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Isolation and Characterization of Hepatotoxic Microcystin Homologs from the Filamentous Freshwater Cyanobacterium *Nostoc* sp. Strain 152

K. SIVONEN,[1]* W. W. CARMICHAEL,[2] M. NAMIKOSHI,[3] K. L. RINEHART,[3] A. M. DAHLEM,[4] AND S. I. NIEMELÄ[1]

Department of Microbiology, University of Helsinki, SF-00710 Helsinki, Finland[1]; Department of Biological Sciences, Wright State University, Dayton, Ohio 45435[2]; School of Chemical Sciences, University of Illinois, Urbana, Illinois 61801[3]; and Merrell Dow Research Institute, Cincinnati, Ohio 45215[4]

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A strain of the filamentous cyanobacterium *Nostoc* sp. isolated from a lake in Finland was found to produce at least nine hepatotoxic peptides with chemical and toxicological properties similar to those of the hepatotoxic hepta- and pentapeptides produced by other cyanobacteria. Toxins were isolated and purified by high-performance liquid chromatography. Amounts available for five of the purified toxins (P6, P14, P15, P16, and P18) were adequate for high-performance liquid chromatography amino acid analysis and determination of molecular weight by fast-atom bombardment-mass spectrometry (FAB-MS). Quantities of three toxins (P14, P15, and P16) were adequate for further analysis by high-resolution FAB-MS, FAB-MS/MS, and ^{1}H-nuclear magnetic resonance. Analysis showed that the toxins are new types of microcystin-LR homologs. Microcystin-LR contains equimolar amounts of D-alanine, L-leucine, D-*erythro*-β-methylaspartic acid, L-arginine, ADDA (3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4,6-decadienoic acid), D-glutamic acid, and *N*-methyldehydroalanine (molecular weight [MW], 994). *Nostoc* sp. strain 152 was found to produce the following microcystin-LR homologs: (i) P6 contains an extra methylene group most probably due to the presence of *N*-methyldehydrobutyrine instead of *N*-methyldehydroalanine (MW, 1,008); (ii) P14 is *O*-acetyl-*O*-demethyl ADDA-microcystin-LR (MW, 1,022); (iii) P15 is 3-demethyl-*O*-acetylADDA-homoarginine-microcystin-LR (MW, 1,036); (iv) P16 is 3-demethyl-*O*-acetyl-*O*-demethylADDA-microcystin-LR (MW, 1,008); (v) P18 is 3-demethyl-*O*-acetyl-*O*-demethylADDA-homoarginine-microcystin-LR (MW, 1,022). The toxicities of the new microcystin homologs were not significantly different from those of microcystin-LR or demethylmicrocystin-LR.

Toxin-producing genera of fresh- and brackish water cyanobacteria are now known to include filamentous *Anabaena*, *Aphanizomenon*, *Nodularia*, and *Oscillatoria*, plus coccoid *Microcystis*. While several other cyanobacterial genera are implicated in animal and human water-based toxicosis (11), species and strains within these five genera are known to contain cyclic hepatotoxic hepta- or pentapeptides and/or neurotoxic alkaloids (1, 2, 4, 5, 8). Cyclic hepatotoxic peptides are the most common offenders worldwide in cases of water-based disease caused by toxic cyanobacteria. We now know that these peptides are a related family of cyclic hepta- and pentapeptides having a molecular weight range of 824 to 1,044. Nine cyclic heptapeptides (termed microcystins) (5, 6) and one cyclic pentapeptide (termed nodularin) (7, 16, 19) have been chemically defined. Microcystins have a dehydroamino acid, a characteristic C_{20} amino acid (ADDA [3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4,6-decadienoic acid]), three D-amino acids, and two L-amino acids. Differences between the microcystins are primarily in the type of L-amino acid present and secondarily in the presence or absence of a methyl group on one of the D-amino acids or on a dehydroamino acid or on both. The general composition for these toxins is as follows: cyclo (-D-Ala^1-L-X^2-D-*erythro*-β-methylAsp^3-L-Z^4-ADDA^5-D-Glu^6-*N*-methyldehydroAla^7). Demethylation can occur on amino acid number 3 and/or 7; X = leucine (L), arginine (R), tyrosine (Y); and Z = arginine (R), alanine (A), methionine (M). Currently, combinations for XZ include LR, LA, YA, YM, YR, and RR. ADDA is necessary for biological activity (A. M. Dahlem, V. R. Beasley, S. B. Hooser, K.-I. Harada, K. Matsura, M. Suzuki, K. L. Rinehart, C. A. Harvis, and W. W. Carmichael, Chem. Res. Toxicol., in press). The pentapeptide nodularin is cyclo (D-*erythro*-β-methylAsp-L-Arg-ADDA-D-Glu-*N*-methyldehydrobutyrine). All of these cyclic peptides are potent hepatotoxins which cause death rapidly in wild and domestic animals drinking from water supplies containing heavy concentrations (water blooms) of these toxigenic genera (3, 10, 24). Studies in Scandinavia over the past 10 years have pointed out the occurrence of these same cyanobacterial toxins (18, 19, 21–23). Genera involved in producing these peptide toxins have been the same as reported elsewhere in the world. Recent studies in Finland have, however, revealed a hepatotoxin-producing strain of the filamentous cyanobacterial genus *Nostoc*. *Nostoc* has previously been reported toxic in only one case (9). In that report, a stock pond near Waco, Tex., contained blooms of *Nostoc rivulare* that were responsible for the deaths of turkeys, ducks, chickens, cattle, frogs, and fish. The toxin(s) present was not defined, but the signs of toxicity reported would indicate the presence of a hepatotoxin(s). This paper presents the results of our studies on the toxicity and structures of the peptide toxins produced by a toxic strain of *Nostoc* sp. isolated from a freshwater lake in Finland.

* Corresponding author.

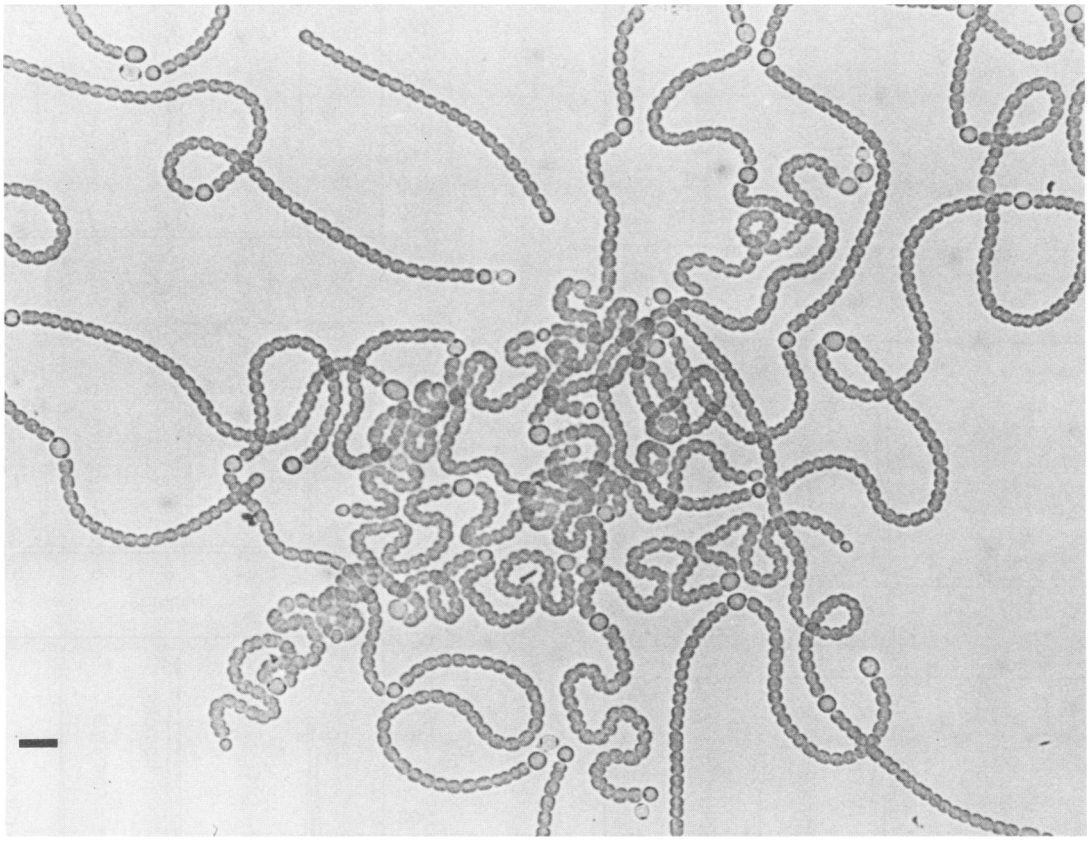

FIG. 1. Photomicrograph of *Nostoc* strain 152 grown in Z8 medium minus nitrogen. Bar, 10 μm.

MATERIALS AND METHODS

Isolation and culture of strain 152. *Nostoc* sp. strain 152 was isolated from a water bloom sample taken on 1 September 1986 at Lake Sääksjärvi, Finland, which is located at latitude 60°50′ N, longitude 26°25′ E (21). When collected, the water bloom sample was dominated by *Aphanizomenon flos-aquae*. Mouse bioassay of the bloom sample indicated that it was nontoxic (50% lethal dose, intraperitoneally [i.p.], mouse, >1,500 mg/kg). *Nostoc* strain 152 was isolated by streaking the bloom sample onto Z8 agar plates minus nitrogen (20). After approximately 2 weeks, *Nostoc* appeared as a single colony, which was transferred to Z8-minus-nitrogen medium and batch cultured for toxicity testing (20). The toxins were then isolated. An axenic clone from the culture was obtained by the method of Vaara et al. (26).

Toxin isolation. Lyophilized cells were extracted twice (2 h and then overnight) with 5% 1-butanol–20% methanol (vol/vol) in water and centrifuged (10,000 rpm; Sorvall rotor GSA). The supernatant was air dried to 0.5 volume and then passed through octadecyl cartridges (Bond Elut C_{18}; Analytichem). The toxic fraction was eluted with 100% methanol. Air-dried toxic fractions in aqueous solution were separated by high-performance liquid chromatography (HPLC), using a C_{18} column (μBondapack; 19 by 150 mm; Waters Associates) according to a procedure modified from that of Siegelman et al. (17) and Krishnamurthy et al. (13). At the first

TABLE 1. Amino acid analyses and molecular weights of toxins isolated from *Nostoc* sp. strain 152

Peak[a]	Retention time (min)[b]	Amino acid analysis[c]	Mol wt (M + H; *m/z*)[d]	Molecular formula[e]
P6	11.5	Glu, β-methylAsp, Arg, Ala, Leu	1,009	
P14	25.3	Glu, β-methylAsp, Arg, Ala, Leu	1,023	$C_{50}H_{75}N_{10}O_{13}$
P15	28.7	Glu, β-methylAsp, Ala, (homoarginine),[f] Leu	1,037	$C_{51}H_{77}N_{10}O_{13}$
P16	30.8	Glu, Asp, Arg, Ala, Leu	1,009	$C_{49}H_{73}N_{10}O_{13}$
P18	36.7	Glu, Asp, Ala, (homoarginine),[f] Leu	1,023	

[a] Peaks 7, 13A and B, and 17 with retention times of 12.8, 22, 22.5, and 33.7 min showed base-line resolution (i.e., were separate peaks) and were hepatotoxic. Chemical analysis was not done for these peaks since their amounts were too small. Analysis of these peaks will be done as more cell material becomes available.

[b] Based on reversed-phase C_{18} HPLC separation with a MicroBondapak column (Waters), 19 by 150 mm.

[c] Analysis by Waters Pico Tag method. Molar ratio of each amino acid was equal to 1. This method does not detect two of the seven amino acids present in these heptapeptides. ADMAdda and *N*-methyldehydroAla are confirmed by ^{1}H-NMR.

[d] Determined by FAB-MS on a ZAB-SE mass spectrometer.

[e] Based on high-resolution FAB-MS.

[f] Confirmed by ^{1}H-NMR.

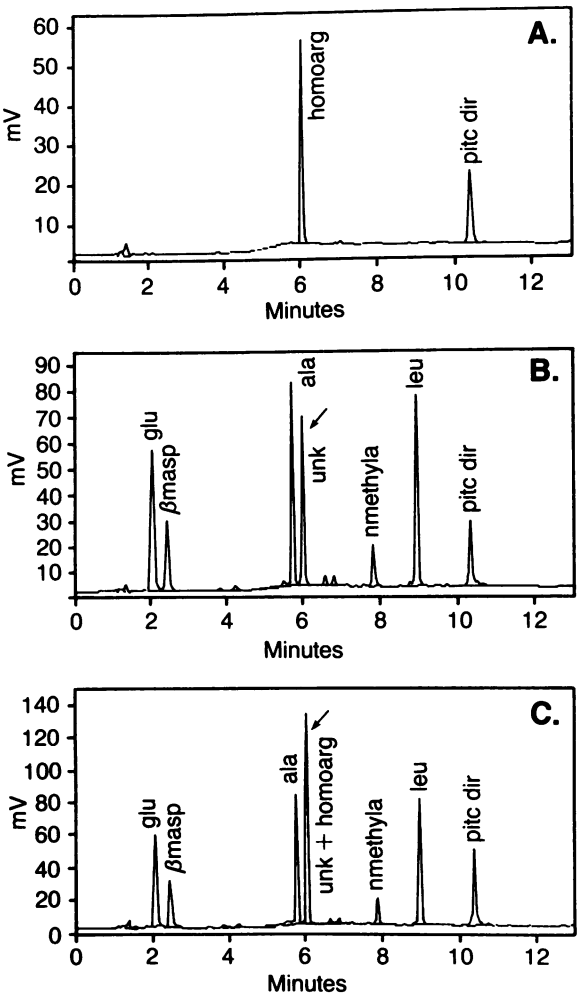

FIG. 2. HPLC amino acid analysis of (A) homoarginine standard (Har; Sigma), (B) *Nostoc* toxin P15, and (C) *Nostoc* toxin P15 plus homoarginine. This analysis shows that the unknown amino acid of P15 has the same retention time as homoarginine. Analysis was by Waters Pico Tag precolumn derivatization with phenylisothiocyanate to yield phenylthiocarbamyl amino acids. pitc dir, Unidentified phenylisothiocyanate derivative produced during sample derivatization; nmethyla, *N*-methylamine, a hydrolysis product of *N*-methyldehydrobutyric acid; βmasp, β-methylaspartic acid; glu, glutamic acid; ala, alanine; leu, leucine; homoarg, homoarginine; unk, unknown.

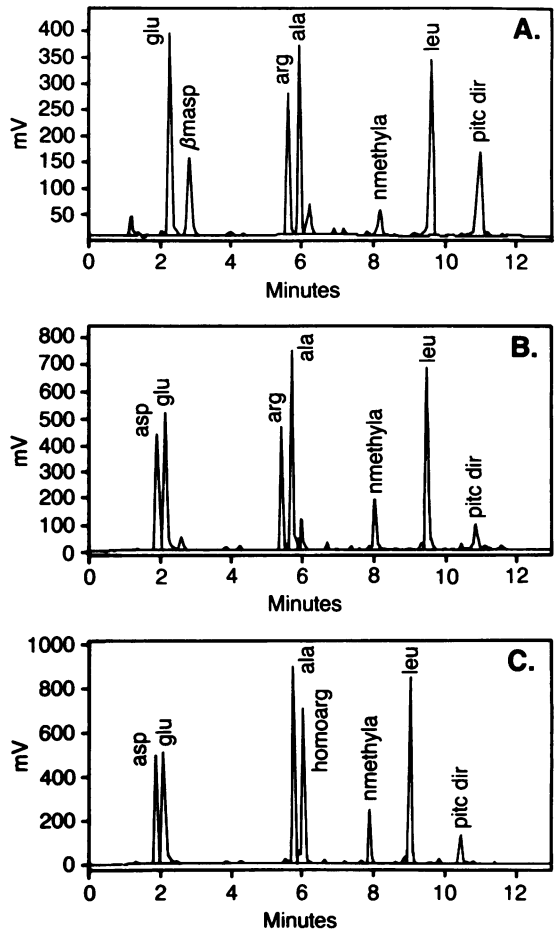

FIG. 3. HPLC amino acid analysis of (A) *Nostoc* toxin P14 ([ADMAdda^5]MCYST-LR), (B) *Nostoc* toxin P16 ([D-Asp^3, ADMAdda^5]MCYST-LR), and (C) *Nostoc* toxin P18 ([D-Asp^3, ADMAdda^5]MCYST-LHar). See legend to Fig. 2 for abbreviations.

HPLC purification step, a 4-ml/min mobile phase of 26% acetonitrile–10 mM ammonium acetate was used. Isolated toxic fractions were further purified by HPLC with an acetonitrile-water gradient of 20% acetonitrile over 5 min followed by a gradient to 30% acetonitrile in 25 min at a flow rate of 4 ml/min and then, if necessary, an isocratic run with a mobile phase of 35% acetonitrile–17 mM orthophosphoric acid, pH 3. Purified fractions were desalted by C_{18} cartridges and lyophilized. Fractions were stored at −80°C.

Toxicity. The i.p. mouse (20 to 25 g; female NMRI; University of Helsinki) assay was used to test toxicity of cultured cells and toxic HPLC fractions. The 50% lethal doses were estimated for three of the purified toxins (20 to 25 g; male ICR Swiss mice; Wright State University). Postmortem necropsies were done and liver weights were determined as a percentage of body weight (20, 21).

Analysis of the toxins. Purified toxins (~95%) from HPLC were hydrolyzed in 6 N HCl at 106°C for 24 h prior to amino acid analysis. The released amino acids were pre-column derivatized with phenyl isothiocyanate, and the phenylthiocarbamyl amino acids were analyzed with a Waters Pico Tag HPLC system. The derivatives were loaded onto a C_{18} column (15 cm by 4.6 mm) and eluted over 8 min by using a 0 to 60% gradient of acetonitrile in 0.138 M aqueous sodium acetate. The column flow rate was 1.0 ml/min, and the compounds eluted were detected by UV absorption at 258 nm (7, 19).

The intact peptides were analyzed at the University of Illinois by low-resolution fast-atom bombardment-mass spectrometry (FAB-MS; ZAB-SE mass spectrometer), using the magic bullet matrix (dithiothreitol-dithioerythritol, 1:3; m/z = 155). The source temperature was 30°C, and the target was bombarded with xenon atoms at 8 keV. This was followed by high-resolution FAB-MS (ZAB-SE), FAB-MS/MS (VG 70-5E4F), and ^{1}H-nuclear magnetic resonance (NMR) (GN 500-MHz FT NMR spectrometer).

RESULTS

A photomicrograph of isolate 152 identified as *Nostoc* sp. is shown in Fig. 1. The culture does not have gas vacuoles

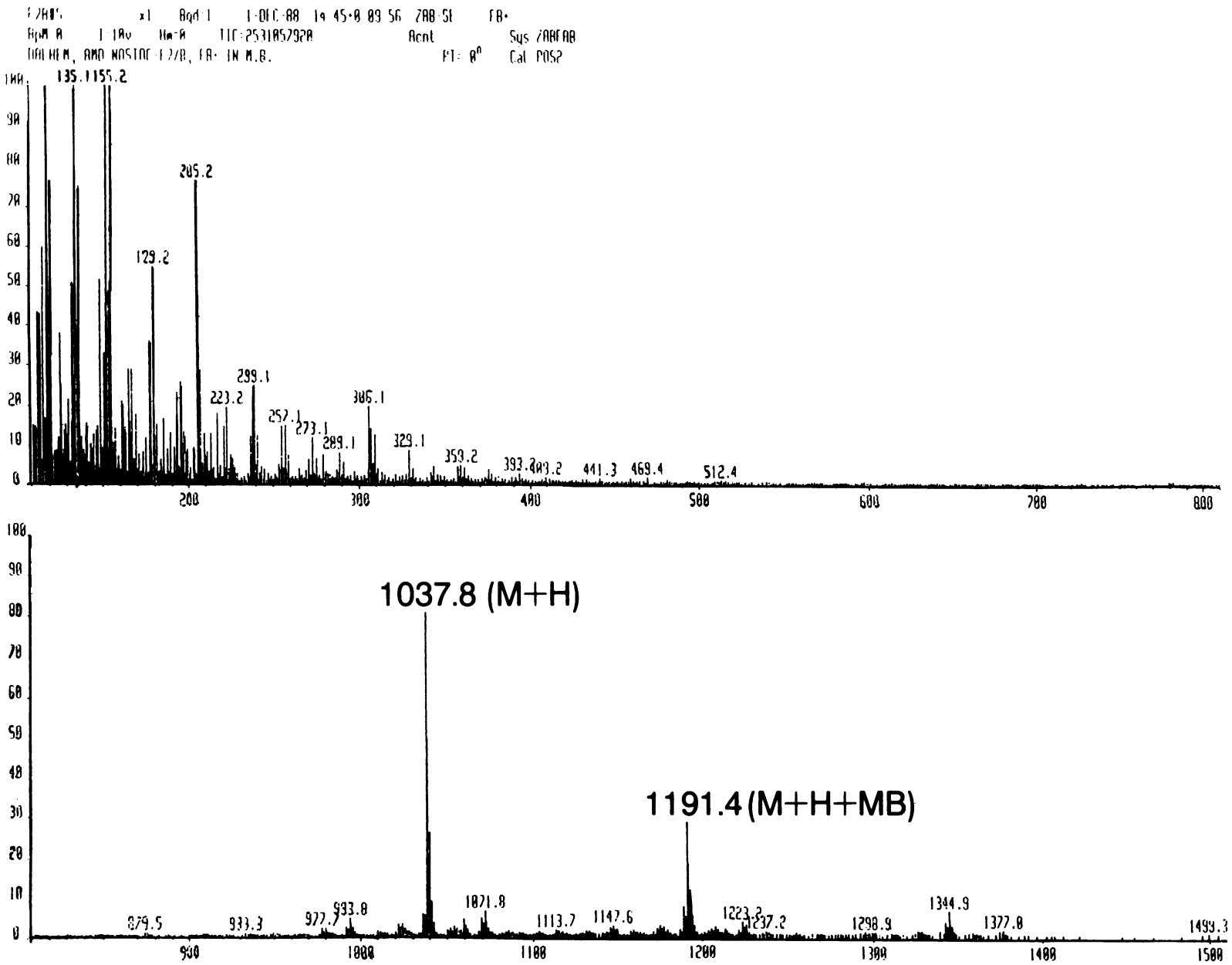

FIG. 4. Low-resolution FAB-MS of *Nostoc* toxin P15 in magic bullet (MB) matrix. M + H = 1,037. M + H + MB matrix = 1,191. Molecular weight based on MCYST-LR (m/z = 994) plus an acetyl group on ADDA (m/z = 28) plus a methylene group on arginine (m/z = 14); total molecular weight, m/z = 1,036 (M + H; m/z = 1,037).

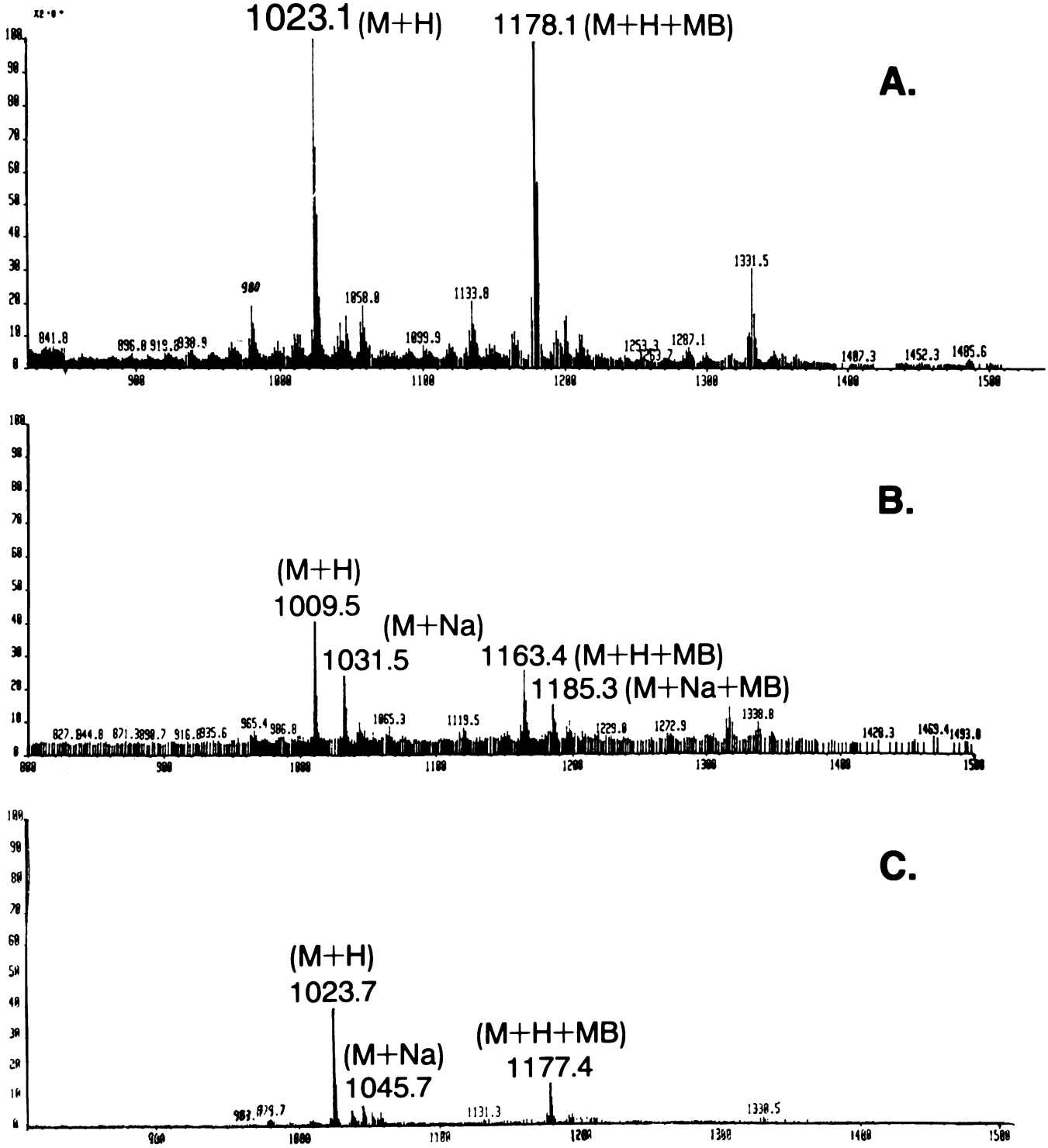

FIG. 5. Low-resolution FAB-MS of (A) *Nostoc* toxin P14, (B) *Nostoc* toxin P16, and (C) *Nostoc* toxin P18 in magic bullet (MB) matrix. Note toxin P14 equals MCYST-LR (m/z = 994) plus an acetyl group (m/z = 28); total m/z = 1,022. Toxin P16 equals MCYST-LR (m/z = 994), minus methylene (m/z = 14), plus an acetyl group (m/z = 28); total m/z = 1,008. Toxin P18 equals MCYST-LR (m/z = 994) minus methylene (m/z = 14) plus an acetyl group (m/z = 28) and a methylene group (m/z = 14); total m/z = 1,022.

and grows forming loose, slightly slimy aggregates. Minimal lethal dose of the cultured cells was 25 mg/kg (i.p., mouse), and the symptoms of poisoning were similar to those seen with hepatotoxins of other cyanobacteria. Upon autopsy, swollen and blood-engorged livers were observed.

HPLC analysis found 12 peaks that were isolated and tested positive for hepatotoxicity by mouse bioassay. Due to peak overlap, it is possible that some of these peaks were not individual compounds. From subsequent peak separation and partial amino acid analysis, the presence of at least nine

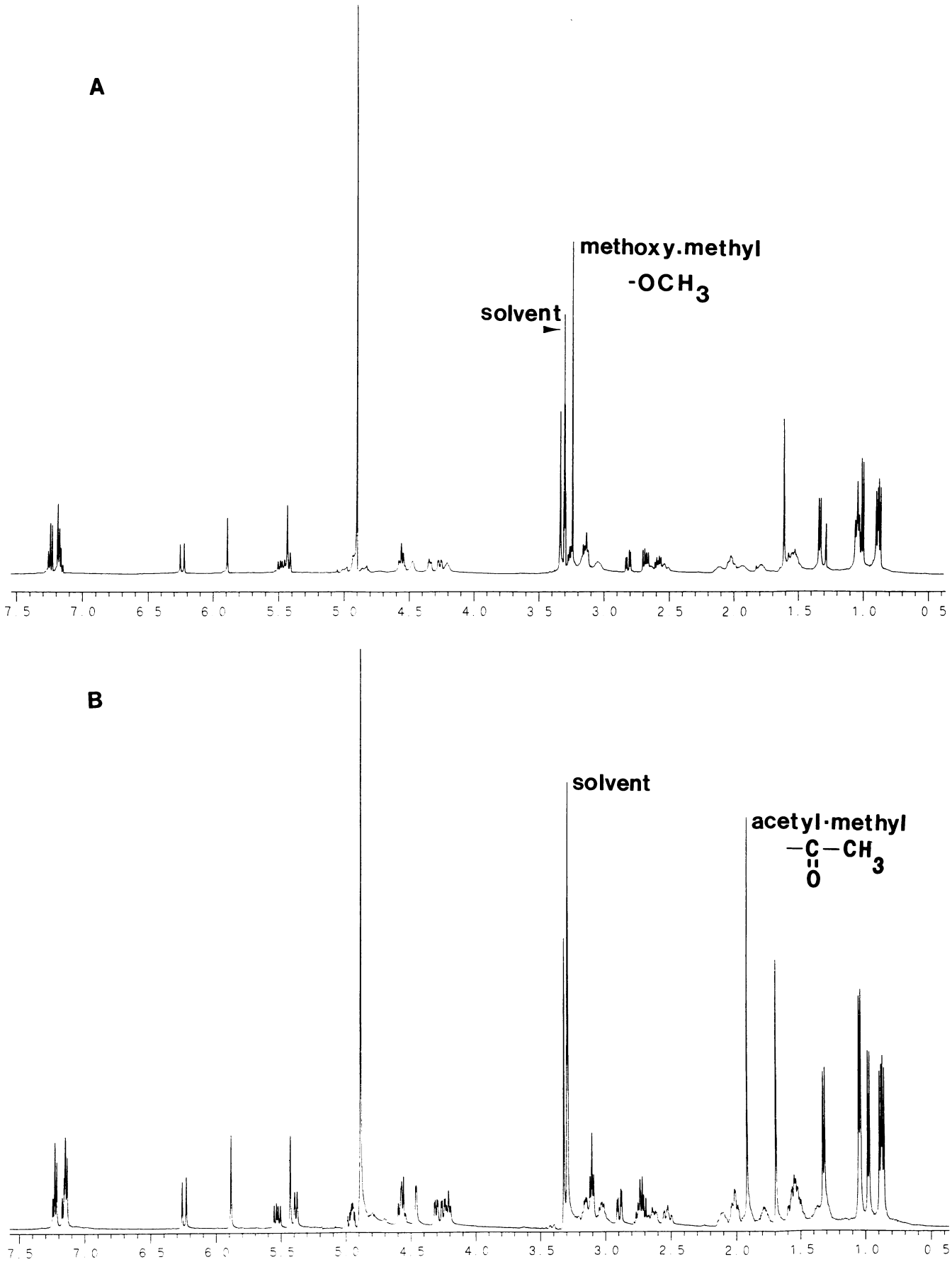

FIG. 6. ^{1}H-NMR spectrum of (A) MCYST-LR and (B) toxin P15. MCYST-LR standard is from *M. aeruginosa* PCC 7820. Note presence of methoxymethyl in panel A (corresponding to normal ADDA) and acetylmethyl in panel B (corresponding to ADMAdda).

chemically different toxins in strain 152 was detected (Table 1). Because of the amounts available, only five of these toxins are discussed further in this paper. Peaks 7, 13A and B, and 17 with retention times of 12.8, 22, 22.5, and 33.7 min, respectively, are omitted here pending additional cell material for analysis.

Amino acid analysis results of these five toxins are given in Table 1 and Fig. 2 and 3. All five toxins were analyzed by low-resolution FAB-MS (Fig. 4 and 5; toxin P6 not shown). Quantities of toxins P6 and P18 were not adequate to continue structure analysis studies, but P14, P15 (the two main toxins), and P16 were further analyzed by high-resolution FAB-MS, FAB-MS/MS (results not shown), and ^{1}H-NMR spectroscopy (Fig. 6).

Amino acid analysis and low-resolution FAB-MS results revealed some interesting points about the toxins in *Nostoc* sp. strain 152. The basic structure of the toxins is that of microcystin-LR (MCYST-LR; *m/z* 994), the most common heptapeptide hepatotoxin in cyanobacteria. MCYST-LR consists of D-Glu, D-*erythro*-β-methylAsp, L-Arg, D-Ala, L-Leu, ADDA, and *N*-methyldehydroAla. Three differences between the toxins of strain 152 and MCYST-LR can be seen in Table 1 and Fig. 2 to 6. First is the presence of Asp instead of β-methylAsp in P16 and P18 (Fig. 3). Second is the presence of an unknown amino acid instead of Arg in P15 and P18 (Fig. 2 and 3). Comparison of homoarginine (Har; Sigma) standard (Fig. 2) with the unknown amino acid in P15 and P18 indicated that this unknown amino acid was most likely Har. This was subsequently verified by ^{1}H-NMR (M. Namikoshi, K. L. Rinehart, R. Sakai, K. Sivonen, and W. W. Carmichael, J. Org. Chem., in press). The third difference is in the molecular weight for each of the five toxins compared with MCYST-LR (Table 1; Fig. 4 and 5). The molecular weight difference of the various toxins of *Nostoc* sp. is based on the presence of one additional methylene group (homoarginine) or demethylation of β-methylAsp or both. The other molecular weight difference was found to be associated with the ADDA part of the compound, giving an acetyl analog of ADDA ($COCH_3$ instead of CH_3) (Fig. 6). The toxicities (50% lethal dose, i.p., mouse) of P14 and P15 did not differ significantly from that of MCYST-LR (50% lethal dose, i.p., mouse, approximately 60 μg/kg), while that of P16 was ca. 160 μg/kg.

DISCUSSION

Identification of *Nostoc* relative to *Anabaena* is based primarily on the shape of the cells and the filaments plus the presence of a gelatinous envelope around the *Nostoc* filaments that is not present in *Anabaena*. A species name was not given to strain 152 because it was not identified from a field sample but rather after growth had occurred on an agar plate. *Nostoc* is not a common cyanobacterium in Finnish waters, and only one species, *N. kihlmanii* Lemmermann (*N. planctonicum* Poretzky & Tschernov), has been identified (25). This points out the importance and usefulness of enrichment culture methods for the detection of previously unknown or rare toxin-producing (or other bioactive) cyanobacteria since this particular toxic *Nostoc* sp. would not have been detected in the original field sample.

The hepatotoxic peptides isolated from strain 152 differ structurally from known microcystins. The basic structure of the cyclic peptides found in strain 152 is that of MCYST-LR. MCYST-LR contains equimolar amounts of D-Ala, L-Leu, D-*erythro*-β-methylAsp, L-Arg, ADDA, D-Glu, and *N*-methyldehydroAla. *Nostoc* strain 152 was found to contain at least five toxins (and probably more) having a modified MCYST-LR structure. The modifications not reported prior to this time include a modified arginine, that is, homoarginine (Har), and *O*-acetyl-*O*-demethylADDA (ADMAdda). While the absolute structure of these MCYST-LR homologs will be published elsewhere (Namikoshi et al., in press), the work presented here shows the following. (i) Although the P6 toxin was not studied by NMR because of an inadequate sample, amino acid analysis and MS results indicate that Har and ADMAdda are absent. The extra methylene group may be due to the presence of *N*-methyldehydrobutyrine instead of *N*-methyldehydroalanine. (ii) P14 contains ADMAdda and, therefore, would be [ADMAdda^5]MCYST-LR (*m/z* = 1,022). (iii) P15 contains ADMAdda and Har and would be [ADMAdda^5]MCYST-LHar (*m/z* = 1,036). (iv) P16 contains ADMAdda and Asp instead of β-methylAsp and, therefore, would be [D-Asp^3, ADMAdda^5]MCYST-LR (*m/z* = 1,008). (v) P18 contains ADMAdda, Har, and Asp instead of β-methylAsp, becoming [D-Asp^3, ADMAdda^5]MCYST-LHar (*m/z* = 1,022). Demethylation of β-methylAsp has been found earlier for certain toxins of *Anabaena* (4, 14), *Oscillatoria* (3, 15) and *Microcystis* (12) spp.

The various microcystins are all hepatotoxic and give similar signs of poisoning. Lethal dose concentrations vary from 50 μg/kg of body weight for MCYST-LR and LA to the least toxic MCYST-RR and its demethylated analogs at 200 to 1,000 μg/kg of body weight (i.p., mouse). Toxicities of the microcystins reported in this paper did not differ significantly from that of MCYST-LR, except for P16, which is about one-half as toxic as MCYST-LR. This is due to the presence of aspartic acid instead of β-methylaspartic acid, since this difference has been reported earlier to decrease the toxicity of MCYST-LR (4).

Microcystins are now known to be produced by four planktonic cyanobacteria genera. Three are filamentous, *Anabaena*, *Oscillatoria*, and now *Nostoc*, while one is coccoid, *Microcystis*. *Microcystis* produces the greatest variety of toxins differing in the L-amino acids variants, i.e., MCYST-LA, YA, LR, YM, RR, and YR (4, 5). The toxic filamentous genera produce mainly MCYST-LR and RR, but the toxins show a great variation in the amount of methylation on the various amino acids present. In the research reported here, these differences in methylation led to the discovery of five new hepatotoxic microcystin analogs based on modifications of the previously known MCYST-LR. It now appears clear that these cyclic peptide microcystins frequently occur among the most common planktonic cyanobacteria. Their role in animal and human water-based disease, including their newly found role as a tumor promoter (Fujiki et al., J. Biol. Chem., in press), deserves closer examination.

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