Neuritogenic Activity-Guided Isolation of a Free Base Form Manzamine A from a Marine Sponge, *Acanthostrongylophora* aff. *ingens* (Thiele, 1899)

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Two manzamine-class alkaloids, manzamine A (1) and 8-hydroxymanzamine (2) were isolated from a Japanese marine sponge *Acanthostrongylophora* aff. *ingens*, together with three known alkaloids manzamine E (3), manzamine F (4), and manzamine X (5). The spectral features of 1 and 2 were different from the reported data. Detailed structure analysis using 2D NMR revealed the structure of 1 and 2 as a free base form of hydrochloric salt. These manzamine-class alkaloids showed neuritogenic activity against Neuro 2a cells.

Key words marine sponge; *Acanthostrongylophora ingens*; manzamine-class alkaloid; free base form; neuritogenic activity; Neuro 2a

Manzamines have been reported to show a number of significant biological activities including insecticidal,[1] cytotoxicity,[2] antibacterial,[3] anti-HIV-I,[4] anti-infective,[5] anti-Alzheimer diseases,[6] and anti-malarial activity.[7] Manzamine A·HCl (**6**) was first isolated from the marine sponge *Haliclona* sp. as an antitumor alkaloid by Higa's group.[2] Histrorically, manzamine A was also isolated from a marine sponge, *Pellina* sp. as an antimicrobial alkaloid, and named keramamine-A by Nakamura's group.[3] Manzamine A and keramamine-A were also isolated as a hydrochloric salt, and these structures were determined by X-ray analysis. In our ongoing research for neuronal differentiation inducers from marine invertebrates,[8] we isolated two manzamine-class alkaloids manzamine A (**1**) and 8-hydroxymanzamine A (**2**)[9] as free base forms, together with three known manzamine alkaloids manzamine E (**3**),[10] manzamine F (**4**),[10] and manzamine X (**5**)[11] from an Okinawan marine sponge *Acanthostrongylophora* aff. *ingens*. ^{1}H- and ^{13}C-NMR spectra of **1** and **2** were quite different from the reported data. We investigated their structure determination by using 2D-NMR extensively. This paper reports the isolation, structural elucidation and neuritogenic activity against mouse neurobrastoma cell lines (Neuro 2a) of these manzamine-class alkaloids.

The Et_2O soluble fraction obtained from the EtOH extract of a Japanese marine sponge, *A. ingens* collected at Hedo Peninsula, Okinawa showed neuritogenic activity against Neuro 2a. Bioassay guided separation of the active fraction by Sephadex LH-20 and silica gel chromatography to give two active alkaloids manzamine A (**1**) and 8-hydroxymanzamine A (**2**) together with three known alkaloids manzamine E (**3**), manzamine F (**4**), and manzamine X (**5**) (Chart 1).

Manzamine A (**1**) was obtained as a yellow solid, positive to Dragendorff reagent. The EI-MS showed a molecular ion peak at *m*/*z* 548, and the molecular formula was determined as $C_{36}H_{44}N_4O$ $[M+H]^+$ from the HR-ESI-MS. The UV spectral showed characteristic absorbance of the β-carboline chromophore at 219, 237, 279, 347, and 357 nm. On the basis of 1D-NMR and COSY, TOCSY, HSQC, HMBC, and HSQC-TOCSY spectral data, **1** consists of a β-carboline substituent, two six-membered rings, a 13-membered macrocycle consisting of a chain of nine carbon atoms bridging two six-membered rings, and an eight-numbered ring. It has been suggested that **1** has the same planar structure as manzamine A·HCl (**6**) (Fig. 1). The comparison of ^{1}H- and ^{13}C-NMR chemical shifts between **1** and **6**, and the large differences at C26—C29 and C32—C34 in eight-numbered rings were ob-

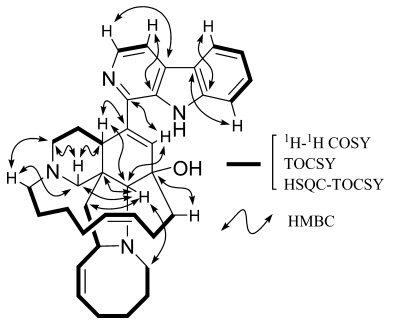

Fig. 1. ^{1}H–^{1}H COSY, TOCSY, HSQC-TOCSY and Selected HMBC Correlations of Compound **1**

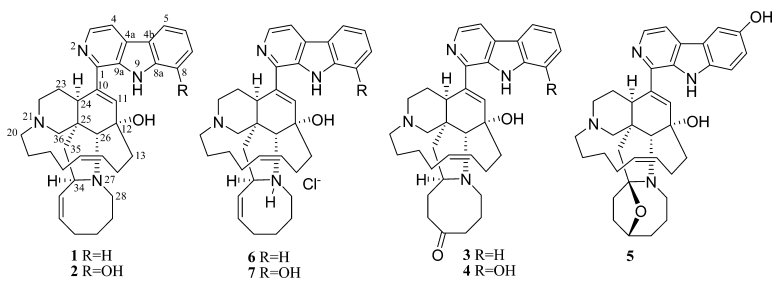

Chart 1. Structures of Compounds **1**—**7**

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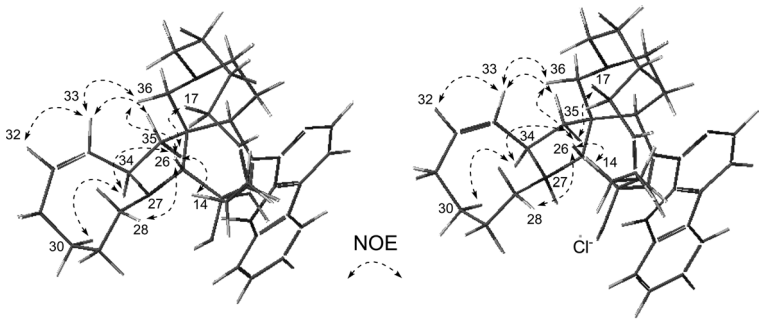
served (Tables 1, 2). Since **1** was thought to be a stereo-, or conformational isomer of **6**, the ROESY and NOESY spectral data of **1** and **6** were analyzed in depth. In the NOESY spectrum of **6**, characteristic NOE correlations were observed from H-34 (δ_H 4.94) to Hα-30 (δ_H 1.95) and Hα-35 (δ_H 2.40), and from H-26 (δ_H 3.72) to H-14 (δ_H 2.15), H-17 (δ_H 1.60, 2.50), H-28 (δ_H 3.27, 4.03), and H-36 (δ_H 2.88), and from H-33 (δ_H 5.39) to H-32 (δ_H 6.29), Hβ-35 (δ_H 1.85), and H-36 (δ_H 2.88). These NOE correlations indicated that **6** has the same conformation as proposed by X-ray crystallographic analysis. On the other hand, characteristic NOE correlations were observed from H-34 (δ_H 4.18) to Hα-30 (δ_H 1.88) and Hα-35 (δ_H 2.29), and from H-26 (δ_H 3.50) to H-14 (δ_H 2.37), H-17 (δ_H 2.53), H-28 (δ_H 3.09), and H-36 (δ_H 2.80), and from H-33 (δ_H 5.27) to H-32 (δ_H 5.91), H-35β (δ_H 1.73) and H-36 (δ_H 2.80) in the NOESY spectrum of **1**. These NOE data suggest that **1** has the same configuration as that of **6**, and their conformation is superimposable as shown in Fig. 2. In the ^{1}H-NMR spectrum of **6**, the H-26 (δ_H 3.72, d, J=6.0 Hz) proton signal was observed as doublet and coupled to H-27 (δ_H 10.62, br s), which was confirmed by the COSY spectrum. While in the ^{1}H-NMR spectrum of **1**, the H-26 proton signal was observed as a singlet, and was not coupled to any other proton signals. These data suggest that the structure of **1** was a free base form of manzamine A.

8-Hydroxymanzamine A (**2**) is obtained as a pale yellow powder. The molecular formula $C_{36}H_{44}N_4O_2$ was deduced by HR-ESI-MS, and an additional oxygen atom by comparison

Table 1. ^{1}H-NMR Spectral Data of Compounds **1**,[a] **2**,[b] **6**[a] and **7**[c] (in $CDCl_3$)

Position	**1**	**6**	**2**	**7**
3	8.42 (d, 5.0)	8.34 (d, 5.2)	8.37 (d, 5.0)	8.33 (d, 5.1)
4a	7.81 (d, 5.0)	7.85 (d, 5.1)	7.77 (d, 5.0)	7.83 (d, 5.1)
5	8.08 (d, 8.0)	8.08 (d, 7.9)	7.56 (d, 7.5)	7.62 (d, 7.2)
6	7.26 (t, 8.0)	7.23 (t, 7.9)	7.04 (t, 7.0)	7.15 (t, 7.5)
7	7.52 (d, 8.0)	7.52 (t, 7.9)	6.95 (d, 7.5)	7.09 (dd, 0.9, 7.5)
8	7.52 (d, 8.0)	7.83 (d, 7.9)	—	—
9	8.57 (br s)	11.75 (br)	9.48 (br)	11.50 (s)
11	6.38 (s)	6.52 (s)	6.54 (s)	6.46 (s)
13	1.78 (m), 2.02 (m)	1.75(m), 2.15 (m)	1.80 (m), 2.07 (m)	1.80 (m), 2.06 (m)
14	2.12 (m), 2.37 (m)	2.15 (2H, m)	2.12 (m), 2.32 (m)	2.23 (2H, m)
15	5.63 (m)	5.57 (m)	5.56 (m)	5.59 (m)
16	5.52 (m)	5.57 (m)	5.49 (m)	5.59 (m)
17	1.72 (m), 2.53 (m)	1.60 (m), 2.50 (m)	1.73 (m), 2.49 (m)	1.64 (m), 2.49 (m)
18	1.40 (m), 1.72 (m)	1.20 (m), 1.45 (m)	1.40 (m), 1.75 (m)	1.23 (m), 1.54 (m)
19	1.72 (m), 1.88 (m)	1.45 (m), 1.81 (m)	1.73 (m), 1.82 (m)	1.23 (m), 1.54 (m)
20	2.42 (m), 2.62 (m)	2.38 (m), 2.58 (m)	2.40 (m), 2.58 (m)	2.43 (m), 2.62 (m)
22	1.95 (m), 2.75 (m)	1.88 (m), 2.93 (m)	1.98 (m), 2.80 (m)	1.88 (m), 2.97 (m)
23	1.60 (m), 1.98 (m)	1.78 (m), 2.95 (m)	1.59 (m), 2.20 (m)	1.86 (m), 2.97 (m)
24	3.08 (m)	2.55 (m)	3.03 (m)	2.53 (m)
26	3.50 (s)	3.72 (d, 6.0)	3.59 (s)	3.77 (d, 6.9)
27	—	10.62 (br s)	—	9.80 (br s)
28	3.09 (2H, m)	3.27 (m), 4.03 (m)	3.15 (2H, m)	3.32 (m), 4.03 (m)
29	1.60 (m), 1.98 (m)	2.00 (m), 2.60 (m)	1.62 (m), 1.92 (m)	2.30 (m), 2.50 (m)
30	1.40 (m), 1.88 (m)	1.45 (m), 1.95 (m)	1.40 (m), 1.85 (m)	1.54 (m), 2.03 (m)
31	2.12 (m), 2.26 (m)	2.30 (2H, m)	2.12 (m), 2.22 (m)	2.35 (2H, m)
32	5.91 (m)	6.29 (m)	5.95 (m)	5.23 (s)
33	5.27 (t, 9.0)	5.39 (t, 9.9)	5.27 (t, 9.6)	5.42 (t, 9.8)
34	4.18 (br s)	4.94 (m)	4.28 (br s)	4.98 (br q, 6.9)
35	1.73 (m), 2.29 (m)	1.85 (m), 2.40 (m)	1.72 (m), 2.30 (m)	1.92 (m), 2.39 (m)
36	2.31 (m), 2.80 (m)	2.32 (m), 2.88 (m)	2.32 (m), 2.80 (m)	2.40 (m), 2.92 (m)

a) 150 MHz, *b*) 125 MHz, *c*) ref. 9.



Free Base Form of Manzamine A (**1**) Hydrochloric Salt of Manzamine A (**6**)

Fig. 2. Stereochemistry of Compounds **1** and **6**, and Selected NOE Correlations

Structures were refined by performing an optimized geometry calculation in MOPAC using PM3 (CAChe) with a crystal structure of **6** as an initial conformation.

Table 2. ^{13}C-NMR Spectral Data of Compounds **1**,[a] **2**,[b] **6**[a] and **7**[c] (in $CDCl_3$)

Position	1	6	2	7
1	143.3 (s)	143.7 (s)	143.1 (s)	143.3 (s)
3	138.7 (d)	137.6 (d)	138.1 (d)	137.9 (d)
4	113.3 (d)	113.9 (d)	113.9 (d)	114.7 (d)
4a	129.3 (s)	129.5 (s)	130.0 (s)	129.8 (s)
5	121.5 (d)	121.0 (d)	112.6 (d)	112.6 (d)
6	120.1 (d)	119.3 (d)	120.7 (d)	120.7 (d)
7	128.5 (d)	128.1 (d)	113.0 (d)	114.3 (d)
8	111.6 (d)	112.9 (d)	143.6 (s)	143.8 (s)
8a	140.0 (d)	141.6 (d)	130.7 (d)	130.6 (d)
9a	133.5 (s)	133.4 (s)	133.3 (s)	132.9 (s)
10	139.9 (s)	141.3 (s)	140.5 (s)	141.9 (s)
11	137.5 (d)	135.2 (d)	135.9 (d)	134.6 (d)
12	70.0 (s)	71.3 (d)	70.4 (d)	71.2 (d)
13	40.9 (t)	39.2 (t)	42.8 (t)	39.2 (t)
14	21.7 (t)	20.8 (t)	21.6 (t)	20.7 (t)
15	128.3 (d)	127.0 (d)	128.3 (d)	126.7 (d)
16	132.3 (d)	132.9 (d)	132.2 (d)	133.0 (d)
17	26.0 (t)	25.0 (t)	25.9 (t)	24.7 (t)
18	26.8 (t)	26.5 (t)	26.8 (t)	26.5 (t)
19	25.7 (t)	24.6 (t)	25.7 (t)	24.5 (t)
20	53.5 (t)	53.6 (t)	53.6 (t)	53.4 (t)
22	49.6 (t)	49.4 (t)	49.7 (t)	49.2 (t)
23	32.7 (t)	33.6 (t)	32.9 (t)	33.4 (t)
24	40.2 (d)	41.2 (d)	40.6 (d)	41.3 (d)
25	47.1 (s)	47.1 (s)	47.1 (s)	47.1 (s)
26	75.2 (d)	78.2 (d)	75.4 (d)	78.3 (d)
28	50.8 (t)	53.5 (t)	51.3 (t)	53.7 (t)
29	31.7 (t)	26.4 (t)	30.9 (t)	26.4 (t)
30	25.7 (t)	24.4 (t)	25.3 (t)	24.2 (t)
31	28.1 (t)	28.5 (t)	28.1 (t)	28.4 (t)
32	134.6 (d)	142.4 (d)	135.9 (d)	142.8 (d)
33	129.9 (d)	123.8 (d)	127.5 (d)	123.3 (d)
34	55.0 (d)	57.2 (d)	55.5 (d)	57.4 (d)
35	44.7 (t)	44.8 (t)	44.6 (t)	44.8 (t)
36	68.6 (t)	70.6 (t)	68.5 (t)	70.2 (t)

a) 150 MHz, *b*) 125 MHz, *c*) ref. 9.

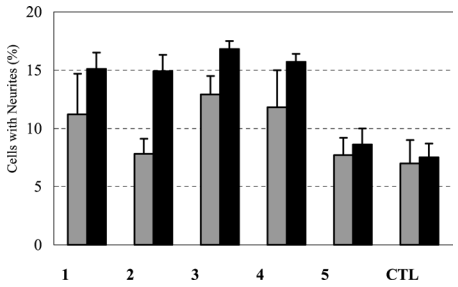


Fig. 3. Neuritogenic Effects of Compounds **1**, **2**, **3**, **4** and **5** against Neuro 2a

Gray bars: treatment for 48 h at 1.0 μM, black bars: treatment for 72 h at 1.0 μM. Each column represents mean±S.D. (n=4).

to **1** in the molecular formula of **2** pointed toward a phenolic hydroxyl. ^{1}H- and ^{13}C-NMR spectral data of **2** were differed from **1** only in the carbocyclic ring of the β-carboline moiety, and the NMR chemical shifts of this moiety were a good agreement of 8-hydroxymanzamine A·HCl (**7**) as shown in Tables 1 and 2. Accordingly, compound **2** was identified as a free base form of 8-hydroxymanzamine A.

Compounds (**1**—**5**) were tested for evaluation of their ability to induce neurite outgrowth against Neuro 2a at the concentration of 1.0 μM for 48 and 72 h treatments. The results are shown in Fig. 3. After 48 h treatment, compounds **1**, **3**, and **4** showed moderate neuritogenic activity, and after 72 h, almost all compounds except for **5** showed the activity about two times stronger than that of the control. Their cytotoxic effect against Neuro 2a and human acute promyelotic cells (HL-60) was also evaluated, and the results are shown in Table 3. Compounds **1**, **2**, and **3** showed cytotoxicity against both cell lines, and their IC_{50} values were under 10 μM.

Table 3. Inhibitory Effects of Compounds **1**, **2**, **3**, **4**, and **5** on the Proliferation of Neuro 2a and HL-60

Compounds	IC_{50} (μM) Neuro 2a	IC_{50} (μM) HL-60
1	3.3	4.2
2	3.2	3.0
3	5.7	8.9
4	>15	>10
5	>15	>10

To our knowledge, this is the first report about the isolation of a free base form manzamine A. The NMR chemical shifts of free base forms and hydrochloric salts were quite different, and the NMR chemical shifts of free base form manzamines were similar to manzamine B[12] and ircinal B,[13] which lacked the C–N bond reductively common to the 5- and 8-membered rings. We used the eluting solvent containing 0.5% diethylamine for the final separation, so that it is speculated that the hydrochloric salt is converted to a free base form. A free base form manzamine A was easily converted to hydrochloric salt in HCl containing solvent.

Treatment of manzamine A displays a predominantly bipolar morphology against Neuro 2a in the same way as lactacystin-induced neurite outgrowth.[14,15] Lactacystin enhances the intracellular cAMP level transiently, and inhibits cell cycle progression. Furthermore, an analog of manzamine alkaloids delayed cell cycle progression at mitosis,[16] so the neuritogenic activity of manzamines may be caused by the same mechanism of lactacystin.

Experimental

UV spectra were recorded on a JASCO U-best 30 spectrometer. IR spectra were recorded on a JASCO FT/IR-410 spectrometer. ^{1}H- and ^{13}C-NMR spectra were recorded on an Varian INOVA 600 (600 MHz) spectrometer. The chemical shifts are reported in parts per million from TMS as an internal standard. HR-ESI-MS data were obtained using a Micromass Q-TOF Ultima LCMS spectrometer. EI-MS data were obtained using a Shimadzu GCMS-QP5050A. Optical rotations were recorded on a JASCO DIP-370 digital polarimeter.

Animal Material The marine sponge *A. ingens* was collected in 2000, at depths of 15 m off Hedo Peninsula, Okinawa Japan. A voucher specimen (ZMAPOR. 19867) is deposited at the Institute for Biodiversity and Ecosystem Dynamics, University of Amsterdam, Netherlands.

Extraction and Isolation Wet specimens (0.775 kg) were macerated in a blender and extracted three times with EtOH (1 l). The EtOH extract was evaporated under reduced pressure, and the resulting aqueous suspension was diluted with H_2O (0.5 l) and extracted with Et_2O (1 l×3). The Et_2O-soluble extract (2.744 g) was subjected to Sephadex LH-20 with $CHCl_3$/MeOH (1 : 1) to give six fractions (Fr. 1—Fr. 5). The active Fr. 3 (475.9 mg) was chromatographed on Silica gel 60 with *n*-hexane/isopropanol/diethylamine (20 : 1 : 0.1) and purified by Sephadex LH-20 with MeOH to give compounds **1** (107.3 mg), **2** (23.9 mg) and **3** (14.8 mg). The Fr. 4 (520.7 mg) was purified in the same procedure as Fr. 3 to give compounds **4** (8.1 mg) and **5** (6.6 mg).

Manzamine A (**1**): Pale yellow amorphous powder. $[\alpha]_D^{25}$ +54.6° (c=0.48, $CHCl_3$). IR ($CHCl_3$) cm^{-1}: 3033, 2931, 1632, 1555, 1454, 1221. UV λ_{max} (MeOH) nm (log ε): 219 (4.38), 237 (4.3), 279 (4.1), 347 (3.78), 357 (3.79). ^{1}H-NMR (600 MHz, $CDCl_3$) and ^{13}C-NMR (150 MHz, $CDCl_3$):

see Tables 1, 2. HR-ESI-TOF-MS: *m/z*: 549.3604 $[M+H]^+$ (Calcd for $C_{36}H_{44}N_4O$: 549.3593). EI-MS: *m/z* 548, 530, 438, 162 (base peak).

8-Hydroxymanzamine A (**2**): Pale yellow amorphous powder. $[\alpha]_D^{25}$ +123.3° (*c*=1.33, $CHCl_3$). IR ($CHCl_3$) cm^{-1}: 3200, 2920, 1580, 1560, 1500, 1420; 1360, 1280. UV λ_{max} (MeOH) nm (log ε): 221 (4.5), 244 (4.43), 264 (4.13), 359 (3.93). ^{1}H-NMR (600 MHz, $CDCl_3$) and ^{13}C-NMR (150 MHz, $CDCl_3$): see Tables 1, 2. HR-ESI-TOF-MS: *m/z*: 565.3566. $[M+H]^+$ (Calcd for $C_{36}H_{44}N_4O_2$: 565.3542). EI-MS: *m/z* 564, 546, 424, 162 (base peak).

Neuritogenic Activity Assay Neuro 2a cells were grown in Dulbecco's modified essential medium (DMEM) with 10% FBS. The cells were kept in an incubator at 37 °C with 5% CO_2. The cells were plated on 24-well plates at a density of 1×10^4/ml per well. Testing samples of 50 μl of an ethanol solution were added to each well. After 48 and 72 h incubation, morphological changes in the cells were observed under a phase contrast microscope. The cells processed longer than the diameter of the cell body were evaluated as neurite-bearing cells. The percentage of the cells with neurites in a particular culture was determined by counting 100 cells at least in the photomicrographs of the areas where the cell density was representative.

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