(6) V. du Vigneaud, G. Winestock, V. V. S. Murti, D. B. Hope, and R. D. Kimbrough, Jr., *J. Biol. Chem.*, **235**, PC64 (1960); D. B. Hope, V. V. S. Murti, and V. du Vigneaud, *ibid.*, **251**, 1563 (1962).
(7) M. Manning, T. C. Wuu, and J. W. M. Baxter, *J. Chromatogr.*, **38**, 396 (1968).
(8) M. Bodanszky and V. du Vigneaud, *J. Amer. Chem. Soc.*, **81**, 5688 (1959).
(9) W. S. Wadsworth, Jr., and W. D. Emmons, *J. Amer. Chem. Soc.*, **83**, 1733 (1961).
(10) R. Albert, Ph.D. Thesis, The Polytechnic Institute of Brooklyn, Brooklyn, N.Y., 1970, p 91.
(11) P. Holton, *Brit. J. Pharmacol. Chemother.*, **3**, 328 (1948).
(12) R. A. Munsick, *Endocrinology*, **66**, 451 (1960).
(13) J. M. Coon, *Arch. Int. Pharmacodyn.*, **62**, 79 (1939).
(14) R. A. Munsick, W. H. Sawyer, and H. B. van Dyke, *Endocrinology*, **66**, 860 (1960).
(15) "The Pharmacopeia of the United States of America," 18th revision, Mack Publishing Co., Easton, Pa., 1970, p 771.
(16) H. O. Schild, *Brit. J. Pharmacol.*, **2**, 189 (1947).
(17) D. F. Dyckes, J. J. Nestor, Jr., M. F. Ferger, and V. du Vigneaud, *J. Med. Chem.*, **17**, 250 (1974).
(18) A. Goldstein, "Biostatistics: An Introductory Text," Macmillan, New York, N.Y., 1964, p 59.
(19) M. F. Ferger, W. C. Jones, Jr., D. F. Dyckes, and V. du Vigneaud, *J. Amer. Chem. Soc.*, **94**, 982 (1972); E. Kaiser, R. L. Colescott, C. D. Bossinger, and P. I. Cook, *Anal. Biochem.*, **34**, 595 (1970).
(20) M. Bodanszky, M. Kondo, C. Y. Lin, and G. F. Sigler, *J. Org. Chem.*, **39**, 444 (1974).
(21) M. Bodanszky and V. du Vigneaud, *J. Amer. Chem. Soc.*, **81**, 2504 (1959).
(22) M. Bodanszky and V. du Vigneaud, *J. Amer. Chem. Soc.*, **81**, 5688 (1959).
(23) W. König and R. Geiger, *Chem. Ber.*, **106**, 3626 (1973).
(24) G. L. Ellman, *Arch. Biochem. Biophys.*, **82**, 70 (1959).
(25) J. Porath and P. Flodin, *Nature (London)*, **183**, 1657 (1959).
(26) D. H. Spackman, W. H. Stein, and S. Moore, *Anal. Chem.*, **30**, 1190 (1958).
(27) S. Moore, *J. Biol. Chem.*, **238**, 235 (1963).

Synthesis of Isosteres of *p*-Amidinophenylpyruvic Acid. Inhibitors of Trypsin, Thrombin, and Pancreatic Kallikrein†

Larry J. Loeffler,* Eng-Chun Mar,‡

Division of Medicinal Chemistry, University of North Carolina School of Pharmacy, Chapel Hill, North Carolina 27514

J. D. Geratz, and Lynda B. Fox

Department of Pathology, University of North Carolina School of Medicine, Chapel Hill, North Carolina 27514.
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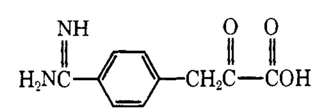
A series of amino acids, amidino acids, and amidino esters was synthesized and the compounds were evaluated for their inhibitory activity against bovine trypsin, bovine thrombin, and porcine pancreatic kallikrein and as anticoagulants. Among these compounds, ethyl 4-amidino-2-iodophenoxyacetate was found to be the most effective inhibitor of the enzymes in question, with a potency ($K_i = 3.16 \times 10^{-6}$ *M vs.* trypsin; $K_i = 4.8 \times 10^{-5}$ *M vs.* thrombin) similar to that of *p*-amidinophenylpyruvic acid ($K_i = 6.0 \times 10^{-6}$ *M vs.* trypsin; $K_i = 2.0 \times 10^{-5}$ *M vs.* thrombin). Ethyl 4-amidino-2-iodophenoxyacetate was also found to be the most effective in blocking the clotting activity of plasma, as indicated by significant prolongation of the partial thromboplastin time. This paper reports the synthetic methods, the enzyme inhibitory activity, and the structure–activity relationships observed.

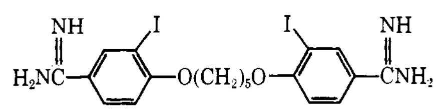
In view of the fact that serine proteinases of physiopathological importance are being discovered whose inhibition may be of therapeutic value, chemical modification of known serine proteinase inhibitors is of considerable importance in a search for a more effective or selective inhibitor.

The development of reversible trypsin, thrombin, and kallikrein inhibitors was greatly stimulated by Mares-Guia and Shaw's discovery of the considerable potency of benzamidine and *p*-aminobenzamidine.[1] Since then many substituents have been introduced into the benzene ring of benzamidine,[2] leading to equal or greater inhibitory activity against proteinases. *p*-Amidinophenylpyruvic acid (I) has been found to be an excellent inhibitor of thrombin, plasmin, and trypsin.[2,3] More recently, aromatic diamidines such as pentamidine[4-6] and 4′,4″-diamidino-2′,2″diiodo-1,5-diphenoxypentane (II)[7] have been studied and shown to be even more effective serine proteinase inhibitors.

Due to the strong *in vitro* effectiveness of diamidino compounds, the aromatic diamidines have been investi-



I



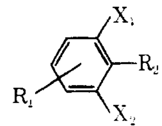
II

gated for *in vivo* use in disease states where the kallikrein–kinin system is considered to play an important role. Such pathological conditions are inflammatory edema, shock, and arthritis. However, an obstacle found to the systemic application of those diamidines is the fact that they may also dramatically lower the blood pressure, promoting hypotensive shock.[8] On the other hand, *p*-amidinophenylpyruvic acid, which has shown to be less toxic,[9] is a potent and interesting inhibitor of serine proteinases and is a compound of possible clinical use. Therefore, systematic modification of this molecule seemed highly desirable to us. The compounds reported here represent part of such an effort, in particular a systematic study of some isosteric and isoelectronic analogs of *p*-amidinophenylpyruvic acid.

Chemistry. Synthesis of the new amino and amidino acids and esters listed in Table II–IV, prepared as isosteres

† Kallikrein is a registered trademark assigned to Farbenfabriken Bayer AG, Leverkusen, Federal Republic of Germany.
‡ Taken in part from a thesis presented by Mr. E. C. Mar in Nov 1973 to the Graduate School of the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the Master of Science in Medicinal Chemistry degree.

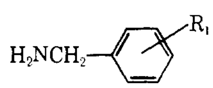
Table I. Cyano Esters



Compd	R_1	R_2	X_1	X_2	Mp or bp (mm), °C	Yield, %	Formula	Analyses
1	3-CN	$OCH_2COOC_2H_5$	H	H	140–142 (0.35)	78	$C_{11}H_{11}NO_3$	C, H
2	4-CN	$OCH_2COOC_2H_5$	H	H	53–54.5	81	$C_{11}H_{11}NO_3$	C, H
3	4-CN	$OCH(CH_3)COOC_2H_5$	H	H	72–75	74	$C_{12}H_{13}NO_3$	C, H
4	4-CN	$OCH_2COOC_2H_5$	Cl	H	64–65	90	$C_{11}H_{10}ClNO_3$	C, H
5	4-CN	$OCH_2COOC_2H_5$	Cl	Cl	87–88	84	$C_{11}H_9Cl_2NO_3$	C, H
6	4-CN	$OCH_2COOC_2H_5$	Br	H	51–52	96	$C_{11}H_{10}BrNO_3$	C, H
7	4-CN	$OCH_2COOC_2H_5$	Br	Br	85–87	95	$C_{11}H_9Br_2NO_3$	C, H
8	4-CN	$OCH_2COOC_2H_5$	I	H	75–76	70	$C_{11}H_{10}INO_3$	C, H
9	4-CN	$OCH_2COOC_2H_5$	I	I	128–129	75	$C_{11}H_9I_2NO_3$	C, H
10	4-CN	$OCH_2COOC_2H_5$	NO_2	H	96–97	77	$C_{11}H_{10}N_2O_5$	C, H
11	3-CN	$CH{=}CHCOOC_2H_5$	H	H	69–70.5	89	$C_{12}H_{11}NO_2$	C, H
12	4-CN	$CH{=}CHCOOC_2H_5$	H	H	59–63	95	$C_{12}H_{11}NO_2$	C, H
13	3-CN	$NHC{=}OCOOC_2H_5$	H	H	145–146.5	57	$C_{11}H_{10}N_2O_3$	C, H
14	4-CN	$NHC{=}OCOOC_2H_5$	H	H	184–185[a]	55	$C_{11}H_{10}N_2O_3$	C, H

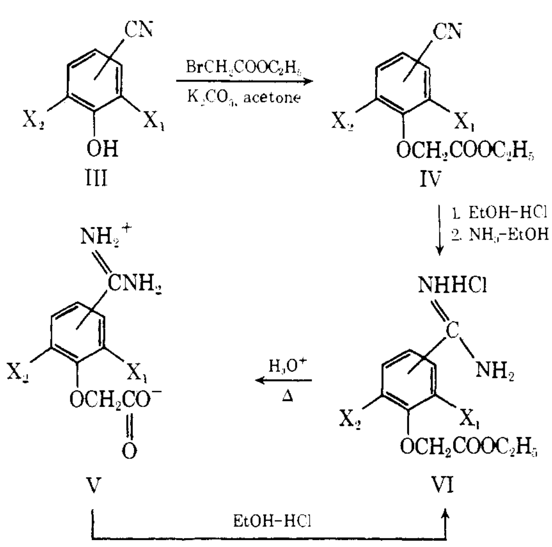
[a] Lit.[23] mp 189–190°.

Table II. Amino Acids



Compd	R_1	Mp, °C	Yield, %	Formula	Analyses	Inhibitory act., K_i, M: Trypsin	Thrombin	Kallikrein
15	$3\text{-}CH_2CH_2COOH$	234–236	75	$C_{10}H_{13}NO_2$	C, H	$\gg 10^{-3}$	$\gg 10^{-3}$	$\gg 10^{-3}$
16	$4\text{-}CH_2CH_2COOH$	254–257	60	$C_{10}H_{13}NO_2$	C, H, N	9.31×10^{-3}	$\gg 10^{-3}$	1.54×10^{-2}
17	$4\text{-}OCH_2COOH$	277–279	24	$C_9H_{11}NO_3$	C, H, N	3.15×10^{-3}	$\gg 10^{-3}$	1.58×10^{-2}

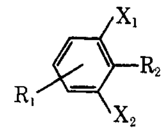
Scheme I



of *p*-amidinophenylpyruvic acid, proceeded in a fairly straightforward manner as indicated in Schemes I–III. However, one unusual observation should be noted; namely, all of the derivatives of 4-amidinophenoxyacetic acid hydrochloride (**18–27**) were spontaneously transformed into free bases (zwitterion form) by loss of hydrogen chloride during purification by recrystallization from water or even 3 *N* HCl.

Structure–Activity Relationships. As indicated in Table II, the amino acids 3- and 4-aminomethylphenylpropionic acid and 4-aminomethylphenoxyacetic acid (compounds **15**, **16**, and **17**, respectively) were weak proteolytic inhibitors. As shown in Tables III and IV, it was found that all esters of 4-amidinocinnamic acid and halogen-substituted analogs of 4-amidinophenoxyacetic acids were more potent proteolytic enzyme inhibitors than their corresponding amidino acids by approximately a factor of 10. The enhanced biological activity of these simple ester derivatives might be explained by the increase of hydrophobicity but more probably results from the susceptibility of the ester bond to enzymatic hydrolysis *via* the formation of a moderately stable, inactive covalent intermediate (*i.e.*, an acyl enzyme[10–12]) which is hydrolyzed to regenerate the active enzyme. In our work, it was also found that 4-amidino-2-bromophenoxyacetamide (**44**) was less active than the corresponding ester, ethyl 4-amidino-2-bromophenoxyacetate (**36**), on trypsin and thrombin by factors of about 26- and 22-fold, respectively. This indicates that in the case of esters, the rate of acylation is greater than that of deacylation (in contrast to the corresponding amide).[10] Ethyl 4-ami-

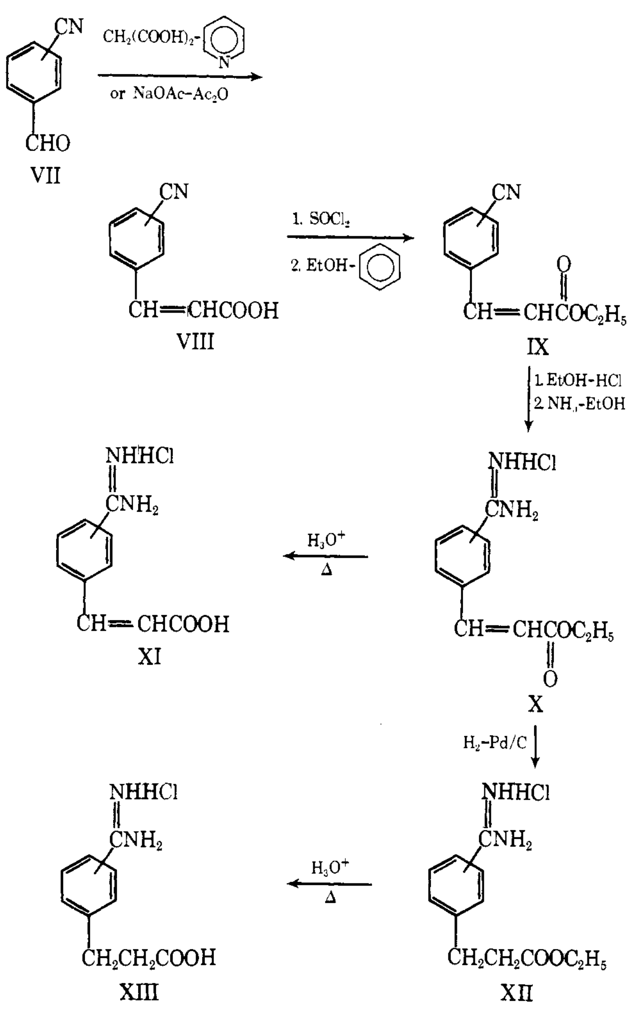
Table III. Amidino Acids



Compd	R_1	R_2	X_1	X_2	Mp, °C	Yield, %	Formula	Analyses	Inhibitory act., K_i, M[a] Trypsin	Thrombin	Kallikrein
18	3-Am$^+$	OCH_2COO^-	H	H	283–285	64	$C_9H_{10}N_2O_3$	C, H	–	–	–
19	4-Am$^+$	OCH_2COO^-	H	H	334–335	80	$C_9H_{10}N_2O_3$	C, H, N	1.60×10^{-4}	1.77×10^{-3}	4.60×10^{-3}
20	4-Am$^+$	$OCH(CH_3)COO^-$	H	H	335–337	72	$C_{10}H_{12}N_2O_3$	C, H	–	–	–
21	4-Am$^+$	OCH_2COO^-	Cl	H	288–289	87	$C_9H_9ClN_2O_3$	C, H	8.10×10^{-4}	1.31×10^{-2}	1.04×10^{-2}
22	4-Am$^+$	OCH_2COO^-	Cl	Cl	281–282	60	$C_9H_8Cl_2N_2O_3$	C, H	3.69×10^{-4}	5.38×10^{-3}	3.66×10^{-3}
23	4-Am$^+$	OCH_2COO^-	Br	H	295–296	80	$C_9H_9BrN_2O_3$	C, H	6.20×10^{-4}	3.63×10^{-3}	9.63×10^{-3}
24	4-Am$^+$	OCH_2COO^-	Br	Br	253–255	78	$C_9H_8Br_2N_2O_3$	C, H	–	–	–
25	4-Am$^+$	OCH_2COO^-	I	H	243–246	27	$C_9H_9IN_2O_3 \cdot 0.5H_2O$	C, H	–	–	–
26	4-Am$^+$	OCH_2COO^-	I	I	228–230	44	$C_9H_8I_2N_2O_3$	C, H	–	–	–
27	4-Am$^+$	OCH_2COO^-	NO_2	H	256–258	64	$C_9H_9N_3O_5$	C, H	–	–	–
28	4-Am$^+$Cl$^-$	CH=CHCOOH	H	H	327–329[b]	51	$C_{10}H_{22}ClN_2O_2$	C, H	*b*	*b*	–
29	4-Am$^+$Cl$^-$	CH_2CH_2COOH	H	H	293–295[c]	78	$C_{10}H_{13}ClN_2O_2$	C, H	6.60×10^{-4}[d]	2.94×10^{-3}[d]	8.40×10^{-3}
30[e]	4-Am$^+$	$NHC(=O)COO^-$	H	H					1.75×10^{-5}	2.82×10^{-4}	1.15×10^{-4}

[a]–: data unavailable because of insolubility of compounds under conditions of incubation. [b]Lit.[24] mp 340°. Lit.[25] reports 5×10^{-5} *M vs.* trypsin; 5×10^{-3} *M vs.* thrombin. [c]Lit.[24] mp 300–303°. [d]Lit.[25] reports 5×10^{-4} *M vs.* trypsin; 2.9×10^{-3} *M vs.* thrombin. [e]Prepared in a separate investigation by L. J. Loeffler and M. Pittmann.

Scheme II



dino-2-nitrophenoxyacetate (**40**), prepared by introduction of the nitro group into the parent compound ethyl 4-amidinophenoxyacetate (**32**), exhibits a slight reduction of antithrombin activity (about threefold). However, a hydroxy group introduced onto the amidino moiety results in an inactive inhibitor (compound **45**).

The introduction of a halogen (Cl, Br, or I) group into the benzene nucleus, as in ethyl 4-amidino-2-chloro-(bromo-, or iodo-) phenoxyacetate (**34, 36, 38**), increases the inhibitory activity against trypsin by approximately tenfold when compared with the parent compound, ethyl 4-amidinophenoxyacetate (**32**). The inhibitory activity also increases in the order I > Br > Cl. Therefore, it seems that monohalogen-substituted inhibitors, and particularly that with the large iodo group, fit the steric requirements for binding with the enzyme trypsin (**38**) very well. On the other hand, dihalogen-substituted compounds such as ethyl 4-amidino-2,6-dichloro- (dibromo-, or diodo-) phenoxyacetate (**35, 37, 39**) were found to be of lower activity by approximately a factor of 10. This seems to indicate

Scheme III

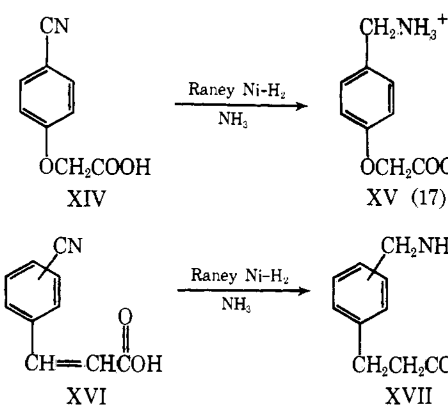
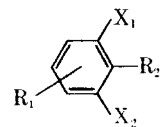


Table IV. Amidino Esters and Analogs



Compd	R_1[a]	R_2	X_1	X_2	Mp, °C	Yield, %	Formula	Analyses	Inhibitory act., K_i, M: Trypsin	Thrombin	Kallikrein
31	3-Am·HCl	$OCH_2COOC_2H_5$	H	H	108–110	58	$C_{11}H_{15}ClN_2O_3 \cdot 0.5H_2O$	C, H	5.20×10^{-6}	3.33×10^{-5}	1.52×10^{-3}
32	4-Am·HCl	$OCH_2COOC_2H_5$	H	H	186–187	71	$C_{11}H_{15}ClN_2O_3$	C, H	1.10×10^{-5}	8.33×10^{-5}	7.40×10^{-4}
33	4-Am·HCl	$OCH(CH_3)COOC_2H_5$	H	H	148–152	72	$C_{12}H_{17}ClN_2O_3$	C, H	5.63×10^{-5}	1.56×10^{-4}	1.06×10^{-3}
34	4-Am·HCl	$OCH_2COOC_2H_5$	Cl	H	50–52	77	$C_{11}H_{14}Cl_2N_2O_3$	C, H	6.78×10^{-6}	6.93×10^{-5}	2.71×10^{-4}
35	4-Am·HCl	$OCH_2COOC_2H_5$	Cl	Cl	158–159	70	$C_{11}H_{13}Cl_3N_2O_3$	C, H	1.25×10^{-5}	1.61×10^{-4}	4.40×10^{-4}
36	4-Am·HCl	$OCH_2COOC_2H_5$	Br	H	135–136	61	$C_{11}H_{14}BrClN_2O_3$	C, H	3.51×10^{-6}	7.22×10^{-5}	1.71×10^{-4}
37	4-Am·HCl	$OCH_2COOC_2H_5$	Br	Br	253–255	78	$C_{11}H_{13}Br_2ClN_2O_3$	C, H	2.05×10^{-5}	5.20×10^{-4}	4.40×10^{-4}
38	4-Am·HCl	$OCH_2COOC_2H_5$	I	H	85–87	85	$C_{11}H_{14}ClIN_2O_3 \cdot H_2O$	C, H	3.16×10^{-6}	4.80×10^{-5}	1.42×10^{-4}
39	4-Am·HCl	$OCH_2COOC_2H_5$	I	I	235–237	90	$C_{11}H_{13}ClI_2N_2O_3$	C, H	1.99×10^{-5}	3.85×10^{-5}	5.00×10^{-4}
40	4-Am·HCl	$OCH_2COOC_2H_5$	NO_2	H	193–195	93	$C_{11}H_{14}ClN_3O_5$	C, H	1.64×10^{-5}	2.38×10^{-4}	7.20×10^{-4}
41	4-Am·HCl	$CH{=}CHCOOC_2H_5$	H	H	233–234	82	$C_{12}H_{15}ClN_2O_2$	C, H	3.40×10^{-5} [b]	2.71×10^{-4} [b]	1.88×10^{-4}
42	4-Am·HCl	$CH_2CH_2COOC_2H_5$	H	H	133	54	$C_{12}H_{17}ClN_2O_2$	C, H	2.30×10^{-5} [c]	6.64×10^{-4} [c]	6.15×10^{-5}
43[d]	4-Gu picrate	$CH_2CH_2COOC_2H_5$	H	H	193–194.5	41	$C_{18}H_{20}N_6O_9$				
44	4-Am·HCl	$OCH_2C({=}O)NH_2$	Br	H	247–248	85	$C_9H_{11}BrClN_3O_2$	C, H	9.09×10^{-5}	1.63×10^{-3}	6.10×10^{-4}
45[e]	4-N(OH)=C(NH_2)·HCl	$OCH_2COOC_2H_5$	H	H	159–161	67	$C_{11}H_{15}ClN_2O_4$	C, H, N	10^{-3}	10^{-3}	10^{-3}
46[f]	4-Am·HCl	$NHC({=}O)COOC_2H_5$	H	H					4.50×10^{-5}	9.30×10^{-4}	8.25×10^{-4}

[a] Am = $H_2NC({=}NH)$; Gu = $H_2NC({=}NH)NH$. [b] Lit.[25] reports 2×10^{-5} *M* *vs.* trypsin; 2.1×10^{-4} *M* *vs.* thrombin. [c] Lit.[25] reports the H_2SO_4 salt with K_i values 1.4×10^{-5} *M* *vs.* trypsin; 9.1×10^{-5} *M* *vs.* thrombin. [d] The procedure of Mares-Guia, *et al.*,[26] was employed. [e] Its free base, mp 128–130°. *Anal.* ($C_{11}H_{14}N_2O_4$) C, H, N. [f] Prepared in separate investigation by L. J. Loeffler and M. Pittmann.

Table V. Inhibitory Effect of Amidines on the Partial Thromboplastin Time of Human Plasma[a]

Compd no.	Formula	Concentration of inhibitor, *M*			
		5×10^{-4}	10^{-4}	5×10^{-5}	10^{-5}
31	Am(3)-$ROCH_2C(=O)OC_2H_5$	234.8	112.9	89.5	66.1[a]
32	Am$ROCH_2C(=O)OC_2H_5$	178.7	84.7	75.8	64.1
34	AmRCl(2)-$OCH_2C(=O)OC_2H_5$	158.2	99.3	73.9	*b*
36	AmRBr(2)-$OCH_2C(=O)OC_2H_5$	362.8	110.9	82.6	69.0
37	AmRBr_2(2,6)-$OCH_2C(=O)OC_2H_5$	89.0	*b*	*b*	*b*
38	AmRI(2)-$OCH_2C(=O)OC_2H_5$	494.6	151.2	134.1	75.6
41	Am$RCH{=}CHC(=O)OC_2H_5$	95.3	70.4	66.8	*b*
42	Am$RCH_2CH_2C(=O)OC_2H_5$	144.8	91.0	73.1	*b*
44	AmRBr(2)-$OCH_2C(=O)NH_2$	70.1	*b*	*b*	*b*

[a]Control = 61.0 ± 0.9 sec; R = benzene ring. The numbers placed in parentheses after certain amidino (Am) groups and after the halogens indicate the location on the respective benzene rings. Amidino groups without numbers are present in the para position (4) with respect to the ester linkages. [b]No inhibition.

that the configuration of dihalogen-substituted amidino esters fits the space and orientation of the active site in trypsin and kallikrein more poorly, resulting in less effective binding. This could imply that there is no "bulk tolerance" in the region of the second substituent.[13]

Results obtained in a biochemical test procedure measuring the ability of these compounds to prolong blood clotting (partial thromboplastin time measurement in human plasma) indicated behavior completely parallel and consistent with data obtained in the inhibition of thrombin (see Table V).

Conclusions

In summary, we have succeeded by our approach in preparing inhibitors such as ethyl 4-amidino-2-iodophenoxyacetate (**38**) which appear to be about as potent as *p*-amidinophenylpyruvic acid (I) in the inhibition of bovine trypsin, bovine thrombin, and porcine pancreatic kallikrein. Although not as potent as certain inhibitors reported, such as the bis(amidine) derivatives, these compounds might be of special interest because of the probability of a lower toxicity relative to the bis(amidino) compounds. Certain reported observations are of interest in this respect. Markwardt and associates[14] have reported evidence that *p*-amidinophenylpyruvic acid (I) lacks the profound hypotensive effects of certain bis(amidines). Geratz and coworkers[8] have observed that the hypotensive effect appears to be characteristic of bis(amidino) compounds, rather than simple monoamidines. The high toxicity of bis(amidines) such as the trypanocidal pentamidine has been well known for years. It appears likely, therefore, that amidinophenoxyacetic acids, prepared as isosteres of *p*-amidinophenylpyruvic acid, will probably be less toxic and have less effect on blood pressure than the bis analogs. This awaits experimental proof.

Increased stability and ease of preparation might also be positive factors when the phenoxyacetic acid analogs are compared with *p*-amidinophenylpyruvic acid. We have observed that commercial samples of *p*-amidinophenylpyruvic acid are often highly impure and, through personal experience, very difficult to purify. Pyruvic acid derivatives are often subject to oxidation and decomposition in solution; in contrast, none of the compounds we have reported here have been excessively difficult to obtain pure or show any detectable instability up to periods of a year or more.

No dramatic separations of enzyme inhibitory or anticoagulant activities have been achieved with the compounds synthesized; in fact, activity appears to run parallel in this series of compounds *vs.* all systems tested. However, these and similar compounds are of potential interest in areas where the counteraction of pathological proteolytic activity is desired, *i.e.*, in the areas of thrombosis, excessive fibrinolysis, shock, allergic and inflammatory conditions, etc.

Experimental Section

Enzyme Inhibition Studies. Amidase Assay. The inhibitory effects of the various amino acids, amidino acids, and amidino esters were conveniently measured in an assay system using *N*-benzoyl-DL-arginine-*p*-nitroanilide hydrochloride (BANA) as substrate for bovine trypsin, bovine thrombin, and procine pancreatic kallikrein. The detailed biochemical procedures, including sources and purity of enzymes, have been reported previously.[7]

Partial Thromboplastin Time (PTT). The assay was carried out essentially as described by Nye, *et al.*[15] To 0.1 ml of human plasma incubated for 1 min at 37° were added 0.1 ml of 0.154 *M* NaCl (with or without inhibitor) and 0.1 ml of partial thromboplastin solution (Thrombofax reagent, Ortho Diagnostics). After subsequent addition of 0.1 ml of 0.02 *M* $CaCl_2$ the time until formation of a clot was measured.

Organic Syntheses. Melting points were determined with a Thomas-Hoover capillary apparatus and are uncorrected. Infrared (ir) spectra were determined in KBr disks, except as specified, with a Perkin-Elmer 257 grating infrared spectrophotometer. Elementary analyses were performed by Atlantic Microlab, Inc., Atlanta, Ga. Where analyses are indicated only by symbols of the elements, analytic results obtained for those elements were within ±0.4% of the theoretical values.

2-Bromo-4-cyanophenol,[16] 2,6-dibromo-4-cyanophenol,[17] 2-chloro-4-cyanophenol,[7] 2,6-dichloro-4-cyanophenol,[17] 2-iodo-4-cyanophenol,[16] 2,6-diiodo-4-cyanophenol,[16] 2-nitro-4-cyanophenol,[16] 3-cyanocinnamic acid,[18] 4-cyanocinnamic acid,[19] and 4-cyanophenoxyacetic acid[20] were prepared by literature methods.

Cyano Esters (Table I). The following procedures are typical of the reaction conditions employed.

Ethyl 4-Cyanophenoxyacetate (2). A mixture of 5.95 g (0.05 mol) of 4-cyanophenol, 10 g (0.06 mol) of ethyl bromoacetate, 6.7 g (0.05 mol) of anhydrous potassium carbonate, and 80 ml of dry acetone was refluxed for 24 hr. The acetone was then distilled off and water (100 ml) was added to give a white precipitate. Recrystallization of the product from *n*-hexane or 50% ethanol yielded the corresponding esters.

Ethyl 4-Cyanocinnamate (12). 4-Cyanocinnamic acid was refluxed with an excess of thionyl chloride for 16 hr. After evaporation of the excess of thionyl chloride the residue was dissolved in benzene and absolute ethanol and refluxed overnight. Evaporation of the solvent gave a solid. Recrystallization of the crude product from 50% EtOH yielded the corresponding ester.

Ethyl 4-Cyanooxanilic Acid (14). To a suspension of 4-aminobenzonitrile (1.2 g, 0.01 mol) and potassium carbonate (1.4 g, 0.01 mol) in 50 ml of dry benzene was added dropwise ethyloxalyl chloride (1.4 g, 0.01 mol) during a period of 10 min. The mixture was refluxed for 1 hr and was stirred at room temperature overnight. After evaporation of the solvent, H_2O (100 ml) was added and the product collected by filtration and washed thoroughly with water. Recrystallization of the crude product from 50% ethanol gave the desired compound.

Amino Acids (Table II). The procedure of Kazmirowski, *et al.*,[21] was followed. A mixture of cyano acid in methanol–concentrated ammonium hydroxide (2:1) and W-2 Raney nickel was hydrogenated at room temperature at an initial pressure of 45 psi for 5 hr. The filtrate of the reaction mixture was concentrated to dryness and the residue was recrystallized from water to yield the corresponding amino acid.

Amidino Esters (Table IV). The procedure of Dox[22] was employed.

Ethyl 4-Amidinocinnamate (41). Into a solution of ethyl 4-cyanocinnamate (1.02 g, 5 mmol) in 20 ml of absolute ethanol cooled to 0–5° was passed dry HCl gas for 5 min. The solution was stirred overnight. Evaporation of the ethanol and hydrogen chloride *in vacuo* at room temperature gave a white solid which was collected and washed with ether. The product, an imino ether, was kept in a desiccator over alkali for several days to remove excess HCl. The imino ether was then treated with 1 equiv of alcoholic ammonia solution with stirring overnight. Evaporation of the excess of alcoholic ammonia gave the product, ethyl 4-amidinocinnamate hydrochloride. Recrystallization of the product from ethanol–ether yielded the pure compound.

Ethyl 4-Hydroxyamidinophenoxyacetate (45). To a solution of $NH_2OH \cdot HCl$ (1.05 g, 0.015 mol) and K_2CO_3 (1.04 g, 0.0075 mol) in H_2O (9 ml) was added a solution of ethyl 4-cyanophenoxyacetate (2.05 g, 0.01 mol) in ethanol (40 ml). The mixture was stirred at reflux for 3 hr. After the mixture was cooled to room temperature the solvent was stripped off *in vacuo*. Water (100 ml) was added to the residue. The solids were filtered and washed with water. The crude crystals were recrystallized from 50% EtOH to give ethyl 4-hydroxyamidinophenoxyacetate, which was dissolved in an excess of absolute ethanol saturated with HCl gas. After evaporation of the solvent the residue was recrystallized from EtOH–Et_2O to give compound **45**: mp 159–161° (1.85 g, 67%).

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References and Notes

(1) M. Mares-Guia and E. Shaw, *J. Biol. Chem.*, **240**, 1579 (1965).
(2) F. Markwardt, H. Landmann, and P. Walsmann, *Eur. J. Biochem.*, **6**, 502 (1968).
(3) J. D. Geratz, *Arch. Biochem.*, 118, 90 (1967).
(4) J. D. Geratz, *Experientia*, **25**, 1254 (1969).
(5) J. D. Geratz, "Pulmonary Emphysema and Proteolysis," C. Mittman, Ed., Academic Press, New York, N.Y., 1972, p 325.
(6) G. E. Davies and J. S. Lowe, *Advan. Exp. Med.*, **8**, 453 (1970).
(7) J. D. Geratz, A. C. Whitemore, M. C.-F. Cheng, and C. Piantadosi, *J. Med. Chem.*, **16**, 970 (1973).
(8) J. D. Geratz and W. P. Webster, *Arch. Int. Pharmacodyn. Ther.*, **194**, 359 (1971).
(9) J. D. Geratz, unpublished observation.
(10) G. Glover, C. C. Wang, and E. Shaw, *J. Med. Chem.*, **16**, 62 (1973).
(11) S. J. Singer, *Advan. Protein Chem.*, **22**, 1 (1967).
(12) K. Tanizawa, S. Ishii, and Y. Kanaoka, *Chem. Pharm. Bull.*, **18**, 2247 (1970).
(13) T. Inagami, *J. Biol. Chem.*, **239**, 787 (1964).
(14) F. Markwardt, H.-P. Klocking, and G. Nowak, *Experientia*, **27**, 812 (1971).
(15) S. W. Nye, T. B. Graham, K. M. Brinkhous, *Amer. J. Med. Sci.*, **243**, 279 (1962).
(16) S. S. Berg and G. Newberg, *J. Chem. Soc.*, 642 (1949).
(17) French Patent Specification 1,375,311 (patent assigned to May and Baker Ltd.) (The Patent Office, Paris, 1965); *Chem. Abstr.*, **62**, 3982*h* (1965).
(18) R. H. Wiley and N. R. Smith, *J. Amer. Chem. Soc.*, **70**, 1560 (1948).
(19) (a) N. Moses, *Chem. Ber.*, **33**, 2623 (1900); (b) H. Rapport, A. R. Williams, O. G. Lorve, and W. W. Spooner, *J. Chem. Soc.*, 1125 (1953).
(20) N. V. Hages and G. E. K. Branch, *J. Amer. Chem. Soc.*, **65**, 1555 (1943).
(21) P. N. Kazmirowski, H. Landmann, and F. Markwardt, *Pharmazie*, **22**, 465 (1967).
(22) A. W. Dox, "Organic Syntheses," Collect. Vol. I, Wiley, New York, N.Y., 1932, p 5.
(23) IBA Ltd., French Patent 1,517,896 (March 22, 1968); *Chem. Abstr.*, **72**, 12413m (1970).
(24) G. Wagner, Ch. Garbe, and P. Richter, *Pharmazie*, **28**, 724 (1973).
(25) P. Walsmann, F. Markwardt, P. Richter, J. Sturzebicher, G. Wagner, and H. Landmann, *Pharmazie*, **29**, 333 (1974).
(26) M. Mares-Guia, E. Shaw, and W. Cohen, *J. Biol. Chem.*, **242**, 5777 (1967).

Base-Catalyzed and Cholinesterase-Catalyzed Hydrolysis of Acetylcholine and Optically Active Analogs

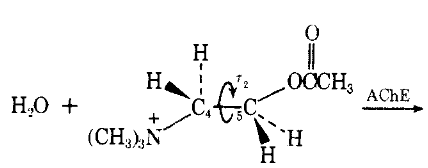
Katharine B. Schowen,* Edward E. Smissman, and William F. Stephen, Jr.

Department of Medicinal Chemistry, School of Pharmacy, The University of Kansas, Lawrence, Kansas 66044. *Received May 16, 1974*

The base- and cholinesterase-catalyzed hydrolyses of the following optically active analogs of acetylcholine were studied: 3(a)-trimethylammonium-2(a)-acetoxy-*trans*-decalin iodide, *threo*- and *erythro*-α,β-dimethylacetylcholine iodide, α-methylacetylcholine, and β-methylacetylcholine. Evidence that the optimum dihedral ⁺N-C-C-O angle in the transition state for acetylcholinesterase hydrolysis of acetylcholine analogs is positive and anticlinal is given. The data obtained suggest that acetylcholine undergoes a geometrically flexible mode of attachment to the enzyme.

Acetylcholine [ACh (1)] is well known as the chemical transmitter of nerve impulses in cholinergic neural systems.[1] Once it has accomplished its function at a receptor site it is rapidly destroyed in a hydrolytic reaction catalyzed by the enzyme acetylcholinesterase (AChE, E.C. 3.1.1.7) as given in eq 1.

The work reported herein is part of a continuing investigation[2-7] into the structural requirements for cholines-



1

$$(CH_3)_3\overset{+}{N}CH_2CH_2OH + CH_3COOH \quad (1)$$