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AROMATIC TRIS-AMIDINES

A NEW CLASS OF HIGHLY ACTIVE INHIBITORS OF TRYPSIN-LIKE PROTEASES

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Summary

A number of novel aromatic Tris-amidines have been synthesized and investigated for their antiproteolytic property. The basic structure of the compounds is that of mesitylene where each of the methyl groups has been substituted with a 3- or 4-amidinophenoxy moiety. The compounds displayed considerable activity against trypsin (EC 3.4.21.4) and thrombin (EC 3.4.21.5), but proved most effective against porcine pancreatic kallikrein (EC 3.4.21.8). With this enzyme a K_i value of $2.43 \cdot 10^{-8}$ M was recorded for α,α',α''-tris(4-amidino-2-bromophenoxy)mesitylene at pH 8.1 and 37°C. The most potent thrombin inhibitor, α,α',α''-tris(3-amidinophenoxy)mesitylene, had a K_i value of $6.51 \cdot 10^{-7}$ M and was also a strong overall anticoagulant. The inhibitors were able to interfere with the kinin release by human plasma kallikrein at concentrations as low as $1 \cdot 10^{-10}$ M. However, despite this remarkable antikallikrein effect and the known importance of plasma kallikrein in the activation of Hageman factor (factor XII), the compounds had only little influence on the early stages of blood coagulation.

Introduction

The development of synthetic inhibitors of trypsin (EC 3.4.21.4) has become greatly accelerated since Mares-Guia and Shaw [1] reported on the effectiveness of benzamidine whose protonated amidino group is attracted to the anionic carboxyl moiety of Asp 177 at the active site and whose benzene

Abbreviations: Bz-Arg-Nal · HCl, N^{α}-benzoyl-DL-arginine-*p*-nitroanilide hydrochloride; Bz-Pro-Phe-Arg-Nal · HCl, N^{α}-benzoyl-L-prolyl-L-phenylalanyl-L-arginine-*p*-nitroanilide hydrochloride.

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ring insures considerable additional, hydrophobic bonding to the enzyme molecule. Since the recognition of its potency benzamidine has been used extensively as the key building block for the production of new inhibitors, reversible ones as well as irreversible ones. The interest in such compounds has been stimulated by the fact that other arginine- or lysine-specific proteases besides trypsin are also inhibited, and that their inhibition may be of therapeutic value.

A significant advance occurred when it was found that link-up of two benzamidine moieties by an aliphatic or arylaliphatic chain markedly enhanced inhibitory strength beyond that of mono-amidines [2,3]. Systematic studies of the resulting aromatic diamidines have culminated in the discovery of the most potent low-molecular-weight inhibitors yet of not only trypsin, but also of thrombin (EC 3.4.21.5), pancreatic kallikrein (EC 3.4.21.8) and urokinase (EC 3.4.99.26) [4—6].

The success of the diamidines turned our attention to even more highly protonated compounds, and consequently we have devised a series of Tris-benzamidine derivatives of mesitylene. This communication deals with the synthesis of these novel compounds and also reports on their remarkable activity, especially against pancreatic and plasma kallikrein.

Experimental Procedures

Materials

Porcine pancreatic Kallikrein® (635 Frey units/mg) and Trasylol®, the polyvalent bovine pancreatic trypsin-kallikrein inhibitor, were obtained from Farbenfabriken Bayer AG. Bovine trypsin (twice crystallized, salt-free) was purchased from Schwarz-Mann. Bovine thrombin (topical) was a product of Parke, Davis and Co. Bz-Arg-Nal · HCl [7] was obtained from Bachem, Inc., while Bz-Pro-Phe-Arg-Nal · HCl, a derivative of the carboxyl terminal tripeptide of bradykinin, was synthesized by Vega-Fox Biochemicals. Partial thromboplastin (Thrombofax®) was obtained from Ortho Diagnostics and Celite from Johns-Manville Company.

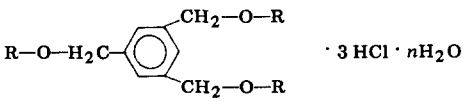
Human plasma and dog plasma were prepared from blood which had been anticoagulated by mixing 8 parts with 1 part of 0.109 M sodium citrate solution. Contact activation of the plasma was avoided by employing only materials with nonwettable surfaces. Dog serum was obtained by clotting the plasma through addition of $CaCl_2$ (6.25 ml of 0.5 M $CaCl_2$ solution/100 ml plasma). Plasma and serum samples were stored at —16° until being used in the clotting or kininogenase assays, respectively.

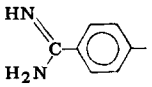
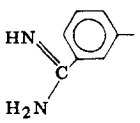
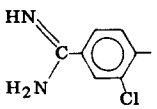
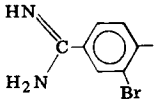
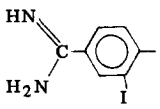
Synthesis of Tris-amidines

1,3,5-Tris-bromomethyl-benzene was prepared by the bromination of mesitylene according to the method of Vögtle et al. [8]. The tribrominated intermediate was converted to the desired tri-cyanophynoxy-ether by the standard Williamson ether synthesis. The target amidino compounds were then prepared by slight modification of a procedure developed by Ashley et al. [9] and by Berg and Newbery [10]. The following chemicals were prepared by known methods: 4-cyano-2-iodophenol, 2-chloro-4-cyanophenol and 2-bromo-4-cyanophenol [10]. The melting points were determined on a Thomas-Hoover capillary melt-

TABLE I

PHYSICAL DATA ON TRIS-AMIDINES



Compound No.	n	R	m.p. °C	Analysis calculated/found		
				C	H	N
I	1.5		223–225	54.68/54.55	5.50/5.49	12.75/12.72
II	1		125	55.43/55.28	5.43/5.75	12.93/12.76
III	2		260 (decomposed)	46.71/46.86	4.43/4.50	10.89/10.77
IV	0		240–243	41.48/41.24	3.48/3.50	9.58/9.58
V	2		234	34.45/34.57	3.27/3.32	8.03/7.84

ing point apparatus and are uncorrected. Elemental analyses were carried out by Galbraith Laboratories, Inc., Knoxville, Tenn. The structures, yields, melting points, analytical data and water of hydration of the target compounds are given in Table I.

Methods

Amidase assays. For all three purified enzymes examined, the interaction with the Tris-amidines followed the pattern of a reversible competitive inhibitory process, and therefore rate assays and Dixon's graphical method [11] could be used to obtain the dissociation constants (K_i values). In each instance the reaction mixtures consisted of 1.6 ml and included 0.09 M Tris and 10% by volume dimethyl sulfoxide. To the assays with trypsin there was added also 0.02 M $CaCl_2$. Bz-Arg-Nal · HCl in concentrations of $3 \cdot 10^{-3}$ M and $1 \cdot 10^{-3}$ M served as substrate for thrombin and trypsin, while Bz-Pro-Phe-Arg-Nal · HCl in concentrations of $5 \cdot 10^{-4}$ M and $2 \cdot 10^{-4}$ M was used with pancreatic kallikrein [5]. The effect of the inhibitors was determined at three different concentrations with each of the two substrate concentrations. The amounts of enzyme employed in the incubation mixtures were as follows: pancreatic kal-

likrein 0.41—5.0 Frey units; thrombin 44.4 NIH units; trypsin 3.33 μg. The enzymatic reactions were halted by acidification, and the amount of nitroaniline released was determined by measuring adsorption at 410 nm.

Kininogenase activity of contact-activated human plasma. This test was based on the fact that prekallikrein in plasma can be activated by surface contact and that the resulting active enzyme and its susceptibility to inhibition can be analyzed in a sensitive kininogenase assay. The procedure involved three steps: (1) activation, (2) kallikrein-induced kinin release from dog serum kininogen, and (3) biological measurement of the kinin formed. The inhibitors were added in the second step thus making it possible to evaluate their blocking effect on the kininogenase activity. Activation was brought about by stirring 0.4 ml of citrated plasma with 0.4 ml of Celite suspension (10 mg Celite/ml of 0.1 M Tris · HCl buffer, pH 8.1) for precisely 1 min at room temperature. In the immediately following second step of the assay, 0.6 ml of the Celite-plasma mixture was added to test tubes containing 1.6 ml of dog serum, 0.6 ml of L-cysteine solution (2.9 g of L-cysteine · HCl · H_2O/100 ml of 0.1 M Tris · HCl buffer, pH adjusted to 7.3) and 0.4 ml of 0.154 M NaCl solution. A second set of tubes was furnished with only 0.3 ml of the Celite-plasma mixture and the volumes were brought to par by including an additional 0.15 ml of 0.154 M NaCl solution and 0.15 ml of Celite suspension. The high-plasma as well as the low-plasma assays were incubated for 24 min at 37°C. The loose fibrin clots which formed in the tubes were compacted with an applicator stick in the initial phase of the incubation period. They sank to the bottom of the tubes where they did not further interfere with the handling of the specimens. Kinin liberation was terminated by adding to each of the assay mixtures 8000 KI units of Trasylol. The tubes were then centrifuged and the kinin content of the supernatant was determined in a standard manner by its ability to induce isometric contractions of a guinea pig ileum suspended in a muscle bath [12]. As small amounts of kinin were already released during the initial 1-min activation step, i.e. before the kininogenase could be subjected to inhibition by the amidino compounds, those amounts were measured with the help of a set of controls, and the values were taken into consideration when calculating the kininogenase activities in the second step of the test. To determine the potency of a given inhibitor, various amounts of the compound were added to the high plasma-serum kininogen mixture and that concentration of inhibitor was established which would suppress kininogenase activity to the same level as in the inhibitor-free low-plasma assay. The inhibitor concentration at this point represented the I_{50} value.

Kininogenase activity of pancreatic kallikrein. This assay was similar to the preceding test except that there was no need for an activation step and that the activated plasma in the incubation mixtures was replaced by a solution of pancreatic kallikrein. The test tubes contained 1.6 ml dog serum, 0.6 ml L-cysteine solution, 0.8 ml of 0.154 M NaCl solution (with or without inhibitor) and 0.2 ml kallikrein solution containing either 0.0416 or 0.0208 Frey units. To obtain the I_{50} value of a given Tris-amidine, the compound was added to one set of the high-kallikrein mixtures and that concentration of the inhibitor was determined which would reduce kinin liberation to the same level as in the inhibitor-free mixtures containing only half the amount of kallikrein.

Partial thromboplastin time test. The assay was slightly modified from the method of Nye et al. [13]. Citrated human plasma (0.1 ml) was mixed in regular glass tubes with 0.1 ml of partial thromboplastin solution and 0.1 ml of 0.154 M NaCl solution or 0.154 M NaCl/inhibitor solution. After an incubation period of 30 s at 37°C, 0.1 ml of 0.02 M $CaCl_2$ solution was added, and the time until formation of a clot was recorded.

Contact activation of coagulation. This assay represented a two-stage partial thromboplastin time test in which the first stage was given to contact activation of the previously non-activated plasma, while the second stage involved the clotting process proper. In one set of tubes inhibitor was included already in the activation stage, and further inhibitor was added in the second stage to keep the inhibitor concentration constant. In a second set of tubes inhibitor was present only in the second stage. The activation mixtures were composed of 0.1 ml of non-activated plasma, 0.1 ml Celite suspension (5 g Celite/100 ml 0.154 M NaCl/0.05 M Tris solution, pH 7.4) and 0.1 ml of 0.154 M NaCl solution (with or without inhibitor). After repeated agitation at 37°C for 1 min or 5 min, respectively, there were added 0.1 ml of 0.154 M NaCl solution (with or without inhibitor) and 0.1 ml partial thromboplastin solution. After elapse of another 30 s, 0.1 ml of 0.02 M $CaCl_2$ solution were pipetted into the tubes and the clotting times recorded.

Results

Inhibition of the amidase activity of pancreatic kallikrein, thrombin and trypsin

In Table II the five Tris-amidines are listed together with the dissociation constants for the three enzymes investigated. The order of the inhibitors is the same as in Table I, i.e., compound I possesses the amidino groups in the para position while compound II is the corresponding meta-amidino derivative. Compounds III, IV and V are obtained from compound I by substituting on each of the amidinobenzene moieties chlorine, bromine or iodine, respectively. Comparison of the K_i values shows that compound I produced considerable in-

TABLE II

INHIBITORY EFFECT OF TRIS-AMIDINES ON THE AMIDASE ACTIVITY OF PANCREATIC KALLIKREIN, THROMBIN AND TRYPSIN

Values are means ± S.D., $n = 3$.

Compound No.	K_i (μM)		
	Pancreatic kallikrein *	Thrombin **	Trypsin **
I	0.387 ± 0.054	1.44 ± 0.11	0.97 ± 0.04
II	0.105 ± 0.005	0.651 ± 0.105	0.901 ± 0.11
III	0.0328 ± 0.007	7.14 ± 0.73	0.725 ± 0.08
IV	0.0243 ± 0.013	7.79 ± 0.82	0.648 ± 0.12
V	0.0393 ± 0.019	11.17 ± 2.9	0.416 ± 0.1

* Bz-Pro-Phe-Arg-Nal · HCl

** Bz-Arg-Nal · HCl

TABLE III

INHIBITORY EFFECT OF TRIS-AMIDINES ON THE KININOGENASE ACTIVITY OF PANCREATIC KALLIKREIN AND PLASMA KALLIKREIN

Compound	I_{50} (M)	
	Pancreatic kallikrein	Plasma kallikrein
I	$3 \cdot 10^{-6}$	$5 \cdot 10^{-7}$
II	$5 \cdot 10^{-7}$	$5 \cdot 10^{-8}$
III	$2 \cdot 10^{-6}$	$2 \cdot 10^{-8}$
IV	$7 \cdot 10^{-7}$	$1 \cdot 10^{-10}$
V	$3 \cdot 10^{-7}$	$1 \cdot 10^{-10}$

hibition of all three proteases and that meta positioning of the amidino groups (compound II) favorably affected the affinity for kallikrein and thrombin. With trypsin, on the other hand, the location of the amidino groups appeared of no importance for inhibitory potency. Discrimination by the inhibitors between the enzymes became even more notable with the halogenated derivatives. Compared with their parent compound I, these inhibitors were endowed with an activity 10—15 times greater against kallikrein, but with at most only twice the activity against trypsin. With regard to thrombin, halogenation resulted even in a marked loss in inhibitory effect, a loss which increased from the chlorinated compound over the brominated compound to the iodinated compound.

Inhibition of the kininogenase activity of pancreatic and plasma kallikrein

Tris-amidines also effectively blocked kinin release by pancreatic kallikrein as well as by contact-activated human plasma. The kininogenase activity of the latter can be attributed chiefly to plasma kallikrein [14]. As can be seen from Table III, the potency of the inhibitors increased in a similar order as their affinity for pancreatic kallikrein in Table II. The only exception in the case of pancreatic kallikrein was compound II which moved ahead of compound IV, while with plasma kallikrein the iodinated compound V was now equal to or slightly more potent than the brominated compound IV. From a comparison

TABLE IV

INHIBITORY EFFECT OF TRIS-AMIDINES ON THE PARTIAL THROMBOPLASTIN TIME OF HUMAN PLASMA

Values are means ± S.D., $n = 22$ for the control and $n = 3$ for the inhibitor assays.

Compound No.	Partial thromboplastin time (s, control 59.4 ± 2.1 s)		
	$5 \cdot 10^{-5}$ M inhibitor	10^{-5} M inhibitor	$5 \cdot 10^{-6}$ M inhibitor
I	278 ± 10.6	101 ± 4.1	61 ± 2.7
II	586 ± 31.8	173 ± 12.5	117 ± 5.1
III	94 ± 7.7	66 ± 0.9	62 ± 1.3
IV	125 ± 1.1	72 ± 2.9	63 ± 1.1
V	72 ± 2.5	63 ± 2.0	60 ± 1.4

TABLE V

EFFECT OF TRIS-AMIDINES ON THE CONTACT ACTIVATION OF COAGULATION AS STUDIED BY A TWO-STAGE PARTIAL THROMBOPLASTIN TIME TEST

Values are means ± S.D., n = 3. Without activation and in the absence of inhibitor the partial thromboplastin test time was 130 s.

Compound No.	Activation time (min)	Assay system		
		No inhibitor	Inhibitor in post-activation step only *	Inhibitor in activation and post-activation step **
II	1	63.9 ± 1.1	155.3 ± 7.2	155.6 ± 10.3
	5	53.4 ± 0.6	111.5 ± 9.2	119.1 ± 9.6
V	1	65.6 ± 3.6	89.6 ± 7.5	86.6 ± 4.1
	5	52.5 ± 1.6	79.3 ± 2.0	83.1 ± 5.2

* Inhibitor concn. in post-activation step $5 \cdot 10^{-6}$ M.
** Inhibitor concn. during activatin and in post-activation step $5 \cdot 10^{-6}$ M.

of the two kininogenases, it is evident that the activity of the plasma enzyme was more readily suppressed than the activity of the glandular enzyme.

Inhibition of the partial thromboplastin time

The partial thromboplastin time test as used was sensitive to inhibition of any of the multiple enzymatic steps occurring during clotting, and it was therefore a convenient indicator of the overall anticoagulant activity of a given compound. The data in Table IV show that compound II, the most effective antithrombin agent in the amidase assay, was also the leading inhibitor in the partial thromboplastin time test producing a marked prolongation of the coagulation process, even at the low concentration of $5 \cdot 10^{-6}$ M. The strength of the other inhibitors in the partial thromboplastin time test correlated generally with their K_i values for thrombin. Only the brominated derivative IV did not follow this pattern by proving slightly more active than compound III.

Inhibition of contact activation of the clotting process

The influence of Tris-amidines on the activation process was judged by the difference in the clotting times between two sets of assay mixtures, one set containing inhibitor in the activation and clotting phase and another set containing inhibitor only in the clotting phase. As can be seen from a comparison of the values in the last two columns in Table V, neither of the two compounds examined interfered with activation, and all prolongation of the partial thromboplastin time test appears to have occurred in the second phase. This absence of any effect on contact activation was found with the short 1-min activation period as well as with the long 5-min period.

Discussion

Our investigations have shown that Tris-amidinophenoxy derivatives of mesitylene are highly potent inhibitors of pancreatic and plasma kallikrein and that

they inhibit also, though somewhat less affectively, at least two other arginine-specific esteroproteases, thrombin and trypsin. In α,α',α''-Tris(4-amidino-2-bromophenoxy)mesitylene we have discovered the most potent synthetic low molecular weight inhibitor of pancreatic kallikrein reported to date, and with its K_i value of $2.43 \cdot 10^{-8}$ M this inhibitor approaches the antikallikrein activity of the polyvalent protease inhibitor from bovine organs whose dissociation constant has been determined as $1.2 \cdot 10^{-8}$ M [15]. It is of special interest that the compound is endowed not only with remarkable inhibitory strength but also with considerable specificity being 230 and 26 times more active against pancreatic kallikrein than against thrombin and trypsin, respectively. A similar selectivity of some compounds for kallikrein was noted earlier during a study of aromatic diamidines [5] and also during a comparative evaluation of irreversible inhibitors of the lysyl chloromethyl ketone type [16]. Those results, together with our findings above, attest to the fact that a high degree of discrimination between related enzymes can be achieved with simple organic compounds and that one enzyme can be inhibited preferentially in the presence of another similar enzyme.

Tris-amidines of the type described here can be expected to be arranged in a single plane with each amidinobenzene moiety being separated from the other two by an angle of 120° and thus establishing the greatest possible distance between the mutually repulsive cationic groups. It appears that such an arrangement is especially fortuitous with kallikrein. While only one of the amidino groups can be directed into the depth of the active site of the enzyme, the extended position of the other two side chains must owe its favorable influence on affinity to improved hydrophobic bonding and/or to positioning of the cationic groups into the vicinity of additional anionic groups on the enzyme molecule. Further modifications in the composition of the inhibitors, such as replacement of one or two of the cationic groups, may help to decide on the actual mode of enzyme-inhibitor interaction and may contribute information about the three-dimensional environment of the active site.

Though in the kininogenase assays only the pancreatic enzyme was employed in a purified form, it appears safe to conclude from our results that susceptibility of the plasma enzyme to inhibition is by several orders of magnitude greater than that of the glandular enzyme. These findings are in agreement with observations of Markwardt et al. [17] who noted a similar dissociation in sensitivity of the enzymes to blockage by a series of monoamidines. The different susceptibility of the two types of kallikrein to inhibition is not surprising, however, in view of their known divergent behavior with some of the natural trypsin inhibitors and their differences in enzymatic specificity [18].

In recent years it has become evident that plasma kallikrein serves not only a kinin-releasing function, but is also involved in the early stage of blood coagulation by contributing to the activation or action of Hageman factor (factor XII) [19,20]. Hereditary deficiency of Fletcher factor which is identical with pre kallikrein [21], thus finds its expression in a considerable prolongation of the partial thromboplastin test time (22). In the face of these facts it came as a surprise that Tris-amidines had no effect on the activation phase of clotting, even at concentrations several thousand times greater than the one able to suppress 50% of the kininogenase activity of the plasma. As of now we can only

speculate as to the reason for the unexpected result. Could the affinity of plasma kallikrein for Hageman factor be so great that the inhibitors at the concentrations used were unable to compete successfully for the active site of the enzyme? Or could it be that participation of kallikrein in the contact activation process does not involve enzymatic activity and thus is in no need of a free active site? This latter alternative seems unlikely in view of the findings of Cochrane et al. [19] that prekallikrein of diisopropylphosphofluoridate-kallikrein cannot substitute for the active enzyme in the fluid-phase activation of Hageman factor. Observations of Katori et al. [23] may also have some bearing on the interpretation of our results. Those investigators have demonstrated the likelihood that the mediation of contact activation by plasma kallikrein is not limited to a positive feedback mechanism but that a negative feedback is also involved. The latter process is contingent upon the release by kallikrein of a large peptide fragment from kininogen, and the fragment has been shown to retard the activation of Hageman factor. In the light of this report one might consider whether blocking of the active site of kallikrein would not eliminate both the activating and retarding properties of the enzyme and thus cancel out any obvious effect on the partial thromboplastin test time. Such an interpretation, however, would appear not to be in keeping with the known prolongation of the partial thromboplastin test time in patients with Fletcher factor deficiency, a condition where one would also expect both activities of the enzyme to be missing.

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