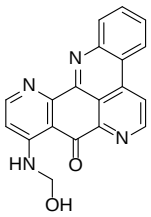
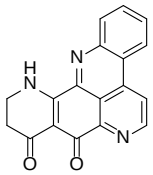
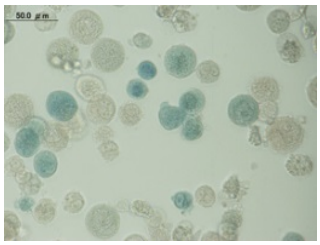
Graphical Abstract



1: *N*-hydroxymethyl isocystodamine



2: neolabuanine A



Two Cell Differentiation Inducing Pyridoacridines from a Marine Sponge *Biemna* sp. and Their Chemical Conversions.

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Keywords: sponge, cell differentiation, K562, pyridoacridine

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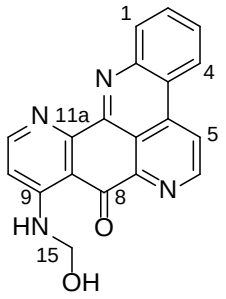
ABSTRACT

Two pyridoacridines, *N*-hydroxymethylisocystodamine (**1**) and neolabuanine A (**2**), together with the known ecionine A (**3**), ecionine B (**4**), isocystodamine (**5**), *N*-methylisocystodamine (**6**), 9-hydroxyisoascididemin (**7**), and biemnadin (**8**), were isolated from a marine sponge *Biemna* sp. Several of these compounds were shown to induce cell differentiation of K562 leukemia cells into erythrocytes. Following inspection of the NMR data, and comparison of these data with literature values, we demonstrated that neolabuanine A (**2**) had the structure previously reported as labuanine A (**2**), and that the compound initially reported under the name of labuanine A possessed the structure assigned to ecionine A (**3**). We found that both neolabuanine A (**2**) and ecionine A (**3**) were gradually converted to 9-hydroxyisoascididemin (**7**), indicating that **2** and **3** can be considered both as precursors of **7**.

1. Introduction

Induction of differentiation from acute myelogenous leukemia tumor cells into mature normal cells by chemical inducers has been considered as a strategy for cancer chemotherapy, because early hematopoietic progenitors fail to give birth to cell lineage restricted phenotype in leukemias due to blockade of differentiation.[1] Against this background we searched for metabolites that exhibit induction of cell differentiation in K562, human myelogenous leukemia cells. We found activity in the organic extract of the marine sponge *Biemna* sp. In this paper we report the isolation, structure elucidation and cell differentiation activity of the isolated constituents.



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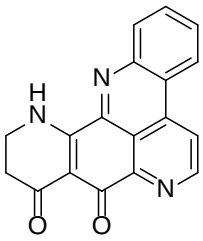
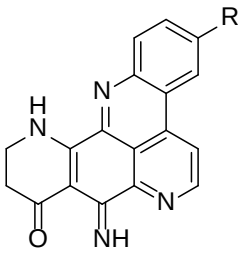
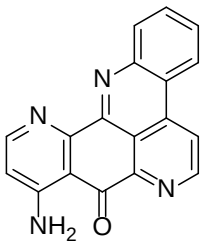


2: neolabuanine A



3: R=H ecionine A
4: R=OH ecionine B



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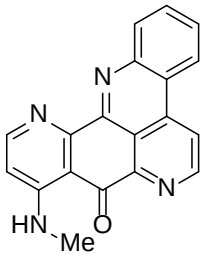

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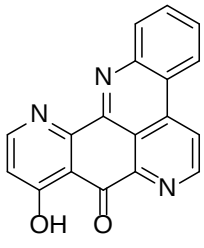
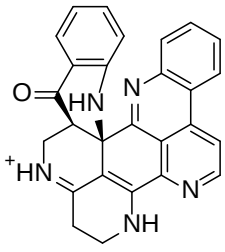


7: 9-hydroxyisoacididemin



8: biemnadin

2. Results and discussion

2.1. Isolation of cell differentiation inducers

The sponge (500 g) was extracted with MeOH and the extract was partitioned between $CHCl_3$ and H_2O. The organic fraction was further partitioned between *n*-hexane and 90% MeOH. The bioassay-guided fractionation of the 90% MeOH fraction by ODS flash chromatography and RP-HPLC afforded two new pyridoacridines, *N*-hydroxymethyl isocystodamine (**1**) and neolabuanine A (**2**), as well as the previously reported natural products, ecionine A (**3**),[2] ecionine B (**4**),[2] isocystodamine (**5**),[3] *N*-methylisocystodamine (**6**),[4] 9-hydroxyisoascididemin (**7**),[5] and biemnadin (**8**).[6]

2.2. Structure elucidation of *N*-hydroxymethyl isocystodamine (1)

Compound **1** was isolated as an yellow amorphous solid with a molecular formula of $C_{19}H_{12}N_4O_2$ as determined by HRESIMS [*m/z* 329.1013, $(M+H)^+$]. ^{1}H NMR and HSQC spectra of **1** showed eight aromatic CH (δ_H 9.27/δ_C 149.5, 9.05/120.2, 8.96/124.1, 8.59/152.6, 8.38/131.1, 8.03/131.9, 7.92/129.3, and 7.21/109.5), one hydroxymethyl group (δ_H 4.94/δ_C 65.5), and an exchangeable amino proton at δ_H 10.31 (Table 1). The ^{1}H-^{1}H coupling constants, characteristic carbon chemical shifts, and HMBC correlations suggested the presence of one disubstituted benzene ring (partial structure A) and two trisubsituted pyridine rings (partial structures B and C) (Fig 1). These partial structures and the molecular formula implied that **1** was closely related to isocystodamine.[3] Further HMBC correlation from H_2-15 (δ_H 4.94) to C-9 (δ_C 155.1) indicated that aromatic primary amino group found in the structure of isocystodamine was substituted by a hydroxymethyl group, which was supported by a COSY correlation between H_2-15 and NH-14 (δ_H 10.31). LCMS analysis revealed that **1** gradually converted to isocystodamine (**5**) in solution (Fig S1), further supporting our structural assignment.

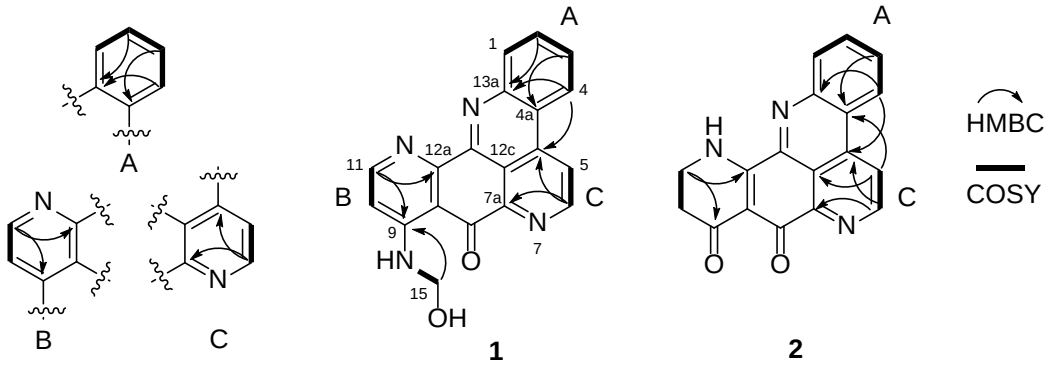


Fig 1. Partial structures A-C in **1**, and key COSY (bold) and HMBC (arrows) correlations observed in **1** and **2**.

Table 1. NMR Spectroscopic data (600 MHz, DMSO-d_6) for **1** and **2**

Position	**1**			Position	**2**		
	^{13}C	^{1}H (*J* in Hz)	HMBC		^{13}C	^{1}H (*J* in Hz)	HMBC
1	131.1, CH	8.38, d (7.4)	3, 4a	1	130.5, CH	8.33, d (7.6)	3, 4a
2	131.9, CH	8.03, t (7.4)	4, 13a	2	132.0, CH	8.08, t (7.6)	4,13a
3	129.3, CH	7.92, t (7.4)	1, 4a	3	130.1, CH	7.99, t (7.6)	1, 4a
4	124.1, CH	8.96, d (7.4)	2,4b,13a	4	124.3, CH	8.97, d (7.6)	2,4b,13a
4a	121.7, C			4a	123.0, C		
4b	136.9, C			4b	136.1, C		
5	120.2, CH	9.05, d (5.3)		5	118.4, CH	8.91, d (5.5)	4a,6,12c
6	149.5, CH	9.27, d (5.3)	4b, 7a	6	150.1, CH	9.18, d (5.5)	4b,5,7a
7a	146.6, C			7a	147.3, C		
8	ND			8	174.8, C		
8a	ND			8a	107.7, C		
9	155.1, C			9	188.9, C		
10	109.5, CH	7.21, d (5.9)		10	36.1, CH_2	2.58, t (7.6)	9
11	152.6, CH	8.59, d (5.9)	9,12a	11	40.4, CH_2	3.86, t (7.6)	9,10,12a
12a	153.2, C			12-NH		9.72, s	
12b	ND			12a	156.6, C		
12c	ND			12b	145.0, C		
13a	145.4, C			12c	116.8, C		
14		10.31, t (5.8)		13a	143.8, C		
15	65.5, CH_2	4.94, d (5.8)	9				

ND: not determined

2.3. Structure elucidation of neolabuanine A (2)

Compound **2** was isolated as a yellowish amorphous solid with a molecular formula of $C_{18}H_{11}N_3O_2$ [*m/z* 324.0747 $(M+Na)^+$] as assigned by HRESIMS. Interpretation of the ^1H NMR and 2D NMR spectra of **2** suggested the presence of partial structures A and C. There were two methylene protons (δ_H 3.86 and 2.58), an exchangeable proton (δ_H 9.72), and one ketone (δ_C 188.9), belonging to a 2,3-dihydropyridin-4(1H)-one moiety as determined by COSY and HMBC correlations (Table 1). These three partial structures and the three unassigned carbons (δ_C 174.8, 145.0, and 143.8) implied that the structure of compound **2** could be represented by the tautomeric form of the previously proposed structure of labuanine A. Labuanine A was first isolated from *Biemna fortis* by Aoki *et al.*[3] and while the molecular formula of this compound was determined by HR-FABMS analysis, the coincidence of the ^1H and ^{13}C chemical shifts between ecionine A (**3**) and labuanine A suggested that the structure of one of these molecules had been mis-assigned. Careful analysis of the NMR data for both ecionine A and labuanine A suggested that the chemical structure of the latter marine natural product had been mis-assigned (Table S1). In particular, the chemical shift of C-8 (δ_C 157.6-157.8) assigned as an enolic carbon in labuanine A contradicts the value of the latent iminoquinone partial structure, but is in accordance with a diiminoquinone moiety. In compound **2**, C-8 appeared at δ_C 174.8 in agreement with the iminoquinone equivalent. Altogether we concluded that compound **2** had the structure identical with the one proposed as labuanine A, and that the labuanine A isolated by Aoki *et al*. had the structure assigned several years later for ecionine A. Since the compound with the iminoquinone moiety had been named as labuanine A, we gave compound **2** the trivial name, neolabuanine A.

2.4. Chemical transformations of ecionine A (3) and cystodimine A

Compound **3** exhibited the ^{1}H NMR spectrum in DMSO-d_6 very similar to ecionine A[2] and cystodimine A[7] (Table S2), both of which gave remarkably close ^{1}H NMR spectra in DMSO-d_6. In order to confirm the individuality of the two previously reported compounds and to firmly establish the structure of **3**, we directly compared the three compounds by LCMS. An authentic sample of ecionine A gave a major UV-active HPLC peak with the $(M+H)^+$ ion at *m*/*z* 301, together with two minor UV-active peaks with $(M+H)^+$ ions at *m*/*z* 300 (Fig 2). The major UV-active peak was assigned as ecionine A, whereas the other peaks were suggested to be the isolable tautomeric forms of 9-hydroxyisoascididemin, **7** and **9**, because the last peak coincided with 9-hyroxyisoascididemin we isolated. In the LCMS pattern of an authentic sample of cystodimine A,[7] the major UV-active peak with the $(M+H)^+$ ion at *m*/*z* 301 and with a slightly longer retention time than that of ecionine A was assigned as cystodimine A, whereas the UV peak with the $(M+H)^+$ ion at *m*/*z* 299 was suggested to be dehydrocystodimine A, and the two peaks with *m*/*z* 300 were assigned as the tautomeric forms of 11-hydroxyascididemin (Fig S6).[8] Compound **3** gave the HPLC profile identical with that of authentic ecionine A, permitting us to confirm the identity of **3** with ecionine A. In our structure elucidation of the TFA salt of **3** (Fig 3), a COSY correlation was observed between two exchangeable protons at δ_H 10.83 and 11.12, both of which correlated to a N atom (^{1}H-^{15}N-HSQC) with an identical chemical shift. Therefore, both signals at δ_H 10.83 and 11.12 were assigned to N-8 ($=NH_2^+$) (Fig 3).

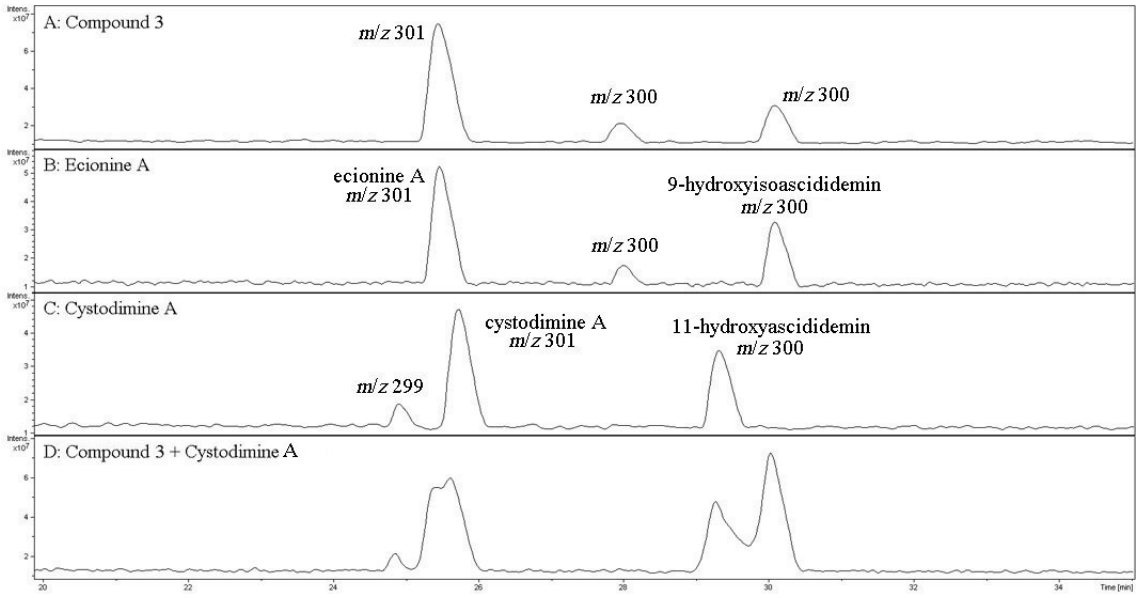


Fig 2. LCMS analyses for purified compound **3** (A), authentic ecionine A (B), authentic cystodimine A (C), and co-injection of **3** and authentic cystodimine A (D).

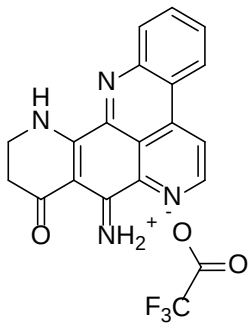


Fig 3. Structure of ecionine A TFA salt.

In this study, we found that neolabuanine A (**2**) gradually converted to 9-hydroxyisoascididemin (**7**) (Fig S2), and ecionine A (**3**) to 9-hydroxyisoascididemin (**7**) and two other compounds **9** and **10** (Fig 2, Fig S3, Scheme).[9] LCMS analyses showed that 9-hydroxyisoascididemin was in equilibrium between two peaks (Fig S4); **10** (*m*/*z* 299) and isocystodamine (**5**) gave different retention times (Fig S5). Due to the

instability and paucity of the samples, it was not possible to firmly establish the structures of **9** and **10** by NMR. We tentatively assigned the structures of **9** and **10** as shown in scheme based on the molecular weight and the reactivity of the compounds. The hydrolysis of the imine, the oxidation of the 2,3-dihydropyridin-4(1H)-one moiety, and interconversion between isolable tautomers were observed in ecionine A (**3**) and its reaction products. Corresponding reactions took place with cystodimine A, but 8,9-dihydro-11-hyroxyascididemin was not detected (Fig S6). The propensity for the facile reaction suggests that ecionine A and cystodimine A are the precursors of other pyridoacridines (Scheme) as previously envisaged by Bontemps *et al.* that proposed a biosynthetic relationship between cystodimine A and 11-hydroxyascididemin.[7]

2.5. Cytotoxicity and cell-differenciation inducing activity of 1–5, 7, and 8

Cytotoxicity and inductions of cell differentiation in K562 cells by **1**-**5**, **7**, and **8** were evaluated. They exhibit cytotoxicity at 5 μg/mL, but at lower concentration these compounds induced differentiation in K562 cells (Fig 4, Table 2). The study by Aoki *et al*. showed that isocystodamine (**5**) exhibited by far the most potent neuronal differentiation inducing activity compared with those of labuanine A (now assigned to ecionine A), 9-hydroxyisoascididemin, or biemnadin,[3] whereas Ueoka *et al*. reported that the cell differentiation inducing activity of isocystodamine **5** was equipotent with its *N*-methyl or *N*-methoxymethyl derivatives.[4] In this study, we demonstrated that **1** was as potent as **5**, while **2** and **3** had reduced activity, and **4**, **7**, and **8** showed significantly less activity, which is in agreement with the previous studies.[3,4] We also examined an authentic 11-hydroxyascididemin and found that it does not show cell-differentiation inducing activity (Fig S7).

Table 2. Percentage of differentiated cells induced by pyridoacridines 1-8 (*N*=2).

Compound	Concentration (ng/mL)	Differentiation %
1	5	36
2	50	53
3	25	42
4	500	17
5	5	28
7	500	19
8	500	17
Doxorubicin	5	37

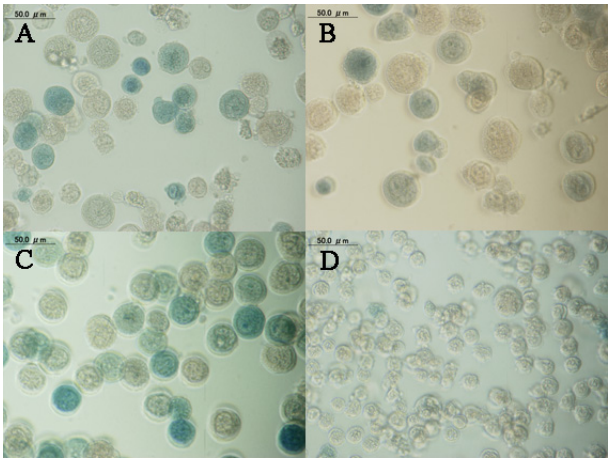
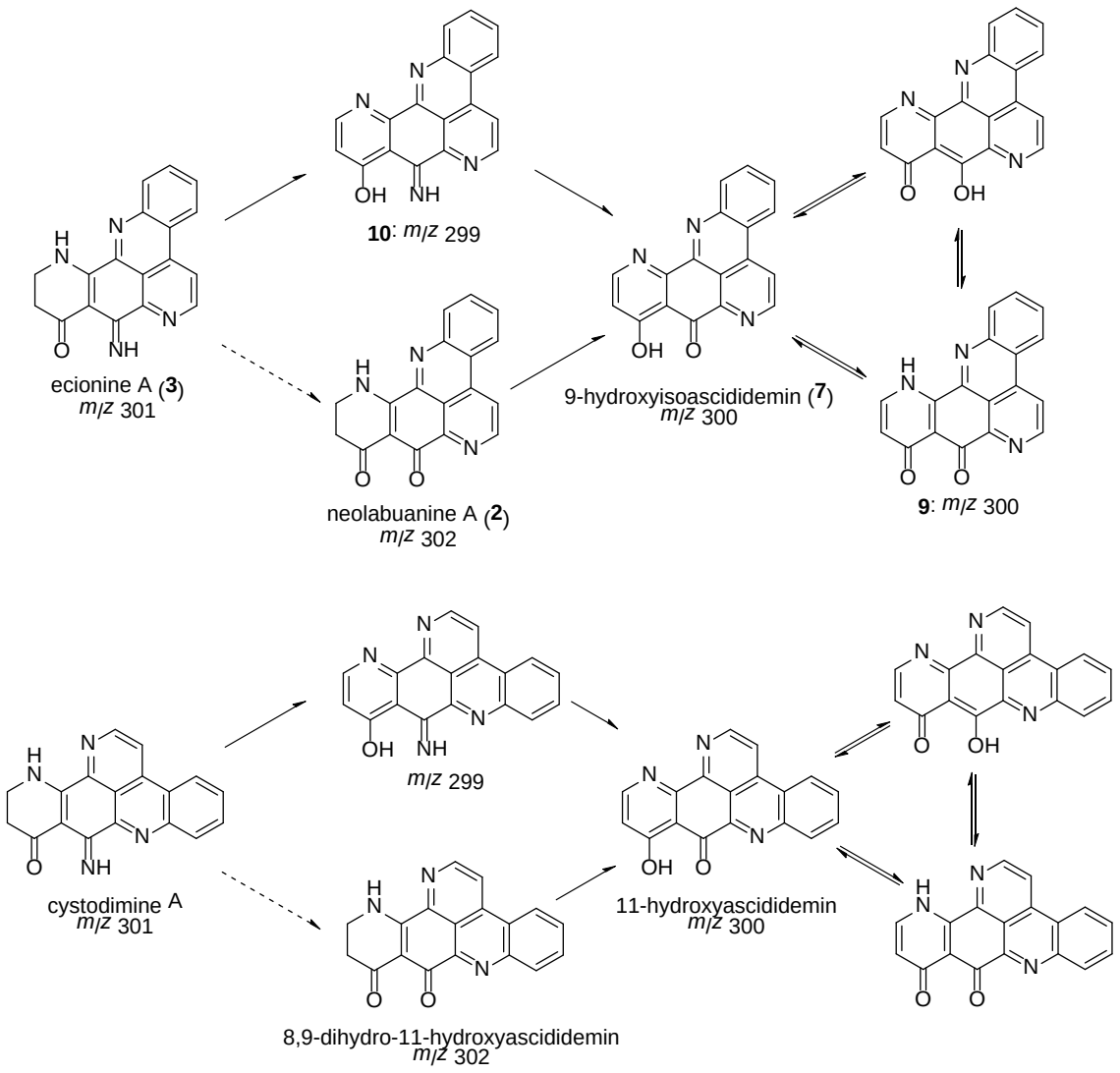


Fig 4. Induction of differentiation in K562 cells as detected by diaminofluorene. (A) *N*-hydroxymethylisocystodamine (**1**, 0.5 ng/mL), (B) neolabuanine A (**2**, 50 ng/mL), (C) doxorubicin (5 ng/mL), and (D) DMSO. Differentiated cells producing hemoglobin were clearly identified by the light blue or blue coloration.



Scheme. Reaction of ecionine A (**3**) and cystodimine A. The conversion from ecionine A (**3**) to neolabuanine A (**2**), and cystodimine A to 8,9-dihydro-11-hydroxyascididemin were not observed in this study.

3. Experimental

3.1. General experimental procedures

UV data were measured with SHIMADZU Biospec-1600. NMR spectra were recorded on a JEOL delta 600 NMR spectrometer at 600 MHz for ^{1}H and 150 MHz for

^{13}C. The chemical shifts in ^{1}H and ^{13}C were referenced to the solvent peaks at δ_H 2.49 and δ_C 39.5 for DMSO-d_6. LC-MS data were measured using a Bruker amaZon SL-TA. HPLC was conducted on a Shimadzu LC-20AT with an SCL-10Avp controller and SPD-10Avp detector. Fluorescence for the diaminofluorene assay was measured with a Molecular Device Spectra MAX GEMINI apparatus.

3.2. Collection and identification of the sponge

The sponge *Biemna* sp. was collected during the NT-09-17 cruise of the R/V 'Natsushima" of the Japan Agency for Marine-Earth Science and Technology (JAMSTEC), 29 September 2009. The sponge was collected by the Remotely Operated Vehicle (ROV) 'Hyper-Dolphin' (Dive#1058) at a depth of 162 m at a seamount "Oshima-Shinsone" (28º52.97′N, 129º32.382′E), southern Japan. Sponge description: huge, massive, circular cushion-shaped with hollow area on the upper surface; color dark brown on the whole surface, pale ocher inside the sponge; oscule invisible; surface smooth; texture soft; skeleton composed of style, sigmas in three size classes, raphide and microstrongyle. Up to now, 54 species have been reported as valid for the genus *Biemna* according to World Porifera database.[10] Of these, only one species *Biemna microstrongyla*[11] (Hentschel, 1912) has microstrongyle as one of its microscleres and thus can be comparable to our specimen. However *B. microstrongyla* has microxea as important spicule component that is completely absent in our specimen. The specimen used for the identification (NSMT-Po-248) is deposited at National Museum of Nature and Science, Tokyo.

3.3. Extraction and Isolation

The sponge (500 g) was blended in MeOH and the extract was partitioned between H_2O and $CHCl_3$. The organic layer was evaporated and partitioned between *n*-hexane and 90% MeOH. The 90% MeOH layer was separated by ODS-flash chromatography to obtain six fractions. The active fractions were purified by RP-HPLC using Cosmosil AR-II column (20-100% MeCN + 0.5% AcOH) to give **1** (3.1 mg) , **2** (16.4 mg), ecionine A (**3,** 8.4 mg), ecionine B (**4,** 1.8 mg), isocystodamine (**5,** 2.5 mg), *N*-methylisocystodamine (**6,** 2.2 mg), 9-hydroxyisoascididemin (**7,** 4.2 mg) and biemnadin (**8,** 17.3 mg). To compare the chemical shifts with those in the literature, a small portion of ecionines A and B were further purified by RP-HPLC using the method reported by Barnes, E. *et al.*[2] in order to generate the TFA salts of these compounds.

3.3.1. *N-hydroxymethylisocystodamine* (1)**.** yellow solid; UV (MeOH) λ_{max} (log ε) 210 (3.7), 364 (2.9); ^{1}H NMR (DMSO-d_6) and ^{13}C NMR (DMSO-d_6) data, see Table **1**; HRESIMS *m/z* 329.1013 $[M+H]^+$ (calcd for $C_{19}H_{13}N_4O_2$, 329.1038).

3.3.2. *Neolabuanine A* (**2**). yellow solid; UV (MeOH) λ_{max} (log ε) 215 (4.1), 374 (3.6); ^{1}H NMR (DMSO-d_6) and ^{13}C NMR (DMSO-d_6) data, see Table **1**; HRESIMS *m/z* 324.0747 $[M+Na]^+$ (calcd for $C_{18}H_{11}N_3NaO_2$, 324.0749).

3.3.3. *Ecionine A (**3**).* yellow solid; UV (MeOH) λ_{max} (log ε) 216 (3.7), 282 (3.4), 378 (3.2) nm; ^{1}H NMR (DMSO-d_6, 600 MHz) data see Table S2; HREISMS *m/z* 301.1122 $[M+H]^+$ (calcd for $C_{18}H_{13}N_4O_1$, 301.1089)

3.4. LC-MS analyses for pyridoacridines

Purified pyridoacridines and authentic samples were analyzed by LC-MS, with a column (Cosmosil 2.5C_{18}-MSII, ϕ 2.0 × 100 mm) a solvent system 0-100 % MeCN containing 0.5% acetic acid for 50 min, at a flow rate of 0.5 mL/min.

3.5. Cell culture and cell differentiation assay for human leukemia K562 cells

Human leukemia K562 cells were cultured in Ham's F-12 medium containing 10% calf bovine serum with penicillin and streptomycin at 37°C under an atmosphere of 5% CO_2. The assay was conducted in a 96-well micro-plate containing 200 μL of K562 cell suspension of 5.0×10^4 cells/mL. A portion of the sample (10 μg) dissolved in DMSO (2 μL) was added to the micro-plate and serial dilution (1/10) was performed. The plate was incubated at 37°C for 96 h. After the addition of 50 μL of diaminofluorene solution in each well, the plate was incubated at room temperature in the dark for 5 min.[12] The blue cells observed under the microscope due to the production of hemoglobin were quantified. Doxorubicin and DMSO were used as the positive and negative control, respectively. The number of differentiated cells was counted under the microscope.

3.6. Cell viability assay

K562 cells were prepared as described above. After incubation for 96 h, a solution of XTT (3-[1-phenylnocarbyl)-3,4-tetrazolium-bis(4-methoxy-6-nitro) benzenesulfonic acid hydrate, 50 μL, 1 mg/mL) and 4% of the total volume with PMS (phenazine methosulfate 0.15 mg/mL) solution were added in each well, incubated for 4 h and the absorbance at 450 nm was read.

Acknowledements

We thank Prof. Motomasa Kobayashi, Osaka University, for valuable discussion. The sponge was collected during the NT-09-17 cruise of Natsushima, JAMSTEC Japan Agency For Marine-Earth Science and Technology. This work was partly supported by a Grant-in-Aid for Scientific Research on Innovative Areas "Chemical Biology of Natural Products"[13] and JSPS KAKENHI Grant Numbers 25252037, 25712024, and 25660163 from The Ministry of Education, Culture, Sports, Science and Technology, Japan.

Supplementary data

LCMS data of pyridoacridines, ^{1}H and 2D NMR data for **1**-**3** are available at http://.

References and notes

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Fig legends.

Fig 1. Partial structures A-C in **1**, and key COSY (bold) and HMBC (arrows) correlations observed in **1** and **2**.

Fig 2. LCMS analyses for purified compound **3** (A), authentic ecionine A (B), authentic cystodimine A (C), and co-injection of 3 and authentic cystodimine A (D)

Fig 3. Structure of ecionine A TFA salt.

Fig 4. Induction of differentiation in K562 cells as detected by diaminofluorene. (A) *N*-hydroxymethylisocystodamine (**1**, 0.5 ng/mL), (B) neolabuanine A (**2**, 50 ng/mL), (C) doxorubicin (5 ng/mL), and (D) DMSO. Differentiated cells producing hemoglobin were clearly identified by the light blue or blue coloration.

Scheme. Reaction of ecionine A (**3**) and cystodimine A. The conversion from ecionine A (**3**) to neolabuanine A (**2**), and cystodimine A to 8,9-dihydro-11-hydroxyascididemin were not observed in this study.