Organic &
Biomolecular
Chemistry

Cite this: *Org. Biomol. Chem.*, 2011, **9**, 604

www.rsc.org/obc

PAPER

Wilsoniamines A and B: novel alkaloids from the temperate Australian bryozoan, *Amathia wilsoni*†

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Received 4th August 2010, Accepted 23rd September 2010
DOI: 10.1039/c0ob00538j

Two novel alkaloids, wilsoniamines A and B, both possessing a hexahydropyrrolo[1,2-*c*]imidazol-1-one ring system that has not previously been found in nature, together with a new alkaloid, amathamide H and a known alkaloid, amathamide C were isolated from the temperate Australian bryozoan, *Amathia wilsoni*. MS and NMR analysis established the structure of the new compounds and indicated that the structure of amathamide C and several related compounds be revised. Amathamides C and H showed moderate anti-malarial and anti-trypanosomal activity.

Introduction

Marine invertebrates from the phylum Ectoprocta (Bryozoans) show great diversity and wide geographic distribution.[1] Medical interest in this group of animals was inspired by the discovery of the potently active antitumor compounds, the bryostatins, from the fouling species *Bugula neratina* collected in the USA.[2] Naturally occurring bryostatins and synthetic analogues of these complex macrolides have been the focus of numerous anticancer clinical trials and a selection of compounds show good efficacy particularly as combination therapies.[3] These findings have prompted further research into the natural products chemistry of other bryozoan species and this has yielded a modest number of compounds, mainly alkaloids, many possessing novel ring systems and interesting biological activities.[4] It is however surprising, given their rich species diversity (estimated to be > 5000 species worldwide) that more natural products haven't been isolated from species from this phylum. For this reason we have initiated a program to identify bioactive natural products from Australian bryozoans. We have developed efficient methods to identify biota species that contain natural products with lead-like properties.[5] These procedures were applied to bryozoans collected from Tasmania, New South Wales and Queensland. One sample *Amathia wilsoni* Kirkpatrick, collected from Port Arthur in Tasmania was highlighted for further analysis and this resulted in four alkaloids, wilsoniamines A and B and amathamides C and H, being isolated. Two of the alkaloids, wilsoniamines A and B possess a novel hexahydropyrrolo[1,2-*c*]imidazol-1-one ring system. Herein the isolation, structural determination and biological activity of these novel bryozoan compounds are reported.

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† Electronic supplementary information (ESI) available: 1D and 2D NMR spectra for wilsoniamines A and B and amathamide C and H. See DOI: 10.1039/c0ob00538j

Results and discussion

Purification of the MeOH extract of *A. wilsoni* by HPLC on C_{18} silica gel eluting with a linear gradient from H_2O (containing 1% TFA) to MeOH (containing 1% TFA) over 60 min. yielded wilsoniamine B (**2**), a mixture of wilsoniamines A and B (**1** and **2**), amathamide H (**3**) and amathamide C (**4**) (Fig. 1). The mixture of wilsoniamines A and B was further separated by repeated C_{18}

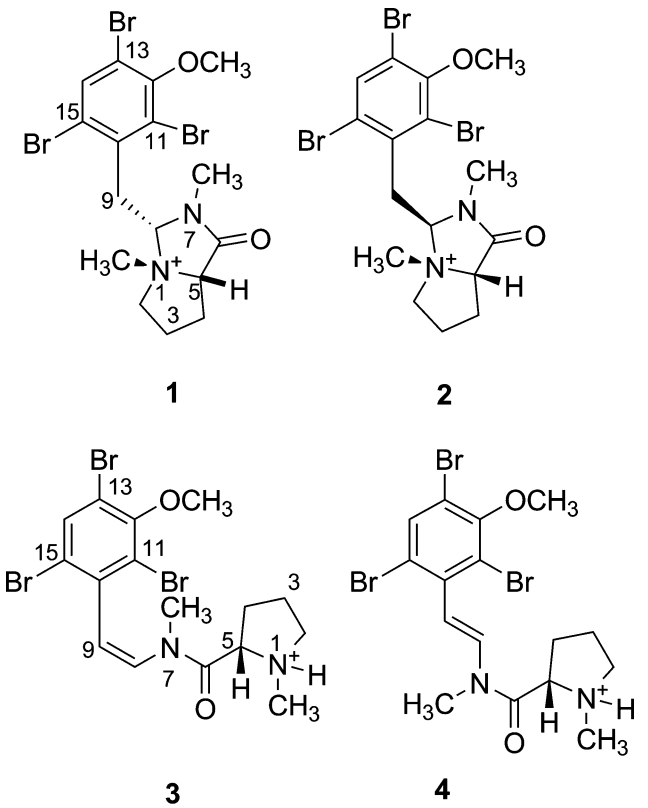


Fig. 1 Alkaloids isolated from *Amathia wilsoni*.

Table 1 ^{1}H and ^{13}C NMR data for wilsoniamines A and B and amathamide H (**1–3**) in DMSO-d_6

	Wilsoniamine A (**1**)		Wilsoniamine B (**2**)		Amathamide H (**3**)[d]	
Position	δ_C[a,b]	δ_H[c] (mult, *J* in Hz, int.)	δ_C[a,b]	δ_H[c] (mult, *J* in Hz, int.)	δ_C[a,b]	δ_H[c] (mult, *J* in Hz, int.)
1-NMe	46.8	3.33 (s, 3H)	43.9	3.37 (s, 3H)	41.2	2.82 (s, 3H)
2	58.4	3.86 (m, 2H)	65.7	3.70 (m, 1H) 3.98 (ddd, 3.9, 8.8, 12.7, 1H)	55.6	3.12 (m, 1H) 3.60 (m, 1H)
3	21.1	2.17 (m, 1H) 2.25 (m, 1H)	21.0	2.06 (m, 1H) 2.25 (m, 1H)	22.2	1.90 (m, 1H) 2.11 (m, 1H)
4	23.4	2.25 (m, 1H) 2.46 (m, 1H)	23.4	2.35 (m, 1H) 2.41 (m, 1H)	27.9	1.94 (m, 1H) 2.59 (m, 1H)
5	74.0	4.69 (dd, 3.1, 9.8, 1H)	70.8	5.12 (dd, 0.1, 7.8, 1H)	67.4	4.75 (t, 7.3, 1H)
6	168.9	—	169.2	—	167.4	—
7-NMe	27.8	2.60 (s, 3H)	29.8	2.30 (s, 3H)	33.2	2.61 (s, 3H)
7-NH		—		—		9.79 (bs, 1H)
8	80.9	5.76 (dd, 3.7, 9.2, 1H)	80.2	5.31 (dd, 3.9, 10.7, 1H)	129.6	6.89 (d, 9.8, 1H)
9	34.6	3.72 (dd, 9.2, 15.3, 1H) 3.93 (dd, 3.7, 15.3, 1H)	38.0	3.74 (dd, 3.9, 12.7, 1H) 3.80 (dd, 10.7, 12.7, 1H)	113.6	5.82 (d, 9.8, 1H)
10	133.7	—	133.4	—	137.9	—
11	120.4	—	120.6	—	118.5	—
12	154.4	—	153.9	—	153.5	—
12-OMe	60.3	3.83 (s, 3H)	60.1	3.82 (s, 3H)	60.5	3.81 (s, 3H)
13	118.3	—	119.3	—	116.9	—
14	136.1	8.18 (s, 1H)	135.8	8.17 (s, 1H)	134.6	8.08 (s, 1H)
15	123.3	—	123.4	—	120.1	—

[a] 150 MHz. [b] ^{13}C Chemical shifts obtained from correlations observed in gHSQC and gHMBC spectra [c] 600 MHz. [d] NMR data for the major isomer, NMR data for the minor isomer is presented in the experimental section.

silica gel HPLC to yield **1** and **2**. All molecules were isolated as their TFA salts.

Wilsoniamine A (**1**) was obtained as a colourless gum. A $[M]^+$ ion cluster at *m/z* 509, 511, 513, 515 in the LRESIMS suggested the molecule contained three bromine atoms. Analysis of (+) HRESIMS data at *m/z* 510.9044 (Δ 1.0 ppm) allowed the molecular formula $C_{16}H_{20}{}^{79}Br_2{}^{81}BrN_2O_2$ to be assigned to **1**. Absorption bands at 1731 and 1684 cm^{-1} in the IR spectrum pointed to the molecule containing amide, and aromatic functionality. The ^{1}H NMR spectrum of **1** (Table 1) contained one aromatic singlet, a methoxyl singlet, two *N*-methyl singlets, two heterosubstituted methine proton doublet of doublets at δ_H 5.76 and 4.69 and a further four one proton multiplets between 2.15 and 4.00 ppm. HSQC correlations allowed one protonated aromatic methine, two heteroatom substituted methine, one aromatic methoxyl, one amino-methylene, two *N*-methyl and three aliphatic methylene carbons to be assigned. Analysis of the COSY spectrum and inspection of ^{1}H–^{1}H coupling constants indicated that **1** possessed two discrete coupled proton spin systems, a CH_2CH and a $CHCH_2CH_2CH_2$. Correlations obtained from a HMBC spectrum provided evidence to assemble these identified partial structures. The *N*-methyl protons 1-NCH_3 correlated to three carbons (C-2, C-5 and C-8) affirming that **1** contained a quaternary nitrogen and a pyrrolidine ring. The second *N*-methyl protons, 7-NCH_3, also showed a correlation to C-8 as well as to an amide carbon, C-6, at δ_C 168.9 and this meant that C-8 was an *N*,*N*-aminal. A correlation was also observed between H-5 and the amide carbon, C-6, and this established the presence of a bicyclic hexahydropyrrolo[1,2-*c*]imidazol-1-one ring system. Correlations were observed from the methylene protons H_2-9 to three quaternary aromatic carbons (C-10, C-11, and C-15) indicating that the hexahydropyrrolo[1,2-*c*]imidazol-1-one ring system was attached to an aromatic moiety *via* a methylene bridge. The aromatic proton, H-14, also showed correlations to two of these carbons, C-10 (strong correlation) and C-15 (weak correlation), and this suggested that these carbons were *meta* and *ortho* to C-14 respectively. H-14 also showed strong correlations to aromatic quaternary carbons at δ_C 154.4 and 118.3, while the methoxy proton 13-OCH_3 also correlated to the carbon at δ_C 154.4. This combined data indicated that bromine atoms substituted C-11 and C-14, and left the methoxy and the third bromine atom to be attached to either C-12 or C-13. Two distinguishing ^{13}C NMR features allowed the correct regiochemical assignment to be established for these substituents. Firstly, the ^{13}C chemical shift of a carbon adjacent to an oxygenated aromatic carbon is consistently shifted ~15 ppm upfield relative to that expected for benzene (*i.e.* upfield of 128 ppm) and secondly, aromatic methoxy carbons that do not have *ortho* proton substituents resonate downfield of 60 ppm, while aromatic methoxyl carbons that are *ortho* to either one or two aromatic protons resonate at ~ 55 ppm.[6] Therefore the data observed for **1** was consistent with the methoxy group being attached at C-12 and the bromine attached at C-13. The relative configuration of the three stereogenic centers in **1** was determined from correlations observed in a ROESY spectrum (Fig. 2). Mutual correlations observed between 1-NCH_3, H-5 and H-8 confirmed that these protons all reside on the β-face of the bicyclic ring system.

Wilsoniamine B (**2**) was also isolated as a colourless oil. HRESIMS data (*m/z* 508.9136, Δ 1.3 ppm) established a molecular formula of $C_{16}H_{20}{}^{79}Br_3N_2O_2$ suggesting that **2** was an isomer of **1**. NMR analysis (Table 1) showed that the major difference between the ^{1}H NMR spectra for **2** compared to **1** were the significant (~0.3 ppm) upfield shift of 7-NCH_3 and H-8 and downfield shift of H-5. Subtle changes in the chemical shifts of the remaining protons were also observed. Analysis of 2D NMR data (COSY, HSQC and HMBC) confirmed that **2** was diastereomeric with **1**. ROESY correlations observed between H-5 and H-9a and

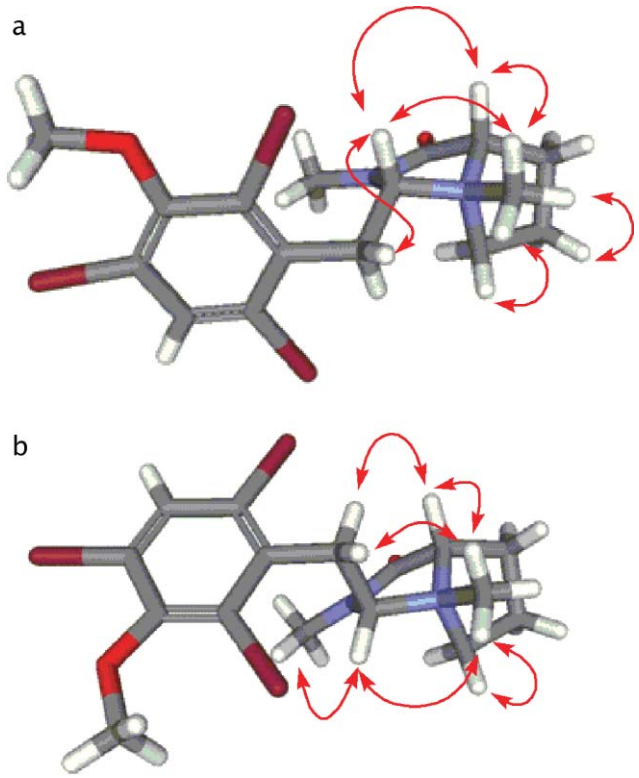


Fig. 2 ROESY correlations observed for **1** (a) and **2** (b).

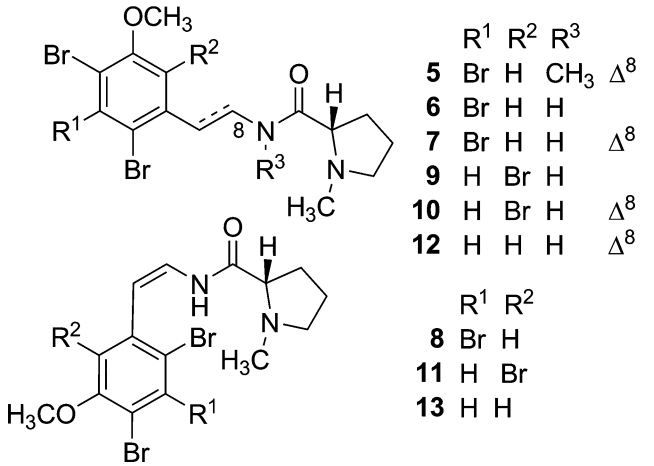
1-NCH_3 (Fig. 2) allowed the relative configuration of the three stereogenic centers in **2** to be assigned, indicating that **2** was the C-8 epimer of **1**.

Amathamide H (**3**) was isolated as a colourless gum. Although its HRESIMS analyzed for a molecule with the same molecular formula as **1** and **2** (*m/z* 508.9093, Δ 4.5 ppm, $C_{16}H_{20}{}^{79}Br_3N_2O_2$) the NMR data for **3** was very different. A prominent feature of the ^{1}H NMR spectrum of **3** was the presence of doubled signals throughout the spectrum, each possessing the same splitting pattern and in a ratio of 2 : 3, and this suggested that the sample was a mixture of two closely related compounds. Attempts to separate this mixture by C_{18} HPLC failed and so 2D NMR analysis (COSY, HSQC and HMBC) was carried out on the mixture. The ^{1}H NMR spectrum possessed features indicative that each molecule contained a pentasubstituted aromatic ring, a *cis* double bond (J = 9.8 Hz), an aromatic methoxy group, two *N*-methyl singlets, a number of aliphatic multiplets and a downfield exchangeable proton. The HSQC spectrum confirmed the presence of *O*-methyl and two *N*-methyl groups, two protonated olefinic carbons, a protonated aromatic carbon, an N or O substituted methine, an amino-methylene and two aliphatic methylenes. COSY data and analysis of ^{1}H–^{1}H coupling constants supported the presence of two coupled spin systems, a *cis* 1,2-disubstituted olefin and $CHCH_2CH_2CH_2N$. HMBC correlations between 1-NCH_3 and C-2 and C-5 established the presence of an *N*-methyl-pyrrolidine ring. The second *N*-methyl protons (7-NCH_3) correlated to the olefinic carbon, C-8, and an amide carbonyl carbon, C-6, suggesting that each molecule contained an *N*-methyl enamide. This was corroborated by the observation of correlations from the olefinic proton, H-8, to the *N*-methyl carbon 7-NCH_3, and the amide carbonyl carbon C-6. HMBC correlations between the olefinic proton H-9, the aromatic proton H-14 and the methoxy protons 12-OCH_3 to carbons of very similar chemical shift to those observed for correlations from 9-CH_2, 12-OCH_3 and H-14 in **1** and **2** suggested that **3** also contained a 2,4,6-tribromo-3-methoxyphenyl group attached, in this case, to a disubstituted olefin. The addition of a bond between C-6 and C-5 established the structure of **3** and demonstrated that both sets of signals in the NMR spectrum could be assigned to the same molecular structure. It is well recognized that tertiary amides commonly exist in solution as interconverting *cis–trans* isomers about the amide bond.[7] This isomerism is slow on the NMR time scale but quick enough to prevent purification of individual isomers. The doubled sets of signals present in the NMR spectra for **3** could therefore be assigned to interconverting amide bond tautomers. Comparison of ^{13}C shifts of *N*-methyl groups *syn* and *anti* to the amide carbonyl have shown that *syn* methyls always resonate upfield of the *anti* methyl.[8] Therefore the major isomer present in DMSO solutions of **3** is the *syn* isomer.

Analysis of the spectroscopic data (MS, 1D and 2D NMR) acquired for **4** indicated that it too was a molecule that was present in solution as two interconverting *cis–trans* amide bond isomers. The only difference between the structure of **3** compared to **4** was that **4** contained a *trans* olefin (J = 15.3 Hz) instead of a *cis* olefin. A review of the literature suggested that **4** could be isomeric with amathamide C (**5**) isolated from a previous collection of the Tasmanian bryozoan *A. wilsoni*.[9] Comparison of the NMR data published for **5** with that we obtained for **4** however showed remarkable similarities suggesting that both molecules possessed the same structure. Therefore structure **5** proposed by Blackman and Green is incorrect and should be revised to **4**. Compelling evidence for structural revision came from inspection of the published chemical shift data for the methoxyl carbon (δ_C 60.4) and the protonated aromatic carbon (δ_C 136.2) since these chemical shifts are not consistent with a 2,3,4-tribromo-5-methoxyphenyl group, but are consistent with a 2,4,6-tribromo-3-methoxyphenyl group. By analogy, the structures of amathamides D–F (**6–8**), each proposed to possess 2,3,4-tribromo-5-methoxyphenyl residues, should also be revised to possess 2,4,6-tribromo-3-methoxyphenyl residues (**9–11**), since all compounds have almost identical ^{13}C NMR shifts for the aromatic and methoxyl carbon resonances when compared to those observed for **1**, **2**, **3** and **4** (Fig. 3).

The absolute configuration of the stereogenic centre, C-5, in amathamide A (**12**) and B (**13**) was proposed to be *S* based upon CD analysis and this has been confirmed by asymmetric total synthesis.[10,11] Since **12** and **13** differ from **3** and **4** only by the removal of a bromine atom at C-11 and a methyl group at N-7, and since the optical rotations recorded for **3** and **4** and synthetic **12** and **13** were all similar in magnitude and sign, this suggested that **3** and **4** also both possessed 5*S* configuration. The absolute configuration of the three stereogenic centres in **1** and **2** remain unassigned.

Since Tasmanian collections of *A. wilsoni* have been extensively studied in the past it is surprising that **1** and **2** have not previously been isolated and characterised.[9,10] A plausible explanation for why these alkaloids have not been detected previously is related to the extraction and purification methods previously employed. Amathamides A–F were previously isolated using a classic acid/base extraction partitioning protocol. The alkaloids were first extracted



	R^1	R^2	R^3	
5	Br	H	CH_3	Δ^8
6	Br	H	H	
7	Br	H	H	Δ^8
9	H	Br	H	
10	H	Br	H	Δ^8
12	H	H	H	Δ^8

	R^1	R^2
8	Br	H
11	H	Br
13	H	H

Fig. 3 Published and revised structures of amathamides A–F.

with MeOH, and then subsequently partitioned into 2M H_2SO_4 from which lipids were removed by hexane extraction and then re-extracted into CH_2Cl_2 after basification with conc. ammonium hydroxide. The resultant CH_2Cl_2 soluble alkaloid fraction was then purified by normal phase silica gel chromatography to yield amathamides A–F in low yield (~0.02%). Wilsoniamines A and B are both quaternary amine salts that are not CH_2Cl_2 soluble. Application of an acid/base partitioning protocol would result in both molecules remaining in the aqueous layer even after addition of ammonium hydroxide and therefore **1** and **2** would not have been detected using the previous purification methods. Our purification protocol has been specifically designed to avoid such losses, and employs direct and rapid C_{18} HPLC purification of crude extracts to limit the exposure of compounds present in crude extracts to conditions likely to cause loss. Since wilsoniamines A and B are epimeric at C-8, there is a possibility that these compounds may not be made enzymatically and indeed could be artefacts generated by acid catalyzed cyclization of amathamide C or H. Several pieces of evidence however suggest that **1** and **2** are true natural products. One would anticipate that if **1** and **2** were artefacts produced upon acid catalyzed cyclization of **3** and **4** that conversion of **3** and **4** into **1** and **2** would have to be a rapid reaction that occurs immediately upon exposure to TFA on the HPLC column. This is clearly not the case, since evaporation of the acidic HPLC solvent from fractions containing amathamide C and H at 50 °C resulted in no trace of wilsoniamine A or B being detected. Amathamide C and H were both isolated in pure form after HPLC purification. Furthermore, treatment of amathamide C with 1% TFA/MeOH overnight resulted in no reaction. Finally, NMR analysis of the crude MeOH extract of *A. wilsoni* clearly demonstrated that **1** and **2** were present in the crude extract. These combined data suggest that **1** and **2** are true natural products.

The new compounds, wilsoniamines A and B, both possess a bicyclic hexahydropyrrolo[1,2-*c*]imidazol-1-one ring system that, to the best of our knowledge, has not previously been encountered in nature. The closest structurally related natural product is the tricyclic alkaloid dysibetaine PP, isolated from the sponge *Dysidea herbacea*.[12] Even synthetic pyrrolo[1,2-*c*]imidazol-1-ones are very rare and have been produced only as intermediates or by products en route to other compounds,[13] and as catalysts for enantioselective Strecker reactions.[14]

Compounds **1–4** were tested for their ability to inhibit the growth of chloroquine sensitive (3D7) and resistant (Dd2) strains of the malarial parasite, *Plasmodium falciparum*, and the protozoan parasite *Typanosoma brucei brucei.* Human cell cytotoxicity was assessed using the normal mammalian cell line HEK-293 and the HeLa cancerous cell line. In all assays compounds **3** and **4** were more active compared to **1** and **2**. Against the Dd2 strain **3** and **4** displayed IC_{50} values of 10.2 and 8.0 μM, respectively and against the 3D7 strain IC_{50} values of 28.0 and 14.9 μM, respectively were obtained. In comparison, compounds **1** and **2** only showed growth inhibition against these two strains at significantly higher doses, reaching ~100% inhibition only at the highest dose tested (120 μM). Similarly, only compounds **3** and **4** exhibited minimal inhibition of *T. brucei brucei* growth (IC_{50} values 32 and 57.1 μM, respectively). None of the compounds displayed cytotoxic activity up to and inclusive of the highest concentration tested (120 μM). Wilsoniamines A and B are both quaternary amine salts and their lower bioactivity might be associated with the decreased cell permeability of charged molecules. The markedly higher anti-malarial activity obtained for amathamide C and H compared to wilsoniamines A and B could in part be due to their ability to form uncharged species.

Conclusions

In summary, four alkaloids have been isolated from the Tasmanian bryozoan *A. wilsoni*. Two of the alkaloids, wilsoniamines A and B, both possess a bicyclic ring system not previously encountered in nature. Detailed analysis of spectroscopic data acquired for the new compound amathamide H, and the known compound amathamide C, has shown that both compounds possess a 2,4,6-tribromo-3-methoxyphenyl moiety, and not a 2,3,4-tribromo-5-methoxyphenyl moiety as had previously been assigned to amathamide C. This result indicates that the structures previously proposed for amathamides C–F isolated from *A. wilsoni*, should be revised to each possess a 2,4,6-tribromo-3-methoxyphenyl moiety. Modest anti-malarial and anti-trypanosomal activity was obtained for amathamides C and H, but not for wilsoniamines A and B.

Experimental

General procedures

NMR spectra were recorded at 30 °C on a Varian 600 MHz spectrometer equipped with a triple resonance cold probe. The ^1H 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 HRESIMS were recorded on an Applied Biosystems Mariner Biospectrometry TOF workstation using positive electrospray ionization, mobile phase 1 : 1 MeOH : H_2O. 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 bryozoan extract prior to HPLC separation. A Merck Hitachi L7100 pump equipped with a Merck Hitachi L7455 PDA detector and a Merck Hitachi L7250 autosampler were used for HPLC. A Betasil C_{18} 5 μm 120 Å column (21.2 mm × 150 mm)

was 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 bryozoan

The bryozoan sample *Amathia wilsoni* (phylum Ectoprocta, class Gymnolaemata, order Ctenostomata, family Vesiculariidae) was collected by scuba diving at a depth of 16 m at Point Peur near Port Arthur on the Tasman Peninsula southeast Tasmania, Australia, in October 2002. A voucher sample, TAS00150, was lodged at Aquenal Pty Ltd, Hobart, Australia.

Extraction and isolation

The freeze dried and ground bryozoan sample (7.8 g) was extracted exhaustively with MeOH (5 × 200 mL) to yield a dark brown residue (195 mg). This extract was dissolved in a small amount of MeOH (10 mL) and C_{18} silica gel (1.4 g) was added. The solvent was evaporated and the extract adsorbed on to the gel was transferred to a refillable HPLC column (10 mm × 20 mm). This column was connected in series to a C_{18} HPLC column and combined columns eluted with a linear gradient of water 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 mass ions were further analysed by ^{1}H NMR spectroscopy and fractions containing similar signals were combined. Fraction 34 was pure wilsoniamine B (**2**, 16.4 mg, 0.21%), fraction 43 was pure amathamide H (**3**, 12.5 mg, 0.16%) and fraction 45 was pure amathamide C (**4**, 14.1 mg, 0.18%). Fractions 35–37 were a mixture of wilsoniamine A and B and were combined and chromatographed on C_{18} HPLC using a linear gradient from 85% water/15% MeOH (each solvent containing 1% TFA) to 40% water/60% MeOH (each solvent containing 1% TFA) over 30 min. Sixty 20 s fractions were collected. (+) LRESIMS analysis of these fractions indicated fractions 25–40 contained peaks at *m/z* 509, 511, 513, 515 and these fractions were further analysed by ^{1}H NMR spectroscopy. Fractions 25–32 were pure wilsoniamine A (**1**, 15.2 mg, 0.19%) and fractions 36–38 were pure wilsoniamine B (**2**, 4.5 mg, 0.06%).

Wilsoniamine A (1). colourless gum; $[\alpha]_D^{23}$ +7.1 (*c* 0.063, MeOH);. UV λ_{max} (MeOH)/nm (log ε) 210 (4.42), 230sh (3.96), 284 (313), 294 (3.12); IR υ_{max} (film)/cm^{-1} 3418, 2928, 1731, 1684, 1455, 1416, 1206, 1032; ^{1}H and ^{13}C NMR data (DMSO-d_6) see Table 1; (+)-ESIMS *m/z* 509 (M^+ 15%), 511 (M^+ 90%), 513 (M^+ 100%), 515 (M^+ 30%); (+)-HRESIMS *m/z* 510.9044 (calcd for $C_{16}H_{20}{}^{79}Br_2{}^{81}BrN_2O_2$, 510.9049).

Wilsoniamine B (2). colourless gum; $[\alpha]_D^{23}$ +36.0 (*c* 0.065, MeOH);. UV λ_{max} (MeOH)/nm (log ε) 210 (4.60), 230sh (4.11), 292 (3.17); IR υ_{max} (film)/cm^{-1} 3385, 2972, 1717, 1684, 1653, 1576, 1456, 1128, 1015; ^{1}H and ^{13}C NMR data (DMSO-d_6) see Table 1; (+)- ESI MS *m/z* 509 (M^+ 20%), 511 (M^+ 95%), 513 (M^+ 100%), 515 (M^+ 33%); (+)-HRESIMS *m/z* 508.9136 (calcd for $C_{16}H_{20}{}^{79}Br_3N_2O_2$, 508.9069).

Amathamide C (4). colourless gum; $[\alpha]_D^{23}$ −12.8 (*c* 0.13, MeOH);.^{1}H NMR data (DMSO-d_6) major isomer δ_H 1.90 (m, 1H, H-3a), 1.92 (m, 1H, H-4a), 2.11 (m, 1H, H-3b), 2.62 (m, 1H, H-4b), 2.80 (s, 3H, 1-NCH_3), 3.10 (m, 1H, H-2a), 3.26 (s, 3H, 7-NCH_3), 3.60 (m, 1H, H-2b), 3.81 (s, 3H, 12-OCH_3), 4.80 (t, 7.3 Hz, 1H, H-5), 6.02 (d, 15.3 Hz, 1H, H-9), 7.09 (d, 15.3 Hz, 1H, H-8), 8.05 (s, 1H, H-14), 9.77 (bs, 1H, 1-NH), minor isomer δ_H 1.90 (m, 1H, H-3a), 1.95 (m, 1H, H-4a), 2.11 (m, 1H, H-3b), 2.67 (m, 1H, H-4b), 2.84 (s, 3H, 1-NCH_3), 3.16 (m, 1H, H-2a), 3.25 (s, 3H, 7-NCH_3), 3.65 (m, 1H, H-2b), 3.81 (s, 3H, 12-OCH_3), 4.80 (t, 7.3 Hz, 1H, H-5), 6.08 (d, 15.3 Hz, 1H, H-9), 7.63 (d, 15.3 Hz, 1H, H-8), 8.02 (s, 1H, H-14), 9.73 (bs, 1H, 1-NH); ^{13}C NMR data (DMSO-d_6) major isomer δ_C 22.3 (C-3), 27.9 (C-4), 29.1 (7-NCH_3), 40.4 (1-NCH_3), 56.0 (C-2), 60.3 (12-OCH_3), 67.0 (C-5), 111.0 (C-9), 116.1 (C-13), 118.9 (C-11), 120.8 (C-15), 133.4 (C-8), 135.2 (C-14), 137.6 (C-10), 153.6 (C-12), 167.2 (C-6), minor isomer δ_C 22.3 (C-3), 27.8 (C-4), 30.1 (7-NCH_3), 40.4 (1-NCH_3), 56.0 (C-2), 60.3 (12-OCH_3), 67.3 (C-5), 111.2 (C-9), 115.5 (C-13), 118.4 (C-11), 120.5 (C-15), 132.7 (C-8), 135.4 (C-14), 137.5 (C-10), 153.7 (C-12), 167.3 (C-6); (+)-ESIMS *m/z* 509 (MH^+ 18%), 511 (MH^+ 88%), 513 (MH^+ 100%), 515 (MH^+ 25%); (+)-HRESIMS *m/z* 512.9262 (calcd for $C_{16}H_{20}{}^{79}Br^{81}Br_2N_2O_2$, 512.9030).

Amathamide H (3). colourless gum; $[\alpha]_D^{23}$ −25.3 (*c* 0.12, MeOH);. UV λ_{max} (MeOH)/nm (log ε) 214 (4.33), 268 (3.74); IR υ_{max} (film)/cm^{-1} 3505, 2937, 1697, 1682, 1239, 1202, 1187, 1033, 1013; ^{1}H NMR and ^{13}C NMR data (DMSO-d_6) see Table 1 for major isomer; ^{1}H NMR data (DMSO-d_6) for minor isomer δ_H 1.84 (m, 1H, H-3a), 1.94 (m, 1H H-4a), 2.07 (m, 1H, H-3b), 2.62 (m, 1H, H-4b), 2.70 (s, 3H, 7-NCH_3), 2.79 (s, 3H, 1-NCH_3), 3.17 (m, 1H, H-2a), 3.54 (m, 1H, H-2b), 3.81 (s, 3H, 12-OCH_3), 4.65 (t, 7.3 Hz, 1H, H-5), 5.89 (d, 9.8 Hz, 1H, H-9), 7.03 (d, 9.8 Hz, 1H, H-8), 8.06 (s, 1H, H-14) 9.68 (bs, 1H, 1-NH); ^{13}C NMR data (DMSO-d_6) for minor isomer δ_C 21.9 (C-3), 27.1 (C-4), 34.9 (7-NCH_3), 41.2 (1-NCH_3), 55.7 (C-2), 60.5 (12-OCH_3), 67.4 (C-5), 113.9 (C-9), 116.9 (C-13), 118.6 (C-11), 120.2 (C-15), 130.5 (C-8), 134.5 (C-14), 138.3 (C-10), 153.6 (C-12), 167.6 (C-6); 509 (MH^+ 15%), 511 (MH^+ 92%), 513 (MH^+ 100%), 515 (MH^+ 20%); (+)-HRESIMS *m/z* 508.9093 (calcd for $C_{16}H_{20}{}^{79}Br_3N_2O_2$, 508.9069).

Anti-malarial assay

Compounds were incubated in the presence of 2 or 3% parasite (3D7 or Dd2) and 0.3% hematocrit in a total assay volume of 50 μL, for 72 h at 37 °C and 5% CO_2, in Poly-D-lysine coated CellCarrier Imaging plates. After incubation plates were stained with DAPI (4′,6-diamidino-2-phenylindole) in the presence of Saponin and Triton X-100 and incubated for a further 5 h at RT in the dark before imaging on the OPERA™ HTS confocal imaging system. The digital images obtained were then analyzed using the PerkinElmer Acapella spot detection software where spots which fulfill the criteria established for a stained parasite are counted. The % inhibition of parasite replication was calculated using DMSO and Artemisinin control data.

Trypanosome viability estimation assay

The *T.b.brucei* viability assay was carried out as previously described,[15] with a modification of the compound dilution medium. Briefly, 55 μL $well^{-1}$ of 2000 cells mL^{-1} in HMI-9 medium supplemented with 10% FCS and 100 IU mL^{-1} Penicillin/Streptomycin were added to 384 well plates with a Multidrop liquid handler (Thermo Scientific, USA). Plates were incubated

for 24 h prior to addition of 5 μL of pre-diluted compounds or DMSO for control wells. Compounds or controls in 100% DMSO were pre-diluted 1 : 20 in high glucose DMEM medium with L-glutamine supplemented with 1x non essential amino acids and 1 mM sodium pyruvate (Invitrogen, USA), by a Minitrack robotic liquid handler (PerkinElmer, USA). Plates were incubated for a further 48 h at 37 °C and 5% CO_2 before addition of 10 μL of Alamar Blue™ (Biosource, USA). Plates were then incubated for 2 h under the same conditions, then for a further 22 h at room temperature. Wells were read at excitation 535 nm, emission 590 nm on a Victor II Wallac plate reader (PerkinElmer, USA). Reference drugs were pentamidine (Sigma, USA), diminazene aceturate (Sigma, USA) and puromycin (Calbiochem, USA).

Cytotoxicity assay

Compounds were added to assay wells containing 3000 adherent cells well^{-1} (HEK 293) or 2000 cells well^{-1} HeLa in an assay volume of 45 μL. The plates were incubated for 72 h at 37 °C and 5% CO_2. After incubation the supernatant was "flicked" out of the wells and 40 μL of 10% Alamar blue added per well. Plates were incubated for a further 5–6 h and measured for fluorescence at 535 nm excitation and 590 nm emission.

IC_{50} values were obtained by plotting % inhibition against log dose using Prizm4 graphing package using non-linear regression with variable slope plot.

Acknowledgements

We thank Mr. D. Shields and his team from Aquenal Pty Ltd for collection and identification of the bryozoan.

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