Reaction of Serine Proteases with Substituted 3-Alkoxy-4-chloroisocoumarins and 3-Alkoxy-7-amino-4-chloroisocoumarins: New Reactive Mechanism-Based Inhibitors†

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ABSTRACT: The time-dependent inactivation of several serine proteases including human leukocyte elastase, cathepsin G, rat mast cell proteases I and II, and human skin chymase by a number of 3-alkoxy-4-chloroisocoumarins, 3-alkoxy-4-chloro-7-nitroisocoumarins, and 3-alkoxy-7-amino-4-chloroisocoumarins at pH 7.5 and the inactivation of several trypsin-like enzymes including human thrombin and factor XIIa by 7-amino-4-chloro-3-ethoxyisocoumarin and 4-chloro-3-ethoxyisocoumarin are reported. The 3-alkoxy substituent of the isocoumarin is likely interacting with the S_1 subsite of the enzyme since the most reactive inhibitor for a particular enzyme had a 3-substituent complementary to the enzyme's primary substrate specificity site (S_1). Inactivation of several enzymes including human leukocyte elastase by the 3-alkoxy-7-amino-4-chloroisocoumarins is irreversible, and less than 3% activity is regained upon extensive dialysis of the inactivated enzyme. Addition of hydroxylamine to enzymes inactivated by the 3-alkoxy-7-amino-4-chloroisocoumarins results in a slow ($t_{1/2} > 6.7$ h) and incomplete (32–57%) regain in enzymatic activity at pH 7.5. Inactivation by the 3-alkoxy-4-chloroisocoumarins and 3-alkoxy-4-chloro-7-nitroisocoumarins on the other hand is transient, and full enzyme activity is regained rapidly either upon standing, after dialysis, or upon the addition of buffered hydroxylamine. The rate of inactivation by the substituted isocoumarins is decreased when substrates or reversible inhibitors are present in the incubation mixture, which indicates active site involvement. The inactivation rates are dependent upon the pH of the reaction mixture, the isocoumarin ring system is opened concurrently with inactivation, and the reaction of 3-alkoxy-7-amino-4-chloroisocoumarins with porcine pancreatic elastase is shown to be stoichiometric. The results are consistent with a scheme where 3-alkoxy-7-amino-4-chloroisocoumarins react with the active site serine of a serine protease to give an acyl enzyme in which a reactive quinone imine methide can be released. Irreversible inactivation could then occur upon alkylation of an active site nucleophile (probably histidine-57) by the acyl quinone imine methide. The finding that hydroxylamine slowly catalyzes partial reactivation indicates that several inactivated enzyme species may exist. The 3-alkoxy-substituted 4-chloroisocoumarins and 4-chloro-7-nitroisocoumarins are simple acylating agents and do not give stable inactivated enzyme structures. Substituted isocoumarins are some of the most potent inactivators reported for many of the enzymes tested and may be quite useful as inhibitors of proteolysis both in vivo and in vitro.

The design of serine protease inhibitors in general and mechanism-based inhibitors in particular has received considerable attention recently. This interest stems from the fact that serine proteases play critical roles in the development of numerous disease states including emphysema, adult respiratory distress syndrome, arthritis, and certain degenerative skin disorders. For example, human leukocyte (HL)[1] elastase, and to some extent cathepsin G, is thought to be responsible for the degradation of lung elastin that occurs during chronic pulmonary emphysema.

A variety of heterocyclic structures including ynenol lactones (Tam et al., 1984), haloenol lactones (Daniels et al., 1983), 6-chloropyrones (Westkaemper & Abeles, 1983; Gelb & Abeles, 1984), isatoic anhydride and oxazine-2,6-diones (Moorman & Abeles, 1982; Weidmann & Abeles, 1984), benzopyran-1,4-diones (Hemmi et al., 1985), and benzoxazin-4-ones (Hedstrom et al., 1984; Teshima et al., 1982) have recently been reported as inactivators of serine proteases. While the ynenol and haloenol lactones contain masked functional groups capable of alkylating an active site nucleophile upon enzyme acylation, the chloropyrones contain a masked acid chloride. The oxazine-2,6-diones and benzoxazin-4-ones do not contain masked reactive functionalities but react with some serine proteases to give stable acyl enzymes.

Recently, we reported the discovery of 3,4-dichloroisocoumarin, a general mechanism-based inhibitor for serine proteases (Harper et al., 1985; Harper et al., 1983). This structure is related to the 6-chloropyrones in that it contains a masked acid chloride capable of acylating an active site nucleophile to give an inactivated enzyme. While this structure inactivates a wide variety of serine proteases, it reacts most rapidly with HL elastase. Further consideration of the iso-

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[1] Abbreviations: Aala, $-NHN(CH_3)CO-$; Boc, *tert*-butyloxycarbonyl; Cat G, cathepsin G; ChyT, chymotrypsin A_α; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HL, human leukocyte; HLE, human leukocyte elastase; HSC, human skin chymase; MeO-Suc, methoxysuccinyl; Mes, 2-(*N*-morpholino)ethanesulfonic acid; NA, 4-nitroanilide; NAD^+, oxidized nicotinamide adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide; Np, 4-nitrophenyl; PP, porcine pancreatic; PPE, porcine pancreatic elastase; RMCP I, rat mast cell protease I; RMCP II, rat mast cell protease II; SBzl, $-SCH_2C_6H_5$; SGPA, *Streptomyces griseus* protease A; Suc, succinyl; Tris, tris(hydroxymethyl)aminomethane; Tris·HCl, tris(hydroxymethyl)aminomethane hydrochloride; Z, benzyloxycarbonyl.

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coumarin ring structure indicated that other 3-substituted 4-chloroisocoumarins containing substituents complementary to the active site of a particular enzyme could act as selective acylating agents for serine proteases. Such structures are related to the 2-substituted 4*H*-3,1-benzoxazin-4-ones described previously (Teshima et al., 1982). During the course of this investigation, we noted that the 7-amino-4-chloroisocoumarin ring system might represent a new class of mechanism-based irreversible inhibitors for serine proteases. It was envisioned that acylation of the active site serine could result in the release of a reactive 4-aminobenzyl chloride or the corresponding quinone imine methide at the active site, which could then alkylate another active site nucleophile to give an irreversibly inactivated enzyme.

Here, we report the inactivation of a variety of serine proteases including HL elastase, mammalian chymotrypsin-like enzymes (chymases), and trypsin-like enzymes by a number of 3-alkoxy-substituted 4-chloroisocoumarins, 4-chloro-7-nitroisocoumarins, and 7-amino-4-chloroisocoumarins. The results indicate that 3-alkoxy-7-amino-4-chloroisocoumarins are potent mechanism-based inhibitors for serine proteases and give extremely stable inactivated enzyme structures while the 3-alkoxy-4-chloroisocoumarins and 3-alkoxy-4-chloro-7-nitroisocoumarins are simple acylating agents and give acyl enzymes of varying stability. The 3-alkoxy-7-amino-4-chloroisocoumarins should be useful in the inhibition of proteolysis due to serine proteases both in vivo and in vitro. A portion of this work has appeared in an earlier communication (Harper & Powers, 1984).

Materials and Methods

HL elastase and cathepsin G were generous gifts from Dr. James Travis and his research group at the University of Georgia. Rat mast cell proteases I and II were kindly provided by Dr. Richard Woodbury and Dr. Hans Neurath of the University of Washington. Bovine factor Xa and human β-factor XIIa were gifts from Dr. Kotoku Kurachi, Dr. Kazuo Fujikawa, and Dr. Earl Davie of the University of Washington. *Streptomyces griseus* protease A was provided by Dr. Michael James of the University of Alberta. Human skin chymase was a gift from Dr. Norman M. Schectner and Dr. Gerald S. Lazarus of the University of Pennsylvania. Bovine chymotrypsin A_α, human thrombin, human plasmin, porcine pancreatic elastase, bovine thrombin, porcine pancreatic kallikrein, bovine trypsin, leucine aminopeptidase, Tris·HCl, glutathione, and leucine-4-nitroanilide hydrochloride (leucine-NA·HCl) were obtained from Sigma Chemical Co., St. Louis, MO, and were of the highest purity available. Hepes was purchased from Aldrich Chemical Co., Milwaukee, WI. Thermolysin was a product of Bachem Inc., Torrence, CA. Furanacryloyl-Gly-Leu-NH_2 was purchased from Chemical Dynamics Corp., South Plainfield, NJ. MeO-Suc-Ala-Ala-Pro-Val-NA (Nakajima et al., 1979), Suc-Val-Pro-Phe-NA (Tanaka et al., 1985), Suc-Ala-Ala-Ala-NA (Bieth et al., 1974), MeO-Suc-Ala-Ala-Pro-Val-SBzl (Castillo et al., 1979), Z-Phe-Gly-Arg-NA·HCl (Cho et al., 1984), Z-Arg-SBzl·HCl (McRae et al., 1981), CF_3CO-Lys-Ala-4-methylanilide (Renaud et al., 1983), 3-(heptafluoropropyl)-4*H*-3,1-benzoxazin-4-one (**7g**), and 3-benzyl-4*H*-3,1-benzoxazin-4-one (**7d**) (Teshima et al., 1982) were prepared as previously described.

Enzyme Inactivation: Incubation Method. Inactivation was initiated by adding a 5–50-μL aliquot of inhibitor in Me_2SO to 0.3–0.5 mL of a buffered enzyme solution (0.1–2.0 μM) such that the final Me_2SO concentration was 8–12% v/v at 25 °C. Aliquots were removed with time and diluted into substrate solution (40–200-fold dilution), and the residual activity was measured spectrophotometrically as described below. Unless otherwise noted, 0.1 M Hepes and 0.5 M NaCl, pH 7.5, buffer was utilized throughout, and inhibitor concentrations are shown in the appropriate table. All spectrophotometric measurements were carried out on either a Beckman 25, Beckman 35, or Varian DMS-90 spectrophotometer.

Chymotrypsin A_α, cathepsin G, RMCP I, RMCP II, and *S. griseus* protease A were assayed with Suc-Val-Pro-Phe-NA (0.075–0.125 mM; Tanaka et al., 1985). HL elastase was assayed with MeO-Suc-Ala-Ala-Pro-Val-NA (0.1–0.125 mM; Nakajima et al., 1979), and PP elastase was assayed with Suc-Ala-Ala-Ala-NA (0.6–1.2 mM; Bieth et al., 1974). Trypsin was assayed with Z-Phe-Gly-Arg-NA·HCl (0.030 mM; Cho et al., 1984). Bovine thrombin, human thrombin, bovine factor Xa, human β-factor XIIa, human plasmin, and porcine pancreatic kallikrein were assayed with Z-Arg-SBzl·HCl (0.05–0.2 mM; McRae et al., 1981). Leucine aminopeptidase was assayed with Leu-NA·HCl (0.18 mM). Thermolysin was assayed with furanacryloyl-Gly-Leu-NH_2 (1.6 mM; Nishino & Powers, 1978). All peptide thio ester hydrolysis rates were measured with assay mixtures containing either 4,4′-dithiodipyridine (ϵ_{324} = 19 800 M^{-1} cm^{-1}; Grassetti & Murray, 1967) or 5,5′-dithiobis(2-nitrobenzoic acid) (ϵ_{410} = 13 600 M^{-1} cm^{-1}; Ellman, 1959). Peptide 4-nitroanilide hydrolysis was measured at 410 nm (ϵ_{410} = 8800 M^{-1} cm^{-1}) (Erlanger et al., 1961).

First-order inactivation rate constants (k_{obsd}) were obtained from plots of ln v_t/v_0 vs. time; the correlation coefficients were greater than 0.98, and most were greater than 0.99. Inactivation rate constants shown in Table I are typically the average of duplicate or triplicate experiments while those in Table II are based on single determinations.

Determination of Inactivation Rates in the Presence of Substrate: Progress Curve Method. In some cases (see Table I), $k_{obsd}/[I]$ values were determined in the presence of substrate as described by Tian & Tsou (1982). For example, the reaction of HL elastase (8 nM final concentration) with 4-chloro-3-ethoxyisocoumarin (**1b**) was studied by adding a 0.025-mL aliquot of enzyme to a buffered solution of MeO-Suc-Ala-Ala-Pro-Val-NA (0.171 mM) containing between 0.2 and 0.6 μM inhibitor and 10% Me_2SO. The increase in absorbance was monitored (410 nm) with time until no further release of 4-nitroaniline was observed. The $k_{obsd}/[I]$ values were calculated from plots of log ($[P]_\infty - [P]_t$) vs. time, where $[P]_\infty$ and $[P]_t$ are the concentrations of 4-nitroaniline at total inactivation and at time t, respectively, as previously described (Tian & Tsou, 1982). Correlation coefficients were greater than 0.99. Inactivation of HL elastase (8 nM) by 7-[(*N*-tosylphenylalanyl)amino]-4-chloro-3-methoxyisocoumarin (55 nM) was carried out similarly. The reaction of 3-(benzyloxy)-4-chloroisocoumarin (0.3–1.7 μM) and 4-chloro-3-[(4-fluorobenzyl)oxy]isocoumarin (1.2 μM) with chymotrypsin A_α (22 nM) was measured in the presence of Suc-Val-Pro-Phe-NA (0.25 mM). Inactivation of RMCP I (5 nM) by 4-chloro-3-ethoxyisocoumarin (0.147 mM), 3-(benzyloxy)-4-chloroisocoumarin (1.6 μM), 4-chloro-3-[(4-fluorobenzyl)-oxy]isocoumarin (0.7 μM), and 7-amino-4-chloro-3-(2-phenethoxy)isocoumarin (0.022 mM) was carried out in the presence of Suc-Val-Pro-Phe-NA (0.1 mM). Inactivation of RMCP II (0.19 μM) by 4-chloro-3-ethoxyisocoumarin (0.012 mM) was measured in the presence of Suc-Val-Pro-Phe-NA (0.13 mM). Inactivation of human skin chymase (1 nM) was measured by using Suc-Val-Pro-Phe-NA (0.1 mM) with the inhibitor concentrations shown in Table I.

Reactivation Kinetics. The reactivation of enzymes inactivated by 3-ethoxy-4-chloroisocoumarin and the 3-alkoxy-4-chloro-7-nitroisocoumarins was studied by monitoring enzymatic activity (as above) upon standing of the incubation solution at 25 °C without removal of excess inhibitor. Reactivation half-lives were determined from plots of % activity vs. time. Deacylation rates of slowly reactivated enzymes were determined after dialysis of the inactivated enzymes against 50 mM potassium phosphate buffer, pH 7.5, at 5 °C for 3 h or against 0.1 M phosphate buffer, pH 6.8, for 24–48 h at 5 °C. Enzymatic activity upon further standing at 25 °C of the dialyzed enzyme was assayed as described above. Hydroxylamine-catalyzed reactivation was measured likewise upon addition of buffered hydroxylamine (0.47–0.047 M final concentration) to inactivated enzyme (<5% activity) at 25 °C. Enzyme controls lost less than 10% activity after standing in the presence of hydroxylamine for 24 h.

Determination of Spontaneous Hydrolysis Rates of Inhibitors in Buffer and in the Presence of Glutathione. An aliquot of isocoumarin derivative (0.015–0.10 mM final concentration) in Me_2SO was added to the appropriate buffer solution such that the final Me_2SO concentration was 10% v/v, and the spontaneous hydrolysis was monitored by following the decrease in absorbance at 350 (3-alkoxy-4-chloroisocoumarin derivative) or 385 nm (7-amino- and 7-nitroisocoumarin derivatives). The hydrolysis products [except for 3-(benzyloxy)-4-chloro-7-nitroisocoumarin] had negligible absorbance at the wavelength utilized. First-order hydrolysis rate constants were obtained from plots of ln $[(A_t - A_\infty)/(A_0 - A_\infty)]$ vs. time and these constants converted to half-lives. All plots gave correlation coefficients of 0.99 or greater.

Determination of Enzymatic Ring Opening Rate Constants: Spectrophotometric Method. The enzymatic ring opening rates of the 3-alkoxy-7-amino-4-chloroisocoumarins were measured spectrophotometrically by monitoring the absorbance decrease at 385 nm. Typically, an aliquot (0.025–0.1 mL) of inhibitor in Me_2SO (0.018–0.051 mM final concentration) was added to 1.9–1.95 mL of a buffered enzyme solution (6.4–12.6 μM enzyme, 0.1 M Hepes, 0.5 M NaCl, pH 7.5, 10% Me_2SO) and the decrease in absorbance at 385 nm measured. Under these conditions, extinction coefficients of 3330 M^{-1} cm^{-1} for 3-methoxy- and 3-ethoxy-7-amino-4-chloroisocoumarin derivatives and 2710 M^{-1} cm^{-1} for 7-amino-4-chloro-3-(2-phenethoxy)isocoumarin were determined. The active site concentrations of PP elastase and chymotrypsin A_α were determined by using Ac-Ala-Ala-Aala-ONp (Powers et al., 1984) and 4-nitrophenyl acetate (Bender et al., 1966), respectively. First-order ring opening rate constants were obtained from plots of ln $[(A_0 - A_\infty)/(A_t - A_\infty)]$ vs. time, where A_∞ and A_t are the absorbance values at total inactivation and at time t, respectively, and correlation coefficients were 0.993 or greater. All reported values are based on between three and five determinations. Enzymatic ring opening of 4-chloro-3-ethoxyisocoumarin (0.066 mM; ϵ_{349} = 2920 M^{-1} cm^{-1}) by chymotrypsin (0.050 mM) and 3-(benzyloxy)-4-chloro-7-nitroisocoumarin (0.045 mM; ϵ_{385} = 6600 M^{-1} cm^{-1}) by chymotrypsin (3 μM) was measured similarly.

Fluorescence Method. A Perkin-Elmer spectrophotofluorometer, standardized daily with quinine sulfate (2.5 μM) in 0.1 N H_2SO_4 (λ_{ex} = 365 nm, λ_{em} = 460 nm) as 100% fluorescence and buffer as 0% fluorescence, respectively, was used for all fluorescence measurements. The enzymatic ring opening rates of 7-amino-4-chloro-3-methoxyisocoumarin (0.031–0.063 mM) were measured by monitoring the decrease in fluorescence emission at 510 nm (λ_{ex} = 400 nm) upon the addition of PP elastase (8.9–13.0 μM; buffer was 0.1 M potassium phosphate, pH 6.8, 10% Me_2SO) or chymotrypsin (12.6 μM; buffer was 0.1 M Hepes, 0.5 M NaCl, pH 7.5, 10% Me_2SO). The spontaneous hydrolysis product of 7-amino-4-chloro-3-methoxyisocoumarin (0.03 mM), measured after standing in buffer for 28 h, has negligible fluorescence at 510 nm under these conditions. Ring opening rate constants were determined from plots of ln $[(F_0 - F_\infty)/(F_t - F_\infty)]$ vs. time, where F represents arbitrary fluorescence units and correlation coefficients were 0.994 or greater.

Determination of Ethanol Release. Chymotrypsin (0.27 mM) was incubated with 7-amino-4-chloro-3-ethoxyisocoumarin (0.33 mM) in 0.1 M phosphate buffer (pH 7.0) for 1 h (less than 5% activity remained) and then diluted 2-fold into a solution of liver alcohol dehydrogenase (1 μM) and NAD^+ (1.5 mM) in 0.1 M phosphate buffer (pH 8.8). The final pH was 7.8. Alcohol dehydrogenase activity was measured by the increase in absorbance at 340 nm (ϵ = 6200 M^{-1} cm^{-1}) upon reduction of NAD^+ to NADH. Control measurements, performed by adding aliquots of ethanol to reaction mixtures containing dehydrogenase (0.5 μM), NAD^+ (0.75 mM), and inactivated chymotrypsin (0.13 mM), indicated that as little as 2 μM ethanol could have been detected.

Amino Acid Analysis. Samples of chymotrypsin A_α (2–5 mg) were dissolved in 10 mL of 0.1 M Hepes buffer (pH 7.5) containing 0.5 M NaCl, and 0.6 mL of 7-amino-4-chloro-3-methoxyisocoumarin (8 mM) was added. After a 70-min incubation period (0.2% residual activity), the samples were dialyzed against two changes of 1 mM HCl (2 L) and then lyophilized. The inactivated enzyme samples were hydrolyzed in 6 N HCl in a sealed tube for 20 h and the hydrolyzed samples subjected to amino acid analysis using a Beckman 120C instrument. Uninhibited samples were treated similarly.

Synthesis. Homophthalic acid, 4-fluorobenzyl alcohol, and 2-phenylethanol were obtained from Aldrich Chemical Co., Milwaukee, WI. All common chemicals and solvents were reagent grade or better. The purity of each compound was checked by NMR, IR, mass spectroscopy, melting point, thin-layer chromatography (TLC) (silica gel plates), and elemental analysis, and results were consistent with the proposed structures. The TLC solvent systems were as follows: chloroform/methanol (9:1) (system 1); benzene (system 2); methylene chloride (system 3). The NMR spectra were recorded on either a Varian T-60 or a Bruker 360-MHz instrument. Mass spectra were recorded on a Varian MAT 112S spectrometer. Infrared spectra were measured on a Perkin-Elmer 299 instrument. Elemental analyses were performed by Atlantic Microlabs of Atlanta, GA.

7-Amino-4-chloro-3-methoxyisocoumarin (**3a**), 7-amino-4-chloro-3-ethoxyisocoumarin (**3b**), 4-chloro-3-methoxy-7-nitroisocoumarin (**2a**), 4-chloro-3-ethoxy-7-nitroisocoumarin (**2b**) (Choksey & Usgaonkar, 1976), and 4-chloro-3-ethoxyisocoumarin (**1b**) (Tiradkar & Usgaonkar, 1969) were prepared by cyclization of the appropriate alkyl 2-carboxyphenylacetate derivative with PCl_5 as described earlier with some modifications. The ring closure reactions were carried out for 45–120 min instead of 4 h. After removal of the reaction solvent, the residue was triturated with isopropyl ether or hexane to remove remaining phosphorus oxychloride and the crude product purified by silica gel chromatography using benzene as the eluent. The 7-amino derivatives were purified by silica gel chromatography using methylene chloride as the eluent. Concentration of the appropriate fractions generally gave pure products (one spot on TLC) as pale yellow or orange solids that had physical properties consistent with those pre-

viously reported. All 2-carboxyphenylacetate derivatives were prepared by refluxing homophthalic acid or 4-nitrohomophthalic acid (Ungnade et al., 1945) with the appropriate alcohol in the presence of sulfuric acid as described below for 2-phenylethyl 2-carboxy-4-nitrophenylacetate. Most of the new substituted isocoumarins were prepared by cyclization with PCl_5 (Tiradkar & Usgaonkar, 1969; Choksey & Usgaonkar, 1976), and typical syntheses as well as the physical constants of all new structures are given below.

4-Chloro-7-nitro-3-(2-phenethoxy)isocoumarin (**2f**). 4-Nitrohomophthalic acid (6.0 g, 26.5 mmol) was suspended in 2-phenylethanol (20 mL), H_2SO_4 (3 drops) added, and the reaction mixture heated at 120–130 °C for 3.5 h. The mixture was diluted with ethyl acetate (200 mL) and washed with 4% $NaHCO_3$ (2 × 300 mL). Thin-layer chromatography indicated that the product was contained primarily in the ethyl acetate layer. After concentration of the ethyl acetate layer, the solid was triturated with isopropyl ether to give 2-phenylethyl 2-carboxy-4-nitrophenylacetate (2.0 g) as a tan solid and was used without further purification. 2-Phenylethyl 2-carboxy-4-nitrophenylacetate (1.3 g, 3.9 mmol) was added slowly to a solution of PCl_5 (2.05 g, 9.9 mmol) in benzene (30 mL) and the mixture refluxed at 80 °C for 1 h. The benzene was removed and the residue triturated with petroleum ether. The crude product was purified by silica gel chromatography using benzene as the eluent to give **2f** (0.1 g) as an orange solid: mp 95–100 °C dec; one spot on TLC, R_f (system 2) = 0.5; IR (nujol) 1752 cm^{-1}. Anal. Calcd for $C_{17}H_{12}ClNO_5$: C, 59.05; H, 3.50. Found: C, 58.9; H, 3.55.

7-Amino-4-chloro-3-(2-phenethoxy)isocoumarin (**3f**). 4-Chloro-7-nitro-3-(2-phenethoxy)isocoumarin (**2f**, 50 mg, 0.14 mmol) was dissolved in absolute ethanol (50 mL) and hydrogenated with Pd–C (50 mg) as catalyst for 2 h. After filtering over celite, the solvent was removed and the residue chromatographed on silica gel with methylene chloride as the eluent to give **3f** (35 mg) as yellow plates: mp 105–107 °C; one spot on TLC, R_f (system 3) = 0.45; IR (CH_2Cl_2) 1745 cm^{-1}; mass spectrum m/e 315 (M^+). Anal. Calcd for $C_{17}H_{14}ClNO_3$: C, 64.66; H, 4.47. Found: C, 64.56; H, 4.51.

3-(Benzyloxy)-4-chloroisocoumarin (**1d**). This compound was prepared by cyclization of benzyl 2-carboxyphenylacetate (0.3 g, 1.1 mmol) with PCl_5 as described above for **2f**. The crude product was purified by silica gel chromatography using benzene as the eluent to give **1d** (125 mg) as a pale yellow solid: mp 92 °C; single spot on TLC, R_f (system 2) = 0.63; IR (CH_2Cl_2) 1742 cm^{-1}; mass spectrum m/e 286 (M^+). Anal. Calcd for $C_{16}H_{11}O_3Cl$: C, 67.02; H, 3.84. Found: C, 66.83; H, 3.86.

3-[(4-Fluorobenzyl)oxy]-4-chloroisocoumarin (**1e**). This compound was obtained by cyclization of 4-fluorobenzyl 2-carboxyphenylacetate (0.8 g, 2.8 mmol) with PCl_5 as described above for **2f**. After removal of the reaction solvent, the residue was dissolved in diethyl ether and filtered. The filtrate was concentrated and collected with isopropyl ether to give **1e** (500 mg) as pale yellow needles: mp 127 °C; one spot on TLC, R_f (system 2) = 0.7; IR (CH_2Cl_2) 1743 cm^{-1}; mass spectrum m/e 304 (M^+). Anal. Calcd for $C_{16}H_{10}ClFO_3$: C, 63.07; H, 3.31. Found: C, 62.82; H, 3.36.

3-(Isobutyloxy)-4-chloroisocoumarin (**1c**). This compound was obtained by cyclization of isobutyl 2-carboxyphenylacetate (0.7 g, 3 mmol) with PCl_5 as described above for **2f**. The crude product was purified by silica gel chromatography using benzene as the eluent to give **1c** (340 mg) as pale yellow needles: mp 48 °C; one spot on TLC, R_f (system 2) = 0.5; IR (CH_2Cl_2) 1740 cm^{-1}. Anal. Calcd for $C_{13}H_{13}ClO_3$: C, 61.79; H, 5.19. Found: C, 61.64; H, 5.22.

3-(Benzyloxy)-4-chloro-7-nitroisocoumarin (**2d**). This compound was prepared by cyclization of benzyl 2-carboxy-4-nitrophenylacetate (3.0 g, 9.5 mmol) using PCl_5 as described above for **2f**. The crude product was triturated with isopropyl ether to give 575 mg of a yellow solid, which was purified further by silica gel chromatography using methylene chloride as the eluent. Crystallization from methylene chloride gave **2d** (500 mg) as yellow needles: mp 153–154 °C dec; one spot on TLC, R_f (system 3) = 0.8; IR (CH_2Cl_2) 1758, 1622 cm^{-1}; mass spectrum m/e 331 (M^+). Anal. Calcd for $C_{16}H_{10}ClNO_5$: C, 57.94; H, 3.04; N, 4.22. Found: C, 58.04; H, 3.09; N, 4.18.

3-Methoxy-7-nitroisocoumarin (**4a**). Methyl 2-carboxy-4-nitrophenylacetate (2.0 g, 8.4 mmol) (Choksey & Usgaonkar, 1976) was suspended in methylene chloride (50 mL), and trifluoroacetic anhydride (1.42 mL, 10 mmol in 5 mL of methylene chloride) was added dropwise with stirring at 5 °C. After the reaction mixture was stirred overnight at room temperature, the precipitate was filtered and crystallized from methanol/petroleum ether to give (**4a**) (1.7 g) as a pale yellow solid: mp 148–150 °C dec; one spot on TLC, R_f (system 2) = 0.4; IR (CH_2Cl_2) 1741 cm^{-1}; mass spectrum m/e 221 (M^+). Anal. Calcd for $C_{10}H_7NO_5$: C, 54.30; H, 3.19. Found: C, 54.23; H, 3.24.

7-Amino-3-methoxyisocoumarin (**5a**). 3-Methoxy-7-nitroisocoumarin (**4a**, 500 mg, 2.2 mmol) was dissolved in methanol (20 mL) and hydrogenated with 10% Pd–C (200 mg) as catalyst for 45 min at 25 °C. After filtration over Celite, the product was recrystallized from methanol/isopropyl ether to give **5a** (250 mg) as yellow needles: mp 160–161 °C dec; one spot by TLC, R_f (system 3) = 0.3; IR (CH_2Cl_2) 1722 cm^{-1}; mass spectrum m/e 191 (M^+). Anal. Calcd for $C_{10}H_9NO_3$: C, 62.82; H, 4.75. Found: C, 62.71; H, 4.76.

7-[(N-Tosylphenylalanyl)amino]-4-chloro-3-methoxyisocoumarin (**6**). *N*-Tosylphenylalanine acid chloride (77 mg, 0.3 mmol) and 7-amino-4-chloro-3-methoxyisocoumarin (50 mg, 0.2 mmol) were suspended in a mixture of methylene chloride/tetrahydrofuran (1:1), and triethylamine (0.037 mL, in 2 mL of methylene chloride) was added dropwise with stirring. After the mixture was stirred at 25 °C for 2 h, the reaction solvents were removed, and the residue wase dissolved in ethyl acetate. After washing with 10% citric acid (3 × 30 mL) and 4% $NaHCO_3$ (2 × 30 mL), the ethyl acetate layer was dried over magnesium sulfate and concentrated. The crude product was purified by silica gel chromatography using a 1% mixture of methanol in methylene chloride and **6** (22 mg) collected from methanol/isopropyl ether as a pale yellow solid: mp 222–224 °C dec; one spot on TLC, R_f (system 3) = 0.3; IR (nujol) 1750 cm^{-1}; mass spectrum m/e 527 (M + 1). Anal. Calcd for $C_{26}H_{23}ClN_2O_6S \cdot {}^1/_2H_2O$: C, 58.26; H, 4.53. Found: C, 58.28; H, 4.50.

Results

Inactivation Kinetics. Several substituted 3-alkoxyisocoumarins (Figure 1) were synthesized and tested as inactivators of a number of serine proteases including HL elastase, HL cathepsin G, rat mast cell protease I, rat mast cell protease II, *Streptomyces griseus* protease A, and human skin chymase. Attempts to prepare 7-amino-3-(benzyloxy)-4-chloroisocoumarin from the 7-nitro derivative **2d** (one of the better inhibitors of chymotrypsin-like enzymes) by reduction with either H_2/Pd–C or $NaBH_4$ were unsuccessful.

The second-order inactivation rate constants ($k_{obsd}/[I]$) are reported in Table I. In most cases, first-order inactivation plots were linear for greater than four half-lives. Inactivation

Table I: Rate Constants (k_{obsd}/[I]) for Inactivation of Serine Proteases by Substituted Isocoumarins[a]

inactivator	enzyme							
	HLE[b]	PPE[c]	Cat G[d]	ChyT[e]	RMCP I[f]	RMCP II[g]	SGPA[h]	HSC[i]
4-chloro-3-ethoxyisocoumarin (**1b**)	43000[j]	1440	190	610	190[j]	3050[j]	820	
4-chloro-3-(isobutyloxy)isocoumarin (**1c**)	9500	NI[k]	190	750	985	920	6400	230
3-(benzyloxy)-4-chloroisocoumarin (**1d**)	1525	6	1140	16000[j]	29560[j]	9600	5500	19500
4-chloro-3-[(4-fluorobenzyl)oxy]isocoumarin (**1e**)	2300	NI	220	32000[j]	46000[j]	3200	3350	11500
4-chloro-3-methoxy-7-nitroisocoumarin (**2a**)	>2580	>580	NI	>5300	>2000			
3-methoxy-7-nitroisocoumarin (**4**)	>800	>1000	NI	>3200				
4-chloro-3-ethoxy-7-nitroisocoumarin (**2b**)	>2800	>1300	NI	>4300	>4200	>7000	>2700	
3-(benzyloxy)-4-chloro-7-nitroisocoumarin (**2d**)	>890	NI	>2600	>10500	>8100	>10500	>5100	
4-chloro-7-nitro-3-(2-phenethoxy)isocoumarin (**2f**)	>2600	NI	NI	>5000			>5000	
7-amino-4-chloro-3-methoxyisocoumarin (**3a**)	10000	1035	17	110	80	130	40	60
7-amino-3-methoxyisocoumarin (**5**)	200	10	NI	4[l]			5[l]	
7-amino-4-chloro-3-ethoxyisocoumarin (**3b**)	9420	700	195	270	590	880	220	320
7-amino-4-chloro-3-(2-phenethoxy)isocoumarin (**3f**)	40	NI	70	1170	910[j]	30	380	210
7-[(*N*-tosylphenylalanyl)amino]-4-chloro-3-methoxyisocoumarin (**6**)	190000[j]	6480	NI	150	NI		NI	

[a] Conditions were as follows: 0.1 M Hepes, 0.6 M NaCl, pH 7.6, and 10% Me_2SO at 25 °C. Rate constants were obtained by the incubation method (see Materials and Methods) unless otherwise noted. The units of k_{obsd}/[I] are M^{-1} s^{-1}. [b] Inhibitor concentrations were as follows: **1c**, 0.007 mM; **1d**, 0.010 mM; **1e**, 0.011 mM; **2a**, 0.016 mM; **4**, 0.036 mM; **2b**, 0.034 mM; **2d**, 0.036 mM; **2f**, 0.037 mM; **3a**, 0.006 mM; **5**, 0.112 mM; **3b**, 0.004 mM; **3f**, 0.036 mM. [c] Inhibitor concentrations were as follows: **1b**, 0.037 mM; **1c**, 0.027 mM; **1d**, 0.011 mM; **1e**, 0.020 mM; **2a**, 0.040 mM; **4**, 0.033 mM; **2b**, 0.030 mM; **2d**, 0.068 mM; **2f**, 0.033 mM; **3a**, 0.016 mM; **5**, 0.091 mM; **3b**, 0.019 mM, **3f**, 0.033 mM; **6**, 0.009 mM. [d] Inhibitor concentrations were as follows: **1b**, 0.037 mM; **1c**, 0.067 mM; **1d**, 0.009 mM; **1f**, 0.026 mM; **2a**, 0.031 mM; **4**, 0.036 mM; **2b**, 0.033 mM; **2d**, 0.022 mM; **2f**, 0.073 mM;, **3a**, 0.098 mM; **5**, 0.093 mM; **3b**, 0.113 mM; **3f**, 0.036 mM; **6**, 0.047 mM. [e] Inhibitor concentrations were as follows: **1b**, 0.013 mM; **1c**, 0.020 mM; **2a**, 0.031 mM; **4**, 0.036 mM; **2b**, 0.032 mM; **2d**, 0.022 mM; **2f**, 0.071 mM; **3a**, 0.098 mM; **5**, 0.089 mM; **3b**, 0.023 mM; **3f**, 0.016 mM; **6**, 0.047 mM. [f] Inhibitor concentrations were as follows: **1c**, 0.033 mM; **1e**, 0.031 mM; **2b**, 0.033 mM; **2d**, 0.034 mM, **3a**, 0.023 mM; **3b**, 0.039 mM; **6**, 0.067 mM. [g] Inhibitor concentrations were as follows: **1c**, 0.020 mM; **1d**, 0.009 mM; **1e**, 0.013 mM; **2b**, 0.033 mM; **2d**, 0.022 mM; **3a**, 0.098 mM; **3b**, 0.022 mM; **3f**, 0.090 mM. [h] Inhibitors concentrations were as follows: **1b**, 0.047 mM; **1c**, 0.006 mM; **1d**, 0.013 mM; **1e**, 0.086 mM; **2b**, 0.086 mM; **2d**, 0.036 mM; **2e**, 0.019 mM; **3a**, 0.060 mM; **5**, 0.094 mM; **3b**, 0.044 mM; **3f**, 0.009 mM; **6**, 0.067 mM. [i] Rate constants obtained by progress curve method as described under Material and Methods. Inhibitor concentrations were as follows: **1c**, 0.011 mM; **1d**, 0.0016 mM; **1e**, 0.73 μM; **3a**, 0.028 mM; **3b**, 0.049 mM; **3f**, 0.022 mM. [j] Progress curve method as described under Materials and Methods. [k] NI, less than 10% inactivation observed over 1 h. [l] Less than 40% inhibition observed after incubation for 1 h.

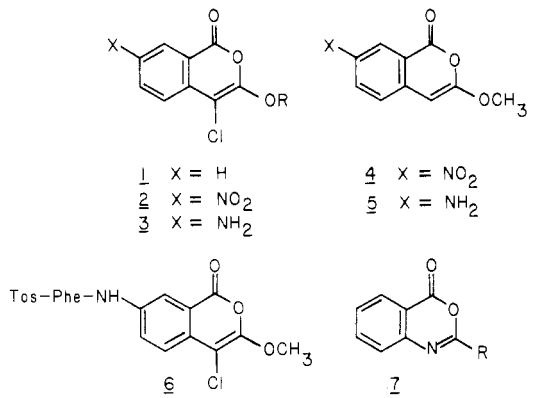

FIGURE 1: Structures of the substituted isocoumarins investigated as serine protease inactivators. The substituents (R) on the 3-alkoxy-4-chloroisocoumarins (**1**) are as follows: **1b**, CH_2CH_3; **1c**, $CH_2CH(CH_3)_2$; **1d**, $CH_2C_6H_5$; **1e**, $CH_2C_6H_4$-4-F. The substituents (R) on the 3-alkoxy-4-chloro-7-nitroisocoumarins (**2**) are as follows: **2a**, CH_3; **2b**, CH_2CH_3; **2d**, $CH_2C_6H_5$; **2f**, $CH_2CH_2C_6H_5$. The substituents (R) on 3-alkoxy-7-amino-4-chloroisocoumarins (**3**) are as follows: **3a**, CH_3; **3b**, CH_2CH_3; **3f**, $CH_2CH_2C_6H_5$. 3-Methoxy-7-nitroisocoumarin and 7-amino-3-methoxyisocoumarin have structures **4** and **5**, respectively. 7-[(*N*-Tosylphenylalanyl)amino]-4-chloro-3-methoxyisocoumarin is structure **6**. The substituents (R) on the 4*H*-3,1-benzoxazin-4-ones (**7**) are as follows: **7d**, $CH_2C_6H_5$; **7g**, $CF_2CF_2CF_3$.

by the 3-alkoxy-4-chloro-7-nitroisocoumarins (**2**) was very rapid, and typically less than 20% activity was observed at the first time point examined (0.2–0.3 min) under the conditions given in Table I. Thus, the inactivating rate constants calculated from this residual activity should be considered to be lower limits. Attempts to decrease the inactivation rate by using lower levels of inhibitor were not successful due to the rapid spontaneous decompositon of the 7-nitroisocoumarins in aqueous solution and the relatively rapid reactivation of the inhibited enzyme. In some cases where inactivation was extremely rapid or when limited quantities of enzyme were available, rate constants were determined by using the progress curve method of Tian & Tsou (1982). Total inactivation (>99%) was generally observed, except in the cases of inactivation of HL and PP elastase by 3-alkoxy-4-chloro-7-nitroisocoumarins and 7-amino-3-methoxyisocoumarin, where a maximum of 60–90% inactivation was observed.

In general, the most reactive inhibitors toward all of the enzymes studied were the 3-alkoxy-4-chloroisocoumarins, which had k_{obsd}/[I] values as high as 43 000 M^{-1} s^{-1} with HL elastase. The 3-alkoxy-4-chloro-7-nitroisocoumarins (**2**) and 3-alkoxy-7-amino-4-chloroisocoumarins (**3**) were intermediate in reactivity while the 7-aminoisocoumarin derivative **5** inactivated serine proteases quite slowly or not at all. PP elastase was inactivated poorly or not at all by compounds containing bulky or aromatic 3-substituents (R = isobutyl, benzyl, 4-fluorobenzyl) but was effectively inhibited by isocoumarins with 3-ethoxy or 3-methoxy substituents. HL elastase, on the other hand, was effectively inhibited by isocoumarins with both types of 3-alkoxy substituents. Cathepsin G was most effectively inhibited by two isocoumarins (**1d** and **2d**) with 3-(benzyloxy) substituents. The same two inhibitors most effectively inhibited chymotrypsin A_α, RMCP I, RMCP II, SGPA, and human skin chymases. Changing the 3-(benzyloxy) substituent to a 3-[(4-fluorobenzyl)oxy] substituent led to an increased k_{obsd}/[I] with chymotrypsin, RMCP I, and HL elastase, but not with other serine proteases. 7-[(*N*-Tosylphenylalanyl)amino]-4-chloro-3-methoxyisocoumarin (**6**) was the most effective HL elastase and PP elastase inhibitor and was quite selective since it did not inhibit several of the other enzymes tested and inhibited chymotrypsin quite slowly. Cathepsin G was inert to more different isocoumarin structures than the other serine proteases. This is particularly true with the 7-nitroisocoumarins where cathepsin G was only inhibited by the 3-(benzyloxy) derivative **2d**.

Inactivation of several mammalian blood coagulation and trypsin-like serine proteases by 4-chloro-3-ethoxyisocoumarin

Table II: Inactivation of Trypsin-like Serine Proteases and Metalloproteases by Substituted Isocoumarins[a]

enzyme	$k_{obsd}/[I]$ (M^{-1} s^{-1}) 4-chloro-3-ethoxy-isocoumarin (**1b**)[b]	7-amino-4-chloro-3-ethoxy-isocoumarin (**3b**)[c]
human thrombin	13	3
bovine thrombin	NI[d]	0.6
bovine factor Xa	8	
human β-factor XIIa	340	55
human plasmin	11	5
PP kallikrein	370	78
bovine trypsin	140	10
leucine aminopeptidase		NI[d,e,f]
thermolysin		NI[d,g]

[a] Inactivation rate constants were determined as described under Materials and Methods. Unless otherwise noted, conditions were as follows: 50 mM Tris, 0.2 M NaCl, 5 mM $CaCl_2$, pH 7.8, and 8–10% Me_2SO at 25 °C. [b] Inhibitor concentration was 0.038–0.040 mM. [c] Inhibitor concentration was 0.090–0.098 mM unless otherwise noted. [d] NI, less than 5% inactivation observed after 1 h. [e] Buffer was 0.1 M Hepes, 0.5 M NaCl, pH 7.5, and 8–10% Me_2SO. [f] Inhibitor concentration was 0.49 mM. [g] Inhibitor concentration was 0.1 mM.

(**1b**) and 7-amino-4-chloro-3-ethoxyisocoumarin (**3b**) was investigated at pH 7.8 (pH 7.5 with bovine trypsin), and the $k_{obsd}/[I]$ values are reported in Table II. In general, the trypsin-like enzymes were inhibited by the isocoumarins at slower rates than other serine proteases. The 4-chloro-3-ethoxyisocoumarin was 2.2–14-fold more reactive than the corresponding 7-amino derivative **3b**, which is consistent with the results obtained with elastases and chymotrypsin-like enzymes.

The isocoumarins seem to be specific for serine proteases since neither **3b** (Table II) nor 7-amino-4-chloro-3-(2-phenethoxy)isocoumarin (**3f**, 0.06–0.09 mM) inactivated the metalloproteases leucine aminopeptidase or thermolysin. The related general serine protease inhibitor 3,4-dichloroisocoumarin has previously been shown not to react with leucine aminopeptidase, the cysteine protease papain, or β-lactamase (type II) but slowly inhibited acetylcholinesterase (Harper et al., 1985).

Substrate Protection and pH Dependence. Addition of substrates or reversible competitive inhibitors to enzyme incubation mixtures resulted in significant decreases in inactivation rate constants. Inactivation of HL elastase (0.4 μM) by 3-(benzyloxy)-4-chloroisocoumarin (**1d**, 0.010 mM) in the presence of 0.03 and 0.15 mM MeO-Suc-Ala-Ala-Pro-Val-SBzl gave $k_{obsd}/[I]$ values of 1230 and 700 M^{-1} s^{-1}, respectively, compared to 1510 M^{-1} s^{-1} in the absence of the thio ester substrate. HL elastase (0.4 μM) preadsorbed onto the substrate bovine elastin (2 mg/mL) was inhibited 1.2-fold more slowly by 7-amino-4-chloro-3-methoxyisocoumarin (**3a**, 15 μM), than in the absence of elastin. Inactivation of PP elastase (1 μM) by **3a** (0.016 mM) in the presence of the reversible competitive inhibitor CF_3CO-Lys-Ala-4-methylanilide (0.015 mM) (Renuad et al., 1983) gave a $k_{obsd}/[I]$ value of 76 M^{-1} s^{-1}, which is 11-fold lower than in the absence of the reversible inhibitor. Thus, inactivation by isocoumarins occurs at elastase's active site.

The pH dependence of inactivation of HL elastase (0.4 μM) by 3-(benzyloxy)-4-chloroisocoumarin (**1d**, 0.01 mM) was investigated from pH 6.7–9.5 by utilizing 0.1 M Hepes buffer (except at pH 9.5 where 50 mM Tris was used). As shown in Figure 2, the maximum observed inactivation rate was near 4200 M^{-1} s^{-1} at pH 8.7, and this rate was 2.5-fold higher than

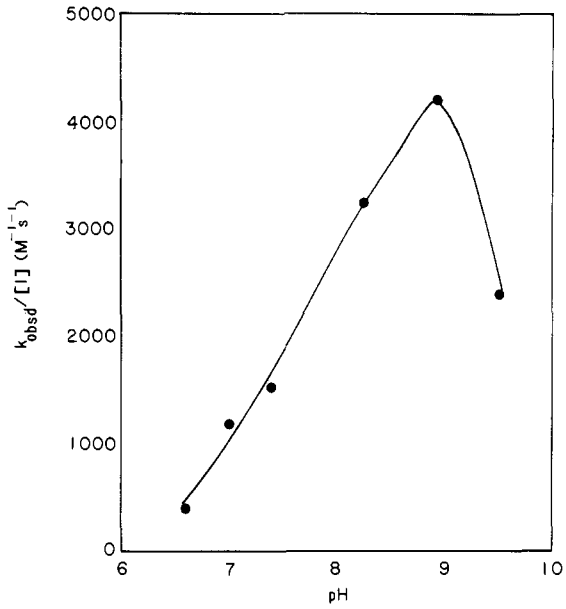

FIGURE 2: The pH dependence for inactivation of HL elastase by 3-(benzyloxy)-4-chloroisocoumarin (**1d**). Buffer was 0.1 M Hepes, 0.5 M NaCl, and 10% Me_2SO except at pH 9.5 where a buffer containing 50 mM Tris, 0.5 M NaCl, and 10% Me_2SO was used. The inhibitor concentration was 0.01 mM.

that observed at pH 7.4. This pH maximum agrees closely with the pH maximum observed for substrate hydrolysis (pH 8.5; Barrett, 1981) and adds further support for the interaction of these inhibitors with the catalytic apparatus of the enzyme. Inactivation of HL elastase by 7-amino-4-chloro-3-methoxyisocoumarin (**3a**, 5 μM) was also pH-dependent, and rate constants of 1580, 10000, and 14200 M^{-1} s^{-1} were determined at pH values of 6.3, 7.5, and 8.2, respectively (0.1 M Hepes, 0.5 M NaCl, 10% Me_2SO).

Reactivation Kinetics. Enzymes inactivated by substituted isocoumarins showed differential stability toward spontaneous reactivation. All enzymes inactivated by the 7-nitroisocoumarin derivatives (**2** and **4**) and 4-chloro-3-ethoxyisocoumarin (**1b**) underwent transient inactivation and rapidly regained full activity upon standing in the incubation mixture at 25 °C. The half-lives for reactivation in the presence of excess inhibitor are summarized in Table III. These values indicated the relative ease of maintaining inhibited enzyme under similar conditions and are not deacylation rates since any enzyme formed by deacylation can be reinhibited by excess inhibitor until the inhibitor itself is destroyed by spontaneous hydrolysis. Preliminary results with the 3-(isobutyloxy)-, 3-(benzyloxy)-, and 3-[(4-fluorobenzyl)oxy]-4-chloroisocoumarins (**1c**, **1d**, and **1e**) and with the 3-alkoxy-7-amino-4-chloroisocoumarin derivatives **3a** and **3b** indicated that little or no enzymatic activity was regained upon standing overnight and in some cases after several days. For example, RMCP I, RMCP II, and SGPA inhibited by **3a**, **3b**, and **3f** were all inhibited (>98%) after standing for 100–120 h in the presence of inhibitor (see Table I for the concentrations). Therefore, reactivation of several enzymes, including HL elastase and cathepsin G, inactivated by 4-chloro-3-(isobutyloxy)isocoumarin and 3-(benzyloxy)-4-chloroisocoumarin and the two 7-amino-4-chloroisocoumarin derivatives was investigated after dialysis of the inactivated enzyme against 50 mM potassium phosphate (pH 7.5) for 3 h at 5 °C or against 0.1 M phosphate buffer (pH 6.8) for 24–48 h at 5 °C (Table IV). The finding that less than 8% of the specific activity [14% with cathepsin G inactivated by 4-chloro-3-(isobutyloxy)isocoumarin] was regained during the dialysis period is consistent with formation

Table III: Half-Lives for Reactivation of Serine Proteases Inactivated by Substituted Isocoumarins[a]

inactivator	$t_{1/2}$ (h)						
	HLE	PPE	Cat G	ChyT	RMCP I	RMCP II	SGPA
4-chloro-3-ethoxyisocoumarin (**1b**)	1.0[b]	1.2		6.3			
4-chloro-3-(isobutyloxy)isocoumarin (**1c**)	20[c]		>48[d]	>48[e]		>48[f]	>48[g]
3-(benzyloxy)-4-chloroisocoumarin (**1d**)	20[h]		30[i]	>100[e]		>48[j]	
4-chloro-3-[(4-fluorobenzyl)oxy]isocoumarin (**1e**)	20[j]			24[j]			>24[j]
4-chloro-3-methoxy-7-nitroisocoumarin (**2a**)	0.3	0.3		1.2	1.7		
3-methoxy-7-nitroisocoumarin (**4**)	0.8	0.8		2.3			
4-chloro-3-ethoxy-7-nitroisocoumarin (**2b**)	0.6	0.2			3.8		1.7
3-(benzyloxy)-4-chloro-7-nitroisocoumarin (**2d**)	0.7		0.05	0.7	1.7	1.2	3.8
4-chloro-7-nitro-3-(2-phenethoxy)isocoumarin (**2f**)	0.7			1.0			0.8

[a] Enzyme activity was followed in the presence of excess inactivator as described under Materials and Methods. Inactivator concentrations are as shown in Table I unless otherwise noted. [b] Inhibitor concentration was 0.013 mM. [c] Inhibitor concentration was 7 μM. [d] Inhibitor concentration was 0.07 mM. [e] Inhibitor concentration was 0.02 mM. [f] Inhibitor concentration was 0.03 mM. [g] Inhibitor concentration was 5 μM. [h] Inhibitor concentration was 0.05 mM. [i] Inhibitor concentration was 0.04 mM. [j] Inhibitor concentration was 0.01 mM.

Table IV: Deacylation Rate Constants for Enzymes Inactivated by 3-Alkoxy-4-chloroisocoumarins and 3-Alkoxy-7-amino-4-chloroisocoumarins[a]

enzyme	inhibitor	% act. after dialysis	k_{deacyl} (s^{-1})
HL elastase	**1c**	6.8	0.14×10^{-3}
	1d	7.6	0.15×10^{-3}
	3a	<1	0
	3b	<1	0
cathepsin G	**1c**	14.0	0.15×10^{-3}
	1d	1.0	0.15×10^{-3}
	3d	<1	0
chymotrypsin A	**1c**	3.0	0.02×10^{-3}
	1d	1.6	0.06×10^{-3}
	3a	<1	0
	3b	<1	0
PP elastase	**3a**	<1	0
	3b	<1	0

[a] Inactivated enzymes (<4% activity) were dialyzed against 50 mM phosphate buffer (pH 7.5) for 3 h at 5 °C except with **3a** and **3b**, which were dialyzed for 24 and 48 h, respectively, against 0.1 M phosphate buffer (pH 6.8) at 5 °C. Enzymic activity and deacylation rate constants were measured upon standing of the dialyzed enzyme at 25 °C as described under Materials and Methods.

of a covalent enzyme–inhibitor adduct. Further standing of the dialyzed enzymes at 25 °C resulted in slow reactivation of enzymes inactivated by the 3-alkoxy-4-chloroisocoumarins while little (<10% with chymotrypsin and cathepsin G) or no (HL and PP elastases) reactivation was observed with enzymes inactivated by the 3-alkoxy-7-amino-4-chloroisocoumarins after standing for at least 48 h.

Reactivation with Hydroxylamine. Rapid and essentially complete (>80%) reactivation was observed upon the addition of buffered hydroxylamine (0.26–0.5 M final concentration) to enzymes inactivated by 3-(benzyloxy)-4-chloroisocoumarin (**1d**) and 7-amino-3-methoxyisocoumarin (**5**). HL elastase totally inactivated (>99%) by **1d** (0.01 mM) regained activity with a half-life of 42 min upon addition of hydroxylamine (0.39 M). Similarly, chymotrypsin inactivated by **1d** (0.025 mM) regained activity with a half-life of 5 min. Addition of hydroxylamine (0.26 M) to HL elastase and PP elastase inactivated by 7-amino-3-methoxyisocoumarin (**5**, 0.9–0.11 mM) (36% and 10% activity, respectively) resulted in a rapid reactivation with half-lives of 12 and 10 min, respectively. Hydroxylamine (0.3–0.4 M) also catalyzed the total reactivation of HL elastase, PP elastase, and chymotrypsin A_α inactivated by 7-[(*N*-tosylphenylalanyl)amino]-4-chloro-3-methoxyisocoumarin (**6**; 0.01, 0.02, and 0.05 mM, respectively), and reactivation rate constants of 0.2×10^{-3} s^{-1} ($t_{1/2}$ = 50 min), 0.4×10^{-3} s^{-1} ($t_{1/2}$ = 30 min), and 0.6×10^{-3} s^{-1} ($t_{1/2}$ = 20 min), respectively, were determined.

Addition of buffered hydroxylamine to elastases and chymotrypsin inactivated by 3-alkoxy-7-amino-4-chloroisocoumarins **3a** and **3b** resulted in a slow ($t_{1/2}$ > 6.7 h) and incomplete (15–43%) regain in enzymatic activity at pH 7.5. The reactivation half-lives along with the extent of reactivation are summarized in Table V. Hydroxylamine (0.37 M) completely destroys the isocoumarin ring system of the inhibitor itself within 1 min as measured by the decrease in absorbance at 385 nm (see below). The ratio of inhibitor to enzyme had negligible effect on either the rate or extent of reactivation. With HL elastase and chymotrypsin, the 7-amino-4-chloro-3-ethoxyisocoumarin-inactivated enzymes were 1.5–2-fold more stable toward hydroxylamine reactivation than were the 7-amino-4-chloro-3-methoxyisocoumarin-inactivated enzymes.

The rate of hydroxylamine reactivation of PP elastase inactivated by 7-amino-4-chloro-3-methoxyisocoumarin was pH-dependent while the extent of reactivation remained relatively constant (30–47%) over the pH range examined (Table VI) except when an acetate buffer was used. The half-lives for reactivation decreased from 46 h at pH 5.0 (Mes buffer) to 4.7 h at pH 8 (Tris buffer) while the extent of reactivation increased from 32% to 43%. Reactivation half-lives and the extent of reactivation were also dependent upon the hydroxylamine concentration utilized. At 0.46 M hydroxylamine, a half-life of 6.2 h with a maximum activation of 42% was observed, while at 0.045 M hydroxylamine, a maximum of 16% activity was regained with a half-life of >54 h. Essentially identical reactivation parameters were obtained after allowing

Table V: Hydroxylamine Reactivation of Serine Proteases Inactivated by 3-Alkoxy-7-amino-4-chloroisocoumarins[a]

enzyme	7-amino-4-chloro-3-methoxyisocoumarin (**3a**)			7-amino-4-chloro-3-ethoxyisocoumarin (**3b**)		
	[I]/[E]	$t_{1/2}$ (h)	% act. regained	[I]/[E]	$t_{1/2}$ (h)	% act. regained
PP elastase	1.5	8.1	36	2.4	7.3	34
	50	9.4	43	49	6.7	39
HL elastase	4.3	17.1	28	5.1	29.6	26
	43	17.4	29	15	31.2	25
chymotrypsin	12	11.0	26	14	20.9	15
	118	11.2	24	42	25.0	14

[a] Conditions were as follows: 0.47 M hydroxylamine, 0.1 M Hepes, and 0.5 M NaCl, pH 7.5 at 25 °C. Enzymatic activity was measured as described under Materials and Methods.

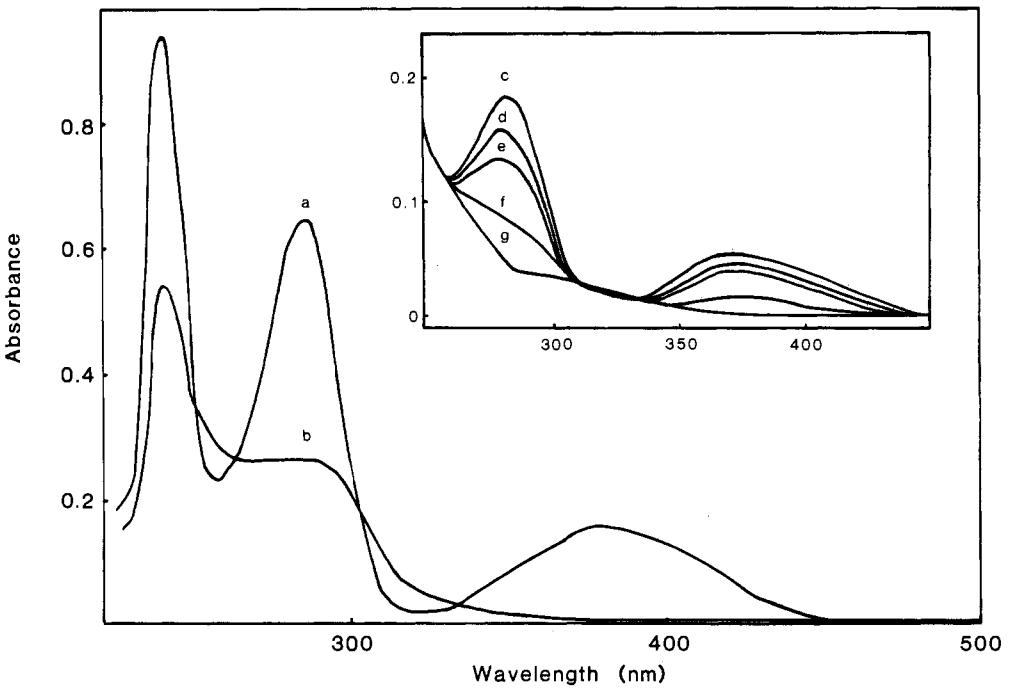

FIGURE 3: Reaction of chymotrypsin A_α with 7-amino-4-chloro-3-ethoxyisocoumarin (**3b**): Spectra of 7-amino-4-chloro-3-ethoxyisocoumarin (0.05 mM) (curve a) and its spontaneous hydrolysis product (curve b) recorded in 0.1 M Hepes, 0.5 M NaCl, and 10% Me_2SO at 25 °C. Inset: Spectra of 7-amino-4-chloro-3-ethoxyisocoumarin (0.015 mM) (curve c) recorded upon the addition of chymotrypsin (0.013 mM) at 1.6 min (curve d), at 3.1 min (curve e), at 10.15 min (curve f), and at 37 min (curve g).

Table VI: pH Dependence of Hydroxylamine Reactivation of PP Elastase Inactivated by 7-Amino-4-chloro-3-methoxyisocoumarin[a]

pH	buffer	$t_{1/2}$ (h)	% act. regained
5.0	Mes		32[b]
5.0	citrate	15.2	40[b]
5.0	acetate	9.7	57[b]
5.5	Mes	15.8	41[c]
6.0	Mes	10.0	38[c]
6.5	Mes	6.3	43[c]
7.0	Hepes	5.5	47[c]
7.5	Hepes	6.2	42[c]
8.0	Tris	4.7	43[c]

[a] Reactivation conditions were as follows: 0.46 M hydroxylamine, 0.1 M buffer, and 0.5 M NaCl at 25 °C. Enzymic activity was measured as described under Materials and Methods. [b] Activity was measured at 24 h. [c] Activity was measured at 17 h and remained constant after 48 h at pH 7.5.

the enzyme–inhibitor complex to stand for 4 h before addition of hydroxylamine.

These reactivation data suggest that, following treatment with hydroxylamine, the observed enzymatic activity is due to either a modified enzyme that has approximately 15–43% activity as compared to the native enzyme or a mixture of fully active enzyme (15–43%) and irreversibly inactivated enzyme (57–85%). To distinguish between these two possibilities, we determined the kinetic parameters for hydrolysis of Suc-Ala-Ala-Ala-NA by PP elastase that had been inactivated by 7-amino-4-chloro-3-methoxyisocoumarin and subsequently partially reactivated by hydroxylamine. The enzyme preparation used for the kinetic analysis had 36% activity after 20.3 h. The native enzyme gave values of K_M = 7.9 mM and k_{cat} = 47 s^{-1} while the modified enzyme gave values of K_M = 5.4 mM and k_{cat} = 34 s^{-1}. The observation that the K_M value of the hydroxylamine-reactivated PP elastase is essentially unchanged from that of native enzyme suggests that native elastase is generated in the hydroxylamine reaction since one would expect a markedly different K_M value for a chemically modified, but still active, form of the enzyme.

Spectral Changes. The UV–visible spectra of 7-amino-4-chloro-3-ethoxyisocoumarin (0.05 mM) and its spontaneous hydrolysis product (measured after 24 h, 12 half-lives) in 0.1 M Hepes and 0.5 M NaCl, pH 7.5, at 25 °C are shown in Figure 3. Time-dependent spectral changes occurred upon the addition of chymotrypsin A_α (0.013 mM) to 7-amino-4-chloro-3-ethoxyisocoumarin (0.015 mM) (Figure 3, inset), and less than 5% activity was detected after 30 min. Most significant are the absorbance decreases at 385 (ϵ = 3330 M^{-1} cm^{-1}) and 286 nm (ϵ = 13 000 M^{-1} cm^{-1}), since these chromophores are due to the isocoumarin ring system and are not present in the spontaneous hydrolysis product. The UV–visible spectra of HL elastase, PP elastase, and chymotrypsin inactivated by 7-amino-4-chloro-3-ethoxyisocoumarin after dialysis against 0.1 M phosphate buffer for 24 h at 5 °C were essentially indistinguishable from those of the enzymes alone. The isocoumarin ring chromophore of 4-chloro-3-ethoxyisocoumarin (**1b**, 0.25 mM, ϵ_{349} = 2920 M^{-1} cm^{-1}) was completely destroyed within 2 min after addition of chymotrypsin A_α (0.25 mM) at which time the enzyme showed <2% activity.

The structural similarity of 7-amino-4-chloro-3-methoxyisocoumarin to the fluorescent 7-amino-4-methylcoumarin, a widely used fluorescent leaving group in protease substrates, prompted us to investigate the fluorescence characteristics of the isocoumarin derivative. The fluorescence excitation spectrum of 7-amino-4-chloro-3-methoxyisocoumarin (0.03 mM, in 0.1 M Hepes, 0.5 M NaCl, pH 7.5, 10% Me_2SO) contains peaks at 300 and 400 nm with the former having about half the intensity of the latter. Excitation at 300 or 400 nm gives an emission spectrum (425–780 nm) that contains a single peak at 510 nm, while the spontaneous hydrolysis product has negligible fluorescence under these conditions (Figure 4). The emission intensity is dependent upon the excitation wavelength with excitation at 400 nm giving rise to a 2-fold more intense signal than excitation at 300 nm. Addition of PP elastase (0.009 and 0.013 mM) to excess 7-amino-4-chloro-3-methoxyisocoumarin (0.031 mM) resulted in a decrease in fluorescence emission at 510 nm (spectra recorded after 6 and 7 min, respectively; >99% inactivation

Table VII: Ring Opening Rates of 3-Alkoxy-7-amino-4-chloroisocoumarins by PP Elastase and Chymotrypsin A_α[a]

inactivator	PP elastase [I] (μM)	PP elastase $k_{obsd} \times 10^3$ (s^{-1})	PP elastase inact stoich[d]	chymotrypsin A_α [I] (μM)	chymotrypsin A_α $k_{obsd} \times 10^3$ (s^{-1})	chymotrypsin A_α inact stoich
Spectrophotometric Method						
7-amino-4-chloro-3-methoxyisocoumarin (**3a**)	51	56.4	1.05	51	4.3	1.53
7-amino-4-chloro-3-ethoxyisocoumarin (**3b**)	30	30.7	1.03	30	7.6	1.31
7-amino-4-chloro-3-(2-phenethoxy)isocoumarin (**3f**)				18	23.0[b]	1.41
Fluorescence Method						
7-amino-4-chloro-3-methoxyisocoumarin (**3a**)	31	18.2[c]	1.06	63	5.0	1.59

[a] Rate constants were determined as described under Materials and Methods. Conditions were as follows: 0.1 M Hepes, 0.5 M NaCl, pH 7.5, and 10% Me_2SO; the concentrations of PP elastase and chymotrypsin were 6.4 and 12.6 μM, respectively, unless otherwise noted. [b] Enzyme concentration was 10.5 μM. [c] Enzyme concentration was 8.9 μM, and buffer was 0.1 M phosphate, pH 6.8, and 10% Me_2SO. [d] inact stoich, inactivation stoichiometry.

observed) (Figure 4). PP elastase (8.9 μM) inactivated by **3a** (0.03 mM) was dialyzed against 0.1 M phosphate buffer (pH 6.8) for 24 h at 5 °C, and the fluorescence emission and excitation spectra were found to be essentially identical with that of enzyme alone and contained negligible fluorescence at 510 nm (Figure 4). Addition of excess chymotrypsin (12.6 μM) to 7-amino-4-chloro-3-methoxyisocoumarin (10.5 μM) gave an emission spectrum that was the same as that of enzyme alone (measured after 60 min; 50% inactivation). Thus, inactivation results in the formation of no new fluorescent species.

These spectral changes allowed determination of PP elastase and chymotrypsin A_α catalyzed ring opening rate constants and inactivation stoichiometry with the 3-alkoxy-7-amino-4-chloroisocoumarins. The results are summarized in Table VII. First-order reaction rate constants were determined as described under Materials and Methods by measuring the decrease in absorbance at 385 nm or the decrease in emission fluorescence at 510 nm (λ_{ex} = 400 nm) with time. Inactivation stoichiometry was calculated from the total absorbance change or fluorescence change accompanying inactivation by using the experimentally determined extinction coefficients or specific fluorescence changes. Enzyme concentrations were based on active site titrations using either Ac-Ala-Ala-Aala-ONp (PP elastase; Powers et al., 1984) or 4-nitrophenyl acetate (chymotrypsin; Bender et al., 1966). While inactivation of PP elastase was essentially stoichiometric, inactivation of chymotrypsin was slightly less efficient, and between 1.3 and 1.6 equiv of inactivator are required for total inactivation, depending on the inhibitor in question. The $k_{obsd}/[I]$ values calculated from these ring opening rates (corrected for inactivation stoichiometry) are in good agreement with those determined by using the incubation method. For example, inactivation of PP elastase (8.9 μM) by 7-amino-4-chloro-3-methoxyisocoumarin (0.031 mM) under the conditions of the fluorescence measurements (pH 6.8) gave a second-order inactivation rate constant of 540 M^{-1} s^{-1}, which is in excellent agreement with that calculated from the ring opening rate constant (550 M^{-1} s^{-1}).

Enzymatic ring opening of other isocoumarin derivatives was investigated at pH 7.5. Reaction of chymotrypsin (0.05 mM) with 4-chloro-3-ethoxyisocoumarin (0.066 mM) resulted in a rapid ($t_{1/2}$ < 20 s) decrease in absorbance at 349 nm (ϵ = 2920 M^{-1} cm^{-1}), and 0.97 equiv of inactivator were destroyed upon total (>99%) inactivation. Addition of chymotrypsin A_α (3 μM) to 3-(benzyloxy)-4-chloro-7-nitroisocoumarin (0.045 mM, ϵ_{385} = 6600 M^{-1} cm^{-1}) resulted in a rapid ($t_{1/2}$ < 10 s) turnover of 4.3 equiv of inactivator followed by a slower steady-state turnover ($k = 0.5 \times 10^{-3}$ s^{-1}, corrected for spontaneous hydrolysis of the inactivator). PP elastase (7 μM) catalyzed the hydrolysis of 7-amino-3-methoxyisocoumarin (0.054 mM, ϵ_{385} = 4300 M^{-1} cm^{-1}) with a pre-steady-state rate of 0.75×10^{-3} s^{-1} and a steady-state rate of 0.054×10^{-3} s^{-1} (corrected for spontaneous hydrolysis, $k_{hyd} = 0.03 \times 10^{-3}$ s^{-1}). The pre-steady-state burst corresponded to 65% of the total enzyme concentration, which is in reasonable agreement with the maximum inactivation observed with this compound in the incubation reaction.

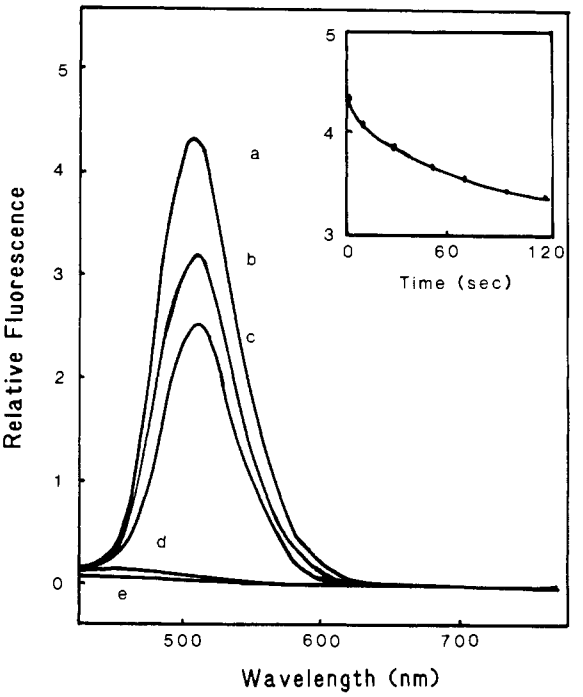

FIGURE 4: Fluorescence emission spectra of 7-amino-4-chloro-3-methoxyisocoumarin (**3a**) in the presence and absence of PP elastase: 7-amino-4-chloro-3-methoxyisocoumarin (0.03 mM; 0.1 mM phosphate buffer, pH 6.8, 10% Me_2SO) (curve a), 7-amino-4-chloro-3-methoxyisocoumarin (0.03 mM) in the presence of PP elastase (9 μM) (curve b), 7-amino-4-chloro-3-methoxyisocoumarin (0.03 mM) in the presence of PP elastase (13 μM) (curve c), and inactivated PP elastase (13 μM) dialyzed against 0.1 M phosphate (pH 6.8) for 24 h at 5 °C (curve d). The spectrum of the spontaneous hydrolysis product of 7-amino-4-chloro-3-methoxyisocoumarin (0.03 mM) is also shown (curve e). Spectra b and c were measured at 6 and 7 min, respectively, after the addition of enzyme, and less than 2% activity was determined at that time. The inset shows a plot of relative fluorescence with respect to time for the reaction of excess 7-amino-4-chloro-3-methoxyisocoumarin (0.03 mM) with PP elastase (9 μM).

Amino Acid Analysis and Ethanol Release. The amino acid compositions of two separate samples of chymotrypsin A_α inactivated by 7-amino-4-chloro-3-methoxyisocoumarin were determined after acid hydrolysis and compared to an uninhibited enzyme treated similarly. In neither case was there any evidence for loss of a histidine or methionine residue upon inactivation. Both histidine and methionine side chains have

Table VIII: Half-Lives for Hydrolysis of Substituted Isocoumarins in Buffer and in the Presence of Glutathione and Albumin[a]

	$t_{1/2}$ (min)			
	Hepes[b]	phosphate[c]	glutathione[d]	albumin[e]
4-chloro-3-ethoxyisocoumarin (**1b**)	140		20[f]	
3-(benzyloxy)-4-chloroisocoumarin (**1d**)	70			
4-chloro-3-[(4-fluorobenzyl)oxy]isocoumarin (**1e**)	50	60		
4-chloro-3-ethoxy-7-nitroisocoumarin (**2b**)	10			
3-(benzyloxy)-4-chloro-7-nitroisocoumarin (**2d**)	17			
7-amino-3-methoxyisocoumarin (**5**)	380	2000		
7-amino-4-chloro-3-methoxyisocoumarin (**3a**)	200	815	28[f]	855
7-amino-4-chloro-3-(2-phenethoxy)isocoumarin (**3f**)	390		50	270

[a] Hydrolysis rates were determined spectrophotometrically as described under Materials and Methods. [b] Conditions were as follows: 0.1 M Hepes, 0.5 M NaCl, and 10% Me_2SO, pH 7.5. [c] Conditions were as follows: 0.02 M potassium phosphate, 0.15 M NaCl, and 10% Me_2SO, pH 7.4. [d] As in footnote *c* with [glutathione] = 0.2 mM. [e] As in footnote *c* with [albumin] = 0.5 mg/mL. [f] As in footnote *b* with [glutathione] = 0.2 mM.

been shown to react irreversibly with other active site directed inhibitors of chymotrypsin (Bechet et al., 1977; Alazard et al., 1973). Inactivation of chymotrypsin A_α (0.27 mM) by 7-amino-4-chloro-7-ethoxyisocoumarin (0.33 mM) resulted in release of less than 1% ethanol as measured by a coupled assay with horse liver alcohol dehydrogenase.

Spontaneous Hydrolysis of Substituted Isocoumarins. The spontaneous hydrolysis rate constants of a number of the substituted isocoumarins were determined under a variety of conditions (Table VIII). The most hydrolytically stable inhibitors were the 7-aminoisocoumarins while the most unstable were the 7-nitroisocoumarins. Hydrolysis rates were dependent upon the buffer composition, and hydrolysis in Hepes (pH 7.5) was significantly faster than that in phosphate buffer (pH 7.4). Addition of glutathione (0.2 mM) resulted in significant increases in decompositions rates while addition of albumin (0.5 mg/mL) affected the decomposition of the 7-aminoisocoumarins only slightly.

Benzoxazin-4-ones. In earlier work from this laboratory, it was shown that 2-substituted 4*H*-3,1-benzoxazin-4-ones (**7**) (Figure 1) and quinazolin-4-ones are potent inhibitors of several serine proteases including HL elastase and that inhibitor potency is dependent upon the infrared stretching frequency of the inhibitor carbonyl group (Teshima et al., 1982). During that study, we found no evidence for enzyme-catalyzed benzoxazinone ring opening in the presence of catalytic levels of enzyme (10^{-8}–10^{-9} M). As it became clear in the present study that many isocoumarins were stoichiometrically reacting with serine proteases, we decided to examine the possibility that benzoxazinones were indeed inhibiting by a similar process. We reinvestigated the inactivation of HL elastase (0.5 μM) by 3-(heptafluoropropyl)benzoxazin-4-one (**7g**, 0.024 mM) and 3-benzylbenzoxazin-4-one (**7d**, 0.024 mM) at pH 7.5 in the presence of excess inhibitor (I/E = 50). These inhibitors are structurally related to the 3-alkoxy-4-chloroisocoumarins (**1**), which are potent inactivators of HL elastase. With the heptafluoropropyl derivative, a maximum inactivation of 55% was observed at 0.25 min and enzymatic activity was regained rapidly ($t_{1/2}$ = 7 min) under these conditions. The benzyl derivative gave a maximum inactivation of 80% after 5 min and regained full activity with a half-life of 60 min. With HL elastase the benzoxazin-4-ones appear to be reacting nonstoichiometrically with the formation of unstable acyl enzymes. In contrast, it has recently been shown that other substituted benzoxazin-4-ones inactivate chymotrypsin by forming stable acyl enzymes (Hedstrom et al., 1984). Further experiments will be required to demonstrate whether the deacylation process with HL elastase results in the regeneration of the benzoxazin-4-one structure.

Discussion

A clearer understanding of the critical roles played by serine proteases in the development of disease states has resulted in a renewed interest in the design of clinically useful serine protease inhibitors. Two types of irreversible inhibitors that appear to have potential for use in vivo are acylating agents and mechanism-based inactivators. The latter have variously been referred to as enzyme-activated inhibitors, suicide inhibitors, suicide substrates, and k_{cat} inhibitors. Acylating agents such as isatoic anhydride and oxazine-2,6-diones (Moorman & Abeles, 1982; Weidman & Abeles, 1984), N-substituted saccharins (Zimmerman et al., 1980), *N*-arylbenzoisothiazolinone 1,1-dioxides (Ashe et al., 1981), 4*H*-3,1-benzoxazin-4-ones (Teshima et al., 1982; Hedstrom et al., 1984), and benzopyran-1,4-diones (Hemmi et al., 1985) react with serine proteases to give slowly hydrolyzed acyl enzymes. Such compounds, which can also be considered mechanism based, are often relatively unstable in aqueous solution and in the presence of plasma or cellular nucleophiles such as albumin (Lawson et al., 1982). Acylating agents also have the potential for reacting with other proteins, and their use in vivo may be somewhat limited unless they are highly specific. Suicide inhibitors, which may form an additional covalent bond with the enzyme during the inactivation reaction, are generally thought to be more resistant to regeneration of enzyme activity when compared to acylating agents and should have more potential for use in vivo. Examples of enzyme-activated inhibitors for serine protease include halomethylcoumarins (Bechet et al., 1973, 1977), haloenol lactones (Daniels et al., 1983), ynenol lactones (Tam et al., 1984), 6-chloro-2-pyrones (Westkaemper & Abeles, 1983; Gelb & Abeles, 1984), and 3-chloroisocoumarins (Harper et al., 1983, 1985). The 3-chloroisocoumarins react with serine proteases to give slowly hydrolyzed diacyl enzyme structures and therefore are, in effect, acylating agents. In contrast, the 6-chloro-2-pyrones form a stable monoacylated enzyme. Halomethylcoumarins, haloenol lactones, and ynenol lactones, on the other hand, apparently react with serine proteases to give stable alkylated enzyme structures. The halomethylcoumarins have the disadvantage of containing a reactive benzyl halide moiety on the parent structure that can nonspecificially alkylate cellular nucleophiles prior to enzyme activation of the inhibitor structure. None of the mechanism-based inhibitors have been tested in animals, and their utility in vivo remains to be determined.

With these results in mind, we set out to design a class of serine protease inhibitors that could easily be modified to give specificity toward a particular serine protease of physiological importance, that would give stable inactivated enzyme structures upon reaction, and that would be stable toward spontaneous hydrolysis and reaction with plasma or cellular nucleophiles. The results presented here indicate that 3-alkoxy-7-amino-4-chloroisocoumarins largely satisfy these goals.

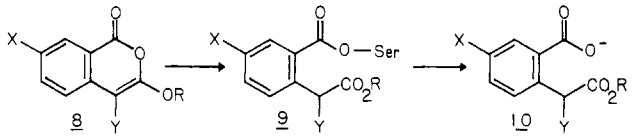

FIGURE 5: Proposed mechanism of inactivation of serine proteases by 3-alkoxy-4-chloroisocoumarins (**1**) and 3-alkoxy-4-chloro-7-nitroisocoumarins (**2**).

Reaction of Serine Proteases with 3-Alkoxy-4-chloroisocoumarins. Two isocoumarins, 3-chloroisocoumarin and 3,4-dichloroisocoumarin, have previously been shown to react with a number of serine proteases and form acyl enzymes of differing stabilities (Harper et al., 1985). 3,4-Dichloroisocoumarin is a general serine protease inhibitor and inhibits at least 18 different enzymes. The finding that isocoumarin itself was not an inhibitor and that 3,4-dichloroisocoumarin was more reactive than 3-chloroisocoumarin is consistent with a mechanism involving initial attack of the serine protease on the isocoumarin carbonyl to form an acyl enzyme. This process can result in the unmasking of varioius functional groups hidden in the isocoumarin structure. The acylation reaction requires electronegative groups on the isocoumarin ring, and isocoumarins containing other electronegative functional groups would be expected to retain the ability to acylate serine proteases. Introduction of the appropriate substituents on the isocoumarin ring would also be expected to give additional specificity toward particular target serine proteases. The alkoxy substituent seemed to provide both the necessary inductive electronegativity and enough variability in size to achieve specificity.

Since our primary interest is elastases and chymases, we initially investigated 3-alkoxy-4-chloroisocoumarins that contained small alkyl or bulky aromatic 3-substituents. The 3-alkoxy substituents in these structures are complementary to the S_1 binding site[2] of elastases and chymases, respectively. The 3-alkoxy-4-chloroisocoumarins and 7-nitro-4-chloroisocoumarins are potent inactivators of the elastases and chymotrypsin-like enzymes studied. The best HL elastase inhibitor was 4-chloro-3-ethoxyisocoumarin, which displayed reasonable selectivity since inhibition of other enzymes, including several trypsin-like enzymes such as human β-factor XIIa and human thrombin, occurred from 14- to 5000-fold more slowly. The best chymotrypsin and chymase inhibitors were the 4-chloroisocoumarins with either a 2-(benzyloxy) or 2-[(4-fluorobenzyl)oxy] substituent. HL elastase was inhibited 20-fold slower by these two inhibitors.

The mechanism of inhibition by 3-alkoxy-4-chloroisocoumarin **8** involves formation of a relatively stable acyl enzyme **9** by reaction with the active site serine of a serine protease (Figure 5). Evidence for the involvement of the active site and the catalytic residues is the observation that rate of inactivation of HL elastase by 3-(benzyloxy)-4-chloroisocoumarin (**1d**) is decreased significantly in the presence of the substrate, MeO-Suc-Ala-Ala-Pro-Val-SBzl, and that the reaction is pH-dependent with a maximum rate near pH 8.7. This value is similar to the pH maximum reported earlier for substrate hydrolysis (pH 8.5) (Barrett, 1981). Chymotrypsin catalyzes the ring opening and loss of the isocoumarin ultraviolet chromophore of 4-chloro-3-ethoxyisocoumarin and 3-(benzyloxy)-4-chloro-7-nitroisocoumarin, and inactivation by 4-chloro-3-ethoxyisocoumarin is essentially

[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 & Berger (1967).

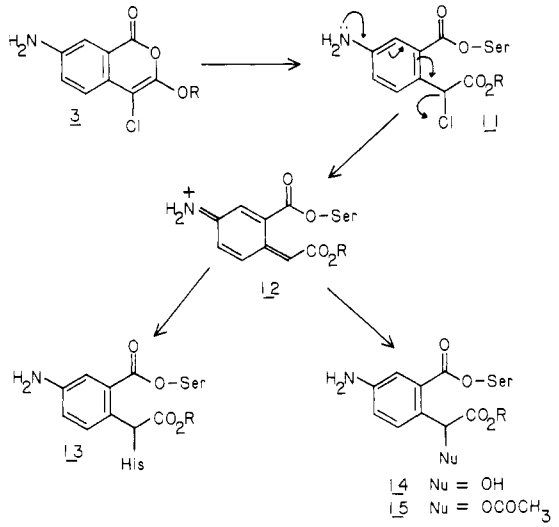

FIGURE 6: Proposed mechanism of inactivation of serine proteases by 3-alkoxy-7-amino-4-chloroisocoumarins (**3**).

stoichiometric as measured by the UV absorption change. This indicates that the enzyme is not displacing halide or alkoxide from the isocoumarin with retention of the heterocyclic system as has been observed in the reaction of chymotrypsin with 5-benzyl-6-chloro-2-pyrone (Westkaemper & Abeles, 1983). Enzyme activity is regained upon standing of the inactivated enzymes, after dialysis, or upon the addition of hydroxylamine. This is consistent with the formation of the acyl enzyme **9**, which hydrolyzes to regenerate active enzyme **10**. The deacylation reaction also indicates that no irreversible alkylation of an active site nucleophile by the the benzyl halide functional group in **9** is involved in the inactivation process since it is unlikely that an alkylated enzyme would regain activity under these conditions. An inactivated chymotrypsin derivative formed by alkylation of the active site histidine residue by a peptide chloromethyl ketone (Z-Gly-Leu-Phe-CH_2Cl) was stable to reactivation by hydroxylamine (Hemmi et al., 1985).

The stability of the acyl enzymes derived from the 3-alkoxy-4-chloroisocoumarins (**1**) and the 3-alkoxy-4-chloro-7-nitroisocoumarins (**2**) is extremely dependent upon the structure of the 3-substituent and the enzyme in question. All of the 7-nitro derivatives deacylated very quickly with reactivation half-lives of 3.8 h or less. This is likely due to the presence of the electronegative nitro substituent, which would destablize these acyl enzymes toward hydrolysis. Enzymes inhibited with the 3-isobutoxy (**1c**) and the 3-benzyl (**1d**) derivatives of 4-chloroisocoumarin regained activity much more slowly. With HL elastase and cathepsin G, the deacylation rate constants were 0.15×10^{-3} s^{-1} or less, while deacylation with chymotrypsin was 2–7-fold slower. The stability of these acyl enzymes could be attributed to steric effects that hinder attack of water on the acyl serine carbonyl group or geometric effects that result in a twist of the acyl serine carbonyl away from the geometric optimum for deacylation. Similar geometric effects have been proposed to account for the stability of other acyl enzymes including carbamoylchymotrypsin (Robillard et al., 1972), indolylacryloylchymotrypsin (Henderson, 1970), and azapeptide derivatives of serine proteases (Gupton et al., 1984). Hydrogen bonding between the enzyme and the unmasked ester moiety could also provide additional stability to the acyl enzymes derived from 3-alkoxyisocoumarins.

7-Amino-4-chloroisocoumarins. The finding that substituted 4-chloroisocoumarins are potent acylating agents for serine proteases indicated that similar structures containing

masked reactive functionalities could be effective mechanism-based inactivators for serine proteases. One such structure is 3-alkoxy-7-amino-4-chloroisocoumarin (**3**). The proposed reaction pathway (Figure 6) involves initial acylation by the active site serine residue of a serine protease to form the acyl enzyme **11** with the unmasking of a 4-aminobenzyl chloride functional group. Similar aminobenzyl halides are known to be quite unstable and decompose rapidly to 4-quinone imine methide structures, which are potent alkylating agents of nucleophiles such as imidazole (Wakselman & Dome, 1975; Dome & Wakselman, 1975). The inherent reactivity of the 4-aminobenzyl chloride is due to the strong electron-releasing properties of the free amino group ($\sigma^+ = -1.3$) (Clementi & Linda, 1973). Elimination of chloride from acyl enzyme **11** would result in the formation of the reactive acyl quinone imine methide **12** tethered at the active site. This quinone imine methide could either react with an active site residue such as the imidazole side chain of histidine-57 to give an alkylated enzyme (**12**) or react with the solvent water to give an acyl benzyl alcohol structure (**14**) (nucleophilic attack on the quinone imine methide structure can occur at several places, and **13** and **14** are representative of two of the most likely families of products). Since formation and alkylation of serine proteases by quinone imine methides appeared to be reasonable possibilities, we prepared several 3-alkoxy-7-amino-4-chloroisocoumarins and found them to be potent mechanism-based inhibitors for a variety of serine proteases.

Inhibition by 3-alkoxy-7-amino-4-chloroisocoumarins is active site directed as evidenced by the substrate protection and the pH dependence of the inhibition reaction. Enzyme inactivation occurs concurrently with opening of the isocoumarin ring system as measured by the loss in both the ultraviolet chromophore and the fluorescence of the inhibitor, with no new ultraviolet or fluorescent products being formed. The inactivation of PP elastase by 7-amino-4-chloro-3-ethoxy- and 7-amino-4-chloro-3-methoxyisocoumarins is stoichiometric. With chymotrypsin, inactivation by the 3-alkoxy-7-amino-4-chloroisocoumarins is slightly less efficient, and between 1.3 and 1.6 equiv of inhibitor is required for total inactivation. The loss of the UV and fluorescence chromophores indicates that the reaction does not involve displacement of the chloride or alkoxide by the active site serine as has been observed in the case of the reaction of chymotrypsin with 5-benzyl-6-chloro-2-pyrone (Westkaemper & Abeles, 1983). The retention of the alkoxy group in the inhibited enzyme is indicated by the absence of ethanol release in the reaction of chymotrypsin with 7-amino-4-chloro-3-ethoxyisocoumarin.

The finding that elastases and chymotrypsin-like enzymes inactivated by the 3-alkoxy-7-amino-4-chloroisocoumarins are extremely stable to reactivation either upon standing or after dialysis suggests that inactivation is irreversible and is consistent with the formation of an alkylated enzyme such as **13**. Many of the other isocoumarins investigated, including the 7-nitro derivatives and 3-alkoxy-4-chloroisocoumarin derivatives such as **1c** and **1d**, inhibited serine proteases but reactivated quite rapidly either in the presence of excess inhibitor or after dialysis.

Further evidence for alkylation of the enzymes was obtained by study of the reactivation using hydroxylamine. Simple acyl enzymes are rapidly reactivated by nucleophiles such as hydroxylamine, while many alkylated enzymes do not ordinarily reactivate upon treatment with nucleophiles. For instance, chymotrypsin inactivated by a peptide chloromethyl ketone, which alkylates the active site histidine, does not reactivate upon exposure to hydroxylamine. With the exception of enzymes inhibited by 3-alkoxy-7-amino-4-chloroisocoumarins, all inhibited enzymes that were investigated regained activity complete (>80%) and quickly ($t_{1/2} < 1$ h) upon addition of buffered hydroxylamine. Included are enzymes inhibited by 3-alkoxy-4-chloroisocoumarins, which form very stable acyl enzymes, and by 7-amino-3-methoxyisocoumarin, an analogue of the 3-alkoxy-7-amino-4-chloroisocoumarins lacking the 4-chlorine atom. Thus both the 4-chloro and the 7-amino substituents are needed to produce inhibited enzymes that are not reactivatable either upon standing or upon treatment with hydroxylamine, and this is further support for the formation of the quinone imine methide.

The reaction of hydroxylamine with serine protease inactivated by 3-alkoxy-7-amino-4-chloroisocoumarins indicated the presence of stable inhibited enzyme species since almost no activity was regained upon standing for 1 h. However, treatment of the inhibited enzymes for extended times led to a slow and incomplete reactivation of enzymatic activity. With chymotrypsin, a maximum of 26% activity was observed, while with HL elastase and PP elastase between 25% and 43% activity was observed. The half-lives for reactivation were 6.7 h or greater at pH 7.5. The finding that the kinetic constants for hydrolysis of Suc-Ala-Ala-Ala-NA by PP elastase after partial reactivation of the 7-amino-4-chloro-3-methoxyisocoumarin–elastase complex are similar to those of native PP elastase suggests that the observed reactivation is due to formation of a mixture of native PP elastase and a nonreactivatable enzyme structure and not to formation of a reactivated enzyme species with modified activity.

The hydroxylamine reactivation results indicate that inactivation of serine proteases by 3-alkoxy-7-amino-4-chloroisocoumarins yields at least two inactivated enzyme species. The nonreactivatable portion (57–85%) of the inhibited enzyme most likely has a structure such as **13**, and the reactivatable enzyme (15–43%) is most likely **14**, which would be formed by reaction of the quinone imine methide **12** with water. If the acyl enzyme **14** represents one of the inactivated enzyme species, it is extremely stable. Hydroxylamine reactivation rates of serine proteases inhibited by 3-alkoxy-7-amino-4-chloroisocoumarins ($6.1–28 \times 10^{-6}$ s^{-1}) are much slower than the uncatalyzed deacylation of acyl enzymes formed by inhibition of serine proteases by 3-alkoxy-4-chloroisocoumarins ($20–190 \times 10^{-6}$ s^{-1}). The increased stability of the acyl enzyme could be due to steric interference imposed by the hydroxyl group, or hydrogen bonding between the enzyme and the hydroxyl group or amino group of the inhibitor could stabilize the inhibited enzyme. Alternatively, it is possible that only one inhibited species such as **13** is formed, which upon reaction with hydroxylamine partially reactivates.

The structure of PP elastase inhibited by 7-amino-4-chloro-3-methoxyisocoumarin has been determined by X-ray crystallography (Meyer et al., 1985). Crystals were grown and inhibited in an 0.1 M acetate buffer at pH 5.0, and the structure was shown to be **15**. A single ester bond links the enzyme to the inhibitor, and an acetate has added to the quinone imine methide intermediate. The acetate is located in the S_1 binding pocket of the enzyme, and the carbomethoxy group is hydrogen-bonded to His-57. At pH 5, the active site histidine of elastase would be protonated and therefore less likely to react with the quinone imine methide. Acetate ion, which is present at fairly high concentration (0.1 M), could react to give the acyl enzyme **15**. Hydroxylamine reactivation of PP elastase inactivated by 7-amino-4-chloro-3-methoxyisocoumarin at pH 5 in the same acetate buffer resulted in the largest regain in activity that was observed in any buffer

Table IX: Relative Rates for Inactivation of Serine Proteases by 3-Alkoxy-7-amino-4-chloroisocoumarins and 3,4-Dichloroisocoumarin[a]

inhibitor	HLE	PPE	Cat G	RMCP I	RMCP II	SGPA	chymase	ChyT	trypsin
7-amino-4-chloro-3-methoxyisocoumarin (**3a**)	93	9.5	0.16	0.75	1.2	0.37	0.56	1.0	
7-amino-4-chloro-3-ethoxyisocoumarin (**3b**)	87	6.5	1.8	5.4	8.1	2.0	3.0	2.5	0.9
7-amino-4-chloro-3-(2-phenethoxy)isocoumarin (**3f**)	0.39	0	0.63	8.4	0.28	3.5	1.9	10.8	0
3,4-dichloroisocoumarin[b]	83	23	0.26	2.4	5.4	2.8	0.25	5.24	1.8

[a] The inactivation rate of chymotrypsin by 7-amino-4-chloro-3-methoxyisocoumarin (**3a**) is set at 1.0. [b] Inactivation rates previously reported (Harper et al., 1985).

or pH and is consistent with the formation of **15** as the major product in solution under these conditions.

A structure such as **13** in which His-57 has added to the quinone imine methide probably best represents the nonreactivatable inhibited enzyme. There are other nucleophiles at the active site of serine protease (Met-192 in chymotrypsin, Gln-192 in elastase), but His-57 is close by and, in the X-ray structure of **15**, is hydrogen-bonded to the ester. Our inability to obtain evidence for enzyme alkylation (no loss of His or Met) from duplicate amino acid analyses of chymotrypsin inactivated by 7-amino-4-chloro-3-methoxyisocoumarin indicates that enzyme alkylation does not occur or that the enzyme–inhibitor adduct is unstable in concentrated HCl. Bechet et al. (1973) have reported a similar case where 3-bromo-6-(imidazol-1-ylmethyl)coumarin, a model for the reaction product of mechanism-based inhibitor with His-57 of chymotrypsin, was decomposed in HCl to imidazole. Thus, further proof for enzyme alkylation by 3-alkoxy-7-amino-4-chloroisocoumarin and identification of the alkylation site will require peptide mapping by mild degradative techniques or X-ray crystallographic studies at higher pH.

The inhibition mechanism proposed for 3-alkoxy-7-amino-4-chloroisocoumarins is similar to that proposed for the inhibition of several serine proteases by halomethylcoumarins (Bechet et al., 1973). A reactive quinone methide appears to be responsible for irreversible inactivation, but the inhibitors contain a benzyl halide functional group with the electron-donating phenol released upon enzyme acylation. The halomethylcoumarins thus contain a reactive alkylating functional group in the parent structure, while the alkylating functional group is only released from the 3-alkoxy-7-amino-4-chloroisocoumarin structure upon enzyme activation.

7-[(N-Tosylphenylalanyl)amino]-4-chloro-3-methoxyisocoumarin. This structure (**6**) was synthesized as a possible example of a pro-mechanism-based inhibitor. A pro-mechanism-based inhibitor can be envisioned to be a structure that would require two enzyme activation steps before irreversible inhibition could take place. The structural relationship between 3-alkoxy-7-amino-4-chloroisocoumarin and 7-amino-4-methylcoumarin, a widely used fluorescent leaving group in serine protease substrates, suggested that the appropriate peptide containing a P_1' 7-amino-4-chloro-3-methoxyisocoumarin moiety might be hydrolyzed by a target serine protease releasing the potent 7-amino-4-chloro-3-methoxyisocoumarin inhibitor moiety. The inhibitor portion of the pro-mechanism-based inhibitor could be targeted for the same enzyme that carried out the initial hydrolysis or for an entirely different enzyme. Pro-mechanism-based inhibitors appear to be an attractive method for delivering a general or specific inhibitor to regions of high protease activity while leaving proteases at other sites untouched and might be useful in inflammatory disease therapy where HL elastase, cathepsin G, chymases, and tryptases are often found in association. A number of other protease-activated drugs have been described (Marquisee & Kauer, 1978; Carl et al., 1981).

The inhibitor **6** contains a peptide structure that would be expected to be recognized by chymotrypsin-like enzymes, but it did not inhibit cathepsin G or RMCP I and inhibited chymotrypsin only poorly. Surprisingly, this compound was the most effective elastase inhibitor that was discovered. The results indicate that initial enzymatic attack occurs at the isocoumarin ring carbonyl to give an acyl derivative since the enzymes are rapidly reactivated upon addition of hydroxylamine, whereas enzymes directly inactivated with 7-amino-4-chloro-3-methoxyisocoumarin are essentially stable to such treatment. The increased reactivity of **6** toward HL elastase and PP elastase probably results from additional hydrophobic interactions of the tosylphenylalanyl residue with some part of the active site of these two enzymes, and reactivation indicates that acylation of the 7-amino group has decreased the ability of the inhibitor to alkylate serine proteases. The pro-mechanism-based inhibitor approach warrants further study since a longer and more specific peptide sequence might prevent initial enzyme attack at the isocoumarin carbonyl group and increase the probability of initial peptide bond hydrolysis and release of the inhibitor moiety.

Specificity and Reactivity. The isocoumarins investigated here are excellent inactivators of many of the serine proteases, with the most reactive enzymes being HL elastase, PP elastases, chymotrypsin, and RMCP I. In general, inactivation rates closely parallel the primary specificities of the enzymes studied. HL elastase, which prefers small alkyl amino acid residues at P_1 in peptide substrates, is inactivated most rapidly by structures that contain small alkyl 3-substituents, and inhibitors containing aromatic substituents were from 19- to 250-fold less reactive. Similarly, chymotrypsin-like enzymes showed a clear preference for 3-alkoxy substituents with aromatic groups, which indicates that the 3-substituent is probably interacting with the S_1 subsite of the enzyme.

The relative reactivities of the 3-alkoxy-7-amino-4-chloroisocoumarins are shown in Table IX and are compared to those of 3,4-dichloroisocoumarin, one of the few mechanism-based inhibitors that has been tested with all the enzymes studied here (Harper et al., 1985). In general, isocoumarins are much better inhibitors for HL elastase than any of the other serine proteases. The most selective inhibitor for HL elastase is 7-amino-4-chloro-3-methoxyisocoumarin, which is 80–580-fold more reactive with HL elastase than with the chymotrypsin-like enzymes. 7-Amino-4-chloro-3-ethoxyisocoumarin is similar to 3,4-dichloroisocoumarin in both selectivity and reactivity and is most reactive toward HL elastase. The 7-amino-4-chloro-3-(2-phenethoxy)isocoumarin derivative is more reactive toward all the chymotrypsin-like enzymes than 3,4-dichloroisocoumarin (excluding RMCP II) and is somewhat selective for RMCP I and chymotrypsin. Several trypsin-like serine proteases react with 3-alkoxy-4-chloroisocoumarins but at rates as much as 4 orders of magnitude lower than with HL elastase. The metalloproteases thermolysin and leucine aminopeptidase are not inhibited by any of the inhibitors tested.

The 7-nitro-substituted and the unsubstituted 3-alkoxy-4-chloroisocoumarins are generally more reactive inhibitors than the 7-aminoisocoumarins. The 4-chloro group has a significant effect on reactivity since inactivation by 7-amino-3-methoxyisocoumarin is 9–100-fold slower than with the corre-

sponding 4-chloro derivative. A similar effect was observed with 3-chloroisocoumarin where the addition of a 4-chloro substituent resulted in a significant inactivation rate enhancement (Harper et al., 1985).

Summary. Here we report that 3-alkoxy-7-amino-4-chloroisocoumarins are potent mechanism-based inhibitors for serine proteases and give rise to extremely stable inactivated enzyme structures. Stable acyl enzymes are also obtained from some 3-alkoxy-4-chloroisocoumarins. The 7-amino-4-chloroisocoumarins are quite stable to spontaneous hydrolysis in aqueous solution and in the presence of albumin unlike many other types of serine protease inhibitors (Lawson et al., 1982) and should have considerable utility as serine protease inactivators both in vivo and in vitro. Extension of these structures to other serine proteases and further mechanistic studies are currently under way.

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REFERENCES

Alazard, R., Bechet, J., Dupaix, A., & Yon, J. (1973) *Biochim. Biophys. Acta 309*, 379–396.

Ashe, B. M., Clark, R. L., Jones, H., & Zimmerman, M. (1981) *J. Biol. Chem. 256*, 11603–11606.

Barrett, A. (1981) *Methods Enzymol. 80*, 581–588.

Bechet, J.-J., Dupaix, A., Yon, J., Wakelman, M., Robert, J.-C., & Vilkas, M. (1973) *Eur. J. Biochem. 35*, 527–539.

Bechet, J.-J., Dupaix, A., & Blagoeva, I. (1977) *Biochimie 59*, 231–239.

Bender, M. L., Beque-Canton, M. L., Blakely, R. L., Brukacher, L. J., Feder, J., Gunter, C. R., Kezdy, F. J., Killheffer, J. V., Marshal, T. H., Miller, C. G., Roeske, R. W., & Stoops, J. K. (1966) *J. Am. Chem. Soc. 88*, 5890–5913.

Bieth, J., Spiess, B., & Wermuth, C. G. (1974) *Biochem. Med. 11*, 350–357.

Carl, P. L., Chakravarty, P. K., & Katzenellenbogen, J. A. (1981) *J. Med. Chem. 24*, 479–480.

Castillo, M. J., Nakajima, K., Zimmerman, M., & Powers, J. C. (1979) *Anal. Biochem. 99*, 53–64.

Cho, K., Tanaka, T., Cook, R. R., Kisiel, W., Fujikawa, K., Kurachi, K., & Powers, J. C. (1984) *Biochemistry 23*, 644–650.

Choksey, I., & Usgaonkar, R. N. (1976) *Indian J. Chem., Sect. B 14B*, 596–598.

Clementi, S., & Linda, P. (1973) *J. Chem. Soc., Perkin Trans. 2*, 1887–1888.

Daniels, S. B., Cooney, E., Sofia, M. J., Chakravarty, P. K., & Katzenellenbogen, J. A. (1983) *J. Biol. Chem. 258*, 15046–15053.

Dome, M., & Wakselman, M. (1975) *Bull. Soc. Chim. Fr.*, 576–582.

Ellman, G. L. (1959) *Arch. Biochem. Biophys. 82*, 70–77.

Erlanger, B. F., Kokowsky, N., & Cohen, W. (1961) *Arch. Biochem. Biophys. 95*, 271–278.

Gelb, M. H., & Abeles, R. H. (1984) *Biochemistry 23*, 6596–6604.

Grassetti, D. R., & Murray, J. F. (1967) *Arch. Biochem. Biophys. 119*, 41–49.

Gupton, B. F., Carroll, D. L., Tuhy, P. M., Kam, C. M., & Powers, J. C. (1984) *J. Biol. Chem. 259*, 4279–4287.

Harper, J. W., & Powers, J. C. (1984) *J. Am. Chem. Soc. 106*, 7618–7619.

Harper, J. W., Hemmi, K., & Powers, J. C. (1983) *J. Am. Chem. Soc. 105*, 6518–6520.

Harper, J. W., Hemmi, K., & Powers, J. C. (1985) *Biochemistry 24*, 1831–1841.

Hedstrom, L., Moorman, A. R., Dobbs, J., & Abeles, R. H. (1984) *Biochemistry 23*, 1753–1759.

Hemmi, K., Harper, J. W., & Powers, J. C. (1985) *Biochemistry 24*, 1841–1848.

Henderson, R. (1970) *J. Mol. Biol. 54*, 341–354.

Lawson, W. B., Valenty, V. B., Wos, J. D., & Lobo, A. P. (1982) *Folia Haematol. (Leipzig) 109*, 52–60.

Marquisee, M. J., & Kauer, J. C. (1978) *J. Med. Chem. 21*, 1188–1194.

McRae, B. J., Kurachi, K., Heimark, R. L., Fujikawa, K., Davie, E. W., & Powers, J. C. (1981) *Biochemistry 20*, 7196–7206.

Meyer, E. F., Jr., Presta, L. G., & Radhakrishman, R. (1985) *J. Am. Chem. Soc. 107*, 4091–4093.

Moorman, A. R., & Abeles, R. H. (1982) *J. Am. Chem. Soc. 104*, 6785–6786.

Nakajima, K., Powers, J. C., Ashe, B. M., & Zimmerman, M. (1979) *J. Biol. Chem. 254*, 4027–4032.

Nishino, N., & Powers, J. C. (1978) *Biochemistry 17*, 2846–2850.

Powers, J. C., Boone, R., Carroll, D. L., Gupton, B. F., Kam, C.-M., Nishino, N., Sakamoto, M., & Tuhy, P. M. (1984) *J. Biol. Chem. 259*, 4288–4294.

Renaud, A., Lestienne, P., Hughes, D. L., Bieth, J. G., & Dimicoli, J. L. (1983) *J. Biol. Chem. 258*, 8312–8316.

Robillard, G. T., Powers, J. C., & Wilcox, P. E. (1972) *Biochemistry 11*, 1773–1784.

Schechter, I., & Berger, A. (1967) *Biochem. Biophys. Res. Commun. 27*, 157–162.

Tam, T.-F., Spencer, R. W., Thomas, E. M., Copp, L. J., & Krantz, A. (1984) *J. Am. Chem. Soc. 106*, 6849–6851.

Tanaka, T., Minematsu, Y., Reilly, C. F., Travis, J., & Powers, J. C. (1985) *Biochemistry 24*, 2040–2047.

Teshima, T., Griffin, J. C., & Powers, J. C. (1982) *J. Biol. Chem. 257*, 5085–5091.

Tian, W.-X., & Tsou, C.-L. (1982) *Biochemistry 21*, 1028–1032.

Tirodkar, R. B., & Usgaonkar, R. N. (1969) *Indian J. Chem. 7*, 1114–1116.

Ungnade, H. E., Nightingale, D. V., & French, H. E. (1945) *J. Org. Chem. 10*, 533–536.

Wakselman, M., & Dome, M. (1975) *Bull. Soc. Chim. Fr.*, 571–575.

Weidmann, B., & Abeles, R. H. (1984) *Biochemistry 23*, 2373–2376.

Westkaemper, R. B., & Abeles, R. H. (1983) *Biochemistry 22*, 3256–3264.

Zimmerman, M., Morman, H., Mulvey, D., Jones, H., Frankshun, R., & Ashe, B. M. (1980) *J. Biol. Chem. 255*, 9848–9851.