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The cyanobacterium *Nodularia* PCC 7804, of freshwater origin, produces [L-Har^2]nodularin

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Abstract

The cyanobacterium *Nodularia* PCC 7804, an axenic, non gas-vacuolate strain from a freshwater source, produces several metabolites with cyanobacterial hepatotoxin characteristics. The most abundant is a cyclic pentapeptide, [L-Homoarginine^2]nodularin. [L-Har^2]nodularin is of similar toxicity, in terms of bioassay in vivo, and the inhibition of protein phosphatase-1 in vitro to nodularin, which was present in lesser amounts in the cultures.

Keywords: *Nodularia* PCC 7804; *Nodularia sphaerocarpa*; Cyanobacteria; Blue–green algae; Nodularin

1. Introduction

The cyclic pentapeptide nodularin, a potent hepatotoxin and tumour-promoter, is commonly produced by environmental samples and laboratory strains of the filamentous bloom- and scum-forming cyanobacterium (blue–green alga), *Nodularia spumigena*. The toxin has been identified from brackish waters, including many parts of the Baltic Sea (e.g. Eriksson et al., 1988) and from brackish coastal sites in New Zealand (Carmichael et al., 1988), Australia (Jones et al., 1994), South Africa (Harding et al., 1995) and the UK (Twist and Codd, 1997). Animal poisonings have long been associated with the ingestion of hepatotoxic *N. spumigena* scums and blooms (Francis, 1878) and nodularin is now assumed to be a primary causative factor (Codd, 1994; Harding et al., 1995).

The structure of nodularin is cyclo-(D-Masp^1-L-Arginine^2-Adda^3-D-Glutamic acid^4-Mdhb^5) (Rinehart et al., 1988). D-Masp is D-*erythro*-β-methylaspartic acid. Adda is (2*S*,3*S*,8*S*,9*S*)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4(*E*),6(*E*)-dienoic acid. Mdhb is *N*-methyldehydrobutyrine. In contrast to the wide diversity of the related hepatotoxic cyclic heptapeptides (microcystins) of cyanobacteria, of which over 65 structural variants are described, only five naturally occurring nodularin variants have been characterised, including nodularin itself (Sivonen and Jones, 1999). Three of these have been characterised as minor components from a bloom and laboratory cultures of *N. spumigena* from Lake Ellesmere, New Zealand, namely [6(*Z*)-Adda^3]nodularin, [D-Asp^1]nodularin and a demethylated-Adda form, [DMAdda^3]nodularin (Namikoshi et al., 1994). The remaining known variant, [L-valine^2]nodularin, also known as motuporin, has been characterised from the marine sponge *Theonella swinhoei* (de Silva et al., 1992). It is not known whether motuporin is a product of the animal cells of the sponge or of associated cyanobacteria.

Extracts of *Nodularia* PCC 7804, originally from a freshwater thermal source and with a benthic (mat-forming) habit (Komarek et al., 1993) were found by the Dundee laboratory to be acutely hepatotoxic (see

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Table 1
$^{1}H^{a}$- and $^{13}C^{b}$-NMR data for [L-Har^{2}]nodularin in MeOH-d_4

^{1}H-NMR			^{13}C-NMR	
Proton	Multiplicity[c]	δ (J in Hz)	Carbon	δ (ppm)
[Masp]			[Masp]	
H-2	*d*	4.13 (3.0)	C-1	177.1
H-3	*m*		C-2	60.5
H-5	*d*	1.16 (7.0)	C-3	41.2
			C-4	178.3
			C-5	16.6
[Har]			[Har]	
H-2	*dd*	4.37 (10.4, 4.0)	C-1	177.4
H-3	*m*	1.35	C-2	52.2
	m	2.00	C-3	31.3
H-4	*m*	1.24	C-4	23.6
	m	1.35	C-5	29.0
H-5	*m*	1.38	C-6	42.2
	m	1.51	C-7	158.7
H-6	*m*	3.09		
[Adda[d]]			[Adda[d]]	
H-2	*m*	3.03	C-1	175.7
H-3	*t*	4.49 (10.4)	C-2	42.3
H-4	*dd*	5.57 (15.5, 9.4)	C-3	56.5
H-5	*d*	6.17 (15.5)	C-4	127.3
H-7	*d*	5.34 (9.8)	C-5	138.7
H-8	*m*	2.54	C-6	134.3
H-9	*m*	3.24	C-7	136.4
H-10	*dd*	2.64 (14.0, 8.0)	C-8	37.7
	m	2.78	C-9	88.4
H-11	*d*	0.98 (6.8)	C-10	39.0
H-12	*s*	1.59	C-11	16.1
H-13	*d*	0.95 (6.8)	C-12	13.0
H-14	*s*	3.21	C-13	16.5
H-16, 20	*d*	7.14 (7.0)	C-14	58.7
H-17, 19	*dd*	7.20 (7.0, 7.3)	C-15	140.6
H-18	*t*	7.11 (7.0)	C-16,20	130.5
			C-17,19	129.2
			C-18	129.8
[Glu]			[Glu]	
H-2	*dd*	4.47 (6.4, 8.9)	C-1	177.1
H-3	*m*	1.70	C-2	55.5
	m	2.26	C-3	28.8
H-4	*m*	1.99	C-4	29.7
	m	2.73	C-5	172.2
[Mdhb[e]]			[Mdhb[e]]	
H-3	*q*	6.88 (7.0)	C-1	165.6
H-4	*d*	1.68 (7.0)	C-2	137.8
NCH_3	*s*	3.03	C-3	136.6
			C-4	13.2
			NCH_3	37.7

[a] 500 MHz.
[b] 125 MHz.
[c] *s*: singlet, *d*: doublet, *dd*: doublet of doublet, *t*: triplet, *q*: quartet, *m*: multiplet.
[d] Adda: 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid.
[e] Mdhb: methyldehydrobutyrine.

Rippka and Herdman, 1992). In a recent survey of *Nodularia* strains, PCC 7804 was found to contain a compound with UV absorption spectrum characteristic of nodularin, but with a different HPLC retention time (Bolch et al., 1999). Here we report that strain PCC 7804 produces a previously unreported nodularin variant as a major component, in addition to lesser quantities of nodularin. Chemical structure, toxicity and protein phosphatase-inhibition data are presented.

2. Results and discussion

Analysis of extracts of 10 g dry wt of *Nodularia* PCC 7804 by HPLC with photodiode array detection revealed four peaks with similar UV absorption spectra to nodularin. A minor peak (R_T 8.34 min) was seen to be nodularin when compared to purified reference nodularin by HPLC and FABMS (data not shown), whereas a more hydrophobic, larger peak (R_T 8.89 min: Nod-X) was unknown. Nod-X was recovered at a nodularin-equivalent concentration of 4.2 μg mg^{-1} dry wt, and (when included in the estimation of the total intracellular nodularin-equivalent pool concentration) accounted for 91% of total nodularin content.

Nod-X was purified by semi-preparative HPLC to give a colourless amorphous solid. In the positive HR FAB mass spectrum using glycerol as the matrix, the $[M + H]^+$ ion was observed at m/z 839.4670. From the results, the molecular formula was established as $C_{42}H_{62}O_{10}N_{18}$ (calcd. for $C_{42}H_{63}O_{10}N_{18}$: 839.4667, Δ −0.3 mmu). The spectral data (Table 1) of ^{1}H- and ^{13}C-NMR of Nod-X suggested that it was a variant of nodularin. Amino acid analysis of the hydrolysate (6 M HCl, 110°C, 24 h) indicated the presence of 1 mol each of L-Homoarginine (L-Har), D-Glutamic acid (D-Glu) and β-methylaspartic acid (Masp).

In the ^{1}H-NMR spectra of Nod-X and nodularin obtained from Sigma, the spectrum of Nod-X closely resembled that of nodularin. In the ^{1}H–^{1}H COSY and HOHAHA spectra of Nod-X, an extra two protons of CH_2 in Arg were observed at 1.24 and 1.38 ppm. Also, an extra carbon in Arg was observed at 23.6 ppm in the HSQC spectrum. From these results, Nod-X was shown to contain L-Har instead of L-Arg. The chemical shifts and close peaks of protons and carbons of Masp, Adda, D-Glu and Mdhb in the ^{1}H–^{1}H COSY, HOHAHA, HSQC and HMBC spectra were very similar to those of nodularin (Fig. 1). The sequence of Nod-X was mostly deduced by HMBC correlation from an α-H to a carbonyl carbon. β-H of Adda correlated to the carbonyl carbon of Harg. From the results, Nod-X was identified as [L-Homoarginine^{2}]nodularin ([L-Har^{2}]nodularin; Fig. 2).

[L-Har^{2}]nodularin was shown to be acutely hepato-

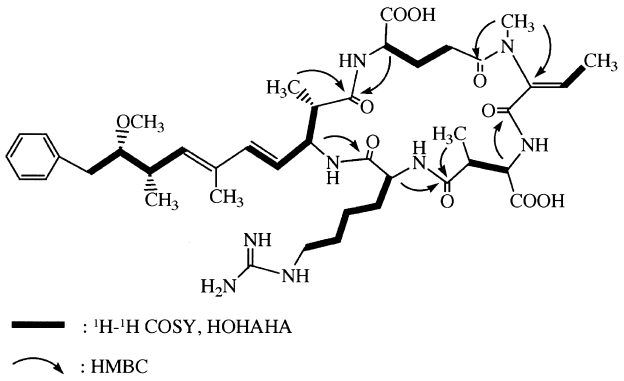

Fig. 1. ^{1}H–^{1}H COSY, HOHAHA and HMBC correlations of [L-Homoarginine^{2}]nodularin in methanol-d_4.

toxic by mouse bioassay with signs typical of nodularin and microcystin poisoning. The minimum lethal dose (intraperitoneal route) for [L-Har^{2}]nodularin was found to be 75 μg kg^{-1} body wt. This is similar to the published LD_{50}s for nodularin (60 μg kg^{-1}, Carmichael et al., 1988), [DMAdda^{3}]nodularin and [D-Asp^{1}]nodularin (150 and 75 μg kg^{-1}, respectively; Namikoshi et al., 1994).

The ability to inhibit protein phosphatase-1 (PP1) activity in a colorimetric assay was also examined. [L-Har^{2}]nodularin inhibited the enzyme with an IC_{50} of 4.5 nM. This compares to an IC_{50} of 5.0 nM for nodularin and 3.5 nM for microcystin-LR (this study), showing [L-Har^{2}]nodularin to be a potent inhibitor of PP1.

Nodularin production, with the exception of the toxin associated with the sponge *T. swinhoei*, is a common, though variable, characteristic of brackish water blooms and strains of the planktonic, gas vacuolate cyanobacterium, *N. spumigena* (Sivonen and Jones, 1999; Bolch et al., 1999). The production of [L-Har^{2}]-nodularin and nodularin by axenic, monocyanobacterial cultures of *Nodularia* PCC 7804 provides clear evidence that the extent of production of this family of potent hepatotoxic pentapeptides is not limited to planktonic populations of *N. spumigena*. Strain PCC 7804 was originally isolated from a freshwater thermal spring in France and deposited as *N. harveyana* (Rippka and Herdman, 1992), although Komarek et al. (1993) have designated this strain as *N. sphaerocarpa*. A recent genetic study of *Nodularia* strains indicates that PCC 7804 is indeed genetically distinct from *N. spumigena* but also *N. sphaerocarpa* (Bolch et al., 1999). In all other studies where nodularins have been found, nodularin has been the most abundant component. In this study, nodularin accounts for only 2% of total nodularin content, with about 90% being accounted for by [L-Har^{2}]nodularin. Nodularin has been shown to be a liver carcinogen, in addition to tumour promoter (Ohta et al., 1994). [L-Har^{2}]nodularin from *Nodularia* PCC 7804 may be useful in the further study of structure-activity relationships among the nodularins and related naturally-occurring toxins.

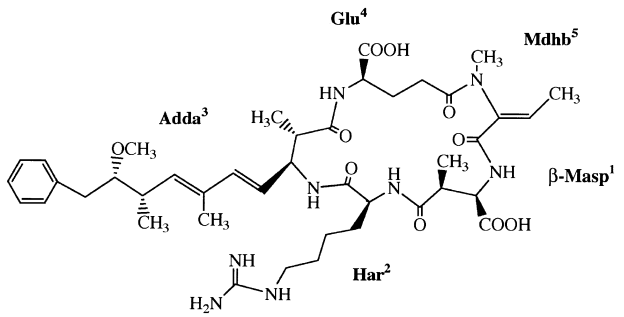

Fig. 2. Chemical structure of [L-Homoarginine^{2}]nodularin isolated from *Nodularia* PCC 7804. **β-Masp**: D-*erythro*-β-methylaspartic acid. **Har**: Homoarginine. **Adda**: (2*S*,3*S*,8*S*,9*S*)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4(*E*),6(*E*)-dienoic acid. **Glu**: Glutamic acid. **Mdhb**: *N*-methyldehydrobutyrine.

3. Experimental

3.1. Organism, culture conditions and reference toxins

Nodularia PCC 7804 was obtained from the Pasteur Culture Collection of Cyanobacterial Strains, Paris. It was grown aseptically and photoautotrophically in 8 l batch cultures of BG-11 growth medium containing nitrate (Stanier et al., 1971). Cultures were sparged with sterile air and maintained at 20–25°C under constant illumination provided by cool white fluorescent tubes at an irradiance of about 20 μmol m^{-2} s^{-1} incident on the surface of the growth vessels. Cells were harvested during stationary phase by centrifugation (4000 × *g*, 20 min), lyophilised and stored at −20°C until required. Purified nodularin was purchased from Sigma. Microcystin-LR was purified from *Microcystis* PCC 7820 (Lawton et al., 1994).

3.2. Extraction and purification

Ten grams of freeze-dried cells were extracted twice in 70% (v/v) MeOH in Milli-RO water (Millipore Corp).

Supernatants were then pooled and diluted with Milli-RO water to give a final MeOH concentration of <10% (v/v). This suspension was passed through a Whatman GF/C filter disc and the eluant applied to a pre-conditioned C-18 SPE cartridge and eluted with MeOH. The nodularin was isolated from this fraction using C-18 reversed-phase HPLC (NovaPak HR, 25 cm × 10 mm i.d., radial compressed cartridge) with a linear gradient of 20–25% MeCN in 0.1% NH_4OAc over 15 min at 5 ml min^{-1}.

3.3. NMR and FABMS Analysis

^{1}H-NMR spectra were recorded at 500 MHz, and ^{13}C-NMR spectra at 125 MHz using a JEOL JNMA-500 spectrometer. ^{1}H and ^{13}C chemical shifts are referenced to TMS. Homonuclear ^{1}H connectivities were determined by COSY and HOHAHA experiments and heteronuclear ^{1}H–^{13}C connectivities were determined by HSQC and HMBC experiments. The conditions were as follows: ^{1}H: Pulse delay (PD), 3.7 s; Pulse width (PW), 3.5 μs; Date point (DP), 32K; Frequency, 10 kHz; Scan, 200; Probe, 3 mm; Solvent volume (SV), 0.2 ml. ^{13}C: PD, 0.7 s; PW, 3.0 μs; DP, 32K; Frequency, 34 kHz; Scan, 20,000; Probe, 3 mm; SV, 0.2 ml. HMBC: F1 point, 256; F2 point, 2K; PD, 1.0 s; Scan, 380. Low and high resolution FABMS were performed on a JEOL JMS-700 spectrometer using Xe atoms (accelerating potential 8 kV) and glycerol as matrix.

3.4. Amino acid analysis

The nodularin variants were hydrolysed with 6 M HCl at 110°C for 24 h and evaporated to dryness under nitrogen. Residues were reacted with Marfey's reagents (Marfey, 1984) and liberated amino acids were analysed on a reversed-phase C-18 column (Spheri-5, 10 cm × 4.6 mm i.d.) using a linear gradient over 60 min of 10–50% MeCN in 0.05 M triethylamine-Pi (pH 3) (flow rate 2 ml min^{-1}, UV detection at 340 nm).

3.5. Colorimetric protein phosphatase (PP1) inhibition assay

The assay was essentially that of Ward et al. (1997), except that the PP1 enzyme (α-isoform, rabbit muscle, recombinant, *Escherichia coli*) was obtained from Calbiochem and 0.02 units of this was used per assay. The rate of production of *p*-nitrophenol was measured over 30 min at 410 nm at 3 min intervals using a VERSAmax plate reader with temperature control set at 30°C. Data were collected and analysed using SOFTmax PRO software. All assays were carried out in triplicate.

3.6. Toxicity assessment

Toxicity assessment was by intraperitoneal mouse bioassay (Namikoshi et al., 1994).

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