Mechanistic Studies on the Inactivation of Papain by Epoxysuccinyl Inhibitors[1]

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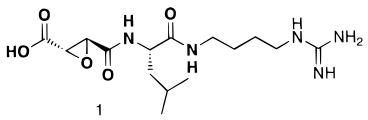
Received June 15, 1995[⊗]

Analogs of the epoxysuccinyl peptide cysteine proteinase inhibitor, EP-475 (**2a**), in which the free carboxylate has been replaced by hydroxamic acid, amide, methyl ketone, hydroxyl, and ethyl ester functionalities, have been synthesized. Individual rate constants of inhibition of papain were determined for these inhibitors. The results show that a carbonyl-containing functionality is necessary for good activity. The pH dependence of the inhibition of papain was determined for a nonionizable EP-475 (**2a**) analog; inhibition was found to depend on two acidic ionizations (pK_as of 3.93 and 4.09) of papain. Implications for the mechanism of action of epoxysuccinyl peptides with papain are discussed.

Introduction

Cysteine proteinases play important roles in human physiology and pathology as demonstrated by their involvement in protein processing and turnover,[2] apoptosis,[3] platelet aggregation,[4] tumor metastasis,[5] muscular dystrophy,[6] arthritis,[7] malaria,[8] and various viral infections.[9] Papain, the most studied cysteine proteinase, shares many features with physiologically important mammalian cysteine proteinases such as cathepsins B, H, L, and S, and the calpains. The X-ray structure for human cathepsin B[10] and earlier comparisons[11] of the crystal structure of papain to models of cathepsins B and H demonstrated that the mammalian enzymes show nearly identical folding patterns to papain, especially around the active site. In the active site of papain, Cys25 and His159 are thought to be catalytically active as a thiolate−imidazolium ion pair. The γ-N of Asn175 is hydrogen-bonded to the His159 imidazole and helps to position it for catalysis. An "oxyanion hole" composed of the Cys25 backbone NH and the Asn19 side chain NH_2 is positioned to hydrogen bond to the oxyanion of the tetrahedral intermediate during hydrolysis. These features are general for the papain superfamily which includes mammalian cathepsins and calpains.



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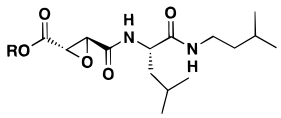
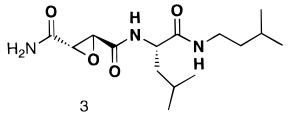


2a, R = H
2b, R = Et



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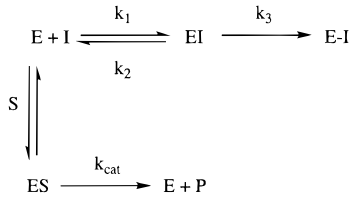
Epoxysuccinyl inhibitors such as E-64[12] (**1**) and EP-475[13] (**2a**) are specific irreversible inhibitors of cysteine proteinases and act by alkylating the catalytic cysteine thiol.[14,15] They have been used as probes for cysteine proteinase activity and may also have therapeutic potential. Although a number of enzyme−inhibitor X-ray structures have been determined,[16,17] there has been remarkably little study of the mechanism of action of epoxysuccinyl inhibitors. A number of researchers have looked at structure−activity relationships for various epoxysuccinyl peptides versus papain, calpain, and cathepsins B, H, and L by measuring their apparent second-order inhibition constants (k_i').[14,18,19,20] However, previously published studies have not examined the individual rate constants of inhibition for epoxysuccinyl inhibitors nor the pH dependence of epoxysuccinyl inhibitors. Such information, presented in this paper, complements the structural information presently available, and offers additional insight into the specificity and activity of epoxysuccinyl inhibitors of cysteine proteinases. After completion of this work, Bihovsky and co-workers reported apparent second-order rate constants of inactivation with papain and cathepsin B for several of the compounds presented here.[21]

Scheme 1



Scheme 1 shows the minimal scheme for irreversible inhibition in the presence of substrate. (The substrate reaction is represented in simplified form.) Previously we reported the first measurement of k_3 and K_i for any epoxysuccinyl inhibitor, EP-475 (**2a**).[22] In order to more closely examine the role of the epoxysuccinyl carboxylate, several analogs of **2a** in which the carboxylate was replaced by other functionalities were synthesized, and their individual rate constants of inhibition of papain were measured.

It has been postulated that during inactivation of papain by epoxysuccinyl inhibitors the oxirane would be protonated by His159.[23] This would be somewhat analogous to protonation by His159 of the oxygen of the thiohemiacetal formed by reaction of papain and peptide aldehydes.[24] However, based on the crystal structure of papain alkylated by E-64 (**1**), Varughese *et al.* argued

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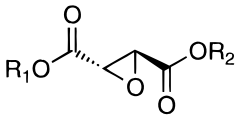
⊗ Abstract published in *Advance ACS Abstracts,* July 15, 1996.

S0022-2623(95)00445-6 CCC: $12.00

that the epoxide was more likely protonated by water because of the distance of the resulting hydroxyl from His159.[16] In order to resolve this question and demonstrate that pH−activity profiles could be a useful tool in the investigation of specific irreversible inhibitors of cysteine proteinases, we synthesized **3**, the primary amide derivative of **2a**, and measured the pH dependence of inhibition of papain.

Results

Synthesis. The preparation of EP-475 (**2a**) and its carboxylate-replacement analogs commenced with the synthesis of the diethyl ester of (*S,S*)-epoxysuccinic acid **4** by a modification of the method of Mori and Iwasawa,[25] in which a syringe pump was used to give slow addition of sodium ethoxide, which suppressed byproduct formation. In addition, use of DBU in place of sodium ethoxide gave more reproducible yields. The diester **4** was saponified with 1 equiv of KOH in ethanol to give the desired half-acid **5** in 89−93% yield. Approximately 5−10% of the starting diester could be recovered and recycled to give a higher conversion efficiency. The epoxysuccinyl diacid **6** was produced from **5** by further saponification with NaOH.

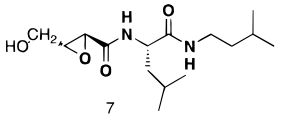


4, R_1=R_2= Et
5, R_1=Et; R_2= H
6, R_1=R_2= H

Coupling of ethyl epoxysuccinate to a peptide is the most practical convergent approach to obtain the desired inhibitor. (*S,S*)-Ethyl epoxysuccinate **5** was coupled to L-leucyl-3-methylbutanamide (Leu-Mba) by the mixed anhydride method to give **2b** in 75−77% yield after recrystallization. This procedure is superior to previous methods using diethylphosphoryl cyanide[12b] as the coupling agent, the active ester approach (*p*-nitrophenol),[26] and DCC/HOBt coupling of similar compounds.[18,20] It was our experience that HOBt[27] and phosphorus-based reagents such as BOP decomposed the epoxysuccinate moiety during long exposures (*e.g.*, overnight). Recently Korn *et al.*[28] reported the use of *N*-hydroxysuccinimide and pentafluorophenol esters of ethyl epoxysuccinate in the synthesis of D-epoxysuccinate dipeptides. EP-475 (**2a**) was easily synthesized in up to 84% yield by saponification of ethyl ester **2b**. The diastereomeric and enantiomeric purity of the epoxy half-acid was confirmed to be ≥98% by NMR studies of its methyl benzylamide derivative.

Altered carboxyl derivatives of **2a** were prepared efficiently by modifying the ester functionality in **2b**. Reaction of **2b** with ammonia in methanol at 0 °C produced carboxamide **3** in 77−82% yield. Treatment of **2b** in ethanol at 0 °C with sodium borohydride gave the desired alcohol **7** in 68% yield. In contrast, borane reduction of **2a** resulted in an intractable mixture of products.

Synthesis of the hydroxamic acid derivative **8** was achieved in 73−75% yield by coupling *O*-benzylhydroxylamine with **2a** by use of EDCI/HOBt. Ester **8** was hydrogenolyzed in 63% yield to give the hydroxamic acid analog **9**.



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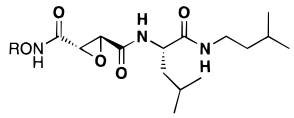
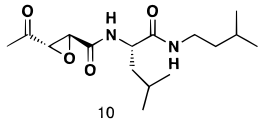


8, R = $C_6H_5CH_2$
9, R = H



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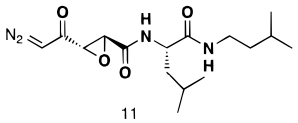

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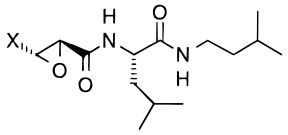
Synthesis of the methyl ketone **10** was problematic because the epoxide moiety proved incompatible with traditional methods of methyl ketone formation utilizing organometallic reagents.[29] A variety of routes were tried, but ultimately the method of Pojer *et al.* proved successful.[30] The diazomethyl ketone **11** of **2a** was prepared (71% via mixed anhydride) and exposed to 3 equiv of HI at 5 °C for 30 min to give a 58% yield of compound **10** after flash chromatography. Temperatures colder than 5 °C increased the amount of iodomethyl ketone left unreduced. This method has also been used by Scholtz and Bartlett in the synthesis of a δ-methyl ketone glutamate derivative.[31]

Inhibition Kinetics. The apparent binding constant (K_i), first-order rate of inactivation (k_3), and the apparent second-order rate of inactivation (k_i') were determined for inhibition of papain by the inhibitors shown in Table 1 by a method previously described.[22] In this method, inhibition is measured in the presence of substrate, and no assumptions are made concerning the rate of k_3. Therefore, based on the model depicted in Scheme 1, $K_i = (k_2 + k_3)/k_1$ and $k_i' = k_3/K_i$. The potency of each inhibitor was judged by the magnitude of k_i' because it directly reflects the observed rate of inhibition. The value of k_i' for **2a** was the highest of the inhibitors and similar to previously published values obtained under slightly differing conditions.[14] The weakly acidic hydroxamic acid analog proved to be nearly as good, and HPLC experiments (not shown) confirmed that no hydrolysis to the carboxylate took place under the assay conditions. The carboxamide analog and ketone analogs were 2 orders of magnitude slower than **2a** while the hydroxy analog was 5 orders of magnitude slower. The data for the ethyl ester were somewhat scattered and less reproducible than the other analogs. Ester hydrolysis was suspected, and HPLC experiments were carried out on the ethyl and methyl esters of **2a**. From these experiments, the half-lives are estimated to be approximately 6 h for the methyl ester and 8.4 h for the ethyl ester.

pH Dependence of Inhibition. The activity of compound **3** was determined over the pH range 3.3−

Table 1. Kinetic Constants Determined for the Inhibition of Papain by Carboxylate-Replaced Analogs of EP-475[a]



compd	X	k_i' (M^{-1} s^{-1})	k_3 (s^{-1})	K_i ($\times 10^{-6}$ M)
2a[b]	−COOH	$(2.9 \pm 0.15) \times 10^5$	0.71 ± 0.25	2.4 ± 0.9
9	−CONHOH	$(1.0 \pm 0.06) \times 10^5$	0.67 ± 0.08	6.6 ± 1.2
3	$-CONH_2$	$(6.8 \pm 0.45) \times 10^3$	0.35 ± 0.04	51 ± 6.7
10	$-COCH_3$	$(2.3 \pm 0.08) \times 10^3$	0.47 ± 0.13	170 ± 53
2b	$-CO_2CH_2CH_3$	$(7.5 \pm 1.2) \times 10^2$	0.059 ± 0.017	52 ± 29
7	$-CH_2OH$	4.1 ± 0.22	0.019 ± 0.0017	4400 ± 630

[a] Assays conducted in 0.1 M phosphate, pH 6.8, 2 mM DTT, and 1 mM EDTA, 30 °C. [b] Data taken from reference 22.

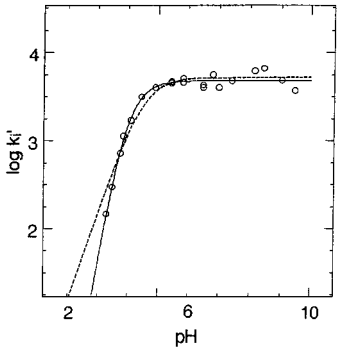
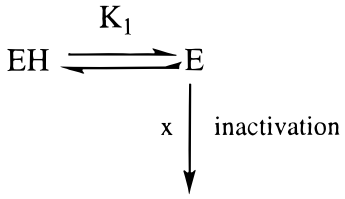


Figure 1. pH dependence of the apparent second-order rate of inhibition (k_i') of papain by inhibitor **3**. The dashed line is the fit to eq 1, describing a single enzyme ionization, and the solid line is the fit to eq 2, describing two enzyme ionizations. Assays were performed at 30 °C at constant ionic strength (0.3 M) in the presence of DTT (2 mM) and EDTA (1 mM); papain was preincubated for 5 min in assay buffer prior to addition to inhibitor and substrate.

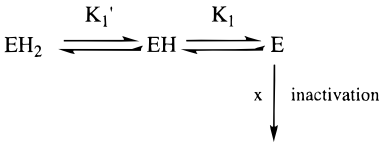
9.5 at constant ionic strength (0.3 M). Five or more inhibitor concentrations were tested at each pH value in order to examine the pH dependence of k_3 and K_i as well as k_i'. The pH dependence of the substrate (Z-Phe-Arg-AMC) was measured as a control. The data were fit as before to recover the kinetic constants. However, there was not always enough structure in the data to determine values for k_3 and K_i, especially at the extremes of the pH range. The kinetic constants were then fit to equations as described in the Experimental Section. Each scheme assumes that only a single protonation state of papain is active. To determine the enzyme pK_as involved in inactivation, the inhibitor constants were fit to equations for one or two ionizations (eqs 1 and 2), corresponding to Schemes 2 and 3. The pH dependence of substrate hydrolysis by papain was analyzed by fitting the substrate kinetic constants to eqs 3 or 4, corresponding to Schemes 4 or 5, respectively. Scheme 4 predicts a bell-shaped curve resulting from two ionizations; the monoprotonation state of the enzyme is the active one. Scheme 5 is similar except that two deprotonations must take place rather than one to activate the enzyme. The results for the substrate are in accord with the literature;[32] the data fit more accurately to eq 4 (three pK_as) than to eq 3 (two pK_as). The pK_a values obtained were 3.78 ± 0.33, 4.51 ± 0.21, and 8.00 ± 0.11.

A plot of log(k_i') versus pH (Figure 1) shows that the inactivation of papain by epoxide **3** depends only on groups of acid pK_a and not alkaline pK_a. Therefore,

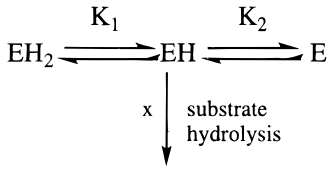
Scheme 2



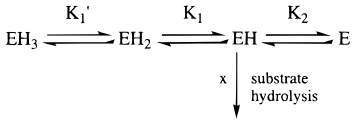
Scheme 3



Scheme 4



Scheme 5



His159 is not necessary for protonation of the epoxide. The inhibitor data could be fit by eq 1 to yield a pK_a of 4.34 ± 0.14 or by eq 2 to give pK_as of 3.93 ± 0.22 and 4.09 ± 0.15. By examining the log plots of the two possibilities in Figure 1, it is seen by inspection that the data are more accurately fit by eq 2 to yield a slope of 2 in the acid limb. This result is therefore consistent[33] with the existence of at least two acid groups on papain which are necessary for the activity of the inhibitor.

Discussion

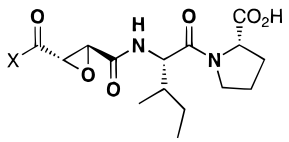
Effect of Inhibitor Structure on Inhibitor Binding and Rate of Enzyme Inactivation. Comparison of the individual kinetic constants for various epoxysuccinate derivatives (Table 1) shows that first-order rate constant of inactivation (k_3) is relatively constant for inhibitors containing a free carboxylate or at least a carbonyl-containing group. Therefore, the intrinsic reactivity of the epoxysuccinyl moiety with the papain catalytic thiolate is in large part responsible for the extremely high reactivity of the better epoxysuccinyl inhibitors. Since alkylation occurs at carbon-3 (the carbon α- to the carboxylate group),[15] the changes in inhibitor structures reported here are adjacent to the normal site of alkylation. In the case of the carbonyl-containing inhibitors (**2a**, **9**, **3**, **10**), k_3 is comparable to that of EP-475 (**2a**), whereas K_i is slightly higher. Inhibitors **2b** and **7** inactivate papain at much slower rates, but **2b** represents a special case that will be discussed in a later section. However, the dramatic drop in the rate of alkylation for alcohol **7** is consistent with the need for a carbonyl group adjacent to the reactive epoxide moiety.

The changes in inhibitor structure produce more substantial effects on the apparent prealkylation dis-

sociation constant (K_i) for the inhibitors; K_i varies by up to 3 orders of magnitude depending on the structure. Although K_i may not be an equilibrium binding constant for fast epoxysuccinyl inhibitors (*vide infra*), if Scheme 1 is correct then K_i should closely reflect changes in K_d (the dissociation constant). The differences in rates of inactivation are primarily due to the changes in K_i brought on by structural changes. Here again, the carbonyl-containing analogs (**9**, **3**, **10**) bind much better to the enzyme than the alcohol analog **7**.

The hydroxamic acid **9** is the most potent of the analogs, displaying approximately one-third of the activity of the parent compound **2a**, which results from a 3-fold increase in K_i. Hydroxamic acid **9** is also the only acidic analog of **2a**, with a pK_a expected to be within the range of a typical aliphatic hydroxamic acid pK_a of 8.5−9.5.[34] At pH 6.8, the hydroxamic acid **9** should be highly protonated whereas **2a** itself is ionized. The fact that the protonated form of hydroxamic acid **9** and the neutral amide **3** and ketone **10** all bind well to the enzyme suggests that complexes with neutral inhibitors are stabilized appreciably by interactions with the inhibitor carbonyl oxygen.[35] Since the primary amide nitrogen of **3** is a hydrogen bond donor rather than an acceptor (as is the carboxylate oxygen of **2a**), the higher K_i may result from the absence of a second hydrogen bond which the carboxylate of **2a** can form with the enzyme. The methyl ketone **10** is similar to the amide, in that it can hydrogen bond only to the carbonyl oxygen. Its K_i is even higher, reflecting the nonpolar nature of the methyl group and perhaps the methyl group's slightly larger bulk as well. Taking into account the X-ray structure of the alkylated enzyme,[16,17] our data suggest that the interaction between the acidic moiety and the catalytic histidine may be closer to a hydrogen-bonding interaction than an electrostatic interaction.

CA074 [*N*-[[L-*trans*-3-(*n*-propylcarbamoyl)oxiran-2-yl]-carbonyl]-L-isoleucylproline, **12**] is a specific inhibitor of cathepsin B lacking the epoxysuccinyl carboxylate.[18] Before the X-ray structure of cathepsin B was available, the dipeptidyl portion of these inhibitors was assumed to bind in the S subsites in analogy to **1** and **2a** with papain, and the epoxysuccinyl carboxyl ester and amide extended into the S′ sites. Gour-Salin *et al.*[19] argued persuasively based on the cathepsin B X-ray structure that CA074 **12** and related inhibitors may in fact bind in the S′ subsites of cathepsin B. Very recently, Turk *et al.*[36] have confirmed that CA030 [*N*-[[L-*trans*-3-(ethoxycarbonyl)oxiran-2-yl]carbonyl]-L-isoleucylproline, **13**] does indeed bind on the S′ side of the cathepsin B active site. These results show that an electrostatic interaction of the epoxysuccinyl moiety is not an absolute requirement if another part of the molecule can stabilize the enzyme−inhibitor interaction. Based on our results, we would expect k_3 for inhibitors like **12** to be similar to those reported in this work.



12, CA074: X=$CH_3(CH_2)_2HN$
13, CA030: X=CH_3CH_2O

The free carboxylates of epoxysuccinyl inhibitors play a key role in the *selectivity* of these compounds for cysteine proteases over other biological sulfur nucleophiles. In the active site of the cysteine protease, hydrogen bonding to the carboxylate may activate the epoxide toward nucleophilic attack by Cys25 by neutralizing the negative charge of the carboxylate. This is consistent with the fact that the first-order rate of alkylation (k_3) is comparable for **2a** (0.71 s^{-1}), its amide derivative (0.35 s^{-1}), and its methyl ketone derivative (0.47 s^{-1}). These values of k_3 are in contrast to the pseudo-first-order rates of reaction observed for methanethiolate with α,β-epoxy carbonyl compounds[37] at alkaline pH; the rates of attack by thiolate on the α-carbon decreased by 3 orders of magnitude in the order ketone > amide > acid. The interaction of His159 with the carboxylate of an inhibitor has been previously invoked to explain the unexpectedly high rate of alkylation of papain at neutral pH by chloroacetate relative to chloroacetamide,[38] and by *N*-(*N*′-acetyl-L-Phe)-γ-aminocrotonic acid relative to the crotonamide and the crotonic nitrile derivatives.[39] In the former case, a pH−activity profile of chloroacetate with papain was bell-shaped like that of a substrate, clearly indicating the importance of a group with an alkaline pK_a, most likely His159. A pH−activity profile for **2a** with papain would be expected to show an alkaline pK_a of about 8 if this hypothesis is correct. In solution, outside the enzyme active site, the negative charge of the carboxylate helps prevent reaction with low molecular weight thiols, in a fashion analogous to the slower rate of alkylation of chloroacetate versus chloroacetamide by simple thiols.

Instability of Esters of EP-475 (2a). The relatively high error of the kinetic constants for ethyl ester **2b** and increasing potency with increasing incubation time in the buffer suggested that **2b** was spontaneously hydrolyzing to the acid. HPLC experiments confirmed that this occurred (*vide infra*), so that the kinetic constants calculated for **2b** must be regarded as upper limits at best. Remarkably, hydrolysis of ester **2b**, despite the seemingly long half-life (8.4 h), is sufficiently fast to produce 0.1% of the acid by the start of the assay (inhibitor was added 1 min prior to enzyme), and up to 0.3% over the course of a typical 2 min assay. Thus, the concentrations of the acid **2a** would be 35−375 nM and could account for most or all of the observed inhibition. Hence, even the low literature value for k_i' (4.6 M^{-1} s^{-1})[26] measured for ethyl ester **2b** under similar conditions to this study may merely reflect inhibition by a tiny quantity of **2a**. Also, it now seems likely that the early studies[40] of ester and diester derivatives of epoxysuccinate were measuring the inhibition of papain caused by hydrolysis products.

Effect of pH on Epoxide Inhibition Rates. The pH dependencies of the catalytic Cys25 and His159 have been examined by substrate reaction,[41,42] NMR titration,[43] fluorescence titration,[44] reaction with small nonspecific inhibitors,[38,45] and other methods.[46] These studies reveal a bell-shaped pH−activity curve for papain with substrate. The bell shape results from three pK_as: one at pH 8−8.5, assigned to His159, and two between pH ∼3.6−4.5, one of which is assigned to Cys25. The third pK_a has not been assigned and may be due to more than a single group.[47,48]

The pH dependence of inhibition of papain varies with inhibitor structure. Generally, studies have found inhibition to depend on one (e.g., chloroacetamide[38a]) or two (*e.g.*, 2,2′-bipyridyl disulfide,[45a] *N*-acetylphenylalanylglycinal[24]) enzymatic pK_as. However, in most of this early work, the data are insufficient to distinguish two acid pK_as. The pH dependence of relatively specific irreversible inhibitors of cysteine proteinases such as peptidyl fluoromethyl ketones and arylacyloxymethyl ketones has not been determined. Shaw and colleagues reported that the activity of the diazomethyl ketone of Z-Phe with papain reached a plateau at pH 6.5 and rapidly fell off at alkaline pH.[49] However, the study was not systematic, and values for the pK_as were not reported. The pH dependence of epoxysuccinyl-based inhibitors has not been previously determined.

Compound **3** was chosen for pH studies for several reasons. It has no ionizable groups which would alter the pH−activity profile and make interpretation of the results more difficult. In addition, its moderate k_i' (6800 M^{-1} s^{-1}) makes it likely that the K_i closely approximates the dissociation constant if the mechanism of eq 1 holds. More specifically, $K_i = (k_2 + k_3)/k_1$, where k_1 is approximately 10^6−10^8 M^{-1} s^{-1}, the value for association of enzymes with small molecules. If k_3 makes a significant contribution (e.g., $k_2 \approx k_3$), then k_1 would be significantly less than this value.[50] In the case of compound **3**, k_1 would need to be on the order of 10^4 M^{-1} s^{-1} in order for k_3 to significantly affect K_i. Under the conditions of the assay, there is no reason to expect the value of k_1 to be so small, and it is reasonable to assume that $K_i \approx k_2/k_1$. E-64, **1**, on the other hand, has a k_i' approaching 10^6 M^{-1} s^{-1}, and this approximation may no longer be a safe one.

Within experimental error, the pK_a values for papain with the inhibitor match those of papain with the substrate in the acidic region and are likely to be of the same groups in both cases. By prior work, one of these pK_as must be for the Cys25 thiol.[42−44,51] Papain does not exhibit an alkaline pH dependence for inhibition with inhibitor **3**. Therefore, the alkylation of papain by epoxysuccinyl-based inhibitors is modulated by at least two groups on the enzyme, neither of which is His159.

Because sufficient data could not be collected at the pH extremes, quantitative analysis did not define the pK_as of K_i and k_3. Nevertheless, qualitative pH profiles of k_3 and K_i (Figure 2) show that k_3 is dependent on groups with acid pK_as, whereas K_i is pH independent. This confirms that if the mechanism of Scheme 1 holds, K_i is a good approximation of the dissociation constant, k_2/k_1. If it were not, then one would expect to see an acid pH dependence because of the contribution from k_3.

The question remains as to how the epoxide is protonated during inactivation. Water, specific acid catalysis, and buffer catalysis have all been reported to affect the hydrolysis of simple epoxides such as 1,3-cyclohexadiene oxide.[52] The plot shown in Figure 1 is essentially flat from pH 5.5 to 9.5, but cannot be extended much higher because papain is irreversibly denatured above pH 10. This result rules out specific acid catalysis because protonation does not appear to be rate-limiting at pHs up to 9.5. Since increasing phosphate concentration up to 0.3 M at pH 6.8 and constant ionic strength (1 M) also did not alter the rate of inactivation, catalysis by buffer can also be ruled out. Hence, it seems likely that water is indeed the predominant source of protons in the protonation of the epoxide.

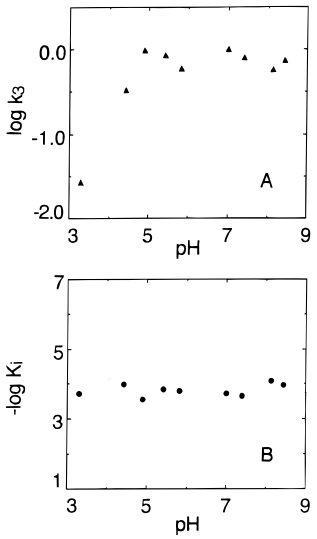


Figure 2. Panel A: pH dependence of the first-order rate of inactivation (k_3) of papain by inhibitor **3**. Panel B: pH dependence of the binding constant (K_i) of inhibitor **3** to papain. Assay conditions as in Figure 1.

There exists the possibility that papain may hydrogen bond to the epoxide oxygen prior to ring opening. Preliminary molecular modeling studies of **2a** binding to papain prior to inactivation place the epoxide oxygen within 2.8 Å of the backbone carbonyl of Asp158.[53] If protonation of the oxirane by water or buffer were concurrent with nucleophilic attack of the sulfur, then Asp158 O_1 is in a position to hydrogen bond to the nascent hydroxyl. Although no hydrogen bond between Asp158 O_1 and the inhibitor hydroxyl was observed in the **2a**/papain complex initially reported,[17a−c] such a bond was observed in the second form of the complex reported by the same group.[17d]

The above results with carboxylate-replaced analogs showed that the increased potency of **2a** compared to compound **3** results from the additional hydrogen bonding or electrostatic interaction in which the carboxylate of **2a** engages. Therefore, if His159 is required to be protonated for electrostatic interaction with **2a**, then one would expect a loss of inhibition by **2a** at alkaline pH, dependent on a pK_a of approximately 8. The results for the pH dependence of papain with compound **3** have laid the groundwork for such an experiment with **2a**; any observed alkaline pH dependence may now be unambiguously interpreted in terms of binding rather than protonation of the epoxide.

Conclusions

In summary, we have synthesized carboxylate-replaced derivatives of **2a** and measured their individual rate constants of inhibition as well as their apparent second-order rate constants of inhibition. The results suggest that the carboxylate of **2a** positions and activates the epoxide for alkylation of Cys25 by a hydrogen-bonding interaction with His159 or the oxyanion hole. We have also shown by means of a pH−activity profile of the primary amide derivative of **2a** that His159 is not involved in protonation of the epoxide during inactivation. These results are likely to be general for

all epoxysuccinyl inhibitors with a free carboxylate α to the epoxide.

Experimental Section

General Methods. ^{1}H and ^{13}C NMR spectra were obtained on a Bruker AM300 spectrometer at 300 and 75 MHz, respectively. ^{1}H chemical shifts are reported in parts per million (ppm) relative to tetramethylsilane (TMS) as an internal standard, and ^{13}C chemical shifts are reported relative to the central solvent peak relative to TMS. ^{13}C NMR spectra were taken in the same solvents as indicated for the ^{1}H spectra. Coupling constants (*J*) for ^{1}H NMR are reported in hertz. Infrared spectra were recorded on a Perkin-Elmer 599B spectrometer with NaCl solution cells or KBr disks. Optical rotations were recorded on a Perkin-Elmer 241 polarimeter at the sodium D line. Melting points were obtained on a Fisher−Johns apparatus and are uncorrected. Mass spectrometry samples were analyzed by the University of Wisconsin School of Pharmacy (Finnegan) or Chemistry Department (Kratos) mass spectrometry facilities. Elemental analyses were performed by Galbraith Laboratories (Knoxville, TN).

Tetrahydrofuran was freshly distilled from sodium/benzophenone under inert atmosphere. Ethyl ether (Mallinckrodt) was either used from a freshly opened can without further purification or distilled from sodium/benzophenone. Dioxane and dimethylformamide were Aldrich anhydrous grade. Dichloromethane was distilled over CaH_2 for anhydrous reactions. Triethylamine and *N*-methylmorpholine were stored over KOH and used without further purification. All other reagents and solvents were reagent grade and used without further purification. Ethereal diazomethane (∼0.3 M) was prepared from Diazald (Aldrich) according to the manufacturer's directions. Brine refers to a saturated aqueous solution of NaCl.

Flash chromatography was conducted on Merck silica gel 60 (230−400 mesh). Thin-layer chromatography was performed using Merck 60 F-254 silica gel plates; visualization was either by UV illumination, 7% phosphomolybdic acid/EtOH dip and heat, or by ninhydrin spray and heat. TLC solvent systems were (A) EtOAc/hexanes 1:1, (B) EtOAc/hexanes 3:7, or (C) CH_2Cl_2/MeOH 9:1.

HPLC was carried out using Waters Model 510 pumps, an Automatic Gradient Controller, a Model 441 detector (214 nm), and a BBC Servogor 120 chart recorder. Analytical work utilized a Water μ-Bondapak C-18 reverse phase column (30 × 0.39 cm) or a Vydac C-18 reverse phase column (25 × 0.39 cm) at a flow rate of 1.1 mL/min. Mobile phase: A = 95:5 H_2O/acetonitrile, 0.045% TFA; B = acetonitrile, 0.036% TFA. All solvents were HPLC grade and filtered and degassed before use.

General Procedure: Coupling via Mixed Anhydride. One equivalent of carboxylic acid in ethyl acetate (0.1−0.5 M) or another appropriate solvent was cooled under an inert gas in a −20 to −25 °C bath. To this solution was added 1 equiv of *N*-methylmorpholine followed by dropwise addition of 1 equiv of IBCF. The solution was stirred for 10−20 min, during which time a white precipitate formed. One equivalent of the amino component was added, either neat or as a solution in EtOAc or DMF. If necessary, an additional equivalent of NMM was added to neutralize the amine salt. The reaction was stirred 1−2 h in the cold and 1−18 h at room temperature. The reaction mixture was diluted with ethyl acetate and washed successively with equal volumes of water, 5% citrate, saturated sodium bicarbonate, and brine. The organic layer was dried over $MgSO_4$, filtered, and concentrated. The product was purified by recrystallization from a suitable solvent system or by flash chromatography.

***N*-(*tert*-Butyloxycarbonyl)-L-leucyl-3-methylbutanamide (Boc-L-Leu-Mba).** Boc-L-Leu hydrate (2.50 g, 10.0 mmol) was azeotropically dried by dissolution in dry toluene followed by evaporation of the solvent under reduced pressure. This process was repeated 3 times and was followed by storage of the compound over P_2O_5 *in vacuo*, overnight. The residue was taken up in 50 mL of EtOAc and treated with NMM (1.10 mL, 10.0 mmol), isobutyl chloroformate (1.30 mL, 10.0 mmol), and isoamylamine (1.16 mL, 10.0 mmol) according to the general procedure. Workup and removal of solvent yielded 2.61 g (87%) of an analytically pure white powder: mp 90.5−92 °C (lit.[54] 89−90 °C); TLC(A) R_f 0.54; $[\alpha]_D$ −24.8° (*c* 1.01, MeOH) [lit.[50] $[\alpha]_D$ −25° (*c* 0.80, MeOH)]; IR ($CHCl_3$) 3460, 3360, 2975, 1710, 1680, 1505 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 0.89−0.96 (m, 12 H), 1.34−1.73 [m, 16 H including 1.49 (s, 9 H)], 3.26 (m, 2 H), 4.05 (m, 1 H), 4.89 (m, 1 H), 6.05 (m, 1 H); ^{13}C NMR δ 21.93, 22.42, 23.03, 24.76, 25.74, 28.30, 37.73, 38.37, 41.31, 53.09, 80.05,155.36, 172.41. Anal. ($C_{16}H_{32}N_2O_3$) C, H, N.

Diethyl (2*S*,3*S*)-Oxirane-2,3-dicarboxylate. D-Diethyl tartrate was converted to diethyl (2*R*,3*S*)-2-bromo-3-hydroxybutanedioate by the method of Mori.[25] The bromohydrin (1.08 g, 4.01 mmol) was dissolved in 4 mL of absolute EtOH under Ar and cooled to −5 °C. To this was added 1.65 mL of 21% NaOEt (2.68 M) in EtOH (4.4 mmol) dropwise over 30 min. (Faster addition caused lower yields.) The reaction was stirred 50 min, during which time the temperature of the bath rose to 6 °C. The milky white reaction mixture was then quenched with 23 μL of AcOH (0.4 mmol), and the solvent was removed at low temperature (<45 °C). The residue was diluted with 25 mL of ice−water and extracted with 2 × 25 mL of ether. The combined organics were washed with 30 mL of water and 30 mL of brine, dried over $MgSO_4$, and concentrated to a yellow oil. Purification by flash chromatography (30% EtOAc/hexane) followed by Kugelrohr distillation (42 °C, 0.03 mmHg) gave 595 mg of colorless oil (79%): TLC(B) R_f 0.27; $[\alpha]_D$ +118.0° (*c* 1.483, ether) [lit.[24] +108.49° (*c* 1.413, ether)]; IR ($CHCl_3$) 3000, 1755, 1380, 1335, 1195, 1030, 900 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.33 (t, 6 H), 3.68 (s, 2 H), 4.19−4.36 (m, 4 H); ^{13}C NMR δ 14.04, 52.04, 62.26, 166.80; MS (M^+ + 1) 189, 161 (−C_2H_4, +H^+), 143 (−OEt), 127 (−COOEt). Anal. ($C_8H_{12}O_5$) C, H.

Ethyl (2*S*,3*S*)-Oxirane-2,3-dicarboxylate (5). To diethyl ester **4**[25] (1.69 g, 8.98 mmol) in 12 mL of EtOH cooled to 4 °C (lower temperatures decreased the yield) was added 8.3 mL of 1.08 N KOH in EtOH over 3 h via syringe pump. During this time, a white precipitate formed. The mixture was then stored overnight in a freezer. After warming to room temperature, the volatiles were removed under reduced pressure, and the residue was taken up in 35 mL of water, washed with 2 × 25 mL of EtOAc, and cooled in an ice bath. The pH was adjusted to 2 with 3 N HCl, and the solution was extracted with 6 × 25 mL of EtOAc. The combined organics were dried over $MgSO_4$ and concentrated to 1.24 g of an oil (89%) which solidified upon standing. This material was used without further purification. Recovered starting material (0.17 g) was recycled to achieve 96% conversion: TLC(C) R_f 0.51; ^{1}H NMR (DMSO-d_6) δ 1.22 (t, 3 H, *J* = 7.1), 3.60 (d, 1 H, *J* = 1.9), 3.69 (d, 1 H, *J* = 1.9), 4.17 (q, 2 H, *J* = 7.1); ^{13}C NMR δ 13.87, 51.28, 51.57, 61.61, 166.75, 167.97.

***N*-[[L-*trans*-3-(Ethoxycarbonyl)oxiran-2-yl]carbonyl]-L-leucyl-3-methylbutanamide (2b).** Boc-L-Leu-3-methylbutanamide (1.00 g, 3.33 mmol) was deprotected by stirring in 4 N HCl/dioxane at room temperature for 45 min. The solvent was then evaporated and the residue repeatedly (3×) taken up in dichloromethane and concentrated on a rotary evaporator. The HCl salt was then stored *in vacuo* over KOH and P_2O_5 for 4 h. The yield was quantitative and the salt used without further purification. Epoxy acid **5** (533 mg, 3.33 mmol) in 12 mL of EtOAc was treated with 370 μL of NMM (3.33 mmol), 430 μL of isobutyl chloroformate, IBCF (3.33 mmol), HCl·Leu-Mba, and 370 μL of NMM following the general procedure. Two recrystallizations from EtOAc/hexane (1:3) gave 880 mg of a white powder (77% yield): mp 123−124 °C [lit.[21] 126.2 °C]; TLC(A) R_f 0.31; $[\alpha]_D$ +51.3° (*c* 0.849, EtOH) [lit.[25] $[\alpha]_D$ +51.7° (*c* 1.00, EtOH)]; IR (KBr) 3300, 3100, 2960, 1760, 1645, 1570−1540, 905 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 0.90−0.95 (m, 12 H), 1.32 (t, 3 H, *J* = 7.1), 1.39 (q, 2 H, *J* = 7.3), 1.47−1.72 (m, 4 H), 3.24 (m, 2 H), 3.46 (d, 1 H, *J* = 1.8), 3.68 (d, 1 H, *J* = 1.8), 4.22−4.40 (m, 3 H), 5.92 (m, 1 H), 6.56 (d, 1 H, *J* = 8.6); ^{13}C NMR δ 14.05, 22.16, 22.38, 22.42, 22.81, 24.83, 25.85, 38.01, 38.27, 41.18, 51.31, 52.96, 53.78, 62.28, 166.00, 166.43, 170.69. Anal. ($C_{17}H_{30}N_2O_5$) C, H, N.

***N*-[(L-*trans*-3-Carboxyoxiran-2-yl)carbonyl]-L-leucyl-3-methylbutanamide (2a, EP-475).** Ester **2b** (300 mg, 0.876 mmol) in 5 mL of EtOH, cooled by an ice bath, was treated with 0.96 mL of 1 N NaOH. The reaction was stirred 1 h in

the cold and then at ambient temperature until TLC indicated complete consumption of starting material. The reaction was quenched with 85 μL of AcOH. The solvent was removed under reduced pressure and the residue taken up in water. The solution was cooled in an ice bath and the pH adjusted to 3 with 3 N HCl. The solution was extracted 5 times with equal volumes of EtOAc, and the combined organics were washed with brine, dried over $MgSO_4$, filtered, and concentrated. Trituration of the product with ether gave 184 mg of a white powder. Concentration of the supernatant and trituration of the resulting residue yielded an additional 48 mg of analytically pure product (84% total): mp 153−154 °C (lit.[14] 157−160 °C); TLC(C) R_f 0.54; $[\alpha]_D$ +43.1° (*c* 0.2248, EtOH) [lit.[14] $[\alpha]_D$ +45.5° (*c* 1.00, EtOH)]; IR (KBr) 3280, 3110, 2960, 1720, 1690, 1630, 1570 cm^{-1}; ^1H NMR (DMSO-d_6) δ 0.80−0.96 (m, 12 H), 1.27 (q, 2 H, J = 7.0), 1.43 −1.63 (m, 4 H), 3.05 (m, 2 H), 3.45 (d, 1 H, J = 1.7), 3.66 (d, 1 H, J = 1.7), 4.29 (m, 1 H), 8.02 (t, 1 H, J = 5.4), 8.56 (d, 1 H, J = 8.4); ^{13}C NMR δ 21.66, 22.31, 22.85, 24.25, 25.08, 36.72, 37.94, 41.10, 51.18, 52.64, 164.83, 168.76, 170.93. Anal. ($C_{15}H_{26}N_2O_5$) C, H, N.

***N*-[(L-*trans*-3-Carbamoyloxiran-2-yl)carbonyl]-L-leucyl-3-methylbutanamide (3).** Ester **2b** (72.9 mg, 0.213 mmol) was dissolved in 5 mL of MeOH and cooled in an ice bath. A stream of ammonia was led gently over the stirred solution for 40 min; then Ar was bubbled through the reaction to remove the ammonia. The solution was concentrated, azeotropically dried with CH_2Cl_2, and subjected to flash chromatography (92:8 CH_2Cl_2/MeOH) to give 55 mg (82%) of white solid: mp 82−85 °C; TLC(C) R_f 0.44; $[\alpha]_D$ +51.7° (*c* 0.2145, DMF); IR (KBr) 3360, 2960, 1650, 1540 cm^{-1}; ^1H NMR (DMSO-d_6) δ 0.85−0.93 (m, 12 H), 1.27 (q, 2 H, J = 7.1), 1.40−1.63 (m, 4 H), 3.10 (m, 2 H), 3.44 (d, 1 H, J = 1.8), 3.61 (d, 1 H, J = 1.8), 7.50 (bs, 1 H), 7.77 (bs, 1 H), 8.05 (t, 1 H, J = 5.3), 8.56 (d, 1 H, J = 7.9); ^{13}C NMR δ 21.63, 22.31, 22.35, 22.87, 24.25, 25.10, 36.72, 37.93, 41.09, 52.51, 52.59 (br, 2 oxirane *C*H), 165.40, 167.98, 171.03. Anal. ($C_{15}H_{27}N_3O_4$) C, H, N.

***N*-[[L-*trans*-3-(Hydroxymethyl)oxiran-2-yl]carbonyl]-L-leucyl-3-methylbutanamide (7).** Ester **2b** (100.1 mg, 0.292 mmol) in 1.5 mL of EtOH was cooled in an ice bath and treated with 12.3 mg of $NaBH_4$ (0.325 mmol). The reaction was stirred for 2 h in the cold and 2 h at room temperature. The solution was again cooled in an ice bath and quenched with 40 μL of acetone followed by 25 μL of AcOH. The solvent was removed *in vacuo*, and the residue was taken up in 5 mL of dichloromethane, washed with 5 mL of brine, dried over $MgSO_4$, filtered, and concentrated. The resulting oil was purified by flash chromatography (9:1 CH_2Cl_2/MeOH). Further purification was performed with a second flash column, eluting first with 1% and then 10% MeOH in EtOAc. The resulting oil was triturated from CH_2Cl_2/hexane to give 60 mg (68%) of white solid: mp 99.5−101.5 °C; TLC(C) R_f 0.54; $[\alpha]_D$ −17.6° (*c* 0.2097, EtOH); IR (KBr) 3600−3200, 2960, 1660, 1550 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.89−0.95 (m, 12 H), 1.39 (q, 2 H, J = 7.2), 1.46−1.69 (m, 4 H), 1.86 (dd, 1 H, J = 7.8, 5.5), 3.18 (m, 1 H), 3.26 (m, 2 H), 3.53 (d, 1 H, J = 2.1), 3.72 (ddd, 1 H, J = 13.3, 7.7, 3.9), 4.02 (ddd, 1 H, J = 13.3, 5.5, 2.1), 4.30−4.39 (m, 1 H), 5.94 (m, 1 H), 6.58 (d, 1 H, J = 9); ^{13}C NMR δ 22.09, 22.39, 22.42, 22.83, 24.81, 25.85, 37.98, 38.26, 40.85, 51.08, 52.20, 59.15, 60.43, 168.27, 171.00. Anal. ($C_{15}H_{28}N_2O_4$) C, H, N.

***N*-[[L-*trans*-(*O*-Benzylhydroxylamino)oxiran-2-yl]carbonyl]-L-leucyl-3-methylbutanamide (8).** Compound **2a** (0.200 g, 0.638 mmol) was dissolved in 3 mL of DMF under nitrogen and cooled in an ice bath. To this solution was added 86.2 mg of HOBt (0.638 mmol) and 122 mg of ethyl dimethylpropylamine carbodiimide (0.638 mmol). After the reaction had stirred for 30 min, 112 mg of $BnONH_2$·HCl (0.703 mmol) was added followed by dropwise addition of 77 μL of NMM (0.700 mmol). The heterogeneous reaction mixture was stirred for 5 h, during which time it was permitted to come to room temperature. The reaction mixture was diluted with 10 mL of EtOAc, washed with 10 mL each of water, 5% citrate, saturated $NaHCO_3$, and brine, and dried over $MgSO_4$. Concentration, purification by flash chromatography (2% followed by 10% MeOH/CH_2Cl_2), and 48 h drying *in vacuo* yielded 200 mg of white solid (75%): mp 125.5−127.5 °C; TLC(C) R_f 0.64; $[\alpha]_D$ +2.82° (*c* 1.10, $CHCl_3$); IR ($CHCl_3$) 3410, 1670, 1535−1530 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.89−0.96 (m, 12 H), 1.39 (q, 2 H, J = 7.3), 1.48−1.70 (m, 4 H), 3.12−3.35 (m, 3 H), 3.48 (bs, 1 H), 3.51 (bs, 1 H), 4.41 (m, 1 H), 4.91 (s, 2 H), 6.24 (m, 1 H), 6.95 (d, 1 H, J = 8.5), 7.37 (s, 5 H), 9.12 (s, 1 H); ^{13}C NMR δ 22.36, 22.47, 22.75, 24.80, 25.88, 38.03, 38.17, 41.64, 51.62, 52.89, 54.03, 78.54, 128.96, 129.25, 134.54, 163.63, 165.61, 171.39. Anal. ($C_{22}H_{33}N_3O_5$) C, H, N.

***N*-[[L-*trans*-(Hydroxylamino)oxiran-2-yl]carbonyl]-L-leucyl-3-methylbutanamide (9).** The *O*-benzylhydroxamate **8** (183 mg, 0.436 mmol) from the previous step was hydrogenolyzed in 4 mL of MeOH with 16 mg of 10% palladium on charcoal, under a static atmosphere of hydrogen, for 1 h. The solution was then filtered through a pad of Celite, washed with additional MeOH, and concentrated *in vacuo*. The resulting brown oil was triturated with ether to give 90 mg of white powder (63%): mp 142−145 °C; TLC(CH_2Cl_2/MeOH/AcOH, 9:1:0.5) R_f 0.47; $[\alpha]_D$ +58.3° (*c* 0.2400, EtOH); IR (KBr) 3390, 3290, 1700, 1650, 1530 cm^{-1}; ^1H NMR (DMSO-d_6) δ 0.83−0.90 (m, 12 H), 1.27 (q, 2 H, J = 7.1), 1.40−1.59 (m, 4 H), 2.97−3.15 (m, 2 H), 3.37 (d, 1 H, J = 1.8), 3.65 (d, 1 H, J = 1.8), 4.30 (m, 1 H), 8.01 (t, 1 H, J = 5.5), 8.57 (d, 1 H, J = 8.3), 9.20 (bs, 1 H), 11.10 (bs, 1 H); ^{13}C NMR δ 21.61, 22.33, 22.36, 22.88, 24.26, 25.11, 36.74, 37.95, 41.05, 51.13, 51.46, 52.11, 162.41, 165.38, 171.02. Anal. ($C_{15}H_{27}N_3O_5$) C, H, N.

***N*-[[L-*trans*-[(Diazomethyl)carbonyl]oxiran-2-yl]carbonyl]-L-leucyl-3-methylbutanamide (11).** Compound **2a** (315 mg, 1.00 mmol) was dissolved in 5 mL of EtOAc to which 0.2 mL of anhydrous DMF was added. The solution was cooled to −15 to −20 °C. To this was added 0.110 mL of NMM (1.00 mmol) followed by slow addition of 0.130 mL of IBCF (1.00 mmol). After 10 min, the reaction mixture was cooled in a −78 °C bath for 20 min, and then filtered under nitrogen through a sintered funnel into a flask cooled to −20 °C. The sintered funnel was washed with several small portions of cold EtOAc. Seven milliliters of ice-cold diazomethane in ether (∼0.3 M, ∼2.1 mmol) was poured into the clear solution of mixed anhydride. The reaction mixture was stirred for 30 min in the cold; then nitrogen was bubbled through the solution at room temperature until the bright yellow color faded to pale yellow (30 min). The reaction solution was washed with 50% brine and then brine, and dried over $MgSO_4$. Concentration afforded 360 mg of a dark yellow oil. Trituration of this product with EtOAc/hexane gave a gelatinous precipitate which was filtered by suction to yield 240 mg (71%) of a pale yellow amorphous solid which was used without further purification: TLC(A) R_f 0.14; ^1H NMR ($CDCl_3$) δ 0.86−1.03 (m, 12 H), 1.40 (q, 2 H, J = 7.2), 1.51−1.69 (m, 4 H), 3.15−3.36 (m, 2 H), 3.49 (bs, 1 H), 3.55 (bs, 1 H), 4.37 (m, 1 H), 5.52 (s, 1 H, CHN_2), 6.15 (m, 1 H), 6.78 (d, 1H, J = 8.5).

***N*-[[L-*trans*-(Methylcarbonyl)oxiran-2-yl]carbonyl]-L-leucyl-3-methylbutanamide (10).** The diazomethyl ketone **11** (180 mg, 0.532 mmol) from the previous step was dissolved in 5.3 mL of chloroform and cooled to 5 °C. To this was added 240 μL of 57% HI (aqueous) (1.6 mmol, 3 equiv) dropwise. The reaction was stirred in the cold for 30 min, and then quenched with 25 mL of 5% sodium thiosulfate. After the solution had changed from brown to colorless, it was transferred to a separatory funnel and diluted with 35 mL of EtOAc. The aqueous layer was removed; the organic layer was washed with 25 mL each of 5% thiosulfate, saturated bicarbonate, and brine and dried over $MgSO_4$. After concentration, the residue was purified by flash chromatography (first column: EtOAc; second column: 4:1 EtOAc/hexane) and recrystallized (1:7 EtOAc/hexane) to give 97 mg of a white solid (58%): mp 130−135 °C (dec.); TLC(EtOAc/hexane 4:1) R_f 0.49; $[\alpha]_D$ +47.3° (*c* 0.3254, $CHCl_3$); IR ($CHCl_3$) 3440, 3400, 2960, 1710, 1670, 1515 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.90−0.95 (m, 12 H), 1.40 (q, 2 H, J = 7.3), 1.48−1.67 (m, 4 H), 2.12 (s, 3 H), 3.20−3.31 (m, 2 H), 3.43 (d, 1 H, J = 1.9), 3.61 (d, 1 H, J = 1.9), 4.30−4.39 (m, 1 H), 5.87 (m, 1 H), 6.54 (d, 1 H, J = 8.6); ^{13}C NMR δ 22.19, 22.37, 22.42, 22.80, 24.37, 24.82, 25.84, 38.00, 38.25, 41.32, 51.39, 53.60, 58.65, 165.95, 1270.88, 202.09. Anal. ($C_{16}H_{28}N_2O_4$) C, H, N.

^1H NMR Determination of Enantiomeric and Diastereomeric Purity. The enantiomeric purity of **5** was verified by coupling it with racemic α-methylbenzylamine and its *S*-enantiomer by the mixed anhydride method. Comparison

of the ^{1}H NMR ($CDCl_3$) spectra of the epoxide methine protons of the resulting compounds revealed that the epoxysuccinate moiety retains ≥98% ee under the conditions of mixed anhydride coupling. The diastereomeric purity of **2a** was confirmed by coupling ethyl (*S*,*S*)-epoxysuccinate to (*R*)-Leu-Mba followed by saponification and examination of the ^{1}H NMR spectra of the ester (in $CDCl_3$) and of the acid (in DMSO-d_6). Comparison of the epoxide methine protons for the (*S*,*S*,*S*) and (*S*,*S*,*R*) diastereomers of **2a** again revealed ds > 98% for each diastereomer.

Compound Stability. Although **2a** and the carboxylate-replaced derivatives are all reasonably stable at room temperature for several days at a time, they are best stored under argon in a −20 °C freezer. Under these conditions, most of the inhibitors have been stable indefinitely. However, over the course of a year, ester **2b** was found to slowly hydrolyze to the extent of a few percent, and **2a** underwent slow loss of activity. The decomposition product of EP-475 is unknown, but seems to be dependent on the carboxylate; amide derivative **3** showed no decomposition after more than 2 years.

Inhibition Kinetics. Inhibition rate constants (K_i, k_3, k_i') were determined as described previously.[22]

HPLC Assay of Hydrolysis of Esters of 2a. The standard reverse-phase HPLC equipment and conditions described under General Methods were utilized. The mobile phase was isocratic with A = 66%. Inhibitors were dissolved in acetonitrile (20 mg/mL) and diluted in duplicate to 0.2 mg/mL in the standard kinetic assay buffer (0.1 M phosphate, pH 6.8, 2 mM DTT, 1 mM EDTA). These solutions were incubated at 30 °C for 24 h during which time 20 μL aliquots were removed and injected onto the HPLC. For comparison, **5** was also incubated in pure water in the same way. The appearance and disappearance of compounds were monitored by measuring peak height and area.

The retention times were 7.6 and 6.2 min for the ethyl and methyl esters respectively. During the course of the incubations, a peak arose at 4.2 min which coeluted with an authentic sample of **2a**. From these experiments, the half-lives are estimated to be approximately 6 h for the methyl ester and 8.4 h for the ethyl ester.

In the above chromatograms, another peak appeared at 5.9 min and may be an addition product between **2a** and DTT. In pure water, the ethyl ester had hydrolyzed only about 10% in 21 h, and no peak at 5.9 min was present. When DTT was added to the ethyl ester in water, the **5** peak at 7.6 min decreased by 6% relative to the peak at 4.6 min (**2a**) over the course of 3 h, and a new peak appeared at 5.9 min. This apparent slow reaction with DTT accounted for approximately 40% of the decrease in **5** incubated in phosphate buffer; the remainder of the decrease was mostly due to ester hydrolysis.

pH−Activity Profiles with Papain. The following buffers were used to span the pH range of 3.3−9.5: citrate (pH 3.3−4.4), acetate (pH 4.4−5.5), phosphate (pH 5.8−7.5), and borate (pH 8.0−9.5). The assay solution was identical to that used in the standard assay except that buffer concentration was 50 mM, and ionic strength was adjusted to 300 mM with NaCl. The previously described procedures for measuring substrate and inhibitor kinetics were employed.[22] At each pH, seven different concentrations of inhibitor **3**, the amide analog of **2a**, were tested with papain (2.0 nM) in the presence of substrate (10 μM). The values of pH for the assay solutions were checked before and after the assays; values were identical within experimental error.

Data Analysis. Inhibitor data from the pH−activity profile were fit to two different schemes in the manner recommended by Cleland.[33b] In Scheme 2, two protonation states are represented, but only unprotonated enzyme species E can be inactivated by the inhibitor. This scheme is described by eq 1 where x may be k_3/K_i, k_3, or K_i, and $[H^+]$ is the proton concentration.

$$\ln(x_{obs}) = \ln\{x/[1 + [H^+]/K_1 + [H^+]^2/K_1']\} \quad (1)$$

In Scheme 3, three protonation states are represented, but again only the unprotonated enzyme species may be inactivated by inhibitor. This scheme is described by eq 2:

$$\ln(x_{obs}) = \ln\{x/[1 + [H^+]/K_1 + [H^+]^2/K_1']\} \quad (2)$$

Similarly, substrate pH−activity data were fit to Schemes 4 and 5, in which only the singly protonated enzyme, EH, may carry out catalysis. In Schemes 4 and 5, there are three and four protonation states represented, and these are described by eqs 3 and 4, respectively:

$$\ln(x_{obs}) = \ln\{x/[1 + [H^+]/K_1 + K_2/[H^+]]\} \quad (3)$$

$$\ln(x_{obs}) = \ln\{x/[1 + [H^+]/K_1 + [H^+]^2/K_1' + K_2/[H^+]]\} \quad (4)$$

Acknowledgment. Thanks go to Karen Rebholz for critically reading the manuscript, to Dexter Northrop for the use of RAGASSEK during the preliminary stages of this work, and to Petr Kuzmic for the use of KineTic. J.P.M. was a fellow of the American Foundation for Pharmaceutical Education and a Squibb Fellow. D.H.R. was the recipient of NIH Grant GM40092.

Note Added in Proof. Recent work by Martichonok et al.[55] demonstrates an acid and an alkaline dependence for inaction of papain by E-64. In conjunction with the work presented here, this shows that His159 is indeed necessary for interaction with the free carboxylate of EP-475 and related inhibitors.

References

(1) (a) Abstracted in part from the Ph.D. Thesis of J.P.M., University of Wisconsin, 1993. (b) Abbreviations: AcOH, acetic acid; AMC, 7-amino-4-methylcoumarin; Bn, benzyl; Boc, butyloxycarbonyl; BOP, benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate; DBU, 1,7-diaminobicyclo[0.4.5]undec-7-ene; DCC, dicyclohexylcarbodiimide; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; E-64, *N*-(L-*trans*-carboxyoxirane-2-carbonyl)-L-leucyl-4-guanidinobutanamide; EDCI, ethylpropylaminecarbodiimide; EP-475, *N*-(L-*trans*-carboxyoxirane-2-carbonyl)-L-leucyl-3-methylbutanamide; EtOAc, ethyl acetate; EtOH, ethanol; HOBt, 1-hydroxybenzotriazole; HPLC, high-pressure liquid chromatography; IBCF, isobutyl chloroformate; K_i, apparent binding constant; k_3, first-order rate of inactivation; k_i', apparent second-order rate of inactivation; Mba, 4-methylbutanamine; MeOH, methanol; MeCN, acetonitrile; NaOEt, sodium ethoxide; NMM, *N*-methylmorpholine; TFA, trifluoroacetic acid; Z, benzyloxycarbonyl.

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JM950445B