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Halo Enol Lactones: Studies on the Mechanism of Inactivation of α-Chymotrypsin†

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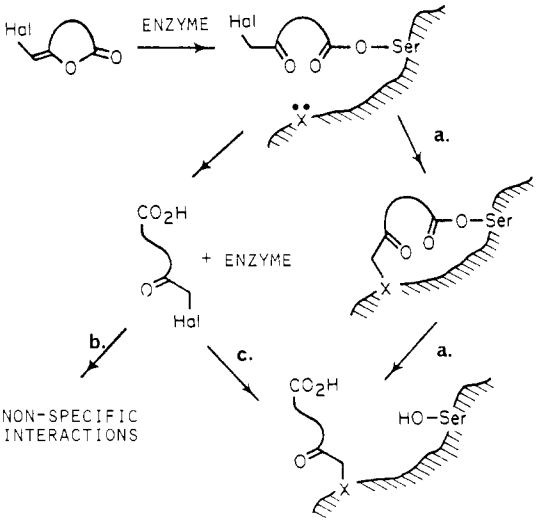
ABSTRACT: In a previous investigation [Daniels, S. B., Cooney, E., Sofia, M. J., Chakravarty, P. K., & Katzenellenbogen, J. A. (1983) *J. Biol. Chem. 258*, 15046–15053], we demonstrated that α-aryl-substituted five- and six-membered ring halo enol lactones were effective inhibitors of chymotrypsin, and we proposed that they reacted by an enzyme-activated mechanism: acyl transfer to the active site serine generates a halomethyl ketone that remains tethered in the catalytic site until it alkylates an accessible nucleophilic residue. In this study, we have investigated in greater detail the process of chymotrypsin inactivation by an α-naphthyl-substituted five- and six-membered bromo enol lactone. Inactivation by both compounds appears to be active site directed, since the time-dependent inactivation is retarded by competing substrate. The possible involvement of a paracatalytic mechanism for inactivation (generation of a free, rather than active site bound, inactivating species) was investigated by comparing the inactivation efficiencies of the lactones with that of the bromomethyl keto acid hydrolysis products. The bromomethyl ketone derived from the five-membered lactone is ineffective, whereas that derived from the six-membered lactone is highly efficient. However, the possible involvement of the free keto acid in chymotrypsin inactivation by the six-membered lactone is ruled out by experiments involving selective scavenging. The long-term inactivation of chymotrypsin requires the presence of the bromine substituent and appears to involve an alkylation rather than an acylation reaction (hydrazine resistant). Furthermore, a 1:1 lactone:enzyme stoichiometry is demonstrated with the ^{14}C-labeled six-membered lactone. These results are consistent with the mechanism-based inactivation process previously presented.

In previous studies, we described the preparation of halo enol lactones (Krafft & Katzenellenbogen, 1981) and their activity as active site directed irreversible inhibitors of chymotrypsin (Chakravarty et al., 1982; Daniels et al., 1983). On the basis of their structure, we proposed that these lactones were acting as mechanism-based inactivators: Acyl transfer to the active site serine would generate a halomethyl ketone that would remain tethered in the catalytic site and, during the lifetime of the acyl enzyme, would alkylate an accessible nucleophilic residue; this covalent attachment, at or near the active site, would then impair the catalytic activity of the enzyme, even after deacylation (Scheme I, pathway a).

In order for this mechanism-based or "suicide" inactivation process to achieve high efficiency and selectivity, it is important that the activated inhibitor undergo covalent attachment more rapidly than it departs from the active site. Otherwise, the released halomethyl keto acid can also react with components outside of the catalytic site, compromising the selectivity of its inactivation (Scheme I, pathway b). The assessment of the

Scheme I



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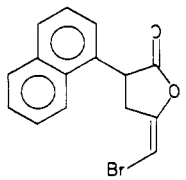
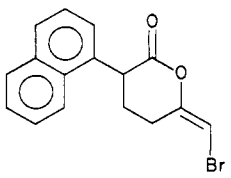
inactivation mechanism may be complicated further by the fact that any halomethyl keto acid that has escaped from the active site by hydrolysis of the acyl enzyme may return to inactivate the enzyme ("pseudosuicide" or paracatalytic inactivation) (Walsh, 1982) (Scheme I, pathway c).

In order to establish that a compound is acting as a true mechanism-based irreversible inactivator as proposed (Scheme I, pathway a), it is necessary to establish (1) that it is acting at the active site, (2) that the inactivating species remains in the catalytic site prior to inactivation (not pseudo-suicide), (3) that it forms a permanent, covalent attachment to the enzyme (not a tight binding or acyl–enzyme complex), and (4) that reaction proceeds with a 1:1 enzyme to inactivator stoichiometry.

Previously we prepared an extended series of aryl-substituted halomethylenedihydrofuranones and -tetrahydropyranones and developed a comprehensive kinetic analysis for their inactivation of chymotrypsin (Daniels et al., 1983). We found that the potency and efficiency of these compounds as suicide inactivators depended largely on the lactone ring size and to a much lesser extent on the nature of the aryl groups and halogen. In this study, we have selected a single lactone representative of each ring size, (*E*)-5-(bromomethylene)dihydro-3-(1-naphthalenyl)-2(3*H*)-furanone (Np5Br)[1] and (*E*)-6-(bromomethylene)tetrahydro-3-(1-naphthalenyl)-2*H*-pyran-2-one (Np6Br), and we have shown through studies of



Np5Br



Np6Br

the kinetics and stoichiometry of their inactivation and the stability of the enzyme product that they are behaving as true enzyme-activated irreversible inhibitors of chymotrypsin.

Experimental Procedures

Chemical Synthesis

General. Proton magnetic resonance (^{1}H NMR) spectra were obtained on a Varian Associates Model HR-220 (220 MHz) or Nicolet Model NT (360 MHz) spectrometer and are expressed as parts per million downfield from tetramethylsilane as an internal standard (δ scale). The ^{1}H NMR data are presented in the form δ value of signal [peak multiplicity, coupling constant (if applicable), integrated number of protons]. Infrared (IR) spectra were obtained on a Perkin-Elmer Model 1320 IR spectrometer, and the data reported for the important diagnostic absorptions. Mass spectra were obtained on a Varian Associates MAT CH-5 spectrometer at 10 and 70 eV. Data are reported in the form *m/z* (intensity relative to base peak = 100). Elemental analyses were performed by the Microanalytical Service Laboratory of the University of Illinois School of Chemical Sciences.

Analytical thin-layer chromatography (TLC) was carried out on 0.25 mm Merck silica gel 60 F-254 glass backed plates, and compounds were visualized by ultraviolet light or iodine vapor. All flash chromatography was performed with Woelm silica gel (32–63 μm) (Still et al., 1978). High-performance liquid chromatographic (HPLC) separations were performed by using a Spectra Physics model 8700 solvent delivery system, and compounds were detected at 280 nm with a Beckman Model 153 UV detector.

Solvents and reagents used were purchased as analytical reagent grade. Tetrahydrofuran (THF) was dried immediately prior to use by distillation from sodium benzophenone ketyl. Diisopropylamine was purified by distillation from calcium hydride. *n*-Butyllithium as a hexane solution was purchased from Alfa (Ventron) and titrated for organic base content (Kofron & Baclawski, 1976). 1-Naphthyl[*carboxy*-^{14}C]acetic acid was purchased from Pathfinder Laboratories. Air- and moisture-sensitive reactions were carried out in oven-dried glassware and under an atmosphere of dry nitrogen. The preparation of 4-bromo-1-butyne, α-2-propynyl-1-naphthaleneacetic acid, α-3-butynyl-1-naphthaleneacetic acid, Np5Br, and Np6Br has been reported in a previous publication (Daniels et al., 1983).

5-Methylenedihydro-3-(1-naphthalenyl)-2(3H)-furanone (Np5H). Mercuric trifluoroacetate (19 mg, 0.045 mmol) and α-2-propynyl-1-naphthaleneacetic acid (100 mg, 0.45 mmol) in 25 mL of methylene chloride were stirred for 1 h at room temperature. The reaction mixture was washed with water and then saturated sodium chloride and dried over anhydrous magnesium sulfate. The solvent was removed and the crude oil purified by flash chromatography using 20% ether in hexane to yield 64 mg (64%) of a clear oil: ^{1}H NMR ($CDCl_3$) δ 3.27 (AB quartet, $\Delta\nu_{AB}$ = 172.8 Hz, J = 30.4 Hz with each peak split into a dt, upfield portion J = 16.4, 2.3 Hz, downfield portion J = 20.6, 2.1 Hz, 2 H), 4.38 (dd, J = 2.3, 2.1 Hz, 1 H), 4.70 (dd, J = 20.6, 16.4 Hz, 1 H), 4.89 (dd, J = 2.3, 2.1 Hz, 1 H), 7.36–7.62 (m, 4 H), and 7.79–7.95 (m, 3 H); IR (nujol mull) 1800 cm^{-1}; mass spectrum (10 eV), *m/z* 224 (84.5, M^+), 181 (16.8), 154 (100), 58 (24.8), 43 (52.6), 32 (26.9), 31 (29.1). Anal. Calcd for $C_{15}H_{12}O_2$: C, 80.34; H, 5.39. Found: C, 80.63; H, 5.40.

6-Methylenetetrahydro-3-(1-naphthalenyl)-2H-pyran-2-one (Np6H). Mercuric trifluoroacetate (0.17 g, 0.40 mmol) and α-3-butynyl-1-naphthaleneacetic acid (0.95 g, 4.0 mmol) in 50 mL of methylene chloride were stirred for 18 h at room temperature. Workup and purification as for Np5H gave 0.41 g (43%) of a clear oil: ^{1}H NMR ($CDCl_3$) δ 2.25–2.32 (m, 2 H), 2.62–2.69 (m, 2 H), 4.41 (s, 1 H), 4.55 (dd, J = 8.6, 6.9 Hz, 1 H), 4.82 (s, 1 H), 7.26–7.65 (m, 4 H), and 7.78–7.97 (m, 3 H); IR (nujol mull) 1750 cm^{-1}; mass spectrum (10 eV), *m/z* 238 (100, M^+), 210 (51.7), 168 (11.8), 167 (13.8), 154 (31.8), 152 (21.3). Anal. Calcd for $C_{16}H_{14}O_2$: C, 80.65; H, 5.92. Found: C, 80.59; H, 6.11.

α-(3-Bromo-2-oxopropyl)-1-naphthaleneacetic Acid (Np5BMK). A solution of bromine (12 μL, 0.22 mmol) in 1 mL of a buffered acetic acid solution (1.4 g of sodium acetate in 25 mL of glacial acetic acid) was added dropwise to Np5H (50 mg, 0.22 mmol) in 5 mL of buffered acetic acid at 0 °C. The solution was stirred at room temperature for 1 h, poured into ice water, extracted with ether, washed with water and then saturated sodium chloride, and dried over anhydrous

[1] Abbreviations: Lactones [a nomenclature shorthand for the lactones, previously proposed (Daniels et al., 1983), has been used here: the first designation (Np) indicates that a 1-naphthyl substituent is on carbon 3; the second designation (5 or 6) indicates the lactone ring size, and the third designation (H, Br, or I) indicates the nature of the enol substituent]: Np5Br, (*E*)-5-(bromomethylene)dihydro-3-(1-naphthalenyl)-2-(3*H*)-furanone; Np6Br, (*E*)-6-(bromomethylene)tetrahydro-3-(1-naphthalenyl)-2*H*-pyran-2-one; [^{14}C]Np6I, (*E*)-6-(iodomethylene)tetrahydro-3-(1-naphthalenyl)-2*H*-[2-^{14}C]pyran-2-one; Np5H, 5-methylenedihydro-3-(1-naphthalenyl)-2(3*H*)-furanone; Np6H, 6-methylenetetrahydro-3-(1-napthalenyl)-2*H*-pyran-2-one. Bromomethyl ketones: Np5BMK, α-(3-bromo-2-oxopropyl)-1-naphthaleneacetic acid; Np6BMK, α-(4-bromo-3-oxobutyl)-1-naphthaleneacetic acid. Substrates: BzTyrOEt, *N*-benzoyl-L-tyrosine ethyl ester; AcTrpONP, *N*-acetyl-L-tryptophan *p*-nitrophenyl ester.

magnesium sulfate. The solvent was removed, and the crude oil was purified by flash chromatography using 20% ether in hexane to yield 41 mg (57%) of a clear oil: ^{1}H NMR ($CDCl_3$) δ 3.00 (dd, J = 29.4, 5.7 Hz, 2 H), 3.89 (s, 2 H), 5.02 (dd, J = 18.1, 6.1 Hz, 1 H), 7.37–7.61 (m, 4 H), 7.77–7.91 (m, 3 H), 8.05 (d, J = 6.8 Hz, 1 H); mass spectrum (10 eV), m/z 322 (2.10, M^+), 320 (1.95, M^+), 304 (18.3), 302 (16.9), 195 (38.2), 168 (63.1), 167 (100). Anal. Calcd for $C_{15}H_{13}BrO_3$: C, 56.10; H, 4.08; Br, 24.88. Found: C, 55.96; H, 3.81; Br, 25.00.

α-(4-Bromo-3-oxobutyl)-1-naphthaleneacetic Acid (Np6BMK). A solution of bromine (16 μL, 0.31 mmol) in 1 mL of buffered acetic acid (see Np5BMK) was added dropwise to Np6H (74 mg, 0.31 mmol) in 5 mL of buffered acetic acid at 0 °C. The mixture was stirred at room temperature for 1 h. Workup and purification as for Np5BMK gave 32 mg (31%) of a clear oil: ^{1}H NMR ($CDCl_3$) δ 2.65–2.93 (m, 4 H), 4.02 (s, 2 H), 4.58 (dt, J = 9.3, 0.5 Hz, 1 H), 7.39–7.58 (m, 4 H), 7.77–7.93 (m, 3 H), 8.30 (d, J = 0.5 Hz, 1 H); mass spectrum (10 eV), m/z 336 (1.02 M^+), 334 (1.01, M^+), 318 (15.6), 316 (14.4), 237 (19.5), 236 (16.2), 209 (48.3), 168 (50.6), 167 (100). Anal. Calcd for $C_{16}H_{15}BrO_3$: C, 57.33; H, 4.51; Br, 23.84. Found: C, 57.42; H, 4.49; Br, 24.08.

α-3-Butynyl-1-naphthalene[carboxy-^{14}C]acetic Acid. *n*-Butyllithium (0.45 mL of a 2.4 M solution, 1.1 mmol) was added dropwise to a solution of diisopropylamine (0.16 mL, 1.2 mmol) in 2 mL of dry THF at 0 °C under nitrogen and stirred for 30 min. This solution of lithium diisopropylamide was added dropwise over 15 min to 1-naphthyl[*carboxy*-^{14}C]acetic acid (100 mg, 0.53 mmol, 0.27 mCi) in 2 mL of dry THF at −78 °C under nitrogen and stirred for 2 h. To the orange solution of the dianion was added dropwise a solution of 4-bromo-1-butyne (70.4 mg, 0.53 mmol) in 2 mL of THF, and the reaction mixture was allowed to warm to room temperature. After 16 h the reaction was quenched with 1 mL of 1 N hydrochloric acid, 5 mL of ether was added, the organic layer was washed with another 1-mL portion of acid, and the combined acid washes were extracted with three 5-mL portions of ether. All the organic extracts were combined, washed with saturated sodium chloride, dried over anhydrous magnesium sulfate, and filtered, and the solvent was removed to yield 109 mg (86.5%) of a light yellow oil. TLC using 30% ether in hexane showed that the product had an identical R_f with that of an authentic sample of the unlabeled compound (Daniels et al., 1983). The product was used in the next reaction without further purification.

(E)-6-(Iodomethylene)tetrahydro-3-(1-naphthalenyl)-2H-[2-^{14}C]pyran-2-one ([^{14}C]Np6I). A mixture of α-3-butynyl-1-naphthalene[*carboxy*-^{14}C]acetic acid (109 mg, 0.46 mmol, 0.23 mCi), potassium bicarbonate (50 mg, 0.50 mmol), and iodine (119 mg, 0.50 mmol) in 10 mL of dry acetonitrile was stirred in the dark at room temperature for 24 h. The solvent was removed under a stream of nitrogen and the residue taken up in ether, washed with 5% sodium thiosulfate and then saturated sodium chloride, and dried over anhydrous magnesium sulfate. The solvent was removed, and the crude product was purified by flash chromatography using 20% ether in hexane to give 79 mg (47%) of colorless crystals with a melting point of 109.5–111 °C and a specific activity of 1130 dpm/nmol. TLC (using 20% ether in hexane) and HPLC (using a gradient of 50% 0.01 N sodium acetate/acetic acid, pH 4.2, in acetonitrile to 100% acetonitrile over 10 min on a 3.9 mm × 30 cm Waters μBondapak column with a flow rate of 2 mL/min) showed that the product had an identical R_f and retention time, respectively, with those of an authentic sample of the unlabeled compound (Daniels et al., 1983).

Biochemical Procedures

General. Spectrophotometric data were obtained on a Varian Associates Model 635 UV–vis or Hewlett-Packard Model 8451A spectrophotometer. A phosphate buffer used in all inactivation studies was 0.1 M in total potassium phosphate, pH 7.2.

α-Chymotrypsin (three times crystallized and free of autolysis products and low molecular weight contaminants) was obtained from Worthington Biochemical and was shown to have 88–96% of theoretical activity by active site titration with *N-trans*-cinnamoylimidazole (Schonbaum et al., 1961). *N*-Benzoyl-L-tyrosine ethyl ester (BzTyrOEt) was obtained from Sigma. *N*-Acetyl-L-tryptophan *p*-nitrophenyl ester (AcTrpONP) was obtained from Vega Biochemicals.

The procedures for the time-dependent inactivation assays of chymotrypsin by halo enol lactones with and without added substrate are described elsewhere (Daniels et al., 1983).

Determination of Ultimate Enzyme Activity. Incubations were carried out in triplicate by using inhibitor concentrations that would not totally inactivate the enzyme. The remaining chymotrypsin activity was determined 15–24 h after the addition of inhibitor by transferring a 25-μL aliquot of the incubation mixture to a cuvette containing a 1:1 mixture of 1.5 mM BzTyrOEt (in 50% methanol/water) and 0.1 N phosphate buffer, pH 7.2. After an initial lag period of 0.3–3 min (presumed to arise from deacylation of enzyme not yet inactivated) the rate of change in the UV absorbance at 256 nm, with respect to a cuvette containing only BzTyrOEt/buffer solution, gave a straight line with a slope proportional to the amount of active enzyme remaining. The enzyme activity is expressed relative to a chymotrypsin control solution that was treated the same as the incubation mixture except that no inhibitor was added.

Ultimate Activity of Np5Br and Np6Br and Their Hydrolysis Products. Stock solutions of the inhibitors in acetonitrile were added to test tubes, the solvent was blown off, and 1 mL of 2.0 μM chymotrypsin in pH 7.2 phosphate buffer was added to give final inhibitor to enzyme molar ratios from 1.5 to 13.5 for Np5Br, 1.5 to 13.5 for Np5BMK, 0.5 to 3.5 for Np6Br, and 0.25 to 1.0 for Np6BMK. After an incubation of 15 h at 25 °C, the ultimate enzyme activity was determined at each concentration for each inhibitor and plotted vs. the initial inhibitor to enzyme molar ratio.

HPLC of Np6Br and Np6BMK in the Presence of Nucleophiles. A 1:1 mixture of 0.5 mM Np6Br and 0.5 mM Np6BMK in acetonitrile was mixed with an equal volume of a 1 M solution of a nucleophile in pH 7.2 phosphate buffer. After 15 s, 10 μL of the mixture was injected onto a 3.9 mm × 30 cm Waters μBondapak column using a separation gradient from 50% 0.01 N sodium acetate/acetic acid, pH 4.2, at 0 min to 100% acetonitrile at 10 min with a flow rate of 2 mL/min. The retention times for Np6Br and Np6BMK were 7.9 and 6.7 min, respectively. The ratio of Np6Br to Np6BMK peak heights is expressed relative to the same ratio from a blank in which phosphate buffer was added instead of the nucleophile solution.

Scavenging Assay: Ultimate Activity in the Presence of Methylamine. Various amounts of a stock solution of methylamine in pH 7.2 phosphate buffer was added to a 6 μM solution of chymotrypsin in buffer; the pH was adjusted back to 7.2 with 1 mM hydrochloric acid in buffer, and the solutions were diluted with buffer to 3.0 μM in enzyme to give final concentrations of methylamine from 0 to 20 mM. Each

concentration (1 mL) of the enzyme/methylamine was added to two series of test tubes. To each tube in one series was added 25 μL of 0.14 mM Np6Br in acetonitrile and to each tube in the other 25 μL of 0.10 mM Np6BMK in acetonitrile. After an 18-h incubation at 25 °C, the ultimate enzyme activity was determined and expressed relative to blanks containing the same concentrations of enzyme and methylamine but no inhibitor. These results were plotted vs. the initial concentration of methylamine.

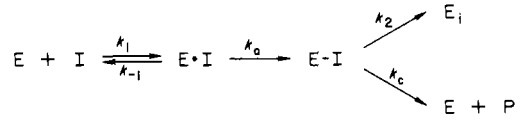
Reactivation Assay. Stock solutions of the inhibitors in acetonitrile were added to test tubes; the solvent was blown off, and 0.5 mL of 4.0 μM chymotrypsin in pH 7.2 phosphate buffer was added to give final inhibitor to enzyme molar ratios from 1.5 to 9 for Np5Br and from 0.5 to 3 for Np6Br. After incubation at 25 °C for 16 h, the ultimate enzyme activity was determined and plotted vs. the initial inhibitor to enzyme molar ratios. The remaining incubation mixture was put onto a 0.5 × 5 cm Sephadex G-25 column (void volume = 1.0 mL) which was preequilibrated and eluted with buffer. To the first 2.0 mL of eluent was added 50 μL of a 4 M hydrazine solution in buffer to give a final deacylator concentration of 100 mM. After 1 h the enzyme activity was determined and expressed relative to a blank that was treated identically but did not contain any inhibitor. These results were also plotted vs. the initial inhibitor to enzyme molar ratio.

Stoichiometry of Inactivation. Various amounts of a stock solution of [^{14}C]Np6I (1130 dpm/nmol) in acetonitrile were added to two sets of test tubes, and the solvent was blown off. To one set of tubes was added 1 mL of 9.26 μM chymotrypsin in pH 7.2 phosphate buffer to give final inhibitor to enzyme molar ratios of 0.25 to 1.75 and to the other set only 1 mL of buffer. After the tubes were incubated for 19 h at 25 °C, a 25-μL aliquot was removed from each tube, and the ultimate enzyme activity was determined for those containing chymotrypsin. All of the samples were lyophilized, taken up in 150 μL of water, and applied to 1.5-cm disks of Whatman No. 3 filter paper (Katzenellenbogen et al., 1975). Each tube was rinsed with two 100-μL portions of water, and these washes were also applied to the disks. The disks were boiled twice in 500-mL portions of 95% ethanol, washed twice with 500-mL portions of 1:1 ethanol/ether and twice with 500-mL portions of ether, put in vials with scintillation fluid, and each counted for 10 min. The counts from the disks spotted only with inhibitor solution were subtracted from those counts on the disks containing enzyme and the same concentration of inhibitor. This number was further corrected by taking into account the counts removed from the sample for the determination of the enzyme activity (2.5% of the total sample), and the 96.5% counting efficiency of the scintillation counter. The amount of enzyme-bound inhibitor could be determined by using the specific activity of the inhibitor (1130 dpm/nmol). The molar ratio of covalently bound inhibitor to initial amount of enzyme was plotted vs. the ultimate enzyme activity.

Results

Kinetic Models for the Inactivation of Chymotrypsin by Halo Enol Lactones. Earlier, we presented an inactivation scheme and a kinetic model for the analysis of chymotrypsin inhibition by the halo enol lactones (Daniels et al., 1983). In this simplified analysis, the inactivation step proceeded directly from the reversible enzyme–inhibitor binding complex to the covalently modified enzyme. According to Scheme I, however, acyl transfer to the serine hydroxyl is required to unmask the reactive halo methyl ketone moiety prior to the inactivation step. We reasoned that this acylation step could be ignored in the kinetic analysis, since it is known to be very fast in the case of most ester substrates of chymotrypsin (Hess, 1971). If the covalent attachment of the inhibitor is assumed to be relatively slow, making it the rate-limiting step, then the simplified kinetic analysis would be valid. It became apparent to us in further studies, however, that in some assay systems with very potent and efficient irreversible inhibitors the acylation step can be very important and can significantly alter the interpretation of the inactivation kinetics.

A more complete inactivation scheme that includes the acylation step is



E is the enzyme, I the inhibitor, and k_{-1}/k_1 the dissociation constant for the reversible binding complex, E•I, which is processed to the acyl enzyme–inhibitor complex, E–I, at a rate described by k_a. The acyl intermediate can either deacylate to regenerate free enzyme and an inhibitor hydrolysis product, P, at a rate described by k_c or form the irreversibly inactivated enzyme, E_i, at a rate described by k_2.

Steady-state analysis of the kinetics for this overall process, assuming a large excess of inactivator over enzyme (so that inactivator concentration does not change significantly during the inactivation period), gives

$$\ln\left(\frac{E_0 - E_i}{E_0}\right) = \frac{-At}{(B/I_0) + 1} = -k_{obsd}t \qquad (1)$$

where

$$A = \frac{k_a k_2}{k_a + k_2 + k_c}$$

$$B = \left(\frac{k_{-1} + k_a}{k_1}\right)\left(\frac{k_2 + k_c}{k_2 + k_c + k_a}\right)$$

and E_0 and I_0 are the initial enzyme and inhibitor concentrations, respectively (Waley, 1980; Tatsunami et al., 1981). The constants A and B are roughly analogous to the maximum velocity and Michaelis binding constant in Michaelis–Menten kinetics. According to eq 1, a semilogarithmic plot of the fraction of active enzyme vs. time after the addition of the suicide inactivator should give a straight line with a slope equal to the observed rate constant, k_{obsd}.

Kinetics of Time-Dependent Inactivation of Chymotrypsin by Np5Br and Np6Br: Evidence for Active Site Interaction. Chymotrypsin was incubated with a 50-fold excess of Np5Br; aliquots were removed at various times; the inactivation process was stopped by dilution into a concentrated substrate solution, and after allowing for the deacylation of the acyl enzyme-inhibitor complex, the remaining enzyme activity was determined. The semilogarithmic plot of the enzyme activity vs. time after inhibitor addition, shown in Figure 1A (solid line), is a straight line from which the observed rate constant was determined from the slope to be 0.26 min^{-1}. The inclusion of the chymotrypsin substrate in the incubation mixture slowed the inactivation process by competing with the inactivator for access to the enzyme's catalytic site (Figure 1A, broken line). This substrate protection and the linearity of the semilogarithmic time-dependent plots are indicative of an active site directed process.

The rearrangement of eq 1 gives

$$\frac{1}{k_{obsd}} = \frac{B}{A}\frac{1}{I_0} + \frac{1}{A} \qquad (2)$$

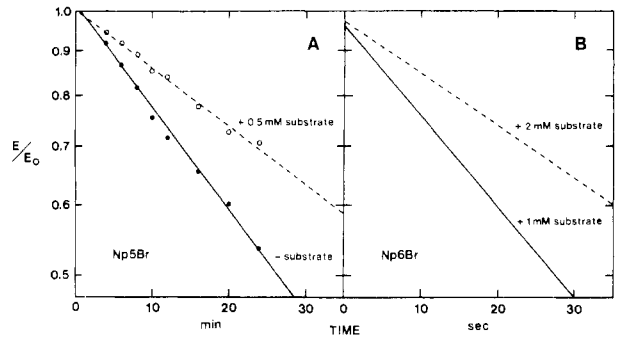


FIGURE 1: Time-dependent loss of activity and substrate protection. (Panel A) Chymotrypsin (1 μM) was incubated with 50 μM Np5Br (solid line) or 50 μM Np5Br and 0.50 mM BzTyrOEt (broken line) at pH 7.2 and 25 °C, and the remaining enzyme activity was determined in aliquots removed at regular time intervals. A semilogarithmic plot of the fraction of enzyme activity vs. time gives a straight line with a slope of $-k_{obsd}$. (Panel B) Chymotrypsin (25 μL of a 2.42 μM solution) was added to 1 mL of a solution of inhibitor Np6Br and 1 (solid line) or 2 μM (broken line) AcTrpONP at pH 7.2 and 25 °C. As the enzyme interacts with the inhibitor, the rate of substrate hydrolysis decreases, producing an inhibition progress curve (not shown). The first derivative of the progress curve was obtained directly from a computer-assisted spectrophotometer and indicates the velocity of substrate hydrolysis as a function of time. A semilogarithmic plot of the fraction of enzyme activity vs. time gives a straight line with a slope of $-\rho$.

When chymotrypsin was incubated with different concentrations of Np5Br, a series of observed rate constants were obtained. A double-reciprocal plot of k_{obsd} vs. the initial inhibitor concentration is shown in Figure 2A. Extrapolation to infinite inhibitor concentration ($1/I_0 = 0$) gives the value for $1/A$, while the x intercept gives the value for $-1/B$. From these intercepts, binding constant B was found to be 17 μM and inactivation rate constant A was found to be 0.036 min^{-1}. If, as was the case in the simplified kinetic analysis utilized in our earlier publication, the value of k_a is assumed to be large compared with the values of k_2 and k_c (i.e., $k_a + k_2 + k_c \cong k_a$), then the value of A would closely approximate that of the true inactivation rate constant, k_2. The presence of nonzero intercepts on a double-reciprocal plot of this type is characteristic of the saturation kinetics associated with the binding of a competitive inhibitor prior to an inactivation step.

When the time-dependent assay for the inhibition of chymotrypsin by Np6Br was attempted, the inactivation of the enzyme appeared to be complete before an aliquot could be removed from the incubation mixture. Our solution to the rapidity of this process was to include a chymotrypsin substrate in the incubation mixture to slow the rate of enzyme inactivation by substrate protection (Daniels et al., 1983; Main, 1973). The decrease in the rate of substrate hydrolysis can then be continuously followed as the enzyme becomes inhibited. The resulting inactivation progress curve for Np6Br was analyzed and replotted to give Figure 1B (solid line). The observed rate constant in the presence of substrate, ρ, was found to be 0.025 s^{-1}. At higher substrate concentrations, the inhibition is slowed further (Figure 1B, broken line), indicating an active site directed process.

According to the earlier, simplified kinetic scheme, this observed rate should be proportional to the inactivation rate. This assumption, however, is not valid because this assay procedure is continuous and does not allow for deacylation before the enzyme activity is measured as in the discrete aliquot method previously described. In addition, it has been observed that deacylation is slow in comparison to the assay period, so that essentially no enzyme is regenerated during the assay. Therefore, according to this assay, the acyl enzyme–

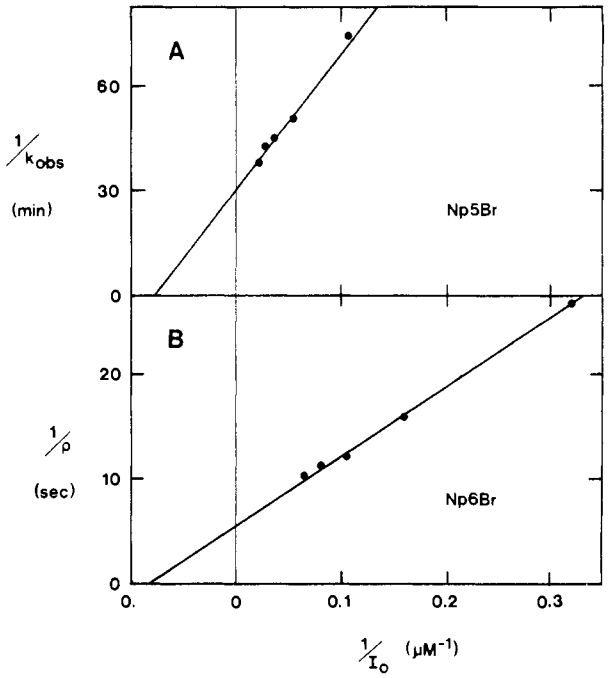


FIGURE 2: Determination of binding and rate constants. (Panel A) Chymotrypsin (1 μM) was incubated with different concentrations of Np5Br at pH 7.2 and 25 °C, and the enzyme activity was determined in aliquots removed at regular time intervals. From semilogarithmic plots, similar to those in Figure 1A, the observed inactivation rate constant, k_{obsd}, was determined for each inhibitor concentration. A double-reciprocal plot of k_{obsd} vs. the initial concentration of Np5Br gives a straight line with an x intercept of $-1/B$ and a y intercept of $1/A$, where B is the binding constant and A is an inactivation rate constant (see text). (Panel B) Chymotrypsin (59 nM) was inactivated in the presence of substrate (AcTrpONp) with different concentrations of Np6Br at pH 7.2 and 25 °C, and an inhibition progress curve was obtained for each inhibitor concentration. These curves were analyzed and replotted to give semilogarithmic plots, similar to those in Figure 1B, from which a series of observed "inactivation" rate constants ρ were obtained. A double-reciprocal plot of ρ vs. the initial concentration of Np6Br gives a straight line with an x intercept of $-1/C$ and a y intercept of $1/K_a$, where C is the binding constant.

inhibitor complex appears to be irreversibly inactivated, and the inactivation equation reduces to

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} E{\cdot}I \xrightarrow{k_a} E\text{–}I$$

Steady-state analysis of this equation gives

$$\frac{1}{\rho} = \frac{C}{k_a}\frac{1}{I_0} + \frac{1}{k_a} \qquad (3)$$

where

$$C = \frac{k_{-1} + k_a}{k_1}$$

The competitive assay was performed at different inactivator concentrations, and the observed rate constant ρ was plotted vs. the initial inhibitor concentration on a double-reciprocal plot as shown in Figure 2B to give a y intercept of $1/k_a$ and an x intercept of $-1/C(1 + K_m/S_0)$ where K_m and S_0 are the Michaelis binding constant and the initial concentration of the substrate, respectively. From these intercepts, binding constant C was found to be 0.0978 μM and acylation rate constant k_a to be 7.69 min^{-1}. Although this kinetic treatment gives no information about inactivation rate constant k_2, it does indicate that Np6Br is behaving in an active site directed manner.

In the previous study we used this competitive assay to determine the inactivation rate constants, k_2, for the aryl-

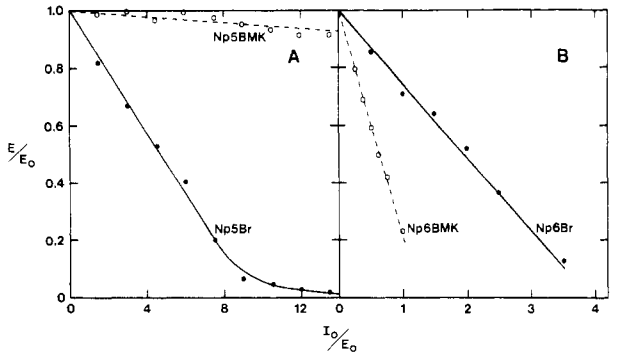


FIGURE 3: Ultimate activity assays of the lactones and their hydrolysis products. Chymotrypsin (2 μM) was incubated with different concentrations of inhibitor at pH 7.2 and 25 °C. After all of the inhibitor had been consumed, the remaining enzyme activity was determined. Panel A shows the plot of the initial inhibitor to enzyme molar ratios vs. the fraction of enzyme activity remaining for Np5Br (solid line) and its hydrolysis product, Np5BMK (broken line). Panel B shows the same plot for Np6Br (solid line) and its hydrolysis product, Np6BMK (broken line). The initial slope of the plots approximates the number of turnovers of inhibitor per inactivation event.

substituted halo enol valerolactones (Daniels et al., 1983). It is now apparent that the values we reported as inactivation rate constants are actually the values for the acylation rate constants k_a. Although there can be no quantitative comparison between the inactivation rates of the five- and six-membered ring lactones, qualitatively the valerolactones are still much more potent irreversible inactivators of chymotrypsin than the butyrolactones (see below).

Inactivation Efficiency. Ultimate Activity Assay. Because of the problems associated with the time-dependent assay systems, a time-independent assay was developed from which the efficiency of the inactivation process can be determined for every inactivator. This assay involves incubating chymotrypsin with inhibitor concentrations that will not totally inactivate the enzyme. After all of the inhibitor has been consumed, the remaining or ultimate enzyme activity is determined and plotted vs. the initial inhibitor to enzyme molar ratios.

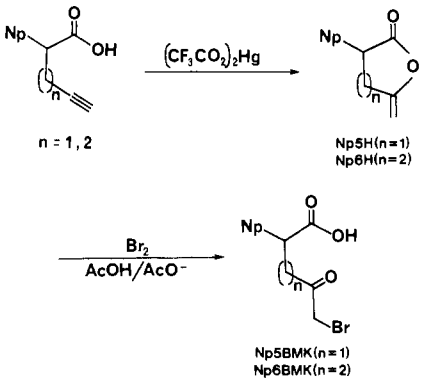
The derivation of an analytical expression that describes the ultimate activity curves has been presented in the previous publication (Daniels et al., 1983). A more detailed treatment that takes into account the acylation step gives a similar expression

$$\frac{I_0}{E_0} = \left(\frac{k_2 + k_c}{k_2}\right)\left(1 - \frac{E}{E_0}\right) - \frac{k_h C}{k_a E_0}\left(\frac{k_2 + k_c}{k_2}\right) \ln\left(\frac{E}{E_0}\right) \quad (4)$$

where k_h is the rate constant that describes the spontaneous hydrolysis of the inhibitor in buffer. This hydrolysis rate constant is small for Np5Br and Np6Br (0.00538 and 0.00098 min^{-1}, respectively); so, the logarithmic term in eq 4 is negligible until the enzyme activity reaches low levels [i.e., ln $(E/E_0) \rightarrow \infty$]. Therefore, the slope of the initial linear portion of the ultimate activity curves is equal to $(k_2 + k_c)/k_2$, which is a partition ratio that describes the number of catalytic turnovers of inhibitor per inactivation event. Ultimate activity curves for Np5Br and Np6Br are presented in Figure 3 (solid lines). From the initial slopes, the number of turnovers per inactivation was found to be 11 for Np5Br and 4.0 for the more efficient Np6Br.

Catalytic vs. Paracatalytic Mechanisms. Once it has been shown that a mechanism-based inhibitor is active site directed,

Scheme II



it is necessary to prove that it remains anchored in the catalytic site prior to its irreversible covalent attachment. If the activated inhibitor deacylates and leaves the active site before covalent bond formation, it can react with external nucleophilic components (Scheme I, pathway b) or return to inactivate the enzyme (pathway c). Evidence for this type of pseudosuicide inactivation can be indicated by the presence of a time lag preceding the linear portion of a time-dependent inactivation curve. Apparently Np5Br does not inactivate chymotrypsin by a paracatalytic mechanism since there is no time lag in the time-dependent inactivation curve in Figure 1A. However, if the production and release of the inactivating species in a pseudosuicide mechanism are fast compared to the inactivation rate, a time lag may not be detectable.

Inactivation by the Bromomethyl Ketones Np5BMK and Np6BMK. One approach to rule out the involvement of a paracatalytic process is to synthesize the proposed free activated inhibitor and to test its inactivating potential toward the enzyme. If this synthetic product does not inactivate the enzyme, then the possibility of a pseudosuicide mechanism is unlikely.

The synthesis of the bromomethyl keto acids derived from Np5Br and Np6Br is shown in Scheme II. α-2-Propynyl-1-naphthaleneacetic acid and α-3-butynyl-1-naphthaleneacetic acid were cyclized to the corresponding methylene butyrolactone (Np5H) and valerolactone (Np6H) by using a catalytic amount of mercuric trifluoroacetate in methylene chloride at room temperature. The cyclization to Np5H was complete within an hour and gave a purified yield of 64%, while the cyclization to Np6H was much slower (18 h), giving a purified yield of 43%. The methylene lactones were brominated to the ring-opened bromomethyl keto acids, Np5BMK and Np6BMK, in a buffered acetic acid solution of bromine at 0 °C. The reaction was complete within an hour and gave purified yields of 57% for Np5BMK and 31% for Np6BMK.

It is interesting to note that neither Np5H nor Np6H showed any inactivation of chymotrypsin in an ultimate activity assay, confirming the fact that the halogen (and presumably the generated halomethyl ketone) is necessary for the irreversible inactivation of the enzyme. The ultimate activity of chymotrypsin with Np5BMK is shown in Figure 3A (broken line). It can be seen that Np5BMK shows little inactivation of the enzyme compared to the parent lactone, Np5Br. Therefore, it is unlikely (assuming the mechanisms in Scheme I) that Np5Br is acting as a pseudosuicide inactivator of the enzyme (pathway c).

The ultimate activity of chymotrypsin after inactivation by Np6BMK is shown in Figure 3B (broken line). It was discovered to be an even more efficient inhibitor than its parent lactone, Np6Br, with only 1.3 turnovers per inactivation. Since no acylation step is possible, the inactivation rate and binding

Table I: Selectivity of Nucleophilic Scavengers toward the Lactone (Np6Br) and Bromomethyl Ketone (Np6BMK)[a]

nucleophile	[Np6Br]/[Np6BMK]	nucleophile	[Np6Br]/[Np6BMK]
imidazole	0.74	ethanolamine	1.10
mercaptoethanol	0.88	sodium azide	1.14
sodium thiocyanate	0.88	*n*-butylamine	1.54
none	[1]	propanolamine	1.57
ethylamine	1.07	methylamine	3.49

[a] A mixture of Np6Br and Np6BMK was added to a solution of nucleophile and assayed, after 15 s, by HPLC. The ratio [Np6Br]/[Np6BMK] is the ratio of peak heights expressed relative to a control incubation (no scavenger). For details, see Experimental Procedures.

constants can be determined from the competitive assay (Main, 1973). From the intercepts of the double-reciprocal plot the binding constant $(k_2 + k_{-1})/k_1$ was found to be 0.0262 μM and the inactivation rate constant, k_2, 7.87 min^{-1}. Even though the proposed enzyme hydrolysis product of Np6Br inactivates chymotrypsin, this does not prove that the valerolactone is a pseudosuicide inactivator; Np6BMK may simply be a potent affinity inactivator itself. Further studies were needed to distinguish between these mechanistic pathways.

Selective Scavenging of Np6BMK vs. Np6Br. Since a paracatalytic mechanism (Scheme I, pathway c) involves the release of the inactivating species from the catalytic site before enzyme modification, then the presence of a scavenger that can intercept the free halomethyl keto acid should prevent enzyme inactivation. To perform this experiment, it was first necessary to find a scavenger that reacted quickly and preferentially with the hydrolysis product but not (or at least not to the same extent) with the parent lactone. This was somewhat difficult, since the lactone is quite sensitive to nucleophiles itself.

A solution of the nucleophilic scavenger was added to a mixture of Np6Br and Np6BMK and immediately injected onto an HPLC column. The relative disappearance of each compound was quantitated with respect to a blank that contained no nucleophile. Table I shows the relative reactivity of the nucleophile with Np6Br and Np6BMK. In general both the lactone and its hydrolysis product were sensitive to added nucleophile to a similar extent. However, methylamine was found to be 3.5 times more reactive toward Np6BMK than Np6Br and was chosen as the scavenger for subsequent studies.

The incubation mixture for the scavenging assay contained constant amounts of enzyme but varying concentrations of methylamine. A constant amount of either Np6Br or Np6BMK was added to these solutions and the ultimate enzyme activity determined. A plot of the enzyme activity vs. the scavenger concentration is shown in Figure 4. The presence of methylamine had little effect on the enzyme inactivation by Np6Br (solid line) but markedly decreased the inactivation potential of Np6BMK (broken line). This result strongly suggests that the inactivating species generated from Np6Br remains in the catalytic site, protected from the scavenger, during the inactivation process.

Stability and Stoichiometry of Chymotrypsin Inactivation. It is important to show that the inactive enzyme is irreversibly modified and not simply a stable reversible complex. If the inactive enzyme were a stable acyl enzyme–inhibitor intermediate, then the addition of a potent deacylating agent of chymotrypsin such as hydrazine should lead to regeneration of enzyme activity (Fastrez & Fersht, 1973).

Chymotrypsin was incubated with varying concentrations of Np5Br and Np6Br; an aliquot was removed, and the ultimate enzyme activity was determined (Figure 5, solid lines). The remaining incubation mixture was passed through a

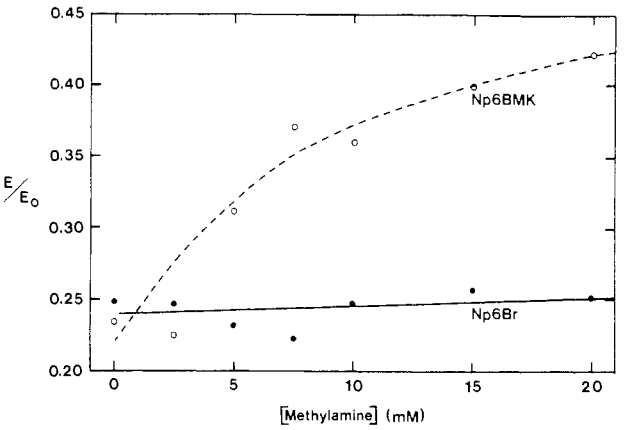


FIGURE 4: Inactivation in the presence of a scavenger. Chymotrypsin (3.0 μM) was incubated with Np6Br or Np6BMK in the presence of different concentrations of methylamine at pH 7.2 and 25 °C. After all of the inhibitor had been consumed, the remaining enzyme activity was determined. The plot shows the concentration of the scavenger vs. the ultimate enzyme activity of Np6Br (solid line) and Np6BMK (broken line).

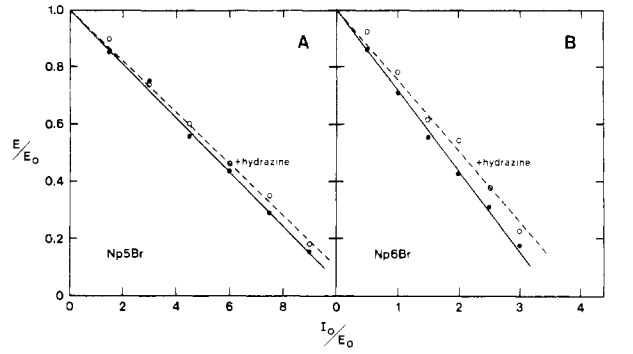


FIGURE 5: Regeneration of enzyme activity in the presence of a deacylating agent. (Panel A) Chymotrypsin (4.0 μM) was inactivated with various concentrations of Np5Br at pH 7.2 and 25 °C, and the ultimate enzyme activity was determined (solid line). The remaining incubation mixture was passed through a Sephadex G-25 column to remove any unbound inhibitor, and hydrazine was added to a final concentration of 100 mM. After 1 h the enzyme activity was redetermined and plotted vs. the initial inhibitor to enzyme molar ratios (broken line). (Panel B) A chymotrypsin solution was treated as above with Np6Br as the inactivator, and the molar ratios of inhibitor to enzyme were plotted vs. the enzyme activity before (solid line) and after (broken line) hydrazine treatment.

Sephadex G-25 column to remove any excess inhibitor or inhibitor byproducts. Hydrazine was then added to the eluent, which now contained only free and inactive enzyme, and the enzyme activity was determined again (Figure 5, broken lines). It can be seen that there was little or no regeneration of enzyme activity with either the Np5Br- or Np6Br-inactivated enzyme. Therefore, it is unlikely that the only attachment of these inactivators to the enzyme is through an acyl linkage.

Another possibility is that the inactive enzyme species is a noncovalent tight binding enzyme–inhibitor complex. Only by denaturing the enzyme and showing that the inhibitor is still bound can this possibility be eliminated.

For this study a carbon-14 labeled inhibitor [^{14}C]Np6I was prepared by standard methods for the synthesis of halo enol lactones (see Experimental Procedures). In the previous report, Np6I was found to be the most potent and efficient inhibitor of chymotrypsin in the series of aryl-substituted halo enol lactones (Daniels et al., 1983). It differs from Np6Br only in the replacement of bromine with iodine and should presumably follow the same inactivation mechanism.

Chymotrypsin was inactivated with varying amounts of [^{14}C]Np6I, and the ultimate activity was determined. The

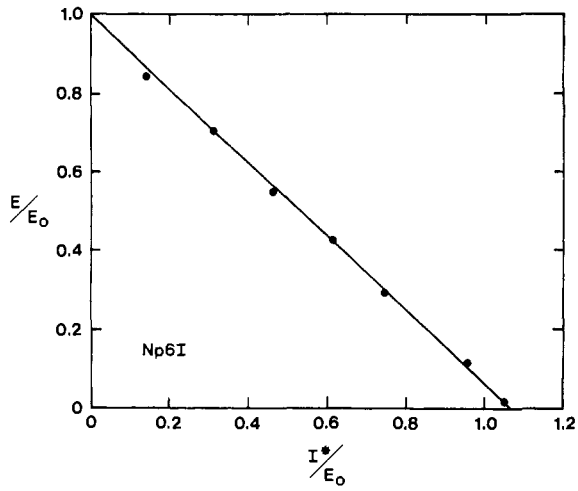


FIGURE 6: Stoichiometry of inactivation. Chymotrypsin (9.26 μM) was incubated with different concentrations of [^{14}C]Np6I at pH 7.2 and 25 °C, and the ultimate enzyme activity was determined. The remainder of the incubation mixture was spotted on filter paper disks, and the enzyme was immobilized by denaturation in boiling ethanol. The excess inactivator was washed away, and the amount of remaining radiolabel due to covalently bound inhibitor was quantitated. The plot shows the molar ratio of covalently bound inhibitor vs. the fraction of enzyme activity remaining.

remaining enzyme was immobilized on filter paper disks by denaturation in boiling ethanol, and the excess inhibitor was washed away, so that only the covalently bound inactivator remained. The amount of radiolabel bound to the disks was determined and the molar quantity of inhibitor calculated from its specific activity. A plot of the ultimate enzyme activity vs. the molar ratio of covalently bound inhibitor to enzyme is shown in Figure 6.

As can be seen, inhibitor does remain bound to the denatured enzyme, indicating the presence of a covalent linkage between the inactivator and the protein. In addition, the slope and the x intercept of the plot are nearly equal to 1, showing that only a single molecule of [^{14}C]Np6I per catalytic site is needed to inactivate chymotrypsin irreversibly. This one to one stoichiometry of inactivation proves that there is no covalent binding of the inhibitor at extraneous sites distant from the active site and lends additional support to our hypothesis that the activated species remains tethered in the catalytic site during the inactivation process.

Discussion

The proposed mechanism for irreversible inactivation of chymotrypsin by halo enol lactones is shown in Scheme I (pathway a). Two lactones, Np5Br and Np6Br, representative of the five- and six-membered ring series prepared earlier, were selected to test the validity of this mechanistic scheme further.

The first step of the proposed inactivation pathway involves the reversible binding of the inhibitor at the enzyme's active site. Kinetic studies in which the accumulation of inactive enzyme was measured over the assay period gave the observed inactivation rate constants from the slope of semilogarithmic plots (see Figure 1). The assay method used for the inhibition of enzyme with Np5Br directly measured the amount of irreversibly inactivated enzyme in aliquots removed from the incubation mixtures at various time intervals. The inactivation by Np6Br, however, was too rapid to use this discrete aliquot method. A system was used in which a substrate was included in the incubation mixture both to slow the inactivation process and to provide a means of continuously monitoring the amount of free enzyme. Since deacylation of the acyl enzyme intermediate was slow compared to the assay period, this acylated complex, not the irreversibly modified product, was the "inactive" enzyme species observed by this method.

A double-reciprocal plot of the initial inhibitor concentrations vs. the observed inactivation rates gave a straight line for both Np5Br and Np6Br with nonzero intercepts (see Figure 2). This type of plot is typical for inhibitors in which there is a reversible binding step prior to an actual or apparent inactivation event.

A single assay was utilized to evaluate the extent of irreversible inactivation of chymotrypsin by both lactones. This ultimate activity assay entails the incubation of the enzyme with concentrations of inhibitor that will not totally inactivate the enzyme followed by the determination of the enzyme activity after all of the inhibitor has been consumed. From the initial slope of the plot of the ultimate enzyme activity vs. the initial inhibitor to enzyme molar ratios, the number of inhibitor turnovers per inactivation is obtained and was found to be 11 for Np5Br and 4.0 for Np6Br (see Figure 3, solid lines).

The next step along the proposed inactivation pathway is the formation of the acyl enzyme–inhibitor intermediate in which the reactive bromomethyl ketone is tethered to the enzyme by the acyl–serine linkage. However, if a pseudosuicide or paracatalytic mechanism ensues, the reactive species may deacylate and then leave the catalytic site in order to achieve a more favorable orientation for bond formation (Scheme I, pathway c). By leaving the active site, the bromomethyl keto acid also becomes available for reaction with nucleophiles outside of the catalytic site, thus compromising both the selectivity and efficiency of the inactivator.

The experimental evidence obtained in this study leads us to the conclusion that the halo enol lactones, Np5Br and Np6Br, are behaving as true suicide inactivators. The time-dependent plot of the inhibition of chymotrypsin with Np5Br shows no time lag in the initiation of inactivation (see Figure 1a), indicating that the formation and release of the reactive species prior to the irreversible modification, as in a paracatalytic mechanism, are unlikely. The time-dependent assay of Np6Br with chymotrypsin, however, gives no information about the irreversibly inactivated product; so, the absence or presence of a time lag is irrevelant in this context.

The proposed inactivating species in a paracatalytic scheme would be the free bromomethyl keto acids, Np5BMK and Np6BMK. These analogues were synthesized and tested as inactivators for chymotrypsin. Since no acyl linkage to the enzyme is possible, the absence of inactivation supports a true suicide mechanism where the reactive moiety must be tethered to the enzyme during the formation of the irreversible attachment. Np5BMK did not significantly lower the enzyme activity, but Np6BMK proved to be a nearly ideal irreversible inactivator with 1.3 turnovers per inactivation (see Figure 3, broken lines). This result does not necessarily prove that Np6Br is a pseudosuicide inhibitor, since Np6BMK could simply be a potent inactivator itself.

The difference in inactivation potential of the two bromomethyl ketones Np5BMK and Np6BMK suggests that the relationship between the aryl group and the alkylating moiety is very important. We have used such arguments previously to account for the widely differing reactivity of halo enol lactones of differing structure (Daniels et al., 1983; Sofia & Katzenellenbogen, 1986).

Methylamine was found to react more quickly and preferentially with Np6BMK than with Np6Br. Therefore, if methylamine were present in the incubation mixture, Np6BMK would be scavenged more readily than Np6Br. If a paracatalytic mechanism were in operation, then the extent of

inactivation of enzyme by the lactone (Np6Br) and its hydrolysis product (Np6BMK) would be equally affected by added scavenger (since the inactivating species is proposed to be the same bromomethyl keto acid). However, the inclusion of the methylamine scavenger in the inactivation mixture led to a significant decrease in the extent of enzyme inactivation with Np6BMK, but not with Np6Br (see Figure 4). This indicates that the reactive species generated from Np6Br remains in the catalytic site, inaccessible to scavenger, prior to irreversible covalent modification.

The ultimate step leading to the irreversibly inactivated enzyme is the formation of the irreversible covalent attachment between the inhibitor and the enzyme. There is the possibility that the inactive enzyme is a stable acyl enzyme–inhibitor complex. Although these species might appear irreversible under simple assay conditions, components in biological systems could displace the inhibitor leading to regeneration of the enzyme activity.

The possibility of a stable acyl enzyme–inhibitor complex can be tested by treating the inactivated enzyme with a deacylating agent to see if any enzyme activity is recovered. Chymotrypsin inactivated by Np5Br or Np6Br was filtered to remove any excess inhibitor and treated with hydrazine, and the enzyme activity was redetermined. There was little or no regeneration of enzyme activity, indicating that the inactive enzyme species is not (or is not only) an acyl enzyme (see Figure 5).

The possibility of a tight binding enzyme–inhibitor complex can be tested by inactivating the enzyme with a radiolabeled inhibitor, denaturing the enzyme, removing any unbound inactivator or byproducts, and determining if any radiolabel remains covalently attached to the protein. If the inhibitor was reversibly bound at the catalytic site, then denaturation should release it, and no counts would remain bound to the protein. Chymotrypsin was inactivated with [^{14}C]Np6I (which should follow the same inactivation mechanism as Np6Br) and treated as above. It was found that there was a direct linear correlation between the enzyme activity and the amount of enzyme-bound inhibitor, showing a 1:1 stoichiometry of enzyme inactivation (see Figure 6). This result indicates that there is a stable covalent bond formed between the enzyme and inhibitor leading to irreversible impairment of the enzyme's catalytic activity. In addition, the 1:1 stoichiometry of inactivation proves that there is no covalent binding of the inactivator outside of the catalytic site further, ruling out the possibility of a pseudosuicide inactivation mechanism.

On the basis of molecular modeling studies, we have proposed that His-57 is the most likely site for covalent attachment (Naruto et al., 1985). Preliminary studies attempting to identify the modified amino acid obtained upon degradation of the irreversibly inactivated enzyme seem to support this proposal, but a more definitive determination may require crystallographic examination of the modified enzyme.

In this study we have attempted to provide insight into the mechanism of inactivation of a serine protease by a class of halo enol lactones. The results obtained have led us to the conclusion that these two lactones, Np5Br and Np6Br, and, most likely the extended series of halo enol lactones prepared earlier (Daniels et al., 1983), are behaving as true enzyme-activated irreversible inhibitors of chymotrypsin.

Registry No. Np5BMK, 100208-00-2; Np5Br, 88070-94-4; Np5H, 100207-99-6; Np6BMK, 100208-02-4; Np6Br, 88070-98-8; Np6H, 100208-01-3; Np6I, 88070-99-9; [^{14}C]Np6I, 100231-57-0; L-AcTrpOC$_6$H$_4$NO$_2$-*p*, 14009-92-8; F_3CCO_2Hg, 13257-51-7; L-PhCOTyrOEt, 3483-82-7; HC≡CCH$_2$CH$_2$Br, 38771-21-0; α-2-propynyl-1-napthaleneacetic acid, 88071-00-5; α-3-butynyl-1-naphthaleneacetic acid, 88071-02-7; α-3-butynyl-1-naphthalene-[*carboxy*-^{14}C]acetic acid, 100208-03-5; 1-naphthyl[*carboxy*-^{14}C]acetic acid, 80792-65-0; α-chymotrypsin, 9004-07-3.

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