Synthesis and Pharmacological Profile of a Series of 1-substituted-2-Carbonyl Derivatives of Diphenidol: Novel M_4 Muscarinic Receptor Antagonists

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Abstract: Novel 2-carbonyl analogues of diphenidol (**1**) - bearing lipophylic 1-substituents (**2**) - were synthesized starting from previously investigated diphenidol derivatives acting as M_2-selective muscarinic antagonists. These compounds were tested for receptor binding affinity versus human muscarinic M_1-M_5 receptors stably expressed in CHO-K1 cells. Their activity in functional assays carried out on CHO-K1 cells expressing human M_4 receptors (CHO-hM_4) and on classical models of M_1-M_3 receptors, in guinea pig and rabbit tissue preparations, was also evaluated. Compound **2d** showed an affinity of pK_i = 7.73 at the human M_4-receptor subtype with selectivity ratios ranging from 31-fold (M_4/M_5) to 60-fold (M_4/M_2). Interestingly this compound, in CHO-hM_4 cells, blocked the inhibition of forskolin-activated cAMP accumulation produced by carbachol (IC_{50}= 61 nM) whereas it was a weak muscarinic antagonist in functional tests carried out in guinea-pig and rabbit tissue expressing M_1 (pK_b = 5.96), M_2 (pK_b = 6.43) and M_3 (pK_b = 6.09) receptors. In conclusion, the modifications performed in this work on reference compounds led us to obtain surprisingly a M_4 selective antagonist. Considering the therapeutic indications for M_4 selective antagonists, compound **2d** may serve as a novel lead compound for further optimization.

Key Words: Muscarinic antagonists, subtype selectivity, diphenidol derivatives, binding studies, functional studies, cAMP.

INTRODUCTION

Muscarinic receptors play important roles both in the peripheral 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 play a crucial role in neurodegenerative diseases [1]. To date, five subtypes (M_1-M_5) of muscarinic receptors have been identified, which are variable in both the tissue distribution and signal transduction mechanisms [1c]. The identification of the physiological roles of muscarinic receptor subtypes has been hindered by the poor subtype selectivity of available ligands and by the fact that most tissues and cell types express multiple muscarinic receptor subtypes [1c,2]. Knowledge of the specific roles of the muscarinic receptor subtypes is of considerable interest because they may represent important therapeutic targets for various diseases. For example, M_1 and M_2 receptors are linked to Alzheimer's disease and cognitive impairment, M_2 receptors are linked also to cardiac arrhythmias, M_3 receptors are linked to urinary incontinence, irritable bowel syndrome and chronic obstructive pulmonary disease, and M_4 receptor antagonists may ameliorate Parkinson's disease [3].

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In previous papers [4], we described our investigations on muscarinic antagonists derived from diphenidol (1,1-diphenyl-4-piperidin-1-yl-butan-1-ol, **1,** Fig. **1**). Diphenidol, an antiemetic agent used in the treatment of vomiting and vertigo, has been reported to bind to M_1-M_4 receptors with superimposable affinity [5]. However, it represents an useful model to obtain derivatives with different selectivity profiles for muscarinic receptor subtypes, which makes these compounds useful tools to investigate further muscarinic receptor heterogeneity [6]. The introduction of a carbonyl group in position 2 of the butyl chain of diphenidol led to compound **2a** (R = hydroxy group, OH) with enhanced affinity for the M_2 and the M_3 receptor subtypes and a better selectivity ratio M_3/M_2 [4b]. Substituents with lipophilic properties, such as a

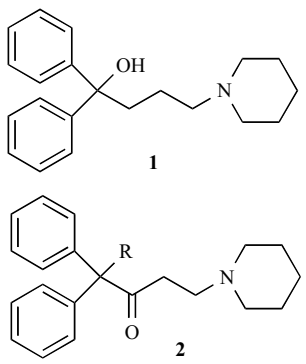

Fig. (1). Structure of diphenidol (**1**) and 2-carbonyl analogues (**2**).

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phenyl (Ph) (**2b**) or an ethylthio group (SEt) (**2c**) in position 1 of the 2-carbonyl derivative [4c] led to compounds with M_2-selective antimuscarinic profile.

In an attempt to further assess the structural requirements for the muscarinic receptor selectivity of diphenidol derivatives, starting from compound **2a**, we synthesized and assayed some 1-substituted-2-carbonyl derivatives with improved lipophilic properties. Thus, considering that by a structure-activity analysis it appeared that the effect of 1-substituents on selectivity was much stronger than that predicted on the basis of lipophilicity alone for ethylthio, and for methoxy and ethoxy groups [4c] we synthesized a series of ethers (**2d-f**) and the tioether **2g**. Moreover, in replacement of the phenyl substituent we introduced in position 1 an isosteric ring bearing a sulfur atom (**2h**).

In the present paper we report the synthesis, the radioligand binding studies on human cloned muscarinic receptor subtypes expressed in CHO cells and *in vitro* functional assays of the title compounds.

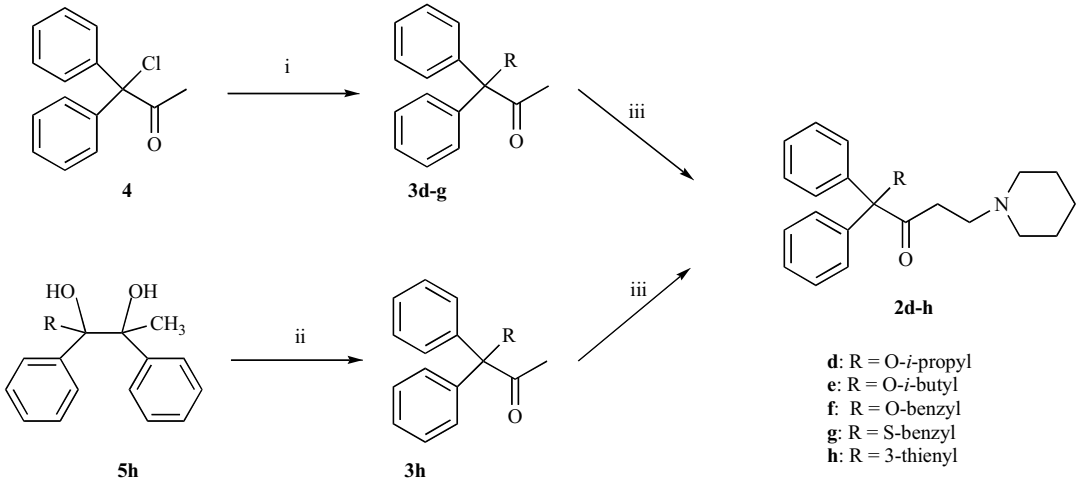
CHEMISTRY

The title compounds **2d-h** were synthesized by means of the Mannich reaction on the appropriate 1-substituted-1,1-diphenylpropan-2-one (**3d-h**) and piperidine hydrochloride, as reported in Scheme (**1**). Because of the better stability and solubility, the compounds were obtained as hydrochloride salts (**2d-f**, **2h**) or oxalate salts (**2g**). Ketones **3d-h** were prepared according to standard methods or by reaction of 1-chloro-1,1-diphenylpropan-2-one (**4**) with an excess of the proper alcohol, in the presence of anhydrous calcium carbonate (**3d-g**), or submitting the 1,2-diphenyl-1-thiophen-3-yl-propane-1,2-diol (**5h**) to pinacol rearrangement (**3h**). Compound **5h** was synthesized by means of two Grignard reactions, as outlined in Scheme (**2**). Particularly, the 1,2-diphenyl-ethane-1,2-dione (**6**) was submitted to Grignard reaction with 3-bromothiophene in the presence of the entrainer 1,2-dibromoethane, then the obtained 2-hydroxy-1,2-diphenyl-2-thiophen-3-yl-ethanone (**7h**) was reacted with methylmagnesium iodide.

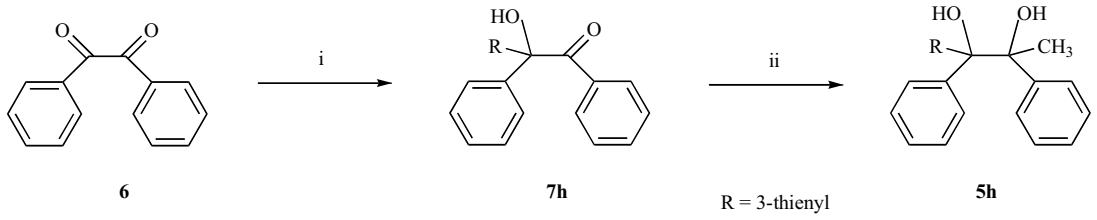
RESULTS AND DISCUSSION

All the synthesized compounds were tested for affinity versus human M_1-M_5 stable transfectants of CHO-K1 cells, using [^{3}H]-N-methylscopolamine chloride ([^{3}H]-NMS) as labelled ligand. Binding profile of the reference compounds **2a-c** was also investigated in the present study. Binding data are reported as pK_i values (affinity) in Table **1**. The following K_d ([^{3}H]-NMS affinity) and B_{max} (receptor density) were determined: CHO-hM_1 K_d=0.32 ± 0.01 nM, B_{max}= 2818 ± 125 fmol mg^{-1} protein; CHO-hM_2 K_d=0.23 ± 0.04 nM, B_{max}= 2145 ± 21 fmol mg^{-1} protein; CHO-hM_3 K_d=0.12 ± 0.02 nM, B_{max}=1840 ± 40 fmol mg^{-1} protein; CHO-hM_4 K_d=0.11 ± 0.05 nM, B_{max}= 2615 ± 182 fmol mg^{-1} protein, CHO-hM_5 K_d= 0.23 ± 0.05 nM, B_{max}=2.1 ± 0.09 pmol mg^{-1} protein. All the competition curves obtained with assayed compounds were compatible with competitive inhibition of tracer binding.

As illustrated in Table **1**, reference compound **2a** showed a significant affinity to human muscarinic receptors; however, it was not able to discriminate among the different



Scheme (1). (i) ROH, $CaCO_3$, reflux; (ii) H_2SO_4/H_2O, reflux; (iii) CH_2O, piperidine hydrochloride, $CH_3OCH_2CH_2OH$, reflux.



Scheme (2). (i) 3-Br-thiophene, Mg, 1,2-dibromoethane, Et_2O/THF; (ii) CH_3I, Mg, Et_2O/THF.

Table 1. Binding Affinities (pK_i) at Human Muscarinic Receptors of the Antagonists 2a-h

Compd	R	pK_i ± S.E.M.[a]				
		hM_1	hM_2	hM_3	hM_4	hM_5
2a	OH	7.52 ± 0.11	7.12 ± 0.09	7.48 ± 0.06	7.84 ± 0.26	6.72 ± 0.10
2b	Ph	5.74 ± 0.21	6.10 ± 0.22	5.45 ± 0.33	5.92 ± 0.11	6.28 ± 0.13
2c	SEt	5.68 ± 0.29	5.85 ± 0.15	5.38 ± 0.35	6.27 ± 0.29	5.67 ± 0.17
2d	O-*i*Pr	6.11 ± 0.10	5.95 ± 0.18	6.21 ± 0.12	7.73 ± 0.11	6.24 ± 0.15
2e	O-*i*Bu	5.61 ± 0.44	5.74 ± 0.43	5.80 ± 0.29	6.62 ± 0.09	6.10 ± 0.13
2f	OCH_2Ph	5.41 ± 0.10	5.15 ± 0.24	5.11 ± 0.16	5.11 ± 0.14	5.47 ± 0.18
2g	SCH_2Ph	5.67 ± 0.32	5.56 ± 0.28	5.84 ± 0.32	5.90 ± 0.10	5.71 ± 0.19
2h	3-thienyl	5.96 ± 0.34	5.97 ± 0.23	6.22 ± 0.12	6.67 ± 0.31	6.31 ± 0.17

[a] Obtained in CHO-K1 cells expressing the human muscarinic M_1-M_5 receptors (mean of three experiments performed in triplicate).

muscarinic receptor subtypes. Replacements of the OH group in compound **2a** significantly decreased the affinities for muscarinic receptor subtypes hM_1, hM_2, hM_3 and hM_5. As regards the hM_4 subtype, only the isopropoxy derivative (O-*i*Pr, **2d**) maintained a relevant affinity for this subtype similar to that of **2a** while, in comparison to it, it showed a decrease in affinity at hM_1 (26-fold), hM_2 (15-fold), hM_3 (18-fold) and hM_5 (3-fold). Therefore, compound **2d** can represent a good M_4 receptor ligand capable to discriminate the different receptor subtypes. The other derivatives showed lower affinities than compound **2d** at the hM_4 receptor subtype, ranging from 10-fold (**2e** and **2h**) to 400-fold (**2f**).

The effects of the substitutents at hM_1-hM_5 receptor subtypes are different with regard to reference compounds **2b** and **2c**. Considering compound **2b**, the isosteric replacement of a phenyl ring by a thienyl ring resulted in a small increment (about 5-fold) in potency at hM_3 and hM_4 subtypes. As concerns reference compound **2c**, the substitution of the ethylthio group with a benzylthio group (**2g**) caused no significant difference in affinity at hM_1-hM_5 receptor subtypes. The oxygen analogue of compound **2g** (**2f**) showed lower values of affinity at all the five receptor subtypes. Finally, considering compound **2a** with respect to the subset of the parent compounds **2d**, **2e** and **2f**, a decrease in affinity at hM_1-hM_5 receptor subtypes is present for all compounds and this fall appears to increase with the bulkiness of the alkyl group linked to the oxygen. As aforementioned, the only exception is compound **2d** at the hM_4 subtype.

The affinity profile of the O-*i*Pr derivative **2d** towards the M_4 muscarinic receptor subtype was confirmed in functional assays. First, we evaluated the regulation of cAMP formation in CHO-hM_4 cells. Forskolin (10 μM) itself increased the rate of cAMP more than 12-fold in these cells. The muscarinic agonist carbachol (10^{-12}-10^{-4} M) inhibited in a concentration-related manner the forskolin-stimulated synthesis of cAMP (EC_{50}= 1870 ± 128 nM). Compound **2d** (from 0.1 nM to 1 μM) reversed the inhibitory effect elicited by 5 μM carbachol (IC_{50}= 61 nM; 95% confidence intervals 45-83 nM). Compound **2a** showed a similar affinity (IC_{50}= 96 nM; 95% confidence intervals: 64-143 nM) whereas the other compounds were less effective (IC_{50} >1 μM) (Fig. **2**). These compounds (from 0.1 nM to 1 μM), added to CHO-hM_4 cells in absence of carbachol did not modify forskolin-stimulated cAMP accumulation; thus, they do not exhibit any activity as muscarinic agonists (data not shown).

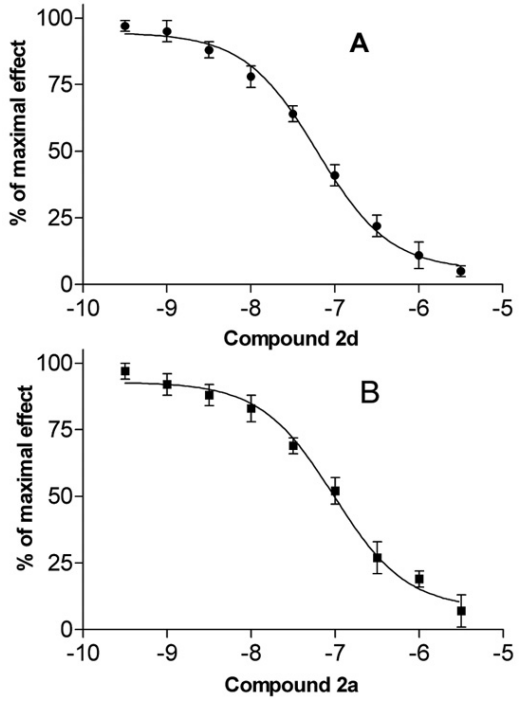


Fig. (2). Antagonism of O-*i*Pr derivative (**2d**) (panel A) and of compound **2a** (panel B) of carbachol inhibition of forskolin-stimulated cAMP accumulation in CHO cells stably expressing hM_4 receptors. Abscissa: log of the concentration (M) of the assayed compound. Ordinate: Percent of maximal inhibition of the accumulation of cAMP induced by carbachol (5μM). Data are means (± S.E.M.) of three experiments with incubations performed in duplicates.

Finally, we investigated the functional activity of these novel compounds on classical *in vitro* models of M_1, M_2 and

M_3 receptors in guinea pig and rabbit tissues.The rabbit vas deferens was taken as a M_1 model [7], however, it should be mentioned that there is still some question in how far M_4 receptors are involved [1c,8]. The guinea pig stimulated left atria was used to measure M_2 receptor mediated actions [9], and the guinea pig ileum served as M_3 model [10]. Arecaidine propargyl ester (APE) at M_2 and M_3 subtypes, and *p*-Cl-McN-A-343 at rabbit vas deferens receptors were used as reference agonists. The affinity values of the new compounds, expressed as dissociation constant (pK_b), together with the corresponding data of the previously published reference compounds **2a**-**c** [4c] are reported in Table **2**. The functional assay data show that all the synthesized compounds behave as weak muscarinic antagonists. Particularly, compound **2d** did not display any relevant antagonist activity in the three functional assays.

Binding data generally are fairly similar to that obtained in functional assays. Minor discrepancies might be due to several factors [11]; mainly to the finding that tissues may express more than one muscarinic subtype and all may contribute to the functional response [3b].

The binding studies of the present work confirm the receptor profile of compound **2a** (R = OH), even if the M_3/M_2 selectivity is lower with respect to that previously reported for the functional assays [4b], but a relevant M_4 affinity value emerges. The introduction in position 1 of a O-*i*Pr group preserves the affinity at M_4 subtype with respect to the OH substituent, while it causes a fall in potency at the other subtypes. As a consequence, compound **2d** shows a M_4 binding selectivity, with selectivity ratios ranging from 31 (M_4/M_5) to 60 (M_4/M_2).

The introduction in position 1 of substituents with higher steric hindrance (compounds **2e**-**2h**) is not well-tolerated and causes a reduction of affinity for hM_4 subtype, suggesting that the steric parameter of the substituent in position 1 may play an important role in binding to hM_4 subtype, even if other substituent properties might be involved.

Following our early hypothesis [4b], the bioactive conformation of carbonyl-derivatives of diphenidol might be one which particularly exposes the R groups to the receptor, still allowing for other external interactions, eventually involving both the carbonyl and the charged nitrogen (Fig. **3**).

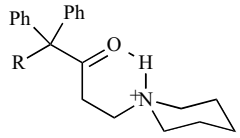


Fig. (3). Structure of the proposed bioactive conformation.

At the light of the present results, namely the effect of the 1-substituents on affinity and selectivity towards receptor subtypes, we can confirm that hypothesis and point out the critical role of R in recognizing and binding the different muscarinic receptor subtypes.

In conclusion, the modifications performed in this work on reference compounds **2a**-**c** led us to obtain surprisingly a M_4 selective compound while our aim of developing potent and selective M_2 antagonists failed. Considering the therapeutic indications for M_4 selective antagonists, compound **2d** may serve as a novel lead compound for further drug development.

EXPERIMENTAL SECTION

Chemistry

Melting points were taken on electrothermal open capillary apparatus and are uncorrected. Elemental analyses (C, H, N) were performed for compounds **2d-h** and the results were within ±0.4% of the theoretical values. Infrared spectra (IR) were recorded on a Nicolet Avatar 320 FT IR instrument and only the principle sharply defined bands are reported; ν_{max} is expressed in cm^{-1}. ^1H NMR spectra were registered on a Varian VXR 300 spectrometer, the chemical shifts are expressed in parts per million (δ) downfield from

Table 2. Affinity Values (pK_b) for Muscarinic Antagonists 2a-h on Rabbit Vas Deferens (Putative M_1), Guinea-Pig Atria Force (M_2) and Guinea-Pig Ileum (M_3)

Compd.	pK_b ± S.E.M.[a]		
	Putative M_1	M_2	M_3
2a[b]	n.d.	7.48 ± 0.05	8.12 ± 0.03
2b[c]	< 5	7.06 ± 0.14[d]	5.85 ± 0.14
2c[c]	< 5	7.19 ± 0.10[d]	5.40 ± 0.02
2d	5.96 ± 0.17	6.43 ± 0.14	6.09 ± 0.11
2e	6.14 ± 0.16	5.77 ± 0.05	6.25 ± 0.16
2f	5.16 ± 0.05	5.28 ± 0.20	6.00 ± 0.11
2g	6.21 ± 0.10	5.37 ± 0.06	6.28 ± 0.02
2h	6.59 ± 0.14	5.86 ± 0.12	6.66 ± 0.04

[a]Affinity constants calculated from the equation log(DR-1) = log[ant] - logK_b for a single concentration of the antagonist are reported, according to van Rossum [16]. [b][4b]. [c][4c]. [d] pA_2.

TMS with reference to internal solvent. Chromatographic separations were performed on a silica gel column by gravity chromatography (Merck silica gel 60, 70-230 mesh) or flash chromatography (Merck silica gel 60, 230-400 mesh). Thin-layer chromatography (Merck silica gel 60 F_{254} analytical plates) was used to monitor reactions. Visualization was performed by UV light and/or iodine vapor. Commercial grade solvents and reagents were used without further purification. Anhydrous diethyl ether (Et_2O) and tetrahydrofuran (THF) were purchased from Aldrich. The term "dried" refers to the use of anhydrous sodium sulfate.

General Procedure for the Synthesis of 1-substituted-1,1-diphenyl-4-piperidin-1-ylbutan-2-ones (2d-h)

A solution of the proper 1-substituted-1,1-diphenylpropan-2-one (**3d-h**) (8.80 mmol), paraformaldehyde (0.64 g) and piperidine hydrochloride (1.48 g, 12.17 mmol) in 2-methoxyethanol (10 mL) was refluxed with stirring at 140 °C for 10 min. A suspension of paraformaldehyde (0.64 g) in 2-methoxyethanol (3 mL) was added during 10 min. Concentrated hydrochloric acid (0.5 mL) was added and refluxing continued for a further 10 min to produce a clear solution. In the case of compounds **2d-g**, the reflux was kept for further 30 min and the reaction was performed in a mild acidic medium, without the addition of concentrated hydrochloric acid which led to the hydrolysis of the ethereal function. The cooled solution was poured into brine (10 mL) and extracted with chloroform (2 × 30 mL). The combined organic layers were extracted with dilute hydrochloric acid (2 × 30 mL), the aqueous extracts were made basic with sodium hydroxide solution and treated with chloroform (2 × 30 mL). The chloroform was washed with brine, dried and evaporated. The residue was purified on a silica gel column by flash chromatography eluting with chloroform /methanol (95/5). The oily tertiary amine was dissolved in anhydrous Et_2O and converted into the corresponding hydrochloride (**2d-f**, **2h**) and oxalate (**2g**) by treatment with a hydrochloric acid saturated diethyl ether solution and one equivalent of oxalic acid in absolute EtOH, respectively. The obtained solid was filtered and recrystallized.

1-isopropoxy-1,1-diphenyl-4-piperidin-1-ylbutan-2-one hydrochloride (2d)

From 1-isopropoxy-1,1-diphenylpropan-2-one (**3d**) (2.36 g): 1.24 g (35%); m.p. 142-143 °C ($CHCl_3/Et_2O$). IR (Nujol) 3490, 3424, 2644, 2556, 1704, 1620, 1311, 1062, 758, 700. ^{1}H NMR (DMSO-d_6) δ: 0.86 (d, J = 6 Hz, 6H, CH(CH_3)$_2$,), 1.32 (m, 1H, piperidine proton), 1.62-1.65 (m, 5H, piperidine protons), 2.69 (m, 2H, 3-CH_2), 3.09-3.18 (m, 4H, piperidine protons), 3.25 (m, 2H, 4-CH_2), 3.65 (hep, J = 6 Hz, 1H, CH), 7.36-7.38 (m, 10H, ArH), 10.42 (bs, 1H, NH D_2O exch.). Anal. Calcd for $C_{24}H_{32}ClNO_2$: C, 71.71; H, 8.02, N, 3.48. Found: C, 71.50; H, 7.98; N, 3.35.

1-isobutoxy-1,1-diphenyl-4-piperidin-1-ylbutan-2-one hydrochloride (2e)

From 1-isobutoxy-1,1-diphenylpropan-2-one (**3e**) (2.48 g): 1.10 g (30%); m.p. 103-105 °C ($CHCl_3/Et_2O$). IR (Nujol) 3476, 3410, 2626, 2524, 1718, 1623, 1307, 1069, 722, 700. ^{1}H NMR (DMSO-d_6) δ: 0.89 (d, 6H, CH(CH_3)$_2$), 1.29 (m, 1H, piperidine proton), 1.61-1.68 (m, 5H, piperidine protons), 1.85 (m, 1H, CH), 2.70 (m, 2H, 3-CH_2), 2.83 (d, 2H, CH_2CH(CH_3)$_2$), 3.14-3.18 (m, 4H, piperidine protons), 3.28 (m, 2H, 4-CH_2), 7.30-7.39 (m, 10H, ArH), 10.50 (bs, 1H, NH D_2O exch.). Anal. Calcd for $C_{25}H_{34}ClNO_2$: C, 72.18; H, 8.24, N, 3.37. Found: C, 72.56; H, 8.30; N, 3.51.

1-benzyloxy-1,1-diphenyl-4-piperidin-1-ylbutan-2-one hydrochloride (2f)

From 1-benzyloxy-1,1-diphenylpropan-2-one (**3f**) (2.78 g) : 0.40 g (10%); m.p. 147-148 °C ($CHCl_3/Et_2O$). IR (Nujol) 2652, 2559, 1710, 1620, 1055, 727, 698. ^{1}H NMR (DMSO-d_6) δ: 1.34 (m, 1H, piperidine proton), 1.61-1.64 (m, 5H, piperidine protons), 2.75 (m, 2H, 3-CH_2), 3.15-3.20 (m, 4H, piperidine protons), 3.32 (m, 2H, 4-CH_2), 4.17 (s, 2H, OCH_2), 7.32-7.40 (m, 15H, ArH), 9.95 (bs, 1H, NH D_2O exch.). Anal. Calcd for $C_{28}H_{32}ClNO_2$: C, 74.73; H, 7.17, N, 3.11. Found: C, 74.35; H, 7.02; N, 3.22.

1-benzylsulfanyl-1,1-diphenyl-4-piperidin-1-ylbutan-2-one oxalate (2g)

From 1-benzylsulfanyl-1,1-diphenylpropan-2-one (**3g**) (2.93 g): 0.69 g (15%); m.p. 174-176 °C (abs. EtOH). IR (Nujol) 3410, 2635, 1706, 1620, 1598, 1158, 1107, 728, 697. ^{1}H NMR (DMSO-d_6) δ: 1.50-1.62 (m, 6H, piperidine protons), 2.94-2.98 (m, 6H, 4 × piperidine protons and 3-CH_2), 3.17 (m, 2H, 4-CH_2), 3.28 (s, 2H, SCH_2), 7.19-7.53 (m, 15H, ArH). Anal. Calcd for $C_{30}H_{33}NO_5S$: C, 69.34; H, 6.40, N, 2.70. Found: C, 69.56; H, 6.62; N, 2.98.

1-thiophen-3-yl-1,1-diphenyl-4-piperidin-1-ylbutan-2-one hydrochloride (2h)

From 1,1-diphenyl-1-thiophen-3-yl-propan-2-one (**3h**) (2.57 g): 0.67 g (18%); m.p. 160-161 °C ($CHCl_3/Et_2O$). IR (Nujol) 3420, 2724, 2334, 1711, 1621, 1307, 1158, 1106, 968, 835, 721. ^{1}H NMR (DMSO-d_6) δ: 1.28 (m, 1H, piperidine proton), 1.58-1.64 (m, 5H, piperidine protons), 2.68 (m, 2H, 3-CH_2), 3.04-3.19 (m, 6H, 4 × piperidine protons and 4-CH_2), 6.86 (dd, 1H, ArH), 7.16-7.39 (m, 11H, ArH), 7.53-7.57 (m, 1H, ArH), 10.05 (bs, 1H, NH D_2O exch.). Anal. Calcd for $C_{25}H_{28}ClNOS$: C, 70.48; H, 6.62, N, 3.29. Found: C, 70.81; H, 6.77; N, 3.57.

General Procedure for the Synthesis of Compounds 3d-g

2.45 g (10.01 mmol) of 1-chloro-1,1-diphenylpropan-2-one (**4**) [12], 1.00 g (10.01 mmol) of anhydrous calcium carbonate and an excess of the appropriate alcohol (30 mL) were heated in a sealed tube for 48 h at a temperature slightly higher with respect to the b.p. of the alcohol. After cooling, chloroform was added, the solid formed filtered off and the solution washed with water. The organic layer was dried and evaporated. The excess of alcohol was removed by means of a short-path distillation apparatus connected to a mechanical pump and the residue was purified on a silica gel column eluting with toluene to give the proper ketone.

1-isopropoxy-1,1-diphenylpropan-2-one (3d)

0.62 g (23%); m.p. 44-45 °C (petroleum ether). IR (Nujol) 3424, 1713, 1600, 1322, 1038, 751, 702. ^{1}H NMR (DMSO-d_6) δ: 0.85 (d, J = 6.1 Hz, 6H, CH(CH_3)$_2$,), 2.15 (s, 3H, $COCH_3$), 3.64 (hep, J = 6.1 Hz, 1H, CH,), 7.34-7.40 (m, 10H, ArH).

1-isobutoxy-1,1-diphenylpropan-2-one (3e) [13]

1.98 g (70%); oil. IR (Neat) 3411, 1715, 1609, 1351, 1165, 1033, 752, 703. ^{1}H NMR (DMSO-d_6) δ: 0.90 (d, J = 6.6 Hz, 6H, CH(CH_3)$_2$), 1.85 (hep, J = 6.6 Hz, 1H, CH), 2.19 (s, 3H, $COCH_3$), 2.87 (d, J = 6.5 Hz, 2H, CH_2), 7.33-7.35 (m, 10H, ArH).

1-benzyloxy-1,1-diphenylpropan-2-one (3f) [13]

0.51 g (16%); m.p. 65 °C (Et_2O/petroleum ether). IR (Nujol) 1698, 1603, 1149, 754, 703. ^{1}H NMR (DMSO-d_6) δ: 2.26 (s, 3H, CH_3), 4.17 (s, 2H, CH_2), 7.36-7.40 (m, 15H, ArH).

1-benzylsulfanyl-1,1-diphenylpropan-2-one (3g)

1.56 g (47%); m.p. 73-74 °C (petroleum ether). IR (Nujol) 3430, 1691, 1605, 1148, 756, 705. ^{1}H NMR (DMSO-d_6) δ: 2.18 (s, 3H, CH_3), 3.29 (s, 2H, CH_2), 7.18-7.51 (m, 15H, ArH).

1,1-diphenyl-1-thiophen-3-yl-propan-2-one (3h)

1,2-diphenyl-1-thiophen-3-yl-propane-1,2-diol (**5h**) (0.62 g, 2.12 mmol) was treated with 25% sulfuric acid (5 mL) and the mixture was refluxed with stirring for 30 min. After cooling, the solution was diluted with ice water and extracted with chloroform (2 × 30 mL). The chloroform was washed with brine, dried and evaporated. The residue was purified on a silica gel column by gravity chromatography eluting with toluene to obtain 0.34 g of a white solid (58%) with m.p. 116-118 °C (petroleum ether). IR (Nujol) 3405, 1712, 1673, 1155, 779, 745, 701. ^{1}H NMR (DMSO-d_6) δ: 2.08 (s, 3H, $COCH_3$), 6.79 (dd, 1H, ArH), 7.12-7.17 (m, 4H, ArH), 7.23-7.37 (m, 7H, ArH), 7.49-7.53 (m, 1H, ArH).

1,2-diphenyl-1-thiophen-3-yl-propane-1,2-diol (5h)

Several drops of methyl iodide were added to a stirred suspension of Mg turnings (0.48 g, 0.02 g-atom) in anhydrous Et_2O (15 mL). After reaction was initiated, 20.00 mmol (2.84 g) of methyl iodide were added at a rate to maintain reflux. After addition was complete, the mixture was stirred and refluxed for 1 h, then a solution of 2-hydroxy-1,2-diphenyl-2-thiophen-3-yl-ethanone (**7h**) (1.47 g, 5.00 mmol) in anhydrous THF (10 mL) was added during 0.5 h. The mixture was refluxed for an additional 8 h, cooled to room temperature, poured into a 27% aqueous solution of ammonium chloride (20 mL) and extracted with Et_2O (2 × 30 mL). The combined organic layers were washed with brine, dried and evaporated. The residue was purified on a silica gel column by gravity chromatography (toluene/acetone, 98/2) to afford 0.32 g (35%) of a colorless oil, which was used in the next step without stereochemical characterization because intermediate of compound **3h**, where no chiral center is present. IR (Neat) 3542, 3440, 1673, 1597, 1494, 1446, 1245, 1186, 847, 763, 700. ^{1}H NMR (DMSO-d_6) δ: 1.61 (s, 3H, CH_3), 5.32 (s, 1H, OH D_2O exch.), 5.67 (s, 1H, OH D_2O exch.), 6.98-7.42 (m, 13H, ArH).

2-hydroxy-1,2-diphenyl-2-thiophen-3-yl-ethanone (7h)

To a stirred suspension of Mg turnings (3.02 g, 0.12 g-atom) in anhydrous Et_2O (10 mL) about 5 mL of a solution of 22.92 g (122.00 mmol) of 1,2-dibromoethane in anhydrous Et_2O (30 mL) was added until the reaction started. The residual 1,2-dibromoethane solution was mixed with 3-bromothiophene (1.96 g, 12.00 mmol) and added dropwise at a rate to maintain reflux. After addition was complete, the mixture was stirred and refluxed for 1 h, then a solution of 1,2-diphenyl-ethane-1,2-dione (**6**) (1.68 g, 8.00 mmol) in anhydrous THF (10 mL) was added during 0.5 h. The mixture was cooled to room temperature, treated with a 27% aqueous solution of ammonium chloride (30 mL) and extracted with Et_2O (2 × 50 mL). The combined organic layers were washed with brine, dried and evaporated. The oily residue was purified on a silica gel column by gravity chromatography (toluene) to give 2.00 g (85%) of a white solid with m.p. 84-86 °C ($CHCl_3$/petroleum ether). IR (Nujol) 3476, 1667, 1590, 1223, 1029, 835, 768, 739, 717. ^{1}H NMR (DMSO-d_6) δ: 7.03-7.10 (m, 2H, ArH), 7.24 (s, 1H, OH D_2O exch.), 7.31-7.48 (m, 9H, ArH), 7.89 (d, 2H, ArH).

Pharmacology

Binding Studies

Cloned human M_1–M_5 muscarinic receptors inserted in pCD vector [8] were obtained from the National Institutes of Health (a kind gift of Dr T.I. Bonner). CHO-K1 cells were grown in Ham F12 medium supplemented with 10% foetal calf serum, 100 u mL^{-1} penicillin G and streptomycin in a humidified environment of 95% air and 5% CO_2 at 37°C. Cells were transfected using a modified calcium phosphate procedure (Gibco, Life Technologies). Cells were plated in 100 mm dishes. 3 h before transfection, medium was changed with fresh whole medium. A calcium solution containing 20 µg of DNA (10 µg of plasmid DNA and 10 µg of carrier salmon sperm DNA) was mixed with a phosphate solution in 1xHBSS (Hanks balanced salt solution; 8xHBSS contains in mM: 0.44 KH_2PO_4, 5.37 KCl, 0.34 Na_2HPO_4, 136.89 NaCl, 5.55 di-glucose) and let sit at 25°C for 20 min. The suspension was subsequently added to cells and incubated at 37°C in humidified air containing 5% CO_2 for 24 h. Following incubation, medium was changed with fresh whole medium. 48 h after, medium was changed and selection started with 400 µg mL^{-1} G418. Selection with G418 continued for about three weeks. During the selection the medium was changed every 3 days. Clonal cell lines were assayed with [^{3}H]-N-methyl-scopolamine ([^{3}H]-NMS) (Perkin-Elmer). Cells were grown to about 80% confluence, washed with phosphate buffered saline (per 1000 ml: NaCl 9.0 g; potassium dihydrogen phosphate 0.144 g; sodium hydrogen phosphate eptahydrate 0.795 g; pH = 7.4), scraped into ice-cold binding buffer and homogenized using a Thomas teflon pestle tissue homogenizer 2 mL (10 strokes). Membranes were centrifuged at 35,000x*g*, 15 min at 4°C. The pellet was suspended in 5 mL ice-cold binding buffer, the membranes were homogenized a second time and aliquots were stored at -80°C until used.

Competition binding studies were performed in duplicate using homogenates of the indicated cells in incubation buffer (25 mM phosphate buffer, pH 7.4, 25°C, enriched with 2 mM $MgCl_2$). Homogenates (100 µg of protein) were incubated for 2 h at 25°C in 1 mL of incubation buffer with 0.2 nM [^{3}H]-NMS. Non specific binding was determined with 10 µM atropine. Binding assays were terminated by

filtration on Whatman GF/C glass-fibre filters previously soaked in 0.1% polyethylenimine and then rinsed four times with 5 mL of ice-cold incubation buffer. Saturation studies were performed as indicated above, in presence of [^{3}H]-NMS (25−4000 pM) and in the absence or presence of atropine. The results were analysed according to the method of Scatchard [14].

Adenylyl Cyclase Assay

hM_4 receptor coupling to adenylyl cyclase was investigated by measuring, in whole CHO-K1 cells stably expressing hM_4 receptors (CHO-hM_4), the concentration-dependent inhibitory effect of carbachol on forskolin-stimulated cAMP accumulation [15]. Three days before the experiments, ≈50,000 CHO-hM_4 cells were seeded into 24-well plates. On the day of experiments, the culture medium was removed and the cells were washed with 0.5 mL of PBS (in mM: 137 NaCl, 2.7 KCl, 0.9 $CaCl_2$, 0.5 $MgCl_2$, 6.5 Na_2HPO_4, 1.5 KH_2PO_4, pH 7.2) and incubated at 37 °C for 30 min with 0.5 mL assay buffer (in mM: 150 NaCl, 5 KCl, 2.5 $CaCl_2$, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 25 $NaHCO_3$, 10 HEPES, 10 mg/mL BSA, pH 7.4) containing 0.5 mM 3-isobutyl-1-methyl-xanthine (IBMX; Sigma-Aldrich) and in the presence of the investigated muscarinic ligand dissolved in assay buffer or in the presence of the assay buffer alone. Then, the cells were exposed to 10 μM forskolin without and with carbachol (5 μM) for 10 min at 37 °C. Previously, concentration-response curves of carbachol (10^{-12} – 10^{-4} M) in absence of antagonists were determined. The reaction was stopped by addition of 1 volume of ice-cold 0.2 M NaOH. Cells were further disrupted by sonication and suspensions were centrifuged at 14,000 × g for 15 min. The resulting supernatants were stored at -20°C until determination of cAMP by radioimmunoassay (Amersham-Pharmacia). The experiments were carried out in duplicate and repeated at least three times. Concentration-response curves were analyzed by a least square curve-fitting computer program and IC_{50} values were determined (GraphPad, San Diego, CA, USA).

Isolated Organ Assays

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 3-fold 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 30 min, and a new dose-response curve to the agonist was obtained. Contractions were recorded by means of a transducer connected to a MacLab System PowerLab/800. In all cases, parallel experiments in which tissues received only the reference agonist were run in order to check any variation in sensitivity.

All animal testing was carried out according to the European Community Council Directive of 24 November 1986 (86/609/EEC).

Guinea-Pig Ileum

Two-centimeter-long portions of terminal ileum were taken at about 5 cm from the ileum-cecum junction and mounted in PSS/1, 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.

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/1 (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.

Rabbit Stimulated Vas Deferens

This preparation was set up according to Eltze [7]. 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/2 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 α_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.

Determination of Antagonist Potency

To quantify antagonist potency, pK_b values were calculated from the equation pK_b = log(DR-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] [16]. Values are given as mean ± standard error (S.E.M.) 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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