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Synthesis and pharmacological characterization of new chiral derivatives of muscarine and *allo*-muscarine

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Dedicated to Professor Pietro Pratesi

Abstract

Novel derivatives of natural muscarine and *allo*-muscarine, i.e. the benzyl ethers (−)-**10** and (−)-**12** and the benzoate (−)-**13**, were synthesized in very high enantiomeric excess. Target compounds were tested in vitro on guinea pig tissues, and their muscarinic potency was evaluated at M_2 (heart force and rate) and M_3 (ileum and bladder) receptor subtypes. The derivatives under study were also assayed in vivo on pithed rat. In addition, muscarinic receptor heterogeneity was investigated by determining the affinity and the relative efficacy of compounds (−)-**10**, (−)-**12** and (−)-**13** at M_2 (heart force and rate) and M_3 (ileum and bladder) receptor subtypes.

Keywords: Muscarine derivatives; *allo*-Muscarine derivatives; Muscarinic receptor subtypes

1. Introduction

Muscarinic receptors are a family of G protein coupled receptors widely distributed both in the central and peripheral nervous systems, where they mediate the actions of endogenous acetylcholine [1,2]. Muscarinic receptors have been pharmacologically classified into four different subtypes (M_1–M_4). In addition, parallel molecular cloning studies have demonstrated the presence, in the brain and peripheral tissues, of a fifth subtype (m_5) [1], which is waiting for a pharmacological identification. Current research in the field of muscarinic ligands is addressed to the design of subtype selective agents as potential targets to disorders of intestinal motility, cardiac and urinary bladder functions, asthma, analgesia, Parkinson's and Alzheimer's diseases [3,4].

In the last decade we studied in depth the structure–activity relationships of a set of chiral muscarinic agonists related to the alkaloid muscarine (+)-**1**, whose absolute configuration is reported in Fig. 1. Well-defined stereochemical requirements in the molecular structure of such muscarinic ligands revealed essentials for a productive interaction with the complementary receptor subsites. Among the tested chiral muscarinic agonists, the four chiral isomers of muscarone [5] and methylenemuscarone [6] showed, in fact, a high value of the eudismic ratio (ER) and a spatial arrangement around the chiral centers matching those of (+)-**1**. The absolute configuration of the most potent stereoisomer of muscarone [(−)-**2**] and methylenemuscarone [(−)-**3**] is represented in Fig. 1. The nature of the interaction of the hydroxy group of muscarine with the complementary receptor subsite ('muscarinic subsite') was further investigated by synthesizing and testing fluoromuscarine (+)-**4** [7] and the enantiomers of both difluoromuscarine **5** [8] and desoxymuscarine **6** [9,10], whose eutomers are depicted in Fig. 1. The pharmaco-

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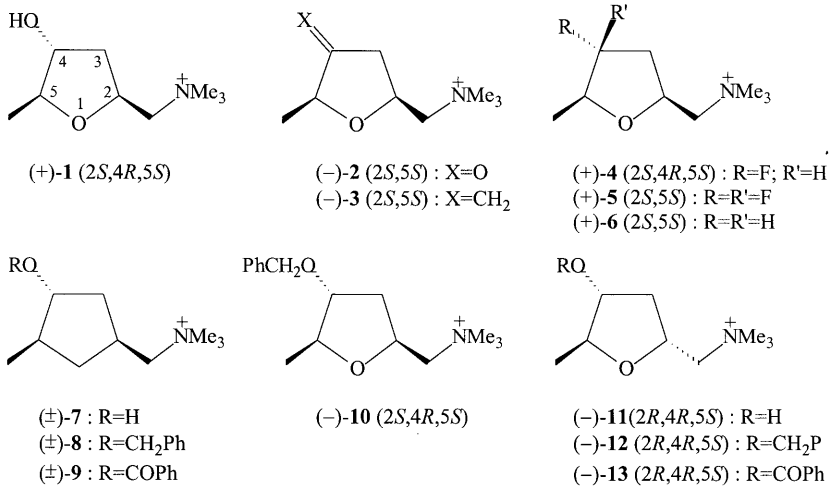


Fig. 1. Structures of the muscarinic ligands under study.

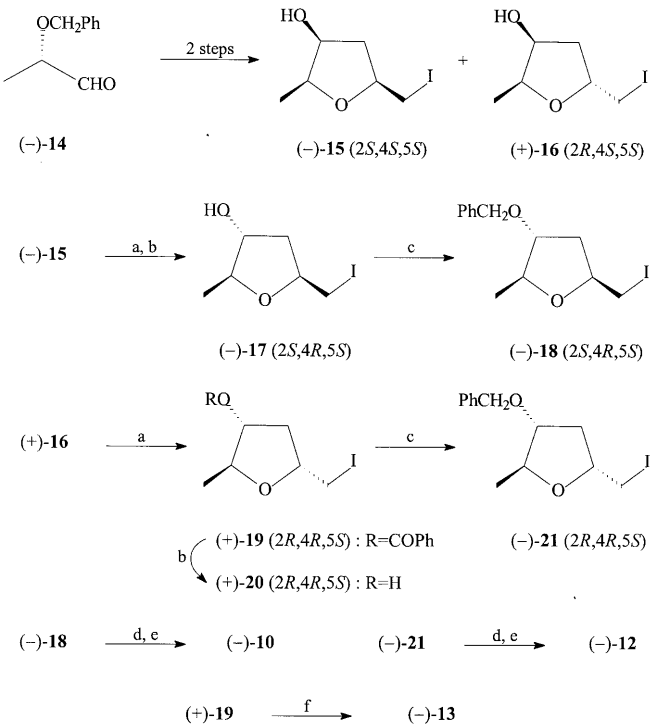
logical profile of these new chiral muscarinic agonists was studied by means of in vitro and in vivo assays, and the results compared to those of natural muscarine (+)-**1** [10]. The replacement of the hydroxy group of muscarine with a fluorine atom, i.e. (+)-**4**, brought about significant changes at heart force potency and at heart rate affinity and efficacy, whereas the introduction of a second fluorine atom at C-4 greatly affected the responses at the M_2 subtype regulating heart rate, where (+)-**4** displays a 240-fold higher affinity and a 120-fold lower efficacy than (+)-**5**. Furthermore, the presence of two fluorine or hydrogen atoms at C-4, as in **5** and **6**, caused an overall drop of the enantioselectivity, when compared to muscarine **1** and muscarone **2**.

In the course of parallel studies on the nature of the interaction at the 'muscarinic subsite', interesting results were reported by Brasili et al., who synthesized and tested some ether derivatives of deoxamuscarine (±)-**7** [11]. Among them, benzyldeoxamuscarine (±)-**8** (Fig. 1) behaved as a muscarinic agonist with a pronounced selectivity for the ileum M_3 receptor subtype. On the other hand, Giannella and co-workers have recently reported [12] that the benzoate of deoxamuscarine (±)-**9** (Fig. 1) is a muscarinic agonist with some degree of selectivity towards the cardiac M_2 subtype.

In view of these outcomes, we designed new chiral derivatives, such as the benzyl ether of natural muscarine (+)-**1** and the benzyl ether and the benzoate of *allo*-muscarine (−)-**11** [13], characterized by the presence of lipophylic and sterically hindered groups at the 'muscarinic subsite'. This paper reports the synthesis and the pharmacological investigation of (−)-**10**, (−)-**12** and (−)-**13** (Fig. 1) at M_2 (heart) and M_3 (ileum and bladder) muscarinic receptor subtypes.

2. Chemistry

The desired target compounds (−)-**10**, (−)-**12** and (−)-**13** were prepared following the reaction sequences reported in Scheme 1. The two stereoisomeric iodoalcohols (−)-**15** and (+)-**16** were obtained in a 1:1 ratio from (*S*)-*O*-benzyl lactic aldehyde (−)-**14**, according to a previously described procedure [13,14]. HPLC analysis of the (*R*)-(+)-MTPA esters [15] of (−)-**15** and (+)-**16** showed an enantiomeric excess higher than



Scheme 1. (a) TPP/DEAD/PhCOOH, THF. (b) K_2CO_3, MeOH/H_2O. (c) $C_6H_5CH_2C(NH)CCl_3/CF_3SO_3H$, CH_2Cl_2. (d) $(CH_3)_2NH$, MeOH. (e) CH_3I, acetone/ether. (f) $N(CH_3)_3$, MeOH.

Table 1
Potencies and intrinsic activities of compounds (+)-**1**, (±)-**7**, (±)-**8**, (±)-**9**, (−)-**10**, (−)-**11**, (−)-**12** and (−)-**13** at M_2 and M_3 muscarinic receptors

	Tissue							
	Guinea pig heart (M_2)				Guinea pig ileum (M_3)		Guinea pig bladder (M_3)	
	Force		Rate					
	pD_2 [a]	α [b]	pD_2 [a]	α [b]	pD_2 [a]	α [b]	pD_2 [a]	α [b]
(+)-**1**	7.68 ± 0.10	1.0	7.07 ± 0.14	1.0	7.05 ± 0.10	1.0	5.58 ± 0.05	1.0
(±)-**7** [c]	5.93 ± 0.05	1.0			6.13 ± 0.07	1.0	4.79 ± 0.05	0.95
(±)-**8** [c]	5.59 ± 0.08	0.92			6.81 ± 0.08	1.0	4.82 ± 0.02	0.23
(±)-**9** [d]	6.56 ± 0.15	0.96			5.41 ± 0.11	0.86		
(−)-**10**	6.87 ± 0.05	1.0	6.80 ± 0.07	1.0	6.74 ± 0.13	1.0	4.87 ± 0.14	0.86
(−)-**11** [e]	5.27 ± 0.06	1.0			5.66 ± 0.07	1.0		
(−)-**12**	7.55 ± 0.12	0.36	7.07 ± 0.19	0.23	4.98 ± 0.09	1.0		0.0
(−)-**13**	5.07 ± 0.15	1.0	4.51 ± 0.11	1.0	4.91 ± 0.12	0.91		

[a] $-\log ED_{50}$. The results are the mean ± SEM, and the number of observations varies between six and ten.
[b] Intrinsic activity, measured by the ratio between the maximum response of the compound and the maximum response of (+)-muscarine.
[c] Data taken from Ref. [11].
[d] Data taken from Ref. [12].
[e] Data taken from Ref. [13].

98% for both derivatives [13]. A complete inversion of the configuration of the hydroxy group of (−)-**15** and (+)-**16** was achieved by means of the Mitsunobu reaction [16], leading to the two iodoalcohols (−)-**17** and (+)-**20**, respectively. Stereoisomeric iodobenzyl ethers (−)-**18** and (−)-**21** were smoothly prepared by reacting (−)-**17** and (+)-**20** with benzyl 2,2,2-trichloroacetimidate [17,18], under the catalysis of trifluoromethanesulfonic acid. The use of a more conventional procedure, that is benzyl bromide in the presence of sodium hydride, substantially lowered the reaction yield. Finally, the reaction of (−)-**18** and (−)-**21** with dimethylamine followed by treatment with methyl iodide afforded the desired quaternary ammonium salts (−)-**10** and (−)-**12**, respectively. Methiodide (−)-**13** was in turn prepared by reaction of the intermediate iodobenzoate (+)-**19** with trimethylamine. Based on the kind of reactions performed and our previous experience in the field [9,13], we believe that each stereoisomer has completely retained the enantiomeric purity induced by the initial iodoetherification process.

3. Results and discussion

Natural muscarine (+)-**1** and compounds (−)-**10**, (−)-**12** and (−)-**13** were tested in vitro on guinea pig tissues, and their muscarinic potency (expressed as pD_2) was evaluated at M_2 (heart force and rate) and M_3 (ileum and bladder) receptor subtypes. The results obtained, gathered in Table 1, were compared to those, known from literature, of deoxamuscarine (±)-**7**, benzyl deoxamuscarine (±)-**8**, deoxamuscarine benzoate (±)-**9** and *allo*-muscarine (−)-**11**.

Inspection of the data of Table 1 shows a decrease of the potency values on passing from natural muscarine (+)-**1**, the most potent compound in the series, to the corresponding benzyl ether (−)-**10**, which still behaves as a relatively potent full agonist at the investigated tissues. Such a trend is more pronounced at heart force and bladder (7- and 5-fold, respectively) than at heart rate and ileum (2-fold at both tissue preparations). In any case, the substitution pattern does not increase the selectivity for ileal M_3 muscarinic receptors observed on passing from deoxamuscarine (±)-**7** to its benzyl ether (±)-**8** [M_3(ileum)/M_2(force) selectivity = 17].

On the other hand, the replacement of the hydroxy group of *allo*-muscarine (−)-**11** with the benzyloxy function, i.e. (−)-**12**, causes a sharp variation of the pharmacological profile at cardiac receptors. Indeed, the benzyl ether (−)-**12**, at variance with the parent compound, is a potent partial agonist at cardiac M_2 receptors ($pD_2 = 7.55$, $\alpha = 0.36$ and $pD_2 = 7.07$, $\alpha = 0.23$), whereas, like (−)-**11**, it behaves as a weak full agonist at ileal M_3 receptors ($pD_2 = 4.98$, $\alpha = 1$). Conversely, the profile of the benzoate of (−)-**13** is very similar to that of *allo*-muscarine (−)-**11**, both in terms of potency and intrinsic activity. In this instance, the introduction of the ester function does not affect subtype selectivity, at variance with what observed on moving from deoxamuscarine (±)-**7** to the corresponding benzoate (±)-**9** [M_2(force)/M_3(ileum) selectivity = 14].

Agonists (+)-**1**, (−)-**10**, (−)-**12** and (−)-**13** were also assayed in vivo on pithed rat (Table 2), in order to

evaluate their muscarinic activity (expressed as ED_{50}) at ganglionic M_1 and cardiac (heart rate) M_2 receptors. The in vivo results, obtained according to a previously described protocol [8], confirm that natural muscarine (+)-**1** is the most potent ligand at both receptor subtypes. The results reported in Table 2 suggest that all the tested compounds are equally active at the ganglionic M_1 and the heart rate M_2 receptors, with the exception of (−)-**10**, which is about two times more active at the M_1 subtype. A conceivable explanation for the different activity order at heart rate between the in vitro and the in vivo assays could be once again attributed to differences in metabolism and pharmacokinetics among the agonists in the two preparations [7,8,10].

Since the comparison of the sole potency of agonists does not allow any speculation on differences among receptor subtypes [19], muscarinic receptor heterogeneity was investigated by determining the affinity (K_D, K_B, or K_P) and the relative efficacy (e_r) in vitro of the compounds under study at M_2 (heart force and rate) and M_3 (ileum and bladder) receptors (Table 3).

A vertical analysis of the data reported in Table 3 shows that, among the compounds examined, natural muscarine (+)-**1** displays the highest values of affinity at heart force and ileum, whereas derivative (−)-**12** shows its peak values at heart rate and bladder. Compound (−)-**10**, the benzyl ether of natural muscarine, has an affinity profile which is not significantly different from that of the parent compound at all the investigated tissues. Conversely, (−)-**10** is about six times more efficacious than (+)-**1** at the M_2 heart rate receptors. Furthermore, inspection of the data of (±)-**7** and (±)-**8** reveals that the ileum/heart (force) selectivity observed for benzyl deoxamuscarine (±)-**8** is due to a sharp difference in the relative efficacy values at the ileal and cardiac receptors. Indeed, (±)-**8** is 130 times more efficacious at the ileum M_3 receptors than at the heart force M_2 ones. On the contrary, the benzyl ether (−)-**10** displays comparable efficacies at the same receptor subtypes (0.63 and 0.98, respectively).

Table 2
Potencies of compounds (+)-**1**, (−)-**10**, (−)-**12** and (−)-**13** at ganglionic M_1 receptors mediating tachycardia and at cardiac M_2 receptors mediating bradycardia in the pithed rat

	ED_{50} (μg/kg) [a], pithed rat	
	Increase in heart rate (M_1)	Decrease in heart rate (M_2)
(+)-**1**	3.0 ± 0.50	4.5 ± 0.75
(−)-**10**	4.0 ± 1.6	9.1 ± 4.7
(−)-**12**	66.8 ± 21.8	64.2 ± 20.8
(−)-**13**	21.7 ± 3.1	30.1 ± 11.8

[a] The results are the mean ± SEM, and the number of observations varies between five and nine.

Interestingly, the inversion (from *S* to *R*) of the absolute configuration at C-2, as in the two stereoisomeric benzyl ethers (−)-**10** and (−)-**12**, brings about a significant change of the affinity profile at both M_2 and M_3 receptor subtypes. In fact, on passing from (−)-**10** to (−)-**12**, a 190-fold higher affinity at the M_2 heart rate receptor subtype is observed, whereas the affinity at the M_2 heart force subtype is almost unaffected. On the contrary, a 140-fold lower affinity is measured at M_3 ileal receptors, while the affinity at bladder is about 4-fold higher.

A horizontal analysis of the data of Table 3 suggests that derivatives (+)-**1**, (−)-**10** and (−)-**12** are able to discriminate within M_2 and M_3 receptor populations since, according to Furchgott's receptor theory [20], differences in $-\log K_D$ of at least 0.5 may be taken as an evidence of a receptor heterogeneity. In particular, (+)-**1** and (−)-**10** show remarkable differences between the affinity values at heart force and rate (43- and 68-fold, respectively), whereas (−)-**12** displays a 100-fold higher affinity at bladder than at ileum M_3 receptors. In addition, (−)-**12** behaves as a weak full agonist at ileum and as an antagonist at bladder.

On the whole, the replacement of the hydroxy group of natural muscarine with the benzyloxy function gives a muscarinic agonist which retains the overall pharmacological profile of the parent compound, while showing no selectivity between M_2 heart force and M_3 ileal receptors and an improved capability to discriminate the populations of the M_2 receptor subtype. This result is different from that emerged from the study of the related pair deoxamuscarine/deoxamuscarine benzyl ether, in which the same structural variation brought about a discrimination between M_2 heart force and M_3 ileal receptors. The presence of the benzyloxy function at the muscarinic subsite (C-4) coupled with the presence of a substituent at C-2 with the (*R*) configuration caused major variations of the pharmacological profile, predominantly at cardiac M_2 receptors. Once again [7,8,21], our studies on the structure–activity relationships of a set of chiral muscarinic ligands structurally related to muscarine put in evidence distinct structural requirements of the receptor subtypes regulating heart force and rate on one side, and those mediating the contraction of ileum and bladder on the other.

4. Experimental

4.1. Chemistry

Melting points were determined on a model B 540 Büchi apparatus and are uncorrected. Liquid compounds were characterized by the oven temperature for Kugelrohr distillations. ^{1}H NMR spectra were recorded with a Bruker AC-E 200 (200 MHz) spectrometer in

Table 3
Pharmacological parameters of (+)-**1**, (±)-**7**, (±)-**8**, (±)-**9**, (−)-**10**, (−)-**11**, (−)-**12** and (−)-**13** in the guinea pig heart force and rate (M_2) and ileum and bladder (M_3)

	Tissues											
	Heart		Ileum	Bladder	Heart		Ileum	Bladder	Heart		Ileum	Bladder
	Force	Rate			Force	Rate			Force	Rate		
	$-\log ED_{50}$ [a]	$-\log ED_{50}$ [a]	$-\log ED_{50}$ [a]	$-\log ED_{50}$ [a]	$-\log K_D$ [a]	$-\log K_D$ [a]	$-\log K_D$ [a]	$-\log K_D$ [a]	e_r [b]	e_r [b]	e_r [b]	e_r [b]
(+)-**1**	7.68 ± 0.10	7.07 ± 0.14	7.05 ± 0.10	5.58 ± 0.05	5.30 ± 0.12	3.67 ± 0.32	5.74 ± 0.23	4.81 ± 0.09	1.0	1.0	1.0	1.0
(±)-**7** [c]	5.93 ± 0.05		6.13 ± 0.07	4.79 ± 0.05	4.47 ± 0.16		4.38 ± 0.12	4.30 ± 0.12	0.13		2.8	0.59
(±)-**8** [c]	5.59 ± 0.08		6.81 ± 0.08	4.82 ± 0.02	4.58 ± 0.08		4.67 ± 0.11	4.85 ± 0.09 [d]	0.05		6.5	
(±)-**9** [e]	6.56 ± 0.15		5.41 ± 0.11									
(−)-**10**	6.87 ± 0.05	6.80 ± 0.07	6.74 ± 0.13	4.87 ± 0.14	4.50 ± 0.08	2.67 ± 0.07	5.65 ± 0.16	4.88 ± 0.18 [f]	0.98	5.7	0.63	0.28
(−)-**11** [g]	5.27 ± 0.06		5.66 ± 0.07									
(−)-**12**	7.55 ± 0.12	7.07 ± 0.19	4.98 ± 0.09		4.71 ± 0.15 [d]	4.95 ± 0.08 [d]	3.50 ± 0.25	5.50 ± 0.25 [d]			1.5	
(−)-**13**	5.07 ± 0.15	4.51 ± 0.11	4.91 ± 0.12									

[a] The results are the mean ± SEM, and the number of observations varies between six and ten.
[b] Relative efficacy [(+)-**1** = 1].
[c] Data are taken from Ref. [11].
[d] $-\log K_B$.
[e] Data are taken from Ref. [12].
[f] $-\log K_P$.
[g] Data are taken from Ref. [13].

$CDCl_3$ (unless otherwise specified) solution; chemical shifts (δ) are expressed in ppm and coupling constants (J) in Hertz. Rotary power determinations were carried out with a Perkin–Elmer 241 polarimeter coupled with a Haake N3-B thermostat. TLC analyses were performed on commercial silica gel 60 F_{254} aluminum sheets: spots were further evidenced by spraying with a dilute alkaline potassium permanganate solution. Microanalyses (C, H, N) of new compounds agreed with the theoretical value within $\pm 0.4\%$.

*4.1.1. 2-(Iodomethyl)-4-hydroxy-5-methyltetrahydrofuran isomers (−)-**17** and (+)-**20** and 2-(iodomethyl)-4-benzoyloxy-5-methyltetrahydrofuran (+)-**19***

(A) To a magnetically stirred ice-cooled solution of (−)-**15** [13] (1.2 g, 4.96 mmol), triphenylphosphine (5.2 g, 19.84 mmol) and benzoic acid (1.21 g, 9.92 mmol) in dry THF (80 ml) a solution of diethylazodicarboxylate (3.14 ml, 19.92 mmol) in dry THF (20 ml) was added dropwise. At the disappearance of the starting material, the solvent was evaporated off and the residue was column chromatographed (eluant: 10% ethyl acetate–cyclohexane) to yield the benzoate of (−)-**17** (1.47 g, 86%), colorless prisms from petroleum ether, m.p. 67–68°C. R_f 0.43 (eluant: 10% ethyl acetate–cyclohexane); $[\alpha]_D^{20}$ −11.75 (*c* 0.950, $CHCl_3$).

(B) To a solution of the above reported crude benzoate (1.3 g, 3.76 mmol) in methanol (75 ml) a 20% aqueous solution of potassium carbonate (40 ml) was added. The reaction mixture was stirred until disappearance of the starting material. Methanol was evaporated off and the residual aqueous phase was extracted with ethyl acetate (4 × 25 ml). The pooled organic extracts were dried over anhydrous sodium sulfate. The solvent was removed at reduced pressure and the crude iodoalcohol (−)-**17** was distilled at 110–115°C/0.5 mmHg (0.855 g, 94%). R_f 0.14 (eluant: 20% ethyl acetate–*n*-hexane); $[\alpha]_D^{20}$ −34.08 (*c* 0.9, $CHCl_3$), lit. [13] $[\alpha]_D^{20}$ −33.72 (*c* 0.874, $CHCl_3$).

The same procedure carried out on a comparable amount of (+)-**16** [13] afforded benzoate (+)-**19** and iodoalcohol (+)-**20** in 82 and 92% yields, respectively.

(2*R*,4*R*,5*S*) 2-(Iodomethyl)-4-benzoyloxy-5-methyltetrahydrofuran (+)-**19**: colorless oil, b.p. 180–185°C/0.5 mmHg; R_f 0.38 (eluant: 10% ethyl acetate–cyclohexane); ^{1}H NMR 1.30 (d, 3H, CH_3, J = 6.7); 2.15 (ddd, 1H, H-3′, J = 3.4, 3.4 and 14.3); 2.65 (ddd, 1H, H-3, J = 6.8, 7.2 and 14.3); 3.36 (m, 2H, CH_2I); 4.31–4.47 (m, 2H, H-2 and H-5); 5.18 (m, 1H, H-4); 7.46 (t, 2H, arom., J = 7.9); 7.59 (t, 1H, arom., J = 7.9), 8.02 (d, 2H, arom., J = 7.9); $[\alpha]_D^{20}$ + 32.85 (*c* 1.064, $CHCl_3$).

(+)-**20**: colorless oil, b.p. 110–115°C/0.5 mmHg; R_f 0.21 (eluant: 20% ethyl acetate–*n*-hexane); $[\alpha]_D^{20}$ + 13.22 (*c* 0.9, $CHCl_3$), lit. [13] $[\alpha]_D^{20}$ +13.51 (*c* 1.061, $CHCl_3$).

*4.1.2. 2-(Iodomethyl)-4-benzyloxy-5-methyltetrahydrofuran isomers (−)-**18** and (−)-**21***

To a magnetically stirred solution of (−)-**17** (0.850 g, 3.51 mmol) and benzyl 2,2,2-trichloroacetimidate (980 μl, 5.27 mmol) in dichloromethane (30 ml) trifluoromethanesulfonic acid (50 μl) was added at 0°C under N_2. The cooling bath was removed and the temperature of the reaction mixture raised at 30°C with the concomitant formation of a colorless precipitate. After about 2 h, a further 490 μl of the benzylating agent and 25 μl of the acid catalyst were added at 0°C. The reaction was stirred for additional 2 h, then filtered and the filtrate was washed with an aqueous saturated solution of $NaHCO_3$. After the usual work up, the residue of the organic phase was submitted to a silica gel column chromatography (eluant: 2% ethyl acetate–petroleum ether) to afford 0.966 g (83% yield) of the desired benzyl ether.

(2*S*,4*R*,5*S*) 2-(Iodomethyl)-4-benzyloxy-5-methyltetrahydrofuran (−)-**18**: pale yellow oil, b.p. 175–180°C/0.1 mmHg; R_f 0.28 (eluant: 5% ethyl acetate–cyclohexane); ^{1}H NMR 1.28 (d, 3H, CH_3, J = 6.5); 1.82 (ddd, 1H, H-3′, J = 6.5, 9.1 and 13.3); 2.18 (ddd, 1H, H-3, J = 2.6, 5.9 and 13.3); 3.28 (dd, 1H, CH_2I, J = 6.1 and 10.1); 3.31 (dd, 1H, CH_2I, J = 4.9 and 10.1); 3.77 (m, 1H, H-4); 4.03–4.19 (m, 2H, H-2 and H-5); 4.51 and 4.53 (d, 2H, CH_2Ph, J = 11.9); 7.35 (m, 5H, arom.); $[\alpha]_D^{20}$ −40.08 (*c* 1.038, $CHCl_3$).

The above reported procedure carried out on a comparable amount of (+)-**20** gave benzyl ether (−)-**21** in 74% yield.

(2*R*,4*R*,5*S*) 2-(Iodomethyl)-4-benzyloxy-5-methyltetrahydrofuran (−)-**21**: pale yellow oil, b.p. 150–155°C/0.1 mmHg; R_f 0.30 (eluant: 5% ethyl acetate–cyclohexane); ^{1}H NMR 1.20 (d, 3H, CH_3, J = 6.5); 2.02 (ddd, 1H, H-3′, J = 4.6, 4.6 and 13.5); 2.37 (ddd, 1H, H-3, J = 6.9, 6.7 and 13.5); 3.33 (m, 2H, CH_2I); 3.78 (m, 1H, H-4); 4.18–4.36 (m, 2H, H-2 and H-5); 4.50 and 4.55 (d, 2H, CH_2Ph, J = 11.7); 7.35 (m, 5H, arom.); $[\alpha]_D^{20}$ −20.79 (*c* 1.068, $CHCl_3$).

*4.1.3. 2-[(Dimethylamino)methyl]-4-benzyloxy-5-methyltetrahydrofuran methiodides (−)-**10** and (−)-**12** and 2-[(dimethylamino)methyl]-4-benzoyloxy-5-methyltetrahydrofuran methiodide (−)-**13***

A sealed metal container, filled with a solution of (−)-**18** (0.810 g, 2.44 mmol) in methanol (10 ml) and a 10-fold excess anhydrous dimethylamine was heated overnight at 120°C. The container was cooled at 0°C, the solution was acidified by addition of 3 N HCl, and the volatiles were evaporated under vacuum. The residual aqueous phase was treated with ether (4 × 10 ml), made alkaline by a portionwise addition of solid K_2CO_3, then extracted with dichloromethane (4 × 15 ml). After the usual work up, the residue was column chromatographed (eluant: 5% methanol–dichloro-

methane), affording 0.328 g (54%) of the corresponding tertiary base as a pale yellow oil.

(2*S*,4*R*,5*S*) 2-[(Dimethylamino)methyl]-4-benzyloxy-5-methyltetrahydrofuran: b.p. 175–180°C/0.2 mmHg; R_f 0.38 (eluant: 20% methanol–chloroform); ^{1}H NMR 1.25 (d, 3H, CH_3, $J = 6.5$); 1.70 (ddd, 1H, H-3′, $J = 6.8$, 9.8 and 13.3); 2.07 (ddd, 1H, H-3, $J = 1.9$, 5.8 and 13.3); 2.30 (s, 6H, NMe_2); 2.39 (dd, 1H, CH_2NMe_2, $J = 4.6$ and 12.7); 2.49 (dd, 1H, CH_2NMe_2, $J = 7.5$ and 12.7); 3.68 (m, 1H, H-4); 3.98 (m, 1H, H-2); 4.22 (m, 1H, H-5); 4.48 and 4.53 (d, 2H, CH_2Ph, $J = 11.7$); 7.33 (m, 5H, arom.); $[\alpha]_D^{20}$ −32.93 (*c* 1.154, $CHCl_3$).

A solution of the tertiary amine in acetone/ether (1:1) was treated with a 5-fold excess methyl iodide. The quaternary salt precipitated quantitatively as a gummy oil.

(2*S*,4*R*,5*S*) 2-[(Dimethylamino)methyl]-4-benzyloxy-5-methyltetrahydrofuran methiodide (−)-**10**: m.p. 107–108°C (colorless prisms from acetone/ethyl acetate); ^{1}H NMR 1.21 (d, 3H, CH_3, $J = 6.6$); 1.78 (ddd, 1H, H-3′, $J = 6.2$, 10.6 and 13.3); 2.32 (ddd, 1H, H-3, $J = 1.5$, 5.1 and 13.3); 3.44 (m, 2H, CH_2NMe_3); 3.48 (s, 9H, NMe_3); 3.75 (m, 1H, H-4); 4.13 (dq, 1H, H-5, $J = 2.1$ and 6.6); 4.44 and 4.52 (d, 2H, CH_2Ph, $J =$ 11.6); 4.53 (m, 1H, H-2); 7.33 (m, 5H, arom.); $[\alpha]_D^{20}$ −25.52 (*c* 0.960, $CHCl_3$).

The above reported procedure performed on the iodo derivative (−)-**21** afforded the corresponding tertiary base in 47% yield.

(2*R*,4*R*,5*S*) 2-[(Dimethylamino)methyl]-4-benzyloxy-5-methyltetrahydrofuran: b.p. 185–190°C/0.2 mmHg as a pale yellow oil; R_f 0.32 (eluant: chloroform/20% methanol); ^{1}H NMR 1.18 (d, 3H, CH_3, $J = 6.4$); 1.69 (ddd, 1H, H-3′, $J = 1.3$, 6.5 and 13.0); 2.20–2.37 (m, 2H, H-3 and CHNMe$_2$); 2.25 (s, 6H, NMe_2); 2.57 (dd, 1H, CHNMe$_2$, $J = 8.0$ and 12.6); 3.68 (m, 1H, H-4); 4.07 (dq, 1H, H-5, $J = 2.0$ and 6.4); 4.18 (m, 1H, H-2); 4.44 and 4.50 (d, 2H, CH_2Ph, $J = 11.8$); 7.29 (m, 5H, arom.); $[\alpha]_D^{20}$ −50.59 (*c* 1.018, $CHCl_3$).

(2*R*,4*R*,5*S*) 2-[(Dimethylamino)methyl]-4-benzyloxy-5-methyltetrahydrofuran methiodide (−)-**12**: m.p. 88.5–90°C (colorless prisms from acetone/ethyl acetate); ^{1}H NMR 1.14 (d, 3H, CH_3, $J = 6.6$); 1.86 (ddd, 1H, H-3′, $J = 3.7$, 3.7 and 13.7); 2.52 (ddd, 1H, H-3, $J = 1.8$, 7.0 and 13.7); 3.32–3.51 (m, 2H, CH_2NMe_3); 3.45 (s, 9H, NMe_3); 3.78 (m, 1H, H-4); 4.23 (m, 1H, H-5); 4.46 and 4.52 (d, 2H, CH_2Ph, $J = 11.8$); 4.71 (m, 1H, H-2); 7.31 (m, 5H, arom.); $[\alpha]_D^{20}$ −42.42 (*c* 1.002, $CHCl_3$).

A sealed metal container, filled with a solution of (+)-**19** (0.790 g, 2.28 mmol) in dichloromethane/methanol 1:1 (30 ml) and a 15-fold excess anhydrous trimethylamine was heated overnight at 70°C. The container was cooled at room temperature and the reaction mixture was evaporated under vacuum at 20°C. The crude quaternary ammonium salt was obtained as pale yellow leaflets after treatment with ether/methanol (0.508 g, 55% yield).

(2*R*,4*R*,5*S*) 2-[(Dimethylamino)methyl]-4-benzoyloxy-5-methyltetrahydrofuran methiodide (−)-**13**: m.p. 169–170°C (colorless prisms from 2-propanol); ^{1}H NMR (D_2O) 1.30 (d, 3H, CH_3, $J = 6.4$); 2.03 (m, 1H, H-3′); 2.84 (m, 1H, H-3); 3.20 (s, 9H, NMe_3); 3.54 (dd, 1H, CH_2NMe_3, $J = 1.5$ and 13.8); 3.61 (dd, 1H, CH_2NMe_3, $J = 8.0$ and 13.8); 4.52 (dq, 1H, H-5, $J = 1.9$ and 6.4); 4.89 (m, 1H, H-2); 5.21 (m, 1H, H-4); 7.53 (t, 2H, arom., $J = 7.9$); 7.67 (t, 1H, arom., $J = 7.9$); 8.02 (d, 2H, arom., $J = 7.9$); $[\alpha]_D^{20}$ −31.54 (*c* 0.964, MeOH).

4.2. Pharmacology

4.2.1. In vitro tests: general considerations

Male guinea pigs (200–300 g) were killed by cervical dislocation, and the organs required were set up rapidly under 1 g of tension in 20 ml organ baths containing physiological salt solution (PSS) kept at an appropriate temperature (see below) and aerated with 5% CO_2–95% O_2. Two dose–response curves were constructed by cumulative addition of the reference agonist [(+)-muscarine]. 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, a new dose–response curve to the agonist under study was obtained. Responses were expressed as a percentage of the maximal response obtained in the control curve. The results are expressed in terms of pD_2, which is the $-\log ED_{50}$, the concentration of agonist required to produce 50% of the maximum contraction. Contractions were recorded by means of a force transducer connected to a two-channel Gemini polygraph (U. Basile). In all cases, parallel experiments in which tissues received only the reference agonist were run in order to check any variation in sensitivity.

4.2.1.1. Determination of dissociation constants. Dissociation constants (K_D) and relative efficacies (e_r) were determined as previously described according to the method of Furchgott and Bursztyn for full agonists ($\alpha = 1$) [22]. For partial agonists ($\alpha < 1$), the affinity constants (K_B) were calculated from the equation $\log(DR - 1) = \log(\text{partial agonist}) - \log K_B$, where DR (dose ratio) is the ratio of the ED_{50} values of (+)-muscarine after and before incubation (1×10^{-4} M) with the partial agonist [23]. The partial agonist K_P value for compound (−)-**10** at guinea pig bladder ($\alpha = 0.86$) was determined according to Waud [24].

4.2.1.2. Guinea pig ileum. Portions of terminal ileum (2 cm) were taken at about 5 cm from the ileum–cecum junction and mounted in PSS at 37°C. The composition

of PSS was the following (mM): NaCl (118), $NaHCO_3$ (23.8), KCl (4.7), $MgSO_4 \cdot 7H_2O$ (1.18), KH_2PO_4 (1.18), $CaCl_2$ (2.52), glucose (11.7). Tension changes were recorded isotonically. Tissues were equilibrated for 30 min, and dose–response curves to (+)-muscarine were obtained at 30 min intervals, the first one being discarded and the second one being taken as the control.

4.2.1.3. Guinea pig bladder. A 2 mm wide longitudinal strip of bladder from urethra to the apex of the bladder was cut, excluding the portion under the urethra orifice, and mounted in PSS (the same used for ileum) at 37°C. Contractions were recorded isometrically. Tissues were equilibrated for 30 min (see protocol for ileum).

4.2.1.4. 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 curve to (+)-muscarine was constructed.

4.2.1.5. Right atria. Right atria were equilibrated for 1 h at the above conditions (see guinea pig stimulated left atria for PSS and temperature). Contractions were recorded isometrically.

4.2.2. In vivo tests: pithed rat

Male normotensive rats (270–330 g) were housed five per cage and maintained on a 12 h light/dark cycle. Food and water were available ad libitum. The animals were anaesthetized with equithesin (9.6 g nembutal sodium, 42.6 g chloral hydrate, 21.2 g $MgSO_4$, 400 ml propylene glycol, 50 ml ethyl alcohol and water to 1000 ml) 3 ml/kg of body weight i.p. The right jugular vein was cannulated (PE 10 polyethylene tubing) for drug administration. Blood pressure was measured from the left common carotid artery through a PE 50 catheter connected to a pressure transducer (P23 ID, Statham, Hato Rey, Puerto Rico). The heart rate was measured continuously by means of a rate meter (Basile) which was triggered by the blood pressure pulse in the carotid artery.

After catheterization of the trachea, heparin (150 IU/kg) was given i.v. to prevent blood coagulation. Temperature was maintained at approximately 37°C throughout the experiment by means of an overhead-heating lamp. The rats were then pithed by insertion of a steel rod (1.5 mm in diameter) through the skull and foramen magnum down into the spinal canal [25]. The animals were respired artificially by means of a Harvard Apparatus model 681 rodent respirator at a frequency of 60 cycles/min with a volume of 1 ml/100 g. The preparation was allowed to equilibrate for at least 30 min before drug administration, until mean heart rate had stabilized. The basal heart rate amounted to 300 ± 8 beats/min ($n = 50$). Changes in heart rate were measured for individual doses of the agonist given i.v. (0.1 ml/100 g). Full recovery from the pressor and cardiac effects with return to preinjection values was allowed between successive doses. After drug injection, the venous cannula was flushed with 50 μl of isotonic saline solution.

4.2.2.1. Experimental protocol. All drugs were dissolved in saline (0.9% w/v) and injected i.v. in a volume of 0.1 ml/100 g. Because of desensitization phenomena, when compounds (+)-**1**, (−)-**10**, (−)-**12** and (−)-**13** were employed, only one single dose–response curve was assessed in each preparation. ED_{50} values were determined graphically from the resultant dose–response curves and represent the dose causing 50% of the maximum response of the compound under study. Pretreatment (i.v.) with antagonists was carried out 20 min before the administration of the agonist. This interval was selected because preliminary experiments showed that after this time the antagonistic effects of pirenzepine (50 μg/kg i.v.) and tripitramine (30 μg/kg i.v.), respectively, were constant during the whole experiment.

4.2.3. Statistical analysis

Data are presented as means ± SEM of n experiments. Student's t-test was used to assess the statistical significance of the difference between two means.

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