# Nodularin-Har: A New Nodularin from *Nodularia*

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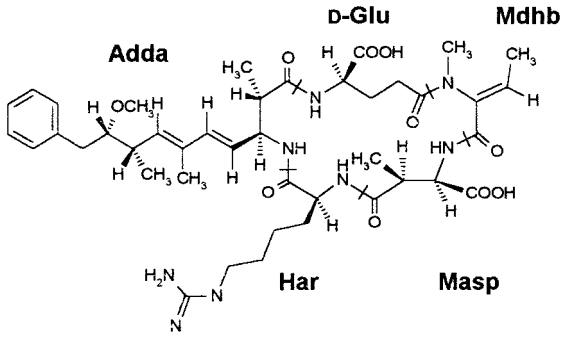
A cyanobacterial hepatotoxin, nodularin-Har, having a homoarginine instead of an arginine in nodularin, was isolated from *Nodularia* PCC7804. The structure was elucidated as **1** on the basis of 2D NMR and FABMS. The $LD_{50}$ (mouse ip) value of **1** was 70 $\mu$g/kg.

Cyanobacterial hepatotoxins, microcystins and nodularins, occur throughout the world. These toxins show potent hepatotoxicity for vertebrates and are also toxic in many other organisms.[1] The toxicity is associated with the inhibition of protein phosphatases 1 and 2A in the same manner as for okadaic acid. Microcystins and nodularins affect hepatocytes, resulting in the destruction of hepatocytoskelton and in the promotion of liver cancer.[2] Microcystins, with more than 60 homologues reported to date, are produced mainly by the genera *Microcystis, Anabaena,* and *Oscillatoria,*[3] whereas nodularins are produced by *Nodularia*.[4] In addition, motuporin ([Val$^2$]nodularin), which has a structure similar to nodularin, was isolated from marine sponge.[5] The cyanobacterial species producing microcystins and nodularins often form blooms, and these toxins consequently cause problems in eutrophic lakes and marine environments. The toxins cause death not only of wild and domestic animals, but also of humans.[6] It is, furthermore, suggested that endemic primary liver cancer in China might be caused by microcystin.[7] Taking these reasons into consideration, the World Health Organization (WHO) has established guidelines for safe levels of cyanobacterial toxins in managing recreational and potable water. Study of these toxins is, thus, essential for preserving human health.

One substance in *Nodularia* PCC7804 (Pasteur Culture Collection, aka *Nodularia harveyana*, *Nodularia sphaerocarpa*) isolated from a thermal spring in France showed hepatotoxicity to mice on ip injection ($LD_{50}$ = 70 $\mu$g/kg), and caused increase in liver weight and intrahepatic hemorrhages. The UV $\lambda_{max}$ of the substance and nodularin occurred at 236 nm, and their UV spectra completely accorded, although the retention time was delayed by 1 min compared to nodularin on reversed-phase HPLC/PDA. The result clearly shows the existence of a conjugated double bond(s) in the substance. From these observations, the substance was considered to be homologous to nodularin with the Adda and Mdhb groups conserved. In this study, the structure of **1** was determined as a new nodularin having homoarginine instead of arginine, and is referred to as nodularin-Har.[8]

A pseudo-molecular ion at 837.4529 ($\Delta$ 1.9 mmu) in the negative HRFABMS indicated that nodularin-Har had the molecular formula $C_{42}H_{62}N_8O_{10}$. This result shows that




**1**

nodularin-Har contains either an additional methyl or methylene group compared with nodularin. The $^1$H NMR spectrum of nodularin-Har showed no additional methyl signal when compared to nodularin.[9] Nodularin-Har was, therefore, assumed to contain an additional methylene. 2D NMR spectra, including H−H COSY, H−H TOCSY, HSQC, and HMBC of nodularin-Har, indicated it to be similar in structure to nodularin except for the arginine side chain. H−H COSY and HMBC spectra showed an additional methylene resonance of two protons and a carbon observed at $\delta_H$ 1.57 and $\delta_C$ 29.0, respectively, in the arginine residue (Figure 1, Table 1). This result was also confirmed by H−H TOCSY. Furthermore, the positive FABMS showed a fragment ion from a homoarginine residue at *m*/*z* 84 instead of *m*/*z* 70 from an arginine residue (Figure 2). From these results, the arginine in nodularin was replaced with a homoarginine in nodularin-Har.

The specific rotation of nodularin-Har was $[\alpha]^{27}_D$ −81.9° in MeOH, which was almost the same as that of nodularin (−86.1°),[9] suggesting that the absolute configuration of nodularin-Har would be the same as nodularin. This was corroborated by identical hepatotoxicity in mice for nodularin[4] and nodularin-Har. It is well accepted that changes in the configuration result in inactivation of their bioactivity in microcystins and nodularins. It is also reported that an amino acid group close to Adda is related to the toxicity in microcystins.[10] The change from arginine to homoarginine does not influence the toxicity.

Until now the only known nodularin producer was *Nodularia spumigena*. It is interesting that substitution of homoarginine for arginine occurs in nodularin-Har and that nodularin is not detected in the PCC7804, which is not identical to *N. spumigena*. The mode of its biosynthesis will be an aim of the future.

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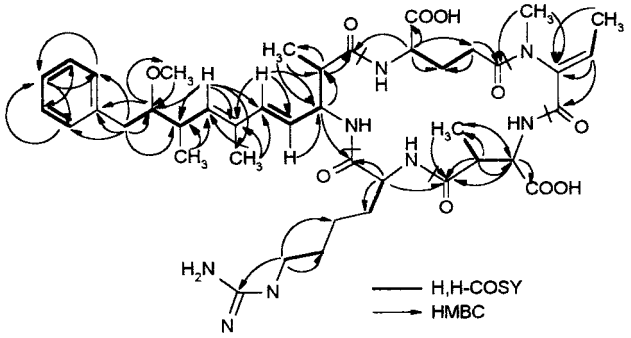


**Figure 1.** H−H COSY and HMBC correlations of nodularin-Har.

**Table 1.** $^1$H and $^{13}$C NMR Spectral Data for Nodularin-Har

| position | | $^1$H [a] ($J$ in Hz) | $^{13}$C [b] | HMBC correlations (from $^1$H to $^{13}$C) |
|---|---|---|---|---|
| Masp | 1 | | 176.6 | |
| | 2 | 2.98 (m, 2.4) | 40.0 | Masp 1, 3, 5 |
| | 3 | 4.38 (d, 2.4) | 57.9 | Masp 1, 4, 5 |
| | 4 | | 176.3 | |
| | 5 | 1.29 (d, 6.8) | 16.7 | Masp 1, 2 |
| Har | 1 | | 171.7 | |
| | 2 | 4.44 (dd, 10.3, 3.9) | 52.5 | Har 1, 3; Masp 1 |
| | 3 | 1.43 (m) | 31.2 | |
| | | 2.08 (m) | | |
| | 4 | 1.32 (m) | 24.0 | |
| | | 1.59 (m) | | |
| | 5 | 1.57 (m, 7.1) | 29.0 | |
| | 6 | 3.17 (m, 7.1) | 42.4 | Har 4, 5, 7 |
| | 7 | | 158.6 | |
| Adda | 1 | | 176.1 | |
| | 2 | 2.73 (m, 10.7, 6.8) | 45.3 | Adda 1, 3, 17 |
| | 3 | 4.55 (dd, 10.7, 9.3) | 56.3 | Adda 2, 5; Har 1 |
| | 4 | 5.46 (dd, 15.6, 9.3) | 126.2 | Adda 3 |
| | 5 | 6.25 (d, 15.6) | 139.6 | Adda 3, 4, 6 |
| | 6 | | 133.9 | |
| | 7 | 5.42 (d, 9.8) | 137.3 | Adda 5, 6, 8, 9, 18 |
| | 8 | 2.16 (m, 9.8, 7.0, 6.6) | 37.9 | Adda 19 |
| | 9 | 3.18 (m, 7.0, 6.8, 4.8) | 88.4 | Adda 11, 20 |
| | 10 | 2.68 (dd, 14.1, 6.8) | 39.1 | Adda 8, 9, 11,12, 16 |
| | | 2.81 (dd, 14.1, 4.8) | | |
| | 11 | | 140.6 | |
| | 12, 16 | 7.18 (m, 6.8) | 130.6 | Adda 14 |
| | 13, 15 | 7.24 (m, 7.3) | 129.2 | Adda 13, 15 |
| | 14 | 7.16 (m, 7.3) | 127.1 | Adda 12, 16 |
| | 17 | 1.05 (d, 6.8) | 16.6 | Adda 2, 3 |
| | 18 | 0.98 (d, 1.2) | 13.1 | Adda 5, 6, 7 |
| | 19 | 1.60 (d, 6.8) | 16.7 | Adda 7, 8 |
| | 20 | 3.24 (s) | 58.9 | Adda 9 |
| Glu | 1 | | 174.5 | |
| | 2 | 4.70 (dd) | 52.1 | Glu 1, 3, 4; Adda 1 |
| | 3 | 2.07 (m) | 28.0 | |
| | | 2.16 (m) | | |
| | 4 | 2.04 (m) | 29.5 | Glu 3, 5 |
| | | 2.49 (m) | | |
| | 5 | | 174.3 | |
| Mdhb | 1 | | 165.6 | |
| | 2 | | 137.1 | |
| | 3 | 7.00 (q, 7.1) | 138.1 | Mdhb 1, 2 |
| | 4 | 1.76 (d, 7.1) | 13.5 | Mdhb 1, 2 |
| | N-Me | 3.11 (s) | 35.2 | Mdhb 2; Glu 1 |

[a] 500 MHz; $CD_3OD$ ($\delta$ 3.30). [b] 125 MHz; $CD_3OD$ ($\delta$ 49.3).

## Experimental Section

**General Methods.** HPLC system consisted of a Waters 600E solvent-delivery system, a model 996 photodiode array detector, and a model 717 autosampler. HPLC separation was achieved using the method developed by Lawton et al. (1994).[11] A column temperature of 30 °C and a Waters Symmetry $C_{18}$ column (250 × 4.6 mm i.d., 5-$\mu$m pore size) was used for separation. $^1$H and $^{13}$C NMR spectra were measured and recorded on a Varian Unity 500 in $CD_3OD$. The resonance of $CD_3OD$ at $\delta_H$ 3.30 ppm and $\delta_C$ 49.3 ppm were used as internal standards for NMR spectra. FABMS were recorded on a JEOL JMS-SX-102. The optical rotation was determined on a Horiba SEPA-300 polarimeter at ambient temperature. Preparative nodularin was purchased from Wako Pure Chemical Industries.

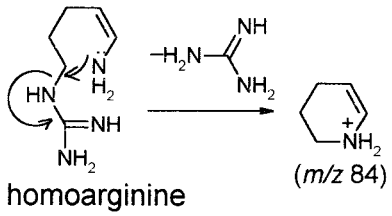


**Figure 2.** Fragmentation scheme of an ion with homoarginine on the positive FABMS.

**Culture Conditions.** *Nodularia* PCC7804 was obtained from the Pasteur Culture Collection (Pasteur Institute, Paris, France). The strain was grown in modified BG-11 medium [$NaNO_3$ 17.65 mM, $K_2HPO_4$ 0.18 mM, $MgSO_4 \cdot 7H_2O$ 0.30 mM, $CaCl_2 \cdot 2H_2O$ 0.25 mM, citric acid$\cdot H_2O$ 0.03 mM, ammonium iron citrate 0.03 mM, EDTA disodium 0.003 mM, and A5+Co trace metal mixture[12] 1 mL/L, pH 8.0 with NaOH before autoclaving] in 19-L bottles with bubbling air at 24 °C under continuous illumination with fluorescence lamps of 120 $\mu E \cdot s^{-1} \cdot m^{-2}$ on a surface of the bottle. Cells were grown for three weeks, harvested by centrifugation at 10 000 *g*, lyophilized, and stored at −30 °C until used.

**Isolation.** Lyophilized cells (13.2 g) were extracted twice with 500 mL of 70% aqueous MeOH−0.1% TFA by stirring for 12 h. The crude extracts were centrifuged at 12 000 *g*, for 10 min, at 4 °C. The supernatants were combined and filtered through a Whatman GF/C glass filter. The filtrate was rotary vacuum evaporated at 40 °C to dryness. The residue was suspended in MeOH, filtered, evaporated, and then dissolved in 10 mL of MeOH, diluted with 180 mL of $H_2O$, filtered with a GF/C filter, and rotary evaporated. The extract was chromatographed with a TSK gel DEAE-Toyopearl 650M column (18 × 4.0 cm i.d.) with linear gradient from 0.05 M MES−KOH (pH 5.5) to 0.05 M $NH_4HCO_3$−0.05 M MES−KOH (pH 5.5). The absorbance of eluate was monitored at 238 nm, and the fractions containing **1** were passed through a Waters Sep-Pak Vac $C_{18}$ cartridge (500 mg). The cartridge was successively rinsed with $H_2O$ and 30% (v/v) aqueous MeOH, and then eluted with 40, 50, and 60% MeOH. The eluate was pooled and evaporated. The residue was dissolved in 20 mL of 0.1 M NaCl−0.05 M MOPS−KOH (pH 7.0), applied to a TSK gel Toyopearl HW-40F gel filtration column (25 × 4.0 cm i.d.), and eluted with the same solvent. Fractions containing **1** were collected and desalted with a Sep-Pak Vac $C_{18}$ cartridge. Crystalline and colorless nodularin-Har (15.5 mg) was finally obtained with 97% purity. The UV spectrum was measured in HPLC/PDA (in aqueous MeCN), $\lambda_{max}$ 236 nm. Approximately 1.5 mg of nodularin-Har was analyzed: $[\alpha]^{27}_D$ −81.9° (*c* 0.12, MeOH); HRFABMS *m*/*z* 837.4529 [M − H]$^-$, calcd for $C_{42}H_{61}N_8O_{10}$, 837.4511 (+1.9).

**Mouse Bioassay.** Twenty ddy male mice (20 g) were injected intraperitoneally with nodularin-Har. Dead mice were dissected, and their liver weight compared with that of the control.

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(12) Composition of A5+Co mixture: $H_3BO_3$ 2.86 g, $MnCl_2 \cdot 4H_2O$ 1.18 g, $ZnSO_4 \cdot 7H_2O$ 0.222 g, $Na_2MoO_4 \cdot 2H_2O$ 0.390 g, $CuSO_4 \cdot 5H_2O$ 0.079 g, $Co(NO_3)_2 \cdot 6H_2O$ 0.0494 g, $H_2O$ to make 1 L.

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