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Pteridine-, thymidine-, choline- and imidazole-derived alkaloids from the Australian ascidian, *Leptoclinides durus*†

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Four new acylated pteridine alkaloids, duramidines A–D, two new acylated thymidine alkaloids, leptoclinidines A and B, two new 1-acylglyceryl-3-(*O*-carboxyhydroxymethylcholine) alkaloids, durabetaines A and B, three new 1,3-dimethyl-5-methylsulfanylimidazole alkaloids, leptoclinidamines D–F, and the known alkaloids leptoclinidamines B and C and 6-bromo-1*H*-indolo-3-yl-oxoacetic acid methyl ester were isolated from the Australian ascidian *Leptoclinides durus*. The duramidines are the first pteridine alkaloids, possessing a three carbon side chain esterified at C-1′ with a 4-hydroxy-2′-methoxycinnamic acid, and are either hydroxylated or sulfated at C-2′. The leptoclinidines are the first 3′-indole-3-carboxylic acid ester derivatives of thymidine to be reported in the literature. The durabetaines are the first glyceryl-3-(*O*-carboxyhydroxymethylcholine) alkaloids to be reported from an animal source and are also the only known derivatives from this class to be acylated with aromatic carboxylic acids. MS and NMR data analysis established the structures of the new compounds. All compounds were shown to be inactive when tested for cytotoxic activity against prostate (LNCaP) and breast (MDA-MB-231) cancer cell lines and antimicrobial activity against *Pseudomonas aeruginosa* and *Staphylococcus aureus*.

Introduction

In a previous paper we reported on the alkaloid chemistry of the colonial ascidian *Leptoclinides durus* from the Great Barrier Reef, which included three indole derivatives, leptoclinidamines A–C.[1] At the time of isolating these compounds a number of other minor components were observed in HPLC separations, however insufficient quantities prevented structure identification. A subsequent recollection of *L. durus* from the Swain Reefs region of the Great Barrier Reef has now allowed the isolation of 14 compounds [11 of which are new (Fig. 1, **1–8** and **10–12**)] from this ascidian. In parallel, 143 crude organic extracts derived from Australian ascidians belonging to the family Didemnidae were investigated using analytical HPLC and MS[2] and another specimen of *L. durus*, was shown to contain three of the same novel compounds (**3, 4** and **11**) along with (−)-leptoclinidamine C (**9**). In this paper we report the isolation and structure elucidation of compounds **1–8** and **10–12**. These compounds were tested for cytotoxicity against prostate (LNCaP) and breast (MDA-MB-231) cancer cell lines as well as a panel of microbes known to be associated with nosocomial infections.

Results and discussion

Purification of the crude MeOH extract from the freeze-dried and ground ascidian by HPLC on C_{18} silica gel eluting with a linear gradient from H_2O to MeOH (containing 1% TFA) yielded many fractions eluting between 30% and 70% MeOH that contained mixtures of compounds that displayed aromatic signals in their ^1H NMR spectra. In addition, many of these fractions displayed molecular ions in the (+)-LRESIMS characteristic of brominated compounds. Further purification of pooled fractions containing similar ^1H NMR spectra and/or MS signals, by gradient C_{18} HPLC with mixtures of H_2O and MeOH afforded compounds **1–14** in low yield. Analysis of NMR and MS indicated that three of these compounds were the known natural products, (−)-leptoclinidamine C (**9**) and (+)-leptoclinidamine B (**13**) isolated previously from *L. durus*[1] and 6-bromo-1*H*-indolo-3-yl-oxoacetic acid methyl ester (**14**) that was previously purified from the Korean sponge *Spongosorites* sp.[3]

NMR analysis of the 11 new compounds highlighted that they represented groups of compounds from four different

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structure classes that we have named duramidines, leptoclinidines, durabetaines and leptoclinidamines.

Duramidines

Duramidine A (**1**) was obtained as an optically active yellow gum. Analysis of (+)-HRESIMS data at *m*/*z* 523.1133 (Δ 0.4 ppm) allowed a molecular formula, $C_{21}H_{22}N_4O_{10}S$ to be assigned to **1**. A fragment ion at *m*/*z* 443 in the (+)-LRESIMS (loss of 80 amu) suggested that the compound contained a sulfate ester group.

Absorption bands at 3190, 1720 and 1700 cm^{-1} in the IR spectrum were consistent with the molecule containing alcohol, and carbonyl functionality while absorption bands at 285 and 320 nm in the UV spectrum indicated the molecule contained an extended conjugated chromophore.

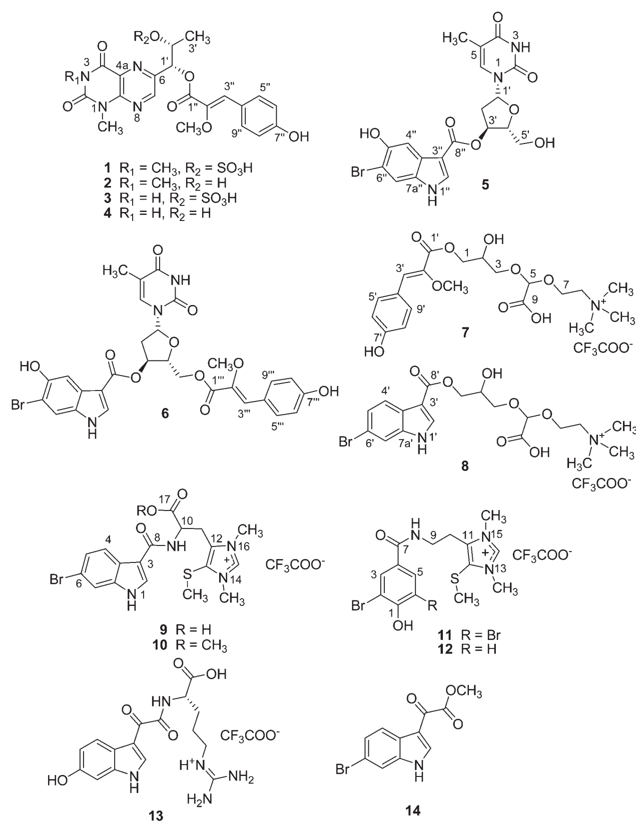

Fig. 1 Alkaloids isolated from *Leptoclinides durus*.

The ^{1}H NMR spectrum of **1** (Table 1) contained a methyl doublet at δ_H 1.22, three methyl singlets at δ_H 3.31, 3.54 and 3.72, a methine multiplet at δ_H 4.71, a methine doublet at δ_H 5.99 and two sp^2 hybridised methine singlets at δ_H 7.12 and 8.77. In addition, two *ortho* coupled aromatic doublets that each integrated to two protons at δ_H 6.80 and 7.66 and an exchangeable proton at δ_H 9.87 suggested that **1** contained an oxygenated *para*-disubstituted phenyl group. Edited HSQC correlations confirmed the presence of 10 protonated carbon resonances (two of which, δ_C 115.8 and δ_C 132.3 represented the four protonated carbons of the oxygenated *para*-disubstituted phenyl group), indicating that **1** contained an additional nine quaternary carbons. The chemical shifts of the carbons attached to the methyl protons at δ_H 3.54 (δ_C 29.2) and δ_H 3.31 (δ_C 28.5) indicated that they were substituted by nitrogen atoms, while the carbon attached to the methyl protons resonating at δ_H 3.72 (δ_C 58.8) was oxygenated.

A 1,2-dioxygenated propyl group was assigned based upon COSY and HSQC correlations and a 4-hydroxy-2′-methoxycinnamate group was identified from analysis of HMBC correlations. In particular, correlations were observed from a benzylic olefinic methine singlet at δ_H 7.12 to an oxygenated non-protonated sp^2 carbon at δ_C 142.4, an unsaturated ester carbonyl carbon at δ_C 163.4 and the aromatic protonated carbon resonance at δ_C 132.3 assigned to C-5″ and C-9″ of the *para*-disubstituted phenyl group, while the methoxy proton signal at δ_H 3.72 also correlated to the carbon at δ_C 142.4. The regiochemistry of the double bond was established to be *Z* based on the observation that the methoxy carbon in *E* isomers resonates at 56 ppm while in *Z* isomers it resonates at 59 ppm.[4] This regiochemistry was further supported by a strong ROESY correlation between the methoxy protons and the aromatic protons H-5″ and H-9″ in duramidine B (**2**). A HMBC correlation between H-1′ and the ester carbonyl carbon at δ_C 163.4 provided evidence to link the 4-hydroxy-2′-methoxycinnamate group to C-1′ of the propyl group. The chemical shift of H-2′ (δ_H 4.77) suggested that it was the site of attachment of the sulfate ester group.

Table 1 ^{1}H NMR data (600 MHz) for duramidines A–D (**1–4**) in DMSO-d$_6$

Position	1	2	3	4
1-CH$_3$	3.54 (s, 3H)	3.53 (s, 3H)	3.45 (s, 3H)	3.46 (s, 3H)
3	—	—	11.92 (s, 1H)	11.95 (s, 1H)
3-CH$_3$	3.31 (s, 3H)	3.30 (s, 3H)	—	—
7	8.77 (s, 1H)	8.83 (s, 1H)	8.74 (s, 1H)	8.82 (s, 1H)
1′	5.99 (d, 3.9, 1H)	5.71 (d, 5.5, 1H)	5.99 (d, 3.4, 1H)	5.71 (d, 6.0, 1H)
2′	4.71 (dq, 3.9, 6.4, 1H)	4.19 (dq, 5.5, 6.2, 1H)	4.72 (dq, 3.4, 6.1, 1H)	4.20 (dq, 6.0, 6.0, 1H)
3′	1.22 (d, 6.4, 3H)	1.17 (d, 6.2, 3H)	1.22 (d, 6.1, 3H)	1.22 (d, 6.1, 3H)
2″-OCH$_3$	3.72 (s, 3H)	3.68 (s, 3H)	3.72 (s, 3H)	3.69 (s, 3H)
3″	7.12 (s, 1H)	7.03 (s, 1H)	7.12 (s, 1H)	7.12 (s, 1H)
5″, 9″	7.66 (d, 8.4, 2H)	7.65 (d, 8.4, 2H)	7.67 (d, 8.4, 2H)	7.67 (d, 8.4, 2H)
6″, 8″	6.80 (d, 8.4, 2H)	6.80 (d, 8.4, 2H)	6.80 (d, 8.4, 2H)	6.80 (d, 8.4, 2H)
7″-OH	9.87 (bs, 1H)	9.90 (bs, 1H)	9.91 (bs, 1H)	9.96 (bs, 1H)

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The remaining molecular fragment, $C_8H_7N_4O_2$, represented seven degrees of unsaturation. A coupling of 186 Hz from the proton at δ_H 8.77 to the carbon at δ_C 146.5 was consistent with it being a protonated aromatic carbon substituted by a nitrogen atom.[5] Intense HMBC correlations were observed from this proton to two additional N substituted aromatic non-protonated carbons at δ_C 147.9 and 147.1 and a weak $^4J_{CH}$ correlation was also observed to a non-protonated sp^2 carbon at δ_C 127.0. The propyl protons H-1′ and H-2′ correlated to the carbon at δ_C 147.1 while H-1′ correlated to the protonated heteroaromatic carbon at δ_C 146.5, suggesting that the propyl side chain was attached to a carbon adjacent to C-7. The *N*-methyl protons at δ_H 3.54 also correlated to the carbon at δ_C 147.9 in addition to a carbon at δ_C 150.6, while the remaining *N*-methyl protons at δ_H 3.31 also correlated to the carbon at δ_C 150.6 and the last remaining unassigned quaternary carbon at δ_C 159.8. These correlations in combination with chemical shift comparisons with literature data were consistent with **1** containing a 1,3-dimethylpteridine moiety substituted at C-6 by the propyl side chain.[6]

Duramidine A (**1**) was unstable in DMSO and readily converted to a molecule (**2**) with a molecular weight 80 amu smaller. (+)-HRESIMS analysis of its molecular ion at *m*/*z* 443.1583 allowed a molecular formula $C_{21}H_{22}N_4O_7$ to be assigned, indicating that **2** was the desulfated derivative of **1**. The ^{1}H NMR spectrum of this product was identical to that obtained for a second compound, duramidine B (**2**), isolated by HPLC purification of the crude extract. The most prominent difference in the ^{1}H NMR spectrum of **2** compared to that of **1** was the 0.28 and 0.52 ppm upfield shifts of the proton signals assigned to H-1′ and H-2′, respectively, and this was in full agreement with the molecule lacking a sulfate ester group at C-2′. Full 2D NMR analysis confirmed this assignment.

Schmid has analysed chemical shift and coupling constant data for a series of 1-aryl-1,2-disubstituted propanes and concluded that vicinal coupling constants should be used cautiously when assigning either *threo* or *erythro* configuration and analysis of a combination of data provides a more useful insight.[7] There are six potential staggered conformations (three for *erythro* E_T, E_{G1}, E_{G2} and three for *threo* T_T, T_{G1}, T_{G2}) for diastereomeric 1,2-disubstituted-1-aryl propanes (Fig. 2).

Four of these conformations have the vicinal protons in a gauche relationship and two in a *trans* relationship. Since small vicinal coupling constants were observed between H-1′ and H-2′ for **1** (3.9 Hz) and **2** (5.5 Hz) this suggests that H-1′ and H-2′ occupy a gauche conformation thus ruling out the two *trans* conformations (E_T and T_T). In the ROESY spectrum for **2**, a strong ROE was observed between the methyl protons H$_3$-3′ and H-1′ indicating that H$_3$-3′ and H-1′ must also be in a gauche relationship. This observation also rules out the *threo* (E_{G1}) and *erythro* (T_{G1}) conformations. To distinguish between the two remaining conformations, ROESY correlations observed between H-2′ and H-7 were only possible in the *threo* isomer (T_{G2}) since these protons occupy gauche positions, while in the *erythro* isomer (E_{G2}) these protons are *trans* to each other and so are too far apart to show ROE correlations. Therefore the dioxygenated propyl side chain in **2** (and logically in **1** as well) was assigned *threo* relative configuration. Unfortunately the absolute configuration for **2** could not be determined since the compound decomposed before any chemical derivatisation could be attempted.

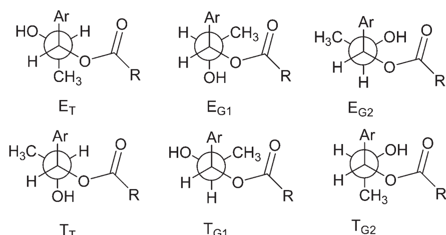

Fig. 2 Conformations of 1,2-disubstituted-1-arylpropanes.

Duramidine C (**3**) was isolated as an optically active yellow gum. The molecular formula, $C_{20}H_{20}N_4O_{10}S$ was determined by interpretation of (−)-HRESIMS in conjunction with the NMR data. Analysis of the ^{1}H and ^{13}C NMR (Table 2), together with HSQC and HMBC data for **3**, showed that it had very similar chemical shifts and correlations to that obtained for duramidine A (**1**), the major difference being the replacement of the *N*-methyl signals at δ_H 3.31/δ_C 28.5 with an exchangeable proton signal at δ_H 11.92. The (−)-HRESIMS result corroborated that the molecular weight difference was due to loss of one of the methyl groups in **1**. Since the exchangeable proton at δ_H 11.92 correlated to both C-4 and C-4a in an HMBC spectrum this indicated that **3** was the *N*-3 demethyl derivative of **1**. ROESY correlations were identical with those observed for **2** suggesting that **3** could also be assigned *threo* relative configuration of the propyl side chain. Duramidine C was unstable and readily hydrolysed to its desulfated derivative duramidine D (**4**).

Table 2 ^{13}C NMR data (150 MHz) for duramidines A–D (**1–4**) in DMSO-d$_6$

Position	**1**[a]	**2**[a]	**3**[b]	**4**[b]
1-CH$_3$	29.2 (CH$_3$)	29.0 (CH$_3$)	28.0 (CH$_3$)	28.2 (CH$_3$)
2	150.6 (C)	150.3 (C)	149.6 (C)	150.0 (C)
3-CH$_3$	28.5 (CH$_3$)	28.5 (CH$_3$)	—	—
4	159.8 (C)	159.7 (C)	160.0 (C)	159.8 (C)
4a	127.0 (C)	126.2 (C)	127.5 (C)	127.6 (C)
6	147.1 (C)	147.2 (C)	147.0 (C)	147.2 (C)
7	146.5 (CH)	146.8 (CH)	146.5 (CH)	146.5 (CH)
8a	147.9 (C)	147.9 (C)	149.8 (C)	149.2 (C)
1′	77.0 (CH)	78.5 (CH)	76.9 (CH)	78.5 (CH)
2′	72.7(CH)	67.4 (CH)	72.4 (CH)	67.3 (CH)
3′	16.2 (CH$_3$)	19.2 (CH$_3$)	15.8 (CH$_3$)	19.1 (CH$_3$)
1″	163.4 (C)	163.4 (C)	163.3 (C)	163.2 (C)
2″	142.4 (C)	142.2 (C)	142.4 (C)	142.4 (C)
2″-OCH$_3$	58.8 (CH$_3$)	58.8 (CH$_3$)	58.6 (CH$_3$)	58.7 (CH$_3$)
3″	125.0 (CH)	124.8 (CH)	125.2 (CH)	124.7 (CH)
4″	124.0 (C)	123.4 (C)	124.0 (C)	124.0 (C)
5″, 9″	132.3 (CH)	132.1 (CH)	132.1 (CH)	132.0 (CH)
6″, 8″	115.8 (CH)	115.7 (CH)	115.7 (CH)	115.6 (CH)
7″	159.0 (C)	158.8 (C)	158.8 (C)	158.7 (C)

[a] Chemical shifts determined from HSQC and HMBC data. [b] Chemical shifts obtained from a ^{13}C NMR spectrum of a mixture of **3** and **4** and assigned following analysis of HSQC and HMBC data.

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Duramidine D (**4**) was isolated as an optically active yellow gum. The molecular formula, $C_{20}H_{20}N_4O_7$ was determined by interpretation of (−)-HRESIMS in conjunction with the NMR data. This data suggested that **4** was the desulfated derivative of **3**. The major difference in the ^{1}H NMR data for **4** compared to **3** was the upfield shift of the resonances associated with H-1′ and H-2′ since these were shifted 0.28 and 0.52 ppm upfield, respectively. These chemical shift differences were completely analogous to those observed between **1** and **2** and therefore duramidine D (**4**) was deduced to be the 2′-desulfated derivative of **3**. ROESY correlations were identical with those observed for **2** and **3** suggesting that **4** could also be assigned *threo* relative configuration of the propyl side chain.

6-Propylpteridines have been isolated previously from insect,[8] polychaete worm,[9] sponge,[6,10] ascidian[11,12] and coral sources,[13] however no sulfated pteridines, nor pteridines esterified with aromatic carboxylic acids, have been reported previously.

Leptoclinidines

Leptoclinidine A (**5**) was isolated as an optically active yellow gum. The (+)-LRESIMS spectrum for **5** had two equally intense peaks at *m/z* 502 and 504 suggesting that the molecule contained one bromine atom. Analysis of (+)-HRESIMS data obtained for the sodiated adduct ion at *m/z* 502.0226 established a molecular formula $C_{19}H_{18}BrN_3O_7$ for **5**.

The ^{1}H NMR spectrum of **5** contained signals for two olefinic/aromatic singlets at δ_H 7.57 and 7.58, one aromatic doublet at δ_H 8.06, one olefinic quartet at δ_H 7.79, three downfield exchangeable protons at δ_H 11.32, 11.80 and 9.81 and one upfield exchangeable proton at δ_H 5.22, five heterosubstituted aliphatic proton multiplets at δ_H 6.29, 5.43, 4.16 and 3.71 (2H), two additional aliphatic multiplets at δ_H 2.36 and 2.40 and one allylic methyl doublet at δ_H 1.79. Edited HSQC correlations established that **5** contained three protonated sp^2 carbons at δ_C 133.8, 115.9 and 105.5, three heterosubstituted methine carbons at δ_C 83.8, 73.8 and 84.7, one oxygenated methylene carbon at δ_C 61.4, one aliphatic methylene carbon at δ_C 37.0 and one methyl carbon at δ_C 12.2. A COSY correlation between the exchangeable proton at δ_H 11.80 to the aromatic proton at δ_H 8.06 was indicative of H-1″ and H-2″ being part of an indole moiety.

HMBC correlations from δ_H 11.80 to carbons at δ_C 133.8, 105.5, 126.3 and 131.5 and from δ_H 8.06 to δ_C 105.5, 126.3 and 131.5 further supported the presence of an indole group. The aromatic singlet at δ_H 7.57 correlated to the carbon at δ_C 131.5 while the aromatic singlet at δ_H 7.58 correlated to the carbon at δ_C 126.3 suggesting that these protons could be assigned to H-4″ and H-7″ of the indole. These protons further correlated to carbons at δ_C 149.0 and 106.1 while the phenolic proton at δ_H 9.81 correlated to both δ_C 149.0 and carbons resonating at δ_C 105.5 and 106.1 indicating that C-5″ was substituted by a hydroxy group and C-6″ by a bromine atom. An additional HMBC correlation from H-2″ to a carbonyl carbon at δ_C 163.6 indicated that the indole was substituted at C-3″ by a carboxyl group. COSY and HMBC correlations demonstrated that the seven aliphatic multiplets were part of a 2-deoxypentose moiety. This sugar group was established to be deoxyribose since strong ROESY correlations were observed between H-1′ and H-4′ and between H-3′ and H$_2$-5′. The olefinic proton at δ_H 7.79 (attached to a carbon at δ_C 135.9) showed a COSY correlation to the methyl protons at δ_H 1.79 confirming that the methyl was allylic. HMBC correlations from δ_H 1.79 and 7.79 to carbons at δ_C 163.5 and 109.6, from δ_H 7.79 to carbons at δ_C 150.4 and 83.8, from the anomeric proton, δ_H 6.29, to carbons at δ_C 135.9 and 150.4 and from the exchangeable proton at δ_H 11.32 to carbons at δ_C 109.6 and 163.5 were consistent with the presence of a thymidine moiety. Finally, a HMBC correlation from H-3′ to the carbonyl carbon at δ_C 163.6 indicated that **5** was a 3′-indole-3-carboxylic acid ester derivative of thymidine. The absolute configuration of the stereogenic centres in **5** was determined by comparison of its optical rotation with that reported for a series of (−)-3′-acyl-thymidine derivatives.[14] In particular the sign and magnitude of the optical rotation for **5** was very similar to that reported for ester derivatives with greater than two carbons in the ester chain and was almost identical with the optical rotation reported for (−)-3′-benzoylthymidine suggesting that **5** contained the natural enantiomer of thymidine.

Leptoclinidine B (**6**) was isolated as an optically active yellow gum. The molecular ion in the (+)-LRESIMS spectrum for **6** also had two equally intense peaks suggesting that one bromine atom was present. Analysis of (+)-HRESIMS data obtained for a sodiated molecular ion at *m/z* 678.0669 established a molecular formula, $C_{29}H_{26}BrN_3O_{10}$, for **6**. The ^{1}H NMR spectrum of **6** (Table 3) contained all of the same signals observed for **5** except the exchangeable signal at δ_H 5.22 which was replaced in **6** with signals indicative of a 4-hydroxy-2′-methoxycinnamate moiety and in addition the oxygenated methylene protons, H$_2$-5′ were shifted 0.79 ppm downfield. Leptoclinidine B was therefore assigned as the 5′-(4-hydroxy-2′-methoxycinnamate) ester of leptoclinidine A. Detailed 2D NMR analysis confirmed this assignment.

The leptoclinidines are the first examples of thymidine derivatives esterified by an indole-3-carboxylic acid to be reported in the literature. Leptoclinidine B is also the first example of a 4-hydroxy-2′-methoxycinnamic acid ester of thymidine.

Durabetaines

Durabetaine A (**7**) was isolated as a colourless gum. A prominent molecular ion at *m/z* 428.1918 in the (+) HRESIMS allowed a molecular formula of $C_{20}H_{30}NO_9^+$ to be assigned to **7**. Analysis of ^{1}H NMR data for **7** identified signals indicative of a 4-hydroxy-2′-methoxycinnamate, in addition to ten heteroatom substituted methine and methylene protons between δ_H 5.07 and 3.60, and a nine proton singlet at δ_H 3.11. Edited HSQC correlations identified four methylene carbons between δ_C 60.4 and 68.3, an oxygenated methine carbon at δ_C 67.3, a dioxygenated methine carbon at δ_C 97.4 and a methyl carbon signal at δ_C 53.1 that could be assigned to three equivalent methyl groups attached to a quaternary nitrogen atom. A

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Table 3 ^{1}H (600 MHz) and ^{13}C (150 MHz) NMR data for leptoclinidines A and B (**5** and **6**) in DMSO-d$_6$

Position	δ_C	δ_H (mult, J in Hz, int.)	HMBC	δ_C	δ_H (mult, J in Hz, int.)	HMBC
2	150.4 (C)	—		150.4 (C)	—	—
3	—	11.32 (s, 1H)	4, 5	—	11.37 (s, 1H)	—
4	163.5 (C)	—		163.6 (C)	—	—
5	109.6 (C)	—		110.0 (C)	—	—
5-CH$_3$	12.2 (CH$_3$)	1.79 (d, 1.0, 3H)	4, 5, 6	11.9 (CH$_3$)	1.72 (d, 1.0, 3H)	4, 5, 6
6	135.9 (CH)	7.79 (q, 1.0, 1H)	2, 4, 5, 5-CH$_3$	136.0 (CH)	7.55 (q, 1.0, 1H)	2, 4, 5, 5-CH$_3$
1′	83.8 (CH)	6.29 (dd, 6.2, 8.5, 1H)	2, 6	84.3 (CH)	6.32 (dd, 6.8, 7.7, 1H)	2, 6
2′	37.0 (CH$_2$)	2.36 (ddd, 2.1, 6.2, 14.4, 1H)	3′, 4′	36.3 (CH$_2$)	2.47 (ddd, 2.1, 6.4, 15.1, 1H)	3′, 4′
		2.40 (ddd, 6.3, 8.5, 14.4, 1H)	1′, 3′		2.57 (ddd, 7.8, 7.8, 15.1, 1H)	1′, 3′
3′	73.8 (CH)	5.43 (ddd, 2.1, 2.2, 6.3, 1H)	1′, 4′, 5′, 8″	73.3 (CH)	5.54 (ddd, 2.6, 2.1, 7.8, 1H)	1′, 4′, 5′, 8″
4′	84.7 (CH)	4.16 (ddd, 2.2, 3.4, 3.4, 1H)	3′	81.3 (CH)	4.44 (ddd, 2.6, 4.5, 4.5, 1H)	3′
5′	61.4 (CH$_2$)	3.71 (m, 2H)	3′	64.5 (CH$_2$)	4.50 (m, 2H)	3′
5′-OH		5.22 (t, 5.1, 1H)	4′, 5′		—	—
1″	—	11.80 (d, 3.1, 1H)	2″, 3″, 3a″, 7a″		11.83 (d, 2.5, 1H)	—
2″	133.8 (CH)	8.06 (d, 3.1, 1H)	3″, 3a″, 7a″, 8″	134.0 (CH)	8.09 (d, 2.5, 1H)	3″, 3a″, 7a″
3″	105.1 (C)	—	—	104.9 (C)	—	—
3a″	126.3 (C)	—	—	126.1 (C)	—	—
4″	105.5 (CH)	7.57 (s, 1H)	6″, 7a″	105.6 (CH)	7.59 (s, 1H)	6″, 7a″
5″	149.0 (C)	—	—	149.1 (C)	—	—
5″-OH		9.81 (s, 1H)	4″, 5″, 6″		9.82 (s, 1H)	4″, 5″, 6″
6″	106.1 (C)	—	—	106.1 (C)	—	—
7″	115.9 (CH)	7.58 (s, 1H)	3a″, 5″	116.0 (CH)	7.59 (s, 1H)	3a″, 5″
7a″	131.5 (C)	—		131.0 (C)	—	—
8″	163.6 (C)	—		163.5 (C)	—	—
1‴				163.6 (C)	—	—
2‴				142.4 (C)	—	—
2‴-OCH$_3$				58.6 (CH$_3$)	3.67 (s, 3H)	2‴
3‴				124.4 (CH)	6.92 (s, 1H)	1‴, 2‴, 5‴, 9‴
4‴				123.7 (C)	—	—
5‴, 9‴				131.9 (CH)	7.57 (d, 8.4, 2H)	3‴, 5‴, 7‴, 9‴
6‴, 8‴				115.6 (CH)	6.77 (d, 8.4, 2H)	4‴, 6‴, 8‴
7‴				158. (C)	—	—
7‴-OH				—	9.92 (s, 1H)	6‴, 7‴, 8‴

glycerol group was assigned from COSY correlations between the methylene protons at δ_H 4.12, 4.20 and 3.63 (2H) and a methine proton at δ_H 3.96, while an ethyl group was assigned from COSY correlations between methylene protons at δ_H 3.97, 4.04 and 3.60 (2H). The presence of a choline group was deduced from HMBC correlations between the trimethyl signal at δ_H 3.11 and the methylene carbon at δ_C 64.4 and the methyl carbon at δ_C 53.1 and from the methylene proton signal at δ_H 3.60 to the methyl carbon at δ_C 53.1. HMBC correlations from the dioxygenated methine proton at δ_H 5.07 to oxygenated methylene carbons of the glycerol (δ_C 68.3) and choline (δ_C 60.4) as well as to a carboxylic acid carbon at δ_C 168.4 showed that a 2,2-dioxyacetic acid moiety formed an acetal linkage to both the choline and glycerol. HMBC correlations from the methylene protons, H-1a and H-1b, to the carbonyl carbon of the 4-hydroxy-2′-methoxycinnamate indicated that the glycerol was esterified at C-1 by the cinnamate group. The structure of durabetaine A was therefore assigned as 1-(4-hydroxy-2′-methoxycinnamoyl)-glyceryl-3-(*O*-carboxyhydroxymethylcholine) (**7**).

The structure of durabetaine B (**8**) was similar to that of durabetaine A except that **8** contained a different ester group. A molecular formula $C_{19}H_{26}BrN_2O_7^+$ was deduced from (+)-HRESIMS analysis. The region of the ^{1}H NMR spectrum upfield of δ_H 5.10 in **8** (Table 4) was almost identical to that of **7** however the signals associated with the 4-hydroxy-2′-methoxycinnamate were missing from the spectrum of **8** and replaced with signals that were almost identical to those observed for the indole moiety in leptoclinidamine C (**9**). This suggested that durabetaine B was 1-(6-bromoindole-3-carboxy)-glyeryl-3-(*O*-carboxyhydroxymethylcholine) (**8**).

Detailed 2D NMR analysis confirmed this structure assignment. The flexible nature of the glyeryl-3-*O*-carboxyhydroxymethylcholine moiety precluded assignment of the relative configuration of the two stereogenic centres, C-2 and C-5 in both **7** and **8** by NMR analysis. Unfortunately **7** and **8** were both isolated in very low yield which restricted our ability to derivatise or degrade the compounds. It should be noted that even if sufficient quantity were available, hydrolysis of either **7** or **8** would yield the achiral products, glycerol and 2-oxo-acetic acid and Mosher ester analysis would only assign the chirality at C-2 without providing evidence to assign the chirality at C-5. The configuration at C-2 and C-5 remains unresolved for both compounds.

Durabetaines A and B are related to lipid esters that have previously been isolated from algae.[15–17] The significant difference between these algal products and the durabetaines is the replacement of the long chain fatty acid attached at C-1 by aromatic esters. Furthermore the algal derivatives reported to date have all contained a second long chain fatty acid attached at C-2. Significantly, the durabetaines are the first compounds from this structure class to be isolated from an animal source.

Table 4 ^{1}H (600 MHz) and ^{13}C (150 MHz) NMR data for durabetaines A and B (**7** and **8**) in DMSO-d$_6$

Position	δ_C^{a}	$^1\delta_H$ (mult, J in Hz, int.)	HMBC	δ_C^{a}	δ_H (mult, J in Hz, int.)	HMBC
1	66.1 (CH_2)	4.12 (dd, 6.0, 11.1, 1H)	2, 3, 1′	64.9 (CH_2)	4.19 (dd, 5.8, 11.2, 1H)	2, 3, 1′
		4.20 (dd, 4.3, 11.1, 1H)	2, 3, 1′		4.25 (dd, 4.4, 11.2, 1H)	2, 3, 1′
2	67.3 (CH)	3.96 (m, 1H)	—	67.7 (CH)	4.00 (m, 1H)	—
3	68.3 (CH_2)	3.63 (m, 2H)	1, 5	68.7 (CH_2)	3.67 (m, 2H)	1, 2, 5
5	97.4 (CH)	5.07 (s, 1H)	3, 7, 9	97.6 (CH)	5.06 (s, 1H)	3, 7, 9
7	60.4 (CH_2)	3.97 (m, 1H)	—	60.6 (CH_2)	3.95 (m, 1H)	—
		4.04 (m, 1H)	—		4.03 (m, 1H)	—
8	64.4 (CH_2)	3.60 (m, 2H)	7, 8-$N(CH_3)_3^+$	64.6 (CH_2)	3.57 (m, 2H)	7, 8-$N(CH_3)_3^+$
8-$N(CH_3)_3^+$	53.1 (3× CH_3)	3.11 (s, 9H)	8, 8-$N(CH_3)_3^+$	53.4 (3× CH_3)	3.09 (s, 9H)	8, 8-$N(CH_3)_3^+$
9	168.4 (C)	—	—	168.7 (C)	—	—
1′	163.9 (C)	—	—	—	11.99 (bs, 1H)	3′, 3a′
2′	142.7 (C)	—	—	133.9 (CH)	8.12 (d, 2.6, 1H)	3′, 3a′, 7a′, 8′
2′-OCH_3	58.7 (CH_3)	3.67 (s, 3H)	C-2′	—	—	—
3′	124.1 (CH)	6.91 (s, 1H)	C-1′, C-2′, C-5′, C-9′	106.9 (C)	—	—
3a′	—	—	—	124.9 (C)	—	—
4′	124.0 (C)		—	122.5 (CH)	7.92 (d, 8.5, 1H)	6′, 7a′
5′	132.0 (CH)	7.62 (d, 8.4, 1H)	3′, 7′, 9′	124.5 (CH)	7.32 (dd, 1.3, 8.5, 1H)	3a′, 7′
6′	115.7 (CH)	6.80 (d, 8.4, 1H)	4′, 8′	115.6 (C)	—	—
7′	158.8 (C)	—	—	115.3 (CH)	7.69 (d, 1.3, 1H)	3a′, 5′
7a′	—	—	—	137.9 (C)	—	
8′	115.7 (CH)	6.80 (d, 8.4, 1H)	4′, 6′	164.3 (C)	—	
9′	132.0 (CH)	7.62 (d, 8.4, 1H)	3′, 5′, 7′	—	—	

^{a} Chemical shifts determined from HSQC and HMBC data.

Table 5 ^{1}H (600 MHz) and ^{13}C (150 MHz) NMR data for leptoclinidamine D (**10**) in DMSO-d$_6$

Position	δ_C^{a}	δ_H (mult, J in Hz, int.)	HMBC
1	—	11.72 (d, 2.8, 1H)	2, 3, 3a, 7a
2	129.2 (CH)	8.03 (d, 2.8, 1H)	3, 3a, 7a
3	109.8 (C)	—	—
3a	125.2 (C)		—
4	122.5 (CH)	7.94 (d, 8.4, 1H)	6, 7a
5	123.6 (CH)	7.22 (dd, 1.8, 8.4, 1H)	3a, 7
6	115.1 (C)	—	—
7	114.7 (CH)	7.63 (d, 1.8, 1H)	3a, 5
7a	137.2 (C)	—	—
8	164.5 (C)	—	—
9-NH	—	8.50 (d, 9.1, 1H)	8
10	50.0 (CH)	4.91 (dt, 5.6, 9.1, 1H)	8, 11, 12, 17
11	25.6 (CH_2)	3.29 (m, 1H)	10, 12, 13, 17
		3.43 (dd, 5.5, 15.2, 1H)	10, 12, 13, 17
12	136.3 (C)	—	—
13	126.3 (C)	—	—
13-SCH_3	18.6 (CH_3)	2.29 (s, 3H)	13
14-CH_3	32.6 (CH_3)	3.77 (s, 3H)	13, 15
15	138.5 (CH)	9.16 (s, 1H)	12, 13
16-CH_3	34.3 (CH_3)	3.87 (s, 3H)	12, 15
17	171.4 (C)	—	—
17-OCH_3	52.4 (CH_3)	3.68 (s, 3H)	17

^{a} Chemical shifts determined from HSQC and HMBC data.

Leptoclinidamines

Leptoclinidamine D (**10**) was isolated as a colourless gum. (+)-HRESIMS analysis of the molecular ion at m/z 465.0571 in combination with NMR analysis indicated that **10** was a quaternary amine salt with a formula $C_{19}H_{22}BrN_4O_3S^+$ for the cation and with trifluoroacetate as the counter ion. The ^{1}H NMR spectrum of **10** (Table 5) was almost identical to that reported previously for leptoclinidamine C (**9**),[1] the only difference being an additional methyl resonance at δ_H 3.68. These protons correlated to a carbon at δ_C 52.4 in the HSQC spectrum and a carbon at δ_C 171.4 in the HMBC spectrum and this supported the assignment of **10** as the methyl ester of **9**. Full 2D NMR analysis confirmed this assignment.

NMR analysis of the remaining two new compounds (**11** and **12**) indicated that each contained a 1,3-dimethyl-5-methylsulfanylimidazole moiety similar to that found in **9** and **10**, but differences were clearly evident in the downfield region of their spectra compared to **9** and **10**. Leptoclinidamine E (**11**) was isolated as its TFA salt and was a pale yellow gum. The (+)-LRESIMS of **11** showed three ions at m/z 462, 464, 466 (1 : 2 : 1) which indicated that the molecule contained two bromine atoms. The molecular formula, $C_{15}H_{18}Br_2N_3O_2S^+$ was determined from analysis of (+)-HRESIMS data.

^{1}H NMR data for **11** (Table 6) pointed to the presence of two mutually coupled methylene groups, an amide triplet, an aromatic singlet that integrated to two protons, suggestive of a symmetrical 1,2,3,5-tetrasubstituted phenyl group, in addition to signals for a 1,3-dimethyl-5-methylsulfanylimidazole group. COSY, HSQC and HMBC correlations confirmed the presence of a substituted histamine in **11** since correlations from the methylene protons at δ_H 3.02 of an ethylamido group were observed to C-11 and C-12 of the imidazole moiety. Strong HMBC correlations from the aromatic resonance at δ_H 7.96 to downfield carbons at δ_C 163.7, 153.5 and 131.2 suggested **11** contained a *para*-hydroxy benzoyl moiety. An additional correlation from these protons to a carbon resonance at δ_C 111.7 supported the positioning of the two bromine atoms at C-2 and C-6 of the benzoyl system on the basis of the diagnostic carbon chemical shift at δ_C 111.7 being similar to that reported for the previously isolated compounds, cadiolides A and B.[18] HMBC cross-peaks observed from both the methylene protons

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Table 6 ^{1}H (600 MHz) and ^{13}C (150 MHz) NMR data for leptoclinidamines E and F (**11** and **12**) in DMSO-d_6

Position	δ_C[a]	δ_H (mult, *J* in Hz, int.)	HMBC	δ_C[a]	δ_H (mult, *J* in Hz, int.)	HMBC
1	153.5 (C)	—	—	157.5 (C)	—	—
1-OH	—	10.66 (br s, 1H)	—		NO[b]	—
2	111.7 (C)	—	—	109.1 (C)	—	—
3	131.2 (CH)	7.96 (s, 2H)	1, 2, 4, 5, 7	132.0 (CH)	7.92 (d, 2.0, 1H)	1, 5, 7
4	131.4 (C)	—	—	126.7 (C)	—	—
5	131.2 (CH)	7.96 (s, 2H)	1, 3, 4, 6, 7	128.3 (CH)	7.63 (dd, 2.0, 8.5, 1H)	1, 3, 7
6	111.7 (C)	—	—	115.9 (CH)	6.96 (d, 8.5, 1H)	2, 4
7	163.7 (C)	—	—	163.7 (C)	—	—
8-NH	—	8.69 (t, 6.0, 1H)	7	—	8.52 (t, 6.0, 1H)	7
9	37.2 (CH_2)	3.48 (dt, 6.0, 6.6, 2H)	7, 11	37.1 (CH_2)	3.47 (dt, 6.0, 6.9, 2H)	7, 11
10	26.6 (CH_2)	3.02 (t, 6.6, 2H)	9, 11, 12	23.9 (CH_2)	3.01 (t, 6.9, 2H)	9, 11, 12
11	137.3 (C)	—	—	137.9 (C)	—	—
12	125.7 (C)	—	—	125.9 (C)	—	—
12-SCH_3	18.7 (CH_3)	2.28 (s, 3H)	12	18.9 (CH_3)	2.27 (s, 3H)	12
13-CH_3	33.8 (CH_3)	3.81 (s, 3H)	12, 14	33.5 (CH_3)	3.80 (s, 3H)	12, 14
14	138.2 (C)	9.19 (s, 1H)	11, 12, 13-CH_3, 15-CH_3	138.5 (C)	9.17 (s, 1H)	11, 12
15-CH_3	33.3 (CH_3)	3.85 (s, 3H)	11, 14	34.0 (CH_3)	3.84 (s, 3H)	14, 15

[a] Chemical shifts determined from HSQC and HMBC data. [b] Not Observed.

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at δ_H 3.48 and the amide proton at δ_H 8.69 to the benzoyl carbon at δ_C 163.7 (C-7) indicated that an amide bond linked the benzoyl group to the histamine moiety. Leptoclinidamine E was therefore assigned structure **11**.

Leptoclinidamine F (**12**), isolated as a TFA salt, displayed two peaks of equal intensity at *m/z* 384 and 386 in the (+)-LRESIMS suggesting that it was the debromo derivative of **11**. (+)-HRESIMS analysis of the molecular ion peak at *m/z* 384.0393 established a molecular formula of $C_{15}H_{19}BrN_3O_2S^+$ for **12**. The 1D and 2D NMR data obtained for **12** (Table 6) was consistent with it being the 6-debromo derivative of **11** since the only differences were associated with the replacement of the two proton aromatic singlet with three aromatic protons with splitting patterns and coupling constants consistent with a 1,2,4-trisubstituted phenyl group.

The duramidines (**1–4**), leptoclinidines (**5**, **6**), durabetaines (**7**, **8**) and leptoclinidamines (**9–13**) and compound **14** have been screened against the breast cancer cell line MDA-MB-231 and the prostate cancer cell line LNCaP, but showed no cytotoxicity at 10 μM after 72 hours. The compounds (**1–14**) have also been screened against the bacteria *Pseudomonas aeruginosa* and *Staphylococcus aureus* at concentrations up to 330 μM but showed no antimicrobial activity after 18 hours.

Conclusions

In summary, 14 alkaloids have been isolated from the Australian ascidian *L. durus.* Four of the alkaloids, duramidines A–D (**1–4**) are first examples of propylpteridine alkaloids substituted by either a 4-hydroxy-2′-methoxycinnamate and/or sulfate esters. Leptoclinidines A and B (**5**, **6**) are the first 3′-indole-3-carboxylic acid ester derivatives of thymidine. Durabetaine A and B (**7**, **8**) are the first glyceryl-3-(*O*-carboxyhydroxymethylcholine) alkaloids to be reported from an animal source and are also the only known derivatives from this class to be acylated with aromatic carboxylic acids. The new leptoclinidamines (**11–12**) are benzamide derivatives of histamine in which the imidazole is substituted by 5-methylsulfanyl and 1,3-dimethyl groups.

Experimental

General procedures

NMR spectra were recorded at 30 °C on a Varian 600 MHz spectrometer equipped with a triple resonance cold probe. The ^{1}H and ^{13}C chemical shifts were referenced to the solvent peak for DMSO-d_6 at δ_H 2.49 and δ_C 39.5. LRESIMS and some HRESIMS were recorded on a Applied Biosystems Mariner Biospectrometry TOF workstation using positive electrospray ionization, mobile phase 1 : 1 MeOH : H_2O. HRESIMS were recorded on a Bruker Daltronics Apex III 4.7e Fourier-transform mass spectrometer. IR and UV spectra were recorded on a Bruker Tensor 27 spectrometer and a Shimadzu UV-1800 UV spectrophotometer, respectively. Optical rotations were measured on a JASCO P-1020 polarimeter and $[\alpha]_D$ values are given in 10^{-1} deg cm^2 g^{-1}. Alltech Davisil 30–40 μm 60 Å C_{18} bonded silica was used to adsorb the ascidian extract prior to HPLC separation. A Merck Hitachi L7100 pump equipped with a Merck Hitachi L7455 PDA detector and a Merck Hitachi L7250 autosampler or a Waters 600 pump equipped with a Waters 996 PDA detector and a Waters 717 autosampler were used for HPLC. A Betasil C_{18} 5 μm 143 Å column (21.2 mm × 150 mm) and a Phenomenex Luna C_{18} 5 μm 100 Å column (21.2 × 250 mm) were used for semi-preparative HPLC separations. All solvents used for chromatography, UV, and MS were Lab-Scan HPLC grade, and the H_2O was Millipore Milli-Q PF filtered.

Collection and identification of the ascidians

The ascidian *Leptoclinides durus* Kott, 2001 (Didemnidae) was collected by SCUBA diving from the Swain Reefs region of the southern Great Barrier Reef in 2005. A voucher specimen, G325084, has been deposited at the Queensland Museum,

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South Brisbane, Queensland, Australia. A second sample of *L. durus* was collected by SCUBA diving at a depth of 23 m between Surprise and Swain Reefs in 2001. A voucher sample G317469 has also been deposited at the Queensland Museum.

Extraction and isolation

Ascidian sample 1 (G325084). The freeze-dried and ground ascidian sample (144.9 g) was extracted exhaustively with MeOH (5 × 300 mL) to yield a dark brown residue (7.6 g). This extract was dissolved in a small amount of MeOH (20 mL) and C_{18} silica gel (8 g) was added. The solvent was evaporated and the extract, adsorbed on to the gel, was transferred to four refillable HPLC columns (30 mm × 10 mm). These columns were connected in series to a C_{18} HPLC column and combined columns eluted with a linear gradient of H_2O containing 1% TFA to MeOH containing 1% TFA at a flow rate of 10 mL min^{-1} over 60 min. Sixty 1 min fractions were collected and all fractions were analyzed by (+)-LRESIMS. Fractions containing similar ions were further analysed by ^1H NMR spectroscopy and fractions containing similar signals were combined. Fractions 31–37 were combined and further separated by C_{18} HPLC with a gradient from 80% H_2O containing 1% TFA/20% MeOH containing 1% TFA to 20% H_2O containing 1% TFA/80% MeOH containing 1% TFA at a flow rate of 10 mL min^{-1} over 60 min yielding leptoclinidamine B (**13**, 9.4 mg, 0.007%) and durabetaine A (**7**, 0.7 mg, 0.005%) respectively. Fractions 38–40 were combined and repurified by C_{18} HPLC with a gradient from 80% H_2O containing 1% TFA/20% MeOH containing 1% TFA to 20% H_2O containing 1% TFA/80% MeOH containing 1% TFA at a flow rate of 10 mL min^{-1} over 60 min, collecting 30 s fractions. Fractions eluting between 24 min and 32 min were combined and further purified by C_{18} HPLC with a gradient from 90% H_2O containing 1% TFA/10% CH_3CN containing 1% TFA to 75% H_2O containing 1% TFA/25% CH_3CN containing 1% TFA at a flow rate of 10 mL min^{-1} over 60 min, collecting 30 s fractions yielding leptoclinidamine F (**12**, 0.4 mg, 0.0003%). Fractions 41–51 from the first HPLC separation were combined and repurified by C_{18} HPLC with a gradient from 70% H_2O containing 1% TFA/30% MeOH containing 1% TFA to 30% H_2O containing 1% TFA/70% MeOH containing 1% TFA at a flow rate of 10 mL min^{-1} over 60 min, collecting 30 s fractions yielding leptoclinidamine E (**11**, 0.3 mg, 0.0002%), duramidine C (**3**, 1.0 mg, 0.0007%) and 6-bromo-1*H*-indolo-3-yl-oxoacetic acid methyl ester (**14**, 1.8 mg, 0.001%). Fractions eluting between 18 min and 23 min were combined and further purified by C_{18} HPLC with a gradient from H_2O containing 1% TFA to 60% H_2O containing 1% TFA/40% CH_3CN containing 1% TFA at a flow rate of 10 mL min^{-1} over 60 min, collecting 30 s fractions yielding duramidine A (**1**, 3.1 mg, 0.002%), leptoclinidine A (**5**, 5.2 mg, 0.004%), leptoclinidamine C (**9**, 13.5 mg, 0.009%), duramidine D (**4**, 1.1 mg, 0.0008%), duramidine B (**2**, 5.3 mg, 0.004%) and leptoclinidamine D (**10**, 1.2 mg, 0.0008%) respectively. Fractions 52–60 from the first HPLC separation were combined and purified further by C_{18} HPLC with a gradient from 90% H_2O containing 1% TFA/10% MeOH containing 1% TFA to 60% H_2O containing 1% TFA/40% MeOH containing 1% TFA at a flow rate of 10 mL min^{-1} over 60 min yielding pure leptoclinidine B (**6**, 1.7 mg, 0.001%) and durabetaine B (**8**, 1.3 mg, 0.0009%).

Ascidian sample 2 (G317469). The second freeze-dried and ground ascidian sample (10 g) was transferred to a conical flask (1 L); *n*-hexane (250 mL) was added and the flask was shaken at 200 rpm for 2 h. The *n*-hexane extract was filtered under gravity, and then discarded. CH_2Cl_2/MeOH (4 : 1, 250 mL) were added to the de-fatted ascidian sample in the conical flask and shaken at 200 rpm for 2 h. The resulting extract was filtered under gravity, and set aside. MeOH (250 mL) was added to the ascidian sample, and the flask shaken at 200 rpm for 2 h before filtration. Another volume of MeOH (250 mL) was then added and the MeOH/ascidian mixture shaken for a further 16 h at 200 rpm, followed by gravity filtration. All CH_2Cl_2/MeOH extracts were combined and dried under reduced pressure to yield a dark brown gum (1 g). This extract was resuspended in CH_2Cl_2/MeOH and adsorbed to C_{18} silica then packed into a stainless steel refillable HPLC column (10 × 30 mm) that was connected to a semi-preparative C_{18} Betasil HPLC column. Isocratic conditions of 10% MeOH (0.1% TFA)/90% H_2O (0.1% TFA) were initially performed for the first 10 min, followed by a linear gradient to MeOH (0.1% TFA) in 40 min, then isocratic conditions of MeOH (0.1% TFA) were maintained for a further 10 min, all at a flow rate of 9 mL min^{-1}. Sixty fractions (60 × 1 min) were collected from time = 0 min, and analysed by (±)-LRESIMS. Fractions 33, 34 and 36 were further purified by semi-preparative HPLC using a C_{18} Luna column. Separation of both fractions 33 and 34 involved isocratic conditions of 5% MeOH (0.1% TFA)/95% H_2O (0.1% TFA) for 10 min, followed by a linear gradient to 60% MeOH (0.1% TFA)/40% H_2O (0.1% TFA) in 10 min, and then another linear gradient to 90% MeOH (0.1% TFA)/10% H_2O (0.1% TFA) for 30 min, followed by MeOH (0.1% TFA) for 10 min, all at a flow rate of 4 mL min^{-1}. Sixty fractions (60 × 1 min) were collected from time = 0 min, and analysed by (±)-LRESIMS. HPLC of fraction 34, afforded fractions 36 and 42 which following lyophilisation yielded compounds **11** (0.77 mg, 0.008%) and **3** (0.67 mg, 0.007%), respectively. HPLC of fraction 33 yielded fractions 43 and 44 that were combined and purified using the same conditions described above to yield compound **4** (0.35 mg, 0.004%). Fraction 36 obtained from the first isolation step was purified at a flow rate of 4 mL min^{-1} using a linear gradient starting at 10% MeOH (0.1% TFA)/90% H_2O (0.1% TFA) and finishing at 50% MeOH (0.1% TFA)/50% H_2O (0.1% TFA) in 5 min. This was followed by a linear gradient to 80% MeOH (0.1% TFA)/20% H_2O (0.1% TFA) in 30 min, and then a gradient to MeOH (0.1% TFA) in 10 min, which was held at these conditions for a further 5 min. This afforded the known compound leptoclinidamine C (**9**, 1.3 mg, 0.013%), which eluted at 43–44 min.

Duramidine A (1) yellow gum; $[\alpha]_D^{23}$ +74 (c 0.025, MeOH); UV λ_{max} (MeOH)/nm (log ε) 216 (4.56), 285 (3.69), 320 (3.49); IR ν_{max} (film)/cm^{-1} 3190, 1720, 1700, 1683, 1653, 1544, 1457; ^1H and ^{13}C NMR data (DMSO-d_6) see Tables 1 and 2; (+)-LRESIMS m/z 545 (MNa^+ 15%), 523 (MH^+ 90%), 443 (MH^+-SO_3 10%), 515

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(M^+ 30%); (+)-HRESIMS *m/z* 523.1133 (calcd for $C_{21}H_{23}N_4O_{10}S$, 523.1135).

Duramidine B (2) yellow gum; $[\alpha]_D^{23}$ +94 (*c* 0.05, MeOH); UV λ_{max} (MeOH)/nm (log *ε*) 214 (4.52), 284 (3.65), 318 (3.48); IR ν_{max} (film)/cm^{-1} 1721, 1698, 1682, 1651, 1561, 1456; ^{1}H and ^{13}C NMR data (DMSO-d$_6$) see Tables 1 and 2; (+)-LRESIMS *m/z* 443 (MH^+ 100%); (+)-HRESIMS *m/z* 443.1583 (calcd for $C_{21}H_{23}N_4O_7$, 443.1567).

Duramidine C (3) yellow gum; $[\alpha]_D$ +93 (*c* 0.044, MeOH); UV λ_{max} (MeOH)/nm (log *ε*) 233 (4.12), 250 sh (3.98), 316 (4.24); ^{1}H and ^{13}C NMR data (DMSO-d$_6$) see Tables 1 and 2; (−)-LRESIMS *m/z* 427 ($M\text{-}SO_3H^-$ 100%); (−)-HRESIMS *m/z* 507.0808 (calcd for $C_{20}H_{19}N_4O_{10}S$, 507.0827).

Duramidine D (4) yellow gum; $[\alpha]_D^{23}$ +7 (*c* 0.023, MeOH); UV λ_{max} (MeOH)/nm (log *ε*) 230 (3.77), 249 sh (3.65), 313 (3.75); ^{1}H and ^{13}C NMR data (DMSO-d$_6$) see Tables 1 and 2; (−)-LRESIMS *m/z* 427 ($M\text{-}H^-$ 100%); (−)-HRESIMS *m/z* 427.1244 (calcd for $C_{20}H_{19}N_4O_7$, 427.1244).

Leptoclinidine A (5) yellow gum; $[\alpha]_D^{24}$–29 (*c* 0.26, MeOH); UV λ_{max} (MeOH)/nm (log *ε*) 216 (4.55), 252 (4.08), 282 (3.49); IR ν_{max} (film)/cm^{-1} 1720, 1700, 1683, 1653; ^{1}H and ^{13}C NMR data (DMSO-d$_6$) see Table 3; (+)-LRESIMS *m/z* 504 (MNa^+ 50%), 502 (MNa^+ 50%); (+)-HRESIMS *m/z* 502.0224 (calcd for $C_{19}H_{18}{}^{79}BrN_3O_7Na$, 502.0226).

Leptoclinidine B (6) colourless gum; $[\alpha]_D^{24}$ −4.6 (*c* 0.3, MeOH); UV λ_{max} (MeOH)/nm (log *ε*) 216 (5.18), 252 (4.86), 306 (4.81); IR ν_{max} (film)/cm^{-1} 2946, 1718, 1698, 1682, 1674, 1456, 1204; ^{1}H and ^{13}C NMR data (DMSO-d$_6$) see Table 3; (+)-LRESIMS *m/z* 680 (MNa^+ 50%), 678 (MNa^+ 50%); (+)-HRESIMS *m/z* 678.0669 (calcd for $C_{29}H_{26}{}^{79}BrN_3O_{10}Na$, 678.0699).

Durabetaine A (7) colourless gum; $[\alpha]_D^{23}$ −64 (*c* 0.02, MeOH); UV λ_{max} (MeOH)/nm (log *ε*) 216 (4.38), 302 (3.71); IR ν_{max} (film)/cm^{-1} 2919, 2849, 1735, 1686, 1651, 1542; ^{1}H and ^{13}C NMR data (DMSO-d$_6$) see Table 4; (+)-LRESIMS *m/z* 428 (M^+ 100%); (+)-HRESIMS *m/z* 428.1918 (calcd for $C_{20}H_{30}NO_9$, 428.1921).

Durabetaine B (8) colourless gum; $[\alpha]_D^{24}$ −77 (*c* 0.008, MeOH); IR ν_{max} (film)/cm^{-1} 2972, 1733, 1701, 1681; ^{1}H and ^{13}C NMR data (DMSO-d$_6$) see Table 4; (+)-LRESIMS *m/z* 475 (M^+ 50%), 473 (M^+ 50%); (+)-HRESIMS *m/z* 473.0908 (calcd for $C_{19}H_{26}{}^{79}BrN_2O_7$, 473.0923).

Leptoclinidamine D (10) colourless gum; $[\alpha]_D^{23}$ −18 (*c* 0.04, MeOH); UV λ_{max} (MeOH)/nm (log *ε*) 216 (4.34), 286 (3.65); IR ν_{max} (film)/cm^{-1} 1701, 1686, 1653; ^{1}H and ^{13}C NMR data (DMSO-d$_6$) see Table 5; (+)-LRESIMS *m/z* 467 (M^+ 50%), 465 (M^+ 50%); (+)-HRESIMS *m/z* 465.0571 (calcd for $C_{19}H_{22}{}^{79}BrN_4O_3S$, 465.0596).

Leptoclinidiamine E (11) yellow gum; UV λ_{max} (MeOH)/nm (log *ε*) 224 (4.24), 286 (3.30); IR ν_{max} (film)/cm^{-1} 3430, 2952, 1684, 1653; ^{1}H and ^{13}C NMR data (DMSO-d$_6$) see Table 6; (+)-LRESIMS *m/z* 462 (M^+ 50%), 464 (M^+ 100%), 466 (M^+ 50%); (+)-HRESIMS *m/z* 461.9484 (calcd for $C_{15}H_{18}{}^{79}Br_2N_3O_2S$, 461.9481).

Leptoclinidamine F (12) yellow gum; UV λ_{max} (MeOH)/nm (log *ε*) 218 (4.41), 284 (3.26); IR ν_{max} (film)/cm^{-1} 3421, 2923, 1701, 1651; ^{1}H and ^{13}C NMR data (DMSO-d$_6$) see Table 6; (+)-LRESIMS *m/z* 386 (M^+ 50%), 384; (+)-HRESIMS *m/z* 384.0393 (calcd for $C_{15}H_{19}{}^{79}BrN_3O_2S$, 384.0381).

Anti-microbial assay

Antibacterial activity was determined using cellular bioassays, and quantified by the spectroscopic analysis of the metabolic dye, resazurin. The bioassays were conducted in 500 μL, 96 well microtiter plates, with each well containing broth (75 μL), microbial inoculate stock solution (37.5 μL), and Milli-Q H_2O (32.5 μL). Finally DMSO (5 μL) stocks of the pure compounds (**1–14**), or positive controls (Piperacillin for *P. aeruginosa* MIC 2 μg mL^{-1} and *S. aureus* MIC 0.13 μg mL^{-1}), were transferred to the wells in triplicate and incubated for 18 h. Microbial inoculate stock solutions of *Pseudomonas aeruginosa* and *Staphylococcus aureus* were prepared by transferring a single colony from a plate to a sterile falcon tube, containing 1 mL of single strength Luria bertani broth (prepared by dissolving 25 g of powdered broth in l L of distilled H_2O and sterilised by autoclaving for 20 min at 121 °C). The stock solution was incubated for 18 h, at 37 °C while being shaken at 100 rpm. After the incubation, 40 μL was transferred to a new sterile falcon tube and adjusted to 2 mL, with single strength broth. 100 μL of this solution was then transferred to a 15 mL sterile tube and adjusted to 10 mL with single strength broth. A resazurin stock solution was prepared by dissolving powdered resazurin (16.14 mg) in sterilised water (100 mL). After the microbial solutions were incubated with the tests or control solutions for 18 h resazurin stock solution (20 μL) was added to all wells, and incubated for a further 1 h at 37 °C while being shaken at 100 rpm. Inhibition was quantified by determining the percentage inhibition of test compounds in comparison with positive and solvent controls using a Fluoroskan Ascent fluorospectrometer (excitation wavelength 570 nm and emission wavelength 620 nm).

Cytotoxicity assay

All compounds were tested against the prostate cancer cell line LNCaP and the breast cancer cell line MDA-MB-231 using a real time cell analyser (xCELLigence, Roche Applied Systems). LNCaP and MDA-MB-231 cells were routinely grown in RPMI or DMEM medium, respectively, supplemented with 10% (v/v) FBS (Invitrogen, USA) at 37 °C in the presence of 5% CO_2. These cells were seeded in a 96-well E-plate™ at a density of 10 × 10^3 cells (LNCaP) or 5 × 10^3 cells (MDA-MB-231) in a final volume of 150 μL. The attachment of the cells was monitored for 4 h every 2 min. After this period, the conditions of the cells were analysed every 1 h. After 24 h, the cells were treated with compounds **1–14** at a final concentration of 10 μM. The biological status of the cells were monitored for 2 h every minute and then for 70 h every hour. Doxorubicin (5 μM) and DMSO (0.1%) were used as controls.

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