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Molecular Properties of the Adrenergic α Receptor. 2. Optimum Covalent Inhibition by Two Different Prototypes of Polyamine Disulfides

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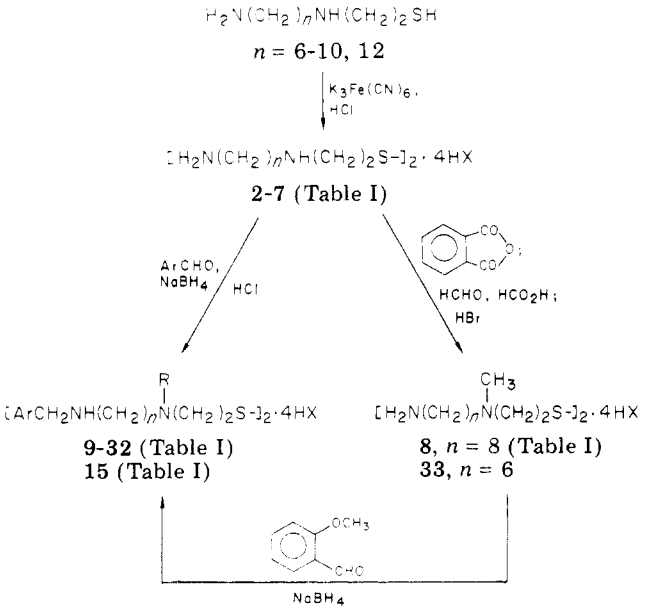
In order to further improve the α-adrenoreceptor blocking activity of analogues of *N,N'*-bis(5-aminopentyl)cystamine (APC), the effect of distance between the nitrogens of the all-carbon chains together with the effects of benzyl substituents on the terminal nitrogens was studied. It was discovered that with *o*-methoxybenzyl substituents on the terminal nitrogen, optimum α-blocking activity is associated with a six-carbon chain (structure **12**), whereas, in the absence of substituents, the eight-carbon analogue **4** had optimum activity. Evidence is given that these two prototype antagonists involve two distinct binding-site topographies on the α receptor. The effects of N-methylation of the common cystamine segments of **4** and **12** on blocking potency support this conclusion. Polyamine disulfides **4** and **12** (BHC) are shown to be specific irreversible blockers of the α receptor. Complete inactivation of the latter by either one of the inhibitors left intact tissue responses (rat vas deferens and rabbit aortic tissue) elicited by 5-hydroxytryptamine and histamine. However, the response of guinea pig ileum to acetylcholine was weakly inhibited but in a completely reversible manner in contrast to the α receptor. The receptor saturation mechanism for **4** and especially **12** may involve cooperative interactions. The more potent α blocker **12** displayed an affinity and specificity reminiscent of the neurotoxin class of nicotinic receptor inhibitors. The results of receptor protection experiments by norepinephrine (NE) were unusual in that at a ratio as low as 1:10 of BHC to NE, complete protection was achieved. When blockade of the α receptor by **12** was monitored with epinephrine (E) instead of NE, it was observed that the effectiveness of the antagonist was reduced to one-third on rat vas deferens and one-tenth on aortic tissue. This discriminatory power of **12** against NE was not shared by **4** or some close analogues of **12**. It is concluded that **12** is a novel, powerful, and selective pharmacological tool.

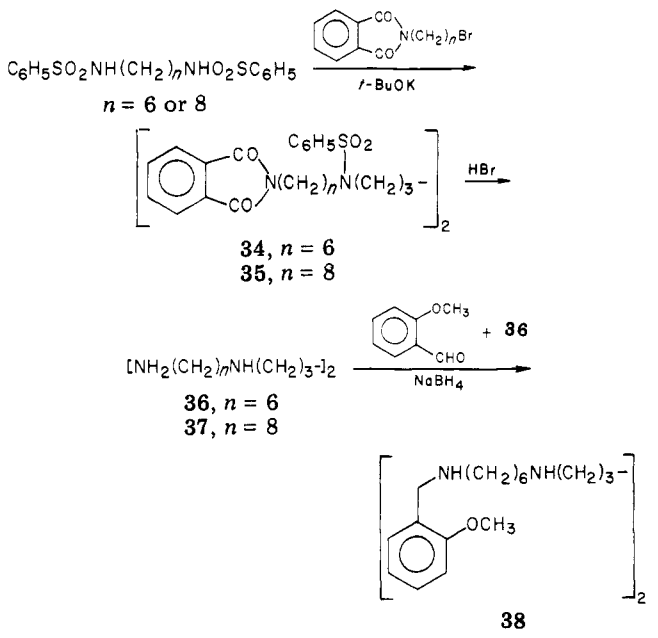
In part 1 of this series[2] we have shown that, among a variety of *N,N'*-bis(5-aminopentyl)cystamine (APC) derivatives and analogues, optimum α-adrenoreceptor blocking activity was obtained when the terminal nitrogens of APC carried benzyl or substituted benzyl groups. In order to further improve α-blocking activity, it was necessary to study the effect of distance between the nitrogens of the all-carbon chains together with the effects of benzyl substituents on the terminal nitrogens. It was previously inferred by others[3] that optimum activity was associated with a five-carbon chain, but this conclusion was based on the biological evaluation of the corresponding diaminothiol *S*-phosphate salts. Disulfides other than APC were not reported and since we have already shown that it is the disulfides that constitute the active species at the receptor level,[4] it was essential to make the other homologous tetramine disulfides available for testing. In addition, each new homologue was substituted on the terminal nitrogens by benzyl groups carrying various substituents on the aromatic ring. Finally, the all-carbon analogues of the best prototype antagonists were synthesized in order to clarify the mechanism of α-receptor inactivation by polyamine disulfides.

Chemistry. The structures of the compounds synthesized are given in Table I. They were prepared according to Schemes I and II. The *N*-(ω-aminoalkyl)-cysteamines (Table I, n = 6–10, 12) were obtained in 50–55% yield by the procedure already reported[2] for n = 6 and were easily converted to the corresponding disulfides (**2–7**, Table I) in 80–85% yields by potassium ferricyanide

Scheme I



Scheme II



oxidation.[2] Methylation of the inner nitrogens was carried out starting from **2** and **4**. Phthalic anhydride was used to protect the primary amino group followed by Eschweiler–Clarke methylation[5] and finally acid hydrolysis (Scheme II). The *N,N'*-dimethyl analogues **8** and **15** thus obtained were purified by column chromatography and were obtained in overall yields of 25–35%. These compounds (**8** and **15**) were characterized as the oxalate salts because of difficulties at crystallizing the hydrochloride or hydrobromide salts The substituents on the terminal nitrogens were easily introduced by condensation of **1–4** and **33** with the appropriate aromatic aldehyde and subsequent reduction of the intermediate Schiff bases.[2] The *N,N*-bis[ω-(arylamino)alkyl]cystamine tetrahydrochlorides **9–32** were thus obtained in 65–75% yields.

Finally, the synthesis of the all-carbon analogues **37** and **38** of **4** and **12**, respectively, was accomplished as shown in Scheme II.

Pharmacology and Discussion. (A) α-Adrenergic Blocking Activities. Relative potencies of the polyamine disulfides as irreversible inhibitors of norepinephrine (NE)-induced contractions of the rat vas deferens are assembled in Table I. Complete concentration–inhibition curves were constructed for the most active and other relevant analogues (**4**, **8**, **12**, and **15**) using both vas deferens and rabbit aortic tissue (Figures 1 and 2). It is worth noting that at ~50% blockade of the receptors by the most active member [*N,N'*-bis(*o*-methoxybenzylamino-*n*-hexyl)cystamine (BHC, **12**, Table I)], the slope of the curves was very steep, indicating a mechanism of blockade which is a general characteristic of cooperative interactions in ordered systems. Such a mechanism, if applicable, has not previously been observed with other classes of α blockers and accordingly deserves further investigation. The effectiveness of BHC (**12**) as an α blocker was such that at a bath concentration of 0.3 μM it caused complete, irreversible blockade of the response of aortic tissue to NE after a 90-min incubation. The time course of this inactivation is shown in Figure 3. The blocking effects of 1 and 3 μM BHC against NE on the rabbit aorta persisted and were unchanged after 18 h (Figure 4). As the most active member of the series, BHC (**12**) was selected for further pharmacological studies. Its α-receptor selectivity

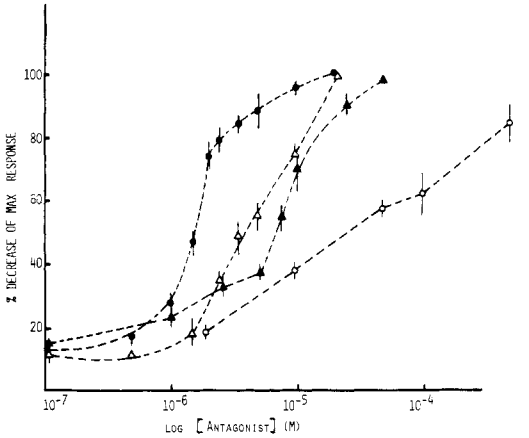


Figure 1. Course of α-receptor covalent occupancy (rat vas deferens) by **4** (Δ—Δ), **8** (▲—▲), **12** (●—●), and **15** (○—○). The percent decrease of maximum response to NE was measured after a 30-min incubation for each concentration of antagonist followed by washing with the bath solution for 30 min. The results are expressed as the mean ± SEM of 8–10 independent observations (see Experimental Section for general protocol).

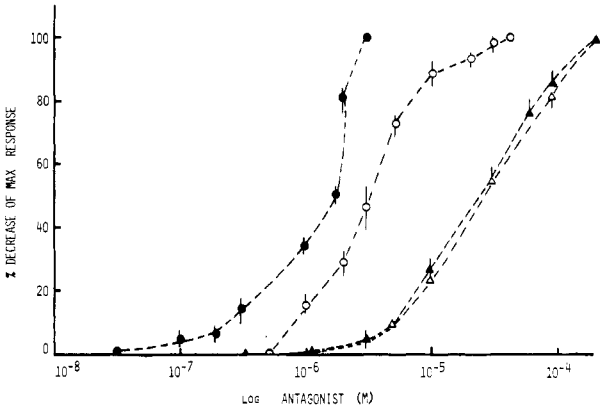


Figure 2. Course of α-receptor covalent occupancy of rabbit aortic strips by **4** (▲—▲) and **12** (●—●). The other two curves (○—○ and Δ—Δ) are for E as the agonist (see text and Figure 9). See explanation for Figure 1 and the Experimental Section for detailed protocol.

is illustrated in Figure 5 where is is evident that complete α-receptor inactivation in aortic tissue left intact tissue responses elicited by 5-hydroxytryptamine (5-HT), histamine, and isoprenaline in potassium-contracted tissue. It may be recalled that α-adrenergic blocking drugs generally antagonize in some measure muscarinic responses[7,8] apart from their known lack of discrimination against the 5-HT receptor. While excess BHC (**12**) had no detectable effect on the latter, it had an effect on the former. In contrast to the α-adrenergic blockade with its slow onset and long persistance, onset and offset of the muscarinic blockade were fast. Thus a 20- or 40-min incubation with 7 μM BHC reduced the contractile effect of acetylcholine on the guinea pig ileum by 50%, and following a 40-min incubation with 30 μM BHC (**12**), a 50% recovery of the tissue response was observed after washing for 20 min. In the guinea pig atrium, a 30- or 60-min incubation with 0.24 μM BHC reduced the inotropic and chronotropic potency of carbachol by 50%, and following a 60-min incubation with 30 μM BHC, a 50% recovery of the response was obtained in 26 min. These results are summarized in Figure 6. The all-carbon analogue of BHC (**38**, Scheme II) behaved similarly: a 30- or 60-min incubation with a 0.17 μM concentration reduced the inotropic and chronotropic effects of ace-

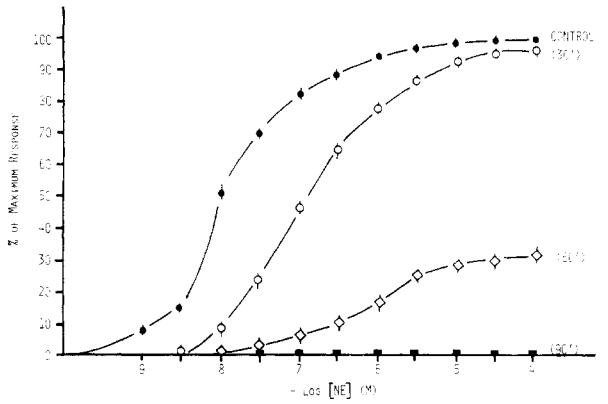


Figure 3. Time course of α-receptor blockade (rabbit aortic strips) by **12** at a bath concentration of 0.3 μM as measured by the tissue responses to cumulative concentrations of NE: control (●—●), after 30 min (○—○), after 60 min (◇—◇), and after 90 min (■—■). Incubations were followed by washing prior to assaying with NE (see the Experimental Section for general protocol). The results are expressed as the mean ± SEM of 8–10 independent observations.

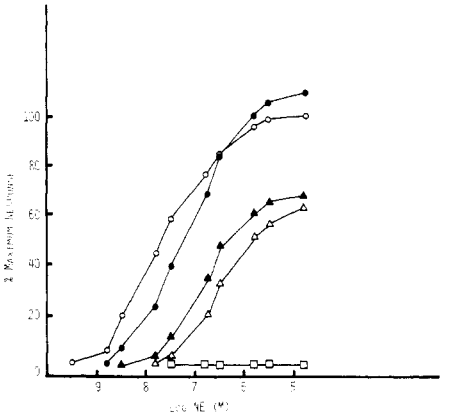


Figure 4. Effects of norepinephrine on contractile response of rabbit aorta (means of two experiments): control (○); control after 18 h (●); 30-min incubation with 1 μM BHC (△); 18 h after a 30-min incubation with 1 μM BHC (▲); immediately after and 18 h after a 30-min incubation with 3 μM BHC (□).

tylcholine on the guinea pig atrium 50%, and following a 60-min incubation with a 10 μM concentration, a 50% recovery was observed after 17 min (Figure 6).

In contrast to BHC (**12**), the irreversible α-adrenoreceptor blocking drug, phenoxybenzamine, has a muscarinic blocking activity of slow onset and long duration.[7] Furthermore, phenoxybenzamine inhibited the nicotinic effect of butyrylcholine on rate and contractility of the guinea pig atrium.[9] In contrast, 10 or 30 μM BHC did not antagonize the nicotinic effect of butyrylcholine on the spontaneously beating guinea pig atrium. Also, phenoxybenzamine inhibited the inotropic and chronotropic effects of tyramine and potentiated the effects of norepinephrine on guinea pig atrium,[9] effects which are based on inhibition of neuronal uptake of sympathomimetic amines by phenoxybenzamine;[10] in contrast, BHC at 10 or 30 μM did not inhibit the inotropic and chronotropic effects of tyramine and did not potentiate the effects of noradrenaline on the guinea pig atrium. It remains that the α-receptor selectivity of BHC (**12**) as regards *covalent* inhibition is unprecedented, which suggests applications in the selective affinity labeling of the α receptor (manuscript in preparation). Of considerable interest was the observation that NE at a bath concentration of 30 μM afforded *complete* protection of the α receptors against

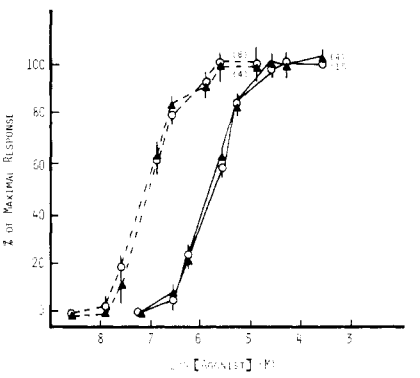


Figure 5. Cumulative log concentration–response curves of aortic strips to 5-HT [control (○--○); after exposure to **12** at 10 μM for 30 min followed by washing for 30 min (▲--▲)] and to histamine [control (○—○); after exposure to **12** at 10 μM for 30 min followed by washing for 30 min (▲—▲)]. The results are presented as the mean ± SEM and the number of observations is shown in parentheses. See the Experimental Section for detailed general protocol.

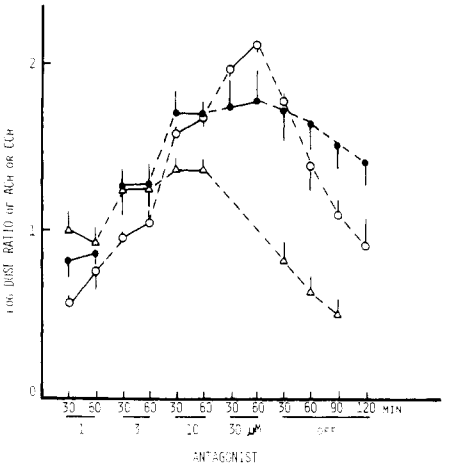


Figure 6. Guinea pig atrium. Dose ratio of acetylcholine (●) and carbachol (○) in the presence of BHC (**12**) and of acetylcholine in the presence of the carbon analogue **38** (Scheme II) of BHC (△). Blockade of acetylcholine by BHC has fast onset and fairly slow offset, and blockade by 30 μM BHC is not much greater than by 10 μM. The effect of **38** reaches a limit at 3 μM (against acetylcholine). "Off" means washing with the bath solution for 30–120 min. Vertical bars are the standard error of the mean. The means were obtained from 4–6 experiments.

BHC (Figure 7). This may have special significance because such effectiveness at a relatively low concentration of NE is unprecedented for an α agonist. Thus 30 μM norepinephrine afforded 100% protection against 3 μM BHC, whereas 30 μM norepinephrine gave only 56% protection against 0.147 μM phenoxybenzamine, and the effect of 300 μM norepinephrine was no greater.[11]

(B) Topographical Dualism. From the data assembled in Table I, it is evident that the α receptor responds divergently to terminal *N*-benzyl substitution and to distance between the nitrogens of the carbon chains. Thus, the same N-substituents (*o*-methoxybenzyl) that confer optimum activity to the *six*-carbon homologue **12** (BHC) markedly impair the activity of the eight-carbon analogue **4** which by itself is a markedly effective blocker (compare **14** with **4**). The specificity of these two chain lengths in promoting optimum activity is illustrated in Figure 8 for the two distinct classes of polyamine disulfides. The sharp peaks at six- and eight-carbon chains show that the two prototypes **4** and **12** involve distinct topographies in their interaction with the receptor. This remarkable

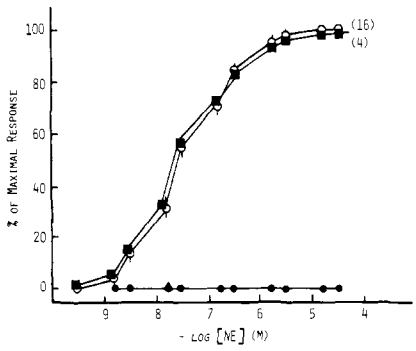


Figure 7. Cumulative log concentration–response curves for aortic tissue after exposure to **12** at 3 μM in the presence and absence of NE in the baths: control (O—O); after **12** at 3 μM for 30 min followed by washing (●—●); after prior addition of NE at 30 μM and, 10 min later, addition of **12** at 3 μM for 30 min followed by washing (□—□). The results are the mean ± SEM, and the number of observations is in parentheses.

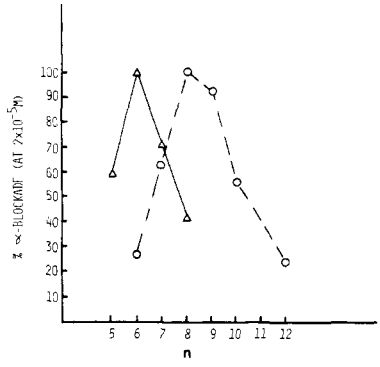


Figure 8. Comparative potencies as α-adrenergic blockers of the unsubstituted series of tetramine disulfides (O--O) and the ω-(*o*-methoxybenzyl) series (Δ—Δ) vs. length of the carbon chains separating their nitrogens (data from Table I).

dualism may have two main sources: the two prototype antagonists may react with two distinct sets of sites or they may bind on a single set which selectively adapts conformationally to either one of the two structures. These alternatives raise the question at first of whether the common cystamine segments of **4** and **12** contribute not only equally well to the observed potencies but also independently of their respective aminoalkyl appendages. The fact that N-methylation of the cystamine segments (see **8** and **15**, Table I) had only a slight effect on the blocking potency of **4** but a marked detrimental effect on the activity of **12** (Figure 1) demonstrates that the binding sites for the terminal nitrogens of BHC (**12**) are *not* indifferent to receptor interactions with the cystamine segment, whereas the reverse is true of **4** (insofar as N-methylation is concerned). Accordingly, two distinct binding site topographies must be involved in the binding of **4** and **12** but whether they are conformationally interchangeable or sterically unconnected cannot be answered at this time. Ongoing experiments with labeled analogues should help clarify this key point.

(C) Interaction Chemistry. The time-dependent and irreversible nature of α-receptor inactivation by BHC (Figure 3) and its analogues is clearly consistent with covalent bond formation with a protein thiol through a disulfide–thiol interchange reaction. Concrete evidence that the chemical reactivity of the disulfide part of BHC is indeed responsible for receptor inactivation is found in the fact that the all-carbon analogue of BHC (**38**, Scheme II) caused no inactivation under the same conditions. The possibility that a thiol group may be involved in the makeup of the α receptor and as a target site for alkylating blockers and organomercury reagents has been previously suggested.[12-14] However, on the basis that methylmercuric iodide inhibition of noradrenergic responses is not receptor specific[15] (responses to acetylcholine and histamine being blocked equally well), it was suggested that thiol groups play no part in the binding-site chemistry of the α receptor and that the effects of the organomercuric reagent would be the result of interference with the contractile process.[15] While this may well be true, the possibility was overlooked that one or more receptor thiols sterically protected from alkylating and organomercuric reagents may nevertheless be present. As we have previously reported,[4] α-receptor responses to NE are unaffected by prior exposure of the tissues to common thiol oxidizing reagents (such as ferricyanide and Ellman's reagent), thus suggesting that the target thiol for BHC and its analogues may not be exposed on the surface. Moreover, the covalent product of the receptor reaction with prototype **1** (Table I) was previously shown[4] to be stable to high concentrations of mercaptoethanol, thus indicating that the target receptor thiol may reside under the surface or at the interface between protomers or other protein constituents. These observations suggest that the multiple cation–anion interactions in the initial addition complex with BHC would promote by way of a conformational change accessibility of the disulfide part of the inhibitor to a target receptor thiol. After reaction, only half the cationic charges of BHC would be retained by the receptor which raises the possibility that the product conformation may differ from that which is initially stabilized by four changes.

The intriguing possibility emerges that an α-receptor transition involving an "inside–outside" thiol conformation may play a functional role in receptor-associated events. This hypothesis is under investigation.

(D) Substituent Effects. It is worth noting that the *o*-methoxy groups of BHC appear essential for optimum α-blocking activity although the presence of 3,4-dihydroxy substituents (**30**, Table I) also contribute markedly to activity. Whether this means that the binding sites for the terminal nitrogens may also be involved in the binding of NE cannot be answered at this time. The NE analogue of **30** when available should give useful information in this regard. The α agonist, methoxamine, also incorporates an *o*-methoxy substituent which also raises the possibility that the binding sites for the terminal nitrogens of BHC might be involved in the binding of α agonists.

(E) Discrimination between NE- and E-Induced Responses. An intriguing discovery was the unique ability of BHC (**12**) to block NE-induced responses more efficiently than those induced by epinephrine (E) (Figures 2 and 9). This discrimination is especially marked with aortic tissue (Figure 2). The other prototype antagonist **4**, as well as a few other analogues of BHC (**9**, **20**, **23**, and **31**; see Figure 10 for **9** as an example), displayed no such discriminatory power. This is also known to be true of phenoxybenzamine which was used as control. In no way can the 10-fold excess of BHC required to achieve 100% blockade of E be due to experimental error. Detailed studies of the mechanism underlying this discriminatory phenomenon are under way.[19]

Conclusions

The tetramine **12** (BHC) is a novel, powerful, and selective pharmacological tool which has allowed the unraveling of previously unsuspected molecular properties of the α receptor. Structure–activity relationship studies

Table I. α-Adrenergic Blocking Results

compd no.	n	R_1	R_2	mp, °C[a]	purifn solvent[b]	formula	analyses[c]	% α blockade[d]
				$[R_1HN(CH_2)_nNR_2(CH_2)_2S-]_2 \cdot 4HX$				
1[e]	5	H	H					10 (4)
2	6	H	H	271-271.5	A	$C_{16}H_{38}N_4S_2 \cdot 4HCl$	C, H	27 (4)
3	7	H	H	272-272.5	A	$C_{18}H_{42}N_4S_2 \cdot 4HCl$	C, H	63 (4)
4	8	H	H	275-275.5	A	$C_{20}H_{46}N_4S_2 \cdot 4HCl$	C, H, N	100 (4)[h]
5	9	H	H	278-278.5	A	$C_{22}H_{50}N_4S_2 \cdot 4HCl$	C, H, N	92 (4)
6	10	H	H	286-287	A	$C_{24}H_{54}N_4S_2 \cdot 4HCl$	C, H	56 (4)
7	12	H	H	285-286	A	$C_{28}H_{62}N_4S_2 \cdot 4HCl$	C, H	24 (4)
8	8	H	CH_3	indefinite	A	$C_{22}H_{56}N_4S_2 \cdot 4H_2C_2O_4$[g]	C, H, N	90 (4)
9	6	Bzl	H	287-288	A	$C_{30}H_{50}N_4S_2 \cdot 4HCl$	C, H	90 (4)
10	7	Bzl	H	278-278.5	A	$C_{32}H_{54}N_4S_2 \cdot 4HCl$	C, H	58 (4)
11[f]	5	2′-CH_3O-Bzl	H					59 (4)
12	6	2′-CH_3O-Bzl	H	227-228	C	$C_{32}H_{54}N_4O_2S_2 \cdot 4HCl \cdot H_2O$	C, H, N	100 (6)[h]
13	7	2′-CH_3O-Bzl	H	191-192	C	$C_{34}H_{58}N_4O_2S_2 \cdot 4HCl \cdot H_2O$	C, H, N	71 (4)
14	8	2′-CH_3O-Bzl	H	185-186	C	$C_{36}H_{62}N_4O_2S_2 \cdot 4HCl \cdot H_2O$	C, H, N	41 (4)
15	6	2′-CH_3O-Bzl	CH_3	indefinite	A-B	$C_{34}H_{62}N_4O_2S_2 \cdot 4H_2C_2O_4 \cdot H_2O$	C, H, N	48 (4)
16	6	2-EtO-Bzl	H	240-241	A-B	$C_{34}H_{58}N_2O_2S_2 \cdot 4HCl$	C, H, N	81 (4)
17	6	2′-allyloxybenzyl	H	210-211	A-B	$C_{36}H_{58}N_4O_2S_2 \cdot 4HCl$	C, H, N	71 (4)
18	6	3′-CH_3O-Bzl	H	259-259.5	A	$C_{32}H_{54}N_4O_2S_2 \cdot 4HCl \cdot H_2O$	C, H, N	72 (4)
19	6	4′-CH_3O-Bzl	H	298-298.5	C-H_2O	$C_{32}H_{54}N_4O_2S_2 \cdot 4HCl$	C, H, N	81 (4)
20	6	2′-CH_3-Bzl	H	258.5-259	A	$C_{32}H_{54}N_4S_2 \cdot 4HCl$	C, H	69 (4)
21	7	2′-CH_3-Bzl	H	240-241	A-B	$C_{34}H_{58}N_4S_2 \cdot 4HCl$	C, H, N	56 (4)
22	8	2′-CH_3-Bzl	H	241-242	A	$C_{36}H_{62}N_4S_2 \cdot 4HCl$	C, H, N	49 (4)
23	6	2′-Cl-Bzl	H	241-242	A-B	$C_{30}H_{46}N_4S_2 \cdot 4HCl$	C, H	44 (4)
24	7	2′-Cl-Bzl	H	225.5-226	A-B	$C_{32}H_{52}N_4S_2 \cdot 4HCl$	C, H	36 (4)
25	6	2′,3-$(OCH_3)_2$-Bzl	H	218.5-219	A-B	$C_{34}H_{58}N_4O_2S_2 \cdot 4HCl$	C, H, N	26 (4)
26	6	2′,4′-$(OCH_3)_2$-Bzl	H	193-194	A-B	$C_{34}H_{58}N_4O_2S_2 \cdot 4HCl$	C, H, N	26 (3)
27	6	2′,5′-$(OCH_3)_2$-Bzl	H	183-184	A-B	$C_{34}H_{58}N_4O_4S_2 \cdot 4HCl$	C, H, N	76 (4)
28	6	3′,5′-$(OCH_3)_2$-Bzl	H	255-255.5	A	$C_{34}H_{58}N_4O_4S_2 \cdot 4HCl$	C, H, N	41 (4)
29	6	3′,4′-$(OCH_3)_2$-Bzl	H	257-258	A-B	$C_{34}H_{58}N_4O_4S_2 \cdot 4HCl$	C, H, N	24 (4)
30	6	3′,4′-$(OH)_2$-Bzl	H	183-185	A-B	$C_{34}H_{50}N_4O_4S_2 \cdot 4HBr$	C, H	98 (6)
31	6	2′,6′-Cl_2-Bzl	H	265-266	A-B	$C_{30}H_{44}Cl_4N_4S_2 \cdot 4HCl$	C, H	40 (4)
32	7	2′,6′-Cl_2-Bzl	H	255-255.5	B-C	$C_{32}H_{50}Cl_4N_4S_2 \cdot 4HCl$	C, H	39 (4)

[a] All compounds were recrystallized at least three times and decolorized with charcoal when necessary. Their purity was uniformly checked by TLC (silica) using concentrated NH_4OH-CH_3OH in appropriate ratios and/or $CHCl_3$-CH_3OH-HCO_2H (6.5:3:0.5) as mobile phases. Their NMR spectra (D_2O) were in agreement with the expected structures. The heating rate was 1°/min for melting point determinations. [b] A, CH_3OH; B, 2-propanol; C, ethanol; D, acetone. [c] Analyses for the elements indicated were within ±0.4% of the theoretical values required. [d] Potencies as irreversible inhibitors of NE-induced responses in rat vas deferens using compounds **4** and **12** as standards. The number of experiments is in parentheses and the percent inhibition is accurate to within ±5%. For compounds **4**, **8**, **9**, **12**, and **15** complete saturation curves are given in Figures 1 and 2. See the Experimental Section for detailed protocols. [e] See ref 1 for synthesis. [f] See part 1, ref 2, for synthesis. [g] Tetraoxalate salts exhibiting broad melting points. [h] At low concentrations (<20 μM), compound **4** is less active than **12** as shown in Figure 1. On rabbit aorta, **4** is much less active than **12** (see Figure 2).

have unveiled some topographical properties of the receptor protein which deserve closer scrutiny. It should be mentioned that there are decisive differences between tetramine disulfides and β-haloalkylamine: (1) no inhibition of histamine, 5-HT, or nicotine effects; (2) virtually no effect on muscarinic receptors at effective α-blocking concentrations; some effects generally reversible at high concentrations; (3) no inhibition of tyramine effects, indicating no inhibition of neuronal uptake processes; (4) stable in aqueous solution; (5) not lipid soluble. The role of molecular symmetry in the receptor–tetramine disulfide interactions is an intriguing aspect which will be evaluated and discussed in a forthcoming publication.

Experimental Section

Chemistry. All melting points were taken in sealed capillaries on a Büchi SMP-20 apparatus and are uncorrected. IR spectra were recorded with a Perkin-Elmer 127 spectrophotometer. NMR spectra were measured on a Varian T-60 spectrometer using Me_4Si or DSS as internal standards. Although the IR and NMR spectral data are not included (because of the lack of unusual features), they were obtained for all compounds reported and were all consistent with the assigned structures. Chromatographic separations were performed on a silica gel column (Silicar CC7 special, Mallinckrodt).

The microanalyses were performed by Spang Microanalytical Laboratory, Eagle Harbor, Mich. The physical characteristics of all biologically relevant disulfides are listed in Table I.

***N*-(6-Aminohexyl)cysteamine.** It was synthesized according to Wineman et al.[16] This general procedure allowed the preparation of the following aminothiols in yields of 50–55%: *N*-(7-aminoheptyl)cysteamine, bp 130–132 °C (0.6 mm); *N*-(8-aminooctyl)cysteamine, bp 140–142 °C (0.8 mm); *N*-(9-aminononyl)cysteamine, bp 134–136 °C (0.2 mm); *N*-(10-aminodecyl)cysteamine, bp 154–156 °C (0.35 mm); *N*-(12-aminododecyl)cysteamine, bp 168–170 °C (0.3 mm). These compounds solidified on standing and they were used in the next step without further purification.

***N*,*N*′-Bis(ω-aminoalkyl)cystamine Tetrahydrochlorides 1–7.** They were synthesized starting from the appropriate *N*-(ω-aminoalkyl)cysteamine (free base) by potassium ferricyanide oxidation as reported for *N*,*N*′-bis(5-aminopentyl)cystamine tetrahydrochloride.[2] The yields were 80–85%.

***N*,*N*′-Bis(ω-aminoalkyl)-*N*,*N*′-dimethylcystamines 8 and 15.** A mixture of **2** or **4** as free bases (2.0 mmol) and phthalic anhydride (4.0 mmol) in dry toluene (50 ml) was heated under reflux for 8 h while continuously removing the water. The solution was then filtered, the filtrate evaporated in vacuo, the residue treated with 2 N HCl (10 mL), and the solution extracted with $CHCl_3$ (3 × 50 mL). The combined extracts were washed with water (2 × 10 mL) and evaporated to yield a residue which was treated with 20% NaOH (20 mL), and the mixture was extracted with $CHCl_3$ (3 × 50 mL). After washing the extract with H_2O and drying (Na_2SO_4), the solvent was evaporated in vacuo to give the *N*-phthaloyl derivatives. They were treated with HCOOH (5 mL) and 36% HCHO (5 mL) for 6 h at reflux temperature and, after cooling, the mixture was made basic with 40% NaOH (20

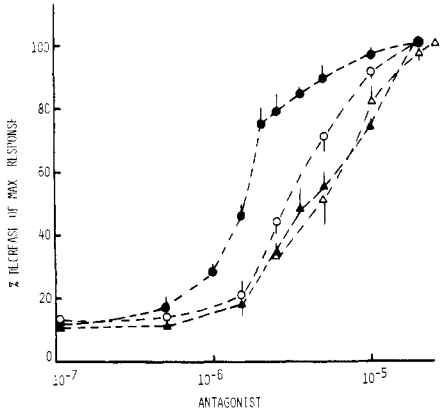


Figure 9. Comparison of relative α-receptor occupancy (vas deferens) by **4** and **12** when respectively measured with NE and E as the agonists: **12** vs. NE (●--●); **12** vs. E (○--○); **4** vs. NE (▲--▲); **4** vs. E (△--△). The protocol was the same as that under Figure 1; see Figure 2 for a similar comparison using aortic tissue.

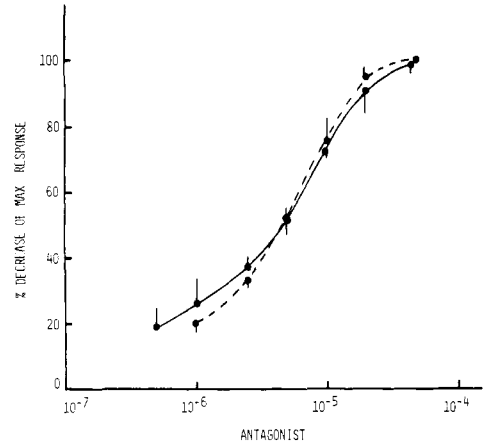


Figure 10. Comparison of relative α-receptor occupancy (vas deferens) by **9** (Table I) when respectively measured with NE (●--●) and E (●—●) as the agonists. The protocol was the same as that under Figure 1.

mL) and extracted with $CHCl_3$ (3 × 50 mL). Removal of the washed (H_2O) and dried (Na_2SO_4) extract afforded a residue which was heated under reflux with AcOH (5 mL) and 48% HBr (20 mL) for 24 h under nitrogen. The solution was diluted with 20 mL of water, cooled, filtered, and finally washed with $CHCl_3$ (3 × 50 mL). The acidic solution was made basic with NaOH pellets and extracted with $CHCl_3$ (4 × 30 mL). Evaporation of washed (NaCl solution) and dried (Na_2SO_4) extract gave crude **8** or **33** as free bases which were purified by column chromatography (concentrated NH_4OH–CH_3OH) (15:85 as the eluant). The overall yields ranged from 25 to 35%. Intermediate **33** was used as the free base for benzylation of the terminal nitrogens as described below, whereas **8** was converted into its oxalate salt by treating an alcoholic solution with 4 equiv of oxalic acid dihydrate. After cooling, the solid was collected, washed with ether, and recrystallized several times to give pure **8** (Table I).

***N,N'*-Bis[ω-(arylamino)alkyl]cystamine Tetrahydrochlorides 9–29, 31, and 32.** They were synthesized starting from the appropriate disulfides **1–4** or **33** (as free bases) and arylaldehyde following the procedure described for other *N,N*-bis(5-arylaminopentyl)cystamine tetrahydrochlorides.[2] Yields were in the range of 65–75%. Compound **15** was obtained as the tetraoxalate salt by adding 4 equiv of oxalic acid dihydrate in ether to the corresponding free base. Substance **12** (BHC), the adopted α-blocking agent, was nonextractable by 1-octanol from physiological buffer, thus establishing its nonlipophilic character.

***N,N'*-Bis[6-(3',4'-dihydroxybenzylamino)hexyl]cystamine Tetrahydrobromide (30).** *N,N'*-Bis[6-(3',4'-dimethoxybenzylamino)hexyl]cystamine (**29**) as free base (1.17 g, 1.6 mmol) was heated under reflux in 48% HBr (15 mL) for 4 h under nitrogen. The solvent was evaporated in vacuo and the residue recrystallized several times to give pure **30** in 60% yield (Table I).

***N,N'*-Bis(6-amino-*n*-hexyl- and 8-amino-*n*-octyl)-1,6-hexanediamine Tetrahydrochlorides (36 and 37, Scheme II).** To a solution of 5 mmol of *N,N'*-bis(phenylsulfonyl)-1,6-hexanediamine[17] and 10 mmol of potassium *tert*-butoxide in dry DMF (10 mL) was added dropwise a DMF solution (10 mL) of 10 mmol of *N*-(ω-bromohexyl- or ω-bromooctyl)phthalimide,[18] and the mixture was stirred under reflux for 5 h. It was cooled and filtered, the filtrate evaporated in vacuo, the residue shaken with water–chloroform, and the aqueous phase extracted with chloroform. The combined extracts were decolorized with Norit, dried, and evaporated in vacuo to give the crude tetramine derivatives **34** and **35** as yellow oils which were used as such in the next step. They were respectively taken up in a mixture of 48% HBr (70 mL), acetic acid (10 mL), and phenol (4.0 g), and the solution was stirred under nitrogen for 15 h followed by heating under reflux for 30 h. After cooling, the mixture was filtered, and the filtrate was washed repeatedly with methylene chloride, made strongly basic (NaOH), and extracted with methylene chloride. The extract was dried and evaporated, the residue was mixed with excess ethanolic HCl, and after evaporation the tetrachloride salts were recrystallized from methanol–2-propanol to give pure **36** (mp 309–311 °C dec. Anal. C, H, N) and **37** (mp >315 °C dec. Anal. C, H, N).

***N,N'*-Bis[6-(*o*-methoxybenzylamino)hexyl]-1,6-hexanediamine Tetrahydrochloride (38).** This was prepared from **36** (free base) and *o*-methoxybenzaldehyde according to the procedure already described for the analogous cystamine analogue,[2] mp 242–243 °C (EtOH). Anal. C, H, N.

Pharmacology. The following protocol was applied for the relative potencies listed in Table I. Male rats weighing 175–200 g were killed by a sharp blow on the head and both vasa deferentia were isolated. These were mounted individually in organ baths of 10-mL vol containing Krebs bicarbonate buffer (118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.2 mM KH_2PO_4, 12.5 mM $NaHCO_3$, and 11.1 mM dextrose). All solutions were made 0.05% in metabisulfite in order to prevent oxidation of catecholamines. The medium was maintained at 37 °C while being aerated with 95% O_2–5% CO_2. The loading tension was 0.40 g and the contractions were recorded by means of force transducers connected to a Harvard polygraph. The tissues were allowed to equilibrate for 1 h and the medium was changed prior to addition of the antagonists. All compounds listed in Table I were tested at a concentration of 2×10^{-5} M. After a 30-min incubation, the bath was drained and the tissues were washed with the bath solution for 30 min. Cumulative concentration–response curves for NE (and E in some cases) were constructed after treatment with each antagonist. The decrease in maximum response was expressed in percent of the control value. The percent blockade for each compound is expressed as the mean ± SEM of four to six separate experiments.

Alternatively, segments of spirally cut rabbit aortic strips were submitted to a 2-g tension in a medium consisting of 116 mM NaCl, 4.6 mM KCl, 2.4 mM $CaCl_2$, 1 mM $MgSO_4$, 1.0 mM NaH_2PO_4, 21 mM $NaHCO_3$, 45 mM dextrose, and 0.26 mM Na_2EDTA. Cumulative concentration responses to NE, 5-HT, or histamine were obtained after equilibration of the strips for a 2-h period. Muscarinic blockade was determined on the isolated guinea pig atrium and ileum according to the method of Benfey and Grillo;[7] dose–response curves for acetylcholine and carbachol were determined cumulatively. The effects of antagonists **4** or **12** on the tissue responses to agonists were evaluated after a 30-min incubation at concentrations in excess of those required for complete inactivation on the α receptor followed by repeated washing for 30 min with the bath solutions. The results of five to eight independent observations were used to estimate the degree of agonist inhibition. The protochol for guinea pig atrium effects was as given in ref 7.

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(19) We have recently obtained concrete evidence that the discriminatory effect of BHC reflects the existence of two α-receptor subspecies displaying different affinities for BHC and consequently for NE and E, respectively.

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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