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Aza vinyl sulfones: Synthesis and evaluation as antiplasmodial agents

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ABSTRACT

A series of novel aza vinyl sulfones were designed, synthesized in good yields and evaluated as antiplasmodial agents. Tested compounds did not show activity against papain or the *Plasmodium falciparum* cysteine protease falcipain-2. However, a number of the new compounds effectively inhibited the in vitro development of *P. falciparum*. Compounds containing a squaramide group were the most active, with IC_{50} values between 0.95 and 4.5 μM, suggesting that these are potential lead compounds for the development of new antimalarial agents.

1. Introduction

Malaria is one of the most important infectious diseases in the world. The most severe form of malaria in humans is caused by *Plasmodium falciparum*. Although several antimalarial drugs are known, resistance to available drugs is widespread and increasing.[1] As a consequence, there is an urgent need to find new antimalarial agents. A promising target for antimalarial drug design is falcipain-2, a cysteine protease from *P. falciparum*, that has received considerable attention due to its key role in the life cycle of the parasite.[2]

Falcipain-2 belongs to the papain family or CA clan, which is one of the largest subfamilies of cysteine proteases. Several peptidyl vinyl sulfone cysteine protease inhibitors have been described as potent irreversible falcipain-2 inhibitors.[3–5] These compounds act as Michael acceptors of the catalytic cysteine residue.[6] However, peptidyl inhibitors generally exhibit low bioavailability because of their susceptibility to degradation by other proteases. In biologically active peptide analogs, the replacement of the alpha carbon by a nitrogen atom to give azapeptides has led to enhanced activity and selectivity as well as improved properties, such as prolonged duration of action and metabolic stability.[7]

Several cysteine protease inhibitors containing azapeptide scaffolds have already been reported,[8–11] but this strategy has not, to our knowledge, been applied to the P_1 position of peptidyl vinyl sulfones. As part of our ongoing interest in the design and synthesis of Michael acceptors as cysteine protease inhibitors,[12–14] herein we report the synthesis of novel antiplasmodial agents **1** based on the aza vinyl sulfone scaffold (Fig. 1). These compounds were designed in order to explore the impact of CH/N exchange in the P_1 position of dipeptide vinyl sulfones on enzyme–inhibitor interaction and on antiplasmodial activity.

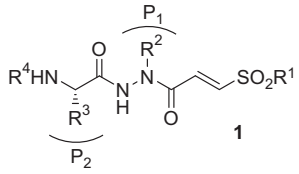

Figure 1. Chemical structure of aza vinyl sulfones **1**.

2. Results and discussion

The required methyl and phenyl vinyl sulfones acids were synthesized from propiolic acid and the corresponding thiol sodium salts, followed by oxidation of the *trans*-isomer with excess of *m*CPBA.[15] The next stage was the design of selective falcipain-2 inhibitors, by incorporating an appropriate recognition moiety to the double bond. We started by studying the reaction of Boc protected hydrazine **2a** with methyl and phenyl vinyl sulfones acids. Compounds **3a–b** were obtained in good yields (62% and 71%

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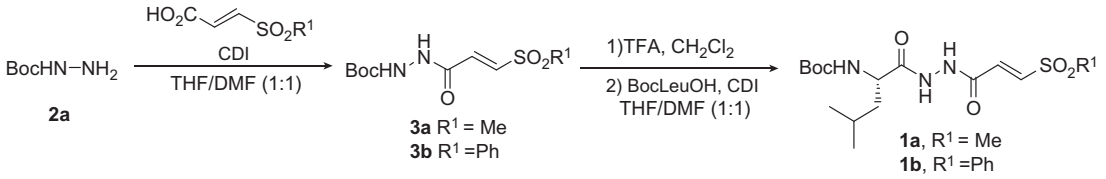
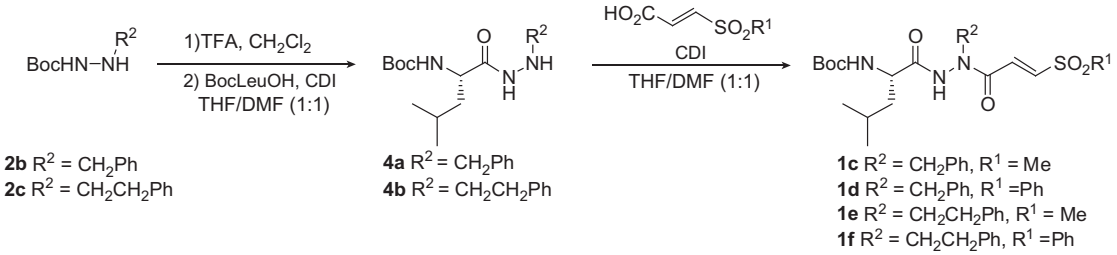


Scheme 1. Synthesis of aza vinyl sulfones **1a–b**.



Scheme 2. Synthesis of aza vinyl sulfones **1c–f**.

yields). Deprotection of the Boc group, followed by coupling with Leu led to aza vinyl sulfones **1a–b** containing Gly in P_1 (Scheme 1).

The aza vinyl sulfones containing Phe and homoPhe in P_1 were synthesized starting from the corresponding Boc protected hydrazines **2b–c**. Deprotection of the Boc group with TFA, followed by coupling with BocLeuOH led to azadipeptides **4a–b**. Finally, coupling with the acid derivative of the corresponding vinyl sulfones led to aza vinyl sulfones **1c–f** in 62–78% yields (Scheme 2).

Compounds **1a–f** were then screened against the parasite cysteine protease falcipain-2 and against the chloroquine-resistant W2 strain of *P. falciparum* (Table 1). Compounds **1a–f** were devoid of enzyme inhibitory activity. However, most of the compounds demonstrated modest activity against cultured *P. falciparum*. Probably, falcipain-2 inhibition is not the primary mode by which derivatives **1a–f** exert antiplasmodial activities. The most active compound in this series, **1f**, contained aza-homoPhe in P_1 and a phenyl vinyl sulfone, and thus we decided to prepare a new series of compounds containing these features and Cbz-protected amino acids in P_2. Leu, Phe and Gly residues were selected in order to modulate the interaction with P_2 (Scheme 3).[16] Two compounds were also synthesized with a Mu-protected Leu at P_2 and an aza-Gly or aza-homoPhe in P_1 (Scheme 4).

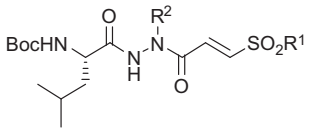
For the Cbz series, the recognition moiety was obtained starting with different *N*-Cbz amino acids (Leu, Gly and Phe) and Boc protected hydrazine **2a** in the presence of CDI and NEt_3. Deprotection of the Boc group with TFA, followed by reaction with $PhCH_2CHO$ led to azadipeptides **6a–c**. Reduction of the double bond with $NaBH_3CN$ in the presence of sulfonic acid led to azadipeptides **7a–c**.[16] Finally, coupling with the acid derivative of the corresponding vinyl sulfones led to aza vinyl sulfones **1g–i** in 73–86% yields (Scheme 3). Despite the fact that compounds **1g–i** belong to the same class of compounds **1a–f**, a different synthetic pathway was adopted in order to avoid the formation of the dialkylated subproduct of **2b–c**, observed during the preparation of **1c–f**.

Compounds **1j** and **1l** were obtained from aza vinyl sulfones **1b** and **1f** in 72% and 87% yields, respectively, by deprotection of the Boc group with TFA, followed by reaction with 4-morpholinecarbonyl chloride (Scheme 4).

All compounds were evaluated for activity against papain, the prototype for clan CA cysteine proteases. As observed before for falcipain-2, our compounds were not active against papain (Table 2). Compounds **1g–l** were also evaluated against cultured *P. falciparum*. Inspection of the data in Table 2, allows the following observations to be made:

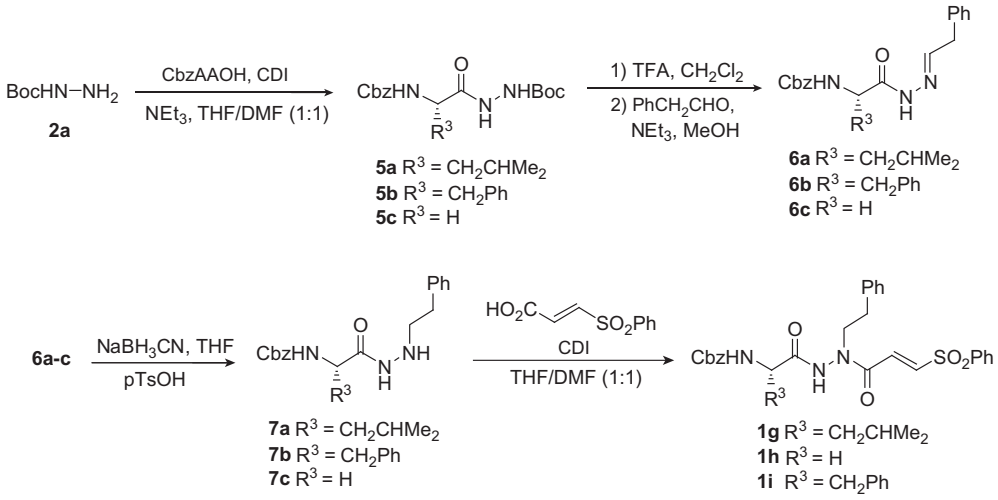
1. For the Cbz-protected compounds containing an aza-homoPhe in P_1 and a phenyl vinyl sulfone, that is, vinyl sulfones **1g–i**, the presence of a Cbz-protected Phe in P_2 (i.e., vinyl sulfone **1i**) improved inhibitory activity against cultured parasites when compared to the compound with Boc-protected Leu in P_2, that is, **1f**. The presence of a Leu or Gly in P_2 resulted in lost of antiparasitic activity vinyl sulfones **1g** and **1h**).

Table 1
Antiplasmodial activity for compounds **1a–f**

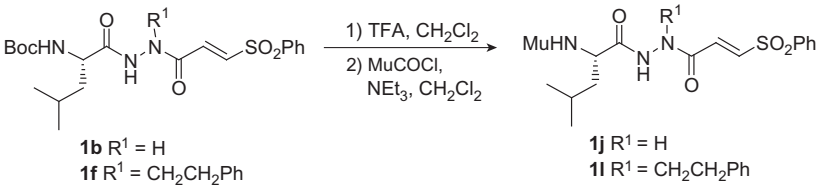


Compds	R^1	R^2	Falcipain-2 IC_{50}[a](μM)	*P. falciparum* W2 IC_{50} (μM)
1a	Me	H	ND	34.2 ± 4.2
1b	Ph	H	ND	33.5 ± 1.4
1c	Me	CH_2Ph	>50	21.6 ± 1.8
1d	Ph	CH_2Ph	>50	>50
1e	Me	CH_2CH_2Ph	>50	46.6 ± 1.6
1f	Ph	CH_2CH_2Ph	>50	15.4 ± 0.4
E-64			0.11 ± 0.025	1.94 ± 0.004

[a] ND = not done.

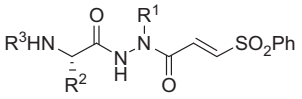


Scheme 3. Synthesis of aza vinyl sulfones **1g–i**.



Scheme 4. Synthesis of aza vinyl sulfones **1j–l**.

Table 2
Papain inhibition and antiplasmodial activity for compounds **1g–l**



Compds	R^1	R^2	R^3	Papain IC_{50} (μM)	*P. falciparum* W2 IC_{50} (μM)
1g	CH_2CH_2Ph	CH_2CHMe_2	Cbz	>50	>10
1h	CH_2CH_2Ph	H	Cbz	>50	>10
1i	CH_2CH_2Ph	CH_2Ph	Cbz	>50	9.0 ± 0.9
1j	H	CH_2CHMe_2	Mu	>50	>10
1l	CH_2CH_2Ph	CH_2CHMe_2	Mu	>50	3.4 ± 0.04
E-64				0.002 ± 0.0001	1.94 ± 0.004

2. For compounds containing an aza-Gly in P_1 and Leu in P_2 the presence of a Mu protective group resulted in lost of activity against cultured parasites (i.e., vinyl sulfones **1j**, versus their Boc-protected counterpart **1b**). The opposite result was observed for inhibitors containing an aza-homoPhe in P_1 and Leu in P_2. In this case a Mu protective group (i.e., aza vinyl sulfone **1l**) increased the activity ~5-fold compared to that of its Boc-protected counterpart **1f**.

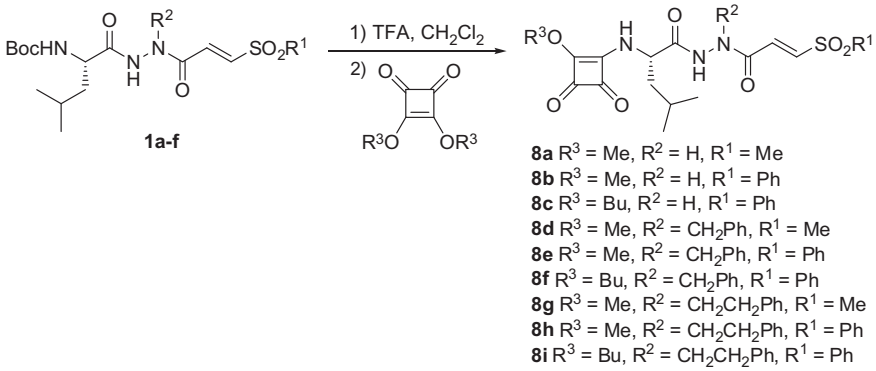
To further study aza vinyl sulfones as antiplasmodial agents we prepared compounds **8a–i**, in which the aza vinyl sulfone scaffold is coupled to a squarate moiety. In fact, since the first synthesis of squaric acid a number of derivatives have been reported in the fields of bioorganic and medicinal chemistry.[17–20] Very recently, a few compounds containing a squarate moiety were described in the GSK database as antimalarials.[21]

The synthesis of derivatives **8a–i** involved the deprotection of the Boc group of aza vinyl sulfones **1a–f**, followed by reaction with dimethoxy or dibutoxy squaric acid in 61–69% yields (Scheme 5).

Compounds **8a–i** were screened against the parasite cysteine protease falcipain-2 and against the cultured *P. falciparum* (Table 3). Most of the compounds had low micromolar antiplasmodial activity, with IC_{50} values ranging from 0.95 to 4.5 μM. Except for the aza-homoPhe derivatives **8h** and **8i**, the presence of a methoxy or a butoxy group in the squaric moiety did not markedly change activity against *P. falciparum* (compounds **8b** and **8e** vs compounds **8c** and **8f**, respectively). For the phenyl vinyl sulfones, the order of inhibitory activity against *P. falciparum* depended on the nature of the P_1 residue and varied in the order aza-Phe > aza-Gly > aza-homoPhe. In the case of methyl vinyl sulfones, inhibitory activity was similar for aza-Gly (**8a**) and aza-homoPhe (**8g**), but in the presence of aza-Phe (**8d**) activity against *P. falciparum* was lost. As observed before, all compounds were devoid of enzyme inhibitory activity.

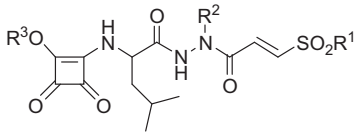
3. Conclusion

Dipeptide aza vinyl sulfones incorporating a recognition moiety for Falcipain-2 inhibition were inactive for cysteine proteases of



Scheme 5. Synthesis of aza vinyl sulfones squaramates **8a–i**.

Table 3
Antiplasmodial activity for compounds **8a–i**



Compds	R^1	R^2	R^3	Falcipain-2 IC_{50} (μM)	*P. falciparum* W2 IC_{50} (μM)
8a	Me	H	Me	>50	2.3 ± 0.1
8b	Ph	H	Me	>50	3.5 ± 0.02
8c	Ph	H	Bu	>50	2.6 ± 0.5
8d	Me	CH_2Ph	Me	>50	>10
8e	Ph	CH_2Ph	Me	>50	0.95 ± 0.06
8f	Ph	CH_2Ph	Bu	>50	1.2 ± 0.1
8g	Me	CH_2CH_2Ph	Me	>50	2.5 ± 0.5
8h	Ph	CH_2CH_2Ph	Me	>50	>10
8i	Ph	CH_2CH_2Ph	Bu	>50	4.5 ± 0.2
E-64				0.11 ± 0.025	1.94 ± 0.004

clan CA but displayed antiplasmodial activity against a chloroquine-resistant strain of *P. falciparum*, with IC_{50} values ranging from 13 to 47 μM. In fact, the exchange of CH/N in the P_1 position led to much poorer antiplasmodial activity compared to classical peptidyl vinyl sulfone cysteine protease inhibitors previously reported.

Antiplasmodial activity was significantly improved when Mu was used as protective group or a squaric moiety was coupled to the dipeptide Leu-aza vinyl sulfones. The most active compounds, squaric-Leu-azaPhe-VSPh, **8e** and **8f**, presented IC_{50} values of 0.95 and 1.2 μM, suggesting that these are potential lead compounds for the development of new antimalarial agents. Since the squaric moiety is a known zinc binding group, it is possible that these compounds target the heme polymerization.[22] Further studies are underway to identify the molecular targets responsible for the activity of the most potent compounds.

4. Experimental

4.1. Chemistry

All reagents and solvents were obtained from commercial suppliers and were used without further purification. Melting points were determined using a Kofler camera Bock monoscope M and are uncorrected. The infrared spectra were collected on a Nicolet Impact 400 FTIR infrared spectrophotometer. High resolution mass spectra (HMRS) were performed in Unidade de Espectrometria de Masas, Santiago de Compostela. Merck Silica Gel 60 F254 plates were used as analytical TLC; flash column chromatography was performed on Merck Silica Gel (200–400 mesh). ^1H and ^{13}C NMR spectra were recorded on a Bruker 400 Ultra-Shield (400 MHz). ^1H and ^{13}C chemical shifts are expressed in δ (ppm) referenced to the solvent used and the proton coupling constants (J) in hertz.

4.1.1. General preparation of vinyl sulfones 3a–b

To a solution of the suitable vinyl sulfone acid (1 equiv) in a mixture of THF/DMF (1:1) was added CDI (1 equiv) and the mixture stirred at rt for 30 min. After this time, the *t*-butyl carbazate **2a** (1 equiv) was added. The mixture was stirred overnight at rt. The reaction mixture was then diluted with water and extracted with AcOEt and the organic layers combined, dried with anhydrous Na_2SO_4, filtered and concentrated to dryness. The obtained residue was purified by column chromatography (Hexane/AcOEt 1:1) affording the corresponding product as a white solid.

4.1.2. General preparation of Boc Leu aza-Gly vinyl sulfones 1a–b

To a solution of the suitable Boc aza vinyl sulfone **3** (1 equiv) in DCM was added TFA (4 equiv). After 2 h the solvent was removed under reduced pressure affording quantitatively a slightly yellow oil.

To a solution of BocLeuOH (1 equiv) in a mixture of THF/DMF (1:1) was added CDI (1 equiv) and the mixture stirred at rt for 30 min. After this time, the deprotected aza vinyl sulfone (1 equiv) was added followed by NEt_3 (2 equiv). The mixture was stirred overnight at rt. The reaction mixture was then diluted with water and extracted with AcOEt and the organic layers combined, dried with anhydrous Na_2SO_4, filtered and concentrated to dryness. The obtained residue was purified by column chromatography

(Hexane/AcOEt 3:2) affording the corresponding product as a white solid.

4.1.2.1. BocLeuazaGlyCOVSMe 1a. Obtained in 64% yield. Mp 88–89 °C. IR (cm^{-1}) 3442 (NH), 2929, 2858, 1743 (C=O), 1718 (C=O), 1637 (C=O), 1461, 1404, 1303, 1144. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.84 (d, J = 16 Hz, 1H), 7.19 (d, J = 16 Hz, 1H), 6.87 (d, J = 8 Hz, 2H), 4.52 (t, J = 8 Hz, 1H), 3.85 (s, 1H), 2.93 (s, 3H), 2.18 (m, 1H), 1.72 (t, J = 8 Hz, 1H), 1.45 (sl, 10H), 1.01 (d, J = 8 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.66 (C=O), 168.56 (C=O), 159.31 (C=O), 146.86 ($CHSO_2CH_3$), 131.71 (CH=$CHSO_2CH_3$), 80.65 ($C(CH_3)_3$), 50.58 (CH), 40.43 (CH_2), 37.99 (CH=$CHSO_2CH_3$), 28.41 ($C(CH_3)_3$), 25.38 (CH), 23.14 ($2CH_3$). HRMS-ESI-TOF: m/z calcd $C_{15}H_{27}N_3O_6SNa$ (M^++Na) 400.1518, found 400.1509.

4.1.2.2. BocLeuazaGlyCOVSPh 1b. Obtained in 73% yield. Mp 91–92 °C. IR (cm^{-1}) 3358 (NH), 3051, 2969, 2914, 1715 (C=O), 1654 (C=O), 1525, 1456, 1368, 1231, 1150. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.95 (t, J = 4 Hz, 2H), 7.64–7.58 (m, 4H), 7.17 (s, 1H), 7.04 (d, J = 16 Hz, 1H), 6.81 (s, 1H), 4.62 (t, J = 4 Hz, 1H), 4.29 (s, 1H), 1.64 (m, 2H), 1.46 (sl, 10H), 1.01 (d, J = 4 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.66 (C=O), 168.56 (C=O), 158.31 (C=O), 140.93 (C_q), 137.57 ($CHSO_2Ph$), 133.05 (CH_{Ar}), 130.28 (CH=CH-SO_2Ph), 129.10 (CH_{Ar}), 128.17 (CH_{Ar}), 80.65 ($C(CH_3)_3$), 50.58 (CH), 40.43 (CH_2), 28.41 ($C(CH_3)_3$), 25.38 (CH), 23.14 ($2CH_3$). HRMS-ESI-TOF: m/z calcd $C_{20}H_{29}N_3O_6SNa$ (M^++Na) 462.1675, found 462.1668.

4.1.3. General preparation of N-Boc azahomoPhe and azaPhe vinyl sulfones 1c–f

To a solution of the suitable vinyl sulfone acid (1 equiv) in a mixture of THF/DMF (1:1) was added CDI (1 equiv) and the mixture stirred at rt for 30 min. After this time, the suitable azadipeptide (1 equiv) was added. The mixture was stirred overnight at rt. The reaction mixture was then diluted with water and extracted with AcOEt and the organic layers combined, dried with anhydrous Na_2SO_4, filtered and concentrated to dryness. The obtained residue was purified by column chromatography (Hexane/AcOEt 3:2) affording the corresponding product as a white solid.

4.1.3.1. BocLeuazaPheCOVSMe 1c. Obtained in 62% yield. Mp 98–99 °C. IR (cm^{-1}) 3409 (NH), 3340 (NH), 3065, 2969, 2914, 1722 (C=O), 1675 (C=O), 1518, 1368, 1265. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.65 (d, J = 16 Hz, 1H), 7.40 (d, J = 16 Hz, 1H), 7.25 (m, 5H), 6.87 (s, 1H), 5.06 (s, 1H), 4.89 (t, J = 8 Hz, 1H), 4.32 (s, 2H), 2.95 (s, 3H), 1.76 (m, 1H), 1.53 (m, 11H), 1.03 (d, J = 4 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.87 (C=O), 165.32 (C=O), 158.31 (C=O), 147.17 ($CHSO_2CH_3$), 136.21 (C_q), 133.52 (CH=$CHSO_2CH_3$), 128.77 (CH_{Ar}), 128.43 (CH_{Ar}), 127.60 (CH_{Ar}), 80.65 ($C(CH_3)_3$), 53.18 (CH_2), 51.09 (CH), 40.43 (CH_2), 37.99 (SO_2CH_3), 28.41 ($C(CH_3)_3$), 25.38 (CH), 23.14 ($2CH_3$). HRMS-ESI-TOF: m/z calcd $C_{22}H_{33}N_3O_6SNa$ (M^++Na) 490.1988, found 490.1969.

4.1.3.2. BocLeuazaPheCOVSPh 1d. Obtained in 71% yield. Mp 101–102 °C. IR (cm^{-1}) 3299 (NH), 3051 (NH), 2969, 2955, 1715 (C=O), 1647 (C=O), 1531, 1225, 1156. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.33 (s, 1H), 7.86 (m, 2H), 7.57 (m, 3H), 7.42 (m, 2H), 7.31 (m, 2H), 7.23 (m, 1H), 6.96 (d, J = 16 Hz, 1H), 6.50 (d, J = 16 Hz, 1H), 4.49 (t, J = 8 Hz, 1H), 4.16 (s, 2H), 3.06 (s, 1H), 1.50 (m, 12H), 1.36 (t, J = 8 Hz, 1H), 1.01 (d, J = 8 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.87 (C=O), 165.32 (C=O), 158.31 (C=O), 140.93 (CH_{Ar}), 139.58 ($CHSO_2Ph$), 136.21 (CH_{Ar}), 133.05 (CH_{Ar}), 130.05 (CH_{Ar}), 130.14 (CH=$CHSO_2Ph$), 129.10 (CH_{Ar}), 128.77 (CH_{Ar}), 128.43 (CH_{Ar}), 128.17 (C_q), 127.60 (C_q), 80.65 ($C(CH_3)_3$), 53.18 (CH_2), 51.09 (CH), 40.43 (CH_2), 28.41 ($C(CH_3)_3$), 25.38 (CH), 23.14 ($2CH_3$). HRMS-ESI-TOF: m/z calcd $C_{27}H_{35}N_3O_6SNa$ (M^++Na) 552.2144, found 552.2140.

4.1.3.3. BocLeuazahomoPheCOVSMe 1e. Obtained in 73% yield. Mp 103–104 °C. IR (cm^{-1}) 3340 (NH), 3244 (NH), 3051, 2969, 2914, 1722 (C=O), 1647 (C=O), 1538, 1416, 1265. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.16 (d, J = 16 Hz, 1H), 7.29 (m, 3H), 7.17 (m, 3H), 6.57 (s, 1H), 4.88 (sl, 1H), 3.84 (s, 1H), 3.45 (t, J = 8 Hz, 1H), 3.33 (t, J = 8 Hz, 1H), 2.93 (s, 3H), 2.82 (t, J = 8 Hz, 2H), 1.86 (m, 1H), 1.60 (dd, J = 8 Hz, J = 4 Hz, 1H), 1.54 (dd, J = 8 Hz, J = 4 Hz, 1H), 1.43 (s, 9H), 1.00 (d, J = 4 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.87 (C=O), 161.64 (C=O), 158.31 (C=O), 147.17 ($CHSO_2CH_3$), 138.54 (C_q), 133.52 (CH=$CHSO_2CH_3$), 129.20 (CH_{Ar}), 129.09 (CH_{Ar}), 126.37 (CH_{Ar}), 80.65 ($C(CH_3)_3$), 51.09 (CH), 49.77 (CH_2), 40.43 (CH_2), 37.99 (SO_2CH_3), 30.94 (CH_2), 28.41 ($C(CH_3)_3$), 25.38 (CH), 23.14 ($2CH_3$). HRMS-ESI-TOF: m/z calcd $C_{23}H_{35}N_3O_6SNa$ (M^++Na) 504.2144, found 504.2137.

4.1.3.4. BocLeuazahomoPheCOVSPh 1f. Obtained in 78% yield. Mp 112–113 °C. IR (cm^{-1}) 3409 (NH), 3326 (NH), 3051, 2969, 2928, 1715 (C=O), 1668 (C=O), 1511, 1272, 1150. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.02 (s, 1H), 7.96 (m, 2H), 7.59 (m, 3H), 7.25 (m, 5H), 7.14 (d, J = 16 Hz, 2H), 6.69 (s, 1H), 4.77 (t, J = 8 Hz, 1H), 4.36 (s, 1H), 3.56 (t, J = 8 Hz, 1H), 3.47 (t, J = 8 Hz, 1H), 2.89 (m, 2H), 1.59 (m, 2H), 1.50 (m, 1H), 1.43 (s, 9H), 0.89 (d, J = 4 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.87 (C=O), 161.64 (C=O), 158.31 (C=O), 140.93 (C_q), 139.58 ($CHSO_2Ph$), 138.54 (CH_{Ar}), 133.0 (CH_{Ar}), 130.14 (CH=$CHSO_2Ph$), 129.20 (CH_{Ar}), 129.09 (CH_{Ar}), 128.17 (CH_{Ar}), 126.37 (CH_{Ar}), 80.65 ($C(CH_3)_3$), 51.09 (CH), 49.77 (CH_2), 40.44 (CH_2), 30.95 (CH_2), 28.41 ($C(CH_3)_3$), 25.39 (CH), 23.14 ($2CH_3$). HRMS-ESI-TOF: m/z calcd $C_{28}H_{37}N_3O_6SNa$ (M^++Na) 566.2301, found 566.2297.

4.1.4. General preparation of Cbz aza vinyl sulfones 1g–i

To a solution of the suitable vinyl sulfone acid (1 equiv) in a mixture of THF/DMF (1:1) was added CDI (1 equiv) and the mixture stirred at rt for 30 min. After this time, the suitable azapeptide (1 equiv)[16] was added. The mixture was stirred overnight at rt. The reaction mixture was then diluted with water and extracted with AcOEt and the organic layers combined, dried with anhydrous Na_2SO_4, filtered and concentrated to dryness. The obtained residue was purified by column chromatography (Hexane/AcOEt 3:2) affording the corresponding product as a white solid.

4.1.4.1. CbzLeuazahomoPheCOVSPh 1g. Obtained in 73% yield. Mp 115–116 °C. IR (cm^{-1}) 3299 (NH), 3051, 2969, 1715 (C=O), 1647 (C=O), 1531, 1450, 1361, 1225, 1156. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.96 (d, J = 8 Hz, 2H), 7.75 (d, J = 16 Hz, 1H), 7.61 (m, 3H), 7.29 (m, 8H), 7.17 (m, 3H), 6.49 (s, 1H), 5.38 (s, 2H), 4.87 (t, J = 4 Hz, 1H), 3.61 (s, 1H), 3.49 (t, J = 4 Hz, 1H), 3.14 (t, J = 4 Hz, 1H), 2.75 (t, J = 4 Hz, 2H), 1.70 (m, 2H), 1.57 (t, J = 4 Hz, 1H), 1.05 (d, J = 4 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.87 (C=O), 161.64 (C=O), 159.04 (C=O), 140.93 (C_q), 139.58 ($CHSO_2Ph$), 138.54 (CH_{Ar}), 136.99 (CH_{Ar}), 133.05 (CH_{Ar}), 130.14 (CH=$CHSO_2Ph$), 129.20 (CH_{Ar}), 129.09 (CH_{Ar}), 128.33 (CH_{Ar}), 128.20 (CH_{Ar}), 128.18 (CH_{Ar}), 126.37 (CH_{Ar}), 67.05 (CH_2), 51.09 (CH), 4977 (CH_2), 40.43 (CH_2), 30.94 (CH_2), 25.38 (CH), 23.14 ($2CH_3$). HRMS-ESI-TOF: m/z calcd $C_{31}H_{35}N_3O_6SNa$ (M^++Na) 600.2144, found 600.2137.

4.1.4.2. CbzGlyazahomoPheCOVSPh 1h. Obtained in 86% yield. Mp 114–115 °C. IR (cm^{-1}) 3292 (NH), 3285 (NH), 2969, 1722 (C=O), 1647 (C=O), 1518, 1450, 1375, 1238, 1150. ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.08 (s, 1H), 7.91 (m, 2H), 7.58 (m, 3H), 7.24 (m, 11H), 6.46 (d, J = 16 Hz, 1H), 5.39 (s, 2H), 4.13 (s, 1H), 3.97 (s, 1H), 3.84 (s, 1H), 3.48 (t, J = 8 Hz, 1H), 3.20 (t, J = 4 Hz, 1H), 2.86

(t, J = 8 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.16 (C=O), 161.64 (C=O), 157.43 (C=O), 140.75 (C_q), 139.58 (*C*HSO$_2$Ph), 138.64 (CH_{Ar}), 137.08 (CH_{Ar}), 133.04 (CH_{Ar}), 130.14 (*C*H=CHSO$_2$Ph), 129.19 (CH_{Ar}), 129.08 (CH_{Ar}), 129.06 (CH_{Ar}), 128.31 (CH_{Ar}), 128.16 (CH_{Ar}), 128.15 (CH_{Ar}), 126.36 (CH_{Ar}), 66.68 (CH_2), 49.77 (CH_2), 43.68 (CH_2), 31.42 (CH_2). HRMS-ESI-TOF: m/z calcd $C_{27}H_{27}N_3O_6SNa$ (M^++Na) 544.1518, found 544.1510.

4.1.4.3. CbzPheazahomoPheCOVSPh 1i. Obtained in 77% yield. Mp 126–127 °C. IR (cm^{-1}) 3305 (NH), 2971, 1727 (C=O), 1657 (C=O), 1523, 1242, 1153, 1031. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.94 (m, 3H), 7.52 (m, 3H), 7.29 (m, 15H), 7.10 (d, J = 16 Hz, 1H), 6.70 (s, 1H), 5.39 (s, 2H), 5.14 (sl, 1H), 3.71 (s, 1H), 3.43 (t, J = 4 Hz, 1H), 3.36 (t, J = 4 Hz, 1H), 3.23 (dd, J = 12 Hz, J = 4 Hz, 1H), 2.84 (t, J = 8 Hz, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 168.38 (C=O), 161.64 (C=O), 159.04 (C=O), 140.93 (C_q), 139.58 (*C*HSO$_2$Ph), 138.54 (CH_{Ar}), 137.59 (CH_{Ar}), 136.99 (CH_{Ar}), 133.05 (CH_{Ar}), 130.14 (*C*H=CHSO$_2$Ph), 129.42 (CH_{Ar}), 129.20 (CH_{Ar}), 129.10 (CH_{Ar}), 129.07 (CH_{Ar}) 128.33 (CH_{Ar}), 128.20 (CH_{Ar}), 128.18 (CH_{Ar}), 127.17 (CH_{Ar}), 126.37 (CH_{Ar}), 67.05 (CH_2), 56.18 (CH), 49.77 (CH_2), 38.45 (CH_2), 30.94 (CH_2). HRMS-ESI-TOF: m/z calcd $C_{34}H_{33}N_3O_6SNa$ (M^++Na) 634.1988, found 634.1973.

4.1.5. General preparation of Mu aza vinyl sulfones 1j–l

To a solution of the suitable Boc azadipeptide vinylsulfone **1** (1 equiv) in DCM was added TFA (4 equiv). After 2 h the solvent was removed under reduced pressure affording quantitatively a slightly yellow oil. The residue was dissolved in DCM and NEt_3 (2 equiv) was added followed by 4-morpholinecarbonyl chloride (1 equiv) under N_2. The reaction mixture was stirred at rt for 2 h. The solvent was then removed under reduced pressure and the obtained residue was purified by column chromatography (Hexane/AcOEt 3:2) affording the corresponding product as a white solid.

4.1.5.1. MuLeuGlyCOVSPh 1j. Obtained in 72% yield. Mp 98–99 °C. IR (cm^{-1}) 3312 (NH), 2941, 1661 (C=O), 1593, 1456, 1381, 1197. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.26 (s, 1H), 7.93 (m, 2H), 7.59 (m, 5H), 6.99 (d, J = 16 Hz, 1H), 4.76 (t, J = 4 Hz, 1H), 4.03 (s, 1H), 3.90 (t, J = 4 Hz, 2H), 3.79 (t, J = 4 Hz, 4H), 3.57 (t, J = 4 Hz, 2H), 1.87 (m, 2H), 1.53 (t, J = 4 Hz, 1H), 1.08 (d, J = 8 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.66 (C=O), 168.56 (C=O), 157.25 (C=O), 140.93 (C_q), 137.57 (*C*HSO$_2$Ph), 133.05 (CH_{Ar}), 130.28 (*C*H=CHSO$_2$Ph), 129.10 (CH_{Ar}), 128.17 (CH_{Ar}), 67.21 ($2CH_2$), 51.33 (CH), 46.36 ($2CH_2$), 40.43 (CH_2), 25.38 (CH), 23.14 ($2CH_3$). HRMS-ESI-TOF: m/z calcd $C_{20}H_{28}N_4O_6SNa$ (M^++Na) 475.1627, found 475.1619.

4.1.5.2. MuLeuhomoPheCOVSPh 1l. Obtained in 87% yield. Mp 93–94 °C. IR (cm^{-1}) 3370 (NH), 3051, 2969, 2914, 1722 (C=O), 1642 (C=O), 1518, 1450, 1368, 1313, 1259, 1143. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.94 (t, J = 4 Hz, 2H), 7.58 (m, 3H), 7.49 (d, J = 16 Hz, 1H), 7.31 (m, 5H), 6.73 (d, J = 16 Hz, 1H), 4.56 (sl, 1H), 3.88 (s, 1H), 3.81 (m, 6H), 3.57 (sl, 1H), 3.52 (sl, 2H), 3.26 (t, J = 4 Hz, 1H), 2.93 (t, J = 4 Hz, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.87 (C=O), 161.64 (C=O), 157.71 (C=O), 140.75 (C_q), 139.58 (*C*HSO$_2$Ph), 138.64 (C_q), 133.04 (C_q), 130.14 (*C*H=CHSO$_2$Ph), 129.19 (CH_{Ar}), 129.08 (CH_{Ar}), 129.06 (CH_{Ar}), 128.14 (CH_{Ar}), 126.36 (CH_{Ar}), 66.10 ($2CH_2$), 51.98 (CH), 49.77 (CH_2), 46.58 ($2CH_2$), 40.44 (CH_2), 31.42 (CH_2), 25.39 (CH), 23.14 ($2CH_3$). HRMS-ESI-TOF: m/z calcd $C_{28}H_{36}N_4O_6SNa$ (M^++Na) 579.2253, found 579.2245.

4.1.6. General procedure for the preparation of squaric aza vinyl sulfones derivatives 8a–i

To a solution of the suitable Boc azapeptide vinylsulfone **1** (1 equiv) in DCM was added TFA (4 equiv). After 2 h the solvent was removed under reduced pressure affording quantitatively slightly yellow oil. The residue was dissolved in methanol and NEt_3 (2 equiv) was added followed by the addition of the suitable squaric acid derivative (1 equiv) under N_2. The reaction mixture was stirred overnight at rt. The solvent was then removed under reduced pressure and the obtained residue was purified by column chromatography (Hexane/AcOEt 3:2) affording the corresponding product as a white solid.

4.1.6.1. SQMeLeuazaGlyCOVSMe 8a. Obtained in 61% yield. Mp 126–127 °C. IR (cm^{-1}) 3436 (NH), 3051, 2955, 1715 (C=O), 1661 (C=O), 1525, 1272, 1156, 1040. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.81 (d, J = 16 Hz, 1H), 7.41 (s, 1H), 7.22 (m, 2H), 5.07 (t, J = 8 Hz, 1H), 4.97 (s, 1H), 3.93 (s, 3H), 2.95 (s, 3H), 1.60 (t, J = 8 Hz, 1H), 1.46 (m, 2H), 1.01 (sl, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 191.89 (C=O), 179.35 and 178.94 (C_q and C=O), 173.66 (C=O), 168.56 (C=O), 161.68 (C_q), 146.86 (*C*HSO$_2$CH$_3$), 131.71 (*C*H=CHSO$_2$CH$_3$), 61.45 (OCH_3), 57.78 (CH), 40.43 (CH_2), 37.99 (SO_2CH_3), 25.38 (CH), 23.14 ($2CH_3$). HRMS-ESI-TOF: m/z calcd $C_{15}H_{21}N_3O_7SNa$ (M^++Na) 410.0998, found 410.0988.

4.1.6.2. SQMeLeuazaGlyCOVSPh 8b. Obtained in 67% yield. Mp 147–148 °C. IR (cm^{-1}) 3354 (NH), 3051, 2969, 2914, 1722 (C=O), 1654 (C=O), 1525, 1456, 1381, 1313, 1136, 1040. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.93 (d, J = 16 Hz, 1H), 7.87 (m, 2H), 7.58 (m, 3H), 7.25 (s, 1H), 6.88 (d, J = 16 Hz, 1H), 6.10 (s, 1H), 4.67 (t, J = 8 Hz, 1H), 3.91 (s, 3H), 3.00 (s, 1H), 1.59 (m, 3H), 1.03 (sl, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 191.95 (C=O), 179.41 and 179.00 (C=O and C_q), 173.71 (C=O), 168.61 (C=O), 161.73 (C_q), 140.99 (CH_{Ar}), 137.62 (*C*HSO$_2$Ph), 133.11 (C_q), 130.34 (*C*H=CHSO$_2$Ph), 129.15 (CH_{Ar}), 128.23 (CH_{Ar}), 61.51 (OCH_3), 57.84 (CH), 40.49 (CH_2), 25.44 (CH), 23.19 ($2CH_3$). HRMS-ESI-TOF: m/z calcd $C_{20}H_{23}N_3O_7SNa$ (M^++Na) 472.1154, found 472.1147.

4.1.6.3. SQBuLeuazaGlyCOVSPh 8c. Obtained in 66% yield. Mp 132–133 °C. IR (cm^{-1}) 3340 (NH), 3051 (NH), 2969, 2914, 1709 (C=O), 1668 (C=O), 1518, 1470, 1245, 1081. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.95 (m, 3H), 7.62 (m, 3H), 7.23 (s, 1H), 6.90 (d, J = 16 Hz, 1H), 6.17 (s, 1H), 4.70 (t, J = 4 Hz, 1H), 4.42 (t, J = 8 Hz, 2H), 3.34 (s, 1H), 1.78 (m, 2H), 1.66 (m, 3H), 1.53 (m, 2H), 1.05 (m, 9H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 191.89 (C=O), 181.91 (C_q), 180.01 (C=O), 173.66 (C=O), 168.56 (C=O), 164.00 (C_q), 140.93 (CH_{Ar}), 137.57 (*C*HSO$_2$Ph), 133.05 (CH_{Ar}), 130.28 (*C*H=CHSO$_2$Ph), 129.10 (CH_{Ar}), 128.17 (CH_{Ar}), 73.09 (OCH_2), 57.78 (CH), 40.43 (CH_2), 31.14 (CH_2), 25.38 (CH), 23.14 ($2CH_3$), 19.94 (CH_2), 14.01 (CH_3). HRMS-ESI-TOF: m/z calcd $C_{23}H_{29}N_3O_7SNa$ (M^++Na) 514.1624, found 514.1615.

4.1.6.4. SQMeLeuazaPheCOVSMe 8d. Obtained in 68% yield. Mp 148–149 °C. IR (cm^{-1}) 3340 (NH), 3244 (NH), 3051, 2969, 1722 (C=O), 1647 (C=O), 1538, 1265, 1150, 1088, 1034. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.24 (m, 7H), 6.48 (s, 1H), 5.02 (t, J = 4 Hz, 1H), 4.33 (s, 1H), 4.16 (s, 1H), 3.85 (s, 3H), 3.46 (s, 1H), 2.91 (s, 3H), 1.90 (m, 1H), 1.69 (m, 1H), 1.54 (m, 1H), 1.09 (m, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 191.89 (C=O), 179.35 and 178.94 (C_q and C=O), 170.87 (C=O), 165.33 (C=O), 161.68 (C_q), 147.17 (*C*HSO$_2$CH$_3$), 136.21 (CH_{Ar}), 133.52 (*C*H=CHSO$_2$CH$_3$), 128.77 (CH_{Ar}), 128.44 (CH_{Ar}), 127.61 (CH_{Ar}), 61.46 (OCH_3), 58.31 (CH), 53.18 (CH_2), 40.44 (CH_2), 37.99 (SO_2CH_3), 25.39 (CH), 23.14 ($2CH_3$). HRMS-ESI-TOF: m/z calcd $C_{22}H_{27}N_3O_7SNa$ (M^++Na) 500.1467, found 500.1456.

4.1.6.5. SQMeLeuazaPheCOVSPh 8e. Obtained in 67% yield. Mp 143–144 °C. IR (cm^{-1}) 3392 (NH), 3218 (NH), 3070, 1703 (C=O), 1644 (C=O), 1600, 1466, 1311, 1192, 1125. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.84 (m, 3H), 7.54 (m, 1H), 7.47 (m, 2H), 7.18 (m, 5H), 7.05 (d, J = 16 Hz, 1H), 6.22 (s, 1H), 5.00 (t, J = 8 Hz, 1H),

4.39 (s, 1H), 3.94 (s, 1H), 3.89 (s, 3H), 3.55 (s, 1H), 1.74 (t, J = 8 Hz, 1H), 1.65 (t, J = 8 Hz, 1H), 1.52 (m, 1H), 1.01 (sl, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 191.89 (C=O), 179.35 and 178.94 (C_q and C=O), 170.87 (C=O), 165.32 (C=O), 161.68 (C_q), 140.93 (C_q), 139.58 (*C*HSO$_2$Ph), 136.21 (CH_{Ar}), 133.05 (CH_{Ar}), 130.14 (*C*H=CH-SO$_2$Ph), 129.10 (CH_{Ar}), 128.77 (CH_{Ar}), 128.43 (CH_{Ar}), 128.17 (CH_{Ar}), 127.60 (CH_{Ar}), 61.45 (OCH_3), 58.31 (CH), 53.18 (CH_2), 40.43 (CH_2), 25.38 (CH), 23.14 ($2CH_3$). HRMS-ESI-TOF: m/z calcd $C_{27}H_{29}N_3O_7S$-Na (M^++Na) 562.1624, found 562.1617.

4.1.6.6. SQBuLeuazaPheCOVSPh 8f. Obtained in 63% yield. Mp 138–139 °C. IR (cm^{-1}) 3177 (NH), 2945, 2765, 1797 (C=O), 1644 (C=O), 1574, 1421, 1300, 1146, 1044. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.85 (d, J = 8 Hz, 2H), 7.79 (d, J = 16 Hz, 1H), 7.55 (m, 3H), 7.30 (m, 5H), 6.86 (s, 1H), 6.61 (d, J = 16 Hz, 1H), 5.19 (t, J = 4 Hz, 1H), 4.46 (m, 3H), 4.33 (s, 1H), 3.26 (s, 1H), 1.81 (m, 5H), 1.52 (m, 2H), 1.07 (sl, 9H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 191.89 (C=O), 181.91 (C_q), 180.01 (C=O), 170.87 (C=O), 165.33 (C=O), 164.01 (C_q), 140.94 (C_q), 139.59 (*C*HSO$_2$Ph), 136.21 (CH_{Ar}), 133.05 (CH_{Ar}), 130.14 (*C*H=CHSO$_2$Ph), 129.10 (CH_{Ar}), 128.77 (CH_{Ar}), 128.44 (CH_{Ar}), 128.18 (CH_{Ar}), 127.61 (CH_{Ar}), 73.09 (OCH_2), 58.31 (CH), 53.18 (CH_2), 40.44 (CH_2), 31.15 (CH_2), 25.39 (CH), 23.14 ($2CH_3$), 19.95 (CH_2), 14.02 (CH_3). HRMS-ESI-TOF: m/z calcd $C_{30}H_{35}N_3O_7SNa$ (M^++Na) 604.2093, found 604.2087.

4.1.6.7. SQMeLeuazahomoPheCOVSMe 8g. Obtained in 69% yield. Mp 138–139 °C. IR (cm^{-1}) 3409 (NH), 3299 (NH), 3051, 2969, 1722 (C=O), 1647 (C=O), 1511, 1368, 1265, 1156, 1034. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.25 (m, 6H), 7.07 (d, J = 16 Hz, 1H), 6.47 (s, 1H), 4.87 (t, J = 4 Hz, 1H), 3.76 (s, 3H), 3.53 (t, J = 4 Hz, 1H), 3.37 (t, J = 4 Hz, 1H), 3.30 (s, 1H), 2.93 (s, 3H), 2.85 (t, J = 4 Hz, 2H), 1.87 (m, 1H), 1.66 (m, 1H), 1.53 (m, 1H), 1.09 (d, J = 4 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 191.89 (C=O), 179.35 (C_q), 178.94 (C=O), 170.87 (C=O), 161.68 and 161.64 (C=O and C_q), 147.17 (*C*HSO$_2$CH$_3$), 138.54 (CH_{Ar}), 133.52 (*C*H=CHSO$_2$CH$_3$), 129.20 (CH_{Ar}), 129.09 (CH_{Ar}), 126.37 (CH_{Ar}), 61.45 (OCH_3), 58.31 (CH), 49.77 (CH_2), 40.43 (CH_2), 37.99 (SO_2CH_3), 30.94 (CH_2), 25.38 (CH), 23.14 ($2CH_3$). HRMS-ESI-TOF: m/z calcd $C_{23}H_{29}N_3O_7S$-Na (M^++Na) 514.1624, found 514.1617.

4.1.6.8. SQMeLeuazahomoPheCOVSPh 8h. Obtained in 63% yield. Mp 134–135 °C. IR (cm^{-1}) 3299 (NH), 3051 (NH), 2983, 1709 (C=O), 1681 (C=O), 1647 (C=O), 1525, 1422, 1265, 1129, 1040. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.89 (d, J = 8 Hz, 2H), 7.55 (m, 3H), 7.36 (d, J = 16 Hz, 1H), 7.20 (m, 5H), 7.03 (d, J = 16 Hz, 1H), 6.26 (s, 1H), 4.92 (t, J = 8 Hz, 1H), 3.78 (s, 3H), 3.60 (t, J = 8 Hz, 1H), 3.39 (t, J = 8 Hz, 1H), 3.31 (s, 1H), 2.82 (t, J = 8 Hz, 2H), 1.86 (m, 1H), 1.70 (m, 1H), 1.67 (m, 1H), 1.06 (d, J = 4 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 191.89 (C=O), 179.35 (C_q), 178.94 (C=O), 170.87 (C=O), 161.68 (C=O), 161.64 (CH_{Ar}), 148.14 (*C*HSO$_2$Ph), 140.93 (CH_{Ar}), 139.58 (CH_{Ar}), 138.54 (CH_{Ar}), 133.05 (CH_{Ar}), 130.14 (*C*H=CHSO$_2$Ph), 129.20 (CH_{Ar}), 129.10 (CH_{Ar}), 128.17 (CH_{Ar}), 126.37 (CH_{Ar}), 61.45 (OCH_3), 58.31 (CH), 49.77 (CH_2), 40.43 (CH_2), 30.94 (CH_2), 25.38 (CH), 23.14 ($2CH_3$). HRMS-ESI-TOF: m/z calcd $C_{28}H_{31}N_3O_7SNa$ (M^++Na) 576.1780, found 576.1768.

4.1.6.9. SQBuLeuazahomoPheCOVSPh 8i. Obtained in 65% yield. Mp 142–143 °C. IR (cm^{-1}) 3409 (NH), 3065 (NH), 2969, 2914, 1722 (C=O), 1675 (C=O), 1518, 1368, 1313, 1265, 1143, 1040. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.90 (m, 3H), 7.59 (m, 3H), 7.21 (m, 6H), 6.60 (s, 1H), 5.17 (t, J = 4 Hz, 1H), 4.40 (t, J = 8 Hz, 2H), 4.23 (s, 1H), 3.65 (m, 2H), 2.86 (t, J = 8 Hz, 2H), 1.63 (m, 7H), 1.02 (sl, 9H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 191.89 (C=O), 180.91 (C_q), 179.01 (C=O), 169.87 (C=O), 163.00 (C_q), 160.64 (C=O), 139.93 (CH_{Ar}), 138.58 (*C*HSO$_2$Ph), 137.54 (CH_{Ar}), 132.05 (CH_{Ar}), 129.14 (*C*H=CHSO$_2$Ph), 128.20 (CH_{Ar}), 128.10 (CH_{Ar}), 127.17 (CH_{Ar}), 125.37 (CH_{Ar}), 72.09 (CH_2), 57.31 (CH), 48.77 (CH_2), 39.43 (CH_2), 30.14 (CH_2), 29.94 (CH_2), 24.38 (CH), 22.14 ($2CH_3$), 18.94 (CH_2), 13.01 (CH_3). HRMS-ESI-TOF: m/z calcd $C_{31}H_{37}N_3O_7SNa$ (M^++Na) 618.2250, found 618.2243.

4.2. Pharmacology

4.2.1. Falcipain-2 assays

Falcipain-2 was assayed at 25 °C using 25 μM Z-Leu-Arg-AMC as substrate in 100 mM sodium acetate pH 6.0, 5 mM DTT, 0.75% DMSO. One microliter of serial dilutions of each compound dissolved in DMSO (highest concentration was 50 μM diluted 1:5 in eight steps resulting in the lowest concentration used 0.65 nM) was diluted into 100 μL of assay buffer. Enzyme (1 nM) was added, the mixture was incubated for 10 min, the reaction was initiated by addition of 50 μL of assay buffer containing the substrate, and fluorescence was read immediately in a Fluoroskan Ascent microplate spectrofluorometer. IC_{50} values were determined by plotting percentage inhibition relative to controls without inhibitor using GraphPad Prism 4 (GraphPad Software).

4.2.2. In vitro antiplasmodial activity in human red blood cells

Human red blood cells infected with ~1% parasitemia of ring stage *P. falciparum* synchronized with 5% sorbitol were incubated with tested compounds in 96 well plates at 37 °C for 48 h in RPMI-1640 medium, supplemented with 25 mM HEPES pH 7.4, 10% heat inactivated human serum (or 0.5% Albumax, 2% human serum), and 100 μM hypoxanthine under an atmosphere of 3% O_2, 5% CO_2, 91% N_2. After 48 h the cells were fixed in 2% HCHO in PBS, transferred into PBS with 100 mM NH_4Cl, 0.1% Triton X-100, 1 nM YOYO-1, and then analyzed in a flow cytometer (FACSort, Beckton Dickinson; EX 488 nm, EM 520 nm). IC_{50}s were calculated using GraphPad PRISM software.

4.2.3. Papain assays

Assays were carried out in 200 μL assay buffer (10 mM PBS, pH 7, 4, 5 mM DTT) containing 20 μL of papain activated in assay buffer at 5 μg/mL, and 5 μL of each concentration of tested inhibitors. Reactions were initiated by the addition of 30 μM fluorogenic substrate (Z-Leu-Leu-Arg-AMC, from Bachem, Germany) and activity was monitored (excitation 355 nm; emission 460 nm) for 30 min, at 37 °C on a Fluorescence Microplate Reader Tecan infinite M200 (Tecan, Switzerland). The *K*m of this substrate of papain was previously determined to be 0.4 μM (data not shown). For all assays, saturated substrate concentrations were used in order to obtain linear fluorescence curves. Inhibitors stock solutions were prepared in DMSO, and serial dilutions were made in DMSO. Controls were performed using enzyme alone, substrate alone, enzyme with DMSO and a positive control (*trans*-epoxysuccinyl-L-leucyl-amido(4-guanidino)butane E64, Calbiochem, Germany). The IC_{50} values were determined by non-linear regression analysis based on the log of inhibitors concentrations versus the percentage of activity using GraphPad PRISM software. Assays were performed in triplicate.[23]

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.10.018.

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