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Two new betaines from the Australian bryozoan *Amathia lamourouxii*

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ABSTRACT

A chemical investigation of the eastern Australian endemic bryozoan *Amathia lamourouxii* led to the isolation of two unique betaine molecules, lamouroic acid trifluoroacetate and lamourimidazolium trifluoroacetate. The structures of these molecules were determined using (+)-HRESIMS, 2D NMR and ECD analysis. The new compounds were screened for antiparasitic, cytotoxic and antibacterial activity but were inactive at 40 μ M. Lamouroic acid trifluoroacetate is structurally related to the ubiquitous marine compound homarine, that has previously been shown to possess feeding deterrent properties, and this suggests that the new compound may also have an ecological role in the bryozoan.

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Bryozoans are sessile, soft-bodied animals that, like other marine invertebrates, often lack any physical defences and thus rely on secondary metabolites for protection [1]. These metabolites represent a diverse source of interesting chemical structures, many of which have been shown to play important ecological roles and may be of interest as drug leads [1-2]. Compared to other marine invertebrates such as sponges and ascidians, the chemistry of the bryozoan phylum is still vastly unexplored, with only 1% of all marine invertebrates collected being bryozoans [3]. This is surprising as bryozoan metabolites have a chemical profile very similar to that of approved drugs, while the chemical profiles of other marine invertebrate phyla are less similar [4]. The few bryozoans that have been studied have yielded a variety of biologically interesting new compounds, including brominated compounds and a variety of alkaloids with unique structures and properties. These natural products include the bryostatins, a series of highly oxygenated marine macrolides with a unique polyacetate backbone, which were originally isolated from a Gulf of Mexico collection of the bryozoan *Bugula neritina* [5]. The bryostatins have shown bioactivity against various cancer types. They show a specific interaction with protein kinase C and other enzymes. The bryostatins also enhance the cytotoxicity of other anticancer compounds that have been investigated in phase II clinical trials [6]. The continuing study of *B. neritina* has also yielded various new compounds such as the ceramides, neritinaceramides A-E, and two new sterols, all of which display selective cytotoxicity against several different human tumor cell lines [7-8].

A large proportion of the bryozoans that have been chemically studied come from Australasia and exhibit potent antimicrobial, antitrypanosomal and antitumor activity [9-10]. The *Amathia* genus, which has a cosmopolitan distribution, is a prolific producer of a diverse range of bioactive natural products, including novel alkaloids such as dibrominated and tribrominated indole alkaloids [11-14]. *Amathia lamourouxii* was first described in 1987 by Chimonides and is mainly found off the coast of southeastern Australia [15]. Unlike the closely related bryozoan species *A. tortuosa*, *A. wilsoni* and *A. convoluta*, which contain bioactive molecules of high interest, the chemistry of *A. lamourouxii* has never been studied before [13-14,16].

Analysis of the LCMS data for the crude extract of an *A. lamourouxii* specimen collected at Korora Beach, New South Wales, Australia suggested it contained brominated alkaloids. Bioassays on the crude extract also showed activity against a methicillin susceptible strain of *Staphylococcus aureus* (MSSA; ATCC25923) and a methicillin resistant strain (MRSA; ATCC43300). Partitioning of the crude extract in water/CH₂Cl₂ (1:1) yielded a CH₂Cl₂ fraction with brominated compounds, showing bioactivity against *S. aureus* and an aqueous fraction showing no bioactivity. However, NMR data analysis suggested that the aqueous fraction contained interesting natural products. This paper describes the successful isolation and structure identification of two new betaine compounds lamouroic acid trifluoroacetate (**1**) and lamourimidazolium trifluoroacetate (**2**) from the aqueous fraction of the bryozoan species *A. lamourouxii* (Fig. 1) [17]. Due to the usage of TFA during separation, both compounds were isolated as their TFA salts, as evidenced by the resonances of TFA in the carbon spectra.

Figure 1. New betaines isolated from the bryozoan *A. lamourouxii*: lamouroic acid trifluoroacetate (**1**) and lamourimidazolium trifluoroacetate (**2**).

Table 1. NMR data for lamouroic acid trifluoroacetate (**1**) recorded in DMSO-*d*₆ at 25 °C.

Position	δ_C^a , type	δ_H (mult., <i>J</i> in Hz) ^b	COSY ^c	HMBC ^d
1-N	---	---	---	---
1-N-CH ₃	46.2, CH ₃	4.35 (s)	---	2, 6
2	148.6, C	---	---	---
3	126.7, CH	8.18 (dd, 7.8, 1.0)	4	2, 4, 5, 7
4	146.4, CH	8.69 (dd, 7.8, 7.7)	3, 5	2, 3, 5, 6
5	128.3, CH	8.21 (ddd, 7.5, 6.1, 1.0)	4, 6	3, 6
6	147.1, CH	9.08 (d, 6.0)	5	2, 4, 5, 1
7	160.5, C	---	---	---
8-NH	---	9.56 (bs)	9	---
9	40.4, CH ₂	3.98 (bd)	8	7, 10
10	170.0, C	---	---	---
TFA	117.4, CF ₃	---	---	---
TFA	157.7, COO ⁻	---	---	---

^a Spectra recorded at 200 MHz, ^b Spectra recorded at 800 MHz, ^c COSY correlations are from the proton(s) stated to the indicated proton and ^d HMBC correlations are from the proton(s) stated to the indicated carbon.

A charged molecule at *m/z* 195.0772 was observed in the (+) HRESIMS for **1** that was consistent with a molecular formula of C₉H₁₁N₂O₃⁺ (Δ 4.1 ppm) [18]. The ¹H NMR spectrum of **1** (Table 1) contained seven proton resonances. These were an aromatic doublet (δ_H 9.08, H-6), two aromatic doublets of doublets (δ_H 8.18, H-3; 8.69, H-4), an aromatic doublet of doublet of doublets (δ_H 8.21, H-5), an NH singlet (δ_H 9.56; H-8), a methyl singlet (δ_H 4.35; H₃-1) and a broad methylene doublet (δ_H 3.98; H₂-9). The ¹³C NMR spectrum showed the presence of nine carbons: a carboxylic acid (δ_C 170.0, C-1), an amide (δ_C 160.5, C-7), five aromatics (δ_C 148.6, C-2; 126.7, C-3; 146.4, C-4; 128.3, C-5; 147.1, C-6), one *N*-methyl (δ_C 46.2, C-1) and one aliphatic methylene (δ_C 40.4, C-9).

COSY correlations indicated a spin system consisting of four aromatic protons (δ_H 8.18, H-3; 8.69, H-4; 8.21, H-5 and 9.08, H-6) (Fig. 2). The aromatic carbon C-2 (δ_C 148.6) was identified as a quaternary carbon. H-4 and H-6 ³*J*_{CH} HMBC correlate to the aromatic carbon resonating at δ_C 148.6 (C-2), suggesting that this carbon is *meta* to positions 4 and 6. The downfield chemical shifts of CH-6 (δ_C 147.1, δ_H 9.08) and C-2 (δ_C 148.6) indicated the substitution of a nitrogen atom in between these positions and that a pyridine ring was present in the structure. HMBC correlations between H-6 and δ_C 46.2 (C-1) and from H₃-1 (δ_H 4.35) to C-2 indicated an *N*-methylated pyridine (Fig. 2).

Figure 2. Key HMBC correlations used to identify the structure of **1**.

The aliphatic methylene proton resonance at δ_H 3.98 (H-9) showed a COSY correlation to the NH singlet resonance at δ_H 9.56 (H-8). They further showed HMBC correlations from H-8 and H-9 to a secondary amide carbon resonance at δ_C 160.5 (C-7) and a carboxylic acid carbon resonance at δ_C 170.0 (C-10). An HMBC correlation from δ_H 8.18 (H-3) to the secondary amide carbon (δ_C 160.5, C-7) indicated that the amide carbon is directly connected to the pyridine ring at the C-2 position. The combined spectroscopic evidence was consistent with **1** being *N*-[(1-methyl-2-pyridinyl)carbonyl]glycine trifluoroacetate.

Table 2. NMR data for lamourimidazolium trifluoroacetate (**2**) recorded in DMSO- d_6 at 25°C.

Position	δ_C^a , type	δ_H (mult., J in Hz) ^b	COSY ^c	HMBC ^d
1	170.2, C	---	---	---
2	63.1, CH	4.39 (dd, 7.0, 12.5)	6a, b	1, 6
3-N	---	---	---	---
4	165.8, C	---	---	---
5-N	---	---	---	---
6a	53.8, CH ₂	3.98 (dd, 12.5, 11.2)	2	1, 2
6b	53.8, CH ₂	3.81 (dd, 7.0, 11.2)	2	1, 2
7	33.1, CH ₃	3.11 (s)	---	2, 4
8	10.3, CH ₃	2.25 (s)	---	4
9	33.3, CH ₃	3.04 (s)	---	4, 6
TFA	117.8, CF ₃	---	---	---
TFA	157.9, COO ⁻	---	---	---

^a Spectra recorded at 125 MHz, ^b Spectra recorded at 500 MHz, ^c COSY correlations are from the proton(s) stated to the indicated proton and ^d HMBC correlations are from the proton(s) stated to the indicated carbon.

Lamourimidazolinine (**2**) was isolated as its trifluoroacetate salt as a colorless gum. A charged molecule at m/z 157.0974 was observed in the (+) HRESIMS for **2**, which was consistent with a molecular formula of $C_7H_{13}N_2O_2^+$ (Δ 1.3 ppm) [19]. Six proton resonances were observed in the 1H NMR spectrum of **2** (Table 2) including two *N*-methyl singlets (δ_H 3.11, H₂-7; 3.04, H₃-9), one methyl singlet (δ_H 2.25, H₃-8), a methine doublet of doublets (δ_H 4.39, H-2), and a diastereotopic methylene group (δ_H 3.81, H-6b and δ_H 3.98; H-6a). The ^{13}C NMR spectrum in combination with HSQC data analysis indicated that **2** contained seven carbon resonances that could be assigned to a carboxylic acid (δ_C 170.2, C-1), an aliphatic methine carbon (δ_C 63.1, C-2), a heterosubstituted olefin (δ_C 165.8, C-4), an aliphatic methyl carbon (δ_C 10.3, C-8), two *N*-methyl carbons (δ_C 33.1, C-7; 33.3, C-9), and one aliphatic methylene carbon (δ_C 53.8, C-6).

Figure 3. Key HMBC correlations used to identify the structure of **2**.

COSY correlations observed between δ_H 4.39 (H-2) and H-6a (δ_H 3.98) and H-6b (δ_H 3.81) indicated that **2** contained a CH_2CH moiety. The carbon chemical shifts of C-2 (δ_C 63.1) and C-6 (δ_C 53.8) suggested that they were both bonded to nitrogen atoms [20]. An HMBC correlation from the *N*-methyl proton resonance at δ_H 3.11 (H₃-7) to C-2 and an HMBC correlation from the second *N*-methyl proton resonance at δ_H 3.04 (H₃-9) to C-6 further substantiated that C-2 and C-6 are substituted by *N*-methylated nitrogen atoms (Fig. 3). Additional HMBC correlations were observed from the two *N*-methyl proton resonances (H₃-7 and H₃-9) to the heterosubstituted olefinic carbon resonance at δ_C 165.8 (C-4) indicating that **2** contains a dihydroimidazole ring. The methyl proton resonance at δ_H 2.25, (H₃-8) showed a strong HMBC correlation to C-4 and no further HMBC correlations, indicating that this methyl group is directly attached to C-4. HMBC correlations from H-2 (δ_H 4.39) and H₂-6 (δ_H 3.98; 3.81) to the carbonyl carbon resonance at δ_C 170.2 (C-1) indicated that CH-2 was substituted by a carboxylic acid. The combined spectroscopic evidence was consistent with **2** being 2-carboxy-6-hydro-3,4,5-methyl-1*H*-imidazolinium trifluoroacetate.

The absolute configuration of the stereogenic center in **2** was determined by comparison of experimental and predicted Electronic Circular Dichroism (ECD) spectra of the two possible enantiomers (*2S*)-**2** and (*2R*)-**2**. The predicted ECD spectra were calculated using the Time-Dependent Density Functional Theory (TDDFT) method. The calculated ECD spectrum of (*2S*)-**2** at the B3LYP/6-31G(d) level showed an almost perfect match with the experimental data, implying a *2S* absolute configuration in **2** (Fig. 4).

Figure 4. Comparison of the experimental ECD spectrum of compound **2** with those calculated at the B3LYP/6-31G(d) level for enantiomers *2S* and *2R*.

Compound **1** and **2** were screened against a Methicillin-susceptible *S. aureus* strain (MSSA; ATCC 25923) and a Methicillin-resistant *S. aureus* strain (MRSA; ATCC 43300) and were found to be inactive at 100 μ g/mL. Both compounds were also tested for activity against the chloroquine sensitive strain (3D7) of the malaria parasite *Plasmodium falciparum* and normal human embryonic kidney cells (HEK293) but were also inactive at the highest doses (40 μ M) tested.

The structure of **1** closely resembles the common marine metabolite homarine. Homarine was first described in 1933 when Hoppe-Seyler extracted the compound from the muscles of a

lobster [21]. It has since been found in various marine invertebrate species, including bryozoans, crustaceans, molluscs, gastropods and cnidarians [22-27].

Due to its high prevalence in marine invertebrates, the biological significance of homarine has been intensively studied, with various papers concluding it possesses antifouling and feeding deterrent properties [24,26]. One study compared the antifouling properties of homarine, isolated from the cnidarian *Leptogorgia virgulata*, against the benthic marine diatom *Navicula salinicola*, to structurally related compounds, such as nicotinic acid, picolinic acid and pyridine [26]. Their findings showed that the activity is most likely due to the carboxyl group on the pyridine ring and not the *N*-methylation [26]. In another study of homarine from shrimp muscles showed that glycine was the main precursor for its biosynthesis [22]. This study also found that homarine might be involved in the primary metabolism of crustaceans, acting as a metabolic methyl donor and potential reservoir of methyl groups [22]. Newer studies describe homarine as an organic osmolyte thus regulating the osmotic balance of the invertebrate, and this hypothesis is supported by the fact that it is often found in large quantities within various classes of invertebrates [28-30].

Further, 3-hydroxy pyridine-2-carbonyl glycine, which is a close analogue to lamouroic acid trifluoroacetate (**1**), was found to act as a hypoxia-inducible factor (HIF) prolyl hydroxylase (PH) inhibitor [31]. HIF-PH inhibitors were recently found to be a potential treatment for anemia in patients with chronic kidney disease, since they can play a significant role in the improvement of iron metabolism and transportation to the bone marrow and are reported to stimulate production of red blood cells [32].

Since lamouroic acid trifluoroacetate (**1**) has a relatively similar structure to homarine it is likely to have similar properties of being a feeding deterrent, an antifouling agent, an osmolyte, metal chelator or a reservoir for methylation.

Several molecules related to **2** such as clathridine have been found to have metal chelation properties, suggesting that they may be involved in the uptake and transport of essential metal ions present in the aquatic environments [33]. Clathridine, which is a guanidine alkaloid, was isolated from the sponge *Clathrina clathrus* and also found as a 2:1 zinc complex [34]. Many compounds containing imidazolines have been shown to possess anticancer, antibacterial and anti-inflammatory activity [35-38]. Spongotines, which are sponge derived brominated natural products, sensitise cancer cells to the chemotherapeutic agent camptothecin, making it more efficient [36,39]. Further, a synthetic imidazolinium carboxylate salt analogue closely related to lamourimidazolinium trifluoroacetate was found to synergistically inhibit corrosion of mild steel in aqueous chlorine solutions [40-41]. Interestingly most of the molecules structurally related to **2** are fully synthetic compounds.

The chemical investigation of *A. lamourouxii* and the discovery of two new compounds emphasizes the importance of future studies of marine natural products. The limited studies on bryozoans showed that they produce a variety of interesting compounds revealing different activities and research should be continued.

Acknowledgments

The *A. lamourouxii* specimen (ACENV637) was collected under the NSW DPI Fisheries Scientific collection permit P09/0031-1.1.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.tetlet.2019.151047>.

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- [17] *A. lamourouxii* (ACENV637) (25.01 g) was exhaustively extracted in MeOH yielding a brown gum (1.46 g). This crude extract was partitioned between water, basified with ammonium hydroxide (0.7%, 50 mL), and CH₂Cl₂ (50 mL). The separated water fraction was further extracted with CH₂Cl₂ (50 mL) and both CH₂Cl₂ fractions were combined, and dried yielding a CH₂Cl₂ fraction as a gum (0.32 g) and a water fraction as a brown solid (0.84 g). Bioassay testing indicated that the CH₂Cl₂ fraction possessed the majority of the antimicrobial activity. The water fraction (0.84 g) was adsorbed onto C₁₈ silica gel (0.84 g), loaded into a refillable HPLC guard column (20 mm x 10 mm) and connected in series to a preparative C₁₈ HPLC column. The combined columns were then eluted with a solvent gradient from 0% MeOH, 100% water to 100% MeOH, 0% water over 60 min, at 9 mL/min and fractions were collected every min. Both HPLC solvents contained 0.1% TFA. Fractions were analysed by ¹H NMR spectroscopy and fractions containing similar resonances were combined and further purified using normal phase HPLC separations. Combined fractions 4-10 were separated with a gradient from 90% CH₂Cl₂, 10% MeOH to 70% CH₂Cl₂, 30% MeOH, over 60 min at 9 mL/min and fractions were collected every min. After the second normal phase separation **1** (2.7 mg / 0.002%) was found pure in fractions 31-35 and **2** (4.0 mg / 0.003%) was found pure in fraction 14.
- [18] Lamouroic acid trifluoroacetate (**1**): light brown gum; UV λ_{max} MeOH (log ε) 210 (3.89), 268 (2.51) nm; IR ν_{max} (film) 3400, 2920, 1680, 1200, 1130, 669 cm⁻¹; ¹H and ¹³C NMR data (DMSO-*d*₆) see Table 1; (+)-HRESIMS m/z 195.0772 (calcd for C₉H₁₁N₂O₃⁺, 195.0764).
- [19] Lamourimidazolinium trifluoroacetate (**2**): light brown gum; [α]_D²³ +2.74 (c 0.115, MeOH); UV λ_{max} MeOH (log ε) 239 (2.84) nm; ECD (c 0.115), λ_{max} MeOH (Δε) 296 (0), 243 (+14.7), 220 (0), 200 (-7.8) nm; IR ν_{max} (film) 3380, 1680, 1420, 1200, 802, 723, 667 cm⁻¹; ¹H and ¹³C NMR data (DMSO-*d*₆) see Table 2; (+)-HRESIMS m/z 157.0974 (calcd for C₇H₁₃N₂O₂⁺, 157.0972).
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