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

Keiji Hemmi, J. Wade Harper, and James C. Powers*
School of Chemistry, Georgia Institute of Technology, Atlanta, Georgia 30332
Received May 9, 1984

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.

† This research was supported by a grant to the Georgia Institute of Technology (HL 29307) from the National Institutes of Health and by the Council for Tobacco Research.

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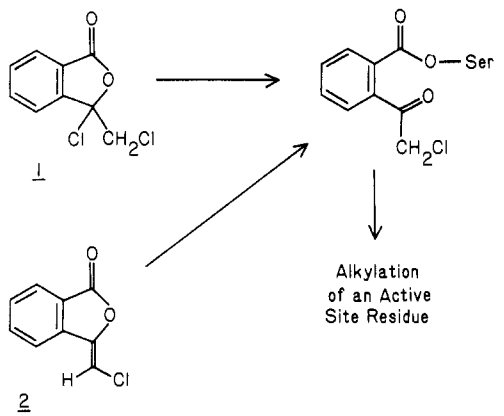

FIGURE 1: Proposed mechanism for the inactivation of serine protease by masked halo ketones.

The finding that 3,4-dichloroisocoumarin and 3,3-dichlorophthalide are potent mechanism-based inhibitors of human leukocyte (HL)[1] elastase and cathepsin G (Harper et al., 1985) led us to investigate several isobenzofuranones and benzopyran-1-ones (isocoumarins) as inhibitors of these enzymes. It was envisioned that the enzymatic ring opening of structures such as 3-chloro-3-(chloromethyl)-1(3*H*)-isobenzofuranone (**1**) or 3-(chloromethylene)-1(3*H*)-isobenzofuranone (**2**) would lead to the formation of a reactive halo ketone which could alkylate an active site residue (Figure 1). Here, we report the inactivation of several serine proteases by a number of 3-halo-3-(1-haloalkyl)-1(3*H*)-isobenzofuranones, 3-(1-haloalkylidene)-1(3*H*)-isobenzofuranones, and 3-(bromomethyl)isocoumarin derivatives. We show that some 3-halo-3-(1-haloalkyl)-1(3*H*)-isobenzofuranones rearrange to 1*H*-2-benzopyran-1,4(3*H*)-diones which are potent inhibitors of serine proteases. The results indicate that inactivation occurs by the formation of a stable acyl enzyme and alkylation of a second active site nucleophile does not occur.

Materials and Methods

Human leukocyte elastase and cathepsin G were generous gifts from Dr. James Travis and his research group at the University of Georgia. Porcine pancreatic elastase and bovine chymotrypsins A_α and A_γ were purchased from Sigma Chemical Co., St. Louis, MO. HEPES was obtained from Aldrich Chemical Co., Milwaukee, WI. MeO-Suc-Ala-Ala-Pro-Val-NA (Nakajima et al., 1979), Suc-Phe-Pro-Phe-NA (Yoshida et al., 1980), Suc-Ala-Ala-Ala-NA (Bieth et al., 1974), and 3-(bromomethyl)isocoumarin (**26**) (Chatterjea et al., 1981) were prepared as described previously.

Synthesis. The 3-(phenylmethylene)-1(3*H*)-isobenzofuranone and pentafluorobenzaldehyde were purchased from Aldrich Chemical Co., Milwaukee, WI. The 3-butylidene-1(3*H*)-isobenzofuranone was purchased from ICN Pharmaceuticals, Plainview, NY. All common chemicals and solvents were reagent grade. The NMR spectra for all new compounds were obtained on a Varian T60 instrument using $CDCl_3$ as solvent and tetramethylsilane as internal standard. The IR spectra were measured on a Perkin-Elmer 299 instrument. The mass spectra were obtained on a Varian MAT 112S. Melting points were determined with a Buchi melting point apparatus and are uncorrected. Elemental analyses were performed by Atlantic Microlab of Atlanta, GA. The synthesis of the best two inhibitors is given in this section. All other new compounds are reported in the supplementary material (see paragraph at end of paper regarding supplementary material).

3-Bromo-3-(α-bromobenzyl)-1(3H)-isobenzofuranone (**13**). A solution of 3-(phenylmethylene)-1(3*H*)-isobenzofuranone (500 mg) in methylene chloride (25 mL) was brominated by addition of a slight excess of bromine for 30 min at 25 °C. After removal of the solvent, the residue was purified on a short column of silica gel with benzene as eluent to yield 3-bromo-3-(α-bromobenzyl)-1(3*H*)-isobenzofuranone (370 mg), which was recrystallized from benzene–hexane: mp 145–148 °C; IR (Nujol) 1785 cm^{-1}; NMR δ 5.68 (s, 1 H), 7.2–8.1 (m, 4 H). Anal. Calcd for $C_{15}H_{10}O_2Br_2$: C, 47.15; H, 2.64; Br, 41.83. Found: C, 47.19; H, 2.67; Br, 41.75.

Isolation of 3-Propyl-1H-2-benzopyran-1,4(3H)-dione (**31**). A solution of 3-bromo-3-(1-bromobutyl)-1(3*H*)-isobenzofuranone (500 mg) in Me_2SO (15 mL) was added dropwise to a solution of 0.1 M HEPES (pH 7.5, 50 mL) containing 0.5 M NaCl and Me_2SO (35 mL). After the mixture was stirred for 15 min at room temperature, the mixture was diluted with water (100 mL) and extracted with ethyl acetate (50 mL × 2). The extracts were combined, washed with water (50 mL × 5), dried over magnesium sulfate, and concentrated under reduced pressure. The residue was chromatographed on silica gel with benzene as eluent to yield the decomposition product (**31**) (170 mg), which was recrystallized from cyclohexane: mp 44–45 °C; IR (Nujol) 1730, 1700 cm^{-1}; NMR δ 1.00 (t, J = 6 Hz, 3 H), 1.2–2.3 (m, 4 H), 5.08 (t, J = 6 Hz, 1 H), 7.8–8.4 (m, 4 H); mass spectrum, m/e 204 (M+), 162, 132, 104. Anal. Calcd for $C_{12}H_{12}O_3$: C, 70.57; H, 5.92. Found: C, 70.55; H, 5.96.

Inactivation Kinetics. Inactivation experiments were performed as described in Harper et al. (1985). Briefly, a 5–50-μL aliquot of inhibitor in Me_2SO was added to an enzyme solution (0.1 M HEPES, 0.5 M NaCl, pH 7.5) such that the final concentration of Me_2SO was 10%. Aliquots were removed at various times, and residual enzymatic activity was measured spectrophotometrically. Chymotrypsin and cathepsin G were assayed with Suc-Phe-Pro-Phe-NA (0.3–0.9 mM) or Suc-Val-Pro-Phe-NA (0.2–0.8 mM), HL elastase with MeO-Suc-Ala-Ala-Pro-Val-NA (0.1–0.4 mM), and PP elastase with Suc-Ala-Ala-Ala-NA (0.6–1.2 mM). All nitroanilide kinetics were measured at 410 nm (ϵ = 8800 M^{-1} cm^{-1}) (Erlanger et al., 1961). Most inactivation rate constants were calculated by using a method described earlier for unstable inhibitors (Ashani et al., 1972). Inactivation rates of stable inhibitors were obtained from first-order plots as described in the preceding paper (Harper et al., 1985). All plots gave correlation coefficients of 0.98 or greater. Reactivation kinetics were performed as described in the preceding paper (Harper et al., 1985) except that a 2-h dialysis period against 0.1 M phosphate, pH 6.8, was used.

Results

Synthesis. The structures of the inhibitors investigated are shown in Figure 2. Chlorination of 2-acetylbenzoic acid with sulfuryl chloride gave a complex reaction mixture, from which were isolated 3-chloro-3-(dichloromethyl)-1(3*H*)-isobenzofuranone (**4**) and 3-(chloromethylene)-1(3*H*)-isobenzofuranone (**2**) in low yield (<5%). The expected reaction product, 3-chloro-3-(chloromethyl)-1(3*H*)-isobenzofuranone (**1**) was not obtained. An alternate procedure was used for the synthesis of **1**. 2-Acetylbenzoic acid was converted to 3-methylene-1-(3*H*)-isobenzofuranone and reacted with chlorine to yield 3-chloro-3-(chloromethyl)-1(3*H*)-isobenzofuranone (**1**). The synthesis of substituted 3-alkylidene-1(3*H*)-isobenzofuranones gave mixtures of *Z* and *E* isomers. Addition of bromine to

[1] Abbreviations: HL, human leukocyte; PP, porcine pancreatic; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MeO-Suc, methoxysuccinyl; Me_2SO, dimethyl sulfoxide; C_6F_5, pentafluorophenyl.

Table I: Rate Constants for Inactivation of Human Leukocyte Elastase and Porcine Pancreatic Elastase by 3-Halo-3-(1-haloalkyl)-1(3*H*)-isobenzofuranones[a]

inhibitor[b]				HL elastase			PP elastase		
X	R_1	R_2	compd no.	[I] (μM)	k_{obsd}/[I] (M^{-1} s^{-1})	reactivation (%, h)	[I] (μM)	k_{obsd}/[I] (M^{-1} s^{-1})	reactivation (%, h)
C	H	H	**1**	520	NI[c]		360	NI	
Br	H	H	**3**	960	0.49	40, 16	960	NI	
Cl	Cl	H	**4**	510	2.9	43, 24	880	0.54	71, 18.5
Br	Cl	H	**5**	300	25	45, 24	530	5.4	72, 20
Br	Br	H	**6**	470	2.2	25, 18	502	NI	
Br	Me	H[d]	**7**	76	505	74, 16	160	16.4	66, 16
Br	Me	H[e]	**8**	72	175	ND[h]	ND		
Br	Et	H[f]	**9**	108	337	45, 16	220	12.7	60, 21
Br	Et	H[g]	**10**	68	153	ND	ND		
Br	Me	Me	**11**	210	NI		210	NI	
Br	Pr	H	**12**	58	220	50, 6	868	1.6	45, 18
Br	Ph	H	**13**	9.3	1320	41, 15	320	72	51, 6.5
Br	C_6F_5	H	**14**	196	NI		229	NI	

[a] Incubation conditions were 0.1 M HEPES, 0.5 M NaCl, and 10% Me_2SO, pH 7.5, at 25 °C. [b] Structure of the isobenzofuranone inhibitor is shown in Figure 2 (left). [c] NI, no inhibition after incubation for at least 30 min. [d] Prepared from (*Z*)-3-ethylidene-1(3*H*)-isobenzofuranone, mixture of (3*R*)-3-[1(*R*)-bromoethyl] and (3*S*)-3-[1(*S*)-bromoethyl] isomers. [e] Prepared from (*E*)-3-ethylidene-1(3*H*)-isobenzofuranone, mixture of (3*R*)-3-[1(*S*)-bromoethyl] and (3*S*)-3-[1(*R*)-bromoethyl] isomers. [f] Prepared from (*Z*)-3-propylidene-1(3*H*)-isobenzofuranone, mixture of (3*R*)-3-[1-(*R*)-bromopropyl] and (3*S*)-3-[1(*S*)-bromopropyl] isomers. [g] Prepared from (*E*)-3-propylidene-1(3*H*)-isobenzofuranone, mixture of (3*R*)-3-[1(*S*)-bromopropyl] and (3*S*)-3-[1(*R*)-bromopropyl] isomers. [h] ND, not determined.

Table II: Rate Constants for Inactivation of Chymotrypsin A_α and Cathepsin G by 3-Halo-3-(1-haloalkyl)-1(3*H*)-isobenzofuranones[a]

inhibitor[b]				chymotrypsin A_α		cathepsin G		
X	R_1	R_2	compd no.	[I] (μM)	k_{obsd}/[I] (M^{-1} s^{-1})	reactivation (%, h)	[I] (μM)	k_{obsd}/[I] (M^{-1} s^{-1})	reactivation (%, h)
Cl	H	H	**1**	520	NI[c]		460	NI	
Br	H	H	**3**	960	0.98	70, 23	960	NI	
Cl	Cl	H	**4**	510	2.3	22, 19	400	NI	
Br	Cl	H	**5**	630	6.7	32, 20	530	3.3	4, 8
Br	Br	H	**6**	470	1.6	11, 15	502	21	10, 1.5
Br	Me	H[d]	**7**	120	76	54, 16	152	24	22, 3
Br	Me	H[e]	**8**	130	114	ND	150	24	ND[h]
Br	Et	H[f]	**9**	123	74	56, 18	130	17	63, 6
Br	Et	H[g]	**10**	93	105	ND	161	87	ND
Br	Me	Me	**11**	38	1385	100, 16	210	NI	
Br	Pr	H	**12**	71	110	45, 18	294	22	11, 3
Br	Ph	H	**13**	7.5	580	60, 15	72	76	9, 4
Br	C_6F_5	H	**14**	178	NI		209	NI	

[a] Incubation conditions were 0.1 M HEPES, 0.5 M NaCl, and 10% Me_2SO, pH 7.5, at 25 °C. [b] Structure of the isobenzofuranone inhibitor is shown in Figure 2 (left). [c] NI, no inhibition. [d] Prepared from (*Z*)-3-ethylidene-1(3*H*)-isobenzofuranone, mixture of (3*R*)-3-[1(*R*)-bromoethyl] and (3*S*)-3-[1(*S*)-bromoethyl] isomers. [e] Prepared from (*E*)-3-ethylidene-1(3*H*)-isobenzofuranone, mixture of (3*R*)-3-[1(*S*)-bromoethyl] and (3*S*)-3-[1(*R*)-bromoethyl] isomers. [f] Prepared from (*Z*)-3-propylidene-1(3*H*)-isobenzofuranone, mixture of (3*R*)-3-[1(*R*)-bromopropyl] and (3*S*)-3-[1-(*S*)-bromopropyl] isomers. [g] Prepared from (*E*)-3-propylidene-1(3*H*)-isobenzofuranone, mixture of (3*R*)-3-[1(*S*)-bromopropyl] and (3*S*)-3-[1(*R*)-bromopropyl] isomers. [h] ND, not determined.

the isolated *Z* and *E* isomers of 3-alkylidene-1(3*H*)-isobenzofuranones gave mixtures of *RR*, *SS* and *RS*, *SR* diastereoisomers of 3-halo-3-(1-haloalkyl)-1(3*H*)-isobenzofuranones which were used without further separation. The 3-(1-bromoalkylidene)-1(3*H*)-isobenzofuranones (**15**, **24**, and **25**) were prepared from the corresponding dibromides (**3** and **12**) by treatment with triethylamine or 1,8-diazabicyclo-[5.4.0]undeca-7-ene.

Inactivation of Serine Proteases by 3-Halo-3-(1-haloalkyl)-1(3H)-isobenzofuranones, 3-Alkylidene-1(3H)-isobenzofuranones, and Isocoumarins. The rate constants for inactivation of HL elastase and PP elastase by 3-halo-3-(1-haloalkyl)-1(3*H*)-isobenzofuranones are shown in Table I. The most potent inhibitor for HL elastase was 3-bromo-3-(α-bromobenzyl)-1(3*H*)-isobenzofuranone (**13**). The haloalkyl derivatives **7**–**10** and **12** were moderate inhibitors of HL elastase. The inhibitors were at least 20-fold less effective with PP elastase. Neither HL elastase nor PP elastase was inhibited by 3-chloro-3-chloromethyl-1(3*H*)-isobenzofuranone (**1**), and PP elastase was not inhibited by 3-bromo-3-bromomethyl-1-(3*H*)-isobenzofuranone (**3**). The 3-halo-3-(1-haloalkyl)-1-(3*H*)-isobenzofuranones were also found to be potent inhibitors of chymtrypsin A_α (Table II) but were only moderate inhibitors of cathepsin G. The most potent inhibitors for chymotrypsin A_α contained large alkyl groups or an aromatic substituent. Cathepsin G was not inhibited by 3-chloro-3-

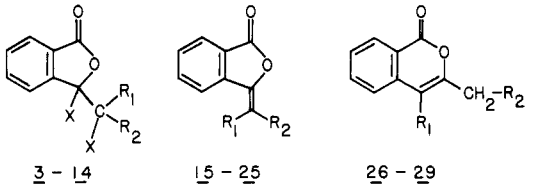

FIGURE 2: Structures of the 3-alkyl-1(3*H*)-isobenzofuranones (**3**–**14**), 3-alkylidene-1(3*H*)-isobenzofuranones (**15**–**25**), and isocoumarins (**26**–**29**) investigated as serine protease inhibitors. The substituents (X, R_1, R_2) on the 3-alkyl-1(3*H*)-isobenzofuranones are as follows: **3**, Br, H, H; **4**, Cl, Cl, H; **5**, Br, Cl, H; **6**, Br, Br, H; **7**, Br, Me, H (mixture of *RR* and *SS*); **8**, Br, Me, H (mixture of *RS* and *SR*); **9**, Br, Et, H (mixture of *RR* and *SS*); **10**, Br, Et, H (mixture of *RS* and *SR*); **11**, Br, Me, Me; **12**, Br, Pr, H; **13**, Br, Ph, H; **14**, Br, C_6F_5, H. The substituents (R_1, R_2) on the 3-alkylidene-1(3*H*)-isobenzofuranones are as follows: **15**, H, Br; **16**, H, Me; **17**, Me, H; **18**, Me, Me; **19**, H, Et; **20**, Et, H; **21**, H, Pr; **22**, H, Ph; **23**, H, C_6F_5; **24**, Br, Pr; **25**, Pr, Br. The substituent on the isocoumarins are as follows: **26**, H, Br; **27**, Br, H; **28**, Br, Br; **29**, COOMe, Br.

Table III: Rate Constants for Inactivation of Serine Proteases by Substituted Isocoumarins[a]

compd	compd no.	$k_{obsd}/[I]$ (M^{-1} s^{-1}) HL elastase[b]	PP elastase[c]	chymotrypsin A_α[d]
3-(bromomethyl)isocoumarin	**26**	0.47	0.11	2.5
4-bromo-3-methylisocoumarin	**27**	NI[e]	ND[f]	NI
3-(bromomethyl)-4-bromoisocoumarin	**28**	NI	ND	NI
3-(bromomethyl)-4-(methoxycarbonyl)isocoumarin	**29**	NI	ND	NI

[a] Enzyme (0.5–2.0 μM) was incubated with inhibitor in buffer containing 0.1 M HEPES, 0.5 M NaCl, and 10% Me_2SO, pH 7.5 at 25 °C. Aliquots were removed with time, and enzymatic activity was measured as described under Materials and Methods. [b] Inhibitor concentrations were the following: **26**, 1.1 mM; **27**, 0.32 mM; **28**, 0.20 mM; **29**, 0.28 mM. [c] Concentration of **26** was 1.1 mM. [d] Inhibitor concentrations were the following: **26**, 0.73 mM; **27**, 0.32 mM; **28**, 0.47 mM; **29**, 0.3 mM. [e] NI, no inactivation. [f] ND, not determined.

chloromethyl- (**1**), 3-bromo-3-(bromomethyl)- (**3**), or 3-chloro-3-(dichloromethyl)-1(3*H*)-isobenzofuranone (**4**) at concentrations greater than 0.4 mM, and chymotrypsin A_α was not inhibited by 3-chloro-3-chloromethyl-1(3*H*)-isobenzofuranone (**1**) (0.52 mM).

The concentration dependence of the inactivation of HL elastase and chymotrypsin A_α by 3-bromo-3-(α-bromobenzyl)-1(3*H*)-isobenzofuranone (**13**) was studied by using the method of Kitz & Wilson (1962), and K_I values of 25 μM and 29 μM and k_3 values of 6.4×10^{-2} s^{-1} and 2.1×10^{-2} s^{-1} were determined, respectively.

The 3-alkylidene-1(3*H*)-isobenzofuranones (Figure 2, middle) were, in general, not inhibitors of the serine proteases investigated. However, HL elastase and chymotrypsin A_α were inhibited very slowly by the *Z* and *E* isomers of 3-(1-bromobutylidene)-1(3*H*)-isobenzofuranone (**24** and **25**). The half-life of inactivation of HL elastase and chymotrypsin A_α by **25** (0.26 mM) was 175 ($k_{obsd}/[I]$ = 0.2 M^{-1} s^{-1}) and 230 min ($k_{obsd}/[I]$ = 0.4 M^{-1} s^{-1}), respectively. The maximum inhibition observed with HL elastase at an inhibitor concentration of 0.26 mM was 92%, while chymotrypsin A_α was inhibited 70% under similar conditions. The decomposition of 3-(1-bromobutylidene)-1(3*H*)-isobenzofuranone (**25**) in buffer solution was measured by following the decrease in absorbance at 318 nm (ε = 7960 M^{-1} cm^{-1}) and was found to have a half-life of ca. 1.5 h.

The finding that 3-alkylidene-1(3*H*)-isobenzofuranones did not inactivate several serine proteases led us to investigate a number of isocoumarins (Figure 2, right), which also contained masked halo ketone functionalities, as inhibitors of HL elastase, PP elastase, and chymotrypsin A_α. The rate constants for inactivation are shown in Table III. 3-(Bromomethyl)-isocoumarin (**26**) was found to be a poor inhibitor of these enzymes ($k_{obsd}/[I]$ < 2.5 M^{-1} s^{-1}) while 4-bromo-3-methylisocoumarin (**27**), 3-(bromomethyl)-4-bromoisocoumarin (**28**), and 3-(bromomethyl)-4-(methoxycarbonyl)isocoumarin (**29**) showed no inhibition of HL elastase and chymotrypsin A_α at concentrations as high as 0.2 mM. Rapid changes in the ultraviolet spectrum of 3-(bromomethyl)-4-bromoisocoumarin (**28**) indicated that it decomposed rapidly ($t_{1/2}$ < 2 min) in aqueous solution. The structure of the decomposition product was not determined.

Reactivation of Enzymes Inhibited by 3-Halo-3-(1-haloalkyl)-1(3H)-isobenzofuranones. In most cases, serine proteases inhibited by 3-halo-3-(1-haloalkyl)-1(3*H*)-isobenzofuranones were fairly resistant to reactivation upon incubation without the removal of excess inhibitor (Tables I and II). With a few exceptions the half-lives for reactivation were greater than 14–16 h. Cathepsin G appeared to undergo reactivation more rapidly than HL elastase or chymotrypsin A_α.

Addition of buffered hydroxylamine (0.3–0.45 M) to enzymes inhibited by 3-halo-3-(1-haloalkyl)-1(3*H*)-isobenzofuranones resulted in a rapid regain of enzymatic activity (Table IV). The half-lives for reactivation of HL elastase were 1.8–10.5 times lower than those of chymotrypsin except in the case of 3-bromo-3-(1-bromomethyl)-1(3*H*)-isobenzofuranone (**10**) where both half-lives were near 6.0 min. Hydroxylamine reactivation indicated that labile acyl moieties were present in the inhibited enzyme structure and that no alkylation of residues such as histidine had occurred. Chymotrypsin (2 μM) inactivated with the peptide chloromethyl ketone Boc-Ala-Gly-PheCH_2Cl (0.3 mM) contains a functional group similar to that which would be formed if the unmasked bromoketone in the isobenzofuranone inhibitor alkylated His-57 of chymotrypsin. Addition of buffered hydroxylamine (final concentration 0.5 M) to Boc-Ala-Gly-PheCH_2-chymotrypsin resulted in no regain in enzymatic activity after 20 h at pH 7.5 and 25 °C.

Inhibition of Serine Proteases by 3-Bromo-3-(1-bromobutyl)-1(3H)-isobenzofuranone. We discovered in the course of measuring the inhibition kinetics that the inactivation of HL elastase and chymotrypsin A_α by 3-bromo-3-(1-bromobutyl)-1(3*H*)-isobenzofuranone (**12**) is a biphasic process (Figure 3) with the initial slow inactivation ($k_{obsd}/[I]$ ~35 M^{-1} s^{-1}) being followed by a more rapid inactivation rate. The rapid phase gave $k_{obsd}/[I]$ values of 370 M^{-1} s^{-1} and 130 M^{-1} s^{-1} with HL elastase and chymotrypsin A_α, respectively. Similar lag phases were observed with the ethyl (**7**) and propyl (**9**) derivatives, but no lag phase was observed with the benzyl (**13**) derivative. The absence of an observable inactivation lag phase with 3-bromo-3-(α-bromobenzyl)-1(3*H*)-isobenzofuranone (**13**) is probably due to its rapid decomposition in buffer ($t_{1/2}$ = 16 s). The kinetic constants reported in Tables I and II are based on observations over an extended time period and are not affected by the lag phase. With the slow inhibitors, the lag phase (if present) was rapid compared to inactivation

Table IV: Reactivation of HL Elastase and Chymotrypsin A_α Inhibited by 3-Halo-3-(1-haloalkyl)-1(3*H*)-isobenzofuranones in the Presence of Hydroxylamine[a]

inhibitor[b] X	R_1	R_2	compd no.	HL elastase 10^3k_{deacyl} (s^{-1})	HL elastase $t_{1/2}$ (min)	chymotrypsin A_α 10^3k_{deacyl} (s^{-1})	chymotrypsin A_α $t_{1/2}$ (min)
Br	Cl	H	**5**	1.1	10.5	0.6	19.3
Br	Me	H	**7**	1.9	6	2.0	5.8
Br	Et	H	**9**	1.2	9.6	0.5	23.1
Br	Pr	H	**12**	1.8	6	0.5	23.1
Br	Ph	H	**13**	2.1	5.5	0.2	58.0

[a] Hydroxylamine (in 0.1 M HEPES and 0.5 M NaCl, pH 7.5; final concentration 0.3–0.45 M) was added to 0.3–0.5 mL of inactivated enzyme (less than 5% activity), and the time course for recovery of enzymatic activity was measured. [b] Structure of the inhibitor is shown in Figure 2 (left).

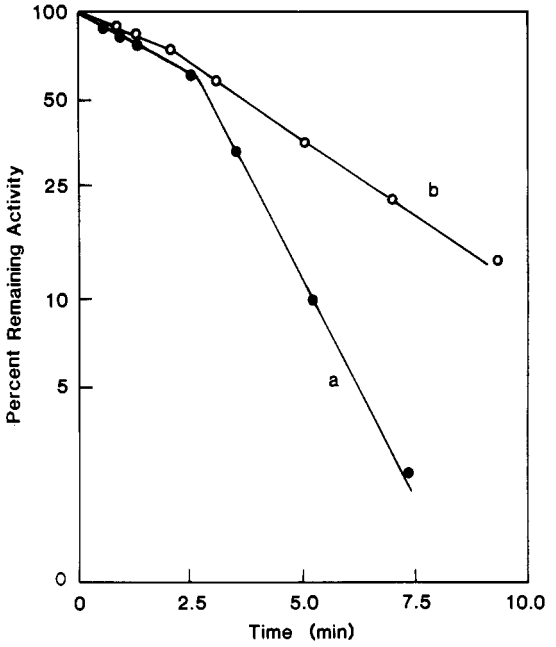

FIGURE 3: Inactivation of HL elastase (a, [E] = 0.4 μM, [I] = 57.6 μM) and chymotrypsin A_α (b, [E] = 2 μM, [I] = 70.5 μM) by 3-bromo-3-(1-bromobutyl)-1(3*H*)-isobenzofuranone in 0.1 M HEPES, 0.5 M NaCl, pH 7.5, and 10% Me_2SO at 25 °C.

rate and was not observable. With several of the more effective inhibitors, no lag could be observed during the brief course of the reaction.

Ultraviolet Spectral Studies. The presence of a lag phase in the inactivation process indicated that the 3-bromo-3-(1-bromoalkyl)-1(3*H*)-isobenzofuranones were being converted, either spontaneously or enzymatically, to a more reactive inhibitor. Therefore, we investigated the ultraviolet spectral changes which occurred upon spontaneous and enzymatic decomposition of 3-bromo-3-(1-bromobutyl)-1(3*H*)-isobenzofuranone (12).

Addition of 3-bromo-3-(1-bromobutyl)-1(3*H*)-isobenzofuranone (**12**) (0.017 mM) to buffer resulted in a rapid change (ca. 2 min) in the ultraviolet spectrum with a notable increase in absorbance in the region of 320–350 nm (Figure 4). The decomposition of **12** (0.017 mM) was followed spectrophotometrically at 340 nm (ϵ = 2150 M^{-1} cm^{-1}), and a decomposition rate constant (k_{obsd}) of 26 × 10^{-3} s^{-1} was obtained. Similar experiments in the presence of chymotrypsin A_α (0.018 mM) gave a k_{obsd} of 33 × 10^{-3} s^{-1}, which indicates that chymotrypsin enhances decomposition only slightly if at all. The ethyl (**7**) (ϵ_{335} = 2200 M^{-1} cm^{-1}) and propyl (**9**) (ϵ_{330} = 1900 M^{-1} cm^{-1}) derivatives decomposed with rates constants of 25 × 10^{-3} s^{-1} and 21 × 10^{-3} s^{-1}, respectively, while the benzyl derivative (**13**) (ϵ_{348} = 6770 M^{-1} cm^{-1}) and 3-bromo-3-(1-bromomethyl)-1(3*H*)-isobenzofuranone (**3**) (ϵ_{304} = 1880 M^{-1} cm^{-1}) decomposed more rapidly (k_{obsd} = 43 × 10^{-3} s^{-1} and 140 × 10^{-3} s^{-1}, respectively).

Spontaneous and enzymatic hydrolysis rates of 3-chloro-3-chloromethyl-1(3*H*)-isobenzofuranone (**1**) and 3-(chloromethylene)-1(3*H*)-isobenzofuranone (**2**) were also measured. 3-Chloro-3-chloromethyl-1(3*H*)-isobenzofuranone (**1**) (86 μM; ϵ_{304} = 1880 M^{-1} cm^{-1}) decomposed more slowly than the bromo derivative (**3**) in buffer (k_{obsd} = 1.1 × 10^{-3} s^{-1}), and chymotrypsin A_α (4.7 μM) catalyzed the hydrolysis of **1** (86 μM) 2-fold (k_{obsd} = 2.6 × 10^{-3} s^{-1}). 3-(Chloromethylene)-1-(3*H*)-isobenzofuranone (**2**) is, however, stable in buffer and chymotrypsin A_α (molar ratio 1/1) had no effect on the ultraviolet absorbance (λ_{max} 310 nm, ϵ_{310} = 5700 M^{-1} cm^{-1}) over

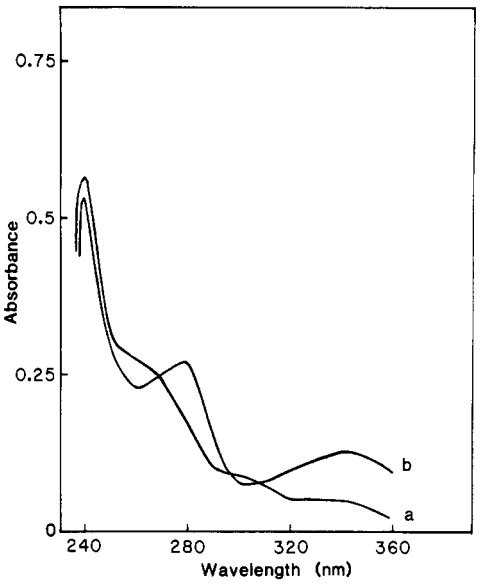

FIGURE 4: Ultraviolet spectra of 3-bromo-3-(1-bromobutyl)-1-(3*H*)-isobenzofuranone in buffer. Inhibitor (55.8 μM) was rapidly added to 0.1 M HEPES, 0.5 M NaCl, pH 7.5, and 10% Me_2SO, and the spectra were recorded immediately (a) and after 5 min (b).

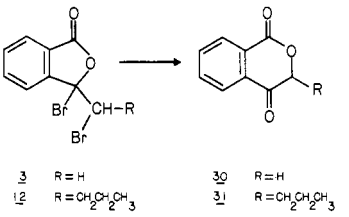

FIGURE 5: Rearrangement of 3-bromo-3-(1-bromoalkyl)-1(3*H*)-isobenzofuranones to 3-alkyl-1*H*-2-benzopyran-1,4(3*H*)-diones.

a period of 1 h. These results indicate that **1** is at best a poor substrate of chymotrypsin A_α while **2** is not a substrate.

The spontaneous and enzymatic hydrolysis of isocoumarins was measured spectrophotometrically by following the decrease in absorbance at 325 nm. The spontaneous hydrolysis rate of 3-(bromomethyl)isocoumarin (**26**; 54 μM, ϵ_{325} = 4000 M^{-1} cm^{-1}) was found to be 20 × 10^{-6} s^{-1}. The hydrolysis rate of **26** (54 μM) in the presence of chymotrypsin A_γ (60 μM) is 45 × 10^{-6} s^{-1}, which indicated that this inhibitor is at best a poor substrate for chymotrypsin under these conditions. 4-Bromo-3-methylisocoumarin (**27**) was also found not be be hydrolyzed by chymotrypsin A_γ under similar conditions. These results indicated that inhibition of serine proteases by 3-(bromomethyl)isocoumarin (**26**) was due to simple alkylation of an enzyme active site residue.

Isolation of the Decomposition Product (**31**) *of 3-Bromo-3-(1-bromobutyl)-1(3H)-isobenzofuranone* (**12**). The previously described kinetic and spectrophotometric measurements indicated that 3-bromo-3-(1-bromobutyl)-1(3*H*)-isobenzofuranone (**12**) decomposes rapidly in buffer solution to an extremely potent inhibitor of HL elastase and chymotrypsin A_α. A decomposition product was isolated by column chromatography in 50% yield after stirring **12** for 15 min in buffer (50% Me_2SO), and was shown to be 3-propyl-1*H*-2-benzopyran-1,4(3*H*)-dione (**31**) by its NMR, IR, and mass spectra and by elemental analysis (Figure 5). Addition of an aliquot of the purified decomposition product (in Me_2SO) to buffer resulted in a rapid increase (<2 min) in absorbance at 340 nm (ϵ = 2150 M^{-1} cm^{-1}) which was reversibly shifted to λ_{max} 310 nm (ϵ = 3580 M^{-1} cm^{-1}) at pH 9.0. The spectrum in Me_2SO alone has no absorbance at 340 nm. This absorbance

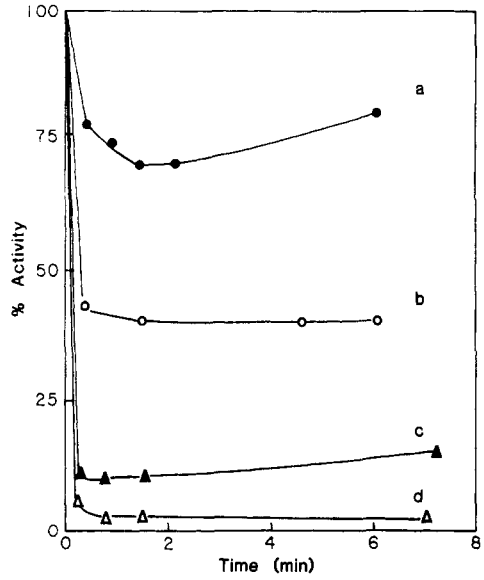

FIGURE 6: Inactivation of HL elastase (0.4 μM) by 3-propyl-1*H*-2-benzopyran-1,4(3*H*)-dione (a, 2.2 μM; b, 11 μM; c, 28.5 μM; d, 143 μM) in 0.1 M HEPES, 0.5 M NaCl, and 10% Me_2SO, pH 7.5 at 25 °C.

change appears to be due to partial tautomerization of the ketone to the enol form, which has absorbance near 340 nm. Also consistent with this result is the finding that, in Me_2SO-d_6, the NMR spectrum is that of the ketone form of 3-propyl-1*H*-2-benzopyran-1,4(3*H*)-dione. The decomposition product (**30**) of 3-bromo-3-(bromomethyl)-1(3*H*)-isobenzofuranone (**3**) was also isolated, and the physical properties are consistent with those reported by Knott (1963) for 1*H*-2-benzopyran-1,4(3*H*)-dione.

Inactivation of Serine Proteases by 2-Benzopyran-1,4-diones. 3-Propyl-1*H*-2-benzopyran-1,4(3*H*)-dione (**31**) rapidly inactivated HL elastase (Figure 6) and chymotrypsin A_α (data not shown), and the maximum inhibition obtained was dependent upon the inhibitor concentration. HL elastase (0.4 μM) was inhibited 97% at [I] = 143 μM, and chymotrypsin A_α (2 μM) was inhibited 98% at [I] = 199 μM. The unsubstituted 1*H*-2-benzopyran-1,4(3*H*)-dione (**30**) was a less effective inhibitor, and HL elastase and chymotrypsin A_α were only inhibited 70% at inhibitor concentrations of 0.22 and 0.26 mM, respectively. Neither PP elastase nor cathepsin G was inhibited by **30** or **31**. HL elastase inhibited by **31** (0.04 mM) recovered activity gradually upon standing with a half-life of 6 h. Dialysis of HL elastase and chymotrypsin inactivated by **31** (0.09 mM) against 0.1 M phosphate buffer (pH 6.8) for 2 h at 5 °C resulted in <1% and 8% reactivation, respectively. Further standing resulted in total reactivation with rate constants of 36×10^{-6} s^{-1} ($t_{1/2}$ = 5.3 h) and 44×10^{-6} s^{-1} ($t_{1/2}$ = 4.4 h), respectively. The 3-propyl derivative (**31**) is fairly stable in buffer ($t_{1/2} \sim 2.5$ h, followed spectrophotometrically at 340 nm), and chymotrypsin A_α (62 μM) failed to catalyze the hydrolysis of **31** (59 μM; $t_{1/2} \sim 6$ h). The hydrolysis product, 2-(2-hydroxypentanoyl)benzoic acid was prepared by stirring 3-bromo-3-(1-bromobutyl)-1(3*H*)-isobenzofuranone (**12**) at pH 9 for 3 h at room temperature. This compound (0.2 mM) did not inhibit HL elastase or chymotrypsin A_α.

DISCUSSION

Several aryl- and naphthyl-substituted haloenol butyrolactones and valerolactones, which contain masked halo ketone functionalities, have been shown previously to inactivate chymotrypsin by an enzyme-mediated process (Daniels et al., 1983; Chakravarty et al., 1982). These compounds either react with the active site serine of chymotrypsin to give a rapidly hydrolyzed acyl enzyme (inhibitor turnover) or react with the active site serine in a process that gives rise to a stable inhibited enzyme structure. Chymotrypsin that has been inactivated by (*E*)-2-phenyl-5-(1-bromoethylidene)tetrahydro-2-furanone, one of the more reactive haloenol butyrolactones, is stable to gel filtration. Neither the non-halogen-containing lactone (*Z*)-5-ethylidene-2-phenyltetrahydro-2-furanone nor the noncyclic bromo ketone 5-bromo-4-oxo-2-phenylhexanoic acid (hydrolyzed form of the lactone) inactivated chymotrypsin. While the results clearly show that the inhibitory potency of this series of inhibitors depends largely on certain structural characteristics of the lactone or masked halo ketone moiety, there is no evidence that alkylation of an active site residue is involved in the inactivation. Therefore, our initial goals in this investigation were to extend the masked halo ketone concept to structures that would react with other serine proteases such as HL elastase and to obtain evidence for involvement of an active site residue in addition to serine in the inhibition process.

Initially, we investigated 3-(chloromethylene)-1(3*H*)-isobenzofuranone (**2**) and 3-(1-bromobutylidene)-1(3*H*)-isobenzofuranones (**25**) (Figure 2, center), which are the benzo relatives of the haloenol furanones previously studied as serine protease inhibitors (Daniels et al., 1983, Chakravarty et al., 1982). In contrast to the haloenol furanones, we find that these haloenol isobenzofuranones inactivate HL elastase and chymotrypsin A_α extremely poorly or not at all. In addition, 3-(chloromethylene)-1(3*H*)-isobenzofuranone (**2**) was not hydrolyzed by chymotrypsin A_α at equimolar concentrations. It is likely that the extremely slow inactivation observed with a few compounds such as 3-(1-bromobutylidene)-1(3*H*)-isobenzofuranone (**25**) is due to a decomposition product since the inhibition rate was on the same order as spontaneous hydrolysis. We also found that several 3-(bromomethyl)isocoumarin derivatives and 4-bromo-3-methylisocoumarin (Figure 2, right), which contained masked halo ketone moieties, inactivated HL elastase, PP elastase, and chymotrypsin either very slowly or not at all. The ring system contained in these inhibitors (isocoumarin or benzofuran) is also present in other types of mechanism-based inhibitors (Harper et al., 1985), but it is obvious that the addition of the haloenol lactone moiety is not a sufficient condition to obtain inhibition.

The lack of activity of these haloenol benzofuranones led us to investigate 3-halo-3-(1-haloalkyl)-1(3*H*)-isobenzofuranones as inhibitors of serine proteases. These compounds (Figure 2, left), which are relatives of the haloenol lactones discussed above, contain a previously unreported form of a masked halo ketone. Enzymatic acylation by these structures would be expected to release bromide ion to give the corresponding halo ketone. Preliminary results indicated that these compounds were potent inhibitors of HL elastase, the most reactive of which was 3-bromo-(3α-bromobenzyl)-1(3*H*)-isobenzofuranone (**13**). However, we discovered during further kinetic and spectrophotometric measurements that these 3-halo-3-(1-haloalkyl)-1(3*H*)-isobenzofuranones were decomposing rapidly upon addition to a pH 7.5 HEPES buffer solution to give extremely potent inhibitors of HL elastase and chymotrypsin. A decomposition rate of 26×10^{-3} s^{-1} ($t_{1/2}$ = 26 s) was measured spectrophotometrically at 340 nm for 3-bromo-3-(1-bromobutyl)-1(3*H*)-isobenzofuranone (**12**). Many of the other inhibitors decomposed rapidly and gave similar kinetics. The major decomposition product, 3-propyl-1*H*-2-benzopyran-1,4(3*H*)-dione (**31**), was isolated

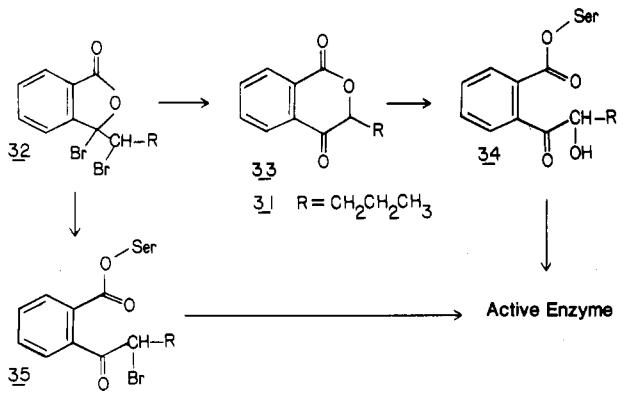

FIGURE 7: Mechanisms of inactivation of serine proteases by 3-bromo-3-(1-bromoalkyl)-1(3*H*)-isobenzofuranones and 1*H*-2-benzopyran-1,4(3*H*)-diones.

from the buffer solution and characterized by its NMR, infrared, and mass spectra and with elemental analysis (Figure 5). This compound has considerable structural resemblance to the 2-alkyl- and 2-(perfluoroalkyl)-4*H*-3,1-benzoxazin-4-ones which we have previously shown to be potent inhibitors of HL elastase and chymotrypsin (Teshima et al., 1982). Pure 3-propyl-1*H*-2-benzopyran-1,4(3*H*)-dione (**31**) inactivated HL elastase and chymotrypsin A_α very rapidly at the concentrations investigated (Figure 6), and $k_{obsd}/[I]$ values could not be calculated. This indicates that the $k_{obsd}/[I]$ values reported to Tables I and II simply reflect the rate of cyclization of 2-(2-bromopentanoyl)benzoic acid to the 2-benzopyran-1,4-dione, and the actual $k_{obsd}/[I]$ must be at least 800 M^{-1} s^{-1}.

These results are consistent with the mechanism of inactivation shown in Figure 7. Attack of the active site serine residue on the lactone moiety of the 3-alkyl-1*H*-2-benzopyran-1,4-dione (**33**) results in the formation of an acyl enzyme structure (**34**) in which a hydroxy ketone has been released. Deacylation of the acyl enzyme (**34**) would give rise to active enzyme.

The kinetics of inhibition of HL elastase by 3-propyl-1*H*-2-benzopyran-1,4-dione (**31**) indicate that an equilibrium might exist between enzyme, inhibitor, and the enzyme–inhibitor complex since the fraction of enzyme inactivated depends upon the inhibitor concentration. The finding that no enzyme-catalyzed loss of the benzopyran-1,4-dione ring system occurred except after long incubation suggests that deacylation is occurring by a process that ordinarily results in the reformation of the inhibitor. A similar situation has been found for inhibition of chymotrypsin by 5-butyl-3*H*-1,3-oxazine-2,6-dione (Weidmann & Abeles, 1984) and 2-hydroxy-5-nitro-α-toluenesulfonic acid sultone (Berg & Kaiser, 1975). The deacylation reaction is slow since little or no activity was regained upon dialysis of the inactivated enzyme, and the half-lives for deacylation of HL elastase and chymotrypsin were respectively 5.3 and 4.4 h. Although we consider it unlikely, we cannot completely eliminate the possibility that our analytically pure sample of 3-propyl-1*H*-2-benzopyran-1,4(3*H*)-dione contains <1% of a very potent stoichiometric inhibitor of HL elastase. It should also be noted that only one enantiomeric isomer of (**31**) is likely to be involved in the inhibition process.

Alternate inhibition pathways may be involved. One such pathway would involve direct attack of the active site serine residue on the lactone of 3-bromo-3-(1-bromoalkyl)-1(3*H*)-isobenzofuranones (**32**) to give acyl enzyme **35** in which bromo ketone has been released. This acyl bromo ketone could hydrolyze to the same acyl hydroxy ketone (**34**; Figure 7) formed upon reaction with the benzopyrandione (**33**). HL elastase and chymotrypsin A_α, which have been inactivated by either 3-bromo-3-(1-bromobutyl)-1(3*H*)-isobenzofuranone (**12**) or 3-propyl-1*H*-2-benzopyran-1,4(3*H*)-dione (**31**) have similar half-lives for reactivation, and this indicates that the inhibited enzyme structure is similar in both cases. However, any such direct pathway is of minor importance since the initial rate of inactivation is slow compared to the subsequent rate once the benzopyrandione is formed (Figure 3).

The observation that PP elastase and cathepsin G are not inhibited by the purified 2-benzopyran-1,4-diones (**30** and **31**) indicates that a second pathway for inactivation must be entirely responsible for the slow inhibition of these enzymes by 3-bromo-3-(1-bromoalkyl)-(3*H*)-isobenzofuranones. As shown in Tables I and II, extremely high concentrations of inhibitor are required for inactivation. Therefore, the most likely possibility is that unrearranged quantities of 3-bromo-3-(1-bromoalkyl)-1(3*H*)-isobenzofuranones react slowly with the active site serine to give **35** (Figure 7). While no other decomposition products could be detected by TLC, we cannot exclude the possibility that other trace decomposition products are responsible for inhibition of PP elastase and cathepsin G. We did find, however, that 3-(1-bromobutylidene)-(3*H*)-isbenzofuranone (**25**), a likely minor decomposition product of 3-bromo-3-(1-bromobutyl)-(3*H*)-isobenzofuranone (**12**), and the hydrolysis product 2-(2-hydroxypentanoyl)benzoic acid did not inhibit PP elastase or cathepsin G.

Stable Acyl Enzymes. Our results clearly indicate that these inhibitors are forming stable acyl enzymes, and we obtained no evidence that these structures are alkylating an active site residue. The absence of alkylation is indicated by the finding that addition of hydroxylamine to inhibited enzyme resulted in full regain in enzymatic activity with a $t_{1/2}$ of 6–60 min. Long incubation without hydroxylamine also resulted in regeneration of enzyme activity. Alkylation of the side chain of a residue such as histidine or methionine by the inhibitor would have formed an irreversibly inhibited enzyme structure. An inactivated chymotrypsin derivative formed by alkylation of the active site histidine residue by a peptide chloro ketone was stable to reactivation by hydroxylamine.

A number of different types of structures including azapeptides (Gupton et al., 1984; Powers et al., 1984), alkyl isocyanates (Ardelt et al., 1976), 4-nitrophenyl carbamate (Robilland et al., 1972), *N*-aryl- and *N*-acylsaccharins (Zimmerman et al., 1980; Ashe et al., 1981), isatoic anhydride (Moorman & Abeles, 1982), and benzoxazinones (Hedstrom et al., 1984) react with serine proteases to give stable acyl enzymes. Electronic effects that deactivate the carbonyl of the acyl enzyme toward deacylation are thought to be responsible for the stability of the acyl enzymes derived from azapeptides and isatoic anhydride. Geometric effects, which result in a twist of the acyl enzyme carbonyl away from an optimal transition state for deacylation, have been proposed to account for the stability of indolylacryloylchymotrypsin (Henderson, 1970) and carbamoylchymotrypsin (Robillard et al., 1972). In addition, we have proposed that steric features in the acyl enzyme structure may interfere with the attack of a water molecule on the acyl carbonyl group, thus stabilizing the acyl enzyme (Harper et al., 1985).

It is unlikely that the acyl enzymes reported here are stabilized by electronic effects since electron-donating groups are absent, and indeed, the acyl enzyme structures contain electronegative groups which should increase susceptibility toward deacylation. The stability of these acyl enzymes is possibly due to interactions of the inhibitor structure with the enzyme's binding site(s) which results in a twist in the acyl carbonyl,

as has been observed with indolylacryloylchymotrypsin. Additional acyl enzyme stability could also result from hydrogen bonding of the hydroxy ketone moiety of the acyl enzyme with residues such as histidine-57 and the peptide N-H group of residue 216, which are present in all serine proteases, and the sides chains of groups such as glutamine-192 (PP elastase), which are present in some of the enzymes investigated. Other heterocyclic structures such as *N*-aryl- and *N*-acylsaccharins and isobenzthiazolinones have been shown to give stable acyl enzymes with serine proteases (Zimmerman et al., 1980; Ashe et al., 1981) and may derive their stability from similar interactions.

Conclusion. Here we report that several novel structures such as 3-bromo-3-(α-bromobenzyl)-1(3*H*)-isobenzofuranone, react with a number of serine proteases, including HL elastase and cathepsin G, to give stable acyl enzymes but do not alkylate the enzymes. We have also shown that some 3-halo-3-(1-haloalkyl)isobenzofuranones rearrange in buffer solution to give the corresponding 3-alkyl-1*H*-2-benzopyran-1,4-(3*H*)-diones, which are extremely potent inhibitors of serine proteases. In addition, they are very specific since the 2-benzopyran-1,4-diones inactivated HL elastase and chymotrypsin A_α but did not react with the related serine proteases PP elastase and cathepsin G. These 2-benzopyran-1,4-diones are a new and unique class of serine protease inhibitors which we are actively investigating.

ACKNOWLEDGMENTS

We are grateful to Dr. E. Meyer and Leonard Presta at Texas A&M for helpful discussions on the active site structure of PP elastase.

SUPPLEMENTARY MATERIAL AVAILABLE

Syntheses and physical properties of the substituted isobenzofuranones and isocoumarins reported in the paper (5 pages). Ordering information is given on any current masthead page.

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