Further Pterocellins from the New Zealand Marine Bryozoan *Pterocella vesiculosa*

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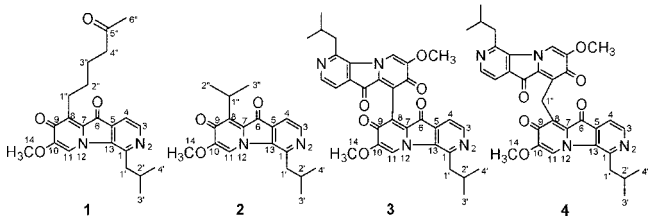
Four new alkaloids, pterocellins C−F (**1**–**4**), have been isolated from the New Zealand marine bryozoan *Pterocella vesiculosa*. Structural elucidation was achieved through NMR spectroscopic and mass spectrometric analysis and comparison of spectroscopic data with that of the known compounds pterocellins A and B. The biological activities of **1**–**4** were assessed in a number of different assay systems and compared with those of pterocellins A and B.

Despite the fact that comparatively little research has been undertaken into the chemistry of bryozoans as compared with that 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 compounds are the bryostatins,[2] which are macrocyclic polyketides; however the vast majority of bryozoan metabolites isolated to date have been alkaloids[3] such as the euthyroideones[4] and the amathaspiramides.[5]

As part of our ongoing search for bioactive and/or novel compounds from New Zealand marine bryozoans, we undertook an investigation of an extract of *Pterocella vesiculosa* (Lamarck, 1816) (order Cheilostomatida, suborder Ascophorina, family Catenicellidae). We have previously reported the isolation and structural elucidation of the alkaloids pterocellins A and B[6] from the bryozoan and proposed tentative structures for two further analogues.[1] Pterocellins A and B possess relatively potent antitumor, antibacterial, and antifungal activity in vitro but were inactive in the in vivo hollow fiber antitumor assay at the NCI.[6] We now report the isolation and complete structural elucidation and bioactivity determination of four additional pterocellins, C−F (**1**–**4**), two of which, pterocellins E and F, are dimeric.



1 **2** **3** **4**

P. vesiculosa is currently known only from waters off the North Island of New Zealand and South Eastern Australia.[6] The bryozoan was collected by scuba from the Alderman Islands, off the North Island of New Zealand, and identified as *P. vesiculosa*. An extract of the bryozoan ($MeOH/CH_2Cl_2$ (3:1)) was subjected to reversed-phase flash column chromatography and gel permeation chromatography, with final purification by HPLC, to give the red pterocellins C−F (**1**–**4**) in 2.6×10^{-4}, 1.5×10^{-4}, 3.3×10^{-4}, and 4.3×10^{-4}% yield, respectively (based on bryozoan wet weight). The extract also contained pterocellins A and B at considerably higher levels.

Structures of the new pterocellins were determined by comparison of their ^1H and ^{13}C NMR spectra with those of pterocellins A and B and by extensive use of 2-D NMR techniques such as COSY, HSQC, and HMBC. Mass spectrometric analysis was the key step in the determination of the structures of the dimers **3** and **4**.

Positive ion ESIMS of pterocellin C contained peaks at 383 $[M + H]^+$, 405 $[M + Na]^+$, 765 $[2\ M + H]^+$, and 787 $[2\ M + Na]^+$. HRESIMS of pterocellin C (**1**) contained peaks at *m/z* 383.1987 $[M + H]^+$, 405.1812 $[M + Na]^+$, and 787.3709 $[2\ M + Na]^+$, consistent with a molecular formula of $C_{22}H_{26}N_2O_4$. Comparison of the ^1H NMR spectrum of **1** (Table 1) in $CDCl_3$ with that of pterocellin A indicated that it contained many similar features to the spectrum of pterocellin A, namely, a methoxyl proton resonance, and resonances suggestive of the presence of an isobutyl group. There was also one less aromatic proton singlet than in the spectrum of pterocellin A, suggesting that pterocellin C was substituted at either C-8 or C-11. Other resonances in the ^1H NMR spectrum of **1** (Table 1) were indicative of the presence of further aliphatic protons. The ^{13}C NMR spectrum acquired in $CDCl_3$ (Table 2) contained 21 resonances, of which all but six could be accounted for as belonging to the heterocyclic core indicative of pterocellin A and to an isobutyl group. The remaining six carbon resonances including a ketone resonance at 209.6 ppm therefore represented a side chain that must be attached at either C-8 or C-11. Atom connectivities were established by COSY, $^1H-^{13}C$ HSQC, and $^1H-^{13}C$ HMBC NMR experiments. The results of these experiments confirmed the presence of the heterocyclic core and isobutyl C-1 substituent as for pterocellin A and established the presence of a 5-keto-hexanoyl side chain at C-8 or C-11. The side chain was placed at C-8 through analysis of the $^1H-^{13}C$ HMBC results (Table 2). For example, the aromatic singlet proton at 7.97 ppm displayed correlations to C-7, C-9, and C-13. This last correlation could only be rationalized if this proton was assigned as H-11. In addition, HMBC correlations from H-1″ to C-7, C-8, and C-9 and from H-2″ to C-8 confirmed the side-chain placement.

The ^1H NMR spectrum of pterocellin D (**2**) in $CDCl_3$ (Table 1) contained similar resonances to that of pterocellin C (**1**) but lacked the 5-keto-hexanoyl group resonances. Instead it contained a methine septet at 4.36 ppm and a doublet at 1.42 ppm, which integrated as six protons. The ^{13}C NMR spectrum of **2** in $CDCl_3$ (Table 2) also resembled that of **1** in most respects but lacked the 5-keto-hexanoyl group resonances and instead contained a methine carbon signal at 24.8 ppm and a methyl carbon signal at 19.3 ppm. These spectra implied that the structure of **2** was very similar to that of **1** but that an isopropyl group was attached at C-8 instead of a 5-keto-hexanoyl group. $^1H-^{13}C$ HMBC and HSQC NMR experiments facilitated assignment of all ^1H and ^{13}C NMR signals, while HRESIMS of pterocellin D (**2**) contained peaks at *m/z* 327.1735 $[M + H]^+$, 349.1560 $[M + Na]^+$, and 675.3192 $[2\ M + Na]^+$, consistent with a molecular formula of $C_{19}H_{22}N_2O_3$ and thus confirming the structure as **2**.

The ^1H NMR spectrum of pterocellin E (**3**) in $CDCl_3$ (Table 1) strongly resembled that of pterocellin A. It lacked the H-8 signal, yet contained no additional resonances that could be assigned to a substituent at this position. The ^{13}C NMR spectrum of **2** in $CDCl_3$ (Table 2) also resembled that of pterocellin A and also contained no additional resonances. These spectra implied that the structure of **3** was very similar to that of pterocellin A but that there was an

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Table 1. ^{1}H NMR Spectroscopic Data (400 MHz, $CDCl_3$) for Pterocellins C−F (**1**–**4**)

position	pterocellin C (**1**) δ_H (*J* in Hz)	pterocellin D (**2**) δ_H (*J* in Hz)	pterocellin E (**3**) δ_H (*J* in Hz)	pterocellin F (**4**) δ_H (*J* in Hz)
3	8.64, d (4.5)	8.56, d (4.6)	8.61, d (4.5)	8.58, d (4.6)
4	7.70, d (4.5)	7.52, d (4.6)	7.48, d (4.5)	7.58, d (4.6)
11	7.97, s	7.83, s	7.94, s	7.77, s
14	3.93, s	3.91, s	3.98, s	3.82, s
1′	3.15, d (7.2)	3.12, d (7.3)	3.17, d (6.7)	3.10, d (7.0)
2′	2.25, m	2.26, m	2.32, m	2.22, m
3′	1.11, d (6.6)	1.09, d (6.6)	1.14, d (6.3)[a]	1.09, d (6.5)
4′	1.11, d (6.6)	1.09, d (6.6)	1.13, d (6.2)[a]	1.09, d (6.5)
1″	3.14 (obscured)	4.36, septet (7.0)		5.16, s
2″	1.57, pentet (6.4)	1.42, d (7.0)		
3″	1.69, pentet (7.2)	1.42, d (7.0)		
4″	2.52, t (7.3)			
6″	2.16, s			

[a] Values are interchangeable.

Table 2. ^{13}C NMR Data (400 MHz, $CDCl_3$) for Pterocellins C-F (**1–4**)

	pterocellin C (**1**)		pterocellin D (**2**)		pterocellin E (**3**)		pterocellin F (**4**)	
position	δ_C, mult	HMBC[a]	δ_C, mult	HMBC[a]	δ_C, mult	HMBC[a]	δ_C, mult	HMBC[a]
1	146.4, qC	3, 1′	146.7, qC	3, 1′	146.6, qC	3, 1′	146.2, qC	3, 1′
3	146.5, CH	4	146.3, CH	4	147.1, CH	4	146.6, CH	4
4	116.4, CH	3	115.4, CH	3	116.3, CH	3	116.2, CH	3
5	131.2, qC	3	131.0, qC	3, 4	130.5, qC	3	131.1, qC	3
6	184.8, qC	4	186.1, qC		183.4, qC	4	185.0, qC	4
7	131.6, qC	11, 1″	131.6, qC	11, 1″	133.5, qC	11	133.0, qC	11, 1″
8	135.5, qC	1″, 2″	140.1, qC	2″, 3″	119.2, qC		130.1, qC	1″
9	173.2, qC	11, 1″	173.3, qC	11, 1″	170.5, qC	11	173.1, qC	11, 1″
10	149.0, qC	14	149.0, qC	11, 14	150.1, qC	11, 14	148.9, qC	11, 14
11	115.6, CH		115.8, CH		115.6, CH		115.1, CH	
13	139.9, qC	4, 11, 1′	139.7, qC	4, 11, 1′	140.3, qC	4, 11, 1′	139.9, qC	4, 11
14	56.6, CH_3		56.8, CH_3		56.8, CH_3		56.5, CH_3	
1′	44.3, CH_2	3′	45.1, CH_2	3′, 4′	44.6, CH_2	2′, 3′, 4′	44.8, CH_2	3′, 4′
2′	28.7, CH	1′, 3′, 4′	28.5, CH	1′, 3′, 4′	28.6, CH	1′, 3′, 4′	28.5, CH	1′, 3′, 4′
3′	22.4, CH_3	1′, 2′	22.5, CH_3	1′, 2′	[b]22.4, CH_3	1′, 2′	22.5, CH_3	1′
4′	22.4, CH_3	1′, 2′	22.5, CH_3	1′, 2′	[b]22.5, CH_3	1′, 2′	22.5, CH_3	1′
1″	22.6, CH_2	2″	24.8, CH	2″, 3″			18.3, CH_2	
2″	28.0, CH_2	1″, 3″, 4″	19.3, CH_3	1″				
3″	23.5, CH_2	1″, 2″, 4″	19.3, CH_3	1″				
4″	43.2, CH_2	2″, 3″, 6″						
5″	209.6, qC	3″, 4″, 6″						
6″	29.0, CH_3							

[a] HMBC correlations are from proton(s) stated to the indicated carbon. [b] Values are interchangeable.

unknown substituent at C-8. ^{1}H−^{13}C HMBC and HSQC NMR experiments confirmed the substructure of the pterocellin A nucleus substituted at C-8 but gave no additional information that could be used to assign a structure to pterocellin E. Mass spectrometric analysis was the key to the determination of the structure. Positive ion ESIMS of pterocellin E yielded ions at 567 and 589, which appeared to represent an $[M + H]^+$ ion and an $[M + Na]^+$ ion, respectively. This was confirmed by HRESMS of pterocellin E (**3**), which yielded ions at *m/z* 567.2299 $[M + H]^+$ and 589.2107 $[M + Na]^+$, consistent with a molecular formula of $C_{32}H_{30}N_4O_6$. These data established that pterocellin E was a dimer of pterocellin A with a direct C−C bond between the two C-8 carbons, as shown in structure **3**.

The ^{1}H NMR spectrum of pterocellin F (**4**) in $CDCl_3$ (Table 1) was very similar to that of pterocellin E (**3**) but contained an additional singlet resonance at 5.16 ppm. The ^{13}C NMR spectrum of **2** in $CDCl_3$ (Table 2) also resembled that of pterocellin F, but it contained an additional resonance at 18.3 ppm. A ^{1}H−^{13}C HSQC NMR experiment revealed that the additional proton resonance at 5.16 ppm correlated to the carbon signal at 18.3 ppm. ^{1}H−^{13}C HMBC NMR experiments showed that the proton resonance at 5.16 ppm correlated to C-7 and C-9 of the pterocellin nucleus, which placed it as a substituent attached to C-8. A correlation from this proton to a carbon at 130.1 ppm implied that this carbon must be C-8. As for pterocellin E, mass spectrometric analysis was the key to the determination of the structure. Positive ion ESIMS of pterocellin F yielded ions at 581 and 603, which again appeared to represent an $[M + H]^+$ ion and an $[M + Na]^+$ ion, respectively, while HRESIMS of pterocellin F (**4**) yielded ions at *m/z* 581.2428 $[M + H]^+$ and 603.2273 $[M + Na]^+$, consistent with a molecular formula of $C_{33}H_{32}N_4O_6$. These data established that pterocellin F was again a dimer of pterocellin A but that in this case there was a methylene group joining the two C-8 carbons as shown in structure **4**.

The possibility that pterocellins E and F (**3**, **4**) were artifacts of isolation was considered but discounted on the basis of the following evidence. Both were consistently present in extracts (from certain sites), even when different extraction methods and solvent systems were utilized. To further investigate the possibility of artifact formation, samples of pterocellin A were dissolved in CD_2Cl_2 and $CDCl_3$, respectively, in NMR tubes and heated, and then monitored by ^{1}H NMR spectroscopy over time. There was no evidence of any changes in the spectra after 4 days; in fact pterocellin A appears to be very stable in solution. It is therefore most probable that pterocellins E and F are indeed naturally occurring metabolites.

Pterocellins C−F (**1**–**4**) were assayed in P388 murine leukemia, antiviral/cytotoxicity, and antimicrobial assay systems[7,8] (Supporting Information). Pterocellins C(**1**), E (**3**), and F (**4**) were essentially inactive against the P388 murine leukemia cell line with IC_{50} values of >6250 ng/mL, while pterocellin D (**2**) displayed modest activity with an IC_{50} value of 4773 ng/mL. This is in marked contrast to pterocellins A and B, which possess relatively potent activity against the same cell line (477 and 323 ng/mL, respectively).[6] Pterocellins

C (**1**), E (**3**), and F (**4**) were also inactive against the BSC-1 cell line (derived from African Green Monkey kidney cells) used in the antiviral/cytotoxicity assay (Supporting Information), unlike pterocellins A and B, which were strongly cytotoxic to this cell line.[6] Pterocellin D (**2**) was not tested due to unavailability of the assay. Pterocellins C−F (**1**–**4**) exhibited variable levels of activity against the Gram-positive bacterium *Bacillus subtilis*, with **1** and **2** exhibiting reasonably strong activity (only slightly less active than pterocellins A and B), whereas **3** and **4** were considerably less active. Pterocellin D (**2**) exhibited modest activity against *Trichophyton mentagrophytes*, while pterocellins C (**1**), E (**3**), and F (**4**) were all inactive. As for pterocellins A and B, pterocellins C−F (**1**–**4**) were inactive against the other two bacteria, *Escherichia coli* and *Pseudomonas aeruginosa*, and two fungi, *Candida albicans* and *Cladisporum resinae*, that they were tested against. The overall pattern of pterocellins A and B exhibiting much stronger cytotoxicity than pterocellins C−F suggests that H-8 may be crucial for the observed bioactivity. For the antimicrobial activity, it would appear that size may be a factor and that it is still the region of the molecule near C-8 that is important for activity.

Experimental Section

General Experimental Procedures. UV–visible spectra were acquired in methanol on a Varian CARY 100 UV/visible spectrophotometer. All NMR spectra were determined on a Bruker Avance 9.4 T instrument, operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C. Spectra were acquired in $CDCl_3$. ^{1}H NMR and ^{13}C NMR chemical shifts were referenced to residual chloroform (7.26 and 77.0 ppm, respectively). Heteronuclear ^{1}H−^{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. ESIMS mass spectral data were measured on a Fisons VG Platform II machine operating in positive ion mode with the cone voltage set between 20 and 80 eV. High-resolution mass spectral data were obtained in the ES mode on a Bruker Daltonics MicrOTOF mass spectrometer. HPLC was carried out utilizing a Waters system with a Waters 515 pump, a Waters 996 PDA detector, and Millenium software. Details of the assay procedures have been reported elsewhere.[7,8]

Collection of *P. vesiculosa*. Colonies of bryozoans (2316 g wet weight) were collected by scuba at the Alderman Islands off the North Island of New Zealand and stored frozen. A voucher specimen, 01-FI-01-06, is held at the Department of Chemistry, University of Waikato. The bryozoan was identified by Dr. Dennis Gordon.

Extraction, Isolation, and Characterization. The bryozoan (1390 g wet weight) was macerated in a blender and exhaustively extracted with MeOH/CH_2Cl_2 (3:1) (4 L). The combined extract was filtered and the solvent removed in vacuo. The crude extract (40 g) was crushed and exhaustively extracted with CH_2Cl_2 and then was again filtered and the solvent removed in vacuo. The CH_2Cl_2 extract (4.2 g) was fractionated by reversed-phase flash column chromatography on C_{18} silica using a stepped gradient from H_2O to MeOH to CH_2Cl_2. Fractions from this column containing pterocellins on the basis of TLC analysis (pink or red spots, silica, EtOAc/MeOH (5:1)) were subjected to further chromatography on C_{18} silica and gel permeation on Sephadex LH-20 in MeOH to yield a number of fractions containing crude pterocellins. These were purified by HPLC on an Alltech semipreparative C18 column, with a H_2O to MeOH gradient at a flow rate of 5 mL/min to yield pure pterocellins C (**1**) (4 mg), D (**2**) (2 mg), E (**3**) (5 mg), and F (**4**) (6 mg).

Pterocellin C (1): amorphous, orange-red solid; UV (MeOH) λ_{max} (ϵ) 203 (4.87), 224 sh (4.76), 257 (4.74), 290 (5.02), 489 (4.06) nm; ^{1}H NMR data, see Table 1; ^{13}C NMR data, see Table 2; HRESIMS *m/z* found 383.1987 $[MH]^+$ (calcd for $C_{22}H_{27}N_2O_4$, 383.1965), 405.1812 $[MNa]^+$ (calcd for $C_{22}H_{26}N_2O_4Na$, 405.1785), and 787.3709 $[2MNa]^+$ (calcd for $C_{44}H_{52}N_4O_8Na$, 787.3677).

Pterocellin D (2): amorphous, red-orange solid; UV (MeOH) λ_{max} (ϵ) 203 (4.29), 255 (4.01), 290 (4.11), 490 (3.08) nm; ^{1}H NMR data, see Table 1; ^{13}C NMR data, see Table 2; HRESIMS *m/z* found 327.1735 $[MH]^+$ (calcd for $C_{19}H_{23}N_2O_3$, 327.1703), 349.1560 $[MNa]^+$ (calcd for $C_{19}H_{22}N_2O_3Na$, 349.1523), and 675.3192 $[2MNa]^+$ (calcd for $C_{38}H_{44}N_4O_6Na$, 675.3153).

Pterocellin E (3): amorphous, rose pink solid; UV (MeOH) λ_{max} (ϵ) 201 (4.65), 230 sh (4.48), 258 sh (4.48), 286 (4.57), 480 (3.61) nm; ^{1}H NMR data, see Table 1; ^{13}C NMR data, see Table 2; HRESIMS *m/z* found 567.2299 $[MH]^+$ (calcd for $C_{32}H_{31}N_4O_6$, 567.2238) and 589.2107 $[MNa]^+$ (calcd for $C_{32}H_{30}N_4O_6Na$, 589.2058).

Pterocellin F (4): amorphous, dark red solid; UV (MeOH) λ_{max} (ϵ) 202 (3.80), 286 (3.36), 494 (2.20) nm; ^{1}H NMR data, see Table 1; ^{13}C NMR data, see Table 2; HRESIMS *m/z* found 581.2428 $[MH]^+$ (calcd for $C_{33}H_{33}N_4O_6$, 581.2395) and 603.2273 $[MNa]^+$ (calcd for $C_{33}H_{32}N_4O_6Na$, 603.2214).

Variable-Temperature NMR Spectroscopy. Pterocellin A (4 mg) was dissolved in CD_2Cl_2 in an NMR tube. The ^{1}H NMR spectrum was recorded at 30 °C, and signal integrals were measured. The sample was then heated to 38 °C, and ^{1}H NMR spectra were obtained at times 15, 150, and 270 min and 4 days with integration of the resulting spectra. A similar procedure was repeated for pterocellin A in $CDCl_3$ with heating to 50 °C.

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Supporting Information Available: ^{1}H NMR spectra of **1**–**4**. P388, antiviral/cytotoxicity, and antimicrobial/antifungal assay results for **1**–**4**. This material is available free of charge via the Internet at http://pubs.acs.org.

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