Two (*Z*)-Dehydrobutyrine-Containing Microcystins from a Hepatotoxic Bloom of *Oscillatoria agardhii* from Soulseat Loch, Scotland

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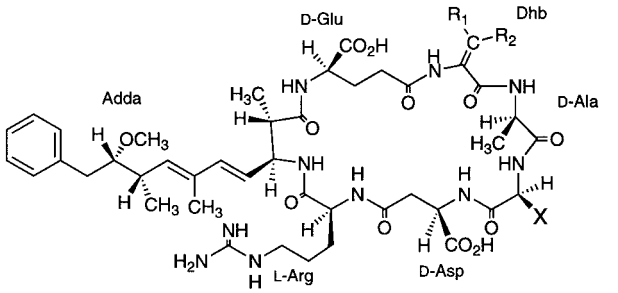
Two (*Z*)-dehydrobutyrine(Dhb)-containing microcystins, [D-Asp^3, (*Z*)-Dhb^7]microcystin-HtyR (**1**) and [D-Asp^3, (*Z*)-Dhb^7]microcystin-LR (**2**), were isolated from a hepatotoxic bloom of the cyanobacterium *Oscillatoria agardhii* from a freshwater lake in Scotland. The geometrical structure of the Dhb units in the microcystins was determined as *Z* on the basis of NOE and ROESY experiments.

Several of the genera and species of cyanobacteria (blue-green algae) that form massive growths (blooms and scums) in eutrophic lakes and reservoirs can produce cyclic heptapeptide hepatotoxins, named microcystins.[1,2] The general structure of microcystins 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*-methylaspartic 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. The two acidic amino acids, D-MeAsp and D-Glu, are connected by isolinkages. In our previous studies on cyanobacterial hepatotoxins,[3−5] we identified two 2-amino-2-butenoic acid dehydrobutyrine (Dhb)-containing microcystins (Dhb-microcystins) from *Oscillatoria agardhii* (**3** and **4**) and from a *Nostoc* sp. The geometrical structure of the Dhb units in these Dhb-microcystins was deduced to be (*E*).[5] During our investigations of the toxic compounds in cyanobacterial blooms associated with cattle hepatotoxicosis and deaths at a Scottish freshwater lake, we found two new (*Z*)-Dhb-microcystins (**1** and **2**). Here, we describe the isolation and structure elucidation of **1** and **2**.



1: R_1 = Me, R_2 = H, X = CH_2CH_2-*p*-OH-Ph ; [D-Asp^3, (*Z*)-Dhb^7]microcystin-HtyR
2: R_1 = Me, R_2 = H, X = $CH_2CH(CH_3)_2$; [D-Asp^3, (*Z*)-Dhb^7]microcystin-LR
3: R_1 = H, R_2 = Me, X = CH_2CH_2-*p*-OH-Ph ; [D-Asp^3, (*E*)-Dhb^7]microcystin-HtyR
4: R_1 = H, R_2 = Me, X = $CH_2CH(CH_3)_2$; [D-Asp^3, (*E*)-Dhb^7]microcystin-LR

Bloom material from Soulseat Loch contained apparently healthy and intact filaments of *Oscillatoria agardhii* as the only cyanobacterium discernible by light microscopy. The bloom material was toxic and contained two microcystins (**1** and **2**), which were isolated from lyophilized bloom material (10 g). After purification by HPTLC, microcystins **1** (5.4 mg) and **2** (1.5 mg) were obtained as colorless amorphous solids with a λ_{max} (MeOH) at 239 nm.

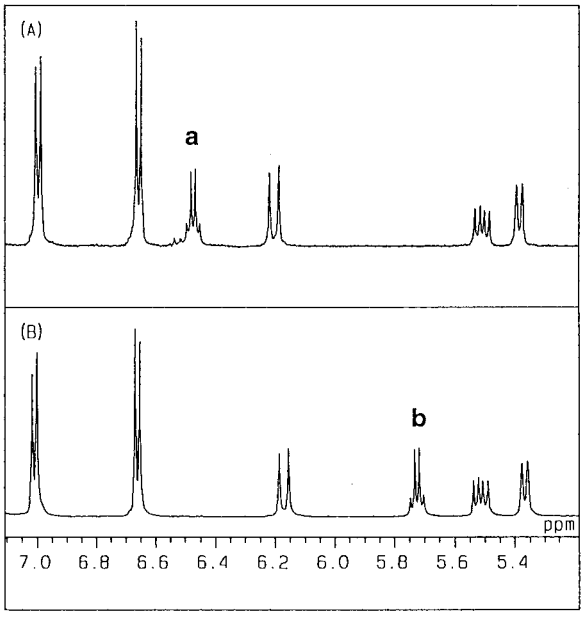


Figure 1. Parts of ^{1}H NMR spectra of (*Z*)- and (*E*)-Dhb-microcystins. (A) [D-Asp^3, (*Z*)-Dhb^7]-microcystin-HtyR (**1**); (B) [D-Asp^3, (*E*)-Dhb^7]microcystin-HtyR (**3**). Peaks **a** and **b** were the olefin proton signals of the Dhb units.

From a positive HRFABMS spectrum, the molecular formula of **1** was established as $C_{52}H_{72}N_{10}O_{13}$. The amino acids detected after acid hydrolysis (6 M HCl, 110 °C, 21 h) were D-Ala, L-homotyrosine (Hty), D-Asp, L-Arg, and D-Glu. The molecular formula and amino acid composition were identical with those of [D-Asp^3, (*E*)-Dhb^7]microcystin-HtyR [**3**, (*E*)-Dhb-microcystin-HtyR].[5] However, the ^{1}H NMR spectrum of **1** was clearly different from that of (*E*)-Dhb-microcystin-HtyR (**3**) (Figure 1). In the ^{1}H NMR spectrum of **1** (Table 1), a quartet appeared at 6.49 ppm (Figure 1A, signal a), while the quartet of (*E*)-Dhb-microcystin-HtyR (**3**) ap-

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Table 1. ^{1}H and ^{13}C NMR Spectra Data for **1** in CD_3OD at 500 MHz

position		^{1}H	*J* (Hz)	^{13}C	position		^{1}H	*J* (Hz)	^{13}C
Dhb	1			167.0	Adda	1			176.5
	2			131.9		2	2.98	(m)	45.1
	3	6.49	(q, 7.3)	129.3		3	4.52	(dd, 8.4, 9.2)	56.4
	4	1.79	(d, 7.3)	13.1		4	5.51	(dd, 8.4, 15.6)	127.0
Ala	1			175.4		5	6.20	(d, 15.6)	138.3
	2	4.63	(q, 7.3)	50.0		6			134.0
	3	1.31	(d, 7.3)	17.2		7	5.38	(d, 9.8)	136.8
Hty	1			174.3		8	2.58	(m)	37.7
	2	4.11	(dd, 6.7, 11.0)	56.0		9	3.24	(m)	88.4
	3	2.19	(m)	34.1		10	2.81	(dd, 4.6, 13.7)	39.0
		2.12	(m)				2.68	(dd, 8.0, 13.7)	
	4	2.70	(m)	32.6		11	1.07	(d, 7.0)	16.1
		2.52	(m)			12	1.61	(s)	13.0
	5			133.1		13	0.99	(d, 6.7)	16.6
	6, 10	7.01	(d, 8.6)	130.7		14	3.23	(s)	58.7
	7, 9	6.66	(d, 8.6)	116.2		15			140.5
	8			156.5		16, 20	7.17	(d, 7.3)	130.5
Asp	1			176.8		17, 19	7.23	(t, 7.3)	129.2
	2	4.57	(br)	53.1		18	7.15	(t, 7.3)	127.0
	3	2.78	(m)	39.9	Glu	1			178.9
		2.49	(m)			2	4.24	(m)	56.4
	4			175.0		3	2.16	(m)	29.9
Arg	1			172.4			1.98	(m)	
	2	4.44	(m)	53.1		4	2.55	(m)	34.3
	3	2.01	(m)	29.0			2.45	(m)	
		1.55	(m)			5			176.5
	4	1.53	(m)	26.5					
	5	3.11	(m)	42.0					
	6			158.6					

peared at 5.73 ppm (Figure 1B, signal b).[5] In the same manner as **3**, the quartet was deduced to be the olefin proton signal of the Dhb unit by 2D NMR analysis.

Extensive analysis of the 2D NMR spectra of **1** revealed the presence of an Adda unit and the amino acids detected by amino acid analysis. The sequences of **1** were mainly performed by HMBC experiments. From 1D NOE and ROESY experiments in DMSO-d_6, the geometrical structure of the Dhb unit in **1** was deduced to be (*Z*). From these results, the structure of **1** was established to be [D-Asp^3, (*Z*)-Dhb^7]microcystin-HtyR [(*Z*)-Dhb-microcystin-HtyR].

The pseudomolecular ion $[M + H]^+$ of **2** in the FABMS spectrum was observed at *m/z* 981. From the HR-FABMS, the molecular formula of **2** was deduced to be $C_{48}H_{72}N_{10}O_{12}$. After acid hydrolysis of **2**, the amino acids detected were D-Ala, L-Leu, D-Asp, L-Arg, and D-Glu. The molecular formula and amino acid composition were the same as those of [D-Asp^3, (*E*)-Dhb^7]-microcystin-LR [**4**, (*E*)-Dhb-microcystin-LR].[5] In the ^{1}H NMR spectrum of **2**, the quartet assigned as the olefin proton signal of the Dhb unit was observed at 6.49 ppm (Table 2). Extensive analysis of the NMR spectra indicated that the structure of **2** was [D-Asp^3, (*Z*)-Dhb^7]-microcystin-LR [(*Z*)-Dhb-microcystin-LR].

The geometrical structure of the Dhb unit of **1** and **2** is (*Z*),that is, the same as that in the cyclic pentapeptide toxin, nodularin, isolated from *Nodularia spumigena*.[6] The (*Z*)-Dhb units of nodularins are biosynthesized via trans dehydration of Thr.[7] However, the (*E*)-Dhb units of Dhb-microcystins may be the result of trans dehydration of *allo*-Thr or the isomerization of (*Z*)-Dhb. The biosynthetic pathway of the (*Z*)-Dhb units in Dhb-microcystins may be the same as that in nodularin.

Experimental Section

General Procedures. NMR spectra were recorded on a JEOL JNM A-500 spectrometer (500 MHz). ^{1}H and ^{13}C NMR chemical shifts are referenced to TMS. Homonuclear ^{1}H connectivities were determined from the COSY and HOHAHA experiments, and heteronuclear ^{1}H−^{13}C connectivities were determined by HSQC and HMBC experiments. LRMS and HRMS were performed with a JEOL JMS-700 spectrometer. Specific rotations were obtained on a Atago POLAX-D polarimeter.

Cyanobacterial Sample. Cyanobacterial cells were collected from an extensive shoreline scum at Soulseat Loch, southwest Scotland, in June 1995. The bloom material contained apparently healthy and intact filaments of *O. agardhii* as the only discernible cyanobacterium by light microscopy. The scum was frozen at −20 °C, then lyophilized and stored at −20 °C.

Toxin Extraction. Toxins were extracted from 10 g of lyophilized material with 5% (v/v) HOAc aqueous solution, then MeOH. The extract was suspended with 5% aqueous HOAc solution, and the suspension was filtered. The filtrate was fractionated with ODS cartridges (Sep-Pak ODS) using 20% and 80% aqueous MeOH.

Toxin Analysis. The fraction eluted with 80% MeOH was analyzed by reversed-phase HPLC (column, Mightysil RP-18, 4.6 × 150 mm; mobile phase, 60% MeOH in 50 mM phosphate buffer, pH 3.0; flow rate, 1.0 mL/min) and detected with a photodiode array detector.

Toxin Isolation. Toxins in the 80% MeOH eluate were isolated by preparative reversed-phase HPLC (Mightysil RP-18, 20 × 250 mm; flow rate, 10 mL/min) with 60% MeOH containing 50 mM phosphate buffer (pH 3.0). The isolated toxins were further purified by HPTLC (Merck, Si gel 60 F_{254}, 0.25 mm, $CHCl_3$−MeOH−H_2O 6:4:1). The yields of [D-Asp^3, (*Z*)-Dhb^7]-microcystin-HtyR (**1**) and -LR (**2**) were 5.4 mg and 1.5 mg, respectively.

Hydrolysis and Amino Acid Analysis. The microcystins were heated in 6 M HCl at 110 °C for 21 h.

Table 2. ^{1}H and ^{13}C NMR Spectral Data for **2** in CD_3OD at 500 MHz

position		^{1}H	*J* (Hz)	^{13}C	position		^{1}H	*J* (Hz)	^{13}C
Dhb	1			166.6	Adda	1			177.4
	2			131.6		2	3.02	(m)	45.2
	3	6.49	(q, 7.3)	129.6		3	4.54	(t, 9.0, 10.4)	56.5
	4	1.80	(d, 7.3)	13.1		4	5.48	(dd, 9.0, 15.6)	126.7
Ala	1			175.4		5	6.24	(d, 15.6)	138.8
	2	4.60	(q, 7.3)	49.8		6			134.0
	3	1.26	(d, 7.3)	17.1		7	5.41	(d, 9.5)	137.0
Leu	1			174.5		8	2.59	(m)	37.7
	2	4.25	(dd, 4.3, 11.3)	55.4		9	3.26	(m)	88.4
	3	1.94	(m)	40.7		10	2.81	(dd, 4.9, 14.0)	39.0
		1.64	(m)				2.69	(dd, 7.3, 14.0)	
	4	1.73	(m)	26.0		11	1.06	(d, 6.7)	16.1
	5	0.92	(d, 8.4)	23.5		12	(m)	(s)	12.9
	5′	0.88	(d, 6.7)	21.5		13	1.00	(d, 7.0)	16.5
Asp	1			176.8		14	3.24	(s)	58.7
	2	4.70	(dd, 3.7, 7.3)	53.1		15			140.5
	3	2.87	(dd, 7.3, 14.3)	39.3		16, 20	7.18	(m)	130.5
		2.29	(dd, 3.7, 14.3)			17, 19	7.24	(m)	129.2
	4			175.2		18	7.16	(m)	127.1
Arg	1			172.2	Glu	1			176.0
	2	4.43	(dd, 4.0, 9.2)	53.1		2	4.36	(dd, 5.8, 9.2)	54.4
	3	2.02	(m)	29.1		3	2.13	(m)	28.4
		1.52	(m)				2.05	(m)	
	4	1.53	(m)	26.7		4	2.55	(m)	34.1
	5	3.13	(m)	42.0			2.36	(m)	
	6			158.6		5			176.0

The amino acid hydrolysates were heated with 6 M HCl (0.2 mL) and ^{i}PrOH (0.2 mL) at 110 °C for 1 h. The mixtures were evaporated to dryness under a gentle stream of nitrogen (N_2). The residues were treated with trifluoroacetic anhydride (100 μL) and CH_2Cl_2 (100 μL) at 100 °C for 5 min and evaporated under N_2. The mixtures in CH_2Cl_2 were analyzed by GC−MS using a Chirasil-L-Val capillary column (0.25 mm × 25 m) with a column temperature of 40−200 °C at 8 °C/min. Retention times of the standard amino acids (min): D-Ala (6.08), L-Ala (6.93), D-Leu (9.61), L-Leu (10.49), D-Asp (12.51), L-Asp (12.71), D-Glu (14.76), L-Glu (15.17), D-Hty (19.25), L-Hty (19.57). Retention times of the amino acids of **1** (min): Ala (6.10), Asp (12.51), Glu (14.76), Hty (19.55). Retention times of the amino acids of **2** (min): Ala (6.10), Leu (10.48), Asp (12.51), Glu (14.74). Arg were analyzed by Marfey's method.[8] Retention times of the standard Arg (min): L-Arg (8.8), D-Arg (9.4). Retention times of Arg of **1** and **2** (min): Arg of **1** (8.8) and **2** (8.8).

[D-Asp^3, (*Z*)-Dhb^7]microcystin-HtyR (1): colorless amorphous solid; $[\alpha]_D^{25}$ −62° (*c* 0.50, MeOH); UV (MeOH) λ_{max} (log ϵ) 239 (4.5) nm; ^{1}H and ^{13}C NMR Table 1; HRFABMS *m/z* 1045.5377 $[M + H]^+$ (calcd for $C_{52}H_{73}N_{10}O_{13}$ 1045.5358).

[D-Asp^3, (*Z*)-Dhb^7]microcystin-LR (2): colorless amorphous solid; $[\alpha]_D^{25}$ −95° (*c* 0.29, MeOH); UV (MeOH) λ_{max} (log ϵ) 239 (4.5) nm; ^{1}H and ^{13}C NMR Table 2; HRFABMS *m/z* 981.5435 $[M + H]^+$ (calcd for $C_{48}H_{73}N_{10}O_{12}$ 981.5409).

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