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Cell differentiation inducers from a marine sponge *Biemna* sp.

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ABSTRACT

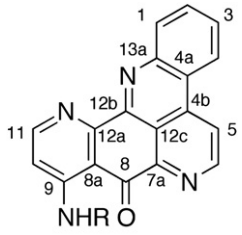
Two pyridoacridines closely related to the known isocystodamine were isolated from the marine sponge *Biemna* sp. together with isocystodamine. Their structures were determined on the basis of spectroscopic data. They induce the erythroid differentiation of human leukemia K562 cells with an ED_{50} value of 5 nM each.

1. Introduction

In spite of the fact that the precise mechanism that leads to the under-differentiated states in most of cancer cells has not been clarified, cell-differentiation inducers are considered as potential anticancer agents,[1] because they can convert cancer cells into less malignant cells as demonstrated by the success of retinoids to treat acute promyelocytic leukemia.[1] Against this background we have launched a program to discover compounds that induce erythroid differentiation of human leukemia K562 cells, expecting to find a wide variety of biologically active compounds including HDAC inhibitors.[2,3] As a result, we have isolated *N*-methylisocystodamine (**2**) and *N*-methoxymethylisocystodamine (**3**) together with the known isocystodamine (**1**)[4] from a marine sponge *Biemna* sp. collected at Oshima-Shinsone. Their structures were elucidated on the basis of NMR and MS data. This paper describes the isolation, structure elucidation, and biological activities of these compounds.

2. Results and discussion

The extract of the sponge was suspended in H_2O and extracted sequentially with $CHCl_3$ and *n*-BuOH. The *n*-BuOH layer was separated by ODS flash chromatography followed by reversed-phase HPLC to give isocystodamine (**1**), *N*-methylisocystodamine (**2**), and *N*-methoxymethylisocystodamine (**3**) (Fig. 1).



1: R = H
2: R = CH_3
3: R = CH_2OCH_3

Fig. 1. Structures of **1**–**3**.

Compound **1** was isolated as yellowish solids with the molecular formula of $C_{18}H_{11}N_4O$ as shown by HRESIMS [*m*/*z* 299.0933, $(M+H)^+$, Δ±0 mmu]. The UV absorption (λ_{max} 365, 277, 247 nm) suggested that **1** contained a highly-conjugated chromophore. The ^1H NMR data was well compared with those of isocystodamine.[4] The nomenclature of 'cystodamine' is confused, because the structure of the compound first reported as cystodamine with structure **4** was later revised by a total synthesis and careful inspection of ^1H NMR data to be identical with 11-hydroxyascidiidemin (**5**) (Fig. 2).[5–7] Therefore, a compound with structure **4** had not been discovered from nature. On the other hand **1** is a nameless compound, an isomer of **4** differing in the orientation of 4-aminopyridine ring. Compound **1** had been synthesized together with **4**,[6] and later isolated from a marine sponge *Biemna* sp.[4]

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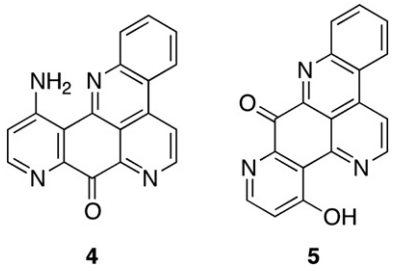
Because compound **4** was called cystodamine by those who first synthesized the compound,[6] we call **1** isocystodamine for the sake of clarity.



4 **5**

Fig. 2. Structures of **4** and **5**.

N-Methylisocystodamine (**2**) had a molecular formula of $C_{19}H_{13}N_4O$ larger than that of **1** by a CH_2 unit. The UV spectrum (λ_{max} 372, 277, 248 nm) suggested that **2** had the same chromophore as that of isocystodamine (**1**). The ^{1}H NMR spectrum contained the same set of spin systems except for one of exchangeable protons, which was replaced by an *N*-methyl group. The structural similarity of **1** and **2** was supported by analysis of ^{1}H NMR spectrum in conjunction with the HSQC data. The three partial structures each comprised of aromatic carbons were conserved judging from both ^{1}H and ^{13}C chemical shifts, and one of exchangeable protons was replaced by a methyl group (δ_H 3.08; δ_C 29.1), which was assigned as an *N*-methyl (Fig. 3). HMBC data was instrumental to confirm the three partial structures (Fig. 3). Vicinal ^{1}H–^{1}H coupling constant of 8.3 Hz observed within unit a suggested that they comprised a part of a benzene ring, which was supported by remarkable $^3J_{CH}$ (H-1/C-3, H-2/C-4, H-3/C-1, H-4/C-2) and hardly detectable $^2J_{CH}$ in the HMBC spectrum.[8] ^{13}C chemical shifts of the remaining carbons in the benzene ring were shown to be 145.0 (C-13a) and 121.6 ppm (C-4a) on the basis of HMBC cross peaks, (H-1 and H-3)/C-4a and (H-2 and H-4)/C-13a. The ^{13}C chemical shift of C-13a suggested that this carbon was connected to a nitrogen atom (Fig. 1). ^{1}H–^{1}H coupling constant of 4.8 Hz observed between H-5 and H-6 as well as the ^{13}C chemical shifts of C-5 (119.8 ppm) and C-6 (149.4 ppm) indicated that they were a part of a pyridine ring.[8] HMBC data permitted us to determine the ^{13}C chemical shifts of the remaining carbons (C-4b, C-7a, C-12c) of the pyridine ring in unit b and to connect between C-4a and C-4b. Similar arguments of vicinal ^{1}H–^{1}H coupling constant and ^{13}C chemical shifts enabled us to form the second trisubstituted pyridine ring (unit c). The chemical shift of C-9 (155.5 ppm) suggested that C-9 was substituted by a nitrogen atom, which was confirmed by an HMBC cross peak between *N*–CH_3 and C-9. Judging from the molecular formula, two carbon and one oxygen atoms remained unassigned. Because it was not possible to obtain ^{13}C NMR spectrum of **2** due to the paucity of material, there remained four candidates as the structure of **2** (Fig. 4). A similar discussion had already been conducted in the structure elucidation of calliactine and cystodamine,[7,9] whose structures were solved only by comparison of NMR data among the candidate structures prepared by chemical synthesis.[7,10] In our case we propose the structure of **2** as *N*-methyl derivative of isocystodamine because of the almost coincidence of the NMR data with **1** and the presence of **1** in this sponge.

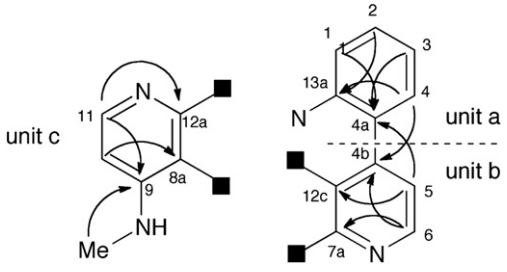


Fig. 3. HMBC correlations in **2** from protons to non-hydrogenated carbons.

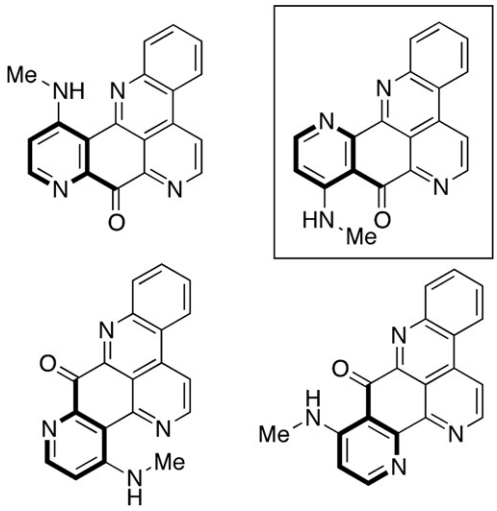


Fig. 4. Four possible structures assigned from the NMR data.

Methoxymethylisocystodamine (**3**), which exhibited a similar UV spectrum as **1** and **2**, was larger than **2** by a unit of CH_2O. The aromatic region of the ^{1}H NMR and HSQC spectra of **3** was almost identical with those of **2**, whereas there were an *O*-methyl (δ_H 3.31; δ_C 54.1) and an isolated methylene (δ_H 4.94; δ_C 73.6) carbons and the *N*-methyl carbon was absent. The above data suggested the replacement of *N*-methyl group in **2** by a methoxymethyl group, which was confirmed by HMBC cross peaks: H-14/(C-9 and C-15) and H-15/C-14. Although the signal to noise ratio was small we were able to measure the ^{13}C NMR spectrum of **3**, which allowed us to assign the chemical shifts of C-8 and C-12b to be 184.4 and 149.0 ppm, respectively.

Compounds **1**, **2**, and **3** induced cell-differentiation of K562 human leukemia cells with ED_{50} value of 5 nM each. The activity at one digit nanomolar concentrations are remarkable and the data is in agreement with the potent neuronal differentiation activity of **1**. Aoki et al. reported that substitution of the amino group by a hydroxyl group caused significant decrease in the activity.[4] Our results demonstrate that substitution of the aromatic amino group by an alkyl group is tolerated, indicating the possibility of preparing chemical probes to study the mechanisms of action of this class of molecules through C-9 nitrogen atom.

3. Experimental section

3.1. General procedures

NMR spectra were recorded on a JEOL delta 600 NMR spectrometer at 600 MHz for ^{1}H and 150 MHz for ^{13}C. ^{1}H and ^{13}C chemical shifts were referenced to the solvent peaks at δ_H 2.50 and δ_C 39.51 for DMSO-d_6. ESI mass spectra were measured on a JEOL JMS-T100LC mass spectrometer. UV spectra were recorded on a Shimadzu BioSpec-1600 spectrophotometer.

3.2. Animal material

The sponge *Biemna* sp. was collected by dredging at a depth of 150 m on a sea knoll named Oshima-Shinsone, southern Japan (28°52′N 129°33′E). The sponge was frozen after collection and

kept frozen until used. The voucher specimen was deposited at the Misaki Marine Biological Station, The University of Tokyo.

3.3. Extraction and isolation

The sample (200 g) was extracted with MeOH (3×1 L) and $CHCl_3$/MeOH (1:1) (1×1 L), and the extracts were combined and concentrated in vacuo. The residue was suspended in H_2O (200 mL) and extracted with $CHCl_3$ (3×200 mL) and *n*-BuOH (2×200 mL). The *n*-BuOH layer was concentrated and separated by ODS flash chromatography to give seven fractions (A–G). The fraction E (90% MeOH fraction) was separated by reversed-phase HPLC (Inertsil $5C_{18}$-ODS-3, 10×250 mm) with a gradient elution from 20% MeOH to 100% MeOH containing 1% AcOH to give 15 fractions (A′–O′). The active fraction F′ was further separated by reversed-phase HPLC (Phenomenex 5-Phenylhexyl, 10×250 mm) with 30% MeOH containing 1% AcOH to give 0.3 mg of isocystodamine (**1**) and 0.2 mg of *N*-methylisocystodamine (**2**). The active fraction C (50% MeOH fraction) was purified by reversed-phase HPLC (COSMOSIL $5C_{18}$-AR-II, 20×250 mm) with a gradient elution from 20% MeOH to 100% MeOH to give 0.3 mg of *N*-methoxymethylisocystodamine (**3**).

*3.3.1. N-Methylisocystodamine (**2**).* Yellow solid; UV (MeOH) 372 nm (ε 3500), 277 nm (ε 8900), 248 nm (ε 11,800); ^{1}H NMR (DMSO-d_6) and ^{13}C NMR (DMSO-d_6) data, see Table 1; HRESIMS *m*/*z* 313.1091 (calcd for $C_{19}H_{13}N_4O$, 313.1089).

Table 1
^{1}H and ^{13}C NMR data for **2** and **3** in DMSO-d_6

	2		3	
No.	^{1}H (ppm)	^{13}C (ppm)	^{1}H (ppm)	^{13}C (ppm)
1	8.38 (d, 7.6)	130.9	8.39 (d, 7.6)	130.9
2	8.04 m	131.6	8.05 (br t, 7.9)	131.6
3	7.93 (br t, 7.6)	129.0	7.94 (br t, 7.9)	129.1
4	8.98 (d, 8.3)	123.7	8.98 (d, 8.2)	123.7
4a		121.6		121.5
4b		136.7		136.7
5	9.06 (d, 4.8)	119.8	9.08 (d, 4.8)	120.0
6	9.28 (d, 4.8)	149.4	9.30 (d, 4.8)	149.5
7a		146.6		146.4
8		—		184.4
8a		112.5		113.4
9		155.5		154.7
10	7.01 (d, 4.8)	107.8	7.21 (d, 4.8)	109.3
11	8.57 (d, 4.8)	152.3	8.63 (d, 4.8)	152.5
12a		152.8		152.7
12b		—		149.0
12c		117.5		117.6
13a		145.0		145.0
NH	9.84 br m		10.33 (br t, 6.6)	
14	3.08 (br d, 4.8)	29.1	4.94 (d, 6.2)	73.6
15			3.31 s	54.1

*3.3.2. N-Methoxymethylisocystodamine (**3**).* Yellow solid; UV (MeOH) 380 nm (ε 5200), 278 nm (ε 14,400), 247 nm (ε 17,700); ^{1}H NMR (DMSO-d_6) and ^{13}C NMR (DMSO-d_6) data, see Table 1; HRESIMS *m*/*z* 365.1026 (calcd for $C_{20}H_{14}N_4O_2Na$, 365.1014).

3.4. Assay for the differentiation of human leukemia K562 cells

Human leukemia K562 cells were cultured in Ham's F-12 medium containing 10% fetal bovine serum and penicillin-streptomycin solution at 37 °C under an atmosphere of 5% CO_2. To each well of the 96-well microplate containing 100 μL of K562 cell suspension (1×10^4 cells/mL), 100 μL of test solution dissolved in Ham's F-12 medium was added and the plate was incubated at 37 °C for 96 h. After addition of 50 μL of benzidine dihydrochloride in aq AcOH containing 10% hydrogen peroxide solution to each well, the plate was incubated for 10 min and the stained hemoglobin-producing cells were detected under a microscope.

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Supplementary data

NMR spectra of compounds **1**–**3** are given. Supplementary data related to this article can be found online at doi:10.1016/j.tet.2011.04.098.

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