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Novel Inactivators of Serine Proteases Based on 6-Chloro-2-pyrone[†]

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ABSTRACT: The interaction of serine protease (esterases) with 6-chloro-2-pyrones was investigated. Time-dependent inactivation of chymotrypsin, α-lytic protease, pig liver elastase, and cholinesterase was found with 3- and 5-benzyl-6-chloro-2-pyrone, as well as 3- and 5-methyl-6-chloro-2-pyrone. No inactivation was observed with the unsubstituted 6-chloro-2-pyrone. The substituted pyrones did not inactivate papain or carboxypeptidase A, as well as a number of other nonproteolytic enzymes. The substituted chloropyrones, therefore, show considerable selectivity toward serine proteases. Analogues in which the 6-chloro substituent is replaced by H or OH do not inactivate. The presence of the halogen is, therefore, essential for inactivation. Chymotrypsin catalyzes the hydrolysis of 3-benzyl-6-chloro-2-pyrone. At pH 7.5, (*E*)-4-benzyl-2-pentenedioic acid is the major product, and 2-benzyl-2-pentenedioic anhydride is a minor product. The ratio of hydrolysis product found to the number of enzyme molecules inactivated varies from 14 to 40. The enzyme inactivated with the 3-benzyl compound does not show a spectrum characteristic of the pyrone ring. This suggests that inactivation by 3-benzyl-6-chloro-2-pyrone occurs in a mechanism-based fashion after enzymatic lactone hydrolysis. When the enzyme is inactivated with the 5-benzyl compound, absorbance due to the pyrone ring is observed. We suggest that inactivation occurs through an active site directed mechanism involving a 1,6-conjugate addition of an active site nucleophile to the pyrone ring.

The concept of suicide substrates or mechanism-based irreversible inactivators has led to the design of inhibitors for a number of enzyme classes (Abeles & Maycock, 1976; Rando, 1975; Seiler et al., 1978; Walsh, 1982). Inactivators of this type contain a latently reactive functional group that is unmasked only at the active site after enzymatic activation. Most mechanism-based irreversible inactivators described to date are for pyridoxal-dependent enzymes.

Serine proteases play a crucial role in many biochemical systems and disease states (Barrett, 1980). Human leucocyte elastase, for example, which causes the destruction of lung elastin, has been implicated in the development of emphysema (Mittman, 1972; Turino et al., 1974; Hance & Crystal, 1975; Boudier et al., 1981). For these reasons, we thought it desirable to explore the possibility of developing additional mechanism-based irreversible inactivators of serine proteases.

While this work was in progress, Chakravarty et al. (1982) reported the discovery of a new type of mechanism-based

Scheme I

Chart I

	R_1	R_2
2	$CH_2\phi$	H
3	H	$CH_2\phi$
4	CH_3	H
5	H	CH_3
1	H	H
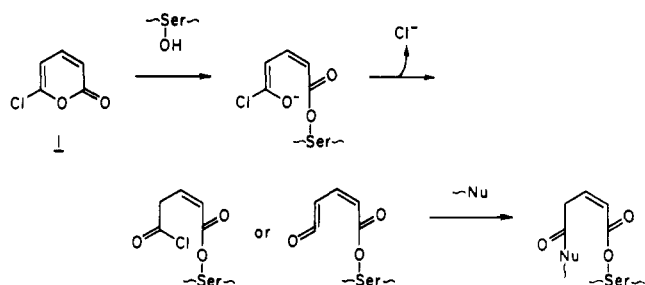
irreversible inactivator for chymotrypsin. This inactivator is an haloenol lactone that reveals an α-halo ketone moiety after enzymatic lactone hydrolysis. Two earlier reports describe the N-nitroso lactam (White et al., 1977), which generates a benzylic carbonium ion on hydrolysis, and halomethylcoumarins (Bechet et al., 1973), which produce a quinone-methide Michael acceptor after enzymatic hydrolysis and nonenzymatic chloride elimination. The instability of these compounds severely limits ultimate use in vivo. Enzyme inactivation caused by 0.2 mM haloenol lactone occurs only 3 times faster than nonenzymatic hydrolysis at neutral pH ($t_{1/2}$ ~ 95 min; Chakravarty et al., 1982). The N-nitrosoamide compounds are quite unstable in aqueous media (White et al., 1977). Inactivation by the halomethylcoumarins is relatively rapid compared to nonenzymatic hydrolysis ($t_{1/2}$ ~ 77 min), but even this degree of reactivity may be unsuitable for use in vivo. A further disadvantage is the necessary presence of a benzyl halide, a reasonably good alkylating agent, even before enzymatic activation.

We report here our progress in the development of mechanism-based irreversible inactivators of serine proteases based on the 6-chloro-2-pyrone (1) nucleus. The sequence of events leading to inactivation of serine proteases, as initially envisioned, is shown in Scheme I. During the course of lactone hydrolysis, acyl-enzyme formation will be accompanied by generation of acyl chloride or a ketene. Subsequent reaction with an active site nucleophile results in irreversible loss of enzyme activity.

Experimental Procedures

Materials. α-Chymotrypsin (type I-S), papain, acetylcholinesterase, trypsin, creatine kinase, L(+)-lactate dehydrogenase, and elastase were obtained from Sigma Chemical Co. Gel filtration media were obtained from Bio-Rad. S-Adenosylhomocysteinase was prepared as described by Palmer & Abeles (1979). O-*trans*-Cinnamoyl-β-phenyllactic acid was prepared as described by McClure & Neurath (1966). The method of Pirkle & Dines (1968) was used to prepare 6-chloro-2-pyrone (1). α-Lytic protease and N-acetyl-L-Ala-L-Pro-Pro-L-Ala-p-nitroanilide were generous gifts from Professor W. Bachovchin. 1-Acetamido-2-phenylethaneboric acid was kindly provided by Professor G. Lienhard.

Methods. All spectrophotometric determinations were made with either a Perkin-Elmer 559 or Lambda 3 UV-vis instrument with 1-cm quartz cells thermostated at 25 °C. NMR spectra were determined on a Brucker FT WH-90 spectrometer with tetramethylsilane as an internal standard. The chymotrypsin concentration was determined spectrophotometrically with $\epsilon_{280} = 5 \times 10^4$ M^{-1} cm^{-1} (Dixon & Neurath, 1957). All thin-layer chromatography was performed on silica gel.

Syntheses. The synthesis of the substituted 6-chloro-2-pyrones was performed by a modification of the procedures described for 3-benzyl-6-chloro-2-pyrone (2) (Thole & Thorpe,
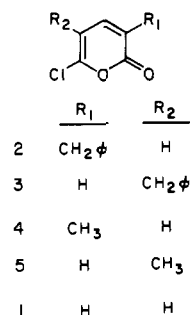
1911) and the isomeric 3-methyl- and 5-methyl-6-chloro-2-pyrones (4 and 5) (Kagen et al., 1975; Weis & Winkler, 1974). The structure of the pyrones synthesized are shown in Chart I.

1,1,3,3-Tetracarbethoxypropene Sodium Salt. Sodium ethoxide was generated in a 2-L three-neck round-bottom flask fitted with an overhead stirrer, a reflux condenser, and a dropping funnel. Sodium metal, 28.7 g (1.25 mol), was dissolved in 700 mL of anhydrous ethanol. To the resulting solution were added 100 g (0.624 mol) of diethyl malonate and 37 g (0.31 mol) of chloroform. After a 1-h reflux, the hot mixture was filtered to remove sodium chloride, the filtrate was allowed to stand for 8 h at room temperature, and 30 g of yellow crystalline 1,1,3,3-tetracarbethoxypropene sodium salt was collected by filtration: NMR (Me_2SO-d_6) δ 1.17 (t, 12 H, $J = 7$ Hz), 2.97 (q, 8 H, $J = 7$ Hz), 8.10 (s, 1 H).

4-Phenyl-1,1,3,3-tetracarbethoxybutene. A suspension of 30 g (0.0851 mol) of sodio-1,1,3,3-tetracarbethoxypropene and 16 g (0.094 mol) of benzyl bromide in 75 mL of anhydrous ethanol was refluxed for 48 h. Most of the solvent was removed on a rotary evaporator, and 150 mL of water was added to the residue. The resulting off-white precipitate was filtered and recrystalized from 175 mL of ethanol to yield 30 g (0.071 mol) of colorless crystalline 4-phenyl-1,1,3,3-tetracarbethoxybutene: NMR ($CDCl_3$) δ 1.27 (t, 12 H), 3.56 (s, 2 H), 4.25 (q, 8 H), 7.27 (br s, 5 H), 7.5 (s, 1 H); TLC[1] R_f 0.37 [ethyl acetate/hexane (1:4)].

(E)-2-Benzyl-2-pentenedioic Acid. A mixture consisting of 20 g (0.048 mol) of 4-phenyl-1,1,3,3-tetracarbethoxybutene, 20 mL of ethanol, 18.6 g (9.285 mol) of potassium hydroxide, and 70 mL of water was refluxed for 4 h. Ethanol was then removed by distillation at atmospheric pressure, and the aqueous reaction mixture was adjusted to pH 1 with concentrated hydrochloric acid. After the vigorous foaming ceased, the mixture was refluxed for 1 h and extracted 4 times with 100 mL of ether. The pooled extracts were dried over sodium sulfate and evaporated to yield 11.75 g of a tan solid. The crude acid was triturated twice with 20 mL of methylene chloride, yielding 7.7 g (0.035 mol) of colorless solid: mp 152-153 °C [lit. mp 153-154 °C (Thole & Thorpe, 1911)]; NMR (CD_3CN) δ 3.53 (d, 2 H, $J = 7.2$ Hz, $HO_2CCH_2^-$), 3.65 (s, 2 H, benzylic), 7.03 (t, 1 H, $J = 7.2$ Hz, olefinic), 7.2 (br s, 5 H, Ar H).

3-Benzyl- and 5-Benzyl-6-chloro-2-pyrone (2 and 3). A mixture of 2 g of (E)-2-benzyl-2-pentenedioic acid (9.1 mmol) and 10 mL of acetyl chloride was refluxed for 3 days and concentrated in vacuo to yield a dark brown oil. Chromatography of the crude mixture on 50 g of silica gel containing

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[1] Abbreviations: BTEE, N-benzoyl-L-tyrosine ethyl ester; TLC, thin-layer chromatography; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; ACH, acetylcholine.

10% (w/w) water with 5% ether/hexane as the eluent and evaporation of fractions containing the desired product (240–400 mL) yielded 0.85 g (3.85 mmol) of 3-benzyl-6-chloro-2-pyrone as a colorless crystalline solid: mp 71–72 °C [lit. mp 74 °C (Thole & Thorpe, 1911)]; NMR (CDCl$_3$) δ 3.76 (d, 2 H, $J = 1.3$ Hz, benzylic), 6.15 (d, 1 H, $J = 7.55$ Hz, H$_5$), 6.94 (dt, $J = 7.55$ and 1.3 Hz, H$_4$), 7.33 (m, 5 H, Ar H); TLC R_f 0.47 [ethyl acetate/hexane (1:4)]. Evaporation of a later fraction (580–880 mL of eluent) yielded 0.18 g of 5-benzyl-6-chloro-2-pyrone as a slightly yellow solid. Recrystallization from a hexane/ether mixture yielded 0.081 g (0.37 mmol) of colorless crystalline solid: mp 50–51 °C; NMR (CDCl$_3$) δ 3.81 (s, 2 H, benzylic), 6.3 (d, 1 H, $J = 9.5$ Hz, H$_3$), 7.26 (d, 1 H, $J = 9.5$ Hz, H$_4$), 7.35 (m, 5 H, Ar H); TLC R_f 0.34 [ethyl acetate/hexane (1:4)]. The assignment of the isomeric structures is based on the fact that coupling constants between H$_3$ and H$_4$ in all 2-pyrone compounds evaluated are between 9 and 10 Hz whereas values from 6 to 7 Hz are observed for the coupling constants between H$_4$ and H$_5$ (Pirkle & Dines, 1969).

2-Benzyl-2-pentenedioic Anhydride (**6**). A mixture consisting of 250 mg (1.13 mmol) of 3-benzyl-6-chloro-2-pyrone (**2**) in 10 mL of tetrahydrofuran and 20 mL of 0.1 N sodium hydroxide was stirred at room temperature for 30 min. The tetrahydrofuran was evaporated in vacuo, and the aqueous residue was adjusted to pH 12 and extracted with ether. The aqueous portion was acidified to pH 1 with 50% HCl and extracted 3 times with 20 mL of ether. The pooled ether extract was dried over Na$_2$SO$_4$, filtered, and evaporated to yield 228 mg (1.13 mmol) of crude anhydride. Recrystallization from CCl$_4$ yielded 105 mg of 2-benzyl-2-pentenedioic anhydride: mp 89–90 °C [lit. mp 90 °C (Thole & Thorpe, 1911)]; NMR (CDCl$_3$) δ 3.52 (dt, 2 H, J 3.96 and 1.9 Hz, methylene), 3.74 (td, 2 H, $J = 1.9$ and 1.9 Hz, benzylic), 6.49 (tt, 1 H, $J = 3.96$ and 1.9 Hz, vinylic), 7.37 (m, 5 H, aromatic). The signal at δ 3.52 disappears on addition of D$_2$O, and the vinylic proton becomes a triplet with $J = 1.9$ Hz.

3-Methyl- and 5-Methyl-6-chloro-2-pyrone (**4** *and* **5**). A mixture of the two isomeric pyrones was obtained by refluxing (*E*)-2-methyl-2-pentenedioic acid and acetyl chloride for 24 h as previously described (Kagen et al., 1975). Chromatography of 500 mg of crude product on 10 g of silica gel containing 10% water (w/w) eluted with 5% ether/hexane yielded 185 mg of 3-methyl-6-chloro-2-pyrone (**4**) (400–600 mL of eluent) and 90 mg of 5-methyl-6-chloro-2-pyrone (**5**) (500–1200 mL of eluent). NMR (CDCl$_3$) for **4**: δ 2.11 (s, 3 H, –CH$_3$), 5.27 (d, 1 H, $J = 6.7$ Hz, =CH–CO$_2$–), 7.29 (dq, 1 H, $J = 6.7$ and 1.4 Hz, =CH–). NMR (CDCl$_3$) for **5**: δ 2.09 (s, 3 H, –CH$_3$), 6.3 (d, 1 H, $J = 9$ Hz, =CH–CO$_2$), 7.33 (d, 1 H, $J = 9$ Hz, =CH–).

3-Benzyl-2-pyrone. The following is a modification of a procedure described for the reduction of 6-chloro-2-pyrone (Pirkle & Dines, 1968). Zinc dust (200 mg, 3.06 mmol) was added to a solution of 45 mg (0.204 mmol) of 3-benzyl-6-chloro-2-pyrone in 2 mL of a 1:1 (v/v) mixture of acetic acid and tetrahydrofuran. The mixture was stirred 15 h at room temperature, 10 mL of ether added, and the zinc dust removed by filtration. Evaporation of the solvent from the filtrate yielded 30 mg (0.16 mmol) of a colorless oil, which darkened on standing. A portion (15 mg) of the crude product was chromatographed on 1 g of silica containing 10% water (v/w) and eluted with a mixture of 5% ether in hexane. Fractions containing pure pyrone (13–23-mL total eluate volume) were pooled, and solvent was evaporated to yield 8 mg (0.043 mmol) of 3-benzyl-2-pyrone as a colorless oil: NMR (CDCl$_3$) δ 3.81

(br s, 2 H, benzylic), 6.23 (dd, $J_{4,5} = 6.67$ Hz, $J_{5,6} = 5.13$ Hz, H$_5$), 6.99 (ddt, $J_{4,5} = 6.67$ Hz, $J_{4,6} = 2.17$ Hz, $J_{benzylic,4} = 1.37$ Hz, H$_4$), 7.34 (br s, 5 H, Ar H), 7.48 (ddt, $J_{5,6} = 5.13$ Hz, $J_{4,6} = 2.17$ Hz, $J_{benzylic,6} = 0.68$ Hz, H$_6$); TLC R_f 0.47 [ethyl acetate/hexane (1:4 v/v)].

Dimethyl Ester of (E)-2-Benzyl-2-pentenedioic Acid. (*E*)-2-Benzyl-2-pentenedioic acid (300 mg, 1.36 mmol) was stirred at room temperature for 48 h in a mixture of 3 mL of methanol and 0.1 mL of concentrated sulfuric acid. The reaction mixture was neutralized with 10% NaHCO$_3$, and the methanol was evaporated in vacuo. The aqueous mixture was extracted with ether after the addition of 15 mL of water. The pooled ether extract was dried over MgSO$_4$, and the solvent was evaporated in vacuo to yield 200 mg (0.81 mmol) of the dimethyl ester as a colorless oil: NMR (CDCl$_3$) δ 3.34 (d, $J = 7.5$ Hz, 2 H, –O$_2$CCH$_2$–), 3.66 (s, 6 H, –OCH$_3$), 3.68 (s, 2 H, benzylic), 7.10 (t, 1 H, $J = 7.5$, Hz, vinylic), 7.19 (br s, 5 H, aromatic).

(E)-4-(Methoxycarbonyl)-5-phenyl-3-pentenoic Acid. The dimethyl ester as prepared above (42 mg, 0.169 mmol) was added to 0.5 mL of methanol containing 0.17 mL of 1 M NaOH (0.17 mmol), and the mixture was stirred at room temperature for 3 h. The methanol was evaporated in vacuo, and 5 mL of water was added to the remaining aqueous mixture. The pH was adjusted to 9, and the aqueous mixture was extracted with ether. After acidification to pH 2, the mixture was again extracted with ether, dried over Na$_2$SO$_4$, and evaporated to yield 19 mg of the monomethyl ester as a colorless oil: NMR (CDCl$_3$) δ 3.40 (d, 2 H, $J = 7.13$ Hz, –O$_2$CCH$_2$–), 3.78 (s, 3 H, –OCH$_3$), 3.80 (s, 2 H, benzylic), 7.23 (t, 1 H, $J = 7.13$ Hz, vinylic), 7.34 (br, s, 5 H, aromatic).

Isolation of Enzymatic Product of 3-Benzyl-6-chloro-2-pyrone (**2**). A solution of 25 mg (0.113 mmol) of 3-benzyl-6-chloro-2-pyrone (**2**) in 2.5 mL acetonitrile was added in portions over a period of 2.5 h to a solution of 100 mg of chymotrypsin in 50 mL of 0.1 M potassium phosphate, pH 7.5. After a total reaction time of 5 h, 100 mL of cold methanol was added, and the mixture was cooled in a dry ice/acetone bath for 5 min. Denatured protein was removed by centrifugation, and methanol was removed from the aqueous supernatant in vacuo. The neutral reaction mixture was extracted with ether, and the extract was dried over sodium sulfate and evaporated in vacuo to yield 9.5 mg (0.043 mmol) of unreacted 3-benzyl-6-chloro-2-pyrone (**2**). After acidification to pH 2 with 1 N HCl, the aqueous reaction mixture was again extracted with ether, and the extract dried over sodium sulfate and evaporated in vacuo to yield 14.3 mg (0.065 mmol) of (*E*)-4-benzyl-2-pentenedioic acid (**7**). Thus,

7
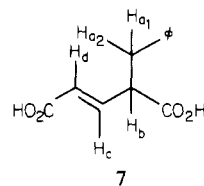
95% of the pyrone (0.113 mmol) can be accounted for as diacid product (57%) and unreacted pyrone (38%). NMR (CD$_3$CN) for **7**: δ 2.97 (d, $J_{a_1,b} = 7.0$ Hz, H$_{a_1}$), 3.07 (d, $J_{a_2,b} = 7.0$ Hz, H$_{a_2}$), 3.53 (dddd, $J_{b,c} = 9$ Hz, $J_{a_1,b} = 7.9$ Hz, $J_{a_2,b} = 7.9$ Hz, $J_{b,d} = 1.0$ Hz, H$_b$), 5.76 (dd, $J_{c,d} = 16.1$ Hz, $J_{b,d} = 1.9$ Hz, H$_d$), 6.8 (dd, $J_{c,d} = 16.1$ Hz, $J_{b,c} = 9$ Hz, H$_c$), 7.23 (br s, Ar H).

Isolation of Enzymatic Product of 2-Benzyl-2-pentenedioic Anhydride (**6**). A solution of 15.35 mg (0.076 mmol) of 2-benzyl-2-pentenedioic anhydride in 2.5 mL of acetonitrile

was added to a solution of 100 mg of chymotrypsin in 0.1 M potassium phosphate buffer, pH 7.5. Disappearance of the anhydride was followd from the decrease in A_{380}. After a 3-h incubation, the reaction was 98% complete. Protein was removed from the mixture by methanol precipitation as described for the isolation of the product from 3-benzyl-6-chloro-2-pyrone. The resulting mixture was extracted with ether, acidified to pH 2, and extracted again with ether. Evaporation of the acidic extract yielded 12 mg (0.054 mmol) of a crystalline solid that was shown by NMR to be a 5:1 mixture of (Z)-2-benzyl-2-pentenedioic acid (8) and (Z)-4-benzyl-2-

8   9
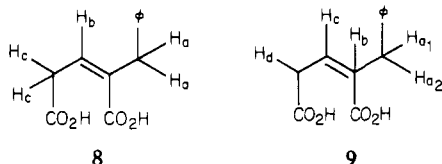
pentenedioic acid (9). NMR (CD$_3$CN) for 8: δ 3.57 (dt, $J_{b,c}$ = 7.3 Hz, $J_{a,c}$ = 0.88 Hz, H$_c$), 3.66 (br s, H$_a$), 6.3 (tt, $J_{b,c}$ = 7.3 Hz, $J_{a,b}$ = 1.2 Hz, H$_b$), 7.35 (br s, aromatic). NMR (CD$_3$CN) for 9: δ 3.01 (d, $J_{a_1,b}$ = 7.6 Hz, H$_{a_1}$), 3.12 (d, $J_{a_2,b}$ = 7.2 Hz, H$_{a_2}$), 4.65 (dddd, $J_{b,c}$ = 9.9 Hz, $J_{a_1,b}$ = 7.6 Hz, $J_{a_2,b}$ = 7.2 Hz, $J_{b,d}$ = 0.9 Hz, H$_b$), 5.95 (dd, $J_{c,d}$ = 11.6 Hz, $J_{b,d}$ = 0.9 Hz, H$_d$), 6.41 (dd, $J_{c,d}$ = 11.6 Hz, $J_{b,d}$ = 0.9 Hz, H$_c$), 7.35 (br s, aromatic).

Enzyme Inactivation. (A) General Method. Inactivation experiments were initiated by the addition of 50 μL of a 10 mM solution (acetonitrile) of inactivator to 0.95 mL of buffer containing enzyme. Aliquots of the above preincubation mixture were added to 1 mL of assay buffer containing substrate (20-fold dilution), and enzyme activity was determined. Preincubation and assay buffers were identical in composition except for the absence of substrate during preincubation. All experiments were done at 25 °C.

α-Chymotrypsin was inactivated in 0.1 M potassium phosphate buffer, pH 7.5, and enzyme activity determined (Hummel, 1959) at various time points by the addition of 50-μL aliquots to 1 mL of 0.1 M potassium phosphate buffer containing 50 μL of a 11.5 mM solution of N-benzoyl-L-tyrosine ethyl ester in acetonitrile. The increase in absorbance at 256 nm was recorded.

Acetylcholinesterase was preincubated with inactivator in 0.1 M potassium phosphate, pH 7.0. Enzyme activity was determined as ΔA_{410}/min after the addition of 50-μL aliquots to 0.95 mL of 0.1 M potassium phosphate, pH 7.0, containing 50 μL of 10 mM p-nitrophenyl acetate in acetonitrile. The velocities (ΔA_{410}/min) determined were corrected for nonenzymatic hydrolysis.

Papain was preincubated with inactivator in 0.05 M Tris-HCl, pH 8.5, containing 5 mM cysteine and 2 mM EDTA. Aliquots (50 μL) were assayed by addition to 0.95 mL of the above buffer containing 1 mM N$^\alpha$-benzoyl-L-arginine-p-nitroanilide and 1% (v/v) Me$_2$SO. The change in absorbance at 410 nm was followed.

Other enzymes, lactate dehydrogenase (Bergmeyer, 1974a), creatine kinase (Bergmeyer, 1974b), and S-adenosylhomocysteinase (Palmer & Abeles, 1979), were determined as previously described. Trypsin was assayed with p-toluenesulfonyl-L-arginine methyl ester (Hummel, 1959), carboxypeptidase A with O-trans-cinnamoyl-β-phenyllactate (McClure & Neurath, 1966), and elastase and α-lytic protease with N-acetyl-L-Ala-L-Pro-L-Ala-p-nitroanilide (Hunkapiller et al., 1976).

(B) Solution Kinetics. Reactions of the chloropyrones with NaOH were followed by measuring the decrease in absorbance

at 304 nm as a function of time at 25 °C. First-order rate constants were determined from plots of ln $[(A_t - A)/(A_0 - A)]$ vs. time. Second-order rate constants were determined from the slopes of plots of k_{obsd} vs. NaOH concentration. The rate constant for reaction of 2 under neutral conditions was estimated from the initial change in absorbance at 304 nm measured in 0.1 M potassium phosphate, pH 7.0, 25 °C, over a period of 7 days.

Results

Reaction of 3-Benzyl-6-chloro-2-pyrone (2) with Chymotrypsin. Compound 2 is a substrate for chymotrypsin. K_m and k_{cat} for substrate disappearance are 9 μM and 0.55 min^{-1} (0.1 M potassium phosphate, pH 7.5/4.5% acetonitrile, 25 °C). K_m and k_{cat} for benzoyltyrosine ethyl ester (BTEE) measured under identical conditions are 85 μM and 115 min^{-1}. Compound 5 is a competitive inhibitor of BTEE hydrolysis with $K_I \sim 4$–10 μM. The only isolatable product of enzymatic hydrolysis is (E)-4-benzyl-2-pentenedioic acid (7). The amount of 7 isolated accounts for 95% of the pyrone that has reacted. A second product was detected spectrophotometrically. Repetitive UV spectra of a mixture of chymotrypsin (25 μM) and 2 (0.13 mM) in 0.1 M potassium phosphate buffer, pH 7.5, showed a rapid decrease in absorbance at 304 nm accompanied by slow formation of a new peak with λ_{max} = 354 nm, corresponding to 2-benzyl-2-pentenedioic acid anhydride (6). The initial rates of disappearance of the chloropyrone and formation of the anhydride were 9 μM min^{-1} and 0.2 μM min^{-1}, respectively. At pH 5.0 (0.1 M potassium acetate), the rate of chloropyrone disappearance decreased from 9 to 2 μM min^{-1}, and the rate of anhydride synthesis increased to 1.34 μM min^{-1}. No anhydride was detected at pH 10 (0.1 M potassium carbonate), and the initial rate of chloropyrone disappearance was 4.7 μM min^{-1}. Nonenzymatic hydrolysis of the chloropyrone and the anhydride and enzymatic hydrolysis of the anhydride were negligible at pH 5 and 7.5. The values obtained at pH 10 were corrected for a small amount ($\sim$10%) of nonenzymatic hydrolysis of substrate.

Incubation of chymotrypsin with 2 causes time-dependent loss of enzyme activity, which is diminished in the presence of the competitive inhibitor 1-acetamido-2-phenylethaneboronic acid (Figure 1). Semilogarithmic plots of percent remaining activity vs. time invariably reveal that inactivation is biphasic. An initial fast phase ($t_{1/2}$ = 32 min) gives way to a slow phase ($t_{1/2}$ = 56 min) after approximately 15% of the enzyme has been inactivated (Figure 1). The time course of inactivation of a fresh aliquot of enzyme (0.3 μM) added to a mixture consisting of 0.18 mM 2 and 0.7 μM enzyme that had been allowed to inactivate for 200 min ($\sim$16% remaining activity) is identical with the time course of inactivation observed in the initial inactivation. This result rules out the possibility that biphasic inactivation is due to the presence of a small amount of a faster inactivating impurity in preparations of 2 that is consumed in the initial fast phase. Since it was noticed that the prominence of the initial fast phase of inactivation varied with age and source of the enzyme, enzyme heterogeneity was suspected to be the cause of the complex kinetics. Further purification (Nakagawa & Bender, 1970; Yapel et al., 1966) of commercially obtained enzyme did not simplify the inactivation kinetics.

The possibility that biphasic kinetics of inactivation results from the presence of different forms of chymotrypsin, each having different susceptibility to inactivation by 2, was tested in the following way. The time course of inactivation of 0.7 μM chymotrypsin in the presence of 0.19 mM 2 was followed as usual. After an 80-min incubation, when 30% of the initial
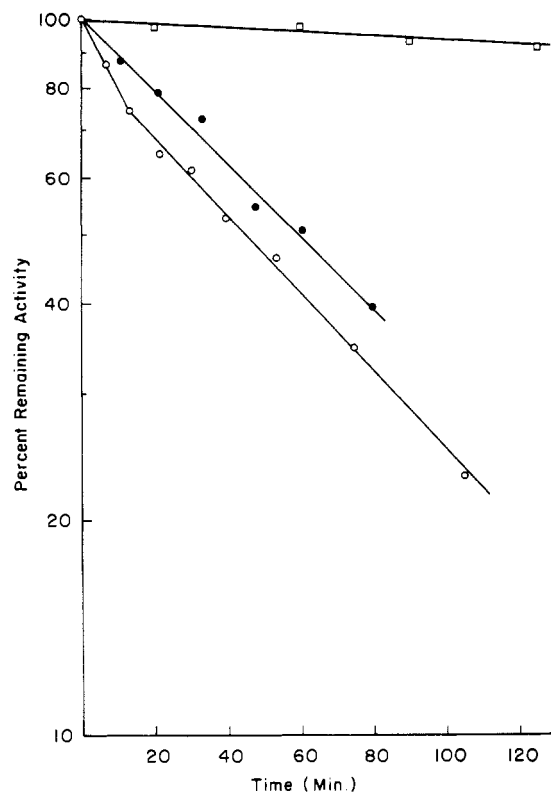
FIGURE 1: Inactivation of chymotrypsin by 3-benzyl-6-chloro-2-pyrone (2). Reacton contained 0.7 μM chymotrypsin, 0.18 mM 3-benzyl-6-chloro-2-pyrone, 0.1 M potassium phosphate, pH 7.5, and 4.5% acetonitrite at 25 °C: (O)) no additions; (□) 2.3 M N-acetylphenylalaninebornic acid; (●) enzyme exposed to inactivator, as above. After 80 min, inactivator was removed by gel filtration, and 0.18 mM inactivator was added again. Rates measured after second addition.

activity remained, the inactivation mixture was passed through an XAD-2 column to remove excess inactivator. This treatment removes 96% of 2 and results in loss of 60% of the enzyme. A fresh aliquot of 2 was added to the partially inactivated enzyme, and the time course of further inactivation was followed. Whereas inactivation in the initial incubation took place in a biphasic fashion ($t_{1/2}$ = 32 and 56 min), the second inactivation proceeded with a single phase ($t_{1/2}$ = 59.7 min, Figure 1).

Further support for the notion that biphasic inactivation kinetics results from enzyme heterogeneity was gained as follows. Chymotrypsin (2 μM) was incubated with a limiting amount of 2 (23 μM). Inactivation was initially pseudo first order ($t_{1/2}$ = 26 min) and, after 100 min, had proceeded to 64% remaining activity when all of the inactivator was consumed. Inactivation was reinitiated by the addition of excess 2 (0.18 mM), and the time course for loss of enzyme activity was followed. A single phase ($t_{1/2}$ = 50 min) was observed. A control inactivation in the presence of 0.18 mM 2 showed two processes; a fast ($t_{1/2}$ = 26 min) and slow phase ($t_{1/2}$ = 52 min) were observed.

The rate of the initial fast phase of inactivation is saturable since rate constants obtained with inactivator between 10 and 500 μM are identical (K_1 < 10 μM).

An experiment was done to determine the moles of product formed from the pyrone per mole of enzyme inactivated. Chymotrypsin (0.7 μM) was incubated with 0.18 mM 2 in phosphate buffer, pH 7.5, and the absorbance at 304 nm was monitored continuously with time. Aliquots of a parallel incubation were assayed for catalytic activity at various time points. It was thus possible to simultaneously measure extent of inactivation and pyrone disappearance. For the initial fast
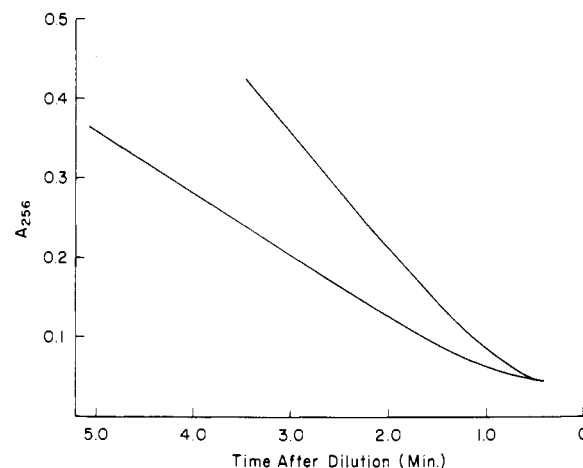

FIGURE 2: Recovery of catalytic activity of chymotrypsin after preincubation with 3-benzyl-6-chloro-2-pyrone (2). Inactivation was carried out as described in Figure 1. Aliquots were removed 0.5 (upper trace) and 70 min (lower trace) after addition of inactivator and added to a reaction mixture (20-fold dilution) containing 0.5 mM N-benzoyltyrosine ethyl ester. Change in optical density, reflecting hydrolysis of substrate, was recorded continuously.

phase ($t_{1/2}$ = 22 min), turnover/inactivation = 14, and, for the slow phase ($t_{1/2}$ = 64 min), turnover/inactivation = 40. Interestingly, the specific activity of the remaining active enzyme with respect to pyrone turnover remains constant throughout the entire course of inactivation. A mean value for eight determinations from 90 to 14% remaining activity was 0.475 ± 0.046 min⁻¹.

The UV spectrum of inactivated enzyme subjected to either Bio-Gel P-6 filtration or extensive dialysis resembles that of native enzyme; no absorbance at 304 nm, the absorbance maximum of inactivator (2), could be detected. Enzyme inactivation at pH 5 (0.1 M KOAc) exhibited a spectrum identical with that of enzyme inactivated at pH 7.5. These results suggest that in the inactivated enzyme the pyrone ring is no longer present. Activity is regained slowly on incubation at pH 7.5 (0.1 M potassium phosphate) with $t_{1/2}$ = 1389 min. Inactivated enzyme is rapidly and completely reactivated by incubation in 1 M hydroxylamine at pH 7.0 with a half-life of 24 min.

When chymotrypsin is preincubated with 2 and an aliquot added to an assay mixture (20-fold dilution) consisting of N-benzoyltyrosine ethyl ester (0.5 mM) in 1 mL of 0.1 M potassium phosphate, pH 7.5, maximal activity is attained only after a short lag time (Figure 2). Return of activity obeys the first-order rate law with k_{obsd} = 2.2 ± 0.2 min⁻¹. This behavior is observed at the earliesttime point, after a 0.5-min preincubation time. The portion of enzyme present in this reversibly inactivated form has been estimated by comparing the initial rate of BTEE hydrolysis measured 30 s after dilution to the maximal rate ultimately attained. After a 30-s preincubation, values from 70 to 80% were obtained. Thus two inactivated forms of chymotrypsin have been identified: one form rapidly regains activity with $t_{1/2}$ = 0.32 min, and the other more slowly regains activity with $t_{1/2}$ = 1389 min.

When an aliquot of a solution of 2 (0.133 mM final concentration) was added to a concentrated solution of enzyme (23 μM in 0.1 M potassium phosphate, pH 7.5), a rapid nonlinear decrease in A_{304} followed by a slower linear decrease in absorbance was observed. Extrapolation of the slower linear rate to A_{304} at zero time gave a value 0.128 optical density units lower than that measured in the absence of enzyme. From this value and $\Delta\epsilon_{304}$ = 8500 M⁻¹ cm⁻¹, it can be estimated that 15.1 μM inactivator and 67% of the enzyme are
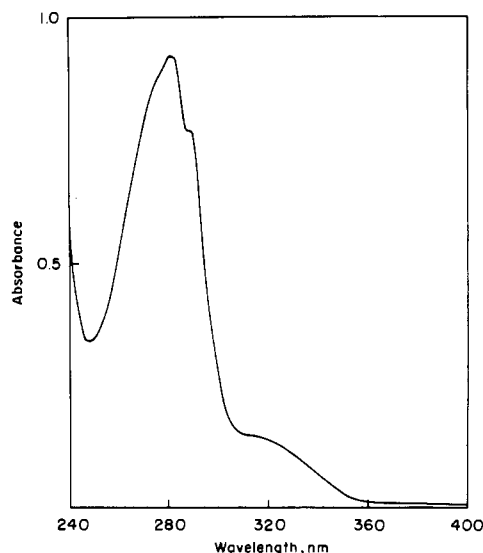
FIGURE 3: Spectrum of chymotrypsin inactivated with 5-benzyl-6-chloro-2-pyrone (3). Chymotrypsin, 200 μm, 5-benzyl-6-chloro-2-pyrone (3), 1.1 mM, and potassium phosphate buffer, 0.1 M, pH 7.5, were allowed to react 70 min at 25 °C (<0.5% activity remaining). The reaction mixture (175 mL) was passed through a Bio-Gel P-6 column (30 × 1.2 cm) and eluted with same buffer. The spectrum of a solution containing 18 μM inactivated chymotrypsin was determined.

Table I: Half-Lives for Inactivation of Proteolytic Enzymes by 6-Chloro-2-pyrones[a]

	$t_{1/2}$ (min)			
compd	α-chymotrypsin	α-lytic protease	elastase	ACH-esterase
2	30, 80[b]	>600	1.4[d]	45
3	3.4	0.4[c]	7	2
4	27, 90[b]	111	0.9[e]	>310
5	62	26	84	4.5
1	>470	>900	>1700	>2000

[a] The inactivator concentration was 0.5 mM throughout. Other conditions are described under Experimental Procedures. Compound numbers refer to Chart I. [b] Biphasic inactivation. [c] $t_{1/2}$ = 1.25 min at 0.1 mM inactivator. Incubation of 10.2 μM enzyme with 5 μM inhibitor results in 43% inactivation after 50 min. [d] Inactivation proceeds to 30% initial activity. [e] Approximately 15% of initial activity remaining at t. The amount of residual activity is independent of enzyme concentration at 5.81, 11.6, and 23.3 μM.

consumed in the initial burst. The approach to steady state is first order with k_{obsd} = 2.7 min^{-1}. In a similar experiment performed at pH 5.0 (0.1 M potassium acetate), an initial burst was not observed; the decrease in A_{304} was linear with time, and the value of A_{304} at zero time was equal to A_{304} in the absence of enzyme.

Reaction of 5-Benzyl-6-chloro-2-pyrone (3) with Chymotrypsin. Incubation of chymotrypsin with 3 causes time-dependent, first-order loss of enzyme activity. Saturation kinetics are observed with K_I = 0.64 mM (1.95 μM enzyme/0.1 M potassium phosphate, pH 7.5) and k_{inact} = 0.70 min^{-1}. The competitive inhibitor N-acetylphenylalanine boronic acid (2.4 mM) protects the enzyme from inactivation in the presence of 0.4 mM 3. Inactivated chymotrypsin (<0.5% remaining activity) could be separated from excess 3 by P-6 gel filtration without detectable reactivation.

The UV spectrum of inactivated enzyme shows a distinct shoulder at 320 nm (ϵ = 7400 M^{-1} cm^{-1}) (Figure 3). When 27 μM chymotrypsin was incubated with 96 μM 3 in a sample cuvette and spectra recorded at fixed time intervals vs. a reference cuvette containing unmixed solutions of enzyme and 3, a time-dependent increase in absorbance at 322 nm was observed. Loss of enzyme activity and the appearance of the 322-nm peak were found to occur with comparable first-order rate constants ($t_{1/2}$ = 13.5 min).

Activity is regained slowly ($t_{1/2}$ = 395 min) on incubation of inactivated enzyme with 0.9 M hydroxylamine, pH 7.0. The ratio A_{320}/A_{280} decreases with increasing extent of reactivation.

Reaction of Analogues of 3-Benzyl-6-chloro-2-pyrone with Chymotrypsin. 2-Benzyl-2-pentenedioic anhydride (6) is an analogue of 3-benzyl-6-chloro-2-pyrone in which Cl at C-6 is replaced by OH. The anhydride is a substrate for chymotrypsin with K_m = 300 μM and k_{cat} = 0.28 min^{-1} (0.1 M potassium phosphate, pH 7.5). Two isomeric dicarboxylic acid products were isolated by ether extraction of an acidified incubation mixture. The major product is (Z)-2-benzyl-2-pentenedioic acid (8), as would be expected from simple acyl oxygen–carbonyl carbon bond cleavage. An unexpected result is the formation of (Z)-4-benzyl-2-pentenedioic acid (9) (25%)

from the anhydride. No inactivation was observed when chymotrypsin was incubated with 1.58 mM anhydride for up to 4 h. A lag time in the hydrolysis of N-benzoyltyrosine ethyl ester by enzyme preincubated with the anhydride similar to that described for 2 was observed on dilution (21-fold) of preincubation mixtures. An estimated 30% of active enzyme was present in this transiently inactive form 0.5 min after dilution. Return of activity was first order with k_{obsd} = 0.85 min^{-1}.

The effect of 3-benzyl-2-pyrone on chymotrypsin was also determined. No time-dependent inactivation of chymotrypsin ($t_{1/2}$ > 600 min^{-1}) could be observed on incubation with 1 mM 3-benzyl-2-pyrone for 1 h (0.1 M potassium phosphate, pH 7.5). The presence of 46 μM 3-benzyl-2-pyrone causes 21% inhibition of the rate of reaction with 0.522 mM BTEE. Assuming that inhibition is competitive and K_m for BTEE is 84 μM, the value for K_I = 34 μM can be calculated. No change in the pyrone spectrum (λ_{max} = 288 nm, ϵ = 9400) was observed when repetitive UV spectra of a mixture of 20 μM chymotrypsin (0.1 M potassium phosphate, pH 7.5) containing 0.2 mM 3-benzyl-2-pyrone were recorded over a period of 30 min. Therefore, hydrolysis probably does not occur.

Reaction of Monomethyl Ester of (E)-4-(Methoxy-carbonyl)-5-phenyl-3-pentenoic Acid. The action of chymotrypsin on the half-ester was examined, since this ester and the chloropyrone could give rise to a similar acyl-enzyme intermediates. The chymotrypsin-catalyzed hydrolysis of the monomethyl ester was followed by adding 0.01 N NaOH with a microburet to a mixture of 2 mL of 33 μM chymotrypsin in water sufficient to maintain pH 7.5. The titration indicated that under these conditions the initial velocity for hydrolysis was 9.3 μM/min. No inactivation was observed when chymotrypsin (2 μM) was preincubated with 1 mM half-ester for up to 6 h.

Inactivation of Enzymes Other Than Chymotrypsin by 3-Benzyl-, 5-Benzyl-, 3-Methyl-, and 5-Methyl-6-chloro-2-pyrones and 6-Chloro-2-pyrone (2, 3, 4, 5, and 1). The effect of various 6-chloro-2-pyrones on several proteolytic enzymes, as well as acetylcholinesterase, is shown in Table I. It should be noted that none of these enzymes are inactivated by the parent compound 6-chloro-2-pyrone (1). No inactivation ($t_{1/2}$ > 300 min) of lactate dehydrogenase, S-adenosylhomocysteinase, creatine kinase, papain, trypsin, or carboxypeptidase occurred on preincubation with 0.5 mM of any of the pyrones.

Nonenzymatic Reactivity of 6-Chloro-2-pyrones. The 6-chloro-2-pyrone nucleus is quite inert to hydrolysis under neutral and acidic conditions. No change in absorbance at

Scheme II
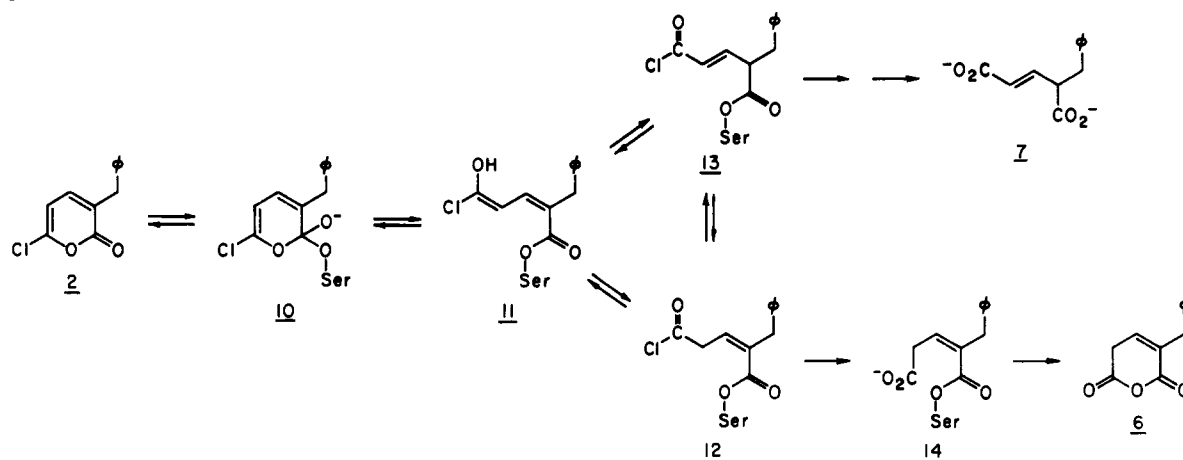
304 nm is observed when **2** is incubated with 1 N H_2SO_4 at 25 °C for 1 h. Since, under the conditions employed, a 1% change in absorbance could have been detected, k_{obsd} must be less than 3×10^{-4} min^{-1}. The rate constant for the hydrolysis of **2** under neutral conditions (0.1 M potassium phosphate buffer, pH 7.0/5% acetonitrile) is approximately 5×10^{-6} min^{-1} as calculated from the initial rate of change is absorbance at 304 nm measured over a period of 7 days.

Hydrolysis of the 6-chloro-2-pyrone nucleus is rapid under alkaline conditions. Second-order rate constants determined in NaOH solutions containing 1% acetonitrile at 25 °C are 29, 27, and 24 M^{-1} min^{-1} for **4, 5,** and **6,** respectively. As alkaline hydrolysis of **2** and **3** proceeds, the characteristic long-wavelength absorbance (304 nm) due to starting material decreases with concomitant appearance of a new spectrum having a λ_{max} at 352 nm, which is identical with the spectrum of the anhydride **6.** The fact that clean isosbestic points are observed at 323.5 and 257.0 nm indicates that the anhydride is the sole hydrolysis product. Hydrolysis of **1** yields 2-pentenedioic anhydride.

Discussion

Specificity of Inactivation. This work was undertaken to test the proposal that the 6-chloro-2-pyrone nucleus could be used as a core structure in the design of suicide inactivators for serine proteases. The unsubstituted parent structure and 3- and 5-benzyl-6-chloro-2-pyrone and 3- and 5-methyl-6-chloro-2-pyrone were chosen for initial screening. The 3-benzyl compound was chosen because it most closely resembles specific substrates for chymotrypsin (N-blocked esters or amides of phenylalanine; Hess, 1971). The 3-methyl compound was chosen because it most closely resembles specific substrates for α-lytic protease and elastase (N-blocked esters or amides of alanine; Gold & Shalitin, 1975; Kaplan et al., 1970). The results obtained indicate an high specificity toward enzymes with hydrolytic activity. None of the nonproteolytic enzymes tested were inactivated by the compounds even at a relatively high concentration (0.5 mM). A further degree of specificity was observed between classes of proteolytic enzymes. Neither trypsin (Keil, 1971), a serine protease that acts almost exclusively on amino acid esters and amides with charged side chains (lysine or arginine), nor carboxypeptidase, which is a metalloexopeptidase and acts on substrates having a free carboxy terminus (Hartsuck & Lipscomb, 1971), was inactivated. Specificity among the remaining serine proteases, α-chymotrypsin, α-lytic protease, acetylcholinesterase, and elastase, is less pronounced. Examination of Table I reveals that, as expected, the alanine analogue 3-methyl-6-chloro-2-

pyrone is a more effective inactivator for α-lytic protease than 3-benzyl-6-chloro-2-pyrone. With elastase, the difference in activity is much less pronounced. It should also be noted that 5-benzyl-2-chloro-pyrone (**3**) is an effective inactivator of α-lytic protease. As far as we know, no active site directed inactivators for α-lytic protease have been reported.

It is unclear why papain, a sulfhydryl protease of broad substrate specificity, is resistant to inactivation. The lack of activity of the parent compound, 6-chloro-2-pyrone, is remarkable. This result suggests that enzyme–side-chain interactions are important for inactivation to occur and that inactivation does not take place by a mechanism involving nonspecific bimolecular alkylation or acylation. Hopefully, by synthesis of compounds in which the chloropyrone nucleus is incorporated into a short polypeptide with a sequence complementary to specific enzyme subsites, greater specificity could be achieved.

The 5-substituted 6-chloro-2-pyrones show similar enzyme specificity (Table I), although they inactivate by different mechanisms. This difference will be further discussed subsequently.

*Reactions of 3-Benzyl-6-chloro-2-pyrone (**2**) and Chymotrypsin.* Consistent with the proposed mechanism of inactivation and the operation of the suicide mode of inactivation, 3-benzyl-6-chloro-2-pyrone is also a substrate for chymotrypsin with $K_m = 9$ μM and $k_{cat} = 0.55$ min^{-1}. The compound is hydrolyzed to (*E*)-4-benzyl-2-pentenedioic acid (**7**) and the anhydride (**6**) (Scheme II). During the hydrolysis of 3-benzyl-6-chloro-2-pyrone, an enzyme–substrate complex, presumably an acyl-enzyme, accumulates. Evidence for this was obtained from two types of experiments. First, when substrate and enzyme are mixed, a burst in substrate disappearance measured as a rapid decrease in the absorbance at 304 nm is observed. The magnitude of the burst indicates that approximately 67% of the enzyme is occupied at steady state. Furthermore, the fact that the absorbance at 304 nm decreases indicates that the pyrone ring is no longer present after the burst. Second, when preincubation mixtures consisting of enzyme and substrate are diluted into assay mixtures containing a second substrate (BTEE), the rate of hydrolysis of the second substrate is initially slow and increases continually over time until a constant maximal rate is attained. Return of activity during this lag phase is first order with a rate constant $k_{react} = 2.2$ min^{-1}. The fraction of the total enzyme present in the transiently inactive form at steady state was estimated to be 70–80%, which is in good agreement with the fraction of enzyme occupied in the initial burst (67%). This fact suggests that the two events represent the decomposition

Scheme III

$$E \overset{S}{\rightleftharpoons} ES \rightleftharpoons EP \overset{P}{\longrightarrow} E$$

$$\downarrow$$

$$EP_{inact}$$
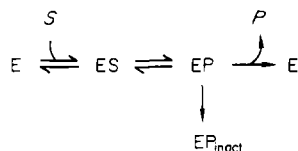
and formation of the same species, most probably an acyl-enzyme.

A minimal reaction scheme for the reaction of 3-benzyl-6-chloro-2-pyrone chymotrypsin is shown in Scheme III. EP is the species that accumulates at steady state and in which the chloropyrone ring is no longer present. It, presumably, is formed after attack of the active site serine on the chloro-pyrone (Scheme I). EP partitions between product formation and formation of the inactive enzyme. It was observed that k_{react}, which represents the rate of disappearance of EP, is 4 times k_{cat}, and we believe that this difference is significant. This difference in the two rate constants requires that EP can decompose to some compound other than the final hydrolysis products, 4-benzyl-2-pentenedioic acid (7) and the anhydride (6). Since the reaction stoichiometry that we have is not consistent with the formation of other products, it must be concluded that EP can also decompose to regenerate the starting chloropyrone (2) (S in Scheme III). So far, we have no experimental evidence in support of this.

The chemical nature of the compound(s) represented by EP in Scheme III needs to be considered. Any intermediates proposed for the hydrolysis of 3-benzyl-6-chloro-2-pyrone must account for the formation of the two hydrolysis products: (E)-4-benzyl-2-pentenedioic acid (7) and the anhydride (6). A possible pathway is shown in Scheme II; clearly, a number of variables of this scheme can be envisioned at 11. Structures 11, 12, and 13 correspond to EP of Scheme III. The reaction is initiated by the general base catalyzed addition of the active site serine to the pyrone. This generates the tetrahedral intermediate (10), which breaks down to the enolized acyl halide (11) as a discrete intermediate. Ketonization of 11 can occur by protonation at C-2 to yield the $trans$-Δ^3-acyl halide (13) or by protonation at C-4 to yield the cis-Δ^2-acyl chloride (12). Once formed, the acylenzyme intermediate (13) can give rise to the observed diacid product (7) by either direct deacylation of 13 followed by release of the corresponding acyl chloride into solution or by acyl chloride hydrolysis at the active site followed by enzymatic deacylation of the resulting carboxylic acid containing acyl enzyme. Because acyl halides are extremely unstable in aqueous media, these two possibilities are indistinguishable. The minor product anhydride (6), is thought to be derived from the acyl-enzyme (12) via a carboxylic acid intermediate (14).

The alternative possibility, a process in which collapse of the tetrahedral intermediate (10) occurs simultaneously with protonation, can account for the formation of 12 but not 13, the precursor of the major product (7). Conversion of 10 to 12 requires 180° rotation about the C_3–C_4 bond. It is highly unlikely that such a rotation could be accommodated in the transition state for concerted ring opening and proton transfer.

In addition to being a substrate, 3-benzyl-6-chloro-2-pyrone is an effective inactivator of chymotrypsin. Loss of enzyme activity is pseudo first order, consistent with either active site directed or mechanism-based inactivation, and occurs in a biphasic fashion (Figure 1). Biphasic kinetics of inactivation are due to the presence of two different catalytically active species in commercial chymotrypsin preparations. The ratio of turnover/inactivation is approximately 14 for the initial rapid phase and 40 for the slower phase. The specific activity,

as measured by the rate of hydrolysis of the chloropyrone, is constant over the course of inactivation. The difference in rate of inactivation between the two forms of enzyme must, therefore, be due to the efficiency with which a reactive intermediate is trapped not how rapidly it is formed. The time dependence of inactivation does not show a lag phase, indicating that inactivation does not occur through enzyme-catalyzed formation of a reactive species that is released into the solvent and subsequently reacts with the enzyme. The rate of inactivation is saturable and inactivation is prevented by a specific competitive inhibitor for chymotrypsin. These observations support the conclusion that inactivation results from selective modification of a functional group of the active site rather than a nonspecific reaction. Since enzyme activity does not return on removal of excess inactivator by gel filtration, it is likely that enzyme modification is covalent.

What is the structure of the inactivated enzyme and how is it formed? We consider it most likely that inactivation occurs from structures 12 or 13 (Scheme II). We have considered several mechanism: (1) Acylation of a nucleophile can occur while the inactivator remains attached to the active site serine as proposed in Scheme I. Examination of a CPK model of the chymotrypsin active site acetylated at serine-195 with acyl chlorides derived from the chloropyrone suggests that the only likely group to be acetylated is the imidazole of His-57. (2) The serine ester of structure 12 or 13 is hydrolyzed and the resulting half-acid chloride can either escape from the active site and form reaction products or acetylate a nucleophile at the active site, which would lead to inactivation. In this event, only one covalent bond would be found between enzyme and inactivator. We have as yet been unable to distinguish these two possibilities.

The intermediate involvement of an acid chloride or its equivalent is supported by the observation that compounds structurally related to the pyrone that do not have a chloride substituent at C-6 such as the anhydride (6) and the unsubstituted 3-benzyl-2-pyrone do not inactivate. It should be noted that the reaction of these compounds, as well as the monomethyl ester of (E)-4-(methoxycarbonyl)-5-phenyl-3-pentenoic acid, involves intermediate formation of α,β-unsaturated acyl-enzymes. The formation of unsaturated acyl-enzymes, per se, is therefore probably not responsible for inactivation.

The absence of a long-wavelength chromophore is the most important result pertaining to the structure of the inactivated enzyme. In order to appreciate the significance of this observation, it is first necessary to consider the chemical reactivity of the 6-chloro-2-pyrone system. Hydrolysis can take place either by addition of water to the carbonyl carbon, ultimately yielding a dicarboxylic acid, or by 1,6-conjugate addition of water to the carbon-bearing chloride, giving rise to an anhydride. If the inactivation would have occurred by Michael addition by an enzyme nucleophile at C-6 the spectrum of the inactive enzyme would show a long-wave absorbance characteristic of the pyrone system. Since the inactivated enzyme has no absorption characteristic of the pyrone system, it is very likely that inactivation involves nucleophilic attack on the carbonyl carbon of the pyrone and subsequent ring opening. The inactivated enzyme regains catalytic activity slowly at neutral pH ($t_{1/2}$ = 1389 min) and rapidly in the presence of NH_2OH ($t_{1/2}$ = 24 min). These results are more consistent with the presence in the inactivated enzyme, of an ester linkage, or a labile amide bond.

Reaction of 5-Benzyl-6-chloro-2-pyrone (3) with Chymotrypsin. Incubation of chymotrypsin with 5-benzyl-6-chloro-2-pyrone causes time-dependent, pseudo-first-order loss of

Scheme IV
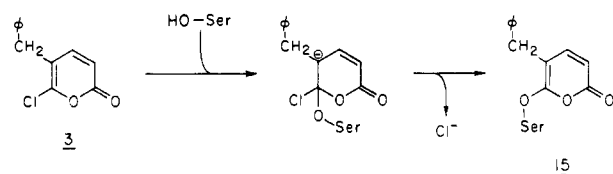
enzyme activity. Gel filtration of the inactivated enzyme does not cause reactivation, consistent with a mechanism of inactivation involving covalent modification of the enzyme. Saturation kinetics are observed ($K_I = 0.65$ mM and $k_{inact} = 0.70$ min^{-1}), excluding the possibility that inactivation occurs by a nonspecific bimolecular mechanism. The observation that inactivation is diminished in the presence of a competitive inhibitor indicated that covalent modification occurs at the enzyme active site.

Unlike the 3-benzyl isomer 5-benzyl-6-chloro-2-pyrone is not a substrate for chymotrypsin. The UV spectrum of the inactivated enzyme is obviously different from that of the native enzyme showing an absorbance maximum at 322 nm with $\epsilon = 7400$ min^{-1} cm^{-1}. The fact that the first-order rate constant for the increase in absorbance at 322 nm on preincubation of 5-benzyl-6-chloro-2-pyrone and chymotrypsin is comparable to that for inactivation indicates that the spectrum observed is due to the formation of the inactivated enzyme and not a secondary process unrelated to loss of enzyme activity. Confirmation of this ascertion was obtained by showing that reactivation on treatment with 0.9 N NH$_2$OH is accompanied by a decrease in the ratio A_{322}/A_{280}. We believe that the spectrum is most consistent with structure **15** (Scheme IV). and suggest by analogy to the known reactivity of 6-chloro-2-pyrone to hydroxide that inactivation is an active site directed process that occurs by the reaction sequence shown in Scheme IV. If this interpretation is correct, it follows that the point of attack of the active site serine on the pyrone nucleus is determined by the side chain. The importance of the side chain in conferring reactivity on the pyrone nucleus has all ready been pointed out.

Registry No. **1**, 20357-65-7; **2**, 85533-80-8; **3**, 85533-81-9; **4**, 53358-25-1; **5**, 53358-24-0; **6**, 85533-82-0; **7**, 85533-83-1; **8**, 85533-84-2; **9**, 85533-85-3; 1,1,3,3-tetracarbethoxypropene sodium salt, 85533-86-4; diethyl malonate, 105-53-3; chloroform, 67-66-3; 4-phenyl-1,1,3,3-tetracarbethoxybutene, 85533-87-5; (E)-2-benzyl-2-pentenedioic acid, 85533-88-6; 3-benzyl-2-pyrone, 85533-89-7; (E)-2-benzyl-2-pentenedioic acid dimethyl ester, 85533-90-0; (E)-4-(methoxycarbonyl)-5-phenyl-3-pentenoic acid, 85533-91-1; 2-pentenedioic acid anhydride, 5926-95-4; chymotrypsin, 9004-07-3; α-lytic protease, 37288-76-9; elastase, 9004-06-2; ACH-esterase, 9000-81-1; serine proteinase, 37259-58-8.

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