Active-Site Specific Inhibitors of Elastase†

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ABSTRACT: A series of five tripeptide and four tetrapeptide chloromethyl ketones containing alanine as the P_1 residue was synthesized as active-site directed irreversible inhibitors of elastase. Most of the compounds were effective elastase inhibitors, being both reactive and specific, with Ac-Ala-Ala-Pro-AlaCH_2Cl being the most reactive. Chymotrypsin and trypsin which are closely related to elastase were not significantly inhibited by these peptide chloromethyl ketones under identical reaction conditions. The tetrapeptide inhibitors were 10–50 times more reactive than the tripeptides. The increased reactivity in terms of k_3/K_I was due both to the better binding of inhibitor to elastase (the K_I of Ac-Ala-Ala-Ala-AlaCH_2Cl is three times lower than that of Ac-Ala-Ala-AlaCH_2Cl) and to the increased rate of reaction within the bound complex (the k_3 for Ac-Ala-Ala-Ala-AlaCH_2Cl is three times higher than that for Ac-Ala-Ala-AlaCH_2Cl). Since peptide substrates exhibit a similar reactivity difference between tripeptides and tetrapeptides, both inhibitors and substrates are probably interacting with the same extended binding site in elastase. Proline and/or leucine are the preferred residues at the P_2 site of an inhibitor, while proline at the P_3 site renders an inhibitor incapable of inhibiting elastase. Several peptide chloromethyl ketone inhibitors have proven useful in studying the biological function of other elastolytic enzymes.

Pulmonary emphysema is currently thought to result from the uninhibited proteolysis of lung tissue by elastase and related neutral proteases derived from leukocytes and macrophages (Mittman, 1972). During infection or inflammation, the normal lung is protected from proteolytic digestion by the protease inhibitor, α_1-antitrypsin. Individuals with an α_1-antitrypsin deficiency, either genetically or due to other causes, are not so protected. Synthetic elastase inhibitors capable of replacing α_1-antitrypsin would therefore be expected to be useful reagents both for the treatment of emphysema and related diseases and for the study of the biological function of elastolytic enzymes.

Synthetic inhibitors with a variety of functional groups have been shown to react stoichiometrically with elastase, including reagents such as diisopropyl phosphorofluoridate, *p*-nitrophenyl diethyl phosphate, and sulfonyl fluorides such as tosyl fluoride. All of these inhibitors react with the strongly nucleophilic active site serine residue of serine proteases, including Ser-188 of elastase (Hartley and Shotton, 1971). Brown and Wold (1973a,b) have recently shown that *n*-butyl isocyanate also inhibits elastase by reaction at Ser-188. This reagent was not specific for elastase since in addition it inhibited chymotrypsin. Stoichiometric alkylation of the γ-carboxyl group of a glutamic acid residue, tentatively identified as Glu-6, by 1-bromo-4-(2,4-dinitrophenyl)butan-2-one inhibits porcine pancreatic elastase (Visser *et al.*, 1971). However, this reagent was incapable of inhibiting a human leukocyte elastolytic enzyme (Janoff, 1969). Thus no suitable inhibitors specific for elastolytic enzymes were available when we began this research.

Peptide chloromethyl ketones appeared to be likely candidates for use as potential elastase inhibitors. These compounds are relatives of Tos-PheCH_2Cl and Tos-LysCH_2Cl, the active site specific inhibitors of chymotrypsin and trypsin developed by Shaw and his coworkers (Shaw, 1970). The crystallographic determinations of the binding modes of peptide chloromethyl ketones to chymotrypsin A_γ (Segal *et al.*, 1971a,b) and to subtilisin BPN′ (Kraut *et al.*, 1971; Robertus *et al.*, 1972) have provided revealing insights into the interactions of inhibitors with these serine proteases. The inhibitors are bound to the enzyme *via* a covalent linkage between the imidazole ring of the active site histidine residue and the methylene group of the chloromethyl ketone moiety. The peptide chain of an extended inhibitor and a section of three residues of the backbone of the enzyme form an antiparallel β-sheet structure. In addition, the rates of inactivation of chymotrypsin A_α (Kurachi *et al.*, 1973) and subtilisin BPN′ (J. C. Powers and J. T. Tippett, unpublished observations) by peptide chloromethyl ketones in solution are dependent upon the interactions between the enzyme and inhibitor both in the region of the primary specificity site and at subsites far removed from the catalytic site.

In order to be both specific for and reactive toward elastase, we felt that a peptide chloromethyl ketone must possess two structural features. First, it would have to be a derivative of alanine chloromethyl ketone since the enzyme has demonstrated a specificity toward alanine residues. Studies with *N*-benzyloxycarbonyl amino acid *p*-nitrophenyl esters (Geneste and Bender, 1969), *N*-benzoyl amino acid methyl esters (Kaplan *et al.*, 1970), oxidized insulin A and B chains (Narayanan and Anwar, 1969), and ribonuclease S peptide (Atlas *et al.*, 1970) have shown that elastase most readily cleaves peptide bonds C terminal to alanine residues. Work with synthetic peptide substrates has also shown that the rate of elastase hydrolysis is strongly dependent on peptide chain length (Atlas *et al.*, 1970; Atlas and Berger, 1972; Thompson and Blout, 1970, 1973c.). Thus the second required structural feature is an extended peptide chain in the inhibitor which also increases the resemblance of the inhibitor to elastase's natural substrate, elastin. Acyl amino acid chloromethyl ke-

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tones lacking an extended peptide chain such as Tos-Ala-CH_2Cl, $C_6H_5SO_2$-AlaCH_2Cl, Tos-GlyCH_2Cl, and Tos-Val-CH_2Cl have been shown to be ineffective elastase inhibitors (Visser *et al.*, 1971; Kaplan *et al.*, 1970).

This paper reports the synthesis of a series of peptide chloromethyl ketones which are both reactive and selective elastase inhibitors. Subsequent to our preliminary communication of these results (Powers and Tuhy, 1972), Thompson and Blout (1973a) independently reported similar studies.

Materials and Methods

Porcine pancreatic elastase (batch 24202) was obtained from Whatman Biochemicals Ltd. and was used without further purification; its substrate, BOC-Ala-ONp, was purchased from Sigma Chemical Co. Chymotrypsin A_α (lot CDIOBK) was obtained from Worthington Biochemical Corp., and its substrate, Ac-Tyr-OEt, was synthesized by standard methods and had mp 80–81°. Trypsin (lot 102C-1920) and its substrate, Bz-Arg-OEt, were obtained from Sigma Chemical Co. The tripeptide acids Ac-Ala-Ala-Ala-OH, Ac-Ala-Ala-Pro-OH, Ac-Ala-Ala-Phe-OH, and Ac-Ala-Pro-Ala-OH were synthesized by Dr. A. Ali. Two peptide chloromethyl ketones, Ac-Ala-Gly-AlaCH_2Cl and Z-Gly-Leu-AlaCH_2Cl, were synthesized by Mr. C. Joiner. All other amino acid derivatives, reagents, and solvents used were analytical grade. Mass spectra were taken on a Varian M-66 instrument and nuclear magnetic resonance (nmr) spectra were taken on a Varian A-60 instrument. Thin-layer chromatography was performed using Merck silica gel G plates.

*N-Benzyloxycarbonyl-*L*-alanine Chloromethyl Ketone* (Z-AlaCH_2Cl). The diazo ketone Z-AlaCHN_2 was prepared from Z-Ala-OH and diazomethane using a mixed anhydride method (Penke *et al.*, 1970). Anhydrous HCl was bubbled through a solution of Z-AlaCHN_2 (*ca.* 60 mmol) in ether–tetrahydrofuran at 5° until it turned colorless. Work-up of the reaction mixture involved evaporation of the ether solvent, extraction of the residue into ethyl acetate, washing with citric acid and $NaHCO_3$ solutions, drying over anhydrous $MgSO_4$, and evaporation to an oil that crystallized upon standing. The product was recrystallized from ethyl acetate–cyclohexane (1:3) to give 9.60 g (63%) of a white solid, mp 88–89°, $[\alpha]_D^{25} = -42°$ (*c* 1.8, MeOH), R_F 0.84 ($CHCl_3$–CH_3OH, 9:1); Thompson and Blout (1973a) report mp 87–88°, $[\alpha]_D^{25}$ −43.6° (*c* 2.8, MeOH). The mass spectrum had major peaks at *m/e* 178 ($C_6H_5CH_2OCONHCH(CH_3)^+$), 134 ($C_6H_5CH_2OCONHCH(CH_3)^+ - CO_2$), and 91 ($C_6H_5CH_2^+$); no M^+ or M − HCl peaks were observed. *Anal.* Calcd for $C_{12}H_{14}ClNO_3$: C, 56.37; H, 5.52; N, 5.48. Found: C, 56.27; H, 5.34; N, 5.60.

L-*Alanine Chloromethyl Ketone Hydrobromide* (HBr·Ala-CH_2Cl). Z-AlaCH_2Cl (5.0 g, 20 mmol) was deblocked by dissolving in 6 ml of a 32% solution of hydrogen bromide in acetic acid. Within 3 min the product crystallized out and was washed with 100 ml of ether. The product was recrystallized from acetone–ethyl acetate (3:2) to give 2.53 g (62%) of a hygroscopic white solid. It was immediately dissolved in 10 ml of dimethylformamide and used in further coupling reactions.

*N-Acetyl-*L*-alanyl-*L*-alanyl-*L*-alanine chloromethyl ketone* (Ac-Ala-Ala-AlaCH_2Cl) was prepared using a mixed anhydride method. The dipeptide acid Ac-Ala-Ala-OH was synthesized from Ac-Ala-OH and HCl·Ala-OMe by standard peptide coupling methods. Ac-Ala-Ala-OH (1.21 g, 6 mmol) was dissolved in 50 ml of dimethylformamide and stirred at −20°, while *N*-methylmorpholine (0.66 ml, 6 mmol) and isobutyl chloroformate (0.78 ml, 6 mmol) were added. After 5 min, HBr·AlaCH_2Cl (1.21 g, 6 mmol, in 10 ml of DMF) and *N*-methylmorpholine (0.66 ml, 6 mmol) were added, and the mixture was allowed to warm to 25° while stirring for 4 hr. Then the solvent was removed *in vacuo* and the product was isolated from the residue after extraction of all other components into 60 ml of hot acetone. The product was recrystallized from 95% ethanol to give 0.45 g (25%) of a white solid, mp 194–195°, R_F 0.70 ($CHCl_3$–CH_3OH, 4:1). The mass spectrum had major peaks at *m/e* 305 (M^+), 269 (M − HCl), 256 (Ac-Ala-Ala-Ala$^+$), 228 (Ac-Ala-Ala-NHCH$(CH_3)^+$), 185 (Ac-Ala-Ala$^+$), 157 (Ac-Ala-NHCH$(CH_3)^+$), 114 (Ac-Ala$^+$ or -Ala-NHCH$(CH_3)^+$), and 86 (Ac-NHCH$(CH_3)^+$). *Anal.* Calcd for $C_{12}H_{20}ClN_3O_4$: C, 47.14; H, 6.59; N, 13.74. Found: C, 46.72; H, 6.53; N, 13.53.

*N-Acetyl-*L*-alanyl-*L*-prolyl-*L*-alanine chloromethyl ketone* (Ac-Ala-Pro-AlaCH_2Cl) was prepared similarly to Ac-Ala-Ala-AlaCH_2Cl from Ac-Ala-Pro-OH (made by standard methods) and HBr·AlaCH_2Cl. In this case the product was isolated from the residue by chromatography on a silica gel column from which it was eluted with chloroform–methanol (19:1). The product was recrystallized from ethanol to give 0.72 g (43%) of a white solid, mp 172–173° (Thompson and Blout (1973a) report mp 176–180°), R_F 0.73 ($CHCl_3$–CH_3OH, 4:1). The mass spectrum had major peaks at *m/e* 331 (M^+), 295 (M − HCl), 254 (Ac-Ala-Pro-NHCH$(CH_3)^+$), 211 (Ac-Ala-Pro$^+$), 183 (Ac-Ala-$C_4H_7N^+$), 140 (-Ala-$C_4H_7N^+$), 114

$$C_4H_7N^+ = N^+{=}(CH_2)_3CH^+$$

(Ac-Ala$^+$), and 86 (Ac-NHCH$(CH_3)^+$). *Anal.* Calcd for $C_{14}H_{22}ClN_3O_4$: C, 50.68; H, 6.68; N, 12.66. Found: C, 50.80; H, 6.86; N, 12.51.

*N-Acetyl-*L*-prolyl-*L*-alanyl-*L*-alanine chloromethyl ketone* (Ac-Pro-Ala-AlaCH_2Cl) was prepared similarly to Ac-Ala-Pro-AlaCH_2Cl from Ac-Pro-Ala-OH (made by standard methods) and HBr·AlaCH_2Cl. The product was recrystallized from ethyl acetate to give 0.92 g (55%) of a white solid, mp 142–143°, R_F 0.73 ($CHCl_3$–CH_3OH, 4:1). The mass spectrum had major peaks at *m/e* 311 (M^+), 295 (M − HCl), 254 (Ac-Pro-Ala-NHCH$(CH_3)^+$), 211 (Ac-Pro-Ala$^+$), 183 (Ac-Pro-NHCH$(CH_3)^+$), 140 (Ac-Pro$^+$ or -Pro-NHCH$(CH_3)^+$), and 112 (Ac-$C_4H_7N^+$). *Anal.* Calcd for $C_{14}H_{22}ClN_3O_4$: C, 50.68; H, 6.68; N, 12.66. Found: C, 50.78; H, 6.73; N, 12.63.

*N-Acetyl-*L*-alanyl-*L*-alanyl-*L*-alanyl-*L*-alanine chloromethyl ketone* (Ac-Ala-Ala-Ala-AlaCH_2Cl) was prepared similarly to Ac-Ala-Ala-AlaCH_2Cl from Ac-Ala-Ala-Ala-OH and HBr·AlaCH_2Cl. The product was recrystallized from methanol to give 1.28 g (57%) of a white solid, mp 252–253°, R_F 0.60 ($CHCl_3$–CH_3OH, 4:1). The mass spectrum had major peaks at *m/e* 340 (M − HCl), 299 (Ac-Ala-Ala-Ala-NHCH$(CH_3)^+$), 256 (Ac-Ala-Ala-Ala-Ala$^+$), 228 (Ac-Ala-Ala-NHCH$(CH_3)^+$), 185 (Ac-Ala-Ala$^+$), 157 (Ac-Ala-NHCH$(CH_3)^+$), 141 ((-Ala-Ala$^+$) − H), and 114 (Ac-Ala$^+$); no M^+ peak was observed. *Anal.* Calcd for $C_{15}H_{25}ClN_4O_5$: C, 47.81; H, 6.69; N, 14.87. Found: C, 48.20; H, 6.83; N, 14.54.

*N-Acetyl-*L*-alanyl-*L*-alanyl-*L*-prolyl-*L*-alanine chloromethyl ketone* (Ac-Ala-Ala-Pro-AlaCH_2Cl) was prepared similarly to Ac-Ala-Ala-AlaCH_2Cl from Ac-Ala-Ala-Pro-OH and HBr·AlaCH_2Cl. The product was recrystallized from 95% ethanol–acetone to give 0.48 g (20%) of a white solid, mp 187–188°, R_F 0.69 ($CHCl_3$–CH_3OH, 4:1). The mass spectrum had major peaks at *m/e* 366 (M − HCl), 325 (Ac-Ala-Ala-Pro-NHCH$(CH_3)^+$), 282 (Ac-Ala-Ala-Pro$^+$), 254 (Ac-Ala-Ala-$C_4H_7N^+$), 185 (Ac-Ala-Ala$^+$), 167 ((-Ala-Pro$^+$) − H), 157 (Ac-Ala-NHCH$(CH_3)^+$), and 114 (Ac-Ala$^+$); no M^+ peak was

observed. *Anal.* Calcd for $C_{17}H_{27}ClN_4O_5$: C, 50.68; H, 6.76; N, 13.91. Found: C, 50.48; H, 6.79; N, 13.52.

N-Acetyl-L-alanyl-L-alanyl-L-phenylalanyl-L-alanine chloromethyl ketone (Ac-Ala-Ala-Phe-AlaCH$_2$Cl) was prepared similarly to Ac-Ala-Ala-AlaCH$_2$Cl from Ac-Ala-Ala-Phe-OH and HBr·AlaCH$_2$Cl. The product was recrystallized from methanol to give 0.56 g (41%) of a white solid, mp 236–237°, R_F 0.76 ($CHCl_3$–CH_3OH, 4:1). The mass spectrum had major peaks at *m/e* 416 (M − HCl), 375 (Ac-Ala-Ala-Phe-NH-$CH(CH_3)^+$), 332 (Ac-Ala-Ala-Phe$^+$), 304 (Ac-Ala-Ala-NH-$CH(CH_2Ph)^+$), 217 ((-Ala-Phe$^+$) − H), 185 (Ac-Ala-Ala$^+$), 157 (Ac-Ala-NHCH$(CH_3)^+$), and 114 (Ac-Ala$^+$); no M^+ peak was observed. *Anal.* Calcd for $C_{21}H_{27}ClN_4O_5$: C, 55.69; H, 6.45; N, 12.37. Found: C, 55.33; H, 6.33; N, 12.20.

N-Acetyl-L-alanyl-L-prolyl-L-alanyl-L-alanine chloromethyl ketone (Ac-Ala-Pro-Ala-AlaCH$_2$Cl) was prepared similarly to Ac-Ala-Pro-AlaCH$_2$Cl from Ac-Ala-Pro-Ala-OH and HBr·AlaCH$_2$Cl. The product was recrystallized from ethanol–ethyl acetate to give 0.27 g (15%) of a white solid, mp 173–174°, R_F 0.69 ($CHCl_3$–CH_3OH, 4:1). The mass spectrum had major peaks at *m/e* 366 (M − HCl), 325 (Ac-Ala-Pro-Ala-NHCH$(CH_3)^+$), 282 (Ac-Ala-Pro-Ala$^+$), 254 (Ac-Ala-Pro-NHCH$(CH_3)^+$), 211 (Ac-Ala-Pro$^+$), 183 (Ac-Ala-$C_4H_7N^+$), 167 ((-Pro-Ala$^+$) − H), and 114 (Ac-Ala$^+$); no M^+ peak was observed. *Anal.* Calcd for $C_{17}H_{27}ClN_4O_5$: C, 50.68; H, 6.76; N, 13.91. Found: C, 50.43; H, 6.87; N, 13.42.

Reaction of Elastase with Inhibitors. Inhibition of elastase with the series of peptide chloromethyl ketones was carried out in solutions which contained at least a tenfold excess of inhibitor over enzyme. The tetrapeptide compounds were relatively insoluble in water and were soluble in methanol only to *ca.* 1–2 mM. Two sets of inhibition conditions were employed: pH 6.5 (0.1 M phosphate buffer) with 100-fold excess of inhibitor for "slow" inhibitors, and pH 5.0 (0.1 M acetate buffer) with tenfold excess of inhibitor for "fast" inhibitors. For compounds used in concentration-dependent determinations, a stock solution of inhibitor was prepared, *ca.* 1–10 mM in methanol, and used within 4 days. Otherwise the inhibitor solution was directly prepared. A solution (10 ml) of inhibitor at the appropriate concentration, buffered at pH 6.5 or 5.0, and in 10% (v/v) methanol, was prepared 60 min before the start of the inhibition run. Its concentration was 1.0 mM, 0.1 mM, or a graded series (concentration-dependent studies, see Table II). A stock solution of elastase prepared in 1 mM HCl was stored at 4° and had a concentration of 9.3 μM or 0.23 mg/ml by uv absorbance. The inhibition reactions were performed at 30° and were started by mixing 1 ml of the inhibitor solution with 1 ml of the enzyme solution. The final concentrations were as follows: inhibitor, 0.5 mM, 0.05 mM, or a graded series (concentration-dependent studies); enzyme, 4.7 μM; and methanol, 5% (v/v). In the concentration-dependent studies the variable inhibitor concentration extended over the range from $0.2x$ to x, where x was the largest inhibitor concentration, usually less than 1.0 mM. Seven or eight aliquots (100 μl) were removed from the inhibition mixture at regular time intervals and the residual elastase activity was measured at each point by means of the BOC-Ala-ONp spectrophotometric assay (Visser and Blout, 1972). Control experiments identical with inhibition runs but without inhibitor showed that the active enzyme concentration did not decrease more than 3% in 90 min. For each inhibition reaction the values of the kinetic parameters k_{obsd} and $k_{2nd} = k_{obsd}/[I]$ were calculated from the equation

$$v = k_{obsd}[E] = k_{2nd}[I][E]$$

using a least-squares computer program. For compounds for which a series of inhibition runs was done at differing concentrations, the values of the kinetic parameters k_3/K_I, k_3, and K_I were calculated from the equation

$$1/k_{obsd} = (K_I/k_3[I]) + (1/k_3)$$

using another least-squares computer program. Correlation coefficients of better than 0.993 were obtained throughout.

Reaction of Chymotrypsin and Trypsin with Inhibitors. Inhibition experiments with chymotrypsin and trypsin were carried out under conditions virtually identical with those employed in the inhibition runs with elastase. Solutions of inhibitors and enzymes were made up to the correct concentrations as required. The inhibition reactions were performed at 30° and were started by mixing 0.5 ml of the inhibitor solution, 0.5 ml of the enzyme solution, and 9 ml of buffer. The final concentrations were as follows: inhibitor, 0.5 mM or 0.05 mM; enzyme, 5 μM; and methanol, 5% (v/v). Enzyme assays were periodically performed using a Radiometer automated pH-stat to measure the initial hydrolysis rate of substrate at pH 7.8. Chymotrypsin was assayed using Ac-Tyr-OEt as the substrate (Wilcox, 1970) and trypsin was assayed using Bz-Arg-OEt as the substrate (Walsh and Wilcox, 1970).

Results

Synthesis of the Inhibitors. A series of nine peptide chloromethyl ketones was prepared using Z-AlaCH$_2$Cl as the key intermediate. The synthesis of Z-AlaCH$_2$Cl utilized a new mixed anhydride procedure (Penke *et al.*, 1970) which proceeded smoothly and in better yield (63%) than the usual acid chloride method. This compound was deblocked and coupled with an assortment of blocked peptide acids to produce the peptide chloromethyl ketone inhibitors listed in Table I. All new compounds were characterized by combustion analysis, mass spectra, ir, nmr, and tlc. Mass spectral analysis was especially valuable for confirming assigned structures since the inhibitors displayed highly characteristic fragmentation patterns. Figure 1 shows how two isomeric peptide chloromethyl ketones could be distinguished on the basis of their mass spectra.

Elastase Inhibition Studies. Elastase was irreversibly inhibited by a series of peptide chloromethyl ketones, and good pseudo-first-order kinetics were observed for all inhibition reactions. The inhibitor solutions were freshly prepared since the peptide chloromethyl ketones underwent a slow hydrolysis upon standing in a buffered aqueous solution as measured by a decreased k_{obsd} of inhibition. Inhibitor solutions contained 5% (v/v) methanol to aid in dissolving some slightly soluble compounds such as Ac-Ala-Ala-Ala-AlaCH$_2$Cl. The reaction conditions were selected in order to obtain reasonable rates of reaction; both the inhibitor concentration and the solution pH were decreased to values as low as practicable so that the rates could be measured easily and accurately. In all experiments the reaction was allowed to proceed through *ca.* 2 half-lives; the half-lives ranged from 5 to 50 min.

Table I shows the results for the fixed concentration experiments in which five tripeptide and four tetrapeptide chloromethyl ketones were used to inhibit elastase. The tripeptide compounds were observed to be "slow" inhibitors and were run at pH 6.5 using a 100-fold excess of inhibitor, while the tetrapeptide compounds were "fast" inhibitors and were run at pH 5.0 using a tenfold excess of inhibitor. Although the pH optimum for elastase (esterase) activity is pH 8.5, fairly rapid

TABLE I: Inhibition of Elastase with Peptide Chloromethyl Ketones.[a]

Inhibitor P_4 P_3 P_2 P_1	pH	[I] × 10^4 (M)	$10^4 k_{obsd}$[b] (sec^{-1})	Half-life (min)	k_{obsd}/[I] (M^{-1} sec^{-1})	k_{obsd}/[I] (rel)[c]
Ac-Ala-Ala-$AlaCH_2Cl$	6.5	5.0	13	8.8	2.6	1.0
	5.0	5.0	2.6	43	0.53	
Ac-Ala-Pro-$AlaCH_2Cl$	6.5	5.0	20	5.7	4.1	1.6
Ac-Pro-Ala-$AlaCH_2Cl$	6.5	5.0	0			
Ac-Ala-Gly-$AlaCH_2Cl$	6.5	5.0	2.3	50	0.47	0.18
Z-Gly-Leu-$AlaCH_2Cl$	5.0	5.0	5.0	23	1.0	1.9
Ac-Ala-Ala-Ala-$AlaCH_2Cl$	5.0	0.5	4.9	24	9.8	18
Ac-Ala-Ala-Pro-$AlaCH_2Cl$	5.0	0.5	19	6.1	38	71
Ac-Ala-Pro-Ala-$AlaCH_2Cl$	6.5	5.0	0			
Ac-Ala-Ala-Phe-$AlaCH_2Cl$	5.0	0.5	4.2	27	8.4	16

[a] Elastase concentration 5 μM, 5% (v/v) methanol, 30°. [b] Averages of at least three runs with a maximum spread of ±5%. [c] These relative values were calculated for pH 6.5 assuming that the k_{obsd}/[I] value measured at pH 5.0 for some of the inhibitors would be *ca.* 20% of that at pH 6.5.

inhibition occurred at pH 6.5, and even lower (pH 5.0) with the "fast" inhibitors. One inhibitor, Ac-Ala-Ala-$AlaCH_2Cl$, was run at both pH 6.5 and 5.0 at the same inhibitor concentration (0.5 mM), to establish a general correlation between the two solution media. This compound had a k_{obsd}/[I] value which was 4.9 times faster at the higher pH. The best elastase inhibitor is Ac-Ala-Ala-Pro-$AlaCH_2Cl$; remarkably, its isomer Ac-Ala-Pro-Ala-$AlaCH_2Cl$ is a noninhibitor.

Kinetics of Inactivation. The kinetics of inhibition of certain enzymes by irreversible inhibitors reveal the presence of a reversible complex between enzyme and inhibitor preceding covalent bond formation. The reaction pathway for the inhibition reaction is expressed by eq 1 and 2 where E·I represents a noncovalently bound complex of enzyme with inhibitor and E–I is the final product with the inhibitor irreversibly bound to the enzyme *via* a covalent linkage. Here K_I is the dissociation constant of the E·I complex and k_3 is the limiting rate of inhibition. Pseudo-first-order kinetics are observed for the inhibition reaction if the initial inhibitor concentration is sufficiently greater than the total enzyme concentration, and k_{obsd}, the observed first-order rate constant, is given by eq 3

$$E + I \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E\cdot I \xrightarrow{k_3} E\text{–}I \qquad (1)$$

$$K_I = [E][I]/[E\cdot I] \qquad (2)$$

$$1/k_{obsd} = (K_I/k_3[I]) + (1/k_3) \qquad (3)$$

(Kitz and Wilson, 1962; Kurachi *et al.*, 1973).

The most appropriate parameter to compare the reactivity of the various inhibitors is the inhibition parameter k_3/K_I analogous to the catalytic parameter k_{cat}/K_M. If the inhibitor concentrations used are much smaller than K_I, then eq 3 reduces to

$$k_{obsd}/[I] = k_3/K_I \qquad (4)$$

and the parameter k_{obsd}/[I] will remain constant over a whole range of inhibitor concentrations. If the inhibitor concentrations used are equal or close to K_I, then k_{obsd}/[I] will vary over a range of inhibitor concentrations and the kinetic constants k_3/K_I, k_3, and K_I may be determined using a reciprocal plot of $1/k_{obsd}$ *vs.* 1/[I].

In the absence of enough information to determine the inhibition parameter k_3/K_I, it is possible to compare the reactivity of a series of inhibitors on the basis of their relative k_{obsd}/[I] values (Table I). The differences in the magnitude of the numbers for related inhibitors reflect mostly the effect of structural changes on the binding of the inhibitor to the enzyme (K_I) and on the rate of reaction within the bound complex (k_3). However, k_{obsd}/[I] values are subject to distortion from nonlinear concentration effects when the inhibitor concentration is close to the inhibitor K_I value, as is the case for many of the inhibitors in Table I. Still, the k_{obsd}/[I] values are useful in providing a rough guide to the relative reactivities of structurally related inhibitors.

Concentration-Dependent Studies. Table II shows the results for the concentration-dependent experiments in which selected peptide chloromethyl ketones in a graded series of concentrations were used to inhibit elastase. For each compound, a set of eight inhibition runs was performed at different concentrations extending over a fivefold range, with the highest inhibitor concentration limited by solubility or rapidity of reaction. For

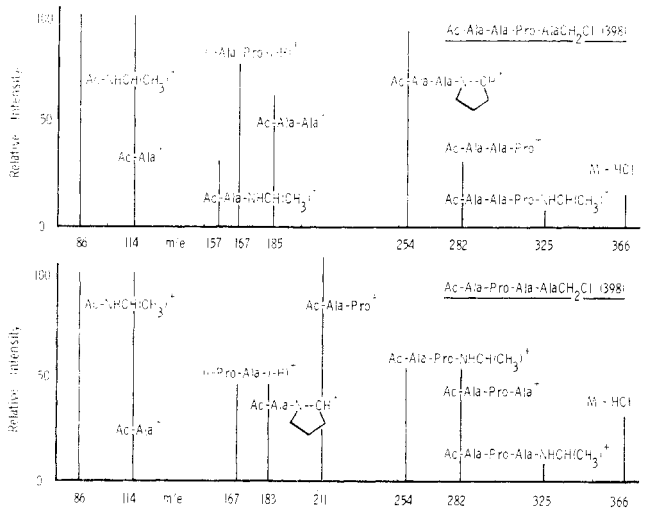

FIGURE 1: A comparison of the mass spectra of the isomeric compounds Ac-Ala-Ala-Pro-$AlaCH_2Cl$ and Ac-Ala-Pro-Ala-$AlaCH_2Cl$.

all compounds except Ac-Ala-Ala-Pro-AlaCH$_2$Cl, the k_{obsd}/[I] values regularly decreased as [I] increased over the range of [I], which shows that the [I] values of the inhibitors were large enough to be in the region of K_I. Pseudo-first-order kinetics were observed for Ac-Ala-Ala-Pro-AlaCH$_2$Cl for 1.5–2.0 half-lives although the [I] to [E] ratio was below 10. Second-order rate constants were calculated from the data obtained with this inhibitor and are listed in parentheses in Table II. Again one inhibitor, Ac-Ala-Ala-AlaCH$_2$Cl, was run at both pH 6.5 and 5.0 over a suitable [I] range, to establish a general correlation between the two solution media. This compound had a k_3/K_I value which was 5.6 times faster at the higher pH.

The effectiveness of the inhibitors may now be compared on the basis of their relative k_3/K_I values, which are superior to k_{obsd}/[I] values for the purpose of correlating the effect of structural changes on reactivities. This is shown in Table III. It is apparent from the k_3/K_I values that the tetrapeptide compounds are more reactive as inhibitors than the tripeptide compounds by a factor of 10 or more, and in general the longer chain length and increased reactivity of the inhibitors are directly related. The best elastase inhibitor in this series is Ac-Ala-Ala-Pro-AlaCH$_2$Cl. The k_3/K_I value for this compound was assumed to be equal to the second-order rate constant for the reaction. This assumption is valid at inhibitor concentrations which are much less than K_I. Since the K_I value is likely to be close to that observed for Ac-Ala-Ala-Ala-AlaCH$_2$Cl (0.39 mM), this condition was met in the range of inhibitor concentrations used with Ac-Ala-Ala-Pro-AlaCH$_2$Cl (0.04 mM and below).

Inhibitor constants K_I (dissociation constants for the enzyme–inhibitor complex) were obtained for three inhibitors. These constants were measurable since k_{obsd}/[I] varied with [I] over a range of inhibitor concentrations where [I] $\geq 0.1K_I$ for almost all [I]. Standard deviations of 5–40% were obtained. No K_I value was obtained for Ac-Ala-Ala-Pro-AlaCH$_2$Cl because its rapid inhibition rate necessitated the use of small values of [I] with [I] $\ll K_I$.

It should be noted here that one inhibitor, Ac-Ala-Pro-AlaCH$_2$Cl, was synthesized in order to compare our results with those reported by Thompson and Blout (1973a). Unfortunately, we were unable to reproduce the k_3/K_I value of 35 M^{-1} sec^{-1} which they report; our experimental value is 3.8 M^{-1} sec^{-1} at pH 6.5. The most likely reason for nonagreement involves differences in the procedures used for quenching the inhibition reaction mixture and measuring the residual elastase activity. In the experiments of Thompson and Blout, aliquots were first quenched by dilution into cold buffer and residual elastase activity was subsequently measured; incomplete quenching of the inhibition reaction or enzyme adsorption onto glass walls could lead to high values for k_{obsd}. In our experiments, aliquots were withdrawn directly from the reaction mixture to measure residual elastase activity.

Studies with Other Enzymes. Table IV shows the results for the experiments in which representative peptide chloromethyl ketones were used in an attempt to measure the rate of reaction with chymotrypsin and trypsin, which are homologous with elastase. Since the inhibition conditions were virtually identical with those used with elastase, a direct comparison of results is possible. All inhibition reactions (except one) produced only slight inactivation over several hours for both chymotrypsin and trypsin, and no kinetic constants could be determined. Control experiments without inhibitor showed that both chymotrypsin and trypsin remained almost fully active in solution over the time periods of the runs. One inhibitor, Ac-Ala-Pro-AlaCH$_2$Cl, did actually inhibit chymotrypsin with k_{obsd}/[I] = 0.119 M^{-1} sec^{-1} and half-life = 3.2 hr under the conditions employed. However, this inhibition rate is only 3% of that with elastase. In general the inhibitor compounds inhibit chymotrypsin and trypsin at rates of 1% or less of the rates at which they inhibit elastase under the same conditions. This result is probably also valid at higher pH values.

TABLE II: Concentration-Dependent Inhibition of Elastase with Peptide Chloromethyl Ketones.[a]

Inhibitor (P_4 P_3 P_2 P_1)	pH	[I] × 10^4 (M)	$10^4 \cdot k_{obsd}$ (sec^{-1})	k_{obsd}/[I] (M^{-1} sec^{-1})
Ac-Ala-Ala-AlaCH$_2$Cl	6.5	1.0	3.97	3.97
		1.25	4.85	3.88
		1.67	6.08	3.64
		2.0	7.23	3.62
		2.5	8.98	3.59
		3.0	10.88	3.62
		3.75	13.06	3.48
		5.0	15.75	3.15
Ac-Ala-Ala-AlaCH$_2$Cl	5.0	4.0	2.24	0.559
		5.0	2.64	0.529
		6.7	3.25	0.485
		8.0	3.58	0.448
		10.0	3.90	0.390
		12.0	4.34	0.361
		15.0	5.08	0.338
		20.0	5.80	0.290
Ac-Ala-Pro-AlaCH$_2$Cl	6.5	0.8	2.94	3.68
		1.0	3.46	3.46
		1.33	4.21	3.17
		1.6	5.09	3.18
		2.0	6.26	3.13
		2.4	7.44	3.10
		3.0	10.16	3.39
		4.0	12.50	3.13
Ac-Ala-Ala-Ala-AlaCH$_2$Cl	5.0	0.4	3.35	8.37
		0.5	3.87	7.74
		0.67	4.82	7.20
		0.8	5.69	7.12
		1.0	7.01	7.01
		1.2	8.73	7.27
		1.5	10.14	6.76
		2.0	11.93	5.96
Ac-Ala-Ala-Pro-AlaCH$_2$Cl	5.0	0.08	2.44	30.6 (42.2)[b]
		0.10	3.10	31.0 (39.9)
		0.133	4.28	32.2 (38.6)
		0.16	5.02	31.4 (36.6)
		0.20	6.42	32.1 (36.3)
		0.24	7.76	32.3 (35.7)
		0.30	10.90	36.3 (39.6)
		0.40	13.65	34.1 (36.5)

[a] Elastase concentration 5 μM, 5% (v/v) methanol, 30°. [b] Values in parentheses are calculated second-order rate constants.

Discussion

X-Ray crystallographic studies of the active site regions of the serine proteases chymotrypsin, subtilisin, trypsin, and elastase have revealed a remarkable degree of homology

TABLE III: Concentration-Dependent Inhibition of Elastase with Peptide Chloromethyl Ketones.[a]

Inhibitor P_4 P_3 P_2 P_1	pH	k_3/K_I[b] (M^{-1} sec^{-1})	k_3/K_I (rel)[c]	$10^3 k_3$ (sec^{-1})	K_I[d] (mM)	[I] range (mM)
Ac-Ala-Ala-$AlaCH_2Cl$	6.5	4.2	1.0	6	1.5 (0.27)	0.10–0.50
	5.0	0.75		0.9	1.2 (0.06)	0.40–2.00
Ac-Ala-Pro-$AlaCH_2Cl$	6.5	3.8	0.9	5	1.2 (0.48)	0.08–0.40
Ac-Ala-Ala-Ala-$AlaCH_2Cl$	5.0	8.9	12	3	0.39 (0.09)	0.04–0.20
Ac-Ala-Ala-Pro-$AlaCH_2Cl$	5.0	38[e]	51			0.008–0.040

[a] Elastase concentration 5 μM, 5% (v/v) methanol, 30°. [b] These values have a maximum standard deviation of ±5%. [c] These relative values were calculated for pH 6.5 assuming that the k_3/K_I value measured at pH 5.0 for some of the inhibitors would be *ca.* 18% that at pH 6.5. [d] Values in parentheses are standard deviations. [e] Average second-order rate constant calculated from the data in Table II.

TABLE IV: Inhibition of Chymotrypsin and Trypsin with Peptide Chloromethyl Ketones.[a]

Inhibitor P_4 P_3 P_2 P_1	pH	[I] × 10^4 (M)	Chymotrypsin Time (hr)	Chymotrypsin Activity[b] (%)	Trypsin Time (hr)	Trypsin Activity[b] (%)
Ac-Ala-Ala-$AlaCH_2Cl$	6.5	5.0	6	62	4	92
Ac-Ala-Pro-$AlaCH_2Cl$	6.5	5.0	6	28	4	87
Ac-Ala-Ala-Ala-$AlaCH_2Cl$	5.0	1.0	6	100	4	92
Ac-Ala-Ala-Pro-$AlaCH_2Cl$	5.0	1.0	6	100	4	88
None[c]	6.5		6	100	4	91

[a] Enzyme concentration 5 μM, 5% (v/v) methanol, 30°, for both. [b] Enzyme activity after t (hr) measured as per cent of initial activity at $t = 0$. [c] Control experiments.

(Robertus *et al.*, 1972; Stroud *et al.*, 1972; Shotton *et al.*, 1972). The catalytic sites are essentially identical, all containing a charge relay system composed of the side chains of a histidine, an aspartic acid, and two serine residues (Blow *et al.*, 1969). Each enzyme contains a pronounced pocket adjacent to the catalytic residues, the dimensions and chemical characteristics of which match the substrate specificity of each enzyme perfectly. Peptide chloromethyl ketones containing a phenylalanine residue at P_1[1] have been shown to react with chymotrypsin and subtilisin by alkylation of the active site histidine residue (Robertus *et al.*, 1972; Segal *et al.*, 1971a). The phenyl group of the inhibitors is located in the hydrophobic binding pocket of chymotrypsin and subtilisin, and the peptide chain of an extended inhibitor and a section of three residues of the backbone of the enzyme form a β-sheet structure.

Our rationale in the design of specific and reactive peptide chloromethyl ketone inhibitors for elastase was to synthesize compounds with an alanine residue at P_1 because of the smaller size of the S_1 binding pocket in elastase compared to chymotrypsin and subtilisin. In addition, most of the inhibitors were designed to contain only alanine and proline residues since elastin, the natural substrate of elastase, contains a high content of alanine, proline, and glycine. The inhibitors were expected to alkylate His-45, the active site histidine residue, by analogy with earlier studies with chymotrypsin and subtilisin. Finally, we chose to synthesize peptide chloromethyl ketones with an extended peptide chain, expecting enhanced reactivity due to an increased interaction with the extended binding region of elastase analogous to the results obtained with chymotrypsin (Kurachi *et al.*, 1973).

The results obtained with P_1 alanine peptide chloromethyl ketones demonstrate that our strategy was successful. Most of the inhibitors synthesized were found to be highly reactive elastase inhibitors even at the low pH values studied. They also possessed a high degree of specificity for this enzyme, being virtually inactive toward the closely related enzymes chymotrypsin and trypsin. This property has made these compounds useful in studies requiring the selective inhibition of elastolytic enzymes in contrast to other proteases.

Although our studies produced no direct evidence to indicate that peptide chloromethyl ketones react with the active site histidine residue (His-45) of elastase, it is virtually certain that this is indeed the case by analogy with related serine proteases. In addition, Thompson and Blout (1973a) have shown that these compounds do react with a histidine residue of elastase.

Extended Binding Site. The reactivity of alanine peptide chloromethyl ketones toward elastase is strongly influenced by the number of amino acid residues in the peptide chain of the inhibitor. Those inhibitors which possess a P_4 residue (tetrapeptides) are in general 10–50 times more reactive than those without (tripeptides). For example, from k_3/K_I values, Ac-Ala-Ala-Ala-$AlaCH_2Cl$ is 55 times more reactive than Ac-Ala-Pro-$AlaCH_2Cl$. This marked difference in the inhibi-

[1] In the discussion of the reactivity of various inhibitors, we have adopted the notation originally proposed by Schechter and Berger (1967) for describing peptide binding subsites for proteolytic enzymes. The individual amino acid residues of a substrate (or inhibitor) are designated P_1, P_2, etc., numbering from the amino acid which supplies the carbonyl group of the peptide bond which is cleaved by the enzyme and numbering in the direction of the amino-terminal end of the substrate. The corresponding subsites of the enzyme which interact with the substrate (or inhibitor) are designated S_1, S_2, etc. For the alanine peptide chloromethyl ketones reported in this paper, the alanine chloromethyl ketone moiety would be referred to as the P_1 residue.

TABLE V: Comparison of Kinetic Parameters for a Series of Inhibitors and Substrates.

	Chloromethyl Ketone ($-CH_2Cl$)		Amide ($-NH_2$)	
P_4 P_3 P_2 P_1	k_3/K_I[a] (M^{-1} sec^{-1})	K_I (mM)	k_{cat}/K_M (M^{-1} sec^{-1})	K_M (mM)
Ac-Ala-Ala-Ala-	4.2 (1.0)[b]	1.5	13[c] (1.0)[b]	2.5
Ac-Ala-Pro-Ala-	3.8 (0.9)	1.2	21[c] (1.6)	4.2
Ac-Ala-Ala-Ala-Ala-	50[d] (12)	0.4	2070[e] (160)	2.9
Ac-Ala-Ala-Pro-Ala-	166[d] (51)		2900[e] (220)	2.1

[a] Values at pH 6.5. [b] Numbers in parentheses are relative values. [c] pH 9.0 (Thompson and Blout, 1973c). [d] Values adjusted to pH 6.5. [e] pH 9.0 (Thompson and Blout, 1973b).

tion rates is evidence for an interaction between the inhibitors and an extended binding site in elastase.

Both K_I and k_3 are affected by the addition of a P_4 residue onto the inhibitor. The K_I value decreases: the K_I of Ac-Ala-Ala-Ala-AlaCH_2Cl is three times lower than that of the tripeptide analog. This is probably caused by increased hydrogen bonding and/or hydrophobic interactions between an inhibitor with a P_4 residue and the S_4 binding subsite of elastase. The k_3 value increases: the k_3 for Ac-Ala-Ala-Ala-AlaCH_2Cl is three times higher than that for the tripeptide analog. The P_4–S_4 interaction in some way increases the rate of the inactivating alkylation reaction within the bound complex, possibly by slightly changing the geometry of the active site or by a slight alteration of the inhibitor conformation in the complex. Thus the increase in the inhibition rate caused by a distal P_4–S_4 interaction is due both to enhanced binding and an increased rate of alkylation within the enzyme–inhibitor complex.

Studies with peptide substrates have shown that elastase possesses an extended substrate binding site composed of at least five subsites near the catalytic residues (Atlas *et al.*, 1970; Atlas and Berger, 1972; Thompson and Blout, 1970, 1973c). The fact that there is a large incremental reactivity difference between tri- and tetrapeptide substrates (Thompson and Blout, 1973c) as well as tri- and tetrapeptide chloromethyl ketones is evidence that peptide chloromethyl ketones are interacting with the same extended substrate binding site in elastase. Since simple analogs of peptide chloromethyl ketones such as Tos-AlaCH_2Cl and Tos-ValCH_2Cl are incapable of inhibiting elastase, it appears that the occupancy of a substantial portion of this binding site by an inhibitor is not simply helpful but is obligatory in order for inhibition to occur.

P_2 Residues. In the tripeptide inhibitors, alanine or proline is equally effective as the P_2 residue since the $k_{obsd}/[I]$ values are very similar. When P_2 is glycine, $k_{obsd}/[I]$ drops by a factor of 5 and when P_2 is leucine it increases by a factor of 2. These approximate rate factors were calculated from the data in Table I. In the tetrapeptide inhibitors, $k_{obsd}/[I]$ increases by a factor of 4 when P_2 alanine is replaced by proline. A change from alanine to phenylalanine leaves $k_{obsd}/[I]$ virtually unchanged. Thus the relative reactivity of inhibitors with a variety of P_2 residues can be represented by Gly < Ala < Pro, Leu > Phe. These results suggest a preference by elastase for inhibitors with an alkyl hydrophobic side chain at the P_2 residue. It is possible that the enzyme exhibits a secondary specificity for inhibitors with P_2 residues such as proline and leucine. The homologous serine protease chymotrypsin also shows a secondary specificity for inhibitors or substrates with P_2 residues possessing alkyl side chains (especially leucine) due to an interaction of the inhibitor with the side chain of Ile-99 (Kurachi *et al.*, 1973). From our limited data, a proline seems to be the optimal residue for the P_2 residue of an elastase inhibitor, and leucine seems to be almost as effective.

P_3 Residues. Two peptide chloromethyl ketones, Ac-Pro-Ala-AlaCH_2Cl and Ac-Ala-Pro-Ala-AlaCH_2Cl, are completely unreactive toward elastase under our standard inhibition conditions. Both inhibitors have a proline as the P_3 residue. Thus an inhibitor with proline in P_3 is incapable of normal binding to the enzyme, or if it does bind, it binds in a conformation which does not lead to inhibition of the enzyme. Substrates have also been shown to be incapable of productive binding when a proline residue would interact with the S_3 binding subsite of elastase (Thompson and Blout, 1973b). In a study of chymotrypsin hydrolysis of a series of peptide esters, a P_3 proline residue caused a 30-fold increase in K_M relative to an isomeric compound with a P_2 proline residue (Segal, 1972). This observation is consistent with the fact that a proline in P_3 should disrupt the antiparallel β-sheet hydrogen bonding formed between an extended peptide substrate (or inhibitor) and part of chymotrypsin's backbone (Segal *et al.*, 1971a,b). Whether the inability of elastase to accept proline in its S_3 subsite is due to a similar structural explanation must await further crystallographic investigation. The possibility of structural homologies in the extended binding sites among this family of enzymes should also be considered in such a crystallographic investigation.

Comparison of Inhibitors with Substrates. Kinetic parameters for a series of comparable peptide chloromethyl ketones and peptide amides are listed in Table V. The K_I values for the inhibitors and the K_M values for the substrates are similar both in magnitude and in the direction of change upon going from tripeptides to tetrapeptides. This indicates that both types of compounds are binding to the enzyme in the same manner. Since the K_M for Bz-Ala-OMe is constant from pH 5.0–10.0 (Hartley and Shotton, 1971), the fact that the measurements were made at different pH's is probably not significant. The relative k_3/K_I and k_{cat}/K_M values for the inhibitors and substrates also show similar general trends. However, in going from tripeptides to tetrapeptides, the inhibition parameter k_3/K_I increases by a factor of *ca.* 20, but the catalytic parameter k_{cat}/K_M increases by a factor of 100. This indicates that the acylation of Ser-188 by peptide amides is more sensitive to the presence of a P_4 residue than is the alkylation of His-45 by peptide chloromethyl ketones. In general, distal interactions influence the rates of reaction of both inhibitors and substrates with elastase in a similar way.

Since our initial report of the synthesis of these alanine peptide chloromethyl ketones and their effectiveness in inhibiting porcine elastase, other elastolytic enzymes have also been shown to be inhibited by these compounds. The digestion of human lung tissue and rat aortic tissue by porcine pancreatic elastase or human polymorphonuclear leukocytic elastase is

totally inhibited by Ac-Ala-Ala-AlaCH$_2$Cl *in vitro* (Janoff, 1972). It has recently been shown that preincubation of human leukocyte lysosomes with Ac-Ala-Ala-Pro-AlaCH$_2$Cl depresses the ability of the lysosomal preparation to promote agglutinability by concanavalin A in mouse fibroblasts (Mosser *et al.*, 1973). In addition, Ac-Ala-Ala-AlaCH$_2$Cl has proven useful in the location of elastolytic enzymes on disc electrophoresis zymograms; this compound inhibits the esterolytic activity of three major neutrophil esterases from human polymorphonuclear granules which exhibit elastase-like behavior (Sweetman *et al.*, 1973). Further studies of the biological behavior of elastolytic enzymes using these inhibitors are currently in progress.

In conclusion, the results presented in this paper demonstrate that peptide chloromethyl ketones with alanine as the P_1 residue are reactive and specific inhibitors of porcine pancreatic elastase and other elastolytic enzymes. Future studies on the effect of structure on reactivity could lead to the design of peptide chloromethyl ketones which are even more reactive and specific elastase inhibitors. The similarities in behavior of these inhibitors and peptide substrates with elastase support the hypothesis that the productive binding mode of this enzyme which leads to alkylation of His-45 is similar to the productive substrate binding mode which leads to acylation of Ser-188. Crystallographic studies of elastase inhibited with peptide chloromethyl ketones should shortly reveal the molecular interactions between these inhibitors and the enzyme (D. M. Shotton, private communication).

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