

STRUCTURE OF A HEPATOTOXIC PENTAPEPTIDE FROM THE CYANOBACTERIUM *NODULARIA SPUMIGENA*

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A. SANDSTRÖM, C. GLEMAREC, J. A. O. MERILUOTO, J. E. ERIKSSON and J. CHATTOPADHYAYA. Structure of a hepatotoxic pentapeptide from the cyanobacterium *Nodularia spumigena*. *Toxicon* **28**, 535-540, 1990.—The structure of a hepatotoxic peptide from the cyanobacterium *Nodularia spumigena* was determined using 1D and 2D proton nuclear magnetic resonance spectroscopy and fast atom bombardment mass spectrometry. The toxin was a cyclic pentapeptide (mol. wt 824.5) with the structure cyclo-(β -methylisoAsp-Arg-Adda-isoGlu-*N*-methyldehydrobutyric acid) (Adda: 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid).

INTRODUCTION

WE HAVE previously purified and characterized toxicologically a hepatotoxin from the cyanobacterium *Nodularia spumigena* (ERIKSSON *et al.*, 1988*a,b*). This potent hepatotoxin (LD₅₀ 50 μ g/kg, mouse, i.p.) induces massive hemorrhages in the liver and causes a disruption of the lobular and sinusoidal structure. The toxicological properties of the *Nodularia* toxin are closely related to microcystins, cyclic heptapeptide toxins from other genera of cyanobacteria. The structure of this toxin has now been determined using one-dimensional and two-dimensional proton nuclear magnetic resonance (NMR) spectroscopy and fast atom bombardment mass spectroscopy (FAB/MS).

MATERIALS AND METHODS

Cyanobacterial material and toxin isolation

The toxic *Nodularia spumigena* material was collected 3 July 1986 from a bloom around northern Öland in the Baltic Sea. The toxin was isolated on reversed phase high performance liquid chromatography (HPLC) as described in ERIKSSON *et al.* (1988). The purity of the isolated toxin was assessed using internal surface reversed phase chromatography (MERILUOTO and ERIKSSON, 1988). The isolated toxin gave a single sharp peak in the purity check.

Nuclear magnetic resonance spectroscopy

The constituent amino acids of the *Nodularia* toxin were determined by proton NMR spectroscopy using a Jeol GX-270 spectrometer operating at 270 MHz. The residues were identified by performing homonuclear

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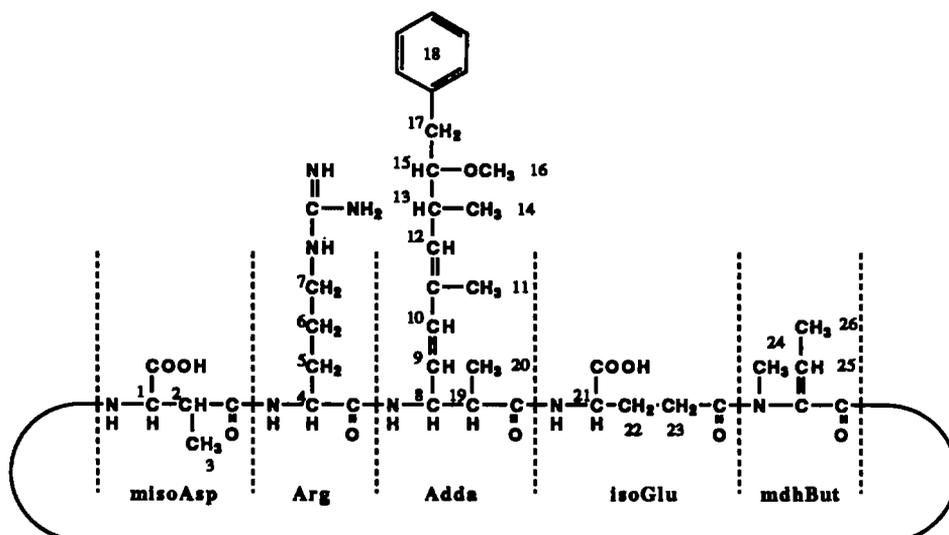


FIG. 1. STRUCTURE OF THE *Nodularia* toxin. $^1\text{H-NMR}$ CHEMICAL SHIFTS δ (REFERENCED TO ACETONITRILE SET AT 2.00 ppm) AND COUPLING CONSTANTS J IN HZ ARE SHOWN BELOW. THE NUMBERING IN THE FIGURE IS ARBITRARY.

misoAsp: δ 4.1 (1H, H-1), 3.1 (1H, H-2), 1.2 (3H, H-3); Arg: δ 4.3 (1H, H-4), 1.9 and 2.1 (2H, H-5), 1.3 (2H, H-6), 3.1 (2H, H-7); Adda: δ 4.4 (1H, H-8), 5.6 (1H, $J_{9,10} = 15.4$ Hz, H-9), 6.3 (1H, $J_{9,10} = 15.4$ Hz, H-10), 1.6 (3H, H-11), 5.5 (1H, H-12), 2.5 (1H, $J_{13,14} = 6.8$ Hz, H-13), 0.9 (3H, $J_{13,14} = 6.8$ Hz, H-14), 3.4 (1H, H-15), 3.2 (3H, H-16), ca 2.5 and 2.7 (2H, H-17), 7.25 (5H, H-18), ca 2.7 (1H, $J_{19,20} = 6.9$ Hz, H-19), 1.0 (3H, $J_{19,20} = 6.9$ Hz, H-20); isoGlu: δ 4.4 (1H, H-21), 1.9 and 2.1 (2H, H-22), 2.6 (2H, H-23); mdhBut: δ 3.0 (3H, H-24), 6.9 (1H, $J_{25,26} = 7.2$ Hz, H-25), 1.7 (3H, $J_{25,26} = 7.2$ Hz, H-26).

proton-proton decoupling experiments and a double quantum filtered (DQF) correlation spectroscopy (COSY) 2D NMR pulse sequence (PIANTINI *et al.*, 1982). The sample size was 0.5 mg in 0.5 ml $^2\text{H}_2\text{O}$ and the sample temperature was 30°C.

Fast atom bombardment mass spectrometry

The FAB/MS spectra were recorded on a Jeol DX-303 instrument connected to a Jeol DA-5000 data system. The FAB gun was operated at 5 or 6 kV producing a beam of 5 or 6 keV xenon neutrals. In the linked scan experiments helium was used as collision gas. The resolution was set to 5000 for the exact mass measurements, 1500 for the magnetic field scans and 500 for the linked scans (magnetic field-to-electric field ratio or B/E constant). The sample (0.5–2 μg) dissolved in water (1 μl was mixed on the stainless steel target with the appropriate matrix). For the cyclic peptide 3-nitrobenzyl alcohol and for the linearized peptide(s) glycerol or a mixture of glycerol and 3-nitrobenzyl alcohol was used as a matrix. For the exact mass measurements polyethylene glycol was used as a matrix and as a mass marker.

RESULTS

The determined chemical shifts and coupling constants are given in Fig. 1. The presence of Adda (3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid) is detected by its phenyl protons at ca 7.2 ppm and its three vinylic protons at 6.3, 5.6 and 5.5 ppm (BOTES *et al.*, 1984; RINEHART *et al.*, 1988). The *N*-methyldehydrobutyric acid (mdhBut) is identifiable in the 2D COSY spectrum by the correlation between the doublet at 1.7 ppm (vinylic methyl, H-26) and the quadruplet at 6.9 ppm (vinylic methine, H-25). The *N*-methyl group of mdhBut appears as a singlet at 3.0 ppm. The remaining methyl group at 1.2 ppm was attributed to the methyl group of methyl(iso)Asp. The 2D COSY spectrum

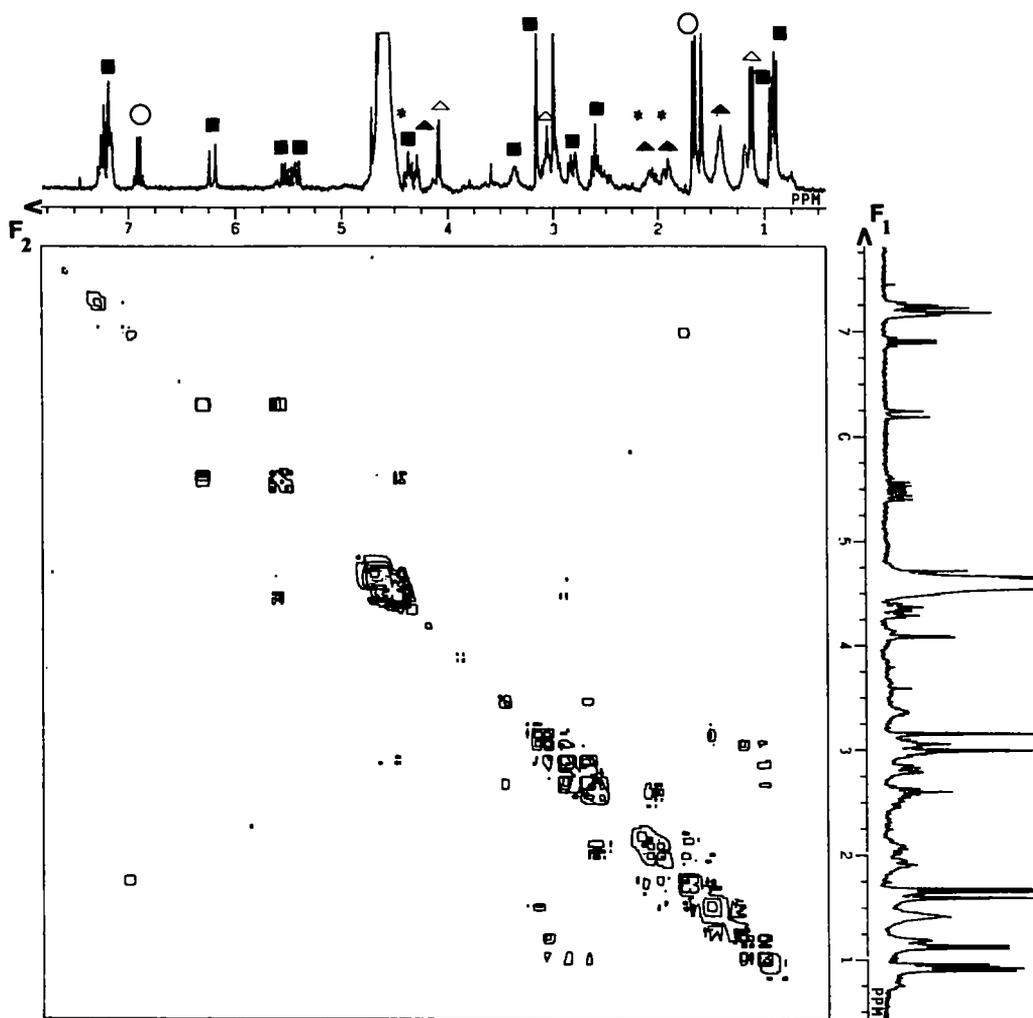


FIG. 2. THE DOUBLE QUANTUM FILTERED CORRELATION SPECTROSCOPY SPECTRUM OF THE *Nodularia* TOXIN.

The 1D spectra are referenced to HO²H peak set at 4.6 ppm. The main signals of the five amino acid residues are shown as follows: (■) Adda, (○) mdhBut, (△) misoAsp, (*) isoGlu and (▲) Arg. Experimental conditions: F1 = F2 = 2000 Hz with a resolution of 3.7 Hz/data point in both directions. 608 scans were acquired for each t_1 value which was changed by 0.250 ms. A sine bell window was applied and the spectrum was symmetrized.

(Fig. 2) shows a correlation between this methyl at 1.2 ppm and a methine (H-2) signal at 3.1 ppm. Additional confirmation of the presence of methyl(iso)Asp has been obtained by selective irradiation of the resonance at 3.1 ppm which converted the doublet at 4.4 ppm (due to methine, H-1) and 1.2 ppm (methyl, H-3) into a singlet. The (iso)glutamic acid and arginine residues were identified as in GROß and KALBITZER (1988) (see Fig. 1).

The protonated molecular ion (M+H)⁺ was observed at m/z (mass-to-charge ratio) 825.4535 (calculated m/z for C₄₁H₆₀N₈O₁₀+H: 825.4511). The absence of fragment ions indicated that the peptide was cyclic (KRISHNAMURTHY *et al.*, 1986; SANTIKARN *et al.*, 1983).

Attempts to hydrolyze the peptide by trifluoroacetic acid (TFA; 24–72 hr, 21–37°C) did not yield the desired intact linearized peptide with an $(M+H)^+$ at m/z 843.46. Instead, a mixture of chemically degraded and unidentified compounds was observed at m/z 811, 793 and 775. We believe that these peaks reflect a chemical degradation since their intensities varied depending on the TFA treatment. *N*-methyldehydroamino acids have been shown to undergo a chemical degradation together with the desired peptide bond cleavage when treated with acid (LIESCH *et al.*, 1976). In our previous work on similar cyclic peptides (MERILUOTO *et al.*, 1989) we did not encounter any difficulties with the TFA hydrolysis of the native peptide. For the *Nodularia* peptide, a hydrolysis product with $(M+H)^+$ at m/z 830 would be expected but it was not observed. However, the observed ions correspond to a species formed as a result of addition of one molecule of water followed by loss of one molecule of methanol yielding a compound with an $(M+H)^+$ ion at m/z 811. The exact mass measurement of this ion indicates that the assumption is correct; found m/z 811.4333, calculated m/z for $C_{40}H_{59}N_8O_{10}$: 811.4354.

In order to achieve the desired hydrolysis of the peptide it was necessary to reduce the *N*-methyldehydroamino acid and this was done essentially according to the method of LIESCH *et al.* (1976). The reaction was complete after 3 hr of sodium borohydride reduction and acetic acid was added to quench the reaction. The solvents were removed by freeze drying and the sample was purified on reversed phase HPLC (column: Spherisorb ODS 10 μ m (HPLC Technology, U.K.), 4.6 \times 250 mm, eluent: 10 mM ammonium acetate in 21.5% acetonitrile/water, detection: 254 nm). The fractions corresponding to the main peak in the u.v. chromatogram were pooled and freeze dried. The mass spectrum of this material showed the $(M+H)^+$ at m/z 827 corresponding to the expected dihydropeptide. Fifty microliters TFA was then added to the dihydropeptide (ca 10 μ g) and this mixture was kept at room temperature for 20 hr. The TFA was removed by nitrogen blowing. The sample was redissolved in water and freeze dried. This sample consisting of reduced and linearized peptides showed the $(M+H)^+$ at m/z 845.5 as expected.

The magnetic field scan spectrum shows some fragment ions from the reduced and linearized peptide misoAsp–Arg–Adda–Glu–mdhBut^R (misoAsp = β -methylisoaspartic acid, mdhBut = *N*-methyldehydrobutyric acid, 'R' denotes that the amino acid is reduced): C_4^+ and C_3^+ , Y_4^+ and Z_4^+ , and the ion $(M-CH_3O)^+$. See Fig. 3.

The linked scan spectra of the reduced and linearized sample (B/E constant) performed several times with various amounts of helium as collision gas confirmed that the linear peptides misoAsp–Arg–Adda–isoGlu–mdhBut^R and mdhBut^R–misoAsp–Arg–Adda–(iso)Glu are the most abundant ones among the linearized peptides. The sample matrix consisted either of a mixture of 3-nitrobenzyl alcohol and glycerol, or magic bullet (dithiothreitol and dithioerythritol, 5:1 v/v). The ions observed are shown in Fig. 3. The iso-linkages are not definitely confirmed by mass spectrometry although the A^*_4 and A^{**}_4 fragments indicate this type of linkage. The positions of isoGlu and misoAsp could possibly be interchanged, but again the A^*_4 and A^{**}_4 fragments support the shown structure.

DISCUSSION

The general structure of *Microcystis aeruginosa* toxins, microcystins, is thought to be cyclo-(D-Ala–L-X-D-erythro- β -methylAsp–Y–Adda–Glu–*N*-methyldehydroAla) (CAR-MICHAEL *et al.*, 1988). In this structure X and Y refer to variable amino acids. Furthermore, the D-erythro- β -methylAsp is demethylated in some toxin variants and the *N*-methyldehydroAla residue may be replaced by dehydroAla. Of this general microcystin

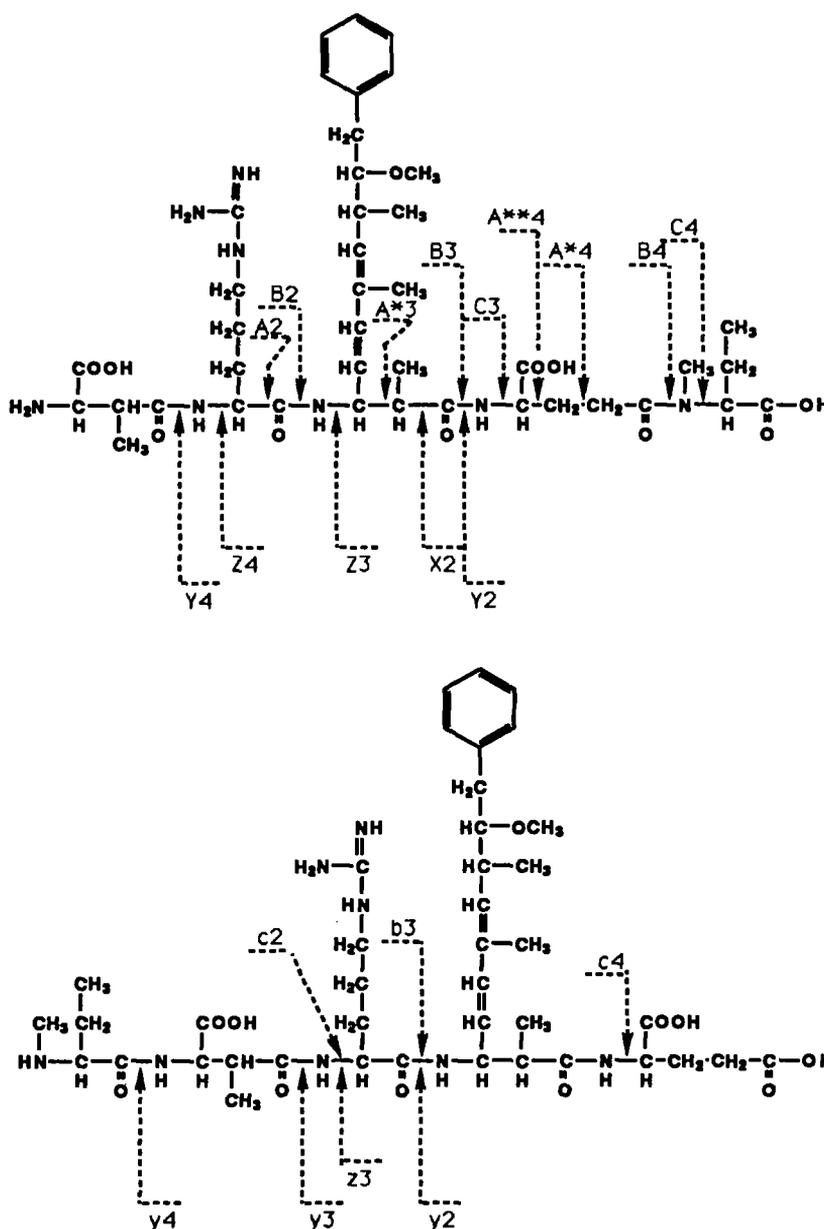


FIG. 3. ASSIGNMENT OF THE SEQUENCE IONS SEEN IN THE MASS SPECTRA.

The notation of ROEPSTORFF and FOHLMAN (1984) is used in a slightly modified manner. The extra possible peptide backbone cleavage sites in the residues misoAsp, isoGlu and Adda are denoted with one or two asterisks (*) together with the common ROEPSTORFF nomenclature. Another difference is that capital letters are used to denote fragments from the linear peptide misoAsp-Arg-Adda-isoGlu-mdhBut^R (R indicates a reduced amino acid) and small letters denote fragments from the linear peptide mdhBut^R-misoAsp-Arg-Adda-(iso)Glu.

structure, the sequence β -methylAsp-Y (Arg)-Adda-Glu is conserved in the *Nodularia* toxin. Additionally, the *N*-methyldehydroAla and *N*-methyldehydrobutyric acid residues are closely related. This link is interesting with respect to evolution.

The structure of the *Nodularia* toxin determined in this study, cyclo-(β -methylisoAsp-

Arg-Adda-isoGlu-*N*-methyldehydrobutyric acid), is consistent with previous reports on the toxic pentapeptide from *Nodularia spumigena*. The same peptide (improbable differences in stereochemistry are not considered), which has been termed nodularin by RINEHART *et al.* (1988), has been found in material from New Zealand (RINEHART *et al.*, 1988) and in the Baltic Sea (SIVONEN *et al.*, 1989; this study). Although these identical structures may be just a pure coincidence, they may indicate less structural variation among the *Nodularia* toxins. *Nodularia* toxin has only one of the two variable residues of microcystins in its amino acid sequence.

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