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STRUCTURE AND TOXICITY OF A PEPTIDE HEPATOTOXIN FROM THE CYANOBACTERIUM *OSCILLATORIA AGARDHII*

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J. A. O. MERILUOTO, A. SANDSTRÖM, J. E. ERIKSSON, G. REMAUD, A. GREY CRAIG and J. CHATTOPADHYAYA. Structure and toxicity of a peptide hepatotoxin from the cyanobacterium *Oscillatoria agardhii*. *Toxicon* 27, 1021–1034, 1989.—A peptide hepatotoxin was isolated by reversed phase liquid chromatography from the cyanobacterium *Oscillatoria agardhii* and characterized structurally and toxicologically. Amino acid analyses, proton nuclear magnetic resonance and fast atom bombardment mass spectrometry showed that the toxin is a cyclic heptapeptide (mol.wt 1023.5) with the structure cyclo-(Ala-Arg-Asp-Arg-Adda-Glu-*N*-methyldehydroAla) (Adda: 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid). In mice the toxic effects were restricted mainly to the liver where the toxin induced massive hemorrhages and a disruption of the lobular and sinusoidal structure. The i.p. LD_{50} of the toxin was 250 μg/kg. The structural and toxic properties of this peptide are very close to those of microcystins, cyclic peptide toxins produced by the cyanobacterium *Microcystis aeruginosa*.

INTRODUCTION

THE FRESHWATER cyanobacterium *Microcystis aeruginosa* produces a family of peptide hepatotoxins that have the structure cyclo-(D-Ala-L-*X-erythro-β*-methyl-D-isoAsp-L-*Y*-Adda-D-isoGlu-*n*-methyldehydroAla) where the residue Adda refers to a *β*-amino acid, 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid, and the residues *X* and *Y* symbolize the variable part of the toxin structure (BOTES *et al.*, 1984, 1985; BOTES, 1986). The toxins injure liver causing massive internal hemorrhages and damage the cytoskeleton as well as cellular organelles in hepatocytes (RUNNEGAR and FALCONER, 1986; FALCONER and RUNNEGAR, 1978; RUNNEGAR *et al.*, 1987; DABHOLKAR and CARMICHAEL, 1987;

Abbreviations used in this paper: Adda, 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid; *B/E*, constant magnetic field-to-electric field ratio; DECSY, double quantum coherence echo correlated spectroscopy; DOSE-SECSY, double quantum spin echo–spin echo correlation spectroscopy; HPLC high performance liquid chromatography; *m/z*, mass-to-charge ratio; NMR, nuclear magnetic resonance; OA-29, *Oscillatoria* toxin studied in this paper.

ERIKSSON *et al.*, 1987). The cyanobacterium *Oscillatoria agardhii* produces peptides that exhibit the same toxic effects as the *Microcystis* toxins in mice and in isolated rat hepatocytes (ERIKSSON *et al.*, 1988*a*,*b*). The structure of toxins from *Oscillatoria* has not been finally characterized. We have elucidated the identity of one of the toxins using two-dimensional proton nuclear magnetic resonance and fast atom bombardment mass spectrometry. The biological effects of the isolated toxin are also considered.

MATERIALS AND METHODS

Cyanobacterial material

The *O. agardhii* material, strain CYA-29 (green), was originally obtained from a laboratory culture at the Norwegian Institute for Water Research. The culture was grown in 50% Z8 medium (STAUB, 1961) under continuous illumination (20–40/μEm^{-2} sec^{-1}) at 20°C. The *Microcystis aeruginosa* material was collected from lake Akersvatn, Norway, and it was kindly provided by Mr Olav Skulberg, the Norwegian Institute for Water Research.

Toxin purification

The unknown toxin from *Oscillatoria agardhii* and microcystin-LR (reference material from *Microcystis aeruginosa*) were purified by high performance liquid chromatography. The purification procedure was modified from earlier described methods. (SIEGELMAN 1984; KRISHNAMURTHY *et al.*, 1986*a*; BROOKS and CODD, 1986). The cyanobacteria were concentrated, frozen and lyophilized. The lyophilized material was extracted by 30 min bath ultrasonication using 50 ml of water–methanol–butanol (75:20:5, v:v:v) per g dry cyanobacteria. The extracts were spun for 1 hr at 48,000 *g* and the pellets were then reextracted once. The pooled supernatants were rotary evaporated (at 40°C) to about 50% of the original volume after which they were concentrated and pre-purified on Bond-Elut C-18 cartridges (Analytichem Inc., U.S.A.). The HPLC purification was made on a Nucleosil 7 C-18, 10 × 250 mm, reversed phase column (Macherey-Nagel, F.R.G.). The extracts were eluted with 27% acetonitrile–73% 0.0135 M ammonium acetate in water, flow rate 3.0 ml/min, detector set at 238 nm (Eriksson *et al.*, 1988*a*). The pooled fractions containing the toxic peak were rotary evaporated to a smaller volume and passed once more through an identical column reserved for the second HPLC stage only. The toxins were desalted by binding them to Bond-Elut C-18 cartridges and flushing with water, after which they were eluted with methanol and dried with nitrogen. Although most ammonium acetate is removed in the lyophilization process, Bond-Elut cartridges were used to minimize the amount of ammonium acetate which can interfere in the NMR spectroscopy. The dry toxins were dissolved in water and lyophilized. The final purity of the toxins was tested by analyzing an aliquot on an internal surface reversed phase column, Pinkerton GFF ISRP, 4.6 × 150 mm, Regis Chemical Co. (MERILUOTO and ERIKSSON, 1988).

Toxicological characterization

Toxicity was tested on male SJL mice (20–25 g, source: Turku University Animal Center) by i.p. injection using five doses and three animals per dose. Probit analysis (FINNEY, 1963) was used to calculate the LD_{50} value. Vital organs were examined histologically immediately after death. The tissue was fixed in 4% (w/v) phosphate-buffered formaldehyde, dehydrated and embedded in paraffin. Sections (4 μm) were stained with hematoxylin–eosin for overall examination of histological damage and with periodic-acid–Schiff with and without diastase digestion for visualization of glycogen (COOK, 1982).

Conventional amino acid analysis

The amino acid composition was determined using an LKB amino acid analyzer and confirmed with a gas chromatograph (GC; column: SE-30, 25 m, internal diameter 0.2 mm, Nordion Oy, Finland) coupled to a mass spectrometer (MS; VG 7070 E, VG Analytical Ltd, U.K.) (ERIKSSON *et al.*, 1988*c*). The GC/MS method was used for qualitative results only and arginine could not be detected by this method. The toxin (50–100 μg) was hydrolyzed at 110°C under nitrogen in 6 M hydrochloric acid with the addition of 0.5% phenol. The hydrolyzed toxin was prepared for the GC analysis by esterification with *n*-butanol followed by acetylation with trifluoroacetic anhydride (ROACH and GEHRKE, 1969; AMICO *et al.*, 1976; NAGY *et al.*, 1979).

Structure determination

Proton NMR spectroscopy was used to determine the constituent amino acids of the toxin OA-29. A qualitative (no accurate chemical shifts and no coupling constants are given) assignment of ^{1}H resonances of the NMR spectrum of OA-29 was done by comparison with a known toxin (microcystin-LR) which possesses a

related structure. The ^{1}H-NMR spectra were recorded on a Jeol GX 270 spectrometer at 270 MHz in 5 mm o.d. tubes. The connectivities between the different groups in the toxins were studied by performing DOSE-SECSY 2D NMR pulse sequence which has been adapted (REMAUD, 1988) from the DECSY pulse sequence (IKURA and HIKICKI, 1984). The main advantage of this technique is the suppression of the center line of the SECSY spectrum. The arrangements for the DOSE-SECSY experiment are described in the legends of Figs 2 and 3.

Mass spectra were recorded on a Jeol DX-303 instrument connected to a Jeol DA-5000 computer system. The fast atom bombardment spectra were recorded at a resolution set to 1500 for magnetic field scans and 500 for linked scans. The samples were prepared by using 0.5–1 μl of an aqueous solution of the peptide (concentration 3–5 μg/μl) with either 0.5 μl of 3-nitrobenzylalcohol (cyclic peptide) or with 0.5 μl of glycerol (linearized peptide) on the stainless steel target. The fast atom bombardment gun was operated at 5 or 6 kV producing a beam of xenon neutrals. Helium was used as the collision gas for the linked scan experiments. Several scans (usually 10–20) were accumulated in order to increase the signal-to-noise ratio at a scan speed of 40 sec (m/z 0-1500). The hydrolysis of the toxin OA-29 was effected by a 10–15 hr treatment with sequencing grade trifluoroacetic acid to give a mixture of linear peptides. The products of the trifluoroacetic acid hydrolysis were studied using both unimolecular decomposition spectrum and collision activated dissociation spectrum.

RESULTS

Toxin purification

The *Oscillatoria* toxin from the strain CYA-29 that was used in this study had a somewhat shorter retention time than the toxin from the strain CYA-38 (ERIKSSON *et al.*, 1988*a*) and it was eluted at about 18 min. The toxic effects could be assigned to this single peak. The toxin yield was about 200 μg/g dry cyanobacteria. Microcystin-LR was eluted at about 13 min (yield 2000 μg/g). The lyophilized toxins (fluffy powder) both gave a single sharp peak in the purity check with the internal surface reversed phase column.

Toxic effects

The LD_{50} of the OA-29 toxin was 250 μg/kg (mouse, i.p.). The symptoms preceding death were very similar to those earlier reported for various microcystins, i.e. piloerection, lethargy, pallor, coldness of legs, ears and tail, paresis of hindlimbs, and tachypnea. Doses close to the LD_{50} value caused death in mice within 3–4 hr. The liver weight of the intoxicated mice had doubled to about 8–10% of the body weight. This increase in liver weight was due to massive hemorrhages which were markedly conspicuous in histological sections (Fig. 1.). The basic lobular and sinusoidal structure was disrupted and most parenchymal cells were more or less detached from each other and surrounded by lacunae filled with blood. However, necrosis was not apparent in the parenchymal tissue since only a few karyorrhectic and karyopyknotic cells could be observed, the majority of cells, although deformed, still being intact. The most likely cause of death was a hemodynamic shock because the bulk of the blood was accumulated in the liver. Periodic-acid–Schiff staining revealed a total depletion of glycogen in hepatocytes (Fig. 1.). The overall toxic effects were almost exclusively restricted to the liver, although signs of hydropic degeneration could be seen in the tubular epithelium of the kidneys. This degeneration could also be due to secondary effects of the intoxication.

Amino acid analysis

Amino acid analysis of OA-29 revealed the presence of aspartic acid (molar ratio 1.1), glutamic acid (1.0), alanine (1.0) and arginine (1.4). Aspartic acid, glutamic acid and alanine were identified in the gas chromatographic separation of the derivatized amino acids.

FIG. 1. THE TOXIC EFFECTS IN MOUSE LIVER INDUCED BY A PEPTIDE TOXIN FROM *Oscillatoria agardhii*. Liver sections were fixed in 4% phosphate-buffered formaldehyde and embedded in paraffin. The 4 μm sections were stained with the periodic-acid–Schiff reaction. (A) Structure of a normal mouse liver. The dark and dense spots within the hepatocytes are glycogen granules which disappeared upon diastase digestion. V = centrilobular vein. Bar 100 μm. (B) Structure of a liver from a mouse intoxicated with 300 μg OA-29 toxin per kg animal weight. Notice the complete disruption of the normal lobular architecture and the absence of glycogen granules. Diastase digested sections looked identical. V = centrilobular vein, L = lacuna filled with blood. Bar 100 μm.

NMR spectroscopy

The structure of microcystin-LR has been reported earlier (BOTES *et al.*, 1985; KRISHNAMURTHY *et al.*, 1986*b*): the sequence of the amino acids has been determined to be cyclo-(Ala-Leu-methylisoAsp-Arg-Adda-isoGlu-*N*-methyldehydroAla). The NMR spectrum of microcystin-LR is shown in Fig. 2. From the one-dimensional spectrum shown on the top of Fig. 2 it is easy to detect the two methyl groups of the isopropyl group of leucine which

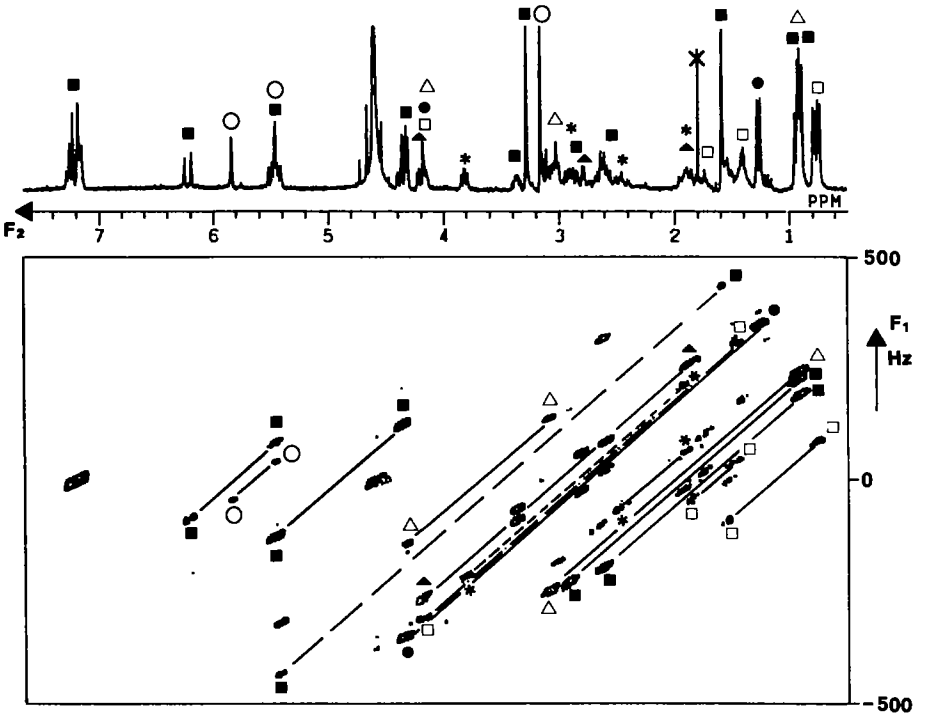

FIG. 2. DOSE-SECSY SPECTRUM OF MICROCYSTIN-LR (0.75 mg IN 0.5 ml ^2H_2O, $T=30°C$). The main correlations of the different amino acids are shown. The one-dimensional spectrum on the top is referenced to the water (HO^2H) peak set at 4.6 ppm. The main signals of each amino acid are marked as follows: (■) Adda, (□) leucine, (●) alanine, (*) isoglutamic acid, (▲) arginine, (○) *N*-methyldehydroalanine and (△) *β*-methylisoaspartic acid. × denotes solvent impurities. Experimental conditions: $F_1=F_2=2000$ Hz, $t_1=0.250$ ms, reading pulse 135°C. The double quantum coherence was created with a delay (γ) of 30 ms. 240 free induction decays were accumulated for the 256 experiments. The spectrum was symmetrized.

appear as doublets at ~0.8 ppm. The connectivities of the whole leucine moiety are shown in Fig. 2 (lower part). Three aliphatic methyl groups absorb at ~0.9 ppm. Two of them belong to the Adda moiety and the third one to the methylisoAsp amino acid. A doublet appears at ~1.3 ppm which corresponds to the methyl group of the alanine residue. The CH-proton coupled to this methyl is found at ~4.2 ppm. The presence of the uncommon amino acid Adda is detected by its phenyl protons (~7.2 ppm) and its three vinylic protons (~5.5 and ~6.2 ppm). Two vinylic protons of *N*-methyldehydroAla absorb in this downfield region of the spectrum. They are weakly coupled to each other since they appear as a singlet in one-dimensional spectrum; however, using DOSE-SECSY such weak correlations are detectable. Among the isolated peaks (singlets with high intensity) two are generated by Adda (CH_3O ~3.3 ppm and CH_3-C=C ~1.6 ppm) and the third one is the methyl (CH_3-N ~3.2 ppm) of *N*-methyldehydroAla. The well resolved quartet at ~3.8 ppm belongs to glutamic acid. The two-dimensional map indicates that this quartet originates from the nonequivalent CH_2 absorbing at ~1.9 ppm which is itself coupled with the second nonequivalent CH_2 of Glu (CH_2-CO_2- ~2.5 and ~2.9 ppm). The arginine residue is somehow more difficult to apprehend without a complete analysis.

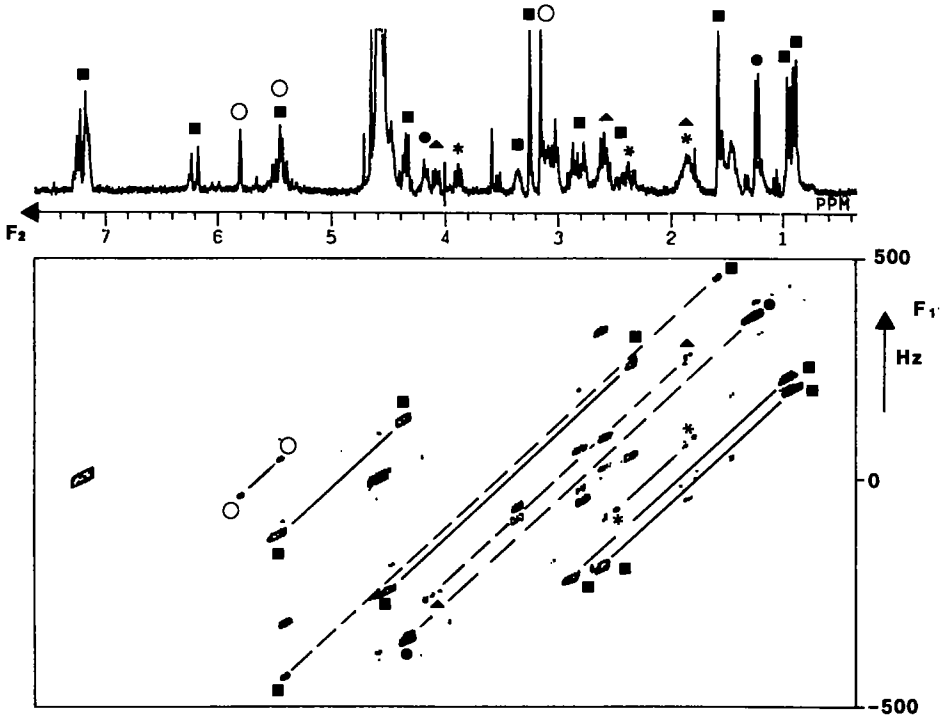

FIG. 3. DOSE-SECSY SPECTRUM OF TOXIN OA-29 (0.65 mg IN 0.5 ml ^2H_2O, $T=30°C$). The main correlations of the different amino acids are shown. The one-dimensional spectrum is shown on the top (referenced to the water (HO^2H) peak set at 4.6 ppm). The main signals of each amino acid are represented as follows: (■) Adda, (●) alanine, (*) glutamic acid, (▲) arginine and (○) *N*-methyldehydroalanine. The spectrum has been recorded using the same experimental conditions as in Fig. 2.

Nevertheless a neat correlation between signals resonating at ~4.2 ppm and others absorbing at ~2.8 ppm is visible.

A similar study can be realized with toxin OA-29 as shown in Fig. 3. Characteristic groups of amino acids are easily pointed out: isolated methyl, aliphatic methyl, vinylic CH, aromatic rings. By comparison between the spectra of toxins microcystin-LR and OA-29 in Figs. 2 and 3, respectively, one can easily conclude that the leucine residue is absent in the toxin OA-29 because of the missing doublets at ~0.8 ppm. Similarly *β*-methylaspartic acid is not a constituent of OA-29: there are only two doublets at ~0.9 ppm. These doublets are due to methyl groups of Adda. The presence of Adda is confirmed by the phenyl ring protons (~7.2 ppm), vinylic protons (~6.2 ppm and ~5.5 ppm) and singlets at ~1.6 and ~3.3 ppm. The CH_3-CH– part of alanine is also clearly seen. The *N*-methyldehydroAla residue is detected by the characteristic two singlets at ~5.8 and ~5.5 ppm linked to each other by a weak coupling as in microcystin-LR. In addition the singlet at ~3.2 ppm is due to N-CH_3 of *N*-methyldehydroAla. The multiplet corresponding to a doublet of doublets at ~3.9 ppm is characteristic of CH– of glutamic acid even if no correlations are detected in Fig. 3 with the delays used to record the 2D spectrum. The quartet which resonates at around ~4.1 ppm for the toxin OA-29 does not appear in the spectrum of microcystin-LR. The position of this signal (~4.1 ppm) corresponds to a signal of a H_2N–CH–COOH part. Furthermore, this signal is correlated

with resonances at ~1.9 ppm: we concluded that aspartic acid is most probably a constituent of the toxin OA-29 as verified by the amino acid analysis. As in microcystin-LR the presence of arginine in OA-29 is revealed by correlations between signals at ~4.2, ~2.8 and ~1.9 ppm.

The qualitative NMR analysis led us to conclude that alanine, arginine, glutamic acid, *N*-methyldehydroalanine, aspartic acid and Adda are the constituents of the toxin OA-29.

Mass spectrometry

The monoisotopic mass (M) of the toxin OA-29 was found to be *m/z* 1023.5 and the $(M+H)^+$ was observed at m/z 1024.5 (Fig. 4A). In order to confirm whether the toxin OA-29 was cyclic it was hydrolyzed with trifluoroacetic acid. The protonated molecular

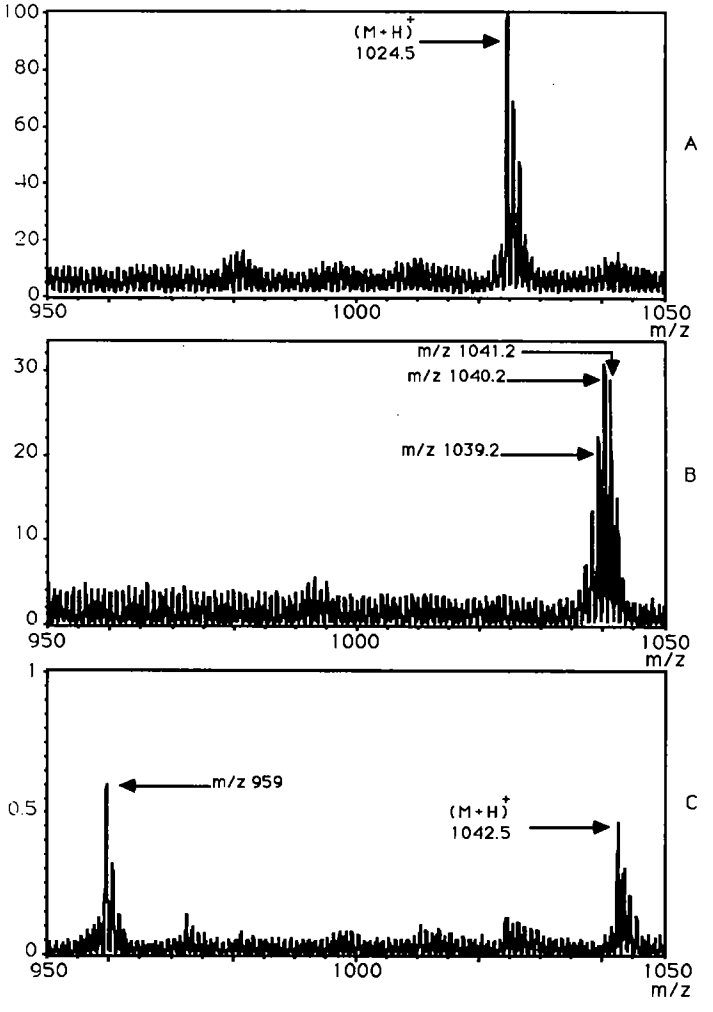

FIG. 4. POSITIVE FAST ATOM BOMBARDMENT SPECTRA OF THE MOLECULAR ION REGION OF THE OA-29 PEPTIDE.

(A) Spectrum of the cyclic OA-29 peptide. (B) Spectrum of the deuterated cyclic OA-29 peptide. (C) Spectrum of the hydrolyzed OA-29 peptide.

ions of the resultant peptides were found to be m/z 1042.5 $(M+H)^+$ (Fig. 4C) which is in agreement with the expected increase of 18 mass units (one molecule of water added). The absence of fragment ions from parent toxin also indicates that its structure is cyclic (KRISHNAMURTHY *et al.*, 1986*a*). The fast atom bombardment spectra of these trifluoroacetic acid hydrolysates showed an intense $(M+H)^+$ and an ion at m/z 959 with the

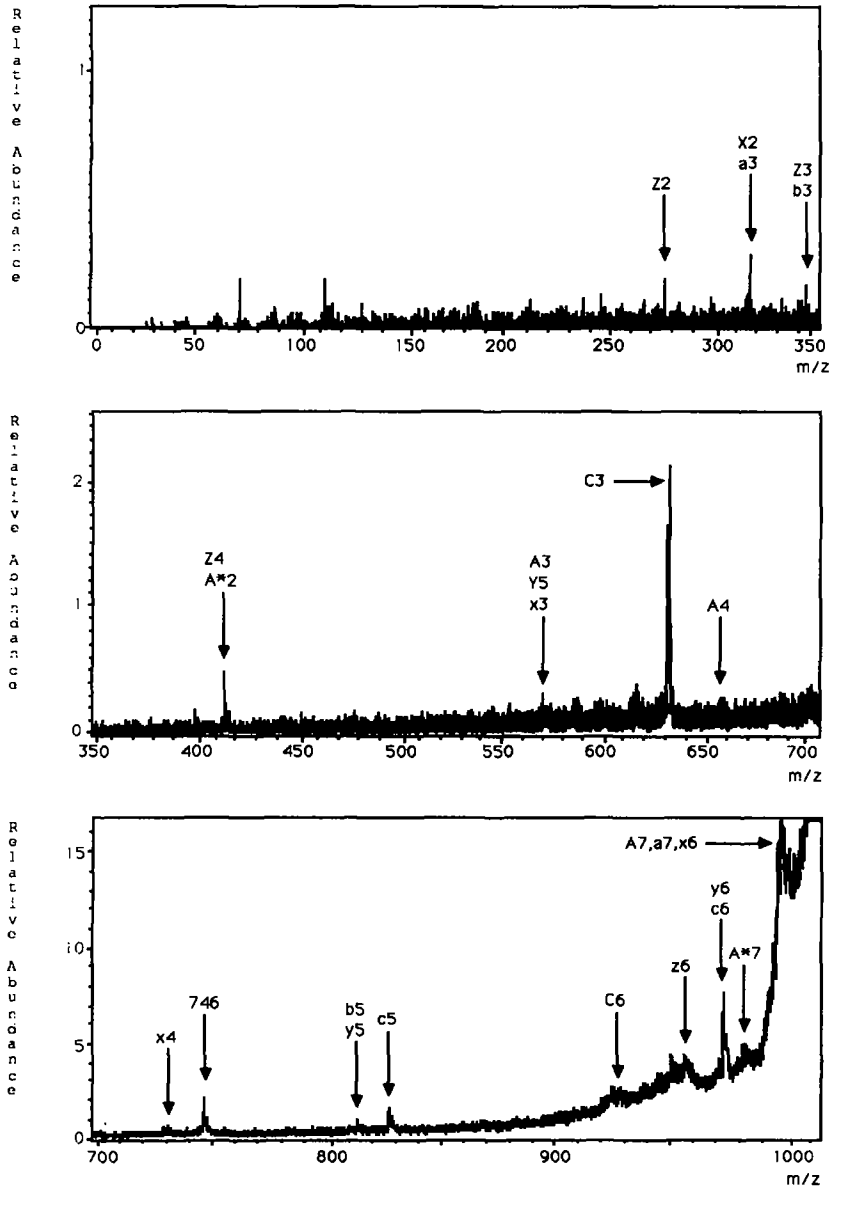

FIG. 5. POSITIVE FAST ATOM BOMBARDMENT *B/E* SPECTRUM OF THE MOLECULAR ION CLUSTER OF THE HYDROLYZED OA-29 PEPTIDE.

No collision gas was used. Capital letters denote fragment ions from the linear peptide no. 2 Arg—isoAsp and small letters denote fragments from the linear peptide no. 4 Ala—*N*-methyldehydroAla (see Fig. 7 for assignments of the fragment ions).

intensity between 25 and 125% compared to that of $(M+H)^+$, depending upon the batch of hydrolysate used. This ion indicates a chemical cleavage of at least one of the two peptide bonds around the *N*-methyldehydroAla. In addition an ion was observed at *m/z* 928 indicating the cleavage of the Asp residue.

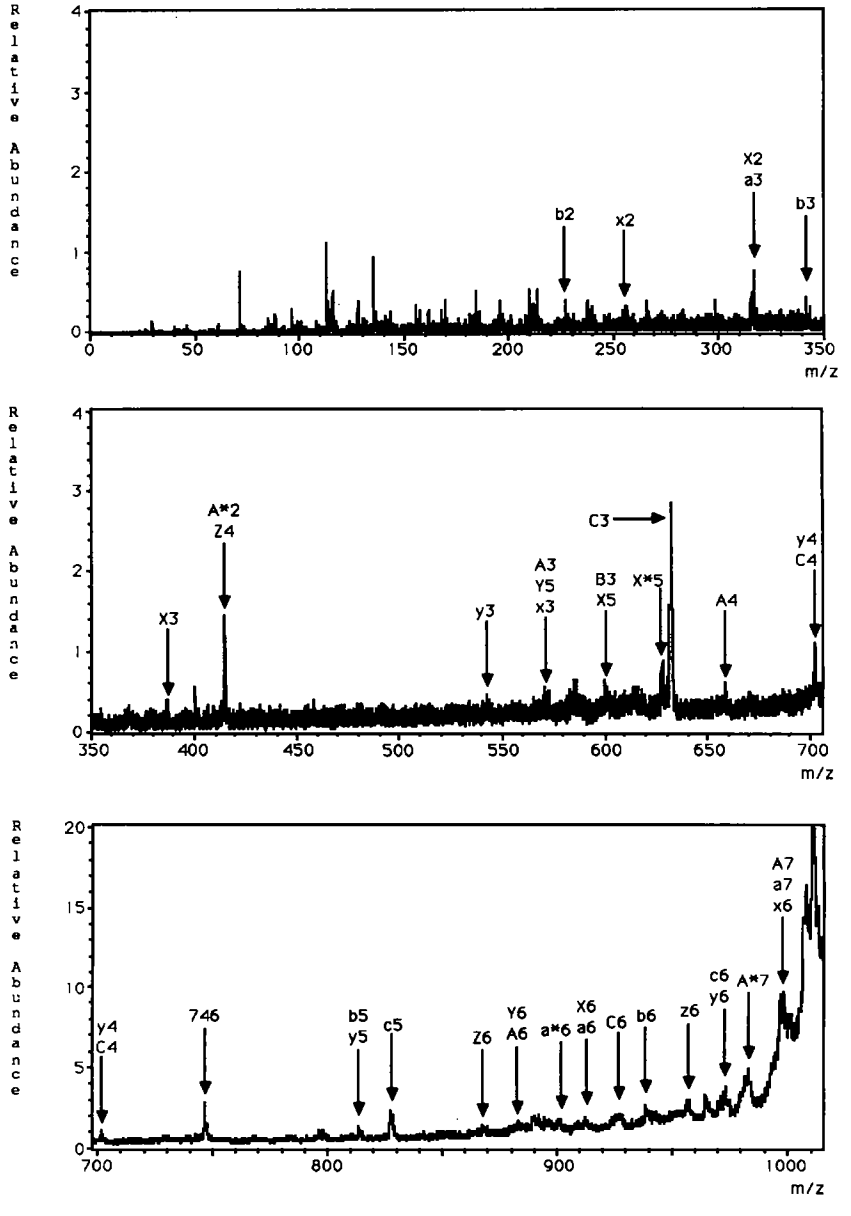

FIG. 6. POSITIVE FAST ATOM BOMBARDMANT *B/E* SPECTRUM OF THE MOLECULAR ION CLUSTER OF THE HYDROLYZED OA-29 PEPTIDE.

Helium was used as collision gas and the pressure was adjusted to reduce the $(M+H)^+$ by 40%. Capital letters denote fragment ions from the linear peptide no. 2 Arg—isoAsp and small letters denote fragments from the linear peptide no. 4 Ala—*N*-methyldehydroAla (see Fig. 7 for assignments of fragment ions).

The number of exchangeable protons in the cyclic toxin OA-29 was investigated by mass spectroscopy of the deuterated toxin. The molecular ion region (Fig. 4B) shows three peaks of approximately equal intensities: m/z 1039.2 [corresponds to $M(^2H_{15})+{}^1H^+$ or $M(^2H_{14})+{}^2H^+$ (22%)], m/z 1040.2 [$M(^2H_{16})+{}^1H^+$ or $M(^2H_{15})+{}^2H^+$ (31%)] and m/z 1041.2 [$M(^2H_{17})+{}^1H^+$ or $M(^2H_{16})+{}^2H^+$ (29%)]. Thus, the sample was a mixture of species containing 14–17 deuterium atoms and the number of exchangeable protons in the toxin is probably 16.

The fragmentation pattern observed in mass spectra indicates that aspartic acid and glutamic acid are bonded with iso-linkages (see Fig. 7 for details). We therefore use the abbreviations 'isoAsp' and 'isoGlu', respectively, in the following presentation. The

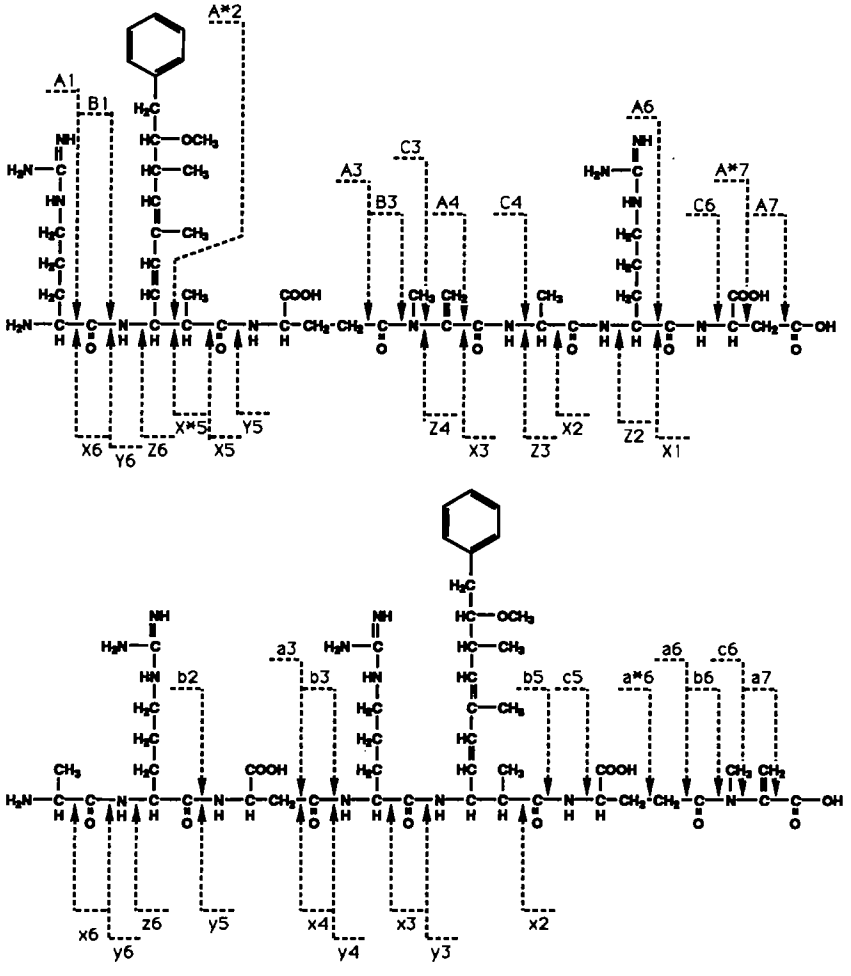

FIG. 7. ASSIGNMENT OF THE SEQUENCE IONS SEEN IN THE SPECTRA IN FIGS 5. AND 6. Capital letters denote fragment ions from the linear peptide no. 2 Arg—isoAsp and small letters denote fragment ions from the linear peptide no. 4 Ala—*N*-methyldehydroAla. The notation of ROEPSTORFF and FOHLMAN (1984) is used in a slightly modified manner. The isoGlu, isoAsp and Adda residues have more than three cleavage sites due to their longer peptide backbone. We have therefore used the symbol * in combination with the common ROEPSTORFF nomenclature. * Indicates that the cleavage occurs at the bond one atom closer to the N-terminal end.

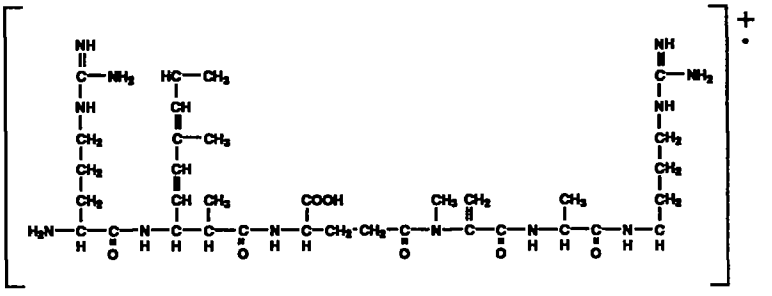

FIG. 8. TENTATIVE STRUCTURE OF THE ION AT *m/z* 746 OBSERVED IN THE SPECTRA SHOWN IN FIGS 4 AND 5.

chemical cleavages observed in the 'normal' magnetic field scan spectra show that the linearized peptide consists of four main fragments due to the cleavage at the peptide bonds (-Arg-isoAsp-), (-isoAsp-Arg-), (isoGlu-mdhAla-) and (-mdhAla-Ala-) to give the following oligopeptides (mdhAla = *N*-methyldehydroalanine):

(1) isoAsp-Arg-Adda-isoGlu-mdhAla-Ala-Arg
(2) Arg-Adda-isoGlu-mdhAla-Ala-Arg-isoAsp
(3) mdhAla-Ala-Arg-isoAsp-Arg-Adda-isoGlu
(4) Ala-Arg-isoAsp-Arg-Adda-isoGlu-mdhAla.

All four peptides have the same elemental composition and the same molecular ion [$(M+H)^+$ at *m/z* 1042.57]. The fragmentation pattern is however different in these four peptides. It has been shown that the cleavage of the –mdhAla–Ala– bond is a common event in the cleavage of cyclic peptides with similar structures (BOTES *et al.*, 1984; KRISHNAMURTHY *et al.*, 1986*b*). This cleavage corresponds to the peptide no. 4, and indeed many of the ions detected in the daughter ion spectra of *m/z* 1042.6 [unimolecular decomposition (Fig. 5) or collision activated dissociation (Fig. 6)] do fit in well with the expected fragmentation. Fragments from the linear peptide no. 2 produced by the cleavage of the –isoAsp–Arg– bond were also observed.

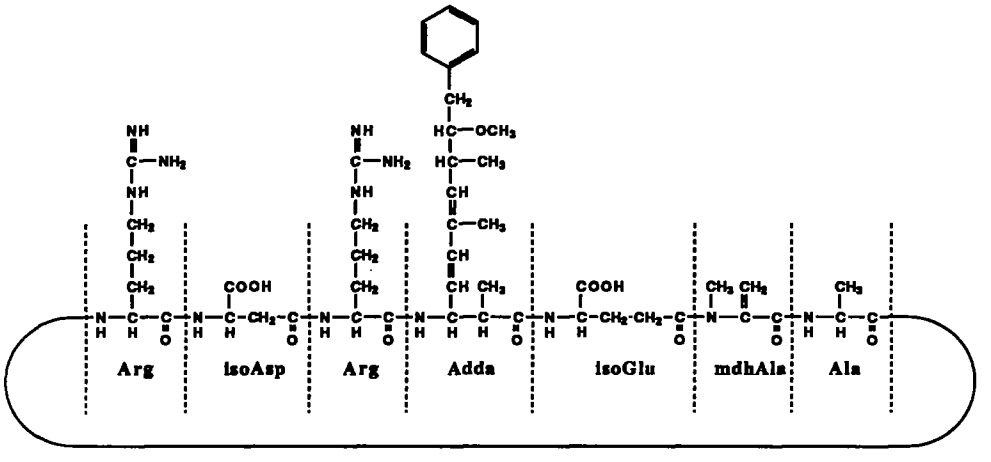

FIG. 9. STRUCTURE OF THE CYCLIC PEPTIDE OA-29.
The aspartic acid and glutamic acid residues are drawn with iso-linkages in the figure (in analogy with microcystins). mdhAla = *N*-methyldehydroalanine.

As shown above, the linearized peptide was a mixture of mainly two linear peptides: Ala-Arg-isoAsp-Arg-Adda-isoGlu-mdhAla and Arg-Adda-isoGlu-mdhAla-Ala-Arg-isoAsp. The major ions seen in the spectra which are not assigned as sequence ions (Fig. 7) can be explained as shown in Fig. 8 or as the immonium ions of the constituent amino acids. We have concluded that the toxin OA-29 is a cyclic peptide, as shown in Fig. 9. The present structural assignment of the toxin OA-29 does not address, however, whether the constituent amino acids are D or L isomers and whether the aspartic and glutamic acid residues are indeed iso-linked. However, the mass spectra suggest that the aspartic acid and glutamic acid are iso-linked. The iso-linkages are also in analogy with previously reported microcystin structures.

DISCUSSION

The analogy between the *Oscillatoria* and *Microcystis* toxins is very clear. The structure of the *Microcystis* toxins has been reported to be cyclo-(D-Ala-L-*X-erythro-β*-methyl-D-isoAsp-L-*Y*-Adda-D-isoGlu-*N*-methyldehydroAla) (BOTES *et al.*, 1984, 1985; BOTES, 1986). According to a proposed nomenclature (CARMICHAEL *et al.*, 1988) cyanobacterial cyclic heptapeptide toxins should be termed microcystin-*XY*, where *X* and *Y* denote the variable amino acid residues. The *Oscillatoria* toxin we are presenting in this paper has an almost identical structure with a previously described *Microcystis* toxin, microcystin-RR (two arginine residues as the variable amino acids) from a Japanese *Microcystis* strain (PAINULY *et al.*, 1988).The single exception is that the *β*-methylaspartic acid residue is replaced by aspartic acid in the *Oscillatoria* toxin (as far as the stereochemistry and the unlikely differences in iso-linkages are not concerned). The OA-29 peptide is thus desmethyl 3-microcystin-RR. The overall results of structure determination agree well with the fast atom bombardment/mass spectrometry data presented by KRISHNAMURTHY *et al.* (1986*b*, also in CARMICHAEL, 1988). The stereochemistry of the OA-29 residues has still to be studied in detail with stereospecific enzymes and chiral chromatography.

The very strong and rapid induction of hemorrhagic liver damage caused by the toxin OA-29 resembles the effects induced by microcystins to such a high degree that the mode of action is likely to be identical. Recently also toxins isolated from other species such as *Nodularia spumigena* and *Anabaena flos-aquae* have been reported to induce similar liver damage (KRISHNAMURTHY *et al.*, 1986*a*; ERIKSSON *et al.*, 1988*b*, 1988*c*).

The macroscopic massive hemorrhagic liver damage observed in this study (and in many previous studies on microcystins) is very similar to the *in vivo* effects of phalloidin. The mushroom toxin phalloidin also induces strong swelling of the liver due to extensive hemorrhages; "the animals bleed to death into their own liver" (WIELAND, 1965; AGOSTINI, 1983). In contrast to phalloidin no apparent necrosis could be seen with the peptide toxin from *Oscillatoria*. It is striking that necrotic cells were sparse in the livers of intoxicated mice although the structure of the parenchymal tissue was totally demolished. This reflects the rapidity of the toxic process and, furthermore, this fact corresponds well with the *in vitro* findings which have shown that microcystins do not have any major effects on the viability or the cell membrane permeability of hepatocytes although the toxins induce strong morphological changes (RUNNEGAR and FALCONER, 1986; ERIKSSON *et al.*, 1987; FALCONER and RUNNEGAR, 1987; ERIKSSON *et al.*, 1986*b*; J. ERIKSSON, G. PAATERO, J. MERILUOTO, G. CODD, G. KASS, P. NICOTERA and S. ORRENIUS, unpublished

results). Thus, the basic mechanism of toxicity is not associated with cell death or cell lysis but rather with changes in the cell morphology. The main target for the OA-29 toxin was the liver which also agree well with the *in vitro* studies which have shown that microcystins exhibit a high degree of cell specificity for hepatocytes (ERIKSSON *et al.*, 1987; FALCONER and RUNNEGAR, 1987). In a previous study with microcystin-YM a rapid activation of phosphorylase a was observed (RUNNEGAR *et al.*, 1987). The very marked glycogen depletion observed in this study could reflect such an activation of phosphorylase a, but could also be due to metabolic disturbances during the process of intoxication.

The obvious similarity in the structure of microcystins and the *Oscillatoria* toxin explains the qualitatively identical biological activities. There is, however, a notable difference in the i.p. LD_{50} values; 43 μg/kg for microcystin-LR (ERIKSSON *et al.*, 1988*a*) and 250 μg/kg for the OA-29 peptide. This difference cannot be explained by difference in the purity of the toxins (spectroscopic methods used in this paper typically demand over 95% purity in the samples). The difference in LD_{50} values could be due to, for example, the stronger hydrophobicity of the toxin from *Oscillatoria*, as reflected by the chromatographic behavior of the toxin, resulting in reduced uptake efficiency or binding site specificity. To clarify this aspect we are going to conduct uptake studies with isotope labeled toxins.

As mentioned above, toxins similar to microcystins have been isolated from several different cyanobacterial species and genera. These cyclic peptide toxins which all have similar toxicological properties and share some common structural features constitute a peculiar link between taxonomically very distinct species. The conservation of the toxin structure is interesting because the role of toxins in cyanobacteria is unknown. They are often produced in great amounts: we have measured very high toxin levels, up to 1% of the dry weight, in certain *Microcystis* strains. Are the toxins involved in some physiological processes or do they have an ecological role in competition with other algae or as defense against predators? This question can perhaps be answered by studying the toxins, toxin precursors and degradation products in different cyanobacteria and environments.

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