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Falcipain Inhibitors Based on the Natural Product Gallinamide A Are Potent in Vitro and in Vivo Antimalarials

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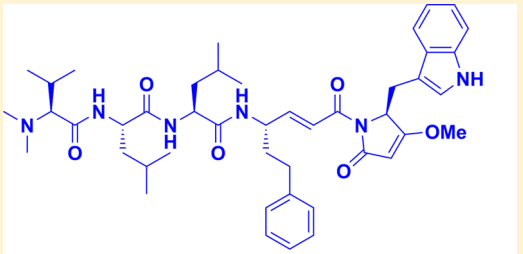
Supporting Information

ABSTRACT: A library of analogues of the cyanobacterium-derived depsipeptide natural product gallinamide A were designed and prepared using a highly efficient and convergent synthetic route. Analogues were shown to exhibit potent inhibitory activity against the *Plasmodium falciparum* cysteine proteases falcipain 2 and falcipain 3 and against cultured chloroquine-sensitive (3D7) and chloroquine-resistant (W2) strains of *P. falciparum*. Three lead compounds were selected for evaluation of in vivo efficacy against *Plasmodium berghei* infection in mice on the basis of their improved blood, plasma, and microsomal stability profiles compared with the parent natural product. One of the lead analogues cured *P. berghei*-infected mice in the Peters 4 day-suppressive test when administered 25 mg kg^{-1} intraperitoneally daily for 4 days. The compound was also capable of clearing parasites in established infections at 50 mg kg^{-1} intraperitoneally daily for 4 days and exhibited moderate activity when administered as four oral doses of 100 mg kg^{-1}.



Falcipain-2: IC_{50} = 33 nM, Falcipain-3: IC_{50} = 112 nM
P. falciparum (in vitro): IC_{50} (3D7) = 1 nM, IC_{50} (W2) = 4 nM
P. berghei (in vivo): curative @ 25 mg/kg/day

INTRODUCTION

Malaria is a mosquito-transmitted disease caused by parasites of the genus *Plasmodium*.[1] Almost half the global population lives in malaria endemic areas and is at high risk of infection. The most virulent human parasite is *Plasmodium falciparum*, which leads to a severe infection that is often deadly, especially for children. Each year there are over 200 million new cases of malaria, and in 2017, the disease was responsible for an estimated 435 000 deaths.[2] A major global health concern is that the currently employed drug regimens for malaria are rapidly losing their effectiveness due to the emergence of drug-resistant parasites. Particularly concerning is the rapid emergence of resistance against the artemisinin (ART)-based combination therapies that serve as the cornerstone of antimalarial therapy.[2,3] The widespread resistance of *Plasmodium* against currently available drugs and the lack of an efficacious vaccine underscores the need for the development of novel antimalarials that operate through unique mechanisms of action. Many therapeutics in clinical use are either natural products or natural product derivatives.[4] The use of natural products as privileged biologically active scaffolds from which to develop new anti-infectives has proven to be a highly successful strategy,[5] e.g., in the development and clinical approval of lipopeptide and glycopeptide antibiotic analogues[6] and antimalarial artemisinins.[7]

We have recently explored analogues of gallinamide A (**1**), a linear depsipeptide natural product isolated independently in 2009 from *Schizothrix*[8] and *Symploca*[9] species of cyanobacteria as antimalarial leads (Figure 1).[10] The natural product and structural analogues have been shown to possess potent inhibitory activity against *P. falciparum* growth in vitro. The mechanism of action of these natural product analogues is due to the inhibition of cysteine proteases, namely, falcipain 2 (FP2) and falcipain 3 (FP3),[11] which are critical for hemoglobin breakdown in the parasitic food vacuole.[12−16] The targeting of falcipains by these compounds has been demonstrated through a continuous kinetic assay with the recombinant enzyme and through the visible accumulation of undegraded hemoglobin inside the parasite's food vacuole, leading to vacuolar swelling and death of the parasite, when erythrocytic *P. falciparum* is incubated with the natural product analogues.[11,17−27]

Several promising gallinamide A analogues have been recently reported.[10] Compounds in the series that exhibited

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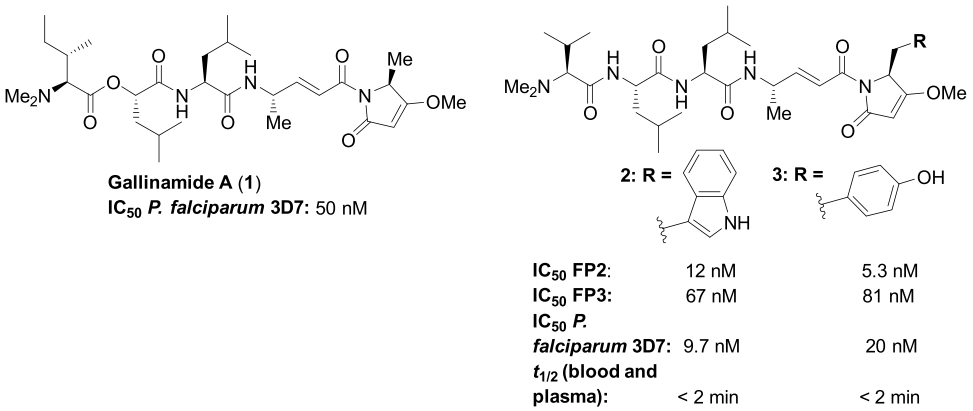
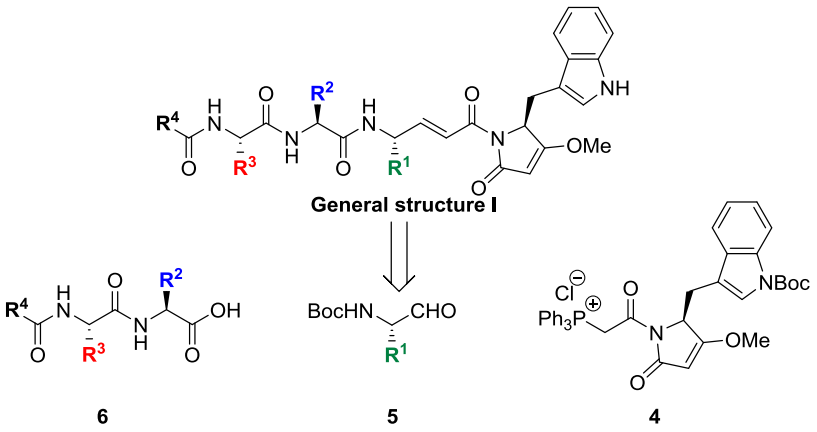


Figure 1. Structures of gallinamide A (**1**) and synthetic analogues **2** and **3** with inhibitory activities against FP2, FP3, and *P. falciparum* in vitro together with in vitro blood and plasma half lives (in mouse and human).

Scheme 1. Retrosynthesis of Target Gallinamide A Analogue Library



the most potent inhibition of FP2, FP3, and *P. falciparum* in vitro included compounds **2** and **3**. Both analogues were designed with a more stable amide bond in place of the labile ester linkage in the natural product (**1**) as well as substitution of the native terminal *N*,*N*-dimethylisoleucine residue with *N*,*N*-dimethylvaline.[10] A key modification that modulated activity involved substitution on the pyrrolinone moiety. Specifically, incorporation of indole (**2**) and phenol (**3**) functionalities derived from tryptophan and tyrosine, respectively, provided potent inhibitors of *P. falciparum*[10] in vitro (IC_{50} 9.7−20 nM against the chloroquine (CQ)-sensitive 3D7 strain and 29−67 nM against the CQ-resistant Dd2 strain), with compound **2** exhibiting the most potent activity against both strains (Figure 1). Despite this potent activity, stability studies revealed rapid degradation in human and mouse blood and plasma in vitro (half-life of **2** <2 min). In addition, these compounds were susceptible to glutathionylation, N-terminal demethylation, and oxidation in liver microsomes, thus limiting their attractiveness as antimalarial lead compounds. In the current work, we aimed to develop a second-generation library of analogues maintaining the key features for inhibitory potency against the FPs and *P. falciparum* in vitro but guided by pharmacological evaluation with the view of generating compounds with antimalarial potential in vivo.

RESULTS AND DISCUSSION

We initially designed a library of gallinamide A analogues containing modifications at R^1−R^4 on the peptide backbone but maintaining the indole-derived pyrrolinone in analogue **2** that possessed the most potent activity in the prior analogue study[10] (see general structure I, Scheme 1). The prior synthetic route to gallinamide A (as well as analogues) relied on a low-yielding imide coupling step between a pyrrolinone and an α,β-unsaturated γ-amino acid that limited the overall efficiency of the synthetic route,[10] as well as the speed at which natural product analogues could be generated for a medicinal chemistry campaign. We therefore proposed a new synthetic route to the target compounds through disconnection at the E-olefin that would necessitate the generation of the triphenylphosphonium salt **4** (Scheme 1) as the key fragment that could undergo Wittig olefination with readily available Boc-protected amino aldehydes (**5**). The target analogues could then be generated through coupling of tripeptides (**6**), which could in turn be prepared via Fmoc-strategy solid-phase peptide synthesis (Fmoc-SPPS) (Scheme 1).

Our synthetic efforts began with the preparation of phosphonium salt **4**. Commercially available Fmoc-Trp-(Boc)-OH (**7**) was first condensed with Meldrum's acid using *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC·HCl) and 4-(dimethylamino)pyridine (DMAP) (Scheme 2). Without purification, the product was heated to reflux in ethyl acetate to effect cyclization of the condensation adduct to the corresponding Fmoc-protected pyrrolin-2,4-dione, which was then O-methylated through a Mitsunobu reaction. Removal of the Fmoc-protecting group using just 1.3 equiv of piperidine in acetonitrile at 0 °C gave **8**

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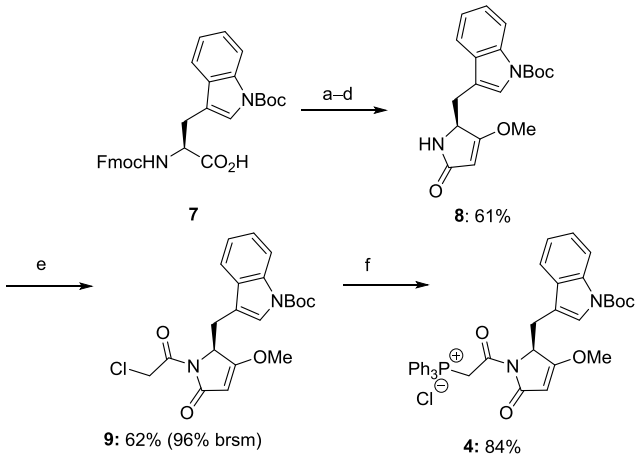
Scheme 2



[a]Reagents and conditions: (a) Meldrum's acid, EDC·HCl, DMAP, CH_2Cl_2, rt, 16 h; (b) EtOAc, reflux, 30 min; (c) PPh_3, diisopropyl azodicarboxylate, MeOH, CH_2Cl_2, 0−10 °C, 3 h; (d) piperidine, MeCN, 0 °C, 30 min; (e) lithium bis(trimethylsilyl)amide (LiHMDS), tetrahydrofuran (THF), chloroacetyl chloride, −78 °C, 15 min; (f) PPh_3, toluene, 35 °C, 5 days.

in an excellent yield (61%) over the four steps without any loss in enantiopurity (as judged by chiral high-performance liquid chromatography (HPLC), see the Supporting Information). It is important to note that those reactions where the Fmoc-group was removed at room temperature (rt) or using an excess of piperidine led to significant racemization of the α-stereogenic center.

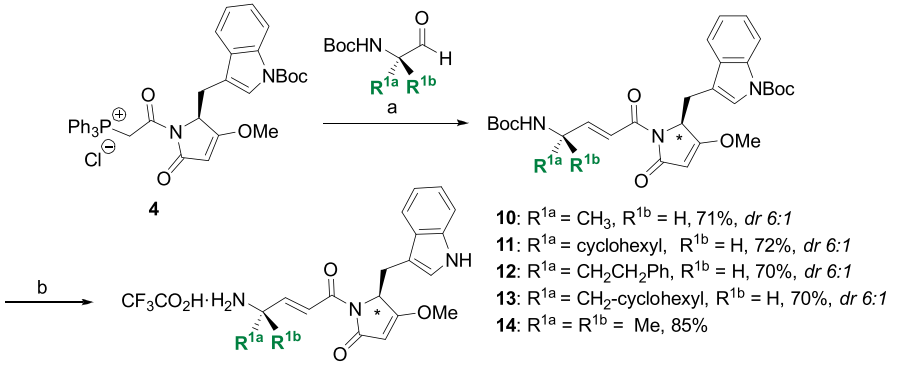
Having generated the desired pyrrolinone **8**, deprotonation of the cyclic amide using LiHMDS at low temperatures followed by acylation with chloroacetyl chloride furnished **9**, in 62% yield (96% based on the recovered starting material). Notably, this yield represented a significant improvement on the imide coupling used in the original synthetic route to the natural product and subsequent analogues.[10] The chloride was subsequently transformed to the desired triphenylphosphonium salt **4** through second-order nucleophilic substitution displacement in excellent yield.

Phosphonium salt **4** was next subjected to the key Wittig olefination with a range of Boc-protected amino aldehydes. Specifically, treatment of **4** with five different Boc-protected amino aldehydes using Hünig's base delivered the protected imide fragments (**10**−**14**) with variation at the γ-position of the α,β-unsaturated γ-amino acid moiety in 70−85% yields. Olefins **10**−**14** were each produced almost exclusively in the E-isomers (11:1−14:1 E/Z); however, pure E-isomers were obtained after flash column chromatography (Scheme 3). Removal of the Boc-protecting groups, followed by reversed-phase HPLC (RP-HPLC) purification, afforded imide fragments (**15**−**19**) in 42% quant. yields. It should be noted that for **16**−**18**, the diastereomers were separable by HPLC at this stage; however, in the case of **15** (R^{1a} = CH_3, R^{1b} = H), we were unable to achieve separation, which therefore remained as a 6:1 diastereomeric ratio (dr) (see the Experimental Section for synthetic details). The synthesis of the tripeptides (**20**−**32**) was accomplished using Fmoc-strategy solid-phase peptide synthesis (Fmoc-SPPS). Specifically, 2-chlorotrityl chloride resin was first loaded with Fmoc-protected amino acids (R^2) followed by an iterative assembly of the linear peptides by Fmoc-SPPS with installation of the desired side chains at R^3 and R^4 (Scheme 4).

After the final Fmoc-deprotection, the peptides were cleaved from the solid support using a mixture of 30 vol % hexafluoroisopropanol (HFIP) in CH_2Cl_2. The crude peptides were then treated with 37 wt % aqueous formaldehyde and sodium cyanoborohydride in a mixture of MeOH/MeCN to facilitate N-terminal dimethylation. Purification by preparative reverse-phase HPLC then afforded the desired library of 13 peptides (**20**−**32**, Table 1) in excellent yields.

With the target building blocks in hand, we could begin the modular assembly of the gallinamide A analogue library. Specifically, imide fragments **15**−**19** were coupled to tripeptides **20**−**32** using a combination of 1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]-pyridinium 3-oxid hexafluorophosphate (HATU), 1-hydroxy-7-azabenzotriazole (HOAt), and *N*-methylmorpholine

Scheme 3



10: R^{1a} = CH_3, R^{1b} = H, 71%, *dr 6:1*
11: R^{1a} = cyclohexyl, R^{1b} = H, 72%, *dr 6:1*
12: R^{1a} = CH_2CH_2Ph, R^{1b} = H, 70%, *dr 6:1*
13: R^{1a} = CH_2-cyclohexyl, R^{1b} = H, 70%, *dr 6:1*
14: R^{1a} = R^{1b} = Me, 85%

15: R^{1a} = CH_3, R^{1b} = H, quant., *dr 6:1*
16: R^{1a} = cyclohexyl, R^{1b} = H, 64%, *dr >99:1*
17: R^{1a} = CH_2CH_2Ph, R^{1b} = H, 71%, *dr >99:1*
18: R^{1a} = CH_2-cyclohexyl, R^{1b} = H, 75%, *dr >99:1*
19: R^{1a} = R^{1b} = Me, 42%

[a]Reagents and conditions: (a) $^i Pr_2NEt$, CH_2Cl_2, 35 °C, 12 h (**5**−**8**), $^i Pr_2NEt$, toluene, 80 °C, 24 h (**9**); (b) trifluoroacetyl (TFA)/CH_2Cl_2 (1:1, v/v), 15 min, rt, then H_2O, 15 h, rt. * indicates center of epimerization.

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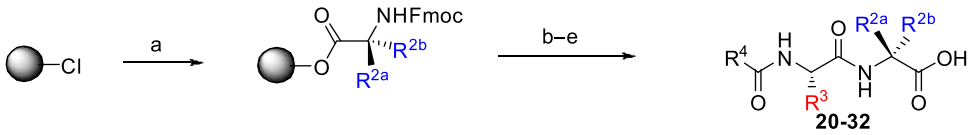
Scheme 4. Solid-Phase Synthesis of Peptide Fragments[a]

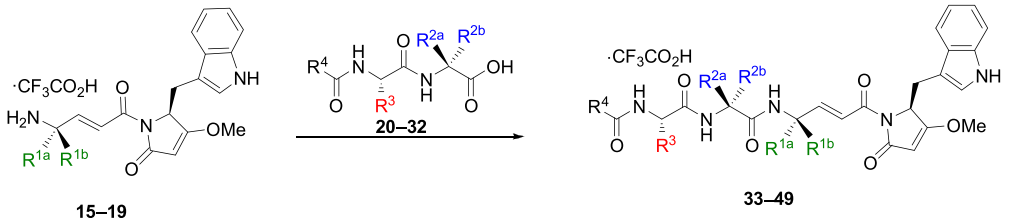


[a]Reagents and conditions: (a) resin loading: Fmoc-AA-OH, $^{i}Pr_2NEt$, dimethylformamide (DMF)/CH_2Cl_2; (b) Fmoc-SPPS: deprotection: 20 vol % piperidine/DMF, coupling: 4 equiv Fmoc-AA-OH, 4 equiv PyAOP, 8 equiv *N*-methylmorpholine (NMM), DMF, capping: 10 vol % Ac_2O/pyridine; cleavage: 30 vol % hexafluoroisopropanol (HFIP)/CH_2Cl_2; (c) (di)methylation: HCHO, $NaCNBH_3$, MeOH/MeCN (1:4 v/v).

Table 1. Yield of Tripeptides Synthesized by SPPS

cpd	R^4	R^3	R^{2a}	R^{2b}	yield (%)
20	$Me_2NCH(^{i}Pr)$–	$-CH_2CH(CH_3)_2$	$-CH_2CH(CH_3)_2$	–H	99
21	$Me_2NCH(^{i}Pr)$–	$-CH_2$-cyclohexyl	$-CH_2CH(CH_3)_2$	–H	98
22	4-(*N*-Me)piperidine–	$-CH_2$-cyclohexyl	$-CH_2CH(CH_3)_2$	–H	94
23	4-(*N*-Me)piperidine–	$-CH_2CH(CH_3)_2$	$-CH_2CH(CH_3)_2$	–H	99
24	$Me_2NCH(^{i}Pr)$–	$-CH_2Ph$	$-CH_2CH(CH_3)_2$	–H	91
25	$Me_2NCH(^{i}Pr)$–	$-CH_2CH_2Ph$	$-CH_2CH(CH_3)_2$	–H	98
26	$Me_2NCH(^{i}Pr)$–	$-CH_2CH(CH_3)_2$	$-CH_2CH_2Ph$	–H	94
27	$Me_2NCH(^{i}Pr)$–	$-CH_2$-cyclohexyl	$-CH_2CH_2Ph$	–H	90
28	$Me_2NCH(^{i}Pr)$–	$-CH_2$-cyclohexyl	$-CH_2$-cyclohexyl	–H	90
29	$Me_2NCH(^{i}Pr)$–	$-CH_2CH(CH_3)_2$	$-CH_2$-cyclohexyl	–H	95
30	4-(*N*-Me)piperidine–	$-CH_2$-cyclohexyl	$-CH_2$-cyclohexyl	–H	81
31	4-(*N*-Me)piperidine–	$-CH_2CH(CH_3)_2$	$-CH_2$-cyclohexyl	–H	98
32	$Me_2NCH(^{i}Pr)$–	$-CH_2CH(CH_3)_2$	$-CH_3$	$-CH_3$	93

Scheme 5. Synthesis of Gallinamide A Analogue Library[a]



[a]Reagents and conditions: HATU, HOAt, DMF/CH_2Cl_2 (1:1 v/v), 30 min.

Table 2. Overview of the Yields of Synthetic Gallinamide A Analogues

cpd	R^4	R^3	R^{2a}	R^{2b}	R^{1a}	R^{1b}	yield (%)
2	$Me_2NCH(^{i}Pr)$–	$-CH_2CH(CH_3)_2$	$-CH_2CH(CH_3)_2$	–H	$-CH_3$	–H	91
33	$Me_2NCH(^{i}Pr)$–	$-CH_2$-cyclohexyl	$-CH_2CH(CH_3)_2$	–H	$-CH_3$	–H	72
34	4-(*N*-Me)piperidine–	$-CH_2$-cyclohexyl	$-CH_2CH(CH_3)_2$	–H	$-CH_3$	–H	86
35	4-(*N*-Me)piperidine–	$-CH_2CH(CH_3)_2$	$-CH_2CH(CH_3)_2$	–H	$-CH_3$	–H	78
36	$Me_2NCH(^{i}Pr)$–	$-CH_2Ph$	$-CH_2CH(CH_3)_2$	–H	$-CH_3$	–H	92
37	$Me_2NCH(^{i}Pr)$–	$-CH_2CH_2Ph$	$-CH_2CH(CH_3)_2$	–H	$-CH_3$	–H	83
38	$Me_2NCH(^{i}Pr)$–	$-CH_2CH(CH_3)_2$	$-CH_2CH_2Ph$	–H	$-CH_3$	–H	90
39	$Me_2NCH(^{i}Pr)$–	$-CH_2$-cyclohexyl	$-CH_2CH_2Ph$	–H	$-CH_3$	–H	87
40	$Me_2NCH(^{i}Pr)$–	$-CH_2$-cyclohexyl	$-CH_2$-cyclohexyl	–H	$-CH_3$	–H	93
41	$Me_2NCH(^{i}Pr)$–	$-CH_2CH(CH_3)_2$	$-CH_2$-cyclohexyl	–H	$-CH_3$	–H	71
42	4-(*N*-Me)piperidine–	$-CH_2$-cyclohexyl	$-CH_2$-cyclohexyl	–H	$-CH_3$	–H	68
43	4-(*N*-Me)piperidine–	$-CH_2CH(CH_3)_2$	$-CH_2$-cyclohexyl	–H	$-CH_3$	–H	95
44	$Me_2NCH(^{i}Pr)$–	$-CH_2CH(CH_3)_2$	$-CH_3$	$-CH_3$	$-CH_3$	–H	81
45	$Me_2NCH(^{i}Pr)$–	$-CH_2CH(CH_3)_2$	$-CH_2CH(CH_3)_2$	–H	$-CH_3$	$-CH_3$	51[a]
46	$Me_2NCH(^{i}Pr)$–	$-CH_2CH(CH_3)_2$	$-CH_2CH(CH_3)_2$	–H	–cyclohexyl	–H	69
47	$Me_2NCH(^{i}Pr)$–	$-CH_2$-cyclohexyl	$-CH_2CH(CH_3)_2$	–H	–cyclohexyl	–H	65
48	$Me_2NCH(^{i}Pr)$–	$-CH_2CH(CH_3)_2$	$-CH_2CH(CH_3)_2$	–H	$-CH_2$-cyclohexyl	–H	88
49	$Me_2NCH(^{i}Pr)$–	$-CH_2CH(CH_3)_2$	$-CH_2CH(CH_3)_2$	–H	$-CH_2CH_2Ph$	–H	83

[a]Combined yield of diastereomers.

(NMM) in a 1:1 v/v mixture of DMF/CH_2Cl_2, which furnished the target library of 17 gallinamide A analogues following reverse-phase HPLC purification (**33**–**49**, Scheme 5 and Table 2).

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With the target library complete, the compounds were screened against recombinant FP2 and FP3 using a fluorescence-based kinetic assay reported previously (Table 3).[10,28] The compounds were also tested for activity against

Table 3. Inhibition of FP2, FP3, 3D7, and W2 Strains of *P. falciparum*[a]

cpd	IC_{50} FP2 (nM)	IC_{50} FP3 (nM)	IC_{50} 3D7 (nM)	IC_{50} W2 (nM)
2	12	67	9.7	ND
33	59	131	14	11
34	31	117	26	28
35	29	79	42	49
36	60	851	24	10
37	57	228	5	7
38	3097	10 235	2593	691
39	3523	8788	1248	478
40	464	459	229	119
41	235	407	72	37
42	455	547	164	55
43	156	480	205	59
44	>50 000	>50 000	>10 000	3729
45	>50 000	>50 000	6994	1017
46	84	484	9	10
47	169	490	83	95
48	270	2571	76	54
49	33	112	1	4
CQ	ND	ND	4	55
ART	ND	ND	32	21
E64	68	136	ND	ND

[a]CQ = chloroquine, ART = artemisinin, E64 = proteinase inhibitor E64. ND = not determined; see ref 10 for raw data on **2**.

CQ-sensitive 3D7 and CQ-resistant W2 strains of *P. falciparum* in vitro (Table 3). The five variations at R^1 provided a relatively flat structure−activity relationship against both the enzyme and the parasite; the majority of substituents were well tolerated, including analogue **49**, bearing a homophenylalanine-derived [R^{1a} = $-CH_2CH_2Ph$, R^{1b} = H] moiety, which possessed more potent activity than CQ, artemisinin, and previously reported gallinamide A analogues against the parasite (IC_{50} 3D7 = 1 nM; W2 = 4 nM). Additionally, the presence of more bulky substituents on R^1 significantly increased the stability in plasma and blood (Table 4), most

Table 4. In Vitro Degradation Half-Lives in Mouse Blood and Plasma

	degradation half-life (min)					
	2	34	35	37	46	49
plasma	<2	126	82	557	>600	373
blood	<2	41	17	110	>600	273

likely owing to the presence of the more sterically demanding substituent, which could hinder hydrolysis and/or Michael addition of erythrocytic glutathione. Compounds bearing a leucyl side chain at R^{2a} = [$-CH_2CH(CH_3)_2$] were the most potent, and variations on R^2 were not well tolerated, culminating in complete loss of activity for analogues having a quaternary carbon at this position (**44**: R^{2a} = R^{2b} = CH_3). However, analogues with R^{2a} = cyclohexylmethyl maintained most of their activity against the W2 strain whereas a drop in activity was observed for the 3D7 strain and against the activities of both FP2 and FP3 enzymes; the reason for these effects is unclear. There appeared to be a relatively large tolerance for modifications at R^3, e.g., analogues **36** and **37** with R^3 = $-CH_2Ph$ and $-CH_2CH_2Ph$ did not show significant differences in antiplasmodial activity.

The screening of our library for the ability to inhibit the activities of FP2 and FP3, as well as the growth of two strains of *P. falciparum*, allowed us to generate important structure−activity information. The data suggest that these analogues exert their antiplasmodial effect by inhibiting FP2 and FP3, which was further supported by the effects of the compounds on parasite morphology, where parasites had swollen food vacuoles with accumulated hemoglobin following compound treatment (see Figure S1, Supporting Information). However, direct correlation between the ability of the compounds to inhibit FPs and their in vitro potency against the parasites indicates that additional factors, such as uptake into the food vacuole, have an impact that requires further investigation. From here, we decided to select compounds for in vitro absorption, distribution, metabolism, and excretion studies to determine their stability in mouse blood and plasma (Table 4) and metabolic stability parameters on the basis of reduced nicotinamide adenine dinucleotide phosphate-dependent degradation profiles in human and mouse liver microsomes (see the Supporting Information for details). The compounds selected for these studies were **34**, **35**, **37**, **46**, and **49**. It should be noted that these compounds were selected with both potency and structural diversity in mind to gauge the effects of structural changes on stability.

Gratifyingly, the stability of all five gallinamide A analogues in mouse blood and plasma was dramatically improved compared with analogue **2**. Specifically, the presence of more sterically encumbered substituents at R^1 and R^3 (**34**, **37**, **46**, and **49**) led to a substantial prolongation of half-life in plasma and blood (Table 4). These were also assessed for cytotoxicity in a human embryonic kidney (HEK293) cell line; compounds showed no measurable cytotoxicity at 25 μm (see Figure S2, Supporting Information). Having established improved plasma and blood stability, we next assessed all five compounds in liver microsomes in vitro to study the generation of any metabolites via liquid chromatography−mass spectrometry (LC−MS). Overall, the compounds exhibited enhanced microsome stability. The main metabolites detected were N-demethylation for **34** and **35**, *N*,*N*-bis-demethylation for **37**, **46**, and **49**, as well as glutathione addition products, although the amounts of the latter addition products were substantially less than those for **2** (see the Supporting Information). On the basis of this stability data, compounds **37**, **46**, and **49**, which showed rapid metabolism in liver microsomes, were triaged and two compounds, namely, **34** and **35**, which showed significantly improved stability profiles when compared with our original lead (compound **2**), were selected for assessment of in vivo antimalarial activity. Despite the higher metabolic instability of **49** (intrinsic clearance = 787 μL min^{-1} mg^{-1} protein in mouse microsomes), we chose to assess the in vivo activity of this analogue given its exquisite potency against parasites in vitro and its reasonable plasma stability.

Toward this end, analogues **34**, **35**, and **49** were next assessed in vivo in a mouse model of cerebral malaria (CM), *Plasmodium berghei* ANKA (PbA) infection (Figure 2A). First, we assessed their efficacy in the Peters 4 day-suppressive test[29] where mice were treated with the test compound 4, 24, 48, and

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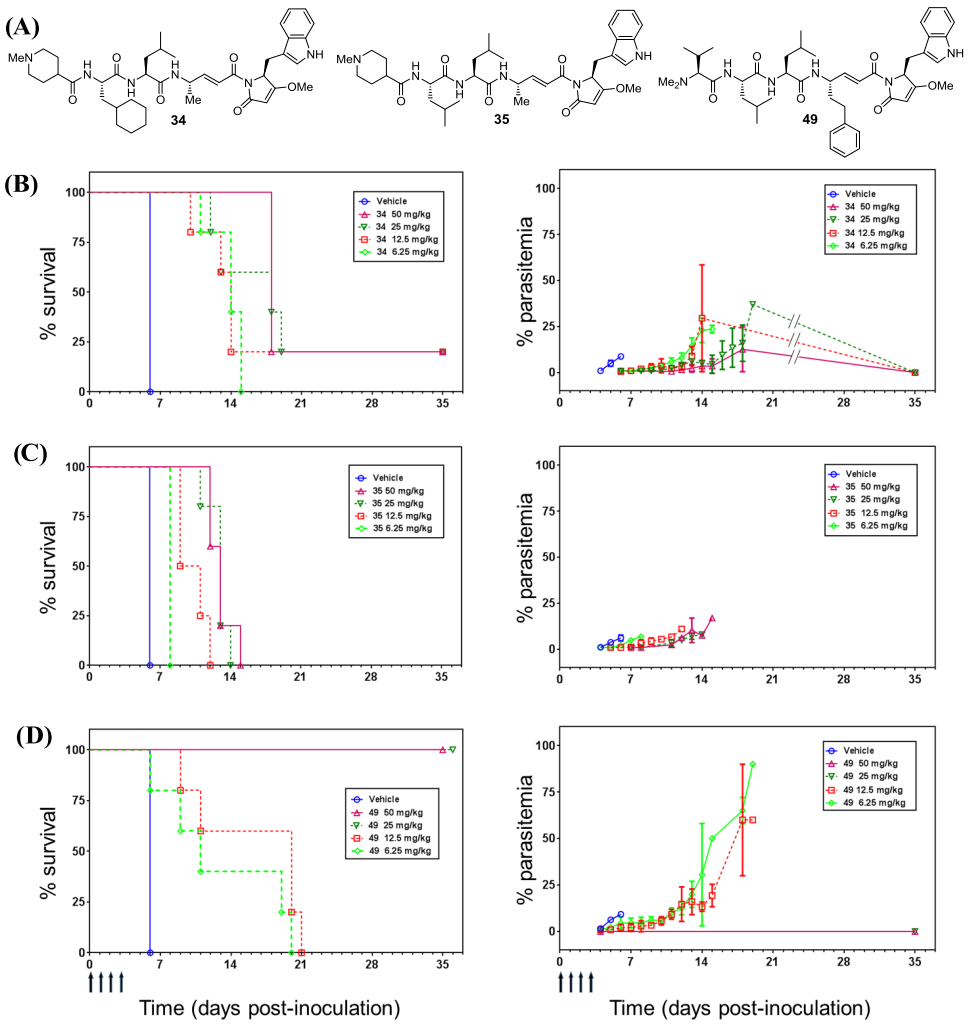

Figure 2. (A) Structures of the three lead gallinamide A analogues (**34**, **35**, and **49**) selected for in vivo antimalarial studies. Antimalarial effect in mice of lead gallinamide A analogue compounds (B) **34**, (C) **35**, and (D) **49**. Mice were inoculated i.p. with 10^6 parasitized red blood cells in 200 μL of normal saline at 0 h. Compounds **34**, **35**, and **49** (50 mg kg^{-1}) in saline, or vehicle alone, were administered i.p. 4, 24, 48, and 72 h later, as indicated by the vertical arrows. Left panels: "survival" was defined as the period before mice were euthanized because they exhibited pronounced signs of disease that inevitably would have led to their death. Right panels: tail vein blood smears were taken, at the times indicated, for determination of % parasitemia (the percentage of red blood cells containing one or more malaria parasites) by microscopy. For parasitemia, values are mean ± standard error of the mean (SEM). N = 5 mice per group.

72 h after *P. berghei* ANKA infection. Compounds were initially dosed through intraperitoneal (i.p.) injection at 50, 25, 12.5, and 6.25 mg kg^{-1}. Analogue **35** showed moderate antimalarial effects when applied at the highest doses (25 and 50 mg kg^{-1}, Figure 2C). In contrast, one out of five mice was cured when treated with compound **34** at concentrations of 12.5, 25, and 50 mg kg^{-1} and the development of detectable parasitemia was delayed in all animals (Figure 2B). The most striking result was achieved in the mice treated with inhibitor **49**, where delayed parasitemia was observed at doses ≥6.25 mg kg^{-1} and all animals treated with concentrations of 25 mg kg^{-1} or higher were cured, i.e., survived >35 days with no detectable malaria parasites in their blood (Figure 2D).

Encouraged by these results, we assessed the ability of **49** to treat an established infection of *P. berghei* ANKA in mice, where animals were treated with four i.p. injections (50 mg kg^{-1} day^{-1}) of the drug candidate at days 4−7 after initiation of the infection (Figure 3). Chloroquine was used as positive control, and inhibitor **49** prevented parasitemia and was able to cure more animals (3/6) than did chloroquine (1/5). Finally, we evaluated the efficacy of candidate **49** in treating an established infection using a four dose regimen (100 mg kg^{-1}) administered orally (see Figure S3, Supporting Information for data). Gallinamide A analogue **49** significantly delayed both the increase in parasitemia and the occurrence of severe signs of disease that necessitated euthanasia, although to a lesser extent than the same dose of chloroquine.

CONCLUSIONS

In summary, we developed a novel and efficient synthetic approach to access analogues of the natural product gallinamide A. We identified compounds that possess potent in vitro activity against the two intravacuole cysteine proteases, FP2 and FP3, as well as *P. falciparum* parasite growth in vitro. This allowed us to gain valuable information on how structural changes influence antiplasmodial activity as well as plasma and metabolic stability. Suitable candidates were selected and evaluated for their efficacy against *P. berghei* malaria infection in mice. One analogue was able to cure murine malaria in a Peters 4 day suppressive test in all animals of the test group at

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J. Med. Chem. XXXX, XXX, XXX−XXX

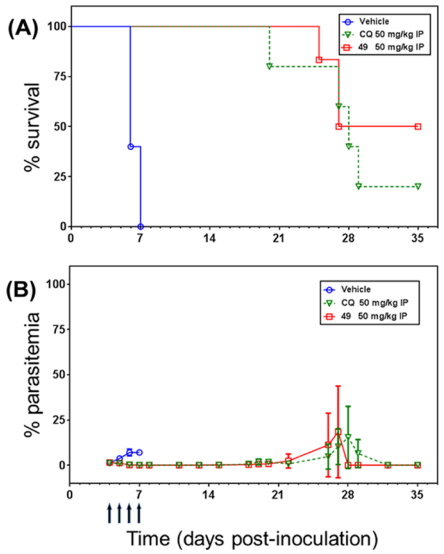

Figure 3. Antimalarial effect of synthetic gallinamide A analogue compound **49** on established murine malaria infection. Mice were inoculated i.p. with 10^6 parasitized red blood cells in normal saline at 0 h. Compound **49** or chloroquine (CQ) (both 50 mg kg^{-1}) in saline, or the vehicle alone, was administered i.p. 4, 5, 6, and 7 days later, as indicated by the vertical arrows. (A) "Survival" was defined as the period before mice were euthanized because they exhibited pronounced signs of the disease that inevitably would have led to their death. (B) Tail vein blood smears were taken, at the times indicated, for determination of % parasitemia by microscopy. For parasitemia, values are mean ± SEM. N = 7 mice per group.

doses of 25 mg kg^{-1} and was also efficacious in a therapeutic model at 50 mg kg^{-1}. This compound also showed promising activity when administered orally at 100 mg kg^{-1} in established infections. Future directions in our laboratory will involve the design and synthesis of peptidomimetic variants of our lead inhibitors to further improve metabolic stability and oral bioavailability, the results of which will be reported in due course.

EXPERIMENTAL SECTION

General Synthetic Chemistry Methods. Unless otherwise stated, reactions were carried out under an argon atmosphere and at room temperature (22 °C). Reactions undertaken at −78 °C utilized a bath of dry ice and acetone. Reactions carried out at 0 °C employed a bath of water and ice. Anhydrous THF, CH_2Cl_2, DMF, and MeCN were obtained using a PureSolv solvent purification system (water <10 ppm). Reactions were monitored by thin-layer chromatography (TLC) on aluminum-backed silica plates (Merck Silica Gel 60 F_{254}). Visualization of TLC plates was undertaken with ultraviolet (UV) light at λ = 254 nm and staining with solutions of vanillin or phosphomolybdic acid, followed by exposure of the stained plates to heat. Silica flash column chromatography (Silica Gel 60 40−63 μm) was undertaken to purify crude reaction mixtures using solvents as specified. Separations were performed using a Biotage Isolera purification system with a diode array detector and a fraction collector.

NMR spectra were obtained using a Bruker DRX 400 or DRX 500 at frequencies of 400 or 500 MHz, respectively, in $CDCl_3$, CD_3OD, CD_3CN, or dimethyl sulfoxide (DMSO)-d_6. Chemical shifts are reported in parts per million (ppm) and coupling constants in hertz (Hz). The residual solvent peaks were used as internal standards ($CDCl_3$: δ_H = 7.26, δ_C = 77.16; CD_3OD: δ_H = 3.31, δ_C = 49.0; CD_3CN: δ_H = 1.94, δ_C = 118.26/1.32; DMSO-d_6: δ_H = 2.50, δ_C = 39.52 ppm).[30,31] ^{1}H NMR data is reported as follows: chemical shift values (ppm), multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet), coupling constant(s), and relative integral. ^{13}C NMR spectra were obtained using a Bruker DRX 400 or DRX 500 at 100.6 or 125.8 MHz in $CDCl_3$, CD_3OD, CD_3CN, or DMSO-d_6. ^{13}C NMR data is reported as chemical shift values (ppm). In the case of diastereomeric mixtures, the signals of the major diastereomer are reported unless otherwise noted.

Mass spectra were recorded on a Shimadzu 2020 (electrospray ionization (ESI)) mass spectrometer operating in positive mode. High-resolution mass spectra were recorded on a Bruker-Daltonics Apex Ultra 7.0 T Fourier transform mass spectrometer.

Optical rotations were measured on a PerkinElmer 341 polarimeter at a wavelength of 589 nm. IR spectra were recorded on a Bruker α Fourier transform infrared (FTIR) spectrometer using a diamond-attenuated total reflection (ATR) unit. Melting points were determined with a SRS OptiMelt melting point apparatus and are uncorrected.

Preparative RP-HPLC was performed using a Waters 600 Multisolvent Delivery System and Waters 500 pump with a 2996 photodiode array detector. The programmable wavelength detector was operated at 210−300 nm. Compounds were purified using an XBridge BEH C_{18} 5 μm (30 × 150 mm^2) column operating at flow rates of 37.5−50.0 mL min^{-1}. A mobile phase of 0.1% trifluoroacetic acid in water (solvent A) and 0.1% trifluoroacetic acid in acetonitrile (solvent B) was used in all cases. LC−MS was performed on a Shimadzu ultra-high-performance LC (UPLC)−MS instrument consisting of an LC-M20A pump and a SPD-M30A diode array detector coupled to a Shimadzu 2020 mass spectrometer (ESI) operating in positive mode. Separations on the UPLC−MS system were performed using a Waters Acquity UPLC BEH C_{18} 1.7 μm column (2.1 × 50 mm^2) at a flow rate of 0.60 mL min^{-1}. Separations were performed using a mobile phase of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B). Chiral HPLC was performed using a RegisPack chiral column (250 × 4.6 mm^2, 5 μm) at a flow rate of 1.50 mL min^{-1}. Separations were performed using a mobile phase of *n*-hexane and 2-propanol.

The purity of all compounds assessed in in vitro and in vivo assays was >97%, as judged by NMR spectroscopy and HPLC.

General Procedures. *General Procedure 1: Solid-Phase Peptide Synthesis. Preloading 2-Chlorotrityl Chloride Resin.* 2-Chlorotrityl chloride resin was swollen in dry CH_2Cl_2 for 30 min, then washed with CH_2Cl_2 (5 × 3 mL). A solution of Fmoc-AA-OH (0.5 mmol g^{-1} of resin) and ^{i}Pr_2NEt (2.0 equiv relative to resin functionalization) in CH_2Cl_2 (final concentration, 100 μM amino acid) was added, and the resin was shaken at rt for 16 h. The resin was washed with DMF (5 × 3 mL) and CH_2Cl_2 (5 × 3 mL). The resin was capped by treating with a solution of CH_2Cl_2/CH_3OH/^{i}Pr_2NEt (17:2:1 v/v/v, 3 mL) for 1 h and washed with DMF (5 × 3 mL), CH_2Cl_2 (5 × 3 mL), and DMF (5 × 3 mL). The resin was subsequently submitted to an iterative peptide assembly (Fmoc-SPPS).

Estimation of Amino Acid Loading. The resin was treated with 20% piperidine/DMF (2 × 3 mL, 3 min), and 50 μL of the combined deprotection solution was diluted to 10 mL using 20% piperidine/DMF in a volumetric flask. The UV absorbance of the resulting piperidine−fulvene adduct was measured (λ = 301 nm, ε = 7800 M^{-1} cm^{-1}) to estimate the amount of amino acid loaded onto the resin.

General Procedure 2: Iterative Peptide Assembly (Fmoc-SPPS). General Amino Acid Coupling. A solution of Fmoc-protected amino acid (4 equiv), benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (4 equiv), and 4-methylmorpholine (NMM, 8 equiv) in DMF (final concentration 100 μM) was preactivated for 5 min before being added to the resin. After 1 h, the resin was washed with DMF (5 × 3 mL), CH_2Cl_2 (5 × 3 mL), and DMF (5 × 3 mL).

Deprotection. The resin was treated with 20% piperidine/DMF (2 × 3 mL, 3 min) and washed with DMF (5 × 3 mL), CH_2Cl_2 (5 × 3 mL), and DMF (5 × 3 mL).

Capping. Acetic anhydride/pyridine (1:9 v/v, 3 mL) was added to the resin. After 3 min, the resin was washed with DMF (5 × 3 mL), CH_2Cl_2 (5 × 3 mL), and DMF (5 × 3 mL).

Cleavage. Hexafluoroisopropanol (HFIP)/CH_2Cl_2 (3:7 v/v) was added to the resin and shaken for 1 h. Then, the resin was washed

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J. Med. Chem. XXXX, XXX, XXX−XXX

with HFIP/CH_2Cl_2 (3:7 v/v, 4 × 4 mL) and then with CH_2Cl_2 (10 × 4 mL). The combined solutions were concentrated in vacuo and purified by preparative RP-HPLC and analyzed by LC−MS (ESI+).

General Procedure 3: N-Terminal Dimethylation of Tripeptides. The crude peptide (200 μmol scale) was suspended in MeCN/MeOH (4:1, 3.0 mL). A solution of 37% aqueous formaldehyde (160 μL, 2.00 mmol, 10 equiv) was added, and the reaction mixture was stirred until the peptide had completely dissolved (10−20 min). $NaBH_3CN$ (126 mg, 2.00 mmol, 10 equiv) was added followed by 100 μL of glacial acetic acid (to reach pH = 5), and the reaction was stirred for 1 h. After complete disappearance of the starting material (LC−MS), the reaction mixture was subsequently concentrated in vacuo and purified by preparative RP-HPLC

*General Procedure 4 for the Synthesis of Tripeptides (**20**−**32**).* Fmoc-AA-OH was loaded onto 2-chlorotrityl chloride resin according to general procedure 1 followed by an iterative assembly of the peptide according to general procedure 2. Following the final Fmoc-deprotection, the peptide was cleaved from the resin according to general procedure 2, then dimethylated according to general procedure 3 and purified by preparative reverse-phase HPLC (RP-HPLC) using a focused gradient [MeCN/H_2O (0.1% TFA) = 0:0 (0.00−1.00 min) → 20:80 (5.00 min) → 30:70 (15.00 min), flow rate: 42 mL min^{-1}, λ = 214 nm, unless otherwise noted] and lyophilized to afford the target tripeptide as the corresponding trifluoroacetate salt as a white solid.

*General Procedure 5 for the Synthesis of α,β-Unsaturated Imide Fragments (**10**−**14**) via Wittig Reaction.* $^iPr_2EtN$ (1.0 equiv) was added to a solution of Wittig salt **4** in CH_2Cl_2, and the mixture was stirred at rt for 10 min. A solution of Boc-protected amino aldehyde (1.0 equiv) in CH_2Cl_2 (final concentration: 100 μM) was subsequently added, and the reaction mixture was heated to 35 °C for 15 h.

*General Procedure 6 for the Synthesis of **15**−**19**.* Boc-protected imides **10**−**14** were dissolved in CH_2Cl_2/CF_3CO_2H (1:1, final concentration: 20 mM) and stirred at rt for 15 min. After the organic solvent had been removed in vacuo, H_2O was added (final concentration: 7.5 mM) and the mixture was stirred at rt for 15 h before the solvent was removed by a stream of N_2.

*General Procedure 7 for the Synthesis of Gallinamide A Analogues (**2**, **33**−**49**).* NMM (4.0 equiv) was added to a solution of the imide fragment **15**−**19** (1.0 equiv, as trifluoroacetate), tripeptide **20**−**32** (1.5 equiv, as trifluoroacetate), HATU (1.5 equiv), and HOAt (3.0 equiv) in DMF/CH_2Cl_2 (1:1 v/v, concentration of the tripeptide = 50 mM), and the reaction mixture was stirred at rt for 30 min. After consumption of the starting material (as judged by LC−MS), the solvent was subsequently removed by a stream of N_2 and the residue was purified by preparative RP-HPLC.

*(S)-(2-(2-((1-(tert-Butoxycarbonyl)-1H-indol-3-yl)methyl)-3-methoxy-5-oxo-2,5-dihydro-1H-pyrrol-1-yl)-2-oxoethyl)-triphenylphosphonium Chloride (**4**).* Triphenylphosphine (472 mg, 1.80 mmol) was added to a stirred solution of *N*-chloroacetyl pyrrolinone **9** (752 mg, 1.80 mmol) in toluene (5.00 mL). The reaction mixture was stirred at 35 °C for 5 days. After cooling to room temperature, *n*-pentane (20 mL) was added and the resulting slurry was stirred for another 30 min before the product was filtered-off as a slightly yellow and sticky solid. After being washed with *n*-pentane (50 mL), the solid was redissolved in CH_2Cl_2 (50 mL). Subsequently, the solvent was removed in vacuo, yielding a slightly yellow foam, which was used in the next step without further purification (1.03 g, 84%, enantiomeric ratio (er) ∼ 6:1). ^1H NMR (400 MHz, $CDCl_3$): δ = 8.07−7.48 (m, 19H), 7.25−7.19 (m, 1H), 7.18−7.12 (m, 1H), 7.05 (t, *J* = 7.5 Hz, 1H), 4.90 (dd, *J* = 5.2, 3.1 Hz, 1H), 4.84 (s, 1H), 3.69 (s, 3H), 3.47−3.31 (m, 1H), 3.17 (dd, *J* = 14.8, 3.1 Hz, 1H), 1.62 (s, 9H) ppm; ^{13}C NMR (125.8 MHz, $CDCl_3$): δ = 179.2, 170.2, 163.3, 135.1, 134.1, 132.3, 130.6, 130.4, 128.7, 124.5, 122.5, 118.8, 115.6, 94.6, 60.0, 59.0, 38.3, 34.9, 31.4, 29.8, 28.4, 24.5 ppm; ^{31}P NMR (202.5 MHz, $CDCl_3$): δ = 21.3 ppm; $[\alpha]_D^{25}$ = +155 (*c* 0.1, CH_2Cl_2); FTIR (ATR): $\tilde{\nu}$ = 1721, 1617, 1453, 1372, 1255, 1155, 1078, 745 cm^{-1}; MS (ESI+): *m*/*z* (%): 645.2 (100) $[M]^+$; high-resolution mass spectrometry (HRMS) (ESI+): calcd for $[C_{39}H_{38}N_2O_5P]$: *m*/*z* = 645.2513, found: 645.2503.

*(S)-5-((1H-Indol-3-yl)methyl)-1-(2-chloroacetyl)-4-methoxy-1,5-dihydro-2H-pyrrol-2-one (**9**).* To a stirred solution of pyrrolinone **8**[10] (2.74 g, 8.00 mmol) in THF (25 mL) at −78 °C was added LiHMDS (8.80 mL, 8.8 mmol, 1 M, THF), and the reaction mixture was stirred for 10 min before chloroacetyl chloride (954 μL, 12.0 mmol) was added in one portion; then, the reaction mixture was stirred at −78 °C for another 30 min. Glacial acetic acid (1.60 mL) was added, and the mixture was allowed to warm to room temperature. After removal of the solvent, the residue was purified by flash chromatography on silica (hexanes/EtOAc = 85:15 → 0:100) to afford **9** as a slightly yellow foam (2.06 g, 62%, 99% enantiomeric excess (ee)). ^1H NMR (400 MHz, $CDCl_3$): δ = 8.08 (d, *J* = 8.3 Hz, 1H), 7.41 (d, *J* = 7.6 Hz, 1H), 7.33−7.14 (m, 3H), 4.93 (dd, *J* = 5.3, 2.7 Hz, 1H), 4.83 (s, 1H), 4.69 (d, *J* = 15.8 Hz, 1H), 4.58 (d, *J* = 15.8 Hz, 1H), 3.80 (s, 3H), 3.70 (dd, *J* = 14.9, 5.3 Hz, 1H), 3.30 (dd, *J* = 14.9, 2.7 Hz, 1H), 1.65 (s, 9H) ppm; ^{13}C NMR (125.8 MHz, $CDCl_3$): δ = 178.7, 169.7, 166.0, 149.6, 135.1, 130.9, 124.9, 124.6, 122.6, 118.4, 115.4, 112.5, 94.5, 83.9, 59.4, 58.8, 45.2, 28.3, 23.8 ppm. $[\alpha]_D^{25}$ = +166 (*c* 0.05, CH_2Cl_2); FTIR (ATR): $\tilde{\nu}$ = 1721, 1700, 1617, 1454, 1375, 1232, 1157, 1127, 766, 745 cm^{-1}; chiral HPLC: t_R = 8.5 min (S-isomer), 7.9 min (R-isomer), *n*-hexanes/2-propanol = 95:5 (0.00 min) → 85:15 (15.00 min), flow rate: 1.50 mL min^{-1}, λ = 294 nm, 98% ee; MS (ESI+): *m*/*z* (%): 419.2 $[M + H]^+$; HRMS (ESI+): calcd for $[C_{21}H_{23}ClN_2O_5 + Na]$: *m*/*z* = 441.1188, found: 441.1186.

*tert-Butyl 3-(((S)-1-((S,E)-4-((tert-Butoxycarbonyl)amino)pent-2-enoyl)-3-methoxy-5-oxo-2,5-dihydro-1H-pyrrol-2-yl)methyl)-1H-indole-1-carboxylate (**10**).* Wittig-salt **4** (409 mg, 0.60 mmol) was treated with $^iPr_2EtN$ (105 μL, 600 μmol) and (*S*)-Boc-alaninal (104 mg, 0.60 mmol) according to general procedure 5 to give crude **10** (E/Z = 12:1), which was purified by flash chromatography on silica (hexanes/EtOAc = 95:5 → 60:40) followed by preparative RP-HPLC to afford **10** as a colorless oil (240 mg, 74%, dr ∼ 6:1). ^1H NMR (400 MHz, $CDCl_3$): δ = 8.04 (d, *J* = 7.2 Hz, 1H, Ar−*H*), 7.43−7.10 (m, 6H, 4 × Ar−*H*, 2 × C*H*), 4.95 (m, 1H, C*H*), 4.83 (s, 1H, C*H*), 4.61 (d, *J* = 8.4 Hz, 1H, N*H*), 4.48 (m_c, 1H, C*H*), 3.76 (s, 3H, CH_3), 3.65 (dd, *J* = 14.7, 5.2 Hz, 1H, C*H*H), 3.29 (dd, *J* = 14.7, 2.4 Hz, 1H, CH*H*), 1.65 (s, 9H, 3 × CH_3), 1.44 (s, 9H, 3 × CH_3), 1.30 (d, *J* = 6.8 Hz, 3H, CH_3) ppm; ^{13}C NMR (100.6 MHz, $CDCl_3$): δ = 178.1, 170.0, 165.0, 155.3, 150.0, 149.7, 135.1, 131.1, 124.8, 124.4, 122.5, 121.7, 118.8, 115.3, 113.2, 95.0, 83.8, 80.1, 59.5, 58.7, 47.5, 28.5, 28.4, 24.4, 20.4 ppm; $[\alpha]_D^{25}$ = +189 (*c* 1.0, CH_2Cl_2); FTIR (ATR): $\tilde{\nu}$ = 2975, 2931, 2872, 1723, 1672, 1627, 1512, 1452, 1369, 1334, 1306 cm^{-1}; LC−MS: t_R = 1.41 min, MeCN/H_2O (0.1% HCO_2H) = 0:100 (0.00−0.30 min) → 100:0 (3.00 min), flow rate: 0.60 mL min^{-1}; MS (ESI+): *m*/*z* (%): 562.1 (100) $[M + Na]^+$; HRMS (ESI+): calcd for $[C_{29}H_{37}N_3O_7 + Na]$: *m*/*z* = 562.2524, found: 562.5218.

*tert-Butyl 3-(((S)-1-((S,E)-4-((tert-Butoxycarbonyl)amino)-4-cyclohexylbut-2-enoyl)-3-methoxy-5-oxo-2,5-dihydro-1H-pyrrol-2-yl)methyl)-1H-indole-1-carboxylate (**11**).* Wittig-salt **4** (341 mg, 500 μmol) was treated with $^iPr_2EtN$ (87.0 μL, 500 μmol) and (*S*)-Boc-cyclohexylglycinal (120 mg, 500 μmol) according to general procedure 5 to give crude **11** (E-isomer only), which was purified by flash chromatography on silica (hexanes/EtOAc = 75:25 → 50:50) to afford the title compound as a slightly yellow oil (220 mg, 72%, E-isomer, dr ∼ 6:1). In this case, the Wittig reaction furnished **11** as pure E-isomer, which was immediately submitted to the next step (Boc-group removal) without further purification. LC−MS: t_R = 6.98 min, MeCN/H_2O (0.1% HCO_2H) = 0:100 (0.00−0.50 min) → 100:0 (8.00 min), flow rate: 0.60 mL min^{-1}; MS (ESI+): *m*/*z* (%): 608.2 (34) $[M + H]^+$, 630.2 (95) $[M + Na]^+$; HRMS (ESI+): calcd for $[C_{34}H_{45}N_3O_7 + Na]$: *m*/*z* = 630.3150, found: 630.3143.

*tert-Butyl 3-(((S)-1-((S,E)-4-((tert-Butoxycarbonyl)amino)-6-phenylhex-2-enoyl)-3-methoxy-5-oxo-2,5-dihydro-1H-pyrrol-2-yl)-methyl)-1H-indole-1-carboxylate (**12**).* Wittig-salt **4** (240 mg, 352 μmol) was treated with $^iPr_2EtN$ (61.0 μL, 352 μmol) and (*S*)-Boc-homophenylalaninal (92.7 mg, 352 μmol) according to general procedure 5 to give crude **12** (E/Z = 14:1), which was purified by flash chromatography on silica [hexanes/EtOAc = 95:5 → 60:40 (10

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J. Med. Chem. XXXX, XXX, XXX−XXX

CV)] followed by preparative RP-HPLC to afford **12** as a colorless oil (158 mg, 71%, E-isomer, dr ~ 6:1). ^{1}H NMR (500 MHz, $CDCl_3$): δ = 8.02 (d, J = 8.7 Hz, 1H), 7.46−7.38 (m, 1H), 7.38−6.99 (m, 10H), 4.96 (dd, J = 5.3, 2.8 Hz, 1H), 4.86 (s, 1H), 4.78−4.67 (m, 1H), 4.49−4.37 (m, 1H), 3.76 (s, 3H), 3.65 (dd, J = 14.8, 5.4 Hz, 1H), 3.30 (dd, J = 14.8, 2.9 Hz, 1H), 2.80−2.60 (m, 2H), 2.03−1.79 (m, 3H), 1.63 (s, 9H), 1.44 (s, 9H) ppm; ^{13}C NMR (125.8 MHz, $CDCl_3$): δ = 178.5, 170.3, 164.9, 155.5, 149.7, 148.8, 141.3, 135.0, 131.1, 128.6, 128.5, 126.2, 124.7, 124.4, 122.5, 122.4, 118.8, 115.3, 113.2, 95.0, 83.4, 80.1, 59.4, 58.6, 51.6, 36.5, 32.2, 28.5, 28.3, 24.3 ppm; $[\alpha]_D^{25}$ = +46 (c 0.1, CH_2Cl_2); FTIR (ATR): $\tilde{\nu}$ = 2978, 1726, 1629, 1453, 1369, 1378, 1255, 1160, 1084, 784 cm^{-1}; LC−MS: t_R = 3.08 min, MeCN/H_2O (0.1% HCO_2H) = 0:100 (0.00−0.50 min) → 100:0 (8.00 min), flow rate: 0.60 mL min^{-1}; MS (ESI+): m/z (%): 652.2 (100) $[M + Na]^+$; HRMS (ESI+): calcd for $[C_{36}H_{43}N_3O_7 + Na]$: m/z = 652.2993, found: 652.2984.

*tert-Butyl 3-(((S)-1-((S,E)-4-((tert-Butoxycarbonyl)amino)-5-cyclohexylpent-2-enoyl)-3-methoxy-5-oxo-2,5-dihydro-1H-pyrrol-2-yl)methyl)-1H-indole-1-carboxylate (**13**).* Wittig-salt **4** (240 mg, 352 μmol) was treated with ^{i}Pr$_2$EtN (61.0 μL, 352 μmol) and (*S*)-Boc-cyclohexylalaninal (90.0 mg, 352 μmol) according to general procedure 5 to give crude **13** (E/Z = 11:1), which was purified by flash chromatography on silica (hexanes/EtOAc = 95:5 → 60:40) followed by preparative RP-HPLC to afford **13** as a colorless oil (154 mg, 70%, E-isomer, dr ~ 6:1). ^{1}H NMR (500 MHz, $CDCl_3$): δ = 8.04 (m_c, 1H), 7.43 (d, J = 7.8 Hz, 1H), 7.31 (d, J = 15.9 Hz, 1H), 7.28−7.09 (m, 4H), 4.95 (dd, J = 5.4, 2.8 Hz, 1H), 4.86 (s, 1H), 4.64−4.43 (m, 1H), 4.27−3.98 (m, 2H), 3.76 (s, 3H), 3.65 (dd, J = 14.8, 5.4 Hz, 1H), 3.30 (dd, J = 14.8, 2.8 Hz, 1H), 1.86−1.68 (m, 4H), 1.65 (s, 9H), 1.45 (s, 9H), 1.45−1.34 (m, 3H), 1.33−1,11(m, 2H), 0.92 (m, 2H) ppm; ^{13}C NMR (125.8 MHz, $CDCl_3$): δ = 178.5, 170.2, 165.1, 155.5, 150.0, 149.7, 135.1, 131.1, 124.8, 124.4, 122.6, 121.6, 118.9, 115.3, 95.0, 83.8, 79.9, 59.4, 58.6, 49.5, 42.8, 34.3, 33.7, 33.0, 28.5, 28.4, 26.6, 26.4, 26.2, 24.4 ppm; $[\alpha]_D^{25}$ = +25 (c 1.0, CH_2Cl_2); FTIR (ATR): $\tilde{\nu}$ = 2924, 1732, 1629, 1507, 1456, 1373, 1339, 1255, 1161 cm^{-1}; LC−MS: t_R = 3.23 min, MeCN/H_2O (0.1% HCO_2H) = 0:100 (0.00−0.30 min) → 100:0 (3.00 min), flow rate: 0.60 mL min^{-1}; MS (ESI+): m/z (%): 644.4 (100) $[M + Na]^+$; HRMS (ESI+): calcd for $[C_{35}H_{47}N_3O_7 + Na]$: m/z = 644.3306, found: 644.3298.

*tert-Butyl (S,E)-3-((1-(4-((tert-Butoxycarbonyl)amino)-4-methylpent-2-enoyl)-3-methoxy-5-oxo-2,5-dihydro-1H-pyrrol-2-yl)methyl)-1H-indole-1-carboxylate (**14**).* Et^{i}Pr$_2$ (24 μL, 137 μmol) was added to a suspension of Wittig salt **4** in toluene (1.0 mL), and the mixture was stirred at room temperature for 10 min. Boc-Aib-H (25.7 mg, 137 μmol) was subsequently added to the solution, and the reaction mixture was heated to 80 °C for 24 h. The solvent was removed in vacuo, and the residue was passed through a short silica column (hexanes/EtOAc = 90:10 → 50:50), furnishing a colorless oil (32 mg, 42%, er 6:1), which was immediately used in the next step.

*(S)-5-((1H-Indol-3-yl)methyl)-1-((S,E)-4-aminopent-2-enoyl)-4-methoxy-1,5-dihydro-2H-pyrrol-2-one·Trifluoroacetate (**15**).* Boc-protected **10** (160 mg, 296 μmol) was deprotected according to general procedure 6 affording **15** as a white solid (134 mg, quant., dr 6:1). ^{1}H NMR (400 MHz, DMSO-d_6): δ = 10.89 (s, 1H, N*H*), 8.21 (s, 3H, NH_3), 7.35 (dd, J = 15.7, 1.3 Hz, 1H, CH=C*H*C=O), 7.31−7.28 (m, 2H, Ar−*H*), 7.05−7.03 (m, 1H, Ar−*H*), 7.00 (dd, J = 15.7, 6.1 Hz, 1H, C*H*=CHC=O), 6.94 (t, J = 7.5 Hz, 1H, Ar−*H*) 6.82−6.80 (m, 1H, Ar−*H*), 5.09 (s, 1H, =C*H*), 4.97 (dd, J = 4.8, 2.6 Hz, 1H, C*H*), 4.10 (br m_c, 1H, C*H*), 3.79 (s, 3H OCH_3), 3.61 (dd, J = 14.6, 4.8 Hz, 1H, CH*H*), 3.22 (dd, J = 14.6, 2.6 Hz, 1H, C*H*H), 1.33 (d, J = 6.6 Hz, 3H, CH_3) ppm; ^{13}C NMR, heteronuclear single quantum coherence (HSQC), heteronuclear multiple bond correlation (HMBC) (100.6 MHz, DMSO-d_6): δ = 179.2 (C_q−OMe), 170.0 (*C*=O), 163.0 (*C*=O), 158.0 (q, J = 32.0 Hz, CF_3CO_2H) 143.8 (*C*H=CHC=O), 135.7 (aryl C_q), 127.9 (aryl C_q), 124.4 (CH=*C*HC=O), 123.9 (aryl *C*H), 120.9 (aryl *C*H), 118.6 (aryl *C*H), 117.8 (aryl *C*H), 111.4 (aryl *C*H), 106.3 (aryl C_q), 94.6 (*C*H), 59.4 (*C*H), 59.1 (O*C*H_3), 47.1 (*C*H), 23.9 (*C*H_2), 18.3 (*C*H_3) ppm; $[\alpha]_D^{25}$ = +193 (c 0.1, MeCN); FTIR (ATR): $\tilde{\nu}$ = 2981, 1721, 1618, 1454, 1374, 1306, 1156, 967, 746 cm^{-1}; LC−MS: t_R = 2.94 min, MeCN/H_2O (0.1% HCO_2H) = 0:0 (0.00−0.50 min) → 100:0 (8.00 min), flow rate: 0.60 mL min^{-1}; MS (ESI+): m/z (%): 340.0 (100) $[M + H]^+$, 679.2 (27), $[2M + H]^+$; HRMS (ESI+): calcd for $[C_{19}H_{21}N_3O_3 + Na]$: m/z = 362.1475, found: 362.1475.

*(S)-5-((1H-Indol-3-yl)methyl)-1-((S,E)-4-amino-4-cyclohexylbut-2-enoyl)-4-methoxy-1,5-dihydro-2H-pyrrol-2-one·Trifluoroacetate (**16**).* Boc-protected **11** (220 mg, 362 μmol, dr 6:1) was deprotected according to general procedure 6 followed by preparative RP-HPLC using a focused gradient [MeCN/H_2O (0.1% TFA) = 0:0 (0.00−1.00 min) → 30:70 (5.00 min) → 40:60 (20.00 min), flow rate: 50 mL min^{-1}] and lyophilized to afford **16** as a white solid as a single diastereomer (121 mg, 64%). ^{1}H NMR (400 MHz, DMSO-d_6): δ = 10.91 (d, J = 2.5 Hz, 1H, N*H*), 8.18 (s, 3H, NH_3), 7.32−7.21 (m, 3H, CH=C*H*C=O, 2 × Ar−*H*), 7.01 (ddd, J = 8.2, 6.9, 1.1 Hz, 1H, Ar−*H*), 6.89−6.79 (m, 3H, C*H*=CHC=O, 2 × Ar−*H*), 5.13 (s, 1H, C*H*), 5.00 (dd, J = 4.9, 2.6 Hz, 1H, C*H*), 3.84 (s, 3H, OCH_3), 3.74 (br s, 1H, C*H*), 3.67 (dd, J = 14.8, 4.9 Hz, 1H, CH*H*), 3.19 (dd, J = 14.8, 2.6 Hz, 1H, C*H*H), 1.83−1.56 (m, 6H, C*H*, CH_2), 1.29−1.07 (m, 3H, CH_2), 1.07−0.72 (m, 2H, CH_2) ppm; ^{13}C NMR, HSQC, HMBC (100.6 MHz, DMSO-d_6): δ = 179.1 (C_q−OMe), 169.9 (*C*=O), 162.7 (*C*=O), 157.9 (q, J = 31.1 Hz, CF_3CO_2H), 140.7 (*C*H=CHC=O), 135.7 (aryl C_q), 127.9 (aryl C_q), 127.1 (CH=*C*HC=O), 124.0 (aryl *C*H), 120.7 (aryl *C*H), 118.3 (aryl *C*H), 117.7 (aryl *C*H), 111.5 (aryl *C*H), 106.1 (aryl C_q), 94.6 (*C*H), 59.4 (*C*H), 59.1 (O*C*H_3), 56.2 (*C*H), 40.0 (*C*H, overlap with DMSO), 28.5 (*C*H_2), 27.4 (*C*H_2), 25.5 (*C*H_2), 25.3 (*C*H_2), 25.3 (*C*H_2), 23.7 (*C*H_2) ppm; $[\alpha]_D^{25}$ = +208 (c 0.05, MeCN); FTIR (ATR): $\tilde{\nu}$ = 1703, 1669, 1618, 1427, 1340, 1198, 1177, 739 cm^{-1}; LC−MS: t_R = 1.70 min, MeCN/H_2O (0.1% HCO_2H) = 0:100 (0.00−0.30 min) → 100:0 (3.00 min), flow rate: 0.60 mL min^{-1}; MS (ESI+): m/z (%): 408.2 (100) $[M + H]^+$; HRMS (ESI+): calcd for $[C_{24}H_{29}N_3O_3 + Na]$: m/z = 430.2101, found: 430.2099.

*(S)-5-((1H-Indol-3-yl)methyl)-1-((S,E)-4-amino-6-phenylhex-2-enoyl)-4-methoxy-1,5-dihydro-2H-pyrrol-2-one·Trifluoroacetate (**17**).* Boc-protected **12** (85 mg, 135 μmol, dr 6:1) was deprotected according to general procedure 6 followed by preparative RP-HPLC using a focused gradient [MeCN/H_2O (0.1% TFA) = 0:0 (0.00−1.00 min) → 30:70 (4.00 min) → 40:60 (13.00 min), flow rate: 50 mL min^{-1}] and lyophilized to afford **17** as a white solid and as a single diastereomer (52.1 mg, 71%). ^{1}H NMR (500 MHz, CD_3CN): δ = 9.49 (s, 1H, N*H*), (7.44 (dd, J = 15.7, 1.1 Hz, 1H, CH=C*H*C=O), 7.41−7.33 (m, 2H, Ar−*H*), 7.33−7.27 (m, 2H, Ar−*H*), 7.24−7.21 (m, 3H. Ar−*H*), 7.10−7.05 (m, 1H, Ar−*H*), 7.02−6.99 (m, 1H, Ar−*H*), 6.96 (dd, J = 15.7, 8.1 Hz, 1H, C*H*=CHC=O), 6.87 (d, J = 2.4 Hz, 1H, Ar−*H*), 4.94 (dd, J = 5.0, 2.6 Hz, 1H, C*H*), 4.82 (s, 1H, C*H*), 4.01−3.93 (m, 1H, C*H*), 3.81 (s, 3H, OCH_3), 3.70 (dd, J = 14.8, 5.0 Hz, 1H, CH*H*), 3.30 (dd, J = 14.8, 2.6 Hz, 1H, C*H*H), 2.76−2.55 (m, 2H, CH_2), 2.20−2.00 (m, 2H, CH_2) ppm; ^{13}C NMR, HSQC, HMBC (125.8 MHz, CD_3CN): δ = 180.6 (C_q−OMe), 171.4 (*C*=O), 164.3 (*C*=O), 141.4 (aryl C_q), 140.7 (*C*H=CHC=O), 137.0 (aryl C_q), 129.6 (aryl *C*H), 129.4 (aryl *C*H), 129.2 (CH=*C*HC=O), 129.1 (aryl C_q), 127.3 (aryl *C*H), 125.4 (aryl *C*H), 122.4 aryl *C*H), 120.1 (aryl *C*H), 119.2 (aryl *C*H), 112.4 (aryl *C*H), 108.1 (*C*H), 95.2 (*C*H), 60.9 (*C*H), 59.9 (O*C*H_3), 53.7 (*C*H), 34.9 (*C*H_2), 31.8 (*C*H_2), 25.0 (*C*H_2) ppm; $[\alpha]_D^{25}$ = +222 (c 0.05, MeCN); FTIR (ATR): $\tilde{\nu}$ = 1705, 1617, 1617, 1340, 1198, 1139, 973, 838, 740 cm^{-1}; LC−MS: t_R = 3.28 min, MeCN/H_2O (0.1% HCO_2H) = 0:100 (0.00−0.50 min) → 100:0 (8.00 min), flow rate: 0.60 mL min^{-1}; MS (ESI+): m/z (%): 430.2 (100) $[M + H]^+$; HRMS (ESI+): calcd for $[C_{26}H_{27}N_3O_3 + H]$: m/z = 430.2125, found: 430.2122.

*(S)-5-((1H-Indol-3-yl)methyl)-1-((S,E)-4-amino-5-cyclohexylpent-2-enoyl)-4-methoxy-1,5-dihydro-2H-pyrrol-2-one·Trifluoroacetate (**18**).* Boc-protected **13** (60 mg, 96 μmol, dr 6:1) was deprotected according to general procedure 6 followed by preparative RP-HPLC using a focused gradient [MeCN/H_2O (0.1% TFA) = 0:0 (0.00−1.00 min) → 25:75 (4.00 min) → 35:65 (13.00 min), flow rate: 50 mL min^{-1}] and lyophilized to afford **18** as a white solid and as a single diastereomer (38 mg, 75%). ^{1}H NMR (500 MHz, CD_3CN): δ = 9.49 (s, 1H, N*H*), 7.42 (dd, J = 15.6, 0.8 Hz, 1H, CH=C*H*C=O), 7.40−7.31 (m, 2H, Ar−*H*), 7.12−7.05 (m, 1H, Ar−*H*), 7.05−6.97 (m, 1H,

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J. Med. Chem. XXXX, XXX, XXX−XXX

Ar−*H*), 6.92 (dd, *J* = 15.6, 8.2 Hz, 1H, C*H*═CHC═O), 6.87 (d, *J* = 2.5 Hz, 1H, Ar−*H*), 4.93 (dd, *J* = 5.0, 2.6 Hz, 1H, C*H*), 4.79 (s, 1H, C*H*), 4.11−4.01 (m, 1H, C*H*), 3.80 (s, 3H, OCH_3), 3.69 (dd, *J* = 14.8, 5.0 Hz, 1H, CH_2), 3.29 (dd, *J* = 14.8, 2.6 Hz, 1H, CH_2), 1.77−1.59 (m, 7H), 1.39−1.13 (m, 4H, C*H*, CH_2), 1.05−0.87 (m, 2H, CH_2) ppm; ^{13}C NMR (125.8 MHz, CD_3CN): δ = 180.5, 171.3, 164.4, 141.7, 136.9, 129.2, 128.5, 125.5, 122.3, 120.1, 119.1, 112.3, 107.9, 95.1, 60.9, 59.9, 51.8, 40.8, 34.3, 34.0, 33.1, 27.0, 26.7, 26.6, 24.9 ppm; $[\alpha]_D^{25}$ = +226 (*c* 0.05, MeCN); FTIR (ATR): $\tilde{\nu}$ = 2928, 1673, 1618, 1350, 1311, 1139, 966, 801, 742, 723 cm^{-1}; LC−MS: t_R = 1.83 min, MeCN/H_2O (0.1% HCO_2H) = 0:100 (0.00−0.30 min) → 100:0 (3.00 min), flow rate: 0.60 mL min^{-1}; MS (ESI+): *m*/*z* (%): 422.1 (100) $[M + H]^+$; HRMS (ESI+): calcd for $[C_{25}H_{31}N_3O_3 + H]$: *m*/*z* = 422.2438, found: 422.2436.

(S,E)-5-((1H-Indol-3-yl)methyl)-1-(4-amino-4-methylpent-2-enoyl)-4-methoxy-1,5-dihydro-2H-pyrrol-2-one·Trifluoroacetate ***(19)***. Boc-protected **14** (32.0 mg, 57.8 μmol) was deprotected according to general procedure 6 followed by preparative RP-HPLC using a linear gradient [MeCN/H_2O (0.1% TFA) = 30:70 (0.00−1.00 min) → 60:40 (26.00 min) flow rate: 37.5 mL min^{-1}] and lyophilized to afford **19** as a white solid (23.0 mg, 85%, er 6:1). ^{1}H NMR (500 MHz, DMSO-d_6 + CD_3CN): δ = 10.85 (s, 1H, N*H*), 8.94 (br s, 3H, NH_3), 7.92−7.84 (m, 3H, CH═C*H*C═O, 2 × Ar−*H*), 7.64 (d, *J* = 15.8 Hz, 1H, C*H*═CHC═O), 7.60 (t, *J* = 7.5 Hz, 1H, Ar−*H*), 7.52 (t, *J* = 7.5 Hz, 1H, Ar−*H*), 7.40 (d, *J* = 2.5 Hz, 1H, Ar−*H*), 5.49 (dd, *J* = 5.0, 2.6 Hz, 1H, C*H*), 5.42 (s, 1H, C*H*), 4.36 (s, 3H, OCH_3), 4.24 (dd, *J* = 14.8, 5.0 Hz, 1H, C*H*H), 3.82 (dd, *J* = 14.8, 2.6 Hz, 1H, CH*H*), 2.02 (s, 3H, CH_3), 2.00 (s, 3H, CH_3) ppm; ^{13}C NMR, HSQC, HMBC (125.8 MHz, DMSO-d_6 + CD_3CN): δ = 179.5 (C_q−OMe), 170.3 (C═O), 163.6 (C═O), 147.3 (*C*H═CHC═O), 136.1 (aryl C_q), 128.2 (aryl C_q), 124.3 (aryl C*H*), 123.0 (aryl C*H*), 121.2 (aryl C*H*), 118.9 (aryl C*H*), 118.2 (CH═C*H*C═O), 111.5 (aryl C*H*), 106.8 (aryl C_q), 94.3 (C*H*), 59.9 (C*H*), 58.9 (CH_3), 54.0 (C_q), 25.2 (CH_3), 25.1 (CH_3), 24.0 (CH_2) ppm; $[\alpha]_D^{25}$ = +30 (*c* 0.1, MeCN); FTIR (ATR): $\tilde{\nu}$ = 1721, 1618, 1374, 1307, 1155, 1078, 967, 746 cm^{-1}; LC−MS: t_R = 2.78 min, MeCN/H_2O (0.1% HCO_2H) = 0:100 (0.00−0.50 min) → 100:0 (8.00 min), flow rate: 0.60 mL min^{-1}; MS (ESI+): *m*/*z* (%): 337.2 (100) $[M-(NH_3) + H]^+$, 354.2 (46) $[M + H]^+$, 707.3 (31) $[2M + H]^+$; HRMS (ESI+): calcd for $[C_{20}H_{23}N_3O_3 + Na]$: *m*/*z* = 376.1632, found: 376.1630.

N,N-Dimethyl-L-valyl-L-leucyl-L-leucine·Trifluoroacetate ***(20)***. Synthesis was performed on a 200 μm scale according to general procedure 4, and a white solid was isolated following HPLC purification and lyophilization (96 mg, 99% yield). ^{1}H NMR (400 MHz, CD_3CN): δ = 8.25 (d, *J* = 8.5 Hz, 1H, N*H*), 7.34 (d, *J* = 7.5 Hz, 1H, N*H*), 4.64−4.54 (m, 1H, Leu α−*H*), 4.36−4.25 (m, 1H, Leu α−*H*), 3.65 (d, *J* = 7.9 Hz, 1H, Val α−*H*), 2.85 (s, 6H, 2 × NCH_3), 2.37−2.22 (m, 1H, Val C*H*), 1.75−1.54 (m, 6H, 2 × Leu C*H*, CH_2), 1.07 (d, *J* = 6.7 Hz, 3H, CH_3), 0.96−0.90 (m, 12H, 4 × CH_3), 0.86 (d, *J* = 6.7 Hz, 3H, CH_3) ppm; ^{13}C NMR (100.6 MHz, CD_3CN): δ = 174.3, 173.1, 166.5, 118.3, 73.2, 52.7, 52.2, 42.1, 40.6, 28.4, 25.6, 25.5, 23.2, 23.1, 22.2, 21.6, 19.4, 18.2 ppm; FTIR (ATR): $\tilde{\nu}$ = 1645, 1550, 1467, 1369, 1190 cm^{-1}; $[\alpha]_D^{25}$ = −30 (*c* 0.1, MeCN); LC−MS: t_R = 2.41 min, MeCN/H_2O (0.1% HCO_2H) = 0:100 (0.00−0.50 min) → 35:65 (3.00 min) → 100:0 (4.00 min), flow rate: 0.60 mL min^{-1}; MS (ESI+): *m*/*z* (%): 372.4 $[M + H]^+$; HRMS (ESI+): calcd for $[C_{19}H_{37}N_3O_4 + H]^+$: *m*/*z* = 372.2862, found: 372.2861.

N,N-Dimethyl-L-valyl-L-cyclohexylalanyl-L-leucine·Trifluoroacetate ***(21)***. Synthesis was performed on a 155 μm scale according to general procedure 4, and a white solid was isolated following HPLC purification and lyophilization (82 mg, 98% yield). ^{1}H NMR (400 MHz, acetonitrile-d_3): δ = 8.30−8.06 (m, 1H), 7.41−7.18 (m, 1H), 4.74−4.53 (m, 1H), 4.30 (dd, *J* = 14.7, 7.6 Hz, 1H), 3.65−3.52 (m, 1H), 2.83 (s, 6H), 2.28 (dp, *J* = 8.3, 6.7 Hz, 1H), 1.81−1.48 (m, 10H), 1.46−1.12 (m, 4H), 1.07 (d, *J* = 6.7 Hz, 3H), 0.96−0.89 (m, 8H), 0.86 (d, *J* = 6.3 Hz, 3H) ppm; ^{13}C NMR (100.6 MHz, acetonitrile-d_3): δ = 174.2, 173.1, 166.6, 73.2, 52.3, 51.9, 41.9, 40.7, 40.5, 34.9, 34.2, 33.3, 28.4, 27.2, 26.9, 26.9, 25.6, 23.2, 21.5, 19.4, 18.3 ppm; $[\alpha]_D^{25}$ = −46 (*c* 0.05, MeCN); FTIR (ATR): $\tilde{\nu}$ = 2924, 1648, 1543, 1198, 1135, 703 cm^{-1}; LC−MS: t_R = 1.46 min, MeCN/H_2O (0.1% HCO_2H) = 0:100 (0.00−0.30 min) → 100:0 (3.00 min), flow rate: 0.60 mL min^{-1}; MS (ESI+): *m*/*z* (%): 412.5 (100) $[M + H]^+$; HRMS (ESI+): calcd for $[C_{22}H_{41}N_3O_4 + Na]$: *m*/*z* = 434.2989, found: 434.2985.

(1-Methylpiperidine-4-carbonyl)-L-cyclohexylalanyl-L-leucine·Trifluoroacetate ***(22)***. Synthesis was performed on a 190 μm scale according to general procedure 4 and a white solid was isolated following HPLC purification and lyophilization (77 mg, 94%). ^{1}H NMR (400 MHz, methanol-d_4): δ = 4.50−4.34 (m, 2H, C*H*), 3.66−3.46 (m, 2H, CH_2), 3.01 (tt, *J* = 13.1, 3.4 Hz, 2H, CH_2), 2.87 (s, 3H, NCH_3), 2.56 (tt, *J* = 12.0, 3.8 Hz, 1H, C*H*), 2.17−1.84 (m, 4H, CH_2), 1.84−1.61 (m, 10H, CH_2), 1.61−1.07 (m, 5H, C*H*, CH_2), 1.07−0.85 (m, 2H, CH_2), 0.96 (d, *J* = 6.4 Hz, 3H, CH_3), 0.91 (d, *J* = 6.3 Hz, 3H, CH_3) ppm; ^{13}C NMR (100.6 MHz, methanol-d_4): δ = 175.7, 175.3, 174.8, 54.9, 52.3, 52.0, 43.9, 41.6, 40.5, 40.4, 35.4, 34.8, 33.5, 27.7, 27.6, 27.4, 27.3, 25.9, 23.4, 21.8 ppm; $[\alpha]_D^{25}$ = −46 (*c* 0.05, MeCN); FTIR (ATR): $\tilde{\nu}$ = 2926, 1644, 1542, 1198, 1136, 703 cm^{-1}; LC−MS: t_R = 1.50 min, MeCN/H_2O (0.1% HCO_2H) = 0:100 (0.00−0.30 min) → 100:0 (3.00 min), flow rate: 0.60 mL min^{-1}; MS (ESI+): *m*/*z* (%): 410.4 (100) $[M + H]^+$; HRMS (ESI+): calcd for $[C_{22}H_{39}N_3O_4 + Na]$: *m*/*z* = 432.2833, found: 432.2829.

(1-Methylpiperidine-4-carbonyl)-L-leucyl-L-leucine·Trifluoroacetate ***(23)***. Synthesis was performed on a 160 μm scale according to general procedure 4 and a white solid was isolated following HPLC purification and lyophilization (77 mg, 99%). ^{1}H NMR (500 MHz, acetonitrile-d_3): δ = 7.27 (d, *J* = 7.7 Hz, 1H, N*H*), 7.20 (d, *J* = 8.1 Hz, 1H, N*H*), 4.45−4.22 (m, 2H, 2 × C*H*), 3.48 (d, *J* = 13.0 Hz, 1H, C*H*), 3.29 (br s, 1H, alkyl−*H*), 2.88−2.83 (m, 2H, alkyl−*H*), 2.75 (s, 3H, NCH_3), 2.52−2.44 (m, 1H), 2.13−1.95 (m, 4H, alkyl−*H*), 1.72−1.45 (m, 6H, alkyl−*H*), 0.92−0.87 (4 × d, 12H, 4 × CH_3) ppm; ^{13}C NMR (125.8 MHz, acetonitrile-d_3): δ = 174.8, 174.5, 173.7, 54.5, 54.5, 53.2, 51.8, 44.1, 41.5, 41.1, 40.2, 27.0, 27.0, 25.6, 25.6, 23.3, 23.2, 22.0, 21.8 ppm; $[\alpha]_D^{25}$ = −35 (*c* 0.1, MeCN); FTIR (ATR): $\tilde{\nu}$ = 1664, 1537, 1194, 722 cm^{-1}; LC−MS: t_R = 2.79 min, MeCN/H_2O (0.1% HCO_2H) = 0:0 (0.00−0.50 min) → 100:0 (8.00 min), flow rate: 0.60 mL min^{-1}; MS (ESI+): *m*/*z* (%): 370.2 (100) $[M + H]^+$; HRMS (ESI+): calcd for $[C_{19}H_{35}N_3O_4 + Na]$: *m*/*z* = 392.2520, found: 392.2518.

N,N-Dimethyl-L-valyl-L-phenylalanyl-L-leucine·Trifluoroacetate ***(24)***. Synthesis was performed on a 160 μm scale according to general procedure 4, and a white solid was isolated following HPLC purification and lyophilization (76 mg, 91%). ^{1}H NMR (400 MHz, CD_3CN): δ = 8.61 (s_{br}, 1H, CO_2H), 8.08 (d, *J* = 9.1 Hz, 1H, Phe N*H*), 7.40 (d, *J* = 7.6 Hz, 1H, Leu N*H*), 7.33−7.15 (m, 5H, aryl−*H*), 4.92 (ddd, *J* = 10.7, 9.1, 4.7 Hz, 1H, Phe α−*H*), 4.37 (q, *J* = 7.4 Hz, 1H, Leu α−*H*), 3.53 (d, *J* = 6.9 Hz, 1H, Val α−*H*), 3.23 (dd, *J* = 13.9, 4.7 Hz, 1H, CH_2Ph), 2.86 (dd, *J* = 13.9, 10.7 Hz, 1H, CH_2Ph), 2.48 (s, 6H, NCH_3), 2.25 (m_c, 1H, Val C*H*), 1.74−1.59 (m, 3H, Leu CH_2, C*H*), 1.00 (d, *J* = 6.8 Hz, 3H, Val CH_3), 0.93 (d, *J* = 6.0 Hz, 3H, Leu CH_3), 0.88 (d, *J* = 5.9 Hz, 3H, Leu CH_3), 0.84 (d, *J* = 6.6 Hz, 3H, Val CH_3); ^{13}C NMR, HSCQ, HMBC (100.6 MHz, CD_3CN): δ = 174.2 (CO_2H), 172.0 (Phe *C*═O), 166.0 (Val *C*═O), 138.0 (aryl C_q), 130.5 (aryl C*H*), 129.5 (aryl C*H*), 127.9 (aryl C*H*), 73.3 (Val C_α), 55.0 (Phe C_α), 52.2 (Leu C_α), 40.8 (2 × NCH_3), 39.1 (CH_2Ph), 28.0 (Val CH), 25.6 (Leu CH), 23.2 (Leu CH_3), 21.7 (Leu CH_3), 19.5 (Val CH_3), 17.5 (Val CH_3) ppm; $[\alpha]_D^{25}$ = −19 (*c* 0.1, MeCN); FTIR (ATR): $\tilde{\nu}$ = 1646, 1188, 1137, 837, 722, 699 cm^{-1}; LC−MS: t_R = 1.35 min, MeCN/H_2O (0.1% HCO_2H) = 0:100 (0.00−0.30 min) → 100:0 (3.00 min), flow rate: 0.60 mL min^{-1}; MS (ESI+): *m*/*z* (%): 406.6 (100) $[M + H]^+$; HRMS (ESI+): calcd for $[C_{22}H_{35}N_3O_4 + Na]$: *m*/*z* = 428.2520, found: 428.2516.

N,N-Dimethyl-L-valyl-L-homophenylalanyl-L-leucine·Trifluoroacetate ***(25)***. Synthesis was performed on a 160 μm scale according to general procedure 4, and a white solid was isolated following HPLC purification and lyophilization (84 mg, 98%). ^{1}H NMR (400 MHz, CD_3CN): δ = 8.50 (d, *J* = 8.2 Hz, 1H), 7.48 (d, *J* = 7.3 Hz, 1H), 7.35−7.03 (m, 5H), 4.61 (q, *J* = 7.4 Hz, 1H), 4.34 (q, *J* = 7.3 Hz, 1H), 3.72 (d, *J* = 8.2 Hz, 1H), 2.86 (s, 6H), 2.66 (t, *J* = 8.2 Hz, 2H), 2.30 (q, *J* = 6.9 Hz, 1H), 2.11−1.90 (m, 2H), 1.77−1.59 (m, 3H), 1.08 (d, *J* = 6.7 Hz, 3H), 0.92 (d, *J* = 6.3 Hz, 6H), 0.87 (d, *J* = 6.1 Hz,

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J. Med. Chem. XXXX, XXX, XXX−XXX

3H) ppm; ^{13}C NMR (100.6 MHz, CD_3CN): δ = 174.5, 172.6, 166.7, 142.1, 129.5, 129.3, 127.0, 73.1, 54.0, 52.5, 41.9, 40.5, 35.5, 32.4, 28.4, 25.7, 23.2, 21.6, 19.4, 18.3; $[\alpha]_D^{25}$ = −1 (*c* 0.5, MeCN); FTIR (ATR): $\tilde{\nu}$ = 2965, 1640, 1535, 1194, 1137, 699 cm^{-1}; LC−MS: t_R = 1.40 min, MeCN/H_2O (0.1% HCO_2H) = 0:100 (0.00−0.30 min) → 100:0 (3.00 min), flow rate: 0.60 mL min^{-1}; MS (ESI+): *m*/*z* (%): 406.6 (100) $[M + H]^+$; HRMS (ESI+): calcd for $[C_{22}H_{35}NaN_3O_4]$: *m*/*z* = 428.2520, found: 428.2516.

*N,N-Dimethyl-L-valyl-L-leucyl-L-homphenylalanine·Trifluoroacetate (**26**).* Synthesis was performed on a 155 μm scale according to general procedure 4, and a white solid was isolated following HPLC purification and lyophilization (77 mg, 94%). ^{1}H NMR (500 MHz, methanol-d_4): δ = 7.30−7.15 (m, 5H, Ar−*H*), 4.62 (dd, *J* = 8.8, 6.4 Hz, 1H, C*H*), 4.35 (dd, *J* = 10.0, 4.2 Hz, 1H, C*H*), 3.66 (d, *J* = 5.7 Hz, 1H, C*H*), 2.90 [s, 6H, N(CH_3)$_2$], 2.81−2.70 (m, 1H, C*H*H), 2.71−2.61 (m, 1H, CH*H*), 2.40 (dq, *J* = 13.3, 6.7 Hz, 1H, C*H*), 2.23−2.10 (m, 1H, C*H*H), 2.05−1.92 (m, 1H, CH*H*), 1.80−1.59 (m, 3H, C*H*, CH_2), 1.14 (d, *J* = 6.8 Hz, 3H, CH_3), 1.01 (2 × d, *J* = 6.6 Hz, 6H, CH_3), 0.98 (d, *J* = 6.4 Hz, 3H, CH_3) ppm; ^{13}C NMR (100.6 MHz, methanol-d_4): δ = 175.0, 173.9, 166.8, 142.2, 129.6, 129.4, 127.1, 74.2, 53.3, 53.0, 41.8, 34.5, 33.0, 28.6, 25.9, 23.2, 22.1, 19.9, 17.8 16.8 ppm; $[\alpha]_D^{25}$ = −32 (*c* 0.1, CH_3CN); FTIR (ATR): $\tilde{\nu}$ = 2927, 1647, 1542, 1183, 721, 699 cm^{-1}; LC−MS: t_R = 1.48 min, MeCN/H_2O (0.1% HCO_2H) = 0:100 (0.00−0.30 min) → 100:0 (3.00 min), flow rate: 0.60 mL min^{-1}; MS (ESI+): *m*/*z* (%): 420.5 (100) $[M + H]^+$; HRMS (ESI+): calcd for $[C_{23}H_{37}N_3O_4 + Na]$: *m*/*z* = 442.2676, found: 434.2672.

*N,N-Dimethyl-L-valyl-L-cyclohexylalanyl-L-homphenylalanine·Trifluoroacetate (**27**).* Synthesis was performed on a 155 μm scale according to general procedure 4 and a white solid was isolated following HPLC purification and lyophilization (82 mg, 92%). ^{1}H NMR (500 MHz, methanol-d_4): δ = 7.44−6.84 (m, 5H, Ar−*H*), 4.68−4.60 (m, 1H, C*H*), 4.35 (dd, *J* = 9.8, 4.3 Hz, 1H, C*H*), 3.69 (d, *J* = 5.8 Hz, 1H, C*H*), 2.91 [s, 6H, N(CH_3)$_2$], 2.80−2.69 (m, 1H, C*H*H), 2.69−2.60 (m, 1H, CH*H*), 2.45−2.33 (m, 1H, C*H*), 2.22−2.10 (m, 1H, C*H*H), 2.08−1.92 (m, 1H, CH*H*), 1.88−1.52 (m, 7H, CH_2), 1.46−1.35 (m, 1H, C*H*), 1.34−1.15 (m, 3H, C*H*, CH_2), 1.12 (d, *J* = 6.8 Hz, 3H, CH_3), 1.03−0.94 (m, 5H, CH_3, CH_2) ppm; ^{13}C NMR (100.6 MHz, methanol-d_4): δ = 175.2, 173.9, 166.8, 142.1, 129.6, 129.4, 127.1, 74.1, 53.0, 52.6, 42.7, 42.1, 40.2, 35.3, 34.5, 34.4, 33.6, 32.9, 28.5, 27.4, 27.21, 27.20, 19.8, 16.9 ppm; $[\alpha]_D^{25}$ = −16 (*c* 0.1, MeCN); FTIR (ATR): $\tilde{\nu}$ = 2931, 1653, 1204, 1147, 724 cm^{-1}; LC−MS: t_R = 1.61 min, MeCN/H_2O (0.1% HCO_2H) = 0:0 (0.00−0.30 min) → 100:0 (3.00 min), flow rate: 0.60 mL min^{-1}; MS (ESI +): *m*/*z* (%): 460.6 (100) $[M + H]^+$; HRMS (ESI+): calcd for $[C_{26}H_{41}N_3O_4 + Na]$: *m*/*z* = 482.2989, found: 482.2984.

*N,N-Dimethyl-L-valyl-L-cyclohexylalanyl-L-cycloheylalanine·Trifluoroacetate (**28**).* Synthesis was performed on a 190 μm scale according to general procedure 4, and a white solid was isolated following HPLC purification and lyophilization (97 mg, 90%). ^{1}H NMR (400 MHz, methanol-d_4): δ = 4.62 (dd, *J* = 9.2, 6.5 Hz, 1H, C*H*), 4.47 (dd, *J* = 10.5, 4.6 Hz, 1H, C*H*), 3.63 (d, *J* = 5.6 Hz, 1H, C*H*), 2.90 (s, 6H, NCH_3), 2.41 (dq, *J* = 13.2, 6.6 Hz, 1H, C*H*), 1.86−1.51 (m, 14H, alkyl−*H*), 1.48−1.32 (m, 2H, alkyl−*H*), 1.32−1.15 (m, 6H, alkyl−*H*), 1.12 (d, *J* = 6.9 Hz, 3H, CH_3), 1.04−0.88 (m, 7H, alkyl−*H*) ppm; ^{13}C NMR (100.6 MHz, methanol-d_4): δ = 175.8, 173.7, 166.4, 74.3, 52.4, 51.2, 42.4. 40.5, 40.1, 35.4, 35.3, 34.9, 34.6, 33.7, 33.0, 28.6, 27.5, 27.4, 27.3, 27.2, 20.0, 16.9 ppm; $[\alpha]_D^{25}$ = −18.5 (*c* 0.2, CH_3CN); FTIR (ATR): $\tilde{\nu}$ = 1617, 1498, 1428, 1387, 1139, 783 cm^{-1}; LC−MS: t_R = 3.61 min, MeCN/H_2O (0.1% HCO_2H) = 0:100 (0.00−0.50 min) → 100:0 (8.00 min), flow rate: 0.60 mL min^{-1}; MS (ESI+): *m*/*z* (%): 452.4 (100) $[M + H]^+$; HRMS (ESI+): calcd for $[C_{25}H_{45}N_3O_4 + Na]$: *m*/*z* = 474.3302, found: 474.3297.

*N,N-Dimethyl-L-valyl-L-leucyl-L-cycloheylalanine·Trifluoroacetate (**29**).* Synthesis was performed on a 190 μm scale according to general procedure 4, and a white solid was isolated following HPLC purification and lyophilization (95 mg, 95%). ^{1}H NMR (400 MHz, methanol-d_4): δ = 8.50 (d, *J* = 8.2 Hz, 1H, N*H*), 4.60 (dd, *J* = 8.8, 6.3 Hz, 1H, C*H*), 4.53−4.37 (m, 1H, C*H*), 3.63 (d, *J* = 5.6 Hz, 1H, C*H*), 2.90 (s, 6H, NCH_3), 2.41 (dq, *J* = 13.4, 6.7 Hz, 1H, C*H*), 1.86−1.55 (m, 10H, alkyl−*H*), 1.50−1.39 (m, 1H, alkyl−H), 1.29−1.16 (m, 3H, alkyl−*H*), 1.13 (d, *J* = 6.8 Hz, 3H), 1.00 (2 × d *J* = 6.5, Hz, 6H), 0.97 (d, *J* = 6.2 Hz, 3H), 0.92−0.85 (m, 1H, alkyl−*H*) ppm; ^{13}C NMR (100.6 MHz, methanol-d_4): δ = 175.8, 173.7, 166.5, 74.3, 53.1, 51.2, 41.9, 40.1, 35.3, 34.9, 33.0, 28.6, 27.5, 27.4, 27.2, 25.9, 23.2, 22.1, 20.0, 16.9 ppm; $[\alpha]_D^{25}$ = −15 (*c* 0.4, MeCN); LC−MS: t_R = 1.54 min, MeCN/H_2O (0.1% HCO_2H) = 0:0 (0.00−0.30 min) → 100:0 (3.00 min), flow rate: 0.60 mL min^{-1}; MS (ESI+): *m*/*z* (%): 420.5 (100) $[M + H]^+$; HRMS (ESI+): calcd for $[C_{22}H_{41}N_3O_4 + Na]$: *m*/*z* = 434.2989, found: 434.2985.

*(1-Methylpiperidine-4-carbonyl)-L-cyclohexylalanyl-L-cyclohexylalanine·Trifluoroacetate (**30**).* Synthesis was performed on a 190 μm scale according to general procedure 4, and a white solid was isolated following HPLC purification and lyophilization (87 mg, 81%). ^{1}H NMR (500 MHz, methanol-d_4): δ = 4.52−4.31 (m, 2H), 3.66−3.40 (m, 2H), 3.10−2.93 (m, 2H), 2.87 (s, 3H), 2.62−2.47 (m, 1H), 2.27−1.96 (m, 2H), 1.96−1.83 (m, 1H), 1.83−1.44 (m, 15H), 1.46−1.38 (m, 2H), 1.35−1.13 (m, 6H), 1.08−0.71 (m, 4H) ppm; ^{13}C NMR (125.8 MHz, methanol-d_4): δ = 176.0, 175.3, 174.8, 54.9, 52.4, 51.3, 43.9, 40.5, 40.2, 35.4, 35.3, 34.8, 34.7, 33.5, 33.2, 27.8, 27.6, 27.4, 27.4, 27.3, 27.2 ppm; $[\alpha]_D^{25}$ = −48 (*c* 0.1, MeCN); FTIR (ATR): $\tilde{\nu}$ = 1684, 1558, 1457, 1200, 811 cm^{-1}; LC−MS: t_R = 3.62 min, MeCN/H_2O (0.1% HCO_2H) = 0:100 (0.00−0.50 min) → 100:0 (8.00 min), flow rate: 0.60 mL min^{-1}; MS (ESI+): *m*/*z* (%): 450.4 (100) $[M + H]^+$; HRMS (ESI+): calcd for $[C_{25}H_{43}N_3O_4 + H]$: *m*/*z* = 450.3326, found: 450.3323.

*(1-Methylpiperidine-4-carbonyl)-L-leucyl-L-cyclohexylalanine·Trifluoroacetate (**31**).* Synthesis was performed on a 190 μm scale according to general procedure 4, and a white solid was isolated following HPLC purification and lyophilization (97 mg, 98%). ^{1}H NMR (400 MHz, methanol-d_4): δ = 4.49−4.36 (m, 2H, 2 × C*H*), 3.65−3.42 (m, 2H), 3.12−2.95 (m, 2H), 2.87 (s, 3H, NCH_3), 2.65−2.50 (m 1H), 2.03 (m, 4H), 1.85−1.49 (m, 10H), 1.48−1.12 (m, 1H), 1.07−0.85 (m, 2H), 0.98 (d, *J* = 6.4 Hz, 3H, CH_3), 0.94 (d, *J* = 6.4 Hz, 3H, CH_3) ppm; ^{13}C NMR (100.6 MHz, Methanol-d_4): δ = 175.4, 174.8, 173.3, 54.8, 53.1, 51.4, 43.9, 41.9, 40.4, 40.2, 35.3, 34.8, 33.2, 27.7, 27.6, 27.4, 27.2, 25.9, 23.4, 22.0 ppm; $[\alpha]_D^{25}$ = −19.5 (*c* 0.2, MeCN); FTIR (ATR): $\tilde{\nu}$ = 2925, 1648, 1542, 1199, 1136, 703 cm^{-1}; LC−MS: t_R = 3.10 min, MeCN/H_2O (0.1% HCO_2H) = 0:100 (0.00−0.50 min) → 100:0 (8.00 min), flow rate: 0.60 mL min^{-1}; MS (ESI+): *m*/*z* (%): 410.4 (100) $[M + H]^+$; HRMS (ESI+): calcd for $[C_{22}H_{39}N_3O_4 + H]$: *m*/*z* = 410.3013, found: 410.3011.

*N,N-Dimethyl-L-valyl-L-leucyl-aminoisobutyric Acid·Trifluoroacetate (**32**).* Synthesis was performed on a 160 μm scale according to general procedure 4, and a white solid was isolated following HPLC purification and lyophilization (68 mg, 93%). ^{1}H NMR (400 MHz, CD_3CN): δ = 9.73 (s_{br}, 1H, CO_2H), 8.51 (d, *J* = 8.9 Hz, 1H, N*H*), 7.47 (s, 1H, N*H*), 4.68−4.57 (m, 1H, Leu α−*H*), 3.64 (d, *J* = 8.5 Hz, 1H, Val α−*H*), 2.85 (s_{br}, 6H, 2 × NCH_3), 2.37−2.22 (m, 1H, Val C*H*), 1.68−1.56 (m, 3H, Leu C*H*, CH_2), 1.41 (s, 6H, 2 × Aib CH_3), 1.10 (d, *J* = 6.8 Hz, 3H, CH_3), 0.95 (d, *J* = 6.4 Hz, 3H, CH_3), 0.92 (d, *J* = 6.8 Hz, 6H, 2 × CH_3) ppm; ^{13}C NMR (100.6 MHz, CD_3CN): δ = 175.8, 172.4, 166.6, 72.8, 56.9, 52.5, 42.3, 28.5, 25.7, 25.5, 24.4, 22.9, 22.4, 19.0, 18.3 ppm; $[\alpha]_D^{25}$ = −20 (*c* 0.1, MeCN); FTIR (ATR): $\tilde{\nu}$ = 1643, 1547, 1467, 1368, 1190 cm^{-1}; LC−MS: t_R = 1.28 min, MeCN/H_2O (0.1% HCO_2H) = 0:100 (0.00−0.30 min) → 100:0 (3.00 min), flow rate: 0.60 mL min^{-1}; MS (ESI+): *m*/*z* (%): 344.4 (100) $[M + H]^+$; HRMS (ESI+): calcd for $[C_{17}H_{33}N_3O_4 + Na]$: *m*/*z* = 366.2363, found: 366.2362.

*(S)-N-((S,E)-5-((S)-2-((1H-Indol-3-yl)methyl)-3-methoxy-5-oxo-2,5-dihydro-1H-pyrrol-1-yl)-5-oxopent-3-en-2-yl)-2-((S)-3-cyclohexyl-2-((S)-2-(dimethylamino)-3-methylbutanamido)propanamido)-4-methylpentanamide·Trifluoroacetate (**33**).* Imide fragment **15** (6.80 mg, 15.0 μmol) was coupled to **21** (11.8 mg, 22.5 μmol) using general procedure 7 and purified by preparative RP-HPLC using a focused gradient [MeCN/H_2O (0.1% TFA) = 0:100 (0.00−1.00 min) → 35:65 (4.00 min) → 45:55 (13.00 min), flow rate: 50 mL min^{-1}], yielding **33** as a white solid (9.2 mg, 72%, dr 6:1). ^{1}H NMR (400 MHz, DMSO-d_6): δ = 10.85 (d, *J* = 2.2 Hz, 1H, N*H*), 9.61 (br s, 1H, N*H*), 8.74 (d, *J* = 8.4 Hz, 1H, N*H*), 8.20 (d, *J* = 7.7 Hz, 1H, N*H*),

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8.16 (d, J = 7.6 Hz, 1H, NH), 7.30−7.28 (m, 2H, 2 × Ar−H), 7.19 (dd, J = 15.6, 1.7 Hz, 1H, CH=CHC=O), 7.03−6.89 (m, 3H, 2 × Ar−H, CH=CHC=O), 6.80 (d, J = 2.2 Hz, 1H, Ar−H), 5.05 (s, 1H, CH), 4.92 (dd, J = 4.9, 2.6 Hz, 1H, CH), 4.58−4.49 (m, 2H, 2 × CH), 4.38−4.32 (m, 1H, CH), 3.78 (s, 3H, OCH_3), 3.65 (br m, 1H, CH), 3.60 (dd, J = 14.7, 4.9 Hz, 1H, CHH), 3.20 (dd, J = 14.7, 2.7 Hz, 1H, CHH), 2.76−2.73 (br m, 6H, 2 × NCH_3), 2.33−2.23 (m, 1H, CH), 1.72−1.54 (m, 6H, 3 × CHH, CH_2, CH), 1.53−1.46 (m, 4H, 2 × CH_2), 1.26−1.20 (m, 1H, CH), 1.21 (d, J = 7.3 Hz, 3H, CH_3), 1.18−1.04 (m, 3H, CHH, CH_2), 1.00 (d, J = 7.3 Hz, 3H, CH_3), 0.93−0.84 (m, 2H, 2 × CHH), 0.85 (2 × d, overlapped, 6H, 2 × CH_3), 0.82 (d, J = 6.3 Hz, 3H, CH_3) ppm; ^{13}C NMR, HSQC, HMBC (100.6 MHz, DMSO-d_6): δ = 178.8 (C_q−OMe), 171.1 (C=O), 170.9 (C=O), 169.7 (C=O), 164.8 (C=O), 163.8 (C=O), 149.6 (CH=CHC=O), 135.7 (aryl C_q), 127.9 (aryl C_q), 123.9 (aryl CH), 121.0 (CH=CHC=O), 120.7 (aryl CH), 118.4 (aryl CH), 117.9 (aryl CH), 111.3 (aryl CH), 106.4 (aryl C_q), 94.7 (CH), 71.7 (CH), 59.3 (CH), 58.9 (OCH_3), 50.8 (CH), 50.3 (CH), 45.3 (CH), 41.7 (NCH_3), 41.0 (CH_2), 40.8 (NCH_3), 39.0 (CH_2), 33.6 (CH), 33.2 (CH_2), 31.7 (CH_2), 26.5 (CH), 26.0 (CH_2), 25.8 (CH_2), 25.6, (CH_2), 24.1 (CH), 24.0 (CH_2), 23.1 (CH_3), 21.3 (CH_3), 19.5 (CH_3), 19.2 (CH_3), 16.5 (CH_3) ppm; $[\alpha]_D^{25}$ = +98 (c 0.1, MeCN); FTIR (ATR): $\tilde{\nu}$ = 2930, 1671, 1618, 1340, 1197, 1138, 800 cm^{-1}; LC−MS: t_R = 3.82 min, MeCN/H_2O (0.1% HCO_2H) = 0:100 (0.00−0.50 min) → 100:0 (8.00 min), flow rate: 0.60 mL min^{-1}; MS (ESI+): m/z (%): 733.5 (100) $[M + H]^+$; HRMS (ESI+): calcd for $[C_{41}H_{60}N_6O_6 + H]$: m/z = 733.4647, found: 733.4640.

*N-((S)-1-(((S)-1-(((S,E)-5-((S)-2-((1H-Indol-3-yl)methyl)-3-methoxy-5-oxo-2,5-dihydro-1H-pyrrol-1-yl)-5-oxopent-3-en-2-yl)amino)-4-methyl-1-oxopentan-2-yl)amino)-3-cyclohexyl-1-oxopropan-2-yl)-1-methylpiperidine-4-carboxamide·Trifluoroacetate (**34**).* Imide fragment **15** (6.80 mg, 15.0 μmol) was coupled to **22** (11.8 mg, 22.5 μmol) using general procedure 7 and purified by preparative RP-HPLC using a focused gradient [MeCN/H_2O (0.1% TFA) = 0:100 (0.00−1.00 min) → 35:65 (4.00 min) → 45:55 (13.00 min), flow rate: 50 mL min^{-1}], yielding **34** as a white solid (10.9 mg, 86%, dr 6:1). ^{1}H NMR (400 MHz, DMSO-d_6): δ = 10.85 (d, J = 2.2 Hz, 1H, NH), 9.34 (br s, 1H, NH), 8.15 (d, J = 7.5 Hz, 1H, NH), 8.09 (d, J = 8.2 Hz, 1H, NH), 7.73 (d, J = 8.2 Hz, 1H, NH), 7.30−7.28 (m, 2H, Ar−H), 7.18 (dd, J = 15.7, 1.7 Hz, 1H, CH=CHC=O), 7.03−6.90 (m, 3H, 2 × Ar−H, CH=CHC=O), 6.80 (d, J = 2.2 Hz, 1H, Ar−H), 5.05 (s, 1H, CH), 4.93 (dd, J = 4.9, 2.6 Hz, 1H, CH), 4.57−4.44 (m, 1H, CH), 4.37−4.24 (m, 2H, 2 × CH), 3.78 (s, 3H, OCH_3), 3.60 (dd, J = 14.7, 4.9 Hz, 1H, CHH), 3.37−3.39 (m, 2H, 2 × CHH), 3.20 (dd, J = 14.7, 2.6 Hz, 1H, CHH), 2.99−2.83 (m, 2H, 2 × CHH), 2.75 (br s, 3H, NCH_3), 2.43 (tt, J = 12.1, 3.6 Hz, 1H, CH), 1.92−1.80 (m, 2H, CH_2), 1.76−1.35 (m, 12H, CH, CH_2), 1.33−1.02 (m, 8H, CH, CH_2, CH_3), 0.97−0.74 (m, 8H, 2 × CH_3, CH_2) ppm; ^{13}C NMR, HSQC, HMBC (100.6 MHz, DMSO-d_6): δ = 178.8, 172.7, 171.7, 171.2, 169.7, 163.8, 149.5, 135.7, 127.9, 123.9, 121.1, 120.8, 118.4, 117.9, 111.4, 106.4, 94.6, 59.3, 59.0, 52.8, 52.8, 50.7, 50.4, 45.3, 42.6, 41.2, 39.0, 38.4, 33.6, 33.2, 31.8, 26.1, 26.0, 25.8, 25.7, 24.2, 24.0, 23.1, 21.5, 19.5 ppm; $[\alpha]_D^{25}$ = +91 (c 0.1, MeCN); FTIR (ATR): $\tilde{\nu}$ = 2938, 1638, 1543, 1458, 1341, 1178 cm^{-1}; LC−MS: t_R = 3.84 min, MeCN/H_2O (0.1% HCO_2H) = 0:100 (0.00−0.50 min) → 100:0 (8.00 min), flow rate: 0.60 mL min^{-1}; MS (ESI+): m/z (%): 731.5 (100) $[M + H]^+$; HRMS (ESI+): calcd for $[C_{41}H_{58}N_6O_6$ + Na]: m/z = 753.4310, found: 731.4302.

*N-((S)-1-(((S)-1-(((S,E)-5-((S)-2-((1H-Indol-3-yl)methyl)-3-methoxy-5-oxo-2,5-dihydro-1H-pyrrol-1-yl)-5-oxopent-3-en-2-yl)amino)-4-methyl-1-oxopentan-2-yl)amino)-4-methyl-1-oxopentan-2-yl)-1-methylpiperidine-4-carboxamide·Trifluoroacetate (**35**).* Imide fragment **15** (6.80 mg, 15.0 μmol) was coupled to **23** (10.9 mg, 22.5 μmol) using general procedure 7 and purified by preparative RP-HPLC using a focused gradient [MeCN/H_2O (0.1% TFA) = 0:100 (0.00−1.00 min) → 35:65 (4.00 min) → 45:55 (13.00 min), flow rate: 50 mL min^{-1}], yielding **35** as a white solid (9.4 mg, 78%, dr 6:1). ^{1}H NMR (400 MHz, DMSO-d_6): δ = 10.85 (d, J = 2.2 Hz, 1H, NH), 9.30 (s, 1H, NH), 8.15 (d, J = 8.2 Hz, 1H, NH), 8.10 (d, J = 8.0 Hz, 1H, NH), 7.73 (d, J = 8.5 Hz, 1H, NH), 7.33−7.25 (m, 2H, Ar−H), 7.20 (dd, J = 15.7, 1.7 Hz, 1H, CH=CHC=O), 7.01−6.90 (m, 3H, 2 × Ar−H, CH=CHC=O), 6.80 (d, J = 2.2 Hz, 1H, Ar−H), 5.05 (s, 1H, CH), 4.93 (dd, J = 4.9, 2.6 Hz, 1H, CH), 4.58−4.44 (m, 1H, CH), 4.36−4.22 (m, 2H, 2 × CH), 3.78 (s, 3H, OCH_3), 3.60 (dd, J = 14.7, 4.9 Hz, 1H, CHH), 3.37−3.39 (m, 2H, 2 × CHH), 3.20 (dd, J = 14.7, 2.6 Hz, 1H, CHH), 2.99−2.83 (m, 2H, 2 × CHH), 2.74 (d, J = 4.2 Hz, 3H, NCH_3), 2.43 (tt, J = 12.1, 3.6 Hz, 1H, CH), 1.93−1.84 (m, 2H, 2 × CHH), 1.80−1.64 (m, 2H, 2 × CHH), 1.62−1.41 (m, 6H, CH, CH_2), 1.21 (d, J = 7.1 Hz, 3H, CH_3), 0.90−0.79 (m, 12H, 4 × CH_3) ppm; ^{13}C NMR (100.6 MHz, DMSO-d_6): δ = 178.8, 172.8, 171.7, 171.2, 169.7, 163.8, 149.5, 135.7, 127.9, 123.9, 121.1, 120.8, 118.4, 117.9, 111.4, 106.4, 94.6, 59.3, 59.0, 52.8, 51.0, 50.7, 45.3, 42.6, 41.2, 40.5, 38.4, 26.1, 25.9, 24.3, 24.2, 24.0, 23.1, 23.0, 21.5, 19.5 ppm; $[\alpha]_D^{25}$ = +93 (c 0.1, MeCN); FTIR (ATR): $\tilde{\nu}$ = 2937, 1638, 1543, 1458, 1341, 1177, 1131 cm^{-1}; LC−MS: t_R = 3.55 min, MeCN/H_2O (0.1% HCO_2H) = 0:100 (0.00−0.50 min) → 100:0 (8.00 min), flow rate: 0.60 mL min^{-1}; MS (ESI+): m/z (%): 691.6 (100) $[M + H]^+$; HRMS (ESI+): calcd for $[C_{38}H_{54}N_6O_6 + H]$: m/z = 691.4178, found: 691.4171.

*(S)-N-((S,E)-5-((S)-2-((1H-Indol-3-yl)methyl)-3-methoxy-5-oxo-2,5-dihydro-1H-pyrrol-1-yl)-5-oxopent-3-en-2-yl)-2-((S)-2-((S)-2-(dimethylamino)-3-methylbutanamido)-3-phenylpropanamido)-4-methylpentanamide·Trifluoroacetate (**36**).* Imide fragment **15** (6.80 mg, 15.0 μmol) was coupled to **24** (11.4 mg, 22.5 μmol) using general procedure 7 and purified by preparative RP-HPLC using a focused gradient [MeCN/H_2O (0.1% TFA) = 0:100 (0.00−1.00 min) → 35:65 (4.00 min) → 45:55 (13.00 min), flow rate: 50 mL min^{-1}], yielding **36** as a white solid (11.6 mg, 92%, dr 6:1). ^{1}H NMR (400 MHz, DMSO-d_6): δ = 10.85 (d, J = 2.2 Hz, 1H, NH), 9.35 (br s, 1H, NH), 8.91 (d, J = 9.2 Hz, 1H, NH), 8.30 (d, J = 8.2 Hz, 1H, NH), 8.27 (d, J = 7.8 Hz, 1H, NH), 7.31−7.14 (m, 8H, 7 × Ar−H, CHC=CHC=O), 7.04−6.98 (m, 2H, Ar−H, CHCHC=O), 6.94−6.92 (m, 1H, Ar−H), 6.80 (d, J = 2.2 Hz, 1H, Ar−H), 5.05 (s, 1H, CH), 4.93 (dd, J = 4.9, 2.7 Hz, 1H, CH), 4.91−4.87 (m, 1H, CH), 4.57−4.50 (m, 1H, CH), 4.41−4.36 (m, 1H, CH), 3.79 (s, 3H, OCH_3), 3.61 (dd, J = 14.7, 4.9 Hz, 1H, CHH), 3.45−3.43 (m, 1H, CH, overlap with water signal), 3.20 (dd, J = 14.7, 2.7 Hz, 1H, CHH), 3.11 (dd, J = 13.8, 3.8 Hz, CHHPh) Hz, 2.72 (dd, J = 13.8, 11.7 Hz, CHHPh), 2.48 (br s, 3H, NCH_3, overlap with DMSO-d_6 signal), 2.22−2.16 (m, 4H, NCH_3, CH), 1.64−1.49 (m, 3H, CH, CH_2), 1.23 (d, J = 6.8 Hz, 3H, CH_3), 0.94 (d, J = 6.9 Hz, 3H, CH_3), 0.88 (d, J = 6.3 Hz, 3H, CH_3), 0.85 (d, J = 6.2 Hz, 3H, CH_3), 0.80 (d, J = 6.7 Hz, 3H, CH_3) ppm; ^{13}C NMR, HSQC, HMBC (100.6 MHz, DMSO-d_6): δ = 178.8 (C_q−OMe), 171.1 (C=O), 170.2 (C=O), 169.8 (C=O), 164.5 (C=O), 163.8 (C=O), 149.7 (CH=CHC=O), 137.6 (aryl C_q), 135.6 (aryl C_q), 129.3 (aryl CH), 128.0 (aryl CH), 127.9 (aryl C_q), 126.3 (aryl CH), 123.9 (aryl CH), 121.0 (CH=CHC=O), 120.8 (aryl CH), 118.4 (aryl CH), 117.9 (aryl CH), 111.4 (aryl CH), 106.4 (aryl C_q), 94.6 (CH), 71.6 (CH), 59.3 (CH), 58.9 (OCH_3), 53.4 (CH), 51.0 (CH), 45.3 (CH), 41.2 (CH_2), 41.1 (NCH_3), 40.5 (NCH_3), 37.8 (CH_2), 26.3 (CH), 24.3 (CH), 24.0 (CH_2), 23.1 (CH_3), 21.2 (CH_3), 19.4 (CH_3), 19.1 (CH_3), 16.4 (CH_3) ppm; $[\alpha]_D^{25}$ = +94 (c 0.1, MeCN); FTIR (ATR): $\tilde{\nu}$ = 1651, 1452, 798, 724 cm^{-1}; LC−MS: t_R = 3.69 min, MeCN/H_2O (0.1% HCO_2H) = 0:100 (0.00−0.50 min) → 100:0 (8.00 min), flow rate: 0.60 mL min^{-1}; MS (ESI+): m/z (%): 727.6 (100) $[M + H]^+$; HRMS (ESI+): calcd for $[C_{41}H_{54}N_6O_6 + H]$: m/z = 727.4178, found: 727.4170.

*(S)-N-((S,E)-5-((S)-2-((1H-Indol-3-yl)methyl)-3-methoxy-5-oxo-2,5-dihydro-1H-pyrrol-1-yl)-5-oxopent-3-en-2-yl)-2-((S)-2-((S)-2-(dimethylamino)-3-methylbutanamido)-4-phenylbutanamido)-4-methylpentanamide·Trifluoroacetate (**37**).* Imide fragment **15** (6.80 mg, 15.0 μmol) was coupled to **25** (12.0 mg, 22.5 μmol) using general procedure 7 and purified by preparative RP-HPLC using a focused gradient [MeCN/H_2O (0.1% TFA) = 0:100 (0.00−1.00 min) → 35:65 (4.00 min) → 45:55 (13.00 min), flow rate: 50 mL min^{-1}], yielding **37** as a white solid (10.6 mg, 83%, dr 6:1). ^{1}H NMR (400 MHz, DMSO-d_6): δ = 10.85 (d, J = 2.2 Hz, 1H, NH), 9.62 (br s, 1H, NH), 8.86 (d, J = 7.0 Hz, 1H, NH), 8.25 (d, J = 8.2 Hz, 1H, NH), 8.18 (d, J = 7.8 Hz, 1H, NH), 7.30−7.24 (m, 4H, 4 × Ar−H), 7.21−7.12 (m, 4H, 3 × Ar−H, CH=CHC=O), 7.03−6.89 (m, 3H, 2 ×

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J. Med. Chem. XXXX, XXX, XXX−XXX

Ar−*H*, C*H*=CHC=O), 6.80 (d, *J* = 2.2 Hz, 1H, Ar−*H*), 5.04 (s, 1H, C*H*), 4.92 (dd, *J* = 4.9, 2.7 Hz, 1H, C*H*), 4.55−4.47 (m, 2H, 2 × C*H*), 4.40−4.32 (m, 1H, C*H*), 3.78 (s, 3H, OCH_3), 3.70 (br m, 1H, C*H*), 3.60 (dd, *J* = 14.7, 4.9 Hz, 1H, CH*H*), 3.19 (dd, *J* = 14.7, 2.7 Hz, 1H, C*H*H), 2.76 (br s, 6H, 2 × NCH_3), 2.63−2.52 (m, 2H, CH_2), 2.33−2.26 (m, 1H, C*H*), 1.98−1.85 (m, 2H, CH_2), 1.67−1.58 (m, 1H, C*H*), 1.53−1.49 (m, 2H, CH_2), 1.20 (d, *J* = 6.9 Hz, 3H, CH_3), 1.04 (d, *J* = 6.7 Hz, 3H, CH_3), 0.87 (d, *J* = 6.4 Hz, 3H, CH_3), 0.86 (d, *J* = 6.2 Hz, 3H, CH_3), 0.83 (d, *J* = 6.5 Hz, 3H, CH_3) ppm; ^{13}C NMR, HSQC, HMBC (100.6 MHz, DMSO-d_6): δ = 178.8 (C$_q$−OMe), 171.2 (*C*=O), 170.3 (*C*=O), 169.7 (*C*=O), 165.1 (*C*=O), 163.8 (*C*=O), 149.6 (CH=CHC=O), 141.3 (aryl C$_q$), 135.7 (aryl C$_q$), 128.4 (aryl CH), 128.2 (aryl CH), 127.9 (aryl C$_q$), 125.9 (aryl CH), 123.9 (aryl CH), 121.0 (CH=*C*HC=O), 120.8 (aryl CH), 118.4 (aryl CH), 117.9 (aryl CH), 111.4 (aryl CH), 106.4 (aryl C$_q$), 94.7 (CH), 71.7 (CH), 59.3 (CH), 58.9 (OCH_3), 52.6 (CH), 50.9 (CH), 45.3 (CH), 41.7 (CH_2), 41.02, 40.96 (2 × NCH_3), 33.8 (CH_2), 31.5 (CH_2), 26.5 (CH), 24.2 (CH), 24.0 (CH_2), 23.1 (CH_3), 21.3 (CH_3), 19.5 (CH_3), 19.2 (CH_3), 16.5 (CH_3) ppm; $[\alpha]_D^{25}$ = +80 (*c* 0.1, MeCN); FTIR (ATR): ṽ = 2930, 1644, 1543, 1354, 1201, 1131, 801, 744, 721 cm^{-1}; LC−MS: t_R = 3.82 min, MeCN/H_2O (0.1% HCO_2H) = 0:100 (0.00−0.50 min) → 100:0 (8.00 min), flow rate: 0.60 mL min^{-1}; MS (ESI+): *m*/*z* (%): 741.7 (100) [M + H]$^+$; HRMS (ESI+): calcd for [$C_{42}H_{56}N_6O_6$ + H]: *m*/*z* = 741.4334, found: 741.4328.

*(S)-N-((S)-1-(((S,E)-5-((S)-2-((1H-Indol-3-yl)methyl)-3-methoxy-5-oxo-2,5-dihydro-1H-pyrrol-1-yl)-5-oxopent-3-en-2-yl)amino)-1-oxo-4-phenylbutan-2-yl)-2-((S)-2-(dimethylamino)-3-methylbutanamido)-4-methylpentanamide·Trifluoroacetate (**38**).* Imide fragment **15** (6.80 mg, 15.0 μmol) was coupled to **26** (12.0 mg, 22.5 μmol) using general procedure 7 and purified by preparative RP-HPLC using a focused gradient [MeCN/H_2O (0.1% TFA) = 0:100 (0.00−1.00 min) → 40:60 (4.00 min) → 50:50 (13.00 min), flow rate: 50 mL min^{-1}], yielding **38** as a white solid (11.5 mg, 90%, dr 6:1). ^{1}H NMR (400 MHz, DMSO-d_6): δ = 10.83 (br s, 1H, N*H*), 9.57 (br s, 1H, N*H*), 8.78 (d, *J* = 8.5 Hz, 1H, N*H*), 8.30 (d, *J* = 7.8 Hz, 1H, N*H*), 8.21 (d, *J* = 7.5 Hz, 1H, N*H*), 7.29−7.12 (m, 8H, 7 × Ar−*H*, CH=C*H*C=O), 7.02−6.87 (m, 3H, 2 × Ar−*H*, C*H*=CHC=O), 6.80 (d, *J* = 2.2 Hz, 1H, Ar−*H*), 5.03 (s, 1H, C*H*), 4.92 (dd, *J* = 4.9, 2.7 Hz, 1H, C*H*), 4.60−4.51 (m, 2H, 2 × C*H*), 4.31−4.26 (m, 1H, C*H*), 3.78 (s, 3H, OCH_3), 3.66 (br m, 1H, C*H*), 3.60 (dd, *J* = 14.7, 4.9 Hz, 1H, CH*H*), 3.19 (dd, *J* = 14.7, 2.7 Hz, 1H, C*H*H), 2.76 (br s, 6H, 2 × NCH_3), 2.64−2.55 (m, 3H), 2.33−2.26 (m, 1H), 2.04−1.97 (m, 1H), 1.62−1.50 (m, 3H), 1.20 (d, *J* = 6.9 Hz, 3H, CH_3), 1.03 (d, *J* = 6.7 Hz, 3H, CH_3), 0.93 (d, *J* = 6.7 Hz, 3H, CH_3), 0.89 (2 × d, 6H overlapped, 2 × CH_3) ppm; ^{13}C NMR (100.6 MHz, DMSO-d_6): δ = 177.8, 170.1, 169.5 168.8, 164, 162.8, 148.4, 140.2, 134.7, 127.3, 127.2, 126.9, 124.7, 122.9, 120.2, 119.8, 117.4, 116.9, 110.4, 105.4, 93.6, 70.7, 58.3, 58.0, 51.2, 50.2, 44.4, 40.8, 39.9, 39.4, 32.7, 30.4, 25.6, 23.3, 23.0, 22.0, 20.3, 18.5, 18.3, 15.5 ppm; $[\alpha]_D^{25}$ = +106 (*c* 0.1, MeCN); FTIR (ATR): ṽ = 2930, 1644, 1544, 1453, 1354, 1201, 1131, 801, 744, 722 cm^{-1}; LC−MS: t_R = 3.93 min, MeCN/H_2O (0.1% HCO_2H) = 0:0 (0.00−0.50 min) → 100:0 (8.00 min), flow rate: 0.60 mL min^{-1}; MS (ESI+): *m*/*z* (%): 741.7 (100) [M + H]$^+$; HRMS (ESI+): calcd for [$C_{42}H_{56}N_6O_6$ + H]: *m*/*z* = 741.4334, found: 741.4328.

*(S)-N-((S,E)-5-((S)-2-((1H-Indol-3-yl)methyl)-3-methoxy-5-oxo-2,5-dihydro-1H-pyrrol-1-yl)-5-oxopent-3-en-2-yl)-2-((S)-3-cyclohexyl-2-((S)-2-(dimethylamino)-3-methylbutanamido)propanamido)-4-phenylbutanamide·Trifluoroacetate (**39**).* Imide fragment **15** (6.80 mg, 15.0 μmol) was coupled to **27** (12.9 mg, 22.5 μmol) using general procedure 7 and purified by preparative RP-HPLC using a focused gradient [MeCN/H_2O (0.1% TFA) = 0:100 (0.00−1.00 min) → 25:60 (4.00 min) → 35:65 (11.00 min) → 40:60 (11.50 min) → 50:50 (20.00 min) flow rate: 50 mL min^{-1}], yielding **39** as a white solid (12.5 mg, 87%, dr 6:1). ^{1}H NMR (400 MHz, DMSO-d_6): δ = 10.85 (d, *J* = 2.0 Hz, 1H, N*H*), 9.67 (br s, 1H, N*H*), 8.81 (d, *J* = 8.5 Hz, 1H, N*H*), 8.30 (d, *J* = 7.8 Hz, 1H, N*H*), 8.23 (d, *J* = 7.5 Hz, 1H, N*H*), 7.29−7.12 (m, 8H, 7 × Ar−*H*, CH=C*H*C=O), 7.02−6.87 (m, 3H, 2 × Ar−*H*, C*H*=CHC=O), 6.81 (d, *J* = 2.2 Hz, 1H, Ar−*H*), 5.03 (s, 1H, C*H*), 4.92 (dd, *J* = 4.9, 2.7 Hz, 1H, C*H*), 4.64−4.52 (m, 2H, 2 × C*H*), 4.32−4.26 (m, 1H, C*H*), 3.78 (s, 3H, OCH_3), 3.68 (br m, 1H, C*H*), 3.60 (dd, *J* = 14.7, 4.9 Hz, 1H, CH*H*), 3.20 (dd, *J* = 14.7, 2.7 Hz, 1H, C*H*H), 2.76 (br s, 6H, 2 × NCH_3), 2.64−2.51 (m, 2H, CH_2), 2.33−2.24 (m, 1H, C*H*), 2.04−1.97 (m, 1H, C*H*H), 1.88−1.81 (m, 1H, CH*H*), 1.72−1.51 (m, 7H, CH_2), 1.35−1.25 (m, 1H, C*H*), 1.20 (d, *J* = 6.9 Hz, 3H, CH_3), 1.20−1.05 (m, 3H, CH_2), 1.03 (d, *J* = 6.7 Hz, 3H, CH_3), 0.98−0.84 (m, 4H, CH_2), 0.89 (d, *J* = 6.7 Hz, 3H, CH_3) ppm; ^{13}C NMR (100.6 MHz, DMSO-d_6): δ = 178.8, 171.2, 170.5, 169.8, 165.0, 163.8, 149.4, 141.2, 135.7, 128.3, 128.2, 127.9, 125.7, 123.9, 121.2, 120.7, 118.4, 117.9, 111.3, 106.4, 94.6, 71.6, 59.3, 58.9, 52.2, 50.4, 45.3, 41.5, 40.9, 33.8, 33.7, 33.2, 31.6, 31.4, 26.5, 26.0, 25.8, 25.6, 24.0, 19.5, 19.3, 16.6 ppm; $[\alpha]_D^{25}$ = +96 (*c* 0.1, MeCN); FTIR (ATR): ṽ = 1644, 1544, 1454, 1353, 1130, 801, 744, 722 cm^{-1}; LC−MS: t_R = 4.09 min, MeCN/H_2O (0.1% HCO_2H) = 0:0 (0.00−0.50 min) → 100:0 (8.00 min), flow rate: 0.60 mL min^{-1}; MS (ESI+): *m*/*z* (%): 781.8 (100) [M + H]$^+$; HRMS (ESI+): calcd for [$C_{45}H_{60}N_6O_6$ + H]: *m*/*z* = 781.4647, found: 781.4639.

*(S)-N-((S)-1-(((S)-1-(((S,E)-5-((S)-2-((1H-Indol-3-yl)methyl)-3-methoxy-5-oxo-2,5-dihydro-1H-pyrrol-1-yl)-5-oxopent-3-en-2-yl)amino)-3-cyclohexyl-1-oxopropan-2-yl)amino)-3-cyclohexyl-1-oxopropan-2-yl)-2-(dimethylamino)-3-methylbutanamide·Trifluoroacetate (**40**).* Imide fragment **15** (6.80 mg, 22.5 μmol) was coupled to **28** (12.7 mg, 22.5 μmol) using general procedure 7 and purified by preparative RP-HPLC using a focused gradient [MeCN/H_2O (0.1% TFA) = 0:100 (0.00−1.00 min) → 40:60 (4.00 min) → 50:50 (13.00 min), flow rate: 50 mL min^{-1}], yielding **40** as a white solid (12.4 mg, 93%, dr 6:1). ^{1}H NMR (400 MHz, DMSO-d_6): δ = 10.85 (d, *J* = 2.4 Hz, 1H, N*H*), 9.66 (br s, 1H, N*H*), 8.76 (d, *J* = 8.1 Hz, 1H, N*H*), 8.19 (d, *J* = 7.8 Hz, 1H, N*H*), 8.15 (d, *J* = 7.8 Hz, 1H, N*H*), 7.30−7.29 (m, 2H, 2 × Ar−*H*), 7.19 (dd, *J* = 15.7, 1.7 Hz, 1H, CH=C*H*C=O), 7.03−6.89 (m, 3H, 2 × Ar−*H*, C*H*=CHC=O), 6.80 (d, *J* = 2.4 Hz, 1H, Ar−*H*), 5.05 (s, 1H, C*H*), 4.92 (dd, *J* = 4.9, 2.6 Hz, 1H, C*H*), 4.53−4.49 (m, 1H, C*H*), 4.33−4.24 (m, 2H, 2 × C*H*), 3.78 (s, 3H, OCH_3), 3.67 (br m, 1H, C*H*), 3.60 (dd, *J* = 14.7, 4.9 Hz, 1H, CH*H*), 3.21 (dd, *J* = 14.7, 2.7 Hz, 1H, C*H*H), 2.75 (br s, 6H, 2 × NCH_3), 2.34−2.24 (m, 1H, C*H*), 1.72−1.44 (m, 14H, CH_2), 1.31 1.17 (m, 4H, C*H*, CH_2, CH_3), 1.13−1.03 (m, 5H, CH_2), 1.00 (d, *J* = 6.6 Hz, 3H, CH_3), 0.94−0.81 (m, 7H, CH_2, CH_3) ppm; ^{13}C NMR, HSQC, HMBC (100.6 MHz, DMSO-d_6): δ = 178.8 (C$_q$−OMe), 171.2 (*C*=O), 170.8 (*C*=O), 169.7 (*C*=O), 164.8 (*C*=O), 163.8 (*C*=O), 149.6 (CH=CHC=O), 135.7 (aryl C$_q$), 127.9 (aryl C$_q$), 123.9 (aryl CH), 121.1 (CH=*C*HC=O), 120.7 (aryl CH), 118.4 (aryl CH), 117.9 (aryl CH), 111.4 (aryl CH), 106.4 (aryl C$_q$), 94.7 (CH), 71.6 (CH), 59.3 (CH), 58.9 (OCH_3), 50.9 (CH), 50.0 (CH), 45.3 (CH), 41.5 (NCH_3), 40.9 (NCH_3), 39.4 (CH_2), 33.6 (CH), 33.5(CH_2), 33.2 (CH_2), 31.7 (CH_2), 31.5 (CH_2), 26.5 (CH), 26.1 (CH_2), 25.8 (CH_2), 25.6 (CH_2), 25.5 (CH_2), 24.0 (CH_2), 19.5 (CH_3), 19.3 (CH_3), 16.6 (CH_3) ppm; $[\alpha]_D^{25}$ = +85 (*c* 0.1, MeCN); FTIR (ATR): ṽ = 2924, 1647, 1542, 1362, 1202, 1134, 802, 722 cm^{-1}; LC−MS: t_R = 1.91 min, MeCN/H_2O (0.1% HCO_2H) = 0:100 (0.00−0.30 min) → 100:0 (3.00 min), flow rate: 0.60 mL min^{-1}; MS (ESI+): *m*/*z* (%): 773.7 (100) [M + H]$^+$; HRMS (ESI+): calcd for [$C_{44}H_{64}N_6O_6$ + H]: *m*/*z* = 773.4960, found: 773.4954.

*(S)-N-((S)-1-(((S,E)-5-((S)-2-((1H-Indol-3-yl)methyl)-3-methoxy-5-oxo-2,5-dihydro-1H-pyrrol-1-yl)-5-oxopent-3-en-2-yl)amino)-3-cyclohexyl-1-oxopropan-2-yl)-2-((S)-2-(dimethylamino)-3-methylbutanamido)-4-methylpentanamide·Trifluoroacetate (**41**).* Imide fragment **15** (6.80 mg, 15.0 μmol) was coupled to **29** (11.8 mg, 22.5 μmol) using general procedure 7 and purified by preparative RP-HPLC using a focused gradient [MeCN/H_2O (0.1% TFA) = 0:100 (0.00−1.00 min) → 35:65 (4.00 min) → 45:55 (13.00 min), flow rate: 50 mL min^{-1}], yielding **41** as a white solid (9.0 mg, 71%, dr 6:1). ^{1}H NMR (400 MHz, DMSO-d_6): δ = 10.85 (d, *J* = 2.6 Hz, 1H, N*H*), 9.58 (br s, 1H, N*H*), 8.74 (d, *J* = 8.1 Hz, 1H, N*H*), 8.17 (m, 2H, N*H*), 7.31−7.26 (m, 2H, 2 × Ar−*H*), 7.19 (dd, *J* = 15.7, 1.7 Hz, 1H, CH=C*H*C=O), 7.03−6.89 (m, 3H, 2 × Ar−*H*, C*H*=CHC=O), 6.80 (d, *J* = 2.6 Hz, 1H, Ar−*H*), 5.05 (s, 1H, C*H*), 4.92 (dd, *J* = 4.9, 2.6 Hz, 1H, C*H*), 4.57−4.48 (m, 2H, 2 × C*H*), 4.40−4.34 (m, 1H, C*H*), 3.78 (s, 3H, OCH_3), 3.65 (br m, 1H, C*H*), 3.60 (dd, *J* = 14.7,

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J. Med. Chem. XXXX, XXX, XXX−XXX

4.9 Hz, 1H, CH*H*), 3.20 (dd, *J* = 14.7, 2.7 Hz, 1H, C*H*H), 2.76−2.73 (br m, 6H, 2 × NCH_3), 2.34−2.24 (m, 1H, C*H*), 1.72−1.44 (m, 10H, C*H*, 3 × C*H*H, 3 × CH_2), 1.26−1.20 (m, 1H, C*H*), 1.21 (d, *J* = 7.9 Hz, 3H, CH_3), 1.15−1.05 (m, 3H, CH*H*, CH_2), 1.00 (d, *J* = 7.0 Hz, 3H, CH_3), 0.95−0.81 (m, 2H, 2 × CH*H*), 0.91 (d, *J* = 6.1 Hz, 3H, CH_3), 0.86 (2 × d, *J* = 6.5 Hz, 6H, 2 × CH_3) ppm; ^{13}C NMR, HSQC, HMBC (100.6 MHz, DMSO-d_6): δ = 178.8 (C$_q$−OMe), 171.2 (*C*=O), 170.8 (*C*=O), 169.7 (*C*=O), 164.8 (*C*=O), 163.8 (*C*=O), 149.6 (CH=CHC=O), 135.7 (aryl C$_q$), 127.9 (aryl C$_q$), 123.9 (aryl CH), 121.0 (CH=CHC=O), 120.7 (aryl *C*H), 118.4 (aryl CH), 117.9 (aryl CH), 111.3 (aryl CH), 106.4 (aryl C$_q$), 94.7 (CH), 71.6 (CH), 59.3 (CH), 58.9 (OCH_3), 50.9 (CH), 50.0 (CH), 45.3 (CH), 41.7 (NCH_3), 40.9 (CH_2), 40.7 (NCH_3), 39.6 (CH_2), 33.5(CH), 33.3 (CH_2), 31.4 (CH_2), 26.5 (CH), 26.1 (CH_2), 25.8 (CH_2), 25.5, (CH_2), 24.3 (CH), 24.0 (CH_2), 23.0 (CH_3), 21.4 (CH_3), 19.5 (CH_3), 19.3 (CH_3), 16.6 (CH_3) ppm; $[\alpha]_D^{25}$ = +93 (*c* 0.1, MeCN); FTIR (ATR): $\tilde{\nu}$ = 2936, 1646, 1541, 1457, 1354, 1201, 1131, 801, 721 cm^{-1}; LC−MS: t_R = 1.81 min, MeCN/H_2O (0.1% HCO_2H) = 0:100 (0.00−0.30 min) → 100:0 (3.00 min), flow rate: 0.60 mL min^{-1}; MS (ESI+): *m*/*z* (%): 733.7 (100) [M + H]$^+$; HRMS (ESI+): calcd for [$C_{41}H_{60}N_6O_6$ + Na]: *m*/*z* = 755.4467, found: 755.4457.

*N-((S)-1-(((S)-1-(((S,E)-5-((S)-2-((1H-Indol-3-yl)methyl)-3-methoxy-5-oxo-2,5-dihydro-1H-pyrrol-1-yl)-5-oxopent-3-en-2-yl)amino)-3-cyclohexyl-1-oxopropan-2-yl)amino)-3-cyclohexyl-1-oxopropan-2-yl)-1-methylpiperidine-4-carboxamide·Trifluoroacetate (**42**).* Imide fragment **15** (6.80 mg, 15.0 μmol) was coupled to **30** (12.7 mg, 22.5 μmol) using general procedure 7 and purified by preparative RP-HPLC using a focused gradient [MeCN/H_2O (0.1% TFA) = 0:100 (0.00−1.00 min) → 37:63 (4.00 min) → 47:53 (13.00 min), flow rate: 50 mL min^{-1}], yielding **42** as a white solid (9.0 mg, 68%, dr 6:1). ^{1}H NMR (400 MHz, DMSO-d_6): δ = 10.85 (d, *J* = 2.2 Hz, 1H, N*H*), 9.31 (br s, 1H, N*H*), 8.13 (d, *J* = 7.7 Hz, 1H, N*H*), 8.09 (d, *J* = 8.5 Hz, 1H, N*H*), 7.72 (d, *J* = 8.2 Hz, 1H, N*H*), 7.30−7.28 (m, 2H, Ar−*H*), 7.18 (dd, *J* = 15.7, 1.7 Hz, 1H, CH=C*H*C=O), 7.03−6.90 (m, 3H, 2 × Ar−*H*, C*H*=CHC=O), 6.80 (d, *J* = 2.2 Hz, 1H, Ar−*H*), 5.04 (s, 1H, C*H*), 4.93 (dd, *J* = 4.9, 2.6 Hz, 1H, C*H*), 4.54−4.45 (m, 1H, C*H*), 4.36−4.23 (m, 2H, 2 × C*H*), 3.78 (s, 3H, OCH_3), 3.60 (dd, *J* = 14.7, 4.9 Hz, 1H, CH*H*), 3.49−3.41 (m, 2H, 2 × C*H*H), 3.20 (dd, *J* = 14.7, 2.6 Hz, 1H, CH*H*), 2.96−2.89 (m, 2H, 2 × CH*H*), 2.75 (d, *J* = 4.6 Hz, 3H, NCH_3), 2.43 (tt, *J* = 12.1, 3.6 Hz, 1H, C*H*), 1.92−1.87 (m, 2H, CH_2), 1.76−1.38 (m, 16H, CH_2), 1.33−1.02 (m, 12H, C*H*, CH_2, CH_3), 0.94−0.76 (m, 4H, CH_2) ppm; ^{13}C NMR, HSQC, HMBC (100.6 MHz, DMSO-d_6): δ = 178.8, 172.7, 171.7, 171.3, 169.7, 163.8, 149.4, 135.7, 127.9, 124.0, 121.2, 120.8, 118.5, 117.9, 111.4, 106.4, 94.6, 59.3, 59.0, 52.8, 50.5, 50.0, 45.3, 42.7, 39.4, 38.4, 33.6, 33.5, 33.2, 33.1, 31.9, 31.7, 26.1, 26.0, 25.9, 25.8, 25.7, 25.6, 24.1, 19.5 ppm; $[\alpha]_D^{25}$ = +83 (*c* 0.1, MeCN); FTIR (ATR): $\tilde{\nu}$ = 2937, 1639, 1543, 1458, 1341, 1178 cm^{-1}; LC−MS: t_R = 4.02 min, MeCN/H_2O (0.1% HCO_2H) = 0:100 (0.00−0.50 min) → 100:0 (8.00 min), flow rate: 0.60 mL min^{-1}; MS (ESI+): *m*/*z* (%): 771.8 (100) [M + H]$^+$; HRMS (ESI+): calcd for [$C_{44}H_{62}N_6O_6$ + Na]: *m*/*z* = 793.4323, found: 793.4617.

*N-((S)-1-(((S)-1-(((S,E)-5-((S)-2-((1H-Indol-3-yl)methyl)-3-methoxy-5-oxo-2,5-dihydro-1H-pyrrol-1-yl)-5-oxopent-3-en-2-yl)amino)-3-cyclohexyl-1-oxopropan-2-yl)amino)-4-methyl-1-oxopentan-2-yl)-1-methylpiperidine-4-carboxamide·Trifluoroacetate (**43**).* Imide fragment **15** (6.80 mg, 15.0 μmol) was coupled to **31** (11.8 mg, 22.5 μmol) using general procedure 7 and purified by preparative RP-HPLC using a focused gradient [MeCN/H_2O (0.1% TFA) = 0:100 (0.00−1.00 min) → 35:65 (4.00 min) → 45:55 (13.00 min), flow rate: 50 mL min^{-1}], yielding **43** as a white solid (12.0 mg, 95%, dr 6:1). ^{1}H NMR (400 MHz, DMSO-d_6): δ = 10.85 (d, *J* = 2.2 Hz, 1H, N*H*), 9.37 (s, 1H, N*H*), 8.11 (m, 2H, N*H*), 7.73 (d, *J* = 8.5 Hz, 1H, N*H*), 7.30−7.28 (m, 2H, Ar−*H*), 7.18 (dd, *J* = 15.7, 1.7 Hz, 1H, CH=C*H*C=O), 7.03−6.90 (m, 3H, 2 × Ar−*H*, C*H*=CHC=O), 6.80 (d, *J* = 2.2 Hz, 1H, Ar−*H*), 5.05 (s, 1H, C*H*), 4.93 (dd, *J* = 4.9, 2.6 Hz, 1H, C*H*), 4.57−4.44 (m, 1H, C*H*), 4.37−4.21 (m, 2H, 2 × C*H*), 3.78 (s, 3H, OCH_3), 3.59 (dd, *J* = 14.7, 4.9 Hz, 1H, CH*H*), 3.37−3.39 (m, 2H, 2 × C*H*H), 3.20 (dd, *J* = 14.7, 2.6 Hz, 1H, CH*H*), 2.99−2.83 (m, 2H, 2 × CH*H*), 2.75 (br s, 3H, NCH_3), 2.43 (tt, *J* = 12.1, 3.6 Hz, 1H, C*H*), 1.92−1.86 (m, 2H, CH_2), 1.80−1.41 (m, 12H, alkyl−*H*), 1.31−1.02 (m, 8H, alkyl−*H*), 0.97−0.74 (m, 8H, alkyl−*H*) ppm; ^{13}C NMR (100.6 MHz, DMSO-d_6): δ = 178.8, 172.7, 171.7, 171.3, 169.7, 163.8, 149.4, 135.7, 127.9, 123.9, 121.2, 120.8, 118.5, 117.9, 111.4, 106.4, 94.6, 59.3, 59.0, 52.8, 51.2, 50.0, 45.3, 42.6, 40.6, 38.4, 33.5, 33.2, 31.7, 26.1, 25.9, 25.6, 24.3, 24.0, 22.9, 21.6, 19.5 ppm; $[\alpha]_D^{25}$ = +89 (*c* 0.1, MeCN); FTIR (ATR): $\tilde{\nu}$ = 2929, 1671, 1618, 1428, 1340, 1199, 1137, 837, 729, 723 cm^{-1}; LC−MS: t_R = 3.64 min, MeCN/H_2O (0.1% HCO_2H) = 0:100 (0.00−0.50 min) → 100:0 (8.00 min), flow rate: 0.60 mL min^{-1}; MS (ESI+): *m*/*z* (%): 731.6 (100) [M + H]$^+$; HRMS (ESI+): calcd for [$C_{41}H_{58}N_6O_6$ + H]: *m*/*z* = 731.4491, found: 731.4482.

*(S)-N-(1-(((S,E)-5-((S)-2-((1H-Indol-3-yl)methyl)-3-methoxy-5-oxo-2,5-dihydro-1H-pyrrol-1-yl)-5-oxopent-3-en-2-yl)amino)-2-methyl-1-oxopropan-2-yl)-2-((S)-2-(dimethylamino)-3-methylbutanamido)-4-methylpentanamide·Trifluoroacetate (**44**).* Imide fragment **15** (9.07 mg, 20.0 μmol) was coupled to **32** (13.7 mg, 30.0 μmol) using general procedure 7 (reaction time = 16 h). The crude mixture was purified by preparative RP-HPLC using a focused gradient [MeCN/H_2O (0.1% TFA) = 0:100 (0.00−1.00 min) → 25:75 (5.00 min) → 35:65 (15.00 min), flow rate: 42 mL min^{-1}], yielding **44** as a white solid (12.8 mg, 81%, dr ∼ 2:1). ^{1}H NMR (500 MHz, CD_3CN): δ = 7.32−7.25 (m, 2H, Ar−*H*, CH=C*H*C=O), 7.06−6.91 (m, 4H, 3 × Ar−*H*, C*H*=CHC=O), 6.83 (s, 1H, Ar−*H*), 4.89 (dd, *J* = 4.9, 2.6 Hz, 1H, C*H*), 4.82 (s, 1H, C*H*), 4.55−4.49 (m, 1H, C*H*), 4.36−4.32 (m, 1H, C*H*), 3.77 (s, 3H, OCH_3), 3.61 (dd, *J* = 14.9, 4.9 Hz, 1H, CH*H*), 3.55 (d, *J* = 6.6 Hz, 1H, C*H*), 3.23 (dd, *J* = 14.9, 2.6 Hz, 1H, C*H*H), 2.78 [br s, 6H, N(CH_3)$_2$], 2.29−2.22 (m, 1H, C*H*), 1.57−1.45 (m, 3H, C*H*, CH_2), 1.36 (s, 3H, CH_3), 1.34 (s, 3H, CH_3), 1.17 (d, *J* = 7.0 Hz, 3H, CH_3), 0.98 (d, *J* = 7.0 Hz, 3H, CH_3), 0.88−0.83 (m, 9H, 3 × CH_3) ppm; ^{13}C NMR, HSQC, HMBC (125.8 MHz, CD_3CN): δ = 181.1, 175.7, 172.73, 172.66, 166.9, 166.4, 150.4, 136.7, 128.9, 125.3, 122.3, 120.0, 119.1, 112.4, 107.6, 95.1, 73.3, 61.1, 60.0, 57.7, 53.2, 47.2, 42.2, 40.8, 27.9, 26.5, 25.4, 24.8, 23.1, 21.8, 19.7, 19.5, 17.2 ppm; $[\alpha]_D^{25}$ = +53.7 (*c* 0.16, MeCN); FTIR (ATR): $\tilde{\nu}$ = 1664, 1624, 1543, 1458, 1337, 1200, 1131, 967, 801, 746, 720 cm^{-1}; LC−MS: t_R = 1.68 min, MeCN/H_2O (0.1% HCO_2H) = 0:100 (0.00−0.30 min) → 100:0 (3.00 min), flow rate: 0.60 mL min^{-1}; MS (ESI+): *m*/*z* (%): 665.7 (100) [M + H]$^+$; HRMS (ESI+): calcd for [$C_{36}H_{52}N_6O_6$ + H]: *m*/*z* = 665.4021, found: 665.4013.

*N-((E)-5-((S)-2-((1H-Indol-3-yl)methyl)-3-methoxy-5-oxo-2,5-dihydro-1H-pyrrol-1-yl)-2-methyl-5-oxopent-3-en-2-yl)-2-((S)-2-((S)-2-(dimethylamino)-3-methylbutanamido)-4-methylpentanamido)-4-methylpentanamide·Trifluoroacetate (**45**).* Imide fragment **19** (17.0 mg, 36.4 μmol) was coupled to **20** (26.5 mg, 54.6 μmol) using general procedure 7 (reaction time = 16 h). The crude mixture was purified by preparative RP-HPLC using a focused gradient [MeCN/H_2O (0.1% TFA) = 0:100 (0.00−1.00 min) → 30:70 (4.50 min) → 40:60 (30.00 min), flow rate: 42 mL min^{-1}], yielding **45** as white solid (9.2 mg, 31%, dr 30:1, NB: 20% yield of the minor diastereomer was also isolated but not characterized; overall yield was 51%). ^{1}H NMR (500 MHz, DMSO-d_6, CD_3CN): δ = 10.47 (br s, 1H, N*H*), 10.26 (br s, 1H, N*H*), 8.21 (d, *J* = 6.7 Hz, 1H, N*H*), 8.10 (d, *J* = 8.5 Hz, 1H, N*H*), 7.88 (s, 1H, N*H*), 7.37−7.34 (m, 2H, 2 × Ar−*H*), 7.25−7.17 (m, 2H, C*H*), 7.09−7.06 (m, 1H, Ar−*H*), 7.00−6.97 (m, 1H, Ar−*H*), 6.86 (d, *J* = 2.5 Hz, 1H, Ar−*H*), 4.92 (dd, *J* = 4.9, 2.7 Hz, 1H, C*H*), 4.87 (s, 1H, C*H*), 4.49−4.43 (m, 1H, C*H*), 4.30−4.26 (m, 1H, C*H*), 3.80 (s, 3H, OCH_3), 3.72 (br s, 1H, C*H*), 3.66 (dd, *J* = 14.7, 4.9 Hz, 1H, C*H*H), 3.25 (dd, *J* = 14.7, 2.7 Hz, 1H, CH*H*), 2.80 [br s, 6H, N(CH_3)$_2$], 2.31−2.24 (m, 1H C*H*), 1.66−1.50 (m, 6H, CH_2), 1.43 (s, 3H, CH_3), 1.42 (s, 3H, CH_3), 1.07 (d, *J* = 7.2 Hz, 3H, CH_3), 0.97−0.86 (m, 15H, 5 × CH_3) ppm; ^{13}C NMR (125.8 MHz, DMSO-d_6, CD_3CN): δ = 179.0, 171.6, 171.5, 170.1, 165.8, 164.6, 153.8, 136.0, 128.1, 124.2, 121.0, 119.9, 118.7, 118.2, 111.4, 106.9, 94.4, 71.7, 59.7, 58.7, 53.3, 52.3, 51.8, 40.7, 40.5, 27.1, 26.6, 26.54, 24.49, 24.1, 22.7, 22.3, 21.2, 20.9, 19.0, 17.2 ppm; $[\alpha]_D^{25}$ = +138 (*c* 0.1, MeCN); FTIR (ATR): $\tilde{\nu}$ = 1655, 1618, 1437, 1342, 1174, 1134, 720 cm^{-1}; LC−MS: t_R = 2.11 MeCN/H_2O (0.1% HCO_2H) = 0:100 (0.00−0.30 min) → 100:0 (3.00 min), flow rate: 0.60 mL min^{-1}; MS (ESI+): *m*/*z* (%): 707.7 (100) [M + H]$^+$; HRMS (ESI+): calcd for [$C_{39}H_{58}N_6O_6$ + H]: *m*/*z* = 707.4491, found: 707.4481.

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J. Med. Chem. XXXX, XXX, XXX−XXX

*(S)-N-((S,E)-4-((S)-2-((1H-Indol-3-yl)methyl)-3-methoxy-5-oxo-2,5-dihydro-1H-pyrrol-1-yl)-1-cyclohexyl-4-oxobut-2-en-1-yl)-2-((S)-2-((S)-2-(dimethylamino)-3-methylbutanamido)-4-methylpentanamido)-4-methylpentanamide·Trifluoroacetate (**46**).* Imide fragment **16** (7.82 mg, 15.0 μmol) was coupled to **20** (10.9 mg, 22.5 μmol) using general procedure 7 and purified by preparative RP-HPLC using a focused gradient [MeCN/H_2O (0.1% TFA) = 0:100 (0.00−1.00 min) → 35:65 (5.00 min) → 45:55 (15.00 min) →55:45 (20.00 min), flow rate: 42 mL min^{-1}], yielding **46** as a white solid (9.04 mg, 69%, single diastereomer). ^{1}H NMR (500 MHz, DMSO-d_6): δ = 10.85 (d, *J* = 2.5 Hz, 1H, N*H*), 9.60 (s, 1H, N*H*), 8.76 (d, *J* = 8.3 Hz, 1H, N*H*), 8.24 (d, *J* = 8.5 Hz, 1H, N*H*), 8.03 (d, *J* = 8.7 Hz, 1H, N*H*), 7.29−7.26 (m, 2H, 2 × Ar−*H*), 7.17 (dd, *J* = 15.4, 1.0 Hz, 1H, CH═C*H*C═O), 7.01−6.98 (m, 1H, Ar−*H*), 6.95 (dd, *J* = 15.5, 6.6 Hz, 1H, C*H*═CHC═O), 6.89−6.86 (m, 1H, Ar−*H*), 6.81 (d, *J* = 2.5 Hz, 1H, Ar−*H*), 5.06 (s, 1H, C*H*), 4.93 (dd, *J* = 4.9, 2.7 Hz, 1H, C*H*), 4.53−4.49 (m, 1H, C*H*), 4.43−4.38 (m, 1H, C*H*), 4.29 (q, *J* = 7.4 Hz, 1H, C*H*), 3.78 (s, 3H, OCH_3), 3.64 (br s, 1H, C*H*), 3.61 (dd, *J* = 14.7, 4.9 Hz, 1H, C*H*H), 3.18 (dd, *J* = 14.7, 2.7 Hz, 1H, CH*H*), 2.76 (br s, 3H, NCH_3), 2.73 (br s, 3H, NCH_3), 2.31−2.25 (m, 1H, C*H*), 1.69−1.45 (m, 12H, C*H*, CH_2), 1.23−1.15 (m, 3H, CH_2), 1.00 (d, *J* = 6.8 Hz, 3H, CH_3), 0.97−0.95 (m, 2H, CH_2), 0.97 (d, *J* = 6.6 Hz, 3H, CH_3), 0.87−0.85 (m, 9H, 3 × CH_3), 0.82 (d, *J* = 6.7 Hz, 3H, CH_3) ppm; ^{13}C NMR, HSQC, HMBC (125.8 MHz, DMSO-d_6): δ = 178.8, 171.3, 170.8, 169.7, 164.9, 163.6, 147.2, 135.7, 127.9, 123.9, 122.7, 120.7, 118.4, 117.9, 111.4, 106.4, 94.7, 71.7, 59.3, 59.0, 54.5, 51.1, 50.9, 41.8, 41.3, 40.9, 40.7, 40.6, 29.2, 28.4, 26.5, 25.8, 25.63, 25.56, 24.3, 24.1, 24.0, 23.1, 23.0, 21.5, 21.4, 19.2, 16.4 ppm; $[\alpha]_D^{25}$ = +79 (*c* 0.1, MeCN); FTIR (ATR): $\tilde{\nu}$ = 2932, 1644, 1542, 1363, 1201, 1135, 801, 722, cm^{-1}; LC−MS: t_R = 2.12 min, MeCN/H_2O (0.1% HCO_2H) = 0:100 (0.00−0.30 min) → 100:0 (3.00 min), flow rate: 0.60 mL min^{-1}; MS (ESI+): *m*/*z* (%): 761.4 (100) $[M + H]^+$; HRMS (ESI+): calcd for $[C_{43}H_{64}N_6O_6 + H]$: *m*/*z* = 761.4960, found: 761.4951.

*(S)-N-((S,E)-4-((S)-2-((1H-Indol-3-yl)methyl)-3-methoxy-5-oxo-2,5-dihydro-1H-pyrrol-1-yl)-1-cyclohexyl-4-oxobut-2-en-1-yl)-2-((S)-3-cyclohexyl-2-((S)-2-(dimethylamino)-3-methylbutanamido)-propanamido)-4-methylpentanamide·Trifluoroacetate (**47**).* Imide fragment **16** (7.82 mg, 15.0 μmol) was coupled to **21** (11.8 mg, 22.5 μmol) using general procedure 7 and purified by preparative RP-HPLC using a focused gradient [MeCN/H_2O (0.1% TFA) = 0:100 (0.00−1.00 min) → 35:65 (5.00 min) → 45:55 (15.00 min) → 55:45 (20.00 min), flow rate: 42 mL min^{-1}], yielding **47** as a white solid (9.50 mg, 65%, single diastereomer). ^{1}H NMR (500 MHz, DMSO-d_6): δ = 10.85 (br s, 1H, N*H*), 9.63 (br s, 1H, N*H*), 8.75 (br s, 1H, N*H*), 8.20 (br s, 1H, N*H*), 8.05 (d, *J* = 8.6 Hz, 1H, N*H*), 7.29−7.26 (m, 2H, 2 × Ar−*H*), 7.18 (d, *J* = 15.3, 1H, CH═C*H*C═O), 7.01−6.93 (m, 2H, Ar−*H*, C*H*═CHC═O), 6.89−6.86 (m, 1H, Ar−*H*), 6.81 (d, *J* = 2.0 Hz, 1H, Ar−*H*), 5.06 (s, 1H, C*H*), 4.93 (dd, *J* = 4.9, 2.7 Hz, 1H, C*H*), 4.54 (q, *J* = 7.9 Hz, 1H, C*H*), 4.40 (td, *J* = 9.1, 5.5 Hz, 1H, C*H*), 4.29 (q, *J* = 7.3 Hz, 1H, C*H*), 3.79 (s, 3H, OCH_3), 3.64 (br s, 1H, C*H*), 3.61 (dd, *J* = 14.7, 4.9 Hz, 1H, C*H*H), 3.18 (dd, *J* = 14.7, 2.7 Hz, 1H, CH*H*), 2.73 [br s, 6H, N(CH_3)$_2$], 2.26 (br s, 1H, C*H*), 1.67−1.46 (m, 16H, alkyl−*H*) 1.23−0.81 (m, 24H, alkyl−*H*) ppm; ^{13}C NMR, HSQC, HMBC (125.8 MHz, DMSO-d_6): δ = 178.8, 171.4 170.9, 169.8, 164.9, 163.7, 147.3, 135.7, 128.0, 123.9, 122.7, 120.7, 118.4, 117.9, 111.4, 106.4, 94.7, 71.7, 59.4, 59.0, 54.5, 50.9, 50.3, 41.6, 41.3, 40.9, 40.8, 39.9, 39.1, 33.6, 33.1, 31.8, 29.2, 28.4, 26.5, 26.0, 25.9, 25.8, 25.64, 25.58, 24.2, 24.0, 23.1, 21.4, 19.3, 16.5 ppm; $[\alpha]_D^{25}$ = +43 (*c* 0.1, MeCN); FTIR (ATR): $\tilde{\nu}$ = 1644, 1543, 1354, 1203, 1146, 810, 721 cm^{-1}; LC−MS: t_R = 2.05 min, MeCN/H_2O (0.1% HCO_2H) = 0:100 (0.00−0.30 min) → 100:0 (3.00 min), flow rate: 0.60 mL min^{-1}; MS (ESI+): *m*/*z* (%): 801.4 (100) $[M + H]^+$; HRMS (ESI+): calcd for $[C_{46}H_{68}N_6O_6 + Na]$: *m*/*z* = 823.5093, found: 823.5080.

*(S)-N-((S,E)-5-((S)-2-((1H-Indol-3-yl)methyl)-3-methoxy-5-oxo-2,5-dihydro-1H-pyrrol-1-yl)-1-cyclohexyl-5-oxopent-3-en-2-yl)-2-((S)-2-((S)-2-(dimethylamino)-3-methylbutanamido)-4-methylpentanamido)-4-methylpentanamide·Trifluoroacetate (**48**).* Imide fragment **18** (8.04 mg, 15.0 μmol) was coupled to **20** (10.9 mg, 22.5 μmol) using general procedure 7 and purified by preparative RP-HPLC using a focused gradient [MeCN/H_2O (0.1% TFA) = 0:100 (0.00−1.00 min) → 35:65 (4.00 min) → 45:55 (13.00 min) → 55:45 (18.00 min), flow rate: 50 mL min^{-1}], yielding **48** as a white solid (11.9 mg, 88%, single diastereomer). ^{1}H NMR (400 MHz, DMSO-d_6): δ = 10.85 (d, *J* = 2.5 Hz, 1H, N*H*), 9.60 (s, 1H, N*H*), 8.72 (d, *J* = 8.1 Hz, 1H, N*H*), 8.23 (d, *J* = 8.7 Hz, 1H, N*H*), 8.11 (d, *J* = 8.3 Hz, 1H, N*H*), 7.29−7.27 (m, 2H, 2 × Ar−*H*), 7.18 (dd, *J* = 15.5, 1.1 Hz, 1H, CH═C*H*C═O), 7.03−6.99 (m, 1H, Ar−*H*), 6.99−6.88 (m, 2H, Ar−*H*, C*H*═CHC═O), 6.80 (d, *J* = 2.5 Hz, 1H, Ar−*H*), 5.05 (s, 1H, C*H*), 4.92 (dd, *J* = 4.9, 2.7 Hz, 1H, C*H*), 4.57−4.48 (m, 2H, 2 × C*H*), 4.40−4.34 (m, 1H, C*H*), 3.78 (s, 3H, OCH_3), 3.65 (br s, 1H, C*H*), 3.60 (dd, *J* = 14.7, 4.9 Hz, 1H, C*H*H), 3.19 (dd, *J* = 14.7, 2.7 Hz, 1H, CH*H*), 2.73 [2 × br s, 6H, N(CH_3)$_2$], 2.33−2.24 (m, 1H C*H*), 1.75−1.12 (m, 18H, alkyl−*H*), 1.00 (d, *J* = 7.3 Hz, 3H, CH_3), 0.97−0.81 (m, 17H, 5 × CH_3, CH_2) ppm; ^{13}C NMR, HSQC, HMBC (100.6 MHz, DMSO-d_6): δ = 178.8, 171.3, 170.7, 179.7, 164.9, 163.8, 158.0, 157.7, 149.1, 135.7, 127.9, 123.9, 121.4, 120.7, 118.7, 118.4, 117.9, 111.4, 106.4, 94.6, 71.6, 59.3, 58.9, 51.1, 50.9, 47.0, 41.7, 41.0, 40.9, 40.6, 33.4, 33.2, 31.7, 26.5, 26.1, 25.8, 25.6, 24.0, 23.1, 23.0, 21.4, 19.2, 16.4 ppm; $[\alpha]_D^{25}$ = +56 (*c* 0.05, MeCN); FTIR (ATR): $\tilde{\nu}$ = 1684, 1543, 1355, 1203, 1131, 726 cm^{-1}; LC−MS: t_R = 2.05 min, MeCN/H_2O (0.1% HCO_2H) = 0:100 (0.00−0.30 min) → 100:0 (3.00 min), flow rate: 0.60 mL min^{-1}; MS (ESI+): *m*/*z* (%): 775.4 (100) $[M + H]^+$; HRMS (ESI+): calcd for $[C_{44}H_{66}N_6O_6 + Na]$: *m*/*z* = 797.4936, found: 797.4925.

*(S)-N-((S,E)-6-((S)-2-((1H-Indol-3-yl)methyl)-3-methoxy-5-oxo-2,5-dihydro-1H-pyrrol-1-yl)-6-oxo-1-phenylhex-4-en-3-yl)-2-((S)-2-((S)-2-(dimethylamino)-3-methylbutanamido)-4-methylpentanamido)-4-methylpentanamide·Trifluoroacetate (**49**).* Imide fragment **17** (10.9 mg, 20.0 μmol) was coupled to **20** (14.5 mg, 30.0 μmol) using general procedure 7 and purified by preparative RP-HPLC using a focused gradient [MeCN/H_2O (0.1% TFA) = 0:100 (0.00−1.00 min) → 35:65 (4.00 min) → 45:55 (13.00 min), flow rate: 50 mL min^{-1}], yielding **49** as a white solid (14.9 mg, 83%, single diastereomer). ^{1}H NMR (400 MHz, DMSO-d_6): δ = 10.84 (d, *J* = 2.5 Hz, 1H, N*H*), 9.5 (s, 1H, N*H*), 8.74 (d, *J* = 8.3 Hz, 1H, N*H*), 8.27 (t, *J* = 8.7 Hz, 2H, 2 × N*H*), 7.31−7.13 (m, 8H, 7 × Ar−*H*, CH═C*H*C═O), 7.03−6.92 (m, 3H, 2 × Ar−*H*, C*H*═CHC═O), 6.81 (d, *J* = 2.5 Hz, 1H, Ar−*H*), 5.05 (s, 1H, C*H*), 4.93 (dd, *J* = 4.9, 2.7 Hz, 1H, C*H*), 4.60−4.47 (m, 1H, C*H*), 4.47−4.31 (m, 2H, 2 × C*H*), 3.78 (s, 3H, OCH_3), 3.64 (br s, 1H, C*H*), 3.60 (dd, *J* = 14.7, 4.9 Hz, 1H, C*H*H), 3.19 (dd, *J* = 14.7, 2.7 Hz, 1H, CH*H*), 2.75 (s, 3H, NCH_3), 2.73 (s, 3H, NCH_3), 2.70−2.52 (m, 2H, CH_2), 2.35−2.20 (m, 1H, C*H*), 1.96−1.71 (m, 2H, CH_2), 1.71−1.39 (m, 6H, C*H*, CH_2), 1.00 (d, *J* = 6.8 Hz, 3H, CH_3), 0.92−0.77 (m, 15H, 5 × CH_3) ppm; ^{13}C NMR, HSQC, HMBC (100.6 MHz, DMSO-d_6): δ = 178.8, 171.5, 170.9, 169.7, 164.9, 163.7, 148.4, 141.4, 135.7, 128.5, 128.2, 127.9, 125.8, 123.9, 121.8, 120.7, 118.4, 117.9, 111.4, 106.4, 94.6, 71.7, 59.3, 58.9, 51.1, 51.0, 49.0, 41.8, 40.8, 40.7, 40.6, 35.3, 31.4, 26.5, 24.24, 24.18, 24.0, 23.1, 23.0, 21.42, 21.40, 19.2, 16.4 ppm; $[\alpha]_D^{25}$ = +91 (*c* 0.1, MeCN); FTIR (ATR): $\tilde{\nu}$ = 2926, 1644, 1542, 1340, 1202, 965, 801, 744, 721 cm^{-1}; LC−MS: t_R = 4.00 min, MeCN/H_2O (0.1% HCO_2H) = 0:100 (0.00−0.50 min) → 100:0 (8.00 min), flow rate: 0.60 mL min^{-1}; MS (ESI+): *m*/*z* (%): 783.7 (100) $[M + H]^+$; HRMS (ESI+): calcd for $[C_{45}H_{62}N_6O_6 + Na]$: *m*/*z* = 805.4623, found: 805.4618.

FP2 and FP3 Assays. FP2 and FP3 were heterologously expressed as previously described,[32,33] and the inhibitory activities of the gallinamide A analogues against these two enzymes were measured on the basis of cleavage of the fluorogenic substrate Z-Leu-Arg-AMC using a previously reported continuous assay.[34]

In Vitro Assays against *P. falciparum* (3D7 and W2). Activities of the gallinamide A analogues were assessed against cultured 3D7 and W2 strains of *P. falciparum*, as previously described.[23] To assess morphological effects, a range of concentrations (4.5−10 000 nM) of leads **34**, **35**, and **49**, a positive control E64 (10 μm), and a negative control 0.1% DMSO were each incubated with cultured, synchronized W2 strain parasites for 36 h, beginning at the ring stage, and thin smears were then made, fixed with methanol, stained with Giemsa, and photographed with a SpotFlex Digital camera (Spot Imaging).

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J. Med. Chem. XXXX, XXX, XXX−XXX

Blood and Plasma Stability Assays. Mouse blood was collected on the day of the experiment from nonfasted male Swiss outbred mice under gaseous isoflurane anesthesia according to protocols approved by the Monash Institute of Pharmaceutical Sciences Animal Ethics Committee. Plasma was separated from whole blood by centrifugation (Heraeus Multifuge 3SR, 4500*g* for 2 min). Aliquots of whole blood and plasma were spiked with a DMSO/acetonitrile/water solution of each compound to a final compound concentration of 1 μg mL^{-1}. Concentrations of DMSO and acetonitrile in the final sample were 0.2% (v/v) and 0.4% (v/v), respectively. Following gentle mixing, blood and plasma samples were aliquoted into individual microcentrifuge tubes, which were maintained at 37 °C. At various time points over a 4 h incubation, individual aliquots of blood or plasma (n = 3 per time point) were removed from the incubator and snap-frozen on dry ice.

For quantitative analysis, acetonitrile was added to thawed blood and plasma samples, followed by centrifugation, to precipitate and remove proteins. The supernatant was analyzed by UPLC−MS (Waters/Micromass Xevo TQ coupled to a Waters Acquity UPLC), with positive electrospray ionization, against calibration standards prepared in blank mouse whole blood or plasma prepared in the same way. Compound concentration versus time data were fitted to an exponential decay function to determine the first-order rate constant (k) for substrate depletion, which was then used to calculate a degradation half-life (half-life = ln(2)/k).

In Vivo Antimalarial Studies. *P. berghei* ANKA (PbA) infection in mice is a well-established model of cerebral malaria (CM).[35,36] Mice-inoculated i.p. with 10^6 red blood cells containing PbA strain malaria parasites develop fatal CM 6−7 days post inoculation (p.i.), with % parasitemia (percentage of red blood cells containing one or more malaria parasites) <20.

Mice: female C57BL/6 mice, from the Australian Resources Centre (Perth, Western Australia), 8−9 week old at the time of infection, were given food and water ad libitum. All experiments were approved by The University of Sydney Animal Ethics Committee (Protocol Number 2015/751) and strictly followed Institutional Animal Care and Use Committee guidelines. Mice were maintained with environmental enrichment in specific pathogen-free conditions at the Medical Foundation Building Animal Facility.

The PbA strain was originally obtained from Josef Bafort (Prince Leopold Institute, Antwerp, Belgium)[37] and maintained by successive infections of CBA/Ca and C57BL/6 mice.[38] The animals were monitored twice daily after inoculation with PbA and euthanized when they exhibited signs of severe disease that invariably would have led to death from CM. If treated with antimalarial compounds, mice do not develop CM and blood parasitemia may rise to ∼70%.[39,40] When showing signs of fatal anemia, mice were euthanized. "Survival" was defined as the period before mice were euthanized.

Peter's 4 Day Test. The antimalarial effects of compounds **34**, **35**, and **49** were initially tested with a dose-ranging protocol.[41] C57BL/6 mice were inoculated i.p. with 10^6 red blood cells containing PbA malaria parasites in 200 μL of Alsever's medium, as described previously.[38] Then, 200 μL of different concentrations of the test compounds (5 mice per group), dissolved initially in DMSO then diluted in 154 mM NaCl ("saline"), or vehicle alone, were injected i.p. 4, 24, 48, and 72 h later. Each day p.i. drops of blood were taken from the tail vein to make thin smears on glass slides for staining with Rapid Stain (ProSciTech, Thuringowa, Australia). The % parasitemia was determined by microscopy. Mice were considered to have been cured if they were healthy with no detectable parasitemia on day 35 p.i.

Therapeutic Model. The antimalarial efficacy of compound **49** was compared to that of the highly active drug chloroquine (CQ). Compound **49** and CQ were dissolved in DMSO, then diluted in saline and injected i.p. as before (7 mice per group), at a dose of 50 mg kg^{-1}, on days 4, 5, 6, and 7 p.i. Controls received vehicle only. Survival and parasitemia were recorded at regular intervals.

Oral Administration. The antimalarial efficacy of compound **49** was compared to that of CQ when the agents were administered orally. Compound **49** and CQ were dissolved as before and administered per os (oral gavage) at 100 mg kg^{-1} on days 4, 5, 6, and 7 p.i. (5−6 mice per group). Controls received vehicle only. Survival and parasitemia were recorded as in the preceding experiments.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jmedchem.9b00504.

NMR spectra, mass spectra, and UPLC−MS data of fragments and final compounds; microsomal stability studies; parasite morphology images; cytotoxicity studies in HEK cells; in vivo antimalarial activity of analogue **49** following oral administration (PDF)

Molecular formula SMILES strings for compounds (CSV)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

AA, amino acid; Aib, 2-aminoisobutryric acid; ART, artemisinin; CQ, chloroquine; CM, cerebral malaria; E64, proteinase inhibitor E64; EDC·HCl, *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride; FP2, falcipain 2; FP3, falcipain 3; HATU, 1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxid hexafluorophosphate; HFIP, hexafluoroisopropanol; HOAt, 1-hydroxy-7-azabenzotriazole; LiHMDS, lithium bis(trimethylsilyl)amide; NMM, *N*-methylmorpholine; p.i., post inoculation; PbA, *Plasmodium berghei* ANKA; PyAOP, (7-azabenzotriazol-1-yloxy)-trispyrrolidinophosphonium hexafluorophosphate; RP, reverse phase; SPPS, solid-phase peptide synthesis; UPLC−MS, ultrahigh-performance liquid chromatography−mass spectrometry

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J. Med. Chem. XXXX, XXX, XXX−XXX