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Isolation and synthesis of *N*-acyladenine and adenosine alkaloids from a southern Australian marine sponge, *Phoriospongia* sp.

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ABSTRACT

Chemical fractionation of the southern Australian marine sponge *Phoriospongia* sp. (CMB-03107) yielded phorioadenine A (**1**) as a nematocidal agent and the first reported example of a 6-*N*-acyladenine natural product. The structure of **1** was confirmed by spectroscopic analysis and the chemical synthesis of racemic (**1a**) and enantiomeric (**1b**) analogues. HPLC–ESIMS analysis of the crude sponge extract with comparisons to the synthetic 6-*N*-acyladenosine **2a** provided evidence that the biosynthetically related adenosine, phorioadenosine A (**2**), was present as a trace co-metabolite. The rare starfish metabolite asterubine (**3**) was also isolated as a co-metabolite, and its structure confirmed by spectroscopic analysis and chemical synthesis. Biological investigations confirmed that natural products **1**–**3** and synthetic analogues **1a**–**e** and **2a** were not cytotoxic to multiple mammalian cancer cell lines, or Gram-positive or -negative bacteria. Nematocidal activity (inhibition of larval development of *Haemonchus contortus*) detected in the *Phoriospongia* sp. extract was attributed to **1** (LD_{99} 31 μg/mL), with preliminary structure–activity relationship investigations confirming the importance of the *N*-acyl side chain.

The ruminant livestock industry is heavily dependent on the use of anthelmintic agents (drenches) to combat gastrointestinal nematode parasites, with the cost of treating infected livestock (i.e., sheep, goats, cattle), combined with parasite-related reductions in productivity, representing a significant economic burden.[1] For much of the last three decades, commercial anthelmintics have been largely limited to the benzimidazoles, levamisoles and avermectins, with widespread multidrug resistance to all three classes demanding the use of double and even triple drenches.[2] In response to the ever growing threat of anthelmintic resistance, in 2009 Novartis introduced Zolvix® (monepantel)[3] as a new synthetic class of amino-acetonitrile anthelmintics, while in 2010 Pfizer launched Startect® (derquantel–abamectin), a formulation of the new fungal anthelmintics 2-deoxyparaherquamide (derquantel) and avermectins (abamectin).[4] While Zolvix® and Startect® were welcome contributions, resistance to these new drugs is inevitable. Given the ongoing nature of the resistance challenge, there remains an urgent and compelling case to continue research aimed at discovering new classes of anthelmintics, in particular those with modes-of-action not compromised by existing resistance pathways. In noting the contribution made by microbial natural product anthelmintics, with the avermectins reigning supreme as the gold standard anthelmintic for many decades, we hypothesised that marine biodiversity may offer an alternative source of natural product anthelmintics.

To test this hypothesis we screened a library of >2500 southern Australian and Antarctic marine invertebrates and algae in a *Haemonchus contortus* larval development assay.[5] This approach has proved very successful in the past, facilitating our discovery of many new classes of nematocides spanning such biosynthetic classes as lipids (thiocyanatins,[6] notheians[7]), alkaloids (esmodil,[8] echinobetaines,[9] bromoindoles[10]), peptides (phoriospongins[11]) and polyketides (amphilactams,[12] geodin A Mg salt,[13] onnamides[14]). In a continuation of these studies, this report describes our investigation of the nematocidal extract obtained from a southern Australian marine sponge, *Phoriospongia* sp. (CMB-03107), collected during scientific SCUBA operations off St Leonards Pier, Port Phillip Bay, Victoria. On subjecting the crude EtOH extract to bioassay guided solvent partitions and triturations, followed by C_{18} SPE and HPLC chromatography, we recovered phorioadenine A (**1**) as the first example of a 6-*N*-acyladenine natural product, and a new nematocidal agent, together with the rare marine metabolite asterubine (**3**) (Fig. 1), first reported in 1935 from a North Sea starfish[15] and patented in 2013 for the treatment of metabolic

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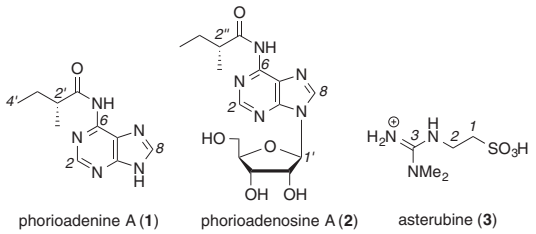


Figure 1. Metabolites from *Phoriospongia* sp. (CMB-03107).

syndrome.[16] The structure elucidation of **1** and **3** were supported by detailed spectroscopic analysis and chemical synthesis, as detailed below. During the course of these investigations we prepared the 6-*N*-acylated adenosine **2a**, and used HPLC–ESIMS with single ion extraction to detect and propose phorioadenosine A (**2**) as a trace co-metabolite.

HRESI(+)MS analysis of **1** returned a pseudo-molecular ion $(M+Na)^+$ consistent with a molecular formula ($C_{10}H_{13}N_5O$, Δmmu +0.4) requiring seven double bond equivalents (DBE). The UV–vis spectra for **1** together with ^{1}H NMR (DMSO-d_6) resonances diagnostic for two deshielded heteroaromatic protons (δ_H 8.41, s; 8.63, s), was suggestive of an adeninyl moiety ($C_5H_4N_5$). Further analysis of the 1D and 2D NMR data (Table 1) revealed resonances consistent with a 2-methylbutyryl amide residue (C_5H_9ON) (δ_H 1.25, d, 2′-Me; 0.99, dd, 4′; 1.55 and 1.81, ddd, H_2-3′; 2.68, ddq, H-2′), accounting for the remaining DBE and satisfying the molecular formula. Given the very low yields of **1** from the *Phoriospongia* sp., the structure assignment and absolute configuration were confirmed by chemical synthesis. Treatment of adenine with (±)-2-methylbutyryl chloride, followed by standard work-up and stepwise gradient C_{18} SPE chromatography yielded the racemic product *rac*-phorioadenine A (**1a**), which was spectroscopically indistinguishable from **1**. Significantly, key 2D ^{1}H–^{15}N HMBC NMR (methanol-d_4) correlations from H-2′ (δ_H 2.68) to 6-N (δ_N −239), from H-2 (δ_H 8.63) to N1 (δ_N −133) and N3 (δ_N −130), and from H-8 (δ_H 8.41) to N7/N9 (δ_N −150/224) and N3 (δ_N −130), confirmed 6-*N*-acyl substitution. Repeating the synthesis using (*S*)-2-methylbutyryl chloride yielded the enantiomer *ent*-phorioadenine A (**1b**) ($[\alpha]_D^{21}$ +10.3), confirming the structure and absolute configuration of phorioadenine A (**1**) ($[\alpha]_D^{22}$ −14.1).

The nematocidal activity against *H. contortus* detected in the crude *Phoriospongia* sp. (CMB-03107) extract was attributed to phorioadenine A (**1**) (LD_{99} 31 μg/mL), being slightly weaker than that of the commercial anthelmintics levamisole and closantel (LD_{99} 5–10 μg/mL). Of note, the synthetic analogues *rac*-phorioadenine A (**1a**) and *ent*-phorioadenine A (**1b**) (Fig. 2) were inactive in the nematode larval development assay. To further explore the importance of the structure–activity relationship (SAR) significance of the *N*-acyl side chain, we synthesised and tested the three achiral phorioadenine homologues **1c**–**e** (Scheme 1), determining that all

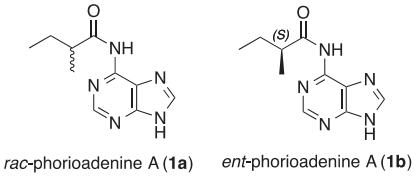
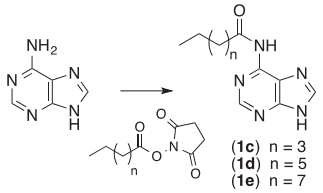


Figure 2. Synthetic phorioadenine analogues **1a**,**b**.



Scheme 1. Synthesis of achiral phorioadenine analogues **1c**–**e**. Conditions: K_2CO_3, MeCN, reflux, overnight.

three were inactive in the nematode larval development assay. While limited, these structure–activity relationship considerations suggest that nematocidal activity is correlated with and is dependent on the nature and chirality of the *N*-acyl side chain.

Having identified the natural product **1**, we speculated that the corresponding adenosine analogue may exist as a co-metabolite. To test this hypothesis we synthesised the 6-*N*-acyladenosine **2a** (Scheme 2), and used it as an authentic standard to probe the HPLC–ESIMS data of the *Phoriospongia* extract, successfully employing single ion extraction technology to detect phorioadenosine A (**2**) as a very minor co-metabolite (Fig. 3).

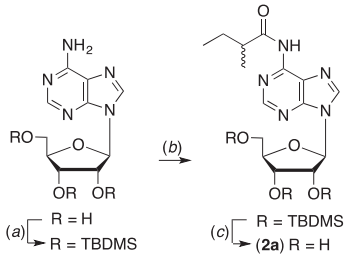
HRESI(+)MS analysis of **3** returned a pseudo-molecular ion (M−H) consistent with a molecular formula ($C_5H_{13}N_3O_3S$, Δmmu −0.8) suggestive of the known marine metabolite asterubine, first reported in 1935 from two North Sea starfish of the genus *Asterias*,[15] and synthesised in the same year.[17] As a detailed spectroscopic characterisation of **3** had not been reported in the scientific literature, to confirm our re-isolation we synthesised and independently characterised this metabolite (Table 2). Optimisation of the condensation of taurine with dimethylcyanamide delivered a simple one-step (66%) synthesis of asterubine that was spectroscopically identical to that isolated from *Phoriospongia* sp. (CMB-03107).

In addition to evaluation in a nematode larval development assay, we determined that the crude extract, and the natural/synthetic compounds **1a**–**e** and **2a**, were not cytotoxic towards the Gram-negative bacteria *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853), or the Gram-positive bacteria *Staphylococcus aureus* (ATCC 25923), *Staphylococcus epidermidis* (ATCC 12228) and *Bacillus subtilis* (ATCC 6633), or human colon (SW620) and lung (NCIH460) cancer cell lines. While of modest potency, the selective cytotoxicity (i.e., nematocidal activity)

Table 1
NMR (400 MHz, CD_3OD) data of phorioadenine A (**1**)[a]

Position	δ_C	δ_H, m (*J* in Hz)
2	153.0	8.63, s
8	146.1	8.41, s
1′	179[b]	—
2′	43.6	2.68, ddq (6.8, 6.8, 6.8)
3′	28.2	a 1.81, ddd (14, 7.4, 6.8) b 1.55, ddd (14, 7.4, 6.8)
4′	12.0	0.99, dd (7.4, 7.4)
2′-Me	17.6	1.25, d (6.8)

[a] Assignments supported by HMQC data.
[b] Detected from the HMBC spectrum.



Scheme 2. Synthesis of 6-*N*-acyladenosine (**2a**). Reagents and conditions: (a) TBDMSCl, DMF, N_2, rt, 21 h, 67%. (b) Pyridine, 2-methylbutanoyl chloride, CH_2Cl_2, N_2, rt, 21 h, 74%. (c) NH_4F, anhyd MeOH, reflux, N_2, 18 h, 40%.

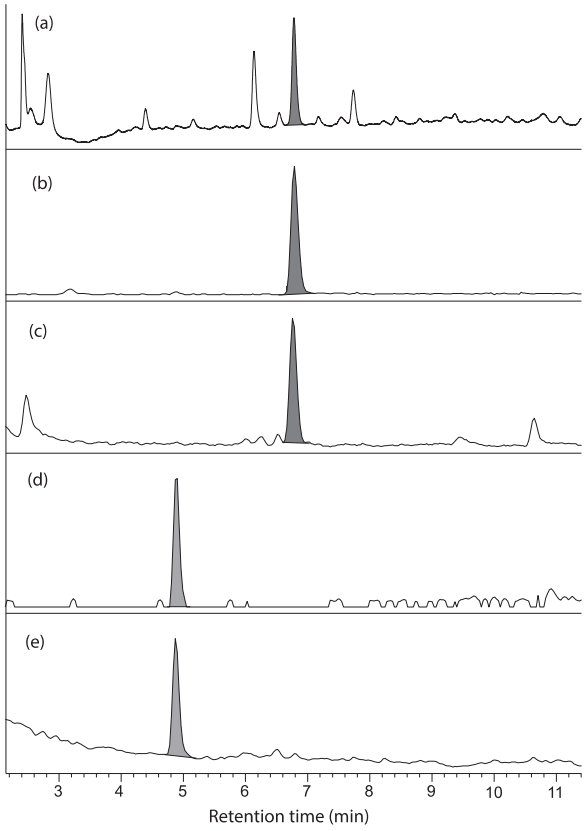


Figure 3. (a) HPLC chromatogram (UV 298 nm) of *Phoriospongia* extract and HPLC-ESI(+)MS chromatograms of *Phoriospongia* extract with single ion monitoring on *m*/*z* (M+H) ions for the natural products (b) **1** and (d) **2**, compared to ESI(+)MS chromatograms for the synthetic authentic standards (c) **1a** and (e) **2a**.

Table 2
NMR (400 MHz, D_2O) data of asterubine (**3**)[a]

Position	δ_C	δ_H, m (*J* in Hz)
1	50.2	3.00, t (6.4)
2	38.8	3.49, t (6.4)
3	157[b]	—
NMe_2	38.5	2.85, s

[a] Assignments supported by HMQC data.
[b] Detected from the HMBC spectrum.

exhibited by the 6-*N*-acyladenine **1** is noteworthy, and may be indicative of a mode of action that deserves closer investigation.

In conclusion, we have isolated the first example of a 6-*N*-acyladenine (**1**) from a marine sponge, and synthesised its enantiomer to confirm its structure and absolute configuration. Compound **1** inhibits larval development of *H. contortus* (LD_{99} 31 μg/mL), and was not cytotoxic to multiple mammalian cancer cell lines, Gram-positive and Gram-negative bacteria. SAR investigations revealed that the chirality at position 2′ is essential for its anthelminthic activity.

Fractionation and characterisation

A portion of the crude EtOH extract of *Phoriospongia* sp. (CMB-03107) was concentrated in vacuo and the residue (270 mg) triturated with CH_2Cl_2, leaving an insoluble residue that was partitioned between *n*-BuOH and H_2O. All three fractions were concentrated in vacuo, and based on bioassay data the *n*-BuOH fraction was selected for chromatographic fractionation (C_{18} SPE with a 20% stepwise gradient elution from 60% H_2O/MeOH to 100% MeOH, followed by a Phenomenex C_{18}, 250 × 9.6 mm, 5 μm column, with a 2 mL/min gradient from 100% H_2O to 100% MeOH over 30 min) to yield phorioadenine A (**1**) (0.9 mg, 0.33%) and asterubine (**3**) (1.1 mg, 0.41%). [Note–% yields are estimates in the crude EtOH extract].

Phorioadenine A (**1**): a colourless amorphous solid. $[\alpha]_D^{22}$ −14.1 (*c* 0.035, MeOH); UV (MeOH) λ_{max} (logε) 209 (3.96), 227 (sh), 256 (sh), 273 (3.63), 281 (3.63), 2.91 (3.48) nm; NMR (MeOH-d_4) data see Table 1; HRESI(+)MS *m*/*z* 242.1016 $[M+Na]^+$ (calculated for $C_{10}H_{13}N_5ONa$, 242.1012, Δmmu +0.4).

Phorioadenosine A (**2**): detected by HPLC–ESIMS (SIE) as a trace metabolite. HRESI(+)MS *m*/*z* 352.1617 $[M+H]^+$ (calculated for $C_{15}H_{22}N_5O_5$, 352.1615, Δmmu +0.2).

Asterubine (**3**): a colourless solid. NMR (400 MHz, D_2O) data see Table 2; ESI(+)MS *m*/*z* 234 $[M+K]^+$, 218 $[M+Na]^+$, 196 $[M+H]^+$; HRESI(−)MS *m*/*z* 194.0597 $[M-H]^-$ (calculated $C_5H_{12}N_3O_3S$, 194.0605, Δmmu −0.8).

Acknowledgments

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Supplementary data

Supplementary data (general experimental, sponge collection and taxonomy, bioassays, synthetic procedures and ^1H and ^{13}C NMR spectra for **1b**) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tetlet.2014.08.116.

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