Effect of Conformational Mobility and Hydrogen-Bonding Interactions on the Selectivity of Some Guanidinoaryl-Substituted 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

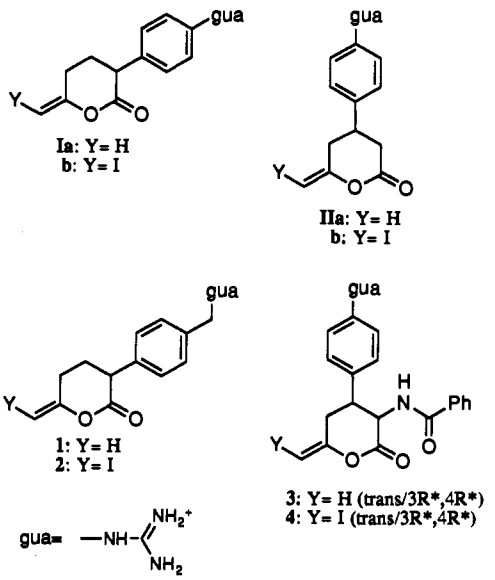
Received June 8, 1992

Previously, we had reported that some guanidino-substituted α- and β-aryl enol lactones **I** and **II** behaved as selective, mechanism-based inhibitors of some trypsin-like proteases (Rai, R.; Katzenellenbogen, J. A. *J. Med. Chem.*, submitted). In this study, we describe the synthesis and kinetic evaluation of some related, guanidino-substituted enol lactones having greater conformational mobility and affording additional hydrogen-bonding sites at the active site. The α-aryl-substituted lactones **1** and **2**, which have greater conformational mobility in the guanidinoaryl linkage than **I**, selectively inhibited the trypsin-like enzymes, and they were relatively poor inactivators of α-chymotrypsin and human neutrophil elastase (HNE). The iodo enol lactone **2** permanently inactivated trypsin, urokinase, tissue plasminogen activator, and plasmin, showing exceptionally high specificity in its interaction with trypsin and urokinase. The selectivity pattern exhibited by the closely related, conformationally less mobile α-aryl-substituted iodo lactone **Ib**, which was previously shown to be a selective suicide substrate of urokinase and plasmin, provides an interesting comparison. The α-benzamido-substituted lactones **3** and **4**, which afford an additional site for active-site hydrogen bonding, were found to be very potent alternate substrate inhibitors of trypsin and urokinase. In addition, the iodo lactone **4** permanently inactivated α-chymotrypsin. The importance of secondary interactions in increasing the specificities in the case of α-chymotrypsin is discussed.

Introduction

Enol lactones have been shown to act as mechanism-based inhibitors of α-chymotrypsin and human neutrophil elastase (HNE).[1] This class of inhibitors acts by initially acylating the active-site serine residue. Concomitant with the formation of the acyl enzyme is the release of a methyl ketone (from protio enol lactones) or an iodo methyl ketone group (from iodo enol lactones). Thus, protio enol lactones may act as alternate substrate inhibitors, that is transient inactivators which form very stable acyl enzymes. In the case of iodo enol lactones, the revealed iodomethyl ketone group may alkylate a suitably positioned nucleophilic residue in the active site. Therefore, these are potential suicide substrates which become permanently tethered at the enzyme active site.[2]

Recently, we have reported that α- and β-(4-guanidinophenyl)-substituted valero enol lactones (**I** and **II**) behave as effective, mechanism-based inhibitors of trypsin-like serine proteases.[3] These guanidino-substituted inhibitors exhibited much greater specificity in their interaction with trypsin-like enzymes, as compared to α-chymotrypsin and HNE. In particular, the α-aryl substituted iodo enol lactone (**Ib**) was a suicide substrate of urokinase, plasmin, tissue plasminogen activator (t-PA), thrombin, and α-chymotrypsin, with an exceptionally high specificity for the former two enzymes (k_a/K_i = 117 and 453 min^{-1} μM^{-1} for urokinase and plasmin, respectively).



Since these enol lactone inhibitors are small and nonpeptidic, they are large enough only to interact at the active site of the enzyme. Thus, the differences in specificity observed in the inhibition of these enzymes must be due to some very subtle differences in the active site of these enzymes. We thought that the major driving force in the interaction of the guanidino-substituted

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

(1) (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) Baek, C-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. (c) Reed, P. E.; Katzenellenbogen, J. A. Proline-Valine Pseudo Peptide Enol Lactones. Effective and Selective Inhibitors of Chymotrypsin and Human Leucocyte Elastase. *J. Biol. Chem.* **1991**, *266*, 13–21.

(2) Rando, R. R. Chemistry and Enzymology of k_{cat} Inhibitors. *Science* **1974**, *185*, 320–324.

(3) Rai, R.; Katzenellenbogen, J. A. Guanidinophenyl-Substituted Enol Lactones as Selective Mechanism-Based Inhibitors of Trypsin-Like Enzymes. *J. Med. Chem.* **1992**, in press.

0022-2623/92/1835-4297$03.00/0

lactones with trypsin-like enzymes would be the salt bridge formation between the positively charged guanidino group on the lactone with the aspartate residue at the bottom of the specificity pocket. Attaching the guanidino group to the phenyl ring through a methylene linker, as in the α-aryl substituted lactones **1** and **2**, would provide flexibility and greater conformational mobility of the inhibitor in the active site. We hoped, thereby, to see a different specificity pattern for inhibition within the trypsin-like enzymes.

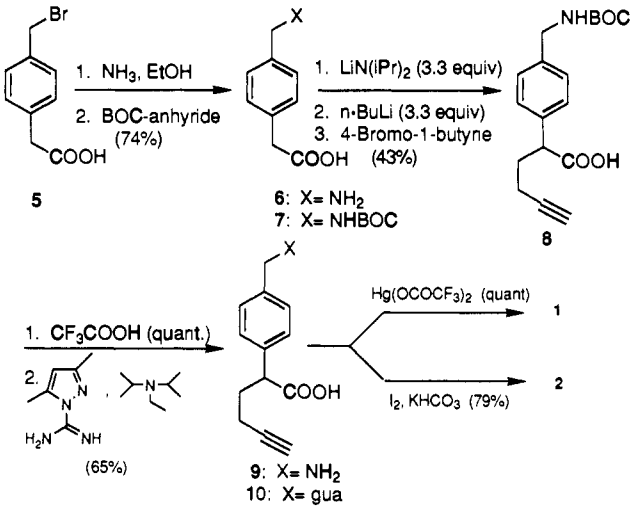
The β-aryl-substituted lactones (**IIab**), reported earlier,[3] were transient, alternate substrate inhibitors, selective for the trypsin-like enzymes, namely, trypsin, urokinase, and plasmin. The potency of these inhibitors (**II**) was ascribed to formation of stable acyl enzymes (low k_d values), and the specificity constant (k_a/K_i) measured in each case was modest. In incubation assays, the inhibition levels of α-chymotrypsin and HNE by **IIab** were low and transient. The iodo enol lactone (**IIb**) did not act as a suicide substrate for any of the enzymes tested. The acyl enzymes were relatively unstable, exhibiting faster rates of deacylation, as compared to the corresponding protio lactone (**IIa**). To extend these studies, we undertook the synthesis and kinetic evaluation of α-benzamido-β-aryl enol lactones **3** and **4**, since such systems would mimic in greater detail the structure of α-amino acids. It was hoped that the α-acylamino substituent in these lactones would provide added hydrogen-bonding interactions between the inhibitor and the peptide backbone of the enzyme at the active site. We wished to study the effect of such a substitution on the inactivation profiles of the enzymes.

In this report, we describe the synthesis of the conformationally mobile α-aryl-substituted lactones **1** and **2**, as well as the α-benzamido-β-aryl-substituted lactones **3** and **4**. These lactones have been tested for inhibitory activity with some trypsin-like enzymes, namely, trypsin, urokinase, t-PA, plasmin, and thrombin, as well as α-chymotrypsin and HNE. The α-aryl iodo lactone **2** is a permanent inactivator of all the trypsin-like enzymes that were tested, with an exceptionally high specificity for trypsin and urokinase. The α-benzamido-substituted lactones **3** and **4** were transient inhibitors of some of the trypsin-like enzymes tested. The iodo lactone **4** permanently inactivated α-chymotrypsin. More importantly, it formed very stable acyl enzymes with trypsin and urokinase. The inactivation profiles of lactones **1–4** are discussed and compared with the profiles obtained for the related lactones **I** and **II**, reported earlier.

Results and Discussion

Synthesis. α-Aryl-Substituted Lactones 1 and 2. The protio and iodo enol lactones **1** and **2**, bearing an α-[*p*-(guanidinomethyl)phenyl] substituent, were synthesized using the bromo acid **5** as starting material (Scheme I). Treatment of **5** with excess ammonia yielded the amino acid **6** as a mixture with ammonium bromide, after evaporation of solvent. The product (**6**) could not be purified from the side product (ammonium bromide) due to the similarity in their solubility properties. Instead, treatment of this crude mixture with aqueous NaOH (to remove excess ammonia), followed by *tert*-butyl pyrocarbonate, afforded the *tert*-butyloxycarbonyl (BOC) derivative of the amino acid **7**. Alkylation of this protected amino acid **7** with 4-bromo-1-butyne,[1a] using 3.3 equiv of LDA, proceeded in low yield, with most of the starting material being recovered. As explained earlier,[3,4] the yield improved somewhat when 3.3 equiv of *n*-BuLi was added prior to addition of the electrophile.

Scheme I



The acetylenic acid **8** was deprotected and the guanidino group was introduced by refluxing the amino acid **9** with 3,5-dimethylpyrazole-1-carboxamidine nitrate in the presence of diisopropylethylamine. Purification of the guanidino acid **10** was achieved by flash chromatography using a polar solvent system. The modest yields obtained are a reflection of the difficulty in isolation and purification of the product, and not of the reaction itself.

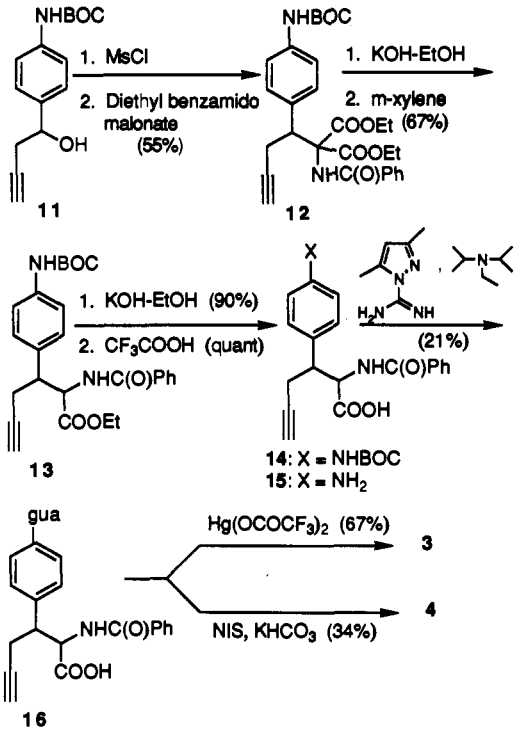
The guanidino acid **10** was insoluble in most organic solvents like acetonitrile, methylene chloride, and even methanol. Cyclization to either lactone **1** or **2** was preceded by solubilization of the guanidino acid **10** in acetonitrile, containing a trace of trifluoroacetic acid. Cyclization to the protio enol lactone **1** proceeded smoothly in presence of mercuric trifluoroacetate.[5] Iodolactonization using $KHCO_3$ and iodine also went in high yield.[5]

β-Aryl-Substituted Lactones 3 and 4. The β-aryl-substituted lactones **3** and **4** were both synthesized from the guanidino acid intermediate **16**, as outlined in Scheme II. The BOC-protected acetylenic alcohol **11**, prepared as described earlier,[3] was used as starting material. This acetylenic alcohol (**11**) was treated with methanesulfonyl chloride to afford an unstable acetylenic chloride. This chloride was used immediately, without purification, to alkylate diethyl benzamidomalonate. The relatively low yield of the product acetylenic diester **12** in the reaction is attributed to the instability of the homopropargylic chloride intermediate, which undergoes facile elmination to the eneyne during chromatography or even upon standing at −5 °C. Saponification of **12** to the corresponding half-ester was followed by decarboxylation in refluxing xylene, to afford the acetylenic ester **13** as a mixture of diastereomers. Separation of the diastereomers could be achieved at this stage by a combination of flash chromatography and recrystallization. However, we continued in the synthesis with the mixture of diastereomers, since some of the subsequent steps resulted in epimer-

(4) Seebach, D. Structure and Reactivity of lithium Enolates. From Pinacolone to Selective C-Alkylation of Peptides. Difficulties and Opportunities Afforded by Complex Structures. *Angew. Chem. Int. Ed. Engl.* **1988**, *27*, 1624–1654.

(5) Krafft, G. A.; Katzenellenbogen, J. A. Synthesis of Halo Enol Lactones. Mechanism-Based Inactivators of Serine Proteases. *J. Am. Chem. Soc.* **1981**, *103*, 5459–5466.

Scheme II



ization. Hydrolysis of the ester **13** was followed by deprotection of the BOC derivative to afford the amino acid **15** in high yield. The guanidino group was introduced by allowing the amino acid **15** to react with 3,5-dimethylpyrazole-1-carboxamidine nitrate in presence of diisopropylethylamine. The reaction was sluggish and did not go to completion, even upon refluxing for 2 days. The amino group in **15** is aromatic and is therefore less nucleophilic. This lack of reactivity is in contrast to the reaction of the benzylic amino acid **9** (which went to completion in 12 h to afford the corresponding guanidino acid **10**). Isolation and purification of the guanidino acid **16** followed a procedure similar to that described for the α-aryl-substituted guanidino acid **10**.

Separation of diastereomers of the guanidino acid **16** was accomplished by reverse-phase HPLC (70:30:0.05 of water/methanol/trifluoroacetic acid). Cyclization to either the protio or the iodo enol lactone was preceded by solubilization of the guanidino acid **16** in acetonitrile, containing a trace of trifluoroacetic acid. A catalytic quantity of mercuric trifluoroacetate was used for protio lactonization.[5] Cyclization to the iodo enol lactone **4** was done using *N*-iodosuccinamide and $KHCO_3$.[5] It turned out that, in both protio and iodolactonization reactions, only one diastereomer of the products **3** and **4** could be isolated. Reaction of the second diastereomer gave decomposition products in each case. The guanidino acid **16** was therefore used as a mixture of diastereomers for the final reaction and a single diastereomer of product (**3** and **4**) was isolated.

Assignment of relative stereochemistry of compounds **3** and **4** was done by an analysis of ^1H NMR coupling constants. The protio enol lactone **3** had a C_3–C_4 ^1H NMR coupling constant of 11.9 Hz. The corresponding coupling constant for the iodo lactone **4** was 12.2 Hz. Therefore, the lactone substituents were assigned as having a trans relationship (i.e., 3*R**,4*R**) in both lactones **3** and **4**. This stereochemical assignment correlates well with similar assignments made for 3-(acrylamido)-4-pheny-6(*E*)-(iodomethylidene)tetrahydro-2-pyranones by Sofia and Katzenellenbogen.[6]

Biochemical Studies. Kinetic Models and Assay Methods. Equation 1 is the kinetic model describing the inactivation process of serine protease by a protio enol lactone. Since protio enol lactones are inhibitors of the

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E{\cdot}I \xrightarrow{k_a} E{\sim}I \xrightarrow{k_d} E + I' \qquad (1)$$

alternate substrate type, this is simply the kinetic model for serine protease substrates. 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 decylation rate constant, k_d.

Haloenol lactones can act as irreversible inhibitors of serine proteases.[2] Equation 2 describes the kinetic scheme

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E{\cdot}I \xrightarrow{k_a} E{\sim}I \begin{cases} \xrightarrow{k_d} E + P \\ \xrightarrow{k_2} E_i \end{cases} \qquad (2)$$

for 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 rate constant of acylation, respectively. Deacylation of the acyl enzyme prior to the alkylation event, or hydrolysis of the iodomethyl ketone to the corresponding hydroxy methyl 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.

The lactones were tested by an incubation assay (Table I) 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 certain time intervals and tested for residual enzyme activity against a suitable chromogenic substrate. After a 2-h incubation period, 0.1 M hydrazine was added to the incubation solution, and the residual enzyme activity was monitored for an additional 2 h. Hydrazine was expected to act as a strong external nucleophile, cleaving any acyl linkage between the enzyme and inhibitor. The results of this 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 results of the hydrazine reactivation study are listed as "*D*" value (relative rate of recovery of enzyme activity) and *E* reflects the percent enzyme activity recovered upon treatment of hydrazine, in the case of suicide substrates. Inactivation profiles which exhibited a high percent inhibition that persisted during the time course of the assay (2 h) were analyzed further to determine the parameters of the inactivation process.

(6) Sofia, M. J.; Katzenellenbogen, J. A. 3-(Acylamido)-4-phenyl-6(E)-(iodomethylidene)tetrahydro-2-pyranones. Synthesis of Novel Amino Acid Analogues. *J. Org. Chem.* **1985**, *50*, 2331–2336.

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

	lactone 1			lactone 2			lactone 3		lactone 4		
Protease (nM)	I_0/E_0	$A/B/C$[a]	D[b]	$A/B/C$[a]	D[b]	E[c]	$A/B/C$[a]	D[b]	$A/B/C$[a]	D[b]	E[c]
trypsin (14.6)	86	45/82/0[f]		0/99/∞		33	0/95/∞	slow	15/96/∞	slow	100
urokinase (300)	50	0/91/∞	fast	0/91/∞		20	0/86/∞	slow	45/85/∞[f]	slow	100
t-PA (19.4)	100	0/17/0		15/90/∞		0	0/77/0		45/41/0		
plasmin (625)	16	30/82/∞	fast	0/98/∞		40	15/93/30		15/93/30		
thrombin (1.93)	518	15/94/∞	fast	15/98/∞	fast	100	0/97/45		0/80/0		
α-chymotrypsin (17)	118	30/19/0		0/34/0			0/98/∞ (0/34/45)[d]	fast	45/96/∞ (0/47/15)[e]		20
HNE (66)	36	45/31/0		15/66/0			15/17/30		15/61/0		

[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] Relative rate of recovery of enzyme activity upon N_2H_4 treatment. "Fast" indicates $t_{1/2} < 1$ h and "slow" indicates $t_{1/2} > 2$ h. [c] Percent activity regained upon N_2H_4 treatment. [e,e] Numbers in parentheses are the $A/B/C$ values obtained for lactones IIa and IIb, respectively (taken from ref 3). [f] Delay method was used in incubation assay (see Experimental Section).

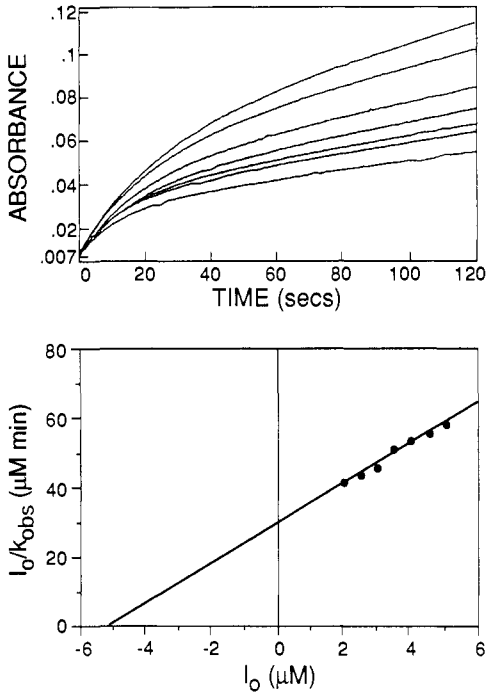


Figure 1. Time dependent and competitive inhibition of thrombin by the iodo enol lactone 2: panel A, progress curves for the inhibition of thrombin by lactone 2 in presence of a chromogenic substrate; panel B, competitive substrate assay of thrombin inhibited by lactone 2. A plot of the initial inhibitor 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 $-K_i$ $(1 + S_0/K_m)$.

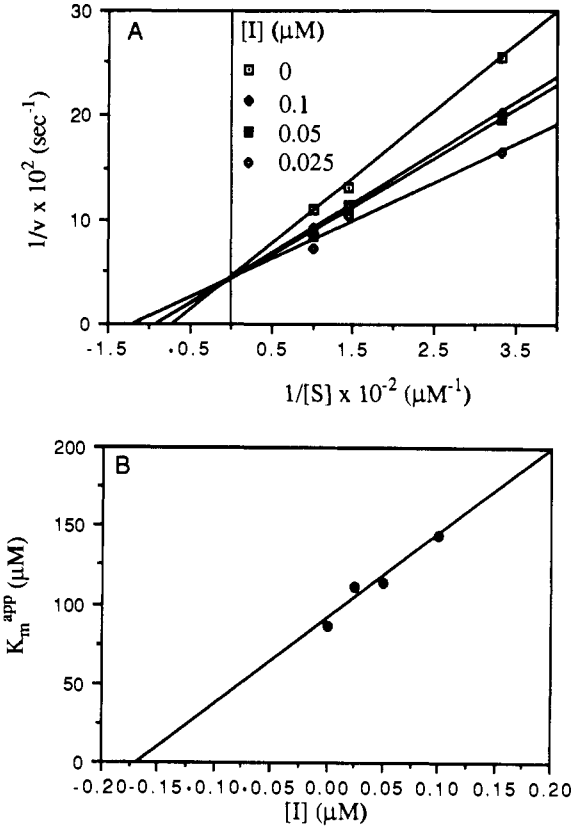


Figure 2. Competitive inhibition assay of α-chymotrypsin with the iodo enol lactone 4: panel A, determination of K_m^{app} at different inhibitor concentrations; panel B, A plot of K_m^{app}) versus [I] gives a straight line with an x-intercept of $-K_i$.

In a competitive substrate assay (method A), the enzyme is added to a mixture of the lactone and a suitable chromogenic substrate. 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 a flat steady-state region is reached (Figure 1). The binding constant, K_s (or K_i), and the rate constant of acylation, k_a, can be determined by this assay.[7] The inhibition of urokinase and α-chymotrypsin by the iodo lactone 4 and of t-PA by the iodo lactone 2 could not be evaluated by this method. In these cases, the burst region observed prior to attainment of the steady state could not be perceived over a wide range of substrate and inhibitor concentrations. This could be due to a fast k_a combined with a low K_i; such a combination would make a measurement of the rate of approach to steady state impractical. An estimated binding constant (K_i) was obtained by the use of the competitive inhibition assay[8] (method B). At different inhibitor concentrations, a K_m^{app} value was obtained by a reciprocal plot (1/velocity vs 1/[substrate]). A replot of inhibitor concentrations vs K_m^{app} allowed the determination of K_i from the x-intercept (Figure 2). In order to be able to estimate a purely competitive binding constant in this assay, the initial reaction velocities were measured during the first 15 s after addition of the enzyme. This minimized the contributions due to "irreversible" inactivation of the enzyme from formation of the acyl enzyme. In some cases, both methods were used to determine K_i values, in order to check the validity of method B.

(7) 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.

(8) Segel, I. H. In Enzyme Kinetics Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems; John Wiley and Sons; 1975; Chapter 3.

Table II. Parameters for Inactivation by Lactones 1–4

	K_s (μM)	k_a (min^{-1})	k_a/K_s (min^{-1} μM^{-1})	k_d (min^{-1})
		Lactone 1		
urokinase	0.35 ± 0.1	9.8 ± 1.3	28	0.044
plasmin	466 ± 173	57 ± 21	0.12	fast[a]
thrombin	37.87 ± 4.7	5.58 ± 0.6	0.15	0.006
		Lactone 2		
trypsin	0.08 ± 0.03	18.4 ± 4	230	
urokinase	0.01[b]	7.56 ± 0.9	756	
t-PA	0.12 ± 0.03	[c]		
plasmin	1.19 ± 0.07	10.2 ± 0.5	9	
thrombin	14.84 ± 8	27.25 ± 15	2	0.013
		Lactone 3[d]		
trypsin	10 ± 2	10.2 ± 1.8	1.02	0.015
	(9.4 ± 4.1)	(91.4 ± 40)	(10)	(0.002)
urokinase	6 ± 1.4	4.83 ± 0.5	0.8	0.002
	(0.12 ± 0.08)	(0.24 ± 0.02)	(2)	(0.009)
α-chymotrypsin	0.46 ± 0.17	9.51 ± 1.1	21	fast[a]
		Lactone 4[e]		
trypsin	0.67 ± 0.4	2.76 ± 0.3	4	0.005
	(4.28 ± 1.1)	(0.54 ± 0.02)	(0.13)	(0.02)
urokinase	18.73 ± 9	[c]		0.0008
	(7.83 ± 0.9)	(2.61 ± 0.2)	(0.33)	(fast)[a]
α-chymotrypsin	0.17 ± 0.03	[c]		

[a] k_d was too fast to measure by the centrifugation method (see Experimental Section). [b] The value reported reflects the higher limit. [c] Method B was used, k_a could not be obtained by this method. [d] Numbers in parentheses are the values for lactone IIa (taken from ref 3). [e] Numbers in parentheses are the values for lactone IIb (taken from ref 3).

In the case of alternate substrate inhibitors, the rate constant of deacylation (k_d) of the acyl enzyme intermediates was determined by monitoring the recovery of enzyme activity with time at room temperature.

In the interaction of an inhibitor and an enzyme, tight binding (low $K_{s/i}$ value) and a fast rate of acylation (high k_a value) contribute to a high specificity ($k_a/K_{s/i}$ value). The potency of an alternate substrate inhibitor is further enhanced by a slow rate of deacylation (low k_d value, i.e., stable acyl enzyme). A good suicide substrate would show efficient partitioning between alkylation (rate = k_2, eq 2) and deacylation (rate = k_d), such that permanent and complete inactivation could be achieved with only a few equivalents of inhibitor. A relative idea of efficiency can be derived from the percent activity regained in the hydrazine reactivation study (*E* value in Table I), a lower number indicating a higher efficiency.

Inhibition by α-Aryl-Substituted Lactones 1 and 2. The α-aryl-substituted lactones 1 and 2 were selective inactivators of trypsin-like enzymes. By contrast, the inhibition levels of α-chymotrypsin and HNE by these lactones was relatively low and transient.

The protio enol lactone 1 showed a very high percent inhibition (*B* value) in inactivating trypsin, urokinase, plasmin, and thrombin (Table I). This high level of inhibition lasted for the duration of the assay in the case of urokinase, plasmin, and thrombin. When hydrazine was added to these incubation solutions, all the enzyme activity was recovered relatively quickly. This was an expected result, since hydrazine cleaves any acyl linkage between the enzyme and inhibitor.

The inhibition of urokinase, plasmin, and thrombin by the protio lactone 1 was analyzed further to determine specificity preferences within the trypsin-like enzymes (Table II). Urokinase was inhibited with a moderately high specificity (k_a/K_s = 18 min^{-1} μM^{-1}) and a low rate of deacylation (k_d = 0.044 min^{-1}). The specificity constant was lower in the case of thrombin, but the corresponding acyl enzyme was very stable (k_d = 0.006 min^{-1}). The inhibition of plasmin by 1 was poorer, with a low specificity and a fast rate of deacylation.

The α-aryl-substituted iodo enol lactone 2 exhibited almost the same selectivity pattern as the corresponding protio enol lactone 1, in addition inhibiting t-PA (Table I). α-Chymotrypsin and HNE showed transient and relatively low levels of inhibition. The hydrazine reactivation study indicated that the iodo lactone 2 was a permanent inactivator of trypsin, urokinase, t-PA, and plasmin, but a transient inactivator of thrombin. The competitive substrate assay performed in each case provided further insight into the inactivation process (Table II). The inactivation of trypsin and urokinase was characterized by exceptionally high specificity constant (236 and 756 min^{-1} μM^{-1}, respectively). The inhibition of t-PA was typified by tight binding (K_i = 0.12 μM), although the rate constant of acylation could not be determined by the method used. Thus, the iodo enol lactone 2 showed good selectivity, even within the trypsin-like enzymes.

Inhibiton by β-Aryl, α-Benzamido Lactones 3 and 4. The β-aryl-substituted lactones 3 and 4 exhibited the same selectivity patterns in their inactivation profiles (Table I). They showed high levels of inhibition when incubated with trypsin, urokinase, α-chymotrypsin, plasmin, and thrombin. Incubation solutions with HNE and t-PA had low levels of inhibition. Plasmin, thrombin, t-PA, and HNE were transiently inactivated, i.e, enzyme activity approached normal levles during the course of the assay. The hydrazine reactivation study revealed that the inactivation of α-chymotrypsin by the iodo lactone 4 was permanent, and its inactivation by the protio lactone 3 was rapidly reversed upon treatment with hydrazine. Some unexpected results were obtained when incubation solutions of lactones 3 and 4 with trypsin and urokinase were treated with 0.1 M hydrazine. A very slow recovery of enzyme activity ($t_{1/2}$ > 2 h) was observed in each case. It seems reasonable that the presence of the benzamido group at the α-position in these inhibitors may be providing added hydrogen-bonding interactions between the lactone and the peptide backbone of the enzyme that may cause the acyl linkage in the acyl enzyme to be less accessible to hydrazine. The difference in behavior between the trypsin-like enzymes (trypsin and urokinase) and α-chymotrypsin in the hydrazine reactivation study must be due to some subtle changes in orientation of the inhibitor in the active site of each enzyme or due to differences in the active site. This is also apparent from the fact that α-chymotrypsin is permanently inactivated by the iodo lactone 4.

The specificity constants and k_d values for inactivation of trypsin, urokinase, and α-chymotrypsin by the protio lactone 3 are listed in Table II. Although lactone 3 inhibited α-chymotrypsin with a relatively high specificity (characterized by tight binding and a fast rate of acylation), it was pleasing to note that the acyl enzymes formed with the trypsin-like enzymes (trypsin and urokinase) were more stable (reflected by low k_d values). The binding constants for the inhibition of trypsin, urokinase, and α-chymotrypsin by the iodo enol lactone 4 were also compared (Table II). Once again, as with the protio lactone 3, the interaction between the iodo lactone 4 and α-chymotrypsin exhibited the tightest binding (low K_i value). Although inhibition

of urokinase and trypsin and trypsin by the iodo lactone 4 was transient in nature, the acyl enzymes formed in each case were extremely stable and resistant to hydrolysis (k_d values were 0.0008 and 0.005 min^{-1}, respectively, Table II).

Importance of Secondary Interactions: Comparison of Inhibition by the β-Aryl-Substituted Lactones IIab and the α-Benzamido Analogs 3 and 4. α-Chymotrypsin, elastase, and trypsin, share a common catalytic mechanism based on an active site serine residue. The catalytic groups implicated in bond cleavage exhibit virtually identical conformation.[9] However, differences in the binding sites are responsible for the differences in selectivities of these enzymes. 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. An asparate residue at the base of the hydrophobic pocket in trypsin-like enzymes accounts for their specificity for cleaving the peptide bond at positively charged basic amino acids like Lys and Arg. In elastase, the hydrophobic pocket is relatively sterically congested, due to the presence of bulky side chains of Val and Thr residues; only small aliphatic side chains such as the methyl group of Ala can be accommodated in this shallow cavity. Thus, the primary specificity qualitatively accounts for selecting the bond that is cleaved by a certain serine protease. Beyond this, secondary interactions (hydrogen bonds between a polypeptide substrate and the enzyme backbone) facilitate the cleavage of the bond in question, i.e., quantitatively increase the specificity rate constant, by additional orientation and stabilization.

The primary specificity of trypsin for arginine or lysine bonds appears to be so stringent that, with small oligopeptide substrates, secondary interactions have relatively little effect on either binding affinity or catalytic efficiency.[10] (This may not be the case in other trypsin-like enzymes, which exhibit much greater specificity toward their target macromolecules).[11] Secondary interactions play a very important role in chymotrypsin and elastase, where the primary specificity is relatively lower.[10]

The difference in the importance of secondary interactions in α-chymotrypsin and trypsin-like enzymes is well-illustrated when we compare the inactivation profiles and inhibition parameters of the β-aryl-substituted lactones **IIab**, studied previously,[3] with the corresponding α-benzamido, β-aryl-substituted lactones **3** and **4**, studied in this report. The β-aryl-substituted protio and iodo enol lactones (**IIab**) caused less than 50% inhibition when incubated with α-chymotrypsin (Table I, values in parentheses). This low level of inhibition did not persist, and enzyme activity approached normal levels very rapidly. By contrast, when lactones **3** and **4** (with the α-benzamido substituent) were incubated with α-chymotrypsin, they showed high levels of inhibition which persisted over the time course of the assay. The inhibition was characterized by tight binding in each case. We attribute this improvement in inhibition of α-chymotrypsin, to secondary interactions between the benzamido group (in **3** and **4**) and the peptide backbone of the enzyme.

The situation with trypsin and urokinase is different: Here the β-substituted protio and iodo enol lactones **IIab** and the corresponding α-benzamido substituted lactones **3** and **4** were transient inhibitors (Table II), and the binding affinity and specificity constants did not change significantly between the unsubstituted and α-benzamido-substituted lactones (see values in parentheses in Table II for comparison). As detailed above, this lack of sensitivity to secondary interactions in trypsin-like enzymes may be due to the extremely high primary specificities of these enzymes.

The potency of alternate substrate inhibitors is attributed to the stability of the acyl enzyme intermediate. When normal substrates interact with serine proteases, hydrolysis of the acyl enzyme is facilitated because the acyl carbonyl is activated by hydrogen bonds in the oxyanion binding hole. In the case of protio enol lactones, formation of the acyl enzyme results in the release of a methyl ketone group. The lack of full stabilizing interactions of the ester carbonyl in the oxyanion hole may render the acyl linkage relatively inactive to hydrolysis.[12] The inertness of the acyl bond in alternate substrate inhibitors may also be due to steric or ionic repulsion of the attacking water molecule, due to hydrogen bonding at the ketone carbonyl.[12]

Formation of the acyl enzyme with an iodo enol lactone results in the release of an iodomethyl ketone group. When iodo enol lactones act as alternate substrate inhibitors, the iodomethyl ketone may undergo hydrolysis to the corresponding hydroxymethyl ketone, which can provide additional modes of acyl enzyme decomposition by intramolecularly attacking the acyl carbonyl. As a result, in most cases,[3,13] when iodo enol lactones act as alternate substrate inhibitors, the acyl enzymes are less stable (higher k_d values) than the corresponding protio enol lactones. However, this was not true in the case of the α-benzamido-substituted lactones **3** and **4**. Both the protio and iodo lactones **3** and **4** were alternate substrate inhibitors of trypsin and urokinase. The k_d values for the iodo lactone **4** for trypsin and urokinase were lower (0.005 and 0.0008 min^{-1}, respectively) than the corresponding k_d values for the protio lactone **3** (0.015 and 0.002 min^{-1}, respectively). In the absence of a crystal structure of the inhibited enzymes, we speculate that a carboxylate group of an aspartate or glutamate residue near the active site may get alkylated by the revealed iodomethyl ketone group (a crystal structure of trypsin with a guanidino-substituted isocoumarin[14] has two aspartate residues besides Asp-189, within 10 Å of the active site Ser-195). This would lead to an additional acyl linkage between the enzyme and inhibitor and might explain why the enzyme–inhibitor

(9) Polgár, L. Structure and Function of Serine Proteases. In Hydrolytic Enzymes, Neuberger, A.; Brockelhurst, K., Eds.; Elsevier, 1989; Chapter 3.

(10) Fruton, J. A. The Specificity of Proteinases Toward Protein Substrates. In Proteases and Biological Control; Reich, E.; Riflein, D. B.; Shaw, E., Eds.; Cold Spring Harbor Laboratory, 1975, Vol 2; pp 33–50.

(11) Vali, Z.; Patthy, L. Location of the Intermediate and High Affinity ω-Aminocarboxylic Acid-binding Sites in Human Plasminogen. *J. Biol. Chem.* **1982**, *257*, 2104–2110.

(12) (a) Reed, P. E.; Katzenellenbogen, J. A. β-Substituted β-Phenylpropionyl Chymotrypsins. Structural and Stereochemical Features in Stable Acyl Enzymes. *J. Med. Chem.* **1991**, *34*, 1162–1176. (b) Bemis, G. W.; Carlson-Golab, G.; Katzenellenbogen, J. A. A Molecular Dynamics Study of the Stability of Chymotrypsin Acyl Enzymes. *J. Am. Chem. Soc.* **1992**, *114*, 570–578.

(13) 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**, *29*, 230–238.

(14) Chow, M. M.; Meyer, E. F., Jr.; Bode, W.; Kane, C-M.; Radhakrishnan, R.; Vijayalaxmi, J.; Powers, J. C. The 2.2-Å Resolution X-ray Crystal Structure of Trypsin Inhibited by 4-Chloro-3-ethoxy-7-guanidinoisocoumarin: A Proposed Model for the Thrombin-Inhibitor Complex. *J. Am. Chem. Soc.* **1990**, *112*, 7783–7789.

complexes formed from iodo enol lactones are so slow to decompose in this case.

Conclusion

Trypsin-like enzymes play important roles in numerous physiological processes. Selective inhibitors for this class of enzymes could be therapeutically useful. Inhibitors of trypsin can be used for treatment of pancreatitis and hyperproteolytic conditions.[15] Trypsin-like enzymes are involved in the blood-coagulation cascade,[16] a process which has been implicated in various thrombotic diseases. Inhibitors of blood clotting enzymes are potential anticoagulants.[15] Fibrinolysis[17] (the process of dissolution of the blood clot) is mediated by plasmin, which is activated from plasminogen by the action of plasminogen activators, namely, urokinase and t-PA. Inhibitors of plasmin, urokinase, and t-PA can be used in the therapy of hyperfibrinolytic states.[18] Recently, the involvement of plasmin and urokinase has been implicated in breast tumors and stomach and colorectal carcinomas. Inhibitors of these enzymes may contribute significantly in the field of cancer research.[19] An amino-substituted isatoic anhydride,[20] and guanidino-substituted isocoumarins[21] are some of the few mechanism-based inhibitors designed for trypsin-like enzymes that have been reported earlier.

Our investigation of guanidino-substituted protio and iodo enol lactones which contain either an α- or β-aryl substituent has provided us with very potent inhibitors. In general, they are selective for the trypsin-like enzymes, over α-chymotrypsin and HNE; moreover, there is some selectivity within the class of trypsin-like enzymes. A notable exception is the α-benzamido-substituted β-aryl iodo lactone 4, a very selective suicide substrate of α-chymotrypsin, which did not permanently inactivate any of the trypsin-like enzymes or HNE; it was, however, a potent transient inactivator of trypsin and urokinase.

Our studies indicated that the α-aryl substituted iodo enol lactones are potent suicide substrates of some trypsin-like enzymes. In contrast, the β-aryl-substituted iodo lactones were transient inactivators of the trypsin-like enzymes tested. We can generalize that the iodo methyl ketone group revealed upon formation of the acyl enzyme with the β-aryl-substituted systems is not suitably positioned to alkylate an active site nucleophile. Among the protio enol lactones, it was observed that although the α-aryl-substituted protio lactones interacted with the trypsin-like enzymes with high specificities, the β-aryl-substituted protio lactones formed more stable acyl enzyme intermediates, and were thus more potent as alternate substrate inhibitors.

Experimental Section

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 and brine), dried ($MgSO_4$), and concentrated. The crude compound was purified by flash chromatography[22] and recrystallization (when applicable). This workup procedure is presented in the following 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 following form: δ value of signal (peak multiplicity, integrated number of protons, coupling constant) where spectra of mixtures of diastereomers are reported, the integration values have been doubled in order to report whole numbers. Mass spectral data were obtained by using electron-impact (EI) ionization on a Varian CH-5 spectrometer for low resolution data on 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 following form: m/z (intensity relative to base peak = 100).

Tetrahydrofuran (THF) was distilled from sodium benzophenone ketyl; all other solvents were distilled from CaH_2.

Chemicals were obtained from the following sources and were used as received: Fischer, Mallinckrodt, Aldrich, Sigma, Fluka, or Eastman. 4-Bromo-1-butyne[1a] and diethyl benzamidomalonate[23] were prepared according to literature procedures.

4-[[[(*tert*-Butyloxy)carbonyl]amino]methyl]phenylacetic Acid (7). The bromo acid 5 (1.61 g, 7.03 mmol) was added in small portions to ethanol (170 mL) previously saturated with anhydrous NH_3. The mixture was stirred at room temperature for 1 h. The volatile components were removed in vacuo and the solid thus obtained was treated with aqueous NaOH (1 M, 21 mL). The water was removed and the resulting solid was dissolved in a 1:1 mixture of dioxane and water (34.5 mL). Aqueous NaOH (6.97 mL of a 1 N solution) was added and the mixture was cooled to 0 °C in an ice/water bath. *tert*-Butyl pyrocarbonate (1.67 g, 7.66 mmol) was added in portions, and subsequently the reaction mixture was allowed to warm up to room temperature and stirred for 12 h. Dioxane was removed in vacuo; the aqueous layer was chilled and acidified to pH 3 with dilute $KHSO_4$ and extracted (ethyl acetate). The crude product was purified (1:2 of ethyl acetate/hexane; ethyl acetate, hexane) to yield 1.37 g (74%) of a white solid: mp 110 °C; NMR ($CDCl_3$) δ 7.25 (br s, 4), 4.90 (br s, 1), 4.30 (d, 2, J = 5.4 Hz), 3.65 (s, 2), 1.50 (s, 9); mass spectrum (EI) m/z 209 (M^+, 23), 208 (39), 164 (27), 150 (64), 149 (23), 106 (23), 91 (27), 57 (100), 41 (32). Anal. ($C_{14}H_{19}O_4N$) C, H, N.

2-[4-[[[(*tert*-Butyloxy)carbonyl]amino]methyl]phenyl]-5-hexynoic Acid (8). Diisopropylamine (1.61 mL, 11.49 mmol) was added dropwise to a solution of *n*-BuLi (1.45 M, 7.76 mL, 11.49 mmol) in THF (11 mL) at 0 °C. The mixture was stirred at 0 °C for 30 min and then cooled to −40 °C. A solution of the acid 7 (0.923 g, 3.48 mmol) in THF (11 mL) was added dropwise and stirring was continued for 1 h. *n*-BuLi (7.76 mL, 11.49 mmol) was added dropwise and the mixture was stirred for an additional 45 min followed by the addition of 4-bromo-1-butyne[1a] (1.62 mL, 17.4 mmol). The reaction mixture was stirred for 2 h at −40 °C

(15) 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, 1989; pp 619–649.

(16) 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.

(17) (a) Castellino, F. J. Recent Advances in the Chemistry of the Fibrinolytic-System. *Chem. Rev.* 1980, *81*, 431–446. (b) Collen, D. Molecular Mechanisms of Fibrinolysis and their Application to Fibrin-Specific Thrombolytic Therapy. *J. Cell. Bioch.* 1987, *33*, 77–86.

(18) Barrett, A. J. Proteinase Inhibitors: Potential Drugs? In Enzume Inhibitors as Drugs; Sandler, M., Ed.; University Park Press, 1980; pp 219–229.

(19) Moscatelli, D.; Rifkin, D. B.; Isseroff, R. R.; Jaffe, E. A. Proteinases and Tumor Invasion, Vol. 6; Raven Press, New York, 1980; pp 143–152.

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

(21) Kam, C-M.; Fujikawa, K.; Powers, J. C. Mechanism-Based Isocoumarin Inhibitors for Trypsin and Blood Coagulation-serine Proteases: New Anticoagulants. *Biochemistry* 1988, *27*, 2547–2557.

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

(23) Sofia, M. J.; Chakravarty, P. K.; Katzenellenbogen, J. A. Synthesis of Five-Membered Halo Enol Lactone Analogues of α-Amino Acids: Potential Protease Suicide Substrates. *J. Org. Chem.* 1983, *48*, 3318–3325.

and then allowed to warm up to 0 °C and quenched with ice/water. Acidification to pH 4 with dilute, aqueous $KHCO_3$ was followed by extraction (ethyl acetate) and purification (3.5:1.5:0.05 of hexane/ethyl acetate/acetic acid; ether, hexane) to yield 0.5 g (46%, 52% if corrected for recovered starting material) of a white solid: mp 92–93 °C; NMR ($CDCl_3$) δ 9.9 (br s), 7.18 (br q, 4), 4.87 (br s, 1), 4.20 (d, 2, J = 5.3 Hz), 3.69 (br t, 1), 2.08 (m, 5), 1.37 (s, 9); mass spectrum (FAB) m/z 318 (M + 1, 10), 262 (40), 216 (35), 201 (57), 103 (100); Anal. ($C_{18}H_{24}NO_4$) exact mass HRFAB.

2-[4-(Aminomethyl)phenyl]-5-hexynoic Acid (9). The BOC-protected amino acid **8** (0.93 g, 2.93 mmol) was dissolved in CH_2Cl_2 (12 ml) and cooled to 0 °C in an ice/water bath. Trifluoroacetic acid (3 mL) was added dropwise and the reaction mixture was stirred at 0 °C for 1 h and at room temperature for an additional 2 h. The volatile components were removed in vacuo, and the semisolid residue thus obtained was purified by recrystallization (ethyl acetate, hexane) to yield 0.6 g (quant.) of a white solid: mp 133 °C; NMR (MeOH-d_4) δ 7.42 (AB q, 4, Δν = 0.027 ppm, J = 8.5 Hz), 4.10 (s, 2), 3.80 (t, 1, J = 7.5 Hz), 2.10 (m, 5); mass spectrum (FABMS) m/z 218 (M + H, 49), 201 (42), 152 (71), 149 (28), 121 (39), 105 (19), 103 (100), 102 (22). Anal. ($C_{13}H_{16}NO_2$) exact mass HRFAB.

2-[4-(Guanidinomethyl)phenyl]-5-hexynoic Acid (10). A mixture of the amino acid **9** (0.63 g, 2.93 mmol) diisopropylethylamine (2.55 mL, 14.65 mmol) and 3,5-dimethylpyrazole-1-carboxamidine nitrate (2.9 g, 14.65 mmol) was refluxed in THF (16 mL) for 12 h. The solvent was removed in vacuo and the crude mixture was purified by flash chromatography, initially eluting with pure chloroform and subsequently eluting with a 20:1 mixture of chloroform/methanol. The proportion of methanol in the eluting solvent was increased (10:1, 2:1, then 1:1) in order to remove side products and any excess left over reagents. The column was finally eluted with pure methanol to yield a solid which was further purified by trituration in methanol to afford 0.49 g (65%) of a white solid: mp 260 °C dec; NMR (CD_3CN, trifluoro acetic acid) δ 7.33 (AB q, 4, J = 8.4 Hz, Δν, = 0.040 ppm), 6.45 (br s), 4.34 (s, 2), 3.74 (t, 1, J = 7 Hz), 2.1 (m, overlapping with CD_3CN signal); mass spectrum (FABMS) m/z 260 (M + H, 16), 149 (23), 121 (36), 105 (17), 103 (100), 102 (21). Anal. ($C_{14}H_{17}O_2N_3 \cdot {}^1/_3\ H_2O$) C, H, N.

3-[4-(Guanidinomethyl)phenyl]-6-methylidenetetrahydro-2-pyranone (1). The acetylenic acid **10** (0.0115 g, 0.04 mmol) was dissolved in acetonitrile (4 mL) and trifluoroacetic acid (2 μL). Mercuric trifluoroacetate (0.002 g, 0.004 mmol) was added and the mixture was stirred at room temperature for 45 min. The volatile components were removed in vacuo, and the crude product was purified by flash chromatography (1:4 of 2-propanol/chloroform) to yield 0.01 g (quant.) of a white foam: NMR (MeOH-d_4) δ 8.42 (AB q, 4, J = 8.4 Hz, Δν = 0.031 ppm), 4.64 (s, 1), 4.40 (s, 3), 3.97 (dd, 1, J = 6.3, 11.0 Hz), 2.70 (m, 2), 2.13 (m, 2); mass spectrum (FABMS) m/z 260 (M + H, 100), 213 (29), 193 (59), 121 (28), 115 (24), 103 (76). Anal. ($C_{14}H_{18}O_2N_3$) exact mass HRFAB.

3-[4-(Guanidinomethyl)phenyl]-6-(*E*)-(iodomethylidene)-tetrahydro-2-pyranone (2). The acetylenic acid **10** (0.017 g, 0.064 mmol) was dissolved in acetonitrile (3.4 mL) and trifluoroacetic acid (2 μL). To this solution were added $KHCO_3$ (0.007 g, 0.07 mmol) and iodine (0.02 g, 0.07 mmol), and the resulting mixture was stirred at room temperature for 1 h. The volatile components were removed in vacuo and purification and isolation were accomplished by flash chromatography. The column was first eluted with chloroform (to remove any excess iodine) and then with a 5:1 chloroform/methanol mixture to yield 0.019 g (79%) of a yellow foam: NMR (CD_3OD) δ 7.30 (AB q, 4, J = 8.5 Hz, Δν = 0.034 ppm), 6.02 (s, 1), 4.39 (s, 2), 3.96 (dd, 1, J = 6.9, 9.9 Hz), 2.90 (m, 1), 2.75 (m, 1), 2.19 (m, 2); mass spectrum (FABMS) m/z 386 (M + H, 15), 347 (21), 307 (69), 193 (29), 103 (100), 102 (21). Anal. ($C_{14}H_{17}N_3O_2I$) exact mass HRFAB.

Diethyl 2-Benzamido-2-[1-[4-[(*tert*-butoxycarbonyl)amino]phenyl]-3-butynyl]malonate (12). Diethyl benzamidomalonate[23] (1.8 g, 6.45 mmol) dissolved in THF (17 mL) was added dropwise to a suspension of sodium hydride (0.26 g, 6.45 mmol) in THF (35 mL) at 25 °C. After stirring for 20 min at 25 °C, the chloride[3] (1.8 g, 6.45 mmol; prepared just prior to use) was added as a THF (22 mL) solution. The reaction mixture was stirred at room temperature for 3 h and subsequently quenched with water and extracted (ethyl acetate). The crude product thus obtained was purified (4:1 of hexane/ethyl acetate; ethyl acetate, hexane) to yield 1.85 g (55%) of a white solid: mp 159 °C; NMR ($CDCl_3$) δ 7.79 (d, 2, J = 7.1 Hz), 7.50 (m, 3), 7.33 (m, 2), 7.16 (d, 2, J = 8.55 Hz), 6.45 (br s, 1), 4.19 (m, 5), 3.49 (t, 1, J = 2.95 Hz), 3.43 (t, 1, J = 2.92 Hz), 2.59 (m, 1), 1.71 (t, 1, J = 2.42 Hz), 1.51 (s, 9), 1.24 (t, 6, J = 7.1 Hz); mass spectrum (EI) m/z 522 (M^+, 0.5), 244 (35), 188 (77), 144 (63), 105 (100), 77 (32), 57 (53). Anal. ($C_{29}H_{34}O_7N_2$) C, H, N.

(2*R,3*R**)- and (2*R**,3*S**)-Ethyl 2-Benzamido-3-[4-[[(*tert*-butyloxy)carbonyl]amino]phenyl]-5-hexynoate (13).** The substituted diethyl benzamidomalonate **12** (3.16 g, 6.05 mmol) was dissolved in ethanol (120 mL). A 20% aqueous solution of KOH (7 mL) was added dropwise and stirred at room temperature for 45 min. The ethanol was removed in vacuo and the water layer was chilled and acidified to pH 3 with dilute aqueous $KHSO_4$. The aqueous layer was extracted (ethyl acetate). The crude product thus obtained was refluxed in *m*-xylene (18 g) for 4 h. The *m*-xylene was removed in vacuo and the residue was dissolved in ethyl acetate. The ethyl acetate layer was washed (saturated $NaHCO_3$, water, brine), dried ($MgSO_4$), and concentrated. The crude product was purified (2:1, hexane/ethyl acetate; ethyl acetate, hexane) to afford a 1:1 mixture of diastereomers of **13** in a 67% (1.82 g) yield: mp 187 °C, NMR (MeOH-d_4) δ 7.84 (d, 2, J = 7.2 Hz), 7.40 (m, 16), 5.08 (d, 2, J = 8.2 Hz), 4.94 (d, 2, J = 9.9 Hz), 4.21 (m, 2), 3.89 (m, 2), 3.40 (m, 2), 2.70 (m, 4), 2.22 (m, 2), 1.51 (s, 9), 1.49 (s, 9), 1.30 (t, 3, J = 7.2 Hz), 0.97 (t, 3, J = 7.2 Hz); mass spectrum (FABMS) m/z 451 (M + H, 16), 395 (35), 279 (15), 105 (55), 103 (100). Anal. ($C_{26}H_{30}O_5N_2$) C, H, N.

(2*R,3*R**)- and (2*R**,3*S**)-2-Benzamido-3-[4-[[(*tert*-butyloxy)carbonyl]amino]phenyl]-5-hexynoic Acid (14).** The ester **13** (1.4 g, 3.11 mmol) was dissolved in ethanol (150 mL), and 20% aqueous KOH (7 mL) was added dropwise. The reaction mixture was stirred for 1.5 h at room temperature. The ethanol was removed in vacuo and the water layer was chilled and acidified to pH 3 with dilute $KHSO_4$. The aqueous layer was extracted (ethyl acetate). The crude product was purified (4:1:0.1; ethyl acetate/hexane/acetic acid; ethyl acetate, hexane) to afford 1.2 g (91%) of a white solid as a 1:1 mixture of diastereomers: mp 110–115 °C dec; NMR ($CDCl_3$) δ 7.4 (m, overlapping with $CDCl_3$), 6.8 (br d, 1), 6.35 (br d, 1), 5.38 (m, 1), 5.21 (m, 1), 3.70 (m, 1), 3.5 (m, 1), 2.8 (m, 4), 2.01 (m, 2), 1.52 (br s, 18); mass spectrum (FABMS) m/z 423 (M + H, 36), 367 (100), 321 (11), 244 (9), 217 (11), 188 (12), 103 (69). Anal. ($C_{24}H_{27}O_5N_2$) exact mass HRFAB.

(2*R,3*R**)- and (2*R**,3*S**)-2-Benzamido-3-(4-aminophenyl)-5-hexynoic Acid (15).** The BOC-protected amino acid **14** (1.78 g, 4.2 mmol) was dissolved in CH_2Cl_2 (31 mL) and cooled to 0 °C in an ice/water bath. Trifluoroacetic acid (7.8 mL) was added dropwise and stirring was continued at 0 °C for 1 h followed by stirring at 25 °C for 2 h. The volatile components were removed in vacuo and the crude product was purified by trituration (ethyl acetate, hexane) to yield an off-white solid 1.7 g (94%) as a mixture of diastereomers: NMR (MeOH-d_4) δ 7.81 (m, 1), 7.40 (m, 14), 7.05 (m, 3), 5.07 (d, 1, J = 8.2 Hz), 4.99 (d, 1, J = 8.6 Hz), 3.50 (m, 2), 2.72 (m, 4), 2.25 (t, 1, J = 2.5 Hz), 2.20 (t, 1, J = 2.5 Hz); mass spectrum (FABMS, spectrum was offscale) m/z 323 (M + H, 60), 287 (29), 177 (53), 167 (25), 165 (26), 144 (26), 105 (100), 103 (100), 102 (78). Anal. ($C_{19}H_{19}O_3N_2$) exact mass HRFAB.

(2*R,3*R**)- and (2*R**,3*S**)-2-Benzamido-3-(4-guanidinophenyl)-5-hexynoic Acid (16).** A mixture of the amino acid **15** (1.35 g, 4.2 mmol), diisopropylethylamine (3.6 mL, 20.96 mmol), and 3,5-dimethylpyrazole-1-carboxamidine nitrate (4.2 g, 20.96 mmol) was refluxed in THF (30 mL) for 48 h. The solvent was removed in vacuo and the crude mixture was purified by flash chromatography, initially eluting with pure chloroform and then with a 8:1 mixture of chloroform/methanol followed by a 1:1 mixture of chloroform/methanol. This procedure removed side products and any excess leftover reagents and starting material. The column was finally eluted with methanol to afford the product (0.32 g, 21%), a light brown foam, as a 1:1 mixture of diastereomers: NMR (MeOH-d_4) δ 7.79 (m, 4), 7.41 (m, 10), 7.18 (m, 4), 4.89 (d, 1, J = 8.0 Hz), 4.75 (d, 1, J = 7.9 Hz), 3.64 (m, 1), 3.50 (m, 1), 2.81 (m, 2), 2.70 (m, 2), 2.15 (t, 1, J = 2.3 Hz), 2.11 (t, 1, J = 2.0 Hz); mass spectrum (FABMS) m/z 365 (M + H, 20), 275 (5), 103 (100), 101 (37). Anal. ($C_{20}H_{21}N_4O_3$) exact mass HRFAB.

(3R*,4R*)-3-Benzamido-4-(4-guanidinophenyl)-6-methylidenetetrahydro-2-pyranone (3). The acetylenic acid **16** (0.0418 g, 0.11 mmol) was dissolved in acetonitrile (12.5 mL) and trifluoroacetic acid (5 μL). Mercuric trifluoroacetate (0.005 g, 0.01 mmol) was added and the reaction mixture stirred at room temperature for 2 h. The volatile components were removed in vacuo, and the crude product was purified by flash chromatography (5:2, chloroform/2-propanol) to afford 0.028 g (67%) of a light colored foam as a single diastereomer: NMR (MeOH-d_4) δ 7.67 (d, 2, J = 7.1 Hz), 7.41 (m, 5), 7.23 (d, 2, J = 8.3 Hz), 5.00 (d, 1, J = 11.9 Hz), 4.74 (s, 1), 4.50 (s, 1), 3.59 (m, 1), 3.09 (m, 1), 2.91 (dd, 1, J = 4.31, 15.1 Hz); mass spectrum (FABMS) m/z 365 (M + H, 35), 307 (100), 289 (28), 193 (39), 149 (28), 105 (31), 103 (89). Anal. ($C_{20}H_{21}O_3N_4$) exact mass HRFAB.

(3R*,4R*)-3-Benzamido-4-(4-guanidinophenyl)-6(E)-(iodomethylidene)tetrahydro-2-pyranone (4). The acetylenic acid **16** (0.03 g, 0.08 mmol) was dissolved in acetonitrile (7.28 mL) and trifluoroacetic acid (5 μL). $KHCO_3$ (0.008 g, 0.08 mmol) and N-iodosuccinamide (0.018 g, 0.08 mmol) was added sequentially, and the reaction 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, initially eluting with $CHCl_3$ (this removed colored impurities) and then eluting with a 5:1 mixture of $CHCl_3$/2-propanol to afford 0.0136 g (34%) of a brown-yellow foam; NMR (CD_3CN) δ 8.97 (br s), 7.65 (d, 2, J = 8.7 Hz), 7.45 (m, 5), 7.22 (d, 2, J = 8.4 Hz), 6.61 (br s, 2), 6.15 (br s, 1), 4.96 (m, 1), 3.70 (m, 1), 3.29 (m, 1), 2.96 (m, 1); NMR (MeOH-d_4) δ 5.11 (d, 1, J = 12.15 Hz); mass spectrum (FABMS) m/z 491 (M + H, 15), 461 (10), 425 (14), 411 (12), 279 (25), 103 (100), 102 (27). Anal. ($C_{20}H_{20}O_3N_4I$) exact mass HRFAB.

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 Calibiochem. 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-valylglycyl-L-arginine p-nitroanilide (Z-Val-Gly-ARg pNA), benzoyl-β-alanylglycyl-L-arginine p-nitroaniide (Bz-(β)-Ala-Gly-Arg pNA), tosylglycyl-L-prolyl-L-arginine p-nitroanilide (Ts-Gly-Pro-Arg pNA) and tosylglycyl-L-prolyl-L-lysine p-nitroanilide (Ts-Gly-Pro-Lys pNA) were obtained from Boehringer Mannheim. (Methylsulfonyl)-D-cyclohexyltyrosylglycyl 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 μL, 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, ionic strength 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 the "Results" section.

The absolute standard deviation values of k_a and $K_{s/I}$ were determined as described by Baek et al.[1b] 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 were the same as those 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.

Acknowledgment. We are grateful for the support of this research through a grant from the National Institutes of Health (PHS 5RO1 DK27526). High resolution mass spectra and FAB mass spectra were obtained in the Mass Spectrometry Laboratory, School of Chemical Sciences, University of Illinois, supported in part by a grant from the National Institute of General Medical Sciences (GM 27029). The ZAB-SE mass spectrometer was purchased in part with grants from the Division of Research Resources, National Institutes of Health (RR 01575), the National Sciences Foundation (PCM 8121494), and the National Institute of General Medical Sciences (GM 27029).