7-Desmethyl-Microcystin-RR, a Hepatotoxin from a Waterbloom of *Microcystis aeruginosa*

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A peptide toxin was isolated from a waterbloom of *Microcystis aeruginosa* from Lake Frøylandsvatn in Norway. The isolation procedure included liquid and solid phase extraction and reversed phase high performance liquid chromatography. Amino acid analysis yielded D-glutamic acid, D-*erythro*-β-methylaspartic acid and D-alanine in equimolar and L-arginine in twofold molar ratios. The presence of dehydroalanine was confirmed by hydrogenation and subsequent amino acid analysis with combined gas liquid chromatography/mass spectrometry. Investigation of the toxin with fast atom bombardment mass spectrometry showed a nominal relative molecular mass of 1023. 3-Amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4,6-decadienoic acid (Adda) was identified by ^{1}H NMR and ^{1}H, ^{1}H COSY spectroscopy. The structure of the toxin was elucidated as 7-desmethyl-microcystin-RR.

Introduction

Cyanobacteria of the genera *Microcystis, Oscillatoria, Anabaena* and *Nostoc* are known to produce hepatotoxic cyclic heptapeptides called microcystins, which cause poisoning of wild animals and livestock in different parts of the world [1–3]. Only hepatocytes are susceptible to microcystins, and competition studies with several peptides and bile acids indicated that the toxins use the multispecific bile acid transport system to enter the cell [4, 5]. Recently, microcystins and the closely related hepatotoxic pentapeptide nodularin from the cyanobacterium *Nodularia spumigena* were found to inhibit strongly and specific protein phosphatases 1 and 2A from different eucaryotic cells [6, 7].

Abbreviations: Adda, 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4,6-decadienoic acid; FAB-MS, fast atom bombardment mass spectrometry; GC, gas-liquid chromatography; GC/MS, combined gas-liquid chromatography/mass spectrometry; HPLC, high performance liquid chromatography; NMR, nuclear magnetic resonance.

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Toxins of the microcystin type can be described as cyclo(-D-Ala^1-X^2-D-*erythro*-β-methyl*iso*Asp^3-Y^4-Adda^5-D-*iso*Glu^6-N-methyldehydroAla^7) where Adda is the abbreviation of 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4,6-decadienoic acid. The positions X and Y refer to distinct pairs of L-amino acids depending on the strain. Additionally N-methyldehydroalanine (position 7) and/or D-*erythro*-β-methylaspartic acid may be present non-methylated. The methylation status of a distinct microcystin variant is described by a prefix to the term "microcystin" where the suffix XY refers to the respective variant amino acids in the single letter code [8].

Materials and Methods

Isolation of toxin, reversed phase HPLC

Cells were harvested from a hepatotoxic waterbloom of *Microcystis aeruginosa* from Lake Frøylandsvatn in Norway. Lyophilized cells (4 g) were extracted twice with 5% acetic acid in water (v/v) and the collected supernatants were applied to C_{18} cartridges (Chromabond, Macherey & Nagel, Düren, F.R.G.). After elution with methanol the C_{18} eluate was diluted with water to 10% methanol

and lyophilized. Reversed phase HPLC was performed using a Waters 600 E multisolvent delivery system (Waters, Eschborn, F.R.G.) consisting of a Waters 600 E controller, a fluid handling unit and a Rheodyne 7125 injection valve. Chromatographical and spectral data were recorded with a Waters 991 photodiode array detector connected to an AT-compatible personal computer (Nec Powermate 386SX plus). Data processing was achieved with Powerline PDA software 6.22a. A Superpac Pep-S ODS column (5 µm, 4.0 × 250 mm; Pharmacia LKB Biotechnology, Uppsala, Sweden) was used for toxin purification at room temperature. The chromatographic conditions were: flow rate 1.0 ml/min; eluent A 25 mM ammonium acetate, pH 6.0; eluent B 25 mM ammonium acetate in methanol/water/acetonitrile (1:2:7, v/v/v), isocratic elution at 36% B.

Catalytic hydrogenation, hydrolysis with trifluoroacetic acid

The peptide (1 mg in 1 ml methanol) was stirred for 1 h at room temperature with palladium/charcoal under a hydrogen stream. Repeated washing of the catalyst with methanol and subsequent drying of the solvent *in vacuo* yielded 0.5 mg of the hydrogenated peptide. For hydrolysis with trifluoroacetic acid, a solution of 0.5 mg toxin in 0.5 ml of the acid was incubated overnight at room temperature, concentrated *in vacuo,* diluted with A. dest. and lyophilized.

Amino acid, GC, and GC/MS analyses

Toxin (200 µg) was hydrolyzed in 400 µl of 6 M HCl at 100 °C for 18 h. Amino acids were separated by ion exchange chromatography on a Biotronic amino acid analyzer (LC 6001) using an automatic sample injector (RT 7040, Biotronic, München, F.R.G.). Elution was monitored at 570 nm and 440 nm (proline) after ninhydrin reaction. For GC and GC/MS, the toxin or its reduction product were hydrolyzed in 200 µl of 6 N HCl at 100 °C for 18 h. After drying *in vacuo* the sample was incubated with 1 ml of HCl/methanol (1.5 N) at 100 °C. The sample was dried in a nitrogen stream and heated with 50 µl trifluoroacetic acid anhydride and 200 µl dichloromethane at 100 °C for 15 min. Excess reagent was removed in a nitrogen stream and the sample was taken up in dichloromethane. The derivatives were analyzed by capillary gas chromatography/mass spectrometry (HP 5840 and HP 5985 A, Hewlett Packard, Palo Alto, U.S.A.) using a fused silica capillary column (SE-54, 25 m) and electron impact at 70 mV. For configurational analysis a capillary column coated with a chiral stationary phase (Lipodex E, 30 m, [9]) was used.

FAB-MS and NMR analyses

FAB-MS spectra were obtained using a VG 70-250 S instrument (VG Analytical, Manchester, United Kingdom). Operation conditions were: 0.1 to 0.3 mg sample in 3-nitrobenzylalcohol or a mixture of glycerol and thioglycerol as matrix, xenon as collision gas, and an accelerating voltage of 8 kV. For NMR-analyses, a Bruker WM 400 spectrometer (Bruker, Karlsruhe, F.R.G.) was used for ^{1}H NMR and ^{1}H, ^{1}H correlation spectroscopy (COSY). The peptide (3 mg) was measured in deuterated methanol (CD_3OD) which was also used as an internal standard (3.30 ppm).

Results

Isolation of the toxin

The HPLC elution profile of the C_{18} eluate recorded at 214 nm shows three major peaks (Fig. 1 A) where only fraction P_2 exhibits the characteristic UV absorption spectrum of Adda (Fig. 1 B). Up to 200 mg crude material were applied repeatedly to reversed phase HPLC and P_2 was collected. Rechromatography of P_2 yielded the pure toxin (Fig. 2) with a retention time of 10.8 min. It was further used for chemical analysis. The yield of the toxin was 2.6 mg per g of cell dry weight.

Amino acid analysis

Amino acid analysis (ion exchange separation/ninhydrin detection) showed the toxin to contain β-methylaspartic acid, glutamic acid and alanine in equimolar amounts, while arginine is present in the two-fold molar amount (Fig. 3). β-Methylaspartic acid, alanine, glutamic acid and arginine were confirmed by GC/MS of their trifluoroacetylated methylester derivatives. β-Methylaspartic acid was identified as the *erythro* diastereomer by comparison with a standard mixture of the authentic *threo*- and *erythro*-forms. Stereo-

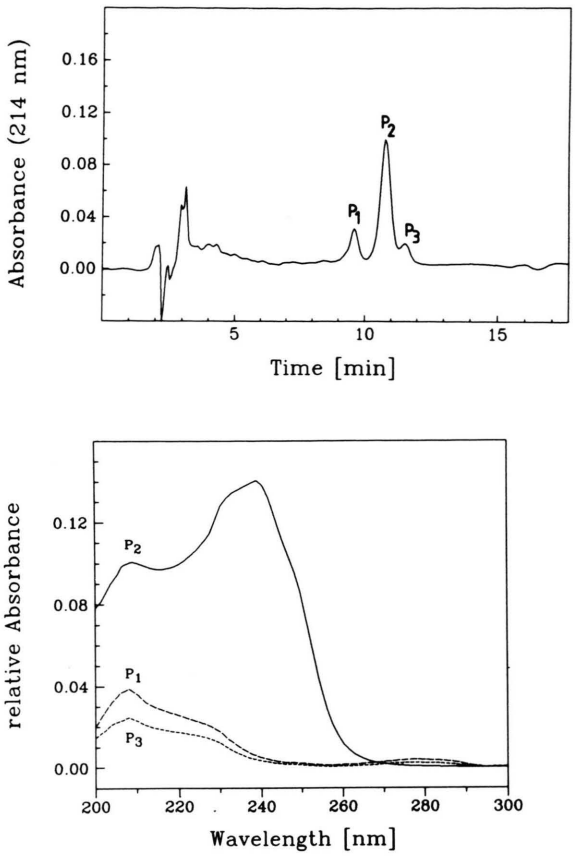

Fig. 1. (A): HPLC elution profile from 10 µg of the C_{18} eluate obtained from solid phase extraction. Column: Pep-S, ODS, 5 m, 4.0 × 250 mm. Elution: isocratic at 36% B where eluent A is 25 mM ammonium acetate, pH 6.0 and eluent B is 25 mM ammonium acetate in methanol/water/acetonitrile (1:2:7, v/v/v). Flow rate: 1 ml/min. Detection: monitor wavelength: 214 nm. (B): UV spectra of P_1, P_2 and P_3, respectively, were recorded on line from 200 to 300 nm with a photodiode array detector.

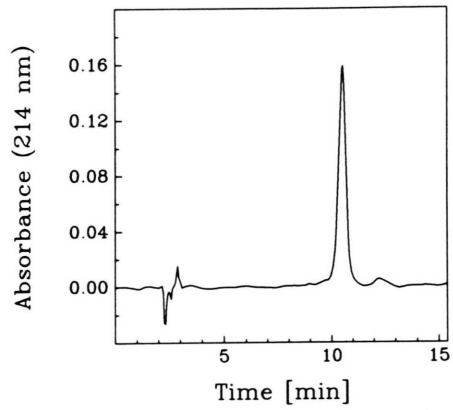

Fig. 2. Pure toxin obtained after rechromatography of P_2 from C_{18} eluate, chromatographic conditions were as described in Fig. 1 A.

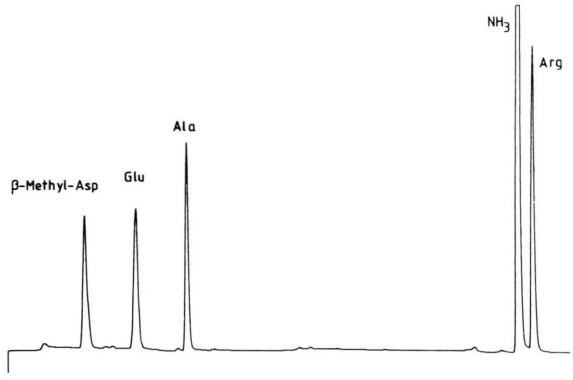

Fig. 3. Elution profile of amino acids after total hydrolysis of the toxin. Separation was performed on a cation exchange column. Detection was done with on line post column derivatization of the eluted amino acids with ninhydrin and subsequent monitoring at 570 nm and 440 nm. The molar amounts of components were determined by comparison with external authentic standards. β-Methyl-Asp, β-methylaspartic acid; Glu, glutamic acid; Ala, alanine; Arg, arginine.

chemical analysis by gas chromatography using a column with a chiral stationary phase yielded D-alanine, D-glutamic acid and L-arginine. After hydrogenation of the toxin the hydrolyzate proved to contain no N-methylalanine but L-alanine and additional D-alanine. This suggests the existence of dehydroalanine in the toxin which gave a mixture of D- and L-alanine by hydrogenation, but not of N-methyldehydroalanine.

Although Adda was not observed by GC/MS, we found a double peak of its methanol elimination products with a molecular mass of 409 (trifluoroacetylated methylester). GC/MS of the hydrogenated sample showed a trace of the elimination products, with a molecular mass of 413 (tetrahydroAdda-methanol).

FAB-MS and NMR spectroscopy

A single large $(M + H)^+$ signal at m/z 1024.8 indicates a relative molecular mass of 1023. The

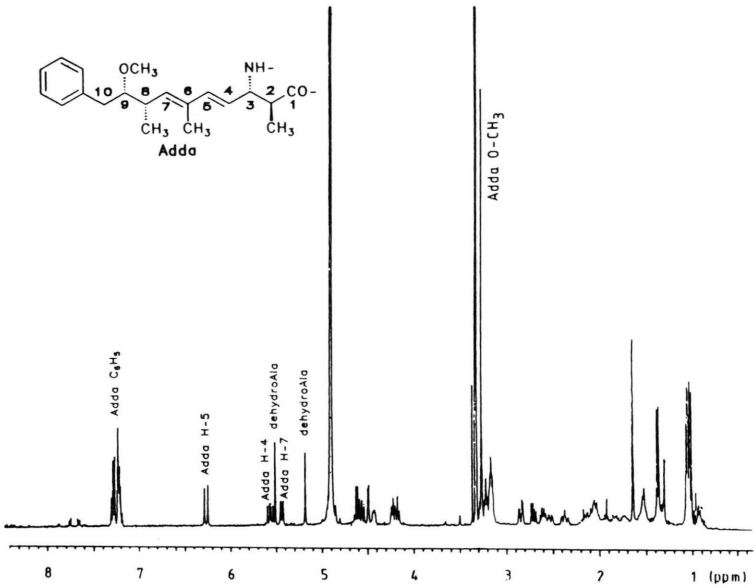

Fig. 4. ^{1}H NMR spectrum of the toxin, 400 MHz, 3 mg sample in CD_3OD (3.30 ppm). The characteristic aromatic and olefinic protons (5–7.5 ppm) are assigned to Adda and dehydroalanine. Adda: 7.21 ppm (C_6H_5-10, m), 6.23 ppm (H-5, d), 5.53 ppm (H-4, dd) and 5.40 ppm (H-7, d); dehydroalanine: 5.47 ppm (H-3, s) and 5.15 ppm (H-3, s), where dehydroalanine shows a geminal coupling constant of 0 Hz (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet).

toxin after trifluoroacetic acid treatment yielded a $(M + H)^+$ ion at m/z 1042.5 which is expected for the linear peptide after hydrolysis of dehydroalanine [10].

The ^{1}H NMR spectrum (Fig. 4) reveals the characteristic signals of the aromatic and olefinic protons of Adda, C_6H_5-10, H-5, H-4 and H-7 at 7.21, 6.23, 5.53 and 5.40 ppm, respectively. The other two olefinic protons (5.47 and 5.15 ppm, singlets) are those of dehydroalanine which have the geminal coupling of 0 Hz. In the COSY spectrum (Fig. 5) the two correlation series of Adda

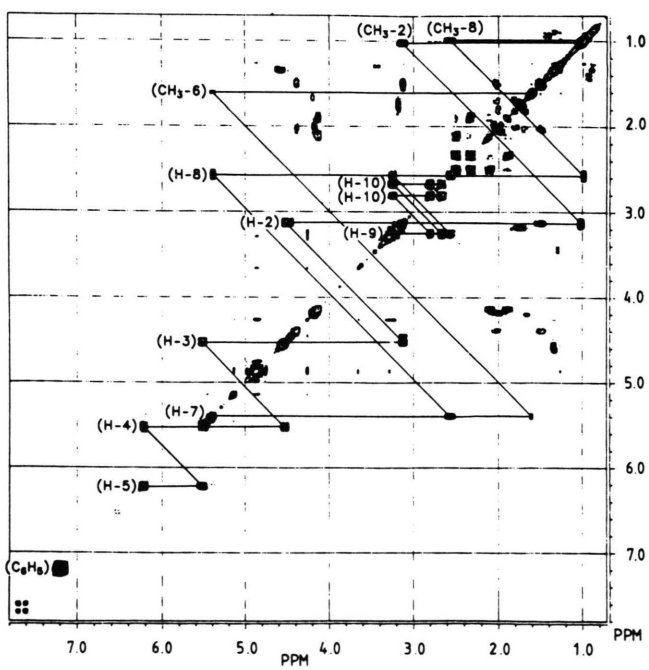

Fig. 5. ^{1}H, ^{1}H COSY spectrum of the toxin, 400 MHz, 3 mg sample in CD_3OD (3.30 ppm). See text for the correlation of Adda protons. The geminal protons of dehydroalanine H-3 (5.47 ppm, s) and H-3 (5.15 ppm, s) hardly show any correlation.

(H-2 to H-5 and H-7 to H-10) can be detected easily. The one is [H-5 (6.23 ppm, d) → H-4 (5.53 ppm, dd) → H-3 (4.53 ppm, dd) → H-2 (3.14 ppm, dq) → CH_3-2 (1.03 ppm, d)]. The other one is [H-10 (2.82 ppm, dd) ↔ H-10 (2.68 ppm, dd) → H-9 (3.26 ppm, ddd) → H-8 (2.58 ppm, ddq) → H-7 (5.40 ppm, d) → CH_3-6 (1.62 ppm, s)] where H-8 has an additional correlation to CH_3-8 (0.99 ppm, d).

The singlet (3.24 ppm) in the ^{1}H NMR spectrum which shows no correlation in the ^{1}H, ^{1}H COSY was assigned to the methoxy protons (OCH_3) of Adda, according to its low field chemical shift. All the Adda protons, including the methoxy group, are almost identical with those of microcystin-RR reported in ref. [11]. The other amino acids could also be characterized by means of ^{1}H NMR and ^{1}H, ^{1}H COSY spectra, and are listed in Table I. The doublet of H-2 in β-methylaspartic acid (4.46 ppm) is additional evidence for the β-methylation of aspartic acid.

Table I. ^{1}H NMR signals of 7-desmethyl-microcystin-RR.

Amino acid	Chemical shift [ppm]	Multiplicity	Integral	Coupling constant [Hz]	Assignment
Adda					
	7.21	m	5H		C_6H_5
	6.23	d	1H	15.4	H-5
	5.53	dd	1H	15.5/9.2	H-4
	5.40	d	1H	9.7	H-7
	4.53	dd	1H	10.4/9.4	H-3
	3.26	ddd	1H	7.5/6.3/4.7	H-9
	3.24	s	3H		OCH_3-9
	3.14	dq	1H	10.4/7.0	H-2
	2.82	dd	1H	13.9/4.8	H-10
	2.68	dd	1H	13.9/7.2	H-10
	2.58	ddq	1H	9.8/6.6/6.6	H-8
	1.62	s	3H		CH_3-6
	1.03	d	3H	6.8 or 6.4	CH_3-2
	0.99	d	3H	6.8	CH_3-8
Alanine					
	4.59	q	1H	7.4	H-2
	1.35	d	3H	7.3	CH_3-3
Dehydroalanine					
	5.47	s	1H		H-3
	5.15	s	1H		H-3
β-Methylaspartic acid					
	4.46	d	1H	4.0	H-2
	3.14	m	1H		H-3
	1.03	d	3H	6.8 or 6.4	CH_3-3
Glutamic acid					
	4.14	dd	1H	7.8/7.8	H-2
	2.49	ddd	1H	15.8/11.8/5.4	H-4
	2.34	ddd	1H	15.6/12.0/3.2	H-4
	2.14	m	1H		H-3
	1.91	m	1H		H-3
Arginine					
	4.18	dd	1H	8.0/7.0	H-2
	3.19	t	2H	6.4	H-5
	2.01	m	2H		H-3
	1.81	m	1H		H-4
	1.71	m	1H		H-4
Arginine					
	4.40	dd	1H	12.6/3.6	H-2
	3.13	m	2H		H-5
	2.05	m	2H		H-3
	1.50	m	2H		H-4

Discussion

Since *Botes* and coworkers had proposed the structure of microcystin-LA [12], the structures of nine different microcystins have been reported [13]. While microcystin-LR and -RR are the most common variants, 7-desmethyl-microcystin-RR seems to be a very rare one. To our knowledge this toxin has been found only in *Oscillatoria agardhii* strain CYA-129 [4]. In our study, 7-desmethyl-microcystin-RR has been found as a single hepatotoxic peptide in the *Microcystis aeruginosa* strain studied.

The lack of fragment ions in FAB-MS indicates that the peptide with a relative molecular mass of 1023 is cyclic. The increase of 18 mass units in the FAB-spectrum of the trifluoroacetic acid hydrolysate was due to the addition of water during the cleavage of the labile C-N bond of dehydroalanine [10]. The components, Adda, β-methylaspartic acid, D-glutamic acid, D-alanine and L-arginine lead to a microcystin variant where the positions X and Y are occupied by L-arginine. The finding of additional D- and L-alanine in the hydrogenated peptide shows the native toxin to contain dehydroalanine instead of N-methyldehydroalanine. This was in agreement with the relative molecular mass of 1023 which differs by 14 mass units from fully methylated microcystin-RR. We conclude that the unknown toxin was 7-desmethyl-microcystin-RR. The structure of 7-desmethyl-microcystin-RR is shown in Fig. 6 according to the established amino acid sequence of microcystin-RR [14]. The stereochemistry of the amino acids except Adda was determined to be identical to that published by other groups.

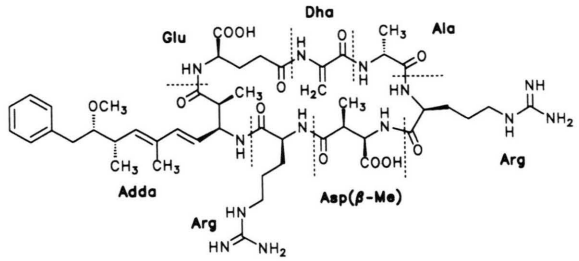

Fig. 6. Structure of 7-desmethyl-microcystin-RR. Dha = dehydroalanine, Asp(β-Me) = β-methylaspartic acid.

The photodiode array detection in HPLC analysis applied in this study is a valuable tool for rapid and reliable detection of even small amounts of microcystins. Absorption spectra of the peaks are taken during the HPLC run and can be automatically compared to a standard spectrum stored in a spectral library. Peak purity can be easily assessed by using the peak contour plot device from the PDA software.

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