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Antiparasitic activity of bromotyrosine alkaloids and new analogues isolated from the Fijian marine Sponge *Aplysinella rhax*

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Ten bromotyrosine alkaloids (**1-10**) were isolated and characterised from the marine sponge *Aplysinella rhax* (de Laubenfels 1954) collected from the Fiji Islands, which included one new bromotyrosine analogue, psammaplins P (**6**) and two other analogues, psammaplins O (**5**) and 4-bromo-6-carbomethoxy salicylic acid (**7**), which have not been previously reported from natural sources. HRESIMS, 1D and 2D NMR spectroscopic methods were used in the elucidation of the compounds. Bisaprasin, a biphenylic dimer of psammaplins A, showed moderate activity with IC₅₀ at 19+/- 5 and 29+/- 6 µM against *Trypanozoma cruzi* Tulahuen C4, and the lethal human malaria species *Plasmodium falciparum* clone 3D7, respectively, while psammaplins A (**1**) and D (**4**) exhibited low activity against both parasites. This is the first report of the antimalarial and antitrypanosomal activity of the psammaplins-type compounds. Additionally, the biosynthesis hypotheses of the three natural products (**5**, **6** and **7**) were proposed.

Keywords: Psammaplins • Chagas disease • Bromotyrosine • Malaria • Sponge •

Introduction

The scourge of tropical diseases caused by kinetoplastid parasites, *Plasmodium falciparum* and *Trypanosoma cruzi* has negatively impacted the health of a substantial group of the world population living mostly in the developing world and has led to the death of millions of people.^[1] Chagas disease otherwise refers to as American Trypanosomiasis, is a chronic parasitic infection caused by *Trypanosoma Cruzi* discovered by the Brazilian physician, Carlos Chagas, in 1909.^[2] Chagas disease is primarily confined to Latin America, and southern parts of North America, but has spread to many developed countries owing to migration.^[2-3] People infected with Chagas disease are usually asymptomatic, and one-third of them later develop a chronic form of the disease which can manifest as cardiac disease and is the leading cause of morbidity and mortality in affected countries.^[2, 4] The recent infection cases reported for malaria and Chagas diseases are estimated to be 228 million and 6-7 million, respectively.^[5-6] To make the matter worse, the *Plasmodium* parasite that causes malaria has developed resistance against the current therapeutic drugs (Artesunate) and Artemisinin-based combination therapies (ACT), which first emerged on Cambodia–Thailand border.^[7-8] Despite the huge resources channelled into vaccine development, no vaccine seems to be in view.^[9] There is a high demand for an affordable and safe drug to help reduce the scourge of infection in the affected countries.^[10-11] Enormous progress is being made to eradicate these diseases a significant effort of which is concentrated on finding less toxic therapeutic agents against Chagas disease.^[12]

Marine Sponges (phylum Porifera) are among the oldest multicellular animals in the world.^[13] They are highly diverse and capable of biosynthesising a greater variety of natural bioactive natural products than other invertebrate phyla.^[14] Investigation of

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compounds isolated from taxa in the class Demospongiae, order Verongida, confirmed the presence of a high number of nitrogen-containing secondary metabolites,^[15] and in particular, a diverse array of brominated tyrosine derivatives,^[16-17] Marine sponges have contributed immensely to natural product discovery and were responsible for nearly 30% of all of the secondary metabolites produced till date.^[18] Compounds isolated from the Verongida showed a wide range of bioactivities such as antifoulant,^[19-22] anticancer,^[23-27] antimicrobial,^[28-31] antifungal,^[32-33] and enzymatic activities^[34-37] with IC₅₀ values at low micromolar levels. Aeropylsinin-I, an optically active bioactive small molecule, was the first brominated metabolite isolated from a sponge *Aplysina aerophoba* (family Aplysinidae) collected from the Mediterranean Sea,^[38] and reported to possess moderate inhibition against *Plasmodium falciparum* and *Trypanosoma cruzi*.^[39] Other marine-derived bromotyrosine alkaloids such as Psammaplysina F, H and 11-hydroxyaerotherionin have shown promising antimalarial activities.^[40-43]

In our search for new metabolites from marine sources against these diseases, the methanolic extract of the sponge *Aplysinella rhax*, collected from the Fiji Islands was subjected to sequential fractionation and purification, indicating that 3 of the 10 compounds have now been isolated for the first time from natural sources. Herein we report the structure elucidation of the three marine-derived compounds and antiparasitic activities of most of the compounds isolated (**1-7, 9-10**).

Results and Discussion

The marine sponge extract was partitioned between water and dichloromethane (50% v/v) using a modified Kupchan method^[44] previously described^[45-46] and further fractionated using reversed-phase solid-phase extraction (SPE). The resulting fractions were purified on reversed-phase HPLC to yield psammaplins A (**1**),^[16, 47, 48-50] B-D(**2-4**),^[16, 49] O (**5**) and P (**6**), 3-bromo-2-hydroxyl-6-carbomethoxy benzoic acid (**7**), 2-(3-bromo-4-hydroxyphenyl)acetonitrile (**8**),^[16, 50] 3-bromo-4-hydroxybenzoic acid (**9**),^[51] and bisaprasin (**10**)^[47-48-50]. All known compounds were identified by comparison of experimental NMR and HRESIMS data with those published.

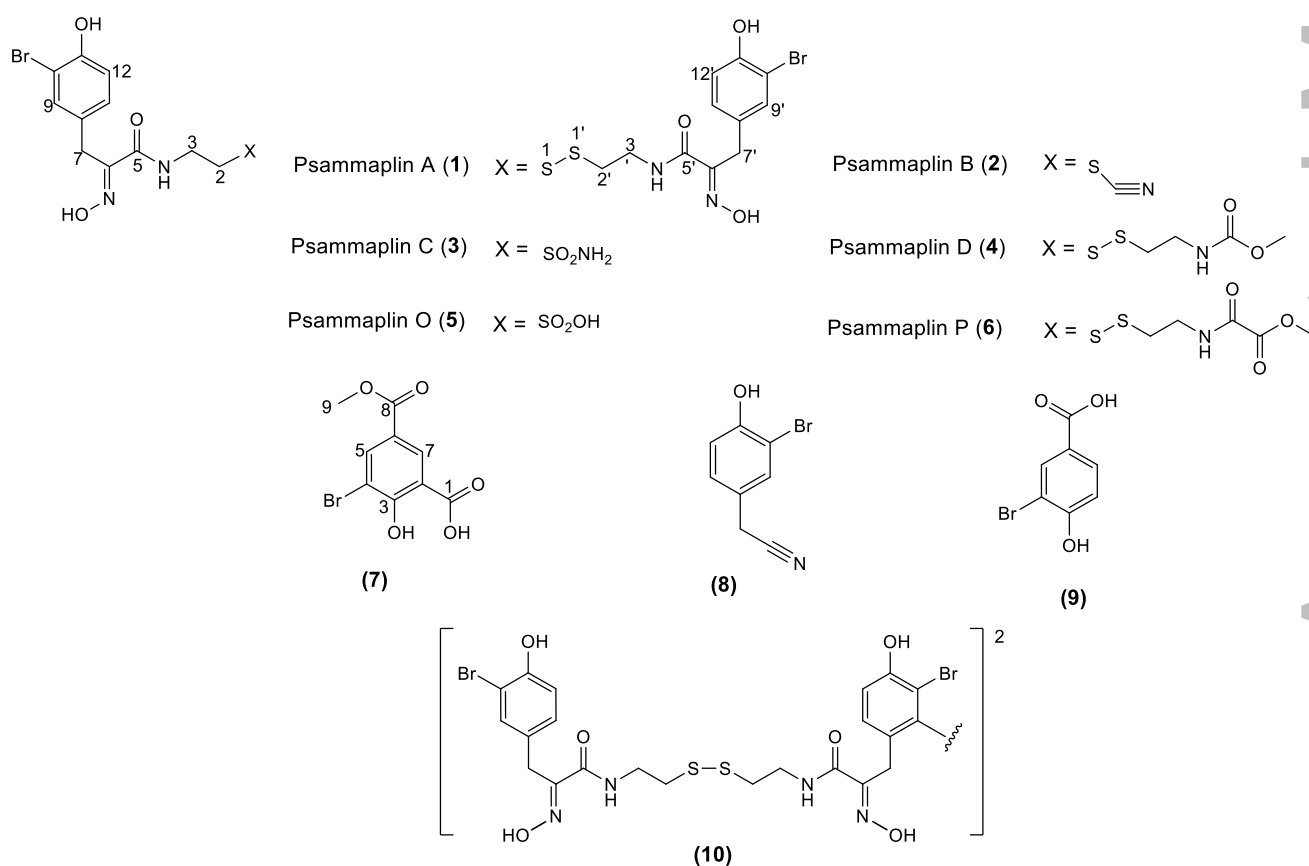


Figure 1. Structure of compounds **1-10**

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Compound **5** was obtained as yellowish oil. High-resolution electrospray ionisation mass spectroscopy HRESI-TOF-MS revealed a protonated 1:1 isotopic cluster indicating the presence of one bromine atom at m/z 380.9746/382.9725 $[M+H]^+$ in accordance with the molecular formula of $C_{11}H_{14}BrN_2O_6S$, requiring 6 degrees of unsaturation (See fig. S21).

The 1H , ^{13}C and HSQC data of **5** (Table 1) showed the presence of five quaternary carbons, three methylene and three methine groups. Detailed analysis of 1H chemical shifts, coupling constants, and COSY data suggested the presence of a 1,3,4 trisubstituted aromatic ring with H-13 (δ_H 7.07) coupling to both H-12 (δ_H 6.76, $J = 8,4$ Hz), and H-9 (δ_H 7.36, $J = 2.0$ Hz). The position of the methylene singlet H-7 was deduced from HMBC correlations to C-9 (δ_C 134.3), and C-13 (δ_C 130.2) in the aromatic ring, C-6 (δ_C 151.3, oxime), and C-5 (δ_C 165.4, carboxamide) units. The unusual ^{13}C downfield chemical shift for the CH_2 group at C-2 (δ_C 50.8 ppm) suggested it was attached to the resonance stabilised electron-withdrawing sulfonate group at this position (Fig. 2). Similar chemical ^{13}C chemical shifts can be found for psammaplin C^[16] that contains the SO_2NH_2 group at C-2 instead of SO_2OH as in **5**. All 1H , HSQC, COSY, and HMBC (Figs. 2 and S22-27, Table 1 and S63) data were consistent with published data for psammaplin type molecules^[16] suggesting that the structure of **5** was correct. Even though this article represents the first report of compound **5** from marine sources, it has been previously described as a semisynthetic product obtained through MCPBA-mediated oxidation of psammaplin I.^[52] The NMR data of **5** were consistent with those reported for the semisynthetic version providing further evidence for the structure of **5**.

The molecular mass of compound **6** was determined by high-resolution ESI-TOF-MS as 494.0065 $[M+H]^+$ corresponding to a molecular formula of $C_{16}H_{20}BrN_3O_6S_2$, requiring 8 degrees of unsaturation (Figs. S28-S29). Analysis of 1D and 2D NMR data (Tables 1 & S63, Figs. 1, S30-35) suggested that the general backbone for **6** was essentially that of psammaplin F^[50] with the exception of the presence of an O-methyl group in **6** to form an ester. The presence of protonated and sodium adducts at m/z 274.9545 $(M+H)^+$ and 296.9364 $(M+Na)^+$ in the ESI-TOF spectrum of **7** suggested a molecular formula of $C_9H_7BrO_5$, requiring 6 degrees of unsaturation (Figs. S36-S37). It also displayed the same isotopic pattern shown by **5** indicating the presence of one bromine atom in the structure of **7**.

Table 1. 1H and ^{13}C N.M.R. spectroscopy data for **5**, **6** and **7** in CD_3OD

Position	5		6		7	
	δ_C , type ^[a]	1H , mult. (J in Hz) ^[b]	δ_C , type ^[a]	1H , mult. (J in Hz) ^[b]	δ_C , type ^[a]	1H , m (J in Hz) ^[b]
1					171.4, C	
2	50.8, CH_2	2.98, t (6.6)	37.4, CH_2	2.86, t (6.6)	114.4, C	
3	35.8, CH_2	3.67, t (6.6)	39.2, CH_2	3.57, t (6.6)	162.3, C	
4					110.2, C	
5	165.4, C		165.6, C		138.4, CH	8.30, d (2.1)
6	151.3, C		152.8, C		121.2, C	
7	28.2, CH_2	3.79, s	28.3, CH_2	3.81, s	131.8, CH	8.50, d (2.1)
8	130.3, C		130.2, C		165.2, C	
9	134.3, CH	7.36, d (2.0)	134.2, CH	7.38, d (2.0)	51.1, CH_3	3.89, s
10	109.6, C		110.3, C			
11	152.4, C		153.5, C			
12	116.7, CH	6.76, d (8.4)	116.8, CH	6.79, d (8.4)		
13	130.2, CH	7.07, dd (8.4, 2.0)	130.2, CH	7.09, dd (8.4, 2.0)		
2'			38.2, CH_2	2.86, t (6.6)		
3'			39.5, CH_2	3.57, t (6.6)		
5'			158.9, C			
6'			161.6, C			
7'			53.3, CH_3	3.88, s		

^[a] Measured at 150 MHz for ^{13}C -NMR data and ^[b] measured at 600 MHz for 1H -NMR data in CD_3OD

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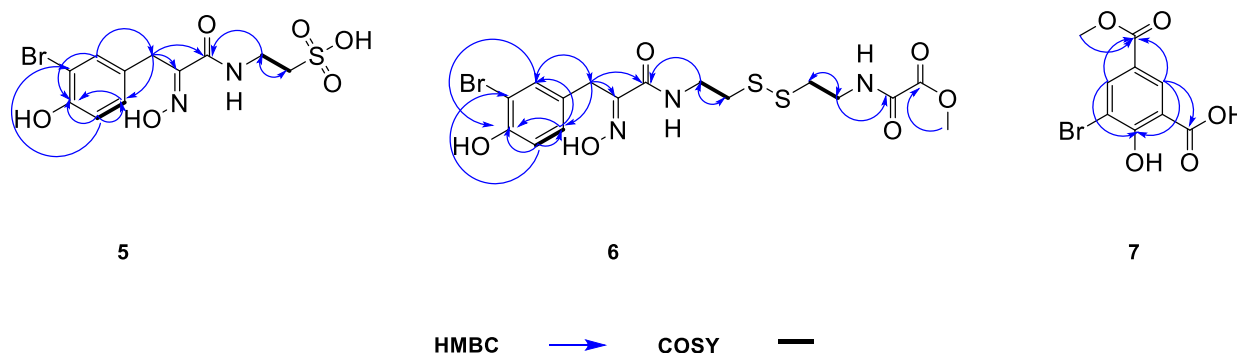
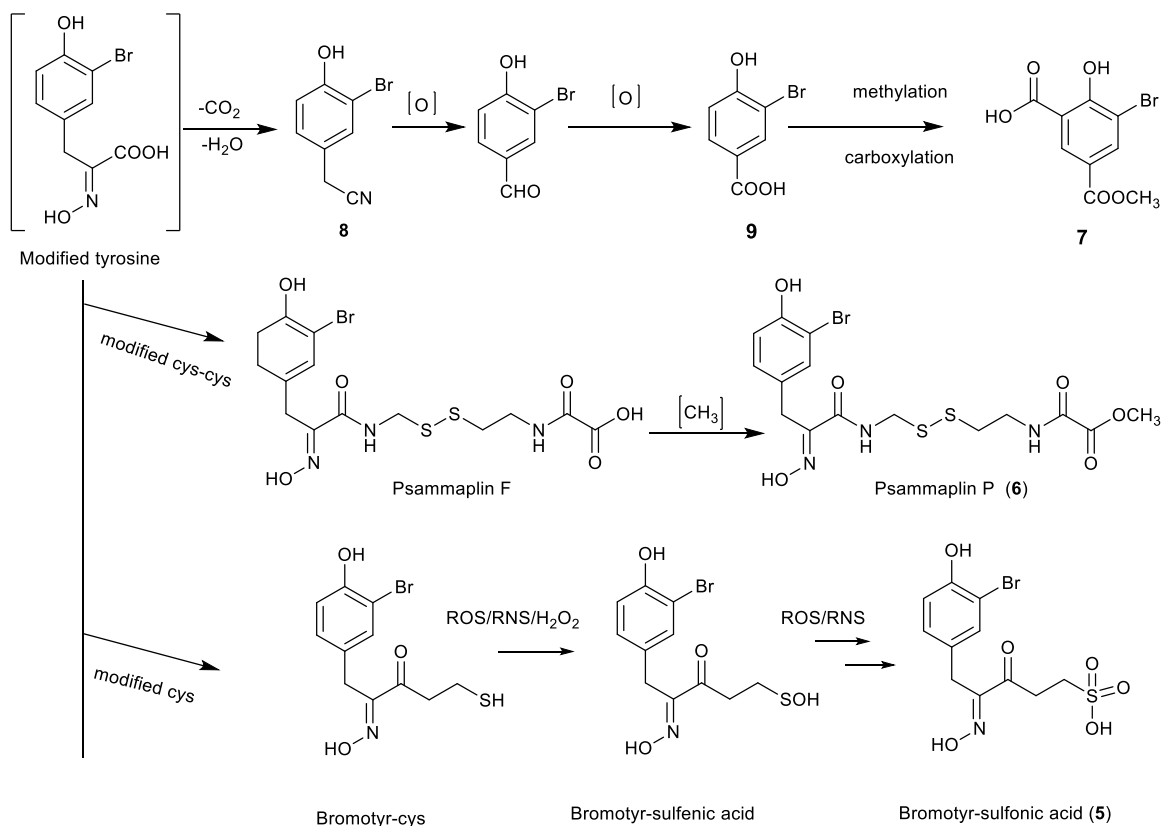


Figure 2. Selected COSY and HMBC correlations for **5**, **6** and **7**

Analysis of ^{13}C and HSQC data (Table 1, Figs. S39) showed the presence of six quaternary carbons, one methoxy group, while ^1H NMR and COSY data (Figs. S38 and S40) suggested the presence of a 1,2,3,5 tetrasubstituted aromatic spin system. The 2.1 Hz coupling of H-5 to H-7 suggested their *meta* relationship. HMBC correlations between H-5 (δ_{H} 8.30) and H-7 (δ_{H} 8.50) to C-6 and C-8, and between H-9 (δ_{H} 3.89) and C-8 confirmed the placement of the methyl ester functionality at C-8. (Figs. 2, S41, Table 1 and S63). There is HMBC strong correlation between both H-5 and H-7 to the oxygenated carbon C-3 (δ_{C} 162.3) and from H-7 to C-1 (δ_{C} 171.4). The resonance signal δ_{C} 110.2 assigned to C-4 was suggestive of the shielding effect of the bromine atom^[53] consistent with the brominated carbon C-10 in both **5** and **6** (See table 1). The structure of **7** was determined by assigning the hydroxyl group and carboxylic acid substituent to C-3 and C-1, respectively, based on HMBC correlation and chemical shift. Interestingly, compound **7** was previously synthesised^[54-55], and there was only ^1H NMR information available in the literature^[55] and was consistent with the data obtained for **7**.



Scheme 1. The proposed biosynthetic hypotheses of **5**, **6** and **7**. ROS (reactive oxygen species), RNS (reactive nitrogen species).

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The stereochemistry of the oxime groups is assigned as *E* for compounds **5** and **6** based on ^{13}C and ^1H data. This is in accordance with the model carried out by Arabshahi and Schmitz^[47] where ^{13}C chemical shifts of the benzylic carbons varied diagnostically with geometry. The modified bromotyrosine and cysteine are the basic structural scaffold of psammaplins A and other derivatives isolated from the sponge extract and the biosynthetic pathway of psammaplins type compounds have been extensively studied.^[16, 50]

As predicted by Pina and co-workers,^[16] the logical precursor for the biosynthetic origin of **8** and **9** was the modification of tyrosine through condensation, decarboxylation process and subsequent oxidation of 3-bromo-4-hydroxybenzaldehyde.^[50] Based on this prediction, the biosynthetic hypothesis of **7** was proposed to be through further methylation of **9**, followed by carboxylation process (Scheme 1). Psammaplins P (**6**) was undoubtedly an esterified derivative of psammaplins F, the biosynthetic pathway of which was proposed to have originated from the formation of cystine^[16] intermediate through oxidation of a dimerised cysteine modification possibly catalysed by cytochrome P450 enzymes in union with bromotyrosine units via a condensation reaction,^[50] followed by methylation of the carboxylic acid functionality (Scheme 1).

On the other hand, compound **5** (Scheme 1) can be rationalised by the reaction of rearranged cysteine as proposed by Jimenez and Crews^[50] with bromotyrosine to form the bromotyrosine-cysteine unit containing a reactive thiol nucleophile (-SH) that undergoes oxidation via reactive oxygen species (ROS) and reactive nitrogen species (RNS) to form sulphenic acid (-SOH) as an intermediate reacting further to form sulfinic acid (-SO₂H) and sulfonic acid (-SO₃H).^[56-57] Cysteine is very susceptible to oxidative enzymatic reactions owing to the electron-rich sulfur atom in its side chain. This mechanism is well established in bacteria,^[57] although the link between psammaplins biosynthesis and bacteria has not been proven to date, there is strong evidence of the presence of metabolite producing microbes (Poribacteria) living in association with Verongida sponges such as *Pseudoceratina purpurea*^[58-59] which produces psammaplins.^[16, 47, 48-50]

Table 2. In vitro biological activity of antiparasitic assays for **1-10**

Compounds	<i>IC</i> ₅₀ μM	
	<i>T. cruzi</i> C2C4 strain	<i>P. falciparum</i> 3D7
1	30 +/- 2	60 +/- 2
2	>35	>70
3	>66	>131
4	43 +/- 2	67 +/- 3
5	>33	>66
6	>32	>64
7	>45	>91
8	NT	NT
9	>57	>115
10	19 +/- 5	29 +/- 6
Benznidazole ^[a]	2.6 +/- 0.5	NA
Chloroquine ^[b]	NA	0.017 +/- 0.002

^[a] Benznidazole as standard for *T. cruzi* C2C4 strain ^[b] Chloroquine as standard for *P. falciparum* 3D7 strain. NT and NA represent 'not tested' and 'not applicable', respectively.

Compounds **1-6**, **9**, and **10** were evaluated for their antiparasitic activity against *T. cruzi* Tulahuen C4, and *P. falciparum* 3D7 strains. Bisaprasin (**10**), a biphenylic dimer of psammaplins A, showed moderate activity with *IC*₅₀ at 19 and 29 μM, respectively, while psammaplins A (**1**) showed activity at 30 and 60 μM for the two respective parasites. Besides, Psammaplins D (**4**) exhibited a lower level of activity in the two assays, but the rest of the compounds did not show activity. All compound activities are shown in Table

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2, including the results for the two standard compounds benznidazole and chloroquine (Figs. S61-S62). The observed activities could be classified as low to moderate when compared to the two standard compounds.

Conclusions

In conclusion, three bromotyrosine derivatives structurally related to the psammoplin family have been structurally characterised where two of the compounds (**5** and **7**) have now been isolated for the first time from the marine sponge, *Aplysinella rhax*. These three compounds present a new finding that contributes further in widening the chemical space of this family of interesting bioactive molecules. Additionally, this study has identified bromotyrosine compounds as potential molecular architectural 'signboards' for new antiparasitic agents. Our results are in line with a recent study on two new bromotyrosine compounds isolated from an Australian marine sponge that showed potent activity against malarial parasites.^[60] After all, nature has continued to inspire drug discovery in this field as out of the 15 compounds used against parasites between 1981-2014, 9 have their origins in natural products.^[61]

Experimental Section

General Experimental Procedures

UV spectra were recorded on an Agilent 1200 HPLC system coupled to a photodiode array detector (DAD). IR spectra were recorded on a PerkinElmer UATR Two, model L1600300. Both 1D and 2D NMR data were recorded on a Bruker AVANCE III HD Prodigy TCI. cryoprobe at 600 and 150 MHz for ¹H and ¹³C, respectively. ¹H and ¹³C chemical shift were referenced to the solvent peak at 3.31 and 49.1 ppm (CD₃OD) respectively. HRESIMS data were obtained using a ThermoScientific LTQ XL/LTQ Orbitrap Discovery coupled to a Thermo instrument Accela HPLC system, and an Agilent 6540 HRESI-TOF-MS coupled to an Agilent 1200 HPLC system. Fractionations were carried out on solid-phase extraction columns using C18-E (Phenomenex, 55 μm, 70 Å, 2 g/12 mL, giga tubes). Purification was done on an Agilent 1200 semi-preparative HPLC system equipped with binary pump, photodiode array detector (DAD)22, Waters Sunfire reversed-phase column C18 (5μm 10 × 250 mm) and Agilent Zorbax C18 (5 μm 9.4×250 mm), and a mobile phase solvent gradient between 95:5% and 20:80% ((H₂O-MeOH).

Collection and Identification

The sponge sample was collected from the Fiji Islands in December 1997, freeze-dried and stored in 4 °C. It was identified as *Aplysinella rhax* by Dr. John Hooper of the Queensland Centre for Biodiversity, Queensland Museum, Australia as described in a previous publication.^[49] A voucher specimen (Voucher number: 9712SD130) is held at the Pacific Regional Herbarium at the University of the South Pacific, Suva, Fiji Islands.

Extraction and Isolation

The sponge sample was extracted with MeOH (3× 300 mL) followed by DCM (3× 200 mL), dried and partitioned following the modified Kupchan liquid-liquid partitioning technique described previously.^[44] The four fractions (WB, FM, FD and FH) were dried and weighed. The FD fraction (0.152 g) was further fractionated on a C-18 SPE using aqueous methanol (25%, 50%, 100% and 100%MeOH with TFA) as the mobile phase yielding two interesting fractions: FD-100%MeOH (80 mg) and FD50%MeOH (52.6 mg) based on ¹H NMR profiles. The fraction FD-100%MeOH was purified on a Sunfire reversed phased column using a gradient solvent system from 80:20 to 0:100% H₂O/MeOH as mobile phase in 30 min to obtain compounds **1** (5.4 mg), **6** (1.3 mg) and **10** (5.8 mg). Using the same gradient system, FD-50%MeOH was purified to yield compounds **5** (0.5 mg), **2** (1.2 mg), **3** (3.2 mg), **4** (4.3 mg) and **8** (0.1 mg). Fractions FM and FB were fractionated by SPE. Using the conditions described for the FD fraction with the WB-25%MeOH purified further by reversed-phase HPLC to yield compounds **7** (1.3 mg) and **9** (2.9 mg).

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Psammaplin O (**5**). Yellowish oil; 0.5 mg; UV (MeOH-H₂O) λ_{\max} (log ϵ) nm, 221 (3.72), 282 (3.14); IR (MeOH cm⁻¹) 3312, 1660, 1538, 1494, 1330, 1144; ¹H, ¹³C, and 2D NMR data (CD₃OD) are given in Table 1, Table S63 and figs. S22-S27. HRESIMS m/z 380.9746 [M+H]⁺ (Fig. S21). calculated for C₁₁H₁₄BrN₂O₆S, 380.9751, Δ = -0.26 ppm. purity of **5** was 97% based on proton NMR baseline peak analysis

Psammaplin P (**6**). Yellowish oil; 1.3 mg; UV (MeOH-H₂O) λ_{\max} (log ϵ) nm, 218 (3.66), 230 (3.73), 281 (3.30); IR (MeOH cm⁻¹) 3197, 2953, 2869, 1691, 1575, 151, 1384, 1296, 1212; ¹H, ¹³C, and 2D NMR data (CD₃OD) are given in Table 1, Table S63 and figs. S30-S35. HRESIMS m/z 494.0065 [M+H]⁺ (figs. S28-S29). calculated for C₁₆H₂₁BrN₃O₆S₂, 494.0050, Δ = -2.8 ppm. Purity of **6** was 95% based on 1H NMR baseline peak analysis

3-Bromo-2-hydroxy-5-(methoxycarbonyl)benzoic acid (**7**). Yellowish oil; 1.3 mg; UV (MeOH-H₂O) λ_{\max} (log ϵ) nm, 227 (3.61), 258 (3.13), 312 (2.79); IR (MeOH cm⁻¹) 3400, 2800, 1703, 1300; ¹H, ¹³C, and 2D NMR data (CD₃OD) are given in Table 1, Table S62 and fig. HRESIMS m/z 274.9542 [M+H]⁺ (Figs. S36-S37). calculated for C₉H₈BrO₅, 274.9550, Δ = -1.81 ppm. Purity of **7** was 97% based on baseline peaks analysis.

β -D-Galactosidase Transgenic Trypanosoma Cruzi in Vitro Assay.

The T. cruzi Tulahuen C2C4 strain, expressing the β galactosidase gene (LacZ) and L6 rat skeletal muscle cells used as host cells were cultured in RPMI-1640 supplemented with 10% iFBS, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μ g/mL streptomycin at 37 °C and 5% CO₂. T. cruzi amastigote infected L6 cell culture (2 × 10³ infected L6 cells per well) were dispensed into 384-well assay plates already containing 5 μ L of the compounds (**1-6, 9-10**). Each compound was tested at least in duplicate using 16 points dose-response curves (1/2 serial dilution), and the starting concentrations were between 31 μ M and 66 μ M. The plates were incubated at 37 °C for 96 h. 1.5 μ L of 100 μ M CRPG and 0.1% NP40 diluted in PBS were subsequently added, and plates were further incubated at 37 °C for 4 h in the dark after which an Envision plate reader (PerkinElmer, Waltham, MA) was used to measure the absorbance at 585nm. The assay was normalised by using the in-plate benznidazole at 10 μ g/mL as negative control and 0.167% DMSO as positive growth control. The method employed in this assay was previously reported by Annang and co-workers.^[62]

Plasmodium Falciparum 3D7 Lactase Dehydrogenase in Vitro Assay.

P. falciparum 3D7 strain parasites were cultured in a freshly type 0 positive (0+) human erythrocyte (Centro Regional de Transfusiones Sanguíneas-Biobanco, Granada) and the preparation for the assay was enabled by using the standard method previously described in 2016 by Pérez-Moreno and co-workers.^[63] The compounds (**1-6, 9-10**) were tested at least in duplicate using a 16 points dose-response curve (1/2 serial dilution) with starting concentrations between 63 and 115 μ M in 384-well plates. Each plate contained a parasite culture medium as a positive growth control and 100 nM of chloroquine as the negative control. The plates were incubated for 72 h after which they were frozen for 4 h and thawed at room temperature for 1h. before LDH activity was measured. To do this, 70 μ L of freshly prepared solution containing 143 mM Sodium L-lactate, 143 μ M APAD, 178.75 μ M NBT, 1 μ g/mL diaphorase, 0.7% Tween 20, and 100 mM Tris-HCL (pH 8.0) were added into the plates. Absorbance was measured at 650 nm after gently shaking the plates to ensure homogeneity and 10 mins incubation at room temperature. The Envision plate reader (Perkin Elmer, Waltham MA) was used to measure the absorbance in this assay.

Supplementary Material

Appendix and Supplementary data related to this article can be found at <http://dx.doi.org/10.1002/MS-number>

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Author Contribution Statement

J.T. and M.J. collected the samples and designed the experiment. E.O. performed extraction, isolation and purification of the compounds. K.S.A. assisted with the extraction process. E.O. and F.R. analysed the MS data and elucidated the structures. F.A. and G.P. performed the biological assays. E.O. and J.T. drafted the initial manuscript, which was edited and approved by all the authors.

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