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1,2,4-Thiadiazole: A Novel Cathepsin B Inhibitor

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Abstract—A novel class of Cathepsin B inhibitors has been developed with a 1,2,4-thiadiazole heterocycle as the thiol trapping pharmacophore. Several compounds with different dipeptide recognition sequence (i.e., **P1′–P2′** = Leu-Pro-OH or **P2–P1** = Cbz-Phe-Ala) at the C5 position and with different substituents (i.e., OMe, Ph, or COOH) at the C3 position of the 1,2,4-thiadiazole ring have been synthesized and tested for their inhibitory activities. The substituted thiadiazoles **3a–h** inhibit Cat B in a time dependent, irreversible manner. A mechanism based on active-site directed inactivation of the enzyme by disulfide bond formation between the active site cysteine thiol and the sulfur atom of the heterocycle is proposed. Compound **3a** ($K_i = 2.6\,\mu M$, $k_i/K_i = 5630\,M^{-1}\,s^{-1}$) with a C3 methoxy moiety and a Leu-Pro-OH dipeptide recognition sequence, is found to be the most potent inhibitor in this series. The enhanced inhibitory potency of **3a** is a consequence of its increased enzyme binding affinity (lower K_i) rather than its increased intrinsic reactivity (higher k_i). In addition, **3a** is inactive against Cathepsin S, is a poor inhibitor of Cathepsin H and is >100-fold more selective for Cat B over papain.

Introduction

The activity of many therapeutically important enzymes depends on the cysteine residues that play essential catalytic or structural functions.[1–5] As cysteine proteases have been implicated in a wide variety of disease processes including cardiovascular, inflammatory, neurological, viral, musculo-skeletal disorders and cancer, the development of cysteine protease inhibitors is believed to have broad potential.[1–8]

Most of the reported cysteine protease inhibitors are derived from peptidic or peptidomimetic scaffolds in which the scissile amide is replaced by an electrophilic functionality or 'warhead'.[5,8] The cysteine thiol in the active site of the enzyme possesses thiolate characteristics and forms a covalent complex with the inhibitor resulting in loss of enzymatic activity.[5,6a] Extensive studies on cysteine protease inhibitors have been reported, in which the enzyme is irreversibly inactivated by modification of the cysteine thiol through nucleophilic displacement or through conjugate addition to the electrophilic warhead of the inhibitors.[5,8,9] Examples include methyl methanethiosulfonates,[10a] epoxides,[10b] haloketones,[10c] α-arylacetyloxy-methyl ketones,[10d] vinyl esters[10e] or vinyl sulphones.[10f] Alternatively, potent and reversible inhibitors based on highly electrophilic functionalities[8] such as aldehydes,[11a] bis-α-amidoketones[11b] or nitriles[11c] have been developed and studied. These efforts, however, have been largely frustrated due to the high reactivity and the lack of thiol specificity of these inhibitors, and may well be the underlying reason for their observed ex vivo and in vivo inefficacy and/or toxicity.[5,12]

In 1997, we disclosed the use of 1,2,4-thiadiazoles as thiol trapping agents.[13] In continuation of our research program, we have examined the application of this class of compounds to the inhibition of cysteine proteases. The heterocyclic nucleus of 1,2,4-thiadiazole provides several advantages. In contrast to most reported inhibitors, these compounds undergo ring opening with thiols but not with amines and/or alcohols.[14] Also, a wide variety of potential inhibitors can be designed by changing the substituents at the C3 and/or the C5 position of the ring (Scheme 1). It is conceivable that the C3 substituent can be tailored to tune the reactivity of the heterocycle, that is increase or decrease the rate of ring opening by

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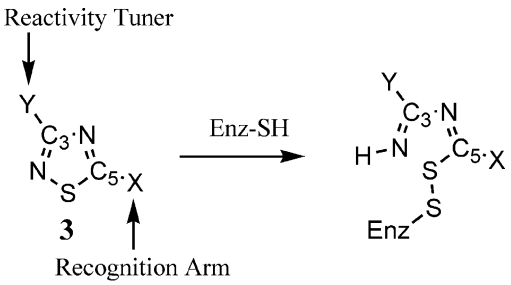


Scheme 1. Proposed mechanism for inhibition of cysteine proteases by thiadiazoles.

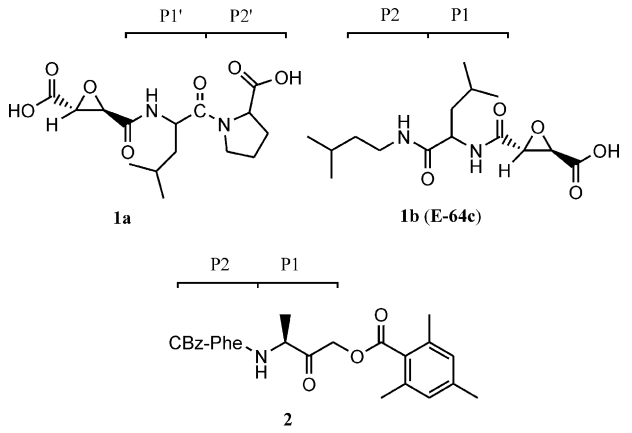
thiols, hence providing the necessary quiescence for the inhibitor to reach the targeted enzyme. Electron withdrawing substituents decrease the electron density on 1,2,4-thiadiazole ring facilitating nucleophilic attack at the sulfur center thus increasing the rate of ring opening. In addition, a peptidyl recognition or peptidomimetic replacement group can be attached at the C5 position of the heterocycle to introduce enzyme selectivity[15] (Scheme 1). The inhibition of tissue-type transglutaminase, a thiol-dependent enzyme, by monocyclic thiadiazoles has recently been reported.[16]

Cathepsin B (Cat B), a lysosomal cysteine protease, has been chosen for the evaluation of the potencies of several rationally designed inhibitors based on the 1,2,4-thiadiazole pharmacophore. Cat B belongs to the papain superfamily of enzymes, and exhibits both endopeptidase and exopeptidase activities.[15b] An interesting feature of the exopeptidase activity is that access to the substrate binding site in the prime region of the enzyme is restricted to two amino acid residues due to the presence of an occluding loop.[17] The peptidyl-dipeptidase activity distinguishes Cat B from the other members of the papain family. Hence, specific inhibitors of Cat B can be designed taking advantage of this feature.[6,15b]

Cat B has been implicated in several pathological processes[1,6] that include cancer[18] and neurodegenerative disorders.[19] Increased amounts of cathepsins B and L have been found in joint tissues of arthritic animals.[20] The inhibition of Cat B has been shown to decrease the severity of joint inflammation and to reduce the destruction of articular tissues in the rat model of antigen adjuvant-induced arthritis.[21] Cat B inhibitors are therefore expected to be useful for the treatment of inflammatory joint disease.[6,22]

Epoxysuccinyl peptides (**1**)[23] and aryloxymethyl ketones (**2**)[24] represent two extensively studied classes of Cat B inhibitors (Scheme 2). The enzyme recognition sequence for **1a** is leucylproline (Leu-Pro-OH), a dipeptide complementary to **S1′**–**S2′** substrate binding subsites, and that for **2** is *N*-carbobenzyloxy-L-phenylalanyl-L-alanyl (Cbz-Phe-Ala) which binds to the **S2**–**S1** subsites. The known inhibitor E-64c (**1b**) with a N^1-isoamylleucinamide (Leu-isoamyl) recognition sequence also binds to the **S2**–**S1** subsites (Scheme 2).[5,25]

In our study, two series of inhibitors have been designed and evaluated for the contribution of C3 and C5 substituents to reactivity and enzyme active site recognition. In the first series (**3a**–**3e**, Table 1), the dipeptide Leu-Pro-OH has been employed as prime site recognition sequence at C5 in a number of 1,2,4-thiadiazoles with different C3 substituents while in the second series (**3f**–**3h**), different dipeptides have been substituted at the C5 position of the thiadiazole ring to probe the non-prime enzyme binding site. In addition, the inhibitory activities of thiadiazole derivatives **4a**–**e**, as illustrated in Figure 1, have also been determined.



Scheme 2. Structure of epoxysuccinyl peptides (**1a**,**b**) and aryloxymetyl ketone (**2**). P denotes substrate residues that bind to corresponding subsites (S) in the enzyme.[25]

Results and Discussion

Chemistry

The synthesis of 5-chloro-1,2,4-thiadiazoles **5** has been accomplished[26] by reacting amidines **4** with perchloromethyl mercaptan (CCl_3SCl). The dipeptide methyl leucylprolinate (H-Leu-Pro-OMe)[27] is then reacted with **5** to give compounds **3a**–**3c** after base hydrolysis followed by acidification (Scheme 3).

Ethyl 2-oxo-1,3,4-oxathiazole-5-carboxylate,[28] obtained from the reaction of ethyl oxamate and chlorocarbonylsulfenyl chloride (ClC(O)SCl), undergoes a [3 + 2] cycloaddition with *p*-toluenesulfonyl cyanide (*p*-TsCN) on heating in 1,2-dichlorobenzene via a nitrile sulfide intermediate to afford 3-carboethoxy-5-*p*-toluenesulfonyl-1,2,4-thiadiazole.[29] It is then reacted with the dipeptide H-Pro-Leu-OMe, and subsequently transformed to compound **3d** through base hydrolysis followed by acidification as shown in Scheme 3.

Compounds **3e**–**3f** have been prepared by reacting 3-methoxy-5-amino-1,2,4-thiadiazole (**6**)[30] with 1,1-carbonyl diimidazole (CDI) to give the carbamoyl imidazole intermediates which are then condensed with peptides H-Leu-Pro-OMe and H-Leu-isoamyl,[31] respectively, to give the ester derivative of **3e,** and **3f**, respectively. Base hydrolysis of the ester followed by acidification afforded the desired carboxylic acid derivative **3e**. Compounds **3g**–**3h** have been prepared by direct peptide coupling of *N*-carbobenzyloxy-L-phenyl-

Table 1. Summary of inhibition kinetic parameters for thiadiazole derivatives **3a–h**

Compd	Y	X	Cat B			Cat S (M^{-1} s^{-1})	Cat H (M^{-1} s^{-1})	Papain (M^{-1} s^{-1})
			K_i[a] (μM)	K_i/K_i[a] (M^{-1} s^{-1})	k_i[a] (s^{-1})			
3a	MeO	–NH-Leu-Pro-OH	2.6	5630	0.015	NI	(200 μM)[b]	49
3b	Ph	–NH-Leu-Pro-OH	74	175	0.013	NI	NI	(120 μM)[b]
3c	Me	–NH-Leu-Pro-OH	447	55	0.026	nd	nd	nd
3d	HOOC	–NH-Leu-Pro-OH	300	293	0.089	nd	nd	nd
3e	MeO	–NHCONH-Leu-Pro-OH	390	36	0.014	NI	NI	NI
3f	MeO	–NHCONH-Leu-isoamyl	367	84	0.032	nd	nd	nd
3g	MeO	Cbz-Phe-NH–	21	658	0.013	(50 μM)[b]	(45 μM)[b]	NI
3h	MeO	Cbz-Phe-Ala-NH–	37	864	0.032	(50 μM)[b]	(50 μM)[b]	(142 μM)[b]

NI, not inhibited at up to 200 μM inhibitor concentration; nd, not determined.
[a]K_i is dissociation constant for non-covalent enzyme-inactivatior complex, k_i is rate constant for conversion of non-covalent complex to covalent adduct.
[b]IC_{50} for reversible inhibition determined at 10 μM substrate concentration.

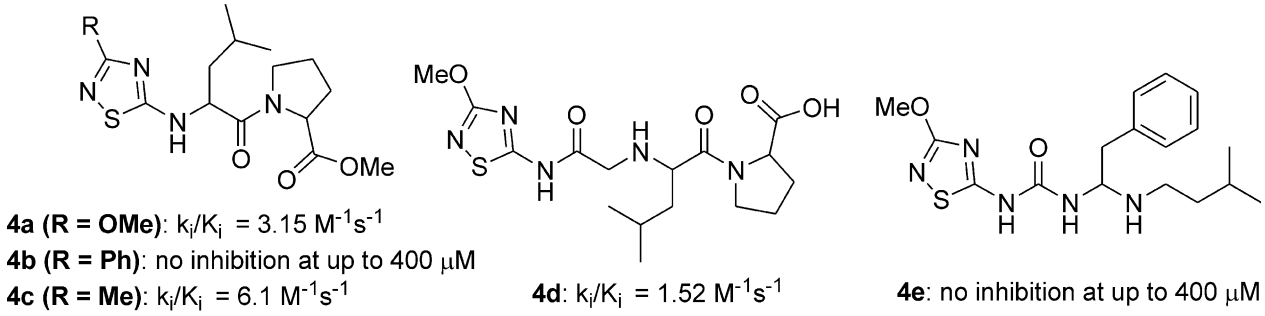
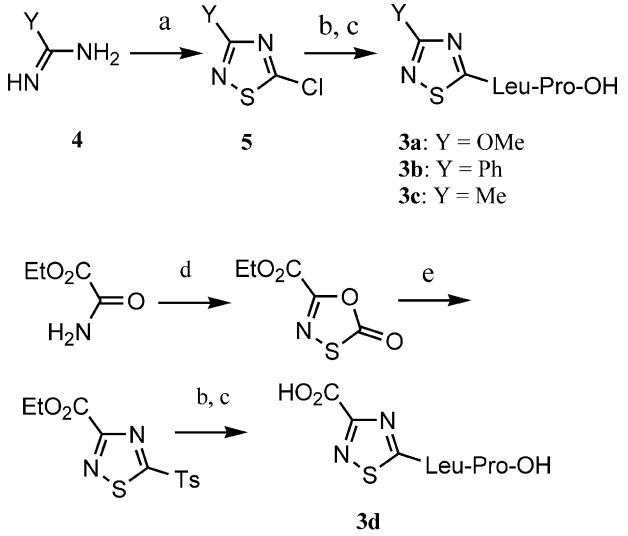


Figure 1. Inhibitory activities of thiadiazole derivatives (**4a–e**) against cathepsin B.

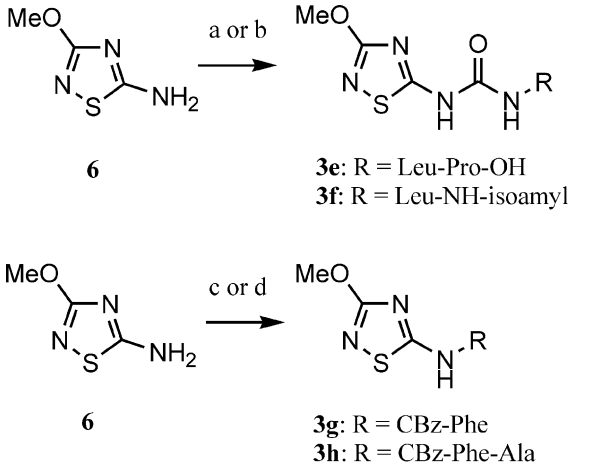
alanine (Cbz-Phe-OH) and *N*-carbobenzyloxy-L-phenylalanyl-L-alanine (Cbz-Phe-Ala-OH) with **6**, respectively, using the dicyclohexylcarbodiimide/1 - hydroxybenzotriazole (DCC/HOBt) procedure (Scheme 4).



Scheme 3. Preparation of substituted thiadiazoles **3a–3d**: (a) CCl_3SCl, NaOH, $CHCl_3$ or CH_2Cl_2/water; (b) H-Leu-Pro-OMe, DMF, Et_3N; (c) aq NaOH then aq HCl; (d) ClC(O)SCl; (e) *p*-TsCN, 1,2-dichlorobenzene, heat.

Enzyme inhibition

The substituted 1,2,4-thiadiazoles **3a–3h** inhibit Cat B in a time dependent, irreversible manner and the loss of enzymatic activity follows pseudo-first order kinetics. The inactivated enzyme does not regain its activity upon exhaustive dialysis. The enzymatic activity can, however, be restored in a time-dependent fashion by the addition of dithioerithritol (DTE) or dithiothreitol



Scheme 4. Preparation of substituted thiadiazoles **3e–3h**: (a) CDI/H-Leu-Pro-OMe, then aq NaOH followed by aq HCl; (b) CDI/H-Leu-isoamyl; (c) DCC/HOBt/Cbz-Phe-OH; (d) DCC/HOBt/ Cbz-Phe-Ala-OH.

(DTT) which are both disulfide bond reducing agents. The Cat B substrate, *N*-Cbz-Phe-Arg 7-amido-4-methylcoumarin, affords protection against the inactivation by 1,2,4-thiadiazoles. Thus, increasing the substrate concentration results in decreasing enzyme inactivation rates. These observations are consistent with the proposed mechanism (Scheme 1) of active-site directed inactivation of the enzyme brought about by the formation of a disulfide bond between the active site cysteine and the sulfur atom of the thiadiazole.[9] The disulfide bond is stable in the absence of reducing agents, but undergoes a disulfide exchange reaction with low molecular weight thiols that are able to access the active site of the enzyme with the inhibitor present.

Kinetic parameters for the inactivation of Cat B by substituted 1,2,4-thiadiazoles **3a–h** are summarized in Table 1. Compound **3a** inactivates Cat B with a second order rate constant of $5630\,M^{-1}\,s^{-1}$. When the methoxy group at the C3 position in **3a** is replaced with phenyl, methyl or carboxylate, the resulting compounds **3b–3d** are 20- to 100-fold less potent against Cat B. Analysis of the data shown in Table 1 indicates that the loss of the inhibitory activity is a result of reduced enzyme binding affinity (increased K_i) rather than a decreased intrinsic reactivity (k_i) of these compounds.

The methyl ester derivative **4a** (Fig. 1; $k_i/K_i = 1.52\,M^{-1}\,s^{-1}$) is significantly less potent than its acid counterpart **3a** (Table 1; $k_i/K_i = 5630\,M^{-1}\,s^{-1}$) at inhibiting Cat B. The energy minimized structure of **3a**-Cat B complex, with the prolyl carboxylate moiety docked to His110 and His111, is shown in Figure 2. The predicted orientation of **3a**, with S–S bond formation between Cys29 of Cat B and the S atom of the thiadiazole moiety in the active site is shown.

The introduction of a urea linker or a glycine residue at the C5 position of **3a** leads to compound **3e** or **4d** with decreased enzyme binding affinity and low overall activity. Similarly, compound **3f**, which has a urea linker inserted between the thiadiazole ring and the Leu-isoamyl (**P2–P1**) peptide recognition sequence inhibits Cat B with a low second order rate constant. Attachment of the **P2–P1** recognition sequence Cbz-Phe-Ala at the C5 position of thiadiazole results in compound **3h** which is 7-fold less active than its '**P1′–P2″**' counterpart **3a**. Interestingly, inhibitor **3g** with a Cbz-Phe substituent was only about 8-fold less active than **3a** against Cat B. Replacement of the thiadiazole moiety of **3e** with a 2-thiazolyl group leads to an inactive compound.[32]

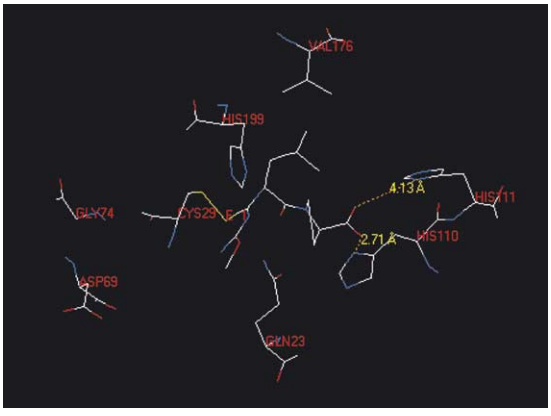
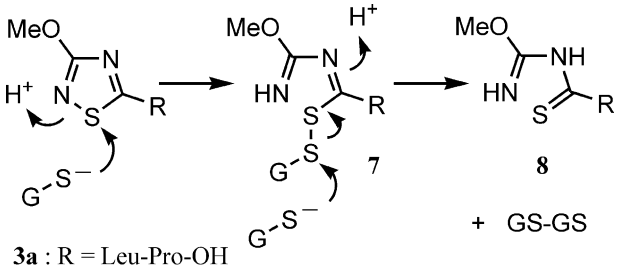


Figure 2. Predicted structure of **3a**–Cat B complex using SYBYL 6.4 from Tripos. The initial structure confined the prolyl carboxylate moiety to within bonding distance of His110 and His111, and the complex was then energy minimized. The salient features include S–S bond formation between Cys29 of Cat B and S atom of the thiadiazole moiety and the salt bridge formation between the prolyl carboxylate moiety with His110 and His111.

We have demonstrated in a model system that the 1,2,4-thiadiazole ring of **3a** is opened by reduced glutathione, presumably via a mechanism as illustrated in Scheme 5. The intermediate **7**, analogous to that proposed in the enzyme inactivation mechanism, undergoes fast disulfide exchange reaction with another molecule of reduced glutathione resulting in product **8** and oxidized glutathione. The molecular weight of **8** ($M^+ + 1 = 345$) has been confirmed by LC–MS technique. Analysis of the integrated chromatographic peak corresponding to compound **8** indicated quantitative conversion of **3a** to **8**. The rate of reaction of **3a** with reduced glutathione has been measured by UV spectroscopy. Quantitative conversion of **3a** to **8** without accumulation of intermediates has been confirmed by analysis of the reaction mixture at various stages of substrate conversion using chromatographic (HPLC) and LC–MS techniques.

The second order rate constant for the ring opening of **3a** with reduced glutathione is $1.7\,M^{-1}\,s^{-1}$ at pH 6.0, 37 °C and ionic strength of 1.0. Thus, the rate constant for enzyme inactivation (Table 1) is over 3000 times higher than the rate of reaction of **3a** with reduced glutathione. Rate acceleration of this magnitude cannot be explained only by increased nucleophilicity (decreased pK_a) of the active site cysteine. Assuming that the environment in the active site of Cat B ensures complete deprotonation of the active site thiol, the maximum achievable rate enhancement is 794-fold, since only 1.26×10^{-3} fraction GSH is in thiolate form at pH 6.0 (pK_a 8.9). Thus, the enhanced rate of reaction of **3a** with Cat B may be, in part, attributed to the orientational effects associated with binding of **3a** to the active site of the enzyme. The analysis of enzyme inactivation kinetics supports the formation of a reversible enzyme-inhibitor complex prior to inactivation. Dissociation constants (K_i) were measured for all inhibitors which could be assayed at concentrations close to their K_i values. The results obtained here strongly indicate that inhibition of Cat B by substituted 1,2,4-thadiazoles is brought about



Scheme 5. Opening of thiadiazole ring with glutathione.

by the formation of a disulfide bond between the sulfur atom of the inhibitor and Cys29 of Cat B.[9]

The reactivity of 1,2,4 thiadiazoles with thiols may contribute to the metabolism of these compounds in the body. For example at pH 6.0 and at a steady state concentration of GSH of 5 μM (approximate concentration in plasma) **3a** has a half-life of 22.6 h. Therefore, future efforts should be directed towards designing 1,2,4-thiadiazoles with decreased reactivity towards thiols and increased binding affinity towards Cat B.

Enzyme selectivity is a very important feature of protease inhibitors intended for therepeutic use. This is especially true for the inhibitors targeted towards enzymes of the papain superfamily. Members of this group have similar tertiary structures and substrate specificities.[6,15b] Therefore, selected thiadiazoles have been tested for the inhibition of Cathepsin L (Cat L), Cathepsin S (Cat S), Cathepsin H (Cat H) and papain. As discussed earlier, thiol reagents that are able to participate in disulfide exchange reaction with the enzyme–inhibitor complex, are capable of regenerating the enzyme activity. Therefore DTT, DTE or cysteine, commonly added to the solutions of thiol-dependent enzymes to protect them against the loss of enzymatic activity due to oxidation of the active site cysteine, can only be used with caution and at low concentration. Unlike Cat B that is stable for several hours in the absence of a thiol reducing agent, we found that Cat S, Cat H and papain, required low concentration of DTE present in solution. However, Cat L is unstable under our experimental conditions and, as a result, its inhibition by thiadiazoles could not be measured.

Compound **3a** is over 100-fold less effective at inactivating papain than Cat B. Cat S is not inhibited by **3a** at up to 200 μM concentration. Cat H shows reversible inhibition with an IC_{50} of 200 μM at 10 μM substrate. Similarly, other thiadiazoles reversibly inhibit some of the enzymes but the IC_{50}s are generally high (Table 1).

Conclusion

The 1,2,4-thiadiazole heterocycle can be added to the arsenal of electrophilic warheads to trap enzyme active site cysteine thiols through the formation of a disulfide bond. The 1,2,4-thiadiazoles **3a–h** irreversibly inhibit Cat B. Substituents at the C3 position of the heterocycle strongly influence the enzyme binding affinity (K_i) of the inhibitors **3a–d** (**3a**, OMe > **3b**, Ph > **3d**, COOH > **3c**, Me). The intrinsic reactivity (k_i) of these inhibitors, with the exception of **3d** which has an electron-withdrawing carboxylate moiety, are not significantly affected by substitution at the C3 position.

The inhibitor **3a** with prime site dipeptide recognition sequence (Leu-Pro-OH) at the C5 position shows a 6-fold better enzyme inhibitory activity (k_i/K_i) and a 14-fold higher binding affinity (K_i) than its non-prime site analogue (**3h**, Cbz-Phe-Ala). The enzyme inhibitory preference of **3a** for Cat B over Cat H, Cat S or papain is consistent with the characteristic exopeptidase activity of Cat B. In contrast, **3h** has marginal selectivity for binding to the active site of Cat B as compared to Cat H, Cat S or papain.

The application of 1,2,4-thiadiazoles to the inhibition of other therapeutically important cysteine proteases will be reported in future communications.

Experimental

Analytical data were recorded for the compounds described below using the following general procedures. ^1H and ^{13}C NMR spectra were recorded on a Bruker 400 MHz spectrometer; the chemical shifts (δ) are reported in ppm from an internal tetramethylsilane standard and the solvents are indicated in the text. Unless otherwise indicated, positive electronspray mass spectra (MS) were recorded on a Perkin-Elmer Sciex LC mass spectrometer, API300 LC/MS/MS system, operating in an open access mode. Positive ion atmospheric pressure chemical ionization (APCI) MS were performed for the more sensitive compounds. Melting points (mp, uncorrected) were determined with an open capillary tube Büchi 535 melting point apparatus.

Chromatography was done using the flash chromatography technique on silica gel (Merck Kieselgel 60: 230–400 mesh) and the mixed solvent systems are given in the text. Reagents were purchased from commercial sources. Commonly used abbreviations are: EtOAc (ethyl acetate), MeOH (methanol), CH_2Cl_2 (dichloromethane), DMF (*N*,*N*-dimethylformamide), DMSO (dimethylsulfoxide), DTE (dithioerithritol), DTT (dithiothreitol).

The following compounds were prepared according to published procedures: **5a–c**,[26] **6**,[30] ethyl 2-oxo-1,3,4-oxathiazole-5-carboxylate,[28] H-Leu-Pro-OMe[27] (methyl leucylprolinate), and H-Leu-isoamyl (N^1-isoamyl-leucinamide).[31]

N-(3-Methoxy-[1,2,4]thiadiazol-5-yl)-L-leucyl-L-proline methyl ester

A solution of the hydrochloride salt of H-Leu-Pro-OMe (5.6 g, 20 mmol) and triethylamine (5.6 mL, 40 mmol) in DMF (60 mL) was stirred at room temperature for 15 min. Then, a solution of **5a** (1.51 g, 10 mmol) in DMF (10 mL) and tetra-*n*-butylammonium bromide (150 mg) were added all at once. The resulting mixture was stirred at room temperature for 24 h. The reaction mixture was then quenched with water and extracted into EtOAc. The organic layer was washed with a saturated solution of sodium bicarbonate, brine, dried (sodium sulfate), filtered and concentrated in vacuo. Purification by column chromatography on silica gel using a mixture of hexane and EtOAc (7/3 and 6/4) afforded the title compound (1.92 g, 53.9%) as an off-white foam. ^1H NMR ($CDCl_3$) δ 7.10 (d, $J = 8.5$ Hz, 1H, N*H*CH), 4.74 (m, 1H, NHC*H*), 4.57 (dd, $J = 8.6$, 4.6 Hz, 1H, $CHCO_2$), 3.92 (s, 3H, OMe), 3.85 (m, 1H), 3.69 (s,

3H, OMe), 3.62 (m, 1H), 2.17–2.23 (m, 1H), 1.95–2.07 (m, 4H), 1.77–1.82 (m, 1H), 1.59–1.66 (m,1H), 0.97 (d, $J=6.5$ Hz, 3H, Me), 0.94 (d, $J=6.7$ Hz, 3H, Me); MS m/z 357 ($M^+ + 1$), 228, 200, 130, 101, 70.

The following compounds were prepared similarly.

***N*-(3-Phenyl-[1,2,4]thiadiazol-5-yl)-L-leucyl-L-proline methyl ester.** A white solid, 85% yield; mp 154.0–154.5 °C. ^{1}H NMR ($CDCl_3$) δ 8.14–8.16 (m, 2H, Ar–H), 7.83 (d, $J=8.7$ Hz, 1H, N*H*CH), 7.40–7.42 (m, 3H, Ar–H), 5.02 (m, 1H, NHC*H*), 4.68 (dd, $J=8.6$, 4.6 Hz, 1H, $CHCO_2$), 4.09–4.14 (m, 1H), 3.77–3.82 (m, 1H), 3.76 (s, 3H, OMe), 1.72–2.29 (m, 7H), 1.09 (d, $J=6.5$ Hz, 3H, Me), 0.98 (d, $J=6.6$ Hz, 3H, Me).

***N*-(3-Methyl-[1,2,4]thiadiazol-5-yl)-L-leucyl-L-proline methyl ester.** A white solid, 30% yield; mp 88–89 °C. ^{1}H NMR ($CDCl_3$) δ 7.80 (d, $J=8.6$ Hz, 1H, N*H*CH), 4.68 (m, 1H, NHC*H*), 4.58 (dd, $J=8.6$, 4.6 Hz, 1H, $CHCO_2$), 3.94–3.98 (m, 1H), 3.62–3.68 (m, 1H), 3.68 (s, 3H, OMe), 2.31 (s, 3H, Me), 2.07–2.22 (m, 1H), 1.96–2.07 (m, 3H), 1.69–1.79 (m, 4H), 0.96 (d, $J=6.6$ Hz, 3H, Me), 0.94 (d, $J=6.7$ Hz, 3H, Me).

***N*-(3-Methoxy-[1,2,4]thiadiazol-5-yl)-L-leucyl-L-proline (3a).** To an ice-cooled solution of *N*-(3-methoxy-[1,2,4]thiadiazol-5-yl)-L-leucyl-L-proline methyl ester (0.68 g, 1.91 mmol) in methanol (10 mL) was added a solution of 1 N sodium hydroxide (2.4 mL, 2.4 mmol). The resulting mixture was stirred in ice for 3 h, then at room temperature for 16 h. Volatile materials were removed in vacuo and the residue was dissolved in water (10 mL) and washed with ethyl acetate. The aqueous layer was collected and acidified with 1 N HCl solution (pH ca. 5.6) as a voluminous white precipitate separated. The mixture was extracted into EtOAc (6 × 25 mL), and the combined organic layers was dried (sodium sulfate), filtered and concentrated to a light yellow foam. Trituration with ether gave the desired product as a white solid (0.58 g, 89%). Mp foamed at 78 °C and melted at 89.0–92.0 °C. ^{1}H NMR ($CDCl_3$) δ 7.65 (br. s, 1H, N*H*CH), 4.70 (br. t, 1H, NHC*H*), 4.49 (t, $J=5.3$ Hz, 1H, $CHCO_2$), 3.97 (s, 3H, OMe), 3.56–3.58 (m, 2H), 2.03–2.21 (m, 3H), 1.60–1.76 (m, 3H), 0.76–0.99 (m, 1H), 0.99 (d, $J=6.5$ Hz, 3H, Me), 0.96 (d, $J=6.2$ Hz, 3H, Me); MS (m/z) 343 ($M^+ + 1$), 228, 200, 102, 83.

The following compounds were similarly prepared.

***N*-(3-Phenyl-[1,2,4]thiadiazol-5-yl)-L-leucyl-L-proline, 3b.** A white solid, 91.8% yield; mp foamed at 112 °C and melted at 136.0–138.0 °C. ^{1}H NMR ($CDCl_3$) δ 8.08–8.12 (m, 2H, Ar–H), 7.58 (br. s, 1H, N*H*CH), 7.38–7.42 (m, 3H, Ar–H), 4.90 (m, 1H, NHC*H*), 4.53 (m, 1H, $CHCO_2$), 4.09–4.14 (m, 1H), 3.52–3.62 (m, 1H), 2.00–2.32 (m, 4H), 1.75–1.95 (m, 2H), 1.55–1.64 (m, 1H), 1.04 (d, $J=5.9$ Hz, 3H, Me), 0.99 (d, $J=6.0$ Hz, 3H, Me), 0.85–0.92 (m, 1H); MS (m/z) 389 ($M^+ + 1$), 333, 274, 246, 190, 116, 70.

***N*-(3-Methyl-[1,2,4]thiadiazol-5-yl)-L-leucyl-L-proline, 3c.** A white solid, 80% yield; mp foamed at 81 °C and melted at 112–115 °C. ^{1}H NMR ($CDCl_3$) δ 7.30 (br. s, N*H*CH), 4.42–460 (m, 1H, NHC*H*), 3.80–4.00 (m, 1H, $CHCO_2$), 3.54–3.65 (m, 1H), 2.37 (s, 3H, Me), 2.07–2.42 (m, 5H), 1.50–1.90 (m, 4H), 0.96–1.10 (m, 6H, 2Me), 0.80–0.95 (m, 1H); MS (m/z) 327 ($M^+ + 1$), 271, 212, 184, 116, 70.

***N*-(3-Carboxy-1,2,4-thiadiazol-5-yl)leucylproline, 3d.** A mixture of ethyl 2-oxo-1,3,4-oxathiazole-5-carboxylate[28] (2.52 g, 14.4 mmol) and *p*-toluenesulfonyl cyanide (10 g, 55.2 mmol) in 40 mL of 1,2-dichlorobenzene was heated to 180 °C pot temperature under an atmosphere of nitrogen for 2 days. On cooling to ambient temperature, the reaction mixture was applied on top of a silica gel column and eluted with a gradient of solvent mixtures of hexanes and EtOAc (9/1, 85/15, 8/2 then 7/3). A product contaminated with some starting materials was obtained (2.3 g). This material was stirred in a mixture of hexanes and EtOAc and after filtration 3-carboethoxy-5-*p*-toluenesulfonyl-1,2,4-thiadiazole was obtained as a beige solid (1.56 g, 35% yield); mp 112–113 °C. ^{1}H NMR ($CDCl_3$) δ 8.00 (d, $J=8.0$ Hz, 2H), 7.40 (d, $J=8.2$ Hz, 2H), 4.44 (q, $J=7.4$ Hz, 2H, OCH_2), 2.43 (s, 3H, Ar–CH_3), 1.39 (t, $J=7.4$ Hz, 3H, CH_3CH_2); ^{13}C NMR ($CDCl_3$) δ 190.3, 165.5, 157.8 (C=O), 147.2 (Cipso), 133.8, 130.5 (two C), 129.4 (two C), 63.1 (CH_2O), 21.8 (Ar–CH_3), 14.0 (CH_3CH_2); MS (m/z) 313 ($M^+ + 1$), 285, 267 (100%), 139.

A solution of the hydrochloride salt of H-Leu-Pro-OMe (1.1 g, 4 mmol) and triethylamine (1.1 mL, 8 mmol) in 40 mL of DMF was stirred at room temperature for 10 min. Then, 3-carboethoxy-5-*p*-toluenesulfonyl-1,2,4-thiadiazole (0.63 g, 2 mmol) was added in one portion and the resulting mixture was heated at a pot temperature of 50 °C for 6.5 h. On cooling to ambient temperature, the reaction mixture was quenched with water and extracted into EtOAc. The organic fraction was collected and was successively washed with water, a 10% citric acid solution and water. It was then dried over sodium sulfate and filtered. The filtrate was evaporated to dryness and the residue was purified by flash chromatography on silica gel using a solvent mixture of hexanes and EtOAc (1/1) as eluent to afford methyl *N*-[3-(ethoxycarbonyl)-1,2,4-thiadiazol-5-yl]leucylprolinate as a light yellow oil (0.35 g, 44% yield). ^{1}H NMR ($CDCl_3$) δ 8.26 (d, $J=8.2$ Hz, 1H, N*H*CH), 4.82 (m, 1H, NHC*H*), 4.60 (dd, $J=8.3$, 4.9 Hz, 1H, $CHCO_2$), 4.40 (q, $J=7$ Hz, 2H, CH_2O), 4.23 (m, 1H), 3.67–3.76 (m, 1H), 3.72 (s, 3H, OMe), 2.24–2.29 (m, 1H), 1.99–2.14 (m, 3H), 1.69–1.84 (m, 3H), 1.38 (t, $J=7.2$ Hz, 3H, Me), 0.98–1.00 (2 d, 6H, 2Me); MS (m/z) 399 ($M^+ + 1$), 270, 242, 130 (100%), 70.

The title compound **3b** was obtained as a light-yellow solid by base saponification of methyl *N*-[3-(ethoxycarbonyl)-1,2,4-thiadiazol-5-yl]leucylprolinate followed by acidification as described before for the preparation of **3a**. Yield of **3b**: 69%; mp foamed at 110 °C and melted at 142.0–145.0 °C. ^{1}H NMR (CD_3OD) δ 5.63 (d, $J=8.2$ Hz, 1H), 4.10–4.52 (m, 2H), 3.65–3.68 (m, 1H), 3.52–3.52 (m, 1H), 2.25–2.34 (m, 1H), 1.57–2.12 (m, 6H), 0.99–1.03 (2 d, 6H, 2Me); MS (m/z) 357 ($M^+ + 1$), 311, 273, 242, 214, 116 (100%), 70.

{3-Methoxy-[1,2,4]-thiadiazol-5-yl}carbamoyl-L-leucyl-L-proline methyl ester. To a solution of **6** (1.31 g, 10 mmol) in CH_2Cl_2 (15 mL) at room temperature was added CDI, (1.78 g, 11 mmol) followed by triethylamine (2.1 mL, 15 mmol). The resulting suspension was stirred under nitrogen for 2.5 h. Volatile materials were removed in vacuo and the residue was dissolved in DMF (20 mL). The latter was then added to a solution of H-Leu-Pro-OMe hydrochloride (4.18 g, 15 mmol) and triethylamine (2.1 mL, 15.1 mmol) in DMF (25 mL). The resulting mixture was heated at 120 °C for 2 h and then allowed to cool to room temperature. Volatile materials were removed in vacuo and the residue was diluted with EtOAc (250 mL) and water (100 mL). The aqueous layer was again extracted with EtOAc (2 × 100 mL). The combined organic layers were dried (sodium sulfate), filtered and evaporated to dryness. Purification by column chromatography on silica gel using a solvent mixture of hexanes and EtOAc (1/1 and 2/3) afforded the title compound as a light yellow foam (3.2 g, 76.6%); mp 88–90 °C. ^{1}H NMR ($CDCl_3$) δ 12.75 (s, 1H, NH), 6.70 (d, J = 12.0 Hz, 1H, NH), 4.79–4.87 (m, 1H, NCHpro), 4.54–4.59 (dd, J = 11.8, 6.0 Hz, 1H, NCH), 4.12 (s, 3H, OMe), 3.64–3.90 (m, 2H, NCH_2pro), 1.56–2.26 (m, 6H, $3CH_2$), 0.94–1.12 (2 d, J = 9.7 Hz, 7H, $2CH_3$ and 1CH); ^{13}C NMR ($CDCl_3$) δ 178.3 (C3), 172.3, 171.1 (CO_2), 165.9 (C5), 153.7, 58.8, 56.6, 52.2, 49.8, 47.0 (CH_2), 42.0 (CH_2), 29.0 (CH_2), 24.8 (CH_2), 24.5, 23.2, 22.0; MS (m/z) 400 ($M^+ + 1$),326, 271, 243, 209,158, 130, 87.

{3-Methoxy-[1,2,4]-thiadiazol-5-yl}carbamoyl-L-leucyl-L-proline (3e). Base saponification of 3-methoxy-5-(*N*-L-leucyl-L-proline)carbamoyl-[1,2,4]-thiadiazole methyl ester using a solution of 1 N sodium hydroxide followed by acidification using a solution of 1 N HCl afforded **3e** as a white solid (55%). Melting point 209–211 °C. ^{1}H NMR (CD_3OD) δ 4.64–4.67 (dd, J = 8.1, 2.3 Hz, 1H, NCH), 4.44–4.45 (m, 1H, NCHpro), 3.94 (s, 3H, OMe), 3.60–3.86 (m, 2H, NCH_2pro), 1.54–2.27 (m, 6H, $3CH_2$), 0.94–1.01 (2 d, J = 6.4 Hz, 7H, $2CH_3$ + 1CH); MS (m/z) 386 ($M^+ + 1$), 271, 229, 158, 116, 86.

***N*^{1}-Isoamyl-*N*^{2}-{[(3-methoxy-1,2,4-thiadiazol-5-yl)amino] carbonyl}leucinamide, (3f).** This was prepared as described above using the CDI coupling procedure starting from **6** and N^1-isoamylleucinamide (H-Leu-isoamyl). White solid; yield: 94%. ^{1}H NMR ($CDCl_3$) δ 12.82 (s, 1H, NH), 6.61 (d, J = 8.5 Hz, 1H, NH), 6.13 (t, J = 5.3 Hz, 1H, NH); 4.37–4.43 (m, 1H), 4.15 (s, 3H, OMe), 3.25–3.34 (m, 2H), 1.66–1.80 (m, 3H), 1.57–1.65 (m, 2H), 1.39–1.44 (q, J = 7.2 Hz, 2H), 0.96–0.99 (2 d, 6H, $2CH_3$), 0.87–0.92 (m + 2 d, 7H, 1H + $2CH_3$).

3-Methoxy-5-(*N*-carbobenzyloxy-L-phenylalaninamido)-[1,2,4]thiadiazole (3g). To an ice-cooled solution of **6** (1.31 g, 10 mmol) and *N*-carbobenzyloxy-L-phenylalanine, (Cbz-L-Phe-OH 2.99 g, 10 mmol) in DMF (100 mL) was added DCC, (2.06 g, 10 mmol) followed by HOBt, (1.35 g, 10 mmol). The resulting mixture was stirred at room temperature for 16 h. The mixture was diluted with EtOAc (250 mL) and water (200 mL). The organic layer was collected and successively washed with a solution of 1 N HCl (15 mL), water, a saturated solution of sodium bicarbonate, and water. The organic layer was dried (sodium sulfate), filtered and concentrated in vacuo. Purification by column chromatography using a solvent mixture of hexanes and EtOAc (3/1) afforded **3g** as a colorless oil. Crystallization from ether and hexanes gave the product as a white solid (1.1 g, 26.7%). ^{1}H NMR ($CDCl_3$) δ 12.50 (s, 1H, NH), 7.06–7.35 (m, 10H, Ar–H), 6.02 (br. d, J = 7.6 Hz, 1H, NH), 5.19–5.22 (m, 1H, CH), 5.11 (s, 2H, OCH_2), 4.05 (s, 3H, OMe), 3.11–3.20 (m, 2H, $CHCH_2$ Ar); MS (m/z) 413 ($M^+ + 1$), 369, 222, 210, 132, 91.

3-Methoxy-5-(*N*-carbobenzyloxy-L-phenylalanyl-L-alaninamido)-[1,2,4]thiadiazole (3h). To a solution of **6** (0.37 g, 2.8 mmol) and *N*-carbobenzyloxy-L-phenylalanyl-L-alanine, (*N*-Cbz-Phe-Ala-OH, 1.0 g, 2.7 mmol) in DMF (25 mL) was added DCC, (0.56 g, 2.7 mmol). After stirring for 30 min, HOBt (0.36 g, 2.7 mmol) was added and the resulting mixture was stirred at room temperature for 20 h. Volatile materials were removed in vacuo and the residue was purified by column chromatography on silica gel using a solvent mixture of CH_2Cl_2 and MeOH (96/4) thereby affording the title compound as a white solid (1.2 g). Further purification by crystallization and chromatography on silica gel gave **3h** (0.87 g, 67% yield); mp 161–162 °C. ^{1}H NMR ($CDCl_3$) δ 7.14–7.38 (m, 12H, 10Ar–H and 2 NH), 5.92 (br. s, 1H, NH), 5.06 (s, 2H, OCH), 4.38 (m, 1H, $CHCH_2$Ar), 4.00 (m, 1H, $CHCH_3$), 3.97 (s, 3H, OMe), 3.00–3.08 (m, 2H, $CHCH_2$Ar), 1.34 (d, J = 8.9 Hz, 3H, Me); MS (m/z) 484 ($M^+ + 1$), 416, 361, 316, 285, 185, 132, 75.

Kinetics

Reaction of 3a with reduced glutathione. All buffers were thoroughly degassed to prevent oxidation of reduced glutathione. Potassium phosphate buffer pH 6.0 (0.2–1.0 M), glutathione (final concentration at least 15-fold higher than **3a**) and KCl (added to adjust the ionic strength to 1.0) were incubated in a final volume of 995 μL in a quartz cell (1 cm light path) at 37 °C for 5 min. The reactions were initiated by the addition of **3a** stock solution (5 μL in DMSO). The progress of the reaction was monitored by following the increase of absorbance at 300 nm. The pseudo-first-order rate coefficients were calculated by fitting the data (absorbance vs time) to a first order rate equation. To correct for buffer catalysis, the rate coefficients were measured at different buffer concentrations. The rate coefficients extrapolated to zero buffer concentration were plotted against the concentration of reduced glutathione to obtain second order rate constant. To ensure complete conversion of the substrate and to identify reaction intermediates and products, the reaction mixture at various stages of progress was analyzed using liquid chromatography coupled with mass spectroscopy (LC–MS).

Enzyme kinetics. Enzymes were regenerated by incubation with 2.5 mM DTE, 2.5 mM EDTA and 0.005% (v/v) Brij-35 (0.01% Triton X-100 was used instead of Brij-35 for Cathepsin S) in 50 mM potassium phosphate

(pH 6.0 for Cat B, pH 6.5 for Cat H and Cat S, and pH 6.8 for papain) for 5 min at 25 °C. Acetate buffer pH 5.5 was used for Cat L. In the case of Cat B, DTE was removed by size exclusion chromatography on a NAP-10 column (Pharmacia) equilibrated with 20 mM sodium acetate pH 5.0 containing 10 mM EDTA. Cat B activated in this way was stable for several hours. Cat S, Cat H and papain were found to rapidly lose activity in the absence of DTE. Therefore, these enzymes were stored and used in the regeneration buffer containing DTE. In cases when reversible inhibition was observed, it was confirmed that it was not due to the regeneration of the inactivated enzyme with DTE. Cat L was unstable under these reaction conditions. Therefore inhibition parameters for this enzyme could not be measured.

Enzyme inhibition was measured using published procedures.[23a,33] Fluorogenic substrates, *Z*-Phe-Arg-NHMec for Cat B, Cat L and papain, *Z*-Val-Val-Arg-NHMec for Cathepsin S, and Arg-NHMec for Cathepsin H were used. Reactions were started by the addition of the enzyme (1–5 μL, 0.6–2.4 μM) to the assay mixture containing substrate (10–100 μM), inhibitor (2.0 μM–4.0 mM) EDTA (2 mM), and DMSO (1–1.5%) in 50 mM potassium phosphate pH 6.0, in a final volume of 3 mL. The reactions were incubated at 25 °C. The progress of inhibition was monitored by following the increase of fluorescence at the excitation wavelength of 383 nm and the emission wavelength of 450 nm. For poorly soluble inhibitors, the inhibition was assayed at the solubility limit.

When time-dependent enzyme inhibition was observed, the reaction progress curves were recorded continuously over five half-lives. No more than 5% of substrate was converted to product. Inactivation parameters were calculated using method of Tian and Tsou.[34] In the cases where reversible inhibition was observed, IC_{50} values were determined at 10 μM substrate concentration.

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