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Synthesis and antagonistic activity at muscarinic receptor subtypes of some derivatives of diphenidol

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Dedicated to the memory of Prof. Piero Pratesi

Abstract

A series of new derivatives, related to diphenidol and to its 2-carbonyl analogue, were designed as antimuscarinic agents. The synthesized compounds were evaluated both as hydrochlorides and as methiodides by functional tests at guinea-pig heart (M_2), guinea-pig ileum (M_3) and rabbit vas deferens (putative M_4). Two derivatives (**3a** and **5a**) showed an M_3-selective profile similar to that of the reference compounds, though they resulted less potent.

Keywords: Receptor; Muscarinic activity; Antagonists; SAR

1. Introduction

Muscarinic receptors are present both in parasympathetic and central nervous systems. In the periphery, muscarinic receptors mediate smooth muscle contraction, glandular secretion, and modulation of cardiac rate and force. In the central nervous system there is evidence that muscarinic receptors are involved in motor control, temperature and cardiovascular regulation, and memory. Interest in the classification of muscarinic receptors involved in functions at different locations has been heightened by the potential therapeutic application of selective ligands. Currently, selective compounds may emerge as therapeutics in several diseases: agonists may be used to treat Alzheimer's disease, nociceptive pain, schizophrenia, while antagonists may be used for Parkinson's disease, urinary incontinence, irritable bowel syndrome, gastric ulceration and chronic obstructive pulmonary disease [1–4].

However, the unambiguous identification of muscarinic receptor M_1–M_5 subtypes mediating many important responses has been impeded by the lack of any selective agonist and the paucity of highly selective antagonists.

As a part of our studies on diphenidol (1,1-diphenyl-4-piperidin-1-yl-butan-1-ol, **1**, Fig. 1) [5–7], we synthesized some analogues in which the intermediate chain connecting the lipophilic head of the molecule to the cationic center was modified, rendering it more polar or less flexible [6]. The introduction of a carbonyl group in position 2 of the butyl chain of diphenidol **1** led to derivative **2a** with enhanced affinity for both the M_2 and the M_3 receptor subtypes and a better M_3/M_2 selectivity ratio.

In order to check our hypothesis that the carbonyl group on the butyl chain might reinforce the ligand-receptor interaction, or affect the conformational behavior of the molecule in such a way as the pharmacophoric groups are exposed to the receptor in a favorable spatial arrangement, we synthesized some derivatives with an additional carbon atom in the alkyl chain (**3**–**7**, Fig. 1). In particular, we examined the effects of the distance between the active functions (lipophilic moiety

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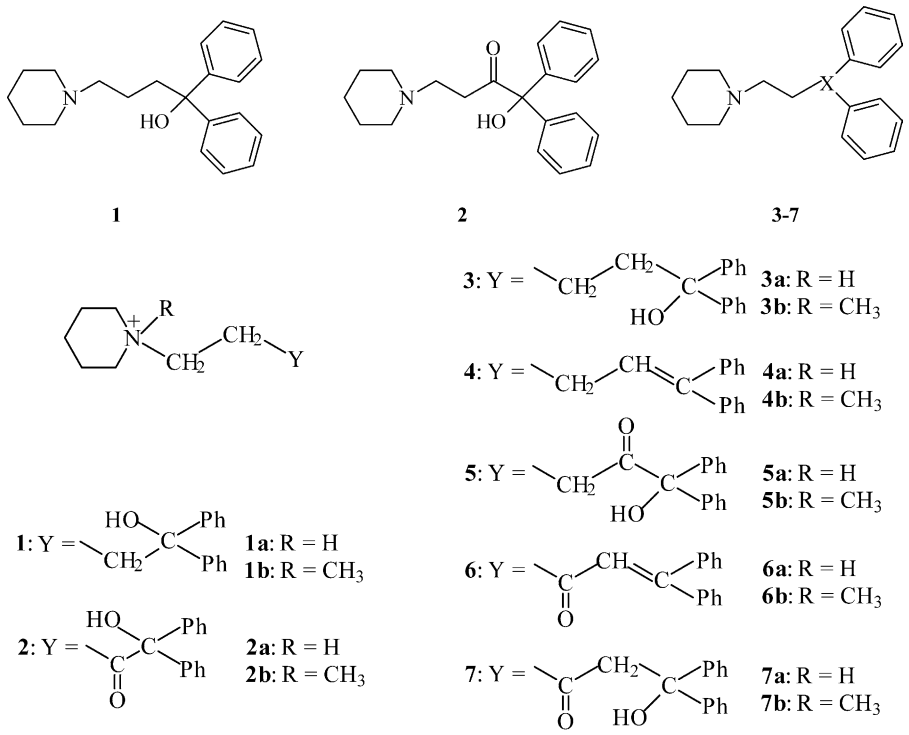


Fig. 1. Structure of diphenidol (**1**), 2-carbonyl analogue (**2**) and target compounds (**3**–**7**).

and cationic head), and of the presence of the carbonyl group at different positions in the pentyl chain. The contribution to the affinity and/or selectivity of the benzylic hydroxy group was also studied.

Considering that Lambrecht et al. [8] observed that the methylation of the basic nitrogen atom of hexahydrodiphenidol and hexahydrosiladiphenidol affects differently the affinity for the M_2 and M_3 receptor subtypes, the designed compounds were prepared and tested as both methiodide and hydrochloride salts.

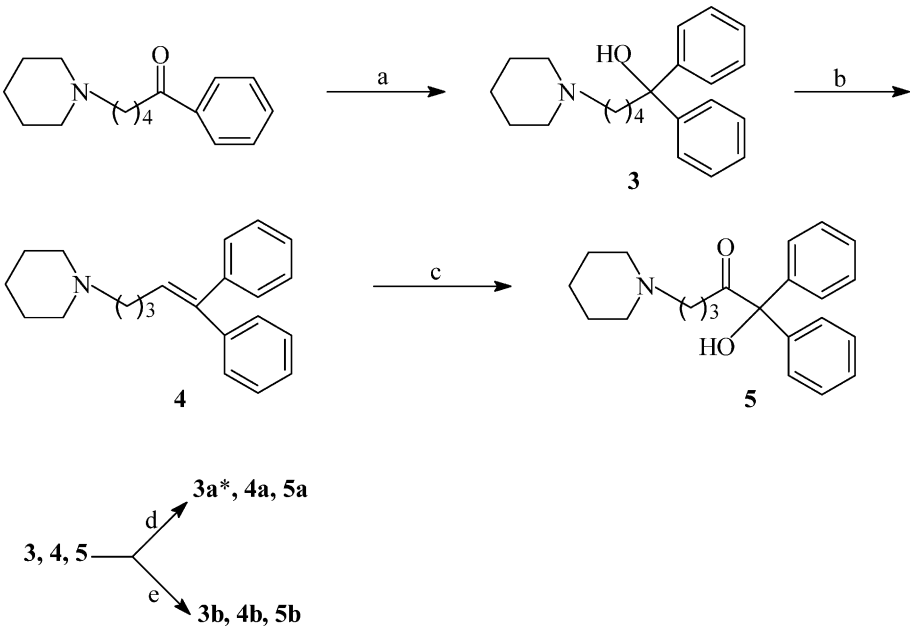
In the present paper, we report on the synthesis and the pharmacological evaluation by functional studies of compounds **3a,b**–**7a** and we discuss their structure–activity relationships.

2. Chemistry

The designed compounds **3a,b**–**7a** were synthesized by standard procedures as reported in Schemes 1 and 2. Treatment in dry conditions and under nitrogen of δ-piperidinovalerophenone with phenyl lithium, prepared by addition of a stoichiometric amount of *tert*-butyl lithium to bromobenzene, afforded the pentanol **3**. This compound was dehydrated with 37% hydrochloric acid in glacial acetic acid giving the corresponding pentene derivative **4**. Oxidation of **4** with potassium permanganate in diluted acetic acid and acetone led to the hydroxy-pentanone **5**. Compounds **3**, **4** and **5** were converted into the corresponding hydrochlorides **3a**, **4a** and **5a** and methiodides **3b**, **4b** and **5b** with a hydrochloric acid saturated solution in ether and methyl iodide in acetone, respectively (Scheme 1). Reaction of silyl enol ether **8** (Scheme 2), prepared from 1-piperidinobutan-3-one and chlorotrimethylsilane, with benzophenone activated by titanium tetrachloride, afforded a mixture of compounds **6** and **7**, which were separated by column chromatography and converted to the hydrochlorides **6a** and **7a** and methiodide **6b**, as described previously. Methiodide **7b** resulted unstable and could not be isolated.

3. Results and discussion

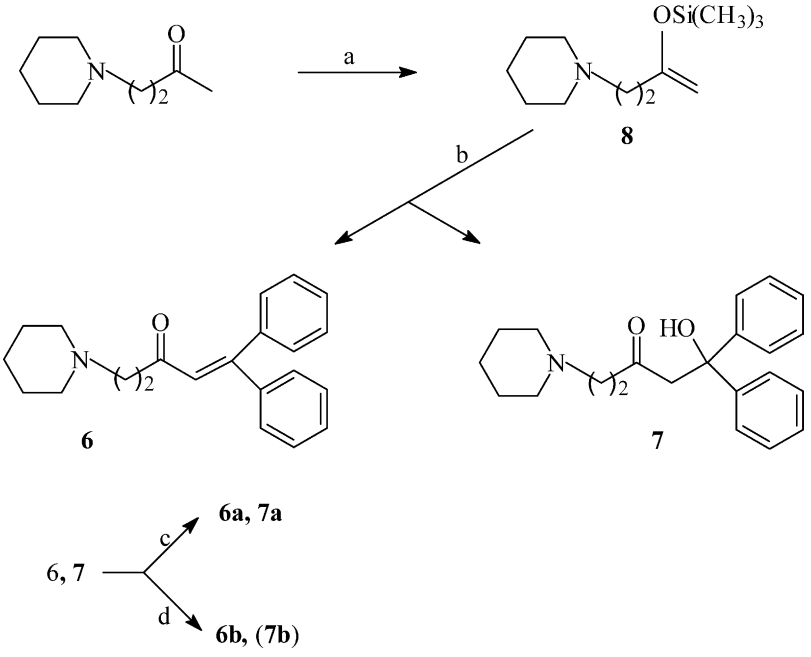
All the synthesized compounds were tested on M_2 (guinea-pig heart), M_3 (guinea-pig ileum) and putative M_4 (rabbit vas deferens) muscarinic receptor subtypes for their antimuscarinic activity, using arecaidine propargyl ester (APE) at M_2 and M_3, and *p*-Cl–McN–A-343 at M_4 receptor, as reference agonists. The antagonistic potency was expressed as dissociation constant (pK_b); the results are reported in Table 1**,** together with the corresponding data at M_2 and M_3 receptor subtypes of the diphenidol **1** and of the previously published diphenidol analogue **2** [6]. Although many authors use rabbit vas deferens for the putative muscarinic M_1 receptors, recently Melchiorre and co-workers [9] showed that a series of polymethylene tetra-amines are able to significantly differentiate muscarinic M_1 and M_4



Scheme 1. (a) C_6H_5Br, *t*-BuLi, ether; (b) HCl, AcOH; (c) $KMnO_4$, $AcOH/H_2O$/acetone; (d) HCl/ether; (e) CH_3I, acetone. *Directly obtained from work up of the reaction mixture.

receptors. In fact, these authors report that affinity values (pA_2) obtained in functional studies on the prostatic portion of rabbit vas deferens for some of these tetra-amines, namely Dipitramine and AM172, most closely correlate with the affinity values (pK_i) obtained at native and human recombinant muscarinic M_4 receptors, suggesting that the muscarinic receptor mediating inhibition of neurogenic contractions of rabbit vas deferens could belong to the M_4 subtype. For this reason in this paper we prefer to appoint the term 'putative M_4 muscarinic receptor subtype' to the receptors active on rabbit vas deferens.

Examination of the pharmacological results reveals that, generally, all the studied compounds present a decrease in affinity with regard to the reference structural analogues **1a**,**b**, **2a**,**b** [6]. Considering the hydrochloride salts, the derivative **3a**, bearing an additional methylene group with respect to diphenidol (**1a**), shows a five and twofold fall in the affinity for the M_2 and M_3 receptor subtypes, respectively, but a slightly improved



Scheme 2. (a) $(CH_3)_3SiCl$, NaI, $(C_2H_5)_3N$, CH_3CN; (b) $(C_6H_5)_2CO$, $TiCl_4$, CH_2Cl_2; (c) HCl/ether; (d) CH_3I; acetone.

Table 1
Affinity values (pK_b) and selectivity ratios for muscarinic antagonists **3a,b**–**7a**, at guinea-pig heart (force) (M_2), guinea-pig ileum (M_3), and rabbit vas deferens (putative M_4)

No.	$pK_b \pm$SEM [a]			Selectivity ratios [b]		
	M_2	M_3	M_4	M_2/M_3	M_2/M_4	M_3/M_4
1a [c]	6.72±0.02	7.02±0.04		0.5		
1b [c]	7.47±0.04	7.26±0.02		1.6		
2a [c]	7.48±0.05	8.12±0.03		0.2		
2b [c]	6.22±0.09	7.40±0.02		0.07		
3a	5.99±0.11	6.72±0.19	6.10±0.21	0.2	0.8	4
3b	6.59±0.05	6.56±0.11	7.34±0.15	1	0.2	0.2
4a	6.51±0.05	6.30±0.16	6.33±0.11	2	1.5	1
4b	7.00±0.08	7.02±0.11	6.74±0.04	1	2	2
5a	6.92±0.06	7.67±0.02	6.69±0.13	0.2	2	9.5
5b	7.49±0.21	7.30±0.06	7.55±0.07	1.5	0.9	0.6
6a	5.94±0.15	<5.52	5.88±0.17	>3	1	>0.4
6b	4.58±0.08	<5.52	<5	>0.1	>0.4	>3
7a	5.57±0.02	5.97±0.25	5.29±0.12	0.4	2	5

[a] Affinity constants calculated from the equation $\log(DR-1) = \log[ant] - \log K_b$ for a single concentration of the antagonist, according to van Rossum [10], are reported.
[b] Antilog of the difference between the pK_b values for M_2, M_3 and putative M_4 muscarinic receptor subtypes.
[c] Ref. [6].

M_3 selectivity. The introduction of a double bond in position 1 (**4a**) affects in a different way the affinity for the M_2 and M_3 receptor subtypes, if compared with compound **3a**: it causes an about threefold increase of affinity at M_2 subtype and an equivalent decrease at the M_3 subtype. Compound **5a**, the most potent M_3 antagonist of the new series, compared with the lower homologue **2a**, shows a behaviour similar to that of compound **3a**, with a decreased affinity at both M_2 and M_3 receptor subtypes, four and threefold, respectively. The introduction of the carbonyl group in β-position (**7a**) instead of α-position (**5a**) with regard to the hydroxy group, leads to a more important fall in the affinity at both M_2 and M_3 receptor subtypes (22- and 50-fold, respectively). Likewise, the introduction of a β-carbonyl group is detrimental for the affinity also in the case of the pentene derivative **6a** when compared with **4a**.

With regard to the methiodide series, a similar trend is evident for compound **3b** when compared with diphenidol methiodide **1b**, with an eight and fivefold lower affinity at M_2 and M_3 receptor subtypes, respectively. Derivative **4b** shows a little increment in the affinity at both M_2 and M_3 subtypes with respect to the hydroxy analogue **3b** and a more relevant increment when referred to the butene analogue (pK_b values of 6.39 and 6.17 at M_2 and M_3 receptor subtypes, respectively) [6]. In the case of compound **5b**, the presence of an additional methylene group compared with compound **2b** displays an increased affinity only at M_2 receptor subtype, without any influence at M_3 one: this causes a complete loss in the selectivity. Finally, as for the corresponding hydrochloride derivative **6a**, the introduction of a β-carbonyl group (**6b**) affords a decrease in the affinity at both M_2 and M_3 receptor subtypes when compared with **4b**.

Compound **3b** proves to be the only antagonist showing a higher affinity (five times) at putative M_4 respect to M_2 and M_3 receptors; moreover, this compound and the α-carbonyl analogue **5a** show the highest selectivity ratios between M_3 and putative M_4 receptors (0.2 and 9.5, respectively).

In conclusion, the results of the present study confirm our hypothesis that the carbonyl group α to the benzylic carbon is the most important feature of this class of diphenidol derivatives. Furthermore, the polar benzylic OH group seems to play a critical role for the affinity and the selectivity at M_3 receptor subtype, on condition that it is correctly oriented [6]. The lack of the hydroxy benzylic group influences the affinity at M_2 and M_3 receptor subtypes in such a way that it causes a loss in selectivity.

Finally, the changes in the length of the intermediate chain connecting the active functions (lipophilic moiety and cationic heads) and in the position of the carbonyl group (α or β), and the presence of the double bond, negatively influence the affinity of compounds **3**–**7** at M_2 and M_3 receptor subtypes.

4. Experimental

4.1. Chemistry

Melting points were taken on Electrothermal open capillary apparatus and are uncorrected. Elemental

analysis was performed for compounds **3a,b**–**7a** and the results (not shown) were within ±0.4% of the theoretical values. Infrared spectra (IR) were recorded on a Perkin–Elmer 683 instrument for all compounds and were consistent with the assigned structures; because of the lack of unusual features, they are not included. ^{1}H and ^{13}C NMR spectra were registered on a Varian VXR 300 spectrometer, peak positions are given in parts per million (δ) relative to the standard chemical shift of the solvent. Merck silica gel 60 (230–400 mesh) was used for column chromatography. Thin-layer chromatography (Merck silica gel 60 F_{254} analytical plates) was used to monitor reactions. Sodium iodide was dried 24 h under atmospheric pressure at 140 °C and stored under nitrogen. Triethylamine was refluxed over potassium hydroxide pellets and then distilled. Methylene chloride was dried over molecular sieves 4 Å. Anhydrous ether and acetonitrile were purchased from Aldrich. The term 'dried' refers to the use of anhydrous sodium sulfate.

4.1.1. 1,1-Diphenyl-5-piperidin-1-yl-pentan-1-ol hydrochloride (*3a*)

A solution of *t*-BuLi (1.7 M in pentane) (11.8 ml) was added (10 min) under nitrogen to a solution of bromobenzene (1.57 g, 10 mmol) in anhydrous ether (60 ml), dropwise and at a temperature of −15 °C. After stirring at −15 °C for 1 h, 1-phenyl-5-piperidino-pentan-1-one [11] (2.45 g, 10 mmol) in anhydrous ether (10 ml) was added dropwise, keeping the temperature at −15 °C. The mixture was stirred for a further 4 h, then allowed to warm to +10 °C, put into iced 2 N HCl (5 ml) and extracted with chloroform (3 × 50 ml). The combined organic layers were washed with brine, dried and evaporated to give a solid product, which was treated with activated carbon and recrystallized from $CHCl_3$/ether, m.p.: 194–195 °C (yield: 55%).

^{1}H NMR (DMSO-d_6): δ 1.18–1.41 (3H, m, 1 × piperidine proton + 3-CH_2), 1.67–1.73 (7H, m, 5 × piperidine protons + 4-CH_2), 2.25 (2H, t, 2-CH_2), 2.75 (2H, m, piperidine protons), 2.89 (2H, t, 5-CH_2), 3.37 (2H, m, piperidine protons), 5.55 (1H, s, OH exch. D_2O), 7.12–7.16 (2H, m, ArH), 7.26 (4H, t, ArH), 7.43 (4H, d, ArH), 10.15 (1H, brs, NH exch. D_2O). ^{13}C NMR (DMSO-d_6): δ 20.75 (C3), 21.41 (C–piperidine), 22.14 (2 × C–piperidine), 23.16 (C4), 40.28 (C2), 51.61 (2 × C–piperidine), 55.45 (C5), 76.19 (C1), 125.56 (4 × C–Ar), 125.83 (2 × C–Ar), 127.55 (4 × C–Ar), 148.10 (2 × C–Ar).

4.1.2. 1-(5,5-Diphenyl-pent-4-eny1)-piperidine hydrochloride (*4a*)

Compound **3** was treated with conc. hydrochloric acid in glacial acetic acid, at reflux for 2 h, according to a standard method. From the basified (NaOH) and worked up reaction mixture, **4** was obtained in 33% yield. A sample was solubilized in ether and treated with hydrochloric acid saturated ethereal solution. A white solid separated, which was collected and recrystallized from $CHCl_3$/ether, m.p.: 223–225 °C (lit. [12] m.p.: 210–212 °C).

^{1}H NMR (DMSO-d_6): δ 1.27 (1H, m, piperidine proton), 1.74–1.87 (7H, m, 5 × piperidine protons + 2-CH_2), 2.07 (2H, q, $J = 6$ Hz, 3-CH_2), 2.78 (2H, m, piperidine protons), 2.91 (2H, t, 1-CH_2), 3.30 (2H, m, piperidine protons), 6.10 (1H, t, $J = 6$ Hz, 4-CH), 7.12–7.44 (10H, m, ArH), 10.42 (1H, brs, NH exch. D_2O). ^{13}C NMR (DMSO-d_6): δ 21.33 (C–piperidine), 22.25 (2 × C–piperidine), 23.24 (C2), 26.43 (C3), 51.67 (2 × C–piperidine), 55.10 (C1), 126.59 (2 × C–Ar), 126.87 (C4), 126.97 (C5), 127.53 (C–Ar), 127.99 (2 × C–Ar), 128.23 (2 × C–Ar), 129.16 (2 × C–Ar), 139.06 (C–Ar), 141.54 (C–Ar), 141.69 (C–Ar).

4.1.3. 1-Hydroxy-1,1-diphenyl-5-piperidin-1-yl-pentan-2-one hydrochloride (*5a*)

To a stirred solution of **4** (0.305 g, 1 mmol) in acetone (7 ml), water (1.5 ml) and glacial acetic acid (0.13 ml) was added, at 0 °C, a solution of powdered potassium permanganate (0.210 g, 1.3 mmol) in acetone containing 20% water (4.5 ml). The stirring was continued for 30 min at room temperature, the formed manganese dioxide was filtered, the solution was concentrated, made alkaline (NaOH) and extracted with ether (3 × 20 ml). The combined organic layers were washed, dried and evaporated. The residue was purified on a silica gel column by flash chromatography eluting first with chloroform and then with $CHCl_3$/MeOH 95:5, giving **5** as pure oil (yield: 25%). Conversion to the HCl salt afforded a white solid, m.p.: 166–167 °C from $CHCl_3$/ether (lit. [13] m.p.: 176 °C).

^{1}H NMR (DMSO-d_6): δ 1.35 (1H, m, piperidine proton), 1.62–1.85 (7H, m, 5 × piperidine protons + 4-CH_2), 2.78–2.85 (6H, m, 2 × piperidine protons + 3-CH_2 + 5-CH_2), 3.30 (2H, m, piperidine protons), 6.89 (1H, s, OH exch. D_2O), 7.27–7.35 (10H, m, ArH), 10.42 (1H, brs, NH exch. D_2O). ^{13}C NMR (DMSO-d_6): δ 17.50 (C4), 21.19 (C–piperidine), 22.06 (2 × C–piperidine), 34.69 (C3), 51.56 (2 × C–piperidine), 54.81 (C5), 84.30 (C1), 126.87 (4 × C–Ar), 127.04 (2 × C–Ar), 127.53 (4 × C–Ar), 142.53 (2 × C–Ar), 210.41 (C2).

4.1.4. 1-(3-Trimethylsilanyloxy-but-3-enyl)-piperidine (*8*)

Sodium iodide (2.25 g, 15 mmol) in anhydrous acetonitrile (15 ml) was added dropwise, under nitrogen, at room temperature, to a solution of 4-piperidin-1-yl-butan-2-one [14] (2.75 g, 10 mmol), triethylamine (1.52 g, 15 mmol) and trimethylchlorosilane (1.63 g, 15 mmol), successively introduced in the reaction flask. An abundant white precipitate formed, while the acetonitrile solution became yellow. The stirring was maintained for 10 min, cold hexane (30 ml) and then ice-

water (30 ml) were added. After decantation, the aqueous layer was rapidly extracted with cold hexane (2 × 30 ml) and the combined organic layers were washed with ice-water, dried and evaporated. ^{1}H NMR spectroscopy of the crude product showed a signal corresponding to enoxysilane ($\delta = 0.19$, s, $(CH_3)_3Si$) while the signal corresponding to trimethylchlorosilane was not detectable. The crude product was immediately submitted to the next reaction without further characterization to prevent hydrolysis.

4.1.5. 1,1-Diphenyl-5-piperidin-1-yl-pent-1-en-3-one hydrochloride (**6a**) and 1-hydroxy-1,1-diphenyl-5-piperidin-1-yl-pentan-3-one hydrochloride (**7a**)

To a mixture of benzophenone (1.82 g, 10 mmol) and titanium tetrachloride (1.90 g, 10 mmol) in dry CH_2Cl_2 (50 ml) was added dropwise a solution of **8** (2.04 g, 9 mmol) in dry CH_2Cl_2 (50 ml), carrying out the reaction under nitrogen at room temperature. The reaction mixture was stirred at room temperature for not more than 10 min. Longer stirring gave only derivative **6**. After hydrolysis, the resulting mixture was purified on a silica gel column by flash chromatography eluting first with chloroform to separate compound **6** (yield 50%) and then with $CHCl_3$/MeOH 9:1 to obtain **7** (yield 15%) as pure oils. Conversion to the HCl salts afforded **6a**, m.p.: 174–175 °C from $CHCl_3$/ether. ^{1}H NMR (DMSO-d_6): δ 1.34 (1H, m, piperidine proton), 1.73 (5H, m, piperidine protons), 2.76 (2H, m, 4-CH_2), 3.10 (4H, m, piperidine protons), 3.24 (2H, m, 5-CH_2), 6.83 (1H, s, 2-CH), 7.14 (2H, m, ArH), 7.30–7.40 (8H, m, ArH), 10.47 (1H, brs, NH exch. D_2O). ^{13}C NMR (DMSO-d_6): δ 21.26 (C–piperidine), 22.23 (2 × C–piperidine), 37.34 (C4), 50.40 (C5), 51.85 (2 × C–piperidine), 124.81 (C2), 128.00 (5 × C–Ar), 128.45 (2 × C–Ar), 128.93 (2 × C–Ar), 129.54 (C–Ar), 138.45 (C–Ar), 140.12 (C–Ar), 152.79 (C1), 196.06 (C3).

7a: m.p.: 169–170 °C from $CHCl_3$/ether. ^{1}H NMR (DMSO-d_6): δ 1.32 (1H, m, piperidine proton), 1.70 (5H, m, piperidine protons), 2.70 (2H, m, 4-CH_2), 3.01–3.14 (6H, m, 4 × piperidine protons + 5-CH_2), 3.54 (2H, s, 2-CH_2), 6.04 (1H, s, OH exch. D_2O), 7.16–7.32 (6H, m, ArH), 7.44 (4H, d, ArH), 10.37 (1H, brs, NH exch. D_2O). ^{13}C NMR (DMSO-d_6): δ 21.05 (C–piperidine), 22.13 (2 × C–piperidine), 37.82 (C4), 49.74 (C5), 51.65 (2 × C–piperidine), 53.27 (C2), 75.66 (C1), 125.27 (4 × C–Ar), 126.12 (2 × C–Ar), 127.56 (4 × C–Ar), 146.96 (2 × C–Ar), 205.54 (C3).

4.1.6. General procedure for the synthesis of 1-(5-hydroxy-5,5-diphenyl-pentyl)-1-methyl-piperidinium iodide (**3b**) and derivatives **4b**–**6b**

Methiodide derivatives were prepared according to the general procedure previously described [5] in 40–60% yields.

3b: m.p. 185 °C from abs.ethanol. ^{1}H NMR (DMSO-d_6): δ 1.21 (2H, m, 3-CH_2), 1.54 (2H, m, piperidine protons), 1.64–1.74 (6H, m, 4 × piperidine protons + 2-CH_2), 2.27 (2H, m, 4-CH_2), 2.92 (3H, s, NCH_3), 3.19–3.23 (6H, m, 4 × piperidine protons + 1-CH_2), 5.55 (1H, s, OH exch. D_2O), 7.15–7.17 (2H, m, ArH), 7.27 (4H, t, $J = 6$ Hz, ArH), 7.42 (4H, d, $J = 6$ Hz, ArH).

4b: m.p. 187–188 °C from abs.ethanol. ^{1}H NMR (DMSO-d_6): δ 1.53 (2H, m, piperidine protons), 1.75–1.86 (6H, m, 4 × piperidine protons + 2-CH_2), 2.10 (2H, q, $J = 7.2$ Hz, 3-CH_2), 2.98 (3H, s, NCH_3), 3.24–3.29 (6H, m, 4 × piperidine protons + 1-CH_2), 6.14 (1H, t, $J = 7.2$ Hz, 4-CH), 7.14–7.45 (10H, m, ArH).

5b: m.p. 173-174 °C from abs.ethanol. ^{1}H NMR (DMSO-d_6): δ 1.51 (2H, m, piperidine protons), 1.72–1.82 (6H, m, 4 × piperidine protons + 2–CH_2), 2.82 (2H, t, 3-CH_2), 2.96 (3H, s, NCH_3), 3.14–3.27 (6H, m, 4 × piperidine protons + 1-CH_2), 6.85 (1H, s, OH exch. D_2O), 7.28–7.37 (10H, m, ArH).

6b: m.p. 140–141 °C from abs.ethanol/ether. ^{1}H NMR (DMSO-d_6): δ 1.50 (2H, m, piperidine protons), 1.72–1.75 (4H, m, piperidine protons), 2.90 (3H, s, NCH_3), 3.24–3.27 (6H, m, 4 × piperidine protons + 1-CH_2), 3.49 (2H, t, 2-CH_2), 6.84 (1H, s, 4-CH), 7.16 (2H, m, ArH), 7.31 (2H, m, ArH), 7.40–7.43 (6H, m, ArH).

4.2. Pharmacology

4.2.1. General considerations

Male guinea pigs (200–300 g) and male New Zealand white rabbits (3.0–3.5 kg) were killed by cervical dislocation. The organs required were set up rapidly under 1 g of tension in 20 ml organ baths containing physiological salt solution (PSS) maintained at an appropriate temperature (see below) and aerated with 5% CO_2–95% O_2. Dose–response curves were constructed by cumulative addition of the reference agonist. The concentration of agonist in the organ bath was increased approximately threefold at each step, with each addition being made only after the response to the previous addition had attained a maximal level and remained steady. Following 30 min of washing, tissues were incubated with the antagonist for 1 h, and a new dose-response curve to the agonist was obtained. Contractions were recorded by means of a force displacement transducer connected to the MacLab system PowerLab/800. In addition parallel experiments in which tissues did not receive any antagonist were run in order to check any variation in sensitivity.

4.2.2. Guinea-pig ileum

Two-centimeter-long portions of terminal ileum were taken at about 5 cm from the ileum-cecum junction. The tissue was cleaned and the ileum longitudinal muscle was separated from the underlying circular muscle, and mounted in PSS, at 37 °C, of the following composition

(mM): NaCl (118), $NaHCO_3$ (23.8), KCl (4.7), $MgSO_4.7H_2O$ (1.18), KH_2PO_4 (1.18), $CaCl_2$ (2.52), and glucose (11.7). Tension changes were recorded isotonically. Tissues were equilibrated for 30 min, and dose-response curves to arecaidine propargyl ester (APE) were obtained at 30-min intervals, the first one being discarded and the second one being taken as the control.

4.2.3. Guinea-pig stimulated left atria

The heart was rapidly removed, and the right and left atria were separately excised. Left atria were mounted in PSS (the same used for ileum) at 30 °C and stimulated through platinum electrodes by square-wave pulses (1 ms, 1 Hz, 5–10 V) (Tetra Stimulus, N. Zagnoni). Inotropic activity was recorded isometrically. Tissues were equilibrated for 2 h and a cumulative dose–response curves to APE was constructed.

4.2.4. Rabbit stimulated vas deferens

This preparation was set up according to Eltze [15]. Vasa deferentia were carefully dissected free of surrounding tissue and were divided into four segments, two prostatic portions of 1 cm and two epididymal portions of approximately 1.5 cm length. The four segments were mounted in PSS with the following composition (mM): NaCl (118.4), KCl (4.7), $CaCl_2$ (2.52), $MgCl_2$ (0.6), KH_2PO_4 (1.18), $NaHCO_3$ (25), glucose (11.1); 10^{-6} M yohimbine and 10^{-8} M tripitramine were included to block alpha$_2$-adrenoceptors and M_2 muscarinic receptors, respectively. The solution was maintained at 30 °C and tissues were stimulated through platinum electrodes by square-wave pulses (0.1 ms, 2 Hz, 10–15 V). Contractions were measured isometrically after tissues were equilibrated for 1 h, then a cumulative dose–response curve to *p*-Cl–McN–A-343 was constructed.

4.2.5. Determination of antagonist potency

To quantify antagonist potency, pK_b values were calculated from the equation $pK_b = \log(DR\text{-}1) - \log[B]$, where DR is the ratio of ED_{50} values of agonist after and before treatment with one or two antagonist concentration [B] [10].

4.2.6. Statistical analysis

Values are given as mean ± standard error of four or five independent observations. Student's *t*-test was used to assess the statistical significance of the difference between two means.

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