

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

Michael J. Sofia and John A. Katzenellenbogen*

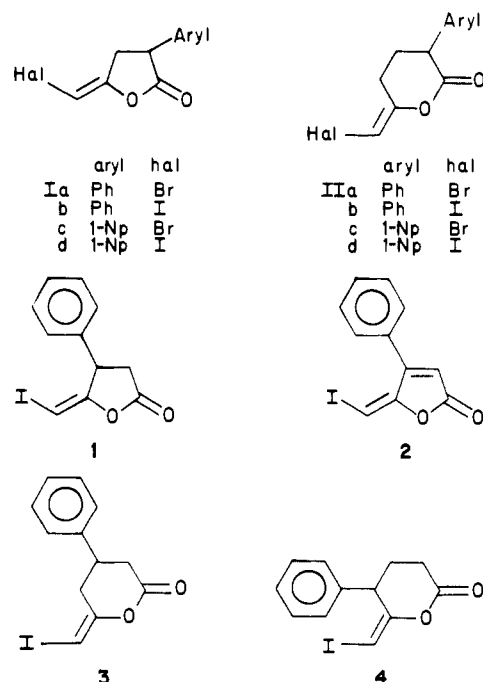
School of Chemical Sciences, University of Illinois, Urbana, Illinois 61801. Received May 3, 1985

We have found that α -aryl-substituted halo enol lactones (I and II) are effective mechanism-based inactivators for chymotrypsin. In this study, we have investigated, for comparative purposes, halo enol lactones with aryl functions situated β and γ to the lactone carbonyl group. We synthesized 4-phenyl-5(*E*)-(iodomethylidene)tetrahydro-2-furanone (1), 4-phenyl-5(*E*)-(iodomethylidene)dihydro-2-furanone (2), 4-phenyl-6(*E*)-(iodomethylidene)tetrahydro-2-pyranone (3), and 5-phenyl-6(*E*)-(iodomethylidene)tetrahydro-2-pyranone (4), using a halolactonization reaction to convert the appropriate phenyl-substituted acetylenic acid precursor into the corresponding 5(*E*)-(halomethylidene)furanone and 6(*E*)-(halomethylidene)pyranone system. The 4-phenylfuranone (1 and 2) and the 5-phenylpyranone (4) proved to be only reversible, competitive inhibitors. By contrast, the 4-phenyltetrahydropyranone (3) inactivated α -chymotrypsin in a time-dependent manner. This inactivation was very rapid but reversible, with regeneration of enzyme activity being spontaneous and hydrazine-accelerated, suggestive of the intermediacy of a stable acyl enzyme. Kinetic comparison of the iodomethylene lactone 3 with the corresponding protio lactone 25 indicates that the iodine accelerates the rate of chymotrypsin acylation but produces an acyl enzyme that is more hydrolytically labile than that formed from lactone 25. From the results of this study, we conclude that a phenyl group situated at C-3 (α to the lactone carbonyl group) in both the 5(*E*)-(iodomethylidene)tetrahydro-2-furanone (I) and 6(*E*)-(iodomethylidene)tetrahydro-2-pyranone (II) series is essential for their activity as mechanism-based irreversible inactivators of chymotrypsin. The corresponding β -aryl-substituted lactones, by contrast, are potent acylating agents that lead to acyl enzymes of high stability.

Recently, we have demonstrated that halo enol lactones act as effective enzyme-activated irreversible inhibitors (suicide substrates) of chymotrypsin, thus confirming Rando's 1974 proposal¹ that compounds with this structure may act as mechanism-based inactivators for serine proteases.²⁻⁴ (The proposed mechanism for this inactivation is shown in Scheme I.) In order to examine how the structural and functional features of the halo enol lactone system affect their potency as enzyme-activated irreversible inhibitors of α -chymotrypsin, we undertook a systematic study of structure-activity relationships. In an earlier report,⁴ we described a comprehensive approach to the kinetic analysis of the inactivation properties of these compounds, and we examined the behavior of a series of (halomethylene)tetrahydrofuranone and -tetrahydropyranone systems substituted with aryl groups α to the lactone carbonyl group (C-3; lactones I and II). The effectiveness of these compounds as inactivators for chymotrypsin depends strongly on the ring size of the lactone (the six-membered lactones being better than five-membered ones) and to a lesser extent on the nature of the aryl group (1-naphthyl being more effective than phenyl) and the halogen (iodine better than bromine).

In this report, we described the synthesis of the 4-phenylfuranones 1 and 2 and the 4- and 5-phenylpyranones 3 and 4. The lactones 1-3 in this series encompass analogues of the hydrocinnamyl and cinnamyl systems that are known to be substrates for chymotrypsin. By comparing the inactivation properties of these lactones with that of the previously studied 3-phenyl substituted series, we have been able to determine how the position of attachment of the aryl group (α or β to the carbonyl group in the tetrahydrofuranones and α , β , or γ in the tetrahydropyranones) affects their capacity for inactivation.

From the results of our studies, it is apparent in both the furanone and pyranone series that an α -aryl substituent (at C-3) is needed for them to act as irreversible inhibitors of chymotrypsin. The β -substituted tetrahydropyranone (3), however, is a potent reversible inactivator, and it and its diprotio analogue (25) appear to act through very stable acyl enzyme intermediates. These structure-activity relationships will be helpful in guiding further efforts in the design of halo enol lactone suicide substrates.

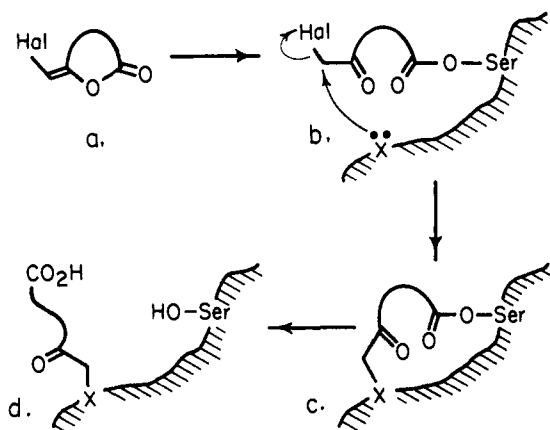


Results

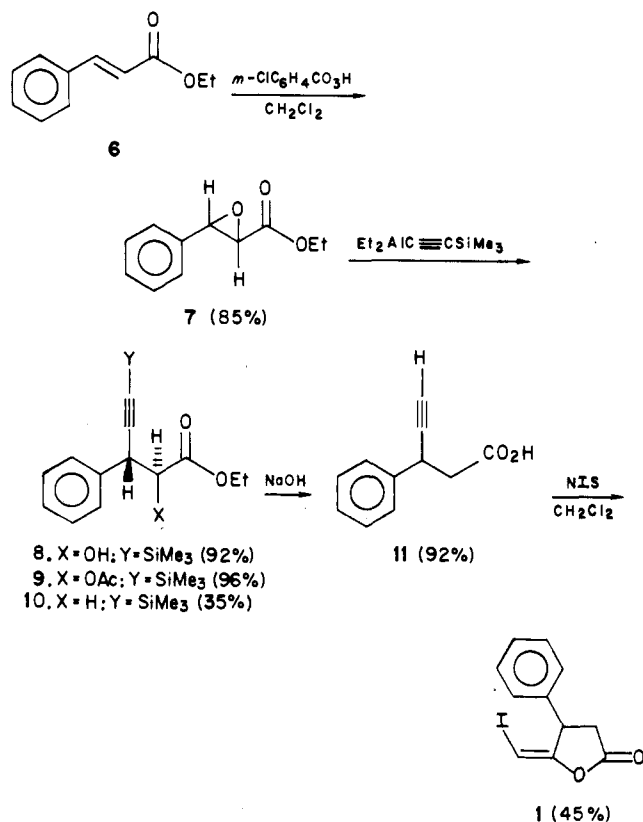
Synthesis of the Halo Enol Lactones. In previous publications,²⁻⁴ we have demonstrated that halo enol lactones can be prepared by an efficient and stereoselective halolactonization of acetylenic acid precursors, with *N*-halosuccinimides. We have used this procedure to prepare

- (1) Rando, R. R. *Science* 1974, 185, 320.
- (2) Chakravarty, P. K.; Krafft, G. A.; Katzenellenbogen, J. A. *J. Biol. Chem.* 1982, 257, 610.
- (3) Krafft, G. A.; Katzenellenbogen, J. A. *J. Am. Chem. Soc.* 1981, 103, 5459.
- (4) Daniels, S. B.; Cooney, E.; Sofia, M. J.; Chakravarty, P. K.; Katzenellenbogen, J. A. *J. Biol. Chem.* 1983, 258, 15046.

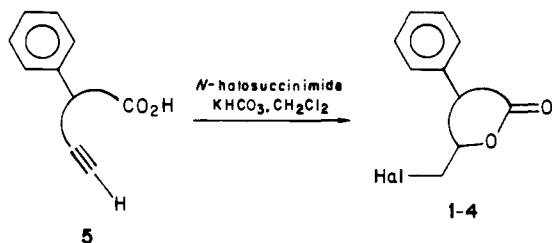
Scheme I



Scheme II



the lactones 1-4. In each case, an appropriately substituted acetylenic acid 5 was required as a precursor.

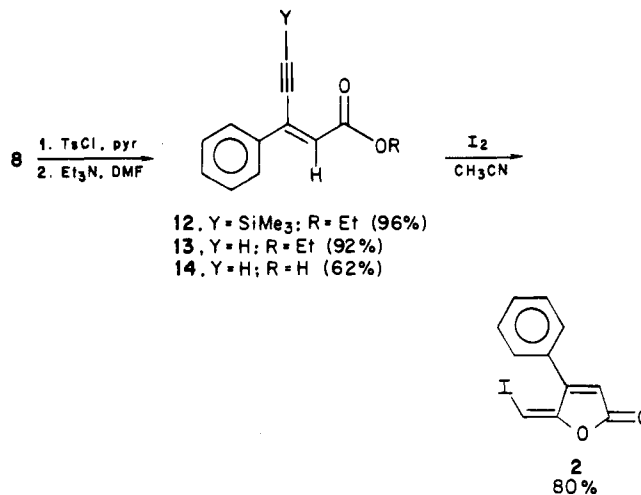


4-Phenyl-5(E)-(iodomethylidene)tetrahydro-2-furanone (1). For the synthesis of the 4-phenylfuranone 1, a β -ethynyldihydrocinnamic acid precursor was required. Attempts to conjugatively ethynylate ethyl cinnamate (6) by utilizing known enone chemistry proved unsuccessful.^{5,6}

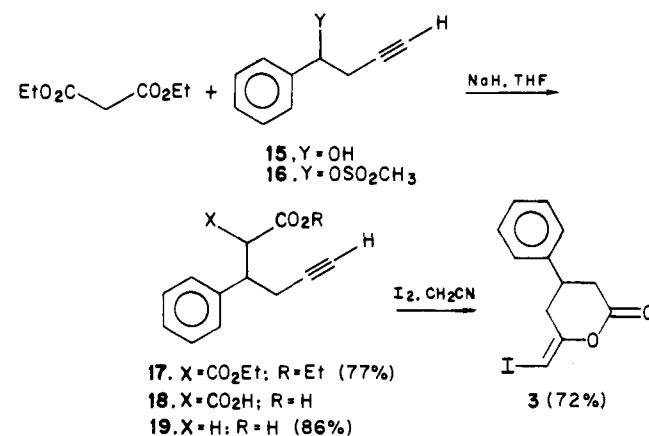
(5) Hooz, J.; Layton, R. *J. Am. Chem. Soc.* 1971, 93, 7320.

(6) Schwartz, J.; Carr, D.; Hansen, R.; Dayrit, F. *J. Org. Chem.* 1980, 45, 3053.

Scheme III



Scheme IV



However, the acetylenic moiety could be introduced β to the ester functionality by regioselective epoxide opening on ethyl epoxycinnamate (7), using diethyl aluminum (trimethylsilyl)acetylene,⁷⁻⁹ to give the β -ethynyl α -hydroxy ester 8 as shown in Scheme II. Cleavage of the α -hydroxyl group was accomplished by careful reductive scission of the acetoxy derivative 9 with Li/NH₃ (liquid).¹⁰ Treatment of the ester 10 with 2 N NaOH resulted in hydrolysis of the ester functionality and cleavage of the trimethylsilyl protecting group, giving the desired acid 11. Lactonization of 11 to the 4-phenylfuranone 1 was accomplished with *N*-iodosuccinimide.

4-Phenyl-5(E)-(iodomethylidene)dihydro-2-furanone (2). To produce the dihydrofuranone 2 (see Scheme III), the hydroxy ester 8 was dehydrated by toluenesulfonylation of the hydroxyl group, followed by base-induced elimination, giving exclusively the *Z* isomer of the ester 12. Treatment with 2 N NaOH in THF resulted in selective cleavage of the trimethylsilyl protecting group, giving 13. Ester hydrolysis was effected with 10% aqueous KOH in methanol, and acid 14 was lactonized to compound 2 with I₂ in CH₃CN.

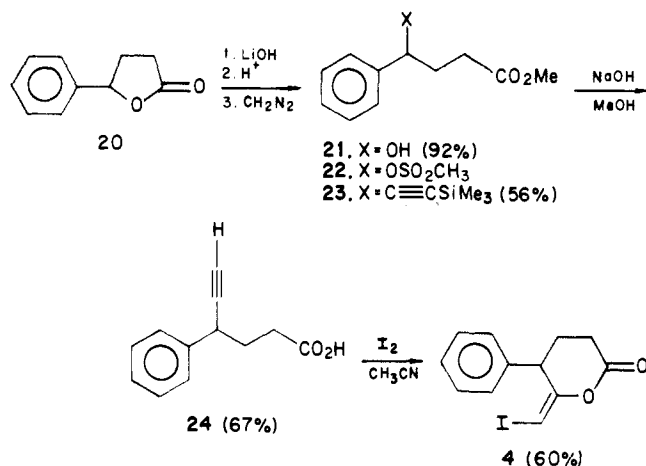
(7) Rousch, W.; Blizzard, T. A.; Basha, F. Z. *Tetrahedron Lett.* 1982, 23, 2331.

(8) Bartlett, P.; Tanzella, D.; Barstow, J. *J. Org. Chem.* 1982, 47, 3941.

(9) Trost, B. M.; McDougal, P. G. *Tetrahedron Lett.* 1982, 23, 5497.

(10) Boar, R. B.; Joukhadar, L.; McGhie, J.; Misra, S.; Barrett, A. G.; Barton, D. H. R.; Prokopiou, P. A. *J. Chem. Soc., Chem. Commun.* 1978, 68.

Scheme V



4-Phenyl-6(*E*)-(iodomethylidene)tetrahydro-2-pyranone (3). The preparation of 4-phenyltetrahydro-2-pyranone 3 utilized the classical malonic ester synthesis (Scheme IV). Diethyl malonate was alkylated with the methanesulfonate 16, which itself was prepared in quantitative yield without purification from the readily available alcohol 15.¹¹ Because methanesulfonate 16 undergoes facile elimination to the enyne during chromatography or even upon standing at -5°C , it must be prepared just prior to use. The modest yield in the preparation of 17 (77%) is attributed to the instability of the electrophile. Saponification in 10% aqueous KOH gave the diacid 18, which was immediately decarboxylated in refluxing *m*-xylene to give the desired acid 19 as a white solid. The desired pyranone 3 was then obtained by iodolactonization of the acid.

5-Phenyl-6(*E*)-(iodomethylidene)tetrahydro-2-pyranone (4). The 5-phenyltetrahydropyranone was prepared according to the route shown in Scheme V. Saponification of the commercially available lactone 20 and in situ esterification with diazomethane after carefully adjusting the pH to 5 furnished the hydroxy ester 21. Reaction of the readily prepared methanesulfonate 22 with diethylaluminum (trimethylsilyl)acetylene at 0°C produced the desired acetylenic ester 23 in 56% overall yield from the alcohol 21.¹² Saponification and desilylation with aqueous NaOH gave the desired acid 24, which was lactonized with iodine in acetonitrile to the crystalline lactone 4.

Chymotrypsin Inactivation Studies. When lactones 1–4 were studied as α -chymotrypsin inhibitors, no new irreversible inhibitors of serine proteases were discovered. However, the temporary inhibition of α -chymotrypsin activity by lactone 3 arises from an interesting mode of halo enol lactone interaction with α -chymotrypsin which had not been appreciated with the lactones I and II studied previously. The results from these investigations are summarized in Table I.

Hydrolysis Rates. The spontaneous rates of hydrolysis (k_h) for lactones 1–4 were determined by following with time the change in the absorbance at the wavelengths corresponding to the peak in the difference spectra (see the Experimental Section).⁴ The rates of hydrolysis were first order, and the k_h values are presented in Table I.

When the rates of hydrolysis for lactones 1 and 4 were observed for lactone solutions containing α -chymotrypsin,

Table I. Binding, Inactivation, and Hydrolysis Rate Constants for Halo Enol Lactones

	K_i , μM	k_c , min^{-1}	k_h , min^{-1}
βPh5I^a (1)	49 ^b		0.0021
βPh6I (3)	0.0053 ^b	0.025 ^c	0.0068
βPh6H (25)			0.0015
Dehydro βPh5I (2)			0.0015
γPh6I (4)	55 ^b		0.0040

^a α,β,γ -position of aryl group relative to lactone carbonyl group; Ph type of aryl substituent; 5,6-lactone ring size; I,H substituent appended in the *E* stereochemistry on the exocyclic methylene unit. ^b Binding constants determined by competitive inhibition assay with BTEE¹⁵ as the substrate for lactones 1 and 4 and Suc-Ala-Ala-Pro-Phe-*p*NA¹⁴ as the substrate for lactone 3. ^c Catalytic hydrolysis rate constant determined from the spontaneous rate of reactivation after Sephadex G-25 chromatography of an α -chymotrypsin incubation solution containing lactone 3.

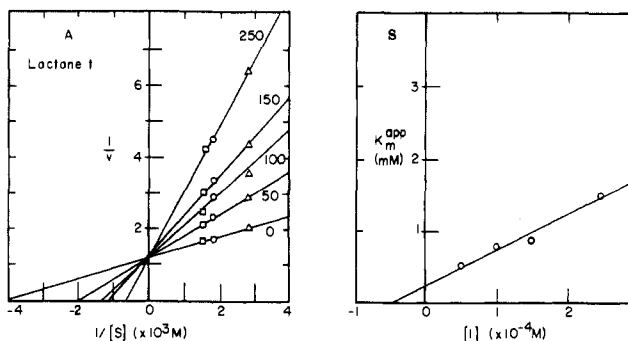


Figure 1. Competitive inhibition assay of α -chymotrypsin with lactone 1. α -Chymotrypsin (50 nM) was added to a solution of BTEE (Hummel, 1959) and inhibitor (50–250 μM) in 80 mM Tris buffer at pH 7.2 and 25°C . Enzyme activity was followed by UV at 256 nm. A. Panel A shows the double-reciprocal plot of $1/\text{velocity}$ vs. $1/[\text{substrate}]$ where at different substrate and inhibitor concentrations, the velocity of BTEE hydrolysis was monitored. The x intercepts are the values of $-1/K_m^{\text{app}}$. B. Panel B shows the plot of the K_m^{app} values from panel A vs. inhibitor concentration. The x intercept is $-K_i$.

no acceleration in the rates of hydrolysis of these lactones was seen. Thus, lactones 1 and 4 do not appear to be substrates for α -chymotrypsin. However, as will be discussed later, lactone 3 did demonstrate a substrate-like interaction with α -chymotrypsin.

Competitive Inhibition Studies. In competitive binding assays, lactones 1–4 all appeared to be active site-directed reagents. Lactones 1 and 4 behaved as competitive inhibitors of α -chymotrypsin. The K_i values for lactones 1 and 4, determined from double-reciprocal plots (see Figure 1 for a representative example), were 49 and 55 μM , respectively. Although lactone 2 did show some substrate protection in a competitive assay, there was no indication that it interacted with α -chymotrypsin as a purely competitive inhibitor.

Because α -chymotrypsin appeared to catalyze the turnover of lactone 3, the binding constant for lactone 3 was estimated by using a competitive assay as suggested for inhibition by competing substrates.¹³ For this assay, enzyme activity was measured with succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide¹⁴ for the first 10–20 s after addition of enzyme to a solution of substrate (0.043–0.10 mM) and inhibitor (50–250 nM). A binding constant ($K_i = K_m$, where K_m is the Michaelis constant for a substrate)

(11) Lauger, P.; Prost, M.; Charlier, R. *Helv. Chim. Acta* 1959, 42, 2379.

(12) Negishi, E.; Baba, S. *J. Am. Chem. Soc.* 1975, 97, 7385.

(13) Cornish-Bowden, A. "Fundamental of Enzyme Kinetics"; Butterworths: Boston, 1979; Chapter 5.

(14) DelMar, E. G.; Largman, C.; Brodrick, J. W.; Geokas, M. C. *Anal. Biochem.* 1979, 99, 316.

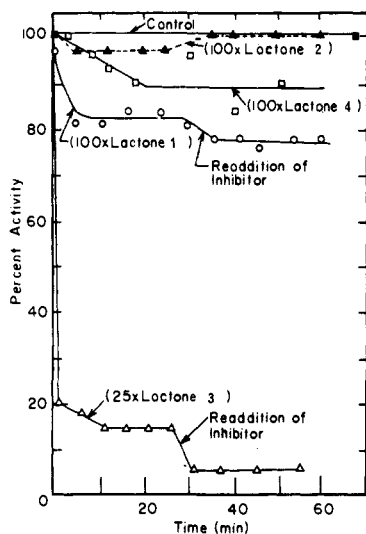


Figure 2. Time-dependent loss of α -chymotrypsin activity (2 μ M) incubated at 25 $^{\circ}$ C in phosphate buffer at pH 7.2 with 100-fold excess of lactone 4 (\square), lactone 1 (\circ), or lactone 2 (\triangle) and 25-fold excess of lactone 3 (Δ). Readdition of inhibitor 1 and 3 to their respective α -chymotrypsin incubation solutions occurred at 30 min. Percent remaining α -chymotrypsin activity is plotted vs. time as assayed with BTEE (535 μ M in 1:1 phosphate buffer and 50/50 MeOH/H₂O). The control contains α -chymotrypsin and 20 μ L of CH₃CN.

of 52.5 nM was determined for lactone 3.

Time-Dependent Inactivation of α -Chymotrypsin by Lactones 1, 2, and 4. Exposure of α -chymotrypsin (2 μ M) to a 100-fold excess of lactone 1, 2, or 4 (200 μ M) resulted, in each case, in only a relatively small time-dependent loss (ca. 20%) of enzymatic activity toward benzoyltyrosine ethyl ester (BTEE)¹⁵ (see Figure 2). Even in the case of the more effective lactone 1, addition of a second aliquot of inhibitor after 30 min caused only a slight additional loss of activity (5%).

Time-Dependent α -Chymotrypsin Inactivation with Lactone 3. In contrast to the inactivations with lactones 1, 2, and 4, when α -chymotrypsin was exposed to only a 25-fold excess of lactone 3, a rapid loss of 85% of the enzyme activity was observed within 1 min, and addition of a second aliquot of inhibitor at 30 min consumed essentially all remaining enzyme activity (see Figure 2).

A leveling off of enzyme activity, as observed in Figure 2 for lactone 3 as well as for lactones 1, 2, and 4, is consistent with a significant amount of enzyme activity regeneration occurring during the inactivation process.¹⁶ In such an inactivation scheme, a steady state is reached when the rate at which the inhibited enzyme breaks down to regenerate free enzyme equals the rate at which enzyme and inhibitor combine to form inactive enzyme. As long as the inhibitor concentration remains constant, the steady state will be reflected by a region in the time course where the activity remains constant (line runs parallel to the time axis), but eventually the activity curve will swing upward, since the inhibitor is being continuously consumed.¹⁶ Consequently, activity regeneration was observed upon continued analysis over 24 h of incubation solutions containing 25-fold or 50-fold excess of inhibitor 3 (see Figure 3). For the incubation solution containing a 25-fold excess of inhibitor, reactivation began after 7.5 h, with 90% of the activity regenerating after 24 h, and for the incubation solution containing 50-fold excess of inhibitor, activity

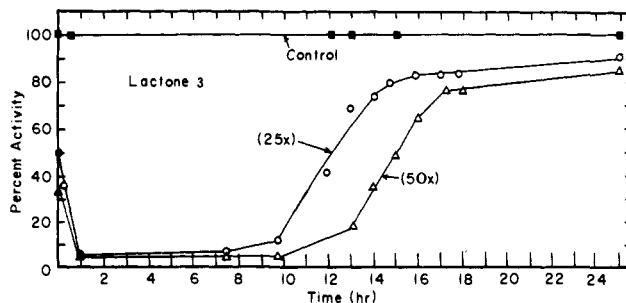


Figure 3. Spontaneous reactivation of α -chymotrypsin inactivated with 25-fold (\circ) and 50-fold (Δ) excess of lactone 3 over a 24-h period at 25 $^{\circ}$ C in 0.1 M phosphate buffer at pH 7.2. Percent remaining enzyme activity is plotted vs. time as assayed with 535 μ M BTEE in phosphate buffer containing 5% v/v CH₃CN. The control solution (\blacksquare) contained only enzyme and 20 μ L of CH₃CN.

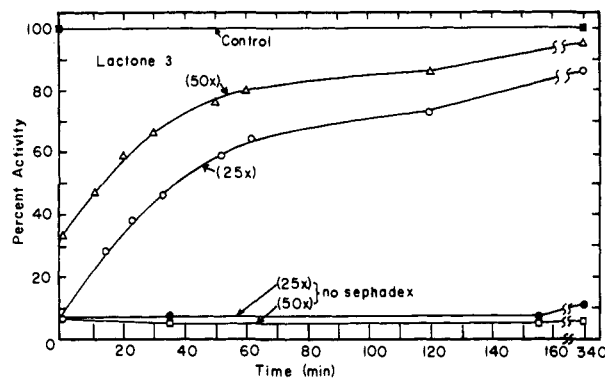


Figure 4. Reactivation of α -chymotrypsin inactivated with 50-fold (Δ) and 25-fold (\circ) excess of inhibitor 3 after passage through a Sephadex G-25 column. The resulting protein eluant was assayed with BTEE in phosphate buffer with 5% v/v CH₃CN (535 μ M). The control solutions containing only enzyme and 40 μ L of CH₃CN were passed through identical Sephadex columns. Parallel enzyme-inhibitor incubation solutions with 50-fold (\square) and 25-fold (\bullet) excess of inhibitor were not passed through Sephadex columns.

regeneration began after 10 h, with 85% of the activity regenerating after 24 h.

Sephadex G-25 Chromatography Reactivation of α -Chymotrypsin Inactivated with Lactone 3. In order to investigate further the nature of the inactivation of α -chymotrypsin by lactone 3, specific reactivation experiments were undertaken. α -Chymotrypsin (4 μ M) was incubated with either 0 μ M (control), 100 μ M (25-fold excess), or 200 μ M (50-fold excess) of lactone 3 for 4 h, at which time the lactone incubations had only 7% the activity of the control.¹⁵ To remove any unreacted inhibitor or hydrolysis product, aliquots of the incubation solutions were then passed through identical Sephadex G-25 columns pre-equilibrated with phosphate buffer. The resulting protein eluants were then assayed with BTEE,¹⁵ and the enzyme activity was compared to that of the control incubation. Reactivation of enzyme activity began with both lactone incubations immediately after passage through the Sephadex columns; the rate constant for reactivation was 0.025 min⁻¹ (Figure 4).

Hydrazine is known to accelerate the deacylation rate of acylchymotrypsin.¹⁷ An accelerated rate of regeneration of enzyme activity was observed (Figure 5) when hydrazine was added to a solution of chymotrypsin that had been treated with a 25-fold excess of inhibitor and then passed through a Sephadex column. With 25 mM hydrazine,

(15) Hummel, B. C. W. *Can. J. Biochem.* 1959, 37, 1393.

(16) Main, A. R. In "Essays in Toxicology"; Hays, W. J., Ed.; Academic Press: New York, 1973; Vol 4.

(17) Fastrez, J.; Fersht, A. R. *Biochemistry* 1973, 12, 2025.

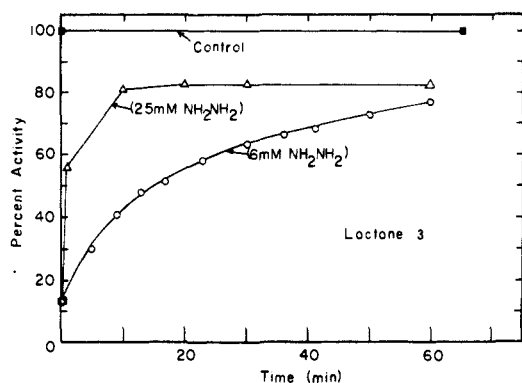
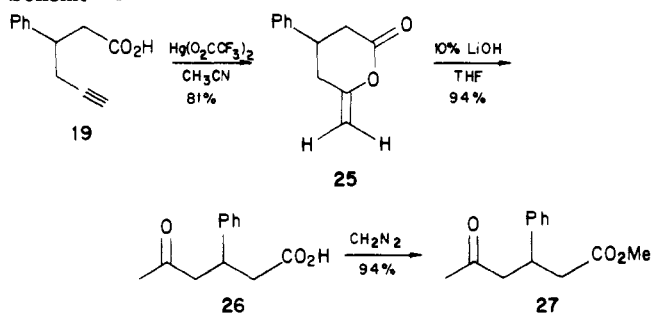


Figure 5. Acceleration of activity regeneration of α -chymotrypsin inactivated with lactone 3 by addition of hydrazine after Sephadex G-25 chromatography of a 4-h preincubation of α -chymotrypsin ($4 \mu\text{M}$) and lactone 3 ($100 \mu\text{M}$), at which time 90% loss of enzyme activity was observed. Equivolumes of protein eluant and 50 mM hydrazine (Δ) or 12 mM hydrazine (\circ) were combined and α -chymotrypsin activity was assayed with $535 \mu\text{M}$ BTEE in phosphate buffer containing 5% v/v CH_3CN . The control containing enzyme and $40 \mu\text{L}$ of CH_3CN was passed through an identical Sephadex column and combined with hydrazine.

Scheme VI



activity regeneration was very rapid, resulting in 83% activity recovery within 10 min; 6 mM hydrazine gave a 76% recovery within 60 min (Figure 5).

A Comparative Inactivation Study: Iodolactone 3 vs. Diprotiolactone 25. In an effort to study further the mode of chymotrypsin inactivation by lactone 3, as well as the role played by the halogen, we prepared lactone 25, the diprotio lactone analogue of 3. As shown in Scheme VI, mercury lactonization of the acetylenic acid 19 provided this diprotio lactone 25 in 81% yield.³ Its spontaneous hydrolysis rate was 0.00148 min^{-1} .

Although no time-dependent loss of activity was expected for lactone 25, a 70% time-dependent loss of enzyme activity was observed over 1 h at 25-fold, 62.5-fold, and 100-fold excess of inhibitor relative to enzyme concentration (see Figure 6). Also, no spontaneous activity regeneration seemed to occur over 22 h (data not shown).

To account for the possible role of the lactone hydrolysis product in the inactivation profile of diprotio lactone 25, the keto acid 26 was prepared by LiOH hydrolysis of lactone 25 (Scheme VI). This acid provided only a 7% time-dependent inactivation of α -chymotrypsin over 1 h and was only a modest competitive inhibitor, with a $K_i = 25 \mu\text{M}$. Therefore, it was discounted as a major contributor to the inactivation produced by lactone 25.

To investigate the intermediacy of a slowly decomposing imine (that might form between the ketone in the inactivator hydrolysis product and lysine residue) as a contributor to the inhibition by lactone 25, the methyl ester 27 of hydrolysis product 26 was prepared (Scheme VI). It was hoped that this ester would act as a substrate and acylate the active serine residue, thereby giving the same

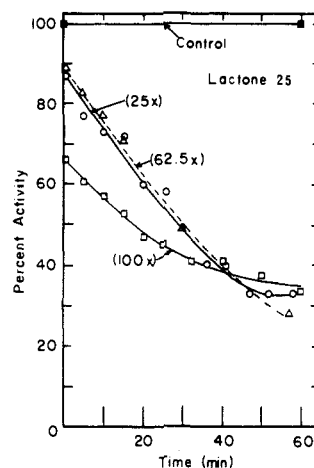


Figure 6. Time-dependent loss of α -chymotrypsin activity when incubated at $2 \mu\text{M}$ with lactone 25 at 25- (\bullet), 62.5- (Δ), and 100-fold (\square) excess in phosphate buffer at pH 7.2 and 25°C . α -Chymotrypsin was assayed with BTEE ($535 \mu\text{M}$ in 1:1 phosphate buffer and 50/50 MeOH/ H_2O).

acyl enzyme as would be produced from lactone 25. However, this ester provided only a 5–7% loss of enzyme activity, which is probably a reflection of the fact that its acylation activity is much less than that of the enol lactone 25.

The possibility that lactone 25 was forming a stable acyl enzyme was investigated by a hydrazine reactivation experiment on the inactivated enzyme.¹⁷ After α -chymotrypsin was exposed to a 50-fold excess of lactone 25 for 1.5 h, 10% of the initial enzyme activity remained. No spontaneous enzyme activity regeneration occurred over 40 min after Sephadex chromatography; however, when equal volumes of the protein fraction and 5 mM hydrazine were combined at 25°C , enzyme activity quickly regenerated (to 95% of a control solution over 30 min; data not shown).

Discussion

In this study, we have investigated the effect that the position of aryl substitution in halo enol lactones has on their character as inactivators of chymotrypsin. It appears from the 3- and 4-phenyltetrahydro-2-furanones (I, 1 and 2) and the 3-, 4-, and 5-phenyltetrahydro-2-pyranones (II, 3 and 4) that have now been studied that only the previously investigated 3-aryl systems (I and II) are capable of acting as true enzyme-activated irreversible inhibitors (suicide substrates). The β -substituted 2-furanones (1 and 2) and the γ -substituted 2-pyranone (4) were only reversible (mostly) competitive inhibitors. The β -substituted 2-pyranones (3), however, did cause a time-dependent but reversible inactivation of chymotrypsin.

From the spontaneous and hydrazine-accelerated reactivation experiments done with both the iodo lactone 3 and the diprotio lactone 25, it appears that the inactivation of α -chymotrypsin by these two compounds results from the formation of slowly deacylating acyl enzymes. It is also evident from these experiments that the diprotio lactone 25 forms a more stable acyl enzyme than does iodo lactone 3. The difference in acyl enzyme stability can only be speculated upon: After formation of the acyl-chymotrypsin, the diprotio lactone 25 reveals a methyl ketone, whereas the iodo lactone 3 reveals an iodo ketone. If the iodo ketone 3 does not react with an active site amino acid, displacement of the iodine would likely proceed to give an α -hydroxy keto acyl enzyme. (Such a hydrolysis in aqueous medium was noted in the lactone hydrolysis rate studies reported earlier.⁴) The hydroxyl group in this

hydroxymethyl ketone could provide additional modes for acyl enzyme decomposition, e.g., by attacking the acyl enzyme intramolecularly or by acting as a general base. These modes of decomposition are not available to the methyl ketone acyl enzyme formed from the diprotio lactone 25.

A qualitative comparison of the rate of inactivation of α -chymotrypsin by lactones 3 and 25 shows that the iodo lactone 3 inactivates α -chymotrypsin more rapidly than does the diprotio lactone 25. This reactivity difference is also mirrored in the rates of spontaneous hydrolysis of lactones 3 and 25: The iodo lactone hydrolyzes 4.6 times faster than the diprotio lactone (Table I). These reactivity differences are presumably due to the electron withdrawing nature of the iodine which increases the electrophilicity of the carbonyl carbon in lactone 3.

The enzyme inactivation results presented in this study may be rationalized by invoking common orientational arguments.^{4,18-20} In the case of lactones 1, 2, and 4, if the aromatic residue is assumed to bind in the hydrophobic pocket of α -chymotrypsin, then the relative orientation of the hydroxyl group of serine-195 and the lactone carbonyl group may be such that acylation cannot occur. Or, if acylation does occur, the revealed α -halo ketone may be held in such an orientation that nucleophilic amino acids are inaccessible. This inaccessibility may be especially pronounced in lactones 1, 2, and 4, because the carbon chain length, from the aromatic residue—a point of effective attachment to the enzyme—to the halo ketone, is relatively short compared to that for lactones I and II.

In the case of lactone 3, active-site acylation of α -chymotrypsin does occur. Again, however, because of possible orientational changes caused by a β -aryl group binding in the hydrophobic region (compared to the α -aryl halo enol lactone acyl enzymes derived from lactones I and II), nucleophilic active-site amino acids may be inaccessible for reaction with the iodo ketone; consequently, the resultant acyl enzyme simply undergoes deacylation to regenerate active enzyme. An issue of considerable interest is the high stability of the acyl enzymes produced from iodo lactone 3 and protio lactone 25.

At the moment, we are not certain why the deacylation rate of the lactone 25 is so slow (no deacylation detected over 22 h in the absence of hydrazine). For example, by contrast, the deacylation rates for cinnamyl- and pivaloylchymotrypsin, under comparable conditions, are ca. 0.21 and 0.006 min⁻¹, respectively.^{21,22} In preliminary studies, we have investigated this issue of deacylation by molecular graphics,²³ and it appears that the carbonyl group of the methyl ketone in the acyl enzyme derived from lactone 25 is involved in a hydrogen-bonding pattern

that appears to block the access of water to the acyl serine linkage. Further investigations of this problem are in progress.

In conclusion, we have demonstrated that the aryl substitution pattern in halo enol lactones has a pronounced effect on their capacity to act as inactivators of α -chymotrypsin, for in the class of simple phenyl-substituted lactones, only the α -aryl lactones I and II behave as true enzyme-activated irreversible inhibitors. Nevertheless, the very stable acyl enzymes formed by the β -aryl-substituted lactones 3 and 25 provide an intriguing basis for further developments in the design of mechanism-based serine protease inhibitors.

Experimental Section

A. Synthetic Methods. General Procedures. Analytical thin-layer chromatography was performed with 0.25-mm silica gel glass-backed plates with F-254 indicator (Merck). Visualization was by ultraviolet light, iodine, or phosphomolybdic acid. All column chromatography was done by using the flash chromatography technique as described by Still et al.²⁴ The column packing was Woelm 32-63- μ m silica gel.

Proton magnetic resonance (¹H NMR) chemical shifts are reported in ppm downfield from a tetramethylsilane internal standard (δ scale), and data are presented in the form: δ value of signal (peak multiplicity, integrated number of protons, coupling constant (if applicable)). Infrared (IR) spectral data are presented as reciprocal centimeters for important diagnostic absorptions. Mass spectra data are reported in the form: m/z (intensity relative to base peak = 100). Melting points are uncorrected.

Chemicals were obtained from the following sources: Aldrich Chemical Co., triethylamine, 4-(dimethylamino)pyridine (DMAP), dimethylformamide, *m*-chloroperoxybenzoic acid (mCPBA), methanesulfonyl chloride, γ -phenyl- γ -butyrolactone; Alfa (Ventron), *n*-butyllithium in hexane, sodium hydride, diethylaluminum chloride; Parish, *N*-iodosuccinimide (NIS); Petrarch (Farchan), (trimethylsilyl)acetylene.

Tetrahydrofuran (THF) was distilled from sodium benzophenone ketyl prior to use. Dimethylformamide (DMF), methylene chloride, and triethylamine were refluxed over calcium hydride and then distilled to ensure dryness. The organolithium reagents were titrated periodically to determine the organic base present, using either the double-titration method²⁵ or the single-titration method with 1,10-phenanthroline as indicator.²⁶

The following compound was prepared according to literature procedures: 1-phenyl-3-butyn-1-ol.¹¹

Ethyl 2,3-epoxy-3-phenylpropionate (7): prepared according to literature procedure with the modification of extended reaction time (4 days) in refluxing CH₂Cl₂/*trans*-ethyl cinnamate (5.0 g, 28 mmol), CH₂Cl₂ (100 mL), CH₂Cl₂ (75 mL) solution of *m*-chloroperoxybenzoic acid (7.33 g, 43 mmol). Purification by flash chromatography, eluting with 1:5.7 acetone/hexane, gave 4.63 g (85%) of the *trans*-epoxide 7²⁷ as an oil: IR (CHCl₃) 1725, 1240 cm⁻¹; NMR (CDCl₃) δ 7.35 (s, 5), 4.30 (q, 2 *J* = 2.0 Hz), 4.10 (d, 1, *J* = 2.0 Hz), 3.50 (d, 1, *J* = 2.0 Hz), 1.30 (t, 3, *J* = 7.0 Hz). Anal. C, H.

Ethyl 2-Hydroxy-3-phenyl-5-(trimethylsilyl)-4-pentynoate (8). (Trimethylsilyl)acetylene (Petrarch; 4.99 g, 51 mmol) was added to 93 mL of hexane at 0 °C under N₂ atmosphere. *n*-Butyllithium (1.44 M in hexane (35.3 mL)) was added to the acetylene solution at 0 °C; sufficient dry Et₂O was added to dissolve the salts. This solution was stirred at -40 °C for 1.5 h to give the lithium acetylide. The lithium acetylide solution was transferred under N₂ atmosphere to a dry reaction flask that contained Et₂AlCl (25% in hexane) (25.4 mL, 51 mmol), producing a white precipitate. This solution was then stirred for 3.5 h at 25 °C; the precipitate was allowed to settle, and the supernatant was

(18) Walsh, C. "Enzyme Reaction Mechanisms"; Freeman: San Francisco, 1979; Chapter 3.

(19) Kraut, J. *Annu. Rev. Biochem.* 1977, 46, 331.

(20) Blow, D. M. In "The Enzymes"; Boyer, P., Ed.; Academic Press: New York, 1971; Chapter 6.

(21) Bender, M. L.; Schonbaum, G. R.; Zomer, B. *J. Am. Chem. Soc.* 1962, 84, 2562.

(22) For other examples of chymotrypsin inhibition by stable acyl enzymes, see: (i) Wang, C.-C.; Shaw, E. *Arch. Biochem. Biophys.* 1972, 150, 259. (b) Geeb, M. H.; Abeles, R. H. *Biochemistry* 1984, 23, 6569. (c) Hestrom, L.; Moorman, A. R.; Dobbs, J.; Abeles, R. H. *Biochemistry* 1984, 23, 1753. (d) Harper, J. W.; Powers, J. C. *J. Am. Chem. Soc.* 1984, 106, 7618.

(23) Naruto, S.; Motoc, I.; Marshall, G. R.; Sofia, M. J.; Katzenellenbogen, J. A., unpublished. Earlier molecular graphics studies on halo enol lactone interactions with chymotrypsin have appeared: Naruto, S.; Motoc, I.; Marshall, G. R.; Daniels, S. B.; Sofia, M. J.; Katzenellenbogen, J. A. *J. Am. Chem. Soc.* 1985, 107, 5262.

(24) Still, W. C.; Kahn, M.; Mitra, A. *J. Org. Chem.* 1978, 43, 2923.

(25) Whitesides, G. M.; Casey, C. P.; Kreiger, J. K. *J. Am. Chem. Soc.* 1971, 93, 1379.

(26) Watson, S. C.; Eastham, J. F. *J. Organomet. Chem.* 1967, 9, 165.

(27) Valente, V.; Wolfhagen, J. *J. Org. Chem.* 1966, 31, 2509.

transferred to a third dry reaction flask. A hexane solution of the epoxide 7 was added to the transferred supernatant at 25 °C. After stirring at 35 °C for 3.5 h, the reaction was cooled in an ice bath and quenched with 0.3 M HCl. The resulting solution was washed with 0.3 M HCl and H₂O, and the organic extract was dried over Na₂SO₄, followed by filtration and solvent removal to give a yellow oil. After purification by flash chromatography, eluting with 1:4 EtOAc/hexane, 6.30 g (92%) of the acetylenic hydroxy ester 8 was obtained: IR (CHCl₃) 3400, 2175, 1725 cm⁻¹; NMR (CDCl₃) δ 7.10 (s, 5), 4.20 (d, 1, *J* = 4.5 Hz), 3.93 (d, 1, *J* = 4.5 Hz), 3.90 (q, 2, *J* = 7.0 Hz), 2.58 (br s, 1), 0.98 (t, 3, *J* = 7.0 Hz), 0.0 (s, 9); mass spectrum, *m/z* (relative intensity) 290 (2.64, M⁺), 217 (5.6), 200 (100), 187 (100). Anal. C, H.

Ethyl 2-Acetoxy-3-phenyl-5-(trimethylsilyl)-4-pentynoate (9). The hydroxy ester 8 (1.0 g, 3.45 mmol) was stirred at 25 °C with CH₂Cl₂ (15 mL). Et₃N (0.384 g, 3.8 mmol) acetic anhydride (0.387 g, 3.8 mmol), and DMAP (0.046 g, 0.38 mmol) were added to the CH₂Cl₂/ester solution. This reaction mixture was stirred for 5 h at 25 °C. All the volatiles were removed, and the residue was solubilized in CH₂Cl₂. The CH₂Cl₂ extract was washed with H₂O and saturated NaHCO₃ solution. The organic extract was dried over MgSO₄ and filtered to give 1.26 g of an oil after solvent removal. Purification by flash chromatography, eluting with 1.5:8.5 EtOAc/hexane, gave 1.10 g (96%) of the acetate 9: IR (CHCl₃) 2150, 1750 (br) cm⁻¹; NMR (CDCl₃) δ 7.10 (s, 5), 5.30 (d, 1, *J* = 6.0 Hz), 4.98 (d, 1, *J* = 6.0 Hz), 4.90 (q, 2, *J* = 7.0 Hz), 1.88 (s, 3), 1.00 (t, 3, *J* = 7.0 Hz), 0.0 (s, 9); mass spectrum, *m/z* (relative intensity) 322 (3.8, M⁺), 273 (100), 259 (100), 217 (56), 187 (100). Anal. C, H.

Ethyl 3-Phenyl-5-(trimethylsilyl)-4-pentynoate (10). Liquid ammonia (36 mL) was distilled from sodium metal into a dry reaction flask at -78 °C under a N₂ atmosphere. Lithium metal (0.026 g, 3.7 mmol) was added to the distilled ammonia at -78 °C. A diethyl ether (6 mL) solution of the acetoxy ester 9 (0.683 g, 2.0 mmol) was added very slowly to the Li/NH₃ (liquid) at -78 °C. After the reaction mixture was stirred for 40 min at -78 °C, it was quenched with solid NH₄Cl (1.7 g) and then stirred at -78 °C for 30 min. The NH₃ was then allowed to evaporate, and the remaining orange residue was solubilized in Et₂O. The Et₂O extract was washed with H₂O and a saturated NaCl solution, dried over MgSO₄, and then filtered to give 0.40 g of an oil after solvent removal. Purification by flash chromatography, eluting with 1:9 EtOAc/hexane, afforded 1.80 g (35%) of the desired ester 10: IR (CHCl₃) 2150, 1720, 1600 cm⁻¹; NMR (CDCl₃) δ 7.00 (m, 5), 3.92 (d, 2, *J* = 7.0 Hz), 3.83 (t, 2, *J* = 7.0 Hz), 2.59 (d, 1, *J* = 7.0 Hz), 2.55 (d, 1, *J* = 7.0 Hz), 0.98 (t, 3, *J* = 7.0 Hz); mass spectrum, *m/z* (relative intensity) 274 (37, M⁺), 200 (11), 187 (11), 73 (100). Anal. High-resolution MS calcd for C₁₆H₂₂O₂Si, 274.1393; found, 274.1391.

3-Phenyl-4-pentynoic Acid (11). The ester 10 (0.17 g, 0.62 mmol) was stirred in methanol (6 mL) with 2 N NaOH (2.55 mL) at 25 °C for 2.5 h. Then the reaction mixture was acidified with concentrated HCl. The methanol was removed under vacuum, and the remaining solution was extracted three times with CH₂Cl₂. The CH₂Cl₂ extract was dried over MgSO₄, and after filtration and solvent removal, 0.10 g (92%) of a yellow oil remained. Purification by preparative thin-layer chromatography, developing with EtOAc/AcOH/hexane (4.2:0.1:5.7), gave 0.047 g (43%) of a white solid 11: mp 85–88 °C; IR (KBr) 3700–2600, 3300, 1730 cm⁻¹; NMR (CDCl₃) δ 10.00 (br s, 1), 7.39 (m, 5), 4.15 (dt, 1, *J* = 2.0 Hz, *J* = 7.0 Hz), 2.84 (d, 1, *J* = 7.0 Hz), 2.80 (d, 1, *J* = 7.0 Hz), 2.20 (d, 1, *J* = 2.0 Hz); mass spectrum, *m/z* (relative intensity) 174 (4, M⁺), 129 (13), 128 (36), 114 (100), 77 (19). Anal. High-resolution MS calcd for C₁₁H₁₀O₂, 174.0680; found, 174.0671.

4-Phenyl-5(E)-(iodomethylidene)tetrahydro-2-furanone (1). The acetylenic acid 11 (0.051 g, 0.29 mmol) was stirred with CH₂Cl₂ at -10 °C under N₂ atmosphere. To the acid solution was added NIS (0.072 g, 0.32 mmol) and solid KHCO₃ (0.029 g, 0.29 mmol), sequentially. This reaction mixture was stirred at -10 °C for 40 min. The reaction was quenched with a 5% Na₂S₂O₃ solution. The CH₂Cl₂ extract was washed with H₂O and dried over Na₂SO₄. After filtration and solvent removal, the resulting white solid was recrystallized twice from Et₂O and hexane to give 0.040 g (45%) of white crystalline lactone 1: mp 84–86 °C; IR (KBr) 3700–3100, 1805, 1650 cm⁻¹; NMR (CDCl₃) δ 7.29 (m, 5), 6.0 (d, 1, *J* = 2.0 Hz), 4.33 (ddd, 1, *J* = 10.5 Hz, *J* = 3.5 Hz, *J*

= 2.0 Hz), 3.24 (dd, 1, *J* = 18 Hz, *J* = 10.5 Hz), 2.69 (dd, 1, *J* = 18 Hz, *J* = 3.5 Hz); mass spectrum, *m/z* (relative intensity) 299.8 (47, M⁺), 173 (14), 140 (1), 131 (39), 128 (3), 77 (8). Anal. High-resolution MS calcd for C₁₁H₉O₂I, 299.9647; found, 299.9648.

Ethyl 3-Phenyl-5-(trimethylsilyl)-2-penten-4-ynoate (12). The hydroxy ester 8 (1.0 g, 3.4 mmol) and dry pyridine (0.5 mL) were stirred with *p*-toluenesulfonyl chloride (0.65 g, 3.4 mmol) at 25 °C for 25 h under N₂ atmosphere. The reaction mixture was then diluted with Et₂O and washed twice with a saturated CuSO₄ solution and once with water. The organic extract was dried over MgSO₄ and filtered to give the tosylate as a clear oil (1.12 g): NMR (CDCl₃) δ 7.30 (d, 2, *J* = 8.0 Hz), 7.08 (s, 5), 6.95 (d, 2, *J* = 8.0 Hz), 4.55 (d, 1, *J* = 7.5 Hz), 3.95 (q, 2, *J* = 7.0 Hz), 3.95 (d, 1, *J* = 7.5 Hz), 2.20 (s, 3), 1.03 (t, 3, *J* = 7.0 Hz), 0.0 (s, 9).

Because the tosylate could not be purified, the unpurified tosylate was warmed in DMF (10 mL) with Et₃N (0.3 mL) at 70 °C under a N₂ atmosphere for 2 h. The reaction mixture was cooled to room temperature and diluted with Et₂O. The diluted reaction mixture was washed several times with H₂O. The Et₂O extracts were dried over MgSO₄ and then filtered to give a yellow oil (0.91 g). Purification of the oil by flash chromatography, eluting with 1:9 EtOAc/hexane, gave 0.899 g (96%) of the unsaturated ester 12 as a clear oil: IR (CHCl₃) 2900, 2125, 1700 (br), 1575, 850, 690 cm⁻¹; NMR (CDCl₃) δ 7.30 (m, 2), 6.97 (m, 3), 6.13 (s, 1), 3.90 (q, 2, *J* = 7.0 Hz), 1.00 (t, 3, *J* = 7.0 Hz), 0.00 (s, 9); mass spectrum, *m/z* (relative intensity) 272 (6.3 M⁺), 257 (70), 99 (51), 97 (33), 73 (100). Anal. High-resolution MS calcd for C₁₆H₂₀O₂Si, 272.1238; found, 272.1235.

Ethyl 3-Phenyl-2-penten-4-ynoate (13). The ester 12 (0.899 g, 3.3 mmol) was stirred in 100 mL of THF at 25 °C. To this mixture was added 2 N NaOH (4.70 mL, 9.4 mmol) and the entire reaction mixture was stirred at 25 °C for 1.5 h. The reaction mixture was then acidified with concentrated HCl to pH 4, and from this mixture the THF was removed under vacuum. To the remaining material was added 30 mL of CH₂Cl₂, and this organic solution was washed with H₂O. The CH₂Cl₂ extract was dried over MgSO₄. After filtration and solvent removal, a white solid remained. Crystallization from Et₂O and hexane afforded 0.61 g (92%) of the ester 13: mp 84–86 °C; IR (CHCl₃) 3230, 2080, 1700, 1590 cm⁻¹; NMR (CDCl₃) δ 7.70 (m, 2), 7.38 (m, 3), 6.65 (s, 1), 4.27 (q, 2, *J* = 7.0 Hz), 3.75 (s, 1), 1.40 (t, 3, *J* = 7.0 Hz); mass spectrum, *m/z* (relative intensity) 200 (1.6 M⁺), 171 (100), 155 (41), 127 (47), 103 (5). Anal. High-resolution MS calcd for C₁₃H₁₂O₂, 200.0837; found, 200.0847.

3-Phenyl-2-penten-4-ynoic Acid (14). The ester 13 (0.573 g, 2.9 mmol) was stirred with MeOH (24 mL) and 10% aqueous KOH (7.0 mL) at 25 °C for 2 h 20 min. The reaction mixture was then acidified with 6 N HCl to pH 1. The MeOH and water were removed under vacuum, and the residue was solubilized in CH₂Cl₂. The CH₂Cl₂ solution was washed with water, and the organic extract was dried over MgSO₄. After filtration and solvent removal, 0.503 g of a yellow solid was obtained. The solid was crystallized from Et₂O and hexane to give 0.307 g (62%) of the acetylenic acid 14: mp 108–110 °C; IR (CHCl₃) 3250, 3500–2400, 2080, 1690, 1600 cm⁻¹; NMR (CDCl₃) δ 9.60 (br s, 1), 7.74 (m, 2), 7.40 (m, 2), 6.67 (s, 1), 3.90 (s, 1); mass spectrum, *m/z* (relative intensity) 172 (22, M⁺), 144 (30), 127 (33), 116 (100), 77 (27). Anal. High-resolution MS calcd for C₁₁H₈O₂, 172.0524; found, 172.0520.

4-Phenyl-5(E)-(iodomethylidene)dihydro-2-furanone (2). A CH₃CN (6 mL) solution of the acetylenic acid 14 (0.070 g, 0.41 mmol) was stirred at 25 °C under a N₂ atmosphere. A CH₃CN (2 mL) solution of iodine (0.31 g, 1.2 mmol) was added dropwise to the acid solution followed by addition of KHCO₃ (0.040 g, 0.41 mmol). The reaction mixture was stirred at 25 °C for 16 h under N₂ atmosphere. The same product isolation procedure as used for lactone 1 furnished 0.10 g of lactone 2 as an oil. The oil was crystallized from Et₂O and hexane three times (0.097 g, 80%): mp 75–76 °C; UV_{max} (CH₃CN) 310 nm (ε 15 850); IR (KBr) 1765, 1620, 1583 cm⁻¹; NMR (CDCl₃) δ 7.40 (m, 5), 6.72 (d, 1, *J* = 2.0 Hz), 6.28 (d, 1, *J* = 2.0 Hz); mass spectrum, *m/z* (relative intensity) 298 (29, M⁺), 171 (77), 140 (12), 115 (100). Anal. High-resolution MS calcd for C₁₁H₇O₂I, 297.9491; found, 297.9497.

Diethyl 2-(1-Phenyl-3-butynyl)malonate (17). Diethyl malonate (3.44 g, 21.5 mmol) was added to a suspension of NaH (0.516 g, 21.5 mmol) in dry THF under N₂ atmosphere at 25 °C.

After stirring the anion for 20 min at 25 °C, the methanesulfonate 16 [5.3 g, 25.9 mmol; prepared from 1-phenyl-3-butyn-1-ol by treatment in CH₂Cl₂ with triethylamine (1 equiv) and methanesulfonyl chloride (1 equiv) for 10 min followed by quenching with saturated NaHCO₃ and isolation by extraction with CH₂Cl₂: NMR (CDCl₃) δ 7.30 (s, 5), 5.53 (t, 1, *J* = 7.0 Hz), 2.80 (m, 2), 2.70 (s, 3), 1.92 (t, 1, *J* = 2.5 Hz)] was then added as a THF (30 mL) solution. The reaction mixture was refluxed at 70 °C for 6.5 h followed by stirring at 25 °C for 18 h. After the reaction was quenched with saturated NH₄Cl solution and THF was removed under vacuum, an Et₂O solution of the residue was washed with saturated NH₄Cl, saturated NaHCO₃ solution, and H₂O. The combined organic extracts were dried over MgSO₄ to give 6.2 g of an oil after filtration and solvent removal. Purification by flash chromatography 20% EtOAc/80% hexane gave 4.76 g (77%) of the desired diester 17: IR (film) 3200, 2930, 1740, 1730 cm⁻¹; NMR (CDCl₃) δ 7.27 (s, 5), 4.20 (q, 2, *J* = 6.0 Hz), 3.90 (q, 2, *J* = 6.0 Hz), 3.70 (m, 2), 2.65 (m, 2), 1.93 (t, 1, *J* = 2.5 Hz), 1.28 (t, 3, *J* = 6.0 Hz), 0.95 (t, 3, *J* = 6.0 Hz); mass spectrum, *m/z* (relative intensity) 288 (1.5, M⁺), 249 (1), 215 (27), 214 (14), 160 (36), 128 (100). Anal. High-resolution MS calcd for C₁₇H₂₀O₄, 288.1361; found, 288.1364.

3-Phenyl-5-hexynoic Acid (19). The diester 18 (1.28 g, 44 mmol) was dissolved in MeOH (18 mL) and stirred with a 10% aqueous KOH solution (45.5 mL) at 25 °C for 16 h. The methanol was then removed under vacuum, and the remaining aqueous solution was extracted with Et₂O. The aqueous solutions were combined and acidified with 6 N HCl to pH 1. The resulting aqueous solution was then extracted several times with EtOAc. The organic extracts were dried over MgSO₄ and filtered to give 0.988 g (96%) of a white solid diacid 18: NMR (CDCl₃) δ 10.60 (s, 2), 7.30 (s, 5), 4.00 (d, 1, *J* = 10.5 Hz), 3.47 (m, 1), 1.90 (t, 1, *J* = 2.5 Hz). The diacid was then stirred in *m*-xylene at 135 °C for 2 h 45 min. The xylene solution was cooled to 25 °C and was then extracted several times with 2 N NaOH. The NaOH extracts were combined and then acidified to pH 1. The acidified solution was extracted several times with CH₂Cl₂, and the combined organic extracts were dried over MgSO₄. The resulting solid, after filtration and solvent removal, was purified by flash chromatography, eluting with 45% EtOAc/1.0% AcOH/54% hexane, to give 0.624 g (88%) of the desired acid 19: mp 86–88 °C; IR (CHCl₃) 3310, 3020, 2400, 1710 cm⁻¹; NMR (CDCl₃) δ 11.10 (br s, 1), 7.30 (s, 5), 3.30 (m, 1), 2.80 (dd, 2, *J* = 11.0 Hz, *J* = 6.0 Hz), 2.50 (dd, 2, *J* = 6.0 Hz, *J* = 2.5 Hz), 1.95 (t, 1, *J* = 2.5 Hz); mass spectrum, *m/z* (relative intensity) 188 (1, M⁺), 149 (31), 143 (15), 142 (11), 128 (100), 107 (98). Anal. High-resolution MS calcd for C₁₂H₁₂O₂, 188.0841; found, 188.0831.

4-Phenyl-6(E)-(iodomethylidene)tetrahydro-2-pyranone (3). The acid 19 (0.100 g, 0.53 mmol) in CH₃CN (15 mL) was stirred with iodine (0.270 g, 1.06 mmol) and KHCO₃ (0.054 g, 0.53 mmol) at 25 °C for 23 h. The reaction was quenched with a 5% Na₂S₂O₃ solution and extracted with CH₂Cl₂. The organic extracts were dried over Na₂SO₄ and filtered to give 0.153 g of an oil. After purification by flash chromatography, eluting with 20% EtOAc/80% hexane, 0.118 g (71%) of the white solid lactone 3 was obtained: mp 88–90 °C; IR (KBr) 1770, 1640, 1210, 1195, 1115 cm⁻¹; NMR (CDCl₃) δ 7.33 (m, 5), 6.00 (d, 1, *J* = 3.0 Hz), 3.20 (m, 2), 2.75 (m, 3); mass spectrum, *m/z* (relative intensity) 314 (21, M⁺), 187 (17), 131 (100), 104 (50), 77 (15). Anal. High-resolution MS calcd for C₁₂H₁₁O₂I, 313.9806; found, 313.9800.

Methyl 4-Hydroxy-4-phenylbutanoate (21). γ -Phenyl- γ -butyrolactone 20 (1.5 g, 9.25 mmol) was stirred with 30 mL of THF at 25 °C. To the THF solution was added 2 N LiOH (23 mL, 46.2 mmol). This solution was stirred at 25 °C for 1 h. The reaction mixture was then cooled to 0 °C and carefully acidified to pH 5 with solid NaH₂PO₄·H₂O. Immediately after acidification, esterification was accomplished by careful addition at 0 °C of diazomethane (excess). Nitrogen was bubbled through the reaction mixture for 10 min. Then the reaction mixture was diluted with H₂O and extracted (three times) with Et₂O. The ether extract was dried over MgSO₄; filtration and solvent removal afforded 1.67 g of an oil. Purification by flash chromatography (30% EtOAc/hexane) gave 1.53 g (85%) of the ester 21 as a clear oil: IR (film) 3400 (br), 1725 cm⁻¹; NMR (CDCl₃) δ 7.30 (s, 5), 4.70 (dt, 1, *J* = 6 Hz, *J* = 3 Hz), 3.63 (s, 3), 2.70 (d, 1, *J* = 3 Hz), 2.41 (m, 2), 2.08 (t, 2, *J* = 6 Hz); mass spectrum, *m/z* (relative intensity)

194 (20, M⁺), 176 (1), 117 (17), 107 (100), 88 (24), 77 (55). Anal. C, H.

Methyl 4-Phenyl-6-(trimethylsilyl)-5-hexynoate (23). Hydroxy ester 21 (0.60 g, 3.1 mmol) was stirred with dry CH₂Cl₂ (16 mL) under N₂ atmosphere and then cooled to 0 °C. Et₃N was added (0.89 mL, 6.2 mmol), and stirring was continued for 10 min. Methanesulfonyl chloride (0.39 g, 3.4 mmol) was then added at 0 °C, and after 15 min, the reaction mixture was quenched with aqueous saturated NaHCO₃ and stirred for 5 min. The quenched reaction mixture was subsequently extracted with CH₂Cl₂, and the CH₂Cl₂ extract was washed with saturated aqueous NaHCO₃, water, and saturated aqueous NaCl solution. The CH₂Cl₂ extract was dried over Na₂SO₄ and gave a yellow oil on solvent removal: NMR (CDCl₃) δ 7.38 (s, 5), 5.55 (m, 1), 3.65 (s, 3), 2.62 (s, 3), 2.30 (m, 4).

The diethylaluminum (trimethylsilyl)acetylene reagent was then prepared as in the preparation of compound 8: *n*-BuLi (3.37 mL, 2.3 M in hexane), hexane (12 mL) solution of (trimethylsilyl)acetylene (0.761 g, 7.75 mmol), hexane solution (10 mL) of Et₂AlCl (3.88 mL, 25% in hexane). After stirring for 3 h at 25 °C, the reagent solution was filtered with use of Schlenk funnel into a dry reaction vessel with a N₂ atmosphere. The hexane was then removed from the filtrate under vacuum (5 mmHg), leaving a white residue to which was added dry 1,2-dichloroethane (30 mL). The dichloroethane solution was cooled to 0 °C, and a dichloroethane (10 mL) solution of the previously prepared mesylate 22 (used without purification) was added. This reaction mixture was stirred at 0 °C for 1 h 20 min and was then poured into 80 mL of ice-cold 3 N HCl. The organic layer was removed, and the aqueous layer was extracted several times with Et₂O. The organic extracts were washed separately with 3 N HCl and H₂O, dried over MgSO₄, filtered, and then combined, giving a clear oil on solvent removal. Purification by flash chromatography (10% EtOAc/hexane) provided 0.473 g (56%) of the desired acetylenic ester 23 as a clear oil: IR (film) 2880, 2150, 1730, 1600, 1480 cm⁻¹; NMR (CDCl₃) δ 7.00 (m, 5), 3.55 (t, 1, *J* = 6.0 Hz), 3.44 (s, 3), 2.25 (m, 2), 2.87 (m, 2), 0.00 (s, 9); mass spectrum, *m/z* (relative intensity) 274 (17, M⁺), 259 (2), 201 (1), 187 (3), 177 (1), 146 (100), 73 (22). Anal. High-resolution MS calcd for C₁₆H₂₂O₂Si, 274.1393; found, 274.1394.

4-Phenyl-5-hexynoic Acid (24). Acetylenic ester 23 (0.448 g, 1.63 mmol) was stirred with methanol (16 mL) at 25 °C. To this solution was added 2 N NaOH (6.8 mL). After 3.5 h at 25 °C, the methanol was removed under vacuum, and the resulting aqueous solution was cooled to 0 °C and acidified to pH 1 with 6 N HCl. Extraction with CH₂Cl₂ and drying of the CH₂Cl₂ extracts with MgSO₄ provided, upon solvent removal, a white solid. Trituration with hexane provided the acetylenic acid 24 (0.300 g, 98%) as white crystals: mp 91–92 °C; IR (KBr) 3700–2200 (br), 3290, 1695, 1495 cm⁻¹; NMR (CDCl₃) δ 10.97 (br s, 1), 7.27 (br s, 5), 3.73 (dt, 1, *J* = 6.0 Hz, *J* = 2.5 Hz), 2.50 (m, 2), 2.27 (d, 1, *J* = 2.5 Hz), 2.07 (t, 2, *J* = 7.0 Hz); mass spectrum, *m/z* (relative intensity) 188 (16, M⁺), 163 (2), 143 (4), 130 (1), 116 (12), 115 (100), 77 (7). Anal. C, H.

5-Phenyl-6(E)-(iodomethylidene)tetrahydro-2-pyranone (4). A solution of the acetylenic acid 24 (0.100 g, 0.53 mmol) in dry CH₃CN (21 mL) was stirred under N₂ atmosphere. To this solution at 25 °C was added I₂ (0.270 g, 1.06 mmol) and KHCO₃ (0.053 g, 0.53 mmol), sequentially. The reaction mixture was stirred for 21 h at 25 °C at which time another equivalent of I₂ was added. The reaction mixture was stirred for an additional 11 h at 25 °C and then quenched with 5% aqueous Na₂S₂O₃. Extraction with CH₂Cl₂ and washing of the extract with 5% Na₂S₂O₃ and H₂O afforded 0.135 g of an oil after the CH₂Cl₂ extract was dried over Na₂SO₄ and the solvent removed. Purification by flash chromatography (20% EtOAc/hexane) and crystallization from Et₂O and hexane afforded the lactone 4 (0.100 g, 60%) as white crystals: mp 51.0–52.5 °C; IR (KBr) 1760, 1630, 1495, 1450 cm⁻¹; NMR (CDCl₃) δ 7.27 (br d, 5), 6.15 (s, 1), 4.42 (t, 1, *J* = 5.0 Hz), 2.28 (m, 4); mass spectrum, *m/z* (relative intensity) 314 (3, M⁺), 187 (100), 146 (13), 140 (2), 127 (3), 77 (27). Anal. C, H, I.

3-Phenyl-6-methylidenetetrahydro-2-pyranone (25). The acid 19 (0.10 g, 0.53 mmol) was stirred at 25 °C under N₂ atmosphere with CH₂Cl₂ (7 mL) and mercuric trifluoroacetate (0.023 g, 0.053 mmol). After the reaction was stirred for 3 h, it was

quenched with saturated aqueous NaHCO_3 and stirred for 10 min. The reaction mixture was then diluted with water and extracted several times with CH_2Cl_2 . The CH_2Cl_2 extract was washed with water and dried over MgSO_4 , to give 0.099 g of an oil after solvent removal. Purification by flash chromatography provided 0.081 g (81%) of a clear oil: IR (CHCl_3) 3020, 1750, 1662, 1600, 1500, 1450 cm^{-1} ; NMR (CDCl_3) δ 7.27 (m, 5), 4.70 (t, 1, $J = 1.0$ Hz), 4.30 (t, 1, $J = 1.0$ Hz), 2.87 (m, 5); mass spectrum, m/z (relative intensity) 188 (7, M^+), 160 (12), 144 (7), 131 (100), 118 (5), 77 (20). Anal. C, H.

5-Oxo-3-phenylhexanoic Acid (26). Lactone **25** (0.078 g, 0.042 mmol) was stirred at 25 °C in THF (2 mL) with 2 N LiOH (1.04 mL) for 1 h. The reaction mixture was then diluted with 1 mL of water and acidified to pH 2 with 6 N HCl followed by extraction with ethyl acetate to give 0.081 g of white solid after drying over MgSO_4 , filtration, and solvent removal. The solid **26** was recrystallized from ether/hexane to give clear needles (94% yield): mp 81.5–83.0 °C; IR (CHCl_3) 3500–2300, 1710, 1500, 1455 cm^{-1} ; NMR (CDCl_3) δ 9.7 (br s), 7.25 (s, 5), 3.67 (quintet, 1, $J = 7.0$ Hz), 2.82 (d, 2, $J = 7.0$ Hz), 2.69 (d, 2, $J = 7.0$ Hz), 2.06 (s, 3); mass spectrum, m/z (relative intensity) 206 (2, M^+), 188 (3), 161 (2), 147 (5), 129 (2), 43 (100). Anal. C, H.

Methyl 5-Oxo-3-phenylhexanoate (27). Diazomethane (excess) was added slowly to a diethyl ether solution (10 mL) of the acid **26** (0.150 g, 0.73 mmol) at 0 °C until a yellow color persisted. The cold bath was removed, and the reaction solution was allowed to warm to 25 °C while N_2 bubbled through the reaction solution. Solvent removal and purification by flash chromatography using 30% ethyl acetate/hexane provided the ester **27** (0.15 g, 94%): mp 34–36 °C; NMR (CDCl_3) δ 7.23 (s, 5), 3.67 (quintet, 1, $J = 7.0$ Hz), 3.57 (s, 3), 2.80 (d, 2, $J = 7.0$ Hz), 2.63 (d, 2, $J = 7.0$ Hz), 2.03 (s, 3); mass spectrum, m/z (relative intensity) 220 (2, M^+), 189 (2), 160 (14), 147 (7), 59 (5), 43 (100). Anal. C, H.

B. Biochemical Methods. General Procedures. Inactivation assays were performed with a Varian 635 UV–vis double-beam spectrophotometer or a Hewlett-Packard 8451A diode array single beam spectrophotometer. α -Chymotrypsin (three times crystallized and free of autolysis products and low-molecular-weight contaminants) was obtained from Worthington Biochemical. *N*-Benzoyltyrosine ethyl ester (BTEE)¹⁵ was obtained from Sigma. *N*-Succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide¹⁴ was obtained from Vega Biochemicals. The phosphate buffer used in the α -chymotrypsin inactivation studies was 0.1 M NaH_2PO_4 – Na_2HPO_4 , pH 7.2.

Time-Dependent Loss of Activity Assay. To 1.0 mL of a 2.0 μM or 60 nM α -chymotrypsin solution in pH 7.2 phosphate buffer in a thermostated water bath at 25 °C was added 20 μL of a solution of halo enol lactone in acetonitrile or dimethyl sulfoxide to give the final concentration of lactone. (The final concentration of acetonitrile or dimethyl sulfoxide had no effect on the inactivation process.) The enzyme activity of this incubation mixture was evaluated at various time intervals by transferring a 25- μL aliquot into a cuvette containing 1.0 mL of a 1:1 mixture of 1.07 mM BTEE (50% water/methanol) and pH 7.2 0.1 M phosphate buffer, a 535 μM BTEE solution in 0.1 M phosphate buffer at pH 7.2 containing 5% v/v CH_3CN , or containing 0.5 mM Suc-Ala-Ala-Pro-Phe-*p*NA in buffer. After an initial lag period of 0.3–3 min that is presumed to arise from deacylation of enzyme not yet inactivated, the rate of change in absorbance at 256 nm, or 410 nm, with respect to a cuvette containing only substrate solution, gives a straight line with a slope proportional to the enzyme activity. The absolute enzyme activity is expressed relative to an α -chymotrypsin solution in buffer, incubated in parallel to the inactivation experiment, to a cuvette containing 1.0 mL of substrate solution. The activity of the blank was tested before and after the inactivation experiment.

General Procedure for a Competitive Inhibition Assay. Into a 1.5-mL cuvette were combined 1.0 mL of the desired concentration of a substrate solution, either BTEE¹⁵ in a 1:1 mixture of 0.1 M phosphate buffer at pH 7.2 and 50% water/

methanol, 1:1 80 mM Tris buffer/0.1 CaCl_2 and 50% water/methanol, or succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide¹⁴ in 0.1 M phosphate buffer at pH 7.2, and the appropriate amount of inhibitor stock solution in CH_3CN or Me_2SO which would give the desired final inhibitor concentration. To this cuvette at 25 °C was then added α -chymotrypsin, either 25 μL or 5 μL of a 2 μM stock solution in the appropriate buffer. The initial rate of change in absorbance at 256 nm (BTEE) or 410 nm (succinyl-Ala-Ala-Pro-Phe-*p*NA) gave the velocity. The change in absorbance was taken against a cuvette containing only the corresponding substrate plus inhibitor solution. The control consisted of the 1.0 mL substrate solution to which was added the appropriate amount of α -chymotrypsin. For additional details on the competitive assay of individual inhibitors, see the text.

Determination of the Hydrolysis Rate Constants, k_h , by UV. The spontaneous hydrolysis of the inhibitor is a first-order process described by

$$dI/dt = -k_h[I]$$

Integration and substitution of $A/\epsilon l$, where A is the absorbance, ϵ the molar absorptivity, and l , the cell path length, gives

$$A = A_0 e^{-k_h t}$$

where A_0 is the absorbance at initial time. The difference spectrum of each inhibitor was taken in pH 7.2 0.1 M phosphate buffer in order to determine a wavelength where there was an appreciable difference between the two. The disappearance of each lactone was then followed at 25 °C at the appropriate wavelength: 230 nm (lactone 1), 310 nm (lactone 2), 235 nm (lactone 3), and 225 nm (lactone 4). Fitting the resulting progress curves to the exponential function gave the hydrolysis rate constants k_h .

Reactivation of α -Chymotrypsin in the Presence and Absence of Hydrazine. A solution of 4 μM α -chymotrypsin (1.0 mL) in 0.1 M phosphate buffer pH 7.2 was incubated at 25 °C with the desired inhibitor (see text for inhibitor concentration). The enzyme activity was monitored by assaying with a 535 μM BTEE solution (1.0 mL) in 0.1 M phosphate buffer at pH 7.2 with 5% v/v CH_3CN according to the procedure described for time-dependent loss of activity assay. A control incubation consisted of 4 μM α -chymotrypsin (1.0 mL) containing the corresponding amount of CH_3CN or Me_2SO added with the inhibitor in the enzyme–inhibitor incubation. After the appropriate incubation period (determined in order to maximize the percent enzyme inhibition), both the control and enzyme–inhibitor incubation solutions were passed through identical Sephadex G-25 columns (5 cm \times 0.5 cm) preequilibrated with 0.1 M phosphate buffer at pH 7.2, and the protein fraction (initial 2 mL) was collected. To 0.5 mL of the protein fraction from each of the Sephadex columns was added 0.5 mL of a NH_2NH_2 solution¹⁷ in 0.1 M phosphate buffer pH 7.2, and the enzyme activity was monitored by assaying 50- μL aliquots with BTEE.¹⁵ Similarly, 0.5 mL of each of the protein fractions was combined with 0.5 mL of buffer followed by monitoring of the enzyme activity with BTEE.¹⁵ Hydrazine and buffer reactivation solutions were brought to ionic strength $\mu = 1$ with NaCl.

Acknowledgment. We are grateful for the support of this work through a grant from the National Institutes of Health (Grant PHS 5R01 AM 25836). High-field ^1H NMR spectra were obtained on instruments in the University of Illinois Regional Instrumentation Facility, supported by the National Science Foundation (Grant NSF CHE 79-16100). Exact mass determinations were performed on mass spectrometers in the University of Illinois Mass Spectrometry Center, supported by the National Institutes of Health (Grant GM 27029). We are grateful to Drs. G. R. Marshall, S. Naruto, and I. Motoc for providing preliminary molecular graphics results on the interaction of lactone **25** with chymotrypsin.