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PAPER

Isothiazolones; thiol-reactive inhibitors of cysteine protease cathepsin B and histone acetyltransferase PCAF†

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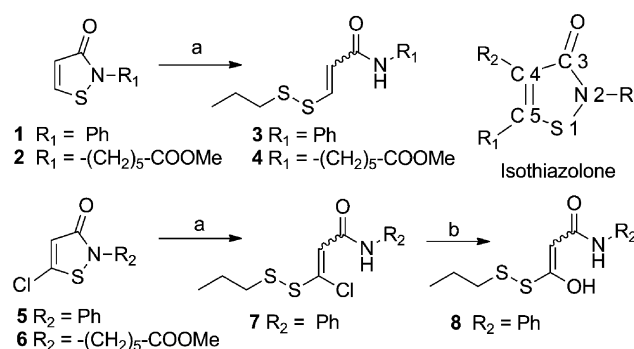
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Isothiazolones and 5-chloroisothiazolones react chemoselectively with thiols by cleavage of the weak nitrogen–sulfur bond to form disulfides. They show selectivity for inhibition of the thiol-dependent cysteine protease cathepsin B and the histone acetyltransferase p300/CBP associated factor (PCAF) based on their substitution pattern. Furthermore, enzyme kinetics and mass spectroscopy indicate covalent binding of a 5-chloroisothiazolone to cathepsin B, which demonstrates their potential utility as probes for activity-based protein profiling.

Introduction

Functional groups that react covalently with thiolates have been used extensively for activity-based protein profiling of cysteine-proteases.¹ This technology depends on covalent labelling of active site thiolates of enzymes in cell-lysates. Labelling is often performed with biotinylated functionalities, which can be employed for purification with immobilized streptavidin. Cleavable inhibitor-biotin linkers have been developed to improve the recovery of the proteins of interest.² Our current study indicates that isothiazolones and 5-chloroisothiazolones are excellent candidates for this purpose, because the disulfides that are formed upon reaction with active site thiolates (Scheme 1) can be cleaved under mild conditions.

The activity of isothiazolones and 5-chloroisothiazolones is expected to originate mainly from the reactivity of these heterocycles towards thiolates.³ In a careful study, Alvarez-Sánchez *et al.*⁴ described that *N*-methyl-5-chloroisothiazolone reacts with oxygen and nitrogen nucleophiles *via* an addition–elimination in the 5-position, thereby substituting the chloride. Treatment of *N*-methyl-5-chloroisothiazolone with thiol-nucleophiles, however, provided ring-opened thioester and dithioester products that can not be explained this way. This indicates that the thiol nucleophiles react through cleavage of the N–S bond (Scheme 1).



Scheme 1 Reaction of isothiazolones and 5-chloroisothiazolones with propane-1-thiolate results in cleavage of the N–S bond. (a) propane-1-thiol, Et₃N, CH₂Cl₂ (b) extended reaction times resulted in hydrolysis of 7.

Cathepsin B is a biologically relevant enzyme that is implicated in tumor formation, angiogenesis, tumor invasion and metastasis.^{5,6} Furthermore, cathepsin B has been considered a potential biomarker for prognosis in cancer.⁷ Biotinylated irreversible inhibitors can be used as a probe in activity-based protein profiling of cathepsin B.^{8,9} In this study we identify isothiazolones and 5-chloroisothiazolones as covalent inhibitors of cathepsin B. Furthermore, we evaluated the selectivity of cathepsin B inhibition compared to the histone acetyltransferase (HAT) p300/CBP associated factor (PCAF). This enzyme plays a role in the nuclear factor κ B (NF κB) pathway, which is an important regulator of several genes that are involved in inflammation.¹⁰ Upon activation, NF κB binds to cofactors that have intrinsic HAT activity, like PCAF, which in turn acetylate lysines in core histone H4. This leads to a less compact chromatin structure, which enables the transcription of proinflammatory genes, including Interleukin 8 (IL-8).^{11,12} Isothiazolones and 5-chloroisothiazolones (Scheme 1) are potent and cell-permeable inhibitors of PCAF, and attempts to optimize their inhibitory

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activity have been made in several studies.^{13–17} However, their mode of action and selectivity remain to be studied.

Herein we describe isothiazolones, 5-chloroisothiazolones and 5-chloroisothiazolone-1-oxides as new classes of cathepsin B inhibitors. Selectivity between cathepsin B and PCAF inhibition can be obtained by variation of the substitution. 5-chloroisothiazolones are non-competitive inhibitors of cathepsin B, and mass spectroscopy demonstrates covalent binding. The reaction products of isothiazolones and 5-chloroisothiazolones that are formed upon thiolate treatment proved to be disulfides, which could be advantageous in activity-based protein profiling.

Results and discussion

Synthesis

Compounds **1**, **5** and **11** were prepared by reaction of the corresponding primary amines with 3,3'-dithiobispropionyl chloride to yield the 3,3'-dithiobispropionic amides as described previously for compounds **9**, **10**, **12–18** and **19–26**.^{15,16} These amides were treated with 3 equiv. of sulfonyl chloride in dichloromethane to yield a mixture of the 5-chloroisothiazolone and isothiazolone, which were separated by column chromatography.

Thiolate reactivity of (5-chloro)isothiazolones

The reaction products that were formed upon treatment with thiolates were identified in order to study the thiol-reactivity of isothiazolones and 5-chloroisothiazolones. Isothiazolones **1** and **2** were treated with 1.0 equiv. of propane-1-thiol and triethylamine in dichloromethane (Scheme 1). The resulting products were isolated and characterized by NMR and mass analysis. In substrate **1** the observed $^3J_{\text{H4,H5}}$ was 6.5 Hz, whereas in product **3** it amounted to 14.1 Hz. Comparably, in **2** $^3J_{\text{H4,H5}}$ was 6.5 Hz, whereas it was 9.7 Hz in **4**. This is a strong indication for an *E*-configuration of the double bond in **3** and **4**, which could be caused by a catalytic amount of thiol that reacts *via* subsequent addition, rotation and elimination. When 5-chloroisothiazolones **5** and **6** were subjected to the same reaction conditions, **7** (Fig. S5†) was isolated upon treatment of **5**, however we were not able to isolate a comparable product after treatment of **6**. Upon extended reaction times for the formation of **7**, a new product **8** was isolated that was identified as the hydrolysis product of **7**. The ^1H and ^{13}C NMR spectra of **8** showed two isomers most likely due to keto–enol tautomerism (Fig. S6†).

The reactivity of isothiazolones originates from the electronic structure of the heterocycle, which is supposed to have considerable aromatic character. This is illustrated by resonance structures II and III in Fig. 1. To provide experimental evidence for the aromaticity of isothiazolones, the crystal structures of **1** and **5** were resolved (Fig. 2, Table S1, CCDC 763228 and 763229†) The

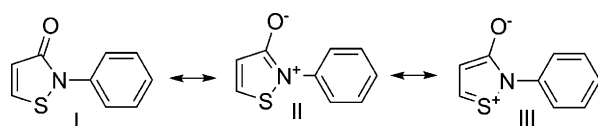


Fig. 1 Three resonance structures of **1**.

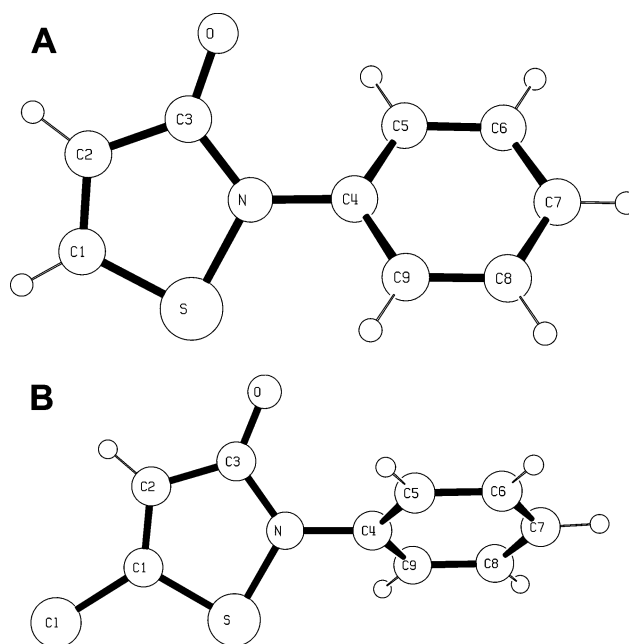


Fig. 2 Crystal structure of (A) 2-phenylisothiazolone **1** and (B) 5-chloro-2-phenylisothiazolone **5**. CCDC 763228 & 763229 contain the supplementary crystallographic data for this paper†. These data can be obtained free of charge at www.ccdc.cam.ac.uk/conts/retrieving.html.

numbering of the carbon atoms in the crystal structure deviates from the IUPAC numbering in Scheme 1. The crystal structure shows a planar isothiazolone ring for both **1** and **5**. In comparison with literature data (Table S1†) the C1–C2 bond length fits to a double bond, C2–C3 fits to a single, conjugated bond and C3–O to a double bond. This indicates that resonance structure I has a major contribution to the structure and that the S–N bond length has single bond character. Nevertheless, the C3–N bond is shorter than expected for a single bond and the C1–S bond is also shorter than observed for a single bond, which indicates that resonance structures II and III also contribute to the structure.

The observation that thiol nucleophiles attack isothiazolones at the sulfur atom can be explained by the strength of the formed sulfur–sulfur bond and the weakness of the sulfur–nitrogen bond. In contrast, isothiazolones are non-reactive towards oxygen or nitrogen nucleophiles, whereas 5-chloroisothiazolones react with these nucleophiles *via* addition–elimination at the 5-position.⁴

Cathepsin B and PCAF inhibition

Cathepsin B inhibition was evaluated by the cleavage of 7-amino-4-methyl coumarin (AMC) from the substrate Cbz-Arg-Arg-AMC.¹⁸ Inhibitory concentration (IC_{50}) values were derived after 15 min of pre-incubation with the inhibitors (Table 1). Since these inhibitors are expected to interact covalently with the enzyme, differences in potency reflect changes in enzyme binding as well as differences in the reaction rate of covalent binding to the enzyme. The assay for the HAT PCAF inhibition and the IC_{50} values of **9**, **10**, **12–18** and **19–26** have been reported previously.^{15,16}

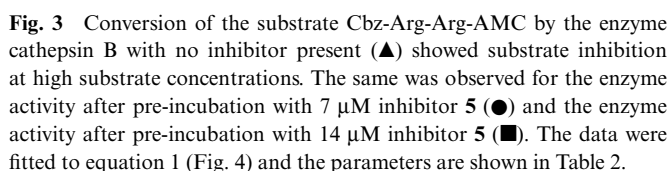
Disulfides **3**, **4** and **7**, isothiazolone **12** and 5-chloroisothiazolone **5** inhibited cathepsin B, whereas no inhibition of PCAF

| | R ₁ | R ₂ | R ₃ | Cat B ^a (μM) | PCAF ^b (μM) |
|-----------|----------------|-----------------|---|-------------------------|------------------------|
| 1 | H | H | -phenyl | >100 | >10 |
| 3 | H | H | -phenyl | 12 ± 1 | >10 |
| 4 | H | H | -(CH ₂) ₅ CO ₂ Me | 24 ± 1 | >10 |
| 5 | Cl | H | -phenyl | 13 ± 1 | >10 |
| 7 | Cl | H | -phenyl | 0.046 ± 0.002 | >10 |
| 9 | H | H | 3-Cl-4-F-phenyl | >100 | 4.2 ± 0.6 |
| 10 | Cl | H | 3-Cl-4-F-phenyl | 42 ± 2 | 2.5 ± 0.1 |
| 11 | Cl | H | 2-Cl-4,5-dimethoxy phenethyl | 4.1 ± 0.2 | 3.9 ± 0.4 |
| 12 | H | H | -pentyl | 13 ± 1 | >10 |
| 13 | Cl | H | -pentyl | 7.3 ± 0.5 | 2.9 ± 0.3 |
| 14 | Cl | H | -(CH ₂) ₂ CO ₂ Me | 12 ± 1 | 1.8 ± 0.2 |
| 15 | Cl | Cl | -(CH ₂) ₂ CO ₂ Me | 8.5 ± 0.7 | 2.6 ± 0.6 |
| 16 | Cl | CH ₃ | -(CH ₂) ₂ CO ₂ Me | 11 ± 1 | >10 |
| 17 | Cl | H | -(CH ₂) ₂ CO ₂ Et | 14 ± 2 | 2.0 ± 0.2 |
| 18 | Cl | H | -(CH ₂) ₃ CO ₂ Me | 11 ± 1 | 2.5 ± 0.3 |

was observed in the applied concentrations. Remarkably, disulfide **7** showed the highest potency for cathepsin B (46 nM). Isothiazolone **9** inhibited HAT PCAF, whereas cathepsin B was not inhibited, which demonstrated that selective PCAF inhibition can be obtained with N-aryl substituents. This was not observed for N-aliphatic substituted isothiazolones and 5-chloroisothiazolones **12–18**. Substitution in the isothiazolone 4-position (R_2), in **14**, **15** and **16**, changed the cathepsin B inhibition modestly. Remarkably, N-aliphatic substituted 5-chloroisothiazolone **11** was more potent for cathepsin B than the other N-aliphatic substituted 5-chloroisothiazolones. Furthermore, 5-chloroisothiazolone-1-oxides also inhibited cathepsin B (Table S2†) and their potency also depended on their substitution pattern. Taking these data together it is concluded that isothiazolones, 5-chloroisothiazolones and 5-chloroisothiazolone-1-oxides are inhibitors of cathepsin B and HAT PCAF and that their potency and selectivity depends on the substitution at the 2-, 4- and 5-position.

$$\begin{array}{ccccc} E + S & \xrightleftharpoons[K_m]{} & E - S & \longrightarrow & E + P \\ & & + S & & \\ & & \updownarrow [K_s] & & \\ & & E - S - S & & \end{array}$$

| [5] (μM) | V_{\max} (nmol s ⁻¹) | K_m (mM) | K_s (mM) | R^2 |
|----------|------------------------------------|------------|--------------|-------|
| 0 | 0.60 ± 0.41 | 1.2 ± 0.92 | 0.11 ± 0.087 | 0.996 |
| 7 | 0.36 ± 0.20 | 1.1 ± 0.68 | 0.11 ± 0.068 | 0.996 |
| 14 | 0.22 ± 0.10 | 1.1 ± 0.60 | 0.12 ± 0.069 | 0.997 |



$$v = \frac{V_{\max} \times [S]}{K_m + [S] \times \left(1 + \frac{[S]}{K_s}\right)} \quad \text{equation 1}$$

Fig. 4 Equation for non-linear curve fitting of enzyme kinetics that is influenced by substrate inhibition according to the model in Scheme 2¹⁹ v is the reaction velocity, V_{\max} is the maximal reaction velocity, $[S]$ is the substrate concentration and K_m is the Michaelis–Menten constant. K_s is the binding constant of a second substrate molecule to the enzyme substrate complex resulting in inhibition of the enzyme activity.

(Table 2). The same was observed for **14** (Fig. S7, Table S3†). This indicated non-competitive inhibition of the cathepsin B activity by 5-chloroisothiazolones. Inhibition of cathepsin B activity by **5** proved to be time dependent (Table S4†) and dilution experiments did not show reactivation of cathepsin B (Table S5†). Pre-steady state kinetic analysis revealed a very fast inactivation of the enzyme (50% inhibition after 20 s by 14 μM **5**, Table 1). The IC_{50} value proved to decrease slightly over time. This suggested a fast initial binding event followed by a slow second binding event (Table S4, S6†). Furthermore, mass-spectroscopy experiments showed an increased mass after incubation of cathepsin B with the inhibitor (Fig. S9, S10†). The mass spectrometry data showed that the inhibitor can bind one or two times to the enzyme. The mass spectrum of the non-reacted enzyme (Fig. S9†) gave m/z 27960 as the $[\text{M}+\text{H}]^+$ peak and 13985 as the $[\text{M}+2\text{H}]^{2+}$ peak. The mass spectrum of the enzyme pre-incubated with the inhibitor (Figure S10†) gave m/z 28111 and 28354 as the $[\text{M}+\text{H}]^+$ and 14082 and 14177 as the $[\text{M}+2\text{H}]^{2+}$ peaks. The difference between reacted and non-reacted enzyme was 151 and 394 for the $[\text{M}+\text{H}]^+$ peaks

and 97 and 192 for the $[M+2H]^{2+}$ peaks. This indicates that 5-chloroisothiazolone **5** binds once or twice to the enzyme to give a product corresponding to **8**, which would mean a mass increase of 194 or 388. Taking these experiments together, it is concluded that the 5-chloroisothiazolone **5** binds covalently to the thiolate in the enzyme active site and/or to another surface exposed cysteine of cathepsin B.

Conclusion

In conclusion, isothiazolones and 5-chloroisothiazolones react with thiolates by disulfide formation and cleavage of the nitrogen–sulfur bond. The crystal structures illustrate the aromatic character of isothiazolones. Whereas this aromaticity provides stability to the compounds, the weak sulfur–nitrogen bond allows a fast reaction with sulfur nucleophiles. Based on their reactivity, 5-chloroisothiazolones and isothiazolones inhibit thiol containing enzymes, such as the cysteine protease cathepsin B and HAT PCAF. Their potency depends on the substitution pattern. Selectivity for inhibition of HAT PCAF was observed for N-aryl substituted isothiazolone **9**. Enzyme kinetics and mass spectroscopy indicate covalent binding of 5-chloroisothiazolone **5** to the active site thiol and/or to another surface exposed cysteine of cathepsin B. The fact that isothiazolones and 5-chloroisothiazolones react with thiolates to form disulfides makes them excellent candidates for activity-based protein profiling of thiol-containing enzymes.

Experimental

General

All reagents and solvents were obtained from commercial suppliers (Sigma-Aldrich, Acros Organics) and were used without further purification. Dichloromethane was distilled over CaH_2 before use. Merck silica gel 60 F₂₅₄ plates were used for analytical thin layer chromatography (TLC) and spots were detected by UV light, or stained using KMnO_4 or ninhydrin solution. Column chromatography was performed with MP Ecochrom silica gel 32–63, 60 Å using the flash chromatography technique. ^1H (200 MHz) and ^{13}C (50 MHz) NMR spectra were recorded on a Varian Gemini 200 spectrometer in CDCl_3 unless otherwise specified. ^1H chemical shifts are reported in ppm (δ) downfield from internal tetramethylsilane (TMS). ^{13}C NMR spectra were recorded using the attached proton test (APT) and chemical shifts are relative to the solvent. Melting points were determined using an Electrocome digital melting point measurement apparatus. High-resolution mass spectra (HR-MS) were recorded using a flow injection method on a LTQ-Orbitrap XL mass spectrometer (Thermo Electron, Bremen, Germany) with a resolution of 60,000 at m/z 400. Protonated testosterone (lock mass m/z = 289.2162) was used for internal recalibration in real time.

The enzyme cathepsin B (human liver) and cathepsin Substrate III, Fluorogenic (Z-RR-AMC) was obtained from Calbiochem. 7-amino 4-methyl coumarin was obtained from Sigma.

Synthetic procedure 1

Isothiazolones and 5-chloroisothiazolones were synthesized according to the procedure described by Dekker *et al.*^{15,16} The 3,3'-dithiobis-propionamide was dissolved in dry CH_2Cl_2 at 0 °C and

SO_2Cl_2 was added dropwise to the solution. The mixture was stirred at 0 °C for 2 h. The mixture was washed with water (2 times) and brine (1 time), dried using Na_2SO_4 , filtered and concentrated under reduced pressure. The residue was purified by column chromatography.

5-chloro-2-phenylisothiazol-3(2H)-one (**5**)

The product was obtained using synthetic procedure 1. Purification was performed using column chromatography with hexane : EtOAc 10 : 1 (v/v) as eluent. Yield 43%. Off-white solid. Crystals (colorless needles) for X-ray diffraction were obtained by crystallization from EtOAc : hexanes 1 : 1 at 4 °C over 2 days. Mp = 118.4 °C. R_f 0.52 (hexane : EtOAc 1 : 1). ^1H NMR (200 MHz, CDCl_3) δ 6.35 (s, 1H), 7.27–7.55 (m, 5H). ^{13}C NMR (50 MHz CDCl_3) δ 114.9, 124.8, 127.8, 129.5, 135.7, 146.4, 165.4. MS (ESI) m/z 211.9 $[M+H]^+$. HRMS (m/z) 211.9931 $[M+H]^+$, calcd 211.9931 $\text{C}_9\text{H}_7\text{ClNOS}^+$.

2-phenylisothiazol-3(2H)-one (**1**)

After elution of product **5** product **1** was eluted from the column with hexane : EtOAc 1 : 1 (v/v) as eluent. The product was purified again using column chromatography using hexane : EtOAc 1 : 1 as eluent. Yield 29%. Off-white solid. Crystals (colorless platelets) for X-ray diffraction were obtained by crystallization from EtOAc : hexanes 3 : 4 at 4 °C over 2 days. Mp = 92.8 °C. R_f 0.16 (hexane : EtOAc 1 : 1). ^1H NMR (200 MHz, CDCl_3) δ 6.32 (d, J = 6.2 Hz, 1H), 7.29–7.61 (m, 5H), 8.18 (d, J = 6.5 Hz). ^{13}C NMR (50 MHz CDCl_3) δ 114.8, 124.8, 127.5, 129.4, 136.5, 139.8, 167.6. MS (ESI) m/z 178.1 $[M+H]^+$. HRMS (m/z) 178.0321 $[M+H]^+$, calcd 178.0321 $\text{C}_9\text{H}_8\text{NOS}^+$.

5-chloro-2-(2-chloro-4,5-dimethoxyphenethyl)isothiazol-3(2H)-one (**11**)

The product was obtained using synthetic procedure 1. Purification was performed using column chromatography with hexane : EtOAc 1 : 1 (v/v) as eluent. Yield 6%. White-yellow solid. Mp = 108.5 °C. R_f 0.20 (hexane : EtOAc 1 : 1). ^1H NMR (200 MHz, CDCl_3) δ 3.04 (t, J = 7.0 Hz, 2H), 3.82 (s, 3H), 3.86 (s, 3H), 3.95 (t, J = 7.0 Hz, 2H), 6.25 (s, 1H), 6.68 (s, 1H), 6.86 (s, 1H). ^{13}C NMR (50 MHz CDCl_3) δ 32.8, 43.3, 56.0, 112.5, 113.2, 114.3, 124.9, 126.4, 145.8, 147.8, 148.5, 166.6. MS (ESI) m/z 334.3 $[M+H]^+$. HRMS (m/z) 334.0065 $[M+H]^+$, calcd 334.0066 $\text{C}_{13}\text{H}_{14}\text{Cl}_2\text{NO}_3\text{S}^+$; (m/z) 199.0521 $[M-\text{C}_3\text{HCINOS}]^+$, calcd 199.0526 $\text{C}_{10}\text{H}_{12}\text{ClO}_2^+$.

Synthetic procedure 2

The 5-chloroisothiazolone (1 mmol) was dissolved in 2 mL CH_2Cl_2 and cooled to 0 °C. A 0.5 M propane-1-thiol solution in CH_2Cl_2 (1 mL) was added, and the reaction was followed by TLC. A 0.5 M triethylamine (TEA) solution in CH_2Cl_2 (1 mL) was added to the mixture and reacted for another 30 min. The mixture was diluted with 20 mL of CH_2Cl_2 and washed with H_2O (2 times 50 mL), brine (1 times 50 mL) and dried using Na_2SO_4 .

N-phenyl-3-(propyldisulfanyl)acrylamide (**3**)

The product was obtained using synthetic procedure 2. Purification was performed using column chromatography with

hexane:EtOAc 15:1 (v/v) as eluent. Yield 21%. White solid. R_f 0.70 (hexane:EtOAc 1:1). ^1H NMR (200 MHz, CD_3CN) δ 1.03 (t, $J = 7.4$ Hz, 3H), 1.72 (m, 2H), 2.80 (t, $J = 7.2$ Hz, 2H), 6.50 (d, $J = 14.1$ Hz, 1H), 7.09 (t, $J = 7.4$ Hz, 1H), 7.32 (t, $J = 7.5$ Hz, 2H), 7.51–7.64 (m, 3H), 8.50 (br. 1H). ^{13}C NMR (50 MHz CD_3CN) δ 13.2, 23.2, 40.4, 120.4, 121.2, 124.7, 129.8, 139.9, 142.9, 163.0. MS (ESI) m/z 254.1 $[\text{M}+\text{H}]^+$. HRMS (m/z) 254.0668 $[\text{M}+\text{H}]^+$, calcd 254.0668 $\text{C}_{12}\text{H}_{16}\text{NOS}_2^+$; (m/z) 178.0323 $[\text{M}-\text{C}_3\text{H}_7\text{S}]^+$, calcd 178.0321 $\text{C}_9\text{H}_8\text{NOS}^+$.

Methyl 6-(3-(propyldisulfanyl)acrylamido)hexanoate (4)

The product was obtained using synthetic procedure 2. Purification was performed using column chromatography with hexane:EtOAc 3:1 (v/v) as eluent. Yield 24%. White solid. R_f 0.36 (hexane:EtOAc 1:1). ^1H NMR (200 MHz, CD_3CN) δ 0.98 (t, $J = 7.2$ Hz, 3H), 1.25–1.70 (m, 10H), 2.77 (t, $J = 7.2$ Hz, 2H), 3.10–3.21 (m, 2H), 3.60 (s, 3H), 5.86 (d, $J = 9.7$ Hz, 1H), 6.59 (br. 1H), 6.99 (d, $J = 9.7$ Hz, 1H). ^{13}C NMR (50 MHz CD_3CN) δ 12.2, 22.1, 24.3, 26.0, 28.9, 33.4, 38.6, 41.0, 50.9, 118.0, 152.1, 165.9, 173.8. MS (ESI) m/z 306.0 $[\text{M}+\text{H}]^+$. HRMS (m/z) 328.1009 $[\text{M}+\text{Na}]^+$, calcd 328.1012 $\text{C}_{13}\text{H}_{23}\text{NNaO}_3\text{S}_2^+$; (m/z) 306.1191 $[\text{M}+\text{H}]^+$, calcd 306.1192 $\text{C}_{13}\text{H}_{24}\text{NO}_3\text{S}_2^+$; (m/z) 230.0844 $[\text{M}-\text{C}_3\text{H}_7\text{S}]^+$, calcd 230.0845 $\text{C}_{10}\text{H}_{16}\text{NO}_3\text{S}^+$.

N-phenyl-3-(propyldisulfanyl)-3-chloro-acrylamide (7)

The product was obtained using synthetic procedure 2. Purification was performed using column chromatography with hexane:EtOAc 20:1 (v/v) as eluent. Yield 60%. White solid. R_f 0.67 (hexane:EtOAc 1:1). ^1H NMR (200 MHz, CD_3CN) δ 0.99 (t, $J = 7.4$ Hz, 3H), 1.72 (m, 2H), 2.79 (t, $J = 7.2$ Hz, 2H), 6.56 (s, 1H), 7.10 (t, $J = 7.4$ Hz, 1H), 7.33 (m, 2H), 7.58 (d, $J = 7.6$ Hz, 2H), 8.50 (br. 1H). ^{13}C NMR (50 MHz CD_3CN) δ 13.2, 22.7, 42.8, 120.3, 122.2, 125.0, 129.8, 139.4, 151.4, 163.3. MS (ESI) m/z 288.1 $[\text{M}+\text{H}]^+$. HRMS (m/z) 288.0277 $[\text{M}+\text{H}]^+$, calcd 288.0278 $\text{C}_{12}\text{H}_{15}\text{ClNOS}_2^+$; (m/z) 211.9931 $[\text{M}-\text{C}_3\text{H}_7\text{S}]^+$, calcd 211.9931 $\text{C}_9\text{H}_7\text{ClNOS}^+$.

N-phenyl-3-(propyldisulfanyl)-3-hydroxy-acrylamide (8)

The product was obtained using synthetic procedure 2 with overnight reaction time. Purification was performed using column chromatography with hexane:EtOAc 5:1 (v/v) as eluent. Yield 30%. Yellow solid. R_f 0.57 (hexane:EtOAc 1:1). ^1H NMR (200 MHz, CDCl_3) δ 0.97 (m, 3H), 1.63 (m, 2H), 2.67 (m, 2H), 7.22 (m, 1H), 7.25 (m, 2H), 7.50 (m, 3H), 8.73 (br. 1H). ^{13}C NMR (50 MHz CDCl_3) δ 13.7, 23.3, 36.7, 37.1, 119.6, 119.7, 120.6, 120.7, 124.4, 125.4, 127.1, 129.2, 129.3, 130.8, 136.9, 137.1, 138.0, 157.4, 163.5. MS (ESI) m/z 270.1 $[\text{M}+\text{H}]^+$.

Cathepsin B inhibition

Enzyme inhibition was measured by the residual enzyme activity after 15 min incubation with the inhibitor. The enzyme activity was determined by conversion of cathepsin B substrate Cbz-Arg-Arg-AMC.¹⁸ The conversion rate was determined by fluorescence detection of 7-amino 4-methyl coumarin with 1 min intervals over 15 min at 25 °C. Detection was performed with an excitation wavelength of 355 nm and an emission wavelength of 450 nm.

The cathepsin B enzyme (0.5 mg mL^{-1}) was diluted 1:4000 with cathepsin B assay buffer (100 mM $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$; 1.25 mM EDTA.2Na; pH = 6.8) containing 1 mM DTT. The inhibitor (10 mM in CH_3CN) was diluted with cathepsin B assay buffer containing 0.1% (v/v) Triton-X to the desired concentration. Per assay 50 μL inhibitor solution was mixed with 50 μL enzyme solution and after 15 minutes incubation 50 μL substrate solution was added. Calculations were performed with Excel 2003 and non-linear curve fitting was performed with Origin 7 software.

Michaelis–Menten enzyme kinetics

The enzyme kinetic experiments were performed using the same assay setup as used for the IC_{50} determinations. Various concentrations of fluorogenic substrate Cbz-Arg-Arg-AMC (7.81–1000 μM) were added to the enzyme. The fluorescence values were converted to concentrations using a 7-amino 4-methyl coumarin calibration curve (0.78–25 μM). The concentration change over time is the reaction velocity (v) that is plotted against the substrate concentration ($[\text{S}]$) in Fig. 3, S7, S8†. The experiments were performed in triplicate and the average triplicate values and their standard deviations are plotted. Calculations were performed with Excel 2003 and non-linear curve fitting with equation 1 (Fig. 4) was performed with Origin 7 software. The results are shown in Table 2 and Table S3.†

Mass spectrometry

Inhibitor 5 (50 mM) and cathepsin B (0.25 mg mL^{-1}) were incubated in phosphate buffer (100 mM $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$; 1.25 mM EDTA.2Na; pH = 6.8) for 20 min at room temperature. This mixture was mixed 1 to 1 with the sinapinic acid matrix and subjected to MALDI TOF mass spectroscopy.

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