Irreversible Enzyme Inhibitors. CXIII.[1,2] Proteolytic Enzymes. III. Active-Site-Directed Irreversible Inhibitors of α-Chymotrypsin Derived from Phenoxyacetamides with an N-Fluorosulfonylphenyl Substituent[3]

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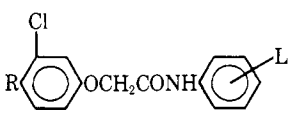
N-(3-Chlorophenoxyacetyl)sulfanilyl fluoride (**11**) and its *ortho* (**7**) and *meta* (**12**) analogs were reversible inhibitors of α-chymotrypsin that also showed rapid irreversible inhibition of the enzyme by the active-site-directed mechanism. In addition to inactivating the enzyme, the *meta* isomer (**12**) was catalytically hydrolyzed to the corresponding sulfonic acid (**15**) by the enzyme, as shown kinetically and by product isolation. In contrast, the sulfonyl fluoride (**12**) was stable to water and bovine serum albumin under the same conditions that the two interactions with α-chymotrypsin occurred, namely, hydrolysis of **12** and inactivation of the enzyme. Similar kinetic evidence was obtained for both the hydrolysis of, and inactivation of α-chymotrypsin by, the *ortho* (**7**) and *para* (**11**) analogs.

In blood serum are a series of proteolytic enzymes involved in antibody-augmented foreign cell rejection, histamine release, blood clotting, and blood clot solution; these areas of research are important for the organ transport and cardiovascular problems.[5] Selective reversible inhibition of one of these myriad of proteases is not apt to be achieved.[5] Active-site-directed irreversible inhibitors[6] that can covalently link to the enzyme outside the active site (*exo* type) have an extra dimension of specificity[7] that could lead to selective inhibition of only one of these many proteases.

Chymotrypsin[3] and trypsin[5] were selected for initial study since no *exo*-type irreversible inhibitors of these two enzymes were known. The first two phases on the design of these *exo*-type irreversible inhibitors for chymotrypsin, namely, (a) the binding points for reversible inhibition, and (b) positioning of bulky groups on the inhibitor that are tolerated within the enzyme–inhibitor complex, have been reported.[3] The third phase, the positioning of a proper leaving group in the bulk tolerance area of the inhibitor to give active-site-directed irreversible inhibitors is the subject of this paper.

The phase II study[3] suggested that a leaving group, L, be positioned on the N-aryl moiety of **1** or **2**. Since the extra chloro group of **2** gave about twofold better binding, derivatives were synthesized where L = *m*-chloroacetyl (**3**), *p*-chloroacetyl (**4**), *p*-sulfonyl fluoride (**5**), *m*-sulfonyl fluoride (**6**), and *o*-sulfonyl fluoride (**7**). The chloroacetyl leaving group attached to the proper carrier that complexes with the active site has been used previously for the inactivation of dihydrofolic reductase,[8] chymotrypsin,[9,10] and trypsin.[10,11] All of these were endo-type irreversible inhibitors, and in the case of trypsin and chymotrypsin, a histidine within the active site was covalently linked. The sulfonyl fluoride leaving group of α-tolylsulfonyl fluoride has been used for active-site-directed irreversible inhibition of chymotrypsin[12] by the endo mechanism; this leaving group has also been used for potent exo-type irreversible inhibitors of dihydrofolic reductase.[13]



1, R = H
2, R = Cl

Enzyme Results.—The initial screening with **3–7** on inhibition of chymotrypsin is collated in Table I; this initial screen involved a two-point inactivation study at 1 and 60 min at 37° in order to separate the irreversible inhibitors from those which were not. Unfortunately, the first four compounds were too insoluble to be measured, but the fifth (**7**) showed strong irreversible inhibition of chymotrypsin; more detailed studies on **7** are discussed below.

Since 3-chlorophenoxy derivatives (**1**) are more water soluble than the 3,4-dichlorophenoxy derivatives,[3] the same five candidate irreversible inhibitors were synthesized in the 3-chlorophenoxy series (**8**, **9**, **11–13**) and, in addition, one bromoacetamido derivative (**10**); as anticipated these were 5–10 times more water soluble. The two chloroacetyl derivatives (**8**, **9**) and the bromoacetamido derivative (**10**) failed to show inactivation of chymotrypsin in the screen, but the three sulfonyl fluorides (**11–13**) did show inactivation of chymotrypsin. Of these four compounds (**7**, **11–13**) that could inactivate chymotrypsin, two (**7**, **12**) were then studied in more detail.

A Dixon reciprocal inhibition plot[14] with the *m*-sulfonyl fluoride (**12**) (Figure **1**) showed "competitive"

(1) This work was supported in part by Grant CA-08695 from the National Cancer Institute, U. S. Public Health Service.

(2) For the previous paper of this series see B. R. Baker and G. J. Lourens, *J. Med. Chem.*, **11**, 39 (1968).

(3) For the previous paper on inhibitors of chymotrypsin see B. R. Baker and J. A. Hurlbut, *ibid.*, **10**, 1129 (1967), paper CVII of this series.

(4) N.D.E.A. predoctoral fellow.

(5) For a discussion and key references see B. R. Baker and E. H. Erickson, *ibid.*, **10**, 1123 (1967), paper CVI of this series.

(6) B. R. Baker, "Design of Active-Site-Directed Irreversible Enzyme Inhibitors. The Organic Chemistry of the Enzymic Active-Site," John Wiley and Sons, Inc., New York, N. Y., 1967.

(7) See ref 6, Chapter IX.

(8) B. R. Baker, P. C. Huang, and A. L. Pogolotti, Jr., *J. Med. Chem.*, **10**, 1134 (1967), paper CVIII of this series.

(9) G. Schoellmann and E. Shaw, *Biochemistry*, **2**, 252 (1963).

(10) See ref 6, Chapter VIII, for more detail.

(11) E. Shaw, M. Mares-Guia, and W. Cohen, *Biochemistry*, **4**, 2219 (1965); P. H. Petra, W. Cohen, and E. N. Shaw, *Biochem. Biophys. Res. Commun.*, **21**, 612 (1965).

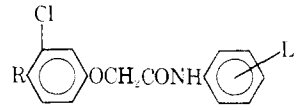
(12) D. E. Fahrney and A. M. Gold, *J. Am. Chem. Soc.*, **85**, 997 (1963).

(13) B. R. Baker and G. J. Lourens, *J. Med. Chem.*, **10**, 1113 (1967), paper CV of this series.

(14) M. Dixon and E. C. Webb, "Enzymes," Academic Press Inc., New York, N. Y., 1958, pp 23–27.

Table I

Inhibition of α-Chymotrypsin by



			Reversible[a]			Irreversible		
No.	R	L	Concn, μM	% inhib.	Estd I_{50},[b] μM	Concn, μM	Time, min	% inactvn[c]
3	Cl	m-$COCH_2Cl$	20[d]	15	190	20	60	0
4	Cl	p-$COCH_2Cl$	8[d]	0	>40[e]	8	60	0
5	Cl	p-SO_2F	5[d]	0	>25	5	60	0
6	Cl	m-SO_2F	10[d]	0	>50	12	60	~10
7	Cl	o-SO_2F	20	50	20[f]	80	60	95
						10	60	0
8	H	m-$COCH_2Cl$	100[d]	34	180	100	60	0
9	H	p-$COCH_2Cl$	100[d]	17	500	100	60	0
10	H	m-$NHCOCH_2Br$	100	50	100	150	60	0
11	H	p-SO_2F	100[d]	23	350	150	60	60
12	H	m-SO_2F	50	50	50[g]	90	60	100
13	H	o-SO_2F	42	50	42	86	1	100
15	H	m-SO_3^-	830	50	830			

[a] Assayed with 200 μM N-glu(aryl-L-phenylalanine p-nitroanilide (GPNA)[31] in 0.05 M Tris buffer (pH 7.4) containing 10% DMSO as previously described.[3] [b] I_{50} = concentration of inhibitor required for 50% inhibition. [c] Inactivation performed in 0.05 M Tris buffer (pH 7.4) containing 10% DMSO at 37° (see Experimental Section). [d] Near the maximum solubility. [e] Since 20% inhibition is readily detectable, the concentration required for 50% inhibition is greater than five times the concentration measured. [f] A Lineweaver–Burk plot[14] gave "competitive" kinetics with $K_i = 2 \times 10^{-5}$ M. [g] A Dixon plot[15] gave "competitive" kinetics with $K_i = 3.6 \times 10^{-5}$ M.

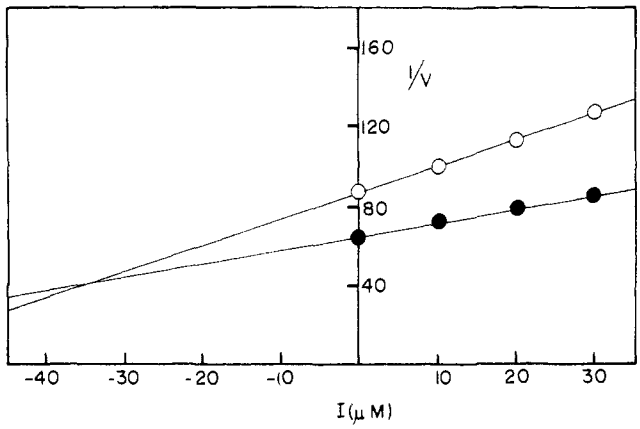


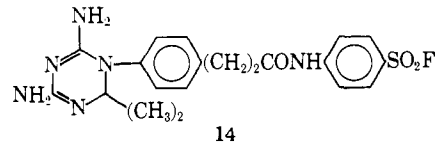
Figure 1.—Dixon plot of inhibition of α-chymotrypsin by **12**: ○, 200 μM GPNA; ●, 300 μM GPNA. Assays performed at pH 7.4 with 0.05 M Tris buffer diluted with 10% DMSO.

kinetics; **12** had $K_i = 36$ μM. The rate of inactivation by an active-site-directed irreversible enzyme inhibitor is dependent upon the concentration of reversible enzyme–inhibitor complex (EI);[10,15] the amount of complex is expressed[10,15] as a function of total enzyme concentration (E_t) by eq **1** where I is the inhibitor concentration.

$$[EI] = \frac{[E_t]}{1 + K_i/[I]} \quad (1)$$

At a concentration of $[I] = K_i$, $[EI] = 0.50[E_t]$, and at a concentration of $[I] = 2K_i$, $[EI] = 0.67[E_t]$; thus, the relative rates of inactivation under these two sets of conditions should be 1.3, as previously observed with active-site-directed irreversible inhibitors of glutamic dehydrogenase,[15,16] lactic dehydrogenase,[15,16] dihydrofolic reductase,[17] and adenosine deaminase.[18,19] Furthermore, these inactivations showed psuedo-first-order kinetics throughout greater than 80% inactivation, that is, a plot of log E *vs.* time gave a straight line.

Time curves for inactivation of chymotrypsin by **12** at concentrations equal to K_i, $2K_i$, and $0.25K_i$ are presented in Figure 2. Note that the rates are linear only for a short part of the reaction; therefore, comparison of the rates of inactivation of different concentrations is subject to large error. Furthermore, the inactivation with a $2K_i$ concentration of inhibitor practically stopped at 70% reaction; at the lower concentration of inhibitor (K_i), the reaction practically stopped at 50% inhibition. Such results have not been previously observed with the bromoacetamido type of irreversible inhibitor,[15-19] but have been observed with inactivation of dihydrofolic reductase by the sulfonyl fluoride type (**14**) of irreversible inhibitor.[13] This instability of **14** was traced to destruction of the inhibitor by the enzyme preparation, since the compound was stable to the incubation conditions in the absence of enzyme; since the dihydrofolic reductase was an impure preparation, it could not be stated with any certainty whether this decomposition of the inhibitor was enzyme catalyzed or was due to some unknown impurities in the enzyme preparation. The observation of the same phenomenon with α-chymotrypsin, a pure enzyme, now opened up for re-examination this question of enzymic-catalyzed decomposition of the inhibitor.



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From Figure 2 it can be seen that little, if any, irreversible inhibitor remains after 1 hr at 37°. If the decomposition of the irreversible inhibitor is caused

(15) B. R. Baker, W. W. Lee, and E. Tong, *J. Theor. Biol.*, **3**, 159 (1962).
(16) See ref 6, Chapter IX, for more detail.
(17) B. R. Baker and J. H. Jordaan, *J. Pharm. Sci.*, **55**, 1417 (1966), paper I,XVII of this series.
(18) H. J. Schaeffer, M. A. Schwartz, and E. Odin, *J. Med. Chem.*, **10**, 686 (1967).
(19) See ref 6, Chapter XII for more detail.

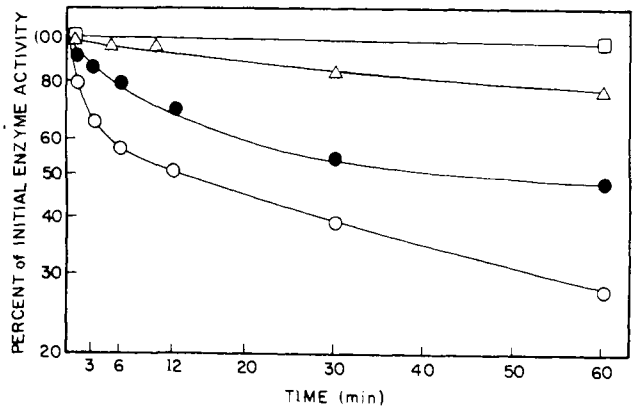


Figure 2.—Rate of irreversible inhibition of α-chymotrypsin by **12** at 37° in 0.05 *M* Tris buffer (pH 7.4) diluted with 10% DMSO: □, enzyme control; ○, 72 μM **12**; ●, 36 μM **12**; △, 9 μM **12**. The initial enzyme activity was 0.01 OD unit/min.

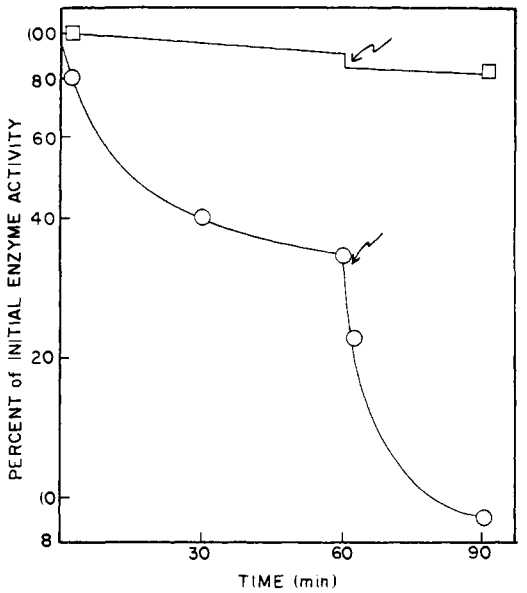
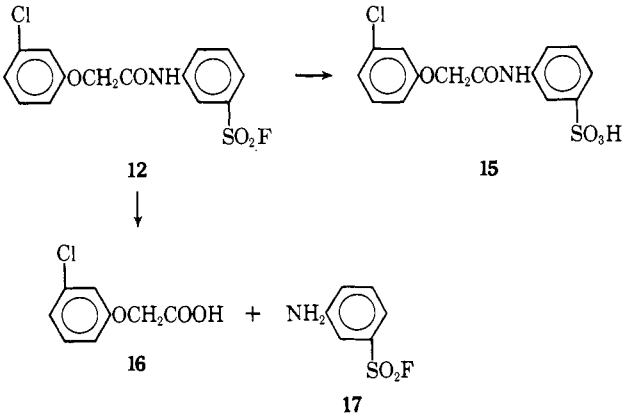


Figure 3.—Irreversible inhibition of α-chymotrypsin by 72 μM **12** at 37° in 0.05 *M* Tris buffer (pH 7.4) containing 5% DMSO. At the broken arrow, an additional 72 μM **12** (in DMSO) was added to the inhibitor solution and a corresponding amount of DMSO to the enzyme control: □, enzyme control; ○, inhibitor. The initial enzyme activity was 0.017 OD unit/min.

by the buffer and/or solvent, then preincubation of the inhibitor in buffer in the absence of enzyme should destroy the inhibitor. That this preincubation with buffer had no effect on **12** at a concentration of $2K_i$ was then shown by addition of enzyme; the enzyme was still inactivated to a level of 30% of the original concentration.

In order to verify further that the enzyme was catalyzing the decomposition of **12**, the experiment in Figure 3 was performed. When the enzyme was treated with **12** at a $2K_i$ concentration, 60% inactivation occurred in 30 min, and 67% in 60 min. At this point an additional $2K_i$ aliquot of inhibitor was added; in another 60 min, the amount of remaining enzyme was decreased another 80% showing that the first aliquot of inhibitor had been destroyed by the enzyme in the first 60 min.

Two mechanisms can be envisioned for the enzyme-catalyzed destruction of the irreversible inhibitor (**12**). Since chymotrypsin can hydrolyze amide linkages, it



was possible that **12** was being hydrolyzed to the acid **16** and arylamine **17**; that such was not the case was shown by a negative Bratton–Marshall test[20] for **17** which in a separate experiment was shown to be sensitive down to a concentration of 1 μM, considerably below the 70 μM concentration of **17** that would have been generated.

The second possibility was that the enzyme was catalyzing the hydrolysis of the sulfonyl fluoride (**12**) to the corresponding sulfonic acid (**15**). That this indeed was the case was shown by a preparative-scale enzymatic reaction. Alternate additions of α-chymotrypsin (700 mg) and **12** (160 mg) to 1 l. of 10% H_2O–DMSO with addition of NaOH to maintain the pH at 7.2–8.0 gave a recovery of only 13% of the sulfonyl fluoride; the sulfonic acid (**15**) could be isolated as its guanidine salt from the solution. Two control reactions were also performed: (a) omission of the enzyme resulted in 81% recovery of the sulfonyl fluoride (**12**), much less base was consumed, and only traces of sulfonic acid (**15**) could be detected by tlc; (b) replacement of the α-chymotrypsin by bovine serum albumin gave the same results as the omission of α-chymotrypsin, that is, 74% of the sulfonyl fluoride was recovered and much less base was consumed.

The failure of bovine serum albumin to hydrolyze the sulfonyl fluoride (**12**) affords strong evidence that a reversible complex between the inhibitor and chymotrypsin is an obligatory intermediate to hydrolysis.[13] Two possible types of complexes can be envisioned: (a) the complex for hydrolysis of **12** is the same complex that leads to enzyme inactivation (eq 2), or (b) the two complexes are not the same (eq 3). If two different complexes, $(E\cdots I)_i$ and $(E\cdots I)_s$, are required

$$\begin{array}{l} E + I \underset{K_i}{\rightleftarrows} (E\cdots I)_i \xrightarrow{k_1} E\cdots I \\ \qquad\qquad \downarrow k_2 \\ \qquad\qquad (E\cdots P) \underset{K_p}{\rightleftarrows} E + P \end{array} \qquad (2)$$

$$E + I \underset{K_s}{\rightleftarrows} (E\cdots I)_s \xrightarrow{k_2} (E\cdots P) \underset{K_p}{\rightleftarrows} E + P \qquad (3)$$

for inactivation of the enzyme or hydrolysis of the inhibitor, respectively, then the kinetic situation is quite complicated as shown.

$$[E_t] = [E] + [EI]_i + [EI]_s + [EP] \qquad (4)$$

(20) B. R. Baker, D. V. Santi, J. K. Coward, H. S. Shapiro, and J. H. Jordaan, *J. Heterocycl. Chem.*, **3**, 425 (1966).

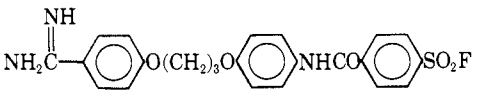
The total active enzyme concentration will be the summation in (4). By use of K_i, K_s, and K_p, eq 4 can be transformed to (5), a form which relates to the rate of enzyme inactivation, since $V_i = k_1[EI]_i$.[10]

$$[EI]_i = \frac{[E_t]}{1 + \frac{K_i}{[I]}\left(1 + \frac{[I]}{K_s} + \frac{[P]}{K_p}\right)} \quad (5)$$

Theoretically, it should be possible to determine K_i, K_s, K_p, k_1, and k_2, but it would require extensive manipulation of kinetic experiments.

The second explanation, that is, the complex $(EI)_i$ can lead either to hydrolysis or to enzyme inactivation as in eq 2, is currently preferred for the following reasons.

The dual reactions of hydrolysis of inhibitor and inactivation of enzyme are seen with **14** and dihydrofolic reductase,[13] **18** and trypsin,[21] **19** and xanthine oxidase,[22] **20** and xanthine oxidase,[23] as well as selected



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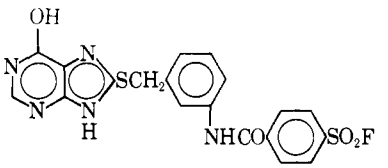

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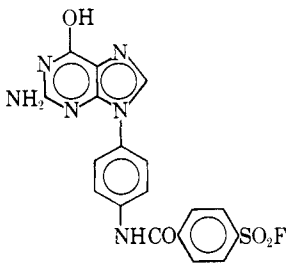

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analogs of **12**, **14**, **18–20**. To assume that each of these many compounds must have both an $(EI)_i$ and $(EI)_s$ complex for the three different enzymes becomes highly improbable. Therefore, a better working hypothesis is eq 2, where only one complex is needed for two different reactions. Working models for this one complex will be discussed later after additional enzyme data are described.

The kinetics for eq 2 are considerably simpler than eq 3 since only protection against inactivation by product **15** must be considered; eq 6 has been previously derived for protection by a reversible inhibitor against enzyme inactivation by an active-site-directed irreversible inhibitor.[10,15]

$$[EI] = \frac{[E_t]}{1 + \frac{K_i}{[I]}\left(1 + \frac{[P]}{K_p}\right)} \quad (6)$$

The hydrolysis product **15**, which was synthesized chemically, was then found to give 50% reversible

(21) B. R. Baker and E. H. Erickson, *J. Med. Chem.*, **11**, 245 (1968); paper CXV of this series.

(22) B. R. Baker and J. A. Kozma, manuscript in preparation.

(23) B. R. Baker and W. F. Wood, manuscript in preparation.

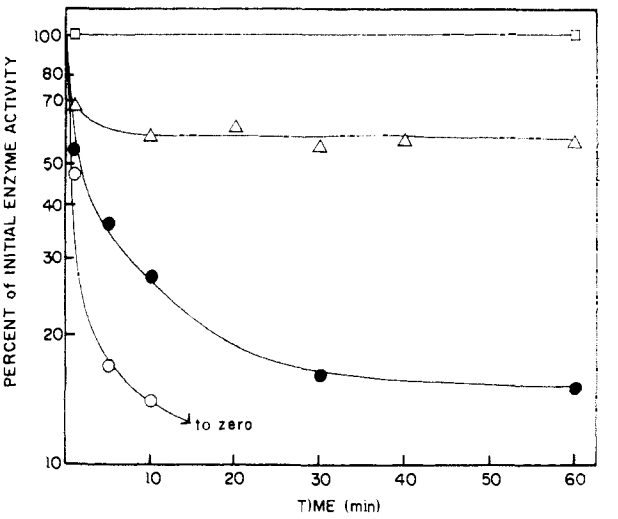


Figure 4.—Rate of irreversible inhibition of α-chymotrypsin at 37° by 72 μ*M* **12** in 10% dimethyl sulfoxide: ○, 0.05 *M* Tris buffer, pH 8.4; ●, 0.05 *M* Tris buffer, pH 7.4; □, 0.05 *M* Tris–maleate buffer, pH 6.5; △, 0.05 *M* citrate buffer, pH 5.0. Since each enzyme control was slightly different, each curve is corrected for the slight enzyme die-off in its control. The initial rates were 0.01–0.017 OD unit/min.

inhibition of chymotrypsin at 830 μ*M*, compared to 50 μ*M* required for the sulfonyl fluoride (**12**); thus it can be estimated that $K_p \cong 17K_i$. In an experiment where the concentration of **12** was 72 μ*M* = $2K_i$, the maximum concentration of **15** that could be generated was 72 μ*M*; thus the term $[P]/K_p$ in eq 6 becomes negligible compared to **1**, and eq 6 reduces to eq **1** in the case of **12** and **15**. Thus the curvature in Figure 2 is most probably due only to a decreasing concentration of irreversible inhibitor with time. It is possible in other cases, such as **14**, that the generated sulfonic acid would be as good a reversible inhibitor as the sulfonyl fluoride, or better; such an inhibitor would give even more line curvature since the concentration of I is decreasing and the concentration of P is increasing with time.

The effect of pH on the rate of inactivation of chymotrypsin and on the shape of the inactivation curve by 72 μ*M* **12** is shown in Figure 4. Even though some of the lines show curvature prior to 50% inactivation, it is clear that decreasing pH decreases the rate of inactivation; at pH 8.4, 7.4, 6.5, and 5.0, the times for 50% inactivation were about 10, 20, 60, and >90 min, respectively. It is also apparent that the rate of enzyme-catalyzed hydrolysis of **12** slows with decreasing pH; little **12** remained at pH 8.4, 7.4, 6.5, and 5.0 after 15, 60, 90, and 120 min, respectively. These results suggest that both the enzyme-catalyzed hydrolysis of the inhibitor and enzyme inactivation by the inhibitor are dependent upon the hydroxide ion concentration, either directly or by an indirect effect on ionization of a participating enzymic group. The significance of such a suggestion is discussed below.

The *o*-sulfonyl fluoride **7** was a "competitive" reversible inhibitor of chymotrypsin; a Lineweaver–Burk reciprocal plot[14] gave K_i = 20 μ*M*. The rate of inactivation of chymotrypsin with three different concentrations of **7** is shown in Figure 5. From eq 1 it can be calculated that 80, 71, and 55% of the enzyme is in the rate-determining (EI) form with 80, 50, and 25 μ*M* of **7**, respectively; thus, if only active-site-directed irre-

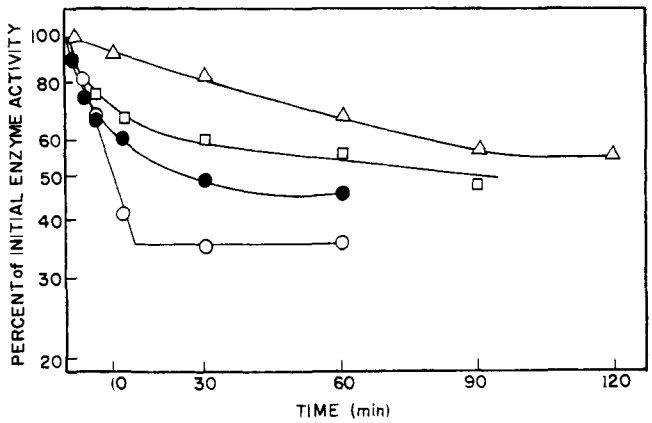


Figure 5.—Rate of irreversible inhibition of α-chymotrypsin by varying concentrations of **7** at 37° in 0.05 *M* Tris buffer (pH 7.4), containing 10% DMSO; □, enzyme control; ○, 80 μ*M* **7**; ●, 50 μ*M* **7**; △, 25 μ*M* **7**. The initial enzyme activity was 0.01 OD unit/min.

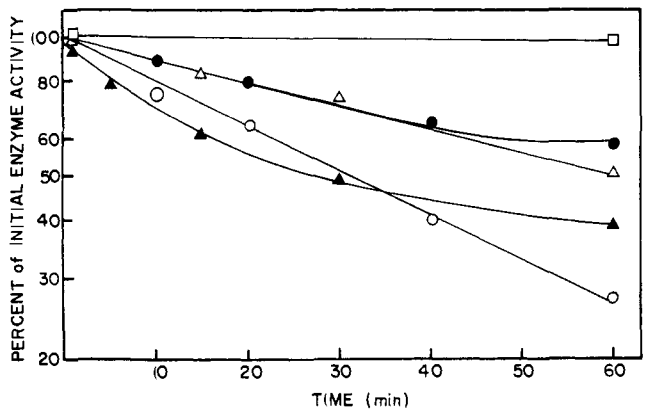
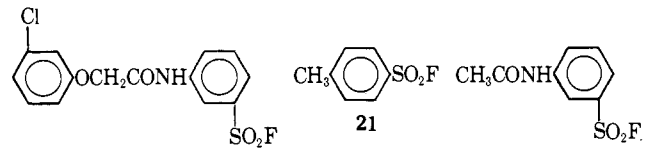


Figure 6.—Rate of inactivation of α-chymotrypsin at 37° in 0.05 *M* Tris buffer (pH 7.4) containing 10% DMSO: □, enzyme control; ○, 100 μ*M* *p*-tolylsulfonyl fluoride; ●, 50 μ*M* *p*-tolylsulfonyl fluoride; △, 72 μ*M* *m*-acetamidobenzenesulfonyl fluoride; ▲, 150 μ*M* *p*-sulfonyl fluoride **11**. The initial enzyme activity was 0.009 OD unit/min. All curves have a 1-min point, but are overlapping.

versible inhibition were occurring, the relative rates for the three concentrations should be 8:7:5.5. It is clear that the initial rates are extremely rapid, but that decomposition of the inhibitor **7** is also taking place, since the lines are not linear and the curvature increases with decreasing inhibitor concentration as seen with the *meta* isomer (**12**) in Figure 2.

In contrast to **12**, the *ortho* isomer (**7**) was unstable to water and even more unstable to Tris buffer at pH 7.4. When a 50 μ*M* concentration of **7** was preincubated in 0.05 *M* Tris buffer (pH 7.4), then the chymotrypsin added, no inactivation of the enzyme occurred; note that with no preincubation, these conditions gave 75% inactivation of the enzyme (Figure 5). When a 100 μ*M* concentration of **7** was preincubated in 10% aqueous DMSO for 1 hr, then an equal volume of buffer containing enzyme was added, only 23% inactivation of the enzyme occurred in 60 min. The significance of the instability of the *ortho* isomer (**7**) is discussed below.

Fahrney and Gold[12] have reported that α-tolylsulfonyl fluoride can inactivate chymotrypsin *via* a reversible complex faster than diisopropyl phosphofluoridate (DFP). They also observed that the enzyme was inactivated by benzenesulfonyl fluoride at about 1% of the rate shown by α-tolylsulfonyl fluoride. The rates of inactivation of chymotrypsin by 50 and 100 μ*M* *p*-tolylsulfonyl fluoride (**21**) under our experimental conditions are shown in Figure 6. Two points are noteworthy: (a) the time for 50% inactivation was 75 and 32 min for 50 and 100 μ*M* *p*-tolylsulfonyl fluoride, respectively, and (b) both concentrations gave essentially linear plots, indicating that the inhibitor was not being decomposed by the enzyme. The time for 50% inactivation of chymotrypsin by 72 μ*M* *m*-sulfonylphenoxyacetanilide (**12**) was 12 min and a rapid change from linearity occurred due to enzyme-catalyzed hydrolysis of **12**; it can be estimated from Figure 6 that a 72 μ*M* concentration of *p*-tolylsulfonyl fluoride would require about 50 min for inactivation of half of the enzyme.



12 **21** **22**

m-Acetamidobenzenesulfonyl fluoride (**22**), which is more closely related to **12** in that the phenoxy group has been removed, was measured as an inhibitor of chymotrypsin. As a reversible inhibitor, a 1.3 m*M* concentration of **22** was needed for 50% inhibition; compared to **12** with I_{50} = 0.05 m*M*, the loss in binding in removing the chlorophenoxy group of **12** to give **22** is 26-fold. Yet at a concentration of 72 μ*M*, **22** inactivated chymotrypsin with a half-life of 60 min (Figure 6), which is close to the inactivation rate shown by *p*-tolylsulfonyl fluoride (**21**) at this concentration, but is only one-fourth the rate shown by **12** (Figure 2).

If **12** and **22** are complexed identically, and in only one possible mode of binding, with chymotrypsin when they inactivate the enzyme, it can be calculated from eq **1** that **12** should inactivate ten times faster than **22** since, at 72 μ*M*, **12** complexes 67% of the enzyme and **22** complexes only 7%. Thus it is apparent that **12** and **22** do not complex to the enzyme in the same way when they inactivate chymotrypsin. It follows that *p*-tolysulfonyl fluoride (**21**) also complexes differently from **12** since **21** and **22** have nearly identical linear inactivation rates.

Since it is highly probable that the benzenesulfonyl moiety of **21** and **12** are complexed differently when giving their most productive mode (best inhibition) of binding, one should consider the possibility that **12** inactivates the enzyme by the less productive mode of reversible binding equivalent to **21**. If two modes of binding for **12** are possible and only one can inactivate the enzyme, then **12** is self-protecting by one of its modes of binding and eq 6 then holds. The less productive mode of binding is likely to be about as effective as **22**, that is, a 26-fold difference, where $K_i = 26K_p$, [P] = [I] = 72 μ*M*, and K_i = 36 μ*M*. With this assumption it can be calculated that the amount of (EI) complex productive for inactivation is only 2.5% of the total enzyme; therefore, this mechanism is not operative, since **22** would inactivate the enzyme three times (7/2.5) faster than **12** in contrast to the observed

reverse order of rates of **12** being four times faster than **22**.

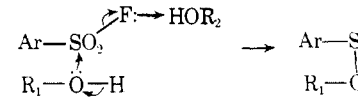
The final compound studied was the phenoxyacetanilide bearing a *p*-sulfonyl fluoride group (**11**); it showed a nonlinear inactivation of chymotrypsin with a half-life of about 35 min at a concentration of 150 μM (Figure 6). Whether **11** inactivates by a mechanism common with either **12** or with **22** cannot be ascertained kinetically due to the poorer I_{50} of **11** than **12**; the nonlinearity tends to favor a mechanism common with **12**.

With an active site defined as those parts of an enzyme necessary to complex the substrate and those parts necessary for the catalytic action,[6] it is probable that the *o*-sulfonyl fluoride group is attacking within the active site (endo mechanism). The *m*-sulfonyl fluoride (**12**) most probably does not have the sulfonyl fluoride near the complexing region, but it still might be sufficiently near the catalytic site to be operating by the endo mechanism. However, the N-phenyl group could be outside of the active site; if such is the case, greater specificity can probably be built into molecules such as **7** or **12** by further substitution on the N-phenyl group, since bulk tolerance adjacent to the active sites may well vary among the proteases.

Studies currently underway have the sulfonyl fluoride group considerably removed from the amide moiety of the inhibitor so that irreversible inhibition, if any, is more apt to occur by the exo mechanism.

Proposed Mechanisms of Hydrolysis and Inactivation.—A thorough kinetic study of the hydrolysis of acetyl fluoride has been performed by Bunton and Fendler.[24] They observed that sodium formate and pyridine catalyzed the hydrolysis by generation of the more reactive acetic–formic anhydride and acetylpyridinium ion, respectively, but that sodium acetate slowed hydrolysis by generation of the less reactive acetic anhydride; these observations show that several nucleophiles attack acetyl fluoride faster than water. Furthermore, the hydrolysis was catalyzed by H^+ below pH 3 or OH^- above pH 7. Although no such studies have yet been performed on a sulfonyl fluoride, some rough projections on the reactivity of sulfonyl fluorides can be made by a combination of the enzyme-inactivation experiments described in the previous section with the known chemistry of acetyl fluoride, arylsulfonyl fluorides,[25] and arylsulfonyl chlorides.

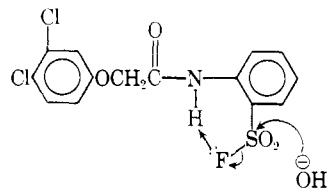
Arylsulfonyl fluorides are stable to pyridine, in contrast to arylsulfonyl chlorides and acetyl fluoride which are hydrolyzed *via* their acylpyridinium ions. Furthermore, arylsulfonyl fluorides are relatively stable to boiling water, boiling ethanol, or boiling 2-methoxyethanol;[13,25] in contrast, sulfonyl chlorides and acetyl fluorides are attacked under these conditions. Even though sulfonyl fluorides are quite stable to water and alcohols, these sulfonyl fluorides will attack hydroxyl groups when absorbed on cellulose, particularly under slightly alkaline conditions;[25] this apparent discrepancy can be resolved if one considers that the probable mechanism of reaction of a sulfonyl fluoride with a nucleophile is envisioned by eq 7. This reaction, similar



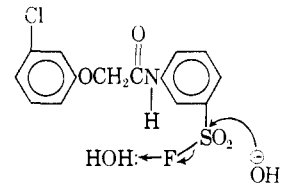
(7)

to the mechanism of hydrolysis of acetyl fluoride, requires assistance by the electron-rich fluorine atom forming a hydrogen bond with the protic solvent (water or alcohol); nucleophilic attack by solvent then occurs. In order for reaction with solvent to occur by this concerted reaction 7, the solvent most probably has to assume a structure around the sulfonyl fluoride that disrupts the solvent's hydrogen bond structure; such a disruption requires energy which slows the reaction of solvent with the sulfonyl fluoride. In contrast, if R_1 is cellulose on which the arylsulfonyl fluoride has been absorbed, the solvent structure involving R_2OH does not have to be disrupted for reaction to occur.

That hydrogen bonding of the fluorine atom is a necessary prerequisite for attack of the sulfonyl fluoride is supported by the observations on the relative stability of **7** *vs.* **12**. The *ortho* isomer (**7**) is internally hydrogen



7a



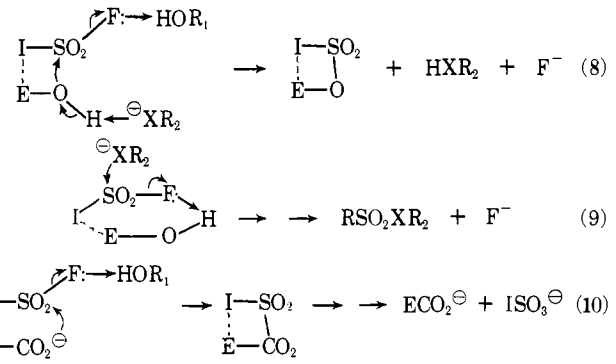
12a

bonded as shown in **7a**; nucleophilic attack by solvent then does not require disruption of solvent and **7a** undergoes hydrolysis probably by attack of the small amount of hydroxide ion present. In contrast, the *meta* isomer (**12**) requires hydrogen bonding with solvent before attack can occur, as shown in **12a**, which is equivalent to eq 7; therefore **12a** is much more stable to water than **7a**.

The pH profile studies of **12** in Figure 4 showed that increasing the pH from 5 to 6.5 to 7.4 to 8.4 gave increased increments in rate of both attack by solvent (as catalyzed by the enzyme) and covalent bond formation with the enzyme; these results indicated that both reactions were dependent upon the hydroxide ion concentration. Of the 15 types of nucleophilic groups on an enzyme surface, eight have the nucleophilic capacity to react with a sulfonyl fluoride; these are (a) the hydroxyl group of serine, threonine, and tyrosine, (b) the carboxylate group of aspartate and glutamate, (c) the imidazole-NH of histidine, and (d) the NH_2 group of any environmentally weakened lysine. All but group b would form a covalent linkage stable at relatively neutral pH; in contrast, the carboxylate group could form a mixed anhydride by nucleophilic attack of the sulfonyl fluoride, which would then be rapidly hydrolyzed by water. The number of histidines in an enzyme are few and are usually less than four per 25,000 molecular weight of protein; similarly, the number of environmentally weakened lysines on the surface are probably few. Thus the main classes of functional groups to be considered are the carboxylates and the hydroxyl groups which, except for perhaps tyrosine, are prevalent on the surface of an enzyme.

In the most generalized sense, the irreversible inhibition of an enzyme by a sulfonyl fluoride can be

(24) T. A. Bunton and J. H. Fendler, *J. Org. Chem.*, **31**, 2307 (1966).
(25) For a review of pertinent references, see ref 13.



depicted by eq 8, which takes into account the above discussed chemistry. If $R_1 = R_2 = H$ and X = O, then only water and OH^- are necessary for covalent bond formation to take place within the reversible E···I complex. The transition in (8) could require little or no disruption of solvent compared to (7) for several reasons: (a) the E–O–H group of (8) has more flexibility for rotation than does the hydrogen-bonded solvent, R_1OH, of (7); (b) the protein already has disrupted water structure molecules on its surface, so-called "ice structure."

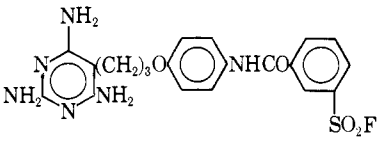
It is also possible that either R_2X^- or HOR_1 or both in eq 8 are part of the protein. For example, R_2X^- could be the carboxylate of aspartate or glutamate; this result could also account for the decreased rate of inactivation by decreasing pH since the carboxylate would be gradually converted to the less nucleophilic COOH group with increasing acidity.

Two mechanisms for enzyme-catalyzed hydrolysis of the inhibitor can be envisioned. The enzymic hydroxyl group of eq 8 may be able to be positioned in the complex as shown in (9), where internal hydrogen bonding of the fluorine has occurred as shown earlier in **7a**. Nucleophilic attack by $R_2X^- = HO^-$ would cause hydrolysis. Again the R_2X^- group could be a properly positioned carboxylate group of the enzyme, which would lead to an easily hydrolyzable mixed anhydride.

The second possibility for hydrolysis is shown in eq 10; here a properly positioned carboxylate of the enzyme surface could serve as the nucleophile and water as the hydrogen bond acceptor from fluorine. Such a nucleophilic attack would lead to a mixed anhydride which would be readily hydrolyzed to a sulfonic acid analog of the inhibitor, then be desorbed from the enzyme surface as indicated in eq 2. The R'OH may be water or an enzymic hydroxyl group; the latter would be the same as eq 9.

As discussed earlier, the combination of enzyme inactivation by, and enzyme-catalyzed hydrolysis of, sulfonyl fluorides has been seen with chymotrypsin, trypsin, dihydrofolic reductase, and xanthine oxidase, suggesting that a common mechanism for all cases is highly probable; the proper positioning of two or more enzymic groups to the sulfonyl fluoride on four different enzymes becomes a highly improbable structural coincidence. Therefore, the most logical mechanism is that only one and the same enzymic group is involved in covalent linkage or hydrolysis of the sulfonyl fluoride or both as depicted in (8) and (9) where R_2X^- is OH^- and HOR_1 is water.

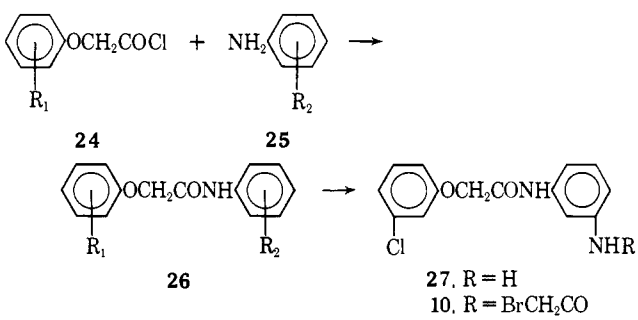
The above-depicted mechanism, even though subject to future reinterpretation as new experimental facts emerge, has already led to a number of practical applications. For example, **23** at 1 μM can rapidly inactivate the dihydrofolic reductase from L1210/FR8 mouse



23

leukemia or Walker 256 rat tumor in a few minutes, with only minimal hydrolysis of the sulfonyl fluoride. In contrast, the rat liver enzyme shows a greater rate of hydrolysis of **23** than inactivation by **23**, and some inactivation is seen only when the concentration of inhibitor is raised to 25 μM. The mouse liver enzyme behaves still differently in that no inactivation is seen even at high concentration.[26] These diverse results, which will be described in a future paper,[26] can be explained by eq 8–10.

Chemistry.—All of the candidate irreversible inhibitors (**26**) in Table I except **10** were prepared by reaction of the appropriate acid chloride (**24**) and arylamine (**25**)



in boiling toluene. In the case of **10**, hydrogenation of the *m*-nitro group to **27** followed by bromoacetylation[20] completed the synthesis. The sulfonic acid (**15**), isolated at the guanidine salt, was prepared by basic hydrolysis of **12**. Acetylation of metanilyl fluoride yielded **22**.

Experimental Section[27]

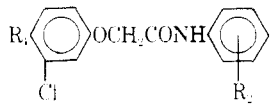
N-(*p*-Chloroacetylphenyl)-3,4-dichlorophenoxyacetamide (4) (Method A).—A mixture of 0.60 g (2.7 mmoles) of 3,4-dichlorophenoxyacetic acid[28] and 4 ml of $SOCl_2$ was refluxed with stirring until gas evolution was complete (about 30 min). The excess $SOCl_2$ was removed by evaporation *in vacuo*. To the residual acid chloride were added 60 ml of toluene and 0.425 g (2.5 mmoles) of 4'-amino-2-chloroacetophenone (Eastman). The mixture was gently refluxed under an air condenser until HCl evolution ceased (about 5 hr). The resulting solution was cooled, then washed successively with 5% HCl, 5% $NaHCO_3$, and H_2O. Dried with $MgSO_4$, the solution was spin evaporated *in vacuo*. Two recrystallizations from EtOH gave 0.50 g (54%) of yellow needles, mp 165–168°, which gave a negative Bratton–Marshall test for aromatic amine and a positive 4-(*p*-nitrobenzyl)pyridine test for activated halogen.[20] Further data on this and other compounds prepared by this method are listed in Table II. For the sulfonyl fluorides, which of course do not respond to the 4-(nitrobenzyl)pyridine test, the presence of the sulfonyl fluoride

(26) B. R. Baker and R. B. Meyer, Jr., manuscript in preparation.

(27) All new compounds had ir spectra and combustion analyses in agreement with their assigned structures; each moved as a single spot on tlc on Brinkmann silica gel GF. Melting points were taken on a Mel-Temp block and are corrected. Where analyses are indicated only by symbols of the elements analytical results obtained for those elements were within ±0.4% of the theoretical values.

(28) For preparation of 3-chlorophenoxy acetic acid needed in preparation A, see B. R. Baker, W. W. Lee, W. A. Skinner, A. P. Martinez, and E. Tong, *J. Med. Pharm. Chem.*, **2**, 633 (1960).

TABLE II
PHYSICAL CONSTANTS OF



No.	R_1	R_2	Method	% yield	Mp, °C	Formula	Analyses
3	Cl	*m*-$COCH_2Cl$	A[a]	75[b]	150–152	$C_{16}H_{12}Cl_3NO_3$	C, H, N
4	Cl	*p*-$COCH_2Cl$	A	54[c]	165–168	$C_{16}H_{12}Cl_3NO_3$	C, H, N
5	Cl	*p*-SO_2F	A	42[c,d]	173–175	$C_{14}H_{10}Cl_2FNO_4S$	C, H, N
6	Cl	*m*-SO_2F	A	25[d,e]	163–164	$C_{14}H_{10}Cl_2FNO_4S$	C, H, N
7	Cl	*o*-SO_2F	A	33[e]	144–146	$C_{14}H_{10}Cl_2FNO_4S$	C, H, N
8	H	*m*-$COCH_2Cl$	A[f]	31[c,f]	117–119	$C_{16}H_{13}Cl_2NO_3$	C, H, N
9	H	*p*-$COCH_2Cl$	A	39[d,e]	141–143	$C_{16}H_{13}Cl_2NO_3$	C, H, N
10	H	*m*-$NHCOCH_2Br$	C[g]	61[a,d,f]	166–169	$C_{16}H_{14}BrClN_2O_3$	C, H, N
11	H	*p*-SO_2F	A	26[h]	144–147	$C_{14}H_{11}ClFNO_4S$	C, H, N
12	H	*m*-SO_2F	A	68[d,i]	119–121	$C_{14}H_{11}ClFNO_4S$	C, H, N
13	H	*o*-SO_2F	A	44[d,h]	129–131	$C_{14}H_{11}ClFNO_4S$	C, H, N
15	H	*m*-$SO_3H \cdot NHCNH_2$ (NH)	D	29[e]	184–186	$C_{15}H_{17}ClN_4O_5S$	C, H, N
27	H	*m*-NH_2	B	83[j,f]	117–119	$C_{14}H_{13}ClN_2O_2$	C, H, N
28	H	*m*-NO_2	E[k]	39[l]	141–143	$C_{14}H_{11}ClN_2O_4$	C, H, N

[a] The starting amine was prepared according to the method of B. R. Baker and B.-T. Ho, *J. Pharm. Sci.*, **56**, 28 (1967). [b] Recrystallized from EtOH. [c] Recrystallized from C_6H_6. [d] Recrystallized from $CH_3C_6H_5$. [e] Recrystallized from EtOH–petroleum ether (bp 60–110°). [f] With the aid of decolorizing carbon. [g] Recrystalized from Me_2CO–H_2O. [h] Recrystallized from $CH_3C_6H_5$–$CHCl_3$–EtOH. [i] Recrystallized from EtOH–H_2O. [j] Recrystallized from MeOEtOH–H_2O, then EtOAc–petroleum ether. [k] By method E in ref 3; no cooling was employed. [l] Recrystallized from $CHCl_3$.

was verified by characteristic ir bands at 1400, 1210, and 750–800 cm^{-1}.[29]

N-(*m*-Aminophenyl)-3-chlorophenoxyacetamide (27) (Method B).—A solution of 1.84 g (6 mmoles) of **28** in 100 ml of MeOEtOH was shaken with H_2 at 2–3 atm in the presence of 50 mg of PtO_2, reduction being completed in about 20 min. The catalyst was removed by filtration through a Celite pad. The filtrate was diluted with 400 ml of H_2O, and the product was collected on a filter. Recrystallization from EtOAc–petroleum ether (bp 60–110°) with the aid of decolorizing carbon gave 1.38 g (83%) of light yellow crystals, mp 117–119°. See Table II for additional data.

N-(*m*-Bromoacetamidophenyl)-3-chlorophenoxyacetamide (10) (Method C).—To a solution of 277 mg (1 mmole) of **27** in 4 ml of Me_2CO was added 390 mg (1.5 mmoles) of bromoacetic anhydride. After 4 hr, during which time the product crystallized from solution, the mixture was filtered and washed with a small quantity of ice-cold Me_2CO, then 5% $NaHCO_3$, and finally H_2O. Three recrystallizations from toluene with the aid of decolorizing carbon, gave 244 mg (61%) of white needles, mp 166–169°, which gave a negative Bratton–Marshall test and a positive 4-(*p*-nitrobenzyl)pyridine test.[20] See Table II for additional data.

Guanidinium N-(3-Chlorophenoxyacetyl)metanilate (15) (Method D).—To a stirred suspension of 1.89 g (5.5 mmoles) of **12** in 6 ml of H_2O at 50° was added a solution of 0.65 g (16 mmoles) of NaOH in 2 ml of H_2O. The mixture was stirred at 60–70° for an additional 20 min, then the hot solution was filtered. After being chilled at 0°, the mixture was filtered. The insoluble sodium salt of **15** was dissolved in 60 ml of warm H_2O. The solution was acidified with HCl to pH 1–2, then a solution of 1.92 g (20 mmoles) of guanidine·HCl in 40 ml of H_2O was added. The filtered solution was kept at 5° for several hours, then the guanidinium salt was collected on a filter. The product was recrystallized from 50 ml of H_2O containing 0.1 g of guanidine·HCl. A second recrystallization from EtOH–petroleum ether (bp 60–110°) gave 0.632 g (29%) of white crystals, mp 184–186°. See Table II for additional data.

N-Acetylmetanilyl Fluoride (22).—To a solution of 2.63 g (15 mmoles) of metanilyl fluoride in 5 ml of Me_2CO was added 2.56 g (25 mmoles) of Ac_2O. After 20 min at ambient temperature, the solution was warmed on a steambath for 5 min, then diluted with 30 ml of H_2O. The product was collected on a filter and washed with 5% HCl, then H_2O. Recrystallization from C_6H_6 with the aid of decolorizing carbon, then two more recrystallizations from C_6H_6, gave 1.55 g (48%) of pure product as white needles, mp 114–116°. *Anal.* ($C_8H_8FNO_3S$) C, H, N.

Enzyme Assays.[30]—The reversible inhibition assays were performed with three-times recrystallized, salt-free α-chymotrypsin from bovine pancreas and 0.2 m*M* N-glutaryl-L-phenylalanine-*p*-nitroanilide (GPNA)[31] as previously described.[2] The irreversible inhibiton assays were performed as follows. The velocity of the enzyme reaction with 0.2 m*M* GPNA was observed to be proportional to the enzyme concentration. The amount of spontaneous enzyme inactivation in 10% DMSO at pH 7.4 and 37° was less than 5%/hr. The buffer employed in the assays was 0.05 *M* Tris (pH 7.4); additional buffers employed in the incubation were 0.05 *M* Tris–maleate (pH 6.5), 0.05 *M* Tris (pH 8.4), and 0.05 *M* citrate (pH 5.0). Bulk enzyme was dissolved in cold 1 m*M* HCl at 6.1 mg/ml and stored at 0–5°. GPNA was stored as a 12.4 m*M* solution in DMSO in a brown bottle.

In two tubes were placed 0.50 ml of enzyme solution and 1.30 ml of 0.05 *M* buffer of the desired pH; after 5 min in a 37° bath, 200 μl of DMSO was added to tube 1 (enzyme control) and 200 μl containing inhibitor was added to tube 2. The contents were mixed and a 1.00-ml aliquot was withdrawn for each tube 1 min after the addition of the DMSO, then stored at 0° until ready for assay. The aliquot from the control tube was labeled C_1 and the aliquot from the inhibitor tube was labeled I_1. The remainder in the two tubes labeled C_2 and I_2 was kept for 1 hr at 37°, then cooled in an ice bath until ready for assay. The amount of remaining enzyme in each aliquot was assayed as follows. In a 3-ml glass cuvette were placed 2.85 ml of 0.05 *M* Tris buffer (pH 7.4) (regardless of the incubation pH) and 50 μl of 12.4 m*M* GPNA in DMSO. The enzyme reaction was then started by addition of 200 μl of C_1 (or other aliquot). The increase in optical density at 410 mμ was followed with a Gilford 2000 recording spectrophotometer; the C_1 aliquot usually gave an optical density change of about 0.01 OD unit/min. The velocities in OD/min, which are proportional to the remaining active enzyme concentration, were plotted on a log scale against time on a linear scale.[15] Each aliquot was run in

(29) N. S. Ham, A. N. Hambly, and R. H. Laby, *Australian J. Chem.*, **13**, 443 (1960).

(30) The technical assistance of Maureen Baker with these assays is acknowledged.

(31) B. E. Erlanger, F. Edel, and A. G. Cooper, *Arch. Biochem. Biophys.*, **115**, 206 (1966).

duplicate, or triplicate if necessary. This procedure is adequate for a routine screen for a plus or minus answer on irreversible inhibition. As many as three inhibitor tubes can be run with one enzyme control in less than 1 working day.

With a positive compound, a larger amount of inhibitor–enzyme mixture can be set up, then a number of aliquots removed at varying times in order to obtain the half-life and extent of irreversible inhibition. For irreversible inhibitors having a fast initial inactivation, a zero-time tube can be set up as previously described for dihydrofolic reductase.[13]

α-Chymotrypsin-Catalyzed Hydrolysis of N-(3-Chlorophenoxyacetyl)metanilyl Fluoride (12). **A.**—To 1 l. of 10% H_2O–DMSO were added with stirring 700 mg (0.028 mmole) of α-chymotrypsin and 160 mg (0.465 mmole) of **12** (in 10 ml of DMSO); the additions were made in six equal aliquots over a period of 9 hr, then stirring was continued an additional 1 hr. The pH was maintained at 7.2–8.0 by addition of 0.01 *N* NaOH, and the temperature was maintained at 30–35°; a total of 78 ml (0.78 mmole) of base was consumed.

The mixture was filtered and the recovered **12** was washed with H_2O; 10 mg. The filtrate was extracted with four 200-ml portions of $CHCl_3$. The combined $CHCl_3$ extracts were washed with 400 ml of H_2O, then evaporated *in vacuo*. The residue was extracted with 25 ml of EtOH. Quantitative uv analyses showed the presence of 11 mg of **12** for a total recovery of 21 mg (13%).

The original aqueous solution was concentrated to about 200 ml by freeze evaporation, then was spin evaporated to residue *in vacuo*. The residue was suspended in 20 ml of H_2O, then adjusted to pH 1–2 with HCl. The separated protein was removed by centrifugation. To the supernatant was added 250 mg (2.5 mmoles) of guanidine hydrochloride. Since the guanidine salt of **12** failed to crystallize from solution, the solution was spin evaporated *in vacuo*. The residue was dissolved in 5 ml of H_2O and applied to four plates (20 × 20 cm) coated with a total of 50 g of silica gel GF. The plates were developed with 2:1 EtOH–petroleum ether (bp 60–110°). The zones of the guanidine salt of **15** were scraped off, combined, and extracted with 20% aqueous Me_2CO. The solution was clarified with carbon, then evaporated *in vacuo*. Crystallization of the residue from 2 ml of H_2O gave 30 mg (16%) of the guanidinium salt of **15**; this material was identical with an authentic sample as shown by ir, uv, and tlc. The filtrate contained additional product as shown by tlc.

B.—A run identical with A, except the α-chymotrypsin was omitted, gave an 81% recovery of **12**, and only traces of **15** could be detected by tlc; only 5.6 ml (0.056 mmole) of 0.01 *N* NaOH was consumed.

C.—A run identical with A, except that lyophilized bovine serum albumin was used in place of α-chymotrypsin, gave a 74% recovery of **12** without $CHCl_3$ extraction; only 5.0 ml (0.05 mmole) of 0.01 *N* NaOH was consumed.

Irreversible Enzyme Inhibitors. CXIV.[1,2] Proteolytic Enzymes. IV.[2] Additional Active-Site-Directed Irreversible Inhibitors of α-Chymotrypsin Derived from Phenoxyacetamides Bearing a Terminal Sulfonyl Fluoride

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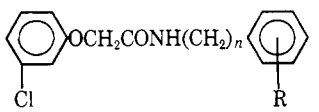
Fifteen candidate irreversible inhibitors of α-chymotrypsin derived from N-phenyl- or N-benzyl-3-chloro- or 3,4-dichlorophenoxyacetamide were synthesized that contained a fluorosulfonylbenzamido or fluorosulfonylphenylureido group on the N-aryl ring. Of these, ten showed irreversible inhibition of α-chymotrypsin; due to lack of solubility compared to their reversible binding constants (K_I), none of these compounds could completely inactivate α-chymotrypsin at their maximum solubility. The kinetics of inactivation indicated that these compounds were being enzymatically hydrolyzed to the corresponding sulfonic acids as well as causing inactivation of α-chymotrypsin by the active-site-directed mechanism.

The selective inhibition of only one of the myriad of blood serum proteases[4] is not apt to be achieved with reversible inhibitors, but can theoretically be achieved with active-site-directed irreversible inhibitors[5] of the exo type that covalently link to the enzyme outside the active site.[6] Inhibitors that use a sulfonyl fluoride group for convalent linkage to enzymes are particularly well suited for exo-type irreversible inhibition since this functional group can rapidly attack the hydroxyl group of a serine or threonine within the reversible enzyme–inhibitor complex;[7] such hydroxyl groups are prevalent on the surface of proteins. In the previous paper of this series[2] it was shown that compounds of type **1** bearing a



1, $n = 0$
2, $n = 1$

sulfonyl fluoride (R = SO_2F) group could rapidly inactivate α-chymotrypsin by the active-site-directed mechanism. It was also established that chymotrypsin could catalyze the hydrolysis of these sulfonyl fluorides to the corresponding sulfonic acids. This paper reports additional studies where the sulfonyl fluoride group of the inhibitor is further removed from the carboxamide group of **1** since the latter is complexed within the active site; this further removal of the sulfonyl fluoride group in compounds of type **1** and **2** is more

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(2) For the previous paper of this series see B. R. Baker and J. A. Hurlbut, *J. Med. Chem.*, **11**, 233 (1968).

(3) N.D.E.A. predoctoral fellow.

(4) For a discussion of the utility of such inhibitors in the cardiovascular and organ transplant areas see B. R. Baker and E. H. Erickson, *J. Med. Chem.*, **10**, 1123 (1967), paper CVI of this series.

(5) B. R. Baker, "Design of Active-Site-Directed Irreversible Enzyme Inhibitors. The Organic Chemistry of the Enzymic Active-Site," John Wiley and Sons, Inc., New York, N. Y., 1967.

(6) (a) The exo type of irreversible inhibitor can have an extra dimension of specificity not present in reversible inhibitors by using the bridge principle of specificity; (b) see ref 5, Chapter IX, for a detailed discussion of this principle.

(7) B. R. Baker and G. J. Lourens, *J. Med. Chem.*, **10**, 1113 (1967), paper CV of this series.