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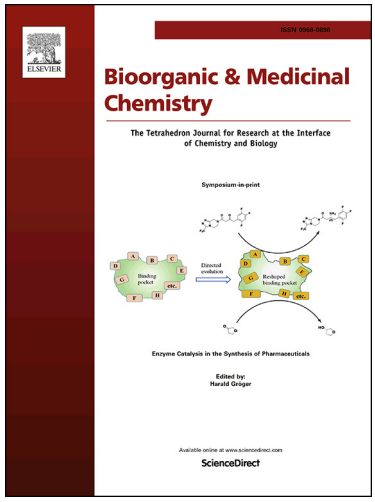

Highly tunable thiosulfonates as a novel class of cysteine protease inhibitors with anti-parasitic activity against *Schistosoma mansoni*

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D.J. Ward, [a] H. Van de Langemheen, [a] E. Koehne, [b] A. Kreidenweiss, [b]* R.M.J. Liskamp. [a]*

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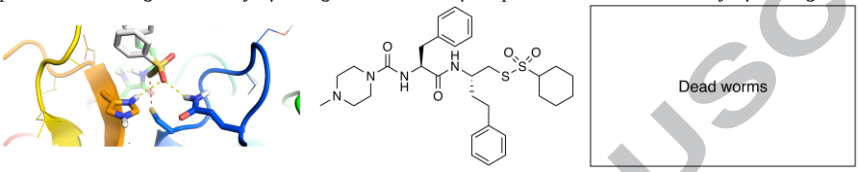

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ABSTRACT

The development of a new class of cysteine protease inhibitors utilising the thiosulfonate moiety as an SH specific electrophile is described. This moiety has been introduced into suitable amino acid derived building blocks, which were incorporated into peptidic sequences leading to very potent *i.e.* sub micromolar inhibitors of the cysteine protease papain in the same range as the vinyl sulfone based inhibitor K11777. Therefore, their inhibitory effect on *Schistosoma mansoni*, a human blood parasite, that expresses several cysteine proteases, was evaluated. The homophenylalanine side chain containing compounds **27** - **30** and especially **36** showed promising activities compared with K11777 and warrant further investigations of these peptidic thiosulfonate inhibitors as new potential anti-parasitic compounds.

1. Introduction

Covalently interacting drugs may form long-lived ties, therefore irreversible inhibitors may provide unique benefits with respect to target occupancy beyond metabolic half-life and kinetic selectivity.[1–5] Often, an active site residue in a crucial enzyme is covalently modified by an electrophilic trap, frequently termed a 'warhead'.[4] We have previously demonstrated that a new warhead can be successfully explored and further developed by drawing inspiration from relatively simple reactive organic compounds such as the sulfonyl fluoride moiety in phenylmethane sulfonyl fluoride (PMSF).[5] This previous work led to the incorporation of the sulfonyl fluoride moiety in (functionalized) peptides leading to powerful peptido sulfonyl fluoride proteasome inhibitors and prolyl oligopeptidase (POP) inhibitors.[6–9] The sulfonyl fluoride warhead in proteasome inhibitors exhibited a unique cross linking mechanism, highlighting the potential of relatively simple reactive moieties.[7,8] This sulfonyl fluoride warhead reacted preferentially with serine and threonine hydroxyl groups. However, from the realm of sulfur compounds and sulfur chemistry we wish to introduce here the *thiosulfonate* moiety as an electrophilic trap, with increased specificity for the highly nucleophilic SH-group in the development of new cysteine protease inhibitors. We anticipate that the thiosulfonate moiety will be a valuable addition to the considerable variety of existing SH selective electrophilic traps. The presently available electrophilic SH trap containing compounds can be roughly divided into three classes: (1) irreversible inhibitors such as the tetrafluorophenoxymethyl ketones,[10] aziridine-2,3-dicarboxylates,[11] epoxyketones, epoxyamides, α-alkoxyketones and diacy-bis-hydrazides;[12] (2) reversible inhibitors such as the nitriles,[13] nitroalkenes,[14] aldehydes[15] and thiosemicarbazones [16] and (3) 1,4-michael acceptors such as fumarates,[17] vinyl α-keto esters, amides and acids [18] and vinyl sulfones.[19]

In this work we have drawn inspiration from the simplest thiosulfonate, methyl methane thiosulfonate (MMTS,), which serves as an SH specific electrophile. MMTS was isolated from cauliflower extracts by Nakamura *et al* in 1996.[20] After its isolation Reddy *et al* went on to show its chemo-preventive effect on colon cancer, demonstrating the link between thiosulfonates in diet and therapeutic potential.[21] MMTS itself is regularly used as a reversible thiolating reagent in studies of the natural thiol-disulfide oxidation state of proteins,[22] for studying protein S-nitrosylation[22,23] and for evaluation of the role of both catalytic and structural cysteine residues on enzyme activities.[24–26] A significant next step was the use of functionalized methane thiosulfonate derivatives by Matsumoto *et al* for modifying mutants of subtilisin.[27]

All of these applications exploited the nature of the thiosulfonate as an excellent thiophile for highly chemoselective targeting of protein thiols. Here, we propose that modification of the primed side residue R' (**Fig**. **1**.) group can be used to control reactivity resulting in highly tunable and specific cysteine

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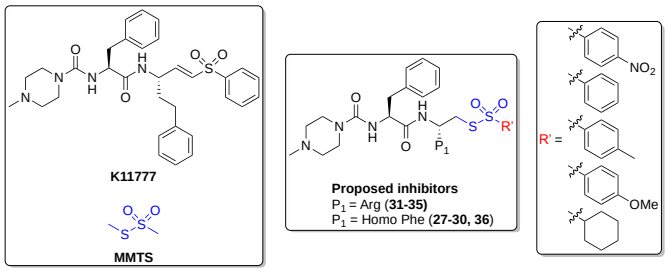


Fig 1. Proposed inhibitor design (centre) based on the backbone of cysteine protease inhibitor K11777 (top left) deriving a novel warhead design inspired by MMTS (bottom left) and controlling the electrophilicity by altering the R' substituent on the hexavalent sulfur (right).

protease inhibitors. In addition, by modifying the substitution of aromatic analogues the electrophilicity of the bivalent sulfur of this novel warhead class can be altered. This reactivity profile will also be further explored by changing the aromatic ring to the aliphatic cyclohexane counterpart for further tuning towards a highly chemo-selective warhead. These novel thiosulfonate warheads will then be incorporated into suitable cysteine protease inhibitors, shown in **Fig**. **1**.

From the currently available inhibitors we identified K11777, a vinyl sulfone based cysteine protease inhibitor, as a starting compound for our study as toxicological, ADME and pharmacodynamics data has been reported in non-human primates.[28] It is also believed that the vinyl sulfone exhibits a high similarity to the thiosulfonate thereby providing an excellent reference compound, since the vinyl sulfone is an excellent Michael acceptor of thiol-groups alike the thiosulfonate moiety. These reactivities can be partly explained by the presence of an oxidized sulfur atom in each warhead, which can provide a H-bond acceptor in the active site of cysteine proteases as is illustrated by modelling studies (**Fig. 2**). Furthermore, by taking the K11777 backbone motif and equipping it with our thiosulfonate warheads we could access a convenient platform for testing of this first inhibitor series to evaluate the effect of altering only the warhead moiety.

This led to very promising inhibitors, which were further evaluated in a bioassay to determine their inhibitory effect on *Schistosoma mansoni*, a human blood parasite that expresses several cysteine proteases.[29]

2. Results and discussion

2.1. Docking study

Initially, modelling was used as a visual cue to assess the potential impact of moving from a vinyl sulfone to a thiosulfonate warhead. Papain was selected as a model cysteine protease having been widely studied. Docking was very useful to visualize the synergistic effect between both the backbone and the warhead design for development of specific inhibitors. Thus, AutoDock Vina[30] was utilised for docking studies of ChemDraw generated inhibitor constructs with 3D coordinates generated through Open Babel software.[31] Generation of ligand constructs and subsequent docking was performed through a Bash script, which facilitated a virtual screen directly from ChemDraw (.cdx) files, thus named *DrawtoDock* [see Supporting Information].

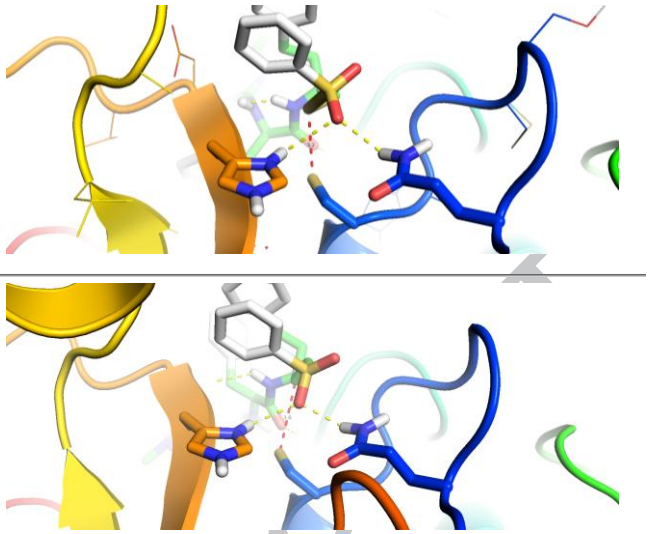


Fig 2. In situ activation of thiosulfonate warhead (top) and vinyl sulfone (bottom) through H bonding to active site residues. H Bonding is highlighted with yellow dash. Distance between the active site nucleophile and site of attack on the warhead (highlighted by red dash) for the thiosulfonates is 3.2Å and for the vinyl sulfone is 3.8Å.

Docking was performed on variants of both the warhead and backbone design to identify any site of potential improvement of the designed inhibitors. Focusing on the warhead design, one of the key findings was that the thiosulfonate warheads may undergo activation in the active site of cysteine proteases through hydrogen bonding to conserved active site residues (**Fig 2**.). Hydrogen bonding of the thiosulfonate warhead oxygen atoms to His-159 may prove particularly important, as this residue forms the catalytic dyad with the active site Cys-25. The combination of this H-bond donor (His-159) and H-bond acceptor character of the thiosulfonate oxygen atoms may in turn polarize the thiosulfonate S-S bond, thereby increasing the rate of cleavage. Importantly, cysteine proteases utilise the catalytic histidine residue as a general acid in their catalytic cycle, facilitating the H-bond donor specifically in cysteine proteases as the His-159 is in its protonated state. This is in contrast to serine proteases which utilise a histidine residue as a general base in their catalytic cycle.[32] As such, these differing catalytic mechanisms are favourable for the inhibitory selectivity of thiosulfonates towards cysteine proteases. In addition, due to the "soft" electrophilic nature, like the disulfide bond, thiosulfonates are thiophiles with an increased selectivity towards thiol based nucleophiles. It is expected that this intrinsic soft electrophilic nature will enhance selectivity towards the cysteine SH of cysteine proteases and possibly also toward, for example, protein kinases having a cysteine-SH in their active site. Therefore, we believe that both of the above characteristics are favourable for development of thiosulfonates as a highly selective warhead class.

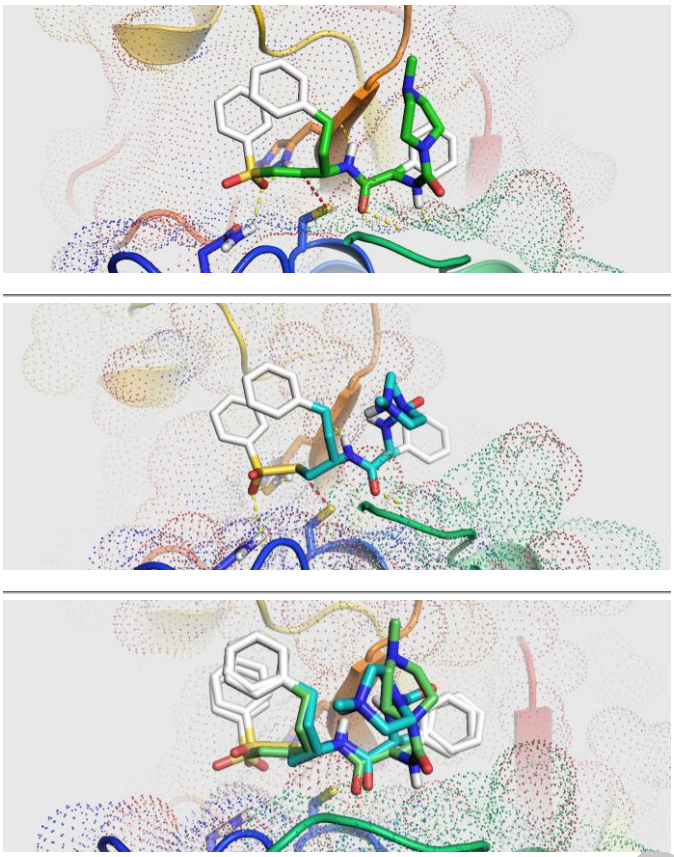


Fig 3. Comparison of binding modes by docking with papain (PDB code 1CVZ). K11777 (top, green carbon backbone) and the thiosulfonate **27** (middle, cyan carbon backbone). Overlayed image (bottom) shows high agreement with the largest discrepancy in the S_3 region, the N-methylpiperazine cap is anticipated to be freely rotatable in a solvated environment.

Furthermore, modelling suggested that the overall thiosulfonate inhibitor binding mode was in agreement with the known binding mode of K11777 in similar cysteine proteases.[33] This was confirmed by also docking K11777 for comparison (**Fig. 3**), for both K11777 and the thiosulfonate inhibitor constructs the aromatic ring of the warhead occupies the S_1' pocket with the homophenylalanine residue occupying the S_1, the phenylalanine residue occupies the S_2 pocket and the N-methyl piperazine cap occupies the S_3 pocket. The N-methyl piperazine cap displays the greatest variability between the thiosulfonate and K11777 models, which was expected as this region is solvent facing. The RMS determined with the Pymol align function was 0.320 Å (overlay of 28 atom pairs specified in the Supporting Information) of the docked thiosulfonate and docked K11777. The largest discrepancy in docking was observed for the N-methyl piperazine cap, which was expected, as this moiety is freely rotatable within the solvated environment. Most notably, the bivalent sulfur was poised in a favourable position to facilitate nucleophilic attack by Cys-25-SH (**Fig. 2**), being 3.2 Å from the active site thiolate compared with the 3.7 Å observed for the vinyl sulfone reference compound K11777 in this modelling study.

Beyond the warhead design, docking studies also showed the likely impact of the backbone on the binding affinity of the potential inhibitors. Modifications of side chain containing residues had the greatest effect with arginine present in the P_1 position (**Fig**) resulting in an increased predicted binding affinity determined by AutoDock Vina. This was apparent from a favourable electrostatic environment created by the backbone

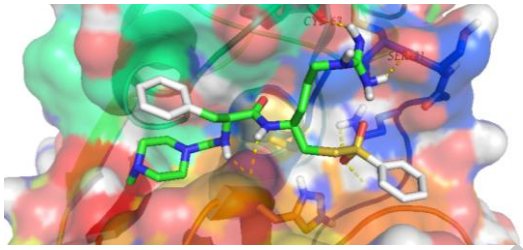
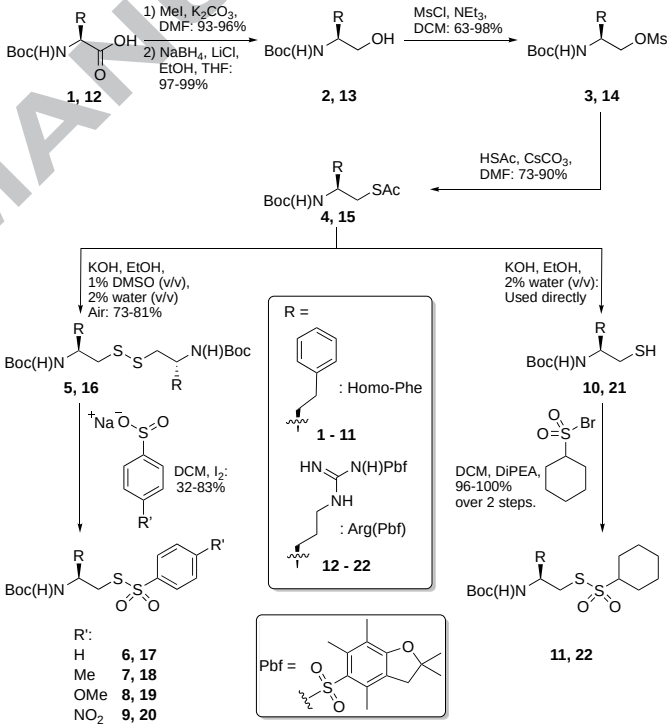


Fig 4. Docking with arginine derived thiosulfonates, H-bonding coordination between arginine side chain and bottom of P_1 binding pocket through the backbone carbonyls of Cys-63 and Ser-21.

carbonyls of Ser-21 and Cys-63, which has been previously proposed by Turk *et al* [34] as being significant. With this information it would be preferable to vary the P_1 position side chain by introduction together with the thiosulfonate warhead. Thus, from these modelling studies it became evident that a flexible warhead synthesis is required, which allows for the introduction of different (functionalised) amino acid residues at the P_1 position and here both Arginine and Homophenylalanine were further investigated.

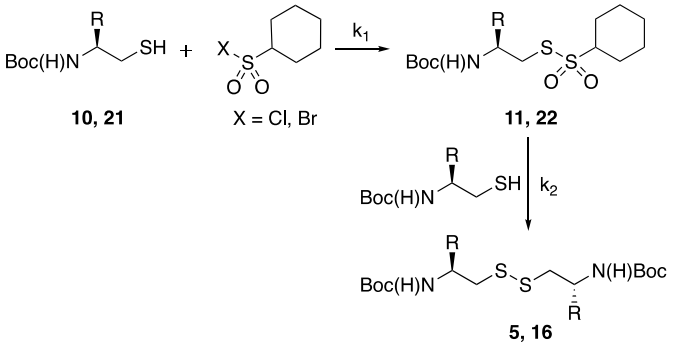
2.2. Thiosulfonate warhead synthesis



Scheme 1. Synthesis of amino acid derived thiosulfonate warheads.

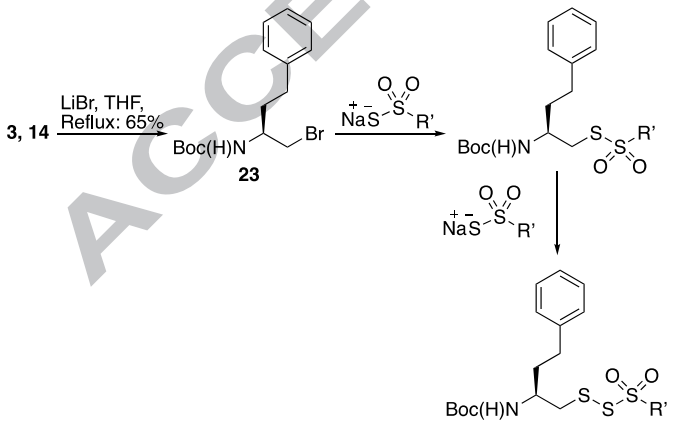
As the warheads were to be incorporated into peptido-mimetic sequences, the first challenge was to devise an efficient synthesis towards amino acid derived thiosulfonates. This was achieved as is outlined in **Scheme 1** and applied to both homophenylalanine and arginine derivatives. Starting from carboxylic acids **1** and **12,** amino alcohols **2** and **13**, respectively, were obtained by esterification followed by reduction. Conversion to mesylates **3** and **14** allowed introduction of a thioacetate moiety leading to **4** and **15.** These thioacetates could then be easily diversified towards either aromatic or aliphatic thiosulfonates. The aromatic thiosulfonates **6-9** and **17-20** were prepared by first hydrolysing the thioacetates to thiols followed by oxidation with DMSO/air to the corresponding disulfides **5** and **16**. Further iodine mediated oxidative cleavage by a sulfinate salt to the thiosulfonates, according to Fujiki *et al*,[35] yielded the aromatic thiosulfonates **6-9** and **17-20** in good to excellent yields. In our hands, the method

of Fujiki *et al* was not suitable for the preparation of aliphatic thiosulfonates. Therefore, In order to obtain the cyclohexane thiol derived thiosulfonates, thioacetates **4** and **15** were hydrolysed under an inert atmosphere to obtain the corresponding thiols, which were then directly reacted with cyclohexane sulfonyl bromide yielding warhead containing amino acid derivatives **11** and **22** in excellent yields. The required sulfonyl bromide was readily accessible from the corresponding thiol by adapting the method of Nishiguchi *et al.* for obtaining sulfonyl chlorides by oxidative chlorination with NCS / HCl in MeCN using NBS/HBr instead.[36] [see Supporting Information]



Scheme 2. Thiosulfonate formation from sulfonyl halides. When X = Cl $k_2 > k_1$ hence no thiosulfonate, only disulfide is isolated. When X = Br, $k_1 >> k_2$ and thiosulfonate is isolated with no detectable disulfide.

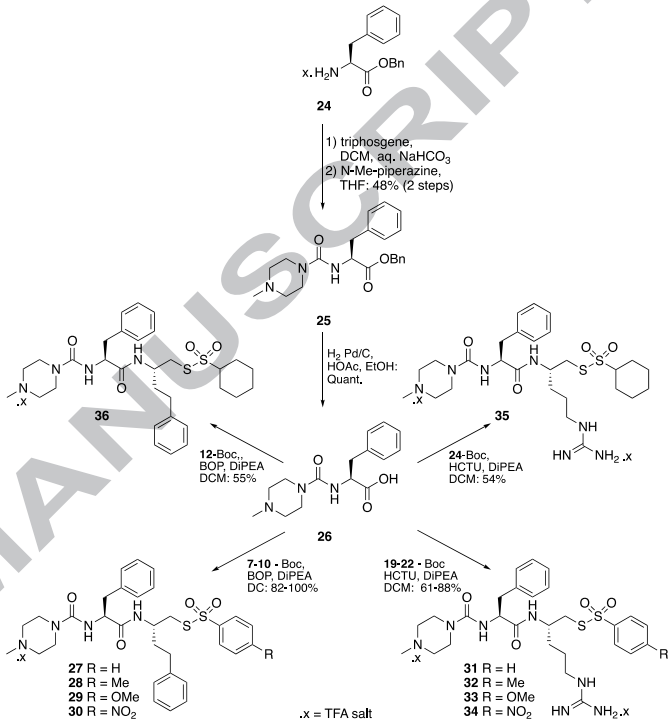
The less reactive corresponding sulfonyl chlorides were explored earlier for the synthesis of aliphatic thiosulfonates. However, in this case only the symmetrical disulfide **5** or **16** was obtained. Hence, it was rationalised that formation of the desired thiosulfonate was competing with its decomposition by the thiol starting material leading to disulfide **5** or **16** (**Scheme 2**.). It was reasoned that the use of the more reactive sulfonyl bromide would lead to a complete consumption of the thiol starting material, before it could decompose the obtained thiosulfonate. Thus, when using a sulfonylbromide: $k_1>>k_2$ and the thiosulfonate is preferentially formed whereas when using a sulfonylchloride: $k_2>k_1$ leading to formation of the disulfide (**Scheme 2**.).



Scheme 3. Thiosulfonate synthesis from alkyl bromide and sodium thiosulfonate salts.

Other early attempts towards synthesis of the thiosulfonate moiety involved generation of the thiosulfonates directly from alkyl bromides via substitution by thiosulfonate salts. This reaction was inefficient and low yielding due to a subsequent nucleophilic attack of the thiosulfonate salt on the obtained thiosulfonate warhead, leading to poor yields due to formation of a product (observed by LC-MS: M+32, having a very similar ^{1}H-NMR as the thiosulfonate) proposed to contain an additional sulfur atom. (**Scheme 3**.).

2.3. Coupling thiosulfonate warheads to backbone



Scheme 4. Generating the backbone and coupling the thiosulfonate warhead fragments under standard peptide coupling conditions.

The core backbone depicted in **Scheme 4**. consisting of phenylalanine with an N-methyl piperazine urea cap at the N terminus, was efficiently constructed by isocyanate formation with triphosgene followed by a nucleophilic reaction with N-methyl piperazine yielding urea **25.** Next, the benzyl ester was removed by hydrogenolysis to yield carboxylic acid **26** in accordance with the literature.[37] Then, the amino acid derived thiosulfonates **6-9, 11, 17-20** and **22** were deprotected with either TFA (arginine derivatives) or HCl (homophenylalanine derivatives) and coupled to the core backbone moiety **26** by standard peptide coupling techniques (**Scheme 4**.). The possibility of using standard peptide coupling reactions was desirable and highlighted the suitability of the thiosulfonate warheads for incorporation into abundantly present peptidomimetic based protease inhibitors. The thiosulfonates were stable to both BOP and HCTU coupling conditions so that in principle large and diverse peptide based libraries containing the thiosulfonate are accessible.

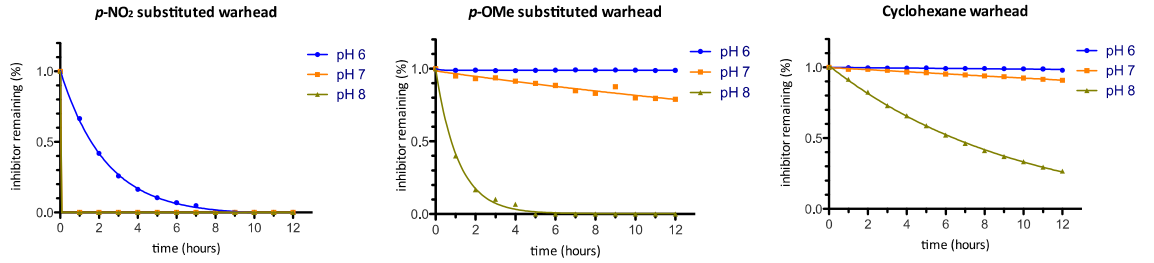
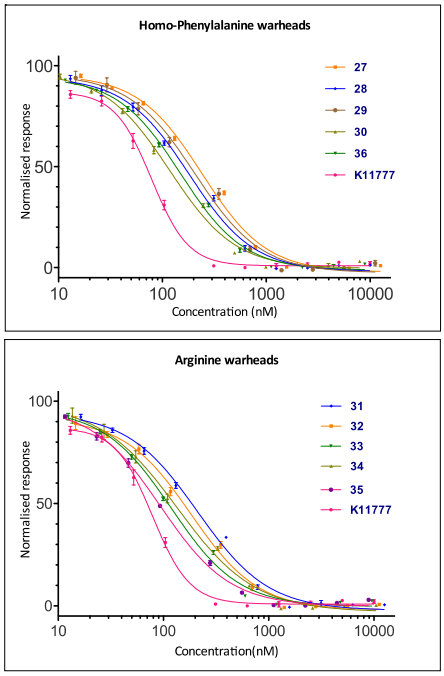


Figure 5. Aqueous stability testing of homophenylalanine derived compounds **30** (left) **29** (centre) and **36** (right). Results were obtained by HPLC quantification in 0.1M phosphate buffer at the described pH and peaks integrated against Ac-Phe-OH as an internal standard.



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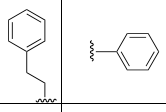
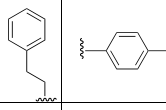
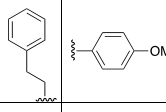
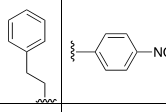
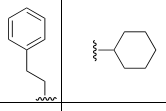
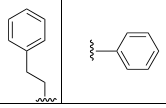
27			190 ± 6
28			182 ± 5
29			148 ± 6
30			186 ± 11
36			168 ± 4
K11777 (vinyl sulfone reference compound)			81 ± 2

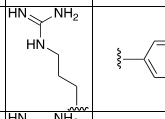
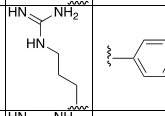
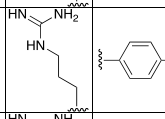
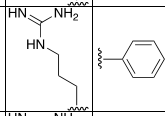
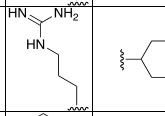
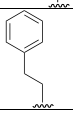
Compound number	R_2	R_1	IC50 (nM)
31			168 ± 3
32			162 ± 18
33			124 ± 1
34			129 ± 2
35			106 ± 2
K11777 (vinyl sulfone reference compound)			81 ± 2

Figure 6. IC_{50} determination of inhibitors against papain. IC_{50} curves (left) are shown for both homophenylalanine (top) and arginine (bottom) derived warheads. Assay pH: pH 6.5 (0.1M Phosphate buffer). Assay time: 1 hr incubation time. Curves have been staggered to prevent overlapping for visualization. Tabulated IC50 values (right) shown. IC50 of K11777 was also determined as a reference compound.

2.4. pH stability testing of thiosulfonate warheads

Obviously, of any newly developed electrophilic trap or warhead, first the aqueous stability profile has to be explored. This was carried out at pH 6,7 and 8 to represent a reasonable physiological pH range. The aqueous stability profiles proved a valuable means for quantifying the effect of substitution of the aromatic ring on the reactivity of the warheads. (**Fig. 5**) Not surprisingly, it was found that electron withdrawing substituents (p-NO_2) greatly increased, and electron donating substituents (p-OMe) decreased the reactivity of the warheads relative to each other. The p-NO_2 substituted warheads were effectively unstable at pH 7 and 8 being degraded completely in less than one hour, with a slightly improved half life of ca. 1 hr 45 min at pH 6. The p-OMe substituted warheads on the other hand were virtually stable at pH 6 and displayed very reasonable half lives of ca. 24 hrs and 1 hr at pH 7 and 8 respectively. Not entirely unexpectedly the aqueous stability was greatly increased when moving to the aliphatic (cyclohexane) based warheads, which were effectively stable at pH 6 and 7 with a greatly increased half life of ca. 6 hrs at pH 8. Having shown the highly tunable nature of the thiosulfonates, it was concluded that the aromatic analogues were more susceptible to pH dependent hydrolysis and that this could be well controlled by the nature of the substituent on the aromatic ring. However by moving towards the aliphatic counterpart the hydrolytic stability of thiosulfonates can be further improved.

2.5. Papain IC_{50} determination

Papain was used as the prototype cysteine protease to evaluate the novel thiosulfonate warhead containing peptide mimics. The well-known cysteine protease inhibitor K11777 (previously known as K777) was used as a reference compound.[38] IC_{50}-values of all inhibitors are tabulated together with the inhibitory curves (**Fig. 6**). Gratifyingly, all tested thiosulfonates inhibited in the submicromolar range. Moreover, the inhibition was of the same order of magnitude as that observed for K11777. Determination of the IC_{50} values also showed that increasingly reactive thiosulfonate warheads (based both on the predicted reactivity and the observed aqueous stability) were less potent. This effect may be due to partial decomposition of the more reactive analogues. It is reasonable to suggest that moving forward to more complex biological systems the effects of stability as such will become less important and the role of other potential nucleophiles, such as circulating thiols, will be more significant. Hence, this information is being used to develop a new, less reactive but more potent inhibitor series. A particularly important finding was that when moving from the aromatic to the aliphatic warheads a significant increase was found in terms of stability (see aqueous stability profiles, **Fig. 5**) with no significant loss in potency (compound **36**, 168 nM) or a slight improvement in potency (compound **35**, 106 nM) when compared with their aromatic counterparts. This suggested that the cyclohexane ring is well tolerated in the S_1' position of

papain. When comparing this with the IC_{50} value of K11777 at 81 nM this was a very encouraging result, highlighting that the thiosulfonate containing inhibitors are able to perform as well as current cysteine protease inhibitors (within the same order of magnitude). With these results in hand we moved forward to testing on schistosomula, the larval stage of *S. mansoni* in humans.

2.6. Inhibitory activity against a human blood fluke

S. mansoni is a trematode blood fluke causing acute and chronic intestinal schistosomiasis (bilharzia) in humans. Schistosomiasis is widely prevalent in Africa, Middle East, and South America affecting more than 200 million people. Large-scale elimination activities are ongoing and praziquantel, the only schistosomiasis medication, is widely deployed by mass drug administration programs. New antischistosomal drug candidates are urgently needed since reduced susceptibility to praziquantel has been ongoing for many decades.[39,40] Research on candidate antischistosomal targets has focused on digestive enzymes in *S. mansoni* since the parasite feeds on the haemoglobin of red blood cells, essential for the parasite's growth, development, and reproduction.[41] Papain like cysteine proteases play a major role in haemoglobin degradation and have become an important drug target.[42–44] Namely, the cathepsin B like cysteine protease SmCB1 for which K11777, the reference compound in this study, showed high efficacy against the *S. mansoni* parasite in the murine model.[45] Furthermore K11777 has been utilised to probe the structural basis for inhibition of SmCB1, providing a foundation for the thiosulfonates derivatives presented.[33] This study evaluated the drug activity of 10 thiosulfonate derivatives against the larval stage of *S. mansoni* in vitro with half of the compounds showing high activity.

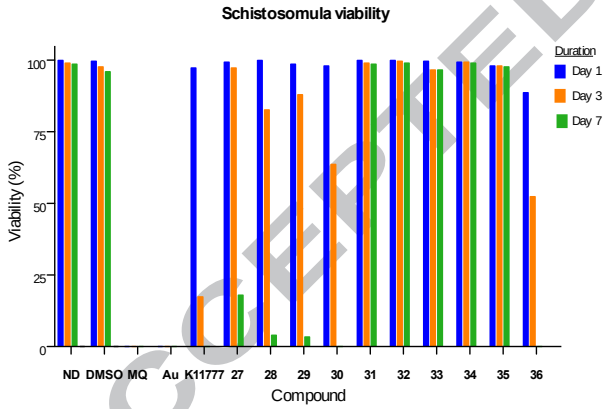


Figure 7. In vitro drug susceptibility screen of schistosomula. Mean % viability of schistosomula after drug exposure for 1 day, 3 days and 7 days. Derivatives (**27** - **36**) and K11777 control were tested at 30 µM, MQ (mefloquine) at 100 µM, and AU (auranofin) at 1 µM. ND (no drug, medium only) and DMSO control for schistosomula viability. Assay was done in triplicate.

Thiosulfonate derivates **27** - **36** and K11777 were tested for inhibition of *S. mansoni* viability. For the *in vitro* drug susceptibility assay, schistosomula - the Schistosoma larval stage that occurs shortly after human skin penetration – were incubated with the compounds at various concentrations and worm viability was determined by morphology and motility assessment. K11777 is a known experimental cysteine protease inhibitor of *S. mansoni* and was included as an active control drug in all assays.[45] Further active assay controls included mefloquine (MQ) and auranofin (AU), respectively.[46] Viability of schistosomula during the culture duration was controlled for 'medium only' and for 1% DMSO (drug solvent), respectively.

Compound	Concentration range [µM] tested	Mean IC50 (SD) in µM	
		3 days	7 days
MQ	200 – 0.27	2.4 (0.6)	0.4 (0.1)
AU	10 – 0.014	0.2 (0.1)	0.1 (0.1)
K11777	100 – 0.14	59.9 (23.5)	7.3 (3.3)
27	300 – 0.14	64.4 (19.3)	21.4 (3.5)
28	300 – 0.14	41.1 (7.6)	26.9 (4.7)
29	300 – 0.14	44.9 (27)	13.6 (1.0)
30	300 – 0.14	29.3 (12.6)	14.7 (1.1)
36	300 – 0.14	25.4 (16.7)	13.5 (1.2)

Table 1. Mean IC_{50} (SD) of compounds assessed in schistosomula *in vitro* drug susceptibility assays. Compounds were tested at the indicated concentration range and IC_{50}-values were determined for 3 days and 7 days of drug exposure. Assays were done 3 times with triplicate measurements. MQ (mefloquine), AU (auranofin).

All derivatives were first screened at 30 µM and schistosomula viability was assessed 1 day, 3 days and 7 days after assay start. Amongst the 10 compounds tested, **27, 28, 29, 30**, and **36** were highly active at 7 days of drug exposure (**Fig. 7**). That compounds **31** - **35** having the arginine side chain in the P_1 position were devoid of any activity in the parasite, may have looked surprising at a first glance, since they were even slightly more active in the papain assay than the homophenylalanine side chain containing compounds **27** - **30** and **36**. However, alike K11777, the hydrophobic homophenylalanine side chain in **27** - **30** and **36** may have allowed for a better penetration of the parasite and subsequent biocidal activity. Active compounds were further tested at a serial, 3-fold diluted concentration range to determine the IC_{50} in schistosomula at 3 days and 7 days drug exposure (**Table 1**). The dose-response (**Fig. 8**) followed an approximate sigmoidal response for all derivatives at 7 days drug exposure. All control compounds (MQ, AU and K11777) were highly active at 3 days and 7 days drug exposure with IC_{50}'s well below 10 µM (Table 1). IC_{50} values of the thiosulfonate derivates **27, 28, 29, 30,** and **36** against larval stage schistosomula ranged between 10 µM and 30 µM and are promising compounds. Compounds **29, 30** and **36** were the most active of the derivatives with IC_{50}'s of about 13.5 µM, while the standard, K11777, had an IC_{50} of 7.3 µM after 7 days showing higher activity against larval *S. mansoni* parasites. Potency again correlated with stability, especially evident with the most stable and potent compounds **29** and **36**, as seen in the earlier papain assay (section 2.5)**.** Observing that the least stable inhibitor **30** displays comparable potency to the most stable **36** may be a result of off target effects by decomposition products, as rapid decomposition of inhibitor **30** is expected in this assay (pH 7.3) based on earlier observed stability data (**Fig. 5**). The increased activity of **36** as compared to **27** -**30**, may reflect the increased stability of the cyclohexane ring containing thiosulfonate warhead (**Fig. 5**). Thus, these new warhead containing compounds are promising starting compounds that deserve further development and investigation as promising potential anti parasitic compounds.

vinyl sulfone based cysteine protease inhibitor K11777 and

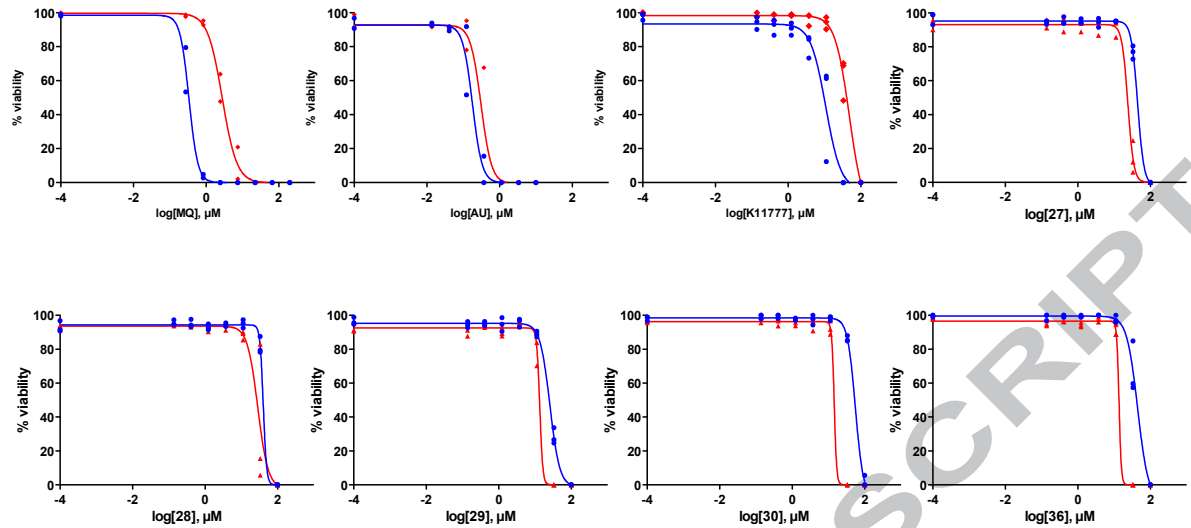


Figure 8. Dose-response curves. Viability (%) is displayed per compound concentration (log10, µM). Drug exposure: blue: 1 day, red: 7days. Dots and triangles represent individual data points per drug concentration (triplicates) from one exemplary assay. Lines represent the modelled curve fit. MQ (mefloquine), AU (auranofin).

3. Conclusions

An efficient synthesis towards amino acid derived thiosulfonates has been developed and applied to molecular construction of peptidomimetic cysteine protease inhibitors. This synthesis allowed for a late stage introduction of the electrophilic trap. It is envisioned that this late stage functionalisation by a wide range of warheads, each with differing reactivity profiles, will prove to be an invaluable feature in future syntheses of warhead containing inhibitors. Mechanistically, the thiosulfonates offer a soft electrophilic centre, which will inherently be more selective for sulfur nucleophiles. In combination with the *in situ* activation, observed in modeling studies through hydrogen bonding to highly conserved catalytic residues, the thiosulfonates may represent a valuable new class of cysteine protease selective electrophilic traps. Furthermore, a synergistic effect upon binding has been observed in modeling studies of both warhead and the backbone. This led to improved inhibitor potency by tuning of the warhead reactivity as well as attempts to optimize binding interactions of the backbone in our test system. The modelling studies predicted potential in situ activation of the novel thiosulfonate warhead and identified improvements to the initial inhibitor binding by varying the side chain utilised, which correlated well with subsequent IC_{50} values. This was combined with aqueous stability profiles, which also correlated with improved IC_{50} values thereby supporting the design of future thiosulfonate warheads containing inhibitors. Modelling successfully predicted the most potent residues, aqueous stability showed the most stable warheads and the correlation between both was reflected in the IC_{50} values leading to the most potent inhibitor **35**. Most notably the large increase in stability of the aliphatic analogues was not detrimental to potency but even met with an improvement. Next, the thiosulfonate derived inhibitors have been found to be effective on *S. mansoni*, a human blood parasite, with **36** displaying the highest potency. Although in this case the predicted most potent inhibitors carrying an arginine residue in the P_1 position (based on modeling and the papain test system) were found to be less active, this is believed to be a permeability issue. Thus, the described inhibitor series can already compete with the known efforts are underway to further down regulate the thiosulfonate warhead reactivity, which should yield more potent and selective warheads. The robust synthesis towards aliphatic thiosulfonates outlined in scheme 1 was the result of several optimizations and should facilitate further diversification of future inhibitors by changing the used sulfonyl bromide. This, together with the possibility of incorporating suitable substituents occupying both P_1, P_2,... and P_1', P_2',... positions is highly promising for obtaining cysteine protease inhibitors with increased specificity.

4. Experimental

4.1. Chemistry

4.1.1. Materials and methods

Reagents: All reagents and starting materials (were applicable) were obtained from either Sigma Aldrich® or Fluorochem Ltd. of the highest available quality, and utilised without further purification.

Solvents: All solvents were obtained from Fisher Scientific. Where necessary (under strict anhydrous conditions) solvents were obtained from a dry source. All deuterated solvents for NMR were obtained from Cambridge Isotope Laboratories, Inc.

Reactions: Air and moisture sensitive reactions were performed under a nitrogen atmosphere. All glassware used was dried under reduced pressure with heating (250°C) from a heat gun. All reactions were conducted in septa sealed vessels with equipped with a nitrogen balloon, unless otherwise stated, and stirred with the use of a magnetic stirrer bead. A glass stopper replaced the rubber septum in strongly acidic reaction conditions, such as Boc removal with TFA.

Thin Layer Chromatography (TLC) All TLC's were conducted on aluminium backed TLC plates coated (0.25 mm), with silica gel 60 F_{254} obtained from Merck. Compounds were then detected by fluorescent quenching at 254 nm, by UV light from a UV Minerallight® lamp. Non-fluorescent quenching substances were visualised by oxidative staining with $KMnO_4$ (2.00 g in 400 mL water), or Ninhydrin (1.5 g in 100 mL n-Butanol, 3 mL AcOH) for staining amines. Stained TLC plates were developed by

heating with a heat gun (250°C), on a hot plate (250°C) or a combination of both as required.

Automated column chromatography: was conducted with the Biotage® Isolera One® automated chromatograph. Products were purified on Biotage® SNAP Ultra cartridges pre packed with Biotage® HP-sphere™ Spherical silica. UV absorption was detected with Biotage® Isolera™ Spektra UV detector at both UV1 (254 nm) and UV2 (280 nm) to identify fractions for collection in combination with TLC analysis.

Preparative HPLC: was conducted with the Agilent Technologies 1260 Infinity Preparative-scale Purification system. Separation was achieved on a Phenomenex Gemini®, 10 µ C18 110˚A AXIA, 250 x 21.2 mm. A linear gradient of 5 → 95% MeCN in Ultra pure water with 0.1% TFA was utilised. All runs were conducted over 80 minutes with a flow rate of 12.5 mL/Min. Fraction collection was based on UV absorption detected at both 214 and 254 nm.

LC-MS: was conducted with a Thermo Scientific, Dionex UltiMate 3000 standard LC system coupled to a Thermo Scientific LCQ Fleet™ Ion trap mass spectrometer. Separations were achieved with a Dr. Maisch GmbH Reprosil Gold 120 C18, 3 µm, 150 x 4 mm column with a flow rate of 1 mL/Min. A linear gradient of 5 → 95% MeCN in Ultra pure water with 0.1% TFA over either 40 min. or 10 min. was utilised and retention times given. UV absorption was detected at 214 nm.

Nuclear Magnetic Resonance (NMR): characterization employed a Bruker® 400 MHz spectrometer measuring ^{1}H, ^{13}C, COSY and HSQC as required. Chemical shifts are given in parts per million (ppm). Shifts were downfield of a TMS reference (δTMS = 0 ppm) and the resonances of the rest protons of the deuterated solvents served as internal standard.[47] $CDCl_3$: 7.26 ppm (^{1}H-NMR), 77.16 ppm (^{13}C-NMR); Methanol-d_4: 3.31 ppm (^{1}H-NMR), 49.0 ppm (^{13}C-NMR). ^{1}H-^{1}H COSY experiments and ^{1}H-^{13}C HSQC experiments were also conducted for correct signal assignment as required.

4.1.2. General procedures

(1) General procedure for amino acid derived methyl esters: the Boc protected amino acid (1.00 equiv.) and K_2CO_3 (3.00 equiv.) were slurried in DMF (3.5 mL/mmol) for 1.5 hrs. under a nitrogen atmosphere. Methyl Iodide (3.00 equiv.) was added dropwise and the solution stirred overnight. TLC (0.1% AcOH in a suitable mixture of EtOAc and Pet-ether 40-60) confirmed reaction completion and the solution was evaporated to dryness. The residue was taken up in EtOAc (10 mL/mmol), washed with water (10 mL/mmol), aqueous back extracted with EtOAc (3 × 10 mL/mmol). The combined organic layers were washed with 5% aq. Na_2SO_3 (3× 20 mL/mmol), brine (50 mL/mmol) and dried over $MgSO_4$. Concentration in vacuo yielded the title compound with no further purification required unless otherwise stated.

(2) General procedure for obtaining amino alcohols by methyl ester reduction: The previously formed methyl ester (1.00 equiv.) and LiCl (2.50 equiv.) were stirred in dry THF (3.00 mL/mmol) and cooled to 0°C for 15 min. under a nitrogen atmosphere. $NaBH_4$ (2.50 equiv.) was added and stirred for 15 min. followed by the addition of EtOH (4 mL/mmol). The solution was stirred for 1 hr. at 0°C before removal of the ice bath. TLC (suitable eluent mixture of EtOAc and pet-ether 40-60) confirmed reaction completion after 3 hrs. Water (1.5 mL/mmol) was added followed by addition of sat. NH_4Cl (2.5 mL/mmol) and EtOAc (10 mL/mmol). The aqueous and organic layers were separated and the aqueous layer was back extracted with EtOAc (3 × 10 mL/mmol). The combined organic layers were washed with brine (50 mL/mmol) and dried over $MgSO_4$. Concentration in vacuo yielded the title compound without further purification unless otherwise stated.

(3) *General procedure for preparation of mesylates*: The previously formed alcohol (1.00 equiv.) was dissolved in DCM (10 mL/mmol) and cooled to 0°C. Triethylamine (5.00 equiv.) was added followed by MsCl (3.50 equiv.) and stirred overnight. Completion of the reaction was confirmed by TLC (suitable concentration of EtOAc, pet-ether) and the solution washed with 1M $KHSO_4$ (2 × 5 mL/mmol), water (2 × 15 mL/mmol), brine (30 mL/mmol) and dried over $MgSO_4$. Concentration in vacuo yielded the title compound with no further purification required unless otherwise stated.

(4*) General procedure for preparation of thioacetates by mesylate substitution*: $CsCO_3$ (1.00 equiv.) was suspended in DMF (10mL/mmol) and thioacetic acid (2.00 equiv.) was added under an N_2 atmosphere before stirring for 15 min. In a separate flask the previously formed mesylate (1.00 equiv.) was dissolved in DMF (2 mL/mmol) and added dropwise to the $CsCO_3$/HSAc solution before DMF (2 mL/mmol) was used to rinse any remaining mesylate into the $CsCO_3$/HSAc solution. The reaction vessel was then covered with aluminium foil and stirred overnight, TLC confirmed completion (suitable mixture of EtOAc and pet-ether 40-60). The reaction mixture was evaporated to dryness and taken up in EtOAc (10 mL/mmol), washed with water (3 × 5 mL/mmol), back extracted with EtOAc (4 × 5 mL/mmol). The combined organic layers were washed with brine (7.5 mL/mmol) and dried over $MgSO_4$. Purification by column chromatography (using a suitable gradient of EtOAc and pet-ether 40-60) yielded the title compound.

(5) *General Procedure for the preparation of a disulfide from a thioacetate*: The previously prepared thioacetate (1.00 equiv.) was dissolved in EtOH (25 mL/mmol) and KOH (3.00 equiv.) was added followed by DMSO (1% by volume) and water (2% by volume). Air was bubbled through the solution for 30 min. before stirring vigorously in an open topped flask overnight. TLC (suitable eluent mixture of EtOAc, pet-ether 40-60) confirmed reaction completion. The thiol formation was always observed as a spot on TLC with a higher R_f value than the disulfide, which tends to have an R_f value close to that of the starting material thioacetate. The solution was then evaporated to dryness, taken up in EtOAc (20 mL/mmol), washed with water (3 × 20 mL/mmol), brine (20 mL/mmol), dried over $MgSO_4$ and concentrated in vacuo to yield the title compound which was used without further purification unless otherwise stated.

(6) *General procedure for preparation of a thiol from a thioacetate*: All solvents used in this procedure were first degassed by bubbling N_2 gas through for 30 min. Under a N_2 atmosphere the previously prepared thioacetate (1.00 equiv.) was dissolved in EtOH (25 mL/mmol) and KOH (3.00 equiv.) was added and stirred overnight. TLC (suitable eluent mixture of EtOAc, pet-ether 40-60) confirmed reaction completion. The solution was then evaporated to dryness on a rotary evaporator fitted with a N_2 balloon to ensure a N_2 atmosphere was maintained upon removal from the rotary evaporator. The resultant residue was taken up in EtOAc (20 mL/mmol), washed with water (3 × 20 mL/mmol), brine (20 mL/mmol), dried over $MgSO_4$ and concentrated in vacuo to yield the title compound which was used without further purification.

(7) *General procedure for thiosulfonate formation from the disulfide*: The previously formed disulfide (1.00 equiv.) was dissolved in DCM (10 mL/mmol) and the respective sodium sulfinate salt (3.20 equiv.) was added followed by iodine

(2.00 equiv.). Completion was confirmed by TLC (relevant concentration of EtOAc, pet-ether) after 1.5 hrs. The reaction mixture was diluted with DCM (10 mL/mmol), washed with 1M Na_2SO_3 (2 × 10 mL/mmol), water (20 mL/mmol), back extract aqueous with DCM (10 mL/mmol), combine organic and wash with brine (10 mL/mmol) and dry over $MgSO_4$. Purification by column chromatography (relevant gradient of EtOAc, pet-ether) yielded the title the title compound.

(8) *Thiosulfonate formation from Sulfonyl bromide*: Cyclohexane sulfonyl bromide which was prepared by modification of the procedure of Nishiguchi et al.[36] (see Supporting Information) (1.2 equiv.) was stirred in DCM (10 mL/mmol) and DiPEA was added (1.2 equiv.). The solution was cooled to 0°C in an ice bath and the relevant thiol dissolved in DCM (10 mL/mmol) was added dropwise. Reaction completion was confirmed by TLC after 30 min., the solution was diluted with 10 mL/mmol DMF and evaporated to dryness before being taken up in EtOAc (10 mL/mmol) and washed with (3 × 10 mL/mmol) 1M $KHSO_4$, Brine (1 × 10 mL/mmol), dried over $MgSO_4$ and concentrated in vacuo to yield the title compound.

(9) *Peptide coupling of the warhead containing derivative to backbone peptides - Method 1*: The free amine hydrochloride salt was prepared from the relevant Boc protected amine by stirring in 2M HCl/dioxane (10 mL/mmol) for 1 hr. and completion confirmed by TLC (15% EtOAc, n-Hexane). The solution was then evaporated to dryness and co-evaporated with toluene (2 × 10 mL/mmol) yielding the unprotected amine as the hydrochloride salt. The amine was then taken up in DCM (10 mL/mmol) and BOP (1.20 equiv.) was added followed by DiPEA (initially 2.00 equiv. with more added as necessary to maintain basic conditions) and reaction progress followed by TLC (10% MeOH in DCM). Coupling was usually complete within 3 hrs. at which point the solution was diluted with EtOAc (100 mL/mmol), washed with water (2 × 50 mL/mmol), brine (2 × 50 mL/mmol), dried over $MgSO_4$ and purified by column chromatography (0 → 10% MeOH, DCM) yielding the title compounds. Purification by preparative HPLC (5 → 95% MeCN, water, 0.1% TFA) yielded the title compounds as fluffy white TFA salts after lyophilisation.

(10) *Peptide coupling of the warhead containing derivative to backbone peptides - Method 2*: The free amine TFA-salt was prepared from the relevant Boc-Arg(Pbf)-warhead by stirring in TFA (10 mL/mmol) with 2% water and 1% TIPS for 2 hr. and completion confirmed by LCMS to ensure both Boc and Pbf removal. The solution was then evaporated to dryness and co-evaporated with toluene (2 × 10 mL/mmol) yielding the unprotected amine as the bis-TFA salt. The amine was then taken up in DCM (10 mL/mmol) and HCTU (1.20 equiv.) was added followed by DiPEA (initially 2.00 equiv. with more added as necessary to maintain basic conditions) and reaction progress followed by TLC (10% MeOH, DCM). Coupling was usually complete within 3 hrs. at which point the solution was diluted with DMF (10mL/mmol) and evaporated to dryness. The residue was taken up in 0.1% TFA in water and freeze dried before purification by reverse phase column chromatography (5 → 40% MeCN, water, 0.1% TFA) yielding the title compounds. Further purification by preparative HPLC yielded compounds as fluffy white TFA salts after lyophilisation.

4.1.3. Compound synthesis

Boc-HomoPhe-Ψ[CH_2OH] (**2**): step 1, methyl ester was prepared according to general procedure 1 on a 1 mmol (279 mg) scale. Yield: 280 mg, 0.955 mmol, 96%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.24 – 7.09 (m, 5H), 5.00 (d, J = 8.4 Hz, 1H), 4.29 (dd, J = 12.5, 7.5 Hz, 1H), 3.64 (s, 3H), 2.65 – 2.55 (m, 2H), 2.14 – 2.03 (m, 1H), 1.93 – 1.82 (m, 1H), 1.38 (s, 9H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 173.16, 155.35, 140.77, 128.48, 128.40, 126.15, 79.94, 53.25, 52.27, 34.40, 31.64, 28.33. LC-MS (Linear gradient 5 → 95% MeCN, 0.1% TFA, 10 min): Rt (min): 6.91 (ESI-MS (m/z): 293.66 (M^+)).

Step 2, reduction to the alcohol was achieved according to general procedure 2 on a 0.933 mmol (272 mg) scale. Yield: 239 mg, 0.901 mmol, 97%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.23 – 7.07 (m, 5H), 4.73 (d, J = 8.0 Hz, 1H), 3.57 (d, J = 10.2 Hz, 2H), 3.48 (br s, 1H), 2.75 (s, 1H), 2.68 – 2.53 (m, 2H), 1.82 – 1.72 (m, 1H), 1.72 – 1.60 (m, 1H), 1.38 (s, 9H).^{13}C NMR (101 MHz, $CDCl_3$) δ 156.47, 141.55, 128.45, 128.36, 125.98, 79.65, 65.71, 52.49, 33.32, 32.40, 28.42. LC-MS (Linear gradient 5 → 95% MeCN, 0.1% TFA, 10 min): Rt (min): 6.21 (ESI-MS (m/z): 265.75 (M^+)). HRMS: calcd. for $C_{15}H_{23}NO_3Na$, 288.1570 [M+Na^+]; found 288.1559.

Boc-HomoPhe-Ψ[CH_2OMs] (**3**): Prepared according to general procedure 3 on a 1 mmol (263 mg) scale. Yield: 335 mg, 0.976 mmol, 98%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.35 – 7.18 (m, 5H), 4.66 (d, J = 8.7 Hz, 1H), 4.29 (dd, J = 9.9, 4.0 Hz, 1H), 4.23 (dd, J = 10.1, 4.1 Hz, 1H), 3.90 (br s, 1H), 3.03 (s, 3H), 2.83 – 2.64 (m, 2H), 1.99 – 1.81 (m, 2H), 1.48 (s, 9H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 155.30, 140.78, 128.57, 128.37, 126.22, 71.14, 49.42, 37.56, 32.98, 32.09, 28.34. LC-MS (Linear gradient 5 → 95% MeCN, 0.1% TFA, 10 min): Rt (min): 6.59 (ESI-MS (m/z): 366.08 (M+Na^+)).). HRMS: calcd. for $C_{16}H_{25}NO_5Na$, 366.1346 [M+Na^+]; found 366.1343.

Boc-HomoPhe-Ψ[CH_2SAc] (**4**): Prepared according to general procedure 4 on a 23.5 mmol (8.07 g) scale. Purified by column chromatography 0 → 10% EtOAc, Hexane. Yield: 5.55 g, 17.2 mmol, 73%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.24 – 7.08 (m, 5H), 4.46 (d, J = 9.1 Hz, 1H), 3.73 (br s, 1H), 3.05 (dd, J = 13.9, 4.8 Hz, 1H), 2.97 (dd, J = 13.9, 7.0 Hz, 1H), 2.69 – 2.53 (m, 2H), 2.28 (s, 3H), 1.81 – 1.70 (m, 1H), 1.70 – 1.61 (m, 1H), 1.38 (s, 9H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 155.58, 141.52, 128.58, 128.49, 126.11, 79.54, 50.45, 36.33, 34.06, 32.49, 30.72, 28.51. LC-MS (Linear gradient 5 → 95% MeCN, 0.1% TFA, 40 min): Rt (min): 22.14 (ESI-MS (m/z): 323.67 (M^+)). HRMS: calcd. for $C_{17}H_{25}NO_3SNa$, 346.1447 [M+Na^+]; found 346.1437.

Boc-HomoPhe-Disulfide (**5**): Prepared according to general procedure 5 on a 2.67 mmol (864 mg) scale. Yield: 605 mg, 1.08 mmol, 81%. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.23 – 7.08 (m, 10H), 4.90 (br s, 2H), 3.81 (br s, 2H), 2.92 (d, J = 11.9 Hz, 2H), 2.72 (dd, J = 14.2, 6.3 Hz, 2H), 2.65 (dd, J = 9.8, 5.0 Hz, 2H), 2.57 (ddd, J = 13.8, 9.8, 6.6 Hz, 2H), 1.86 (br s, 2H), 1.70 (br s, 2H), 1.39 (s, 18H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 155.50, 141.46, 128.44, 125.98, 49.96, 45.05, 35.10, 32.44, 28.49. LC-MS (Linear gradient 5 → 95% MeCN, 0.1% TFA, 10 min): Rt (min): 8.36 (ESI-MS (m/z): 860.89 (M^+), 883.20 (M+Na^+)). HRMS: calcd. for $C_{30}H_{44}N_2O_4S_2Na$, 583.2635 [M+Na^+]; found 583.2610.

Boc-HomoPhe-Ψ[CH_2SH] (**10**): Prepared according to general procedure 6 on a 1.20 mmol (684 mg) scale. Yield: Quant. Due to air sensitivity the thiol was not characterized and used directly in following reaction.

Boc-HomoPhe-Ψ[CH_2SSO_2-Ph] (**6**): Prepared according to general procedure 7 on a 0.196 mmol (110 mg) scale. Purified by automated column chromatography 5 → 25% EtOAc, pet-ether 40-60. Yield: 90 mg, 0.213 mmol, 54%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.90 (dd, J = 8.3, 1.4 Hz, 2H), 7.60 (tt, J = 7.3, 1.3 Hz, 1H), 7.52 (tt, J = 7.5, 1.5 Hz, 2H), 7.29 – 7.23 (m, 2H), 7.18 (tt, J = 7.4, 1.3 Hz, 1H), 7.11 (dd, J = 8.3, 1.5 Hz, 2H), 4.67 (d, J = 8.7 Hz, 1H), 3.86 – 3.74 (m, 1H), 3.22 (dd, J = 13.4, 4.9 Hz, 1H),

3.15 (dd, J = 13.1, 6.3 Hz, 1H), 2.70 – 2.60 (m, 1H), 2.56 (ddd, J = 13.9, 9.2, 7.0 Hz, 1H), 1.83 – 1.67 (m, 2H), 1.43 (s, 9H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 155.24, 144.64, 140.91, 133.83, 129.39, 128.56, 128.37, 127.01, 126.18, 79.79, 49.42, 40.91, 35.62, 32.26, 28.41. LC-MS (Linear gradient 5 → 95% MeCN, 0.1% TFA, 40 min): Rt (min): 23.52 (ESI-MS (m/z): 421.57 (M^+), 444.07($M+Na^+$)). HRMS: calcd. for $C_{21}H_{27}NO_4S_2Na$, 444.1274 [$M+Na^+$]; found 444.1260.

Boc-HomoPhe-Ψ[CH_2SSO_2-(p-Me)Ph] (**7**): Prepared according to general procedure 7 on a 0.212 mmol (119 mg) scale. Purified by automated column chromatography 5 → 25% EtOAc, pet-ether 40-60. Yield: 157 mg, 0.266 mmol, 63%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.78 (d, J = 8.4 Hz, 2H), 7.32 – 7.28 (m, 2H), 7.28 – 7.23 (m, 2H), 7.21 – 7.14 (m, 1H), 7.13 – 7.09 (m, 2H), 4.71 (d, J = 8.7 Hz, 1H), 3.87 – 3.76 (m, 1H), 3.20 (dd, J = 13.1, 4.2 Hz, 1H), 3.14 (dd, J = 13.2, 6.2 Hz, 1H), 2.70 – 2.60 (m, 1H), 2.56 (ddd, J = 13.9, 9.2, 7.0 Hz, 1H), 2.41 (s, 3H), 1.81 – 1.68 (m, 2H), 1.43 (s, 9H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 155.25, 144.92, 141.80, 140.95, 129.94, 128.51, 128.35, 127.08, 126.13, 79.70, 49.41, 40.79, 35.59, 32.25, 28.39, 21.69. LC-MS (Linear gradient 5 → 95% MeCN, 0.1% TFA, 10 min): Rt (min): 7.56 (ESI-MS (m/z): 435.65 (M^+), 458.07($M+Na^+$)). HRMS: calcd. for $C_{22}H_{29}NO_4S_2Na$, 458.1430 [$M+Na^+$]; found 458.1417.

Boc-HomoPhe-Ψ[SSO_2-(p-OMe)Ph] (**8**): Prepared according to general procedure 7 on a 0.212 mmol (119 mg) scale. Purified by automated column chromatography 5 → 25% EtOAc, pet-ether. Yield: 158 mg, 0.350 mmol, 83%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.88 – 7.80 (m, 2H), 7.31 – 7.24 (m, 2H), 7.22 – 7.15 (m, 1H), 7.15 – 7.10 (m, 2H), 7.01 – 6.93 (m, 2H), 4.62 (d, J = 8.7 Hz, 1H), 3.87 (s, 3H), 3.86 – 3.77 (m, 1H), 3.20 (dd, J = 13.6, 4.2 Hz, 1H), 3.15 (dd, J = 13.3, 6.0 Hz, 1H), 2.72 – 2.63 (m, 1H), 2.58 (ddd, J = 13.9, 9.1, 7.1 Hz, 1H), 1.86 – 1.70 (m, 2H), 1.44 (s, 9H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 163.75, 155.25, 140.96, 136.28, 129.43, 128.52, 128.35, 126.13, 114.43, 79.69, 55.80, 49.40, 40.75, 35.61, 32.26, 28.39. LC-MS (Linear gradient 5 → 95% MeCN, 0.1% TFA, 10 min): Rt (min): 7.39 (ESI-MS (m/z): 451.62 (M^+), 474.06 ($M+Na^+$)). HRMS: calcd. for $C_{22}H_{29}NO_5S_2Na$, 474.1379 [$M+Na^+$]; found 474.1366.

Boc-HomoPhe-Ψ[CH_2SSO_2-(p-NO_2)Ph] (**9**): Prepared according to general procedure 7 on a 1 mmol (560 mg) scale. Purified by automated column chromatography 5 → 25% EtOAc, pet-ether. Yield: 520 mg, 1.11mmol, 56%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.34 (d, J = 8.9 Hz, 2H), 8.08 (d, J = 8.9 Hz, 2H), 7.31 – 7.23 (m, 2H), 7.22 – 7.16 (m, 1H), 7.14 – 7.08 (m, 2H), 4.52 (d, J = 8.4 Hz, 1H), 3.82 – 3.69 (m, 1H), 3.27 (dd, J = 13.6, 5.4 Hz, 1H), 3.21 (dd, J = 13.9, 6.2 Hz, 1H), 2.68 (ddd, J = 14.6, 9.1, 5.9 Hz, 1H), 2.59 (ddd, J = 13.9, 9.0, 7.1 Hz, 1H), 1.86 – 1.76 (m, 1H), 1.76 – 1.66 (m, 1H), 1.43 (s, 9H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 155.18, 150.46, 149.72, 140.59, 128.62, 128.35, 128.27, 126.34, 124.72, 80.04, 60.47, 49.38, 41.52, 35.54, 32.18, 28.39, 21.12, 14.28. LC-MS (Linear gradient 5 → 95% MeCN, 0.1% TFA, 10 min): Rt (min): 7.32 (ESI-MS (m/z): 466.31 (M^+)). HRMS: calcd. for $C_{21}H_{26}N_2O_6S_2Na$, 489.1124 [$M+Na^+$]; found 489.1119.

Boc-HomoPhe-Ψ[CH_2SSO_2-cHex] (**11**): Prepared according to general procedure 8 on a 0.500 mmol (141 mg) scale. Purified by column chromatography 3 → 30% EtOAc, pet-ether 40-60. Yield: 214 mg, Quant. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.31 – 7.25 (m, 2H), 7.22 – 7.15 (m, 3H), 4.68 (d, *J* = 8.8 Hz, 1H), 3.90 – 3.82 (m, 1H), 3.39 (dd, *J* = 13.4, 4.1 Hz, 1H), 3.18 (dd, *J* = 13.8, 7.0 Hz, 1H), 3.17 – 3.11 (m, 1H), 2.77 – 2.61 (m, 2H), 2.31 – 2.22 (m, 2H), 1.95 – 1.87 (m, 2H), 1.87 – 1.76 (m, 2H), 1.75 – 1.67 (m, 1H), 1.63 – 1.49 (m, 2H), 1.46 (s, 9H), 1.37 – 1.26 (m, 2H), 1.26 – 1.15 (m, 1H).^{13}C NMR (101 MHz, $CDCl_3$) δ 155.36, 140.96, 128.53, 128.38, 126.14, 71.37, 50.07, 41.17, 35.88, 32.26, 28.38, 26.36, 26.21, 25.17, 25.11, 25.04. LC-MS (Linear gradient 5 → 95% MeCN, 0.1% TFA, 10 min): Rt (min): 7.39 (ESI-MS (m/z): 427.75 (M^+)). HRMS: calcd. for $C_{21}H_{33}NO_4S_2Na$, 450.1743 [$M+Na^+$]; found 450.1746.

Boc-Arg(Pbf)-Ψ[CH_2OH] (**13**): Step 1, methyl ester was synthesized according to general procedure 1 on a 10 mmol (5.27 g) scale. Purified by automated column chromatography 20 → 100% EtOAc, pet-ether 40-60. Yield: 5.02 g, 9.29 mmol, 93%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.02 (s, 1H), 6.18 – 6.04 (m, 2H), 5.26 (d, J = 8.3 Hz, 1H), 4.35 – 4.19 (m, 1H), 3.73 (s, 3H), 3.35 – 3.25 (m, 1H), 3.23 – 3.13 (m, 1H), 2.96 (s, 2H), 2.58 (s, 3H), 2.52 (s, 3H), 2.09 (s, 3H), 1.87 – 1.75 (m, 1H), 1.70 – 1.55 (m, 3H), 1.46 (s, 6H), 1.42 (s, 9H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 173.07, 158.83, 156.29, 156.08, 138.47, 133.05, 132.39, 124.70, 117.59, 86.49, 80.46, 52.81, 52.60, 43.37, 40.85, 30.71, 28.74, 28.45, 25.30, 19.40, 18.03, 12.61. LC-MS (Linear gradient 5 → 95% MeCN, 0.1% TFA, 10 min): Rt (min): 6.70 (ESI-MS (m/z): 541.12 ($M+H^+$)). HRMS: calcd. for $C_{25}H_{40}N_4O_7SNa$, 563.2510 [$M+Na^+$]; found 563.2501.

Step 2, reduction to alcohol was achieved according to general procedure 2 on a 9.29 mmol (5.02 g) scale. Yield: 4.72 g, 9.21 mmol, 99%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.39 – 6.12 (m, 3H), 5.16 (d, J = 7.9 Hz, 1H), 3.64 – 3.51 (m, 3H), 3.31 – 3.15 (m, 3H), 2.95 (s, 2H), 2.56 (s, 3H), 2.50 (s, 3H), 2.09 (s, 3H), 1.64 – 1.51 (m, 4H), 1.46 (s, 6H), 1.41 (s, 9H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 158.92, 156.80, 156.47, 138.46, 132.88, 132.37, 124.77, 117.67, 86.56, 79.74, 65.12, 43.37, 41.20, 29.03, 28.74, 28.55, 25.75, 19.43, 18.09, 12.62. LC-MS (Linear gradient 5 → 95% MeCN, 0.1% TFA, 10 min): Rt (min): 6.15 (ESI-MS (m/z): 513.08 ($M+H^+$)). HRMS: calcd. for $C_{24}H_{40}N_4O_6SNa$, 535.2561 [$M+Na^+$]; found 535.2543.

Boc-Arg(Pbf)-Ψ[CH_2OMs] (**14**): Prepared according to general procedure 3 on a 9.21 mmol (4.72 g) scale. Purified by automated column chromatography 20 → 100% EtOAc, pet-ether 40-60. Yield: 3.42 g, 5.79 mmol, 63%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.20 – 5.98 (m, 3H), 5.03 (d, J = 9.0 Hz, 1H), 4.23 (dd, J = 10.2, 4.2 Hz, 1H), 4.16 (dd, J = 10.2, 4.4 Hz, 1H), 3.90 – 3.79 (m, 1H), 3.32 – 3.15 (m, 2H), 3.04 (s, 3H), 2.96 (s, 2H), 2.57 (s, 3H), 2.51 (s, 3H), 2.10 (s, 3H), 1.70 – 1.53 (m, 4H), 1.46 (s, 6H), 1.42 (s, 9H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 159.07, 156.19, 155.96, 138.53, 132.48, 124.90, 117.74, 86.65, 79.97, 71.52, 49.69, 43.29, 40.95, 37.39, 28.71, 28.67, 28.47, 25.50, 19.42, 18.06, 14.30, 12.59. LC-MS (Linear gradient 5 → 95% MeCN, 0.1% TFA, 10 min): Rt (min): 6.40 (ESI-MS (m/z): 591.08 ($M+H^+$)). HRMS: calcd. for $C_{25}H_{42}N_4O_8S_2Na$, 613.2336 [$M+Na^+$]; found 613.2312.

Boc-Arg(Pbf)-Ψ[CH_2SAc] (**15**): Prepared according to general procedure 5 on a 5.79 mmol (3.42 g) scale. Purified by automated column chromatography 20 → 100% EtOAc, pet-ether 40-60. Yield: 2.98 g, 5.23 mmol, 90%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.19 – 6.02 (m, 3H), 4.71 (d, J = 9.2 Hz, 1H), 3.78 – 3.64 (m, 1H), 3.32 – 3.20 (m, 1H), 3.20 – 3.09 (m, 1H), 2.95 (s, 2H), 3.03 – 2.86 (m, 2H), 2.58 (s, 3H), 2.52 (s, 3H), 2.35 (s, 3H), 2.09 (s, 3H), 1.63 – 1.53 (m, 2H), 1.53 – 1.46 (m, 2H), 1.46 (s, 6H), 1.41 (s, 9H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 158.81, 156.52, 156.21, 138.50, 132.44, 124.69, 117.58, 86.47, 80.04, 50.19, 43.39, 41.12, 34.15, 32.44, 30.72, 28.75, 28.52, 25.60, 19.41, 18.04, 12.63. LC-MS (Linear gradient 5 → 95% MeCN, 0.1% TFA, 10 min): Rt (min): 6.74 (ESI-MS (m/z): 571.08 ($M+H^+$)). HRMS: calcd. for $C_{26}H_{42}N_4O_6S_2Na$, 593.2438 [$M+Na^+$]; found 593.2408.

Boc-Arg(Pbf)-Disulfide (**16**): Prepared according to general procedure 5 on a 4.21 mmol (2.40 g) scale. Purified by automated column chromatography 20 → 100% EtOAc, pet-ether 40-60. Yield: 1.62 g, 1.54 mmol, 73%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.47 – 6.18 (m, 6H), 3.91 – 3.77 (m, 2H), 3.40 – 3.13 (m, 4H), 2.96 (s, 4H), 3.04 – 2.81 (m, 4H), 2.57 (s, 6H), 2.51 (s, 6H), 2.10 (s, 6H), 1.76 – 1.50 (m, 8H), 1.46 (s, 12H), 1.41 (s, 18H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 158.90, 156.76, 156.69, 138.45, 132.98, 132.42, 124.74, 117.65, 86.52, 79.65, 50.68, 43.42, 41.01, 31.53, 28.76, 28.64, 26.04, 19.49, 18.11, 12.65. LC-MS (Linear gradient 5 → 95% MeCN, 0.1% TFA, 10 min): Rt (min): 7.62 (ESI-MS (m/z): 1055.25 ($M+H^+$)). HRMS: calcd. for $C_{48}H_{78}N_8O_{10}S_4Na$, 1077.4616 [$M+Na^+$]; found 1077.4598.

Boc-Arg(Pbf)-Ψ[CH_2SH] (**21**): Prepared according to general procedure 7 on a 3.285mmol scale. Yield: 94%. Thiol was used directly in following reaction due to air sensitivity.

Boc-Arg(Pbf)-Ψ[CH_2SSO_2-Ph] (**17**): Prepared according to general procedure 7 0.331 mmol (349 mg) scale. Purified by automated column chromatography 20 → 80% EtOAc, pet-ether 40-60. Yield: 142 mg, 0.212 mmol, 32%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.94 – 7.89 (m, 2H), 7.65 – 7.59 (m, 1H), 7.58 – 7.51 (m, 2H), 6.30 – 6.02 (m, 3H), 5.01 (d, J = 8.8 Hz, 1H), 3.86 – 3.66 (m, 1H), 3.23 – 3.10 (m, 3H), 3.06 (dd, J = 13.7, 6.8 Hz, 1H), 2.95 (s, 2H), 2.56 (s, 3H), 2.50 (s, 3H), 2.09 (s, 3H), 1.63 – 1.50 (m, 0H), 1.45 (s, 6H), 1.50 – 1.37 (m, 0H), 1.40 (s, 9H).^{13}C NMR (101 MHz, $CDCl_3$) δ 158.79, 156.16, 155.81, 144.37, 138.33, 133.90, 132.80, 132.28, 129.42, 127.02, 124.67, 117.52, 86.43, 79.92, 49.37, 43.23, 40.92, 40.82, 31.27, 28.61, 28.36, 25.60, 19.33, 17.97, 12.50. LC-MS (Linear gradient 5 → 95% MeCN, 0.1% TFA, 10 min): Rt (min): 7.18 (ESI-MS (m/z): 669.02 ($M+H^+$)). HRMS: calcd. for $C_{30}H_{44}N_4O_7S_3Na$, 691.2246 [$M+Na^+$]; found 691.2235.

Boc-Arg(Pbf)-Ψ[CH_2SSO_2-(*p*-Me)Ph] (**18**): Prepared according to general procedure 7 on a 0.383 mmol (404 mg) scale. Purified by automated column chromatography 20 → 80% EtOAc, pet-ether 40-60. Yield: 423 mg, 0.423 mmol, 55%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.79 (d, J = 8.4 Hz, 2H), 7.33 (d, J = 8.1 Hz, 2H), 6.20 – 6.01 (m, 3H), 4.94 (d, J = 9.0 Hz, 1H), 3.83 – 3.72 (m, 1H), 3.25 – 3.08 (m, 3H), 3.04 (dd, J = 13.7, 6.8 Hz, 1H), 2.95 (s, 2H), 2.57 (s, 3H), 2.51 (s, 3H), 2.44 (s, 3H), 2.09 (s, 3H), 1.63 – 1.47 (m, 4H), 1.45 (s, 6H), 1.40 (s, 9H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 158.92, 156.21, 145.25, 141.64, 138.51, 132.47, 130.14, 127.25, 124.78, 117.63, 86.54, 80.16, 49.33, 43.38, 40.98, 40.85, 31.61, 31.60, 28.75, 28.50, 25.65, 21.81, 19.44, 18.08, 12.63. LC-MS (Linear gradient 5 → 95% MeCN, 0.1% TFA, 10 min): Rt (min): 7.33 (ESI-MS (m/z): 683.01 ($M+H^+$)). HRMS: calcd. for $C_{31}H_{46}N_4O_7S_3Na$, 705.2421 [$M+Na^+$]; found 705.2402.

Boc-Arg(Pbf)-Ψ[CH_2SSO_2-(*p*-OMe)Ph] (**19**): Prepared according to general procedure 7 on a 0.319 mmol (336 mg) scale. Purified by automated column chromatography 20 → 80% EtOAc, pet-ether 40-60. Yield: 142 mg, 0.212 mmol, 33%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.85 (d, J = 9.0 Hz, 2H), 7.00 (d, J = 9.0 Hz, 2H), 6.16 – 5.97 (m, 3H), 4.92 (d, J = 9.0 Hz, 1H), 3.88 (s, 3H), 3.85 – 3.73 (m, 1H), 3.29 – 3.08 (m, 3H), 3.04 (dd, J = 13.8, 6.7 Hz, 1H), 2.95 (s, 2H), 2.57 (s, 3H), 2.51 (s, 3H), 2.09 (s, 3H), 1.64 – 1.48 (m, 4H), 1.45 (s, 6H), 1.42 (s, 9H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 163.87, 158.77, 156.02, 138.38, 132.35, 129.49, 124.63, 117.49, 114.50, 86.40, 80.05, 55.81, 43.24, 40.86, 40.64, 31.52, 28.61, 28.36, 25.47, 19.29, 17.93, 12.48. LC-MS (Linear gradient 5 → 95% MeCN, 0.1% TFA, 10 min): Rt (min): 7.18 (ESI-MS (m/z): 699.01 ($M+H^+$)). HRMS: calcd. for $C_{31}H_{46}N_4O_8S_3Na$, 721.2370 [$M+Na^+$]; found 721.2341.

Boc-Arg(Pbf)-Ψ[CH_2SSO_2-(*p*-NO_2)Ph] (**20**): Prepared according to general procedure 7 0.337 mmol (355 mg) scale. Purified by automated column chromatography 20 → 80% EtOAc, pet-ether 40-60. Yield: 172 mg, 0.241 mmol, 36%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.34 (d, J = 8.9 Hz, 2H), 8.11 (d, J = 8.5 Hz, 2H), 6.32 – 6.04 (m, 3H), 5.10 (d, J = 8.9 Hz, 1H), 3.83 – 3.71 (m, 1H), 3.24 – 3.16 (m, 3H), 3.12 (dd, J = 13.6, 7.1 Hz, 1H), 2.96 (s, 2H), 2.55 (s, 3H), 2.49 (s, 3H), 2.09 (s, 3H), 1.63 – 1.48 (m, 4H), 1.46 (s, 6H), 1.38 (s, 9H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 158.96, 156.15, 155.77, 150.46, 149.41, 138.32, 132.44, 132.28, 128.34, 124.82, 124.71, 117.65, 86.57, 80.02, 49.51, 43.19, 41.28, 40.80, 31.21, 28.60, 28.34, 25.69, 19.32, 17.97, 12.49. LC-MS (Linear gradient 5 → 95% MeCN, 0.1% TFA, 10 min): Rt (min): 7.21 (ESI-MS (m/z): 713.99 (M^+)). HRMS: calcd. for $C_{30}H_{43}N_5O_9S_3Na$, 736.2115 [$M+Na^+$]; found 736.2095.

Boc-Arg(Pbf)-Ψ[CH_2SSO_2-*c*Hex] (**22**): Prepared according to general procedure 8 0.500 mmol (264 mg) scale. Purified by automated column chromatography 20 → 100% EtOAc, pet-ether 40-60. Yield: 96%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.28 – 6.06 (m, 3H), 5.12 (d, J = 8.9 Hz, 1H), 3.83 – 3.73 (m, 1H), 3.36 – 3.05 (m, 5H), 2.96 (s, 2H), 2.57 (s, 3H), 2.51 (s, 3H), 2.31 – 2.22 (m, 2H), 2.09 (s, 3H), 1.97 – 1.86 (m, 2H), 1.76 – 1.66 (m, 1H), 1.64 – 1.49 (m, 6H), 1.46 (s, 6H), 1.42 (s, 9H), 1.37 – 1.16 (m, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 158.75, 156.23, 156.00, 138.30, 132.87, 132.25, 124.64, 117.49, 86.41, 79.80, 71.27, 50.15, 43.24, 41.09, 40.84, 31.56, 28.62, 28.39, 26.32, 26.17, 25.71, 25.12, 25.06, 25.02, 19.33, 17.98, 12.50. LC-MS (Linear gradient 5 → 95% MeCN, 0.1% TFA, 10 min): Rt (min): 7.47 (ESI-MS (m/z): 675.17 ($M+H^+$)). HRMS: calcd. for $C_{30}H_{50}N_4O_7S_3Na$, 697.2734 [$M+Na^+$]; found 697.2709.

4.1.4. Backbone Synthesis

Synthesis of phenyl alanine derived Urea **25** according to the literature procedure.[37]: *step 1, Isocyanate formation*: was achieved from the TFA salt TFA.H_2N-Phe-OBn (17.2 g, 48.7 mmol, 1.00 equiv.) and reaction progress was followed by TLC (5% MeOH/DCM). *Step 2: Urea formation*: according to the literature procedure.[37] Reaction progress was followed by TLC (5% MeOH, DCM) and the title compound purified by column chromatography (0 → 6% MeOH, DCM). Yield: (8.84 g, 23.2 mmol, 48%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.35 – 7.30 (m, 3H), 7.30 – 7.26 (m, 2H), 7.21 – 7.17 (m, 3H), 7.02 – 6.97 (m, 2H), 5.16 (d, J = 12.2 Hz, 1H), 5.07 (d, J = 12.2 Hz, 1H), 5.00 (d, J = 7.6 Hz, 1H), 4.82 (dt, J = 7.6, 5.9 Hz, 1H), 3.37 – 3.31 (m, 4H), 3.09 (dd, J = 5.9, 3.1 Hz, 2H), 2.36 – 2.31 (m, 4H), 2.27 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 172.51, 156.47, 136.13, 135.23, 129.34, 128.55, 128.47, 128.43, 128.42, 126.91, 67.06, 54.48, 54.38, 45.98, 43.56, 38.19. LC-MS (Linear gradient 5 → 95% MeCN, 0.1% TFA, 40 min): Rt (min): 14.63 (ESI-MS (m/z): 382.04 ($M+H^+$)).

Carboxylic acid **26** was made according to the literature procedure.[37]: Benzyl ester **25** (8.84 g, 23.2 mmol, 1.00 equiv.) was dissolved in 1% AcOH / EtOH (250 mL) and 10wt. % Pd/C (2.3 g) used. TLC (5% MeOH in DCM) confirmed reaction completion. Yield: 7.36 g, 25.3 mmol, quant. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.18 – 7.08 (m, 5H), 5.88 (s, 1H), 4.48 (dd, J = 12.3, 6.4 Hz, 1H), 3.59 – 3.42 (m, 2H), 3.42 – 3.26 (m, 2H), 3.14 (dd, J = 13.8, 5.0 Hz, 1H), 2.99 (dd, J = 13.8, 7.1 Hz, 1H), 2.73 – 2.49 (m, 4H), 2.39 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 177.12, 156.91, 138.26, 129.71, 128.24, 126.60, 56.12, 53.09, 43.96, 42.02, 38.18.

4.1.5. Coupling warheads to backbone

Inhibitor HomoPhe-Ψ[CH_2SSO_2-Ph] (**27**): Prepared according to general procedure 9 on a 0.577 mmol (243 mg) scale. Purified

by automated column chromatography 0 → 10% MeOH, DCM. Yield: 281 mg, 0.472 mmol, 82%. To ensure high purity for biological testing an 82 mg portion was further purified by preparative HPLC (Linear gradient 5 → 95% MeCN, 0.1% TFA, 80 min), returning 55 mg of the title compound as a TFA salt after lyophilisation which was > 98% pure by HPLC. ^{1}H NMR (400 MHz, Methanol-d_4) δ 7.91 (d, J = 8.4 Hz, 2H), 7.70 – 7.63 (m, 1H), 7.62 – 7.55 (m, 2H), 7.26 (d, J = 4.3 Hz, 4H), 7.23 – 7.18 (m, 3H), 7.16 – 7.13 (m, 1H), 7.12 – 7.07 (m, 2H), 4.44 (dd, J = 9.0, 6.6 Hz, 1H), 4.21 – 4.06 (m, 2H), 4.01 – 3.91 (m, 1H), 3.49 – 3.36 (m, 2H), 3.19 – 3.10 (m, 2H), 3.07 (dd, J = 13.7, 6.6 Hz, 1H), 3.00 (dd, J = 6.4, 4.2 Hz, 2H), 2.92 (dd, J = 13.7, 9.0 Hz, 1H), 2.95 – 2.80 (m, 2H), 2.84 (s, 3H), 2.65 – 2.56 (m, 1H), 2.50 – 2.40 (m, 1H), 1.82 – 1.71 (m, 1H), 1.71 – 1.60 (m, 1H). ^{13}C NMR (101 MHz, Methanol-d_4) δ 174.63, 158.51, 145.98, 142.54, 138.70, 135.16, 130.66, 130.38, 129.53, 129.46, 129.40, 128.05, 127.82, 126.98, 57.98, 54.10, 49.38, 43.61, 42.38, 41.11, 39.12, 36.22, 33.00. LC-MS (Linear gradient 5 → 95% MeCN, 0.1% TFA, 40 min): Rt (min): 17.06 (ESI-MS (m/z): 595.13 ($M+H^+$)). HRMS: calcd. for $C_{31}H_{39}N_4O_4S_2$, 595.2407 [$M+H^+$]; found 595.2397. calcd. for $C_{31}H_{39}N_4O_4S_2Na$, 617.2227 [$M+Na^+$]; found 617.2214.

Inhibitor HomoPhe-Ψ[CH_2SSO_2-(*p*Me)Ph] (**28**): Prepared according to general procedure 9 on a 0.485 mmol (211 mg) scale. Purified by automated column chromatography 0 → 10% MeOH, DCM. Yield: 297 mg, 0.488 mmol, Quant. To ensure high purity for biological testing a 33 mg portion was further purified by preparative HPLC (Linear gradient 5 → 95% MeCN, 0.1% TFA, 80 min), returning 13 mg of the title compound as a TFA salt after lyophilisation which was > 99% pure by HPLC. ^{1}H NMR (500 MHz, Methanol-d_4) δ 8.09 (d, J = 8.4 Hz, 1H), 7.79 (d, J = 8.4 Hz, 2H), 7.40 (d, J = 8.1 Hz, 2H), 7.31 – 7.18 (m, 6H), 7.15 (d, J = 7.5 Hz, 1H), 7.13 – 7.08 (m, 2H), 4.42 (dd, J = 9.0, 6.6 Hz, 1H), 4.25 – 4.04 (m, 2H), 4.01 – 3.89 (m, 1H), 3.52 – 3.35 (m, 2H), 3.21 – 3.07 (m, 2H), 3.03 (dd, J = 13.8, 6.7 Hz, 1H), 2.98 (t, J = 6.6 Hz, 2H), 3.04 – 2.92 (m, 2H), 2.90 (dd, J = 13.8, 9.0 Hz, 1H), 2.87 (s, 3H), 2.65 – 2.57 (m, 1H), 2.48 – 2.43 (m, 1H), 2.42 (s, 3H), 1.81 – 1.73 (m, 1H), 1.69 – 1.60 (m, 1H). ^{13}C NMR (126 MHz, Methanol-d_4) δ 174.69, 158.57, 146.63, 143.29, 142.61, 138.71, 131.14, 130.38, 129.55, 129.45, 129.40, 128.20, 127.84, 126.98, 57.95, 54.20, 43.65, 42.44, 41.10, 39.17, 36.25, 33.05, 21.59. LC-MS (Linear gradient 5 → 95% MeCN, 0.1% TFA, 40 min): Rt (min): 18.29 (ESI-MS (m/z): 609.10 ($M+H^+$)). HRMS: calcd. for $C_{32}H_{41}N_4O_4S_2$, 609.2564 [$M+H^+$]; found 609.2544.

Inhibitor HomoPhe-Ψ[CH_2SSO_2-(*p*-OMe)Ph] (**29**): Prepared according to general procedure 9 on a 0.441 mmol (199 mg) scale . Purified by automated column chromatography 0 → 10% MeOH, DCM. Yield: 315 mg, 0.504 mmol, Quant. To ensure high purity for biological testing an 83 mg portion was further purified by preparative HPLC (Linear gradient 5 → 95% MeCN, 0.1% TFA, 80 min), returning 60 mg of the title compound as a TFA salt after lyophilisation which was > 99% pure by HPLC . ^{1}H NMR (400 MHz, Methanol-d_4) δ 7.84 (d, J = 9.0 Hz, 2H), 7.28 – 7.18 (m, 7H), 7.16 – 7.05 (m, 5H), 4.43 (dd, J = 9.0, 6.5 Hz, 1H), 4.23 – 4.04 (m, 2H), 4.00 – 3.91 (m, 1H), 3.86 (s, 3H), 3.50 – 3.34 (m, 2H), 3.22 – 3.08 (m, 2H), 3.05 (dd, J = 13.7, 6.5 Hz, 1H), 2.98 (t, J = 6.2 Hz, 2H), 3.05 – 2.90 (m, 2H), 2.91 (dd, J = 13.6, 9.0 Hz, 1H), 2.85 (s, 3H), 2.65 – 2.56 (m, 1H), 2.50 – 2.40 (m, 1H), 1.83 – 1.72 (m, 1H), 1.71 – 1.59 (m, 1H). ^{13}C NMR (101 MHz, Methanol-d_4) δ 174.61, 165.47, 158.54, 142.58, 138.72, 137.55, 130.56, 130.38, 129.53, 129.44, 129.39, 127.82, 126.98, 115.65, 57.96, 56.42, 54.13, 43.62, 42.40, 41.02, 39.15, 36.22, 33.04. LC-MS (Linear gradient 5 → 95% MeCN, 0.1% TFA, 40 min): Rt (min): 17.33 (ESI-MS (m/z): 625.11 ($M+H^+$)). HRMS: calcd. for $C_{32}H_{41}N_4O_5S_2$, 625.2513 [$M+H^+$]; found 625.2482, calcd. for $C_{32}H_{40}N_4O_4S_2Na$, 647.2332 [$M+Na^+$]; found 647.2305.

Inhibitor HomoPhe-Ψ[CH_2SSO_2-(*p*-NO_2)Ph] (**30**): Prepared according to general procedure 9 on a 0.204 mmol (95 mg) scale. Purified by automated column chromatography 0 → 10% MeOH, DCM. Yield: 108 mg, 0.169 mmol, 83 %. To ensure high purity for biological testing all 108 mg was further purified by preparative HPLC (Linear gradient 5 → 95% MeCN, 0.1% TFA, 80 min), returning 36 mg of the title compound as a TFA salt after lyophilisation which was > 98% pure by HPLC ^{1}H NMR (400 MHz, Methanol-d_4) δ 8.36 (d, J = 9.0 Hz, 2H), 8.11 (d, J = 8.9 Hz, 2H), 7.32 – 7.15 (m, 7H), 7.13 – 7.04 (m, 3H), 4.42 (dd, J = 8.8, 6.8 Hz, 1H), 4.23 – 4.07 (m, 2H), 3.87 – 3.77 (m, 1H), 3.51 – 3.36 (m, 2H), 3.22 – 3.09 (m, 2H), 3.09 – 3.01 (m, 3H), 3.17 – 2.86 (m, 2H), 2.92 (dd, J = 13.7, 8.8 Hz, 1H), 2.86 (s, 3H), 2.66 – 2.57 (m, 1H), 2.50 – 2.39 (m, 1H), 1.80 – 1.70 (m, 1H), 1.68 – 1.57 (m, 1H). ^{13}C NMR (101 MHz, Methanol-d_4) δ 174.72, 158.55, 151.94, 151.01, 142.37, 138.65, 130.37, 129.58, 129.47, 129.46, 129.35, 127.87, 127.00, 125.89, 57.93, 54.15, 43.63, 42.42, 41.73, 39.10, 36.14, 32.84. LC-MS (Linear gradient 5 → 95% MeCN, 0.1% TFA, 40 min): Rt (min): 17.55 (ESI-MS (m/z): 640.11 ($M+H^+$)). HRMS: calcd. for $C_{31}H_{38}N_5O_6S_2$, 640.2258 [$M+H^+$]; found 640.2232.

Inhibitor HomoPhe-Ψ[CH_2SSO_2-*c*Hex] (**36**): Prepared according to general procedure 9 on a 0.391 mmol (167 mg) scale. Purified by automated column chromatography 0 → 10 % MeOH, DCM. Yield: 163 mg, 0.271 mmol, 69 %. To ensure high purity for biological testing all 163 mg was further purified by preparative HPLC (Linear gradient 5 → 95% MeCN, 0.1% TFA, 80 min), returning 130 mg of the title compound as a TFA salt after lyophilisation which was > 99% pure by HPLC ^{1}H NMR (400 MHz, Methanol-d_4) δ 8.15 (d, J = 8.5 Hz, 1H), 7.34 – 7.10 (m, 10H), 4.48 (dd, J = 9.2, 6.2 Hz, 1H), 4.27 – 4.08 (m, 2H), 4.08 – 3.96 (m, 1H), 3.52 – 3.34 (m, 2H), 3.26 (tt, J = 11.7, 3.4 Hz, 1H), 3.23 – 3.05 (m, 5H), 3.07 – 2.86 (m, 2H), 2.96 (dd, J = 13.8, 9.3 Hz, 1H), 2.86 (s, 3H), 2.71 (ddd, J = 14.4, 9.3, 5.4 Hz, 1H), 2.59 (ddd, J = 13.7, 9.1, 7.0 Hz, 1H), 2.26 – 2.14 (m, 2H), 1.94 – 1.84 (m, 3H), 1.84 – 1.74 (m, 1H), 1.73 – 1.66 (m, 1H), 1.55 – 1.43 (m, 2H), 1.42 – 1.27 (m, 2H), 1.22 (tt, J = 12.6, 3.2 Hz, 1H). ^{13}C NMR (101 MHz, Methanol-d_4) δ 174.76, 158.59, 142.66, 138.84, 130.37, 129.57, 129.55, 129.43, 127.80, 127.03, 71.85, 58.00, 54.17, 50.14, 43.63, 42.44, 41.37, 39.17, 36.65, 33.03, 27.51, 27.45, 26.23, 26.11, 26.07. LC-MS (Linear gradient 5 → 95% MeCN, 0.1% TFA, 40 min): Rt (min): 17.97 (ESI-MS (m/z): 601.08 ($M+H^+$)). HRMS: calcd. for $C_{31}H_{45}N_4O_4S_2$, 601.2877 [$M+H^+$]; found 601.2859, calcd. for $C_{31}H_{44}N_4O_4S_2Na$, 623.2696 [$M+Na^+$]; found 623.2672.

Inhibitor Arg-Ψ[CH_2SSO_2-Ph] (**31**): Prepared according to general procedure 10 on a 0.190 mmol (127 mg) scale. Purification directly by preparative HPLC (Linear gradient 5 → 40% MeCN, 0.1% TFA, 80 min) returned the title compound as a TFA salt after lyophilisation which was >99% pure by HPLC: Yield: 131 mg, 0.167 mmol, 88%. ^{1}H NMR (400 MHz, Methanol-d_4) δ 8.05 (d, J = 8.7 Hz, 1H), 7.98 – 7.93 (m, 2H), 7.77 – 7.70 (m, 1H), 7.69 – 7.61 (m, 2H), 7.27 – 7.15 (m, 5H), 4.33 (dd, J = 9.0, 6.6 Hz, 1H), 4.20 – 4.03 (m, 2H), 4.06 – 3.94 (m, 2H), 3.55 – 3.32 (m, 2H), 3.15 – 3.01 (m, 5H), 2.97 – 2.92 (m, 3H), 2.88 (s, 3H), 1.69 – 1.34 (m, 4H). ^{13}C NMR (101 MHz, Methanol-d_4) δ 174.99, 158.64, 158.56, 145.98, 138.64, 135.26, 130.75, 130.34, 129.56, 128.16, 127.88, 58.21, 54.13, 43.63, 42.40, 41.94, 40.93, 39.03, 31.39, 26.23. LC-MS (Linear gradient 5 → 95% MeCN, 0.1% TFA, 40 min): Rt (min): 12.24 (ESI-MS

(m/z): 590.33 ($M+H^+$)). HRMS: calcd. for $C_{27}H_{40}N_7O_4S_2$, 590.2578 [$M+H^+$]; found 590.2557.

Inhibitor Arg-Ψ[CH_2SSO_2-(*p*Me)Ph] (**32**): Prepared according to general procedure 10 on a 0.180 mmol (124 mg) scale yielding 254 mg of crude material. Purification of a 44 mg portion of crude material directly by preparative HPLC (Linear gradient 5 → 40% MeCN, 0.1% TFA, 80 min) gave the title compound as a TFA salt after lyophilisation which was >99% pure by HPLC: Yield: 20 mg, (0.152 mmol, 84%, assuming all crude was to be purified). ^{1}H NMR (500 MHz, Methanol-d_4) δ 8.03 (d, J = 8.7 Hz, 1H), 7.82 (d, J = 8.4 Hz, 2H), 7.46 (d, J = 8.4 Hz, 2H), 7.28 – 7.17 (m, 5H), 4.33 (dd, J = 9.1, 6.6 Hz, 1H), 4.29 – 3.83 (m, 2H), 4.06 – 3.94 (m, 1H), 3.59 – 3.21 (m, 2H), 3.15 – 3.00 (m, 3H), 2.98 – 2.89 (m, 3H), 2.88 (s, 3H), 2.45 (s, 3H), 1.69 – 1.60 (m, 1H), 1.61 – 1.49 (m, 2H), 1.46 – 1.38 (m, 1H). ^{13}C NMR (126 MHz, Methanol-d_4) δ 174.98, 158.64, 158.57, 146.77, 143.16, 138.66, 131.20, 130.34, 129.56, 128.27, 127.87, 58.21, 54.13, 43.64, 42.40, 41.95, 40.90, 39.04, 31.43, 26.24, 21.59. LC-MS (Linear gradient 5 → 95% MeCN, 0.1% TFA, 40 min): Rt (min): 13.70 (ESI-MS (m/z): 604.33 ($M+H^+$)). HRMS: calcd. for $C_{28}H_{42}N_7O_4S_2$, 604.2734 [$M+H^+$]; found 604.2717.

Inhibitor Arg-Ψ[CH_2SSO_2-(*p*-OMe)Ph] (**33**): Prepared according to general procedure 10 on a 0.094 mmol (66 mg) scale. Purification by preparative HPLC (Linear gradient 5 → 40% MeCN, 0.1% TFA, 80 min) gave the title compound as a TFA salt after lyophilisation which was >99% pure by HPLC: Yield: 50 mg, 0.61 mmol, 65%. ^{1}H NMR (400 MHz, Methanol-d_4) δ 8.05 (d, J = 8.7 Hz, 1H), 7.88 (d, J = 8.9 Hz, 2H), 7.45 – 7.18 (m, 5H), 7.13 (d, J = 8.9 Hz, 2H), 4.33 (dd, J = 9.1, 6.5 Hz, 1H), 4.20 – 4.05 (m, 2H), 4.03 – 3.94 (m, 1H), 3.89 (s, 3H), 3.54 – 3.35 (m, 2H), 3.24 – 3.00 (m, 5H), 2.99 – 2.89 (m, 3H), 2.88 (s, 3H), 1.69 – 1.48 (m, 3H), 1.47 – 1.37 (m, 1H). ^{13}C NMR (101 MHz, Methanol-d_4) δ 175.00, 165.56, 158.61, 158.54, 138.68, 137.43, 130.66, 130.34, 129.54, 127.85, 115.70, 58.27, 56.45, 54.09, 49.02, 43.62, 42.37, 41.93, 40.87, 39.00, 31.46, 26.23. LC-MS (Linear gradient 5 → 95% MeCN, 0.1% TFA, 40 min): Rt (min): 12.88 (ESI-MS (m/z): 620.25 ($M+H^+$)). HRMS: calcd. for $C_{28}H_{42}N_7O_5S_2$, 620.2683 [$M+H^+$]; found 620.2656.

Inhibitor Arg-Ψ[CH_2SSO_2-(*p*-NO_2)Ph] (**34**): Prepared according to general procedure 10 on a 0.217 mmol (155 mg) scale. Purified by reverse phase automated column chromatography (Linear gradient 5 → 40% MeCN, 0.1% TFA, 40 min) using a Biotage® SNAP Ultra C18, 60 g column yielding the title compound as a TFA salt after lyophilisation which was >98% pure by HPLC. Yield: 110 mg, 0.132 mmol, 61%. ^{1}H NMR (400 MHz, Methanol-d_4) δ 8.47 (d, J = 8.5 Hz, 2H), 8.19 (d, J = 8.5 Hz, 2H), 8.10 (d, J = 8.7 Hz, 1H), 7.31 – 7.17 (m, 5H), 4.33 (dd, J = 9.2, 6.4 Hz, 1H), 4.21 – 4.02 (m, 2H), 4.00 – 3.90 (m, 1H), 3.45 (br s, 2H), 3.22 – 2.98 (m, 7H), 2.95 – 2.90 (m, 1H), 2.98 – 2.80 (m, 2H), 2.88 (s, 3H), 1.69 – 1.40 (m, 4H). ^{13}C NMR (101 MHz, Methanol-d_4) δ 175.08, 158.60, 158.54, 152.09, 150.82, 138.65, 130.30, 129.62, 129.56, 127.86, 125.97, 58.21, 54.08, 43.62, 42.36, 41.89, 41.42, 38.93, 31.52, 26.22. LC-MS (Linear gradient 5 → 95% MeCN, 0.1% TFA, 40 min): Rt (min): 13.24 (ESI-MS (m/z): 635.33 ($M+H^+$)). HRMS: calcd. for $C_{27}H_{39}N_8O_6S_2$, 635.2428 [$M+H^+$]; found 635.2407.

Inhibitor Arg-Ψ[CH_2SSO_2-*c*Hex] (**35**): Prepared according to general procedure 10 on a 0.220 mmol (148 mg) scale. Purification directly by preparative HPLC (Linear gradient 5 → 40% MeCN, 0.1% TFA, 80 min) returned the title compound as a TFA salt after lyophilisation which was >99% pure by HPLC Yield: 93 mg, 0.118 mmol, 54%. ^{1}H NMR (400 MHz, Methanol-d_4) δ 8.11 (d, J = 8.8 Hz, 1H), 7.34 – 7.19 (m, 5H), 4.40 (dd, J = 9.6, 5.9 Hz, 1H), 4.18 – 4.02 (m, 3H), 3.51 – 3.38 (m, 2H), 3.32 (tt, J = 11.8, 3.4 Hz, 1H), 3.25 – 3.04 (m, 7H), 2.96 (dd, J = 13.8, 9.6 Hz, 1H), 2.87 (s, 3H), 2.29 – 2.20 (m, 2H), 1.95 – 1.88 (m, 2H), 1.75 – 1.59 (m, 4H), 1.59 – 1.46 (m, 3H), 1.46 – 1.31 (m, 2H), 1.29 – 1.19 (m, 1H). ^{13}C NMR (101 MHz, Methanol-d_4) δ 175.17, 158.63, 158.59, 138.84, 130.32, 129.55, 127.81, 71.96, 58.29, 54.10, 50.12, 42.37, 42.00, 41.29, 39.02, 31.87, 27.57, 27.43, 26.32, 26.22, 26.09, 26.05. LC-MS (Linear gradient 5 → 95% MeCN, 0.1% TFA, 40 min): Rt (min): 13.65 (ESI-MS (m/z): 596.33 ($M+H^+$)). HRMS: calcd. for $C_{27}H_{46}N_7O_4S_2$, 596.3047 [$M+H^+$]; found 596.3028.

4.2. Biology

4.2.1. Papain Assay

4.2.1.1. Materials

All tested inhibitors were prepared as described and were >98% pure as determined by analytical HPLC (chromatograms in Supporting Information). Papain from papaya latex was purchased as a lyophilised powder from Sigma Aldrich®. The substrate *N*α-Benzoyl-L-arginine 4-nitroanilide hydrochloride was purchased from Sigma Aldrich®. As assay buffer sodium phosphate (100 mM, pH 6.5) containing EDTA (1.5 mM) was used. Compounds were dissolved in DMSO (for molecular biology grade) which was purchased from Sigma Aldrich®.

A CLARIOstar microplate reader with Corning® 96 well UV-transparent plates was used.

4.2.1.2. Method

Each inhibitor was screened by three separate experiments, each conducted in duplicate. The thiol independent papain assay was based on our previously reported assay.[48] Papain stock solution was prepared in assay buffer (40 μM), the solution shaken for 10 min. and then centrifuged at 13,200 rpm for 3 min. The substrate stock solution (Bz-L-Arg-pNA) was prepared in DMSO (50 mM).

Stock solutions of inhibitors were prepared in DMSO (9.8 mM) and a 1:1 dilution made with buffer solution (4.95 mM), from which relevant serial dilutions with 1:1 DMSO:Buffer solution were made to achieve the desired concentrations of inhibitor solutions for the assay.

To each well was added inhibitor solution (4.0 μL), buffer solution (172.0 μL) and papain solution (20.0 μL) followed by thorough mixing (15 times by pipette upon papain addition). For the positive control a DMSO/buffer solution (1:1) was used instead of the inhibitor solution and the negative control (blank) was taken by replacing papain solution with assay buffer. After 1 h incubation with shaking (shaker plate set to 100 rpm) a sample (98.0 μL) was taken from each well and added to wells containing substrate solution (2.0 μL), Mixed thoroughly by pipette (15 times), the plate covered with a lid and centrifuged at 1000 rpm for 1 min (to remove any air bubbles) and the subsequent liberation of p-nitroaniline was measured over a 1h. time frame with 1 measurement per well per minute. Final concentrations in the wells were: enzyme: 4 μM; substrate: 1.0 mM; inhibitor: Doubling dilution range starting at 10 μM giving: 10 μM, 5 μM, 2.5 μM, 1.25 μM, 0.625 μM, 0.3125 μM, 156.25 nM, 78.125 nM, 39.0625 nM.

Processing: As the liberation of p-nitro anilide was linear, the response was measured by applying a line of best fit to the blank corrected data and taking the gradient as a measure of the response. The gradients were normalized against the gradient of the positive control (100% response in the absence of inhibitor). The normalized response was used to calculate the IC50 value by

plotting log(inhibitor) vs. normalized response with the GraphPad Prism software suite.

4.2.2. S. mansoni bio-assay

4.2.2.1. Preparation of schistosomula

S. mansoni life cycle is maintained at the Institute of Tropical Medicine, University Hospital Tübingen/Germany. *S. mansoni* infected vector snails were exposed to day-light to induce shedding of cercariae. Schistosomula were obtained by mechanical transformation of cercariae by vortexing following published procedures.[46] Schistosomula were kept in 48-well plates in schistosomula culture medium (SCM; phenol-red free medium 199 [M199; catalog number 11043-023; Gibco], 5.5 mM d-glucose, 200 U/ml penicillin, 200 µg/ml streptomycin, 1% heat-inactivated FCS [iFCS]) at 400 schistosomula/1 ml SCM/well for 24 h to allow maturation before being further processed.

4.2.2.2. Schistosomula in vitro drug susceptibility assay

All compounds were tested *in vitro* against mature schistosomula. Thiosulfonate compounds were dissolved in DMSO at a stock concentration of 10 mM. Mefloquine hydrochloride (MQ, Sigma-Aldrich) and auranofin (AU, Sigma-Aldrich) were dissolved in DMSO at 36 mM and 14.7 mM, respectively. All compounds were stored at -20°C until further use. 96 well, sterile, flat bottom plates were predosed with compounds at respective concentrations. Drug dilutions were done in SCM. Schistosomula were distributed at a density of 100 worms/well. The number of worms was counted for each individual well. Final volume/well was 225 µl. *In vitro* cultures were kept at 37°C and 5% CO_2. Each experiment included MQ and AU to control for schistosomula inhibition. Worm viability during *in vitro* culture was controlled using 'medium only' and DMSO (1%), respectively.

After the described incubation time (1 day, 3 days or 7 days) the viability of worms per drug concentration was analysed by an inverted microscope (Nikon Eclipse Ti) using 40x magnification. On the basis of motility and morphology, the parasites were classified as viable (movement and normal appearance) or dead (no movement within 10 s and/or severe morphological changes of any kind compared to the morphology of untreated parasites, e.g., granularity, blebbing).

Data analysis: Viability of schistosomula is reported as the proportion of viable schistosomula to the total number of schistosomula per respective well. Viability in % and log concentration of the drug were used to estimate the IC50 by GraphPad Prim 6 applying the built-in 4 parametric regression analysis to model curve fit.

4.3. Stability tests of thiosulfonate inhibitors

All stability tests were carried out in 0.1 M sodium phosphate buffer of the corresponding pH with Ac-Phe-OH used as an internal standard. 70 µL of Ac-Phe-OH solution (1 mg/mL, 0.07 mg) in phosphate buffer of the relevant pH was added to 25 µL of inhibitor solution (9.8 mM stock solution in DMSO) and diluted with 880 µL of pH buffer solution giving a final volume of 1 mL with 5% DMSO. The sample was shaken for 10 min. before being centrifuged at 13,200 rpm for 5 min. and transferred to an HPLC vial for sampling. The sample was then analysed over 12 hrs. by HPLC using 12 × 60 min. gradients from (5 → 95% MeCN, 0.1% TFA, 60 min.) yielding 12 measurements 1 h apart, a blank sample containing 5% DMSO in phosphate buffer was run after the analysis for subtraction from the baseline. The Inhibitor peak was integrated against the internal standard, data normalised to show percentage degradation with time and plotted on a scatter graph for visualisation.

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Supplementary Material

Included are the syntheses of starting materials; a description of the used modelling software, procedures and docking studies; ^{1}H-NMR, ^{13}C-NMR, COSY and HSQC-spectra of **2** - **36**; HPLC-chromatograms of **27** - **36**.