Aziridine Analogs of [[*trans*-(Epoxysuccinyl)-L-leucyl]amino]-4-guanidinobutane (E-64) as Inhibitors of Cysteine Proteases†

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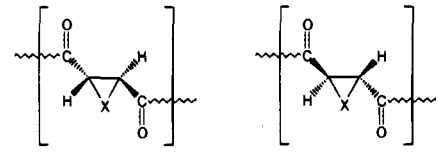
Aziridine derivatives of E-64 have been synthesized, and their characterization against the cysteine proteases cathepsin B, cathepsin L, and papain is reported. The inhibition was found to be strongly pH-dependent, with maximun activity observed at pH 4, indicating that the protonated aziridinium ion form of the inhibitor is the more reactive form. At low pH, the peptide aziridine HO-(L)Az-Leu-NH-iAm inactivated papain with a second-order rate constant, k_{inac}/K_i, of 7.0×10^4 M^{-1} s^{-1}, a value very close to that observed with E-64 or with the corresponding epoxysuccinyl analog HO-(L)Eps-Leu-NH-iAm. This demonstrates that with the correct peptide sequence, aziridine analogs of E-64 can be good irreversible inhibitors of cysteine proteases. Substitution of the epoxysuccinyl moiety by an aziridine does not affect the specificity of inhibition against the three proteases used in this study. The D-diastereomer is the preferred (by 10-fold) diastereomer for the inhibition of cysteine proteases. The reactivity of both diastereomers of iBuNH-Az-LeuPro-OH against cathepsin B was also found to be much lower than that of iBuNH-(L)Eps-LeuPro-OH, which is a potent selective inhibitor of cathepsin B. These differences are attributed mainly to the presence of the protonated aziridine ring, which can modify the binding mode of aziridine analogs at the active site of cysteine proteases.

Introduction

A number of specific cysteine protease inhibitors have been developed on the basis of affinity labeling of the active site cysteine residue.[1] In many cases, these inhibitors were made by attaching a group chemically reactive toward thiols to a peptide portion corresponding to the known substrate specificity preferences of the enzyme, thereby resulting in alkylation of the active site cysteine residue. A somewhat different type of alkylating agent was found by Hanada *et al.* through screening of fungal products.[2] E-64 (**1**), isolated from a culture of *Aspergillus japonicus*, consists of a L-*trans*-epoxysuccinic acid ((L)Eps) linked to L-leucine and an agmatine (Agm) residue.[3] This compound is a potent irreversible inhibitor of cysteine proteases but does not react at an appreciable rate with simple thiol compounds.[4] The reactive entity of E-64 was found to be the oxirane ring of the epoxysuccinyl moiety,[5] and the stereospecificity for inhibition by (L)Eps as opposed to (D)Eps was demonstrated using E-64 analogs.[4] In later work, the importance of the free carboxylate in E-64 was demonstrated[6] and the site of attack of the active site thiol group on the epoxide ring was identified as C-2 from NMR experiments[7] and the crystal structure of a papain–E-64 complex.[8] The crystal structure also clearly showed that, in contrast to earlier predictions, the inhibitor binds in the S subsites of the enzyme in a manner similar to that observed for the peptide chloromethane inhibitors.[9]

Because of the implication of cysteine proteases in a variety of disease states, E-64 has been used as a lead compound for drug design, and a number of E-64 analogs have been synthesized. The agmatine portion of E-64 has been replaced by various alkyl and amino groups.[4,10] To ensure membrane permeability, neutral E-64 analogs were prepared by derivatization of the carboxylate group on the epoxysuccinyl moiety and by replacement of the agmatine by alkyl groups. Loxistatin (**2**), the ethyl ester derivative of Ep-475 (**3**), is another neutral analog of E-64 that has been shown to be effective in animal models of muscular dystrophy.[11] Loxistatin, which is itself a poor inhibitor of cysteine proteases, is a prodrug form of Ep-475. After crossing the cell membrane, loxistatin is hydrolyzed to Ep-475, which then inactivates cellular cysteine proteases.[11c,12] Evidence for potential therapeutic effects of such E-64 analogs has been found against other disease states such as cancer[13] and myocardial infarctions.[14]



X =		
NH	(2*S*,3*S*)Az or (*L*)Az	(2*R*,3*R*)Az or (*D*)Az
O	(2*S*,3*S*)Eps or (*L*)Eps	(2*R*,3*R*)Eps or (*D*)Eps

E-64 analogs with modified peptide portions have also been reported.[5,15] In particular, the leucine side chain in E-64 has been shown to interact in the S_2 subsite of papain,[8] the S_2 site being the major substrate specificity determinant for cysteine proteases of the papain family.[16] Accordingly, E-64 analogs where the leucine residue was replaced by a number of amino acids were prepared to evaluate the contribution of S_2 binding of the leucine residue to inhibition. It was found that the specificity with respect to these analogs does not parallel

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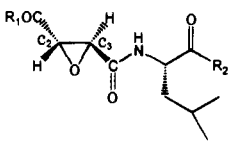
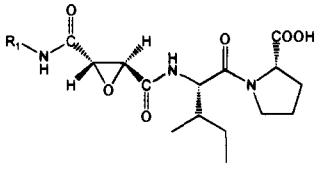


1 R_1 = HO ; R_2 = Agm E-64 (HO-(*L*)Eps-Leu-Agm)
2 R_1 = EtO ; R_2 = NH-iAm loxistatin (EtO-(*L*)Eps-Leu-NH-iAm)
3 R_1 = HO ; R_2 = NH-iAm Ep-475 (HO-(*L*)Eps-Leu-NH-iAm)



4 R_1 = nPr (nPrNH-(*L*)Eps-IlePro-OH)
5 R_1 = iBu (iBuNH-(*L*)Eps-IlePro-OH)

that observed for substrates. Furthermore, selectivity for cathepsin B cannot be achieved on the basis of known specificity for the P_2 residue of substrates.[17] However, an interesting series of synthetic epoxysuccinyl dipeptides, including EtO-(L)Eps-IlePro-OH (CA030) and nPrNH-(L)Eps-IlePro-OH (CA074), were reported to display potent and specific inhibition of cathepsin B over other cysteine proteases of the papain family.[15b,c] Results of two independent studies on CA074 (**4**), iBuNH-(L)Eps-LeuPro-OH (**5**), and their derivatives led to the proposal that, in contrast to E-64, these inhibitors bind in the S′ subsites of cathepsin B.[18,19] On the basis of the cathepsin B structure,[20] the specificity of these inhibitors is attributable to interaction of the proline carboxylate group with histidine residues located on a loop that partly occludes the S′ subsites of cathepsin B.

Very little attention has been given to modification of the chemically reactive portion of the inhibitor, *i.e.*, the oxirane ring. The three-membered aziridine ring is structurally similar to the epoxide, with the exception that the oxygen of the oxirane ring is replaced by a nitrogen atom. Depending on the ring substituents,[21] aziridines have been shown to react with thiols under certain conditions, and we felt that this property could be exploited in designing new cysteine protease inhibitors. In fact, aziridine-2-carboxylic acid has been tested as an inhibitor of the cysteine protease papain and shown to be a very weak irreversible inhibitor of the enzyme.[22] However, the study also demonstrated that, as for the epoxide ring in E-64, the aziridine ring was relatively unreactive toward small thiol compounds at pH 6.5.

No aziridine analogs incorporating specific peptide sequences for the proper recognition of cysteine proteases have previously been reported. In this paper, we describe the synthesis of peptidyl aziridine analogs and their characterization as inhibitors of the cysteine proteases cathepsin B, cathepsin L, and papain. The aziridines prepared are analogs of Ep-475 (**3**) and iBuNH-(L)Eps-LeuPro-OH (**5**), the latter having been shown to selectively inhibit cathepsin B.[19]

Preparation of Inhibitors

The syntheses of the target compounds were performed as indicated in Scheme 1. The stereochemistries shown follow from the literature methodologies applied, as follows: the key diethyl aziridine-2,3-dicarboxylates (2*S*,3*S*)-**9** and (2*R*,3*R*)-**9** were prepared using the strategy of Legters *et al.*[23] Epoxides (2*R*,3*R*)-**6** and (2*S*,3*S*)-**6** were synthesized from diethyl L-(+) and D-(−)-tartrates, respectively, via the three-step procedure described by Mori.[24] Each compound numbered **9**−**16** was synthesized as both the (L)Az (series **a**) and (D)Az (series **b**) derivatives (*e.g.*, **11a**, EtO-(L)Az-NH-iBu; **11b**, EtO-(D)-Az-NH-iBu).

Compounds (2*S*,3*S*)-**12a** and (2*R*,3*R*)-**12b** are aziridine analogs of loxistatin (iAm = isoamyl). Hydrolysis of the ester groups of **12a,b** with LiOH in EtOH gave the acids **14a,b**, the aziridine analogs of Ep-475 (**3**). Hydrolysis of (2*S*,3*S*)-**11a** and (2*R*,3*R*)-**11b** under analogous conditions afforded acids (2*S*,3*S*)-**13a** and (2*R*,3*R*)-**13b**, which were coupled with *N*-L-leucyl-L-proline methyl ester hydrochloride to give (2*S*,3*S*)-**15a** and (2*R*,3*R*)-**15b**, respectively. The steric and/or electronic environments of the proline methyl ester groups in **15a,b** rendered them hydrolytically resistant to LiOH in EtOH, and treatment with NaOH in aqueous MeOH was required to give (2*S*,3*S*)-**16a** and (2*R*,3*R*)-**16b**. The analogous epoxy reference compounds HO-(L)Eps-Leu-NH-iAm (**3a**) and HO-(D)Eps-Leu-NH-iAm (**3b**) were obtained by the standard literature procedures.[4]

Scheme 1

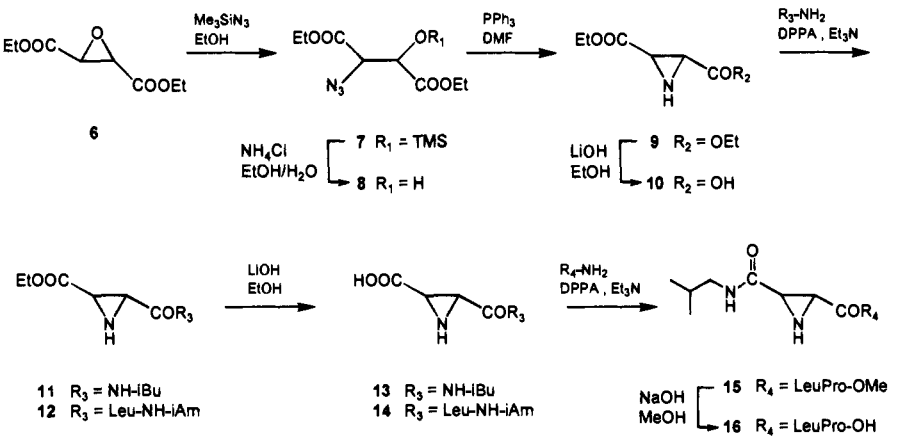


Table 1. Second-Order Rate Constants for Inactivation of Papain at pH 6.5

inhibitor	k_{inac}/K_i (10^3 M^{-1} s^{-1})
HO-(L)Az-Leu-NH-iAm (**14a**)	1.8 ± 0.1
HO-(D)Az-Leu-NH-iAm (**14b**)	14.4 ± 0.7
EtO-(L)Az-Leu-NH-iAm (**12a**)	0.003[a,b]
EtO-(D)Az-Leu-NH-iAm (**12b**)	0.055[b]
HO-(L)Az-NH-iBu (**13a**)	0.0094 ± 0.0004
HO-(L)Eps-Leu-NH-iAm (**3a**)	218 ± 9
HO-(D)Eps-Leu-NH-iAm (**3b**)	13.7 ± 0.3

[a] Obtained by dividing the first-order rate constant, k_{obs}, by [I]. [b] Approximate value since an adduct of papain with the hydrolyzed form of the inhibitor was observed by mass spectrometry.

Results and Discussion

The second-order rate constants for the irreversible inhibition of papain by aziridine analogs of Ep-475 at pH 6.5 are reported in Table 1. For HO-(L)Az-Leu-NH-iAm (**14a**), k_{inac}/K_i is 1.8×10^3 M^{-1} s^{-1}, a value 125-fold lower than that obtained for the corresponding Eps derivative **3a** (218×10^3 M^{-1} s^{-1}). However, in contrast to the Eps series, HO-(D)Az-Leu-NH-iAm (**14b**) is approximately 8-fold more reactive than its (L)-diastereomer **14a** and displays almost the same inhibitory activity as the corresponding Eps itself, HO-(D)Eps-Leu-NH-iAm (**3b**). For both aziridine derivatives, the molecular masses of the enzyme–inhibitor adducts measured by mass spectrometry are 23 735 Da, which correspond to the sum of the molecular masses of the enzyme (23 422 Da) and the full "intact" inhibitor (313 Da). This indicates that the inhibition is not due to a degradation product of the aziridines. The very low inhibitory activities (Table 1) of compounds EtO-(L)Az-Leu-NH-iAm (**12a**), EtO-(D)Az-Leu-NH-iAm (**12b**), and HO-(L)Az-NH-iBu (**13a**) are evidence for the importance of the free carboxylate on the aziridine ring and of the peptide portion Leu-NH-iAm. In that respect, aziridine derivatives behave similarly to E-64 and other epoxysuccinyl derivatives.

The previous observations suggest that the aziridines and the epoxysuccinyl analogs inhibit cysteine proteases by a somewhat similar mechanism. The stereospecificity of the inhibition is one major difference between aziridine derivatives and epoxysuccinyl analogs of E-64. Of probable importance in the mechanism of inhibition by aziridines is the fact that the three-membered ring has the potential to be protonated on the nitrogen atom. Aziridines have been shown to be more reactive at low pH,[25] and in contrast to E-64, this protonation is likely to occur in the pH range where cysteine proteases are active. To investigate if protonation of the aziridine ring modulates the activity of the inhibitor against cysteine proteases, the pH dependency for inhibition of papain by HO-(L)Az-Leu-NH-iAm (**14a**) and HO-(D)Az-Leu-NH-iAm (**14b**) was monitored and compared to that for E-64 (**1**) (Figure 1).

The k_{inac}/K_i values obtained for E-64 display a bell-shaped pH dependency which is almost identical to that observed for substrate hydrolysis reactions (Figure 1a). As the pH is raised, the activity first increases (pK_a of 4.1) and then decreases (pK_a of 8.6), with maximum inhibitory activity exhibited at near-neutral pH. This is attributed to deprotonation of the active site cysteine and histidine residues respectively, corresponding to the reactive form of the enzyme being a thiolate–imidazolium ion pair. The situation is very different with the

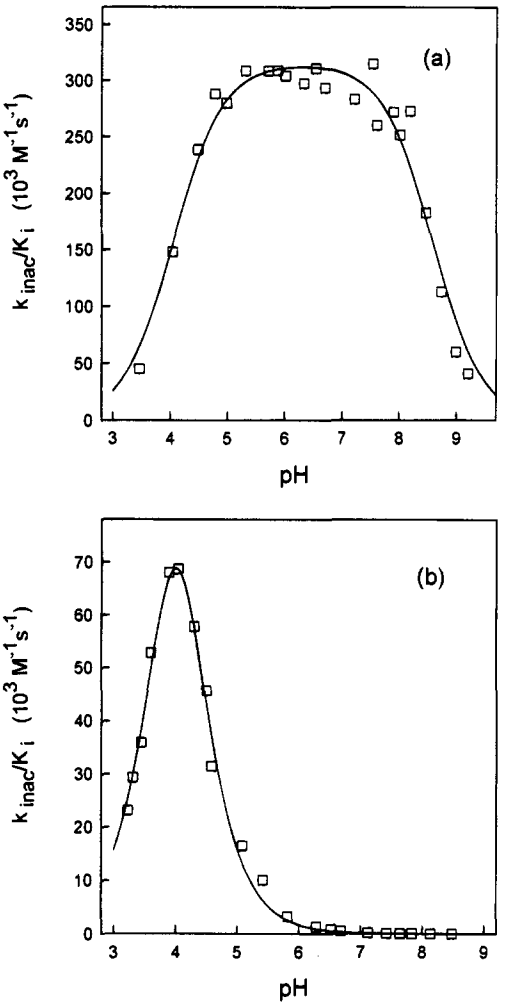


Figure 1. pH dependence of the second-order rate constant (k_{inac}/K_i) for inactivation of papain by (a) E-64 (1) and (b) HO-(L)Az-Leu-NH-iAm (**14a**). The solid line represents the best fit to a model considering that only one form of the inhibitor is active and that two ionizable groups modulate the pH dependence.

inhibitor HO-(L)Az-Leu-NH-iAm (**14a**) (Figure 1b). Maximum activity is obtained at pH 4, where k_{inac}/K_i is now 6.9×10^4 M^{-1} s^{-1}, a value much higher than that observed at pH 6.5. The increase in rate at low pH can be attributed to protonation of the aziridine nitrogen atom, which translates into increased reactivity of the inhibitor toward papain. If the pH is lowered below 4, the rate of inactivation decreases due to protonation of the active site cysteine residue in papain leading to the nonreactive neutral thiol form of the enzyme. It must be noted that the width of this profile is equal to the theoretical minimum width of a bell-shaped pH–activity profile.[26] For this reason, the exact pK_a values describing the profile do not correspond to pH values where the activity is one-half the maximum value. Nonlinear regression analyses of the data give a pK_a value of 4.4 for deprotonation of the papain active site cysteine residue, in agreement with the value obtained with E-64 and substrates,[27] and 3.6 for deprotonation of the aziridinium ion. The data are accommodated very well by a model in which the neutral form of the aziridine is unreactive. If the neutral form reacts at all, within the precision limits of the curve-fitting procedure it must be at least 100-fold less reactive than the protonated form. Another consequence of the narrow width of the

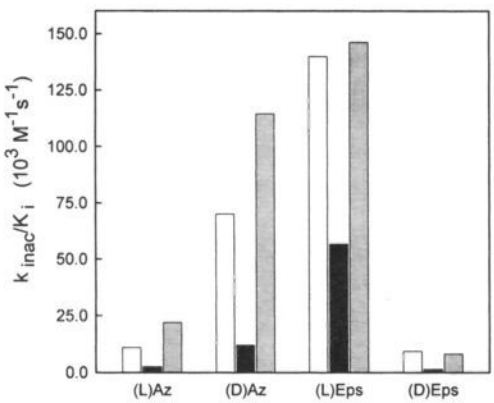


Figure 2. Stereospecificity and enzyme selectivity of aziridine and epoxysuccinyl inhibitors. The inhibitors used are HO-(L)-Az-Leu-NH-iAm (**14a**, (L)Az), HO-(D)Az-Leu-NH-iAm (**14b**, (D)-Az), HO-(L)Eps-Leu-NH-iAm (**3a**, (L)Eps), and HO-(D)Eps-Leu-NH-iAm (**3b**, (D)Eps). The second-order rate constant for enzyme inactivation, k_{inac}/K_i, was measured against papain (white bars), cathepsin B (black bars), and cathepsin L (gray bars).

pH−activity profile is that the true activity of the fully protonated inhibitor will be much higher than the value of 7.0×10^4 M^{-1} s^{-1} observed at the pH of maximum activity. From the fitted data, a limiting value for this inhibitor of approximately 4.0×10^5 M^{-1} s^{-1} was obtained, which is as good as that of E-64 and better than the value obtained for Ep-475. However, in practice we cannot achieve this full inhibitory potential because it occurs at pH values where the enzyme becomes less reactive due to protonation of the active site ion pair. The profile obtained with HO-(D)Az-Leu-NH-iAm (**14b**) is almost identical to that of the (L)-diastereomer, with the exception that the maximum activity observed at pH 4 is approximately 10-fold higher, with $k_{inac}/K_i = 7.2 \times 10^5$ M^{-1} s^{-1}.

The selectivity of the aziridine analogs was investigated by characterizing both HO-(L)Az-Leu-NH-iAm (**14a**) and HO-(D)Az-Leu-NH-iAm (**14b**) against the cysteine proteases cathepsin B, cathepsin L, and papain. For comparison, the rates of inactivation of all three enzymes by the corresponding epoxysuccinyl analogs HO-(L)Eps-Leu-NH-iAm (**3a**) and HO-(D)Eps-Leu-NH-iAm (**3b**) were also determined. Because of the strong pH dependency of the inhibition by aziridine compounds, and of the instability of cathepsin B and particularly cathepsin L at pH above 6, the assays were carried out at a constant pH of 5.5. The results are shown in Figure 2. It must be noted that for all three enzymes, the stereospecificity of the inhibition reaction is conserved, namely that the D-diastereomer is favored over the L-diastereomer for the aziridines, while the situation is opposite for the epoxysuccinyl inhibitors, for which the L-diastereomer is favored over the D-diastereomer. It should also be noted that there is no difference in selectivity in going from the epoxysuccinyl to the aziridine series. In all four cases, the rates of inactivation are somewhat similar for papain and cathepsin L but are lower for cathepsin B. Substitution of the epoxysuccinyl moiety for an aziridine does not affect the selectivity of the inhibitor in these cases.

Finally, both **16a,b**, the L- and D-aziridine analogs of iBuNH-(L)Eps-LeuPro-OH, respectively, were evaluated as cysteine protease inhibitors. (Table 2). In contrast to the epoxysuccinyl derivative **5**, which is a potent and selective inhibitor of cathepsin B, almost no activity was detected for either the L- or D-aziridine compounds against any of the three cysteine proteases, including cathepsin B. Furthermore, the highest k_{inac}/K_i observed for the aziridines **16a,b** for any of the Table 2 enzymes was 13 M^{-1} s^{-1} with iBuNH-(D)Az-LeuPro-OH (**16b**) for cathepsin B, a value still dramatically lower than the k_{inac}/K_i value of 52×10^3 M^{-1} s^{-1} for the oxirane iBuNH-(L)Eps-LeuPro-OH (**5**) for the same enzyme.

Even though many similarities exist between the two classes of inhibitors, the differences noted in stereospecificity, and also in the activity of compounds of the type iBuNH-Az-LeuPro-OH against cathepsin B, are noteworthy. A possible explanation for these differences is that it is only the protonated form of an aziridine inhibitor that reacts with cysteine proteases. In the mechanism of cysteine protease inactivation by E-64, protonation of the epoxide oxygen during ring opening must involve a water molecule,[8] and binding interactions and orientation at the active site must be such as to favor such a reaction. For the aziridine analogs, since the inhibitors will be protonated prior to the attack by the sulfur atom of the active site cysteine, it becomes unnecessary to involve a water molecule in the mechanism. In addition, a number of interactions exist in the papain−E-64 structure between the enzyme and the inhibitor that might be affected by replacement of the oxirane by an aziridine.[8] In particular, the free carboxylate group on the oxirane ring of E-64 interacts with Cys25 and Gln19 in the oxyanion hole and also specifically with a water molecule and the active site histidine residue of papain. When a protonated aziridine binds to the active site, the presence of the positive charge on the three-membered ring might perturb the positioning of the inhibitor, either through interaction with the negatively charged carboxylate on the ring or through other interactions with the enzyme. These, and previous, considerations suggest that the binding mode of aziridine analogs at the active site of cysteine proteases will be different from that of E-64. As a consequence, the site of attack of the cysteine thiolate anion might be different for aziridine inhibitors and occur at the C-3 atom of the three-membered ring instead of the C-2 atom as observed with E-64. Such differences in inhibitor orientation at the active site and in reaction regioselectivity could well be responsible for the opposite

Table 2. Second-Order Rate Constants for Inactivation of Cathepsin B, Cathepsin L, and Papain[a]

	k_{inac}/K_i(10^3 M^{-1} s^{-1})		
inhibitor	cathepsin B	cathepsin L	papain
iBuNH-(L)Az-LeuPro-OH (**16a**)	ni[b]	0.0067 ± 0.0001[c]	0.0018 ± 0.0001[c]
iBuNH-(L)Eps-LeuPro-OH (**5**)[d]	52 ± 6		0.558 ± 0.004
iBuNH-(D)Az-LeuPro-OH (**16b**)	0.013 ± 0.003	0.050 ± 0.001	ni

[a] The assays were done at pH 6.0 for cathepsin B, 5.5 for cathepsin L, and 6.5 for papain. [b] No time-dependent inhibition observed. [c] Obtained by dividing the first-order rate constant, k_{obs}, by [I]. [d] From Gour-Salin *et al.*[19]

epoxide and aziridine stereoselectivities observed in the present study. For the two diastereomers of iBuNH-Az-LeuPro-OH, the lack of activity could again be attributable to different binding modes which do not tolerate substitution of the ring carboxylate by an isobutyl amide function. However, it is also possible that conversion of the negatively charged carboxylate function to an amide might lead to a decrease in the pK_a of the aziridine, therefore decreasing significantly the apparent activity of these analogs. A better understanding of the mechanism of cysteine protease inactivation by aziridine analogs of E-64 will require more detailed experiments and molecular modeling. Nevertheless, the present study clearly demonstrates that, with a suitable peptide sequence, aziridine analogs of E-64 can be relatively good irreversible inhibitors of cysteine proteases of the papain family.

Experimental Section

Unless indicated otherwise, all reactions were performed under nitrogen using oven-dried glassware. Anhydrous reagents and solvents were prepared according to literature procedures.[28] Analytical thin layer chromatography was performed on Merck plates (silica gel F_{254}, 0.25 mm). Compounds that were not visualized by UV light were detected by spraying with a mixture of ninhydrin (0.3 g) and acetic acid (3 mL) in EtOH (100 mL) followed by heating. Preparative flash column chromatography was performed using silica gel 60 (40–63 μm), supplied by Toronto Research Chemicals Inc. Melting points were obtained on a Fisher-Johns melting point apparatus and are uncorrected. Boiling points are uncorrected. Optical rotations were measured in a Perkin-Elmer 243 B polarimeter in a thermostated cell. Infrared (IR) spectra were determined in KBr pellets (for solids) and films (liquids) on a Nicolet 5DX FTIR spectrophotometer. NMR (^{1}H and ^{13}C) spectra were recorded on a Gemini 200 (200 and 50 MHz, respectively) spectrometer unless otherwise indicated. ^{1}H NMR chemical shifts are reported in ppm relative to the $CHCl_3$ peak (δ = 7.24) with $CHCl_3$ as solvent, the DMSO peak (δ = 2.49) in DMSO-d_6, and the HOD peak (δ = 4.80) in D_2O. ^{13}C NMR chemical shifts are reported in ppm relative to the $CHCl_3$ peak (δ = 77.00) with $CHCl_3$ as solvent, the DMSO peak (δ = 39.50) in DMSO-d_6 and external dioxane (δ = 66.50) in D_2O as solvent. Mass spectra were measured on a Bell and Howell 21–490 instrument (low resolution) or an AEI MS3074 instrument (high resolution). Elemental analyses were by Galbraith Laboratories, Knoxville, TN. Reagent grade chemicals were purchased from Aldrich. Boc-L-leucine and L-proline methyl ester were from Sigma. Isobutylamine hydrochloride was prepared from isobutylamine and an ethereal solution of HCl. The reference epoxy inhibitors HO-(L)Eps-Leu-NH-iAm (**3a**) and HO-(D)Eps-Leu-NH-iAm (**3b**) were prepared by the well-established literature methods.[4]

General Procedure for DPPA-Mediated Coupling of Carboxylic Acids and Amines. This basic procedure was applied several times, as follows: To a dry flask purged with nitrogen and containing carboxylic acid (5.00 mmol) and amine hydrochloride (5.2 mmol) was added dry DMF (30 mL), and the stirred solution was cooled to 0 °C. DPPA (diphenyl phosphorazidate; 1.2 mL, 5.5 mmol) was added followed by a solution of Et_3 N (1.43 mL, 10.5 mmol) in DMF (10 mL) and stirring continued at 0 °C for 10 h. The mixture was then diluted with EtOAc (150 mL) and washed successively with H_2O (3 × 25 mL), 5% aqueous $NaHCO_3$ (25 mL), and brine (2 × 25 mL). The organic phase was dried ($MgSO_4$) and the solvent rotoevaporated under reduced pressure to give the product, purified for each individual compound as described below.

L-Series Inhibitors. Ethyl (2S,3S)-3-[[(S)-3-Methyl-1-[(3-methylbutyl)carbamoyl]butyl]carbamoyl]-2-aziridinecarboxylate (12a, EtO-(L)Az-Leu-NH-iAm). To a cooled solution (0 °C) of diethyl (−)-(2R,3R)-2,3-epoxysuccinate[24] ((2R,3R)-**6**; 8.92 g, 47.4 mmol) in dry DMF (50 mL), were added azidotrimethylsilane (12.6 mL, 95.0 mmol) and absolute EtOH (5.6 mL, 94.8 mmol) sequentially. The reaction mixture was stirred at 65 °C (external temperature). After 18 h an additional amount of EtOH (2.8 mL, 47.4 mmol) was added, and the mixture was heated for another 10 h. The solvents were then removed *in vacuo*, and the crude product was purified by chromatography (ether/hexanes, 2:5, elution) giving the corresponding alcohol (2R,3S)-**8** (containing about 10% of the 2R,3R-diastereomer formed as a result of nucleophilic azide exchange under the reaction conditions[23]) as a major product (R_f = 0.23) and small amounts of the silyl ether (2R,3S)-**7** as a minor product (R_f = 0.49). Separation by flash chromatography on silica gel afforded diethyl (+)-(2R,3S)-3-azido-2-[(trimethylsilyl)oxy]succinate ((2R,3S)-**7**; 1.44 g, 10%): $[\alpha]^{23}_D$ = +25.8° (*c* 1.11, EtOH). ^{1}H NMR ($CDCl_3$): δ 0.17 (s, 9 H), 1.28 (t, *J* = 7.2 Hz, 3 H), 1.29 (t, *J* = 7.2 Hz, 3 H), 4.10–4.27 (m, 5 H), 4.57 (d, *J* = 3.4 Hz, 1 H). ^{13}C NMR ($CDCl_3$): δ 13.77, 61.32, 62.05, 63.95, 73.59, 166.94, 169.42.

It also afforded diethyl (+)-(2R,3S)-3-azido-2-hydroxysuccinate ((2R,3S)-**8**; 9.14 g, 83%): $[\alpha]^{23}_D$ = +32.8° (*c* 1.64, EtOH). IR (neat): 3470 (br), 2120, 1747 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 1.29 (t, *J* = 7.2 Hz, 3 H), 1.30 (t, *J* = 7.2 Hz, 3 H), 3.31 (d, *J* = 5.4 Hz, 1 H), 4.21–4.35 (m, 5 H), 4.62 (dd, *J* = 2.7, 5.5 Hz, 1 H). ^{13}C NMR ($CDCl_3$): δ 13.92, 13.96, 62.29, 62.62, 64.35, 71.99, 166.90, 170.70. The silyl ether (2R,3S)-**7** was conveniently hydrolyzed by dissolving in EtOH (15 mL), to which a solution of NH_4Cl (510 mg, 9.5 mmol, 2 equiv) in H_2O (1 mL) was added. After stirring at room temperature for 24 h, an additional 1.06 g of (2R,3S)-**8** was obtained, for a total yield of 93% for (2R,3S)-**8**.

Triphenylphosphine (10.18 g, 38.8 mmol) was slowly added to a stirred ice-cooled solution of (2R,3S)-**8** (8.89 g, 38.5 mmol of the 9:1 2R,3S/2R,3R mixture) in dry DMF (150 mL). After being stirred at room temperature for 1.5 h, the reaction mixture was heated at 90 °C for 3 h. The solvent was evaporated *in vacuo*, and the residue was chromatographed on silica gel (ether/hexanes, 3:2, R_f = 0.38), yielding 3.37 g (47%) of diethyl (2S,3S)-aziridine-2,3-dicarboxylate ((2S,3S)-**9a**, EtO-(L)Az-OEt) containing 10% of the corresponding (2S,3R)-*cis*-aziridine (which was not separated, since the mixture was conveniently purified by recrystallization in the next step): $[\alpha]^{23}_D$ = +126.0° (*c* 1.75, $CHCl_3$). IR (neat): 1747, 1725 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 1.28 (t, *J* = 7.2 Hz, 3 H), 1.76 (bs, 1 H), 2.84 (s, 2 H), 4.21 (q, *J* = 7.1 Hz, 2 H). ^{13}C NMR ($CDCl_3$): δ 13.75, 35.39, 61.60, 169.27.

LiOH monohydrate (1.01 g, 0.24 mmol) was added to an ice-cooled solution of (2S,3S)-**9a** (4.51 g, 24.1 mmol) in EtOH (100 mL), which was then stirred at 0 °C for 2 h and at room temperature for 48 h. After evaporation of the solvent, the residue was taken up in H_2O (50 mL) and extracted with EtOAc (3 × 50 mL). The aqueous solution was acidified with Dowex 50W-X2 (H^+ form) and then lyophilized. The crude monoacid was crystallized until constant rotation from EtOAc (3×) to afford (2S,3S)-3-(ethoxycarbonyl)-2-aziridinecarboxylic acid (**10a**, EtO-(L)Az-OH) 2.15 g (56%) as colorless needles: mp 130–131 °C dec; $[\alpha]^{23}_D$ = +154.6° (*c* 1.12, EtOH). IR (KBr): 3250–2540 (br), 1739, 1725 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 1.30 (t, *J* = 7.2 Hz, 3 H), 2.91 (s, 2 H), 4.24 (q, *J* = 7.2 Hz, 2 H), 5.98 (bs, 2 H). ^{13}C NMR (DMSO-d_6): δ 14.04, 34.82, 36.22, 60.97, 169.78, 171.31. Anal. ($C_6H_9NO_4$) C, H.

Boc-L-leucine (10 mmol) and isoamylamine hydrochloride (10.4 mmol) were coupled using the above general DPPA procedure to give Boc-L-leucine(isoamylamide) (2.6 g, 87%), mp 90–91 °C, after recrystallization from EtOH: $[\alpha]^{23}_D$ = −33.8° (*c* 1.33, $CHCl_3$). ^{1}H NMR ($CDCl_3$): δ 0.83–0.96 (m, 12 H), 1.30–1.82 (m, 6 H), 1.39 (s, 9 H), 3.15–3.28 (m, 2 H), 4.04 (m, 1 H), 4.92 (bd, *J* = 7.9 Hz, 1 H), 6.20 (bs, 1 H). ^{13}C NMR ($CDCl_3$): δ 22.32, 22.36, 22.83, 24.66, 25.68, 28.23, 37.62, 38.27, 41.37, 52.96, 53.01, 79.28, 155.75, 172.53.

Subsequent treatment of Boc-L-leucine(isoamylamide) (73 mmol) by cleavage with 3 M solution of HCl in EtOAc[29] gave L-leucine(isoamylamide) hydrochloride (15.4 g, 90%) as a glasslike solid: mp 90–95 °C; $[\alpha]^{23}_D$ = +9.61° (*c* 0.51, H_2O). ^{1}H NMR (D_2O): δ 0.89–0.99 (m, 12 H), 1.36–1.86 (m, 6 H), 3.13–3.35 (m, 2 H), 4.04–4.19 (m, 1 H). ^{13}C NMR (D_2O): δ

21.37, 22.04 (double peak), 22.73, 24.35, 25.47, 37.19, 37.91, 43.10, 52.85, 173.95.

(2*S*,3*S*)-3-(Ethoxycarbonyl)-2-aziridinecarboxylic acid ((2*S*,3*S*)-**10a**; 5 mmol) was coupled with L-leucine(isoamylamide) hydrochloride (5 mmol) using the general DPPA method. The product was purified by flash chromatography on silica gel (EtOAc/hexanes, 3:1, R_f = 0.40) and then recrystallized from EtOAc/hexanes to give **12a** (EtO-(L)Az-Leu-NH-iAm; 1.0 g, 59%): mp 88–90 °C; $[\alpha]^{23}_D$ = +53.9° (*c* 1.17, EtOH). IR (KBr): 3302–3286 (br), 1743, 1641 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 0.82–1.02 (m, 12 H, 2 C*H*$_3$-Leu, 2 C*H*$_3$-iAm), 1.28 (t, *J* = 7.12 Hz, 3 H, OCH$_2$C*H*$_3$), 1.26–1.80 (m, 7 H, N*H*-Az, C*H*$_2$C*H*CH$_3$-Leu, C*H*$_2$C*H*CH$_3$-iAm), 2.52 (dd, *J* = 2.20, 7.69 Hz, 1 H, C*H*-Az), 2.81 (dd, *J* = 2.23, 9.03 Hz, 1 H, C*H*-Az), 3.15–3.27 (m, 2 H, NHC*H*$_2$-iAm), 4.21 (q, *J* = 7.71 Hz, 2 H, OC*H*$_2$-CH$_3$), 4.30 (m, 1 H, C*H*-Leu), 6.17 (m, 1 H, N*H*-iAm), 6.79 (d, *J* = 8.80 Hz, 1 H, N*H*-Leu). ^{13}C NMR ($CDCl_3$): δ 13.95, 22.05, 22.26, 22.32, 22.72, 24.68, 25.71, 35.66, 37.46, 37.74, 38.10, 41.03, 51.13, 52.19, 167.82, 170.16, 171.26. Anal. ($C_{17}H_{31}N_3O_4$) C, H.

(2*S*,3*S*)-3-[[(*S*)-3-Methyl-1-[(3-methylbutyl)carbamoyl]-butyl]carbamoyl]-2-aziridinecarboxylic Acid (14a, HO-(L)Az-Leu-NH-iAm). This compound was prepared from (2*S*,3*S*)-**12a** (840 mg, 2.46 mmol) by reaction with 1.2 equiv of LiOH in EtOH and subsequent neutralization with Dowex 50W-X2 (H^+ form) as described above for (2*S*,3*S*)-**10a**. The product was recrystallized from aqueous MeOH to give **14a** (407 mg, 52%): mp 190–195 °C dec (without melting); $[\alpha]^{23}_D$ = +43.4° (*c* 0.4, CH_3OH). IR (KBr): 3484–3250 (br), 1721, 1702, 1644 cm^{-1}. ^{1}H NMR (DMSO-d_6): δ 0.80–0.91 (m, 12 H, 2 C*H*$_3$-Leu, 2 C*H*$_3$-iAm), 1.21–1.61 (m, 6 H, C*H*$_2$C*H*CH$_3$-Leu, C*H*$_2$C*H*CH$_3$-iAm), 2.35 (d, *J* = 2.15 Hz, 1 H, C*H*-Az), 2.70 (d, *J* = 2.20 Hz, 1 H, C*H*-Az), 2.97–3.12 (m, 2 H, NHC*H*$_2$-iAm), 4.21–4.33 (m, 1 H, C*H*-Leu), 8.01 (t, *J* = 5.49 Hz, 1 H, N*H*-iAm), 8.28 (bs, 1 H, N*H*-Leu), *H*N-azir and COO*H* were not detected. ^{13}C NMR (DMSO-d_6): δ 21.77, 22.34, 22.37, 22.89, 35.10, 36.65, 36.72, 37.96, 41.38, 51.14, 167.82, 171.24, 171.52. Anal. ($2C_{15}H_{27}N_3O_4{\cdot}1H_2O$): C, H.

(2*S*,3*S*)-3-[(2-Methylpropyl)carbamoyl]-2-aziridinecarboxylic Acid (13a, HO-(L)Az-NH-iBu). DPPA-mediated coupling of (2*S*,3*S*)-**10a** (5.0 mmol) with isobutylamine hydrochloride (5.2 mmol) gave, after purification by flash chromatography on silica gel (EtOAc/hexanes, 2:1 (R_f = 0.38), elution) and recrystallization from Et_2O/hexanes, ethyl (2*S*,3*S*)-3-[(2-methylpropyl)carbamoyl]-2-aziridinecarboxylate (**11a**, EtO-(L)-Az-NH-iBu; 787 mg, 69%): mp 59 °C; $[\alpha]^{23}_D$ = +123.1° (*c* 1.17 EtOH). IR (KBr): 3309–3224 (br), 1729, 1650 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 0.86 (d, *J* = 6.7 Hz, 6 H), 1.28 (t, *J* = 2.3, 9.0 Hz, 1 H), 3.00 (t, *J* = 6.6 Hz, 2 H), 4.20 (q, *J* = 7.2 Hz, 2 H), 6.46 (bs, 1 H). ^{13}C NMR ($CDCl_3$): δ 13.89, 19.82, 28.24, 35.58, 37.60, 45.97, 62.04, 167.53, 170.21.

The ethyl ester (2*S*,3*S*)-**11a** (246 mg, 1.1 mmol) was hydrolyzed with 1.2 equiv of LiOH in EtOH followed by subsequent neutralization with Dowex 50W-X2 (H^+ form), as described above for the preparation of (2*S*,3*S*)-**10a** from (2*S*,3*S*)-**9a**. The product was recrystallized from H_2O/dioxane to give **13a** (HO-(L)Az-NH-iBu; 183 mg, 86%): mp 185–189 °C dec (without melting); $[\alpha]^{23}_D$ = +73.1° (*c* 0.52, H_2O). IR (KBr): 3402–3220 (br), 1735, 1724, 1659 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 0.88 (d, *J* = 6.22 Hz, 6 H, 2 C*H*$_3$-iBu), 1.76 (m, 1 H, C*H*CH$_3$), 2.64 (bs, 1 H, C*H*-Az), 2.94 (bs, 1 H, C*H*-Az), 3.05 (m, 2 H, NHC*H*$_2$), 5.50–7.00 (m, 3 H, COO*H*, N*H*-iBu, N*H*-Az). ^{13}C NMR (D_2O/dioxane, 7:3): δ 19.95 (double peak), 28.52, 37.73, 37.87, 47.98, 167.52, 167.54. Anal. ($3C_8H_{14}N_2O_3{\cdot}1H_2O$) C, H.

1-[*N*-[[(3*S*)-3-[[(2-Methylpropyl)amino]carbonyl]-(2*S*)-2-aziridinyl]carbonyl]-L-leucyl]-L-proline (16a, iBuNH-(L)Az-LeuPro-OH). Boc-L-leucine (5.0 mmol) and L-proline methyl ester hydrochloride (5.2 mmol) were coupled by the general DPPA procedure to give, after purification by flash chromatography on silica gel (ether/hexanes, 3:1 (R_f = 0.42), elution), Boc-*N*-L-leucyl-L-proline methyl ester (Boc-LeuPro-OMe; 1.48 g, 83%) as a colorless syrup: $[\alpha]^{23}_D$ = −67.1° (*c* 0.76, $CHCl_3$). IR (KBr): 3310, 1750, 1707, 1651 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 0.92 (d, *J* = 6.6 Hz, 3 H), 0.97 (*J* = 6.5 Hz, 3 H), 1.39 (s, 9 H), 1.40–2.28 (m, 7 H), 3.51–3.80 (m, 2 H), 3.69 (s, 3 H), 4.38–4.55 (m, 2 H), 5.09 (d, *J* = 7.8 Hz, 1 H). ^{13}C NMR ($CDCl_3$): δ 21.63, 23.20, 24.35, 24.72, 28.15, 28.77, 41.75, 46.51, 50.10, 51.97, 58.48, 79.25, 155.50, 171.61, 172.28.

Boc-*N*-L-leucylproline methyl ester was deprotected with 3 M HCl in EtOH[29] to afford *N*-L-leucyl-L-proline methyl ester hydrochloride (LeuPro-OMe·HCl) in 85% yield: mp 200–202 °C; $[\alpha]^{23}_D$ = −86.9° (*c* 0.51, H_2O). ^{1}H NMR (D_2O): δ 0.92–1.01 (m, 6 H), 1.61–2.36 (m, 7 H), 3.50–3.76 (m, 2 H), 3.67 (s, 3 H), 4.21–4.37 (m, 1 H), 4.42–4.50 (m, 1 H). ^{13}C NMR (D_2O): δ 20.63, 22.28, 23.72, 24.58, 28.62, 38.88, 47.56, 50.33, 52.85, 52.91, 59.54, 168.95, 173.95.

DPPA-mediated coupling of (2*S*,3*S*)-**13a** (1.5 mmol) and *N*-L-leucyl-L-proline methyl ester hydrochloride (1.5 mmol) gave, after purification by flash chromatography on silica gel deactivated with triethylamine (3%, w/w) (ether/MeOH, 10:1 (R_f = 0.54), elution), 1-[*N*-[[(3*S*)-3-[[(2-methylpropyl)amino]-carbonyl]-(2*S*)-2-aziridinyl]carbonyl]-L-leucyl]-L-proline methyl ester (**15a**, iBuNH-(L)Az-LeuPro-OMe; 411 mg, 64%) as a colorless syrup: $[\alpha]^{23}_D$ = −11.7° (*c* 0.75, CH_3OH). IR (KBr): 3346–3300 (br), 1749, 1656, 1631 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 0.80–0.99 (m, 12 H), 1.48–2.36 (m, 10 H), 2.42 (dd, *J* = 2.2, 7.6 Hz, 1 H), 2.70 (dd, *J* = 2.3, 8.8 Hz, 1 H), 3.00 (t, *J* = 6.6 Hz, 1 H), 3.08–3.16 (m, 1 H), 3.56–3.76 (m, 2 H), 3.70 (s, 3 H), 4.42–4.53 (m, 1 H), 4.68–4.90 (m, 1 H), 6.56 (m, 1 H), 7.13 (d, *J* = 8.1 Hz, 1 H). ^{13}C NMR ($CDCl_3$): δ 19.97 (double peak), 21.90, 23.16, 24.54, 24.80, 28.43, 28.93, 36.80, 41.81, 46.15, 46.91, 49.43, 52.13, 58.78, 168.20, 168.52, 170.77, 172.25.

To the methyl ester **15a** (127 mg, 0.3 mmol) was added 1 M aqueous methanolic sodium hydroxide (1.2 equiv). The resulting mixture was stirred at 0 °C for 5 h and then at 20 °C for 24 h. After rotary evaporation, the residue was dissolved in H_2O (10 mL) and thoroughly washed with EtOAc (3 × 10 mL). The resulting aqueous solution was acidified with Dowex W50-X2 (H^+ form) and then lyophilized and the product recrystallized from H_2O/dioxane to give **16a** (iBuNH-(L)Az-LeuPro-OH; 47 mg, 38%): mp 197–203 °C dec (without melting); $[\alpha]^{23}_D$ = −10.1° (*c* 0.63, CH_3OH). IR (KBr): 3465–3225 (br), 1733, 1722, 1656, 1631 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 0.82–0.97 (m, 12 H, 2 C*H*$_3$-Leu, 2 C*H*$_3$-iBu), 1.48–2.32 (m, 10 H, C*H*$_2$C*H*CH$_3$-Leu, C*H*CH$_3$-iAm, NCH$_2$C*H*$_2$C*H*$_2$-Pro, *H*N-Az, COO*H*), 2.49 (dd, *J* = 2.20, 7.65 Hz, 1 H, C*H*-Az), 2.75 (dd, *J* = 2.28, 8.79 Hz, 1 H, C*H*-Az), 3.06–3.18 (m, 2 H, NHC*H*$_2$-iBu), 3.50–3.71 (m, 2 H, NC*H*$_2$-Pro), 4.46–4.50 (m, 1 H, C*H*-Leu), 4.70–4.92 (m, 1 H, C*H*-Pro), 6.59 (m, 1H, N*H*-iBu), 7.19 (d, *J* = 8.14 Hz, 1 H, N*H*-Leu). ^{13}C NMR (DMSO-d_6): δ 19.80 (double peak), 21.86, 23.10, 24.36, 24.51, 28.41, 28.94, 36.73, 41.77, 46.26, 46.94, 49.53, 52.00, 168.40, 168.68, 170.30, 172.04. Anal. ($C_{19}H_{32}N_4O_5$) C, H.

D-Series Inhibitor. The experimental procedures applied, and the IR and NMR data of enantiomeric structures, for each of the D-series compounds were identical to those described above for the corresponding L-series compounds, except that the starting material was diethyl (+)-(2*S*,3*S*)-oxirane-2,3-dicarboxylate ((2*S*,3*S*)-**6**).[24] Their additional properties are as follows.

Ethyl (2*R*,3*R*)-3-[[(*S*)-3-methyl-1-[(3-methylbutyl)carbamoyl]butyl]carbamoyl]-2-aziridinecarboxylate (12b, EtO-(D)Az-Leu-NH-iAm): 55% yield from **10b**: mp 118–120 °C; $[\alpha]^{23}_D$ = −103.9° (*c* 0.44, EtOH). IR (KBr): 3299, 3293, 1743, 1641 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 0.84–0.96 (m, 12 H, 2 C*H*$_3$-Leu, 2 C*H*$_3$-iAm), 1.28 (t, *J* = 7.17 Hz, 3 H, OCH$_2$C*H*$_3$), 1.30–1.75 (m, 7 H, N*H*-Az, C*H*$_2$C*H*CH$_3$-Leu, C*H*$_2$C*H*CH$_3$-iAm), 2.56 (dd, *J* = 2.24, 7.73 Hz, 1 H, C*H*-Az), 2.82 (dd, *J* = 2.22, 8.95 Hz, 1 H, C*H*-Az), 3.16–3.26 (m, 2 H, NHC*H*$_2$-iAm), 4.20 (q, *J* = 7.16 Hz, 2 H, OC*H*$_2$CH$_3$), 4.31 (m, 1 H, C*H*-Leu), 6.01 (m, 1 H, N*H*-iAm), 6.70 (d, *J* = 8.35 Hz, 1 H, N*H*-Leu). ^{13}C NMR ($CDCl_3$): δ 14.00, 22.16, 22.33 (double peak), 22.73, 24.69, 25.73, 35.43, 37.43, 37.73, 38.18, 40.76, 51.25, 62.25, 167.97, 170,31, 171.22. Anal. ($C_{17}H_{31}N_3O_4$) C, H.

The D-inhibitor **12b** was obtained from the intermediates: diethyl (−)-(2*S*,3*R*)-3-azido-2-[(trimethylsilyl)oxy]succinate ((2*S*,3*R*)-**7**), 12% yield from (2*S*,3*S*)-**6**, $[\alpha]^{23}_D$ = −27.1° (*c* 1.11, EtOH); diethyl (−)-(2*S*,3*R*)-3-azido-2-hydroxysuccinate ((2*S*-3*R*)-**8**), 88% yield from (2*S*,3*S*)-**6**, $[\alpha]^{23}_D$ = −32.0° (*c* 1.64, EtOH); diethyl (2*R*,3*R*)-aziridine-2,3-dicarboxylate ((2*R*,3*R*)-**9b**, EtO-(D)Az-OEt), 54% yield of 5:1 mixture of (2*R*,3*R*)-**9b**

and the corresponding *cis*-aziridine, $[\alpha]^{23}_D = -133.4°$ (*c* 1.86, $CHCl_3$); and (2*R*,3*R*)-3-(ethoxycarbonyl)-2-aziridinecarboxylic acid ((2*R*,3*R*)-**10b**, EtO-(D)Az-OH), 52% yield, mp 129–131 °C dec, $[\alpha]^{23}_D = -153.9°$ (*c* 1.21, EtOH). Anal. ($C_6H_9NO_4$) C, H.

(2*R*,3*R*)-3-[[(*S*)-3-Methyl-1-[(3-methylbutyl)carbamoyl]-butyl]carbamoyl]-2-aziridinecarboxylic acid (14b, HO-(D)Az-Leu-NH-iAm): 44% yield from **12b**, mp 202–206 °C dec (without melting); $[\alpha]^{23}_D = -96.2°$ (*c* 0.41, CH_3OH). IR (KBr): 3491–3230 (br), 1720, 1706, 1642 cm^{-1}. ^1H NMR (DMSO-d_6): δ 0.81–0.90 (m, 12 H, 2 CH_3-Leu, 2 CH_3-iAm), 1.21–1.58 (m, 6 H, CH_2CHCH_3-Leu, CH_2CHCH_3-iAm), 2.40 (d, J =1.54 Hz, 1 H, C*H*-Az), 2.72 (d, J = 1.75 Hz, 1 H, C*H*-Az), 2.90–3.12 (m, 2 H, $NHCH_2$-iAm), 3.10–4.00 (broad signal, 2 H, *H*N-Az, COO*H*), 4.25 (m, 1 H, C*H*-Leu), 7.98 (t, J = 5.78 Hz, 1 H, N*H*-iAm), 8.30 (bs, 1 H, N*H*-Leu. ^{13}C NMR (DMSO-d_6): δ 21.75, 22.34, 22.37, 22.82, 24.30, 25.10, 34.80, 36.12, 36.72, 37.96, 41.23, 51.29, 167.88, 171.21, 171.43. Anal. ($3C_{15}H_{27}N_3O_4{\cdot}2H_2O$) C, H.

1-[*N*-[[(3*R*)-3-[[(2-Methylpropyl)amino]carbonyl]-(2*R*)-2-aziridinyl]carbonyl]-L-leucyl]-L-proline (16b, iBuNH-(D)Az-LeuPro-OH): 39% yield from **15b**; mp 205–208 °C dec (without melting); $[\alpha]^{23}_D = -131.5°$ (*c* 0.63, CH_3OH). IR (KBr): 3469–3236 (br), 1736, 1725, 1657, 1631 cm^{-1}. ^1H NMR ($CDCl_3$): δ 0.82–0.96 (m, 12 H, 2 CH_3-Leu, 2 CH_3-iBu), 1.49–2.32 (m, 10 H, CH_2CHCH_3-Leu, $CHCH_3$-iAm, $NCH_2CH_2CH_2$-Pro, *H*N-Az, COO*H*), 2.47 (dd, J = 2.20, 7.65 Hz, 1 H, C*H*-Az), 2.70 (dd, J = 2.28, 8.79 Hz, 1 H, C*H*-Az), 3.01–3.17 (m, 2 H, $NHCH_2$-iBu), 3.53–3.71 (m, 2 H, NCH_2-Pro), 4.46–4.52 (m, 1 H, C*H*-Leu), 4.78–4.90 (m, 1 H, C*H*-Pro), 6.57 (m, 1H, N*H*-iBu), 7.14 (d, J = 8.14 Hz, 1 H, N*H*-Leu). ^{13}C NMR (DMSO-d_6): δ 19.87 (double peak), 21.85, 22.95, 24.30, 24.45, 28.40, 28.91, 36.72, 41.70, 46.25, 46.93, 49.50, 52.06, 168.10, 168.45, 170.30, 171.99. Anal. ($C_{19}H_{32}N_4O_5$) C, H.

The D-inhibitor **16b** was obtained from the intermediates: ethyl (2*R*,3*R*)-3-[(2-methylpropyl)carbamoyl]-2-aziridinecarboxylate (**11b**, EtO-(D)Az-NH-iBu), 69% yield from (2*R*,3*R*)-**10b**, mp 60–61 °C, $[\alpha]^{23}_D = -119.0°$ (*c* 1.26, EtOH); (2*R*,3*R*)-3-[(2-methylpropyl)carbamoyl]-2-aziridinecarboxylic acid (**13b**, HO-(D)Az-NH-iBu), 81% yield from **11b**, mp 187–190 °C dec (without melting), $[\alpha]^{23}_D = -71.8°$ (*c* 0.57, H_2O); and 1-[*N*-[[(3*R*)-3-[[(2-methylpropyl)amino]carbonyl]-(2*R*)-2-aziridinyl]-carbonyl]-L-leucyl]-L-proline methyl ester (**15b**, iBuNH-(D)Az-LeuPro-OMe): 44% yield from **13b**; mp 124–126 °C, $[\alpha]^{23}_D = -142.7°$ (*c* 0.59, CH_3OH). IR (KBr): 3344, 3300, 1747, 1657, 1630 cm^{-1}. ^1H NMR ($CDCl_3$): δ 0.83–0.96 (m, 12 H), 1.47–2.22 (m, 9 H), 2.35 (d, J = 7.5 Hz, 1 H), 2.68 (d, J = 7.8 Hz, 1 H), 2.84–3.11 (m, 2 H), 3.53–3.80 (m, 2 H), 3.68 (s, 3 H), 4.42–4.74 (m, 2 H), 6.54 (t, J = 6.1 Hz, 1 H), 7.4 (d, J = 8.3 Hz, 1 H). ^{13}C NMR ($CDCl_3$): δ 19.97, 23.24, 24.48, 24.80, 28.36, 28.86, 36.92, 37.00, 40.60, 41.14, 46.06, 46.75, 49.83, 52.16, 58.70, 168.47, 171.06, 171.12, 172.13. Anal. ($C_{20}H_{34}N_4O_5$) C, H.

Kinetic and Mass Spectrometric Measurements. Papain and recombinant rat cathepsin B were prepared as described previously.[30] Recombinant human cathepsin L was kindly provided by Dr. John S. Mort (Shriners Hospital for Crippled Children, Montreal, Canada). The assay buffer contained 50 mM buffer (sodium citrate, pH 3.0–5.7, phosphate, pH 5.7–7.9, and borate, pH 7.9–9.0), 0.2 M NaCl, 5 mM EDTA, and 1 mM DTT. All kinetic experiments were done at 25 °C and in the presence of 10% acetonitrile. The inactivation of cysteine proteases was monitored as described previously,[17] and the second-order rate constant for inactivation, k_{inac}/K_i, was determined using the method of Tian and Tsou.[31] For the determination of the pH dependency of inhibition, the rate constant for inactivation at a given pH was measured at [I] << K_i and the k_{inac}/K_i value obtained by dividing k_{obs} by the inhibitor concentration.

Mass spectrometric analyses of enzyme–inhibitor adducts formed between papain and compounds **14a,b** were performed on a triple quadrupole mass spectrometer (the API III LC/MS/MS system; Sciex, Thornhill, Ontario, Canada) as described previously.[19,32]

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References

(1) (a) Rich, D. H. Inhibitors of Cysteine Proteinases. In *Proteinase Inhibitors*; Barrett, A. J., Salvesen, G. S., Eds.; Elsevier Science Publishers B. V.: Amsterdam, The Netherlands, 1986; pp 153–178. (b) Demuth, H.-U. Recent Developments in Inhibiting Cysteine and Serine Proteases. *J. Enzyme Inhib.* **1990**, *3*, 249–278. (c) Shaw, E. Cysteinyl Proteinases and Their Selective Inactivation. *Adv. Enzymol. Rel. Areas Mol. Biol.* **1990**, *63*, 271–347.

(2) Hanada, K.; Tamai, M.; Yamagishi, M.; Ohmura, S.; Sawada, J.; Tanaka, I. Isolation and Characterization of E-64, a New Thiol Protease Inhibitor. *Agric. Biol. Chem.* **1978**, *42*, 523–528.

(3) Hanada, K.; Tamai, M.; Ohmura, S.; Sawada, J.; Seki, T.; Tanaka, I. Studies on Thiol Protease Inhibitors. Part II. Structure and Synthesis of E-64, a New Thiol Protease Inhibitor. *Agric. Biol. Chem.* **1978**, *42*, 529–536.

(4) (a) Barrett, A. J.; Kembhavi, A. A.; Brown, M. A.; Kirschke, H.; Knight, C. G.; Tamai, M.; Hanada, K. L-*trans*-Epoxysuccinylleucylamido(4-guanidino)butane (E-64) and Its Analogues as Inhibitors of Cysteine Proteinases Including Cathepsins B, H and L. *Biochem. J.* **1982**, *201*, 189–198. (b) Tamai, M.; Yokoo, C.; Murata, M.; Oguma, K.; Sota, K.; Sato, E.; Kanaoka, Y. An Efficient Synthetic Method for Ethyl (+)-(2S,3S)-3-[(S)-3-methyl-1-(3-methylbutylcarbamoyl)butylcarbamoyl]-2-oxiranecarboxylate (EST), a New Inhibitor of Cysteine Proteinases. *Chem. Pharm. Bull.* **1987**, *35*, 1098–1104.

(5) Hanada, K.; Tamai, M.; Morimoto, S.; Adachi, T.; Ohmura, S.; Sawada, J.; Tanaka, I. Inhibitory Activities of E-64 Derivatives on Papain. *Agric. Biol. Chem.* **1978**, *42*, 537–541.

(6) Bihovsky, R.; Powers, J. C.; Kam, C.-M.; Walton, R.; Loewi, R. C. Further Evidence for the Importance of Free Carboxylate in Epoxysuccinate Inhibitors of Thiol Proteases. *J. Enzyme Inhib.* **1993**, *7*, 15–25.

(7) Yabe, Y.; Guillaume, D.; Rich, D. H. Irreversible Inhibition of Papain by Epoxysuccinyl Peptides. ^{13}C NMR Characterization of the Site of Alkylation. *J. Am. Chem. Soc.* **1988**, *110*, 4043–4044.

(8) Varughese, K. I.; Ahmed, F. R.; Carey, P. R.; Hasnain, S.; Huber, C. P.; Storer, A. C. Crystal Structure of a Papain-E-64 Complex. *Biochemistry* **1989**, *28*, 1330–1332.

(9) Drenth, J.; Kalk, K. H.; Swen, H. M. Binding of Chloromethyl Ketone Substrate Analogues to Crystalline Papain. *Biochemistry* **1976**, *15*, 3731–3738.

(10) Hanada, K.; Tamai, M.; Morimoto, S.; Adachi, T.; Oguma, K.; Ohmura, S.; Ohzeki, M. A Specific Thiolproteinase Inhibitor, E-64 and Its Derivatives. In *Peptide Chemistry 1979: Proceedings of the 17th Synposium on Peptide Chemistry*; Yonehara, H., Ed.; Protein Research Foundation: Osaka, Japan, 1980; pp 31–36.

(11) (a) Komatsu, K.; Inazuki, K.; Hosoya, J.; Satoh, S. Beneficial Effect of New Thiol Protease Inhibitors, Epoxide Derivatives, on Dystrophic Mice. *Exp. Neurol.* **1986**, *91*, 23–29. (b) Tamai, M.; Omura, S.; Kimura, M.; Hanada, K.; Sugita, H. Prolongation of Life Span of Dystrophic Hamster by Cysteine Proteinase Inhibitor, Loxistatin (EST). *J. Pharmacobio-Dyn.* **1987**, *10*, 678–681. (c) Tamai, M.; Matsumoto, K.; Oguma, K.; Koyama, I.; Omura, S.; Hanada, K.; Ishiura, S.; Sugita, H. EST, a New Analog of E-64, Can Prolong the Life Span of Dystrophic Hamsters, UM-X7. In *Cysteine Proteinases and Their Inhibitors*; Turk, V., Ed.; Walter de Gruyter & Co.: New York, 1986; pp 633–648.

(12) Tamai, M.; Matsumoto, K.; Ohmura, S.; Koyama, I.; Ozawa, Y.; Hanada, K. *In vitro* and *In vivo* Inhibition of Cysteine Proteinase by EST, a New Analogue of E-64. *J. Pharmacobio-Dyn.* **1986**, *9*, 672–677.

(13) Shoji-Kasai, Y.; Senshu, M.; Iwashita, S.; Imahori, K. Thiol Protease-Specific Inhibitor E-64 Arrests Human Epidermoid Carcinoma A431 Cells at Mitotic Metaphase. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 146–150.

(14) (a) Hanada, K.; Tamai, M.; Adachi, T.; Oguma, K.; Kashiwagi, K.; Ohmura, S.; Kominami, E.; Towatari, T.; Katunuma, N. Characterization of the Three New Analogs of E-64 and Their Therapeutic Application. In *Proteinases Inhibitors: Medical and Biological Aspects*; Katunuma, N., Ed.; Springer-Verlag: Berlin, 1983; pp 25–36. (b) Prous, J. R., Ed. NCO-700. *Drugs Future* **1986**, *11*, 941–943.

(15) (a) Yamamoto, D.; Matsumoto, K.; Ohishi, H.; Ishida, T.; Inoue, M.; Kitamura, K.; Hanada, K. The Importance of Val-157 Hydrophobic Interaction for Papain Inhibitory Activity of an Epoxysuccinyl Amino Acid Derivative. A Structure-Activity Relationship Based on the Crystal Structure of the Papain-E-64-c Complex. *FEBS Lett.* **1990**, *263*, 134–136. (b) Murata, M.; Miyashita, S.; Yokoo, C.; Tamai, M.; Hanada, K.; Hatayama, K.; Towatari, T.; Nikawa, T.; Katunuma, N. Novel Epoxysuccinyl Peptides. Selective Inhibitors of Cathepsin B *in vitro*. *FEBS Lett.* **1991**, *280*, 307–310. (c) Towatari, T.; Nikawa, T.; Murata, M.;

Yokoo, C.; Tamai, M.; Hanada, K. Novel Epoxysuccinyl Peptides. A Selective Inhibitor of Cathepsin B, *in vivo*. *FEBS Lett.* **1991**, *280*, 311–315. (d) Hashida, S.; Towatari, T.; Kominami, E.; Katunuma, N. Inhibitions by E-64 Derivatives of Rat Liver Cathepsin B and Cathepsin L *in vitro* and *in vivo*. *J. Biochem. (Tokyo)* **1980**, *88*, 1805–1811.

(16) Baker, E. N.; Drenth, J. The Thiol Proteases: Structure and Mechanism. In *Biological Macromolecules and Assemblies, Vol. 3: Active Sites of Enzymes*; Jurnak, F. A., McPherson, A., Eds.; John Wiley and Sons: New York, 1987; pp 313–368.

(17) Gour-Salin, B. J.; Lachance, P.; Magny, M.-C.; Plouffe, C.; Ménard, R.; Storer, A. C. E64 [*trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane] Analogues as Inhibitors of Cysteine Proteinases: Investigation of S_2 Subsite Interactions. *Biochem. J.* **1994**, *299*, 389–392.

(18) Buttle, D. J.; Murata, M.; Knight, C. G.; Barrett, A. J. CA074 Methyl Ester: A Proinhibitor for Intracellular Cathepsin B. *Arch. Biochem. Biophys.* **1992**, *299*, 377–380.

(19) Gour-Salin, B. J.; Lachance, P.; Plouffe, C.; Storer, A. C.; Ménard, R. Epoxysuccinyl Dipeptides as Selective Inhibitors of Cathepsin B. *J. Med. Chem.* **1993**, *36*, 720–725.

(20) Musil, D.; Zucic, D.; Turk, D.; Engh, R. A.; Mayr, I.; Huber, R.; Popovic, T.; Turk, V.; Towatari, T.; Katunuma, N.; Bode, W. The Refined 2.15 Å X-Ray Crystal Structure of Human Liver Cathepsin B: The Structural Basis for its Specificity. *EMBO J.* **1991**, *10*, 2321–2330.

(21) (a) Okawa, K.; Nakajima, K. Studies on 2-Aziridinecarboxylic Acid Peptides: Their Reaction and Uses. *Biopolymers* **1981**, *20*, 1811–1821. (b) Hata, Y.; Watanabe, M. Reaction of Aziridinecarboxylic Acids with Thiols in Aqueous Solution. The Formation of β-Amino Acid. *Tetrahedron* **1987**, 3881–3888.

(22) Moroder, L.; Musiol, H.-J.; Scharf, R. Aziridine-2-carboxylic Acid. A Reactive Amino Acid Unit for a New Class of Cysteine Proteinase Inhibitors. *FEBS Lett.* **1992**, 51–53.

(23) Legters, J.; Thijs, L.; Zwanenburg, B. Synthesis of Naturally Occurring (2S,3S)-(+)-Aziridine-2,3-Dicarboxylic Acid. *Tetrahedron* **1991**, *47*, 5287–5294.

(24) Mori, K.; Iwasawa, H. Pheromone Synthesis. XXXV. Stereoselective Synthesis of Optically Active Forms of δ-Multistriatin, the Attractant for European Populations of the Smaller European Elm Bark Beetle. *Tetrahedron* **1980**, *36*, 87–90.

(25) Dermer, O. C.; Ham, G. E. *Ethyleneimine and Other Aziridines*; Academic Press: New York, 1969; p 106.

(26) Dixon, H. B. F. Shapes of Curves of pH-Dependence of Reactions. *Biochem. J.* **1973**, *131*, 149–154.

(27) Khouri, H. E.; Vernet, T.; Ménard, R.; Parlati, F.; Laflamme, P.; Tessier, D. C.; Gour-Salin, B.; Thomas, D. Y.; Storer, A. C. Engineering of Papain: Selective Alteration of Substrate Specificity by Site-Directed Mutagenesis. *Biochemistry* **1991**, *30*, 8929–8936.

(28) Perrin, D. D.; Armarego, W. L. F. *Purification of Laboratory Chemicals*, 3rd ed.; Pergamon Press: New York, 1988.

(29) Stahl, G. L.; Walter, R.; Smith, C. W. General Procedure for the Synthesis of Mono-N-Acylated 1,6-Diaminohexanes. *J. Org. Chem.* **1978**, *43*, 2285–2286.

(30) (a) Ménard, R.; Khouri, H. E.; Plouffe, C.; Dupras, R.; Ripoll, D.; Vernet, T.; Tessier, D. C.; Laliberté, F.; Thomas, D. Y.; Storer, A. C. A Protein Engineering Study of the Role of Aspartate 158 in the Catalytic Mechanism of Papain. *Biochemistry* **1990**, *29*, 6706–6713. (b) Fox, T.; de Miguel, E.; Mort, J. S.; Storer, A. C. Potent Slow-Binding Inhibition of Cathepsin B by Its Propeptide. *Biochemistry* **1992**, *31*, 12571–12576. (c) Rowan, A. D.; Mason, P.; Mach, L.; Mort, J. S. Rat Procathepsin B. Proteolytic Processing to the Mature Form *in vitro*. *J. Biol. Chem.* **1992**, *267*, 15993–15999.

(31) Tian, W. X. D.; Tsou, C. L. Determination of the Rate Constant of Enzyme Modification by Measuring the Substrate Reaction in the Presence of the Modifier. *Biochemistry* **1982**, *21*, 1028–1032.

(32) Ménard, R.; Carmona, E.; Plouffe, C.; Brömme, D.; Konishi, Y.; Lefebvre, J.; Storer, A. C. The Specificity of the S_1' Subsite of Cysteine Proteases. *FEBS Lett.* **1993**, *328*, 107–110.

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