The synthesis of lysylfluoromethanes and their properties as inhibitors of trypsin, plasmin and cathepsin B

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The synthesis of two lysylfluoromethanes is described by an extension of the synthesis method of Rauber, Anglikar, Walker & Shaw [(1986) *Biochem. J.* **239**, 633–640]. Ala-Phe-Lys-CH₂F was found to be an active-centre-directed inhibitor of plasmin and trypsin, as is the corresponding chloromethane. However, the rate of covalent-bond formation is about an order of magnitude lower at 25 °C for the fluoro derivative. It was, in addition, an extremely effective inactivator of cathepsin B at pH 5.4 and 6.4. The chemical reactivity of fluoromethanes was compared with that of chloromethanes as alkylators of GSH. At pH 7.4 and 37 °C, a fluoromethane has 1/500th the reactivity of a chloromethane. A comparison of the rates of reaction of the fluoromethane with cathepsin B and with GSH at pH 6.4 revealed an enhancement of 10⁸-fold for the alkylation of the enzyme, ascribable largely to a proximity effect.

INTRODUCTION

Recently we have described a synthetic approach for peptidylfluoromethanes, which were desired for investigation as potential active-site-directed inhibitors of proteinases (Rauber *et al.*, 1986). In spite of the chemical inertness of the fluoro substituent towards nucleophilic displacement, we found that Cbz-Phe-CH₂F inactivated chymotrypsin (EC 3.4.21.1) with formation of a covalent bond to a histidine residue, as had been found initially with Tos-Phe-CH₂Br and Tos-Phe-CH₂Cl (Schoellmann & Shaw, 1962, 1963). However, the rate of inactivation was lower, as was to be expected. On the other hand, in the case of the cysteine proteinase cathepsin B the difference in the effectiveness of the fluoromethyl ketone and chloromethyl ketone derivatives was relatively minor (Rauber *et al.*, 1986). We have now synthesized derivatives of lysylfluoromethane. This has permitted a more extensive comparison of fluoromethanes and chloromethanes as affinity labels for serine proteinases to be made. The same comparison has been made with a cysteine proteinase, cathepsin B (EC 3.4.22.1).

Studies were carried out of the rate of reaction of a fluoromethyl ketone with GSH at several pH values as a measure of the reactivity of fluoromethyl ketones and as a basis for comparison with the rate of alkylation of the active-centre thiol group in cathepsin B.

EXPERIMENTAL

Materials

Trypsin [Tos-Phe-CH₂Cl- ('TPCK')-treated] was purchased from Worthington Biochemicals Corp. (Freehold, NJ, U.S.A.) and plasmin (human) from Boehringer Mannheim (Mannheim, West Germany). Cathepsin B (pig liver) was prepared by the method of Evans & Shaw (1983). D-Phe-Pip-Arg-Nan was purchased from Kabi-Vitrum (Stockholm, Sweden), 5,5'-dithiobis-(2-nitrobenzoic acid) from E. Merck (Darmstadt, West Germany),

Cbz-Phe-Arg-Amc from Bachem Chemicals (Bubendorf, Switzerland) and phthaloyl-Lys(Cbz) from Senn Chemical Co. (Dielsdorf, Switzerland). Cbz-Lys-SBzl was synthesized as described previously (Green & Shaw, 1979).

METHODS

Inhibition studies with plasmin

To determine the *K_i* of Ala-Phe-Lys-CH₂F, various amounts of a 0.1 mM solution of the inhibitor in 1 mM-HCl were added to a volume of 0.1 M-Tris/HCl buffer, pH 8.0, such that a final volume of 1 ml was achieved after completion of all additions. These included portions of 1 mM-Cbz-Lys-SBzl in water to provide the desired final concentration (Fig. 1) and 5,5'-dithiobis-(2-nitrobenzoic acid) (5 µl of a 0.13 M solution). Plasmin was added last as 100 µl of a 10 nM solution in Pipes/NaOH buffer, pH 7.0, containing 0.02% Tween. The stock solution of plasmin was titrated by the method of Jameson *et al.* (1973). The initial velocity was measured at 412 nm in a Shimadzu recording spectrophotometer set with full scale at 0.3 A unit and at 0.7 A unit for low and high substrate concentrations respectively.

To determine the kinetic properties of the time-dependent inactivation produced by Ala-Phe-Lys-CH₂F, studies were carried out in 0.1 M-Pipes/NaOH buffer, pH 7.0, at 25 °C. A reaction mixture composed of plasmin (0.3 ml of 20 nM solution in the Pipes buffer containing 0.02% Tween) and various amounts of inhibitor (from a 0.1 mM solution in 1 mM-HCl containing 0.5% dimethylformamide) was adjusted to a final volume of 0.6 ml. Dimethylformamide was maintained at a constant 0.5%. Timed portions (60 µl) were removed from the reaction and assayed with Cbz-Lys-SBzl (100 µl of 0.01 M aqueous solution) in the presence of 5,5'-dithiobis-(2-nitrobenzoic acid) (5 µl of a 0.13 M solution) made up to a final volume of 1 ml with 0.1 M-Tris/HCl

Abbreviations used: Pip, pipecolic acid; Cbz-, benzyloxycarbonyl-, Boc-, t-butoxycarbonyl-; Tos-, tosyl- (toluene-*p*-sulphonyl-); Bz-, benzoyl-; Bzl-, benzyl-; -Nan, *p*-nitroanilide; -SBzl, benzyl thioester; Amc, 7-amino-4-methylcoumarin.

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buffer, pH 8.0. Changes in the absorbance at 412 nm were recorded with a Shimadzu spectrophotometer.

Inhibition studies with trypsin

Time-dependent inactivations were carried out similar to those described for plasmin except that the buffer contained 0.05 M-CaCl₂ instead of Tween and the enzyme concentration was 0.2 μ M. The substrate was D-Phe-Pip-Arg-Nan at 0.1 mM in the assay.

Inactivation studies with cathepsin B

Portions of inhibitor solution in 1 mM-HCl were added to a solution of cathepsin B (4 ml of a 3.6 nM solution) in either 0.1 M-sodium acetate buffer, pH 5.4, or 0.1 M-Pipes/NaOH buffer, pH 6.4, containing 1 mM-EDTA and 0.02% 2-mercaptoethanol. The reaction mixtures were maintained at 25 °C, and at various time intervals portions (50–100 μ l) were removed and assayed fluorimetrically for residual activity at 37 °C by dilution in the same buffer containing Cbz-Phe-Arg-Amc (10 μ M) (Barrett & Kirschke, 1981). The cathepsin B stock solution was titrated with D-Phe-Phe-Arg-CH₂Cl (Shaw & Kettner, 1981).

Reaction of GSH with peptidylfluoromethanes

GSH at 1–10 mM in a buffer at the desired pH (0.1 M-acetate, pH 5.4, or 0.1 M-Pipes, pH 6.4 or 7.4) was mixed with a volume of the appropriate halomethyl ketone in the same buffer, containing some organic solvent if necessary, to achieve the final conditions listed in Table 1.

The mixture was incubated in an Eppendorf thermostat, and timed portions were removed, diluted with the same buffer and treated with 5,5'-dithiobis-(2-nitrobenzoic acid) as described above. The rate of thioether formation was calculated from the decrease of absorbance at 412 nm.

Synthesis of peptidylfluoromethanes

ϵ -N-Benzoyloxycarbonyl- α -NN-phthaloyl-lysyl diazomethane (2). To ϵ -N-benzoyloxycarbonyl- α -NN-phthaloyl-lysine (2.4 g, 5.8 mmol) was added triethylamine (848 μ l, 6.09 mmol) and isobutyl chloroformate (796 μ l, 6.09 mmol) at –20 °C. After 15 min diazomethane (18 mmol) in diethylether was added, and the mixture was stirred first for 1 h at –20 °C, then for 2 h at room temperature. After the addition of water (50 ml) the reaction mixture was extracted with ethyl acetate; the organic phase was washed with satd. NaHCO₃ and brine (satd. NaCl), dried over MgSO₄, filtered and evaporated, yielding a yellow oil (2.3 g, 90%). I.r. (neat) 2090, 1700 and 1630 cm^{–1}; n.m.r. δ (p.p.m.) (in [³H]chloroform) 1.24–1.42, 1.42–1.66 (4H, m, CH₂CH₂CH₂CH₂), 2.00–2.40 (2H, m, CHCH₂), 3.08–3.26 (2H, m, CH₂NH), 4.68–4.84 (1H, m, CH), 5.04 (2H, s, C₆H₅CH₂O), 5.39 (1H, s, CHN₂), 7.26–7.42 (6H, m, C₆H₅ + NH), 7.72–7.83 and 7.83–7.94 (4H, 2m, phthaloyl).

ϵ -N-Benzoyloxycarbonyl- α -NN-phthaloyl-lysyl fluoromethane (3). A solution of 52% HF in pyridine (97.2 ml) was prepared by slow addition of pyridine (25.2 ml) to 70% HF in pyridine (72 ml) at 0 °C. After 10 min ϵ -N-benzoyloxycarbonyl- α -NN-phthaloyl-lysyl diazomethane (2) (7.8 g, 18 mmol) was added, and the mixture was stirred for 1 h at 0 °C, then for 18 h at room temperature. The reaction mixture was neutralized by addition to satd. NaHCO₃ (500 ml) and solid NaHCO₃.

The solution was extracted twice with ethyl acetate (500 ml); the combined extracts were washed with 2 M-HCl (320 ml) and brine (200 ml), dried over MgSO₄, filtered and evaporated. The resultant oily solid was chromatographed over silica gel with chloroform, yielding a yellow oil (2.33 g, 31%) (Found: C, 64.10; H, 5.64; F, 4.30; N, 6.75; C₂₂H₂₃FN₂O₅ requires C, 63.76; H, 5.60; F, 4.59; N, 6.76%). N.m.r. δ (p.p.m.) (in [³H]chloroform) 1.26–1.44, 1.44–1.66 (4H, 2m, CH₂CH₂CH₂CH₂), 2.12–2.28 (2H, m, CHCH₂), 3.08–3.24 (2H, m, CH₂NH), 4.66–4.80 (1H, m, CH), 4.97 (2H, d of the AB system, *J* = 47 Hz, CH₂F), 5.04 (2H, s, C₆H₅CH₂O), 7.26–7.40 (6H, m, C₆H₅ + NH), 7.72–7.82 and 7.82–7.96 (4H, m, phthaloyl).

3-Amino-7-(N-benzoyloxycarbonylamido)-1-fluoroheptan-2-ol hydrochloride (4). To ϵ -N-benzoyloxycarbonyl- α -NN-phthaloyl-lysyl fluoromethane (3) (1.76 g, 4.13 mmol) suspended in propanol/water (17:3, v/v) (41 ml) was added NaBH₄ (780 mg, 20.6 mmol). Clear solution was obtained after 10 min of stirring and, after additional stirring for 16 h at room temperature, h.p.l.c. showed complete consumption of starting material. Acetic acid (4.1 ml) was added and the solution heated at 80 °C for 24 h. The reaction mixture was then concentrated. The residue was dissolved in water, adjusted to pH 9 with 2 M-NaOH and extracted with ethyl acetate. The aqueous phase was acidified to pH 1 with 1 M-HCl, washed with ethyl acetate and then freeze-dried, yielding a colourless oil (1.04 g, 77%), which on treatment with ninhydrin gave a violet spot on t.l.c.

3-(N-Benzoylphenylalanyl amido)-7-(N-benzoyloxycarbonylamido)-1-fluoroheptan-2-ol (5a). To 3-amino-7-(N-benzoyloxycarbonylamido)-1-fluoroheptan-2-ol hydrochloride (4) (303 mg, 0.904 mmol) in 18 ml of tetrahydrofuran was added N-benzoylphenylalanine (258 mg, 0.904 mmol), N-methylmorpholine (203 μ l, 1.853 mmol) and succinimid-N-yl diphenyl phosphate (299 μ l, 0.949 mmol). The reaction mixture was stirred for 16 h at room temperature. Then 1 M-NaOH (20 ml) and ethyl acetate (40 ml) were added, the phases were separated, and the organic phase was washed with 1 M-HCl (20 ml) and brine (20 ml), dried over MgSO₄, filtered and evaporated, yielding an oily colourless solid (340 mg, 66%; 40:60 diastereomers mixture).

7-(N-Benzoyloxycarbonylamido)-3-(N-t-butoxycarbonylalanyl phenylalanyl amido)-1-fluoroheptan-2-ol (5b). Isobutyl chloroformate (137 μ l, 1.05 mmol) was added to N-t-butoxycarbonylalanyl phenylalanine (336 mg, 1 mmol) and N-methylmorpholine (115 μ l, 1.05 mmol) in tetrahydrofuran (10 ml) at –20 °C. After 10 min 3-amino-7-(N-benzoyloxycarbonylamido)-1-fluoroheptan-2-ol (4) (335 mg, 1 mmol) and N-methylmorpholine (100 μ l, 1 mmol) in tetrahydrofuran (10 ml) were added. The reaction mixture was stirred for 1 h at –20 °C and then for 2 h at room temperature. Ethyl acetate and satd. NaHCO₃ were added, the phases were separated, and the organic phase was washed with 10% citric acid and brine, dried over MgSO₄, filtered and evaporated, yielding a yellowish oil (563 mg, 91%; 40:60 diastereomers mixture).

α -N-Benzoylphenylalanyl- ϵ -N-benzoyloxycarbonyl-lysyl fluoromethane (6a). To a solution of CrO₃ (320 mg,

3.203 mmol) and pyridine (516 μ l, 6.407 mmol) in methylene dichloride (50 ml) was added the alcohol (5a) (302 mg, 0.534 mmol) in methylene dichloride (10 ml). The reaction mixture was stirred for 2 h, and then two more additions of CrO_3 (100 mg, 1 mmol) and pyridine (161 μ l, 2 mmol) in methylene dichloride (20 ml) were made in the next hour because of the persistence of starting material. After a further 2 h the reaction mixture was filtered through a short silica-gel column with ethyl acetate/methylene dichloride (1:1, v/v), and the solution was washed with 1 M-HCl and brine, then dried over MgSO_4 , filtered and evaporated. The resulting oily solid was first purified through a silica-gel column with methylene dichloride/ethyl acetate (1:1, v/v), then through a second silica gel column with chloroform/methanol (99:1, v/v). Recrystallization from ethyl acetate/hexane yielded colourless crystals (25 mg, 8% yield), m.p. 140–165 °C (Found: C, 67.64; H, 6.44; F, 3.41; N, 7.67; $\text{C}_{31}\text{H}_{34}\text{FN}_3\text{O}_5$ requires C, 67.99; H, 6.26; F, 3.47; N, 7.67%). I.r. (KBr) 3410, 3310, 3280, 1745, 1688, 1662, 1628 and 1533 cm^{-1} ; n.m.r. δ (p.p.m.) (in $[\text{H}_6]\text{dimethyl sulphoxide}$) 1.08–1.84 (6H, m, $\text{CHCH}_2\text{CH}_2\text{CH}_2$), 2.9–3.14 (4H, m, $\text{CH}_2\text{NH} + \text{C}_6\text{H}_5\text{CH}_2\text{CH}$), 4.20–4.36 (1H, br s, $[\text{CH}_2]_4\text{CH}$), 4.62–4.78 (1H, m, $\text{C}_6\text{H}_5\text{CH}_2\text{CH}$), 5.00 (2H, s, $\text{C}_6\text{H}_5\text{CH}_2\text{O}$), 5.20 (2H, d of the AB system, $J = 47$ Hz, CH_2F), 7.14–7.58, 7.82 (14H, m, 2H, d, $3\text{C}_6\text{H}_5 + \text{NH}$), 8.51 (1H, d, $J = 8$ Hz, NH), 8.55 (1H, d, $J = 8$ Hz, NH) and 8.66 (1H, d, $J = 8$ Hz, NH).

α -N-t-Butoxycarbonylalanylphenylalanyl- ϵ -N-benzoyloxycarbonyl-lysylfluoromethane (6b). Procedure as for compound (6a) provided colourless crystals (13%), m.p. 125–140 °C (Found: C, 62.39; H, 7.16; F, 3.10; N, 9.11; $\text{C}_{32}\text{H}_{43}\text{FN}_3\text{O}_7$ requires C, 62.52; H, 7.05; F, 3.09; N, 9.11%). I.r. (KBr) 3410, 3320, 3290, 1745, 1685, 1640 and 1533 cm^{-1} ; n.m.r. δ (p.p.m.) (in $[\text{H}_6]\text{dimethyl sulphoxide}$) 1.08 (3H, d, CHCH_3), 1.02–1.80 (6H, m, $\text{CHCH}_2\text{CH}_2\text{CH}_2$), 1.34 [9H, s, $\text{C}(\text{CH}_3)_3$], 2.76–3.06 (4H, m, $\text{C}_6\text{H}_5\text{CH}_2\text{CH} + \text{CH}_2\text{NH}$), 3.78–4.02 (1H, m, CHCH_3), 4.14–4.26 (1H, m, $[\text{CH}_2]_4\text{CH}$), 4.42–4.62 (1H, m, $\text{C}_6\text{H}_5\text{CH}_2\text{CH}$), 4.90 (2H, d of the AB system, $J = 47$ Hz, CH_2F), 4.98 (2H, s, $\text{C}_6\text{H}_5\text{CH}_2\text{O}$), 6.93 (1H, d, $J = 7$ Hz, NH), 7.22, 7.33 (11H, 2br s, $2\text{C}_6\text{H}_5$, NH), 7.96 (1H, d, $J = 7$ Hz, NH) and 8.37 (1H, d, $J = 7$ Hz, NH).

Benzoylphenylalanyl-lysylfluoromethane hydrochloride (7a). Protected fluoromethane 6a (37.3 mg, 66.2 μ mol) was dissolved in trifluoroacetic acid (0.5 ml) and warmed to 50 °C. T.l.c. showed no starting material after 1 h. On treatment with dry diethyl ether saturated with gaseous HCl, a white precipitate was formed. The mixture was centrifuged and the solvent decanted. The residue was washed twice with dry diethyl ether by centrifugation, and then dried on high vacuum, yielding a colourless solid (28.7 mg, yield 93%), which on treatment with ninhydrin gave a violet spot on t.l.c.; positive fast-atom-bombardment m.s. ($M + \text{H}$)⁺ 414 ($\text{C}_{23}\text{H}_{26}\text{FN}_3\text{O}_3$ formula M_r 413.493).

Alanylphenylalanyl-lysylfluoromethane dihydrochloride (7b). Compound 7b was deblocked as described for compound 7a, yielding a colourless solid (98%), which on treatment with ninhydrin gave a violet spot on t.l.c.; positive fast-atom-bombardment m.s. ($M + \text{H}$)⁺ 381 ($\text{C}_{19}\text{H}_{29}\text{FN}_4\text{O}_3$ formula M_r 380.464).

RESULTS AND DISCUSSION

By applying the methods described previously (Rauber *et al.*, 1986) it was possible to convert *N*-phthaloyl-Lys(Cbz) into the fluoromethyl ketone and extend the structure to obtain a dipeptide and a tripeptide derivative, Bz-Phe-Lys- CH_2F and Ala-Phe-Lys- CH_2F respectively. The latter was desirable particularly for comparison with the corresponding chloromethyl ketone studied previously (Kettner & Shaw, 1978).

The synthesis that we are using can give partial or complete racemization, for reasons not yet understood. Other synthetic methods are also available that give racemic products (Rasnick, 1985; Imperiali & Abeles, 1986). It is not apparent from our synthetic procedure that racemization should result. We have shown through n.m.r. studies (Rauber *et al.*, 1986) that Cbz-Phe-Ala- CH_2F ($[\alpha]_D -0.7 \pm 1.1^\circ$) is completely and Cbz-Phe-Phe- CH_2F is partially (60:40) racemized. The signals of the $-\text{NH}-$ groups are each split into two doublets. Furthermore, in the case of Cbz-Phe-Ala- CH_2F the signals of the CH_2- group are also split into two doublets.

We have now extended our n.m.r. studies to Cbz-Phe- CH_2F ($[\alpha]_D + 11.4 \pm 1.0^\circ$) by using the shift reagent 1-(9-anthranil)-2,2,2-trifluoroethanol. Addition of this shift reagent results in splitting the signals of the $\text{CH}_2\text{F}-$ group. Comparison with racemic Bz-Phe- CH_2F ($[\alpha]_D -0.7 \pm 0.9^\circ$, splitting of the $\text{CH}_2\text{F}-$ signals) and optically pure Cbz-Phe- CH_2Cl ($[\alpha]_D + 28.8 \pm 0.9^\circ$, no splitting of the $\text{CH}_2\text{Cl}-$ signal) shows that Cbz-Phe- CH_2F is partially racemized (60:40).

Application of those n.m.r. studies to Bz-Phe-Lys(Cbz)- CH_2F and Boc-Ala-Phe-Lys(Cbz)- CH_2F indicates that they are optically pure. There is no splitting of the $-\text{NH}-$ signals and, on addition of the shift reagent there is no splitting of the $-\text{CH}_2\text{F}-$ signals.

Our initial observations with Cbz-Phe- CH_2F and chymotrypsin established that alkylation of the active-centre histidine residue had taken place, but a kinetic comparison of this reagent and the chloromethyl ketone was not carried out because of the low affinity of the fluoromethyl ketone ($K_i = 1.3 \times 10^{-4}$ M) and the need for organic solvent to provide a useful inhibitor range. We

Fig. 1. Dixon plot of the inhibition of plasmin by Ala-Phe-Lys- CH_2F at 25 °C in 0.1M-Tris/HCl buffer, pH 8.0

The substrate, Cbz-Lys-SBzl, was at either 75 μ M (upper line) or 150 μ M (lower line). The abscissa represents the reciprocal of arbitrary velocity units.

have now been able to examine saturation kinetics with the water-soluble lysine derivatives in the inactivation of trypsin and plasmin.

Ala-Phe-Lys-CH₂F was found to be a competitive inhibitor of plasmin when examined by initial-velocity measurements in the presence of substrate. A Dixon plot is shown in Fig. 1, and from this and similar experiments a $K_i = 5.03 \pm 1.1 \mu\text{M}$ was deduced. In time-dependent inactivations of plasmin at 25 °C at pH 7.0 it was found that pseudo-first-order loss of enzymic activity was produced, characteristic of affinity labels, for example peptidyl-lysylchloromethanes acting on serine proteinases (Coggins *et al.*, 1974). Thus a 20 μM solution of the peptidylfluoromethane gave a $t_{1/2}$ of 49 min. (At 37 °C this was decreased to 17 min.) By determining the k_{app} of inactivation at various inhibitor concentrations it was possible to derive, as in Fig. 2, a double-reciprocal plot (Kitz & Wilson, 1962) whose $1/k_{\text{app}}$ intercept indicated a limiting rate of covalent bond formation of $0.025 \pm 0.0018 \text{ min}^{-1}$ and $K_i = 1.39 \times 10^{-5} \pm 0.03 \times 10^{-5} \text{ M}$. This result indicates that in the enzyme-inhibitor complex covalent-bond formation is about one order of magnitude slower than with the corresponding chloromethane, i.e. 0.025 compared with 0.18 min^{-1} (Kettner & Shaw, 1978). A similar result was obtained with trypsin and Ala-Phe-Lys-CH₂F with $k_2 = 0.030 \pm 0.0035 \text{ min}^{-1}$ at pH 7.0.

We have shown previously (Raubert *et al.*, 1986) that Cbz-Phe-CH₂F alkylates a histidine residue in chymotrypsin, as had been found with the corresponding chloromethane. It has been established by protein chemistry that the histidine residue alkylated in chymotrypsin (Ong *et al.*, 1965), trypsin (Shaw & Springhorn, 1967), thrombin (Glover & Shaw, 1971) and plasmin (Groskopf *et al.*, 1969) by substrate-derived chloromethyl ketones is the conserved active-centre histidine residue of this homologous proteinase family. It seems fair to assume that substrate-derived fluoromethyl ketones will, in general, result in the formation of the same covalent bond as the chloromethyl ketones, though at a lower rate, however. Even in a bacterial serine proteinase not homologous with the mammalian group represented above, namely subtilisin BPN', there is an analogous hydrolytic mechanism and the histidine residue is alkylated by a substrate-derived chloromethyl ketone

Fig. 2. Kitz-Wilson plot for the inactivation of plasmin by Ala-Phe-Lys-CH₂F

The inhibition studies were carried out at 25 °C in 0.1 M-Pipes/NaOH buffer, pH 7.0. The enzyme concentration was 10 nM and the inhibitor concentrations spanned the range 10–100 μM .

(Markland *et al.*, 1968). Remarkably, in all cases it is N-3 (i.e. N-7) of the histidine ring that is alkylated.

It had been shown in the case of Cbz-Phe-CH₂Cl that the reaction with the active-centre histidine residue of chymotrypsin was about 10⁶-fold faster than the reaction with acetylhistidine as a model (Shaw & Ruscica, 1971). This has been taken to be due to the proximity effect afforded by complex-formation before covalent-bond formation. A more recent example (Kettner & Shaw, 1979) achieves an even greater enhancement, reaching 10¹¹-fold, i.e. the second-order rate constant for the inactivation of thrombin by D-Phe-Pro-Arg-CH₂Cl is 10⁵ times that for the inactivation of chymotrypsin by Cbz-Phe-CH₂Cl. It would be interesting to know what the proximity effect is in the case of the inactivation of plasmin by a fluoromethyl ketone, but we have no model rate of histidine alkylation for comparison.

With respect to the cysteine proteinase cathepsin B, it is not surprising that the lysyl derivatives described in the present paper are very effective inactivators. This is due to the relatively rapid alkylation rate for cathepsin B by the peptidylfluoromethanes (Rasnick, 1985; Rauber *et al.*, 1986) as well as the presence of a phenylalanine residue in the penultimate position of the peptide, which promotes binding in this enzyme (cf., among others, Shaw & Kettner, 1981). Thus Bz-Phe-Lys-CH₂F at 12.5 nM inactivated cathepsin B (3.6 nM) with a half-time of about 9 min at pH 5.4, a rate of $6.29 \times 10^6 \pm 0.76 \times 10^6 \text{ M}^{-1} \cdot \text{min}^{-1}$ (at 25 °C). Since attempts to measure the rate of reaction of GSH with fluoromethanes, as discussed below, showed a negligible rate at pH 5.4, inactivations with cathepsin B were carried out at pH 6.4, where the model reaction could be measured. The instability of cathepsin B with increased pH (Mort *et al.*, 1980) was not observed with the pig liver preparation, at least at pH 6.4 over a 2 h period at 25 °C. Under these conditions the rate of inactivation of cathepsin B by Ala-Phe-Lys-CH₂F was $1.80 \times 10^7 \pm 0.12 \times 10^7 \text{ M}^{-1} \cdot \text{min}^{-1}$, considerably faster than for Cbz-Phe-Ala-CH₂F (Rasnick, 1985; Rauber *et al.*, 1986). Presumably alkylation of the active-centre thiol group took place as shown for the inactivation of papain by chloromethanes (Bender & Brubacher, 1966; Whitaker & Perez-Villasenor, 1968).

To obtain data on the chemical reactivity of fluoromethanes for comparison with chloromethanes, the rate of alkylation of GSH was measured by thiol disappearance by using Ellman's (1959) reagent. Observations were made at pH 5.4, 6.4 and 7.4 and at 25 °C and 37 °C in order to have information relative to physiological conditions as well as to experimental ones. Various fluoromethanes were examined. Some required organic solvent to obtain concentrations adequate to promote reaction. The results are listed in Table 1. At pH 5.4, no reaction was detectable in 7 h at 25 °C. However, at more elevated pH values a low order of reactivity was found. Under physiological conditions, alkylation of GSH by a fluoromethane was found to take place at 0.6 $\text{M}^{-1} \cdot \text{min}^{-1}$ compared with 370 $\text{M}^{-1} \cdot \text{min}^{-1}$ for a chloromethane. Thus the relative reactivity was 0.2%.

The rate of alkylation of cathepsin B by Ala-Phe-Lys-CH₂F at pH 6.4 at 25 °C can now be compared with that of GSH, i.e. $1.8 \times 10^7 \text{ M}^{-1} \cdot \text{min}^{-1}$ for the reaction with the enzyme and 0.18 $\text{M}^{-1} \cdot \text{min}^{-1}$ for that with GSH. The enhancement is thus 10⁸-fold and is probably largely due to a proximity effect.

Table 1. Comparative reactivity of fluoromethyl ketones and chloromethyl ketones towards thiols

For full details see the text.

Concn. of GSH (mM)	Haloketone		Concn. of organic solvent (%, v/v)	pH	Temp. (°C)	K_2 (M ⁻¹ ·min ⁻¹)
	Structure	Concn. (mM)				
0.05	Cbz-Phe-CH ₂ Cl	0.1	5*	7.4	37	370
0.05	Cbz-Phe-CH ₂ Cl	0.1	10*	7.4	25	161
0.13	Bz-Phe-CH ₂ F	1.33	13†	5.4	37	0
0.33	Bz-Phe-Lys-CH ₂ F	1.33	13*	6.4	25	0.48
3.3	Ala-Phe-Lys-CH ₂ F	6.7	0	6.4	25	0.18
3.3	Ala-Phe-Lys-CH ₂ F	6.7	0	7.4	25	0.25
3.3	Ala-Phe-Lys-CH ₂ F	6.7	0	7.4	37	0.66

* Ethanol.

† Dimethylformamide.

We undertook the study of peptidylfluoromethanes in the hope of obtaining active-site-directed proteinase inactivators that would retain the specificity of the chloromethanes as inhibitors but be less reactive chemically. It was hoped that this change would diminish side reactions. GSH was used as a reference for chemical reactivity since the thiol group is prevalent in a biochemical environment and is the most reactive nucleophile likely to be encountered. We have shown that this reactivity can be diminished in fluoromethanes to about 1/500th that of chloromethanes. From these initial results it appears that the limiting rate of alkylation of the histidine residue in serine proteinases by a fluoromethane is decreased about 10-fold by this change to a fluoromethane. This slower onset of inactivation may not detract from the utility of this class of reagents for application *in vivo* and may well be worth the diminished reactivity.

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