

Synthesis of Gallinamide A Analogues as Potent Falcipain Inhibitors and Antimalarials

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ABSTRACT: Analogues of the natural product gallinamide A were prepared to elucidate novel inhibitors of the falcipain cysteine proteases. Analogues exhibited potent inhibition of falcipain-2 (FP-2) and falcipain-3 (FP-3) and of the development of *Plasmodium falciparum* *in vitro*. Several compounds were equipotent to chloroquine as inhibitors of the 3D7 strain of *P. falciparum* and maintained potent activity against the chloroquine-resistant Dd2 parasite. These compounds serve as promising leads for the development of novel antimalarial agents.

INTRODUCTION

Malaria, a mosquito-borne disease caused by infection with *Plasmodium* parasites, is the world's most deadly parasitic infection.¹ Almost half the world's population live in malaria endemic areas, and an estimated 1.2 billion people are at high risk of contracting the disease.² This has resulted in hundreds of millions of *P. falciparum* infections each year, causing hundreds of thousands of deaths, primarily in children.² Nearly all of these deaths are caused by *Plasmodium falciparum*, the most virulent human malaria parasite.² Unfortunately, the introduction of a highly effective vaccine against malaria has remained elusive³ and, as a consequence, chemotherapy remains central to control and treatment.⁴ Natural products and their derivatives, including quinine, chloroquine (CQ) and artemisinin and its analogues, have led the way as antimalarial drugs used clinically.^{5,6} However, the control of malaria has been severely compromised in recent years by the widespread resistance of *P. falciparum* to nearly all frontline therapeutics used for both prophylaxis and treatment.⁷ Of growing concern is recently discovered resistance to components of artemisinin-based combination therapies,⁸ the cornerstone of treatment of falciparum malaria.⁹ Consequently, there is an urgent need for the development of new antimalarials that are structurally distinct from existing drugs and operate through novel mechanisms of action.¹⁰

We have an interest in utilizing the privileged biological activity of natural products to elucidate new antimalarial drug leads. In this area we have recently reported the efficient total synthesis¹¹ and stereochemical assignment of the N-terminal isoleucine residue¹² of gallinamide A (also known as symprostatin 4) a depsipeptide natural product that has been isolated independently from a *Schizothrix* species of cyanobacteria from the Caribbean Coast of Panama¹³ and from the *Symploca* genus in Key Largo, Florida¹⁴ (Figure 1). Through our synthetic efforts we have demonstrated that the natural product exhibits potent activity *in vitro* against cultured *P. falciparum*, with an IC₅₀ of 50 nM. Importantly, gallinamide A did not exhibit

hemolytic activity against red blood cells,¹¹ did not inhibit the proteasome and displayed weak or no detectable activity against mammalian Vero cells, NCI-H460 lung tumor cells, or neuro-2a mouse neuroblastoma cell lines.^{13,14}

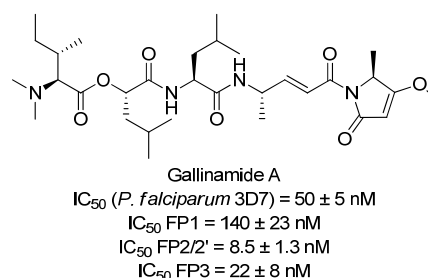


Figure 1. Structure of gallinamide A and inhibitory activity against *P. falciparum*¹¹ and the falcipains (FPs).¹⁵

Recently, the putative mode of antimalarial action of gallinamide A has been revealed in a study by Stolze *et al.*¹⁵ Specifically, the natural product has been shown to inhibit a group of cysteine proteases found in the food vacuole of the parasite, known as the falcipains (FPs). *P. falciparum* is known to possess four falcipains, FP1, FP2, FP2' and FP3, with the last three located in the food vacuole of erythrocytic parasites.¹⁶ All three food vacuole-associated FPs (FP2, FP2' and FP3) were inhibited by gallinamide A at low to mid-nanomolar concentrations. The food vacuole FPs are required for the degradation of hemoglobin and are essential for growth and survival of the organism.¹⁶ Treatment of cultured *P. falciparum* with gallinamide A leads to swelling of the food vacuole, which fills with undegraded hemoglobin; if not halted, falcipain inhibition leads to parasite death.^{15,17-19} Over the past decade several classes of FP inhibitors have been developed,^{17,20-28} some of which have shown efficacy in *in vivo* models of malaria,^{27,29} but no falcipain inhibitors have yet progressed into human clinical trials.³⁰

Gallinamide A possesses a number of unique structural fea-

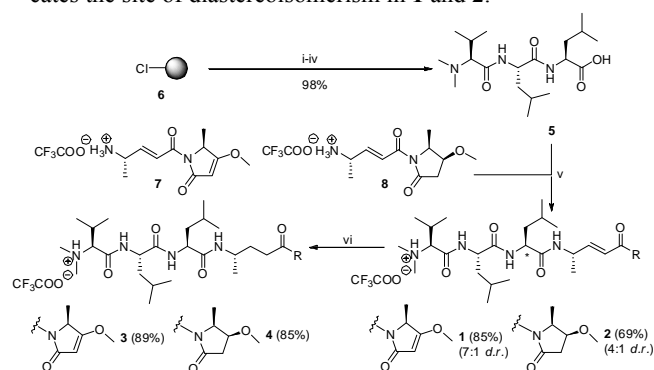
tures, including a dimethyl-terminated aliphatic depsipeptide backbone, an unusual 4(*S*)-amino-2(*E*)-pentenoyl moiety and a C-terminal *N*-acylpyrrolinone unit. Based on these features, it is likely that gallinamide A is a covalent, irreversible inhibitor of the FPs *via* nucleophilic attack by the sulfhydryl side chain of the active site cysteine of the FPs onto one of the two Michael acceptor moieties of the natural product. This hypothesis is supported by a recent study showing that gallinamide A potently inhibits cathepsin L through a covalent, irreversible mechanism.³¹ Given the potent anti-plasmodial activity of gallinamide A, coupled with its general lack of toxicity against human cell lines, we envisaged the development of structurally unique gallinamide A analogues as inhibitors of the food vacuole FPs, which may serve as antimalarial drug leads. We proposed that the C-terminal pyrrolinone moiety and *N*-terminal region of the natural product would be amenable to significant structural change, thus providing scope for dramatic alteration and simplification of the structure to provide the first structure-activity data for this class of natural products.

RESULTS AND DISCUSSION

We were first interested in assessing whether one or both of the olefinic moieties in gallinamide A were crucial for inhibitory activity by serving as Michael acceptors for the active site Cys residue of the FPs. Thus we initially designed and synthesized four analogues of gallinamide A with varied degrees of saturation. This included compound **1** that is structurally identical to gallinamide A, except that the native ester linkage in the depsipeptide natural product had been replaced with an amide bond. We envisioned that this linkage could be formed *en bloc* from commercially available amino acids, negating the use of synthetically challenging preformed amino ester building blocks (as was required for the total synthesis of the natural product^{11,12}). The other modification was the incorporation of a dimethylated valine (Val) residue, which we had previously shown to be an excellent replacement for the *N*-terminal dimethylated isoleucine (Ile) moiety in the natural product (see Supporting Information). The three other proposed analogues were **2**, where the methoxy-enol moiety in the pyrrolinone ring of **1** was reduced, **3** where the olefinic component of the 4(*S*)-amino-2(*E*)-pentenoic acid unit was reduced, and the completely saturated analogue **4**. Preparation of the proposed analogues began with the synthesis of *N*-terminal fragment **5** *via* Fmoc-strategy SPPS. 2-Chloro-Trt-Cl resin (**6**) was first loaded with Fmoc-Leu-OH followed by coupling of Fmoc-Leu-OH and Fmoc-Val-OH. On-resin reductive amination followed by cleavage from the resin using hexafluoroisopropanol (HFIP) provided tripeptide **5** in excellent yield. From here, **5** was coupled to imide fragments **7** and **8**, which were prepared using a similar protocol to that adopted for the total synthesis of gallinamide A (see Supporting Information for synthetic details). The coupling was carried out using HATU at low temperature to minimize epimerization and afforded analogues **1** and **2**, primarily as single diastereoisomers, in good yields. At this stage **1** and **2** were subjected to hydrogenation to provide **3** and **4** in 85% and 89% yields, respectively, following HPLC purification, which also enabled separation of the diastereoisomers of these compounds.

Having prepared the four target gallinamide A analogues, the compounds were next screened against FP-2 and FP-3 using a fluorescence-based kinetic assay.²⁶ The compounds were also

screened against the chloroquine sensitive 3D7 strain of *P. falciparum* using a [³H]-hypoxanthine incorporation assay (Table 1).^{11,12,16} Gallinamide A analogue **1** exhibited potent inhibitory activity against FP-2 (IC₅₀ = 6.78 nM), FP-3 (IC₅₀ = 292 nM) and *P. falciparum* *in vitro* (IC₅₀ = 89.3 nM). Interestingly, while **1** was equipotent to gallinamide A against FP-2, the compound exhibited 10-fold weaker inhibition against FP-3 and an almost 2-fold drop in activity against *P. falciparum*. Reduction of the enol moiety in the acyl-pyrrolinone unit in **2** led to a slight improvement in activity against FP-2 and FP-3, but a two-fold reduction in anti-plasmodial activity (IC₅₀ = 210 nM). In contrast, reduction of the olefin in the α,β-unsaturated imide moiety had a dramatic effect on inhibitory activity. Specifically, analogue **3** exhibited a three orders of magnitude drop in inhibitory potency against FP-2 (IC₅₀ = 3710 nM) and demonstrated no measurable inhibition of FP-3. This compound also showed a marked reduction in activity against *P. falciparum*. Removal of both olefinic moieties in analogue **4** led to a loss of measurable inhibitory activity against both the FPs and the parasite. In addition, when *P. falciparum* trophozoites were treated with compounds **1** and **2**, both caused swollen food vacuole morphology, a hallmark of FP inhibition (see Supporting Information).^{16,32} In contrast, compounds **3** and **4** (that were inactive against the FPs) did not cause swelling of food vacuoles in the parasite. Taken together, these studies strongly suggest that the olefinic functionality within the α,β-unsaturated imide moiety is critical for inhibitory activity against both the FPs and against *P. falciparum*. * indicates the site of diastereoisomerism in **1** and **2**.



Scheme 1. Synthesis of gallinamide A analogues **1-4**.

Reagents and conditions: (i) resin loading: Fmoc-Ile-OH, *i*Pr₂EtN, DMF/CH₂Cl₂, (ii) Fmoc-SPPS (*deprotection*: 10vol.% piperidine/DMF, *coupling*: 4 equiv Fmoc-AA-OH, 4 equiv. PyBOP, 8 equiv. NMM, DMF, *capping*: 10vol.% Ac₂O/pyridine); (iii) HCHO, NaBH(OAc)₃, AcOH, DMF; (iv) 30vol.% HFIP/ CH₂Cl₂; (v) HATU, NMM, 0 °C for 20 min, rt for 2 h. (vi) H₂, Pd/C, MeOH.

Table 1: Inhibition of FP-2, FP-3 and *P. falciparum* by gallinamide A analogues **1-4.** (errors are standard error of the mean of three experiments).

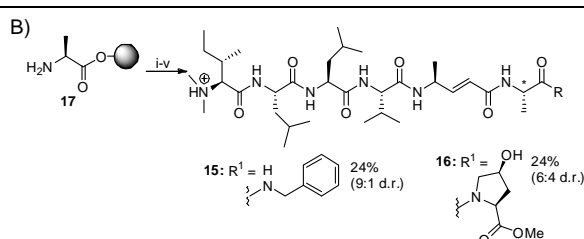
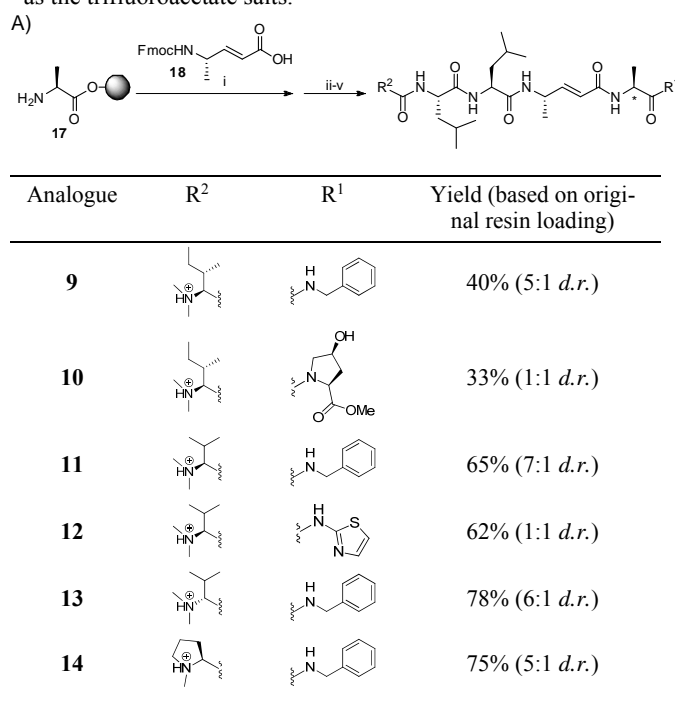
Analogue	IC ₅₀ FP-2 [nM]	IC ₅₀ FP-3 [nM]	IC ₅₀ <i>P. falciparum</i> (3D7) [nM]
1	6.78 ± 0.44	292 ± 1.3	89.3 ± 73
2	2.81 ± 0.39	163 ± 21	210 ± 80
3	3710 ± 420	>50 000	4375 ± 690

Having established the importance of unsaturation in the 4(*S*)-amino-2-(*E*)-pentenoyl unit for FP inhibition and antiparasitic activity, we next explored a small library of gallinamide A analogues (**9-16**) as second generation inhibitors (Scheme 2). The major modification made to these analogues was substitution of the C-terminal *N*-acylpyrrolinone moiety of gallinamide A. Specifically, we proposed that derivatization of a range of scaffolds at the C-terminus (using simple amides to facilitate more rapid syntheses, R¹ in Scheme 2A), would enable investigation of structure-activity relationships for this region of the molecule. Introduction of these modifications also enabled the rapid construction of analogues primarily *via* solid-phase synthesis, without the need for numerous solution-phase fragment condensation and purification steps that were necessary for the synthesis of the natural product (and of **1-4**). It should be noted that, whilst Stolze *et al.* reported that replacement of the C-terminal methylmethoxy-pyrrolinone moiety in gallinamide A with a C-terminal alanine methyl ester moiety significantly decreased activity against the FPs and *P. falciparum*,¹⁵ we were interested in accessing compounds that possessed greater C-terminal functionalization, so as to explore the effect of modifying the acyl-pyrrolinone on FP activity and *P. falciparum* inhibition. We were encouraged by the fact that reduction of the methoxy-enol moiety in analogue **2** did not have a dramatic effect on FP and *P. falciparum* inhibitory activity, suggesting that modifications in this region may be tolerated. A number of other changes were also proposed for the *N*-terminal region, including a range of aliphatic amino acids at the N-terminus (R² in Scheme 2A). Analogues **15** and **16**, extended by one L-Ile residue in the peptide backbone compared to **9-14**, were also proposed in order to probe the importance of the length of the analogues for inhibition of the FPs and *P. falciparum* (Scheme 2B).

Synthesis of **9-14** began from 2-Cl-Trt Cl resin preloaded with Fmoc-Ala (**17**). Coupling of Fmoc-protected α,β -unsaturated amino acid **18** (see Supporting Information for synthesis) followed by elongation *via* standard Fmoc-strategy SPPS provided the desired resin-bound peptide sequences. Following an *en bloc* reductive methylation of the N-terminus with formaldehyde and sodium cyanoborohydride, cleavage of the peptides from the resin using HFIP provided the C-terminal peptide acids (including the extended peptide precursor for **15** and **16**) in moderate to good yields over the ten resin-bound steps following HPLC purification (36-88%, see Supporting Information for full synthetic details and yields). Having assembled the C-terminal peptide acids, we next installed the C-terminal functionality. Benzylamine was coupled using PyBOP at low temperature and, following purification by reverse-phase HPLC, the desired inhibitors **9**, **11**, **13**, **14** and **15** were isolated in excellent yields based on the original resin loading without significant epimerization. 4-(*R*)-Hydroxy-L-proline methyl ester was also coupled to the C-terminus of two peptides, this time with the addition of NMM as a hindered base, to afford inhibitors **10** and **16** in excellent yields following HPLC purification. On this occasion the compounds were isolated as close to 1:1 mixtures of diastereoisomers, reflecting the slower coupling rate of hydroxyproline methyl ester to the C-terminus of the peptides. Finally, aminothiazole was coupled to the C-terminus of one peptide acid using PyBOP at low temperature, which provided **12** as a 1:1

mixture of diastereoisomers in 62% yield following purification, based on the original loading of the resin.

Scheme 2. A) Synthesis of gallinamide A analogues **9-14** and B) synthesis of extended analogues **15** and **16** *via* a solid-phase synthesis approach. NB: compounds **9-16** were isolated as the trifluoroacetate salts.



Reagents and conditions: (i) 4 equiv. **18**, 4 equiv. PyBOP, 6 equiv. NMM (ii) Fmoc SPPS (*deprotection*: 10vol.% piperidine/DMF, *coupling*: 4 equiv Fmoc-AA-OH, 4 equiv. PyBOP, 8 equiv. NMM, DMF, *capping*: 10vol.% Ac₂O/pyridine); (iii) HCHO, NaBH(OAc)₃, AcOH, DMF; (iv) 30vol.% HFIP/DCM, (v) # R¹-NH₂, PyBOP, DMF, 0 °C # NMM was added to the coupling reactions for the preparation of inhibitors **10** and **16**. * indicates the site of diastereoisomerism in **9-16**.

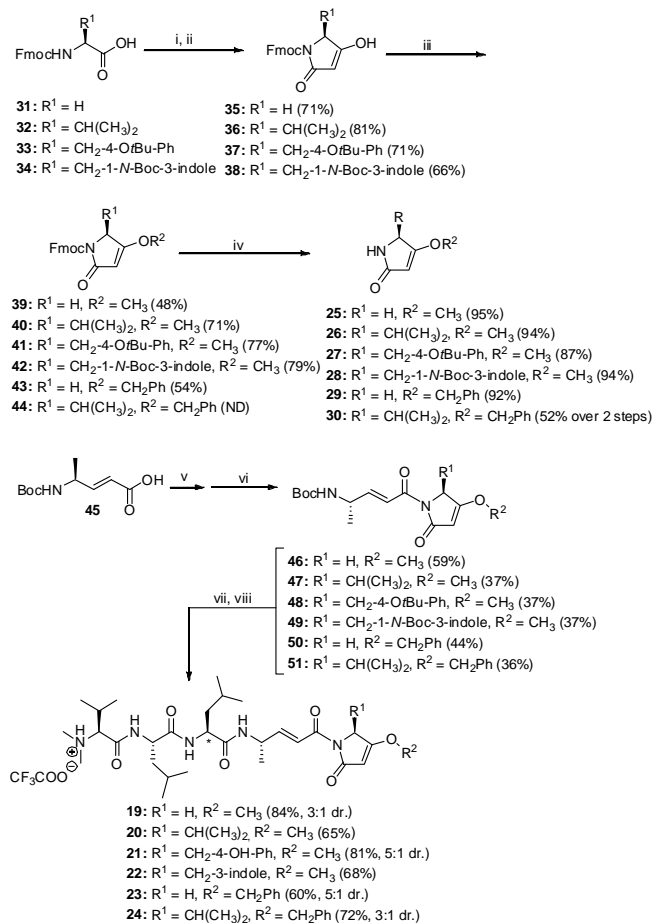
Table 2: Inhibition of FP-2, FP-3 and the 3D7 strain of *P. falciparum* by gallinamide A analogues **9-16.** (errors are standard error of the mean of three experiments).

Analogue	IC ₅₀ FP-2 [μM]	IC ₅₀ FP-3 [μM]	IC ₅₀ <i>P. falciparum</i> (3D7) [μM]
9	10.5 ± 0.64	> 25	0.54 ± 0.21
10	7.66 ± 0.19	> 50	1.90 ± 0.62
11	11.5 ± 0.19	>25	0.32 ± 0.01
12	2.48 ± 0.15	33.9 ± 0.46	1.10 ± 0.80
13	3.44 ± 0.39	>25	0.54 ± 0.38
14	6.00 ± 1.56	46.2 ± 5.35	6.60 ± 3.80
15	> 50	> 50	> 50
16	> 50	> 50	> 50

The gallinamide A analogues **9-16** were next screened for inhibitory activity against FP-2, FP-3 and the 3D7 strain of *P. falciparum* *in vitro* (Table 2). All analogues possessed significant activity, and all led to the swollen food vacuole morphology in trophozoites (see Supporting Information). However, in general the replacement of the N-acyl pyrrolinone moiety in gallinamide A (and analogue **1**) with different C-terminal groups was detrimental to activity against both the FPs and the parasite. Analogues **9**, **11** and **13**, bearing a C-terminal benzylamide moiety, all exhibited similar activity: low micromolar inhibition of FP-2 (IC_{50} = 3.44-11.5 μ M), no measurable inhibition of FP-3 at 25 μ M and nanomolar inhibitory activity against *P. falciparum* (IC_{50} = 320-540 nM). Interestingly, introduction of an N-methylproline functionality at the N-terminus of the peptide, while retaining the C-terminal benzylamide in **14**, led to inhibition of both FP-2 (IC_{50} = 6.00 μ M) and FP-3 (IC_{50} = 46.2 μ M) but exhibited less potent antiparasitic activity (IC_{50} = 6.60 μ M). The loss of activity was particularly striking for **11**, which possesses an identical structure to analogue **1** (Table 1, IC_{50} FP-2 = 6.78 nM, IC_{50} FP-3 = 292 nM, IC_{50} 3D7 89.3 nM), with the exception that the C-terminal acyl pyrrolinone unit has been replaced by a C-terminal benzylamide. The three orders of magnitude drop in activity against the FPs and order of magnitude decrease in antiparasitic activity from **1** to **11** suggests that the N-acyl pyrrolinone unit is important for activity. Introduction of a more highly functionalized and flexible hydroxyproline methyl ester to the C-terminus in **10** provided similar inhibitory activity to the benzylamide-derived compounds against FP-2 and *P. falciparum*. C-terminal functionalization as a thiazole amide in **12** led to moderate inhibitory activity against both FP-2 (IC_{50} = 2.48 μ M) and FP-3 (IC_{50} = 33.9 μ M) as well as low micromolar antiparasitic activity (IC_{50} = 1.10 μ M). Taken together, these data suggest that the N-acyl pyrrolinone moiety is critical for potent FP inhibition and *in vitro* antimalarial activity. The importance of this functionality may lie in the ability of the aminopentenoyl imide motif to serve as a better Michael acceptor for the active site Cys residue in the FPs (Cys42 in FP-2 and Cys51 in FP-3)³³ when compared to the corresponding amide. Specifically, replacement of the imide moiety with the corresponding amide in analogues **9-14** reduces the propensity of the α,β -unsaturated amide unit to serve as a Michael acceptor, presumably due to the less electron withdrawing nature of the amide linkage compared to the imide in the native acylpyrrolinone linkage. Finally, extension of the analogues through the insertion of an additional Val residue in the peptide backbone in **15** and **16** abolished enzyme and parasite inhibitory activity. It is possible that these compounds can no longer be accommodated into the active site of the FPs, as the Leu residue that would be expected to be positioned in the S2 site of the protease³³ has been replaced by a Val, or that the double bond must be positioned at a suitable distance from the N-terminus to exhibit inhibitory activity.

While the replacement of the C-terminal pyrrolinone unit of gallinamide with simplified functionalities provided a number of compounds with activity against FP2, FP3 and *P. falciparum*, these compounds were significantly less potent than **1** and **2** (*vide supra*). As such, we chose to reinstate the C-terminal acyl pyrrolinone moiety in an additional series of

analogues to probe the effect of substitution on the pyrrolinone ring on FP inhibition and antiparasitic activity. In total six analogues (**19-24**) were proposed possessing the identical peptide backbone to **1** and **2** but with variation in the side chain on the pyrrolinone unit and in the substitution of the enol of the pyrrolinone (Scheme 3). The synthesis of **19-24** began with the preparation of the requisite pyrrolinones **25-30** from commercially available Fmoc-protected amino acids **31-34**. Coupling of Meldrum's acid to amino acids **31-34** with EDC in the presence of DMAP followed by reflux of the Meldrum's adduct in ethyl acetate to effect cyclization-condensation provided the corresponding Fmoc-protected pyrrolinones **35-38** in good yield over the two steps. These were each reacted with methanol under Mitsunobu conditions using DIAD and triphenylphosphine to provide O-methylated pyrrolinones **39-42** in 48-79% yields. In addition, **35** and **36** were treated with benzyl alcohol under the same conditions to provide **43** and **44**. All that remained for the synthesis of the target pyrrolinones was removal of the Fmoc group, which was effected smoothly by treatment with piperidine in acetonitrile to pro-



Scheme 3. Synthesis of second generation gallinamide A analogues 26-31. Reagents and conditions: (i) 1. Meldrum's acid, EDC, DMAP, CH₂Cl₂, 0 °C to rt; (ii) EtOAc, 77 °C; (iii) R²-OH, DIAD, PPh₃, CH₂Cl₂, 0 °C to rt; (iv) 1:4 v/v piperidine/MeCN, rt, 15 min; (v) CF₃COOC₆F₅, pyridine, DMF, 0 °C; (vi) n-BuLi, THF, -78 °C; (vii) 1:1 v/v TFA, CH₂Cl₂; (viii) tripeptide **5**, HATU, NMM, DMF, 0 °C. ND = not determined due to co-contamination with DIAD by-product. * indicates the site of diastereoisomerism in **19-21** and **23** and **24**.

vide **25-30** in excellent yields. With each of the required pyrrolinone building blocks in hand, attention turned to the modular assembly of the proposed gallinamide A analogues. To this end, Boc-protected amino acid **45**¹¹ was activated as the corresponding pentafluorophenyl ester by treatment with pentafluorophenyl trifluoroacetate in the presence of pyridine. Separately, pyrrolinones **25-30** were deprotonated with *n*-butyllithium at low temperature before addition of the pentafluorophenyl esters to afford imides **46-51** in moderate yields (36-59%). Unfortunately, despite efforts to improve the reactions by modifying the temperature and base, these yields could not be improved. From here, acidolysis of the Boc group from **46-51** followed by coupling to the *N*-terminal tripeptide **5** using HATU as the coupling reagent and NMM as the base (at low temperature to minimize epimerization), furnished the desired gallinamide A analogues **19-24** in good yields following HPLC purification. As with the synthesis of gallinamide A and analogues **1-4**, epimerization occurred during the final fragment condensation reaction. The minor epimer could be separated by preparative HPLC for analogues **20** and **22**, while **19**, **21**, **23** and **24** were isolated as predominantly one diastereoisomer.

With the small library of pyrrolinone-modified analogues in hand, the compounds were next screened against FP-2, FP-3 and the 3D7 strain of *P. falciparum* (Table 3). Reintroduction of the C-terminal *N*-acyl-pyrrolinone in **19-24** led to reinstatement of potent inhibitory activity against the FPs and *P. falciparum*, as observed for gallinamide A and analogue **1**. All compounds also showed swollen food vacuole morphology at a concentration of less than 5 nM, supporting potent inhibition of the FPs as the mode of antiplasmodial action (see Supporting Information). Compound **19**, bearing no substitution on the pyrrolinone moiety, exhibited similar inhibitory activity to **1** against FP-2 ($IC_{50} = 24.8$ nM), FP-3 ($IC_{50} = 225$ nM) and *P. falciparum* ($IC_{50} = 57.3$ nM). Incorporation of a more hydrophobic substituent on the pyrrolinone ring in **20** led to improvement in inhibitory activity against FP-3 ($IC_{50} = 131$ nM) and *P. falciparum* ($IC_{50} = 16.6$ nM). Indeed, **20** proved to be equipotent to the antimalarial drug CQ against the CQ-sensitive 3D7 strain of the parasite. Introduction of aromatic side chains introduced onto the pyrrolinone ring in **21** and **22** did not lead to a change in activity against FP-2 but, as with **20**, led to a marked increase in inhibitory activity against FP-3 and *P. falciparum*. Compound **22**, bearing an indole side chain on the pyrrolinone ring, proved to be the most potent inhibitor of FP-3 ($IC_{50} = 66.7$ nM) and *P. falciparum* ($IC_{50} = 9.7$ nM) in this series of compounds.

Table 3: Inhibition of FP-2, FP-3 and the 3D7 strain of *P. falciparum* by gallinamide A analogues **1 and **19-24**.** (errors

Analogue	IC_{50} FP-2 [nM]	IC_{50} FP-3 [nM]	IC_{50} <i>P. falciparum</i> (3D7) [nM]
1	6.78 ± 0.44	292 ± 1.30	89.3 ± 73
19	24.8 ± 22.9	225 ± 26.5	57.3 ± 26.0
20	9.52 ± 0.14	131 ± 43.6	16.6 ± 9.0
21	5.25 ± 2.06	81.4 ± 7.73	20.0 ± 8.0
22	12.0 ± 3.19	66.7 ± 25.4	9.7 ± 2.0
23	9.59 ± 0.21	196 ± 7.00	96.0 ± 74.0
24	6.86 ± 2.53	182 ± 15.8	62.0 ± 47.0
CQ	-	-	17.3 ± 3.0

are standard error of the mean of three experiments).

Having elucidated a number of gallinamide A analogues as potent FP and *P. falciparum* inhibitors, we were next interested in investigating whether selected compounds were capable of maintaining activity against a CQ-resistant (Dd2) strain of *P. falciparum* and whether the compounds exhibited selective killing of parasites over human cells by screening against a HEK298 cell line (Table 4). Finally, we were interested in investigating the selectivity of the compounds in inhibiting the FPs over other parasitic proteases. To this end, the compounds were screened against three aminopeptidase (AP) enzymes from *P. falciparum*, namely, AP M1, AP M17 and AP M18. The compounds selected included the potent *N*-acylpyrrolinone-containing analogues **1** and **19-22** as well as the C-terminal amide derivatives, **9**, **11** and **13**, which exhibited IC_{50} values < 600 nM against the 3D7 strain of *P. falciparum* (see Table 2). All of the tested compounds (**1**, **9**, **11**, **13**, **19-22**) exhibited potent inhibition of the CQ-resistant Dd2 strain of *P. falciparum* ($IC_{50} = 29.0 - 421$ nM). Despite exhibiting potent inhibitory activity against human cathepsin, the compounds were selective inhibitors of *P. falciparum* over HEK298 cells, with **9**, **11** and **13**, showing no measurable inhibition of this cell line at a concentration of 50 μ M (see Supporting Information). In addition, none of the analogues displayed any inhibitory activity against the metalloproteases AP M1, AP M17 and AP M18 from *P. falciparum*. (see Supporting Information). Taken together, these data suggest that structural analogues of gallinamide A are promising leads for the pursuit of potent food vacuole FP inhibitors as antimalarial compounds.

Table 4: Inhibition of the CQ-resistant Dd2 strain of *P. falciparum*, HEK298 cells and *P. falciparum* aminopeptidase M1, M17 and M18 (AP M1, M17 and M18) by gallinamide A analogues. (errors are standard error of the mean of three experiments).

Analogue	IC_{50} <i>P. falciparum</i> (Dd2) [nM]	HEK298 [nM]	AP M1, M17 and M18 [nM]
1	302 ± 126	14 200 ± 1050	>10 000
9	421 ± 152	> 50 000	>10 000
11	378 ± 20	> 50 000	>10 000
13	170 ± 68	> 50 000	>10 000
19	419 ± 185	16 300 ± 1700	>10 000
20	165 ± 68.0	9650 ± 1480	>10 000
21	67.0 ± 30.0	18 900 ± 110	>10 000
22	29.0 ± 16.0	8500 ± 124	>10 000

CONCLUSIONS

In summary, a number of potent new inhibitors of the food vacuole FPs, FP-2 and FP-3, were discovered based on the structure of the cyanobacterium-derived natural product gallinamide A. The importance of the α,β -unsaturated imide moiety of the natural product for inhibitory activity was initially demonstrated through the synthesis of selectively reduced analogues and through the synthesis and evaluation of several derivatives bearing a C-

terminal amide in place of the imide functionality found in the natural product. A number of potent inhibitors of FP-2 and FP-3 were elucidated through variation of the side chain on the pyrrolinone ring. Several of these compounds also demonstrated potent inhibition of the CQ-sensitive 3D7 strain of *P. falciparum*, with a number of these proving similarly potent to CQ. Gratifyingly, these analogues maintained potent activity against the CQ-resistant Dd2 strain of *P. falciparum* and did not possess noteworthy toxicity to HEK298 cells. These compounds serve as promising leads for the development of second generation natural-product-based FP inhibitors for the discovery of potential antimalarials, as will be the focus of ongoing research in our laboratories.

EXPERIMENTAL SECTION

Final inhibitors **19–24** were synthesized as detailed in the representative example for compound **20** below. The purity of all final compounds was determined to be $\geq 95\%$ by NMR and HPLC-MS analysis. General methods, full experimental details and original NMR spectra for all analogues can be found in the Supporting Information.

Me₂-Val-Leu-Leu-Apa-pyVal-OMe.TFA (20). Imide **47** (20 mg, 57 μmol), was dissolved in 1:1 v/v TFA/DCM (2 mL) and the reaction was stirred for 15 min before it was concentrated *in vacuo* and the residue was redissolved in DMF (0.3 mL) and cooled to 0 °C. N,N-dimethyltripeptide **5** (46.5 mg, 95 μmol) and HATU (36.1 mg, 95 μmol) were added, followed by NMM (21 μL , 190 μmol), and the reaction was stirred for 20 min at 0 °C, then 2 h at room temperature. The reaction was subsequently quenched with TFA (20 μL), diluted with 1:1 v/v MeCN/H₂O (4 mL) and purified by preparative reverse phase HPLC (gradient: 0-60% MeCN over 40 min) to afford **20** as a white amorphous solid and a single diastereomer (27 mg, 65%). R_f [0-100% MeCN over 30 min] = 20.1 min; $[\alpha]_D = 30.2$ ($c = 0.4$, MeOH); IR (thin film) $\nu_{\text{max}} = 3295, 3073, 2963, 1725, 1670, 1646, 1625, 1550, 1464, 1350 \text{ cm}^{-1}$; ¹H NMR (500 MHz, CDCl₃) δ 8.95 (d, 1H, J 8.0 Hz, NH), 8.26 (s, 1H, NH), 7.37 (d, 1H, J 15.5 Hz, CH), 7.35 (s, 1H, NH), 6.95 (dd, 1H, J 15.5, 5.0 Hz, CH), 5.09 (s, 1H, CH), 4.83-4.70 (m, 3H, 3 x CH), 4.60 (d, 1H, J 2.5 Hz, CH), 3.87 (d, 1H, J 8.5 Hz, CH), 3.84 (s, 3H, CH₃), 3.00 (s, 3H, NCH₃), 2.86 (s, 3H, CH₃), 2.54 (m, 1H, CH), 2.26 (m, 1H, CH), 1.71-1.53 (m, 6H, 2 x CH₂, 2 x CH), 1.26 (d, 3H, J 7.0 Hz, CH₃), 1.12-1.07 (m, 6H, 2 x CH₃), 0.93-0.82 (m, 15H, 5 x CH₃), 0.73 (d, 3H, J 6.5 Hz, CH₃); ¹³C NMR (125 MHz, CDCl₃) δ 180.0, 172.1, 172.0, 170.7, 166.6, 164.5, 148.5, 122.7, 94.7, 71.7, 64.1, 58.7, 51.7, 51.7, 46.4, 41.7, 41.6, 28.9, 28.1, 25.1, 25.0, 23.2, 23.1, 22.2, 21.7, 19.7, 19.3, 18.9, 18.8, 15.4; MS (ESI): m/z 629 [(M+Na)⁺, 100%]; HRMS Calcd for C₃₂H₅₆N₅O₆: M+H⁺, 606.4230 found M+H⁺, 606.4225.

Boc-Apa-pyVal-OMe (47). To a solution of amino acid **45**¹¹ (101 mg, 396 μmol) in DMF (2 mL) at 0 °C was added pentafluorophenyl trifluoroacetate (89 μL , 0.51 mmol), followed by pyridine (32 μL , 396 μmol) and the reaction was allowed to warm to room temperature. The reaction was subsequently stirred for 1 h before diluting with 1:1 v/v Et₂O/EtOAc (20 mL) and washing with 0.2 M aqueous HCl (5 mL), saturated aqueous NaHCO₃ solution (5 mL) and brine. The organic phase was then dried (MgSO₄), before concentrating *in vacuo* to afford the pentafluorophenyl ester as a pale yellow oil which was used immediately in the following reaction. To a solution of pyrrolinone **26** (28.0 mg, 183 μmol) in THF (1.5 mL) at -78 °C was added 2.41 M *n*-butyllithium in hexane (76 μL , 183 μmol) and the reaction was stirred for 10 min. A solution of the freshly prepared pentafluorophenyl ester (99 mg, 235 μmol) in THF (0.5 mL) was subsequently added dropwise over 15 min and the reaction was allowed to stir for a further hour at -60 °C. The reaction was subsequently quenched with AcOH

(50 μL), concentrated *in vacuo*, and the residue was purified by column chromatography (eluent: 2:1 v/v hexane/EtOAc) to afford imide **47** as a colourless oil (24 mg, 37%). R_f [1:1 v/v hexane/EtOAc] = 0.65; $[\alpha]_D = +69$ ($c = 1.0$, CHCl₃); IR (thin film) $\nu_{\text{max}} = 3343, 3102, 2971, 2934, 2877, 1719, 1673, 1621, 1515, 1455, 1365, 1323 \text{ cm}^{-1}$; ¹H NMR (400 MHz, CDCl₃) δ 7.39 (dd, 1H, J 15.8, 1.6 Hz, CH), 7.02 (dd, 1H, J 15.8, 4.4 Hz, CH), 5.08 (s, 1H, CH), 4.69-4.61 (m, 2H, NH, CH), 4.45 (m, 1H, CH), 3.84 (s, 3H, OCH₃), 2.55 (m, 1H, CH), 1.44 (s, 9H, 3 x CH₃), 1.29 (d, 3H, J 7.2 Hz, CH₃), 1.10 (d, 3H, J 7.2 Hz, CH₃), 0.74 (d, 3H, J 7.2 Hz, CH₃); ¹³C NMR (100 MHz, CDCl₃) δ 179.8, 175.7, 170.7, 164.6, 149.5, 122.1, 94.8, 79.8, 64.1, 58.6, 47.5, 28.5, 20.5, 18.9, 15.5; MS (ESI): m/z 375 [(M+Na)⁺, 100%]; HRMS: Calcd for C₁₈H₂₈N₂O₅Na: M+Na⁺, 375.1896 found M+Na⁺, 375.1891.

ASSOCIATED CONTENT

Supporting Information. Experimental procedures and analytical data for all novel compounds including ¹H and ¹³C NMR and analytical HPLC.

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ABBREVIATIONS

CQ, chloroquine; FP, falcipain; NMM, N-methylmorpholine; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; DIAD, diisopropyl azodicarboxylate; PyBOP, (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate; DMAP, 4-dimethylaminopyridine; HATU, 1-[bis(dimethylamino) methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate.

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