Azapeptides as Inhibitors and Active Site Titrants for Cysteine Proteinases

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Ester and amide derivatives of α-azaglycine (carbazic acid, $H_2NNHCOOH$), α-azaalanine, and α-azaphenylalanine (i.e., Ac-L-Phe-NHN(R)CO-X, where X = H, CH_3, or CH_2Ph, respectively) were synthesized and evaluated as inhibitors of the cysteine proteinases papain and cathepsin B. The ester derivatives inactivated papain and cathepsin B at rates which increased dramatically with leaving group hydrophobicity and electronegativity. For example, with **8** (R = H, X = OPh) the apparent second-order rate constant for papain inactivation was 67 600 M^{-1} s^{-1}. Amide and P_1-thioamide derivatives do not inactivate papain, nor are they substrates; instead they are weak competitive inhibitors (0.2 mM < K_i < 4 mM). Inactivation of papain involves carbamoylation of the enzyme, as demonstrated by electrospray mass spectrometry. Active site titration indicated a 1:1 stoichiometry for the inactivation of papain with **8**, and both inactivated papain and cathepsin B are highly resistant to reactivation by dialysis ($t_{1/2}$ > 24 h at 4 °C). Azaalanine derivatives Ac-L-Phe-NHN(CH_3)CO-X inactivate papain ca. 400−900-fold more slowly than their azaglycine analogues, consistent with the planar configuration at N_α of the P_1 residue and the very substantial stereoselectivity of papain for L- vs D- residues at the P_1 position of its substrates. Azaglycine derivative **9** (R = H, X = $OC_6H_4NO_2$-*p*) inactivates papain extremely rapidly (>70 000 M^{-1} s^{-1}), but it also decomposes rapidly in buffer with release of nitrophenol (k_{obs} = 0.13 min^{-1}); under the same conditions **8** shows <7% hydrolysis over 24 h. This nitrophenol release probably involves cyclization to an oxadiazolone since **17** (R = CH_3, X = $OC_6H_4NO_2$-*p*), which cannot form an isocyanate, releases nitrophenol almost as rapidly (k_{obs} = 0.028 min^{-1}). Cathepsin C, another cysteine proteinase with a rather different substrate specificity (i.e., aminopeptidase), was not inactivated by **8**, indicating that the inactivation of papain and cathepsin B by azapeptide esters is a specific process. Their ease of synthesis coupled with good solution stability suggests that azapeptide esters may be useful as active site titrants of cysteine proteinases and probes of their biological function in vivo.

Introduction

The number of reports on the involvement of cysteine proteinases in a wide range of biological processes has grown enormously in the past decade. Cysteine proteinases are found in plants, bacteria, protozoa, and fungi, as well as in mammals where both endogenous and virus-induced forms are known. They play important roles in a multitude of physiological as well as pathological processes including lysosomal function, bone resorption, hormone biosynthesis and inactivation, certain viral and protozoal infections, and even apoptosis or "programmed cell death" (for reviews, see refs 1 and 2). With increased awareness of the widespread and often pivotal involvement of cysteine proteinases in disease processes has come increased interest in the development of inhibitors for these enzymes. As an extension of our own efforts in this direction,[3−5] we wish to report here on the inhibition of two related cysteine proteinases, papain and cathepsin B, by a series of azapeptide analogues of peptide substrates for these enzymes.

Papain is an endoproteinase from plants that cleaves at peptide bonds having a large hydrophobic side chain in their P_2[6,7] position. It also displays considerable kinetic specificity for small substrates based on the Ac-L-Phe-Gly-X motif, where X is an alcohol, amine, or peptide leaving group.[8,9] Cathepsin B is a mammalian lysosomal proteinase which is secreted by some tumors and has been implicated in facilitating tumor metastasis. It has a similar protein folding pattern to that of papain and a somewhat similar active site structure and mechanism. However, because of a small insertion (relative to papain) in its primary sequence that affects the "prime side" of its substrate binding cleft, it has significant dipeptidyl carboxypeptidase activity in addition to its endoproteinase activity.[10,11] Like papain, cathepsin B readily cleaves Ac-L-Phe-Gly-X type substrates, and for both enzymes this kinetic specificity carries over to substrate-like inhibitors as well.[10,12,13] Although papain and cathepsin B are not closely related to serine proteinases in terms of protein structure, they are similar in utilizing an acyl enzyme mechanism of catalysis and in having an active site histidine positioned to help deprotonate the active site nucleophile.[14,15]

Many inhibitors of cysteine and serine proteinases[1,16−19] contain a reactive aldehyde or ketone group that undergoes *reversible* covalent adduct formation with the active site nucleophile group. Because such adducts resemble transition states or reaction intermediates in substrate hydrolysis, they are very tightly

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Table 1. Apparent Second-Order Rate Constants for Inactivation of Papain and Cathepsin B by Azapeptide Esters and Amides

		k_2/K_i (M^{-1} s^{-1})	
compd	structure	papain	cathepsin B
1	AcPheNHNHCOOMe	19	
2	AcPheNHNHCOOEt	47	
3	AcPheNHNHCOOiBu	55	
4	AcPheNHNHCOOCH$_2$CH$_2$Cl	1150	54
5	AcPheNHNHCOOCH$_2$CH$_2$Br	1250	90
6	AcPheNHNHCOOCH$_2$CCl$_3$	11800	730
7	AcPheNHNHCOOCH$_2$Ph	30500	310
8	AcPheNHNHCOOPh	67600	7730
9	AcPheNHNHCOOC$_6$H$_4$NO$_2$-*p*	>70000[a]	
10	AcPheNHNHCONH$_2$	4	0
11	AcPheNHNHCONHC$_6$H$_4$NO$_2$-*p*	1	0
12	AcPheNHNHCSNH$_2$	0.03	
13	AcPheNHN(CH$_3$)COOMe	0.05	
14	AcPheNHN(CH$_3$)COOEt	0.05	
15	AcPheNHN(CH$_3$)COOCH$_2$CCl$_3$	12	0.34[b]
16	AcPheNHN(CH$_3$)COOPh	110	18[c]
17	AcPheNHN(CH$_3$)COOC$_6$H$_4$NO$_2$−*p*	*a*	
18	AcPheNHN(CH$_2$Ph)COOPh	0.3[b]	<0.2[b]

[a] See text. [b] k_{obs}/[I], with [I] = 0.95 mM. [c] k_{obs}/[I], with [I] = 0.20 mM.

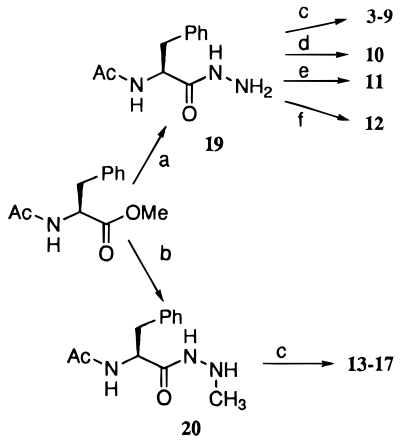
bound; consequently they are also potent competitive inhibitors. The greater nucleophilicity of cysteine (thiolate) vs serine probably accounts for the *selective* inhibition or inactivation of cysteine proteinases by peptidyl nitriles, Michael acceptors, epoxides, diazomethanes, acyloxymethyl ketones, and *O*-acylhydroxamates. Peptidyl halomethanes irreversibly inactivate both cysteine and serine proteinases but by different processes, i.e., alkylation of the active site thiolate[20] vs the active site histidine,[21,22] respectively.

Inhibitor Design

Replacing the α carbon of the P_1 amino acid of a peptide substrate with a nitrogen generates an azapeptide[23−26] in which the P_1 amino acid is formally a derivative of carbazic acid ($H_2NNHCOOH$). This modification decreases the electrophilicity of the P_1 carbonyl group and changes the geometry of the α position from tetrahedral to trigonal (i.e., planar, achiral) so that the P_1 residue is neither D nor L but "in between". For these reasons some azapeptides are more resistant to enzymatic hydrolysis than their normal peptide analogues. On the other hand, azapeptide derivatives with suitably reactive leaving groups acylate serine proteases forming *carbamoyl* enzymes which undergo deacylation very slowly. As a result, enzyme accumulates in a catalytically inactive from. This approach has been widely studied in regard to serine proteinases[24,27−31] and other serine hydrolases such as acetyl cholinesterase for which carbamoylating inhibitors are important therapeutic agents.

A few azapeptide-based inhibitors have been designed for cysteine proteinases, but most have been thiol-alkylating halomethyl ketone analogues rather than carbamoylating agents.[32−35] An exception to this is the work of Magrath and Abeles[36] who reported that azapeptide **1** (structure in Table 1) and several of its analogues were inactivators of papain. The intermediacy of a stable acyl enzyme was inferred on the basis of an increasing rate of inactivation from **1** to **3** to **8** and on the reactivation of the inhibited enzyme by

Scheme 1[a]



[a] (a) H_2N-NH_2; (b) H_2N-$NHCH_3$; (c) ROCOCl; (d) Me_3SiNCO; (e) *p*-$O_2NC_6H_4NCO$; (f) Me_3SiNCS.

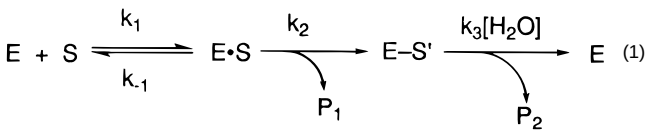
incubating it with valine methyl ester. As their report appeared we were also involved in examining the interaction of a series of azapeptides with papain and cathepsin B. In this manuscript we present results with azapeptides **1**−**18** which both confirm and extend the work of Magrath and Abeles.

Chemistry

Azapeptides **1** and **2** were prepared by mixed anhydride coupling of Ac-L-Phe with commercially available methyl and ethyl carbazates, respectively. For the synthesis of compounds **3**−**9** (Scheme 1), Ac-L-Phe-OMe was condensed with hydrazine and the resulting acetyl-L-phenylalaninehydrazide (**19**) was acylated with the requisite chloroformate esters. Compounds **10**−**12** were prepared by condensation of **19** with trimethylsilyl isocyanate, *p*-nitrophenyl isocyanate, or trimethylsilyl isothiocyanate, respectively; the silyl groups were evidently lost during workup or chromatography. For preparation of **13**−**17**, the requisite hydrazide (**20**) was prepared by condensation of Ac-L-Phe-OMe with methylhydrazine, followed by chromatographic separation of the two regioisomeric hydrazide products, which are easily distinguishable by ^{1}H NMR.[30] In this case the major product (59% yield) was the desired isomer Ac-L-Phe-$NHNHCH_3$. Acylation of the latter with the appropriate chloroformate esters afforded **13**−**17**.

Results and Discussion

The catalytic mechanism of serine and cysteine proteinases involves three principle steps, namely, substrate recognition and noncovalent binding, acylation of the active site nucleophile with release of the leaving group, and deacylation or acyl transfer to water (eq 1).



(1)

With serine proteinases, azapeptide substrates with poor leaving groups fail to acylate the enzyme and hence are at best competitive inhibitors. Azapeptides with more reactive leaving groups acylate the active site serine, but the resulting carbamoyl enzymes are less reactive than the normal acyl enzymes. Thus, depend-

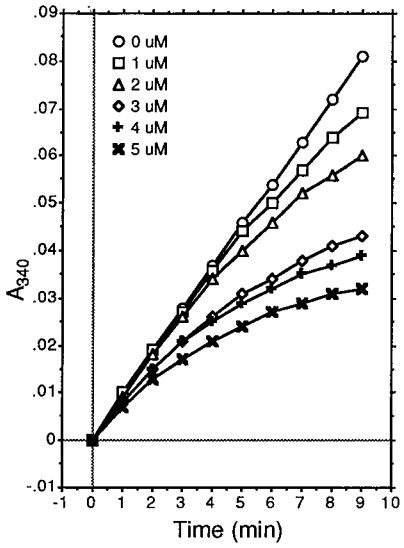


Figure 1. Effect of Ac-L-Phe-AGly-OCH_2CCl_3 (**6**) on progress curves for papain-catalyzed hydrolysis of Cbz-Gly-ONp. The assay substrate (50 μM) and inhibitor (concentrations indicated on figure) were combined in a cuvette, and the reaction was initiated by addition of papain (0.0125 μM).

ing on the particular enzyme and azapeptide, the deacylation rate may be anywhere from somewhat reduced to effectively zero. In the latter case the compounds behave like irreversible affinity-labeling agents for the enzyme.

Azapeptide **1** has been reported[36] to inactivate papain with an apparent second-order rate constant (k_2/K_i) of 13 M^{-1} s^{-1}. Under slightly different experimental conditions with **1**, we observed the same type of behavior and a second-order rate constant of 19 M^{-1} s^{-1} (Table 1). As will be shown below, this inactivation is specific, covalent, stoichiometric, and essentially irreversible on the time scale of our experiments. The time course of this type of enzyme inhibition is illustrated in Figure 1 using compound **6,** which is conveniently faster-acting than **1** but produces the same (inactive) carbamoyl enzyme product. As can be seen, incubation of even low-micromolar concentrations of **6** with papain leads to complete inactivation of the enzyme in a matter of minutes.

Specificity of Inactivation. Papain shows a marked preference for hydrophobic as well as for reactive (i.e., acidic, electronegative) leaving groups.[3,37−40] Across the series of azapeptides **1**−**9** the interplay of these two factors results in a gradual increase of over 3500-fold in the rate of papain inactivation without changing the peptide portion of the molecule (Table 1). The electrophilicity of carbonyl carbons decreases in the order ester > amide > urea. Azapeptide amide **10**, having a urea-like carbonyl, is thus a very poor inactivator and not a substrate for papain. Even adding an electron-withdrawing *p*-nitrophenyl substituent to the potential leaving group nitrogen fails to make the carbonyl carbon in **11** attractive to the cysteine nucleophile of papain. Although papain hydrolyzes simple peptide amides quite well, it does not hydrolyze peptide thioamides unless they have an electron-withdrawing nitrogen substituent such as *p*-nitrophenyl;[3] thus **12** is virtually inert to papain (Table 1). Nevertheless, compounds **10**−**12** still interact noncovalently with papain reasonably well, as shown by the fact that they are reasonable competitive inhibitors (K_i = 0.21, 3.3, and 3.9 mM, respectively).

Table 2. Specificity Constants for Substrates and Inactivators of Papain and Cathepsin B

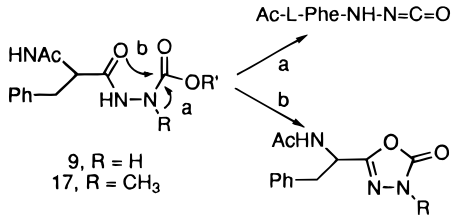
compd	papain	cathepsin B
	k_{cat}/K_m (M^{-1} s^{-1})	
Cbz-Gly-ONp	203 000	123 000
Cbz-L-Ala-ONp	800 000	450 000
Cbz-L-Phe-ONp	120 000	80 000
Cbz-L-Lys-ONp	853 000	776 000
	k_2/K_i (M^{-1} s^{-1})	
Ac-L-Phe-AGly-OPh	67 600	7 730
Ac-L-Phe-AAla-OPh	110	18
Ac-L-Phe-APhe-OPh	0.3	<< 0.2

Compounds **4**−**8** also inactivate cathepsin B, and although there are fewer data (Table 1) and the rates are lower than with papain, the same dependence of inactivation rate on leaving group electronegativity and hydrophobicity is clearly evident suggesting that this pattern of behavior may be a general one. Azapeptide inactivators are still subject to the substrate specificity of the particular enzyme, however. For example, cathepsin C, another member of the papain clan,[15] has considerable sequence similarity to papain and cathepsin B but has a very different substrate specificity. It is an aminopeptidase with broad specificity that also hydrolyzes small dipeptide substrates such as glycyl-L-phenylalanine *p*-nitroanilide.[41,42] Lacking a free amino terminus, neither **6** nor **8** show any tendency to inactivate cathepsin C, suggesting that their inactivation of papain and cathepsin B is a specific process.

S_1−P_1 Interactions. Papain is known to have broad tolerance for different amino acids at the P_1 position of its substrates, as long as they have the L-configuration,[43] and cathepsin B has somewhat similar preferences.[44] This is illustrated by the rate constants for hydrolysis of the Cbz-amino acid derivatives in Table 2. Crystallographic studies on papain[20,45] and cathepsin B[10,11] indicate that the side chains of L-amino acids in the P_1 position either lie along the surface of the protein or project out into solution and suggest that the side chains of D-residues in the P_1 position would give rise to strongly unfavorable steric interactions with the enzyme itself. P_1-Azapeptides have planar geometry at their α-position and hence their side chains would project "in between" those of D- vs L-amino acids at this position. It was therefore of interest to explore this aspect of azapeptides as inactivators of papain. Magrath and Abeles[36] reported that Ac-L-Phe-AAla-OiBu does not inactivate papain, but this ester, like compounds **13** and **14**, has an unreactive leaving group. Thus these compounds are poor inactivating agents and less than ideal as probes for exploring the influence of configuration at the α position.

Upon introducing more reactive leaving groups, as in **15** and **16**, inactivation rates with the azaalanine derivatives become measurable, although they are greatly reduced by comparison to the azaglycine analogues (Table 1). Comparing the ratios of inactivation rate constants for P_1-azaglycine vs P_1-azaalanine derivatives with different leaving groups (−OMe, 380; −OEt, 940; $-OCH_2CCl_3$, 980; −OPh, 615) reveals only little more than a 2-fold variation in this ratio across a 350-fold range of inactivation rate constants. On the

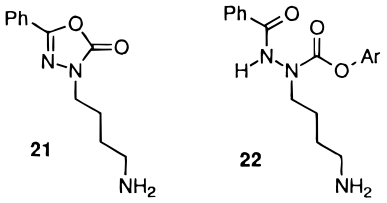
Scheme 2



other hand, as shown in Table 2, for normal peptide substrates with good leaving groups the P_1 side chain has little influence on hydrolysis rate, whereas for azapeptides with good leaving groups there is a huge decrease in inactivation rate constant (225 000-fold) as the size of the side chain increases in the order H < CH_3 < CH_2Ph. Once again, cathepsin B shows parallel behavior to that of papain in both of these respects, and the effect appears to be almost entirely steric in origin, meaning that P_1 substituents larger than hydrogen greatly hinder productive binding to these enzymes so that acylation rates are drastically reduced.

Mechanistic Considerations. Compound **9** contains the most reactive leaving group in the series, and it inactivates papain too rapidly for us to determine k_2/K_i as we did for its congeners. In buffer, **9** also releases *p*-nitrophenol quite rapidly ($k_{obs} = 0.13$ min^{-1}). Because release of *p*-nitrophenol from **9** can generate potentially inhibitory byproducts (see below), we performed several experiments to characterize the inactivation of papain by **9**. In the first experiment compound **9** (5 μM) was added to papain (0.125 μM), and as rapidly as the solution could be mixed an aliquot was removed and diluted 10-fold into assay buffer containing Cbz-G-ONp[46] (50 μM); the observed activity was <2% that of a control reaction in which **9** was omitted, indicating that extremely rapid inactivation had occurred. Next, **9** (5 μM) and Cbz-G-ONp (50 μM) were added simultaneously to prediluted papain (0.0125 μM); the initial rate observed was reduced compared to the control with **9** omitted, and it continued to decline steadily reaching zero within ca. 9−10 min as shown for **6** in Figure 1. In the third experiment **9** (5 μM) was allowed to stand in assay buffer for 40 min (ca. 7.5 times its half-life) before addition of Cbz-G-ONp (50 μM) and papain (0.0125 μM); the initial rate was ca. 90% that of a control reaction in which **9** was omitted altogether, and it declined only slightly over the next 5 min, indicating that nothing with significant inhibitory activity remained in solution. In contrast, HPLC studies showed that **6** and **7** were unchanged after standing in buffer for 24 h at room temperature, while the more reactive **8** had hydrolyzed only to the extent of 4−7%. Thus, while **9** is quite unstable in buffer its decomposition products are not papain inactivators; the other azapeptides are stable in buffer for extended periods of time.

In addition to direct hydrolysis, other possible routes for nitrophenol release from **9** include an E1cb mechanism giving rise to a peptidyl isocyanate (Scheme 2, path a) and an intramolecular cyclization giving rise to an oxadiazolone (Scheme 2, path b). Alkyl and peptidyl isocyanates inactivate serine proteases by carbamoylating the active site serine[47] or, in special circumstances, the active site histidine.[48] On the other hand, oxadiazolone **21**, formed by cyclization of the trypsin-inactivating azapeptide **22**, does not inactivate trypsin.[49]



Oxadiazolones are relatively unreactive toward basic hydrolysis[25,50] and thus do not acylate the enzyme. Replacing the acyl moiety on an azapeptide nitrophenyl ester with an alkoxycarbonyl[27] or dialkylcarbamoyl[49] group prevents oxadiazolone formation and nitrophenol release under mild conditions in buffer.

Azaalanine derivative **17** cannot undergo E1cb elimination to form an isocyanate, yet it still releases nitrophenol quite readily in buffer solution ($k_{obs} = 0.028$ min^{-1}). This suggests that the E1cb route is unlikely to be a significant contributor to either the decompostion of **9** in buffer or its inactivation of papain (unless the enzyme both catalyzes isocyanate formation and traps it very efficiently). Compound **17** also inactivates papain, but much less rapidly than **9**. We did not measure the rate constant for this process, but incubation of **17** (128 μM) and papain (2.5 μM) for 30 min followed by 10-fold dilution into assay mixture gave an initial rate 55% of control. Thus the relative reactivities of **9** and **17** are probably in accord with those of other AGly/AAla ester pairs as discussed above. On the basis of the above considerations, we conclude that **9** and **17** inactivate papain by the same mechanism as their other less reactive analogues (i.e., direct acylation), that formation of isocyanates in solution is not involved in enzyme inactivation, and that release of nitrophenol is due to intramolecular cyclization to a noninhibitory oxadiazolone derivative.

Observations on Carbamoylated Papain. Papain inactivated by **3** was reported[36] to be resistant to reactivation by dialysis, although it could be reactivated by incubation with a high concentration of valine methyl ester (90 mM). This lipophilic substrate-like nucleophile presumably binds to the S_1' region of the active site and attacks the carbamoyl enzyme in a reversal of the k_2 step of eq 1. We also observed that papain inactivated by **6**, which should give the same carbamoyl enzyme as **3**, was extremely slow to regenerate under dialysis conditions ($t_{1/2} \geq 24$ h at 4−5 °C). To demonstrate that inactivation was indeed due to carbamoyl enzyme formation, papain inactivated with excess **6** was dialyzed briefly to remove noncovalently bound inhibitor and lyophilized. Electrospray mass spectrometry showed the resultant protein to have a molecular mass of 23 667.6 ± 3.6 Da, which is in excellent agreement with the calculated mass of the anticipated *S*-carbamoyl enzyme (23 665.3 Da).

Stoichiometry of Inactivation and Active Site Titration of Papain. A common problem in dealing with cysteine proteinases is that of determining the operational normality of a given enzyme preparation or solution. One accepted approach to this is active site titration with [L-*trans*-(epoxysuccinyl)leucinamido](4-guanidino)-butane, also known as E-64.[51] This agent alkylates the active site cysteine of papain and many other cysteine

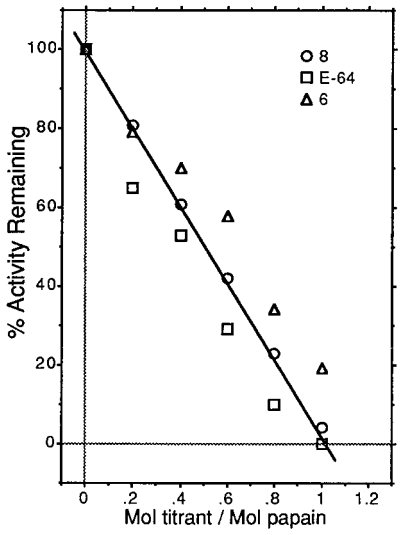


Figure 2. Active site titration of papain with E-64 and azapeptides **6** and **8**. Papain (0.125 μM) was preincubated with **6**, **8**, or E-64 at room temperature for 60 min. Remaining activity was determined by adding 50 μM Cbz-Gly-ONp as substrate. See text for details.

proteinases extremely rapidly and irreversibly. To establish the stoichiometry of the reactions of **6** and **8** with papain, we compared them to E-64 in an active site titration experiment. Variable aliquots of compound (0−125 pmol) were combined with fixed aliquots of papain (125 pmol in 1.0 mL of buffer); substrate was added after 60 min at room temperature, and active enzyme was measured. The results, shown in Figure 2, indicate that **8**, like E-64, inactivates papain with 1:1 stoichiometry. Compound **6** appears to be less efficient, but this is probably a consequence of incomplete reaction with papain during the 60-min reaction period rather than competing hydrolysis of **6** in buffer. Since azapaptides such as **8** are easily synthesized and can be tailored to the specificity requirements of individual cysteine proteinases, they may offer advantages over E-64 as active site titrants for this family of enzymes.

Experimental Section

Mercuripapain, cathepsin B from bovine spleen, cathepsin C from bovine spleen, and [L-*trans*-(epoxysuccinyl)leucinamido]-(4-guanidino)butane (E-64) were purchased from Sigma Chemical Co. (St. Louis, MO). Solvents and other chemicals were obtained from standard commercial sources and used without further purification. All reactions were carried out at room temperature unless otherwise stated. Organic extracts were dried over anhydrous Na_2SO_4 or $MgSO_4$ prior to concentration by rotary evaporation. HPLC analyses were carried out using a C-18 reverse-phase column (Alltech Econosil, 10 μm, 4.6 × 250 mm) eluted with 40% CH_3CN in H_2O at a flow rate of 1 mL/min and monitored at 220 nm. Thin-layer chromatography was performed on glass plates (2.5 × 10 cm) precoated with silica gel GHLF (250 μm; Analtech) eluted with 10% methanol in chloroform. NMR spectra were recorded on a GE QE Plus instrument. Melting points were determined in open capillary tubes using a Thomas-Hoover apparatus and are uncorrected.

***N*-(Acetyl-L-phenylalanyl)azaglycine Methyl Ester (1).** *N*-Ac-L-phenylalanine (0.83 g, 4.0 mmol) was activated with *N*-methylmorpholine (1.05 equiv) and isobutyl chloroformate (1.05 equiv) in THF (15 mL) at −20 °C as described.[4] After stirring for 10 min at −20 °C, methyl carbazate (0.36 g, 4.0 mmol) in 3 mL of THF was added, and the reaction was stirred at room temperature for 3 h. The reaction mixture was filtered and the filtrate evaporated to give a white paste which was partitioned into chloroform (20 mL), washed with $NaHCO_3$ solution and water, and then dried. Recrystallization from ethyl acetate and ether afforded the desired product as a white solid (0.51 g, 46%). Mp: 167−169 °C. ^{1}H NMR ($CDCl_3$): δ 1.95 (s, 3H), 3.05 (dd, 1H), 3.14 (dd, 1H), 3.72 (s, 3 H), 4.74 (q, 1H), 6.25 (d, 1H), 6.70 (s, 1H), 7.25 (m, 5H), 8.27 (s, 1H). MS (CI, NH_3): *m*/*z* 297 ($M{\cdot}NH_4^+$, 19), 280 (MH^+, 100), 190 (58), 120 (26). Anal. ($C_{13}H_{17}N_3O_4$) C, H, N.

***N*-(Acetyl-L-phenylalanyl)azaglycine Ethyl Ester (2).** **2** was synthesized from ethyl carbazate as described above for **1**. The crude product was chromatographed over silica gel eluted with methanol/chloroform (10:90, v/v); recrystallization from ether/hexane gave a white solid (0.56 g, 64%). Mp: 157−159 °C. ^{1}H NMR ($CDCl_3$): δ 0.92 (t, 3H), 1.60 (s, 3H), 2.57 (dd, 1H), 2.85 (dd, 1H), 3.81 (q, 2H), 4.96 (m, 1H), 6.34 (d, 1H), 6.72 (s, 1H), 6.90 (m, 5H), 8.50 (s, 1H). MS (CI, NH_3): *m*/*z* 311 ($M{\cdot}NH_4^+$, 40), 294 (MH^+, 100), 190 (53), 120 (40). Anal. ($C_{14}H_{19}N_3O_4$) C, H, N.

***N*-Acetyl-L-phenylalanine hydrazide (19).** *N*-Ac-L-Phenylalanine (10.4 g, 80 mmol) was dissolved in 80 mL of methanol with 0.5 mL of concentrated H_2SO_4 and refluxed at 80 °C for 6 h. The methanol was removed by rotary evaporation and the residue dissolved in $CHCl_3$ which was then extracted with 5 mL of saturated $NaHCO_3$ and 5 mL of H_2O, dried, and concentrated by rotary evaporation. Recrystallization from $CHCl_3$/hexane yielded Ac-L-Phe-OCH_3 (9.98 g, 90%). Mp: 87−88 °C. ^{1}H NMR ($CDCl_3$): δ 1.95 (s, 3H), 3.10 (dq, 2H), 3.70 (s, 3H), 4.86 (q, 1H), 6.00 (s, 1H), 7.05−7.30 (m, 5H). The latter (2.21 g, 10 mmol) and hydrazine hydrate (5.01 g, 100 mmol) were dissolved in 20 mL of MeOH and kept at room temperature for 2 days. After removal of solvent the residue was washed successively with EtOH, ether, and hexane which yielded a white solid (2.02 g, 92%). Mp: 178−179 °C. ^{1}H NMR ($CDCl_3$): δ 1.92 (s, 3H), 2.97 (m, 2H), 3.74 (s, 2H), 4.53 (q, 1H), 6.04 (d, 1H), 7.04 (s, 1H), 7.20 (m, 6H). MS (CI, NH_3): *m*/*z* 222 (MH^+, 100), 207 (45), 191 (27), 163 (24), 120 (28). Anal. ($C_{11}H_{15}N_3O_2$) C, H, N.

***N*^{1}-(Acetyl-L-phenylalaninyl)-*N*^{2}-methylhydrazine (20).** Ac-L-PheOMe (1.58 g, 7.0 mmol) was dissolved in methylhydrazine (1.38 g, 30.0 mmol) and kept at room temperature for 24 h. After removal of the excess methylhydrazine by rotary evaporation, the residue was chromatographed on silica gel eluted with 10% MeOH in $CHCl_3$. The major product (0.98 g, 59% yield, compound **19**) was a white solid. Mp: 201−204 °C. R_f 0.22 (silica gel, MeOH/$CHCl_3$, 10:90, v/v). ^{1}H NMR ($CDCl_3$): δ 1.96 (s, 3H), 2.46 (s, 3H, −NHNHC*H*$_3$), 2.98 (q, 1H), 3.05 (q, 1H), 4.50 (q, 1H), 6.10 (d, 1H), 7.05 (s, 1H), 7.20 (m, 6H). The minor product (0.26 g, 12% yield, compound **20**) had R_f 0.30 and showed an *N*-methyl peak (−N(C*H*$_3$)NH_2) at 3.05 ppm in the ^{1}H NMR.

General Procedure for Synthesis of Azaglycine Peptides 3−9. The general procedure was to dissolve Ac-L-PheNHNH_2 and 1 equiv of *N*-methylmorpholine in $CHCl_3$ at −10 °C followed by slow dropwise addition of 1 equiv of a chloroformate ester dissolved in 5 mL of $CHCl_3$. The reaction mixture was stirred at −10 °C for 30 min and at room temperature for an additional 6 h. After addition of more $CHCl_3$, the organic phase was extracted with saturated $NaHCO_3$ and then H_2O and dried. Evaporation of the $CHCl_3$ left a white paste as crude product, from which pure products were isolated by chromatography and/or recrystallization.

***N*-(Acetyl-L-phenylalaninyl)azaglycine Isobutyl Ester (3).** Chromatography of the crude product on silica gel gave pure **3** as a white solid. Yield: 84%. Mp: 159−160 °C (lit. mp 157−159 °C).[36] ^{1}H NMR ($CDCl_3$): δ 0.90 (d, 6H), 1.90 (s, 3H), 3.00 (q, 1H), 3.15 (q, 1H), 3.86 (d, 2H), 4.78 (q, 1H), 6.46 (d, 1H), 6.86 (s, 1H), 7.22 (m, 5H), 8.63 (s, 1H). MS (CI, NH_3): *m*/*z* 339 ($M{\cdot}NH_4^+$, 2), 322 (MH^+, 35), 248 (54), 190 (100), 162 (30), 120 (50). Anal. ($C_{16}H_{23}N_3O_4$) C, H, N.

***N*-(Acetyl-L-phenylalaninyl)azaglycine 2-Chloroethyl Ester (4).** **4** was obtained by chromatography on silica gel followed by recrystallization from $CHCl_3$/Et_2O. Yield: 64%. Mp: 158−159 °C. ^{1}H NMR (acetone-d_6): δ 1.80 (s, 3H), 2.82

(q, 1H), 3.15 (q, 1H), 3.72 (t, 2H), 4.26 (t, 2H), 4.70 (m, 1H), 7.22 (m, 5H), 8.32 (s, 1H), 9.20 (s, 1H). MS (CI, NH_3): *m/z* 328 (MH^+, 1), 292 (5), 248 (3), 232 (2), 190 (39), 162 (41), 120 (100). Anal. ($C_{14}H_{18}N_3O_4Cl$) C, H, N.

***N*-(Acetyl-L-phenylalaninyl)azaglycine 2-Bromoethyl Ester (5).** **5** was purified by chromatography and recrystallized from $CHCl_3/Et_2O$. Yield: 94%. Mp: 151−153 °C. NMR ^{1}H NMR (acetone-d_6): δ 1.80 (s, 3H), 3.25 (q, 1H), 3.15 (q, 1H), 3.60 (s, 2H), 4.35 (m, 2H), 4.68 (m, 1H), 7.22 (m, 5H), 8.35 (s, 1H). MS (CI, NH_3): *m/z* 372 (MH^+, 2), 292 (9), 248 (2), 190 (61), 162 (51), 120 (100). Anal. ($C_{14}H_{18}N_3O_4Br$) C, H, N.

***N*-(Acetyl-L-phenylalaninyl)azaglyine Trichloroethyl Ester (6).** **6** was isolated by chromatography on silica gel using 10% MeOH/$CHCl_3$. Recrystallization from $CHCl_3/Et_2O$ gave **6** as a white solid in 76% yield. Mp: 188−189 °C. ^{1}H NMR ($CDCl_3$): δ 1.94 (s, 3H), 3.04 (q, 1H), 3.14 (q, 1H), 4.72 (s, 2H), 4.80 (m, 1H), 6.35 (d, 1H), 7.25 (m, 6H), 8.75 (s, 1H). MS (CI, NH_3): *m/z* 396 (MH^+, 14), 265 (16), 248 (100), 190 (91), 188 (56), 162 (38), 131 (23), 120 (91). Anal. ($C_{14}H_{16}N_3O_3Cl_3$) C, H, N.

***N*-(Acetyl-L-phenylalaninyl)azaglycine Benzyl Ester (7).** **7** was obtained in 54% yield as a white solid. Mp: 145−146 °C. ^{1}H NMR ($CDCl_3$): δ 1.90 (s, 3H), 3.05 (q, 1H), 3.15 (q, 1H), 4.70 (d, 1H), 5.13 (s, 2H), 6.12 (s, 1H), 6.75 (m, 1H), 6.7−7.4 (m, 10H), 8.24 (s, 1H). MS (CI, NH_3): *m/z* 356 (MH^+, 18), 190 (68), 162 (18), 120 (43). Anal. ($C_{19}H_{21}N_3O_4$) H, N; C: calcd, 64.21; found, 63.30.

***N*-(Acetyl-L-phenylalaninyl)azaglycine Phenyl Ester (8).** **8** was isolated by chromatography using 10% MeOH/$CHCl_3$. Recrystallization from $CHCl_3/Et_2O$ gave a white solid in 57% yield. Mp: 138−141 °C (lit. mp 137−140 °C).[36] ^{1}H NMR ($CDCl_3$): δ 1.95 (s, 3H), 3.04 (q, 1H), 3.16 (q, 1H), 4.76 (q, 1H), 6.15 (d, 1H), 7.10 (s, 1H), 7.25 (m, 10H), 8.35 (s, 1H). MS (CI): *m/z* 342 (MH^+, 1), 265 (2), 248 (37), 188 (42), 114 (43), 94 (100), 66 (32), 43 (44).

***N*-(Acetyl-L-phenylalanyl)azaglycine *p*-Nitrophenyl Ester (9).** After acylation of hydrazide **19** with *p*-nitrophenyl chloroformate as described above, the reaction mixture was diluted with ethyl acetate, extracted with 1 N HCl, saturated $NaHCO_3$, and H_2O, and dried. Evaporation of the solvent gave a solid residue, but upon attempted chromatography on silica gel the column turned deep-yellow suggesting extensive hydrolysis. Therefore the product was isolated by crystallization from ether/hexane in 26% yield. This compound is highly unstable in water and was used without further purification. Mp: 144−145 °C. ^{1}H NMR (DMSO): δ 1.72 (s, 3H), 2.72 (q, 1H), 2.96 (q, 1H), 4.54 (m, 1H), 7.22 (m, 5H), 7.40 (d, 2H), 8.20 (d, 1H), 8.25 (d, 2H), 10.02 (s, 1H), 10.22 (s, 1H). MS (CI, NH_3): *m/z* 265 (23), 248 (100), 188 (40), 157 (59), 139 (31), 109 (22), 91 (25), 65 (95). Anal. ($C_{18}H_{18}N_4O_6$) H, N; C: calcd, 55.96; found, 54.60.

***N*-(Acetyl-L-phenylalanyl)azaglycine Amide (10).** Ac-L-PheNHNH$_2$ (0.44 g, 2.0 mmol) in 15 mL of THF/EtOH (1:1) was combined with trimethylsilyl isocyanate (0.54 g, 4.0 mmol), and the mixture was heated to reflux for 20 h, after which water was added and the mixture was stirred for 1 h. After removal of solvent by rotary evaporation, the residue was chromatographed yielding a white solid which was recrystallized from methanol/ether. Yield: 56%. Mp: 123−126 °C. ^{1}H NMR (DMSO): δ 1.72 (s, 3H), 2.72 (q, 1H), 2.95 (q, 1H), 4.38 (m, 1H), 5.78 (s, 2H), 7.22 (m, 5H), 7.74 (s, 1H), 8.16 (d, 1H), 9.74 (s, 1H). MS (CI, NH_3): *m/z* 265 (MH^+, 13), 248 (7), 222 (54), 190 (83), 162 (100), 120 (84). Anal. ($C_{12}H_{16}N_4O_3\cdot 0.5H_2O$) C, H, N.

***N*-(Acetyl-L-phenylalaninyl)azaglycine *p*-Nitroanilide (11)**. Ac-L-PheNHNH$_2$ (0.22 g, 1.0 mmol) in 10 mL of THF was combined with *p*-nitrophenyl isocyanate (0.16 g, 1.0 mmol) in 5 mL of THF for 20 h. Removal of solvent and chromatography on silica gel gave a faintly-yellow solid which was recrystallized from THF and hexane. Yield: 48%. Mp: 193−195 °C. ^{1}H NMR (DMSO): δ 1.78 (s, 3H), 2.78 (m, 1H), 2.97 (m, 1H), 4.40 (s, 1H), 7.22 (m, 6H), 7.72 (d, 2H), 8.12 (d, 2H), 8.32 (s, 1H), 8.53 (s, 1H), 10.07 (s, 1H). MS (CI, NH_3): *m/z* 386 (MH^+, 0.4), 248 (4), 222 (100), 190 (77), 164 (59), 120 (97). Anal. ($C_{18}H_{19}N_5O_5$) C, H, N.

***N*-(Acetyl-L-phenylalaninyl)thiosemicarbazide (12).** Ac-L-PheNHNH$_2$ (0.44 g, 2.0 mmol) in 15 mL of EtOH/THF (1:1) was combined with trimethylsilyl isothiocyanate (0.52 g, 4.0 mmol) as described for compound **10**. After removal of solvent by rotary evaporation, the residue was chromatographed yielding a white solid which was recrystallized from methanol/ether. Yield: 33%. Mp: 197−199 °C. ^{1}H NMR (DMSO): δ 1.74 (s, 3H), 2.75 (q, 1H), 2.96 (q, 1H), 4.28 (m, 1H), 6.88 (s, 1H), 7.22 (m, 5H), 7.92 (s, 1H), 8.24 (d, 1H), 9.32 (s, 1H). MS (CI, NH_3): *m/z* 281 (MH^+, 1), 280 (M^+, 1), 204 (1), 190 (11), 162 (16), 120 (72), 43 (100). Anal. ($C_{12}H_{16}N_4O_2S$) C, H, N.

***N*-(Acetyl-L-phenylalaninyl)azaalanine Methyl Ester (13).** **13** was obtained as a white solid following chromatography followed by recrystallization from EtOH/hexane. Yield: 69%. Mp: 141−142 °C. ^{1}H NMR ($CDCl_3$): δ 1.90 (s, 3H), 2.97 (s, 3H), 3.08 (m, 2H), 3.62 (s, 3H), 4.73 (q, 1H), 6.72 (s, 1H), 7.22 (m, 5H), 8.80 (s, 1H). MS (CI, NH_3): *m/z* 294 (MH^+, 3), 190 (6), 162 (1), 105 (2), 84 (21), 47 (100). Anal. ($C_{14}H_{19}N_3O_4$) C, H, N.

***N*-(Acetyl-L-phenylalaninyl)azaalanine Ethyl Ester (14).** **14** was obtained as a white solid following chromatography followed by recrystallization from EtOH/hexane. Yield: 65%. Mp: 158−160 °C. ^{1}H NMR ($CDCl_3$): δ 1.21 (t, 3H), 1.95 (s, 3H), 3.03 (s, 3H), 3.05 (m, 2H), 4.10 (q, 2H), 4.68 (q, 1H), 6.33 (s, 1H), 7.25 (m, 6H), 8.23 (s, 1H). MS (CI, NH_3): *m/z* 325 ($M\cdot NH_4^+$, 19), 308 (MH^+, 68), 279 (100), 262 (82), 190 (32), 120 (23). Anal. ($C_{15}H_{12}N_3O_4$) C, H, N.

***N*-(Acetyl-L-phenylalaninyl)azaalanine Trichloroethyl Ester (15).** **15** was obtained as a white solid following chromatography and recrystallization from ethyl acetate and ether. Yield: 69%. Mp: 132−134 °C. ^{1}H NMR ($CDCl_3$): δ 1.90 (s, 3H), 3.04−3.08 (m, 5H), 4.68 (m, 3H), 6.08−6.22 (d, 1H), 7.21 (m, 5 H), 8.55 (s, 1H). MS (CI, NH_3): *m/z* 410 (MH^+, 9), 279 (28), 262 (100), 202 (27), 190 (22), 128 (45), 120 (18). Anal. ($C_{15}H_{18}N_3O_4Cl_3$) C, H, N.

***N*-(Acetyl-L-phenylalaninyl)azaalanine Phenyl Ester (16).** **16** was obtained as a white solid following chromatography and recrystallization from methanol and ether. Yield: 45%. Mp: 158−160 °C. ^{1}H NMR (DMSO): δ 1.72 (s, 3H), 2.75 (m, 1H), 2.95 (s, 3H), 3.10 (s, 1H), 4.50 (m, 1H), 7.0−7.4 (m, 10H), 8.30 (d, 1H). MS (CI, NH_3): *m/z* 262 (4), 202 (17), 170 (10), 128 (45), 94 (100). Anal. ($C_{19}H_{21}N_3O_4$) C, H, N.

***N*-(Acetyl-L-phenylalaninyl)azaalaine *p*-Nitrophenyl Ester (17).** **17** was obtained as a white solid following recrystallization from ether and hexane. Yield: 41%. Mp: 168.5−170 °C. ^{1}H NMR (DMSO): δ 1.72 (s, 3H), 2.75 (q, 1H), 2.95 (q, 1H), 2.96 (s, 3H), 4.50 (m, 1H), 7.22 (m, 5H), 7.26 (d, 2H), 7.52 (d, 1H), 8.24 (d, 2H), 8.30 (s, 1H). MS (CI, NH_3): *m/z* 262 (37), 220 (5), 202 (69), 170 (28), 128 (100), 109 (15), 91 (44), 65 (60), 43 (72). Anal. ($C_{19}H_{20}N_4O_6$) C, H, N.

***N*-(Acetyl-L-phenylalaninyl)azaphenylalanine Phenyl Ester (18).** A mixture of Ac-L-PheHNH$_2$ (221 mg, 1.0 mmol) and benzaldehyde (106 mg, 1.25 mmol) in 30 mL of MeOH was refluxed for 8 h under N_2. Concentration and recrystallization from MeOH/ether give a white solid (255 mg, 83% yield). ^{1}H NMR ($CDCl_3$): δ 2.0 (s, 3H), 3.0−3.3 (m, 4H), 4.7 (d, 1H), 7.1−7.4 (m, 10H). MS (CI, NH_3): *m/z* 310 (MH^+, 100), 163 (14), 162 (22), 120 (92), 106 (38).

The above material (Ac-L-PheNHN=CHPh, 255 mg, 0.83 mmol) was dissolved in dry THF, $LiAlH_4$ (76 mg, 2.0 mmol) was added, and the reaction mixture was refluxed for 4 h. After cooling to room temperature, water (1.0 mL) was added cautiously. The white precipitate which formed was removed by filtration and the filtrate evaporated to give a yellow oil. Recrystallization from ether/hexane gave Ac-L-PheNHNH-CH_2-Ph as a soft-yellow solid in 31% yield. MS (CI, NH_3): *m/z* 312 (MH^+, 100).

The latter product (80 mg, 0.26 mmol) was acylated with phenyl chloroformate as described for compound **8**. After silica gel chromatography and recrystallization from ether and hexane, compound **18** was obtained as a white solid in 37% yield. Mp: 141−143 °C. ^{1}H NMR ($CDCl_3$): δ 1.90 (s, 3H),

3.00 (q, 1H), 3.10 (q, 1H), 4.65 (m, 3H), 6.00 (s, 1H), 7.3 (m, 15H), 8.40 (s, 1H). MS (CI, NH_3): *m/z* 449 ($M{\cdot}NH_4^+$, 4), 432 (MH^+, 16), 355 (40), 338 (100), 278 (24), 204 (40), 120 (13). Anal. ($C_{25}H_{25}N_3O_4$) C, H, N.

Enzyme Assays. Papain was purified and assayed as described previously.[4,5,52] Briefly, Hg−Sepharose 4B was prepared according to Sluyterman and Wijdenes[53,54] and found to have a capacity of 2.24 μmol of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB)/mL of resin bed. Commercial mercuripapain (200 mg of crystallized suspension in 11.6 mL of 70% EtOH) was added to 40 mL of column buffer (50 mM NaOAc, 100 mM KCl, pH 5.0) containing 10 mM Na_2SO_3 and gently stirred at room temperature for 45 min to allow the protein to dissolve. (The original procedure[53] calls for adding DMSO (10%, v/v) to prevent protein aggregation, but we find this is not necessary; when organic cosolvents are needed, for example, to improve solubility of test compounds, up to 20% (v/v) acetonitrile may be used with very little adverse effect on enzyme activity.) The mixture was centrifuged at 18000*g* for 10 min, and the supernatant was diluted to 78 mL with standard buffer and loaded onto a Hg−Sepharose-4B column (bed volume 40 mL) at a flow rate of 20 mL/h. The column was washed with 150 mL of column buffer and then with 200 mL of column buffer containing 0.5 mM $HgCl_2$. Active fractions were pooled (ca. 54 mL) and dialyzed against distilled water (4 °C, 10-kDa cutoff membrane, 3 × 4000 mL). Lyophilization afforded mercuripapain as a white solid power which could be stored at −20 °C for long periods of time.

Solutions of active papain were prepared by dissolving 3 mg of Hg-papain into 50 mL of standard buffer (50 mM K_2HPO_4, 1 mM EDTA, pH 6.3) and activating it by addition of L-cysteine·HCl (50 μL of 0.1 M solution/mL of papain solution) at room temperature for 15 min. Papain assays contained 50 μM Cbz-Gly-ONp as substrate and 20% CH_3CN in buffer. The rate of substrate hydrolysis was followed at 340 nm with $\Delta\epsilon$ of 6000 M^{-1} cm^{-1} and corrected for spontaneous chemical hydrolysis of substrate.

Bovine spleen cathepsin B was assayed in 25 mM sodium acetate buffer, pH 5.3, containing 1 mM EDTA as described by Bajkowski et al.[55] Stock cathepsin B was activated by 10 mM dithiothreitol and assayed with 80 μM Cbz-Lys-ONp as substrate in buffer containing 20% CH_3CN (v/v). The rate of substrate hydrolysis was monitored at 326 nm with $\Delta\epsilon$ of 9060 M^{-1} cm^{-1}. The concentration of cathepsin B was determined by titration with protease inhibitor E-64 according to Barrett et al.[56]

Cathepsin C from bovine spleen (Sigma type X) was assayed at 37 °C in 50 mM sodium citrate, pH 5.0, containing 0.5 mM EDTA and 10 mM NaCl. It was activated by 5 mM dithiothreitol at room temperature for 15 min. Enzyme activity was monitored at 410 nm ($\Delta\epsilon$ = 9300 M^{-1} cm^{-1}) at 37 °C with 4 mM Gly-Phe-pNA as substrate.[42]

Inhibitors were evaluated using the method of Kitz and Wilson[57] as described previously.[4] Inactivation rate constants (k_{obsd}, s^{-1}) were determined from the slopes of semilog plots of enzyme activity vs time. At least five time points were used for each plot, and r^2 values were ≥0.94 in most cases. Apparent second-order rate constants (k_2/K_i, M^{-1} s^{-1}) were determined from replots of k_{obs} vs [I] using at least five values of [I] (except as indicated in Table 1); in these plots r^2 values were generally 0.90−0.98. The resulting uncertainty on the rate constants (slope values) is probably in the order of 10% or less for the more active compounds and 20% or more for compounds showing only marginal activity.

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