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 $\mu$M. Benzoyl-Arg-CF₂CF₃ was the best inhibitor for bovine thrombin and human Factor Xla, and inhibited thrombin and Factor Xla competitively with $K_i$ values of 13 $\mu$M and 62 $\mu$M respectively. The best inhibitor for pig pancreatic kallikrein was p-toluoyl-Arg-CF₃, with a $K_i$ value of 35 $\mu$M. Benzoyl-Arg-CF₂ and benzoyl-Arg-CF₂CF₃ inhibited human plasma kallikrein competitively, with $K_i$ values of 50 $\mu$M. 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 fluoroalkanes, 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 $\mu$M and to prolong the APTT 2.4-fold at 90 $\mu$M, whereas 1-naphthoyl-Arg-CF₃ only prolonged the APTT 1.7-fold at 100 $\mu$M.

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 fluoroalkyl 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 $k_a$) 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 fluoroalkanes are potent transition-state inhibitors for serine proteinases and in most cases do not alkylate the enzyme. The inhibitors include Ac-Leu-ambo-Phe-CH₂F for chymotrypsin, Ac-Ala-Ala-Pro-ambo-Ala-CH₂F₃ for pig pancreatic elastase (Imperiali & Abeles, 1986b) and Z-Lys(Z)-Val-Pro-Val-CH₂F₃ for human leucocyte elastase (Stein et al., 1987; Dunlap et al., 1987). Upon binding to the serine proteinase, several of the fluoroalkane 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 (Angliker et al., 1987), Bz-Phe-Arg-CH₂F and 9-Phe-Pro-Arg-CH₂F (Angliker 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 x 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 Xla and Factor XIIa were generously donated by Dr. Kazuo Fujikawa (University of Washington, Seattle, WA, U.S.A.). Adamantan-1-carbonyl chloride, benzoyl chloride, 1-naphthyl chloride, p-toluoyl chloride, trifluoroacetic anhydride, 4-dimethyl-

Abbreviations used: ambo, racemic amino acid residue; Bz-, benzoyl-; Z-, benzoxycarbonyl-; SBel, 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 (Aldrichthiol-4) were products of 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 Thrombax reagent were obtained from Ortho Diagnostic Systems (Raritan, N.J., 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.). Ninyhdrin, 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.). High-resolution mass spectra were recorded on a Varian A-60 instrument. All 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% (v/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.I.c.: Rₚ 0.57 (solvent 1), Rₚ 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 adamantanone-1-carbonyl chloride [yield 26%; m.p. 87 °C (decomp.)]. T.I.c.: Rₚ 0.61 (solvent 1), Rₚ 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.I.c.: Rₚ 0.52 (solvent 1), Rₚ 0.46 (solvent 2), positive Sakaguchi reaction. F.a.b.-m.s.: m/z 329 (M⁺+1).

**Synthesis of arginyfluoroalkanes**

**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 benzyloxyarginine 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.I.c.: Rₚ 0.67 (solvent 3), positive Sakaguchi reaction. F.a.b.-m.s.: m/z 331 (M⁺+1).

**Bz-DL-Arg-CF₃CF₃**. This compound was synthesized from benzoylarginine hydrochloride and pentafluoropropionic anhydride (yield 30%; hygroscopic powder, m.p. 67 °C). T.I.c.: Rₚ 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⁺+1–benzoyl) and 489 (M⁺+1+thioglycerol). ¹⁹F n.m.r.: δ (p.p.m.) ([²H₆]dimeethyl sulfoxide) −81.2 (CF₃), −80.6 (CF₂), −118.0 (CF₃) and −119.9 (CF₃). Analysis: Found: C, 39.11; H, 3.86; N, 9.41; Calc. for C₁₀H₁₂F₂N₂O₄, C₁₁F₃CO₂H·H₂O·C, 38.44; H, 3.58; N, 9.96%.

**Bz-DL-Arg-CF₃CF₂CF₃**. This compound was synthesized from benzoylarginine hydrochloride and heptafluorobutyl chloride (yield 8.4%, oil). T.I.c.: Rₚ 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-CCl₃F₄**. This compound was synthesized from benzoylarginine hydrochloride and chlorodifluoroacetic anhydride (yield 65%; oil). T.I.c.: Rₚ 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.I.c.: Rₚ 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.I.c.: Rₚ 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]dimeethyl sulfoxide) −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.I.c.: Rₚ 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).
Anticoagulant activity of arginylfluoroalkanes

**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′-dithiodipipyridine at 25 °C. The rate was monitored by the increase in the absorbance at 324 nm (ε₂₅₄ 19800 M⁻¹ cm⁻¹ for 4-thiopyridine; Grassetti & Murray, 1967) with a Beckman 35 spectrophotometer when 25 μl of enzyme solution (0.06–1.8 μM) was added to a cuvette containing 2 ml of buffer, 150 μl of 4,4′-dithiodipipyridine, 25 μl of substrate solution and 25 μl of inhibitor solution. Initial-rate measurements were performed 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′-dithiodipipyridine. The Kᵢ values were obtained from Dixon plots at two substrate concentrations (25 and 75 μM) and five inhibitor concentrations at each substrate concentration. For less potent inhibitors actual Kᵢ values were not obtained, but the lower limit of Kᵢ 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 Reformatky 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 peptide-fluoromethanes as chymotrypsin and elastase inhibitors (Imperi & Abeles, 1986a,b). A modified Dakin-West method was also used to prepare Z-Phe-Ala-CH₂F (Rasnick, 1985) and α-benzamidoalkyl mono- di- and trifluoromethyl 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(4H)-oxazolines 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 fluoro ketone 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 (Steiglisch & 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-CF₃Cl). 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, octopamine, glycocyamine, α-guanidinobutyric acid and monomethylguanidine give positive reactions, whereas dimethylguanidine, nitroarginine, creatinine, creatine, αδ-dibenzoxyarginine 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 creatinine derivatives; however, none of these would give positive Sakaguchi reactions.

The mass spectra of the fluorooalkyl 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-toluyl-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 thioglycoler matrix, there were often higher mass peaks corresponding to M + 1 + 108 (thioglycoler) 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, CF₂CF₂CO₂H 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 CF₂CF₂CO₂H, and the peaks around -119 p.p.m. were assigned to the corresponding CF₃ 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
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 CF₃ and CF₂ 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 carbinoamine forms in aqueous solution (Maeda et al., 1971; Shimizu et al., 1972). Similarly, the arginylfluoroalkanes could exist as cyclic carbinoamine 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 guanidine 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 carbinoamine species, and indeed both may be present in the equilibrium in aqueous solution.

**Inhibition kinetics**

The inhibition constants (Ki) 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 Ki 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 Ki values of 0.2–56 μM. 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-CF₃CF₃, p-Toluoyl-Arg-CF₃ and Bz-Arg-CF₃ inhibit pancreatic kallikrein equally well, whereas the thrombin inhibitor Bz-Arg-CF₂CF₃ did not inhibit human Factor XII.

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**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.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Bovine trypsin*</th>
<th>Bovine thrombin</th>
<th>Pig pancreatic kallikrein</th>
<th>Human plasma kallikrein</th>
<th>Human Factor Xa</th>
<th>Human Factor XIa</th>
<th>Human Factor XIIa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bz-Arg-CF₃</td>
<td>0.6</td>
<td>&gt;60</td>
<td>46</td>
<td>50</td>
<td>N.I.†</td>
<td>&gt;410</td>
<td>&gt;380</td>
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<tr>
<td>Bz-Arg-CF₂CF₃</td>
<td>7.4</td>
<td>13</td>
<td>N.I.‡</td>
<td>52</td>
<td>&gt;450</td>
<td>62</td>
<td>&gt;480</td>
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<tr>
<td>Bz-Arg-CF₃CF₂CF₃</td>
<td>6.3</td>
<td>&gt;90</td>
<td>&gt;250</td>
<td>&gt;170</td>
<td>&gt;160</td>
<td>&gt;1130</td>
<td>&gt;320</td>
</tr>
<tr>
<td>p-Toluoyl-Arg-CF₃</td>
<td>56</td>
<td>&gt;210</td>
<td>35</td>
<td>&gt;300</td>
<td>&gt;450</td>
<td>&gt;1130</td>
<td>&gt;250</td>
</tr>
<tr>
<td>Adamantanoxy-Arg-CF₃</td>
<td>1.9</td>
<td>&gt;45</td>
<td>&gt;110</td>
<td>&gt;330</td>
<td>&gt;1400</td>
<td>&gt;1130</td>
<td>&gt;800</td>
</tr>
<tr>
<td>Naphthoyl-Arg-CF₃</td>
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<td>&gt;40</td>
<td>&gt;60</td>
<td>&gt;60</td>
<td>&gt;110</td>
<td>&gt;500</td>
<td>&gt;500</td>
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<td>Bz-Arg-CClF₂</td>
<td>3.8</td>
<td>&gt;56</td>
<td>56</td>
<td>&gt;80</td>
<td>&gt;390</td>
<td>&gt;180</td>
<td>&gt;240</td>
</tr>
</tbody>
</table>

* 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 μM.
‡ No inhibition at 70 μM.
Anticoagulant activity

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

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

not inhibit this enzyme at a concentration of 70 μM. 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 μM. The one effective inhibitor for Factor XIIa is Bz-Arg-CF₃CF₃, with a Kᵢ value of 60 μM. 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-CF₃CF₃, which increases the PT 1.8-fold at 120 μM. This anticoagulant activity is consistent with the observation that Bz-Arg-CF₃CF₃ 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 Xa, Factor IXa, Factor Xa and thrombin are involved. The control value of APTT is 32.4 s. Bz-Arg-CF₃CF₃ and naphthyl-Arg-CF₃ prolong the APTT approx. 1.7–2.4-fold at 90–100 μM. When both the PT and the APTT are considered, Bz-Arg-CF₃CF₃ 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), chymotrypsin, pig pancreatic elastase (Imperiali & Abeles, 1986b) and human leucocyte elastase (Stein et al., 1987). Peptidyltrifluoromethanes are slow-binding inhibitors, having Kᵢ 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-CH₃F (McMurray & Dyckes, 1986), Bz-Phe-Lys-CH₃F and Ala-Phe-Lys-CH₃F (Angliker et al., 1987), Bz-Phe-Pro-Arg-CH₃F and Bz-Phe-Arg-CH₃F (Angliker 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-CH₃F had a Kᵢ value of 1 μM for trypsin (McMurray & Dyckes, 1986) and Ala-Phe-Lys-CH₃F had a Kᵢ value of 5 μM for plasmin (Angliker 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 Kᵢ 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 Kᵢ values towards trypsin are comparable with those of the tripeptidylfluoromethane Lys-Ala-Lys-CH₃F (K, 1 μM). 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 Kᵢ values of these compounds for trypsin were in the range 4–320 μM, and the Kᵢ values for thrombin were in the range 70–2700 μM. 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 $K_\text{m}$ in the thrombin case. The tripeptidyl inhibitor D-Phe-Pro-Arg-CH$_2$F, which contains a thrombin-specific sequence, was a 50-fold better inhibitor than Bz-Arg-CF$_2$CF$_2$ (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 arginyfluoroalkanes showed some selectivity towards various coagulation enzymes. Bz-Arg-CF$_2$CF$_2$ is the best inhibitor for thrombin, plasma kallikrein and Factor XIa, but it did not inhibit pancreatic kallikrein or human Factor XIIa. Bz-Arg-CF$_2$ inhibited pancreatic and plasma kallikreins effectively, but not the other coagulation enzymes. Both $p$-tolyuyl-Arg-CF$_2$ and Bz-Arg-CF$_2$Cl only showed good inhibitory activity towards pancreatic kallikrein. It is apparent that the different acyl groups such as benzoyl, $p$-tolyuyl, adamantanyl and 1-naphthoyl on the $\alpha$-amino group of the arginine residue and the various fluorooalkyl group moieties (trifluoromethyl, pentafluoro and Factor VIIa in the extrinsic pathway. Although these compounds showed good inhibitory activity towards the various coagulation enzymes, the potency and selectivity of these fluorooalkyl ketones would be expected to increase by introducing systematic changes into the structure and by synthesizing longer peptide sequences. Bz-Arg-CF$_2$CF$_2$ and naphthoyl-Arg-CF$_2$ also showed significant anticoagulant activity.

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