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The Cyano-NNO-Azoxy Function in the Design of an Irreversible Label for α_1 Adrenoreceptors

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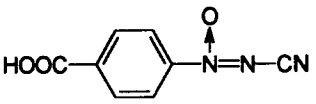
Abstract—A potential α_1-adrenergic irreversible antagonist **6**, containing the cyano-NNO-azoxy function was synthesized and tested. The effects of norepinephrine on rat thoracic aorta were irreversibly blocked by this compound at the concentration of 1×10^{-5} M after 60 minutes. Binding studies showed that **6**, at 1×10^{-6} M, did not modify the K_D of Prazosin and caused a 30% decrease of the B_{max}. Substitution in **6** of the bis (2-chloroethyl)amino moiety for the cyano-NNO-azoxy function afforded **7** which behaves as an irreversible antagonist able to change K_D of Prazosin without influencing B_{max}.

Introduction

The cyano-NNO-azoxy function (-N(O)=N-CN) is present in "Calvatic acid" **1** (*p*-carboxyphenyldiazenecarbonitrile-2-oxide or 4-(cyano-NNO-azoxy)benzoic acid), an antibiotic isolated from the cultural broths of a few Basidiomicetes.[1]



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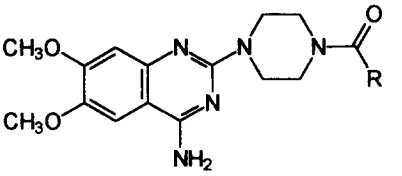
The chemistry of this group has received only scattered interest[2,3] and it is under consideration in our laboratory. Recently we found that phenyldiazenecarbonitrile-2-oxide (C_6H_5-N(O)=N-CN) and its analogues are able to react quickly in physiological conditions with cysteine, but not with serine and lysine.[4] The reactivity of the cyano-NNO-azoxy function with cysteine residues is probably responsible for the ability of Calvatic acid to inhibit cysteine proteinases and glutathione transferase,[5] as well as tubuline polymerisation.[6]

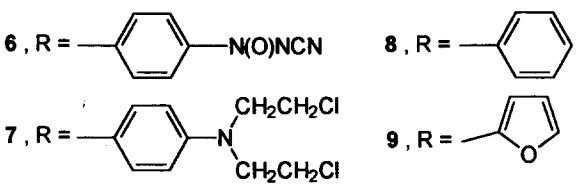
Studies with a series of tetramine disulphides related to benextramine suggest that on the surface of α_1-adrenoceptors a thiol group, able to irreversibly react with the disulphide moiety of these drugs, is present.[7]

On these bases we designed, as potential irreversible α_1-antagonists, the derivatives **6** and **7**, analogues of **8**, structurally related to Prazosin **9**, a well known α_1-adrenoceptor blocking drug.

Chemistry

The synthetic pathways used to obtain the final compounds **6–8**, are reported in Scheme 1.



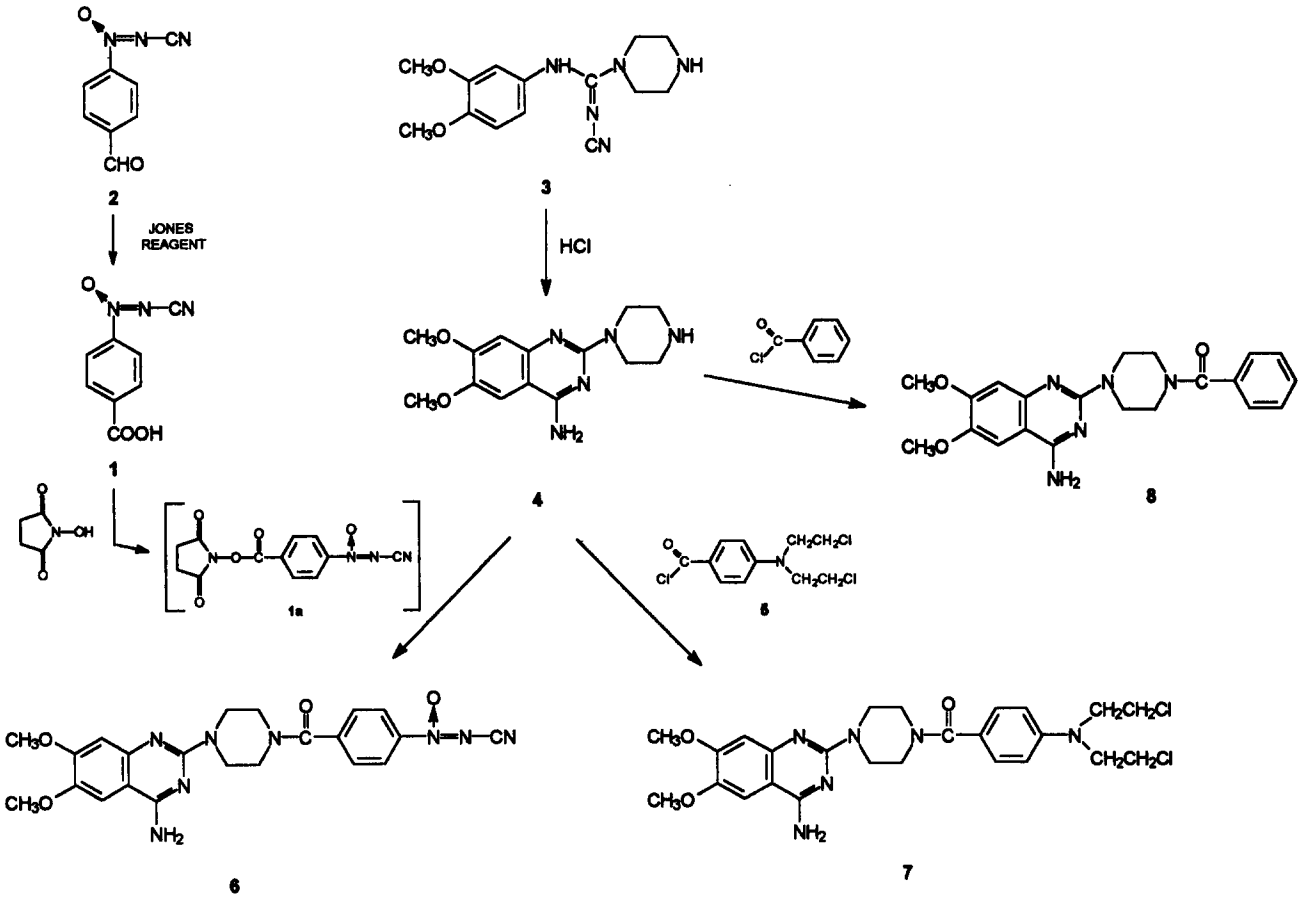


Amine **4**, prepared from compound **3** *via* Houben–Hoesch reaction, was allowed to react either with activated ester **1a**, obtained from Calvatic acid **1**, *N*-hydroxysuccinimide and *N*,*N*'-dicyclohexylcarbodiimide (DCC), or with benzoyl chloride to afford the expected compounds **6** or **8** respectively. Compound **1** was prepared by the oxidation of aldehyde **2** with "Jones' reagent". The final derivative **7** was obtained from **4** and **5** using the classical Schotten–Bauman reaction.

Pharmacology

The pharmacological profiles of compounds **6–8** at α_1-adrenoceptors were assessed on isolated rat thoracic aorta. Irreversible inactivation by **6** and **7** of α_1-binding sites was studied in the presence of either 10 or 30 μM concentrations of antagonists, extending incubation time from 10 min to 1 h. Aortic strips were then washed free of drug and a norepinephrine cumulative dose–response curve was recorded.

The decrease in maximum response to norepinephrine was expressed as a percentage of the control value (see Figs 1 and 2).



Scheme 1.

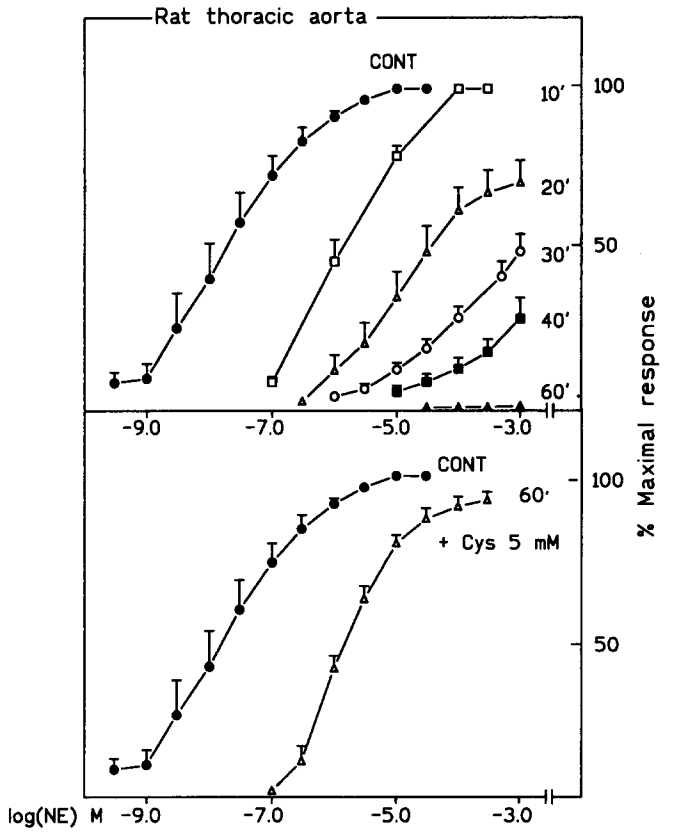


Figure 1. Concentration–response curves for norepinephrine after incubation with compound **6** at 10 μM for different times (top) and in the presence of 5 mM L-cysteine (bottom).

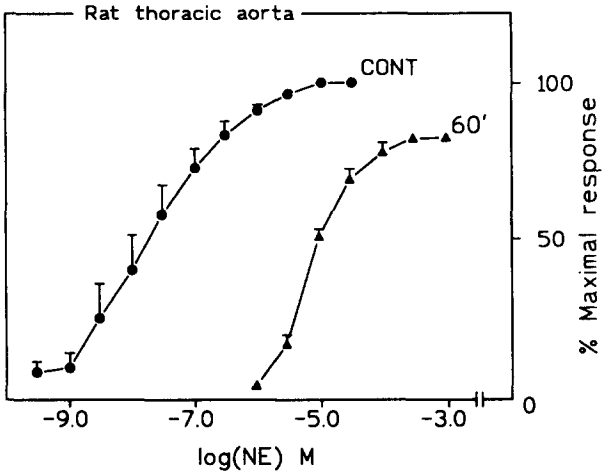


Figure 2. Concentration-response curves for norepinephrine after incubation with compound **7** at 30 μM for 60 min.

The α_2-adrenoceptor blocking activity was assessed on the prostatic portion of the rat vas deferens by antagonism to clonidine which inhibits twitch responses of the field-stimulated tissue by acting on the α_2-receptors.

Experiments of saturation and displacement binding were assessed on rat cerebral cortical membranes using [^{3}H]-Prazosin. Specific binding was defined as the difference between total binding, measured in the absence of any added ligand, and non-specific binding, measured in the presence of 10 μM phentolamine.

Results and Discussion

SAR studies have shown that in Prazosin **9** ($pA_2 \pm 95\%$ CL = 9.81 ± 0.27) the 2,4-diaminoquinazoline substructure, when protonated, behaves as a conformationally restricted form of the norepinephrine cation. It is able to display a high degree of complementarity for the coplanar hydrophobic and anionic sites of the α_1-receptor. The substituent at the quinazoline 2-nitrogen atom provides additional binding.[8]

When in **9** the benzoyl moiety is substituted for the 2-furoyl substructure, the resulting molecule **8** retains high affinity and selectivity for the α_1-receptor ($pA_2 \pm 95\%$ CL = 9.04 ± 0.18).

The covalent binding of an affinity label occurs in two consecutive processes. The first process is a reversible interaction of the drug to the receptor and the second process an irreversible reaction between the reactive part of the molecule (generally electrophilic in nature) with an adjacent group on the receptor.[9] The potential α_1-irreversible antagonist **6** we designed bears at the *p*-position of the phenyl ring of **8** a -N(O)=NCN function, an electrophilic group able to interact quite selectively with thiol moieties. In effect a UV study shows that **6** quickly reacts, at physiological pH and at room temperature, with an excess (1:6) of cysteine, while only minor changes in its UV spectrum were observed after 1 h of interaction with serine, lysine and tyrosine.

Compound **6** at 1×10^{-5} M concentration, after 10 min of incubation with rat aortic strips, was able to irreversibly shift to the right the cumulative concentration–response curve for norepinephrine, without a reduction of the maximal response. After 20 min of incubation, the shift to the right of the curve was accompanied by a 30% irreversible decrease in maximal response. This decrease was time-dependent (see Fig. 1) and after 1 h of incubation any response induced by norepinephrine was irreversibly blocked. This effect disappeared in the presence of 5 mM cysteine (see Fig. 1).

Compound **6** at 3×10^{-5} M concentration was unable to antagonise the clonidine induced depression of the twitch responses of field-stimulated prostatic portion of rat vas deferens.

When in **6** the cyano-NNO-azoxy function was replaced by a bis(2-chloroethyl)amino moiety (derivative **7**), a decrease in the antagonism activity was observed. In fact **7**, at 3×10^{-5} M concentration, after 1 h of incubation with the tissue, was able to irreversibly antagonise only 20% of maximal response of norepinephrine at the α_1-receptor (see Fig. 2).

Preincubation of rat cerebral cortical membranes with **6** followed by extensive washing and then assaying for saturation of [^{3}H]-Prazosin binding established that this compound was able to irreversibly bind the α_1-receptors. Scatchard plots of saturation data showed about 30% of irreversible decrease in the number of α_1-receptors (B_{max}) after membrane treatment with 10^{-6} M **6**, without change in the affinity of the remaining sites for Prazosin (see Table 1 and Fig. 3).

Table 1. Effect of analogues on α_1-adrenoceptor binding of [^{3}H]-Prazosin

Compound	$-\log IC_{50} \pm SE$	% decrease[a] in $B_{max} \pm SE$
6	7.70 ± 0.11	28.2 ± 3.5 (1 μM) 0 (0.1 μM)
7	7.22 ± 0.11	0 (1 μM)[b] 0 (0.1 μM)
8	8.88 ± 0.091	-

[a]Results are the average of at le ast three separate experiments.
The control B_{max} value was 154.34 ± 23.36 fmol mg^{-1} protein (n = 7) and the K_D for [^{3}H]-Prazosin was 0.17 ± 0.021; [b]K_D value for [^{3}H]-Prazosin after treatment with compound **7** was 0.49 ± 0.075.

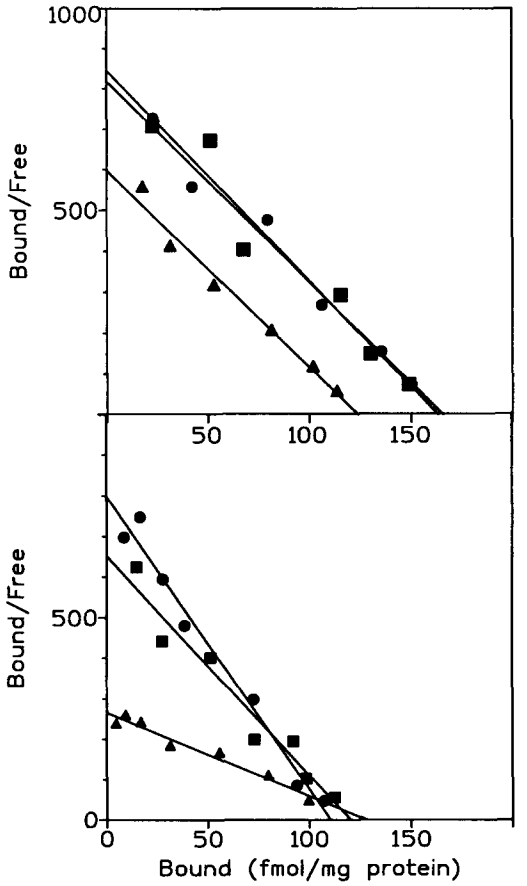
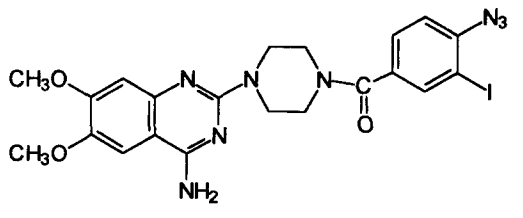


Figure 3. Scatchard plots of specific [^{3}H]-Prazosin binding on membrane preparations from rat cortex pretreated with no drug (●) or with 0.1 μM (■) and 1 μM (▲) concentrations of compounds **6** (top) and **7** (bottom).

IC_{50} values for derivatives **6** and **8** were evaluated by their ability to displace [^{3}H]-Prazosin from its binding sites. In the case of **6** this study was done in a concentration range in which no irreversible activity was detected. The insertion of the -N(O)=NCN moiety into **8** (log $IC_{50} \pm SE$ = 8.88 ± 0.091) to give **6** is accompanied by a relevant decrease of the affinity (log IC_{50} ± SE = 7.70 ± 0.11). Affinity of **6** is about 16-fold less than that of ^{125}ICP65,526 **10**, a well known photoaffinity label of α_1-adrenergic receptors.[10]



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This decrease can be ascribed to the presence in **10** of the iodine atom and to the different receptorial affinity of the azido group compared to azoxycyano moiety.

Derivative **7** displayed a different behaviour in the binding studies. In fact a concentration of 1 μM of **7** was able to modify the K_D of Prazosin without changing B_{max} (see Table 1 and Fig. 3). This means that **7** binds sites intimately associated with the receptor, but distinct from sites for Prazosin. This binding changes the affinity of the Prazosin for its own sites and this could be explained by the high reactivity of the nitrogen mustard group which is able to interact with a variety of nucleophiles present on the receptor protein.

In conclusion this work shows that the cyano-NNO-azoxy function can be a moiety useful to design electrophilic affinity labels with specificity for thiol groups. In addition it confirms previous data showing that in an irreversible antagonist the chemoreactive part of the molecule must have some receptor affinity in order to carry on its irreversible action.[11]

Experimental

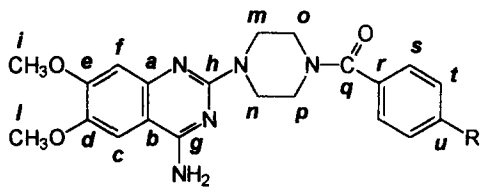
Chemical protocols

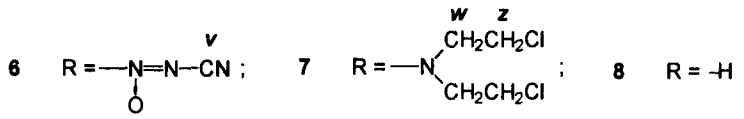
Melting points were measured with a capillary apparatus (Büchi 530) and are uncorrected. The final compounds **6** and **7** decompose by heating; the reported temperatures are the decomposition maxima recorded by DSC analyses (Perkin–Elmer DSC 7). All compounds were routinely checked by IR (Perkin–Elmer 781), ^{1}H and ^{13}C NMR (Bruker AC-200) and mass spectrometry (Finnigan-Mat TSQ-700). The ^{13}C chemical shifts, relative to TMS, of the final compounds in DMSO-d_6 are reported in Table 2. Silica gel (Merck Kieselgel 60), 70–230 mesh ASTM was employed for column chromatography. TLC was carried out on plates precoated with Merck Kieselgel 60 F_{254} layer thickness 0.25 mm. Anhydrous magnesium sulphate was used as the drying agent of the organic extracts. Analyses of the new compounds were performed by REDOX (Cologno M.) and the results are within ± 0.4% of theoretical values.

Compounds **2**,[4] **3**[12] and **5**,[13] were synthesized according to the literature.

4-(Cyano-NNO-azoxy)benzoic acid (1). To a stirred and externally cooled solution of **2** (0.53 g, 3.0 mmol) in 6 mL of acetone, a solution of chromic acid oxidizing reagent (Jones' reagent)[14] was added dropwise in such a way as to ensure that the temperature was maintained between 10–15 °C. The progress of the reaction was checked via TLC (eluent dichloromethane: petroleum ether 40–60 °C 7:3) and was stopped when the brown colouration of the mixture became persistent. The excess of Jones' reagent was destroyed with isopropanol and the mixture was poured, with stirring, into 100 mL of ice-water. After 1 h standing and cooling the solid was filtered, washed with water and dried giving **1** as a white solid. The yield of **1** was 0.52 g (90%), mp = 201–203 °C dec. Lit.[1] 198–199 °C dec. The compound was used without further purification.

Table 2. ^{13}C NMR data for Prazosin analogues





	a	*b*	*c*	*d*	*e*	*f*	*g*	*h*	*i*	*l*
6	148.5	103.1[a]	103.7[a]	145.0[a]	154.3	105.1[a]	161.3	158.2	55.9[a]	55.5[a]
7	148.9	103.2[a]	103.9[a]	147.8[a]	154.5	105.4[a]	161.4	158.5	56.1[a]	55.6[a]
8[c]	135.6[a]	101.8	105.0	147.0	155.4	99.3	161.4	151.5	56.3[a]	56.2[a]

	m, n, o, p	*q*	*r*	*s*	*t*	*u*	*v*	*w*	*z*
6	47.0, 43.8, 43.4, 41.8	167.2	145.2[a]	128.6	123.5	142.9	111.0		
7	44.0[b]	169.7	145.3[a]	129.8	111.2	123.5		41.2	52.1
8[c]	44.5[d]	169.5	136.3[a]	127.2[a]	128.6[a]	129.9			

[a]Tentative of assignment; [b]a sharp and a broad peak overlapped; [c]hydrochloride, [d]broad.

6,7-Dimethoxy-2-(1-piperazinyl)-4-quinazolylamine (4). Compound **3** (10 g, 35 mmol) was stirred mechanically with 20% HCl (20 mL) at room temperature over 5 h. The mixture was filtered and the product obtained was washed with acetone and dried giving a white solid. The yield of **4** hydrochloride was 7.98 g (70%). The free base can be obtained by dissolving the dihydrochloride in 2 N NaOH (35 mL) and scratching while cooling quickly. The solid obtained was filtered then washed with a little ice-water and recrystallised from ethanol, mp 229–230 °C, Lit.[15] 230–232 °C.

1-(4-Amino-6,7-dimethoxy-2-quinazolyl)-4-[4-(cyano-NNO-azoxy)benzoyl]piperazine (**6**). *N*-Hydroxysuccinimide (0.15 g, 1.3 mmol) and DCC (0.27 g, 1.3 mmol) were added to a solution of **1** (0.19 g, 1.0 mmol) in 10 mL of anhydrous THF and the mixture was stirred for 1 h at room temperature. The solution of the active ester **1a** was obtained by filtering off the residue of dicyclohexylurea from the reaction mixture. A solution of **4** (0.29 g, 1.0 mmol) in anhydrous THF (5 mL) and dry DMF (1 mL), was added drop by drop to the solution of **1a** under stirring. After 2 h at room temperature the crude **6** was collected by filtration and purified by column chromatography eluting with ethyl acetate. The solid obtained, after solvent removal, was pure on TLC analyses and the yield was 0.27 g (50%). An analytical sample was recrystallised from ethyl acetate and petroleum ether 40–60 °C, decomposition maximum 219 °C. Anal. for $C_{22}H_{22}N_8O_4$ C, H, N.

1-(4-Amino-6,7-dimethoxy-2-quinazolyl)-4-[4-(bis-β-chloroethylamino)benzoyl]piperazine (7). Solutions of **5** (1.3 g, 5.0 mmol) in dichloromethane (10 mL) and sodium carbonate monohydrate (0.62 g, 5.0 mmol) in water (10 mL) were added portionwise alternatively to a well stirred solution of **4** (1.45 g, 5.0 mmol) in water (10 mL). After 30 min at room temperature the pure precipitate was collected, washed with water and dried; the yield of **7** was 2.27 g (80%). An analytical sample of **7** was dissolved in a large amount of ethanol, filtered and the solvent was partially eliminated under reduced pressure. After cooling, **7** crystallised as a white solid, decomposition maximum 253 °C. Anal. for $C_{25}H_{28}Cl_2N_6O_3$ C, H, N.

1-(4-Amino-6,7-dimethoxy-2-quinazolyl)-4-benzoylpiperazine hydrochloride (8). This compound was synthesised, according to the method reported in a patent,[16] from **4** (0.87 g, 3 mmol) and benzoyl chloride (0.42 g, 3 mmol). Thus to a stirred suspension of **4** in methanol (15 mL), a solution of benzoyl chloride in anhydrous THF (5 mL), was added; in a few minutes the solid was dissolved, then a new precipitate was formed. The reaction mixture was stirred at room temperature for 1 h, after this time the pure solid was collected, washed with a small amount of methanol and dried. The yield of **8** was 1.11 g (90%). An analytical sample was recrystallised from methanol, mp = 297–299 °C dec. Anal. for $C_{21}H_{23}N_5O_3$ HCl 0.3 H_2O C,H,N.

Pharmacological protocols

Functional antagonism at α_1-adrenoceptors. Male Wistar rats (250–300g) were sacrificed by decapitation and the thoracic aortae were rapidly isolated. The vessels were helically cut, the endothelium removed and two strips were obtained from each aorta. The tissues were suspended under a tension of 1 g in organ baths containing a *Krebs–Henseleit* solution of the following composition (mM): NaCl 137; KCl 2.68; $MgCl_2$ 0.5; $CaCl_2$ 5.44; NaH_2PO_4 0.54; $NaHCO_3$ 8.93; glucose 8.3; ascorbic acid 0.1. Desmethylimipramine hydrochloride 1×10^{-7}M,

deoxycorticosterone acetate 5×10^{-6} M, propranolol 5×10^{-6} M, and yohimbine hydrochloride 1×10^{-7} M were added to the solution to prevent neuronal and extraneuronal uptake of norepinephrine and to block β- and α_2-adrenoceptors respectively. The medium was maintained at 37 °C and was gassed with 95% O_2–5% CO_2 (pH 7.4). A stabilization period of 2 h was allowed before a cumulative dose–response curve to norepinephrine (0.001–50 μM) was performed. During this equilibration period the bathing solution was changed every 30 min. Preparations were incubated with antagonists for different times, then washed for 60 min and a concentration–response curve for norepinephrine was repeated.

Functional antagonism at α_2-adrenoceptors. Prostatic portions of the rat vas deferens were mounted in an organ bath containing 30 mL of Krebs solution: NaCl 118.4; KCl 4.7; $CaCl_2$ 2.52; $MgSO_4$ 0.6; KH_2PO_4 1.2; $NaHCO_3$ 25.0; glucose 11.1 mM. The solution was maintained at 37 °C and continuously gassed with 95% O_2–5% CO_2 (pH 7.4). The preparations were stretched to a resting tension of 1 g (isometric transducers) and allowed to equilibrate for at least 60 min before addition of any drug. A first clonidine dose–response curve, taken as control, was obtained cumulatively. Thus, after incubation with antagonist for 30 min, a second dose–response curve was obtained.

Membrane preparation. Cerebral cortices, obtained from male Wistar rats (220–240 g), were homogenised in 20 volumes (w/v) of 5 mM Tris–5 mM EDTA buffer (pH 4 at 4 °C) in an Ultra-Turrax homogeniser and centrifuged at 43000 *g* for 12 min. The pellet was first resuspended in 10 volumes of homogenising buffer, centrifuged and then washed twice with ice-cold 50 mM Tris–0.5 mM EDTA assay buffer (pH 7.4 at 4 °C). The final pellet was resuspended in 20 mL assay buffer (pH 7.4 at 25 °C).

Irreversibility experiments. Aliquots of this suspension were incubated for 1 h at 37 °C with the addition of either only buffer (control) or a solution of compounds **6** and **7** at concentration 0.1 μM or 1 μM. The membranes were then diluted to approximately 30 mL with buffer, incubated a further 10 min on ice and washed by a series of four centrifugations and resuspensions before finally being resuspended for saturation assay of [^{3}H]-Prazosin binding.

Receptor assay. [^{3}H]-Prazosin binding sites were measured in an assay volume of 1 mL containing the following: [^{3}H]-Prazosin (~83 Ci mmol^{-1}, Amersham, 0.2 nM for affinity experiments, 0.03–2.0 nM, seven different concentrations for saturation experiments), rat cortical membranes, 800 μL containing approximately 250 μg of membrane protein per assay and buffer (Tris–HCl, 50 mM, EDTA 0.5 mM, pH 7.4) to make 1 mL. For affinity studies, tubes also contained Prazosin analogues (twelve different concentrations between 3×10^{-11} and 1×10^{-5}M). Specific binding was defined as the difference between total binding (measured in the absence of any added ligand), and non-specific binding (measured in the presence of 10 μM phentolamine). Duplicate tubes were used for each condition. Reaction tubes were incubated for 30 min at 30 °C, then diluted to 5 mL with ice cold buffer and filtered under reduced pressure through Whatman GF/B glass fiber filters, treated with 0.1% polyethylenimine. Tubes and filters were washed three additional times with 5 mL of buffer. The amount of radioactivity retained on the filters was quantitated by liquid scintillation counting, using a Beckman liquid scintillation spectrophotometer.

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References

1. Gasco, A.; Serafino, A.; Mortarini, V.; Bianco, M. A.; Scurti, J.C. *Tetrahedron Lett.* **1974**, *38*, 3431.

2. Calvino, R.; Fruttero, R.; Gasco, A.; Miglietta, A.; Gabriel, L. *J. Antibiotics* **1986**, *39*, 864, and references cited therein reported.

3. Zlotin, G. S.; Podgurskii, I. A.; Airapetova, V. N.; Lukyanoy, O. A. *Izv. Akad. Nauk. SSSR. Seer. Khim.* **1991**, *7*, 1647; *Chem. Abstr.* **1991**, *115*, 232183h.

4. Gasco, A. M.; Di Stilo, A.; Fruttero, R.; Gasco, A.; Budriesi, R.; Chiarini, A. *Med. Chem. Res.* **1993**, *28*, 34.

5. Caccuri, A. M.; Ricci, G.; Desideri, A.; Buffa, M.; Fruttero, R.; Gasco, A.; Ascenzi, P. *Biochem. Mol. Biol. Inter.* **1994**, *32*, 819.

6. (a) Gadoni, E.; Miglietta, A.; Olivero, A.; Gabriel, L. *Biochem. Pharmacol.* **1989**, *38*, 1121; (b) Miglietta, A.; Gadoni, E.; Olivero, A.; Gabriel, L. *Third European Congress on Cell Biology*, Firenze, 2–7 September, 1990.

7. Melchiorre, C. *Trends Pharmacol. Sci.* **1981**, *9*, 209.

8. Campell, S. F. In: *X-Ray Crystallography and Drug Action*, pp 347–366, Horn, A. S.; De Ranter, C. F. Eds; Clarendon; Oxford, 1984.

9. Takemori. A. E.; Portoghese, P. S. *Ann. Rev. Pharmacol. Toxicol.* **1985**, *25*, 193.

10. Seidman, C. E.; Hess, H. S.; Homcy, C. S.; Graham, R. M. *Biochemistry* **1984**, *23*, 3765.

11. Pitha, J.; Szabo, L.; Szuramai, Z.; Buchowiecki, W.; Kusiak, J. W. *J. Med. Chem.* **1989**, *32*, 96.

12. Boschi, D.; Di Stilo, A.; Fruttero, R.; Medana, C.; Sorba, G.; Gasco, A. *Arch. Pharm.* **1994**, *327*, 661.

13. Ederfield, R. C.; Liao, T. K. *J. Org. Chem.* **1961**, *26*, 4996.

14. Eisenbraun, E. J. *Org. Synth. Coll.* **1973**, *5*, 310.

15. Sekiya, T.; Hiranuma, H.; Hata, S; Mizogami, S.; Hanazuka, M.; Yamada, S. *J. Med. Chem.* **1983**, *26*, 411.

16. Hess, H. J. E. *Ger. Offen.* 2,457,911; *Chem. Abstr.* **1975**, *83*, 114486g.

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