Alternate Substrate Inhibitors of an α-Chymotrypsin: Enantioselective Interaction of Aryl-Substituted Enol Lactones†

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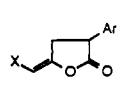
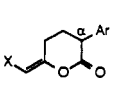
ABSTRACT: Four enol lactones, bearing phenyl or 1-naphthyl substituents on the α or β positions [3-phenyl-6-methylenetetrahydro-2-pyranone (αPh6H, IIc), 3-(1-naphthyl)-6-methylenetetrahydro-2-pyranone (αNp6H, IId), 4-phenyl-6-methylenetetrahydro-2-pyranone (βPh6H, IIIc), and 4-(1-naphthyl)-6-methylenetetrahydro-2-pyranone (βNp6H, IIId)], available as pure *R* and *S* enantiomers, have been studied as alternate substrate inhibitors of chymotrypsin. Kinetic constants for substrate binding (K_s) and acylation (k_a) were determined by a competitive substrate assay, using succinyl-L-Ala-L-Ala-L-Pro-L-Phe *p*-nitroanilide; the deacylation rate constant (k_d) was determined by the proflavin displacement assay. All lactones undergo rapid acylation (k_a varies from 17 to 170 min^{-1}) that shows little enantioselectivity; there is, however, pronounced enantioselectivity in substrate binding for three of the lactones [$K_s(R/S)$ = 40–110]. In each case it is the enantiomer with the *S* configuration that has the higher affinity. In all cases, deacylation rates are slow, and in two cases, acyl enzymes with half-lives of 4.0 and 12.5 h at pH 7.2, 25 °C, are obtained (for βPh6H and αNp6H, respectively). In these cases, high deacylation enantioselectivity is observed [$k_d(S/R)$ = 60–70], and the lactone more weakly bound as a substrate (*R* enantiomer) gives the more stable acyl enzyme. Two hypotheses, involving hindrance of the attack of water or an exchange of the ester and ketone carbonyl groups in the acyl enzyme, are advanced as possible explanations for the high stability of these acyl enzymes.

Proteases play an active role in many normal physiological and pathological processes (Hörl & Heidland, 1984; Cunningham & Long, 1987), and specific inhibitors of proteases are often useful agents of therapy (Seiler et al., 1978; Barrett, 1980; Silverman, 1988). Serine proteases, in particular, are involved in digestion, processing of peptide prohormones, thrombolysis and fibrinolysis, fertilization and blastocyst implantation, inflammation, arthritis, glomerulonephritis, emphysema, and tumor invasion and metastasis. Many strategies—high affinity competitive inhibitors or transition-state analogues, active site alkylating agents, alternate substrate inhibitors, and mechanism-based enzyme inactivators—have been utilized to develop effective and selective inhibitors for serine proteases (Silverman, 1988).

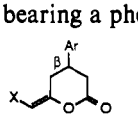
Earlier, we reported that certain aryl-substituted halo enol lactones were effective mechanism-based inactivators of α-chymotrypsin (Daniels et al., 1983; Daniels & Katzenellenbogen, 1986). Their action appeared to involve acyl transfer to the serine-195, generating an acyl enzyme in which a latent alkylating function, a halomethyl ketone, was revealed; subsequently, irreversible inactivation (by alkylation of histidine-57) competed with deacylation. In this series, the most effective inactivators of α-chymotrypsin were halomethylene butyrolactones Iab or valerolactones IIab,[1] bearing a phenyl or 1-naphthyl substituent on the α carbon (Daniels et al., 1983). Certain members of this series (notably Ib and IIb) demonstrated rapid acylation and efficient partitioning between alkylation and deacylation, such that permanent and complete inactivation could be achieved with only a few equivalents of halo enol lactone (Daniels et al., 1983; Daniels & Katzenellenbogen, 1986).



	Ar	X
Ia	Ph	Br, I
Ib	1-Np	Br, I



	Ar	X	
IIa	Ph	Br	αPh6Br
IIb	1-Np	Br	αNp6Br
IIc	Ph	H	αPh6H
IId	1-Np	H	αNp6H



	Ar	X	
IIIa	Ph	Br	βPh6Br
IIIb	1-Np	Br	βNp6Br
IIIc	Ph	H	βPh6H
IIId	1-Np	H	βNp6H

In further exploration of the structural diversity of these lactones, we noted that the halomethylene lactones with the aryl substituent at the β position IIIab showed rapid, but only transient, inhibition of α-chymotrypsin (Sofia & Katzenellenbogen, 1986). The time-dependent recovery of catalytic activity, which could be accelerated by the addition of hydrazine, and the nearly equivalent behavior of the corresponding protio enol lactones IIIcd, which lack the latent alkylating function, suggested that these lactones formed stable acyl enzyme intermediates, their transient inhibitory activity being of an "alternate substrate" character.

In this paper, we describe a comprehensive investigation of the kinetics of inhibition of α-chymotrypsin by a set of four aryl-substituted methylene valerolactones αPh6H, αNp6H (IIcd) and βPh6H, βNp6H (IIIcd), each a pair of enantiomers. The aryl substituents are phenyl and 1-naphthyl groups that are placed at the α and β positions with respect to the lactone carbonyl carbon. Each set has been studied as the racemate and as the individual pure enantiomers, which were resolved by chromatographic separation of a diastereomeric derivative of the precursor acetylenic acid (Baek et al., 1989). The absolute configuration of the α-aryl-substituted lactones is known from chemical correlations studies and of the β-sub-

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[1] We have adopted an abbreviated designation of the four lactones used in this kinetic study: (a) site of substitution, α or β; (b) aryl substituent, phenyl (Ph) or 1-naphthyl (Np); (c) lactone ring size, 6; (d) methylene substituent, H. These designations are given with the structures IIcd and IIIcd.

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stituted lactones by X-ray crystallographic studies (Baek et al., 1989). Two of the lactones (βPh6H, αNp6H) form very stable acyl enzymes (half-lives at 25 °C, pH 7.2, are in excess of 4 and 12 h, respectively), ascribable in each case to the *R* enantiomer. High enantioselectivity in K_s is also demonstrated by three of the lactones, whereas none of the lactones demonstrate significant enantioselectivity in their rates of acylation.

These studies illustrate that high enantioselectivity, characteristic for α-chymotrypsin in its hydrolysis of peptide substrates, can also be demonstrated toward simple synthetic substrates and that in certain cases very long-lived acyl enzymes can be formed. Structural considerations of the unusual stability of these acyl enzymes, which may derive from a conformational reorganization of the acyl enzyme structure or a steric blockade of the access of water, will be discussed in greater detail in a future paper.

Experimental Procedures

General. Inactivation assays were performed by using a Hewlett-Packard 8451A diode array spectrophotometer. α-Chymotrypsin (three times crystallized and free of autolysis products and low molecular weight contaminants) was obtained from Worthington Biochemical. *N*-Succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine *p*-nitroanilide (suc-Ala-Ala-Pro-Phe pNA (DelMar et al., 1979)) was obtained from Vega Biochemicals. The phosphate buffer used in the studies of α-chymotrypsin inactivation was 0.1 M NaH_2PO_4–Na_2HPO_4, pH 7.2. All the kinetics were performed at 25 °C in 0.1 M, pH 7.2, phosphate buffer, and the concentrations shown in the text are the final concentrations after all the incubation solutions are combined.

Determination of K_s and k_a. Into the 1.5-mL cuvette were combined 800 μL of 0.1 M phosphate buffer at pH 7.2, 100 μL of 3 mM solution of suc-Ala-Ala-Pro-Phe pNA in 1:1 methanol/pH 7.2 phosphate buffer (final concentration is 300 μM), 10–50 μL of 500 μM stock solution of lactone in dimethyl sulfoxide (final concentrations are 5–25 μM), and 0–40 μL dimethyl sulfoxide (to give, with stock solution, a total volume of dimethyl sulfoxide of 100 μL). To this cuvette was added 50 μL of a 1 μM solution of α-chymotrypsin in pH 7.2 phosphate buffer (final concentration is 0.05 μM), and the change in absorbance at 410 nm was recorded over a time interval of 60–200 s. After correction for the limiting slope due to turnover,[2] the semilogarithmic plot of this corrected absorbance change against time gave a straight line with a slope of k_{obs}. A plot of the initial inhibitor concentration (I_0) versus I_0/k_{obs} gave a straight line with the slope of $1/k_a$ and an *x* intercept of $-K_s(1 + S_0/K_m)$. With the value of S_0 (300 μM) and K_m (50 μM; determined in our lab by standard methods), K_s was calculated from an expression of *x* intercept (cf. eq 6).

To determine the absolute standard deviations of k_a and K_s (s_x and s_y, respectively), the absolute standard deviations of the slope and *y* intercept (s_a and s_b, respectively) were calculated by the least-squares method. The uncertainty of k_a is equal to the uncertainty of the slope, which is the relative standard deviation of the slope, $(s_a)_r$.

$$(s_a)_r = s_a/\text{slope} = s_x/k_a$$

Thus, the absolute standard deviation s_x is obtained by multiplying k_a and $(s_a)_r$.

K_s is expressed by a division of *y* intercept and slope as follows:

$$K_s = (y \text{ intercept}/\text{slope})[1/(1 + S_0/K_m)]$$

Since the relative variance of K_s, $(s_y)_r^2$, is equal to the sum of the individual relative variances of the slope and *y* intercept [$(s_a)_r^2$ and $(s_b)_r^2$, respectively] (Skoog & West, 1982), the relative standard deviation of K_s was calculated by eq 1.

$$(s_y)_r = \sqrt{(s_a)_r^2 + (s_b)_r^2} \quad (1)$$

Again, the absolute standard deviation s_y is obtained by multiplying K_s and $(s_y)_r$. In Table I, the relative standard deviations (coefficient of variance) of K_s and k_a are given in parentheses.

Proflavin Displacement Assay. Method A. To a mixture of 100 μL of a 100 μM proflavin solution in pH 7.2 phosphate buffer and 1.0 mL of 100 μM α-chymotrypsin stock solution in phosphate buffer was added 10 μL of a 10 mM solution of a lactone in dimethyl sulfoxide at 25 °C. (The concentrations of proflavin, enzyme, and lactone after mixing were 9, 90, and 90 μM, respectively, as initial concentrations.) The change in absorbance was then followed at 466 nm, and the semilogarithmic plot of the increasing progress curve versus time gave a straight line with a slope of k_d.

Method B. To a mixture of 1.0 mL of 100 μM α-chymotrypsin in phosphate buffer and 100 μL of 10 mM lactone in dimethyl sulfoxide, preincubated for 1 min (for single enantiomers) or 1 h (for racemate) at 25 °C, was combined a preequilibrated mixture of 1.0 mL of phosphate buffer, 5 mg of charcoal, 0.5 mg of dextran, and 0.5 mg of gelatin. (Gelatin was used to saturate charcoal–dextran to prevent adsorption of enzyme.) After 3 min, the mixture was passed through Celite, and 50 μL of a 100 μM proflavin solution in phosphate buffer was added to 1.0 mL of the eluent. (The initial concentrations of proflavin, enzyme, and lactone in the mixture were 4.6, 46, 460 μM, respectively.) The k_d value was calculated from the plot of the natural log of the absorbance change versus time. This method is especially useful for a lactone with a high K_s value and/or high solubility in aqueous buffer.

Results

Kinetic Model. The kinetic model shown in eq 2 is sufficient to describe the transient inhibitor activity of these enol lactones

$$E + L \underset{k_{-1}}{\overset{(K_s)\ k_{+1}}{\rightleftharpoons}} E{\cdot}L \xrightarrow{k_a} E{\sim}L \xrightarrow{k_d} E + L' \quad (2)$$

for chymotrypsin. As the enol lactones are inhibitors of the alternate substrate type, this is simply the kinetic model for serine protease substrates: L is the lactone, E·L the Michaelis complex, E∼L the acyl enzyme, and L′ the hydrolyzed lactone (keto acid). Determination of the deacylation rate constant k_d, acylation rate constant k_a, and the substrate binding constant K_s [$(k_a + k_{-1})/k_{+1}$, which approximates k_{-1}/k_{+1} since $k_{-1} \gg k_a$] are described in the sections below.

Binding Constant K_s and Rate Constant of Acylation k_a for Enol Lactone Inhibition. A competitive substrate assay was employed to determine the kinetic constants K_s and k_a for the

[2] This kinetic analysis according to Main (1967) was derived for a competitive assay between the substrate and a time-dependent *irreversible* inhibitor. In our case the inhibitor is an alternate substrate, which is not irreversible; the deacylation rate, however, is so slow that the kinetic model derived by Main can still be used. In practice, we estimate the limiting slope of the progress curves, which is due to deacylation of the lactone acyl enzyme, and subtract this rate from the progress curves, so that the true exponential approach to the steady state (k_{obs}) can be determined accurately. *Note*: In place of the inhibitor binding and rate constants K_i and k_2, we use the equivalent parameters for an alternate substrate K_s and k_a.

Table I: Binding Constants (K_s) and Acylation and Deacylation Rate Constants (k_a and k_d) for Enol Lactones

		K_s[a,b] (μM)	k_a[a,b] (min^{-1})	k_d[d] (min^{-1})
αPh6H	(±)	19.5 ± 7.3 (0.38)	71 ± 27 (0.37)	0.17 (0.098)[e]
(IIc)	(*R*)	680 ± 220 (0.32)	169 ± 55 (0.32)	0.095
	(*S*)	6.1 ± 1.1 (0.18)	40.5 ± 6.7 (0.16)	0.11
αNp6H	(±)	9.0 ± 3.6 (0.40)	106 ± 42 (0.40)	0.039 (~0.001)[e]
(IId)	(*R*)	250 ± 100 (0.42)	129 ± 53 (0.41)	0.00092
	(*S*)	6.1 ± 1.5 (0.25)	98 ± 25 (0.25)	0.067
βPh6H	(±)	18.8 ± 1.6 (0.09)	45.1 ± 3.8 (0.08)	0.13 (0.0039)[e]
(IIIc)	(*R*)	51 ± 14 (0.28) [630 ± 200][c]	35.2 ± 9.5 (0.27) [73 ± 23]	0.0029
	(*S*)	7.7 ± 0.7 (0.09) [91 ± 9]	17.4 ± 1.5 (0.09) [58 ± 6]	0.17
βNp6H	(±)	1.3 ± 0.2 (0.17)	30.2 ± 4.7 (0.16)	0.26 (0.23)[e]
(IIId)	(*R*)	27.9 ± 2.6 (0.09)	23.0 ± 2.1 (0.09)	0.22
	(*S*)	0.40 ± 0.04 (0.10)	20.8 ± 1.5 (0.07)	0.47

[a] Values are given ± SD; coefficient of variance is given in parentheses. Error analysis is discussed under Experimental Procedures. [b] Measured in 10% DMSO–0.1 M phosphate buffer, pH 7.2, 25 °C. [c] Numbers in the square brackets are for the corresponding open-chain keto esters (*R*)- and (*S*)-IVa. [d] Determined by method A (1:1 stoichiometry). [e] Determined by method B (excess lactone).

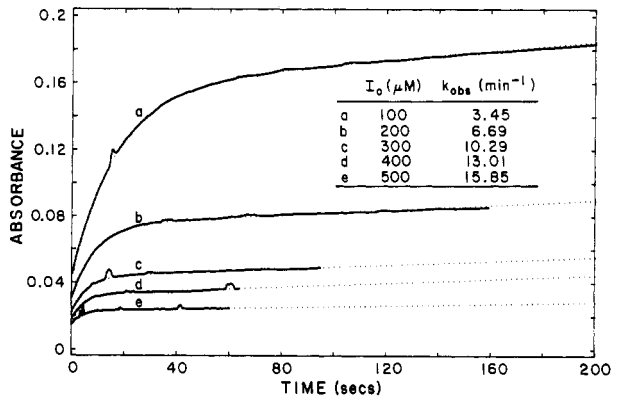


FIGURE 1: Competitive substrate assay. α-Chymotrypsin (50 nM) was added to a solution of (*R*)-αPh6H (IIc) (100–500 μM) and suc-Ala-Ala-Pro-Phe pNO$_2$-anilide (300 μM) in 10% DMSO–0.1 M phosphate buffer, pH 7.2 at 25 °C, and the hydrolysis of the substrate was followed at 410 nm. As the enzyme becomes inhibited, the rate of substrate hydrolysis decreases, producing the inhibition progress curve. A series of these curves is shown for different concentrations of an enol lactone [(*R*)-αPh6H (IIc)]. The dotted lines show the progress curves calculated with the observed rate constants (k_{obs}) (and corrected for steady-state turnover, see text and footnote 2).

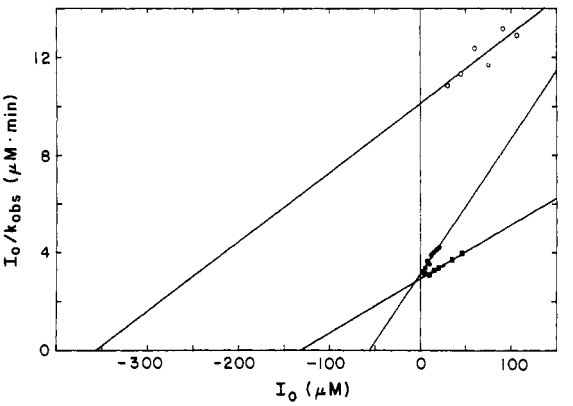


FIGURE 2: Determination of K_s and k_a for βPh6H (IIIc) using the competitive substrate assay (cf. Figure 1 legend). A plot of the initial lactone concentrations (I_0) versus I_0/k_{obs} gives a straight line with a slope of $1/k_a$ and an x intercept of $-K_s(1 + S_0/K_m)$. (■) Racemate; (○) *R* enantiomer; (●) *S* enantiomer.

inhibition of α-chymotrypsin by the enol lactones. When the 6-membered protio enol lactones are incubated with α-chymotrypsin in the presence of a substrate, there is competition between the inhibitor and the substrate to occupy the active site of the enzyme. This interaction is considered to proceed according to the kinetic equations

$$E + L \underset{}{\overset{K_s}{\rightleftharpoons}} E{\cdot}L \xrightarrow{k_a} E{\sim}L \xrightarrow{k_d} E + L' \qquad (3)$$

$$E + S \overset{K_m}{\rightleftharpoons} E^*S \xrightarrow{k_{cat}} E + P \qquad (4)$$

Since the rate of deacylation of the acyl enzyme derived from the enol lactone is very slow, the acylation process can be followed by the decrease in free enzyme, monitored continuously by its consumption of the chromogenic substrate succinyl-L-Ala-L-Ala-L-Pro-L-Phe *p*-nitroanilide. Thus, the progress curves show a decreasing slope until a very low, steady-state rate is reached, where substrate hydrolysis is controlled by the slow deacylation of E~L and the repartitioning of E through E*S. Representative progress curves for the lactone (*R*)-αPh6H (IIc) are shown in Figure 1. During this assay it is assumed that the substrate and inhibitor concentrations remain constant, due to large excess of substrate (6000-fold excess over enzyme) and inhibitor (10–10 000-fold excess over enzyme).

Main has derived an expression to describe the progress curve for substrate consumption in this case of time-dependent inhibition (eq 5; Main, 1973),[2] where S_0 and I_0 are the initial

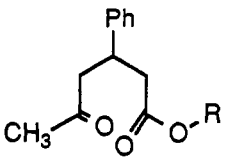
$$\ln \frac{V}{V_0} = \left[\frac{-k_a K_m I_0}{K_s K_m + S_0 K_s + I_0 K_m} \right] t = -k_{obs} t \qquad (5)$$

$$\frac{I_0}{k_{obs}} = \frac{1}{k_a} I_0 + \frac{K_s}{k_a}\left(1 + \frac{S_0}{K_m}\right) \qquad (6)$$

slope = $1/k_a$ $\quad$ x intercept = $-K_s(1 + S_0/K_m)$

concentrations of a substrate and an inhibitor, respectively, K_m is the Michaelis constant for the substrate, and V_0 and V are the velocity of substrate hydrolysis at initial time and time t, respectively. Rearrangement of the definition of k_{obs} gives eq 6. When I_0/k_{obs} is plotted versus I_0, a straight line is obtained according to eq 6, with a slope of $1/k_a$ and an x intercept of $-K_s(1 + S_0/K_m)$. The K_m of the substrate S was determined to be 50 μM by standard methods, so K_s for the lactone can be calculated from an expression for the x intercept, since S_0 and K_m are known; k_a is obtained from the inverse of the slope. The K_s and k_a values of protio enol lactones determined by this method are given in Table I. A representative plot of I_0/k_{obs} versus I_0 for βPh6H (IIIc) is shown in Figure 2.

To investigate what structural features contribute to enantioselectivity in the acylation and deacylation processes of these enol lactones, we have done a limited study of the acylation of chymotrypsin by an acyclic analogue of the enol lactone, active esters of the corresponding keto acid IV. These active esters are chymotrypsin substrates, and they generate



IVa R = *p*-CN-Ph
IVb R = *p*-NO_2-Ph

the same acyl enzyme as the enol lactone βPh6H. However, since they are not conformationally constrained as are the cyclic lactones, they have different possibilities for enantioselective interactions with the enzyme active site during the acylation process.

The *p*-nitrophenyl esters IVb proved to be too reactive to follow the acylation transient (burst) without the use of stopped-flow methods, so we studied the less reactive nonchromogenic *p*-cyanophenyl active ester IVa. This ester could be used together with the chromogenic substrate succinyl-L-Ala-L-Ala-L-Pro-L-Phe *p*-nitroanilide in the competitive substrate assay, where its competitively reduced burst rate led to more conveniently measured progress curves. The values for the binding and acylation constants K_s and k_a are listed in Table I (data in square brackets), and a representative plot is given in Figure 3.

Deacylation Rate Constant (k_d) for Enol Lactone Inhibition. The rate constant for deacylation was determined by the proflavin displacement assay (Bernhard et al., 1966). The maximum difference in absorbance between free proflavin and the enzyme–proflavin complex is at 466 nm, so the measurement of absorbance increase at this wavelength can be used to monitor acyl enzyme decay.

When the inhibitor αPh6H (90 μM) is added to a solution of α-chymotrypsin (90 μM) and proflavin (9 μM) and the time-dependent absorbance change was monitored at 466 nm with time, an initial fast disappearance of the enzyme–dye spectrum was followed by a slow regeneration of the original enzyme–dye spectrum, as seen in Figure 4. The rapid decrease in absorbance is due to enzyme acylation and thus dissociation of proflavin, and the slow increase is generated by deacylation of the acyl enzyme and the rebinding of proflavin to free enzyme. The rate constant of deacylation was calculated directly from the second phase of the progress curve of the enzyme–dye spectrum (method A).

A variation of method A was also employed: α-Chymotrypsin (46 μM) was incubated with 10-fold excess of the lactone inhibitor (460 μM); when acylation of the enzyme by the lactone was complete (e.g., 1 min), the excess lactone was then adsorbed onto dextran-coated charcoal and separated by filtration. After addition of the proflavin (4.6 μM) to the filtrate, the progress curve of increased absorbance at 466 nm was observed, from which a rate constant was calculated (method B).

When the individual enantiomers of the lactones were studied separately, equivalent deacylation rate constants were obtained by either method A or method B. However, when racemic mixtures of lactones that have very different deacylation rates were used, the different methods gave different results: When method A was used, the progress curve for deacylation was biphasic, since it represents the combination of the rates of deacylation of the two different acyl enzymes derived from the two enantiomers. If the difference between the deacylation rates of the two enantiomers is large, such as with βPh6H or αNp6H, the biphasic progress curve is clearly distinguishable (Figure 5), and the rate constants for each enantiomer can be obtained.

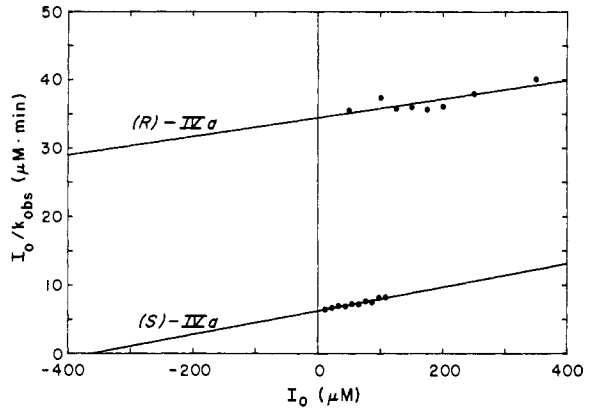


FIGURE 3: Determination of K_s and k_a for the keto esters (*R*)- and (*S*)-IVa, using the competitive substrate assay. For details, see legends of Figures 1 and 2.

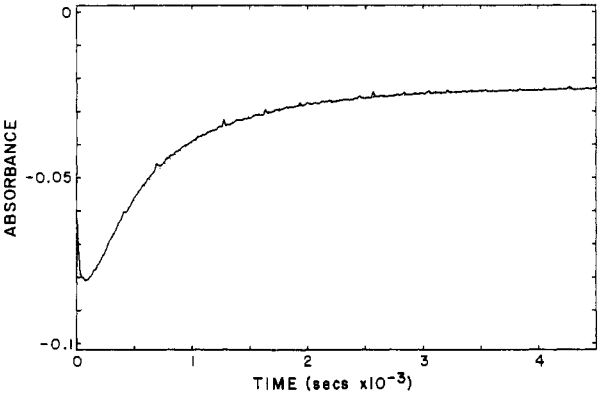


FIGURE 4: Determination of k_d for (*R*)-αPh6H (IIc) by the proflavin displacement assay (method A). A solution of equimolar amounts of a lactone (90 μM) and α-chymotrypsin (90 μM) was assayed with proflavin (9 μM) at 466 nm. The dotted line shows the theoretical exponential function fitted to the observed data.

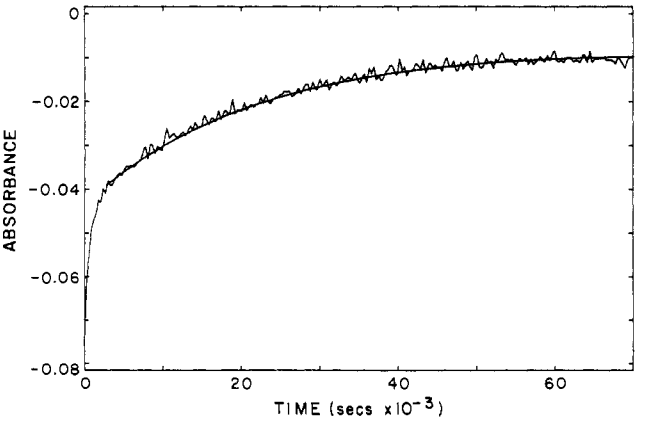


FIGURE 5: Observation of the deacylation of racemic (*R*/*S*)-βPh6H (IIIc) using the proflavin displacement assay (method A). A solution of equimolar amounts of a lactone (90 μM) and α-chymotrypsin (90 μM) was assayed with proflavin (9 μM). The appearance of the enzyme–dye spectrum is biphasic, with an initial fast increase followed by a slow increase. The solid line shows the theoretical curve fitted to the second component, due to the slow deacylating enantiomer [(*R*)-βPh6H (IIIc)].

When method B was used for racemic mixture of lactones, only the slower deacylation rate was observed under most circumstances: According to this protocol, if the enzyme–inhibitor mixture is preincubated long enough for most of the rapidly deacylating enantiomer to be consumed by multiple turnovers, then the initial distribution of rapidly and slowly deacylating acyl enzymes shifts so that only the rate for the slowly deacylating acyl enzyme is observed.

Table II: Stereochemical Comparisons of Kinetic Constants between Enantiomers

	$K_s(R)/K_s(S)$	$k_a(S)/k_a(R)$	$k_d(S)/k_d(R)$
αPh6H (IIc)	111 ± 41	0.24 ± 0.09	1.2
αNp6H (IId)	41 ± 20	0.77 ± 0.36	73
βPh6H (IIIc)	7 ± 2 [7 ± 2][a]	0.50 ± 0.15 [0.77 ± 0.50][a]	59
βNp6H (IIId)	70 ± 9	0.91 ± 0.08	2.1

[a] Numbers in square brackets are for the corresponding open-chain keto esters (*R*)- and (*S*)-IVa.

The rate constants of deacylation of protio enol lactones determined by these methods are given in Table I.

Discussion

This study was prompted by an observation that we made during our investigation of halo enol lactones as mechanism-based serine protease inactivators: Certain protio enol lactones—that did not have the capacity for irreverible covalent modification of α-chymotrypsin—still showed potent, though transient, enzyme inhibition (Sofia & Katzenellenbogen, 1986). Effective inhibition of serine proteases by alternate substrates generally indicates that a stable acyl enzyme intermediate is involved, as proved to be the case with the first system studied βPh6H (IIIc) (Sofia & Katzenellenbogen, 1986).

Beyond their capacity for enzyme inhibition, stable acyl enzymes are interesting, since their stability is generally ascribed to an inherently low hydrolytic reactivity of the ester link or an active site conformation that is unfavorable to catalyzed hydrolysis (Henderson, 1970; Robillard et al., 1972; Zimmerman et al., 1980; Ashe et al., 1981; Moorman & Abeles, 1982; Westkaemper & Abeles, 1983; Wertmann & Abeles, 1984; Gupton et al., 1984; Powers et al., 1984; Hedstrom et al., 1984; Harper et al., 1985; Hemmi et al., 1985; Gelb & Abeles, 1986; Krantz et al., 1987; Bode et al., 1989).

Most examples of stable serine protease acyl enzymes involve achiral substrates; in our case, however, the site of aryl substitution on the enol lactone provides a stereogenic center. In many cases, chymotrypsin shows pronounced enantioselectivity in catalytic activity (Wipff et al., 1983, and references cited therein). Since this has been studied more often with substrates derived from natural amino acids rather than synthetic ones, we were prompted to investigate the enantioselectivity of hydrolysis of our four different enol lactone systems. By relating structure and stereochemistry to kinetic parameters, we hoped to reveal further those structure features that are important in stabilizing the acyl enzyme and to characterize what kinetic features lead to favorable alternate substrate inhibitory properties.

Enantioselectivity of Enol Lactone Hydrolysis. The interaction of chymotrypsin with a substrate involves at least three distinct steps: binding, acylation, and deacylation. Therefore, the effect of variations of substrate structure and stereochemistry can involve all three of these steps and can be manifest in the kinetic parameters K_s (binding), k_a (acylation), and k_d (deacylation). These data for the enol lactones IIcd and IIIcd are summarized in Table I. The binding constant K_s spans approximately 3 orders of magnitude, from about 10^{-7} to 10^{-4} M, but the acylation rate constant k_a shows less than 1 order of magnitude difference, from about 20 to 170 min^{-1}. The deacylation rate constant k_d has a 500-fold range, from about 0.001 to 0.5 min^{-1}. In Table II, these constants are compared among the lactones with regard to enantioselectivity(*R* versus *S*).

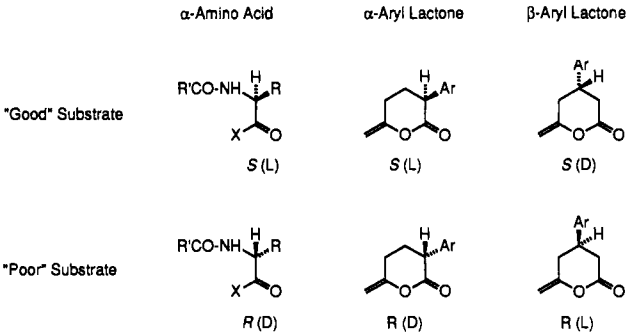


FIGURE 6: Stereochemistry of "good" and "poor" chymotrypsin substrates.

Although the enantioselectivities we have encountered with these enol lactone substrates are, in some cases, pronounced (ca. factors of 100), they are far lower than those observed with more natural chymotrypsin substrates, such as amino acids (factors of 10^5 and greater; Ingles & Knowles, 1967, 1968). This is not surprising, since the natural substrates have several specific interactions that define their stereoselective interaction with the active site of α-chymotrypsin; in particular, the interaction between the α-acylamino group on the substrate as a hydrogen-bond donor with the carbonyl group of serine-214 as the acceptor appears to be the critical determinant in catalytic enantioselectivity (Wipff et al., 1983, and references cited therein).

(*a*) *Binding Constant* (K_s). The binding constant K_s characterizes the affinity of the enol lactone toward the active site of chymotrypsin and is a measure of the stability of the E·L complex. The K_s values for the lactone substrate (Table I) indicate binding affinities that are comparable to, to far superior to those for the better small molecular synthetic substrates for α-chymotrypsin [e.g., benzoyltyrosine ethyl ester (Bz-Tyr-OEt) has a K_s of 64 μM and benzoylphenylalanine methyl ester (Bz-Phe-OMe) a K_s of 52 μM] (Fersht, 1985).

From Table II, it is clear that the *S* enantiomer has a lower to very much lower K_s than the *R* enantiomer. This strong binding of the *S* enantiomer is presumed to arise from a more effective steric complementarity between the enantiomer and the enzyme. Most α-chymotrypsin substrates that show strong binding and high deacylation rates have the L configuration. The *S* enantiomer of α-aryl lactone has a configuration that corresponds to an L-amino acid or specific substrate (see Figure 6); thus, this enantiomer is expected to be the one that binds better in its catalytically reactive orientation. By contrast, the *R* enantiomer has the opposite stereochemistry; so if the hydrolytic ester bond is held in the orientation required for enzyme-catalyzed acylation, then the other three components cannot dispose themselves optimally in the three remaining subregions of the active site. Similar considerations are believed to result in the lower affinity of the *R* enantiomer of the β-aryl lactones; in this case, *S* enantiomer corresponds to a D-β-amino acid (cf. Figure 6). Curiously, it is known from the work of Cohen and Weinstein (1964) that the D or *S* enantiomers of β-amino acids are preferred as substrates for chymotrypsin. It is not clear, however, why the enantioselectivity in K_s is greater for the phenyl versus naphthyl substituent in the α-substituted lactones, whereas the naphthyl exceeds the phenyl in the β-substituted lactones (cf. Tables I and II).

The sense of enantioselectivity in K_s is the same for the active ester of the one acyclic keto acid studied (IVa) (Table II; data in square brackets): *S* enantiomer binds better than the *R*. This indicates that the conformational restriction arising from the cyclic nature of the lactone is not a major factor

in the enantioselectivity of binding in these systems.

It is difficult to make a direct comparison of our results on the enantioselectivity of lactone substrate binding to chymotrypsin, with studies on the enantioselectivity of amino acid derivatives (Knowles, 1965; Ingles & Knowles, 1967): In the latter cases, the unnatural D enantiomers are such poor substrates that K_M or K_s values cannot be determined. The K_i values for the D enantiomers, however, can be measured and are generally slightly lower than the K_s values for the L enantiomers (Knowles, 1965), and a comparison of the values for K_i or K_s for enantiomeric pairs of amino acid substrates for chymotrypsin indicates that binding is affected less by the stereochemistry of the amino acid than by the structure of their residue (e.g., binding affinity increases with the series: Leu, Phe, Trp). While we have not measured the K_i values for our lactones (This would be difficult, since, regardless of stereochemistry, they are all substrates having substantial rates of acylation.), it is clear from comparison of K_s values that high enantioselectivity in binding is apparent.

(*b*) *Acylation Rate Constant* (k_a). The acylation rate constants for the lactones are generally far lower than those for the better small molecular synthetic substrates for α-chymotrypsin (e.g., Bz-Tyr-OEt has a k_a of 15000 min^{-1} and Bz-Phe-OMe a k_a of 2800 min^{-1}) (Fersht, 1985). The effect of the change in stereochemistry on the acylation rate is remarkably modest, with the acylation rates of *R* and *S* enantiomers being equal (βNp6H) to only 4 times faster (αPh6H) for the *R* enantiomer (Table II). Although the differences are nearly too small to discuss at all in terms of free energy, the effect of stereochemistry of the acylation rate seems to be contrary to the effect on the binding affinity; that is, the enantiomer that binds better acylates slightly more slowly. The acyclic keto ester (IVa), as well, shows little enantioselectivity in k_a (Table II; data in square brackets).

It is also curious that in the *R*-lactones, which correspond to the unnatural D-amino acids or the unpreferred L-β-amino acids (Figure 6), the stereochemical incongruity between substrate and enzyme during acylation is more apparent in the value of the binding constant (K_s) than in the value of the acylation rate constant (k_a). Again, comparison of our results with those from other studies on chymotrypsin enantiospecificity with amino acid derivatives is difficult, since acylation rates were not determined with the unnatural or unpreferred enantiomers.

(*c*) *Deacylation Rate Constant* (k_d). Since the deacylation rate is very slow compared to the acylation rate, this step is truly the rate-determining step. In the case of αPh6H and βNp6H, the effect of the orientation of the stereogenic center on the deacylation rates is very small (1–2-fold); however, the change in configuration at the stereogenic center of βPh6H or αNp6H shows a large difference in the deacylation rate, with the *R* enantiomer having about a 60–70-fold slower rate of deacylation than the *S* enantiomer (Table II). Before one considers the enantioselectivity of deacylation, it is important to note that the deacylation rates of all of these enol lactones are very slow: Good substrates (Bz-Tyr-OEt and Bz-Phe-OEt) have k_d values of 7900 and 5500 min^{-1}, respectively (Fersht, 1985).

The deacylation rate of chymotrypsin acyl enzymes depends upon the inherent reactivity of the ester linkage and its proper conformational alignment with the catalytic machinery of the enzyme. There are a number of alternate substrate inhibitors that are derivatives of aromatic acids which produce relatively stable serine protease acyl enzymes. Part of the slow deacylation rate of these esters may be due to the generally lower reactivity of esters of aromatic acids toward hydrolysis (Ashe et al., 1981; Moorman & Abeles, 1982; Hedstrom et al., 1984), but more likely, their catalytic inertness derives either (a) from a steric blockade or ionic repulsion of the attacking water molecule (Westkaemper & Abeles, 1983; Harper et al., 1985; Gelb & Abeles, 1986; Krantz et al., 1987), (b) from a conformational demand that pulls the acyl enzyme carbonyl oxygen out of the oxyanion hole, a site in which its reactivity with nucleophiles is thought to be enhanced (Henderson, 1970; Hemmi et al., 1985), or (c) from a conformational alteration of other portions of the catalytic machinery of the enzyme (e.g., His-57 moving from the active "in" position to the inactive "out" position). These types of conformational alterations have been noted in crystal structures of stable acyl enzymes with chymotrypsin and elastase; in fact, structurally related inhibitors can give acyl enzymes with significantly different conformations of inhibitor and enzyme (Bode et al., 1989).

The relationship between proper stereoelectronic alignment and deacylation rate can be appreciated by a comparison of the deacylation rates of the lactone-derived acyl α-chymotrypsins with various amino acid derivatives (Fersht, 1985). Rapid deacylation is an earmark almost exclusively of the aromatic α-amino acids, L-phenylalanine, L-tyrosine, and L-tryptophan (k_d ca. 5000–8000 min^{-1}; Fersht, 1985; Ingels & Knowles, 1967), which can achieve proper stereoelectronic alignment by placing the aromatic residue in the narrow hydrophobic binding pocket of the enzyme; glycine derivatives are slow to deacylate (k_d ca. 7–70 min^{-1}; Fersht, 1985), presumably because their lack of detailed steric complementarity fails to align the acyl ester as fully with the catalytic machinery of the enzyme, and the unnatural D-amino acids (D-phenylalanine, D-tyrosine, and D-tryptophan) have deacylation rates far slower than the glycine derivatives, presumably because they are forced stereochemically into conformations unfavorable for catalytic activity (Ingles & Knowles, 1967). The glycine derivative deacylation rates are still much greater than those for the lactones, indicating that additional impediments to deacylation are operating in the acyl enzymes derived from the lactones. Nevertheless, the α-aryl lactones that deacylate more rapidly are the *S* enantiomers that correspond to the L-α-amino acids (Figure 6).

Although deacylation rates were not measured, the fact that the D enantiomers of β-amino acids were the preferred substrates (Cohen & Weinstein, 1964) suggests that the *S* stereochemistry at the β carbon leads to more rapid deacylation (Figure 6). In fact, in preliminary studies, we have noted high enantioselectivity in the deacylation of chymotrypsin acyl enzymes derived from various other β-substituted β-phenylpropionates, although the absolute stereochemistry of these systems has not yet been assigned (P. Reed, unpublished results).

Hypotheses for Enantioselectivity of Enol Lactone Alternate Substrate Inhibitors. It is difficult to advance a detailed hypothesis to explain the enantioselectivity that we have observed with the enol lactone systems, without having crystal structure data indicative, at least, of the ground-state conformation of the acyl enzymes. Furthermore, the degree of enantioselectivity, though highly reproducible from experiment to experiment, is not so consistent from system to system, that is, the highest enantioselectivity in deacylation rate is for the α-naphthyl and β-phenyl systems, whereas the β-naphthyl and the α-phenyl systems show little stereochemical dependence in deacylation.

In each of the two systems that demonstrate high enantioselectivity (αNp and βPh), however, the enantiomer that

shows the lowest binding (highest K_s) is the one that has the slower deacylation rate. Thus, one might envisage the following scenario, illustrated for the αNp6H lactones: In these systems, the high binding enantiomer (*S*), which enjoys a relatively normal binding more comparable to that of an L-α-amino acid substrate (cf. Figure 6), with the aryl group positioned in the hydrophobic pocket, undergoes acylation and then deacylates at a favorable rate. In contrast, the low binding enantiomer (*R*) can acylate only when bound in a very poor manner (e.g., with the aryl hydrogen or lactone ring groups exchanged or the aryl groups not fully inserted into the hydrophobic pocket), hence, its higher K_s value. Once in the form of the acyl enzyme, however, with the cyclic constraint of the lactone ring now released, the *R*-lactone-derived acyl enzyme can readjust its subsite interactions, perhaps now placing the aryl group more completely in the hydrophobic pocket. This conformational reorganization that takes place after acylation of the *R* enantiomer causes a marked reduction in its hydrolytic reactivity, perhaps by disposing the keto alkyl group in a position such that it blocks the trajectory of the attacking water or displaces His-57, or, more profoundly, by actually exchanging the positions of the acyl enzyme carbonyl and the methyl ketone carbonyl groups. With these carbonyl groups exchanged, the ester carbonyl is no longer activated toward hydrolysis through interaction with the oxyanion binding hole. A similar situation may be operating with the βPh6H lactone system, which also shows high deacylation enantioselectivity. In a future publication we will present an investigation of these hypotheses using computer graphics–molecular mechanics/dynamics approaches.

Through an analysis of the binding and catalytic activity of a series of amino acid substrates, Knowles (1965) came to the tentative conclusion that the substrates that bound better (had lower K_M values) generally were better substrates (had greater k_{cat} values). While this generalization holds for structural changes within the L-amino acid series, it was obviously not intended to hold for stereochemistry, since the unnatural D-amino acids still bind to α-chymotrypsin with comparable or somewhat greater affinity than the natural L isomers, yet they are not substrates. Nevertheless, in our systems, there is an enantiomeric relationship between binding affinity (K_s) and deacylation rate (k_d), such that the higher affinity enantiomer (*S*) is also the one that undergoes more rapid deacylation.

While this correlation may be pleasing in a mechanistic sense, it is not gratifying in a practical sense in terms of the effectiveness of these lactones as alternate substrate inhibitors. Ideally, a potent alternate substrate inhibitor should bind with high affinity (K_s should be low) and should acylate rapidly (k_a should be high) but deacylate slowly (k_d should be low). In our stereochemical studies here, however, we find that in all cases the enantiomer that is slower to deacylate is always the one with the lower substrate binding affinity. In developing new alternate substrate inhibitors, the challenge will be to design systems in which favorable values of K_s and k_a coordinate with unfavorable values for k_d, rather than the reverse.

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