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Isodiplamine, cystodytin K and lissoclinidine: novel bioactive alkaloids from the New Zealand ascidian *Lissoclinum notti*

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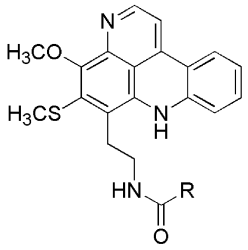
Abstract—A study of the bioactive crude extract of the New Zealand ascidian *Lissoclinum notti* led to the isolation of the new pyridoacridine alkaloids isodiplamine (**4**), cystodytin K (**5**) and lissoclinidine (**6**), as well as the known pyridoacridine alkaloids diplamine (**7**) and cystodytin J (**8**) and the benzopentathiepin varacin (**3**) and related trithiane varacin A. The new alkaloids were characterised using standard spectroscopic techniques, including 2D $^{1}H-^{15}N$ NMR experiments. Pyridoacridine alkaloids **4**–**8** were assayed for a range of biological activities including antitumour and antibiotic properties.

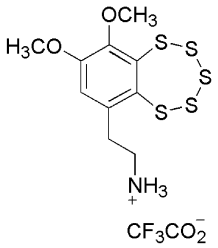
1. Introduction

Since the mid-1980's, studies of ascidians of the genus *Lissoclinum* have led to the discovery of many structurally interesting and extremely bioactive natural products.[1–6] For example, the red thin-encrusting ascidian *L. vareau* found in the Fijian islands, has yielded the sulfur-containing pyridoacridines alkaloids varamines A (**1**) and B (**2**)[1] and the benzopentathiepin alkaloid varacin (**3**).[2] Pyridoacridine alkaloids are well known for their range of bioactivities,[7] including DNA intercalation and topoisomerase II enzyme inhibition[8] while benzopentathiepins are potent cytotoxins and antibiotics.[2–5,9,10]

In this study, we have examined the species *L. notti* collected near Leigh Harbour, Northland, New Zealand and found the crude organic extract to possess a wide range of potent bioactivities. Bioassay guided fractionation led to the isolation and structural elucidation of three new pyridoacridine alkaloids; isodiplamine (**4**), cystodytin K (**5**) and lissoclinidine (**6**). As well as these, the known alkaloids diplamine (**7**), cystodytin J (**8**), varacin (**3**), varacin A and the purine N^2,N^2-7-trimethylguanine[11] were also isolated. The pyridoacridine alkaloids were evaluated in a range of biological assays in an attempt to extend the SAR data known for this series of compounds.



1 R = CH_2CH_3
2 R = CH_3



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2. Results and discussion

2.1. Isolation

Specimens of *L. notti* were collected by hand using SCUBA (depth ~7 m) from Leigh Harbour, Northland, New Zealand and kept frozen until freeze-dried and extracted. The dried ascidians were exhaustively extracted with methanol and dichloromethane to give a brown extract. Although *L. notti* exists in three colour-morphs; deep blue/purple, grey and brown, comparison of analytical C_{18} reversed phase HPLC traces of the crude extract of each of the colour-morphs revealed them to be chemically identical, and so the extracts were combined. The crude extract exhibited potent cytotoxic and antimicrobial properties.

Bioassay guided fractionation using reversed phase C_{18}

Keywords: ascidian metabolites; lissoclinum; pyridoacridine; varacin.
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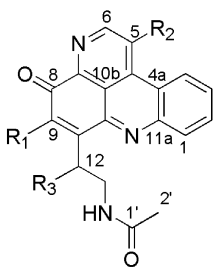
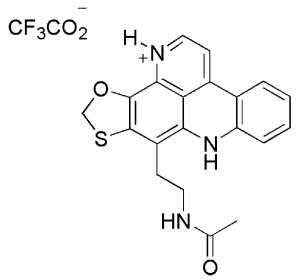
Table 1. ^{1}H NMR data (δ_H (mult, J (Hz)) for alkaloids **4**–**6** (DMSO-d_6)

Atom no	4	5	6
1	8.32 (d, 7.8)	8.25 (d, 8.3)	7.75 (m)
2	8.05 (t, 7.1)	8.03 (t, 8.3)	7.75 (m)
3	8.02 (obsc)	7.96 (t, obsc)	7.31 (m)
4	9.35 (d, 8.1)	8.96 (d, 7.9)	8.24 (d, 8.2)
5	–	9.06 (d, 5.4)	7.44 (d, 6.7)
6	9.13 (s)	9.27 (d, 5.5)	8.18 (d, 6.6)
7	–	–	13.2 (bs)
9	6.85 (s)	6.84 (s)	–
11	–	–	11.71 (bs)
12	3.17 (t, 6.4)	5.28 (t, 4.3)	2.93 (t, 7.3)
13	3.52 (obsc)	3.54 (m)	3.22 (td, 7.3, 5.5)
14	7.95 (obsc)	7.94 (obsc)	8.47 (t, 5.5)
2′	1.74 (s)	1.68 (s)	1.91 (s)
SCH_3	2.97 (s)	–	–
OCH_3	–	3.42 (s)	–
OCH_2S	–	–	6.09 (s)

flash column chromatography concentrated cytotoxic activity into two fractions. A combination of cyanopropyl silica gel chromatography, Sephadex LH20, chemical derivatisation and semi-preparative C_{18} HPLC afforded the known compounds diplamine (**7**),[12] varacin (**3**)[2] and varacin A,[13,14] identified by comparison of their spectroscopic data with published data, and three new pyridoacridine alkaloids, isodiplamine (**4**), cystodytin K (**5**) and lissoclinidine (**6**). In a second collection of *L. notti* from the same area one year later, cystodytin J (**8**)[8] was also isolated. Interestingly in this collection only trace amounts of cystodytin K (**5**) were detected.



4 R_1=H, R_2=SCH_3, R_3=H
5 R_1=H, R_2=H, R_3=OCH_3
7 R_1=SCH_3, R_2=H, R_3=H
8 R_1=H, R_2=H, R_3=H



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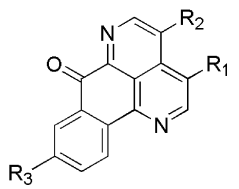
Compound **4** had a molecular formula of $C_{20}H_{17}N_3O_2S$, as deduced by HRFABMS, making it isomeric with diplamine (**7**). A lack of pH dependence of the UV–Vis spectrum of **4** suggested the presence of an iminoquinone skeleton as in diplamine.[15] Inspection of the ^{1}H NMR spectrum also confirmed the presence of an iminoquinone skeleton with an *N*-acetyl ethylamine side chain, however it became obvious that placement of the thiomethyl group in **4** differed from that of diplamine (see Table 1). The diagnostically *ortho*-coupled pyridine protons H-5 and H-6 in **7** were absent in **4** suggestive of placement of the thiomethyl group in pyridine ring-D. A proton at δ 9.13 (1H, s) could be placed at C-6 (δ 145.9) in **4** by $^{1}H-^{13}C$ HMBC correlations to C-4b (δ 132.8), C-5 (δ 138.2) and C-7a (δ 141.7) (see Table 2), while the thiomethyl group could now be placed at C-5 due to an HMBC correlation from the methyl protons to C-5 and a ROESY correlation to H-6. The same HMBC experiment also allowed placement of a proton (δ 6.85 (1H, s)) at C-9 due to correlations being observed from H-9 to C-7a (δ 141.7), C-10a (δ 150.1) and C-12 (δ 31.2), thereby securing the structure of isodiplamine **4**. Further confirmation of the structure of **4** was made by acquiring a $^{1}H-^{15}N$ HMBC NMR spectrum that allowed for the assignment of N-7, N-11 and N-14. The chemical shifts observed (see Table 2) were entirely consistent[16] with the proposed structure of **4**.

To the best of our knowledge, isodiplamine is the first reported structure with β-pyridine ring substitution of a pyrido[4,3,2-*mn*]acridine skeleton. The only other examples of similarly substituted natural products are 3-methoxysampangine (**9**),[17] eupomatidine-2 (**10**) and eupomatidine-3 (**11**)[18] from the plants *Cleistopholis patens* and *Eupomatia bennettii*.



9 R_1=OCH_3, R_2=H, R_3=H
10 R_1=H, R_2=OCH_3, R_3=H
11 R_1=H, R_2=OCH_3, R_3=OCH_3

Table 2. ^{13}C, ^{15}N and HMBC ($^{13}C/^{15}N\rightarrow^{1}H$) data for **4**–**6** (DMSO-d_6)

Atom no.	4	5	6
1	131.7 (3)	131.0 (3)	117.8 (3)
2	131.1 (4)	131.9 (4)	135.0 (4)
3	129.5 (1)	129.7 (1)	123.2 (1)
4	127.5 (2)	124.1 (2)	125.3 (2)
4a	121.8 (1, 3)	121.6 (1, 3, 5)	114.2 (1, 3, 5, 11)
4b	132.8 (4, 6)	137.3 (4, 6)	148.3 (6)
5	138.2 (6, SCH_3)	120.3 (6)	103.6 (6)
6	145.9 (SCH_3)	149.9	143.2 (5)
N-7	300.4 (6)		145.9 (5, 6)
7a	141.7 (6, 9)	146.0 (6)	121.1 (6)
8	182.6	n.o.[a]	134.2 (OCH_2S)
9	131.8 (12)	132.0	137.3 (12, OCH_2S)
10	151.1 (12)	150.5 (13)	109.7 (12)
10a	150.1 (9, 12)	146.2 (9)	131.8 (11, 12)
10b	129.3	117.7 (5)	118.3 (5, 11)
N-11	325.7 (1)		120.9[b]
11a	145.1 (2, 4)	144.5 (2, 4)	140.1 (2, 4)
12	31.2 (9, 13)	76.1 (9, OCH_3)	30.1
13	37.8 (12)	42.4	36.3 (12)
N-14	116.1 (12, 2′)		116.8 (2′)
1′	169.1 (13, 14, 2′)	169.3 (13, 2′)	171.3 (13, 14, 2′)
2′	22.5	22.3	22.4
SCH_3	16.4		
OCH_3		56.8 (12)	
OCH_2S			77.2

Numbers in parentheses are protons to which the carbon or nitrogen correlated to in HMBC NMR experiments. ^{15}N data determined for **4** and **6** only. HMBC data acquisition was optimised for 6 Hz and referenced to liquid NH_3 using urea as an external standard. For solubility reasons, the solvent used for ^{15}N data acquisition was $CDCl_3/CD_3OD$.

[a] Not observed.

[b] Chemical shift determined by $^{1}H-^{15}N$ HSQC, optimized for 87 Hz, in DMSO-d_6.

Table 3. In vitro antitumour, antimicrobial and cytotoxicity activity of alkaloids **4**–**8**

	P388[a]	HCT-116[b]	BSC-1[c]	*B. subtilis*[d]	*E. coli*[d]	*C. albicans*[d]	*T. mentagrophytes*[d]
4	2.1	n.t.[e]	*2+, −	4	0	4	1
5	1.3	n.t.	*4+, −	5	n.t.	n.t.	3
6	4.6	3.0	4+, +	8	6	9	6
7	1.9	<1.4	4+, 4+	9	3	0	12
8	3.5	1.6	4+, +	6	4	10	4

[a] IC_{50} (μM) against the P388 D1 murine leukaemia cell line.
[b] IC_{50} (μM) against HCT-116 human colon tumour cell line. Data for **6** was obtained from testing at the NCI (see text), while data for **7** and **8** were taken from Ref. 8.
[c] The test compound was applied at two dose levels (either 120 μg or *60 μg, and 10 μg) to a 6 mm paper disc and incubated with the BSC-1 African Green Monkey kidney cell line growing in continuous culture in a 16 mm well for 24 h at 36°C in an atmosphere containing 5% CO_2. Zones of cytotoxicity were measured microscopically as excess radii from the disc and indicated by −, none detectable; +, 1–2 mm; 2+, 2–3.5 mm; 3+, 3.5–4.5 mm; 4+, greater than 4.5 mm.
[d] Zone of microbial inhibition against *B. subtilis*, *E. coli*, *C. albicans* and *T. mentagrophytes* for 120 μg of test compound on a 6 mm paper disc. Incubation for 18 h at 35°C. Zones measured as excess radii in mm.
[e] n.t.—not tested.

The optically active alkaloid **5** was isolated in very low yield (0.003% dry weight) and found to have a molecular formula of $C_{20}H_{17}N_3O_3$ by HRFAB mass spectrometry. The UV–Vis, ^1H and ^{13}C NMR data observed for **5** were reminiscent of the cystodytin family of pyridoacridone alkaloids, and in particular close structural similarity with cystodytin J (**8**) was noted.[8,19,20] A triplet observed in the ^1H NMR spectrum at δ_H 5.28 (1H, H-12) and methyl protons at δ 3.42 (OCH_3) which exhibited an HMBC correlation to C-12 (δ 76.1) indicated that the new compound was 12-methoxy cystodytin J (cystodytin K (**5**)).

Like **4**, compound **6** also had a molecular formula isomeric with diplamine. However, unlike **4** and **5**, the colour of **6** was pH dependent suggesting the presence of a reduced pyridoacridine chromophore. Inspection of the ^{13}C NMR spectrum confirmed this with the absence of the quinoid-carbonyl resonance at ~δ 180. Standard 2D NMR experiments revealed the presence of a fused 1,2-disubstituted benzene-2,3,4-trisubstituted pyridine ring and *N*-acetyl ethylamine side chain as present in diplamine (**7**). In addition to these fragments were two exchangeable single proton singlets at δ 11.71 (NH-11) and δ 13.2 (NH-7) and a methylene singlet at δ 6.09 (2H) while the thio-methyl group was absent. The broad exchangeable proton at δ 11.71 could be assigned as H-11 in the acridine ring system due to HMBC correlations to 4a (δ 114.2), 10a (δ 131.8) and 10b (δ 118.3). The methylene singlet at δ 6.09 (δ_C 77.2) exhibited HMBC correlations to C-8 (δ 134.2) and C-9 (δ 137.3) suggesting the presence of a benzoxathiole ring encompassing C-8 and C-9. A similar ring system has been described before as a photoreduction product of related pyridoacridines alkaloids (see Section 2.2).[6] Due to the extremely broad nature of the single proton resonance at δ 13.2 it was not possible to locate the proton directly in the structure by spectroscopic techniques. We presumed the proton to be located at N-7, forming a protonated pyridoacridinium skeleton. This was confirmed with the determination of ^{15}N chemical shifts via $^1H-^{15}N$ HSQC and HMBC NMR experiments. While the chemical shifts observed for N-11 (δ_N 120.9) and N-14 (δ_N 116.8) were consistent with their being indole-like and amide nitrogens, the chemical shift observed for N-7 (δ_N 145.9) indicated it to be a pyridinium cation.[16,21,22] This completed the structural assignment of the trifluoroacetate salt of lissoclinidine (**6**).

2.2. Lissoclinidine (6) presence in the ascidian

Molinski and Searle have shown that the benzoxathiole ring as in **6** can be formed photochemically by irradiation of the corresponding 9-methylthio-iminoquinone.[6] Given this result, it is possible that the presence of lissoclinidine (**6**) in the crude extract was due to exposure to sunlight and subsequent photoreduction of diplamine (**7**) in the extract. Indeed when a pure sample of **7** in acidic aqueous methanol was irradiated with a UV lamp conversion to **6** occurred in several hours. Interestingly, the presence of acid was required for this reaction to proceed.[6]

In order to confirm if **6** was a secondary metabolite of *L. notti* or merely a product of the extraction process, we set out to collect a sample of extract in the field and took care to protect this from sunlight until it was analysed. Live thin encrusting ascidians were cut and the seawater in the immediate area sampled, passed through a leur lock C_{18} cartridge and wrapped in aluminium-foil while still underwater. Analysis of the methanol-eluent of the cartridges by analytical C_{18} HPLC revealed the presence of lissoclinidine (**6**). This suggests that lissoclinidine is most likely a secondary metabolite and not an extraction artifact.

2.3. Biological activity

Pyridoacridine alkaloids **4**–**8** were assayed for a range of biological activities, as summarised in Table 3. All compounds possessed moderate to high activity towards P388 murine leukaemia, HCT-116 human colon tumour and non-malignant African Green Monkey kidney (BSC-1) cells. Diplamine (**7**) was the most cytotoxic of the pyridoacridine compounds towards BSC-1 cells, although interestingly this potency was not so clear-cut for P388 or HCT-116 tumour cells. Movement of the thiomethyl group from C-9 (diplamine, **7**) to C-5 (isodiplamine, **4**) clearly reduces cytotoxicity (towards BSC-1 cells), as also occurs when the thiomethyl group is cyclised into a benzoxathiole ring (**6**). Such results are consistent with the proposed mechanisms of cytotoxicity of diplamine, which include DNA intercalation, inhibition of topoisomerase II and other DNA processing enzymes[8] and bioreductive activation.[23] Lissoclinidine (**6**) was further evaluated against the NCI 60-cell line panel but was found to exhibit only moderate activity and selectivity (panel average values: GI_{50} 1.0 μM,

TGI 6.9 μM, LC_{50} 29 μM). All of the compounds exhibited modest to potent antimicrobial activity towards a variety of microorganisms including the bacteria *Bacillus subtilis* and *Escherichia coli* and the fungi *Candida albicans* and *Trichophyton mentagrophytes*.

3. Experimental

3.1. General

Details of general procedures and analytical HPLC conditions have been reported previously.[24]

3.2. Animal material

Specimens (voucher specimen number 2000LH1-2) of *L. notti* were collected in June 2001 and again in May 2002 near Leigh Harbour, Northland, New Zealand and identified by one of us (G. L.). The voucher sample is held in the Department of Chemistry, The University of Auckland.

3.3. Extraction and isolation

Ascidian specimens were freeze-dried (dry weight 62.07 g) and extracted with MeOH (3×200 mL) then CH_2Cl_2 (1×200 mL). Solvents were removed in vacuo to give a brown extract (5.55 g). A portion of extract (1.90 g) was fractionated using reversed phase C_{18} flash column chromatography with a steep gradient from H_2O (0.05% trifluoroacetic acid (TFA)) to MeOH (0.05% TFA). The 25% MeOH fraction contained almost pure N^2,N^2-7-trimethylguanine (70 mg, 0.47% dry weight) while BSC-1 cell line cytotoxicity was concentrated in the second and third fractions (50 and 75% MeOH respectively). The second fraction was then subjected to cyanopropyl-silica flash column chromatography using a steep gradient from CH_2Cl_2 (0.05% TFA) through to CH_2Cl_2/MeOH (95:5, 0.05% TFA). The yellow CH_2Cl_2/MeOH (99:1) fraction was further purified using Sephadex LH20, eluting with MeOH (0.05% TFA) to give cystodytin K (**5**) (0.8 mg, 0.003% dry weight). The CH_2Cl_2/MeOH (98:2) fraction was a 10:1 mixture of varacin (**3**) (0.087% dry weight) and varacin A (0.008% dry weight) as determined by analytical C_{18} HPLC and NMR. The purple fraction, CH_2Cl_2/MeOH (95:5), after purification using Sephadex LH20 eluting with MeOH (0.05% TFA), gave pure lissoclinidine (**6**) (16 mg, 0.074% dry weight). The third C_{18} fraction (75% MeOH) was subjected to Sephadex LH20 chromatography, eluting with MeOH (0.05% TFA), to give a mixture of two compounds. Semi-preparative HPLC (C_{18}, MeOH/H_2O (70:30), 5 mL/min) of the mixture yielded isodiplamine (**4**) (1.1 mg, 0.005% dry weight) and diplamine (**7**) (2.2 mg, 0.01% dry weight).

A portion (5.95 g) of the crude organic extract of the second collection (ascidian dry weight 104.70 g yielded 7.44 g extract) was fractionated in the same way to yield **4** (5.0 mg, 0.006% dry weight), **6** (27.0 mg, 0.032% dry weight), **7** (13.9 mg, 0.017% dry weight), **8** (9.2 mg, 0.011% dry weight), and a 10:1 mixture (119 mg) of **3** and varacin A.

A portion of the mixture of varacin (**3**) and varacin A (70 mg, ~0.16 mmol as TFA salts) was stirred in MeOH (10 mL) with di-*t*-butyloxycarbonate (51 mg, 0.23 mmol) and Et_3N (65 μL, 0.47 mmol) at room temperature under N_2 for 15 h. CH_2Cl_2 (60 mL) was then added and washed with 0.2N HCl (60 mL), H_2O (60 mL) and brine (60 mL). The solvent was then removed in vacuo. Semi-preparative HPLC (C_{18}, MeOH/H_2O (0.05% TFA), 90:10, 5 mL/min) yielded *N*-*t*-butyloxycarbonyl varacin (29 mg, 48%) and *N*-*t*-butyloxycarbonyl varacin A (6 mg, 10%). Each of the BOC-derivatives were then deprotected by stirring in CH_2Cl_2/TFA (9:1) for 1.5 h to return the separated natural products varacin (**3**) (34 mg, 100%) and varacin A (6.8 mg, 100%).

3.3.1. Isodiplamine (4). Green solid. Mp 208–210°C; UV–Vis (MeOH/TFA) λ_{max} (log ε) 215 (4.68), 274 (4.16), 320 (4.01), 406 (4.00) nm, (MeOH/KOH) 205 (4.57), 273 (4.20), 320 (4.03), 406 (4.04) nm; IR ν_{max} 3297, 2923, 1652, 1566, 1525, 1430, 1192 cm^{-1}; ^1H NMR: see Table 1; ^{13}C NMR: see Table 2; FABMS *m*/*z* 366 $[M+2H+H]^+$, 364 $[M+H]^+$; HRFABMS *m*/*z* 364.1126 (calcd for $C_{20}H_{18}N_3O_2S$ 364.1120).

3.3.2. Cystodytin K (5). Yellow solid. $[\alpha]_D^{20}=-292$ (*c* 0.065, MeOH); UV–Vis (MeOH/TFA) λ_{max} (log ε) 207 (4.57), 272 (4.17), 382 (3.67) nm, (MeOH/KOH) 204 (4.46), 270 (4.17), 380 (3.71) nm; IR ν_{max} 3303, 2926, 1655, 1587, 1465, 1112, 1022 cm^{-1}; ^1H NMR: see Table 1; ^{13}C NMR: see Table 2; FABMS *m*/*z* 350 $[M+2H+H]^+$; HRFABMS *m*/*z* 350.1507 (calcd for $C_{20}H_{20}N_3O_3$ 350.1505).

3.3.3. Lissoclinidine trifluoroacetate salt (6). Hygroscopic purple solid. UV–Vis (MeOH/TFA) λ_{max} (log ε) 218 (4.41), 281 (4.26), 295 (4.21), 310 (4.10), 384 (3.61), 548 (3.40) nm, (MeOH/KOH) 225 (4.17), 264 (4.16), 301 (4.00), 312 (3.99), 374 (3.23), 391 (3.38), 486 (3.32) nm; IR ν_{max} 3442, 2913, 1660, 1590, 1423, 1200, 1129 cm^{-1}; ^1H NMR: see Table 1; ^{13}C NMR: see Table 2; FABMS *m*/*z* 364 $[M+H]^+$; HRFABMS *m*/*z* 364.1122 (calcd for $C_{20}H_{18}N_3O_2S$ 364.1120).

3.4. Photoreduction of diplamine (7)

A solution of diplamine (**7**) (0.75 mg) in MeOH/H_2O (1:1, 1.5 mL) was irradiated with a standard UV lamp at 254 nm. After 3 h no change was detected by analytical C_{18} HPLC. TFA (four drops) was then added and the solution was irradiated for a further 4 h, by which time the solution had changed to purple. Analytical C_{18} HPLC confirmed the presence of lissoclinidine (**6**) and some minor degradation products.

3.5. Collection of extract in field

By SCUBA, specimens of *L. notti* at ~7 m were scratched with a knife while the water was sucked into a syringe from above the cut. This water was immediately eluted through a pre-conditioned leur lock C_{18} cartridge (600 mg). This was repeated three times, each time with a fresh cartridge. The cartridges were all wrapped in aluminium foil before returning to the surface then frozen as soon as possible. The following day the cartridges were eluted with MeOH

(0.05% TFA) and combined. Analytical C_{18} HPLC of the resulting solution was identical to the crude organic extract obtained in Section 3.3.

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