Kaiser, E. T. (1981) *Biochemistry 20*, 602-610.
Kezdy, F. J., & Bender, M. L. (1965) *Annu. Rev. Biochem. 34*, 49-76.
Lincoln, T. M., & Corbin, J. D. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 3239-3243.
Rosevear, P. R., Fry, D. C., Mildvan, A. S., Doughty, M., O'Brian, C., & Kaiser, E. T. (1984) *Biochemistry 23*, 3161-3173.
Rosoff, P. M., & Cantley, L. C. (1985) *J. Biol. Chem. 260*, 9209-9215.
Stadtmauer, L. M., & Rosen, O. M. (1983) *J. Biol. Chem. 258*, 6682-6685.
Takio, K., Wade, R. D., Smith, S. B., Krebs, E. G., Walsh, K. A., & Titani, K. (1984) *Biochemistry 23*, 4207-4218.
Thomas, N. E., Bramson, H. N., Miller, W. T., & Kaiser, E. T. (1987) *Biochemistry* (first of three papers in this issue).
Walter, U., Miller, P., Wilson, F., Menkes, D., & Greengard, P. (1980) *J. Biol. Chem. 255*, 3757-3762.

Extended Binding Inhibitors of Chymotrypsin That Interact with Leaving Group Subsites S_1'-S_3' †

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ABSTRACT: We have synthesized inhibitors of chymotrypsin, based on fluoromethyl ketones, that bind at S and S′ subsites. "Small" inhibitors of serine proteases, which have previously been synthesized, only interact with S subsites. The parent compound is Ac-Leu-*ambo*-Phe-CF_2H (**1**) ($K_i = 25 \times 10^{-6}$ M). This inhibitor was modified by successively replacing H of the $-CF_2H$ group by $-CH_2CH_2CONHCH_3$ (**4**), $-CH_2CH_2CONH$-Leu-NHMe (**5**), $-CH_2CH_2CONH$-Leu-Val-OEt (**6**), and $-CH_2CH_2CONH$-Leu-Arg-OMe (**7**). Corresponding K_i values are 7.8 (**4**), 0.23 (**5**), 0.21 (**6**), and 0.014 (**7**) μM. Extending **5** to **6** by addition of Val-OEt at P_3' does not decrease K_i. In contrast, extension of **5** to **7** by incorporating Arg-OMe at P_3' decreases K_i approximately 15-fold, suggesting interaction between Arg and the S_3' subsite but no corresponding interaction at that subsite with Val. These results are in accordance with results obtained with the homologous family of avian ovomucoid third domain proteins. Proteins with Arg at the P_3' position show highly favorable interactions with the protease at the S_3' subsite [Park, S. J. (1985) Ph.D. Thesis, Purdue University; M. Laskowski, Jr., personal communication]. These results establish that incorporation of residues which interact with S′ subsites significantly increases the efficacy of inhibitors and that valuable information concerning the most effective amino acid composition of small inhibitors can be obtained from the amino acid sequence of protein inhibitors.

Effective inhibitors of the serine proteases chymotrypsin and porcine pancreatic elastase can be obtained by incorporating a difluoro- or trifluoromethyl ketone moiety into substrate analogues (Imperiali & Abeles, 1986a). The fluoromethyl ketones appear to be acting as transition-state analogue inhibitors (Wolfenden, 1976). The inhibitors described to date occupy maximally the S_1-S_4 binding subsites[1] of the proteolytic enzyme. There is reason to believe that K_i could be further reduced by utilizing interactions with binding subsites on the leaving group side of the cleaved peptide (the S_1'-S_3' subsites). Small synthetic inhibitors of serine proteases that interact in this extended manner have not to date been available due to the chemical limitations of the incorporated functional groups (e.g., aldehydes, boronic acid derivatives, etc.) (Westerik & Wolfenden, 1972; Kettner & Shenvi, 1984).

The work of Fersht et al. (1973) clearly establishes the importance of leaving group effects on peptide hydrolysis. It was observed that with the specific acyl-enzyme (acetylphenylalanyl)chymotrypsin the rate constants for attack on the acyl-enzyme vary greatly with different nucleophiles. For example, the ratio of reactivity of alaninamide:glycinamide:hydrazine:water (55 M) is 44:11.5:2:1. Although hydrazine is the best nucleophile of the series of compounds, alaninamide reacted over 20 times faster. It is interesting to note that when the acyl-enzyme is not specific (such as with the furoylchymotrypsin) the reactivity toward nucleophile then parallels the chemical reactivity of the incipient nucleophile. Thus specificity on the leaving group side does influence chymotrypsin-catalyzed peptide hydrolysis. Fersht also examined effects on k_{cat}/K_m and observed that the specificity for better substrates is expressed in the k_{cat} and not in K_m. The binding energy on the leaving group side is used to lower the activation energy of the rate-limiting chemical steps. It is, therefore, reasonable to assume that these interactions will also lower K_i of a transition-state analogue. Further evidence

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[1] 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. We shall refer to inhibitors that interact with S and S′ subsites as extended inhibitors.

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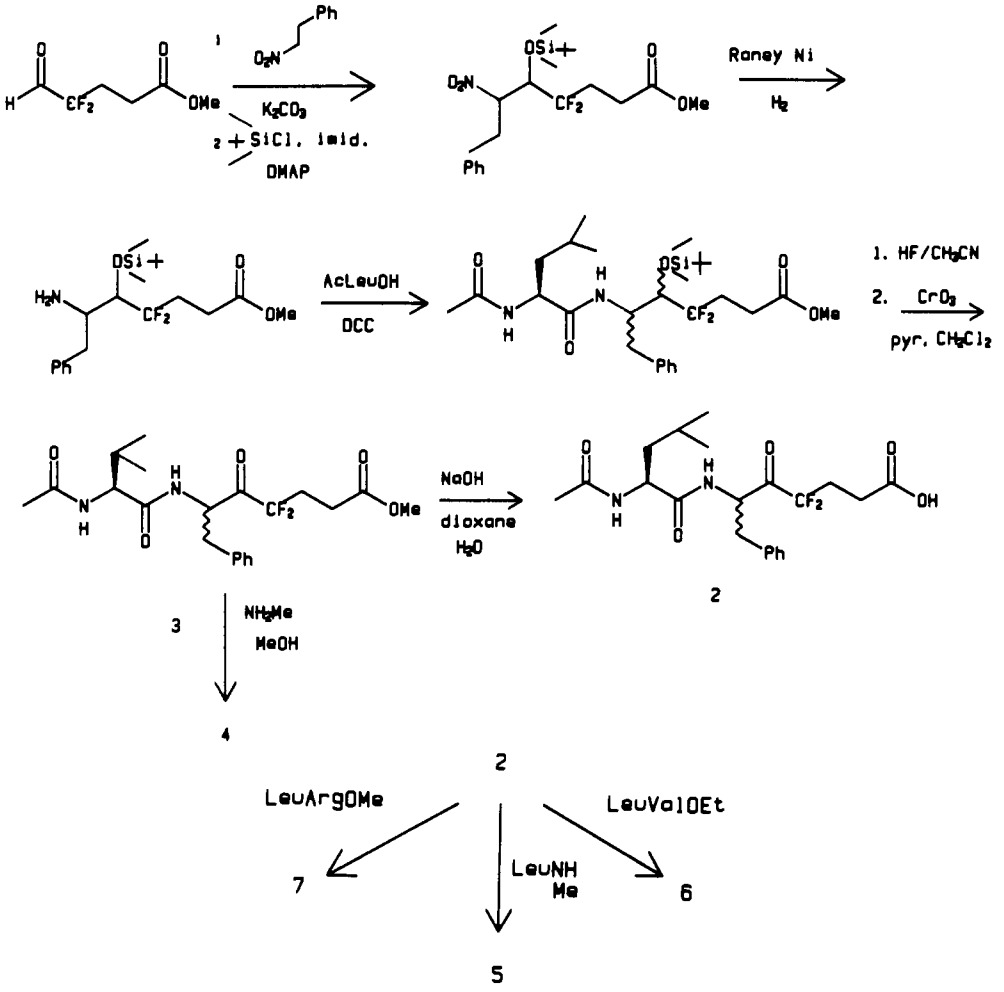
Scheme I



establishing the importance of S′ site interactions comes from the work of Laskowski and co-workers on the homologous family of avian ovomucoid third domain proteins, which are natural inhibitors of serine proteases (Laskowski & Kato, 1980; Park, 1985).

This paper describes the effect of successively extending the chymotrypsin inhibitor Ac-Leu-*ambo*-Phe-CF_2H by replacing the hydrogen in the difluoromethyl ketone with further residues, such that the inhibitors interact with subsites up to and including the S_3' site. Crystallographic studies on serine proteases bound to natural protein inhibitors indicate that the limit of the binding cleft in the direction of the carboxyl terminus is at the S_3' subsite (Bolognesi et al., 1982; Read et al., 1983).

Experimental Procedures

Enzyme Assay. α-Chymotrypsin from bovine pancreas (type II, 3× crystallized) was purchased from Sigma Chemical Co. It was assayed in 100 mM potassium phosphate buffer, pH 7.8, with *N*-benzoyltyrosine ethyl ester (Sigma) by monitoring the increase in absorbance at 256 nm (Hummel, 1959).

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

Evaluation of Kinetic Parameters. When slow-binding kinetics were not observed, the K_i values were determined from the initial inhibited steady-state velocities in the presence of substrate. In the presence of slow-binding kinetics, the K_i values were determined according to the procedure of Cha (1975). The application of this procedure has already been described in detail (Imperiali & Abeles, 1986b). Since the rate of association between these inhibitors and the enzyme is fairly fast, the K_i can also be determined from the final steady-state velocities measured with inhibitor in the presence of substrate. Both values are given in Table I for comparison.

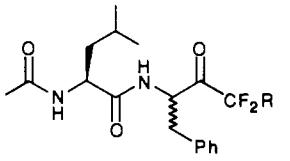
Results and Discussion

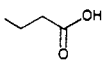
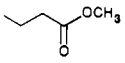
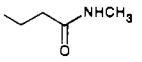
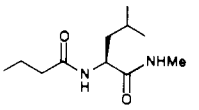
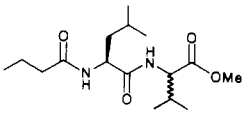
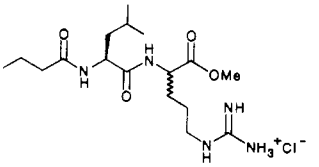
Synthesis. A detailed description of the synthesis, purification, and physical properties of all new compounds is presented in the supplementary material (see paragraph at end of paper regarding supplementary material). The fluoromethyl ketone inhibitors were considered to be pure on the basis of thin-layer chromatography (TLC) analysis (single spot in solvent system described) or by single peak on high-performance liquid chromatography (HPLC) and unambiguous assignment of all ^1H NMR signals.

The synthesis of the extended binding inhibitors involves the same overall synthetic strategy that we developed for the "one-sided" compounds (Imperiali & Abeles, 1986b). The general synthetic route is outlined in Scheme I and deserves some comments.

In the cases of the "one-sided" inhibitors the amino acid analogue that contained the fluoromethyl ketone moiety was constructed by utilizing the condensation of a nitroalkane with a fluorinated aldehyde based on acetaldehyde. To afford the extended binding inhibitors, the α,α-difluoroaldehyde used is more complex and allows for functionality through which to extend to the P′ residues.

Table I: Extended Binding Inhibitors of Chymotrypsin



compd	R	K_i (μM)
1	H	25[a]
2		64[b]
3		1.7[b]
4		7.8[b]
5		0.23
6		0.21[b] 0.30[b-d]
7		0.014[b] 0.009[c,e]

[a] Imperiali and Abeles, 1986a. [b] Determined from steady-state velocities. [c] Slow binding, determined from k_{on} and k_{off} according to the Cha method. [d] k_{on} 6600 s^{-1} M^{-1}; k_{off} 2 × 10^{-3} s^{-1}. [e] k_{on} 470 000 s^{-1} M^{-1}; k_{off} 6.7 × 10^{-3} s^{-1}.

The extended binding inhibitors for α-chymotrypsin described in this paper have been synthesized from a glutarate derivative (five carbons). Thus, the resulting inhibitors are actually one methylene unit longer than the analogous substrate would be. The inhibitors of exact length should actually be synthesized from a succinate derivative (four carbons); however, this modification was adopted initially to avoid anticipated problems such as HF elimination from an α,α-difluorosuccinate derivative. Full experimental details of the synthesis are given in the supplementary material.

Inhibitors—Structure and K_i. The results of the extended binding inhibitor studies are shown in Table I. The K_i for the parent compound Ac-Leu-*ambo*-Phe-CF_2H (**1**), which was determined earlier (Imperiali & Abeles, 1986a), is given for comparison. The K_i value for **1** is 25 × 10^{-6} M. From the wide range of inhibition constants obtained with difluoromethyl ketones **1**–**7**, it is clear that interactions on the leaving group side have a profound effect on inhibitor binding. Effects at the P_1' subsite are rather hard to interpret since it is not yet synthetically feasible to examine variously substituted α-amino acids. What is apparent, however, is that the more hydrophobic R group is preferred. The ester **3** has a K_i of 1.7 × 10^{-6} M while the corresponding acid **2** has a K_i of 64 × 10^{-6} M. The carboxylate negative charge is not well tolerated in the S_1' subsite, and the addition of this residue makes the compound worse than the unsubstituted difluoromethyl ketone **1**. The *N*-methylamide **4** is of intermediate hydrophobicity and has an intermediate K_i of 7.8 × 10^{-6} M. Because of the synthetic limitations it was not possible to manipulate the P_1' residue by addition of further hydrophobic residues, for better interactions. This residue acts basically as a linking unit between the P_1 and P_2' residues, which can be manipulated. The inhibitor **5** can occupy subsites from P_2 to P_2' inclusive. The choice of leucine as the P_2' residue is based on the general observation that in the natural protein inhibitors of chymotrypsin a hydrophobic residue is preferred in this position. It is established that tyrosine and phenylalanine are actually the ideal residues at this subsite (Park, 1985). However, we chose to incorporate the more conservative, nonaromatic hydrophobic residue leucine in order to avoid the possibility of nonproductive binding and amidolysis of the inhibitor which would occur if the P_2' were bound in the primary specificity pocket (S_1) which has such a strong tendency to bind hydrophobic aromatic residues. The addition of the P_2' residue, to afford compound **5**, results in a compound with a K_i of 0.23 × 10^{-6} M, 2 orders of magnitude lower than the unsubstituted compound **1**. The limit of the substrate binding cleft, in the serine proteases, on the leaving group side is at the S_3' subsite (Bolognesi et al., 1982; Read et al., 1983). Therefore, the final residue added to the chymotrypsin inhibitors is in the P_3' position. The choice of residue is based on extensive work by Laskowski and coworkers (M. Laskowski, Jr., personal communication) on the avian ovomucoid third domain proteins. This homologous family of proteins, while highly conserved in the structural regions, exhibit hypervariability around the active site region (residues 14–21, which correspond to P_5–P_3') which binds to the proteases and causes inhibition. Comparison of binding constants between variants that differ in only one binding contact residue can give an idea of what might be a suitable residue to use in a given position for an optimally binding inhibitor. Thus, the $K_{a,obsd}$ for the turkey third domain ovomucoid protein is 1.8 × 10^{11} M^{-1} while that for the Montezuma quail is 4.9 × 10^9 M^{-1}. This 37-fold decrease in affinity arises from the change of Arg-21 for Val-21. Residue 21 corresponds to the P_3' residue interacting at the S_3' subsite. There is one other residue difference between the ovomucoid proteins of these two species, Leu-23 vs. Ile-23; however, this is a very conservative change, and the residues at this position are not thought to be involved in binding. The basis for the higher affinity has been rationalized by structural studies, which demonstrate that the arginine makes several favorable interactions with the protease [notably the guanidinium group with an aspartate residue (64), which is absent in the Val-21 variant (Park, 1985)]. We synthesized two inhibitors that extended to the P_3' residue, one with valine at this position and one with arginine. The K_i for **6** (P_3' valine) is 0.25 × 10^{-6} M, and that for **7** (P_3' arginine) is 0.011 × 10^{-6} M, a difference of 23-fold. The comparison of **6** and **7** with the Montezuma quail and turkey ovomucoid proteins shows very similar differences in binding with the arginine to valine variation. This is indicative of the fact that similar favorable interactions must be available to the small synthetic inhibitors.

The difluoromethyl ketones **6** and **7** both demonstrate slow-binding kinetics [Williams & Morrison, 1979; also see footnote 4 in Imperiali and Abeles (1986a)]. Previously, with the one-sided inhibitors, we noted that slow-binding kinetics would be observed with some peptidyl trifluoromethyl and difluoromethyl ketones only if sufficient binding interactions were available. For instance, a difluoromethyl ketone inhibitor of porcine pancreatic elastase was only slow binding with P_1–P_4 (Ac-Ala-Ala-Pro-*ambo*-Ala-CF_2H) residues and not with just P_1–P_2 (Ac-Pro-*ambo*-Ala-CF_2H). Here, again, similar observations are made, only the more extended inhibitors show the slow-binding property. The k_{on} and k_{off} values for **6** and **7** can be evaluated by the Cha (1975) procedure as described under Experimental Procedures. The large difference between

the two inhibitors is manifested completely in the association rate constants; for **6** k_{on} is 6600 s^{-1} M^{-1}, and for **7** k_{on} is 470000 s^{-1} M^{-1}. Interestingly, Laskowski (personal communication) has made a similar observation in the case of the natural protein protease inhibitors: the change of arginine for the valine manifests most of its effect in the association rate constant. The k_{off} values are very similar and can essentially be considered identical, since there is a large amount of error in this parameter when determined by the Cha method.

In the design of these small synthetic inhibitors of α-chymotrypsin we have attempted (successfully) to mimic the important interactions that have been observed in both good substrates and effective natural protease inhibitors. With this general strategy in mind, and with the viable difluoromethyl ketone functional group at the center of the inhibitor, it should be possible, by observing the sequence of both natural inhibitors and good substrates, to design effective small (M_r <1000) synthetic inhibitors to any of the physiologically important serine proteases.

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SUPPLEMENTARY MATERIAL AVAILABLE

Detailed description of the synthesis, purification, and physical properties of all new compounds used in this study (14 pages). Ordering information is given on any current masthead page.

REFERENCES

Cha, S. (1975) *Biochem. Pharmacol. 24*, 2177.
Bolognesi, M., Gatti, G., Menegatti, E., Guarneri, M., Marquart, M., Papamokos, E., & Huber, R. (1982) *J. Mol. Biol. 162*, 839.
Fersht, A. R., Blow, D. M., & Fastrez, J. (1973) *Biochemistry 12*, 2035.
Hummel, B. C. W. (1959) *Can. J. Biochem. Physiol. 37*, 1393.
Imperiali, B., & Abeles, R. H. (1986a) *Biochemistry 25*, 3760.
Imperiali, B., & Abeles, R. H. (1986b) *Tetrahedron Lett. 27*, 135.
Kettner & Shenvi (1984) *J. Biol. Chem. 259*, 5106.
Laskowski, M., Jr., & Kato, I. (1980) *Annu. Rev. Biochem. 49*, 593.
Park, S. J. (1985) Ph.D. Thesis, Purdue University.
Read, R. J., Fujinaga, M., Sielecki, A. R., & James, M. N. G. (1983) *Biochemistry 22*, 4420.
Westerik & Wolfenden (1972) *J. Biol. Chem. 247*, 8195.
Williams, J. W., & Morrison, J. F. (1979) *Methods Enzymol. 63*, 437.
Wolfenden, R. (1976) *Annu. Rev. Biophys. Bioeng. 5*, 271.

Adduct Formation between the Cupric Site of Phenylalanine Hydroxylase from *Chromobacterium violaceum* and 6,7-Dimethyltetrahydropterin†

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ABSTRACT: The interaction of pterin-dependent phenylalanine hydroxylase from *Chromobacterium violaceum* with the cofactor analogue 5-deaza-6-methyltetrahydropterin and the cofactor 6,7-dimethyltetrahydropterin ($DMPH_4$) has been investigated by multifrequency electron spin resonance (ESR) spectroscopy. 5-Deaza-6-methyltetrahydropterin, which lacks the N-5 nitrogen present in the pyrazine ring of $DMPH_4$, binds tightly to the cupric form of the enzyme; however, no changes are observed in the ESR parameters of the copper center. In contrast, the binding of $DMPH_4$ (or 6-methyltetrahydropterin) shifts the ESR parameters ($g_{\|}$ and $A_{\|}$) associated with the cupric enzyme. In addition, superhyperfine transitions were resolved and assigned to hyperfine splitting from nitrogen ligands. ESR spectra of the enzyme recorded in the presence of [5-^{14}N]$DMPH_4$ or [5-^{15}N]$DMPH_4$ were computer simulated and found to be consistent with pterin serving as a direct donor ligand to the copper center through the N-5 position.

L-Phenylalanine hydroxylase (phenylalanine 4-monooxygenase, EC 1.14.16.1) catalyzes the formation of tyrosine from phenylalanine in the presence of a reduced pterin cofactor and molecular oxygen. In addition, phenylalanine hydroxylase (PAH)[1] from either bacterial or mammalian liver requires a transition metal for activity. While the mammalian liver enzyme contains 1 mol equiv of tightly bound iron (Fisher et al., 1972; Gottschall et al., 1982), the enzyme from *Chromobacterium violaceum* contains 1 mol equiv of copper

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[1] Abbreviations: PAH, phenylalanine hydroxylase; pterin, generic name for 2-aminopteridin-4-one; $DMPH_4$, 6,7-dimethyltetrahydropterin; EPR, electron paramagnetic resonance; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

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