Synthesis and Pharmacological Characterization of Enantiomerically Pure Muscarinic Agonists: Difluoromuscarines

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The four homochiral 4-deoxy-4,4-difluoromuscarine stereoisomers (difluoromuscarines) were prepared in very high enantiomeric excess. A convenient sequence based on the use of natural as well as "unnatural" ethyl lactate allowed the synthesis of target compounds, whose absolute configuration is dictated by that of the starting synthon. Quaternary ammonium salts (+)-**5**, (−)-**5**, (−)-**6**, and (+)-**6** were tested *in vitro* on guinea pig tissues, and their muscarinic potency was evaluated at M_2 (heart) and M_3 (ileum and bladder) muscarinic receptor subtypes. The eutomer (+)-**5** and distomer (−)-**5** were also tested *in vivo* on pithed rat, and their muscarinic activity at the M_1 receptor subtype was compared with those of racemic muscarine [(±)-**1**] and (2*S*,4*R*,5*S*)-4-deoxy-4-fluoromuscarine [(+)-**4**]. Further pharmacological parameters such as affinity, relative efficacy, and enantioselectivity have been determined for compounds (+)-**5** and (−)-**5** at M_2 (heart force and rate) and M_3 (ileum and bladder) receptors in order to investigate muscarinic receptor heterogeneity. The four homochiral difluoromuscarines behave as muscarinic agonists in all the tests with a potency trend which is different from that previously observed with the 4-deoxy-4-fluoromuscarines and (±)-**1**, thus indicating the intervention of the second fluorine atom on the receptor−ligand interaction. Moreover, the second fluorine atom produces significant differences in the affinity and relative efficacy values of compounds (+)-**5** and (−)-**5** at M_2 and M_3 subtypes, which could be attributed to a heterogeneity between the muscarinic receptors mediating heart rate and heart force and those involved in the contraction of ileum and bladder.

A common feature among the major chiral muscarinic agonists is the high value of the eudismic ratio (ER) and a spatial arrangement around the chiral centers matching those of natural muscarine (+)-**1** (Figure 1).

These peculiarities were essentially confirmed by our studies on the eight chiral muscarine stereoisomers,[1] as well as the four chiral isomers of muscarone[2] and methylenemuscarone.[3] The absolute configuration of the most potent stereoisomer of muscarone [(−)-**2**] and methylenemuscarone [(−)-**3**] is depicted in Figure 1. The nature of the interaction involving the binding of the hydroxy group of muscarine with the complementary receptor subsite has been the subject of detailed investigations by us since many years.[4] In 1992 Bravo et al.[5] reported the synthesis and the pharmacological evaluation of four 4-deoxy-4-fluoromuscarine stereoisomers. The overall results suggested that the pharmacological profile of this set of muscarinic agonists was quite similar to that of the reference muscarines. In particular, the replacement of the hydroxy group of muscarine with a fluorine atom, i.e. (+)-**4** (Figure 1) produced a change only in the receptor−ligand interaction at the cardiac M_2 muscarinic receptors controlling rate.

In order to further investigate the role played by a fluorine atom in this critical position we prepared the four chiral 4-deoxy-4,4-difluoromuscarine stereoisomers

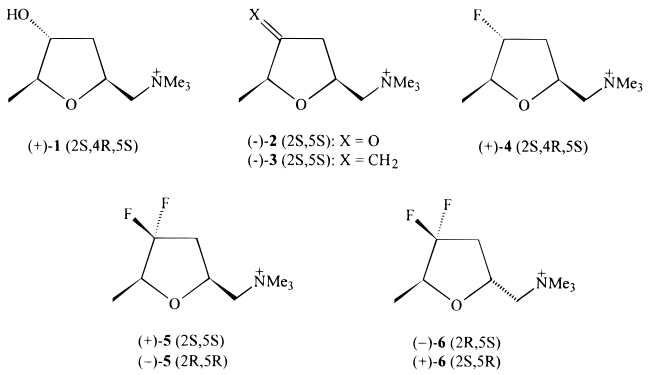

Figure 1.

[(+)-**5**, (−)-**5**, (−)-**6**, and (+)-**6**] (Figure 1) in enantiomeric excess higher than 98%. The compounds were tested *in vitro* on guinea pig tissues and their muscarinic potency evaluated at M_2 (heart) and M_3 (ileum and bladder) muscarinic receptor subtypes.

Chemistry

The synthesis of compounds (+)-**5**, (−)-**5**, (−)-**6**, and (+)-**6** (difluoromuscarines) was achieved following the strategy previously reported for the preparation of homochiral muscarines[1] and muscarones.[2] By employing the commercially available natural [*S*-(−)-] and "unnatural" [*R*-(+)-] ethyl lactate we prepared key diastereomeric iodo ketones **7** and **8** which could be separated by column chromatography (Scheme 1). Intermediates **7** and **8** were then separately reacted with

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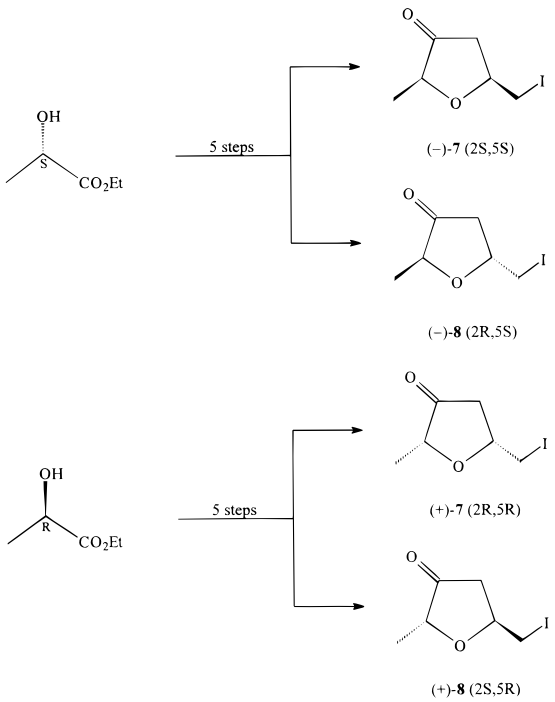
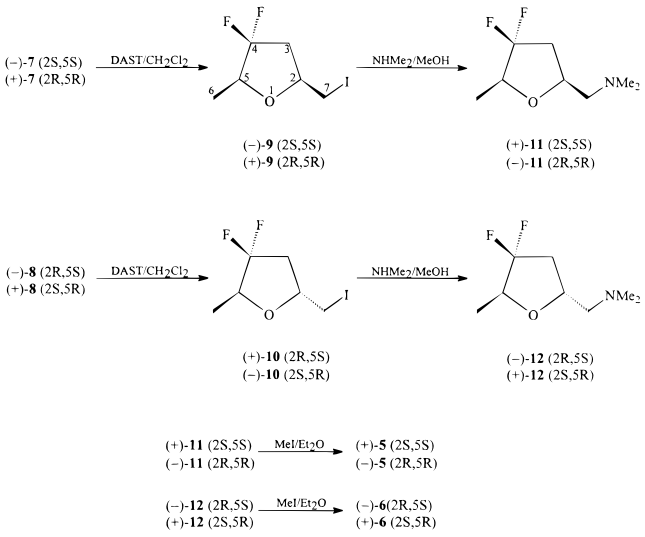
Scheme 1



Scheme 2



a 4-fold excess of (diethylamino)sulfur trifluoride (DAST).[6,7] The reaction was completed in a few hours at room temperature and gave the desired difluoro derivatives **9** and **10** in 60−70% yield (Scheme 2).

Through the conventional transformations of the side chain reported in Scheme 2 we synthesized the two pairs of enantiomeric salts (+)-**5**/(−)-**5** and (−)-**6**/(+)-**6**. Their absolute configuration derives from the absolute configuration of the known iodo ketones **7** and **8**.[2] The enantiomeric excess (ee) of the final derivatives (>98%) is inferred from the ee value of iodo ketones **7** and **8** which was previously determined.[2]

Results and Discussion

The pairs of enantiomers **5** and **6** were tested *in vitro* on guinea pig tissues, and their muscarinic potency was evaluated at M_2 (heart) and M_3 (ileum and bladder) muscarinic receptor subtypes (Table 1). These results were compared with those of (2*S*,4*R*,5*S*)-4-deoxy-4-fluoromuscarine (+)-**4** (fluoromuscarine) and of racemic muscarine (±)-**1** to study the effects of the different substituents at the 4-position on the pharmacological profile of the ligand. Compound (+)-**5**, the most potent isomer in the series, was also tested *in vivo* on pithed rat and its muscarinic activity at M_1 receptor subtype was compared with those of fluoromuscarine (+)-**4** and (±)-**1** (Table 2). In addition, affinity and relative efficacy were calculated for the pair of enantiomers (+)-**5** and (−)-**5** at M_2 (heart force and rate) and M_3 (ileum and bladder) receptors in order to investigate muscarinic receptors heterogeneity (Table 3).

Table 1. Potencies and Intrinsic Activities of Difluoromuscarines (+)-**5**/(−)-**5**, (−)-**6**/(+)-**6**, Fluoromuscarine (+)-**4,** and Muscarine (±)-**1** at M_2 and M_3 Muscarinic Receptors

	tissue					
	guinea pig heart (force) M_2		guinea pig ileum M_3		guinea pig bladder M_3	
compd	pD_2[a]	α[b]	pD_2[a]	α[b]	pD_2[a]	α[b]
(+)-**5**	6.56 ± 0.14	1.00	5.80 ± 0.07	0.99	5.23 ± 0.17	0.83
(−)-**5**	5.76 ± 0.13	0.97	5.53 ± 0.04	0.98	3.82 ± 0.06	0.95
(−)-**6**	6.28 ± 0.20	1.00	5.33 ± 0.20	0.97	4.16 ± 0.13	1.00
(+)-**6**	5.77 ± 0.04	0.98	4.85 ± 0.12	0.99	3.95 ± 0.08	0.67
(+)-**4**	6.73 ± 0.08	0.98	7.36 ± 0.03	0.95	5.66 ± 0.03	0.88
(±)-**1**	6.69 ± 0.05	1.00	7.10 ± 0.11	1.00	5.69 ± 0.06	1.00

[a] −log ED_{50}. The results are the mean ± SEM, and the number of observations varies between 6 and 10. [b] Intrinsic activity, measured by the ratio between the maximum response of the compound and the maximum response of muscarine.

Table 2. Potencies of (+)-**5**, (−)-**5**, (+)-**4**, and (±)-**1** at Cardiac M_2 Receptors Mediating Bradycardia and at Ganglionic M_1 Receptors Mediating Tachycardia in the Pithed Rat

	ED_{50} (μg/kg),[a] pithed rat	
	increase in heart rate (M_1)	decrease in heart rate (M_2)
(+)-**5**	50 ± 5.5	140 ± 15.2
ER[b]	2	2
(−)-**5**	100 ± 8.5	273.5 ± 33.3
(+)-**4**	4.5 ± 0.70	23.5 ± 3.1
(±)-**1**	2.5 ± 0.23	3.7 ± 0.46

[a] The results are the mean ± SEM, and the number of observations varies between five and nine. [b] Eudismic ratio: ratio between the potency of the more potent and the less potent enantiomer.

Since the study of enantioselectivity gives information on the behavior of ligands and on the characterization of receptor subgroups, we also evaluated this parameter in our *in vivo* and *in vitro* studies by inspection of the results obtained with distomer (−)-**5** (Tables 2 and 3).

Table 1 shows that the four chiral difluoromuscarines behave as muscarinic agonists with different degrees of potency at the investigated tissues. Their potency trend (heart > ileum > bladder) is different from that observed with fluoromuscarines and (±)-**1**,[5] suggesting that a further introduction of a fluorine atom in the nucleus has different consequences on the receptor−ligand interaction. As expected, the (2*S*,5*S*)-enantiomer [(+)-**5**)], which has the same absolute configuration of natural muscarine, is the most potent agonist in the new set of derivatives; it displays its higher potency at heart (M_2 subtype) at variance with (+)-**4** and (±)-**1** which are more active at ileum (M_3 subtype). Difluoromuscarine (+)-**5** is in fact 6 and 21 times more potent at M_2 than at M_3 receptor subtypes (ileum and bladder, respectively).

The overall results suggest that the hydrogen bonding

Table 3. Pharmacological Parameters of (+)-**5**, (−)-**5**, (+)-**4**, and (±)-**1** in the Guinea Pig Heart Force and Rate (M_2) and Ileum and Bladder (M_3)

	tissue											
	heart				heart				heart			
	force $-\log ED_{50}$[a]	rate $-\log ED_{50}$[a]	ileum $-\log ED_{50}$[a]	bladder $-\log ED_{50}$[a]	force $-\log K_D$[a]	rate $-\log K_D$[a]	ileum $-\log K_D$[a]	bladder $-\log K_D$[a]	force e_r[b]	rate e_r[b]	ileum e_r[b]	bladder e_r[b]
(+)-**5**	6.56 ± 0.14	6.58 ± 0.13	5.80 ± 0.07	5.23 ± 0.17	4.37 ± 0.21	3.39 ± 0.15	4.70 ± 0.08	4.40 ± 0.16	1.6	23	0.9	0.6
ER[c]	6.3	1.9	1.9	26	5.4	0.8	1.7	4.9	1.2	2.3	1.0	3.0
(−)-**5**	5.76 ± 0.13	6.31 ± 0.15	5.53 ± 0.04	3.82 ± 0.06	3.64 ± 0.14	3.48 ± 0.16	4.47 ± 0.14	3.71 ± 0.06	1.3	10	0.8	0.2
(+)-**4**	6.73 ± 0.08	6.87 ± 0.04	7.36 ± 0.03	5.66 ± 0.03	4.67 ± 0.12	5.77 ± 0.17	5.72 ± 0.11	4.39 ± 0.09	1.2	0.2	3	1.5
(±)-**1**	6.69 ± 0.05	6.42 ± 0.04	7.10 ± 0.11	5.69 ± 0.06	4.70 ± 0.10	4.61 ± 0.19	5.95 ± 0.10	4.62 ± 0.09	1	1	1	1

[a] The results are the mean ± SEM, and the number of observations varies between 6 and 10. [b] Relative efficacy [(±)-muscarine = 1]. [c] Eudismic ratio: ratio between the potency, the affinity and the relative efficacy of eutomer and distomer.

or the dipole−dipole interaction between the fluorine atom of (+)-**4** and the complementary receptor subsite is less effective due to the presence of a second fluorine atom in the geminal position, i.e. (+)-**5**. The extent of this effect is particularly evident in the ileum. A similar decline in potency was previously observed in the muscarinic agonists bearing the oxathiolane sulfoxide and oxathiolane sulfone nucleus.[8]

With the purpose to evaluate the muscarinic activity of enantiomers (+)-**5** and (−)-**5** at ganglionic M_1 and cardiac M_2 receptors, these agonists were tested *in vivo* on a pithed rat preparation, according to the method reported by Angeli et al.,[9] and results were compared with those obtained on compounds (+)-**4** and (±)-**1** (Table 2).[4] In particular, (+)-**5** is 11 and 20 times less active than reference compounds (+)-**4** and (±)-**1** at M_1 receptor subtype, while at M_2 subtype lowerings amount to 6 and 38, respectively. As a consequence, eutomer (+)-**5** is slightly (3-fold) selective for M_1 muscarinic receptors. The same selectivity was observed for distomer (−)-**5**, which displays a reduced potency both at M_1 and M_2 receptors.

A conceivable explanation for the decrease of the ED_{50} values displayed by the pair of enantiomers (+)-**5** and (−)-**5** when compared to those of reference compounds (+)-**4** and (±)-**1** on heart rate *in vivo* (the four agonists show similar potencies on heart rate *in vitro*) could be attributed to differences in metabolism and pharmacokinetics of the agonists in the two preparations, as already postulated for compounds (+)-**4** and (±)-**1**.[5]

According to Furchgott's theory,[10] differences in $-\log K_D$ of at least 0.5 are indicative of receptor heterogeneity; at the same time we know that the comparison of agonist potencies on different tissues is not sufficient to claim for receptor heterogeneity.[8,11] Consequently, further pharmacological parameters such as affinity and relative efficacy have been determined for the pair of enantiomers (+)-**5** and (−)-**5** (Table 3).

A vertical analysis of Table 3 shows that, as far as the M_2 subtype regulating heart force and the M_3 subtype of bladder are concerned, potency, affinity, and relative efficacy of compounds (+)-**5**, (+)-**4**, and (±)-**1** do not significantly differ from each other. On the contrary, at the M_2 subtype regulating heart rate, the same compounds display similar potency values, whereas affinity and relative efficacy show discrete differences. As a matter of fact fluoromuscarine (+)-**4** has a 240-fold higher affinity and a 115-fold lower efficacy than difluoromuscarine (+)-**5**. Moreover, (+)-**5** shows in the ileum (M_3 subtype) a decrease in potency in comparison with (+)-**4** and racemic muscarine (36 and 20 times, respectively), which parallels a decrease in affinity (10 and 18 times, respectively) as well as relative efficacy.

Furthermore, the introduction of two fluorine atoms in the 4-position of muscarine causes a marked drop of the enantioselectivity as reflected by the negligible eudismic ratio values (0.8−26, Table 3) shown by the pair of enantiomers (+)-**5** and (−)-**5**. This outcome does not allow any valuable speculation on the differences among the two muscarinic receptor subtypes but the observation that these values are always slightly higher on heart force (potency and affinity) and bladder (potency, affinity and efficacy) than on heart rate and ileum. The poor enantioselectivity observed for difluoromuscarines is rather unusual when the parallel results obtained on muscarine and other closely related chiral muscarinic agonists are taken into account.[1−4]

The horizontal analysis of the results gathered in Table 3 indicates that compounds (+)-**4** and (±)-**1** have identical profiles at the two receptor subtypes as far as potency is concerned. The two agonists show also similar affinity values except at heart rate, where a 15-fold higher value has been reported[5] for compound (+)-**4**. Remarkably, compounds (+)-**4** and (±)-**1** show the highest potency value on ileum, and compound (+)-**4** has the highest relative efficacy in the same tissue preparation.

Conversely, compound (+)-**5** is more active on heart (both force and rate) where it shows the highest relative efficacy value (e_r = 23) on heart rate. It is worth pointing out that distomer (−)-**5** possesses a pharmacological profile similar to that of (+)-**5**, thus confirming the peculiar interaction of the two fluorine atoms with the complementary muscarinic receptor subsite.

Once again our results with agonists confirm the different structural requirements of the muscarinic receptor subtypes and the role played by the subsite which interacts with the hydroxy function of muscarine (the "muscarinic subsite") in affecting organ selectivity.[4]

In conclusion, our data show that, while the replacement of the hydroxy group for fluorine in the 4 position of muscarine does not greatly influence the behavior of the molecule, the introduction of two fluorine atoms at the same position does affect its pharmacological profile at M_2 (heart rate) and M_3 (ileum) muscarinic receptor subtypes. Difluoromuscarines represent, therefore, a valuable tool to study the heterogeneity among M_2 and M_3 subtypes and to further put in evidence the differences between the muscarinic receptors mediating heart rate and heart force and those of ileum and bladder.[4]

Experimental Section

Material and Methods. (*R*)-(+)- and (*S*)-(−)-ethyl lactate and DAST were obtained from commercial suppliers and were used without further purification. Iodo ketones (−)-**7**, (+)-**7**, (+)-**8**, and (−)-**8** were prepared according to the previously reported procedure;[2] their specific rotations agreed with the value known in the literature[2] for the same compounds. ^{1}H NMR and ^{13}C NMR spectra were recorded with a Bruker AC-E 300 (300 Mhz) spectrometer in $CDCl_3$ or D_2O 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 N-3B thermostat. TLC were performed on commercial silica gel GF_{254} plates; spots were further evidenced by spraying with a dilute alkaline potassium permanganate solution. Liquid compounds were characterized by the oven temperature for Kugelrohr distillations. Melting points were determined on a Büchi apparatus and are uncorrected. Microanalyses of new compounds agreed with the theoretical value to within ±0.3%.

Synthesis of 2-(Iodomethyl)-4,4-difluoro-5-methyltetrahydrofuran Isomers (−)-9, (+)-9, (−)-10, and (+)-10. To a dichloromethane solution (15 mL) of iodoketone (−)-**7**[2] (1.00 g, 4.17 mmol) was added dropwise DAST (2.20 mL, 16.67 mmol). The progress of the reaction was monitored by TLC (eluent: 10% ethyl acetate/cyclohexane). After being stirred overnight at room temperature, the mixture was carefully poured in a slurry of ice and sodium bicarbonate and extracted with ethyl ether (4 × 10 mL). The organic extracts were dried over anhydrous sodium sulfate. Compound (−)-**9** distilled at 75−80 °C/20 mmHg, yield 0.743 g (68%).

The same procedure was applied to iodo ketones (+)-**7**, (+)-**8** and (−)-**8** to produce the corresponding difluoro derivatives (+)-**9**, (−)-**10**, and (+)-**10** in 60−70% yield.

(−)-9 (2*S*,5*S*): ^{1}H NMR ($CDCl_3$) δ 1.29 (dd, 3, H-6; J = 6.4 and 2.3), 2.16 (m, 1, H-3; J = 12.2, 8.7, 18.6 and 12.8), 2.58 (m, 1, H-3′; J = 12.2, 6.7, 14.4, 10.6), 3.24 (m, 1, H-7; J = 10.2, 6.9, 0.8 and 0.8), 3.29 (m, 1, H-7′; J = 10.2, 5.1, 0.8 and 0.9), 4.02 (qdd, 1, H-5; J = 6.4, 12.4 and 12.4), 4.10 (m, 1, H-2; J = 5.1, 6.7, 6.9, 8.7 and 0.8); ^{13}C NMR ($CDCl_3$) δ 6.83 (C-7), 13.59 (C-6; $J_{6,F'}$ = 1.7, $J_{6,F}$ = 6.8), 41.62 (C-3; $J_{3,F'}$ = 24.1, $J_{3,F}$ = 25.2), 75.36 (C-2; $J_{2,F'}$ = $J_{2,F}$ = 5.3), 78.90 (C-5; $J_{5,F'}$ = 26.4, $J_{5,F}$ = 32.5), 128.20 (C-4; $J_{4,F'}$ = 249.0, $J_{4,F}$ = 256.0); R_f 0.56 (cyclohexane/ethyl acetate, 95:5); $[\alpha]^{20}_D$ −9.13 (*c* 0.974, CH_2Cl_2). Anal. ($C_6H_9F_2IO$) C, H.

(+)-9 (2*R*,5*R*): $[\alpha]^{20}_D$ +8.81 (*c* 1.134, CH_2Cl_2). Anal. ($C_6H_9F_2IO$) C, H.

(−)-10 (2*S*,5*R*): ^{1}H NMR ($CDCl_3$) δ 1.27 (dd, 3, H-6; J = 6.4 and 2.2), 2.31 (m, 1, H-3; J = 14.1, 7.5, 0.8, 21.0, and 12.2), 2.59 (m, 1, H-3′; J = 14.1, 7.2, 15.9, and 6.3), 3.30 (m, 1, H-7; J = 10.2, 6.4, 0.8, and 0.8),[12] 3.32 (m, 1, H-7′; J = 10.2, 5.5, 0.8, and 0.8),[12] 4.20 (m, 1, H-5; J = 6.4, 8.7, 14.3, and 0.8),[13] 4.28 (m, 1, H-2; J = 7.2, 7.5, 5.6, and 5.6);[13] ^{13}C NMR ($CDCl_3$) δ 8.83 (C-7), 13.17 (C-6; $J_{6,F'}$ = 1.1, $J_{6,F}$ = 7.4), 40.50 (C-3; $J_{3,F'}$ = 23.3, $J_{3,F}$ = 25.3), 75.10 (C-2; $J_{2,F'}$ = 2.9, $J_{2,F}$ = 7.1), 77.10 (C-5; $J_{5,F'}$ = 26.2, $J_{5,F}$ = 31.5), 129.80 (C-4; $J_{4,F'}$ = 251.4, $J_{4,F}$ = 254.7), R_f 0.59 (cyclohexane/ethyl acetate, 95:5); $[\alpha]^{20}_D$ −19.75 (*c* 1.127, CH_2Cl_2). Anal. ($C_6H_9F_2IO$) C, H.

(+)-10 (2*R*,5*S*): $[\alpha]^{20}_D$ +19.37 (*c* 1.146, CH_2Cl_2). Anal. ($C_6H_9F_2IO$) C, H.

Synthesis of 2-[(Dimethylamino)methyl]-4,4-difluoro-5-methyltetrahydrofuran Isomers (+)-11, (−)-11, (+)-12, and (−)-12. A sealed metal container, filled with a solution of (−)-**9** (0.600 g, 2.29 mmol) in methanol (10 mL) and a 10-fold excess anhydrous dimethylamine was heated at 100 °C for 4 h. The container was cooled at 0 °C, the solution was acidified with dilute HCl, and the volatiles were evaporated under vacuum.The residual aqueous phase was treated with ether (3 × 10 mL), made alkaline by a portionwise addition of solid K_2CO_3, and extracted with dichloromethane (4 × 15 mL). The extracts were dried (Na_2SO_4), the solvent was evaporated, and the residue was distilled at 70−75 °C/18 mmHg to yield 0.230 g (56%) of (+)-**11**.

The same procedure was applied to iodo derivatives (+)-**9**, (+)-**10** and (−)-**10** to produce the corresponding tertiary amines (−)-**11**, (−)-**12**, and (+)-**12** in 55−60% yield.

(+)-11 (2*S*,5*S*): ^{1}H NMR ($CDCl_3$) δ 1.26 (dd, 3, H-6; J = 6.5 and 2.3), 2.06 (m, 1, H-3; J = 13.5, 9.5, 20.0, and 13.5), 2.29 (s, 6, NMe_2), 2.39 (dd, 1, H-7; J = 13.0 and 4.5), 2.43 (m, 1, H-3′; J = 13.5, 6.5, 16.0, and 9.5), 2.54 (dd, 1, H-7′; J = 13.0 and 7.2), 3.89 (m, 1, H-5; J = 6.5, 12.5, and 12.5), 4.15 (m, 1, H-2; J = 4.5, 6.5, 7.2, and 9.5); ^{13}C NMR ($CDCl_3$) δ 13.23 (C-6; $J_{6,F'}$ = 1.4, $J_{6,F}$ = 7.0), 40.14 (C-3; $J_{3,F'}$ = 24.0, $J_{3,F}$ = 24.9), 45.99 (NMe_2), 63.27 (C-7), 74.09 (C-2; $J_{2,F'}$ = $J_{2,F}$ = 4.85), 78.33 (C-5; $J_{5,F'}$ = 26.6, $J_{5,F}$ = 32.65), 128.61 (C-4 $J_{4,F'}$ = 248.6, $J_{4,F}$ = 255.8); R_f 0.53 (dichloromethane/methanol, 9:1); $[\alpha]^{20}_D$ +8.15 (*c* 1.110, CH_2Cl_2). Anal. ($C_8H_{15}F_2NO$) C, H, N.

(−)-11 (2*R*,5*R*): $[\alpha]^{20}_D$ −8.56 (*c* 1.028, CH_2Cl_2). Anal. ($C_8H_{15}F_2NO$) C, H, N.

(+)-12 (2*S*,5*R*): ^{1}H NMR ($CDCl_3$) δ 1.24 (dd, 3, H-6; J = 6.5 and 2.2), 2.15 (m, 1, H-3; J = 13.5, 7.5, and 19.0), 2.29 (s, 6, NMe_2), 2.34 (dd, 1, H-7; J = 12.5 and 4.5), 2.46 (m, 1, H-3′; J = 13.5, 7.5, and 16.5), 2.55 (dd, 1, H-7′; J = 12.5 and 7.5), 4.12 (m, 1, H-5; J = 6.5, 13.0, and 8.7), 4.35 (dddd, 1, H-2; J = 4.5, 7.5, 7.5, and 7.5); ^{13}C NMR ($CDCl_3$) δ 13.80 (C-6; $J_{6,F'}$ = 1.6, $J_{6,F}$ = 7.6), 38.75 (C-3; $J_{3,F'}$ = 21.1, $J_{3,F}$ = 25.2), 45.77 (NMe_2), 63.21 (C-7), 73.43 (C-2; $J_{2,F'}$ = 3.0, $J_{2,F}$ = 6.4), 76.11 (C-5; $J_{5,F'}$ = 26.2, $J_{5,F}$ = 31.5), 128.30 (C-4; $J_{4,F'}$ = 250.9, $J_{4,F}$ = 253.4); R_f 0.51 (dichloromethane/methanol, 9:1); $[\alpha]^{20}_D$ −9.31 (*c* 1.128, CH_2Cl_2). Anal. ($C_8H_{15}F_2NO$) C, H, N.

(−)-12 (2*R*,5*S*): $[\alpha]^{20}_D$ −9.07 (*c* 1.270, CH_2Cl_2). Anal. ($C_8H_{15}F_2NO$) C, N.

Synthesis of 2-[(Dimethylamino)methyl]-4,4-difluoro-5-methyltetrahydrofuran methiodides (+)-5, (−)-5, (−)-6, and (+)-6. An ethereal solution of the tertiary amine was treated with an excess of methyl iodide. The salts precipitated quantitatively and were crystallized from acetone/ethyl ether.

(+)-5 (2*S*,5*S*): ^{1}H NMR (D_2O) δ 1.34 (ddd, 3, H-6; J = 6.5, 2.4, and 1.0), 2.31 (m, 1, H-3; J = 14.0, 9.0, 18.0, and 13.0), 2.80 (m, 1, H-3′; J = 14.0, 7.0, 14.0, and 11.0), 3.30 (s, 9, NMe_3), 3.67 (d, 2, H-7 and H-7′; J = 6.0), 4.20 (m, 1, H-5; J = 6.5, 12.5, and 12.5), 4.79 (m, 1, H-2); ^{13}C NMR (D_2O) δ 13.51 (C-6; $J_{6,F'}$ = 1.5, $J_{6,F}$ = 6.8), 39.52 (C-3; $J_{3,F'}$ = $J_{3,F}$ = 25.6), 54.98 (NMe_3; $J_{Me,F}$ = $J_{Me,F'}$ = 3.7), 69.78 (C-7), 70.92 (C-2; $J_{2,F'}$ = $J_{2,F}$ = 5.3), 79.63 (C-5; $J_{5,F'}$ = 26.6, $J_{5,F}$ = 32.7), 128.50 (C-4; $J_{4,F'}$ = 248.1, $J_{4,F}$ = 253.2); mp 161−162 °C; $[\alpha]^{20}_D$ +25.87 (*c* 0.978, CH_3OH). Anal. ($C_9H_{18}F_2INO$) C, H, N.

(−)-5 (2*R*,5*R*): mp 162−163 °C; $[\alpha]^{20}_D$ −26.40 (*c* 1.00, CH_3OH). Anal. ($C_9H_{18}F_2INO$) C, H, N.

(−)-6 (2*R*,5*S*): ^{1}H NMR (D_2O) δ 1.28 (ddd, 3, H-6; J = 6.4, 2.2, and 0.6), 2.35 (m, 1, H-3; J = 14.4, 7.2, 18.7, and 13.4), 2.82 (m, 1, H-3′; J = 14.4, 7.8, 7.8, and 16.4), 3.21 (s, 9, NMe_3), 3.49 (dd, 1, H-7; J = 2.0 and 14.0), 3.75 (dd, 1, H-7′; J = 10.0 and 14.0), 4.35 (m, 1, H-5; J = 8.8, 13.6 and 6.4), 4.93 (m, 1, H-2); ^{13}C NMR (D_2O) δ 13.10 (C-6; $J_{6,F'}$ = 1.3, $J_{6,F}$ = 7.1), 38.57 (C-3; $J_{3,F'}$ = $J_{3,F}$ = 25.2), 55.02 (NMe_3; $J_{Me,F}$ = $J_{Me,F'}$ = 3.8), 69.04 (C-7), 70.96 (C-2; $J_{2,F'}$ = 3.3, $J_{2,F}$ = 6.9), 77.46 (C-5; $J_{5,F'}$ = 26.4, $J_{5,F}$ = 31.75), 128.30 (C-4 $J_{4,F'}$ = 249.8, $J_{4,F}$ = 252.1); mp 197.5−198.5 °C dec; $[\alpha]^{20}_D$ −22.15 (*c* 1.022, CH_3OH). Anal. ($C_9H_{18}F_2INO$) C, H, N.

(+)-6 (2*S*,5*R*): mp 197.5−198 °C dec; $[\alpha]^{20}_D$ +22.63 (*c* 0.972, CH_3OH). Anal. ($C_9H_{18}F_2INO$) C, H, N.

Pharmacology. *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 (±)-muscarine were run in order to check any variation in sensitivity.

Determination of Dissociation Constants. Dissociation constants and relative efficacies for compounds (+)-**5** and (−)-**5** were determined as previously described[8,10] according to the method of Furchgott and Bursztyn.[14]

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 at 37 °C. The composition of PSS was as follows (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), 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.

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 orefice, 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).

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, 2 Hz, 10−15 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.

Whole Atria. The right and left atria were removed and equilibrated for 1 h at the above conditions (see Guinea Pig Stimulated Left Atria for PSS and temperature). Contractions were recorded isometrically.

***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 of nembutal sodium, 42.6 g of chloral hydrate, 21.2 g of $MgSO_4$, 400 mL of propylene glycol, 50 mL of ethyl alcohol, and water to 1000 mL) 3 mL/kg of body weight ip. 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 iv 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.[15] 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 iv (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.

Experimental Protocol. All drugs were dissolved in saline (0.9% w/v) and injected iv in a volume of 0.1 mL/100 g. Because of desensitization phenomena, when compounds (+)-**5** and (±)-muscarine 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, iv, with antagonists was carried out 20 min before the administration of agonist. This interval was selected because preliminary experiments showed that after this time the antagonistic effects of pirenzepine (50 μg/kg iv) and tripitramine (30 μg/kg iv), respectively, were constant during the whole experiment.

Statistical Analysis. Data are presented as means ± SEM of n experiments. Differences between mean values were tested for significance by the Student's t test.

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