Articles

Chemical Characterization and Toxicity of Dihydro Derivatives of Nodularin and Microcystin-LR, Potent Cyanobacterial Cyclic Peptide Hepatotoxins

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Dihydro derivatives of nodularin (1) and microcystin-LR (4), potent cyclic peptide hepatotoxins isolated from *Nodularia spumigena* and *Microcystis aeruginosa*, respectively, were prepared by sodium borohydride reduction of the dehydroamino acid residues. The two stereoisomers of both dihydronodularin (2 and 3) and dihydromicrocystin-LR (5 and 6), isolated by reversed-phase HPLC, showed similar toxicity to each other [ip in mice, LD_{50} = 150 (2), 150 (3), 85 (5), and 100 (6) μg/kg]. The stereochemistries of the reduced amino acids obtained by acid hydrolysis of dihydronodularin and dihydromicrocystin-LR [respectively, α-(methylamino)butyric acid and *N*-methylalanine] were determined by GC on a permethylated β-cyclodextrin capillary column as their trifluoroacetyl methyl ester derivatives. Authentic L- and DL-*N*-methylamino acids were prepared to compare directly with the natural amino acids. Deuterated derivatives were also prepared using sodium borodeuteride (98 atom % D), and the location (β) and percentage (78–84%) of the deuterium incorporation were determined.

Introduction

Nodularin (Ndn;[1] 1, Scheme I) is a cyclic pentapeptide hepatotoxin produced by a cyanobacterium (blue-green alga), *Nodularia spumigena* (*1*, *2*), in brackish water. The microcystins are cyclic heptapeptides possessing a similar hepatotoxicity, produced by several species of cyanobacteria (*3*–*7*). The structures of the microcystins differ primarily in the two L-amino acids and secondarily in the presence or absence of the methyl groups on D-*erythro*-β-methylaspartic acid (MeAsp) and/or *N*-methyldehydroalanine (MeΔAla) (*7*). Microcystin-LR (LR; 4, Scheme I), containing Leu and Arg as variable L-amino acids, is one of the most potent and commonly found compounds among the microcystins. Ndn (1) has an N-methylated dehydrobutyrine (*8*) residue [α-(methylamino)dehydrobutyric acid (MeΔAbu)] as a structural component similar to the MeΔAla residue in LR (4). The potencies of Ndn (1) and LR (4) are the same, having LD_{50} 60 μg/kg (ip, mice). The recent reports on their inhibitory activity against protein phosphatases 1 and 2A render these compounds quite important also as biochemical tools (*9*–*14*).

These toxins have a novel C_{20} amino acid, (2*S*,3*S*,8*S*,9*S*)-3-amino-9-methoxy-10-phenyl-2,6,8-trimethyldeca-4,6-dienoic acid (Adda), as the common and unusual structural feature (*1*, *15*), which seems to be very important for their activity since an ozonolysis product or a compound hydrogenated at the diene position shows no toxicity and stereoisomers at the Δ^6 double bond have been reported to be inactive (*16*, *17*).

Stereoisomeric mixtures of dihydro derivatives of Ndn and LR obtained by reduction of the dehydroamino acid residues with sodium borohydride (*18*, *19*) have been reported to retain toxicity, and more recently, Meriluoto et al. reported the preparation of tritium-labeled stereoisomers of dihydromicrocystin-LR (H_2-LR) which were separated by reversed-phase (ODS) HPLC (*20*). However, these reports have not characterized the physicochemical properties of the stereoisomers.

We are interested in the structure–activity relationships of these toxins (1 and 4), from both natural and synthetic sources, and have reported the total synthesis of Adda, which showed no toxicity (*21*). We have also reported the structures of three new microcystins possessing an interesting structural change in the Adda unit, from a cultured *Nostoc* sp. (*22*), and nine new microcystins from a water-bloom of *Microcystis* spp. collected from Homer Lake (Illinois) (*23*). The three *Nostoc* toxins have an acetoxyl group at the C-9 position of Adda instead of the methoxyl group in previously known microcystins, and this was the first variant of Adda which retains the toxicity (*22*). A

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[1] Abbreviations: Abu, α-aminobutyric acid; Adda, (2*S*,3*S*,8*S*,9*S*)-3-amino-9-methoxy-10-phenyl-2,6,8-trimethyldeca-4,6-dienoic acid; DH-LR, deuterated dihydromicrocystin-LR; DH-Ndn, deuterated dihydronodularin; FABMS, fast atom bombardment mass spectrometry; FABMS/MS, tandem FABMS; H_2-LR, dihydromicrocystin-LR; H_2-Ndn, dihydronodularin; HRFABMS, high-resolution FABMS; LR, microcystin-LR; MeAbu, α-(methylamino)butyric acid; MeAla, *N*-methylalanine; MeΔAbu, α-(methylamino)dehydrobutyric acid; MeΔAla, *N*-methyldehydroalanine; MeAsp, *erythro*-β-methylaspartic acid; Ndn, nodularin; ODS, octadecylsilanated; Z, benzyloxycarbonyl.

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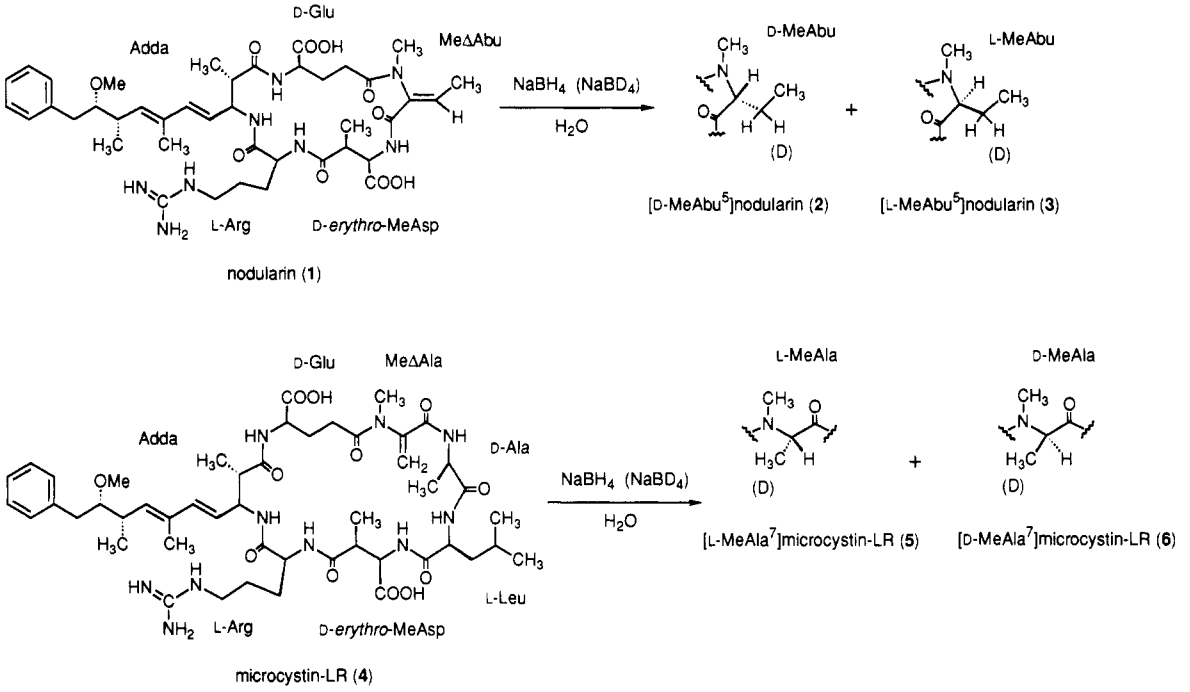
Scheme I



hydroxyl variant at the same position of Adda has been isolated from the mixed *Microcystis* spp., and this also retains the toxicity (*23*).

We have made several derivatives from natural toxins to investigate the toxicity in connection with the structure–activity relationships and wish to report here our results on the chemical characterization and toxicity of the two isolated stereoisomers of dihydronodularin (H_2-Ndn) and of H_2-LR.

Experimental Procedures

General. Mass spectra were run on either a VG ZAB-SE or a VG 70-SE4F mass spectrometer operating in the fast atom bombardment (FAB) mode, using xenon atoms (8 keV energy) and a matrix of dithiothreitol/dithioerythritol ("magic bullet") (*24*). Tandem mass spectra (MS/MS, linked scan at constant *B/E*) in the FAB mode were obtained on a four-sector tandem mass spectrometer (70-SE4F) using helium as a collision gas: resolution of the first and second mass spectrometers, both 1000; accelerating potential, 8 keV; collision energy, 4 keV; attenuation, 90%. High-resolution (HR) FAB mass spectra were acquired at a resolving power of 10 000 (10% valley). About 10 μg of each sample was applied as a methanol solution. ^{1}H NMR spectra were recorded on a GE GN-500 FT NMR spectrometer using CD_3OD as solvent (0.5 mL for each sample) and an internal standard (δ 3.30 ppm). Specific rotations were obtained on a Jasco DIP-370 digital polarimeter using a 3.5-mm × 10-cm cell.

TLC was performed on precoated silica gel plates (Kieselgel 60 F_{254}, 0.25-mm thickness, EM Science, Gibbstown, NJ). Adsorbed spots were detected by UV light at 254 nm and by spraying phosphomolybdic acid (10% in ethanol) followed by heating. The following solvent mixtures were used, and the R_f vales of the toxins are listed in Table I: (A) chloroform/methanol/water (26:14:3), (B) ethyl acetate/2-propanol/water (8:4:3), (C) ethyl acetate/2-propanol/water (4:3:2), and (D) 1-butanol/acetic acid/water (4:1:1).

Algal Material. Cell Culturing. Laboratory cell cultures of filamentous *N. spumigena* L-575 were grown according to the procedure described by Carmichael et al. (*25*). Cultures of unicellular *Microcystis aeruginosa* PCC 7820 were produced as described by Carmichael (*26*) and Codd and Carmichael (*27*). Log to stationary growth phase cultures were harvested by tangential flow centrifugation (Millipore-Pellicon), lyophilized, and stored at −80 °C until used for toxin extraction.

Table I. R_f Values of Compounds 1–6 on Silica Gel TLC (0.25-mm Thickness)

compd	(A)[a]	(B)	(C)	(D)	compd	(A)[a]	(B)	(C)	(D)
1	0.28	0.14	0.34	0.44	4	0.31	0.18	0.42	0.47
2	0.30	0.15	0.38	0.45	5	0.27	0.15	0.37	0.43
3	0.26	0.14	0.35	0.48	6	0.32	0.18	0.44	0.48

[a] Mobile phases: (A) chloroform/methanol/water (26:14:3), (B) ethyl acetate/2-propanol/water (8:4:3), (C) ethyl acetate/2-propanol/water (4:3:2), (D) 1-butanol/acetic acid/water (4:1:1).

Isolation of Toxin. (A) Nodularin. Lyophilized cells of *N. spumigena* L-575 (11 g) were extracted three times with methanol (each 250 mL), and the extract was filtered and evaporated to dryness. The residue was suspended in water (60 mL) and centrifuged (60 min at 9000*g*). The supernatant was passed through an ODS column (12 g, Chromatorex, Fuji-Davison Chemical Ltd., Tokyo, Japan), and the column was washed with water (120 mL) and 5% methanol in water (100 mL) successively and eluted with 80% methanol (100 mL). The eluate was evaporated to dryness, dissolved in methanol, and chromatographed on a Sephadex LH-20 column (13 × 600 mm, Pharmacia, Piscataway, NJ). The toxin fraction obtained (25 mg) was then chromatographed on an ODS HPLC column (Nucleosil 7 C_{18}, 10 × 250 mm, Cobert Associates, St. Louis, MO) with acetonitrile/0.1% ammonium acetate (27:73, 2.0 mL/min) to afford pure Ndn (5.5 mg), $[\alpha]^{26}_D$ −86.1° (*c* 0.18, methanol).

(B) Microcystin-LR. Lyophilized cells of *M. aeruginosa* PCC 7820 were extracted three times with a water/methanol/1-butanol mixture (75:20:5) (25 mL/g of cells) at 25 °C. The extracts were centrifuged (30 min at 13000*g*) and the supernatants combined. The combined supernatants were air-dried at 35 °C to remove methanol and 1-butanol and passed through an ODS column. The column was washed with water followed by 20% methanol in water, and the toxin was eluted with 80% methanol. The methanol was evaporated and the residue chromatographed on an ODS HPLC column (Prep Pak C_{18}, 47 × 300 mm, Waters, Milford, MO) with acetonitrile/0.02 M ammonium acetate (28:72, 30 mL/min). After removal of acetonitrile, the toxin fraction was again chromatographed, employing the same conditions. The toxic fraction was evaporated to dryness, taken up in methanol, and chromatographed on a Toyopearl HW-40F column (25 × 700 mm) to afford the pure toxin (*22*), pure by HPLC and TLC.

Table II. HPLC Retention Times (min) of Compounds 1–6 on Nucleosil 5 C_{18} (4.6 × 250 mm)

compd	(A)[a]	(B)	(C)	(D)	compd	(A)[a]	(B)	(C)	(D)
1	9.0	8.8	8.4	8.9	4	9.7	12.3	12.9	11.4
2	10.4	10.4	10.2	10.0	5	10.2	11.8	14.0	12.0
3	12.1	12.0	10.1	11.1	6	12.7	14.8	13.9	12.4

[a] Mobile phases: (A) methanol/0.7% sodium sulfate (55:45), (B) acetonitrile/0.1% ammonium acetate (25:75), (C) methanol/0.05% trifluoroacetic acid (6:4), and (D) methanol/0.05 M phosphate buffer (pH 3.0) (6:4). Flow rate: 1.0 mL/min.

Dihydronodularin. Sodium borohydride (30 mg) was added in small portions to a solution of Ndn (3.7 mg) in water (1 mL), and the mixture was stirred at room temperature for 27 h. The reaction was quenched by the dropwise addition of 10% acetic acid in water, and the solution was passed through an ODS cartridge (500-mg gel, Bond Elut, Cobert Associates, St. Louis, MO). The cartridge was rinsed with water (30 mL) and eluted with methanol (10 mL). The methanol was evaporated off, and the residue (3.6 mg) was subjected to HPLC separation.

Deuterated Dihydronodularin. Ndn (5.2 mg) was reduced with sodium borodeuteride (45 mg, 98 atom % D, Aldrich Chemical Co., Milwaukee, WI) in water (1.2 mL) for 36 h as above to give deuterated dihydronodularin (DH-Ndn, 5.2 mg).

Dihydromicrocystin-LR. Sodium borohydride (3 mg) was added in small portions to a solution of LR (5.2 mg) in water (0.5 mL), and the mixture was stirred at room temperature. After 3 h, sodium borohydride (2 mg) was added, and the whole solution was further stirred at room temperature for 17 h. The workup as above gave the products (5.1 mg), which were separated by HPLC.

Deuterated Dihydromicrocystin-LR. LR (4.8 mg) was treated with sodium borodeuteride (3 and 2 mg) in water (0.5 mL) as above to afford deuterated dihydromicrocystin-LR (DH-LR, 4.6 mg).

HPLC. A Beckman Model 114M solvent delivery module was used, equipped with a Beckman Model 153 UV detector (254 nm). Isocratic reversed-phase conditions were used with a Nucleosil 5 C_{18} column (4.6 × 250 mm) for analytical and a Nucleosil 7 C_{18} column (10 × 250 mm) for preparative separations. Solvent mixtures (A) methanol/0.7% sodium sulfate (55:45), (B) acetonitrile/0.1% ammonium acetate (25:75), (C) methanol/0.05% trifluoroacetic acid (6:4), and (D) methanol/0.05 M phosphate buffer (pH 3.0) (6:4) were used for analytical separation at a flow rate of 1 mL/min. The retention times of the toxins are listed in Table II.

For the separation of H_2-Ndn and DH-Ndn, methanol/0.7% sodium sulfate (6:4) was used at 2.0 mL/min, and the chromatogram of H_2-Ndn is shown in Figure 1A. DH-Ndn showed a chromatogram similar to that of H_2-Ndn.

H_2-LR was separated with acetonitrile/0.1% ammonium acetate (27:73) as shown in Figure 1B. DH-LR showed a similar chromatogram with the same solvent mixture.

Each peak from preparative HPLC was collected separately, which was concentrated and passed through an ODS cartridge (500-mg gel, Bond Elut). The cartridge was rinsed with water (30 mL), and the product was recovered with methanol (10 mL).

Calculation of Deuterium Incorporation. The percentages (%) of deuterium incorporation in 2′, 3′, 5′ and 6′ were calculated from the intensities of the peaks observed in FABMS as follows:

$$\text{2′ and 3′} = \frac{I_{828}(\text{D}) - I_{827}(\text{D})[I_{828}(\text{H})/I_{827}(\text{H})]}{I_{827}(\text{D}) + I_{828}(\text{D}) - I_{827}(\text{D})[I_{828}(\text{H})/I_{827}(\text{H})]}(100)$$

$$\text{5′ and 6′} = \frac{I_{998}(\text{D}) - I_{997}(\text{D})[I_{998}(\text{H})/I_{997}(\text{H})]}{I_{997}(\text{D}) + I_{998}(\text{D}) - I_{997}(\text{D})[I_{998}(\text{H})/I_{997}(\text{H})]}(100)$$

where $I_{xxx}(\text{D})$ = intensity of the peak at m/z xxx of the deuterated compound and $I_{xxx}(\text{H})$ = intensity of the peak at m/z xxx of the dihydro compound.

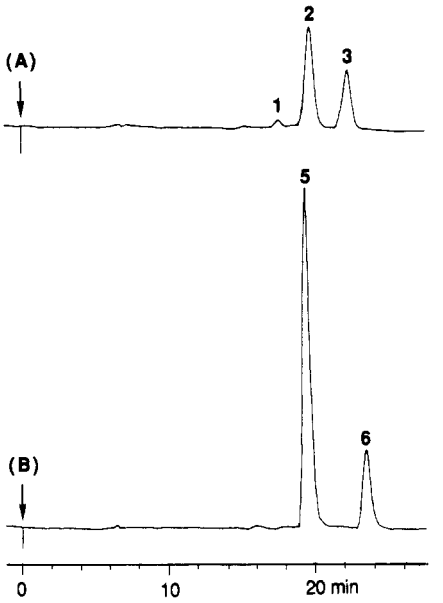


Figure 1. High-performance liquid chromatograms of the reaction mixtures containing stereoisomers of dihydronodularin (A) and dihydromicrocystin-LR (B). Column: Nucleosil 7 C_{18} (10 × 250 mm). Mobile phases: (A) methanol/0.7% sodium sulfate (6:4), (B) acetonitrile/0.1% ammonium acetate (27:73). Flow rate: 2.0 mL/min. Arrows indicate the positions injected.

The calculated percentages of deuterium incorporation were 78% (2′), 79% (3′), 84% (5′), and 83% (6′).

Acid Hydrolysis of Toxin and Derivatization of the Hydrolysate. Each toxin (100 μg) was hydrolyzed with 6 N hydrochloric acid (200 μL, Pierce, Rockford, IL) at 110 °C for 21 h or at 140 °C for 40 min. The dried hydrolysate was dissolved in 300 μL of methanol containing 15% hydrogen chloride, and the solution was heated at 110 °C for 20 min. The mixture was evaporated to dryness by a gentle stream of nitrogen (N_2), trifluoroacetic anhydride (200 μL) was added, and the mixture was heated at 110 °C for 5 min and evaporated by N_2. The residue was dissolved in methylene chloride (50 μL). Each 1 μL of the solution was injected into GC.

Authentic amino acids were derivatized in a similar manner.

GC. A Varian Model 3700 gas chromatograph with a flame ionization detector and a cyclodex-B capillary column (0.25 mm × 30 m, J & W Scientific, Folsom, CA) were operated at 100 °C for detecting Ala, *N*-methylalanine (MeAla), and α-(methylamino)butyric acid (MeAbu) and then at 180 °C (raised at 30 °C/min) for eluting the other amino acid derivatives: helium flow 38 mL/min, split ratio 20:1; injector temperature, 210 °C; detector temperature, 250 °C; makeup gas, helium (20 mL/min).

***N*-Methylamino Acids. (A) Benzyloxycarbonyl(Z)-Amino Acids.** L-α-Aminobutyric acid (L-Abu, 0.51 g, 5 mmol, Aldrich Chemical Co., Milwaukee, WI) was suspended in water (8 mL), and sodium bicarbonate (1.09 g, 13 mmol) was added in small portions to the suspension. Benzyl chloroformate (0.52 mL, 3.25 mmol) in ether (2 mL) was added with stirring at 0 °C, and the mixture was stirred at 0 °C for 1 h and then at room temperature for 2 h. Sodium bicarbonate (0.55 g, 6.5 mmol) and benzyl chloroformate (0.52 mL, 3.25 mmol) were added, and the mixture was further stirred at room temperature for 3 h, diluted with water (10 mL), washed with ether (10 mL × 2), acidified with 6 N hydrochloric acid (ca. 5 mL), and extracted with ethyl acetate (10 mL × 3). The extract was washed with water (10 mL × 2) and saturated NaCl (10 mL), dried over sodium sulfate, and evaporated to dryness. The residue was triturated with petroleum ether and recrystallized from methanol/ether to give 988 mg (83%) of Z-L-Abu.

Z-DL-Abu and Z-L-Ala were obtained in a similar manner from DL-Abu and L-Ala, respectively.

(B) *N*-Methylation (*28*). Iodomethane (500 μL, 8 mmol) was added to a solution of Z-L-Abu (237 mg, 1 mmol) in

Table III. FABMS/MS Data for Compounds 1–6, Obtained on a VG 70-SE4F Spectrometer Using Magic Bullet as a Matrix

sequence	fragment ion, m/z									
	1	2	2′	3	3′	4	5	5′	6	6′
M + H	825	827	828	827	828	995	997	998	997	998
M − 135	689	691	692	691	692	859	861	862	861	862
$C_{11}H_{14}O$-Glu-MeAbu[a]	389[b]	391	392	391	392					
$C_{11}H_{14}O$-Glu-MeAla[a]						375[c]	377	378	377	378
CO-Glu-MeAbu − H	253[b]	255	256	255	256					
CO-Glu-MeAla − H						239[c]	241	242	241	242
Glu-MeAbu + H and MeAbu-MeAsp + H	227[b]	229	230	229	230					
Glu-MeAla + H						213[c]	215	216	215	216
MeAla-Ala + H						155[c]	157	158	157	158
$PhCH_2CH(OCH_3)$	135	135	135	135	135	135	135	135	135	135

[a] $C_{11}H_{14}O$ = $CH_3CH{=}CHC(CH_3){=}CHCH{=}CHCH(CH_3)CO$ − H. [b] Contains MeΔAbu instead of MeAbu. [c] Contains MeΔAla instead of MeAla.

tetrahydrofuran (3 mL) followed by sodium hydride (60% dispersion in mineral oil, 120 mg, 3 mmol) in small portions, with stirring at 0 °C. The mixture was stirred at 0 °C for 1 h and at room temperature for 24 h, diluted with water (20 mL) added dropwise, washed with petroleum ether (10 mL × 2), acidified with 6 N hydrochloric acid to pH 3–4, and extracted with ethyl acetate (10 mL × 3). The extract was washed successively with water (10 mL × 2), 5% sodium sulfite (10 mL), water (10 mL), and saturated NaCl (10 mL), dried (sodium sulfate), and evaporated. The residue was chromatographed on a Sephadex LH-20 column (13 × 600 mm) to afford 185 mg (74%) of Z-L-MeAbu.

Similarly, Z-DL-MeAbu and Z-L-MeAla were prepared.

(C) Deprotection and Derivatization for GC. Each protected *N*-methylamino acid (1 mg) was dissolved in 30% hydrogen bromide/acetic acid (0.2 mL), and the solution was allowed to stand at room temperature for 1 h, evaporated to dryness, triturated with ether, and dried over phosphorus pentoxide under reduced pressure. The amino acids thus obtained and commercial DL-MeAla were derivatized as the trifluoroacetyl methyl esters by the same method used above for authentic amino acids.

Toxicity Testing. The lyophilized toxins were dissolved in water and injected intraperitoneally into four ICR-Swiss male mice (15–25 g; Harlen Sprague-Dawley Inc., Indianapolis, IN) at each toxin concentration. Indications of poisoning, survival times, and body and liver weights were recorded and compared to the effects of known cyanobacterial peptide hepatotoxins. The estimated LD_{50}'s of H_2-Ndn and H_2-LR are listed in Table IV.

Results and Discussion

Synthesis of Dihydro Derivatives. Ndn (1, Scheme I) required a larger excess of sodium borohydride and a longer reaction time relative to LR. When 26 mol (about 100 equiv) of sodium borohydride was used per mole of Ndn, large amounts of the starting material were detected even after 48 h. The reaction was then carried out using about 180 mol (720 equiv) of reagent. About 5–10% of 1 was detected after 24–36 h, but the longer reaction time showed no remarkable change. The starting material remaining in the reaction mixture was easily separated by preparative HPLC on Nucleosil 7 C_{18} (10 × 250 mm) with methanol/0.7% sodium sulfate (6:4) as shown in Figure 1A. The ratio of the isolated stereoisomers **2** [$[\alpha]^{26}_D$ −61.8° (*c* 0.055, methanol); theoretical $(M + H)^+$ for $C_{41}H_{63}N_8O_{10}$ = 827.4667, found 827.4663 (HRFABMS)] and **3** [$[\alpha]^{26}_D$ −34.4° (*c* 0.055, methanol); found 327.4663 (HRFABMS)] was about 2:1. Two stereoisomers of DH-Ndn, **2′** [$[\alpha]^{26}_D$ −54.7° (*c* 0.095, methanol); theoretical $(M + H)^+$ for $C_{41}H_{62}DN_8O_{10}$ = 828.4730, found 828.4712 (HRFABMS)] and **3′** [$[\alpha]^{26}_D$ −28.3° (*c* 0.060, methanol); found 828.4704 (HRFABMS)], were prepared in a manner similar to that employed for **2** and **3**, but using sodium borodeuteride, and purified by HPLC separation with the same solvent mixture.

LR (**4**) is not separated well by HPLC from one of the two stereoisomers of H_2-LR, **5** (Table II). The reduction was, however, complete with 26 mol equiv of the reagent within 20 h in contrast to the reaction of Ndn (**1**). Two stereoisomers, **5** [$[\alpha]^{25}_D$ −73.6° (*c* 0.16, methanol); theoretical $(M + H)^+$ for $C_{49}H_{77}N_{10}O_{12}$ = 997.5722, found 997.5704 (HRFABMS)] and **6** [$[\alpha]^{25}_D$ −38.0° (*c* 0.050, methanol); found 997.5704 (HRFABMS)], were separated by preparative HPLC (Nucleosil 7 C_{18}, 10 × 250 mm) with acetonitrile/0.1% ammonium acetate (27:73), with a ca. 4:1 ratio (Figure 1B). Compounds **5′** [$[\alpha]^{25}_D$ −63.8° (*c* 0.080, methanol); theoretical $(M + H)^+$ for $C_{49}H_{76}DN_{10}O_{12}$ = 998.5785, found 998.5771 (HRFABMS)] and **6′** [$[\alpha]^{26}_D$ −28.0° (*c* 0.025, methanol); found 998.5798 (HRFABMS)] were synthesized by sodium borodeuteride reduction of LR (**4**) followed by preparative HPLC separation as above.

The different reactivity between **1** and **4** with sodium borohydride (borodeuteride) presumably originates from the steric hindrance of the pentapeptide ring and/or the extra methyl group on the double bond of the dehydroamino acid residue in **1**.

Chromatographic Behavior. The isolated compounds **2**, **3**, **5**, and **6** were analyzed by TLC (silica gel, 0.25-mm thickness) and HPLC (Nucleosil 5 C_{18}, 4.6 × 250 mm), and the results are listed in Tables I and II, respectively.

As a result of HPLC and TLC analysis, compounds **1–3** can be separated by HPLC with either methanol/0.7% sodium sulfate or acetonitrile/0.1% ammonium acetate, and compounds **5** and **6** are separated by either HPLC (methanol/0.7% sodium sulfate or acetonitrile/0.1% ammonium acetate) or TLC. The separations of **4** from **5** and of **4** from **6** are achieved by TLC (ethyl acetate/2-propanol/water) and HPLC, respectively.

Tandem FABMS Analysis. Compounds isolated were first analyzed by FABMS followed by HRFABMS using "magic bullet" (*24*) as a matrix. Each molecular ion (detected as a protonated ion, $[M + H]^+$) was subjected to FABMS/MS to observe the product ion peaks. Since the compounds are cyclic peptides, only a few prominent fragment ion peaks were detected, as listed in Table III (*22*, *23*).

We have already reported a method for the structure assignment of microcystins utilizing product ions detected in FABMS/MS (*22*, *23*), and corresponding product ion peaks were observed in FABMS/MS of **5**, **5′**, **6** and **6′**. The fragment ion peaks containing MeAla were shifted 2 Da higher for **5** and **6** (*m/z* 377, 241, 215, and 157) and 3 Da

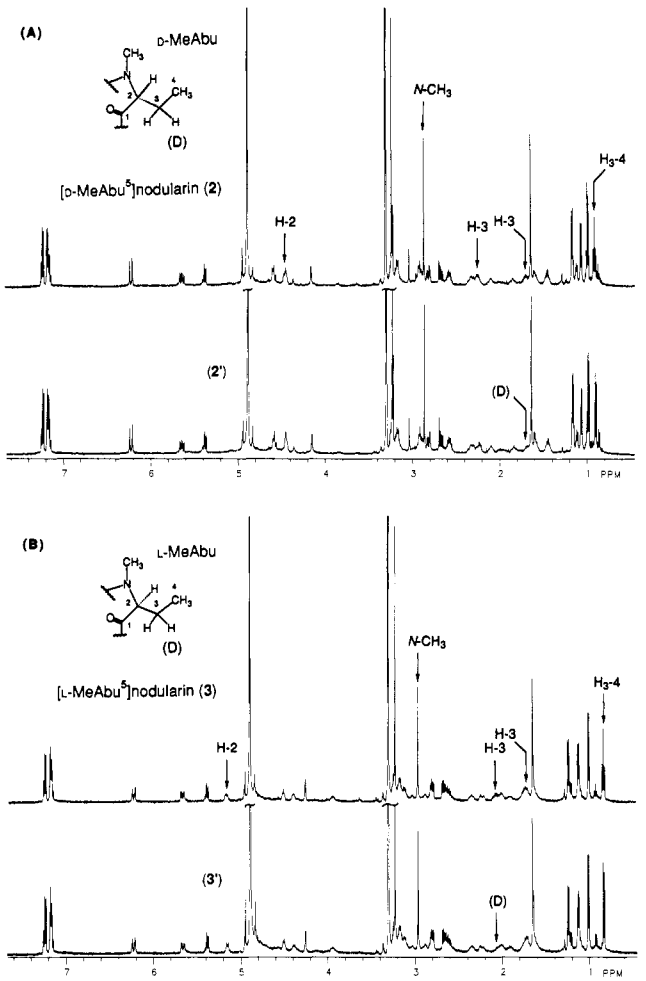


Figure 2. ^{1}H NMR spectra (500 MHz) of **2** [(A), upper trace], **2′** [(A), lower trace], **3** [(B), upper trace], and **3′** [(B), lower trace] obtained in CD_3OD (δ 3.30 ppm) on a GE GN-500. The spectra show the chemical shifts for the MeAbu residue and the position of deuterium incorporation.

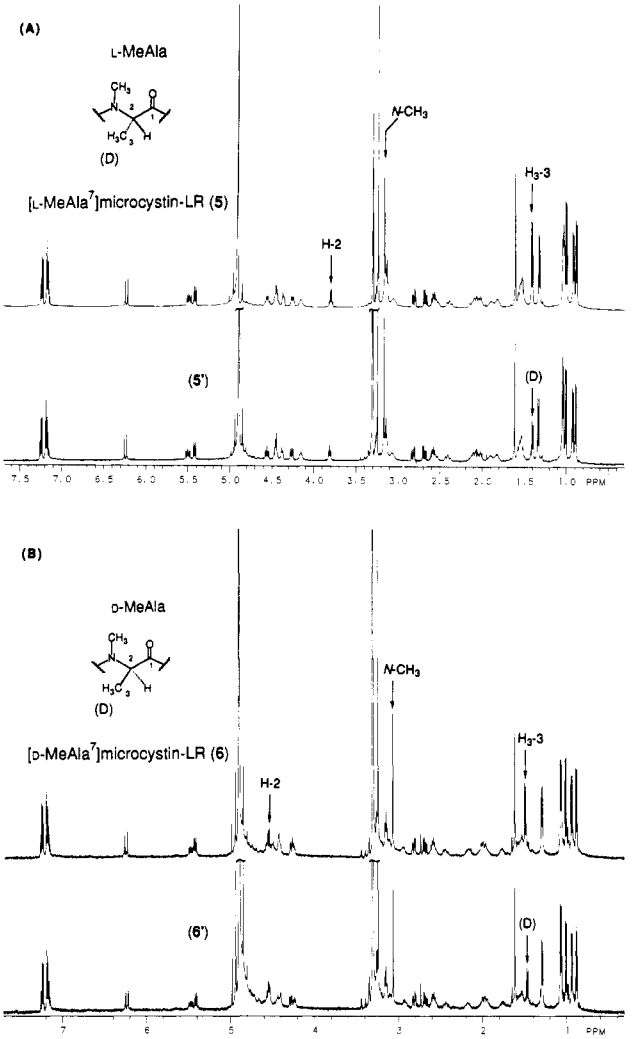


Figure 3. ^{1}H NMR spectra (500 MHz) of **5** [(A), upper trace], **5′** [(A), lower trace], **6** [(B), upper trace], and **6′** [(B), lower trace]. The spectra show the chemical shifts for the MeAla residue and the position of deuterium incorporation.

higher for **5′** and **6′** (*m/z* 378, 242, 216, and 158) than those for LR (**4**) (*m/z* 375, 239, 213, and 155).

Ndn (**1**) gave product ion peaks similar to those for LR (**4**) in the FABMS/MS spectrum (*m/z* 689, 389, 253, 227, and 135).[2] The isotopic compositions of peaks at *m/z* 135, 227, 253, and 689 were confirmed by HRFABMS. Compounds **2** and **3** showed fragment ions containing MeAbu (*m/z* 391, 255, and 229) 2 Da higher than those of **1** (*m/z* 389, 253, and 227). Similarly, the corresponding peaks of **2′** and **3′** (*m/z* 392, 256, and 230) were observed 3 Da higher than those of **1**. The FABMS/MS analysis of these compounds showed that the reductions occurred at the *N*-methyldehydroamino acid residues of Ndn (**1**) and LR (**4**). The percentages of deuterium incorporation in **2′**, **3′**, **5′**, and **6′**, calculated from the FABMS data of the dihydro compounds (**2**, **3**, **5**, and **6**) and the deuterated compounds (**2′**, **3′**, **5′**, and **6′**), were 78, 79, 84, and 83%, respectively.

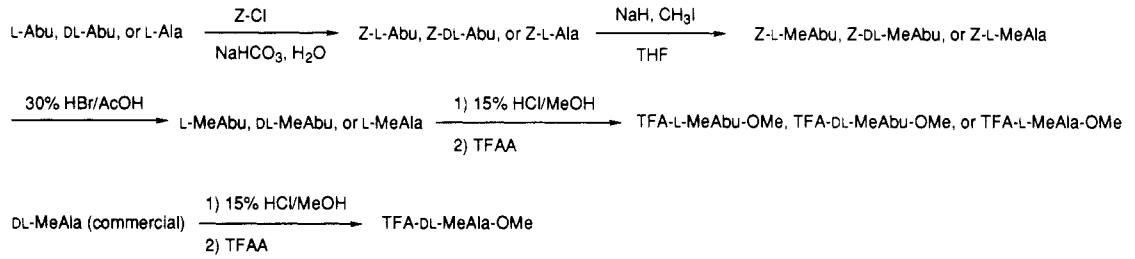
^{1}H NMR and the Position of Deuterium Incorporation. The ^{1}H NMR spectra of **2**, **2′**, **3**, and **3′** are shown in Figure 2. An olefinic one-proton quartet and methyl doublet (respectively, δ 6.95 and 1.74 ppm) of the MeΔAbu residue observed in the ^{1}H NMR spectrum of Ndn (**1**) were not detected in spectra of **2**, **2′**, **3**, and **3′**, which also showed the MeΔAbu residue was saturated. A methyl triplet and an α-proton signal of MeAbu were observed at δ 0.95 and 4.47 ppm, respectively, in the spectrum of **2**. The methyl signal of MeAbu was changed to a doublet, the intensity of the signal at δ 1.68 ppm (H-3) was reduced in that of **2′**, and the shapes of the signals at δ 2.12 (H-3) and 4.47 ppm (H-2) in the spectrum of **2′** were changed by the incorporation of deuterium. These data clearly showed that the deuterium was incorporated at the β-position of MeAbu as expected from the mechanism of the 1,4-addition.

Similar phenomena were observed in the ^{1}H NMR spectra of **3** and **3′** (Figure 2). The α-proton signal (δ 5.17 ppm) of MeAbu in **3** (and **3′**) is observed at much lower field than in that of **2** (and **2′**). The signal of the methylene proton replaced by deuterium was for the proton detected at lower field in contrast to **2** and **2′**, where it was the higher proton.

The ^{1}H NMR spectrum of **5** (Figure 3) showed a methyl doublet and an α-proton signal ascribable to the MeAla residue at δ 1.40 and 3.82 ppm, respectively, instead of three singlets detected at δ 3.33, 5.42, and 5.89 ppm due to MeΔAla in that of LR (**4**). The methyl doublet at δ 1.40 ppm was less intense in the spectrum of **5′** than that of **5**, and the α-proton signal was changed to a triplet from a quartet in consequence of the deuteration at the β-position of MeAla.

[2] M. Namikoshi, B. W. Choi, K. L. Rinehart, and W. W. Carmichael, unpublished data.

Scheme II



Compounds **6** and **6′** showed differences similar to those observed for **5** and **5′** (Figure 3). The α-proton signal of MeAla for **6** (and **6′**) was detected at lower field (δ 4.55 ppm) than for **5** (and **5′**), as observed in the spectra of H_2-Ndn (and DH-Ndn).

Smaller signals detected in each ^1H NMR spectrum of the dihydro derivatives can be ascribed to a stereoisomer at the *N*-methyl group.

The ^1H NMR spectra of these compounds confirmed the site of the reduction and of the hydride (deuteride) incorporation.

Stereochemistry of MeAbu and MeAla. To determine the stereochemistries of MeAbu in **2** and **3** and of MeAla in **5** and **6**, authentic *N*-methylamino acids were synthesized as shown in Scheme II, since only DL-MeAla was commercially available.

L-Abu, DL-Abu, and L-Ala were first converted with benzyl chloroformate to their Z-derivatives, which were N-methylated by the method reported by McDermott and Benoiton (*28*) with slight modification. The Z protecting group was removed in the usual manner, and the *N*-methylamino acid obtained was treated with hydrogen chloride/methanol followed by trifluoroacetic anhydride to give the trifluoroacetyl methyl ester derivative (*22*, *23*).

The D- and L-isomers of *N*-methylamino acids showed no separation on a Chirasil Val III capillary GC column, which is used for separating the D- and L-isomers of amino acids (*22*, *23*, *29*). However, a permethylated β-cyclodextrin capillary GC column (Cyclodex-B, 0.25 mm × 30 m) (*30*) separated the D- and L-isomers of MeAbu and MeAla. Figure 4 shows the chromatograms of MeAbu and MeAla derivatives obtained with 100 °C isothermal operation. DL-Ala was also separated under the same conditions, but Leu, MeAsp, and Glu did not show practical separations. The coelution experiments of L- and DL-isomers showed that the L-isomer elutes faster than the D-isomer for *N*-methylamino acids.

Compounds **2**, **3**, **5**, and **6** were hydrolyzed with 6 N hydrochloric acid, and the hydrolysates were derivatized to the trifluoroacetyl methyl ester derivatives by reaction with hydrochloric acid/methanol followed by trifluoroacetic anhydride. The chromatograms of the derivatized hydrolysates of **2**, **3**, **5**, and **6** are shown in Figure 5. The racemization of *N*-methylamino acids, especially D-isomers, was observed for all compounds, as the purity of each compound was confirmed by HPLC and TLC. Isothermal operation at 100 °C was used to detect Ala, MeAbu, and MeAla derivatives, then the temperature was increased to 180 °C at 30 °C/min to elute the other amino acid derivatives. The stereochemistries of *N*-methylamino acid derivatives were determined by coelution experiments with DL mixtures.

The GC analysis of the hydrolyzate derivatives established the stereochemistries of the two H_2-Ndn derivatives as **2** = [D-MeAbu^5]nodularin and **3** = [L-MeAbu^5]-nodularin. Similarly, the stereochemical relationships of the two H_2-LR derivatives were determined as **5** = [L-MeAla^7]microcystin-LR and **6** = [D-MeAla^7]microcystin-LR.

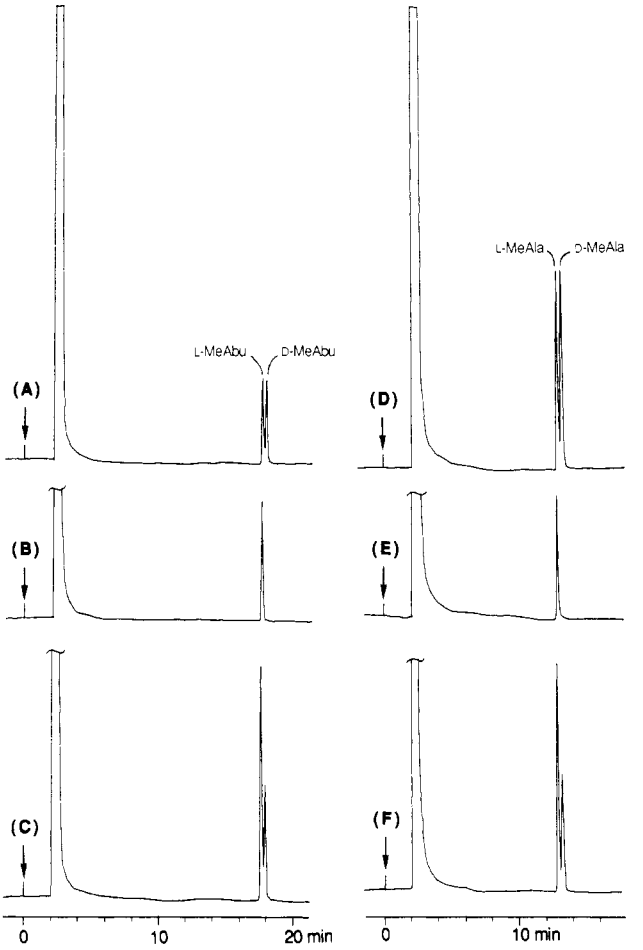


Figure 4. Gas chromatograms of trifluoroacetyl methyl ester derivatives of (A) DL-MeAbu, (B) L-MeAbu, (C) mixture of (A) and (B), (D) DL-MeAla, (E) L-MeAla, and (F) mixture of (D) and (E). Column: Cyclodex-B (0.25 mm × 30 m). Temperature: 100 °C. Carrier gas: He. Arrows indicate the positions injected.

The approximate LD_{50}'s of the isolated compounds **2**, **3**, **5**, and **6** were determined using four male white mice for each sample. Compounds **2** and **3** showed identical toxicity (LD_{50} 150 μg/kg) and are about half as active as Ndn (**1**). The stereoisomers of H_2-LR, **5** and **6**, have toxicity comparable to that of the parent compound, LR (**4**) (Table IV, LD_{50} ca. 100 μg/kg). These data clearly show that the stereoisomerism at the *N*-methylamino acid residue does not affect their toxicity. Probably both stereoisomers settle in a similar conformation to that of the parent compound. Analyses of the three-dimensional structures and solution conformations of microcystins and nodularins are in progress.

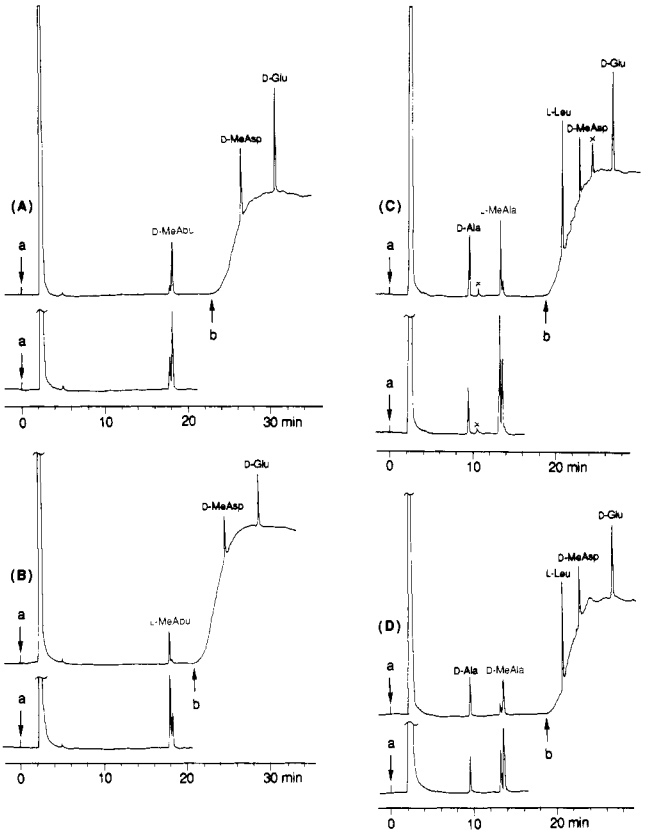


Figure 5. Gas chromatograms of acid hydrolysates of trifluoroacetyl methyl ester derivatives of **2** (A), **3** (B), **5** (C), and **6** (D). Each lower trace shows the coelution experiment with the DL-mixture. Column: Cyclodex-B. Temperature: 100 °C isothermal, raised to 180 °C at 30 °C/min from the position indicated by "b". Arrows with "a" indicate the positions injected.

Table IV. Toxicity of 2, 3, 5, and 6

compd	LD_{50} (ip, mice), μg/kg
2	150
3	150
5	85
6	100

In this study, we prepared two stereoisomers of H_2-Ndn and two of H_2-LR in pure form and determined the stereochemistry of each stereoisomer. The isolated stereoisomers of H_2-Ndn, **2** and **3**, were comparably toxic, about half as toxic as the parent compound. The stereoisomers of H_2-LR, **5** and **6**, and the parent compound were all similarly toxic. The incorporation of deuterium was very high (78–84%). The location of deuterium incorporation into the *N*-methylamino acid residue at H-3 confirmed the reaction mechanism and afforded useful information for tritium labeling and further toxicological studies.

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