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Plasmodium falciparum Subtilisin-like Protease 1: Discovery of Potent Difluorostatone-based Inhibitors

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Currently available drugs to treat malaria are often ineffective due to the acquisition of drug resistance. In this context, drugs with innovative modes of action and no liability to cross-resistance are urgently needed. Recently, subtilisin-like protease 1, a *P. falciparum* serine protease involved in merozoite egress from red blood cells and invasion, has been identified as potential drug target. We describe herein the development of a series of potent PfSUB1 inhibitors. Combining a straightforward synthetic approach, an in depth structure-activity study and in silico investigation, we identified the most potent inhibitors known to date, characterized by an improved enzyme inhibitory potency and a reduced peptidic character over the prototypic peptides.

Introduction

Malaria is one of the most deadly infectious diseases of the world. Despite considerable scientific advances and the allocation of funding by public and private organizations, in 2012 there were an estimated 207 million cases of malaria with 627,000 deaths. 90% of all malaria deaths occur in sub-Saharan Africa. According to WHO data, 97 countries currently have ongoing malaria transmission.[1] Global expansion of the disease has been attributed mainly to the spread of strains of *Plasmodium falciparum* resistant to almost all known antimalarial drugs. *P. falciparum* is the etiological agent of the most virulent form of malaria and despite artemisinin-based combination therapies (ACT), recommended by the WHO as first line treatment in highly endemic malaria regions, parasite resistance to artemisinin has been recently confirmed.[2-4] To reverse this trend and by-pass resistance mechanisms, a

particularly suitable strategy is the development of antimalarials that attack novel parasite pathways, thus avoiding cross resistance with the few commonly used existing drugs.[5]

The lifecycle of *P. falciparum* involves a mosquito and a human stage of development. The human stage can be in turn divided into an asymptomatic exoerythrocytic phase and a symptomatic erythrocytic phase. During this latter phase, the parasite invades and multiplies within a membrane-bound parasitophorous vacuole (PV) in red blood cells (RBCs), eventually producing 16-32 mature merozoites. Upon rupture of the RBC, the merozoites escape in a process known as egress, and invade new RBCs.[6] Both egress and invasion are highly regulated events, essential for proper replication of the parasite.

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†   Electronic Supplementary Information (ESI) available: [Figures S1-S5, ^{1}H NMR spectra]. See DOI: 10.1039/c000000x/

Figure 1 Reference inhibitor **1** and design strategy for inhibitors **2-19** reported in this study.

The serine protease *P. falciparum* subtilisin-like protease 1 (PfSUB1) has recently emerged as a key enzyme in both egress and invasion.[6-9] Although the role of PfSUB1 in parasite survival is not completely understood, it is known that PfSUB1 is released into the PV space just prior to egress where it mediates the proteolytic maturation of a family of proteins called SERA, in turn involved in the molecular events leading to egress.[8] In addition, PfSUB1 processes several merozoite surface proteins (MSP1, MSP6, and MSP7) thus priming the merozoite for the subsequent invasion step.[7]

As part of a program aimed at discovering new molecules targeting erythrocytic *P. falciparum* stages, we became interested in developing PfSUB1 inhibitors as a suitable approach for developing innovative drugs against malaria.[10] We recently reported our rational design concept and the synthesis of a small set of mechanism-based hit inhibitors of PfSUB1.[11] In our initial design approach we replaced the cleavable peptide bond of the decapeptide KITAQ↓DDEES, based on the sequence of the natural PfSUB1 substrate SERA4, with an electrophilic difluorostatone moiety, functionalized at the P1'-end with a carboxylic acid moiety able to interact with K465 located at the P'-side of the substrate binding cleft. Starting from the early hit compound 1[11] (Figure 1) we describe herein the development of the most potent enzyme inhibitor known to date, based on a medicinal chemistry approach through a specific structure-activity relationships study. Accordingly, we modified the difluorostatone warhead in order to assess its role in the inhibitory activity (inhibitors 2,3, Figure 1 and Table 1), we explored different natural and non-natural amino acids at the P side of the molecule in order improve affinity (inhibitors 4-15), and we attempted to reduce the peptidic character of the compounds through introduction of different capping groups in place of P2/P3 (compounds 16,17) or P3 (compounds 18,19) residues. The molecular determinants of binding were carefully analyzed by molecular modeling studies based on the available X-ray crystal structure of PfSUB1.[12]

Chemistry

The synthesis of inhibitors 2,3 is described in Scheme 1. Aldehyde 20[13] was treated with TMSCF3 in the presence of TBAF to obtain alcohol 21.[14] Treatment of this latter compound with TFA afforded the free amine intermediate that was coupled with peptide 26a (prepared through microwave-assisted solid phase synthesis, as described in the Experimental Section) to afford carbinol 22. Oxidation of the secondary alcohol afforded the final compound 2 (Table 1). Aldehyde 20 was also used to synthesize intermediate 23, prepared as previously reported.[11] The ethyl ester group of 23 was converted into the corresponding benzyl ester through a two-step procedure involving alkaline hydrolysis and subsequent esterification of the carboxylic acid. The amino group of the benzyl ester intermediate was deprotected by exposure to TFA and coupled with 26a to afford 24. Oxidation of the secondary alcohol followed by deprotection of the ester group by catalytic
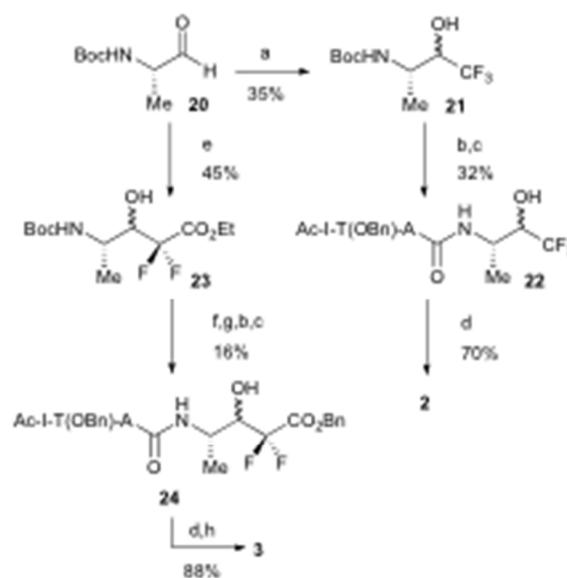
Scheme 1 Synthesis of P1'-modified difluorostatones **2,3**; Reagents and conditions: a) 2M TMSCF3 in THF, TBAF, anhydrous THF, 0 °C, 3 h; b) TFA/DCM, 25 °C, 2 h; c) **26a**, EDC, HOBt, Et3N, anhydrous DMF, 0 to 25 °C, 15 h; d) Dess-Martin periodinane, anhydrous DCM, 0 to 25 °C, 2 h; e) ethyl bromodifluoroacetate, zinc dust, anhydrous THF, 25 °C to reflux, 30 min; f) 0.25 N LiOH solution, MeCN, 25 °C, 2 h; g) benzyl alcohol, DCC, HOBt, anhydrous DCM, 25 °C, 12 h; h) H2, Pd/C 10%, MeOH, 40 °C, 18 h.
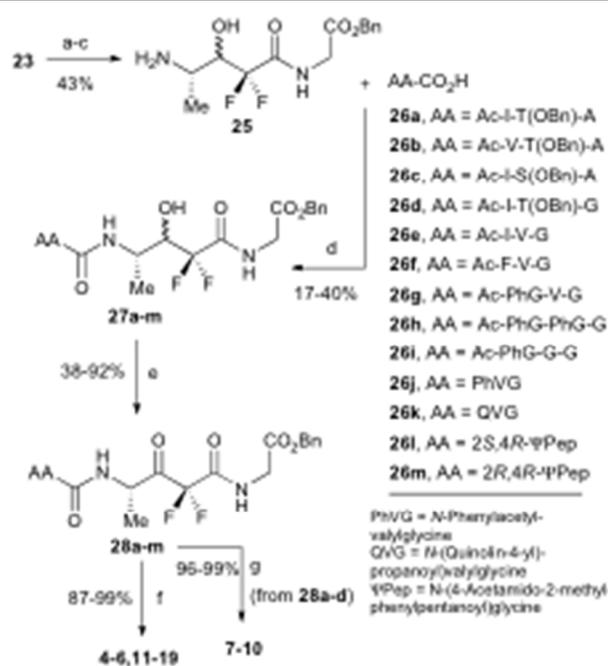
Scheme 2 Reagents and conditions: 0.25 N solution of LiOH, MeCN, 25 °C, 2 h; b) glycine benzyl ester hydrochloride, HATU, DIPEA, dry DMF, 0 to 25 °C, 12 h; c) TFA/DCM, 25 °C, 3 h; d) **26a-m**, EDC, HOBt, DIPEA, dry DMF, 0 to 25 °C, 12-18 h; e) Dess-Martin periodinane, dry DCM or NMP, 0 to 25 °C, 1-24 h; f) H2, Pd/C 10%, MeOH, 40 °C, 18 h; g) Pd/C 10%, 1,4-cyclohexadiene, MeOH, 25 °C, 14 h.
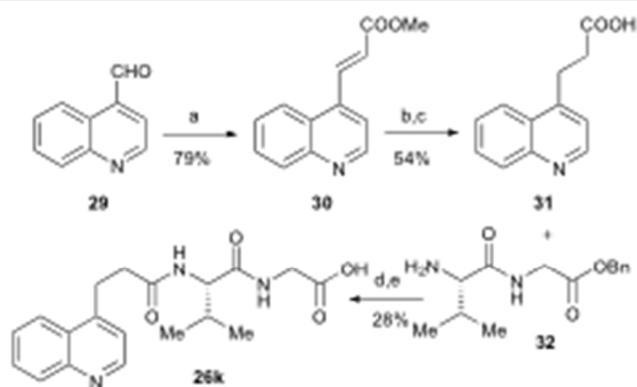
Scheme 3 Reagents and conditions: a) methyl(triphenylphosphoranylidene) acetate, dry toluene, 80 °C, 2.5 h; b) H₂, Pd/C 10%, MeOH, 25 °C, 6 h; c) LiOH, THF/H₂O, 25 °C, 15 h; d) EDC, HOBt, DIPEA, dry DMF, 0 to 25 °C, 15 h; e) H₂, Pd/C 10%, MeOH, 25 °C, 18 h.

hydrogenation resulted in the formation of **3** in good overall yield.

Inhibitors **4-19** were synthesized as reported in Scheme 2. Intermediate **25**, prepared from **23** as above described, was coupled with peptides and peptidomimetics **26a-m** to obtain alcohols **27a-m**. Dess-Martin periodinane-based oxidation of the secondary alcohol furnished the fully protected difluorostatone derivatives **28a-m** in good overall yield. Exposure of compounds **28a-m** to a hydrogen atmosphere in the presence of Pd/C furnished the final compounds **4-6** and **11-19**. Compounds **28a-d** were also submitted to selective deprotection of the benzyl ester functional group by treatment with 1,4-cyclohexadiene in the presence of Pd/C to afford benzyl ether final compounds **7-10**.

Peptides **26a-j** reported in Scheme 2 were prepared through solid phase synthesis assisted by microwave irradiation as described in the Experimental Section. The synthesis of peptidomimetics **26k-m** is described in Schemes 3,4. The quinoline-containing peptide **26k** was synthesized starting from 4-quinolincarboxaldehyde **29** (Scheme 3). Olefination of **29** with methyl(triphenylphosphoranylidene)acetate afforded α,β-unsaturated ester **30**. Hydrogenation followed by alkaline hydrolysis of the methyl ester functionality led to **31**. This latter carboxylic acid was coupled to dipeptide **32**[15] and the benzyl ester was deprotected by catalytic hydrogenation to furnish carboxylic acid **26k**. The synthesis of intermediates **26l,m**, necessary for the synthesis of epimeric derivatives **18** and **19** is reported in Scheme 4. Following a synthetic protocol described by Ullrich et al.,[16] phenylalanine methyl ester **33** was converted into the α,β-unsaturated ethyl ester **34** through a two-step procedure involving reduction of the ester to the corresponding aldehyde followed by olefination reaction.

Compound **34** was then converted into the corresponding menthyl derivative **35**, which was hydrogenated to afford a diastereomeric mixture of **36** and **37**. The two diastereoisomers were separated by flash-chromatography and both of them submitted to the next steps.
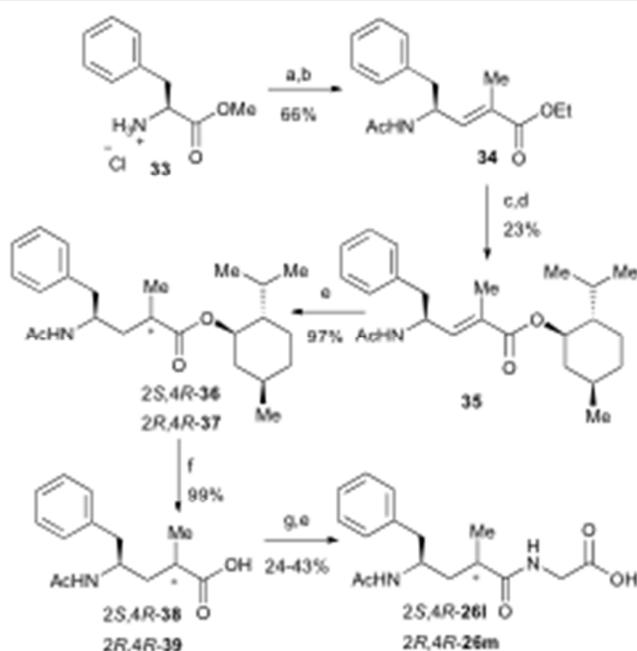
Scheme 4 Reagents and conditions: a) acetic anhydride, Et₃N, DCM, 25 °C, 3 h; b) DIBAL, PPh₃=C(CH₃)COOEt, KOtBu, dry DCM, -78 to 25 °C, 16 h; c) 1 M NaOH solution, 1,4-dioxane, 80 °C, 2 h; d) DCC, DMAP, (1R,2S,5R)-(-)-menthol, dry Et₂O, 0 to 25 °C, 15 h; e) H₂, Pd/C 10%, MeOH, 25 °C, 16 h; f) 6 N HCl solution, 130 °C, 2 h; g) benzylglycine, EDC, HOBt, DIPEA, dry DMF, 0 to 25 °C, 15 h.

Hydrolysis of the menthol chiral auxiliary furnished optically pure carboxylic acids **38** and **39**. These latter compounds were coupled to glycine benzyl ester **33** and deprotected to afford the desired intermediates **26l,m**.

Results and discussion

The inhibitory activity of the synthesized compounds and selected intermediates against recombinant PfSUB1 (Table 1) was assessed using a previously described fluorimetric assay.[17] The binding mode of the developed inhibitors was evaluated by means of docking studies employing GOLD software,[18] performed on the crystal structure of PfSUB1.[12] In our previous work we outlined the following key structural prerequisites necessary for achieving inhibitory activity: i) the difluorostatone moiety, which is hypothesized to be attacked by the nucleophilic active site serine, needs to be accommodated at the right distance from the catalytic S606; ii) the terminal carboxylic acid group at P1' forms key polar contacts with K465 (at S2) and R600 (at S'), thus requiring a fine-tuning of the distance between the electrophilic carbonyl group and the N-terminal P1' carboxylic moiety; and iii) at least three residues (P2-P4) are required at the non-prime side of the inhibitor.[11] To improve potency we further investigated the importance of these key interactions. Accordingly, novel inhibitors endowed with higher inhibitory activity and better physico-chemical properties were identified.

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Table 1. Inhibitory activity (IC$_{50}$, μM), GoldScore (GS), and ΔG of binding (kcal/mol) of inhibitors **2-19**.

Compound	IC$_{50}$ (μM)	GS	ΔG$_{bind}$ (kcal/mol)	Compound	IC$_{50}$ (μM)	GS	ΔG$_{bind}$ (kcal/mol)
2	>50			**11**	0.25	86.35	-123.02
3	>50	68.91	-75.41	**12**	0.55	83.55	-101.42
4	0.9			**13**	0.6	84.96	-108.04
5	1.2	80.97	−107.42	**14**	0.28	84.90	-121.73
6	0.3	87.80	-121.67	**15**	1.7		
7	0.32	88.31	-128.71	**16**	1.0	82.28	-101.67
8	0.95			**17**	n.a.	71.73 (N) 72.59 (P)	-81.29 (N) -79.83 (P)
9	1.45	79.74	-111.39	**18**	n.a	67.34	-71.95
10	0.3	84.03	-119.42	**19**	n.a.		
1[11]	0.6	88.19	-119.10				
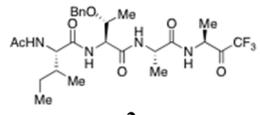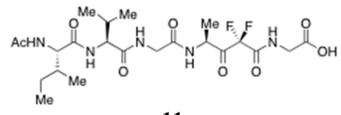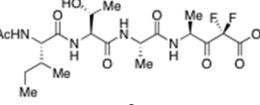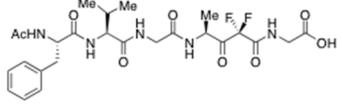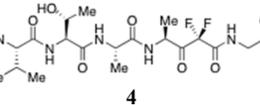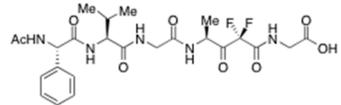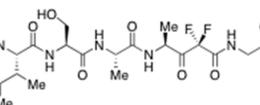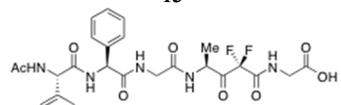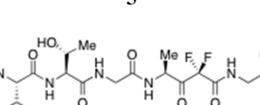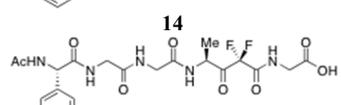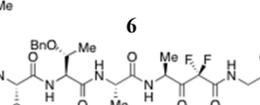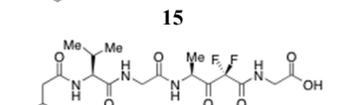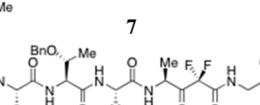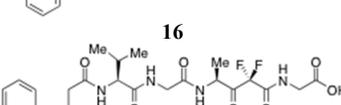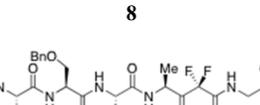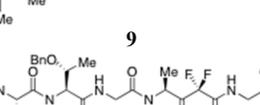
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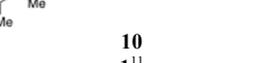
Modifications at the difluorostatone moiety

To assess the role of the difluorostatone moiety and of its substitution pattern on binding, we synthesized and evaluated compounds **2** and **3**. Of particular note, derivative **2** lacks the terminal P1'-glycine carboxylic acid and presents a classical trifluoromethylketone warhead, while in compound **3** we shortened the linker connecting the carboxyl function directly to the difluoromethylene group with respect to previously described inhibitor **1**. In both cases, compounds did not show

appreciable inhibitory activity at concentration up to 50 μM, while the corresponding structural analogues **1** and **7**, bearing an intact C-terminal glycine were both active against the enzyme. As found by docking studies, the C-terminal carboxylic group of compound **3** (Figure S1) forms a H-bond interaction with N603, hindering the correct accommodation of the electrophilic carbonyl group close to the catalytic S606.[11,12] Both the docking score (GoldScore, GS) calculated for compound **3** and the free energy of binding (Table 1) are in agreement with the experimental data.

Modifications at the P4-P2 residues

We next examined modifications of the P4-P2 residues. Replacement of P4 Ile and of P3 Thr residues with the corresponding nor-methyl amino acids Val and Ser, resulted in the synthesis of inhibitors **4** and **5**, showing a minor decrease in inhibitory potency compared to **1**. Replacement of the P2 Ala with a Gly resulted in a small improvement of potency (inhibitor **6**). The IC_{50}s observed for compound **4-6** are in line with calculated affinity parameters (Table 1) as well as their docking outputs. As reported in Figure 2, compound **6** is involved in a strong series of contacts with the binding cleft of the enzyme and is able to reproduce a binding mode comparable to **1**.

In our hit compound **1**, the OH group of the P3 Thr is not involved in H-bond interactions since it is solvent exposed, while in compound **6**, the corresponding Ser residue forms a polar contact with K541 (Figure 2). In order to assess the importance of the free OH group in binding, we tested the P3 benzyl-protected derivatives **7-10**. All the compounds displayed a slightly improved or equal inhibitory potency compared to the corresponding free OH derivatives (**7** vs **1**, **8** vs **4**, **9** vs **5**, **10** vs **6**). As shown in Figure 3 for inhibitor **10** and in Figure S2 for compound **9**, the benzyl group hinders the formation of polar contacts between the inhibitor ether oxygen and the enzyme residues, but the aromatic ring is now able to form a cation-π stacking with K541. Moreover, the formation of this new interaction does not alter, for compounds **7-10**, the key interactions of the P1' carboxylic acid with the enzyme. In fact, the carboxylic moiety engages in H-bond interactions with Y427, and K465, as observed for **1**. Moreover, the P2-P4 residues of **9** and **10** mainly form hydrophobic contacts with residues in the corresponding enzyme sub-sites, in line with Goldscore, ΔG_{bind} and enzyme inhibitory data (Table 1). Due to the low contribution of the free or benzyl-protected Thr OH in binding, we designed and synthesized inhibitor **11**, in which we replaced the Thr residue at P3 with a Val residue, at the same time maintaining a Gly at P2, which was observed to be well-tolerated at this position (**6** vs **1**, **10** vs **7**). Compound **11** is one of the most potent inhibitors known to date. Its inhibitory potency is in line with calculated affinity data (Table 1). Docking studies showed that **11** forms a strong pattern of interactions with the enzyme substrate binding site (Figure 4). The carboxylic acid group at P1' forms an optimized pattern of H-bonds with S' residues Y427 and K465. An additional H-bond is formed between the carbonyl group of the

difluorostatone amide moiety and N603, while the P-side of the inhibitor is anchored to the enzyme through H-bonding with G467 (P4 capping group) and S492 (P3 NH group). In addition, the Ile P3 side chain is deeply accommodated into the S3 hydrophobic pocket (F491, F493, L469 and F500). Starting from the optimized P-side sequence of **11**, we tried to further increase the overall lipophilicity of the molecule, and to maximize hydrophobic interactions with the enzyme sub-sites by introducing the natural amino acid Phe or the unnatural amino acid phenylglycine (PhG) at P4.
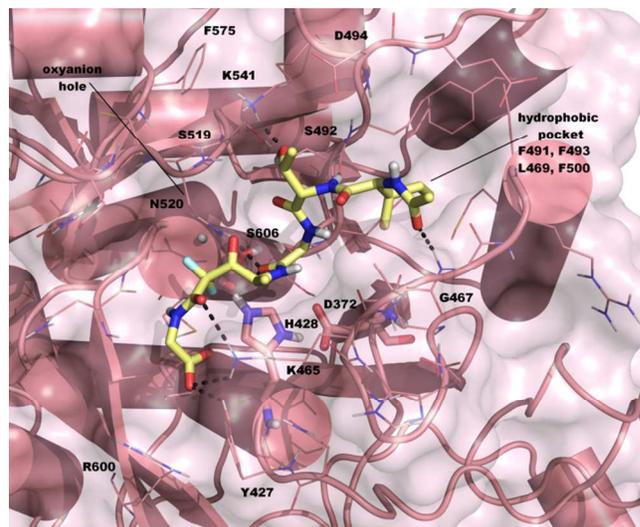
Figure 2 Docked pose of **6** in the cavity of the PfSUB1 (PDB ID: 4LVN). H-bonds are indicated by black dotted lines. The catalytic triad is represented by sticks while the other residues in the binding site are represented by lines. Molecular graphics were generated by PyMOL (The PyMOL Molecular Graphics System, v1.6-alpha; Schrodinger LLC: New York, 2013). Nonpolar hydrogens are omitted for clarity.
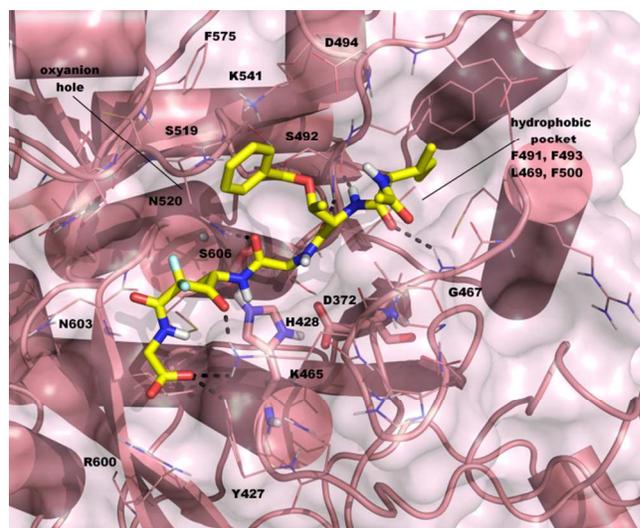
Figure 3. Docked pose of **10** in the cavity of the PfSUB1 (PDB ID: 4LVN). H-bonds are indicated by black dotted lines. The catalytic triad is represented by sticks while the other residues in the binding site are represented by lines. Molecular graphics were generated by PyMOL. Nonpolar hydrogens are omitted for clarity.
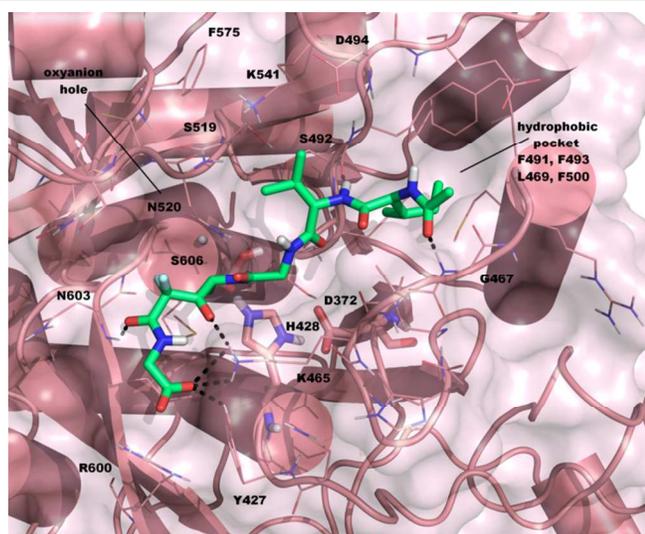
Figure 4. Docked pose of **11** in the cavity of the PfSUB1 (PDB ID: 4LVN). H-bonds are indicated by black dotted lines. The catalytic triad is represented by sticks while the other residues in the binding site are represented by lines. Molecular graphics were generated by PyMOL. Nonpolar hydrogens are omitted for clarity.

The resulting inhibitors **12** and **13** maintained a similar potency compared to our hit compound **1**. Introduction of a PhG residue at both P4 and P3 resulted in the synthesis of compound **14**, endowed with an inhibitory potency comparable to **11**. Accordingly, these compounds showed similar calculated affinity parameters (Goldscore and estimated free binding energy, Table 1), although with subtle differences in their binding modes as highlighted in Figure 5. As expected, while the binding interactions of the P1'-P2 fragment of all three molecules **12-14** were comparable to those for compound **1**, the main differences in binding were observed at the P3-P4 side for compounds **13** and **14** (Figure 5A-C). Replacement of the Ile P4 residue by a Phe (**12**, Figure 5A) did not induce a dramatic difference in the binding mode while introduction of the unnatural amino acid phenylglycine at P4 (inhibitor **13**, Figure 5B) resulted in a marked modification of the binding conformation: the side chain of P3 Ile was forced to assume a different orientation compared to **12** while the H-bond interaction of the acetamide capping group with D494 was lost. When two phenylglycines are placed at both P3 and P4 (inhibitor **14**, Figure 5C) the P4 residue forms contacts with the hydrophobic pocket outlined in Figure 5C (in particular a π-π stacking with F491 and hydrophobic interactions with L466) and the P3 aromatic ring forms a potential π-π stacking with H428. Also for compound **14**, the acetamide capping group did not form any polar contact with the enzyme. The key role of the hydrophobic residue at P3 is evident in compound **15**, bearing a Gly residue at P3 and displaying a decreased inhibitory potency compared to both **11** and **14**.

Modification of the P-capping group

Based on the docking output of compounds **12-15**, we decided to remove the acetamide capping group. Analogue **16**, although displaying a minor loss of enzyme inhibitory potency, is

characterized by a reduction in the number of peptide bonds. The docking output is shown in Figure 6. Compound **16** is able to reproduce the binding mode found for the active difluorostatones of the series.
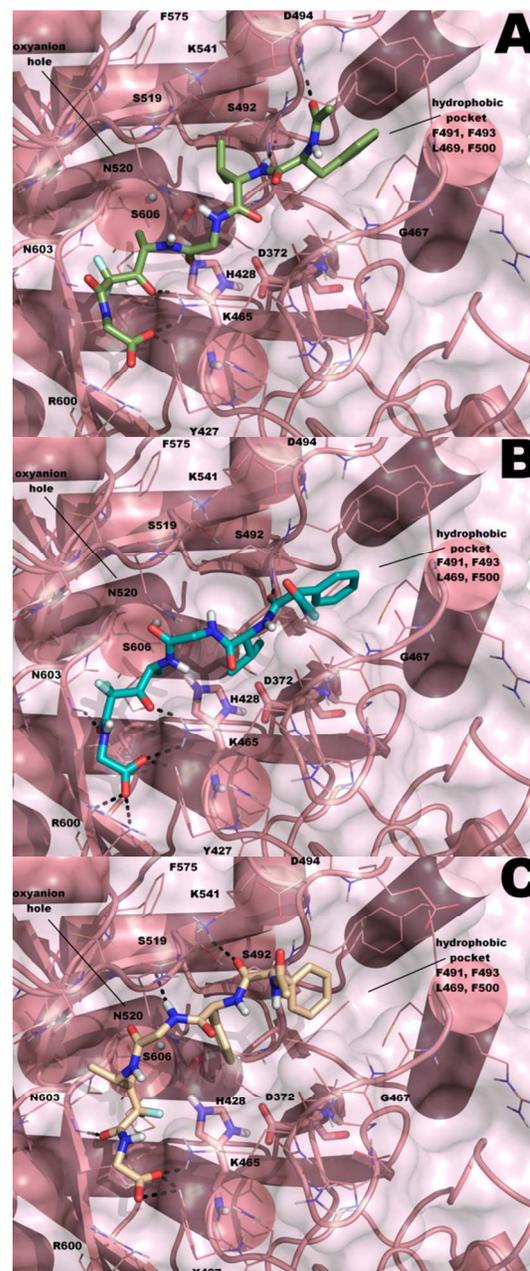
Figure 5. Docked pose of **12** (A), **13** (B), and **14** (C) in the cavity of the PfSUB1 (PDB ID: 4LVN). H-bonds are indicated by black dotted lines. The catalytic triad is represented by sticks while the other residues in the binding site are represented by lines. Molecular graphics were generated by PyMOL. Nonpolar hydrogens are omitted for clarity.
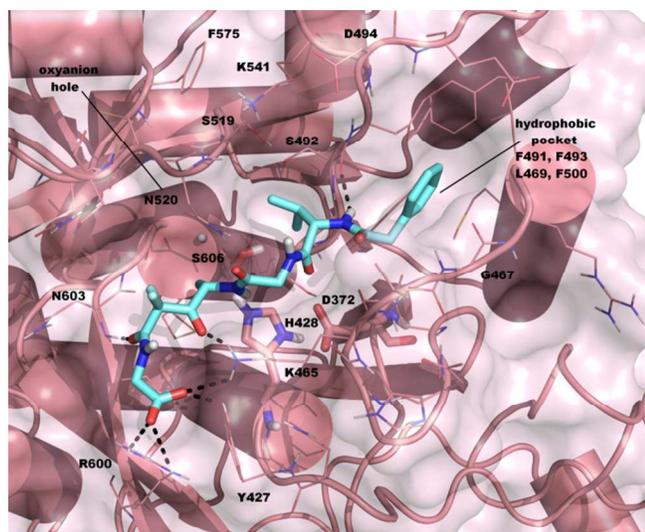
Figure 6. Docked pose of **16** in the cavity of the PfSUB1 (PDB ID: 4LVN). H-bonds are indicated by black dotted lines. The catalytic triad is represented by sticks while the other residues in the binding site are represented by lines. Molecular graphics were generated by PyMOL. Nonpolar hydrogens are omitted for clarity.

Notably, the terminal benzyl-capping group is correctly accommodated into the hydrophobic pocket, interacting with its aromatic residues. Further elaboration of the N-terminal capping group resulted in derivative **17**, in which the benzyl group was replaced by an ethylene-4-quinoline moiety. Unfortunately, poor enzymatic inhibitory activity was observed. This finding is in agreement with our docking output (Figure S3,S4) and computed properties (Table 1). In fact, the 4-quinoline moiety appears to be too large to be correctly accommodated in the hydrophobic pocket, resulting in an unfavorable orientation of the inhibitor inside the binding cleft. Derivatives **18** and **19**, in which the P3/P4 peptide bond was replaced by a methylene linker, also showed poor inhibitory potency as confirmed by the proposed binding mode reported for **18** (Figure S5) which does not meet the above-described requirements for an appropriate binding interaction.

Conclusions

Based on our previously identified PfSUB1 inhibitors, we have extended the structure-activity relationship studies and rationally designed novel and potent inhibitors. The most promising compounds of the series display improved potency against PfSUB1. Compounds **11** and **14**, the latter characterized by the presence of non-natural amino acids in its structure, are the most active PfSUB1 inhibitors reported in the literature. Moreover, the overall good enzymatic potency of inhibitor **16**, possessing only two amino acid residues on the P-side, could pave the way to the development of inhibitors with reduced peptidic character.

Experimental Section

Chemistry

Unless otherwise specified, materials were purchased from commercial suppliers and used without further purification. Reaction progress was monitored by TLC using silica gel 60 F254 (0.040-0.063 mm) with detection by UV. Silica gel 60 (0.040-0.063 mm) was used for column chromatography. Microwave assisted solid-phase peptide synthesis was performed on the Liberty microwave-assisted automatic peptide synthesizer (CEM, Matthews, NC), an additional module of CEM's Discover. ^{1}H NMR spectra were recorded on a Varian 300 MHz spectrometer or a Bruker 400 MHz spectrometer by using the residual signal of the deuterated solvent as internal standard. Splitting patterns are described as singlet (s), doublet (δ), triplet (t), quartet (q), and broad (br); the value of chemical shifts (δ) are given in ppm and coupling constants (*J*) in Hertz (Hz). ESI-MS spectra were performed by an Agilent 1100 Series LC/MSD spectrometer. Yields refer to purified products and are not optimized. All moisture-sensitive reactions were performed under argon using oven-dried glassware and anhydrous solvents.

***tert*-butyl (S)-(1-oxopropan-2-yl)carbamate (20).** The title compound was synthesized according to a literature procedure.[13] Physical and spectroscopic data are consistent with those reported in the literature;[13] ^{1}H NMR (300 MHz, DMSO) δ 1.09 (d, *J* = 7.5 Hz, 3H), 1.36 (s, 9H), 3.83 (t, *J* = 9.9 Hz, 1H), 7.33 (d, *J* = 5.7 Hz, 1H), 9.39 (s, 1H); ^{13}C NMR (CDCl$_3$, 75 MHz) δ 15.0, 28.5, 55.7, 80.3, 155.5, 199.9; ESI-MS: *m/z* 173 (M+H)[+], 196 (M+Na)[+].

***tert*-Butyl ((2S,3R,S)-4,4,4-trifluoro-3-hydroxy-2-butanyl) carbamate (21).**

A 2 M solution of TMSCF$_3$ in anhydrous THF (650 μL, 1.30 mmol) was added to a solution of **20** (150 mg, 0.87 mmol) and TBAF (42 μL, 0.04 mmol) in anhydrous THF (5 mL) cooled to 0 °C under Ar atmosphere. The mixture was stirred at 0 °C until the reaction was complete. Subsequently TBAF (170 μL, 0.17 mmol) was added, the reaction mixture was stirred at 25 °C for 1 h and then quenched by addition of a saturated NH$_4$Cl solution (5 mL). The volatiles were removed and the aqueous phase was extracted 3 times with Et$_2$O. The combined organic layers were washed with a saturated NaCl solution, dried over Na$_2$SO$_4$ and evaporated. The residue was purified by flash chromatography on silica gel (10% EtOAc in *n*-hexane) to afford the title compound **21** as a colorless oil (80 mg, 35 % yield). Physical and spectroscopic data are consistent with those reported in the literature;[19] R$_f$: 0.49 (CHCl$_3$); ^{1}H NMR (300 MHz, CDCl$_3$) δ 1.17 (s, 3H), 1.41 (s, 9H), 3.82 (br s, 1H), 3.89 (br s, 1H), 5.20 (s, 1H), 5.47 (s, 1H).

(2R,S,3S)-3-(*N*-Acetyl-L-isoleucyl-L-(OBzl)threonyl-L-alanylamino)-1,1,1-trifluoro-2-butanol (22).

Compound **21** (80 mg, 0.33 mmol) was deprotected by treatment with a 50:50 v/v TFA/DCM solution at 25 °C for 2 h. The TFA/DCM mixture was evaporated and concentrated to obtain the trifluoroacetate salt of the free amine as a slightly orange oil, in a quantitative amount. The amine was immediately used in the next step. To a stirred solution of **26a**

(150 mg, 0.34 mmol) in anhydrous DMF cooled to 0 °C were added HOBt (52 mg, 0.39 mmol), EDC (74 mg, 0.39 mmol), and Et$_3$N (78 μL, 0.56 mmol) and the mixture was kept at 0 °C for 1 h. Then a solution of the free amine (40 mg, 0.28 mmol) and Et$_3$N (78 μL, 0.56 mmol) in anhydrous DMF was added to the mixture and the reaction was allowed to warm to 25 °C and stirred at the same temperature for 14 h. The organic solvent was evaporated and the residue was purified by flash chromatography on silica gel (2% MeOH in CHCl$_3$) and then on alumina (100 % CHCl$_3$) to afford the title compound **22** as a colorless oil (15 mg, 32% yield); R$_f$: 0.45 (20:1 CHCl$_3$/MeOH). ^{1}H NMR (400 MHz, CD$_3$OD) δ 0.79 - 0.94 (m, 6H), 1.39 (t, J = 6.7 Hz, 3H), 1.25 (s, 2H), 1.51 (t, J = 6.8 Hz, 3H), 1.79 – 1.91 (m, 1H), 1.92 (d, J = 7.1 Hz, 3H), 2.12 (s, 3H), 4.02 – 4.12 (m, 1H), 4.21 (dd, J_1 = 6.4 Hz, J_2 = 1.3 Hz, 1H), 4.41 – 4.62 (m, 4H), 4.91 (s, 2H), 6.90 – 7.44 (m, 5H); ESI-MS: m/z 559 (M-H)$^-$; EA: calcd for C$_{26}$H$_{39}$F$_3$N$_4$O$_6$: C, 55.70; H, 7.01; N, 9.99. Found: C, 56.03; H, 7.05; N, 10.11; HRMS calcd for C$_{26}$H$_{38}$F$_3$N$_4$O$_6$ (M - H)$^-$: 559.2743, found 559.2731.

(3*S*)-3-(*N*-Acetyl-L-isoleucyl-L-(OBzl)threonyl-L-alanylamino)-1,1,1-trifluoro-2-butanone (2).

A stirred solution of **22** (6 mg, 0.01 mmol) in anhydrous DCM (1 mL) was cooled to 0 °C and Dess-Martin periodinane (32 mg, 0.07 mmol) was added. The reaction mixture was warmed to 25 °C, stirred at the same temperature until disappearance of the starting material was observed by LC-MS and then Na$_2$S$_2$O$_3$ (135 mg, 0.48 mmol), in a saturated solution of NaHCO$_3$, was added. The reaction mixture was stirred for 10 min and then the organic solvent was separated, washed 3 times with a saturated solution of Na$_2$S$_2$O$_3$, 3 times with a saturated solution of NaHCO$_3$ and 3 times with H$_2$O, dried over Na$_2$SO$_4$ and evaporated *in vacuo*. The reaction product was purified by flash chromatography on silica gel (2% MeOH in CHCl$_3$) to afford the title compound as a colorless oil (5 mg, 70% yield); ^{1}H NMR (400 MHz, CDCl$_3$) δ 0.82 - 0.97 (m, 6H), 1.39 – 1.58 (m, 8H), 1.81 – 1.89 (m, 1H), 2.03 (d, J = 7.2 Hz, 3H), 2.12 (s, 3H), 4.09 – 4.19 (m, 1H), 4.23 – 4.39 (m, 1H), 4.51 – 4.62 (m, 2H), 4.61 – 4.75 (m, 1H), 5.21 (s, 2H), 6.32 – 6.49 (dd, J_1 = 6.2 Hz, J_2 = 1.5 Hz, 1H), 6.89 – 6.94 (m, 2H), 7.11 – 7.19 (m, 1H), 7.22 – 7.34 (m, 5H); ESI-MS: m/z 559 (M+H)$^+$. EA: calcd for C$_{26}$H$_{37}$F$_3$N$_4$O$_6$: C, 55.90; H, 6.68; N, 10.03. Found: C, 55.60; H, 6.96; N, 9.86; HRMS calcd for C$_{26}$H$_{38}$F$_3$N$_4$O$_6$ (M + H)$^+$: 559.2743, found 559.2740.

(3*R*,*S*,4*S*)-((4-*N*-tert-Butoxycarbonyl)amino)-2,2-difluoro-3-hydroxypentanoic acid ethyl ester (23).

A solution of **21** (2.6 g, 14.9 mmol) and ethyl bromodifluoroacetate (5.7 mL, 44.7 mmol) was added dropwise to a suspension of zinc dust (2.9 g, 44.7 mmol) in anhydrous THF (0.5 mM final concentration of aldehyde). After complete addition, the reaction was heated under reflux for 30 min. The reaction mixture was allowed to cool to 25 °C and partitioned between 1 M KHSO$_4$ and DCM. The organic layer was dried over Na$_2$SO$_4$, filtered, and concentrated. The product was purified by flash chromatography on silica gel (20% EtOAc in

n–hexane) to afford the title compound as a colorless oil (2.0 g, 45% yield). Physical and spectroscopic data are consistent with those reported in the literature;[20,21] R$_f$: 0.61 (1:1 EtOAc/Hex); ^{1}H NMR (400 MHz, CDCl$_3$) δ 1.29 (d, J = 6.5 Hz, 3H), 1.34 (t, J = 7.2 Hz, 3H), 1.42 (s, 9H), 3.89 – 3.97 (m, 2H), 4.32 (q, J = 7.2 Hz, 2H), 4.40 (br s, 1H), 4.96 (d, J = 8.3 Hz, 1H); ^{13}C NMR (Acetone-d_6, 75 MHz) δ 13.4, 17.8, 27.9, 46.0, 62.7, 73.1, 78.5, 115.2, 155.7, 205.5; ESI MS: m/z 320 (M+Na)$^+$.

(4*S*)-4-(*N*-Acetyl-L-isoleucyl-L-(OBzl)threonyl-L-alanylamino)-2,2-difluoro-3-hydroxypentanoic acid benzyl ester (24).

Ester **23** (155 mg, 0.22 mmol) was dissolved in MeCN (2.2 mL) and treated with a 0.25 N solution of LiOH (2.2 mL) for 2 h at 25 °C. The mixture was extracted with EtOAc to remove unhydrolyzed starting material. The aqueous phase was acidified to pH 2 with 1 N HCl and extracted 4 times with an equal volume of EtOAc. The organic layer was dried over Na$_2$SO$_4$ and concentrated to afford the corresponding free carboxylic acid as a colorless oil in quantitative yield, that was immediately used in the next reaction. To a solution of the above compound (140 mg, 0.52 mmol) in DCM were added benzyl alcohol (54 μL, 0.52 mmol), DCC (118 mg, 0.57 mmol) and HOBt (21 mg, 0.16 mmol) and the reaction mixture was stirred under Ar atmosphere at 25 °C for 12 h. The reaction was poured into a NaCl saturated solution, the organic solvent separated, dried over Na$_2$SO$_4$ and evaporated. The residue was purified by flash chromatography on silica gel (2% MeOH in CHCl$_3$) to afford the desired benzyl ester as a whitish oil (80 mg, 40% yield); ^{1}H NMR (300 MHz, CDCl$_3$) δ 1.27 (s, 3H), 1.41 (s, 9H), 3.81 – 4.09 (m, 3H), 4.82 (br s, 1H), 5.28 (s, 2H), 7.23 – 7.41 (m, 5H); ESI-MS: m/z 382 (M+Na)$^+$. EA: calcd for C$_{17}$H$_{23}$F$_2$NO$_5$: C, 56.82; H, 6.45; N, 3.90. Found: C, 56.51; H, 6.27; N, 3.61; HRMS calcd for C$_{17}$H$_{23}$F$_2$NO$_5$Na (M+Na)$^+$: 382.1442, found 382.1433. The benzyl derivative was deprotected of the Boc function and immediately coupled with **26a**, following the procedure described for compound **22**. The residue was purified by flash chromatography on silica gel (2% MeOH in CHCl$_3$) and then on alumina (100 % CHCl$_3$) to afford the title compound **24** as an amorphous white solid (80 mg, 40% yield); R$_f$: 0.40 (CHCl$_3$, alumina); ^{1}H NMR (300 MHz, CD$_3$OD) δ 0.82 – 1.01 (m, 6H), 1.02 – 1.18 (m, 1H), 1.18 – 1.24 (m, 6H), 1.37 (t, J = 6.4 Hz, 3H), 1.51 (br s, 1H), 1.82 (br s, 1H), 1.94 (s, 1H), 3.38 – 3.42 (m, 1H), 3.94 – 4.16 (m, 2H), 4.18 – 4.36 (m, 2H), 4.40 – 4.61 (m, 4H), 5.14 (d, J = 5.9 Hz, 1H), 7.19 – 7.40 (m, 10H); ESI-MS: m/z 675 (M-H)$^-$; EA: calcd for C$_{34}$H$_{46}$F$_2$N$_4$O$_8$: C, 60.34; H, 6.85; N, 8.28. Found: C, 60.48; H, 6.63; N, 8.10; HRMS calcd for C$_{34}$H$_{45}$F$_2$N$_4$O$_8$ (M-H)$^-$: 675.3205, found 675.3215.

(4*S*)-4-(*N*-Acetyl-L-isoleucyl-L-threonyl-L-alanylamino)-2,2-difluoro-3-oxopentanoic acid (3).

Compound **24** was oxidized to the corresponding difluorostatone following the procedure described for the preparation of **2**. The oxidized intermediate (10 mg, 0.01 mmol) and a catalytic amount of 10% Pd/C were stirred in MeOH (2.0

mL) under H_2 atmosphere at 40 °C. The disappearance of the starting material was monitored by TLC and the reaction was cooled to 25 °C, Pd/C was filtered through a pad of Celite which was carefully washed with MeOH (5 mL). The solvent was removed *in vacuo* and the title compound was obtained as a colorless oil without necessity of further purification (6 mg, 88% yield); R_f: 0.05 (5:1 CHCl$_3$/MeOH); ^{1}H NMR (300 MHz, CD$_3$OD) δ 0.84 – 0.95 (m, 6H), 1.12 – 1.19 (m, 4H), 1.32 (s, 3H), 1.48 (d, J = 6.9 Hz, 3H), 1.54 (br s, 1H), 1.82 (br s, 1H), 1.94 (d, J = 7.6 Hz, 3H), 4.09 – 4.23 (m, 3H), 4.30 – 4.41 (m, 2H); ESI-MS: m/z 493 (M-H)$^-$; EA: calcd for C$_{20}$H$_{32}$F$_2$N$_4$O$_8$: C, 48.58; H, 6.52; N, 11.33. Found: C, 48.21; H, 6.68; N, 11.59; HRMS calcd for C$_{20}$H$_{31}$F$_2$N$_4$O$_8$ (M-H)$^-$: 493.2110, found 493.2106.

(3R,S,4S)-(4-Amino-2,2-difluoro-3-hydroxypentanoyl)-glycine benzyl ester (25).

Ethyl ester **23** (890 mg, 3.00 mmol) was hydrolyzed to the corresponding free carboxylic acid following the procedure described for compound **24**. To a 0 °C solution of the above free carboxylic acid (807 mg, 3.00 mmol) and glycine benzyl ester hydrochloride (604 mg, 3.00 mmol) in anhydrous DMF, HATU (1250 mg, 3.30 mmol) and DIPEA (2.6 mL, 15.00 mmol) were added. The reaction mixture was warmed to 25 °C and stirred for 12 h. The solvent was evaporated and the residue was purified by flash chromatography on silica gel (20% EtOAc in *n*–hexane) to afford the desired intermediate as a slightly yellow oil (537 mg, 43% yield); Physical and spectroscopic data are consistent with those reported in the literature.[11] ^{1}H NMR (300 MHz, CDCl$_3$) δ 1.22 (d, J = 6.6 Hz, 3H), 1.38 (s, 9H), 3.88 – 4.13 (m, 6H), 4.93 (br s, 1H), 5.16 (s, 1H), 7.29 – 7.33 (m, 5H), 7.67 (br s, 1H); ESI-MS: m/z 331 (M-Boc+H)$^+$. The above benzyl ester was deprotected of the Boc function by treatment with a 50:50 v/v TFA/DCM solution at 25 °C for 3 h. The TFA/DCM mixture was evaporated and concentrated to obtain the trifluoroacetate salt of the free amine as slightly yellow oil, in a quantitative amount. The salt, dissolved in EtOAc and washed 2 times with a NaHCO$_3$ saturated solution furnished the corresponding free base that was immediately used in the next step.

N-Acetyl-L-isoleucyl-L-(OBn)threonyl-L-alanine (26a).

Microwave assisted solid-phase peptide synthesis was performed starting from Fmoc-Ala-Wang-resin (167 mg based on a loading of 0.6 mmol/g). Each coupling and deprotection reaction was carried out under MW irradiation and N_2 bubbling. Coupling reactions were performed in DMF at 75 °C for 300 s with a power of 28 W, using 5-fold molar excess of Fmoc-L-amino acids, 10-fold molar excess of HOBt/HBTU and 25-fold molar excess of DIEA. For each amino acid a single coupling was applied. Fmoc deprotections were performed with a 20% piperidine solution in DMF at 75 °C for 180 s with a power of 43 W.[22] Acetylation of the free amine functionality was carried out with a 20% acetic anhydride solution in DMF at 65 °C for 30 s with a power of 40 W. After chain assembly, resin was transferred to a gooch filtering apparatus and washed several

times with DCM. Cleavage of the peptide chain was carried out treating the resin with a 95:2.5:2.5 v/v/v TFA/water/TIS mixture for 3 h at 25 °C with N_2 bubbling. The resin was filtered and the filtrate was concentrated under reduced pressure. The resulting peptide was precipitated by treatment with cold Et$_2$O. **26a** was obtained as a white solid in 99% yield; Physical and spectroscopic data are consistent with those reported in the literature.[11] ^{1}H NMR (300 MHz, CD$_3$OD) δ 0.87 – 0.94 (m, 6H), 1.14 – 1.29 (m, 4H), 1.38 (d, J = 7.1 Hz, 3H), 1.48 – 1.57 (m, 1H), 1.80 – 1.88 (m, 1H), 1.95 (s, 3H), 4.05 – 4.08 (m, 1H), 4.25 (d, J = 7.5 Hz, 1H), 4.36 – 4.43 (m, 1H), 4.47 – 4.60 (m, 3H), 7.24 – 7.34 (m, 5H); ESI MS: m/z 434 (M-H)-, 470 (M+Cl)$^-$.

N-Acetyl-L-valyl-L-(OBn)threonyl-L-alanine (26b).

Following the procedure described for **26a**, the title compound was obtained as a white solid in 99% yield (based on a 0.6 mmol/g loading); ^{1}H NMR (300 MHz, CD$_3$OD) δ 0.93 – 0.97 (m, 6H), 1.22 (d, J = 6.2 Hz, 3H), 1.38 (d, J = 7.0 Hz, 3H), 1.96 (s, 3H), 2.03 – 2.14 (m, 1H), 4.02 – 4.10 (m, 1H), 4.21 (d, J = 7.0 Hz, 1H), 4.34 – 4.44 (m, 1H), 4.47 – 4.60 (m, 3H), 7.22 – 7.34 (m, 5H); ESI-MS: m/z 420 (M-H)$^-$; HRMS calcd for C$_{21}$H$_{30}$N$_3$O$_6$ (M-H)$^-$: 420.2135, found 420.2137.

N-Acetyl-L-isoleucyl-L-(OBn)seryl-L-alanine (26c).

Following the procedure described for **26a**, the title compound was obtained as a white solid in 99% yield (based on a 0.6 mmol/g loading); ^{1}H NMR (300 MHz, CD$_3$OD) δ 0.86 – 0.94 (m, 6H), 1.12 – 1.23 (m, 1H), 1.39 (d, J = 6.7 Hz, 3H), 1.52 (br s, 1H), 1.83 (br s, 1H), 1.97 (s, 3H), 3.75 (d, J = 4.8 Hz, 2H), 4.23 (d, J = 7.0 Hz, 1H), 4.35 – 4.42 (m, 1H), 4.53 (s, 2H), 4.61 – 4.65 (m, 1H), 7.24 – 7.32 (m, 5H); ESI-MS: m/z 420 (M-H)$^-$; HRMS calcd for C$_{21}$H$_{30}$N$_3$O$_6$ (M-H)$^-$: 420.2135, found 420.2143.

N-Acetyl-L-isoleucyl-L-(OBn)threonylglycine (26d).

Following the procedure described for **26a**, the title compound was obtained as a white solid in 99% yield (based on a 0.4 mmol/g loading); ^{1}H NMR (300 MHz, CD$_3$OD) δ 0.87 – 0.95 (m, 6H), 1.15 – 1.28 (m, 5H), 1.45 – 1.59 (m, 1H), 1.78 – 1.91 (m, 1H), 1.95 (s, 3H), 3.92 (s, 2H), 4.02 – 4.11 (m, 1H), 4.24 (d, J = 7.5 Hz, 1H), 4.47 – 4.64 (m, 3H), 7.22 – 7.39 (m, 5H); ESI-MS: m/z 420 (M-H)$^-$; HRMS calcd for C$_{21}$H$_{30}$N$_3$O$_6$ (M-H)$^-$: 420.2135, found 420.2126.

N-Acetyl-L-isoleucyl-L-valylglycine (26e).

Following the procedure described for **26a**, the title compound was obtained as a white solid in 99% yield (based on a 0.4 mmol/g loading); ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.76 – 0.87 (m, 6H), 0.90 – 1.00 (m, 7H), 1.37 (br s, 1H), 1. 67 (br s, 1H), 1.81 (s, 3H), 1.88 – 1.96 (m, 1H), 3.58 – 3.85 (m, 2H), 4.12 – 4.19 (m, 2H), 7.68 (d, J = 8.4 Hz, 1H), 7.91 (d, J = 8.3 Hz, 1H), 8.21 (br s, 1H), 12.41 (br s, 1H); ESI-MS: m/z 328 (M-H)$^-$; HRMS calcd for C$_{15}$H$_{26}$N$_3$O$_5$ (M-H)$^-$: 328.1872, found 328.1868.

N-Acetyl-L-phenylalanyl-L-valylglycine (26f).

Following the procedure described for **26a**, the title compound was obtained as a white solid in 99% yield (based on a 0.4 mmol/g loading); ^{1}H NMR (300 MHz, CD$_3$OD) δ 0.92 – 0.98 (m, 6H), 1.88 (s, 3H), 2.03 – 2.13 (m, 1H), 2.85 – 2.95 (m, 1H), 3.06 – 3.13 (s, 1H), 3.78 – 3.96 (m, 2H), 4.26 (d, $J = 6.9$ Hz, 1H), 4.68 – 4.73 (m, 1H), 7.14 – 7.26 (m, 5H); ESI-MS: m/z 362 (M-H)$^-$; HRMS calcd for C$_{18}$H$_{24}$N$_3$O$_5$ (M-H)$^-$: 362.1713, found 362.1718.

N-Acetyl-L-phenylglycyl-L-valylglycine (26g).

Following the procedure described for **26a**, the title compound was obtained as a white solid in 99% yield (based on a 0.4 mmol/g loading); ^{1}H NMR (300 MHz, CD$_3$OD) δ 0.7 – 0.9 (m, 6H), 2.01 (s, 3H), 2.05 – 2.18 (m, 1H), 3.73 – 3.94 (m, 2H), 4.22 – 4.29 (m, 1H), 5.48 – 5.52 (m, 1H), 7.24 – 7.50 (m, 5H); ESI-MS: m/z 348 (M-H)$^-$; HRMS calcd for C$_{17}$H$_{22}$N$_3$O$_5$ (M-H)$^-$: 348.1559, found 348.1554.

N-Acetyl-L-phenylglycyl-L-phenylglycylglycine (26h).

Following the procedure described for **26a**, the title compound was obtained as a white solid in 99% yield (based on a 0.4 mmol/g loading); ^{1}H NMR (300 MHz, CD$_3$OD) δ 1.98 – 2.00 (m, 3H), 3.83 – 4.05 (m, 4H), 7.23 – 7.47 (m, 10H). ESI-MS: m/z 382 (M-H)$^-$; HRMS calcd for C$_{20}$H$_{20}$N$_3$O$_5$ (M-H)$^-$: 382.1403, found 382.1410.

N-Acetyl-L-phenylglycylglycylglycine (26i).

Following the procedure described for **26a**, the title compound was obtained as a white solid in 99% yield (based on a 0.4 mmol/g loading); ^{1}H NMR (300 MHz, CD$_3$OD) δ 2.01 (s, 3H), 3.65 – 4.12 (m, 4H), 5.38 (s, 1H), 7.23 – 7.44 (m, 5H); ESI-MS: m/z 306 (M-H)$^-$; HRMS calcd for C$_{14}$H$_{16}$N$_3$O$_5$ (M-H)$^-$: 306.1090, found 306.1088.

N-Phenylacetyl-L-valylglycine (26j).

Following the procedure described for **26a**, the title compound was obtained as a white solid in 99% yield (based on a 0.4 mmol/g loading); ^{1}H NMR (300 MHz, CD$_3$OD) δ 0.89 – 1.04 (m, 6H), 2.03 – 2.13 (m, 1H), 3.51 – 3.63 (m, 2H), 3.89 (dt, $J_1 = 17.7$ Hz, $J_2 = 29.0$ Hz, 2H), 4.26 (d, $J = 6.9$ Hz, 1H), 7.21 – 7.28 (m, 5H); ESI-MS: m/z 291 (M-H)$^-$; HRMS calcd for C$_{15}$H$_{19}$N$_2$O$_4$ (M-H)$^-$: 291.1345, found 291.1354.

4-Quinolinyl-2-propenoic acid methyl ester (30).

Quinolin-4-carbaldehyde **29** (300 mg, 1.91 mmol) and methyl(triphenylphosphoranylidene)acetate (702 mg, 2.09 mmol) were dissolved in anhydrous toluene (8 mL) and the reaction mixture was stirred at 80 °C for 2.5 h. After cooling the reaction to 25 °C, the organic solvent was extracted 2 times with 1 N HCl. Aqueous extracts were washed with EtOAc, alkalized with 1 N NaOH and extracted 3 times with EtOAc. Combined organic extracts were washed with brine, dried over Na$_2$SO$_4$ and evaporated under reduced pressure to dryness to afford the title product as white solid (320 mg, 79% yield);

Physical and spectroscopic data are consistent with those reported in the literature.[23]

4-Quinolinylpropanoic acid (31).

To solution of **34** (300 mg, 1.40 mmol) in MeOH (5 mL) at 25 °C, a catalytic amount of 10% Pd/C was added and consumption of the starting material was monitored by TLC. After catalyst was filtered off, the filtrate was evaporated under reduced pressure to dryness and the residue was purified by flash chromatography on silica gel (33% n-hexane in EtOAc) to give the pure title product as colorless oil (188 mg, 62% yield) without the necessity of further purification. Subsequently, a solution of LiOH (6 mg, 0.24 mmol) in a 2:1 mixture of THF/H$_2$O (3 mL) was added to a solution of the above compound (50 mg, 0.23 mmol) in THF (1 mL), and the reaction mixture was stirred for 15 h at 25 °C. A small amount of water was added, resulting in the formation of a precipitate, and stirring was continued for approximately 10 min until precipitate dissolved. The aqueous phase was acidified to pH 4 with 1 N HCl and extracted 3 times with EtOAc. The solvent was dried over Na$_2$SO$_4$ and evaporated to dryness to obtain the desired compound as a white solid (40 mg, 86% yield); Physical and spectroscopic data are consistent with those reported in the literature.[24]

N-(4-Quinolinylpropanoyl)-L-valylglycine (26k).

To a stirred solution of **31** (185 mg, 0.92 mmol) and DIPEA (144 μL, 0.83 mmol) in anhydrous DMF cooled to 0 °C were added HOBt (149 mg, 1.10 mmol), EDC (212 mg, 1.10 mmol) as solid, and the mixture was kept at 0 °C for 10 min. Then a solution of **32**[15] (243 mg, 0.92 mmol) and DIPEA (192 μL, 1.10 mmol) in anhydrous DMF was added to the mixture and the reaction was kept at 0 °C for 1 additional hour, allowed to warm to 25 °C and stirred at that temperature for 14 h. The organic solvent was evaporated and the residue was purified by flash chromatography on silica gel (2% MeOH in CHCl$_3$) to afford the desired intermediate **27a** (50 mg, 28% yield); ^{1}H NMR (300 MHz, CD$_3$OD) δ 0.81 – 0.87 (m, 6H), 1.96 – 2.00 (m, 1H), 2.75 (t, $J = 7.2$ Hz, 2H), 3.45 (t, $J = 7.2$ Hz, 2H), 3.96 (q, $J = 16.8$ Hz, 2H), 4.17 (t, $J = 7.5$ Hz, 1H), 5.14 (s, 2H), 7.30 – 7.33 (m, 5H), 7.41 (d, $J = 4.2$ Hz, 1H), 7.65 (t, $J = 7.5$ Hz, 1H), 7.76 (t, $J = 7.5$ Hz, 1H), 8.02 (d, $J = 8.1$ Hz, 1H), 8.22 (d, $J = 8.1$ Hz, 1H), 8.71 (d, $J = 4.5$ Hz, 1H); ESI-MS: m/z 448 (M+H)$^+$, 470 (M+Na)$^+$; EA: calcd for C$_{26}$H$_{29}$N$_3$O$_4$: C, 69.78; H, 6.53; N, 9.39. Found: C, 69.70; H, 6.42; N, 9.15; HRMS calcd for C$_{26}$H$_{29}$N$_3$NaO$_4$ (M+Na)$^+$: 470.2056, found 470.2057. The above compound was deprotected from the terminal benzyl ester following the procedure described for **31** to give the free acid **26k** (40 mg, 99% yield) that was immediately used in the next step.

(S)-4-Acetamido-2-methyl-5-phenylpentenoic acid ethyl ester (34).

Et$_3$N (1.94 mL, 13.91 mmol) was added to a stirred solution of commercially available L-Phenylalanine methyl ester hydrochloride **33** (1.0 g, 4.46 mmol) in anhydrous DCM. The

solution was stirred at 25 °C for 5 min and then acetic anhydride (1.31 mL, 13.91 mmol) was added dropwise. The solution was stirred at 25 °C for 3 h. DCM was washed 2 times with $NaHCO_3$ saturated solution, dried over Na_2SO_4 and concentrated to obtain the desired compound as a white powder (1.06 g, 99% yield). Physical and spectroscopic data are consistent with those reported in the literature.[25] ^{1}H NMR (300 MHz, $CDCl_3$) □□1.02 – 1.11 (m, 6H), 1.47 (s, 9H), 2.72 – 2.78 (m, 1H), 4.27 (dd, J_1 = 6.7 Hz, J_2 = 2.3 Hz, 2H), 4.34 (d, J = 6.2 Hz, 1H), 6.11 (s, 2H), 6.81 (br s, 1H), 6.99 – 7.24 (m, 5H), 7.31 (br s, 1H). A solution of DIBALH in anhydrous DCM (1 M, 2.0 equiv.) was added dropwise at –78 °C to a solution of the above compound (1.06 g, 4.79 mmol) in anhydrous DCM (14.4 mL). The solution was stirred for 30 min at –78 °C and then (carbethoxyethylidene)triphenylphosphorane (3.47 g, 9.58 mmol) and KO*t*Bu (1.08 g, 9.58 mmol), suspended in anhydrous DCM (19.2 mL), were added to the reaction mixture. The cooling bath was removed after 1 h, and the reaction mixture was stirred at 25 °C for 15 h. Afterward it was poured into a saturated potassium sodium tartrate solution (20 mL), and vigorously stirred for 30 min. The aqueous layer was extracted 3 times with EtOAc and the combined organic layers were dried over Na_2SO_4. The reaction mixture was evaporated under reduced pressure and the crude product was purified 2 times by flash chromatography on silica gel (1) 100% $CHCl_3$ and then 2) 100% Et_2O) to afford the title compound as a white powder (870 mg, 66% yield); R_f: 0.75 (Et_2O); ^{1}H NMR (300 MHz, $CDCl_3$) δ□1.27 (t, J = 7.1 Hz, 3H), 1.73 (s, 3H), 1.92 (s, 3H), 2.88 (ddd, J_1 = 6.7 Hz, J_2 = 13.45 Hz, J_3 = 20.9 Hz, 2H), 4.17 (q, J = 7.0 Hz, 2H), 4.94 – 5.04 (m, 1H), 5.41 (d, J = 7.9 Hz, 1H), 6.50 (d, J = 9.3 Hz, 1H), 7.14 – 7.31 (m, 5H); ESI-MS: *m/z* 298 (M+Na)$^+$; HRMS calcd for $C_{16}H_{21}NO_3Na$ (M+Na)$^+$: 298.1419, found 298.1423.

(1R,2S,5RS)-4-Acetamido-2-methyl-5-phenylpentenoic acid (−)-menthyl ester (35).

A mixture of ethyl ester **34** (770 mg, 2.79 mmol) and 1 M aqueous NaOH (3.36 mL, 3.36 mmol) in 1,4-dioxane (28 mL) was heated at 80 °C for 2 h. The solvent was evaporated *in vacuo* and the residue was dissolved in H_2O. The aqueous phase was washed with EtOAc to remove the unhydrolyzed starting material, acidified to pH 2 with 1 M HCl and extracted 2 times with EtOAc. The combined organic layers were dried over Na_2SO_4 and the solvent was evaporated in vacuo to give the corresponding acid as a colorless oil (680 mg, 98% yield); ^{1}H NMR (300 MHz, $CDCl_3$) δ□1.66 (s, 3H), 1.92 (s, 3H), 2.74 – 2.97 (m, 2H), 4.92 – 5.02 (m, 1H), 6.34 (br s, 1H), 6.62 (d, J = 9.2 Hz, 1H), 7.12 – 7.25 (m, 5H), 10.96 (br s, 1H); ESI-MS: *m/z* 246 (M-H)$^-$, 493 (2M-H)$^-$. Subsequently, a solution of DCC (618 mg, 3.00 mmol) in anhydrous Et_2O (5 mL) was added to a suspension of the above carboxylic acid (680 mg, 2.75 mmol), (1R,2S,5R)-(−)-menthol (1.07 g, 6.87 mmol), and DMAP (33 mg, 0.27 mmol) in anhydrous Et_2O (10 mL) cooled to 0 °C. The reaction mixture was allowed to warm to 25 °C and kept at that temperature for 15 h. After filtration of the precipitated urea the crude product was purified by flash chromatography

on silica gel (100% Et_2O) to give the unsaturated menthyl ester **35** as colorless oil (240 mg, 23% yield); R_f: 0.63 (Et_2O); ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.71 (d, J = 6.8 Hz, 3H), 0.80 – 0.94 (m, 6H), 0.98 – 1.08 (m, 2H), 1.22 – 1.44 (m, 3H), 1.62 – 1.68 (m, 5H), 1.78 – 1.97 (m, 5H), 2.84 (ddd, J_1 = 6.6 Hz, J_2 = 13.3 Hz, J_3 = 20.5 Hz, 2H), 4.66 (td, J_1 = 3.8 Hz, J_2 = 10.6 Hz, 1H), 4.92 – 5.03 (m, 1H), 6.24 (d, J = 8.0 Hz, 1H), 6.48 (d, J = 9.3 Hz, 1H), 7.09 – 7.24 (m, 5H); ESI-MS: *m/z* 408 (M+Na)$^+$; HRMS calcd for $C_{24}H_{35}NO_3Na$ (M+Na)$^+$: 408.2515, found 408.2505.

(2S,4R)-4-Acetamido-2-methyl-5-phenylpentanoic acid (-)-menthyl ester (2S,4R-36) and (2R,4R)-4-acetamido-2-methyl-5-phenylpentanoic acid (-)-menthyl ester (2R,4R-37).

A solution of menthyl ester **35** (240 mg, 0.62 mmol) in MeOH (10 mL) was stirred under H_2 with a catalytic amount of Pd/C 10% until complete hydrogenation was observed on TLC. The catalyst was filtered off through a pad of celite and the solvent evaporated. The diastereomers were separated by column chromatography on silica gel (30% EtOAc in *n*-hexane) to give 2S,4R-**36** (120 mg, 50% yield), 2R,4R-**37** (55 mg, 23% yield), and a mixed fraction (60 mg, 24%); (2S,4R-**36**): R_f: 0.67 (1:2 EtOAc/Hex); ^{1}H NMR (300 MHz, $CDCl_3$) δ□0.71 (d, J = 6.9 Hz, 1H), 0.87 (dd, J_1 = 4.6 Hz, J_2 = 6.2 Hz, 6H), 0.94 – 1.07 (m, 3H), 1.13 (d, J = 6.9 Hz, 3H), 1.22 – 1.52 (m, 4H), 1.62 – 1.69 (m, 2H), 1.81 – 2.00 (m, 5H), 2.50 (br s, 1H), 2.71 – 2.85 (m, 2H), 4.15 (br s, 1H), 4.62 (td, J_1 = 4.1 Hz, J_2 = 10.8 Hz, 1H), 5.65 (d, J = 7.5 Hz, 1H), 7.12 – 7.27 (m, 5H); ESI-MS: *m/z* 388 (M+H)$^+$, 410 (M+Na)$^+$, 797 (2M+K)$^+$; HRMS calcd for $C_{24}H_{37}NO_3Na$ (M+Na)$^+$: 410.2671, found 410.2670. (2R,4R-**37**): R_f: 0.44 (1:2 EtOAc/Hex); ^{1}H NMR (300 MHz, $CDCl_3$) δ□0.71 (d, J = 6.9 Hz, 3H), 0.86 (dd, J_1 = 2.9 Hz, J_2 = 6.7 Hz, 6H), 0.90 – 1.06 (m, 3H), 1.11 (d, J = 6.9 Hz, 3H), 1.20 – 1.53 (m, 4H), 1.64 (d, J = 12.6 Hz, 2H), 1.72 – 1.82 (m, 2H), 1.87 – 1.94 (m, 3H), 2.37 (dd, J_1 = 6.8 Hz, J_2 = 13.6 Hz, 1H), 2.77 (ddd, J_1 = 6.4 Hz, J_2 = 13.9 Hz, J_3 = 25.5 Hz, 2H), 4.20 (br s, 1H), 4.60 (td, J_1 = 4.2 Hz, J_2 = 10.8 Hz, 1H), 5.55 (d, J = 8.1 Hz, 1H), 7.12 – 7.28 (m, 5H); ESI-MS: *m/z* 388 (M+H)$^+$, 410 (M+Na)$^+$, 797 (2M+K)$^+$; HRMS calcd for $C_{24}H_{37}NO_3Na$ (M+Na)$^+$: 410.2671, found 410.2665.

(2S,4R)-4-Acetamido-2-methyl-5-phenylpentanoic acid (2S,4R-38).

A solution of 2S,4R-**36** (120 mg, 0.31 mmol) in 6 N HCl (2 mL) was heated at 130 °C for 2 h. The solution was cooled to 25 °C and the solvent evaporated to obtain the desired compound as a colorless oil (76 mg, 99% yield); ^{1}H NMR (300 MHz, $CDCl_3$) δ□0.92 – 1.28 (m, 4H), 1.38 – 1.81 (m, 2H), 1.84 – 2.21 (m, 3H), 2.21 – 2.48 (br s, 1H), 2.61 – 2.93 (m, 2H), 4.15 – 4.23 (br s, 1H), 7.05 – 7.41 (m, 5H), 8.01 (br s, 1H). ESI-MS: *m/z* 248; HRMS calcd for $C_{14}H_{18}NO_3$ (M-H)$^-$: 248.1287, found 248.1296.

(2S,4R)-4-Acetamido-2-methyl-5-phenylpentanoic acid (2R,4R-39).

Starting from 2*R*,4*R*-**37** compound 2*R*,4*R*-**39** was obtained following the procedure described for the preparation of 2*S*,4*R*-**38** (35 mg, 99% yield); ^{1}H NMR (300 MHz, CDCl$_3$) δ☐0.98 – 1.19 (m, 3H), 1.21 – 1.40 (m, 1H), 1.41 – 1.75 (m, 2H), 1.80 – 2.21 (m, 3H), 2.28 – 2.60 (m, 1H), 2.80 (d, J = 2.5 Hz, 2H), 4.23 (br s, 1H), 7.02 – 7.25 (m, 5H), 7.60 (br s, 1H). ESI-MS: *m/z* 248 (M-H)$^-$; HRMS calcd for C$_{14}$H$_{18}$NO$_3$ (M-H)$^-$: 248.1287, found 248.1290.

(2*S*,4*R*)-(4-Acetamido-2-methyl-5-phenylpentanoyl)glycine (2*S*,4*R*-26l).

Starting from 2*S*,4*R*-**38** and glycine benzyl ester hydrochloride, compound 2*S*,4*R*-**26l** was obtained following the procedure described for the preparation of **26k** (41 mg, 43% yield); ^{1}H NMR (300 MHz, CDCl$_3$) δ☐1.11 (d, *J* = 6.9 Hz, 3H), 1.47 (ddd, *J$_1$* = 5.8 Hz, *J$_2$* = 12.1 Hz, *J$_3$* = 20.1 Hz, 1H), 1.82 (s, 3H), 2.25 – 2.48 (m, 1H), 2.73 (ddd, *J$_1$* = 6.6 Hz, *J$_2$* = 13.6 Hz, *J$_3$* = 20.9 Hz, 2H), 3.85 – 4.20 (m, 4H), 5.14 (s, 2H), 5.68 (d, *J* = 8.9 hz, 1H), 6.44 (br s, 1H), 7.13 – 7.33 (m, 10H). ESI-MS: *m/z* 397 (M+H)$^+$, 419 (M+Na)$^+$; HRMS calcd for C$_{23}$H$_{28}$N$_2$O$_4$Na (M+Na)$^+$: 419.1947, found 419.1951. The benzyl derivative (40 mg, 0.10 mmol) was deprotected following the procedure described for the synthesis of 2*S*,4*R*-**38** (31 mg, 99% yield) and used immediately in the next step. R$_f$: 0.35 (20:1 CHCl$_3$/MeOH);

(2*R*,4*R*)-(4-Acetamido-2-methyl-5-phenylpentanoyl)glycine (2*R*,4*R*-26m).

Starting from 2*R*,4*R*-**40** and glycine benzyl ester hydrochloride, compound 2*R*,4*R*-**26m** was obtained following the procedure described for the preparation of **26k** (20 mg, 24% yield); ^{1}H NMR (300 MHz, CDCl$_3$) δ☐1.12 (dd, *J$_1$* = 6.9 Hz, *J$_2$* = 20.3 Hz, 3H), 1.24 – 1.38 (m, 1H), 1.87 (s, 3H), 2.31 – 2.46 (m, 1H), 2.59 – 2.85 (m, 2H), 3.70 – 3.82 (m, 1H), 4.06 (ddd, *J$_1$* = 5.7 Hz, *J$_2$* = 17.9 Hz, *J$_3$* = 22.9 Hz, 2H), 4.45 – 4.56 (m, 1H), 5.11 – 5.21 (m, 2H), 5.72 (d, *J* = 9.2 Hz, 1H), 7.13 – 7.30 (m, 5H). ESI-MS: *m/z* 397 (M+H)$^+$, 419 (M+Na)$^+$; HRMS calcd for C$_{23}$H$_{28}$N$_2$O$_4$ (M+Na)$^+$: 419.1947, found 419.1953. The benzyl derivative (20 mg, 0.05 mmol) was deprotected following the procedure developed for the synthesis of 2*S*,4*R*-**39** (15 mg, 99% yield) and used immediately in the next step.

(3*R*,,S,4*S*)-(4-(*N*-Acetyl-L-isoleucyl-L-(OBn)threonyl-L-alanylamino)-2,2-difluoro-3-hydroxypentanoyl)glycine benzyl ester (27a).

Starting from **25** and **26a** compound **27a** was obtained following the procedure described for the preparation of **26k**. (25 mg, 17% yield); mp (Hex/CHCl$_3$) 206-207 °C; R$_f$: 0.40 (5:1 CHCl$_3$/MeOH); ^{1}H NMR (300 MHz, CD$_3$OD) δ☐0.89 – 0.98 (m, 6H), 1.10 – 1.25 (m, 6H), 1.27 – 1.35 (m, 4H), 1.51 (br s, 1H), 1.85 (br s, 1H), 1.94 (s, 3H), 3.91 – 4.13 (m, 4H), 4.16 – 4.41 (m, 3H), 4.41 – 4.65 (m, 3H), 5.17 (s, 2H), 7.18 – 7.74 (m, 10H); ESI-MS: *m/z* 734 (M+H)$^+$, 756 (M+Na)$^+$; EA: calcd for C$_{36}$H$_{49}$F$_2$N$_5$O$_9$: C, 58.92; H, 6.73; N, 9.54. Found: C, 59.21; H, 6.87; N, 9.22; HRMS calcd for C$_{36}$H$_{50}$F$_2$N$_5$O$_9$ (M+H)$^+$: 734.3577, found 734.3588.

(3*R*,,S,4*S*)-(4-(*N*-Acetyl-L-valyl-L-(OBn)threonyl-L-alanylamino)-2,2-difluoro-3-hydroxypentanoyl)glycine benzyl ester (27b).

Starting from **25** and **26b** compound **27b** was obtained following the procedure described for the preparation of **26k**. (49 mg, 31% yield); R$_f$: 0.20 (20:1 CHCl$_3$/MeOH); ^{1}H NMR (300 MHz, CD$_3$OD) δ☐0.95 – 0.97 (m, 6H), 1.15 – 1.21 (m, 6H), 1.25 – 1.32 (m, 3H), 1.96 (s, 3H), 2.04 – 2.15 (m, 1H), 3.97 – 4.12 (m, 4H), 4.16 – 4.34 (m, 3H), 4.35 – 4.46 (m, 1H), 4.46 – 4.59 (m, 2H), 5.17 (s, 3H), 7.16 – 7.33 (m, 10H); ESI-MS: *m/z* 742 (M+Na)$^+$; EA: calcd for C$_{35}$H$_{47}$F$_2$N$_5$O$_9$: C, 58.40; H, 6.58; N, 9.73. Found: C, 58.20; H, 6.64; N, 9.34; HRMS calcd for C$_{35}$H$_{47}$F$_2$N$_5$O$_9$Na (M+Na)$^+$: 742.3240, found 742.3252.

(3*R*,,S,4*S*)-(4-(*N*-Acetyl-L-isoleucyl-L-(OBn)seryl-L-alanylamino)-2,2-difluoro-3-hydroxypentanoyl)glycine benzyl ester (27c).

Starting from **25** and **26c** compound **27c** was obtained following the procedure described for the preparation of **26k**. (34 mg, 35% yield); R$_f$: 0.20 (20:1 CHCl$_3$/MeOH); ^{1}H NMR (300 MHz, CD$_3$OD) δ☐0.85 – 0.94 (m, 6H), 1.15 – 1.22 (m, 4H), 1.25 – 1.33 (m, 3H), 1.52 (br s, 1H), 1.81 (br s, 1H), 1.97 (s, 3H), 3.65 – 3.79 (m, 2H), 3.96 – 4.04 (m, 2H), 4.11 – 4.35 (m, 4H), 4.46 – 4.63 (m, 3H), 5.17 (s, 2H), 7.25 – 7.34 (m, 10H); ESI-MS: *m/z* 742 (M+Na)$^+$; EA: calcd for C$_{35}$H$_{47}$F$_2$N$_5$O$_9$: C, 58.40; H, 6.58; N, 9.73. Found: C, 58.07; H, 6.49; N, 9.51; HRMS calcd for C$_{35}$H$_{47}$F$_2$N$_5$O$_9$Na (M+Na)$^+$: 742.3240, found 742.3233.

(3*R*,,S,4*S*)-(4-(*N*-Acetyl-L-isoleucyl-L-(OBn)threonylglycylamino)-2,2-difluoro-3-hydroxypentanoyl)glycine benzyl ester (27d).

Starting from **25** and **26d** compound **27d** was obtained following the procedure described for the preparation of **26k**. (44 mg, 35% yield); R$_f$: 0.38 (5:1 CHCl$_3$/MeOH); ^{1}H NMR (300 MHz, CD$_3$OD) δ☐0.81 – 0.95 (m, 6H), 1.08 – 1.29 (m, 7H), 1.51 (br s, 1H), 1.84 (br s, 1H), 1.93 (s, 3H), 3.71 – 3.84 (m, 2H), 3.89 – 4.04 (m, 4H), 4.11 – 4.31 (m, 2H), 4.34 – 4.58 (m, 3H), 5.17 (s, 2H), 7.12 – 7.39 (m, 10H); ESI-MS: *m/z* 720 (M+H)$^+$, 742 (M+Na)$^+$; EA: calcd for C$_{35}$H$_{47}$F$_2$N$_5$O$_9$: C, 58.40; H, 6.58; N, 9.73. Found: C, 58.57; H, 6.63; N, 10.12; HRMS calcd for C$_{35}$H$_{48}$F$_2$N$_5$O$_9$ (M+H)$^+$: 720.3420, found 720.3418.

(3*R*,,S,4*S*)-(4-(*N*-Acetyl-L-isoleucyl-valylglycylamino)-2,2-difluoro-3-hydroxypentanoyl)glycine benzyl ester (27e).

Starting from **25** and **26e** compound **27e** was obtained following the procedure described for the preparation of **26k**. (29 mg, 29% yield); R$_f$: 0.21 (20:1 CHCl$_3$/MeOH); ^{1}H NMR (300 MHz, CD$_3$OD) δ☐0.86 – 0.92 (m, 7H), 0.95 – 0.99 (m, 6H), 1.47 – 1.59 (m, 1H), 1.77 – 1.84 (m, 1H), 1.97 (s, 3H), 2.02 – 2.11 (m, 1H), 3.70 – 3.88 (m, 3H), 3.97 – 4.12 (m, 3H), 4.21 (d, *J* = 8.4 Hz, 1H), 4.26 – 4.28 (m, 1H), 5.19 (s, 2H), 7.30 – 7.35 (m, 5H); ESI-MS: *m/z* 628 (M+H)$^+$, 650 (M+Na)$^+$; EA: calcd for C$_{29}$H$_{43}$F$_2$N$_5$O$_8$: C, 55.49; H, 6.91; N, 11.16. Found: C, 55.87; H, 6.61; N, 10.81; HRMS calcd for C$_{29}$H$_{43}$F$_2$N$_5$O$_8$Na (M+Na)$^+$: 650.2977, found 650.2986.

(3*R*,*S*,4*S*)-(4-(*N*-Acetyl-L-phenylalanyl-L-valylglycylamino)-2,2-difluoro-3-hydroxypentanoyl)glycine benzyl ester (27f).

Starting from **25** and **26f** compound **27f** was obtained following the procedure described for the preparation of **26k**. (53 mg, 40% yield); ^{1}H NMR (300 MHz, CD$_3$OD) δ 0.95 (d, *J* = 6.7 Hz, 6H), 1.21 (d, *J* = 6.7 Hz, 3H), 1.87 (s, 3H), 2.03 – 2.15 (m, 1H), 2.82 – 2.91 (m, 1H), 3.09 – 3.17 (m, 1H), 3.70 – 3.87 (m, 2H), 4.00 – 4.15 (m, 4H), 4.19 – 4.32 (m, 1H), 4.65 – 4.71 (m, 1H), 5.17 (s, 2H), 7.16 – 7.35 (m, 10H); ESI-MS: *m/z* 684 (M+Na)$^+$. EA: calcd C$_{32}$H$_{41}$F$_2$N$_5$O$_8$: C, 58.08; H, 6.25; N, 10.58. Found: C, 58.19; H, 5.91; N, 10.80; HRMS calcd for C$_{32}$H$_{41}$F$_2$N$_5$O$_8$Na (M+Na)$^+$: 684.2821, found 684.2828.

(3*R*,*S*,4*S*)-(4-(*N*-Acetyl-L-phenylglycyl-L-valylglycylamino)-2,2-difluoro-3-hydroxypentanoyl)glycine benzyl ester (27g).

Starting from **25** and **26** compound **27g** was obtained following the procedure described for the preparation of **26k** (55 mg, 39% yield); ^{1}H NMR (300 MHz, CD$_3$OD) δ 0.77 – 0.98 (m, 6H), 1.13 – 1.25 (m, 3H), 2.01 (s, 3H), 2.02 – 2.17 (s, 1H), 3.62 – 3.90 (3H), 3.93 – 4.17 (m, 7H), 4.18 – 4.30 (s, 1H), 5.17 (s, 2H), 5.50 – 5.56 (m, 1H), 7.21 – 7.51 (m, 10H); ESI-MS: *m/z* 670 (M+Na)$^+$. EA: calcd for C$_{31}$H$_{39}$F$_2$N$_5$O$_8$: C, 57.49; H, 6.07; N, 10.81. Found: C, 57.81; H, 6.35; N, 11.00; HRMS calcd for C$_{31}$H$_{39}$F$_2$N$_5$O$_8$Na (M+Na)$^+$: 670.2664, found 670.2654.

(3*R*,*S*,4*S*)-(4-(*N*-Acetyl-L-phenylglycyl-L-phenylglycylglycylamino)-2,2-difluoro-3-hydroxypentanoyl)glycine benzyl ester (27h).

Starting from **25** and **26h** compound **27h** was obtained following the procedure described for the preparation of **26k**. (53 mg, 25% yield); R$_f$: 0.66 (5:1 CHCl$_3$/MeOH); ^{1}H NMR (300 MHz, CD$_3$OD) δ 1.11 – 1.21 (m, 3H), 1.98 (s, 3H), 3.58 – 3.88 (m, 3H), 3.93 – 4.04 (m, 2H), 4.21 – 4.29 (m, 1H), 5.16 (s, 2H), 5.35 – 5.57 (m, 2H), 7.30 – 7.44 (m, 15H); ESI-MS: *m/z* 582 (M+H)$^+$, 704 (M+Na)$^+$; EA: calcd for C$_{34}$H$_{37}$F$_2$N$_5$O$_8$: C, 59.91; H, 5.47; N, 10.27. Found: C, 60.22; H, 5.66; N, 10.30; HRMS calcd for C$_{34}$H$_{37}$F$_2$N$_5$O$_8$Na (M+Na)$^+$: 704.2508, found 704.2499.

(3*R*,*S*,4*S*)-(4-(*N*-Acetyl-L-phenylglycylglycylglycylamino)-2,2-difluoro-3-hydroxypentanoyl)glycine benzyl ester (27i).

Starting from **25** and **26i** compound **27i** was obtained following the procedure described for the preparation of **26k**. (56 mg, 32% yield); ^{1}H NMR (300 MHz, CD$_3$OD) δ 1.20 (s, 3H), 1.99 (s, 3H), 3.71 – 3.96 (m, 4H), 3.96 – 4.04 (m, 3H), 4.28 (br s, 1H), 5.16 (s, 2H), 5.36 (s, 1H), 7.33 – 7.45 (m, 10H); ESI-MS: *m/z* 628 (M+Na)$^+$, 644 (M+K)$^+$; EA: calcd for C$_{28}$H$_{33}$F$_2$N$_5$O$_8$: C, 55.53; H, 5.49; N, 11.56. Found: C, 55.80; H, 5.23; N, 11.23; HRMS calcd for C$_{28}$H$_{33}$F$_2$N$_5$O$_8$Na (M+Na)$^+$: 628.2195, found 628.2189.

((3*R*,*S*,4*S*)-(4-(*N*-Phenylacetyl-L-valylglycylamino)-2,2-difluoro-3-hydroxypentanoyl)glycine benzyl ester (27j).

Starting from **25** and **26j** compound **27j** was obtained following the procedure developed for the preparation of **26k** (42 mg, 23% yield); ^{1}H NMR (300 MHz, CD$_3$OD) δ 0.93 (d, *J* = 5.4 Hz, 6H), 1.15 (dd, *J$_1$* = 6.4 Hz, *J$_2$* = 23.9 Hz, 3H), 2.01 – 2.14 (m, 1H), 3.52 – 3.60 (m, 2H), 3.65 – 3.85 (m, 2H), 3.96 – 4.09 (m, 4H), 4.27 (br s, 1H), 5.16 (s, 2H), 7.22 – 7.31 (m, 5H); ESI-MS: *m/z* 591 (M+H)$^+$; EA: calcd for C$_{29}$H$_{36}$F$_2$N$_4$O$_7$: C, 58.97; H, 6.14; N, 9.49. Found: C, 58.79; H, 5.84; N, 9.69; HRMS calcd for C$_{29}$H$_{37}$F$_2$N$_4$O$_7$ (M+H)$^+$: 591.2630, found 591.2638.

(3*R*,*S*,4*S*)-(4-(*N*-(4-Quinolinylpropanoyl)-L-valylglycylamino)-2,2-difluoro-3-hydroxypentanoyl)glycine benzyl ester (27k).

Starting from **25** and **26k** compound **27k** was obtained following the procedure developed for the preparation of **26k** (50 mg, 28% yield); ^{1}H NMR (300 MHz, CD$_3$OD) δ 0.85 – 0.90 (m, 6H), 1.17 – 1.27 (m, 3H), 1.95 – 2.02 (m, 1H), 2.75 (t, *J* = 7.2 Hz, 2H), 3.45 (t, *J* = 6.9 Hz, 2H), 3.80 (q, *J* = 19.2 Hz, 2H), 3.93 – 4.10 (m, 4H), 4.27 – 4.33 (m, 1H), 5.15 (s, 2H), 7.25 – 7.32 (m, 5H), 7.41 (d, *J* = 3.6 Hz, 1H), 7.64 (t, *J* = 7.5 Hz, 1H), 7.75 (t, *J* = 7.2 Hz, 1H), 8.02 (d, *J* = 8.1 Hz, 1H), 8.21 (d, *J* = 3.6 Hz, 1H), 8.71 (d, *J* = 4.2 Hz, 1H); ESI-MS: *m/z* 678 (M+Na)$^+$; EA: calcd for C$_{33}$H$_{39}$F$_2$N$_5$O$_7$: C, 60.45; H, 6.00; N, 10.68. Found: C, 60.51; H, 5.73; N, 10.32; HRMS calcd for C$_{33}$H$_{39}$F$_2$N$_5$O$_7$Na (M+Na)$^+$: 678.2715, found 678.2722.

(3*R*,*S*,4*S*)-(4-(*N*-(2*S*,4*R*)-(4-Acetamido-2-methyl-5-phenylpentanoyl)glycylamino)-2,2-difluoro-3-hydroxypentanoyl)glycine benzyl ester (27l).

Starting from **25** and **26l** compound **27l** was obtained following the procedure developed for the preparation of **26k** (11 mg, 18% yield); ^{1}H NMR (300 MHz, CDCl$_3$) δ 1.09 (d, *J* = 6.7 Hz, 3H), 1.24 – 1.35 (m, 4H), 1.46 – 1.52 (m, 1H), 1.74 – 1.85 (m, 4H), 2.34 (br s, 1H), 2.67 – 2.80 (m, 3H), 3.71 – 3.85 (m, 2H), 3.92 – 4.33 (m, 6H), 5.16 (s, 2H), 6.01 (t, *J* = 8.2 Hz, 1H), 7.12 – 7.33 (m, 10H), 8.19 (dd, *J$_1$* = 5.8 Hz, *J$_2$* = 12.0 Hz, 1H); ESI-MS: *m/z* 605 (M+H)$^+$, 627 (M+Na)$^+$; EA: calcd for C$_{30}$H$_{38}$F$_2$N$_4$O$_7$: C, 59.59; H, 6.33; N, 9.27. Found: C, 59.91; H, 6.44; N, 9.36; HRMS calcd for C$_{30}$H$_{39}$F$_2$N$_4$O$_7$ (M+H)$^+$: 605.2787, found 605.2792.

(3*R*,*S*,4*S*)-(4-(*N*-(2*R*,4*R*)-(4-acetamido-2-methyl-5-phenylpentanoyl)glycylamino)-2,2-difluoro-3-hydroxypentanoyl)glycine benzyl ester (27m).

Starting from **25** and **26m** compound **27m** was obtained following the procedure developed for the preparation of **26k** (10 mg, 33% yield); ^{1}H NMR (300 MHz, CDCl$_3$) δ 1.09 (dd, *J$_1$* = 6.7 Hz, *J$_2$* = 13.1 Hz, 3H), 1.21 – 1.36 (m, 4H), 1.85 – 1.94 (m, 4H), 1.98 – 2.14 (m, 1H), 2.22 – 2.37 (m, 1H), 2.58 – 2.94 (m, 3H), 3.45 – 3.73 (m, 2H), 3.94 – 4.42 (m, 6H), 5.18 (s, 2H), 5.53 (dd, *J$_1$* = 9.5 Hz, *J$_2$* = 49.3 Hz, 1H), 7.09 – 7.34 (m, 10H), 7.84 (br s, 1H); ESI-MS: *m/z* 605 (M+H)$^+$, 627 (M+Na)$^+$, 643 (M+K)$^+$; EA: calcd for C$_{30}$H$_{38}$F$_2$N$_4$O$_7$: C, 59.59; H, 6.33; N, 9.27. Found: C, 59.33; H, 6.67; N, 8.94; HRMS calcd for C$_{30}$H$_{39}$F$_2$N$_4$O$_7$ (M+H)$^+$: 605.2787, found 605.2780.

(4S)-(N-(N-Acetyl-L-isoleucyl-L-(OBn)threonyl-L-alanyl)-2,2-difluoro-3-oxo-4-aminopentanoyl)glycine benzyl ester (28a).

Following the procedure described for **2**, and using anhydrous NMP as solvent, compound **28a** was obtained from **27a** as a colorless oil (15 mg, 80% yield); ^{1}H NMR (300 MHz, CD$_3$OD) δ 0.82 – 0.94 (m, 6H), 1.12 – 1.24 (m, 6H), 6.25 – 6.37 (m, 4H), 1.53 (br s, 1H), 1.84 (br s, 1H), 1.95 (s, 3H), 3.93 – 4.10 (m, 3H), 4.15 – 4.36 (m, 3H), 4.39 – 4.62 (m, 3H), 5.17 (s, 2H), 7.19 – 7.41 (m, 10H); ESI MS: *m/z* 732 (M+H)$^+$, 754 (M+Na)$^+$, 770 (M+K)$^+$; EA: calcd for C$_{36}$H$_{47}$F$_2$N$_5$O$_9$: C, 59.09; H, 6.47; N, 9.57. Found: C, 59.10; H, 6.30; N, 9.82; HRMS calcd for C$_{36}$H$_{48}$F$_2$N$_5$O$_9$ (M+H)$^+$: 732.3420, found 732.3410.

(4S)-(4-(N-Acetyl-L-valyl-L-(OBn)threonyl-L-alanylamino)-2,2-difluoro-3-oxopentanoyl)glycine benzyl ester (28b).

Following the procedure described for **2**, and using anhydrous NMP as solvent, compound **28b** was obtained from **27b** as a colorless oil (17 mg, 81% yield); ^{1}H NMR (300 MHz, CD$_3$OD) δ 0.94 – 0.96 (m, 6H), 1.18 – 1.19 (m, 6H), 1.25 – 1.38 (m, 3H), 1.96 (s, 3H), 2.03 – 2.16 (m, 1H), 3.96 – 4.05 (m, 3H), 4.16 – 4.22 (m, 1H), 4.27 – 4.40 (m, 2H), 4.44 – 4.59 (m, 3H), 5.16 (s, 2H), 7.27 – 7.33 (m, 10H); ESI-MS: *m/z* 718 (M+H)$^+$, 740 (M+Na)$^+$, 756 (M+K)$^+$; EA: calcd for C$_{35}$H$_{45}$F$_2$N$_5$O$_9$: C, 58.57; H, 6.32; N, 9.76. Found: C, 58.29; H, 6.53; N, 9.45; HRMS calcd for C$_{35}$H$_{46}$F$_2$N$_5$O$_9$ (M+H)$^+$: 718.3264, found 718.3275.

(4S)-(4-(N-Acetyl-L-isoleucyl-L-(OBn)seryl-L-alanylamino)-2,2-difluoro-3-oxopentanoyl)glycine benzyl ester (28c).

Following the procedure described for **2**, and using anhydrous NMP as solvent, compound **28c** was obtained from **27c** as a colorless oil (11 mg, 73% yield); R$_f$: 0.48 (5:1 CHCl$_3$/MeOH); ^{1}H NMR (300 MHz, CD$_3$OD) δ 0.85 – 0.93 (m, 6H), 1.11 – 1.19 (m, 4H), 1.31 – 1.37 (m, 3H), 1.51 (br s, 1H), 1.82 (br s, 1H), 1.98 (s, 3H), 3.66 – 3.75 (m, 2H), 4.05 (s, 2H), 4.14 – 4.36 (m, 3H), 4.46 – 4.61 (m, 3H), 5.16 (s, 2H), 7.24 – 7.33 (m, 10H); ESI-MS: *m/z* 718 (M+H)$^+$, 740 (M+Na)$^+$; EA: calcd for C$_{35}$H$_{45}$F$_2$N$_5$O$_9$: C, 58.57; H, 6.32; N, 9.76. Found: C, 58.92; H, 6.43; N, 9.54; HRMS calcd for C$_{35}$H$_{45}$F$_2$N$_5$O$_9$Na (M+Na)$^+$: 740.3083, found 740.3077.

(4S)-(4-(N-Acetyl-L-isoleucyl-L-(OBn)threonylglycylamino)-2,2-difluoro-3-oxopentanoyl)glycine benzyl ester (28d).

Following the procedure described for **2** compound **28d** was obtained from **27d** as a colorless oil (16 mg, 79% yield); R$_f$: 0.40 (5:1 CHCl$_3$/MeOH); ^{1}H NMR (300 MHz, CD$_3$OD) δ 0.81 – 0.98 (m, 6H), 1.12 – 1.27 (m, 7H), 1.52 (br s, 1H), 1.83 (br s, 1H), 1.96 (s, 3H), 3.75 – 3.91 (m, 2H), 3.92 – 4.08 (m, 3H), 4.15 – 4.22 (m, 1H), 4.24 – 4.40 (m, 1H), 4.41 – 4.58 (m, 3H), 5.14 (s, 2H), 7.13 – 7.41 (m, 10H). ESI-MS: *m/z* 740 (M+Na)$^+$, 756 (M+K)$^+$; EA: calcd for C$_{35}$H$_{45}$F$_2$N$_5$O$_9$: C, 58.57; H, 6.32; N, 9.76. Found: C, 58.33; H, 5.95; N, 10.07; HRMS calcd for C$_{35}$H$_{45}$F$_2$N$_5$O$_9$Na (M+Na)$^+$: 740.3083, found 740.3085.

(4S)-(4-(N-Acetyl-L-isoleucyl-L-valylglycylamino)-2,2-difluoro-3-oxopentanoyl)glycine benzyl ester (28e).

Following the procedure described for **2**, and using anhydrous NMP as solvent, compound **28e** was obtained from **27e** as a colorless oil (14 mg, 87% yield); R$_f$: 0.62 (5:1 CHCl$_3$/MeOH); ^{1}H NMR (300 MHz, CD$_3$OD) δ 0.86 – 0.91 (m, 7H), 0.95 – 0.96 (m, 6H), 1.18 – 1.20 (m, 3H), 1.48 – 1.56 (m, 1H), 1.77 – 1.85 (m, 1H), 1.97 (s, 3H), 2.01 – 2.11 (m, 1H), 3.79 – 3.82 (m, 2H), 4.02 – 4.09 (m, 2H), 4.16 – 4.21 (m, 2H), 4.34 – 4.40 (m, 1H), 5.18 (s, 1H), 7.32 – 7.35 (m, 5H); ESI-MS: *m/z* 648 (M+Na)$^+$; EA: calcd for C$_{29}$H$_{41}$F$_2$N$_5$O$_8$: C, 55.67; H, 6.61; N, 11.19. Found: C, 55.82; H, 6.31; N, 11.38; HRMS calcd for C$_{29}$H$_{41}$F$_2$N$_5$O$_8$Na (M+Na)$^+$: 648.2821, found 648.2828.

(4S)-(4-(N-Acetyl-L-phenylalanyl-L-valylglycylamino)-2,2-difluoro-3-oxopentanoyl)glycine benzyl ester (28f).

Following the procedure described for **2** compound **28f** was obtained from **27f** as a colorless oil (17 mg, 56% yield); ^{1}H NMR (300 MHz, CD$_3$OD) δ 0.92 – 0.97 (m, 6H), 1.14 – 1.29 (m, 3H), 1.88 (s, 3H), 2.11 – 2.12 (m, 1H), 2.83 – 2.92 (m, 1H), 3.06 – 3.17 (m, 1H), 3.67 – 3.87 (m, 2H), 4.05 (s, 2H), 4.08 – 4.20 (m, 1H), 4.27 – 4.41 (m, 1H), 4.63 – 4.69 (m, 1H), 5.17 (s, 2H), 7.16 – 7.34 (m, 10H); ESI-MS: *m/z* 660 (M+H)$^+$, 682 (M+Na)$^+$, 698 (M+K)$^+$; EA: calcd for C$_{32}$H$_{39}$F$_2$N$_5$O$_8$: C, 58.26; H, 5.96; N, 10.62. Found: C, 58.59; H, 6.31; N, 11.02; HRMS calcd for C$_{32}$H$_{40}$F$_2$N$_5$O$_8$ (M+H)$^+$: 660.2845, found 660.2864.

(4S)-(4-(N-Acetyl-L-phenylglycyl-L-valylglycylamino)-2,2-difluoro-3-oxopentanoyl)glycine benzyl ester (28g).

Following the procedure described for **2** compound **28g** was obtained from **27g** as a colorless oil (22 mg, 55% yield); ^{1}H NMR (300 MHz, CD$_3$OD) δ 0.70 – 1.02 (m, 6H), 1.09 – 1.26 (m, 3H), 1.98 – 2.06 (m, 3H), 2.04 – 2.20 (m, 1H), 3.67 – 3.98 (m, 3H), 3.99 – 4.21 (m, 2H), 4.26 – 4.43 (m, 1H), 5.17 (s, 3H), 5.48 – 5.56 (m, 1H), 7.23 – 7.50 (m, 10H); ESI-MS: *m/z* 646 (M+H)$^+$, 668 (M+Na)$^+$, 685 (M+K)$^+$; EA: calcd for C$_{31}$H$_{37}$F$_2$N$_5$O$_8$: C, 57.67; H, 5.78; N, 10.85. Found: C, 58.03; H, 6.17; N, 11.02; HRMS calcd for C$_{31}$H$_{37}$F$_2$N$_5$O$_8$Na (M+Na)$^+$: 668.2508, found 668.2515.

(4S)-(4-(N-Acetyl-L-phenylglycyl-L-phenylglycylglycylamino)-2,2-difluoro-3-oxopentanoyl)glycine benzyl ester (28h).

Following the procedure described for **2** compound **28h** was obtained from **27h** as a colorless oil (46 mg, 92% yield); R$_f$: 0.56 (5:1 CHCl$_3$/MeOH); ^{1}H NMR (300 MHz, DMSO-*d$_6$*) δ 1.15 – 1.31 (m, 3H), 1.86 (d, *J* = 7.8 Hz, 3H), 3.53 – 3.83 (m, 2H), 3.83 – 4.02 (m, 2H), 4.72 (d, *J* = 6.2 Hz, 1H), 5.13 (s, 2H), 5.48 (dd, *J$_1$* = 5.7 Hz, *J$_2$* = 11.5 Hz, 1H), 5.69 (dd, *J$_1$* = 8.0 Hz, *J$_2$* = 16.2 Hz, 1H), 7.14 – 7.47 (m, 15H), 8.27 – 8.65 (m, 3H), 8.92 (dt, *J$_1$* = 6.9 Hz, *J$_2$* = 41.1 Hz, 1H), 9.54 (d, *J* = 5.2 Hz, 1H); ESI-MS: *m/z* 680 (M+H)$^+$, 702 (M+Na)$^+$, 718 (M+K)$^+$; EA: calcd for C$_{34}$H$_{35}$F$_2$N$_5$O$_8$: C, 60.08; H, 5.19; N, 10.30. Found: C, 59.93; H, 5.35; N, 10.01; HRMS calcd for C$_{34}$H$_{35}$F$_2$N$_5$O$_8$Na (M+Na)$^+$: 702.2351, found 702.2345.

(4*S*)-(4-(*N*-Acetyl-ʟ-phenylglycylglycylglycylamino)-2,2-difluoro-3-oxopentanoyl)glycine benzyl ester (28i).

Following the procedure described for **2** compound **28i** was obtained from **27i** as a colorless oil (20 mg, 38% yield); ^{1}H NMR (300 MHz, acetone-*d6*) δ 1.38 (t, *J* = 6.7 Hz, 3H), 2.02 (d, *J* = 7.8 Hz, 3H), 3.73 – 3.93 m, 4H), 4.15 (d, *J* = 5.9 Hz, 2H), 4.77 – 4.87 (m, 1H), 5.20 (s, 2H), 5.39 (d, *J* = 5.1 Hz, 1H), 7.33 – 7.51 (m, 10H), 7.67 (d, *J* = 5.0 Hz, 1H), 7.85 – 7.98 (m, 2H), 8.29 (br s, 1H), 8.70 (br s, 1H). ESI-MS: *m/z* 626 (M+Na)$^+$, 642 (M+K)$^+$; EA: calcd for $C_{28}H_{31}F_2N_5O_8$: C, 55.72; H, 5.18; N, 11.60. Found: C, 55.73; H, 5.03; N, 11.38; HRMS calcd for $C_{28}H_{31}F_2N_5O_8Na$ (M+Na)$^+$: 626.2038, found 626.2026.

(4*S*)-(4-(*N*-Phenylacetyl-ʟ-valylglycylamino)-2,2-difluoro-3-oxopentanoyl)glycine benzyl ester (28j).

Following the procedure described for **2** compound **28j** was obtained from **27j** as a colorless oil (36 mg, 92% yield); ^{1}H NMR (300 MHz, acetone-*d6*) δ 0.93 (d, *J* = 6.0 Hz, 6H), 1.17 – 1.34 (m, 3H), 2.04 – 2.14 (m, 1H), 3.55 – 3.67 (m, 2H), 3.70 – 3.99 (m, 2H), 4.06 – 4.18 (m, 3H), 4.67 – 4.89 (m, 1H), 5.19 (s, 2H), 7.21 – 7.39 (m, 10H), 7.50 – 7.57 (m, 1H), 7.78 – 7.83 (m, 2H), 8.67 (br s, 1H); ESI-MS: *m/z* 611 (M+Na)$^+$, 627 (M+K)$^+$. EA: calcd for $C_{29}H_{34}F_2N_4O_7$: C, 59.18; H, 5.82; N, 9.52. Found: C, 58.81; H, 5.47; N, 9.86; HRMS calcd for $C_{29}H_{34}F_2N_4O_7Na$ (M+Na)$^+$: 611.2293, found 611.2303.

(4*S*)-(4-(*N*-(4-Quinolinylpropanoyl)-ʟ-valylglycylamino)-2,2-difluoro-3-oxopentanoyl)glycine benzyl ester (28k).

Following the procedure described for **2** compound **28k** was obtained from **27k** as a colorless oil (17 mg, 85% yield); ^{1}H NMR (300 MHz, CD$_3$OD) δ 0.84 – 0.89 (m, 6H), 1.18 – 1.24 (m, 3H), 1.95 – 2.04 (m, 1H), 2.76 (t, *J* = 7.6 Hz, 2H), 3.46 (t, *J* = 7.3 Hz, 2H), 3.70 – 3.92 (m, 2H), 3.97 – 4.10 (m, 3H), 4.37 (dd, *J_1* = 6.9 Hz, *J_2* = 13.6 Hz, 1H), 5.15 (s, 2H), 7.26 – 7.32 (m, 5H), 7.41 (d, *J* = 4.0 Hz, 1H), 7.64 (t, *J* = 7.5 Hz, 1H), 7.76 (t, *J* = 7.6 Hz, 1H), 8.02 (d, *J* = 8.4 Hz, 1H), 8.22 (d, *J* = 8.0 Hz, 1H), 8.71 (d, *J* = 4.2 Hz, 1H); ESI-MS: *m/z* 676 (M+Na)$^+$; EA: calcd for $C_{33}H_{37}F_2N_5O_7$: C, 60.63; H, 5.71; N, 10.71. Found: C, 60.70; H, 5.97; N, 10.43; HRMS calcd for $C_{33}H_{37}F_2N_5O_7Na$ (M+Na)$^+$: 676.2559, found 676.2548.

(3*R,S,*4*S*) (4-(*N*-(2*S,*4*R*)-(4-Acetamido-2-methyl-5-phenylpentanoyl)glycylamino)-2,2-difluoro-3-oxopentanoyl)glycine benzyl ester (28l).

Following the procedure described for **2** compound **28l** was obtained from **27l** as a colorless oil (6 mg, 61% yield); ^{1}H NMR (300 MHz, CD$_3$OD) δ 1.06 – 1.10 (m, 3H), 1.20 (d, *J* = 5.6 Hz, 1H), 1.24 – 1.37 (m, 2H), 1.50 – 1.60 (m, 1H), 1.65 – 1.78 (m, 1H), 1.83 (s, 3H), 2.32 – 2.43 (m, 1H), 2.73 (d, *J* = 5.9 Hz, 2H), 3.70 – 3.83 (m, 1H), 4.05 (s, 2H), 4.11 (s, 2H), 4.31 – 4.44 (m, 1H), 5.17 (s, 2H), 7.16 – 7.24 (m, 5H), 7.31 – 7.34 (m, 5H). ESI-MS: *m/z* 603 (M+H)$^+$, 625 (M+Na)$^+$, 641 (M+K)$^+$; EA: calcd for $C_{30}H_{36}F_2N_4O_7$: C, 59.79; H, 6.02; N, 9.30. Found:

C, 59.53; H, 6.17; N, 9.21; HRMS calcd for $C_{30}H_{36}F_2N_4O_7Na$ (M+Na)$^+$: 625.2450, found 625.2452.

(3*R,S,*4*S*) (4-(*N*-(2*R,*4*R*)-(4-Acetamido-2-methyl-5-phenylpentanoyl)glycylamino)-2,2-difluoro-3-oxopentanoyl)glycine benzyl ester (28m).

Following the procedure described for **2** compound **28m** was obtained from **27m** as a colorless oil (5 mg, 58% yield); ^{1}H NMR (300 MHz, CD$_3$OD) δ 1.09 – 1.12 (m, 3H), 1.15 – 1.22 (m, 1H), 1.28 – 1.32 (m, 2H), 1.78 – 1.85 (m, 4H), 1.91 – 2.04 (m, 1H), 2.39 – 2.50 (m, 1H), 2.60 – 2.85 (m, 2H), 3.58 – 3.74 (m, 1H), 3.88 – 4.11 (m, 4H), 4.32 – 4.52 (m, 1H), 5.17 (s, 2H), 7.14 – 7.22 (m, 5H), 7.31 – 7.34 (m, 5H); ESI-MS: *m/z* 603 (M+H)$^+$, 625 (M+Na)$^+$, 641 (M+K)$^+$; EA: calcd for $C_{30}H_{36}F_2N_4O_7$: C, 59.79; H, 6.02; N, 9.30. Found: C, 59.65; H, 6.35; N, 9.12; HRMS calcd for $C_{30}H_{36}F_2N_4O_7Na$ (M+Na)$^+$: 625.2450, found 625.2457.

(4*S*)-(4-(*N*-Acetyl-ʟ-valyl-ʟ-threonyl-ʟ-alanylamino)-2,2-difluoro-3-oxopentanoyl)glycine (4).

Following the procedure described for **3**, compound **4** was obtained from **28b** as a colorless oil (13 mg, 99% yield); ^{1}H NMR (300 MHz, CD$_3$OD) δ 0.96 – 0.97 (m, 6H), 1.13 – 1.26 (m, 5H), 1.26 – 1.38 (m, 5H), 2.00 (s, 3H), 2.08 (br s, 1H), 3.93 – 4.00 (m, 2H), 4.11 – 4.24 (m, 3H), 4.26 – 4.40 (m, 2H); ESI-MS: *m/z* 536 (M-H)$^-$; EA: calcd for $C_{21}H_{33}F_2N_5O_9$: C, 46.92; H, 6.19; N, 13.03. Found: C, 46.75; H, 6.37; N, 13.40; HRMS calcd for $C_{21}H_{32}F_2N_5O_9$ (M-H)$^-$: 536.2168, found 536.2165.

(4*S*)-(4-(*N*-Acetyl-ʟ-isoleucyl-ʟ-seryl-ʟ-alanylamino)-2,2-difluoro-3-oxopentanoyl)glycine (5).

Following the procedure described for **3**, compound **5** was obtained from **28c** as a colorless oil (12 mg, 99% yield); ^{1}H NMR (300 MHz, CD$_3$OD) δ 0.82 – 0.99 (m, 6H), 1.10 – 1.27 (m, 3H), 1.26 – 1.34 (m, 4H), 1.51 (br s, 1H), 1.81 (br s, 1H), 1.99 (s, 3H), 3.70 – 3.83 (m, 2H), 3.91 – 4.01 (m, 2H), 4.12 – 4.21 (m, 1H), 4.24 – 4.48 (m, 3H); ESI-MS: *m/z* 536 (M-H)$^-$; EA: calcd for $C_{21}H_{33}F_2N_5O_9$: C, 46.92; H, 6.19; N, 13.03. Found: C, 47.19; H, 5.95; N, 12.70; HRMS calcd for $C_{21}H_{32}F_2N_5O_9$ (M-H)$^-$: 536.2168, found 536.2167.

(4*S*)-(4-(*N*-Acetyl-ʟ-isoleucyl-ʟ-threonylglycylamino)-2,2-difluoro-3-oxopentanoyl)glycine (6).

Following the procedure described for **3**, compound **6** was obtained from **28d** as a colorless oil (8 mg, 99% yield); ^{1}H NMR (300 MHz, CD$_3$OD) δ 0.82 – 0.96 (m, 6H), 1.11 – 1.24 (m, 7H), 1.52 (br s, 1H), 1.84 (br s, 1H), 1.98 (s, 3H), 3.57 – 3.63 (m, 1H), 3.77 – 3.91 (m, 2H), 3.93 – 4.02 (m, 2H), 4.12 – 4.40 (m, 3H); ESI-MS: *m/z* 536 (M-H)$^-$; EA: calcd for $C_{21}H_{33}F_2N_5O_9$: C, 46.92; H, 6.19; N, 13.03. Found: C, 47.11; H, 6.55; N, 13.03; HRMS calcd for $C_{21}H_{32}F_2N_5O_9$ (M-H)$^-$: 536.2168, found 536.2162.

(4*S*)-(4-(*N*-Acetyl-ʟ-isoleucyl-ʟ-(OBn)threonyl-ʟ-alanylamino)-2,2-difluoro-3-oxopentanoyl)glycine (7).

To a solution of **28a** (10 mg, 0.02 mmol) in absolute EtOH under Ar atmosphere 10 mg of Pd/C 10% and 1,4-cyclohexadiene (15 μL, 0.16 mmol) were added and the reaction mixture was stirred at 25 °C for 14 h.[26] After disappearance of the starting material, Pd/C was filtered and the organic solvent was evaporated to obtain pure compound **7** as a colouless oil (6 mg, 99% yield); ^{1}H NMR (300 MHz, CD$_3$OD) δ 0.87 - 0.94 (m, 6H), 1.18 – 1.22 (m, 6H), 1.25 – 1.38 (m, 4H), 1.54 (br s, 1H), 1.83 (br s, 1H), 3.97 – 4.12 (m, 3H), 4.22 – 4.43 (m, 3H), 4.46 – 4.60 (m, 3H), 7.25 – 7.31 (m, 5H); ESI-MS: *m/z* 640 (M-H)$^-$; EA: calcd for C$_{29}$H$_{41}$F$_2$N$_5$O$_9$: C, 54.28; H, 6.44; N, 10.91. Found: C, 54.46; H, 6.17; N, 11.03; HRMS calcd for C$_{29}$H$_{40}$F$_2$N$_5$O$_9$ (M-H)$^-$: 640.2794, found 640.2804.

(4*S*)-(4-(*N*-Acetyl-L-valyl-L-(OBn)threonyl-L-alanylamino)-2,2-difluoro-3-oxopentanoyl)glycine (8).

Following the procedure described for **7**, compound **8** was obtained from **28b** as a colorless oil (7 mg, 96% yield); ^{1}H NMR (300 MHz, CD$_3$OD) δ 0.95 – 0.96 (m, 6H), 1.10 – 1.24 (m, 5H), 1.26 – 1.40 (m, 4H), 1.98 (d, *J* = 5.8 Hz, 3H), 2.09 (br s, 1H), 3.88 – 4.09 (m, 3H), 4.13 – 4.25 (m, 1H), 4.26 – 4.41 (m, 2H), 4.44 – 4.63 (m, 3H), 7.26 – 7.31 (m, 5H); ESI-MS: *m/z* 626 (M-H)$^-$; EA: calcd for C$_{28}$H$_{39}$F$_2$N$_5$O$_9$: C, 53.58; H, 6.26; N, 11.16. Found: C, 53.36; H, 6.48; N, 10.80; HRMS calcd for C$_{28}$H$_{38}$F$_2$N$_5$O$_9$ (M-H)$^-$: 626.2638, found 626.2632.

(4*S*)-(4-(*N*-Acetyl-L-isoleucyl-L-(OBn)seryl-L-alanylamino)-2,2-difluoro-3-oxopentanoyl)glycine (9).

Following the procedure described for **7**, compound **9** was obtained from **28c** as a colorless oil (10 mg, 98% yield); ^{1}H NMR (300 MHz, CD$_3$OD) δ 0.86 – 0.94 (m, 6H), 1.14 – 1.21 (m, 3H), 1.28 – 1.42 (m, 4H), 1.53 (br s, 1H), 1.83 (br s, 1H), 1.99 (d, *J* = 4.8 Hz, 3H), 3.69 – 3.80 (m, 2H), 3.89 – 3.99 (m, 2H), 4.17 – 4.22 (m, 1H), 4.25 – 4.35 (m, 2H), 4.49 – 4.63 (m, 3H), 7.25 – 7.32 (m, 5H); ESI-MS: *m/z* 626 (M-H)$^-$; EA: calcd for C$_{28}$H$_{39}$F$_2$N$_5$O$_9$: C, 53.58; H, 6.26; N, 11.16. Found: C, 53.43; H, 6.45; N, 11.42; HRMS calcd for C$_{28}$H$_{38}$F$_2$N$_5$O$_9$ (M-H)$^-$: 626.2638, found 626.2645.

(4*S*)-(4-(*N*-Acetyl-L-isoleucyl-L-(OBn)threonylglycylamino)-2,2-difluoro-3-oxopentanoyl)glycine (10).

Following the procedure described for **7**, compound **10** was obtained from **28d** as a colorless oil (8 mg, 97% yield); ^{1}H NMR (300 MHz, CD$_3$OD) δ 0.82 – 0.96 (m, 6H), 1.12 – 1.31 (m, 7H), 1.61 (br s, 1H), 1.85 (br s, 1H), 1.95 (s, 3H), 3.74 – 3.89 (m, 2H), 3.91 – 3.96 (m, 2H), 4.00 – 4.09 (m, 1H), 4.22 (d, *J* = 6.1 Hz, 1H), 4.30 – 4.41 (m, 1H), 4.41 – 4.61 (m, 3H); ESI-MS: *m/z* 626 (M-H)$^-$; EA: calcd for C$_{28}$H$_{39}$F$_2$N$_5$O$_9$: C, 53.58; H, 6.26; N, 11.16. Found: C, 53.32; H, 5.92; N, 10.84; HRMS calcd for C$_{28}$H$_{38}$F$_2$N$_5$O$_9$ (M-H)$^-$: 626.2638, found 626.2644.

(4*S*)-(4-(*N*-Acetyl-L-isoleucyl-L-valylglycylamino)-2,2-difluoro-3-oxopentanoyl)glycine (11).

Following the procedure described for **3**, compound **11** was obtained from **28e** as a colorless oil (9 mg, 99% yield); ^{1}H NMR (300 MHz, CD$_3$OD) δ 0.86 – 0.97 (m, 13H), 1.20 – 1.21

(m, 3H), 1.53 (br s, 1H), 1.81 (br s, 1H), 1.97 (s, 3H), 2.01 – 2.15 (m, 1H), 3.78 – 3.87 (m, 2H), 3.92 – 4.00 (m, 2H), 4.20 (d, *J* = 7.5 Hz, 2H), 4.34 – 4.40 (m, 1H); ESI-MS: *m/z* 534 (M-H)$^-$. EA: calcd for C$_{22}$H$_{35}$F$_2$N$_5$O$_8$: C, 49.34; H, 6.59; N, 13.08. Found: C, C, 49.11; H, 6.78; N, 13.46; HRMS calcd for C$_{22}$H$_{34}$F$_2$N$_5$O$_8$ (M-H)$^-$: 534.2375, found 534.2385.

(4*S*)-(4-(*N*-Acetyl-L-phenylalanyl-L-valylglycylamino)-2,2-difluoro-3-oxopentanoyl)glycine (12).

Following the procedure described for **3**, compound **12** was obtained from **28f** as a colorless oil (6 mg, 99% yield); ^{1}H NMR (300 MHz, CD$_3$OD) δ 0.92 – 0.96 (m, 6H), 1.23 (d, *J* = 6.8 Hz, 3H), 1.89 (s, 3H), 2.03 – 2.20 (m, 1H), 2.81 – 2.94 (m, 1H), 3.06 – 3.18 (m, 1H), 3.69 – 3.86 (m, 2H), 3.93 – 4.04 (m, 2H), 4.08 – 4.23 (m, 1H), 4.31 – 4.42 (m, 1H), 4.62 – 4.73 (m, 1H), 7.16 – 7.26 (m, 5H); ESI-MS: *m/z* 568 (M-H)$^-$; EA: calcd for C$_{25}$H$_{33}$F$_2$N$_5$O$_8$: C, 52.72; H, 5.84; N, 12.30. Found: C, 53.11; H, 5.53; N, 12.47; HRMS calcd for C$_{25}$H$_{32}$F$_2$N$_5$O$_8$ (M-H)$^-$: 568.2219, found 568.2216.

(4*S*)-(4-(*N*-Acetyl-L-phenylglycyl-L-valylglycylamino)-2,2-difluoro-3-oxopentanoyl)glycine (13).

Following the procedure described for **3**, compound **13** was obtained from **28g** as a colorless oil (8 mg, 99% yield); ^{1}H NMR (300 MHz, CD$_3$OD) δ 0.75 – 1.08 (m, 6H), 1.14 – 1.31 (m, 3H), 2.01 (s, 3H), 2.05 – 2.18 (m, 1H), 3.64 – 4.02 (m, 3H), 4.03 – 4.26 (m, 2H), 4.26 – 4.46 (m, 1H), 5.46 – 5.57 (m, 1H), 7.19 – 7.51 (m, 5H); ESI-MS: *m/z* 554 (M-H)$^-$. EA: calcd for C$_{24}$H$_{31}$F$_2$N$_5$O$_8$: C, 51.89; H, 5.62; N, 12.61; Found: C, 51.57; H, 5.36; N, 12.81; HRMS calcd for C$_{24}$H$_{30}$F$_2$N$_5$O$_8$ (M-H)$^-$: 554.2062, found 554.2055.

(4*S*)-(4-(*N*-Acetyl-L-phenylglycyl-L-phenylglycylglycylamino)-2,2-difluoro-3-oxopentanoyl)glycine (14).

Following the procedure described for **3**, compound **14** was obtained from **28h** as a colorless oil (22 mg, 87% yield); ^{1}H NMR (300 MHz, acetone-*d6*) δ 1.25 – 1.41 (m, 3H), 2.00 – 2.03 (m, 3H), 3.83 – 3.97 (m, 2H), 3.97 – 4.09 (m, 2H), 4.78 – 4.89 (m, 1H), 5.46 (dd, *J$_1$* = 4.5 Hz, *J$_2$* = 14.6 Hz, 1H), 5.55 – 5.86 (m, 1H), 7.27 – 7.48 (m, 10H), 7.82 (br s, 1H), 8.00 – 8.16 (m, 2H), 8.38 (br s, 1H), 8.53 – 8.61 (m, 1H), 10.78 (br s, 1H). ESI-MS: *m/z* 588 (M-H)$^-$; EA: calcd for C$_{27}$H$_{29}$F$_2$N$_5$O$_8$: C, 55.01; H, 4.96; N, 11.88. Found: C, 55.04; H, 4.74; N, 11.57; HRMS calcd for C$_{27}$H$_{28}$F$_2$N$_5$O$_8$ (M-H)$^-$: 588.1906, found 588.1911.

(4*S*)-(4-(*N*-Acetyl-L-phenylglycylglycylglycylamino)-2,2-difluoro-3-oxopentanoyl)glycine (15).

Following the procedure described for **3**, compound **15** was obtained from **28i** as a colorless oil (13 mg, 96% yield); ^{1}H NMR (300 MHz, acetone-*d6*) δ 1.38 (t, *J* = 6.9 Hz, 3H), 2.03 (d, *J* = 5.9 Hz, 3H), 3.73 – 3.94 (m, 4H), 4.07 (d, *J* = 5.9 Hz, 2H), 4.76 – 4.85 (m, 1H), 5.42 (d, *J* = 5.6 Hz, 1H), 7.33 – 7.51 (m, 5H), 7.82 (d, *J* = 6.4 Hz, 1H), 7.92 (br s, 1H), 8.01 (dd, *J$_1$* = 7.23 Hz, *J$_2$* = 16.3 Hz, 1H), 8.31 (d, *J* = 5.5 Hz, 1H), 8.59 (br s, 1H). ESI-MS: *m/z* 512 (M-H)$^-$; EA: calcd for C$_{21}$H$_{25}$F$_2$N$_5$O$_8$: C,

49.12; H, 4.91; N, 13.64. Found: C, 48.94; H, 4.64; N, 14.03; HRMS calcd for $C_{21}H_{24}F_2N_5O_8$ (M-H)⁻: 512.1593, found 512.1604.

(4*S*)-(4-(*N*-Phenylacetyl-L-valylglycylamino)-2,2-difluoro-3-oxopentanoyl)glycine (16).

Following the procedure described for **3**, compound **16** was obtained from **28j** as a colorless oil (23 mg, 98% yield); ¹H NMR (300 MHz, acetone-*d6*) δ☐0.92 (d, *J* = 6.8 Hz, 6H), 1.29 (dd, *J₁* = 7.1 Hz, *J₂* = 17.9 Hz, 3H), 2.04 – 2.13 (m, 1H), 3.56 – 3.66 (m, 2H), 3.87 (ddd, *J₁* = 6.0 Hz, *J₂* = 20.1 Hz, *J₃* = 25.90 Hz, 2H), 4.05 (d, *J* = 5.7 Hz, 2H), 4.15 (t, *J* = 7.0 Hz, 1H), 4.69 – 4.85 (m, 1H), 7.18 – 7.33 (m, 5H), 7.62 (t, *J* = 5.7 Hz, 1H), 7.92 – 7.94 (m, 2H), 8.63 (dt, *J₁* = 5.1 Hz, *J₂* = 19.5 Hz, 1H). ESI-MS: *m/z* 497 (M-H)⁻; EA: calcd for $C_{22}H_{28}F_2N_4O_7$: C, 53.01; H, 5.66; N, 11.24. Found: C, 52.70; H, 5.91; N, 11.05; HRMS calcd for $C_{22}H_{27}F_2N_4O_7$ (M-H)⁻: 497.1848, found 497.1855.

(4*S*)-(4-(*N*-(4-Quinolinylpropanoyl)-L-valylglycylamino)-2,2-difluoro-3-oxopentanoyl)glycine (17).

Following the procedure described for **3**, compound **17** was obtained from **28k** as a colorless oil (11 mg, 84% yield); ¹H NMR (300 MHz, CD₃OD) δ☐0.84 – 0.94 (m, 6H), 1.20 – 1.33 (m, 3H), 1.98 – 2.06 (m, 1H), 2.77 (t, *J* = 7.4 Hz, 2H), 3.47 (t, *J* = 7.3 Hz, 2H), 3.72 – 3.92 (m, 3H), 3.96 – 4.17 (m, 3H), 4.33 – 4.39 (m, 1H), 7.43 (d, *J* = 4.3 Hz, 1H), 7.66 (t, *J* = 7.7 Hz, 1H), 7.77 (t, *J* = 7.4 Hz, 1H), 8.03 (d, *J* = 8.2 Hz, 1H), 8.24 (d, *J* = 8.6 Hz, 1H), 8.73 (d, *J* = 3.8 Hz, 1H). ESI-MS: *m/z* 562 (M-H)⁻; EA: calcd for $C_{26}H_{31}F_2N_5O_7$: C, 55.41; H, 5.54; N, 12.43. Found: C, 55.55; H, 5.70; N, 12.82; HRMS calcd for $C_{26}H_{30}F_2N_5O_7$ (M-H)⁻: 562.2113, found 562.2112.

(4*S*)-(4-(*N*-(2*S*,4*R*)-(4-Acetamido-2-methyl-5-phenylpentanoyl)glycylamino)-2,2-difluoro-3-oxopentanoyl)glycine (18).

Following the procedure described for **3**, compound **18** was obtained from **28l** as a colorless oil (4 mg, 99% yield); ¹H NMR (300 MHz, CD₃OD) δ☐1.08 (d, *J* = 5.5 Hz, 3H), 1.18 – 1.41 (m, 4H), 1.42 – 1.61 (m, 1H), 1.64 – 1.80 (m, 1H), 1.85 (s, 3H), 2.36 (br s, 1H), 2.74 (d, *J* = 5.3 Hz, 2H), 3.63 (s, 1H), 3.77 – 3.93 (m, 2H), 4.05 – 4.19 (m, 2H), 7.18 – 7.22 (m, 5H). ESI-MS: *m/z* 511 (M-H)⁻; EA: calcd for $C_{23}H_{30}F_2N_4O_7$: C, 53.90; H, 5.90; N, 10.93. Found: C, 53.67; H, 5.54; N, 10.58; HRMS calcd for $C_{23}H_{29}F_2N_4O_7$ (M-H)⁻: 511.2004, found 511.1998.

(4*S*)-(4-(*N*-(2*R*,4*R*)-(4-Acetamido-2-methyl-5-phenylpentanoyl)-glycylamino)-2,2-difluoro-3-oxopentanoyl)glycine (19).

Following the procedure described for **3**, compound **19** was obtained from **28m** as a colorless oil (3 mg, 99% yield); ¹H NMR (300 MHz, CD₃OD) δ☐1.10 (d, 4.9 Hz, 3H), 1.20 (dd, *J₁* = 5.9 Hz, *J₂* = 15.33 Hz, 2H), 1.27 – 1.44 (m, 2H), 1.84 (d, 5.4 Hz, 3H), 1.92 (m, 1H), 2.47 (br s, 1H), 2.64 – 2.85 (m, 2H), 3.52 – 3.72 (m, 2H), 3.81 (s, 1H), 3.92 – 4.14 (m, 3H), 7.14 – 7.22 (m, 5H). ESI-MS: *m/z* 511 (M-H)⁻; EA: calcd for $C_{23}H_{30}F_2N_4O_7$: C, 53.90; H, 5.90; N, 10.93. Found: C, 54.09; H,

6.02; N, 10.54; HRMS calcd for $C_{23}H_{29}F_2N_4O_7$ (M-H)⁻: 511.2004, found 511.1998.

PfSUB1 inhibition assays for IC₅₀ determination

Inhibitory potency of test compounds against recombinant PfSUB1[12] was assayed as described previously[7,8,17], using fluorogenic substrate SERA4st1F-6R12, which is peptide Ac-CKITAQDDEESC labelled on both cysteine side-chains with tetramethylrhodamine. The intact substrate displays low fluorescence due to non-covalent, concentration-dependent dimerization of the rhodamines. Cleavage within the peptide backbone allows dissociation of the rhodamine dimer and consequent fluorescence increase. One unit (1 U) of recombinant PfSUB1 is defined as the amount of protease that hydrolyses 1 pmol of SERA4st1F-6R12 in 1 min at a substrate concentration of 0.1 µM in digestion buffer (25 mM Tris-HCl pH 8.2, 12 mM CaCl₂, 25 mM CHAPS) at 21 ᵒC. For kinetic assays to determine IC₅₀ values for test compounds, wells of a white 96-well microplate (Nunc) containing 48 µL purified rPfSUB1 (~1 U/mL in digestion buffer), were supplemented in triplicate with 2 µL of various concentrations of the test compounds, freshly diluted in dry DMSO, prior to addition of 50 µL substrate (0.1 µM in digestion buffer). Resulting fluorescence increase was continuously monitored with time at 21 ᵒC using a Cary Eclipse fluorescence spectrophotometer (Varian) equipped with a 96-well microplate reader accessory. Initial hydrolysis rates were calculated from the resulting progress curves and plotted against test compound concentration to obtain IC₅₀ values. Vehicle alone was used to obtain values for uninhibited enzyme activity and *para*-hydroxymercuribenzoate, a potent inhibitor of PfSUB1,[12] was used as a positive control inhibitor.

Computational Details

All calculations performed in this work were carried out on Cooler Master Centurion 5 (Intel Core i5–2400 CPU @ 3.10 GHz Quad; Intel Core i5–2500 CPU @ 3.30 GHz Quad) with Ubuntu 10.04 LTS (long-term support) operating system running Maestro 9.2 (Schrödinger, LLC, New York, NY, 2011) and GOLD software (version 5.2, Cambridge Crystallographic Data Center, UK, 2012).

A) LIGAND PREPARATION.

Three-dimensional structure building for all compounds in this study was carried out by means of Maestro 9.2 (Schrödinger, LLC, New York, NY, 2011). Molecular energy minimizations were performed in MacroModel (MacroModel, version 9.9, Schrödinger, LLC, New York, NY, 2011) using the Optimized Potentials for Liquid Simulations-all atom (OPLS-AA) force field 2005.[27,28] The solvent effects were simulated using the analytical Generalized-Born/Surface-Area (GB/SA) model,[29] and no cutoff for nonbonded interactions was selected. Polak-Ribiere conjugate gradient (PRCG) method with 1000 maximum iterations and 0.001 gradient convergence threshold was employed. All compounds reported in this paper were treated by LigPrep application (version 2.5, Schrödinger, LLC,

New York, NY, 2011), implemented in Maestro suite 2011, generating the most probable ionization state of any possible enantiomers and tautomers at cellular pH value (7 ± 0.5).

b) Protein preparation.

The crystal structure of PfSUB1 (PDB ID:4LVN) was imported into Schrödinger Maestro molecular modeling environment (Maestro, version 9.2; Schrödinger, LLC: New York, 2011). All water molecules and additional proteins necessary for crystallizing the structure were removed and subsequent structure optimization was carried out by protein preparation wizard implemented in Maestro suite 2011 (Protein Preparation Wizard workflow 2011; http://www.schrodinger.com/supportdocs/18/16). This protocol allowed us to obtain a reasonable starting structure of protein for molecular docking calculations by a series of computational steps. In particular, we performed three steps to (1) add hydrogens, (2) optimize the orientation of hydroxyl groups, Asn, and Gln, and the protonation state of His, and (3) perform a constrained refinement with the impref utility, setting the max RMSD of 0.30. The impref utility consists of a cycle of energy minimization based on the impact molecular mechanics engine and on the OPLS_2005 force field.

c) Molecular Docking.

Molecular Docking was carried out using GOLD 5.2 (Genetic Optimization for Ligand Docking)[30] software from Cambridge Crystallographic Data Center, UK, that uses the Genetic algorithm (GA)[30] running under Ubuntu 10.04 LTS OS. This method allows a partial flexibility of protein and full flexibility of ligand. For each of the 100 independent GA runs, a maximum number of 125000 GA operations were performed. The search efficiency values were set on 200% in order to increase the flexibility of the ligands docked. As reported in the Gold user manual this parameters is recommended for large highly flexible ligands. The active site radius of 8 Å was chosen by XYZ coordinates from the center of catalytic triad. Default cutoff values of 2.5 Å (dH-X) for hydrogen bonds and 4.0 Å for van der Waals distance were employed. When the top three solutions attained RMSD values within 1.5 Å, GA docking was terminated. The fitness function GoldScore was evaluated.

d) Estimated free-binding energies.

The Prime/MM-GBSA method implemented in Prime software[31] consists in computing the change between the free and the complex state of both the ligand and the protein after energy minimization. The technique was used on the docking complexes of the selected compounds presented in this study. The software was employed to calculate the free-binding energy (ΔG_{bind}) as previously reported by us.[11,32,33]

Acknowledgements

Notes and references

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† Electronic Supplementary Information (ESI) available: [Figures S1-S5, ^{1}H NMR spectra]. See DOI: 10.1039/b000000x/

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