Suicide Inactivation of Chymotrypsin by Benzoxazinones†

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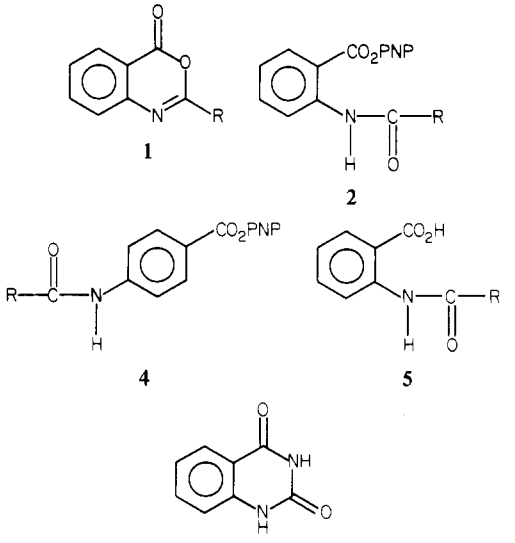
ABSTRACT: The benzoxazinones 2-ethoxy-4*H*-3,1-benzoxazin-4-one (**1a**) and 2-(trifluoromethyl)-4*H*-3,1-benzoxazin-4-one (**1d**) inactivate chymotrypsin. The inactivation is stoichiometric and proceeds with rate constants of 7×10^5 M^{-1} min^{-1} and $>4 \times 10^6$ M^{-1} min^{-1}, respectively. The inactivated enzyme recovers catalytic activity slowly, $k = 2.3 \times 10^{-3}$ min^{-1} and 3.7×10^{-2} min^{-1} (pH 7.1). When the enzyme regains catalytic activity, 2-[*N*-(ethoxycarbonyl)amino]benzoic acid is released from enzyme inactivated with **1a** and *N*-(trifluoroacetyl)anthranilic acid from enzyme inactivated with **1d**. The mechanism of inactivation involves attack of the active site serine on the C-4 carbonyl of the inactivator which leads to ring opening and formation of an ortho-substituted benzoylchymotrypsin, which hydrolyzes slowly due to electron releasing ability of the substituents. The rate of hydrolysis of the benzoylchymotrypsin from **1a** or **1d** is in close agreement with those predicted from the Hammett parameters (σ, ρ) for hydrolysis of their para-substituted analogues [Caplow, M., & Jencks, W. P. (1962) *Biochemistry 1*, 883–893]. The inactivation of chymotrypsin by 2-methyl-4*H*-3,1-benzoxazin-4-one (**1b**) is an equilibrium process ($k_{inact} = 1 \times 10^4$ M^{-1} min^{-1} and $K_{eq} = 2 \times 10^6$ M^{-1}). Formation of a benzoylchymotrypsin is demonstrated by spectral changes and methanol trapping. The benzoylchymotrypsin can also decay by direct hydrolysis to *N*-acetylanthranilic acid. Chymotrypsin is also inactivated by 2-amino-4*H*-3,1-benzoxazin-4-one (**1c**). The inactivation is rapid ($k = 8.7 \times 10^4$ M^{-1} min^{-1}), but reactivation is also relatively fast ($k = 0.14$ min^{-1}). Upon reactivation benzoyleneurea is released. Reaction of **1c** with chymotrypsin leads to the formation of (*o*-ureidobenzoyl)-chymotrypsin, which does not undergo hydrolysis but decomposes through intramolecular attack of the ureido NH_2 upon the carbonyl group of the benzoyl ester. Compound **1a** also inactivates trypsin and elastase. Compound **1b** was a poor inactivator of trypsin and does not inhibit elastase. Both trypsin and elastase catalyze the conversion of **1c** to benzoyleneurea.

We have previously reported the inactivation of chymotrypsin by isatoic anhydride. This compound interacts with chymotrypsin to form CO_2- and *o*-NH_2-substituted benzoylchymotrypsin, which hydrolyzes slowly ($t_{1/2} = 24$ h) (Moorman & Abeles, 1982). The slow hydrolysis is due to the electron-releasing properties of the $-NH_2$ group ($\sigma_p = -0.66$). An important feature of the inhibition is that the $-NH_2$ group is masked and does not become expressed until the benzoyl enzyme is formed. We have now explored the interaction of chymotrypsin and other serine proteases with the benzoxazinones **1a–d** (for structures, see Chart I). It was expected that these compounds could inactivate through a mechanism similar to that which occurs with isatoic anhydride, i.e., formation of an ortho-substituted benzoyl enzyme and concomitant unmasking of an electron-releasing group. A previous study of the interaction of **1b** and **1d** with chymotrypsin reported that these compounds are competitive inhibitors (Teshima et al., 1982). Others have reported that **1b** is an inactivator (Alazard et al., 1973). Additionally, it was reported that other compounds related to **1** (R = CF_2CF_3, $CH_2C_6H_5$, ...) are competitive inhibitors, but not inactivators, of chymotrypsin (Teshima et al., 1982).

In this paper, we report the results of our investigation of the mechanism of inhibition of chymotrypsin and other serine proteases by **1a–d**.

Chart I: Structures of Compounds[a]



[a] a, R = EtO; b, R = CH_3; c, R = NH_2; d, R = CF_3; PNP = $-C_6H_4$-*p*-NO_2.

Experimental Procedures

Materials. Chymotrypsin was supplied by Worthington Biochemicals. Benzoyleneurea was purchased from Aldrich. Trypsin, porcine pancreatic elastase, alcohol dehydrogenase, benzoyltyrosine ethyl ester, *N*-*t*-Boc-L-Ala-*p*-nitrophenyl ester[1] and α-*N*-benzoyl-DL-arginine-*p*-nitroanilide were obtained from Sigma. 2-(Trifluoromethyl)-4*H*-3,1-benzoxazin-4-one (**1d**) was a gift from Dr. T. G. Payne, Sandoz, Ltd.

Enzyme Assays. All spectrophotometric measurements were made with a Perkin-Elmer 559 or Lamda 3 UV–vis spectrophotometer. Chymotrypsin was assayed by following the hydrolysis of benzoyltyrosine ethyl ester (BTEE) spec-

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[1] Abbreviations: *t*-Boc, *tert*-butoxycarbonyl; MOPS, 3-(*N*-morpholino)propanesulfonic acid; Me_2SO, dimethyl sulfoxide; TLC, thin-layer chromatography; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

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trophotometrically at 256 nm (Hummel, 1959). Aliquots of enzyme (0.01 mg) were incubated with 580 μM BTEE in 0.1 M potassium phosphate, pH 6.8, in 5% (v/v) acetonitrile at 25 °C.

Trypsin was assayed by following the hydrolysis of α-*N*-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA) at 410 nm. Aliquots of enzyme in 50 mM potassium MOPS,[1] pH 6.8, and 0.5 mM $CaCl_2$ were incubated with 1 mM BAPNA, 50 mM potassium MOPS, pH 6.8, 0.5 mM $CaCl_2$, and 5% (v/v) Me_2SO.

Elastase was assayed by following the hydrolysis of *N*-*t*-Boc-L-Ala-*p*-nitrophenyl ester at 400 nm. The assay cocktail contained 0.1 M potassium phosphate, pH 6.8, 0.5 mM substrate and 5% (v/v) acetonitrile.

Inactivation Rates. The rate of inactivation was measured by adding inactivator in acetonitrile or Me_2SO to enzyme in 0.1 M phosphate buffer, pH 6.8 (chymotrypsin and elastase), or 50 mM MOPS, pH 6.8–0.5 mM $CaCl_2$ (trypsin) and assaying aliquots at appropriate time intervals as described above.

When enzymes were inactivated with *p*-nitrophenyl esters, release of *p*-nitrophenol was followed by increase in absorbance at 400 nm. The absorption coefficient was 6500 M^{-1} in 0.1 M potassium phosphate, pH 6.8. The inactive enzyme was dialyzed against 0.1 M potassium phosphate buffer, pH 6.8 or 7.1, before spectra were taken or reactivation followed.

The second-order rate constants for inactivation were calculated by using the equation:

$$\frac{1}{[I]^0 - [E]^0} \ln \frac{[E]^0[I]}{[I]^0[E]} = kt$$

where $[I]^0$ and $[E]^0$ are the initial concentrations of inactivator and enzyme respectively (Moore & Pearson, 1981).

Reactivation of Chymotrypsin Inactivated with **1c**. Chymotrypsin, 13 μM, was treated with 620 μM **1c** in 10 mM potassium acetate buffer, pH 4.5, for 15 min. Under these conditions 83% inactivation was observed. The solution (0.5 mL) was applied to a 10-mL Sephadex G-25 column equilibrated with 0.1 M potassium phosphate buffer, pH 6.8 at 4 °C. The protein fractions were collected and assayed.

Determination of Ethanol Released during Inactivation. Chymotrypsin (208 nmol) in 0.1 M phosphate, pH 6.8 (0.5 mL), was treated with **1a** (210 nmol). Under these conditions the enzyme was greater than 99% inactive. The sample was frozen and the solvent collected by bulb to bulb distillation. The distillate was assayed for ethanol with alcohol dehydrogenase (Bergemeyer, 1974).

Synthesis of 2-[N-(Ethoxycarbonyl)amino]benzoic Acid. 2-[*N*-(Ethoxycarbonyl)amino]benzoic acid was synthesized as described previously; mp 127–128.5 °C [lit. mp 126–128 °C (Blank et al., 1968)].

Synthesis of 2-Ethoxy-4H-3,1-benzoxazin-4-one (**1a**). 2-[*N*-(Ethoxycarbonyl)amino]benzoic acid (1 g, 4.8 mmol) was dissolved in 10 mL of dry ethyl acetate and chilled to 0 °C. This solution was treated with dicyclohexylcarbodiimide (1.1 g, 5.3 mmol) in 4 mL of ethyl acetate and stirred 3 h at 0–5 °C. The reaction was stirred overnight at room temperature and then filtered to remove the dicyclohexylurea. The solvent was removed in vacuo. The 2-ethoxy-4*H*-3,1-benzoxazin-4-one was recrystallized from 2-propanol to yield white crystals; mp 91 °C [lit. mp 90–92 °C (Ecsery et al., 1978)]. Anal. Calcd: C, 62.83; H, 4.71; N, 7.33. Found: C, 63.10; H, 4.84; N, 7.38.

Synthesis of p-[N-(Ethoxycarbonyl)amino]benzoic Acid. *p*-Aminobenzoic acid (6.9 g, 50 mmol) was dissolved in chloroform with triethylamine (10.1 g, 100 mmol) and added to excess ethyl chloroformate (16.2 g, 150 mmol) in chloroform at 4 °C with stirring. The solution was stirred overnight at room temperature and extracted with water, and the chloroform was removed in vacuo. The white solid was treated with pyridine/water at room temperature overnight to yield the free acid. Solvent was removed in vacuo, and the product was recrystallized to yield white crystals; mp 198–200 °C [lit. mp 206 °C (Exner & Lakomy, 1970)].

Synthesis of 2-Methyl-4H-3,1-benzoxazin-4-one (**1b**). Compound **1b** was prepared by refluxing *N*-acetylanthranilic acid with acetic anhydride as described by Errede et al. (1977); mp 79–81 °C [lit. mp 78–80 °C (Teshima et al., 1982)] [lit. mp 86–87 °C (Errede et al., 1977)].

Synthesis of 2-Amino-4H-3,1-benzoxazin-4-one (**1c**). Compound **1c** was prepared as described previously; mp 208–210 °C (if heated rapidly to 200 °C) [lit. mp 210–212 °C (Hegarty & Bruice, 1970)].

Synthesis of 2-Ureidobenzoic Acid and 4-Ureidobenzoic Acid. The ureido compounds were obtained by treating the analogous aminobenzoic acids with potassium isocyanate (Hegarty & Bruice, 1970); 2-ureidobenzoic acid mp 170–171 °C (lit. mp 170–171 °C (Hegarty & Bruice, 1970)] and 4-ureidobenzoic acid mp >248 °C [lit. mp >300 °C (Exner & Lakomy, 1970)]. Anal. Calcd: C, 53.33; H, 4.44; N, 15.56. Found: C, 53.16; H, 4.47; N, 15.24.

Synthesis of N-(Trifluoroacetyl)anthranilic Acid. Anthranilic acid (2.8 g, 20 mmol) was treated with trifluoroacetic anhydride (5.3 g, 25 mmol) in trifluoroacetic acid (10 mL) (Teshima et al., 1982). The product was precipitated by addition of water, filtered, redissolved in ethyl acetate, and extracted with 1 N HCl. The solvent was removed in vacuo, and the white solid recrystallized from ethyl acetate–hexane; mp 181–183 °C [lit. mp 180–181 °C (Teshima et al., 1982)].

Synthesis of p-Nitrophenyl Esters. *p*-Nitrophenyl esters of 2-[*N*-(ethoxycarbonyl)amino]benzoic acid, *p*-[*N*-(ethoxycarbonyl)amino]benzoic acid, *p*-(*N*-acetylamino)benzoic acid, and *p*-ureidobenzoic acid were prepared from the free acids by treatment with thionyl chloride and *p*-nitrophenol (Kirsch et al., 1968): *p*-nitrophenyl *p*-[*N*-(ethoxycarbonyl)amino]-benzoate, flaky white crystals, yield 36%, mp 180–182 °C; *p*-nitrophenyl 2-[*N*-(ethoxycarboxyl)amino]benzoate, fibrous white crystals, yield 22%, mp 131–132 °C; *p*-nitrophenyl *p*-(*N*-acetylamino)benzoate, orange crystals, yield 67%, mp 217–219 °C; *p*-nitrophenyl *p*-ureidobenzoate, mp 216–218 °C.

p-Nitrophenyl *N*-acetylanthranilate was synthesized by acetylation of *p*-nitrophenyl anthranilate as described by Cremin & Hegarty (1977); yield 34%; mp 123–126 °C [lit. mp 97–109 °C (Cremin & Hegarty, 1977)].

The identity of the *p*-nitrophenyl ester was confirmed by base hydrolysis. The amount of *p*-nitrophenol released agreed well with that expected (±10%), and the products were identified as the analogous acids by TLC on silica with 1/1 methanol/chloroform. No anthranilic acid was identified by TLC of the *p*-nitrophenyl *N*-acetylanthranilate hydrolysate (system A).

Synthesis of Methyl Esters. Methyl *N*-acetylanthranilate, methyl *N*-(trifluoroacetyl)anthranilate, and methyl 2-[*N*-(ethoxycarbonyl)amino]benzoate were synthesized by treatment of the corresponding acid with excess diazomethane in methanol. The products were recrystallized from methanol: methyl *N*-acetylanthranilate, mp 98.5–100 °C [lit. mp 95–98 °C (Cremin & Hegarty, 1977)]; methyl *N*-(trifluoroacetyl)-anthranilate, mp 63.5–65.5 °C [lit. mp 62.5–64 °C (Sweeny et al., 1971)]; methyl 2-[*N*-(ethoxycarbonyl)amino]benzoate, mp 59–60.5 °C. The nuclear magnetic resonance spectrum

Table I: R_f Values

compound	R_f values for system A	B	C	D
1a	0.85		0.50	
2-([*N*-(ethoxycarbonyl)amino]benzoic acid	0.68		0.88	
1b	0.79		0.65	
N-acetylanthranilic acid	0.63		0.91	
1c		0.62		0.44
2-ureidobenzoic acid		0.20		0.96
benzoyleneurea		0.56		0.63
1d			0.44	
N-(trifluoroacetyl)anthranilic acid	0.66		0.88	

Table II: Rates of Inactivation of Chymotrypsin[a]

inactivator	$k \times 10^{-3}$ (M^{-1} min^{-1})	inactivator	$k \times 10^{-3}$ (M^{-1} min^{-1})
1a	≥700[b]	**2a**	5.5
1b	11[c]	4a	0.5
1c	87	4b	42
1d[d]	>4000	4c[e]	87

[a] Inactivation carried out as described under Experimental Procedures. Chymotrypsin and inactivator at 35 μM, pH 6.8. [b] Inactivation was complete within 2 min under these conditions. The rate constant is, therefore, an estimate. [c] Alazard et al. (1973) reported $k = 9600\ M^{-1}\ min^{-1}$ at pH 7.08 in 0.025 M Hepes–0.5 M NaCl at 25 °C. [d] Chymotrypsin and inactivator at 17 μM. Inactivation complete within 30 s. [e] Solution contained 2.5% Me_2SO in place of acetonitrile.

shows the expected absorbances.

Isolation of the Product of the Reaction of **1c** *with Chymotrypsin.* Compound **1c** (90 μmol, 24 mM in acetonitrile) was added to chymotrypsin (1.2 mg/mL, 100 mL) in 0.1 M potassium phosphate, pH 6.8, in 200-mL aliquots at 2-h intervals to avoid nonenzymatic hydrolysis of **1c**. The solution was treated with KCl and the product isolated by ethyl acetate extraction.

Thin-Layer Chromatography. Four systems were used in the course of this study: system A, 50/50 $CH_3OH/CHCl_3$ on silica gel (Eastman Kodak 13181); system B, CH_3CN on silica gel; system C, 60/40 CH_3OH/H_2O on reverse-phase C_{18} silica gel (Analtech RPS-F); system D, 15/85 CH_3CN/H_2O on reverse-phase C_{18} silica gel. R_f values are summarized in Table I.

Infrared Spectroscopy. Mineral oil mulls of **1c**, 2-ureidobenzoic acid, benzoyleneurea, and **1c**–chymotryspin reaction product were analyzed on a PE 683 infrared spectrophotometer.

Results

Inactivation of Chymotrypsin by 2-Ethoxy-4H-3,1-benzoxazin-4-one (**1a**). Addition of **1a** to chymotrypsin results in rapid inactivation of the enzyme (Table II). When equimolar amounts of **1a** were added to chymotrypsin (17 μM), complete inactivation (>98%) of the enzyme occurred. Reaction of 23 μM chymotrypsin with 13 μM **1a** results in 52% inactivation. The inactivation, therefore, is stoichiometric. The inactivated enzyme does not regain activity after dialysis against 0.1 M phosphate buffer, pH 6.8, at 4 °C for 4 h, or on 10-fold dilution. Activity is recovered after prolonged incubation at 25 °C. Rates of reactivation are listed in Table III. No ethanol is released when the enzyme is inactivated by **1a** or when the inactivated enzyme regains catalytic activity.

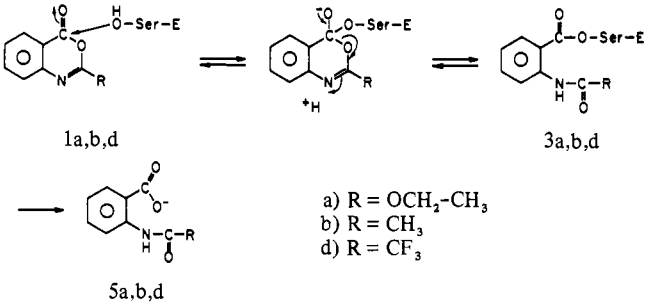
Figure 1 shows the spectrum of chymotrypsin inactivated with **1a** and the spectra of **1a** and the ester methyl 2-[*N*-(ethoxycarbonyl)amino]benzoate. The spectrum of the inactivated enzyme resembles that of the ester; i.e., it has absorbances at 248 (ϵ = 10 000–13 000 M^{-1}) and 312 nm (ϵ = 3300 M^{-1}). The ester absorbs at 242 (ϵ = 10 000 M^{-1}) and 302 nm (ϵ = 3200 M^{-1}).

Table III: Rate of Reactivation of Inactivated Chymotrypsin[a]

inactivator	pH 6.8, $k \times 10^3$ (min^{-1})	pH 7.1, $k \times 10^3$ (min^{-1})	calculated[b] $k \times 10^3$ (min^{-1})
1a	1.0	2.3	
2a	0.8	2.3	
4a	1.8	3.6	4.6
1b	3.1	3.9[d]	
4b[c]	7.7	13	8.8
1c[e]	140		
4c	6.1	7.2	2.8
1d[c]	15	37	
4d			15

[a] Inactivation as described under Experimental Procedures: chymotrypsin, 18 μM; inactivator, 20 μM. The inactivated enzyme was dialyzed for 3 h at 4 °C against 0.1 M potassium phosphate buffer, pH 7.1 or pH 6.8. The reactivations were followed at 25 °C by assaying as described under Experimental Procedures. [b] Rates of reactivation were calculated by using Hammett parameters determined by Jencks & Caplow (1962) for the hydrolysis of substituted benzoylchymotrypsins in 0.1 M potassium phosphate buffer, pH 7.07. The following σ values were used: $-NHCO_2C_2H_5$, $\sigma_p = -0.15$; $-NHCOCH_3$, $\sigma_p = 0.00$; $-NHCONH_2$, $\sigma_p = -0.24$; $-NHCOCF_3$, $\sigma_p = 0.12$ (Hansch et al., 1973). [c] Dialysis after inactivation was omitted. [d] Alazard et al. (1973) reported $k = 3 \times 10^{-3}\ min^{-1}$ at pH 7.50 at 25 °C. [e] See Experimental Procedures.

Scheme I: Mechanism of Inactivation of Chymotrypsin



These observations suggested that inactivation occurs by attack of the active site serine on the carbonyl at position 4 of the inactivator, followed by opening of the heterocyclic ring to form a stable substituted benzoylchymotrypsin as shown in Scheme I.

To test the mechanism shown in Scheme I, *p*-nitrophenyl 2-[*N*-(ethoxycarbonyl)amino]benzoate (**2a**) was added to chymotrypsin. This resulted in inactivation of chymotrypsin and concomitant release of *p*-nitrophenol. The rate of *p*-nitrophenol release closely paralleled the loss of catalytic activity, and the total amount of *p*-nitrophenol released was equivalent to the amount of chymotrypsin present. No *p*-nitrophenol was released in the absence of enzyme. These results indicate that **2a** reacts with chymotrypsin to form a benzoyl enzyme of structure **3a**. The rate constants for the formation and hydrolysis of **3a** are given in Tables II and III. The data in Table III show that chymotrypsin inactivated with **1a** and **2a** recovers activity at the same rate; hence, both **1a** and **2a** react with chymotrypsin to form the same benzoyl enzyme intermediate as required by Scheme I.

When chymotrypsin, which has been inactivated with **1a**, regains catalytic activity, 2-[*N*-(ethoxycarbonyl)amino]benzoic acid (**5a**) should be released according to the mechanism shown in Scheme I. Chymotrypsin (32 μM), inactivated with 1 equiv of **1a**, was incubated at 25 °C in 0.1 M potassium phosphate

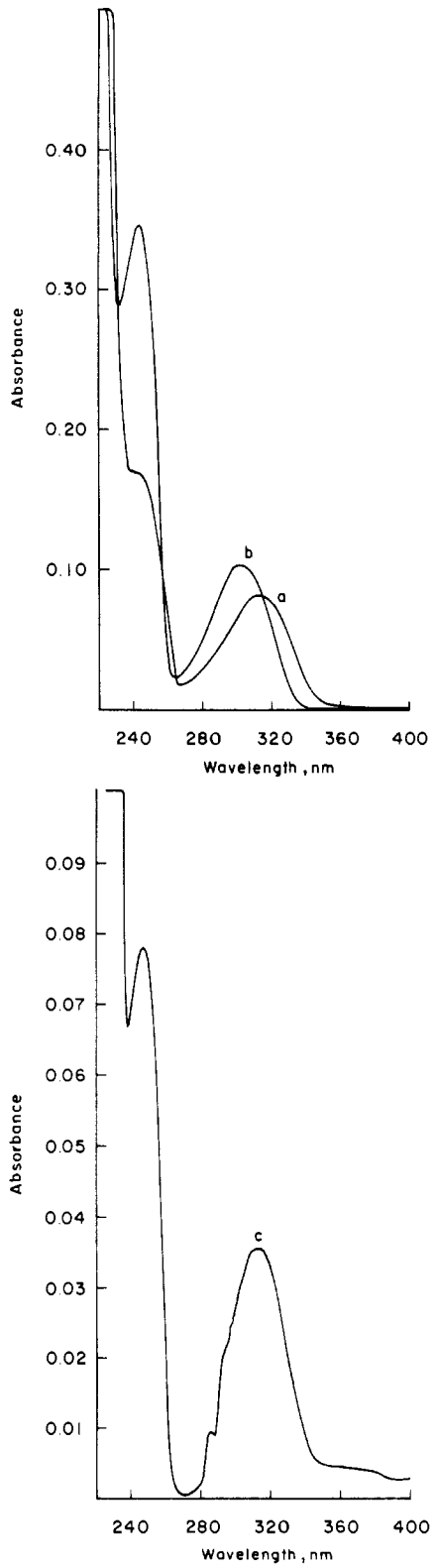


FIGURE 1: Inactivation of chymotrypsin with 2-ethoxy-4*H*-3,1-benzoxazin-4-one (**1a**). (a) 19 μM **1a** in 5% CH_3CN–0.1 M potassium phosphate buffer, pH 6.8. (b) Methyl-2-[*N*-(ethoxycarbonyl)-amino]benzoate, 35 μM, in 5% CH_3CN–0.1 M potassium phosphate buffer, pH 6.8. (c) Chymotrypsin, 7.4 μM, inactivated with **1a** in 0.1 M potassium phosphate buffer, pH 6.8.

buffer, pH 7.1, for 10 h. Under these conditions 65% of the activity was recovered. The protein was precipitated with methanol and the solvent removed in vacuo. The residue was redissolved in H_2O, acidified with HCl, and extracted with ethyl acetate. The product isolated was 2-[*N*-(ethoxycarbonyl)amino]benzoic acid (**5a**) as identified by its ultraviolet spectrum and thin-layer chromatography (systems A and C). The amount of **5a** recovered corresponded to 88% of the reactivated enzyme. No compound **1a** or anthranilic acid was observed. These results taken together provide strong support for the mechanisms of inactivation of chymotrypsin by **1a** shown in Scheme I.

Inactivation by 2-Methyl-4H-3,1-benzoxazin-4-one (**1b**). Compound **1b** is a time-dependent inactivator of chymotrypsin (Table II). Reactivation was slow at 25 °C (Table III). Unlike compound **1a**, the inactivation was not stoichiometric. When chymotrypsin (17 μM) was incubated with an equimolar amount of **1b**, 84% inactivation was observed; treatment of enzyme with 1.5 equiv of **1b** resulted in only 93% inactivation. This suggests that an equilibrium exists between free chymotrypsin, **1b**, and inactive enzyme–**1b** complex. To confirm the existence of an equilibrium, chymotrypsin (17 μM) was treated with equimolar **1b** and then diluted 10-fold. The activity increased from 18% to 40% on dilution, as predicted by an equilibrium reaction. The equilibrium constant was calculated from the observed activity of the enzyme, $K_{eq} = 2 \times 10^6$ M^{-1}, in 0.1 M potassium phosphate buffer, pH 7.1.

Spectral changes were observed when chymotrypsin was inactivated with **1b**. The absorbance shifted from a shoulder at 244 nm (ϵ = 7700 M^{-1}) to a clear peak at 250 nm (ϵ = 16 000 M^{-1}) and from a peak at 302 (ϵ = 3550 M^{-1}) to 308 nm (ϵ = 6700 M^{-1}). These spectral changes suggest that **1b** is chemically modified upon interaction with chymotrypsin.

To further characterize the mechanism of inactivation of chymotrypsin by **1b**, the product(s) formed when inactivated enzyme recovers activity was (were) identified. Chymotrypsin, 0.35 mM, inactivated with equimolar **1b** (98% inactive) was incubated at 25 °C in 0.1 potassium phosphate buffer, pH 7.1, for 10 h. At that time, 68% of the catalytic activity was recovered. The product isolated as described for **1a** was identified as *N*-acetylanthranilic acid (**5b**) by thin-layer chromatography (systems A and C). The amount of *N*-acetylanthranilic acid isolated was 79% of the maximal amount which could have been formed.

Two mechanisms may also be envisioned for the reactivation of chymotrypsin treated with **1b**: (1) the benzoyl enzyme can hydrolyze directly or (2) the complex formed between chymotrypsin and **1b** can dissociate and free **1b** can hydrolyze nonenzymatically. In both cases *N*-acetylanthranilic acid, the observed product, would be formed. The rate constant for nonenzymatic hydrolysis is 3.6×10^{-3} min^{-1} at pH 7.1, which is the same as the observed rate of reactivation. However, at equilibrium, under the experimental conditions employed, the concentration of free **1b** is too small to account for the rate of product formation. Therefore, the benzoylchymotrypsin decomposes predominantly through direct hydrolysis as depicted in Scheme I.

To further demonstrate the existence of a substituted benzoylchymotrypsin, the rate constants for the recovery of enzyme activity and nonenzymatic decay of **1b** were determined in the presence of methanol. Previous work has shown that methanol can compete effectively with water for acyl enzymes (Fastrez & Fersht, 1973). Therefore, it should be possible to trap a benzoylchymotrypsin with methanol, thereby increasing the rate of reactivation, without affecting the nonenzymatic decomposition of **1b**. As shown in Table IV, methanol markedly increased the rate of reactivation but had a smaller effect on the nonenzymatic breakdown of **1b**. This suggests that a benzoylchymotrypsin is indeed formed.

Attempts to use *p*-nitrophenyl *N*-acetylanthranilate to generate benzoylchymotrypsin **3b** were thwarted by the ob-

Table IV: Effect of Methanol on Reaction Rates

reaction	$k \times 10^3$ (min^{-1}) no MeOH	$k \times 10^3$ (min^{-1}) +5% MeOH
hydrolysis of **1b**[a]	3.6	5.0
recovery of activity of chymotrypsin inactivated with **1b**[b]	3.9	10

[a] In 0.1 M potassium phosphate buffer, pH 7.1 at 25 °C. [b] Chymotrypsin, 17 μM; **1b**, 17 μM; in 0.1 M potassium phosphate buffer, pH 7.1 at 25 °C.

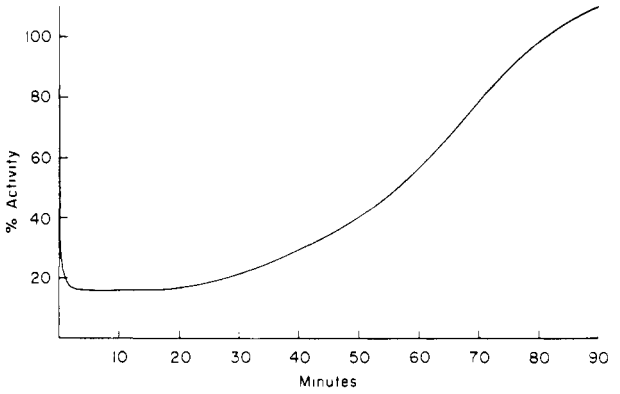


FIGURE 2: Time course of inactivation of chymotrypsin with 2-amino-4*H*-3,1-benzoxazin-4-one (**1c**). Chymotrypsin, 7.4 μM, and **1c**, 74 μM, in 5% Me_2SO–0.1 M potassium phosphate buffer, pH 6.8.

servation that the rate of *p*-nitrophenol release was much faster than the rate of inactivation and occurred equally rapidly in the absence of enzyme. Cremin & Hegarty (1977) have reported that the *p*-nitrophenyl *N*-acetylanthranilate cyclized readily to form **1b** with release of *p*-nitrophenol, which would explain the above observations.

Reaction of Chymotrypsin with 2-Amino-4H-3,1-benzoxazin-4-one (**1c**). Incubation of **1c** with chymotrypsin resulted in rapid, but transient, loss of activity (Table II). As seen in Figure 2, at 10:1 ratios of **1c** (75 μM) to enzyme (7.5 μM) the activity was completely recovered within 90 min. Total inactivation was not observed under these conditions. These results suggested that **1c** is a substrate for chymotrypsin. When the reaction was followed spectroscopically, the absorbances due to **1c** at 256 and 333 nm decreased while a new chromophore with absorbance at 309 nm appeared (Figure 3). The spectrum of the new chromophore was identical with that of benzoyleneurea and distinct from the expected hydrolysis product 2-ureidobenzoic acid.[2] The product resulting from the action of chymotrypsin on **1c** was isolated, as described under Experimental Procedures, and its identity as benzoyleneurea confirmed by thin-layer chromatography (systems B and D) and infrared spectroscopy. Only trace amounts of 2-ureidobenzoic acid consistent with nonenzymatic hydrolysis of **1c** were observed. No benzoyleneurea was formed by incubation of **1c** in the absence of enzyme. It is likely that the reaction of **1c** with chymotrypsin gives rise to 2-ureidobenzoylchymotrypsin. This adduct does not undergo hydrolysis but decomposes through an intramolecular attack of the ureido NH_2 on the carbonyl group as shown in Scheme II.

Inactivation by 2-(Trifluoromethyl)-4H-3,1-benzoxazin-4-one (**1d**). Addition of **1d** (17 μM) to chymotrypsin (17 μM) resulted in instantaneous inactivation (>95%) of the enzyme

[2] 2-Ureidobenzoic acid has an absorbance at 296 nm, $\epsilon = 2800$ M^{-1} in 0.1 M potassium phosphate, pH 6.8.

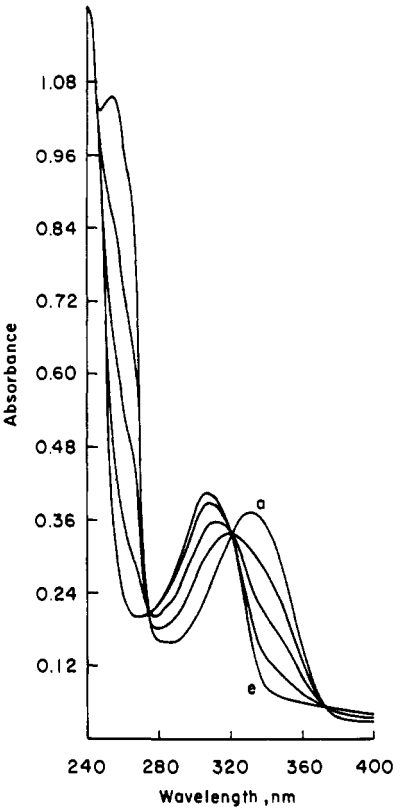
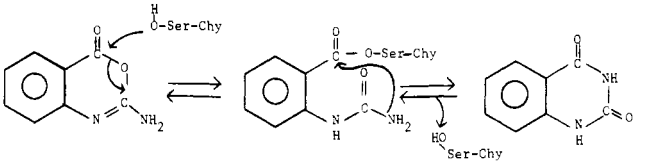


FIGURE 3: Reaction of chymotrypsin with 2-amino-4*H*-3,1-benzoxazin-4-one (**1c**). For conditions see Figure 2. Spectrum taken at 20-min intervals. (a) Spectrum prior to addition of enzyme; (e) final spectrum.

Scheme II: Mechanism of Benzoyleneurea Formation



(Table II). A new chromophore is generated (Figure 4), which resembles the spectrum of the ester methyl *N*-(trifluoroacetyl)anthranilate.[3] The chromophore decays with a first-order rate constant $k = 0.016$ min^{-1}, which is identical with that observed for the recovery of enzymatic activity, $k = 0.015$ min^{-1} (Table III). These results suggest that **1d** also reacts with chymotrypsin to form an ortho-substituted benzoylchymotrypsin as described in Scheme I.

Reaction of Chymotrypsin with Para-Substituted p-Nitrophenyl Benzoates. Interaction of chymotrypsin with **1a** and **1b** most probably gives rise to benzoylchymotrypsin **3a** and **3b**, which hydrolyze slowly. To determine if this slow hydrolysis is primarily due to electronic effects of the substituents, the reaction of chymotrypsin with *p*-nitrophenyl *p*-[*N*-(ethoxycarbonyl)amino]benzoate (**4a**) and *p*-nitrophenyl *p*-(*N*-acetylamino)benzoate (**4b**) was examined. Both of these compounds inactivate chymotrypsin (Table II). The recovery of activity of chymotrypsin inactivated with **4a** was first order with a rate constant of 3.6×10^{-3} min^{-1} at pH 7.1 (Table III). This is comparable to the rate constant of 2.3×10^{-3} min^{-1} observed for the reactivation of chymotrypsin inactivated with **1a**. Compounds **1a** and **4a** give rise to benzoylchymotrypsin

[3] Methyl *N*-(trifluoroacetyl)anthranilate has absorbances at 296 ($\epsilon = 2900$ M^{-1}) and 248 nm ($\epsilon = 8600$ M^{-1}) in 5% CH_3CN–0.1 M potassium phosphate buffer, pH 6.8.

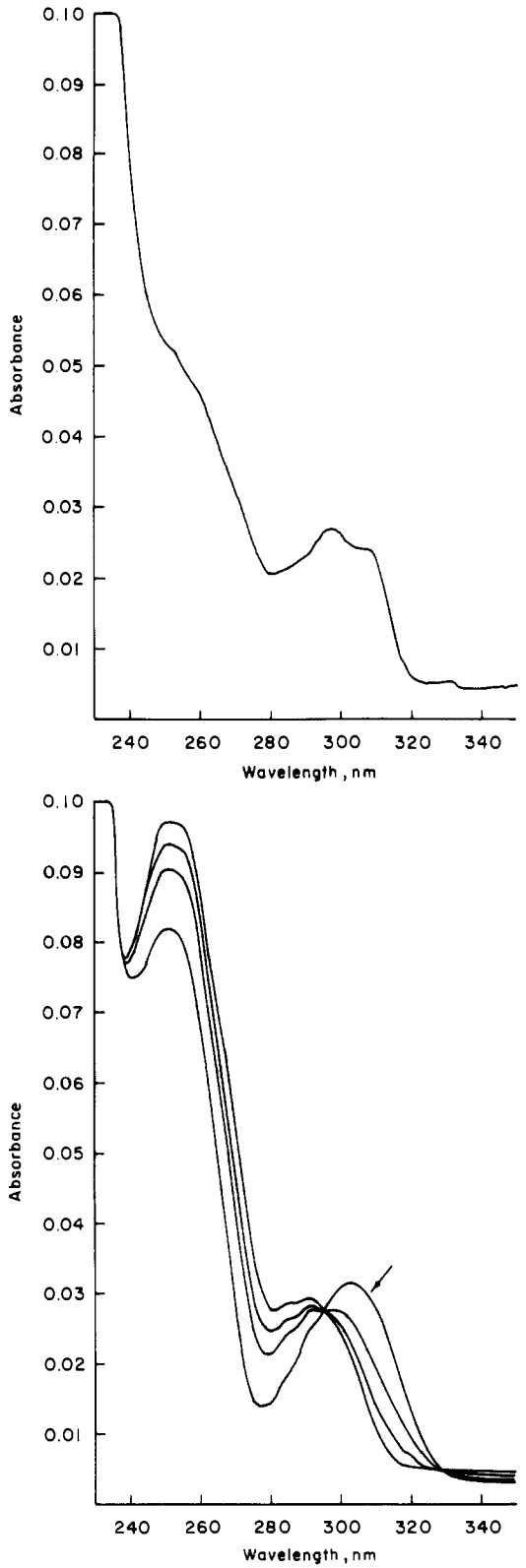


FIGURE 4: Reaction of chymotrypsin with 2-(trifluoromethyl)-4*H*-3,1-benzoxazin-4-one (**1d**). (Top) Spectrum of 8.5 μM **1d** in 0.1 M potassium phosphate buffer, pH 6.8. (Bottom) The arrow denotes the intial spectrum of 8.5 μM **1d** added to 8.5 μM chymotrypsin, in 0.1 M potassium phosphate, pH 6.8. The subsequent spectra are taken at intervals of 30, 60, and 120 min.

with the same substituent on the phenyl ring. With **1a**, the substituent is in the ortho position while **4a** gives rise to a para-substituted adduct. Since both benzoylchymotrypsins hydrolyze at the same rate, the slow hydrolysis rate must be primarily due to the electronic effect of the substituent.

Table V: Inactivation of Other Serine Proteases

compound	rate of inactivation $\times 10^{-3}$ (min^{-1}) trypsin	elastase
1a	90[a]	300[d]
1b	1[b]	*e*
1c	*c*	*f*

[a] 80 μM **1a** and 80 μM trypsin, complete inactivation within 2 min; no activity returned in 2.5 h. [b] 88 μM **1b** and 88 μM trypsin, 60% inactivation observed after 25 min; no activity returned in 2 h. [c] 1.8 mM **1c** and 80 μM trypsin, maximum of 50% inactivation observed after 5 min; full activity recovered within 65 min. [d] 35 μM **1a** and 33 μM elastase in 0.1 M potassium phosphate, pH 6.8; complete inactivation within 2 min; full activity recovered within 2 h. [e] 200 μM **1b** and 24 μM elastase in 0.1 M potassium phosphate, pH 6.8; maximum of 35% inactivation observed after 5 min; full activity was recovered within 65 min. [f] 175 μM **1c** and 33 μM elastase in 0.1 M potassium phosphate, pH 6.8; no significant inactivation observed (<15%).

Chymotrypsin inactivated with **4b** recovered activity with a first-order rate constant of 13×10^{-3} min^{-1} at pH 7.1, which is 3 times larger than that observed for enzyme inactivated with **1b** (Table III). These results suggest that the rate of hydrolysis is influenced by other factors in addition to the electronic effects of the substituent.

Data presented above suggest that (*o*-ureidobenzoyl)chymotrypsin does not undergo the normal hydrolysis reaction. In order to estimate how fast (*o*-ureidobenzoyl)chymotrypsin undergoes hydrolysis, the inactivation of chymotrypsin with *p*-nitrophenyl *p*-ureidobenzoate (**4c**) was investigated (Table II). The enzyme recovered activity with a first-order rate constant of 6.1×10^{-3} min^{-1} at pH 6.8 (Table III). This value is 20 times slower than that observed for the reactivation of **1c**-inactivated chymotrypsin with the formation of benzoyleneurea (Table III). Therefore, decomposition of (*o*-ureidobenzoyl)chymotrypsin through intramolecular attack of the ureido NH_2 predominates.

Caplow & Jencks (1962) have determined the Hammett parameters for the hydrolysis of substituted benzoylchymotrypsins. The data in Table III show that the predicted hydrolysis rates, based on σ_p values, were in reasonable agreement with the observed values.

The rate constant for the hydrolysis of [*p*-[*N*-(trifluoroacetyl)amino]benzoyl]chymotrypsin was not determined experimentally but can be calculated from the Hammett parameters (see Table III). This value is comparable to that observed for the reactivation of **1d**-inactivated chymotrypsin. This suggests that the stability of the benzoylchymotrypsin is due to the CF_3CONH substituent.

Inactivation of Other Serine Proteases. Compounds **1a–c** were tested with trypsin and porcine pancreatic elastase in addition to chymotrypsin (Table V). Trypsin was completely inactivated by stoichiometric quantities of **1a**; no activity (i.e., <3%) was recovered in 2.5 h. Equimolar amounts of **1b** resulted in a maximum of 60% inactivation of trypsin, although no significant recovery of activity was observed in 2 h. Complete inactivation (>90%) could be obtained with 10-fold excess of **1b** (1.6 mM) to enzyme (160 μM). Some activity ($\sim$10%) was recovered on 10-fold dilution of trypsin inactivated with **1b** (10:1 **1b**:enzyme). This suggests that an equilibrium exists between free trypsin, **1b**, and enzyme–**1b** adduct, as observed with chymotrypsin. Compound **1c** did not inactivate trypsin when present in stoichiometric amounts, although some inactivation was observed when **1c** was present in excess (50% inactivation after 5 min with 1.8 mM **1c** to 80 μM trypsin; full activity was recovered in 65 min). The product of the reaction of **1c** and trypsin was benzoyleneurea

as evidenced by UV spectral analysis.

Elastase was completely inactivated by stoichiometric quantities of **1a**, although full activity was recovered within 2 h (Table V). No significant inactivation was observed with **1b** or **1c** (Table V). Elastase catalyzed the conversion of **1c** to benzoyleneurea.

Discussion

The benzoxazinones described here are effective inactivators of chymotrypsin. These compounds react with the active site serine to form ortho-substituted benzoylchymotrypsins (structure **3**, Scheme I). These intermediates are stable due to the electron-donating properties of the ortho substituents. The substituted benzoylchymotrypsins derived from **1a** and **1d** decompose by hydrolysis. The rate of hydrolysis (reactivation) agrees with that predicted by the Hammett parameters (σ, ρ) (Caplow & Jencks, 1962). No reversal of the benzoylation of chymotrypsin by **1a** or **1d** was detected; i.e., the rate of the reverse reaction is at least 10-fold slower than hydrolysis of the benzoyl enzyme. Unlike **1a** and **1d**, the reaction of chymotrypsin with **1b** is reversible. An equilibrium is established between **1b**, enzyme, and **1b**–enzyme adduct ($K_{eq} = 2 \times 10^6$ M^{-1} min^{-1}). Previous work has shown that activated esters of *N*-acetylanthranilic acid readily cyclize to form **1b** (Cremin & Hegarty, 1977). While the **1b**–chymotrypsin adduct can decay by internal cyclization, reactivation most likely results from hydrolysis in our experiments. Under sufficiently dilute conditions reactivation can also occur by reversal of the benzoylation reactions.

The benzoyl enzyme formed by the reaction of **1c** and chymotrypsin decomposes through an internal cyclization reaction to produce benzoyleneurea (Scheme II). The rate of the internal cyclization is 20-fold faster than would be expected for hydrolysis of (*o*-ureidobenzoyl)chymotrypsin.

Our findings that compounds **1a** and **1d** are inactivators of chymotrypsin differ from a previous report that these compounds are reversible inhibitors (Teshima et al., 1982). This discrepancy probably results from the different conditions of the experiments. While this study preincubated equimolar enzyme and inactivator before assay, previous work used large excesses of inactivator to enzyme in the presence of substrate, thus masking the effects noted here.

The inactivation of chymotrypsin by 2-substituted benzoxazinones is an extension of a principle previously illustrated by the inactivation of chymotrypsin by isatoic anhydride (Moorman & Abeles, 1983), i.e., unmasking of an electron-donating substituent concomitant with formation of benzoyl enzyme to generate a stable intermediate. These compounds offer the opportunity to obtain inactivators which can suppress enzyme activity for various periods of time. Chymotrypsin inactivated with isatoic anhydride recovers activity with $t_{1/2}$ = 24 h (Moorman & Abeles, 1982), while $t_{1/2}$ for reactivation of chymotrypsin inactivated with **1a** and **1d** is 11 and 0.8 h, respectively.

We consider chymotrypsin a model for serine proteases, and it now becomes important to determine whether the same approach can be applied to other serine proteases such as elastase- and trypsin-like enzymes, where the control of enzyme activity in vivo is of greater interest. We anticipate that this can be accomplished by introducing structural features into the inactivators which will be recognized by the target enzyme. The ability to control the duration of inactivation may be useful in the in vivo application of these inhibitors.

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