Pergamon

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Bioorganic & Medicinal Chemistry 11 (2003) 733–740

BIOORGANIC & MEDICINAL CHEMISTRY

The Design of Potent Hydrazones and Disulfides as Cathepsin S Inhibitors

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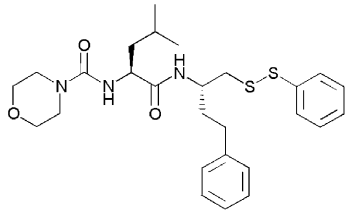
Received 24 July 2002; revised 6 September 2002; accepted 27 September 2002

Abstract—The design and synthesis of dipeptidyl disulfides and dipeptidyl benzoylhydrazones as selective inhibitors of the cysteine protease Cathepsin S are described. These inhibitors were expected to form a slowly reversible covalent adduct of the active site cysteine of Cathepsin S. Formation of the initial adduct was confirmed by mass spectral analysis. The nature and mechanism of these adducts was explored. Kinetic analysis of the benzoyl hydrazones indicate that these inhibitors are acting as irreversible inhibitors of Cathepsin S. Additionally, the benzoylhydrazones were shown to be potent inhibitors of Cathepsin S processing of Class II associated invariant peptide both in vitro and in vivo.

Introduction

Cathepsin S, a cysteine protease, is responsible for the final proteolytic step of MHC Class II invariant chain processing, cleaving the precursor fragment (p10) of invariant chain to CLIP (Class II Invariant chain peptide).[1–4] The MHC Class II-CLIP complex can associate with another protein, HLA-DM, which facilitates the release of CLIP and loading of antigenic peptide. The MHC Class II-peptide complex is transported to the cell surface where it engages $CD4^+$ T cells and elicits an immune response. Inhibition of Cathepsin S should attenuate MHC Class II's ability to load and present antigenic peptide and thus have a beneficial effect in autoimmune diseases. As part of our ongoing efforts to target autoimmune diseases we set out to design novel inhibitors of Cathepsin S. Palmer and Brömme recently reported vinyl sulfones were potent inhibitors of Cathepsin S.[5] However, these inhibitors were irreversible and thus concern for the potential haptenization made the vinyl sulfones less desirable from a drug development point of view.



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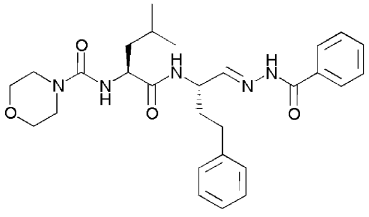

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PII: S0968-0896(02)00468-6

In an attempt to prevent the irreversible binding seen with the vinyl sulfone inhibitors, we investigated replacing the vinyl sulfone moiety with a reactive thiophile capable of forming an adduct, after addition of the cysteine thiol group, that could slowly revert to active enzyme under physiological conditions and thus avoid potential haptenization. We took a two prong approach. First we explored disulfides which can undergo nucleophilic attack by cysteine. There was precedent for inactivating a cysteine protease (papain) with thiolating agents.[6] Target compound **1** could form a disulfide with the Cathepsin S active site cysteine. Inactivation would be expected to be slowly reversible by cleavage with endogenous sulfides such as glutathione (Fig. 1). Compound **1** requires nucleophilic attack by the active site cysteine at the α-sulfur atom. This position adjoins the usual site of attack of a protease on a peptide substrate, but there was precedent for this in other proteases.[7] The second approach involved incorporation of a benzoyl hydrazone which could undergo attack by the cysteine. This would form a thioaminal adduct which theoretically would be reversible or could undergo hydrolysis to the aldehyde (Fig. 2).[8] The proposed peptidyl hydrazone inhibitors, such as **2**, had a potential advantage over the disulfide inhibitors in that the right hand section of the molecule could be capable of binding to the prime side of the active site and unlike the disulfides would be retained after covalent addition of the active site cysteine.

Chemistry

The disulfide inhibitors were synthesized as outlined in Scheme 1. Tosylate **3**, available from known *N*-Boc homophenylalaninol by the reaction of tosyl chloride in pyridine, was reacted with thiolacetic acid to introduce the first sulfur.[9–11] Deacetylation of the intermediate acetate and disulfide formation with 2,2′-dithiodipyridine **4** gives the labile disulfide **5**.[12] The desired disulfides, **1** and **7**, were then produced by displacement with the appropriate thiol followed by removal of the amino protecting group and condensation with known **6** under standard peptide coupling conditions.

Preparation of the hydrazones is outlined in Scheme 2. Condensation of known Weinreb amide **8** with **6** gave the new Weinreb amide **9**.[5] This was converted to aldehyde **10** with lithium aluminum hydride in ether which was then condensed with the appropriate benzoic hydrazide to give the hydrazone inhibitors. Additionally, hydrazone **2** was reduced with sodium cyanoborohydride to give hydrazide **13** (Scheme 3) which was used in mechanistic studies *vide infra*.[5c]

Biology

The potential inhibitors were screened against human recombinant Cathepsin S as the primary assay. Elastase

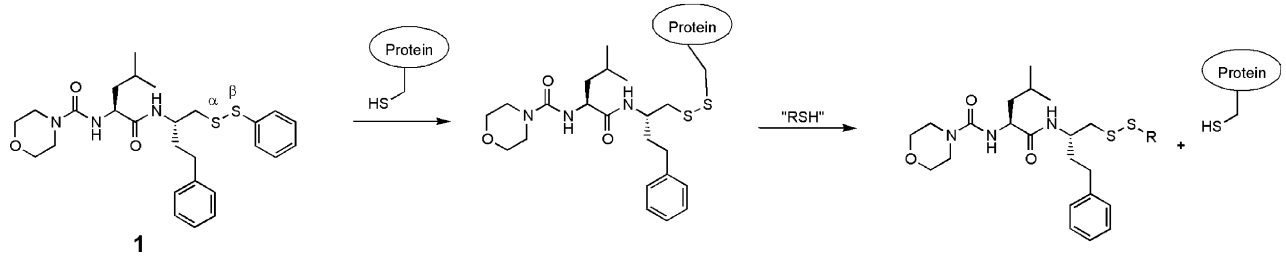


Figure 1.

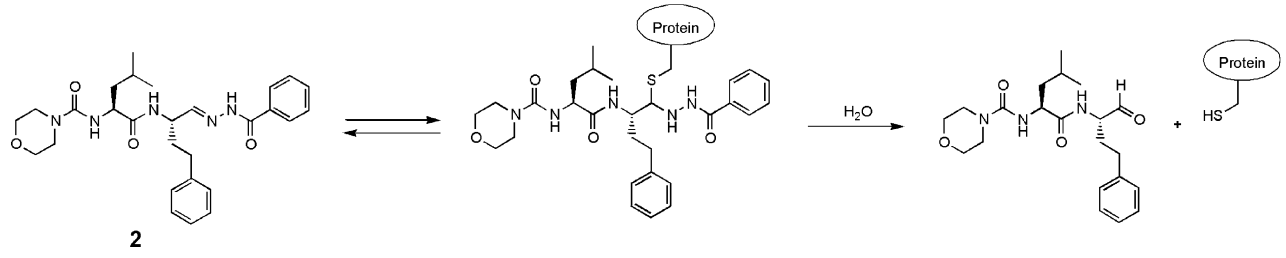
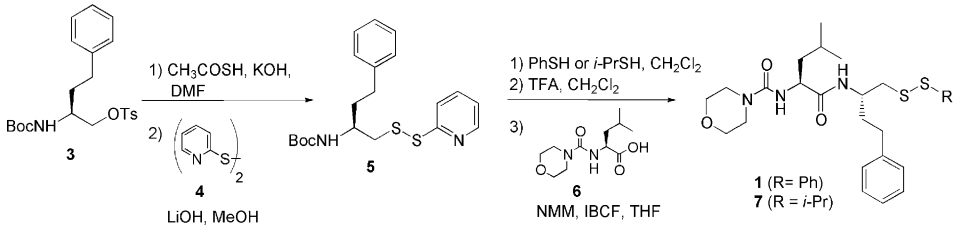
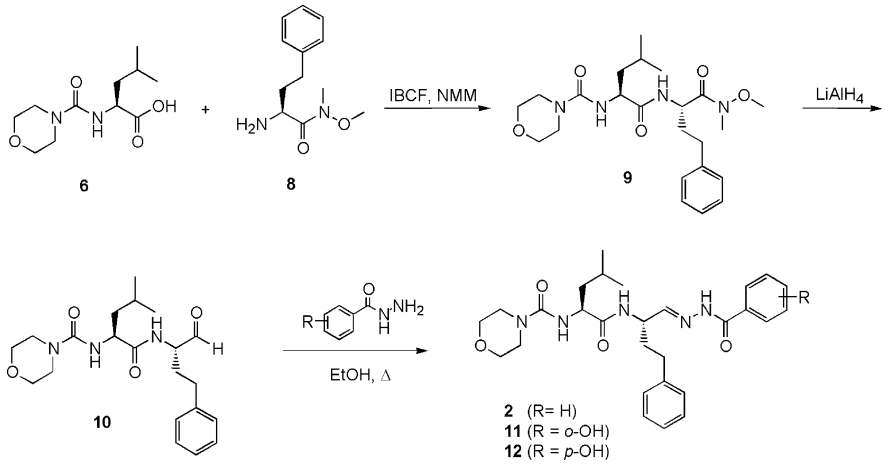


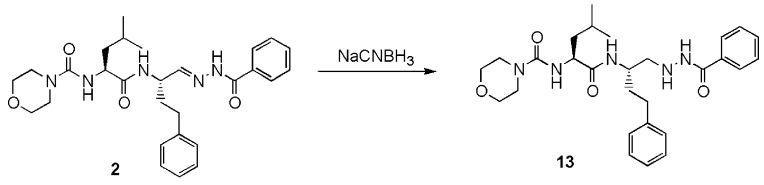
Figure 2.



Scheme 1.



Scheme 2.



Scheme 3.

(porcine pancreatic elastase), as well as the more closely related cysteine protease human Cathepsin B were used as selectivity screens.[13] The primary and selectivity assays were fluorescence based enzymatic assays which provided reproducible IC_{50}'s within 2-fold. Neither **1** nor **7** showed elastase activity and only disulfide **1** showed measurable Cathepsin S activity using standard assay conditions (Table 1). Compound **1** however did show selectivity for Cathepsin S over Cathepsin B. Since the standard assay conditions employ a reducing agent to maximize the free active site cysteine it was possible that the reducing agent could react with the inhibitor. In fact if DTT is removed from the assay, both compound **1** and **7** showed significantly increased potencies. These in vitro results with DTT would likely be mirrored in cellular and in vivo systems by endogenous, ubiquitous thiol nucleophiles such as glutathione.

We next turned our attention to the benzoyl hydrazone series. Compound **2** showed good potency against Cathepsin S in vitro as well as good selectivity over Cathepsin B. By contrast ***ent*-2** (the enantiomer of **2**), prepared as outlined in Scheme 2 starting from enantiomeric starting materials, showed at least a 1000-fold loss in activity. Further evaluation of hydrazone **2** showed it to be selective in a panel of protease assays performed by an independent lab with IC_{50}'s >7 μM observed for Calpain, Angiotensin Converting Enzyme, Cathepsin G, Collagenase IV, neutral endopeptidase and neutrophil elastase.[14]

Table 1. In vitro enzyme inhibition

Compd	Cathepsin S IC_{50} (nM)	Cathepsin B IC_{50} (nM)	Elastase
1	4500 (98)[a]	87,000	NA[b]
7	NA[b] (15,000)[a]	NT[c]	NA[b]
2	11	700	NA[b]
***ent*-2**	13,000	NA @ 500 μM	NA[b]
11	7	180	NA[b]
12	35	720	NA[b]
13	565	30,000	NA[b]
10	10	640	NA[b]

[a]IC_{50}'s in assay without the reducing agent DTT.
[b]NA = not active @ 5 μg/mL.
[c]NT = not tested.

Interestingly, the aldehyde precursor **10** was also active at similar potencies to **2**. This led to speculation that the activity of **2** resulted from hydrolysis to the aldehyde under assay conditions. To test this possibility we developed HPLC conditions to distinguish **2** and **10** in standard assay buffer as shown in Figure 3 graph A. Compound **2** was then assayed for Cathepsin S activity under standard experimental conditions including IC_{50} determination. An aliquot of the assay solution was then analyzed and shown to contain no aldehyde within the limits of detection of the HPLC assay ($\sim$1%, graph B).

Compounds were also tested in a cellular system. The assay measured inhibition of Cathepsin S via the accumulation of substrate invariant chain fragment (p10) and a decrease in product (CLIP) as observed in a human B cell line (HOM2) after treatment with compound.[3] This pulse chase experiment uses ^{35}S methionine, immuno-precipitation, and SDS-PAGE to determine the increase of the physiologic substrate (p10) quantified by a StormR phosphor-imager. In this assay the Minimal Inhibitory Concentration (MIC) of the drug required to detect an increase in p10 is measured. Disulfide **1** showed a minimum inhibitory concentration of 1 μM, similar to the IC_{50} determined with

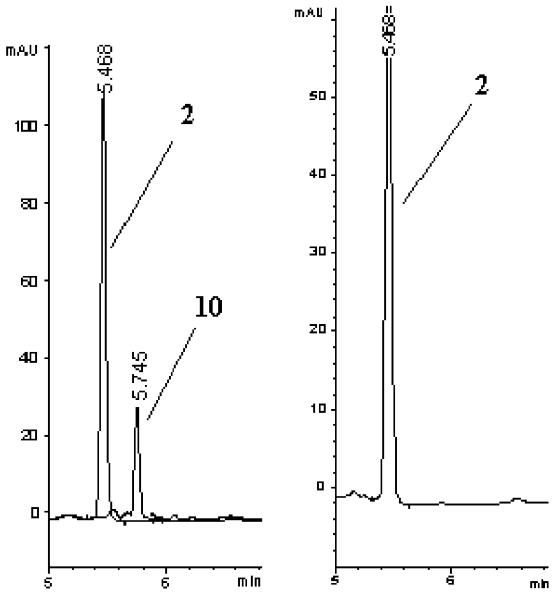


Figure 3. Samples were injected on a 2.1×75 mm, 3.5 μm, XDB-C18 column equilibrated in 90% (0.02% phosphoric acid)/10% acetonitrile. A solvent gradient was run to 10% (0.02% phosphoric acid)/90% acetonitrile. Signals were monitored at 254, 280 and 214 nm. Shown are 254 nm signals. Graph A shows the separation of **2** and **10** under these conditions. Graph B is a sample taken from actual in vitro Cathepsin S assay after determining IC_{50}. Graphs A and B show only the region on interest, from 5–6.8 min.

DTT present. The hydrazones **2**, **11** and **12** show nearly complete reduction of CLIP and an accumulation of p10 at 10 nM as shown in Figure 4. By contrast ***ent*-2** shows a significant CLIP band and no significant accumulation of p10 at 100 nM.

Compound **2** was also evaluated in vivo to determine if an increase in p10 could be observed in several mouse strains including C57Bl/6 and Balb/c (I-Ab and d allele mice).[3,15] Compound **2** showed a significant accumulation of p10 at 100 mg/kg while ***ent*-2** showed no activity at the same dose (Fig. 5).

Although the hydrazone series showed excellent activity in vitro and in vivo the issue of reversibility of these inhibitors was still unresolved. As postulated in Figure 2 addition of the active site cysteine should result in acetal

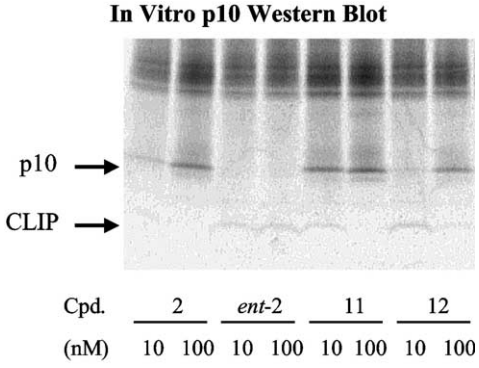


Figure 4. Immunoprecipitation of Class II MHC fragments p10 and CLIP from HOM-2 cells following a 30 min ^{35}S methionine pulse and 4 h chase in the presence of inhibitors at the concentrations shown.

formation which was expected to be reversible. Cathepsin S mediated hydrolysis of the resultant covalent adduct to give the aldehyde was discounted based on our HPLC analysis of **2** after in vitro assay. An irreversibility assay was devised in which the compounds were incubated with Cathepsin S at a 10-fold excess at pH 4.5. The enzyme mixtures were then subjected to reverse phase HPLC. Cathepsin S and/or Cathepsin S/compound complexes were recovered and analyzed using electrospray MS. Irreversible inhibitors such as E-64 result in a new HPLC peak with a molecular weight increase corresponding to addition of the inhibitor.[16] Hydrazone **2, 11** and **12** as well as the disulfide inhibitors **1** and **7** showed new HPLC peaks with corresponding increases in molecular weight that were consistent with the proposed mechanisms in Figures 1 and 2. The hydrazones showed a new HPLC peak with a molecular weight increase corresponding to the addition of the entire molecule. In separate experiments, incubating protein and **2** for 30 min then adding a 10-fold molar excess of E-64 showed no significant displacement. Although this confirmed that the hydrazone, and not the aldehyde, was the active species it also indicated the compound is acting as essentially an irreversible inhibitor. The carbonyl of the benzoyl hydrazone is an alternative site for attack by the active site cysteine as shown in Figure 6 which could result in a more hydrolytically stable covalent adduct. Alternatively it might be expected that the loss of water would result in the acyl enzyme complex instead. However, both scenarios would be inconsistent with the mass spectral data that showed water was not lost. To test the alternative site of attack we assayed compound **13**. It also showed a new HPLC peak with an increase in mass corresponding to the molecular weight of **13**. However, when a 10-fold excess of E-64 was added there was a significant displacement, 82% after 36 h, noted by HPLC with a corresponding change in molecular weight

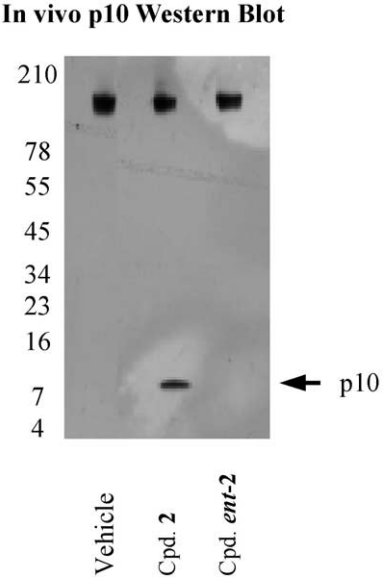


Figure 5. Balb/c male mice were given a single oral dose of 100 mg/kg compound. Splenocytes were collected following dosing, lysed and immunoprecipitated. Western blot analysis for the detection of mouse invariant chain was conducted.

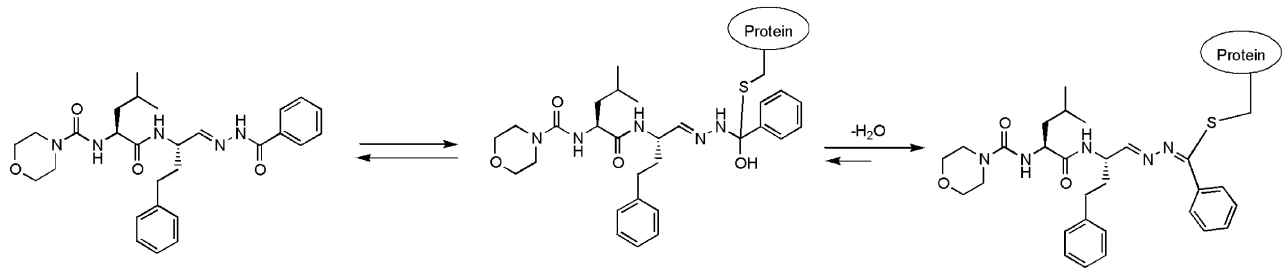


Figure 6.

of the adduct indicating this compound was reversible. The decreased activity of **13** relative to **2** shows a preference for addition at the normal P1 site, but clearly the MS data indicates that attack at the benzoyl group is possible.[17] Additionally, follow-up stop-flow kinetics showed that compound **2** did not have a measurable off rate over a 1.5 h time course (data not shown) confirming these inhibitors are acting in an irreversible fashion.[18] As such, we determined inactivation constants for the more promising hydrazones and the data are reported in Table 2. The fact that these inhibitors remained bound even in denatured protein meant there was still a possibility that, upon dosing of these inhibitors in vivo, haptenization could result. Thus, this series was deemphasized and the pursuit of new rapidly reversible inhibitors continued and will be reported elsewhere.[19]

Table 2. Inhibition kinetics[a]

Compd	Cathepsin S K inactivation (s^{-1})	Cathepsin S K_i (μM)
2	0.035	0.050
11	0.055	0.033
12	0.079	0.11

[a]From Cat S enzymatic assay: Substrate Val-Arg-Coumarin: $K_m = 6.2e{-}6$ M, $k_{cat} = 2.1e{-}2$.

Conclusions

Two new series of Cathepsin S inhibitors have been described with the goal of identifying novel, selective and reversible enzyme inhibitors. The benzoyl hydrazone series was shown to be a potent and selective inhibitor in enzymatic assays. The hydrazones also displayed potent cellular and in vivo activity. The mechanistic aspects of these inhibitors were investigated and both series displayed behavior consistent with irreversible inhibition. Although the possibility that these inhibitors are reversible but with extremely long off-rates has not been completely discounted, the fact that an adduct forms that is not readily reversible or degraded increases the potential risk of haptenization and therefore further synthetic efforts on these series were discontinued.

Experimental

Biology

Assay conditions for the enzymatic assays can be found in ref 19. The cellular assays were performed as outlined below.

Immunoprecipitation of Class II from HOM-2 Cells. Class II MHC was immunoprecipitated as previously reported[3a] with the following changes; HOM-2 cells were preincubated with methionine-free RPMI 1640 (GIBCO) supplemented with 10% FBS, penicillin-streptomycin, and glutamine for 30 min in presence of Cathepsin S inhibitor prior to pulsing for 30 min with 0.25 mCi ^{35}S methionine (Amersham). Cells were centrifuged and complete RPMI 1640 media plus inhibitor was added for a 4 h chase.

In vivo p10 Western. Male Balb/c mice were dosed orally with compound at 100 mg/kg in 30% Cremophor. The mice were sacrificed after 3.5 h and splenocytes were isolated and lysed. Invariant chain bound to MHC Class II was immunoprecipitated with anti-MHC Class II antibody. Proteins were separated by electrophoresis, transferred to 0.2 μm nitrocellulose and probed with invariant chain antibody followed by peroxidase labeled goat anti-rat Ig (Biosource, ari4404). Blots were developed with ECL Detection Reagents (Amersham, RPN 2108).

Reversibility assessment. The compounds were prepared as 5 mg/mL stock solutions in DMSO. Cathepsin S-hexaHis in 100 mM sodium acetate, pH 4.5 with 2 mM DTT and 1mM EDTA was diluted to 1.6 mg/mL with the same buffer containing 10 mM cysteine. Aliquots of Cathepsin S-hexaHis and each of the compound stocks were combined in a ratio of 12 to 1. This results in a final molar ratio for Cathepsin S-hexaHis to compound of approximately 1:10. The samples were incubated for 30 min at 37 °C. The entire sample was injected into a Hewlett Packard HP-1090HPLC operated at RT. A Delta-Pak HPI C4, 300 Å, 2.0×150 mm column (Waters Associates, Milford, MA) was operated at a flow rate of 0.2 mL/min. A 25 min linear gradient from 10 mM ammonium acetate, pH 4.5 to 10 mM ammonium acetate in acetonitrile: water (60:40) was employed. Detection was by UV at 210 nm. Fractions corresponding in retention time to Cathepsin S-hexaHis (15.2–16.6 min) were collected and stored at −80 °C until analyzed. Electrospray ionization mass spectrometry was performed on

a Micromass (Manchester, UK) AutoSpec OATOF mass spectrometer. The recovered fractions were introduced using a syringe pump operated at 0.85 mL/h. The magnet was scanned from $m/z = 3000$–1000 at 8 s/decade and 1000 resolution. Cone voltage was 23 V. On observation of adducts the voltage was increased to 50 V to determine whether the adduction could be removed by this addition of additional collision energy. The multiply charged spectra (+8 to +11) were deconvoluted by application of the Micromass Maxent program from mass 24000 to 26000.

Substrate kinetics. The assay buffer employed in all experiments consisted of 100 mM NaOAc, pH 4.5; 2.5 mM EDTA, 1 mM TCEP, 10% glycerol, 2% DMSO and 0.025% CHAPS. The substrate used in these studies was 7-amino-4-methyl coumarin, CBZ-Val-Arg, hydrochloride salt. Substrate K_m and k_{cat} values were determined using a stopped-flow method. Increase in fluorescence, reflecting product formation, were recorded as a function of time using a KinTek stopped-flow instrument model # SF-2001 (KinTek Corp., Austin, TX). Experiments involved enzyme concentrations of 80, 250 and 750 nM, and substrate concentrations of 2.5, 5, and 10 μM.

Inhibitor kinetics. Inactivation of Cathepsin S occurs in a 2-step process: formation of an equilibrium species (EI), followed by an inactivation step in which the inhibitor irreversibly binds to the enzyme (EI*). Inactivation reactions were initiated by adding Cathepsin S into a solution containing substrate and inhibitor. Fluorescence was measured as a function of time using a spectrofluorimeter (instrumentation: SLM Aminco 8100). A series of progress curves were obtained for each compound (15–260 nM, depending inhibitor potency) at constant enzyme and substrate concentrations (60 nM and 10 μM, respectively). Data were analyzed in a manner similar to the method described by Palmer and Brömme.[5]

Chemistry

General chemical methods. Chromatography was performed on silica gel. Melting points are reported in Celsius (°C) and are uncorrected. ^{1}H NMR spectra were determined at 400 MHz and were referenced to solvent, reported in δ, with J values reported in Hz. Mass spectra were performed by the department of Analytical Sciences of Boehringer Ingelheim Pharmaceuticals. Elemental analyses were performed by Quantitative Technologies, Inc., Whitehouse, N.J.

Preparation of morpholine-4-carboxylic acid [3-methyl-1*S*-(3-phenyl-1*S*-phenyldisulfanylmethyl-propylcarbamoyl)-butyl]-amide (1). (a) To a solution of *N*-Bochomophenylalaninol[9] (10.4 mmol) in 50 mL of CH_2Cl_2 was added 2.636 g (13.8 mmol) of TsCl and 1.20 mL (14.8 mmol) of pyridine. After stirring at rt overnight, the reaction mixture was diluted to 300 mL with EtOAc and washed with 5% aq citric acid (3×50 mL), satd aq $NaHCO_3$ (3×50 mL) and satd aq NaCl (50 mL). The combined organic layers were dried over Na_2SO_4, filtered, concentrated and purified by chromatography (EtOAc/Hexane = 1/9) to yield 1.338 g (31%) of tosylate **3** which was used directly. ^{1}H NMR (DMSO-d_6) 7.76 (d, $J = 8.0$ Hz, 2H), 7.47 (d, $J = 8.0$ Hz, 2H), 7.30–7.20 (m, 2H), 7.14–7.10 (m, 3H), 3.90 (d, $J = 5.0$ Hz, 2H), 3.59–3.51 (m, 1H), 2.61–2.50 (m, 2H), 2.41 (s, 3H), 1.63–1.55 (m, 2H), 1.36 (s, 9H); ESMS 420 (MH^+).

(b) To 10 mL of DMF cooled in an ice bath was added 0.365 g (5.7 mmol) of 87.5% KOH. After the slow addition of 0.60 mL (8.4 mmol) of thiolacetic acid, the mixture was stirred for 15 min. The bath was removed and the mixture was stirred until all the KOH dissolved giving an orange solution. The ice bath was returned and a solution of 1.012 g (2.41 mmol) of **3** in 10 mL DMF was added dropwise. The reaction was allowed to warm to rt overnight. The mixture was then diluted to 300 mL with CH_2Cl_2 and 75 mL of 5% aq citric acid added. The resulting mixture was filtered and the organic layer of the filtrate was washed with 5% citric acid (2×75 mL), satd aq $NaHCO_3$ (3×75 mL) and satd aq NaCl (75 mL). The organics were dried over Na_2SO_4, filtered and concentrated to afford 0.560 g (72%) of the thioacetate which was used directly in the next reaction. ^{1}H NMR ($CDCl_3$) 7.30–7.26 (m, 2H), 7.20–7.16 (m, 3H), 4.51 (br d, $J = 7.0$ Hz, 1H), 3.80 (br s, 1H), 3.13–3.01 (m, 2H), 2.75–2.61 (m, 2H), 2.35 (s, 3H), 1.87–1.74 (m, 2H), 1.45 (s, 9H); CIMS(NH_3) 268 (M-(*t*-Bu)).

(c) A solution of 0.560 g of the thioacetate (1.73 mmol) of **6** in 40 mL of MeOH was degassed by repeatedly subjecting it to vacuum and nitrogen purge. Addition of 0.480 g (2.18mol) of 2,2′-dithiodipyridine **4** was followed by addition of a solution of 0.110 g (2.62 mmol) of $LiOH \cdot H_2O$ in 2 mL water. The reaction was left stirring at rt overnight. MeOH was removed by rotary evaporation and the residue was extracted with EtOAc. After evaporation of the solvent, the residue was chromatographed eluting with EtOAc/Hexane (1:3) to give compound **5** (0.195 g, 29%). ^{1}H NMR ($CDCl_3$) 8.53 (d, $J = 4.5$ Hz, 1H), 7.64–7.55 (m, 2H), 7.29–7.25 (m, 2H), 7.20–7.16 (m, 3H), 7.12–7.09 (m, 1H), 5.55 (br s, 1H), 3.87 (br s, 1H), 3.14–3.04 (m, 2H), 2.74–2.60 (m, 2H), 1.99–1.88 (m, 2H), 1.44 (s, 9H); CIMS(CH_4) 391 (MH^+).

(d) A solution of 0.094 g (0.241 mmol) of **5** and 26 μL (0.25 mmol) of thiophenol in 1 mL of CH_2Cl_2 was stirred for 3 days. The product was diluted with 50 mL of EtOAc and was washed with 3×15 mL of satd aq $NaHCO_3$ and 1×15 mL of satd aq NaCl. The organic solution was dried over Na_2SO_4, filtered and concentrated to a residue that was filtered through SiO_2 eluting 10% ethyl acetate in hexane. This yielded 0.047 g of the phenyl disulfide. ^{1}H NMR ($CDCl_3$) 7.56 (d, $J = 7.5$ Hz, 2H), 7.37–7.17 (m, 6H), 7.17 (d, $J = 7.0$ Hz, 2H), 4.59 (br s, 1H), 3.91 (br s, 1H), 2.98 (br s, 2H), 2.74–2.58 (m, 2H), 2.00–1.91 (m, 1H), 1.81 (br s, 1H), 1.48 (s, 9H); CIMS(NH_3) 390 (MH^+). *N*-deprotection was accomplished by dissolving the phenyldisulfide in 5 mL of 20% trifluoroacetic acid in CH_2Cl_2 and stirring for 45 min. The reaction mixture was concentrated in vacuo and used as its TFA salt directly in the next

reaction. ^{1}H NMR ($CDCl_3$) 7.46 (d, J=6.5 Hz, 2H), 7.37–7.22 (m, 6H), 7.12 (d, J=7.0 Hz, 2H), 3.54 (br s, 1H), 3.07 (br d, J=12.5 Hz, 1H), 2.88 (dd, J=14.6 Hz, 9.0, 1H), 2.78–2.62 (m, 2H), 2.17–2.06 (m, 2H); CIMS(NH_3) 290 (MH^+).

(e) A solution of 0.033 g (0.14 mmol) of *N*-(morpholine-4-carbonyl)-L-leucine (**6**) in 1 mL THF was maintained at −15 to −30 °C. Addition of 0.060 mL (0.55 mmol) of *N*-methylmorpholine (NMM) followed by 0.020 mL (0.15 mmol) of isobutyl chloroformate resulted in a precipitate.[5] After 15 min, a solution of the amine from previous reaction in 1.0 mL of THF was added. After 0.5 h, the reaction mixture was poured into 10 mL of 5% aq citric acid. This mixture was extracted with 50 mL of EtOAc and the extract was washed with 5% aq citric acid (2×10 mL), satd aq $NaHCO_3$ (3×10 mL) and satd aq NaCl (10 mL). The organic solution was dried over sodium sulfate, filtered and concentrated to a residue. The residue, after purification by HPLC (Microsorb C18RP, 21.4 mm×30 cm, 10 mL/min, isocratic 85%AcCN/H_2O, 254 nm) afforded 0.030 g (24% over 3 steps) of product **1**, mp 94–95; ^{1}H NMR ($CDCl_3$) 7.52 (d, J=8.5 Hz, 2H), 7.32 (t, J=7.5 Hz, 2H), 7.28–7.22 (m, 2H), 7.18 (t, J=7.5 Hz, 1H), 7.12 (d, J=6.5 Hz, 2H), 6.33 (d, J=8.5 Hz, 1H), 4.83 (d, J=8.0 Hz, 1H), 4.32–4.26 (m, 1H), 4.21–4.12 (m, 1H), 3.69–3.60 (m, 4H), 3.36–3.33 (m, 4H), 2.98–2.90 (m, 2H), 2.65–2.50 (m, 2H), 2.01–1.91 (m, 1H), 1.87–1.78 (m, 1H), 1.74–1.65 (m, 2H), 1.54–1.46 (m, 1H), 0.96 (d, J=6.5 Hz, 3H), 0.95 (d, J=6.0 Hz, 3H); CIMS(NH_3) 516 (MH^+). Anal. ($C_{27}H_{37}N_3O_3S_2$) C, H, N.

Preparation of morpholine-4-carboxylic acid [1*S*-(1*S*-isopropyldisulfanylmethyl-3-phenyl-propylcarbamoyl)-3-methyl-butyl]-amide (7). The synthesis of **7** was directly analogous to **1** starting from **5** (0.101 g, 0.268 mmol) and isopropyl mercaptan to yield, after final purification by HPLC (Microsorb C18RP, 21.4 mm×30 cm, 10 mL/min, isocratic 85% AcCN/H_2O, 254 nm, retention time 9.5 min), 0.019g (16% over 3 steps) of 7, mp 121–123; ^{1}H NMR ($CDCl_3$) 7.29–7.25 (m, 2H), 7.20–7.16 (m, 3H), 6.35 (d, J=8.5 Hz, 1H), 4.89 (d, J=8.0 Hz, 1H), 4.34–4.29 (m, 1H), 4.20–4.13 (m, 1H), 3.69–3.60 (m, 4H), 3.38–3.29 (m, 4H), 2.98 (septet, J=7.0 Hz, 1H), 2.90 (d, J=5.5 Hz, 2H), 2.70–2.57 (m, 2H), 2.01–1.92 (m, 1H), 1.89–1.79 (m, 1H), 1.75–1.65 (m, 2H), 1.55–1.47 (m, 1H), 1.28 (d, J=7.0 Hz, 6H), 0.96 (d, J=6.5 Hz, 3H), 0.95 (d, J=6.0 Hz, 3H); CIMS(NH_3) 482 (MH^+). Anal. ($C_{24}H_{29}N_3O_3S_2$) C, H, N.

Synthesis of morpholine-4-carboxylic acid {1*S*-[1*S*-(benzoyl-hydrazonomethyl)-3-phenyl-propylcarbamoyl]-3-methyl-butyl}-amide (2). (a) 4-Methyl-2-[(morpholine-4-carbonyl)-amino]-pentanoic acid. A solution of 0.425 g (1.704 mmol) of *N*-(morpholine-4-carbonyl)-L-leucine (**6**) in 15 mL THF was maintained at −15 to −30 °C. Addition of 0.75 mL (6.82 mmol) of *N*-methylmorpholine (NMM) followed by 0.25 mL (1.93 mmol) of isobutyl chloroformate resulted in formation of a precipitate. After 15 min, a solution of the 2*S*-amino-*N*-methoxy-*N*-methyl-4-phenyl-butyramide[5] in 5 mL of THF was added. After 0.5 h of stirring at the reduced temperature, the reaction mixture was poured into 100 mL of 5% aq citric acid. This mixture was extracted with 2×75 mL of EtOAc and the extract was washed with 5% aq citric acid (2×25 mL), satd aq $NaHCO_3$ (3×25 mL) and satd aq NaCl (25 mL). The organic solution was dried over Na_2SO_4, filtered, concentrated to an oil, and purified by chromatography (5%MeOH/CH_2Cl_2) to afford 0.636 g (84%) of product **9**, mp 121–123; ^{1}H NMR (270 MHz, $CDCl_3$) 7.30–7.24 (m, 2H), 7.20–7.15 (m, 3H), 6.74 (d, J=8.4 Hz, 1H), 4.95–4.92 (m, 2H), 4.46–4.39 (m, 1H), 3.69–3.62 (m, 4H), 3.62 (s, 3H), 3.39–3.35 (m, 4H), 3.16 (s, 3H), 2.71–2.61 (m, 2H), 2.10–1.88 (m, 2H), 1.71–1.47 (m, 3H), 0.94 (d, J=5.8 Hz, 6H); CIMS (NH_3) 449 (MH^+). Anal. ($C_{23}H_{36}N_4O_5$) C, H, N.

(b) To a suspension of $LiAlH_4$ in Et_2O (10 mL) at −30 °C was added a solution of **9** in Et_2O/THF (2:1, 6 mL). After warming to −20 °C over 30 min, the mixture was quenched with an aq $KHSO_4$ solution and filtered. The filtrate was diluted with EtOAc (50 mL), washed with 1 N aq HCl (3×10 mL), satd aq $NaHCO_3$ (3×10 mL) and satd aq NaCl (1×10 mL) then dried over Na_2SO_4, filtered, and concentrated in vacuo to afford **10** as a foam (0.118g, 91%). Mp 58–60; ^{1}H NMR ($CDCl_3$) 9.51 (s, 1H), 7.31–7.26 (m, 2H), 7.23–7.17 (m, 3H), 7.01 (br s, 1H), 4.97 (s, 1H), 4.49–4.39 (m, 2H), 3.71–3.63 (m, 4H), 3.43–3.37 (m, 4H), 2.69 (t, J=7.8 Hz, 2H), 2.29–2.20 (m, 1H), 2.00–1.90 (m, 1H), 1.74–1.66 (m, 2H), 1.61–1.55 (m, 1H), 0.97 (t, J=6.4 Hz, 6H); ESMS 390 (MH^+). Anal. ($C_{21}H_{31}N_3O_4$) C, H, N.

(c) A solution of 0.381 g (0.98 mmole) of **10** and 0.132 g (0.97 mmole) of benzoic hydrazide was refluxed for 4 h. The product was concentrated to a foam and chromatographed eluting with a gradient of 0–4% methanol in CH_2Cl_2 afforded 0.282 g (57%) of **2**. Recrystallization from 20% IPA in hexane gave 0.157 g (32%) of purified product, mp 175–176; ^{1}H NMR (DMSO-d_6, 80 °C) 11.28 (s, 1H), 7.84 (app d, J=7.5 Hz, 3H), 7.73 (br s, 1H), 7.57–7.53 (m, 1H), 7.47 (t, J=7.5 Hz, 2H), 7.28 (t, J=7.5 Hz, 2H), 7.22–7.16 (m, 3H), 6.27 (d, J=8.0 Hz, 1H), 4.45–4.39 (m, 1H), 4.27–4.22 (m, 1H), 3.60–3.51 (m, 4H), 3.38–3.28 (m, 4H), 2.73–2.59 (m, 2H), 2.08–1.92 (m, 2H), 1.74–1.62 (m, 1H), 1.58–1.54 (m, 2H), 0.93 (d, J=6.5 Hz, 3H), 0.90 (d, J=6.5 Hz, 3H); CIMS (NH_3) 508 (MH^+). Anal. ($C_{28}H_{37}N_5O_4$) C, H, N.

Synthesis of morpholine-4-carboxylic acid (1*S*-{1*S*-[(2-hydroxy-benzoyl)-hydrazonomethyl]-3-phenyl-propylcarbamoyl}-3-methyl-butyl)-amide (11). Synthesis same as for **2** starting from **10** (201 mg, 0.52 mmol) and *o*-hydroxybenzoic hydrazide (79 mg, 0.52 mmol) yield 12%; mp 107–111; ^{1}H NMR (DMSO-d_6) 11.86 (s, 1H), 11.56 (s, 1H), 8.12 (d, J=8.1 Hz, 1H), 7.79 (d, J=6.5 Hz, 1H), 7.66 (d, J=4.7 Hz, 1H), 7.39 (t, J=7.4 Hz, 1H), 7.27–7.13 (m, 5H), 6.92–6.87 (m, 2H), 6.50 (d, J=8.2 Hz, 1H), 4.38–4.34 (m, 1H), 4.20–4.14 (m, 1H), 3.54–3.49 (m, 4H), 3.30–3.24 (m, 4H), 2.69–2.53 (m, 2H), 1.99–1.84 (m, 2H), 1.67–1.51 (m, 2H), 1.47–1.40 (m, 1H), 0.88 (d, J=6.5 Hz, 3H), 0.84 (d, J=6.5 Hz, 3H); ESMS 524 (MH^+). Anal. ($C_{23}H_{36}N_4O_5$•0.5 equiv H_2O) C, H, N.

Synthesis of morpholine-4-carboxylic acid (1*S*-{1*S*-[(4-hydroxy-benzoyl)-hydrazonomethyl]-3-phenyl-propylcarbamoyl}-3-methyl-butyl)-amide (12). Synthesis same as for **2** starting from **10** (197 mg, 0.51 mmol) and *p*-hydroxybenzoic hydrazide (78 mg, 0.51 mmol) yield 44%; mp 152–155; ^{1}H NMR (DMSO-d_6) 11.31 (s, 1H), 10.05 (s, 1H), 8.13 (d, $J=8.2$ Hz, 1H), 7.73 (d, $J=8.7$ Hz, 2H), 7.64 (br s, 1H), 7.29–7.16 (m, 5H), 6.81 (d, $J=8.7$ Hz, 2H), 6.51 (d, $J=8.3$ Hz, 1H), 4.39–4.32 (m, 1H), 4.22–4.16 (m, 1H), 3.58–3.49 (m, 4H), 3.37–3.23 (m, 4H), 2.70–2.52 (m, 2H), 2.01–1.85 (m, 2H), 1.69–1.53 (m, 2H), 1.48–1.41 (m, 1H), 0.90 (d, $J=6.6$ Hz, 3H), 0.87 (d, $J=6.5$ Hz, 3H); CIMS (CH_4) 524 (MH^+). Anal. ($C_{23}H_{36}N_4O_5 \cdot 0.5$ equiv H_2O) C, H, N.

Synthesis of morpholine-4-carboxylic acid {1-[1S-(*N*-benzoyl-hydrazinomethyl)-3-phenyl-propylcarbamoyl]-3-methyl-butyl}-amide (13). Compound **2** (26 mg, 0.051 mmol) was dissolved in EtOH (1 mL) and treated with $NaCNBH_3$ (7 mg, 0.11 mmol) and the mixture was stirred at rt for 2 h. The mixture was diluted with EtOAc and washed with satd aq $NaHCO_3$ (3×), the organic layer was dried over Na_2SO_4 and concentrated and the residue was purified by HPLC (Microsorb C18RP, 21.4 mm×30 cm, 10 mL/min, isocratic 70%AcCN/H_2O for 10 min then gradient to 95% AcCN/H_2O over 5 min, 254 nm, rentention time 11.29 min), HPLC purity >99%, mp 72–74; ^{1}H NMR ($CDCl_3$) 8.79 (s, 1H), 7.82 (d, $J=7.0$ Hz, 2H), 7.52–7.49 (m, 1H), 7.45–7.42 (m, 2H), 7.26–7.23 (m, 2H), 7.19–7.13 (m, 3H), 6.41(d, $J=8.0$ Hz, 1H), 4.78 (d, $J=7.5$ Hz, 1H), 4.37–4.30 (m, 1H), 4.11–4.06 (m, 1H), 3.69–3.61 (m, 4H), 3.39–3.30 (m, 4H), 3.13 (dd, $J=12.5$ Hz, 4.0, 1H), 2.72–2.60 (m, 3H), 1.84–1.69 (m, 5H), 0.97 (d, $J=6.5$ Hz, 3H), 0.95 (d, $J=6.5$ Hz, 3H); CIMS (NH_3) 510 (MH^+); HRMS (MH^+, $C_{28}H_{40}N_5O_4$) calcd 510.3080 found 510.3078.

Acknowledgements

Lana Smith for the synthesis of the ***ent*-2**, and David Thomson for useful discussions during the preparation of this manuscript.

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14. Performed by MDS Panlabs Pharmacology Services, 11804 North Creek Parkway South, Bothell, WA 98011-8890. The results from this testing are as follows; human Calpain ($IC_{50}=7.6$ μM), rabbit Angiotensin Converting Enzyme (5% inhibition @ 10 μM), human Cathepsin G (6% inhibition @ 30 μM), human Collagenase IV (−8% inhibition @ 100 μM), human neutral endopeptidase (0% inhibition @ 10 μM) and human neutrophil elastase (2% inhibition @ 30 μM).
15. Complete details of this method will be reported elsewhere.
16. E-64 is *N*-(*trans*-epoxysuccinyl)-*L*-leucine 4-guanidinobutylamide from Sigma (Catalog# E 3132).
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