Inactivation of Cysteine Proteases by (Acyloxy)methyl Ketones Using S′−P′ Interactions†

Yong Dai,‡ Lizbeth Hedstrom,* and Robert H. Abeles

Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02454

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ABSTRACT: We have synthesized (acyloxy)methyl ketone inactivators of papain, cathepsin B, and interleukin-1β conversion enzyme (ICE) that interact with both the S and S′ subsites. The value of k_{inact}/K_i for these inactivators is strongly dependent on the leaving group. For example, Z-Phe-Gly-CH_2-X is a poor inactivator of papain when X is $OCOCH_3$ ($k_{inact}/K_i = 2.5\ M^{-1}\ s^{-1}$) but becomes a potent inactivator when X is OCO-L-Leu-Z ($k_{inact}/K_i = 11\ 000\ M^{-1}\ s^{-1}$). Since these leaving groups have similar chemical reactivities, the difference in potency must be attributed to interactions with the S′ sites. The potency of the leaving group correlates with the P′ specificity of papain. Similar results are also observed for the inactivation of cathepsin B by these compounds. A series of inactivators with the general structure Fmoc-L-Asp-CH_2-X were designed to inactivate ICE. No inhibition was observed when X was $OCOCH_3$. In contrast, ICE is inactivated when X is OCO-D-Pro-Z ($k_{inact}/K_i = 131\ M^{-1}\ s^{-1}$). These results demonstrate that S′−P′ interactions can be utilized to increase the efficacy and selectivity of (acyloxy)methyl ketone inactivators.

Affinity labels consist of two components (*1*). One component resembles the substrate, thus providing specificity for the enzyme active site. The second component contains a reactive functionality which covalently modifies an active site residue, inactivating the enzyme. Chloromethyl ketones such as Tos-Phe-CH_2Cl (TPCK)[1,2] (Figure 1, structure **I**) are classical examples of affinity labels. The phenyl group of TPCK directs the inactivator to the S_1 subsite of chymotrypsin where it alkylates His_{57} (*2*). Similar compounds also inactivate cysteine proteases by alkylating the active site cysteine (Scheme 1 and Figure 1, structure **II**) (*3*, *4*). The selectivities of these inactivators are determined by the interactions of the peptidyl moiety (P component) with S subsites [nomenclature of Schecter and Berger (*12*)] and, consequently, match the substrate specificity of the target protease. The efficacy of these inactivators can also be improved by utilizing better leaving groups. Electron-withdrawing groups lower the pK_a of benzoyloxy leaving groups, increasing the reactivity of II, thereby increasing the potency of the inactivator. The values of k_{inact}/K_i for this series of inactivators are strongly dependent on the pK_a of the leaving group (*3*). Such compounds are potent inactivators in vitro, and have proven to be very useful in identifying functional groups at the active sites of proteases. Unfortu-

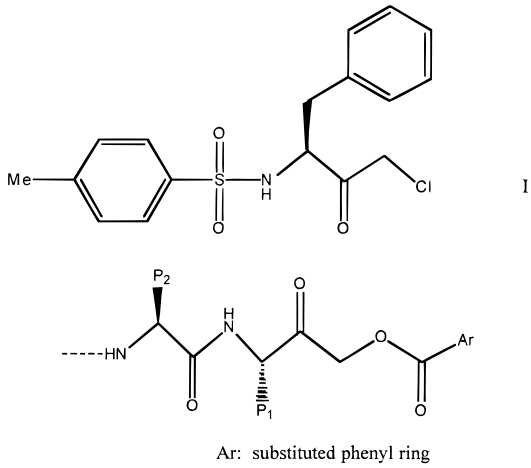
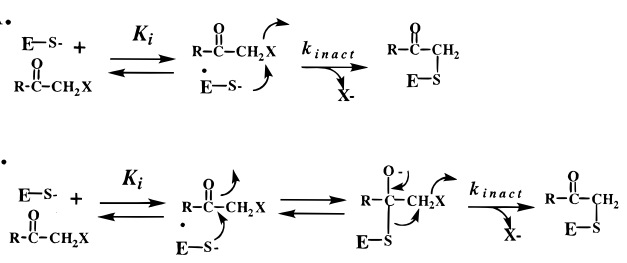


FIGURE 1: Structures of compounds **I** and **II**.

Scheme 1: Inactivation of Cysteine Proteases by (Acyloxy)methyl Ketones



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* To whom correspondence should be addressed: Department of Biochemistry, Brandeis University, MS 009, 415 South St., Waltham, MA 02454. E-mail: hedstrom@brandeis.edu. Phone: (781) 736-2333. Fax: (781) 736-2349.

‡ Present address: Research & Development Department, Instrumentation Laboratory, 526 Route 303, Orangeburg, NY 10962.

[1] Abbreviations: AL, affinity label; Alloc, allyloxycarbonyl; AMC, 7-amido-4-methylcoumarin; CHAPS, 3-[(3-cholaminopropyl)dimethylammonia]-1-propanesulfonate; DTT, dithiothreitol; DMF, dimethylformamide; EDTA, ethylenediaminetetraacetic acid; Fmoc, 9-fluorenylmethyloxycarbonyl; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; ICE, interleukin-1β conversion enzyme; KP_i, potassium phosphate; pNA, *p*-nitroanilide; TFA, trifluoroacetate; TPCK, Tos-Phe-CH_2Cl; Z, benzyloxycarbonyl.

[2] We use nomenclature such as Ac-Phe-$NHCH_2COCH_2$OCO-D-Trp-NH-Ac to denote a peptidyl (acyloxy)methyl ketone inactivator where P_2 is Phe, P_1 is Gly, and P_1' is Ac-D-Trp as shown in Figure 2.

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nately, increasing the nucleofugacity of these compounds also increases their tendency to undergo nonspecific reactions, which can undermine the utility of these inactivators for in vivo applications.

Although not as well studied as the S subsites, the S′ subsites also contribute to protease specificity (*5*−*7*). S′−P′ interactions have been utilized to increase the affinity of transition-state analogues (*5*, *8*). We have previously used S′−P′ interactions to increase the potency of carbonate and azapeptide inactivators (*9*). Therefore, we believed that S′−P′ interactions could be utilized to increase the efficacy and selectivity of (acyloxy)methyl ketones. Here we use this strategy to design selective inactivators for the cysteine proteases papain, cathepsin B, and interleukin-1β conversion enzyme (ICE).

MATERIALS AND METHODS

Enzymes and Substrates. Papain, bovine cathepsin B, and Ac-Tyr-Val-Ala-Asp-AMC were purchased from Sigma Chemical Co. (St. Louis, MO) and used without further purification. Z-Phe-Arg-PNA was obtained from Bachem Bioscience Inc. (King of Prussia, PA). ICE was a gift from Sandoz Research Institute Berne Ltd.

Enzyme Assays. (1) Papain and Cathepsin B. Enzyme activity was determined by monitoring the hydrolysis of Z-Phe-Arg-pNA at 410 nm (k_{cat} = 33 s^{-1} and K_m = 0.14 mM) for papain and (k_{cat} = 10 s^{-1}, K_m = 0.18 mM for cathepsin B). The spectrophotometric assays were performed on a Perkin-Elmer UV/Vis spectrophotometer using 1 cm cuvettes thermostated at 25 °C. Experiments were carried out in 50 mM potassium phosphate (KP_i), 1 mM EDTA, and 2 mM DTT (pH 6.8). For irreversible inhibitors, the reaction was started by mixing the enzyme (5 nM) and varying concentrations of inhibitor. At appropriate time intervals, an aliquot (20 μL) was removed from the reaction mixture and assayed. The values of k_{inact} and K_i were obtained with the method of Kitz and Wilson (*10*). For reversible inhibitors, the values of K_i were obtained from the slope, $K_i^{-1}(1 + [S]/K_m)^{-1}$, by plotting V_o/V_i against inhibitor concentration. V_o and V_i are the rates of catalytic reactions in the absence and presence of inhibitor, respectively.

(2) ICE. Enzyme activity was determined by monitoring the hydrolysis of Ac-Tyr-Val-Ala-Asp-AMC (20 μM) at 37 °C using an excitation wavelength of 380 nm and an emission wavelength of 460 nm with a Hitachi F-2000 spectrofluorimeter. The assay buffer contained 20 mM HEPES (pH 7.2), 10% w/v sucrose, 0.15% w/v CHAPS, and 2 mM DTT. The enzyme (0.5 μM) was incubated with varying concentrations of inhibitor (20−400 μM); aliquots (25 μL) were removed at appropriate time intervals and assayed. The values of K_i and k_{inact} of ICE inhibitors were obtained with the method of Kitz and Wilson (*10*).

Stability Measurements of (Acyloxy)methyl Ketones. The hydrolysis of (acyloxy)methyl ketone **13D** in 50 mM KP_i buffer at pH 6.0, 7.0, and 8.0 was monitored by ^{1}H NMR. Under these conditions, the chemical shift of the methyl protons of the Ala residue of **13D** is 1.40 ppm (doublet) while the chemical shift of the methyl protons of the hydrolysis product Z-Ala is 1.25 ppm (doublet).

Measurement of Chloromethyl Ketone's and (Acyloxy)-methyl Ketone's Susceptibility to Nucleophilic Displacement. The reaction of the TPCK and **8** with cysteine (10 mM) was carried out in 50 mM KP_i buffer (pH 6.5) containing 5 mM EDTA at room temperature under anaerobic conditions. Since both TPCK and **8** inactivate papain stoichiometrically, the concentration of inactivator was determined by titrating with papain. At appropriate time intervals, an aliquot (20 μL) was removed and added to 980 μL of 10 μM papain in assay buffer. The remaining papain activity was determined as described above. The pseudo-first-order rate constants for the reaction of cysteine with TPCK and **8** were obtained from the plot of ln[inhibitor] against time.

Chemicals and Syntheses. All of the amino acids were purchased from Sigma, Aldrich, or Bachem Bioscience Inc. [^{3}H]Acetic anhydride and [^{14}C]iodoacetamide were obtained from DuPont NEN. All other chemicals and solvents were of commercial reagent grade or better. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Varian XL-300 spectrophotometer and were reported in parts per million on the δ scale using TMS (0.00 ppm) or CD_3OD (3.40 ppm) as an internal standard. TLC was performed on EM Science silica gel plates.

(Acyloxy)methyl ketones were synthesized by the method of Krantz (*3*) from the appropriate peptidyl bromomethyl ketone (*11*) and carboxylic acid. [^{3}H]Fmoc-Asp-CH_2-OCO-D-Ala-NH-Ac (specific activity of 0.68 mCi/mmol) was prepared similarly starting with [^{3}H]-Ac-D-Ala. The latter was prepared by acetylation of D-Ala with [^{3}H]acetic anhydride. The TLC and NMR data for compounds **1**−**18** are provided in the Supporting Information.

RESULTS AND DISCUSSION

Inactivation of Papain. (Acyloxy)methyl ketones are much less reactive in displacement reactions than chloromethyl ketones (*3*, *4*). We synthesized a series of (acyloxy)methyl ketones to determine whether the leaving group can be activated by interactions with the S′ subsites (Table 1 and Figure 2). All of these compounds contain the P_2−P_1 residue sequence Ac-Phe-Gly based on the substrate specificity of papain (*12*, *13*) but differ in the leaving group. Compounds **1** and **2** contain the leaving groups $OCOCH_3$ and OCOPh, respectively; these leaving groups should not have optimal interactions with the S′ subsites. As expected, these two compounds are poor inactivators of papain (Table 1). Compounds **3**−**8** contain D-amino acids as the leaving groups. The side chains of the D-amino acid leaving groups are two atoms out of register as compared with an L-amino acid residue of a peptide substrate (Figure 2). However, they have the same spatial orientation as a peptide substrate, and may therefore interact with the S′ subsites in a similar manner. In addition, these D-amino acid compounds should be resistant to enzyme-catalyzed hydrolysis. Compound **9** contains an L-amino acid leaving group for assessing the importance of stereochemistry in the reaction.

Neither compound **3** nor **4** inactivates papain, although both are reversible inhibitors. We suspected that these compounds might form a hemithioacetal adduct with the active site cysteine of papain as observed with aldehyde inhibitors (*15*, *16*). Such an adduct can be detected using inactivators labeled with ^{13}C at the ketone carbonyl. However, the ^{13}C NMR spectrum of Ac-Phe-$NHCH_2{}^{13}COCH_2$-OCO-D-Trp-NH-Ac[2] did not change when it was bound to

Table 1: Rates of Papain and Cathepsin B Inactivation by Peptidyl (Acyloxy)methyl Ketones[a]

Ac-Phe-Gly-CO-CH_2-X		papain			cathepsin B		
no.	X	K_i (μM)	k_{inact} (s^{-1})	k_{inact}/K_i (M^{-1} s^{-1})	K_i (μM)	k_{inact} (s^{-1})	k_{inact}/K_i (M^{-1} s^{-1})
1	$OCOCH_3$	1.0×10^2	0.00025	2.5	4.1	slow-binding inhibitor	
2	OCOPh	66	0.0050	76	1.6×10^2	0.0085	54
3	OCO-D-Asp-NH-Fmoc	5.6	reversible		0.059	reversible	
4	OCO-D-Trp-NH-Ac	0.21	reversible		6.4	0.0022	3.5×10^2
5	OCO-D-Asn-NH-Z	1.6	0.00032	2.0×10^2	29	0.0013	43
6	OCO-D-Phe-NH-Ac	1.0	0.00035	3.5×10^2	45	0.0048	1.1×10^2
7	OCO-D-Lys-Z(HCl)	4.7	0.0037	8.0×10^2	2.7×10^2	0.0042	15
8	OCO-D-Leu-NH-Z	2.6	0.0026	1.0×10^3	27	0.0081	3.0×10^2
9	OCO-L-Leu-NH-Z	1.4	0.015	1.1×10^4	0.78	0.0021	2.7×10^3

[a] The reaction conditions for both papain and cathepsin B include 50 mM KP_i, 1 mM EDTA, and 2 mM DTT at pH 6.8 and 25 °C. Saturation kinetics were observed for all these inhibitors. The values of k_{inact} and K_i were obtained with the method of Kitz and Wilson for irreversible inhibitors. The values of K_i for reversible inhibitors were determined from steady-state velocities.

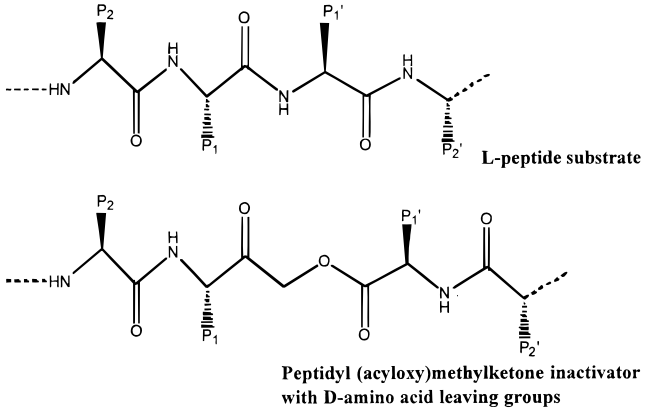

FIGURE 2: Structures of peptide substrate and (acyloxy)methyl ketone inactivators.

papain, which indicates that the tetrahedral adduct did not form.

Compounds **5**−**9** inactivate papain (Table 1). Activity was not recovered upon 100-fold dilution, which suggests that the enzyme is irreversibly inactivated. In addition, the inactive enzyme could no longer be modified by [^{14}C]-iodoacetamide. These results are consistent with the alkylation of the active site cysteine as observed for other (acyloxy)methyl ketone inactivators (*14*) (Figure 2). Table 1 shows that the values of k_{inact}/K_i vary over a range of 50-fold depending on the amino acid leaving group in the following order: L-Leu > D-Leu > D-Lys > D-Phe > D-Asn. Moreover, the value of k_{inact}/K_i for the L-Leu-containing inactivator (**9**) is 4000 times greater than that for **1**. Since these leaving groups presumably have similar pK_a values and therefore similar chemical reactivities, the striking differences in k_{inact}/K_i values must be derived from interactions with S′ subsites. Indeed, a similar relative order and magnitude of k_{cat}/K_m values are observed in the hydrolysis of peptide substrates (*17*).

Interestingly, the best inactivator of papain contains L-Leu (**9**) as the leaving group. The k_{inact}/K_i value of 1.1×10^4 M^{-1} s^{-1} is 10 times greater than that for D-Leu (**8**). This result is perplexing given that the side chain of L-Leu should be in a spatial arrangement different than that of a substrate P_1' residue (Figure 2). However, it is difficult to predict the interactions between the S′ subsites and the leaving group given that the side chain is displaced by two atoms from its position in a peptide substrate and the main chain interactions are also perturbed. Although **9** is the most effective inactivator as judged by the value of k_{inact}/K_i, **8** is the most effective inactivator when measured by the stoichiometry of the inactivator. Complete inactivation of papain can be obtained with 1 equiv of **8**, while a 20-fold excess of **9** is required. This observation suggests that **9** is consumed in the reaction. The L-Leu group of **9** can bind to the S_1 subsite of papain as well as to the S′ site. In this orientation, **9** will be hydrolyzed as an ester substrate to produce Z-L-Leu and Ac-Phe-Gly-CH_2-OH. Although the D-Leu group of **8** can also bind to the S_1 site, it will not be oriented properly with respect to the catalytic residues and will not be hydrolyzed. This conclusion is supported by the observation that Z-L-Leu-SCH_2CH_3 (**18L**) is a papain substrate ($k_{cat} = 0.74$ s^{-1}, $K_m =$ 200 μM). In contrast, Z-D-Leu-SCH_2CH_3 (**18D**) cannot be hydrolyzed by papain. Therefore, although (acyloxy)methyl ketones containing D-amino acid leaving groups appear to be less potent inactivators, they may be more useful in vivo than their L-amino acid counterparts.

Table 2: Inhibition of ICE by Peptidyl (Acyloxy)methyl Ketones[a]

Fmoc-Asp-CO-CH_2-X		interleukin-1β conversion enzyme (ICE)		
no.	leaving group (X)	K_i (μM)	k_{inact} (s^{-1})	k_{inact}/K_i (M^{-1} s^{-1})
11	$OCOCH_3$	no inhibition		
12	OCO-Gly-NH-Z	75	0.00064	8.5
13L	OCO-L-Ala-NH-Z	no inhibition		
13D	OCO-D-Ala-NH-Z	1.7×10^2	0.0055	32
14L	OCO-L-Pro-NH-Z	10	reversible	
14D	OCO-D-Pro-NH-Z	1.4×10^2	0.018	1.3×10^2
15D	OCO-D-Ala-D-Pro-NH-Z	1.0×10^2	0.0083	83
16D	OCO-D-Pro-D-Val-NH-Ac	61	0.0083	1.4×10^2

[a] The reaction conditions for ICE include 20 mM HEPES, 10% w/v sucrose, 0.15% w/v CHAPS, and 2 mM DTT at pH 7.2 and 37 °C. Saturation kinetics were observed in all cases except **11** and **13L**. The values of k_{inact} and K_i were obtained with the method of Kitz and Wilson for irreversible inhibitors. K_i for reversible inhibitor **14L** was determined from steady-state velocities.

Inactivation of Cathepsin B. The S_1 and S_2 subsite specificity of papain is similar to that of cathepsin B (*13*). Therefore, we also determined the effects of compounds **1**−**9** on cathepsin B as shown in Table 1. The value of k_{inact}/K_i varies over a range of 100 depending on the amino acid leaving group in the following order: L-Leu > D-Trp > D-Leu > D-Phe > D-Asn > D-Lys. The value of k_{inact}/K_i for the L-Leu-containing inactivator (**9**) is more than 3000 times greater than that for **1**. Therefore, as in the case of papain, the differences in k_{inact}/K_i for the inactivation of cathepsin B

Table 3: Reaction of Chloromethyl Ketones and (Acyloxy)methyl Ketones with Chymotrypsin,[a] Papain,[b] Ice,[c] and Cysteine[d]

	$k_{inact}/[I]$ (M^{-1} s^{-1})			
inhibitor	chymotrypsin	papain	ICE	cysteine
Tos-Phe-CH_2Cl (TPCK)	95	8.2×10^3	3.5×10^2	0.86
Ac-Phe-Gly-CO-CH_2OCO-D-Leu-NH-Z (**8**)	<0.1	1.0×10^3	<0.1	6.2×10^{-3}
Fmoc-Asp-CO-CH_2OCO-D-Pro-NH-Z (**14D**)	<0.1	<0.1	1.3×10^2	nd[e]

[a] Reaction conditions: 50 mM KP_i and 1 mM EDTA at pH 6.8 and 25 °C. [b] Reaction conditions: 50 mM KP_i, 1 mM EDTA, and 2 mM DTT at pH 6.8 and 25 °C. [c] Reaction conditions: 20 mM HEPES, 10% w/v sucrose, 0.15% w/v CHAPS, and 2 mM DTT at pH 7.2 and 37 °C. [d] Reaction conditions: 50 mM KP_i and 5 mM EDTA at pH 6.8, under anaerobic conditions, and 25 °C. [e] nd, not determined.

must be derived from interactions with S′ subsites. In addition, a similar relative order and magnitude of k_{cat}/K_m values are observed in the hydrolysis of the peptide substrates by cathepsin B (*17*). Therefore, also like papain, the efficacy of the inactivators correlates with the substrate specificity of the protease.

Importantly, some of these compounds can discriminate between cathepsin B and papain. For example, **4** is a potent inactivator of cathepsin B but a poor reversible inhibitor of papain. In contrast, **7** has a 53-fold preference for papain over cathepsin B. These results show that S′ subsite interactions can be utilized to generate selective inhibitors, although these two proteases have the same S_1 and S_2 subsite specificity.

Inactivation of ICE. ICE has a preference for Tyr in P_4, Val in P_3, His in P_2, Asp in P_1, Ala in P_1', and Pro in P_2' (*18*, *19*). We synthesized a series of (acyloxy)methyl ketones containing *N*-Fmoc-aspartyl in the P_1 residue and incorporating D-amino acids into the leaving group (Table 2). As with papain and cathepsin B, the efficacy of the inactivator is dependent on the leaving group and appears to be derived from interactions with the S′ subsites. Compounds in which X = $OCOCH_3$ and X = Z-L-Ala (**11** and **13L)** do not inhibit ICE, while **14L** (X = Z-L-Pro) is a reversible inhibitor. The best inactivators contained Z-D-Pro in the P_1' position of the leaving group (**14D** and **16D**), followed by Z-D-Ala (**13D** and **15D**) and Z-Gly (**12**). The dipetidyl P′ components in **15D** and **16D** do not significantly improve the inhibition potency. Thus, inactivator specificity appears to mimic the S_2' subsite rather than the S_1' subsite. The leaving group of the inactivator is only one atom from the P_2' position of the substrate (Figure 2), and may be positioned to interact with the S_2' subsite in ICE. More potent inactivators might be designed by extending S subsite interactions to include the sequence Tyr-Val-Ala-Asp as P_4-P_1 (*4*).

We performed an experiment to confirm that the (acyloxy)-methyl ketones inactivate the ICE by alkylating the active site cysteine as in Scheme 1 (*4*). ICE was inactivated with Fmoc-Asp-CH_2-OCO-D-Ala-NH-Ac-[^{3}H] (>95% inactive). The reaction mixture was applied to a Bio-Rad P-2 column to separate small molecules from ICE. No radioactivity could be detected in the protein fraction, which confirms that the acyl group is released as shown in Scheme 1.

Stability of (Acyloxy)methyl Ketones. An experiment was performed to evaluate the stability of the (acyloxy)methyl ketones in aqueous buffer. We monitored the hydrolysis of the soluble (acyloxy)methyl ketone Alloc-*N*-Asp-CH_2OCO-Ala-Z (**17**) in deuterated 50 mM KP_i buffer by ^{1}H NMR. The half-time of hydrolysis was >24, 24, and 1.5 h at pH 6.0, 7.0, and 8.0, respectively. In addition, we monitored the reaction of free cysteine with the (acyloxy)methyl ketone (**8)** and the chloromethyl ketone (TPCK) to compare their susceptibility to nucleophillic displacement (Table 3). The second-order rate constants were 0.0062 M^{-1} s^{-1} for **8** and 0.86 M^{-1} s^{-1} for TPCK at pH 6.5. Thus, the (acyloxy)methyl ketone is 140-fold less susceptible to nonspecific nucleophilic displacement than the chloromethyl ketone. These results suggest that (acyloxy)methyl ketones have adequate stability to be used in vivo.

Selectivity of (Acyloxy)methyl Ketones. The second-order reaction rates of TPCK, Ac-Phe-Gly-CH_2OCO-D-Leu-NH-Z (**8**), and Fmoc-Asp-CH_2OCO-D-Pro-NH-Z (**14D**) with chymotrypsin, papain, and ICE were determined as shown in Table 3. Although commonly known as a chymotrypsin inactivator, TPCK is a more potent inactivator of papain and ICE. The poor selectivity of TPCK is probably due to its high intrinsic reactivity. In contrast, none of the (acyloxy)-methyl ketones described in this work inhibits chymotrypsin, and good selectivity is observed between papain and ICE. For example, **8** shows more than 10^4-fold selectivity for papain over ICE, while **14D** shows ~1300-fold selectivity for ICE over papain.

Utilization of S′−P′ Interactions in Inhibitor Design. The S′−P′ interactions are generally underappreciated and have rarely been exploited in inhibitor design. The (acyloxy)-methyl ketone framework permits the utilization of both S−P and S′−P′ interactions to increase inhibitor specificity and potency. We demonstrate that potent affinity reagents with low intrinsic chemical reactivity can be obtained by manipulating S′−P′ interactions. In general, inactivator specificity parallels substrate specificity. These compounds may be useful for in vivo studies of enzyme inhibition.

The mechanism of (acyloxy)methyl ketone inactivation of cysteine proteases is unclear at this time (*20*). Therefore, the mechanism of acceleration of the inactivation reaction due to S′−P′ interactions is also unclear. The active site cysteine residue may be alkylated by direct SN2 displacement of the leaving group (Scheme 1A). In this case, the S′−P′ interactions could be used to activate the leaving group. Alternatively, inactivation may proceed via a thiohemiacetal intermediate as shown in Scheme 1B. In this case, the S′−P′ interactions may also orient the ketone for thiohemiacetal formation. We have shown that the thiohemiacetal intermediate does not form in the case of reversible inhibitors. Unfortunately, this observation does not distinguish between the two possibilities for the inactivators, and further experiments are required to address this question.

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SUPPORTING INFORMATION AVAILABLE

TLC and NMR data for compounds **1**−**18**. This material is available free of charge via the Internet at http://pubs.acs.org.

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