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

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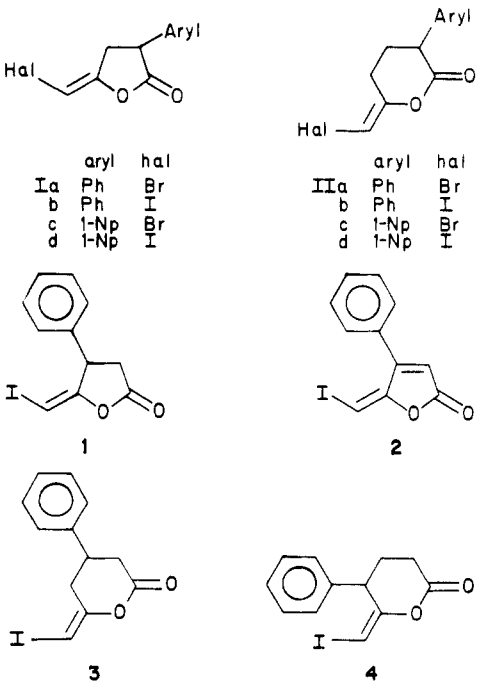
We have found that α-aryl-substituted halo enol lactones (I and II) are effective mechanism-based inactivators for chymotrypsin. In this study, we have investigated, for comparative purposes, halo enol lactones with aryl functions situated β and γ to the lactone carbonyl group. We synthesized 4-phenyl-5(*E*)-(iodomethylidene)tetrahydro-2-furanone (**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 proposal[1] 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.

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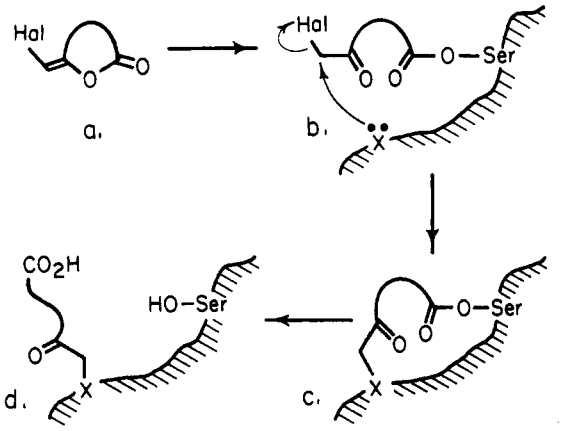
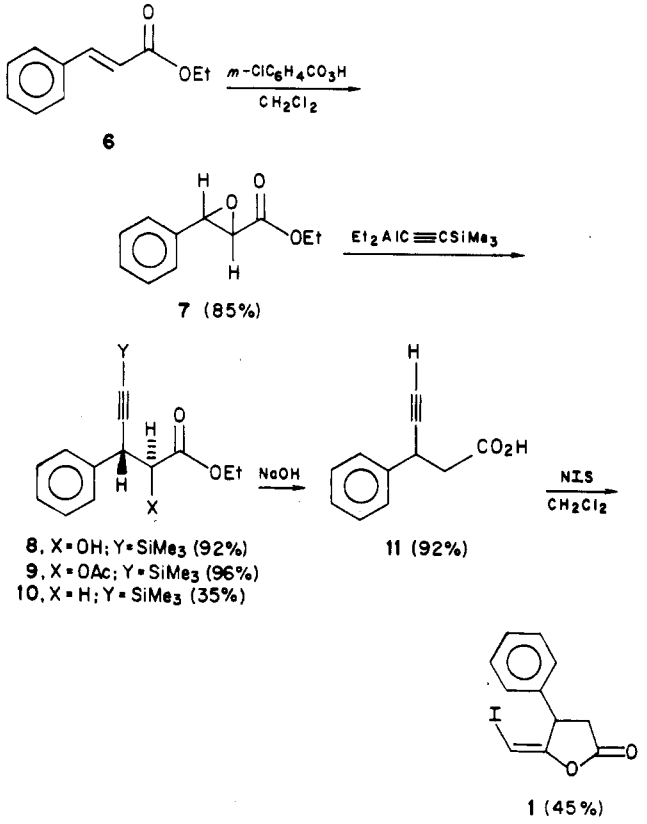
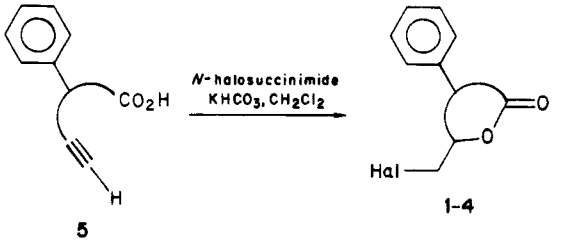
Scheme I



Scheme II

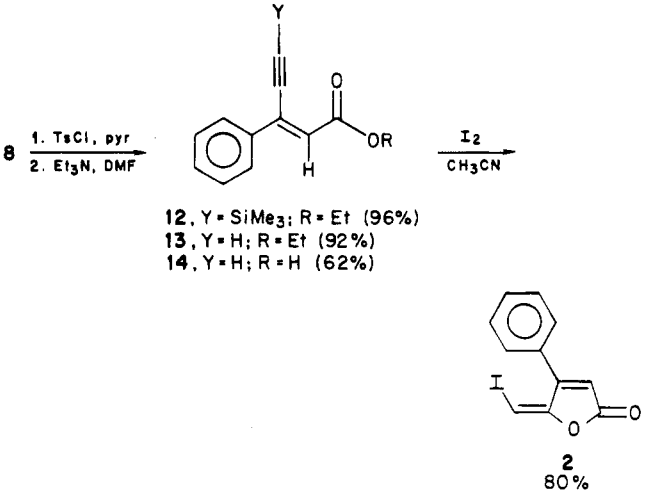


the lactones **1–4**. In each case, an appropriately substituted acetylenic acid **5** was required as a precursor.

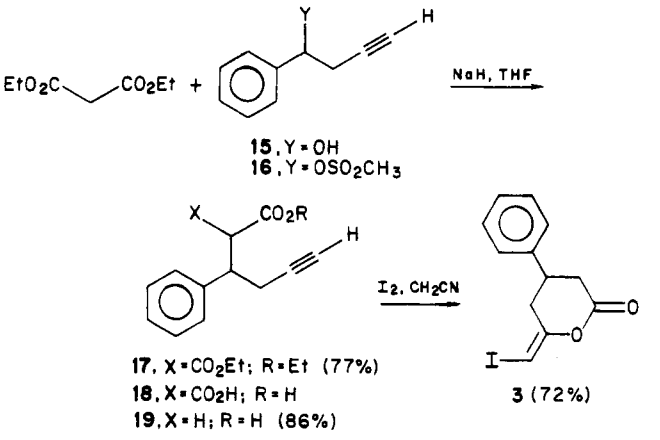


4-Phenyl-5(*E*)-(iodomethylidene)tetrahydro-2-furanone (1). For the synthesis of the 4-phenylfuranone **1**, a β-ethynyldihydrocinnamic acid precursor was required. Attempts to conjugatively ethynylate ethyl cinnamate (**6**) by utilizing known enone chemistry proved unsuccessful.[5,6]

Scheme III



Scheme IV

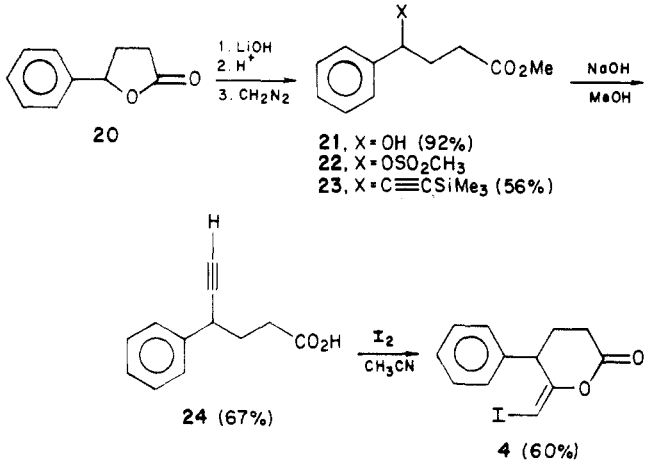


However, the acetylenic moiety could be introduced β to the ester functionality by regioselective epoxide opening on ethyl epoxycinnamate (**7**), using diethyl aluminum (trimethylsilyl)acetylene,[7-9] to give the β-ethynyl α-hydroxy ester **8** as shown in Scheme II. Cleavage of the α-hydroxyl group was accomplished by careful reductive scission of the acetoxy derivative **9** with Li/NH_3 (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 I_2 in CH_3CN.

(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.
(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, 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.

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
DehydroβPh5I (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 BTEE[15] as the substrate for lactones 1 and 4 and Suc-Ala-Ala-Pro-Phe-*p*NA[14] 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.

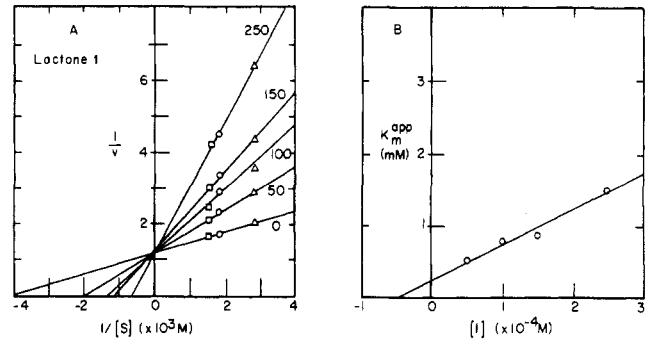


Figure 1. Competitive inhibition assay of α-chymotrypsin with lactone 1. α-Chymotrypsin (50 nM) was added to a solution of BTEE (Hummel, 1959) and inhibitor (50–250 μM) in 80 mM Tris buffer at pH 7.2 and 25 °C. Enzyme activity was followed by UV at 256 nm. A. Panel A shows the double-reciprocal plot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

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*-nitroanilide[14] 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.

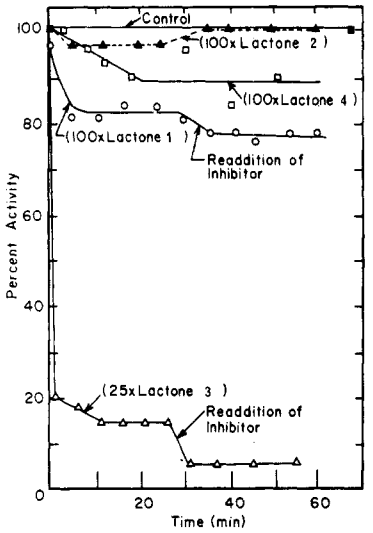


Figure 2. Time-dependent loss of α-chymotrypsin activity (2 μM) incubated at 25 °C in phosphate buffer at pH 7.2 with 100-fold excess of lactone 4 (□), lactone 1 (○), or lactone 2 (▲) and 25-fold excess of lactone 3 (△). Readdition of inhibitor 1 and 3 to their respective α-chymotrypsin incubation solutions occurred at 30 min. Percent remaining α-chymotrypsin activity is plotted vs. time as assayed with BTEE (535 μM in 1:1 phosphate buffer and 50/50 MeOH/H_2O). The control contains α-chymotrypsin and 20 μL of CH_3CN.

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 μM) to a 100-fold excess of lactone 1, 2, or 4 (200 μM) 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

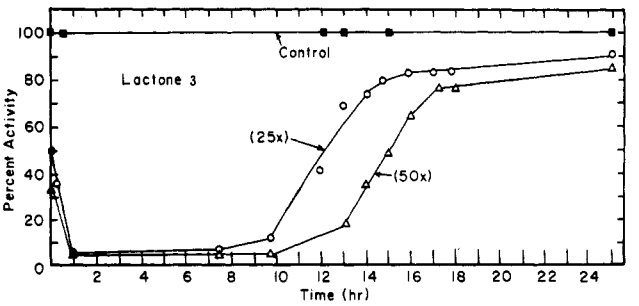


Figure 3. Spontaneous reactivation of α-chymotrypsin inactivated with 25-fold (○) and 50-fold (△) excess of lactone 3 over a 24-h period at 25 °C in 0.1 M phosphate buffer at pH 7.2. Percent remaining enzyme activity is plotted vs. time as assayed with 535 μM BTEE in phosphate buffer containing 5% v/v CH_3CN. The control solution (■) contained only enzyme and 20 μL of CH_3CN.

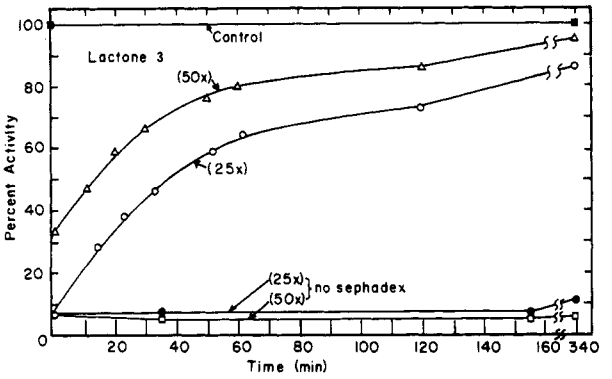


Figure 4. Reactivation of α-chymotrypsin inactivated with 50-fold (△) and 25-fold (○) excess of inhibitor 3 after passage through a Sephadex G-25 column. The resulting protein eluant was assayed with BTEE in phosphate buffer with 5% v/v CH_3CN (535 μM). The control solutions containing only enzyme and 40 μL of CH_3CN were passed through identical Sephadex columns. Parallel enzyme-inhibitor incubation solutions with 50-fold (□) and 25-fold (●) 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 μM) was incubated with either 0 μM (control), 100 μM (25-fold excess), or 200 μM (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 preequilibrated 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.

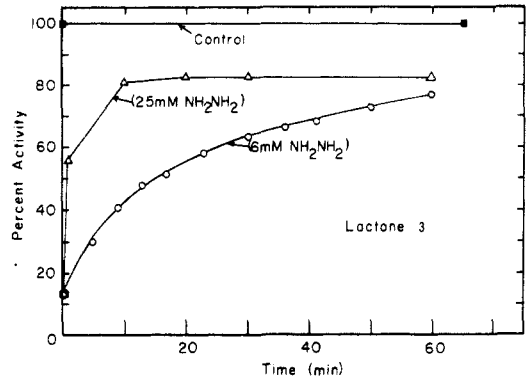
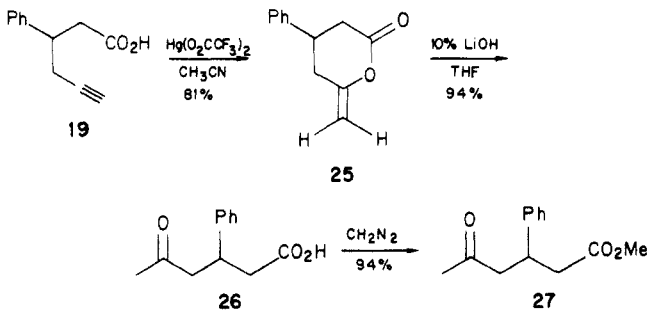


Figure 5. Acceleration of activity regeneration of α-chymotrypsin inactivated with lactone **3** by addition of hydrazine after Sephadex G-25 chromatography of a 4-h preincubation of α-chymotrypsin (4 μM) and lactone **3** (100 μM), at which time 90% loss of enzyme activity was observed. Equivolumes of protein eluant and 50 mM hydrazine (△) or 12 mM hydrazine (○) were combined and α-chymotrypsin activity was assayed with 535 μM BTEE in phosphate buffer containing 5% v/v CH_3CN. The control containing enzyme and 40 μL 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 μM. 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

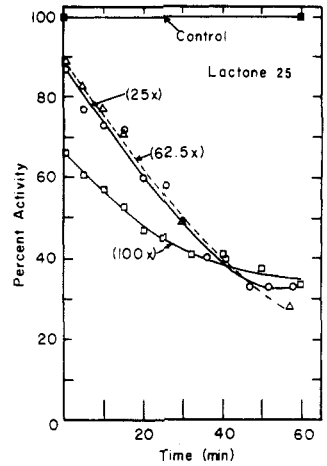


Figure 6. Time-dependent loss of α-chymotrypsin activity when incubated at 2 μM with lactone **25** at 25- (●), 62.5- (△), and 100-fold (□) excess in phosphate buffer at pH 7.2 and 25 °C. α-Chymotrypsin was assayed with BTEE (535 μM in 1:1 phosphate buffer and 50/50 MeOH/H_2O).

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-chymytrypsin 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-μm silica gel.

Proton magnetic resonance (^{1}H 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 method[25] 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 CH_2Cl_2/*trans*-ethyl cinnamate (5.0 g, 28 mmol), CH_2Cl_2 (100 mL), CH_2Cl_2 (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 **7**[27] as an oil: IR ($CHCl_3$) 1725, 1240 cm^{-1}; NMR ($CDCl_3$) 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 N_2 atmosphee. *n*-Butyllithium (1.44 M in hexane (35.3 mL)) was added to the acetylene solution at 0 °C; sufficient dry Et_2O 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 N_2 atmosphere to a dry reaction flask that contained Et_2AlCl (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

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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 H_2O, and the organic extract was dried over Na_2SO_4, 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 ($CHCl_3$) 3400, 2175, 1725 cm^{-1}; NMR ($CDCl_3$) 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 CH_2Cl_2 (15 mL). Et_3N (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 CH_2Cl_2/ester solution. This reaction mixture was stirred for 5 h at 25 °C. All the volatiles were removed, and the residue was solubilized in CH_2Cl_2. The CH_2Cl_2 extract was washed with H_2O and saturated $NaHCO_3$ solution. The organic extract was dried over $MgSO_4$ 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 ($CHCl_3$) 2150, 1750 (br) cm^{-1}; NMR ($CDCl_3$) 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 N_2 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/NH_3 (liquid) at −78 °C. After the reaction mixture was stirred for 40 min at −78 °C, it was quenched with solid NH_4Cl (1.7 g) and then stirred at −78 °C for 30 min. The NH_3 was then allowed to evaporate, and the remaining orange residue was solubilized in Et_2O. The Et_2O extract was washed with H_2O and a saturated NaCl solution, dried over $MgSO_4$, 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 ($CHCl_3$) 2150, 1720, 1600 cm^{-1}; NMR ($CDCl_3$) 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 $C_{16}H_{22}O_2Si$, 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 CH_2Cl_2. The CH_2Cl_2 extract was dried over $MgSO_4$, 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 ($CDCl_3$) 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 $C_{11}H_{10}O_2$, 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 CH_2Cl_2 at −10 °C under N_2 atmosphere. To the acid solution was added NIS (0.072 g, 0.32 mmol) and solid $KHCO_3$ (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% $Na_2S_2O_3$ solution. The CH_2Cl_2 extract was washed with H_2O and dried over Na_2SO_4. After filtration and solvent removal, the resulting white solid was recrystallized twice from Et_2O 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 ($CDCl_3$) 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 $C_{11}H_9O_2I$, 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 N_2 atmosphere. The reaction mixture was then diluted with Et_2O and washed twice with a saturated $CuSO_4$ solution and once with water. The organic extract was dried over $MgSO_4$ and filtered to give the tosylate as a clear oil (1.12 g): NMR ($CDCl_3$) 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 Et_3N (0.3 mL) at 70 °C under a N_2 atmosphere for 2 h. The reaction mixture was cooled to room temeprature and diluted with Et_2O. The diluted reaction mixture was washed several times with H_2O. The Et_2O extracts were dried over $MgSO_4$ 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 ($CHCl_3$) 2900, 2125, 1700 (br), 1575, 850, 690 cm^{-1}; NMR ($CDCl_3$) 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 $C_{16}H_{20}O_2Si$, 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 CH_2Cl_2, and this organic solution was washed with H_2O. The CH_2Cl_2 extract was dried over $MgSO_4$. After filtration and solvent removal, a white solid remained. Crystallization from Et_2O and hexane afforded 0.61 g (92%) of the ester **13**: mp 84–86 °C; IR ($CHCl_3$) 3230, 2080, 1700, 1590 cm^{-1}; NMR ($CDCl_3$) 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 $C_{13}H_{12}O_2$, 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 CH_2Cl_2. The CH_2Cl_2 solution was washed with water, and the organic extract was dried over $MgSO_4$. After filtration and solvent removal, 0.503 g of a yellow solid was obtained. The solid was crystallized from Et_2O and hexane to give 0.307 g (62%) of the acetylenic acid **14**: mp 108–110 °C; IR ($CHCl_3$) 3250, 3500–2400, 2080, 1690, 1600 cm^{-1}; NMR ($CDCl_3$) 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 $C_{11}H_8O_2$, 172.0524; found, 172.0520.

4-Phenyl-5(*E*)-(iodomethylidene)dihydro-2-furanone (2). A CH_3CN (6 mL) solution of the acetylenic acid **14** (0.070 g, 0.41 mmol) was stirred at 25 °C under a N_2 atmosphere. A CH_3CN (2 mL) solution of iodine (0.31 g, 1.2 mmol) was added dropwise to the acid solution followed by addition of $KHCO_3$ (0.040 g, 0.41 mmol). The reaction mixture was stirred at 25 °C for 16 h under N_2 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 Et_2O and hexane three times (0.097 g, 80%): mp 75–76 °C; UV_{max} (CH_3CN) 310 nm (ϵ 15 850); IR (KBr) 1765, 1620, 1583 cm^{-1}; NMR ($CDCl_3$) 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 $C_{11}H_7O_2I$, 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 N_2 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 CH_2Cl_2 with triethylamine (1 equiv) and methanesulfonyl chloride (1 equiv) for 10 min followed by quenching with saturated $NaHCO_3$ and isolation by extraction with CH_2Cl_2: NMR ($CDCl_3$) 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 NH_4Cl solution and THF was removed under vacuum, an Et_2O solution of the residue was washed with saturated NH_4Cl, saturated $NaHCO_3$ solution, and H_2O. The combined organic extracts were dried over $MgSO_4$ 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 ($CDCl_3$) 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 $C_{17}H_{20}O_4$, 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 Et_2O. 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 $MgSO_4$ and filtered to give 0.988 g (96%) of a white solid diacid **18**: NMR ($CDCl_3$) 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 CH_2Cl_2, and the combined organic extracts were dried over $MgSO_4$. 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 ($CHCl_3$) 3310, 3020, 2400, 1710 cm^{-1}; NMR ($CDCl_3$) 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 $C_{12}H_{12}O_2$, 188.0841; found, 188.0831.

4-Phenyl-6(*E*)-(iodomethylidene)tetrahydro-2-pyranone (3). The acid **19** (0.100 g, 0.53 mmol) in CH_3CN (15 mL) was stirred with iodine (0.270 g, 1.06 mmol) and $KHCO_3$ (0.054 g, 0.53 mmol) at 25 °C for 23 h. The reaction was quenched with a 5% $Na_2S_2O_3$ solution and extracted with CH_2Cl_2. The organic extracts were dried over Na_2SO_4 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 ($CDCl_3$) 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 $C_{12}H_{11}O_2I$, 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 $NaH_2PO_4 \cdot H_2O$. 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 H_2O and extracted (three times) with Et_2O. The ether extract was dried over $MgSO_4$; 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 ($CDCl_3$) 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 CH_2Cl_2 (16 mL) under N_2 atmosphere and then cooled to 0 °C. Et_3N 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 $NaHCO_3$ and stirred for 5 min. The quenched reaction mixture was subsequently extracted with CH_2Cl_2, and the CH_2Cl_2 extract was washed with saturated aqueous $NaHCO_3$, water, and saturated aqueous NaCl solution. The CH_2Cl_2 extract was dried over Na_2SO_4 and gave a yellow oil on solvent removal: NMR ($CDCl_3$) 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 Et_2AlCl (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 N_2 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 Et_2O. The organic extracts were washed separately with 3 N HCl and H_2O, dried over $MgSO_4$, 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 ($CDCl_3$) 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 $C_{16}H_{22}O_2Si$, 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 CH_2Cl_2 and drying of the CH_2Cl_2 extracts with $MgSO_4$ 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 ($CDCl_3$) 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 CH_3CN (21 mL) was stirred under N_2 atmosphere. To this solution at 25 °C was added I_2 (0.270 g, 1.06 mmol) and $KHCO_3$ (0.053 g, 0.53 mmol), sequentially. The reaction mixture was stirred for 21 h at 25 °C at which time another equivalent of I_2 was added. The reaction mixture was stirred for an additional 11 h at 25 °C and then quenched with 5% aqueous $Na_2S_2O_3$. Extraction with CH_2Cl_2 and washing of the extract with 5% $Na_2S_2O_3$ and H_2O afforded 0.135 g of an oil after the CH_2Cl_2 extract was dried over Na_2SO_4 and the solvent removed. Purification by flash chromatography (20% EtOAc/hexane) and crystallization from Et_2O 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 ($CDCl_3$) 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 N_2 atmosphere with CH_2Cl_2 (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*-nitroanilide[14] 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 BTEE[15] 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*-nitroanilide[14] 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

$$A = A_0\epsilon^{-k_h t}$$

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 × 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 solution[17] 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 μ = 1 with NaCl.

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