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THREE DEHYDROBUTYRINE-CONTAINING MICROCYSTINS FROM *NOSTOC*

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Key Word Index—*Nostoc*; cyanobacterium; microcystin; dehydrobutyrine microcystins.

Abstract—Three cyclic heptapeptide hepatotoxins, [Asp^3, ADMAdda^5, Dhb^7] microcystin RR, [Asp^3, ADMAdda^5, Dhb^7] microcystin HtyR and [Asp^3, ADMAdda^5, Dhb^7] microcystin LR, were isolated from a *Nostoc* species. Their structures were assigned on the basis of ^{1}H and ^{13}C NMR, HR FAB mass spectrometry and amino acid analysis using GC-mass spectrometry on a chiral capillary column. All three microcystins contained dehydrobutyrine (Dhb), instead of dehydroalanine (Dha), D-aspartic acid, instead of D-*erythro*-β-methylaspartic acid (D-MeAsp), and 9-acetoxy-3-amino-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid (ADMAdda), instead of the corresponding 9-methoxyl derivative (Adda). This is the first report on the identification of Dhb-containing microcystins from *Nostoc*.

INTRODUCTION

The cyanobacterium, *Nostoc*, has been used as a traditional food in several countries, e.g., Bolivia, China, Japan, Mongolia and Russia [1]. However, it has been found to contain hepatotoxic cyclic heptapeptides (microcystins) [2–4]. The general structure of microcystins is cyclo(-D-Ala-X-D-MeAsp-Y-Adda-D-Glu-Mdha-), in which X and Y 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. Demethyl variants have been found at D-MeAsp (i.e., D-Asp) and/or Mdha (i.e., dehydroalanine, Dha). Also, acetyl-demethyl variants have been located at the C-9 of Adda (i.e., ADMAdda) from *Nostoc* species [4]. Recently, dehydrobutyrine(Dhb)-containing microcystin RR has been identified from the cyanobacterium, *Oscillatoria agardhii* [5].

During investigations into toxins from a newly-isolated *Nostoc* strain, we have identified novel Dhb-containing microcystins. In this paper, we report on the chemical structures and toxicities of these new microcystins.

RESULTS AND DISCUSSION

The microcystins were separated by HPLC using a preparative reverse-phase column; five major peaks were detected. The most rapidly eluting, large peak was a cyclic peptide, nostocyclin, which has been previously identified [6]. The last eluted major peak was non-toxic using the mouse bioassay, but we have so far been unable to identify it.

The compounds obtained for the other peaks (**1**–**3**) were acutely hepatotoxic by mouse bioassay, with signs of poisoning and gross liver pathology typical of microcystins [8]. The LD_{50} values (intraperitoneal mouse bioassay) for **1** and **2** were 200 and 100 μg kg^{-1} body wt, respectively. Insufficient amounts of **3** were available for quantitative estimation of toxicity, although this material was again acutely hepatotoxic. The isolated compounds were further purified by HPTLC.

The yields of **1**–**3** were 6.0 mg, 1.9 mg and 0.8 mg, respectively. All were colourless amorphous solids. In the positive HR FAB mass spectrum using glycerol as a matrix, the $[M+H]^+$ of **1** was observed at *m/z* 1052.5576, establishing the molecular formula as $C_{49}H_{73}N_{13}O_{13}$. The $[M+H]^+$ of **2** and **3** were observed at *m/z* 1073.5434 and 1009.5265, and their molecular formulae established to be $C_{53}H_{72}N_{10}O_{14}$ and $C_{49}H_{72}N_{10}O_{13}$, respectively. The ^{1}H and ^{13}C NMR (Tables 1 and 2) of **1**–**3**, suggested that they contained 9-acetoxy-3-amino-2,6,8-trimethyl-10-phenyldeca-4(*E*), 6(*E*)-dienoic acid (ADMAdda) and dehydrobutyrine (Dhb, 2-amino-2-butenoic acid). From amino acid analysis of the hydrolysate (6M HCl, 110°, 20 h) of **1**, alanine (Ala), aspartic acid (Asp), glutamic acid (Glu) and arginine (Arg) were detected. In the case of **2**, Ala, Asp, Glu, Arg and homotyrosine (Hty)

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Table 1. ^{1}H NMR data for compounds **1**–**3** in MeOH-d_4[a]

Proton (mult)[b]	δ (*J* in Hz) 1	2	3	Proton (mult)[b]	δ(*J* in Hz) 1	2	3
[Ala^1]				[ADMAdda^5][c]			
H-2 (*t*)	4.56 (7.6)	4.52 (8.2)	4.54 (7.6)	H-2 (*m*)	3.14	3.13	3.15
H-3 (*d*)	1.31 (7.6)	1.37 (7.3)	1.32 (7.6)	H-3 (*m*)	4.54	4.54	4.54
[Arg^2]				H-4 (*dd*)	5.52 (8.9, 15.5)	5.52 (8.9, 15.5)	5.59 (8.9, 15.5)
H-2 (*t*)	4.21 (7.6)			H-5 (*d*)	6.22 (15.3)	6.22 (15.3)	6.20 (15.5)
H-3 (*m*)	2.00			H-7 (*d*)	5.43 (10.1)	5.43 (10.1)	5.34 (9.5)
H-5 (*m*)	1.79			H-8 (*m*)	2.75	2.75	2.75
(*m*)	1.72			H-9 (*m*)	4.96	4.96	4.96
H-5 (*m*)	3.21			H-10 (*dd*)	2.90 (4.9, 8.9)	2.90 (4.9, 8.9)	2.90 (9.5, 13.7)
[Hty^2]				(*m*)	2.74	2.74	2.74
H-2 (*dd*)		4.17 (4.3, 10.7)		H-11 (*d*)	1.04 (7.0)	1.04 (7.0)	1.09 (6.7)
H-3 (*m*)		2.12		H-12 (*s*)	1.68	1.68	1.69
H-4 (*m*)		2.70		H-13 (*d*)	1.02 (6.7)	1.02 (6.7)	0.97 (7.0)
(*m*)		2.49		H-15 (*s*)	1.91	1.91	1.91
H-6, H-10 (*d*)		6.62 (8.3)		H-17 *m*)	7.18	7.18	7.16
H-7, H-9 (*d*)		6.96 (8.3)		H-18 (*m*)	7.23	7.23	7.23
[Leu^3]				H-19 (*m*)	7.15	7.15	7.15
H-2 (*dd*)			4.30 (11.6, 7.6)	H-20 (*m*)	7.23	7.23	7.23
H-3 (*m*)			2.03	H-21 (*m*)	7.18	7.18	7.16
(*m*)			1.61	[Glu^6]			
H-4 (*m*)			1.75	H-2 (*dd*)	4.19 (8.5, 7.0)	4.19 (8.5, 7.0)	4.24 (9.5, 5.2)
H-5 (*d*)			0.89 (6.4)	H-3 (*m*)	2.04	2.04	2.13
H-6 (*d*)			0.93 (6.4)	(*m*)	1.96	1.96	1.96
[Asp^3]				H-4 (*m*)	2.45	2.45	2.40
H-2 (*t*)	4.64 (4.3)	4.58 (4.3)	4.56 (4.3)	(*m*)	2.24	2.24	2.22
H-3 (*dd*)	2.90 (4.8, 13.9)	2.72 (4.8, 13.9)	2.98 (7.0, 9.2)	[Dhb^7][d]			
(*m*)	2.23	2.14	2.22	H-3 (*q*)	5.68 (7.3)	5.58 (7.3)	5.72 (7.3)
[Arg^4]				H-4 (*d*)	1.86 (7.3)	1.86 (7.3)	1.86 (7.3)
H-2 (*m*)	4.42	4.39	4.44				
H-3 (*m*)	2.03	1.98	2.03				
H-4 (*m*)	1.54	1.56	1.56				
H-5 (*m*)	3.14	3.15	3.16				

[a] 500 MHz.
[b] *s* = singlet, *d* = doublet, *dd* = doublet of doublet, *t* = triplet, *q* = quartet, *m* = multiplet.
[c] ADMAdda, *O*-acetyl-*O*-demethylAdda.
[d] Dhb, Dehydrobutyrine (2-amino-2-butenoic acid).

were detected. Also, Ala, Asp, Glu, Arg and leucine (Leu) were detected in the hydrolysate of **3**. Ala, Asp and Glu in all three compounds were identified in the D-configuration, and Arg, Hty and Leu in the L-configuration, by chiral GC-mass spectrometric analysis [5]. These results show that the isolated compounds are all acetylated Adda- and Dhb-containing microcystins; detection of Asp, instead of MeAsp, shows the presence of demethyl types. Furthermore, in NMR analyses, HMBC correlations were observed between the α-protons and the carbonyl carbons of amino acids in these microcystins [5]. From these results, **1** and **3** were identified as [Asp^3, ADMAdda^5, Dhb^7] microcystin RR, [Asp^3, ADMAdda^5, Dhb^7] microcystin HtyR and [Asp^3, ADMAdda^5, Dhb^7] microcystin LR, respectively. The ^{1}H NMR spectra of Dhb-microcystins are different from those of normal microcystins which contain *N*-methyl dehydroalanine (MDha) or dehydroalanine (Dha). In the spectra of the former, the C-3 proton of Dhb was observed at ca δ 5.6 as a quartet, and the protons at C-4 of Dhb appeared at δ 1.86 as a doublet. In contrast, the protons at C-3 of MDha and Dha showed singlets at δ 5.88 and 5.53. From the chemical shifts of the ^{1}H NMR spectra, normal and Dhb-microcystins are readily identified. However, the analytical results of the both types of microcystins by FAB mass spectrometry, amino acid analysis and UV absorption, are identical. NMR analysis is essential for the identification of microcystin variants.

[ADMAdda^5] microcystins are unstable and hydrolyze at room temperature in aqueous solution and/or in MeOH. In the case of [Asp^3, ADMAdda^5, Dhb^7] microcystin RR (**1**), the microcystin was converted to [Asp^3, DemethylAdda^5, Dhb^7] microcystin RR within one week. The arginine moiety of the microcystin probably acts as a catalyst.

Dhb-microcystin-RR has recently been found in

Table 2. ^{13}C NMR data for compounds **1**–**3** in MeOH-d_4[a]

Carbon	δ 1	2	3	Carbon	δ 1	2	3	Carbon	δ 1	2	3
[Ala[1]]				[Leu[2]]				C-8	37.6	37.6	37.6
C-1	175.1	175.1	175.5	C-3			40.7	C-9	79.8	79.8	79.8
C-2	49.6	49.6	49.6	C-4			25.9	C-10	39.4	39.4	39.4
C-3	17.3	17.4	17.3	C-5			21.3	C-11	16.1	16.1	16.1
[Arg[2]]				C-6			23.7	C-12	13.1	13.1	13.1
C-1	173.7			[Asp[3]]				C-13	17.0	17.0	17.0
C-2	57.0			C-1	176.6	176.7	176.6	C-14	172.4	172.4	172.4
C-3	29.6			C-2	52.9	53.4	52.9	C-15	20.9	20.9	20.9
C-4	26.6			C-3	39.7	39.3	39.7	C-16	138.2	138.2	138.2
C-5	42.0			C-4	175.1	175.1	175.1	C-17	130.5	130.5	130.5
C-6	158.7			[Arg[4]]				C-18	129.3	129.3	129.3
[Hty[2]]				C-1	172.1	173.3	172.1	C-19	127.4	127.4	127.4
C-1		172.1		C-2	52.9	53.1	52.9	C-20	129.3	129.3	129.3
C-2		55.5		C-3	29.2	29.5	29.2	C-21	130.5	130.5	130.5
C-3		34.3		C-4	26.5	26.3	26.5	[Glu[6]]			
C-4		32.5		C-5	41.9	41.9	41.9	C-1	179.5	179.5	179.5
C-5		127.7		C-6	158.7	158.7	158.7	C-2	56.2	56.2	56.2
C-6		117.0		[ADMAdda[5]][b]				C-3	29.5	29.5	29.5
C-7		130.6		C-1	176.6	176.6	176.6	C-4	34.3	34.3	34.3
C-8		156.6		C-2	45.0	45.0	45.0	C-5	175.5	175.5	175.5
C-9		130.6		C-3	56.5	56.5	56.5	[Dhb[7]][c]			
C-10		117.0		C-4	127.7	127.7	127.7	C-1	166.8	166.9	166.8
[Leu[2]]				C-5	138.2	138.2	138.2	C-2	132.1	132.2	132.1
C-1			175.5	C-6	134.8	134.8	134.8	C-3	123.3	123.1	123.3
C-2			55.4	C-7	135.2	135.2	135.2	C-4	13.3	13.4	13.3

[a] 125 MHz.
[b] ADMAdda, *O*-Acetyl-*O*-demethylAdda.
[c] Dhb, dehydrobutyrine (2-amino-2-butenoic acid).

Oscillatoria agardhii (NIES-610 = CCAP-1459/22 = NIVA CYA18) [5]. Other strains of *O. agardhii* (CCAP-11A, -11B, -14 and -16) also contain Dhb-microcystins [9]. However, there are no reports of these microcystin variants from other cyanobacterial genera. In the present study, we have found three Dhb-microcystins in a *Nostoc* isolated from a brackish water lake. The biological significance of the presence of Dhb in the microcystins, in place of Mdha in previously described microcystins [10], is unclear at present.

EXPERIMENTAL

Culture conditions

Nostoc (University of Dundee, strain DUN901) was isolated from a bloom dominated by the cyanobacterium, *Nodularia spumigena*, collected from brackish water of Barrow Ski Club Lake, U.K. It was grown axenically in batch culture in 8 of Z8 minus nitrate medium [6], containing 25% (v/v) seawater, at 20–25°. Cultures were sparged with air at ca 7 l h^{-1} and light was supplied by white fluorescent tubes giving an irradiance incident on the surface of the vessels of ca 20 mmol photon m^{-2} s^{-1}. Cells were harvested at stationary phase by centrifugation at 10,000 *g* for 20 min, to give a pellet which was freeze-dried [7].

Extraction and purification

The MeOH extract from 10 g of freeze-dried cells was evapd under red. press. The remaining residue was suspended in 5% aq. HOAc. The suspension was centrifuged at 2000 rpm for 20 min and the supernatant retained. The toxic fr. was isolated by solid-phase extraction using preconditioned C-18 cartridges (Sep-pak). Microcystins were isolated from the fr. by reverse-phase HPLC (Mightysil RP-18, 20 mm I.D. × 25 cm) with HPLC-grade MeOH (60%) containing 0.05 M Pi buffer (pH 3) at 10 ml min^{-1} and further purified by HPTLC (Kieselgel 60 F_{254}) using $CHCl_3$–MeOH–H_2O (6:4:1).

Analysis

^1H NMR were recorded at 500 MHz and ^{13}C NMR at 125 MHz. CHD_2OD was used as int. standard (^1H, δ 3.30; ^{13}C, δ 49.0). Protons and carbons were assigned by ^1H-^1H COSY, DEPT, HSQC and HMBC. FAB MS were using glycerol as matrix.

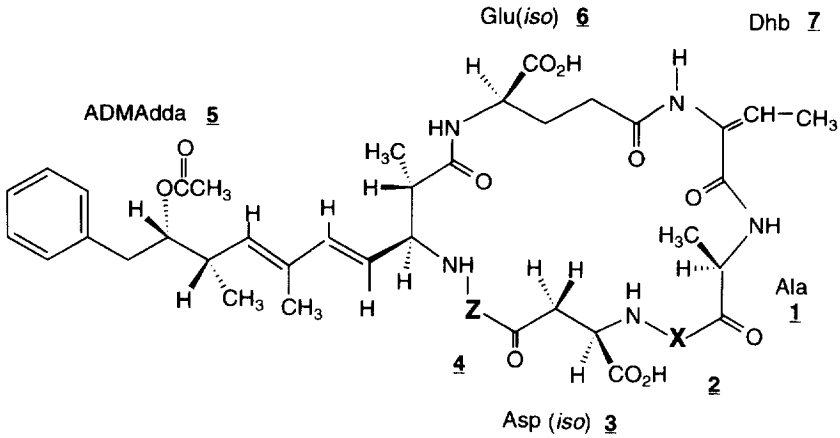

Fig. 1. Chemical structures of dehydrobutyrine(Dhb)-containing microcystins isolated from *Nostoc* sp. DUN901. ADMAdda: 9-Acetoxy-3-amino-10-phenyl-2,6,8-trimethyldeca-4.6-dienoic acid. Dhb: Dehydrobutyrine (2-Amino-2-butenoic acid). **1**, [Asp^3, ADMAdda^5, Dhb^7] microcystin RR (X = Arg, Z = Arg); **2**, [Asp^3, ADMAdda^5, Dhb^7] microcystin HtyR (X = Htyr, Z = Arg); **3**, [Asp^3, ADMAdda^5, Dhb^7] microcystin LR (X = Leu, Z = Arg).

Amino acid analysis

Microcystins were hydrolyzed with 6 M HCl at 110° for 20 h. Liberated amino acids were converted to their corresponding *N*-trifluoroacetyl isopropyl esters [5]. These were separated by GC on a chrasil-L-Val column and identified by EIMS.

Toxicity test

Microcystins were assayed using the intraperitoneal mouse test [8].

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