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Structure–Activity Relationships for the Inhibition of Acrosin by Benzamidine Derivatives

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A series, consisting of 52 benzamidine derivatives, was evaluated for inhibitory activity against homogeneous boar sperm acrosin. All of the compounds in the series proved to be more potent than benzamidine ($K_i = 4.0 \times 10^{-6}$ M), with one of the derivatives, α-(4-amidino-2,6-diiodophenoxy)-3-nitrotoluene (compound **16**), showing outstanding potency with a K_i value of 4.5×10^{-8} M. Although all of the derivatives were effective acrosin inhibitors, structural specificity was observed within homologous groups of compounds. The information gained from this preliminary study should prove extremely beneficial in the design and synthesis of future acrosin inhibitors.

Prior communications have described the development of aromatic amidines as potent and specific inhibitors of a number of proteolytic enzymes, i.e., thrombin, trypsin, and kallikrein.[1] During the course of these studies a sizable series of structurally diverse amidine derivatives has been prepared. Using many of these known amidines, as well as an equal number of novel derivatives, this paper reports the initial structure–activity studies of another biologically important protease, acrosin.

Acrosin (E.C. 3.4.21.10), a trypsin-like protease in spermatozoa, functions to digest a path for the spermatozoon through the zona pellucida of the ovum.[2] The critical dependency of reproduction on acrosin was clearly demonstrated when both in vivo[3,4] and in vitro[5,6] fertilization were blocked by acrosin inhibitors. Thus, acrosin inhibition possesses a powerful potential to serve in an antienzymatic approach to fertility control. Although human acrosin is the ultimate target enzyme for utilization of this potential, only limited studies have been undertaken, due to the small quantities of material available and to the lability of the human enzyme.[7,8] However, boar acrosin has recently been purified to homogeneity[9] from the purified zymogen precursor, proacrosin, is now available in sufficient quantities, and is sufficiently stable, so that large-scale testing of synthetic inhibitors is now feasible. Amidine derivatives were chosen for this study because they were not only proven, potent antiproteolytic agents but also because benzamidine has previously been determined to inhibit crude preparations of both rabbit[10] and bull[11] acrosin.

Results and Discussion

The dissociation constants of the amidine derivatives with acrosin were determined from rate assays employing *N*-α-benzoyl-L-arginine ethyl ester (BzArgOEt) as the substrate. In each instance the reaction followed Michaelis–Menton kinetics, and inhibition was strictly competitive and reversible. The compounds are listed in Tables I–IV with their structural formulas and respective K_i values. The K_i values for thrombin, trypsin, and kallikrein have been included for comparison in Table IV.

Benzyl Phenyl Ethers (Table I). The series of compounds comprising Table I includes 17 novel derivatives and five known compounds. This extensive homologous series, with its wide range of antiacrosin activities, serves best to illustrate the specificity of acrosin for amidine-type inhibitors. With the exception of the *p*-cyano derivative (compound **8**) and the *p*-nitro derivative (compound **9**), monosubstitution of the leadoff compound, **1**, at either the meta (R_5) or para (R_4) position of the benzyl

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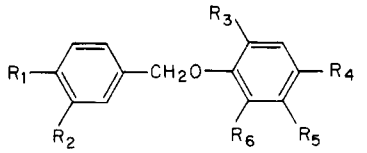
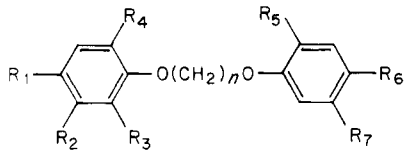
Table I. Acrosin Inhibition by Benzyl Phenyl Ether Derivatives



compd no.	R_1	R_2	R_3	R_4	R_5	R_6	K_i, μM	ref
benzamidine							4.0 ± 0.1	
1	Am[a]	H	H	H	H	H	2.6 ± 0.9	this work
2	Am	H	H	H	CF_3	H	1.7 ± 0.4	this work
3	Am	H	H	H	$N(CH_3)_2$	H	1.9 ± 0.2	this work
4	Am	H	H	Cl	H	H	0.95 ± 0.04	this work
5	Am	H	H	$CH(CH_3)_2$	H	H	1.1 ± 0.05	this work
6	Am	H	H	OCH_3	H	H	1.6 ± 0.3	this work
7	Am	H	H	NH_3	H	H	2.3 ± 0.4	this work
8	Am	H	H	CN	H	H	3.9 ± 0.1	this work
9	Am	H	H	NO_2	H	H	2.6 ± 0.5	this work
10	Am	H	H	H	NO_2	H	0.61 ± 0.04	this work
11	H	Am	H	NO_2	H	H	0.76 ± 0.06	this work
12	H	Am	H	H	NO_2	H	0.86 ± 0.05	this work
13	NO_2	H	H	Am	H	H	1.6 ± 0.1	this work
14	NO_2	H	I	Am	H	H	0.30 ± 0.09	this work
15	H	NO_2	H	Am	H	H	2.4 ± 0.2	this work
16	H	NO_2	I	Am	H	I	0.045 ± 0.006	this work
17	Am	H	H	H	Am	H	0.29 ± 0.02	1b
18	Am	H	H	Am	H	H	0.48 ± 0.02	1b
19	Am	H	Br	Am	H	H	0.68 ± 0.06	1b
20	Am	H	I	Am	H	H	0.45 ± 0.02	this work
21	H	Am	Br	Am	H	H	1.1 ± 0.1	1b
22	H	Am	Cl	Am	H	Cl	0.44 ± 0.06	1b
23	H	Am	I	Am	H	I	0.19 ± 0.01	1b

[a] Am = $-C(=NH)NH_2$.

Table II. Acrosin Inhibition by α,ω-Dioxyalkane Derivatives

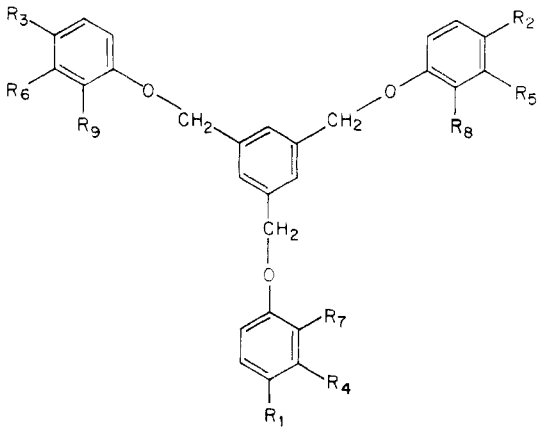


compd no.	n	R_1	R_2	R_3	R_4	R_5	R_6	R_7	K_i, μM	ref
24	3	Am[a]	H	H	H	H	Am	H	3.5 ± 0.3	1a
25	3	Am	H	I	H	H	Am	H	0.68 ± 0.3	1a
26	3	Am	H	Br	H	Br	Am	H	1.7 ± 0.3	1a
27	3	Am	H	Br	Br	H	Am	H	0.57 ± 0.3	1a
28	5	Am	H	H	H	H	H	H	0.28 ± 0.08	1a
29	5	Am	H	H	H	H	Am	H	2.3 ± 1.3	this work
30	5	Am	H	H	H	H	NH_2	H	2.0 ± 0.1	1a
31	5	Am	H	H	H	H	NO_2	H	0.86 ± 0.2	this work
32	5	Am	H	I	H	I	Am	H	1.0 ± 0.1	this work
33	5	Am	H	H	H	H	H	NO_2	1.1 ± 0.2	1a
34	5	Am	H	I	H	H	H	NO_2	0.27 ± 0.07	this work
35	5	H	Am	H	H	H	NO_2	H	0.68 ± 0.1	this work
36	5	H	Am	H	H	H	NH_2	H	0.56 ± 0.07	this work
37	5	Am	H	I	H	H	H	Am	1.0 ± 0.4	this work
38	8	Am	H	H	H	H	H	H	1.0 ± 0.02	this work
39	8	Am	H	H	H	H	Am	H	1.2 ± 0.2	1a
40	8	Am	H	I	H	I	Am	H	0.26 ± 0.02	1a
41	10	Am	H	H	H	H	Am	H	0.56 ± 0.04	1a
42	10	Am	H	Br	H	Br	Am	H	0.26 ± 0.09	1a
43	12	Am	H	H	H	H	Am	H	0.44 ± 0.1	1a
44	14	Am	H	H	H	H	Am	H	0.13 ± 0.01	1a

[a] Am = $-C(=NH)NH_2$.

ring (compounds **2–12**, **17**, and **18**) resulted in an increase in antiacrosin potency. The most effective substituent was an amidino group placed in either the meta or para position (compounds **17** and **18**), where a nine- and fivefold increase in potency, respectively, was observed when compared to compound **1**. Halogenation of compound **18** at the R_3 position (compounds **19** and **20**) produced little change in antiacrosin activity. But, when the amidino group at the R_1 position of compound **19** is moved to the R_2 position (compound **21**), a twofold decrease in antiacrosin potency was observed, relative to compounds **18–20**. Of considerable interest was the diverse effect that nitro substitution had on acrosin binding affinity. For example, replacement of one of the amidino moieties on

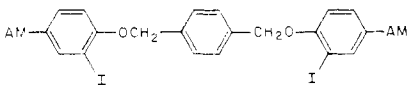
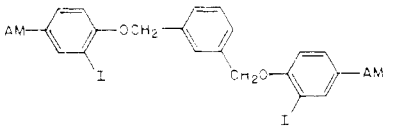
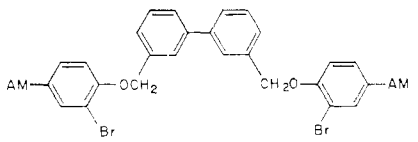
Table III. Acrosin Inhibition by α,α′,α″-Tris(phenoxy)mesitylene Derivatives



compd no.	R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8	R_9	K_i, μM	ref
45	Am[a]	Am	Am	H	H	H	H	H	H	1.3 ± 0.3	1d
46	Am	Am	Am	H	H	H	I	I	I	0.64 ± 0.3	1d
47	NO_2	Am	Am	H	H	H	H	H	H	1.6 ± 0.4	this work
48	NO_2	Am	Am	H	H	H	H	Cl	Cl	1.6 ± 0.2	this work
49	H	H	H	Am	Am	Am	H	H	H	0.56 ± 0.2	1d

[a] Am = —C(=NH)NH_2.

Table IV. Comparison of Acrosin Inhibition with Potent Protease Inhibitors

compd no.	structure[a]	K_i, μM acrosin	thrombin	kallikrein	trypsin	ref
50		0.28 ± 0.02	0.11 ± 0.03	0.61 ± 0.22	2.3 ± 0.55	1c
51		2.3 ± 0.3	1.2 ± 0.1	0.031 ± 0.01	0.67 ± 0.14	1c
52		0.21 ± 0.04	0.27 ± 0.02	2.2 ± 0.77	0.25 ± 0.53	1c

[a] Am = —C(=NH)NH_2.

the unsubstituted diamidines (compounds **9**, **10**, and **13**) by a nitro group resulted in a decrease in antiacrosin potency. However, the nitro replacement of the amidino group at the R_2 position of the diiodo-substituted derivative (compound **23**) produced an increase in potency and, in fact, resulted in the most effective acrosin inhibitor (compound **16**) of the entire series.

α,ω-Diphenoxyalkanes (Table II). The data in Table II show that lengthening the dioxyalkane chain of 4,4′-diamidino-α,ω-diphenoxyalkanes from 1 to 14 carbons led to a progressive increase in inhibitory strength against acrosin. Halogen substitution on either one or both of the amidinophenoxy moieties increased the inhibitory potency of a given compound. One of the most interesting findings was the considerable augmentation of antiacrosin activity produced when pentamidine (compound **29**) was stripped of one amidino group (compound **28**). This observation is in contrast to previous findings for inhibition of thrombin, trypsin, and kallikrein, where the loss of an amidino group resulted in a significant decrease in inhibitory potency.[1e] Surprisingly, the same alteration of the 8-carbon chain bisamidino derivative (compound **39**) resulted in no significant change in acrosin inhibition. Finally, the substitution of a nitro group for one of the amidine groups of compounds **31** and **34** resulted in more powerful antiacrosin agents.

α,α′,α″-Tris(phenoxy)mesitylenes (Table III). The compounds in Table III were selected for acrosin inhibition studies because they have been reported to be highly selective inhibitors of thrombin (compound **49**, $K_i = 6.51 \times 10^{-7}$ M), kallikrein (compound **46**, $K_i = 3.93 \times 10^{-8}$ M), and trypsin (compound **45**, $K_i = 9.7 \times 10^{-7}$ M).[1d] The overall potency of the tris derivatives (Table III) against the previously studied proteolytic enzymes was considerably greater when compared to the antiproteolytic effect of the compounds listed in Tables I and II.[1a,b,d] Against acrosin, however, the compounds in Table III show no significant improvement over the derivatives represented in the preceeding tables. In fact, the most potent acrosin inhibitors are to be found in Tables I and II. Unlike the two preceding series, the nitro substituent seemed to have little effect on the antiacrosin potency of the tris derivatives.

Aromatically Linked Bis(benzamidines) (Table

Table V. Analytical Data, Melting Points, and Yields of Amidine Derivatives

compd no.	mp, °C	yield,[a] %	formula	analyses
1	113-114	41	$C_{14}H_{14}N_2O \cdot HCl \cdot H_2O$	C, H, N
2	174-176	13	$C_{15}H_{13}F_3N_2O \cdot HCl \cdot 0.5H_2O$	C, H, N
3	131-133	28	$C_{16}H_{19}N_3O \cdot 2HCl \cdot 2H_2O$	C, H, N
4	230-232	51	$C_{14}H_{13}ClN_2O \cdot HCl$	C, H, N
5	180-182	66	$C_{17}H_{20}N_2O \cdot HCl$	C, H, N
6	175-177	16	$C_{15}H_{16}N_2O_2 \cdot HCl \cdot 0.25H_2O$	C, H, N
7	288-290	20[b]	$C_{14}H_{15}N_3O \cdot 2HCl \cdot H_2O$	C, H, N
8	141-142	10[c]	$C_{15}H_{13}N_3O \cdot HCl \cdot 2H_2O$	C, H, N
9	221-224	84	$C_{14}H_{13}N_3O_3 \cdot HCl$	C, H, N
10	202-204	33	$C_{14}H_{13}N_3O_3 \cdot HCl$	C, H, N
11	190-192	11	$C_{14}H_{13}N_3O_3 \cdot HCl \cdot H_2O$	C, H, N
12	126-128	59	$C_{14}H_{13}N_3O_3 \cdot HCl \cdot H_2O$	C, H, N
13	262 dec	52	$C_{14}H_{13}N_3O_3 \cdot HCl$	C, H, N
14	288 dec	82	$C_{14}H_{12}IN_3O \cdot HCl$	C, H, N
15	208-210	74	$C_{14}H_{13}N_3O_3 \cdot HCl$	C, H, N
16	228 dec	70	$C_{14}H_{11}I_2N_3O_3 \cdot HCl$	C, H, N
20	265-268	65	$C_{15}H_{15}IN_4O \cdot 2HCl$	C, H, N
28	105-107	17	$C_{18}H_{22}N_2O_2 \cdot HCl \cdot H_2O$	C, H, N
30	125-126	60[b]	$C_{18}H_{23}N_3O_2 \cdot HCl \cdot H_2O$	C, H, N
31	86-90	95	$C_{18}H_{21}N_3O_4 \cdot HCl \cdot H_2O$	C, H, N
33	135-137	72	$C_{18}H_{21}N_3O_4 \cdot HCl \cdot 0.5H_2O$	C, H, N
34	134-135	92	$C_{18}H_{20}IN_3O_4 \cdot HCl$	C, H, N
35	125-126	65	$C_{18}H_{21}N_3O_4 \cdot HCl$	C, H, N
36	144-147	58	$C_{18}H_{23}N_3O_2 \cdot HCl \cdot 0.75H_2O$	C, H, N
38	99-101	60[b]	$C_{21}H_{22}N_2O_2 \cdot HCl \cdot 0.5H_2O$	C, H, N
47	193	56	$C_{29}H_{27}N_5O_5 \cdot 2HCl \cdot 1.5H_2O$	C, H, N
48	262 dec	45	$C_{29}H_{25}Cl_2N_5O_5 \cdot 2HCl$	C, H, N

[a] All yields are calculated from the corresponding cyano derivatives unless so noted. [b] Yields calculated from the reduction of corresponding nitro derivatives. [c] Yield determined from etherization of *p*-cyanophenol with α-bromo-4-amidinotoluene.

IV). The three compounds in Table IV were chosen from a previously reported series of 20 aromatically linked bis(benzamidino) compounds.[1c] These compounds, like the preceding derivatives in Table III, were selected for antiacrosin testing because of the high potency they displayed against other proteases. While two of the derivatives (compounds **50** and **51**) were potent inhibitors of acrosin, the relative potencies of these compounds, when compared to the best antiacrosin derivatives in Tables I and II, were less than anticipated from prior antiprotease activity. Comparison of the inhibitory data for the four enzymes in Table IV indicates that acrosin inhibition is more correlative with antithrombin activity than with the inhibition of the other two enzymes. However, this is not always the case, as evidenced by the fact that the best acrosin inhibitor of all the compounds reported (compound **16**) was a relatively weak thrombin inhibitor ($K_i = 3.24 \times 10^{-5}$ M).

In summary, acrosin is a proteolytic enzyme that is very susceptible to inhibition by benzamidine derivatives. This point is demonstrated by the finding that benzamidine is 100-fold more effective against acrosin than against thrombin or kallikrein and 10-fold more potent against acrosin than trypsin. It is also noteworthy that all the reported compounds were better antiacrosin agents than benzamidine. The best inhibitor of the series (compound **16**) was 90 times more potent than benzamidine. It should also be stressed that there was very little, if any, correlation between acrosin inhibition by amidines and the reported inhibition by amidines on other proteolytic enzymes. Thus, it would appear that the topography in and around the active site of acrosin is far from analogous to the active site areas of either thrombin, trypsin, or kallikrein, a fact that should prove fortuitous in the development of future specific inhibitors of acrosin.

Experimental Section

Acrosin Inhibition Assays. Acrosin was prepared from ejaculated boar spermatozoa as previously described.[9] The preparation was judged to be homogeneous, for only one band of protein was observed in sodium dodecyl sulfate (SDS) disc gel electrophoresis[12] with or without the addition of mercaptoethanol. A comparison of the migration of this band to that of standard proteins resulted in a molecular weight estimation of 34 000. When the sample was subjected to pH 4.3 analytical disc gel electrophoresis[13] and the gels were stained for either protein or enzymatic activity,[14] only a single band was observed and this had a relative migration of 0.54. Acrosin activity was measured spectrophotometrically by monitoring the hydrolysis of BzArgOEt[15] at 253 nm at 30 °C. Protein was measured spectrophotometrically at 280 nm.[16] The assay mixtures (total volume 3 mL) consisted of 0.05 M Tris/HCl, 0.05 M calcium chloride, 1% dimethyl sulfoxide, 0.3 μg of acrosin, two concentrations of BzArgOEt, and various concentrations of the appropriate inhibitors. The acrosin preparations were stable for several weeks when stored at 4 °C in 0.001 M HCl (pH 3.0). When acrosin was incubated under the conditions used for the rate assays there was no degradation detected by SDS or analytical disc gel electrophoresis. The interaction of the amidine derivatives with acrosin followed the pattern of reversible competitive inhibition and, therefore, rate assays and the graphical method of Dixon[17] were used to obtain the dissociation constants (K_i values). K_i values are expressed as means ± mean deviation for at least two determinations.

Organic Synthesis. All melting points were determined on a Thomas-Hoover capillary melting point apparatus and are uncorrected. Elemental analyses for all the amidino derivatives were carried out by Galbraith Laboratories Inc., Knoxville, Tenn. Analyses were accepted if the determined values were within ±0.4% of the theoretical values. The melting points, yields, and analytical data for the novel amidino derivatives are listed in Table V. The references for the synthetic data on the previously reported amidines are included in Tables I–IV. With the exception of compounds **7**, **8**, **30**, and **36**, the target amidino derivatives were prepared from the corresponding cyano derivatives using a modification of the Pinner reaction.[18,19] Compound **7** was prepared by the stannous chloride reduction of the corresponding nitro derivative (compound **9**), while the two analine derivatives in Table II (compounds **30** and **36**) were prepared by the catalytic hydrogenation (10% Pd/C) of their respective nitro-substituted precursors (compounds **31** and **35**). 4-Amidino-α-(4-cyanophenoxy)toluene (compound **8**) was formed by the etherization of *p*-amidino-α-bromotoluene with *p*-cyanophenol. The cyano intermediates of the amidine products in Tables I and II were prepared according to the standard etherization procedures described previously.[1a,b] The following chemicals were not available from commercial sources and were prepared by previously described methods: 4-cyano-2-iodophenol,[19] 2-bromo-4-cyanophenol,[19] and 2,6-dibromo-4-cyanophenol.[20] The cyano intermediates of the two nitro derivatives in Table III (compounds **47** and **48**) were prepared by dietherization of α,α′-dibromo-α″-(4-nitrophenoxy)mesitylene with the appropriate phenol. α,α′-Dibromo-α″-(4-nitrophenoxy)mesitylene was synthesized in the following manner.

A mixture of α,α′,α″-tribromomesitylene[21] (5 g, 14 mmol), *p*-nitrophenol (1.94 g, 14 mmol), and sodium ethoxide/ethanol solution (28 mL, 0.5 N) in 30 mL of acetone was stirred at room temperature for 24 h. The solution was cooled to 5 °C, and the precipitated salts were removed by filtration. The acetone was evaporated under reduced pressure, and the solid residue was recrystallized twice from ethanol to give 2.4 g (41%) of α,α′-dibromo-2″-(4-nitrophenoxy)mesitylene: mp 102–104 °C. Anal. ($C_{15}H_{13}Br_2NO_3$) C, H.

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