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Antimalarial Peptides from Marine Cyanobacteria: Isolation and Structural Elucidation of Gallinamide A

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Abstract

As part of a continuing program to identify novel treatments for neglected parasitic diseases the Panama International Cooperative Biodiversity Group (ICBG) program has been investigating the antimalarial potential of secondary metabolites from Panamanian marine cyanobacteria. From over 60 strains of cyanobacteria evaluated in our biological screens, the organic extract of a *Schizothrix* species from a tropical reef near Piedras Gallinas (Caribbean coast of Panama) showed potent initial antimalarial activity against the W2 chloroquine resistant strain of *Plasmodium falciparum*. Bioassay guided fractionation followed by 2D NMR analysis afforded the planar structure of a new and highly functionalized linear peptide, gallinamide A. Subsequent degradation and derivatization methods were used to determine the absolute configuration at most chiral centers in this unusual new metabolite.

Antimalarial therapy continues to suffer from a lack of international attention in comparison to diseases of developed countries such as cancer and heart disease. The for-profit nature of most commercial drug discovery programs precludes malaria as a viable therapeutic target because the majority of the victims of malaria live in countries with very low per capita incomes.¹ By contrast, academic research efforts are driven by different motivations and can thus investigate and hopefully contribute important lead molecules for treatment of this disease.² In line with this concept the Panama ICBG program is exploring the natural products diversity of plants, endophytic fungi and marine cyanobacteria in the search for new antimalarial drug leads using an assay against *Plasmodium falciparum*.^{3,4}

While many of the current frontline antimalarial drugs are originally of plant origin (e.g. quinine, artemisinin), there has been relatively little investigation of the contributions that marine natural products may play in the discovery of lead compounds against malaria.^{5–11}

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Supporting Information Available. ¹H and ¹³C NMR spectra of **1**, interpretation of ROESY correlations for the Apa subunit, complete biological screening data and a color photograph of the field collected *Schizothrix* sp. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Given the level of success that has been achieved from terrestrial natural products and the tremendous untapped biosynthetic resource that the marine environment represents, we hypothesize that antimalarial drug discovery from marine organisms has the potential to produce potent and selective lead compounds. As an extension of this hypothesis, we are more broadly evaluating cyanobacterial extracts against a panel of tropical parasites, including malaria, leishmaniasis, Chagas disease, and dengue fever, as well as for cytotoxicity to cancer cells. Initial hits are prioritized by their profile of activities across this panel of screens prior to further chemical and biological investigation.

During a field expedition to the Portobelo Marine Park on the Caribbean coast of Panama, a small quantity of a red-tipped *Schizothrix* sp. was collected by hand using SCUBA from a shallow reef near Piedras Gallinas. The organic extract from this material (556 mg) was subjected to our standard prefractionation strategy as described previously.⁴ These fractions were screened against a panel of tropical parasites including the W2 chloroquine-resistant strain of *Plasmodium falciparum*. The fraction eluting with 2:8 hexanes/EtOAc showed activity against the malaria parasite ($IC_{50} = 1 \mu\text{g/mL}$) and was thus evaluated by HPLC-MS and subsequently fractionated by HPLC to give 3.4 mg of compound **1** as a clear glass, which was given the common name gallinamide A. Pure gallinamide A was evaluated in the *P. falciparum* assay and shown to possess moderate antimalarial activity ($IC_{50} = 5.0 \mu\text{g/mL}$).

The HRESITOFMS spectrum of gallinamide A (**1**) gave an $[M+H]^+$ ion at m/z 593.3907 that was consistent with the pseudomolecular formula $C_{31}H_{53}N_4O_7$ (calcd for $C_{31}H_{53}N_4O_7$, 593.3909). ^1H and ^{13}C NMR analysis of metabolite **1** showed that it was peptidic in nature; however, there were several resonances that were not attributable to the common ribosomally-encoded amino acids, implying that **1** possessed a highly functionalized and interesting structure. The presence of a sharp singlet proton resonance (δ_H 2.25, δ_C 41.7) that integrated for 6 protons and showed self-correlation in the HMBC spectrum strongly suggested that **1** possessed an *N,N*-dimethyl terminal residue, a motif with significant precedence in cyanobacterial natural products chemistry.¹² Interpretation of the 2D NMR data confirmed this assignment and identified the presence of an *N,N*-dimethyl isoleucine residue (subunit **a**, figure 1). Standard 2D NMR analyses using a combination of COSY, HSQC, HMBC and TOCSY experiments identified the presence of an isocaproic acid moiety (subunit **b**, figure 1). Consideration of the multiplicity edited HSQC experiment revealed two broad doublets not attached to carbon that integrated for one proton each (δ_H 6.70, 6.77). By COSY, the resonance at δ_H 6.77 could be attributed to the amide NH proton of a leucine residue (subunit **c**, figure 1). The second exchangeable resonance at δ_H 6.70 gave a COSY correlation to a multiplet at δ_H 4.55 which in turn showed COSY correlations to a methyl doublet at δ_H 1.22 and a doublet of doublets at δ_H 6.88. Finally this spin system was completed by a COSY correlation from δ_H 6.88 to a sharp doublet at δ_H 7.33. Interpretation of these connectivities in conjunction with HMBC information allowed assignment of this subunit as 4-(*S*)-amino-2-(*E*)-pentenoic acid (Apa), as depicted in subunit **d** of figure 1. The geometry for the olefin in subunit **d** was determined by coupling constant analysis of the olefinic protons at δ_H 6.88 and 7.33 ($3J_{HH} = 15.7 \text{ Hz}$), which was consistent with *E* geometry. This was confirmed by ROESY correlations from Apa-5 to both olefinic protons (Apa-2 and Apa-3), which is only possible for the *E* configuration (for the *Z* isomer, the Apa-2 to Apa-5 distance is greater than 5 Å for all possible conformations, see Supporting Information).

Consideration of the molecular formula by subtracting the atoms comprising subunits **a** – **d** identified the remaining subunit (**e**) to be of $C_6H_8NO_2$ composition. The presence of a quaternary carbonyl carbon at δ 170.8, a methine carbon at δ 93.7 and a quaternary carbon at δ 182.1 were strongly indicative of a trisubstituted β -oxygenated α,β -unsaturated amide-type carbonyl group. HMBC correlations from a methyl doublet at δ 1.42 (Mmp-5) and a methine quartet at δ 4.56 (Mmp-4) to the quaternary carbon at δ 182.1 suggested the presence of a

modified alanine residue, and an additional HMBC correlation from a methyl singlet at δ 3.85 to the quaternary carbon at δ 182.1 provided evidence for the presence of a methoxy group attached to this latter quaternary center. Consideration of the remaining atoms of this subunit required the presence of a nitrogen atom which could only be installed between carbons Mpp-1 and Mpp-4 to form a methyl-methoxypyrrolinone moiety (Mmp) as the C-terminal residue of gallinamide A (**1**).

Subunits **a** – **e** were connected using a combination of HMBC correlations from the α -protons and amide NH resonances to the amide carbonyls and ROESY correlations between α -protons and amide NH resonances to give the assembled planar structure of **1** as depicted in figure 1.

Stereoanalysis of **1** was accomplished using two complimentary approaches. Determination of the configuration for subunits **c**, **d** and **e** was achieved by oxidative ozonolysis followed by acid hydrolysis and Marfey's analysis which showed the exclusive presence of L-alanine and L-leucine. Configuration of subunit **b** was determined by sequential basic and acidic hydrolyses followed by chiral HPLC and comparison with commercially available standards of *R*- and *S*-isocaproic acid. Co-injections showed **1** to contain exclusively *S*-isocaproic acid. Due to lack of material a similar analytical approach was not successful in determining the configuration of subunit **a** despite exploring numerous conditions; however, based on the precedent that all marine natural products containing an *N,N*-dimethyl terminal amino acid residue possess the L configuration at this center^{13–21} (e.g. belamide A (**2**)) and that all but one of these have been shown to derive from cyanobacteria, it is likely that the stereoconfiguration at this position in **1** is also L.

Gallinamide A (**1**) was tested for its antimalarial activity against the W2 chloroquine-resistant strain of the malaria parasite. Compound **1** showed moderate *in vitro* activity against *Plasmodium falciparum* (IC_{50} = 8.4 μ M), cytotoxicity to mammalian Vero cells (TC_{50} = 10.4 μ M) and activity against *Leishmania donovani* of (IC_{50} = 9.3 μ M). Compound **1** was inactive up to the highest tested concentrations against *Trypanosoma cruzi* (16.9 μ M) (for positive controls and activities for all bioassays see supporting information).

Marine cyanobacteria are proving a valuable source of antimalarial lead compounds of diverse structure types, including alkylated phenols, alkaloids, cyclic peptides,⁴ and linear peptides.³ Of these, gallinamide A most closely resembles the structures of the linear peptides dolastatin 10 (**3**) and 15 (**4**) which have been shown to exhibit both antimalarial and mammalian cell antiproliferative effects.²² Intriguingly, despite showing moderate cytotoxicity to Vero cells (TC_{50} = 10.4 μ M) gallinamide A shows no *in vitro* cytotoxicity toward NCI-H460 human lung tumor and neuro-2a mouse neuroblastoma cell lines up to the highest tested concentrations (16.9 μ M). This is in stark contrast to dolastatin 15 (**4**) which exhibits both nanomolar antimalarial activity and a TC_{50} against the NCI-H460 human lung tumor cell line of 2.4 ± 1.2 nM. In addition we have recently found symplostatin 1,²³ a close analog to dolastatin 10, to be active in our Panama-based antimalarial assays (data not shown). Thus, it appears that linear peptides with terminating *N,N*-dimethyl-valine or *N,N*-dimethyl-isoleucine groups are a class of therapeutic agents which exert antiparasitic as well as antiproliferative activities. In the case of gallinamide A, activity against both *P. falciparum* and human host cells is significantly lower in potency than either dolastatin 15 or symplostatin 1. One possible rationalization of this observation is that these compounds work by fundamentally different modes of action; however the number of antimalarial lead compounds isolated by our program from the marine environment which possess dolastatin-like structural motifs suggests that this class of compounds share a degree of commonality in their interactions with *Plasmodium falciparum*. As previously reported by Fennell *et al.*²² dolastatins 10, 15 and synthetic analogs exhibited varying relative levels of activity against mammalian cells and *P. falciparum*, however the study failed to identify any compounds with greater potency against *P.*

falciparum than host cells.²² By contrast, gallinamide A is a reasonably effective antimalarial whose relative potency against parasites (8–10 μ M) versus mammalian cells (generally > 17 μ M) suggests that this structural framework could be an attractive foundation for further SAR investigations.

The isolation of gallinamide A (**1**) represents an important addition to the current understanding of cyanobacterial secondary metabolite chemistry.^{12,24–26} Compound **1** contains the unusual 4-(*S*)-amino-2-(*E*)-pentenoic acid subunit that has little precedent in nature^{27–29} and has never been previously isolated from the marine environment. The presence of a methyl-methoxypyrrolinone moiety at the *C*-terminus is also relatively unusual, with only a handful of natural products containing this subunit having been reported.^{30–32} It is also intriguing to recognize a tandem repeat of ketide extended alanine residues (residues 4 and 5) in gallinamide A, the first of which is reduced and dehydrated from its intermediate β -carbonyl, and the second is enolyzed, *O*-methylated and then cyclized to the pyrrolinone system. It may be that a gene duplication event was involved in the creation of these tandem NRPS-PKS modules. The inclusion of structural motifs from several cyanobacterial natural products in a single compound makes gallinamide an interesting discovery from a biosynthetic perspective whose origins it would be intriguing to investigate.

Experimental Section

General Experimental Procedures

Optical rotations were measured with a Rudolf Research Analytical Autopol II polarimeter. UV spectra were acquired on a Shimadzu UV2401-PC spectrophotometer. IR spectra were obtained on a Thermo Electron Nicolet IR100 spectrophotometer. NMR spectra were acquired on a JEOL Eclipse 400 MHz spectrometer and referenced to residual solvent proton and carbon signals (δ_{H} 1.94, δ_{C} 118.2 for CD_3CN). Low resolution APCI mass spectra were acquired on a JEOL LC-mate mass spectrometer (INDICASAT). Accurate mass ESI mass spectra were acquired on an Agilent ESI-TOF mass spectrometer (The Scripps Research Institute). HPLC purifications were performed on an Agilent 1100 series HPLC system employing a G1312A binary gradient pump, a G1322A degasser, a G1314A variable wavelength detector tuned to 210 nm with a Phenomenex Jupiter C_{18} (4.6 $\times$ 250 mm) RP-HPLC column. All solvents were HPLC grade and were used without further purification.

Collection

The cyanobacterium *Schizothrix* sp. (44.3 g dry wt) was collected by hand using SCUBA from a depth of 12–15 m near Piedras Gallinas (09° 33.799' N 79° 41.642' W) in the Portobelo National Marine Park, Colon Province on the North coast of Panama. The cyanobacterium was strained through a mesh bag to remove excess seawater, frozen on site, and stored at -4°C until workup. The taxonomy was identified by comparison with characteristics described by Geitler. A voucher was deposited at the Smithsonian Tropical Research Institute, Panama (voucher number PAP-04-OCT-05-2).

Extraction and Isolation

Freshly thawed material was extracted exhaustively with $\text{CH}_2\text{Cl}_2/\text{MeOH}$ (2:1, 6 $\times$ 500 mL) and the combined organic extracts partitioned against H_2O (300 mL) and concentrated to dryness *in vacuo* to give 556 mg of a dark brown gum. This material was subjected to flash Si gel CC (Aldrich, Si gel 60, 230–400 mesh, 40 $\times$ 180 mm) eluting with 100% hexanes (300 mL); 9:1 hexanes/EtOAc (300 mL); 8:2 hexanes/EtOAc (300 mL); 6:4 hexanes/EtOAc (300 mL); 4:6 hexanes/EtOAc (300 mL); 2:8 hexanes/EtOAc (300 mL); 100% EtOAc (300 mL); 3:1 EtOAc/MeOH (300 mL); 100% MeOH (300 mL). The fraction eluting with 2:8 hexanes/EtOAc showed strong antimalarial activity (1 $\mu\text{g/mL}$) and so was passed through a C_{18} SPE

cartridge coupled to a 0.22 μm nylon filter eluting with 100% MeOH. The eluent was concentrated in vacuo and the resulting brown gum subjected to C18 RP-HPLC (Phenomenex Jupiter C18 4.6 $\times$ 250 mm RP-HPLC column, 5 μm , 67% MeOH/33% H₂O, 210 nm, 1 mL/min, t_{R} 32.6 min) to give **1** as a colorless glass (3.4 mg, 0.6% of crude extract).

Gallinamide A (1)

colorless glass; $[\alpha]_{\text{D}}^{25} -22.5$ (c 0.001, MeOH); UV (MeOH) λ_{max} (log ϵ) 218 (3.95), 247 (3.92) nm; IR (CH₂Cl₂) γ_{max} 3282, 2960, 1731, 1653, 1622 cm⁻¹; for ¹H and ¹³C NMR data, see Table 1; HRESIMS m/z [M+H]⁺ 593.3907 (calcd for C₃₁H₅₃N₄O₇, 593.3909).

Ozonolysis of 1

A stream of ozone gas (4% O₃ in O₂, 0.0625 L/min) was bubbled through a solution of **1** (0.1 mg in 200 μL CH₂Cl₂) at room temperature for 5 min. The solvent was removed under N₂ and dried *in vacuo* for one hour. Subsequent hydrolysis and Marfey's analysis of the corresponding ozonate was performed as described below.

Marfey's Analysis of 1

The ozonate of **1** (0.1 mg, 0.2 μmol) was treated with 6N HCl in a sealed vial at 120°C for 18 h. The solution were concentrated to dryness *in vacuo* and treated with a solution of 1-fluoro-2,4-dinitrophenyl-5-L-valine-amide (FDVA) (0.25 mg, 0.8 μmol) in acetone (50 μL) and a solution of 0.1 M NaHCO₃ (100 μL) in a sealed vial at 90°C for 5 min. The reaction mixture was neutralized with 2N HCl (50 μL) and diluted with CH₃CN (100 μL). The resulting solution was analyzed by RP-HPLC employing a HP LiChrospher 100 RP-18 (5 μm , 4 $\times$ 125 mm) column and a gradient elution profile of 15% CH₃CN/85% H₂O (acidified with 0.05% HCOOH) to 50% CH₃CN/50% H₂O (acidified with 0.05% HCOOH) over 45 min at a flow of 0.8 mL/min, monitoring at 340 nm. Comparison with commercially available amino acid standards derivatized using identical methodology established **1** as containing exclusively L-alanine (17.8 min) and L-leucine (25.2 min). Retention times in min for the derivatized amino acid standards were as follows: L-alanine 17.8; D-alanine 21.6; L-leucine 25.2; D-leucine 31.3.

Chiral HPLC analysis of 1

An authentic sample of **1** (0.1 mg, 0.2 μmol) was treated with 1M NaOH-MeOH (1:1) for 18 h at 25 °C, concentrated to dryness *in vacuo* and then treated with 6N HCl in a sealed vial at 120 °C for 18 h. The resulting hydrolysate was analyzed by chiral HPLC employing a Phenomenex Chirex-D column (4.6 $\times$ 50 mm) eluting with 85% 2 mM CuSO₄, 15% MeCN at a flow of 0.8 mL/min, monitoring at 228 nm. Comparison with commercially available standards established **1** as containing exclusively *S*-isocaproic acid (6.4 min). The identity of *S*-isocaproic acid in **1** was confirmed by co-injection. Retention times in minutes for commercially available standards were as follows: *S*-isocaproic acid 6.4; *R*-isocaproic acid 8.1.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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1

2

3

4

Figure 1.
Subunits **a** – **e** and selected ROESY correlations for **1**.

Table 1NMR data for gallinamide A (**1**) (CD₃CN).

Residue	Position	δ_H^a	Mult. (Hz)	δ_C^b
<i>N,N</i> -diMe-Ile	1	-	-	171.8, qC
	2	2.91	d, 10.6	72.9, CH
	3	1.80	m	34.1, CH
	4	1.12, 1.65	m	25.6, CH ₂
			m	
	5	0.87	d, 7.7	10.6, CH ₃
	6	0.82	d, 7.0	16.2, CH ₃
	NMe	2.25	s	41.7, CH ₃
Ica	1	-	-	171.1, qC
	2	4.99	m	73.2, CH
	3	1.52, 1.73	m	41.6, CH ₂
			m	
	4	1.66	m	23.4, CH
	5	0.92 ^c		25.3 ^c , CH ₃
	5'	0.94 ^c		25.6 ^c , CH ₃
Leu	1	-	-	172.1, qC
	2	4.33	dd, 8.0, 14.6	52.3, CH
	3	1.56, 1.60	m m	42.0, CH ₂
			m	
	4	1.65	m	25.5, CH
	5	0.92	d, 7.0	21.8, CH ₃
	5'	0.92	d, 7.0	21.9, CH ₃
	NH	6.77	d, 8.1	-
Apa	1	-	-	164.8, qC
	2	7.33	dd, 1.8, 15.7	123.0, CH
	3	6.88	dd, 5.1, 15.7	149.4, CH
	4	4.55	m	46.9, CH
	5	1.22	d, 7.0	20.1, CH ₃
	NH	6.70	d, 7.7	-
Mmp	1	-	-	170.8, qC
	2	5.09	s	93.7, CH
	3	-	-	182.1, qC
	4	4.56	m	56.4, CH
	5	1.42	d, 6.6	17.3, CH ₃
	OMe	3.85	s	59.6, CH ₃

^aRecorded at 400 MHz.^bRecorded at 100 MHz.

^cResonances interchangeable.