Novel Bis(benzamidino) Compounds with an Aromatic Central Link. Inhibitors of Thrombin, Pancreatic Kallikrein,[1] Trypsin, and Complement

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A series of novel aromatic diamidines was synthesized and evaluated for antiproteolytic activity. The compounds were distinguished by inclusion of an aromatic ring structure—either benzene or bisbenzene or naphthalene—in the link between two amidinobenzene moieties. A highly potent inhibitor of bovine thrombin was discovered in α,α'-bis(4-amidino-2-iodophenoxy)-*p*-xylene with a K_i value of 1.1×10^{-7} M (pH 8.1, 37°), while α,α'-bis(4-amidino-2-iodophenoxy)-*m*-xylene was found to be an outstanding inhibitor of porcine pancreatic kallikrein ($K_i = 3.1 \times 10^{-8}$ M). Several of the compounds investigated also demonstrated a considerable blocking effect on trypsin and on the complement-dependent immune lysis of red cells.

For more than 30 years aromatic diamidines have been of considerable pharmacologic and medicinal interest. The main reason for the attention has been their antiprotozoal and antifungal activity, and numerous compounds have been synthesized and tested in this respect. In the recent past it has become evident, however, that diamidines are also potent, competitive, reversible inhibitors of a number of biologically important arginine- or lysine-specific esteroproteases including enzymes involved in blood coagulation,[2,3] kinin release,[4] and complement activation.[5] Recognition of these inhibitory properties with their implication for new clinical uses of diamidines has led us to a systematic search for ever more active compounds. Previously we reported on a large number of agents where two benzamidine moieties were linked by dioxyalkane[6,7] or monooxyalkane[7] chains, and we showed the influence of a variety of substitutions and other modifications on inhibitory potency. In a further attempt to improve the fit of the inhibitors to the active sites of the enzymes under study, i.e., thrombin, pancreatic kallikrein, and trypsin, and thus to increase hydrophobic bonding, we have now introduced various aromatic ring structures into the central link of the compounds. As will be shown below, this resulted in a remarkable gain in antiprotease activity and also led to several diamidines with considerable anticomplement effects. With respect to the reversible inhibition of thrombin and pancreatic kallikrein, some of the new compounds surpass in potency all previously reported aromatic diamidines as well as monoamidines.[8-10]

Results and Discussion

Inhibition of Bovine Thrombin, Porcine Pancreatic Kallikrein, and Bovine Trypsin. Amidase Assay. The dissociation constants of the new compounds with the enzymes were determined from rate assays employing synthetic amide substrates. The reactions followed Michaelis–Menten kinetics, and inhibition was strictly competitive and reversible for all agents examined.

In Table I we have listed the compounds synthesized with their structural formulas and their respective K_i values. According to the aromatic moiety introduced into the central link three main groups of inhibitors can be distinguished, those with a benzene ring, those with naphthalene, and those with bisbenzene. In the case of the first and third class of compounds the ether bonds of the interposed methoxy groups were always directed toward the terminal benzamidine moieties, while in the second class the ether bonds of a number of compounds were established with the central naphthalene.

In the lead-off compound **1** in the benzene series the central configuration was that of *p*-xylene, and both terminal amidino groups were in the para position also. This arrangement produced considerable affinity for trypsin, less for thrombin, and least for kallikrein. Shifting both amidino groups into the meta position (compound **2**) or replacing *p*-xylene by *m*-xylene (compound **3**) resulted in a decrease in antithrombin and antitrypsin activity, but led to a doubling of the affinity for kallikrein. The combination of the *m*-xylene arrangement with a bis-*m*-amidine grouping (compound **4**) further augmented antikallikrein activity while for the other two enzymes the K_i values were nearly identical with those of compound **3**. A significant increase in potency against thrombin and kallikrein was observed when one bromine or iodine was substituted on each of the benzamidine moieties of compound **1**, thus arriving at compounds **5** and **6**, respectively. Compound **6** with its inhibition constant of 1.1×10^{-7} M represents the strongest reversible thrombin inhibitor of the amidine type reported so far. The rather exacting requirements for optimal accommodation of the inhibitors within the active sites of the individual enzymes are well demonstrated by our experience with compound **7**, the *m*-xylene analogue of compound **6**. The seemingly minor rearrangement of the methyl group from the para into the meta position resulted in a loss of antithrombin activity by a factor of 10, a gain in antitrypsin effect by a factor of 3, and, most remarkably, a rise in antikallikrein activity by a factor of 20. The inhibitors in class I were rounded out by two compounds which possessed four chlorine substitutions on the central benzene ring. For compound **8**, the *p*-xylene derivative, only the affinity for kallikrein was improved over the unsubstituted parent compound. For compound **9**, the *m*-xylene derivative, on the other hand, binding to thrombin and trypsin was facilitated, while the K_i value for kallikrein remained nearly the same as for the unsubstituted analogue **3**.

For the naphthalene derivatives in class II the dissociation constants were of about the same order of magnitude as for the benzene-linked inhibitors in class I. Again it is well demonstrated that the position of the amidino groups and also the location of the bonds on the central naphthalene moiety were of importance for the inhibitory activity. Switching the amidino groups from their para position in compound **10** to the meta position in compound **11**, for example, significantly improved the inhibitory performance against all three enzymes. A similar observation was made with compounds **12** and **13**, respectively, though here only the potency against thrombin and kallikrein was bolstered, while the K_i value for trypsin remained unaffected. It is also noteworthy that the inward or outward location of the ether bonds influenced the potency of otherwise identical compounds. Thus the dioxynaphthalene derivative **13** was twice as effective against thrombin and trypsin as the dimethylnaphthalene derivative **14**.

Proceeding now to the bisbenzene derivatives in class

III, we can see that the parent compound **18** was a respectable inhibitor of all three enzymes, though it was most active against thrombin. Bromine substitution on each of the amidinobenzene moieties (compound **19**) potentiated the affinity for thrombin and trypsin, but not against kallikrein, an experience quite different from the findings with the benzene-linked compounds in class I. Meta positioning of both amidino groups in compound **20** did not alter the antikallikrein effect, yet reduced antithrombin and antitrypsin activity to one-third of the value for the di-*p*-amidino compound **18**.

Inhibition of the Clotting Activity of Human Thrombin and of the Overall Coagulation Process of Human Plasma. In previous experiments we had shown that the inhibitory effect of diamidines on the amidase activity of bovine thrombin did not always truly reflect their effect on the clotting activity of human thrombin.[3,6,7] The basis for this divergent behavior appeared to lie less in the species differences between the two thrombins than in the difference between the two substrates employed, a low-molecular-weight amide with the bovine enzyme and a high-molecular-weight protein, i.e., fibrinogen, with the human enzyme. In addition, we had observed earlier[3,7] that results from the thrombin–fibrinogen clotting test did not allow accurate predictions as to the effectiveness of a given inhibitor in a more encompassing coagulation test such as the partial thromboplastin time (PTT). The reason for this can be sought in the fact that the PTT is sensitive to interference not only at the final thrombin-fibrinogen reaction but also at earlier steps in the clotting cascade. Thus, diamidines can be expected to block at least one other well-characterized arginine-specific clotting enzyme besides thrombin, i.e., factor Xa.[11] Furthermore, electrostatic binding of diamidines to negatively charged phospholipid micelles may eliminate such micelles as sites for the interaction of factors Xa and V with prothrombin.

From the preceding it did not come as a surprise that also for our current compounds the inhibitory activities in the amidase assay, in the thrombin–fibrinogen clotting test and in the PTT, did not necessarily parallel each other. This becomes evident from a comparison of the K_i values in Table I with the coagulation results in Table II and from a comparison between the two coagulation tests themselves. For example, the biphenyl compound **18**, though having a dissociation constant five times greater than the benzene derivative **6**, was more effective than the latter in the thrombin–fibrinogen clotting test. And compound **1**, though considerably less active than its halogen-substituted counterparts **5** and **6** in the thrombin–fibrinogen clotting test, was by far superior to those two inhibitors in the PTT. As an overall in vitro anticoagulant compound **1** proved to be second only to the previously reported 1-(4-amidino-2-iodophenoxy)-5-(3-amidinophenoxy)pentane.[7] Here it should be added that we also prepared and tested α-(4-amidinophenoxy)-α'-(4-nitrophenoxy)-*p*-xylene (compound **21**), the mononitro derivative of compound **1**. In agreement with earlier results the replacement of one of the two amidino groups led to a significant loss of inhibitory strength, the K_i value against thrombin increasing from 3.4×10^{-6} to 5.2×10^{-6} M. Even more striking, however, was the poor showing of the nitro compound in the PTT where there was no inhibition at all at concentrations of 5×10^{-6} and 1×10^{-5} M and where the clotting time at 5×10^{-5} M was prolonged only to 81.5 s. A general impression we can take away from an evaluation of the data in Table II is that benzene substitution in the central link of the diamidines appears to be more conducive to anticoagulant activity than naphthalene substitution.

In Vivo Anticoagulant Effects of Diamidines. Having demonstrated the considerable in vitro anticoagulant activity of compound **1** we then proceeded to investigate its in vivo effectiveness. As an experimental animal we chose the rat after we had ascertained that the PTT of rat plasma was prolonged by diamidines to a similar degree as the PTT of human plasma. To our surprise, intravenous administration of the inhibitor did not alter the PTT of the postinjection plasma though the amount of 1.9 mg/kg of body weight given should have been sufficient to bring about a significant retardation. The dose had been estimated from in vivo results with 1-(4-amidinophenoxy)-5-(3-amidinophenoxy)pentane dihydrochloride. This latter compound is about half as potent as compound **1** in the PTT, and when 3 mg/kg of body weight was injected intravenously into the rat the activated PTT at the end of the 10-min injection period was lengthened to 73 s from a control value of 31 s. The study of additional diamidines revealed for some of them a similar absence of in vivo activity as found with compound **1**. An explanation for the phenomenon is only speculative at this time, but the seeming ineffectiveness might have been the result of release of clotting activators from the vessel walls into the bloodstream as a consequence of the more or less pronounced vasodilating properties of diamidines.[4] Acceleration of coagulation by the activators might then have compensated for the retarding influence of the inhibitors. It is interesting, however, that in the dog even marked hypotension following injection of some diamidines did not obviate the anticoagulant effect,[4] and also that in the rat—where the drop in pressure did not exceed 30 mmHg from the mean pressure of 100 mmHg—the loss of activity did not appear to be directly related to the degree of hypotension produced.

Anticomplement Activity of Diamidines. During activation of the complement system, initial binding of C1 to the antigen–antibody complex is followed by an interaction of C1r and C1s. Both these subcomponents of C1 are potential esteroproteases, and in its active form C1r is known to convert C1s to C1 esterase which in turn will split C4 and C2. Asghar et al.[5] have shown that esterolysis by C1r and C1s and also the activation of C1s by C1r are susceptible to inhibition by aromatic diamidines. Among the limited number of compounds tested they found the strongest inhibitors in 2,2'-dibromopropamidine [1,3-bis(4-amidino-2-bromophenoxy)propane diisethionate] and M & B 4596 [2,7-di(*m*-amidinophenyldiazoamino)-10-ethyl-9-phenylphenanthridinium chloride dihydrochloride]. As M & B 4596 shares with our present compounds the incorporation of a central aromatic moiety, we thought it of considerable interest to extend our inhibition studies to the complement system. In this investigation we made use of a hemolytic assay, the results of which reflect the overall inhibition of the complement system, and for comparative purposes we included in the evaluation M & B 4596, 2,2'-dibromopropamidine, 2,2'-dibromopentamidine [1,5-bis(4-amidino-2-bromophenoxy)pentane], and 2,2'-diiodopentamidine. The data in Table III demonstrate that with only one exception (no. **10**) all compounds exerted an inhibitory influence on the hemolytic reaction. In general, the central naphthalene moiety appeared more effective than the benzene moiety in imparting inhibitory strength to the diamidines. It is also evident that the position of the amidino groups was important for the activity of some compounds (no. **10** vs. no. **11**) but not for

Table I. Aromatic Diamidines with a Central Aromatic Link. Inhibition Constants with Thrombin, Pancreatic Kallikrein, and Trypsin

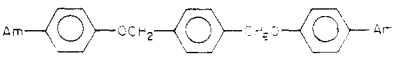
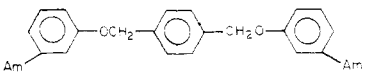
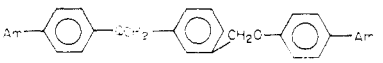
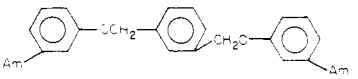
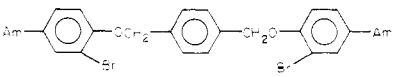
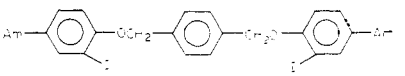
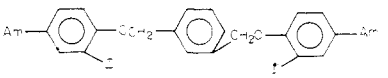
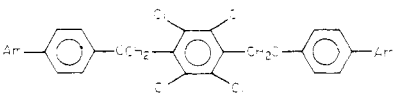
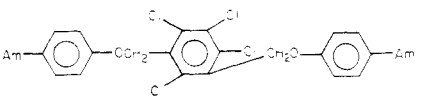
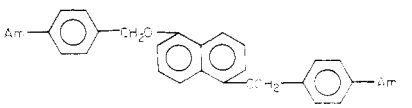
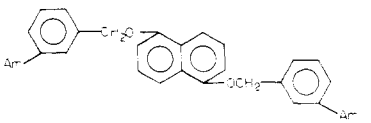
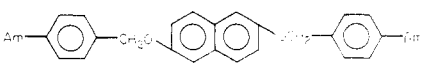
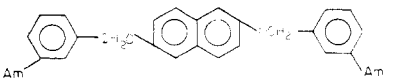
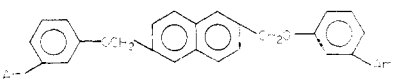
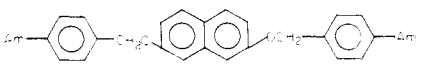
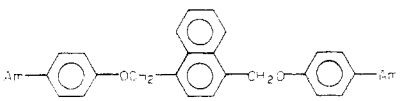
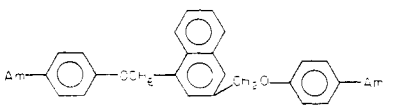
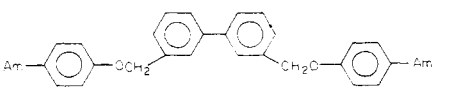
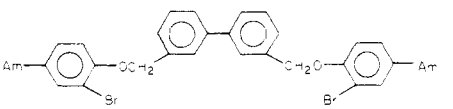
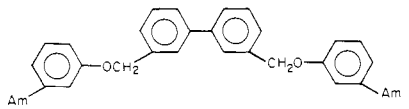
Compd no.	Structure[a]	K_i, μM[b] Thrombin	Kallikrein	Trypsin
	I. Benzene in Central Chain			
1		3.4 ± 0.9	11.4 ± 0.6	1.3 ± 0.32
2		5.0 ± 0.8	5.8 ± 1.71	6.1 ± 0.14
3		4.8 ± 0.4	6.7 ± 0.47	3.5 ± 0.35
4		4.6 ± 0.4	3.4 ± 0.79	3.2 ± 0.2
5		0.16 ± 0.01[c]	0.71 ± 0.1	1.1 ± 0.13
6		0.11 ± 0.03[c]	0.61 ± 0.22[d]	2.3 ± 0.55
7		1.2 ± 0.1	0.031 ± 0.01[d]	0.67 ± 0.14
8		6.7 ± 0.9	3.4 ± 1.1	1.2 ± 0.11
9		1.5 ± 0.2	5.2 ± 1.14	1.3 ± 0.3
	II. Naphthalene in Central Chain			
10		5.1 ± 0.8	8.6 ± 0.5	2.8 ± 0.4
11		2.9 ± 0.1	2.2 ± 0.4	0.42 ± 0.02
12		6.0 ± 1.5	9.7 ± 2.1	3.2 ± 0.3
13		2.4 ± 0.6	5.5 ± 1.0	3.2 ± 0.9
14		5.1 ± 0.1	5.5 ± 1.2	5.9 ± 0.6
15		4.9 ± 0.4	2.8 ± 0.5	1.8 ± 0.5
16		6.6 ± 0.5	2.5 ± 0.2	1.9 ± 0.4
17		5.2 ± 0.8	1.04 ± 0.1	1.8 ± 0.3
	III. Bisbenzene in Central Chain			
18		0.57 ± 0.05[c]	1.6 ± 0.2	1.4 ± 0.13
19		0.27 ± 0.021[c]	2.2 ± 0.77	0.25 ± 0.053

Table I (*Continued*)

Compd no.	Structure[a]	K_i, μM[b] Thrombin	Kallikrein	Trypsin
20		1.67 ± 0.33	1.76 ± 0.32	4.01 ± 1.03

[a] Am, amidino group. [b] K_i, dissociation constant of the enzyme-inhibitor complex. Values are means ± SD; $n = 3$. [c] BPVANA used as substrate. [d] BPPANA used as substrate.

Table II. Inhibitory Effect of Diamidines on the Thrombin Clotting Test and on the Partial Thromboplastin Time

Compd no.	Thrombin clotting test,[a] concn of inhibitor producing 50% inhibn, μM	Partial thromboplastin time,[b] s (control 59 ± 2.6 s), at inhibitor concn (M) 10^{-4}	10^{-5}	5×10^{-6}
1	17	725 ± 6.9	176 ± 1.0	147 ± 0.6
2	13	391 ± 16.7	122 ± 0.8	80 ± 3.6
3	25		117 ± 2.1	87 ± 4.3
4	6.5	714 ± 17.2	112 ± 1.1	92 ± 0.6
5		258 ± 11.1	110 ± 3.6	96 ± 3.6
6	1.0		92 ± 0.7	80 ± 0.7
7		369 ± 8.6	70 ± 0.6	64 ± 0.9
9	8		76 ± 3.1	62 ± 0.4
10	80	141 ± 1.2	61 ± 0.3	60 ± 0.3
11	20		75 ± 1.0	65 ± 0.9
13	30	277 ± 2.8	72 ± 2.3	61 ± 1.6
14	72			
15	20		66 ± 0.7	63 ± 1.0
16		203 ± 2.0	71 ± 0.7	64 ± 1.5
17	15	147 ± 2.0	67 ± 0.9	62 ± 0.2
18	0.73	412 ± 3.7	83 ± 0.6	72 ± 0.4
19	10			62 ± 0.4
20	4		93 ± 4.4	73 ± 1.6

[a] Values are the means of two determinations. [b] Values are means ± SD; $n = 60$ for the control and $n = 3$ for the inhibitor assays.

Table III. Inhibitory Effect of Diamidines on the Complement-Dependent Immune Hemolysis

Compd no.	Inhibn, %, at inhibitor concn (M)[a] 10^{-4}	3×10^{-5}	10^{-5}
1	25.9	0	0
2	19.1	14	5.6
3	24.9	10.3	0
4	34	13.7	0
6		29.8	0
7	>50	32.6	0
8		>50	0
9	>50	>50	5.9
10	0	0	0
11	39	8.9	0
13	>50	>50	11
14		47	36.6
15	>50	37.4	12.8
16		10.8	2.9
17	>50	15.3	5.6
M & B 4596	>50	>50	50
2,2'-Dibromopropamidine	>50	26.9	6.3
2,2'-Dibromopentamidine	>50	28.5	20.8
2,2'-Diiodopentamidine		>50	26.9

[a] All values are the means of three separate determinations.

others (no. 3 vs. no. 4). One of the most significant findings was the considerable augmentation of potency produced by the introduction of halogen on either the terminal or central benzene moieties. These results suggest that halogen substitution on the naphthalene derivatives would probably raise their strength to the level of M & B 4596 which has not yet been reached by any of the present inhibitors. Finally, a comparison of the three dioxyalkane derivatives which round out Table III reveals that lengthening of the central chain from three to five carbons advanced inhibitory strength and, also, that substitution of iodine for bromine brought about a further increase in activity.

Experimental Section

Amidase Assays. The dissociation constants (K_i) of the inhibitors with the three enzymes were obtained from amidase assays employing generally N^{α}-benzoyl-DL-arginine-*p*-nitroanilide hydrochloride[12] (BANA, Bachem, Inc.) as substrate. When K_i values for thrombin decreased below 1×10^{-6} M, a more sensitive substrate, *N*-benzoyl-L-phenylalanyl-L-valyl-L-arginine-*p*-nitroanilide hydrochloride[13] (BPVANA, A. B. Bofors, Nobel Division) was substituted for BANA. Similarly, in the case of pancreatic kallikrein K_i values in the range of 5×10^{-7} M or less could be determined accurately only with a substrate which allowed reduction of the amounts of enzyme in the assay mixtures. For those instances we turned to *N*-benzoyl-L-prolyl-L-phenylalanyl-L-arginine-*p*-nitroanilide hydrochloride (BPPANA, Fox Chemical Co.), a derivative of the carboxyl terminal tripeptide of bradykinin. The K_m value for BPPANA and pancreatic kallikrein of 2.75×10^{-4} M (pH 8.1, 37°) was only one-third that for BANA, and the catalytic rate was more than 60 times greater than with the latter material. The K_i values for the inhibitors were determined graphically according to Dixon[14] by plotting the reciprocals of the initial reaction velocities at two different substrate concentrations against different inhibitor concentrations.

For each enzyme the reaction mixtures consisted of 1.6 ml and included 0.09 M Tris, 10% by volume dimethyl sulfoxide, and either BANA (3×10^{-3} or 1×10^{-3} M) or BPVANA (5×10^{-4} or 2.5×10^{-4} M) or BPPANA (5×10^{-4} or 2×10^{-4} M). In the assays for trypsin there was, in addition, 0.02 M $CaCl_2$. The following amounts of enzymes were included in the respective assay mixtures: thrombin, 44.44 NIH units in the tests with BANA and 0.22 NIH units in the tests with BPVANA; pancreatic

Table IV. Diamidino Derivatives

Compd no.	Mp, °C	Yield, %	Formula	Analyses
1	277-279	15	$C_{22}H_{22}N_4O_2 \cdot 2HCl$	C, H, N
2	286-288	12	$C_{22}H_{22}N_4O_2 \cdot 2HCl$	C, H
3	210-212	20	$C_{22}H_{22}N_4O_2 \cdot 2HCl \cdot 0.5H_2O$	C, H, N
4	290	55	$C_{22}H_{22}N_4O_2 \cdot 2HCl \cdot H_2O$	C, H, N
5	285 dec	10	$C_{22}H_{20}Br_2N_4O_2 \cdot 2HCl$	C, H, N
6	237-239	25	$C_{22}H_{20}I_2N_4O_2 \cdot 2HCl$	C, H
7	270-272	65	$C_{22}H_{20}I_2N_4O_2 \cdot 2HCl$	C, H, N
8	299-301	23	$C_{22}H_{18}Cl_4N_4O_2 \cdot 2HCl \cdot H_2O$	C, H, N
9	247-249	20	$C_{22}H_{18}Cl_4N_4O_2 \cdot 2HCl \cdot H_2O$	C, H, N
10	>300	28	$C_{26}H_{24}N_4O_2 \cdot 2HCl$	C, H, N
11	240 dec	5	$C_{26}H_{24}N_4O_2 \cdot 2HCl$	C, H, N
12	>300	10	$C_{26}H_{24}N_4O_2 \cdot 2HCl$	C, H
13	>300	29	$C_{26}H_{24}N_4O_2 \cdot 2HCl$	C, H, N
14	>300	20	$C_{26}H_{24}N_4O_2 \cdot 2HCl$	C, H
15	175	40	$C_{26}H_{24}N_4O_2 \cdot 2HCl \cdot 2H_2O$	C, H, N
16	280	17	$C_{26}H_{24}N_4O_2 \cdot 2HCl$	C, H
17	238-240	58	$C_{26}H_{24}N_4O_2 \cdot 2HCl$	C, H, N
18	279-281 dec	23	$C_{28}H_{26}N_4O_2 \cdot 2HCl \cdot 0.5H_2O$	C, H, N
19	262-264	33	$C_{28}H_{24}Br_2N_4O_2 \cdot 2HCl$	C, H, N
20	150-152	30	$C_{28}H_{26}N_4O_2 \cdot 2HCl$	C, H, N
21	208-210	4	$C_{21}H_{19}N_3O_4 \cdot HCl$	C, H

kallikrein, 18.1–72.2 Frey units in the tests with BANA and 0.41–8.13 Frey units in the tests with BPPANA; trypsin, 3.33 μg. All reactions were carried out at pH 8.1 and 37°, and the length of the incubation periods was 15–40 min for thrombin, 15–180 min for kallikrein, and 15–30 min for trypsin.

Thrombin (bovine, topical) was purchased from Parke, Davis & Co. Porcine pancreatic kallikrein was obtained from Farbenfabriken Bayer AG, Wuppertal-Elberfeld, West Germany. The preparation possessed 635 Frey units/mg. Trypsin (bovine, twice crystallized, salt-free) was a product of Schwarz-Mann containing 57% active trypsin as determined by active-site titration.

Thrombin Clotting Test. This assay has been described in detail elsewhere.[2b,3] A solution of stabilized human thrombin was produced by clotting plasma in the presence of 20% v/v of ethanol, thus preventing inactivation of the thrombin formed during coagulation.[15] After separation from the fibrin clot by centrifugation, the supernatant solution containing the active enzyme could be stored for long periods in the refrigerator without any loss of thrombin activity. The diluent employed for the thrombin solution was composed of 20% by volume ethanol and 80% by volume 0.05 M Tris–HCl buffer (pH 7.4), the latter including also 0.05 M NaCl and 0.005 M $CaCl_2$. For the clotting test, the control consisted of a mixture of 0.1 ml of 0.154 M NaCl solution and 0.2 ml of human fibrinogen solution (400 mg of AB KABI fibrinogen per 100 ml of 0.146 M NaCl–0.0055 M sodium citrate solution) to which 0.2 ml of a 1:4 dilution of the stabilized thrombin solution was added. The test was carried out at 37°, and clotting occurred in approximately 50 s. In the assays including inhibitors the amount of thrombin was doubled (0.2 ml of a 1:2 dilution) over the control, and then that concentration of inhibitor was determined empirically which would retard clotting to a time matching the control value. At that point there would be inhibition of 50% of the thrombin activity in the inhibitor-containing assay.

Partial Thromboplastin Time Test (PTT). The test with human plasma was modified from the method of Nye et al.[16] Citrated plasma (0.1 ml) was incubated for 30 s at 37° together with 0.1 ml of partial thromboplastin solution (Thrombofax, Ortho Diagnostics) and 0.1 ml of 0.154 M NaCl solution or 0.154 M NaCl–inhibitor solution. At the end of the incubation period 0.1 ml of 0.02 M $CaCl_2$ solution was added, and the time until formation of a firm clot represented the partial thromboplastin time.

To determine the in vivo anticoagulant effect of the inhibitors in the rat use was made of an activated PTT test. Blood was obtained by cardiac puncture, and eight parts were mixed with one part of 0.109 M sodium citrate solution. Plasma was separated from the cells by centrifugation and 0.1 ml was added to 0.1 ml of Celite suspension (5 g of Celite/100 ml of H_2O), 0.1 ml of 0.154 M NaCl solution, and 0.1 ml of Thrombofax. After a 3-min period of repeated agitation at 37° 0.1 ml of 0.02 M $CaCl_2$ was added and the clotting time recorded.

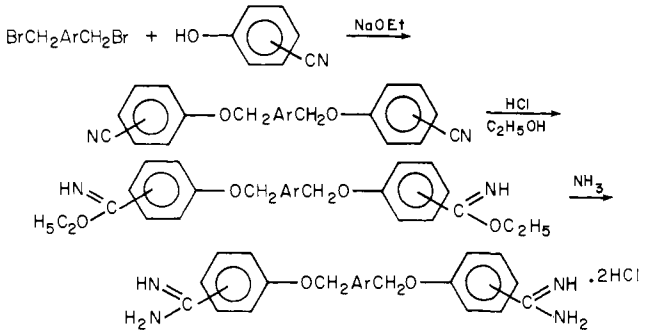
In Vivo Studies. Female rats of a Wistar strain weighing approximately 300 g were employed for these experiments. They were anesthetized by a subcutaneous injection of sodium pentobarbital (25 mg/kg of body weight). The blood pressure was recorded continuously from the femoral artery with the help of a transducer and a Grass Model 7C polygraph. Infusions of diamidines were injected into the external jugular vein, and the inhibitors were given in a volume of 0.19 ml of 0.154 M NaCl solution over a 10-min period. Blood for the clotting studies was obtained by cardiac puncture immediately after the completion of the infusion.

Complement Assay. The procedure was slightly modified from the methods of Kabat and Mayer[17] and Baker and Erickson.[18] Sheep red blood cells suspended in Alsever's solution were purchased from Granite Diagnostics, Inc. They were separated from the solution by centrifugation, washed twice in buffer, and then resuspended in buffer at a concentration of 1×10^9 cells/ml. Rabbit antibodies against the red cells (hemolysin) and guinea pig whole complement were obtained from Cordis Laboratories. The lyophilized hemolysin was reconstituted with 1 ml of cold H_2O and further diluted 1:800 for use in the assay. The lyophilized complement was dissolved in 5 ml of H_2O giving a minimal strength of 200 CH 50 units/ml, and buffer was employed for an additional 1:100 dilution.

The buffer contained 5 mM Tris, 0.5 mM $MgCl_2$, 0.15 mM $CaCl_2$, 0.15 M NaCl, and 1 g/l. of gelatin. The citrate–saline solution included 0.15 M sodium citrate and 0.12 M NaCl and was kept refrigerated at 3–4°.

The assay proper was carried out as follows. Four duplicate sets of tubes were run at a time. Into each of the tubes were placed 0.1 ml of buffer or buffer plus inhibitor and 0.4 ml of either complement solution or buffer. Following an incubation period of 15 min at 37° there was added 0.5 ml of a 1:1 mixture of red cell suspension and hemolysin, the mixture having been made up 10 min earlier and allowed to stand at room temperature. The four sets of tubes represented (1) a complement control free of inhibitor which served as the standard and against which the degree of complement inhibition was determined, (2) a control for spontaneous lysis of red cells containing neither complement nor inhibitor, (3) a control for lysis by inhibitor in the absence of complement, and (4) a set containing complement as well as inhibitor. All tubes were incubated for 12 min at 37°, and then the reaction was stopped by the addition of 2.75 ml of cold citrate–saline solution. The tubes were centrifuged, and the concentration of hemoglobin in the supernatant was determined spectrophotometrically at 524 nm. The percentage of inhibition was calculated from a standard curve which had been prepared with varying concentrations of complement. Due to the sigmoid shape of the curve accurate percentages of inhibition could be determined only in the range from 0 to 50%. Spontaneous lysis of red cells by the inhibitors was always taken into consideration in calculating the inhibition values.

Scheme I



Organic Synthesis. All melting points were determined on a Thomas-Hoover capillary melting point apparatus. Elemental analyses for all compounds were carried out by either Atlantic Microlab, Inc., Atlanta, Ga., or Galbraith Laboratories, Inc., Knoxville, Tenn. Analyses were accepted if the determined values were within ±0.4% of the theoretical values. M & B 4596 [2,-7-di(*m*-amidinophenyldiazoamino)-10-ethyl-9-phenylphenanthridinium chloride dihydrochloride] and 2,2'-dibromopropamidine [1,3-bis(4-amidino-2-bromophenoxy)propane diisethionate] were supplied by May & Baker, Ltd., Dagenham, England. The synthesis of 2,2'-dibromopentamidine [1,5-bis-(4-amidino-2-bromophenoxy)pentane dihydrochloride] and 2,-2'-diiodopentamidine [1,5-bis(4-amidino-2-iodophenoxy)pentane dihydrochloride] has been described earlier.[6] The chemical intermediates, 2-bromo-4-cyanophenol and 4-cyano-2-iodophenol, were prepared by previously described methods.[19] The general procedure for the preparation of target compounds is shown in Scheme I. The dicyano intermediates were prepared according to methods described earlier.[6,7] The diamidino derivatives were synthesized from the corresponding dicyano compounds by procedures described by Ashley et al.[20] and by Berg and Newbery.[19] Purification of the diamidines was accomplished by recrystallization in ethanol, dilute hydrochloric acid, or a mixture of the two solvents. Yields for the conversion of the dicyano intermediates to the diamidines as well as melting points and analyses for the diamidines are given in Table IV. The monocyano intermediate used in the preparation of α-(4-amidinophenoxy)-α'-(4-nitrophenoxy)-*p*-xylene (compound **21**) was prepared in two steps from α,α'-dibromo-*p*-xylene. The two-step ether synthesis was similar to the previously described method for the synthesis of unsymmetrical aliphatic dicyano diether intermediates.[7]

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Synthesis and Anticancer Activity of 5-Diethylaminomethyl Derivatives and Nitrogen Mustards of Uracil and 2-Thiouracils

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Several 5-diethylaminomethyl derivatives and nitrogen mustards of uracil and 2-thiouracil have been synthesized and tested for their potential anticancer activity in vitro on KB cells and in vivo on Ehrlich carcinoma. Among the alkylating derivatives tested several showed cytotoxic activity in vitro and compound V [5-[bis(2-chloroethyl)amino]methyl-6-propyluracil hydrochloride] showed both in vitro and in vivo anticancer activity.

Nitrogen mustard I has been independently synthesized by three research teams[1-3] as an alkylating agent related to the clinically used uracil mustard.[4] The structural difference between these two substances is the methylene bridge separating the alkylating functional group of compound I from its uracil moiety. Thus, the enamine conjugation present in the uracil mustard is no longer present in compound I and therefore the alkylation mechanisms displayed by these two substances are quite different. Recently, it has been reported that compound I inhibits the mitotic division in HeLa cells by at least two mechanisms: (i) its incorporation into DNA, and (ii) its