The Pterocellins, Novel Bioactive Alkaloids from the Marine Bryozoan *Pterocella vesiculosa*

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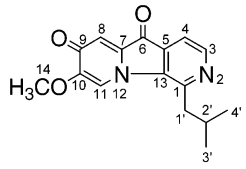
Two new alkaloids, pterocellins A and B, have been isolated from the New Zealand marine bryozoan *Pterocella vesiculosa*. Structural elucidation was achieved through NMR and mass spectral analysis in conjunction with a single-crystal X-ray diffraction study of pterocellin A. The pterocellins possess a novel heterocyclic skeleton and exhibit potent antitumor activity and antimicrobial activity in vitro but only modest activity in the in vivo hollow fiber assay at the National Cancer Institute.

Although comparatively little research has been undertaken into the secondary metabolites of bryozoans as compared with those of other marine invertebrates, bryozoans have proven to be an excellent source of novel and/or biologically active compounds.[1] The most well known of these metabolites are the bryostatins,[2] but other examples include the tambjamines,[3,4] the securamines,[5,6] the euthyroideones,[7] and the amathaspiramides.[8] The majority of bryozoan metabolites isolated to date have been alkaloids.[9]

In our continuing search for bioactive and/or novel compounds from New Zealand marine bryozoans, we undertook an investigation of an extract of *Pterocella vesiculosa* (order Cheilostomatida, suborder Ascophorina, family Catenicellidae) which possessed activity against P388 murine leukemia cells. We report here the isolation and structural elucidation of two novel alkaloids, pterocellins A−B (**1** and **2**) from the bryozoan. An X-ray crystal structure of pterocellin A (**1**) was obtained. The biological activity of the alkaloids was also examined, and the results of these studies are reported, including those from the in vitro 60 cell line panel and in vivo hollow fiber assays at the National Cancer Institute (NCI).



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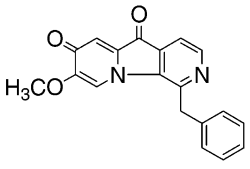

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Results and Discussion

P. vesiculosa is currently known only from waters off the North Island of New Zealand (both east and west coasts) and Southeastern Australia. The bryozoan was collected by scuba from the Hen and Chicken Islands, off the North Island of New Zealand and identified as *P. vesiculosa*. Frozen bryozoan was extracted with $MeOH/CH_2Cl_2$ (3:1). The extract was subjected to reversed-phase flash column chromatography and gel permeation chromatography to give the red pterocellins A and B (**1**, **2**) in 2.4×10^{-5} and 8.9×10^{-6} % yield, respectively (based on bryozoan wet weight).

HREIMS of pterocellin A (**1**) contained a molecular ion peak at *m/z* 284.11609, consistent with a molecular formula of $C_{16}H_{16}N_2O_3$. The ^1H NMR spectrum of **1** (Table 1) in $CDCl_3$ contained four aromatic proton resonances (two singlets and two doublets), a methoxyl proton resonance, and resonances at 3.12, 2.25, and 1.10 ppm, which were suggestive of the presence of an isobutyl group. The ^{13}C NMR spectrum acquired in $CDCl_3$ (Table 2) contained 15 resonances, comprising an aliphatic methyl signal, an aliphatic methane signal, an aliphatic methylene signal, a methoxyl signal, four aromatic methine signals, and seven quaternary carbon signals. Atom connectivities were established by COSY, $^1H-^{13}C$ HSQC, $^1H-^{13}C$ HMBC, and natural abundance $^1H-^{15}N$ HMBC[10] NMR experiments. For example, $^1H-^{13}C$ HMBC correlations from H-1′ to C-1 and C-13 and from H2′ to C-1, in addition to $^1H-^{15}N$ HMBC correlations from H-1′, H-3, and H-4 to N-2 (δ 342.7), established the attachment of the isobutyl group at C-1 of the aromatic ring. $^1H-^{15}N$ HMBC correlations from the two isolated protons H-8 and H-11 to N-12 (δ 140.4) along with $^1H-^{13}C$ HMBC correlations from these two protons established that they were positioned *para* to each other and established the positions of the carbon atoms within the heterocyclic ring (Table 2). Chemical shifts of N-2 and N-12 were within the range expected for similar systems.[10]

The NMR data reveal a pyrido[4,3-*b*]indolizine skeleton with an isobutyl group at position 1, carbonyl groups at positions 6 and 9, and a methoxyl group at position 10. The results of a series of 1D NOE experiments were consistent with the proposed structure (Table 1).

The NMR spectroscopic data were not entirely unambiguous, so the structure of pterocellin A (**1**) was confirmed with the aid of a single-crystal X-ray diffraction study. The structure was solved by direct methods. The crystal structure showed three independent molecules in the asymmetric unit, together with one molecule of water, which is hydrogen-bonded to N-2 of molecule 3. The molecules differ mainly in the relative conformation of the isobutyl side chain. An ORTEP plot of the crystal structure of molecule 1 of **1** is shown in Figure 1. The main core of the compound in all three molecules is planar to within a maximum of ±0.08 Å, with only the side chain significantly involved in the third dimension. This allows efficient π-stacking of the heterocyclic rings in the crystal, with the isobutyl groups interlocking in a channel between the stacks. The C-6−O-1 bond length of 1.21 Å is significantly shorter than the C-9−O-2 length of 1.24 Å, suggesting the former is more

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Table 1. ^{1}H NMR (δ, mult, J), COSY, and NOE Data for Pterocellins A and B (**1**, **2**) in $CDCl_3$

	1			**2**		
proton	^{1}H	COSY	NOE	^{1}H	COSY	NOE
3	8.61 (d, 4.6)	H-4	H-4	8.71 (d, 4.6)	H-4	H-4
4	7.53 (d, 4.6)	H-3	H-3	7.64 (d, 4.6)	H-3	H-3
8	7.07 (s)			7.03 (s)		
11	7.81 (s)		H-14, H-1′			H-14, H-1′
14	3.94 (s)		H-11	3.50 (s)		H-11
1′	3.11 (d, 7.3)	H-2′	H-11, H-2′, H-3′/H-4′	4.69 (s)		H-11, H-3′
2′	2.25 (m)	H-1′, H-3′/H-4′	H-1′, H-3′/H-4′	7.49 (s)		
3′[a]	1.10 (d, 6.6)	H-2′	H-1′, H-2′	7.16 (d, 7.0)	H-4′	H-1′, H-4′, H-5′
4′[a]	1.10 (d, 6.6)	H-2′	H-1′, H-2′	7.34 (m)	H-3′, H-5′	H-3′
5′				7.34 (m)	H-4′	H-3′

[a] For pterocellin A (**1**), H-3′ and H-4′ are equivalent in solution.

Table 2. ^{13}C and ^{15}N[a] NMR Data (δ) for Pterocellins A and B (**1**, **2**) in $CDCl_3$

	1			**2**	
atom	^{13}C	^{15}N	HMBC (H to C or N)	^{13}C	HMBC (H to C or N)
1	146.7		H-3, H-1′, H-2′	144.5	H-3, H-1′
N-2		342.7	H-3, H-4, H-1′		
3	147.3		H-4,	147.7	H-4
4	116.1		H-3	116.8	H-3
5	130.3		H-3	130.8	H-3
6	184.3		H-4, H-8	184.2	H-4, H-8
7	135.9		H-8, H-11	135.6	H-8, H-11
8	114.8			114.6	
9	173.0		H-8, H-11	173.0	H-8, H-11
10	151.6		H-8, H-11, H-14	151.6	H-8, H-11, H-14
11	115.3			115.3	
13	140.5		H-4, H-11, H-1′	141.4	H-4, H-11, H-1′
14	56.6			56.5	
N-12		140.4	H-8, H-11		
1′	44.7		H-2′, H-3′/H-4′[b]	42.1	H-3′
2′	28.5		H-1′, H-3′/H-4′[b]	136.2	H-1′, H-4′
3′	22.4		H-2′	127.7	H-1′, H-4′, H-5′
4′	22.4		H-2′	129.6	H-3′, H-5′
5′				127.8	H-3′, H-4′

[a] ^{15}N data were determined for **1** only. [b] H-3′ and H-4′ are equivalent in solution.

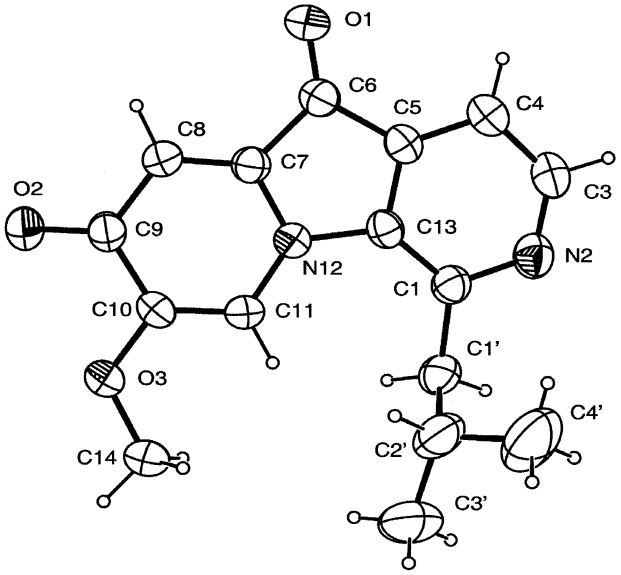

Figure 1. Structure of one independent molecule of pterocellin A (**1**) showing the numbering system.

ketonic with less conjugation with the associated ring. An examination of the individual bond lengths suggests that there is little delocalization of π-electron density across the middle ring since the C-5−C-6, C-6−C-7, and N-12−C-13 bonds are long and are all essentially single. The butyl-substituted pyridine ring is aromatic, but the electron density within the substituted pyridone ring is localized in short C-7−C-8 and C-10−C-11 bonds to give a dienone moiety (as in simpler 4-pyridone molecules).[11−14] Otherwise bond lengths and angles fall within usual ranges, with the main deviation of some angles arising from the strain of fusing five- and six-membered rings.

The ^{1}H NMR spectrum of pterocellin B (**2**) in $CDCl_3$ resembled that of pterocellin A (**1**) but lacked the isobutyl group resonances. Instead it contained a methylene singlet at 4.69 ppm and a two-proton doublet and a three-proton multiplet at 7.16 and 7.35 ppm, respectively. The ^{13}C NMR spectrum of **2** in $CDCl_3$ (Table 2) also resembled that of **1** in most respects. It lacked the isobutyl group resonances and instead contained four additional aromatic resonances compared with the ^{13}C NMR spectrum of **1**. Of these, one was a quaternary carbon signal while the other three were methine carbon signals, but two of these were approximately twice the height of the other methine signals in the spectrum so were likely due to two equivalent methine carbons each. The spectrum also contained a methylene carbon signal at 42.1 ppm, similar to the 44.7 ppm resonance in the spectrum of **1**. These spectra implied that the structure of **2** was very similar to that of **1** but that a benzyl group was attached to position 1 of the 8-methoxypyrido[4,3-*b*]indolizine-5,7-dione nucleus instead of an isobutyl group. ^{1}H−^{13}C HMBC and HSQC NMR experiments facilitated assignment of all ^{1}H and ^{13}C NMR spectral signals, while HREIMS established a molecular formula of $C_{19}H_{14}N_2O_3$. Again, the results of a series of 1D NOE experiments were consistent with the proposed structure (Table 1).

Pterocellins A (**1**) and B (**2**) were assayed in P388 murine leukemia, antiviral/cytotoxicity, and antimicrobial assay systems (Table 3). Pterocellins A and B possessed relatively potent activity against the P388 murine leukemia cell line with IC_{50} values of 477 and 323 ng/mL, respectively. They were also strongly cytotoxic to the BSC-1 cell line (derived from African Green Monkey kidney cells) used in the antiviral/cytotoxicity assay (Table 3). The pterocellins both showed strong activity against the Gram-positive bacterium *Bacillus subtilis* and the fungus *Tricophyton mentagrophytes*, with each having minimum inhibitory doses against *Bacillus subtilis* and *Tricophyton mentagrophytes* of 0−0.3 and 3.9−7.5 μg/disk, respectively (Table 3).

The cytotoxicity of pterocellins A and B was also evaluated by the NCI in their 60 cell line panel, which represents a variety of human tumor cell types: leukemia, non-small cell lung, colon, central nervous system (CNS), melanoma, ovarian, renal, prostate, and breast cancers (Supporting Information).[15] Evaluation of compounds **1** and **2** at the NCI revealed that both possessed potent cytotoxicity overall with panel average values of GI_{50} 1.4 μM, TGI 4.8 μM, LC_{50}

Table 3. P388, Antiviral/Cytotoxicity, and Antimicrobial/Antifungal Assay Results for Pterocellins A and B (**1**, **2**)

		organism[a]								
compound	P388 IC_{50}[b]	HSV[c]	PV1[d]	Cyt[e]	Ec	Bs	Pa	Ca	Tm	Cr
1	477	?	?	ww	>60[f]	0−0.3	>60	>60	3.9−7.5	>60
2	323	?	?	ww	>60	0−0.3	>60	>60	3.9−7.5	>60

[a] Key: ? = indeterminate activity. ww = antiviral/cytotoxic zone over whole well. Ec = *Escherichia coli*, Bs = *Bacillus subtilis*, Pa = *Pseudomonas aeruginosa*, Ca = *Candida albicans*, Tm = *Trichophyton mentagrophytes*, Cr = *Cladisporum resinae* (all strains developed and held in the Department of Plant and Microbial Sciences, University of Canterbury, 1984). [b] The concentration of sample in ng/mL required to reduce the cell growth of the P388 leukemia cell line (ATCC CCL 46) by 50%. [c] *Herpes simplex* Type 1 (strain F, ATCC VR 733) virus grown on the BSC-1 cell line (ATCC CCL 26). [d] *Polio* virus (Pfizer vaccine strain) grown on the BSC-1 cell line. [e] Cytotoxicity to BSC-1 cells at 40 μg/disk. [f] Activities expressed as minimum inhibitory doses in μg/disk.

17.0 μM for pterocellin A (**1**) and GI_{50} 0.7 μM, TGI 2.1 μM, LC_{50} 6.9 μM for pterocellin B (**2**). The leukemia cell line (CCRF-CEM) was the most sensitive cell line to pterocellin A (**1**) with a GI_{50} of 0.05 μM and a TGI of 0.8 μM, although the high LC_{50} value of >100 μM implies that pterocellin A is cytostatic rather than cytotoxic to this cell line. The most sensitive cell line to pterocellin B (**2**) was the melanoma cell line MALME-3M, with a GI_{50} of 0.03 μM and a TGI of 0.1 μM. Cell lines that were particularly sensitive to both agents were non-small cell lung NCI-H23, melanoma MALME-3M, M14, and SK-MEL-5, and breast MDA-MB-435 and MDA-N.

On the basis of these data, both compounds were recommended by the NCI Biological Evaluation Committee for preliminary in vivo antitumor evaluation in the mouse hollow fiber assay, but given the similarity in overall cytotoxicity profiles of the two agents and amounts available of each, only pterocellin A (**1**) was tested. The hollow fiber assay involves cultivation of human tumor cells in polyvinylidene hollow fibers and implantation of these into mice, both intraperitoneally (IP) and subcutaneously (SC). The test compound is then administered via the IP route, and its effect on the reduction of the viable cancer cell mass compared to controls is examined. A value of 2 is assigned for each compound dose that results in a 50% or greater reduction in cell mass, and IP and SC results are scored separately. Compounds with a combined IP + SC score of 20, an SC score of 8, or a total cell kill of one or more cell lines are referred for further study.[15] Pterocellin A (**1**) had an IP score of 0, an SC score of 4, a combined IP + SC score of 4, and no cell kill. These results show that pterocellin A was not effective in vivo.

Pterocellins A and B (**1**, **2**) contain a unique heterocyclic skeleton and exhibit potent cytotoxicity in vitro but poor cytotoxicity in vivo. These alkaloids appear to be unique to the *Pterocella* species, as we have carried out studies on other members of the Catenicellidae and all examined to date contain β-carboline alkaloids,[16,17] rather than pterocellin-like compounds.

Experimental Section

General Experimental Procedures. Melting points were determined on a Reichert Thermovar hotstage microscope and were uncorrected. All NMR spectra, except the $^1H-^{15}N$ HMBC spectrum, were determined on a Bruker Avance 9.4 T instrument, operating at 400 MHz for ^1H and 100 MHz for ^{13}C. Spectra were acquired in $CDCl_3$. ^1H NMR and ^{13}C NMR chemical shifts were referenced to residual chloroform (7.26 and 77.0 ppm, respectively). Heteronuclear $^1H-^{13}C$ connectivities were determined by gradient-selected HSQC and HMBC experiments run with shortened relaxation delays and acquisition times. COSY NMR experiments employed standard pulse sequences. NOE correlations were determined by a series of one-dimensional experiments. $^1H-^{15}N$ NMR data were collected on a Bruker Avance 9.4 T spectrometer equipped with a 5 mm triple-resonance HCN inverse detection probe. Standard Bruker pulse sequences were utilized. The $^1H-^{15}N$ HMBC was optimized for 6.0 Hz. Data were referenced to liquid NH_3 using urea as an external standard.[18] High-resolution mass spectral data were obtained in the EI mode on a VG 7070E mass spectrometer. UV−visible spectra were acquired in methanol on a Hewlett-Packard 8453 single-beam UV−visible diode-array spectrophotometer.

Collection of *P. vesiculosa*. Colonies of bryozoans (370 g wet weight) were collected by scuba in 1999 from the Hen and Chicken Islands to the north of New Zealand and stored frozen. A voucher specimen, 99-AI-01-02, is held at the Department of Chemistry, University of Waikato. The bryozoan was identified by one of us (D.P.G.). *P. vesiculosa* is an erect, bushy species, rooted to the substratum and composed of jointed chains of single or paired feeding zooids, typical of the family Catenicellidae.

Extraction, Isolation, and Characterization. The bryozoan (370 g wet weight) was macerated in a blender and extracted with $MeOH/CH_2Cl_2$ (3:1) (400 mL) three times. The combined extract was filtered and the solvent removed in vacuo. The crude extract (12 g), which possessed activity against P388 murine leukemia cells, was fractionated by reversed-phase flash column chromatography on C_{18} silica using a steep-stepped gradient from H_2O to MeOH to CH_2Cl_2. Three fractions from this column contained a red spot by TLC (silica, EtOAc/MeOH (5:1)) and also possessed activity against P388 murine leukemia cells. These three fractions were combined (382 mg) and subjected to further chromatography on C_{18} silica. Three fractions from this column were combined (59 mg) on the basis of TLC analysis and P388 activity and subjected to gel permeation on Sephadex LH-20 in MeOH. Fractions 9 and 11 from this column yielded the red amorphous solids pterocellin A (**1**) (8 mg) and pterocellin B (**2**) (3 mg), respectively. Further extractions of larger samples of bryozoan resulted in isolation of sufficient quantities of pterocellin A (**1**) for in vitro and in vivo testing at the NCI.

Pterocellin A (1): dark red needles (toluene); mp 172−173°C; UV (MeOH) λ_{max} (ϵ) 202 (3.83), 256 (3.70), 280 (3.87), 483 (2.93); ^1H NMR data, see Table 1; ^{13}C NMR and ^{15}N NMR data, see Table 2; HREIMS *m/z* found 284.11598 (calcd for $C_{16}H_{16}N_2O_3$, 284.11609).

Pterocellin B (2): UV (MeOH) λ_{max} (ϵ) 202 (4.08), 287 (3.63), 485 (2.63); ^1H NMR data, see Table 1; ^{13}C NMR data, see Table 2; HREIMS *m/z* found 318.10019 (calcd for $C_{19}H_{14}N_2O_3$, 318.10044).

X-ray Crystallography of Pterocellin A (1). Crystals suitable for data collection were grown by diffusion of heptane into a toluene solution of **1**. Intensity data were collected on a Siemens SMART diffractometer with a CCD area detector, using Mo Kα radiation, and processed routinely with the SAINT software.[19] Data were retained only to $2\theta = 50°$ because of a drop off in intensity above this value. The structure was solved by direct methods and refined on F^2 with the SHELX programs.[20] A penultimate difference map showed one significant residual peak, which was assigned to the oxygen atom of a water of crystallization in the lattice.

Crystal data: $C_{16}H_{14}N_2O_3 \cdot 0.33\ H_2O$, $M = 290.31$, monoclinic, $a = 12.4450(2)$ Å, $b = 19.6646(1)$ Å, $c = 17.9777(2)$ Å, $\beta = 99.79(1)°$, $U = 4348.06(9)$ Å^3, $T = 200$ K, space group $P2_1/n$, $Z = 12$, μ(Mo Kα) = 0.094 mm^{-1}, 22 713 reflections collected,

7640 unique (R_{int} 0.0316) used after correction for absorption ($T_{max, min}$ = 1.000, 0.782). Crystal dimensions 0.46 × 0.22 × 0.14 mm^3. Refinement gave $R_1 = 0.0475$ [5292 data with $I > 2\sigma(I)$] and $wR_2 = 0.1273$ (all data). Hydrogen atoms were included in calculated positions, except for those on the lattice water molecule.[21]

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Supporting Information Available: ^1H and ^{13}C NMR spectra of **1** and **2**, tables of X-ray crystallographic data for **1**, and a full table of cytotoxicity data for **1** and **2** in the NCI 60 cell line panel. This material is available free of charge via the Internet at http://pubs.acs.org.

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(21) Crystallographic data for the structures reported in this paper have been deposited with the Cambridge Crystallographic Data Centre (Accession No. CCDC 210629). Copies of the data can be obtained, free of charge, on application to the Director, CCDC, 12 Union Rd., Cambridge, CB2 1EZ, UK (fax: +44-(0)1223-336033 or e-mail: deposit@ccdc.cam.ac.uk).

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