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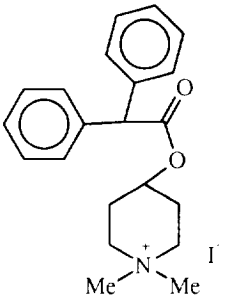
DESIGN, SYNTHESIS AND BIOLOGICAL ACTIVITY OF SOME 4-DAMP-RELATED COMPOUNDS[1]

Vincenzo Tumiatti, Santi Spampinato,[&] Maurizio Recanatini, Anna Minarini
Carlo Melchiorre,* Alberto Chiarini and Roberta Budriesi

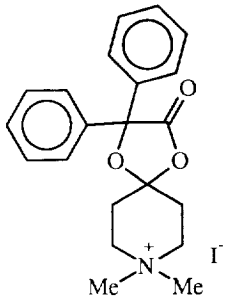
Departments of Pharmaceutical Sciences and [&]Pharmacology,
University of Bologna, Via Belmeloro 6, I-40126 Bologna, Italy

Abstract. Two constrained analogues (**4a** and **4b**) of both 4-DAMP (**1**) and spiro-DAMP (**2**) were designed, and their biological profiles at muscarinic receptor subtypes were assessed by functional and binding assays. It turned out that these constrained analogues display a modest, if any, M_2/M_3 selectivity ratio. However, most interestingly, they proved to be highly selective for muscarinic M_1 receptors.

The identification of multiple muscarinic receptor subtypes has stimulated the search for ligands with selectivity for a given receptor subtype.[2-4] Five different subtypes (m_1-m_5) have been identified so far by molecular cloning. Muscarinic receptors that have been characterized pharmacologically and classified as M_1-M_4, appear to correspond to cloned m_1-m_4 receptors. At present, little information is available about the nature and the cellular location of the m_5 subtype.



1 (4-DAMP)



2 (spiro-DAMP)

Achievement of selectivity is of paramount importance not only for receptor subtype characterization but also for the development of therapeutically useful muscarinic agonists and antagonists.[3,5] For instance, muscarinic M_1 receptor agonists and muscarinic M_2 receptor antagonists may be useful to enhance cognitive function whereas muscarinic M_3 receptor antagonists have potential for the treatment of airway obstruction. Furthermore, muscarinic M_2 receptor antagonists may have application in the treatment of bradycardic

disorders. Presently, several antagonists which bind selectively to the pharmacologically characterized muscarinic receptors are available. For example, pirenzepine has high affinity for muscarinic M_1 receptors which are mainly located in the central nervous system and peripheral ganglia;[6] polymethylene tetraamines, exemplified by methoctramine and tripitramine,[7-9] display high affinity for muscarinic M_2 receptors of cardiac cells, whereas 4-DAMP (**1**) shows high affinity for muscarinic M_3 receptors located in smooth muscle and exocrine glands.[10]

In an attempt to define the structural elements which confer muscarinic receptor subtypes selectivity, we modified the structure of **1** by incorporating its acyl portion into an oxodioxolane ring affording spiro-DAMP (**2**).[11] The finding that there are two distinct binding modes (Figure 1) shared by **2** and other related muscarinic antagonists,[12] prompted us to further constrain spiro-DAMP structure, in order to verify if the low energy conformations of **2** correspond to the active conformations at different muscarinic receptors subtypes. Thus, we describe here the synthesis and the pharmacological profile of **4a** and **4b**, designed by including an ethyl bridge between positions 2 and 6 of the piperidine ring of **2**.

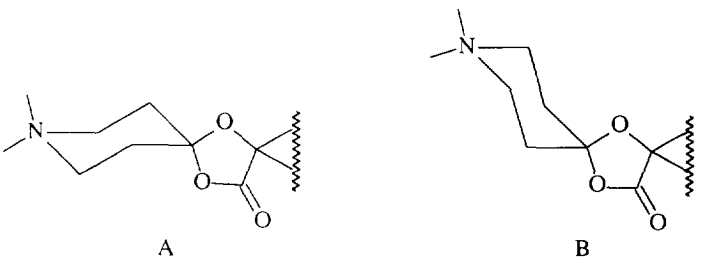


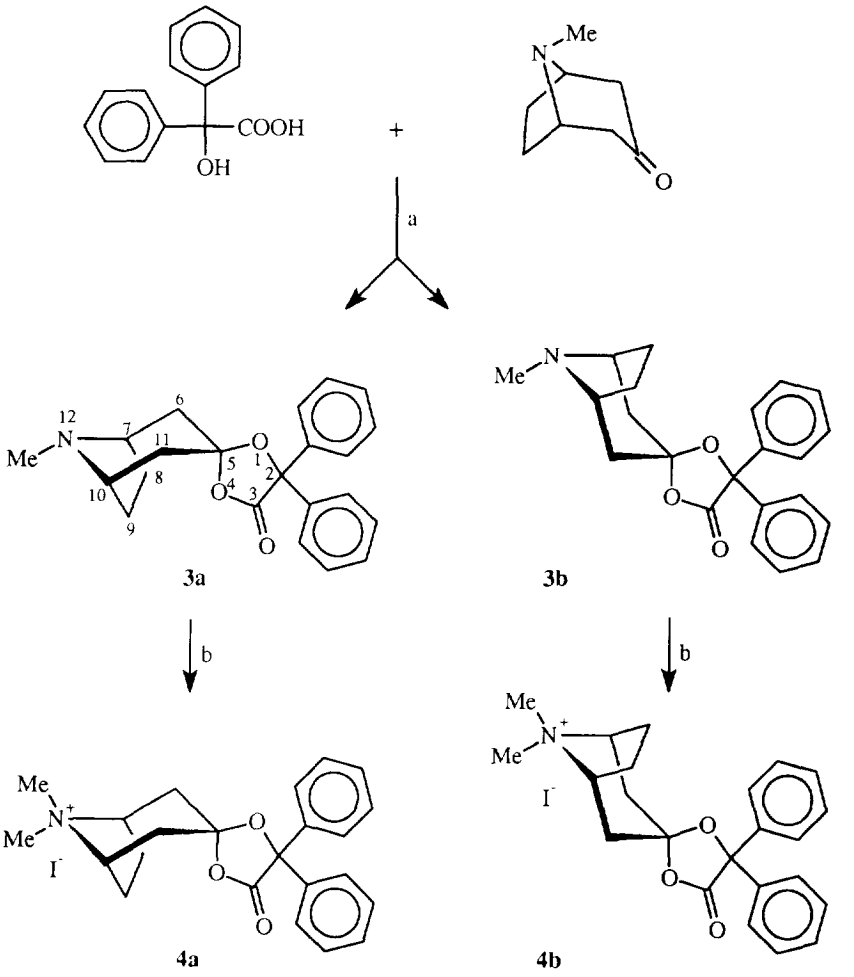
Figure 1. Representation of the two flipped "chair" conformations of the piperidine ring of **2** corresponding to low energy conformations affording two possible binding modes to muscarinic receptors.

The compounds were synthesized by standard procedures (Scheme 1) and were characterized by ^{1}H NMR, ^{13}C NMR and elemental analysis.[13] The two spiro compounds **3a** and **3b**[14] were synthesized through ketalization of 4-tropinone with benzilic acid in the presence of *p*-toluenesulfonic acid as catalyst. The two isomers were separated by medium pressure chromatography and then transformed into quaternary salts with methyl iodide to give **4a**[15] and **4b**.[16]

The structures of isomers **3a** and **3b** were assigned by COSY, DEPT, heteronuclear correlation (HETCOR) and NOE experiments. ^{1}H NMR spectra of **3a** and **3b** are almost identical, except the four ethylenic bridge protons signal (two 8-H and two 9-H) which is a broad singlet at δ 1.98 for **3a** while is split into two multiplets at δ 2.08-2.02 (2H) and 2.21-2.17 (2H) for **3b**. 2D ^{1}H-^{13}C HETCOR spectra confirmed the assignments of the four protons made by ^{1}H NMR spectra of the two isomers. In fact, in **3a** the carbon peak at δ 25.38 is coupled with one broad singlet peak at δ 1.98, whereas in **3b** the carbon peak at δ 25.58 is coupled with two multiplets at δ 2.08-2.02 and 2.21-2.17. The cross peak connectivities in the 2D ^{1}H-^{1}H correlation COSY spectrum of **3b** confirmed that there is a geminal coupling between the hydrogens at positions 8 and 9 in multiplets at δ 2.08-2.02 (2H) and 2.21-2.17 (2H). Similarly, it was also verified the coupling of hydrogens at positions 8 and 9 of both **3a** and **3b** with the hydrogens at positions 7 and 10 but not with those at positions

6 and 11. Furthermore, ^{13}C DEPT spectra showed that the signal at δ 25.38 in **3a** and δ 25.58 in **3b** is due to a methylene carbon atom. The irradiation of the more deshielded multiplet signal at δ 2.21-2.17 (2H) produced by the two hydrogen atoms linked to C8 and C9 in **3b** resulted in a positive NOE signal corresponding to aromatic hydrogen atoms, whereas the irradiation of the broad singlet peak at δ 1.98 (4H) in **3a** did not show any NOE effect in the aromatic region of the spectrum. The structure of **3a** was also confirmed by X-ray crystallography.[17]

Scheme 1



(a) toluene-4-sulfonic acid, benzene, reflux, 36 h, silica gel; (b) CH_3I, CH_2Cl_2, 5 °C, 12 h.

Table I. Affinity Values for Inhibiting [^{3}H]Pirenzepine Binding in Rat Cortex Homogenates (M_1), and [^{3}H]N-Methylscopolamine Binding in Rat Heart (M_2), Rat Submaxillary Gland (M_3) and NG 108–15 Cell (M_4) Homogenates

	pK_i[a]				
Compound	M_1	M_2	M_3	M_4	Affinity Profile[b]
4–DAMP (**1**)	9.23±0.09	8.40±0.10	8.94±0.07	9.29±0.11	$M_4 = M_1 = M_3 \geq M_2$
spiro-DAMP (**2**)	8.30±0.13	8.56±0.18	8.53±0.08	7.95±0.09	$M_2 = M_3 = M_1 = M_4$
Pirenzepine[c]	8.19±0.08	6.10±0.09	6.76±0.10	7.46±0.17	$M_1 > M_4 \geq M_3 \geq M_2$
4a	9.40±0.21	7.48±0.07	7.98±0.07	8.33±0.11	$M_1 > M_4 = M_3 \geq M_2$
4b	9.10±0.15	7.92±0.08	7.30±0.04	8.36±0.12	$M_1 > M_4 = M_2 \geq M_3$

[a] Values are the mean ± SE of at least three separate experiments performed in triplicate. All Hill number (nH) were not significantly different from unity ($p > 0.05$). Equilibrium dissociation constants (K_i) were derived using the Cheng-Prusoff equation.[20] [b] Differences in antagonistic affinities for muscarinic receptor subtypes by a factor of ≤3, >3÷5, and >5 are indicated by =, ≥, and >, respectively. [c] Data from ref 18.

The muscarinic receptor subtype selectivity was assessed by employing receptor binding assays as reported previously.[9, 18] [^{3}H]N-Methylscopolamine ([^{3}H]NMS; specific activity 79.5 Ci/mmol) was used to label M_2, M_3 and M_4 muscarinic receptors binding sites of rat heart (K_d 0.32±0.042 nM; B_{max} 77.8±15.3 fmol/mg of protein) and submaxillary gland (K_d 0.485±0.035 nM; B_{max} 1102±85 fmol/mg of protein), and NG 108-15 cell homogenates (K_d 0.544±0.032 nM; B_{max} 19±4 fmol/mg of protein), respectively. [^{3}H]Pirenzepine (specific activity 86.2 Ci/mmol) was the tracer to label M_1 muscarinic receptors binding sites of the rat cerebral cortex (K_d 2.15±0.19 nM; B_{max} 49±13 pmol/mg of protein). Binding affinities were expressed as pK_i values. 4-DAMP (**1**), spiro-DAMP (**2**) and pirenzepine were used as the standard compounds.

Functional activity at muscarinic receptor subtypes was determined by the use of the 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 ileum longitudinal muscle. These methods have been described in detail earlier.[12, 19] The agonist was arecoline propargyl ester (APE).

An analysis of results shown in Table I reveals that both **4a** and **4b** show a remarkable M_1/M_4 selectivity ratio, while, as regards the M_2/M_3 preference, **4a** is M_3-selective and **4b** is M_2-selective. These results point out the substantial effect caused by the complete freezing of the structure of **2** into two rigid conformational mimics. We observe that, with respect to **2** which is equipotent towards the four subtypes, **4a** and **4b** are less active towards muscarinic M_2 and M_3 receptors, slightly more active towards the M_4 subtype and definitely more active towards M_1 receptors. The functional data of Table II do not reveal any preference for **4a** and **4b** for one of the M_2 and M_3 subtypes, but confirm the drop of activity with respect to **2** observed in binding assays. This loss of affinity is smaller for **4a** than for **4b**, suggesting that, of the two possible binding modes of **2** represented by the rigid analogues, the one mimicked by **4b** is less favoured. Considering the affinity profile of **4a** and **4b** as evidenced by the binding data (Table I), it is now possible to associate each of the two

antimuscarinic pharmacophoric structures we proposed in a previous work[12] with each of the two muscarinic

Table II. Antagonist Affinities of 4-DAMP and Related Analogues at Muscarinic Receptor Subtypes in Guinea Pig Left Atria (M_2) and Ileum (M_3)

	pA_2[a]	
Compound	M_2	M_3
4-DAMP (**1**)	8.57 ± 0.13	9.17 ± 0.15
spiro-DAMP (**2**)	8.91 ± 0.05	9.03 ± 0.10
4a	8.38 ± 0.01	8.44 ± 0.03
4b	7.76 ± 0.02	7.68 ± 0.05

[a] pA_2 values were calculated by Schild analysis[21] using at least three different antagonist concentrations, and each concentration was tested from four to six times. The slopes of the Schild plots were not significantly different from unity ($P > 0.05$).

M_2 and M_3 receptor subtypes. The two pharmacophores were based on low energy conformations of **2** (Fig. 1) and allowed to rationalize the SAR of a series of antagonists. From freezing the conformations as in **4a** and **4b**, it results that conformation A (e.g. **4a**) is the preferred one at the M_3 subtype, while conformation B (e.g. **4b**) is associated to the M_2 subtype. Functional data (Table II) are not significantly different, indicating only weak preferences for one of the M_2 or M_3 subtypes. The lower affinity of **4a** and **4b** with respect to **2** in both binding and functional assays might be due to some unfavourable steric effect exerted by the ethylene bridge and/or to some negative entropic effect associated with the loss of conformational freedom caused by the structural constraints. The most striking feature of **4a** and **4b** is their remarkable selectivity for muscarinic M_1 receptors. Their affinity profile is comparable to that of pirenzepine which is classified as a highly M_1-selective antagonist and resembles that of spirotramine,[22] a recently reported tetraamine whose affinity profile is $M_1 >> M_4 = M_2 = M_3$. The fact that **4a** and **4b** as well as spirotramine carry similar spiro moieties lends support to the hypothesis that such groups play an "address" role in conferring M_1 subtype selectivity.

In conclusion, we designed, synthesized and tested two new conformationally blocked derivatives of both 4-DAMP (**1**) and spiro-DAMP (**2**) that appear to be valuable tools in the study of structure-selectivity relationships of muscarinic antagonists. In fact, **4a** and **4b** proved to be highly selective for muscarinic M_1 receptors, representing rigid templates useful for the identification of the spatial characteristics of the M_1 ligand-binding site.

Acknowledgment

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13. ^{1}H, ^{13}C and 2D NMR experiments were recorded on a Varian VXR 300 instrument. Chemical shifts are reported in parts per million (ppm) relative to tetramethylsilane (TMS), and spin multiplicities are given as s (singlet), d (doublet), t (triplet), and m (multiplet). The elemental compositions of the compounds agreed within ± 0.4% of the calculated value. Medium pressure chromatography was performed on silica gel column (Kieselgel 60 H, Merck) by a Jobin Yvon Chromatospac Prep apparatus eluting with chloroform-petroleum ether-toluene-methanol-triethylamine (5.5:2:2:0.5:0.05).
14. A mixture of benzilic acid (4.5 g, 0.02 mol), tropinone (1.39 g, 0.01 mol) and *p*-toluenesulfonic acid (6 g, 0.03 mol) in anhydrous benzene (50 ml) was heated under reflux and the water formed continuously removed overnight. The cooled mixture (5 °C) was made basic with a 10% Na_2CO_3 solution; the organic layer was removed and the aqueous phase was extracted with benzene-ether (1:1) (3 x 30 ml). The organic extracts were combined, washed with a saturated NaCl solution (2 x 15 ml) and dried. Removal of the solvent gave 3.4 g of crude product that was purified by chromatography to give 1.49 g of **3a** and 0.27 g of **3b**.
endo-12-Methyl-3-oxo-2,2-diphenyl-spiro[8-azabicyclo[3.2.1]octane-3,2'-[1,3]dioxolane] (**3a**): ^{1}H NMR ($CDCl_3$) δ 7.58-7.45 (4H, m, Ar), 7.41-7.25 (6H, m, Ar), 3.25-3.12 (2H, m, 7- and 10-H), 2.34 (3H, s, NCH_3), 2.29 (2H, d, 6- and 11-H, J = 2.9 Hz), 1.98 (4H, br s, 8- and 9-H), 1.76 (2H, d, 6- and 11-H, J = 13.8 Hz).^{13}C NMR($CDCl_3$) δ 171.68 (CO), 139.98, 128.37, 128.27, 126.20 (Ar), 109.45 (C5), 82.40 (C2), 59.65 (C7,C10), 42.93 (C6,C11), 38.96 (NCH_3), 25.38 (C8,C9); MS *m/z* (rel intensity) 349 ([M]$^+$, 51), 216 (100), 82 (82).
exo-12-Methyl-3-oxo-2,2-diphenyl-spiro[8-azabicyclo[3.2.1]octane-3,2'-[1,3]dioxolane] (**3b**): ^{1}H NMR ($CDCl_3$) δ 7.58-7.51 (4H, m, Ar), 7.41-7.30 (6H, m, Ar), 3.25-3.17 (2H, m, 7- and 10-H), 2.33 (3H, s, NCH_3), 2.29-2.25 (2H, dd, 6- and 11-H, J = 13.6 Hz and J = 2.7 Hz), 2.21-2.17 (2H, m, 8- and 9-H), 2.08-2.02 (2H, m, 8- and 9-H), 1.82 (2H, d, 6- and 11-H, J = 13.7 Hz).^{13}C NMR($CDCl_3$) δ 170.79 (CO), 140.51, 128.89, 128.39, 128.17, 128.09, 126.00 (Ar), 110.05 (C5), 84.12 (C2), 59.56 (C7,C10), 40.88 (C6,C11), 38.55 (NCH_3), 25.58 (C8,C9); MS *m/z* (rel intensity) 349 ([M]$^+$, 17), 216 (46), 82 (100).
15. Iodomethane (4.9 ml, 64 mmol) was added to a solution of **3a** (1.11 g, 3.2 mmol) in dichloromethane (20 ml). The resulting solution was left overnight in a refrigerator; the precipitated crystals were collected and washed with ether to give *endo*-12,12-dimethyl-3-oxo-2,2-diphenyl-spiro[8-azoniabicyclo[3.2.1]octane-3,2'-[1,3]dioxolane] iodide (**4a**): 0.98 g, mp 239-240 °C (from acetone/ether). ^{1}H NMR (DMSO-d_6) δ 7.58-7.35 (10H, m), 4.05-3.95 (2H, m), 3.25 (3H, s), 3.10 (3H, s), 2.79 (2H, d, J = 18 Hz), 2.38-2.12 (6H, m). Anal. ($C_{23}H_{26}INO_3$) C, H, N.
16. Iodomethane (0.47 ml, 7.6 mmol) was added to a solution of **3b** (0.13 g, 0.37 mmol) in CH_2Cl_2 (10 ml). The resulting solution was left overnight in a refrigerator; the precipitated crystals were collected and washed with ether to give *exo*-12,12-dimethyl-3-oxo-2,2-diphenyl-spiro[8-azoniabicyclo[3.2.1]octane-3,2'-[1,3]dioxolane] iodide (**4b**): 0.13 g, mp 250-251 °C (from acetone/ether). ^{1}H NMR (DMSO-d_6) δ 7.48-7.35 (10H, m), 4.05-3.95 (2H, m), 3.31 (3H, s), 3.09 (3H, s), 2.76 (2H, d, J = 14 Hz), 2.40 (4H, br s), 2.11 (2H, d, J = 16 Hz). Anal. ($C_{23}H_{26}INO_3$) C, H, N.
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