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Synthesis of lysine-containing sulphonium salts and their properties as proteinase inhibitors

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Some sulphonium salts derived from lysine were synthesized with the general structure R-Lys-CH_2S^+-$(alkyl)_2$. They were examined as inhibitors of the cysteine proteinase clostripain, which has a preference for cleaving peptide bonds at the carboxy group of basic amino acids, and of a number of trypsin-related serine proteinases. Clostripain was irreversibly inactivated by all reagents examined, but in the case of the serine proteinases, depending on the reagent structure, irreversible and reversible inhibitions were observed. These were kinetically characterized.

INTRODUCTION

Peptidylmethanesulphonium salts of the general structure R–CO–$CH_2S^+(CH_3)_2$ were viewed as promising affinity-labelling reagents for proteinases in view of the widespread function of *S*-adenosylmethionine as an enzymically guided alkylating agent. Initial observations with peptides containing hydrophobic side chains such as Cbz-Phe-Ala-$CH_2S^+(CH_2)_2$ showed considerable effectiveness for the inactivation of cathepsin B by transfer of the peptidylmethyl group rather than by methylation (Shaw, 1988). In fact, the active form of the agent may not be the sulphonium salt, but the derived ylide, in view of the increasing effectiveness with increasing pH.

Derivatives containing basic side chains were then synthesized and examined. Cbz-Lys-$CH_2S^+(CH_3)_2$ was found to have no effect at 0.1 mM on trypsin and related enzymes, giving the impression that this type of inhibitor might be effective only on cysteine proteinases (Walker & Shaw, 1985). However, the properties of the dipeptide derivatives such as Cbz-Phe-Lys-$CH_2S^+(CH_3)_2$ reported in the present paper indicate that the pattern of responses of the two classes of proteinases is more complex.

EXPERIMENTAL

Materials

Trypsin (tosylphenylanalylchloromethane-treated) was purchased from Worthington Biochemicals Corp., Freehold, NJ, U.S.A.; plasmin (human) and clostripain were from Boehringer, Mannheim, West Germany; plasma kallikrein was from Kabi-Vitrum, Stockholm, Sweden. Fluorogenic substrates were obtained from Bachem Chemicals, Bubendorf, Switzerland.

Syntheses

Cbz-Lys(-Boc)-CH_2Br. This was prepared from the diazomethyl ketone as described for the chloro compound (Coggins *et al.*, 1974) and obtained as crystals, m.p. 68–69 °C, from ethyl acetate/hexane (Found: C, 52.57; H, 6.42; N, 6.24; Br, 17.24. Calc. for $C_{20}H_{29}BrN_2O_5$ (457.4): C, 52.52; H 6.39; N, 6.13; Br, 17.47%).

Cbz-Lys(-Boc)-CH_2S–CH_2Ph. The foregoing bromomethyl ketone (4.5 g) in methanol (80 ml) was stirred at room temperature for 2 h with sodium methoxide (0.56 g) and benzyl mercaptan (1.2 ml). After removal of methanol under reduced pressure, the crude product was taken up in ethyl acetate, washed with water and then aq. satd. $NaHCO_3$, and dried. The oily residue was chromatographed on silica gel with methylene chloride/ethanol (9:1, v/v) and eventually crystallized from ethyl acetate/hexane. The yield was 4.34 g (88%), m.p. 66–67 °C (Found: C, 64.79; H, 7.39; N, 5.65; S, 6.39. Calc. for $C_{27}H_{26}N_2O_5S$ (500.6): C, 64.73; H, 7.25; N, 5.59; S, 6.40%).

Cbz-Lys(-Boc)-$CH_2S^+(CH_3)$–CH_2Ph,BF_4^-. The benzyl thioether thus obtained (670 mg) and methyl iodide (0.5 ml) were stirred in nitromethane (5 ml) with $AgBF_4$ (260 mg) for 36 h. Methylene chloride was added and the suspension was filtered over a small volume of Fluorosil. The filtrate was concentrated and the residue treated with diethyl ether, yielding 210 mg (27%) of crystals melting unsharply near 100 °C (Found: C, 55.79; H, 6.57; F, 12.39; N, 4.86; S, 5.51. Calc. for $C_{28}H_{40}BF_4N_2O_5$ (599.5): C, 56.02; H, 6.73; F, 12.01; N, 4.67; S, 5.32%).

Cbz-Lys-$CH_2S^+(CH_3)$–CH_2Ph,$2BF_4^-$. The t-butoxycarbonyl derivative (100 mg) in acetic acid (1.5 ml) was stirred at room temperature with BF_3 etherate (80 μl). The precipitate that formed was collected by centrifugation, washed with diethyl ether, and dried. The yield was 43 mg, m.p. 177–179 °C (Found: C, 46.91; H, 5.59; F, 25.35; N, 4.86; S, 5.53. Calc. for $C_{23}H_{32}B_2F_8N_2O_3S$ (590.2): C, 46.80; H, 5.47; F, 25.75; N, 4.75; S, 5.43%).

Cbz-Lys-(Boc)-$CH_2S^+(CH_3)_2$,Br^-. Cbz-Lys(Boc)-CH_2Br (2.0 g) in benzene (40 ml) at 50 °C was treated with dimethyl sulphide (5 ml). The cooled mass was thinned with diethyl ether, and the precipitate collected and washed liberally with diethyl ether to remove unused bromo ketone, yielding 1.4 g, m.p. 107–108 °C (Found: C, 51.04; H, 6.71; Br, 15.04; S, 6.08. Calc. for

Abbreviations used: Cbz, benzyloxycarbonyl; Boc, t-butoxycarbonyl; Mec, 4-methylcoumarin-7-yl; OSu, succinimidate.

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$C_{22}H_{35}BrN_2O_5S$ (519.6): C, 50.86; H, 6.79; Br, 15.38; S, 6.17%).

Cbz-Lys-$CH_2S^+(CH_3)_2,CF_3CO_2^-$. The blocked salt (1 g) was treated at 0 °C with methylene chloride/trifluoroacetic acid (1:1, v/v) mixture (10 ml) for 40 min. At this time t.l.c. in chloroform/methanol (2:1, v/v) revealed complete conversion into unblocked sulphonium salt. The solvents were removed under reduced pressure.

Cbz-Phe-Lys(-Boc)-CH_2Br. Cbz-Phe-OSu (1.3 g, 3.3 mmol), *N*-methylmorpholine (0.4 ml, 3.6 mmol) and H-Lys(-Boc)-OH (0.89 g, 3.6 mmol) were stirred in dimethylformamide (25 ml) at room temperature for 22 h. The filtrate was concentrated under reduced pressure to a syrup, which was taken up in ethyl acetate, extracted with aq. 10% (w/v) citric acid then water, and dried with anhydrous $MgSO_4$. The residue was crystallized from ethyl acetate/hexane, yielding 1.43 g, m.p. 108–110 °C (Found: C, 63.84; H, 6.88; N, 7.88. Calc. for $C_{28}H_{37}N_3O_7$ (527.5): C, 63.74; H, 7.07; N, 7.95%).

The acid (1.0 g) was converted into the diazomethyl ketone by reaction of the mixed anhydride with diazomethane (Shaw & Green, 1981) at room temperature for 1.5 h and the usual isolation, yielding 930 mg (88%). This was converted directly into the bromomethyl ketone by solution in methylene chloride (10 ml) and treatment at 0 °C with a solution of HBr in acetic acid (0.34 ml) for 1 min followed by partitioning between ice-cold aq. satd. $NaHCO_3$ and methylene chloride. The organic layer was washed and dried in the usual way, and the residue was crystallized from ethyl acetate/hexane, yielding 0.76 g (74%), m.p. 156–157 °C (Found: C, 57.66; H, 6.38; Br, 13.04; N, 6.91. Calc. for $C_{29}H_{38}BrN_3O_6$ (604.5): C, 57.62; H, 6.34; Br, 13.22; N, 6.95%).

Cbz-Phe-Lys(-Boc)-CH_2S–CH_2Ph. The bromomethyl ketone described above (730 mg) was converted into the benzyl thioether by treatment with benzyl mercaptan as described above under 'Cbz-Lys(-Boc)-CH_2S–CH_2Ph'. The dipeptide analogue was obtained in 93% yield, m.p. 120–121 °C (Found: C, 67.05; H, 6.87; N, 6.34; S, 4.91. Calc. for $C_{36}H_{45}N_3O_6S$ (647.7): C, 66.75; H, 7.00; N, 6.49: S, 4.95%).

Cbz-Phe-Lys-$CH_2S^+(CH_3)$–CH_2Ph,BF_4^-. The blocked benzyl thioether above (318 mg) in nitromethane (2.5 ml) and acetone (1 ml) was treated with a solution of $AgBF_4$ (110 mg) in nitromethane (1 ml). There was rapid precipitation. After about 3 h at room temperature the mixture was centrifuged. Examination of the supernatant solution by t.l.c. showed the absence of starting material, R_F 0.7 in ethyl acetate/hexane (1:1, v/v), and conversion into a non-migrating iodine-staining zone at the origin. Removal of the solvent under reduced pressure left a residue, 0.38 g. This was deblocked in trifluoroacetic acid at room temperature in 15 min. Removal of the solvent and trituration of the residue with diethyl ether gave a product, 91% pure by h.p.l.c.

KINETIC TECHNIQUES

Inactivation studies

(1) Inactivation of plasma kallikrein with Cbz-Phe-Lys-$CH_2S^+(CH_3)_2$. By using the same procedure as described 0.1 μM stock solution) was added to a solution (1 ml) of Cbz-Phe-Arg-NH-Mec (100 μM) and inhibitor (100–300 μM) in 200 mM-Tris/HCl buffer, pH 7.8, containing 20 mM-NaCl and 1 mM-$CaCl_2$ maintained at 37 °C. The rate of hydrolysis of substrate was monitored continuously by measuring the rate of increase of fluorescence at 455 nm ($\lambda_{exc.}$ 383 nm) in a Perkin–Elmer 650-10S spectrophotometer.

(2) Inactivation of plasma kallikrein with Cbz-Phe-Lys-$Ch_2S^+(CH_3)$–CH_2Ph. The same procedure was used as in the preceding subsection (1); however, the concentration range was 100–400 μM.

(3) Inactivation of plasmin with Cbz-Phe-Lys-$CH_2S^+(CH_3)_2$. By using the same procedure as described in subsection (1), human plasmin (20 μl of an approx. 0.6 μM stock solution was assayed in the presence of Boc-Val-Leu-Lys-NH-Mec (100 μM) and inhibitor (54–350 μM).

(4) Inactivation of plasmin with Cbz-Phe-Lys-$CH_2S^+(CH_3)$–CH_2Ph. The procedure described in subsection (3) was employed but the concentration of inhibitor spanned the range 1–35 μM.

(5) Inactivation of trypsin with Cbz-Phe-Lys-$CH_2S^+(CH_3)_2$. A solution of inhibitor in 1 mM-HCl was added to a solution of trypsin (1–10 μM) in 200 mM-Tris/HCl buffer, pH 7.8, containing 20 mM-NaCl and 1 mM-$CaCl_2$ at 37 °C such that the final concentration of inhibitor was 100–800 μM. Samples were removed at timed intervals and assayed for residual activity with Cbz-Phe-Arg-NH-Mec. The dilution involved in setting up the assay effectively stopped further reaction with inhibitor.

(6) Inactivation of clostripain with Cbz-Lys-$CH_2S^+(CH_3)_2$. Clostripain (1 nM) was assayed at 37 °C with PhCO-Phe-Val-Arg-NH-Mec (40 μM) in the presence of the sulphonium salt (50–300 nM) and Tris buffer as above.

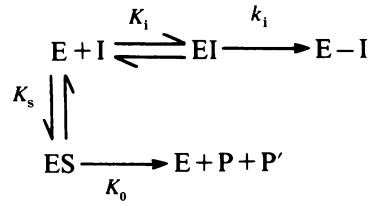
(7) Inactivation of clostripain with Cbz-Phe-Lys-$CH_2S^+(CH_3)$–CH_2Ph. By using the procedure described in subsection (6), clostripain (0.36 nM) was assayed in the presence of 0.5–4.0 nM inhibitor.

Determination of K_m and $V_{max.}$ for fluorogenic substrates

To determine the K_m and $V_{max.}$ for the substrates used in the present studies, substrate concentrations spanning the range 0.2–5 times the K_m were used. For each enzyme–substrate pair studied, it was ensured that the determinations of the kinetic constants were carried out under the exact conditions used to monitor the inactivation processes described in the preceding section. The rate of substrate hydrolysis was determined by measuring the rate of increase of fluorescence at 455 nm ($\lambda_{max.}$ 383 nm). K_m and $V_{max.}$ values were determined by using the least-squares method (Roberts, 1977).

Determination of the operation molarity of the serine proteinase solutions

The operational molarity of trypsin, plasmin and plasma kallikrein was determined by spectrofluorimetric titration with 4-methylumbelliferyl *p*-guanidinobenzoate (Jameson *et al.*, 1973). Clostripain was titrated with



Scheme 1.

accurately determined amounts of D-Phe-Phe-Arg-CH_2Cl. This molarity was then related to the steady-state hydrolysis of a solution (1 ml) of PhCO-Phe-Val-Arg-NH-Mec (40 μM) in order to have a more convenient measure of enzyme concentration from day to day.

Evaluation of kinetic parameters

The kinetic constants for the irreversible inactivation of kallikrein, plasmin and clostripain were determined as previously described (Walker & Elmore, 1984). In this method, data from progress curves (see, e.g., Fig. 1) for the hydrolysis of a suitable substrate obtained in the presence of an inhibitor are utilized to determine the inhibition constant K_i and the first-order rate constant k_i for the reaction scheme shown in Scheme 1, where E–I is the irreversibly inactivated enzyme.

The kinetic constants for the slow-binding reversible inhibition were determined essentially as described by Williams & Morrison (1979). In this method, data from the progress curves (see, e.g., Fig. 2) for the hydrolysis of substrate in the presence of inhibitor are fitted by non-linear regression analysis (Duggleby *et al.*, 1982; Williams *et al.*, 1980; Schloss *et al.*, 1980) to the following integrated equation:

$$[P] = V_s t + (V_0 - V_s)(1 - e^{-k_{obs.}t})/k_{obs.} + [P]_0$$

where V_s is the final steady-state velocity, V_0 is the initial velocity, $k_{obs.}$ is the apparent first-order rate constant for the process and $[P]_0$ is the concentration of product at $t = 0$. The parameters V_s, V_0 and $k_{obs.}$ were evaluated for at least four inhibitor concentrations, and from a plot of $k_{obs.}$ against [I] the inhibitor constant K_i and association rate constant for the slow binding process under study were evaluated.

In the case of reversible competitive inhibition, the K_i values were determined from Lineweaver–Burk plots for reactions monitored in the presence of inhibitor. Initial velocities were computed in accordance with Elmore *et al.* (1963) and a replot of these against inhibitor concentrations afforded the K_i value.

RESULTS AND DISCUSSION

Inhibition studies with plasma kallikrein

The progress curves for the kallikrein-catalysed hydrolysis of Cbz-Phe-Arg-NH-Mec in the presence of Cbz-Phe-Lys-$CH_2S^+(CH_3)_2$ or Cbz-Phe-Lys-$CH_2S^+(CH_3)$–CH_2Ph are shown in Figs. 1 and 2 respectively. These curves clearly represent two distinct types of inhibition. The *SS*-dimethylpeptidylmethanesulphonium salt caused irreversible inactivation of plasma kallikrein, whereas the analogous *S*-benzyl-*S*-methylpeptidylmethanesulphonium derivative functioned as a slow-binding reversible inhibitor.

Analysis of the data points from progress curves in Fig. 1 by the method of Walker & Elmore (1984) yielded

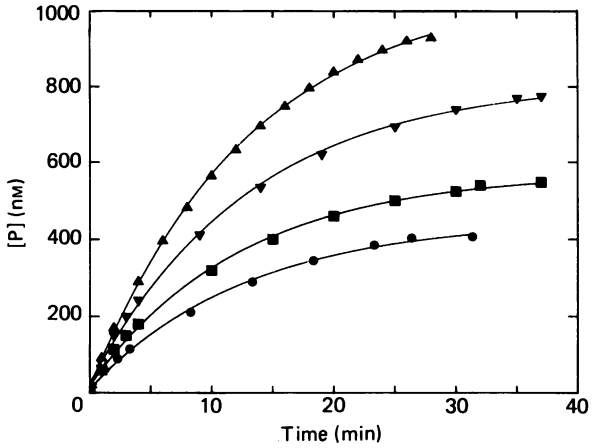


Fig. 1. Progress curve for the generation of 7-amino-4-methylcoumarin from the kallikrein-catalysed hydrolysis of Cbz-Phe-Arg-NH-Mec in the presence of Cbz-Phe-Lys-$CH_2S^+(CH_3)_2$

The curves represent the best fit of the data to the equation for the irreversible inhibition (Walker & Elmore, 1984) of kallikrein by Cbz-Phe-Lys-$CH_2S^+(CH_3)_2$ in the presence of Cbz-Phe-Arg-NH-Mec. The inactivation studies were carried out with a fixed concentration of substrate (100 μM) and the following concentrations of inhibitors: ▲, 107 μM; ▼, 161 μM; ■, 269 μM; ●, 326 μM.

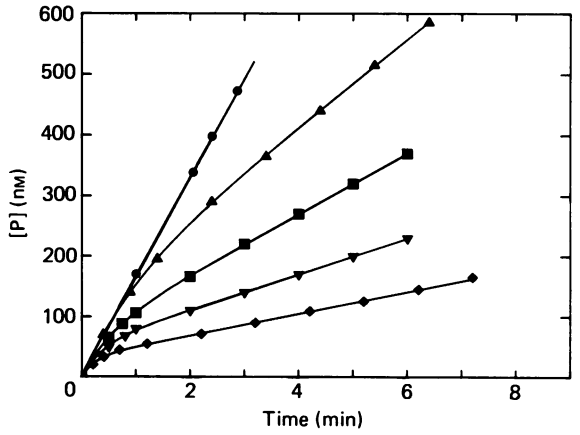


Fig. 2. Progress curves for the generation of 7-amino-4-methylcoumarin from the kallikrein-catalysed hydrolysis of Cbz-Phe-Arg-NH-Mec in the presence of Cbz-Phe-Lys-$CH_2S^+(CH_3)$–CH_2Ph

The curves represent the best fit of the data to the equation for slow-binding reversible inhibition (eqn. 1) of kallikrein in the presence of Cbz-Phe-Arg-NH-Mec. The inhibition studies were carried out with a fixed concentration of substrate (100 μM) and the following concentrations of inhibitor: ●, zero; ▲, 100 μM; ■, 200 μM; ▼, 300 μM; ◆, 400 μM.

values for the inhibition constant K_i and first-order rate constant k_i (see Scheme 1) of $6.0 \pm 0.7\ 10^{-5}$ M and $0.11 \pm 0.01\ min^{-1}$ respectively.

Non-linear regression analysis of the data points from the progress curves in Fig. 2 by the method of Duggleby *et al.* (1982) yielded a set of values for the apparent first-order rate constant ($k_{obs.}$) for the establishment of a final steady-state hydrolysis of substrate in the presence of

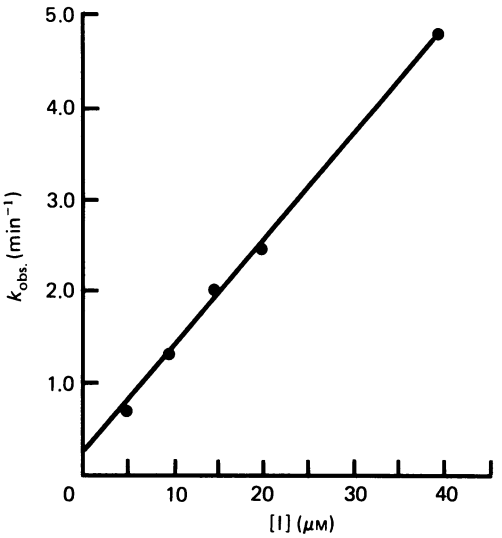
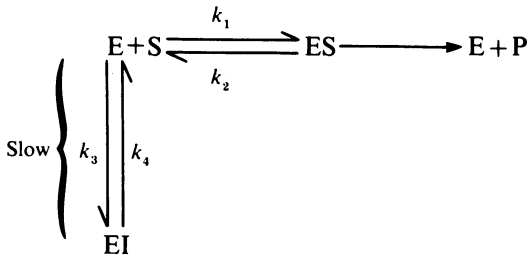


Fig. 3. Relationship between $k_{obs.}$ and [I] for the slow-binding inhibition of kallikrein by Cbz-Phe-Lys-$CH_2S^+(CH_3)$–CH_2Ph

The $k_{obs.}$ values were obtained by non-linear-regression analysis of the progress curves depicted in Fig. 2 for the inhibition of kallikrein by Cbz-Phe-Lys-$CH_2S^+(CH_3)$–CH_2Ph in the presence of Cbz-Phe-Arg-NH-Mec.



Scheme 2.

various concentrations of inhibitor. The resulting plot of $k_{obs.}$ against [I] is shown in Fig. 3. The linear relationship between $k_{obs.}$ and [I] implies that the inhibitor interacts with the enzyme in the manner shown in Scheme 2 (after Morrison, 1982; Williams & Morrison, 1979). In this reaction scheme the formation of the enzyme–inhibitor complex occurs slowly and an initial uninhibited rate for substrate hydrolysis is observed. The velocity of substrate hydrolysis gradually decreases (see Fig. 2) to a final inhibited rate, at which time equilibrium or steady state has been established.

The association rate constant k_3, the dissociation rate constant k_4 and hence K_i (k_4/k_3) for the inhibition of kallikrein by Cbz-Phe-Lys-$CH_2S^+(CH_3)$–CH_2Ph can be evaluated from the plot of $k_{obs.}$ versus [I] in Fig. 3 by utilizing the relationship (see Morrison, 1982):

$$k_{obs.} = k_4 + k_3[I]/\left(1 + \frac{[S]}{K_m}\right)$$

Thus, K_m having been determined in a separate experiment in the absence of inhibitor, k_4 and k_3 can be calculated from the intercept and slope respectively of the plot in Fig. 3. Values of $1.3 \times 10^{-5} \pm 0.1 \times 10^{-5}$ M^{-1}·min^{-1}, 0.25 ± 0.06 min^{-1} and $1.8 \times 10^{-6} \pm 0.4 \times 10^{-6}$ M were obtained for k_3, k_4 and k_i respectively. Although Cbz-Phe-Lys-$CH_2S^+(CH_3)$–CH_2Ph functioned as a potent slow-binding inhibitor of kallikrein, the analogue lacking the phenylalanine residue, Cbz-Lys-$CH_2S^+(CH_3)$–CH_2Ph, had no effect on the enzyme at concentrations up to 300 μM.

Inhibition studies with plasmin

Incubation of plasmin with Cbz-Phe-Lys-$CH_2S^+(CH_3)_2$ in the presence of Boc-Val-Leu-Lys-NH-Mec resulted in progress curves for product formation that were indicative of competitive irreversible inhibition (results not shown). Analysis of the data yielded values for the kinetic constants K_i and k_i of $2.7 \times 10^{-5} \pm 0.3 \times 10^{-5}$ M and 0.033 ± 0.004 min^{-1} respectively.

In contrast with this, incubation of plasmin with Cbz-Phe-Lys-$CH_2S^+(CH_3)$–CH_2Ph in the presence of Boc-Val-Leu-Lys-NH-Mec resulted in reaction traces for formation of product that were indicative of simple competitive reversible inhibition. Fig. 4 shows the hydrolysis of the substrate (100 μM) in the presence of inhibitor (0–35 μM). The K_i for the interaction of the Cbz-Phe-Lys-$CH_2S^+(CH_3)$–CH_2Ph with plasmin was determined to be $1.0 \times 10^{-5} \pm 0.15 \times 10^{-5}$ M.

Inhibition studies with trypsin

Incubation of trypsin with Cbz-Phe-Lys-$CH_2S^+(CH_3)_2$ resulted in complete irreversible inactivation of the enzyme. The kinetic constants K_i and k_i for the inhibition process were found to be $7.1 \times 10^{-4} \pm 0.8 \times 10^{-4}$ M and 0.2 ± 0.02 min^{-1} respectively, as evaluated by using the Kitz–Wilson method outlined in the Experimental section (Kitz & Wilson, 1962). In comparison with this inhibitor, the corresponding *S*-benzyl-*S*-methylpeptidylmethanesulphonium salt functioned as a very poor slow-binding inhibitor. Since very high concentrations (near 1 mM) were required before any inhibition was observed, the kinetic constants for this process were not determined.

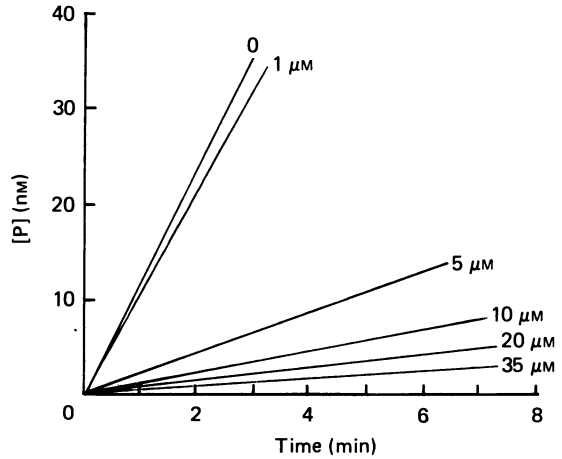


Fig. 4. Traces of increase in fluorescence due to the plasmin-catalysed hydrolysis of Boc-Val-Leu-Lys-NH-Mec in the presence of Cbz-Phe-Lys-$CH_2S^+(CH_3)$–CH_2Ph

The rate of formation of 7-amino-4-methylcoumarin generated by the hydrolysis of a solution (1 ml) of Boc-Val-Leu-Lys-NH-Mec (100 μM) was monitored continuously at 455 nm ($\lambda_{exc.}$ 383 nm) in the absence and in the presence of 1 μM-, 5 μM-, 10 μM-, 20 μM- and 35 μM-Cbz-Phe-Lys-$CH_2S^+(CH_3)$–CH_2Ph.

Table 1. Kinetic characteristics of the inhibition of serine proteinases by various sulphonium salts derived from Cbz-Phe-Lys

Enzyme	Cbz-Phe-Lys-$CH_2S^+(CH_3)_2$	Cbz-Phe-Lys-$CH_2S^+(CH_3)$–CH_2Ph
Plasma kallikrein	Irreversible $K_i = 6.0 \times 10^{-5} \pm 0.7 \times 10^{-5}$ M $k_i = 0.11 \pm 0.01$ min^{-1}	Slow-binding, reversible $K_i = 1.9 \times 10^{-6} \pm 0.4 \times 10^{-6}$ M $k_3 = 1.3 \times 10^5 \pm 0.1 \times 10^5$ M·min^{-1} $k_4 = 0.25 \pm 0.06$ min^{-1}
Plasmin	Irreversible $K_i = 2.7 \times 10^{-5} \pm 0.3 \times 10^{-5}$ M $k_i = 0.033 \pm 0.004$ min^{-1}	Reversible $K_i = 1.0 \times 10^{-5} \pm 0.15 \times 10^{-5}$ M
Trypsin	Irreversible $K_i = 7.1 \times 10^{-4} \pm 0.8 \times 10^{-4}$ M* $k_i = 0.2 \pm 0.02$ min^{-1}*	Slow-binding, reversible Kinetic constants not determined

* Determined by the Kitz–Wilson (1962) method.

The results with serine proteinases are summarized in Table 1.

Inactivation studies with clostripain

Both Cbz-Lys-$CH_2S^+(CH_3)$–CH_2Ph and Cbz-Phe-Lys-$CH_2S^+(CH_3)$–CH_2Ph were found to be irreversible inactivators of clostripain, as was expected, since clostripain has a trypsin-like specificity (Porter *et al.*, 1971) although it is a cysteine proteinase. The progress curves for the hydrolysis of PhCO-Phe-Val-Arg-NH-Mec in the presence of various concentrations of the dipeptide derivative are shown in Fig. 5. Analysis of these data yielded values for K_i and k_i that are given in Table 2 along with those obtained for the lysine derivative. The major feature to note is that the lysine derivative binds the enzyme before covalent-bond formation with a much higher affinity than does the dipeptide derivative; the respective K_i values are 7.8 nM and 280 nM. However, counteracting this decrease in affinity of the dipeptide derivative is the very much greater rate of alkylation of the enzyme by this derivative, i.e. 23-fold more rapidly than the lysine compound. The overall effect of these conflicting differences is that the lysine derivative is marginally the more potent inhibitor, as reflected in the second-order rate constants for the inactivation of clostripain.

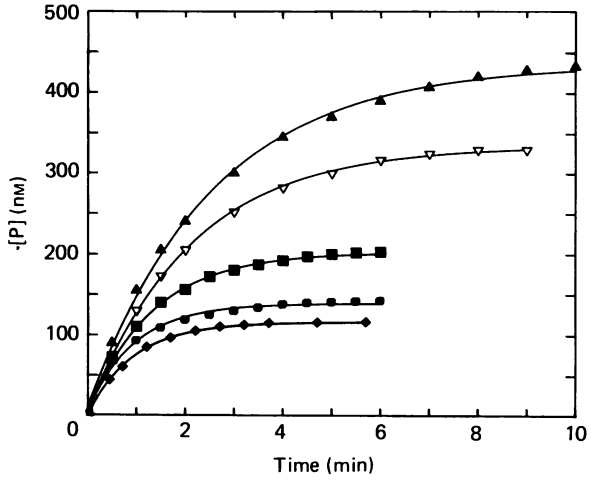


Fig. 5. Progress curves for the generation of 7-amino-4-methylcoumarin from the clostripain-catalysed hydrolysis of PhCO-Phe-Val-Arg-NH-Mec with Cbz-Phe-Lys-$CH_2S^+(CH_3)$–CH_2Ph present

The curves represent the best fit of the data to the equation for the irreversible inhibition (Walker & Elmore, 1984) of clostripain by Cbz-Phe-Lys-$CH_2S^+(CH_3)$–CH_2Ph in the presence of PhCO-Phe-Val-Arg-NH-Mec. The inactivation studies were carried out with a fixed concentration of substrate (10 μM) and the following inhibitor concentrations: ▲, 0.15 μM; ▽, 0.20 μM; ■, 0.30 μM; ●, 0.40 μM; ◆, 0.50 μM.

Comparing the selectivity of these lysine-derived sulphonium salts with respect to the inactivation of serine or cysteine proteinases, the observations available are still limited. However, in the case of *SS*-dimethyl sulphonium salts the affinity of Cbz-Lys-$CH_2S^+(CH_3)_2$ for clostripain is in the nanomolar range (Table 2), whereas for the more susceptible members of the trypsin family the affinities lie in a range near 10 μM for a dipeptide derivative (Table 1). This comparison fortifies the impression obtained with cathepsin B (Shaw, 1988) that peptidylmethanesulphonium salts are particularly effective as cysteine proteinase inactivators. The change from *SS*-dimethyl to an *S*-benzyl-*S*-methyl sulphonium salt provided an unexpected example of changing the nature of the inhibition of the three serine proteinases examined from irreversible to reversible. It seems unlikely that this will be a general finding; other substituents may

Table 2. Behaviour of clostripain towards peptidylmethanesulphonium salts

Inhibitor	Kinetic constants ...	K_i (nM)	k_i (min^{-1})	k_i/K_i (M^{-1}·min^{-1})
Cbz-Lys-$CH_2S^+(CH_3)_2$		7.8 ± 1.2	0.078 ± 0.012	$1.0 \times 10^7 \pm 0.15 \times 10^7$
Cbz-Phe-Lys-$CH_2S^+(CH_3)$–CH_2Ph		280 ± 40	1.8 ± 0.3	$6.4 \times 10^6 \pm 1.1 \times 10^6$

conceivably introduce some selectivity within a closely related group of proteinases such as the trypsin-related enzymes.

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