Ecionines A and B, two new cytotoxic pyridoacridine alkaloids from the Australian marine sponge, *Ecionemia geodides*

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Abstract

Chemical investigations of the Australian marine sponge *Ecionemia geodides* resulted in the isolation of two new pyridoacridine alkaloids, ecionines A (**1**) and B (**2**), along with the previously isolated marine natural products, biemnadin (**3**) and meridine (**4**). Compounds **1** and **2** both contain an imine moiety, which is rare for the pyridoacridine structure class. The chemical structures of **1** and **2** were determined by extensive 1D and 2D NMR, and MS data analyses. All compounds were tested against a panel of human bladder cancer cell lines, the increasingly metastatic TSU-Pr1 series (TSU-Pr1, TSU-Pr1-B1 and TSU-Pr1-B2) and the superficial bladder cancer cell line 5637. Ecionine A (**1**) displayed cytotoxicity against all cell lines, with IC₅₀ values ranging from 3 to 7 μ M. This is the first report of chemistry from the sponge genus *Ecionemia*.

1. Introduction

Marine sponges often produce secondary metabolites for such uses as predator deterrents, settlement cues, and as anti-fouling agents.^{1,2} As such, marine sponges have proven to be a rich source of bioactive natural products, many of which have been shown to be active towards numerous human therapeutic targets. One particular scientific area in which sponge metabolites have played a major role is cancer research.³ Examples of cytotoxic sponge-derived metabolites include tedanolide C (HCT-116, IC₅₀ 95 nM),⁴ peloruside A (P388, IC₅₀ 18 nM),⁵ renieramycin J (P388, IC₅₀ 0.53 nM),⁶ jaspamide M (MCF-7, IC₅₀ 100 nM),⁷ and microcionamide A (SKBR-3, IC₅₀ 95 nM).⁸

As part of our continuing efforts to discover new anticancer natural products from Australian marine organisms, we decided to undertake a detailed chemical analysis of the hitherto underinvestigated sponge, *Ecionemia geodides* (family Ancorinidae). Sponges belonging to the family Ancorinidae have yielded several biologically active compounds, examples of which include the MT1-matrix metalloproteinase inhibitor ancorinoside A (*Ancorina* sp.),^{9,10} the potent actomyosin ATPase activator,¹¹ penaresidin A (*Penares* sp.), and schulzeines A-C (*Penares schulzei*), which inhibits α -glucosidase with IC₅₀ values between 48 and 170 nM.¹² However, no chemistry has been reported from sponges belonging to the genus *Ecionemia*.

This paper reports the isolation and structure elucidation of two new pyridoacridine alkaloids, which we have named ecionines A (**1**) and B (**2**), as well as the known compounds biemnadin (**3**)¹³ and meridine (**4**)¹⁴ (Figure 1). The cytotoxicity of **1-4** towards the metastatic human bladder cancer cell line TSU-Pr1 series (TSU-Pr1, TSU-Pr1-B1 and TSU-Pr1-B2), and the superficial bladder cancer cell line 5637, is also reported.

2. Results and discussion

The freeze-dried and ground *E. geodides* was exhaustively extracted with sequential washes of *n*-hexane, CH₂Cl₂/CH₃OH (4:1) and CH₃OH. The CH₂Cl₂/CH₃OH extracts were all combined

and chromatographed using preparative C₁₈ bonded silica HPLC (CH₃OH/H₂O/0.1% TFA) to yield two fractions of interest. Both these fractions were further purified by semi-preparative C₁₈ bonded silica HPLC (CH₃OH/H₂O/0.1% TFA) to yield the TFA salts of the new compounds ecionines A (**1**, 23.7 mg, 0.035% dry wt) and B (**2**, 5.1 mg, 0.008% dry wt), as well as the known compounds biemnadin (**3**, 1.8 mg, 0.003% dry wt) and meridine (**4**, 11.3 mg, 0.016% dry wt).

The TFA salt of ecionine A (**1**) was obtained as a light brown solid. Compound **1** was assigned the molecular formula C₁₈H₁₃N₄O on the basis of HRESIMS and NMR data (Table 1). The IR spectrum for **1** suggested the presence of a ketone functional group (1666 cm⁻¹). The ¹H NMR spectrum of **1** showed six aromatic signals between δ_H 8.09 and 9.28, three exchangeable signals at δ_H 10.83, 11.57, and 11.12, and two mutually-coupled methylene signals at δ_H 2.92 and 4.11. The ¹³C NMR spectrum of **1** showed 18 resonances, 15 of which resonated between δ_C 116 and 194. These data suggested that **1** belonged to the pyridoacridine structure class.¹³⁻¹⁶

Four of the aromatic resonances in the ¹H NMR spectrum were indicative of a 1,2-disubstituted benzene ring system [δ_H 8.41 (H-1, d, *J* = 7.8 Hz), 8.17 (H-2, dd, *J* = 7.8, 7.2 Hz), 8.09 (H-3, dd, *J* = 7.8, 7.2 Hz), and 9.07 (H-4, d, *J* = 7.8 Hz)]. The coupling constant between the remaining two aromatic protons [δ_H 9.16 (H-5, d, *J* = 5.4 Hz), 9.28 (H-6, d, *J* = 5.4 Hz)] and a HSQC correlation from the proton at δ_H 9.28 to a carbon at δ_C 149.5, suggested a pyridine ring.^{14,15} HMBC correlations from H-6 (δ_H 9.28) and H-4 (δ_H 9.07) to C-4b (δ_C 136.8) and H-1 (δ_H 8.41), H-3 (δ_H 8.09), and H-5 (δ_H 9.16) to C-4a (δ_C 122.8) allowed the benzene and pyridine ring systems to be linked via a carbon-carbon bond between C-4a and C-4b. This linkage was further supported by a strong ROESY correlation between H-4 (δ_H 9.07) and H-5 (δ_H 9.16) (Figure 2). Furthermore, a nitrogen was attached to C-13a (δ_C 144.1) of the benzene moiety on the basis of the ¹³C chemical shift of this carbon.¹³⁻¹⁶ The two methylene signals at δ_H 2.92 (H-10, t, *J* = 7.8 Hz) and 4.11 (H-11, dt, *J* = 7.2, 7.8 Hz) showed strong COSY correlations to each other. Both these signals showed HMBC correlations to a carbon at δ_C 193.2, which allowed the positioning of a carbonyl group at C-9. A COSY correlation between an exchangeable proton at δ_H 11.57 and the protons at H-11 in

conjunction with the chemical shifts of C-11 (δ_C 40.5) and H-11 (δ_H 4.11) suggested the presence of a NH group at position 12. At this stage, and on the basis of the HRESIMS data, it was determined that ecionine A required an extra NH_2 unit. The two remaining unassigned exchangeable protons at δ_H 11.12 and δ_H 10.83 accounted for the hydrogen atoms in this NH_2 moiety. The proton at δ_H 11.12 showed HMBC correlations to C-8a (δ_C 99.0) and C-7a (δ_C 142.5), which suggested that it was part of an imine group attached to C-8. COSY and ROESY correlations between these two exchangeable signals (δ_H 11.12 and 10.83) allowed the proton at δ_H 10.83 to be placed at position 7. Hence, structure **1** was assigned to ecionine A.

Compound **1** possesses the 11*H*-pyrido[4,3,2-*mn*]acridine skeleton (**5**)² that has been identified in a number of marine natural products including meridine,¹⁴ ascididemin,¹⁵ and amphimedine.¹⁷ The only reported natural product pyridoacridine possessing an imine moiety is the anemone pigment calliactine (**6**).¹⁸ Synthetic studies on **6** confirmed the structure of this unique pyridoacridine.¹⁹⁻²² The synthetic compound, 11-hydroxy-10-imino-10*H*-benzo[*i*]quino[2,3,4-*kl*]acridine (**7**), also possesses an imine substituted pyridoacridine skeleton.¹⁶ ¹³C NMR data comparison between similar substructures found in **1**, **6** and **7** provided additional support for our structural assignment of **1**. Encionine A is also structurally related to plakinidines A (**8**), B (**9**) and D (**10**).²³⁻²⁶ Plakinidines A and B were isolated from the sponge genus *Plakortis*,^{23,24} while plakinidine D was first isolated from ascidians belonging to the genus *Didemnum*.^{25,26}

The TFA salt of ecionine B (**2**) was isolated as a light brown solid. Compound **2** was assigned the molecular formula $C_{18}H_{13}N_4O_2$ on the basis of HRESIMS and NMR data. The NMR data for **2** (Table 1) was very similar to that of **1**; the only major differences between the ¹H NMR spectra of **1** and **2** were that the latter had an extra exchangeable proton at δ_H 10.61, and was missing one aromatic proton. Following 1D and 2D data analysis it was possible to construct the same pyridoacridine skeleton as **1** (Figure 3). The extra exchangeable proton at δ_H 10.61 was assigned to a hydroxyl group at C-1, as it showed HMBC correlations to C-1 (δ_C 155.8), C-2 (δ_C 115.1) and C-13a (δ_C 133.5) (Figure 3). The chemical shift of C-1 (δ_C 155.1), and the observed

bathochromic shift seen in the UV spectrum of **2** on the addition of base, provided further proof of the phenol. Hence structure **2** was assigned to ecionine B.

The previously isolated marine natural products, biemnadin and meridine, were assigned to compounds **3** and **4**, respectively, after spectroscopic data comparison with literature values.^{13,14}

Compounds **1-4** all belong to the pyridoacridine class of natural products. Pyridoacridines are often cytotoxic, a trait that has been attributed to their DNA binding abilities.^{27,28} These compounds can, however, also show selectivity in living systems,²⁷ and have been reported to have a range of other biological activities, including anti-bacterial,²⁹ anti-fungal,³⁰ insecticidal,³¹ and topoisomerase II inhibition.³²

Due to our interest in the discovery of anti-cancer natural products,^{8,33} and the previously reported cytotoxicity of several pyridoacridine compounds,^{2,13-15,28} we tested **1-4** against a panel of human bladder cancer cell lines. Toxicity for **1-4** towards the increasingly metastatic TSU-Pr1 series (TSU-Pr1, TSU-Pr1-B1 and TSU-Pr1-B2)³⁴ and the superficial bladder cancer cell line 5637 are reported in Table 2. Preliminary toxicity towards human cancer cells was investigated by measuring changes in cell membrane permeability (Yo-Pro-1 staining). Cell nuclei were stained with Hoechst 33342 stain and the proportion of cells with compromised cell membranes (Yo-Pro-1 positive) counted using a high content screening strategy. Compound **1** showed moderate cytotoxicity against all cell lines, with IC₅₀ values of 6.48 μ M (TSU-Pr1), 6.49 μ M (TSU-Pr1-B1), 3.55 μ M (TSU-Pr1-B2) and 3.66 μ M (5637). Compound **2** had a modest cytotoxic effect on 5637 and TSU-Pr1-B2 cells at 10 μ M, with cell growth inhibitions of 54% and 51% cells respectively, but did not have an effect on TSU-Pr1-B1 cells at 10 μ M. Compound **4** had comparable activity to **1** against the invasive bladder cancer TSU-Pr1 cell line series with IC₅₀ values of 3.77 μ M (TSU-Pr1), 4.56 μ M (TSU-Pr1-B1), and 3.76 μ M (TSU-Pr1-B2) but had a lesser effect on the superficial bladder cancer cell line 5637. Compound **3** displayed weak cytotoxic effects against 5637 cells (10 μ M induced cell death in 22% cells), but had no effect on TSU-Pr1 cells at the screening concentrations used in this study. The cytotoxicity data in Table 2 identifies these *Ecionemia* -

derived compounds as having specific toxicity profiles against the four human bladder cancer cell lines used in these investigations.

In conclusion, this is the first report of chemistry from the sponge genus *Ecionemia*, and ecionines A and B are only the second and third examples, respectively, of imine substituted natural product pyridoacridines.

3. Experimental section

3.1. General Experimental Procedures.

NMR spectra were recorded at 30 °C on either a Varian 500 MHz or 600 MHz Unity INOVA spectrometer. The latter spectrometer was equipped with a triple resonance cold probe. The ^1H and ^{13}C chemical shifts were referenced to the solvent peaks for DMSO- d_6 at δ_{H} 2.49 and δ_{C} 39.5. LRESIMS was recorded on a Mariner Time-of-Flight spectrometer equipped with a Gilson 215 eight probe injector. IR and UV spectra were recorded on a Bruker Tensor 27 spectrophotometer and a Jasco V-650 UV/Vis spectrophotometer, respectively. An Edwards Instrument Company Bio-line orbital shaker was used for large-scale sponge extractions. For the HPLC work a Waters 600 pump equipped with a Waters 966 PDA detector and Gilson 715 liquid handler were used. A ThermoElectron C₁₈ Betasil 5 μm 143Å (50 mm $\times$ 150 mm) preparative HPLC column was used for large-scale separations. A ThermoElectron C₁₈ Betasil 5 μm 143Å (21.2 mm $\times$ 150 mm) column was used for semi-preparative HPLC separations. All solvents used for chromatography, UV, IR and MS were Lab-Scan HPLC grade, and the H₂O was Millipore Milli-Q PF filtered.

3.2. Animal Material.

The sponge *E. geodides* was collected by SCUBA (-10 m) in Moorina Bay, Tasmania, Australia, during April of 2002, and kept frozen prior to freeze-drying and extraction. A voucher sample (G319669) has been lodged at the Queensland Museum, South Brisbane, Australia.

3.3. Extraction and Isolation.

The freeze-dried and ground sponge (68 g) was poured into a conical flask (1 L), *n*-hexane (250 mL) was added and the flask was shaken at 200 rpm for 2 h. The *n*-hexane extract was filtered under gravity then discarded. CH₂Cl₂:CH₃OH (4:1, 250 mL) was added to the de-fatted sponge material in the conical flask and shaken at 200 rpm for 2 h. The resulting extract was filtered under gravity, and set aside. CH₃OH (250 mL) was added and the CH₃OH/sponge mixture was shaken for a further 2 h at 200 rpm. Following gravity filtration, the sponge material was extracted with another volume of CH₃OH (250 mL), while being shaken at 200 rpm for 16 h. All CH₂Cl₂/CH₃OH extractions were combined and dried under reduced pressure to yield a light brown solid (6.3 g). This crude extract was pre-adsorbed onto Betasil C₁₈ silica (~ 6 g), packed into a stainless steel cartridge (25 mm × 50 mm), and subjected to preparative HPLC using a C₁₈ Betasil column (50 mm × 150 mm) at a flow rate of 40 mL/min and isocratic conditions of 10% CH₃OH (0.1% TFA)/90% H₂O (0.1% TFA) for 10 min, followed by a linear gradient to CH₃OH (0.1% TFA) in 45 min, then isocratic conditions of CH₃OH (0.1% TFA) for 20 min. One hundred and fifty fractions (150 × 0.5 min) were collected from time = 0 min. UV-active fractions were analysed by (+)-LRESIMS and ¹H NMR spectroscopy, and resulted in two semi-pure alkaloidal fractions. Fraction 1 (34.4 mg) was further fractionated using a semi-preparative C₁₈ Betasil column (21.2 mm × 150 mm) and the same conditions as those detailed above except at a flow rate of 9 mL/min. Sixty fractions (60 × 1 min) were collected from time = 0 min, then analysed by (+)-LRESIMS and ¹H NMR spectroscopy. Fractions 30 and 32 contained the TFA salts of ecionines A (**1**, 23.7 mg, 0.035% dry wt) and B (**2**, 5.1 mg, 0.008% dry wt), respectively. Fraction 2 (18.1 mg) from the preparative separation was also further fractionated using identical C₁₈ HPLC conditions to those detailed above, and ¹H NMR analysis of all UV-active peaks resulted in the identification of the two previously reported natural products, biemnadin (**3**, 1.8 mg, 0.003% dry wt) and meridine (**4**, 11.3 mg, 0.016% dry wt), which were both isolated as TFA salts.

3.4. TFA salt of ecionine A (1)

Light brown solid; UV (CH₃OH) $\lambda_{\max}$ (log ϵ): 215 (4.23), 228 (4.26), 284 (4.00), 319 (3.70), 376 (3.80), 443 (3.28); IR $\nu_{\max}$ (KBr) 1666, 1462, 1201, 1136 cm⁻¹; ¹H and ¹³C NMR data (DMSO-*d*₆) see Table 1; (+)-LRESIMS *m/z* (rel. int.) 301 (100) [M - CF₃COO]⁺. (+)-HRESIMS *m/z* 301.1079 (C₁₈H₁₃N₄O [M - CF₃COO]⁺ requires 301.1084).

3.5. TFA salt of ecionine B (2)

Light brown solid; UV (CH₃OH) $\lambda_{\max}$ (log ϵ): 211 (3.66), 235 (3.74), 286 (3.48), 321 (3.20), 382 (3.20), 447 (3.00); UV (CH₃OH+KOH) $\lambda_{\max}$ nm (log ϵ): 212 (3.97), 241 (3.73), 293 (3.40), 366 (3.08), 431 (2.97), 541 (2.77); IR $\nu_{\max}$ (KBr) 3410, 1674, 1547, 1432, 1203, 1135, 1025, 994 cm⁻¹; ¹H and ¹³C NMR data (DMSO-*d*₆) see Table 1; (+)-LRESIMS *m/z* (rel. int.) 317 (100) [M - CF₃COO]⁺; (+)-HRESIMS *m/z* 317.1039 (C₁₈H₁₃N₄O₂ [M - CF₃COO]⁺ requires 317.1033).

3.6. Cytotoxicity Assays.

TSU-Pr1, TSU-Pr1-B1, TSU-Pr1-B2 and 5637 bladder cancer cell lines were routinely cultured in Dulbecco's Modified Eagles medium (Gibco) supplemented with 5% foetal bovine serum (ICP Biologicals). Cells were split from flasks using 0.25% trypsin (Gibco) and plated into 96-well black walled/clear bottom plates (Costar) at a density of 5000 cells per well in 100 μ L of medium. Medium was removed prior to addition of compound. All compounds were initially solubilised in DMSO. Compounds were diluted, along with DMSO (negative control, 0.1% working concentration), media (negative control), doxorubicin (positive control, 1 μ M working concentration), in media (to obtain a working concentration of 0.1% DMSO v/v) before 100 μ L of each dilution was transferred into the cell plate. Concentration response curves were constructed using 5 concentrations of each compound, and each treatment was performed in duplicate. Each assay was repeated 2-3 times. Cells were incubated with each compound for 24 h at 37 °C, 95%

humidity, 5% CO₂. Cells were examined and images collected for analysis using a Cellomics Arrayscan following incubation with fluorescent dyes Yo-Pro-1 (Invitrogen) and Hoechst 33342 (Invitrogen). Hoechst 33342 staining was used to determine total cell number, and Yo-Pro-1 positivity was used to quantitate IC₅₀ values.

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Supplementary data

NMR (¹H and ¹³C), (+)-LRESIMS, IR, UV data for ecionines A (1) and B (2).
Supplementary data associated with this article can be found, in the online version at doi:

Figure 1. Chemical structures for 1-10.

Figure 2. Key HMBC (→) and ROESY (↔) correlations for 1.

Figure 3. Key HMBC (→) and ROESY (↔) correlations for 2.

Table 2. Cytotoxicity data for compounds **1-4**.^a

Compound	IC ₅₀ (μM)			
	TSU-Pr1	TSU-Pr1-B1	TSU-Pr1-B2	5637
1	6.48 (1.5 to 9.2) ^b	6.49 (3.6 to 11.7) ^b	3.55 (1.3 to 9.6) ^b	3.66 (1.2 to 10.9) ^b
2	not tested	not active @ 10 μM	51% @ 10 μM ^c	54% @ 10 μM ^c
3	not active @ 10 μM	not tested	not tested	22% @ 10 μM ^c
4	3.77 (1.4 to 10.1) ^b	4.56 (1.8 to 11.8) ^b	3.76 (1.1 to 13.5) ^b	37% @ 10 μM ^c
Doxorubicin ^d	96% @ 1 μM	95% @ 1 μM	99% @ 1 μM	99% @ 1 μM

^aIC₅₀ values calculated based on % Yo-Pro-1 positive cells (Total cells determined using Hoechst 33342). ^b95% confidence intervals. ^cIC₅₀ could not be calculated, % Yo-Pro-1 positive cells at highest compound concentration indicated. ^dpositive control, only tested at 1 μM.

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Table 1. NMR data for ecionines A (**1**) and B (**2**)^a

Position	Ecionine A (1)				Ecionine B (2)			
	¹³ C	¹ H (mult., <i>J</i> in Hz, int.)	gCOSY	gHMBC	¹³ C	¹ H (mult., <i>J</i> in Hz, int.)	gCOSY	gHMBC
1	131.1	8.41 (d, 7.8, 1H)	2	3, 4a	155.8			
1-OH						10.61 (s, 1H)		1, 2, 13a
2	132.9	8.17 (dd, 7.8, 7.2, 1H)	1, 3	4, 13a	115.1	7.51 (d, 7.8, 1H)	3	4, 13a
3	131.6	8.09 (dd, 7.8, 7.2, 1H)	2, 4	1, 4a, 13a ^w	133.2	7.96 (dd, 7.8, 8.4, 1H)	2, 4	1, 4a
4	124.7	9.07 (d, 7.8, 1H)	3	2, 4b, 13a	114.6	8.46 (d, 8.4, 1H)	3	2, 4a ^w , 4b, 13a
4a	122.8				123.6			
4b	136.8				136.9			
5	121.3	9.16 (d, 5.4, 1H)	6	4a, 4b, 6, 7a ^w , 12c	121.6	9.08 (d, 5.4, 1H)	6	4a, 4b ^w , 6, 12c
6	149.5	9.28 (d, 5.4, 1H)	5	4b, 5, 7a, 12c ^w	149.4	9.26 (d, 5.4, 1H)	5	4b, 5, 7a, 12c ^w
7		10.83 (s, 1H)	8-NH ^w			10.86 (s, 1H)	8-NH ^w	
7a	142.5				142.6			
8	157.7				157.6			
8-NH		11.12 (s, 1H)	7 ^w	7a ^w , 8a ^w		11.10 (s, 1H)	7 ^w	
8a	99.0				98.9			
9	193.2				193.4			
10	34.6	2.92 (t, 7.8, 2H)	11	8a, 9, 11	34.5	2.95 (t, 7.8, 2H)	11	8a, 9, 11
11	40.5	4.11 (dt, 7.8, 7.2, 2H)	10, 12	9, 10, 12a	39.9	4.20 (dt, 7.8, 7.8, 2H)	10, 12	9, 10, 12a
12		11.57 (br s, 1H)	11			11.61 (s, 1H)	11	10
12a	157.9				157.7			
12b	142.0				139.2			
12c	116.2				116.4			
13a	144.1				133.5			

^a Spectra were recorded in DMSO-*d*₆ at 30 °C. ^w Weak correlation.