Irreversible Enzyme Inhibitors. CVI.[1] Proteolytic Enzymes. I. Bulk Tolerances in Trypsin–Inhibitor Complexes[2]

B. R. BAKER AND EDWARD H. ERICKSON

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

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Phenylguanidine is known to be a good inhibitor of trypsin, and benzamidine is an excellent inhibitor. Substitution of an alkyl, aryl, or aralkyl group on one of the nitrogens led to a large loss in binding, indicating that there was not bulk tolerance by the enzyme in this area of the inhibitor. Substitution of phenoxyalkloxy groups on either the *meta* or *para* position of benzamidine gave a slight increase in binding, thus showing that these groups are tolerated within the inhibitor–enzyme complex. In contrast, substitution of phenoxyalkyloxy groups on the *para* position of phenylguanidine led to a loss in binding, but substitution on the *meta* position did show bulk tolerance and no loss in binding. Thus further substitution of leaving groups on the terminal phenyl group of *m*-phenoxyalkyloxyphenylguanidine or *m*- or *p*-phenoxyalkyloxybenzamidine should make good candidates for *exo*-type active-site-directed irreversible inhibitors of "tryptic" enzymes.

At least fifteen distinct proteolytic enzymes from mammalian sources have been characterized.[3] These can be divided into three classes: (a) digestive-type enzymes such as trypsin, chymotrypsin, and pepsin;[3] (b) blood serum proteins such as plasmin and thrombin involved in blood clotting,[4,5] C'1a enzyme of the complement system involved in rejection of foreign mammalian cells,[6] and the enzyme involved in antigen-induced histamine release;[6] and (c) others such as insulinase that are not easily classified. The detailed enzyme systems for blood clotting, clot dissolving, complement, and histamine release are not yet completely characterized; these systems are complicated by zymogen forms of the enzymes as well as by activators and inhibitors in the serum. Nevertheless, sufficient information is known about these serum enzyme systems to begin studies on their inhibition for such important problem areas as cardiovascular diseases and organ transplantation.

Since all of these proteinases hydrolyze peptide linkages, they are closely related mechanistically;[7] the relative specificity of the enzymes resides mainly in the type of acylated amino acid amide preferred for complexing.[3] For example, chymotrypsin prefers to hydrolyze the carboxamide end of peptides derived from phenylalanine, tryptophan, and tyrosine; in contrast, trypsin prefers to hydrolyze the carboxamide end of peptides derived from lysine and arginine. Yet the C'1a complement enzyme shows little preference for tyrosine derivatives over arginine derivatives[8] and thus can be classified as both "tryptic" and "chymotryptic."

To design a specific inhibitor for a single one of these enzymes with no effect on the others is not a simple problem. Classical substrate analogs might be sufficiently specific to inhibit only tryptic or chrmotryptic enzymes, but there are several enzymes in each of these two classes. For example, *trans*-4-aminomethylcyclohexanecarboxylic acid, an analog of lysine, is a potent inhibitor of fibrinolysis,[9] but it most probably will inhibit one or more other tryptic enzymes.

Active-site-directed irreversible enzyme inhibitors[10,11] can be divided into two classes: (a) those operating by the endo mechanism, that is, the inhibitor becomes covalently linked to the enzyme within the active site; and (b) those operating by the exo mechanism, that is, the inhibitor becomes covalently linked outside the active site.[12] The irreversible inhibitors operating by the endo mechanism do not have sufficient specificity since the catalytic part of the active sites of proteinases are so mechanistically similar.[13]

For example, diisopropyl fluorophosphate (DFP) is an endo-operating active-site-directed irreversible inhibitor[14] that can attack esterases[15,16] such as aliesterase and acetylcholinesterase, as well as proteinases[15,17] such as trypsin, chymotrypsin, thrombin, plasmin, C'1a complement,[6] and antigen-induced histamine release.[18] Similarly a series of O-*p*-nitrophenyl O-ethyl phosphonates showed little specificity as endo-operating active-site-directed irreversible inhibitors of C'1a complement, antigen-induced histamine release, trypsin, chymotrypsin, and acetylcholinesterase; the effectiveness on these enzymes did vary with the R group of the phosphonate to give characteristic inhibition profiles, but all of the compounds inhibited each enzyme to some degree.

Chloromethyl ketones derived from appropriate N-tosylamino acids are sufficiently specific to attack within the active site of tryptic, but not chymotryptic, enzymes and *vice versa;* again, there are several enzymes within each class. For example, 7-amino-1-chloro-3-

(1) For the previous paper of this series see B. R. Baker and G. J. Lourens, *J. Med. Chem.*, **10**, 1113 (1967).

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

(3) M. Dixon and E. C. Webb, "Enzymes," Academic Press Inc., New York, N. Y., 1958, pp 185–188, 247–266.

(4) W. H. Seegers, "Blood Clotting Enzymology," Academic Press Inc., New York, N. Y., 1967.

(5) (a) C.-B. Laurell, *Blood*, **7**, 555 (1952); (b) J. H. Milstone, *Medicine*, **31**, 411 (1952).

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

(7) T. C. Bruice and S. J. Benkovic, "Bioorganic Mechanisms," Vol. 1, W. A. Benjamin, Inc., New York, N. Y., 1966, p 212.

(8) A. L. Haines and I. R. LePow, *J. Immunol.*, **92**, 456 (1964).

(9) S. Okamoto and V. Okamoto, *Keio J. Med.*, **11** (3), 105 (1962).

(10) 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.

(11) B. R. Baker, *J. Pharm. Sci.*, **53**, 347 (1964), a review.

(12) For this discussion, the active site is defined to include the region necessary for complexing the substrate and the region catalyzing the conversion of substrate to product.

(13) B. S. Hartley in "Structure and Activity of Enzymes," T. W. Goodwin, J. I. Harris, and B. S. Hartley, Ed., Academic Press Inc., New York, N. Y., 1964, pp 57–59.

(14) A. R. Main, *Science*, **144**, 992 (1964).

(15) Reference 3, pp 381–386, 490, 498.

(16) D. F. Heath, "Organophosphorus Poisons," Permagon Press, Ltd., Oxford, 1961.

(17) B. S. Hartley, *Ann. Rev. Biochem.*, **29**, 45 (1960).

(18) K. F. Austen and W. E. Brocklehurst, *J. Exptl. Med.*, **113**, 521 (1961).

tosylamido-2-heptanone (TLCK), a lysine analog, not only attacks the active site of trypsin[19] by the endo active-site-directed mechanism, but also could inactivate papain and thrombin.[19] Similarly, the phenylalanine analog, 1-chloro-4-phenyl-3-tosylamido-2-butanone (TPCK),[20] not only inactivates chymotrypsin, but attacks papain.[19] It is not unlikely[21] that TLCK would also inactivate other tryptic enzymes such as plasmin, kallikrein, ficin, and C1'a complement enzyme; similarly, TPCK would also probably inactivate C1'a complement enzyme and the antigen-induced histamine-releasing enzyme.

In order to design a highly specific enzyme inhibitor that will inhibit only one of these many proteinases in a mammal, it is essential to consider the following two parameters.

(a) Complexing of the inhibitor with the active site will only separate out a single class of enzymes. For example, proteinases could be readily inhibited without effect on dehydrogenases; somewhat more specifically the effect on tryptic enzymes could be separated from effect on chymotryptic enzymes.

(b) In order to separate the effect further within a class of enzymes that are inhibited, differences in enzyme structure outside the active site should be utilized; two ways are known at present to accomplish this.[10] If a hydrophobic bonding region can be found adjacent to the active site, its combination with analog complexing within the active site can lead to specificity sufficient to show high isozyme specificity.[22] The second way utilizes the exo type of active-site-directed irreversible enzyme inhibitor that covalently links the enzyme outside the active site;[10,11,23] an extra dimension of specificity known as the bridge principle of specificity[10,11,23-25] resides in this exo type of irreversible inhibitor that is not present with reversible inhibitors or endo-operating irreversible inhibitors. Still higher isozyme specificity can be obtained when both hydrophobic bonding and exo bond formation outside the active site are combined in one properly constructed irreversible inhibitor.[1,26,27]

von Kaulla[28] has found that salicylic acids bearing hydrocarbon groups such as a 4-(*p*-isopropylbenzyl) or chlorobenzyloxy are good fibrinolytic agents that apparently operate by activation of the fibrinolytic system. Further modification of the hydrocarbon groups with particular consideration of ground-state *vs.* binding conformation[26,29] and the conformational requirements of the hydrophobic bonding region,[26] could well lead to a useful drug; this type of molecule most probably utilizes a hydrophobic bonding region not completely part of the active site.

The major objective of this laboratory with active-site-directed irreversible enzyme inhibitors has been selective inhibition of nucleic acid precursors for chemotherapy of cancer and infectious diseases.[10] The same concepts should be applicable to specific inhibition of serum proteinases. For initial attack on this problem, two common proteinases, trypsin and chymotrypsin, were selected for study since no irreversible inhibitors operating by the exo mechanism are known for these enzymes. The first stage in the design of such exo-irreversible inhibitors of trypsin is the subject of this paper; the first stage with chymotrypsin is the subject of the paper that follows.[30]

Table I

Inhibition of Trypsin by Aralkylamines and -guanidines

No.	Compd	I_{50}, [a] m*M*	Source
1	*n*-$C_4H_9NH_2$	1.5	Eastman
2	$C_6H_5CH_2NH_2$	0.38	Aldrich
3	$C_6H_5(CH_2)_2NH_2$	5.1	Eastman
4	$C_6H_5(CH_2)_3NH_2$	30[b]	Aldrich
5	$C_6H_5(CH_2)_4NH_2$	10	Aldrich
6	$C_6H_5NHC(NH_2)=NH\cdot HNO_3$	0.13	[c]
7	$C_6H_5CH_2NHC(NH_2)=NH\cdot 0.5H_2SO_4$	5.9	[d]
8	$C_6H_5(CH_2)_2NHC(NH_2)=NH\cdot 0.5H_2SO_4$	6.7	[e]
9	$C_6H_5(CH_2)_3NHC(NH_2)=NH\cdot 0.5H_2SO_4$	2.6	[f, g]
10	$C_6H_5(CH_2)_4NHC(NH_2)=NH\cdot 0.5H_2SO_4$	2.7	[g]

[a] Concentration for 50% inhibition with 0.05 m*M* DL-Benzoylarginine-*p*-nitroanilide as substrate. [b] Estimated from 22% inhibition at 8 m*M*. [c] Prepared according to ref 43. [d] Prepared according to H. King and I. M. Tonkin, *J. Chem. Soc.*, 1063 (1946). [e] Prepared according to ref 44. [f] Available from a previous study; see B. R. Baker, G. J. Lourens, and J. H. Jordaan, *J. Heterocyclic Chem.*, **4**, 39 (1967). [g] Prepared according to C. E. Brown and W. M. Randall, *J. Am. Chem. Soc.*, **56**, 2134 (1934).

Enzyme Results.[31] A definite *modus operandi* for the design of exo-type active-site-directed irreversible inhibitors has been developed;[10,11] although it cannot be stated flatly that the *modus operandi* cannot be shortened, usually shorter approaches have not led to the desired irreversible inhibitors. The first step is to determine binding points of the substrate or close substrate analogs. The main binding points for trypsin have been found to be a terminal cationic group attached to a hydrocarbon residue about four carbons long, the latter complexing to the enzyme by hydrophobic bonding.[32] *n*-Butylamine,[33] benzylamine,[33] phenylguanidine,[34] and benzamidine[34] are inhibitors that increase in effectiveness in that order; benzamidine binds more effectively[34] than the substrate, DL-benzoylarginine-*p*-nitroanilide.[35]

The reported inhibition of trypsin by *n*-butylamine (**1**), benzylamine (**2**), phenylguanidine (**6**), and benzamidine (**13**) was first checked, as recorded in Tables I and II. Benzamidine (**13**) (Table II) was an excellent inhibitor that was complexed about three times better than the L isomer of the substrate; phenylguanidine (**6**) was complexed nearly as well as L sub-

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

(20) G. Schoellmann and E. Shaw, *ibid.*, **2**, 252 (1963).

(21) Reference 10, Chapter VIII.

(22) For such a study on the dihydrofolic reductases see (a) B. R. Baker, *J. Med. Chem.*, **10**, 912 (1967), paper XCVII of this series; (b) B. R. Baker and B.-T. Ho, *J. Pharm. Sci.*, **55**, 470 (1966); (c) G. H. Hitchings and J. J. Burchall, *Advan. Enzymol.*, **27**, 417 (1965).

(23) Reference 10, Chapter IX.

(24) B. R. Baker, *J. Med. Pharm. Chem.*, **5**, 654 (1962); *Biochem. Pharmacol.*, **11**, 1155 (1962).

(25) B. R. Baker and R. P. Patel, *J. Pharm. Sci.*, **53**, 744 (1964).

(26) See ref 10, Chapter X.

(27) B. R. Baker and J. H. Joshaan, *J. Pharm. Sci.*, **56**, 660 (1967), paper LXXXVIII of this series.

(28) K. N. von Kaulla, *J. Med. Chem.*, **8**, 164 (1965); K. N. von Kaulla, *Experientia*, **21**, 439 (1965).

(29) B. R. Baker and W. Rzeszotarski, *J. Med. Chem.*, **10**, 1109 (1967), paper CIV of this series; B. R. Baker and W. F. Wood, *ibid.*, **10**, 1106 (1967), paper CIII of this series.

(30) B. R. Baker and J. A. Hurlbut, *ibid.*, **10**, 1129 (1967), paper CVII of this series.

(31) The technical assistance of Maureen Baker and Pepper Caseria with these assays is acknowledged.

(32) For a review on the mode of binding to trypsin see ref 10, Chapter III.

(33) T. Inagami, *J. Biol. Chem.*, **239**, 787 (1964).

(34) M. Mares-Guia and E. Shaw, *ibid.*, **240**, 1579 (1965).

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

TABLE II

INHIBITION OF TRYPSIN BY N-SUBSTITUTED BENZAMIDINES AND PHENYLGUANIDINES

No.	Compd	mM concn	% inhib	Estd I_{50},[a] mM	Source
6	$C_6H_5NHC(NH_2)=NH\cdot HNO_3$	0.13	50	0.13	[b]
11	$C_6H_5NHCNHCNH_2\cdot HCl$ (NH, NH)	6	39	9.6	Aldrich
12	$C_6H_5NHCNHC_6H_5$ (NH)	7.5	0	>30	J. T. Baker
13	$C_6H_5C(NH_2)=NH\cdot HCl$	0.032	50	0.032	K & K
14	$C_6H_5CNHC_4H_9$-n (NH)	5	0	>20	[c]
15	$C_6H_5CNHC_6H_5$ (NH·HCl)	10	0	>40	Aldrich
16	$C_6H_5CNHCH_2C_6H_5$ (NH·HCl)	10	31	20	[d]
17	C_6H_5C (N—CH_2, NH—CH_2)	1.0	0	>4	[e]
18	$C_6H_5C(OC_2H_5)=NH\cdot HCl$	2.5	0	>10	

[a] Concentration for 50% inhibition with 0.05 mM DL-benzoylarginine-*p*-nitroanilide as substrate. [b] See Table I. [c] Prepared according to D. F. Kutepow, A. A. Potashnik, and V. V. Shelvehenko, *Zh. Obshch. Khim.*, **33,** 579 (1963). [d] Prepared according to ref 40. [e] Prepared from ethyl benzoate and ethylenediamine; see H. L. Morrill, U. S. Patent 2,508,415; *Chem. Abstr.*, **45,** P668*e* (1951). [f] Prepared according to ref 39.

strate, but benzylamine (**2**) only about one-quarter as well.

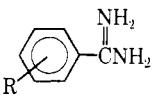
A terminal phenyl group makes a good place to position a variety of leaving groups for design of the exo-type irreversible inhibitor.[10] The higher phenylalkyl analogs (**3**–**5**) of benzylamine (**2**) were therefore assayed, but binding was unfortunately considerably less effective (Table I). Similarly, insertion of methylene groups (**7**–**10**) between the phenyl and guanidine moieties of phenylguanidine (**6**) was detrimental to binding.

The placement of a group on a second nitrogen of phenylguanidine (**6**) and benzamidine (**13**) was then investigated; introduction of an alkyl (**14**), aryl (**15**), or aralkyl (**16**) group was again detrimental to binding (Table II), indicating that there was no bulk tolerance for a group in this area. Replacement of the NH_2 group of benzamidine (**13**) by an ethoxy (as in **18**) was also detrimental to binding; since only one basic nitrogen is necessary for binding, as in benzylamine (**2**), it would appear that trypsin does not have bulk tolerance even for the ethoxy group of **18** in place of an amino group of **13**.

Removal of the carbomethoxy group of tosyl-L-arginine methyl ester results in a 16-fold loss in binding;[36] other evidence that the carbonyl group of trypsin substrates is complexed to the enzyme has been reviewed.[32] Furthermore, *p*-carbethoxybenzamidine has been reported[34] to be a substrate, but its binding constant was not recorded. Therefore, two analogs of benzamidine bearing a *p*-carbonyl group were synthesized for evaluation. Introduction of the *p*-acetyl group (**19**) (Table III) led to an 11-fold loss in binding; even more loss in binding occurred with the N-methylcarbanilide group of **20**. Similarly, introduction of *m*-acetyl (**24**) or *p*-acetyl (**25**) groups on benzamidine (**6**) (Table IV) led to a loss in binding. Since molecular models indicated that derivatives of *p*-carboxymethylbenzamidine should more closely approximate

(36) S. Benzer and B. Weisblum, *Nature*, **190**, 722 (1961).

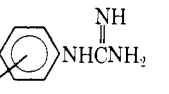
TABLE III

INHIBITION OF TRYPSIN BY 

No.	R	Salt	I_{50},[a] mM
13[b]	H	HCl	0.032
19	*p*-CH_3CO	HI	0.36
20	*p*-$C_6H_5N(CH_3)CO$	HI	0.79
21	*p*-$C_6H_5O(CH_2)_3O$	HNO_3	0.015
22	*p*-$C_6H_5(CH_2)_3O$	HNO_3	0.0079
23	*m*-$C_6H_5O(CH_2)_3O$	TsOH	0.0075

[a] Concentration for 50% inhibition with 0.05 mM DL-benzoylarginine-*p*-nitroanilide as substrate. [b] From K & K Co.

TABLE IV

INHIBITION OF TRYPSIN BY 

No.	R	Salt	I_{50},[a] mM
6	H	HNO_3	0.13
24	*m*-CH_3CO	HNO_3	0.97
25[b]	*p*-CH_3CO	HCl	4.7
26	*p*-$CH_2CON(CH_3)C_6H_5$	HNO_3	0.84
27	*m*-$C_6H_5O(CH_2)_3O$	HCl	0.12
28	*m*-$C_6H_5(CH_2)_3O$	HNO_3	0.074
29	*p*-$C_6H_5(CH_2)_2O$	HNO_3	0.67
30	*p*-$C_6H_5(CH_2)_3O$	HNO_3	0.60

[a] Concentration for 50% inhibition with 0.05 mM DL-benzoylarginine-*p*-nitroanilide as substrate. [b] Prepared according to H. King and I. M. Tonkin, *J. Chem. Soc.*, 1063 (1946).

the dimensions of an arginine substrate, the N-methylanilide (**26**) was synthesized and evaluated, but **26** showed a sevenfold loss in binding.

These binding results with **19**, **20**, and **24**–**26** were difficult to rationalize until new binding data on substrates was obtained by Shaw.[37] Although *p*-carbethoxybenzamidine was a substrate,[34] it was complexed 36-fold less effectively than DL-benzoylarginine-*p*-

(37) We wish to thank Professor E. Shaw for sending us his data prior to publication.

nitroanilide, thus explaining the loss in binding by **19** and **20**. Furthermore, he reported that *p*-carbethoxyphenylguanidine was complexed one-seventh as effectively as phenylguanidine, thus explaining the poor binding of **25**. However, *p*-carbethoxymethylphenylguanidine was complexed fivefold better than phenylguanidine. Since **26** has the carbonyl placed in the same way as *p*-carbethoxymethylphenylguanidine, the loss in binding observed by **26** is most likely due to a lack of bulk tolerance for the N-methylanilino moiety; this moiety was selected in order to decrease potential substrate properties of an amide function in this position.[38]

Since phenylguanidine (**6**) and benzamidine (**13**) are sufficiently good inhibitors of trypsin, studies were then made to see if long groups could be substituted on the benzene ring and still be tolerated by the enzyme. Substitution on the *meta* position of benzamidine (**13**) with a phenoxypropyloxy group (**23**) gave fourfold better binding to trypsin (Table III), indicating that not only was bulk tolerance present in this area, but the effect on binding was favorable; similar results were seen with the *p*-phenoxypropyloxy group (**21**) or a *p*-phenylpropyloxy group (**22**).

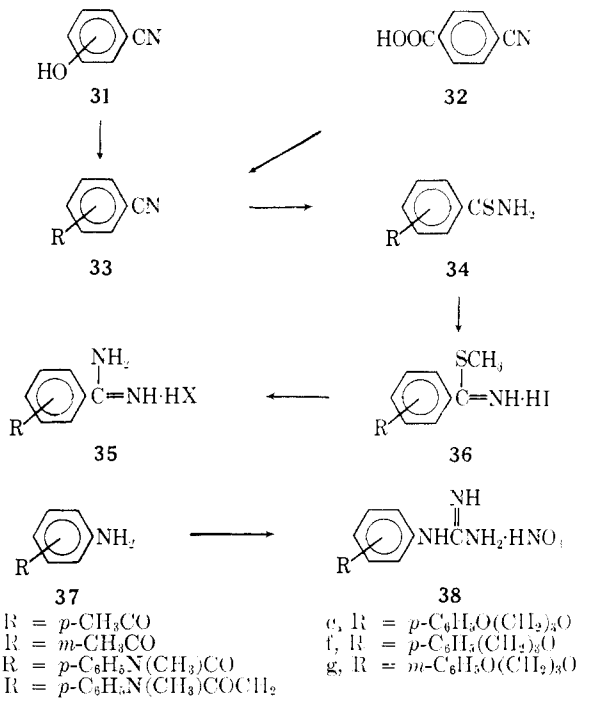
A similar study was made with phenylguanidine (**6**) (Table IV). Introduction of large substituents at the *meta* position such as phenoxypropyloxy (**27**) and phenylpropyloxy (**28**) had no detrimental effect on binding, indicating bulk tolerance for these groups. In contrast, introduction of groups at the *para* position of phenylguanidine (**6**) such as phenethyloxy (**29**) or phenylpropyloxy (**30**) led to about a fivefold loss in binding. The fact that large *para* substituents on phenylguanidine (**6**) cannot be tolorated by the enzyme, but similar substitution on benzamidine (**13**) can be tolerated, gives support to the proposed differences in binding conformations of **6** and **13** to trypsin.[32]

Thus phase II of the *modus operandi* for design of exo-type active-site-directed irreversible inhibitors has been completed. Phase III studies, the proper positioning of a leaving group for covalent bond formation within the enzyme–inhibitor complex, are being pursued; the terminal phenyl group of molecules such as *m*- and *p*-(phenoxypropyloxy)benzamidine (**21** and **23**) and *m*-(phenoxypropyloxy)phenylguanidine (**27**) is a logical place to position these leaving groups.

Chemistry.—There are two major routes for the conversion of substituted benzonitriles (**33**) to substituted benzamidines (**35**). The classical routes proceed by dry HCl catalyzed addition of ethanol to the nitrile to give the imino ether hydrochloride[39] followed by displacement with ammonia or an amine.[40] This alcohol addition is most frequently performed in 1 equiv of alcohol, sometimes in a dry solvent, but suffers when the nitrile is not sufficiently soluble; for example, **33c** was too insoluble to react.

A more recent process involves solvents with good dissolving power (Scheme I). Addition of H_2S to **33c** in pyridine containing triethylamine[41,42] afforded the thioamide **34c** in near quantitative yield. Methylation

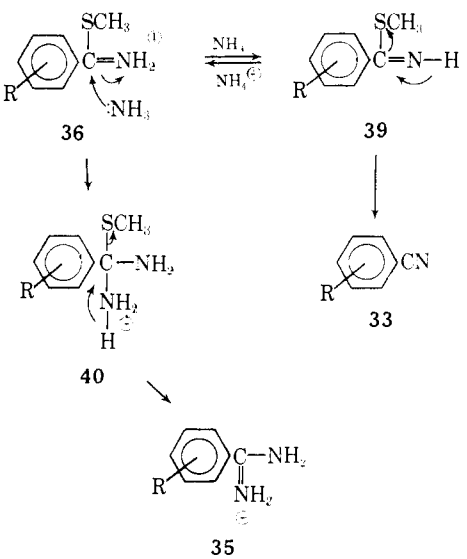
Scheme I



a, R = *p*-CH_3CO
b, R = *m*-CH_3CO
c, R = *p*-$C_6H_5N(CH_3)CO$
d, R = *p*-$C_6H_5N(CH_3)COCH_2$
e, R = *p*-$C_6H_5O(CH_2)_3O$
f, R = *p*-$C_6H_5(CH_2)_3O$
g, R = *m*-$C_6H_5O(CH_2)_3O$

of **34c** with methyl iodide in acetone proceeded smoothly to the imino thioether **36c**.[42] Attempts to convert[42] the imino thioether to the amidine **35c** with alcoholic ammonia led to elimination of methyl mercaptan with regeneration of the benzonitrile **33c**, rather than displacement of the methylthio group with formation of the desired amidine **35c**. It was reasoned that the free base **39** could be formed with ammonia, then could undergo elimination, but that the salt from **36** could undergo displacement (Scheme II). In order to

Scheme II

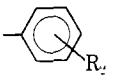


decrease the basicity of the ammonia, ammonium acetate was employed which was sufficiently dissociated to ammonia and acetic acid to allow conversion of **36** to **35**, but not sufficiently basic to form the free base **39** which could undergo elimination to **33**.

This did indeed prove to be the case; **36c** reacted smoothly with ammonium acetate in boiling ethanol in 45 min to give **35c** in 76% yield; a minor amount of **33c** was detectable on tlc. It is probable that this

(38) H. T. Huang and C. Niemann, *J. Am. Chem. Soc.*, **73**, 3223 (1951).
(39) A. W. Dox, "Organic Syntheses," Coll. Vol. I, John Wiley and Sons, Inc., New York, N. Y., 1941, p 6.
(40) F. L. Pyman, *J. Chem. Soc.*, **123**, 3379 (1923).
(41) (a) O. Wallach, *Ber.*, **32**, 1872 (1899); (b) *Org. Syn.*, **36**, 23 (1956).
(42) F. M. Bercot-Vatteroni, *Ann. Chim.* (Paris), **7**, 303 (1962).

TABLE V

PHYSICAL CONSTANTS OF 

No.	R_1	R_2	Method	% yield	Mp, °C	Calcd, % C	Calcd, % H	Calcd, % N	Found, % C	Found, % H	Found, % N
19	*p*-CH_3CO	$HN{=}C(NH_2)\cdot HI$	D[a]	27	228–231	37.3	3.83	9.66	37.5	3.80	9.60
20	*p*-$C_6H_5N(CH_3)CO$	$HN{=}C(NH_2)\cdot HI$	D[a]	76	280–284	47.3	4.23	11.0	47.6	4.47	11.0
21	*p*-$C_6H_5O(CH_2)_3O$	$HN{=}C(NH_2)\cdot HNO_3$	D[a,b]	60	127–134	57.7	5.75	12.6	57.9	5.80	12.4
22	*p*-$C_6H_5(CH_2)_3O$	$HN{=}C(NH_2)\cdot HNO_3$	D[b]	28	141–148	60.6	6.03	13.2	60.3	6.06	13.1
23	*m*-$C_6H_5O(CH_2)_3O$	$HN{=}C(NH_2)\cdot TsOH$[c]	D[d]	26	141–144	62.4	5.92	6.33	62.2	5.82	6.27
24	*m*-CH_3CO	$NHC(NH)NH_2\cdot HNO_3$	E[e]	33	190–192	45.0	5.04	23.3	44.9	4.93	23.0
26	*p*-$C_6H_5N(CH_3)COCH_2$	$NHC(NH)NH_2\cdot HNO_3$	E[e]	40	142–144	55.7	5.55	20.3	55.5	5.45	20.4
27	*m*-$C_6H_5O(CH_2)_3O$	$NHC(NH)NH_2\cdot HCl$	E[e]	16	210–214	58.1[f]	6.40	12.7	58.0	6.26	12.8
28	*m*-$C_6H_5(CH_2)_3O$	$NHC(NH)NH_2\cdot HNO_3$	F[g]	6	141–143	57.8	6.07	16.9	57.6	6.00	16.6
29	*p*-$C_6H_5(CH_2)_2O$	$NHC(NH)NH_2\cdot HNO_3$	F[g]	4	175–177	56.6	5.70	17.6	56.9	5.78	17.8
30	*p*-$C_6H_5(CH_2)_2O$	$NHC(NH)NH_2\cdot HNO_3$	F[g]	9	140–142	57.8	6.07	16.9	58.0	6.19	17.1
33c	*p*-$C_6H_5N(CH_3)CO$	CN	Exptl	33	101–102	76.3	5.12	11.9	76.4	4.97	11.7
33e	*p*-$C_6H_5O(CH_2)_3O$	CN	A[g]	100	72–73[h]	75.9	5.97	5.53	75.6	6.20	5.60
33f	*p*-$C_6H_5(CH_2)_3O$	CN	A[n]	60	50–51	81.0	6.37	5.90	81.2	6.40	6.02
33g	*m*-$C_6H_5O(CH_2)_3O$	CN	A		Oil						
34a	*p*-CH_3CO	$CSNH_2$	B[b]	72	174–176	60.3	5.06	7.82	60.1	5.16	7.66
34c	*p*-$C_6H_5N(CH_3)CO$	$CSNH_2$	B[g]	99	223–226	66.7	5.22	10.4	66.8	5.31	10.4
34e	*p*-$C_6H_5O(CH_2)_3O$	$CSNH_2$	B[g]	93	137–139	66.9	5.96	4.87	66.7	5.92	4.82
34f	*p*-$C_6H_5(CH_2)_3O$	$CSNH_2$	B[g]	86	157–162	70.8	6.32	5.16	70.9	6.45	5.25
34g	*m*-$C_6H_5O(CH_2)_3O$	$CSNH_2$	B[e]	100[i]	119–123	66.9	5.96	4.87	66.8	5.82	4.77
36a	*p*-CH_3CO	$C(SCH_3){=}NH\cdot HI$	C[j]	50	148–150	37.4	3.77	4.36	37.5	3.86	4.27
36c	*p*-$C_6H_5N(CH_3)CO$	$C(SCH_3){=}NH\cdot HI$	C[k]	75	189–191	46.6	4.16	6.80	46.4	4.07	6.93
36e	*p*-$C_6H_5O(CH_2)_3O$	$C(SCH_3){=}NH\cdot HI$	C[l]	60	135–142	47.6	4.70	3.26	47.3	4.66	43.3
36f	*p*-$C_6H_5(CH_2)_3O$	$C(SCH_3){=}NH\cdot HI$	C[m]	87	149–150	49.4	4.89	3.39	49.2	5.00	3.24
36g	*m*-$C_6H_5O(CH_2)_3O$	$C(SCH_3){=}NH\cdot HI$	C[l]	46	112–118	47.6	4.92	3.26	47.4	4.78	3.32

[a] Recrystallized from EtOH–petroleum ether (bp 60–110°). [b] Recrystallized from H_2O. [c] Evaporated reaction mixture dissolved in *i*-PrOH, excess *p*-toluenesulfonic acid added. [d] Recrystallized from i-PrOH. [e] Recrystallized from aqueous EtOH. [f] Hemihydrate. [g] Recrystallized from EtOH. [h] J. D. Brooks, P. T. Charleton, P. E. Macey, D. A. Peak, and W. F. Short, *J. Chem. Soc.*, 452 (1950), report mp 72–73°. [i] Over-all yield from *m*-cyanophenol. [j] Recrystallized from acetone–petroleum ether. [k] Recrystallized from acetone. [l] Recrystallized from EtOAc. [m] Recrystallized from aqueous acetone. [n] Recrystallized from petroleum ether.

base-catalyzed elimination with **39c** only happens when the imino thioether is base-weakened by a *para* electron-withdrawing group.

Other amidines (**35**) were also made by this route. The starting benzonitrile (**33c**) for **35c** was prepared from N-methylaniline and *p*-cyanobenzoic acid (**32c**) *via* its acid chloride. The intermediate cyanophenyl ethers (**33e–g**) were synthesized by alkylation of *m*- or *p*-hydroxybenzonitrile with the appropriate bromide in DMF with K_2CO_3 as an acid acceptor.

The phenylguanidines (**38**) were synthesized from the appropriate arylamine (**37**) by reaction either with cyanamide[43] or with 3,5-dimethylpyrazole-1-carboxamidine nitrate.[44] The syntheses of the required phenyl ethers (**37c–h**) have been previously described in another study from this laboratory.[45] As is common for these syntheses of phenylguanidines, yields were low due to formation of purple by-products.[43]

Experimental Section[46]

Enzyme Assays.—Crystalline trypsin was purchased from Sigma Chemical Co. DL-Benzoylarginine-*p*-nitroanilide (BANA) was purchased from Mann Research Laboratories and dissolved in DMSO to give a 3.1 m*M* solution. The buffer employed was 0.05 *M* Tris, pH 7.4. The following assay method, a modification of the method of Erlanger, Kowkowsky, and Cohen,[35] was employed.

(43) S. R. Safir, S. Kushner, L. M. Brancone, and Y. SubbaRow, *J. Org. Chem.*, **13**, 924 (1948).

(44) F. L. Scott, D. G. Donovan, and J. Reilly, *J. Am. Chem. Soc.*, **75**, 4053 (1953).

(45) B. R. Baker and G. J. Lourens, *J. Pharm. Sci.*, **56**, 871 (1967), paper LXXXVII of this series.

(46) Melting points were determined in capillary tubes on a Mel-Temp block and are uncorrected. Infrared spectra were determined in KBr pellet with a Perkin-Elmer 137B or 337 spectrophotometer; ultraviolet spectra were determined in 10% EtOH with a Perkin-Elmer 202 spectrophotometer. Tlc was performed on Brinkmann silica gel GF and spots were detected by examination under uv light. All analytical samples had ultraviolet and infrared spectra compatible with their assigned structures and all moved as a single spot on tlc.

Trypsin (4.5 mg) was dissolved in 5 ml of 1 m*M* HCl and stored at 0–3°; this solution was renewed at least once a week. In a 3-ml glass cuvette were placed 2.75 ml of buffer, 50 μl of 3.1 μ*M* BANA, and 250 μl of DMSO with or without inhibitor. When the system had balanced, 100 μl of trypsin solution was added and the rate of increase of optical density at 410 mμ was recorded on a 0–0.1 OD slide wire of a Gilford spectrophotometer; the cuvette concentration of BANA was 0.05 m*M*. Without inhibitor, the velocity was about 0.012 OD unit/min. Several concentrations of inhibitor were measured that gave a V_0/V_1 of 1.4–2.5, where V_0 = velocity without inhibitor and V_1 equals velocity with inhibitor. V_0/V_1 *vs.* [I] was then plotted; where $V_0/V_1 = 2$, [I] is the I_{50} concentration.[47]

***p*-Phenoxypropyloxybenzonitrile (33e) (Method A).**—A mixture of 2.38 g (20 mmoles) of *p*-cyanophenol, 4.50 g (21 mmoles) of phenoxypropyl bromide, 2.76 g (20 mmoles) of anhydrous K_2CO_3, and 50 ml of DMF was stirred at 73–78° for 6 hr. Addition of several volumes of water gave a solid which was collected on a filter; yield 5.07 g (99%), mp 59–63°, moved as a single spot in 3:1 petroleum ether (bp 60–110°)–ethyl acetate and was suitable for further transformation. A sample was recrystallized twice from ethanol to give white crystals, mp 72–73°; see Table V for additional data.

***p*-Cyano-N-methylbenzanilide (33c).**—A mixture of 5.0 g (33 mmoles) of **32** and 10 ml of $SOCl_2$ was refluxed for 50 min when solution was complete. The solution was diluted with petroleum ether, then cooled at −15°. The intermediate acid chloride (C=O at 1750 cm^{-1}) (2.88 g) was collected on a filter and washed with petroleum ether. By evaporation of the filtrate was obtained an additional 2.89 g (total 100%). To a solution of 15 ml of N-methylaniline in 150 ml of benzene was added 2.88 g of the acid chloride. The mixture was heated to boiling for 3 min on a steam bath. The solution was washed successively with 15 ml of H_2O, two 25-ml portions of 3 *N* HCl, 25 ml of 3 *N* NaOH, and 15 ml of H_2O. The $MgSO_4$-dried solution was spin-evaporated *in vacuo* leaving an oil which soon solidified; yield 2.8 g (72%). Recrystallization from ethanol gave 1.7 g (44%) of product that was suitable for further transformation; tlc in 3:1 petroleum ether–EtOAc showed one spot. For analysis (see Table V), the compound was recrystallized once more from ethanol to give white crystals, mp 101–102°.

***p*-Nitrophenylacet-N-methylanilide** was prepared similarly from *p*-nitrophenylacetic acid in 30% yield, mp 90–92°, after recrystallization from 80% ethanol.

Anal. Calcd for $C_{15}H_{14}N_2O_3$: C, 66.7; H, 5.22; N, 10.4. Found: C, 66.8; H, 5.23; N, 10.4.

Catalytic hydrogenation of the nitro group in the presence of Pd–C gave **37d** isolated as its hydrochloride, mp 170–172°, after recrystallization from ethanol–petroleum ether.

Anal. Calcd for $C_{15}H_{15}ClN_2O$: C, 65.0; H, 6.17; N, 10.1. Found: C, 65.0; H, 6.20; N, 9.98.

***p*-(Aceto)thiobenzamide (34a) (Method B).**—Through a solution of 718 mg (4.95 mmoles) of **33a**[48] in 7.5 ml of reagent pyridine and 0.6 ml of triethylamine was passed a slow stream of H_2S for 2 hr. After standing at ambient temperature for 24 hr, the solution was diluted with water and the product was collected on a filter; yield 637 mg (72%) that moved as one spot on tlc in 3:1 petroleum ether–EtOAc and was suitable for the next step. For analysis (Table V), a sample was recrystallized from water to give light yellow crystals, mp 174–176°.

***p*-Acetobenzimino Methyl Thioether Hydriodide (36a) (Method C).**—To a solution of 537 mg (3.04 mmoles) of **34a** in 10 ml of reagent acetone was added 0.35 ml (5.7 mmoles) of MeI. After standing for about 18 hr in a stoppered flask, the solution was diluted with petroleum ether to turbidity then cooled at −15°. The product was collected on a filter; yield 475 mg (50%) that was uniform on tlc in 1:1 ethyl acetate–petroleum ether and was suitable for the next step. For analysis (Table V), a sample was recrystallized from acetone–petroleum ether. If tlc after 18 hr showed the reaction was incomplete, a second portion of MeI was added.

***p*-Acetobenzamidine Hydriodide (19) (Method D).**—A solution of 357 mg (1.2 mmoles) of **36a** and 100 mg (1.4 mmoles) of ammonium acetate in 10 ml of EtOH was refluxed for 45 min, then spin-evaporated *in vacuo* to a small volume and diluted with petroleum ether. The product was collected on a filter; yield 131 mg (27%) that moved as a single spot on tlc in 10:5:1 *n*-C_4H_9OH–HOAc–H_2O. Recrystallization from ethanol–petroleum ether gave 52 mg (11%) of the analytical sample (Table V), mp 228–231°.

***m*-Acetophenylguanidine Nitrate (24) (Method E).**—A solution of 1.00 g (5.8 mmoles) of *m*-aminoacetophenone and 0.75 g (17 mmoles) of cyanamide in 15 ml of EtOH, 2 ml of H_2O, and 1 ml of 12 *N* HCl was refluxed for 6 hr, then spin-evaporated to a small volume *in vacuo* and made strongly basic with 6 *N* NaOH. The mixture was extracted with an equal volume of $CHCl_3$. The dried extract was evaporated *in vacuo* to give an oil which was treated with 14% HNO_3. The brown product was collected and recrystallized from 80% aqueous EtOH; yield 0.46 g (34%), mp 190–192°. See Table V for analytical data.

***m*-(Phenylpropyloxy)phenylguanidine Nitrate (28) (Method F).**—A solution of 2.0 g (8.7 mmoles) of **37**[49] and 1.75 g (8.7 mmoles) of 3,5-dimethylpyrazole-1-carboxamidine nitrate (Aldrich) in 20 ml of EtOH was refluxed for 20 hr, then allowed to stand at −15°. The crude product, mp 112–122°, was collected on a filter and washed (EtOH); tlc in 10:5:1 *n*-BuOH–HOAc–H_2O showed two spots. Three recrystallizations from ethanol gave 0.185 g (6%) of white crystals, mp 141–143°, that moved as a single spot on tlc; see Table V for additional data.

(47) B. R. Baker, W. W. Lee, W. A. Skinner, A. P. Martinez, and E. Tong, *J. Med. Pharm. Chem.*, **2**, 633 (1960).

(48) L. Friedman and H. Shechter, *J. Org. Chem.*, **26**, 2522 (1961).