Irreversible Enzyme Inhibitors. CXLIV.[1,2] Proteolytic Enzymes. VII.[3] Additional Active-Site-Directed Irreversible Inhibitors of Trypsin Derived from *m*- and *p*-(Phenoxyalkoxy)benzamidines with a Terminal Sulfonyl Fluoride[4]

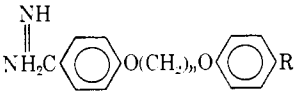
B. R. Baker and Edward H. Erickson

Department of Chemistry, University of California at Santa Barbara, Santa Barbara, California 93106

Received August 5, 1968

Twenty-four *m*- and *p*-(phenoxyalkoxy)benzamidines bearing a terminal sulfonyl fluoride moiety were synthesized and evaluated as irreversible inhibitors of trypsin; all were excellent reversible inhibitors with K_i = 0.7–3.6 μM. Eight (**2**, **3**, **5**, **6**, **19**–**21**, **24**) were excellent active-site-directed irreversible inhibitors when assayed at a K_i concentration giving 88–100% inactivation. Four (**13**, **15**–**17**) showed no irreversible inhibition when assayed at an $8K_i$ concentration. The remaining twelve were poor irreversible inhibitors at a $>K_i$ concentration. One of the excellent irreversible inhibitors of trypsin was *p*-[*p*-(*p*-fluorosulfonylbenzamido)phenoxypropoxy]benzamidine (**2**), which showed no irreversible inhibition of a related "tryptic" enzyme, namely, thrombin; this specificity is presumably due to the probability that the SO_2F moiety of **2** forms a covalent bond "outside" the active site where structural differences between trypsin and thrombin are apt to be present. The possibility of design of inhibitors of opposite specificity, that is, inactivation of thrombin with no inactivation of trypsin, by appropriate modification of benzamidine and phenylguanidine is discussed.

The chemotherapy of cardiovascular diseases and organ transplantation should be approachable by selective blockage of key serum proteases.[5] Our initial studies have used trypsin as a model since a number of these serum proteases are "tryptic" in character. In our first paper on trypsin,[5] it was established that phenoxyalkoxy groups could be substituted on the good reversible inhibitor, benzamidine,[6] with some gain in reversible binding. Later studies[4] described the discovery of active-site-directed irreversible inhibitors[7] of trypsin derived from *p*-(phenoxyalkoxy)benzamidine (**1**, R = H) by insertion of a fluorosulfonylphenyl group on the terminal phenyl.



1

Although five of these six candidate active-site-directed irreversible inhibitors at $2–4K_i$ could inactivate trypsin with a half-life of 3–6 min, inactivation was only 30–55% at a K_i concentration of inhibitor, which is sufficient to convert 50% of the enzyme to the rate-limiting reversible enzyme–inhibitor complex;[8] that the inactivation reaction was incomplete, due in part to concomitant enzyme-catalyzed hydrolysis of the SO_2F group to SO_3^-, was established.[4,9] Therefore a further study has now been made to determine the relationship of structure to efficient irreversible inhibition.

Enzyme Results—The results obtained earlier with the five active-site-directed irreversible inhibitors (**2**–**6**) are listed in Table I, the enzyme inactivation being measured with N-benzoyl-*dl*-arginine *p*-nitroanilide (BANA);[10] since BANA has a low V_{max} compared to ester substrates such as N-tosyl-L-arginine methyl ester (TAME),[11] a high concentration of enzyme is needed in the BANA assay. Thus compounds **2**–**6** were incubated with 3–9 μM trypsin;[4] in a few runs,[4] the trypsin concentration was lower than the inhibitor concentration, such a ratio making total inactivation stoichiometrically impossible.[12] When the extent of irreversible inhibition of trypsin was measured with TAME with its higher V_{max}, the concentration of the enzyme could be reduced to 0.1–0.3 μM (see Experimental Section); it was then readily ascertained which compounds still underwent enzyme-catalyzed hydrolysis of the SO_2F function to SO_3H[9] at a K_i concentration of inhibitor.

The five compounds (**2**–**6**) previously examined as irreversible inhibitors of trypsin with the BANA assay were reexamined at K_i concentration with the TAME assay where the concentration of trypsin was <0.3 μM (Table I). At a K_i concentration (1.6–3.1 μM), all five (**2**–**6**) were excellent irreversible inhibitors of trypsin, showing 83–94% inactivation. One of the compounds (**2**) was then examined as an irreversible inhibitor at less than K_i concentration; with 0.5 and $0.25K_i$ concentrations of **2**, which are still in excess over trypsin and able to reversibly complex 33 and 20%,[8] respectively, of the available trypsin, total irreversible inhibition was reduced to 75 and 40%. Thus enzyme-catalyzed hydrolysis of the SO_2F group is still observable[9] as the inhibitor concentrations approach these lower enzyme concentrations.

Since **6** was an excellent irreversible inhibitor at a K_i = 1.6 μM concentration, several analogs were synthesized for enzymic evaluation. Introduction of

(1) This work was generously supported by Grant CA-08695 from the National Cancer Institute, U. S. Public Health Service; some of the enzyme measurements were made on a Gilford 2400 spectrophotometer purchased with funds from U. S. Public Health Service Grant FR07099.

(2) For the previous paper in this series see B. R. Baker and R. B. Meyer, Jr., *J. Med. Chem.*, **12**, 108 (1969).

(3) For the previous paper on proteolytic enzymes see B. R. Baker and J. A. Hurlbut, *ibid.*, **11**, 1054 (1968), paper CXXXII of this series.

(4) For the previous paper on trypsin see B. R. Baker and E. H. Erickson, *ibid.*, **11**, 245 (1968), paper CXV of this series.

(5) See B. R. Baker and E. H. Erickson, *ibid.*, **10**, 1123 (1967), paper CVI of this series, for a more detailed discussion of the inhibition of these enzymes.

(6) M. Mares-Guia and E. Shaw, *J. Biol. Chem.*, **240**, 1579 (1965).

(7) 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.

(8) For the kinetics of irreversible inhibition see ref 7, Chapter 8.

(9) B. R. Baker and J. A. Hurlbut, *J. Med. Chem.*, **11**, 233 (1968), paper CXIII of this series.

(10) B. F. Erlanger, N. Kokowsky, and W. Cohen, *Arch. Biochem. Biophys.*, **95**, 271 (1961).

(11) B. C. W. Hummel, *Can. J. Biochem. Physiol.*, **37**, 1393 (1959).

(12) We wish to thank Dr. Howard J. Schaeffer for pointing out this stoichiometry.

a Me (**7**), MeO (**8**), or EtO (**9**) group *ortho* to the SO_2F function gave little change in K_i. Although these structural changes still allowed irreversible inhibition, the total irreversible inhibition at a $1–4K_i$ concentration was low, indicating that these structural changes had a detrimental effect on the ratio of the rate of enzyme inactivation *vs.* the rate of enzyme-catalyzed hydrolysis. Substitution of Cl (**10**) or MeO (**11**) *para* to the SO_2F function of **6** was also detrimental to irreversible inhibition, but not reversible inhibition; substitution of a Cl (**12**) on the central phenyl group of **6** gave similar results.

When the *m*-SO_2F function of **6** was moved to *para* (**13**), reversible inhibition was enhanced about twofold; however, this structural change destroyed the irreversible inhibitory properties. When **13** was further substituted by Me (**16**) or Cl (**17**) on the central phenyl group, irreversible inhibition was still not seen. Substitution of *o*-Me (**15**) to the SO_2F function of **13** gave similar negative results. In contrast, substitution of Cl (**14**) *meta* to the SO_2F function of **13** gave a good irreversible inhibitor, although reversible inhibition was not changed. When the bridging in **13** to the benzamidine was changed from *para* to *meta*, the resultant **18** showed improvement over the nonirreversible **13**; **18** was still a poor irreversible inhibitor since a $4K_i$ concentration showed irreversible inhibition but a K_i concentration gave essentially no irreversible inhibition.

When the *p*-(fluorosulfonylbenzamido) moiety of **4** was moved to the *meta* position, the resultant **19** was an even better irreversible inhibitor than **4** when both were compared at K_i concentration.

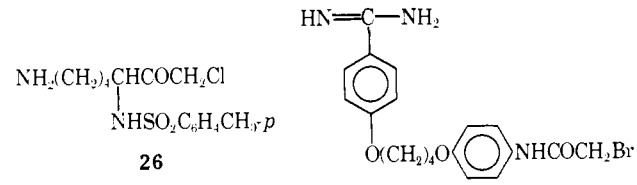
A series of irreversible inhibitors derived from *p*-(*m*-aminophenoxyethoxy)benzamidine were then investigated. The *m*-fluorosulfonylphenylureido derivative (**20**) was an excellent irreversible inhibitor of trypsin, as was **21** with a Cl *para* to the SO_2F moiety. Insertion of an *o*-Me (**22**) on **20** again gave a poorer irreversible inhibitor, presumably due to a less favorable ratio of enzyme inactivation to enzyme-catalyzed hydrolysis.

When the *m*-SO_2F moiety of **20** was moved to the *para* (**23**), little change in reversible inhibition occurred; however, the effect on irreversible inhibition was more dramatic, **23** now being a poor irreversible inhibitor; removal of an NH of the ureido bridge of **23** to give **24** resulted in recouping irreversible inhibition.

The last compound investigated was the lower homolog (**25**) of **13** which was a poor irreversible inhibitor, but was better than **25** which failed to show any irreversible inhibition. The best irreversible inhibitors in Table I are **2**, **3**, **5**, **6**, **19–21**, and **24**, all of which gave >88% inactivation at a K_i concentration. In order to compare these best inhibitors in speed of inactivation, in contrast to total inactivation in 60 min, time studies were performed. The half-lives of irreversible inhibition by these eight compounds at a K_i concentration were compared; these half-lives varied between 1–7 min. Three of the compounds (**19–21**) had half-lives of 2 min or less at a K_i concentration of 0.9–2.2 μM and gave essentially 100% inactivation in 60 min.

Although it is likely that these SO_2F-type irreversible inhibitors in Table I form a covalent bond with a serine or threonine of trypsin, it is unlikely that the same amino acid in trypsin is attacked by all these irreversible inhibitors. The location of the amino acid covalently linked by these inhibitors is a challenging endeavor worthy of pursuit.[13]

One of the objects of synthesizing the compounds in Table I was to gain better specificity among the numerous "tryptic" enzymes. For example, thrombin[14] is defined as a "tryptic" enzyme since it can use N-tosyl-L-arginine methyl ester (TAME) as a substrate. Therefore one of compounds (**2**) in Table I was examined as a reversible and irreversible inhibitor of thrombin. Reversibly, **2** had an I_{50} = 24 μM when assayed with 1 mM TAME at pH 8.4. When thrombin was incubated for 1 hr at 37° at pH 7.4 with 30 μM **2**, no irreversible inhibition was observed. Note that 3.1 μM **2** can give 91% inactivation of trypsin under the same conditions. This selectivity in irreversible inhibition by **2** between trypsin and thrombin should be contrasted with the results of irreversible inhibition of these two enzymes by the chloromethyl ketone from N-tosyl-L-lysine (**26**) (TLCK); the latter could irreversibly inhibit both thrombin and trypsin.[15] TLCK specifically alkylates histidine-46 when complexed



26 **27**

with trypsin;[16] this histidine is most likely part of the active site.[15]

Since thrombin and trypsin are both "tryptic," their catalytic sites and complexing sites for a lysine moiety would be expected to be similar; hence, both should be inactivated by TLCK. In contrast, **2** was designed to complex the benzamidine moiety in the active site of trypsin then extend the SO_2F moiety outside the active site where differences in structure between trypsin and other "tryptic" enzymes should be apparent.[17] Thus, the specificity shown by **2** between trypsin and thrombin can be accounted for by covalent bond formation outside the active site, the so-called exo mechanism of active site-directed ir-

(13) The SO_2F moiety also has the ability to form a stable covalent bond with histidine or tyrosine. Since there are only three histidines in trypsin and the number of tyrosines on the surface of an enzyme is apt to be small, linkage to one of these amino acids is not likely.

(14) A number of commercial crude thrombin preparations contained much water-insoluble protein and gave a low velocity of reaction with TAME which was highly variable. Good activity and duplicatability were achieved with a water-soluble thrombin preparation, No. BT2000, purchased from Sigma Chemical Co. TAME showed an apparent K_m of 3 mM in 0.05 M Tris buffer, pH 8.4.

(15) E. Shaw, M. Mares-Guia, and W. Cohen, *Biochemistry*, **4**, 2219 (1965).

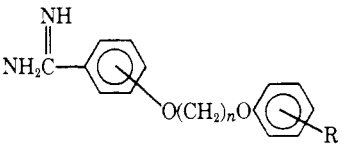
(16) E. Shaw and S. Springhorn, *Biochem. Biophys. Res. Commun.*, **27**, 391 (1967).

(17) An active site is defined as containing those amino acids in contact with the substrate and those amino acids involved in the catalytic process.[18] Since it is not yet known how many amino acid residues at the carboxyl end of a lysine or arginine in a protein are in contact with a protease such as trypsin, the dimensions of the active site are impossible to estimate by substrate size when the substrate is a protein. However, even if two or three amino acid units at the carboxyl terminus were in contact with two different proteases, the farther removed from the catalytic site are these contacts, the greater difference could be expected in primary and tertiary structure of the two proteases.

(18) See ref 7, p 188.

Table I

Inhibition[a] of Trypsin by



No.	Bridge position (n)	R	Reversible: I_{50},[b] μM	Reversible: Estd K_i,[c] μM	Irreversible: Method[d]	Inhib, μM	% E···I[e]	Time, min	% inactvn
2[f]	*para* (3)	*p*-NHCOC$_6$H$_4$SO$_2$F-*p*	6.2	3.1	BANA	25	88	3, 15[g]	50, 100
					BANA	7.5	~70	5, 30[g]	50, 88
					BANA	3.1		4, 15, 30[g]	44, 44
					TAME	3.1[h]	50	60	91
					TAME	1.5[h]	33	60	75
					TAME	0.75[h]	20	60	40
					TAME	3.1[h]	50	7	50[g]
3[f]	*para* (3)	*p*-NHCOC$_6$H$_4$SO$_2$F-*m*	3.4	1.7	BANA	6.5	~80	4, 12[g]	50, 100
					BANA	1.7		14, 30[g]	54, 54
					TAME	1.7[h]	50	60	94
					TAME	1.7[h]	50	4, 10, 60[g]	50, 65, 92
4[f]	*para* (4)	*p*-NHCOC$_6$H$_4$SO$_2$F-*p*	4.1	2.0	BANA	40	95	3, 12[g]	50, 100
					BANA	10	83	3, 30[g]	50, 70[i]
					BANA	2.0		8, 30[g]	31, 31
					TAME	2.0[h]	50	60	83
5[f]	*para* (4)	*p*-NHCOC$_6$H$_4$SO$_2$F-*m*	4.5	2.2	BANA	11	~83	6, 30[g]	50, 80[i]
					BANA	2.2		8, 30[g]	40, 40
					TAME	2.3[h]	50	60	92
					TAME	2.3[h]	50	7	50[g]
6[f]	*para* (4)	*p*-NHCONHC$_6$H$_4$SO$_2$F-*m*	3.2	1.6	BANA	7.0	~80	6, 30[g]	50, 82[i]
					BANA	1.6		8, 30[g]	37, 37
					TAME	1.6[h]	50	60	88
					TAME	1.6[h]	50	7	50[g]
7	*para* (4)	*p*-NHCONHC$_6$H$_3$-4-Me-3-SO$_2$F	2.3	1.1	BANA	14	92	60	87
					BANA	3.8		60	23
					TAME	1.1	50	60	38
8	*para* (4)	*p*-NHCONHC$_6$H$_3$-4-MeO-3-SO$_2$F	2.5	1.2	BANA	7.0	~85	15, 60[g]	24, 24
					BANA	2.0		60[a]	0
					TAME	2.5	67	60[h]	49
9	*para* (4)	*p*-NHCONHC$_6$H$_3$-4-EtO-3-SO$_2$F	2.2	1.1	BANA	10	90	60	74
					BANA	4.0		60[g]	0
					TAME	4.0	79	60	41
10	*para* (4)	*p*-NHCONHC$_6$H$_3$-2-Cl-5-SO$_2$F	1.5	0.75	BANA	9.5	93	60	93
					BANA	2.4		60	28
					TAME	0.75	50	60	76
11	*para* (4)	*p*-NHCONHC$_6$H$_3$-2-MeO-5-SO$_2$F	2.8	1.4	BANA	13	90	60	80
					BANA	5.2		10, 60[g]	41, 41
					TAME	1.4	50	60	6
12	*para* (4)	*o*-Cl-*p*-NHCONHC$_6$H$_3$SO$_2$F-*m*	2.5	1.2	BANA	5.0		60	42
					TAME	1.2	50	60	41
13	*para* (4)	*p*-NHCONHC$_6$H$_4$SO$_2$F-*p*	1.4	0.70	BANA	5.6	89	60	0
					TAME	0.70	50	60	0
14	*para* (4)	*p*-NHCONHC$_6$H$_3$-2-Cl-4-SO$_2$F	1.6	0.80	BANA	7.0	~90	60	95
					BANA	2.8		5, 60[g]	40, 40
					TAME	0.80	50	60	78
15	*para* (4)	*p*-NHCONHC$_6$H$_3$-3-Me-4-SO$_2$F	1.6	0.80	BANA	6.8	~89	60	0
					TAME	1.6	67	60	0
16	*para* (4)	*m*-Me-*p*-NHCONHC$_6$H$_3$SO$_2$F-*p*	1.4	0.70	BANA	5.6	89	60	0
17	*para* (4)	*o*-Cl-*p*-NHCONHC$_6$H$_3$SO$_2$F-*p*	2.4	1.2	BANA	10	89	60	0
18	*meta* (4)	*p*-NHCONHC$_6$H$_4$SO$_2$F-*p*	7.2	3.6	BANA	15	81	20, 60[g]	50, 87[i]
					BANA	7.4		60[a]	0
					TAME	3.6	50	60	6
19	*para* (4)	*m*-NHCOC$_6$H$_4$SO$_2$F-*p*	4.4	2.2	BANA	8.8	~80	2, 8[g]	72, 90[i]
					BANA	2.2		8, 30[g]	45, 45
					TAME	2.2	50	60	96
					TAME	1.1	33	60	78
					TAME	2.2	50	1, 10, 60	50, 92, 97[g]
20	*para* (2)	*m*-NHCONHC$_6$H$_4$SO$_2$F-*m*	3.6	1.8	BANA	7.2	~80	2, 8[g]	75, 87[i]
					BANA	1.8		4, 30[g]	42, 42
					TAME	1.8	50	60	96
					TAME	1.8	50	2, 10, 60	50, 78, 100[g]

TABLE I (*Continued*)

No.	Bridge position (*n*)	R	Reversible: I_{50},[b] μM	Reversible: Estd K_i,[c] μM	Irreversible: Method[d]	Inhib, μM	% E···I[e]	Time, min	% inactvn
21	*para* (2)	*m*-NHCONHC$_6$H$_3$-2-Cl-5-SO$_2$F	1.8	0.90	BANA	3.0		60	54
					TAME	0.90	50	60	100
					TAME	0.90	50	1, 10, 60	50, 86, 100[g]
22	*para* (2)	*m*-NHCONHC$_6$H$_3$-4-Me-3-SO$_2$F	4.1	2.0	BANA	9.2	82	60	86
					BANA	2.3		60	23
					TAME	2.0	50	60	76
23	*para* (2)	*m*-NHCONHC$_6$H$_4$SO$_2$F-*p*	4.8	2.4	BANA	10.6	82	60	59
					BANA	5.3		60	12
					TAME	2.4	50	60	15
24	*para* (2)	*m*-NHCOC$_6$H$_4$SO$_2$F-*p*	3.0	1.5	BANA	6.0		2, 30[g]	50, 83[i]
					BANA	1.5		10, 30[g]	47, 47
					TAME	1.5	50	60	94
					TAME	1.5	50	3, 10, 60	50, 68, 91[g]
25	*para* (3)	*p*-NHCONHC$_6$H$_4$SO$_2$F-*p*	4.0	2.0	BANA	13	87	60	41
					TAME	2.0	50	60	0

[a] The technical assistance of Susan Black, Maureen Baker, Jean Reeder, and Julie Leseman with these assays is acknowledged. [b] I_{50} = concentration necessary for 50% inhibition when assayed with 50 μM DL-benzoylarginine *p*-nitroanilide (BANA) in pH 7.4 Tris buffer containing 10% DMSO as previously described.[5] [c] Estimated as $0.5I_{50}$; see ref 4. [d] Enzyme incubated at 37° in pH 7.4 Tris buffer containing 10% DMSO;[4] method A used 3–9 μM trypsin and was assayed with BANA. Method B used 0.1–0.3 μM trypsin and was assayed with N-tosyl-L-arginine methyl ester (TMAE) (see Experimental Section). [e] Calculated from [EI] = $[E_t]/(1 + K_i/[I])$ where [EI] = amount of reversible complex expressed as a percentage of total enzyme (E_t);[8] this calculation is valid only when inhibitor is in excess of enzyme. [f] Data from ref 4 unless otherwise indicated. [g] From six-point time study.[4] [h] New data. [i] Near maximum irreversible inhibition.

reversible inhibition.[7] Similarly, the bromoacetamidobenzamidine (**27**), which at 14 μM shows slow irreversible inhibition of trypsin with a half-life of 4 hr, showed no irreversible inhibition of thrombin at 30 μM.

The trypsin inhibitors, benzamidine (**28**) and phenylguanidine (**29**), are also reversible inhibitors of thrombin; when thrombin was assayed with 1 mM TAME, **28** and **29** had I_{50} = 0.46 and 0.68 mM, respectively. Thus it should be possible to convert **28** or **29** to a specific irreversible inhibitor of thrombin by utilizing the *modus operandi* developed for trypsin[4,5] and other enzymes.[7] Similarly, it should be possible to develop selective irreversible inhibitors of other serum proteases such as the complement system involved in rejection of organ transplants.[19]

$C_6H_5C(=NH)NH_2$ **28** $\qquad$ $C_6H_5NHC(=NH)NH_2$ **29**

Chemistry.—All of the candidate irreversible inhibitors in Table I can be generalized either as a benzamide (**30**) or phenylurea (**31**); these were synthesized from the appropriate arylamines (**32**, **33**, **39–42**, **47**) by acylation with the appropriate fluorosulfonylbenzoyl chloride in DMF in the presence of Et_3N or with the appropriate O-(*p*-nitrophenyl) carbamate[20] in the absence of Et_3N. Of the required amines, **32** and **33** have been previously synthesized.[4] The remainder were synthesized as follows.

The alkylation of *p*-hydroxybenzamidine (**34**) with the appropriate ω-bromoalkyl *m*-nitrophenyl ether[21] in DMF containing K_2CO_3 gave **35** and **36** by the previously described general method.[4] Catalytic hydrogenation of the NO_2 group afforded the requisite amines **39** and **40**; the amine (**41**) was prepared from **34** in a similar fashion with 4-bromobutyl 3-methyl-4-nitrophenyl ether *via* **37** (Scheme I). Similarly, alkylation of *p*-hydroxybenzamidine (**34**) with the appropriate bromide gave **38**. However, trace impurities in **38** made catalytic reduction to **42** a quite variable reaction that failed at times; therefore **38** was synthesized by the alternate route of alkylation of *p*-cyanophenol to **43** which was more readily purified. Reaction of **43** with EtOH and HCl in $CHCl_3$ converted the CN to an imino ether which was treated *in situ* with ammonia to give the amidine **38**, isolated as its benzenesulfonate salt. Catalytic reduction of **38** then proceeded smoothly to **42**. In a similar fashion, *m*-cyanophenol (**44**)[22] was converted to **47**.

Experimental Section

All analytical samples had ir spectra in agreement with their assigned structures, moves as a single spot on tlc on Brinkmann silica gel GF or polyamide MN, and gave combustion values for C, H, and N or F within 0.4% of theoretical unless otherwise indicated. Melting points were taken in capillary tubes on a Mel-Temp block and are uncorrected.

4-Ethoxy-3-fluorosulfonylacetanilide (48).—To 60 ml of $ClSO_3H$ was added portionwise with stirring 30 g (0.17 mole) of *p*-ethoxyacetanilide with water-bath cooling to keep the temperature at 30–35°. After 2 hr at 40° the solution was cooled in an ice bath, then poured into about 350 g of ice and H_2O with vigorous stirring. The mixture was extracted with two 250-ml portions of $CHCl_3$. Dried ($MgSO_4$), the $CHCl_3$ solution was evaporated *in vacuo* leaving an oily sulfonyl chloride which gradually solidified and had the proper ir spectrum. The oil was dissolved in 60 ml of dioxane. To the solution was added 30 g of KF (0.52 mole) in 30 ml of H_2O, then the mixture was refluxed for 40 min. The cooled mixture was diluted with 300 ml of H_2O and extracted with two 200-ml portions of $CHCl_3$. The combined extracts were dried ($MgSO_4$), then evaporated *in vacuo*, leaving 16.9 g (38%) of product that moved as single spot on tlc with EtOAc and was suitable for the next step. Recrystallization of a sample from $CHCl_3$ gave white crystals, mp 157–159°. *Anal.* ($C_{10}H_{12}FNO_4S$) C, H.

O-(*p*-Nitrophenyl) N-(4-Ethoxy-3-fluorosulfonylphenyl)carbamate (49).—A mixture of 16.9 g (66 mmoles) of **48**, 35 ml of EtOH, 14 ml of H_2O, and 20 ml of 12 N HCl was refluxed for 90 min, then diluted with 450 ml of H_2O. The solution was clarified by filtration, then neutralized with $NaHCO_3$ and extracted

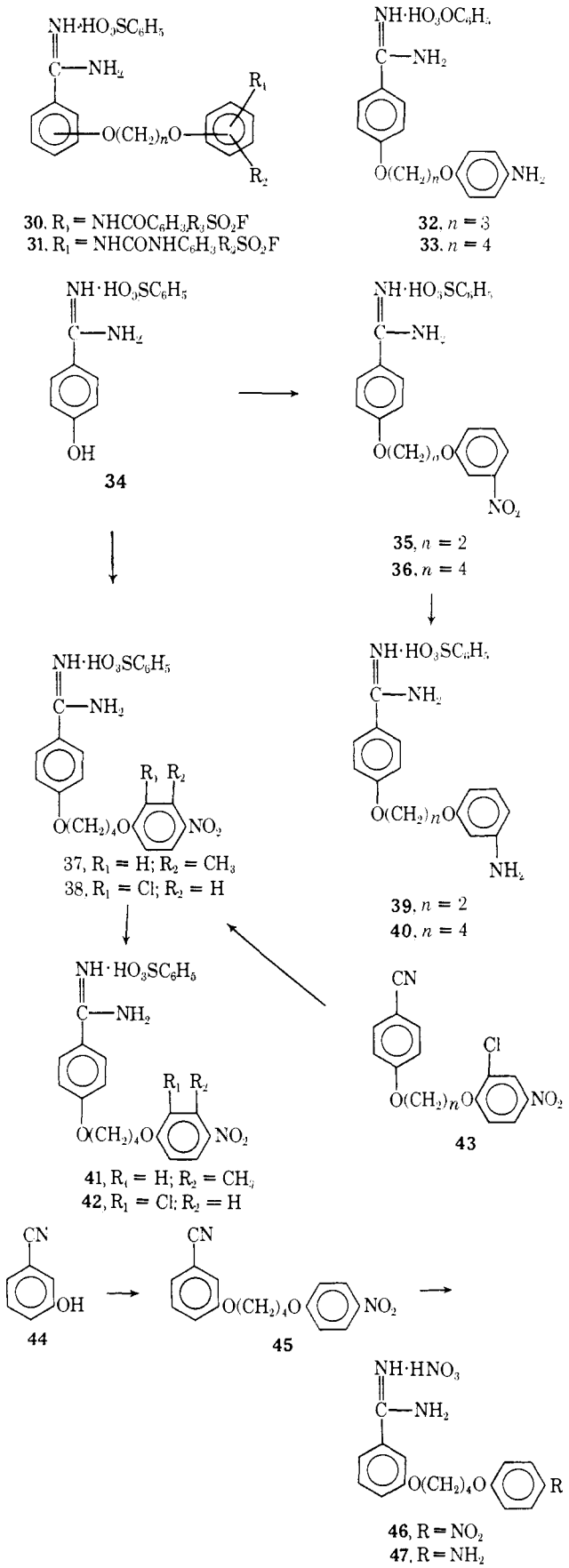
(19) Ciba Foundation Symposium, Complement, G. E. W. Wolstenholme and J. Knight, Ed., Little, Brown and Co., Boston, Mass., 1965.

(20) B. R. Baker and N. M. J. Vermeulen, *J. Med. Chem.*, **12**, 74 (1969), paper CXXXIV of this series.

(21) B. R. Baker and G. J. Lourens, *ibid.*, **11**, 26 (1968), paper CIX of this series.

(22) Prepared by the procedure of T. van Es, *J. Chem. Soc.*, 1564 (1965)

Scheme I



with two 250-ml portions of $CHCl_3$. Dried ($MgSO_4$), the combined extracts were evaporated *in vacuo* leaving 6.8 g (47%) of 2-ethoxy-5-metanilyl fluoride, mp 55–68°, which was not readily purified.

A solution of 2.19 g (10 mmoles) of the crude metanilyl fluoride and 2.2 g (11 mmoles) of *p*-nitrophenyl chloroformate in 20 ml of C_6H_6 was gently refluxed under an air condenser for 3 hr.[29] C_6H_6 was evaporated *in vacuo* and the residue was recrystallized three times for CH_2Cl_2; yield 0.88 g (23%), mp 154–155°. *Anal.* ($C_{13}H_{10}FN_2O_5S$) C, H, N. An additional 2.2 g (58%) of less pure material could be isolated from the combined filtrates.

2-Chloro-4-nitrophenyl 4-Bromobutyl Ether (50).—A mixture of 34.8 g (0.2 mole) of 2-chloro-4-nitrophenol, 174 g (0.8 mole) of 1,4-dibromobutane, 50 ml of DMF, and 28 g (0.2 mole) of K_2CO_3 was heated on a steam bath for 5 hr. The cooled solution was diluted with 250 ml of $CHCl_3$ and washed successively with 250 ml of 5% NaOH and two 250-ml portions of H_2O. Dried ($MgSO_4$), the $CHCl_3$ solution was evaporated to about 100 ml *in vacuo*, then diluted with 200 ml of MeOH and kept overnight at −15°. The insoluble bisphenoxybutane (6.3 g) was removed by filtration. Evaporation *in vacuo* with removal of the 1,4-dibromobutane gave an oil which could be crystallized from MeOH with large loss; yield 13.6 g (22%), mp 40–43°, suitable for further transformation. Two recrystallizations of a sample from MeOH gave the analytical sample, mp 41–43°. *Anal.* ($C_{10}H_{11}BrClNO_3$) C, H, N.

The corresponding 3-methyl-4-nitrophenyl ether was prepared similarly, but melted below room temperature.

4-(2-Chloro-4-nitrophenoxy)-1-(*p*-cyanophenoxy)butane (43).—A stirred mixture of 2.4 g (20 mmoles) of *p*-cyanophenol, 6.25 g (20 mmoles) of **50**, 2.8 g (20 mmoles) of K_2CO_3, and 25 ml of DMF was heated for 5 hr in a bath at 60°. The mixture was diluted with 100 ml of H_2O and extracted with two 100-ml portions of $CHCl_3$. Dried ($MgSO_4$), the combined extracts were evaporated *in vacuo*. The residual oil was crystallized by the addition of petroleum ether (bp 30–60°), then recrystallized from MeOEtOH; yield, 5.3 g (75%), mp 134–136°. *Anal.* ($C_{17}H_{15}ClN_2O_4$) C, H, N.

Similarly, **45** was prepared in 68% yield, mp 70–79°. Recrystallization from MeOH gave the analytical sample, mp 73–75°. *Anal.* ($C_{17}H_{20}N_2O_5$) C, H, N.

***p*-(2-Chloro-4-nitrophenoxybutoxy)benzamidine Benzenesulfonate (38) (Method F).**—Through a solution of 3.47 g (10 mmoles) of **43** and 0.46 g (10 mmoles) of EtOH in 40 ml of $CHCl_3$ cooled in an ice bath was passed a slow stream of HCl gas for 90 min, the system being protected from moisture. After standing 48 hr at room temperature, the solution was further treated with 0.23 g (5 mmoles) of EtOH and HCl gas for 60 min since ir of an aliquot still showed a C≡N band. After 24 hr the mixture was treated with 25 ml of EtOH saturated with NH_3 at 0°. The mixture was stirred for 48 hr, then filtered and evaporated *in vacuo*. The residue was then heated with a solution of 2.5 g (14 mmoles) of $C_6H_5SO_3H$ in 25 ml of H_2O when the product crystallized. Recrystallization from *n*-PrOH gave 1.8 g (35%) of product, mp 198–203°. See Table II for additional data.

Similarly, **46** was prepared from **45** except the product was crystallized from 50 ml of *i*-PrOH plus 3 ml of concentrated HNO_3; yield 1.08 g (40%), mp 152–158°, single spot on tlc with MeOH on Brinkmann polyamide MN. Recrystallization of a sample from EtOH gave the analytical sample, mp 160–163°. *Anal.* ($C_{17}H_{20}N_4O_7$) C, H, N.

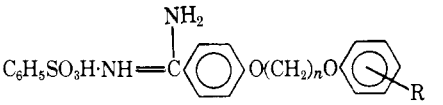
***m*-[*p*-(*p*-Aminophenoxybutoxy)benzamidine nitrate (47)** was prepared from **46** by method B.[4] Two recrystallizations from H_2O gave 0.35 g (40%) of white crystals, mp 198–200°. *Anal.* ($C_{17}H_{22}N_4O_5$) C, H, N.

***m*-[*p*-(*p*-Fluorosulfonylphenylureido)phenoxybutoxy]benzamidine Nitrate (18) (Method C).**—A solution of 175 mg (0.51 mmole) of **47** and 181 mg (0.50 mmole) of O-(*p*-nitrophenyl) N-(*p*-fluorosulfonylphenyl)carbamate[29] in 1 ml of DMF was magnetically stirred at ambient temperature for 1 hr, then diluted with Et_2O (9 ml). An oil separated that soon solidified. Three recrystallizations from MeOH gave 133 mg (48%) of product, mp 188–190°. *Anal.* ($C_{24}H_{28}FN_5SO_9$) C, H, F. See Table II for other compounds prepared by this method. When the oily product did not crystallize, the solvent was decanted and the oil crystallized from the solvent indicated.

2-Fluorosulfonyl-4-methoxybenzoic Acid (51).—To 10 ml of FSO_3H in a Teflon beaker was added in portions over 15 min 5.3 g of anisic acid. The solution was heated in a bath at 100° for 4 hr, then cooled and poured into ice with stirring. The mixture was extracted with Et_2O. The combined extracts, dried with $MgSO_4$, were evaporated *in vacuo* to give 3.5 g (46%) of product, mp 158–165°. Several recrystallizations (C_6H_6) gave white crystals, mp 177–179°. *Anal.* ($C_8H_7FO_5S$) C, H.

TABLE II

PHYSICAL PROPERTIES OF



No.	n	R	Method[a]	% yield	Mp, °C	Formula	Analyses
7	4	*p*-NHCONHC$_6$H$_3$-4-Me-3-SO$_2$F	C	54[b]	230–233	$C_{31}H_{33}FN_4O_8S_2$	C, H, F
8	4	*p*-NHCONHC$_6$H$_3$-4-MeO-3-SO$_2$F	C	38[b]	216–218 dec	$C_{31}H_{33}FN_4O_9S_2$	C, H, F
9	4	*p*-NHCONHC$_6$H$_3$-4-EtO-3-SO$_2$F	C	28[b]	216–219	$C_{32}H_{35}FN_4O_9S_2$	C, H, F
10	4	*p*-NHCONHC$_6$H$_3$-2-Cl-5-SO$_2$F	C	64[c]	180–181	$C_{30}H_{30}ClFN_4O_8S_2$	C, H, F
11	4	*p*-NHCONHC$_6$H$_3$-2-MeO-5-SO$_2$F	C	40[b]	183–185	$C_{31}H_{33}FN_4O_9S_2$	C, H, F
12	4	*o*-Cl-*p*-NHCONHC$_6$H$_4$SO$_2$F-*m*	C	44[b]	213–215	$C_{30}H_{30}ClFN_4O_8S_2$	C, H, F
13	4	*p*-NHCONHC$_6$H$_4$SO$_2$F-*p*	C	38[d]	251–253 dec	$C_{30}H_{31}FN_4O_8S_2$	C, H, F
14	4	*p*-NHCONHC$_6$H$_3$-2-Cl-4-SO$_2$F	C	34[e]	178–180	$C_{30}H_{30}ClFN_4O_8S_2$	C, H, F
15	4	*p*-NHCONHC$_6$H$_3$-3-Me-4-SO$_2$F	C	30[b]	240–241 dec	$C_{31}H_{33}FN_4O_8S_2$	C, H, F
16	4	*m*-Me-*p*-NHCONHC$_6$H$_4$SO$_2$F-*p*	C	68[f]	239–241 dec	$C_{31}H_{33}FN_4O_8S_2$	C, H, F
17	4	*o*-Cl-*p*-NHCONHC$_6$H$_4$SO$_2$F-*p*	C	44[g]	245–249	$C_{30}H_{30}ClFN_4O_8S_2$	C, H, F
19	4	*m*-NHCOC$_6$H$_4$SO$_2$F-*p*	D	13[h]	161–165	$C_{30}H_{30}FN_3O_8S_2$	C, H, N
20	2	*m*-NHCONHC$_6$H$_4$SO$_2$F-*m*	E	26[i]	233–235	$C_{28}H_{27}FN_4O_8S_2$	C, H, F
21	2	*m*-NHCONHC$_6$H$_3$-2-Cl-5-SO$_2$F	C	42[b]	216–219 dec	$C_{28}H_{26}ClFN_4O_8S_2 \cdot H_2O$	C, H, F
22	2	*m*-NHCONHC$_6$H$_3$-4-Me-3-SO$_2$F	C	16[b]	247–249	$C_{29}H_{29}FN_4O_8S_2$	C, H, F
23	2	*m*-NHCONHC$_6$H$_4$SO$_2$F-*p*	C	24[j]	245–248	$C_{28}H_{27}FN_4O_8S_2$	C, H, F
24	2	*m*-NHCOC$_6$H$_4$SO$_2$F-*p*	D	10[g]	270–272	$C_{28}H_{26}FN_3O_8S_2$	C, H, F[e]
25	3	*p*-NHCONHC$_6$H$_4$SO$_2$F-*p*	C	38[b]	243–245	$C_{29}H_{29}FN_4O_8S_2$	C, H, F
35	2	*m*-NO$_2$	A	47[b]	203–205	$C_{21}H_{21}N_3O_7S$	C, H, N
36	4	*m*-NO$_2$	A	46[j]	187–190	$C_{23}H_{25}N_3O_7S$	C, H, N
37	4	3-Me-4-NO$_2$	A	26[k]	174–178	$C_{24}H_{27}N_3O_7S$	C, H, N
38	4	2-Cl-4-NO$_2$	A	61	197–203[l]	$C_{23}H_{24}ClN_3O_7S$	C, H, N
			F	35[l]	198–203		
39	2	*m*-NH$_2$	B	80[b]	238–240	$C_{21}H_{23}N_3O_5S$	C, H, N
40	4	*m*-NH$_2$	B	66[m]	173–180	$C_{23}H_{27}N_3O_5S$	C, H, N
41	4	3-Me-4-NH$_2$	B	42	175–178[n]	$C_{24}H_{29}N_3O_5S$	C, H, N
42	4	2-Cl-4-NH$_2$	B	90[o]	205–207	$C_{23}H_{26}ClN_3O_5S$	C, H, N

[a] For methods A, B, D, and E, see ref 4; for methods E and F, see Experimental Section. [b] Recrystallized from EtOH–H$_2$O. [c] Recrystallized from EtOH. [d] Recrystallized from DMF. [e] *Anal.* Calcd: C, 54.6; H, 4.26; F, 3.09. Found: C, 53.9; H, 4.03; F, 3.76. [f] Reprecipitated from DMF with Et$_2$O. [g] Reprecipitated from MeOEtOH–Et$_2$O. [h] Recrystallized from MeOH–Et$_2$O. [i] Recrystallized from MeOEtOH–H$_2$O. [j] Recrystallized by solution in acetone by adding the minimum of H$_2$O, then addition of Et$_2$O. [k] After one recrystallization from *i*-PrOH, mp 159–170°, suitable for the next step. [l] Recrystallized from *n*-PrOH. [m] Recrystallized from EtOH–Et$_2$O. [n] Recrystallized from H$_2$O. [o] Triturated with Me$_2$CO. [p] Recrystallized from MeOH.

This compound was also prepared from anisic acid by chlorosulfonation[23] followed by treatment with KF in aqueous dioxane as described for **48**; the over-all yield was 21%, mp 175–178°. Attempts to acylate several aminophenoxybenzamidines by method D with the acid chloride of **51** gave mixtures difficult to purify.

Enzyme Assays.—The reversible and irreversible inhibition assays of trypsin using N-benzoyl-DL-arginine *p*-nitroanilide (BANA)[10] have been described previously;[4] the earlier irreversible incubations[4] and those recorded in Table I used 3–9 μM trypsin in the incubation. The incubation concentration of typsin could be reduced to 0.1–0.3 μM by use of the more sensitive N-tosyl-L-arginine methyl ester (TAME);[11] the incubations were run in 0.05 M pH 7.4 Tris buffer, then assayed by 1:10 dilution with 0.05 M pH 8.4 Tris buffer containing 15 mM $CaCl_2$ with 1 mM TAME.[11]

(23) M. S. Shah, C. T. Bhatt, and D. D. Kanga, *J. Chem. Soc.*, 1375 (1933).