Leptoclinidamines A−C, Indole Alkaloids from the Australian Ascidian *Leptoclinides durus*

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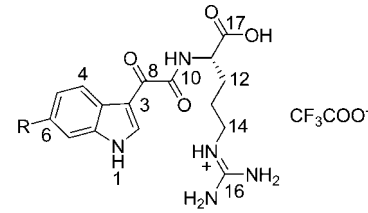
Three new indole alkaloids, leptoclinidamines A−C (**1**−**3**), were isolated from the Australian ascidian *Leptoclinides durus*. Their structures were determined by analysis of 2D NMR spectra. Leptoclinidamines A and B both contain an indoleglyoxylic acid attached to an L-arginine. The structure of leptoclinidamine A was confirmed by total synthesis. Leptoclinidamine C contains the naturally rare 1,3-dimethyl-5-(methylthio)histidine attached to a 6-bromoindole-3-carboxylic acid. Leptoclinidamine C (**3**) and both enantiomers of leptoclinidamine A (**1**) were tested for antimalarial, antitrypanosomal, and cytotoxic activity, but none of the compounds were bioactive.

Lipinski et al. have proposed that restriction of four physicochemical properties below predefined levels (molecular weight < 500, octanol/water partition coefficient (log *P*) < 5, number of hydrogen bond donors < 10, and number of hydrogen bond acceptors < 5) provides important predictors for oral bioavailability. These properties can thus be considered drug-like properties.[1] In a previous study we demonstrated that a library of pure drug-like natural products could be generated by, first, analyzing the Dictionary of Natural Products to identify published drug-like natural products, second, selecting species known to contain these published drug-like natural products from a diverse biota collection, third, analyzing the selected biota extracts by (+)-ESIMS to identify extracts that contain the desired compounds, and finally purifying the identified components rapidly by reversed-phase HPLC using mass-directed purification.[2] This approach successfully led to the isolation of 488 known drug-like natural products and in addition 317 new compounds, and 8 compounds possessing novel ring structures were also isolated. Significantly, most of the additional compounds isolated also met the criteria of possessing drug-like properties. An important lesson learned from this study was that two parameters, knowledge of a compound's molecular weight and knowledge of the relationship between compound retention on reversed-phase HPLC and log *P*, allowed for a good prediction of drug-like properties. Since application of this methodology was successful in isolating known drug-like natural products from known biota sources, it followed that it could possibly also be applied to isolate new and/or novel drug-like natural products from previously unexplored species. Ascidians in particular are rich sources of novel amino acid-derived alkaloids and peptides.[3,4] The biological activity of many ascidian compounds is significant, and a number of compounds of ascidian origin have entered preclinical or clinical development as therapeutic agents to treat human diseases.[5,6] Notably, ecteinascidin 743 (marketed as Yondelis), an ascidian alkaloid developed by PharmaMar, is now marketed to treat soft tissue sarcomas in Europe and South Korea.[7] The Eskitis Institute at Griffith University has amassed a substantial collection of Australian marine organisms (over 10 000 specimens) collected from Queensland and Tasmanian waters, and this collection contains 759 ascidian specimens representing 299 species. Most of these ascidian species have not been studied chemically before. The availability of such a rich collection of unexplored ascidian species prompted us to undertake a systematic (+)-LRESIMS analysis of MeOH extracts from the ascidian collection to search for unique drug-like compounds. The LCMS analysis has identified many alkaloid-containing species, and the previously unexplored ascidian *Leptoclinides durus,* Kott 2001 (Didemnidae) was selected for chemical analysis on the basis of the observation of ions at *m/z* 346, 362, and 451/453 in the LRESIMS of the crude MeOH extract. Retention times for the compounds displaying these ions on C_{18} HPLC suggested that the compounds had log *P*'s < 5. This paper reports on the chemistry of *L. durus* collected from the Great Barrier Reef. Three new indole alkaloids, leptoclinidamines A (**1**), B (**2**), and C (**3**), were isolated. The compounds were tested for bioactivity against chloroquine-sensitive and chloroquine-resistant strains of the malarial parasite *Plasmodium falciparum*, trypanosomal activity against *Trypanosoma brucei,* and cytotoxicity against the cancerous cell line HeLa and against noncancerous HEK 293 cells.



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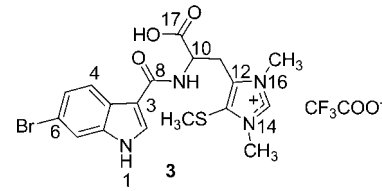
2 R = OH



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Results and Discussion

Extraction of the freeze-dried ground ascidian with MeOH, fractionation of the extract on C_{18} MPLC, and selection of fractions for further C_{18} HPLC purification by MS analysis eventually led to leptoclinidamines A−C (**1**−**3**) being purified.

Leptoclinidamine A (**1**) was isolated as its TFA salt as a colorless oil. A pseudomolecular ion in the (+)-HRESIMS at *m/z* 346.15238 allowed the molecular formula $C_{16}H_{19}N_5O_4$ to be assigned to **1**. The IR spectrum had absorption bands at 1683 and 1636 cm^{-1}, and this suggested that **1** contained amide and/or carboxylic acid and conjugated ketone functionality. The UV spectrum had an absorption maxima at 283 nm, which was indicative of an indole. The ^{13}C NMR spectrum (Table 1) contained signals for all 16 carbons including carbonyl carbon signals at δ_C 182.1, 172.9, and 164.2. Nine protonated carbons were identified in the HSQC spectrum. The ^1H NMR spectrum (Table 1) had signals for five

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Table 1. ^{1}H (600 MHz), ^{13}C (150 MHz), and HMBC NMR Data for Leptoclinidamines A (**1**)[a] and B (**2**)[a]

leptoclinidamine A (**1**)				leptoclinidamine B (**2**)		
δ_C (mult)	δ_H (*J* in Hz)	HMBC[b]	position	δ_C (mult)[c]	δ_H (*J* in Hz)	HMBC[b]
–	12.32, bs	2, 3, 3a, 7a	1		11.86, bs	
136.5, CH	8.67, d (3.0)	3, 3a, 7a, 8	2	137.0, CH	8.63, d (2.9)	3, 3a
112.4, qC			3	112.3, qC		
126.6, qC			3a	119.0, qC		
121.4, CH	8.21, dd (2.4, 6.0)	3, 6, 7a	4	121.4, CH	7.97, d (7.9)	3, 6, 7a
122.7, CH	7.26, m	3a, 7	5	112.1, CH	6.73, dd (1.8, 7.9)	3a, 7
123.6, CH	7.26, m	4, 7a	6	154.3, qC		
			6-OH		9.29, s	5, 6, 7
112.8, CH	7.54, dd (1.8, 6.0)	C-3a, C-5, C-7a	7	97.5, CH	6.86, d (1.8)	5, 3a
138.5, qC			7a	137.2, qC		
182.1, qC			8	181.7, qC		
164.2, qC			9	n.o.[d]		
	8.83, d (7.8)	8, 9, 11, 12, 17	10		8.42, bd (7.8)	11, 9
51.9, CH	4.32, ddd (4.8, 7.8, 7.8)	9, 12, 13, 17	11	52.3, CH	4.05, m	
27.8, CH_2	1.78, m	11, 14, 13, 17	12	28.1, CH_2	1.74, m	17
	1.88, m	11, 14, 13, 17			1.79, m	17
25.5, CH_2	1.54, tt (7.2, 7.2)	11, 12, 14	13	24.6, CH_2	1.51, tt (7.2,7.2)	11, 14, 12
40.4, CH_2	3.12, dt (5.4, 7.2)	12, 13, 16	14	41.7, CH_2	3.10, td (7.2, 7.8)	12, 13, 16
	7.66, t (5.4)	16, 14	15		8.10, bs	
157.2, qC			16	157.7, qC		
	7.22, bs		16-N_2H_4		7.12, bs	
172.9, qC			17	173.1,qC		

[a] Spectra recorded in d_6-DMSO at 30 °C. [b] HMBC correlations, optimized for 8.3 Hz, are from proton(s) stated to the indicated carbon. [c] ^{13}C chemical shifts obtained from gHSQC and gHMBC spectra. [d] Carbon not observed.

Table 2. ^{1}H (600 MHz), ^{13}C (150 MHz), HMBC, and COSY NMR Data for Leptoclinidamine C (**3**)[a]

atom	δ_C (mult)	δ_H (*J* in Hz)	HMBC[b]	COSY
1		11.74, bs	2, 3, 3a, 7a	H-2
2	129.0, CH	8.08, d (2.9)	3, 3a, 7a, 8	H-1
3	109.4,qC			
3a	124.9, qC			
4	122.9, CH	7.96, d (7.9)	3, 6, 7a	H-5
5	123.4, CH	7.24, dd (1.8, 7.9)	3a, 7	H-4, H-7
6	114.6, qC			
7	114.8, CH	7.65, d (1.8)	5, 7a	H-5
7a	137.0, qC			
8	164.1, qC			
9		8.42, d (7.8)	8, 10	H-10
10	50.0, CH	4.87, ddd (5.6, 7.8, 9.8)	8, 11, 12, 17	H-9, H-11a, H-11b
11	25.7, CH_2	3.27, dd (9.8, 15.6)	10, 12, 13, 17	H-10, H-11b
		3.43, dd (5.6, 15.6)	10, 12, 13, 17	H-10, H-11a
12	136.4, qC			
13	125.9, qC			
13-SCH_3	18.6, CH_3	2.31, s	13	
14-NCH_3	33.5, CH_3	3.79, s	13, 15	
15	138.1, CH	9.17, s	13, 12	
16-NCH_3	34.2, CH_3	3.89, s	12, 15	
17	172.2, qC			

[a] Spectra recorded in d_6-DMSO at 30 °C. [b] HMBC correlations, optimized for 8.3 Hz, are from proton(s) stated to the indicated carbon.

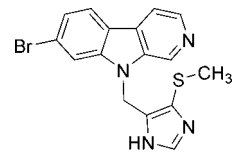
aromatic protons, six methylene protons, a methine proton, sharp exchangeable protons at δ_H 12.32, 8.83, and 7.66, and four broad exchangeable protons at δ_H 7.22. Analysis of COSY correlations indicated that four of the aromatic protons were part of a 1,2-disubstituted phenyl ring. A second spin system was deduced to be H-1 and H-2 of an indole. The third spin system was a 2,5-diaminopentanoic acid. Correlations observed in the HMBC spectrum allowed the indole to be defined. Correlations were observed from H-4 to C-3, C-6, and C-7a, from H-5 to C-3a and C-7, from H-6 to C-4 and C-7a, and from H-7 to C-5 and C-3a. The indole proton H-2 also showed a correlation to C-3, C-3a, and C-7a. The indole exchangeable proton H-1 showed correlations to C-2, C-3, C-3a, and C-7a. Correlations observed from the methylene protons CH_2-12 to a carbonyl carbon at δ_C 172.9 and from the methylene protons CH_2-14 to a carbon at δ_C 157.2 in the HMBC spectrum indicated that the molecule contained an arginine. The exchangeable proton at δ_H 7.66 was assigned to the guanidino proton H-15 because it showed correlations to CH_2-14 in the COSY spectrum and to C-17 in the HMBC spectrum. The broad exchangeable protons at δ_H 7.22 were assigned to the other four guanidino protons. The amide proton H-10 and arginine α proton H-11 showed HMBC correlations to the carbonyl carbons C-9 and C-17. The chemical shifts of H-4 and H-2/C-2 were significantly further downfield than would be expected for a 3-alkyl-substituted indole. This suggested a carbonyl-substituted C-3 of the indole, and a HMBC correlation was observed from H-2 to an unsaturated ketone carbonyl carbon C-8 at δ_C 182.1. The amide proton H-10 also correlated to C-8, indicating that **1** contained a 3-indoleglyoxylyl group directly attached to arginine. To determine the configuration of the arginine and confirm the identity of the assigned structure, both enantiomers of **1** were prepared by reacting 3-indolegyloxylyl chloride with either L- or D-arginine in DMF/pyridine. Comparison of the spectral properties of natural **1** with its synthetic counterparts confirmed the gross structure of leptoclinidamine A. The optical rotation for the synthetic compound containing L-arginine was identical with that of the natural product.

Leptoclinidamine B (**2**) was isolated as its TFA salt as a colorless oil. A pseudomolecular ion in the (+)-HRESIMS at *m*/*z* 362.1447

allowed the molecular formula $C_{16}H_{19}N_5O_5$ to be assigned to **2**. The spectroscopic properties for **2** were very similar to those of **1**. Leptoclinidamine B was not isolated in sufficient quantity to obtain a ^{13}C NMR spectrum, but heteronuclear 2D NMR analysis (Table 1) allowed all but one of the carbon signals to be identified. The only differences observed in the ^1H NMR spectrum of **2** compared to **1** were the signals associated with the indole. COSY analysis indicated that three of the aromatic protons were part of a 1,2,4-trisubstituted aromatic ring. Correlations observed in the HMBC spectrum allowed a 6-hydroxyindole to be defined. Correlations were observed from H-4 to C-3, C-6, and C-7a, from H-5 to C-3a and C-7, and from H-7 to C-5 and C-3a. The indole proton H-2 also showed a correlation to C-3 and C-3a. The chemical shift of C-6, δ_C 154.3, was indicative of a hydroxyl group being directly attached. This was supported by correlations observed from an exchangeable proton, 6-OH, δ_H 9.29, to C-5, C-6, and C-7. Two-dimensional NMR correlations observed for protons and carbons of the arginine in **2** were similar to those observed for **1**. C-9 was not observed in the HMBC spectrum, but the IR absorption bands at 1683 and 1636 cm^{-1} were supportive of an indole-glyoxylamide. The optical rotation for **2** suggested that it contained an L-arginine by analogy with the optical rotation observed for (+)-leptoclinidamine A (**1**).

Leptoclinidamine C (**3**) was isolated as a colorless gum. The (+)-LSESIMS for **3** had two equally intense peaks at *m/z* 451 and 453 that suggested that the molecule contained a bromine atom. A pseudomolecular ion at *m/z* 451.0422 in the (+)-HRESIMS allowed the molecular formula $C_{18}H_{20}BrN_4O_3S$ to be assigned to **3**. All 18 carbons were visible in the ^{13}C NMR spectrum (Table 2), and HSQC analysis allowed 17 carbon-bound protons to be assigned (five aromatic methines, one aliphatic methine, one methylene, and three methyls). The chemical shifts of the protons and carbons associated with two of the methyl groups, CH_3-14 and CH_3-16, indicated that they were *N*-methyl groups. The third methyl group, a singlet in the ^1H NMR spectrum at δ_H 2.31 attached to a carbon at δ_C 18.6, was either an aromatic, olefinic, or *S*-methyl. The three remaining protons were exchangeable. Correlations observed in the COSY spectrum indicated that the molecule contained a 3,6-disubstituted indole and a β-substituted alanine. The alanine nitrogen is an amide since a correlation was observed in the COSY spectrum between the downfield exchangeable proton at δ_H 8.42, 9-NH, and the α proton H-10. Correlations observed in the HMBC spectrum confirmed the presence of an indole since H-1, H-2, and H-4 all correlated to C-3. The chemical shift of the quaternary carbon, C-6, δ_C 114.6, indicated that the bromine substituted this position. Correlations were observed from H-2, H-9, and H-10 to the amide carbonyl carbon C-8, indicating that the indole is substituted at C-3 by an amide carboxyl group. Correlations were observed between CH_2-11 and two aromatic quaternary carbons at δ_C 136.4 (C-12) and 125.9 (C-13). The aromatic methine proton, H-15, also showed HMBC correlations to these aromatic quaternary carbons. The chemical shift of this proton, δ_H 9.17, and its attached carbon, δ_C 138.1, indicated a diheteroatom-substituted sp^2-hybridized methine. The molecule contained a 1,3-dimethylimidazole since correlations were observed in the HMBC spectrum from the *N*-methyl protons CH_3-14 to C-13 and C-15 and from CH_3-16 to C-15 and C-12. A correlation from H-10 to C-12 indicated that the β-substituted alanine is an *N,N′*-dimethylhistidine. Consideration of the molecular formula and the observation that the remaining unassigned methyl singlet at δ_H 2.31 showed only one HMBC correlation, to C-13, suggested that this methyl was a thioether attached at C-13. Leptoclinidamine C was therefore assigned structure **3**. Since TFA was used throughout the purification process, the counterion for leptoclinidamine C (**3**) is trifluoroacetate.

Indol-3-ylcarbonylhistidine and indol-3-ylcarbonylarginine, which are similar to leptoclinidamines A−C, have been isolated previously from *Leptoclinides dubius* from New Caledonia.[8] Methylthioimidazoles, however, are rarely encountered in nature. The mildly cytotoxic didemnolines A (**4**)−D isolated from *Didemnum* sp.,[9] the immunosuppressive agent hyrtiomanzamine (**5**) isolated from the sponge *Hyrtios erecta*,[10] and related compounds isolated from *Dragmacidon* sp. and a Thorectidae sponge[11,12] are the only examples. Indole glyoxylamides are also rarely encountered in nature. The only previous reports of indole glyoxylate derivatives were the polyandrocarpamides A (**6**)−C isolated from the ascidian *Polyandrocarpa* sp.,[13] coscinamides A−C isolated from the sponge *Coscinoderma* sp.,[14] and 6-bromo-5-hydroxyindolyl-3-glyoxylate isolated from the Far Eastern ascidian *Syncarpa oviformis*.[15]



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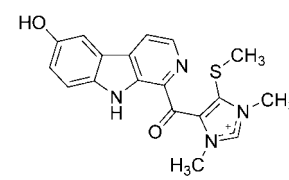

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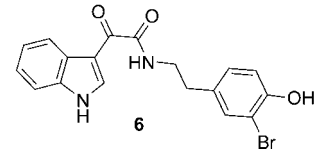

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Lipinski's rule of five was developed to identify potential bioavailability problems for compounds if two or more of the MW (>500), log *P* (>5), hydrogen bond donor (>10), or hydrogen bond acceptor (>5) criteria were violated.[1] Leptoclinidamines A (**1**) and B (**2**) possess only one Lipinski violation (hydrogen bond donors), while **3** possesses no Lipinksi violations, indicating that all three compounds possess drug-like properties. This study has shown that it is possible to find drug-like compounds from unexplored species by applying the methodology developed previously.

Leptoclinidamine C (**3**) and both enantiomers of leptoclinidamine A (**1**) were tested for bioactivity against chloroquine-sensitive (3D7) and chloroquine-resistant (Dd2) strains of the malarial parasite *Plasmodium falciparum*, trypanosomal activity against *Trypanosoma brucei,* cytotoxicity against the cancerous cell line HeLa, and cytotoxicity to noncancerous HEK 293 cells. The three compounds were inactive in all bioassays when tested up to 40 μM in the antimalarial and cytotoxicity assays and 250 μM in the antitrypanosomal assay. The compounds isolated in this study, although inactive in the limited number of screens tested so far, have been incorporated into a library for screening against therapeutically important targets.

Experimental Section

General Experimental Procedures. Optical rotations were measured on a JASCO P-1020 polarimeter. UV and FTIR spectra were recorded on a Camspec MS01 single beam scanning UV/vis spectrophotometer and a Bruker Tensor 27 IR spectrophotometer, respectively. NMR spectra were recorded at 30 °C on a Varian 600 MHz Unity INOVA at 599.926 MHz for ^1H and 149.98 MHz for ^{13}C. The ^1H and ^{13}C chemical shifts were referenced to the proto-deutero solvent peak (d_6-DMSO) at δ_H 2.49 and δ_C 39.5, respectively. Standard parameters were used for the 2D NMR spectra obtained, which included gCOSY, gHSQC ($^1J_{CH}$ = 140 Hz), and gHMBC ($^{3-2}J_{CH}$ = 8.3 Hz). HRESIMS were recorded on either a Bruker BioAPEX 47e mass spectrometer equipped with a Bradford CT 06405 electrospray ion source or a Mariner Biospectrometry TOF workstation using positive electrospray ionization, with mobile phase 1:1 MeOH/H_2O containing 0.1% formic acid. A Waters 600 pump equipped with a Waters 996 PDA detector and a Waters 717 autosampler were used for HPLC separations. Alltech Davisil 30−40 μm 60 Å C_{18} was used for MPLC work. A Hypersil BDS 5 μm 120 Å C_{18} HPLC column (10 mm × 250 mm) was used for HPLC semipreparative separations. All solvents used for HPLC, UV,

$[\alpha]_D$, and MS were Merck Omnisolv grade, and the H_2O used was Millipore Milli-Q PF filtered.

Animal Material. A specimen of *Leptoclinides durus,* Kott, 2001 (Didemnidae) was collected by scuba (−18 m) off Heron Island, Central Queensland, Australia, in August 1996 and kept frozen prior to freeze-drying and extraction. A voucher specimen, G307395, has been deposited at the Queensland Museum, South Brisbane, Queensland, Australia.

Extraction and Isolation. The ground ascidian sample (50 g) was extracted exhaustively with MeOH (5 × 100 mL) followed by water (5 × 100 mL). The combined MeOH and water extracts were evaporated, and the aqueous residue was partitioned with CH_2Cl_2. The aqueous layer was filtered through C_{18} (100 g), and then fractions were eluted off the C_{18} with a gradient of water containing 1% TFA to MeOH containing 1% TFA over 60 min. Sixty 1 min fractions were collected, and all fractions were analyzed by (+)-LRESIMS. Fractions containing similar mass ions were combined. Fractions 31−52 were combined and chromatographed on C_{18} HPLC with a linear gradient from 70% water/30% CH_3CN (each solvent containing 1% TFA) to 40% water/60% CH_3CN (each solvent containing 1% TFA) over 15 min. Sixty 10 s fractions were collected. (+)-LRESIMS analysis of these fractions indicated fractions 21−25 were pure leptoclinidamine B (**2**), fractions 27−28 were pure leptoclinidamine A (**1**), and fractions 30−32 were pure leptoclinidamine C (**3**).

Leptoclinidamine A (1): colorless gum (4.5 mg, 0.009%); $[\alpha]_D^{23}$ +14 (*c* 0.05, MeOH); UV (MeOH) λ_{max} (log ϵ) 214 nm (4.33), 283 (4.06), 318 (3.88); IR (film) ν_{max} 3408, 2928, 1683, 1636, 1442, 1402, 1204, 1135 cm^{-1}; ^1H and ^{13}C NMR (see Table 1); (+)-ESIMS *m/z* 346 (MH^+ 100%); (+)-HRESIMS *m/z* 346.15238 (calcd for $C_{16}H_{20}N_5O_4$, 346.15153).

Leptoclinidamine B (2): colorless gum (0.7 mg, 0.00014%); $[\alpha]_D^{23}$ +26 (*c* 0.035, MeOH); UV (MeOH) λ_{max} (log ϵ) 214 nm (4.33), 283 (4.06), 318 (3.88); IR (film) ν_{max} 3408, 2928, 1683, 1636, 1442, 1402, 1204, 1135 cm^{-1}; ^1H and ^{13}C NMR (see Table 1); (+)-ESIMS *m/z* 362 (MH^+ 100%); (+)-HRESIMS *m/z* 362.1447 (calcd for $C_{16}H_{20}N_5O_5$, 362.1464).

Leptoclinidamine C (3): colorless gum (2.9 mg, 0.0058%); $[\alpha]_D^{23}$ −39.4 (*c* 0.036, MeOH); UV (MeOH) λ_{max} (log ϵ) 232 nm (4.17), 249 (4.06), 281 (3.83); IR (film) ν_{max} 3432, 2928, 1659, 1635 cm^{-1}; ^1H and ^{13}C NMR (see Table 2); (+)-ESIMS *m/z* 451 (M^+ 50%), 453 (M^+ 50%); (+)-HRESIMS *m/z* 451.04122 (calcd for $C_{18}H_{20}{}^{79}BrN_4O_3S$, 451.0434).

Synthesis of (+)-Leptoclinidamine A. Indoleglyoxylyl chloride (40 mg) and L-arginine hydrochloride (30 mg) were added together in DMF-d_7 (1 mL) and pyridine-d_5 (100 μL) and heated to 80 °C for 40 min until the arginine dissolved. The reaction mixture was then kept at room temperature for a further 18 h. NMR analysis after 18 h indicated that the reaction was complete. The DMF was evaporated and the residue partitioned repeatedly between 1% aqueous TFA and 30% MeOH/70% DCM. The aqueous layer was then purified by C_{18} HPLC with a gradient from 1% TFA/99% water to 1% TFA/99% MeOH over 60 min, yielding the TFA salt of (+)-leptoclinidamine A (60 mg, 67%), $[\alpha]_D^{23}$ +14 (*c* 0.05, MeOH).

Synthesis of (−)-Leptoclinidamine A. The synthesis of (−)-leptoclinidamine A was achieved using the same reaction and purification conditions described above. Reaction of indoleglyoxylyl chloride (35 mg) and D-arginine hydrochloride (20 mg) yielded the TFA salt of (−)-leptoclinidamine A (54 mg, 69%), $[\alpha]_D^{23}$ −13 (*c* 0.05, MeOH).

Biological Assays. Detailed descriptions of the assay procedures are provided in the Supporting Information.

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Supporting Information Available: 1D and 2D NMR spectra for leptoclinidamines A−C and detailed descriptions of the biological assay procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

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