method A at −5 to 0° except Et_3N was used as the acid acceptor.

Method C. N-(p-Aminomethylphenyl)-3-chlorophenoxyacetamide Hydrochloride (23).—A solution of 2.86 g (10 mmoles) of **24** and 1.5 g (15 mmoles) of $EtSO_3H$ in 200 ml of 95% aqueous methoxyethanol was stirred with 0.10 g of decolorizing carbon, then filtered. After the addition of 50 mg of PtO_2, the mixture was shaken with H_2 at 2–3 atm. When about one-fourth of the calculated amount of H_2 had been consumed, hydrogenation ceased. The mixture was re-treated with decolorizing carbon and filtered, and then 50 mg of fresh PtO_2 was added. After being shaken with H_2 at 2–3 atm for an additional 24 hr, hydrogenation was essentially complete. The filtered solution was diluted with 600 ml of H_2O and a small amount of **24** was removed by filtration. The filtrate was adjusted to pH 11–13 with 10% NaOH, then extracted with three 50-ml portions of $CHCl_3$. The combined extracts were dried ($MgSO_4$), then saturated with HCl gas. The product was collected on a filter and recrystallized from EtOH; yield, 1.97 g (60%) of white crystals, mp 253–256 dec. See Table II for additional data.

Method D.—A mixture of 2.11 g (5 mmoles) of **32**, 50 ml of MeOEtOH, and 1.0 ml (26 mmoles) of 85% $N_2H_4 \cdot H_2O$ was refluxed with stirring for 20 min when solution was complete. Solvent was removed by spin evaporation *in vacuo*. The residue was stirred with 60 ml of 1 N HCl at about 85° for 15 min; the insoluble phthalhydrazide containing some **32** was removed by filtration and washed with H_2O. The phthalhydrazide containing some **32** was again treated with N_2H_4 for 20 min as above. The combined HCl solutions were brought to about pH 13 with 50% NaOH. The mixture was extracted with two 100-ml portions of Et_2O. The combined extracts were dried ($MgSO_4$), then treated with HCl gas until no more product separated. The product was collected and recrystallized from EtOH; yield 0.15 g (10%), mp 251–256° dec.

Method E was the same as method C except no H_2O or $EtSO_3H$ was added to the reduction mixture.

Method F. N-(m-Bromoacetamidobenzyl)-3,4-dichlorophenoxyacetamide (4).—To a solution of 325 mg (1 mmole) of **48** in 10 ml of Me_2CO was added 390 mg (1.5 mmoles) of bromoacetic anhydride.[17] The mixture was stirred for 4 hr, then diluted (H_2O) if the product had not separated. The product was collected on a filter, then washed (Me_2CO–H_2O, 5% $NaHCO_3$, H_2O). Recrystallization from EtOH afforded 300 mg (67%) of white needles, mp 155–157°, that gave a negative Bratton–Marshall test for arylamine and a positive 4-(p-nitrobenzyl)pyridine test for activated halogen.[17] See Table II for additional data.

Method G. 3-(m-Chlorophenoxyacetamidomethyl)-3'-fluorosulfonyldiphenylurea (13).—To a solution of 291 mg (1 mmole) of **48** in 15 ml of $CHCl_3$ was added 220 mg (1.1 mmoles) of m-fluorosulfonylphenyl isocyanate in 15 ml of $CHCl_3$. After 20 min, the mixture was filtered and the product was washed with $CHCl_3$. Recrystallization from EtOH gave 319 mg (63%) of white crystals, mp 178–181°, which gave a negative Bratton–Marshall test for arylamine.[17] See Table II for additional data.

Irreversible Enzyme Inhibitors. CXV.[1,2] Proteolytic Enzymes. V. Active-Site-Directed Irreversible Inhibitors of Trypsin Derived from p-(Phenoxyalkoxy)benzamidines with a Terminal Sulfonyl Fluoride

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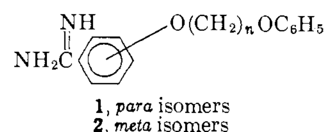
Eight candidate irreversible inhibitors of trypsin have been synthesized from p-phenoxypropoxybenzamidine (**3**) and p-phenoxybutoxybenzamidine by insertion of a leaving group on the *para* position of the phenoxy moiety. The p-bromoacetamido derivative (**6**) of **3** was a good reversible inhibitor of trypsin, but failed to show any irreversible inhibition. When the methylene bridge of **6** was increased to butyl, the resultant bromoacetamide (**11**) showed slow irreversible inhibition of trypsin with a half-life of about 5 hr. Much more dramatic results were obtained by insertion of a m- or p-fluorosulfonylbenzamido or m-fluorosulfonylphenylureido group on the phenoxy moiety. Five irreversible inhibitors of this type could inactivate trypsin with a half-life of 3–6 min. The kinetics indicated that not only did these sulfonyl fluorides rapidly inactivate trypsin, but trypsin could catalyze the hydrolysis of the sulfonyl fluorides to the irreversibly ineffective sulfonic acids.

Selective inhibitors for serum proteases could have utility for chemotherapy of cardiovascular diseases and of organ transplantation.[3] Since a number of these enzymes are "tryptic" in character, our initial studies have concentrated on trypsin as a model. Furthermore, active-site-directed irreversible inhibitors[4] of the exo type could be expected to have the necessary selectivity in inhibition of these closely related serum proteases. The design of such exo-type irreversible inhibitors is best performed stepwise: (a) the binding points of an inhibitor should be determined; (b) a position on the inhibitor should be found where large groups can be tolerated without interfering with formation of a reversible enzyme–inhibitor complex, a necessary intermediate in enzyme inactivation by the active-site-directed mechanism; (c) a properly positioned leaving group should be placed on this large moiety which becomes juxtaposed to a nucleophilic group when complexed to the enzyme and has the proper electrophilicity to react with this enzymic nucleophile; (d) the irreversible inhibitor is further modified to give selective inhibition of closely related enzymes or isozymes.

Since benzamidine is an excellent reversible inhibitor of trypsin,[5] it was selected for further study in order to convert it to an active-site-directed irreversible inhibitor of the exo type. In our first paper on trypsin,[3] we established that phenoxyalkoxy groups could be substituted on benzamidine at either the *para* (**1**) or *meta* positions (**2**) with retention of binding; in fact



1, *para* isomers
2, *meta* isomers

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

(2) For the previous paper see B. R. Baker and J. A. Hurlbut, *J. Med. Chem.*, **11**, 241 (1968).

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

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

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

Table I

Inhibition[a] of Trypsin by

$NH_2C(=NH)-C_6H_4-O(CH_2)_nO-C_6H_4-R$

			Reversible		Irreversible			
No.	n	R	I_{50},[b] μM	Estd K_i,[c] $\times 10^6 M$	Concn, μM	E···I,[d] %	Time, min	Inactn,[e] %
3	3	H	15[f]	7.5				
4	3	$NHCOC_6H_4SO_2F$-*p*	6.2	3.1[g]	25	88	3, 15[h]	50,100
					7.5	71	5, 30[h]	50, 88
					3.1	50	15, 30[h]	44, 44[i]
5	3	$NHCOC_6H_4SO_2F$-*m*	3.4	1.7	13	88	4, 15[h]	50, 100
				1.2[g]	6.5	80	4, 12[h]	50,100
					1.7	50	14, 30[h]	54, 54[i]
6	3	$NHCOCH_2Br$	9.2	4.6	17	79	60	0
7	4	$NHCOC_6H_4SO_2F$-*p*	4.1	2.0	40	95	3, 12[h]	50, 100
					10	83	3, 30[h]	50, 70[i]
					2.0	50	8, 30[h]	31, 31[i]
8	4	$NHCOC_6H_4SO_2F$-*m*	4.5	2.2	11	83	6, 30[h]	50, 80[i]
					2.2	50	8, 30[h]	40, 40[i]
9	4	$NHCONHC_6H_4SO_2F$-*m*	3.2	1.6	7	80	6, 30[h]	50, 82[i]
					1.6	50	8, 30[h]	37, 37[i]
10	4	*p*-$NHCOC_6H_4NHCOC_6H_4SO_2F$-*p*	600[j]	300				
11	4	$NHCOCH_2Br$	5.4	2.7	14	84	60, 120[h]	18, 25[k]

[a] The technical assistance of Maureen Baker and Susan Black with these assays is acknowledged. [b] I_{50} = concentration necessary for 50% inhibition when assayed with 50 μM DL-benzoylarginine *p*-nitroanilide at pH 7.4 Tris buffer in 10% DMSO as previously described. [c] Estimated as $0.5I_{50}$ since **4** has $I_{50} = 2K_i$. [d] Calculated from $[EI] = [E_t]/(1 + K_i/[I])$ where [EI] = amount of reversible complex expressed as a percentage of the total enzyme, $[E_t]$, [I] = inhibitor concentration, and K_i = enzyme–inhibitor dissociation constant.[6] [e] Per cent inactivation of the enzyme when incubated at 37° in pH 7.4 Tris buffer containing 10% DMSO; see Experimental Section. [f] Data from ref 3. [g] Determined by a Dixon plot of $1/V$ *vs.* I with 50 and 75 μM substrate. [h] From six-point time study; see Experimental Section. [i] Near maximum inactivation and the plot of log $[E_t]$ *vs.* time was curved. [j] Estimated from 35% inhibition at 300 μM. [k] Half-life estimated at 300 min.

compounds of type **1** and **2** were 2–4-fold better inhibitors of trypsin than the parent benzamidine, thus meeting the bulk-tolerance requirement in b above. The placement of suitable electrophilic groups on the terminal phenoxy moiety of **1** that have given some new types of active-site-directed irreversible inhibitors of trypsin is the subject of this paper.

Enzyme Results.—*p*-(Phenoxypropyloxy)benzamidine (**3**)[3] with a K_i near 7 μM (Table I) was first selected for conversion to candidate irreversible inhibitors. Introduction of the *p*-bromoacetamido moiety gave a compound (**6**) that was reversibly complexed to trypsin slightly better than the parent **3**. When trypsin was incubated at 37° with 17 μM **6**, which is sufficient to convert 79% of the enzyme to the rate-determining species [EI],[6] no irreversible inhibition occurred in 60 min.

The discovery of the terminal sulfonyl fluoride group for inactivation of dihydrofolic reductase by suitably substituted 4,6-diamino-1,2-dihydro-*s*-triazines[7] suggested that the terminal sulfonyl fluoride group be attached to *p*-(phenoxypropyloxy)benzamidine (**3**). The first compound of this series to be investigated was the *p*-fluorosulfonylbenzamido derivative (**4**) of **3**, **4** being about a twofold better reversible inhibitor than **3**. At a concentration of **4** (25 μM) sufficient to complex 88% of the trypsin, the enzyme was rapidly inactivated; 50% inactivation occurred in 3 min and total inactivation in 15 min. That a reversible complex between **4** and trypsin was an obligatory intermediate to inactivation of the enzyme was shown by the effect of **12**: at a concentration of 25 μM, **12** showed no inactivation of trypsin in 15 min, whereas a simultaneously run incubation with the same concentration of **4** for the same time showed 100% irreversible inhibition. If **4** had inactivated trypsin by a bimolecular reaction, then **12** should have inactivated the enzyme at as fast a rate or faster;[6] thus **4** inactivates trypsin by initial reversible complex formation with trypsin, the so-called active-site-directed mechanism.[4]

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If the inactivation of an enzyme occurs by the active-site-directed mechanism, then one of the requirements is that the rate of inactivation be dependent upon the amount of [EI] reversible complex, *i.e.*, when the concentration of inhibitor is sufficient to complex all of the enzyme, a further increase in inhibitor concentration will not increase the rate, a "rate-saturation" effect.[6] Furthermore, when the log of remaining enzyme is plotted against time, the line is linear through 80–90% reaction due to "pseudo"-first-order kinetics.[6] Thus, if **4** inactivates trypsin by the active-site-directed mechanism, then reducing the inhibitor concentration from 25 to 3.1 μM should reduce the rate only by a factor of 50/88, the ratio of the respective [EI] concentrations. Actually the sulfonyl fluoride (**4**) at 3.1 μM gave a rate plot that showed immediate curvature and the inactivation stopped at 44% after 15 min. This rate curvature at a K_i concentration of irreversible inhibitor was previously seen in studies with the sulfonyl flouride type of inhibitor on inactivation of di-

(6) See ref 4, pp 122–129, for a discussion of the kinetics of irreversible inhibition.

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

hydrofolic reductase[7] and chymotrypsin.[2] In the latter case, this line curvature was proven to be due to a combination of inactivation of the enzyme and enzyme-catalyzed hydrolysis of the sulfonyl fluoride by kinetic observations and by isolation of the corresponding sulfonic acid. Similar kinetics have now been observed with **4** and trypsin.

That the sulfonyl fluoride (**4**) was not hydrolyzed by buffer was shown by preincubation of **4** at 37° in the absence of enzyme for 1 hr; when enzyme was added, the rate curve for inactivation was the same as a standard without preincubation.

The effect of pH on the relative rate of enzyme-catalyzed hydrolysis of the sulfonyl fluoride and to the rate of inactivation of the enzyme by the sulfonyl fluoride was then determined.

(a) At pH 8.4, 3.1 μM of **4** rapidly inactivated the enzyme; 50% inactivation occurred in 1 min and 87% in 15 min. This contrasts with pH 7.4 where 44% inactivation occurred in 15 min, then no further inactivation occurred. These results indicate that the inactivation is more rapid at pH 8.4 and the ratio of inactivation to hydrolysis is also increased.

(b) At pH 6.5, 3.1 μM of **4** inactivated the enzyme more slowly than at pH 7.4; 30% inactivation occurred in 30 min and 48% in 90 min. Since inactivation was still proceeding at 60 min, the sulfonyl fluoride was still present, in contrast to pH 7.4, where all of the sulfonyl fluoride was hydrolyzed in 15 min. Thus, both inactivation of the enzyme and hydrolysis of the sulfonyl fluoride are slower than at pH 7.4.

(c) At pH 5.0, no inactivation of the enzyme was detectable in 2 hr. Whether this lack of inactivation was due to failure of irreversible inhibition to occur or that enzyme-catalyzed hydrolysis was extremely rapid cannot be separated by the current methodology; the latter is unlikely in view of the slower irreversible inhibition at pH 6.5 than pH 7.4. These pH profile results indicate that both the rate of inactivation of the enzyme and the rate of enzyme-catalyzed hydrolysis are dependent upon the OH^- concentration as previously observed with chymotrypsin;[2] whether this increasing OH^- concentration effects these reactions directly or effects ionization of enzymic groups cannot be stated.

When the inactivated enzyme was treated with 10 mM mercaptoethanol at pH 7.4 no detectable regeneration of activity occurred in 10 min; whether or not other thiols such as thioacetate,[8] glutathione, mercaptoethylamine,[9] or thioglycollic acid[10] could regenerate the activity by nucleophilic displacement of the covalently bound inhibitor deserves investigation, particularly in alkaline solution[10] or 8 M urea.[9]

Additional analogs of **4** were then synthesized for investigation (Table I). When the sulfonyl fluoride of **4** was moved to the *meta* position, the resultant **5** was about a twofold better reversible inhibitor; furthermore, **5** gave kinetics of irreversible inhibition similar to **4** when compared at similar [EI] concentrations.

Five analogs (**7-11**) were then synthesized where the methylene bridge was increased from three to four carbons. The *p*-bromoacetamide (**11**) at 14 μM, which gives 84% EI complex, showed slow inactivation of trypsin with a half-life of about 5 hr; this result contrasts with the lower homolog (**6**) where no inactivation was seen at 17 μM (79% EI complex). Three of the sulfonyl fluorides (**7-9**) in this series were both good reversible and irreversible inhibitors of trypsin with kinetic parameters similar to **4** and **5**. However, when the chain of **7** was further extended by insertion of a *p*-aminobenzoyl residue, the resultant **10** was a 150-fold poorer reversible inhibitor than **7**; this result indicates that the coplanar $C_6H_4NHCOC_6H_4NHCOC_6H_4$ system of **10** cannot be tolerated within the enzyme-inhibitor complex.

With the varying dimensions from the benzamidine moiety that complexes in the active site to the sulfonyl fluoride that attacks the enzyme, it is highly unlikely that the identical amino acid on trypsin is attacked by all of the sulfonyl fluorides in Table I. However, it is likely that a serine or threonine is attacked in each case;[2,9] to prove such an attack, a study of the displacement of the sulfonate with mercaptoethylamine[9] or other mercaptans,[10] followed by hydrolysis and identification of the S-substituted cysteine or S-substituted β-mercapto-α-aminobutyric acid, would be worthwhile.

Another problem results from the current study; can the ratio of enzyme inactivation by a sulfonyl fluoride to enzyme-catalyzed hydrolysis of this sulfonyl fluoride be changed by substitution on the phenylsulfonyl moiety? Such an approach on sulfonyl fluoride irreversible inhibitors of several enzymes is being pursued with some successes already being achieved with dihydrofolic reductase.[11]

The question of whether or not compounds of the type in Table I will or will not irreversibly inhibit some of the serum "tryptic" enzymes[3] is also being pursued; if one of these sulfonyl fluorides inactivates two or more of these "tryptic" enzymes, can the irreversible inhibition be made selective by the bridge principle of specificity?[12] The bridge principle of specificity is dependent upon covalent bond formation between the inhibitor and a nucleophilic group on the enzyme that is outside of the active site. Unfortunately, the size of the active site with a proteolytic enzyme using a protein as a substrate is difficult to ascertain since it is not yet known how many monomeric units of the substrate are in beneficial contact with the enzyme; therefore, it is also difficult to guess whether or not the sulfonyl fluorides in Table I attack exo or endo with respect to the active site. However, it would not be too unreasonable to expect the active sites of proteolytic enzymes to differ at a distance from the catalytic part of the active site, particularly where the second or third amino acid from the bond cleavage may be held; if such differences that are still part of the active site do indeed exist, they should be detectable by appropriate irreversible inhibitors such as those in Table I.

Chemistry.—Two routes to the key intermediates, *p*-(*p*-nitrophenoxyalkoxy)benzamidines (**16**), were investigated which differed only in the order of reactions starting with *p*-cyanophenol (**13**) (see Scheme I). Alkylation of **13** with 3-bromopropyl *p*-nitrophenyl ether in DMF in the presence of K_2CO_3 at 70° afforded

(8) (a) K. E. Neet and D. E. Koshland, Jr., *Proc. Natl. Acad. Sci. U. S.*, **56**, 1606 (1966); (b) L. Polgar and M. L. Bender, *J. Am. Chem. Soc.*, **88**, 3153 (1966).

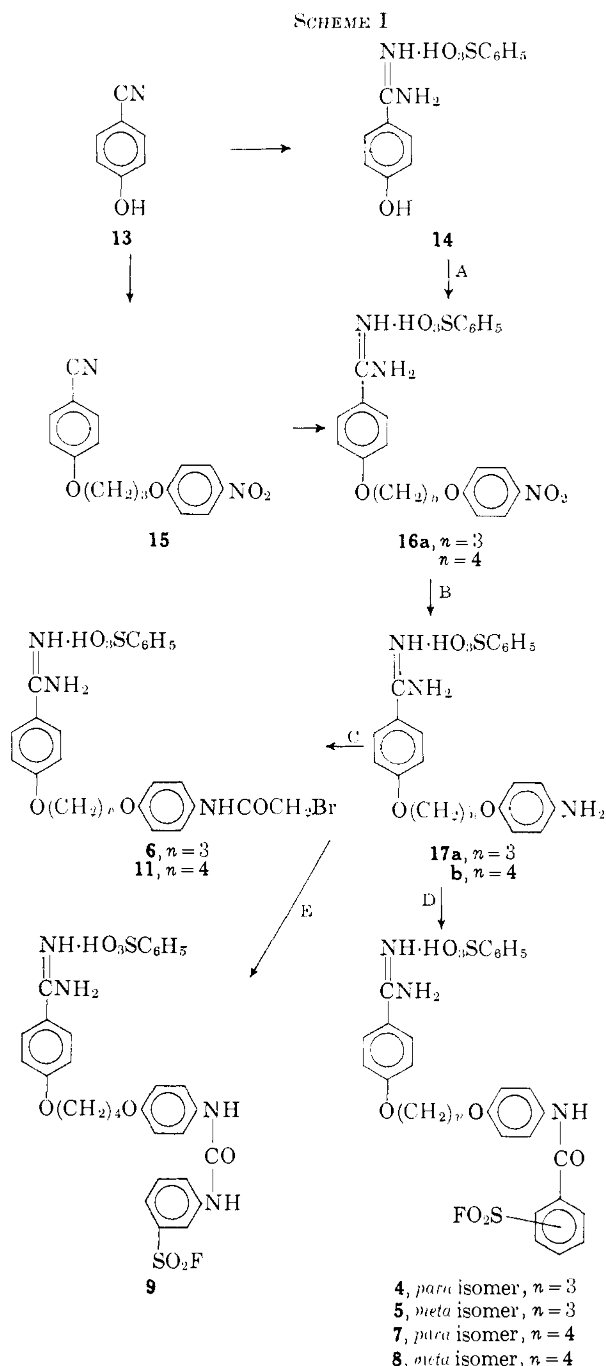
(9) A. M. Gold and D. Fahrney, *Biochemistry*, **3**, 783 (1964).

(10) D. H. Strumeyer, W. N. White, and D. E. Koshland, Jr., *Proc. Natl. Acad. Sci. U. S.*, **50**, 931 (1963).

(11) B. R. Baker and G. J. Lourens, unpublished.

(12) See ref 4, pp 172-190, for a discussion of the bridge principle of specificity.

Scheme I



15 in 66% yield. The cyano group of **15** was converted to the amidine (**16a**) *via* its imino ether hydrochloride in 43% yield for the two steps. Alternately, *p*-cyanophenol (**13**) was converted to *p*-hydroxybenzamidine (**14**) in 47% yield by the imino ether route,[13] which was found superior to the method of Partridge and Short[14] involving fusion of **13** with NH_4SCN. Alkylation of **14** with the appropriate bromide in DMF–K_2CO_3 afforded **16a** in 66% yield that was identical with **16a** prepared *via* **15**; thus alkylation of **14** occurred primarily on the phenolic oxygen and not the amidine. The alkylation of **14** in EtOH was more complex; a mixture of the desired product (**16a**), some **15a**, and an unidentified product that did not move on tlc was obtained.

(13) A. P. T. Easson and F. L. Pyman, *J. Chem. Soc.*, 2991 (1931).
(14) M. W. Partridge and W. F. Short, *ibid.*, 390 (1947).

The method *via* *p*-hydroxybenzamidine (**14**) is preferable since the position of the nitro group and the chain length (n) of **16** is readily varied from one common intermediate; for example, the higher homolog (**16b**) was prepared from **14** in 39% yield.

Hydrogenation of the nitro group of **16** with a Pd–C catalyst proceeded smoothly to **17**; a PtO_2 catalyst gave a mixture of products. Reaction of **17** with bromoacetic anhydride[15] in DMF afforded the bromoacetamides **6** and **11**. Treatment of **17b** with *m*-fluorosulfonylphenyl isocyanate in DMF gave the irreversible inhibitor **9** in 50% yield; similarly, reaction of **17a** and **b** with *m*- or *p*-fluorosulfonylbenzoyl chloride in DMF–Et_3N afforded the irreversible inhibitors **4**, **5**, **7**, and **8** in about 20% yields of analytically pure material.

Experimental Section[16]

p-**Hydroxybenzamidine Benzenesulfonate (14).**—A solution of 24 g (0.2 mole) of **13** and 9.20 g (0.2 mole) of absolute EtOH in 75 ml of $CHCl_3$ cooled in an ice bath was treated with a slow stream of HCl gas for 90 min, during which time the imino ether hydrochloride began to separate. After standing for about 18 hr at ambient temperature, the almost solid cake was thinned with 50 ml of $CHCl_3$. The stirred mixture was cooled in an ice bath, then a solution of 14.5 g of NH_3 in 200 ml of absolute EtOH was added dropwise over a period of 60 min. After being stirred at ambient temperature for about 18 hr, the mixture was filtered to remove NH_4Cl. The combined filtrate and washings were evaporated to about 30 ml *in vacuo*, then a solution of 36 g (0.21 mole) of $C_6H_5SO_3H \cdot H_2O$ in 25 ml of *i*-PrOH was added. The solution was evaporated *in vacuo* and the residue was triturated with $CHCl_3$. The product (24.3 g) was collected on a filter, but tlc showed the presence of **13** which was removed by extraction with hot Me_2CO; yield 20.1 g (34%), mp 180–181°, that was suitable for further transformation (lit.[14] mp 187°). From the chloroform solution was isolated 6.7 g (28%) of cyanophenol; the overall yield of **14** based on unrecovered **13** was 47%.

1-(*p*-Cyanophenoxy)-3-(*p*-nitrophenoxy)propane (15).—A mixture of 6.00 g (50 mmoles) of **13**, 15.0 g (58 mmoles) of 3-bromopropyl *p*-nitrophenyl ether,[17] 7.00 g (50 mmoles) of K_2CO_3, and 50 ml of DMF was stirred in a bath at 70° for 4 hr, then diluted with several volumes of H_2O. The product was collected on a filter and recrystallized from MeOEtOH–*i*-PrOH; yield 9.85 g (66%), mp 140–144°, that was suitable for further transformations. Two recrystallizations from the same solvent pair gave white crystals, mp 143–145°. *Anal.* ($C_{16}H_{14}N_2O_4$) C, H, N.

p-(*p*-**Nitrophenoxypropoxy)benzamidine Benzenesulfonate (16a). Method A.**—A mixture of 2.94 g (10 mmoles) of **14**, 3.00 g (10 mmoles) of 3-bromopropyl *p*-nitrophenyl ether,[17] 1.40 g (10 mmoles) of K_2CO_3, and 25 ml of DMF was stirred in a bath at 70° for 6 hr. The cooled mixture was filtered, the filtrate was spin evaporated *in vacuo* to about 5 ml, then a solution of 1.76 g (10 mmoles) of $C_6H_5SO_3H \cdot H_2O$ in 20 ml of H_2O was added. The product was collected on a filter and washed with hot acetone; yield 3.10 g (66%), mp 184–189°, that was suitable for further transformation. An analytical sample, mp 187–192°, was obtained after two recrystallizations from EtOH; see Table II for additional data.

This compound was also prepared in 43% yield, mp 186–194°, from **15** by the method described for **14**, except the reaction residue was treated with $C_6H_5SO_3H$ in H_2O.

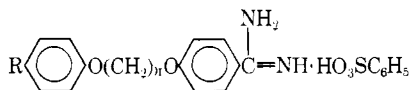
p-(*p*-**Aminophenoxybutoxy)benzamidine Benzenesulfonate (17b). Method B.**—A solution of 1.50 g (3.1 mmoles) of **16b** in

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

(16) All analytical samples had ir spectra in agreement with their assigned structures and gave combustion values within 0.4 of the theoretical percentage; each moved as a single spot on Brinkmann silica gel GF with EtOAc–petroleum ether (bp 60–110°) (1:3) or BuOH–HOAc–H_2O (10:5:1) when viewed under uv light. Melting points were determined in capillary tubes on a Mel-Temp block and those below 230° 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.

(17) P. G. Gagnon, G. Nadeau, and R. Coté, *Can. J. Chem.*, **30**, 592 (1952).

TABLE II
PHYSICAL PROPERTIES OF



No.	n	R	Method	% yield	Mp, °C	Formula	Analyses
4	3	$NHCOC_6H_4SO_2F$-*p*	D	25[a]	268–270	$C_{29}H_{28}FN_3O_8S_2$	C, H, N
5	3	$NHCOC_6H_4SO_2F$-*m*	D	15[b]	>165 dec	$C_{29}H_{28}FN_3O_8S_2$	C, H, N
6	3	$NHCOCH_2Br$	C	60[c]	155–167	$C_{24}H_{26}BrN_3O_6S$	C, H, N
7	4	$NHCOC_6H_4SO_2F$-*p*	D	18[a]	280–283	$C_{30}H_{30}FN_3O_8S_2$	C, H, N
8	4	$NHCOC_6H_4SO_2F$-*m*	D	20[d]	210–213	$C_{30}H_{30}FN_3O_8S_2$	C, H, N
9	4	$NHCONHC_6H_4SO_2F$-*m*	E	50[e]	230–233	$C_{30}H_{31}FN_4O_8S_2$	C, H, N
10	4	*p*-$NHCOC_6H_4NHCOC_6H_4SO_2F$-*p*	D	15[f]	294–296	$C_{37}H_{35}FN_4O_9S_2$	C, H, N
11	4	$NHCOCH_2Br$	C	34[g]	198–200	$C_{25}H_{28}BrN_3O_6S$	C, H, N
16a	3	NO_2	A	66	187–192[a]	$C_{22}H_{23}N_3O_7S$	C, H, N
16b	4	NO_2	A	39	225–228[a]	$C_{23}H_{25}N_3O_7S$	C, H, N
17a	3	NH_2	B	99	173–174	$C_{22}H_{25}N_3O_5S$	C, H, N
17b	4	NH_2	B	80	197–200[g]	$C_{23}H_{27}N_3O_5S$	C, H, N
18	4	$NHCOC_6H_4NO_2$-*p*	D	30[h]	275–284	$C_{30}H_{30}N_4O_8S$	C, H, N
19	4	$NHCOC_6H_4NH_2$-*p*	B	70	209–212	$C_{30}H_{32}N_4O_6S\cdot 0.5H_2O$	C, H, N

[a] Recrystallized from MeOH. [b] Recrystallized from *i*-PrOH–Et_2O. [c] Recrystallized from EtOH–Et_2O. [d] Recrystallized from PrOH. [e] Recrystallized from EtOH–H_2O. [f] Recrystallized from DMF–EtOH. [g] Recrystallized from EtOH. [h] Recrystallized from MeOEtOH–H_2O.

100 ml of 9:1 MeOEtOH–H_2O was shaken with H_2 at 2-3 atm in the presence of 250 mg of 5% Pd–C for about 90 min when reduction was complete. The filtered solution was evaporated *in vacuo*. Recrystallization from EtOH gave 0.82 g (58%) of product, mp 197–200°; an additional 0.30 g (total 80%) was isolated from the filtrate. See Table II for additional data.

***p*-(*p*-Bromoacetamidophenoxybutoxy)benzamidine Benzenesulfonate** (11). **Method C.**—To a solution of 288 mg (0.5 mmole) of **17b** in 1 ml of DMF stirred in an ice bath was added a solution of 192 mg (0.75 mmole) of bromoacetic anhydride in 2 ml of DMF.[15] The ice bath was removed and after 1 hr the solution was diluted with several volumes of Et_2O. The solvent was decanted from the oil and the latter was crystallized by addition of EtOH–Et_2O. Recrystallization from EtOH containing 100 mg of $C_6H_5SO_3\cdot H_2O$ and a second recrystallization from EtOH gave 100 mg (34%) of white crystals, mp 198–200°, that gave a positive 4-(*p*-nitrobenzyl)pyridine test for activated halogen.[15] See Table II for additional data.

***p*-[*p*-(*p*-Fluorsulfonylbenzamido)phenoxybutoxy]benzamidine Benzenesulfonate** (7). **Method D.**—To a stirred solution of 228 mg (0.05 mmole) of **17b** in 1 ml of DMF was added 0.75 ml of 1 *M* Et_3N in DMF. Then a solution of 150 mg (0.67 mmole) of *p*-fluorosulfonylbenzoyl chloride in DMF was added dropwise over a period of about 5 min, the flask being cooled with a 15–20° H_2O bath. After 90 min at ambient temperature, the solution was treated with 175 mg (1 mmole) of $C_6H_5SO_3H\cdot H_2O$ in 8 ml of H_2O. The mixture was warmed on a steam bath, then *i*-PrOH was added until a clear solution was obtained. The solution was kept at −15° for several hours. The gummy solid was recrystallized from MeOH; yield 60 mg (18%), mp 280–283°. See Table II for additional data.

Method E was the same with omission of Et_3N.

4-Fluorosulfonyl-4'-methoxybenzanilide (12).—To a stirred solution of 615 mg (5 mmoles) of *p*-anisidine and 0.51 g (5 mmoles) of Et_3N in 10 ml of $CHCl_3$ cooled in an ice bath was added dropwise a solution of 1.23 g (5.5 mmoles) of *p*-fluorosulfonylbenzoyl chloride in 10 ml of $CHCl_3$ over a period of 20 min. The product (mp 195–199°) was collected on a filter and washed with $CHCl_3$. Two recrystallizations from $CHCl_3$ gave 0.55 g (36%) of analytical sample as white crystals, mp 199–200°. *Anal.* ($C_{14}H_{12}FNO_4S$) C, H, N.

Enzyme Assays.—The reversible inhibition assays were performed with crystalline trypsin from bovine pancreas (Sigma Chemical Co.) and 50 μM N-benzoyl-DL-arginine *p*-nitroanilide (BANA)[18] in pH 7.4 Tris buffer containing 10% DMSO as previously described. The irreversible inhibition assays were performed as follows.

The velocity of the enzyme reaction with 50 μM BANA was observed to be proportional to the enzyme concentration. The amount of spontaneous enzyme inactivation in 10% DMSO at pH 7.4 was about 10%/hr; the inhibitor inactivations in Table I were corrected for this thermal inactivation of an enzyme control that was run simultaneously. The buffer employed was 0.05 *M* Tris (pH 7.4); additional buffers employed 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 0.9 mg/ml and stored at 0–5° BANA was stored as a 3.1 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 0.90 ml was withdrawn from one tube, labeled I_0, and placed in an ice bath; then 100 μl of a DMSO solution of inhibitor was added to the remainder in a 37° bath, which was labeled I_2, and the time was noted. To the second tube serving as a control was added 200 μl of DMSO, then 1.00 ml was withdrawn, placed in an ice bath, and labeled C_1. The tubes labeled I_2 and C_2 were then heated in the 37° bath for the specified time, then placed in an ice bath until ready for assay. The amount of remaining enzyme in each aliquot, except the I_0 tube, was assayed as follows.

In a 3-ml glass cuvette were placed 2.85 ml of pH 7.4 Tris buffer (regardless of the incubation pH) and 50 μl of 3.1 m*M* BANA. 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 spectrophotometer; the C_1 aliquot gave an optical density change of 0.007–0.012 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. Each aliquot was run in duplicate, or in triplicate if necessary. Just before assay, the still ice-cold I_0 aliquot was treated with 100 μl of the DMSO solution of inhibitor, then assayed immediately.

This procedure is satisfactory for a routine screen for a plus or minus answer. With a positive compound, the size of the inhibitor incubation is increased threefold, the I_0 aliquot is removed as before, then the remainder is aliquoted at appropriate times such as 2, 4, 8, 16, and 30 min.

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