

[D-Leu¹] microcystin-LR, a new microcystin isolated from waterbloom in a Canadian prairie lake

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Abstract

A previously undescribed cyclic heptapeptide hepatotoxin was isolated from a cyanobacteria waterbloom collected in Pakowki Lake, Alberta, Canada (49°20'N and 110°55'W). The compound was characterized by amino acid analysis, ESIMS/CID/MS, ¹H and ¹³C NMR, and UV spectroscopy. Structure of the new microcystin was assigned as [D-Leu¹]microcystin-LR (**1**). The amino acid composition is the same as microcystin-LR (**2**) except for D-Leu and L-Leu in **1** instead of D-Ala and L-Leu in **2**. This is the first microcystin identified, among the 64 known microcystins, that has both a D- and L-Leu amino acid. Toxicity as measured by the protein phosphatase inhibition activity of **1** is similar to microcystin-LR. The presence of microcystins in waterblooms from this lake is discussed in relation to the almost yearly bird mortalities that have occurred there since 1995. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Cyanobacteria; Cyanotoxins; Microcystin; [D-Leu¹]microcystin-LR; Lake Pakowki; Canada

1. Introduction

Toxic cyanobacteria waterblooms in eutrophic lakes, rivers and reservoirs have been reported in many countries (Skulberg et al., 1984; Gorham and Carmichael, 1988; Carmichael, 1992; Park et al., 1998; Chorus and Bartram, 1999). These toxic waterblooms cause the death of wild and domestic animals, plus illness and death in humans (Chorus and Bartram, 1999). Over fifty patients at a dialysis center in Caruaru, Brazil, died from intravenous exposure to cyanotoxins, primarily microcystins in 1996 (Jochimsen et al., 1998).

Microcystis aeruginosa is the most common toxic cyanobacterium found worldwide, and is the producer of potent cyclic peptide hepatotoxins, termed microcystins (MCYSTs) (Carmichael, 1992, 1997; Carmichael et al., 1988; Park and Watanabe, 1996). Sixty-four MCYST variants are now described (Rinehart et al., 1994; Chorus and Bartram, 1999;

Sano and Kaya, 1995, 1998; Brittain et al., 2000). MCYSTs are also produced by *Microcystis viridis* (Watanabe et al., 1986; Kusumi et al., 1987), *Anabaena flos-aquae* (Krishnamurthy et al., 1986), *Oscillatoria agardhii* (Meriluoto et al., 1989), *Nostoc* sp. (Sivonen et al., 1990), *Aphanocapsa cumulus* (Domingos et al., 1999) and *Oscillatoria tenuis* (Brittain et al., 2000). The chemical structure of these hepatotoxins, contained in *M. aeruginosa*, was first described by Botes et al. (1984, 1985). These cyclic heptapeptides are composed of five common amino acids with variations occurring within a pair of L-amino acids. Desmethyl derivatives have been reported (Harada et al., 1991), in which methyl groups of *N*-methyldehydroalanine and *N*-methyl aspartic acid are replaced by hydrogen atoms. MCYSTs derive their toxicity from inhibition of protein phosphatases, especially types 1 and 2A, in a manner similar to that of the toxin causing diarrhetic shellfish poisoning, okadaic acid (Matsushima et al., 1990; Yoshizawa et al., 1990; Mackintosh et al., 1990).

It was estimated that in 1995, over 200,000 waterfowl died in the Canadian prairie lake, Pakowki Lake, owing in part to an outbreak of avian botulism. In 1996, a further 12,225 dead ducks were collected. In 1997, another 45,000

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birds were found and it was estimated that up to 2–10 times this number actually died. In 1998, over 4000 dead ducks were collected (T. Bollinger, Canadian Cooperative Wildlife Health Centre, University of Saskatchewan, personal communication). Documentation of the cause of death in these birds, or in other cases where the prime suspect is avian cholera, does not show that all these birds died of avian cholera. These problems are discussed by Ciplef and Wobeser (1993). The possible contribution of cyanobacteria toxins (cyanotoxins) is generally not considered in these cases of bird mortalities. In Pakowki Lake, waterblooms of *Aphanizomenon flos-aquae* occurred just prior to the outbreak of avian botulism. Waterblooms of *Microcystis aeruginosa* are also common from this lake but sampling for toxic algae was too infrequent to allow a direct relationship between bird mortalities and toxic cyanobacteria to be established. However, mouse bioassays, performed in 1997, using waterblooms from Pakowki Lake were acutely lethal in the mouse bioassay. Signs of poisoning were similar to those reported in the literature for MCYSTs (Chorus and Bartram, 1999). Here we report the isolation and characterization of a new MCYST from a cyanobacteria waterbloom in Pakowki Lake, Alberta, Canada that may have contributed to the death of waterfowl in 1997.

2. Materials and methods

2.1. Waterbloom material

The sample of cyanobacteria waterbloom was collected from Pakowki Lake, Alberta, Canada, on September 2, 1997. Waterbloom material, dominated by the colonial cyanobacterium *Microcystis aeruginosa*, was concentrated by pouring the sample through a 30- μ m nitex mesh. The concentrated biomass was rinsed off the mesh with lake-water and transported to the laboratory in a cooler (4°C). The sample was lyophilized and the dried biomass stored at –80°C pending isolation of MCYSTs.

2.2. Microcystin purification

Purification of MCYST was carried out according to a method modified from that of Krishnamurthy et al. (1986). Freeze-dried cells were extracted twice (3 h and overnight) at 22°C while stirring. The extracts were centrifuged at 10,000 rpm for 30 min. The supernatants were combined and dried with an airstream to remove the organic solvents. The aqueous extract was then applied to a preconditioned silica C18 SPE cartridge (YMC, Inc., Wilmington, NC, USA). The column was washed with 10 column volumes of water, 20% methanol (aq.), and MCYSTs were eluted with 80% methanol (aq.). Lyophilized *Microcystis* cells were sonicated in and extracted twice with solvent (water/methanol/1-butanol 15:4:1 v/v) overnight at room temperature, while stirring. Supernatant was pooled and dried under a stream of air overnight. The remaining aqueous cell free extract was applied to a packed C18 column (YMC, Inc.,

Wilmington, NC), washed with water, 20% aqueous methanol (v/v), and 85% aqueous methanol (v/v). Analytical HPLC with photodiode array detection of eluted fractions revealed that the 85% methanol fraction contained a single peak that had characteristics resembling MCYSTs. This peak did not correspond to MCYST-RR, -YR, -LR, -FR, -WR, -LA or nodularin (NODLN) by retention time (Fig. 2), but did display an identical ultraviolet spectrum (200–300 nm) to that of MCYST-LR. This fraction was then rotary evaporated to remove methanol, reconstituted, and chromatographed by HPLC.

The unknown MCYST was first crudely separated on a preparative LC system (Waters DeltaPrep™ System, Milford, MA), which serves as a capture step, and is designed to remove bulk matrix contaminant from the cell free extract. The MCYST fraction was then applied to preconditioned divinylbenzene (DVB) resin (1000 Å, >20 μ m, Jordi Associates, Bellingham, MA), washed with 25% aqueous methanol (v/v), and eluted with 10 column volumes of 80% methanol (aq.). Finally, the toxin fraction was repeatedly separated on a Zorbax SB-C18 column (9.4 × 250 mm², MacMod Analytical, Chadds Ford, PA) and monitored at 238 nm using gradient elution (A: 20 mM NH₄HPO₄ pH = 3, B: acetonitrile, 20–40% B in 30 min, 5 ml/min).

The purified compound was desalted using Bond Elut C18 solid phase extraction (SPE) cartridges, by flushing with Millipore water before eluting with HPLC grade methanol. The MCYST was determined to be approximately 98% pure by analytical HPLC, yielding 26.9 mg used for structural elucidation.

2.3. Amino acid analysis

Amino acid composition was determined using a pre-column fluorescent labeling chemistry package (Waters AccQ-Tag™, Milford, MA), with subsequent comparison to amino acid standard. Isolated MCYST (250 ng) was hydrolyzed (6 M HCl, 110°C) for 24 h, derivatized with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AGC) and analyzed by HPLC.

2.4. Enzyme linked immunosorbent and protein phosphatase inhibition assay

The procedure for the enzyme linked unisorbent assay (ELISA) and protein phosphatase inhibition assay (PPIA) used in this study is as described by An and Carmichael (1994). The ELISA is used to identify and quantitate MCYSTs in a sample and the PPIA is an indirect method for estimating toxicity since the MCYSTs act as potent inhibitors of protein phosphatases 1 and 2A.

2.5. Mass spectrometry and nuclear magnetic resonance

Electrospray ionization (ESI) mass spectra were recorded on a Finnigan TSQ 700 triple quadrupole mass spectrometer with ESIF mode equipped with DEC 2100 data system. Full

scan spectra were acquired in the positive ion peak centroid or profile modes over the mass range 50–1200 at 1.2–2.0 s. Samples were dissolved in methanol–water (1:1) v/v, containing 5% acetic acid and introduced into the ion source by direct infusion or flow injection. ESIMS/CID/mass spectra were measured using Ar as a collision gas (collision energy, 40–50 eV) in the range of 1.2–3.0 mTorr and scanned at a rate of 2–3 s/decade through the required mass range, and 10–25 scans were accumulated and averaged.

^1H and ^{13}C NMR spectra were measured on a JEOL GMX-500 instrument. ^1H and ^{13}C NMR chemical shifts are referenced to DMSO- d_6 (solvent) signals: ^1H 2.49 and ^{13}C 39.5.

Table 1
ESIMS/CID/MS data for **1**

Ion composition	<i>m/z</i>	Fig. 3
M + H	1037	–
Ph–CH ₂ CH(OMe)	135	a
CH(Me)–CH=C(Me)–	163	a
CH=CH–CH ₂ –CH(Me)–CO		
163-Glu-Mdha	375	a
163-Glu-Mdha-Leu	488	a
163-Glu-Mdha-Leu-Leu	601	a
MeAsp-Arg-NH ₂ + 2H	303	b
Leu-MeAsp-Arg-NH ₂ + 2H	416	b
Mdha-Leu-Leu-MeAsp-Arg-NH ₂ + 2H	612	b
Glu-Mdha-Leu-Leu-MeAsp-Arg-NH ₂ + 2H	741	b
Leu-Leu-NH ₂ + 2H	244	–
Mdha-Leu + H	197	c-1
Mdha-Leu-Leu + H	310	c-1
Mdha-Leu-Leu-MeAsp + H	439	c-1
Mdha-Leu-Leu-MeAsp-Arg + H	595	c-1
Leu-MeAsp + H	243	c-2
Leu-MeAsp-Arg + H	399	c-2
Leu-MeAsp-Arg-Adda + H	712	c-2
Leu-MeAsp-Arg-Adda-Glu + H	841	c-2
Leu-MeAsp-Arg-Adda-Glu-Mdha + H	924	c-2
MeAsp-Arg + H	286	c-3
MeAsp-Arg-Adda + H	599	c-3
MeAsp-Arg-Adda-Glu + H	728	c-3
MeAsp-Arg-Adda-Glu-Mdha-Leu + H	924	c-3
Arg-Adda + H	470	c-4
Arg-Adda-Glu + H	599	c-4
Arg-Adda-Glu-Mdha + H	682	c-4
Glu-Mdha + H	213	–
Leu-Leu + H	227	–
Leu-Leu-MeAsp-Arg + H	512	–
Adda-Glu-Mdha-Leu-Leu-MeAsp-Arg + H	881	–
Mdha-Leu-CO + H	169	–
MeAsp-Arg-CO + H	258	–
Arg-Adda-CO + H	571	–

2.6. Determination of the stereochemistry of Leu

Compound **1** (200 μg) was treated with 6 M HCl (200 μl) at 110°C for 16 h. The reaction products were lyophilized and then reacted with a 10% acetone solution, (50 μl) of L-FDAA (1-fluoro-2,4-dinitrophenyl-5-L-alanine amide) and 1 M NaHCO₃ (100 μl) at 50°C for 1 h. After cooling to room temperature, the reaction mixture was neutralized with 2 M HCl (50 μl). The solution was diluted with 50% MeCN (1 ml) and subjected to HPLC analysis (10 μl/injection).

The L-FDAA derivatives of L- and D-Leu were prepared as above. The FDAA derivatives of hydrolysate of **1** and standard Leu were subjected to HPLC analysis using an ODS column (Cosmosil 5C18-MS, 4.6 × 250 mm², flow rate of 1.0 ml/min, and detection at 340 nm) and the gradient elution as follows: 0–60% (1%/min) MeCN containing 0.05% TFA.

The assignment of peaks resulting from L- and D-Leu was made by coinjection with the standard samples. The retention times (min) of FDAA derivatives of L-Leu and D-Leu were 52.4 and 56.2, respectively.

3. Results and discussion

The only MCYST compound found in the waterbloom samples was that identified as **1** (Fig. 1). Total amount as measured by ELISA was 0.35 mg/g (using MCYST-LR as the calibrator), while that estimated from HPLC was 0.4 mg/g (Fig. 2). The molecular weight (1036) and formula (C₅₂H₈₀N₁₀O₁₂) of **1** was determined based on ESIMS [(M + H)⁺, *m/z* 1037.6] and amino acid analysis data, which showed the presence of two Leu and one each of MeAsp, Arg, and Glu. ESIMS/CID/MS data for **1** (Table 1) revealed the Adda unit as an amino acid component of **1** by the characteristic fragment ion peaks at *m/z* 135 and 163 (Namikoshi et al., 1992, 1995, 1998; Yuan et al., 1999a). The seventh amino acid unit of **1** was deduced to be Mdha from the fragment ion

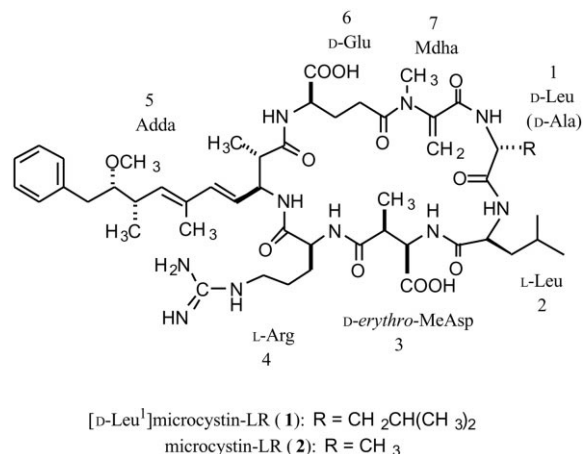


Fig. 1. Structures of a new microcystin ([D-Leu¹] MCYST-LR) (**1**) and MCYST-LR (**2**).

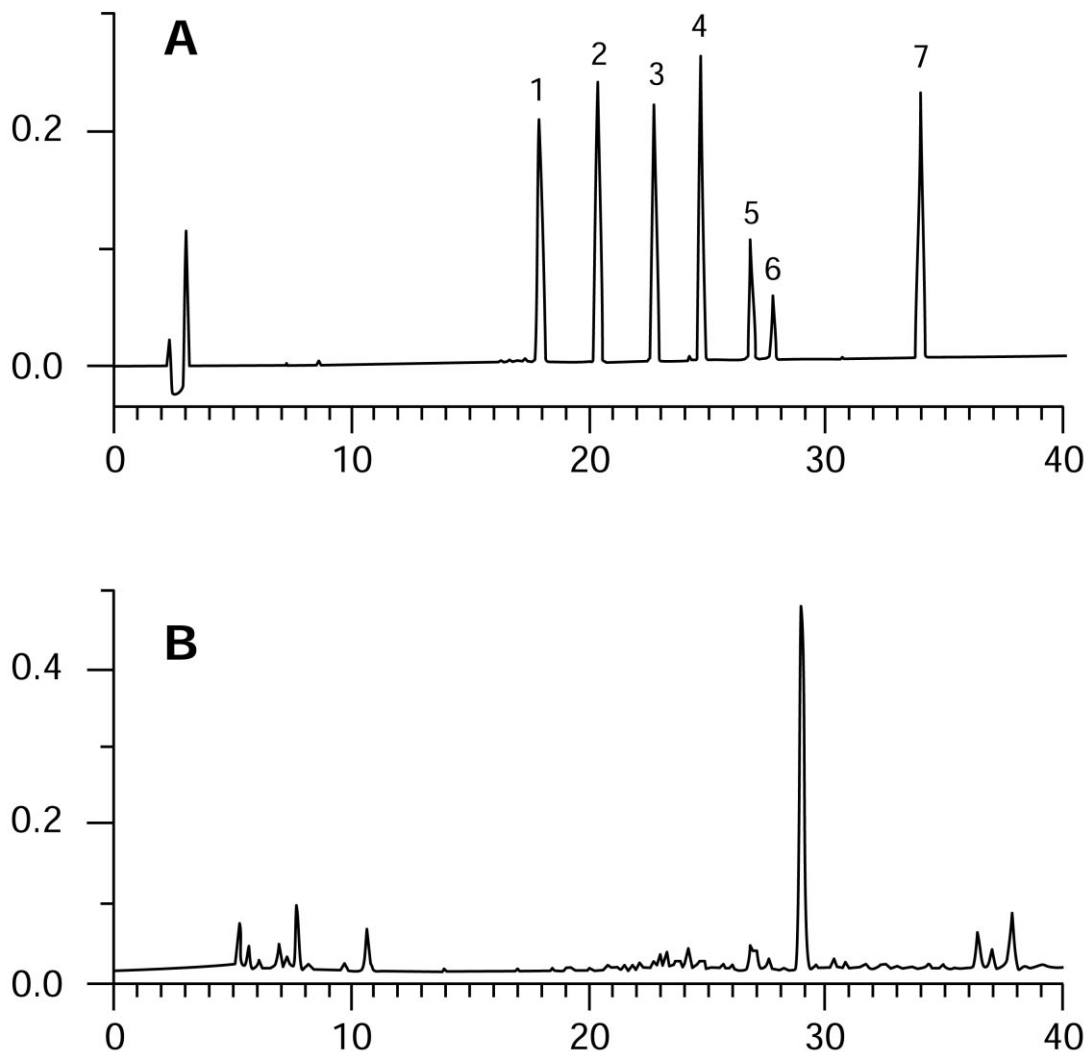


Fig. 2. Analytical HPLC chromatograms of: (A) test mixture for several microcystin (MCYST)/nodularin (NODLN) standards — (1) MCYST-RR, (2) NODLN, (3) MCYST-YR, (4) MCYST-LR, (5) MCYST-FR, (6) MCYST-WR, (7) MCYST-LA; (B) [D-Leu¹] MCYST-LR from Pakowki Lake, Alberta, Canada (retention time = 29 min).

peaks at m/z 213, 375, 197, etc. (Table 1 and Fig. 3) (Namikoshi et al., 1992, 1995, 1998; Yuan et al., 1999a).

The amino acid components of **1** resembled those of MCYST-LR (**2**) except for the presence of two Leu in **1** instead of Ala and Leu in **2**. The difference in the amino acid unit between **1** (Leu) and **2** (Ala) revealed the difference in the molecular weight (42 mass units) and formula (C_3H_6) between **1** and **2** ($M_r = 994$, $C_{49}H_{74}N_{10}O_{12}$).

These data suggested that **1** is the Leu variant of **2** at the [D-Ala¹] unit. The stereochemistry of the two Leu, in the acid hydrolysis products of **1**, was then determined by Marfey analysis (Marfey, 1984; Matsunaga and Fusetani, 1995), which showed the presence of D- and L-Leu together with D-MeAsp, L-Arg, and D-Glu.

The sequence of the amino acid components in **1** was determined by ESIMS analysis (Namikoshi et al., 1998; Yuan et al., 1998, 1999a,b). ESIMS/CID/MS data listed in Table 1 showed the complete sequence of the seven amino acid components of **1**.

Three remarkable fragmentation patterns were detected in the ESIMS/CID/mass spectrum of $[M + H]^+$ ion of **1** as similar to those observed in the spectrum of **2** (Yuan et al., 1999a). These fragment ion peaks were useful to assign the structure of **1** (Fig. 3). The fragment ion peak at m/z 135 was derived from the Adda residue and is the characteristic ion for MCYSTs possessing the ordinary Adda unit (Namikoshi et al., 1992, 1995; Yuan et al., 1998, 1999a). Cleavages of the above unit and C–N bond in the Adda residue

Table 2
 ^{13}C and ^1H NMR data for **1** in $\text{DMSO}-d_6$ (δ_{C} : 39.5 ppm, δ_{H} : 2.49 ppm)

AA	^{13}C	^1H mult. (<i>J</i> in Hz)	AA	^{13}C	^1H mult. (<i>J</i> in Hz)
D-Leu			Adda		
1	171.9	–	1	173.9	–
2	50.0	4.50 m	2	43.1	2.78 m
3	39.4 ^a	1.12 m	3	54.4	4.35 m
		1.78 m	4	126.3	5.32 dd (15.8, 8.0)
4	24.5	1.43 m	5	135.4	6.08 d (15.8)
5	21.0	0.77 d (6.9)	6	131.9	–
5'	21.0	0.77 d (6.9)	7	135.3	5.44 d (9.6)
NH	–	7.13 d (9.2)	8	35.3	2.54 m
			9	85.8	3.24 m
L-Leu			10	37.0	2.66 dd (14.0, 7.0)
1	171.1	–			2.73 dd (14.0, 5.0)
2	53.5	4.00 m	11	139.2	–
3	39.1 ^a	1.48 m	12	129.2	7.18 m
		1.75 m	13	128.0	7.26 m
4	24.2	1.65 m	14	125.8	7.17 m
5	23.1	0.78 d (6.5)	15	128.0	7.26 m
5'	23.0	0.84 d (6.5)	16	129.2	7.18 m
NH	–	7.63 d (7.3)	17	16.0	0.91 d (6.9)
			18	12.5	1.54 s
D-MeAsp			19	15.8	0.97 d (6.9)
1	174.4	–	20	57.4	3.18 s
2	55.4	4.10 m	NH	–	7.73 br d (7.7)
3	39.6 ^a	3.12 m	D-Glu		
4	178.3	–	1	172.8	–
5	15.1	0.87 d (7.3)	2	52.8	4.34 m
NH	–	7.87 d (9.2)	3	25.8	1.75 m
					1.86 m
L-Arg			4	31.2	2.21 m
1	170.2	–			2.30 m
2	51.6	4.11 m	5	175.1	–
3	28.4	1.31 m	NH	–	9.19 d (8.0)
		1.96 m	Mdha		
4	25.8	1.31 m	1	163.0	–
		1.37 m	2	144.8	–
5	40.5	3.00 m	3	113.8	5.38 s
6	156.5	–			5.80 s
2-NH	–	8.83 d (8.8)	N-CH ₃		
5-NH	–	7.38 br s		37.3	3.11 s

^a These signals were overlapped with solvent and calibrated the chemical shifts from 2D spectra.

gave the fragment $\text{CH}(\text{Me})-\text{CH}=\text{C}(\text{Me})-\text{CH}=\text{CH}-\text{CH}_2-\text{CH}(\text{Me})-\text{CO}$ (Fig. 3a). This fragment ion peak was detected in the ESIMS/CID/mass spectrum of **1** at m/z 163, and the fragment ion peaks containing this unit were observed at m/z 375, 488, and 601. These fragment ion peaks revealed the sequence of Adda-Glu-Mdha-Leu-Leu (Fig. 3a). The second pattern is the "C"-series fragmentation as shown in Fig. 3b, which gives an amide moiety at the C-terminus (Biemann and Martin, 1987). The fragment ion peaks of this pattern at m/z 303, 416, 612, and 741 showed the sequence of Glu-Mdha-Leu-Leu-MeAsp-Arg (Fig. 3b). The third pattern is the B-series fragmentation

(Biemann and Martin, 1987) showing the whole sequence of **1** (Fig. 3c). This type of cleavage gives the most abundant fragment ions detected in the ESIMS/CID/mass spectra of MCYSTs containing one Arg residue (Yuan et al., 1999a). Consequently, the structure of **1** was assigned as [D-Leu¹]microcystin-LR as shown in Fig. 1, and the accepted abbreviations apply (Carmichael et al., 1988).

The structure of **1** was confirmed by ^1H and ^{13}C NMR spectra measured in $\text{DMSO}-d_6$. Interpretation of COSY, HOHAHA (Edwards and Bax, 1986), HMQC (Summers et al., 1986), and HMBC (Bax et al., 1986) spectra allowed the complete assignment of ^1H and ^{13}C signals (Table 2). Fig. 4

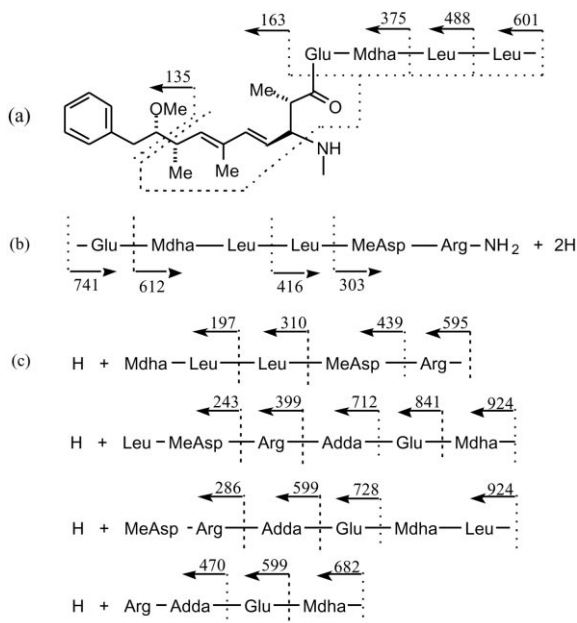


Fig. 3. Three characteristic fragmentation patterns (a–c) observed in the ESIMS/CID/MS of $[M + H]^+$ ion of **1**. The ion series were derived (a) from the Adda residue by loss of the 135 unit and C–N bond cleavage between Adda and Arg residues, (b) via the “C”-type fragmentation, and (c) by the “B”-type cleavage, which gave the most abundant fragment ions.

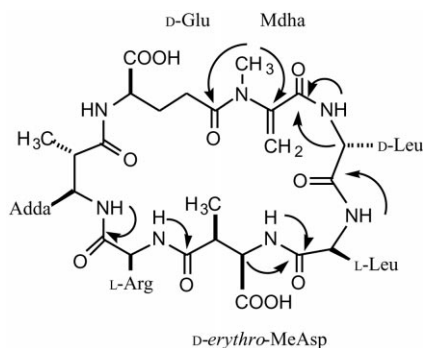


Fig. 4. Correlations showing the sequence information of **1** observed in the HMBC spectrum of **1**.

shows the HMBC correlations utilized to confirm the sequence of **1**.

Compound **1** is the second example containing a different amino acid unit at the D-Ala unit (amino acid position 1) of MCYSTs. The first example, [D-Ser¹,ADMAAdda⁵]-microcystin-LR, was obtained from *Nostoc* sp. strain 152 (Sivonen et al., 1992). It is the D-Ser variant at position 1 and it also has the 9-*O*-acetyl-9-*O*-demethylAdda unit instead of the Adda unit. Compound **1** is therefore the second MCYST example of a D amino acid variation in position 1 and the first example of a variation at position 1 that has a normal Adda.

Compound **1** showed similar toxicity to MCYST-LR as measured by using the PP1 assay ($IC_{50} = 0.3$ nM for both compounds). MCYSTs are known to cause deaths of waterfowl. However, their presence during times of bird losses in Pakowki Lake was not monitored as closely as other, more well-known pathogens such as *Clostridium* that causes fowl botulism. For this reason it is not known whether MCYSTs have contributed to the repeated bird losses in Pakowki and other lakes with a similar pattern of waterfowl poisonings, but based upon their presence during times of waterfowl losses and known toxicity to birds, it is certainly possible.

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