The synthesis of arginylfluoroalkanes, their inhibition of trypsin and blood-coagulation serine proteinases and their anticoagulant activity

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Seven arginylfluoroalkanes ('arginine fluoroalkyl ketones') were synthesized by using a modified Dakin–West procedure. The structure of benzoyl-Arg-CF₂CF₃ was analysed by ¹⁹F-n.m.r. spectroscopy and m.s. and the compound was shown to exist primarily as a hydrate or cyclic carbinolamine. Arginylfluoroalkanes are good inhibitors of blood-coagulation serine proteinases and were found to be slow-binding inhibitors for bovine trypsin with *K*_i values of 0.2–56 μM. Benzoyl-Arg-CF₂CF₃ was the best inhibitor for bovine thrombin and human Factor XIa, and inhibited thrombin and Factor XIa competitively with *K*_i values of 13 μM and 62 μM respectively. The best inhibitor for pig pancreatic kallikrein was *p*-toluoyl-Arg-CF₃, with a *K*_i value of 35 μM. Benzoyl-Arg-CF₃ and benzoyl-Arg-CF₂CF₃ inhibited human plasma kallikrein competitively, with *K*_i values of 50 μM. None of the seven arginylfluoroalkanes was a good inhibitor of human factor Xa or of Factor XIIa. The arginylfluoroalkanes were tested in the prothrombin time (PT) and activated partial thromboplastin time (APTT) coagulant assays. Two fluoroketones, benzoyl-Arg-CF₂CF₃ and 1-naphthoyl-Arg-CF₃, had significant anticoagulant activity. Benzoyl-Arg-CF₂CF₃ was found to prolong the PT 1.8-fold at 120 μM and to prolong the APTT 2.4-fold at 90 μM, whereas 1-naphthoyl-Arg-CF₃ only prolonged the APTT 1.7-fold at 100 μM.

INTRODUCTION

A large number of peptidylchloromethanes ('peptide chloromethyl ketones') have been described as irreversible inhibitors for serine proteinases [for reviews see Powers (1977) and Powers & Harper (1986)]. In contrast, only a handful of peptidylfluoromethanes ('peptide fluoromethyl ketones') have been prepared as inhibitors for cysteine proteinases and serine proteinases. The first reported peptidylfluoromethane was the cathepsin B irreversible inhibitor Z-Phe-Ala-CH₂F (Rasnick, 1985). It alkylated cathepsin B more slowly (lower *k*₃) than the corresponding chloromethyl ketone Z-Phe-Ala-CH₂Cl, but was bound more tightly by the enzyme (lower *K*_i). Subsequently, Z-Phe-CH₂F was found to inactivate chymotrypsin with the formation of a covalent bond to the active-site histidine residue (Rauber *et al.*, 1986). However, the inhibition rate was much lower than with the chloromethyl ketone Tosyl-Phe-CH₂Cl (Schoellmann & Shaw, 1962, 1963).

More highly fluorinated fluoroketones are potent transition-state inhibitors for serine proteinases and in most cases do not alkylate the enzyme. The inhibitors include Ac-Leu-ambo-Phe-CF₃ for chymotrypsin, Ac-Ala-Ala-Pro-ambo-Ala-CF₃ for pig pancreatic elastase (Imperiali & Abeles, 1986b) and Z-Lys(-Z)-Val-Pro-Val-CF₃ for human leucocyte elastase (Stein *et al.*, 1987; Dunlap *et al.*, 1987). Upon binding to the serine proteinase, several of the fluoroketone inhibitors have been shown to react with the hydroxy group of the active-site serine residue to form a hemiketal structure resembling the

transition state for peptide-bond hydrolysis (Takahashi *et al.*, 1988).

Several monofluoromethyl ketone derivatives of lysine and arginine peptides, including Lys-Ala-Lys-CH₂F (McMurray & Dyckes, 1986), Bz-Phe-Lys-CH₂F and Ala-Phe-Lys-CH₂F (Anglikar *et al.*, 1987), Bz-Phe-Arg-CH₂F and D-Phe-Pro-Arg-CH₂F (Anglikar *et al.*, 1988), have been synthesized as inhibitors for trypsin and trypsin-like enzymes. The monofluoromethyl ketone derivatives of lysine peptides, like their corresponding chloromethyl ketones, were found to be active-site-directed irreversible inhibitors of trypsin. In the present paper we report the syntheses of arginylpolyfluoroalkanes. These compounds are good reversible inhibitors for trypsin and several blood-coagulation serine proteinases. Two of the arginylfluoroalkanes also showed anticoagulant activity.

EXPERIMENTAL

Materials

L-Arginine, bovine trypsin (2 × crystallized), bovine thrombin, pig pancreatic kallikrein and normal citrated human plasma were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Human plasma kallikrein, Factor Xa, Factor XIa and Factor XIIa were generously donated by Dr. Kazuo Fujikawa (University of Washington, Seattle, WA, U.S.A.). Adamantane-1-carbonyl chloride, benzoyl chloride, 1-naphthoyl chloride, *p*-toluoyl chloride, trifluoroacetic anhydride, 4-dimethyl-

Abbreviations used: ambo, racemic amino acid residue; Bz-, benzoyl-; Z-, benzyloxycarbonyl-; -SBzl, thiobenzyl ester; f.a.b.-m.s., fast-atom-bombardment mass spectrometry; PT, prothrombin time; APTT, activated partial thromboplastin time.

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aminopyridine and 4,4'-dithiodipyridine (Aldrich Chemical Co. (Milwaukee, WI, U.S.A.)). Chlorodifluoroacetic anhydride, pentafluoropropionic anhydride and heptafluorobutyl chloride were products of PCR (Gainesville, FL, U.S.A.). Orthobrain thromboplastin, 0.02 M-CaCl₂ solution and activated Thrombafax reagent were obtained from Ortho Diagnostic Systems (Raritan, NJ, U.S.A.). Z-Arg-SBzl was prepared as previously described (McRae *et al.*, 1981). All common chemicals and solvents were reagent grade or better.

Synthesis

The purity of all compounds was checked by t.l.c. on Baker Si 250F t.l.c. plates with the following solvent systems: solvent 1, chloroform/methanol (5:1, v/v); solvent 2, methanol; solvent 3, butan-1-ol/acetic acid/pyridine/water (4:1:1:2, by vol.). Ninhydrin, H₂SO₄ and the Sakaguchi reagent were used as detection agents. Purification was carried out by using silica-gel t.l.c. with a Chromatotron (Harrison Research, Palo Alto, CA, U.S.A.). ¹H-n.m.r. spectra were recorded on a Varian A-60 instrument. ¹⁹F-n.m.r. spectra were recorded on a Varian 400 MHz instrument. Mass spectra were obtained with a Varian MAT 112S spectrometer. Elemental analysis was performed by Atlantic Microlab (Atlanta, GA, U.S.A.).

Synthesis of acylarginines

General procedure. Acylarginine derivatives were synthesized according to the procedure of Bergmann *et al.* (1939), with some modification. The synthesis of *p*-toluoylarginine hydrochloride is described as an example. *p*-Toluoyl chloride (3.5 ml, 26 mmol) and Na₂CO₃ (5.11 g, 48 mmol) in water (50 ml) were added (one-fifth portion every 10 min) to a vigorously stirred mixture of diethyl ether and a solution of arginine hydrochloride (4.22 g, 20 mmol) in 25 ml of water. The reaction mixture was stirred for another 2.5 h, and excess acid chloride was removed by extraction with diethyl ether. The pH of the aqueous layer was adjusted to pH 2 with 5% (w/v) HCl and the precipitate was filtered. The filtrate was mixed with saturated NaCl, and the product was dissolved in ethyl acetate/2-methylpropan-2-ol (1:1, v/v), washed with saturated NaCl, dried over MgSO₄ and evaporated to dryness giving a white powder (yield 40%; m.p. 57–59 °C). T.l.c.: *R_F* 0.57 (solvent 1), *R_F* 0.47 (solvent 2), positive Sakaguchi reaction. F.a.b.-m.s.: *m/z* 293 (*M* + 1).

Adamantanoylarginine hydrochloride. This compound was synthesized from arginine hydrochloride and adamantane-1-carbonyl chloride [yield 26%; m.p. 87 °C (decomp.)]. T.l.c.: *R_F* 0.61, (solvent 1), *R_F* 0.56 (solvent 2), positive Sakaguchi reaction. F.a.b.-m.s.: *m/z* 337 (*M* + 1).

1-Naphthoylarginine hydrochloride. This compound was synthesized from arginine hydrochloride and 1-naphthoyl chloride [yield 14%; m.p. 77 °C (decomp.)]. T.l.c.: *R_F* 0.52 (solvent 1), *R_F* 0.46 (solvent 2), positive Sakaguchi reaction. F.a.b.-m.s.: *m/z* 329 (*M* + 1).

Synthesis of arginylfluoroalkanes

General procedure. A modified Dakin–West procedure was used (Kolb *et al.*, 1986), and the synthesis of Bz-DL-

Arg-CF₃ is described as an example. To a solution of benzoylarginine hydrochloride (1.40 g, 5 mmol) and 4-dimethylaminopyridine (37 mg, 0.3 mmol) in tetrahydrofuran was added trifluoroacetic anhydride (10.5 ml, 74 mmol) at 40 °C under an N₂ atmosphere. The reaction was continued under the same conditions for 5 h. The solvent was removed *in vacuo*, and the residual oil was purified by using a Chromatotron, with elution first with dichloromethane and then with dichloromethane/methanol (9:1, v/v). The fractions containing the desired compound were collected and evaporated to dryness giving a hygroscopic powder [yield 59%; m.p. 97 °C (decomp.)]. T.l.c.: *R_F* 0.67 (solvent 3), positive Sakaguchi reaction. F.a.b.-m.s.: *m/z* 331 (*M* + 1).

Bz-DL-Arg-CF₂CF₃. This compound was synthesized from benzoylarginine hydrochloride and pentafluoropropionic anhydride (yield 30%; hygroscopic powder, m.p. 67 °C). T.l.c.: *R_F* 0.76 (solvent 3), positive Sakaguchi reaction. F.a.b.-m.s.: *m/z* 381 (*M* + 1), 399 (*M* + 18 + 1), 363 (*M* + 1 – H₂O), 343 (*M* + 1 – HF – H₂O), 311 (*M* – CF₃), 293 (*M* + 18 – benzoyl) and 489 (*M* + 1 + thioglycerol). ¹⁹F n.m.r.: δ (p.p.m.) ([²H₆]dimethyl sulphoxide) –81.2 (CF₃), –80.6 (CF₂), –118.0 (CF₂) and –119.9 (CF₂). Analysis: Found: C, 39.11; H, 3.86; N, 9.41; Calc. for C₁₅H₁₇F₅N₄O₂, C₂F₅CO₂H, H₂O: C, 38.44; H, 3.58; N, 9.96%.

Bz-DL-Arg-CF₂CF₂CF₃. This compound was synthesized from benzoylarginine hydrochloride and heptafluorobutyl chloride (yield 8.4%, oil). T.l.c.: *R_F* 0.80 (solvent 3), positive Sakaguchi reaction. F.a.b.-m.s.: *m/z* 431 (*M* + 1), 449 (*M* + 18 + 1) and 413 (*M* – 18 + 1).

Bz-DL-Arg-CClF₂. This compound was synthesized from benzoylarginine hydrochloride and chlorodifluoroacetic anhydride (yield 65%; oil). T.l.c.: *R_F* 0.76 (solvent 3), positive Sakaguchi reaction. F.a.b.-m.s.: *m/z* 347 (*M* + 1), 364 (*M* + 1 + H₂O), 276 (*M* – Cl), 255 (*M* – Cl – F – 2), 236 (*M* – Cl – 2F – 2) and 455 (*M* + 1 + thioglycerol).

Adamantanoyl-DL-Arg-CF₃. This compound was synthesized from adamantanoylarginine hydrochloride and trifluoroacetic anhydride (yield 65%; m.p. 62 °C). T.l.c.: *R_F* 0.76 (solvent 3), positive Sakaguchi reaction. F.a.b.-m.s.: *m/z* 389 (*M* + 1), 407 (*M* + 18 + 1), 371 (*M* – H₂O), 351 (*M* – H₂O – HF) and 497 (*M* + 1 + thioglycerol).

1-Naphthoyl-DL-Arg-CF₃. This compound was synthesized from 1-naphthoylarginine hydrochloride and trifluoroacetic anhydride (yield 20%; m.p. 112.5–116 °C). T.l.c.: *R_F* 0.75 (solvent 3), positive Sakaguchi reaction. F.a.b.-m.s.: *m/z* 381 (*M* + 1), 399 (*M* + 18 + 1), 363 (*M* + 1 – H₂O), 343 (363 – HF), 323 (343 – HF), 305 (323 – H₂O) and 489 (*M* + 1 + thioglycerol). ¹⁹F-n.m.r.: δ (p.p.m.) ([²H₆]dimethyl sulphoxide) –73.2 and –79.9.

***p*-Toluoyl-DL-Arg-CF₃.** This compound was synthesized from *p*-toluoylarginine hydrochloride and trifluoroacetic anhydride according to the general procedure (yield 62%, m.p. 93 °C). T.l.c.: *R_F* 0.71 (solvent 3), positive Sakaguchi reaction. F.a.b.-m.s.: *m/z* 345 (*M* + 1), 363 (*M* + 18 + 1), 327 (*M* + 1 – H₂O) and 307 (*M* + 1 – H₂O – HF).

Kinetics measurements

Z-Arg-SBzl was used as a substrate for trypsin and the coagulation serine proteinases (McRae *et al.*, 1981). The hydrolysis rates of Z-Arg-SBzl were measured in 0.01 M-CaCl₂/0.1 M-Hepes buffer, pH 7.5, containing 9% (v/v) dimethyl sulphoxide and 0.34 mM-4,4'-dithiodipyridine at 25 °C. The rate was monitored by the increase in the absorbance at 324 nm (ϵ_{324} 19800 M⁻¹·cm⁻¹ for 4-thiopyridone; Grassetti & Murray, 1967) with a Beckman 35 spectrophotometer when 25 μ l of enzyme solution (0.06–1.8 μ M) was added to a cuvette containing 2 ml of buffer, 150 μ l of 4,4'-dithiodipyridine, 25 μ l of substrate solution and 25 μ l of inhibitor solution. Initial-rate measurements were used with all enzymes except for trypsin, which shows a slow-binding type of inhibition. Therefore the steady-state rates were measured for trypsin by preincubation of trypsin with inhibitor for 5 min before addition of substrate and 4,4'-dithiodipyridine. The K_i values were obtained from Dixon plots at two substrate concentrations (25 and 75 μ M) and five inhibitor concentrations at each substrate concentration. For less potent inhibitors actual K_i values were not obtained, but the lower limit of K_i was estimated from the percentage inhibition.

Coagulant assay

The prothrombin time (PT) (Quick *et al.*, 1935) and activated partial thromboplastin time (APTT) (Nye *et al.*, 1962) blood-coagulation assays were measured with an MLA Electra 750 coagulation timer. The PT was measured in the presence of inhibitor by incubating normal citrated human plasma (0.1 ml) with 0.02 ml of inhibitor solution at 37 °C for 3 min. Orthobrain thromboplastin (0.2 ml) was then added and the clotting time was determined. The APTT was measured by incubating citrated human plasma (0.1 ml) with 0.02 ml of inhibitor solution and 0.1 ml of activated Thrombofax reagent at 37 °C for 4 min, followed by the addition of 0.1 ml of 0.02 M-CaCl₂ (preincubated at 37 °C), and the clotting time determined. Inhibitor stock solutions were prepared in dimethyl sulphoxide. For determination of PT and APTT the inhibitor solution was further diluted 10-fold with 0.5 M-NaCl/0.1 M-Hepes buffer, pH 7.5, and thus the dimethyl sulphoxide concentration was lowered to 10% before addition to the assay system. No prolongation of the PT or APTT was observed at this dimethyl sulphoxide concentration.

RESULTS

Synthesis

Several methods for the synthesis of α -fluoroalkyl ketones have been described in the literature. The Reformatsky reaction was used in the synthesis of difluorostatone-containing peptides, which are potent renin inhibitors (Thaisrivongs *et al.*, 1985; Fearon *et al.*, 1987). Condensation of nitroalkanes with the appropriate fluorinated aldehyde hydrate was used to prepare peptidyl-fluoromethanes as chymotrypsin and elastase inhibitors (Imperiali & Abeles, 1986a,b). A modified Dakin–West method was also used to prepare Z-Phe-Ala-CH₂F (Rasnack, 1985) and α -benzamidoalkyl mono-, di- and tri-fluoromethyl ketones (Kolb *et al.*, 1986).

Seven arginylfluoroalkanes were synthesized from the corresponding *N*-acylated arginine by using a convenient

one-pot Dakin–West reaction. The *N*-acylated arginine hydrochloride salts (1 equiv.) were reacted with 15 equiv. of trifluoroacetic anhydride or various other fluorinated anhydrides and a catalytic amount of 4-dimethylaminopyridine at 40 °C under N₂ for 5 h. Under the conditions of the reaction, the anhydrides reacted with the various 2-phenyl-5(4*H*)-oxazolones generated *in situ* from the corresponding acylarginine derivatives to form keto acid intermediates. With amino acids having stable side chains, decarboxylation of the acylated oxazolone to the fluoroketone product is often accomplished by heating with anhydrous oxalic acid at 110–120 °C for 10–15 min (Kolb *et al.*, 1986). To avoid possible side reactions of the arginyl fluoroalkanes at high temperature and avoid the necessity of removing the oxalic acid from the product, we utilized 4-dimethylaminopyridine to accomplish the decarboxylation during the reaction. With 4-dimethylaminopyridine the Dakin–West reaction can be accomplished at room temperature (Steglich & Hofle, 1969; Hofle *et al.*, 1978; synthesis of Bz-Ala-CH₂F, Rasnick, 1985). The yields of the reaction varied from 8% (Bz-DL-Arg-CF₂CF₂CF₃) to 65% (Bz-DL-Arg-CF₂Cl). The final products were purified by chromatography with a Chromatotron and are racemic at the arginine α -carbon atom owing to the formation of the intermediate oxazolone.

Structure

The structures of the arginyl fluoroalkanes were confirmed by m.s., the Sakaguchi reaction and ¹⁹F-n.m.r. spectra. The final products were single spots on t.l.c. and gave positive Sakaguchi reactions. The Sakaguchi reaction involves formation of a red colour upon treatment of an alkaline solution of a monosubstituted guanidine and α -naphthol with sodium hypobromite. Arginine, octopine, glycoylamine, α -guanidinobutyric acid and monomethylguanidine give positive reactions, whereas dimethylguanidine, nitroarginine, citrulline, creatinine, creatine, $\alpha\delta$ -dibenzoylarginine and canavanine are unreactive (Greenstein & Winitz, 1961; Sakaguchi, 1925). Possible alternative structures for the Dakin–West reaction products include derivatives with acylated guanidino groups, ornithine derivatives (Rink *et al.*, 1984) or citrulline derivatives; however, none of these would give positive Sakaguchi reactions.

The mass spectra of the fluoroalkyl ketones were consistent with their assigned structures. Most of the ketones, including Bz-Arg-CF₂CF₃, Bz-Arg-CF₂CF₂CF₃, adamantanoyl-Arg-CF₃, naphthoyl-Arg-CF₃ and *p*-toluoyl-Arg-CF₃, had peaks at *M* + 1 and *M* + 18 + 1 in the mass spectra. The latter corresponds to the hydrated form of the ketone. Since the f.a.b. mass spectra were measured in a thioglycerol matrix, there were often higher mass peaks corresponding to *M* + 1 + 108 (thioglycerol) or fragments of this ion. However, there were no peaks corresponding to any of the alternative structures listed above.

The ¹⁹F-n.m.r. spectrum of Bz-Arg-CF₂CF₃-CF₃CF₂CO₂H had two peaks at –80.6 and –81.2 p.p.m. and another two peaks at –118.0 and –119.9 p.p.m. The peaks around –80 p.p.m. were assigned to the two CF₃ groups of Bz-Arg-CF₂CF₃ and CF₃CF₂CO₂H, and the peaks around –119 p.p.m. were assigned to the corresponding CF₂ groups. The ¹⁹F-n.m.r. spectra of several substituted Bz-Phe-CF₃ compounds have been reported (Kolb *et al.*, 1986). Bz-Phe-CF₃ had two peaks at –76.6

Fig. 1. Structures of Bz-Arg-CF₂CF₃ in aqueous solution

(I) Cyclic carbinolamine; (II) keto form; (III) hydrate form.

and -82.4 p.p.m., which correspond to the CF₃ group of the ketone and the hydrate form respectively. The chemical shifts of the ketone form were 3–7 p.p.m. downfield from the hydrate form, depending on the structure. Bz-*p*-guanidino-Phe-CF₃ showed one peak at -83.0 p.p.m., which corresponds to the CF₃ group of the hydrated form of the fluoromethyl ketone. Bz-Val-CF₃ was synthesized in this laboratory and the ¹⁹F-n.m.r. spectrum of this compound showed two peaks at -74.4 and -80.0 p.p.m., which again correspond to the CF₃ group of the ketone and the hydrated form. Therefore the lack of additional peaks for the CF₂ and CF₃ groups in Bz-Arg-CF₂CF₃ and their chemical shifts clearly indicate the absence of any ketone form of this compound in solution.

Previous studies with leupeptin, a Leu-Leu-argininal derivative, indicated the existence of an equilibrium mixture of aldehyde, hydrate and carbinolamine forms in aqueous solution (Maeda *et al.*, 1971; Shimizu *et al.*, 1972). Similarly, the arginylfluoroalkanes could exist as cyclic carbinolamine species [(I) in Fig. 1], which can be easily formed from the keto form [(II) in Fig. 1] by attack of the δ -NH of the guanidino group on the ketone carbonyl group. Alternately, the hydrate form [(III) in Fig. 1] can be formed from the ketone by the addition of one water molecule. The ¹⁹F-n.m.r. spectral data do not

differentiate between the hydrate and the cyclic carbinolamine species, and indeed both may be present in the equilibrium in aqueous solution.

Inhibition kinetics

The inhibition constants (K_i) of several arginylfluoroalkanes with bovine trypsin, bovine thrombin, pig pancreatic kallikrein, human plasma kallikrein, human Factor Xa, human Factor XIa and human Factor XIIa are shown in Table 1. With trypsin all the arginylfluoroalkanes were slow-binding inhibitors, and one example (inhibition of trypsin by Bz-Arg-CF₃) is shown in Fig. 2. Initial rates of substrate hydrolysis were different if the order of addition of the substrate, inhibitor and trypsin was changed. However, the same steady-state rate was eventually reached, and this was used for calculation of the K_i values. With the other enzymes initial rates were used, since there was no indication of the slow-binding type of inhibition.

All seven arginylfluoroalkanes inhibit trypsin quite well, with K_i values of 0.2–56 μ M. The best inhibitors are 1-naphthoyl-Arg-CF₃ and Bz-Arg-CF₃, whereas the poorest one is *p*-toluoyl-Arg-CF₃. The only effective inhibitor for thrombin is Bz-Arg-CF₂CF₃. *p*-Toluoyl-Arg-CF₃ and Bz-Arg-CF₃ inhibit pancreatic kallikrein equally well, whereas the thrombin inhibitor Bz-Arg-CF₂CF₃ did

Table 1. Inhibition of trypsin-like serine proteinases by arginylfluoroalkanes

Inhibition constants were measured in 0.01 M-CaCl₂/0.1 M-Hepes buffer, pH 7.5, containing 9% (v/v) dimethyl sulphoxide at 25 °C. Z-Arg-SBzl was used as the substrate.

Inhibitor	K_i (μ M)						
	Bovine trypsin*	Bovine thrombin	Pig pancreatic kallikrein	Human plasma kallikrein	Human Factor Xa	Human Factor XIa	Human Factor XIIa
Bz-Arg-CF ₃	0.6	> 60	46	50	N.I.†	> 410	> 380
Bz-Arg-CF ₂ CF ₃	7.4	13	N.I.‡	52	> 450	62	> 480
Bz-Arg-CF ₂ CF ₂ CF ₃	6.3	> 90	> 250	> 170	> 160	> 1130	> 320
<i>p</i> -Toluoyl-Arg-CF ₃	56	> 210	35	> 300	> 450	> 1130	> 250
Adamantanoyl-Arg-CF ₃	1.9	> 45	> 110	> 330	> 1400	> 1130	> 800
Naphthoyl-Arg-CF ₃	0.2	> 40	> 60	> 60	> 110	> 500	> 500
Bz-Arg-CClF ₂	3.8	> 56	56	> 80	> 590	> 180	> 240

* All inhibitors showed a slow-binding type of inhibition and rates were measured after incubation of trypsin with inhibitor for 5 min.

† No inhibition at 100 μ M.

‡ No inhibition at 70 μ M.

Fig. 2. Inhibition of trypsin by Bz-Arg-CF₃.

Curve A, trypsin was added to the buffer solution containing Z-Arg-SBzl (75 μ M) and 4,4'-dithiodipyridine (0.34 mM). The trypsin concentration was 0.6 nM. Curve B, trypsin was added to the buffer solution containing Z-Arg-SBzl, 4,4'-dithiodipyridine and Bz-Arg-CF₃ (54 μ M). Curve C, trypsin and Bz-Arg-CF₃ were incubated at room temperature for 5 min, then Z-Arg-SBzl and 4,4'-dithiodipyridine were added.

not inhibit this enzyme at a concentration of 70 μ M. With plasma kallikrein Bz-Arg-CF₃ and Bz-Arg-CF₃ are the only two inhibitors showing reasonable inhibition constants. None of the seven arginylfluoroalkanes is an effective inhibitor of Factor Xa and Factor XIIa at concentrations lower than 100 μ M. The one effective inhibitor for Factor XIa is Bz-Arg-CF₂CF₃, with a K_i value of 60 μ M. The hybrid chlorofluoroketone Bz-Arg-CCIF₂ was tested for time-dependent inhibition with bovine trypsin, but none was observed.

Anticoagulant activity

The anticoagulant activity of the seven arginylfluoroalkanes in human plasma was measured. The PT measures the coagulant activity of the extrinsic pathway, where Factor VIIa, Factor Xa and thrombin are involved. The control value of PT is 11.1 s. The only compound that has a significant effect is Bz-Arg-CF₂CF₃, which increases the PT 1.8-fold at 120 μ M. This anticoagulant activity is consistent with the observation that Bz-Arg-CF₂CF₃ is the most effective thrombin inhibitor in this series of fluoroalkyl ketones. The APTT measures the coagulant activity of the intrinsic pathway, where Factor XIIa, Factor XIa, Factor IXa, Factor Xa and thrombin are involved. The control value of APTT is 32.4 s. Bz-Arg-CF₂CF₃ and naphthoyl-Arg-CF₃ prolong the APTT approx. 1.7–2.4-fold at 90–100 μ M. When both the PT and the APTT are considered, Bz-Arg-CF₂CF₃ is the most effective anticoagulant in this series of fluoroalkyl ketones.

DISCUSSION

Peptidylfluoroalkanes (monofluoro, difluoro and trifluoro) are excellent inhibitors for serine hydrolases, including acetylcholinesterase (Gelb *et al.*, 1985), chymo-

trypsin, pig pancreatic elastase (Imperiali & Abeles, 1986b) and human leucocyte elastase (Stein *et al.*, 1987). Peptidyltrifluoromethanes are slow-binding inhibitors, having K_i values in the submicromolar range, with chymotrypsin and pig pancreatic elastase (Imperiali & Abeles, 1986b), and are nanomolar or subnanomolar inhibitors for human leucocyte elastase when appropriate peptide sequences are used. An X-ray structure of the complex of a peptidyltrifluoromethane with pig pancreatic elastase showed that the active-site Ser-195 of the enzyme has added to the fluoroketone carbonyl group to give a tetrahedral complex with interactions between the oxy-anion of the inhibitor and two hydrogen-bond donors in the oxy-anion hole of the enzyme (Takahashi *et al.*, 1988).

The fluoromethyl ketones Lys-Ala-Lys-CH₂F (McMurray & Dyckes, 1986), Bz-Phe-Lys-CH₂F and Ala-Phe-Lys-CH₂F (Anglikar *et al.*, 1987), D-Phe-Pro-Arg-CH₂F and Bz-Phe-Arg-CH₂F (Anglikar *et al.*, 1988) have been synthesized as inhibitors for trypsin-like enzymes. These lysyl- and arginyl-monofluoromethanes irreversibly inactivated several enzymes, although the inactivation rates are much lower than those of the corresponding chloromethyl ketones. Lys-Ala-Lys-CH₂F had a K_i value of 1 μ M for trypsin (McMurray & Dyckes, 1986) and Ala-Phe-Lys-CH₂F had a K_i value of 5 μ M for plasmin (Anglikar *et al.*, 1987) if the initial rates of substrate hydrolysis were used. Formation of the hemiketal intermediate between the ketone carbonyl group of the inhibitor and the hydroxy group of the active-site serine residue before the alkylation of the enzyme has been proposed for the inactivation of trypsin by these compounds (McMurray & Dyckes, 1986).

The seven new arginylfluoroalkanes described in this paper are reversible inhibitors for trypsin and several blood-coagulation enzymes. None showed time-dependent inhibition: even the chlorodifluoromethyl ketone Bz-Arg-CCIF₂ was purely a reversible competitive inhibitor. The inhibitors showed slow-binding inhibition toward trypsin and the K_i values were measured by using steady-state rates. However, these arginylfluoroalkanes did not show slow-binding inhibition towards any of the coagulation enzymes. It has been demonstrated previously with chymotrypsin that peptidylfluoroalkanes showed slow-binding inhibition behaviour, whereas the amino acid derivatives were simple reversible inhibitors. For example, Bz-Phe-CF₃ was only a competitive inhibition of chymotrypsin, whereas Bz-Leu-Phe-CF₃ was a slow-binding inhibitor (Imperiali & Abeles, 1986b). It is clear that the presence of slow-binding inhibition behaviour depends both on the structure of the inhibitor and on the enzyme under investigation.

Although these arginylfluoroalkanes contain only one amino acid residue, their K_i values towards trypsin are comparable with those of the tripeptidylfluoromethane Lys-Ala-Lys-CH₂F (K_i 1 μ M). Several amidinophenyl alkyl ketones and the corresponding chloromethyl ketones have also been synthesized previously as inhibitors for trypsin-like enzymes (Sturzebecher *et al.*, 1976). The K_i values of these compounds for trypsin were in the range 4–320 μ M, and the K_i values for thrombin were in the range 70–2700 μ M. Arginylfluoroalkanes are more effective inhibitors towards trypsin and are comparable with thrombin when compared with these amidinophenyl alkyl ketones. Although the trifluoromethyl group is a better electron-withdrawing group compared with an

alkyl or chloromethyl group, this effect did not produce a lower K_i in the thrombin case. The tripeptidyl inhibitor D-Phe-Pro-Arg-CH₂F, which contains a thrombin-specific sequence, was a 50-fold better inhibitor than Bz-Arg-CF₂CF₃ (Angliker *et al.* 1988). The tripeptidylfluoromethane is much less potent than would be expected on the basis of results with the corresponding chloromethyl ketone.

Interestingly, the arginylfluoroalkanes showed some selectivity towards various coagulation enzymes. Bz-Arg-CF₂CF₃ is the best inhibitor for thrombin, plasma kallikrein and Factor XIa, but it did not inhibit pancreatic kallikrein or human Factor XIIa. Bz-Arg-CF₃ inhibited pancreatic and plasma kallikreins effectively, but not the other coagulation enzymes. Both *p*-toluoyl-Arg-CF₃ and Bz-Arg-CF₂Cl only showed good inhibitory activity towards pancreatic kallikrein. It is apparent that the different acyl groups such as benzoyl, *p*-toluoyl, adamantanoyl and 1-naphthoyl on the α -amino group of the arginine residue and the various fluoroalkyl group moieties (trifluoromethyl, pentafluoro or heptafluoro) will affect the inhibitory behaviour towards the different coagulation enzymes. The potent thrombin inhibitor 4-methyl-1-[N²-(3-methyl-1,2,3,4-tetrahydroquinoline-8-sulphonyl)-L-arginyl]piperidine-2-carboxylic acid, an arginine derivative, was developed by gradual changes in the inhibitor structure on both the α -amino blocking group and arginine amide group (Kikumoto *et al.*, 1984), and a similar strategy should succeed with arginylfluoroalkanes.

The arginylfluoroalkanes Bz-Arg-CF₂CF₃ and 1-naphthoyl-Arg-CF₃ were moderately effective as anticoagulants. Bz-Arg-CF₂CF₃ doubled the PT and APTT at 90–120 μ M, and 1-naphthoyl-Arg-CF₃ prolonged APTT approx. 1.7-fold at 100 μ M. Since Bz-Arg-CF₂CF₃ inhibited thrombin, plasma kallikrein and Factor XIa, it was expected that this compound would inhibit clotting of both the intrinsic and the extrinsic pathways. Although 1-naphthoyl-Arg-CF₃ was not a good inhibitor of any of the coagulation enzymes tested, it was an effective inhibitor of intrinsic-pathway coagulation. Several coagulation factors, such as Factor IXa in the intrinsic pathway and Factor VIIa in the extrinsic pathway, have not been tested with these fluoroalkyl ketones, and it is possible that they are effectively inhibited by several of the arginylfluoroalkanes. Until the inhibition of all the coagulation factors is checked with these compounds, it is difficult to explain their anticoagulant behaviour rationally.

The potent thrombin inhibitors (2*R*,4*R*)-4-methyl-1-[N²-(3-methyl-1,2,3,4-tetrahydroquinoline-8-sulphonyl)-L-arginyl]piperidine-2-carboxylic acid (K_i 0.02 μ M; Kikumoto *et al.*, 1984) and naphthalene-2-sulphonylglycine 4-amidinophenylalanypiperidinylamide (K_i 0.006 μ M; Hauptmann *et al.*, 1985) are also excellent anticoagulants. These two inhibitors can double the PT and APTT at 1 μ M concentration with human plasma (Green *et al.*, 1985; Hauptmann *et al.*, 1985). The potent Factor Xa inhibitor 1,2-bis-(5-amidinobenzofuran-2-yl)ethane also increases the APTT of human plasma 2-fold at 1.5 μ M (Tidwell *et al.*, 1980). Two other types of compounds that have been tested recently as antithrombotic agents are *p*-amidinophenyl esters (Turner *et al.*, 1986; Pizzo *et al.*, 1986) and isocoumarins. The *p*-amidinophenyl esters inhibit human thrombin and Factor Xa with $k_{obs}/[I]$ values of 0.2×10^4 – 2×10^4 M⁻¹·s⁻¹ and double

the PT at 500 μ M. Recently we have reported that 4-chloro-3-ethoxy-7-guanidinoisocoumarin can inhibit coagulation enzymes quite efficiently with $k_{obs}/[I]$ values of 2×10^4 – 50×10^4 M⁻¹·s⁻¹ and prolong the PT approx. 7-fold at 44 μ M in human plasma (Kam *et al.*, 1988).

In conclusion, we have successfully synthesized the first arginylperfluoroalkanes. These compounds showed reasonably good inhibitory activity towards trypsin and several blood-coagulation enzymes. Although they only contained a single amino acid residue (arginine), these fluoroalkyl ketones already showed some selectivity towards the various coagulation enzymes. The potency and selectivity of these fluoroalkyl ketones would be expected to be increased by introducing systematic changes into the structure and by synthesizing longer peptide sequences. Bz-Arg-CF₂CF₃ and naphthoyl-Arg-CF₃ also showed significant anticoagulant activity.

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