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Kottamides A−D: Novel Bioactive Imidazolone-Containing Alkaloids from the New Zealand Ascidian *Pycnoclavella kottae*

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Abstract: Kottamides A−D (**1**−**4**), novel 2,2,5-trisubstituted imidazolone-containing alkaloids, were isolated from the New Zealand endemic ascidian *Pycnoclavella kottae* and structurally characterized using ^{15}N natural abundance 2-D NMR in addition to standard spectroscopic methods. The kottamides exhibited anti-inflammatory and anti-metabolic activity as well as cytotoxicity toward tumor cell lines.

Ascidians have proven to be a rich source of amino acid-derived compounds, some with potent biological activities.[1] Such compounds vary in structure from simple peptide analogues to more complex cyclic peptides and alkaloids.[2]

As part of our ongoing study of New Zealand ascidians as sources of novel biologically active secondary metabolites,[3] we now report the isolation and structural elucidation, including the use of ^{15}N natural abundance 2-D NMR, of kottamides A−D (**1**−**4**) from the endemic ascidian *Pycnoclavella kottae* (Millar**,** 1960) (order Aplousobranchia, family Pycnoclavellidae)[4] collected at the Three Kings Islands, New Zealand. The kottamides are novel imidazol-4-one-containing alkaloids possessing unprecedented substitution. While imidazol-4-one-bearing alkaloids have been reported as synthetic precursors,[5] only the rhopaladins from ascidians,[6] and the fused examples luciferin[7] and coelenterazine,[8] have been reported from natural sources. The kottamides are the first examples of 2,2,5-trisubstituted imidazolone natural products. To the best of our knowledge, this is the first reported study on chemistry from an ascidian of the genus *Pycnoclavella*.

Fractionation of the cytotoxic organic extract using C_{18} reversed-phase column chromatography followed by repeated semipreparative C_{18} HPLC afforded four related compounds that were characterized using spectroscopic techniques.

A molecular formula of $C_{21}H_{24}Br_2N_4O_2$ for **1** was established by HREIMS with the observed isotope pattern supporting the presence of two bromine atoms. UV absorbances of 284 and 236 nm suggested an aromatic chromophore. Inspection of the ^1H NMR spectrum and COSY data established the presence of four independent ^1H spin systems, two aromatic/olefinic and two alkyl, in addition to an isolated exchangeable proton at δ 7.46 (H-3). A broad ^1H NMR resonance at δ 8.43 (H-12) coupled to δ 7.19 (H-11) and $^1H-^{13}C$ HMBC correlations from H-11 to the quaternary carbons at δ 135.5 (C-13) and 127.1 (C-18) suggested an indole moiety,[9,10] while proton singlets at δ 7.73 (H-14) and 7.85 (H-17) indicated that the indole was 5,6-disubstituted. ^1H NMR resonances at δ 5.93 (H-9), 6.93 (H-8), and 8.23 (NH-7) and $^1H-^{13}C$ HMBC correlations from H-8 to C-10 (δ 110.8) and from H-9 to C-10, C-11 (δ 123.5), and C-18 indicated that the indole moiety was substituted at C-10 with a *Z*-geometry enamide fragment (J_{HH} 9.1 Hz).[9] The ^{13}C carbonyl resonance at δ 166.0 was assigned to C-6 by way of $^1H-^{13}C$ HMBC correlations from H-7 and H-8. The two alkyl spin systems were established as 2-propyl and 2-butyl groups on the basis of interpretation of COSY, TOCSY, $^1H-^{13}C$ HSQC, and $^1H-^{13}C$ HMBC data. The 2-propyl group was placed at the quaternary C-2 (δ 88.5) by $^1H-^{13}C$ HMBC correlations from H-1′ (δ 0.83, d), H-2′ (δ 2.42, septuplet), and H-3′ (δ 1.05, d) to C-2 and from H-2′ to C-6 while the 2-butyl group was placed at C-5 (δ 176.1) by $^1H-^{13}C$ HMBC correlations from H-1″ (δ 1.12, d) and H-3″ (δ 1.45, 1.32) to C-5. The remaining atoms, CHN_2O, required three degrees of unsaturation, suggesting a ring and two double bonds. Assignment was achieved by interpretation of $^1H-^{13}C$ HMBC, $^1H-^{15}N$ HSQC and HMBC data[11] (see the Supporting Information). Crucial $^1H-^{15}N$ HMBC correlations from H-2′ to N-1 (δ 326.1) and N-3 (δ 132.2), and from H-2″ (δ 2.76) to N-1, combined with $^1H-^{13}C$ HMBC correlations from H-2″ to C-4 (δ 164.0) and from the broad proton resonance at ^1H δ 7.46 (H-3) to C-2, C-4, and C-5 established the presence of a 2,2,5-trisubstituted imidazol-4-one ring. Chemical shifts of C-4 and C-5 were comparable to those of similar ring-systems.[5] The planar structure of kottamide A could

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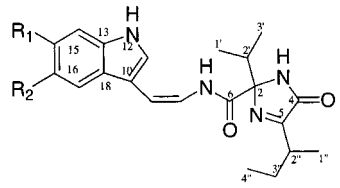
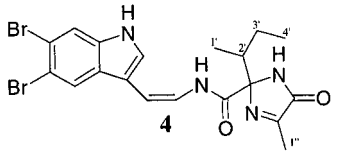
therefore be assigned as **1**. Stereochemistry at C-2 and C-2″ (δ 34.3) was not deduced.

Kottamides B (**2**) and C (**3**) were obtained as an inseparable mixture in 0.8:1.0 ratio as determined by NMR and analytical HPLC. FABMS indicated the presence of only one bromine atom in each of **2** and **3**. Inspection of the ^{1}H NMR spectrum and 2D-NMR data for the mixture confirmed the presence of two different mono-brominated analogues of kottamide A (**1**). An ^{1}H−^{13}C HMBC correlation from H-16 (δ 7.28) to C-18 (δ 125.2) for the minor compound in the mixture, while a correlation from H-15 (δ 7.36) to C-13 (δ 134.6) for the major compound, confirmed **2** and **3** as the 16-debromo and 15-debromo analogues, respectively.



1 R_1=Br, R_2=Br
2 R_1=Br, R_2=H
3 R_1=H, R_2=Br



4

HREIMS of **4** gave a molecular formula of $C_{19}H_{20}Br_2N_4O_2$. NMR data observed for **4** were almost identical to those observed for kottamide A (**1**). Differences, however, were noted in the nature of the substituents on the imidazolone ring and the points of their attachment. Imidazolone ring carbon C-2 was shifted downfield slightly to δ 89.2, while C-5 was shifted upfield to δ 169.3. The 2-propyl group present in **1** was absent in **4**, while the 2-butyl could be placed at C-2 by way of ^{1}H−^{13}C HMBC correlations from δ 0.97 (3H, d, J = 6.8 Hz, H-1′) and δ 1.51, 0.88 (each 1H, m, H-3′) to C-2. The final substituent on the imidazolone ring was established as a methyl group (δ_H 2.17, δ_C 14.4), which was placed at C-5 by virtue of the observation of strong ^{1}H−^{13}C HMBC correlations from H-1″ to C-4 (δ 164.5) and C-5.

Comparison of analytical reversed-phase C_{18} HPLC traces of the crude organic extract of *P. kottae* and each of the isolated kottamides, **1**, **2/3**, and **4**, confirmed them to be the major components of the extract.

Kottamide D (**4**) was investigated for anti-inflammatory and anti-metabolic properties in microplate assays using the cell-impermeable tetrazolium salt WST-1 (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium, monosodium salt) and the intermediate electron acceptor 1-methoxy phenazinemethosulfate (PMS).[12] Compound **4** exhibited potent anti-metabolic activity (IC_{50} 6−10 μM) with both anti-proliferative and anti-inflammatory activity in the 2−200 μM range. In a short-term metabolic assay over 1.25 h, **4** inhibited the reduction of WST-1 by human HL60 and Jurkat cells by 83−92% at 40 μM. While, over 48 h, **4** inhibited proliferation of HL60 cells by 66% at 20 μM and 9% at 2 μM. Similar results were obtained with the nontransformed interleukin-3-dependent murine cell line, 32Dcl23. Under the same conditions, the tubulin-stabilizing anti-cancer drug Taxol inhibited the reduction of WST-1 by only 3−5% in the short-term assay but after 48 h inhibited proliferative responses by 64−81% at 1 μM. In an anti-inflammatory assay using activated human peripheral blood neutrophils, **4** inhibited superoxide production generated in response to the inflammation promoting agents *N*-formylmethionylleucylphenylalanine (fMLP) and phorbol myristate acetate (PMA) in the range 95−100% (200 μM concentration).[13] In contrast, Taxol did not inhibit the fMLP response and only weakly inhibited PMA-induced superoxide production.

Kottamides A−D (**1**−**4**) were also assayed for a range of cytotoxic and antimicrobial properties. All four kottamides exhibited moderate P388 activity with IC_{50} values of 20, 14, and 36 μM for compounds **1**, the **2**/**3** mixture, and **4**, respectively. Further evaluation of **1** at the NCI revealed modest cytotoxicity (panel average values: GI_{50} 15.1, TGI 33.9, and LC_{50} 67.6 μM). Compound **1** was also tested for cytotoxicity/antiviral activity against the African Green Monkey kidney cell line (BSC-1) infected with the RNA virus PV1[10] and was found to have moderate cytotoxicity (zone size >4.5 mm, 240 μg loading) and some antiviral activity (zone size 1−2 mm). Compound **1** exhibited no antimicrobial activity in disk assays against the bacteria *Bacillus subtilis* and *Escherichia coli* or the fungi *Candida albicans* and *Trichophyton mentagrophytes* at 240 μg loading.

A plausible biogenesis of **1**−**4** involves stereospecific imidazolone ring formation from modified Trp-Val-Ile and Trp-Ile-Ala tripeptide precursors.

Experimental Section

General Experimental Procedures. Details of general procedures, analytical HPLC conditions,[14] and biological assays[10] have been reported previously. ^{1}H−^{15}N NMR data were collected on a 400 MHz spectrometer equipped with a 5 mm triple-resonance HCN inverse detection probe. Standard Bruker pulse sequences were utilized. ^{1}H−^{15}N HMBC: Number of scans 112, increments 256, optimized for 6.0 Hz, experiment time 14 h. ^{1}H−^{15}N HSQC: Number of scans 24, increments 256, optimized for 87 Hz, experiment time 1.5 h. Data were referenced to liquid NH_3 using urea as an external standard.

Collection, Extraction, and Isolation Procedures. Specimens (collection no. 99MNP0103) of *P. kottae* were collected from the Three Kings Islands, New Zealand, and identified by one of us (G.L.). The ascidians were freeze-dried (dry weight 31.54 g) and exhaustively extracted with MeOH and CH_2Cl_2. The solvents were removed in vacuo yielding a brown/orange extract (7.44 g). A portion (4.13 g) of crude extract was fractionated using C_{18} reversed-phase flash column chromatography using a steep gradient from MeOH/H_2O (60:40) to MeOH. Compounds of interest were concentrated in the 75% MeOH fraction. Repeated semipreparative HPLC (C_{18}, MeOH/H_2O (85:15); 5 mL/min) yielded kottamide A (**1**) (3.9 mg, 0.020% dry wt) and a mixture of related compounds. The related compounds were then separated using further semipreparative HPLC (C_{18}, MeOH/H_2O (80:20); 5 mL/min) yielding an inseparable mixture of kottamides

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TABLE 1. ^{1}H NMR Data ($CDCl_3$) (δ, Mult, J) for Kottamides A (1), B (2), C (3), and D (4)

atom	1	2	3	4
3	7.46 (bs)	7.23 (bs)	7.23 (bs)	7.25 (bs)
7	8.23 (bd, 11.2)	8.25 (bd, 10.5)	8.27 (bd, 10.9)	8.13 (bd, 11.2)
8	6.93 (dd, 11.3, 9.1)	6.92 (dd, 11.3, 9.2)	6.92 (dd, 11.3, 9.2)	6.91 (dd, 11.2, 9.2)
9	5.93 (d, 9.1)	6.00 (d, 9.9)	5.97 (d, 10.0)	5.95 (d, 9.1)
11	7.19 (d, 1.9)	7.18 (d, 2.4)	7.20 (d, 2.3)	7.20 (d, 2.3)
12	8.43 (bs)	8.32 (bs)	8.35 (bs)	8.39 (bs)
14	7.73 (s)	7.58 (d, 1.6)	7.30 (d, 8.6)	7.73 (s)
15			7.36 (dd, 8.6, 1.8)	
16		7.28 (dd, 8.6, 1.7)		
17	7.85 (s)	7.43 (d, 8.4)	7.72 (d, 1.6)	7.81 (s)
1′	0.83 (d, 6.8)	0.83 (d, 6.8)	0.85 (d, 6.8)	0.97 (d, 6.8)
2′	2.42 (sep, 6.9)	2.41 (sep, 6.9)	2.41 (sep, 6.9)	2.08 (m)
3′	1.05 (d, 6.8)	1.01 (d, 6.7)	1.05 (d, 6.7)	1.51, 0.88 (m)
4′				0.91 (t, 6.8)
1″	1.12 (d, 7.0)	1.14 (d, 7.2)	1.12 (d, 7.1)	2.17 (s)
2″	2.76 (m)	2.76 (m)	2.76 (m)	
3″	1.45, 1.32 (m)	1.49, 1.35 (m)	1.49, 1.35 (m)	
4″	0.67 (t, 7.5)	0.72 (t, 7.5)	0.67 (t, 7.5)	

B (**2**) and C (**3**) (2.6 mg, 0.015% dry wt; 0.8:1.0) and kottamide D (**4**) (2.1 mg, 0.012% dry wt).

Kottamide A (1). Compound **1** was obtained as a white amorphous solid: $[\alpha]^{20}_D$ +160 (*c* 0.20, MeOH); UV (MeOH) λ_{max} (log ϵ) 236 (4.46), 284 (4.14) nm; IR (smear) ν_{max} 3271, 2925, 1712, 1531, 1490, 1452 cm^{-1}; ^{1}H NMR data, see Table 1; ^{13}C NMR and ^{15}N NMR data, see Table 2; EIMS *m*/*z* (rel int) 526/524/522 $[M]^+$ (2/4/2), 344/342/340 (2/3/2), 320/318/316 (3/6/3), 305/303/301 (7/13/6), 304/302/300 (5/10/5); HREIMS *m*/*z* 526.0215 (calcd for $C_{21}H_{24}{}^{81}Br_2N_4O_2$ 526.0225), 524.0231 (calcd for $C_{21}H_{24}{}^{79}Br^{81}BrN_4O_2$ 524.0246), 522.0257 (calcd for $C_{21}H_{24}{}^{79}Br_2N_4O_2$ 522.0266).

Kottamides B (2) and C (3). The mixture of compounds **2** and **3** was obtained as a white amorphous solid (0.8:1 ratio): $[\alpha]^{20}_D$ +245 (*c* 0.20, MeOH); UV (MeOH) λ_{max} (log ϵ) 232 (4.44), 286 (4.13) nm; IR (smear) ν_{max} 3291, 2968, 1714, 1682, 1533, 1493, 1456 cm^{-1}; ^{1}H NMR data, see Table 1; ^{13}C NMR data, see Table 2; FABMS *m*/*z* 447/445 $[M + H]^+$, 446/444 $[M]^+$; HR-FABMS *m*/*z* 446.1150 (calcd for $C_{21}H_{25}{}^{81}BrN_4O_2$ 446.1140), 444.1163 (calcd for $C_{21}H_{25}{}^{79}BrN_4O_2$ 444.1161).

Kottamide D (4). Compound **4** was obtained as a white amorphous solid: $[\alpha]^{20}_D$ +150 (*c* 0.20, MeOH); UV (MeOH) λ_{max} (log ϵ) 237 (4.39), 286 (4.11) nm; IR (smear) ν_{max} 3293, 2968, 1715, 1671, 1531, 1489, 1452 cm^{-1}; ^{1}H NMR data, see Table 1; ^{13}C NMR data, see Table 2; EIMS *m*/*z* (rel int) 498/496/494 $[M]^+$ (4/8/4), 344/342/340 (12/24/12), 316/314/312 (3/7/3), 289/287/285 (4/8/4); HREIMS *m*/*z* 497.9898 (calcd for $C_{19}H_{20}{}^{81}Br_2N_4O_2$ 497.9912), 495.9930 (calcd for $C_{19}H_{20}{}^{79}Br^{81}BrN_4O_2$ 495.9933), 493.9930 (calcd for $C_{19}H_{20}{}^{79}Br_2N_4O_2$ 493.9953).

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TABLE 2. ^{13}C and ^{15}N[a] NMR and HMBC Data ($CDCl_3$) for Kottamides A (1), B (2), C (3), and D (4)

atom	1	2	3	4
N-1	326.1 (2′, 2″)			
2	88.5 (3, 1′, 2′, 3′)	88.5 (3, 1′, 2′, 3′)	88.5 (3, 1′, 2′, 3′)	89.2 (1′, 3′, 1″)
N-3	132.2 (2′)			
4	164.0 (3, 2″)	164.0 (3, 2″)	164.0 (3, 2″)	164.5 (1″)
5	176.1 (3, 1″, 2″, 3″)	176.0 (3, 1″, 2″, 3″)	176.0 (3, 1″, 2″, 3″)	169.3 (3, 1″)
6	166.0 (7, 8, 2′)	165.9 (8, 2′)	165.9 (8, 2′)	165.8
N-7	132.1 (8, 9)			
8	120.4 (9)	119.9 (9)	119.9 (9)	120.2 (9)
9	103.4 (7, 8)	104.0 (8)	103.9 (8)	103.5 (8)
10	110.8 (8, 9, 11, 17)	111.4 (8, 11)	110.8 (8, 11)	110.8 (8, 9, 11, 17)
11	123.5 (9)	122.2 (9)	122.9 (9)	123.4 (9)
N-12	123.0 (11, 14)			
13	135.5 (11, 17)	136.7 (11, 17)	134.6 (11, 15, 17)	135.5 (11, 17)
14	116.0	114.2 (16)	112.7	116.1
15	118.4 (14, 17)	116.7 (14, 17)	126.0 (17)	118.4 (14, 17)
16	115.95 (14, 17)	123.8 (14)	113.8 (14, 17)	115.9 (14, 17)
17	123.7	120.5	112.7 (15)	123.7
18	127.1 (9, 11, 14)	125.2 (9, 11, 14, 16)	128.0 (9, 11, 14)	127.1 (9, 11, 14)
1′	15.5 (2′, 3′)	15.6 (2′, 3′)	15.6 (2′, 3′)	13.2
2′	35.7 (1′, 3′)	35.6 (1′, 3′)	35.6 (1′, 3′)	42.4 (1′, 3′, 1″)
3′	16.9 (1′, 2′)	16.9 (1′, 2′)	16.9 (1′, 2′)	22.4 (1′, 4′)
4′				11.9 (3′)
1″	17.0 (2″, 3″)	17.1 (3″)	17.1 (3″)	14.4
2″	34.3 (1″, 3″, 4″)	34.3 (1″, 3″, 4″)	34.3 (1″, 3″, 4″)	
3″	26.1 (1″, 2″, 4″)	26.1 (1″, 4″)	26.1 (1″, 4″)	
4″	11.3 (2″, 3″)	11.4 (2″, 3″)	11.4 (2″, 3″)	

[a] ^{15}N data were determined for **1** only.

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Supporting Information Available: ^{1}H and ^{13}C NMR spectra of **1**−**4**. COSY, ^{1}H−^{13}C HSQC, ^{1}H−^{13}C HMBC, ^{1}H−^{15}N HMBC, and ^{1}H−^{15}N HSQC spectra of **1**. This material is available free of charge via the Internet at http://pubs.acs.org.

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