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Toxicity and Partial Structure of a Hepatotoxic Peptide Produced by the Cyanobacterium *Nodularia spumigena* Mertens emend. L575 from New Zealand

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A clonal isolate, termed L575, of the filamentous brackish-water cyanobacterium *Nodularia spumigena* Mertens emend. was found to produce a potent hepatotoxic peptide (50% lethal intraperitoneal dose for the mouse, 60 μg/kg) with chemical and toxicological properties similar to those of the hepatotoxic heptapeptides produced by other freshwater planktonic cyanobacteria. The isolate was made from a water sample collected in Lake Ellesmere, New Zealand, in 1980. The toxin, isolated and purified by high-performance liquid chromatography (HPLC) and analyzed by HPLC amino acid analysis, contained glutamic acid, β-methylaspartic acid, and arginine units in equivalent amounts. The fast-atom-bombardment mass spectrum of the toxin indicated the molecular weight to be 824. Batch cultures of strain L575 showed that the toxin content varied between 1.96 and 2.99 mg/g of cells and that a positive correlation between toxin content and chlorophyll *a*, but not biomass, was present.

In 1878, *Nodularia spumigera* [sic] (*spumigena*) became the first cyanobacterium (blue-green alga) to be reported in the scientific literature as toxic to livestock (7). The first isolation and characterization of a toxin from a cyanobacterium was not from filamentous *Nodularia* but instead from the related genus *Anabaena*. Strains of different *Anabaena* spp. are now known to produce potent neurotoxic alkaloids as well as hepatotoxic cyclic heptapeptides (2, 3). Both groups of toxins cause intermittent but repeated cases of wild and domestic animal poisonings from municipal and recreational water supplies (9). Species of the colonial genus *Microcystis* are the most common toxic cyanobacteria found worldwide and are the worst offenders in cases of water-based diseases due to cyanobacterial toxicosis (8, 9, 18). The peptide toxins of *Microcystis*, *Anabaena*, and *Oscillatoria* spp. are a related group of cyclic heptapeptides with a similar effect on the liver (3). More recent reports of toxic *Nodularia* spp. have come from Australia (15), the German Democratic Republic (10, 12), Denmark (14), Sweden (4), and Finland (5, 16). Recently some information on the structure of *Nodularia* toxin has been presented by Rinehart (R. Soc. Chem., Annu. Chem. Congress, Swansea, U.K., 13 to 16 April 1987, paper A-12) for waterbloom material collected from Lake Forsythe, New Zealand, in 1984, by Eriksson et al. (5) from waterbloom material collected in the Baltic Sea in 1986, and by Runnegar et al. (17) for a field isolate from the Peel Inlet, Perth, Australia. All of these reports indicate that *N. spumigena* produces a peptide hepatotoxin with toxicological properties similar to those of the cyclic heptapeptides. The work of Rinehart, however, indicates that the toxin is a cyclic pentapeptide with an amino acid composition of β-methylaspartic acid, glutamic acid, arginine, dehydrobutyrine, and ADDA (3-amino-9-methoxy-10-phenyl-2,6,8-trimethyl-deca-4,6-dienoic acid) (M_r 824). ADDA is the β-amino acid common to all the cyclic peptide cyanobacterial toxins isolated to date (3).

Published reports of toxic cyanobacteria are not as common from New Zealand as they are from other countries, but some are available, most notably that of Flint (6). These cases involve mostly *Microcystis* and *Anabaena*. Nevertheless, Lake Forsythe waterblooms, now known to contain toxic *N. spumigena*, did cause animal deaths in 1970 (1). The signs of poisoning were indicative of a hepatotoxin, but no identification of the alga was made.

Our work presented here gives results for the toxicity and growth of a toxic strain of *N. spumigena* from Lake Ellesmere, New Zealand, plus the isolation and partial chemical characterization of its toxin.

MATERIALS AND METHODS

Isolation and culture of L575. *N. spumigena* L575 (Fig. 1) was isolated from a waterbloom sample collected by I. W. Lineham at Lake Ellesmere (latitude S43°48′, longitude E172°25′), New Zealand, on 24 March 1980. The isolate was maintained on Tris-buffered Bold basal medium plus 1% NaCl. Toxic *N. spumigena* has only been reported from brackish-water sources, i.e., Lake Ellesmere (Fig. 2), Lake Forsythe, and the Baltic Sea, and therefore requires NaCl supplement to the medium. For toxicity growth experiments at the University of Hawaii, L575 (nonaxenic) was grown in nitrate-supplemented A_3M_7 (A_3M_7 + N) (Table 1) plus 1% NaCl at 25 to 27°C under cool-white fluorescent bulbs, providing a maximum of 500 microeinsteins/m^2 per s of photosynthetically active irradiation (measured at the culture surface with a model LI-185A LI-Cor quantum meter). At the initial stages of culture, 1 to 10 days, 200 microeinsteins/m^2 per s was used. To measure growth and toxicity in batch culture, four (three test plus one control) 20-liter Pyrex bottles, fitted with sterile aeration, containing 15 liters of sterile A_3M_7 plus 1% NaCl were inoculated with a stationary-phase culture of L575 (10% [vol/vol] inoculum) to give an initial concentration of 50 mg of biomass per liter. At 2- to 4-day intervals, 1 liter of culture was aseptically removed from the three test bottles (control bottle volume was kept

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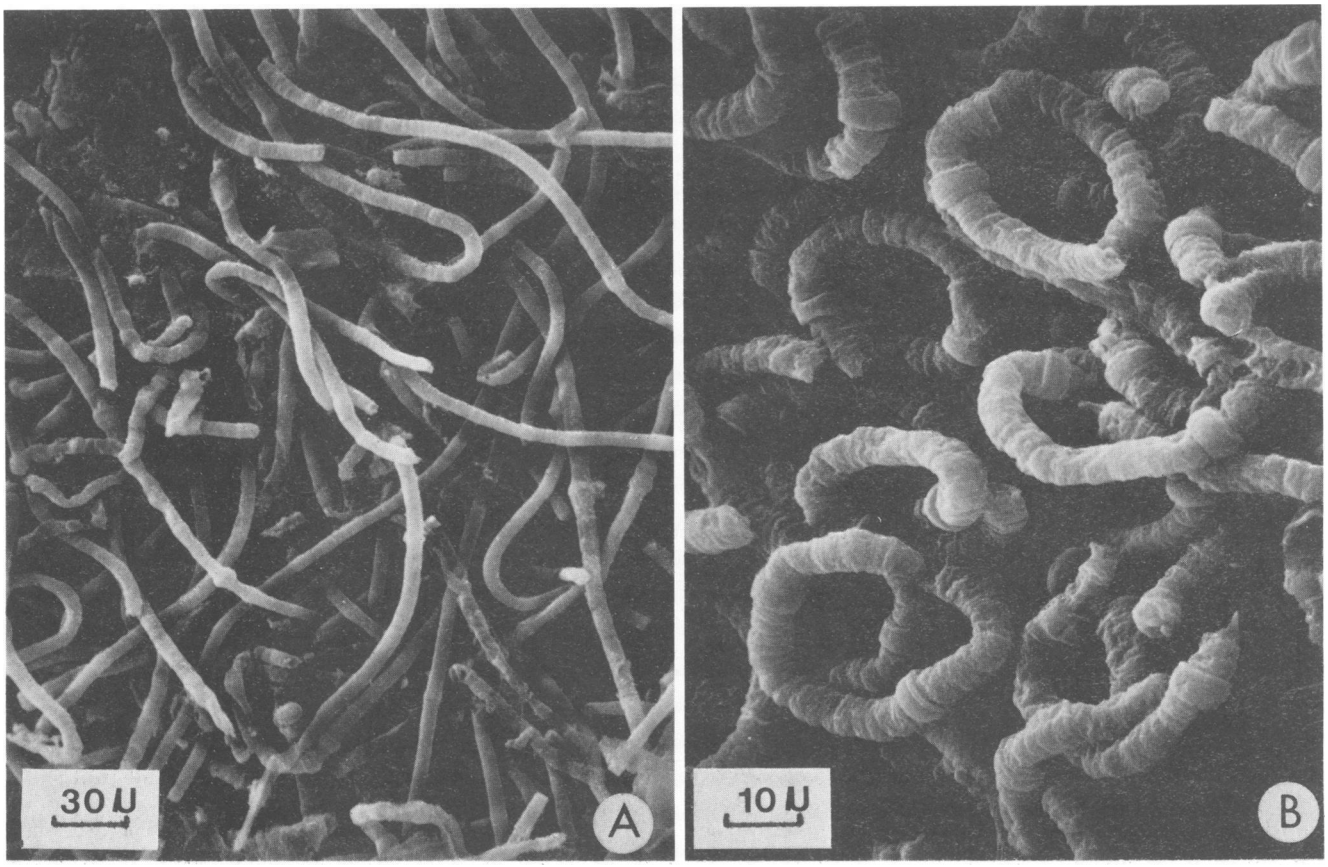

FIG. 1. Scanning electron micrographs of (A) hepatotoxic *N. spumigena* L575 isolated from Lake Ellesmere, New Zealand, and (B) hepatotoxic *N. spumigena* waterbloom sample collected from Lake Forsythe, New Zealand, in 1984. Note the longer, less coiled filaments of the laboratory-cultured material compared with the field sample. The large band-shaped cells in the filaments are heterocysts.

constant except for 100-ml samples to measure OD_{420} and chlorophyll a) for determination of biomass dry weight, chlorophyll *a*, OD_{420} (turbidity measurement), and toxin content per gram (dry weight) of cells. Chlorophyll *a* was determined by the method of Odum (11).

Toxin isolation. Toxin was isolated from lyophilized cells by a modification of the procedure used by Harada and Rinehart (personal communication) for their waterbloom-collected material of *N. spumigena* and by Krishnamurthy et al. (13) for *Microcystis* and *Anabaena* toxins. After extraction with methanol-water (1:1) and centrifugation to remove cell debris, the aqueous extract was partially purified on C-18 Bond Elut Cartridges (Analytichem Inc., Harbor City, Calif.). The toxin was eluted from Bond Elut by using 30% methanol. The partially purified concentrated aqueous extracts were separated by high-pressure liquid chromatography (HPLC) with either Alltech semipreparative C-18 (10 by 250 mm) or analytical (4.6 by 250 mm) columns. The toxin was eluted with 26% acetonitrile in 0.01 M ammonium acetate (flow rate, 3 and 1 ml/min for preparative and analytical columns, respectively). Detection was at 240 nm. The toxin was desalted by the same HPLC column procedure with 25% acetonitrile in water, lyophilized, and stored at −80°C.

Toxicity and toxin content. The intraperitoneal (i.p.) mouse (18 to 23 g; male Swiss Webster [University of Hawaii] and ICR Swiss [Wright State University]) assay was used to assess toxicity of cells and purified toxins. The 50% lethal dose (LD_{50}) was estimated with five dose levels and six animals per level. After death, mice were autopsied and liver weights were determined as a percentage of body weight. This allowed comparison of the liver weight with known cyclic peptide microcystins, all of which cause a characteristic increase to 8 to 12% compared with normal weights of 4 to 6%. Representative liver pieces from selected mice were processed and sectioned for routine histopathological examination with a hematoxylin and eosin (H&E) stain. The toxin content for the growth experiment tests was measured by determining the area of the toxin peak (determined by use of a Hewlett-Packard model 3393A integrator) generated by analytical HPLC. Replicate injections ($n = 4$) of purified toxin at six concentration levels (0.25 to 5 µg/10-µl injection) were used. Areas at each concentration level were averaged, and the total area units were divided by the total amount of toxin injected. The constant obtained (micrograms per unit area) was used to determine concentration of toxin for each of the three replicate culture bottles (and after 32 days for the control bottle) used in the growth and toxicity experiments.

Analysis of the toxin. Purified toxin (95% pure) from HPLC was 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 phenylisothiocyanate, and the phenylthiocarbamyl amino acids were analyzed with a Waters Pico Tag HPLC system. The derivatives were loaded onto a C-18 (15 cm by 4.6 mm) column and eluted over 8 min with a 0 to 60% gradient of acetonitrile in 0.138 M aqueous sodium acetate. The column flow rate was maintained at 1.0 ml/min. The eluted compounds were detected by UV absorption at 254

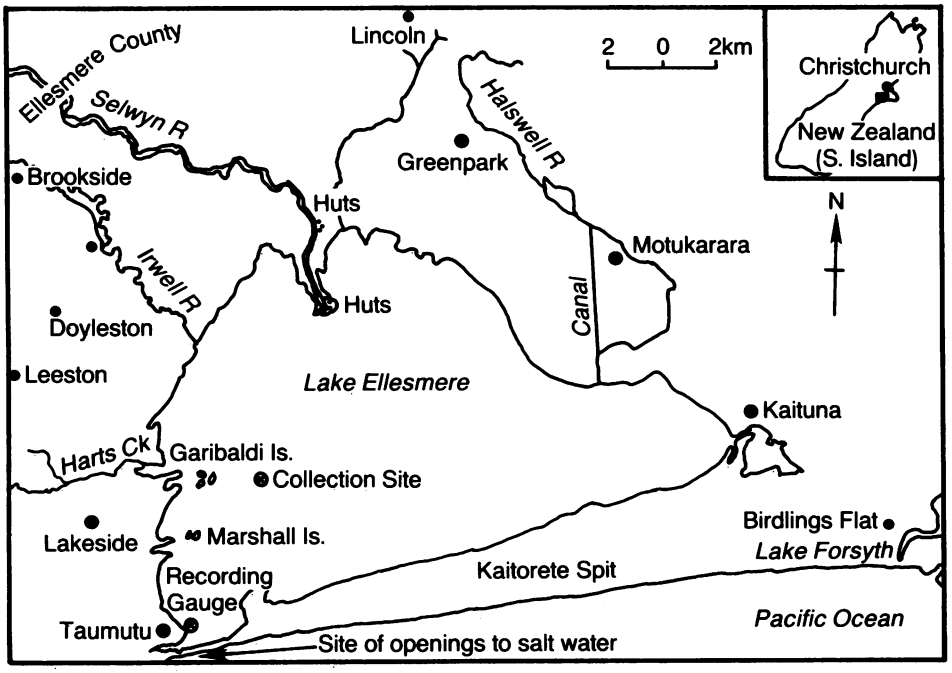

FIG. 2. Map showing location of Ellesmere Lake, South Island, New Zealand. This is the lake from which *N. spumigena* L575 was isolated. Note site of opening to saltwater during seasonal breakthroughs, which account for part of the brackish-water nature of the lake.

nm. The intact peptide was analyzed by fast-atom-bombardment mass spectrometry (ZAB 10 kV mass spectrometer) with the Magic Bullet matrix (1:3, dithiothreitol-dithioerythritol). The source temperature was 30°C, and the target was bombarded with xenon atoms at 9 kV.

RESULTS

Batch cultures of *N. spumigena* harvested at the stationary growth phase (25 to 35 days) yielded 0.5 to 1 g (dry weight) of biomass per liter of culture. Mouse toxicity of different batch cultures varied between 30 and 50 mg/kg (i.p. mouse). Signs of poisoning were similar to that seen with hepatotoxins (i.e., microcystin-LR) of other cyanobacteria (2, 5). Livers were swollen (8 to 12% of body weight vs. 4 to 5% for controls) and blood engorged. Histological examination of the liver (H&E stain) revealed large areas of hemorrhage that were more panlobular than that seen with the hepatotoxic microcystins of other cyanobacteria. In addition, survival times were 2 to 3 h at the minimal lethal dose range, compared with 1 to 1.5 h for the microcystins (19).

Extraction of the toxin showed a single toxic peak both during gel filtration and on HPLC. The toxin (termed nodularin [NODLN]) was found to be more polar than microcys-

TABLE 1. Composition of media used to culture *N. spumigena* L575[a]

Ingredient	Amount (g/liter) in medium[a]	
	A_3M_7 + N[b]	BG-11
$NaNO_3$	0.800	1.500
NH_4Cl	0.010	
K_2HPO_4 ($3H_2O$)	0.065	0.040
$MgSO_4$ ($7H_2O$)	0.050	0.075
$CaCl_2$ ($2H_2O$)	0.013	0.036
Na_2CO_3		0.020
MOPS[c]	0.625	
Tricine[d]		0.026
Disodium EDTA		0.001
Minor elements[e]	1	1

[a] The final pH was 7.2 for both media. Both were supplemented with 1% NaCl. BG-11 is now used routinely to culture L575.
[b] The normal level of nitrogen in A_3M_7 is 0.2 g/liter.
[c] MOPS, 3-[*N*-morpholino]propanesulfonic acid.
[d] Tricine, *N*-Tris[hydroxymethyl]methylglycine.
[e] Minor elements included the following (in grams per liter). A_3M_7. $FeCl_3 \cdot 6H_2O$, 0.54; disodium EDTA, 3.0; H_3BO_3, 0.62; $MnCl_2 \cdot 4H_2O$, 1.40; $ZnCl_2$, 0.10; $CoCl_2 \cdot 6H_2O$, 0.005; $CuCl_2 \cdot 2H_2O$, 0.000034. BG-11: H_3BO_3, 2.86; $MnCl_2 \cdot 4H_2O$, 1.81; $ZnSO_4 \cdot 7H_2O$, 0.222; $Na_2MoO_4 \cdot 2H_2O$, 0.39; $CuSO_4 \cdot 5H_2O$, 0.079; $CO(NO_3)_2 \cdot 6H_2O$, 0.049. The mixtures were added at 1 ml/liter.

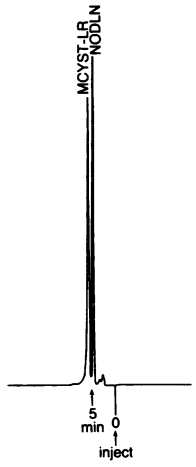

FIG. 3. Comparative HPLC profiles and retention times for purified toxins from *N. spumigena* L575 (NODLN; 8 μg) and *M. aeruginosa* PCC7820 (MCYST-LR; 5 μg) (13). Column conditions were: column, Alltech C-18, 4.6 mm by 25 cm; solvent, 0.01 M ammonium acetate in 26% acetonitrile–water; flow rate, 1 ml/min; wavelength, 240 nm; absorbance units full scale, 1.0.

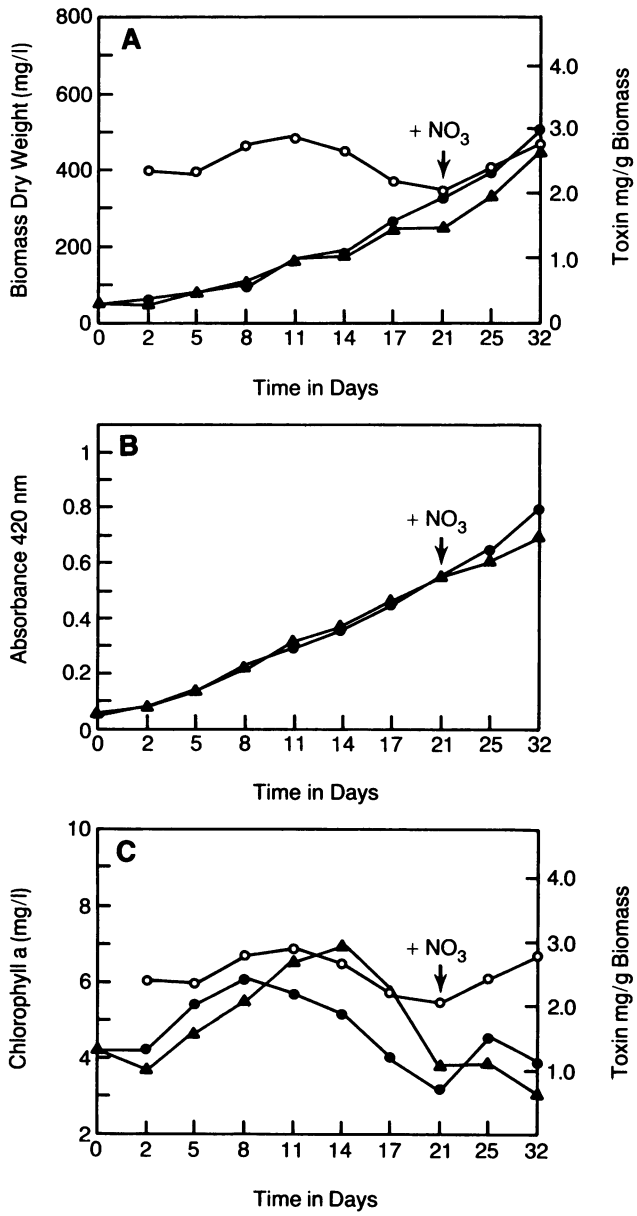

FIG. 4. Growth of *N. spumigena* L575 batch cultures in A_3M_7 + N medium. (A) Biomass (●, mean of three test bottles; ▲, control bottle) and toxin content (○) versus time. Additional $NaNO_3$ to give 1.5 g/liter was added at day 21. (B) Absorbance versus time; mean of three test cultures (●) and control culture (▲). (C) Chlorophyll *a* (●, mean of three test bottles; ▲, control bottle) and toxin content (○) versus time. Additional $NaNO_3$ to give 1.5 g/liter was added at day 21.

tin-LR (MCYST-LR) (cyanoginosin-LR) from *Microcystis aeruginosa* PCC7820. NODLN eluted from C-18 Bond Elut Cartridges with 30% methanol compared with 80 to 90% for MCYST-LR. On C-18 HPLC, NODLN eluted slightly ahead of MCYST-LR (Fig. 3), again indicating it is more polar.

Batch-cultured L575 grown over a 32-day period showed a time-dependent growth curve (Fig. 4). Dry weight (Fig. 4A) of the biomass rose to about 600 mg/liter, compared with 400 in the control bottle. Absorbance turbidity increased in a similar manner as did dry weight. Chlorophyll *a* showed a peak, about midway through the culture cycle, in the three bottles sampled for toxin content and in the control bottle. Both groups showed an increase in chlorophyll *a* content at 21 days when extra nitrogen (as $NaNO_3$) was added. Toxin content (Fig. 4A and C) showed an increase following the increase in chlorophyll *a*, both before and after additional nitrogen.

A constant of $k = 1.068 \times 10^6$ μg/area unit was found for purified toxin on analytical C-18 HPLC. At day zero a starting inoculum of 241 mg/liter was added to each of the four culture bottles at 10%. This inoculum contained 1.96 μg of toxin per g of cells. At day 32, when the growth test was terminated due to depletion of the culture volume in the sample bottles, the average toxin content for the three test bottles was 2.57 μg/g of cells and in the control bottle it was 2.33 μg/g of cells.

Amino acid analysis of NODLN (Fig. 5) showed the presence of three of the five amino acids found in the microcystins, glutamic acid, β-methylaspartic acid, and arginine. Methylamine, which is seen in the hydrolysate of other cyanobacteria peptide toxins when *N*-methyldehydroalanine is present, was also found. The Magic Bullet matrix revealed a single main peak at an *m/z* + H of 825 daltons (Fig. 6).

DISCUSSION

N. spumigena L575, isolated from brackish water of Lake Ellesmere, New Zealand, was found to produce a peptide toxin with properties similar to that reported for the heptapeptide hepatotoxins isolated from *Anabaena*, *Microcystis*, and *Oscillatoria* spp. Mouse toxicity of the lyophilized cells (LD_{50}, 30 to 50 mg/kg) was similar to that of other cyclic peptide hepatotoxin producing cyanobacteria. The LD_{50} i.p. for the mouse of the purified toxin (60 μg/kg) was also similar to that reported for the cyclic heptapeptide toxins of other cyanobacteria (3). The longer survival time, for toxic cells or purified toxin, in test animals (2 to 3 h vs. 1 to 1.5 h) at the minimum lethal dose range indicated that the toxin was more slowly absorbed or metabolized than the microcystins. HPLC purification of the toxic component indicated that a single toxin was accounting for the toxic signs observed in test animals. This toxin had an absorbance maximum at 238 to 240 nm, which is the same as that for the microcystins. The HPLC retention time of NODLN compared to that of MCYST-LR indicated that NODLN was more polar (Fig. 3).

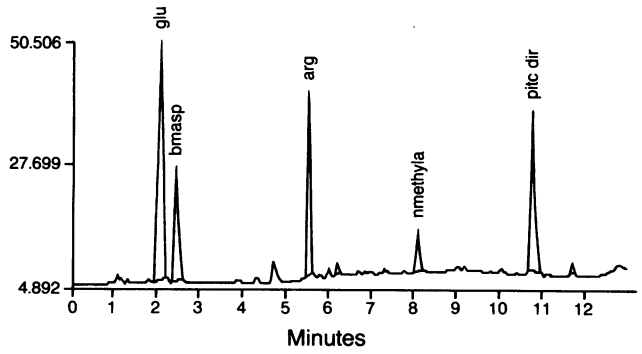

FIG. 5. HPLC and amino acid profile for *N. spumigena* nodularin (NODLN) (10 μg). Analysis was by Waters Pico Tag precolumn derivatization with phenylisothiocyanate, to yield phenylthiocarbomyl amino acids. Pitc dir, Unidentified phenylisothiocyanate derivative produced during sample derivatization; nmethyla, *n*-methylamine, a breakdown product of an unidentified amino acid; bmasp, β-methylaspartic acid. Ordinate units are in millivolts.

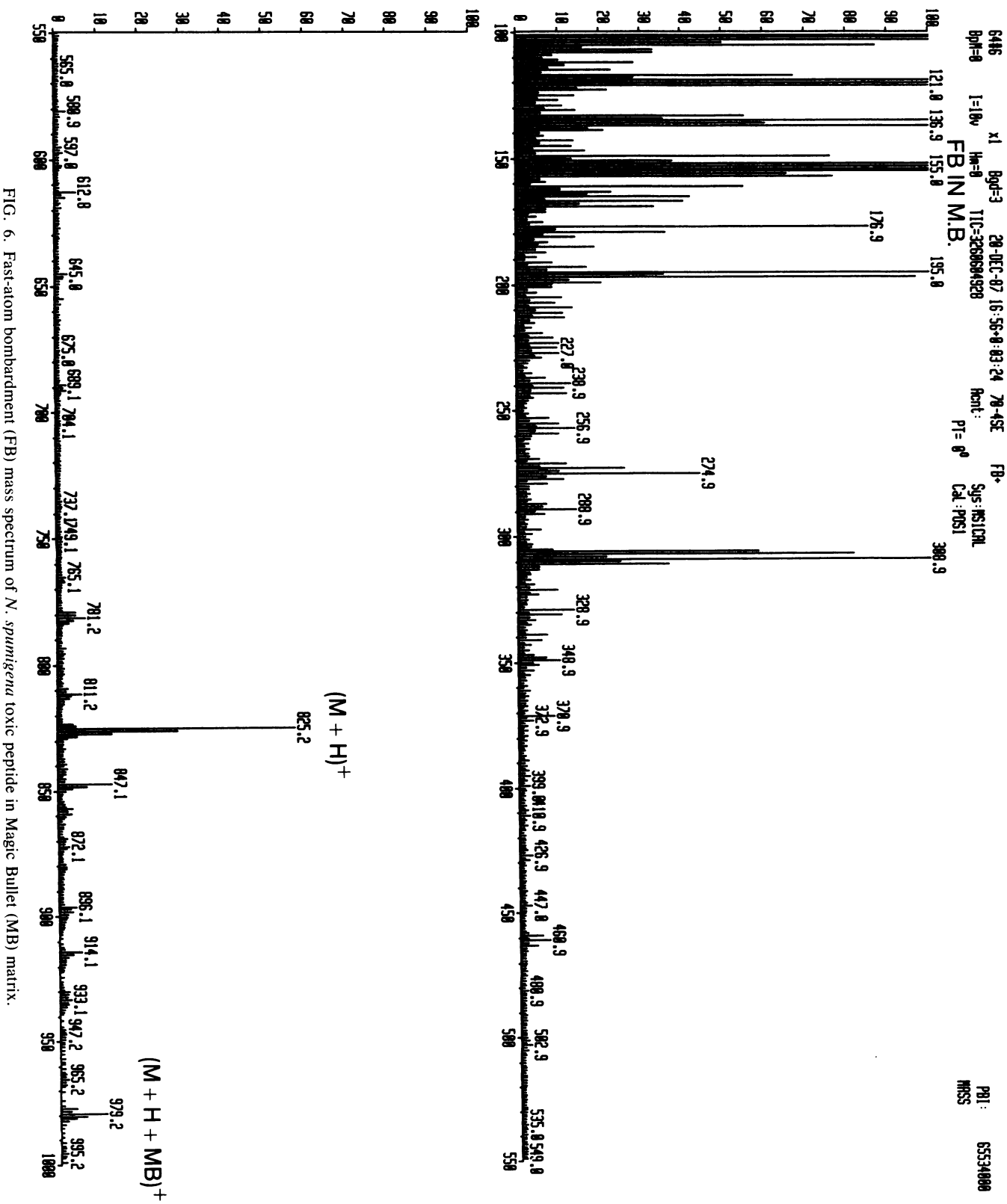

FIG. 6. Fast-atom bombardment (FB) mass spectrum of *N. spumigena* toxic peptide in Magic Bullet (MB) matrix.

Amino acid analysis (HPLC precolumn derivatization method) showed the presence of equivalent molar amounts of glutamic acid, β-methylaspartic acid, and arginine in the acid hydrolysate of the purified toxin. This compares with five amino acids that would have been detected by this method for the heptapeptide microcystins (13). The remaining two modified amino acids in the microcystins have so far proven to be *N*-methyldehydroalanine and ADDA. These two modified amino acids would not have been detected by our amino acid analysis method. Nevertheless, the possible presence of *N*-methyldehydroalanine could be inferred from the methylamine peak at 8.1 min (Fig. 5), and the UV absorbance maximum at 238 to 240 mm for the toxin suggested that ADDA is also present (13). The molecular weight of 824 found for the toxin in strain L575, however, is the same as that of the field material reported by Rinehart, suggesting that an aminobutyrine unit and not a methyldehydroalanine unit is part of the toxic peptide. The molecular weight of 824 and the amino acid analysis data clearly show that the toxin is not a heptapeptide. The results of our chemical analysis show that NODLN is a peptide, most likely cyclic, with an amino acid composition smaller than but similar to that of the known microcystins. It also suggests that the toxin is very similar and perhaps the same as that of the toxin partially characterized by Eriksson et al. (5) from *N. spumigena* bloom material collected in the Baltic Sea and that of Runnegar et al. (17) for a strain isolated from Peel Inlet, Perth, Australia. Based on this different composition, we are using the term nodularin (NODLN), as proposed by Rinehart (personal communication), to name the toxins.

Growth of batch-cultured *N. spumigena* L575 in A_3M_7 medium supplemented with nitrogen showed a typical growth curve over 32 days. Unfortunately, the size of the culture vessel used (20 liters) did not allow continuation of the growth experiment into the stationary phase of growth. The growth conditions used in these experiments did not test for effects of varying temperature, light, pH, or aeration on growth and toxin production. They were instead designed to allow quantitation of the toxin during conditions used under a typical culture cycle for other cyanobacteria, being grown for analysis of secondary chemicals, at the University of Hawaii and Wright State University. The use of HPLC to quantitate the toxin in the cells was more accurate but required more time than using the mouse toxicity assessment method.

Production of toxin did not parallel biomass of the growing culture. While biomass of the three test bottles and that of the control bottle continued to increase over the 32-day period, the toxin content per gram of cells began to increase at day 5 and then decline at day 11 (Fig. 4A). This pattern reflected that seen with chlorophyll *a* content, which began to increase at day 2 and then decrease beginning at day 8 for the test bottles and day 14 for the control bottle. The continued rise in chlorophyll *a* for the control bottle could reflect the greater light penetration of the test cultures as the volumes decreased and the resulting photoinhibition at the higher light intensity. At day 21, when additional nitrogen ($NaNO_3$) was added, both chlorophyll *a* content and toxin content increased while rate of biomass increase was not affected. These results, while not conclusive, suggest that toxin production could be closely tied to primary energy processes of the cell and suggest one possible way for improving toxin production in the culture. An interesting point in the production of toxin was the amount of toxin present in cells of strain L575. Over the 32-day culture period, the amount of toxin varied from 1.96 mg/g of cells (amount in the inoculum added at day 0) to 2.99 mg/g of cells (at day 11). This amount is greater than the 1 to 2 mg/g of cells for microcystin, typically present in laboratory cultures of *M. aeruginosa*, and the 0.5 mg/g of cells isolated from waterbloom samples of *N. spumigena* collected in the Baltic Sea (5).

The results in this study with a laboratory culture (nonaxenic) of *N. spumigena* show that it produces a potent hepatotoxic peptide of similar composition but lower molecular weight than the heptapeptide microcystins produced by strains of *Microcystis*, *Anabaena*, and *Oscillatoria* (3).

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