Mirabilins revisited: polyketide alkaloids from a southern Australian marine sponge, *Clathria* sp.†

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Received 30th July 2009, Accepted 16th October 2009
First published as an Advance Article on the web 30th October 2009
DOI: 10.1039/b915624k

Chemical investigation of a southern Australian marine sponge, *Clathria* sp., yielded the known mirabilins C, F and G, together with three new analogues, mirabilins H–J. For the first time mirabilins C and F are documented as the underivatized natural products, and a complete absolute stereochemistry is assigned to mirabilin F. Mirabilin I represents the first member of this structure class to incorporate a *trans*-fused ring junction. Structures for all mirabilins are assigned on the basis of detailed spectroscopic analysis. A plausible polyketide origin is proposed, linking all mirabilins and related sponge alkaloids. Mirabilin cytotoxicity against several human cancer cell lines is discussed.

Introduction

Marine sponges are a prolific source of polycyclic guanidine-containing compounds, including the rare class of tricyclic (5,6,8b)-triazaperhydroacenaphthylene heterocycles (*i.e.* ptilocaulins, mirabilins and netamines). Known examples of this structure class are restricted to marine sponges and can be collectively grouped on the basis of unsaturation and double bond regiochemistry, with pyrimidine (**1–7**), $\Delta^{11,12}$ (**8–12**), $\Delta^{9,10}$ (**13**), $\Delta^{10,11}$ (**14–17**) or saturated (**18–21**) heterocycles (Fig. 1). The first account of this heterocyclic system appeared in a 1981 report of ptilocaulin (**14**) and isoptilocaulin (**13**) from a Caribbean *Ptilocaulis* sp.[1] Subsequent reports appeared in 1995, 8b-hydroxyptilocaulin (**15**) from a Brazilian *Monanchora arbuscula*,[2] and 1996, mirabilins A–F (**1–3**, **11**, **8**, **17**) from a southern Australian *Arenochalina mirabilis*.[3] A 1997 report described 8a,8b-dehydroptilocaulin (renamed 7-epineoptilocaulin)[4] (**9**), 8a,8b-dehydro-8-hydroxyptilocaulin (renamed 8α-hydroxy-7-epineoptilocaulin)[4] (**12**) and 1,8a;8b,3a-didehydro-8β-hydroxyptilocaulin (renamed 8α-hydroxymiribilin B)[4](**6**) from a Bahamas *Batzella* sp.,[5] while a 2001 report described mirabilin G (**16**) from a southern Australian *Clathria* sp.[6] More recent accounts appeared in 2004, 1,8a;8b,3a-didehydro-8α-hydroxyptilocaulin (renamed 8β-hydroxymiribilin B)[4] (**7**) from a Jamaican *Monanchora unguifera*,[7] and 2006, netamines A–G (**18–21**, **10**, **5**, **4**) from a Madagascan *Biemna laboutei*.[8] Examples of this class of marine sponge alkaloid have been reported as possessing cytotoxic properties,[1,7] as well as antibacterial,[1,3,6] antifungal,[5,6] antimalarial[5] and antiprotozoal[5] activity.

A recent 2008 synthetic investigation by Snider *et al*. suggested revision to trivial nomenclature (see above), provided total syntheses for 7-epineoptilocaulin, mirabilin B, isoptilocaulin and netamines E and G, proposed stereo revision to netamines (*cis* to *trans* disposed sidechains), and assigned absolute configurations to 7-epineoptilocaulin, mirabilin B, and netamines E and G (and by inference related co-metabolites).[4] Given the history of trivial nomenclature and structure revisions, a summary of the current ptilocaulin/mirabilin/netamine natural products (including all revised trivial names and stereochemistry), grouped against common heterocyclic systems, is shown in Fig. 1. All structures are nominally displayed as belonging to the antipodal series defined by Snider *et al*. for **2**, **4**, **9** and **10**. Several examples of this heterocyclic class have unresolved relative configuration assignments, including mirabilin E (**8**),[3] mirabilin D (**11**),[3] and mirabilin F (**17**).[3] This report seeks to resolve some of these assignments, and describes the isolation and structure elucidation of known and new members of this structure class, including mirabilins H–J (**22–24**) (Fig. 2).

In our ongoing search for cytotoxic metabolites from southern Australian marine sponges we had cause to examine an extract derived from a *Clathria* (*Isociella*) sp. prompted by the observation of significant cytotoxicity against human colon (HT29), lung (A549) and breast (MDA-MB-231) cancer cell lines. Chemical fractionation of the *Clathria* extract using a sequence of solvent partitioning and trituration steps, followed by reverse phase HPLC, yielded the known mirabilins C (**3**),[3] F (**17**),[3] and G (**16**),[6] together with three new analogues, mirabilins H (**22**), I (**23**) and J (**24**). Structures for the known compounds were confirmed by spectroscopic analysis, and comparison to literature data. Our characterization and report of mirabilins C (**3**) and F (**17**) represents the first documented account of the underivatized (unacetylated) natural products, and resulted in assignment of a complete stereochemistry to **17**. Spectroscopic analysis leading to structure elucidation of these known mirabilins, and the new mirabilins H–J (**22–23**), is presented below.

Results and discussion

Mirabilin G (**16**) isolated during this study was identified by comparison with authentic and published spectroscopic data.[6] Mirabilins C (**3**) and F (**17**) were isolated for the first time as their TFA salts (previously only characterized as their corresponding acetates **3a** and **17a**), and their structures confirmed by spectroscopic analysis.

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† Electronic supplementary information (ESI) available: ^{1}H NMR spectra, and tabulated 1D and 2D NMR data, for mirabilins C (**3**) and F–J (**16–17**, **22–24**). See DOI: 10.1039/b915624k

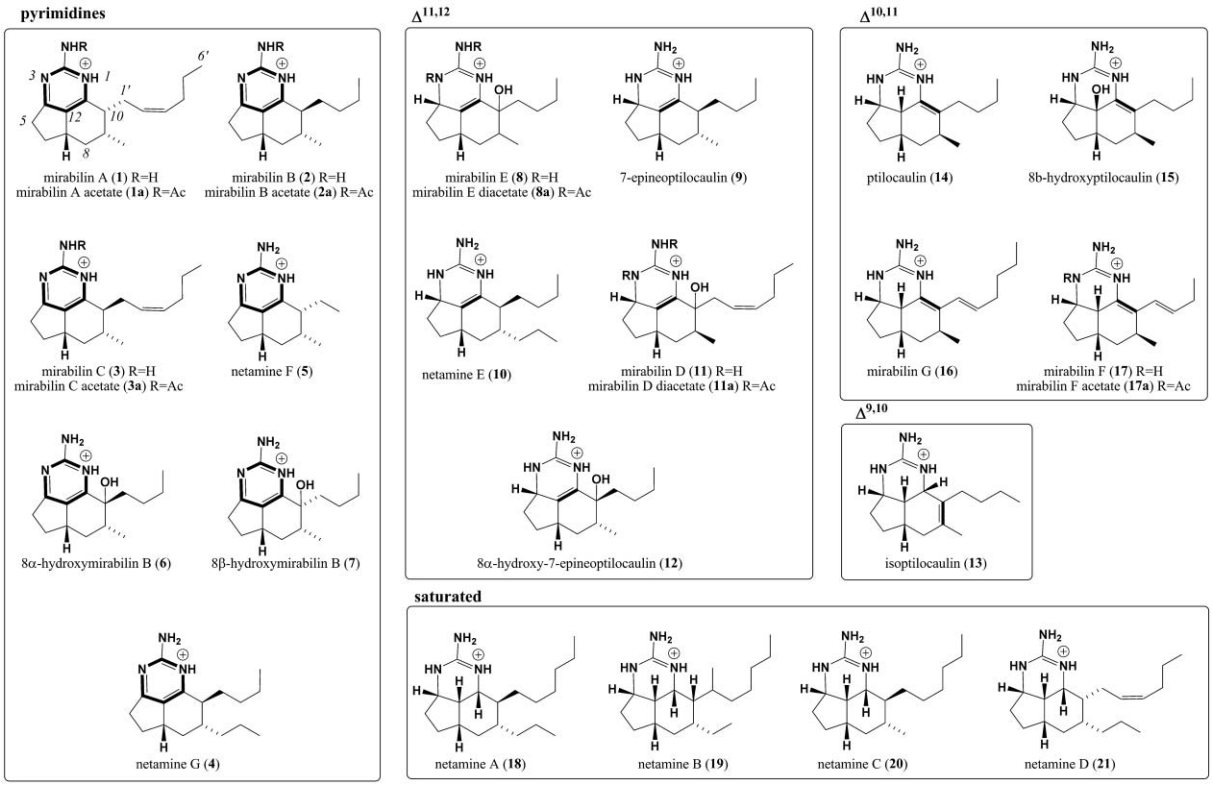

Fig. 1 Known mirabilins, ptilocaulins and netamines.

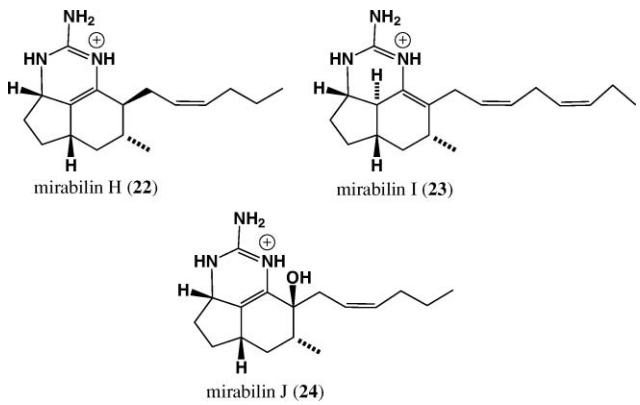

Fig. 2 New mirabilins.

The (+)HRESIMS data for **3** revealed a molecular formula ($C_{17}H_{26}N_3$, Δmmu –0.3) which, together with analysis of the 1D and 2D NMR data (see ESI†), including comparisons with reported NMR data for mirabilin A acetate (**1a**)[3] and C acetate (**3a**),[3] was consistent with mirabilin C. In differentiating between the possible isomers **1** and **3** it is noteworthy that the ^{1}H NMR chemical shift for H-9 in **3** (δ_H 1.92) compared well with that reported for **3a** (δ_H 1.90),[3] rather than **1a** (δ_H 2.20),[3] while key correlations in the ROESY data for **3**, between H-7 (δ_H 2.93) and H-9 (δ_H 1.92), and between 9-Me (δ_H 1.14) and H-10 (δ_H 2.59), supported the proposed mirabilin C stereochemistry over that of mirabilin A. Final confirmation was secured when a sample of **3** was acetylated to yield a monoacetate identical with **3a**.[3] As the reported $[\alpha]_D$ for **3a**[3] is of the same sign (+ve) as that recorded for **3a** prepared from **3**, they possess the same absolute stereochemistry.

The (+)HRESIMS data for **17** revealed a molecular formula ($C_{15}H_{24}N_3$, Δmmu –0.5) consistent with mirabilin F or an isomer. Analysis of the 1D and 2D NMR data for **17** (see ESI†) revealed resonances and correlations consistent with those expected for mirabilin F, based on reported data for mirabilin F acetate (**17a**),[3] while NOESY correlations between H-4 (δ_H 3.76), H-7 (δ_H 2.48) and H-12 (δ_H 2.57) confirmed a mirabilin F relative configuration about these centres. Although the absence of an NOE from H-4, H-7 or H-12, to H-9, precluded an earlier literature (and current) assignment of relative configuration about C-9 in **17a**,[3] our isolation of mirabilin F (**17**) permitted a direct spectroscopic comparison between **17** and the published data for mirabilin G (**16**), which possesses a related heterocyclic core of known total relative configuration. NMR chemical shift comparisons about the four chiral centres in **17** *vs.* **16**;[6] C-4 (δ_H 3.76 *vs.* 3.79; δ_C 53.7 *vs.* 53.4), C-7 (δ_H 2.48 *vs.* 2.47; δ_C 33.6 *vs.* 33.7 (note a correction to the reported ^{13}C NMR chemical shift for C-7 in **16** from δ_C 26.3 to δ_C 33.7), C-9 (δ_H 2.72 *vs.* 2.73; δ_C 27.9 *vs.* 27.5) and C-12 (δ_H 2.57 *vs.* 2.57; δ_C 37.3 *vs.* 37.2); and extending to the C-9 methyl (δ_H 1.18 *vs.* 1.18; δ_C 22.5 *vs.* 22.5, respectively); are supportive of a common relative configuration, in which H-9 occupies the opposite face of the molecule compared to H-4, H-7 and H-12 (consistent with the observed NOEs). Regrettably, **17** decomposed before acetylation could be attempted. However, based on the structural arguments presented above we nevertheless propose the assignment of a complete relative configuration of mirabilin F (**17**),

which given co-occurrence with **3**, is attributed a common absolute stereochemistry.

The (+)HRESIMS data for **22** revealed a molecular formula ($C_{17}H_{28}N_3$, Δmmu 1.0) consistent with an ammonium species with six double bond equivalents. Analysis of the NMR data for **22** (see ESI†) revealed exchangeable protons (δ_H 9.35 (NH), 7.40 (NH_2) and 8.22 (NH)) and a deshielded sp^2 hybridized carbon (δ_C 154.0) consistent with a guanidine moiety, and resonances for a tetrasubstituted (δ_C 117.7, 127.4) and a 1,2-disubstituted (δ_H 5.21, 5.44, δ_C 124.5, 132.7) double bond, requiring that **22** be tricyclic. Comparison of the NMR data for **22** with that reported for 7-epineoptilocaulin (**9**)[4] confirmed the presence of a common hetero/carbocyclic moiety, while the (*Z*)-1′-hex-2′-enyl moiety was evidenced from a C-1′ to C-6′ 2D NMR correlation sequence, $J_{2',3'}$ (10.6 Hz), and comparisons to mirabilin A acetate (**1a**) and C acetate (**3a**).[3] Further 2D NMR correlations (see ESI†) extended this side chain structure fragment to include the sequence from C-4 to C-10, incorporating $\Delta^{11,12}$, the guanidine moiety and a 9-Me. NOESY correlations positioned H-4, H-7 and H-9 on a common face, and 9-Me and H-10 on the opposite face, of the molecule. The observations listed above define the relative stereostructure for mirabilin H (**22**) as shown. Given its co-occurrence with mirabilin C (**3**), we suggest a common absolute stereochemistry.

The (+)HRESIMS data for **23** revealed a molecular formula ($C_{19}H_{30}N_3$, Δmmu 1.1) consistent with an ammonium species with seven double bond equivalents. Analysis of the NMR data for **23** (see ESI†) revealed exchangeable protons (δ_H 8.89 (NH), 7.36 (NH_2) and 8.26 (NH)) and a deshielded sp^2 hybridized carbon (δ_C 153.7) consistent with a guanidine moiety, and resonances for a tetrasubstituted (δ_C 118.1, 127.5) and two 1,2-disubstituted (δ_H 5.26, 5.44; δ_C 124.9, 131.2; and δ_H 5.26, 5.38; δ_C 126.9, 132.4, respectively) double bonds, requiring that **23** be tricyclic. Comparison of the NMR data for **23** with that reported for ptilocaulin (**14**)[2] suggested the presence of a common hetero/carbocyclic moiety, while the (2′*Z*,5′*Z*)-1′-oct-2′,5′-dienyl sidechain was evidenced from a C-1′ to C-8′ 2D NMR correlation sequence, as well as $J_{2',3'}$ (10.5 Hz) and $J_{5',6'}$ (10.6 Hz) (see ESI†). NOESY correlations positioned H-4, H-7 and H-9 on the same face of the molecule. An overlapping ^{1}H NMR resonance for H-12 precluded an unambiguous determination of relative configuration about this centre and adjacent chiral centres, however reacquisition of the NMR data in benzene-d_6 (see ESI†) did resolve the H-12 resonance. A ROESY NMR (benzene-d_6) correlation between H-12 and the 9-Me confirmed occupancy of a common face of the molecule, opposite to that of H-4, H-7 and H-9 (consistent with the observed NOE correlations). Thus mirabilin I (**23**) was assigned the relative configuration as indicated, and on the basis of co-occurrence with mirabilin C (**3**) was attributed a common absolute configuration. It is noteworthy that mirabilin I (**23**) represents the first member of this class of alkaloids to incorporate a *trans* fused ring junction.

The (+)HRESIMS data for **24** revealed a molecular formula ($C_{17}H_{28}N_3O$, Δmmu 0.2) consistent with an ammonium species with six double bond equivalents. Analysis of the NMR data for **24** (see ESI†) revealed exchangeable protons (δ_H 9.05 (NH), 7.02 (NH_2), and 8.30 (NH)) and a deshielded sp^2 hybridized carbon (δ_C 151.9) consistent with a guanidine moiety, and resonances for a tetrasubstituted (δ_C 122.7, 129.5) and one 1,2-disubstituted (δ_H 5.12, 5.42; δ_C 125.6, 132.2) double bonds, requiring that **24** be tricyclic. Analysis of 2D NMR data indicated connectivity sequences from C-1′ to C-6′ and from H-4 to H-9, including the 9-Me, and a $J_{2',3'}$ of 10.1 Hz for a (2′*Z*)-1′-hex-2′-enyl sidechain (see ESI†), consistent with mirabilin D (**11**) or a stereoisomer.[3] Furthermore, the ^{13}C NMR chemical shift for C-10 (δ_C 70.3) confirmed the presence of an sp^3 carbon with an oxygen substituent, while the NMR (DMSO-d_6) data for **24** (see ESI†) revealed a 10-OH resonance (δ_H 5.42) with HMBC correlations to C-10 and C-12 (δ_C 123.8). Diagnostic ROESY NMR (DMSO-d_6) correlations (see ESI†) positioned H-4, H-7 and 10-OH on a common face of the molecule, while correlations between H-4 and H-6a, between 9-Me and H-6b, and between 9-Me and H-1a′ and H-1b′, positioned 9-Me as indicated (on the opposite face to H-4 and H-7). Thus mirabilin J (**24**) was determined to be a new stereoisomer of mirabilin D (**11**), and an oxidation product of the co-metabolite mirabilin H (**22**). Given its co-occurrence with mirabilin C (**3**), and likely relationship to mirabilin H (**22**), we propose a common absolute stereochemistry. In this regard we note that the new co-metabolites mirabilins H–J (**22–24**) belong to the same antipodal series and possess common relative stereochemistry about C-4, C-7 and C-9.

The mirabilins (**1–3**, **6–8**, **11**, **16–17**, **22–24**), ptilocaulins (**9**, **12–15**) and netamines (**4–5**, **10**, **18–21**) possess structural features suggestive of polyketides. Plausible polyketide carbon skeletons are shown in Fig. 3, and incorporate either six, seven, eight or nine acetate residues, or in the case of **19** six acetate and two propionate residues.

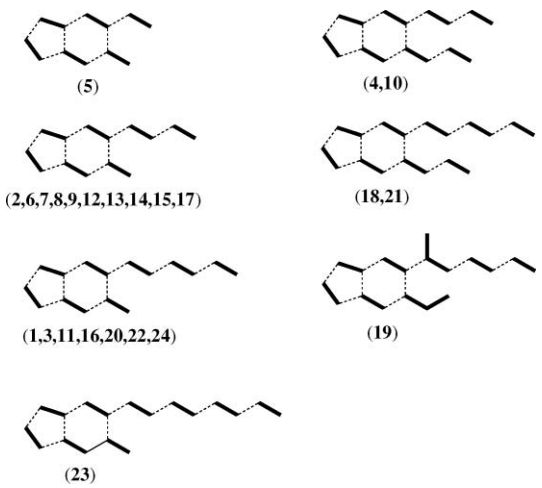

Fig. 3 Plausible polyketide skeletons.

In addition to polyketide chain assembly any biosynthesis must also incorporate a key cyclization event (to form the fused 5,6-carbocycle) and a guanidine addition (to generate the nitrogen heterocycle). While the order and nature of these biosynthetic carbocycle and heterocycle formations has yet to be tested experimentally, a plausible process (Fig. 4) could mirror that of the syntheses described by Snider *et al*.,[4] in which guanidine adds to an α,β-unsaturated ketone precursor *via* an initial Michael addition, and then cyclises to form the heterocycle by a Schiff's base process. The stereochemistry of the initial α,β-unsaturated ketone precursor, together with double bond migrations of the

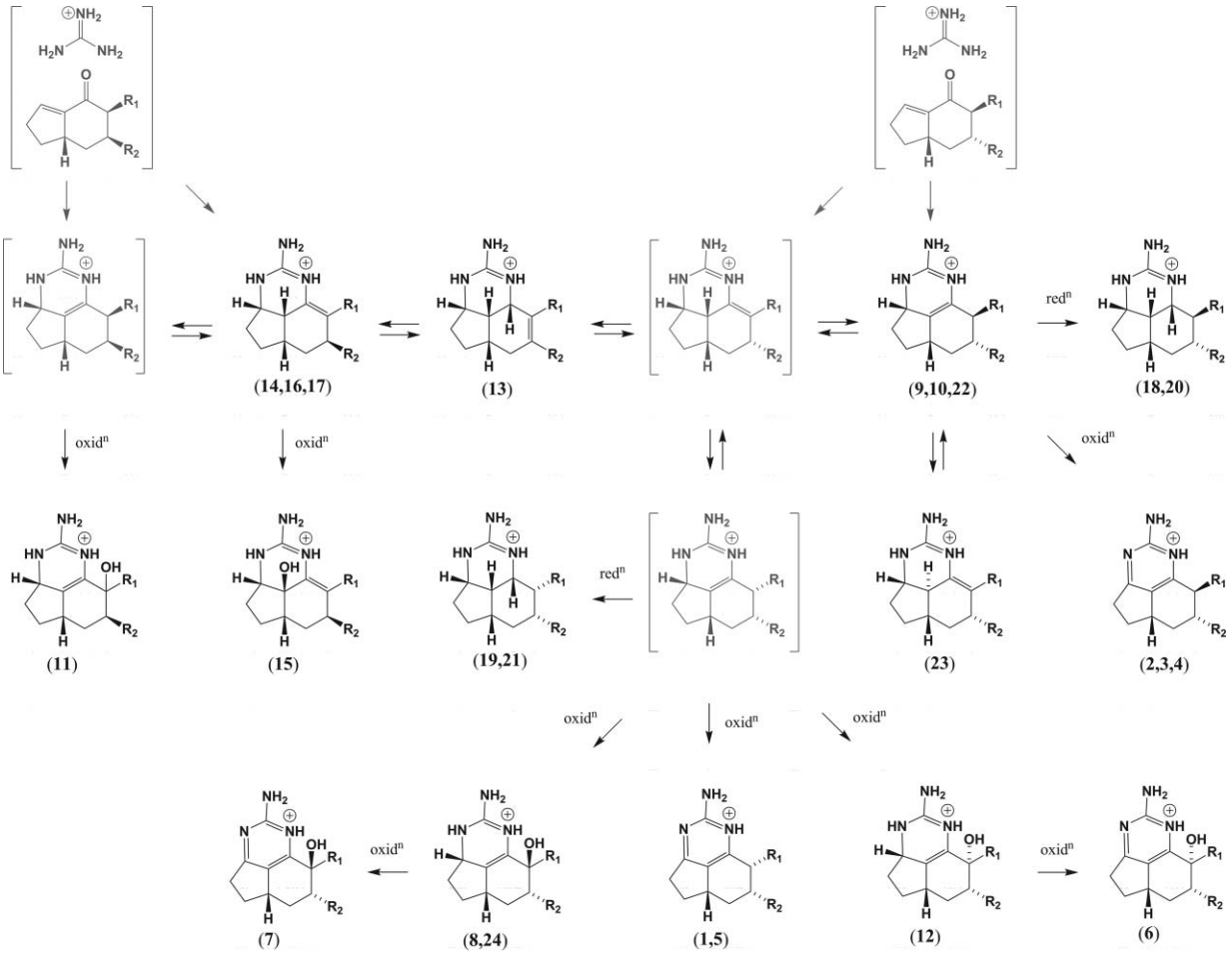

Fig. 4 Plausible chemical transformations (hypothetical species in brackets).

enamine heterocycles could lead to interconversion between $\Delta^{11,12}$, $\Delta^{9,10}$ and $\Delta^{10,11}$ systems, which would facilitate access to the range of observed relative stereochemistries. Oxidations (to pyrimidines) and reductions (to netamines) could expand the repertoire to the full array of observed natural products (**1–24**) (see Fig. 4). Since the full array of regio and stereoisomers are not observed in all sponges known to produce such alkaloids, these processes are presumably enzyme mediated. While speculative, acknowledgement of these transformations provides a plausible chemical basis linking the stereo-diverse array of mirabilin, ptilocaulin and netamine alkaloids.

With access to an array of natural mirabilins, we undertook comparative measures of cytotoxicity against neuroblastoma (SH-SY5Y), gastric (AGS), colorectal (HT29) and intestinal (Intestine-407) cancer cell lines. These studies revealed that **3**, **16–17** and **22–24** were modest cytotoxic agents with LD_{50} values >30 μM. This observation suggests the high cytotoxicity observed for the extract of *Clathria* sp. (CMB-02002) was due to the cumulative yield of mirabilins in the *n*-BuOH soluble partition (~25%). This analysis highlights a dilemma faced in biodiscovery, in that a biological response in any given extract is both a function of the potency and concentration of each individual bioactive metabolite, and the cummulative effect of *all* bioactive co-metabolites.

Experimental

General experimental procedure

Chiroptical measurements were measured on a Jasco *P*-1010 polarimeter with a 10 cm quartz cell. UV-Vis spectra were measured on a SmartSpecTM 3000 spectrophotometer in a 1 cm quartz cell. Melting points were measured on a Buchi 3538 melting point apparatus and are uncorrected. NMR spectra were recorded on a Bruker Avance 600 MHz spectrometer, in $CDCl_3$ unless otherwise noted. Chemical shifts are reported in ppm using residual solvent signal as an internal reference. Electrospray ionization mass spectra (ESIMS) were acquired using an Agilent 1100 series separations module equipped with an Agilent 1100 Series LC/MSD mass detector in both +ve and –ve ion modes. High resolution (HR) ESIMS measurements were obtained on a Bruker micrOTOF-Q or a Bruker micrOTOF high resolution mass spectrometer equipped with an ESI probe by direct infusion in acetonitrile at 3 μL min^{-1} using sodium formate clusters as an internal calibrant. HPLC was performed using an Agilent 1100 series separations module equipped with Agilent 1100 series diode array detector, and Agilent 1100 series fraction collector, controlled using ChemStation Rev.9.03A and Purify version A.1.2 software.

Collection

The sponge sample (UQ code: CMB-02002) was collected in 1995 by epibenthic sled at a depth of 60 m in the Great Australia Bight (116° 52′E, 35° 7′S). Freshly collected material was frozen for shipping to the laboratory, where it was thawed, catalogued, diced and steeped in aqueous EtOH at −30° C for prolonged storage. The specimen was identified by L. Goudie and a voucher sample was deposited with Museum Victoria (Reg No. MVF80004).

Bioassay

The *in vitro* cytotoxicity screening carried out on marine extracts made use of previously described methodology,[9] on lung (A549), colorectal (HT29) and breast (MDA-MB-231) cancer cell lines. The *in vitro* cytotoxicity screening carried out on pure mirabilins was adapted from previously described methodology,[10] on neuroblastoma (SH-SY5Y), gastric (AGS), colorectal (HT29) and intestinal (Intestine-407) cancer cell lines.

Extraction and isolation

The aqueous EtOH extract of specimen CMB-02002 (genus *Clathria*) was decanted and concentrated *in vacuo*, and the residue (1.1 g) partitioned between H_2O and *n*-BuOH. The *n*-BuOH soluble fraction was concentrated *in vacuo* (0.78 g) and the residue further triturated into light petroleum (12.8 mg) and CH_2Cl_2 (758 mg) soluble materials. A portion of the CH_2Cl_2 soluble fraction (250 mg) was subjected to HPLC fractionation (Zorbax SB–C_{18} 5 µm 250 × 9.4 mm column, 4 mL min^{-1} isocratic elution with 60% H_2O–MeCN over 20 min) to yield Fractions A–E (52.8 mg, 91.5 mg, 50.1 mg, 25.3 mg and 10.6 mg respectively). HPLC fractionation of Fraction A (52.8 mg) (Zorbax SB-phenyl column 5 µm 250 × 9.4 mm column, 4 mL min^{-1} gradient elution with 80% H_2O–MeOH (+ 0.01% TFA) to 60% H_2O–MeOH (+ 0.01% TFA) over 20 min) afforded mirabilin J (**24**) (12.4 mg, 4.9%). HPLC fractionation of Fraction D (25.3 mg) (Zorbax SB-CN 5 µm 250 × 9.4 mm column, 4 mL min^{-1} gradient elution with 55% H_2O–MeOH (+ 0.01% TFA) to MeOH (+ 0.01% TFA) over 20 min) afforded mirabilin G (**16**) (8.2 mg, 3.2%) and mirabilin H (**22**) (15 mg, 6.0%). HPLC purification of Fraction E (10.6 mg) (Zorbax SB-phenyl 5 µm 250 × 9.4 mm column, 4 mL min^{-1} gradient elution 60% H_2O–MeOH to MeOH over 20 min) afforded mirabilin C (**3**) (4.5 mg, 1.8%) together with mirabilin I (**23**) (5.2 mg, 2.0%). HPLC fractionation of Fraction B (91 mg) (Zorbax SB-CN 5 µm 250 × 9.4 mm column, 4 mL min^{-1} isocratic elution with 60% H_2O–MeOH (+ 0.01% TFA) over 20 min, followed by Zorbax SB-phenyl 5 µm 250 × 9.4 mm column, 4 mL min^{-1} isocratic elution with 60% H_2O–MeCN (+ 0.01% TFA) over 14 min) afforded mirabilin F (**17**) (13.7 mg, 5.4%). (Note - % yields are expressed as an estimated mass to mass % against the *n*-BuOH solubles).

Mirabilin C (3). Yellow oil; $[\alpha]_D$ +102 (*c* 0.075, $CHCl_3$); UV-vis (MeOH) λ_{max} (log ε) 205 (3.72), 230 (3.59), 320 (3.12) nm; NMR (600 MHz, $CDCl_3$) see ESI†; (+)HRESIMS *m/z* 272.2118 (calcd $C_{17}H_{26}N_3$ 272.2121).

Mirabilin F (17). Yellow solid; $[\alpha]_D$ +90 (*c* 1.26, $CHCl_3$); UV-vis (MeOH) λ_{max} (log ε) 205 (3.94), 260 (3.73) nm; NMR (600 MHz, $CDCl_3$) see ESI†; (+)HRESIMS *m/z* 246.1960 (calcd $C_{15}H_{24}N_3$ 246.1965).

Mirabilin G (16). Yellow solid; $[\alpha]_D$ +66 (*c* 0.325, $CHCl_3$); UV-vis, ^{1}H NMR (600 MHz, $CDCl_3$) see ESI†; and ESI(+)MS data consistent with published data.[6]

Mirabilin H (22). Yellow oil; $[\alpha]_D$ +135 (*c* 0.71, $CHCl_3$); UV-vis (MeOH) λ_{max} (log ε) 205 (3.54), 235 (3.33) nm; 1D and 2D NMR (600 MHz, MeOH-d_4) see ESI†; (+)HRESIMS *m/z* 274.2268 (calcd $C_{17}H_{28}N_3$ 274.2278).

Mirabilin I (23). Colorless oil; $[\alpha]_D$ +92 (*c* 0.41, $CHCl_3$); UV-vis (MeOH) λ_{max} (log ε) 250 (3.19), 207 (3.03) nm; 1D and 2D NMR (600 MHz, $CDCl_3$) and (600 MHz, benzene-d_6) see ESI†; (+)HRESIMS *m/z* 300.2423 (calcd $C_{19}H_{30}N_3$ 300.2434).

Mirabilin J (24). Yellow oil; $[\alpha]_D$ +110 (*c* 0.70, $CHCl_3$); UV-vis (MeOH) λ_{max} (log ε) 202 (4.25), 230 (4.16) nm; ^{1}H NMR (600 MHz, $CDCl_3$) and (600 MHz, DMSO-d_6) see ESI†; (+)HRESIMS *m/z* 290.2225 (calcd $C_{17}H_{28}N_3O$ 290.2227).

Conclusions

In revisiting the mirabilins we have characterized the known mirabilins C (**3**) and F (**17**), previously only recorded as their respective acetate derivatives, and have assigned a complete absolute stereochemistry to **17**. In addition, we report three new members of this rare structure class, mirabilins H–J (**22–24**), of which mirabilin I (**23**) is the first to feature a *trans*-fused ring junction. Our studies extended to assessment of cytotoxic properties against a range of human cancer cell lines, as well as commentary on a plausible polyketide biosynthesis and chemical "transformation" for all known members of this structure class. Finally, a comprehensive analysis of the state of knowledge of this rare class of marine sponge alkaloid is presented.

Acknowledgements

We would like to thank L. Goudie (Museum Victoria) for taxonomic analysis; researchers at PharmaMar for anticancer screening of marine extracts; and The Egyptian Ministry of Higher Education for a PhD scholarship (M. El-N.). This research was funded in part by the Australian Research Council and PharmaMar (Madrid, Spain).

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