Antimalarial Bromotyrosine Derivatives from the Australian Marine Sponge *Hyattella* sp.

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A drug discovery program aimed at identifying new antimalarial leads from a prefractionated natural product library has resulted in the identification of a new bromotyrosine alkaloid, psammaplysin G (**1**), along with the previously isolated compound, psammaplysin F (**2**). When tested against two different strains of the parasite *Plasmodium falciparum* (Dd2 and 3D7), **2** displayed IC₅₀ values of 1.4 μ M and 0.87 μ M, respectively, while **1** showed 98% inhibition at 40 μ M against the chloroquine-resistant (Dd2) strain of *P. falciparum*.

Introduction

Malaria is an infectious disease caused by parasites belonging to the genus *Plasmodium*. The latest statistics show that two billion people live in areas at risk from the disease, and annually up to one million people die from malaria infection.¹ Although several drugs are currently available for the treatment of malaria, the emergence of drug-resistant *Plasmodium* strains means that new therapies are urgently needed to treat this devastating disease.² Historically, medicinal plants have played a very important role in combating this disease. The stem bark of *Cinchona succiruba*, known as “fever tree” from South America and the young leaves of *Artemisia annua*, a traditional Chinese plant, commonly known as “Qinghao” have been used to treat malaria for centuries.³ Subsequent chemical investigations of *Cinchona succiruba* and *Artemisia annua*, identified the major active metabolites to be quinine and artemisinin, respectively.⁴ Further research on these two important compounds has led to the development of numerous antimalaria drugs.⁴ However, the prevalence of resistance of the malaria parasite to known drugs and lack of efficacy of some of these drugs, has highlighted the need for the discovery and development of new antimalarial drugs.

As part of our continuing research into the discovery of new antimalarial leads,^{5, 6} we undertook high-throughput screening (HTS) of a prefractionated natural product extract library. From the HTS data we identified one fraction derived from the sponge *Hyattella* sp. (Spongiidae) that showed parasitic growth inhibition in the antimalarial imaging assay, and no cytotoxicity towards a human embryonic kidney cell line (HEK293). Chemical analysis of the active fraction from the prefractionated library identified ion clusters in the (+)-LRESIMS spectrum at m/z 744/746/748/750/752 and 787/789/791/793/795. Mass-directed fractionation on the large-scale organic extract of *Hyattella* sp. resulted in the purification of a new bromotyrosine derivative, psammaplysin G (**1**), along with the previously isolated compound, psammaplysin F (**2**). Herein we report the isolation, structure elucidation of psammaplysin G (**1**) and antimalarial activity for compounds **1** and **2**.

The freeze-dried and ground *Hyattella* sp. was extracted with *n*-hexanes, 80:20

CH₂Cl₂/CH₃OH, and CH₃OH. The CH₂Cl₂/CH₃OH extracts were combined and chromatographed using C₁₈ bonded silica HPLC (MeOH/H₂O/0.1% TFA) to yield psammalyisin F (**2**, 35 mg, 0.350% dry wt) and a less-polar fraction. This later material was further purified by preparative silica TLC (CHCl₃/CH₃OH, 12:1) to yield psammalyisin G (**1**, 1.1 mg, 0.011% dry wt). Compound **2** was determined to be the TFA salt of psammalyisin F following spectroscopic data comparison with literature values.⁷

The TFA salt of psammalyisin G (**1**) was isolated as an optically active gum. The (+)-LRESIMS spectrum of **1** revealed a cluster of ions at m/z 787/789/791/793/795 [M+H]⁺, which indicated the molecule contained four bromine atoms. The molecular formula of **1** was determined to be C₂₃H₂₆Br₄N₄O₇ by (+)-HRESIMS of the [M+Na]⁺ ion at m/z 808.8449. The characteristic maximum absorptions at 219, 255 and 280 nm in the UV spectrum suggested that **1** was a bromotyrosine derivative.⁸ The ¹H NMR and ¹H-¹H COSY spectra of **1** displayed isolated aromatic protons at δ_H 7.54 (2H, s) and 7.28 (1H, s), two mutually coupled protons at δ_H 6.98 (1H, d, J = 7.8 Hz) and 4.93 (1H, dd, J = 7.8 Hz), three mutually coupled methylenes at δ_H 3.96 (2H, t, J = 6.0 Hz), 3.42 (2H, m) and 2.02 (2H, m), due to a -CH₂CH₂CH₂O- moiety, a methoxy group at δ_H 3.56 (3H, s), two geminal protons at δ_H 3.34 (1H, d, J = 16.0 Hz) and 3.04 (1H, d, J = 16.0 Hz), attributed to a methylene adjacent to an asymmetric carbon, a -CH₂CH₂- moiety at δ_H 3.32 (2H, t, J = 7.2 Hz, H-20), 2.68 (2H, t, J = 7.2 Hz, H-19), as well as a *N*-methyl singlet at δ_H 2.73 (3H). Comparison of the ¹H and ¹³C NMR data of **1** with those of psammalyisin F (**2**)⁶ revealed a high degree of structural similarity between the two metabolites. The main spectroscopic differences were that **1** had an MW that was 43 Da larger than **2**, and that **1** contained an additional quaternary carbon at δ_C 159.1, corresponding to a CONH₂ moiety, inferred from the HRESIMS data. The HSQC and HMBC data analysis allowed the construction and confirmation of the psammalyisin skeleton of **1** (Fig. 1). The long range HMBC correlations of 21-CH₃ and H-20 to the quaternary carbon at δ_C 159.1 supported the presence of the *N*-methylurea moiety (-N(CH₃)CONH₂) (Fig. 1). The relative configuration at C-6 and C-7 in psammalyisin A was assigned by X-ray analysis.⁹ This same configuration was

assumed in psammaplysin F because the two compounds had very similar specific rotations (psammaplysin A, $[\alpha]_D^{22} - 65.2$ (0.5, CH₃OH); psammaplysin F, $[\alpha]_D^{25} - 62.3$ (1.2, CH₃OH)).⁶ The C-6 and C-7 relative configuration for psammaplysin G (**1**) was also assigned as the same as that present in psammaplysin A and F, based on comparison of specific rotation data and the key ROESY correlations (Fig. 1). Therefore, the structure of **1** was assigned to psammaplysin G. To the best of our knowledge, psammaplysin G is the first example of a bromotyrosine analogue with a terminal *N*-methylurea moiety.

Compounds **1** and **2** were tested against a chloroquine-resistant (Dd2) *Plasmodium falciparum* strain. Preliminary toxicity towards human cells was investigated using a human embryonic kidney cell line (HEK293). Psammaplysin G (**1**) displayed 98% inhibition at 40 μ M against this strain, and showed no cytotoxicity against the HEK293 cell line up to concentrations of 40 μ M. Due to the Dd2 antimalarial activity of **2**, this natural product was also tested against a chloroquine-sensitive *P. falciparum* strain (3D7). Compound **2** displayed IC₅₀ values of 1.4 μ M (Dd2) and 0.87 μ M (3D7), respectively, and was shown to have an IC₅₀ value of 11 μ M against the HEK293 mammalian cell line.

To date the marine environment has been the source of some interesting antimalarial natural products. Examples include manzamine A,¹⁰⁻¹¹ lepadins D-F,¹² 6-bromoaplysinopsin,¹³ and venturamides A and B.¹⁴ The above listed natural products, in conjunction with these current studies, further support the continued research into marine natural products and their potential application as new antimalarial leads or drugs.

In conclusion, we have isolated a new bromotyrosine alkaloid psammaplysin G (**1**) with a known analogue, psammaplysin F (**2**) from the marine sponge *Hyatella* sp., and shown that psammaplysin F inhibits the growth of two *Plasmodium falciparum* strains (Dd2 and 3D7) with IC₅₀ values of 1.4 μ M and 0.87 μ M, respectively. The terminal *N*-methyl group of psammaplysin F appears to be important and critical for antimalarial activity. However, more analogues are required

for SAR studies. Further chemical investigations on other marine sponges known to contain bromotyrosines are underway.

Experimental Section

General Experimental Procedures. Optical rotations were recorded on a Jasco P-1020 polarimeter. UV and IR spectra were recorded on a Jasco V-650 UV/Vis spectrophotometer, and a Bruker Tensor 27 spectrometer, respectively. NMR spectra were recorded at 30 °C on Varian INOVA 500 and 600 MHz NMR spectrometers. The latter spectrometer was equipped with a triple resonance cold probe. The ^1H and ^{13}C NMR chemical shifts were referenced to the solvent peak for DMSO- d_6 at δ_{H} 2.49 and δ_{C} 39.5. Standard parameters were used for the 2D experiments, which included gradient gCOSY, gHSQC ($^1J_{\text{CH}} = 140$ Hz) and gHMBC ($^nJ_{\text{CH}} = 8.3$ Hz). LRESIMS were recorded on a Waters ZQ mass spectrometer. HRESIMS were measured on a Bruker Daltonics Apex III 4.7e Fourier Transform Mass Spectrometer, fitted with an Apollo API source. A ThermoElectron C₁₈ Betasil 5 μm 143 Å column (21.2 mm $\times$ 150 mm) was used for semi-preparative HPLC. A Waters 600 pump fitted with a 996 Photodiode Array Detector and 717 plus Autosampler was used for the semi-preparative HPLC separations. End-capped Septra C₁₈ bonded silica (Phenomenex) was used for preadsorption work. Silica gel 60 F₂₅₄ precoated Al sheets (0.25 mm) and silica gel 60 GF₂₅₄ plates (1.00 mm) (both Merck) were used for TLC and preparative TLC, respectively. A BIOLINE orbital shaker was used for the large-scale extraction of sponge material. Water was Millipore Milli-Q PF filtered, while all other solvents used were Lab-Scan HPLC grade. Parasite strains 3D7 and Dd2 were from the Queensland Institute of Medical Research. O+ Erythrocytes were obtained from the Australian Red Cross Blood Service. Cell Carrier polylysine coated imaging plates were from PerkinElmer. 4',6-Diamidino-2-phenylindole (DAPI) stain and Alamar Blue were from Invitrogen. Triton-X, saponin, puromycin, and artemisinin were all from Sigma Aldrich. HEK 293 cells were purchased from the American Tissue Culture Collection. The 384-well Falcon sterile tissue culture treated plates were from BD.

Animal Material. The sponge *Hyattella* sp. (Spongiidae) was collected by SCUBA diving (-19 m) at Hervey Bay, Little Woody, Sponge Garden, Queensland, Australia during November of 1995, and kept frozen prior to freeze-drying and extraction. A voucher sample (G306299) has been lodged at the Queensland Museum, Brisbane, Australia. Sponge description: The sponge has a massive, subspherical growth form. It is pale beige alive, pinkish beige on deck, and brownish-grey in EtOH. It contains numerous oscules, and several are at either end on the upper surface. The sponge has an arenaceous, fibrous, firm and compressible texture. The surface ornamentation is arenaceous, conulose, with tangential surface fibres forming distinctive cob-webbed appearance. The ectosomal skeleton is membranous. The choanosomal skeleton is fibrous and arenaceous, fibres look like algal filaments without distinctive arrangement, mesohyl collagen is very thick, and it is cavernous.

Extraction and Isolation. The freeze-dried and ground sponge (10 g) was poured into a conical flask (1 L), *n*-hexane (250 mL) was added and the flask was shaken at 200 rpm for 2 h. The *n*-hexane extract was filtered under gravity then discarded. CH₂Cl₂/CH₃OH (4:1, 250 mL) was added to the de-fatted sponge in the conical flask and shaken at 200 rpm for 2 h. The resulting extract was filtered under gravity, and set aside. CH₃OH (250 mL) was added and the CH₃OH/sponge mixture was shaken for a further 2 h at 200 rpm. Following gravity filtration the biota was extracted with another volume of CH₃OH (250 mL), while being shaken at 200 rpm for 16 h. All CH₂Cl₂/CH₃OH extractions were combined and dried down under reduced pressure to yield a brown solid (0.87 g). This extract was subsequently pre-adsorbed to C₁₈-bonded silica (1.0 g), then packed into a stainless steel cartridge (10 × 30 mm) and attached to a C₁₈ semi-preparative HPLC column. Isocratic HPLC conditions of 90% H₂O (0.1% TFA)/10% CH₃OH (0.1% TFA) were initially employed for the first 10 min, then a linear gradient to CH₃OH (0.1% TFA) was run over 40 min, followed by isocratic conditions of CH₃OH (0.1% TFA) for a further 10 min, all at a flow rate of 9 mL/min with the Betasil C₁₈ 5 µm (21.2 mm x 150 mm i.d.) column. Sixty fractions (60 × 1 min) were collected from

time = 0 min then analysed by (+)-LRESIMS. Fractions 37-39 contained one ion cluster of interest (m/z 744/746/748/750/752) and fractions 40-43 contained the other ion cluster of interest (787/789/791/793/795). Fractions 37-39 were combined and evaporated and to afford psammaplysin F (**2**, 35 mg, 0.350% dry wt). Fractions 23-27 were also combined and evaporated to yield a brown gum (3.5 mg), which was further separated by preparative TLC ($\text{CHCl}_3/\text{CH}_3\text{OH}$ 12 : 1) to yield psammaplysin G (**1**, 1.1 mg, 0.011% dry wt).

TFA Salt of Psammaplysin G (1): isolated as a brown gum; $[\alpha]_{\text{D}}^{25} - 66$ (c 0.1, CH_3OH); UV (CH_3OH) λ_{max} ($\log \epsilon$) 280 sh (3.91), 255 sh (4.03), 219 (4.09) nm; IR ν_{max} (film) 3364, 2926, 2851, 1668, 1652, 1558, 1540, 1458, 1265 cm^{-1} ; ^1H NMR ($\text{DMSO}-d_6$, 600 MHz): δ_{H} 8.72 (1H, m, 9-NH), 7.54 (2H, s, H-15, 17), 7.28 (1H, s, H-1), 6.98 (1H, d, $J = 7.8$ Hz, 7-OH), 4.93 (1H, d, $J = 7.8$ Hz, H-7), 3.96 (2H, t, $J = 6.0$ Hz, H-12), 3.56 (3H, s, 3-OMe), 3.42 (2H, m, H-10), 3.34 (1H, d, $J = 16.0$ Hz, H-5 α), 3.04 (1H, d, $J = 16.0$ Hz, H-5 β), 3.32 (2H, t, $J = 7.2$ Hz, H-20), 2.68 (2H, t, $J = 7.2$ Hz, H-19), 2.73 (3H, s, 21-Me), 2.02 (2H, m, H-11); ^{13}C NMR ($\text{DMSO}-d_6$, 150 MHz): 159.1 (C-22), 158.7 (C-9), 158.6 (C-8), 151.3 (C-13), 148.4 (C-3), 146.2 (C-1), 139.7 (C-16), 133.8 (C-15, 17), 118.1 (C-14, 18), 117.9 (C-6), 103.8 (C-4), 102.2 (C-2), 79.8 (C-7), 71.8 (C-12), 59.3 (3-OMe), 49.8 (C-20), 37.7 (C-5), 37.0 (C-10), 34.9 (C-21), 32.8 (C-19), 30.0 (C-11); (+)-LRESIMS m/z (rel. int.) 787 (12), 789 (56), 791 (100), 793 (62), 795 (27) $[\text{M}+\text{H}]^+$; (+)-HRESIMS m/z 808.8449 $[\text{M}+\text{Na}]^+$ (calcd for $\text{C}_{23}\text{H}_{26}^{79}\text{Br}_4\text{N}_4\text{O}_7\text{Na}$, 808.8427; Δ 2.67 ppm).

Antimalarial Assay. Compounds were incubated in the presence of 2 or 3% parasitemia (3D7 or Dd2) and 0.3% hematocrit in a total assay volume of 50 μL , for 72 h at 37 $^\circ\text{C}$ and 5% CO_2 , in poly-*D*-lysine coated CellCarrier Imaging plates. After incubation plates were stained with DAPI 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 (PerkinElmer). The digital images obtained were analyzed using the Perkin Elmer Acapella spot detection software where fluorescent spots which fulfill the criteria established for a stained parasite are counted. The percent inhibition of

parasite replication was calculated using DMSO and 2 μ M artemisinin control data. Artemisinin [IC_{50} = 0.021 μ M (Dd2); IC_{50} = 0.021 μ M (3D7)] and chloroquine [IC_{50} = 0.130 μ M (Dd2); IC_{50} = 0.025 μ M (3D7)] were used as positive controls.

Cytotoxicity Assay. Compounds were added to Falcon 384 well black/clear tissue treated assay plates containing 3000 adherent cells/well (HEK293) in an assay volume of 45 μ L. The plates were incubated for 72 h at 37 $^{\circ}$ C and 5% CO_2 . After incubation the supernatant was aspirated out of the wells and 40 μ L 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 using a VICTOR II (PerkinElmer, Waltham, MA, USA). The percent inhibition of cell growth was calculated using DMSO and 10 μ M puromycin control data. IC_{50} values were obtained by plotting % inhibition against log dose using Prism4 graphing package and non-linear regression with variable slope plot.

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Supporting Information Available: A photo of the sponge and NMR spectra for psammaphysin G (1). This material is available free of charge via the Internet at <http://pubs.acs.org>.

References and Notes

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TOC Graphic

- 1 R=CONH₂ *P. falciparum* 98% @ 40 μM (Dd2)
 2 R=H *P. falciparum* IC₅₀ 1.4 μM (Dd2); 0.87 μM (3D7)