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[D-Leu[1]]Microcystin-LR, from the cyanobacterium *Microcystis* RST 9501 and from a *Microcystis* bloom in the Patos Lagoon estuary, Brazil

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Abstract

[D-Leu[1]]Microcystin-LR was identified as the most abundant microcystin from a laboratory strain of the cyanobacterium *Microcystis* sp. isolated from a hepatotoxic *Microcystis* bloom from brackish waters in the Patos Lagoon estuary, southern Brazil. Toxicity of [D-Leu[1]]microcystin-LR, according to bioassay and protein phosphatase inhibition assay, was similar to that of the commonly-occurring microcystin-LR, which was not detectable in the Patos Lagoon laboratory isolate. This is the first report of a microcystin containing [D-Leu[1]] in the cyclic heptapeptide structure of these potent cyanobacterial toxins.

Keywords: Microcystin; Hepatotoxin; *Microcystis*; Cyanobacteria

1. Introduction

Microcystins, of which over 65 structural variants are known, are cyclic heptapeptides, which include potent hepatotoxins and tumour-promoters (Rinehart et al., 1994; Codd et al., 1999; Sivonen and Jones, 1999). They are produced by cyanobacteria in aquatic and terrestrial environments and have been involved, or implicated, in acute and chronic health problems in animals and humans (Carmichael, 1997; Falconer and Humpage, 1996; Codd et al., 1999). The generic structure of the microcystins is cyclo-(-D-Ala[1]-*X*[2]-D-Masp[3]-*Y*[4]-Adda[5]-D-Glu[6]-Mdha[7]-), where *X* and *Y* are variable L-amino acids, D-Masp 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-4,6-dienoic acid. The two acidic amino acids, D-Masp and D-Glu are connected by an isolinkage.

Structural variations are now known to occur at all seven positions of the generic heptapeptide microcystin ring, the most numerous found to date being at *X* and *Y*, with at least 22 L-amino acid permutations (Sivonen and Jones, 1999; Kaya et al., 2000). The combined presence of the two L-amino acids is used in the nomenclature of the variants, thus microcystin-LR contains L-Leu[2] plus L-Arg[4]. Variations arise elsewhere (positions 3, 5, 6 and 7) by demethylations, changes in isomerization and acylation. Position 1 is an apparently relatively conserved position in these toxins (Sivonen and Jones, 1999). Only two exceptions to D-Ala[1] are known: [D-Ser[1], ADMAdda[5]]microcystin-LR, from the cyanobacterium *Nostoc*, contains serine and a modified Adda (*O*-acetyl-*O*-demethylAdda) (Sivonen et al., 1992) and a microcystin variant from a *Microcystis* bloom, in which D-Ala[1] was replaced by an unknown, but more hydrophobic, amino acid (Craig et al., 1993) . There is at least a 24-fold range in toxicity among the known microcystins according to intraperitoneal (i.p.) mouse bioassay. Microcystin-LR is among the most toxic of the known microcystins by this criterion and is among the most

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commonly identified of these toxins in environmental samples (lethal dose to kill 50% of a mouse population via i.p., LD_{50}: about 50 μg kg^{-1} body wt) (Rinehart et al., 1994; Sivonen and Jones, 1999).

Here, we report on the chemical structure and toxicity of the first microcystin found to contain D-Leu[1] (Fig. 1). This toxin is produced by a *Microcystis* strain isolated and cultured from a *Microcystis* bloom in the estuary of the Patos Lagoon, southern Brazil, a major waterbody with a history of extensive nuisance blooms and scums of hepatotoxic *Microcystis* (Yunes et al., 1996, 1998).

2. Results and discussion

Analysis, by HPLC with photodiode array detection, of 70% (v/v) aq. MeOH extracts of lyophilized *Microcystis* bloom material collected from the Patos Lagoon estuary in December 1996, and of lyophilized cells of *Microcystis* strain RST 9501, isolated from the same location in 1995, revealed one major peak and three minor peaks with UV spectra characteristic of microcystins, λ_{max} 238–239 nm. The major peak had a R_T of 15.22 min. None of the HPLC peaks had a R_T of that of microcystin-LR (13.28 min), purified from *Microcystis* PCC 7813, under the conditions used.

For microcystin purification, [D-Leu[1]]microcystin-LR was isolated from 1 g of lyophilized *Microcystis* RST 9501 cells using 5% (v/v) aq. HOAc and HPLC, and purified by HPTLC using $CHCl_3$–MeOH–H_2O (6:4:1). The yield of [D-Leu[1]]microcystin-LR was 0.13 mg. The purified material was a colourless amorphous solid with the same UV absorption spectrum as microcystin-LR: λ_{max} (H_2O) 239 nm. The $[M+H]^+$ ion of the positive HR FAB mass spectrum was observed at m/z 1037.5955. From the results, the molecular formula of [D-Leu[1]]microcystin-LR was established to be C_{52} $H_{80}O_{12}N_{10}$ (calcd for $C_{52}H_{81}O_{12}N_{10}$: 1037.6035, Δ−0.8 mmu).

In the amino acid analyses of [D-Leu[1]]microcystin-LR using reversed-phase HPLC, D-Glu (R_T, 21.03 min) and D-β-Masp (R_T, 24.54 min) were detected from the acidic amino acid fraction, and L-Arg (R_T, 11.44 min), L-Leu (R_T, 34.62 min) and D-Leu (R_T, 40.62 min) were detected in the neutral and basic amino acid fraction. However, D-Ala (R_T, 24.52 min), a characteristic component of microcystins, was not detected. These results suggested that the D-Ala of the majority of microcystins was replaced by D-Leu in the *Microcystis* RST 9501 strain.

Amino acid units of the toxin were assigned by ^1H–^1H–COSY and HOHAHA spectra. ^1H-NMR data for [D-Leu[1]]microcystin-LR are shown in Table 1. Of the chemical shifts of the [D-Leu[1]]microcystin-LR amino acid α-protons, those of Leu-1, Leu-2, D-Masp, L-Arg and D-Glu were located at 4.56, 4.30, 4.33, 4.35 and 4.11 ppm, respectively. In the case of microcystin-LR, chemical shifts of α-protons of D-Ala, L-Leu, D-Masp, L-Arg, and D-Glu were located at 4.60, 4.29, 4.33, 4.36 and 4.10 ppm, respectively. The chemical shifts of the D-Masp, L-Arg and D-Glu of [D-Leu[1]]microcystin-LR agreed well with those of microcystin-LR, and the chemical shift of Leu-2 (Table 1) agreed well with that of the L-Leu of microcystin-LR. Moreover, the chemical shift of the α-proton of the Leu-1 of [D-Leu[1]]microcystin-LR agreed well with the simulated result of the sequence [-Mdha-Leu 1- Leu 2-] using computer software (CS ChemDraw Ultra). From these findings, the structure of [D-Leu[1]]microcystin-LR is deduced to be cyclo-(-Leu[1]-Leu[2]-D-Masp[3]-L-Arg[4]-Adda[5]-D-Glu[6]-Mdha[7]). From the amino acid positions 1 and 2 of all known microcystin variants, D- and L-amino acids would be at positions 1

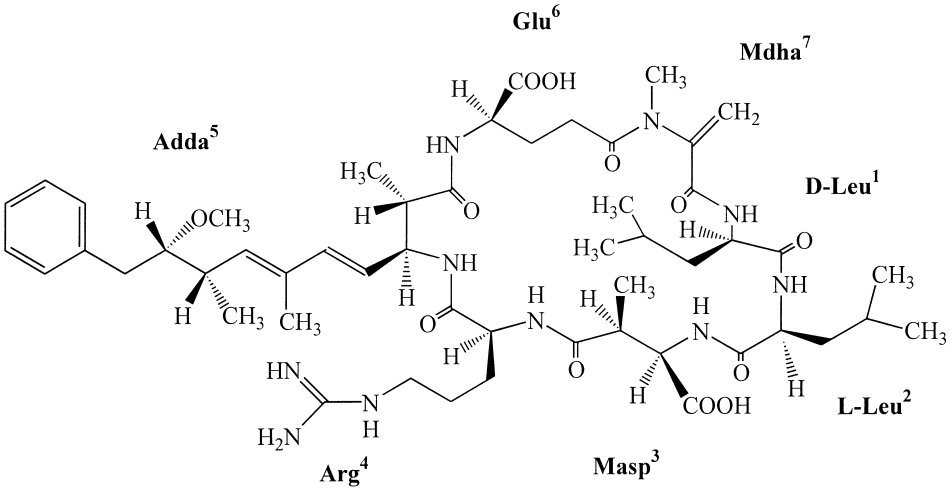

Fig. 1. Chemical structure of [D-Leu[1]]microcystin-LR shown to be present in a bloom of *Microcystis* from the Patos Lagoon, Brazil and in a laboratory strain isolated from hepatotoxic bloom material from the same source. **Masp**: D-*erythro*-β-methylaspartic acid. **Adda**: (2*S*,3*S*,8*S*,9*S*)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid. **Mdha**: *N*-methyldehydroalanine.

Table 1
^{1}H NMR[a] data for [D-Leu^1]microcystin-LR in methanol-d_4

Proton	Multiplicity[b]	δ (*J* in Hz)	Proton	Multiplicity[b]	δ (*J* in Hz)
[Leu-1]			[Adda[d]]		
H-2	*dd*	4.56 (3.7, 11.0)	H-2	*m*	3.10
H-3	*m*	1.50	H-3	*t*	4.58 (8.8)
	m	1.79	H-4	*dd*	5.50 (8.8, 15.2)
H-4	*m*	1.58	H-5	*d*	6.23 (15.6)
H-5	*d*	0.86 (7.0)	H-7	*d*	5.41 (9.3)
H-6	*d*	0.88 (7.0)	H-8	*m*	2.58
[Leu-2]			H-9	*m*	3.24
H-2	*dd*	4.30 (3.7, 11.0)	H-10	*dd*	2.67 (7.3, 14.0)
H-3	*m*	1.56		*dd*	2.81 (4.9,.14.0)
	m	2.05	H-11	*d*	1.03 (7.1)
H-4	*m*	1.78	H-12	*s*	1.62
H-5	*d*	0.87 (7.0)	H-13	*d*	0.99 (6.8)
H-6	*d*	0.89 (7.0)	H-14	*s*	3.21
[Masp[c]]			H-16, 20	*d*	7.18 (7.0)
H-2	*d*	4.33 (3.7)	H-17, 19	*dd*	7.23 (7.0, 7.6)
H-3	*m*	3.16	H-18	*t*	7.17 (7.0)
H-5	*d*	1.02 (7.0)	[Glu]		
[Arg]			H-2	*dd*	4.11 (6.7, 8.6)
H-2	*dd*	4.35 (3.5, 9.0)	H-3	*m*	1.83
H-3	*m*	1.54		*m*	2.08
	m	2.03	H-4	*m*	2.52
H-4	*m*	1.52		*m*	2.62
H-5	*m*	3.12	[Mdha[e]]		
			H-3	*s*	5.41
				s	5.85
			NCH_3	*s*	3.33

[a] 500 MHz.
[b] *s*: singlet, *d*: doublet, *dd*: doublet of doublet, *t*: triplet, *m*: multiplet.
[c] Masp: *erythro*-β-methylaspartic acid.
[d] Adda: 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid.
[e] Mdha: *N*-methyldehydroalanine.

and 2 respectively (Rinehart et al., 1994; Sivonen and Jones, 1999). In the present case, D-Leu and L-Leu would be located at positions 1 and 2 respectively. When the configurations of Leu at positions 1 and 2 are fitted as for all known microcystin variants, the deduced structure of the microcystin is [D-Leu^1]microcystin-LR (Fig. 1).

ChemDraw Ultra does not take into account the amino acid stereochemistry and global conformation of the microcystin and insufficient quantities of the new microcystin were available for ^{13}C NMR studies. However, pulse hydrolysis was carried out with dipeptide and tripeptide amino acid characterization. In positive HR FAB mass spectra analysis using glycerol as the matrix, the $[M+H]^+$ ions were observed at *m*/*z* 261 and 374, respectively, consistent with the identification of Leu and Masp in the dipeptide and two Leu molecules plus Masp in the tripeptide. From the overall findings, the partial amino acid sequence was deduced to be D-Leu-L-Leu-D-Masp, with D-Leu at position 1 in the cyclic peptide, in place of the D-Ala which occurs in the majority of microcystins.

Purified [D-Leu^1]microcystin-LR was acutely hepatotoxic by intraperitoneal mouse bioassay, with signs of poisoning and gross liver pathology (swollen, engorged with blood) typical of microcystins (Carmichael, 1997; Sivonen and Jones, 1999). The minimum lethal dose by mouse bioassay was 100 μg kg^{-1} body wt for [D-Leu^1]-microcystin-LR and microcystin-LR. This indicates that, as with microcystin-LR, [D-Leu^1]microcystin-LR is among the microcystins of highest toxicity (Rinehart et al., 1994; Sivonen and Jones, 1999). The kinetics of inhibition of protein phosphatase activity in vitro by the two purified toxins gave almost the same IC_{50} values (4.43 nM for [D-Leu^1]microcystin-LR and 3.12 nM for microcystin-LR), confirming earlier findings with the latter toxin (MacKintosh et al., 1990). The relatively high toxicity of this microcystin variant, and it amounting to approximately 90% of the intracellular microcystin pool in *Microcystis* RST 9501 and 1996 Patos Lagoon *Microcystis* bloom material, indicate that [D-Leu^1]microcystin-LR can account for the majority of the hepatotoxicity of *Microcystis* blooms under certain conditions.

3. Experimental

3.1. Sample collection and strain culture conditions

Microcystis sp. bloom samples were collected from the estuary of the Patos Lagoon in 1995 and 1996 (Yunes et al., 1996, 1998). *Microcystis* strains RST 9501 (UPC Culture Collection, Fundacao Universidade Federal do Rio Grande) and PCC 7813 (Institut Pasteur, Paris) were grown in BG-11 medium with nitrate (Stanier et al., 1971) as detailed earlier (Beattie et al., 1998), Cells were harvested by centrifugation at 10 000 ***g*** from stationary phase batch cultures for 20 min. Cell pellets were frozen, lyophilized and stored at −20°C.

3.2. Microcystin extraction and purification

Initial extraction of microcystins from environmental material and liquid cultures of *Microcystis* RST 9501 was by 100% MeOH (Lawton et al., 1995). The extraction of microcystin for purification was performed from 1 g of lyophilized cells using 5% (v/v) aq. HOAc, as detailed earlier (Beattie et al., 1998). Toxin purification from RST 9501 was subsequently by reversed-phase HPLC (Mightysil RP-18, 25 cm×20 mm i.d.) with HPLC-grade MeOH (60%) containing 0.05 M Pi buffer (pH 3.0) at 10 ml min^{-1}, followed by HPTLC (Kieselgel 60 F_{254}) using $CHCl_3$–MeOH–H_2O (6:4:1). The R_f values of microcystin-LR and [D-Leu[1]]microcystin-LR were 0.35 and 0.38, respectively. Microcystin-LR (>98% purity), for comparative purposes, was purified from *Microcystis* PCC 7813 (Lawton et al., 1995).

3.3. Analysis

For microcystin analysis from environmental samples and laboratory cultures, 50 mg samples of lyophilized material were extracted in 1 ml 70% aq. MeOH. HPLC analysis was carried out using a C-18 reversed-phase column (Symmetry, 15 cm×3.9 mm i.d., 5 μm particle size) at 40°C with UV detection at 238 nm. The mobile phase was Milli-Q water/MeCN, both containing 0.1% (v/v) trifluoroacetic acid, initially at 75:25 and using a linear gradient over 30 min of 25–55% MeCN at 1 ml min^{-1}.

For amino acid hydrolysis, about 70 μg of purified microcystin were hydrolyzed in 6 M HCl at 110°C for 24 h. The hydrolysate was reacted with Marfey's reagent (Marfey, 1984; Wako Pure Chemicals) in 1 M $NaHCO_3$ at 40°C for 1 h. 2 M HCl was then added and the mixture degassed. Aliquots were analyzed by reversed-phase HPLC (Spheri-5, RP-18, 10 cm×4.6 mm i.d.) using a linear gradient over 60 min of 10–50% MeCN in 0.05 M triethylamine Pi (pH 3.0) (flow rate 2.0 ml min^{-1}, UV detection detection at 340 nm). The HPLC R_T of the D-β-Masp derivative (24.54 min) agreed completely with that of D-*erythro*-β-Masp isolated from microcystin-LR obtained commercially (Wako Pure Chemicals).

Pulse hydrolysis was performed by dissolving 200 μg [D-Leu[1]]microcystin-LR in 6 M HCl (*ca.* 0.2 ml), heating at 100°C under N_2 for 7 min, then drying in vacuo.

Acidic peptides were isolated by dissolving dried hydrolysate in 1 ml H_20 and applying to a DEAE–Sephadex column (bed volume, 0.5 ml). The column was prepared by washing with 10 ml 2 M ammonium carbonate followed by 20 ml H_20. Acidic peptides were eluted with 2 ml 0.2 M ammonium carbonate and the eluted solution dried in vacuo. After further purification by HPTLC (Kieselgel 60 F_{254}) with $CHCl_3$–MeOH–H_20 (5.5:4:1) as solvent, the acidic di- and tripeptides had R_f values of 0.31 and 0.40 respectively and were scraped off the plate and eluted in MeOH. Di- and tripeptides were hydrolyzed by the amino acid hydrolysis procedure and amino acids identified according to Marfey (1984).

^1H NMR spectra were recorded at 500 MHz using a JEOL JNMA-500 spectrometer. MeOH-d_4 was used as a solvent and chemical shifts were referenced to TMS. Homonuclear ^1H connectivities were determined by COSY and HOHAHA experiments. Low and high resolution FABMS spectra were recorded on a JEOL JMS-700 spectrometer using Xe atoms (accelerating potential 8 kV) and glycerol as a matrix.

3.4. Toxicity assessment

Toxicity assessment was by intraperitoneal mouse bioassay (Codd and Carmichael, 1982) and in vitro protein phosphatase (PP1) inhibition assay (Ward et al., 1997).

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