Guanidinophenyl-Substituted Enol Lactones as Selective, Mechanism-Based Inhibitors of Trypsin-like Serine Proteases

Roopa Rai and John A. Katzenellenbogen*

Department of Chemistry, University of Illinois, 1209 West California Street, Urbana, Illinois 61801

We have synthesized four guanidinophenyl-substituted protio enol and iodo enol lactones (3-(4-guanidinophenyl)-6-methylidenetetrahydro-2-pyranone (1), 3-(4-guanidinophenyl)-6-(E)-(iodomethylidene)tetrahydro-2-pyranone (2), 4-(4-guanidinophenyl)-6-methylidenetetrahydro-2-pyranone (3), and 4-(4-guanidinophenyl)-6-(E)-(iodomethylidene)tetrahydro-2-pyranone (4)) and tested them for inhibitory activity against some trypsin-like enzymes, namely trypsin, urokinase, tissue plasminogen activator (t-PA), plasmin, and thrombin, as well as α-chymotrypsin and human neutrophil elastase (HNE). The β-aryl-substituted protio lactone 3 was a potent alternate substrate inhibitor of trypsin and urokinase. The α-aryl-substituted iodo lactone 2 was a permanent inactivator of urokinase, plasmin, t-PA, thrombin, and α-chymotrypsin, exhibiting a relatively high specificity for the former two enzymes. In general, these compounds showed a preference for inactivating trypsin-like enzymes over α-chymotrypsin and HNE. Also, within the class of trypsin-like enzymes, there was generally good selectivity of inhibition.

Introduction

Proteases have attracted a growing interest due to the vital role they play in a number of biological processes. Among the serine proteases, those with trypsin-like specificity are most frequently involved in physiological regulation. Trypsin itself is a digestive enzyme, but serves also to activate itself as well as the other zymogens of the pancreatic tissue. Thus, inhibitors of trypsin could potentially be used for treatment of pancreatitis and hyperproteolytic conditions.[1] Most of the dozen enzymes involved in the coagulation of blood exhibit trypsin-like selectivity, but with much greater specificity than trypsin itself.[2] Thus, specific inhibitors of blood clotting enzymes are of therapeutic potential as anticoagulants.[1]

Fibrinolysis,[3,4] the process of blood clot dissolution, is also mediated by an enzyme cascade, with the activation of plasminogen to plasmin being mediated by plasminogen activators (PA), namely, tissue-type plasminogen activators (t-PA) or urokinase-type plasminogen activators (u-PA). Thus, inhibitors of plasmin, urokinase, or t-PA might be used in the therapy of hyperfibrinolytic states.[5] PA is secreted by malignant tumors[6] and plays a role in tumor invasion and metastasis. PA is also involved in some normal invasive and degradative events in reproduction, such as follicle release during ovulation, sperm penetration of the ovum, and blastocyst implantation in the uterus, as well as in some tissue remodeling events, such as involution of the lactating mammary gland.

A number of heterocyclic compounds have been shown to act as mechanism-based inactivators of serine proteases,[7] e.g., isatoic anhydrides,[8] isocoumarins,[9] ynenol lactones,[10] 6-chloro-2-pyrones,[11] and halo enol lactones.[12] Such compounds utilize the normal catalytic machinery of the enzyme by initially acylating the active-site serine. Some act simply as alternate substrate inhibitors, i.e., as transient inactivators which form very stable acyl enzymes. By contrast, with others, the formation of the acyl enzyme reveals a reactive functional group, which alkylates a suitably positioned active-site residue and becomes permanently tethered to the enzyme. These latter agents are often termed suicide substrates.

We have reported aryl-substituted valerolactones as mechanism-based inactivators of α-chymotrypsin.[12,13] No-

* Address correspondence to John A. Katzenellenbogen, Department of Chemistry, University of Illinois, 461 Roger Adams Laboratory, Box 37, 1209 W. California St., Urbana IL 61801. Telephone: (217) 333-6310. FAX: (217) 244-8068. BITNET: KATZENELL@UIUCSCS. INTERNET: KATZENELL@B.SCS.UIUC.EDU.

(1) Markwardt, F.; Stürzebecher, J. Inhibitors of Trypsin and Trypsin-like Enzymes With a Physiological Role. In *Design of Enzyme Inhibitors as Drugs*; Sandler, M., Smith, H. J., Eds.; Oxford University Press: Oxford, 1989; pp 619-649.

(2) Davie, E. W.; Fujikawa, K.; Kurachi, K.; Kisiel, W. The Role of Serine Proteases in the Blood Coagulation Cascade. *Adv. Enzymol.* 1978, 48, 277-318.

(3) Castellino, F. J. Recent Advances in the Chemistry of the Fibrinolytic System. *Chem. Rev.* 1980, 81, 431-446.

(4) Collen, D. Molecular Mechanisms of Fibrinolysis and their Application to Fibrin-Specific Thrombolytic Therapy. *J. Cell. Biochem.* 1987, 33, 77-86.

(5) Barrett, A. J. Proteinase Inhibitors: Potential Drugs? In *Enzyme Inhibitors as Drugs*; Sandler, M., Ed.; University Park Press: Baltimore, 1980; pp 219-229.

(6) Moscatelli, D.; Rifkin, D. B.; Isseroff, R. R.; Jaffe, E. A. *Proteinases and Tumor Invasion*; Raven Press: New York, 1980; Vol. 6, pp 143-152. (b) Jänicke, F.; Schmitt, M.; Graeff, H. Clinical Relevance of the Urokinase-Type and Tissue-Type Plasminogen Activators and of Their Type 1 Inhibitors in Breast Cancer. *Semin. Thromb. Hemostasis* 1991, 17 (3), 303-312.

(7) Silverman, R. B. *Mechanism-Based Enzyme Inactivation. Chemistry and Enzymology*; CRC Press: Boca Raton, FL, 1988; Vol. 1 and 2.

(8) Gelb, M. H.; Abeles, R. H. Substituted Isatoic Anhydrides: Selective Inactivators of Trypsin-like Serine Proteases. *J. Med. Chem.* 1986, 29, 585-589.

(9) Kam, C-M.; Fujikawa, K.; Powers, J. C. Mechanism-Based Isocoumarin Inhibitors for Trypsin and Blood Coagulation Serine Proteases. *Biochemistry* 1988, 27, 2547-2557.

(10) Copp, L. J.; Krantz, A.; Spencer, R. W. Kinetics and Mechanism of Human Leucocyte Elastase Inactivation by Ynenol Lactones. *Biochemistry* 1987, 26, 169-178.

(11) Westkaemper, R. B.; Abeles, R. H. Novel Inactivators of Serine Proteases Based on 6-Chloro-2-Pyrone. *Biochemistry* 1983, 22, 3256-3264.

(12) (a) Daniels, S. B.; Cooney, E.; Sofia, M. J.; Chakravarty, P. K.; Katzenellenbogen, J. A. Halo Enol Lactones. Potent Enzyme-Activated Irreversible Inhibitors for α-Chymotrypsin. *J. Biol. Chem.* 1983, 258, 15046-15053. (b) Sofia, M. J.; Katzenellenbogen, J. A. Enol Lactone Inhibitors of Serine Proteases. The Effect of Regiochemistry on the Inactivation Behavior of Phenyl Substituted (Halomethylene)tetra- and -dihydrofuranones and (Halomethylene)tetrahydropyranones toward α-Chymotrypsin: Stable Acyl Enzyme Intermediate. *J. Med. Chem.* 1986, 39, 230-238.

(13) Baek, D-J.; Reed, P. E.; Daniels, S. B.; Katzenellenbogen, J. A. Alternate Substrate Inhibitors of an α-Chymotrypsin: Enantioselective Interaction of Aryl-substituted Enol Lactones. *Biochemistry* 1990, 29, 4305-4311.

tably, the α-phenyl- and α-naphthyl-substituted iodo enol lactones (Ia and Ib, respectively) act as suicide substrates, permanently inactivating α-chymotrypsin. The β-aryl-substituted valerolactones (II), on the other hand, showed rapid but transient inactivation of α-chymotrypsin, thus acting as alternate substrate inhibitors.

Scheme I
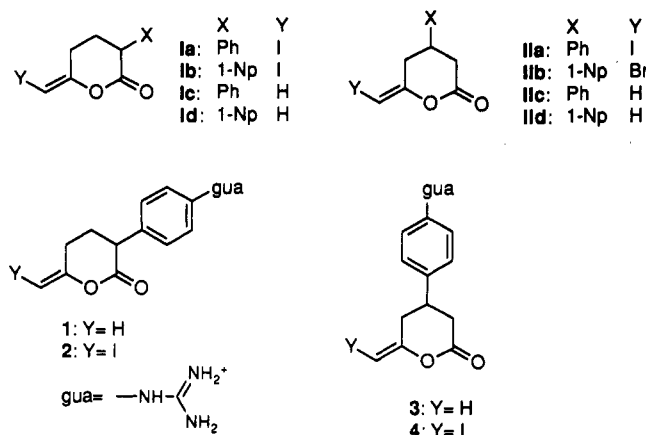
We wished to study the effect of substituting a guanidino group at the 4-position of the phenyl ring in valerolactones Iac, and IIac, on their inactivation profiles with some serine proteases. Since trypsin-like enzymes are specific in cleaving peptide bonds at the positively charged residues of lysine and arginine, we hoped that the presence of a positively charged group on the inhibitor would provide us with compounds which show some selectivity in inhibiting the trypsin-like enzymes over other serine proteases like α-chymotrypsin and human neutrophil elastase (HNE).

In this paper, we describe the synthesis and kinetic evaluation of guanidino-substituted lactones 1–4 as inhibitors of some trypsin-like enzymes, namely, trypsin, urokinase, t-PA, plasmin, and thrombin. For comparison, the inactivation of α-chymotrypsin and HNE by these compounds has also been studied. These compounds were selective in inactivating the trypsin-like enzymes. The β-aryl protio lactone 3 was a potent alternate substrate inhibitor, and the α-aryl iodo lactone 2 was an irreversible inhibitor of some of the target enzymes.

Results

Synthesis of α-Aryl-Substituted Lactones 1 and 2. The protio and iodo enol lactones 1 and 2, bearing an α-(p-guanidinophenyl) substituent, were synthesized using 4-aminophenylacetic acid (5) as starting material (Scheme I). The amino group was protected as the *tert*-butyloxycarbonyl (BOC) derivative. Alkylation of this protected amino acid (6) with 4-bromo-1-butyne[12a] using 3.3 equiv of LDA in presence of HMPA proceeded in low yield to give the acetylenic acid 7, with 80% of the starting material being recovered. The yield improved considerably when 3.3 equiv of n-BuLi was added to the reaction mixture prior to addition of the electrophile. This suggests that diisopropylamine that is generated during enolate formation may be reprotonating the anion.[14]

The acetylenic acid 7 was deprotected, and the guanidino group was introduced by refluxing the amino acid 8 with
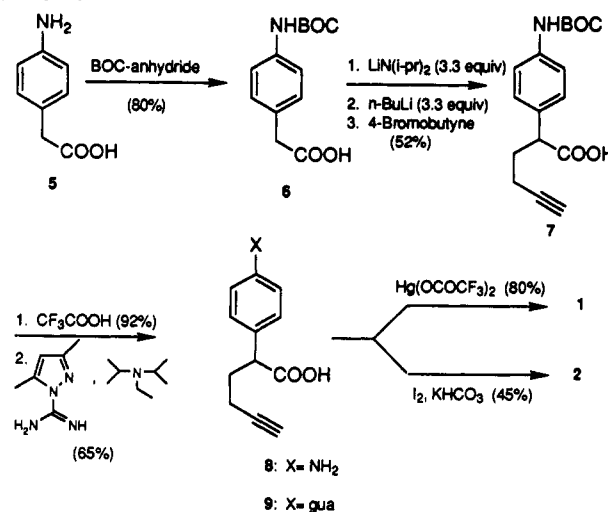
3,5-dimethylpyrazole-1-carboxamidinium nitrate in presence of diisopropylethylamine. The reaction was sluggish and did not go to completion even upon refluxing for 4 days. Isolation of the guanidino acid 9 was achieved by flash chromatography using a very polar solvent system. The lack of reactivity of the amino acid 8 and the difficulty in isolation of the extremely polar guanidino acid 9 are reflected in the modest yields obtained.

Initial attempts to cyclize 9 to either the protio lactone 1 or the iodo lactone 2 proved unsuccessful, due to the insolubility of starting material in most non-hydroxylic solvents like acetonitrile and methylene chloride. A suspension of 9 in acetonitrile was solubilized by adding a trace of trifluoroacetic acid. Subsequently, cyclization to the protio enol lactone 1 proceeded smoothly in the presence of a catalytic quantity of mercuric trifluoroacetate. Cyclization to the iodo enol lactone 2 was achieved by reaction with iodine and $KHCO_3$. This was a slow reaction, and stirring for long periods of time caused isomerization of the double bond to the corresponding internal alkene. Addition of a trace amount of water to the reaction mixture accelerated the rate of reaction and improved the yields considerably.[15] Purification of the lactones 1 and 2 was achieved by flash chromatography using $CHCl_3$ and 2-propanol in the solvent system. (Use of MeOH in the solvent system caused decomposition or double-bond isomerization of the product.)

Synthesis of β-Aryl-Substituted Lactones 3 and 4. The β-aryl lactones 3 and 4 were both synthesized from the guanidino acid intermediate 18, as is outlined in Scheme II. 4-Aminobenzyl alcohol was protected as its BOC derivative and then was oxidized to the aldehyde 12. Reaction with triethyl phosphonoacetate in the Wadsworth–Emmons modification of the Wittig reaction[16] yielded the alkenoic ester 13. Conjugate addition of allyl cuprate to the unsaturated ester 13 afforded the alkene 14. The reaction required the addition of TMSCl to the cuprate preparation prior to the addition of the enoate.[17] Bromination to the dibromide 15 went in poor yield, the
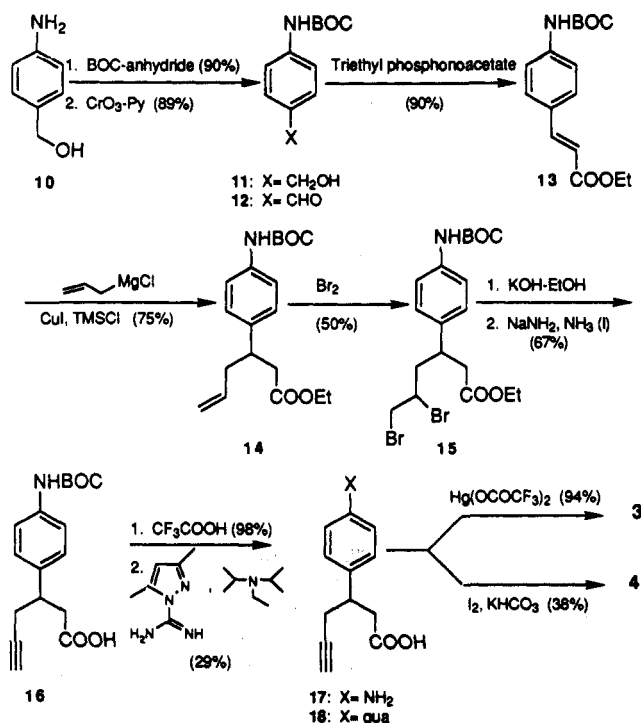
(14) Seebach, D. Structure and Reactivity of Lithium Enolates. From Pinacolone to Selective C-Alkylations of Peptides. Difficulties and Opportunities Afforded by Complex Structures. *Angew. Chem. Int. Ed. Engl.* **1988**, *27*, 1624–1654.

(15) Dai, W.; Katzenellenbogen, J. A. Stereoselective Z- and E-Bromo Enol Lactonization of Alkynoic Acids. *J. Org. Chem.* **1991**, *56*, 6893–6896.

(16) Wadsworth, W. S., Jr.; Emmons, W. D. The Utility of Phosphonate Carbanions in Olefin Synthesis. *J. Am. Chem. Soc.* **1961**, *83*, 1733–1738.
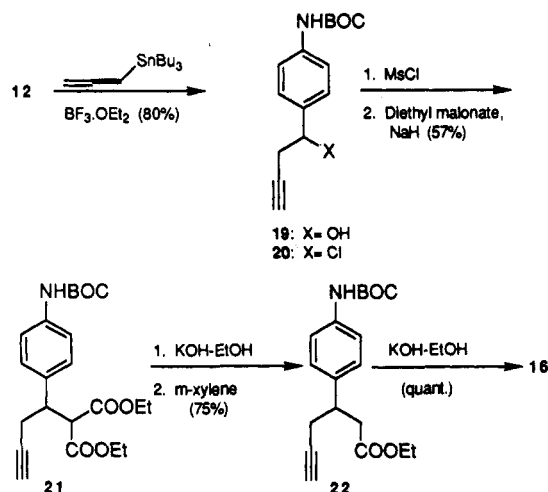
(17) Alexakis, A.; Berlan, J.; Besace, Y. Organocopper Conjugate Addition Reaction in the Presence of Trimethylchlorosilane. *Tetrahedron Lett.* **1986**, *27*, 1047–1050.

Scheme II

Scheme III

major side product being a highly polar, polymeric species. Treatment of the dibromo ester **15** with KOH–EtOH effected hydrolysis and elimination to a vinyl bromo acid. This material was not characterized, but was allowed to react with sodium amide in liquid ammonia to complete the elimination, yielding the alkynoic acid **16**.

An equally successful, but less cumbersome route to the alkynoic acid **16** was also examined (Scheme III). The BOC-protected aldehyde **12** was treated with allenyltributyltin[18] in the presence of boron trifluoride etherate to afford the alcohol **19**. Diethyl malonate was alkylated with the chloride **20**, which itself was prepared (just prior to use, without purification), from the alcohol **19** under mesylation conditions. The relatively low yield of this reaction is attributed to the instability of the chloro intermediate **20**, which undergoes facile elimination to the
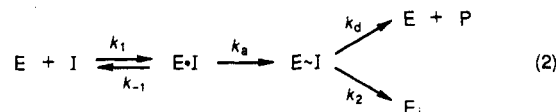
eneyne during chromatography or even upon standing at –5 °C. Saponification in 20% aqueous KOH gave the corresponding half-ester, which was decarboxylated in refluxing xylene to the alkynoic ester **22**. Further saponification of this ester gave the BOC-protected amino acid **16** which after deprotection afforded the amino acid **17** (Scheme II). Introduction of the guanidino group and subsequent purification, handling, and cyclization of guanidino acid **18** to the β-aryl lactones **3** and **4** followed the same procedure as used for conversion of acetylenic acid **9** to the α-aryl lactones **1** and **2**.

Biochemical Studies. Protio enol lactones have the potential to act as alternate substrate inhibitors of serine proteases. They interact with the enzyme in the same manner as a normal substrate, except that the acyl enzyme that is formed is stable, resulting in a slow rate of deacylation. Eq 1 is the kinetic model describing the

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E \cdot I \xrightarrow{k_a} E \sim I \xrightarrow{k_d} E + I' \tag{1}$$
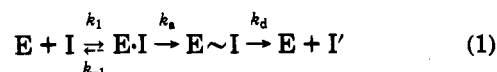
inactivation process: I is the lactone, E·I the Michaelis complex, E∼I the acyl enzyme, and I' the product keto acid. The reaction is described by the binding constant K_s (i.e., k_{-1}/k_1), the acylation rate constant k_a, and the rate constant of deacylation k_d.

Halo enol lactones can act as irreversible inhibitors of serine proteases.[19] Eq 2 describes the kinetic scheme for

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E \cdot I \xrightarrow{k_a} E \sim I \overset{k_d}{\underset{k_2}{<}} \begin{matrix} E + P \\ E_i \end{matrix} \tag{2}$$

the inactivation process. The formation of the acyl enzyme (E∼I) is accompanied with the release of a halomethyl ketone group, which in the ideal case, would alkylate a suitably positioned nucleophile in the active site at a rate k_2. K_i (i.e., k_{-1}/k_1) and k_a describe the binding constant and the rate constant of acylation, respectively. Deacylation of the acyl enzyme prior to the alkylation event, or hydrolysis of the iodomethyl ketone followed by deacylation, would result in transient inactivation of the serine protease. In that case, the kinetic model would be described by eq 1.

Incubation Assay. The lactones were tested by an incubation assay for inhibition with some trypsin-like serine proteases, namely, trypsin, urokinase, t-PA, plasmin, and thrombin, as well as α-chymotrypsin and HNE. In this assay, the enzyme was incubated with an excess of the inhibitor, and then aliquots were removed at specific time intervals and tested for residual enzyme activity against a suitable substrate. A plot of absorbance (A) vs time (t) was obtained, and the slope of this straight line was directly proportional to the residual enzyme activity.

The lactones were tested as a mixture of enantiomers and in some cases the A vs t output was a line with an increasing slope. This is attributed to the fact that one of the enantiomeric pair forms an unstable acyl enzyme that is undergoing deacylation during the initial period of the incubation assay. Method B (see Experimental Section) was used in these cases. The 10-min deacylation delay ensured the decomposition of all unstable acyl

(18) Boaretto, A.; Marton, D.; Tagliavini, G. Preparation of α-Allenic and β-Acetylenic Alcohols by Treatment of a Mixture of Bu₃SnCH=C=CH₂ and RCHO with Bu₂SnCl₂ and Water. *J. Organomet. Chem.* 1985, 297, 149–153.

(19) Rando, R. R. Chemistry and Enzymology of k_cat Inhibitors. *Science* 1974, 185, 320–324.
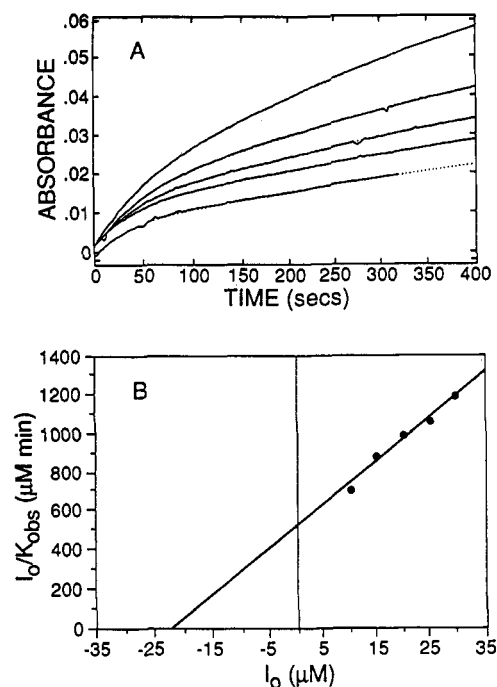
Figure 1. Time-dependent and competitive inhibitor of urokinase by the iodo enol lactone 4. Panel A: Progress curves for the inhibition of urokinase by lactone 4 in presence of a chromogenic substrate. The dotted lines show the progress curves calculated with the experimentally determined rate constants, k_{obs}. Panel B: Competitive substrate assay of urokinase inhibited by lactone 4. A plot of the initial inhibitor concentration I_0 (μM) versus I_0/k_{obs} (μM min) gives a straight line with a slope of $1/k_a$ and an x-intercept of $-K_i$ $(1 + S_0/K_m)$.
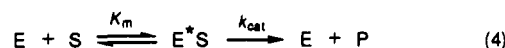
enzyme species; percent enzyme inactivation measured in these cases was solely due to stable acyl enzyme intermediates. After a 2-h incubation period in the case of iodo enol lactones, 0.1 M hydrazine was added to the incubation solution. This was expected to act as a strong external nucleophile, cleaving any acyl linkage between the enzyme and inhibitor.

The results of the incubation assay are summarized in Table I in a convenient format: The time required to reach the maximum level of inhibition (A), the maximum percent inhibition (B), and the time period over which this level of inhibition persists (C) are reported. The percent of enzyme activity regained upon treatment with hydrazine is also listed (D) for the iodo enol lactones 2 and 4. This protocol allowed us to determine (a) whether the lactone is acting as an inhibitor (preliminary qualitative conclusions could be drawn concerning the selectivity of a particular lactone in inhibiting the enzymes that were tested), (b) how efficiently the lactone is acting to inhibit the enzyme, i.e., what percent of the enzyme activity is lost during the course of the assay, and (c) the period of time during which the enzyme is inhibited. Finally, the addition of hydrazine to the incubation solution should cause rapid deacylation and recovery of enzyme activity in the case of protio enol lactones. Thus, we could readily distinguish between a halo enol lactone acting as an alternate substrate inhibitor or as a suicide substrate.

Inactivation profiles which showed a high percent inhibition that lasted for more than 30 min were analyzed further to determine the parameters of the inactivation process. This is described in the following sections.

Binding Constant K_s(K_i for Irreversible Inhibition) and Rate Constant of Acylation k_a for Enol Lactone

Inhibition. Method A: The binding constant (K_s or K_i) and the rate constant of acylation (k_a) were determined by a competitive substrate assay in which the enzyme was incubated with the lactone in the presence of a chromogenic substrate. The lactone and the substrate compete for the enzyme active site, as described by the kinetic eqs 3 and 4. During the time course of the assay, E~L accumulates

$$E + L \underset{K_{s/i}}{\rightleftharpoons} E{\cdot}L \xrightarrow{k_a} E{\sim}L \overset{k_d \nearrow E + L'}{\underset{k_2 \searrow E_i}{\big\langle}} \qquad (3)$$

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

to a steady-state concentration. The acylation process is followed by a decrease in free enzyme, which is monitored continuously by its consumption of the chromogenic substrate. The progress curve shows a decreasing slope until the flat steady-state region is reached. The success of this assay depends upon the fact that the rate of approach to the steady-state level of E~L is slow enough to be measured. Before the steady-state level is attained, there is a burst of P formation (initial 150 s in Figure 1, panel A). In some cases, this burst region, critical to this method, could not be perceived over a wide range of [S] and [I]. This may be due to a very high k_a combined with a low K_i. In these cases, method B (described below) was used.

Main[20a] has derived an expression for a competitive assay between a substrate and a time-dependent irreversible inhibitor (eq 5),where S_0 and I_0 are the initial substrate

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

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

and inhibitor concentrations, respectively. V_0 and V are the velocity of substrate hydrolysis at the initial time and at time t, respectively, and K_m is the Michaelis constant of the substrate. Rearrangement of the definition of k_{obs} in eq 5 yields eq 6, which is that of a straight line with a slope of $1/k_a$ and an x-intercept of $-K_i$ $(1 + S_0/K_m)$. K_m of the substrate used was determined separately in a standard assay. Since S_0 and I_0 were known (substrate and inhibitor were used in large excess and the concentrations were therefore assumed to remain constant during the assay), k_a and K_i could be determined.

When dealing with reversible alternate substrate inhibitors, k_d is slow, and the kinetic model derived by Main can still be used. The steady-state region now has a slope associated with it due to slow deacylation of E~L and repartitioning of E through E*S. A correction is made for this limiting slope so that the true exponential approach to the steady state can be determined accurately. The progress curves for the β-aryl-substituted iodo lactone 4 with urokinase are shown in Figure 1.

(20) (a) Main, A. R. Kinetics of Active-Site-Directed Irreversible Inhibition. In *Essays in Toxicology*; Hays, W. J., Ed.; Academic Press: New York, 1973; Vol 4, pp 59–105. (b) Segel, I. H. In *Enzyme Kinetics. Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems*; John Wiley and Sons: New York, 1975; Chapter 3.

Table I. Preliminary Screen of the Time-Dependent Inhibition of Some Serine Proteases by Lactones 1–4 Using the Incubation Assay

protease (nM)	I_0/E_0	lactone 1: A/B/C[a]	lactone 2 A/B/C[a]	D[b]	lactone 3: A/B/C[a]	lactone 4 A/B/C[a]	D[b]
trypsin (14.6)	86	0/97/45	0/80/0	–	20/98/∞	0/99/∞	100
urokinase (300)	50	0/97/∞	0/98/∞	0	0/99/∞	0/98/∞	100
t-PA (19.4)	100	0/29/0	15/48/∞	0	15/24/30	0/22/15	–
plasmin (625)	16	0/99/0	0/95/∞	14	0/79/∞[c]	60/97/∞	100
thrombin (1.93)	518	0/7/0[c]	0/93/∞[c]	85	15/40/0	90/38/0[c]	–
α-chymotrypsin (17)	118	45/58/0	15/78/∞[c]	32	0/34/45[c]	0/47/15[c]	–
HNE (66)	36	15/22/0	30/38/90	–	30/27/30	15/67/15	–

[a] A = time (min) to reach maximum percent inhibition; B = maximum percent inhibition; C = time (min) for which maximum inhibition persists (∞ indicates that no enzyme activity was recovered over 2 h, the time course of the assay). [b] D = percent activity regained after N_2H_4 treatment. [c] Delay method was used in incubation assay (see Experimental Section).
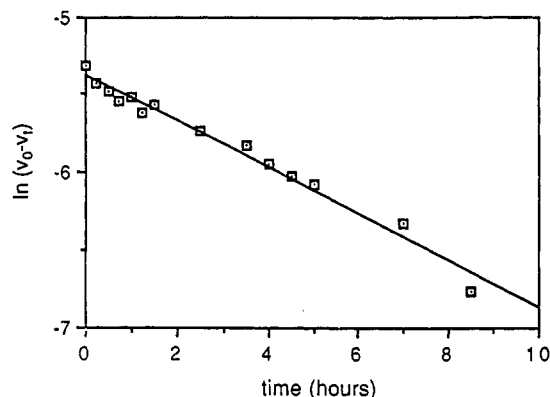
Figure 2. Determination of rate constant of deacylation, k_d, of the protio enol lactone 3 in its inactivation of trypsin.

Method B: The inhibition of t-PA by the iodo lactone 2 could not be determined by the method described above. An estimated binding constant (K_i) was obtained by the use of a competitive inhibition assay.[20b] At different inhibitor concentrations, a K_m^{app} value was obtained by a reciprocal plot. A replot of inhibitor concentrations versus K_m^{app} allowed the determination of K_i from the x-intercept. An assumption in the use of this assay is that all enzyme species are reversibly connected. In order to minimize contributions due to formation of the acyl enzyme, the initial reaction velocities were measured during the first 15 s after addition of enzyme. In some cases, binding constants were obtained by both methods, and shown to be close, thus justifying the use of method B to obtain an estimated binding constant in these cases, where method A cannot be used. A limitation in the use of method B is that the rate constant of acylation (k_a) cannot be determined.

Rate Constant of Deacylation (K_d) for Alternate Substrate Inhibitors. Deacylation of acyl enzyme intermediates was studied by monitoring the recovery of enzyme activity with time at room temperature. The enzyme was incubated with a large excess of the lactone. [The incubation time that was used was the "A" value of the incubation assay; see Discussion and Tables I–III]. Excess lactone was removed from the solution by two 1-h centrifugations at 0 °C, using Amicon Centricon-10 microconcentrator. Assay for evaluating the remaining enzyme activity at various time intervals was done as described in the incubation assay. A control (which contained no inhibitor) was run in parallel, and a plot of the natural log of the remaining enzyme activity (as compared to the control) vs time gave a straight line. The value for k_d was obtained from the slope of this line (see Figure 2).

A disadvantage of this method was that the k_d values of the lactones which formed a relatively unstable acyl

enzyme (high k_d) could not be determined. Complete deacylation occurred during the time required for centrifugation, even though it was done at 0 °C. Since such inhibitors were not as potent as those which exhibited very low k_d's, no further attempt was made to evaluate k_d values by a different method.

Discussion

Protio and halo enol lactones have been extensively studied in our group as mechanism-based inhibitors of α-chymotrypsin[11,12] and HNE.[21] The hydrophobic primary specificity pocket of α-chymotrypsin accounts for its preference for cleaving the peptide bond at aromatic amino acid residues like Phe and Trp. In elastase, the hydrophobic pocket is relatively sterically congested, due to the presence of bulky side chains of Val-216 and Thr-226. (Both of these residues are Gly in α-chymotrypsin and trypsin.) Therefore, only small aliphatic side chains such as the methyl group of Ala can be accommodated in this shallow cavity. In contrast to α-chymotrypsin and elastase, the base of the hydrophobic S_1[22] site of trypsin contains the negatively charged carboxylate side chain of Asp-189. This accounts for the specificity of these enzymes for cleaving the peptide bond at positively charged basic amino acids like Lys and Arg.

Studies have shown that trypsin-like enzymes are inhibited by aromatic compounds containing positively charged groups like benzamidine and phenylguanidine.[23] It was thought that the presence of a positively charged guanidino group on the α-chymotrypsin inhibitors Iac and IIac would complement the specificity preference of trypsin-like enzymes and increase the selectivity in inhibition studies. The present paper describes the syntheses and kinetic evaluation of α- and β-(4-guanidinophenyl)-substituted valerolactones 1–4. These guanidino-substituted enol lactones were tested as inhibitors of trypsin, urokinase, t-PA, plasmin, and thrombin, as well as α-chymotrypsin and HNE.

Protio Enol Lactones. Incubation assays conducted on the protioenol lactones 1 and 3 provided us with a preliminary screen of the potency and selectivity of these compounds as potential inhibitors (Table I). A high level of inhibition, which was attained rapidly and which persisted for at least 30 min, was desirable for the target enzymes. In the case of α-chymotrypsin and HNE, it was hoped that if these enzymes were inhibited, the inhibition

(21) (a) Reed, P. E.; Katzenellenbogen, J. A. Proline-Valine Pseudo Peptide Enol Lactones. *J. Biol. Chem.* **1991,** *266,* 13–21. (b) Dai, W.; Katzenellenbogen, J. A., manuscript in preparation.
(22) Schechter, I.; Berger, A. On the Size of the Active-Site in Proteases. I. Papain. *Biochem. Biophys. Res. Commun.* **1967,** *27,* 157–162.
(23) Mares-Guia, M.; Shaw, E. Studies on the Active Center of Trypsin. *J. Biol. Chem.* **1965,** *240,* 1579–1585.

Table II. Alternate Substrate Binding Constant and Acylation and Deacylation Rates of Protio Enol Lactones 1 and 3 with Selected Proteases

protease	K_s (μM)	k_a (min^{-1})	k_a/K_s (min^{-1} μM^{-1})	k_d (min^{-1})
		Lactone 1		
trypsin	0.23 ● 0.07	14 ● 3	62	fast[a]
urokinase	0.33 ● 0.07	391 ± 70	1185	fast[a]
		Lactone 3		
trypsin	9.4 ± 4.1	91 ± 40	10	0.002
urokinase	0.12 ± 0.08	0.24 ± 0.02	2	0.009
plasmin	63.7 ● 38.0	97 ± 61	1.5	fast[a]

[a] k_d was too fast to measure by the centrifugation method (see Experimental Section).

Table III. Binding Constant and Acylation and Deacylation Rate Constants of Iodo Enol Lactones 2 and 4 with Selected Proteins

protease	$K_{s/i}$ (μM)	k_a (min^{-1})	k_a/K_s (min^{-1} μM^{-1})	k_d (min^{-1})
		Lactone 2		
urokinase	0.16 ± 0.03	18.6 ± 3.4	117	–
t-PA	0.67 ± 0.20	c	–	–
plasmin	0.03[a]	13.6 ± 7.0	453	–
thrombin	0.93 ± 0.20	12.9 ± 3.2	14	–
α-chymotrypsin	2.4 ± 0.6	7.22 ± 0.06	3	–
		Lactone 4		
trypsin	4.28 ± 1.1	0.54 ± 0.02	0.13	0.02
urokinase	7.83 ± 0.9	2.61 ± 0.20	0.33	fast[b]
plasmin	11[a]	11 ± 1	0.9	fast[b]

[a] The value reported reflects the higher limit. [b] k_d was too fast to measure by centrifugation method (see Experimental Section). [c] Method B was used; k_a could not be determined by this method.

would be transient. The α-aryl-substituted lactone 1 inhibited trypsin and urokinase effectively. The inhibition of t-PA, plasmin, thrombin, α-chymotrypsin, and HNE was transient and less efficient. The β-aryl-substituted lactone 3 inactivated trypsin, urokinase, and plasmin effectively. t-PA, thrombin, α-chymotrypsin, and HNE showed relatively lower levels of inhibition, which was also transient in character. Therefore, we had been successful in making compounds that were selective in inhibiting the trypsin-like enzymes, rather than α-chymotrypsin and HNE.

Among the trypsin-like enzymes, the α-substituted lactone 1 showed a preference for inactivating trypsin and urokinase (Table II). It exhibited a high binding affinity (low K_s) for these enzymes, and the second order rate constant of acylation (k_a/K_s) for urokinase was exceptionally high. However, deacylation of the acyl enzymes formed was rapid. The β-aryl-substituted protio enol lactone 3 showed a similar selectivity pattern within the trypsin-like enzymes as did 1 (Table II). Although the preliminary incubation assay indicated that 3 may additionally be a good inhibitor of plasmin, further analysis revealed a relatively fast rate of deacylation of the initially formed acyl enzyme. The second order rate constant of acylation of trypsin and urokinase by the β-substituted lactone 3 was lower than that by the α-substituted lactone 1, but the extremely low k_d values of the corresponding acyl enzymes formed by 3 allowed us to classify it as a very potent alternate substrate inhibitor of trypsin and urokinase. The α-aryl substituted lactone 1, on the other hand, interacted with these enzymes more in the manner of a normal substrate, binding tightly and being hydrolyzed relatively fast.

Iodo Enol Lactones. The incubation assay (Table I) included studies to determine the permanence of inactivation of the enzymes tested by the α- and β-substituted iodo enol lactones 2 and 4. This involved measuring the percent of enzyme activity recovered upon addition of hydrazine (see Table I, D, and Experimental Section and Results). The β-aryl-substituted lactone 4 inactivated >90% of the activity of trypsin, urokinase, and plasmin, but when subjected to the above protocol, 100% of the enzyme activity was recovered (Table I). Lactone 4 was therefore classified as an alternate substrate inhibitor of trypsin, urokinase, and plasmin; it showed the same selectivity pattern as did the corresponding protio enol lactone 3. Although it was not a permanent inactivator as desired, it was pleasing to note its preference in inactivating the trypsin-like serine proteases over α-chymotrypsin and HNE.

Formation of the acyl enzyme from an iodo enol lactone is concomitant with the unmasking of an iodomethyl

ketone group. In the ideal case, a suitably positioned nucleophilic residue in the active site would react with the revealed iodomethyl ketone and become alkylated, leading to permanently inactivated enzyme species. We anticipate that our β-guanidino-substituted lactones are effectively "attached" to the enzyme at two points—the acyl linkage with Ser-195 and the salt bridge formed between the guanidino group and Asp-189. This may restrict the iodomethyl ketone from suitably reorienting itself to get within bonding distance of a nucleophilic residue.

It was observed that the acyl enzymes formed by the iodo lactone 4 with trypsin, urokinase, and plasmin, had faster deacylation rates as compared to the corresponding acyl enzymes formed by the protio enol lactone 3. In cases like this, we have speculated[12b] that hydrolysis of the iodomethyl ketone to the corresponding hydroxymethyl ketone occurs prior to the alkylation event. The hydroxyl group could provide additional modes of acyl enzyme decomposition, which are not available to the methyl ketone derived from the corresponding protio enol lactone.

The α-aryl-substituted iodo enol lactone 2 permanently inactivated some of the enzymes tested, and in some cases, partial recovery of enzyme activity was observed upon addition of hydrazine (Table I). Partitioning of the acyl enzyme (E~I) through deacylation (rate = k_d) and alkylation (rate = k_2) (see eq 2) would explain such an observation. Furthermore, since our inhibitors constitute a mixture of enantiomers, it is possible that one enantiomer alkylates the enzyme (suicide substrate) and the other enantiomer does not (alternate substrate inhibitor). Addition of hydrazine to such an incubation solution could result in partial recovery of enzyme activity. In either case, the percent of enzyme activity recovered would depend on the incubation time prior to addition of hydrazine and the kinetic parameters of the inactivation process.

The α-aryl-substituted iodo enol lactone 2 was a permanent inactivator of urokinase, t-PA, plasmin, and thrombin, as well as α-chymotrypsin (Table I). These preliminary results were disappointing in that 2 did not show the same preference in inactivating the trypsin-like enzymes as did 1, 3, and 4. The binding constant (K_i) and the rate constant of acylation (k_a) was determined in each case. A comparison of the second order rate constant of acylation (k_a/K_i) shows that 2 was 40 and 150 times more specific in inactivating the trypsin-like enzymes, namely urokinase and plasmin, respectively, than α-chymotrypsin (Table III). The inhibition of t-PA by 2 was characterized by tight binding ($K_i = 0.67 \mu$M), although the rate constant

of acylation could not be determined in this case. The inactivation of thrombin and α-chymotrypsin by 2 was not as efficient.

Conclusion

Serine proteases play important roles in numerous physiological processes. In order to be therapeutically useful, an inhibitor should be specific for a targeted enzyme or a group of enzymes, either irreversibly inactivating the enzyme or exhibiting a low rate of deacylation. Since there are numerous enzymes in physiological systems which show trypsin-like specificity, achieving selectivity within this class of enzymes is a difficult task.

In the interaction of an inhibitor with an enzyme, a high specificity is desirable (i.e., tight binding combined with a fast rate of acylation). The α-aryl iodo lactone 2 showed high specificity in inactivating urokinase and plasmin (k_a/K_i values were 117 and 453 min^{-1} μM^{-1}, respectively; cf. Table III). The corresponding protio lactone 1 exhibited an exceptionally high k_a/K_i value of 1185 min^{-1} μM^{-1} (Table II) in its inactivation of urokinase. An amino-substituted isatoic anhydride[7] and guanidino-substituted isocoumarins[9] are the only other examples of mechanism-based inhibitors designed for the trypsin-like enzymes. Among these reported examples, only the isocoumarin inhibitors have been studied extensively enough to make comparisons. Their most potent specificities were in the range of 30 min^{-1} μM^{-1}.[9] In addition, we compared these values with the specificity constants obtained for the inactivation of α-chymotrypsin by the α-substituted lactones Iac and the β-substituted lactones IIac, which ranged from 2.4 to 17.5 min^{-1} μM^{-1}.[12,13] Thus, placing a guanidino group on some α-chymotrypsin inhibitors has provided us with compounds that are not only selective in inhibiting the trypsin-like enzymes, but are more specific in their interaction with the target enzymes (relative to the corresponding α-chymotrypsin inhibitors). It is proposed that the α-chymotrypsin inhibitors interact at the active site through hydrophobic contacts. In the trypsin-like enzymes, these hydrophobic interactions are supplemented with proposed salt-bridge formation between the positively charged guanidino group and the negatively charged aspartate residue.

Our endeavor to prepare compounds which exhibited a higher selectivity in inhibiting trypsin-like serine proteases over α-chymotrypsin and HNE was very successful. Within the trypsin-like enzymes that were tested, good selectivity was observed in the inactivation profiles. The protio enol lactones were designed as potential alternate substrate inhibitors of serine proteases. The β-aryl-substituted protio lactone 3 was a good alternate substrate inhibitor of trypsin and urokinase, its potency being attributed to the slow rate of deacylation (low k_d) of the corresponding acyl enzymes. The selectivity exhibited by these inhibitors within the trypsin-like enzymes was pleasing; the specificity in each case was low, but comparable to other examples in the literature. The α-aryl-substituted protio lactone 1 inhibited urokinase with a relatively much higher specificity, albeit with a faster rate of deacylation. This provides a challenge to design inhibitors which combine a high specificity for target enzymes with a low rate of deacylation. The α-aryl-substituted iodo lactone 2 permanently inactivated urokinase, plasmin, thrombin, and α-chymotrypsin, showing an exceptionally high specificity in inactivating the former

two enzymes. It would be intriguing to study how structural changes in these inhibitors would alter the specificity preference within the trypsin-like enzymes.[24]

Experimental Section

A. Chemical Synthesis. General. Melting points are uncorrected. Reaction progress was monitored by analytical thin-layer chromatography (TLC), and visualization of TLC was done by UV light, iodine vapor, or ninhydrin stain. All reactions using nonaqueous reagents were run under a dry nitrogen atmosphere.

Unless otherwise stated, quenching of the reaction was followed by the following extraction protocol: the aqueous layer was washed three times with the appropriate solvent, the organic extracts were pooled, washed (water, brine), dried (MgSO₄), and concentrated. The crude compound was purified by flash chromatography[25] and recrystallization (when applicable). This workup procedure is presented in the form: extraction (solvent) and purification (eluent in flash chromatography; recrystallizing solvent).

Proton magnetic resonance (¹H NMR) spectra were recorded on a 300-MHz spectrometer and are presented in the form: δ value of signal (peak multiplicity, integrated number of protons, coupling constant). Mass spectral data were obtained by using electron-impact (EI) ionization on a Varian CH-5 spectrometer for low resolution data or a Varian MAT-731 spectrometer for high resolution data (HREI); fast atom bombardment (FAB) spectra were run on a ZAB-SE mass spectrometer employing a dithiothreitol matrix for EI spectra. The reported data is for an electron energy of 70 eV and is presented in the form: m/z (intensity relative to base peak = 100).

Tetrahydrofuran (THF) was distilled from sodium benzophenone ketyl; all other solvents were distilled from CaH₂. Chemicals were obtained from the following sources and were used as received: Fischer, Mallinckrodt, Aldrich, Sigma, Fluka, or Eastman. Allenyltributyltin[18] and 4-bromo-1-butyne[12a] were prepared according to literature procedures.

4-[N-(tert-Butyloxycarbonyl)amino]phenylacetic Acid (6). A solution of 4-aminophenylacetic acid (2 g, 13.24 mmol) in a mixture of dioxane (26 mL), water (26 mL), and sodium carbonate (1.4 g in 13 mL of water) was stirred and cooled in an ice bath. Di-tert-butyl pyrocarbonate (BOC-anhydride, 3.12 g, 14.57 mol) was added in one portion, and stirring was continued at room temperature for 4 h. The dioxane was removed in vacuo and the aqueous layer chilled, covered with a layer of ethyl acetate, and acidified to pH 4 with dilute KHSO₄. This was followed by extraction (ethyl acetate) and purification (1:1:0.1 of ethyl acetate–hexane–acetic acid; ethyl acetate) to yield 2.5 g (76%) of a white solid: mp 153–154 °C; NMR (CDCl₃) δ 7.30 (d, 2, J = 8.5 Hz), 7.19 (d, 2, J = 8.5 Hz), 6.55 (br s, 1), 3.6 (s, 2), 1.5 (s, 9); mass spectrum (EI) m/z 251 (M⁺, 6), 195 (29), 151 (14), 106 (40), 59 (15), 57 (100), 41 (25). Anal. (C₁₃H₁₇NO₄) C, H, N.

2-[4-[(tert-Butyloxycarbonyl)amino]phenyl]-5-hexynoic Acid (7). Diisopropylamine (0.123 mL, 0.88 mmol) was added dropwise to a solution of n-BuLi (0.59 mL, 0.88 mmol) in THF (0.53 mL) at −23 °C. The mixture was stirred at 0 °C for 15 min and then recooled to −23 °C. A solution of the acid 6 (0.1 g, 0.39 mmol) in THF (0.53 mL) was added dropwise to this mixture, and stirring was continued for 1 h. n-BuLi (0.59 mL, 0.88 mmol) was added dropwise to the mixture and allowed to stir for 0.5 h. 4-Bromo-1-butyne[12a] (0.12 g, 0.88 mmol) was added dropwise, stirring was continued at −23 °C for an additional 2 h, and the reaction mixture was allowed to warm to 0 °C. The reaction was quenched with ice and carefully acidified to pH 4 with a dilute solution of KHSO₄. Extraction (ethyl acetate) and purification (1:2:0.1 of ethyl acetate–hexane–acetic acid) yielded 0.065 g (54%, 72% after accounting for recovered starting material) of a white solid: mp 111–113 °C; NMR (CDCl₃) δ 7.31 (d, 2, J = 8.3 Hz), 7.25 (d, 2, J = 8.3 Hz), 6.9 (br s), 3.78 (t, 1, J = 7 Hz), 2.22 (m,

(24) Rai, R.; Katzenellenbogen, J. A. Effect on Conformational Mobility and Hydrogen Bonding Interactions on the Selectivity of Some Guanidino-Aryl Substituted Mechanism-Based Inhibitors of Trypsin-Like Serine Proteases. *J. Med. Chem.*, in press.

(25) Still, W. C.; Kahn, M.; Mitra, A. Rapid Chromatographic Technique for Preparative Separations With Moderate Resolution. *J. Org. Chem.* 1978, 43, 2923–2925.

2), 2.05 (m, 3), 1.51 (s, 9); mass spectrum (EI) m/z 303 (M$^+$, 4), 247 (22), 202 (15), 158 (20), 57 (100). Anal. (C$_{17}$H$_{21}$NO$_4$) C, H, N.

2-(4-Aminophenyl)-5-hexynoic Acid (8). The acetylenic acid 7 (0.11 g, 0.35 mmol) was dissolved in CH$_2$Cl$_2$ (3 mL) at 0 °C. Trifluoroacetic acid (0.75 mL) was added dropwise, and stirring was continued at 0 °C for 2 h. The reaction mixture was allowed to warm up to room temperature and stirred for an additional 1 h. The volatile components were removed in vacuo, and the crude product was purified (ethyl acetate; ethyl acetate) to yield 0.1 g (90%) of a white solid: mp 170 °C dec; NMR (CD$_3$OD) δ 7.14 (d, 2, J = 8.0 Hz), 6.80 (d, 2, J = 8.0 Hz), 3.61 (t, 1, J = 6.8 Hz), 2.1 (m, 5); mass spectrum (EI) m/z 203 (M$^+$, 46), 158 (100), 150 (36), 119 (71), 118 (17), 106 (29), 77 (14). Anal. (C$_{12}$H$_{13}$NO$_2$·CF$_3$COOH) C, H, N.

2-(4-Guanidinophenyl)-5-hexynoic Acid (9). A mixture of 8 (0.82 g, 4.04 mmol), diisopropylethyl amine (3.52 mL, 20.2 mmol), and 3,5-dimethylpyrazole-1-carboxamidinium nitrate (4.06 g, 20.2 mmol) was refluxed in THF (20 mL) for 4 days. The volatile components were removed in vacuo, and the crude mixture was purified by flash chromatography, initially eluting with a 9:1 mixture of chloroform–methanol (this removed side products and any excess reagents and starting material), slowly increasing the proportion of methanol and finally eluting with a 1:1 mixture of chloroform–methanol, to yield 0.65 g (65%) of a white solid which was further purified by recrystallization (methanol): mp 183–185 °C dec; NMR (CD$_3$OD) δ 7.40 (d, 2, J = 8.3 Hz), 7.12 (d, 2, J = 8.3 Hz), 3.61 (t, 1, J = 7.3 Hz), 2.2 (m, 4), 1.89 (m, 1); mass spectrum (EI) m/z 245 (84), 200 (75), 161 (46), 158 (64), 150 (45), 119 (100), 77 (36), 43 (58). Anal. (C$_{13}$H$_{15}$N$_3$O$_2$) exact mass HREI.

3-(4-Guanidinophenyl)-6-methylidenetetrahydro-2-pyranone (1). The acetylenic acid 9 (0.05 g, 0.2 mmol) was dissolved in acetonitrile (10 mL) containing trifluoroacetic acid (5 μL). Mercuric trifluoroacetate (0.008 g, 0.02 mmol) was added, and the mixture was stirred at room temperature for 2 h. The volatile components were removed in vacuo, and the crude product was purified by flash chromatography (5:1 of CHCl$_3$–MeOH) to yield 0.04 g (80%) of a white foam: NMR (CD$_3$CN) δ 9.55 (br s), 7.35 (d, 2, J = 8.4 Hz), 7.23 (d, 2, J = 8.4 Hz), 6.83 (br s), 4.63 (s, 1), 4.39 (s, 1), 3.93 (dd, 1, J = 6.2, 11.3 Hz), 2.68 (m, 2), 2.10 (m, 2); mass spectrum (FAB) m/z 246 (M + 1, 20), 193 (13), 103 (51). Anal. (C$_{13}$H$_{15}$N$_3$O$_2$) exact mass HRFAB.

3-(4-Guanidinophenyl)-6-(E)-(iodomethylidene)tetrahydro-2-pyranone (2). The acetylenic acid 9 (0.0164 g, 0.067 mmol) was dissolved in CH$_3$CN (4 mL) and trifluoroacetic acid (2 μL). To this solution were added KHCO$_3$ (0.007 g, 0.073 mmol), iodine (0.02 g, 0.073 mmol), and water (10 μL), and the resulting mixture was stirred at room temperature for 4 h. The volatile components were removed in vacuo, and purification and isolation was accomplished by flash chromatography. The column was first eluted with chloroform (to remove any excess iodine) and then with a 5:1 CHCl$_3$–2-propanol mixture to yield 0.01 g (45%) of a yellow oil: NMR (CD$_3$OD) δ 7.39 (d, 2, J = 8.2 Hz), 7.28 (d, 2, J = 8.2 Hz), 6.08 (s, 1), 4.00 (t, 1, J = 8.7 Hz), 3.00 (m, 1), 2.80 (m, 1), 2.22 (m, 2); mass spectrum (FAB) m/z 371 (M + H, 4), 279 (26), 157 (12), 149 (23), 103 (62). Anal. (C$_{13}$H$_{14}$N$_3$O$_2$I) exact mass HRFAB.

4-[N-(tert-Butyloxycarbonyl)amino]benzyl Alcohol (11). A solution of 4-aminobenzyl alcohol (5 g, 40.65 mmol) in dioxane (26 mL), water (26 mL), and 1 N NaOH (40 mL) was stirred and cooled in an ice bath. Di-tert-butyl pyrocarbonate (13.3 g, 60.97 mmol) was added in one portion, and the mixture was stirred at room temperature for 6 h. The dioxane was removed in vacuo and extraction (ethyl acetate) and purification (1:1 of ethyl acetate–hexane) afforded 8 g (90%) of a light brown oil: NMR (CDCl$_3$) δ 7.31 (d, 2, J = 8.3 Hz), 7.23 (d, 2, J = 8.3 Hz), 6.72 (br s, 1), 4.57 (s, 2), 2.35 (s, 1), 1.51 (s, 9); mass spectrum (EI) m/z 223 (M$^+$, 5), 123 (15), 122 (11), 106 (10), 94 (9), 57 (100). Anal. (C$_{12}$H$_{17}$NO$_3$) C, H, N.

4-[N-(tert-Butyloxycarbonyl)amino]benzaldehyde (12). Chromium trioxide (0.135 g, 1.35 mmol) was added in portions to pyridine (1.6 mL) with stirring, while the temperature was maintained at 0–10 °C. A yellow suspension resulted when the addition was complete. A solution of the alcohol 11 (0.1 g, 0.45 mmol) in pyridine (1.5 mL) was added dropwise, and the mixture

was stirred at room temperature for 0.5 h. The reaction mixture was poured into water, extracted (ethyl acetate), and purified (2:1, ethyl acetate–hexane; ethyl acetate, hexane) to yield 0.085 g (85%) of a white solid: mp 138 °C; NMR (CDCl$_3$) δ 9.89 (s, 1), 7.83 (d, 2, J = 8.5 Hz), 7.54 (d, 2, J = 8.5 Hz), 1.54 (s, 9); mass spectrum (EI) m/z 221 (M$^+$, 5), 165 (18), 121 (16), 59 (12), 57 (100), 41 (27). Anal. (C$_{12}$H$_{15}$NO$_3$) C, H, N.

Ethyl 3-[4-[N-(tert-Butyloxycarbonyl)amino]phenyl]-propenoate (13). Triethyl phosphonoacetate (0.64 g, 2.85 mmol) was added dropwise to a suspension of sodium hydride (0.1 g, 3.7 mmol) in THF (8 mL) at 0 °C, and stirring was continued for 1.5 h. A solution of the aldehyde 12 (0.42 g, 1.9 mmol) in THF (6 mL) was added and the reaction mixture stirred at room temperature for 1 h. The reaction was quenched with ice, and the chilled aqueous layer was acidified to pH 3 with diluted aqueous KHSO$_4$. Extraction (ethyl acetate) and purification (1:3 of ethyl acetate–hexane; ethyl acetate, hexane) yielded 0.47 g (85%) of a white solid: mp 92–94 °C; NMR (CDCl$_3$) δ 7.62 (d, 1, J = 16.0 Hz), 7.47 (br s, 4), 6.35 (d, 2, J = 16.0 Hz), 4.24 (q, 2, J = 7.0 Hz), 1.53 (s, 9), 1.34 (t, 3, J = 7.0 Hz); mass spectrum (EI) m/z 291 (M$^+$, 7), 235 (40), 191 (13), 190 (11), 146 (10), 119 (12), 57 (100), 41 (32). Anal. (C$_{16}$H$_{21}$NO$_4$) C, H, N.

Ethyl 3-[4-[N-(tert-Butyloxycarbonyl)amino]phenyl]-5-hexenoate (14). Diisopropyl sulfide (0.67 g, 5.74 mmol) was added to CuI (0.26 g, 1.38 mmol) in a 25-mL round-bottom flask under nitrogen, and the mixture was stirred at room temperature until the solid dissolved. Ether (10 mL) was added, and the solution was cooled to –78 °C. Allyl magnesium chloride (1.87 mL, 2.53 mmol) was added dropwise, and stirring was continued for 1 h. Subsequently, addition of TMSCl (0.25 g, 2.30 mmol) was followed by addition of an ether (5 mL) solution of the α,β-unsaturated ester 13 (0.33 g, 1.15 mmol). The reaction mixture was stirred at –78 °C for 1 h and then allowed to warm to 0 °C. The reaction was quenched with dilute KHSO$_4$. Extraction (ether) and purification (1:4 of ethyl acetate–hexane) afforded 0.23 g (62%; 75% if left over starting material is accounted for) of an oil: NMR (CDCl$_3$) δ 7.28 (d, 2, J = 8.6 Hz), 7.12 (d, 2, J = 8.6 Hz), 6.52 (br s, 1), 5.65 (m, 7), 4.95 (m, 2), 4.02 (q, 2, J = 7.0 Hz), 3.17 (m, 1), 2.65 (dd, 1, J = 6.7, 15.2 Hz), 2.58 (dd, 1, J = 8.6, 15.2 Hz), 2.32 (t, 2, J = 6.9 Hz), 1.51 (s, 9), 1.15 (t, 3, J = 7.0 Hz); mass spectrum (EI) m/z 333 (M$^+$, 4.40), 236 (52), 194 (19), 192 (33), 158 (14), 119 (15), 57 (100), 41 (30). Anal. (C$_{19}$H$_{22}$NO$_4$) C, H, N.

Ethyl 3-[4-[N-(tert-Butyloxycarbonyl)amino]phenyl]-5,6-dibromohexanoate (15). Bromine (0.17 g, 1.05 mmol) was added dropwise to a solution of the alkene 14 (0.176 g, 0.52 mmol) in ether (15 mL) at 0 °C, and the reaction mixture was stirred at that temperature for 1 h. The reaction was quenched with aqueous sodium bisulfite, extracted (ether), and purified (4:1 of hexane–ethyl acetate) to yield 0.13 g (50%) of an oil as a mixture of diastereomers: NMR (CDCl$_3$) δ 7.34 (m, 2), 7.18 (m, 2), 6.48 (br s, 1), 4.06 (m, 2), 3.34–3.84 (m, 4), 2.42–2.63 (m, 3), 1.9–2.2 (m, 1), 1.55 (s, 9), 1.15 (m, 3); mass spectrum (EI) m/z 495 (M + 4, 0.82), 493 (M + 2, 1), 491 (M$^+$, 0.68), 437 (10), 235 (13), 192 (26), 119 (14), 57 (100), 41 (26). Anal. (C$_{19}$H$_{27}$NO$_4$Br$_2$) exact mass HREI.

3-[4-[N-(tert-Butyloxycarbonyl)amino]phenyl]-5-hexynoic Acid (16). The dibromoester 15 (0.32 g, 0.65 mmol) was dissolved in EtOH, and KOH (20% aqueous, 2 mL) was added. The mixture was stirred at room temperature for 1 h (until complete disappearance of starting material). The ethanol was removed in vacuo, and the aqueous layer was chilled and acidified to pH 3 with aqueous KHSO$_4$ and extracted (ethyl acetate). An ether solution of the crude acid was added to sodium amide (3.25 mmol) in liquid ammonia (15 mL). After stirring the reaction mixture for 1 h, the ammonia was blown off, and the slurry was carefully quenched with ice–water. The aqueous layer was acidified to pH 3 with KHSO$_4$, extracted (ethyl acetate), and purified (1:2:0.03 of ethyl acetate–hexane–AcOH; ethyl acetate, hexane) to yield 0.13 g (67%) of a white solid: mp 103–105 °C; NMR (CDCl$_3$) δ 7.30 (d, 2, J = 8.5 Hz), 7.19 (d, 2, J = 8.52 Hz), 3.23 (m, 1), 2.85 (dd, 1, J = 6.4, 16.0 Hz), 2.6 (dd, 1, J = 8.7, 16.0 Hz), 2.5 (d, 1, J = 2.6 Hz), 2.48 (d, 1, J = 2.5 Hz), 2.25 (t, 1, J = 2.5 Hz), 1.5 (s, 9); mass spectrum (EI) m/z 303 (M$^+$, 3), 2.09 (12), 108 (24), 165 (10), 164 (21), 57 (100), 40 (23). Anal. (C$_{17}$H$_{21}$NO$_4$) C, H, N.

The acetylenic ester **22** (0.35 g, 1.06 mmol) was dissolved in EtOH (10 mL), and 20% aqueous KOH (1.5 mL) was added dropwise. The reaction mixture stirred at room temperature for 2 h. The ethanol was removed in vacuo, and the aqueous layer was chilled and acidified to pH 3 with aqueous KHSO₄. Extraction, purification, and characterization was done as described above.

3-(4-Aminophenyl)-5-hexynoic Acid (17). The BOC-protected amino acid **16** (0.322 g, 1.06 mmol) was dissolved in CH₂Cl₂ (5 mL) and cooled to 0 °C. Trifluoroacetic acid (1.25 mL) was added dropwise. The reaction mixture was allowed to warm up to room temperature and stirred for an additional 2 h. The volatile components were removed in vacuo, and crude solid thus obtained was recrystallized (ether, hexane) to yield 0.29 g (86%) of a white solid: mp 126–128 °C; NMR (MeOH-d_4) δ 7.34 (d, 2, J = 8.4 Hz), 7.13 (d, 2, J = 8.4 Hz), 3.3 (m, overlapping with MeOH-d_4), 2.87 (dd, 1, J = 6.2, 16.0 Hz), 1.64 (dd, 1, J = 8.9, 16.0 Hz), 2.53 (d, 1, J = 2.4 Hz), 2.5 (d, 1, J = 2.5 Hz), 2.27 (t, 1, J = 2.5 Hz); mass spectrum (FABMS) 204 (M + H, 49), 157 (23), 137 (41), 103 (100), 102 (30). Anal. ($C_{12}H_{13}NO_2$) exact mass HRFAB.

3-(4-Guanidinophenyl)-5-hexynoic Acid (18). A mixture of **17** (1.61 g, 7.9 mmol), diisopropylethylamine (7 mL, 39.6 mmol), and 3,5-dimethylpyrazole-1-carboxamidinium nitrate (7.97 g, 39.6 mmol) was refluxed in THF (30 mL) for 5 days. The solvent was removed in vacuo, and the crude mixture was purified by flash chromatography, initially eluting with pure chloroform and then with a 9:1 mixture of chloroform–methanol (this removed side products and any excess reagents or remaining starting material) and finally eluting with a 1:1 mixture of chloroform–methanol to yield 0.56 g (29%) of a light brown foam: NMR (MeOH-d_4) δ 7.36 (d, 2, J = 8.3 Hz), 7.12 (d, 2, J = 8.3 Hz), 3.3 (m, overlapping with MeOH-d_4), 2.69 (dd, 1, J = 6.1, 14.3 Hz), 2.5 (m, 3), 2.19 (t, 1, J = 2.4 Hz); mass spectrum (FABMS) 246 (M + H, 50), 154 (48), 153 (52), 137 (29), 103 (100). Anal. ($C_{13}H_{16}O_2N_3$) exact mass HRFAB.

4-(4-Guanidinophenyl)-6-methylidenetetrahydro-2-pyranone (3). The acetylenic acid **18** (0.0424 g, 0.173 mmol) was dissolved in acetonitrile (10 mL) and trifluoroacetic acid (5 μL). Mercuric trifluoroacetate (0.007 g, 0.017 mmol) was added, and the mixture was stirred at 25 °C for 2 h. The volatile components were removed in vacuo and the crude product was purified by flash chromatography (4:1, chloroform-2-propanol) to yield 0.04 g (94%) of a foam: NMR (CD₃CN) δ 9.88 (br s), 7.35 (d, 2, J = 8.3 Hz), 7.23 (d, 2, J = 8.3 Hz), 6.98 (br s); 4.63 (s, 1), 4.37 (s, 1), 3.28 (m, 1), 2.78 (m, 4); mass spectrum (FABMS) 264 (M⁺·H₂O + 1, 100), 246 (M⁺, 2), 193 (10), 162 (13). Anal. ($C_{13}H_{15}N_3O_2$·H₂O) exact mass HRFAB.

4-(4-Guanidinophenyl)-6-(E)-(iodomethylidene)tetrahydro-2-pyranone (4). The acetylenic acid **18** (0.018 g, 0.07 mmol) was dissolved in acetonitrile (5 mL) and trifluoroacetic acid (5 μL). KHCO₃ (0.009 g, 0.09 mmol), H₂O (10 μL), and I₂ (0.023 g, 0.09 mmol) were added sequentially, and the reaction mixture was stirred at room temperature for 4 h. The volatile components were removed in vacuo, and the crude product was purified by flash chromatography, initially eluting with CHCl₃ (this removed excess I₂) and then eluting with a 4:1 mixture of CHCl₃–2-propanol to afford 0.001 g (38%) of a brown-yellow foam: NMR (CD₃CN) δ 9.01 (br s, 1), 7.39 (d, 2, J = 8.0 Hz), 7.29 (d, 2, J = 8 Hz), 6.62 (br s, 3), 6.03 (s, 1), 3.35 (m, 1), 3.11 (m, 1), 2.70 (m, 3); mass spectrum (FABMS) m/z 372 (M + 1, 13), 177 (11), 157 (23), 149 (29), 105 (23), 103 (100). Anal. ($C_{13}H_{14}N_3O_2I$) exact mass HRFAB.

4-[4-[N-($tert$-Butyloxycarbonyl)amino]phenyl]-4-hydroxy-1-butyne (19). Boron trifluoride etherate (2.6 mL, 21.16 mmol) was added dropwise to a solution of the aldehyde **12** (2.13 g, 9.62 mmol) in CH₂Cl₂ (61 mL), and the resulting mixture was stirred for 15 min. A solution of allenyltributyltin[18] (4.74 g, 14.43 mmol) in CH₂Cl₂ (31 mL) was added dropwise, and stirring was continued for 4.5 h. The reaction was quenched with water and extracted three times with ethyl acetate. The organic extracts were combined and concentrated. Saturated KF solution (100 mL) and ether (100 mL) were added and stirred at room temperature for 1 h. The precipitates were removed by filtration, and extraction (ethyl acetate) and purification (3:1 of hexane–ethyl acetate) yielded 2.13 g (85%) of an oil: NMR (CDCl₃) δ 7.32 (AB quartet $\Delta\nu$ = 0.0387 ppm, J = 8.7 Hz), 6.60 (br s, 1), 4.82 (m, 1),

2.6 (dd, 2, J = 2.6, 6.4 Hz), 2.50 (d, 1, J = 3.41 Hz), 2.06 (t, 1, J = 2.6 Hz), 1.51 (s, 9); mass spectrum (EI) m/z 261 (M⁺, 2), 166 (67), 122 (39), 57 (100), 41 (23). Anal. ($C_{15}H_{19}NO_3$) exact mass HREI.

Diethyl 2-[1-[4-[N-($tert$-Butyloxycarbonyl)amino]phenyl]-3-butynyl]malonate (21). Diethyl malonate (2.9 mL, 19.1 mmol) was added to a suspension of NaH (1.15 g, 28.6 mmol) in THF (57 mL) at 25 °C. After stirring the anion for 20 min at 25 °C, the chloride **20** [2.6 g, 9.54 mmol; prepared from 1-[4-[(tert-butyloxycarbonyl)amino]phenyl]-3-butyn-1-ol (2.5 g, 9.72 mmol) by treatment in CH₂Cl₂ (134 mL) with triethylamine (2 mL, 14.6 mmol) and methanesulfonyl chloride (1.13 mL, 14.6 mmol) for 1 h at 0 °C followed by quenching with ice–water and isolation by extraction with CH₂Cl₂: NMR (CDCl₃) δ 7.36 (br s, 4), 6.51 (br s, 1), 4.98 (t, 1, J = 7.17 Hz), 2.96 (m, 2), 2.04 (t, 1 J = 2.3 Hz), 1.52 (s, 9)] was added as a THF (20 mL) solution. The reaction mixture was stirred at room temperature for 12 h and subsequently quenched with water, extracted (ethyl acetate), and purified (5.5:1 of hexane–ethyl acetate; ether, hexane) to yield 2.18 g (57%) of a white solid: mp 101 °C; NMR (CDCl₃) δ 7.29 (d, 2, J = 8.5 Hz), 7.23 (d, 2, J = 8.5 Hz), 6.47 (br s, 1), 4.23 (q, 2, J = 7.1 Hz), 3.95 (q, 2, J = 6.4 Hz), 3.88 (d, 1, J = 10.8 Hz), 3.56 (m, 1), 2.65 (m, 2), 1.96 (t, 1, J = 2.0 Hz), 1.51 (s, 9), 1.28 (t, 3, J = 7.1 Hz), 1.01 (t, 3, J = 7.1 Hz); mass spectrum (EI) m/z 403 (M⁺, 3), 308 (18), 264 (17), 187 (20), 146 (18), 57 (100), 41 (24). Anal. ($C_{22}H_{29}NO_6$) C, H, N.

Ethyl 3-[4-[($tert$-Butyloxycarbonyl)amino]phenyl]-5-hexynoate (22). The diester **21** (0.98 g, 2.44 mmol) was dissolved in ethanol (25 mL) and 30% aqueous KOH (10 mL) was added dropwise. The resulting mixture was stirred at room temperature for 2 h. Ethanol was removed in vacuo, and the aqueous layer was extracted (ethyl acetate). The half-ester thus obtained was taken up in m-xylene (18 g) and refluxed for 6 h. The m-xylene was removed, and the resulting concentrate was taken up in ethyl acetate and extracted (ethyl acetate).

The crude product was purified (5:1 of hexane–ethyl acetate; ether, hexane) to yield 0.45 g (75%) of a white solid: mp 78 °C; NMR (CDCl₃) δ 7.29 (d, 2, J = 8.5 Hz), 7.17 (d, 2, J = 8.5 Hz), 6.51 (br s, 1), 4.05 (q, 2, J = 7.2 Hz), 3.33 (m, 1), 2.88 (dd, 1, J = 6.6, 15.7 Hz), 2.63 (dd, 2, J = 8.4, 15.7 Hz), 2.50 (m, 2), 1.98 (t, 1, J = 2.6 Hz), 1.51 (s, 9), 1.16 (t, 3, J = 7.2 Hz); mass spectrum (EI) m/z 331 (M⁺, 44), 275 (71), 236 (100), 192 (58), 57 (44). Anal. ($C_{19}H_{25}NO_4$) C, H, N.

B. Biochemical Procedures. General. Kinetic assays were performed at 25 °C with a Hewlett-Packard 8451A diode array spectrophotometer. α-Chymotrypsin was obtained from Worthington Biochemical. HNE, bovine trypsin, and human kidney urokinase were purchased from Sigma. Human plasmin, t-PA (human, single chain), and human thrombin were purchased from Calbiochem. N-Methoxysuccinyl-L-alanyl-L-alanyl-L-prolyl-L-valine-p-nitroanilide (MeO-Suc-Ala-Ala-Pro-Val-pNA) and N-succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine-p-nitroanilide (Suc-Ala-Ala-Pro-Phe-pNA) were obtained from Sigma. (Carbobenzyloxy)-L-valyl-glycyl-L-arginine-p-nitroanilide (Z-Val-Gly-Arg-pNA), benzoyl-β-alanyl-glycyl-L-arginine-p-nitroaniide (Bz-(β)-Ala-Gly-Arg-pNA), tosyl-glycyl-L-prolyl-L-arginine-p-nitroanilide (Ts-Gly-Pro-Arg-pNA) and tosyl-glycyl-L-prolyl-L-lysine-p-nitroanilide (Ts-Gly-Pro-Lys-pNA) were obtained from Boehringer Mannheim. (Methylsulfonyl)-D-cyclohexyltyrosyl-glycyl-arginine-p-nitroanilide acetate (Spectrozyme t-PA) was obtained from American Diagnostica Inc. Dimethyl sulfoxide (DMSO) was obtained from Fisher Scientific.

Incubation Assay Method A. No Deacylation Delay. Enzyme inactivation was initiated by the addition of a DMSO stock solution of the lactone to a buffered enzyme solution (final concentration of DMSO = 10%). The inhibitor and enzyme concentrations are shown in Table I. Aliquots were withdrawn at various time intervals over the course of 2 h and diluted into a cuvette containing a buffered solution of the appropriate substrate. Trypsin was assayed with Z-Val-Gly-Arg-pNA (155 μM, assay buffer was 0.2 M phosphate, pH 7.2). α-Chymotrypsin was assayed with Suc-Ala-Ala-Pro-Phe-pNA 315 μM, assay buffer was 0.2 M phosphate, pH 7.2). Urokinase was assayed with Bz-(β)-Ala-Gly-Arg-pNA (171 μM, assay buffer was 0.05 M phosphate, 0.1 M NaCl, pH 8.0). t-PA was assayed with spectrozyme t-PA (0.2 mM, assay buffer was Tris-imidazole, I 0.3, pH 8.4).

Plasmin was assayed with Ts-Gly-Pro-Lys-pNA (157.4 μM, assay buffer was 0.05 M phosphate, pH 7.8). Thrombin was assayed with Ts-Gly-Pro-Arg-pNA (75 μM, assay buffer was 0.05 M phosphate, 0.01 M EDTA, 10 g/L polyethylene glycol (8000), 10 mg/L aprotinin, pH 8.0). HNE was assayed with MeO-Suc-Ala-Ala-Pro-Val-pNA (150 μM, assay buffer was 0.1 M phosphate, 0.5 M NaCl, pH 7.4). Measurement of substrate hydrolysis at 410 nm (for α-chymotrypsin and HNE), 404 nm (trypsin, urokinase, plasmin), 405 nm (t-PA) or 408 nm (thrombin) proceeded immediately and was used to determine the remaining enzyme activity relative to a control containing enzyme but no lactone.

Incubation Assay Method B. Deacylation Delay. In this case, aliquots from the "inactivated" enzyme solution were diluted into a cuvette containing buffer, but no substrate. Subsequent to a 10-min delay, substrate was added, and the rate of substrate hydrolysis was measured as before. The 10-min incubation following dilution ensured the decomposition of any unstable acyl enzyme species.

Hydrazine Reactivation Study. Those iodo enol lactones, which did not show any recovery of enzyme activity relative to control at the end of 2 h, were treated with 100 mM hydrazine, and enzyme activity was monitored for an additional 2 h. The percent of enzyme activity regained is reported in Table I.

Competitive Substrate Assay ($K_{s/i}$, k_a). Method A: The buffers and substrates used in this assay were the same as those in the incubation assays described above. The appropriate amount of substrate and lactone (in DMSO to give a final concentration of 10% v/v) were combined with buffer in a 1.5-mL cuvette. The buffered enzyme was added to this cuvette, and the change in absorbance at the appropriate wavelength (same as in the incubation assay, above) was recorded over a time interval, depending upon how long it took to reach the steady-state level. After being corrected for the limiting slope due to turnover (caused by deacylation), the semilogarithmic plot of this corrected absorbance change against time gave a straight line with a slope of k_{obs}. The initial inhibitor concentration (I_0) was plotted against I_0/k_{obs} to obtain a straight line. K_m (for each substrate and enzyme used) was determined in a separate experiment by standard methods. $K_{i/s}$ and k_a were determined from eq 6 as described in Results.

The absolute standard deviation of k_a and $K_{s/i}$ were determined as described by Baek et al.[13] and are reported alongside the binding constant and acylation rate constant. In some rare cases, when the binding constant was low, and/or both the x- and y-intercept were small, the absolute standard deviation of $K_{s/i}$ was slightly higher than the value itself. In these cases, only the upper limit is reported.

Method B: The buffers and substrates used in this assay were the same as those used in the incubation assay. Into a 1.5-mL cuvette were combined the appropriate amount of substrate and lactone (in DMSO, to give a final concentration of 10% v/v) with buffer. The resulting solution was referenced at the appropriate wavelength and finally, the enzyme was added to the above solution and the initial change in absorbance (over 15 s) gave the velocity. At different inhibitor concentrations, a $K_m{}^{app}$ value was obtained from a reciprocal plot ($1/v$ versus $1/S$). A replot of $K_m{}^{app}$ versus inhibitor concentration allowed the determination of K_i from the x-intercept.

Determination of the Rate Constant of Deacylation (k_d). The enzyme was incubated with an excess of lactone (I_0/E_0 was the same as in the incubation assay) for 30–45 min. Subsequently, excess inhibitor was removed from the solution by centrifugation twice at 0 °C for 1 h using Amicon Centricon-10 microconcentrators. The remaining enzyme activity was monitored at certain time intervals and compared to a control which was subjected to the same treatment, except devoid of inhibitor. The final concentration of enzyme and substrate and the method of analysis was the same as that described in the incubation assays above. The k_d was obtained from the slope of a plot of the natural log of the remaining enzyme activity [ln ($v_0 - v_t$)] vs time.