Mechanism-Based Isocoumarin Inhibitors for Trypsin and Blood Coagulation Serine Proteases: New Anticoagulants†

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ABSTRACT: Trypsin, porcine pancreatic kallikrein, and several blood coagulation enzymes, including bovine thrombin, bovine factor Xa, human factor Xa, human plasma factor XIa, human plasma factor XIIa, and human plasma kallikrein, were inactivated by a number of substituted isocoumarins containing basic functional groups (aminoalkoxy, guanidino, and isothiureidoalkoxy). 3-Alkoxy-4-chloro-7-guanidinoisocoumarins were found to be the most potent inhibitors for the coagulation enzymes tested with $k_{obsd}/[I]$ values in the range of 10^3–10^5 M^{-1} s^{-1}. 4-Chloro-3-isothiureidoalkoxyisocoumarins show high inhibitory potency toward porcine pancreatic kallikrein, human plasma kallikrein, human factor XIa, human factor XIIa, and trypsin with $k_{obsd}/[I]$ values of the order of 10^4–10^5 M^{-1} s^{-1}. The inhibition of these serine proteases by the substituted isocoumarins are time dependent, and the inactivation of trypsin by 3-alkoxy-4-chloro-7-guanidinoisocoumarins and 7-amino-4-chloro-3-(3-isothiureidopropoxy)isocoumarin occurred concurrently with the loss of the isocoumarin absorbance. The complex formed from inactivation of trypsin by these two types of inhibitors was very stable and regained less than 4% activity in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (pH 7.5) after 1 day at 25 °C and regained 8–45% activity upon addition of buffered 0.29 M hydroxylamine. Trypsin inactivated by other inhibitors regained full activity upon standing or addition of hydroxylamine. Thrombin inactivated by 3-alkoxy-4-chloro-7-guanidinoisocoumarins was also quite stable and only regained 9–15% activity under similar conditions. These results are consistent with a proposed mechanism, where serine proteases inactivated by aminoalkoxyisocoumarins or isothiureidoalkoxyisocoumarins form acyl enzymes that will deacylate upon standing or addition of hydroxylamine. However, the acyl enzymes formed from 3-alkoxy-4-chloro-7-guanidinoisocoumarins or 7-amino-4-chloro-3-(3-isothiureidopropoxy)-isocoumarin will decompose further, probably through a quinone imine methide, to give an irreversibly inactivated enzyme by reaction with an active-site nucleophile such as His-57. The quinone imine methide intermediate may also react with a solvent nucleophile to give an acyl enzyme that can be reactivated by hydroxylamine. The inhibitors 4-chloro-7-guanidino-3-methoxyisocoumarin and 4-chloro-3-ethoxy-7-guanidinoisocoumarin have been tested as anticoagulants in human plasma and were effective at prolonging the prothrombin time. However, they are unstable in plasma ($t_{1/2}$ = 4–8 min), and their in vivo utility may be limited.

The blood coagulation system consists of a number of inactive enzymes (zymogens) that are activated in a cascade of enzymatic reactions. The final step in coagulation is the formation of the fibrin clot from fibrinogen by the action of the protease thrombin, which in turn is generated from prothrombin by the action of factor Xa. The intrinsic and extrinsic systems of the blood coagulation pathway converge with the activation of factor X and involve a number of other enzymes such as factor XIIa, factor XIa, factor IXa, and factor VIIa. All the blood coagulation enzymes are serine proteases with trypsin-like specificity; however, they have much more complex structures and are more specific than trypsin. Unlike trypsin, blood coagulation serine proteases are specific for arginine residues and in general cleave only one or a few bonds in their natural substrates.

Although intravascular clotting is a major health problem in the United States, almost no new anticoagulant drugs have been developed in recent years (MacKenzie, 1979; Cross, 1982). The major anticoagulant drug in use today, heparin, acts by accelerating the rate at which the natural protease inhibitor antithrombin III inhibits the coagulation factors. Recent anticoagulant research has concentrated on the preparation of heparin analogues or the synthesis of aromatic guanidines and amidines as inhibitors for thrombin and/or factor Xa. Relatively few of these inhibitors have been tested with blood coagulation serine proteases other than thrombin or factor Xa.

A number of heterocyclic compounds such as ynenol lactones (Copp et al., 1987), haloenol lactones (Daniels et al., 1983), 6-chloropyrones (Weskaemper & Abeles, 1983; Gelb & Abeles, 1984), 3,4-dichloroisocoumarin (Harper et al., 1985), isatoic anhydride and oxazine-2,6-diones (Moorman & Abeles 1982; Weidman & Abeles, 1984), benzopyran-1,4-diones (Hemmi et al., 1985), benzoxazin-4-ones (Hedstrom et al., 1984; Teshima et al., 1982), 3-alkoxy-7-amino-4-chloroisocoumarins (Harper & Powers, 1985), and β-lactams (Doherty et al., 1986) have been shown to act as mechanism-based inactivators of serine proteases. The various inhibitors initially react by acylating the active-site serine (Ser-195), which in many cases results in the unmasking of a reactive functional group (a halo ketone in the case of haloenol lactones, an acyl chloride in the case of chloropyrones and 3,4-di-

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chloroisocoumarin, or a quinone imine methide in the case of 3-alkoxy-7-amino-4-chloroisocoumarins). The unmasked functional group can then react with other active-site residues such as His-57. Several of the inhibitors such as benzoxazin-4-ones and isatoic anhydride react with serine proteases simply to form stable acyl enzymes. Although mechanism-based inhibitors have been widely studied with serine proteases such as chymotrypsin and elastase, heterocyclic compounds substituted with guanidino or amidino groups have not yet been reported as inhibitors for trypsin or trypsin-like enzymes. The only example of a mechanism-based inhibitor for trypsin-like enzymes is 7-(aminomethyl)-1-benzylisatoic anhydride, which inactivates thrombin and trypsin (Gelb & Abeles, 1986).

In this paper we report the synthesis of several new guanidino-, aminoalkoxy-, and isothiureidoalkoxy-substituted isocoumarins that are potent mechanism-based inhibitors for blood coagulation serine proteases and other trypsin-like enzymes. In many cases the inhibited enzyme derivatives are very stable and regain little activity upon long standing or treatment with buffered hydroxylamine. Several of the isocoumarins are effective anticoagulants in human plasma since they significantly prolong the prothrombin time. A preliminary report of this work appeared in an earlier paper (Kam et al., 1987).

Materials and Methods

Human plasma prekallikrein was purified by the method described previously (Chung et al., 1986) and activated by factor XIIa. The remaining factor XIIa activity was neutralized by popcorn trypsin inhibitor. Human factor X was purified by the method of Miletich et al. (1980) and activated by a Russell's viper venom enzyme as described previously (Fujikawa et al., 1972). Human factor XI was purified according to the previous method (Fujikawa et al., 1986) and activated by immobilized bovine trypsin. Human factor XII was prepared as previously described (Fujikawa & Davie, 1980) and activated by trypsin (Fujikawa & McMullen, 1983). Partially activated factor XII was prepared by the previous method (Fujikawa & Davie, 1980). Bovine trypsin, bovine thrombin, bovine factor Xa, porcine pancreatic kallikrein, acetylcholinesterase, papain, thermolysin, leucine aminopeptidase, DTNB,[1] FA-Gly-Leu-NH_2, and Leu-NA·HCl were obtained from Sigma Chemical Co., St. Louis, MO. Hepes was purchased from Research Organics Inc., Cleveland, OH. Bovine liver cathepsin B and Z-Ala-Arg-Arg-AFC were obtained from Enzyme Systems Products, Livermore, CA. Acetylthiocholine was obtained from Aldrich Chemical Co., Milwaukee, WI. Benzoyl-L-Arg-NA·HCl was a product of Bachem Inc., Torrence, CA. Ortho brain thromboplastin was obtained from Ortho Diagnostic Systems, Inc., Raritan, NJ. Protein concentrations were determined by using the appropriate extinction coefficient: 15.2 for β-factor XIIa (Fujikawa & McMullen, 1983); 13.4 for factor XIa (Kurachi & Davie, 1977); and 10.9 for kallikrein (Heimark & Davie, 1979). The concentration of bovine trypsin, bovine thrombin, and porcine pancreatic kallikrein were determined by titration with NPGB (Andrews & Ballillie, 1979). The substrates Z-Phe-Phe-Arg-NA·HCl and Z-Phe-Gly-Arg-NA·HCl (Cho et al., 1984), Z-Arg-SBzl·HCl, Z-Gly-Arg-SBu-*i*·HCl, Z-Trp-Arg-SBzl·HCl, and Z-Phe-Arg-SBu-*i*·HCl (McRae et al., 1981) were prepared as previously described.

Enzyme Inactivation: Incubation Method. An aliquot of inhibitor (25 or 50 μL) in Me_2SO was added to 0.25–0.5 mL of a buffered enzyme solution (0.06–2.3 μM) to initiate the inactivation. Aliquots (50 μL) were withdrawn at various intervals, and the residual enzymatic activity was measured as described below. A 0.1 M Hepes and 0.01 M $CaCl_2$, pH 7.5, buffer was utilized for trypsin and the coagulation enzymes. The Me_2SO concentration in the reaction mixture was 8–12% (v/v). The inhibitor concentrations are shown in the appropriate table. Trypsin was assayed with Z-Phe-Gly-Arg-NA·HCl (0.07 mM) or Z-Phe-Phe-Arg-NA·HCl (0.03 mM; Cho et al., 1984). Bovine thrombin was assayed with Z-Arg-SBzl·HCl (0.07 mM) or Boc-Phe-Phe-Arg-SBzl (0.02 mM; McRae et al., 1981). Bovine factor Xa and porcine pancreatic kallikrein were assayed with Z-Gly-Arg-SBu-*i*·HCl (0.07 mM; McRae et al., 1981). Human factor Xa was assayed with Z-Arg-SBzl·HCl (0.075 mM). Human factor XIIa and human plasma kallikrein were assayed with Z-Arg-SBzl·HCl (0.075 mM) or Z-Trp-Arg-SBzl·HCl (0.09 mM; McRae et al., 1981). Human factor XIa was assayed with Z-Arg-SBzl·HCl (0.075 mM) or Z-Phe-Arg-SBu-*i*·HCl (0.08 mM; McRae et al., 1981). Acetylcholinesterase was assayed with acetylthiocholine (0.36 mM, assay buffer was 0.1 M phosphate, pH 8.0) in the presence of 0.47 mM DTNB (Ellman et al., 1961). Papain was assayed with Bz-L-Arg-NA·HCl (0.57 mM, assay buffer was 50 mM Tris-HCl, 5 mM cysteine, and 2 mM EDTA, pH 8.2). Cathepsin B was assayed with Z-Ala-Arg-Arg-AFC (0.12 mM, assay buffer was 0.1 M phosphate, 1.3 mM EDTA, and 2.7 mM cysteine, pH 6.0). Thermolysin was assayed with FA-Gly-Leu-NH_2 (1 mM, assay buffer was 0.1 M Hepes and 0.01 M $CaCl_2$, pH 7.2). Leucine aminopeptidase was assayed with Leu-NA·HCl (0.6 mM). All peptide thio ester hydrolysis rates were measured with assay mixtures containing 4,4′-dithiodipyridine (ϵ_{324} = 19800 M^{-1} cm^{-1}; Grasetti & Murray, 1967). Peptide 4-nitroanilide hydrolysis was measured at 410 nm (ϵ_{410} = 8800 M^{-1} cm^{-1}; Erlanger et al., 1961).

Pseudo-first-order inactivation rate constants (k_{obsd}) were obtained from plots of ln v_t/v_0 vs time, and the correlation coefficients were greater than 0.98. Apparent second-order inactivation rate constants ($k_{obsd}/[I]$) shown in Table I are typically the average of duplicate or triplicate experiments. In some cases, inactivation rates were too fast to be measured under the first-order rate conditions ([I] > [E]). Therefore, equal concentrations of enzyme and inhibitor were used, and the half-life ($t_{1/2}$) for the second-order reaction was determined. The second-order rate constant (k_{2nd}) was calculated from the equation $k_{2nd} = 1/([E]t_{1/2})$.

Determination of Inactivation Rates in the Presence of Substrate: Progress Curve Method. In several cases, $k_{obsd}/[I]$ values were determined in the presence of substrate as described by Tian and Tsou (1982). For example, the inhibition of human plasma kallikrein (8 nM) with 4-chloro-7-guanidino-3-methoxyisocoumarin (7) was studied by adding a 25-μL aliquot of enzyme to a buffered solution of Z-Trp-Arg-SBzl (0.09 mM) containing 4,4′-dithiodipyridine (0.3 mM), inhibitor (0.05–0.2 μM), and 13% Me_2SO. The increase in absorbance was monitored at 324 nm with time until no further release of benzyl thiol was observed. Inactivation of trypsin (85 nM) and thrombin (90 nM) by 4-chloro-7-

[1] Abbreviations: AFC, 7-amino-4-(trifluoromethyl)coumarin; Boc, *tert*-butyloxycarbonyl; ADMP, 3,5-dimethylpyrazole-1-carboxamidine nitrate; Me_2SO, dimethyl sulfoxide; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); FA-Gly-Leu-NH_2, *N*-[3-(2-furyl)acryloyl]glycyl-L-leucinamide; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; NA, *p*-nitroanilide; NPGB, *p*-nitrophenyl *p*-guanidinobenzoate; SBzl, benzyl thio ester; SBu-*i*, isobutyl thio ester; Z, benzyloxycarbonyl; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; TLC, thin-layer chromatography; THF, tetrahydrofuran; DMF, dimethylformamide; Dns, dansyl.

guanidino-3-methoxyisocoumarin (0.1–0.4 μM) in the presence of the substrate Z-Phe-Phe-Arg-NA (0.05 mM) and inactivation of porcine pancreatic kallikrein (0.1 μM) by 4-chloro-7-guanidino-3-methoxyisocoumarin (0.2–0.4 μM) in the presence of the substrate Z-Gly-Arg-SBu-*i* (70 μM) were carried out similarly.

Pseudo-first-order inhibition constants (k_{obsd}) at various inhibitor concentrations were obtained from the slopes of plots of log ($[P]_\infty - [P]_t$) vs time by using eq 1, where $[P]_\infty$ and $[P]_t$

$$\log ([P]_\infty - [P]_t) = \log [P]_\infty - 0.43k_{obsd}t \quad (1)$$

are the concentrations of substrate hydrolysis product formed at infinite time (total inactivation) and at time t (Tian & Tsou, 1982). Correlation coefficients for such plots were always greater than 0.98.

For inhibitors that form a reversible E·I complex prior to the irreversible formation of a E–I complex, the dissociation constant of the enzyme–inhibitor complex (K_I) and the rate of formation of E–I from E·I (k_2) are related to the apparent second-order rate constant ($k_{obsd}/[I]$) by eq 2 (Tian & Tsou, 1982). The k_2 values were calculated from the slopes of

$$1/(k_{obsd}/[I]) = (1 + [S]/K_M + [I]/K_I)/(k_2/K_I) \quad (2)$$

$1/(k_{obsd}/[I])$ vs [I] plots, while K_I values were calculated from the y-axis intercept. Calculated k_2/K_I values are reported in Table I along with $k_{obsd}/[I]$ values determined for other inhibitors by the incubation method. If the K_I value for an inhibitor is much greater than the chosen inhibitor concentration ([I]) and [I] is greater than the enzyme concentration, then $k_{obsd}/[I]$ values are equal to k_2/K_I (Knight, 1986).

Reactivation and Deacylation Kinetics. The reactivation of enzymes inhibited by substituted isocoumarins was studied by monitoring the recovery of enzymatic activity with time upon standing of the inhibited solution at 25 °C without removal of excess inhibitors. Deacylation rates of inactivated enzymes were measured after the removal of excess inhibitors from the solution either by centrifugation twice at 0 °C for 1 h using Amicon Centricon-10 microconcentrators (in the case of trypsin) or by dialysis against 0.1 M Hepes and 0.01 M $CaCl_2$, pH 7.5, buffer for 3 h (in the case of thrombin). The enzymatic activity of the solution was assayed at various time intervals as described above. Hydroxylamine-catalyzed reactivation rates were measured likewise after addition of buffered hydroxylamine (0.29 M). The first-order reactivation rates (k_{obsd}) or deacylations rates (k_{deacyl}) were obtained from plots of ln ($v_0 - v_t$) vs time, where v_0 is the enzyme-catalyzed substrate hydrolysis rate of the solution under the same conditions in the absence of inhibitor. The correlation coefficients were greater than 0.98.

Determination of Spontaneous Hydrolysis Rates of Inhibitors in Buffer. An aliquot of the isocoumarin derivatives in Me_2SO was added to 0.1 M Hepes and 0.5 M NaCl, pH 7.5, buffer such that the inhibitor concentration was 0.05–0.07 mM and the Me_2SO concentration was 10% v/v. The spontaneous hydrolysis rate was monitored by following the decrease in absorbance at 350 nm [for isothiureidoalkoxyisocoumarins and 3-(aminoalkoxy)isocoumarins], 360 nm (for 3-alkoxy-7-guanidinoisocoumarins), or 380 nm [for 7-amino-4-chloro-3-(3-isothiureidopropoxy)isocoumarin]. The hydrolysis products give negligible absorbance at the wavelengths utilized. First-order rate constants were obtained from plots of ln ($A_t - A_f$) vs time, where A_f is the absorbance of the hydrolysis product. All the plots gave correlation coefficients of 0.99 or greater.

Detection of Enzymatic Ring Opening: Spectrophotometric Method. The enzymatic ring opening of 7-guanidino-3-alkoxyisocoumarins was detected spectrophotometrically by monitoring the absorbance decrease at 360 nm. For example, an aliquot (50 μL) of an enzyme solution was added to 2.05 mL of a buffered solution containing inhibitor ([E] = 30 μM, [I] = 24–50 μM, 0.1 M Hepes, 0.01 M $CaCl_2$, pH 7.5, and 2.5% Me_2SO) and the decrease in absorbance at 360 nm measured. Under these conditions, extinction coefficients of 3650, 3380, and 3850 M^{-1} cm^{-1} were determined for 4-chloro-7-guanidino-3-methoxyisocoumarin, 4-chloro-3-ethoxy-7-guanidinoisocoumarin, and 4-chloro-7-guanidino-3-(2-phenylethoxy)isocoumarin. Enzymatic ring opening of 4-chloro-3-(3-isothiureidopropoxy)isocoumarin (ϵ_{350} = 3550 M^{-1} cm^{-1}), 7-amino-4-chloro-3-(3-isothiureidopropoxy)isocoumarin (ϵ_{375} = 3550 M^{-1} cm^{-1}), and 7-guanidino-4-methoxyisocoumarin (ϵ_{350} = 3400 M^{-1} cm^{-1}) by trypsin were measured similarly.

Fluorescence Method. A Perkin-Elmer spectrophotofluorometer was standardized with quinine sulfate (3 μM) in 0.1 N H_2SO_4 (λ_{ex} = 350 nm, λ_{em} = 440 nm) as 100% fluorescence and buffer as 0% fluorescence. The enzymatic ring opening of 3-ethoxy-7-guanidinoisocoumarin (18 μM), 7-guanidino-3-(2-phenylethoxy)isocoumarin (18 μM) was detected by monitoring the decrease in fluorescence emmission at 450 nm (λ_{ex} = 300 and 290 nm, respectively) upon the addition of trypsin (16 μM). Similarly, the decrease in fluorescence emmission at 500 nm (λ_{ex} = 400 nm) was monitored upon addition of trypsin (58 μM) to a buffered solution (0.1 M Hepes and 0.01 M $CaCl_2$, pH 7.5) containing 7-amino-4-chloro-3-(3-isothiureidopropoxy)isocoumarin (55 μM).

Coagulant Assay. The prothrombin time (Quick, 1935) was used to measure the time needed for plasma to clot after the addition of calcium and tissue factor. The complex formed between plasma factor VII and tissue factor in the presence of Ca^{2+} directly activates factor X. Thus, the prothrombin time measures the clotting ability of the extrinsic system. Ortho brain thromboplastin and human plasma were incubated separately at 37 °C for 10–15 and 3 min, respectively, and then 0.2 mL of thromboplastin was added to 0.1 mL of human plasma. Simultaneously, a stopwatch was started and the test tube was shaken to well mix the reactants. The test tube was removed from the incubation bath and gently tilted back and forth until a clot first formed, and the time was recorded as the prothrombin time. The prothrombin time of plasma was measured similarly in the presence of inhibitor with or without preincubation of inhibitor and plasma at 37 °C for 3 min.

Synthesis. Homophthalic acid, 2-bromoethanol, 3-bromopropanol, 3-aminopropanol, 3,5-dimethylpyrazole-1-carboxamidine nitrate (ADMP), and phosphorus pentachloride were obtained from Aldrich Chemical Co., Milwaukee, WI. Thiourea was obtained from Fisher Scientific. *N*-Chlorosuccinimide was obtained from Eastman Kodak Co. All common chemicals and solvents are of reagent grade. Each new compound was checked by UV, NMR, melting point, mass spectroscopy, thin-layer chromatography, and elementary analysis, and the results are consistent with their proposed structures. The solvent systems used for thin-layer chromatography are benzene (system 1), methylene chloride (system 2), methylene chloride–methanol 5:1 (system 3), butanol–acetic acid–pyridine–water 4:1:1:2 (system 4), and butanol–acetic acid–water 6:1:5 (system 5). The NMR spectra were recorded on Varian T-60, Bruker WM-300, or Varian 400 instruments. The mass spectra were recorded on a VG-ZAB mass spectrometer. Elementary analyses were performed by Atlantic Microlabs of Atlanta, Ga. The syntheses of three of the more important inhibitors are given in this section; all

Table I: Inactivation Rates for Inhibition of Trypsin-like Serine Proteases by Substituted Isocoumarins[a]

	$k_{obsd}/[I]$ (M^{-1} s^{-1})							
inhibitor	bovine thrombin[b]	bovine factor Xa[c]	human factor Xa[d]	porcine pancreatic kallikrein[e]	human plasma kallikrein[f]	human factor XIa[g]	human factor XIIa[h]	bovine trypsin[i]
1	2.7	NI[j]		4.7	29	28	3.2	1 200
2	350	160		860	1 400	380	190	7 600
3	4 700	5 600		12 000	>280 000[k]	44 000	39 000	32 000
4	1 400	220		19 000	>112 000[k]	47 000	27 000	46 000
5	630	1 600	61	110 000[l]	1 100	22 000	6 200	410 000[m]
6	4 300	460		1 900	13 000	1 400	520	3 300
7	290 000[m]	3 100	11 000	45 000[m]	240 000[m]	36 000	20 000	310 000[m]
8	3 700	2 700		16 000	44 000	3 100	1 300	20 000
9	55 000[l]	27 000	11 000	200 000[l]	>500 000[k]	60 000	22 000	>110 000[l]
10	5 700	11 000		16 000	62 000	1 200	690	45 000
11	30 000[l]	96 000	11 000	200 000[l]	>270 000[k]	20 000	26 000	>110 000[l]

[a] Conditions were as follows: 0.01 M Hepes, 0.01 M $CaCl_2$, pH 7.5, and 8–12% Me_2SO at 25 °C. Rate constants were obtained by the incubation method (see Materials and Methods) unless otherwise noted. [b] Inhibitor concentrations were as follows: **1**, 400 μM; **2**, 52 μM; **3**, 5 μM; **4**, 8 μM; **5**, 14 μM; **6**, 8 μM; **7**, 0.3 μM; **8**, 16 μM; **9**, 5 μM; **10**, 17 μM; **11**, 45 μM. [c] Inhibitor concentrations were as follows: **1**, 310 μM; **2**, 240 μM; **3**, 0.5 μM; **4**, 15 μM; **5**, 38 μM; **6**, 43 μM; **7**, 8 μM; **8**, 5 μM; **9**, 0.4 μM; **10**, 1.5 μM; **11**, 0.4 μM. [d] Inhibitor concentrations were as follows: **5**, 105 μM; **7**, 6 μM; **9**, 6 μM; **11**, 5 μM. [e] Inhibitor concentrations were as follows: **1**, 300 μM; **2**, 9 μM; **3**, 1.3 μM; **4**, 0.9 μM; **5**, 1.7 μM; **6**, 9 μM; **7**, 0.4 μM; **8**, 8 μM; **9**, 1.7 μM; **10**, 9 μM; **11**, 1.7 μM. [f] Inhibitor concentrations were as follows: **1**, 330 μM; **2**, 9.2 μM; **3**, 0.3 μM; **4**, 0.5 μM; **5**, 4.1 μM; **6**, 3.1 μM; **7**, 0.4 μM; **8**, 3.3 μM; **9**, 0.5 μM; **10**, 1.7 μM; **11**, 0.43 μM. [g] Inhibitor concentrations were as follows: **1**, 330 μM; **2**, 28 μM; **3**, 3 μM; **4**, 4 μM; **5**, 8 μM; **6**, 5 μM; **7**, 4 μM; **8**, 9.2 μM; **9**, 6 μM; **10**, 11 μM; **11**, 5 μM. [h] Inhibitor concentrations were as follows: **1**, 330 μM; **2**, 28 μM; **3**, 3 μM; **4**, 4 μM; **5**, 8 μM; **6**, 5 μM; **7**, 4 μM; **8**, 9.2 μM; **9**, 6 μM; **10**, 11 μM; **11**, 5 μM. [i] Inhibitor concentrations were as follows: **1**, 12 μM; **2**, 4.5 μM; **3**, 3 μM; **4**, 1 μM; **5**, 0.14 μM; **6**, 11 μM; **7**, 0.24 μM; **8**, 3.4 μM; **9**, 1.5 μM; **10**, 3 μM; **11**, 2.5 μM. [j] No inhibition. [k] Inactivation was extremely rapid, and the $k_{obsd}/[I]$ values were based on the residual enzymatic activity at 0.2 min. [l] The second-order rate constant was determined by using equal molar concentrations of inhibitor and enzyme. [m] Inactivation rate constant using the progress curve method (see Materials and Methods).

other new compounds are reported in the supplementary material.

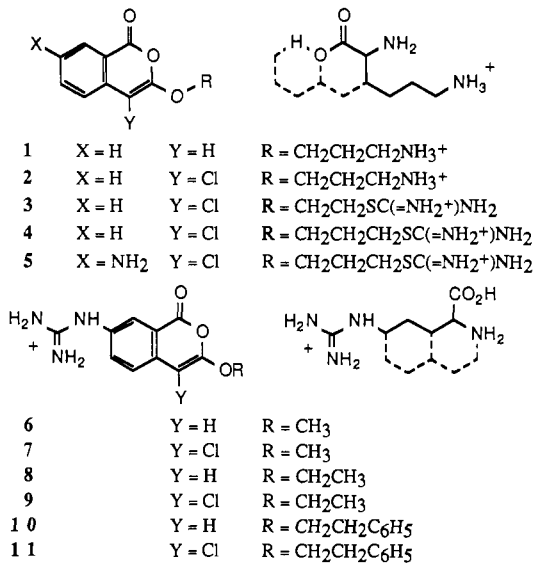
4-Chloro-3-(2-isothiureidoethoxy)isocoumarin (**3**). 2-Bromoethyl 2-carboxyphenylacetate was prepared from heating 10 g of homophthalic acid (56 mmol) and 21 g of 2-bromoethanol (167 mmol) in 175 mL of benzene with a few drops of concentrated sulfuric acid at 90–110 °C for 2 h (yield, 64%). The ester was pure by TLC. Cyclization of 2-bromoethyl 2-carboxyphenylacetate with PCl_5 was performed by a modification of the method of Tirodkcar and Usganokas (1969). 2-Bromoethyl 2-carboxyphenylacetate (1.15 g) was heated with 2.1 g of PCl_5 in 90 mL of benzene at 70 °C for 2 h. The benzene was removed and the residue triturated with petroleum ether. The crude product was purified by silica gel column chromatography with methylene chloride as an eluent to give 560 mg of 3-(bromoethyl)-4-chloroisocoumarin (yield, 46%). IR and NMR spectra show it to be the desired product. 3-(Bromoethyl)-4-chloroisocoumarin (100 mg, 0.3 mmol) was heated with 60 mg of thiourea (0.8 mmol) in 5 mL of THF at 70 °C for 2 days to give a yellow solid, 50 mg (yield, 40%): mp 167–169 °C dec; one spot on TLC, $R_f = 0.7$ (system 5); NMR spectrum (DMSO-d_6) δ 9.1 (2 b, 4 H), 7.5–8.1 (m, 4 H), 4.6 (t, 2 H), 3.6 (t, 2 H); mass spectrum (FAB$^+$), *m/e* 299 (M^+ − Br). Anal. Calcd for $C_{12}H_{12}N_2O_3Br_1Cl_1S_1$: C, 37.96; H, 3.19, N, 7.38. Found: C, 37.81; H, 3.28; N, 7.71.

7-Guanidino-3-methoxyisocoumarin (**6**). Methyl 2-carboxy-4-nitrophenylacetate was prepared from 2-carboxy-4-nitrophenylacetic acid and methanol by the esterification procedure described above. Hydrogenation of this nitro compound gives methyl 4-amino-2-carboxyphenylacetate (yield, 90%). The guanidination of the amino compound with ADMP was carried out by the method of Tsunematsu and Makismi (1980). The amino compound (2.2 g, 10 mmol), 1.9 g of triethylamine (19 mmol), and 3.0 g of ADMP (15 mmol) were heated in 50 mL of THF at reflux for 18 h. The white precipitate was filtered and washed with cold methanol to give 1.5 g of methyl 2-carboxy-4-guanidinophenylacetate (yield, 46%): one spot on TLC, $R_f = 0.6$ (system 4), orange color when sprayed with Sakaguchi reagent; NMR spectrum (CF_3COOH) δ 8.4, 7.7 (b, 4 H), 6.6 (b, 4 H), 4.4 (s, 2 H), 4.1 (s, 3 H). Anal. Calcd for $C_{11}H_{13}N_3O_4 \cdot {}^1/_2H_2O$: C, 50.77; H, 5.42; N, 16.15. Found: C, 51.03; H, 5.38; N, 16.19. Methyl 2-carboxy-4-guanidinophenylacetate (0.9 g, 3 mmol) was heated with 1.5 g of PCl_5 (7.2 mmol) at 70–80 °C for 2 h. A white solid that precipitated during the heating, was filtered, and purified by silica gel column chromatography with methylene chloride and methanol (5:1) as an eluent to give 0.5 g of 7-guanidino-3-methoxyisocoumarin (yield, 59%): one spot on TLC, $R_f = 0.7$ (system 4); mp 185–186 °C dec; NMR spectrum (DMSO-d_6) δ 7.9, 7.6 (b, 3 H), 7.7 (b, 4 H), 6.1 (s, 1 H), 3.9 (s, 3 H); mass spectrum (FAB$^+$), *m/e* 234 (M^+ − Cl). Anal. Calcd for $C_{11}H_{12}N_3O_3Cl_1 \cdot {}^1/_2H_2O$: C, 47.40; H, 4.67; N, 15.08; Cl, 12.75. Found: C, 47.42; H, 4.74; N, 15.05; Cl, 12.68.

4-Chloro-7-guanidino-3-methoxyisocoumarin (**7**). 7-Guanidino-3-methoxyisocoumarin (0.27 g, 1 mmol) was chlorinated with 0.15 g of *N*-chlorosuccinimide (1.1 mmol) in 5 mL of DMF at room temperature overnight. The reaction mixture was evaporated to dryness and the product purified by silica gel column chromatography with methylene chloride and methanol (5:1) as an eluent to give 0.1 g of 4-chloro-7-guanidino-3-methoxyisocoumarin (yield, 34%): one spot on TLC, $R_f = 0.75$ (system 4); the NMR spectrum is similar to that of compound **6** except there was no peak at 6.1 ppm; mass spectrum (FAB$^+$), *m/e* 268 (M^+ − Cl). Anal. Calcd for $C_{11}H_{11}N_3O_3Cl_2 \cdot {}^1/_2H_2O$: C, 42.17; H, 3.83; N, 13.41; Cl, 22.68. Found: C, 42.65; H, 3.72; N, 13.28; Cl, 22.32.

Results

Inactivation Kinetics. Several substituted isocoumarins (Figure 1) containing basic functional groups (guanidino, aminoalkoxy, isothiureidoalkoxy) were synthesized and tested as inhibitors of the blood coagulation enzymes bovine thrombin, bovine factor Xa, human factor Xa, human factor XIa, human factor XIIa, and human plasma kallikrein. Inhibition kinetics were also measured with bovine trypsin and porcine pancreatic kallikrein. The second-order inactivation rate constants $k_{obsd}/[I]$ are reported in Table I. In most cases, the first-order rate (k_{obsd}) plots were linear for a least 2 half-lives.



1	X = H	Y = H	R = $CH_2CH_2CH_2NH_3^+$
2	X = H	Y = Cl	R = $CH_2CH_2CH_2NH_3^+$
3	X = H	Y = Cl	R = $CH_2CH_2SC(=NH_2^+)NH_2$
4	X = H	Y = Cl	R = $CH_2CH_2CH_2SC(=NH_2^+)NH_2$
5	X = NH_2	Y = Cl	R = $CH_2CH_2CH_2SC(=NH_2^+)NH_2$
6		Y = H	R = CH_3
7		Y = Cl	R = CH_3
8		Y = H	R = CH_2CH_3
9		Y = Cl	R = CH_2CH_3
10		Y = H	R = $CH_2CH_2C_6H_5$
11		Y = Cl	R = $CH_2CH_2C_6H_5$

FIGURE 1: Structures of substituted isocoumarin inhibitors of trypsin-like enzymes.

The inhibition rates with many of the isocoumarin inactivators were so fast that they could not be measured accurately under pseudo-first-order reaction conditions of [I] > [E] by using the incubation method. For instance, the inactivation of human plasma kallikrein by 4-chloro-3-isothiureidoalkoxyisocoumarins **3** and **4** was very rapid, and less than 20% of residual enzymatic activity was observed at the first time points (0.2–0.3 min). Therefore, in these cases, the calculated inactivation rate constants should be considered to be lower limits. Two alternate kinetics methods were used to measure the inactivation rates with some of the fast inhibitors. The progress curve method (Tian & Tsou, 1982) involved the addition of substrates to the inhibition mixture to slow the inactivation rate, and several of the rate constants listed in Table I were determined by this method. With the most potent inhibitors even this method did not yield acceptable results. An alternate method involved the determination of the second-order inactivation rate constant, k_{2nd}, under conditions where the inhibitor and enzyme concentrations were equal. The rate constant could then be calculated from the equation $k_{2nd} = 1/(t_{1/2}[E])$. The rate of inactivation of porcine kallikrein and human plasma kallikrein by 4-chloro-7-guanidino-3-methoxyisocoumarin (**7**) was measured both with the incubation method, $k_{obsd}/[I]$ values of 27 000 and 240 000 M^{-1} s^{-1}, respectively, and with the progress curve method, k_2/K_I values of 45 000 and 240 000 M^{-1} s^{-1}, respectively, were obtained.

It is likely that all the isocoumarin inhibitors are inhibiting via an intermediate noncovalent enzyme inhibitor complex according to the mechanism (Knight, 1986)

$$E + I \underset{}{\overset{K_I}{\rightleftharpoons}} E{\cdot}I \xrightarrow{k_2} E\text{–}I$$

The reaction is described by the dissociation constant (K_I) of the enzyme–inhibitor complex (or all steady-state complexes formed prior to k_2) and the first-order rate constant for the formation of the inactivated enzyme (k_2). Using the progress curve method, values for K_I and k_2 for several of the more rapid reactions were determined. The inhibition of trypsin by 7-amino-4-chloro-3-(3-isothiureidopropoxy)isocoumarin (**5**) gave values of 11 nM and 0.0043 s^{-1} for K_I and k_2, respectively. The corresponding values obtained with 4-chloro-7-guanidino-3-methoxyisocoumarin (**7**) were 10 nM and 0.0031 s^{-1} for bovine thrombin and 30 nM and 0.0085 s^{-1} for trypsin.

Table II: Deacylation Rate Constants for Trypsin Inactivated by Substituted Isocoumarins[a]

inhibitors	$t_{1/2}$ (min)	k_{deacyl} (s^{-1})	act. regained (%)
1	22	5.3×10^{-4}	86
2	100	1.2×10^{-4}	100
3	214	0.5×10^{-4}	100
4	312	0.4×10^{-4}	100
5			4[b]
6	21	5.5×10^{-4}	100
7			4[b]
8	62	1.9×10^{-4}	100
9			3[b]
10	30	3.9×10^{-4}	100
11			1[b]

[a] Inactivation trypsin solution (in 0.1 M Hepes and 0.01 M $CaCl_2$, pH 7.5, buffer) was centrifuged at 0 °C for 1 h twice; Centricon-10 microconcentrator was used to remove excess inhibitors. The deacylation rates were measured at 25 °C. [b] The enzyme activity was measured after 1 day.

Table III: Reactivation of Trypsin Inactivated by Substituted Isocoumarins in the Presence of NH_2OH[a]

inhibitor	$t_{1/2}$ (min)	act. regained (%)
1	0.9	96
2	1.3	90
3	7.3	100
4	10.3	100
5		45[b]
6	8.7	100
7		16[b]
8	54	94
9		8[b]
10	6.0	96
11		11[b]

[a] The reactivation rates were measured in 0.1 M Hepes, 0.01 M $CaCl_2$, pH 7.5, and 0.29 M NH_2OH, 25 °C, without removal of excess inhibitor. [b] The enzyme activity was measured after 1 day in the presence of 0.22 M NH_2OH after removal of excess inhibitor, which was removed by centrifugation using a Centricon-10 microconcentrator.

Aminopropoxyisocoumarins **1** and **2** show reasonable inhibition toward trypsin with $k_{obsd}/[I]$ values of 10^3 M^{-1} s^{-1}; however, they are 1–2 orders of magnitude less reactive toward other coagulation enzymes. 3-Alkoxy-7-guanidinoisocoumarins inhibit trypsin-like enzymes with $k_{obsd}/[I]$ values of 10^2–10^4 M^{-1} s^{-1}, which are 1–2 orders of magnitude less reactive than their chloro derivatives. In general, 3-alkoxy-4-chloro-7-guanidinoisocoumarins are potent inhibitors for all the trypsin-like enzymes tested in this study. 4-Chloro-3-(isothiureidoalkoxy)isocoumarins showed their most potent inhibitory activity toward human plasma kallikrein, followed by human factor XIa and human factor XIIa.

Reactivation Kinetics. Trypsin inactivated by substituted isocoumarins showed differential stability toward spontaneous reactivation. Trypsin inactivated by 3-aminopropoxyisocoumarins **1** and **2**, 4-chloro-3-(isothiureidoalkoxy)isocoumarins **3** and **4**, and 3-alkoxy-7-guanidinoisocoumarins **6**, **8**, and **10** regained full activity upon standing at 25 °C. The deacylation rate constants, half-lives for deacylation, and percent activity regained are shown in Table II. Excess inhibitor in the enzyme–inhibitor mixtures was removed by centrifugation using a Centricon-10 microconcentrator before deacylation rates were measured but was not removed prior to determining the percent enzyme activity regained upon standing. Trypsin inhibited by 7-amino-4-chloro-3-(3-isothiureidopropoxy)isocoumarin (**5**) or 3-alkoxy-4-chloro-7-guanidinoisocoumarin **7**, **9**, and **11** only regained a few percent activity upon standing at 25 °C for 1 day.

The reactivation of inhibited trypsin was determined in the presence of excess inhibitors after addition of buffered hy-

Table IV: Half-Lives for the Spontaneous Hydrolysis of Isocoumarin Derivatives in Hepes Buffer[a] and in Human Plasma

isocoumarin inhibitor	$t_{1/2}$ (min)	
	Hepes	plasma
1	606	
2	123	
3	83	
4	99	0.5
5	90	165
6	252	
7	44	6.7
8	136	
9	39	8.2
10	140	
11	36	4.2

[a] Conditions were 0.1 M Hepes, 0.5 M NaCl, pH 7.5, and 9% Me_2SO at 25 °C.

droxylamine. Trypsin inactivated by compounds **1–4**, **6**, **8**, and **10** regained 90–100% activity upon addition of buffered hydroxylamine (0.29 M) with the various half-lives listed in Table III. However, trypsin inactivated by 7-amino-4-chloro-3-(3-isothiureidopropoxy)isocoumarin (**5**) regained only 45% activity, and trypsin inactivated by 3-alkoxy-4-chloro-7-guanidinoisocoumarins **7**, **9**, and **11** regained 8–16% activity under the same conditions.

Reactivation experiments with thrombin were performed after removal of excess inhibitor by dialysis instead of centrifugation, since thrombin bound to the Centricon membrane and lost most its activity during the process. However, bovine thrombin still lost 30% activity during the dialysis experiment. Thrombin inhibited by the 3-alkoxy-4-chloro-7-guanidinoisocoumarin regained less than 9% activity upon standing at 25 °C for 18 h and regained 15% activity upon addition of buffered hydroxylamine (0.29 M), while the same enzyme inactivated by other inhibitors regained 25–91% activity upon standing and 73–100% activity upon addition of hydroxylamine.

Spontaneous Hydrolysis. The rate constants for spontaneous hydrolysis of the substituted isocoumarins were determined in Hepes buffer, and the half-lives are listed in Table IV. 3-(Aminopropoxy)isocoumarin was the most hydrolytically stable inhibitor. Isocoumarins with 4-chloro substituents were hydrolyzed 3–6-fold more rapidly than those without the chlorine atom. The stability of a number of the inhibitors was checked in human plasma and with one exception was found to be much less stable than in Hepes. The 7-guanidinoisocoumarins were found to decompose with half-lives of a few minutes. Interestingly, 7-amino-4-chloro-3-(3-isothiureidopropoxy)isocoumarin was the most stable of any of the isocoumarin inhibitors and was slightly more stable in plasma than in buffer.

Spectra Changes. The UV–visible spectra of 4-chloro-3-ethoxy-7-guanidinoisocoumarin (**9**, 30 μM) and its hydrolysis product (after 20 h, 28 half-lives) in 0.1 M Hepes and 0.5 M NaCl buffer (pH 7.5) at 25 °C are shown in Figure 2. The other 3-alkoxy-4-chloro-7-guanidinoisocoumarins and their hydrolysis products have similar spectra. Addition of trypsin (30 μM) to a buffered solution of **4** (ϵ_{345} = 3550 M^{-1} cm^{-1}, 28 μM), **5** (ϵ_{375} = 3550 M^{-1} cm^{-1}, 38 μM), **7** (ϵ_{360} = 3650 M^{-1} cm^{-1}, 24 μM), **8** (ϵ_{355} = 3840 M^{-1} cm^{-1}, 35 μM), **9** (ϵ_{360} = 3380 M^{-1} cm^{-1}, 30 μM), or **11** (ϵ_{360} = 3850 M^{-1} cm^{-1}, 27 μM) resulted in loss of the isocoumarin ring chromophore within 0.3 min (results with **9** shown in Figure 2). Simultaneously, an aliquot was removed from the mixture of trypsin and inhibitor at 0.2–0.3 min to monitor the residual enzyme activity, and no trypsin activity was found with compounds **5**, **7**, **9**, and **11**. An attempt was made to use these spectral changes to determine the trypsin-catalyzed ring-opening rate constants. However, due to the fast reaction of the inhibitor with trypsin, the ring-opening rate constants could not be determined under these conditions. The inactivation stoichiometry was calculated from the total absorbance change accompanying inactivation by using the experimentally determined extinction coefficients. The results are shown in Table V. The inactivation by trypsin by compounds **5**, **7**, **9**, and **11** was essentially stoichiometric.

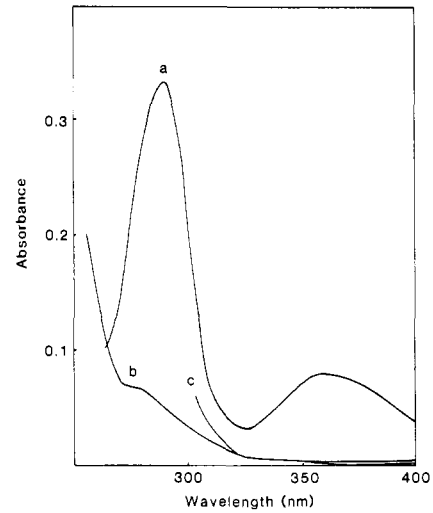


FIGURE 2: Absorption spectra of 4-chloro-3-ethoxy-7-guanidinoisocoumarin (**9**, 30 μM) (curve a) and its hydrolysis product (curve b) in 0.1 M Hepes and 0.5 M NaCl, pH 7.5, buffer and 9% Me_2SO at 25 °C. (Curve c) Spectrum for trypsin (30 μM) was added to the inhibitor solution, and the absorption spectra was taken after 0.3 min.

Table V: Stoichiometry of Ring Opening of Substituted Isocoumarins by Trypsin[a]

inactivator	trypsin	
	[I] (μM)	inactivation stoichiometry
5	31	1.1
7	28	1.04
9	28	1.04
11	26	0.96

[a] Conditions were as follows: 0.1 M Hepes, 0.01 M $CaCl_2$, pH 7.5, and 2.5% Me_2SO. The concentration of trypsin was 27 μM.

Most isocoumarin inhibitors including 3-alkoxy-4-chloro-7-guanidinoisocoumarins are not very fluorescent; however, 7-amino-4-chloro-3-(3-isothiureidopropoxy)isocoumarin (**5**), 3-ethoxy-7-guanidinoisocoumarin (**8**), and 7-guanidino-3-(2-phenylethoxy)isocoumarin (**10**) do show moderate fluorescence spectra. The fluroescence excitation spectrum of compound **5** (55 μM) contains a peak at 400 nm. Excitation at 400 nm gives an emission spectrum (420–700 nm) that contains a single peak at 500 nm, whereas the hydrolysis product has negligible fluorescence. Addition of trypsin (58 μM) to a solution of **5** resulted in a decrease in fluorescence emission at 500 nm within 0.3 min (Figure 3). Excitation of compound **8** at 300 nm gave a peak in fluorescence emission spectra at ca. 450 nm. Addition of trypsin (16 μM) to a solution of **8** (18 μM) resulted in a 92% decrease in fluorescence at 450 nm. Excitation of **10** at 290 nm gave a fluorescence emission peak at ca. 450 nm, and addition of trypsin (16 μM) to a solution of **10** (18 μM) resulted in loss of 97% of the fluorescence at 450 nm within 0.3 min.

Inactivation of Other Enzymes. The cysteine proteases papain and cathepsin B and the metalloprotease thermolysin were not inhibited by 3-alkoxy-4-chloro-7-guanidinoisocoumarins **7**, **9**, and **11** at inhibitor concentrations of 0.2–0.6

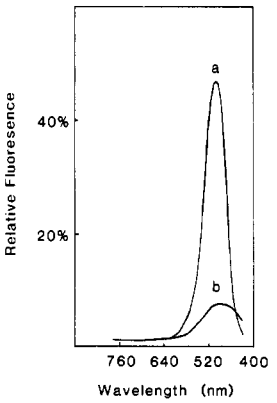


FIGURE 3: Fluorescence emission spectra for the reaction of 7-amino-4-chloro-3-(3-isothiureidopropoxy)isocoumarin with trypsin in 0.1 M Hepes and 0.01 M $CaCl_2$, pH 7.5, buffer and 2% Me_2SO. (Curve a) Inhibitor in buffer, [I] = 55 μM; (curve b) trypsin ([E] = 58 μM) was added to the inhibitor solution, and the spectrum was taken after 0.3 min. The excitation and emission wavelengths were 400 and 500 nm, respectively.

Table VI: Prothrombin Time for Human Plasma in the Presence of Substituted Isocoumarins[a]

inhibitor	[I] (μM)	prothrombin time (s) without preincubation	prothrombin time (s) with preincubation
none		11–14	11–14
3,4-dichloroisocoumarin	330	120	14
4	38	15	13
5	31	14	15
7	33	168	23
9	44	>240	80
11	38	19	18

[a] The prothrombin time (Quick et al., 1935) is the time until first appearance of a clot after calcium and tissue factor are added to plasma or plasma containing an inhibitor. Preincubation involved adding the inhibitor to human plasma 3 min prior to the addition of calcium and tissue factor.

mM after incubation for 30 min. However, another metalloprotease leucine aminopeptidase was inhibited ca. 15–30% at the same inhibitor concentration. Acetylcholinesterase was inhibited 40% by compound **7**, **9**, or **11** at the relatively high inhibitor concentrations of 0.2–0.6 mM after incubation for 5 min; however, compound **7** does not show any inhibition toward this enzyme at 0.03 mM. 3-(Aminopropoxy)-4-chloroisocoumarin (**2**) and 4-chloro-3-(isothiureidopropoxy)-isocoumarins **4** and **5** inhibit acetylcholinesterase up to 90% at concentrations of 0.3–0.5 mM. Other compounds with basic guanidino functional groups such as benzamidine and NPGB also show 40–50% inhibition of acetylcholinesterase at inhibitor concentrations of 0.4–0.5 mM.

Coagulant Assay. The prothrombin time of human plasma in the presence of several isocoumarin inhibitors are shown in Table VI. Without preincubation of the inhibitor in plasma, the prothrombin time was prolonged from 12 s to 2.8 min in the presence of 33 μM 4-chloro-7-guanidino-3-methoxyisocoumarin (**7**) and prolonged to more than 4 min in the presence of 44 μM 4-chloro-3-ethoxy-7-guanidinoisocoumarin (**9**). However, 4-chloro-7-guanidino-3-(2-phenylethoxy)isocoumarin (**11**, 38 μM), 7-amino-4-chloro-3-(3-isothiureidopropoxy)isocoumarin (**5**, 31 μM), and 4-chloro-3-(3-isothiureidopropoxy)isocoumarin (**4**, 38 μM) do not effect the prothrombin time under the same conditions. After preincubation of the inhibitor in human plasma for 3 min, the prothrombin time was prolonged to 23 and 80 s respectively, in the presence of 33 μM **7** and 44 μM **9**. The general serine protease inhibitor 3,4-dichloroisocoumarin (330 μM) prolonged the prothrombin time to 2 min but had no effect after incubation for 3 min in plasma.

Discussion

Trypsin-like serine proteases play important roles in numerous physiological processes including blood coagulation, complement activation, fibrinolysis, kinin formation, digestion, and reproduction. The blood coagulation cascade in particular requires the concerted action of a series of trypsin-like enzymes including thrombin, factor Xa, factor XIa, factor XIIa, factor IXa, factor VIIa, and plasma kallikrein. Inhibitors for these enzymes could potentially be utilized as anticoagulant drugs. Indeed, the major anticoagulant in use today, heparin, accelerates the rate of inhibition of many of these trypsin-like enzymes by the natural plasma inhibitor antithrombin III. Ideally, a therapeutically useful inhibitor would be highly reactive, would be fairly stable in plasma, would be orally active, would react irreversibly with the target enzyme or have a low dissociation rate, and would be specific for the targeted enzyme or group of enzymes. Combination of these features into a single molecule is a considerable challenge that has rarely been accomplished. Due to the large number of trypsin-like enzymes in physiological systems that cleave arginine bonds, the specificity requirement is a particularly difficult challenge.

One approach to the design of specific inhibitors for serine proteases involves the use of specific peptide sequences that resemble sequences found in the natural substrate(s) for the targeted enzyme. Incorporation of a reactive group into these structures has led to a number of useful classes of inhibitors including peptide chloromethyl ketones, peptide aldehydes, peptide boronic acids, and peptide trifluoromethyl ketones [see Powers and Harper (1986) for a review of serine protease inhibitors]. Only the first two classes have thus far been applied to coagulation serine proteases. An alternate approach involves the use of heterocyclic serine protease acylating inhibitors and mechanism-based (suicide) inhibitors. Such heterocyclic inhibitors offer a greater variety of structures and more numerous opportunities for incorporating masked reactive functional groups. However, it is more difficult to obtain specificity with such molecules.

Several classes of heterocyclic acylating inhibitors for serine proteases have been reported, including N-substituted saccharins (Zimmerman et al., 1980), *N*-arylbenzoisothiazolinones (Ashe et al., 1981), benzoxazin-4-ones (Teshima et al., 1982; Hedstrom et al., 1984), 3-alkoxy-4-chloroisocoumarins (Harper & Powers, 1985), isatoic anhydride (Moorman & Abeles, 1982), oxazine-2,6-diones (Weidman & Abeles, 1984), and benzopyran-1,4-diones (Hemmi et al., 1985). These inhibitors react with Ser-195 of serine proteases to give acyl enzymes with varying stabilities toward deacylation. A substituted isatoic anhydride, 7-(aminomethyl)-1-benzylisatoic anhydride (Gelb & Abeles, 1986), is the only heterocyclic acylating inhibitor designed for trypsin-like enzymes, although some of the other heterocycles such as benzoxazinones (Spencer et al., 1986) will react slowly with trypsin and thrombin. Heterocyclic mechanism-based inhibitors that may form additional covalent bonds with serine proteases during the inactivation process include halomethylcoumarins (Bechet et al., 1977a,b), haloenol lactones (Daniels al., 1983), ynenol lactones (Copp et al., 1987), 3,4-dichloroisocoumarin (Harper et al., 1985), and 3-alkoxy-7-amino-4-chloroisocoumarins (Harper & Powers, 1985) and β-lactams (Doherty et al., 1986). A few of these inhibitors, such as 3,4-dichlorisocoumarin and 7-amino-4-chloro-3-ethoxyisocoumarin, are poor inhibitors for various trypsin-like enzymes.

In order to improve the reactivity of heterocyclic mechanism-based inhibitors toward blood coagulation enzymes and other trypsin-like serine proteases, a series of isocoumarins containing aminoalkoxy, guanidino, and isothiureidoalkoxy basic side-chain groups have been synthesized (Figure 1). Elastases and chymotrypsin-like enzymes have previously been shown to be effectively inhibited by 3-alkoxy-4-chloroisocoumarins and 3-alkoxy-7-amino-4-chloroisocoumarins (Harper & Powers, 1985). In contrast to elastase and chymotrypsin, the primary specificity pocket (S_1)[2] of trypsin and related enzymes contains an aspartic acid residue (Asp-189) that interacts with the charged amino and guanidino side-chain groups of lysine and arginine residues in natural substrates. Two different ways of introducing basic groups into the isocoumarin structure were utilized. In the first (compounds **1–5**), the charged group was incorporated into the structure of the 3-alkoxy substituent, and in the second (compounds **6–11**), a guanidino group was attached to the 7-position of the guanidino group. In several cases (Figure 1), the spacing between the reactive isocoumarin ring carbonyl group and the positively charged side-chain group is similar to that found in either lysine or arginine. The isothiureidoalkoxy group was utilized since it is a more easily synthesized analogue of a guanidino group.

Reactivity. The introduction of basic groups into the isocoumarin structure increases the reactivity of these inhibitors toward trypsin-like enzyme by several orders of magnitude. Trypsin and a number of coagulation serine proteases are inactivated slowly by 4-chloro-3-ethoxyisocoumarin and 7-amino-4-chloro-3-ethoxyisocoumarin with $k_{obsd}/[I]$ values of 0.6–370 M^{-1} s^{-1}. Changing the 3-ethoxy group to a 3-aminopropoxy group [3-(aminopropoxy)-4-chloroisocoumarin, **2**] resulted in a 54-fold increase in reactivity with trypsin and introduction of thrombin inhibitory activity (the 3-ethoxy compound does not inhibit thrombin). As expected, isothiureidoalkoxyisocoumarins were even better inhibitors and inhibited trypsin-like serine proteases 2–200-fold faster than 3-(aminopropoxy)-4-chloroisocoumarin. This was expected since the isothiureidoalkoxy group can make additional hydrogen bonds with Asp-189 in the S_1 pocket and has a greater resemblance to arginine. All blood coagulation enzymes cleave arginyl bonds in their natural substrates and have a definite preference for arginine residues over lysine in synthetic substrates.

The 3-alkoxy-4-chloro-7-guanidinoisocoumarins are the most powerful group of isocoumarin inactivators of the blood coagulation and other trypsin-like enzymes that have been studied to date. The 3-alkoxy-4-chloro-7-guanidinoisocoumarins inhibit bovine thrombin, bovine factor Xa, human factor Xa, human plasma kallikrein, porcine pancreatic kallikrein, human factor XIa, human factor XIIa, and trypsin with $k_{obsd}/[I]$ values of 10^4–10^5 M^{-1} s^{-1}. These inhibitors are 2–5 orders of magnitude more reactive than 7-amino-4-chloro-3-ethoxyisocoumarin, which does not contain a basic substituent.

Compared with other heterocyclic inhibitors, 3-alkoxy-4-chloro-7-guanidinoisocoumarins are more reactive than the substituted benzoxazinones, which inhibit thrombin with k_{on} values of 30–1000 M^{-1} s^{-1} (Spencer et al., 1986). 7-(Aminomethyl)-1-benzylisatoic anhydride inhibits trypsin and thrombin with $k_{obsd}/[I]$ values of 50 and 460 M^{-1} s^{-1}, respectively (Gelb & Abeles, 1986), which is 3–4 orders of magnitude less reactive than 3-alkoxy-4-chloro-7-guanidinoisocoumarins.

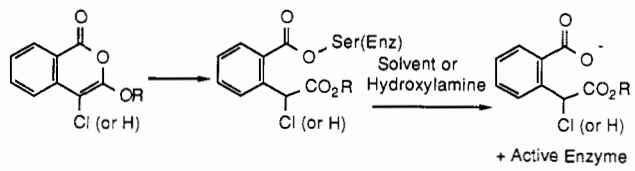


FIGURE 4: Proposed mechanism of inactivation of serine proteases by 3-(aminoalkoxy)isocoumarins **1** and **2**, 4-chloro-3-(isothiureidoalkoxy)isocoumarins **3** and **4**, and 3-alkoxy-7-guanidinoisocoumarins **6**, **8**, and **10**.

The most reactive and most specific irreversible inhibitors for many trypsin-like enzyme are peptide chloromethyl ketones. For example, thrombin, human plasma kallikrein, and factor Xa are inhibited, respectively, by D-Phe-Pro-ArgCH_2Cl, D-Phe-Phe-ArgCH_2Cl, and Dns-Glu-Gly-ArgCH_2Cl with $k_{obsd}/[I]$ values of 1.2×10^7, 3.8×10^5, and 3.7×10^5 M^{-1} s^{-1} (Kettner & Shaw, 1981). Thus, the isocoumarins are equal or slightly more reactive with human plasma kallikrein and less reactive with thrombin and factor Xa. In contrast, the isocoumarins appear to be significantly more reactive with porcine pancreatic kallikrein, human factor XIa, and human factor XIIa. The best chloromethyl ketone inhibitors of porcine pancreatic kallikrein and human factor XIIa are Gly-Val-ArgCH_2Cl ($k_{obsd}/[I]$ = 21 M^{-1} s^{-1}; Fiedler et al., 1977) and Pro-Phe-ArgCH_2Cl (2100 M^{-1} s^{-1}; Silverberg & Kaplan, 1982), and no chloromethyl ketone inhibitor appears to have yet been tested with factor XIa.

Mechanism. The proposed first step in the inhibition mechanism for trypsin-like enzymes by 3-alkoxy-4-chloroisocoumarins is acylation of the active-site serine (Ser-195) with the formation of an acyl enzyme (Figure 4). Addition of trypsin to a solution of the 3-(3-isothioureidopropoxy)isocoumarins **4** and **5** and to the 7-guanidinoisocoumarins **7–9** and **11** resulted in loss of the isocoumarin absorbance, which is consistent with the ring-opening reaction that takes place upon acylation of Ser-195 in the proposed mechanism. In each case, inactivation of trypsin occurred concurrently with the absorbance change. The loss of the UV chromophore also indicates that the inhibition reaction does not involve the displacement of chloride or alkoxide from the isocoumarin by the active-site serine as has been observed with the reaction of chymotrypsin with 5-benzyl-6-chloro-2-pyrone (Westkaemer & Abeles, 1983). Further evidence for the loss of the isocoumarin ring system was obtained by measuring the fluorescence spectra of 7-amino-4-chloro-3-(3-isothioureidopropoxy)isocoumarin (**5**) and the 7-guanidinoisocoumarins **8** and **10** after the addition of trypsin. In each case, the fluorescence of the inhibitor completely disappeared.

The presence of a 4-chloro substituent significantly increases the inhibitory potency of the isocoumarins. For example, 3-(3-aminopropoxy)-4-chloroisocoumarin is 60–170-fold more reactive than 3-(3-aminopropoxy)isocoumarin, and 3-alkoxy-4-chloro-7-guanidinoisocoumarins are 2–100-fold more potent inhibitors than 3-alkoxy-7-guanidinoisocoumarins toward the various enzymes. This increased reactivity is attributed to the chlorine substitution that results in a more electronegative isocoumarin ring system and thus increases the reactivity of the lactone carbonyl group toward nucleophilic attack.

The acyl enzymes derived from all the isocoumarins, with the exception of **5**, **7**, **9**, and **11**, regained complete enzymatic activity upon standing. The deacylation rates varied, with the acyl enzymes derived from 3-alkoxy-7-guanidinoisocoumarins and 3-(3-aminopropoxy)isocoumarin being less stable ($t_{1/2}$ = 22–100 min) than those derived from 3-(isothiureidoalk-

[2] The nomenclature for the individual amino acid residues (P_1, P_2, P_3) of a substrate and for the subsites (S_1, S_2, S_3) of the enzyme is that of Schechter and Berger (1967).

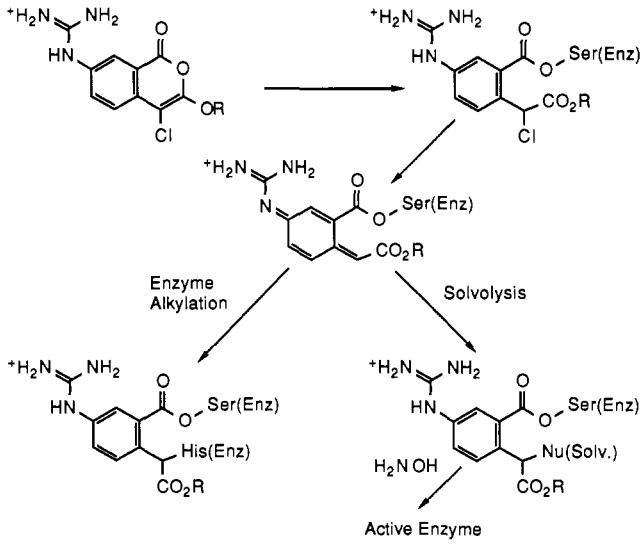


FIGURE 5: Proposed mechanism of inactivation of serine proteases by 3-alkoxy-4-chloro-7-guanidinoisocoumarins **7**, **9**, and **11**.

oxy)isocoumarins ($t_{1/2}$ = 200–300 min). Full enzymatic activity was also recovered more quickly ($t_{1/2}$ < 11 min) after the addition of buffered hydroxylamine. These results are again consistent with the formation of an acyl enzyme (Figure 4) that can be deacylated either slowly in water or more rapidly with an external nucleophilic such a hydroxylamine.

Four isocoumarins, 7-amino-4-chloro-3-(3-isothiureidopropoxy)isocoumarin (**5**) and the 3-alkoxy-4-chloro-7-guanidinoisocoumarins **7**, **9**, and **11**, formed acyl enzymes with trypsin with insignificant deacylation rates. The inactivated enzymes were very stable and regained only 1–4% activity after removal of excess inhibitor and standing for 1 day. Even incubation of the inactivated enzyme derivatives with hydroxylamine for 1 day did not result in recovery of full enzyme activity. For example, trypsin inactivated by 3-alkoxy-4-chloro-7-guanidinoisocoumarins only regained 8–16% activity upon addition of hydroxylamine (0.29 M), while trypsin inactivated by 3-alkoxy-7-guanidinoisocoumarins regained full activity under the same conditions. Treatment of trypsin inhibited by 7-amino-4-chloro-3-(3-isothiureidopropoxy)isocoumarin (**5**) with hydroxylamine resulted in recovery of 45% activity. Similarly, bovine thrombin, inhibited by the 3-alkoxy-4-chloro-7-guanidinoisocoumarins **7**, **9**, and **11**, regained less than 9% activity upon standing for 18 h and only 15% activity upon addition of buffered hydroxylamine.

All the results obtained with 7-amino-4-chloro-3-(3-isothiureidopropoxy)isocoumarin (**5**) and the 3-alkoxy-4-chloro-7-guanidinoisocoumarins **7**, **9**, and **11** are consistent with the mechanism shown in Figure 5. The proposed mechanism initially involves the formation of an acyl enzyme with the unmasking of a 4-aminobenzyl chloride functional group (not shown) in the case of 7-amino-4-chloro-3-(3-isothiureidopropoxy)isocoumarin (**5**) or a 4-guanidinobenzyl chloride functional group (shown in Figure 5) in the case of the 3-alkoxy-4-chloro-7-guanidinoisocoumarins **7**, **9**, and **11**. These intermediates can directly alkylate a nearby enzyme nucleophile or can eliminate chloride to give a quinone imine methide structure. The quinone imine methide intermediate can further react either with an enzyme nucleophile such as His-57 to give an alkylated enzyme or with solvent water to give a new acyl enzyme structure that can then deacylate to regenerate active enzyme. Although there is no evidence for the identity of the enzyme nucleophile, the imidazole ring of His-57 is the only reasonable nucleophile in the vicinity of Ser-195 in the three-dimensional structures of bovine trypsin and porcine pancreatic kallikrein.

The proposed mechanism of inactivation of trypsin-like serine proteases is similar to the mechanism proposed to account for the stability of the derivatives formed upon inhibition of elastases and chymotrypsin-like enzymes by 3-alkoxy-7-amino-4-chloroisocoumarins (Harper & Powers, 1985). Indeed, the inhibition mechanism for 7-amino-4-chloro-3-(3-isothiureidopropoxy)isocoumarin (**5**) is identical with that previously proposed for 3-alkoxy-7-amino-4-chloroisocoumarins since it involves unmasking of an aminobenzyl chloride functional groups. In the case of the 3-alkoxy-4-chloro-7-guanidinoisocoumarins **7**, **9** and **11**, the mechanism involves generation of a 4-guanidinobenzyl chloride function group that then decomposes to an amidino-substituted quinone imine methide. This is the first demonstration that such a functional group generated from a 4-guanidinobenzyl chloride structure can be a reactive moiety. It is not immediately obvious that this should be the case since a 7-guanidino group is much more electronegative than an 7-amino group.

At first, it does not seem possible to distinguish direct displacement of chloride from the acyl enzyme by an enzyme nucleophile or formation of a quinone imine methide intermediate followed by alkylation. However, simple aminobenzyl halides are unstable and decompose rapidly to 4-quinone imine methides, which are potent alkylation agents of nucleophiles such as imidazole (Wakselman & Dome, 1975; Dome & Wakselman, 1975). It has previously been shown that 3-alkoxy-4-chloroisocoumarins and 3-alkoxy-4-chloro-7-nitroisocoumarins acylate serine proteases but deacylate rapidly (Harper & Powers, 1985). Since the unmasked benzyl halide and 4-nitrobenzyl halide functional groups in these isocoumarins are potential alkylating agents, the lack of irreversible inhibition is strong evidence for the formation of a quinone imine methide structure in the case of isocoumarins with a 4-chloro and a 7-amino or 7-guanidino functional group.

The partitioning between enzyme alkylation to form a stable derivative and solvolysis of the quinone imine methide (or benzyl halide) to form a reactivatable acyl enzyme is dependent on the structure of the isocoumarin inhibitor. Trypsin and thrombin inhibited by the 3-alkoxy-4-chloro-7-guanidinoisocoumarins **7**, **9**, and **11** regained very little activity upon treatment with hydroxylamine, indicating that enzyme alkylation was the major pathway. However, trypsin inhibited by 7-amino-4-chloro-3-(3-isothiureidopropoxy)isocoumarin (**5**) regained 45% activity upon treatment with hydroxylamine, indicating that solvolysis was almost as important as enzyme alkylation with this inhibitor. In the case of the inhibition of porcine pancreatic elastase by 7-amino-4-chloro-3-methoxyisocoumarin, the ratio of solvolysis to alkylation was dependent on both pH and buffer (Harper & Powers, 1985). At low pH in an acetate buffer, solvolysis predominated, and a crystal structure of this product has been reported (Meyer et al., 1985). Under these conditions, the quinone imine methide has reacted with acetate to give an acyl enzyme in which the acetate moiety sits in the S_1 pocket and the newly formed carbomethoxy group is hydrogen bonded to His-57. Moreover, the hydroxylamine reactivation reaction may not be an absolute measure of the solvolysis reaction since it is possible that hydroxylamine is cleaving the histidine from the inhibitor in the alkylated enzyme structure. Indeed, the hydroxylamine regeneration of activity from trypsin inactivated by **5**, **7**, **9**, or **11** takes place with a much longer half-life compared to the other reactivation reactions.

Specificity and Binding Mode. It is clear that none of the isocoumarins substituted with basic functional groups are

specific for any of the enzymes tested and are best considered to be general inhibitors for trypsin-like enzymes. However, considerable rate differences between individual enzymes were often observed. The 3-(isothiureidoalkoxy)isocoumarins **3** and **4** were, respectively, 6.3- and 2.3-fold more reactive with human plasma kallikrein than with any of the other enzymes. In contrast, 7-amino-4-chloro-3-(3-isothiureidopropoxy)isocoumarin (**5**) was 5-fold more reactive with porcine pancreatic kallikrein than any other enzyme except trypsin and discriminated between porcine pancreatic kallikrein and human plasma kallikrein by a factor of 100. In addition to its effect on the irreversibility of the inhibition, the 7-amino group of 7-amino-4-chloro-3-(3-isothiureidopropoxy)isocoumarin (**5**) exerted a considerable effect on specificity. The corresponding isocoumarin (**4**) lacking the 7-amino group was, respectively, a 100-, 4.4-, 2.1-, and 2.2-fold more potent inhibitor of human plasma kallikrein, human factor XIIa, human factor XIa, and bovine thrombin than the 7-amino compound. In contrast, the 7-amino compound **5** was, respectively, a 7.3-, 5.8-, and 8.9-fold better inhibitor of bovine factor Xa, porcine pancreatic kallikrein, and bovine trypsin.

The 3-alkoxy-4-chloro-7-guanidinoisocoumarins **7**, **9**, and **11** are the most powerful group of inactivators for the blood coagulation enzymes, and the various 3-alkoxy groups (methoxy, ethoxy, or 2-phenylethoxy) often had a significant effect on reactivity. The $k_{obsd}/[I]$ values for inhibition of bovine factor Xa increased 31-fold upon going from methoxy to ethoxy to 2-phenylethoxy, while with bovine thrombin the $k_{obsd}/[I]$ values increased 9.7-fold in going in the opposite direction. Thus, 4-chloro-7-guanidino-3-methoxyisocoumarin (**7**) discriminates between bovine thrombin and bovine factor Xa by a factor of 94, while 4-chloro-7-guanidino-3-(2-phenylethoxy)isocoumarin (**11**) discriminates in favor of bovine factor Xa by a factor of 3.2. In contrast, the nature of the 3-alkoxy group had little effect with human factor Xa, human plasma kallikrein, human factor XIIa, human factor XIa, and bovine trypsin. In the case of porcine pancreatic kallikrein the longer alkoxy groups were 4.4-fold more potent inhibitors.

It is interesting to note that isocoumarins with either a guanidino group or a guanidine-like group (isothiureidoalkoxy group) on opposite sides of the heterocyclic ring are effective inhibitors. It appears likely that both groups of compounds bind with the basic group interacting with Asp-189 in the S_1 pocket of trypsin and related enzymes. This indicates that the isocoumarin carbonyl group has quite different orientations with respect to the active-site serine and histidine in the transition states leading to inhibition with the two groups of compounds. Thus, heterocyclic compounds such as isocoumarins may have more than one productive mode of interacting with the catalytic triad of serine proteases during inhibition reactions. Bovine thrombin is an exception and reacted well only with the 7-guanidino inhibitors. It is hoped that future crystallographic and molecule modeling studies with these inhibitors will lead to an improved understanding of their binding mode.

Anticoagulant Activity. A number of isocoumarins were tested as anticoagulants in human plasma by using the prothrombin time as a measure of effectiveness. Two inhibitors, 4-chloro-7-guanidino-3-methoxyisocoumarin (**7**) and the corresponding ethoxy compound (**9**), significantly prolonged the prothrombin time and were effective anticoagulants. It is interesting that the corresponding 4-chloro-7-guanidino-3-(2-phenylethoxy)isocoumarin (**11**) had no effect in human plasma, even though it was equally stable toward hydrolysis. The different behavior of the two isocoumarin inhibitors in the coagulant assay may be an indication that the two compounds have different reactivities toward some other component of the coagulation system such as factor VIIa, which has not yet been studied. Alternately, the isocoumarins may bind differently or react different with some other component of human plasma. Radiolabeled inhibitor will be required to distinguish these possibilities. The most stable inhibitor in plasma was 7-amino-4-chloro-3-(3-isothiureidopropoxy)isocoumarin (**5**), but this inactivator had no affect on the prothrombin time, probably because it is a relatively poor inhibitor of thrombin and factor Xa. The general serine protease inhibitor 3,4-dichloroisocoumarin (Harper et al., 1985) was also a good anticoagulant, although at higher concentration. The 7-guanidino compounds are not very stable in human plasma and have half-lives of 4–8 min. Although the two 7-guanidinoisocoumarins **7** and **9** had significant anticoagulant activity after preincubation in plasma for 3 min, their plasma instability may limit their usefulness as in vivo anticoagulants.

Summary. Isocoumarins substituted with 7-guanidino and 3-(isothiureidoalkoxy) groups are potent inhibitors for blood coagulation serine proteases and other trypsin-like enzymes. They are the first mechanism-based inhibitors reported for most of the enzymes studied, often form very stable enzyme–inhibitor derivatives, and are the most potent inhibitors for several of the enzymes examined. The two 7-guanidinoisocoumarins, 4-chloro-7-guanidino-3-methoxyisocoumarin (**7**) and 4-chloro-3-ethoxy-7-guanidinoisocoumarin (**9**), are effective anticoagulants in human plasma and can prolong the prothrombin time. Future work will be directed at improving the specificity of isocoumarin inhibitors, understanding their mode of binding to serine proteases, and improving their stability and reactivity in human plasma as anticoagulants.

Supplementary Material Available

Syntheses of compounds **1**, **2**, **4**, **5**, and **8**–**11** (3 pages). Ordering information is given on any current masthead page.

Registry No. **1**, 113251-04-0; **1** (free base), 113251-23-3; **2**, 113251-05-1; **2** (free base), 113251-24-4; **3**, 113251-06-2; **4**, 113273-56-6; **5**, 113251-07-3; **6**, 113301-72-7; **7**, 113251-08-4; **8**, 113251-09-5; **9**, 113251-10-8; **10**, 113251-11-9; **11**, 112901-86-7; ADMP, 38184-47-3; 2-bromoethyl 2-carboxyyphenylacetate, 109532-12-9; homophthalic acid, 89-51-0; 2-bromoethanol, 540-51-2; 3-(bromoethyl)-4-chloroisocoumarin, 113251-12-0; methyl 2-carboxy-4-nitrophenylacetate, 62252-24-8; 2-carboxy-4-nitrophenylacetic acid, 3898-66-6; methanol, 67-56-1; methyl 4-amino-2-carboxyphenylacetate, 109532-14-1; methyl 2-carboxy-4-guanidinophenylacetate, 109532-15-2; 3-(benzyloxycarbonylamino)-1-propanol, 34637-22-4; 3-(benzyloxycarbonylamino)propyl 2-carboxyphenylacetate, 113251-13-1; 3-aminopropyl 2-carboxyphenylacetate·HAc, 113251-15-3; 3-bromopropyl 2-carboxyphenylacetate, 113251-16-4; 3-bromopropanol, 627-18-9; 3-bromopropoxy-4-chloroisocoumarin, 113251-17-5; thiourea, 62-56-6; 3-bromopropyl 2-carboxy-4-nitrophenylacetate, 113251-18-6; 3-bromopropoxy-4-chloro-7-nitroisocoumarin, 113251-19-7; 7-amino-3-bromopropoxy-4-chloroisocoumarin, 113251-20-0; ethyl 2-carboxy-4-nitrophenylacetate, 62252-28-2; ethanol, 64-17-5; ethyl 4-amino-2-carboxyphenylacetate, 113251-21-1; ethyl 2-carboxy-4-guanidinophenylacetate, 113251-22-2; phenylethyl alcohol, 60-12-8; trypsin, 9002-07-7; kallikrein, 9001-01-8; thrombin, 9002-04-4; factor Xa, 9002-05-5; factor XIa, 37203-61-5; factor XIIa, 37203-62-6; serine protease, 37259-58-8; hydroxylamine, 7803-49-8.

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