Inhibition of Chymotrypsin by Fluorinated α-Keto Acid Derivatives†

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ABSTRACT: A series of fluorinated α-keto acid derivatives [$PhCHFCOCO_2R$, $PhCH_2CHFCOCO_2R$, $PhCF_2COCO_2R$, and $PhCH_2CF_2COCO_2R$ (R = H, Me, and Et)] was synthesized. They were inhibitors of chymotrypsin, with K_i values ranging from 4700 to 15 μM. Benzylpyruvic derivatives were generally more potent than the corresponding phenylpyruvic analogs. Esters of the first series were also more potent than their corresponding acids, and potency increased with the number of fluorine atoms. By replacing the ethoxy group of $PhCH_2CF_2COCO_2Et$ (**15b**) with an amino acid chain (i.e., alanyl-leucyl-arginine methyl ester hydrochloride and alanyl-leucyl-valine ethyl ester), the resultant peptides $PhCH_2CF_2COCO$-Ala-Leu-Arg-OMe·HCl·H_2O (**20**) and $PhCH_2CF_2COCO$-Ala-Leu-Val-OEt·H_2O (**23**) were found to be slow-binding inhibitors of chymotrypsin with considerably lower K_i values (0.19 and 3.6 μM, respectively). ^{19}F NMR studies indicate, in the case of **20**, the presence of an enzyme–inhibitor complex with a hemiketal structure similar to those observed between trifluoromethyl ketones and chymotrypsin. The results illustrate that effective protease inhibitors can be designed by enhancing the electrophilic character of the reactive carbonyl group (with an electron-withdrawing group placed on each side of the carbonyl group). Their potency and/or selectivity can also be improved by taking advantage of binding interactions at S′ subsites of the protease.

Many reversible inhibitors of serine and cysteine proteases exhibit two common features. The first feature is a central structure at the position P_1 (according to the nomenclature of Schecter and Berger (1967)) which reacts covalently with the active-site serine or cysteine to form an adduct resembling a transition-state or a reaction intermediate. This structure must meet the specificity requirements of the S_1 binding site of the target enzyme. The second feature is a peptide component, attached to the central unit, which interacts with enzyme subsites to increase binding.

Examples of functional groups which have been incorporated in the central structure are the following: α-keto acids (Geratz, 1965, 1967; Markwardt et al., 1974; Stürzebecher et al., 1976; Tanizawa et al., 1985; Hori et al., 1985), fluoromethyl ketones (Imperiali & Abeles, 1986: Rauber et al., 1986), boronic acids (Koehler & Lienhard, 1971; Lindquist & Terry, 1974), and aldehydes (Westerik & Wolfenden, 1972; Thompson, 1973). All of these inhibitors contain an electrophilic center. The recurring approach used to improve their effectiveness, apart from a few examples (i.e., "extended binding inhibitors", Imperiali and Abeles, 1987), has generally consisted of the addition of a peptide chain in the $P_2 \rightarrow P_n$ direction to form peptidyl α-keto esters (Hori et al., 1985; Peet et al., 1990: Mehdi et al., 1990), peptidyl fluoromethyl ketones (Imperiali & Abeles, 1986; Stein et al., 1987; Peet et al., 1990), peptidyl boronic acids (Kettner & Shenvi, 1984), and peptidyl aldehydes (Thompson & Bauer, 1979). This unidirectional extension of the P_1 unit of synthetic inhibitors is dictated by the structure itself (i.e., aldehydes and boronic acids), which does not allow extension in the P′ direction.

We have synthesized inhibitors of the general structure RCX_2COCO_2R[1] (R = Ph, $PhCH_2$; X = H, F; R[1] = H, Me, and Et) in which the reactive carbonyl (i.e., α-keto group) is flanked by two electron-withdrawing groups. These compounds combine, in their reactive part, the characteristic features of two known classes of serine protease inhibitors (fluoromethyl ketones and α-keto acids). Subsequently, we added a peptide chain in the P′ direction so that binding interactions with the S′ subsites of the enzyme could be utilized. We have chosen chymotrypsin as prototypical target.

MATERIALS AND METHODS

α-Chymotrypsin (type II, from bovine pancreas), elastase (type I, from bovine pancreas), papain (from papaya latex), trypsin (type XI, from bovine pancreas), L-BAPNA,[1] MeO-Suc-AAPV-pNA, Suc-AAPF-pNA, and Cbz-Ala-Leu-OH were purchased from Sigma Chemical Co. Methyl benzoylformate and sodium phenylpyruvate monohydrate were obtained from Aldrich Chemical Co.

Inhibitor Synthesis. The procedure, developed by Ayi et al. (1981), for the synthesis of methyl 3-fluoro-3-phenyl- and methyl 3-fluoro-3-benzylpyruvate (**4a,b**) and the corresponding acids **5a,b** is outlined in Scheme I. According to this strategy, epoxy esters **2a,b** were obtained by Darzen glycidic ester condensation of benzaldehyde (**1a**) and phenylacetaldehyde (**1b**) with methyl chloroacetate. Ring opening of **2a,b** with a hydrogen fluoride–pyridine solution produced the β-fluoro-α-hydroxy esters **3a,b**, which were oxidized to **4a,b** and then hydrolyzed to the α-keto acids **5a,b**. Methyl phenylpyruvate (**9b**) was obtained by methylation of the

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[1] Abbreviations: L-BAPNA, N^{α}-benzoyl-L-arginine *p*-nitroanilide hydrochloride; MeOSuc-AAPV-pNA, *N*-methoxysuccinyl-L-alanyl-L-alanyl-L-prolyl-L-valine *p*-nitroanilide; Suc-AAPF-pNA, *N*-succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine *p*-nitroanilide; Tris, tris(hydroxymethyl)aminomethane; 4-APPA, (4-amidinophenyl)pyruvic acid; Ac, acetyl; Bz, benzoyl; Cbz, carbobenzyloxy; DCC, 1,3-dicyclohexylcarbodiimide; DME, dimethoxyethane.

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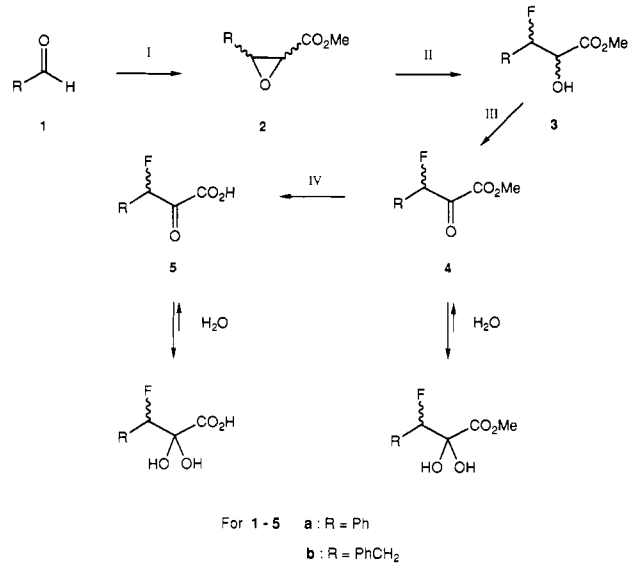
Scheme I [a]



[a] Reagents: (I) $ClCH_2CO_2Me$, ^tBuOK, ^tBuOH; (II) 70% HF–pyridine (w/w %), CH_2Cl_2; (III) Dess–Martin periodinane, CH_2Cl_2; (IV) aqueous $NaHCO_3$–Me_2CHOH (1/1).

corresponding acid (**8**) with diazomethane. Condensation of the Grignard reagent derived from (2-bromoethyl)benzene with diethyl oxalate afforded ethyl benzylpyruvate (**6**), which was then hydrolyzed, under acidic conditions, to benzylpyruvic acid (**7**). 3,3-Difluoro-3-phenyl- and 3,3-difluoro-3-benzylpyruvic acid (**16a,b**) and their ethyl esters **15a,b** were synthesized according to Scheme II. In this sequence, the difluoro esters **10a,b** obtained by the Middleton (1980) method were reduced to the hemiacetals **11a,b** and then treated with potassium cyanide. The cyanohydrins **12a,b** were then converted via their imino ester hydrochlorides **13a,b** to the hydroxy esters **14a,b**, which were finally oxidized to the keto esters **15a,b** and hydrolyzed to yield the keto acids **16a,b**. $PhCH_2CF_2COCO$-Ala-Leu-Arg-OMe·HCl·H_2O (**20**) and $PhCH_2CF_2COCO$-Ala-Leu-Val-OEt·H_2O (**23**) were prepared by the *N*-hydroxysuccinimide ester method (Anderson et al., 1964) in the presence of 1,3-dicyclohexylcarbodiimide, condensing the keto acid **16b** with Ala-Leu-Arg-OMe·HCl (**19**) and Ala-Leu-Val-OEt (**22**), respectively. The tripeptides **19** and **22** had been previously prepared from Cbz-Ala-Leu-OH (**17**) (McDermott & Benoiton, 1973) and Arg-OMe·2HCl by the *N*-hydroxysuccinimide ester method and from Cbz-Ala-Leu-OH (**17**) and Val-OEt·HCl by the mixed carbonic anhydride method (Anderson et al., 1967), respectively (Scheme III). The synthesis of difluoro α-keto acids **16a,b** will be reported elsewhere. The detailed synthetic procedures for the other compounds are available as supplementary material. All compounds were fully characterized by ^1H, ^{13}C, and ^{19}F NMR, and their purity was established by elemental analysis or thin-layer chromatography. Spectral and analytical data for all compounds used in this study (including the difluoro keto acids) are also available as supplementary material.

Enzyme Assays. α-Chymotrypsin assays were conducted spectrophotometrically with a Perkin-Elmer λ-3 UV/vis instrument, using 1-cm cells held at 25 °C. The enzymatic activity was measured by monitoring the increase of absorbance at 410 nm due to release of *p*-nitroaniline during the enzymatic hydrolysis of the peptide substrate Suc-AAPF-pNA (DelMar et al., 1979). Typically, assay mixtures in 0.1 M potassium phosphate buffer at pH 7.8 contained α-chymotrypsin (5.0 nM, concentration based on weight), substrate (0.55–0.05 nM), and inhibitors (concentration ranges given in Table I) in a total volume of 1.0 mL. Stock solutions of the inhibitors described below were prepared in phosphate buffer in the case of keto acids and peptide **20**. Acetonitrile was used as solvent for keto esters and peptide **23** due to their limited solubility in aqueous solution. In all cases, 4% (v/v) acetonitrile was present in the final assay mixture and had no effect on the enzyme. Similarly, all other enzyme assays, carried out to test the specificity of **20**, were also conducted spectrophotometrically by monitoring the release of *p*-nitroaniline from the appropriate substrate. Elastase assays were performed in 0.1 M sodium phosphate buffer at pH 7.8 containing 0.5 M sodium chloride using MeOSuc-AAPV-pNA as substrate. Papain and trypsin were assayed in the presence of L-BAPNA in 0.5 M potassium phosphate buffer, pH 7.0, containing 5 mM EDTA and 5 mM cysteine and in 50 mM Tris-HCl buffer, pH 8.2, containing 20 mM calcium chloride, respectively.

Kinetic Procedures. K_i values for reversible competitive inhibitors were estimated by the method of Lineweaver and Burk. Data were fitted to the best straight line by the least-squares procedure. In the case of **20** and **23**, two slow-binding inhibitors (Morrison, 1982; Morrison & Walsh, 1988) of chymotrypsin, the inhibition constants were calculated according to the procedure of Cha (1975). Under these conditions the release of product *P* with time is described by

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

where v_s, is the final steady-state velocity, v_0 is the initial velocity, and k_{obsd} is the apparent first-order rate constant for the approach to the steady state. The value of k_{obsd} is related to the individual association (k_{on}) and dissociation (k_{off}) constants by eq 3. The assumed mechanism is shown in eq 2. Both k_{on} and k_{off} values can be calculated from the replot

$$E + S \underset{}{\overset{K_m}{\rightleftharpoons}} E{\cdot}S \xrightarrow{k_e} E + P \quad (2)$$

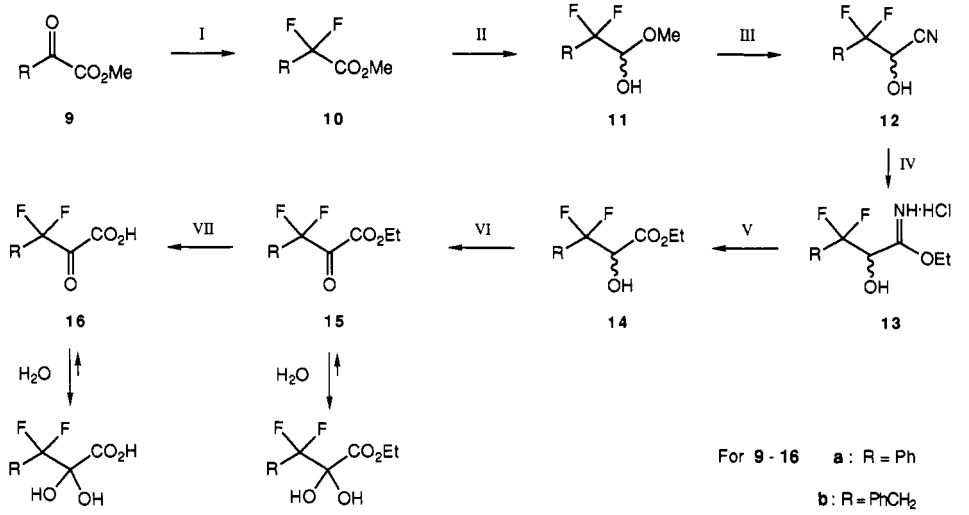
$$E{\cdot}I \quad (k_{on}[I] \uparrow\downarrow k_{off})$$

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

of k_{obsd} vs [I] (eq 3); K_i was calculated from k_{on} and k_{off} and from the steady-state velocity of the progress curve. In addition, k_{on} was also determined under pseudo-first-order conditions ([I] > 10[E]) from the rate of onset of slow-binding inhibition. In this procedure, aliquots of an enzyme–inhibitor mixture, in phosphate buffer, were incubated for various time periods and diluted into an assay mixture containing substrate, and the remaining enzymatic activity was measured. The dissociation rate constant (k_{off}) of **20** was also determined by monitoring the return of activity from a fully inactivated enzyme–inhibitor complex. Such a complex was freed from excess inhibitor by rapid gel filtration (Penefsky, 1979), and aliquots of the eluate were then added to an assay mixture containing substrate to monitor the rate of return of activity.

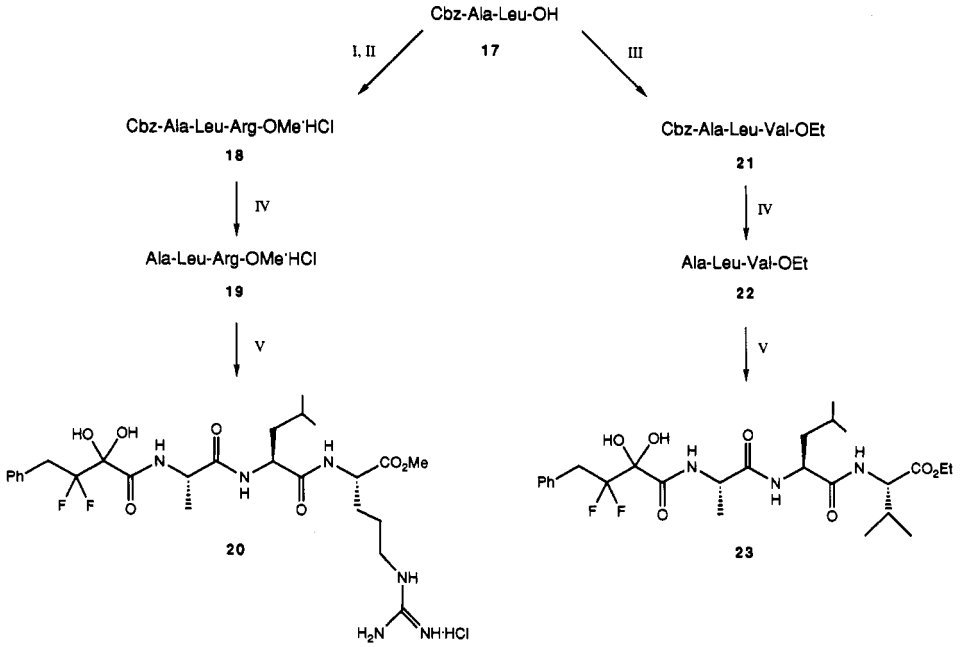
NMR Spectra. All of the NMR spectra were recorded with a Varian XL-300 instrument at 75.5 MHz for ^{13}C under noise proton decoupling conditions and at 282.2 MHz for ^{19}F without proton decoupling. ^{19}F chemical shifts (ϕ) are given in ppm from trifluoroacetic acid (positive for upfield shifts). For the studies on the formation of enzyme–inhibitor complex **20**, the ^{19}F NMR samples contained 3 mM **20**, 3 mM

Scheme II [a]



[a] Reagents: (I) DAST; (II) $NaBH_4$, MeOH; (III) KCN, KH_2PO_4, H_2O; (IV) HCl_{gas}, anhydrous EtOH; (V) H_2SO_4–H_2O; (VI) Dess–Martin periodinane, CH_2Cl_2; (VII) aqueous $NaHCO_3$–Me_2CHOH (1/1).

Scheme III [a]



[a] Reagents: (I) *N*-hydroxysuccinimide, DCC, DME; (II) L-Arg-OMe·2HCl, Et_3N, H_2O; (III) *N*-methylmorpholine, ^{i}BuOCOCl, L-Val-OEt·HCl, THF; (IV) H_2, 10% Pd/C, MeOH; (V) $PhCH_2CF_2COCO_2H \cdot H_2O$, *N*-hydroxysuccinimide, DCC, dioxane.

α-chymotrypsin,[2] 0.1 M potassium phosphate buffer (725 μL) at pH 7.8, and ^2H_2O (75 μL), and trifluoroacetic acid (50 μM) was added as internal standard. Spectra were recorded under the following conditions: 20 000 Hz spectral width, 12K time-domain data points, 0.3 s acquisition time, 60° pulse, and 3000–10 000 scans for each spectrum.

RESULTS

Inhibition. We have synthesized a number of aromatic derivatives of pyruvic acid and tested them as inhibitors of α-chymotrypsin. The results are presented in Table I. All compounds are rapidly reversible competitive inhibitors with the exception of **20** and **23**, which are slow-binding. All benzylpyruvic derivatives are more potent inhibitors than the corresponding phenylpyruvic analogs (the only exception being $PhCH_2CH_2COCO_2H$). Furthermore, two general patterns emerge in the benzylpyruvic series: (a) difluorinated analogs ≥ monofluorinated > unfluorinated, and (b) keto esters > keto acids.

We had previously observed that incorporation of components which can interact with the S′ subsites of chymotrypsin lowered the K_i values of fluoromethyl ketone inhibitors (Imperiali & Abeles, 1987). We therefore replaced the ethoxy group of **15b** with a tripeptide moiety (i.e., Ala-Leu-Arg-OMe and Ala-Leu-Val-OEt). Both extended inhibitors **20** and **23** (K_i, 0.19 and 3.6 μM, respectively) showed slow-binding behavior. Kinetic constants were evaluated as described in the Materials and Methods section. Alternative measurements (see kinetic procedures) of k_{on} and k_{off} in the case of **20** were in good agreement with those determined from the Cha plot (Table I).

Hydrate/Ketone Ratio. Since it is likely that the keto form of each inhibitor reacts with the enzyme to form the enzyme–

[2] Omitted in the reference spectrum.

Table I: Inhibitors of Chymotrypsin

compd	concn range (mM)	K_i (mM)
$PhCH_2COCO_2Me$ (**9b**)	2.0–0.5	1.7
$PhCH_2COCO_2H$ (**8**)	2.0–0.5	2.2[a]
$PhCHFCOCO_2Me$ (**4a**)	2.0–0.5	0.9
$PhCHFCOCO_2H$ (**5a**)	5.0–2.0	3.2
$PhCF_2COCO_2Et$ (**15a**)	5.0–2.0	2.5
$PhCF_2COCO_2H$ (**16a**)	5.0–2.0	4.7
$PhCH_2CH_2COCO_2Et$ (**6**)	2.0–0.5	0.4
$PhCH_2CH_2COCO_2H$ (**7**)	2.0–0.5	2.7
$PhCH_2CHFCOCO_2Me$ (**4b**)	0.5–0.15	0.035
$PhCH_2CHFCOCO_2H$ (**5b**)	1.0–0.25	0.15
$PhCH_2CF_2COCO_2Et$ (**15b**)	0.5–0.15	0.015
$PhCH_2CF_2COCO_2H$ (**16b**)	2.0–1.0	0.27
$PhCH_2CF_2COCO$-Ala-Leu-Arg-OMe[b] (**20**)	0.02–0.006	0.00019[c] 0.00016[d]
$PhCH_2CF_2COCO$-Ala-Leu-Val-OEt[b] (**23**)	0.25–0.075	0.0036[e]

[a] Geratz (1965) has reported for **8** a K_i value of 0.86 mM in a 0.1 M imidazole buffer at pH 7.0. [b] Slow-binding reversible competitive inhibition. [c] k_{on} = 5509 M^{-1} s^{-1}, k_{off} = 1.04 × 10^{-3} s^{-1}; determined from the method of Cha (1975). [d] k_{on} = 5865 M^{-1} s^{-1}, k_{off} = 9.5 × 10^{-4} s^{-1}; determined by alternate method (see Kinetic Procedures). [e] k_{on} = 875 M^{-1} s^{-1}, k_{off} = 3.17 × 10^{-3} s^{-1}; determined by the method of Cha.

inhibitor complex, the extents of hydration of the inhibitors were determined. By ^1H and ^{13}C NMR analysis, the unfluorinated compounds in deuterium oxide–0.1 M potassium phosphate solution at p^{2}H 7.8 were found to be in the keto form. On the other hand, in 0.1 M potassium phosphate at pH 7.8 containing 10% ^2H_2O, the ^{19}F NMR spectra showed that both mono- and difluoro acids and esters were in the hydrated form (>98%; data not shown). This type of analysis was possible by first recording the ^{13}C NMR spectra, which allowed us to assign unambiguously the resonances due to the C=O and $C(OH)_2$ groups and correlate them with the signals recorded in the ^{19}F NMR spectra, which then could be accurately integrated.

Because the ^{19}F NMR signals due to the keto ester **15b** (ϕ 113.2 ppm, triplet) and the corresponding keto acid **16b** (ϕ 114.6 ppm, triplet) are distinguishable, it was also possible to investigate whether ester hydrolysis took place under the experimental conditions used in the enzymatic assay. Upon incubation of keto ester **15b** (1 mM) with α-chymotrypsin (10 nM) (for 60 min) in 0.1 M potassium phosphate buffer (900 μL) containing ^2H_2O (100 μL) and trifluoroacetic acid as internal standard, the ^{19}F NMR spectrum did not show any traces of α-keto acid formation (spectral data not shown). Thus **15b**, is stable to enzymatic hydrolysis under the conditions used for at least 60 min. We have also shown that the amide **20** is stable to enzymic hydrolysis.

^{19}F NMR Studies on Enzyme–Inhibitor Complex. Because of high sensitivity and lack of background interference from protein, ^{19}F NMR spectroscopy is a useful tool for the detection of enzyme–inhibitor interactions (Gorenstein & Shah, 1982; Shah & Gorenstein, 1983; Liang & Abeles, 1987).

In order to investigate the structure of enzyme–inhibitor complex **20**, we determined the ^{19}F NMR spectra (in a 0.1 M potassium phosphate buffer solution at pH 7.8) of inhibitor alone and of inhibitor and enzyme (Figure 1). In the former case, the two diastereotopic fluorine atoms of **20** gave rise to a multiplet at 113.7 ppm which, on the basis of the ^{13}C NMR chemical shift of the adjacent carbonyl [δ_c 93.7 (t, J_{F-C} = 28 Hz) ppm, in H_2O], was assigned to the hydrate rather than the ketone form of the inhibitor (Figure 1A). Addition of a 1/1 molar ratio of α-chymotrypsin to this sample caused the disappearance of the multiplet at 113.7 ppm, due to free inhibitor, and the formation of an AB system (ϕ 108.6 and

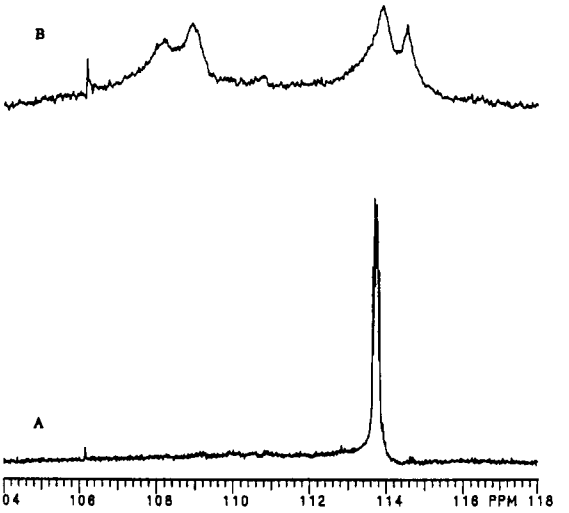


FIGURE 1: ^{19}F NMR spectra (282.2 MHz) of inhibitor **20** and the enzyme–inhibitor **20** complex: (A) 3 mM **20** in aqueous 0.1 M potassium phosphate buffer (725 μL) at pH 7.8, ^2H_2O (75 μL), and trifluoroacetic acid (50 μM) added as internal standard; (B) spectrum A after addition of 3 mM chymotrypsin. Spectral conditions described under Materials and Methods.

114.2 ppm, J_{F-F} = 182 Hz) with broad components[3] (Figure 1B). This new signal is consistent with the formation of an enzyme-bound hemiketal derived from nucleophilic attack of the serine hydroxyl of chymotrypsin on the reactive carbonyl of **20**. The change in splitting pattern, from the multiplet of **20** to the AB system of the final incubation mixture, is probably the result of local asymmetry due to the presence of a new chiral center, next to the difluoromethylene group, of a newly formed hemiketal adduct. In contrast with the case of free inhibitor, where the chemical shifts of the two diastereotopic fluorine atoms are identical (or nearly so), in the spectrum of the enzyme–inhibitor complex two different chemical shifts can be observed. One of these, in relation to the original value, has moved slightly ($\Delta\phi$ = +0.5 ppm), while the other has undergone significant shift ($\Delta\phi$ = –5.1 ppm). The latter shift is consistent with that observed in the case of trifluoromethyl ketones upon hemiketal adduct formation (Imperiali & Abeles, 1986; Liang & Abeles, 1987). It should be noted that the presence of an AB system in the ^{19}F NMR spectrum of the enzyme–inhibitor complex could in principle just arise as a result of the "asymmetric environment" induced by the enzyme active site on the two diastereotopic fluorine atoms of **20**. In consequence, the possibility that **20** is bound to the enzyme as hydrate cannot entirely be ruled out, but we consider this unlikely, given the structural similarity of **20** to trifluoromethyl ketones, which have been shown, by NMR and X-ray studies, to form tetrahedral intermediates (Liang & Abeles, 1987; Brady et al., 1990).

In a model study we also tested, by ^{19}F NMR, the ability of the hydrated carbonyl of **20** to undergo hemiketalization by an incoming nucleophile. In this experiment **20** was dissolved in methanol-d_4, and spectra were recorded after various time periods (Figure 2). After 4 h the original multiplet (ϕ 114.1 ppm) disappeared, and instead new peaks, consisting of two sets of AB systems, were observed. Each of these two sets of AB systems is due to one of the two diastereomeric

[3] A broad line width is generally indicative of a macromolecule-associated signal. The broadness of the peaks in Figure 1B obscures H–F coupling. In contrast, H–F coupling can be seen in the spectra of the free inhibitor and in the mixture of the two diastereomeric hemiketals of the model study.

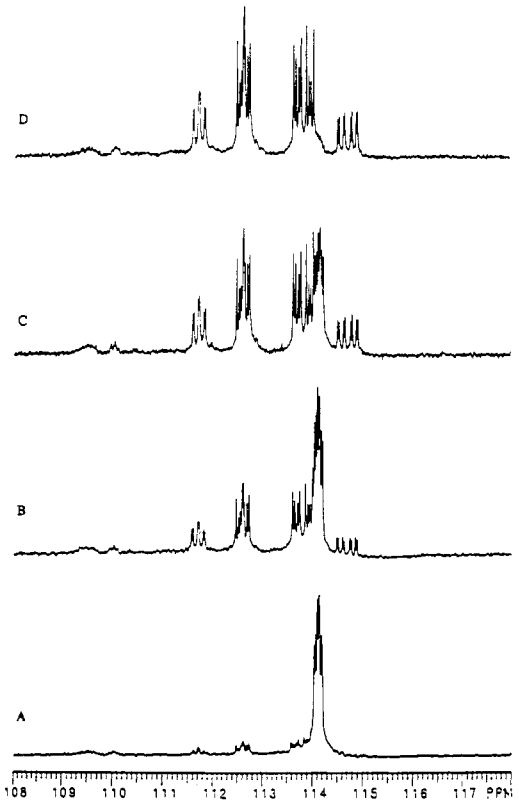


FIGURE 2: ^{19}F NMR spectra (282.2 MHz) of inhibitor **20** in methanol-d_4. Spectra were recorded at increasing lengths of time: (A) time, 15 min; (B) time, 1 h; (C) time, 2 h; (D) time, 4 h. Spectral conditions were 20 000 Hz spectral width, 12K time-domain data points, 0.3 s acquisition time, and 3000 scans for each spectrum.

hemiketals derived from addition (in the two possible modes) of methanol-d_4 to **20**. Under the experimental conditions in which ^{19}F NMR spectra were recorded (282.2 MHz, without proton decoupling), the two sets of AB systems are partially overlapping, and the overall look of the spectrum is that of four highly coupled signals. This result excludes the presence of the ketone or the hydrate form of **20** in the solution.

Inhibitor Specificity. Inhibitor **20** showed a weak effect in the inhibition of papain ($K_i > 2.5$ mM) and elastase ($K_i > 2$ mM).

20 weakly inhibits trypsin as a "competitive substrate". Figure 3 illustrates transient inhibition of the enzymatic hydrolysis of a subsaturating concentration of L-BAPNA by **20**. The inhibition is transient because of the enzymatic hydrolysis of the methyl ester moiety of **20** to an essentially noninhibitory acid form of **20** (K_i = 1 mM). By ^1H NMR, we were able to confirm the enzymatic cleavage of the methyl ester group, from the arginine residue of **20**, by monitoring the disappearance of the singlet at 3.51 ppm due to the addition of trypsin (50 nM) to a sample of **20** (600 μM) in deuterium oxide. From experiments such as that shown in Figure 3, we found that trypsin acts on **20** with a turnover number of 25 s^{-1} and a K_m value of 70 μM. From the initial velocities of reactions such as that shown in Figure 3, **20** was found to exhibit a K_i value of 75 μM as a competitive inhibitor of the hydrolysis of BAPNA by trypsin.

DISCUSSION

The results of these studies indicate that fluorinated α-keto acids and esters are effective inhibitors of chymotrypsin. ^{19}F NMR data indicate that the inhibitors form a tetrahedral intermediate by addition of the active-site serine to the keto

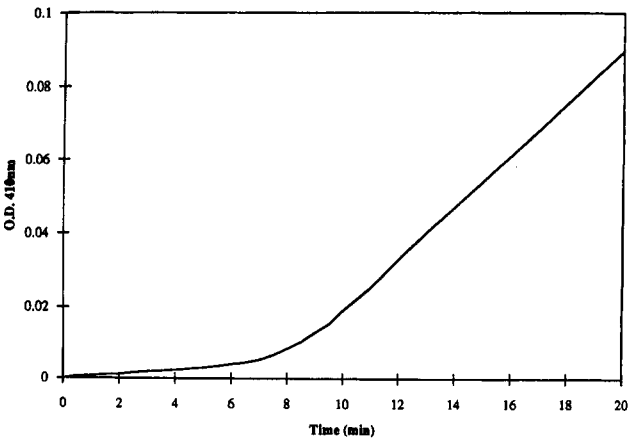


FIGURE 3: Inhibition of trypsin L-BAPNA hydrolysis by the competitive substrate **20**. Progress curve for hydrolysis of L-BAPNA (measured at 410 nm) by trypsin in the presence of **20**. Reaction solution contained 50 nM trypsin, 500 μM L-BAPNA, and 600 μM inhibitor **20** in a 50 mM Tris-HCl buffer at pH 8.2 containing 20 mM $CaCl_2$. Reaction was initiated with addition of enzyme.

group. The K_i value (15 μM) of the difluoro keto ester **15b** is comparable to the K_i values for the trifluoromethyl ketone and lower than the K_i of the α-keto ester analogs of phenylalanine [*N*-Ac-D,L-Phe-CF_3, K_i = 40 μM (Imperiali & Abeles, 1986), and *N*-Bz-D,L-Phe-CO_2Et, K_i = 280 μM (Hori et al., 1985)]. The K_i of 4-APPA for trypsin [K_i = 0.65 μM (Tanizawa et al., 1985); K_i = 1.6 μM (Markwardt et al., 1974)], on the other hand, is considerably lower than the K_i for the corresponding chymotrypsin inhibitor **16b** (K_i = 270 μM). In the last case, however, it is important to bear in mind that some interactions with the active site available to 4-APPA are not available to **16b**. In the former, the amidino moiety is symmetrically bound to the Asp^{189} carboxylate group of trypsin, and it can also form hydrogen bonds with $Gly^{219}O$ on one side and with H_2O 416 and/or Ser Oγ on the other side (as shown by X-ray studies; Walter & Bode, 1983: Marquart et al., 1983).

An important requirement for inhibition is an appropriate length of the aryl aliphatic chain of the inhibitor. An evaluation of the K_i values of the phenylpyruvic (i.e., **4a**, **5a**, **8**, **9b**, **15a**, and **16a**) and the benzylpyruvic (i.e., **4b**, **5b**, **6**, **7**, **15b**, and **16b**) derivatives shows that when the reactive carbonyl group is separated from the aromatic ring by a difluoromethylene group and a methylene group, rather than by a methylene group alone, the inhibitor is more potent. A similar observation has also been reported (Walsmann et al., 1976; Stürzebecher et al., 1976) for the inhibition of trypsin and plasmin by *p*-amidinophenyl ketones; i.e., inhibitors with two methylene units between the carbonyl function and the *p*-amidinophenyl ring are more effective than those with only one. This finding is not surprising, considering the closer resemblance (in terms of distance between the aromatic ring and the reactive carbonyl group) of these benzylpyruvic acid derivatives to phenylalanine than phenylpyruvic derivatives. It reflects the known specificity of chymotrypsin (Bauer, 1978; Nakajima et al., 1979) for substrates containing a Phe residue in the P_1 position.

The data in Table I show that keto esters are more potent inhibitors than the corresponding keto acids. Hori et al. (1985) have reported a comparable trend in the inhibition of human leukocyte and porcine pancreatic elastase by Bz-D,L-Ala-CO_2R (R = H, Et). Conversion of a carboxylic acid group to the ester involves the loss of ionic interactions with His^{57}. On the other hand, a carboalkoxyl group is more electron-withdrawing than an ionized carboxyl group. This effect must more than

compensate for the loss of the ionic interaction. The importance of electron-withdrawing groups α to the reactive carbonyl group is shown by the effect of fluorine on K_i (see below).

Introduction of one fluorine atom α to the carbon group reduces K_i for the benzylpyruvate esters 12-fold, but only 2-fold for the phenylpyruvate esters. Introduction of two fluorine atoms in the same molecule reduces K_i approximately 28-fold.[4] Our results are in agreement with the results obtained with fluoromethyl ketone inhibitors of chymotrypsin (20-fold; Imperiali & Abeles, 1986). Introduction of the second fluorine atom lowered K_i only 2-fold (**4b** → **15b**), whereas in the fluoromethyl ketone case an 8-fold decrease was observed. The reason for this lower than expected effect of fluorine substitution is not clear; possibly it represents a steric effect. The carbon atom bearing the two fluorines in **15b** is "inside" the S_1 binding pocket, while the two fluorine atoms of the difluoromethyl ketone inhibitor are on the α-carbon on the opposite side of the reactive carbonyl group. It should be noted that introduction of a second fluorine in the corresponding carboxylic acids actually raises K_i slightly (**5b** → **16b**; Table I). In the phenylpyruvic series, the introduction of two fluorine atoms actually caused a 2-fold increase in K_i (compare **9b** and **8** with **15a** and **16a**). The increase in electrophilicity of the keto group due to the introduction of one fluorine atom is also reflected in the extent of hydration in the inhibitor. The unfluorinated inhibitor **6** is unhydrated, whereas the monofluorinated inhibitor **4b** is essentially hydrated in aqueous solution. The change in hydration state as well as the decrease in K_i reflects the electron-withdrawing effect of fluorine.

The efficacy of protease inhibitors can be enhanced by increasing the number of favorable interactions between inhibitor and enzyme. It was previously shown that extension of difluoro ketone inhibitor sufficiently to allow for occupation of the S'_2 and S'_3 subsites decreased K_i 2500-fold (Imperiali & Abeles, 1987). Particularly important in lowering K_i was an arginine residue in P'_3. We have extended inhibitor **15b** in a similar manner by replacing its ethoxy group with a peptide moiety (i.e., alanyl-leucyl-arginine methyl ester and alanyl-leucyl-valine ethyl ester). In the peptide inhibitors **20** and **23**, the distances between the reactive keto group and the amide carbonyl groups of the peptide chain are approximately the same as those of the previously synthesized "extended binding inhibitors" (Imperiali & Abeles, 1987) (Chart I). Extension of **15b** to **20** causes a decrease in K_i of about 80-fold. Substitution in the extended peptide inhibitor of an arginine residue by a valine residue (**20** vs **23**) increases K_i 20-fold. The effect of extending **15b** with a tripeptide was smaller than that observed when a difluoro ketone inhibitor of chymotrypsin was extended with a very similar peptide. On the other hand, the effect of replacing a valine in the P'_3 position was the same for both inhibitors. This suggests that there may be some unfavorable interaction of **20**, but that both inhibitors interact identically with the S'_3 subsite.

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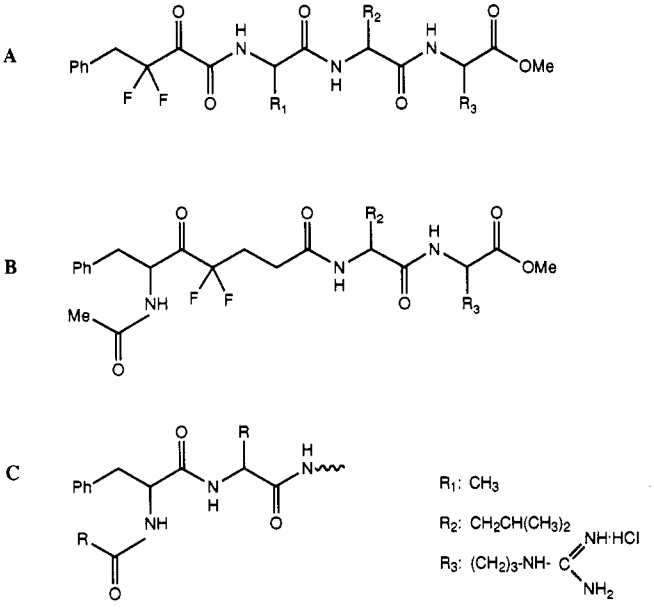
[4] It should be pointed out that the K_i determined for difluoro ketones is an apparent K_i based on the total concentration of difluoro ketone which is largely hydrated in solution. The actual inhibitor is the ketone, which is present at 1% to 0.2% of the total inhibitor concentration. The introduction of two fluorine atoms actually lowers K_i 2800–14 000-fold (Allen & Abeles, 1989).

Chart I: Comparison of the Structures of Inhibitors: Inhibitor **20** (A), Imperiali and Abeles (1987) "Extended Binding Inhibitor" (B), and a Generic N-Protected Phenylalanyl-Peptide (C)



SUPPLEMENTARY MATERIAL AVAILABLE

Listings of physical properties of all new compounds and a detailed description of their syntheses, with the exception of **16a,b** and precursors which will be reported elsewhere (10 pages). Ordering information is given on any current masthead page. Detailed synthetic procedures for compounds **16a,b** can be obtained from R.H.A.

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