New and Biologically Active Imidazole Alkaloids from Two Sponges of the Genus *Leucetta*

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Chemical investigation of two sponges, *Leucetta chagosensis* and *Leucetta* cf *chagosensis*, collected from the Great Barrier Reef and the Fiji Islands, respectively, has led to the isolation of three new imidazole alkaloids (**1**−**3**), along with the known compounds isonaamine B (**4**) and naamine A (**5**). The structures of the new compounds (**1**−**3**) were elucidated by employing spectroscopic techniques (NMR, MS, UV, and IR). The structures of the known compounds **4** and **5** were determined by comparison of their ^{1}H and ^{13}C NMR spectroscopic data with published values. Compounds **1** and **2** were found to be cytotoxic toward several tumor cell lines (GI_{50} values ranged from 1.3 to 7.0 μg/mL).

Sponges of the genera *Leucetta* and *Clathrina* have previously been shown to be a rich source of imidazole alkaloids including some stable zinc complexes.[1−4] These compounds were also found in associated predatory nudibranchs of the genus *Notodoris*, which are known to sequester these alkaloid pigments.[5−7] Some of these alkaloids have been reported to be antimicrobial, antifungal, and cytotoxic.[8−11] In this paper the details of the isolation and structure elucidation of three new analogues of members of the aforementioned imidazole alkaloid family, together with their in vitro cytotoxicity data, are presented.

The current samples of *L. chagosensis* Dendy, 1913 and *L.* cf *chagosensis*, were collected from Bougainville Reef, Australia, and the Fiji Islands, respectively. After extraction with CH_2Cl_2 and MeOH the organic extracts of the two sponges were evaluated for biological activity. Simultaneous with these assays, investigation the secondary metabolite chemistry of the sponge samples was started. Chromatographic separation of the CH_2Cl_2 and MeOH extracts using C_{18} reversed-phase SPE, VLC, and HPLC yielded two new compounds, **1** and **2**, from the Australian sample and one new compound, **3**, together with the known compounds isonaamine B (**4**) and naamine A (**5**) from the Fiji Island sample.

Mass spectral analysis of compound **1**, a yellow amorphous solid, indicated it to have the molecular formula $C_{24}H_{25}N_5O_5$, and so to have 15 degrees of unsaturation. Its ^{13}C NMR data contained a total of 24 resonances for four methyl, two methylene, and eight methine groups and 10 quarternary carbons. These data also revealed the presence of 11 double bonds (7 × CC; 2 × CN; 2 × CO); **1** was thus tetracyclic. The ^{1}H NMR spectrum of **1** (see Table 1) contained three singlet resonances at δ 3.78, 3.84, and 3.85, for three methoxyl groups, and one singlet resonance at δ 3.19, indicating a N-CH_3 group. In addition, the ^{1}H NMR spectrum contained resonances consistent with the presence of two aromatic rings: one of them 1,3,4-substituted, and the other 1,4-substituted. Remaining in the proton NMR spectrum were resonances for a further olefinic proton (δ 6.50 [H-5]) and for two methylene groups (H_2-7 and H_2-14). After assignment of all protons to their directly bonded carbon atoms via a ^{1}H−^{13}C 2D NMR shift correlated measurement (HSQC), it was possible to deduce major fragments of the molecule from the results of a long-range ^{1}H−^{13}C 2D NMR shift correlated measurement (HMBC; see Table 2). Thus, long-range CH couplings observed between C-11 and H-9, H-10, H-12, H-13, and H_3-21, together with the one observed between H_2-7 and C-8, showed one of the two aromatic rings to be a para-methoxy benzyl group. The low-field chemical shift of H_2-7 (δ 5.29) meant that it was not only benzylic but also α to nitrogen. Further long-range couplings, this time between H_3-22 and C-18, H_3-23 and C-17, and H_2-14 and C-15, revealed the second aromatic ring also to be benzylic: a 3,4-dimethoxybenzyl moiety. On the basis of the HMBCs seen between the resonances for H_2-14 and those for C-4 and C-5 it was evident that CH_2-14 was directly bonded to C-4. The chemical shifts for C-2, C-4, and C-5 together with that for H-5 (δ 6.50) were indicative for a 2-amino-1,4-dibenzylimidazole,[11] meaning the second benzylic group bonded to N-1, a deduction supported by the long-range CH coupling seen between C-2 and H_2-7. At this stage of the structural analysis, the N-CH_3, two amide carbonyls, a C=N, and a NH group required assignment. Both C-26 and C-28 long-range CH coupled with H_3-29. This fact together with the chemical shifts of C-24, C-26, and C-28 (see Table 2) indicated all of the remaining groups to form an *N*-imidazolidinedionyl moiety,[6] which was attached to the 2-amino-1,4-dibenzylimidazolyl part of **1** via N-6. Compound **1** is thus a new member of the 2-amino-1,4-dibenzylimidazole family of alkaloids related to isonaamidine A,[1] for which the trivial name isonaamidine E is proposed.

Compound **2** analyzed for $C_{20}H_{23}N_3O_3$ by HREIMS. Inspection of the ^{1}H NMR spectral data of compound **2** revealed them to be extremely similar to those of compound **2**, the major differences being the chemical shifts for H-5, H_2-7, and H_2-14. These differences could readily be accounted for by the presence of a primary amine function in compound **2** instead of the *N*-imidazolidinedionyl group present in compound **1**, a deduction that was supported by all of the remaining spectral data for **2** (see Tables 1 and 2, and the Experimental Section). Compound **2** is the third member of a group known as isonaamines[1] and is thus named isonaamine C.

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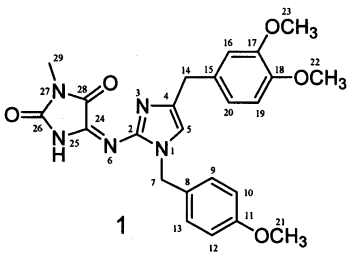

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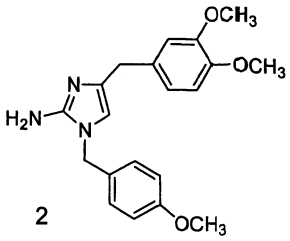

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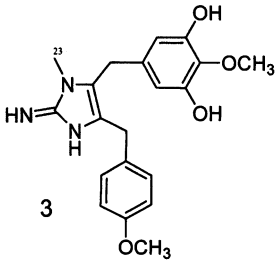

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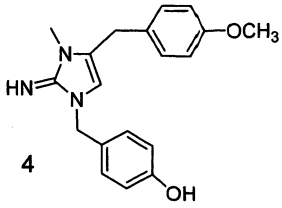

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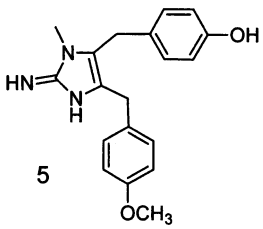

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The molecular formula $C_{20}H_{23}N_3O_4$ was deduced for compound **3** by accurate mass measurement. Comparison of its ^1H and ^{13}C NMR spectral data with those for compounds **1** and **2** (see Tables 1 and 2) clearly showed it to belong to the group of alkaloids known as naamines.[1] In particular, the chemical shift of H_2-7 (δ 3.28), together with the absence of the proton associated with the imidazole ring, the presence of an N-CH_3 group (δ 3.32), and the CH coupling seen between C-5 and H_2-7, all indicated the 4-methoxybenzyl group present in compound **3** to bond to C-5, and not to N-1. In contrast to the para-methoxybenzyl group found at C-4 in compounds **1** and **2**, compound **3** contained a symmetrically (possibly 1,3,4,5) substituted benzyl group. The deduction that this group was symmetrically substituted came from the fact that for C-17 and C-19 (both δ 152.2), C-16 and C-20 (both δ 108.4), and H-16 and H-20 (both δ 6.19) only single resonances were observed in their respective NMR spectra. A long-range CH coupling observed between C-18 and H_3-22 positioned the methoxyl group at C-18, while ROESY cross-peaks seen between the resonances for H_2-14 and those for H-16 and H-20 placed the remaining two substituents (2 × OH) at C-17 and C-19 and so completed the structure of confirmed compound **3**. For **3**, the fifth naamine to be reported, the trivial name naamine E is proposed.

Together with compound **3** the two previously reported compounds isonaamine B (**4**) and naamine A (**5**) were also isolated from the second sponge sample.

The cytotoxic effects of compounds **1**−**5** against HM02, HepG2, and Huh7 cell lines were investigated, and compounds **1** and **2** were found to be active. For compound **1** 50% growth inhibition (GI_{50}) values of 7.0 (HM02), (HepG2), and 1.3 μg/mL (Huh7) were determined, and 5.3 (HM02), 2.2 (HepG2), and 2.1 μg/mL (Huh7) for compound **2**. In antimicrobial, antifungal, and antialgal assays compounds **1**−**5** were all found to be inactive at the 50 μg level. As imidazole alkaloids have been reported to have cytotoxic, antimicrobial, and antifungal properties,[8−11] it was not surprising to find some of the isolates to be active in at least some of the applied test systems. However, it is certainly noteworthy that despite the close structural similarity of compounds **1**−**5**, only compounds **1** and **2** were found to be cytotoxic.

Experimental Section

General Experimental Procedures. UV and IR spectra were obtained employing Perkin-Elmer Lambda 40 and Perkin-Elmer Spectrum BX instruments, respectively. HPLC was carried out using a Merck-Hitachi system consisting of a L-6200 A pump, a L-4500 A photodiode array detector (PDA), and a D-6000 A interface, and a Waters system consisting of a 600 pump, a 996 PDA, and a 717 plus autosampler. ^1H and ^{13}C NMR spectra were recorded on Bruker Avance 500 DMX and Bruker Avance 300 DPX spectrometers in $CDCl_3$ and CD_3OD. Spectra were referenced to residual solvent signals with resonances at $\delta_{H/C}$ 7.26/77.0 ($CDCl_3$) and $\delta_{H/C}$ 3.35/49.0 (CD_3OD). HREIMS were recorded on a Kratos MS 50 spectrometer. All other experimental details were as previously reported.[12]

Animal Material. The sponge sample *L. chagosensis* from Bougainville Reef, the Great Barrier Reef, Australia, was collected in March of 1995 and stored at −20 °C until it was freeze-dried. The sponge sample *L.* cf *chagosensis* from the Fiji Islands was collected in 1999 and stored in EtOH at −20 °C until workup. Voucher specimens have been deposited at the Queensland Museum, voucher number QMG313955 (Australian sample) and voucher number QMG316276 (Fiji Island sample).

Extraction and Isolation. The freeze-dried sponge *L. chagosensis* from Australia (dry weight 11.5 g) was extracted with CH_2Cl_2 (3 × 0.5 L) to yield 0.3 g (2.6%) of yellow extract. This material was fractionated over a solid-phase extraction material (Bakerbond SPE SiOH), using gradient elution from petroleum ether containing increasing proportions of CH_2Cl_2, followed by MeOH, to yield three fractions. ^1H NMR investigations of these fractions indicated fraction 1 to be of further interest due to the large number of aromatic type resonances in its proton spectrum. Fraction 1 was further fractionated by SPE (Macherey-Nagel C_{18f}), using gradient elution from MeOH (100%) to CH_2Cl_2 (100%), to yield two fractions. HPLC separation of the first of these fractions (column: Knauer Diol Eurospher-100, 250 × 8 mm, 5 μm; petroleum ether−CH_2Cl_2 (3:7), 2.0 mL/min) yielded semipure compound **1**. Further purification of **1** by reversed-phase (RP) HPLC (column: Phenomenex Max C_{12}, 250 × 4.6 mm, 5 μm; MeOH−H_2O (8:2), 0.8 mL/min) afforded 21.0 mg of compound **1**. HPLC separation of fraction 2 (column: Knauer Diol Eurospher-100, 250 × 8 mm, 5 μm; petroleum ether−CH_2Cl_2 (1:9), 2.0 mL/min) yielded semipure compound **2**, which was rechromatographed by RP HPLC (column: Phenomenex Max C_{12}, 250 × 4.6 mm, 5 μm; MeOH−H_2O (3:7), 0.8 mL/min) to yield 13.4 mg of pure material.

Table 1. ^{1}H NMR Spectral Data for Compounds **1**−**3** (δ in ppm, J in Hz)[a]

proton	**1**[b]	**2**[c]	**3**[d]
5	6.50 (1H, brs)	5.86 (1H, brs)	
7	5.29 (2H, brs)	4.97 (2H, brs)	3.28 (2H, brs)
9	7.17 (1H, d, J = 8.4)	7.18 (1H, d, J = 8.1)	7.16 (1H, d, J = 8.8)
10	6.84 (1H, d, J = 8.4)	6.81 (1H, d, J = 8.1)	6.91 (1H, d, J = 8.8)
12	6.84 (1H, d, J = 8.4)	6.81 (1H, d, J = 8.1)	6.91 (1H, d, J = 8.8)
13	7.17 (1H, d, J = 8.4)	7.18 (1H, d, J = 8.1)	7.16 (1H, d, J = 8.8)
14	3.84 (2H, brs)	3.66 (2H, brs)	3.85 (2H, brs)
16	6.81 (1H, d, J = 2.0)	6.83 (1H, d, J = 2.0)	6.19 (1H, s)
19	6.79 (1H, d, J = 8.4)	6.76 (1H, d, J = 8.1)	
20	6.77 (1H, dd, J = 2.0, 8.4)	6.74 (1H, dd, J = 2.0, 8.1)	6.19 (1H, s)
21	3.78 (3H, s)	3.75 (3H, s)	3.80 (3H, s)
22	3.85 (3H, s)	3.83 (3H, s)	3.82 (3H, s)
23	3.84 (3H, s)	3.81 (3H, s)	3.32 (3H, s)
29	3.19 (3H, s)		

[a] All assignments are based on extensive 1D and 2D NMR measurements (HMBC, HSQC, COSY). [b] $CDCl_3$, 500 MHz. [c] $CDCl_3$, 300 MHz. [d] CD_3OD, 300 MHz.

Table 2. ^{13}C NMR Spectral Data for Compounds **1**−**3** (δ in ppm)

	1[a,c]		**2**[b,c]		**3**[c,d]	
carbon	^{13}C	HMBC[f]	^{13}C	HMBC[f]	^{13}C	HMBC[f]
2	144.0 (s)[e]	7	146.7 (s)	5	147.8 (s)	7, 23
4	138.7 (s)	14	127.3 (s)	5	124.5 (s)	23
5	116.1 (d)	7, 14	111.0 (d)		123.9 (s)	7, 14
7	48.8 (t)	9, 13	49.1 (t)	9, 13	29.7 (t)	
8	127.6 (s)	7, 10, 12	125.3 (s)	10, 12	130.3 (s)	10, 12
9	129.4 (d)	7, 13	129.8 (d)	7	130.5 (d)	13
10	114.3 (d)	12	114.5 (d)	12	115.3 (d)	9, 12, 13
11	159.6 (s)	9, 10, 12, 13, 21	159.9 (s)	9, 10, 12, 13, 21	160.3 (s)	9, 10, 12, 13, 21
12	114.3 (d)	10	114.5 (d)	10	115.3 (d)	9, 10, 13
13	129.4 (d)	7, 9	129.8 (d)	7	130.5 (d)	9, 10, 12
14	33.5 (t)	16, 20	31.0 (t)	20	28.7 (t)	16, 20
15	130.5 (s)	14, 20	128.3 (s)	14, 16	134.1 (s)	14
16	112.1 (d)	14, 20	112.0 (d)	14	108.4 (d)	14
17	147.8 (s)	16, 23	148.2 (s)	16, 19, 23	152.2 (s)	16, 20
18	149.0 (s)	20, 22	149.2 (s)	19, 20, 22	136.1 (s)	16, 20, 22
19	111.3 (d)		111.5 (d)		152.2 (s)	16, 20
20	120.7 (d)	16, 19	120.8 (d)	14, 16, 19	108.4 (d)	14
21	55.3 (q)		55.3 (q)		55.8 (q)	
22	55.9 (q)		56.1 (q)		60.9 (q)	
23	55.9 (q)		55.9 (q)		30.0 (q)	
24	148.8 (s)					
26	154.4 (s)	29				
28	161.4 (s)	29				
29	24.8 (q)					

[a] $CDCl_3$, 125.75 MHz. [b] $CDCl_3$, 75.5 MHz. [c] CD_3OD, 75.5 MHz. [d] Assignments are based on extensive 2D NMR measurements (HMBC, HSQC, COSY). [e] Implied multiplicities determined by DEPT (C = s; CH = d; CH_2 = t; CH_3 = q). [f] Numbers refer to proton resonances.

After removal of the EtOH used for preservation the Fiji Island sample (wet weight 0.53 kg) was extracted with CH_2Cl_2 (6 × 1.0 L) and MeOH (3 × 1.0 L). The combined CH_2Cl_2 extracts were evaporated to dryness to yield 0.4 g of brown gum. This material was fractionated by vacuum liquid chromatography (VLC) over Si gel (Merck, 5−40 μm) employing gradient elution from hexane containing increasing proportions of EtOAc, followed by MeOH, to yield eight fractions. The considerable number of aromatic resonances seen in the ^{1}H NMR spectrum of fraction 7 showed it to be the only fraction of further interest. It was thus further fractionated by SPE (Bakerbond C_{18}) using gradient elution from H_2O−MeOH (6:4) to MeOH (100%) to yield six more fractions. Fractions 3 and 4 were combined, based on TLC comparisons, and rechromatographed by RP HPLC (column: Knauer C_{18} Eurospher-100, 250 × 8 mm, 5 μm; MeOH−H_2O (6:4), 1.5 mL/min) to yield compounds **3** and **4** in a semipure form. Further purification of these two compounds by RP HPLC (column: Phenomenex Polar C_{18}, 250 × 4.6 mm, 5 μm; MeOH−H_2O (3:7), 0.9 mL/min) afforded 3.4 mg of compound **3** and 5.2 mg of compound **4**. The EtOH and MeOH extracts were combined and evaporated to yield 7.0 g of a salty, brown extract. This extract was fractionated by VLC over Polygoprep 60-50 C_{18} material (Macherey-Nagel), using gradient elution from H_2O−MeOH (7.5:2.5) to MeOH (100%), to yield four fractions. Purification of fraction 1 by RP HPLC (column: Phenomenex Polar C_{18}, 250 × 4.6 mm, 5 μm; MeOH−H_2O (6:4), 1.0 mL/min) afforded 1.7 mg of compound **5**.

Isonaamidine E, 5-{[4-(3,4-dimethoxybenzyl)-1-(4-methoxybenzyl)-1*H*-imidazol-2-yl]imino}-3-methyl-2,4-imidazolidinedione (1): yellow amorphous solid (21.0 mg, 0.18%); UV ($CHCl_3$) λ_{max} 383 nm (ϵ 11 340), 279 nm (ϵ 5550), 237 nm (ϵ 23 840); IR (ATR) ν_{max} 2906, 1797, 1738, 1673, 1512, 1442, 1390, 1248, 1179, 1116, 1026, 822 cm^{-1}; ^{1}H and ^{13}C NMR data (see Tables 1 and 2); EIMS m/z (rel int) 463 (21), 121 (100); HREIMS m/z 463.1851 (calcd for $C_{24}H_{25}N_5O_5$, 463.1856).

Isonaamine C, 4-(3,4-dimethoxybenzyl)-1-(4-methoxybenzyl)-1*H*-imidazol-2-ylamine (2): yellow amorphous solid (13.4 mg, 0.12%); UV ($CHCl_3$) λ_{max} 379 nm (ϵ 1210), 276 nm (ϵ 6450), 251 (ϵ 6080); IR (ATR) ν_{max} 3103, 1663, 1513, 1463, 1250, 1026, 815 cm^{-1}; ^{1}H and ^{13}C NMR data (see Tables 1 and 2); EIMS m/z (rel int) 353 (18), 121 (100); HREIMS m/z 353.1741 (calcd for $C_{20}H_{23}N_3O_3$, 353.1739).

Naamine E, 5-{[2-imino-4-(4-methoxybenzyl)-1-methyl-1,2-dihydro-1*H*-imidazol-5-yl]methyl}-2-methoxy-1,3-benzenediol (3): yellow amorphous solid (3.4 mg, 0.0006%); UV (MeOH) λ_{max} 277 nm (ϵ 6090), 233 (ϵ 21 600); IR (ATR) ν_{max} 3500−2800 (br), 1668, 1609, 1511 cm^{-1}; ^{1}H and ^{13}C NMR data (see Tables 1 and 2); EIMS m/z (rel int) 369 (100), 121 (68); HREIMS m/z 369.1682 (calcd for $C_{20}H_{23}N_3O_4$, 369.1689).

Biological Assays. Activity of compounds **1**−**5** was tested in agar diffusion assays against the bacteria *Bacillus mega-*

terium and *Escherichia coli*, the fungi *Microbotryum violaceum*, *Eurotium repens*, and *Mycotypha microsporum*, and the green microalga *Chlorella fusca*.[13] ELISA-based enzyme inhibition assay against HIV-1 reverse transcriptase was performed as previously described.[14] Cytotoxicity tests against the cell lines HM02 (stomach carcinoma), HepG2 (liver carcinoma), and Huh7 (liver carcinoma with mutated p53) followed the standards of the NCI.[15]

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