method A at -5 to 0° except Et₈N 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 EtSO₃H 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 H₂ had been consumed, hydrogenation ceased. The mixture was re-treated with decolorizing carbon and filtered, and then 50 mg of fresh PtO2 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 CHCl₃. 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% N₂H₄·H₂O 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 H₂O. The phthalhydrazide contain

ing some 32 was again treated with N₂H₄ 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 Et₂O. The combined extracts were dried (MgSO₄), 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 Me₂CO was added 390 mg (1.5 mmoles) of bromoacetic anhydride.¹⁷ The mixture was stirred for 4 hr, then diluted (H₂O) if the product had not separated. The product was collected on a filter, then washed (Me₂CO-H₂O, 5% NaHCO₃, H₂O). 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 CHCl₃ was added 220 mg (1.1 mmoles) of *m*fluorosulfonylphenyl isocyanate in 15 ml of CHCl₃. After 20 min, the mixture was filtered and the product was washed with CHCl₃. 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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Received November 30, 1967

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 inhibitors⁴ 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-sitedirected 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



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

⁽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.



INHIBITION^a OF TRYPSIN BY



			Reversible		,			
N 1		T	150, ⁴	Estd K_1^e $\simeq 106M$	Conen,	$\mathbb{E} \cdot \cdot \cdot \mathbb{L}^d$	Time,	Inacin."
NO.	n	R	μ . μ	X 10.50	μ.,η	. (11111	1
3	3	II	1.5^{7}	7.5				
4	3	$\rm NHCOC_6H_4SO_2F-p$	6, 2	3.1''	25	88	$3, 15^{k}$	50,100
					7.5	71	$5, 30^{6}$	50, 88
					3.1	.50	$15, 30^{h}$	44, 44/
5	3	$\rm NHCOC_6H_4SO_2F$ -m	3.4	1.7	13	88	$4, 15^{h}$	50, 100
				1.2^{a}	6.5	80	$4, 12^{h}$	50,100
					1.7	50	$14, 30^{h}$	$54, 54^{i}$
6	3	$\rm NHCOCH_2Br$	9.2	4.6	17	79	60	0
7	4	$\rm NHCOC_6H_4SO_2F$ - p	4.1	2.0	40	95	$3,12^{k}$	50, 100
					10	83	$3, 30^{h}$	50, 70'
					2.0	50	$8, 30^{k}$	31, 31
8	4	NHCOC ₆ H ₄ SO ₂ F-m	4.5	2.2	11	83	$6, 30^{h}$	$50, 80^{\circ}$
					2.2	50	$8, 30^{h}$	40, 40'
9	4	$\rm NHCONHC_6H_4SO_2F$ -m	3.2	1.6	7	80	$6, 30^{b}$	$50, 82^{\circ}$
					1.6	50	$8, 30^{L}$	37, 37/
10	4	p-NHCOC ₆ H ₄ NHCOC ₆ H ₄ SO ₂ F- p	600 <i>i</i>	300				
11	4	NHCOCH ₂ Br	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_{30} = concentration necessary for 50% inhibition when assayed with 50 μ M pL-benzoylarginine *p*-nitroanilide at pH 7.4 Tris buffer in 10% DMSO as previously described. ^c Estimated as 0.51₅₀ since 4 has $I_{50} = 2K_1$. ^d Calculated from [EI] = $[E_4]/(1 + K_4/[1])$ where [EI] = amount of reversible complex expressed as a percentage of the total enzyme, [E₄], [I] = inhibitor concentration, and K_4 = enzyme-inhibitor dissociation constant.⁶ CPer centinactivation of the enzyme when incubated at 37° in pH 7.4 Tris buffer containing 10% DMSO; see Experimental Section. ^d Data from ref 3. ^d Determined by a Dixon plot of 1/V vs. I with 50 and 75 μ M substrate. ^b From six-point time study; see Experimental Section. ^d Near maximum inactivation and the plot of log [E₄] vs. time was curved. ^d Estimated from 35% inhibition at 300 μ M. ^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-triazines⁷ 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** ($25 \mu M$) 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

$$CH_{3}O\langle O \rangle NHCO\langle O \rangle SO_2F$$

If the inactivation of an enzyme occurs by the activesite-directed mechanism, then one of the requirements is that the rate of inactivation be dependent upon the amount of [E1] 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 [E1] concentrations. Actually the sulforyl 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.

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hydrofolic reductase⁷ and chymotrypsin.² In the latter case, this line curvature was proven to be due to a combination of inactivation of the enzyme and enzymecatalyzed 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 sulfort 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 enzymecatalyzed hydrolysis of the sulfonyl fluoride and to the rate of inactivation of the enzyme by the sulforvl 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 sulforyl 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 acid¹⁰ could regenerate the activity by nucleophilic displacement of the covalently bound inhibitor deserves investigation, particularly in alkaline solution¹⁰ 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 car-

(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).

bons. 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 paminobenzoyl 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 molety that complexes in the active site to the sulforyl 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 threenine is attacked in each case;^{2,9} to prove such an attack, a study of the displacement of the sulfonate with mercaptoethylamine⁹ 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 sulforvl 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" enzymes³ 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 sulforyl 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

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

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



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 Short¹⁴ involving fusion of 13 with NH₄SCN. Alkylation of 14 with the appropriate bromide in DMF-K₂CO₃ 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 the was obtained. 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 PtO₂ catalyst gave a mixture of products. Reaction of **17** with bromoacetic anhydride¹⁵ in DMF afforded the bromo-acetamides **6** and **11**. Treatment of **17b** with *m*-fluoro-sulfonylphenyl 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 = Et₃N afforded the irreversible inhibitors **4**, **5**, **7**, and **8** in about 20% yields of analytically pure material.

Experimental Section¹⁶

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 7.5 ml of CHCl₃ 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 CHCl₃. The stirred mixture was cooled in an ice bath, then a solution of 14.5 g of $\rm NH_3$ 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 NH₄Cl. The combined filtrate and washings were evaporated to about 30 ml in vacuo, then a solution of 36 g (0.21 mole) of C₆H₅SO₃H · H₂O in 25 ml of *i*-PrOH was added. The solution was evaporated in vacuo and the residue was triturated with CH-Cl₃. The product (24.3 g) was collected on a filter, but the showed the presence of 13 which was removed by extraction with hot Me₂CO; 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,⁴⁷ 7.00 g (50 mmoles) of K₂CO₃, and 50 ml of DMF was stirred in a bath at 70° for 4 hr, then diluted with several volumes of H₂O. 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. (C₁₆H₁₄N₂O₄) 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 K₂CO₃, 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 C₆H₅SO₈H·H₂O in 20 ml of H₂O 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 C₆H₃SO₃H in H₂O.

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

(1952),

⁽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).

⁽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 it 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-peroleum ether (bp 60-110⁹) (1:3) or BuOH-HOAc-H2O (10:5:1) when viewed under uv light. Melting points were determined in capillary tubes on a Mel-Temp block and those below 230⁹ are corrected. Where analyses are indicated only by symbols of the elements analytical results obtained for those elements were within $\pm 0.4\%$ of the theoretical values. (17) P. G. Gagnon, G. Nadeau, and R. Coté, Can. J. Chem., **30**, 592

X 1 T T

TABLE I

PHYSICAL PROPERTIES OF

				-1									
$R(\bigcirc)O(CH_2)_{20}O(\bigcirc)C=NH\cdot HO_2SC_2H_5$													
					- 110 3~ 065								
				%	Mp,								
No.	n	R	Method	yield	°C	Formula	Analyses						
4	3	$\mathrm{NHCOC}_{6}\mathrm{H}_{4}\mathrm{SO}_{2}\mathrm{F}$ - p	D	25^a	268 - 270	$C_{29}H_{28}FN_3O_8S_2$	С, Н, N						
5	3	$\mathrm{NHCOC_6H_4SO_2F}$ -m	D	15^{b}	$>165~{ m dec}$	$C_{29}H_{28}FN_3O_8S_2$	С, Н, N						
6	3	$\rm NHCOCH_2Br$	\mathbf{C}	60^{c}	155 - 167	$\mathrm{C}_{24}\mathrm{H}_{26}\mathrm{BrN}_{3}\mathrm{O}_{6}\mathrm{S}$	С, Н, N						
7	4	$\mathrm{N}\mathrm{H}\mathrm{C}\mathrm{O}\mathrm{C}_{6}\mathrm{H}_{4}\mathrm{S}\mathrm{O}_{2}\mathrm{F}$ - p	D	18^a	280 - 283	${ m C}_{30}{ m H}_{30}{ m FN}_{3}{ m O}_{8}{ m S}_{2}$	С, Н, Х						
8	4	$\mathrm{N}\mathrm{HCOC_{6}H_{4}SO_{2}F}$ -m	D	20^d	210 - 213	$\mathrm{C}_{30}\mathrm{H}_{30}\mathrm{FN}_{3}\mathrm{O}_8\mathrm{S}_2$	С, Н, N						
9	4	$\rm NHCONHC_6H_4SO_2F$ -m	\mathbf{E}	50^{e}	230 - 233	$\mathrm{C}_{30}\mathrm{H}_{31}\mathrm{FN}_{4}\mathrm{O}_8\mathrm{S}_2$	С, Н, N						
10	4	p-NHCOC ₆ H ₄ NHCOC ₆ H ₄ SO ₂ F- p	D	15^{f}	294 - 296	$C_{37}H_{35}FN_4O_9S_2$	С, Н, N						
11	4	$\rm NHCOCH_2Br$	\mathbf{C}	34^{g}	198 - 200	$\mathrm{C}_{25}\mathrm{H}_{28}\mathrm{BrN}_{3}\mathrm{O}_{6}\mathrm{S}$	С, Н, N						
16a	3	${ m NO}_2$	Α	66	$187 - 192^{g}$	$\mathrm{C}_{22}\mathrm{H}_{23}\mathrm{N}_{3}\mathrm{O}_{7}\mathrm{S}$	С, Н, N						
16b	4	NO_2	A	39	$225 - 228^{a}$	$\mathrm{C}_{23}\mathrm{H}_{25}\mathrm{N}_{3}\mathrm{O}_{7}\mathrm{S}$	C, H, N						
17a	3	$\rm NH_2$	В	99	173 - 174	${ m C}_{22}{ m H}_{25}{ m N}_{3}{ m O}_{5}{ m S}$	С, Н, N						
17b	4	$ m NH_2$	в	80	$197 - 200^{g}$	$C_{28}H_{27}N_{3}O_{5}S$	С, Н, N						
18	4	$\mathrm{N}\mathrm{H}\mathrm{C}\mathrm{O}\mathrm{C}_{6}\mathrm{H}_{4}\mathrm{N}\mathrm{O}_{2}\text{-}p$	D	30^{h}	275 - 284	$C_{30}H_{30}N_4O_8S$	С, Н, N						
19	4	$\mathrm{N}\mathrm{H}\mathrm{C}\mathrm{O}\mathrm{C}_{6}\mathrm{H}_{4}\mathrm{N}\mathrm{H}_{2}$ - p	В	70	209 - 212	$C_{30}H_{32}N_4O_6S\cdot 0.5H_2O$	С, Н, 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-H₂O was shaken with H₂ 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 Et₂O. The solvent was decanted from the oil and the latter was crystallized by addition of EtOH-Et₂O. Recrystallization from EtOH containing 100 mg of C₄H₅SO₃·H₂O 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

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 Et₂N 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° H₂O bath. After 90 min at ambient temperature, the solution was treated with 175 mg (1 mmole) of C₆H₃SO₃H · H₂O in 8 ml of H₂O. 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 Et₃N in 10 ml of CHCl₃ 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 CHCl₃ over a period of 20 min. The product (mp 195-199°) was collected on a filter and washed with CHCl₃. Two recrystallizations from CHCl₃ gave 0.55 g (36%) of analytical sample as white crystals, mp 199-200°. *Anal.* (C1₁₄H₁₂FNO₄S) 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 mM HCl at 0.9 mg/ml and stored at 0-5° BANA was stored as a 3.1 mM 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 I₀, and placed in an ice bath; then 100 μ l of a DMSO solution of inhibitor was added to the remainder in a 37° bath, which was labeled I₂, and the time was noted. To the second tube serving as a control was added 200 μ l of DMSO, then 1.00 ml was withdrawn, placed in an ice bath, and labeled C₁. The tubes labeled I₂ and C₂ 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 I₀ 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 μ l of 3.1 mM BANA. The enzyme reaction was then started by addition of 200 μ l of C₁ or other aliquot. The increase in optical density at 410 m μ was followed with a Gilford 2000 spectrophotometer; the C₁ 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 I₀ aliquot was treated with 100 μ l 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).