Inhibition of Serine Proteases by Peptidyl Fluoromethyl Ketones†

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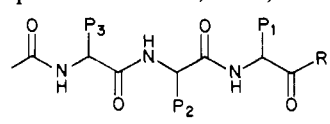
ABSTRACT: We have synthesized peptidyl fluoromethyl ketones that are specific inhibitors of the serine proteases α-chymotrypsin and porcine pancreatic elastase. By analogy with the corresponding aldehydes it is assumed that the fluoromethyl ketones react with the γ-OH group of the active site serine to form a stable hemiacetal [Lowe, G., & Nurse, D. (1977) *J. Chem. Soc., Chem. Commun.*, 815; Chen, R., Gorenstein, D. G., Kennedy, W. P., Lowe, G., Nurse, D., & Schultz, R. M. (1979) *Biochemistry 18*, 921; Shah, D. O., Lai, K., & Gorenstein, D. G. (1984) *J. Am. Chem. Soc. 106*, 4272]. ^{19}F NMR studies of the chymotrypsin-bound trifluoromethyl ketone inhibitors Ac-Leu-*ambo*-Phe-CF_3[1] and Ac-*ambo*-Phe-CF_3 clearly indicate that the carbonyl carbon is tetrahedral at the active site of the enzyme. The inhibitor is bound as either the stable hydrate or the hemiacetal, involving the active site serine. The effect of varying the number of amino acid residues in the peptidyl portion of the inhibitor and the number of fluorines in the fluoromethyl ketone moiety is examined. In the series of trifluoromethyl ketone elastase inhibitors, the lowering of K_i concomitant with the change from a dipeptide analogue to a tetrapeptide analogue (Ac-Pro-*ambo*-Ala-CF_3, $K_i = 3 \times 10^{-3}$ M; Ac-Ala-Ala-Pro-*ambo*-Ala-CF_3, $K_i = 0.34 \times 10^{-6}$ M) correlates well with the variation in V/K for hydrolysis of the corresponding amide substrates. This trend is indicative of the inhibitors acting as transition-state analogues [Bartlett, P. A., & Marlowe, C. K. (1983) *Biochemistry 22*, 4618; Thompson, R. C. (1973) *Biochemistry 12*, 47]. In addition to chain length, the number of fluorine substituents also affects the K_i. In the case of chymotrypsin, the K_i for Ac-Leu-*ambo*-Phe-CF_3 is 30-fold lower than that for Ac-Leu-*ambo*-Phe-CF_2H (0.88×10^{-6} M vs. 25×10^{-6} M). With elastase this trend is not as profound. In all cases, however, the difluoro- and trifluoromethyl ketones are better inhibitors than the monofluoromethyl and nonfluorinated analogues. This improvement must be associated with both the degree of hydration of the fluoromethyl ketones and the significant effect that fluorine substitution has on lowering the first pK_a of the hemiacetal hydroxyl. The latter change would cause the more fluorinated inhibitor to be able to interact better with the anionic hole near the active site. Fluorine substitution also lowers the k_{off} values for the inhibitors. With elastase the trifluoromethyl ketone tetrapeptide has a k_{off} of 1.25×10^{-4} s^{-1}, while the corresponding difluoromethyl compound has a k_{off} of 0.007 s^{-1}. The monofluoromethyl ketone inhibitor of chymotrypsin, Ac-Leu-*ambo*-Phe-CFH_2, is a weak competitive inhibitor ($K_i = 200 \times 10^{-6}$ M). It also demonstrates time-dependent irreversible inhibition with a second-order rate constant of 1.7 M^{-1} s^{-1}. The irreversible inhibition is accompanied by covalent modification of a histidine residue and by fluoride ion release as detected by ^{19}F NMR spectroscopy.

Recently it has been demonstrated that the incorporation of tri- and difluoromethyl ketone moieties into substrate analogues has led to some extremely potent transition-state analogue (Wolfenden, 1976) inhibitors for a variety of hydrolytic enzymes, including acetylcholinesterase (Gelb et al., 1985). This enzyme is very similar in its mode of action to serine proteases. It was, therefore, expected that effective inhibitors, based on trifluoromethyl ketones, could be obtained for serine proteases. By analogy with peptidyl aldehydes (Westerik & Wolfenden, 1972; Thompson, 1973) it would be expected that these compounds form stable adducts with the active site serine.

We have now developed an efficient and general method to synthesize a class of inhibitors incorporating this functional group into a peptide analogue, as illustrated in the general structure **1** (Imperiali & Abeles, 1986). Inhibitors can be



1, R = CF_3, CF_2H, or CFH_2

targeted against any serine proteases of known specificity by replacing the amide, at the cleavage site of the protease substrate, by a fluoromethyl ketone, while simultaneously leaving the determinants of secondary binding interactions (P_1, P_2, etc.)[2] unchanged. Specificity is dictated by the nature of the amino acid residues in the peptide.

In this report we investigate the salient features of the inhibition of serine proteases by this novel class of inhibitors. We have examined the effect of replacing the trifluoromethyl group by a difluoro- or monofluoromethyl group as well as the effect of varying the number of amino acid substituents in the inhibitor peptide.

Experimental Procedures

Enzyme Assays. α-Chymotrypsin from bovine pancreas (type II, 3× crystallized) was purchased from Sigma Chemical Co. It was assayed in 100 mM potassium phosphate, pH 7.8, with *N*-benzoyltyrosine ethyl ester (Sigma) by monitoring the

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[1] The abbreviated designation of the peptidyl inhibitors is as defined in the IUPAC–IUB Joint Commission on Biochemical Nomenclature (1985). The term *ambo* designates the residue in front of which it appears as being racemic.

[2] The terminology used to describe the residues was originally proposed by Schecter and Berger (1967). The amino acid residues of substrate (or in this case, inhibitor) are designated P_1, P_2, etc., numbering from the carbonyl of the scissile amide bond in the direction of the amino terminal. The corresponding subsites are termed S_1, S_2, etc. The residues in the direction of the carboxyl group from the scissile bond are designated P_1', P_2', etc., and the corresponding subsites S_1', S_2', etc.

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increase in absorbance at 256 nm (Hummel, 1959). Elastase from porcine pancreas was from Worthington Biochemicals (lyophilized powder, 8 units/mg). Elastase assays were performed in 100 mM potassium phosphate, pH 7.8, either by monitoring the increase in absorbance at 360 nm accompanying the hydrolysis of the tetrapeptide [(succinyl-L-alanyl-L-alanyl-L-propyl-L-valyl)amino]methylcoumarin or by fluorometric methods (Castillo et al., 1978). The substrate was a gift from Dr. T. Payne, Sandoz Ltd. Trypsin from bovine pancreas (3× crystallized) was obtained from Worthington Biochemicals. It was assayed in 50 mM potassium phosphate, pH 8.0, containing 5 mM calcium chloride with α-*N*-benzoyl-DL-arginine-*p*-nitroanilide by monitoring the increase in absorbance at 412 nm. Papain from papaya latex was from Worthington and was purchased as a crystalline suspension (100 mg in 2 mL of 0.03 M cysteine). It was assayed in 50 mM Tris-HCl[3] buffer, pH 7.5, containing 20 mM EDTA and 5 mM cysteine hydrochloride, by monitoring the change in absorbance at 412 nm that occurs concomitant with the cleavage of the substrate α-*N*-benzoyl-DL-arginine-*p*-nitroanilide.

Inhibitors and substrates were dissolved in Me_2SO or CH_3CN when necessary. The content of the organic solvent in the assays did not exceed 5% v/v. All spectrophotometric enzyme assays were performed on a Perkin-Elmer λ-3 UV/vis spectrophotometer using 1-cm quartz cells thermostated at 25 °C.

Evaluation of Kinetic Parameters. In the case of reversible competitive inhibition the K_i values were determined from Lineweaver–Burk plots for reactions monitored in the presence and absence of inhibitor, using the initial velocities measured from the linear portion of the absorbance vs. time progress curves. A replot of the slopes of these plots against inhibitor concentrations afforded the K_i value.

The K_i's for the slow-binding inhibitors[4] (Williams & Morrison, 1979) of chymotrypsin and elastase were examined according to the procedure of Cha (1975). In this method, data from reaction progress curves (e.g., Figure 1a) obtained in the presence of a slow-binding inhibitor are utilized to determine rate constants for the formation and dissociation of enzyme–inhibitor complexes. In the competitive inhibition by a slow-binding inhibitor the rate equation is

$$v = v_s + (v_0 - v_s)e^{-kt} \quad (1)$$

where v = observed velocity at any time, v_0 = initial velocity, and v_s = steady-state velocity. Equation 1 can be integrated to afford eq 2. This equation describes the observed absorbance (A) as a function of the initial (v_0) and final (v_s) steady-state velocities and the first-order rate constant (k_{obsd}) for equilibration to the final steady state. The k_{obsd} is related to the association and dissociation rate constants by eq 3. Thus a replot of k_{obsd} vs. [I] will afford these constants. For elastase,

$$A = v_s t - (v_s - v_0)[1 - \exp(-k_{obsd}t)]/k_{obsd} + A_0 \quad (2)$$

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

the K_i was also determined by combining known amounts of enzyme and inhibitor and allowing them to reach equilibrium (typically 10 h) and periodically assaying aliquots for free enzyme until no change is apparent. The exact concentration of elastase was determined by the use of a burst experiment. The reaction of elastase with *p*-nitrophenyl trimethylacetate (in potassium phosphate buffer, 100 mM at pH 7.8) results in the rapid formation of a relatively stable acyl enzyme and the release of an equivalent of *p*-nitrophenolate (λ_{max} = 415 nm, ϵ_{max} = 18 600) proportional to the amount of active elastase (Bender & Marshall, 1968).

In addition to the procedure utilizing the Cha plot, association rate constants (k_{on}) were determined under pseudo-first-order conditions ([I] > 10[E]) from the rate of onset of slow-binding inhibition. This was measured by preincubating a mixture of enzyme and inhibitor in the appropriate buffer and periodically removing aliquots and diluting into an assay mixture containing substrate. The final inhibitor concentration in the assay was such that no rapidly reversible inhibition would be observed. Dissociation rate constants (k_{off}) were obtained by monitoring the rate of return of activity from a completely inhibited enzyme–inhibitor complex. This was carried out by fully inactivating the enzyme and then either removing excess inhibitor by rapid gel filtration (Penefsky, 1979) or diluting the enzyme–inhibitor complex, which had been inactivated with stoichiometric amounts of inhibitor. The return of activity was then monitored by periodically assaying portions of the mixture.

^{19}F NMR Studies. The interaction of the enzymes with some of the fluorinated inhibitors was examined by ^{19}F NMR. This was conducted on a Varian XL 300-MHz instrument at 282 MHz in a 5-mm broad-band probe tuned to fluorine. ^{19}F NMR shift values are set relative to $CFCl_3$ at 0 ppm.

^{19}F NMR was also utilized to estimate the degree of hydration of mono-, di-, and trifluoromethyl ketones, since hydrate and ketone signals are well distinguished and can be integrated.

Proton-Release Experiment. Chymotrypsin (1.0 mL, 20 mM in 1 mM HCl) was added to 2.9 mL of distilled water and the pH of the solution adjusted to 8.3 with 0.1 N NaOH by using a microburet. A 0.1-mL aliquot of inhibitor in CH_3CN (0.5 mM final concentration) was then added to the solution, and the effect on pH was followed. The experiment was carried out under a stream of argon. Under these conditions negligible pH drift due to uptake of CO_2 from the atmosphere was observed. All pH measurements were carried out on an Orion Research Model 701A digital ionalyzer.

pH Dependence Studies. The k_{on} for **3** was observed at varying pH by using the following buffers in the preincubation mixture: at pH 6.0, PIPES, 50 mM; at pH 7.0, MOPS, 50 mM; at pH 8.0, TAPS, 50 mM; and at pH 9.0, TAPS, 50 mM. The preincubation was followed by dilution into the standard assay. The K_i for competitive inhibitor **2** at varying pH was determined by using MOPS (pH 7.0 and 7.5, 50 mM) and TAPS (pH 8.0, 8.5, and 9.0, 50 mM) buffer substituted for the standard assay buffer in Lineweaver–Burk plots.

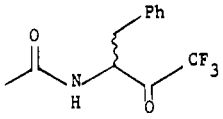
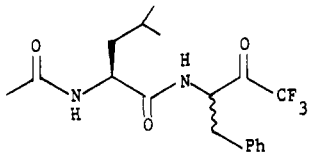
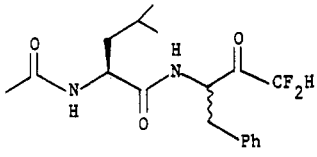
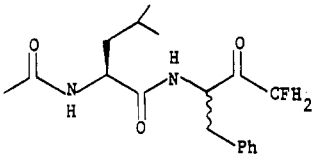
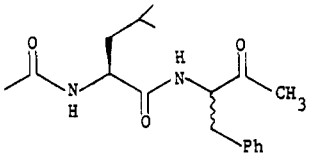
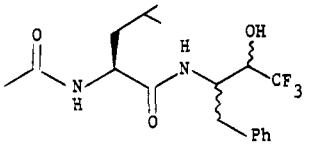
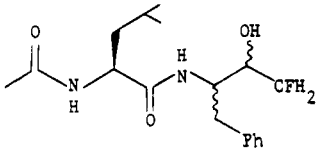
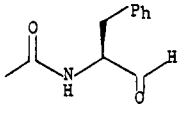
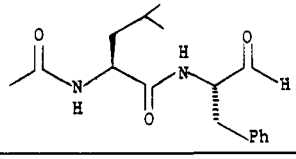
Amino Acid Analysis. The amino acid composition of the protein (native and modified) was determined after acid hydrolysis (6 N HCl, 80 °C, 12 h) on a Durrum D-500 amino acid analyzer.

Synthesis. A detailed description of the synthesis and purification of all new compounds and their physical properties is presented in the supplementary material (see paragraph at

[3] Abbreviations: Me_2SO, dimethyl sulfoxide; CH_3CN, acetonitrile; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)-aminomethane; TAPS, 3-[*N*-[tris(hydroxymethyl)methyl]amino]-propanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; PIPES, 1,4-piperazinebis(2-ethanesulfonic acid); TLC, thin-layer chromatography; HPLC, high-performance liquid chromatography.

[4] We use the term slow binding as defined by Williams and Morrison (1979). Slow-binding inhibitors exhibit the following characteristics: When enzyme is added to a solution containing substrate and inhibitor, initially an uninhibited rate is observed. The velocity gradually decreases to a final inhibited velocity; i.e., equilibrium is reached slowly. We believe it would be more useful to define these inhibitors in terms of k_{on} and k_{off}. All "slow-binding" inhibitors described here have a slow k_{on} (considerably less than diffusion controlled) and k_{off} <0.046 s^{-1} ($t_{1/2}$ > 15 s).

Table I: Inhibitors of Chymotrypsin

compd no.	compd	K_i (μM)	type of inhibitor[a]
2		40	A
3		1.2,[b] 0.56[c]	B
4		25	A
5		200[d]	A and C
6		500	A
7		200	A
8		>5000	nd[g]
9		700[e]	A
10		17[f]	A

[a] A, rapidly reversible competitive inhibition; B, slow-binding reversible competitive inhibition; C, irreversible inhibition. [b] k_{on}, 733 s^{-1} M^{-1}; k_{off}, 9.0 $\times$ 10^{-4} s^{-1}; determined by the method of Cha (1975). [c] k_{on}, 770 s^{-1} M^{-1}; k_{off}, 4.3 $\times$ 10^{-4} s^{-1}; determined by alternate methods. [d] $k_{obsd}/[I]$, 1.67 M^{-1} s^{-1}, pH 7.8, 25 °C. [e] Chen et al., 1979. [f] Breaux & Bender, 1975. [g] nd, not determined.

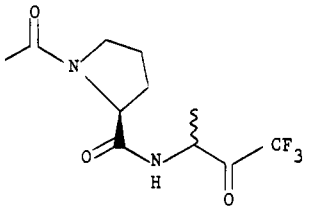
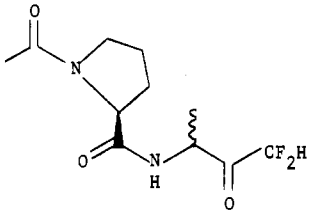
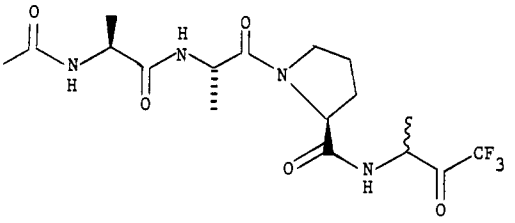
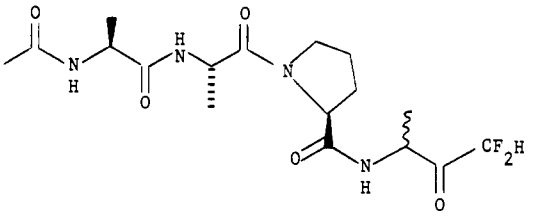
end of paper regarding supplementary material). All fluoromethyl ketone inhibitors were judged as pure on the basis of TLC analysis (single spot in the solvent system described in the supplementary material) and unambiguous assignment of all ^1H NMR signals.

Results

Inhibition of α-Chymotrypsin. We have synthesized a variety of peptidyl fluoromethyl ketones as chymotrypsin inhibitors. The choice of P_1 residue[2] as phenylalanine is in keeping with the well-established specificity of chymotrypsin for preferentially cleaving peptides with hydrophobic aromatic residues on the carbonyl side of the scissile amide bond. The choice of leucine as the P_2 residue is based on work by Breaux and Bender (1975). The results obtained are shown in Table I. The single N-blocked amino acid analogue **2** (racemic) is a simple competitive inhibitor of chymotrypsin with a K_i of 40 $\times$ 10^{-6} M. This fluoro ketone is approximately 20-fold more efficient than the corresponding aldehyde **9** (Chen et al., 1979).

In order to enhance the specificity of the inhibitors and obtain more mechanistic information, we synthesized the (*N*-acetylleucyl-*ambo*-phenylalanyl)mono-, di-, and trifluoromethane peptide analogues. Thus the effect of a varying number of fluorines on binding can be examined. This series

Table II: Inhibitors of Porcine Pancreatic Elastase

compd no.	compd	K_i (μM)	type of inhibitor[a]
11		3000	A
12		1000	A
13		9 0.43,[b] 0.25[c]	A B
14		0.27[d] (isomer **14a**) 3.3 (isomer **14b**)	A and B A and B
15	*N*-Ac-Pro-Ala-Pro-alaninal	0.8[e]	A

[a] A, rapidly reversible competitive inhibition; B, slow-binding reversible competitive inhibition. [b] k_{on}, 290 s^{-1} M^{-1}; k_{off}, 1.25×10^{-4} s^{-1}. [c] Equilibrium method. [d] k_{off}, 0.007 s^{-1}. [e] Thompson, 1973.

of compounds exhibits very different behavior. The trifluoromethyl ketone **3** was again more effective than the corresponding aldehyde **10** and is slow binding.[4] The K_i was determined according to the procedure of Cha (1975) as described under Experimental Procedures. Progress curves for chymotrypsin and **3** are shown in Figure 1a. The replot of the k_{obsd} vs. [I] (Figure 1b) affords values for k_{on} of 733 s^{-1} M^{-1} and k_{off} of 8.7×10^{-4} s^{-1}. Independently measured, the association rate constant (k_{on}) determined under pseudo-first-order conditions was 770 s^{-1} M^{-1}. The dissociation rate constant of the enzyme bound to the trifluoromethyl dipeptide analogue **3** was measured by separating inactivated enzyme from excess inhibitor by rapid gel filtration (Penefsky, 1979). The return of activity was monitored by periodically assaying portions of the mixture. The return of activity was first order with a value for k_{off} of 4.3×10^{-4} s^{-1}. The association and dissociation constants are in good agreement and afford the value for K_i shown in Table I.

The difluoromethyl ketone **4** is a rapid reversible competitive inhibitor with very similar K_i to the corresponding aldehyde **10**. The monofluoromethyl ketone and methyl ketone **5** and **6** and the alcohol **7** are all competitive inhibitors of similar potency, suggesting the absence of tetrahedral adduct formation with **5**. The monofluoromethyl ketone also shows irreversible time-dependent inhibition of chymotrypsin: $k_{obsd}/[I] = 1.67$ M^{-1} s^{-1} at pH 7.8 (25 °C). The rate of inhibition is pH-dependent, being almost nonexistent at pH 5.0. Analogous behavior is observed with the corresponding chloromethyl ketone compound (Kurachi et al., 1973). Observation of the interaction of the enzyme with **5** by ^{19}F NMR shows time-dependent release of F^- (sharp signal at −120 ppm) concomitant with the irreversible inactivation of the enzyme. The fully derivatized enzyme remained inactive after dialysis against 2 × 2 L of 10 mM potassium phosphate buffer, pH 7.5. The site of alkylation was shown to be histidine by amino acid analysis of the fully inactivated acid-hydrolyzed product. The peptidyl difluoro- and trifluoromethyl ketones did not exhibit time-dependent irreversible inactivation.

Inhibition of Porcine Pancreatic Elastase. The results of our studies on porcine pancreatic elastase are shown in Table II. Elastase-like serine proteases are markedly different from the trypsin- and chymotrypsin-like enzymes, in that the binding contact area is far more extended. Thus, this enzyme is far more specific for tetra- and pentapeptide sequence and shows very little affinity for mono- and dipeptides. The peptide sequence chosen is based on extensive studies of the specificity of this enzyme (Thompson, 1973; Powers & Tuhy, 1973).

Comparison of the dipeptides **11** and **12** and the tetrapeptides **13** and **14** clearly demonstrates the importance of the extended binding site in elastase as an improvement of 10^3–10^4 in binding is observed. Both tetrapeptide inhibitors show slow-binding inhibition. The trifluoroketone **13** shows *both* rapid- and slow-binding components in its inhibition. This is in contrast with **3** for which the rapid binding K_i could not

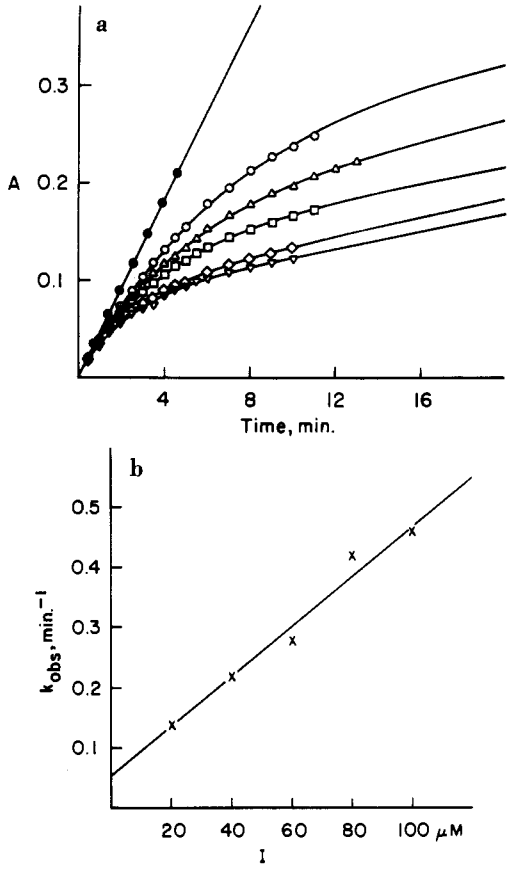


FIGURE 1: Inhibition of chymotrypsin by trifluoromethyl ketone **3**. (a) Progress curves of substrate hydrolysis (measured at 256 nm) by chymotrypsin in the presence of various concentrations of trifluoromethyl ketone **3**. The curves are predicted by fitting the data to the integrated rate equation (eq 2) by nonlinear regression. Reactions contained 0.3 mM substrate, 8 nM enzyme, and increasing inhibitor under the standard assay conditions; the reactions were initiated by enzyme addition. The following inhibitor concentrations were used: (●) 0, (○) 20, (△) 40, (□) 60, (◇) 80, and (▽) 100 μM. (b) Replot of k_{obsd} vs. inhibitor concentration, where k_{obsd} is derived from the progress curves as described in the legend to panel a.

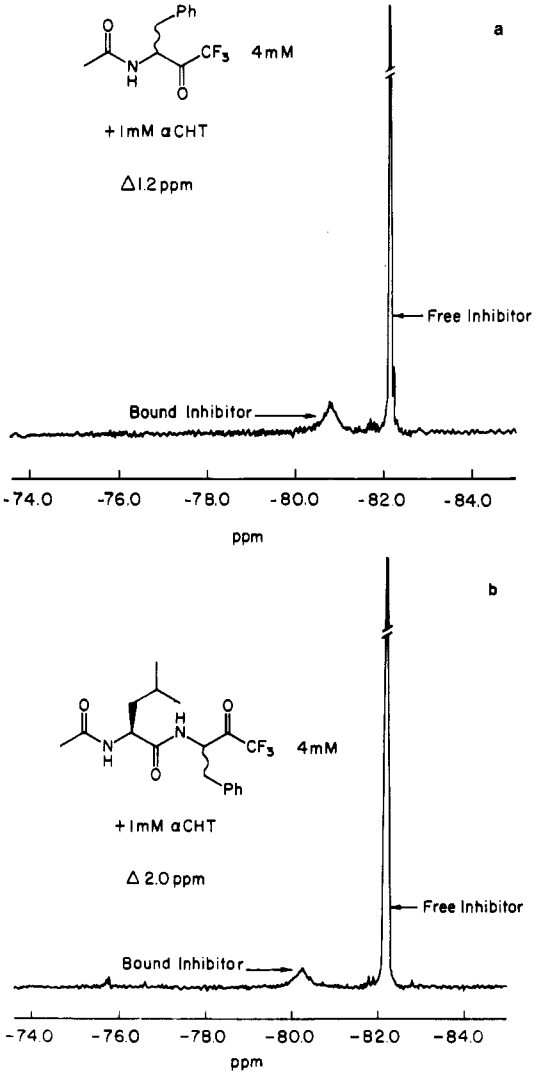


FIGURE 2: ^{19}F NMR (282-MHz) spectrum of chymotrypsin and trifluoromethyl ketone inhibitors. (a) Inhibitor **2**, 4 mM, and chymotrypsin, 1 mM, in 40 mM TAPS buffer, pH 8.5, and 20% [^{2}H]H_2O. (b) Inhibitor **3**, 4 mM; other conditions as in panel a.

be measured (i.e., $K_i > 10^{-4}$ M). The K_i for the rapid-binding complex was determined by conventional methods. Use of the Cha (1975) method resulted in hyperbolic replots for k_{obsd} vs. [I] which could not be readily interpreted. The K_i for slow binding was, therefore, determined by establishing an equilibrium between enzyme and inhibitor, as described under Experimental Procedures, and found to be 0.25×10^{-6} M. Establishment of equilibrium was slow at concentrations from 1.0×10^{-6} to 0.2×10^{-6} M, taking several hours to develop. The association constant (k_{on}) was determined by measuring activity loss as a function of time at various inhibitor concentrations and constructing a series of first-order plots. The slopes of these plots were proportional to [I] at low inhibitor concentration. The k_{on} was determined to be 290 s^{-1} M^{-1}. When [I] approached 10^{-5} M, which corresponds closely to K_i (rapid binding), the slopes were no longer proportional to [I] and approached a limiting value. The k_{off} was measured by dissociation of the EI complex and found to be 1.25×10^{-4} s^{-1}. The K_i can be estimated at 0.43×10^{-6} M from the values of k_{on} and k_{off}, which is in fair agreement with the value determined by the equilibrium method.

For elastase there is very little difference in the potency of equivalent trifluoro- and difluoromethyl ketones. This result may be indicative of more subtle differences in the specificity of chymotrypsin and elastase at the leaving group site.

The tetrapeptide difluoromethyl ketone **14** was separated into two constituent diastereomers by HPLC. One isomer is clearly superior; while it is expected that this is the isomer that would be analogous to the L amino acid on the basis of substrate specificities, this has not been verified. In all other cases fluoromethyl ketones examined are epimeric at the α-carbon of the fluorine-containing amino acid analogue. The K_i of a similar aldehyde **15** is of the same order of magnitude as both trifluoro- and difluoromethyl ketones.

^{19}F NMR Studies of Trifluoromethyl Ketone Inhibitors of Chymotrypsin. The interaction of **2** and **3** with α-chymotrypsin was examined by ^{19}F NMR to address the question of hemiketal vs. hydrate binding to the enzyme. ^{19}F NMR signals are known to be sensitive to such changes in the adjacent carbon atom (Guthrie, 1975). Hemiketalization in free alcoholic solution is generally accompanied by a downfield shift of the ^{19}F NMR signal relative to hydrate; addition of methanol to a solution of trifluoroacetone hydrate causes the appearance of a new signal 2.8 ppm downfield. The fluorine signals in the ketone resonate 8 ppm downfield from the hydrate. ^{19}F NMR studies clearly demonstrate enzyme-bound inhibitors (Figure 2). It is notable that the downfield shift of the more specific inhibitor is almost twice that for the single amino acid analogue: 2.0 vs. 1.2 ppm. The chemical shift of the new, broad signal, while not inconsistent with a hemiacetal

structure involving the serine hydroxyl, does not completely exclude binding of hydrate with downfield shifts due solely to the "new" environment of the fluorine atoms. The slow irreversible binding of *N*-(trifluoroacetyl)$(Ala)_3CH_2Cl$ to elastase (Renaud et al., 1978), via alkylation on histidine, was accompanied by a downfield shift of 0.5 ppm due to the changed environment of the fluorine atoms. It is also possible that the broad signal for enzyme-bound inhibitor, since it is in relatively rapid equilibrium with free inhibitor, represents an average signal and is thus at a higher field than expected. Unfortunately, fluorine NMR signals are rather susceptible to changes in solvent polarity and ionic strength. Hence, while it seems fair to say that the ^{19}F NMR signals observed for the two chymotrypsin inhibitors indicate tighter binding of the dipeptide inhibitor because of the magnitude of the downfield shift, it would be necessary to observe ^{13}C-enriched inhibitors to make more conclusive statements about the exact nature of the enzyme-bound intermediate. Studies are currently in progress to see if anhydrochymotrypsin shows any specific binding to the fluoromethyl ketone inhibitors. Thus one could distinguish the ^{19}F NMR signal changes resulting from environment effects from other effects. The ^{19}F NMR studies exclude the possibility of the inhibitor binding in the ketone form.

^{19}F NMR of **3** and **4** show these inhibitors to be essentially completely hydrated (>100:1, hydrate to ketone) in aqueous solution. Conversely, the monofluoromethyl ketone **5** is only 50% hydrated in water.

Proton Release and pH Dependence. We questioned whether the increasing fluorine substitution caused greater stabilization on the enzyme because of the lower first pK_a of the hemiacetal (or hydrate) adduct, making anion stabilization in the oxyanion hole, by the enzyme, more facile. A typical first ionization of a peptidyl trifluoromethyl ketone hydrate was found to be 9.65, substantially lower (3.5 pK_a units) than the corresponding methyl analogue. In the binding of the inhibitor to chymotrypsin, we found that the addition of inhibitor **3** did not result in observable proton release. However, there is now substantial evidence that the pK_a of the imidazole ring of histidine (57) can be perturbed considerably in inhibitor complexes. This effect is thought to arise from the favorable electrostatic interaction between the positively charged imidazole ring and the oxyanion of the hemiketal hydroxyl (Malthouse et al., 1985).

The k_{on} for the tight-binding inhibitor was observed at varying pH and found to be almost invariant from pH 6 to pH 9. This result indicated that the enzyme did not bind directly to the hydrate since pH might be expected to have an effect on k_{on} if an ionizable form of the inhibitor (such as hydrate) was involved in binding. In addition, no pH dependence of K_i was observed for the trifluoromethyl ketone **2** when examined from pH 7 to pH 9. The insensitivity to pH effects is indicative of a nonionizable species such as the ketone binding to enzyme. The corresponding aldehydic peptide inhibitors are also rather unaffected by pH changes; in the case of aldehyde inhibitors saturation-transfer NMR studies have identified the aldehyde as the species that binds to the enzyme (Chen et al., 1979).

Specificity. In all cases inhibitors were highly specific toward the targeted serine protease. The best chymotrypsin inhibitor, **3**, had negligible effect on bovine trypsin, porcine pancreatic elastase, or the sulfhydryl protease papain, K_i >5 mM, which is the limit of solubility for this compound. Similarly, the trifluoromethyl and difluoromethyl tetrapeptide inhibitors of elastase had no effect on papain, trypsin, or chymotrypsin at levels below 10 mM (K_i >5 mM).

Discussion

The data in Tables I and II show that effective inhibitors of serine proteases can be obtained by the incorporation of a fluoromethyl ketone moiety into substrate analogues of proteolytic enzymes. By analogy with the peptidyl aldehyde inhibitors, where hemiacetal formation is established by X-ray crystallography (Brayer et al., 1979) and by NMR spectroscopy (Lowe & Nurse, 1977; Chen et al., 1979; Shah et al., 1984), it is reasonable to assume that the fluoromethyl ketone inhibitors react with the γ-OH of the active site serine to form a stable hemiacetal. ^{19}F NMR data are consistent with formation of a hemiacetal and exclude the possibility that the complex contains the inhibitor in its pure ketone form. In aqueous solution both the difluoro- and trifluoromethyl ketones are essentially completely hydrated. Therefore, if hemiacetal formation occurs, this step cannot in itself be directly responsible for the low K_i of this class of inhibitors, since complex formation would merely involve replacement of the hydroxyl group of the hydrate by that of a serine residue. Since the interaction of the peptide portion of the inhibitor with the extended binding site of the enzyme makes hemiacetal formation essentially an intramolecular process, while hydrate formation is a bimolecular process, strong binding is largely an entropic effect. This effect has been discussed extensively (Jencks, 1975; Thompson & Bauer, 1979).

It is evident that increasing the number of amino acid residues leads to a decrease in K_i. For instance, the K_i for the trifluoromethyl ketone Ac-Pro-*ambo*-Ala-CF_3 (**11**, Table II) is 1.2×10^4 higher than the K_i for Ac-Ala-Ala-Pro-*ambo*-Ala-CF_3 (**13**, Table II). The incrementing number of secondary binding interactions on K_i has a very similar effect to analogous changes in V/K for hydrolysis of the corresponding amide substrates. The V/K for the amide corresponding to **13** is 4×10^4 higher than the V/K for the substrate corresponding to **11**. The correlation between K_i and V/K suggests that the enzyme–inhibitor interactions are more similar to those that the enzyme possesses for the substrate at its transition state than in the ground state and is suggestive of the inhibitors being transition-state analogues (Thompson & Blout, 1973; Bartlett & Marlowe, 1983). The correlation also provides further evidence that the inhibitors form adducts with the active site serine.

In addition to chain length, the number of fluorine substituents affects the K_i. Increasing the number of fluorines α to the carbonyl group decreases the K_i. The results with elastase differ somewhat from those with chymotrypsin in this respect. With chymotrypsin the K_i for the trifluoromethyl ketone **3** is 30-fold lower than that for the difluoromethyl ketone **4**, while with elastase the K_i's for the corresponding inhibitors are nearly identical. In view of the fact that the difluoro- and trifluoromethyl ketones are both essentially completely hydrated in aqueous solution, it is rather surprising that the K_i decreases to such a large extent from **4** to **3**. Therefore, it is apparent that fluorine substitution must affect K_i through an effect other than simply on the bond strength of the carbonyl serine bond. Fluorine substitution also has a profound effect on the K_i's of the corresponding alcohols, which can form no covalent adduct with the enzyme. Comparison of the monofluoro alcohol **8** with the trifluoro alcohol **7** (both compounds are a racemic mixture of *threo* and *erythro* diastereomers) shows a significant decrease in the K_i on the introduction of more fluorine atoms. This effect could be due to the increased potential for fluorine hydrogen bonding with proton-donating substituents at the active site or more likely

due to the significant effect that fluorine substitution has on lowering the pK_a of the hydroxyl group of the hemiacetal or alcohol. The increased negative charge density at oxygen may facilitate hydrogen-bond formation or in the case of the hemiacetal may actually lead to formation of a discrete alkoxide ion. This oxygen could thus interact more favorably with the "anionic hole" formed by the amide nitrogens of Gly-193 and Ser-195.

Within a series of inhibitors containing the same number of amino acid residues, fluorine substitution also lowers the k_{off} for the inhibitor. In the case of chymotrypsin only the dipeptidyl trifluoromethyl ketone **3** shows slow binding with a k_{off} of 6×10^{-4} s^{-1}. The limit of detection of the slow off-rate, without recourse to rapid-mixing experiments, is $k_{off} < 0.046$ s^{-1} ($t_{1/2} > 15$ s). Therefore, for all the inhibitors termed "rapidly reversible" $k_{off} > 0.046$ s^{-1}. The off-rates represent a useful parameter to delineate whether or not slow binding is demonstrated and are easier to compare than rates of equilibration in the presence of the substrate, which may or may not be observed depending on the inhibitor concentrations used. For elastase both the trifluoromethyl ketone **13** and the difluoromethyl ketone **14** have measurable k_{off}'s, being 1.25×10^{-4} s^{-1} and 0.007 s^{-1}, respectively. The rate of dissociation of the carbon–oxygen bond of the hemiacetal decreases with fluorine substitution. This is consistent with higher k_{assoc} for the hydration of the corresponding ketones; the higher k_{assoc} is associated with lower dissociation of adducts. This is not an independent effect, since the binding of the rest of the inhibitor also contributes to the off-rate; thus the trifluoromethyl ketone **2** and the difluoromethyl ketone **4** do not demonstrate measurable off-rates.

In addition to the slow-binding complex, the peptide analogue **13** also forms a rapidly equilibrating complex with the enzyme. The nature of the intermediate complex and the steps involved in its formation and the formation of the tightly bound complex were not known. This complex could be an intermediate formed prior to the formation of a slow-binding complex as illustrated in eq 4a. Thus the second slow step

$$E + I \underset{}{\overset{K_1}{\rightleftharpoons}} EI \overset{K_2}{\rightleftharpoons} EI^* \qquad (4a)$$

$$E + I \overset{K_2}{\rightleftharpoons} EI^* \qquad (4b)$$

$$K_1 \downarrow\uparrow$$

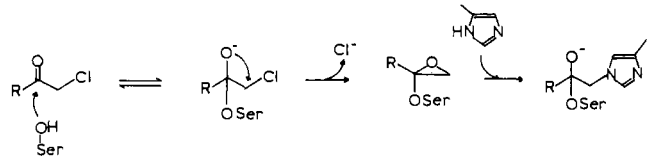
$$EI$$

EI = loosely bound complex
EI* = tightly bound complex

might be represented by a slow chemical step or a conformational change of the protein after inhibitor binding. In this scheme, K_1 corresponds to K_i for the rapidly equilibrating complex, and K_1K_2 corresponds to K_i for the slow-binding complex. Alternatively, as illustrated in eq 4b, the loosely bound enzyme–inhibitor complex might be the result of nonproductive binding and *not* on the reaction path to tightly bound inhibitor EI*. Such a complex might be represented by the binding of the ketone hydrate to the enzyme, which must dissociate before ketone can bind to the enzyme and form a hemiacetal with the serine. Thus dehydration cannot take place within the enzyme active site. A third possibility is that since the inhibitor is in fact a mixture of diastereomers, the rapid- and slow-binding complexes may be attributable to different kinetic behavior by the two constituent isomers.

The possibility was considered in the mechanism in eq 4a that EI is a complex between enzyme and ketone and that the second step involves the reaction of the serine hydroxyl to form the hemiacetal EI*. However, in light of the fact that $K_2 = k_2/k_{-2} = 26$ for inhibitor **13** and that the K_{hyd} for trifluoromethyl ketones is a minimum of 100 (potentially far more with this peptidyl compound favorably positioned at the active site), this alternative has been excluded.

Scheme I: Mechanism of Inactivation of Chymotrypsin by Chloromethyl Ketones



Inhibitors containing di- and trifluoromethyl ketones form reversible enzyme–inhibitor complexes. The monofluoromethyl ketone **5** forms both a weak reversible complex and an irreversible, covalent complex by alkylating a histidine residue of chymotrypsin. The second-order rate constant for the alkylation reaction is 1.7 M^{-1} s^{-1} (pH 7.8, 25 °C); the analogous chloromethyl ketone reacts 15 times faster (pH 7.8, 30 °C), with a rate constant of 25.6 M^{-1} s^{-1} (Kurachi et al., 1973). The value for the chloromethyl ketone has been extrapolated from the value given at different pH in order to facilitate the comparison of the rates. This result was rather surprising in light of the vastly differing rates for bimolecular nucleophilic substitution for the two leaving groups. The average leaving group susceptibility of chloride vs. fluoride is 200-fold (Streitweiser, 1962). Furthermore, preliminary studies using *N*-acetylhistidine as nucleophile with typical chloromethyl and fluoromethyl ketones have indicated that this difference in leaving group susceptibility is a minimum of 200-fold with the halide leaving groups α to a carbonyl as in these inhibitors (unpublished results). Thus in this case it appears that a simple bimolecular model for inactivation might not be pertinent. Alternative schemes for inactivation in which dissociation of the carbon–halogen bond is either not, or only partially, rate determining are more attractive. One such model has been proposed by Powers (1977) and involves the intermediacy of an enzyme-bound epoxide resulting from displacement of the halide by an internal S_Ni reaction, as depected in Scheme I. In this mechanism, the importance of the halide in the rate-determining step might be reduced. Alternatively, because of the possibility for fluorine–hydrogen bonding (for which no analogous effect is present with chlorine) displacement of fluorine could be assisted by an acidic group at the active site and thus subject to acid catalysis. It has been demonstrated with studies on anhydrochymotrypsin, which lacks the active site serine, that alkylation by α-halo ketone inhibitors cannot take place (Weiner et al., 1966); this fact also indicates involvement of the serine hydroxyl group as an integral part of the inactivation mechanism. Peptidyl monofluoromethyl ketones have recently been reported to be irreversible inhibitors of the cysteine protease cathepsin B (Rasnick, 1985).

The demonstration of slow-binding inhibition by some tri- and difluoromethyl ketone inhibitors of serine proteases is noteworthy. Thus far the only other synthetic inhibitors of these proteases that display this property are the peptide α-aminoboronates (Kettner & Shenvi, 1984). We have found no record in the literature of peptidyl aldehydes displaying slow-binding kinetic behavior as defined by Williams and Morrison (1979). However, the slow-binding inhibition of cathepsin B (a cysteine protease) by the naturally occurring peptidyl aldehyde leupeptin (Baici & Gyger-Marazzi, 1982) has been demonstrated. This observation probably reflects the more favorable K_{assoc} of the aldehydes with thiols as compared to alcohols (Lienhard & Jencks, 1966).

Supplementary Material Available

Detailed description of the synthesis, purification, and physical properties of peptidyl fluoromethyl ketones used (20 pages). Ordering information is given on any current masthead page.

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