each compound, except that inactive compounds were generally examined only twice. In assays performed at 30 °C, magnesium-free glutamate was used, the final $MgCl_2$ concentration was 0.25 mM, and both the preincubation without GTP and the incubation in the spectrophotometers was performed at 30 °C.

Colchicine Binding Assay. The DEAE-cellulose filter assay was used.[46] Each 0.1-mL reaction mixture contained 0.1 mg/mL tubulin (1.0 μM), 5 μM [^{3}H]colchicine, 5 μM inhibitor (if present), 5% (v/v) dimethyl sulfoxide, and either 1.0 M commercial monosodium glutamate and 1.0 mM $MgCl_2$ (for 37 °C experiments) or 1.0 M magnesium-free monosodium glutamate and 0.25 mM $MgCl_2$ (for 30 °C experiments). Incubation was for 10 min at both temperatures. In individual experiments, triplicate samples were obtained for all data points, and each potential inhibitor was evaluated in at least three independent experiments.

Inhibition of Growth of L1210 Murine Leukemia Cells and Evaluation of Cultures for Increased Numbers of Cells Arrested in Mitosis. These experiments were performed as described previously.[23]

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Structure–Activity Relationships for Inhibition of Papain by Peptide Michael Acceptors

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Two series of peptidyl Michael acceptors, *N*-Ac-L-Phe-NHCH$_2$CH=CH-E with different electron withdrawing groups (E = CO_2CH_3, **1a**; SO_2CH_3, **1b**; CO_2H, **1c**; CN, **1d**; $CONH_2$, **1e**; and C_6H_4-*p*-NO_2, **1f**) and R-NHCH$_2$CH=CHCOOCH$_3$ with different recognition and binding groups (R = *N*-Ac-D-Phe, **2a**; *N*-Ac-L-Leu, **3a**; *N*-Ac-L-Met, **4a**; $PhCH_2CH_2CO$, **5a**; PhCO, **6a**), were synthesized and evaluated as inactivators against papain. It was found that the inhibition of papain by peptidyl Michael acceptors is a general phenomenon and that the intrinsic chemical reactivity of the E group in the Michael acceptors has a direct effect on the kinetics of the inactivation process as reflected in k_2/K_i. At pH 6.2, the reactivity of papain toward the Michael acceptors is about 283 000-fold higher than the reactivity of the model thiol 3-mercaptopropionate. This large increase in reactivity is attributable to at least 2 factors; one is the low apparent pK_a of Cys-25 of papain, and the other is the recruitment of catalytic power by specific enzyme–substrate interactions. The unexpectedly high reactivity of **1c** (E = COOH) was rationalized by proposing a direct interaction of the acid group with His-159 in the active site of papain. The unexpected inactivity of **1f** (E = C_6H_4-*p*-NO_2) as a Michael acceptor and its very powerful competitive inhibition of papain were rationalized by molecular graphics which showed the nitrophenyl moiety rotated out of conjugation with the olefin and interacting instead with the hydrophobic S_1' region of papain. A plot of log (k_2/K_i) for **1a–6a** vs log (k_{cat}/K_m) for analogous R-Gly-*p*-NA substrates was linear (r = 0.98) with slope of 0.83, suggesting that binding energy from specific enzyme–ligand interactions can be used to drive the self-inactivation reaction to almost the same extent as it is used to drive catalysis.

Proteinase enzymes regulate many physiological functions such as digestion of dietary protein, blood coagulation, activation of physiologically active peptides from their inactive forms, and others.[1] Under physiological conditions, the activity of proteinase enzymes is controlled by means of storage in proenzyme forms, through their sequestration in subcellular vesicles, and by the presence of endogenous protein inhibitors.[2] Imbalances in these control mechanisms can result in excessive proteolytic activity with consequent alteration in physiological processes leading to pathological states. Thus agents which can inhibit selectively the action of a given proteinase without causing toxicity could potentially be developed into useful drugs. For example, synthetic inhibitors of angiotensin converting enzyme are being used clinically for the treatment of hypertension.[3] In addition, synthetic low molecular weight inhibitors have been used as investigative tools to characterize the catalytic functional groups, recognition specificity, catalytic mechanism and transition-state structure for proteinase enzymes.

The cysteine proteinase family includes the plant thiol proteinases such as papain, ficin, and actinidin, the mammalian lysosomal thiol proteinases such as cathepsin B, H, and L, the mammalian calcium-activated neutral proteinases (calpains I and II), and certain viral-induced cysteine proteinases. The cathepsins play an important role in intracellular degradation of proteins and possibly in the activation of some peptide hormones.[4] Enzymes similar to cathepsins B and L are released from tumors and may be involved in tumor metastasis.[5–7] Defects in the regulation of cysteine proteinase activity by their endogenous inhibitors (cystatins) have been reported in connection with several disease processes, including cancer,[8] amyloidosis,[9] and muscular dystrophy.[10] Release of

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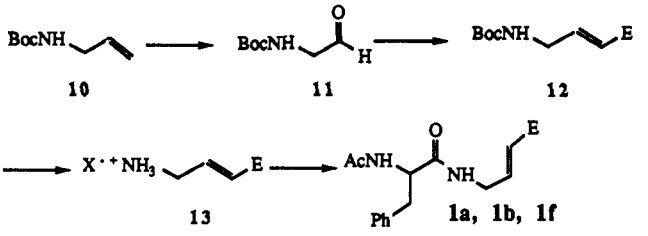
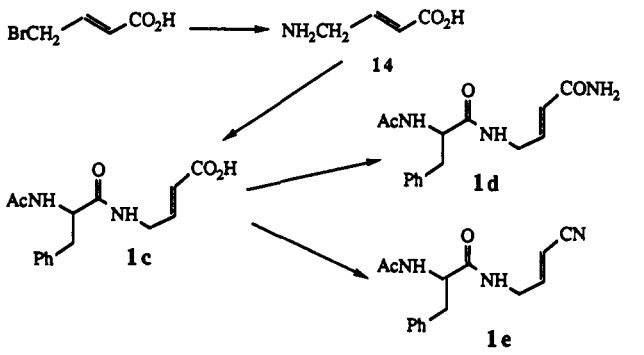
cathepsin B and other lysosomal proteinases from polymorphonuclear granulocytes and macrophages has been observed in trauma and inflammation,[11] and cathepsin L is present in diseased human synovial fluid.[12] Many picornaviruses including poliovirus, foot and mouth disease virus, and rhinovirus encode for cysteine proteinases that are essential for cleavage of viral polyproteins.[13-16] Selective inhibitors of cysteine proteinases are thus of significant interest as potential therapeutic agents and have been widely sought after.[17-25]

Cysteine proteinases are selectively inhibited by several types of peptide-derived inhibitors, including irreversible affinity labeling peptidyl(acyloxy)methyl, halomethyl, and

Scheme I



Scheme II



diazomethyl ketones and various epoxysuccinyl peptide derivatives.[26] In contrast peptidyl aldehydes[27] and peptidyl nitriles[28] represent two groups of potent but fully reversible cysteine proteinase inhibitors. Our laboratory has been active in developing new types of inhibitors for cysteine proteinase enzymes, and recently reported that the vinylogous amino acid ester **1a**, a peptidyl Michael acceptor analogue of substrate, showed strong selectivity for inactivation of papain among several other proteinase enzymes.[29,30] Kinetic studies with this compound showed time- and inhibitor concentration-dependent enzyme inactivation with a second-order rate constant of 70 M^{-1} s^{-1}. Saturated (**7**) and decarboxylated (**8**) derivatives of this compound showed only weak competitive inhibition with K_i values of 19.5 and 9.2 mM, respectively, which indicated that the Michael acceptor moiety was essential for the inhibition. Compound **9**, which is a Michael acceptor analogue of *N*-acetyl-L-phenylalanine, did not show time dependent inactivation of papain. This observation reflects the known substrate specificity of papain for hydrophobic side chains at the P_2 position in its substrates and inhibitors. With compound **9**, placing the benzyl moiety in the hydrophobic S_2 subsite of papain would prevent the Michael acceptor moiety from reaching over to the sulfhydryl of Cys-25.

As an extension of this previous work two series of new peptidyl Michael acceptors **1a–f** and **1a–6a** were designed to test the generality of the peptidyl Michael acceptor concept and to investigate the chemical basis of their efficiency by exploring the quantitative relationships be-

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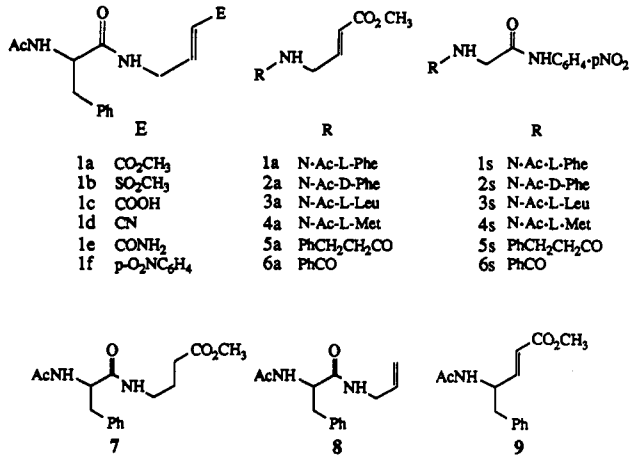

tween their chemical properties, the kinetic specificity of related substrates and their enzyme inhibition reactivity. In this paper, we report the synthesis of compounds **1a–f** and **2a–6a** and their evaluation as inactivators against papain. Within the series **1a–f**, in which the chemical reactivity of the Michael acceptor moiety is varied keeping the "recognition and binding" portion of the molecule constant, there is indeed an excellent correlation between chemical and enzymatic reactivity, with papain at pH 6.2 being ca. 283 000 times more reactive toward the Michael acceptors than the model thiol 3-mercaptopropionate. For two of the compounds the activity observed was unexpected, but molecular graphics analysis of their interaction with papain provided plausible explanations for their observed activity. Within the series **1a–6a**, in which the "recognition and binding" portion of the molecule is varied keeping the chemical reactivity of the Michael acceptor moiety constant, a strong correlation is found between the inactivation rate constants (k_2/K_i) and kinetic constants (k_{cat}/K_m) for the analogous substrates (i.e. **1s–6s**).

Results and Discussion

Synthesis. Two basically different routes were used to synthesize Michael acceptors **1a–f**. The synthesis of **1b** and **1f** followed the Horner–Emmons–Wittig (HEW) approach used previously for **1a** and outlined in Scheme I. *N*-Boc-allylamine (**10**) was ozonized and reduced with Me_2S to give crude *N*-Boc-glycinal (**11**).[30] Attempts to isolate and purify the latter led only to extensive decomposition. Instead, crude **11** was dissolved in $CHCl_3$, washed quickly with dilute HCl and then $NaHCO_3$ solution and dried; the aldehyde content of the material was estimated by ^1H NMR. Generally, crude **11** containing 30–50% aldehyde was used immediately for the HEW couplings, although *solutions* of **11** could be stored in a refrigerator for 1–2 days if necessary. Condensation of aldehyde **11** with HEW reagents, preformed in tetrahydrofuran (THF) from $(CH_3O)_2P(O)CH_2$-E (where E = $-CO_2Me$, $-SO_2Me$, or $-C_6H_4NO_2$-*p*) and NaH, afforded the Boc-protected Michael acceptor fragments **12** in low to good yields. Deprotection with trifluoroacetic acid (TFA) gave the TFA salts **13** in 67–92% yield, and mixed-anhydride coupling to *N*-acetyl-L-phenylalanine gave the desired inhibitors **1a**, **1b**, and **1f** as crystalline compounds.

The synthesis of compounds **1c–e** followed a different route (Scheme II). γ-Aminocrotonic acid (**14**), prepared by amination of γ-bromocrotonic acid as described by Pinza and Pifferi,[31] was purified by chromatography on Dowex 50W-X8 cation exchange resin and coupled to *N*-acetyl-L-phenylalanine to give **1c** directly. The latter was then converted to amide **1d** and nitrile **1e** using mixed anhydride and polyphosphoric ester activation,[32,33] respectively.

The Michael acceptor fragment methyl γ-aminocrotonate was synthesized as described by Thompson et al.[30] and coupled with *N*-acetylamino acids (for **1a–4a**) and dihydrocinnamic acid (for **5a**) by using the mixed anhydride procedure of Anderson et al.[34] Methyl *N*-benzoyl-γ-aminocrotonate (**6a**) was prepared by treating methyl γ-aminocrotonate with benzoyl chloride in the presence of base.

All final products were fully characterized by ^1H NMR, mass spectrometry, polarimetry, and elemental analysis. The yields reported should be regarded as minimum yields since no attempt was made to optimize the procedures.

Enzyme Inhibition Studies. The evaluation of compounds **1a–f** as inhibitors of papain followed the method of Kitz and Wilson.[35] Compounds **1a–e** and **2a–6a** (but

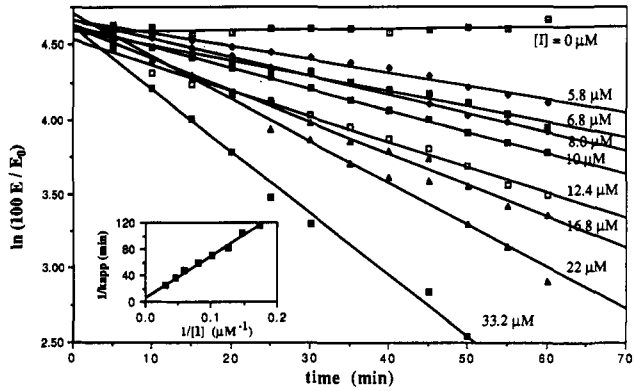

Figure 1. Irreversible inhibition kinetics of compound **1a**. *E* is the remaining enzyme activity measured after incubation with **1a** and dilution with buffer, E_0 is the enzyme activity without inhibitor and k_{app} is the slope of the pseudo-first-order kinetic plot.

Table I. Kinetics of Papain Inactivation by *N*-Ac-L-PheNHCH$_2$CH=CH-E

compd	E group	[I], μM	k_2/K_i, M^{-1} s^{-1}
1a	$COOCH_3$	6–35	26.1
1b	SO_2CH_3	2–30	18.7
1c	COOH	30–450	5.0
1d	CN	60–500	1.7
1e	$CONH_2$	60–1000	1.1
1f	$PhNO_2$	80–160	

Table II. Kinetics of Papain Inactivation by R-NHCH$_2$CH=CH-CO_2CH_3

compd	R group	[I], mM	k_2/K_i, M^{-1} s^{-1}
1a	*N*-Ac-L-Phe	0.006–0.035	26.1
2a	*N*-Ac-D-Phe	1.0–8.0	0.25
3a	*N*-Ac-L-Leu	0.056–1.08	2.46
4a	*N*-Ac-L-Met	0.07–0.58	2.06
5a	dihydrocinnamoyl	0.87–10	0.23
6a	benzoyl	1.42–10	0.05

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not 1f) all showed concentration- and time-dependent inhibition which could be analyzed using eqs 1–3. Enzyme

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E\cdot I \xrightarrow{k_2} E\text{-}I \quad (1)$$

$$K_i = (k_{-1} + k_2)/k_1 \quad (2)$$

$$\frac{1}{k_{app}} = \frac{K_i}{k_2}\frac{1}{[I]} + \frac{1}{k_2} \quad (3)$$

inhibition activity was measured at various times in the presence of different initial concentrations of inhibitor with the latter always being in excess over enzyme concentration (50–5000-fold). All buffer solutions contained acetonitrile (20% v/v) to solubilize the test compounds. In agreement with observations of Lucas and Williams,[36] control experiments showed this amount of acetonitrile had little apparent affect on the activity of papain toward Cbz-Gly-ONp. Plots of ln (E/E_0) vs time were linear and gave the slopes k_{app}, and replots of the reciprocal of these slopes vs $[I]^{-1}$ were used with eq 3 to determine the apparent binding constant (K_i), the apparent inactivation rate constant (k_2) and the apparent second order rate constant for papain inactivation (k_2/K_i). Representative plots for one such analysis with compound **1a** are shown in Figure 1. The results for all 11 compounds (**1a–f** and **2a–6a**) are given in Tables I and II. Note that the kinetic constant for **1a** reported here differ slightly from that mentioned above; this is due to the use of less cosolvent (7.5% v/v) in the earlier study. It is also important to note that the inhibitors were evaluated at concentrations below their apparent K_i values. This is because at higher concentrations inactivation was too fast to measure. According to eq 4, a reciprocal form of eq 3, when $[I] \ll K_i$ the reaction is essentially first order in inhibitor concentration (but overall second order, eq 5) and there is no indication of saturable binding. Consequently plots of $(k_{app})^{-1}$ vs $[I]^{-1}$

$$k_{app} = \frac{k_2[I]}{K_i + [I]} \quad (4)$$

$$k_{app} = \frac{k_2}{K_i}[I] \quad (5)$$

intercept the axes too close to the origin to allow k_2 and K_i to be determined with good statistical precision (see inset, Figure 1). Nevertheless, the apparent second-order rate constant k_2/K_i, obtained from the slope of the inset plot, is not affected by the inhibitor concentration range used. Indeed, plots of k_{app} vs [I] (eq 5) gave the same k_2/K_i values as determined using plots of $(k_{app})^{-1}$ vs $[I]^{-1}$ (eq 3).

E-Group Series. For compounds **1a–e** there was a 25-fold variation in k_2/K_i, the overall second-order rate constant for their (irreversible) reaction with papain. This clearly shows that as anticipated, the inhibition of papain by Michael acceptor peptide analogs of **1a** is indeed a general phenomenon and that the electron-withdrawing ability of the E group does affect their relative inhibition activity toward papain. The marked reactivity of acid **1c** as a time-dependent irreversible inhibitor was unexpected, based on the assumption that a negatively charged ionized carboxylate moiety would not activate the C=C double bond for a Michael addition reaction. However, while **1c** in solution at pH 6.2 is probably more than 95% ionized, it is possible that the form bound by papain is the neutral acid, which would be a good Michael acceptor. We will return to this point later.

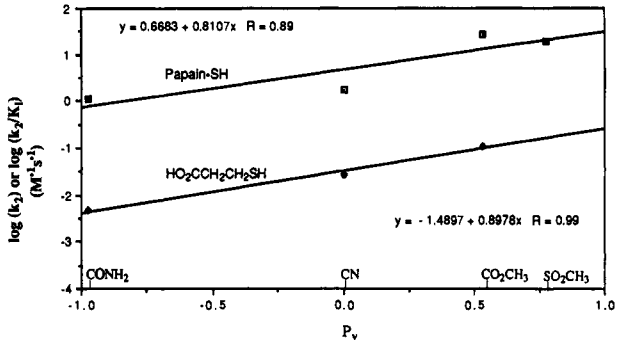

Figure 2. Relative reactivity of enzymatic and chemical Michael addition reactions.

An important question we sought to answer is whether varying the electron-withdrawing group in **1** would affect enzymatic reactivity to the same extent as it affects the purely chemical reactivity of these Michael acceptors. Evidence to the contrary would suggest differential interaction of the E groups with the enzyme. Friedman and Wall[37] devised a scale for expressing the relative reactivity of a series of Michael acceptors toward the amine nucleophile glycine using the parameter P_v as defined in eq 6. In this equation the k values represent second-order

$$P_v = \log [k_{(CH_2=CH\text{-}E)}/k_{(CH_2=CH\text{-}CN)}] \quad (6)$$

rate constants for the Michael addition of glycine to the activated olefin (CH_2=CH-E) vs that for addition to the reference compound acrylonitrile CH_2=CH-CN. The P_v scale is thus based on a linear free energy relationship analogous to the Hammett σ scale. Friedman and Wall also applied this approach to the thiol nucleophile 3-mercaptopropionate (at pH 8.1), and showed that for methyl acrylate, acrylonitrile, and acrylamide the ratio of second-order rate constants for Michael addition of 3-mercaptopropionate vs. glycine was ca. 150 in all three cases.[38] This result is summarized by the bottom line in the plot shown in Figure 2. The fact that the slope of this line is less than unity indicates that the thiol addition reaction is slightly less sensitive to changes in the E group than is the amine addition reaction, perhaps because of an earlier transition state for addition of the more reactive thiol nucleophile.

Application of this approach to the k_2/K_i data for Michael addition of papain to **1a**, **1b**, **1d**, and **1e** (at pH 6.2) results in the upper line shown in Figure 2. The slope of this line is only slightly less than that for the purely chemical model reaction over nearly a 100-fold range in olefin reactivity as reflected by the P_v scale. That the slopes of these two plots are very similar provides strong evidence that the reactivity of the Michael acceptors toward papain is governed almost entirely by chemical factors intrinsic to the Michael moiety per se, i.e. there are no significant differences in binding or enzymatic activation (if any) among the various E groups. The vertical separation of the two lines at the midpoint where $P_v = 0$

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is about 2.15 log units, corresponding to an apparent 144-fold difference in reactivity between these two thiol reactants. However to appreciate the difference in reactivity between papain and 3-mercaptopropionate more fully, allowance must be made that *trans*-crotonate derivatives are 25 times less reactive that acrylate derivatives as Michael acceptors,[37] and the rates for the 3-mercaptopropionate reaction should be corrected from pH 8.1 to pH 6.2. Since only the ionized thiolate form of a mercaptan shows significant reactivity toward electrophiles[39-41] the ratio of reactivities of a given thiol/thiolate compound at two different pH values is equal to the ratio of their fractional degrees of ionization at those pH values, as given by eq 7. Accordingly, the S nucleophilic reactivity of 3-mercaptopropionate (pK_a = 10.05) is predicted to fall by 78.6-fold from pH 8.1 to pH 6.2. Considering both the acrylate/crontonate difference (25-fold) and the pH difference (78.6-fold), the intercept of the lower line in Figure 2 should drop an additional 3.28 log units. The apparent difference in reactivity between papain and 3-mercaptropropionate, both at pH 6.2, would thus be 5.45 log units or 283 000-fold.

$$\frac{\text{rate at pH}_1}{\text{rate at pH}_2} = \frac{K_a + [H^+]_2}{K_a + [H^+]_1} \qquad (7)$$

The extraordinary difference in reactivity between papain and 3-mercaptopropionate may be attributed to two primary factors. One is the special environment of the sulfhydryl group of Cys-25 in papain. Abundant evidence suggests that the sulfhydryl group of Cys-25 in papain has a very low pK_a and exists, at pH 6 (the pH optimum for papain), predominantly as a thiolate–imidazolium ion pair with the adjacent His-159 residue.[42-46] Thus the greater nucleophilic reactivity of papain vs simple model thiols is in part due simply to the fact that at pH 6 or 6.2 the sulfhydryl group of papain is ionized to a much greater extent than that of the thiols. A second factor which may be quite important is the advantage accrued via specific binding of the electrophile (Michael acceptor) to the nucleophile (papain) prior to the irreversible Michael addition step. It is well known that the kinetic specificity of papain toward ligands, both substrates and inhibitors, is enhanced by having an L aromatic amino acid at position P_2 of the

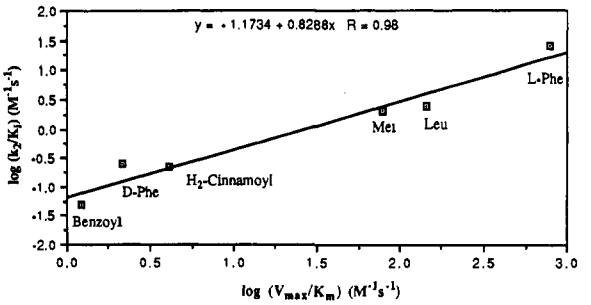

Figure 3. Linear free energy correlation of catalysis and inhibition efficiency. Inhibition data are from Table II; substrate kinetic data are from ref 27.

ligand structure, and that ligands based on the *N*-Ac-L-Phe-Gly-X motif (viz. **1a–f**) are particularly favored by papain.[47] In fact these compounds were designed with this specificity in mind in order to (1) hold constant those aspects of structure supposedly related to recognition and binding while attempting to vary chemical reactivity through changes in the E group in 1 and (2) take advantage of possible recruitment of catalytic power to the inactivation reaction in the same way as other specific substrates and inhibitors of papain. This later aspect of experimental design is specifically addressed below.

R-Group Series. From studies of the hydrolysis of diastereomeric peptides of L- and D-alanine, Schechter and Berger concluded that papain has an active center capable of accommodating up to seven amino acid residues of the substrate.[47-49] Kinetic studies[50] show that the specificity of papain is directed toward substrates with large hydrophobic sidechains at position P_2 (e.g. phenylalanine). Crystallographic studies on enzyme–inhibitor combinations[51] showed that papain consists of a single polypeptide chain folded to form two domains connected by a hinge; the active site is a cleft whose two sides are formed from the two protein domains. The side chain of the P_2 Phe residue is accommodated so that C(β) lies between the side chains of Pro-68 and Ala-160 while the phenyl ring is adjacent to the side chains of Val-133 and Val-157 which, together with Tyr-67, Trp-69 and Phe-207, form the S_2 "hydrophobic pocket" of the enzyme.

Extensive studies of the binding of reversible covalent inhibitors (*N*-acylglycine nitriles and *N*-acylglycinals) have revealed striking changes in binding strength depending on the structure of the acyl moieties.[27,28,52-54] Parallel

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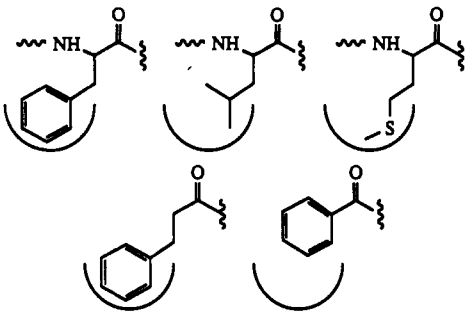
studies with *N*-acylglycine *p*-nitroanilides[27,28,59] have revealed virtually identical trends in their steady-state kinetic parameters, suggesting that peptide nitriles and aldehydes bind to papain as transition state analogues despite the obvious differences in the geometry of their adducts (i.e. sp^2 thioimidates vs sp^3 hemithioacetals).

Steady-state kinetic studies by Lowe and Yuthavong[50,52] have shown that the specificity of papain toward several *N*-acylglycine *p*-nitroanilides and *p*-nitrophenyl esters is manifest mainly in the acylation step, which begins with attack of Cys25-SH on the carbonyl carbon of P_1. Since peptide Michael acceptors were expected to undergo a similar nucleophilic attack, it was of interest to determine whether variations in the side chains known to affect kinetic substrate specificity would have similar consequences for the kinetic properties of analogous inhibitors. This led to the design of inhibitors **1a–6a**, in which the E group (and thus their purely chemical reactivity) was held constant while the "recognition and binding" R group corresponding to the P_3–P_2 portion of normal substrates was varied. The side chains in **1a–6a** (and **1s–6s**) were chosen, on the basis of previous studies of the reversible covalent binding of peptide nitriles and peptide aldehydes to papain, to examine specifically the roles of P_2 hydrophobicity, P_2 stereochemistry, and enzyme–ligand hydrogen bonding involving the P_2NH and/or $P_3C{=}O$ groups. As shown by the k_2/K_i data in Table II, changing the R group has a profound affect on the kinetic properties of the inhibitors.

The specificity of an enzyme for a substrate (or an inactivator) is best measured kinetically by the parameter k_2/K_i (or k_{cat}/K_m), the apparent second-order rate constant for the overall reaction. The changes in inhibition kinetics reported in Table II for **1a–6a** parallel closely those for the kinetic properties of analogous substrates (**1s–6s**) as strikingly demonstrated by the linear free energy correlation shown in Figure 3. The fact that the slope of this correlation (0.83) is less than unity may indicate a slightly earlier transition state for sulfhydryl addition to C=CH-E compared to C=O. However, the major conclusions which may be drawn from this correlation are (1) that peptidyl Michael acceptors are indeed *specific* inhibitors of papain and (2) that binding energy from specific enzyme–ligand interactions can be used to drive self-inactivation by Michael acceptor peptides in the same way as it is used to facilitate substrate hydrolysis. This latter observation may be contrasted to the situation with trypsin-like serine proteinases,[55] where there is only a poor correlation (r = 0.25) between k_{cat}/K_m for tripeptide *p*-nitroanilide substrates and k_2/K_i for the analogous chloromethyl ketones. It is apparent then that ligand structural changes which affect binding interactions may well influence the two distinct reaction types (hydrolysis vs alkylation) in rather different ways, leading to the low correlation observed. Conversely, the strong correlation observed with papain (r = 0.98, Figure 3) implies considerable similarity in the mechanisms leading to hydrolysis and inactivation. This anticipated similarity was in fact the basis of the design of peptide Michael acceptors as inhibitors for cysteine proteinases.[30]

The data in Table II illustrate that two important structure–activity relationships for papain substrates and transition-state analogue inhibitors also apply to irreversible inhibition by peptidyl Michael acceptor analogues.

Scheme III



First, reducing the size of the P_2 side chains makes them less hydrophobic and less able to reach fully into the S_2 hydrophobic pocket (Scheme III); in turn this leads to significant decreases in inhibitor potency. Second, the importance to inhibitor specificity of the putative $P_2NH\cdots O{=}C$(Gly66) hydrogen bond inferred from the crystal structure of a papain–inhibitor complex[51] is clearly illustrated by comparing k_2/K_i values for **1a**, **5a**, and **2a**. Thus removing the acetamido group from **1a** to generate **5a** decreases k_2/K_i by 112-fold, but adding an acetamido group to **5a** to generate **2a**, the enantiomer of **1a**, has almost no effect. The $P_2NH\cdots O{=}C$(Gly66) hydrogen bond is clearly not possible with **5a**, and for this hydrogen bond to form with **2a** the benzyl moiety of the side chain cannot occupy P_2 properly (at least not in the same way as **1a** does). Thus only with the L enantiomer **1a** can *both* interactions be achieved; hence **1a** shows the greatest kinetic specificity. Finally, the hippuryl analogue **6a** can be seen as deficient in two important ways. It lacks the amide group with which to form the $P_2NH\cdots O{=}C$(Gly66) hydrogen bond, and the P_2 side chain (if it may be called that) is much too short to interact effectively with S_2.

Molecular Graphics. In contrast to **1a–e**, compound **1f** showed only competitive inhibition of papain, but compared to simple amide substrate analogues (e.g. *N*-Ac-L-Phe-Gly-NH_2, K_i = 4.7 mM), **1f** was a much more potent inhibitor (K_i = 0.06 mM). Since there is ample precedent for the Michael addition of sulfhydryl nucleophiles to *p*-nitrostyrene[56,57], both the reversibility and the potency of **1f** were unexpected. Analysis of the interaction of **1f** with papain by means of molecular graphics revealed a logical explanation for this result. To begin with **1f** was superimposed onto the Z-Phe-AlaCH_2 moiety in this derivative of papain, whose crystal structure was reported by Drenth et al.[51] The Z-Phe-AlaCH_2 moiety was removed, a hydrogen was replaced on Cys-25, and the conformation compound **1f** was varied to seek an energy minimum. The result of this shown in Figure 4 where two significant findings are immediately apparent. First, the planes of the C=C bond and the phenyl ring form a dihedral angle of ca. 129°. Since the nitrophenyl moiety in bound **1f** is rotated out of coplanarity with the C=C plane, it is prevented from activating the olefin moiety as in *p*-nitrostyrene in solution; thus compound **1f** does not behave as a Michael acceptor when bound to papain. Its potency as a competitive inhibitor probably derives from hydrophobic

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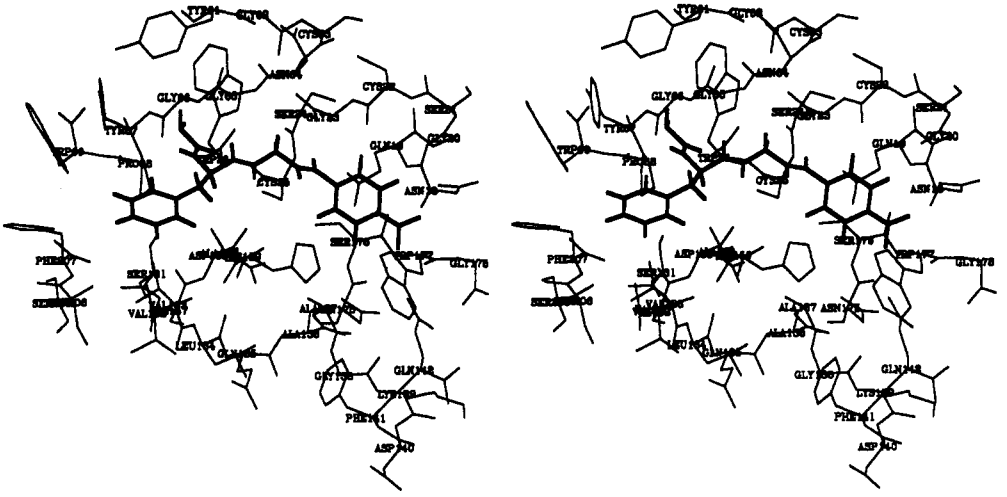

Figure 4. Stereoview of inhibitor **1f** (dark lines) in the active site of papain. See text for discussion.

interaction of the nitrophenyl moiety with the S_1' subsite of papain, which is known to be quite hydrophobic.[58]

As mentioned above it was expected that acid **1c** would be extensively ionized in solution and therefore not very active as an irreversible inhibitor. A similar molecular graphic analysis (not shown) reveals that the carboxylate moiety is very close to N-3 of the imidazole ring of His-159. We hypothesize that hydrogen bonding (–COOH··· imidazole) or ion pairing ($-CO_2^-$···imidazolium) results in the carboxyl moiety of bound **1c** having much greater ability to activate the olefinic bond in a Michael sense than a fully ionized carboxylate group in solution. A similar interaction was also suggested for the inhibition of papain and cathepsin B by peptidyl α-ketocarboxylate derivatives,[59] for the interaction of the acid moiety of the epoxysuccinyl inhibitor E-64 bound in the active site of papain[60] and to explain the abnormally rapid inactivation of papain (via S alkylation) by chloroacetic acid vs chloroacetamide.[40,41] Unfortunately, as there is no simple way to test this hypothesis in a nonenzymatic model, it must remain just a hypothesis. The observation itself is quite encouraging, however, because incorporation of the carboxyl group renders **1c** much more water soluble than the other Michael acceptors, and much less reactive in simple solution (or in vivo?) where no specific hydrogen bonding exists. This suggests that compounds like **1c** might have much better properties for use as in vivo enzyme inactivators.

Conclusion

It has been shown that the inhibition of papain by peptidyl Michael acceptors is a general phenomenon and that both the intrinsic chemical reactivity of the E group in the Michael acceptors and the nature and stereochemistry of the P_2 side chain directly affect the kinetics of the inactivation process. From the point of view of drug design, it is encouraging that the kinetic properties of the irreversible inhibitors are strictly dependent on the chemical reactivity of the Michael acceptor moieties. However, from the range of compounds studied it is not obvious that a great enhancement in k_2/K_i can be achieved by means of E-roup manipulation without incurring the risk of rendering the compounds too reactive to be tolerated by living organisms. Although the reactivity of the inactivators (k_2/K_i) is directly proportional to the chemical reactivity of their particular Michael moiety, variations in the inactivation rate constant associated with changes in the side chain (i.e. "binding group specificity") are in general much larger, and correlate directly with changes in k_{cat}/K_m for analogous substrates. The latter observation suggests that binding energy from specific enzyme–ligand interactions can be used to drive the self-inactivation reaction to almost the same extent as it is used to drive catalysis.

Experimental Section

Solvent and reagents were purchased from commercial sources and were used without further purification except as noted below. Thin-layer chromatography was carried out on glass slides 2.5- × 10-cm precoated with silica gel (Analtech). Melting points were measured in glass capillary tubes on a Thomas Hoover capillary melting point apparatus and are uncorrected. NMR spectra were run on Varian XL-300 and Bruker AM-500 spectrometers (values measured in ppm, J values in Hertz). Electron-impact mass spectra (EIMS) were obtained on Ribermag R10-10b quadrupole instrument. Optical rotations were measured with a Perkin-Elmer Model 141 polarimeter. Elemental analyses were performed on a Hewlett-Packard Model 185b and are within ±0.4% of the theoretical values. Spectrophotometric enzyme assays were performed on a Varian Cary 118 spectrophotometer.

Synthesis. *N*-Boc-glycinal (11). N-Boc-allylamine (2.13 g, 13.6 mmol) was dissolved in 50 mL of dry methanol and cooled to –78 °C. Ozone was bubbled through the solution until it became blue. After the excess of ozone was bubbled out with nitrogen, dimethyl sulfide (1.5 mL, 20 mmol) was added and the solution was kept at –78 °C for 5 h, and then slowly warmed to room temperature. After 1 h at room temperature, the solvent was evaporated. The residue was dissolved in chloroform, washed with 2% HCl and saturated $NaHCO_3$, and dried with $MgSO_4$. After filtration and evaporation, a colorless oil was obtained and used immediately for Horner–Wadsworth–Emmons reactions with no further purification: ^{1}H NMR ($CDCl_3$) δ 1.24 (9 H, s), 3.79 (2 H, d), 5.67 (1 H, s), 9.41 (1 H, s).

Horner–Wadsworth–Emmons Reactions (Method A). NaH (50% in mineral oil) was washed twice with hexane and suspended in anhydrous THF, to which a solution of the Horner reagent (1 equiv) in THF was added. After the reaction solution was stirred for 1 h, *N*-Boc-glycinal (11) (1 equiv) in THF was added and the reaction mixture was stirred at room temperature for 2 h. The reaction was then quenched with H_2O, and the solvents were removed under reduced pressure. The residue was dissolved in $CHCl_3$, washed with 2% HCl and saturated $NaHCO_3$, dried over $MgSO_4$, and evaporated to a yellow product, which was

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purified by chromatography or sublimation.

Removal of the *tert*-Butoxycarbonyl Group (Method B). The *N*-Boc compound was dissolved in trifluoroacetic acid (20–50 equiv) and allowed to stand for 0.5–1 h at room temperature. The trifluoroacetic acid was removed under reduced pressure and the residue was purified by recrystallization from MeOH/ether.

General Procedure for Mixed-Anhydride Couplings (Method C). The *N*-acetyl-protected amino acid was dissolved in THF and *N*-methylmorpholine (NMM) (1 equiv) was added. After the solution was cooled to −15 °C, isobutyl chloroformate (1 equiv) was added and the reaction mixture was stirred at −15 °C for 2 min. The amine trifluoroacetate and NMM (1 equiv) in THF were added and the reaction solution was stirred at −15 °C for 1–2 h and then warmed to room temperature for another 2 h. The solvent was removed under reduced pressure. The residue was dissolved in $CHCl_3$ and washed with dilute HCl and saturated $NaHCO_3$ and dried over $MgSO_4$. Then the solvent was removed to give the crude product.

Methyl *N*-Boc-γ-aminocrotonate (12a). Trimethyl phosphonoacetate (1.32 g, 7.25 mmol) was reacted (method A) with *N*-Boc-glycinal (11) to give the crude product, which was purified by chromatography (10% EtOAc in hexane): yield 840 mg (56%); mp 40–42 °C (lit.[30] mp 45 °C); ^{1}H NMR ($CDCl_3$) δ 1.43 (9 H, s), 3.71 (3 H, s), 3.89 (2 H, m), 4.81 (1 H, s), 5.92 (1 H, d, J_{trans} = 16), 6.88 (1 H, dt, J_{trans} = 16).

Methyl γ-Aminocrotonate Trifluoroacetate (13a). Methyl *N*-boc-γ-aminocrotonate (**12a**) (143 mg, 0.67 mmol) was deprotected (method B) to give 148 mg (97%) methyl γ-aminocrontonate trifluoroacetate (**13a**): mp 122–123 °C (lit.[30] mp 120–120.5 °C); ^{1}H NMR (acetone-d_6) δ 3.67 (3 H, s), 4.62 (2 H, m), 6.08 (1 H, d, J_{trans} = 15.9), 6.96 (1 H, dt, J_{trans} = 15.9).

Methyl *N*-(*N'*-Acetyl-L-phenylalanyl)-4-amino-2-pentenoate (1a). *N*-Acetyl-L-phenylalanine (127 mg, 0.61 mmol) was coupled (method C) to methyl γ-aminocrotonoate trifluoroacetate (**13a**) (140 mg, 0.61 mmol). Recrystallization of the crude product from EtOH/$CHCl_3$/hexane (1:5:80) gave 146 mg (79%) of pure compound **1a**: mp 150–152 °C; $[\alpha]^{25}_D$ +4.83° (*c* 1, MeOH); ^{1}H NMR ($CDCl_3$) δ 1.96 (3 H, s), 3.07 (2 H, m), 3.73 (3 H, s), 3.93 (2 H, d), 4.65 (1 H, m), 5.72 (1 H, d, J_{trans} = 15.8), 6.19 (2 H, m), 6.75 (1 H, dt, J_{trans} = 15.8), 7.27 (5 H, m); EIMS 304 (M^+, 1.6), 261 (1.7), 245 (2.5), 213 (1.7), 190 (4), 162 (17), 131 (17), 120 (90), 91 (100). Anal. ($C_{16}H_{20}N_2O_4$) C, H, N.

3-(*N*-Boc-amino)-1-(methylsulfonyl)-1-propene (12b). Dimethyl [(methylsulfonyl)methyl]phosphonoate (Alfa Products, 1.01 g, 5 mmol) was reacted (method A) with *N*-Boc-glycinal (11) to give the crude product, which was purified with silica gel chromatography (10% EtOAc in hexane): yield 376 mg (32%); mp 69–70 °C; ^{1}H NMR ($CDCl_3$) δ 1.46 (9 H, s), 2.95 (3 H, s), 4.01 (2 H, m), 4.81 (1 H, s), 6.54 (1 H, d, J_{trans} = 15), 6.93 (1 H, dt, J_{trans} = 15); EIMS 235 (M^+, 0.1), 220 (0.9), 178 (10), 162 (19), 135 (17), 120 (17), 57 (100). Anal. ($C_{14}H_{18}N_2O_4$) C, H, N.

3-Amino-1-(methylsulfonyl)-1-propenyl Methanesulfonate (13b). 3-(*N*-Boc)amino-1-(methylsulfonyl)-1-propene (**12b**) (155 mg, 0.48 mmol) was deprotected (method B) to give 77 mg (77%) of **13b**: mp 159–161 °C; ^{1}H NMR (acetone-d_6) δ 2.96 (3 H, s), 4.72 (2 H, m), 6.93 (2 H, m); EIMS 136 (MH^+, 5.8), 120 (6.8), 56 (76), 55 (100). Anal. ($C_5H_{13}NO_5S_2$) C, H, N.

N-(N'-Acetyl-L-phenylalanyl)-3-amino-1-(methylsulfonyl)-1-propene (1b). *N*-Acetyl-L-phenylalanine (35 mg, 0.17 mmol) was coupled (method C) with 3-amino-1-(methylsulfonyl)-1-propenyl methanesulfonate (**13b**) (42 mg, 0.17 mmol). Recrystallization of the product from EtOH/hexane gave 14.2 mg (26%) of pure **1b**: mp 160 °C; $[\alpha]^{25}_D$ +10.5° (*c* = 1.2, MeOH); ^{1}H NMR (acetone-d_6) δ 1.87 (3 H, s), 2.89 (3 H, s), 3.0 (2 H, m), 4.04 (2 H, m), 4.66 (1 H, m), 6.50 (1 H, d, J_{trans} = 15.2), 6.76 (1 H, dt, J_{trans} = 15.2), 7.30 (5 H, m), 7.51 (1 H, d, *J* = 5.7), 7.74 (1 H, s); EIMS 324 (M^+, 0.1), 282 (0.3), 265 (0.6), 245 (0.5), 202 (0.6), 162 (20), 120 (81), 111 (27), 91 (31), 43 (100). Anal. ($C_{15}H_{20}N_2SO_4$) C, H, N.

γ-Aminocrotonic Acid (14). Crotonic acid (20 g, 0.23 mol) and *N*-bromosuccinimide (NBS) (46 g, 0.25 mol) were mixed in dry benzene (200 mL). After the solution was heated to reflux under N_2, azobis(isobutyronitrile) (AIBN) (500 mg, 3.7 mmol) was added and refluxing was continued for 2 h. Then the reaction solution was cooled to 10 °C and filtered. The filtrate was evaporated and the residue was dissolved in cold CCl_4, filtered, and then evaporated. The residue in 50 mL of MeOH was then added into a solution of ammonium hydroxide (29%, 1.3 L) and ammonium chloride (130 g, 2.43 mol) at 0 °C. After the mixture stirred at room temperature for 48 h, one-third of the solvent was removed under reduced pressure and the solution was filtered. The filtrate was passed through the Dowex (50W-X8) cation exchange resin and eluted with ammonium hydroxide solution (gradient from 1% to 5%). The 4.6-g product (20%) obtained by precipitating from cold ethanol solution was recrystalized from EtOH–H_2O solution: mp 173–174 °C (lit.[31] mp 174–176 °C); ^{1}H NMR (D_2O) δ 3.69 (2 H, m), 6.04 (1 H, d, J_{trans} = 16.5), 6.96 (1 H, dt, J_{trans} = 16.5).

N-(N'-Acetyl-L-phenylalanyl)-γ-aminocrotonic Acid (1c). *N*-Acetyl-L-phenylalanine (518 mg, 2.5 mmol) was coupled (method C) with γ-aminocrotonic acid (**14**) (257 mg, 2.5 mmol) in chloroform. Recrystalization from H_2O gave 390 mg (26%) of pure product **1c**: mp 198 °C; $[\alpha]^{25}_D$ +0.15° (*c* = 2, MeOH); ^{1}H NMR (DMSO-d_6) δ 1.85 (3 H, s), 2.88 (2 H, m), 3.85 (2 H, s), 4.48 (1 H, m), 5.74 (1 H, d, J_{trans} = 15.7), 6.72 (1 H, dt, J_{trans} = 15.7), 7.35 (5 H, m), 8.30 (2 H, m), 12.3 (1 H, s); EIMS 291 (MH^+, 100), 273 (7.5), 231 (11), 190 (32), 202 (0.6), 162 (29), 131 (20), 120 (100). Anal. ($C_{15}H_{18}N_2O_4$) C, H, N.

N-Acetyl-L-phenylalanyl-γ-aminocrotonitrile (1d). *N*-(*N'*-Acetyl-L-phenylalanyl)-γ-aminocrotonic acid (**1c**) (580 mg, 2 mmol) and polyphosphoric acid ester (PPE,[32,33] 1.2 g) were mixed, chloroform (2 mL) and dimethyl acetamide (1 mL) were added, the mixture was cooled to 0 °C, and the flask was connected with an ammonia-filled balloon. After stirring at 0–5 °C for 30 min, it was warmed to room temperature and continued stirring for another 2 h. The ammonia balloon was removed and PPE (2 g) was added into the reaction solution. The reaction solution, which was very viscous, was then heated at 80 °C for 11 h. After the reaction, the product was purified by prep. TLC and crystallized from methanol–ethyl ether solution to give 114 mg (21%) of **1d**: mp 169 °C; $[\alpha]^{25}_D$ +0.15° (*c* = 1.3, MeOH); ^{1}H NMR (DMSO-d_6) δ 8.35 (1 H, m), 8.25 (1 H, d), 7.3 (5 H, m), 6.8 (1 H, dt, J_{trans} = 16.6), 5.25 (1 H, d, J_{trans} = 16.6), 4.45 (1 H, m), 3.81 (2 H, m), 2.88 (2 H, m), 1.79 (3 H, s); EIMS 272 (MH^+, 18), 271 (M^+, 23), 162 (17), 138 (27), 131 (29), 120 (100), 104 (12), 103 (12), 91 (16). Anal. ($C_{15}H_{17}N_3O_2$) C, H, N.

N-(N'-Acetyl-L-phenylalanyl)-γ-aminocrotonamide (1e). *N*-(*N'*-Acetyl-L-phenylalanyl)-γ-aminocrotonic acid (**1c**) (2.9 g, 10 mmol) in dimethyl acetamide (6 mL) was coupled (method C) with ammonium hydroxide solution (29%, 50 mL, 850 mmol). The crude product was purified by silica gel chromatography (EtOH/$CHCl_3$ = 4:50). The pure **1e** (1.42 g, 49%) was obtained as white crystals: mp 173–174 °C; $[\alpha]^{25}_D$ +0.20° (*c* = 1.0, MeOH); ^{1}H NMR (DMSO-d_6) δ 8.20 (1 H, m), 7.40 (1 H, s), 7.3 (5 H, m), 7.0 (1 H, s), 6.5 (1 H, dt, J_{trans} = 15.6), 5.9 (1 H, d, J_{trans} = 15.6), 4.5 (1 H, m), 3.8 (2 H, s), 2.9 (2 H, m), 1.75 (3 H, s); EIMS 290 (MH^+, 52), 207 (42), 190 (42), 162 (13), 120 (40), 101 (100), 84 (83), 56 (19). Anal. ($C_{15}H_{19}N_3O_3$) C, H, N.

N-Boc-3-(*p*-nitrophenyl)allylamine (12f). Sodium (207 mg, 9 mmol) was dissolved in 10 mL of absolute ethanol. Diethyl (*p*-nitrobenzyl)phosphonate (1.62 g, 6 mmol) was added. After *N*-Boc-glycinal (11) was added, the reaction mixture was stirred at room temperature for 3 h and then treated following the general procedure (method B). The crude compound was purified by sublimation to give 66.7 mg (4%) of product: mp 123–124 °C. ^{1}H NMR ($CDCl_3$) δ 1.45 (9 H, s), 3.93 (2 H, m), 4.71 (1 H, m), 6.37 (1 H, dt, J_{trans} = 16.0), 6.55 (1 H, d, J_{trans} = 16.0), 7.46 (2 H, d, *J* = 8.8), 8.15 (2 H, d, *J* = 8.8); EIMS 279 (MH^+, 10), 223 (M^+, 18), 179 (100), 161 (41), 116 (13). Anal. ($C_{14}H_{18}N_2O_4$) C, H, N.

1-(*p*-Nitrophenyl)-3-aminopropenyl Trifluoroacetate (13f). 1-(*p*-Nitrophenyl)-3-(*N*-Boc-amino)propene (**12f**) (27.8 mg, 0.1 mmol) was deprotected (method B) to give 18 mg (62%) of **13f**: mp 139–139.5 °C; ^{1}H NMR (acetone-d_6) δ 4.66 (2 H, m), 6.77 (1 H, dt, J_{trans} = 16.2), 6.96 (1 H, d, J_{trans} = 16.2); EIMS 179 (MH^+, 14), 178 (M^+, 65), 162 (12), 132 (65), 77 (37), 56 (100). Anal. ($C_{11}H_{11}F_3N_2O_4$) C, H, N.

N-(N'-Acetyl-L-phenylalanyl)-3-amino-1-(*p*-nitrophenyl)-1-propene (1f). *N*-Acetyl-L-phenylalanine (57 mg, 0.27 mmol) was coupled (method C) with 1-(*p*-nitrophenyl)-3-aminopropene trifluoroacetate (**13f**) (79 mg, 0.27 mmol). Recrystallization from acetone gave 80 mg (80%) of pure product **1f**: mp 217–217.5 °C. $[\alpha]^{25}_D$ +18.6° (*c* = 1.0, $CHCl_3$); ^{1}H NMR

(acetone-d_6) δ 1.89 (3 H, s), 3.0 (2 H, m), 3.16 (1 H, s), 4.02 (2 H, m), 4.67 (1 H, m), 6.51 (1 H, m), 6.60 (1 H, m), 7.28 (5 H, m), 7.50 (1 H, s), 7.65 (2 H, m), 8.22 (2 H, m); EIMS 367 (M^+, 6), 217 (8), 178 (61), 612 (35), 132 (5), 120 (100), 91 (50). Anal. ($C_{20}H_{21}N_3O_4$) C, H, N.

Methyl *N*-(*N'*-Acetyl-D-phenylalanyl)-γ-aminocrotonate (2a). *N*-Acetyl-D-phenylalanine (414 mg, 2 mmol) was coupled to methyl γ-aminocrotonate trifluoroacetate (458 mg, 2 mmol). Recrystallization of the crude product from EtOH/$CHCl_3$/hexane (1:5:80) gave 500 mg (79%) of pure compound **2a**: mp 150–151 °C. $[\alpha]^{25}_D$ −4.93° (c = 1, MeOH); ^{1}H NMR (DMSO-d_6) 2.0 (3 H, s), 3.10 (2 H, m), 3.9 (3 H, s), 4.1 (2 H, m), 4.7 (1 H, m), 6.07 (1 H, d, J_{trans} = 15.8), 6.19 (2 H, m), 7.02 (1 H, dt, J_{trans} = 15.8), 7.45 (5 H, m), 8.5 (m, 1 H). Anal. ($C_{16}H_{20}N_2O_4$) C, H, N.

Methyl *N*-(*N'*-Acetyl-L-leucyl)-γ-aminocrotonate (3a). *N*-Acetyl-L-leucine (173 mg, 1 mmol) was coupled to methyl γ-aminocrotonate trifluoroacetate (214 mg, 1 mmol). Recrystallization of the crude product from ethyl acetate/hexane solution gave 165 mg (61%) of pure compound **2a**: mp 93–94.5 °C; $[\alpha]^{25}_D$ −30.2° (c = 2, MeOH); ^{1}H NMR ($CDCl_3$) 0.95 (6 H, m), 1.62 (3 H, m), 2.02 (3 H, s), 3.73 (3 H, s), 4.03 (2 H, m), 4.5 (1 H, m), 5.88 (1 H, m, J_{trans} = 15.7), 6.15 (1 H, d, J_{trans} = 7.9), 6.89 (1 H, dt, J_{trans} = 15.7); EIMS 271 (MH^+, 1), 214 (2), 171 (1), 128 (42), 86 (100), 43 (65). Anal. ($C_{13}H_{22}N_2O_4$) C, H, N.

Methyl *N*-(*N'*-Acetyl-L-methionyl)-γ-aminocrotonate (4a). *N*-Acetyl-L-methionine (191 mg, 1 mmol) was coupled to methyl γ-aminocrotonate trifluoroacetate (214 mg, 1 mmol). Recrystallization of the crude product from ethyl acetate/hexane solution gave 145 mg (50%) of pure compound **4a**: mp 118–120 °C, $[\alpha]^{25}_D$ −20° (c = 1.7, MeOH); ^{1}H NMR ($CDCl_3$) 2.01 (3 H, s), 2.03 (2 H, m), 2.11 (3 H, s), 2.56 (2 H, m), 3.73 (3 H, s), 4.05 (2 H, m), 4.65 (1 H, m), 5.90 (1 H, d, J_{trans} = 15.8), 6.78 (1 H, d, J = 7.9), 6.89 (1 H, dt, J_{trans} = 15.8), 7.36 (1 H, m); EIMS 289 (MH^+, 1), 241 (2), 214 (41), 172 (12), 155 (15), 116 (35), 104 (36), 84 (36), 56 (100). Anal. ($C_{12}H_{20}N_2O_4S$) C, H, N.

Methyl *N*-Dihydrocinnamoyl-γ-aminocrotonate (5a). Hydrocinnamic acid (601 mg, 4 mmol) was coupled to methyl γ-aminocrotonate trifluoroacetate (916 mg, 4 mmol). Recrystallization of the crude product from ethyl acetate/hexane solution gave 420 mg (43%) of compound **5a**: mp 55 °C; ^{1}H NMR ($CDCl_3$) 2.52 (2 H, t, J = 7.62), 2.98 (2 H, t, J = 7.62), 3.73 (3 H, s), 4.05 (2 H, m), 5.57 (1 H, s), 5.78 (1 H, d, J_{trans} = 15.8), 6.85 (1 H, dt, J_{trans} = 15.8), 7.24 (5 H, m); EIMS 248 (MH^+, 23), 247 (M^+, 19), 131 (7), 114 (67), 104 (86), 91 (100). Anal. ($C_{14}H_{17}NO_30$ C, H, N.

Methyl *N*-Benzoyl-γ-aminocrotonate (6a). Methyl γ-aminocrotonate trifluoroacetate (916 mg, 4 mmol) and NMM (808 μL, 8 mmol) were dissolved in THF (800 μL) and cooled to 0 °C, and benzoyl chloride (514 μL, 4.4 mmol) was added. After the reaction solution was stirred at 0 °C for 2.5 h, the solvent was evaporated and the residue was dissolved in $CHCl_3$ (50 mL), washed with 2% HCl and saturated $NaHCO_3$ and dried over $MgSO_4$. After filtration and evaporation, the residue was recrystallized from ethyl acetate and hexane to give 670 mg (77%) of **6a**: mp 74–75.5 °C; ^{1}H NMR ($CDCl_3$) 7.61 (5 H, m), 6.97 (1 H, dt, J_{trans} = 17.6), 6.54 (1 H, s), 5.97 (1 H, d, J_{trans} = 17.6), 4.23 (2 H, m), 3.72 (3 H, s); EIMS 220 (MH^+, 100), 219 (M^+, 31), 114 (76), 105 (99), 77 (84). Anal. ($C_{12}H_{13}NO_3$) C, H, N.

Assay of Inhibition of Papain. Mercurial papain was obtained from Sigma (type IV) and purified by chromatography over mercurial sepharose as described by Sluyterman and Wijdenes.[61] This yielded an enzyme with a turnover number = 5.37 s^{-1} toward Cbz-Gly-ONp. Active papain solution was prepared by dissolving 6 mg of mercurial papain into 100 mL of buffer (50 mM PO_4, 1 mM EDTA, pH 6.2) and activating it by incubating with L-cysteine hydrochloride solution (0.1 M, 5 mL) at 25 °C for 45 min. The stock substrate solution was prepared by dissolving Cbz-Gly-ONp (Sigma, 9.9 mg, 0.03 mmol) in 100 mL of dry acetonitrile. The rate of substrate hydrolysis was followed at 340 nm ($\Delta\epsilon$ = 6800 M^{-1} cm^{-1}). The observed rates were corrected for spontaneous chemical hydrolysis of the substrate.

For irreversible inhibitors, time-dependent inhibition was evaluated as follows. Solutions of papain (0.11 μM) with varying concentrations of inhibitor (2–1000 μM) were incubated at 25 °C in buffer solution (pH = 6.2) containing 20% CH_3CN. At various time intervals (usually every 10 min), 0.3 mL of reaction solution was removed and diluted to 3 mL with buffer solution containing 20% CH_3CN and substrate (Cbz-Gly-ONp; 2 mM). The remaining enzyme activity was then measured immediately. These data were plotted by the method of Kitz and Wilson.[35]

Molecular Modeling. The molecular modeling studies were accomplished by using the program SYBYL. The structure of active site of papain was from the crystal structure[51] of the Cbz-L-Phe-L-Ala-CH_2-(ZPACK) derivative recorded in the Brookhaven Protein Data Bank file 6PAD. To create the active site, all residues except residues 18–26, 61–69, 131–142, 157–160, 175–178, and 205–207 were deleted and the remaining empty covalences were filled with hydrogens. The initial geometry of the peptide Michael acceptor in the active site was created by superimposing a compound such as **1c** or **1f** on the coordinates of the ZPACK moiety and then deleting the latter. The empty covalence of sulfur atom of Cys-25 was then also filled with hydrogen. An energy minimization was performed for the non-covalently bound inhibitor using the parameters in the Tripos Force Field in the absence of solvent, holding the structure of the active site fixed. The energy minimization processes stopped when the root mean square (rms) values of 0.378 kcal/(mol Å) for compound **1c** and 0.295 kcal/(mol Å) for compound **1f** were achieved. Measurement of distances and dihedral angles was done using the SYBYL program COMPARISON.

Acknowledgment. We thank Professor Michael Doughty for helpful discussions. Financial support was provided in part by grants from the American Heart Association, Kansas Affiliate, the University of Kansas General Research Fund, and the NIH (RR-5606).

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