Synthesis and properties of peptidyl derivatives of arginylfluoromethanes

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Two peptide derivatives of arginylfluoromethane (Arg-CH₂F), namely Bz(benzoyl)-Phe-ArgCH₂F and D-Phe-Pro-Arg-CH₂F, have been synthesized by extension of available methods, i.e. the Dakin–West reaction [Rasnack (1985) *Anal. Biochem.* **149**, 461–465] or synthesis of a phthaloyl-blocked C-terminal fluoromethane [Rauber, Angliker, Walker & Shaw (1986) *Biochem. J.* **239**, 633–640; Angliker, Wikström, Rauber & Shaw (1987) *Biochem. J.* **241**, 871–875] with subsequent elongation. The guanidino group of arginine was protected as the bis-Cbz (benzyloxycarbonyl) derivative. The products were examined as active-site-directed inhibitors of some trypsin-related serine proteinases as well as a pair of cysteine proteinases. The results extend previous observations that the rate of alkylation of serine proteinases by fluoromethanes may be considerably slower than by chloromethanes. As expected, the amino acid sequence of the inhibitors influenced their relative effectiveness. Thus the rate of inactivation of a number of trypsin-like proteinases by D-Phe-Pro-Arg-CH₂F varied by more than two orders of magnitude.

INTRODUCTION

Peptidylfluoromethanes as proteinase inhibitors have been the subject of a number of recent investigations (Rasnack, 1985; Imperiali & Abeles, 1986*a,b*, 1987; Kolb *et al.*, 1986; Rauber *et al.*, 1986; Angliker *et al.*, 1987). This work had several purposes. Although fluoride is very difficult to displace from peptidylfluoromethanes, it was anticipated that a rate enhancement of this process would result within an enzyme–inhibitor complex, whereas side reactions elsewhere would be diminished. This advantage was considered likely to produce less toxic inhibitors for use *in vivo*. Several methods of synthesis of the mono derivatives have been introduced. In a modification of the Dakin–West reaction, a blocked peptide or amino acid derivative is heated with fluoroacetic anhydride to yield a racemic fluoromethane (Rasnack, 1985). In the second approach the phthaloyl derivative of the C-terminal amino acid becomes a fluoromethane derivative through the intermediate diazomethane (Rauber *et al.*, 1986; Angliker *et al.*, 1987). By a somewhat different synthetic route the amino acid structure is built up by a condensation reaction (Imperiali & Abeles, 1986*b*), a route that does not readily lend itself to the synthesis of arginine derivatives. We have made use of the first two methods to obtain samples of peptides with a C-terminal arginylfluoromethane (Arg-CH₂F) group (Scheme 1), and their properties as proteinase inhibitors have been examined.

EXPERIMENTAL

Materials

Human plasma kallikrein and Factor Xa were purchased from Kabi-Vitrum (Molndal, Sweden); tosylphenylalanylchloromethane ('TPCK')-treated trypsin was from Worthington (Freehold, NJ, U.S.A.);

clostripain, human plasmin and porcine pancreatic kallikrein were from Boehringer (Mannheim, Germany). Cathepsin B was prepared from pig liver as described by Evans & Shaw (1983) and thrombin was prepared from human plasma by the method of Hofsteenge *et al.*, (1986). Bz-Pro-Phe-Arg *p*-nitroanilide acetate and D-Pro-Phe-Arg *p*-nitroanilide were purchased from Kabi-Vitrum; Cbz-Phe-Arg 7-aminomethylcoumarylamide (*N*-Mec), Boc-Ile-Glu-Gly-Arg-*N*-Mec and Cbz-Lys thiobenzyl ester were from Bachem (Bubendorf, Switzerland); 5,5'-dithiobis-(2-nitrobenzoic acid) was from Merck (Darmstadt, Germany). All other chemicals were from Fluka (Buchs, Switzerland).

General

Analytical h.p.l.c. (column 1) was performed on a Whatman RAC II ODS-3 100 mm-long × 4.6 mm-internal-diameter column using a gradient mixture of 0.1% trifluoroacetic acid in water and acetonitrile with a flow rate of 1.5 ml/min (gradient 1: linear gradient from 40% acetonitrile to 100% acetonitrile within 7 min; gradient 2: linear gradient from 10% acetonitrile to 100% acetonitrile). Preparative h.p.l.c. (column 2) was performed on a Whatman magnum 9 ODS-3 250 mm-long × 9.4 mm-internal-diameter column using the same gradient mixture with a flow rate of 4 ml/min (gradient 3: same gradient as gradient 1 within 15 min; gradient 4: same gradient as gradient 2 within 15 min). Peaks were detected at wavelength 254 nm (except where another wavelength is mentioned).

Syntheses (Scheme 1)

Phthaloyl- $\delta\omega$ -NN-(dibenzoyloxycarbonyl)arginine (4, Scheme 1). $\delta\omega$ -NN-Arginine (22.6 g, 51 mmol) was stirred with *N*-ethoxyphthalimide (11.35 g, 51 mmol) in dimethylformamide (300 ml) and triethylamine (14 ml, 102 mmol) for 18 h at room temperature (Nefkens *et al.*,

Abbreviations used: -CH₂F, fluoromethane; Bz, benzoyl; Boc-, *t*-butoxycarbonyl-; Cbz-, benzyloxycarbonyl-; Mtr, 4-methoxy-2,3,6-trimethylphenylsulphonyl; *N*-Mec, 7-aminomethylcoumarylamide; Pht, phthaloyl.

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Scheme 1. Synthesis of peptides with a C-terminal arginylfluoromethane group

Key to reagents: i, *N*-ethoxyphthalimide, triethylamine; ii, isobutyl chloroformate, *N*-methylmorpholine; iii, pyridinium trifluoroacetate, *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodi-imide; iv, *N*-hydroxysuccinimide, *NN'*-dicyclohexylcarbodi-imide; v, *H*-Arg(Cbz)₂-OH, *N*-methylmorpholine; vi, trifluoroacetic acid; vii, benzoyl chloride, NaOH. Full names of the compounds 1–13 are given under 'Syntheses' in the Experimental section.

1960). The solvent was removed under reduced pressure and the residue taken up in ethyl acetate for washing with 1 M-HCl then with satd. NaCl. After drying and concentration, a crystalline residue remained. Recrystallization from propan-2-ol and hexane provided a colourless granular solid (25.5 g, 89% yield), m.p. 136–138 °C. (Found: C, 62.90; H, 4.81; N, 9.81; C₃₀H₂₈N₄O₈ requires C, 62.93; H, 4.93; N, 9.78.)

Phthaloyl- $\delta\omega$ -*NN*-(dibenzoyloxycarbonyl)arginyldiazomethane (5, Scheme 1). The phthaloyl derivative 4 was converted into the diazomethane in the usual manner (Green & Shaw, 1981) to provide a foamy solid in quantitative yield.

Phthaloyl- $\delta\omega$ -*NN*-(dibenzoyloxycarbonyl)arginylfluoromethane (6, Scheme 1). The diazomethane 5 (1.3 g, 2.2 mmol) in ether and dichloromethane (15 ml, 1:1, v/v) was added to a mixture of 70% HF in pyridine (5 ml) and NaF (1.42 g, 33.8 mmol, predried by heating 5 h at 190 °C *in vacuo* over P₂O₅) at –78 °C. The mixture was stirred at room temperature for 16 h, then poured into excess aqueous NaHCO₃ and extracted with ethyl acetate. The extracts were washed with 2 M-HCl and satd. NaCl, dried over anhydrous MgSO₄, filtered and evaporated. Purification on silica gel with chloroform yielded; after recrystallization from ethyl acetate/hexane, a colourless solid (150 mg, 12% yield), m.p. 113–115 °C (Found: C, 63.30; H, 5.05; N, 9.71; F, 3.16; C₃₁H₂₈FN₄O₇ requires C, 63.26; H, 4.97; N, 9.52; F, 3.23).

3-Amino-1-fluoro-6-(*N'*-benzyloxycarbonyl)guanidylhexan-2-ol hydrochloride (7, Scheme 1). The method of Angliker *et al.* (1987) was used; the phthaloyl derivative (0.8 g, 1.3 mmol) in aqueous propan-2-ol was treated

with NaBH₄ (0.32 g, 8.5 mmol) for 24 h, then refluxed with acetic acid (2.5 ml) at 80 °C for a second period of 24 h. The crude product was isolated by removal of the solvent under reduced pressure, solution of the residue in water (20 ml), adjustment to pH 10 and extraction with ethyl acetate. The aqueous phase was then adjusted to pH 1 with HCl and freeze-dried, yielding a colourless solid (0.45 g, 85% yield). One benzyloxycarbonyl group was consistently removed by this procedure.

***t*-Butoxycarbonyl-D-phenylalanylprolyl-3-amino-1-fluoro-6-(*N'*-benzyloxycarbonyl)guanidylhexan-2-ol (8, Scheme 1).** Isobutyl chloroformate (131 μ l, 1 mmol) was added to *t*-butoxycarbonyl-D-phenylalanylproline (362 mg, 1 mmol) and *N*-methylmorpholine (110 ml, 1 mmol) in tetrahydrofuran (10 ml) at –20 °C. After 15 min a cold solution of 7 (0.25 g, 0.7 mmol) and triethylamine (94 μ l) in dimethylformamide (7 ml) was added and the mixture stirred at –20 °C for 4 h, then at room temperature overnight. The solvent was evaporated, the residue taken up in ethyl acetate (50 ml) and washed with water, 10% (w/v) citric acid, satd. NaHCO₃ and satd. NaCl. After drying over MgSO₄, the solvent was removed, leaving 0.37 g of crude product. The product (0.14 g) was chromatographed on silica-gel with 5% (v/v) ethanol in chloroform. Further purification by h.p.l.c. (column 2, gradient 3) provided a colourless oil [2.6 mg, 93% pure on h.p.l.c. (column 1, gradient 1)]. N.m.r. δ (p.p.m.) ([²H₆]dimethyl sulphoxide) (owing to low concentration, only the characteristic signals are reported) 5.04 (s, 2H, C₆H₅CH₂O), 5.13 (2H, d, *J* 47 Hz, CH₂F), 5.20–5.26 (1H, d, *J* 5 Hz, OH, exchangeable with ²H₂O); positive fast-atom-bombardment m.s. (*M* + *H*⁺) 671 (C₃₄H₄₆N₆O₇F formula *M*_r 669.775).

t-Butoxycarbonyl-D-phenylalanylprolyl- ω -N-(benzyloxycarbonyl)arginylfluoromethane (9, Scheme 1). Compound 8 (400 mg, 0.58 mmol) in dimethyl sulphoxide (8 ml) was stirred for 6 h with pyridinium trifluoroacetate (135 mg, 0.77 mmol) and *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodi-imide (589 mg, 3.1 mmol) at room temperature. The solvent was removed and the residue partitioned between water (20 ml) and ethyl acetate (80 ml). The organic layer was washed with water, 10% citric acid, satd. NaHCO_3 and satd. NaCl , dried over anhydrous MgSO_4 and filtered. The resulting oil was chromatographed on silica-gel with ethyl acetate and hexane (3:1, v/v). Further purification by preparative h.p.l.c. (column 2, gradient 3) yielded a colourless solid [49 mg, 13% yield, 85% pure on h.p.l.c. (column 1, gradient 1)].

D-Phenylalanylprolylarginylfluoromethane (1, Scheme 1). Compound 9 (66 mg, 0.1 mmol) and anisole (0.5 ml) was treated with liquid HF (5 ml) at 0 °C for 60 min. After evaporation of the solvent, the residue was dried over P_2O_5 and KOH *in vacuo* overnight. The residue in water (30 ml) was extracted with diethyl ether, then applied to a column of sulphopropyl-Sephadex (C-25, H^+ form) (5 ml) and washed with water (100 ml). The product was eluted with 0.5 M-HCl (60 ml) which, on freeze-drying, provided a colourless residue [26 mg, 60% yield, 97% pure on h.p.l.c. (column 1, gradient 2)]. A single ninhydrin-positive spot was found on chromatography on a silica plate in butanol/acetic acid/water (4:1:1, by vol.). It had n.m.r. δ (p.p.m.) ($[\text{H}_6]$ dimethyl sulphoxide) (owing to low concentration only the characteristic signal is reported) 5.16 (2H, d of the AB system, J_{HF} 47 Hz, CH_2F); positive fast-atom-bombardment m.s. ($M + \text{H}$)⁺ 435 ($\text{C}_{21}\text{H}_{31}\text{N}_6\text{O}_3\text{F}$ formula M_r 434.516).

t-Butoxycarbonylphenylalanyl-DL- $\delta\omega$ -NN-(dibenzoyloxycarbonyl)arginine (11, Scheme 1). To t-Butoxycarbonylphenylalanine (5.31 g, 20 mmol) and *N*-hydroxysuccinimide (2.30 g, 20 mmol) in dimethylformamide (200 ml) was added dicyclohexylcarbodi-imide (4.13 g, 20 mmol) at 0 °C. After 1 h the cooling bath was removed and the reaction mixture stirred at room temperature overnight (15 h). A suspension of $\delta\omega$ -NN-(dibenzoyloxycarbonyl)arginine (8.61 g, 20 mmol) and *N*-methylmorpholine (4.4 ml, 40 mmol) in dimethylformamide (100 ml) was added. After stirring for 5.5 h at room temperature the reaction mixture was concentrated under high vacuum, taken up in ethyl acetate (400 ml) and filtered through Celite. The ethyl acetate solution was washed with 10% citric acid (100 ml), satd. NaHCO_3 (100 ml) and satd. NaCl (100 ml), dried over anhydrous MgSO_4 , filtered and evaporated, yielding a colourless solid foam (13.1 g, 96% yield).

Benzoyloxy- $\delta\omega$ -NN-(dibenzoyloxycarbonyl)arginine (12, Scheme 1). t-Butoxycarbonyl- $\delta\omega$ -NN-(dibenzoyloxycarbonyl)arginine (1.02 g, 1.48 mmol) was stirred in trifluoroacetic acid (4 ml) for 5 min. The solution was evaporated and the residue dissolved in water (15 ml) and dimethylformamide (15 ml). NaOH (1 M, 4.44 ml, 4.44 mmol) and benzoyl chloride (172 μl , 1.48 mmol) was added and stirred overnight. To the reaction mixture was added ethyl acetate (150 ml) and 1 M-HCl (50 ml) and then separated; the organic phase was washed with satd. NaHCO_3 (50 ml), satd. NaCl (50 ml), dried

over anhydrous MgSO_4 , filtered and evaporated. The resulting colourless oil was chromatographed on silica-gel with dichloromethane/ethyl acetate (1:2, v/v) to yield a colourless viscous oil (168 mg, 16% yield). It had n.m.r. δ (p.p.m.) (in $[\text{H}_6]$ dimethyl sulphoxide) 1.44–1.80 (4H, m, $\text{CHCH}_2\text{CH}_2\text{CH}_2\text{NH}$), 2.86–3.22 (2H, m, $\text{C}_6\text{H}_5\text{CH}_2\text{CH}$), 3.80–4.02 (2H, m, $\text{CHCH}_2\text{CH}_2\text{CH}_2\text{NH}$), 4.14–4.28 (1H, m, CH_3CH), 4.68–4.83 (1H, m, $\text{C}_6\text{H}_5\text{CH}_2\text{CH}$), 5.05, 5.25 (4H, 2s, $2\text{OCH}_2\text{C}_6\text{H}_5$), 7.12–7.82 (21H, m, $4\text{C}_6\text{H}_5$, NH), 8.36 (1H, d, J 7 Hz, NH, exchangeable with $^2\text{H}_2\text{O}$), 8.55 (1H, d, J 7 Hz, NH, 9.18 (1H, broad s, NH, exchangeable with $^2\text{H}_2\text{O}$), 12.20 (1H, broad s, CO_2H , exchangeable with $^2\text{H}_2\text{O}$), field desorption m.s. (M)⁺ 693 ($\text{C}_{38}\text{H}_{39}\text{N}_5\text{O}_8$ formula M_r 693.757).

Benzoylphenylalanyl-DL- $\delta\omega$ -NN-(dibenzoyloxycarbonyl)arginylfluoromethane (13, Scheme 1). Benzoylphenylalanyl- $\delta\omega$ -NN-dibenzoyloxycarbonylarginine (1.93 g, 2.78 mmol), fluoroacetic anhydride (844 mg, 6.12 mmol) and triethylamine (775 μl , 5.56 mmol) were combined and cooled to 0 °C. 4-Dimethylaminopyridine (34 mg, 0.278 mmol) and methylene chloride (2 ml) were added. After 15 min the cooling bath was removed and the reaction was stirred for 16 h at room temperature. To the clear light-orange solution was added 100 ml of ethyl acetate. The solution was washed with 1 M-HCl (2 $\times$ 20 ml), 1 M- Na_2CO_3 (2 $\times$ 20 ml) and satd. NaCl (2 $\times$ 20 ml) and then dried over anhydrous MgSO_4 , filtered and evaporated. The resulting solid yellow foam was chromatographed over silica gel with hexane/ethyl acetate (3:2, v/v). The resulting colourless viscous oil was further purified by h.p.l.c. (column 2, gradient 1) to yield a colourless solid (36 mg, 2% yield), m.p. 155–157 °C (Found: C, 65.86; H, 5.68; F, 2.70; N, 9.77; $\text{C}_{39}\text{H}_{40}\text{FN}_5\text{O}_7$ requires C, 66.00; H, 5.68; F, 2.68; N, 9.87%). It had n.m.r. δ (p.p.m.) (in $[\text{H}_6]$ dimethyl sulphoxide) 1.33–1.76 (4H, m, $\text{CHCH}_2\text{CH}_2\text{CH}_2\text{NH}$), 2.95–3.12 (2H, m, $\text{C}_6\text{H}_5\text{CH}_2\text{CH}$), 3.72–3.97 (2H, m, $\text{CHCH}_2\text{CH}_2\text{CH}_2\text{NH}$), 4.27–4.37 (1H, m, CH_3CH), 4.62–4.72 (1H, m, $\text{C}_6\text{H}_5\text{CH}_2\text{CH}$), 4.93–5.29 (6H, 2m, CH_2F , $2\text{OCH}_2\text{C}_6\text{H}_5$), 7.09–7.83 (21H, m, $4\text{C}_6\text{H}_5$, NH), 8.55 (1H, d, J 7 Hz, NH), 8.67 (1H, d, J 7 Hz, NH), 9.17 (1H, broad s, NH); field desorption m.s. (M)⁺ 709 ($\text{C}_{39}\text{H}_{40}\text{FN}_5\text{O}_7$ formula M_r 709.775).

Benzoylphenylalanyl-DL-arginylfluoromethane hydrochloride (2, Scheme 1). HF (2 ml) was distilled into benzoylphenylalanyl-DL- $\delta\omega$ -NN-(dibenzoyloxycarbonyl)arginylfluoromethane (13) (17 mg, 0.024 mmol) and anisole (13 μl , 0.12 mmol). After 1.5 h stirring at 0 °C, the HF was distilled off. The light yellow–orange residue was taken up in water (15 ml) and washed twice with 50 ml of diethyl ether/ethyl acetate (1:1, v/v). The aqueous phase was put on a sulphopropyl-Sephadex (C-25, H^+ form) ion-exchange column and eluted with 0.5 M-HCl. The eluant was freeze-dried and the resulting solid was further purified by h.p.l.c. (column 2, gradient 4, 230 nm), yielding a colourless solid [2 mg, 93% pure on h.p.l.c. (column 1, gradient 2, 230 nm)]. It gave an orange spot with the Sakaguchi reagent (Jepson & Smith, 1953).

Enzyme assays

(a) **u.v. spectroscopic methods.** Plasma kallikrein was assayed at 37 °C in 0.05 M-Tris/HCl buffer, pH 7.8, which contained 0.1 M-NaCl and 0.1% poly(ethylene

glycol) (M_r 6000) with 200 μM -D-Pro-Phe-Arg *p*-nitroanilide. The release of *p*-nitroaniline from this substrate was monitored at 405 nm, ϵ_{410} 9920 $\text{M}^{-1}\cdot\text{cm}^{-1}$; Lottenberg & Jackson, 1983). Under the conditions of assay, the Michaelis constant of this substrate with plasma kallikrein was determined, as described previously (Stone & Hofsteenge, 1985), to be $272 \pm 38 \mu\text{M}$. Clostripain was assayed at 37 °C in 0.05 M-Tris/HCl, pH 7.4, containing 0.05 M- CaCl_2 , 0.1% poly(ethylene glycol) (M_r 6000) and 5.0 mM-dithiothreitol with Bz-Pro-Phe-Arg *p*-nitroanilide (400 μM). Under the assay conditions, the Michaelis constant was $348 \pm 35 \mu\text{M}$. Before assay, clostripain was activated by preincubation in the above buffer for 1 h at 37 °C.

Cathepsin B was assayed in 0.1 M-acetate buffer, pH 5.4, 1 mM-EDTA and 5 mM-dithiothreitol with 200 μM -Bz-Pro-Phe-Arg *p*-nitroanilide. Plasmin was assayed in 0.05 M-Tris (pH 8.0)/0.1 M-NaCl, using Cbz-Lys thiobenzyl ester (Green & Shaw, 1979) and 5,5'-dithiobis-(2-nitrobenzoic acid), the A_{410} (ϵ 13600 $\text{M}^{-1}\cdot\text{cm}^{-1}$; Ellman, 1959) being monitored.

(b) Fluorimetric methods. Trypsin and plasma kallikrein were assayed in 0.05 M-Tris/HCl buffer, pH 7.5, 1 mM in Ca^{2+} and 0.1 M-NaCl using Cbz-Phe-Arg-*N*-Mec (2×10^{-5} M). Thrombin was assayed in the same buffer with 4×10^{-5} M-Boc-Val-Pro-*N*-Mec as substrate and 0.1% dimethyl sulphoxide. For Factor Xa, Boc-Ile-Glu-Gly-Arg-*N*-Mec at 10^{-4} M was the substrate and the foregoing buffer contained 1% dimethyl sulphoxide. Cathepsin B was assayed as described by Barrett & Kirschke (1981).

RESULTS AND DISCUSSION

Two approaches were applied to the synthesis of peptides containing arginylfluoromethane. Both give low yields owing to multiple steps or the formation of complex mixtures. However, pure material was obtainable, permitting enzymic observations and comparisons with other classes of irreversible inhibitors. In both

synthetic approaches the guanidino group of arginine was protected as the bis-Cbz derivative. In one attempt employing the Mtr group (Fujino *et al.*, 1981) for blocking, application of the Dakin-West procedure yielded a product with two fluorine atoms, suggesting that this form of protection was inadequate under these reaction conditions.

In the approach using a C-terminal phthaloyl-amino acid to obtain a fluoromethane in the early stages of the synthesis (Rauber *et al.*, 1986), the removal of the phthaloyl (Pht) group by the present method appears to be a step that would merit further study, since Pht-Arg- CH_2F was relatively easily accessible and well characterized. The reductive and hydrolytic deblocking for peptide elongation requires a subsequent re-oxidation for which the consistently most satisfactory procedure is not evident. A procedure for the efficient removal of the phthaloyl group without the temporary sacrifice of the carbonyl group would be a considerable improvement in this approach which has the potential of providing optically pure isomer.

The Dakin-West reaction for the synthesis of fluoromethanes (Rasnick, 1985) has the advantage that it may be applied to the completed peptide structure, requiring only purification of the final product and, in some cases, deblocking. In a number of applications it has been relatively direct and useful, although the product is racemic.

The two new arginine-containing fluoromethanes were investigated for inhibitory activity against two cysteine proteinases, cathepsin B and clostripain (only the latter is strictly specific for cleavage at arginine residues), and a group of serine proteinases homologous with trypsin including, in addition to trypsin, thrombin, tissue and plasma kallikrein, Factor Xa and plasmin. In a few cases it was possible to carry out studies at a variety of inhibitor concentrations and obtain data revealing saturation kinetics and permitting the determination of affinity, K_i , and rate of inhibitor-enzyme conversion into alkylated enzyme, k_i . These results are included in Table 1, which also contains analogous data obtained with

Table 1. Kinetic properties of peptidyl chloromethanes and fluoromethanes

Enzyme and inhibitor	pH	K_i (μM)	k_i (s^{-1})	Reference
Cathepsin B				
Cbz-Phe-Phe- CH_2Cl	5.4	0.23	0.21	Rauber <i>et al.</i> (1986)
Cbz-Phe-Phe- CH_2F	5.4	0.14	0.055	
Cbz-Phe-Ala- CH_2Cl	6.5	1.9	0.086	Rasnick (1985)
Cbz-Phe-Ala- CH_2F	6.5	0.57	0.009	
Thrombin				
D-Phe-Pro-Arg- CH_2Cl	8.0	0.025	0.115	Walker <i>et al.</i> (1985)
	7.3	0.037	0.4	Collen <i>et al.</i> (1982)
D-Phe-Pro-Arg- CH_2F	7.0	0.25	0.0015	The present paper
Human plasma kallikrein				
Ala-Phe-Arg- CH_2Cl	7.0	0.078	0.0058	Kettner & Shaw (1979)
Pro-Phe-Arg- CH_2Cl	7.0	0.24	0.0060	
Bz-Phe-Arg- CH_2F	7.0	35.7	0.002	The present paper
Plasmin				
Ala-Phe-Lys- CH_2Cl	7.0	0.83	0.0030	Kettner & Shaw (1979)
Ala-Phe-Lys- CH_2F	7.0	5	0.00042	Angliker <i>et al.</i> (1987)
Clostripain				
Bz-Phe-Arg- CH_2F	7.5	0.022	0.25	The present paper

Table 2. Inhibitory properties of Bz-Phe-Arg-CH₂F

Abbreviations: a, spectroscopic assay; b, fluorogenic assay; Pan. kal., pancreatic kallikrein; N.I., no inhibition.

(a) Reactivity comparisons

Enzyme	Method	[I] (μM)	$10^{-3} \times t_{1/2}$ (s)	$10^3 \times k_i$ (s^{-1})	$k_i/[I]$ ($\text{M}^{-1} \cdot \text{s}^{-1}$)
Cathepsin B	b	0.1			$(39 \pm 1.3) \times 10^4$
Thrombin	b	0.8	4.32 ± 0.18	0.161 ± 0.007	200.6 ± 8.1
Trypsin	b	4	2.46 ± 0.18	0.284 ± 0.021	70.9 ± 5.1
Pan. kal.	a	100	N.I.		
Factor Xa	b	5	N.I.		
Plasmin	a	4	N.I.		

(b) Demonstration of saturation kinetics for the process

Enzyme	K_i	k_i (s^{-1})	k_i/K_i ($\text{M}^{-1} \cdot \text{s}^{-1}$)
Plasma kallikrein	$35.7 \pm 2.0 \mu\text{M}$	$(2.04 \pm 0.07) \times 10^{-3}$	$(5.7 \pm 0.13) \times 10$
Clostripain	$22.4 \pm 4.1 \text{ nM}$	$(25 \pm 3.0) \times 10^{-3}$	$(1.11 \pm 0.08) \times 10^6$

other fluoromethanes and chloromethanes. It should be borne in mind that most of the fluoromethanes are racemic in position P₁. The unnatural isomer is unlikely to be an irreversible inhibitor [cf. the inertness of D-Tos-Lys-CH₂Cl (D-tosyl-lysylchloromethane) to trypsin (Shaw & Glover, 1970)], and in a Kitz-Wilson plot would be unlikely, therefore, to alter the intercept, indicating the $1/k_i$ value even if functioning as a competitive inhibitor. Therefore the k_i values in Table 1 are considered an accurate reflection of reactivity differences, at saturation, of fluoromethanes and chloromethanes. On the other hand, the K_i values are likely to be influenced by the presence of the unnatural isomer.

The alkylation rates in Table 1 indicate a range for fluoromethanes in the inactivation of serine proteinases from 0.00042 to 0.0015 s^{-1} , whereas the values for cysteine proteinases are invariably higher, i.e., 0.025 – 0.055 s^{-1} . However, as the number of observations has increased, the quantitative variations have become more conspicuous. Thus, in the case of human plasma kallikrein, the differences between fluoromethanes and chloromethanes is less than an order of magnitude, whereas in the case of D-Phe-Pro-Arg-CH₂ derivatives as inactivators of thrombin, bond formation in the complex with the fluoromethane is about two orders of magnitude slower than with the chloromethane (Table 1).

In most cases an attempt to establish saturation kinetics has not been made and observations have been limited to examination of the scope of reactivity of the reagents against a number of proteinases. These additional results are described in Tables 2 and 3. Among the serine proteinases, the sensitivity to Bz-Phe-Arg-CH₂F was expected to reflect the ability of the proteinase to bind phenylalanine in subsite S₂. For some, such as plasma kallikrein, phenylalanine is a preferred residue and promotes sensitivity to arginylchloromethanes (Kettner & Shaw, 1978). Bz-Phe-Arg-CH₂F readily inactivated plasma kallikrein and provided saturation kinetics. On the other hand, pancreatic kalli-

Table 3. Inhibitory properties of D-Phe-Pro-Arg-CH₂F

Enzyme	[I] (μM)	$10^{-2} \times t_{1/2}$ (s)	$10^4 \times k_i$ (s^{-1})	$k_i/[I]$ ($\text{M}^{-1} \cdot \text{s}^{-1}$)
Thrombin	2	4.98 ± 0.12	13.92 ± 0.33	696 ± 16.7
Trypsin	2	7.44 ± 0.24	9.32 ± 0.33	466 ± 16.7
Plasma kallikrein	5	12.18 ± 0.18	5.69 ± 0.17	114 ± 1.7
Factor Xa	40	11.82 ± 0.24	5.86 ± 0.17	15 ± 0.2
Plasmin	400	9.78 ± 0.18	7.09 ± 0.17	1.8 ± 0.02

krein showed no sensitivity, even at an inhibitor concentration of 10^{-4} M . This difference in susceptibility between plasma and tissue kallikreins is not related to sequence preferences, but to a quantitative difference in susceptibility to affinity labelling of these two distinct proteinase groups (Fiedler *et al.*, 1977; Kettner *et al.*, 1980). Sequence preferences, however, are considered to be important in the case of Factor Xa, for which glycine is the appropriate residue in P₂, and a phenylalanine-containing inhibitor is less reactive (Kettner & Shaw, 1981), probably accounting for the lack of susceptibility to Bz-Phe-Arg-CH₂F (Table 2).

Cysteine proteinases show many orders of magnitude greater sensitivity to Bz-Phe-Arg-CH₂F than serine proteinases. The results extend previous observations (Anglikar *et al.*, 1987) with derivatives of Lys-CH₂F. For example the rate of inactivation of cathepsin B by Ala-Phe-Lys-CH₂F, $3 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$, may be contrasted with that for the inactivation of plasmin, $1.8 \text{ M}^{-1} \cdot \text{s}^{-1}$, a difference of 10^5 .

In the case of D-Phe-Pro-Arg-CH₂F (Table 3) the responses of a group of trypsin-like serine proteinases show a spread of about 10^3 in susceptibility; thrombin, as expected, is the most readily inactivated. This was anticipated from the previous results with the chloromethane (Kettner & Shaw, 1979), which is an extremely

Fig. 1. Kitz-Wilson (1962) plot for the inactivation of thrombin by D-Phe-Pro-Arg-CH₂F

effective proteinase inhibitor, inactivating thrombin even at 10^{-10} M. The order of reactivity of the fluoromethane towards the proteinases examined is similar to that of the chloromethane [cf. Table III in Kettner & Shaw (1981)]. The inactivation of thrombin was examined at a number of inhibitor concentrations, and evidence was obtained for the formation of an intermediate complex (Fig. 1), the data indicating a K_i of $0.25 \mu\text{M}$ and a k_i of 0.0015 s^{-1} (pH 7). As reviewed in Table 1, this represents a loss of affinity of about one order of magnitude, but a decrease in the alkylation rate of about two orders. This is considerably greater than in the other cases involving serine proteinases and chloromethanes, on one hand, or fluoromethanes. We expect that further studies will reveal more quantitative differences in the response of serine proteinases to peptidylfluoromethanes, since the displacement of fluoride may be more sensitive to small differences in geometry within the various enzyme-inhibitor complexes than in the case of the bulkier chloromethanes. Connected with this is the additional possibility of influencing affinity and orientation within the complex by taking advantage of the departing-group region of the active centre (Imperiali & Abeles, 1987).

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