Nodularin, Microcystin, and the Configuration of Adda[1,2]

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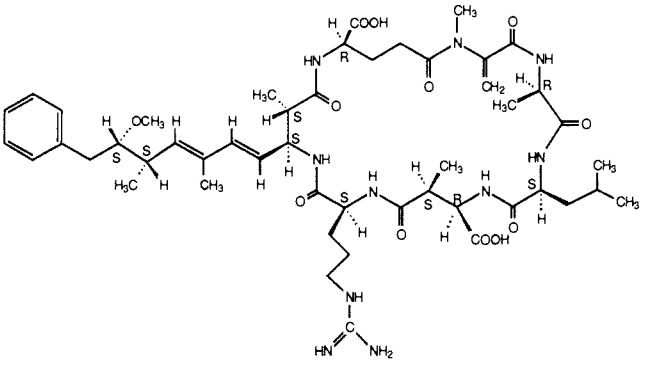
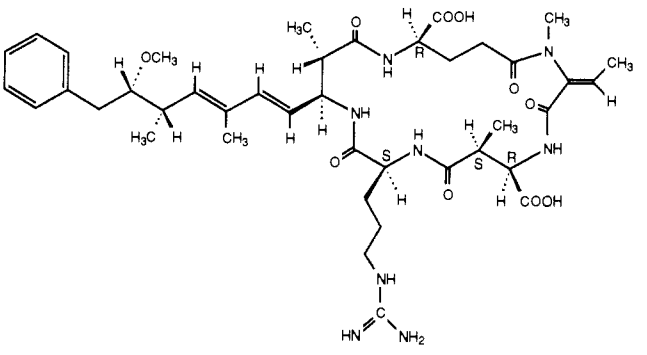
Closely related toxins, all cyclic heptapeptides, have been reported recently from cyanobacteria—microcystin from *Microcystis aeruginosa*;[5] the cyanoginosins[6] and Akerstox[7] also from *M. aeruginosa*; and cyanoviridin from *M. viridis*,[8] as illustrated by microcystin LR[9] (**1**, presumably identical with cyanoginosin LR[6a]).



Microcystin - LR (1)

In other microcystins (cyanoginosins)[9] the Leu unit is replaced by Tyr or Arg and the Arg unit by Met or Ala, but they all contain a uniquely characteristic C_{20} amino acid, 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4,6-decadienoic acid (Adda). We assign here the stereochemistry of Adda as well as the structure (**2**) of the first cyclic *penta*peptide in this class, nodularin,[1] whose hepatotoxicity approximates that of microcystin LR.[9]



Nodularin (**2**)

Reports that *Nodularia spumigena* causes stock losses in New Zealand[10] led us to examine the *N. spumigena* algal mat, which was extracted with methanol–water; purification, capped by HPLC on an ODS column, yielded the pure toxin (**2**, $C_{41}H_{61}N_8O_{10}$, M + H, Δ3.2 mmu, HRFABMS, 0.015% yield from freeze-dried alga). Total acid hydrolysis (6 N HCl, 110 °C) of **2** yielded equimolar quantities of Arg, Glu, β-methylaspartic acid (MeAsp), and methylamine. The stereochemistry of the amino acids was established as D-Glu, D-*erythro*-MeAsp, and L-Arg by comparison of retention times of the butyl esters of their derivatives—*N*-trifluoroacetyl (TFA) of the first two, dimethylpyrimidyl of the last—with those of authentic samples on a chiral GC column, and the peptide linkages were assigned as γ-Glu and β-MeAsp by incorporation of one deuterium atom in each on heating of **2** with acetic anhydride–pyridine in deuterium oxide.

Similar hydrolysis of dihydronodularin ($C_{41}H_{63}N_8O_{10}$, M + H, Δ1.2 mmu, HRFABMS, from sodium borohydride reduction of **2**) yielded Arg, Glu, MeAsp, and *N*-methylbutyrine (MeBut). The presumption that MeBut was derived from an *N*-methyldehydrobutyrine unit (MeDebut, which yielded methylamine on hydrolysis) in **2** was confirmed by the presence in the ^1H NMR spectrum of **2** of cis *N*-methyl and olefinic methyl groups [δ 3.08 and 1.82, $-N(CH_3)C(=CHCH_3)CO-$, *Z* isomer by NOE enhancement (1.4%)] and of an olefinic proton (δ 6.95, $J = 7.1$ Hz) coupled to the latter. The ^1H and ^{13}C NMR spectra also demonstrated the presence of Adda in nodularin, with chemical shifts, coupling patterns, and NOE behavior essentially identical with those previously reported,[6b] assigning both the Δ^4 and Δ^6 double bonds as *E*.

Ozonolysis (MeOH, −78 °C) of dihydromicrocystin LR [$C_{49}H_{76}N_{10}O_{12}$, M + H, Δ2.3 mmu, HRFABMS, obtained by sodium borohydride reduction of **1** ($C_{49}H_{74}N_{10}O_{12}$, M + H, Δ1.5 mmu, HRFABMS) isolated from natural and PCC 7820 *M. aeruginosa*], followed by oxidation (H_2O_2) and vigorous hydrolysis, gave a mixture of D-*erythro* and a stereoisomeric MeAsp, recently reported to be L-*erythro*.[6c] In our hands, however, hydrolysis (5.6 N HCl, 110 °C) of the oxidized product for a limited time gave only D-*erythro*-MeAsp, arguing 2*S*,3*S* stereochemistry at C-2, C-3 of Adda. We then reduced the ozonide from microcystin

(1) Portions of the structural work described here on nodularin were presented by K.L.R. at the following: (a) The 6th Nordic Conference on Mass Spectrometry, Borgholm, Öland, Sweden, May 25–28, 1986. (b) The Royal Society of Chemistry Annual Chemical Congress, Swansea, U.K., April 13–16, 1987, Paper A12. (c) The 16th International Symposium on the Chemistry of Natural Products, IUPAC, Kyoto, May 29–June 3, 1988.

(2) This paper is dedicated to the memory of our colleague (C.A.H.), who carried out the early studies on nodularin in Urbana.

(3) On leave from Meijo University, Nagoya, Japan, 1985–1986.

(4) Deceased.

(5) Konst, H.; McKercher, P. D.; Gorham, P. R.; Robertson, A.; Howell, J. *Can. J. Comp. Med. Vet. Sci.* **1965**, *29*, 221–228.

(6) (a) Botes, D. P.; Wessels, P. L.; Kruger, H.; Runnegar, M. T. C.; Santikarn, S.; Smith, R. J.; Barna, J. C. J.; Williams, D. H. *J. Chem. Soc., Perkin Trans. 1* **1985**, 2747–2748. (b) Botes, D. P.; Tuinman, A. A.; Wessels, P. L.; Viljoen, C. C.; Kruger, H. *J. Chem. Soc., Perkin Trans 1* **1984**, 2311–2318 and earlier papers. (c) Painuly, P.; Perez, R.; Fukai, T.; Shimizu, Y. *Tetrahedron Lett.* **1988**, *29*, 11–14.

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(10) Blunt, J. W.; Mulligan, P. E.; Munro, M. H. G., New Zealand Institute of Chemistry Combined Conference, Hamilton, New Zealand Aug. 23–26, 1983; Paper T2.

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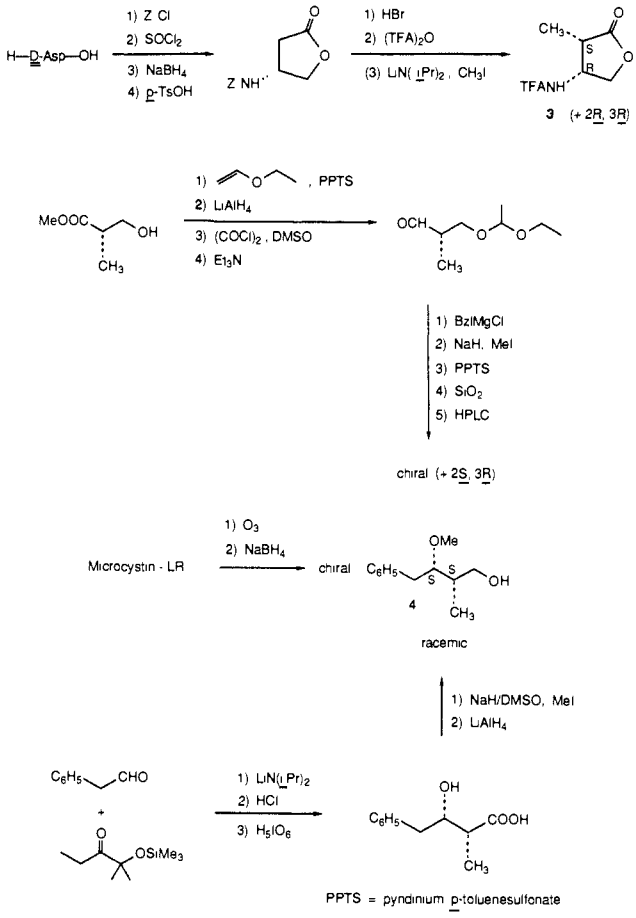
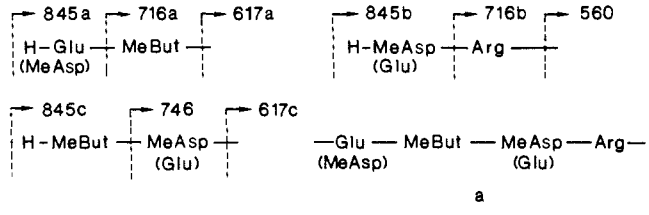
Scheme I



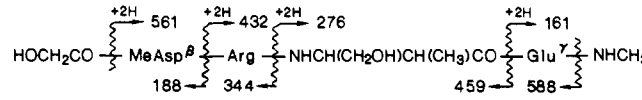
Scheme II



(MeOH, −78 °C, 10 min) with sodium borohydride and hydrolyzed (TFA/5.6 N HCl 1:2, 110 °C, 90 min) to give 3-amino-2-methyl-γ-butyrolactone, characterized as its *N*-TFA derivative **3** ($C_7H_9F_3NO_3$, M + H, Δ0.1 mmu, HRCIMS). The stereochemistry of **3** was assigned as 2*S*,3*R* by comparison with the four stereoisomers prepared from D- and L-aspartic acids (by the route shown for D-Asp) and characterized by NOE experiments (irradiating H-2, H-3, and the methyl proton signals), GC retention times on a chiral column, and mass spectral patterns. The stereochemistry of Adda is therefore 2*S*,3*S*.

To assign the C-8 and C-9 configurations, the ozonide was reduced to 3-methoxy-2-methyl-4-phenyl-1-butanol (**4**, $C_{12}H_{19}O_2$, M + H, Δ0.2 mmu, HRCIMS) whose four stereoisomers were synthesized (Scheme I) in chiral form from commercially available (*R*)- and (*S*)-methyl 3-hydroxy-2-methylpropanoates and in racemic form from phenylacetaldehyde and 2-methyl-2-((trimethylsilyl)oxy)pentan-3-one[11,12] and characterized by HPLC and ^{1}H NMR spectra with added (*R*)-(−)-2,2,2-trifluoro-1-(9-anthryl)ethanol.[13] Adda is then 2*S*,3*S*,8*S*,9*S* in microcystin LR (**1**),[14] completing the latter's structure.

Scheme III



To assign the peptide sequence of nodularin, the trifluoroacetolysis product of dihydronodularin (CF_3COOH, room temperature, 24 h) was studied by MS/MS, employing VG ZAB 4F and VG 70 SE 4F four-sector instruments to demonstrate the major fragmentation pathways shown in Scheme II. The M + H ion (*m/z* 845) must be a mixture of at least three hydrolysis products (partial structures shown in Scheme II), which can lose N-terminal units with either 99 (MeBut) or 129 amu (Glu or MeAsp) to give *m/z* 746 and 716 ions. The latter is also a mixture, since an MS/MS study of *m/z* 716 shows it can lose either N-terminal 156 (Arg) or 99 amu to give *m/z* 560 or 617, while *m/z* 746 fragments by loss of 129 amu to give a different *m/z* 617. These fragments can be combined to sequence a (Scheme II), leaving only Adda to complete the ring.

To distinguish between **2** and the alternative structure in which the isomeric Glu and MeAsp units are exchanged, nodularin was ozonized (MeOH, −78 °C, 30 min) and reduced with sodium borohydride to **5**, whose FAB-generated M + H ion (*m/z* 619) fragmented by collisionally induced decomposition MS/MS as shown in Scheme III, in precisely the pattern predicted for the acyclic peptide.[15] N-Protected peptide **5** ($C_{24}H_{42}N_8O_{11}$, M + H, Δ1.4 mmu) was oxidized with lead tetraacetate (HOAc, room temperature, 6 h) to remove the hydroxyacetyl group, then treated with dansyl chloride (CH_3CN), and hydrolyzed (6 N HCl, 110 °C) to give Dns-MeAsp (M + H, 381.1105, Δ1.5 mmu), R_f 0.23 in EtOAc/MeOH/HOAc 20:1:1 (and no Dns-Glu, R_f 0.38), defining the N-terminus of **5** and completing the sequence of **2** (nodularin).[16]

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Supplementary Material Available: ^{1}H and ^{13}C NMR spectra of nodularin, MS/MS on compound **5** (*m/z* 619) and physical properties of synthetic compounds in Scheme I (7 pages). Ordering information is given on any current masthead page.

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(13) Pirkle, W. H.; Rinaldi, P. L. *J. Org. Chem.* **1978**, *43*, 4475–4480.

(14) The ^{1}H NMR identity establishes the same relative stereochemistry of Adda in nodularin and microcystin; it is highly unlikely that every asymmetric center would be reversed in this complex subunit.

(15) Similar fragmentation of the borohydride-reduced ozonolysis product from microcystin LR confirmed its previously assigned sequence.[6]

(16) Toxins from Australian and Baltic *Nodularia* species which may be the same as nodularin have been partially described: Runnegar, M. T. C.; Jackson, A. R. B.; Falconer, I. R. *Toxicon* **1988**, *26*, 143–151. Eriksson, J. E.; Meriluoto, J. A. O.; Kujari, H. P.; Osterlund, K.; Fagerlund, K.; Hallbom, L. *Toxicon* **1988**, *26*, 161–166. These reports identified only Glu, Arg, MeAsp (or Asp), and methylamine and assigned no stereochemistry; the authors may have been unaware of our earlier partial structural assignments.[1]