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THE PEPTIDE TOXIN OF THE CYANOBACTERIUM *MICROCYSTIS AERUGINOSA* PCC 7941

ISOLATION AND ANALYSIS BY NUCLEAR MAGNETIC RESONANCE AND FAST ATOM BOMBARDMENT MASS SPECTROSCOPY

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SUMMARY

Toxin was obtained from the cyanobacterium *Microcystis aeruginosa* PCC 7941 by extracting freeze-dried cells with water-saturated, acidified *n*-butanol, diethyl ether–water distribution, reversed-phase thin-layer chromatography and silica high-performance liquid chromatography (HPLC). Two toxic peptide fractions resulted from HPLC. One of these fractions was analyzed by UV and NMR spectroscopy, amino acid analysis and fast atom bombardment mass spectroscopy. The following amino acids were identified: β-methyl-Asp, Thr, Glu, Ala, Val, Leu, Phe, Arg, N-methyldehydro-Ala and 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid. Yet the mass spectroscopic data showed that the fraction was still composed of several, most likely cyclic peptides that did not stain with ninhydrin.

INTRODUCTION

Microcystis aeruginosa, an ubiquitous, waterbloom-forming cyanobacterium, is known to produce hepatotoxins. Death of livestock and game due to these toxins has been reported from Australia[1,2], South Africa[3–6], North America[7–12], Europe[13–18] and Asia[19,20]. The lethal dose of the toxins to mice is reportedly of the order of 70 μg/kg or less[1].

Pathological findings include swollen, blood-engorged liver with haemorrhargic necrosis, oedematous lung and loss of architecture of the hepathocystes. Except for lung congestion, other organs seem not to be significantly affected. Under lethal doses, mice usually die within 30 min to 4 h, and death is preceded by pallor and prostation with terminal episodes of unprovoked leaping and twitching. Sublethal doses cause

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respective chronic damage. Gastroenteritis and liver damage have been observed in humans as well[1,12,21–25].

Attempts to identify the structure of the toxins from *Microcystis aeruginosa* had been rather confusing[1,3,7,11,26–32] until 1984, when Botes *et al.*[27] published a cyclic heptapeptide structure for a toxin isolated from a laboratory clone of *Microcystis aeruginosa* that originated from a natural bloom in Witbank Dam, South Africa. This toxin consists of D-Ala–L-X–*erythro*-β-methyl-D-iso-Asp–L-Y–3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,-dienoic acid (Adda)–D-iso-Glu–N-methyldehydro-Ala (X and Y represent variable L-amino acids) giving a total molecular mass of 909. The authors assumed some variation with respect to the two L-amino acids in the toxins of other *Microcystis* sp. isolates.

This report describes the isolation and analysis of the toxin from *Microcystis aeruginosa* strain PCC 7941 (syn. NRC-1[11]). On comparison with the toxins described by Botes *et al.*[27,28], the structural similarity with only minor modification becomes obvious. Furthermore, the presence of non-toxic peptides, having properties very similar to those of the toxin, will be pointed out.

EXPERIMENTAL

Materials and chemicals

All solvents and chemicals were of analytical grade. Uvasol grade methanol and chloroform were used when necessary. Ninhydrin spray and thin-layer chromatography (TLC) plates (RP_{18} F 254 S and cellulose) were obtained from Merck (Darmstadt, F.R.G.). β-Methyl-Asp was from Sigma (München, F.R.G.) analytical (25 cm × 0.8 cm) and preparative (25 cm × 3 cm) high-performance liquid chromatographic (HPLC) columns (Partisil-5) from Knaur (Bad Homburg, F.R.G.) and Sephadex G-15 and G-25 from (Pharmacia, Freiburg, F.R.G.). Sep-Pack C_{18} cartridges were from Waters (Eschborn, F.R.G.). The unialgal strain of *Microcystis aeruginosa* PCC 7941 (syn. NRC-1), originating from Little Rideae Lake, Ontario[11], was from the strain collection of the Institut für Biologie II, Mikrobiologie, Universität Freiburg. The cultures were cultivated in BG 11 medium[33] at 25°C in 1-l erlenmeyer flasks or in a 10-l fermentor. Male mice (10–11 weeks old, mean weight 33.3 ± 3 g) were kindly supplied by H. Mönig, Institut für Biophysik und Strahlenbiologie, Universität Freiburg. The mice received a normal laboratory diet from Altromin, Lage/Lippe, and acidified tap-water (pH 3) *ad libitum*.

Buffers

The following buffers were used: (A) 0.1 *M* sodium phosphate pH 8.0; (B) 27 m*M* Tris, 142 m*M* glycine pH 8.2–8.5; (C) 150 m*M* Tris–HCl pH 8.8.

Toxin isolation

Microcystis was harvested from cultures at the end of their exponential growth phase. Lyophilized cells (10–30 g) were extracted for 20 min at room temperature with water-saturated *n*-butanol (300 ml) containing 1% acetic acid. After centrifugation (1830 *g*, 15 min), the pellet was reextracted and the pooled supernatants were dried under reduced pressure (35°C). The residue was redissolved in water, the pH adjusted to 8 and lipids were extracted into diethyl ether by gentle mixing and subsequent

centrifugation (1830 *g*, 4°C, 5 min). The aqueous phase was separated from the non-toxic interlayer and the ether phase. It was dried under reduced pressure (35°C) and the residue was applied to silica gel RP_{18} thin-layer plates in methanol. The plates were developed in methanol–water–acetic acid (60:38:2, v/v/v), and the fluorescence-quenching band at R_F 0.2 was extracted into propan-2-ol. The extract was dried, the residue was redissolved in chloroform–methanol (7:3, v/v) and the solution was filtered through an HV 0.45 filter (Millipore, Eschborn, F.R.G.) prior to HPLC separation on Partisil-5.

Native polyacrylamide gel electrophoresis (PAGE)

Gradient PAGE was performed on slabs (1 mm × 100 mm × 100 mm, 11.5–18% acrylamide) at 15 mA in buffers B (electrode) and C (separation). The developed gels were cut into 2-mm sections, and the sections were extracted overnight with buffer A. Protein in the extracts was determined spectrophotometrically (mg protein per ml = $1.55\ E_{280} - 0.76\ E_{260}$), and toxin by its maximum at 240 nm. The toxicity of the extracts was tested by the mouse bioassay.

HPLC

Separation on Partisil-5 by HPLC (Beckman or Waters) was accomplished by a linear gradient made up from 30 to 80% methanol–acetic acid (99:1, v/v) in chloroform–acetic acid (99:1, v/v) at a flow-rate of 2 ml/min (analytical column) or 10 ml/min (preparative column). The elution was monitored at 254 nm.

Spectrometry

Ultraviolet spectra were recorded on a Kontron UVIKON 810 photometer. ^1H (300 MHz) and ^{13}C NMR (75 MHz) spectra were recorded on a Bruker WM 300 spectrometer (Max-Planck-Institut für Immunobiologie, Freiburg, F.R.G.), using tetramethylsilane (TMS) as an internal reference. Fast atom bombardment mass spectroscopy (FAB-MS) was performed on a Kratos MS 50 RF spectrometer equipped with a Kratos source (Gesellschaft für Biotechnologische Forschung, Braunschweig, F.R.G.). Glycerol was used as a matrix.

Amino acid analysis

Toxin (*ca.* 50 μg), corresponding to the HPLC fraction with t_R 16.45 min, was hydrolyzed in 6 *M* hydrochloric acid (0.5 ml) for 24 h at 105°C in sealed tubes, and the samples were subsequently subjected to amino acid analysis in a Biotronik LC 6001 amino acid analyzer with an automatic sample injector BT 7040. Elution of the amino acid derivatives was monitored at 570 and 440 nm (proline).

RESULTS

Toxin purification

The toxin present in *Microcystis aeruginosa* PCC 7941, which caused rapid death of mice in the bioassay, was extracted completely from the lyophilized cells by one extraction with acidified, water-saturated *n*-butanol, as revealed by control extractions of the extracted cells with aqueous buffers or acidified *n*-butanol. Furthermore, the

butanol extracts contained comparatively little protein. Purification of the toxins was therefore started routinely from these extracts.

Lipids were subsequently removed from the *n*-butanol extracts by a gentle diethyl ether water (1:1, v/v) distribution. A clear, slightly yellow aqueous phase and a clear, dark green ether phase, separated by a solid orange interphase, resulted. The toxin, recovered in the aqueous phase (approximately 90% of total), was subjected to gel chromatography on Sephadex G-15. The entire toxic activity was eluted with the void volume, indicating a molecular weight exceeding 1500 daltons. Upon re-chromatography on Sephadex G-25, the toxin was slightly retarded compared with the void volume, suggesting a molecular weight of roughly 4000 daltons.

The toxic material was subjected to TLC on silica RP_{18} and the toxin recovered from a fluorescence-quenching band at R_F 0.2 revealed by irradiation at 254 nm. This chromatography replaced the previously used filtration through Sep-Pak C_{18} cartridges and elution of the toxin with *n*-butanol. The compounds in the fluorescent band showed no colour reaction with ninhydrin, and subsequent HPLC on Partisil-5 revealed three major ultraviolet-absorbing fractions (Fig. 1). Fractions at t_R 9.99 and 16.45 min, respectively, contained the toxic activity. Rechromatography by HPLC under the same conditions of either of these toxic fractions again resulted in two toxic fractions. Various modifications of the elution conditions did not significantly change this result nor was the degree of purification improved.

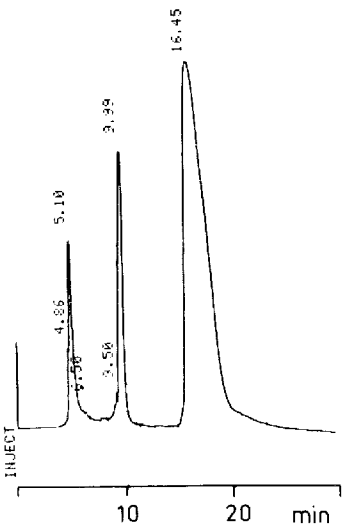

Fig. 1. UV absorbance profile (at 254 nm, in methanol) of the HPLC separation of the toxin of *Microcystis aeruginosa* PCC 7941. The fractions with t_R 9.99 and 16.45 min showed toxic activity.

Analysis of the toxic fractions

The UV absorbance spectra of either of the toxic fractions from HPLC showed maxima at 232 and 238 nm (Fig. 2). These maxima are similar to those described by Botes *et al.*[3] and Eloff[4,34] for the cyanoginosins from a South African *Microcystis aeruginosa* strain and might result from a conjugated diene.

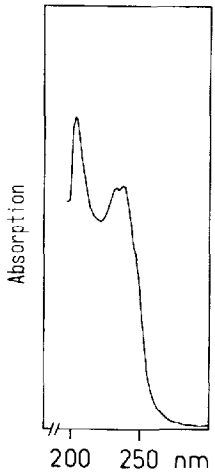

Fig. 2. UV absorbance spectrum (in methanol) of the toxin (fraction at t_R 16.45 min in Fig. 1). Maxima: 203, 232 and 238 nm.

Also the ^1H NMR spectra (Fig. 3), particularly the aromatic and olefinic resonance patterns between 5.1 to 7.3 ppm, were very similar to those reported previously by Botes *et al.*[3]. Resonance signals for the unusual amino acid Adda (H-4, quartet at 5.4 ppm; H-7, doublet at 5.50 ppm; H-5, doublet at 6.3 ppm; phenyl residue,

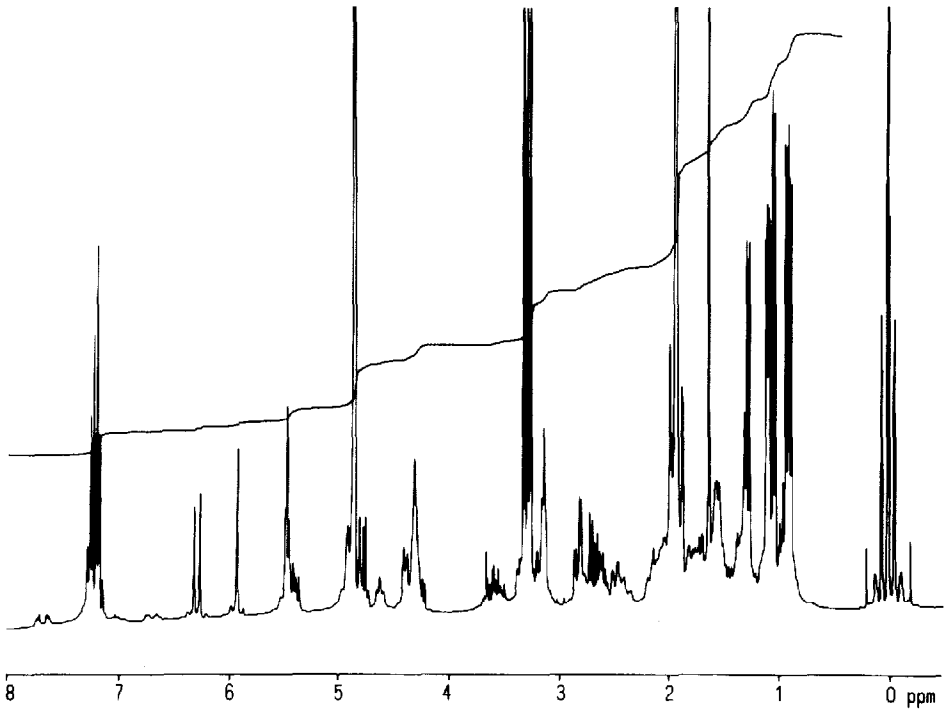

Fig. 3. ^1H NMR spectrum (in ^2H_2O; TMS, $\delta = 0$) of the toxin (fraction at t_R 1.45 min in Fig. 1).

7.0–7.3 ppm; 6-O-methyl, singlet at 1.62 ppm) and N-methyldehydro-Ala (H-3, singlets at 5.50 and 5.94 ppm) were apparent in the spectra. Homonuclear proton–proton decoupling experiments revealed the coupling of H-4 (5.4 ppm) with H-5 (6.3 ppm) and of H-4 (5.4) with H-3 (4.6 ppm) in the Adda moiety. Furthermore, the methoxy group of Adda was identified by ^{1}H NMR (3.28 ppm) and ^{13}C-NMR spectroscopy in $^{2}H_2O$ (58.4 ppm), as well as in $C^{2}H_3O^{2}H$ (58.9 ppm) (^{13}C NMR data not shown). The N-methyl group of N-methyldehydro-Ala was apparent at 3.25 ppm in the ^{1}H NMR spectrum and at 38.5 ppm in the ^{13}C NMR spectrum in $C^{2}H_3O^{2}H$. A characteristic signal at 88.4 ppm in the ^{13}C NMR spectrum in $C^{2}H_3O^{2}H$ or at 88.0 ppm in $^{2}H_2O$ might be assigned to C-9 of Adda. The poor spectral resolution between 1.8 and 5.0 ppm in the ^{1}H NMR spectrum (Fig. 3) did not allow the assignment of individual signals. The presence of Ala was, however, apparent by coupling of the respective methyl resonance (1.25 ppm) with that of the α-proton, as well as by the methyl signal in the ^{13}C NMR spectrum (17.3 ppm) (coupling data not shown). The doublet at 0.9 ppm and the CH_3 resonances at 21.2 and 23.3 ppm in the ^{1}H NMR spectrum, as well as a signal at 40.5 ppm in the ^{13}C NMR spectrum in $C^{2}H_3O^{2}H$ (C-3) were assigned to Leu, which had been identified by the amino acid analyzer. The additional four methyl doublets in the NMR spectrum most likely originated from Adda (two doublets), β-methyl-Asp and Val. The identification of β-methyl-Asp was corroborated by signals at 16.9 (CH_3) and 36.9 ppm (C-3) in the ^{13}C NMR spectrum in $^{2}H_2O$. The small signal at 1.55 ppm perhaps originated from the methyl group of Thr. This resonance was coupled with a signal at 4.4 ppm, which most likely must be assigned to H-3 of Thr.

Analysis of the amino acids, after complete hydrolysis of the peptides, showed β-methyl-Asp (3–4), Thr (1), Glu (4), Ala (5), Val (3), Leu (6), Phe (2) and Arg (4), the values in parentheses representing molar ratios. The presence of β-methyl-Asp was

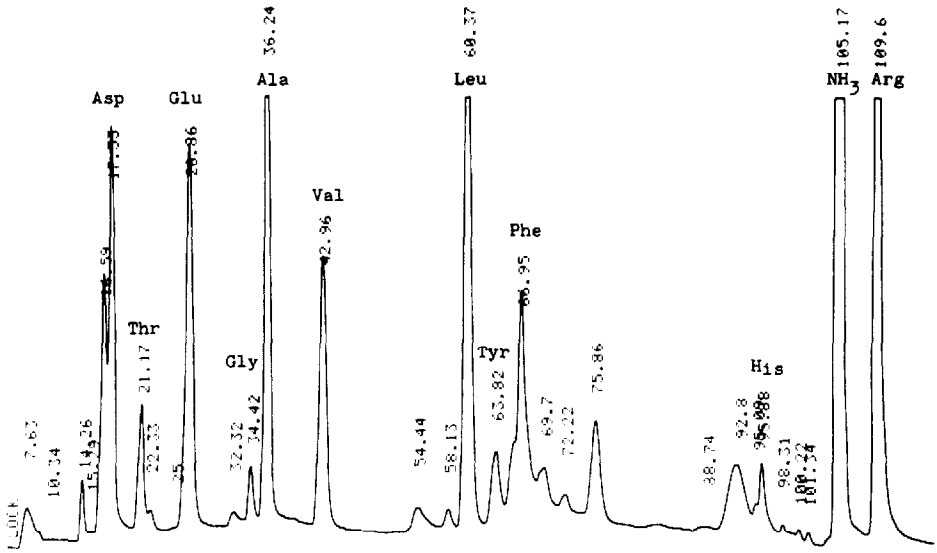

Fig. 4. Analysis by the automatic amino acid analyzer of the toxin (fraction at t_R 16.45 min in Fig. 1).

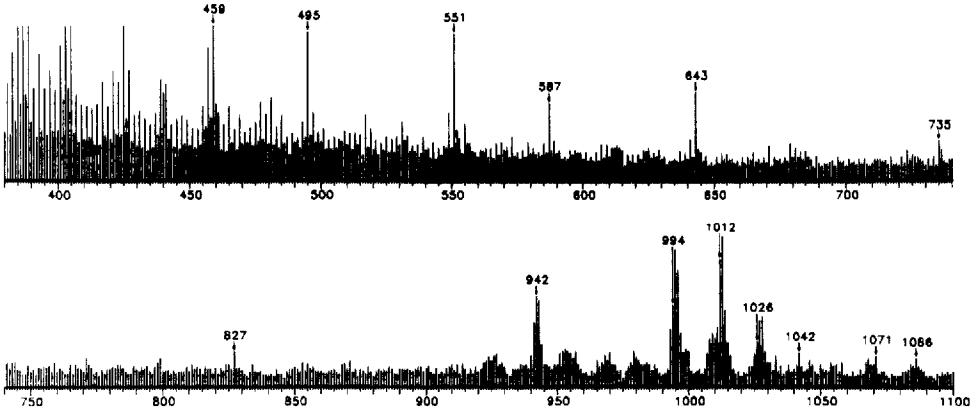

Fig. 5. Positive FAB mass spectrum of the toxin (fraction at t_R 16.45 min in Fig. 1).

derived from the double peak eluted at the Asp position in the amino acid analyzer (Fig. 4), which very likely arises from *threo-* and *erythro*-configurations from β-methyl-Asp during hydrolysis[26]. Since no reference samples of Adda and N-methyl-dehydro-Ala were available, these amino acids could not be identified in the hydrolysis mixture. Nevertheless, the large molecular weights, determined by FAB-MS, *m/z* 943 and higher, might accommodate also these unusual amino acids.

The molecular weights, determined by FAB-MS (Figs. 5 and 6), differ considerably from those extrapolated from the above mentioned gel filtrations. This might be due to either an unusual molecular conformation or to aggregation of the toxins in aqueous solution, a further aspect being here the lack of homogeneity of the HPLC-purified toxin fraction, as became obvious from the FAB-MS data. It should be noted that molecular weights ranging over 600 to 20 000 daltons had been reported for toxins from various *Microcystis sp.* strains[7,35]. Gel exclusion profiles in propanol-containing buffers may provide an answer to this point.

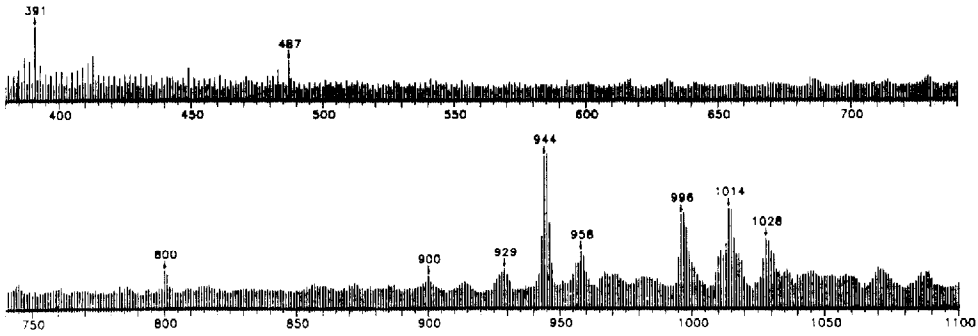

Fig. 6. Negative FAB mass spectrum of the toxin (fraction at t_R 16.45 min in Fig. 1). Glycerol signals at 459, 551, 643, 735 and 827 nm.

DISCUSSION

The solubility of the toxin from *Microcystis aeruginosa* PCC 7941 in alcohols and in slightly alkaline aqueous buffers, but not in diethyl ether or chloroform, indicated its amphilic character. This was also confirmed by the relative mobility (R_F 0.2) of the toxin observed in reversed-phase TLC. Gel electrophoresis at pH 8.2–8.5 revealed the negative charge of the toxin.

The unusual aromatic amino acid "Adda", which had been reported previously as part of the cyanoginosins[27], was also identified in the toxic fraction from *Microcystis aeruginosa* PCC 7941. The identification was based, in particular, on the resonance signals between 5.3 and 7.5 ppm in the ^{1}H NMR spectrum (Fig. 3) and on the signal in ^{13}C NMR spectrum assigned to carbon 9 (88.4 ppm in $C^2H_3O^2H$; 88.0 ppm in ^2H_2O), as well as on the ultraviolet absorbance with maxima at 232 and 238 nm[3,4]. Furthermore, the presence of N-methyldehydro-Ala and β-methyl-Asp, which also form part of the cyanoginosins[27], was detected by ^{1}H and ^{13}C NMR spectroscopy. It should be noted, however, that the material used for spectroscopy consisted of at least four peptides, which at present does not allow us to assign Adda, N-methyldehydro-Ala and β-methyl-Asp to one toxic peptide of *Microcystis aeruginosa* PCC 7941.

The peak eluted from an HPLC column (Fig. 1) indicates heterogeneity (for comparison see ref. 36) of the toxin fraction. Also, the ultraviolet absorbance spectrum of the sample does not prove its purity. Nevertheless, we demonstrated that the same amino acids that had been described as "invariant" in the cyanoginosins from a South African strain[27] are present in the toxin fraction from the North American strain of *Microcystis aeruginosa* used in this work. An homologous structural composition of the toxins from the two strains can, therefore, be anticipated. The failure to stain with ninhydrin as well as the lack of typical peptide fragmentation in FAB-MS point to cyclic structures for all peptides present in the toxic fraction. This is also supported by their solubility in organic solvents.

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