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Isolation of Linear Peptides Related to the Hepatotoxins Nodularin and Microcystins

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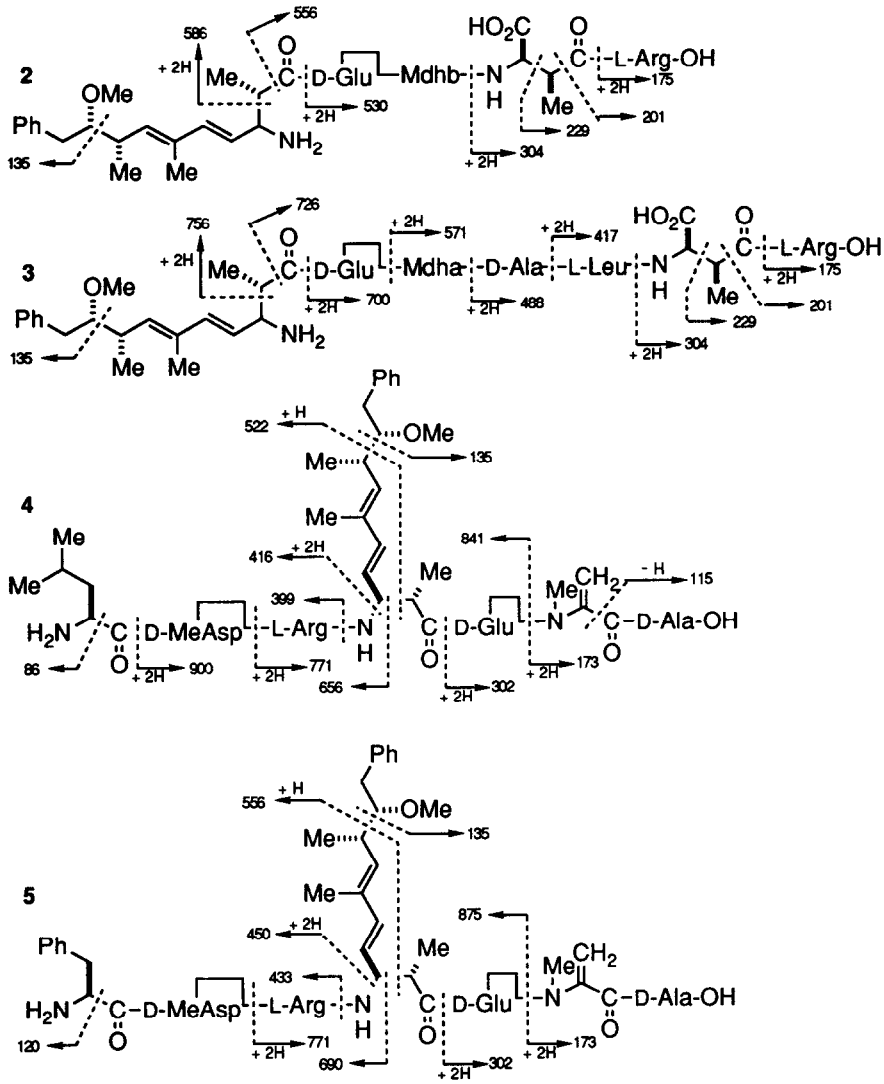
Abstract: Linear peptide **2**, Adda-D-Glu(γ)–Mdhb-D-MeAsp(β)–L-Arg-OH, was isolated from cultured *Nodularia spumigena* and was analyzed in the cells after one week's (1·2 : 30 1) to eight week's (>100 1) cultivation Three linear peptides, Adda-D-Glu(γ)–Mdha-D-Ala–L-Leu-D-MeAsp(β)–L-Arg-OH (**3**), L-Leu-D-MeAsp(β)–L-Arg–Adda-D-Glu(γ)–Mdha-D-Ala-OH (**4**), and L-Phe-D-MeAsp(β)–L-Arg–Adda-D-Glu(γ)–Mdha-D-Ala-OH (**5**) were obtained from a water bloom of *Microcystis* spp. collected from Homer Lake (Illinois) Some of these linear peptides (**2, 3**) are thought to be biogenetic precursors of nodularin and microcystins Linear peptides **2, 3,** and **4** did not show apparent toxicity at 1 0, 1 1, and 2 25 mg/kg, respectively, in a mouse bioassay (ip). Feeding experiments using ^{13}C-labeled precursors established that the 2-, 6- and 8-methyl and 9-methoxy carbons of the unusual (2*S*,3*S*,8*S*,9*S*)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4,6-decadienoic acid (Adda) unit of **1** were clearly derived from L-methionine.

Certain genera of cyanobacteria (blue-green algae) produce potent cyclic peptide hepatotoxins--nodularin (**1**),[1] a cyclic pentapeptide produced by *Nodularia spumigena*, and microcystins,[2] cyclic heptapeptides found in *Microcystis, Anabaena, Nostoc*, and *Oscillatoria* species. Nodularin and microcystins have also been reported to be effective inhibitors of protein phosphatases 1 and 2A[3] and tumor promoters.[4] Until now linear peptides related to **1** and the microcystins have not been observed in the algae, but we report here isolation of linear peptides **2, 3, 4,** and **5** (Scheme I), some of which are presumed biogenetic precursors of nodularin and microcystins The peptides (**2-5**) contain all of the amino acids of their cyclic analogs but are essentially non-toxic.

Linear peptide **2** (Scheme I), $[\alpha]^{25}_D$ –58.2° (*c* 0 0015, MeOH), $C_{41}H_{63}N_8O_{11}$, M + H, Δ +0.5 mDa, HRFABMS, was isolated during our biosynthetic studies on nodularin, described below It was obtained in 0.002% yield (**1** = 0.08%) from dried cells of cultured *N spumigena* L-575 by repeated HPLC and TLC separations. Compound **2** was ninhydrin positive and showed broad signals in its ^1H NMR spectrum compared with those of **1**. The presence of the (2*S*,3*S*,8*S*,9*S*)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4,6-decadienoic acid (Adda) and α-(methylamino)dehydrobutyric acid (Mdhb) units in **2** was indicated by the ^1H NMR spectrum The stereochemistries of other amino acid components were determined by chiral capillary GC of the trifluoroacetyl methyl ester derivatives of the acid hydrolyzate Tandem FABMS



Scheme I

(FABMS/CID/MS, B/E scan) revealed the sequence of **2** by the fragment ion peaks shown in Scheme I. The monoisotopic compositions were confirmed by HRFABMS for the fragment ion peaks at *m/z* 827 (M – NH_2, $C_{41}H_{61}N_7O_{11}$, Δ +0.6 mDa), 692 (M – NH_2 – 135, $C_{32}H_{50}N_7O_{10}$, Δ –3.7 mDa), 586 (Δ –0.1 mDa), 556 (–5.5 mDa), and 530 (–3.2 mDa). The ratio of **1** and **2** in the cells was calculated by harvesting the cells on every 7th day from a continuous culture, to examine the possibility of **2** as a biogenetic precursor of **1**. After

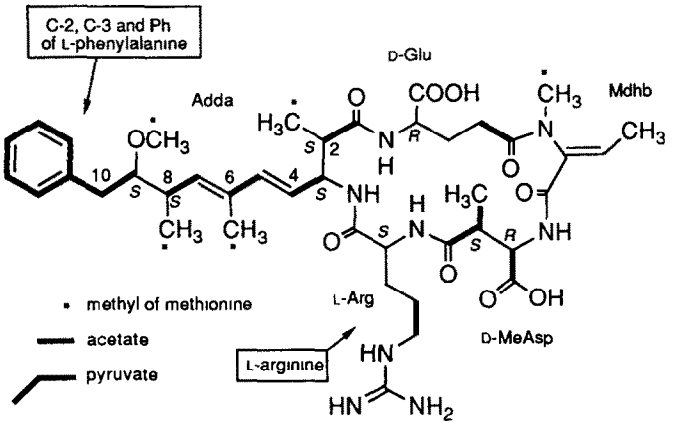
one week **2** was detected in a 1:30 (**2**:**1**) ratio, which increased to 1:80 (two weeks) and 1:>100 (three and eight weeks). The result argues that **2** is a biogenetic precursor to be cyclized to **1**.

Microcystin-LR (LR) was isolated as the main hepatotoxin component (0.1% of dried cells) from a water bloom of *Microcystis* spp. collected from Homer Lake (Illinois).[7] Acyclo-LR (**3**, $[\alpha]^{25}_{D}$ –46.2° (*c* 0.001, MeOH), $C_{49}H_{77}N_{10}O_{13}$, M + H, Δ +1.4 mDa, HRFABMS) was isolated from the above cells (0.0001% yield). FABMS/CID/MS of **3** showed the presence of the Adda and *N*-methyldehydroalanine (Mdha) units by the fragment ion peaks at *m/z* 135, 155, 213, 239, 375, and 877,[7] and other amino acid components were assigned by chiral capillary GC. A FABMS/CID/MS spectrum of **3** resembled that of **2** and revealed the sequence of **3** as shown in Scheme I. The position of ring opening at the Adda-Arg bond was the same as that of **2**. Compound **4** (0.003% yield, $[\alpha]^{26}_{D}$ –34.0° (*c* 0.020, MeOH), $C_{49}H_{77}N_{10}O_{13}$, M + H, Δ +1.1 mDa, HRFABMS) was isolated from the same cells and was also positive to ninhydrin. A ^{1}H NMR spectrum of **4** showed broad signals except for several signals due to the Adda unit, and signals due to the Mdha unit were detected. Dansylation of **4** with dansyl chloride gave the dansyl-peptide (**6**, $C_{61}H_{88}N_{11}O_{15}S$, M + H, Δ –1.7 mDa, HRFABMS). The acid hydrolysis of **6** afforded dansyl-Leu, identified by TLC and FABMS/CID/MS compared to an authentic sample, together with Ala, Arg, Glu, MeAsp, and a decomposition product ($C_{19}H_{26}NO_2$, M + H, Δ +0.4 mDa, HRFABMS)[8] from the Adda unit. FABMS/CID/MS of **4** and **6** indicated the sequence of **4** shown in Scheme I. Compound **5** ($C_{52}H_{75}N_{10}O_{13}$, M + H, Δ –0.3 mDa, HRFABMS) was obtained as a very minor component (0.0001% yield). Chiral capillary GC revealed the presence of L-Phe together with D-Ala, L-Arg, D-Glu, and D-MeAsp. FABMS/CID/MS of **5** gave a spectrum similar to that of **4**, and peaks with the Phe unit were detected 34 Da higher than corresponding peaks of **4**, arguing the structure of **5** shown in Scheme I.

Linear peptide **2** is proposed to be a biogenetic precursor of **1**, as suggested from the cultivation experiment. Peptide **3** is also regarded as a likely precursor of LR, but this is not yet clear, since the cell material used in this study was a mixture of three *Microcystis* spp. (*M. aeruginosa*, *M. viridis*, and *M. wesenbergii*) from a bloom.[7,9] Isotope-labeled linear peptides are being prepared for feeding experiments both by chemical synthesis and by isolation from cultured cells enriched with ^{14}C- and ^{13}C-labeled precursors.

The linear peptides **2**, **3**, and **4** did not show apparent toxicity to mice (i. p.) at 1.0 (**2**), 1.1 (**3**), and 2.25 mg/kg (**4**), which shows that the cyclic structure is necessary for the activity of nodularin and microcystins.

Our biosynthetic studies on nodularin involved feeding potential ^{14}C-labeled precursors followed by ^{13}C-labeled precursors. The results of feeding experiments using L-[*methyl*-^{13}C]Met, [*1*-^{13}C]-, [*2*-^{13}C]-, and [*1,2*-$^{13}C_2$]acetate, L-[*3*-^{13}C]Phe, [*2*-^{13}C]pyruvate, and L-[6-^{13}C,2',3'-$^{15}N_2$]Arg are summarized in Scheme II, and were nearly the same as those for the biosynthesis of the corresponding amino acid components in LR.[5] The Me group on C-2 of the Adda unit in LR was reported earlier to be enriched by two precursors, L-methionine and propionate, depending upon the culture conditions.[5] However, in our feeding experiments, the C-2 Me group of Adda in **1** was enriched by L-[*methyl*-^{13}C]Met to the same extent as the Me groups on Adda's C-6 and C-8, i.e. not by exogenous propionate.[6] When [*1*-^{13}C]propionate was fed, most of the carbons of **1** were enriched, suggesting that propionate underwent intense metabolism and was used as a carbon source rather than a specific precursor. The incorporations of L-[*U*-^{14}C]Thr and L-[*U*-^{14}C]Glu, presumably into the Mdha and D-Glu subunits, were also observed in the radioactive feeding experiments.



nodularin (1)

Scheme II

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