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RESEARCH ARTICLE

Optimization of peptidyl allyl sulfones as clan CA cysteine protease inhibitors

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Abstract

This research investigates the synthesis and inhibitory potency of a series of novel dipeptidyl allyl sulfones as clan CA cysteine protease inhibitors. The structure of the inhibitors consists of a R_1-Phe-R_2-AS-Ph scaffold (AS = allyl sulfone). R_1 was varied with benzyloxycarbonyl, morpholinocarbonyl, or N-methylpiperazinocarbonyl substituents. R_2 was varied with either Phe of Hfe residues. Synthesis involved preparation of vinyl sulfone analogues followed by isomerization to allyl sulfones using *n*-butyl lithium and *t*-butyl hydroperoxide. Sterics, temperature and base strength were all factors that affected the formation and stereochemistry of the allyl sulfone moiety. The inhibitors were assayed with three clan CA cysteine proteases (cruzain, cathepsin B and calpain I) as well as one serine protease (trypsin). The most potent inhibitor, (*E*)-Mu-Phe-Hfe-AS-Ph, displayed at least 10-fold selectivity for cruzain over clan CA cysteine proteases cathepsin B and calpain I with a $k_{obs}/[I]$ of 6080 ± 1390 $M^{-1}s^{-1}$.

Keywords: Cruzain, Chagas disease, peptidyl vinyl sulfone

Introduction

Proteases are a group of enzymes that are responsible for a variety of metabolic functions based on their ability to cleave peptide bonds[1–4]. They are grouped by catalytic mechanism into five major classes: metallo, aspartate, threonine, serine and cysteine proteases[5]. Cysteine proteases catalyze the cleavage of peptide bonds using a catalytic dyad of Cys and His residues[6]. Each of the five major protease classes is further divided into clans and families[7]. Clan CA (C indicates cysteine) is the largest of the cysteine protease clans and contains a variety of enzymes including calpains, cathepsins and cruzain.

Calpains are members of family C2, requiring Ca^{2+} for activation[8]. They have been linked to a number of different cellular functions including signal transduction, cell fusion, mitosis and protein turnover. Mutations and other defects in calpains have been implicated in hypertension and muscular dystrophy as well as neurodegeneration[9]. To date, no highly selective and potent inhibitors of calpains have been synthesized.

Cathepsins are members of family C1. Eleven cathepsins are currently known including cathepsin B, C, F, H, K (O2), L, O, S, V (L2), W and X (parentheses indicate secondary names). Cathepsin B is the most abundant of the cathepsins, serving a variety of cell degradation processes[10] in addition to potentially functioning as a defence mechanism against amyloid plaque aggregation[11]. Cathepsin B has been associated with tumour progression[6,12,13]. More generally, cathepsins have been linked to rheumatoid arthritis, osteoarthritis, neurological disorders and lysosomal storage diseases[14,15]. Because of their role in these diseases, cathepsins have been targets for drug development[16–22]. Continued investigation of new cathepsin inhibitors provides a route for expanding treatment of these diseases.

Cruzain is another member of family C1. It is a key enzyme in the life cycle of *Trypanosoma cruzi*, the parasitic vector of Chagas disease[23–25]. Chagas disease is an increasing problem in Latin America with 10 million people suffering from infection and millions more at

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Abbreviations

AMC, 7-aminomethyl coumarin
AS, allyl sulfone
Cbz, benzyloxycarbonyl
DTT, dithiothreitol
EDCl, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
Elim, eliminated sulfone (-C(O)-N=C(R)-CH=CH_2)
Hfe, homo-phenylalanine residue
HOBT, hydroxybenzotriazole
*i*BCF, isobutyl chloroformate
Mp, *N*-methylpiperazinocarbonyl
Mu, morpholinocarbonyl
NMM, *N*-methylmorpholine
VS, vinyl sulfone

risk[26]. Current treatment utilizes benznidazole (a nitroimidazole derivative)[27] and nifurtimox (a 5-nitrofuran derivative)[28], which are effective in the acute phase of the disease; however, these treatments are plagued with severe side effects and increasing resistance[29,30]. As a result, demand for developing new treatments for this disease remains prevalent.

To date, a number of cysteine protease inhibitors have been developed using a variety of reactive functional groups[31], also referred to as "warheads," including aldehydes[20], fluoromethyl ketones[21], nitriles[32], α-ketoesters, amides, and acids[33], tetrafluorophenoxymethyl ketones[34], epoxysuccinates[35], thiosemicarbazones[36], hydrazone derivatives[37,38], vinyl sulfones[39] and allyl sulfones[40]. These warheads are attached to either a peptidyl or peptidomimetic scaffold[16,41].

We selected cruzain as our primary target due to the high demand for the development of new treatments for Chagas disease. Currently, the most effective known inhibitor of cruzain, referred to as K11777, is a dipeptidyl vinyl sulfone (Mp-Phe-Hfe-VS-Ph)[42]. Both metabolic[43] and animal studies[44,45] have confirmed that K11777 is a highly selective and potent inhibitor of cruzain *in vivo* and is currently entering clinical trials[46]. We have demonstrated previously that use of an allyl sulfone in place of the vinyl sulfone moiety showed enhancements in inhibitory potency for clan CA cysteine proteases (Figure 1)[40].

In order to optimize the lead allyl sulfones for inhibition of cruzain, we chose to modify the P3 and P1 positions of the scaffold. The peptidyl scaffold was maintained because no peptidomimetic scaffolds have been able to achieve *in vivo* potency equivalent to K11777. The goal of the P3 modification was to vary solubility and polarity inside the active site using Cbz, Mp and Mu groups. Based on previous kinetic data, the P2 and P1′ positions were kept constant, as Phe and SO_2Ph, respectively, in all analogues[39]. The P1 residue was varied, using either Phe or Hfe residues, in order to examine the effect of flexibility of the phenyl group after the introduction of the planar allyl sulfone moiety.

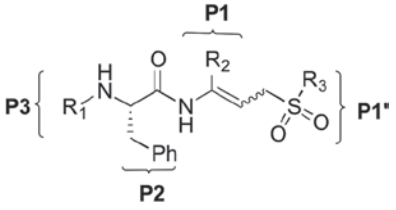


Figure 1. Basic scaffold of dipeptidyl allyl sulfone inhibitors with enzyme subsite designations P3, P2, P1 and P1′.

We have previously synthesized allyl sulfones from vinyl sulfone precursors and determined that stereochemistry of the P1 residue of the vinyl sulfone influenced the stereochemistry of the allyl sulfone product, although the contributing effect was not determined. To further investigate this, both enantiomers of the P1 amino acid residue were used during the synthesis of the dipeptidyl allyl sulfones.

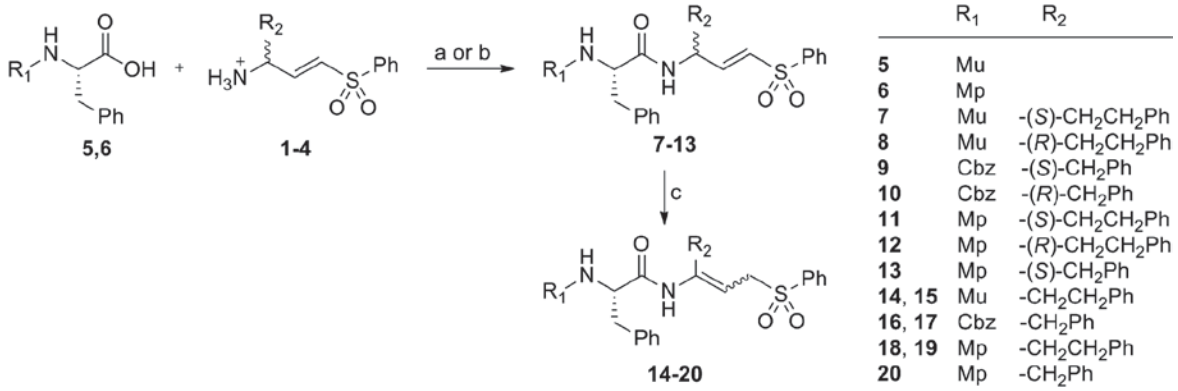
Materials and methods

Materials

Starting materials and reagents were purchased from VWR International (Radnor, Pennsylvania) and used without further purification. Amino acids were purchased from Advanced ChemTech (Louisville, Kentucky) and enzymes and substrates from Calbiochem (San Diego, California) and Enzo Life Sciences (Ann Arbor, Michigan). Compound purity was confirmed by MS electron spray ionization (ESI) and NMR (^{13}C and ^{1}H with chemical shifts reported in ppm relative tetramethyl silane). MS analysis was performed at the University of Arkansas High Performance Mass Spectrometry Laboratory using a Bruker Esquire-LC Ion Trap LC/MS. NMR experiments were conducted using a BrukerAvance III 400 MHz UltrashieldPlus Spectrometer. Compounds were purified via gravity column chromatography using Sigma-Aldrich Fluka analytical silica (60 Å, 70–230 mesh). Reaction progress was monitored using EMD 60 F_{254} TLC plates. Fluorogenic enzyme assays were performed on a BioTek Flx800 Plate Reader. Molecular modelling data was acquired using chemical computing group's Molecular Operating Environment (MOE) software package.

General synthesis of inhibitors

Trans-vinyl sulfones **1–4** were prepared as previously described via Wadsworth-Emmons chemistry using chiral amino acid aldehydes and a sulfonylphosphonate[40,47]. Phe-OMe was reacted with morpholinocarbonyl chloride and saponified to carboxylic acid **5** (Mu-Phe-OH) in 71% overall yield (Scheme 1). Phe-OBz was reacted with N-methylpiperazinocarbonyl chloride and hydrogenated to give carboxylic acid **6** (Mp-Phe-OH) in 89% yield. Trifluoroacetic acid (TFA) salts **1** and **2** (TFA·*L/D*-Hfe-VS-Ph) were coupled to **5** using 1-ethyl-3-(3-dimethylaminopropyl)



	R_1	R_2
5	Mu	
6	Mp	
7	Mu	-(*S*)-CH_2CH_2Ph
8	Mu	-(*R*)-CH_2CH_2Ph
9	Cbz	-(*S*)-CH_2Ph
10	Cbz	-(*R*)-CH_2Ph
11	Mp	-(*S*)-CH_2CH_2Ph
12	Mp	-(*R*)-CH_2CH_2Ph
13	Mp	-(*S*)-CH_2Ph
14, 15	Mu	-CH_2CH_2Ph
16, 17	Cbz	-CH_2Ph
18, 19	Mp	-CH_2CH_2Ph
20	Mp	-CH_2Ph

Scheme 1. (a) EDCl, HOBT, Et_3N, DCM, overnight; (b) *i*BCF, NMM, THF/DMF, overnight; (c) 1 eq.*n*-BuLi, 4.5 eq. *t*-BuOOH, –20°C for 70 min, 9 h or 18 h.

carbodiimide (EDCl) and hydroxybenzotriazole (HOBT) to produce dipeptidyl vinyl sulfones **7** and **8** (Mu-Phe-*L/D*-Hfe-VS-Ph). TFA salts **3** and **4** (TFA·*L/D*-Phe-VS-Ph) were coupled to Cbz-Phe-OH using EDCl and HOBT to yield dipeptidyl vinyl sulfones **9** and **10** (Cbz-Phe-*L/D*-Phe-VS-Ph). TFA salts **1–3** were coupled to **6** using *i*BCF and *N*-methylmorpholine (NMM) to give dipeptidyl vinyl sulfones **11–13** (Mp-Phe-*L/D*-Hfe-VS-Ph, Mp-Phe-*L*-Phe-VS-Ph). Dipeptidyl vinyl sulfones **7–13** were then isomerized to allyl sulfone analogues **14–20** using *t*-BuOOH and *n*-BuLi.

Detailed synthesis

Preparation of Amino Acid Vinyl Sulfone TFA Salts

TFA salts **1–4** were synthesized using previously reported methods[40].

Synthesis of (2S)-2-(morpholine-4-carboxamido)-3-phenylpropanoic acid (5, Mu-Phe-OH) The hydrochloride salt of Phe-OMe (7.00 g, 32.5 mmol) was dissolved in THF (150 mL) under argon at –15°C. NMM (7.25 g, 71.4 mmol) was added followed by the dropwise addition of morpholinecarbonylchloride (5.34 g, 35.7 mmol). The reaction was allowed to warm to room temperature and stirred overnight. The reaction was poured into aq. HCl (1 N, 100 mL) and extracted with EtOAc (3 × 60 mL). The organic extract was then washed with aq. HCl (1 N, 3 × 60 mL) and brine (3 × 60 mL) and dried ($MgSO_4$). The solvent was removed under reduced pressure. The product was initially isolated as a syrup that spontaneously crystallized into white crystals with a 92% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.14 (t, 2H, CHCH_2Ph), 3.33 (m, 4H, CH_2NCH_2), 3.75 (t, 4H, CH_2OCH_2), 4.81 (m, 1H, α-H), 4.87 (d, 1H, NH), 7.11–7.12 (m, 2H, Ph), 7.28–7.32 (m, 3H, Ph). Mu-Phe-OMe (9.47 g, 32.5 mmol) was dissolved in methanol (150 mL). Sodium hydroxide (2 N, 2.62 g, 48.5 mmol) was added. After 4 h, the solvent was removed under reduced pressure and the mixture was suspended in water (100 mL). The solution was acidified to pH 2 by addition of aq. HCl (3 N, approx. 20 mL). The product was extracted with EtOAc (3 × 30 mL), washed with brine (2 × 25 mL) and dried ($MgSO_4$). The solvent was removed under reduced pressure to give **5** as a white powder in a 76.5% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 2.90 (dd, 1H, CH_2Ph J = 13 Hz, 10 Hz), 3.03 (dd, 1H, CH_2Ph, J = 15 Hz, 5 Hz), 3.23 (m, 4H, CH_2NCH_2), 3.49 (m, 4H, CH_2OCH_2), 4.23 (m, 1H, α-H), 6.73 (d, 1H, NH), 7.17–7.30 (m, 5H, Ph), 12.53 (s, broad, 1H, COOH).

Synthesis of (2S)-2-(N-methylpiperazine-4-carboxamido)-3-phenylpropanoic acid (6, Mp-Phe-OH) The hydrochloride salt of N-methylpiperazinecarbonyl chloride (1.50 g, 7.54 mmol) was dissolved in THF (50 mL) under argon at –15°C. Et_3N (0.839 g, 8.29 mmol) was added and the reaction was stirred for 15 min. The hydrochloride salt of Phe-OBz (2.00 g, 6.84 mmol) was dissolved in THF (50 mL) under argon at –15°C. Et_3N (0.839 g, 8.29 mmol) was added and stirred for 15 min. The two reactions were combined and kept at –15°C for 15 min, then allowed to warm to room temperature and stirred overnight. The reaction was quenched with water (80 mL) and the product was extracted with EtOAc (3 × 50 mL). The organic layer was washed with water (3 × 50 mL) and brine (2 × 50 mL) and dried ($MgSO_4$). The solvent was removed under reduced pressure. The crude product was purified via column chromatography (silica, 5% MeOH in DCM, 0.5% Et_3N). The pure product (Mp-Phe-OBz) was isolated as a clear colorless syrup with a yield of 88.5%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.31 (s, 3H, CH_3N), 2.37 (t, 4H, $CH_2N(CH_3)CH_2$), 3.14 (m, 2H, CH_2Ph), 3.36 (m, 4H, CH_2NCH_2), 3.88 (broad, 2H, α-H and NH), 7.00–7.03 (m, 2H, Ph), 7.21–7.41 (m, 8H, Ph). Mp-Phe-OBz was deprotected using a procedure adapted from Maring et al.[48]. Mp-Phe-OBz (0.600 g, 1.52 mmol) was dissolved in ethanol (100 mL) and degassed. Ammonium formate (0.957 g, 15.171 mmol) and Pd/C (120 mg) were added. The reaction was refluxed for 40 min. The resulting solution was filtered over Celite and the solvent was removed under reduced pressure. The product was isolated as a white powder with a yield of 85.3%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.37 (s, 3H, CH_3N), 2.54 (broad, 4H, $CH_2N(CH_3)CH_2$), 3.01 (m, 1H, CH_2Ph), 3.18 (m, 1H, CH_2Ph), 3.34–3.45 (m, 4H, CH_2NCH_2), 4.44 (m, 1H, α-H), 5.73 (d, 1H, NH), 7.12–7.26 (m, 5H, Ph). Acid peak not observed.

General procedure for peptide coupling reaction using Mu-Phe-OH. Phenyl (3S)-3-(N-morpholine-4-carboxamido phenylalanyl)amino-5-phenylpent-1-enylsulfone(7,Mu-Phe-L-Hfe-VS-Ph) TFA salt **1** (TFA·*D*-Hfe-VS-Ph) (2.89 g, 6.95 mmol) was dissolved in DCM (120 mL) and Et_3N (1.06 g, 10.4 mmol) was added. Compound **5** (Mu-Phe-OH) (1.99 g, 6.95 mmol) was dissolved in DCM (80 mL) and added to the reaction followed by HOBT (1.03 g, 7.64 mmol) and EDCl (1.19 g, 7.64 mmol). Additional Et_3N was added to adjust the pH to between 9 and 10. The reaction was stirred overnight. The mixture was washed with aq. HCl (3N, 3 × 50 mL), aq. $NaHCO_3$ (10%, 3 × 50 mL), brine (3 × 50 mL) and dried ($MgSO_4$). The solvent was removed under reduced pressure. The product was purified via column chromatography (silica, 1% MeOH in DCM) and isolated as a white powder with an 86.1% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.79–1.89 (m, 2H, CH_2CH_2Ph), 2.57 (m, 2H, CH_2CH_2Ph), 3.05 (m, 2H, CH_2Ph), 3.28 (m, 4H, CH_2NCH_2), 3.62 (m, 4H, CH_2OCH_2), 4.47 (q, 1H, α-H), 4.62 (s, broad, 1H, α-H), 4.96 (m, 1H, NH), 6.10 (dd, 1H, CH=*CH*-SO_2, *J* = 15 Hz, 2 Hz), 6.39 (d, 1H, NH), 6.80 (dd, 1H, *CH*=CH-SO_2, *J* = 15 Hz, 8 Hz), 7.01–7.33 (m, 10H, 2 × Ph), 7.50–7.91 (m, 5H, Ph-SO_2). ^{13}C NMR (100 MHz, $CDCl_3$) δ 31.71, 35.68, 38.14, 43.96, 49.13, 56.19, 66.31, 126.35, 127.31, 127.68, 128.33, 128.58, 128.62, 128.92, 129.15, 129.21, 129.30, 130.65, 133.50, 136.62, 145.26, 157.13, 171.51.

Synthesis of phenyl (3R)-3-(N-morpholine-4-carboxamidophenylalanyl)amino-5-phenylpent-1-enyl sulfone. (8, Mu-Phe-D-Hfe-VS-Ph) TFA salt **2** (TFA·*L*-Hfe-VS-Ph) was used as the starting material and the general peptide coupling procedure was followed. The product was purified via recrystallization (1:3 Hex: EtOAc) and isolated as white crystals with an 84.6% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.68–1.82 (m, 2H, CH_2CH_2Ph), 2.41 (t, 2H, CH_2CH_2Ph), 3.07 (m, 2H, CH_2Ph), 3.25 (m, 4H, CH_2NCH_2), 3.63 (m, 4H, CH_2OCH_2), 4.42 (q, 1H, α-H), 4.62 (s, broad, 1H, α-H), 4.88 (d, 1H, NH), 6.18 (d, 1H, NH), 6.52 (d, 1H, CH=*CH*-SO_2), 6.79 (dd, 1H, CH=*CH*-SO_2, *J* = 15 Hz, 5 Hz) 7.05–7.37 (m, 10H, 2 × Ph), 7.50–7.93 (m, 5H, Ph-SO_2). ^{13}C NMR (100 MHz, $CDCl_3$) δ 31.69, 35.49, 37.86, 43.89, 49.20, 56.25, 66.29, 126.31, 127.21, 127.64, 128.34, 128.57, 128.84, 129.21, 129.29, 130.73, 133.44, 136.67, 140.26, 140.32, 145.42, 157.21, 171.54.

General procedure for peptide coupling reaction using Cbz-Phe-OH. Phenyl (3S)-3-(N-carbobenzyloxyphenylalanyl) amino-4-phenylbut-1-enyl sulfone (9, Cbz-Phe-L-Phe-VS-Ph) TFA salt **3** (TFA·*L*-Phe-VS-Ph) (0.946 g, 2.36 mmol) was dissolved in DCM (75 mL). Et_3N (0.572 g, 2.76 mmol) was added, followed by Cbz-Phe-OH (0.776 g, 2.59 mmol), EDCl (0.497 g, 2.59 mmol) and HOBT (0.350 g, 2.59 mmol). Additional Et_3N was added to adjust the pH to between 9 and 10. The reaction was stirred overnight. The reaction was washed with citric acid (10%, 3 × 25 mL), aq. $NaHCO_3$ (10%, 3 × 25 mL), brine (2 × 25 mL) and dried ($MgSO_4$). The solvent was removed under reduced pressure. The product was purified via column chromatography (silica, 1:1 Hex: EtOAc) as a white powder with a 36.4% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.81 (d, 2H, CH_2Ph), 2.95–3.05 (m, 2H, CH_2Ph), 4.29 (q, 1H, α-H), 4.93 (q, 1H, α-H), 5.07 (s, 2H, OCH_2Ph), 5.14 (m, 1H, NH), 5.73 (d, 1H, NH), 5.95 (d, 1H, CH=*CH*-SO_2), 6.80 (dd, 1H, *CH*=CH-SO_2, *J* = 15 Hz, 5 Hz), 6.99–7.46 (m, 15H, 3 × Ph), 7.52–7.83 (m, 5H, *Ph*-SO_2).

Synthesis of phenyl (3R)-3-(N-carbobenzyloxyphenylalanyl) amino-4-phenylbut-1-enyl sulfone (10, Cbz-Phe-D-Phe-VS-Ph) See the above procedure for peptide coupling reaction using Cbz-Phe-OH. TFA salt **4** (TFA·*D*-Phe-VS-Ph) was used as starting material. The product was purified via column chromatography (silica, 1:1 Hex: EtOAc) as a white powder with a 64.1% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.61–3.14 (m, 4H, 2 × CH_2Ph), 4.33 (q, 1H, α-H), 4.89–5.15 (m, 3H, α-H and CH_2OPh), 5.34 (m, 1H, NH), 6.19 (d, 1H, NH), 6.32 (d, 1H, CH=*CH*-SO_2), 6.80–7.44 (m, 16 H, 2 × CH_2Ph, O*Ph*, *CH*=CH-SO_2), 7.47–7.87 (m, 5H, Ph-SO_2). ^{13}C NMR (100 MHz, $CDCl_3$) δ 38.16, 39.90, 40.16, 50.27, 56.54, 67.22, 127.21, 127.26, 127.67, 128.07, 128.11, 128.32, 128.60, 128.71, 128.81, 129.16, 129.27, 129.31, 131.15, 133.48, 135.28, 136.06, 140.03, 144.75, 170.48.

General procedure for peptide coupling using Mp-Phe-OH. Phenyl (3S)-3-(N-(N-methylpiperazine)-4-carboxamidophenylalanyl)amino-5-phenylpent-1-enyl sulfone (11, Mp-Phe-L-Hfe-VS-Ph) Compound **6** (Mp-Phe-OH) (0.874 g, 1.23 mmol) was dissolved in 3:1 THF/DMF (40 mL) at –10°C. NMM (0.137 g, 1.35 mmol) was added and the reaction was stirred for 20 min with the gradual addition of *i*BCF (0.185 g, 1.35 mmol). The reaction was stirred for 1 h maintaining a temp below –5°C. TFA salt **1** (TFA·*L*-Hfe-VS-Ph) was added at –10°C. Additional NMM (0.137g, 1.35 mmol) was added and the reaction was stirred for 1 h at –10°C. The reaction was raised to room temperature and stirred overnight. Aq. $NaHCO_3$ (10%, 200 mL) was added and the product was extracted using EtOAc (3 × 40 mL). The organic layers were combined and washed with brine (2 × 50 mL) and dried ($MgSO_4$). The solvent was removed under reduced pressure and the crude product was purified by column chromatography (silica, 5% MeOH in DCM, 0.5% Et_3N). The product was isolated as a clear colorless syrup with a 32.2% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.74 (m, 1H, CH_2CH_2Ph), 1.85 (m, 1H, CH_2CH_2Ph), 2.25 (s, 3H, CH_3N), 2.31 (m, 4H, $CH_2N(CH_3)CH_2$), 2.54 (m, 2H, CH_2CH_2Ph), 3.00 (m, 2H, CH_2Ph), 3.28 (m, 4H, CH_2NCH_2), 4.58 (broad, 2H, α-H, α-H), 5.23 (d, 1H, NH), 6.10 (dd, 1H, CH=*CH*-SO_2, *J* = 15 Hz, 2 Hz), 6.77 (dd, 1H, *CH*=CH-SO_2, *J* = 15 Hz, 8 Hz), 7.01–7.33 (m, 11H, 2 × Ph, NH), 7.50–7.91 (m, 5H, Ph-SO_2). ^{13}C NMR (100 MHz, $CDCl_3$) δ 31.77, 35.67, 38.57, 43.73, 46.02, 49.07, 54.45, 55.96, 126.22, 127.08, 127.63, 128.38, 128.52, 128.64, 129.28, 129.30, 130.32, 133.48, 136.67, 140.18, 140.47, 145.83, 156.95, 172.01. MS (ESI) *m/z* 575.2 $[M+H]^+$.

Synthesis of phenyl (3R)-3-(N-(N-methylpiperazine)-4-carboxamidophenylalanyl)amino-5-phenylpent-1-enyl sulfone (12, Mp-Phe-D-Hfe-VS-Ph) See general procedure for peptide coupling using Mp-Phe-OH. TFA salt **2** (TFA·*D*-Hfe-VS-Ph) was used as the starting material. The crude product was purified using column chromatography (silica, 5% MeOH in DCM, 0.5% Et_3N). The product was isolated as a clear colorless syrup with a 22% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.68 (m, 1H, CH_2CH_2Ph), 1.80 (m, 1H, CH_2CH_2Ph), 2.32 (s, 3H, CH_3N), 2.41 (m, 6H, $CH_2N(CH_3)CH_2$ and CH_2CH_2Ph), 3.07 (m, 2H, CH_2Ph), 3.32 (m, 4H, CH_2NCH_2), 4.41 (m, 1H, α-H), 4.62 (m, 1H, α-H), 4.91 (d, 1H, NH), 6.33 (d, 1H, NH) 6.52 (dd, 1H, CH=*CH*-SO_2, *J* = 15 Hz, 2 Hz), 6.85 (dd, 1H, *CH*=CH-SO_2, *J* = 15 Hz, 8 Hz), 7.01–7.33 (m, 10H, 2 × Ph), 7.50–7.91 (m, 5H, Ph-SO_2). ^{13}C NMR (100 MHz, $CDCl_3$) δ 31.7, 35.52, 37.76, 43.65, 46.00, 49.17, 54.41, 56.28, 60.41, 126.27, 127.16, 127.64, 128.36, 128.56, 128.82, 129.23, 129.28, 130.68, 133.43, 136.76, 140.37, 145.5, 157.04, 171.68.

Synthesis of phenyl (3S)-3-(N-(N-methylpiperazine)-4-carboxamidophenylalanyl)amino-5-phenylbut-1-enyl sulfone (13, Mp-Phe-L-Phe-VS-Ph) See general procedure for peptide coupling using Mp-Phe-OH. TFA salt **3** (TFA·*L*-Phe-VS-Ph) was used as the starting material. The crude product was purified using column chromatography (silica, 5% MeOH in DCM, 0.5% Et_3N). The product was isolated as a clear colorless syrup with a 45.1% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.33 (m, 7H, $CH_2N(CH_3)CH_2$ and $CH_2N(CH_2)CH_2$), 2.85 (dd, 2H, CH_2Ph, *J* = 6 Hz and 3 Hz), 3.01 (m, 2H, CH_2Ph), 3.28 (m, 4H, CH_2NCH_2), 4.41 (q, 1H, α-H), 4.76 (d, 1H, NH), 4.93 (m, 1H, α-H), 6.03 (dd, 1H, CH=*CH*-SO_2, *J* = 15 Hz, 2Hz), 6.22 (d, 1H, NH), 6.84 (dd, 1H, *CH*=CH-SO_2), *J* = 15 Hz, 5 Hz), 7.01–7.30 (m, 10H, 2 × Ph), 7.50–7.83 (m, 5H, *Ph*-SO_2). ^{13}C NMR (100 MHz, $CDCl_3$) δ 37.97, 40.17, 43.67, 54.48, 55.89, 127.07, 127.22, 127.68, 128.62, 128.68, 128.87, 129.16, 129.24, 129.29, 129.35, 130.86, 133.44, 135.51, 136.73, 140.11, 144.76, 156.78, 171.36.

Preparation of allyl sulfones: General procedure for vinyl sulfone isomerization. Phenyl (E)-3-(N-morpholine-4-carbox amidophenylalanyl)amino-5-phenylpent-2-enyl sulfone (14, E-Mu-Phe-Hfe-AS-Ph) To dry THF (40 mL), *t*-BuOOH (4.01 mmol, 1.2 mL, 3.3 M in toluene) was added dropwise under argon at –78°C, followed by *n*-BuLi (1.02 mmol, 0.65 mL, 1.6 M in hexane). Compound **7** (Mu-Phe-*L*-Hfe-VS-Ph) (0.500 g, 0.890 mmol) was dissolved in dry THF (5 mL) and added dropwise. The reaction was allowed to warm to –20°C and stirred for 9 h. The reaction was quenched with aq. NH_4Cl (10%, 50 mL) and the aqueous layer was extracted with EtOAc (3 × 50 mL). The organic layers were combined and washed with water (3 × 50 mL), brine (2 × 50 mL) and dried ($MgSO_4$). The solvent was removed under reduced pressure and the crude product was purified via column chromatography (silica, 1% MeOH in DCM). The product was isolated as a white powder with a 6.7% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.71 (t, 2H, CH_2CH_2Ph), 2.87 (t, 2H, CH_2CH_2Ph), 3.21 (m, 2H, CH_2Ph), 3.37 (m, 4H, CH_2NCH_2), 3.60 (dd, 2H, CH_2SO_2, *J* = 2 Hz, 8 Hz), 3.67 (m, 4H, CH_2OCH_2), 4.64 (m, 2H, α-H and C=CH), 4.84 (d, 1H, NH), 7.13–7.40 (m, 10H, 2 × Ph), 7.45–7.66 (m, 5H, Ph-SO_2), 8.67 (s, broad, 1H, NH). ^{13}C NMR (100 MHz, $CDCl_3$) δ 33.71, 35.77, 37.72, 43.79, 54.47, 56.29, 66.30, 103.83, 126.00, 127.41, 128.27, 128.43, 128.63, 129.07, 129.13, 129.28, 133.83, 136.59, 138.33, 141.15, 145.45, 156.89, 171.05. MS (ESI) *m/z* 562.2 $[M+H]^+$.

Synthesis of phenyl (Z)-3-(N-morpholine-4-carboxamido phenylalanyl)amino-5-phenylpent-2-enyl sulfone (15, Z-Mu-Phe-Hfe-AS-Ph) See above procedure for vinyl sulfone isomerization. Compound **8** (Mu-Phe-*D*-Hfe-VS-Ph) was used as the starting material. The crude product was purified via column chromatography (silica, 1% MeOH in DCM) and isolated as a white powder with an 8.6% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.71 (t, 2H, CH_2CH_2Ph), 2.87 (t, 2H, CH_2CH_2Ph), 3.21 (m, 2H, CH_2Ph), 3.37 (m, 4H, CH_2NCH_2), 3.60 (dd, 2H, CH_2SO_2, *J* = 1 Hz, 8 Hz), 3.67 (m, 4H, CH_2OCH_2), 4.64 (m, 2H, α-H and C=CH), 4.84 (d, 1H, NH), 7.13–7.40 (m, 10H, 2 × Ph), 7.45–7.66 (m, 5H, *Ph*-SO_2), 8.67 (s, broad, 1H, NH). ^{13}C NMR (100 MHz, $CDCl_3$) δ 33.71, 35.77, 37.72, 43.79, 54.47, 56.29, 66.30, 103.83, 126.00, 127.41, 128.27, 128.43, 128.63, 129.07, 129.13, 129.28, 133.83, 136.59, 138.33, 141.15, 145.45, 156.90, 171.06. MS (ESI) *m/z* 562.2 $[M+H]^+$.

Synthesis of phenyl (E)-3-(N-carbobenzyloxyphenylalanyl) amino-4-phenylbut-2-enyl sulfone (16, E-Cbz-Phe-Phe-AS-Ph) See above procedure for vinyl sulfone isomerization. Compound **9** (Cbz-Phe-*L*-Phe-VS-Ph) was used as the starting material. The reaction was stirred for 70 min at –20°C. The crude product was purified via column chromatography (silica, 2:1 Hex: EtOAc). The final compound was isolated as a white powder with a 15.8% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.03 (m, 2H, CH_2Ph), 3.67–3.89 (m, 4H, $PhCH_2C{=}CHCH_2SO_2$), 4.35 (d, 1H, α-H), 4.83 (t, 1H, C=CH), 5.12 (s, broad, 3H, NH, OCH_2Ph), 7.09–7.39 (m, 15H, 3 × Ph), 7.45–7.75 (m, 5H, *Ph*-SO_2). ^{13}C NMR (100 MHz, $CDCl_3$) δ 37.75, 40.14, 54.47, 56.77, 67.40, 106.61, 126.76, 127.29, 128.24, 128.26, 128.29, 128.48, 128.53, 128.98, 129.16, 129.17, 129.24, 133.93, 135.95, 135.97, 137.36, 138.31, 144.45, 156.02, 170.11.

Synthesis of phenyl (Z)-3-(N-carbobenzyloxyphenylalanyl) amino-4-phenylbut-2-enyl sulfone (17, Z-Cbz-Phe-Phe-AS-Ph) See above procedure for vinyl sulfone isomerization. Compound **10** (Cbz-Phe-*D*-Phe-VS-Ph) was used as the starting material. The reaction was stirred for 70 min at –20°C. The product was purified by column chromatography (silica, 2:1 Hex: EtOAc) and isolated as a white powder with a 16.7% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.03 (m, 2H, CH_2Ph), 3.67–3.89 (m, 4H, $PhCH_2C{=}CHCH_2SO_2$), 4.35 (d, 1H, α-H), 4.83 (t, 1H, C=CH), 5.12 (s, broad, 3H, NH, OCH_2Ph), 7.09–7.39 (m, 15H, 3 × Ph), 7.45–7.75 (m, 5H, Ph-SO_2). ^{13}C NMR (100 MHz, $CDCl_3$) δ 37.76, 40.16,

54.49, 56.80, 67.38, 106.67, 126.77, 127.28, 128.24, 128.29, 128.48, 128.54, 128.60, 128.62, 128.97, 129.16, 129.18, 129.25, 133.94, 136.00, 137.37, 138.31, 144.38, 156.07, 170.1.

Synthesis of phenyl (E)-3-(N-(N-methylpiperazine)-4-carboxamidophenylalanyl)amino-5-phenylpent-2-enyl sulfone (18, E-Mp-Phe-Hfe-AS-Ph) See above procedure for vinyl sulfone isomerization. Compound **11** (Mp-Phe-*L*-Hfe-VS-Ph) was used as the starting material. The reaction was stirred for 8 h at –20°C and stored at –20°C for an additional 12 h. The crude product was purified via column chromatography (silica, 3% MeOH in DCM, 0.5% Et_3N) and isolated as a white powder with a yield of 8.2%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.29 (s, 3H, $CH_2N(CH_3)CH_2$), 2.37 (m, 4H, $CH_2N(CH_3)CH_2$), 2.69 (t, 3H, CH_2CH_2Ph), 2.83 (t, 3H, CH_2CH_2Ph), 3.18 (m, 2H, CH_2Ph), 3.41 (m. 4H, CH_2NCH_2), 3.59 (m, 2H, CH_2-SO_2), 4.61 (q, 1H, α-H), 4.67 (t, 1H, C=*CH*), 4.86 (d, 1H, NH), 7.13–7.40 (m, 10H, 2 × Ph), 7.44–7.68 (m, 5H, *Ph*-SO_2), 8.61 (s, 1H, NH).^{13}C NMR (100 MHz, $CDCl_3$) δ 33.73, 35.88, 37.72, 43.62, 46.06, 54.44, 54.49, 56.37, 103.91, 125.97, 127.32, 128.30, 128.42, 128.62, 129.01, 129.10, 129.32, 133.78, 136.68, 138.44, 141.16, 145.20, 156.84, 171.13. MS (ESI) *m/z* 575.2 $[M+H]^+$.

Synthesis of phenyl (Z)-3-(N-(N-methylpiperazine)-4-carboxamidophenylalanyl)amino-5-phenylpent-2-enyl sulfone (19, Z-Mp-Phe-Hfe-AS-Ph) See above procedure for vinyl sulfone isomerization. Compound **12** (Mp-Phe-*D*-Hfe-VS-Ph) was used as the starting material. The reaction was stirred for 8 h at –20°C and stored at –20°C for an additional 12 h. The crude product was purified via column chromatography (silica, 5% MeOH in DCM, 0.5% Et_3N) and isolated as a white powder with a 6.7% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.28 (s, 3H, $CH_2N(CH_3)CH_2$), 2.37 (m, 4H, $CH_2N(CH_3)CH_2$), 2.68 (t, 3H, CH_2CH_2Ph), 2.83 (t, 3H, CH_2CH_2Ph), 3.18 (m, 2H, CH_2Ph), 3.39 (m, 4H, CH_2NCH_2), 3.59 (m, 2H, CH_2-SO_2), 4.61 (q, 1H, α-H), 4.67 (t, 1H, C=*CH*), 4.86 (d, 1H, NH), 7.13–7.40 (m, 10H, 2 × Ph), 7.44–7.68 (m, 5H, *Ph*-SO_2), 8.61 (s, 1H, NH). ^{13}C NMR (100 MHz, $CDCl_3$) δ 33.74, 35.88, 37.71, 43.62, 45.92, 53.44, 54.44, 56.37, 103.89, 125.97, 127.32, 128.30, 128.42, 128.62, 129.01, 129.10, 129.32, 133.78, 136.68, 138.45, 141.16, 145.20, 156.84, 171.12. MS (ESI) *m/z* 575.2 $[M+H]^+$.

Synthesis of phenyl (E)-3-(N-(N-methylpiperazine)-4-carboxamidophenylalanyl)amino-5-phenylbut-2-enyl sulfone (20, E-Mp-Phe-L-Phe-AS-Ph) See above procedure for vinyl sulfone isomerization. Compound **13** (Mp-Phe-*L*-Phe-VS-Ph) was used as the starting material. The reaction was stirred for 3 h at –20°C. The product was purified via column chromatography (silica, 3% MeOH in DCM, 0.5% Et_3N) and isolated as a white powder with a yield of 7.2%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.27 (s, 3H, $CH_2N(CH_3)CH_2$), 2.34 (m, 4H, $CH_2N(CH_3)CH_2$), 2.93–3.18 (m, 2H, CH_2Ph), 3.32 (m, 4H, CH_2NCH_2), 3.61–3.91 (m, 4H, CH_2-SO_2 and CH_2Ph), 4.46 (q, 1H, α-H), 4.79 (m, 2H, α-H and NH), 7.06–7.41 (m, 10H, 2 × Ph), 7.46–7.85 (m, 5H, *Ph*-SO_2), 8.45 (s, 1H, NH). ^{13}C NMR (100 MHz, $CDCl_3$) δ 37.58, 40.20, 43.60, 52.93, 54.40, 54.63, 56.25, 105.78, 126.65, 127.16, 128.29, 128.42, 128.89, 129.12, 129.21, 129.24, 133.84, 136.64, 137.57, 138.45, 144.39, 156.83, 171.15. MS (ESI) *m/z* 561.2 $[M+H]^+$.

Procedure for elimination of Mu-Phe-Hfe-VS-Ph. (2S)-N-(5-phenylpent-1-en-3-ylidene)-2-(N-morpholine-4-carboxamidophenylalanyl)amino-3-phenylpropanamide. (21, Mu-Phe-Hfe-Elim) See general procedure for isomerization of the vinyl sulfone. Compound **7** was used as the starting material. The reaction was allowed to warm to room temperature and instead of adding both *n*-BuLi and *t*-BuOOH at –78°C, only *n*-BuLi was added. The product was purified by column chromatography (silica, 3% MeOH in DCM) and isolated as a white powder with a 6.4% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.13 (m, 1H, CH_2CH_2Ph), 2.44 (m, 2H, 1H of CH_2CH_2Ph, 1H of CH_2CH_2Ph), 2.63 (m, 1H, CH_2CH_2Ph), 2.92 (m, 2H, CH_2NCH_2), 3.11 (m, 1H, CH_2Ph), 3.26 (m, 3H, 1H of CH_2Ph, 2H, of CH_2NCH_2), 3.52 (m, 2H, CH_2OCH_2), 3.63 (m, 2H, CH_2OCH_2), 4.50 (t, 1H, α-H), 5.02 (m, 2H, CH=CH_2), 6.11 (dd, 1H, *CH*=CH_2, *J* = 19 Hz, 11 Hz), 7.10–7.35 (m, 10H, 2 × Ph), 7.60 (s, broad, 1H, NH). ^{13}C NMR (100 MHz, $CDCl_3$) δ 29.60, 37.41, 37.71, 46.80, 61.97, 66.38, 78.62, 114.24, 126.25, 127.02, 128.32, 128.60, 129.77, 129.82, 136.55, 140.60, 140.97, 159.09, 172.24. MS (ESI) *m/z* 442.2 $[M+Na]^+$.

Enzyme assays

General assay parameters

All assays used the incubation method to measure the irreversible inhibition of the specified enzyme[49]. Enzyme activity was observed via monitoring hydrolysis of various fluorogenic 7-amino-4-methylcoumarin (AMC) substrates using a BioTek Flx800 Plate Reader (λ_{ex} = 360 nm, λ_{em} = 460 nm). Enzyme activity was measured at the specified incubation temperature. All assay conditions contained less than 8.3% dimethylsulfoxide (DMSO). Pseudo first-order inactivation rate constants were obtained from ln v_t/v_0 versus time plots.

Cathepsin B Assay[40] Human liver cathepsin B (0.259 mg/mL) was purchased from Calbiochem. A D-10 dilution of cathepsin B was prepared with a 5 µL aliquot of the stock enzyme and 45 µL of deionized water. A D-100 activation dilution was prepared by combining 267 µL kinetic buffer (0.1 M potassium phosphate, 1.25 mM EDTA, 0.01% Brij 35 at pH 6.0), 30 µL of the D-10 solution and 3 µL of 0.1 M dithiothreitol (DTT) (freshly prepared). The D-100 activation dilution was allowed to activate for 1 h at 0°C. The incubation solution contained 30 µL of inhibitor stock (10 µM-5 mM in DMSO), 300 µL kinetic buffer and 30 µL of D-100 enzyme prep at 25°C. Aliquots (50 µL) of the incubation solution were withdrawn at varying intervals and combined with 200 µL of substrate buffer (500 µM Cbz-Arg-Arg-AMC [Calbiochem Cathepsin B Substrate III],

0.25% DMSO v/v, 0.1 M potassium phosphate, 1.25 mM EDTA, 0.01% Brij at pH 6.0,) for assaying.

Calpain I Assay[40] Calpain I from porcine erythrocytes (1.9 mg/mL) was purchased from Calbiochem. The incubation buffer was prepared from 2883 µL of HEPES pH 7.5 buffer, 39 µL of $CaCl_2$ (0.5 M) and 78 µL of 0.5 M cysteine (freshly prepared). The incubation solution contained 30 µL inhibitor stock (10 µM–5 mM in DMSO), 30 µL enzyme stock (1.9 mg/mL) and 300 µL of incubation buffer at 25°C. Aliquots (50 µL) of the incubation solution were withdrawn at varying intervals and combined with 200 µL of substrate buffer (9524 µL HEPES pH 7.5 buffer, 80 µL 0.2 M Suc-Leu-Tyr-AMC in DMSO [Calbiochem Calpain I Substrate II], 214 µL 0.5 M cysteine and 107 µL 0.5 M $CaCl_2$) for assaying.

Cruzain Assay The enzyme stock (50 µM) was diluted 2000 fold (25 nM) using assay buffer (100 mM sodium acetate pH 5.5, 5 mM DTT, 0.001% Triton X-100, freshly prepared). The incubation solution contained 12.5 µL inhibitor stock (10 µM-5 mM in DMSO), 12.5 µL D-2000 enzyme solution and 350 µL assay buffer. Aliquots (50 µL) of the incubation solution were withdrawn at varying intervals and combined with 200 µL of substrate buffer (4998 µL assay buffer and 2 µL 20 mMCbz-Phe-Arg-AMC in DMSO [Enzo Life Sciences]) for assaying.

Trypsin Assay[50] Trypsin from bovine pancreases was acquired from Sigma (Milwaukee, WI). The incubation solution contained 230 µL of buffer (50 mM sodium phosphate buffer, pH 8.0), 60 µL of trypsin (2.5 µM in buffer) and 10 µL inhibitor stock (10 µM-5 mM in DMSO) at 37°C. Aliquots (50 µL) of the incubation solution were withdrawn at varying intervals and combined with 200 µL of substrate buffer (4 µL of 22.7 mMCbz-Phe-Arg-AMC in DMSO [Enzo Life Sciences] in 2400 µL of buffer) for assaying.

Enzyme expression and purification

Cruzain

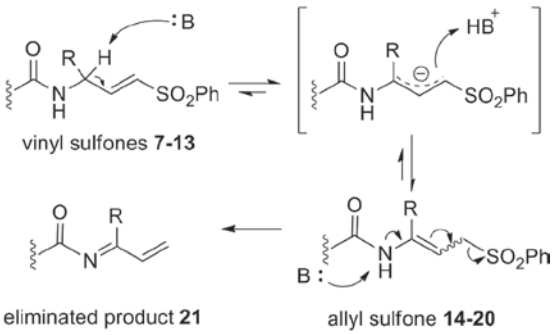
Cruzain was expressed and purified using a modified version (Lee G., Craik CS, unpublished results) of a previously published protocol[51], activated and purified as recently described[32].

To prevent self-degradation, active cruzain was inhibited with S-methyl methanethiosulfonate (MMTS), a covalent reversible inhibitor. MMTS inhibited cruzain was further concentrated to 50 µM in 100 mM MES pH 5.8, 500 mMNaCl and stored at –80 °C.

Results and discussion

Formation of the allyl sulfone

One of the key challenges in synthesizing the inhibitors was finding the correct vinyl-allyl sulfone isomerization conditions. The proposed mechanism of allyl isomerization occurs by deprotonation of the α-proton forming a delocalized intermediate (Scheme 2). Subsequent protonation of the carbon adjacent to the sulfone furnishes allyl sulfones **14**–**20**[52]. It was found that the choice of base, temperature and reaction duration were all significant factors affecting the isomerization. Previously reported methods of isomerization used KO*t*Bu[52], DBU[53] or *n*-BuLi/*t*-BuOOH[40]. The use of 1 eq. of *n*-BuLi and 4.5 eq. of *t*-BuOOH at –20°C resulted in nearly complete isomerization while the other bases, regardless of duration or temperature, produced a mixture of compounds which were not purified further. Hine et al.[52,54] demonstrated that with a relatively low free energy barrier of isomerization, an equilibrium exists between the two isomers (vinyl and allyl) resulting in incomplete isomerization. Along with this equilibrium, we observed an additional side product, formed by elimination of the sulfonyl moiety (Scheme 2). Elimination occurs after formation of the allyl sulfone, when the adjacent nitrogen is deprotonated, eliminating the sulfonyl group and forming an extended conjugated system. Higher temperatures (>–20°C) or fewer equivalents of *t*-BuOOH favoured elimination. Elimination was only observed for the Hfe-based compounds[7,8,11–13]. We purified one analogue side product (Mu-Phe-Hfe-Elim) and added it to the selection of compounds to be tested for inhibitor potency with the various proteases.



Scheme 2. Isomerization equilibrium and elimination mechanism.

We suspect that the acidic property of the *t*-BuOOH partially attenuates the strength of the *n*-BuLi, giving rise to a milder, buffered environment. This has not previously been reported in the literature. The mild conditions seem to favour the formation of the allyl sulfone while minimizing further elimination of the sulfonyl group. Addition of *n*-BuLi in absence of *t*-BuOOH exclusively results in complete elimination of the sulfonyl group, with no allyl sulfone intermediate observed. Chelation of Li^+[55] as well as the "syn-effect," forming a homoaromatic system between Li^+ and the delocalized anionic intermediate[53,56], may be factors that explain the requirement of *n*-BuLi instead of DBU or KO*t*Bu, assisting in the stabilization of the delocalized intermediate.

Reaction time was also a key factor in successful isomerization. Compounds **9**, **10** and **13** (*R*-Phe-*Phe*-VS-Ph) with a Phe residue in the P1 position required less

than two hours to isomerize while the remaining vinyl sulfones (**7**, **8**, **11** and **12**; *R*-Phe-*Hfe*-VS-Ph) with a Hfe residue in the P1 position required more than nine hours. This variation in reaction rate has been primarily attributed to sterics. The increased mobility introduced by the additional methylene of the Hfe residue, compared to the Phe side chain, allows the phenyl ring to impede access of the base to the α-proton, slowing the reaction rate and extending the required isomerization time.

Stereochemistry of allyl sulfones

Following the synthesis of allyl sulfones **14** and **15** (Mu-Phe-*L/D*-Hfe-AS-Ph), the compounds were assayed for inhibitory potency with cathepsin B and cruzain. Although the two compounds have identical 1D ^{1}H NMR spectra (all spectroscopic data is provided in the Supporting Appendix), they differ in their ability to inhibit the enzymes by an order of magnitude, indicative of a significant difference in structure (see Enzyme Kinetics section, Table 2). The only variation in structure that would be undetectable by ^{1}H NMR is the stereochemistry of the allyl sulfone double bond. Because of the presence of only a single proton on the double bond, no distinctive *J*-coupling is produced with either *E* or *Z* configurations. This difference in stereochemistry arises during the isomerization process. With a different spatial orientation, the steric bulk of the P1 residue side chain can either favour *E* or *Z* configuration.

In an effort to deduce the stereochemistry of both analogues, we used Nuclear Overhauser Effect Spectroscopy (NOESY) experiments based on the difference in the spatial arrangement around the double bond, which should produce unique spatial magnetic coupling. The *E* and *Z* isomers of the Mu-Phe-Hfe-AS-Ph structure were modelled using molecular dynamics calculations by MOE to predict Nuclear Overhauser Effect (NOE) potentials. Jones et al.[57] have demonstrated that using energetically minimized models to predict NOE effects produced less than 4% error from the experimentally observed NOE. An MMFF94x potential was used to minimize the energetics of the model molecules using a solvation parameter based on distance with a relative dielectric constant of 4.801 to match the solvation of the samples in chloroform during NMR acquisition. Distances were measured between the alkene vinyl proton and the benzyl methylene of the Hfe side chain. Inter-proton distances of 4.01 and 2.22 Å were calculated for the *E* and *Z* isomers, respectively (Figure 2). Based on this information, a weak NOE should be observed for the *E*-isomer (3.5–5 Å) while the *Z*-isomer should display a strong NOE (<2.5 Å)[58].

We performed 2D NOESY experiments on the P1 Hfe-based allyl sulfones (**14**, **15**, **18** and **19**). This method could not be applied to the P1 Phe-based compounds (**16**, **17** and **20**). With only a single methylene group directly adjacent to the allyl sulfone double bond, long range *J*-coupling between the vinyl proton and benzyl methylene of the Phe made the NOE indistinguishable from the residual COSY cross peaks. However, with an additional carbon present between the vinyl proton and the benzyl methylene in the P1 Hfe-based compounds, long range *J*-coupling did not disrupt the resolution of the NOE.

We focused on the cross peak magnitude between the 2.71 and 4.64 ppm resonances, representing the spatial coupling between the protons of benzyl methylene of the Hfe residue and the vinyl proton, respectively. The difference in spectra is shown in Figure 3 (**14** in blue and **15** in red). The individual peaks were integrated using equivalent contour levels and normalized against multiple residual COSY peaks present in each spectrum (Table 1). The allyl sulfones formed from the *L*-Hfe vinyl sulfones **14** and **18** display a 30% weaker NOE compared with their *D*-Hfe analogues **15** and **19**. This allowed us to conclude that the allyl sulfones derived from the *D*-Hfe vinyl sulfone analogues exhibit *Z* configuration while those derived from the *L*-Hfe vinyl sulfone analogues possess *E* configuration.

Enzyme kinetics

The synthesized dipeptidyl allyl sulfones **14**–**20** were assayed with cathepsin B, cruzain, calpain I and trypsin using fluorogenic enzyme assays and the incubation method. Pseudo-first order kinetics were applied to calculate $k_{obs}/[I]$ values (Table 2)[59]. The Cbz-containing compounds **16,17** unfortunately were insoluble in the

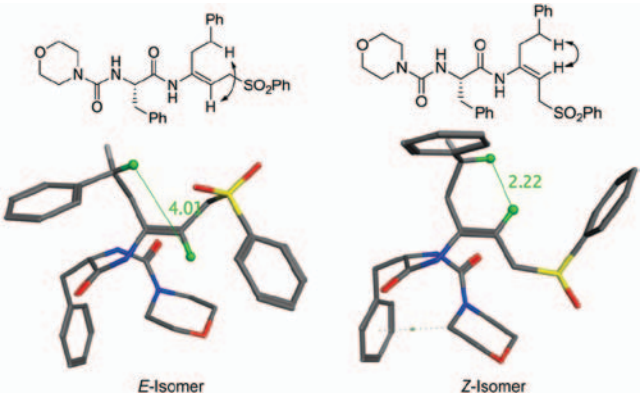


Figure 2. Energy minimized models of the *E* and *Z* isomers of Mu-Phe-Hfe-AS-Ph.

Table 1. Quantification of relative NOE magnitude and the deduced stereochemistry.

Compound	Structure	Vinyl sulfone precursor	Normalized NOE	Configuration
14	Mu-Phe-Hfe-AS-Ph	Mu-Phe-*L*-Hfe-VS-Ph	0.688	*E*
15	Mu-Phe-Hfe-AS-Ph	Mu-Phe-*D*-Hfe-VS-Ph	1.00	*Z*
18	Mp-Phe-Hfe-AS-Ph	Mp-Phe-*L*-Hfe-VS-Ph	0.749	*E*
19	Mp-Phe-Hfe-AS-Ph	Mp-Phe-*D*-Hfe-VS-Ph	1.00	*Z*

assays. All the remaining compounds **14,15,18–21** displayed time dependent, irreversible inhibition.

All inhibitors **14,15,18–21** demonstrated specificity for cysteine proteases, with no inhibition observed for trypsin, a serine protease with similar substrate specificity as cruzain. The same fluorogenic substrate, Cbz-Phe-Arg-AMC, was used to assay both enzymes. Additionally, specificity for clan CA, family C1 was observed with negligible inhibition (<10 $M^{-1}s^{-1}$) of calpain I (family C2). Within clan CA, all of the allyl sulfones (**14**, **15**, **18** and **20**) showed approximately 10-fold selectivity for cruzain over cathepsin B except compound **19** (*Z*-Mp-Phe-Hfe-AS-Ph), which demonstrated no selectivity between the two enzymes (200 ± 40 $M^{-1}s^{-1}$ for cathepsin B and 130 ± 10 $M^{-1}s^{-1}$ for cruzain).

Comparing the potencies of inhibitors **14** (P3 = Mu) versus **18** (P3 = Mp), the P3 group exhibited almost negligible effect on potency for cathepsin B (420 ± 110 $M^{-1}s^{-1}$ for **14** versus 570 ± 20 $M^{-1}s^{-1}$ for **18**). In contrast, the Mu substituent achieved a 3-fold increase in potency for cruzain compared with the Mp group (6080 ± 1390 $M^{-1}s^{-1}$ for **14** versus 2310 ± 230 $M^{-1}s^{-1}$ for **18**).

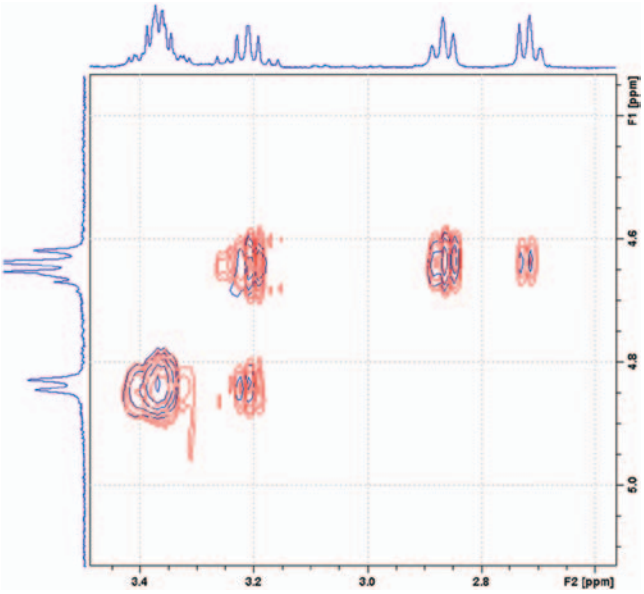


Figure 3. NOESY comparison of **14** (blue) and **15** (red).

The allyl sulfone double bond stereogenicity also contributed significantly to inhibitory potency. Examining *E*-allyl sulfones (**14** and **18**) and *Z*-allyl sulfones (**15** and **19**), the *E* stereochemistry resulted in an order of magnitude greater $k_{obs}/[I]$ values for all cysteine proteases tested. Comparing isomers **14** and **15**, $k_{obs}/[I]$ values for cruzain were 6080 ± 1390 $M^{-1}s^{-1}$ and 260 ± 80 $M^{-1}s^{-1}$, respectively. A similar difference in potency was observed for cathepsin B. Diastereomers **18** and **19** displayed the same trend as well.

The P1 amino acid residue selection also played a key role in inhibitor potency. Although the stereochemistry of compound **20** (Mp-Phe-Phe-AS-Ph) could not be determined using the previously discussed NOE method (section 2.2), the kinetic data provides a clear indication that regardless of double bond stereogenicity, the Phe residue in the P1 position resulted in a decrease in potency. For cruzain, compound **20** had an inhibition rate constant of 45 ± 1 $M^{-1}s^{-1}$. With the assumption that compound **20** would follow the same isomerization mechanism as other allyl sulfones formed from vinyl sulfones with *L*-stereochemistry in their P1 residue, it should likely have *E* configuration. In that case, it is 100-fold less potent than its P1 Hfe analogue **18** (2310 ± 230 $M^{-1}s^{-1}$). Even if the above assumption is not valid and compound **20** adopted *Z* stereochemistry, the *Z*-Hfe analogue **19** still remains more potent (130 ± 10 $M^{-1}s^{-1}$). The same trends were observed with cathepsin B with an even greater decrease in potency with the presence of the P1 Phe residue. Further optimization of additional analogues with P1 Phe residues as well as investigation into absolute stereochemistry was abandoned.

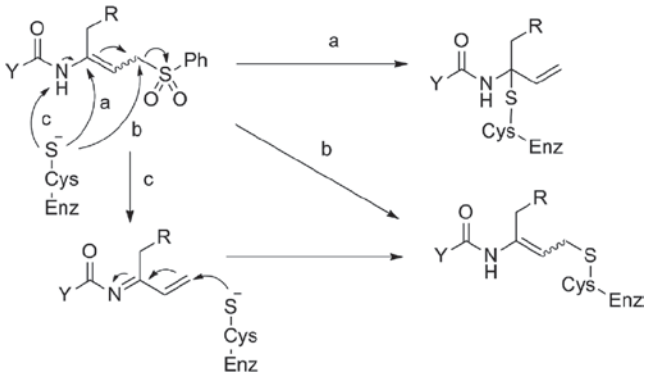
The most potent of the synthesized inhibitors was compound **14** (*E*-Mu-Phe-Hfe-AS-Ph) with a $k_{obs}/[I]$ of 6080 ± 1390 $M^{-1}s^{-1}$ for cruzain. For comparative purposes, compound **11** (Mp-Phe-*L*-Hfe-VS-Ph, also K11777)[42] was assayed with cruzain (221000 ± 8000 $M^{-1}s^{-1}$) and displayed 10-fold selectivity over cathepsin B (39300 ± 1300 $M^{-1}s^{-1}$). Although none of the inhibitors were able to achieve the potency of K11777, equivalent selectivity was maintained.

The precise mechanism of inhibition of the allyl sulfone moiety remains unknown. We have previously proposed possible irreversible mechanisms (Scheme 3)[40]

Table 2. Inhibitor potencies for proteases.

Compound	Inhibitor	$k_{obs}/[I]$ ($M^{-1}s^{-1}$)			
		Cathepsin B	Cruzain	Calpain I	Trypsin
14	*E*-Mu-Phe-Hfe-AS-Ph	420 ± 110	6080 ± 1390	<10	NI
15	*Z*-Mu-Phe-Hfe-AS-Ph	<10	260 ± 80	<10	NI
16	Cbz-Phe-Phe-AS-Ph (isomer A)	NS	NS	NS	NS
17	Cbz-Phe-Phe-AS-Ph (isomer B)	NS	NS	NS	NS
18	*E*-Mp-Phe-Hfe-AS-Ph	570 ± 20	2310 ± 230	<10	NI
19	*Z*-Mp-Phe-Hfe-AS-Ph	200 ± 40	130 ± 10	<10	NI
20	Mp-Phe-Phe-AS-Ph (isomer A)	13 ± 1	45 ± 1	<10	NI
21	Mu-Phe-Hfe-Elim	< 10	< 10	<10	NI

NS, not soluble; NI, no inhibition.
isomer A = from *L*-P1 vinyl sulfone residues, isomer B = from *D*-P1 vinyl sulfone residue.



Scheme 3. Potential mechanisms of inhibition.

including a S_N2 attack by the active site thiolate on the carbon adjacent to the sulfone, resulting in the elimination of the sulfone (a); a Michael addition to the double bond and the resulting elimination of the sulfone (b); and the direct elimination of the sulfone group, forming the enamide, which is then subject to a Michael addition via the thiolate on the terminal alkene carbon (c).

Compound **21**, a side product in our synthesis, contains the same conjugated π-system as the proposed intermediate of mechanism c (Scheme 3). When compound **21** was assayed with the four enzymes, it showed limited inhibition of the three clan CA cysteine proteases ($<10\,M^{-1}s^{-1}$) and no inhibition of trypsin. We speculated that based on the much lower inhibition of **21** as compared to allyl sulfones**14** and **15**, mechanism c can be ruled out as the mechanism of enzyme inhibition. Crystallization of an allyl sulfone bound to cruzain or cathepsin B remains unsuccessful and future work will hopefully elucidate the mechanism.

Conclusion

The successful synthesis of a series of novel dipeptidyl allyl sulfones was achieved by base catalyzed isomerization of peptidyl vinyl sulfones. It was found that stereochemistry of the vinyl sulfone P1 residue controlled the resulting stereochemistry of the allyl sulfones during isomerization. Stereogenic conformations of the two isomers of the P1 Hfe allyl sulfones were resolved using a combination of 2D NOESY experiments and computational modelling. Kinetic assays of dipeptidyl allyl sulfones revealed inhibitor selectivity for family C1 of the clan CA cysteine proteases, with 10-fold intra-family specificity for cruzain over cathepsin B. The Mu substituent was found to be the optimal P3 group while the P1 Hfe residue was crucial in maintaining inhibitory potency. Stereogenic configuration of the allyl sulfone played the most substantial role in inhibitory potency. Overall, (*E*)-Mu-Phe-Hfe-AS-Ph[14] was shown to be the most potent inhibitor. Partial insight into the inhibition mechanism was gained by analysis of the eliminated product of the Hfe-based allyl sulfones (enamide **21**), ruling out a potential mechanism-based inhibitor pathway. Future work in co-crystallization of a bound allyl sulfone inhibitor to cruzain or cathepsin B will be necessary to extend our understanding of the allyl sulfone inhibition mechanism. In addition, further modification of the current design will be necessary to achieve or exceed the potency of K11777.

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Declaration of interest

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