Mechanism for Slow-Binding Inhibition of Human Leukocyte Elastase by Valine-Derived Benzoxazinones

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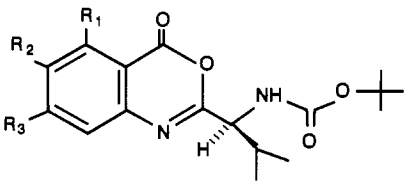
ABSTRACT: Valine-derived benzoxazinones have been synthesized and found to be competitive, slow-binding inhibitors of human leukocyte elastase (HLE). Steady-state inhibition constants K_i* are dependent on aryl substitution and reach a maximum of potency of 0.5 nM with the 5-Cl compound **6**. UV-spectral data for the interaction of HLE and the unsubstituted inhibitor **3** indicate that the stable complex formed between enzyme and inhibitor is an acyl-enzyme that can either undergo ring closure, to reform intact benzoxazinone, or hydrolysis, to liberate an *N*-acylanthranilic acid. "Burst" kinetic data, derived from the direct observation of the interaction of HLE and **3**, are consistent with results of the inhibition of catalysis experiments.

Human leukocyte elastase is thought to be the principal pathogenic agent in a number of connective tissue disorders, most notably, pulmonary emphysema and rheumatoid arthritis (Stein et al., 1985). Thus, the administration of potent inhibitors of this protease may be of therapeutic value in these, and possibly other, disease processes. The search for suitable inhibitors of HLE[1] has been intense during the past 5–10 years and has recently been reviewed (Stein et al., 1985).

Several classes of HLE inhibitors act by forming stable acyl-enzymes (Stein et al., 1985). These inhibitors include *N*-peptide-azaamino acid esters (Powers et al., 1984), isocoumarins (Harper et al., 1983), and benzoxazinones (Teshima et al., 1982).

In this paper we wish to report a new class of potent, competitive inhibitors of HLE: valine-derived benzoxazinones (compounds **1**–**6**). These compounds are structurally related



	R_1	R_2	R_3
1	H	OCH_3	OCH_3
2	H	Cl	H
3	H	H	H
4	H	H	Cl
5	CH_3	H	H
6	Cl	H	H

to the benzoxazinones reported by Powers and co-workers (Teshima et al., 1982) and work by a mechanism involving initial acylation of the enzyme with ring opening of the inhibitor, followed by deacylation by either (i) simple hydrolysis or by (ii) reclosure to regenerate inhibitor.

Materials and Methods

MeOSuc-Ala-Ala-Pro-Val-pNA[1] was prepared according to the method of Nakajima et al. (1979). HLE was prepared as previously described (Stein, 1985a; Viscarello et al., 1983). Buffer salts and Me_2SO were of analytical grade from several sources. Buffer solutions in H_2O and D_2O (99% from Sigma Chemical Co.) were prepared as outlined previously (Stein, 1983). Other reagents were purchased from Aldrich Chemical Co. and Bachem.

Synthesis. Procedures for the synthesis of compounds **1**–**6** are provided as supplementary material (see paragraph at end of paper regarding supplementary material).

Kinetic Procedures. Reaction progress was measured spectrophotometrically by monitoring the release of *p*-nitroaniline at 410 nm. In a typical experiment, a cuvette containing 2.89 mL of buffer and 50 μL each of Me_2SO solutions of inhibitor and MeOSuc-Ala-Ala-Pro-Val-pNA was brought to thermal equilibrium (5–10 min) in a jacketed holder in the cell compartment of a Cary-210 spectrophotometer. The temperature was maintained by water circulated from a Lauda K-RD bath. Injection of 10 μL of enzyme solution initiated the reaction. Absorbances were continuously measured, digitized, and stored in an Apple II microcomputer or a Digital Electronics Corp. PDP 11/23 minicomputer. Progress curves were composed of from 300 to 1000 {absorbance, time} pairs.

Results

Compounds **1**–**6** were tested as inhibitors of HLE. Reaction progress curves reveal that inhibition of HLE develops slowly, as illustrated in Figure 1 for inhibition by **3**. These curves are characterized by initial velocities that are identical with control velocities and final, steady-state velocities that are significantly depressed relative to control. Inhibitors producing these sorts of progress curves have become known as "slow-binding" inhibitors (Williams & Morrison, 1979; Morrison, 1982).

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[1] Abbreviations: HLE, human leukocyte elastase; Me_2SO, dimethyl sulfoxide; MeOSuc, *N*-methoxysuccinyl; ONP, *p*-nitrophenyl ester; pNA, *p*-nitroanilide; Tricine, *N*-[tris(hydroxymethyl)methyl]glycine; Z, *N*-carbobenzoxy.

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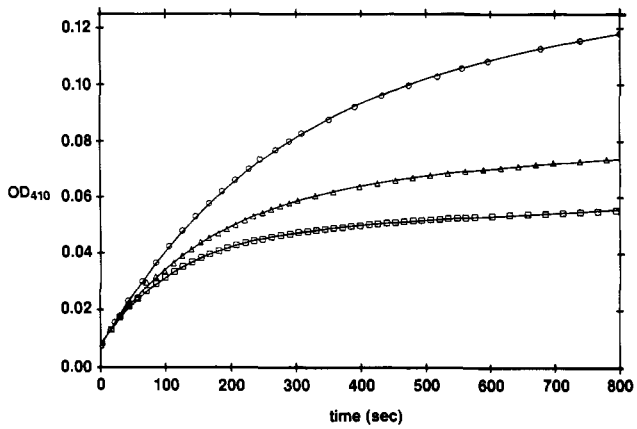


FIGURE 1: Progress curves for inhibition of HLE by **3**. Reaction conditions: [HLE] = 0.020 μM, [MeOSuc-Ala-Ala-Pro-Val-pNA] = 267 μM, 50 mM succinate/50 mM Tricine, 500 mM NaCl, pH 7.0, 3.3% Me_2SO, 25 °C. Inhibitor concentrations: ○, 21 μM; △, 35 μM; □, 52 μM. Reactions were initiated by the addition of enzyme to a thermally equilibrated solution of substrate and inhibitor. Solid lines were calculated by nonlinear least-squares fit of the data to eq 1.

In practice, progress curves for slow-binding inhibition are fit, by nonlinear least-squares, to the integrated expression

$$P = v_s t + (v_0 - v_s)[1 - \exp(-k_{obsd} t)]/k_{obsd} + d \quad (1)$$

where P is the product concentration (in this case related to the absorbance by an extinction coefficient of 8800), v_0 is the initial velocity at $t = 0$, v_s is the final steady-state velocity, k_{obsd} is the observed first-order rate constant for the approach to steady state, and d is the displacement of P from zero at $t = 0$. Equation 1 is a general expression and describes any mechanism of inhibition in which the steady state is reached by a first-order process.

For competitive inhibitors, Scheme I and eq 2 and 3 obtain. In this mechanism, k_{on} is the second-order rate constant for formation of E·I, the complex of inhibitor and enzyme, and k_{off} is the first-order rate constant for decomposition of this complex. K_i, the dissociation constant for E·I, is, of course, the ratio of k_{off}/k_{on}.

Scheme I

$$\begin{array}{ccccc} E + S & \overset{K_m}{\rightleftharpoons} & E\cdot S & \xrightarrow{k_c} & E + P \\ {\scriptstyle k_{on}[I]}\big\downarrow\big\uparrow{\scriptstyle k_{off}} & & & & \\ E\cdot I & & & & \end{array}$$

$$K_i = k_{off}/k_{on} \quad (2)$$

$$k_{obsd} = k_{on}[I]/(1 + [S]/K_m) + k_{off} \quad (3)$$

According to this simple mechanism of inhibition, the observed rate constant for the approach to steady state, k_{obsd}, is given by eq 3 and predicts that a plot of k_{obsd} vs. $[I]/(1 + [S]/K_m)$ will be linear with slope and intercept of k_{on} and k_{off}, respectively. Such a plot appears in Figure 2 for the inhibition of HLE by **3**, and its linearity is in good accord with the mechanism of Scheme I.

Scheme I proposes that the stable, enzyme/inhibitor complex, E·I, forms directly from a bimolecular combination of enzyme and inhibitor. This is probably an oversimplification since E·I is almost certainly preceded by a preassociation complex, as shown in Scheme II.

Scheme II

$$E + I \overset{K_i'}{\rightleftharpoons} (EI)' \underset{k_{-i}}{\overset{k_i}{\rightleftharpoons}} E\cdot I$$

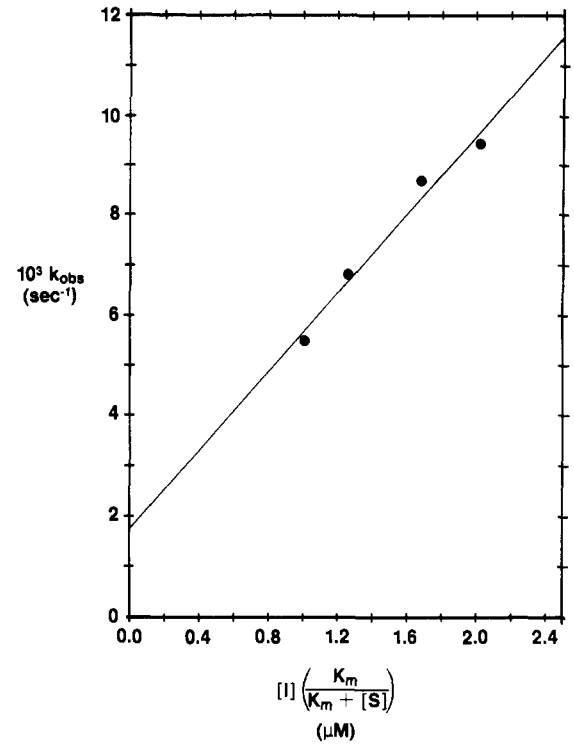


FIGURE 2: Kinetics of inhibition of HLE by **3**. First-order rate constants for the approach to steady state were calculated from inhibition progress curves determined at several concentrations of inhibitor (10 mM sodium phosphate, 500 mM NaCl, pH 7.6, 3.3% Me_2SO, 25 °C, [MeOSuc-Ala-Ala-Pro-Val-pNA] = 165 μM, [HLE] = 0.010 μM). The solid line was drawn from the best fit parameters K_m = 54 μM, k_{on} = 3900 M^{-1} s^{-1}, k_{off} = 0.0018 s^{-1}, and eq 3.

According to this scheme, K_i' is the dissociation constant for the preassociation complex (EI)′, k_i is the first-order rate constant governing the conversion of (EI)′ to E·I, and k_{-i} is the first-order rate constant for the reversal of inhibition. The mechanistic rate constants of Schemes I and II are related by

$$k_{on} = k_i/K_i' \quad (4)$$

$$k_{off} = k_{-i} \quad (5)$$

$$K_i = K_i'(k_{-i}/k_i) \quad (6)$$

Finally, k_{obsd} takes on the slightly more complex form:

$$k_{obsd} = k_i[I]/\{K_i'(1 + [S]/K_m) + [I]\} + k_{-i} \quad (7)$$

Two facts indicate that a preassociation complex does not accumulate in the inhibitor concentration range used in these experiments: (1) the plot of Figure 2 is linear and (2) the initial velocities of Figure 1 are all identical and equal the initial velocity in the absence of inhibitor. This indicates that, under the conditions of these experiments, the inhibitor concentration is much less than K_i'. If (E–I)′ did accumulate, the plot of Figure 2 would be curved and the initial velocities of Figure 1 would decrease with increasing [I] (Morrison, 1982). Although a preassociation complex probably does exist, we were unable to detect its presence in these experiments.

The mechanism of Scheme I assumes competitive inhibition. To test this assumption, we determined steady-state velocities for the inhibition of HLE by **3** at two inhibitor concentrations and over a range of substrate concentrations. The data are presented as a double-reciprocal plot in Figure 3 and indicate competitive inhibition. Analysis of the data by nonlinear least-squares yielded a K_i^{*2} of 0.24 ± 0.05 μM.

Table I: Inhibition of Human Leukocyte Elastase by Benzoxazinones[a]

compd	$[HLE]_0$ (nM)	[I] range (μM)	K_i*[b] (nM) 15% Me_2SO	K_i*[b] (nM) 3.3% Me_2SO	$10^3 k_{hyd}$[c] (s^{-1})
1	10	45–290	140000		0.7
2	10	3.2–21	800		22
3	10	5–80	750	280 ± 23	6
4	10	1.1–22	390		13
5	3	0.09–0.58	23	1.3 ± 0.2	1.5
6	3	0.05–0.12	3.6 ± 0.2	0.46 ± 0.06	22

[a] 10 mM sodium phosphate, 500 mM NaCl, pH 7.6, plus the indicated total concentration of cosolvent. 25 ± 0.1 °C. [b] Error estimates are standard deviations of the mean of three or four independent K_i* determinations. Other values represent a single determination. Standard deviations of these K_i* values were derived from the nonlinear least-squares fitting routine and were less than 25% in all cases. [c] Rate constants for the hydrolysis of benzoxazinones were determined in a pH 10.0 buffer composed of 50 mM sodium carbonate/50 mM sodium borate and 0.83% Me_2SO at 25 °C by monitoring the absorbance change at a convenient wavelength and fitting the progress curves to a first-order rate law.

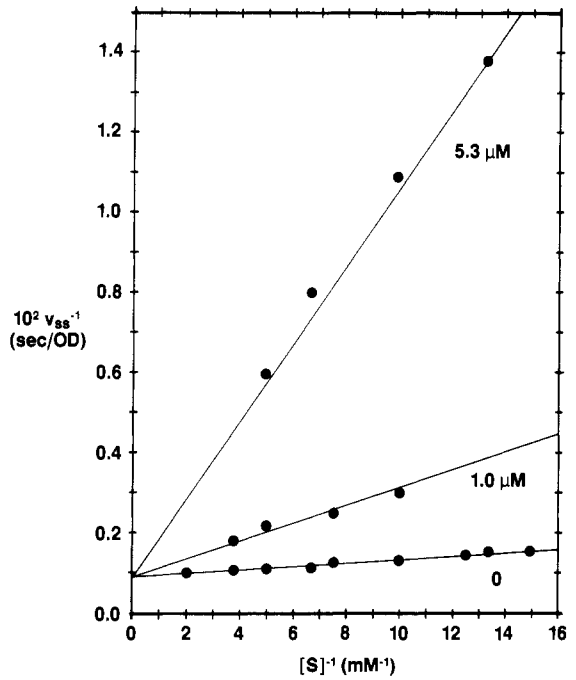


FIGURE 3: Double-reciprocal plot for competitive inhibition of HLE by **3**. Steady-state velocities for the hydrolysis of MeOSuc-Ala-Ala-Pro-Val-pNA (10 mM sodium phosphate, 500 mM NaCl, pH 7.6, 3.3% Me_2SO, 25 °C, [HLE] = 0.010 μM) were calculated from inhibition progress curves recorded at the indicated concentration of inhibitor. Solid lines were drawn from the best fit parameters V_{max} = 0.011 OD/s, K_m = 46 μM, and K_i* = 0.24 μM and the equation $1/v_s = (K_m/V_{max})(1 + [I]/K_i^*)(1/[S]) + 1/V_{max}$.

Assuming competitive inhibition for all valine-derived benzoxazinones, K_i* values were determined by the method of Dixon (1953) for several substituted derivatives of **3**. For the two most potent compounds, **5** and **6**, K_i* was calculated according to

$$K_i^* = \{[I]/(v_0/v_s - 1)\}/(1 + [S]/K_m) \quad (8)$$

[2] We use the term K_i* to represent the inhibition constant measured at the steady state. K_i* differs from the more familiar K_i in that the latter reflects situations of true thermodynamic equilibrium in which the enzyme–inhibitor complex reversibly dissociates to free enzyme and inhibitor. As we will discuss later under Results, the stable complex formed from interaction of benzoxazinones with HLE not only dissociates to enzyme and intact inhibitor but can also decompose hydrolytically to form enzyme and an altered form of the inhibitor. We see that, in this case, a true equilibrium between enzyme and inhibitor cannot be established. Nonetheless, a steady-state condition is reached, and during this steady-state, we can define K_i* to reflect the quantity [E][I]/[EI]. As long as the inhibitor concentration is not depleted due to its turnover by HLE, K_i*, calculated by standard methods, will accurately reflect the dissociation of all enzyme-bound inhibitor complexes that accumulate in the steady state.

Table II: Kinetic Parameters for Inhibition of Human Leukocyte Elastase by Benzoxazinones[a]

inhibitor	$10^{-3}k_{on}$ (M^{-1} s^{-1})	$10^3 k_{off}$ (s^{-1})	k_{off}/k_{on} (nM)	K_i* (nM)
3	4.1 ± 0.4[b]	1.9 ± 0.6	460 ± 160	280 ± 23
5	770 ± 40	1.1 ± 0.5	1.4 ± 0.7	1.3 ± 0.2

[a] 10 mM sodium phosphate, 500 mM NaCl, pH 7.6, 3.3% Me_2SO, 25 ± 0.1 °C. Same enzyme and inhibitor concentrations as Table I. [b] Error estimates are the standard deviations of three or four independent kinetic experiments.

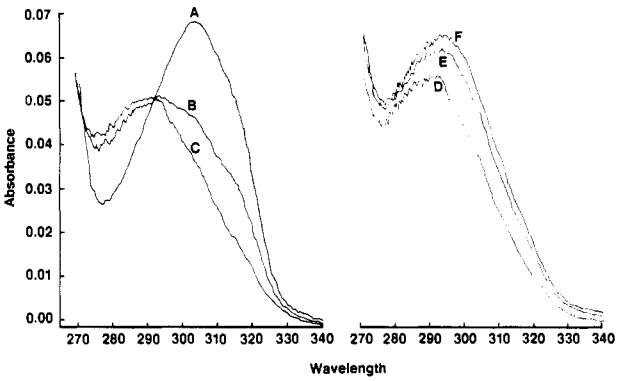


FIGURE 4: UV spectra of reaction of HLE with **3**. (A) Ultraviolet absorbance spectrum of a 23 μM solution of **3** in phosphate buffer (10 mM sodium phosphate, 500 mM NaCl, pH 7.6, 3.3% Me_2SO, 25 °C). (B) HLE was added to the solution of spectrum (A), and a new spectrum was recorded 30 s later ([HLE] = 23 μM). (C) t = 2 min after addition of HLE. (D) An aldehyde inhibitor of HLE was added to the solution of spectrum (C), and a new spectrum was immediately recorded ([aldehyde] = 100 μM). Spectra (E) and (F), t = 10 and 20 min, respectively, after addition of aldehyde.

where v_0 is the control velocity in the absence of inhibitor and v_s is the final, steady-state velocity in the presence of inhibitor. In all the experiments, a substrate concentration of 160 μM (=3K_m) was used. According to this method, values of K_i* are calculated at each of several inhibitor concentrations. From these, a final average K_i* can be computed. This method was required for the more potent compounds since Dixon plots gave x intercepts too close to the origin for accurate estimation of their magnitudes. These values all appear in Table I in order of increasing potency for reaction solutions containing 15% and 3.3% (v/v) Me_2SO. K_i* values in 3% Me_2SO are less than those in 15% Me_2SO and simply reflect the greater solubility of these hydrophobic inhibitors in solutions of higher cosolvent concentration (Maurel, 1978). Compound **6** inhibits HLE with a K_i* value of 0.5 nM and represents the most potent, nonpeptide inhibitor known for this protease.

The kinetics of binding and dissociation are consistent with the K_i* values and a competitive mechanism of inhibition. Table II contains values of k_{on} and k_{off} for the inhibition of HLE by **3** and **5**. These values were obtained from replicate

experiments in which data sets similar to those of Figure 2 were analyzed by linear least squares to give best fit values of k_{on} and k_{off}. The ratio k_{off}/k_{on}, for inhibition by **3** and **5**, agrees well with values of K_i* and points to the general validity of Scheme I.

To investigate the chemical mechanism of inhibition of HLE by these compounds, the interaction of HLE and **3** was studied directly by UV spectroscopy. Tracing A of Figure 4 is the UV spectrum of 23 μM **3** in phosphate buffer. When an equal molar amount of HLE was added to this solution, we observed a time-dependent decrease in the absorbance band centered at 305 nm and a blue shift of the UV maxima, as indicated in spectra B and C. These spectral changes are identical with those observed when the compound is subjected to alkaline hydrolysis (data not shown; hydrolysis of **3** under the conditions of these spectral experiments occurs with an observed first-order rate constant of about 10^{-5} s^{-1}, while at pH 10 this rate constant is 6×10^{-3} s^{-1}). Alkaline hydrolysis of 2-substituted 4*H*-3,1-benzoxazin-4-ones occurs by a mechanism involving attack of hydroxide at the carbonyl carbon, C-2, and subsequent ring opening to generate *N*-acylanthranilic acids (Williams and Salvadori, 1971). Combined, these results indicate that **3** undergoes enzyme-catalyzed ring opening.

Ring closure and the reversibility of inhibition are demonstrated in spectra D–F of Figure 4, in which the spectral characteristics of the intact benzoxazinone are partially regained upon addition of 100 μM of a competitive, peptide-derived aldehyde inhibitor of HLE [MeO-retroLeu-C(O)-Val-Pro-Val-H, K_i = 30 nM; Dutta et al., 1984]. As ring closure occurs, the free enzyme that is generated is trapped by the aldehyde and the intact benzoxazinone that is formed displays its spectral features. Significantly, even upon long incubation of the solution of spectra D–F, characteristics of spectrum A were never fully regained, suggesting that the ring-open species that is formed on the enzyme surface is also able to dissociate from the enzyme in an open form.

We propose that this ring-open species is, in fact, an acyl-enzyme formed from the attack of the active-site serine on the C-4 carbonyl carbon.[3] Thus, we suggest that the interaction of benzoxazinones **1**–**6** occurs according to Scheme III in which E–I is the acyl-enzyme, I′ is an *N*-acylanthranilic acid, k_1 is the second-order rate constant for acylation, and k_{-1} and k_2 are first-order rate constants reflecting ring closure and deacylation, respectively. Kinetically, Schemes I and III are related by the expressions $k_{on} = k_1$ and $k_{off} = k_{-1} + k_2$.

Scheme III

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E\text{–}I \xrightarrow{k_2} E + I'$$

This mechanism was validated in a series of kinetic experiments in which we took advantage of the spectral changes that occur upon interaction of HLE with benzoxazinones. Figure 5 is a reaction progress curve recorded at 314 nm for the interaction of 3 μM HLE with 30 μM **3** at pH 7.0. This curve is characterized by a rapid, first-order disappearance of **3** followed by a slow, linear, steady-state disappearance and is identical with the "burst" kinetics that are commonly seen with serine protease catalyzed reactions (Stein, 1985a). Analysis of these curves was according to standard methods (Stein, 1985a).

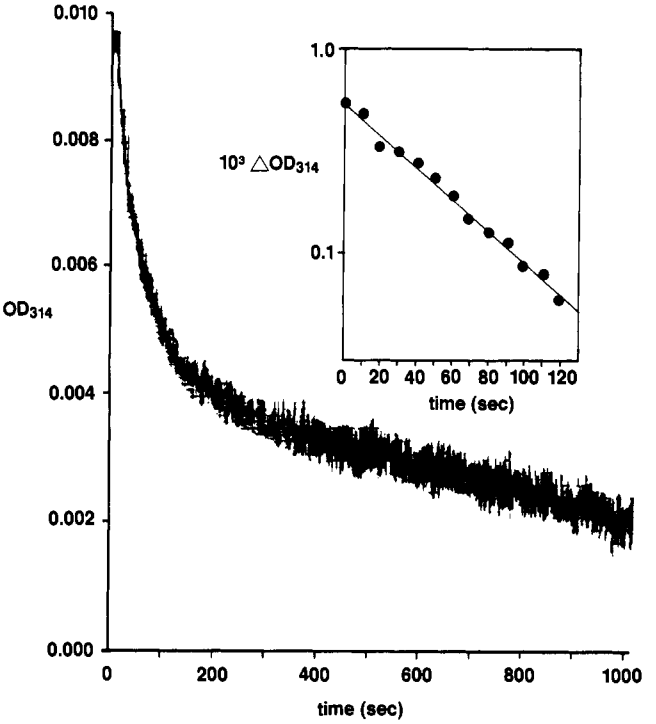


FIGURE 5: Burst kinetics for reaction of HLE with **3**. HLE was added to a thermally equilibrated (25 °C) solution of **3** (50 mM succinate/50 mM Tricine, 500 mM NaCl, pH 7.0, 3.3% Me_2SO, [**3**] = 30 μM, [HLE] = 3 μM). Reaction progress was monitored at 314 nm.

Extrapolation of the linear steady-state portion of the curve to the absorbance at time zero yields the burst size which is related to enzyme concentration. Division of the absorbance, $(4.3 \pm 0.3) \times 10^{-3}$ (n = 5), by a molar extinction coefficient of 1600 yields 2.7 ± 0.2 μM, a value that is identical with the enzyme concentration used in these experiments. The rate constant for the approach to steady state in these experiments is equal to the product k_1[I]. In replicate experiments at [I] = 30 μM, k_1 was found to be 620 ± 35 M^{-1} s^{-1}, in excellent agreement with the k_{on} value of 660 ± 40 M^{-1} s^{-1} determined at pH 7.0 in inhibition of catalysis experiments (data not shown).

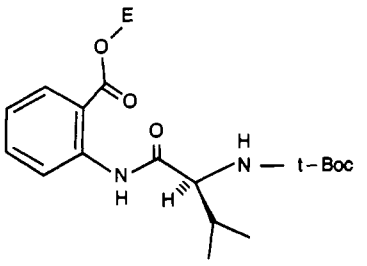
The steady-state velocity in burst experiments of this sort is equal to $k_2[E]_0$. In replicate experiments, k_2 was found to be $(4.5 \pm 0.2) \times 10^{-4}$ and $(1.7 \pm 0.3) \times 10^{-4}$ s^{-1}, in light water and heavy water, respectively. This yields a solvent isotope effect on k_2 of 2.6 ± 0.5 and suggests that deacylation of E–I is a general-acid/general-base catalyzed process.

Finally, it is important to consider how Scheme I is able to account for so much of our kinetic results despite its obvious inability to account for all our observations. The success of Scheme I is principly due to the fact that, in the course of the inhibition experiments, very little of the inhibitor is actually turned over. Thus, the two conditions that $[E]_0 \ll [I]_0$ and $[I]_t = [I]_0$ are always met. Furthermore, k_2 is a small fraction of k_{off} ($=k_{-1} + k_2$) and, to a first approximation, allows us to equate k_{off} and k_{-1}.

Discussion

The results of this study indicate that valine-derived benzoxazinones are competitive, slow-binding inhibitors of HLE and interact with this protease according to a mechanism involving the intermediacy of the acyl-enzyme, E–I. This intermediate can either undergo ring closure, to generate the

[3] In principle, covalent interaction of HLE with these benzoxazinones could also occur at the C-2 position. If the attacking nucleophile is the active-site serine, a substituted imidate would result. However, in a personal communication from Dr. Edgar Meyer (Texas A&M), we learned that recent X-ray crystallographic experiments with porcine pancreatic elastase and a structurally related benzoxazinone established that (i) the site of nucleophilic attack on the inhibitor is the C-4 carbonyl carbon and (ii) the nucleophile is the active-site serine.



E—I

intact benzoxazinone, or hydrolysis, to form an *N*-acylanthranilic acid.

These compounds are observed to be slow-binding inhibitors due to their sluggish kinetics of inhibition. For example, k_{on} and k_{off} for compound **3** are 4100 M^{-1} s^{-1} and 0.002 s^{-1}, respectively, while a classical inhibitor of K_i equal to 10^{-6} M would have values of k_{on} and k_{off} approaching 10^8 M^{-1} s^{-1} and 10^2 s^{-1}, respectively. At a concentration of 10^{-6} M, the classical inhibitor approaches the steady state with a half-time of about 0.003 s, while compound **3** requires a half-time of 120 s.

Formally, these compounds are substrates for HLE, since they generate kinetically competent acyl-enzymes. Their classification as inhibitors, while in part sematic, is based on their long residence times (defined as $0.69/k_{off}$) on the enzyme surface. Compounds **3** and **5** have residence times of about 360 and 630 s, respectively, while substrates generally have residence times less than 0.2 s. In large part, the long residence times for the benzoxazinones are due to their slow rates of deacylation. For example, the acyl-enzyme derived from compound **3** hydrolyzes with a first-order rate constant of about 0.00045 s^{-1} and should be compared with a value of around 3 s^{-1} for the acyl-enzyme derived from Z-Val-ONP (Stein, 1985a,b). Significantly, both compounds rapidly acylate HLE with second-order rate constants of 770 000 and 480 000 M^{-1} s^{-1} for the benzoxazinone and ester, respectively.

The relationship between structure and activity for this series of compounds is complex. Although one may have expected a correlation between reactivity toward basic hydrolysis and potency as an HLE inhibitor, this is clearly not the case, as indicated by the data of Table I. For example, although compounds **2** and **6** hydrolyze in basic solution with identical rate constants, they differ in HLE inhibitory potency by a factor of 200. It appears that the most important influence on potency involves steric bulk at position 5. Large substituents are favorable for potency. Detailed structure activity studies of these effects on a wide selection of analogues will be reported elsewhere.

In a previous study, Powers and his co-workers (Teshima et al., 1982) demonstrated that 2-propyl-3,1-benzoxazin-4-one is an inhibitor of HLE with a k_i of 2 μM. In our laboratory, we obtained a k_i of 11 μM (15% Me_2SO; data not shown), and unlike the valine-derived benzoxazinones, this compound showed no evidence of interacting covalently with HLE. However, in studies with the 2-trifluoromethyl and 2-ethoxy derivatives of 3,1-benzoxazinone, Abeles' group (Hedstrom et al., 1984) was able to demonstrate that these compounds interact covalently with chymotrypsin according to a mechanism identical with that proposed here.

Supplementary Material Available

Procedures for synthesis of compounds **1**–**6** (5 pages). Ordering information is given on any current masthead page.

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