LR. Only, the retention time of **1** by HPLC analysis differed from that of microcystin-LR. Therefore, HPLC analysis, in addition to NMR was important for identification of this microcystin variant.

Table 1
^1H and ^{13}C NMR spectral data for Dhb-microcystin-HilR (**1**) in CD_3OD

Position		^1H	*J* (Hz)	^{13}C
Ala	1			175.1
	2	4.57	q (7.5)	49.7
	3	1.29	d (7.5)	17.3
Hil	1			174.9
	2	4.32	dd (10.5, 5.5)	55.2
	3	1.81	m	39.0
	4	1.58	m	32.2
	5	1.45	m	28.9
		1.10	m	
	6	0.88	t (8.0)	11.2
	7	0.91	d (7.0)	19.8
Asp	1			176.7
	2	4.68		52.3
	3	2.88	m	39.3
		2.28	m	
	4			175.0
Arg	1			172.2
	2	4.42	dd (9.0, 4.0)	53.0
	3	2.00	m	29.2
		1.53	m	
	4	1.51	m	26.5
	5	3.13	m	42.0
	7			159.8
Adda	1			177.2
	2	3.04	m	45.1
	3	4.54	t (10.0)	56.5
	4	5.48	dd (15.5, 9.0)	126.8
	5	6.23	d (16.0)	138.7
	6			134.0
	7	5.41	d (10.0)	136.9
	8	2.58	m	37.7
	9	3.26	m	88.4
	10	2.81	dd (14.0, 5.0)	39.0
		2.68	dd (14.0, 7.0)	
	11	1.05	d (7.0)	16.1
	12	1.61	s	12.9
	13	1.00	d (7.0)	16.5
	14	3.24	s	58.7
	15			140.5
	16, 20	7.20	m	130.5
	17, 19	7.25	m	129.2
	18	7.18	m	127.1
Glu	1			176.3
	2	4.30		54.6
	3	2.05	m	28.6
	4	2.47	m	34.2
		2.22	m	
	5			179.8
Dhb	1			166.4
	2			131.8
	3	5.75	q (7.5)	124.5
	4	1.90	d (7.5)	13.5

3. Experimental

3.1. General experimental procedures

NMR spectra were recorded on a JEOL JNM A-500 spectrometer (500 MHz). ^1H and ^{13}C chemical shifts

were referenced to solvent (CD_3OD) peak (^1H, δ 3.30 ppm; ^{13}C, δ 49.0 ppm). Homonuclear ^1H connectivities were determined with COSY and HOHAHA experiments, and heteronuclear ^1H–^{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 rotation was obtained on a Horiba SEPA-300 polarimeter.

3.2. Culture conditions

P. rubescens CCAP 1459/14 was kindly provided by Dr. John G. Day of CCAP, Scotland. *P. rubescens* was grown in CT medium as described earlier (Sano and Kaya, 1998). Cells were harvested by continuous flow centrifugation at 10 000*g*, with collected cells frozen, lyophilised and stored at −20 °C until needed.

3.3. Extraction and isolation

The lyophilized cells (41.1 g) were extracted with aq. 5% HOAC solution, then with MeOH. The supernatants were combined and evaporated in vacuo. The extract was suspended with aq. 5% HOAC solution. The suspension was centrifuged, and the supernatant was passed through Sep-Pak ODS cartridges. The cartridges were washed with aq. MeOH–H_2O (1:4) and eluted with aq. MeOH–H_2O (4:1). The MeOH–H_2O (4:1) fraction was separated by reversed-phase HPLC (Mightysil RP-18, 25 cm × 10 mm i.d., flow rate; 4 ml/min) with 60% MeOH (3:2) in 50 mM phosphate buffer (pH 3.0). Further purification with HPTLC (Silicagel 60 F_{254}, 0.25 mm, 10 × 25 cm, Merck, developed with $CHCl_3$:MeOH:H_2O (6:4:1)) yielded (*E*)-Dhb-microcystin-HilR ([D-Asp^3, (*E*)-Dhb^7]microcystin-HilR, **1**, 1.5 mg, 0.004%) as a colourless amorphous solid: λ_{max}^{MeOH} nm (log ε): 239 (4.5); $[\alpha]_D^{25}$ −65° (MeOH; *c* 0.15); FABMS (pos. glycerol) *m/z* 995 $[M+H]^+$; HRFABMS (pos. glycerol) *m/z* 995.5553 $[M+H]^+$ (calcd for $C_{49}H_{75}N_{10}O_{12}$, 995.5565). ^1H and ^{13}C NMR: Table 1.

3.4. Hydrolysis and amino acid analysis

Dhb-microcystin (100 μg) in 6 M HCl was heated at 110 °C for 20 h, then the amino acid hydrolysate was treated with 6 M HCl (0.2 ml) and iPrOH (0.2 ml) at 110 °C for 1 h. The mixture was evaporated to dryness under a gentle stream of N_2. The residue was treated with trifluoroacetic anhydride (100 μl) and CH_2Cl_2 (100 μl) at 100 °C for 5 min and evaporated with a N_2 stream, the CH_2Cl_2 solution was 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 40–200 °C at 8 °C/min.

3.5. Protein phosphatase 2A inhibition assay

Inhibition activity of **1** was performed according to Fontal's method (Fontal et al., 1999) using a microplate reader. The substrate was 6,8-difluoro-4-methylunbelliferyl phosphate (10 μM), and the enzymatic reaction was performed at pH 7.0. The microplate was incubated at 37 °C, and the reaction was monitored for 30 min using a microplate reader (Fluostar, BMG labtechnologies, Germany) at ex. 355 nm and em. 460 nm.

3.6. Synthesis and analysis of homoisoleucine isomers

The mixtures of (2*S*, 4*S*)- and (2*R*, 4*S*)-homoisoleucine were synthesized from *S*-(+)-1-bromo-2-methylbutane coupled with diethyl acetamidomalonate following decarbonylation. The mixtures of all four isomers were synthesized from (±)-1-bromo-2-methylbutane in the same way as (4*S*)-homoisoleucine (Namikoshi et al., 1995). The retention times of four isomers were found to be 10.72 min (2*R*, 4*S*), 10.86 min (2*R*, 4*R*), 11.49 min (2*S*, 4*S*), and 11.69 min (2*S*, 4*R*) by a chiral GC analysis in the same way as the amino acid analysis. The homoisoleucine derivative from **1** was coeluted with the third peak (2*S*, 4*S*).

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