Proc. Natl. Acad. Sci. USA
Vol. 86, pp. 770–774, February 1989
Chemistry

Structural characterization of toxic cyclic peptides from blue-green algae by tandem mass spectrometry

(liquid secondary-ion mass spectrometry/*Microcystis aeruginosa*)

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Communicated by Fred W. McLafferty, November 14, 1988 (received for review September 6, 1988)

ABSTRACT **Combined use of chemical degradation, derivatization, and tandem mass spectrometry for rapid structural characterization of toxic cyclic peptides from blue-green algae at the nanomole level is described. Previously, all blue-green algal toxins were thought to belong to a family of seven-residue cyclic peptides, having the general structure cyclo-D-Ala-L-Xaa-*erythro*-β-methyl-D-isoaspartic acid-L-Yaa-Adda-D-isoglutamic acid-*N*-methyldehydroalanine, where Xaa and Yaa represent variable amino acids of the L configuration and Adda is 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid. Structural characterization of two additional toxins indicates that further variability can exist within this family of naturally occurring toxic cyclic peptides. Isoaspartic acid and dehydroalanine can substitute for β-methylisoaspartic acid and *N*-methyldehydroalanine, respectively.**

Production of a family of toxic cyclic peptides in freshwater supplies by the cyanobacterium *Microcystis aeruginosa* has been linked to the death of livestock in both Australia and South Africa (1, 2). Growth of toxic blooms from the above blue-green algae is enhanced in stagnant water enriched in minerals and organic nutrients and depleted of dissolved oxygen (1). Since the toxins are unaffected by normal chlorination, flocculation, and filtration procedures used by water treatment facilities, growth of the cyanobacterium in reservoirs represents a potentially serious threat to the human population.

Structural studies involving extensive use of both NMR and mass spectrometry on five of the toxins indicated that they all belonged to a family of seven-residue cyclic peptides having the general structure cyclo-D-Ala-L-Xaa-*erythro*-β-methyl-D-isoaspartic acid-L-Yaa-Adda-D-isoglutamic acid-*N*-methyldehydroalanine, where Xaa and Yaa represent variable amino acids of the L configuration and Adda is 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid (3–5).

Cyclic peptides in which the Xaa and Yaa residues were identified as Leu and Ala and Tyr and Met, respectively, were characterized fully in earlier papers (4, 5). For three other toxins, only four of seven residues were placed unambiguously. Compositional analysis, however, confirmed the presence of Adda and Glu in each of the samples. The combined data suggested strongly that the three additional toxins all belonged to the above family of cyclic peptides. The two L-amino residues, Xaa and Yaa, in each of these toxins were assigned tentatively as Leu and Arg, Tyr and Arg, and Tyr and Ala, respectively (5).

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Here we describe methodology based on tandem mass spectrometry that facilitates rapid characterization of toxic cyclic peptides from blue-green algae at the nanomole level. Toxin containing the two L-amino acids Leu and Arg (Fig. 1) has been isolated from freshwater lakes in five countries and characterized by tandem mass spectrometry. Structures for two additional peptide hepatotoxins from blue-green algae blooms are also reported.

MATERIALS AND METHODS

Isolation and purification of toxic peptides from blue-green algae have been described (6). Final HPLC separations were performed on an Applied Biosystems model 130A system. Sample dissolved in 0.1% trifluoroacetic acid was injected onto a microbore RP300 Aquapore column (2.1 mm × 3 cm) and eluted with a 40-min linear gradient of 0–60% acetonitrile (0.085% trifluoroacetic acid) in 0.1% trifluoroacetic acid. Column effluent was monitored at 214 nm and fractions were collected by hand. The Waters Pico-Tag system was used for amino acid analysis of the purified toxins. Amino acid chirality was determined by HPLC analysis of diastereoisomers formed on reaction of the amino acids with 1-fluoro-2,4-dinitrophenyl-5-(L-alaninamide) (Pierce) (7). Standard solutions of peptide in either 5% acetic acid or methanol (1 nmol/μl) were stored at 0°C.

Mass Spectrometry. Mass spectra were recorded on both a triple quadrupole mass spectrometer (8) and a Fourier transform mass spectrometer (9). Operation of these instruments has been described (8, 9). Samples for mass analysis were prepared by adding 1 μl of a methanol solution containing peptide at the 0.5–1.0 nmol/μl level to a matrix containing 0.5 μl of monothioglycerol and 0.5 μl of a 1:1 mixture of dimethyl sulfoxide/6 M HCl on a gold-plated stainless steel probe tip 2 mm in diameter. The resulting mixture was then subjected to bombardment by a beam of 6- to 8-keV Cs^+ ions generated from a cesium ion gun (Antek, Palo Alto, CA). Collision-activated dissociation mass spectra were recorded on the triple quadrupole instrument with argon as the collision gas (2.5–3.0 millitorr; 1 torr = 133.3 Pa) and with collision energies between 20 and 30 eV.

Hydrolysis of the Cyclic Peptide Toxins with Trifluoroacetic Acid. An aliquot of the standard peptide solution (10 μl) was lyophilized, treated with trifluoroacetic acid (100 μl), and

Abbreviations: Adda, 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid; MAsp, β-methylaspartic acid; Mdha, *N*-methyldehydroalanine.

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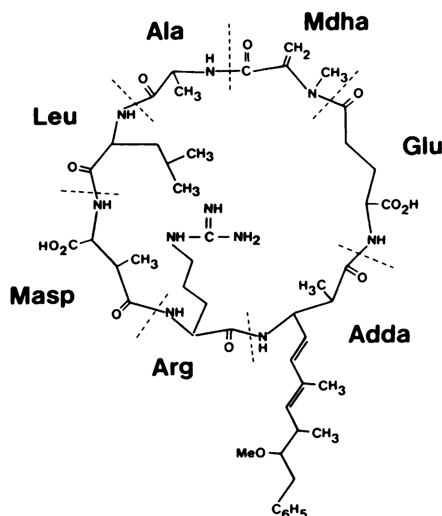

FIG. 1. Hepatotoxin from blooms of blue-green algae *M. aeruginosa*.

allowed to stand at room temperature overnight. The solution was then lyophilized and the residue was dissolved in 20–40 μl of methanol.

Esterification. A standard solution of 2 M HCl in methanol was prepared by adding acetyl chloride (160 μl) dropwise with stirring to methanol (1 ml) and by allowing the resulting solution to stand for 10 min at room temperature. Peptides at the 10–20 nmol level were esterified by treating lyophilized sample with 2 M methanolic HCl (100 μl) and by allowing the reaction mixture to stand at room temperature for 2 hr. Samples were then lyophilized and dissolved in either methanol or 5% acetic acid.

Deuterium Labeling of Amino Acid Residues Containing Free α-Carboxylic Acid Groups (10, 11). Peptide (10–20 nmol) was treated with 60 μl of a 2:1 mixture of acetic anhydride and 100% (vol/vol) formic acid. After 10 min at room temperature, the mixture was lyophilized and the residue was allowed to stand for 3–60 hr at room temperature in a solution containing 100 μl of deuterium oxide, 200 μl of pyridine, and 50 μl of acetic anhydride. Reagents were removed by lyophilization and deuterium incorporated on oxygen, and nitrogen was then back-exchanged by treating the residue with 2 × 200 μl of water and by lyophilizing the resulting solutions. Treatment of the residue with 2 M methanolic HCl for 2 hr converted the labeled peptide sample to the corresponding methyl ester.

Derivatization with *N-t*-Butyloxycarbonylprolylhydroxysuccinimide. Linear peptide (5–10 nmol) was dissolved in 75 μl of 50% aqueous pyridine and then treated with a 5-fold molar excess of reagent in pyridine. After 1 hr at 75°C, the mixture was treated with a second 5-fold molar excess of reagent, allowed to stand for an additional 1 hr at 75°C, and then lyophilized. The residue was taken up in 50 μl of water and the resulting mixture was lyophilized. To remove the *t*-butyloxycarbonyl group, the dried residue was treated with 50 μl of neat trifluoroacetic acid. The resulting solution was heated at 37°C for 10 min and then lyophilized. Traces of acid were removed by dissolving the sample in 50 μl of water and by lyophilizing the mixture. Derivatized peptides were separated from excess reagent by HPLC.

RESULTS AND DISCUSSION

Characterization of Toxin I Isolated from Akersvatn, Norway. Isolation and purification of a hepatotoxic peptide produced by blooms of the blue-green algae *M. aeruginosa* in Akersvatn, Norway, has been described (6). Amino acid analysis on the purified peptide indicated the presence of Ala, Arg, Glu, Leu, and β-methylaspartic acid (β-MAsp) in equimolar quantities. Only Leu and Arg were found to have the L-configuration by HPLC analysis of diastereomeric derivatives (7).

The summed masses of the above residues total 598 Da. Analysis of the peptide by liquid secondary ion mass spectrometry afforded a spectrum containing a strong signal for an $(M + H)^+$ ion at m/z 995. Previous work indicated that the toxic peptides from blue-green algae all belonged to a family of seven-residue cyclic peptides, termed cyanoginosin-XY, that contain two unusual amino acid residues, Adda and Mdha (*N*-methyldehydroalanine) (4, 5), not detected in the normal amino acid analysis scheme. Inclusion of masses for Adda (313 Da) and Mdha (83 Da) in the above calculation affords a mol mass of 994 that is in agreement with that determined by mass spectrometry. Thus, it seemed likely that the isolated toxin was a member of the above family of cyclic peptides. The variable amino acids could be either Leu and Arg or Arg and Leu.

Collision-activated dissociation mass spectra recorded on the toxin $(M + H)^+$ ion contained a large number of fragment ions that could not be assigned unambiguously to a single sequence of amino acids. Results of this type are not atypical for complex cyclic peptides, although successful characterization of several cyclic peptides by this approach has been achieved (12).

To convert the cyclic peptide to a linear structure, toxin was treated directly with neat trifluoroacetic acid. Progress of the reaction was monitored by mass spectrometry. Two products formed in about equal abundance. The $(M + H)^+$ ion for product I occurred at m/z 1013, a number consistent with that expected for a linear structure resulting from the addition of water to the cyclic peptide of mol mass 994. The mass spectrum of product II showed an $(M + H)^+$ ion at m/z 930. This is the expected m/z value for a peptide formed by loss of the Mdha residue (83 Da) from the linear peptide.

Shown in Fig. 2*A* is the collision-activated dissociation mass spectrum recorded on the ion at m/z 930. Nomenclature (13) and mechanisms of formation (14) for the fragment ions observed in this type of spectrum have been discussed. Daughter ions present in Fig. 2*A* are consistent with the partial sequence Ala-Leu-MAsp-Arg-Adda-Glu. Masses for fragments of type B and Y″ expected for this sequence are shown above and below the structure at the top of Fig. 2*A*. Those observed in the spectrum are underlined.

The ion at m/z 783 corresponds to B_5 and results from loss of water and the C-terminal residue (129 Da) from the $(M + H)^+$ ion. Glu and MAsp both have a mol mass of 129, so either could be located at the C terminus. Assignment of Glu to this position is based on the observation that this residue invariably occupies a site on the C-terminal side of Adda within the family of toxic cyclic peptides from blue-green algae. That Adda is adjacent to Glu is indicated by the characteristic mass difference of 313 Da between the B_5 and B_4 fragment ions at masses 783 and 470, respectively. The appearance of the Y_2'' ion at m/z 461 is also consistent with the above assignment. Placement of Arg on the N-terminal side of Adda is dictated by the finding that Y_3'' appears at m/z 617, 156 Da higher than Y_2''. MAsp is assigned to the next position in the chain because the Y_4'' ion appears 129 Da higher than Y_3'' and because this location is invariably occupied by MAsp in all blue-green algal peptides shown to contain this residue. Amino acid analysis on the parent cyclic peptide and the mass difference between Y_4'' and the $(M + H)^+$ ion (184 Da) both support assignment of Ala and Leu to the remaining two positions in the peptide although the fragment ion Y_5'' required to prove this is missing in the daughter ion spectrum of m/z 930.

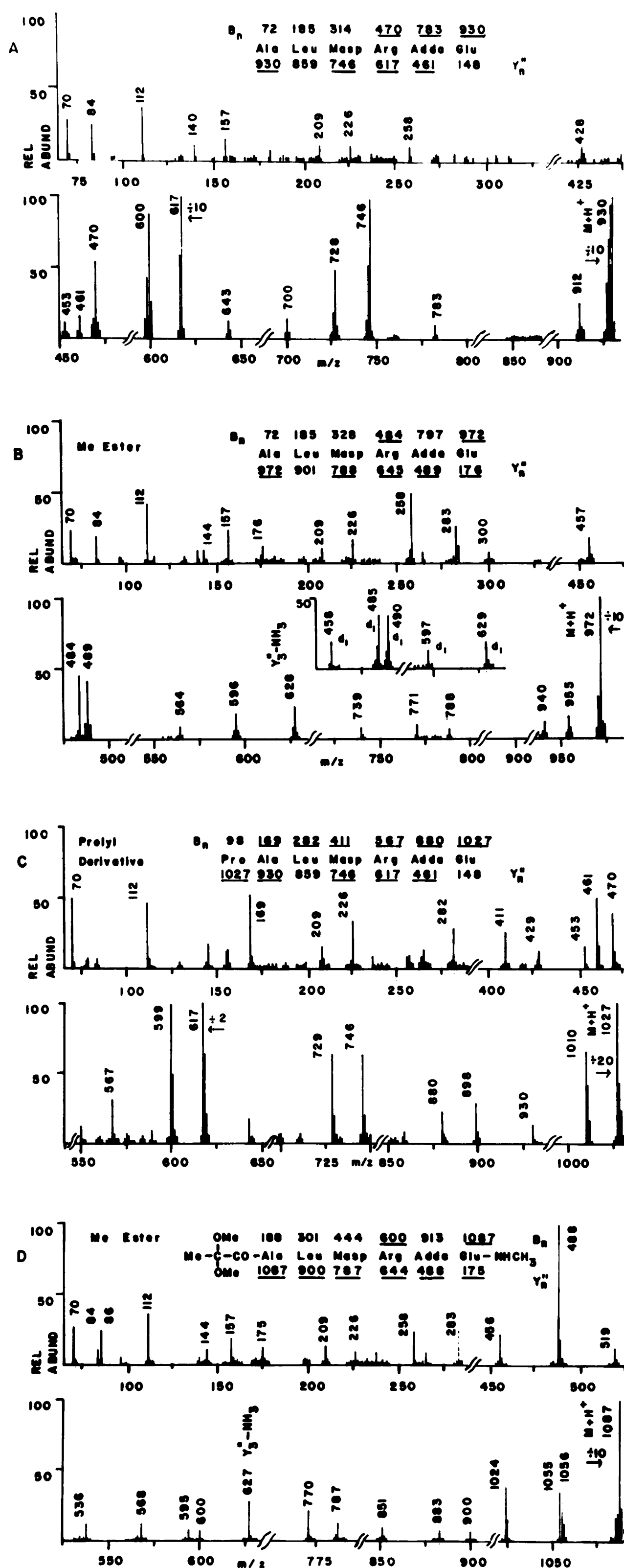

FIG. 2. Collision-activated dissociation mass spectra recorded on toxin I. Spectra generated from the $(M + H)^+$ ion of product II (*A*), product II methyl ester (*B*), prolylated derivative of product II (*C*), and product I methyl ester (*D*).

To confirm the fragment ion assignments made in Fig. 2*A*, products I and II obtained from acid hydrolysis of the cyclic peptide were converted to the corresponding methyl esters. Product II was also derivatized by adding a prolyl moiety to the unblocked N terminus. Collision-activated dissociation mass spectra of the $(M + H)^+$ ions corresponding to the methyl ester and prolyl derivative of the linear peptide of mol mass 929 are shown in Fig. 2 *B* and *C*, respectively. Note that all fragment ions predicted to contain one or more free carboxylic acid groups in Fig. 2*A* do indeed shift to higher mass in the methyl ester spectrum by the expected multiple of 14 Da per COOH group. In Fig. 2*C*, daughter ions designated as being of type B shift by 97 Da as a result of adding a prolyl moiety to the N terminus of the peptide sample. Note also that Fig. 2*C* contains a complete series of ions of type B. Introduction of the strongly basic prolyl group at the N terminus of the peptide promotes formation of fragment ions containing the amino terminus and allows the complete sequence of the peptide to be determined. The abundant ion at m/z 169 dictates that the order of the first two amino acids in underivatized product II be Ala-Leu rather than Leu-Ala.

Collision-activated dissociation of m/z 1013, the $(M + H)^+$ ion observed for product I in the acid catalyzed hydrolysis of toxin I, affords a series of ions of type Y″, all of which occur at m/z values that are 13 Da higher than those seen in Fig. 2*A*. Conversion of product I to the corresponding methyl ester shifted the $(M + H)^+$ from m/z 1013 to 1087, an increase of 74 Da. Incorporation of three methyl groups and one molecule of methanol into the linear peptide accounts for the observed mass shift. The collision-activated dissociation mass spectrum of m/z 1087 is shown in Fig. 2*D*. Particularly noteworthy is the observation of a series of fragment ions of type Y″ that occur at m/z values that are 1 Da lower than those in Fig. 2*B*. We conclude that product I is formed by protonation of the enamine moiety in the Mdha residue, as shown in Fig. 3. This is then followed by acid-catalyzed hydrolysis of the resulting Schiff base to afford a linear peptide blocked at the N terminus and C terminus with α-ketoacyl and *N*-methyl amide groups, respectively. Treatment of this molecule with 2 M methanolic HCl would be expected to esterify the two carboxylic acid side chains in MAsp and Glu and also to convert the keto group to the corresponding dimethyl ketal. The result of these transformations would be to increase the mass of the molecule by the observed 74 Da. Note that the presence of a complete series of Y″ ions in Fig. 2*D* allows all residues except MAsp and Glu to be placed in the proposed structure unambiguously.

Once the above sequence information had been obtained, additional experiments were performed to determine which of the two carboxyl groups in MAsp and Glu were involved in the amide linkages along the backbone of the cyclic peptide. Base-catalyzed incorporation of deuterium selectively onto carbons adjacent to all free α-carboxyl groups in

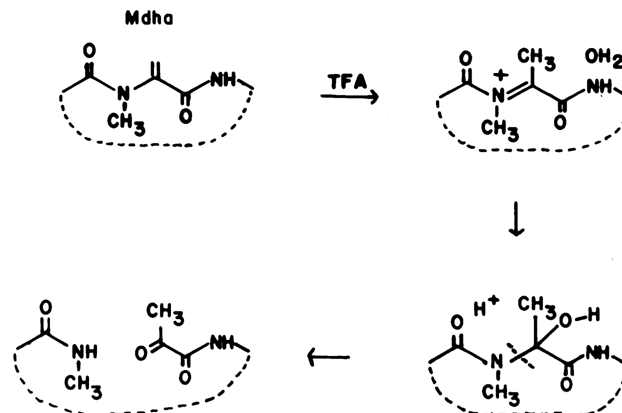

FIG. 3. Acid-catalyzed cleavage of cycloheptapeptide toxins from blue-green algae. TFA, trifluoroacetic acid.

the peptide was used to obtain this information (4, 9, 10). Labeling of MAsp and Glu is expected even when they are in non-C-terminal positions, provided that these residues are connected by an iso-linkage and thus have a free α-carboxyl group.

When the above experiment was performed on the mixture of two products obtained in the acid-catalyzed hydrolysis of the toxin I and the labeled peptides were then converted to the corresponding methyl esters, the m/z values for the $(M + H)^+$ ions of products I and II shifted from m/z 1087 and 972 to 1091–1092 and 974, respectively. Product I incorporates up to five deuterium atoms, and product II incorporates two deuterium atoms. Fig. 2*B* (*Inset*) shows a portion of the collision-activated dissociation mass spectrum recorded on the $(M + H)^+$ at m/z 974 for product II. Note that m/z values for the major fragment ions containing either MAsp or Glu in Fig. 2*C* all shift by 1 Da to higher mass in the spectrum of the labeled peptide. This result requires that MAsp be connected to Arg by an iso-linkage. Incorporation of deuterium into Glu results because this residue is C terminal in the six-residue fragment. That this residue is also part of an iso-linkage in the intact cyclic peptide is strongly suggested by the observation that the methyl ester of the linear peptide in Fig. 2*D* incorporates up to five deuterium atoms, three on the carbon α to the ketoacyl moiety and one each α to the free carboxyl groups of MAsp and Glu. Further support for the presence of isoGlu was obtained from a liquid secondary ion mass spectrum of the deuterium-labeled methyl ester of product I recorded on the tandem quadrupole Fourier transform instrument. Fragment ions corresponding to Y_1''–Y_3'' all showed deuterium incorporation to an extent greater than 25%. We conclude that the toxic heptapeptide isolated from blooms of the blue-green algae *M. aeroginosa* in Akersvatn, Norway, has the structure cyclo-D-Ala-L-Leu-D-MAsp-L-Arg-Adda-D-isoGlu-Mdha. Further work in our laboratory has identified this same toxin in blooms of blue-green algae taken from waters in South Africa, Scotland, Canada, and the United States.

Characterization of Toxin II from Canada. Mass spectra recorded on toxin II from Canada showed $(M + H)^+$ ions at both m/z 981 and 995. HPLC was used to separate the mixture and the sample of mol mass 994 was then hydrolyzed with neat trifluoroacetic acid. Products of this reaction were converted to methyl esters and prolylated as described in *Materials and Methods*. Collision-activated dissociation spectra recorded on the resulting derivatives confirmed that the material of mol mass 994 was identical in structure to toxin I described above.

Amino acid analysis on the sample of mol mass 980 indicated that Ala, Arg, Glu, Leu, and Asp were present in equimolar quantities. Only Leu and Arg were found to have the L-configuration by HPLC analysis of diastereoisomer derivatives. Mass spectra recorded on the sample after treatment with neat trifluoroacetic acid showed two $(M + H)^+$ ions at m/z 916 and 999, respectively. Conversion of the two products to the corresponding methyl esters shifted the observed $(M + H)^+$ ions to m/z 958 and 1073, respectively. Shown in Fig. 4*A* is the collision-activated dissociation spectrum recorded on the $(M + H)^+$ ion of m/z 1073. Comparison of these data with that obtained on the same derivative of toxin I (Fig. 2*D*) shows that all fragment ions assumed to contain the MAsp residue in Fig. 2*D* are now shifted to lower mass by 14 units in Fig. 4*A*. Thus, sample of mol mass 980 in toxin II contains Asp in place of the MAsp residue in toxin I. Fragment ions of type B and Y″ predicted for open-chain derivative of the Asp-containing peptide are shown above and below the structure in Fig. 4*A*. Those observed in the collision-activated dissociation spectra are underlined. Deuterium labeling experiments conducted on the linear peptide derivatives confirm that both Asp and Glu

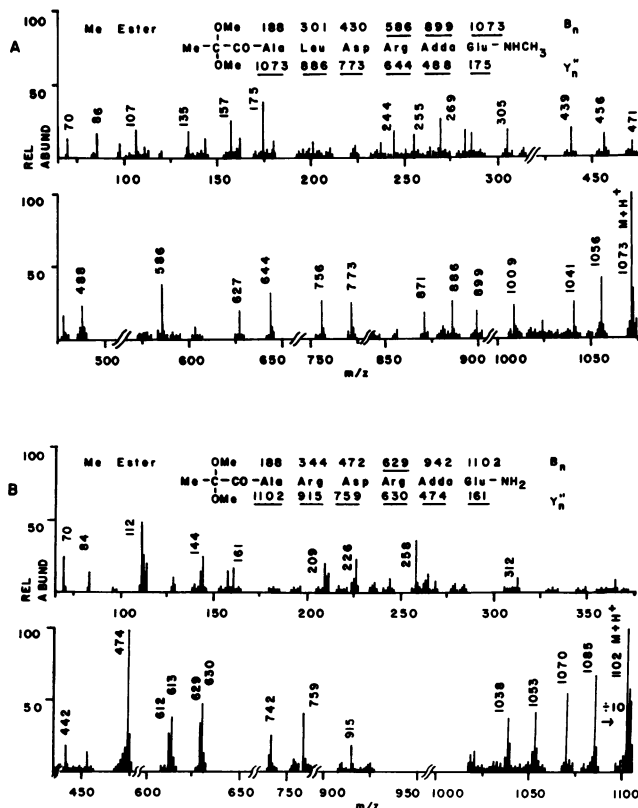

FIG. 4. Collision-activated dissociation mass spectrum generated from the $(M + H)^+$ ions of the linear peptides derived from toxin II (*A*) and toxin III (*B*).

residues are attached to the adjacent amino acids by iso-linkages. We conclude that the sample of mol mass 980 in toxin II has the structure, cyclo-D-Ala-L-Leu-D-isoAsp-L-Arg-Adda-D-isoGlu-*N*-Mdha.

Characterization of Toxin III from Norway. A mass spectrum recorded on the toxin III from Norway showed an $(M + H)^+$ ion at m/z 1010. Amino acid analysis confirmed the presence of Ala, Asp, Glu, and Arg in the molar ratio 1:1:1:2. Only Arg was found to have the L configuration by HPLC analysis of diastereomeric derivatives (7). Treatment of the sample with neat trifluoroacetic acid produced a single product of mol mass 1027. Unlike toxins I and II above, toxin III does not eliminate Mdha to form a second product in the acid hydrolysis reaction. Mass spectra recorded on the single hydrolysis product and the corresponding methyl ester showed $(M + H)^+$ ions at m/z 1028 and 1102, respectively. The observed shift of 74 mass units is analogous to that observed for toxins I and II and suggests that toxin III also suffers hydrolysis in trifluoroacetic acid to a linear peptide blocked at the *N* terminus and C terminus with α-ketoacyl and amide groups, respectively.

Shown in Fig. 4*B* is the collision-activated dissociation spectrum recorded on the $(M + H)^+$ ion of m/z 1102. Comparison of this spectrum to that obtained for the same derivative of toxin II (Fig. 4*A*) shows that the first four fragment ions of type Y″ are all shifted to lower mass by 14 units. Combined with the results of amino acid analysis, these data indicate that the C terminus of the linear peptide is blocked with an unsubstituted amide group. We conclude that toxin III contains dehydroalanine in place of the usual Mdha residue. Placement of Asp and two Arg residues within the peptide is facilitated by the presence of a complete series of Y″ type fragment ions in Fig. 4*B*. The proposed sequence for the linear hydrolysis product is shown at the top of Fig. 4*B*. Predicted fragment ions of types B and Y″ appear above

and below the structure, respectively. Those observed in the collision-activated dissociation spectrum are underlined. Deuterium incorporation into the product of acid hydrolysis confirms that the Asp and Glu residues contain free α-carboxylic acid groups and are therefore attached to adjacent residues by *iso* linkages. We conclude that toxin III has the structure, cyclo-D-Ala-L-Arg-D-isoAsp-L-Arg-Adda-D-isoGlu-dehydroalanine.

The results presented in this paper indicate that tandem mass spectrometry, in combination with specific chemical degradation and derivatization techniques, is ideally suited for the direct characterization of toxic cyclic peptides isolated as mixtures at the nanomole level from blue-green algae. Samples are treated with neat trifluoroacetic acid to convert the cyclic peptides to linear structures, which are then either prolylated or converted to the corresponding methyl esters. Tandem mass spectrometry provides the necessary structural information to deduce the sequence of amino acids in the derivatized linear peptides. Deuterium labeling of the peptides facilitates detection of amino acid residues linked through side-chain carboxyl groups. In the present work, the above methodology was used to determine structures for two toxic cyclic peptides isolated from blue-green algae.

A contract awarded to D.F.H. from the U.S. Army and instrument development funds awarded to D.F.H. from the Monsanto Co., Center for Innovative Technology (Richmond, VA) (BIO-87006), and the National Science Foundation (CHE-8618780) are gratefully acknowledged.

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