Reaction of Serine Proteases with Substituted Isocoumarins: Discovery of 3,4-Dichloroisocoumarin, a New General Mechanism Based Serine Protease Inhibitor†

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ABSTRACT: The mechanism-based inactivations of a number of serine proteases, including human leukocyte (HL) elastase, cathepsin G, rat mast cell proteases I and II, several human and bovine blood coagulation proteases, and human factor D by substituted isocoumarins and phthalides which contain masked acyl chloride or anhydride moieties, are reported. 3,4-Dichloroisocoumarin, the most potent inhibitor investigated here, inactivated all the serine proteases tested but did not inhibit papain, leucine aminopeptidase, or β-lactamase. 3,4-Dichloroisocoumarin was fairly selective toward HL elastase ($k_{obsd}/[I] = 8920\ M^{-1}\ s^{-1}$); the inhibited enzyme was quite stable to reactivation ($k_{deacyl} = 2 \times 10^{-5}\ s^{-1}$), while enzymes inhibited by 3-acetoxyisocoumarin and 3,3-dichlorophthalide regained full activity upon standing. The rate of inactivation was decreased dramatically in the presence of reversible inhibitors or substrates, and ultraviolet spectral measurements indicate that the isocoumarin ring structure is lost upon inactivation. Chymotrypsin A_γ is totally inactivated by 1.2 equiv of 3-chloroisocoumarin or 3,4-dichloroisocoumarin, and approximately 1 equiv of protons is released upon inactivation. These results indicate that these compounds react with serine proteases to release a reactive acyl chloride moiety which can acylate another active site residue. These are the first mechanism-based inhibitors reported for many of the enzymes tested, and 3,4-dichloroisocoumarin should find wide applicability as a general serine protease inhibitor.

Serine proteases are involved in a number of important physiological processes including blood coagulation, the complement system, fertilization, and protein turnover, and many of these enzymes are believed to be involved in diseases such as emphysema, arthritis, and tumorogenesis. Two examples are human leukocyte (HL)[1] elastase and cathepsin G, which are primarily responsible for the destruction of lung elastin which occurs in chronic emphysema (Powers, 1983; Boudier et al., 1982). Selective regulation of these and other proteases in the disease state is an important clinical problem.

Recently, significant progress has been made in the design of mechanism-based irreversible inhibitors for serine proteases. These include haloenol lactones (Daniels et al., 1983; Chakravarty et al., 1982), substituted 6-chloro-2-pyrones (Westkaemper & Abeles, 1983), *N*-nitroso lactams (White et al., 1981), halomethylcoumarins (Bechet et al., 1973, 1977a,b), and 2-bromomethyl-3,1-benzoxazin-4-one (Alazard et al., 1973). 6-Chloro-2-pyrones react with serine proteases to give an acyl enzyme and release an extremely reactive acyl chloride or ketene which is capable of acylating another active site residue. Most of the studies carried out previously have used pancreatic enzymes such as bovine chymotrypsin A_α or porcine elastase. Mechanism-based inhibitors for physiologically important serine proteases such as HL elastase have received little attention thus far.

In this paper, we report the inactivation of a number of serine proteases including human leukocyte elastase, cathepsin G, human thrombin, human β-factor XII_a, bovine factor XI_a, porcine pancreatic kallikrein, human factor D, and several mammalian chymotrypsin-like proteases of mast cell origin by substituted isocoumarin and phthalide derivatives. We have shown that 3-chloroisocoumarin (3-Cl) and 3,4-dichloroisocoumarin (3,4-DCI) are extremely potent inhibitors of several of the enzymes tested and the inactivation is an enzyme-mediated process. We have discovered that 3,4-dichloroisocoumarin is a general serine protease inhibitor, since it inactivated a wide variety of serine proteases but did not react with the thiol protease papain, the metalloprotease leucine aminopeptidase, or β-lactamase. This inhibitor should find wide applicability in the prevention of proteolysis due to serine proteases. A portion of this work has been communicated earlier (Harper et al., 1983).

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 X_a, bovine factor XI_a, and human β-factor XII_a were gifts from Dr. Kotoku Kurachi and Dr. Earl Davie of the University of Washington. *Streptomyces griseus* protease A was provided by Dr. Michael James of the University of Alberta. The human skin chymase was a gift from Dr. Norman M. Schechter and Dr. Gerald

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[1] Abbreviations: HL, human leukocyte; HLE, human leukocyte elastase; PP, porcine pancreatic; PPE, porcine pancreatic elastase; Cat G, cathepsin G; ChyT, chymotrypsin; SGPA, *Streptomyces griseus* protease A; V-8, *Staphylococcus aureus* protease V-8; RMCP I, rat mast cell protease I; RMCP II, rat mast cell protease II; 3-CI, 3-chloroisocoumarin; 3,4-DCI, 3,4-dichloroisocoumarin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Boc, *tert*-butyloxycarbonyl; Z, benzyloxycarbonyl; NA, 4-nitroaniline; SBzl, $-SCH_2C_6H_5$; MeO-Suc, methoxysuccinyl; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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S. Lazarus of the University of Pennsylvania. Human factor D was a gift from Dr. John Volanakis of the University of Alabama. Bovine chymotrypsin A_α, chymotrypsin A_γ, human thrombin, human plasmin, porcine pancreatic elastase, bovine thrombin, porcine pancreatic kallikrein, bovine trypsin, β-lactamase (type II), *Staphylococcus aureus* protease V-8, acetylcholinesterase, subtilisin, leucine aminopeptidase, papain, Tris-HCl, ethylenediaminetetraacetic acid, glutathione, human albumin, Leu-NA·HCl, and phenoxymethylpenicillinic acid were obtained from Sigma Chemical Co., St. Louis, MO, and were of the highest purity available. Homophthalic acid, 2-carboxybenzaldehyde, 4-nitrophenyl acetate, and HEPES were purchased from Aldrich Chemical Co., Milwaukee, WI. Benzoyl-L-Arg-NA·HCl was a product of Bachem Inc., Torrence, CA. MeO-Suc-Ala-Ala-Pro-Val-NA (Nakajima et al., 1979), Suc-Ala-Ala-Ala-NA (Bieth et al., 1974), Suc-Phe-Pro-Phe-NA (Yoshida et al., 1980), Suc-Ala-Ala-Pro-Phe-SBzl (Harper et al., 1981), Boc-Ala-Ala-Glu-SBzl (Harper et al., 1984), Z-Phe-Phe-Arg-NA·HCl, and Z-Phe-Gly-Arg-NA·HCl (Cho et al., 1984), Z-Arg-SBzl·HCl and Z-Lys-Arg-SBu^i·2HCl (McRae et al., 1981), CF_3CO-Lys-Ala-4-methylanilide (Renaud et al., 1983), and 2-(heptafluoropropyl)-4*H*-1,3-benzoxazin-4-one (Teshima et al., 1982) were prepared as previously described. The concentrations of chymotrypsin A_α and chymotrypsin A_γ were determined by active site titration using 4-nitrophenyl acetate (Bender et al., 1966).

Synthesis. Isocoumarin (Narasimhan & Mali, 1975), 3,3,4-trichloro-3,4-dihydroisocoumarin (Milevskaya et al., 1973a,b) 3,3-dichlorophthalide (Ott, 1943), 3-chlorophthalide (Bhatt et al., 1980), 3-acetoxyisocoumarin (Schnekenburger & Kiaser, 1971), and homophthalic anhydride (Grummitt et al., 1955) were prepared as reported earlier. The 3-chloroisocoumarin (Davies & Poole, 1928) and 3,4-dichloroisocoumarin (Milevskaya et al., 1973a,b) were prepared as described previously with some modifications in the purification methods. Pure 3-chloroisocoumarin was obtained only after successive column chromatograhy on silica gel, first by using methylene chloride as the eluent (this step removes most of the polar impurities) followed by a second chromatography using benzene as the eluent (this step removes small quantities of 3,4-dichloroisocoumarin and 3,3,4-trichloro-3,4-dihydroisocoumarin). Some preparations required a third chromatography with benzene. The final product was obtained as a crystalline solid (mp 95–96 °C) upon evaporation of the solvent. The 3,4-dichloroisocoumarin was purified by silica gel chromatography using benzene as the eluent and could be crystallized from several solvents including methylene chloride/petroleum ether (mp 97–98 °C). The purity of each compound was checked by NMR, IR, mass spectrum, melting point, thin-layer chromatography (silica gel plates), and elemental analysis, and the properties were consistent with the physical constants previously published. Elemental analyses were performed by Atlantic Microlabs, Atlanta, GA.

3-Fluorophthalide [3-Fluoro-1(3H)-isobenzofuranone]. A solution of 2-carboxybenzaldehyde (750 mg) in a mixture of methylene chloride (3 mL) and tetrahydrofuran (1 mL) was added to a solution of (diethylamino)sulfur trifluoride (1.3 mL) in methylene chloride (5 mL) at −25 °C, and the mixture was stirred for 2 h, then allowed to warm to 25 °C, and stirred for an additional 40 h. The reaction mixture was poured into ethyl acetate (50 mL) and washed with three 50-mL portions of ice–water. After the mixture was dried over magnesium sulfate, the residue was chromatographed on silica gel by using methylene chloride, and the product recrystallized from hexane to give a white solid (440 mg); mp 65–66 °C; IR (nujol), 1845, 1790 cm^{-1}. Anal. Calcd for $C_8H_5O_2F$: C, 63.16; H, 3.31. Found: C, 63.18; H, 3.33.

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–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 II, and *S. griseus* protease A were assayed with either Suc-Val-Pro-Phe-NA (0.2–0.8 mM) or Suc-Phe-Pro-Phe-NA (0.3–0.9 mM). Human skin chymase, subtilisin, and RMCP I were assayed with Suc-Ala-Ala-Pro-Phe-SBzl (0.095 mM). HL elastase was assayed with MeO-Suc-Ala-Ala-Pro-Val-NA (0.1–0.4 mM), and PP elastase was assayed with Suc-Ala-Ala-Ala-NA (0.6–1.2 mM). *S. aureus* protease V-8 was assayed with Boc-Ala-Ala-Glu-SBzl (0.1 mM). Trypsin was assayed with Z-Phe-Phe-Arg-NA·HCl (0.030 mM). Papain was assayed with benzoyl-L-Arg-NA·HCl (0.49 mM, assay buffer was 50 mM Tris-HCl, 5 mM cysteine, and 2 mM ethylenediaminetetraacetic acid, pH 8.2). Bovine thrombin, human thrombin, human β-factor XII_a, bovine factor XI_a, human plasmin, and porcine pancreatic kallikrein were assayed with Z-Arg-SBzl (0.05–0.2 mM). Human factor D was assayed with Z-Lys-Arg-SBu^i·2HCl (0.48 mM). Factor X_a was assayed with Z-Phe-Gly-Arg-NA·HCl (0.12 mM). β-Lactamase was assayed with phenoxymethylpenicillinic acid (3.6 mM) (Fisher et al., 1978). Acetylcholinesterase was assayed with 4-nitrophenyl acetate (0.50 mM). Leucine aminopeptidase was assayed with Leu-NA·HCl (0.18 mM). All peptide thio ester hydrolysis rates were measured by using either 4,4′-dithiodipyridine (ϵ_{324} = 19800 M^{-1} cm^{-1}) (Grassetti & Murray, 1967) or 5,5′-dithiobis(2-nitrobenzoic acid) (ϵ_{410} = 13600 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).

Second-order inactivation rate constants (k_{obsd}/[I]) were obtained by plotting ln $[E]_t/[E]_0$ vs. time and dividing the slope (k_{obsd}) by the inhibitor concentration. All inactivation rates shown in Tables I and II are the average of duplicate or triplicate experiments, and the correlation coefficients were greater than 0.98 and most were greater than 0.99.

Determination of Inactivation Rates in the Presence of Substrate: Progress Curve Method. In some cases (see Tables I and II), k_{obsd}/[I] values were obtained in the presence of substrate as described by Tian & Tsou (1982). In the case of the reaction of HL elastase (78 nM, final concentration) with 3-CI, a 0.025-mL aliquot of enzyme was added to a buffered solution of MeO-Suc-Ala-Ala-Pro-Val-NA (0.171 mM) which contained between 7 and 35 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. k_{obsd}/[I], K_I, and k_{+3} values were calculated as previously described (Tian & Tsou, 1982). Inactivation of HL elastase (8 nM) by 3,4-DCI (0.4–4.0 μM) was carried out similarly. Inactivation of PP elastase (8.7 nM) by 3-CI (7–35 μM) was carried out with Suc-Ala-Ala-Ala-NA (1.63 mM)

Table I: Inactivation Rates (k_{obsd}/[I]) for Inhibition of Serine Proteases by Substituted Isocoumarin and Phthalide Derivatives[a]

compound	enzyme							
	HLE[b]	PPE[c]	Cat G[d]	ChyT$_\alpha$[e]	RMCP I[f]	RMCP II[g]	SGPA[h]	V-8[i]
isocoumarin	NI[j]	NI		NI				NI
3-chloroisocoumarin	3900	510	NI	330	84	85[k]	200	510
3,4-dichloroisocoumarin	8920[l]	2500	28	570	260	580[m]	310	2765
3-acetoxyisocoumarin	690[n]	190	NI	2600		460		
3,3-dichlorophthalide	>5000	>670	>800	>2000				
3-chlorophthalide	NI	NI	NI	NI				
3-fluorophthalide	NI	NI	NI	NI				

[a] Inactivation kinetics were performed by using the incubation method in 0.1 M HEPES, 0.5 M NaCl, pH 7.5, and 8–12% Me_2SO unless otherwise specified. Residual activity was measured as described under Materials and Methods. Units of k_{obsd}/[I] are M^{-1} s^{-1}. [b] Inhibitor concentrations were the following: isocoumarin, 0.3 mM; 3-CI, 0.013 mM; 3,3-dichlorophthalide, 0.009 mM; 3-chlorophthalide, 0.14 mM; 3-fluorophthalide, 0.24 mM. [c] Inhibitor concentrations were the following: isocoumarin, 0.3 mM; 3-CI, 0.005 mM; 3,4-DCI, 0.008 mM; 3-acetoxyisocoumarin, 0.16 mM; 3,3-dichlorophthalide, 0.069 mM; 3-chlorophthalide, 2.5 mM; 3-fluorophthalide, 0.24 mM. [d] Inhibitor concentrations were the following: 3-CI, 0.23 mM; 3,4-DCI, 0.05 mM; 3-acetoxyisocoumarin, 0.086 mM; 3,3-dichlorophthalide, 0.052 mM; 3-chlorophthalide, 0.05 mM; 3-fluorophthalide, 0.28 mM. [e] Inhibitor concentrations were the following: isocoumarin, 0.013 mM; 3-CI, 0.009 mM; 3,4-DCI, 0.009 mM; 3-acetoxyisocoumarin, 0.047 mM; 3,3-dichlorophthalide, 0.009 mM; 3-chlorophthalide, 2.6 mM; 3-fluorophthalide, 0.15 mM. [f] Inhibitor concentrations were the following: 3-CI, 0.11 mM; 3,4-DCI, 0.038 mM. [g] Concentration of 3-acetoxyisocoumarin was 0.17 mM. [h] Inhibitor concentrations were the following: 3-CI, 0.11 mM; 3,4-DCI, 0.14 mM. [i] Inhibitor concentrations were the following: isocoumarin, 0.13 mM; 3-CI, 0.055 mM; 3,4-DCI, 0.018 mM. Buffer was 0.1 M HEPES, pH 7.5, and 10% Me_2SO. [j] NI, no inactivation. [k] Progress curve method with 3-CI = 0.61 mM. [l] Progress curve method with 3,4-DCI = 1.1 μM. [m] Progress curve method with 3,4-DCI = 0.011 mM. [n] Progress curve method with 3-acetoxyisocoumarin = 0.008 mM.

as the substrate. Reaction rates of RMCP II (0.19 μM) with 3-CI (0.61 mM) and 3,4-DCI (0.011 mM) were measured in the presence of Suc-Val-Pro-Phe-NA (0.13 mM). Inhibition of bovine factor XI_a (3.6 nM) by 3,4-DCI (0.239 mM) was measured with Z-Arg-SBzl·HCl (0.05 mM) was a substrate.

Reactivation Kinetics. Enzymatic activity (measured as described above) was followed upon incubation of the inactivated enzyme at 25 °C under the conditions specified without removal of any residual inhibitor. Controls lost less than 10% activity over the time periods investigated. Reactivation half-lives were calculated from plots of percent activity vs. times. Deacylation rate constants of enzymes inactivated by 3-CI and 3,4-DCI were determined after dialysis of the inactivated enzymes against 0.1 M phosphate (pH 7.5) for 3 h at 5 °C from plots of ln (percent activity) vs. times. Deacylation rates of HL elastase and PP elastase inhibited by homophthalic anhydride (0.01 and 0.1 mM, respectively) were determined from the substrate hydrolysis progress curve measured upon dilution (100–200-fold) of inactivated enzymes into substrate solutions as described above. Plots of ln (v/v_0) vs. times, where v is the tangent of the substrate hydrolysis curve at time t and v_0 is the velocity in the absence of inhibitor, gave correlation coefficients of 0.98 or greater.

Determination of Spontaneous Hydrolysis Rates of Inhibitors in Buffer and in the Presence of Glutathione, Human Albumin, or Plasma. An aliquot of the isocoumarin derivative (0.05–0.15 mM final concentration) in Me_2SO was added to the appropriate buffered solution or plasma such that the final Me_2SO concentration was 10% v/v and the spontaneous hydrolysis monitored by following the decrease in absorbance at 325 nm. The hydrolysis product had negligible absorbance at 325 nm. First-order hydrolysis constants were obtained from a 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.

The spontaneous hydrolysis of 3-chlorophthalide and 3-fluorophthalide (29 and 66 μM, respectively) in buffer (10% Me_2SO) were measured by following the absorbance increase at 295 nm. The hydrolysis product, 2-carboxybenzaldehyde, has an extinction coefficient of 1800 M^{-1} cm^{-1} at 295 nm while the halo derivatives have negligible absorbance. The first-order hydrolysis rates were determined from plots of ln $[(A_\infty - A_0)/(A_\infty - A_t)]$ vs. time.

Determination of Enzyme-Catalyzed Rates of Inhibitor Ring Opening. Rates of enzyme-catalyzed ring opening of 3-CI and 3,4-DCI were measured spectrophotometrically as described above for hydrolysis in buffer. In a typical experiment, a 0.050-mL aliquot of inhibitor (0.069 mM final concentration) was added to 1.98 mL of a buffered enzyme solution (0.015 mM final concentration) and the decrease in absorbance at 325 nm recorded. Under these conditions, extinction coefficients of 3330 and 3500 M^{-1} cm^{-1} were obtained for 3,4-DCI and 3-CI, respectively. Ring opening rate constants were determined as described under Results. Enzymatic hydrolysis rates of 3-chlorophthalide and 3-fluorophthalide were measured similarly by following an increase in absorbance at 295 nm.

Proton Release Experiments. Active site titrated chymotrypsin A_γ (5.0 mL, 1.0 mM in 1 mM HCl) was added to a mixture of distilled water (13.0 mL) and Me_2SO (1.5 mL) and the pH of the solution adjusted to 7.5 with 0.1 N NaOH by using a pH stat. A 0.50-mL aliquot of inhibitor in Me_2SO was rapidly added (0.25–0.261 mM final concentration) and the release of protons at pH 7.5 measured with time by titration with 0.1 N NaOH utilizing an automatic buret (buret volume = 0.250 mL) and chart recorder (full scale = 0.250 mL). All measurements were carried out under a nitrogen atmosphere, and control experiments showed negligible uptake of CO_2 from the atmosphere during the time span of the experiments (by titration with 0.1 N NaOH). Residual enzymatic activity was measured by removing 1-μL aliquots from the reaction mixture and assaying with Suc-Val-Pro-Phe-NA as described above. Measurements at pH 8.5 were done similarly. All pH stat measurements were carried out on a Radiometer/Copenhagen automatic titration apparatus.

Results

Inactivation of Serine Proteases by Substituted Isocoumarins and 3,3-Dichlorophthalide. The structures of the new inhibitors investigated are shown in Figure 1. Incubation of 3-chloroisocoumarin (3-CI), 3,4-dichloroisocoumarin (3,4-DCI), 3-acetoxyisocoumarin, and 3,3-dichlorophthalide with a number of serine proteases resulted in a time-dependent and complete elimination of enzymatic activity (Table I). Unsubstituted isocoumarin did not inactivate several enzymes at concentrations as high as 0.3 mM. In addition, the hydrolysis product of 3-CI, homophthalic acid (1 mM), showed no inhibition of HL elastase, PP elastase, cathepsin G, or chymotrypsin A_α. In every case, 3,4-DCI gave higher k_{obsd}/[I] values (1.5–6.8 fold) than 3-CI, and in every case except

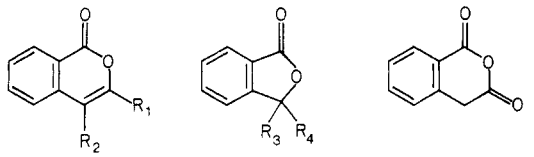

FIGURE 1: Structures of the new inhibitors investigated. The isocoumarins (left) include isocoumarin ($R_1 = R_2 = H$), 3-chloroisocoumarin (3-CI; $R_1 = Cl$, $R_2 = H$), 3,4-dichloroisocoumarin (3,4-DCI; $R_1 = R_2 = Cl$), and 3-acetoxyisocoumarin ($R_1 = OCOCH_3$, $R_2 = H$). The phthalides (center) include 3,3-dichlorophthalide ($R_3 = R_4 = Cl$), 3-chlorophthalide ($R_3 = Cl$, $R_4 = H$), and 3-fluorophthalide ($R_3 = F$, $R_4 = H$). The structure of homophthalic anhydride is shown on the right.

chymotrypsin A_α, 3,4-DCI was significantly more reactive than 3-acetoxyisocoumarin. Cathepsin G was the least reactive of the enzymes shown in Table I and only reacted with 3,4-DCI and 3,3-dichlorophthalide.

Inhibition of serine proteases by 3,3-dichlorophthalide was very rapid ($t_{1/2} < 0.25$ min) at the concentrations employed, and the $k_{obsd}/[I]$ values in Table I are based on approximate half-lives for inactivation. 3-Chlorophthalide and 3-fluorophthalide did not inhibit HL elastase, PP elastase, cathepsin G, or chymotrypsin A_α at concentrations as high as 0.24–2.6 mM. HL and PP elastase were, however, inhibited rapidly ($t_{1/2} < 0.5$ min) by homophthalic anhydride at inhibitor concentrations of 10 and 107 μM, respectively. 3,3,4-Trichloro-3,4-dihydroisocoumarin (0.011–0.040 mM) also inhibited HL elastase, cathepsin G, PP elastase, chymotrypsin A_α, and *S. aureus* protease V-8 rapidly ($t_{1/2} < 0.5$ min) but also decomposed to 3,4-DCI rapidly upon addition to buffer solution ($t_{1/2} = 1$ min) and was therefore difficult to analyze kinetically.

In most cases, inactivation rates were determined by using the incubation method (see Materials and Methods), and the first-order plots were always linear for more than four half-lives. When reaction rates were extremely fast or when small amounts of enzyme were available, $k_{obsd}/[I]$ values were obtained by the progress curve method (Tain & Tsou, 1982). In this method, substrate hydrolysis is monitored in the presence of an irreversible inhibitor. Reaction rates were calculated by using the expression

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

where $[P]_\infty$ and $[P]_t$ are the concentrations of product (4-nitroaniline) formed at infinite time (total inactivation) and at time t, respectively. Correlation coefficients for such plots were always greater than 0.994.

The reaction of HL elastase with 3,4-DCI (0.5–4.0 μM) was carried out by using the progress curve method, and from seven determinations, an average $k_{obsd}/[I]$ value of 9170 ± 800 M^{-1} s^{-1} was obtained. The finding that $k_{obsd}/[I]$ changed negligibly over this concentration range indicated that $K_I > 4$ μM. Estimation of $k_{obsd}/[I]$ for inactivation of HL elastase (1 μM) with a low excess of 3,4-DCI (10 μM) using the incubation method gave 7000 M^{-1} s^{-1} which is in reasonable agreement with the value obtained by using the progress curve method considering the inherent difficulties in measuring fast inactivation rates by the former method. The reaction of HL and PP elastase with 3-CI (7–35 μM) was also analyzed by using the progress curve method. We obtained K_I values of 55 μM and 65 μM, and k_2 values of 0.59 s^{-1} and 0.22 s^{-1}, respectively.

Inactivation of HL elastase (0.5 μM) by increasing concentrations of 3,4-DCI (0.38–4.0 μM) using the incubation method allowed estimation of the number of equivalents of inhibitor required for total inactivation. When I/E was 3.1, a residual activity of 3.7% was obtained while 10 equiv resulted in total inactivation (<0.5%). Therefore, between 3.1 and 10 equiv of 3,4-DCI is required for total inactivation of HL elastase. Similar studies on the inactivation of HL and PP elastase by 3-CI indicated that at least 15 and 4 equiv of inhibitor, respectively, is required for complete inactivation of these enzymes.

Table II: Inactivation of Proteases by 3,4-Dichloroisocoumarin[a]

enzyme	inhibitor concn (μM)	$t_{1/2}$ (min)	$k_{obsd}/[I]$ (M^{-1} s^{-1})
human leukocyte elastase[b]	1.1	1.2	8920
porcine pancreatic elastase	8.1	0.57	2500
human leukocyte cathepsin G	49.0	8.4	28
rat mast cell protease I	38.0	1.2	260
rat mast cell protease II[b]	11.0	1.8	580
human skin chymase	92.0	4.7	27
bovine chymotrypsin A_α	13.0	1.6	570
S. griseus protease A	136.0	0.3	310
subtilisin			substrate
human thrombin[c]	340.0	3.4	10
bovine thrombin	127.0	3.7	25
human plasmin	203.0	0.4	133
porcine pancreatic kallikrein[c]	127.0	3.4	27
bovine factor X_a[c]	422.0	133.0	0.2
bovine factor XI_a[d]	239.0	1.8	27
human factor XII_a[c]	135.0	1.3	64
human factor D	109.0	0.6	192
bovine trypsin	127.0	0.5	198
S. aureus protease V-8[e]	18.0	0.3	2765
acetylcholinesterase[f]	157.0	>120	<0.6
β-lactamase[f]	385.0		NI[g]
papain	422.0		NI
leucine aminopeptidase[e]	400.0		NI

[a] Inactivation measurements were performed by using the incubation method in 0.1 M HEPES, 0.5 M NaCl, and 8–10% Me_2SO, pH 7.5, unless otherwise noted, and the data were analyzed as described under Materials and Methods. [c] Incubation and assay buffer was 0.1 M HEPES, 5 mM $CaCl_2$, and 8–10% Me_2SO, pH 7.5. [d] Data obtained by using the progress curve method (0.1 M HEPES, 5 mM $CaCl_2$, and 10% Me_2SO, pH 7.5). [e] Buffer was 0.1 M HEPES and 10% Me_2SO, pH 7.5. [f] Buffer was 0.1 M phosphate and 10% Me_2SO, pH 7.0. [g] NI, no inactivation.

Inactivation rates were decreased dramatically when substrates or reversible inhibitors were included in the incubation mixture. The reaction of HL elastase with 3,4-DCI decreased 4-fold ($k_{obsd}/[I] = 2100$ M^{-1} s^{-1}) when MeO-Suc-Ala-Ala-Pro-Val-NA (0.69 mM) was included in the incubation mixture, and the inactivation rate of chymotrypsin A_α decreased 2.5-fold ($k_{obsd}/[I] = 230$ M^{-1} s^{-1}) when Suc-Val-Pro-Phe-NA (0.33 mM) was added to the incubation mixture. Inactivation of HL elastase by 3-CI in the presence of the reversible inhibitor 2-(heptafluoropropyl)-4*H*-3,1-benzoxazin-4-one (0.054 mM) (Teshima et al., 1982) gave a $k_{obsd}/[I]$ value of 8.6 M^{-1} s^{-1}. By use of equations reported previously (Kitz & Wilson, 1962), a $k_{obsd}/[I]$ value of 4300 M^{-1} s^{-1} for inactivation in the absence of the competitive inhibitor was calculated, which is in good agreement with the experimentally determined inactivation rate (3900 M^{-1} s^{-1}). Inclusion of CF_3CO-Lys-Ala-4-methylanilide (0.022 mM) (Renaud et al., 1983) in the incubation mixture containing PP elastase and 3-CI (5 μM) resulted in a 26-fold decrease in inactivation rate. These results indicate that inactivation occurs at the enzyme active site.

Since 3,4-dichloroisocoumarin was found to be quite reactive with a number of elastase-like and chymotrypsin-like serine proteases, we decided to try it with a wide variety of serine proteases including bovine blood coagulation factors X_a and XI_a, human blood coagulation factor XII_a and thrombin, porcine pancreatic kallikrein, and the human complement enzyme factor D. All the trypsin-like enzymes were inhibited but with a rather wide range of rate constants (Table II). The

Table III: Half-Lives for Reactivation of Serine Proteases Inactivated by Substituted Isocoumarins and 3,3-Dichlorophthalide[a]

	3-chloroisocoumarin		3,4-dichloroiso-coumarin		3-acetoxyisocoumarin		3,3-dichlorophthalide	
enzyme	[I] (μM)	$t_{1/2}$ (h)	[I] (μM)	$t_{1/2}$ (h)	[I] (μM)	$t_{1/2}$ (h)	[I] (μM)	$t_{1/2}$ (h)
HL elastase	30	20	40	>70[b]	40	>2.5[c]	10	1.3
PP elastase	200	20[d]	40	40	170	2.8	70	0.3
	8	8.3[e]	6	25[e]				
cathepsin G			40	30			50	1.0
chymotrypsin A_α	50	1.0	40	90	47	1.8	150	1.2
	8	6.4[e]	6	>25[e,f]				
S. griseus protease A	30	25	40	55				
RMCP II	50	>100[g,h]	50	>100[h,i]	170	3.8		
S. aureus protease V-8	55	15	18	>24[j]				
PP kallikrein			130	>20[k]				
trypsin			130	24				

[a] The half-lives for reactivation in the presence of residual inhibitor were determined as described under Materials and Methods. Conditions were 0.1 M HEPES, pH 7.5, 0.5 M NaCl, and 10% Me_2SO at 25 °C, unless otherwise indicated. [b] 20% activity at 70 h. [c] 14% activity at 2.5 h. [d] Buffer was 0.1 M HEPES, pH 7.0, 0.5 M NaCl, and 10% Me_2SO. [e] Buffer was 0.1 M phosphate, pH 7.5. [f] 25% activity at 25 h. [g] 10% activity at 100 h. [h] Buffer was 0.1 M phosphate, pH 7.3. [i] 5% activity at 100 h. [j] 15% activity at 24 h. [k] 40% activity at 20 h.

Table IV: Reactivation of Serine Proteases Inhibited by 3-Chloroisocoumarin and 3,4-Dichloroisocoumarin in the Presence of Hydroxylamine[a]

	3-chloroiso-coumarin		3,5-dichloroiso-coumarin	
enzyme	k_{react} ($\times 10^3$ s^{-1})	$t_{1/2}$ (min)	k_{react} ($\times 10^3$ s^{-1})	$t_{1/2}$ (min)
HL elastase	8	1.5	0.3	40
S. griseus protease A	0.5	22	0.1	120
chymotrypsin A_α	2	7	0.04	320[b]

[a] Hydroxylamine (in 0.1 M HEPES and 0.5 M NaCl, pH 7.5; final concentration 0.45–0.47 M) was added to 0.45 mL of inactivated enzyme (less than 5% activity) and the regain in enzymatic activity measured as described under Materials and Methods. [b] Estimated from the reactivation curve with 40% activity regained after 240 min.

Table V: Deacylation Rate Constants for Serine Proteases Inactivated by 3-Chloroisocoumarin and 3,4-Dichloroisocoumarin[a]

	3-chloroiso-coumarin		3,4-dichloroiso-coumarin	
enzyme	k_{deacyl} ($\times 10^3$ s^{-1})	$t_{1/2}$ (h)	k_{deacyl} ($\times 10^3$ s^{-1})	$t_{1/2}$ (h)
HL elastase	0.1	2.0	0.02	9.6
PP elastase	0.085	2.2	0.03	6.3
chymotrypsin A_α	0.2[b]	1.0	0.025	8.1
trypsin			0.02[c]	8.7

[a] Enzymes (0.4–1.5 M) were incubated with 3-CI (0.008–0.04 mM) or 3,4-DCI (0.006–0.019 mM) in phosphate buffer (pH 7.5) for 15–40 min and then dialyzed for 3 ± 0.5 h against 0.1 M phosphate buffer, pH 7.5 at 5 °C. Deacylation rate constants were determined as described under Materials and Methods. [b] Based on reactivation of chymotrypsin (0.25 mM) inactivated by 3-CI (0.25 mM). [c] Inhibitor concentration was 0.04 mM.

most reactive trypsin-like enzyme was bovine trypsin while the least reactive was bovine factor X_a. The trypsin-like enzymes, excluding factor X_a, were 45–890-fold less reactive toward 3,4-DCI than HL elastase.

The metalloprotease leucine aminopeptidase, the cysteine protease papain, and β-lactamase (type II) were not inhibited by 3,4-DCI at inhibitor concentrations of 0.385 mM or greater after incubation for at least 1 h. Acetylcholinesterase was inhibited very poorly. 3,4-DCI is a substrate for subtilisin, and a k_{cat}/K_M of 50 M^{-1} s^{-1} was obtained from initial hydrolysis rates (measured as described subsequently) with 3,4-DCI concentrations of 0.15 mM or less.

Reactivation of Serine Proteases Inhibited by Substituted Isocoumarins and 3,3-Dichlorophthalide. The half-lives for reactivation of serine proteases inactivated by substituted isocoumarins and 3,3-dichlorophthalide obtained without removal of residual inhibitor are given in Table III. In most cases, enzymes inactivated by 3-CI and 3,4-DCI regained activity much more slowly than those inactivated by 3-acetoxyisocoumarin and 3,3-dichlorophthalide under the conditions used. RMCP II gave the longest reactivation half-lives with both 3-CI and 3,4-DCI. In addition, HL and PP elastase, which had been inactivated by homophthalic anhydride under conditions specified above, regained activity quite rapidly and had first-order deacylation rate constants of 3×10^{-3} s^{-1} ($t_{1/2}$ = 3.9 min) and 2.5×10^{-3} s^{-1} ($t_{1/2}$ = 4.6 min), respectively.

Addition of buffered hydroxylamine (0.5 M final concentration) to inhibited enzyme resulted in a rapid and complete regain in enzymatic activity (Table IV). With all enzymes tested, reactivation rates were higher with 3-CI than with 3,4-DCI. While addition of buffered hydrazine (0.1–0.2 M) to the incubation mixture also resulted in reactivation of enzymatic activity, the rates were much slower than with hydroxylamine; its use was therefore discontinued.

The rate constants for deacylation of several enzymes inactivated by 3-CI and 3,4-DCI are shown in Table V. After the dialysis period, specific activities of 1.3, 3.9, 1, and 10% were determined with HL elastase, PP elastase, chymotrypsin A_α, and trypsin, respectively. Deacylation of enzymes inactivated by 3,4-DCI were 2.5–10-fold slower than with 3-CI. The finding that the 3,4-DCI forms an enzyme–inhibitor complex which is more stable toward deacylation than the enzyme complex formed with 3-CI is consistent with the trends observed with hydroxylamine reactivation.

Ultraviolet Spectral Changes. In order to gain a clearer understanding of the mechanism of inactivation, we investigated the ultraviolet spectral changes that occur upon inactivation of chymotrypsin A_γ by 3-CI and 3,4-DCI. As shown in Figure 2 (curve a), 3,4-DCI has a long wavelength absorbance band near 325 nm (ϵ_{325} = 3330 M^{-1} cm^{-1}; 0.1 M HEPES, 0.5 M NaCl, and 10% Me_2SO, pH 7.5) which is associated with the isocoumarin ring system. The hydrolysis product, α-chlorohomophthalic acid, has negligible absorbance at this wavelength and a substantially decreased absorbance near 280 nm (Figure 2, curve c).

Addition of 3,4-DCI (25 μM final concentration) to a buffered chymotrypsin A_γ solution (18 μM) resulted in an ultraviolet spectrum, after 2 min (<2% enzymatic activity), which was distinctly different from that of inhibitor alone (Figure 2, curve b). The chromophore at 325 nm was almost totally absent, and the absorbance near 280 nm was about 30% of inhibitor alone. Addition of excess chymotrypsin A_γ (13.4

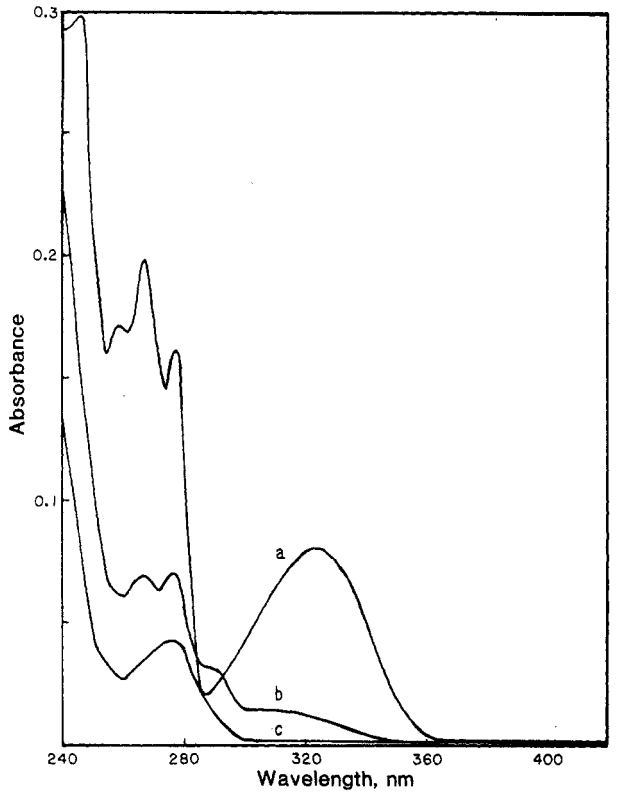

FIGURE 2: Ultraviolet spectrum of 3,4-dichloroisocoumarin in the presence and absence of chymotrypsin A_γ. The isocoumarin 3,4-DCI (0.025 mM) (curve a), its spontaneous hydrolysis product (substituted homophthalic acid, curve c) in 0.1 M HEPES, 0.5 M NaCl, pH 7.5, and 10% Me_2SO, and 3,4-DCI (0.025 mM) in the presence of chymotrypsin A_γ (curve b). Spectrum in the presence of enzyme (0.018 mM) was recorded 2.0 min after addition of inhibitor to an enzyme solution. Absorbance due to enzyme alone is subtracted from the final spectrum.

μM) to 3,4-dichloroisocoumarin (9 μM) gave a spectra after 1.8 min (45% activity) which was indistinguishable from that of enzyme alone. Thus, no new chromophores were observed upon inactivation. Reaction of the active site serine with 3,4-DCI in a 1,6-conjugate addition would have given the same chromophore present in 3-ethoxy-4-chloroisocoumarin which has an extinction coefficient of 2920 M^{-1} cm^{-1} at 350 nm under these conditions. Similar results (not shown) were obtained with 3-CI and chymotrypsin A_γ.

Chymotrypsin-Catalyzed Rates of Ring Opening of Isocoumarin and Phthalide Derivatives. The decrease in absorbance at 325 nm was used to measure the enzymatic ring opening rates of 3-CI and 3,4-DCI. As shown in Figure 3 (curve b), addition of 3,4-DCI (0.069 mM final concentration) to chymotrypsin A_γ (14.9 μM) resulted in a rapid (<60 s) decrease in absorbance at 325 nm, which was followed by a slow decrease in absorbance (ΔA/min = 2.5×10^{-3} min^{-1}). The slow decrease is exactly the same as that of the inhibitor alone (Figure 3, curve a). After 1.05 min, less than 1% residual enzymatic activity was detected. Extrapolation of the slower absorbance decrease to zero time allowed the determination of the number of equivalents of inhibitor reacted per inactivation. Three duplicate determinations gave an average value of 1.20 ± 0.05 3,4-DCI's reacted per inactivation. A plot of ln $(A_0 - A_\infty)/(A_t - A_\infty)$ vs. time, where A_∞ is the absorbance value at total inactivation, gave k_{obsd} which in this case was the rate of enzymatic ring opening. Such a plot gave a straight line with a correlation coefficient of 0.997 and a k_{obsd} of 0.048 ± 0.001 s^{-1}. Division of this rate constant by the inhibitor concentration and 1.2 3,4-DCI's reacted per inactivation gave a $k_{obsd}/[I]$ value of 580 M^{-1} s^{-1} which is in excellent agreement with the value obtained by the incubation method (570 M^{-1} s^{-1}).

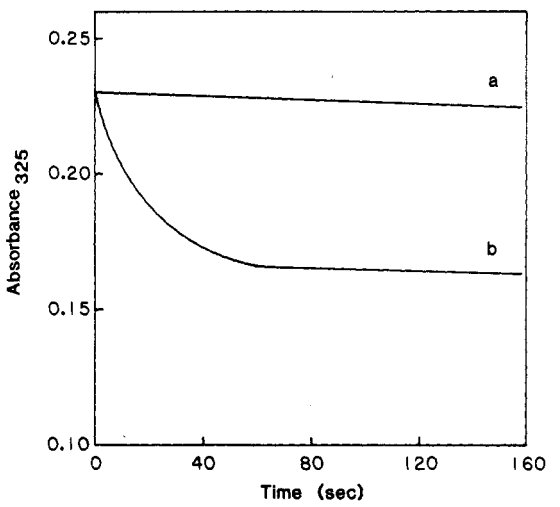

FIGURE 3: Chymotrypsin-catalyzed ring opening of 3,4-dichloroisocoumarin. 3,4-DCI (0.069 mM) was rapidly added to a solution of chymotrypsin A_γ (0.015 mM) in 0.1 M HEPES, 0.5 M NaCl, pH 7.5, and 10% Me_2SO and the decrease in absorbance at 325 nm measured with time (curve b). The decrease in absorbance of 3,4-DCI (0.069 mM) at 325 nm was also measured in the absence of enzyme (curve a).

Reaction of 3-CI (0.060 mM) with chymotrypsin A_γ under similar conditions gave 1.25 ± 0.05 3-CI's reacted per inactivation and a $k_{obsd}/[I]$ value of 290 M^{-1} s^{-1}, which is in reasonable agreement with that obtained by the incubation method (330 M^{-1} s^{-1}).

Interestingly, chymotrypsin A_γ (0.030 mM) did not enhance the decomposition of isocoumarin (0.19 mM) as measured by a decrease in absorbance at 325 nm. This indicates that unsubstituted isocoumarin is at best an extremely poor substrate for chymotrypsin and is probably not hydrolyzed at all.

The chymotrypsin A_γ catalyzed hydrolysis of 3-chlorophthalide and 3-fluorophthalide was investigated by monitoring an absorbance increase at 295 nm. The hydrolysis product, 3-carboxybenzaldehyde, has an extinction coefficient of 1800 M^{-1} cm^{-1} at 295 while the 3-halophthalide derivatives have negligible absorbance. The first-order rate constants, k_{obsd}, for hydrolysis of 3-chlorophthalide (0.029 mM) increased from 1.1×10^{-3} to 2.7×10^{-3} s^{-1} in the presence of 27 μM chymotrypsin A_γ while k_{obsd} for the hydrolysis of 3-fluorophthalide (0.066 mM) increased from 1.2×10^{-4} to 1.7×10^{-3} s^{-1} under similar conditions. These results indicate that the 3-halophthalide ring system is a perfectly adequate substrate for chymotrypsin A_γ.

Proton Release Experiments. The mechanism of inactivation was also investigated by determining the number of equivalents of protons released upon inactivation. As shown in Figure 4A, the addition of 3-CI (0.25 mM) to an aqueous solution of chymotrypsin A_γ (0.25 mM) in a pH stat at pH 7.5 resulted in a rapid release of 0.92 equiv of protons after 6 min, at which time the enzymatic activity was found to be 9.8% (Figure 4B). This rapid release of protons was followed by a gradual release of a total of 3.1 equiv of protons (theoretical 3.0), which was paralleled by a regain in enzymatic activity (Figure 4B). At pH 8.5 under similar conditions, 0.95 proton was released within 3 min, and the additional protons released slowly (results not shown). Again, the enzymatic activity paralleled proton release.

The reaction of 3,4-DCI (0.26 mM) with chymotrypsin A_γ (0.25 mM) was also investigated at pH 7.5 by utilizing a pH

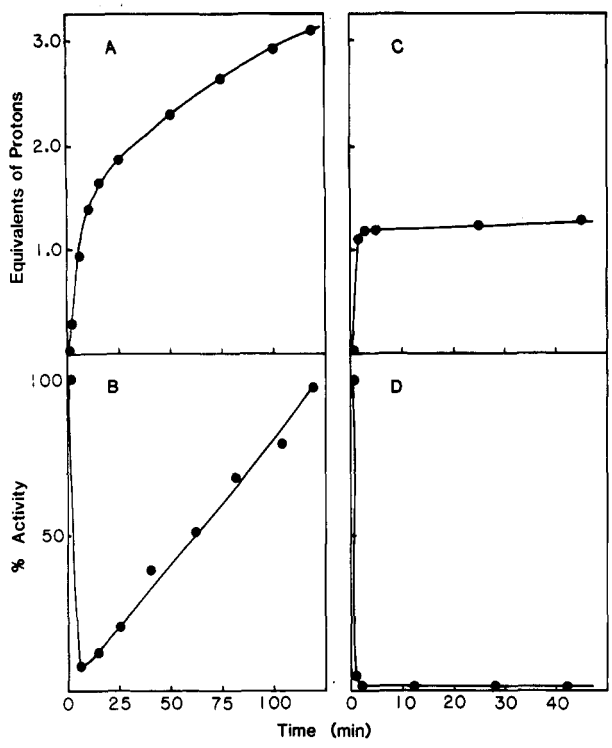

FIGURE 4: Proton release during inactivation of chymotrypsin A_γ by 3-chloroisocoumarin and 3,4-dichloroisocoumarin. (A) A reaction mixture containing chymotrypsin A_γ (0.25 mM) and 3-CI (0.25 mM) in aqueous solution (10% Me_2SO) was titrated with 0.1 N NaOH at a constant pH of 7.5. (B) Residual enzymatic activity was measured with Suc-Val-Pro-Phe-NA (0.125 mM). (C) Titration of a reaction mixture containing chymotrypsin A_γ (0.25 mM) and 3,4-DCI (0.26 mM) at pH 7.5. (D) Residual enzymatic activity measured as in (B).

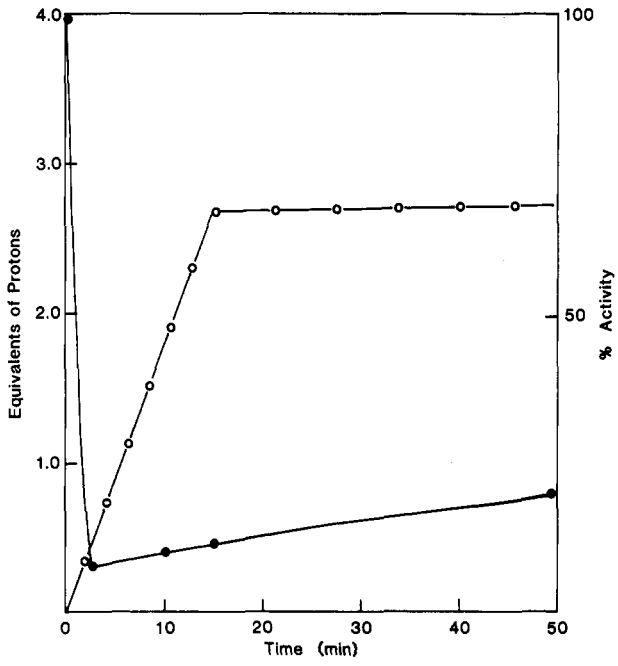

FIGURE 5: Proton release during inactivation of chymotrypsin by 3,3-dichlorophthalide. Titration of reaction mixture containing chymotrypsin A_γ (0.25 mM) and 3,3-dichlorophthalide (0.25 mM) at pH 7.5 with 0.1 N NaOH (open circles). Residual enzymatic activity (measured as in Figure 4B) (closed circles).

stat. A rapid release (<3 min) of 1.2 equiv of protons was observed, which was followed by a gradual release of 0.15 equiv over a 40-min period (Figure 4C). Enzymatic activity was found to be less than 1% at 2, 40, and 60 min (Figure 4D).

The reaction of chymotrypsin A_γ (0.25 mM) with 3,3-dichlorophthalide (0.25 mM) at pH 7.5 resulted in a rapid release of 2.7 equiv of protons after 15 min at which time the residual enzymatic activity was 8% (Figure 5).

Table VI: Half-Lives for the Spontaneous Hydrolysis of Isocoumarin Derivatives in Buffer and in the Presence of Albumin and Glutathione[a]

		$t_{1/2}$ (min)			
compound	$\nu_{C=O}$[b]	HEPES[c]	phosphate[d]	albumin[e]	glutathione[f]
isocoumarin	1730	1200			
3-chloroisocoumarin	1770	140	360	20	1.5
3,4-dichloroisocoumarin	1785	18	48	10	1.0
3-acetoxyisocoumarin	1750	30			

[a] Hydrolysis rates were measured by following the decrease in absorbance at 325 nm as described under Materials and Methods. [b] IR spectra were recorded in methylene chloride. [c] Conditions were 0.1 M HEPES, 0.5 M NaCl, and 10% Me_2SO, pH 7.5. [d] Conditions were 0.02 M K_2HPO_4, 0.15 M NaCl, and 10% Me_2SO, pH 7.4. [e] Conditions were 0.4 mg/mL human albumin, 0.1 M HEPES, 0.5 M NaCl, and 10% Me_2SO, pH 7.5. [f] Conditions were 0.2 mM glutathione, 0.1 M HEPES, 0.5 M NaCl, and 10% Me_2SO, pH 7.5.

In all cases the inactivation rates were much faster than rates of proton release. When protons are rapidly released, the pH stat lags, and therefore, we have not attempted to correlate initial proton release rates to inactivation rates. These proton release experiments do, however, allow estimates of the number of protons released during the inactivation process. Due to the large amounts of enzyme required for these experiments, we were able to carry them out only with chymotrypsin A_γ. The longer lag phase observed wtih 3,3-dichlorophthalide is partially due to the particular instrumental conditions chosen for that experiment but may reflect other steps involved in the inhibition mechanism.

Spontaneous Hydrolysis Rates of Isocoumarin Derivatives. As mentioned earlier, the hydrolysis of isocoumarins are conveniently measured spectrophotometrically by following the decrease in absorbance at 325 nm. We investigated the hydrolysis rates of several inhibitors under a variety of conditions (Table VI). The most stable isocoumarin derivative was found to be isocoumarin itself and had a half-life of greater than 20 h in HEPES buffer, pH 7.5. Both 3,4-DCI and 3-acetoxyisocoumarin were relatively unstable to spontaneous hydrolysis in HEPES buffer at pH 7.5 and had half-lives of 18 and 30 min, respectively. 3-CI was intermediate in stability. The composition of the buffer had a significant effect on the hydrolysis rates of 3-CI and 3,4-DCI. In phosphate-buffered saline, pH 7.4, the half-lives were (2.6 ± 0.1)-fold higher than in HEPES buffer. Glutathione (0.2 mM) and human albumin (0.4 mg/mL) were also found to increase inhibitor decomposition rates significantly (Table V). The decomposition of 3-CI in human plasma was found to be extremely rapid, and the inhibitor had a half-life of 1.8 min. The finding that 3-CI decomposes 11 times faster in plasma than in albumin (0.4 mg/mL) is consistent with previous studies (Lawson et al., 1982) where it was shown that several thrombin-acylating agents are rapidly destroyed in plasma, primarily because of the high levels of albumin present (30–45 mg/mL). The decomposition rates of 3-CI and 3,4-DCI were too rapid to measure accurately with physiological concentrations of albumin. Rapid decomposition of 3,4-DCI ($t_{1/2}$ < 10 s) also occurred upon addition to buffer containing 5 mM cysteine (50 mM Tris and 2 mM EDTA, pH 8.2). The half-lives for spontaneous hydrolysis correlate well with the infrared carbonyl-stretching frequencies; the higher the frequency, the faster the spontaneous hydrolysis rate.

Discussion

A number of heterocyclic inhibitors have been reported for serine proteases. Substituted 2-benzoxazinones and quinazolinones have been shown to be extremely potent inhibitors of HL elastase, cathepsin G, and chymotrypsin, and these structures inhibit by interacting with the enzyme active site serine residue (Teshima et al., 1982; Hedstrom et al., 1984). N-substituted benzisothiazolinone 1,1-dioxides and benzisothiazolinones are potent inhibitors of several serine proteases including HL elastase, cathepsin G, and chymotrypsin (Zimmerman et al., 1980; Ashe et al., 1981). These compounds inhibit by forming stable acyl enzymes with the active site serine residue, and the inhibitor reactivity is dependent upon the electronegativitiy of the *N*-acyl or *N*-aryl substituent. Isatoic anhydride and substituted 3*H*-1,3-oxazine-2,6-diones have been shown to react with serine proteases to give a stable acyl enzyme, and this stability was attributed to the electron-donating capability of an unmasked amino group in the inhibitor structure (Moorman & Abeles, 1982; Weidmann & Abeles, 1984). More recently, substituted 6-chloro-2-pyrones have been shown to acylate chymotrypsin and release a reactive acid chloride moiety which could react with another active site nucleophile, but further acylation by this acid chloride has not yet been proven (Westkaemper & Abeles, 1983).

The finding that heterocyclic structures with fused aromatic ring systems such as substituted benzoxazinones are potent inhibitors of HL elastase and cathepsin G indicated that substituted isocoumarins might act as mechanism-based inhibitors of these enzymes. It was envisioned that enzymatic hydrolysis of the lactone of 3-chloroisocoumarin or 3,4-dichloroisocoumarin would lead to the formation of a reactive acid chloride (or ketene) functionality, which could acylate an active site nucleophile to give an inactivated enzyme. The results of our investigation indicate that inactivation of serine proteases by 3-CI and 3,4-DCI is indeed a mechanism-based process and is consistent with the proposed mechanism.

Mechanism of Inactivation. Enzyme inactivation is a time-dependent process, and the finding that the rate of inactivation of HL elastase, PP elastase, and chymotrypsin A_α by 3-CI and 3,4-DCI is decreased significantly when reversible competitive inhibitors or substrates are included in the incubation mixture indicates that inhibition occurs at the enzyme active site. Spectrophotometric measurements at 325 nm utilizing chymotrypsin A_γ indicate that enzymatic opening of the isocoumarin ring system occurs during the inactivation process, and the rate of loss of the inhibitor chromophore is similar to the rate of inactivation. The fact that enzymatic activity is regained slowly upon standing, after dialysis, or more rapidly in the presence of 0.5 M hydroxylamine indicates that labile acyl moieties are present in the inhibited enzyme. In the case of chymotrypsin, the inactivation is nearly stoichiometric, and approximately 1.2 equiv of 3-CI and 3,4-DCI is reacted per inactivation. With HL elastase, inactivation is not as efficient as it is with chymotrypsin, and at least 15 and 3.1 turnovers per inactivation are required for complete inhibition with 3-CI and 3,4-DCI, respectively. Inactivation of chymotrypsin A_γ results in the release of 0.92 and 0.95 equiv. of protons with the 3-CI at pH 7.5 and 8.5, respectively, and 1.25 equiv with 3,4-DCI at pH 7.5.

These results are consistent with the mechanism shown in Figure 6. After formation of a reversible enzyme–inhibitor complex, the active site serine reacts with the carbonyl of the inhibitor to give the acyl enzyme (**1**) in which a reactive acid chloride (or ketene) has been released. It is this enzyme-catalyzed ring opening process that results in loss of the

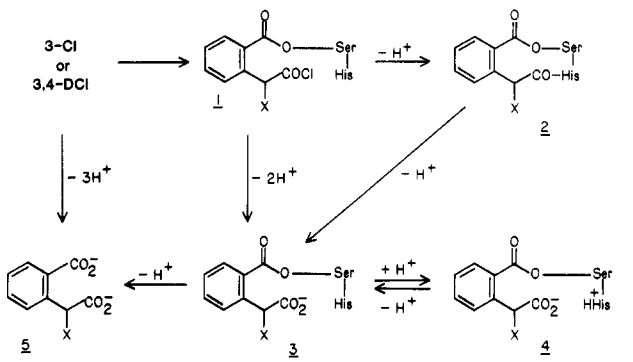

FIGURE 6: Mechanism of inactivation of serine proteases by 3-chloroisocoumarin derivatives (X = H, Cl).

chromophore at 325 nm. The acid chloride (**1**) can then follow one of two reaction pathways: (1) it can react with an active site nucleophile such as histidine-57 (using chymotrypsin numbering system) to give diacylated species **2** and release 1 equiv of protons, or (2) it can be hydrolyzed by water to give **3** and release 2 equiv of protons. While methionine-192 in chymotrypsin and glutamine-192 in PP elastase are potentially available for reaction with the unmasked acid chloride (**1**), the most likely nucleophile in the active site of most serine proteases is histidine-57. The observation that about 1 equiv of protons is released upon inactivation of chymotrypsin by 3-CI and 3,4-DCI rules out structure **3** as a possibility and indicates that structure **2** is responsible for inactivation of serine proteases by 3-CI and 3,4-DCI.

The pH stat measurements at pH 7.5 do not rule out the possibility of **4** being responsible for inactivation since its formation would result in the net release of 1 equiv of protons. Structure **4** could be formed if the inhibitor carboxylate caused a significant increase in the pK_a of the histidine side chain, such that a stable salt link between the inhibitor carboxylate and the imidazolium ion was possible. An example of such an interaction is the hydrogen bond formed between the carboxylate of (4-amidinophenyl)pyruvic acid and the NH of His-57 of trypsin which has been observed in the crystal structure of the (4-amidinophenyl)pyruvic acid–trypsin complex (Walter & Bode, 1983). The finding that 0.95 equiv of protons is released at pH 8.5 argues against **4** being responsible for inhibition since one would expect that, at this pH, the equilibrium would shift toward **3** and additional protons would be released. Structure **4** cannot be completely ruled out by pH stat studies at pH 8.5 alone, since some tetrahedral intermediates of chymotrypsin and trypsin appear to have histidine-57 pK_a values as high as 9.5 (Robillard & Shulman, 1974a,b; Kossiakoff & Spencer, 1981). The finding that homophthalic anhydride inhibits HL and PP elastase but deacylates very rapidly argues against structure **4** being responsible for inhibition of serine proteases by 3-CI and 3,4-DCI. However, additional spectroscopic and/or crystallographic experiments will be required to definitely distinguish between structures **2** and **4**, and such studies are currently in progress. The formation of **3** is probably responsible for the small amount of turnover observed with PP elastase and HL elastase, since this acyl enzyme could deacylate rapidly to give active enzyme. Complete hydrolysis of the diacylated product would result in the total release of 3 protons to give **5** and totally active enzyme (Figure 4A,B). The finding that the rate of proton release (after 6 min) of chymotrypsin inhibited by 3-CI is not perfectly synchronous with the rate of enzyme reactivation (see Figure 4A,B) indicates that one of the acyl bonds is hydrolyzed faster than the other, but the resulting monoacylated enzyme remains catalytically inactive. We were

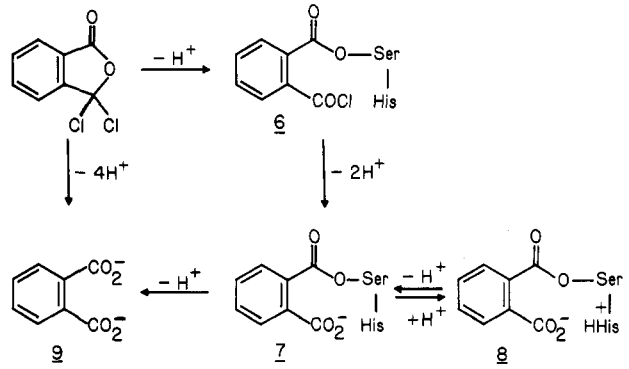

FIGURE 7: Mechanism of inactivation of serine proteases by 3,3-dichlorophthalide.

unable to differentiate between initial acyl histidine hydrolysis and acyl serine hydrolysis.

An alternate mechanism for inactivation by 3-Cl and 3,4-DCI can be envisioned in which the active site serine residue attacks the C-3 position of the inhibitor in a 1,6-conjugate addition, and this adduct decomposes to give a vinyl serine derivative. This inactivation mechanism can be ruled out on the basis of the loss of the isocoumarin ring chromophore at 325 nm and the absence of any new long wavelength chromophores upon inactivation. However, we cannot rule out the possibility that the histidine rapidly attacks the lactone moiety of such a 3-alkoxy-4-chloroisocoumarin derivative to give a diacylated enzyme isomeric with the one we propose. As reported earlier, 5-benzyl-6-chloro-2-pyrone reacts with the active site serine of chymotrypsin in a 1,6-conjugate addition, and a chromophore at 320 nm is retained in the inhibited enzyme (Westkaemper & Abeles, 1983).

The proposed mechanism for inactivation of serine proteases by 3,3-dichlorophthalide is shown in Figure 7. Initially, the active site serine residue reacts with the lactone carbonyl of the inhibitor to give the acyl acid chloride **6** and release of 1 equiv of protons. The acid chloride in structure **6** is then hydrolyzed, releasing two additional equivalents of protons, to give **7**. The apparent lag in proton release observed with 3,3-dichlorophthalide (Figure 5) is partially due to the inability of the pH state to keep up with the proton release under the particular experimental conditions chosen for the dichlorophthalide experiment. However, the lag may also indicate formation of a transiently stable E·I complex prior to acylation and/or a slow rate of hydrolysis of the unmasked acid chloride in **6**. The finding that a total of 2.7 equiv of protons was released upon inactivation of chymotrypsin A_γ by 3,3-dichlorophthalide indicates that **7** is responsible for inhibition since its formation would result in the release of a total of 3 equiv of protons. Structure **8** is ruled out since its formation would result in the net release of 2 equiv of protons at pH 7.5. Deacylation of **7** would give **9** and totally active enzyme. The carboxyl group released upon reaction of a serine protease with 3,3-dichlorophthalide is apparently important for inactivation since compounds such as 3-chloro- and 3-fluorophthalide containing a masked aldehyde instead of a carboxylate react with serine proteases but do not inhibit. The observation that **8** is not responsible for inhibition of chymotrypsin by 3,3-dichlorophthalide is also further evidence against structure **4** being responsible for inactivation of serine proteases by 3-Cl and 3,4-DCI. Furthermore, the acyl enzymes formed from the 3-chloroisocoumarins are generally more resistant to hydrolysis than those formed from 3,3-dichlorophthalide which is also consistent with one additional covalent bond being formed between the enzymes and the chloroisocoumarins. It is interesting to note that the distance from the acyl serine moiety to the acid chloride (see structures **1** and **6**) is apparently critical for the formation of a diacylated product (**2**). 3-Chloroisocoumarin derivatives, which have one more methylene than does 3,3-dichlorophthalide, apparently react to give diacylated products while the monoacyl structure (**7**) is formed with the phthalide derivative.

The finding that enzymes inhibited by 3-acetoxyisocoumarin reactivate rapidly indicates that a monoacyl enzyme is formed upon inactivation. Monoacylation could result if the unmasked mixed anhydride, formed upon enzymatic ring opening, either was sterically hindered toward reaction with an active site nucleophile or was hydrolyzed rapidly to form structure **3** in Figure 6. Alternatively, 3-acetoxyisocoumarin may simply acetylate the active site serine, releasing homophthalic anhydride or the corresponding diacid.

Specificity and Reactivity. Both 3-chloroisocoumarin and 3,4-dichloroisocoumarin are fairly specific toward HL elastase and are [(4–45) × 10^3]-fold more reactive with this enzyme than with PP elastase and a number of chymotrypsin-like and trypsin-like serine proteases (Tables I and II). Little or no specificity was achieved with the chymotrypsin-like enzymes investigated here, except with cathepsin G and human skin chymase which were 10–20-fold less reactive with 3,4-DCI than the other chymotrypsin-like enzymes tested. With the trypsin-like enzymes investigated, however, considerable specificity was observed and the $k_{obsd}/[I]$ values ranged from 198 M^{-1} s^{-1} with bovine trypsin to 0.2 M^{-1} s^{-1} with bovine factor X_a (factor X_a is also quite unreactive toward DFP ($k_{obsd}/[I]$ = ~0.05 M^{-1} s^{-1}) and phenylmethanesulfonyl fluoride (Fujikawa et al., 1972)]. Human β-factor XII_a was 2-fold more reactive than bovine thrombin, PP kallikrein, and bovine factor XI_a, all of which had $k_{obsd}/[I]$ values near 27 M^{-1} s^{-1}. 3,4-Dichloroisocoumarin appears to be quite specific for serine proteases since it did not inactivate the cysteine protease papain, the metalloprotease leucine aminopeptidase, or β-lactamase and inhibited acetylcholinesterase quite poorly.

3,4-Dichloroisocoumarin is 1.5–7 times more reactive than 3-chloroisocoumarin toward a number of serine proteases (Table I). This increased reactivity can be attributed to the substitution of chlorine in the 4-position of the isocoumarin ring, which results in a more electronegative ring system and a more reactive lactone moiety. This increase in inherent reactivity is clearly shown by the spontaneous hydrolysis rates given in Table VI. The spontaneous hydrolysis rate of 3,4-DCI is 7.6 times faster than that of the 3-chloro derivative.

The reactivity of the inhibitors reported here toward several of the enzymes tested compares favorably with other active site directed irreversible and mechanism-based irreversible inhibitors of these serine proteases. The most reactive peptide chloromethyl ketone (MeO-Suc-Ala-Ala-Pro-ValCH$_2$Cl, $k_{obsd}/[I]$ = 1560 M^{-1} s^{-1}) (Powers et al., 1977) and sulfonyl fluoride [2-[(pentafluoropropionyl)amino]benzenesulfonyl fluoride $k_{obsd}/[I]$ = 1700 M^{-1} s^{-1}] (Yoshimura et al., 1982) inhibitors for HL elastase are 5–6-fold less reactive toward this enzyme than is 3,4-DCI. The most reactive cathepsin G inhibitor reported previously, Z-Gly-Leu-PheCH$_2$Cl ($k_{obsd}/[I]$ = 51 M^{-1} s^{-1}) (Powers et al., 1977), is only 1.9 times more reactive than 3,4-DCI. One of the most reactive mechanism-based PP elastase inhibitors is 3-methyl-6-chloro-2-pyrone ($k_{obsd}/[I]$ = 26 M^{-1} s^{-1}) (Westkaemper & Abeles, 1983), which is 96-fold less reactive than 3,4-DCI. The inhibitor 3,4-dihydro-6-chloromethylcoumarin is also fairly reactive toward PP elastase ($t_{1/2}$ < 1 min; [I] = 0.195 mM) (Bechet et al., 1977a,b). Dichloroisocoumarin is 516-fold less reactive

than the most reactive mechanism-based chymotrypsin inhibitor yet reported, (*E*)-3-(1-naphthyl)-6-(iodomethylene)-tetrahydropyran-2-one (Daniels et al., 1983), but is 735-fold more reactive than 3-benzyl-6-chloro-2-pyrone (Westkaemper & Abeles, 1983), a compound with a masked reactive functionality and mechanism of inactivation which may be similar to that of 3,4-DCI.

The 4-chloro group has a significant effect on deacylation rates. In most cases, enzymes inhibited by 3,4-DCI had k_{deacyl} values 2–10-fold lower than with enzymes inactivated by 3-CI. This is probably a steric effect since modeling the binding of the 3-chloroisocoumarins to the active site of PP elastase indicates that a 4 substituent may hinder nucleophilic attack on the acyl carbonyl groups. Thus, it is apparent that substituents such as chlorine not only affect acylation but also affect deacylation of the inhibited enzyme.

3,4-Dichloroisocoumarin as a General Serine Protease Inhibitor. We have discovered that 3,4-DCI is an extremely potent general serine protease inhibitor and could prove to be very useful in experiments such as protein purification and generation of monoclonal antibodies when proteolytic degradation can be a significant problem. 3,4-DCI has several advantages over other commonly used general serine protease inhibitors. It is much easier to handle than the highly toxic reagent DFP and is generally much more reactive toward a number of serine proteases than the commonly used phenylmethanesulfonyl fluoride (Lively & Powers, 1978; James, 1978). For example, 3,4-DCI is 926- and 425-fold more reactive toward HL elastase and PP elastase, respectively, than is phenylmethanesulfonyl fluoride. Unlike phenylmethanesulfonyl fluoride, 3,4-DCI does not react with β-lactamase (Shvyadas et al., 1977), which is often used as a marker enzyme in a variety genetic engineering procedures. This compound has the advantage of being reversible either upon long standing or in the presence of hydroxylamine and, therefore, could also be used as a reversible active site blocking group for serine proteases. The slow deacylation rates coupled with inhibitor stability results in long half-lives for reactivation of enzyme activity in the presence of excess inhibitor. Thus, enzyme activity can be controlled over long periods of time by choosing the appropriate inhibitor concentration.

Summary. 3-Chloroisocoumarin and 3,4-dichloroisocoumarin have been found to be potent mechanism-based inhibitors of a variety of serine proteases and are highly reactive toward HL elastase, the enzyme that is primarily responsible for the degradation of elastin which occurs in emphysema (Powers, 1983). The isocoumarins 3-CI and 3,4-DCI are some of the first mechanism-based inhibitors reported for several of the enzymes studied including HL elastase and cathepsin G, the blood coagulation proteases bovine factor XI_a, human thrombin, β-factor XII_a, plasmin, the complement protease factor D, and several chymotrypsin-like proteases of mast cell origin. Dichloroisocoumarin (3,4-DCI) should have numerous applications as a general serine protease inhibitor and could be used in the elucidation of the physiological roles of serine proteases. While this compound is readily synthesizable, it has the disadvantage of being unstable in the presence of albumin and glutathione. The results reported here should prove valuable in the further design of therapeutically useful serine protease inhibitors.

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Registry No. HLE, 9004-06-2; Cat G, 56645-49-9; RMCP I, 82599-73-3; RMCP II, 82599-74-4; ChyT, 9004-07-3; SGPA, 55326-50-6; V-8, 66676-43-5; 3-CI, 51050-54-5; 3,4-DCI, 51050-59-0; 3-acetoxyisocoumarin, 31913-57-2; 3,3-dichlorophthalide, 601-70-7; human skin chymase, 75496-62-7; subtilisin, 9014-01-1; porcine pancreatic kallikrein, 9001-01-8; bovine factor X_a, 9002-05-5; bovine factor XI_a, 37203-61-5; bovine trypsin, 9002-07-7; acetylcholinesterase, 9000-81-1; thrombin, 9002-04-4; plasmin, 9001-90-5; human factor II_a, 37203-62-6; human factor D, 37213-56-2; 3-fluorophthalide, 95249-86-8; 2-carboxybenzaldehyde, 119-67-5; (diethylamino)sulfur trifluoride, 38078-09-0; serine proteinase, 37259-58-8.

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Inhibition of Human Leukocyte Elastase, Cathepsin G, Chymotrypsin A_α, and Porcine Pancreatic Elastase with Substituted Isobenzofuranones and Benzopyrandiones†

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ABSTRACT: Several 3-halo-3-(1-haloalkyl)-1(3*H*)-isobenzofuranones, 3-(1-haloalkylidene)-1(3*H*)-isobenzofuranones, and 3-bromomethyl-1*H*-2-benzopyran-1-ones containing masked halo ketone functional groups were synthesized and tested as inhibitors of several serine proteases including human leukocyte (HL) elastase and cathepsin G. While many of the 3-halo-3-(1-haloalkyl)-1(3*H*)-isobenzofuranones were quite potent inhibitors of the enzymes tested, the alkylideneisobenzofuranones and benzopyran-1-ones inhibited poorly or not at all. The 3-halo-3-(1-haloalkyl)-1(3*H*)-isobenzofuranones decomposed rapidly upon addition to buffer to give the corresponding 3-alkyl-1*H*-2-benzopyran-1,4(3*H*)-diones. The pure benzopyran-1,4-diones were extremely potent inhibitors of HL elastase and chymotrypsin A_α but did not inactivate porcine pancreatic elastase or cathepsin G. Enzymes inhibited by the isobenzofuranones and benzopyran-1,4-diones regained activity slowly upon standing or after dialysis ($t_{1/2}$ = 5–16 h) and more rapidly in the presence of 0.5 M hydroxylamine, which indicated the presence of labile acyl moieties in the inhibited enzyme. These results are consistent with a scheme in which the active site serine of the protease reacts with the lactone carbonyl of these inhibitors to give a stable acyl enzyme and alkylation of another active site residue by the unmasked halo ketone functional group does not occur.

In one of the first reviews of suicide enzyme inhibitors, Rando (1974) proposed the use of haloenol lactones as mechanism-based inhibitors of serine proteases. Only recently, however, have these compounds been synthesized and tested as inhibitors of serine proteases. Aryl-substituted halomethylenetetrahydropyranones and -tetrahydrofuranones have been shown to be potent mechanism-based inhibitors of chymotrypsin (Daniels et al., 1983; Chakravarty et al., 1982). Acylation of the active site serine residue of the lactone moiety of these inhibitors results in the release of a halo ketone which may be alkylating an active site nucleophile to give an irreversibly inactivated enzyme.

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