The reaction was controlled by the ninhydrin test of Kaiser, *et al.*[11] Ile was the only residue that had to be recoupled under these conditions. Washes included DMF, dioxane, CH_2Cl_2, and MeOH. A 1-hr HF treatment at 0° in the presence of anisole deblocked and cleaved the peptide resin. HF was thoroughly eliminated under high vacuum. The crude peptides mixed with the resin were extracted with 0.2 *N* AcOH that was lyophylized. Purification was achieved in one step by partition chromatography (system 6) (100 mg of crude mixture) on a Sephadex G25 column (1.2 × 100 cm). Cuts emphasizing purity more than yield were made on the basis of a ninhydrin test.

Asp-Arg-Val-Tyr-Ile-1-MeHis-Pro-Phe-OH. Amino acid analysis gave Asp, 1.00; Arg, 1.05; Val, 1.00; Tyr, 1.00; Ile, 0.90; 1-MeHis, 1.00; Pro, 0.95; Phe, 1.10. R_f systems: 0.34, 1; 0.56, 2; 0.32, 3; 0.71, 4; 0.40, 5; 0.39, 6; 0.26, 7. $[\alpha]^{25}D$ −55° (*c* 0.40, 1% AcOH).

Asp-Arg-Val-Tyr-Ile-3-MeHis-Pro-Phe-OH. Amino acid analysis gave Asp, 1.00; Arg, 1.05; Val, 0.95; Tyr, 1.00; Ile, 1.00; 3-MeHis, 1.10; Pro, 0.95; Phe, 1.20. R_f systems: 0.35, 1; 0.57, 2; 0.33, 3; 0.67, 4; 0.38, 5; 0.40, 6; 0.27, 7. $[\alpha]^{25}D$ −54° (*c* 0.66, 1% AcOH).

Nmr showed for both compounds sharp absorptions for im-2-H and im-4-H; sharp AB system for the four aromatic protons of tyrosine and one singlet for the five protons of phenylalanine; α-CH_2-Gly and the CH_3 groups of Ileu were also easily recognizable. All protons integrated properly. Acetyl-Asn-Arg-Val-Tyr-Val-His-Pro-Phe-OH was prepared from [Asn[1],Val[5]]-AII (Hypertensin, Ciba, the generous gift of Dr. B. Riniker) by acetylation according to Riordan and Vallee.[12] The material was purified by preparative thin-layer chromatography (Analtech) in system 6.

Methods. Isolated uterine strips were prepared as previously described.[3] The tissue was then suspended in a 5-ml tissue bath at room temperature in de Jalon's solution[13] (calcium 0.5 m*M*) and aerated with either 95% O_2–5% CO_2 (pH 6.8) or with 100% O_2 (pH 8.1).

Blood Pressure Determinations. Blood pressure was measured in albino rats (150–200 g) anesthetized with sodium pentobartibal (30 mg/kg iv) and treated with phenoxybenzamine (30 mg/kg) and propranolol (15 mg/kg) by recording from the carotid artery (P-1000 A, linear-core physiograph transducer). The agent (50 μl) was injected into the cannulated jugular vein. An AII dose–response curve was determined before and after the administration of each analog.

Prostaglandin Release. The release of prostaglandin-like substance (PLS) was measured from isolated rabbit kidney perfused with Krebs solution at 37° and at a constant flow of 8 ml/min. The venous effluent was superfused over a series of isolated assay tissues chosen for their sensitivity to prostaglandins (chick rectum and rat stomach strip) and to AII (rat stomach strip) as previously described.[14]

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Diamidino-α,ω-diphenoxyalkanes. Structure–Activity Relationships for the Inhibition of Thrombin, Pancreatic Kallikrein,† and Trypsin

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A series of diamidino-α,ω-diphenoxyalkanes was synthesized and examined for inhibitory activity against thrombin, pancreatic kallikrein, and trypsin. Modification of the compounds included lengthening of the alkane chain, variation in the position of the amidino groups, and the substitution of halogen on the benzene rings. The compounds function as active-site-directed reversible inhibitors, and each molecule appears to bind to only a single enzyme molecule. In an amidase assay at pH 8.1 and 37°, bovine thrombin was most effectively inhibited by 2′,2″-diiodo-4′,4″-diamidino-1,8-diphenoxyoctane (K_i = 1.1 × 10^{-6} *M*). Under the same conditions, 2′,2″-diiodo-4′,4″-diamidino-1,5-diphenoxypentane proved to be the most active inhibitor of pancreatic kallikrein and trypsin with K_i values of 4.5 × 10^{-7} and 3.4 × 10^{-7} *M*, respectively. The latter compound also was most effective in blocking the clotting activity of human thrombin.

In body tissues and body fluids proteases play a causative or adjunctive role in a number of important disease processes, such as inflammation, thrombosis, and complement-dependent immune reactions. Control of the hyperproteolytic activity offers itself as an obvious approach to alleviation of the abnormal conditions, and efforts in this direction have been made especially in the prevention and treatment of thromboembolic disorders which are among the leading causes of morbidity and mortality in man. To reduce the clotting tendency two methods have been

† Kallikrein is a registered trademark assigned to Farbenfabriken Bayer AG, Leverkusen, Federal Republic of Germany.

‡ Taken in part from a dissertation presented by Dr. M. C.-F. Cheng in Dec 1972 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.

chiefly employed: first, lowering of the levels of activatable prothrombin and of other clotting factors by vitamin K antagonists and, second, bolstering the reactivity of the natural thrombin and factor Xa inhibitor in plasma by injections of heparin. Both modes of therapy have their drawbacks, however. With vitamin K antagonists it is the long interval between application of the materials and onset of the effect; with heparin it is the necessity for intravenous administration. Furthermore, success of the treatment has often been equivocal.

For those reasons, research in recent years has chosen another route for solving the problem of hypercoagulability, *i.e.*, the development of low molecular weight active-site-directed inhibitors of thrombin. These new agents are active against thrombin itself and are designed for oral as well as parenteral use. Advances in this field have been rapid, and among the many compounds tested aromatic diamidines have turned out to be the most effective inhibitors so far.[1-3] The promising early results have prompted us to continue our investigation of diamidines, and we now wish to report on the synthesis of a large number of new compounds and on their structure-activity relationships for the inactivation of thrombin. The method of preparation of the diamidines was essentially that used by Ashley, *et al.*,[4] and by Berg and Newbery[5] which involves the preparation of the dinitriles and the conversion of the dinitriles into diamidines.

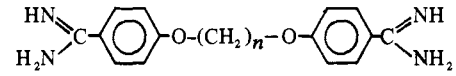
In our study we have included also pancreatic kallikrein and trypsin, two enzymes which like thrombin catalyze the hydrolysis of bonds involving the carboxyl groups of arginine or lysine. Both proteases are thought important in certain pathologic conditions and both of them have previously been shown to be susceptible to inhibition by diamidines.[6-8] Pancreatic kallikrein is a representative of a group of enzymes which are able to release vasoactive kinins from plasma globulins and thus to contribute to the vasodilatation seen in inflamed tissues or encountered in the general circulation in certain shock states. Trypsin, on the other hand, probably plays a central role in the pathogenesis of pancreatitis where autodigestion of the gland is one of the characteristic features. Trypsin has also served us as a readily available model for exploration of the mode of action of low molecular weight inhibitors, and conclusions reached for this enzyme can probably be applied equally well to thrombin and kallikrein.

Results

Protease Inhibition. Amidase Assay. Aromatic diamidines consist of two amidinobenzene moieties bound to one another by molecular chains of varied composition. In this investigation we have restricted ourselves to the study of compounds with a dioxyalkane bridge, and we have examined the influence of the following chemical modifications: first, lengthening of the hydrocarbon chain; second, alteration of the position of the amidino groups on the benzene rings; and third, substitution of halogen on the benzene rings. The inhibitory strength of the diamidines against the three enzymes was generally determined in rate assays employing the synthetic substrate N^{α}-benzoyl-DL-arginine-*p*-nitroanilide (BANA).[9] With each compound Lineweaver-Burk plots conformed to a pattern of straightforward competitive inhibition, and therefore Dixon's graphical method[10] could be used to obtain the K_i values as a measure of potency of the inhibitors.

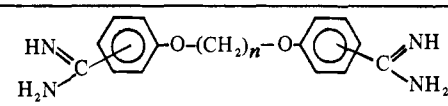
The data in Table I show that progressive elongation of

Table I. Inhibitory Effect of 4',4''-Diamidino-α,ω-diphenoxyalkanes on the Hydrolysis of BANA by Thrombin, Pancreatic Kallikrein, and Trypsin



		K_i, μM		
Compd no.	*n*	Thrombin	Kallikrein	Trypsin
1	3	11.8	16.0	3.1
2	5	8.4	8.5	2.0
3	6	7.4	4.5	1.9
4	8	6.2	3.7	1.8
5	10	13.3	3.8	1.9
6	12	16.5	1.7	1.1
7	14	29.6	3.7	3.3
8	16		10.9	7.9

Table II. Effect of Variation in the Position of the Amidino Groups on the Inhibitory Strength of Diamidino-α,ω-diphenoxyalkanes



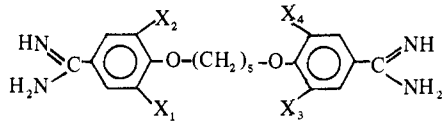
				K_i, μM		
Compd no.	*n*	Am_1	Am_2	Thrombin	Kallikrein	Trypsin
2	5	4'	4''	8.4	8.5	2.0
9	5	3'	4''	38.1	5.7	3.5
10	5	3'	3''	13.0	5.6	2.6
5	10	4'	4''	13.3	3.8	1.9
11	10	3'	3''	16.2	3.1	4.0
6	12	4'	4''	16.5	1.7	1.1
12	12	3'	3''	19.3	3.6	3.1

the central bridge in 4',4''-diamidino-α,ω-diphenoxyalkanes increased the inhibitory power against thrombin and kallikrein up to a point beyond which it began to decline again. It is noteworthy that the 8-carbon chain of compound **4** was optimal for thrombin, while for kallikrein the 12-carbon link of compound **6** proved most effective. With trypsin, compound **6** was also the most active compound in this series, but in contrast to the findings with the other two enzymes, there was only little increase in potency from the 5-carbon chain through the 10-carbon chain compound.

Next we addressed ourselves to the question of how a change in the location of the amidino groups would affect inhibitory activity. For this purpose we prepared the 3',4''-diamidino analog **9** and the 3',3''-diamidino analog **10** of the dioxypentane compound **2** and also the di-*m*-diamidino derivatives **11** and **12** of the 10-carbon and 12-carbon chain members, respectively. From a comparison of the K_i values of the new inhibitors with those of their parent compounds (Table II), it is evident that for thrombin and trypsin any shift of one or both amidino groups into the meta position resulted in a loss of potency. Against kallikrein, on the other hand, all meta derivatives, except the dodecane compound, were more effective than the corresponding di-*p*-diamidines.

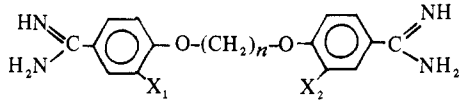
The introduction of bromine on one or both benzene rings of 4',4''-diamidino-1,5-diphenoxypentane (compound **2**) led to compounds that brought out further significant differences between the three enzymes in their susceptibility to inhibition (Table III). In the case of thrombin, only compound **14** with its single bromine substitution on each benzene moiety was more active than the unsubstituted parent compound **2**. Compound **14** turned out to be also the most active inhibitor of trypsin in the bromine series, but in contrast to thrombin, compounds **13** and **16** were also superior

Table III. Effect of Halogen Substitution on the Inhibitory Strength of 4′,4″-Diamidino-1,5-diphenoxypentane



Compd no	X_1	X_2	X_3	X_4	K_i, μM Thrombin	Kallikrein	Trypsin
			A. Bromine Substitution				
2	H	H	H	H	8.4	8.5	2.0
13	Br	H	H	H	12.6	3.6	0.97
14	Br	H	Br	H	6.17	1.74	0.52
15	Br	Br	H	H	14.1	4.22	2.17
16	Br	Br	Br	Br	9.58	0.81	0.78
			B. Chlorine and Iodine Substitution				
17	Cl	H	Cl	H	14.3	2.07	0.92
18	I	H	I	H	3.34	0.45	0.34

Table IV. Effect of Variation in the Length of the Hydrocarbon Chain on the Inhibitory Strength of 2′,2″-Dihalo-4′,4″-diamidino-α,ω-diphenoxyalkanes



Compd no.	n	K_i, μM Thrombin	Kallikrein	Trypsin
	A. Bromine Substitution (X_1, X_2 = Br)			
19	3	12.0	1.8	0.75
14	5	6.17	1.74	0.52
20	8	16.0	1.6	1.65
21	10	>10	5.96	1.44
22	12	35.0	3.8	3.37
	B. Iodine Substitution (X_1, X_2 = I)			
18	5	3.34	0.45	0.34
23	8	1.1	0.98	0.74
24	12	17.3	8.94	1.89
25	16	40.8	11.8	>5

to compound **2**. As to kallikrein, all bromine-bearing inhibitors surpassed the potency of compound **2**, and the leading compound in this instance was number **16** with its two double substitutions. A comparison of the dichlorine compound **17** with the dibromine compound **14** and the diiodine compound **18** reveals clearly that inhibitory activity against all three enzymes increased with size of the halogen. In fact, compound **18** (2′,2″-diiodo-4′,4″-diamidino-1,5-diphenoxypentane) was the most effective inhibitor of trypsin and kallikrein in this study and the second best inhibitor of the amidase activity of thrombin.

Having shown that a single halogen substitution on each of the benzene moieties markedly enhanced the inhibitory power of 4′,4″-diamidino-1,5-diphenoxypentane, we then examined the importance of the length of the alkane chain for the potency of homologous series of dibromine and diiodine compounds. From the findings the interesting fact emerged (Table IV) that the optimal chain length for the halogenated diamidines was generally less than for the corresponding unsubstituted inhibitors listed in Table I. In the dibromine series, for example, the 5-carbon chain compound **14** was now the leading inhibitor of thrombin and trypsin, while in the earlier unsubstituted series the octane and dodecane compounds, respectively, had been most active. Similar observations were also made with the diiodine compounds as regards their activity against kallikrein and trypsin. An exception occurred with thrombin, however. Here, greatest inhibitory strength remained with the 8-carbon chain derivative **23**, and this inhibitor outranked all others in its ability to block the hydrolysis of BANA by thrombin.

Table V. Inhibitory Effect of Diamidino-α,ω-diphenoxyalkanes on the Clotting of Human Fibrinogen by Ethanol-Stabilized Human Thrombin

Compd no.	Formula[a]	Concn producing 50% inhibition, μM
	A. Varying Length of the Hydrocarbon Chain	
1	Am-R-O-$(CH_2)_3$-O-R-Am	60
2	Am-R-O-$(CH_2)_5$-O-R-Am	22
3	Am-R-O-$(CH_2)_6$-O-R-Am	15
4	Am-R-O-$(CH_2)_8$-O-R-Am	20
5	Am-R-O-$(CH_2)_{10}$-O-R-Am	10
	B. Variation in the Position of the Amidino Groups	
9	Am(3′)-R-O-$(CH_2)_5$-O-R-Am	10
10	Am(3′)-R-O-$(CH_2)_5$-O-R-Am(3″)	25
11	Am(3′)-R-O-$(CH_2)_{10}$-O-R-Am(3″)	20
	C. Halogen Substitutions	
17	Am-RCl(2′)-O-$(CH_2)_5$-O-RCl(2″)-Am	13
15	Am-RBr(2′)-O-$(CH_2)_5$-O-RBr(2″)-Am	6.5
18	Am-RI(2′)-O-$(CH_2)_5$-O-RI(2″)-Am	3.0
23	Am-RI(2′)-O-$(CH_2)_8$-O-RI(2″)-Am	5.0

[a] Am = amidino group; 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′ or 4″) with respect to the central linkages.

Inhibition of the Clotting Activity of Human Thrombin. In view of the potential application of diamidines as anticoagulants in man, we evaluated the strength of the inhibitors also against human thrombin. In the assay we induced clotting of purified human fibrinogen by the addition of the ethanol-stabilized human enzyme[2] and determined the inhibitor concentration necessary to reduce thrombin activity by 50%. In Table V we have listed representative diamidines for each of the main classes of compounds studied, together with their respective I_{50} values. Generally, the potency against human thrombin was similar to that against bovine thrombin in the amidase test. There were a few significant differences, however. In group A with the varying length of the hydrocarbon chain the decane compound was most effective rather than the octane homolog. In group B it came as a surprise that the presence of one of the amidino moieties in the meta position was responsible for a doubling of the inhibitory strength of compound **9** as compared with compound **2** which carries both positive groups in the para location. Finally, in the halogen-substituted series (group C) 2′,2″-diiodo-4′,4″-diamidino-1,5-diphenoxypentane (compound **18**) was superior to the octane homolog and was the leading inhibitor with an I_{50} value of $3 \times 10^{-6} M$.

Gel Filtration of Trypsin in the Presence of Diamidine. The effectiveness of cationic compounds as enzyme inhibitors is based to a large degree on the attraction of the positive charge to the anionic aspartic acid moiety in the active site of the respective proteases. The symmetrical arrangement of the positive groups at each end of the aromatic diamidines suggested the possibility that the inhibitor molecules might bind to two enzyme molecules and thus produce dimerization of the enzyme. Such an occurrence would have explained some of the remarkable potency of diamidines. To decide on this point and to gain an insight

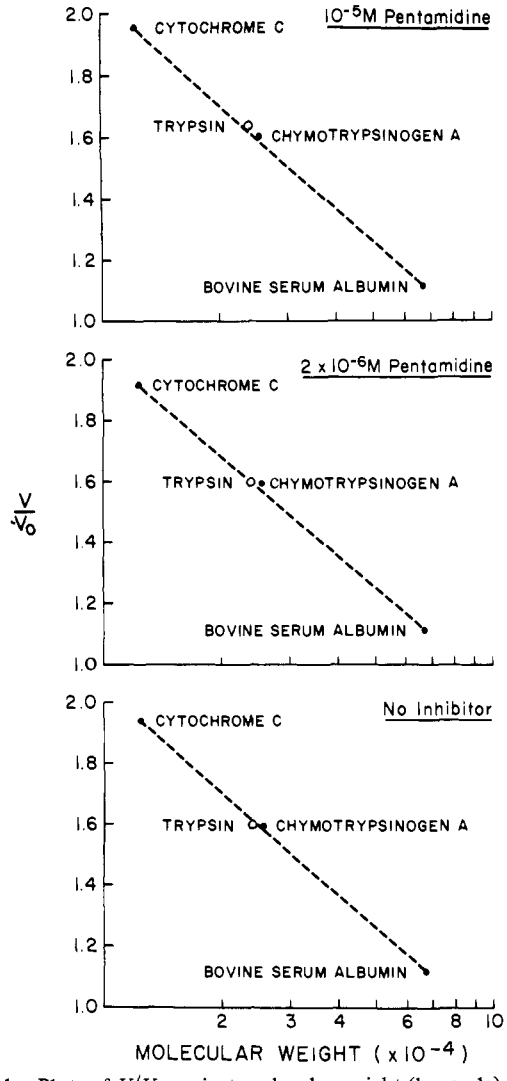


Figure 1. Plots of V/V_0 against molecular weight (log scale) for the elution of trypsin and three marker proteins from a Sephadex G-75 column in the absence or presence of pentamidine (**2**, 4',4''-diamidino-1,5-diphenoxypentane). Failure of the diamidine to alter the elution pattern. V, elution volume; V_0, void volume of the column.

into the mode of action of the inhibitors, we employed a gel filtration technique to look for evidence of dimerization of trypsin in the presence of 4',4''-diamidino-1,5-diphenoxypentane (compound **2**, pentamidine). In the control set of experiments equilibration and elution of the Sephadex column were carried out with buffer only, while in another set the buffer included inhibitor at a concentration of either 2×10^{-6} or $1 \times 10^{-5} M$. Plots of V/V_0 against molecular weight (log scale) (Figure 1) show that there was no change in the elution pattern of trypsin at either inhibitor concentration; *i.e.*, there was no evidence of dimerization of the enzyme.

Conclusions

Our results have reaffirmed the leading role of aromatic diamidines as low-molecular-weight reversible inhibitors of thrombin, pancreatic kallikrein, and trypsin, and for each of the three enzymes we have found inhibitors more powerful than those previously reported. As compared with the single-ring monoamidino compound benzamidine, our compound **23** was approximately 200 times more active against bovine thrombin in the amidase assay, and compound **18** surpassed benzamidine by the same margin in the clotting test employing human thrombin. Compound **18** was also 100 times more potent than benzamidine as a trypsin inhibitor, and, furthermore, it represented a 1300-fold increase in strength over benzamidine as a kallikrein inhibitor. The considerable inhibitory power of several of the diamidines against thrombin would recommend them for therapeutic consideration, and studies are in progress to evaluate the toxicity of the most promising compounds.

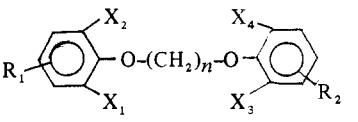
The findings made during this investigation also give a clearer picture of the interaction between aromatic diamidines and trypsin-like proteases. As each inhibitor molecule binds to only one enzyme molecule and as only one of the two cationic groups can lead the advance into the tosyl hole of the active center of the enzymes, the proven beneficial effect of the second amidino groups has probably to be sought in a stabilizing influence on the linear arrangement of the inhibitor molecules. There is electrostatic repulsion between the two positively charged ends of the diamidines and the molecules tend to minimize the repulsion by orienting themselves in an extended fashion. In this way the positive groups will be separated the farthest apart and in their lowest energy conformation. This relative rigidity of the diamidino compounds is advantageous, presumably because it provides optimal hydrophobic interaction between enzyme and inhibitor. With increasing length of the alkane chain the hydrophobic bonding forces increase, thus leading to a rise in the inhibitory strength of the compounds. Beyond a certain extent of the chain, however, the electrostatic repulsive force between the two amidino groups becomes too small for sufficient stabilization and the resulting easier distortion of the linear configuration of the inhibitor molecules is accompanied by a decrease in hydrophobic binding. Furthermore, the presence of electron-rich halogens on the benzene rings can be expected to have a shielding effect on the two positively charged amidino moieties and therefore to reduce the electrostatic repulsion between these two cationic groups. Consequently, the halogenated aromatic diamidines will have a greater tendency to divert from the linear arrangement than their nonhalogenated counterparts of equal hydrocarbon-chain length. The validity of this assumption is supported by the observation that the optimal extent of the alkane chain for the halogen-bearing compounds was generally distinctly less than for the nonsubstituted series.

Experimental Section

Enzyme Inhibition Studies. Amidase Assay. The inhibitory effects of diamidines on thrombin, kallikrein, and trypsin were determined in an amidase assay[9] employing N^{α}-benzoyl-DL-arginine-*p*-nitroanilide hydrochloride (BANA) as substrate. The amounts of nitroaniline released during hydrolysis were measured by recording absorption at 410 nm. The K_i values for the inhibitors were obtained graphically according to Dixon[10] by plotting the reciprocals of the reaction velocities at two different substrate concentrations against different inhibitor concentrations.

Thrombin (bovine, topical) was a product of Parke, Davis and Co. Trypsin (bovine, twice crystallized, salt-free) was obtained from Schwarz-Mann. Active-site titration revealed the preparation to contain 56.6% active trypsin by weight. The material was not further purified; *i.e.*, the active component probably represented a mixture of α- and β-trypsin. Pancreatic kallikrein was kindly supplied by Farbenfabriken Bayer AG, Leverkusen, Germany. The preparation contained 900 Frey units per milligram. The composition of the assay mixtures was as follows. The reaction mixtures with thrombin amounted to 1.6 ml including 0.0875 *M* Tris-HCl (pH 8.1), 50 NIH units of the enzyme, 0.02 *M* $CaCl_2$, 10% by volume dimethyl sulfoxide, 2.5% by volume ethanol, 3×10^{-3} or $1 \times 10^{-3} M$ BANA, and various concentrations of inhibitor. For pancreatic kallikrein and

Table VI. Dicyano-α,ω-diphenoxyalkanes



Compd	n	R_1	R_2	X_1	X_2	X_3	X_4	Reflux[a] time	Mp, °C	Yield, %	Formula	Analyses
26	5	3'-CN	4''-CN	H	H	H	H	24	82–84	59	$C_{19}H_{18}N_2O_2$	C, H
27	5	4'-CN	4''-CN	Cl	H	Cl	H	16	147–148[b]	40	$C_{19}H_{16}Cl_2N_2O_2$	
28	5	4'-CN	4''-CN	Br	H	H	H	48	109–110[c]	66	$C_{19}H_{17}BrN_2O_2$	
29	5	4'-CN	4''-CN	Br	Br	H	H	16	70–71	68	$C_{19}H_{16}Br_2N_2O_2$	C, H
30	5	4'-CN	4''-CN	Br	H	Br	H	16	136–137[d]	66	$C_{19}H_{16}Br_2N_2O_2$	
31	5	4'-CN	4''-CN	Br	Br	Br	Br	48	141–142	43	$C_{19}H_{14}Br_4N_2O_2$	C, H
32	5	4'-CN	4''-CN	I	H	I	H	48	142–143[e]	66	$C_{19}H_{16}I_2N_2O_2$	
33	8	4'-CN	4''-CN	Br	H	Br	H	24	119–120	44	$C_{22}H_{22}Br_2N_2O_2$	C, H
34	8	4'-CN	4''-CN	I	H	I	H	48	130–131	53	$C_{22}H_{22}I_2N_2O_2$	C, H
35	10	3'-CN	3''-CN	H	H	H	H	16	84–85	60	$C_{24}H_{28}N_2O_2$	C, H
36	10	4'-CN	4''-CN	H	H	H	H	16	121–123[f]	37	$C_{24}H_{28}N_2O_2$	
37	10	4'-CN	4''-CN	Br	H	Br	H	24	99–101	48	$C_{24}H_{26}Br_2N_2O_2$	C, H
38	12	3'-CN	3''-CN	H	H	H	H	16	91–92	55	$C_{26}H_{32}N_2O_2$	C, H
39	12	4'-CN	4''-CN	H	H	H	H	16	101–102	56	$C_{26}H_{32}N_2O_2$	C, H
40	12	4'-CN	4''-CN	Br	H	Br	H	48	90–91	49	$C_{26}H_{30}Br_2N_2O_2$	C, H
41	12	4'-CN	4''-CN	I	H	I	H	48	117–119	43	$C_{26}H_{30}I_2N_2O_2$	C, H
42	14	4'-CN	4''-CN	H	H	H	H	24	104–105	70	$C_{28}H_{36}N_2O_2$	C, H
43	16	4'-CN	4''-CN	H	H	H	H	24	95–97	42	$C_{30}H_{40}N_2O_2$	C, H
44	16	4'-CN	4''-CN	I	H	I	H	48	84–85	44	$C_{30}H_{38}I_2N_2O_2$	C, H

[a]Total reflux time in hours. [b]Lit.[5] mp 147–148°. [c]Lit.[5] mp 112°. [d]Lit.[5] mp 136–137°. [e]Lit.[5] mp 137°. [f]Lit.[4] mp 123°

Table VII. Diamidino-α,ω-diphenoxyalkanes

Compd	Mp, °C dec	Yield, %	Formula	Analyses
5	251–253[a]	56	$C_{24}H_{34}N_4O_2 \cdot 2HCl$	C, H
6	228–229	60	$C_{26}H_{38}N_4O_2 \cdot 2HCl$	C, H, N
7	213–215	45	$C_{28}H_{42}N_4O_2 \cdot 2HCl$	C, H, N
8	205–206	65	$C_{30}H_{46}N_4O_2 \cdot 2HCl$	C, H, N
9	133–135	64	$C_{19}H_{24}N_4O_2 \cdot 2HCl$	C, H, N
11	198–200	69	$C_{24}H_{34}N_4O_2 \cdot 2HCl$	C, H, N
12	195–197	71	$C_{26}H_{38}N_4O_2 \cdot 2HCl$	C, H, N
13	230–233[b]	86	$C_{19}H_{23}BrN_4O_2 \cdot 2HCl$	C, H
14	251–252[c]	60	$C_{19}H_{22}Br_2N_4O_2 \cdot 2HCl$	C, H
15	250	55	$C_{19}H_{22}Br_2N_4O_2 \cdot 2HCl$	C, H
16	278	11	$C_{19}H_{20}Br_4N_4O_2 \cdot 2HCl$	C, H
17	249–251[d]	55	$C_{19}H_{22}Cl_2N_4O_2 \cdot 2HCl$	C, H
18	259–261[e]	85	$C_{19}H_{22}I_2N_4O_2 \cdot 2HCl$	C, H
20	225–227	21	$C_{22}H_{28}Br_2N_4O_2 \cdot 2HCl$	C, H, N
21	240–241	92	$C_{24}H_{32}Br_2N_4O_2 \cdot 2HCl$	C, H, N
22	238–239	82	$C_{26}H_{36}Br_2N_4O_2 \cdot 2HCl$	C, H, N
23	251–253	64	$C_{22}H_{28}I_2N_4O_2 \cdot 2HCl$	C, H, N
24	219–221	62	$C_{26}H_{36}I_2N_4O_2 \cdot 2HCl$	C, H, N
25	217–219	92	$C_{30}H_{44}I_2N_4O_2 \cdot 2HCl$	C, H, N

[a]Lit.[4] mp 254° dec. [b]Lit.[5] 241° dec. [c]Lit.[5] mp 264° dec. [d]Lit.[5] mp 260° dec. [e]Lit.[5] mp 276–277° dec.

trypsin the composition of the mixtures was the same as with thrombin, except for the omission of $CaCl_2$ in the case of kallikrein. The amount of kallikrein per assay was 21.3–64 Frey units, and the amount of trypsin was 3.3 μg of the commercial preparation. All tests were carried out at 37°, and the length of the incubation varied from 15 min for trypsin to 40 min with thrombin and to 40–120 min with kallikrein.

Thrombin Clotting Test. A solution of human thrombin stabilized by the presence of 20% ethanol was prepared from human plasma as described previously.[2] Of this solution 0.2 ml was mixed with 0.1 ml of 0.154 *M* NaCl solution (with or without inhibitor), and then 0.2 ml of human fibrinogen solution (400 mg of fibrinogen/100 ml of 0.146 *M* NaCl–0.0055 *M* sodium citrate solution) was added. The assay was carried out at 37°, and the time from the addition of the fibrinogen to the formation of a clot was recorded. A thrombin standard curve was used to determine the percentage of inhibition.

Gel Filtration Experiments with Trypsin. The column had a size of 0.9 × 50 cm and was packed with Sephadex G-75 preswollen in H_2O. For one series of runs the column was equilibrated and eluted with 0.1 *M* Tris–HCl buffer (pH 8.0) and for another series with buffer containing either 2×10^{-6} or 1×10^{-5} *M* 4',4''-diamidino-1,5-diphenoxypentane (pentamidine). The protein samples were applied in 2-mg amounts dissolved in 1 ml of the equilibration solution. Elution was carried out at 25°. The flow rate was 0.45 ml/min and the fraction size was 0.67 ml. The void volume of the column was obtained as the elution volume of purified human fibrinogen (mol wt 340,000), a protein which is excluded from the G-75 gel. Albumin, chymotrypsinogen, and cytochrome C in the eluate were determined by the method of Lowry, *et al.*[11] Trypsin was localized by its activity against N^{α}-benzoyl-L-arginine ethyl ester. Schwert and Takenaka's spectrophotometric assay was used for this purpose.[12] The assay involved a 1:8 dilution of the eluate fraction to be tested. This, together with a substrate concentration of 1×10^{-3} *M*, was sufficient to overcome any inhibition of trypsin by the pentamidine also contained in the eluate and to allow accurate measurement of the enzyme. The trypsin preparation was the same as that employed in the amidase assays. All three marker proteins (bovine serum albumin, bovine chymotrypsinogen A, and horse heart cytochrome C) were obtained from Schwarz-Mann.

Organic Syntheses. Melting points were determined on a Mel-Temp apparatus and are uncorrected. 2-Iodo-4-cyanophenol,[5] 2,6-dibromo-4 cyanophenol,[13] and 5,4'-cyanophenoxylamyl bromide[5] were prepared by literature methods. Aromatic diamidines **1–4**, **10**, and **19** were kindly provided by May & Baker Ltd. A slight modification of the method developed by Ashley, *et al.*,[4] and by Berg and Newbery[5] was adopted for the preparation of aromatic dinitriles and aromatic diamidines. When several compounds were prepared by comparable procedures, only one representative example is included in this section. Reference should be made to Tables VI and VII for supplementary information for each compound. Elemental analyses were performed by Atlantic Microlab, Inc., Atlanta, Ga. Where analyses are indicated only by symbols of the elements, analytical results obtained for those elements were within ±0.4% of the theoretical values.

2-Chloro-4-cyanophenol. A solution of 2.05 g (17 mmol) of potassium chlorate in 100 ml of H_2O was added dropwise into a well-stirred mixture of 5.95 g (50 mmol) of 4-cyanophenol in 200 ml of concentrated HCl and 70 ml of H_2O over a period of 90 min at ambient temperature. The precipitate formed was collected through filtration. Recrystallization of the solid from H_2O gave 5.2 g (68% yield) of the title compound, mp 151–152° (lit.[5] mp 155°). *Anal.* (C_7H_4ClNO) C, H.

2-Bromo-4-cyanophenol. The reaction was carried out in an analogous manner as above with 4-cyanophenol, fuming HBr, and potassium chlorate. Recrystallization of the solid gave 66% yield of the title compound, mp 154–155° (lit.[5] 156–157°).

4',4''-Dicyano-1,12-diphenoxydodecane (39). To an ethanolic sodium ethoxide solution prepared by dissolving 0.92 g (40 mg-atoms) of Na in 100 ml of absolute ethanol was added 4.8 g (40 mmol) of 4-cyanophenol and 6.6 g (20 mmol) of 1,12-dibromododecane. The mixture was allowed to stir at reflux for 16 hr. The

resulting hot solution was filtered. After standing overnight at ambient temperature, the filtrate gave 4.5 g (56% yield) of the title compound, mp 101–102°. *Anal.* ($C_{26}H_{32}N_2O_2$) C, H.

3',4''-Dicyano-1,5-diphenoxypentane (26). To an ethanolic sodium ethoxide solution prepared by dissolving 0.23 g (10 mg-atoms) of Na in 50 ml of absolute ethanol was added 1.19 g (10 mmol) of 3-cyanophenol and 2.52 g (10 mmol) of 5,4'-cyanophenoxylamyl bromide. The mixture was allowed to stir at reflux for 24 hr. The resulting hot solution was filtered. After standing at ambient temperature overnight, the filtrate gave 1.8 g (59% yield) of the title compound, mp 82–84°. *Anal.* ($C_{19}H_{18}N_2O_2$) C, H.

4',4''-Diamidino-1,12-diphenoxydodecane Dihydrochloride (6). A solution of 0.8 g (1.96 mmol) of **39**, in 3 ml of absolute ethanol and 70 ml of benzene, was bubbled with HCl gas at 0° for 15 min. The resulting solution was kept at 4° for 7 days. At the end of the period, the solvent was removed under reduced pressure. To the solid residue was added 30 ml of 0.97 *N* NH_3 in ethanol. The mixture was kept at 50–60° for 2 hr and at ambient temperature overnight. Any precipitate formed was filtered off. Anhydrous ether was then added to this clear filtrate until complete precipitation was attained. The precipitate was recrystallized from dilute HCl, collected, and dried under reduced pressure (0.05 mm) at 60° for 2 hr to give 0.6 g (60% yield) of the title compound, mp 228–229° dec. *Anal.* ($C_{26}H_{38}N_4O_2 \cdot 2HCl$) C, H, N.

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Solid Phase Synthesis of [1-Deamino,4-valine]-8-D-arginine-vasopressin (DVDAVP), a Highly Potent and Specific Antidiuretic Agent Possessing Protracted Effects

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In further exploring the selective enhancement of antidiuretic activity exhibitied by certain [4-threonine]-substituted arginine-vasopressin analogs, we have synthesized [1-deamino,4-valine]-8-D-arginine-vasopressin (DVDAVP) by the Merrifield method with an overall yield of 45%. DVDAVP has an antidiuretic potency of 1230 ± 170 units/mg when assayed by intravenous injection into ethanol-anesthetized rats, about four times that of arginine-vasopressin (AVP). Its antidiuretic effect in conscious diabetes insipidus rats is also greatly prolonged when compared to AVP. It has undetectable vasopressor activity, <0.01 unit/mg or less than $^1/_{40,000}$ that of AVP. The antidiuretic/pressor ratio (A/P) of DVDAVP is thus greater than 125,000, a value higher than that of any other peptide known to date. Its oxytocic potency on the rat uterus is about half the potency of AVP. Most of the undesired side effects of AVP derive from its effects on vascular and visceral smooth muscles. Thus, DVDAVP with its high, specific, and protected antidiuretic properties may offer some advantages over lysine- or arginine-vasopressin in the treatment of hypothalamic diabetes insipidus. In addition, studies on this and related peptides may be helpful in further characterizing the receptors that mediate and the enzymes that terminate antidiuretic responses.

During the course of an investigation of the phylogeny of the neurohypophysial hormones, we synthesized [4-threonine]oxytocin.[1,2] This peptide was shown to be a highly potent and specific oxytocic agent. In subsequent studies we found that substitution of threonine for glutamine in the basic neurohypophysial peptides[3] and in their 1-deamino analogs[4] gave rise to peptides possessing specific and in some instances highly potent antidiuretic properties. These 4-threonine-substituted basic peptides all exhibited varying degrees of enhancement of the antidiuretic/pressor ratio (A/P) which in arginine-vasopressin (AVP) has a value of ~1. These later findings were analogous to those previously observed for other basic peptide analogs in other laboratories.[5-11] It seemed worthwhile therefore to further explore this interesting phenomenon of enhanced antidiuretic/pressor selectivity in the hope of uncovering clues to the design of peptides with even greater selectivity than had heretofor been encountered.

Analysis of our own findings[3,4] and those of others[5-11] revealed how a number of different structural alterations in the arginine-vasopressin (AVP) molecule, individually or in combination, could bring about antidiuretic/pressor selectivity in the resulting AVP analogs. AVP has the following structure in which the numbers indicate the positions of the individual amino acid residues. The individual structural alterations of AVP which bring about enhancement of antidiuretic/pressor (A/P) selectivity in the resulting peptides

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