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[4-[[*N*-(3-Chlorophenyl)carbamoyl]oxy]-2-butynyl]-trimethylammonium (McN-A-343)-Related Compounds. Effect of the Butynyl Chain Inclusion into an Aromatic Unit on the Potency for Muscarinic Receptors

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Abstract—A series of derivatives of the known M_1 selective muscarinic receptor agonist McN-A-343 (**1**) was designed with the aim of investigating the effects of structural variations on both the butynyl chain and the phenyl ring of **1**. The butynyl chain was replaced with an aromatic spacer, and the effects of such a modification on the stereoelectronic properties of the molecules were theoretically studied and considered compatible with muscarinic receptor affinity. Substituents on the phenyl ring of **1** were selected so as to vary their electronic and hydrophobic properties. This design strategy did not produce muscarinic M_1 receptor agonists more potent than the prototype **1**, even if some analogues displayed functional selectivity for different muscarinic receptor subtypes. Compounds **3** and **7** were selective agonists towards muscarinic M_3 receptors, while compounds **14**, **16** and **18** were selective muscarinic M_2 receptor agonists. The most interesting derivative was **8**, a full agonist at muscarinic M_3 receptors devoid of activity at both muscarinic M_1 and M_2 subtypes. The pharmacological profile of the series was further characterized by studying the anticholinesterase and miotic activities of some representative compounds. Compounds **3**–**8** turned out to be weak acetylcholinesterase inhibitors, while derivatives **4**, **6**, **8** and **11** were able to significantly reduce the pupillary diameter in rabbit, indicating **8** as an effective miotic agent.

Introduction

Senile dementia is the most common psychiatric disorder in elderly patients, and Alzheimer's disease[1,2] (AD) is the most common cause. AD is a progressive neurodegenerative disease characterized by significant cell pathology in discrete brain regions such as forebrain cholinergic neurons.[3] It has been demonstrated that presynaptic muscarinic receptors are significantly reduced in these brain regions whereas postsynaptic muscarinic receptors are not significantly affected in patients with AD. Therefore, cholinergic ligands that directly or indirectly stimulate postsynaptic receptors should restore cholinergic function in the central nervous system and ameliorate cognitive deficits thus providing a palliative treatment of AD.

Many efforts were devoted to the development of selective muscarinic M_1 receptor agonists because muscarinic receptors of this type are located postsynaptically and are not significantly reduced in AD.[4] An additional reason is that M_1 selective agonists should not cause serious peripheral side effects since muscarinic M_1 receptors do not predominate in peripheral effector organs such as heart and smooth muscle tissues. However, pharmacotherapy and practical medicine are still waiting for the possible advantages arising from enhancing the cholinergic system with agonists. To date, only few inhibitors of acetylcholinesterase, which enhance indirectly the cholinergic transmission, are used to alleviate the symptoms of AD.[5,6]

[4-[[*N*-(3-Chlorophenyl)carbamoyl]oxy]-2-butynyl]trimethylammonium chloride (McN-A-343, **1**) is an effective agonist in stimulating muscarinic receptors in sympathetic ganglia.[7] It was advanced that the selective actions of **1** are mediated by the muscarinic M_1 receptor

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subtype.[8,9] McN-A-343 actually served as one of the earliest indicators of the existence of muscarinic receptor subtypes,[7] though the selectivity displayed by **1** in functional assays was not retained in binding experiments, as only minor differences were observed in the affinity for different muscarinic receptor subtypes.[10–12] Several analogues of **1** have been studied to improve affinity and selectivity for muscarinic M_1 receptors and to improve the lipophilicity,[13–22] because **1** and related quaternary ammonium salts are not able to penetrate the blood–brain barrier. It derives that compounds related to **1** containing a quaternary ammonium group are not useful in AD. On the other hand, the majority of the analogues of **1** bearing a tertiary amino moiety were inactive as ganglion stimulants.[21]

The aim of this project was to obtain McN-A-343-related compounds, which display selectivity for muscarinic receptor subtypes. The starting point was the observation that the butynyl chain of **1** can be reduced to a butenyl unit, which, in turn, can be oxidized to the corresponding epoxide without losing ganglion-stimulant properties.[14–16] However, only *trans* isomers were active, whereas *cis* isomers were not. It was advanced that extended conformations of **1** and of the olefin analogue **2** (Fig. 1), in which the ether oxygen and the quaternary nitrogen are 5.7 Å apart, are responsible for activity.[14–16]

An analysis of stereomodels reveals that the butynyl chain of **1** or of the corresponding trans olefin unit of **2** can be replaced by an aryl unit without altering dramatically the distance between the ether oxygen and the basic nitrogen. Thus, we speculated that it might be possible to generate centrally acting muscarinic ligands, displaying hopefully M_1 selectivity, by replacing the butynyl chain of **1** with an aryl moiety. Furthermore, we attempted to

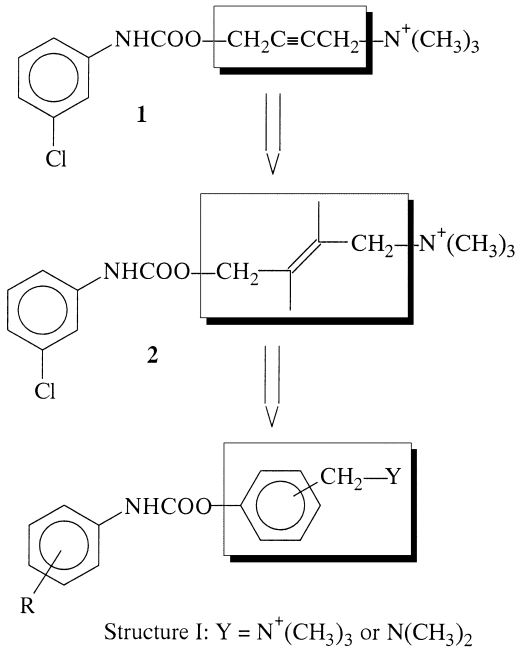


Figure 1. Design strategy for the synthesis of carbamates **3**–**18** by replacing the butynyl chain of McN-A-343 (**1**) by a benzyl moiety.

improve the lipophilicity of the new compounds by replacing the quaternary ammonium group with a tertiary amine function, considering that a similar modification performed on the 4-fluoro analogue of **1** did not modify the potency for muscarinic M_1 receptors.[17–20] Moreover, the presence of a phenyl ring afforded the opportunity to examine the effect of substituents on both potency and selectivity for muscarinic receptor subtypes. In the present study, our aim was to determine only whether electronic and/or lipophilic properties of substituents in the *para* position of the phenyl of the carbamoyl moiety of **1** could exert any favorable effect on muscarinic receptor selectivity and affinity, rather than assess a quantitative relationship. It seemed this could be determined with a few properly chosen substituents, which were selected in such a way as to have σ and π values in a positive or negative direction, in all combinations.[23] Comparison of the activity of these substituted derivatives with either the parent compound **1** or the unsubstituted analogue **3** should reveal the importance, if any, of one or both of these parameters. The compounds used were the fluoro ($+\pi$, $+\sigma$), methyl ($+\pi$, $-\sigma$), nitro ($-\pi$, $+\sigma$) and methoxy ($-\pi$, $-\sigma$) derivatives. The choice of the *para* position was dictated by the observation that the insertion of substituents in this position resulted in an increase in potency for muscarinic M_1 receptors in comparison with McN-A-343 (**1**) and, what is more important, the tertiary amino derivative of the 4-fluoro analogue of **1** retained affinity for muscarinic receptors.[17–20] This finding may have relevance for the design of ligands that have to cross the blood–brain barrier.

Thus, we designed structure I (Fig. 1), which incorporates the butynyl chain of **1** and the butenyl unit of **2** as well into an aromatic ring. Compound **3** and its corresponding quaternary ammonium derivative **4** were synthesized as analogues of McN-A-343 (**1**). To verify the role of the substituents at position 4 of the phenyl ring of the carbamoyl moiety, compounds **5**–**14** were also obtained. Finally, compounds **15**–**18** were included in this study to determine the effect of varying the distance between the ether oxygen and the basic nitrogen on the potency for muscarinic receptor subtypes. All the compounds were tested in isolated rabbit vas deferens (M_1), guinea pig left atria (M_2) and ileum (M_3) to assess their biological profile at muscarinic receptor subtypes. Furthermore, they were tested on acetylcholinesterase (AChE) owing to their structural kinship with covalent inhibitors of this enzyme. Few compounds were also tested in vivo to assess their miotic properties.

Chemistry

The compounds used in the present study were synthesized by standard procedures and characterized by ^{1}H NMR and elemental analysis. The syntheses of the compounds are outlined in Scheme 1.

Carbamates **3, 5**,[24,25] **7**, **9**, **11** and **13** were synthesized through the reaction of the corresponding (un)substituted phenylisocyanate and *N*,*N*-dimethyl-3-hydroxybenzylamine.[24,25] Similarly, carbamates **15** and **17** were

obtained from the corresponding substituted phenylisocyanate and *N*,*N*-dimethyl-4-hydroxybenzylamine.[24,25] Methiodides **4**, **6**, **8**, **10**, **12**, **14**, **16** and **18** were prepared by methylation of the corresponding tertiary amine with an excess of methyl iodide.

Biology

In vitro studies

Functional activity at muscarinic receptor subtypes was determined by the use of the muscarinic M_1 receptor–mediated inhibition of neurogenic twitch contractions of rabbit vas deferens (2 Hz), muscarinic M_2 receptor–mediated negative inotropism in driven guinea pig left atria (1 Hz) and muscarinic M_3 receptor–mediated contraction of guinea pig longitudinal muscle. These methods have been described in detail earlier.[26,27] The agonist at muscarinic M_2 and M_3 receptors was arecaidine propargyl ester (APE), whereas at muscarinic M_1 receptors McN-A-343 (**1**) was used as the reference agonist. The potencies and apparent efficacies of the agonists were expressed by their pEC_{50} and intrinsic activity (ia) values, whereas the apparent affinities (pK_b) of antagonists were calculated at only one concentration (100 μM) according to Arunlakshana and Schild.[28]

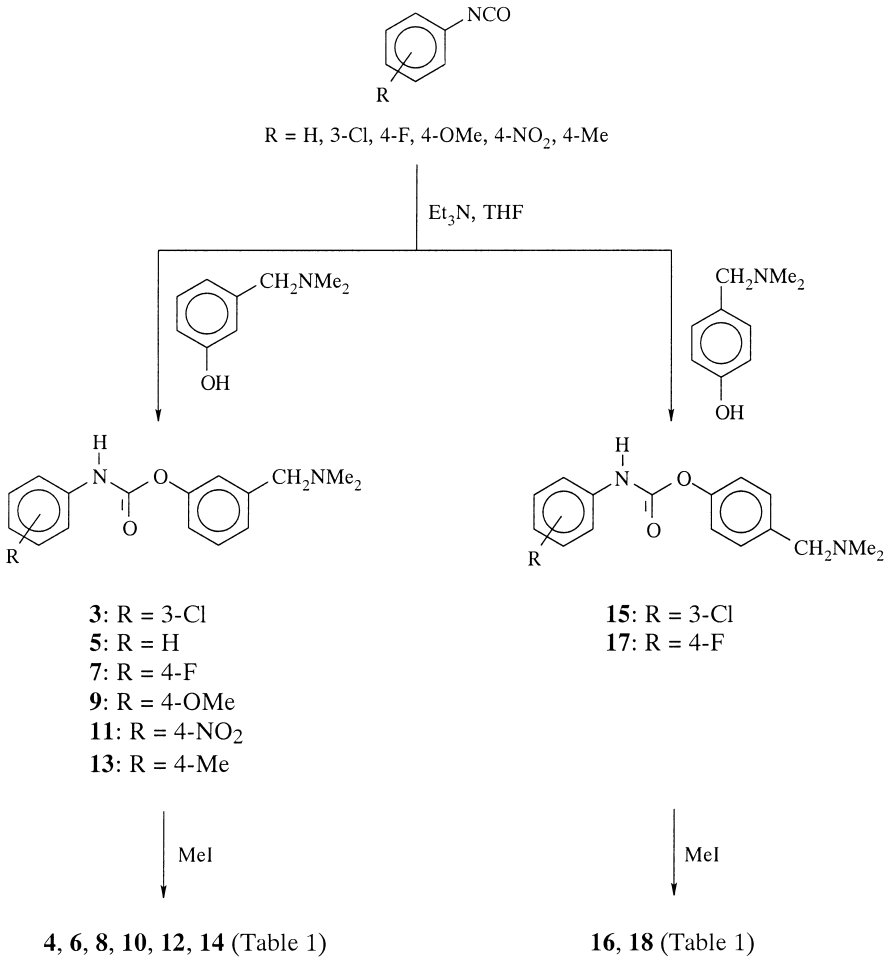
Anticholinesterase activity was assessed on acetylcholinesterase derived from human erythrocytes and is expressed as pIC_{50} values using physostigmine as the reference compound.

Miotic activity in the rabbit

The miotic properties of selected McN-A-343–related compounds were assessed in the conscious rabbit by determining the pupillary diameter following topical administration of the compound under investigation. Pilocarpine was used as the reference drug.

Results and Discussion

The stereoelectronic properties of the lead compound **1** as well as of the analogue **4** were theoretically studied, in order to verify the basic ideas that led to the design of the series. The electrostatic potential maps for the molecular skeleton of McN-A-343 (**1**) and of compounds **3**–**18** were generated and they are shown in Figure 2. It appears that the ethynyl (Fig. 2a) and phenyl (Fig. 2b) spacers do not alter substantially the negative (white) potential zone connecting the carbamic and the methylammonium groups. This seems to confirm the electronic similarity of the molecules and their



Scheme 1.

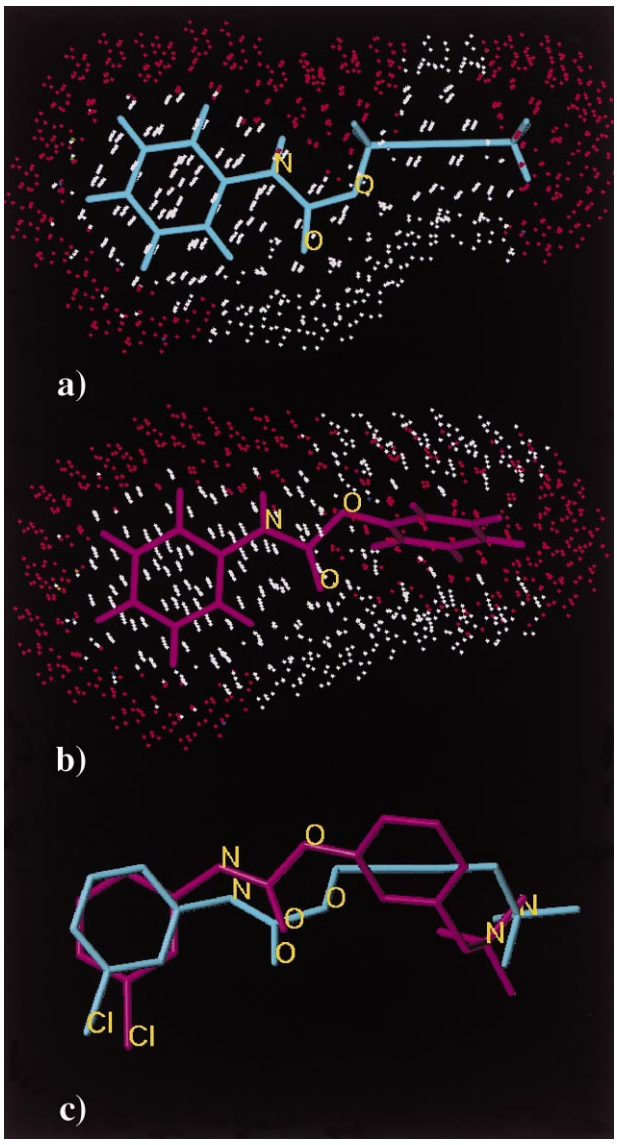


Figure 2. Electrostatic potential maps of the molecular skeleton of compounds **1** (a) and **4** (b), and superimposition of the two molecules (c). In the maps, the dots are color-coded red in the zones of positive potential ($>7.5\times10^{-2}$ kcal/mol) and white in the zones of negative potential ($<-7.5\times10^{-2}$ kcal/mol). The molecules in (c) are **1** (cyan) and **4** (magenta).

consequent possibility to be recognized by the same receptors. The presence of the ammonium head affects in the same way the potential distribution over the two molecules (not shown), while different substituents might induce local changes of the electron density able to influence the receptor affinity of different analogues.

In Figure 2c, the superimposition of compounds **1** and **4** is shown, allowing a comparison of the spatial features of these molecules. Low energy conformations of each molecule were selected and fitted onto each other by using corresponding representative atoms of the common functional groups (the phenyl ring, the carbamate function, and the quaternary ammonium head). It is evident that the overlap is not perfect for the two molecules, even if a good overall superimposition is shown by **1** (cyan) and **4** (magenta). It appears also that most of the oxygen atoms of the −COO- groups (the main negative potential zone of the molecules, see Figure 2a–b) fall in the same area, with the exception of the ethereal O atom of **4**.

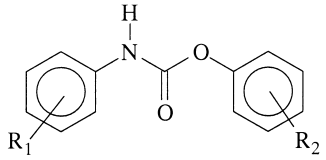
The activity at muscarinic receptor subtypes of compounds **3**–**18** is reported in Table 1 together with that of the reference agonists APE and McN-A-343 (**1**). An analysis of the results reveals that McN-A-343–related compounds did not display the same biological profile of the prototype and that the differences are more pronounced if tertiary amino or trimethylammonium derivatives are considered. A first preliminary conclusion is that the replacement of the butynyl chain of **1** with an aryl moiety produced a significant decrease in the potency towards muscarinic M_1 receptors relative to the prototype **1**.

A comparison between the new compounds and **1** should begin by analysing the biological activity displayed by the trimethylammonium derivatives, because they are structurally closer to **1** rather than the corresponding tertiary amino derivatives. Thus, compound **4**, although showing a different potency, was like **1** a full agonist at both muscarinic M_1 and M_3 receptor subtypes (pEC_{50} values of 5.33 ± 0.21 (M_1) and 5.14 ± 0.16 (M_3) for **4** and pEC_{50} values of 6.29 ± 0.01 (M_1) and 4.86 ± 0.19 (M_3) for **1**), whereas, in contrast with **1**, it was devoid of activity at muscarinic M_2 receptors up to 100 μM concentration. It derives that the structural modification performed on **1** leading to **4** may be a starting point to modulate the functional selectivity for muscarinic receptor subtypes. Next, to verify the effect of varying the distance between the ether oxygen and the trimethylammonium head, compound **16** can be compared to both **1** and **4**. Interestingly enough, compound **16**, bearing a 4-trimethylammonium head, turned out to be a full agonist at all muscarinic receptor subtypes investigated and, what is more important, displayed selectivity for muscarinic M_2 receptors, owing to a low potency for both muscarinic M_1 and M_3 receptors, as revealed by the pEC_{50} values of 5.64 ± 0.19 (M_2), 4.31 ± 0.13 (M_1) and 4.52 ± 0.08 (M_3).

Since it has been claimed that the 4-fluoro analogue of **1** is more potent than the prototype,[17–20] we investigated the 4-fluoro analogue **18**. It turned out to be a partial agonist at muscarinic M_1 and M_2 receptors and a weaker, albeit full, agonist at muscarinic M_3 receptors relative to **16**.

To verify the effect of the substituents on the potency for muscarinic receptor subtypes we investigated derivatives **6**, **8**, **10**, **12** and **14**. Clearly, the potency at muscarinic M_3 receptors was not dependent on the type of substituent, as these compounds showed an activity similar to that of both **1** and **4**, although the intrinsic activity (ia) was lower than unity in some cases. On the contrary, a significant effect on the potency for both muscarinic M_1 and M_2 receptors was observed for the above 4-substituted compounds. The unsubstituted analogue **6** was a weak partial M_1 agonist and a weak

Table 1. In vitro functional activity of **3**–**22** at muscarinic receptor subtypes in rabbit vas deferens (M_1), guinea pig left atria (M_2) and ileum (M_3)



3 - 18

			M_1		M_2			M_3			AChE
No.[a]	R_1	R_2	pEC_{50}[b]	ia[c]	pEC_{50}[b]	ia[c]	pK_b[d]	pEC_{50}[b]	ia[c]	pK_b[d]	pIC_{50}[e]
APE			7.14 ± 0.18	1	8.67 ± 0.04	1	—	7.64 ± 0.09	1	—	nd[g]
1 (McN-A-343)			6.29 ± 0.01	1	4.61 ± 0.12	0.5	—	4.86 ± 0.19	0.8	—	nd
3	3-Cl	3-CH_2NMe_2	4.10 ± 0.09	1	4.97 ± 0.23	1	—	5.94 ± 0.30	0.3	—	3.99
4	3-Cl	3-$CH_2N^+Me_3$	5.33 ± 0.21	1	na[f]	—	—	5.14 ± 0.16	1	—	4.06
5	H	3-CH_2NMe_2	3.64 ± 0.14	1	—	—	4.58 ± 0.10	na	—	—	4.32
6	H	3-$CH_2N^+Me_3$	4.03 ± 0.27	0.3	—	—	4.22 ± 0.05	4.93 ± 0.22	0.9	—	4.10
7	4-F	3-CH_2NMe_2	na	—	4.25 ± 0.24	1	—	6.31 ± 0.30	0.3	—	3.55
8	4-F	3-$CH_2N^+Me_3$	na	—	na	—	—	5.03 ± 0.17	1	—	3.45
9	4-OMe	3-CH_2NMe_2	4.57 ± 0.04	1	5.0 ± 0.26	1	—	—	—	5.15 ± 0.10	nd
10	4-OMe	3-$CH_2N^+Me_3$	4.47 ± 0.07	1	—	—	4.55 ± 0.23	4.87 ± 0.01	0.3	—	nd
11	4-NO_2	3-CH_2NMe_2	4.73 ± 0.03	1	—	—	4.10 ± 0.13	na	—	—	nd
12	4-NO_2	3-$CH_2N^+Me_3$	4.61 ± 0.16	1	4.59 ± 0.21	1	—	5.07 ± 0.14	1	—	nd
13	4-Me	3-CH_2NMe_2	4.10 ± 0.11	1	4.56 ± 0.22	0.8	—	na	—	—	nd
14	4-Me	3-$CH_2N^+Me_3$	4.27 ± 0.08	0.8	6.20 ± 0.06	0.7	—	5.03 ± 0.01	1	—	nd
15	3-Cl	4-CH_2NMe_2	3.84 ± 0.16	1	—	—	4.82 ± 0.04	na	—	—	nd
16	3-Cl	4-$CH_2N^+Me_3$	4.31 ± 0.13	1	5.64 ± 0.19	1	—	4.52 ± 0.08	1	—	nd
17	4-F	4-CH_2NMe_2	3.29 ± 0.29	1	—	—	4.99 ± 0.27	na	—	—	nd
18	4-F	4-$CH_2N^+Me_3$	4.71 ± 0.27	0.5	6.40 ± 0.10	0.3	—	3.99 ± 0.02	1	—	nd

[a]Tertiary amino compounds were oxalate salts, whereas quaternary ammonium compounds were iodides.
[b]pEC_{50} ± SE values are the negative logarithm of the agonist concentration that caused 50% of the maximum response attainable in that tissue and are the mean of at least four independent experiments.
[c]The intrinsic activity (ia) is the maximum response to arecaidine propargyl ester (APE), which was taken equal to 1.
[d]Apparent pK_b values ± SE were calculated according to Arunlakshana and Schild[28] with the following equation: $pK_b = -\log K_b = \log (DR-1) - \log [B]$. The log (DR−1) was calculated from one antagonist concentration, which was tested four times. Dose–ratio (DR) values represent the ratio of the potency of the agonist (EC_{50}) in the presence of the antagonist and in its absence.
[e]AChE was from human erythrocytes. pIC_{50} values ($-\log IC_{50}$ (μM)) represent the concentration of inhibitor required to decrease enzyme activity by 50% and are the mean of two independent measurements, which agreed within ± 10%.
[f]na, not active up to 100 μM concentration either as agonist or as antagonist.
[g]nd, not determined.

M_2 antagonist. The 4-methoxy analogue **10** displayed a biological profile similar to that of **6**. On the other hand, **12** and **14**, which bear substituents with electronic and lipophilic properties opposite to each other, exhibited a similar biological profile, though **14** was significantly more potent at the muscarinic M_2 receptor. This finding may indicate that the effect of substituents on muscarinic potency can be hardly rationalized with our series of compounds. Attempts to correlate the different muscarinic receptor subtype affinities to physico-chemical properties via multivariate analysis failed, despite the screening of several hydrophobic, electronic, and steric descriptors.

Since our aim was to generate centrally acting muscarinic agonists, we investigated also the tertiary amino derivatives **3**, **7**, **9**, **11**, **13**, **15** and **17**. Although the potency displayed by these tertiary amino derivatives was markedly lower than that of **1**, they retained the ability to fully activate (ia = 1) rabbit vas deferens muscarinic M_1 receptors, reaching one of the objectives of this study. The only exceptions were the tertiary amino derivatives bearing a 4-fluoro substituent, which turned out to be inactive (**7**) or very weak agonist (**17**). This finding was somewhat surprising because the tertiary amino derivative of McN-A-343 bearing a 4-fluoro substituent has been claimed to be as active as the prototype **1** at muscarinic M_1 receptors.[17–20] Clearly, a 4-fluoro substituent is not able to contribute to the activation of muscarinic M_1 receptors when the butynyl chain is replaced by an aryl unit, as in **7** and **17**.

In conclusion, it can be said that our design strategy to modify McN-A-343 structure did not produce muscarinic M_1 receptor agonists more potent than the prototype **1**. However, some analogues of **1** displayed functional selectivity for different muscarinic receptor subtypes. For example, the tertiary amino derivatives **3** and **7** were markedly selective agonists for muscarinic M_3 receptors, whereas trimethylammonium derivatives **14**, **16** and **18** were highly selective muscarinic M_2 receptor agonists. However, a most interesting finding was the observation that **8** was a full agonist at muscarinic M_3 receptors, while being devoid of activity at both muscarinic M_1 and M_2 receptors. Thus, it was selected for further pharmacological investigation to

assess its miotic properties in vivo. In fact, topically applied muscarinic agonists cause contraction of the iris sphincter muscle acting through pharmacologically defined muscarinic M_3 receptors.[29] The results obtained with **8** are reported in Table 2 together with those of **4** and the unsubstituted analogue **6**. It resulted that the unilateral instillation of 2% solutions (w/v), ca. 4.5 μM solutions, of compounds **4**, **6** and **8** significantly reduced the rabbit pupillary diameter (PD) in a time-dependent manner. The most effective compound of the set was **8**, which maximally reduced PD 60 min after instillation (about 40% versus basal value). After 3 h, PD returned to baseline (Table 2). This compound behaved like pilocarpine (1% solution, ca. 4.1 μM concentration), a standard miotic cholinergic agent,[30] that produced a potent and long-lasting miosis for 3 h with a maximal drop (about 54%) 60 min after treatment. This study demonstrated that **8**, although less potent than pilocarpine, was an effective miotic agent; after topical instillation, it can penetrate through the cornea producing a significant reduction of PD.

Since one objective of this study was to produce novel agents able to enhance cholinergic transmission, compounds **3**–**8** were also studied as inhibitors of AChE activity, owing to their evident structural kinship with covalent AChE inhibitors such as, for example, neostigmine. The results are shown in Table 1. It is evident that all the compounds tested were very weak AChE inhibitors, though bearing functional moieties supposed to favor the AChE inhibitory activity. The reason for such a behaviour and possible structural modifications are currently under study.

Experimental

Chemistry

Melting points (MP) were taken in glass capillary tubes on a Büchi 530 apparatus and are uncorrected. Electron impact (EI) mass and ^{1}H NMR spectra were recorded on VG 7070E and Varian VXR 300 instruments, respectively. Chemical shifts are reported in parts per million (ppm) relative to tetramethylsilane (TMS), and spin multiplicities are given as s (singlet), br s (broad singlet), or m (multiplet).

General procedure for the synthesis of 3, 5, 7, 9, 11, 13, 15 and 17. An excess of the appropriate phenylisocyanate (47.6 mmol) was added to a solution of *N*,*N*-dimethyl-3-hydroxybenzylamine[24,25] (4.76 mmol) or *N*,*N*-dimethyl-4-hydroxybenzylamine[24,25] (4.76 mmol) under a nitrogen stream, followed by the addition of NEt_3 (1.99 mL, 14.28 mmol). After standing 24 h at room temperature, the mixture was treated with water (20 mL) and extracted with chloroform (3×20 mL). The extracts were washed with water (3×10 mL) and dried over Na_2SO_4. Removal of the solvent gave a residue that was purified by flash chromatography. Eluting with MeOH:CH_2Cl_2 (1:1) gave the desired carbamate as the free base, which was transformed into the oxalate salt and recrystallized from 2-PrOH:EtOH to give the desired compound in 60–65% yield.

3-[(Dimethylamino)methyl]phenyl *N*-(3-chlorophenyl)carbamate oxalate (3). Mp 132–134 °C; ^{1}H NMR (DMSO-d_6) δ 2.65 (s, 6, NMe_2), 4.20 (s, 2, NCH_2), 4.60–6.10 (br s, 2, 2COOH), 6.80–7.20 (m, 8, Ar), 10.55 (s, 1, NH); EI MS *m*/*z* 304 (M^+). Calcd for $C_{18}H_{19}ClN_2O_6$: C, 54.76; H, 4.85; N, 7.10. Found: C, 54.80; H, 4.80; N, 7.01.

3-[(Dimethylamino)methyl]phenyl *N*-(phenyl)carbamate oxalate (5). Mp 164–165 °C; ^{1}H NMR (DMSO-d_6) δ 2.15 (s, 6, NMe_2), 4.15 (s, 2, NCH_2), 4.25–4.90 (br s, 2, 2COOH), 6.90–7.60 (m, 9, Ar), 10.30 (s, 1, NH); EI MS *m*/*z* 270 (M^+). Calcd for $C_{18}H_{20}N_2O_6$: C, 59.99; H, 5.59; N, 7.77. Found: C, 59.80; H, 5.62; N, 7.55.

3-[(Dimethylamino)methyl]phenyl *N*-(4-fluorophenyl)carbamate oxalate (7). Mp 197–198 °C; ^{1}H NMR (DMSO-d_6) δ 2.60 (s, 6, NMe_2), 4.20 (s, 2, NCH_2), 4.70–6.00 (br s, 2, 2COOH), 7.10–7.60 (m, 8, Ar), 10.40 (s, 1, NH); EI MS *m*/*z* 288 (M^+). Calcd for $C_{18}H_{19}FN_2O_6$: C, 57.14; H, 5.06; N, 7.40. Found: C, 57.20; H, 5.15; N, 7.15.

3-[(Dimethylamino)methyl]phenyl *N*-(4-methoxyphenyl)carbamate oxalate (9). Mp 173–175 °C; ^{1}H NMR (DMSO-d_6) δ 2.85 (s, 6, NMe_2), 3.80–4.40 (br s, 7, 2COOH, NCH_2, OCH_3), 7.15–7.80 (m, 8, Ar), 10.75 (s, 1, NH); EI MS *m*/*z* 300 (M^+). Calcd for $C_{19}H_{22}N_2O_7$: C, 58.46; H, 5.68; N, 7.18. Found: C, 58.50; H, 5.75; N, 7.01.

3-[(Dimethylamino)methyl]phenyl *N*-(4-nitrophenyl)carbamate oxalate (11). Mp 159–161 °C; ^{1}H NMR (DMSO-

Table 2. Effect of compounds **4**, **6** and **8** on ipsilateral pupillary diameter (PD, mm) of rabbit

No.	PD before treatment	PD at various time intervals after drug administration[a]				
		0.5 h	1 h	2 h	3 h	4 h
PL[b]	7.83 ± 0.16	4.16 ± 0.16[c]	3.66 ± 0.16[c]	5.16 ± 0.16[c]	6.16 ± 0.15[c]	7.83 ± 0.16
8	7.83 ± 0.28	5.50 ± 0.28[d]	4.83 ± 0.16[d]	6.33 ± 0.16[d]	7.0 ± 0.16	7.66 ± 0.33
4	7.66 ± 0.57	6.67 ± 0.26	6.50 ± 0.28[d]	7.83 ± 0.20	8.0 ± 0.16	7.33 ± 0.33
6	7.66 ± 0.57	6.33 ± 0.33	6.33 ± 0.33[d]	6.50 ± 0.28[e]	7.66 ± 0.33	7.66 ± 0.33

[a]Compounds were instilled as 2% solutions (w/v), whereas the reference compound pilocarpine was instilled as a 1% solution.
[b]PL, pilocarpine hydrochloride.
[c]$P < 0.01$ versus basal values and versus other treatments at the same time.
[d]$P < 0.01$ versus basal value.
[e]$P < 0.05$ versus basal value.

d_6) δ 2.80 (s, 6, NMe_2), 3.75–5.25 (br s, 4, 2COOH, NCH_2), 7.30–8.40 (m, 8, Ar), 11.05 (s, 1, NH); EI MS *m/z* 315 (M^+). Calcd for $C_{18}H_{19}N_3O_8$: C, 53.33; H, 4.72; N, 10.37. Found: C, 53.35; H, 4.90; N, 10.10.

3-[(Dimethylamino)methyl]phenyl *N*-(4-methylphenyl)-carbamate oxalate (13). Mp 142–143 °C; ^{1}H NMR (DMSO-d_6) δ 2.60 (s, 6, NMe_2), 3.15 (s, 3, *p*-CH_3), 3.40–3.95 (br s, 2, 2COOH), 4.15 (s, 2, NCH_2), 7.10–7.60 (m, 8, Ar), 10.20 (s, 1, NH); EI MS *m/z* 284 (M^+). Calcd for $C_{19}H_{22}N_2O_6$: C, 60.95; H, 5.92; N, 7.48. Found: 61.02; H, 5.80; N, 7.25.

4-[(Dimethylamino)methyl]phenyl *N*-(3-chlorophenyl)carbamate oxalate (15). Mp 164–165 °C; ^{1}H NMR (DMSO-d_6) δ 2.40 (s, 6, NMe_2), 3.50–4.45 (br s, 4, 2COOH, NCH_2), 7.0–7.50 (m, 8, Ar), 10.65 (s, 1, NH); EI MS *m/z* 304 (M^+). Calcd for $C_{18}H_{19}ClN_2O_7$: C, 54.76; H, 4.85; N, 7.10. Found: C, 54.85; H, 4.82; N, 7.05.

4-[(Dimethylamino)methyl]phenyl *N*-(4-fluorophenyl)carbamate oxalate (17). Mp 175–176 °C; ^{1}H NMR (DMSO-d_6) δ 2.42 (s, 6, NMe_2), 3.45–4.30 (br s, 4, 2COOH, NCH_2), 7.01–7.50 (m, 8, Ar), 10.45 (s, 1, NH); EI MS *m/z* 288 (M^+). Calcd for $C_{18}H_{19}FN_2O_6$: C, 57.14; H, 5.06; N, 7.40. Found: C, 57.23; H, 5.11; N, 7.12.

General procedure for the synthesis of methiodides 4, 6, 8, 10, 12, 14, 16 and 18. A solution of carbamate **3**, **5**, **7**, **9**, **11**, **13**, **15** or **17** as the free base (1.22 mmol) in acetone (5 mL) was treated with methyl iodide (12.2 mmol). After standing overnight at room temperature, the solvent was removed and the residue was triturated with ether to give a solid, which was filtered and recrystallized from acetone:EtOAc to give the desired compound in 55–60% yield.

(3-{[(3-Chloroanilino)carbonyl]oxy}benzyl)(trimethyl)ammonium iodide (4). Mp 137–138 °C; ^{1}H NMR (DMSO-d_6) δ 3.0 (s, 9, NMe_3), 4.40 (s, 2, NCH_2), 6.80–7.70 (m, 8, Ar), 9.75 (s, 1, NH). Calcd for $C_{17}H_{20}ClIN_2O_2$: C, 45.71; H, 4.51; N, 6.27. Found: C, 45.60; H, 4.55; N, 6.20.

{3-[(Anilinocarbonyl)oxy]benzyl}(trimethyl)ammonium iodide (6). Mp 201–202 °C; ^{1}H NMR (DMSO-d_6) δ 3.0 (s, 9, NMe_3), 4.45 (s, 2, NCH_2), 6.85–7.70 (m, 9, Ar), 10.35 (s, 1, NH). Calcd for $C_{17}H_{21}IN_2O_2$: C, 49.53; H, 5.13; N, 6.79. Found: C, 49.40; H, 5.22; N, 6.68.

(3-{[(4-Fluoroanilino)carbonyl]oxy}benzyl)(trimethyl)ammonium iodide (8). Mp 135–137 °C; ^{1}H NMR (DMSO-d_6) δ 3.0 (s, 9, NMe_3), 4.55 (s, 2, NCH_2), 6.60–7.70 (m, 8, Ar), 10.35 (s, 1, NH). Calcd for $C_{17}H_{20}FIN_2O_2$: C, 47.46; H, 4.69; N, 6.51. Found: C, 47.35; H, 4.80; N, 6.35.

(3-{[(4-Methoxyanilino)carbonyl]oxy}benzyl)(trimethyl)ammonium iodide (10). Mp 171–173 °C; ^{1}H NMR (DMSO-d_6) δ 3.0 (s, 9, NMe_3), 3.75 (s, 3, OCH_3), 4.55 (s, 2, NCH_2), 6.40–7.60 (m, 8, Ar), 10.50 (s, 1, NH). Calcd for $C_{18}H_{23}IN_2O_3$: C, 48.88; H, 5.24; N, 6.33. Found: C, 48.90; H, 5.30; N, 6.22.

(3-{[(4-Nitroanilino)carbonyl]oxy}benzyl)(trimethyl)ammonium iodide (12). Mp 211–213 °C; ^{1}H NMR (DMSO-d_6) δ 2.95 (s, 9, NMe_3), 4.35 (s, 2, NCH_2), 6.50–8.05 (m, 8, Ar), 10.55 (s, 1, NH). Calcd for $C_{17}H_{20}IN_3O_4$: C, 44.65; H, 4.41; N, 9.19. Found: C, 44.70; H, 4.08; N, 8.95.

(3-{[(4-Methylanilino)carbonyl]oxy}benzyl)(trimethyl)ammonium iodide (14). Mp 176–177 °C; ^{1}H NMR (DMSO-d_6) δ 2.10 (s, 3, *p*-CH_3), 3.0 (s, 9, NMe_3), 4.40 (s, 2, NCH_2), 6.40–7.15 (m, 8, Ar), 9.80 (s, 1, NH). Calcd for $C_{18}H_{23}IN_2O_2$: C, 50.72; H, 5.44; N, 6.57. Found: C, 50.55; H, 5.70; N, 6.35.

(4-{[(3-Chloroanilino)carbonyl]oxy}benzyl)(trimethyl)ammonium iodide (16). Mp 207–208 °C; ^{1}H NMR (DMSO-d_6) δ 3.10 (s, 9, NMe_3), 4.55 (s, 2, NCH_2), 7.10–7.70 (m, 8, Ar), 10.55 (s, 1, NH). Calcd for $C_{17}H_{20}ClIN_2O_2$: C, 45.71; H, 4.51; N, 6.27. Found: C, 45.66; H, 4.60; N, 6.15.

(4-{[(3-Fluoroanilino)carbonyl]oxy}benzyl)(trimethyl)ammonium iodide (18). Mp 179–181 °C; ^{1}H NMR (DMSO-d_6) δ 3.25 (s, 9, NMe_3), 4.45 (s, 2, NCH_2), 7.20–7.80 (m, 8, Ar), 10.35 (s, 1, NH). Calcd for $C_{17}H_{20}FIN_2O_2$: C, 47.46; H, 4.69; N, 6.51. Found: C, 47.27; H, 4.70; N, 6.30.

Pharmacology

General considerations. Male guinea pigs (200–300 g) and male New Zealand white rabbits (3.0–3.5 kg) were killed by cervical dislocation. The organs required were set up rapidly under 1 g of tension in 20 mL organ baths containing physiological salt solution (PSS) maintained at an appropriate temperature (see below) and aerated with 5% CO_2–95% O_2. Two concentration–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, a new concentration–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 pEC_{50}, which is the $-\log EC_{50}$, the concentration of agonist required to produce 50% of the maximum contraction. When studying antagonists, these were allowed to equilibrate with the tissue for 30 min, then a new concentration–response curve to the agonist was obtained. 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.

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, of the following composition (mM): NaCl, 118; $NaHCO_3$, 23.8; KCl, 4.7; $MgSO_47H_2O$, 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 concentration-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 (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 concentration–response curve to APE was constructed.

Rabbit stimulated vas deferens. This preparation was set up according to Eltze.[27] Vasa deferentia were carefully dissected free of surrounding tissue and were divided into four segments, two prostatic portions of 1 cm and two epididymal portions of approximately 1.5 cm length. The four segments were mounted in PSS with the following composition (mM): NaCl, 118.4; KCl, 4.7; $CaCl_2$, 2.52; $MgCl_2$, 0.6; KH_2PO_4, 1.18; $NaHCO_3$, 25; glucose, 11.1. Yohimbine (1 μM) and tripitramine (0.01 μM) were included to block α_2-adrenoceptors and muscarinic M_2 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 McN-A-343 was constructed.

Pupillary diameter (PD) measurements. Male New Zealand albino rabbits (1.8–2.2 kg) with no signs of ocular inflammation or gross abnormality were used. Animal procedures followed the guidelines of the Animal Care and Use Committee of the University of Bologna and conformed to the Association for Research in Vision and Ophthalmology resolution on the use of animals in research.

Conscious rabbits were placed in restraint boxes to which they had been accustomed, with unrestricted head or eye movements. PD (mm) was measured with a Castroviejo caliper under constant light 10 min before and 0.5, 1, 2, and 4 h after treatment. PD are expressed as mean ± SE in mm. Topically administered compounds were dissolved in phosphate buffer solution (pH 7.4; vehicle) and 50 μL/eye were instilled. The reference compound was pilocarpine hydrochloride.

Antiacetylcholinesterase activity. AChE derived from human erythrocytes was employed. Anticholinesterase activity was assessed by measuring the hydrolysis of acetylthiocholine and the subsequent reaction of thiocholine with 4,4-dithiopyridine to form 4-thiopyridine.[31] The results are expressed as IC_{50} values, which represent the concentration required to inhibit enzyme activity by 50%.

Statistical analysis

In functional studies responses were expressed as percentage of the maximal contraction observed in the agonist concentration–response curve taken as a control. The agonist concentration–response curves were analyzed by pharmacological computer programs.[32] pEC_{50} values are expressed as the mean ± SE. Student's *t*-test was used to assess the statistical significance of the difference between two means. pK_b values were calculated according to Arunlakshana and Schild[28] by the formula: $pK_b = \log([B]/(DR-1))$, where B was the antagonist concentration (100 μM) and the dose ratio (DR) was the ratio of the potency of the agonist (EC_{50}) in the presence of the antagonist and in its absence.

The effect on ipsilateral pupillary diameter is expressed as the mean ± SE in mm of three different experiments. Statistical comparisons were made by analysis of variance (ANOVA) for repeated measures and post hoc Dunnett's multiple comparison test with differences of $P < 0.05$ being considered significant (GraphPAD Software, San Diego, CA, USA).

Molecular modeling

Three-dimensional models of compounds **1** and **4** were built and studied by means of the molecular modeling package SYBYL Version 6.4[33] running on a Silicon Graphics Indigo2 workstation. The molecules were built by assembling fragments from the SYBYL standard library, and the conformational search on the rotatable bonds was performed by means of the RANDOMSEARCH procedure. The conformations generated by the search were grouped into families, and from each family a representative one was selected. The selected conformers were then energy optimized using the AM1[34] Hamiltonian and from them the ones to be used in the fitting procedure were selected. The points used for fitting the molecules were the carbons 3 and 5 of the phenyl ring, the C and O atoms of the carbonyl group and the nitrogen of the cationic head. Molecular electrostatic potentials were calculated for the fragments corresponding to the main skeleton of the molecules (phenyl substituents and methylammonium group were eliminated). The calculation was performed at the semiempirical level employing the AM1 wavefunctions; it has been shown that the electrostatic potential based on AM1 wavefunction correlates sufficiently well with ab initio results.[35]

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