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

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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 stereogenic 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 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.

Results and Discussion

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 protocol.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 (IC50 = 1 µg/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 (IC50 = 8.4 µM).

The HRESITOFMS spectrum of gallinamide A (1) gave an [M + H]+ ion at m/z 593.3907 that was consistent with the pseudo-molecular formula C31H53N4O7 (calcld for C31H53N4O7, 593.3909), 1H and 13C NMR analysis 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 (δH 6.70, 6.77). By 2D NMR data confirmed this assignment and identified the presence of 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-dimethylisoleucine 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 (δH 6.70, 6.77). By COSY, the resonance at δH 6.77 could be attributed to the amide NH proton of a leucine residue (subunit c, Figure 1). The second exchangeable resonance at δH 6.70 gave a COSY correlation to a multiplet at δH 4.55, which in turn showed COSY correlations to a methyl doublet at δH 1.22 and a doublet of doublets at δH 6.88. Finally, this spin system was completed by a COSY correlation from δH 6.88 to a sharp doublet at δH 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 E geometry for the olefin in subunit d was determined by coupling constant analysis
Consideration of the molecular formula by subtracting the atoms comprising subunits a–d identified the remaining subunit (e) to be of C₁₈H₂₀N₂O₄ 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 was 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 methylmethoxypyrrolinone 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 complementary 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. The 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 configuration 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 (IC₅₀ = 8.4 µM), cytotoxicity to mammalian Vero cells (TC₅₀ = 10.4 µM), and activity against Leishmania donovani of (IC₅₀ = 9.3 µM). Compound 1 was inactive up to the highest tested concentrations against Trypanosoma cruzi (16.9 µM) (for positive controls and activities for all bioassays see the Supporting Information).

Marine cyanobacteria are proving a valuable source of antimalarial lead compounds of diverse structure types, including alkylated phenols, alkaloids, and cyclic 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 (TC₅₀ = 10.4 µM) 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 µM). This is in stark contrast to dolastatin 15 (4), which exhibits both nanomolar antimalarial activity and a TC₅₀ against the NCI-H460 human lung tumor cell line of 2.4 ± 1.2 nM. In addition we have recently found symplostatin 1, a close analogue 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-dimethylvaline or N,N-dimethylisoleucine groups are a class of therapeutic agents that 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 that possess dolastatin-like structural motifs suggests that this class of compounds share a degree of...
commonality in their interactions with *P. falciparum*. As previously reported by Fennell et al., 22 donolastins 10 and 15 and synthetic analogues 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 µM) versus mammalian cells (generally >17 µM) 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. 1,2,4,24–26 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-thiophyryrrolinone 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 enolized, 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 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 IR1000 spectrophotometer. NMR spectra were acquired on a JEOL Eclipse 400 MHz spectrometer and referenced to residual solvent proton and carbon signals (δH 1.94, δC 118.2 for CD3CN). Low-resolution APCl mass spectra were acquired on a JEOL LC-mate mass spectrometer (INDI-CASAT). Accurate mass ESI mass spectra were acquired on an Agilent 11000 series HPLC system employing a G1312A binary gradient pump, a G1322A degasser, and a G1314A variable wavelength detector tuned to 210 nm with a Phenomenex Jupiter C18 (4.6 × 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 identified by comparison with characteristics described by Geitler. 33 A voucher was deposited at the Smithsonian Tropical Research Institute, Panama (voucher number PAP-04-OCT-05).

**Extraction and Isolation.** Freshly thawed material was extracted exhaustively with CH2Cl2/MeOH (2:1, 5 × 500 mL), and the combined organic extracts were partitioned against H2O (300 mL) and concentrated to dryness in *vacuo* to give 556 mg of a dark brown gum. This was confirmed by chiral HPLC employing a Phenomenex Chirex-D column (4.6 × 250 mm RP-HPLC column, 5 µm, 67% MeOH/33% H2O, 210 nm, 1 mL/min, τ = 32.6 min) to give 1 as a colorless glass (3.4 mg, 0.6% of crude extract).

**Table 1. NMR Data for Gallinamide A (1) (CD3CN)**

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<th>residue</th>
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<th>mult. (Hz)</th>
<th>δC (ppm)</th>
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<td>4</td>
<td>1.12, 1.65 m</td>
<td>25.6, CH2</td>
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<tr>
<td>5</td>
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<td>41.7, CH1</td>
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Table 1. NMR Data for Gallinamide A (1) (CD3CN)

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<thead>
<tr>
<th>residue</th>
<th>position</th>
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<tr>
<td>NH</td>
<td>6.77 d</td>
<td>8.1</td>
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| Apa | 1 | 7.33 dd, 8.0, 14.6 | 52.3, 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, CH1 |
| NH | 6.70 d | 7.7 |

| Mmp | 1 | 5.09 s | 93.7, CH |
| 3 | 5.05 s | 182.1, CH |
| 4 | 4.56 m | 56.4, CH |
| 5 | 1.42 d, 6.6 | 17.3, CH1 |
| OMe | 3.85 s | 59.6, CH |

a Recorded at 400 MHz. b Recorded at 100 MHz. c Resonances interchangeable.

**Gallinamide A (1):** colorless glass; [α]D20 = −22.5 (c 0.001, MeOH); UV (MeOH) λmax (log ε) = 218 (3.95), 247 (3.92) nm; IR (CHCl3) νmax 3282, 2960, 1731, 1653, 1622 cm−1; for H and 13C NMR data, see Table 1; HREIMS m/z [M + H]+ 593.3907 (calcld 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 1 h. 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 6 N HCl in a sealed vial at 120°C for 18 h. The solution was 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 sealed vial at 90°C for 5 min. The reaction mixture was neutralized with 2 N HCl (50 µL) and diluted with CH2CN (100 µL). The resulting solution was analyzed by RP-HPLC employing a HP LiChrospher 100 RP-18 (5 µm, 4 × 125 mm) column and a gradient elution profile of 15% CH2CN/85% H2O (acidified with 0.05% HCOOH) to 50% CH2CN/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 minutes 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 1 M NaOH/MeOH (1:1) for 18 h at 25°C, concentrated to dryness in *vacuo*, and then treated with 6 N 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 × 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-isosarcosic acid (6.4 min). The identity of S-isosarcosic acid in 1 was confirmed by co-injection. Retention times in minutes for commercially available standards were as follows: S-isosarcosic acid 6.4; R-isosarcosic acid 8.1.
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Supporting Information Available: 1H and 13C NMR spectra of I, 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.

References and Notes

(33) Geitler, L. Cyanophyceae; Koeltz Scientific Books: Koenigstein, Germany, 1932.

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