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Muscarinic antagonists with multiple stereocenters: Synthesis, affinity profile and functional activity of isomeric 1-methyl-2-(2,2-alkylaryl-1,3-oxathiolan-5-yl)pyrrolidine sulfoxide derivatives

Silvia Dei,[a,*] Cristina Bellucci,[a] Michela Buccioni,[b] Marta Ferraroni,[c] Luca Guandalini,[a] Dina Manetti,[a] Gabriella Marucci,[b] Rosanna Matucci,[d] Marta Nesi,[d] Maria Novella Romanelli,[a] Serena Scapecchi[a] and Elisabetta Teodori[a]

[a]*Laboratorio di Progettazione, Sintesi e Studio di Eterocicli Biologicamente Attivi (HeteroBioLab), Dipartimento di Scienze Farmaceutiche, Università di Firenze, Via U. Schiff 6, 50019 Sesto Fiorentino (FI), Italy*
[b]*Dipartimento di Scienze Chimiche, Università di Camerino, Via S. Agostino 1, 62032 Camerino (MC), Italy*
[c]*Dipartimento di Chimica, Università di Firenze, Via della Lastruccia 5, 50019 Sesto Fiorentino (FI), Italy*
[d]*Dipartimento di Farmacologia Preclinica e Clinica, Università di Firenze, Viale G. Pieraccini 6, 50139 Firenze, Italy*

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Abstract—Completing a long-lasting research on 1,3-oxathiolane muscarinic ligands, we have synthesized a set of isomeric 1-methyl-2-(2,2-alkylaryl-1,3-oxathiolan-5-yl)pyrrolidine 3-sulfoxide derivatives, containing three or four stereogenic centers. In general the compounds are very potent antagonists even if, unlike the corresponding agonists, they show modest subtype selectivity.

1. Introduction

The five muscarinic acetylcholine receptors (m1–m5) are found in the central and peripheral nervous system and mediate a large number of vital functions.[1] Four of these subtypes have also been characterized from a pharmacological point of view (M_1–M_4).[2] As a consequence, muscarinic drugs have potential for therapeutic use in several pathological states.[3,4] Fundamental information on muscarinic receptor subtypes has been collected from a variety of more or less selective natural and synthetic ligands,[5] transgenic mice lacking genes encoding each of the muscarinic receptor subtypes,[6] and muscarinic toxins from snake venoms.[7] Unfortunately, several ligands (mainly antagonists) show some subtype selectivity, but none displays high selectivity for one subtype to the relative exclusion of all others[2–4]; this fact, together with the evidence that the muscarinic subtypes identified so far are widely distributed in the body, limits the number of muscarinic drugs that have been introduced in therapy.[3] Apparently, this is due to the high sequence conservation within the orthosteric domain across all five muscarinic subtypes.[8] This situation could be dramatically improved if fundamental aspects of each subtype such as the structure of the recognition site, the mechanisms of activation, and G-protein coupling were clarified, but until now the detection of the atomic structure of protein G-coupled receptors remains a difficult task and homology models present obvious limits.

Studies with muscarinic antagonists have been the first and main source of evidence on the existence of multiple muscarinic receptor subtypes. In fact, several antagonists do show some subtype selectivity,[9] even if none displays high selectivity for one subtype to the relative exclusion of all others. Also the therapeutic potential of muscarinic antagonists, which has been exploited for many decades for heart, eye, urinary bladder, circulation and airway diseases, is now of growing interest. In fact, new possible fields for mAChR antagonists in clinical therapy are emerging, for instance in tissue

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* Corresponding author. Tel.: +39 055 4573689; fax: +39 055 4573780; e-mail: silvia.dei@unifi.it

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regeneration and carcinogenesis by non-neuronal cholinergic mechanism.[10] Therefore, the discovery of new potent and selective muscarinic antagonists remains the main instrument to study this class of receptors and to find new molecules for therapeutic applications.

For several years we have been working on cholinergic ligands, both agonists and antagonists, characterized by a 1,3-oxathiolane cycle, and on the corresponding sulfoxides.[11,12] More recently, we have designed and studied new compounds of the same class, whose structure has been sterically complicated[13–16] by insertion of the cationic chain in a pyrrolidine ring. We reasoned that chiral compounds bearing multiple stereocenters could selectively interact with the highly conserved recognition sites of muscarinic receptor subtypes. Recently, we reported the synthesis and pharmacological profile of the antagonist derivatives of general structure **A**[16] (Chart 1). This new series of compounds show very high affinity and activity on muscarinic subtypes but, despite the presence of two or three stereocenters, subtype selectivity remains poor. Therefore, building on our hypothesis, we decided to insert a further stereogenic center, by oxidation to the corresponding sulfoxides. This approach, when applied to the analog series of agonist compounds, has led to the discovery of a potent M_2 partial agonist.[15] Accordingly, we designed and synthesized, by oxidation of the sulfur atom of the 1,3-oxathiolane-2-pyrrolidine derivatives **A** (**1**–**3**, Scheme 1), the corresponding sulfoxides **4**–**15** reported in Chart 1, that carry three or four stereogenic centers. Here we report the synthesis and pharmacological characterization of those compounds in comparison with the parent compound **B**, that was one of the most potent among those studied previously.[12]

2. Chemistry

The starting material for the synthesis of sulfoxides **4**–**15** was oxathiolanes **1**–**3** that, in turn, were obtained from commercially available (*R*)- and (*S*)-prolinol, as reported previously.[16] The following sulfoxidation reaction was performed on all the isomers, as shown in Scheme 1 for the 2*S* series; obviously, the reactions proceed in the same way when working with the 2*R* series. Each isomer was oxidized with H_2O_2 to give the expected mixture of two diastereomeric sulfoxides that were separated by column chromatography, yielding compounds **4**–**9**. Under the reaction conditions here reported, no formation of the corresponding sulfones was observed. The *N*-methyl derivatives were then transformed into the corresponding dimethyl pyrrolidinium iodides **10**–**15** with CH_3I.

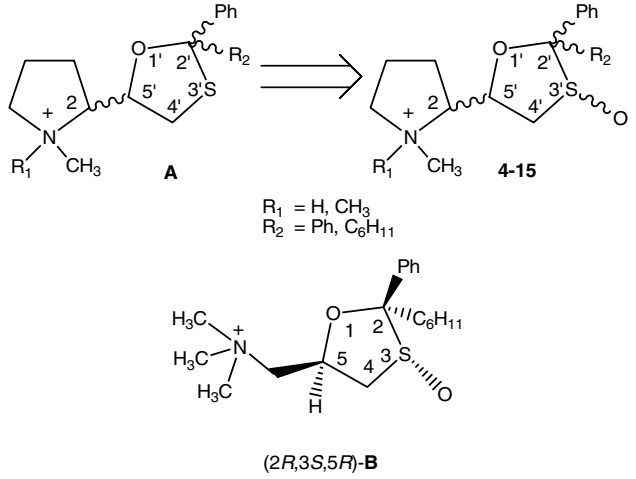
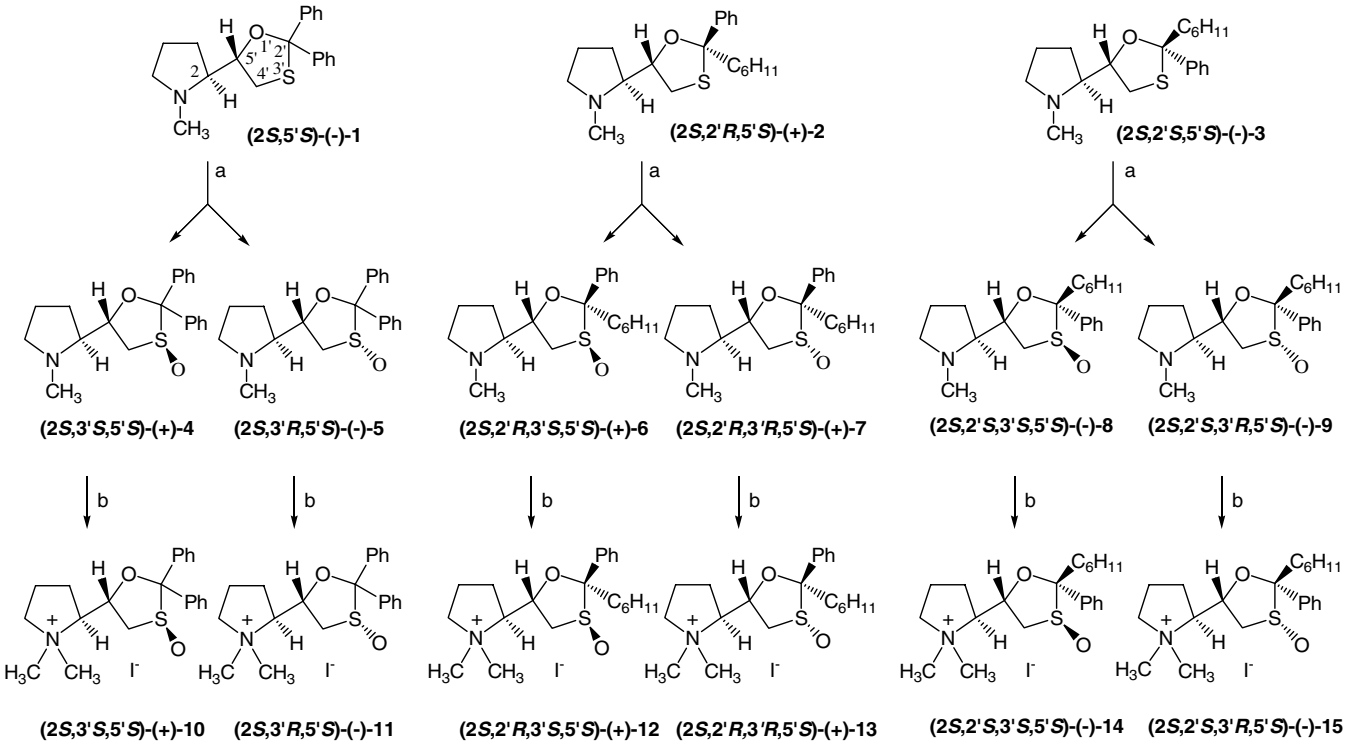


Chart 1. Lead and newly designed compounds.

The relative configuration of the new stereocenter in position 3′ was established by comparison of the properties of the diastereomeric couples **4/5**, **6/7**, as well as **8/9**, with those of the corresponding 1,3-oxathiolane 3-sulfoxide agonists[15]; in each case, analysis of the 1D and 2D ^1H and ^{13}C NMR spectra of the first-eluted isomer showed that the peaks relative to H5′ (0.5–0.9 ppm) and C5′ (2–4.5 ppm) were deshielded with respect to the corresponding diastereoisomers. As confirmed by X-ray crystallography analysis,[15] the more deshielded protons are in position *cis* with respect to the sulfoxide moiety, as already seen in a preceding work.[12] On this basis, knowing the absolute configuration of the other stereocenters, it was possible to attribute the absolute configuration of the optical isomers, as shown in Scheme 1. This attribution was eventually confirmed by the X-ray crystallography of (+)-**4** oxalate that, as shown in Figure 1, is indeed 2*S*,3′*S*,5′*S*.

3. Pharmacology

Muscarinic receptor affinity was evaluated in CHO cells expressing the five human muscarinic subtypes (hm1–hm5). Functional activity was evaluated in vitro on classical preparations: rabbit stimulated vas deferens, guinea pig stimulated left atria (M_2), guinea pig ileum (M_3), and guinea pig lung strips, following the previously reported methods.[17] In this respect, it is fair to recall that, for a long time, the contraction of rabbit vas deferens was considered an effect mediated by M_1-receptor subtypes, whereas more recent studies attribute the same effect to an M_4-activation.[2,18] Analogously, the validity of the guinea pig lung strips as a M_4 model[19] has been questioned.[20] For this reason, in the present work, these two preparations are indicated as putative M_1 and M_4 receptor models. Carbachol, arecaidine propargyl ester (APE) and 4-Cl-McN-A-343 were used as agonists. Results are expressed as pK_i values (affinity), and as pK_b values calculated from the equation $pK_b = \log(DR - 1) - \log[B]$, where DR is the ratio of ED_{50} values of agonist after and before treatment with one or two antagonist concentrations [B].[21] In selected cases, antagonist potency is expressed in terms of pA_2, estimated by Schild plots constrained to slope −1.0, as required by the theory.[22] Results are shown in Table 1, where the data of *N*-methylscopolamine and compound **B** (Chart 1) are reported for comparison.



Scheme 1. Reagents: (a) H_2O_2, CH_3COOH, chromatographic separation; (b) CH_3I, anhyd diethyl ether. For the synthesis of the corresponding isomers of the 2*R*-series, the same procedure was followed, starting from (2*R*,5′*R*)-(+)-**1**,(2*R*,2′*S*,5′*R*)-(−)-**2**,(2*R*,2′*R*,5′*R*)-(+)-**3**, respectively.

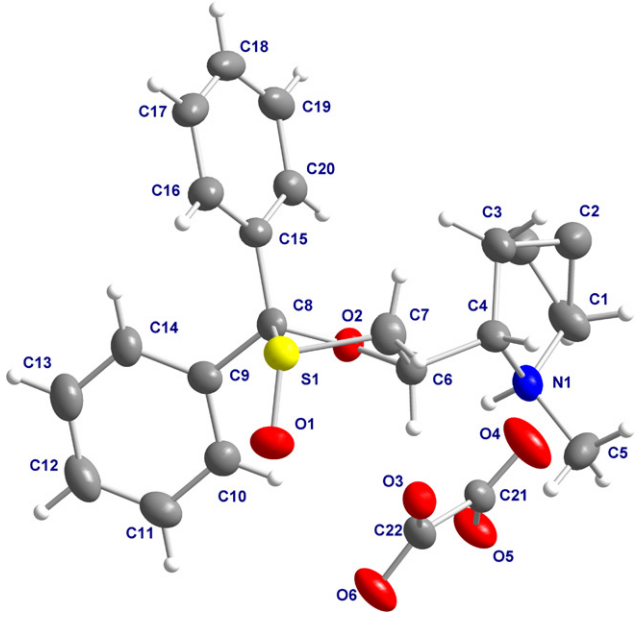


Figure 1. Thermal ellipsoid plot (50% ellipsoids) of compound (+)-**4** oxalate.

4. Results and discussion

The results of binding studies and functional activity of compounds **4**–**15** are reported in Table 1, compared with *N*-methylscopolamine as a general reference and with compound (2*R*,3*S*,5*R*)-**B** which was the most interesting among the parent compounds studied previously.[12] Its pharmacological profile has been evaluated anew, using the same protocols of the new series.

From Table 1 it can be observed that all compounds bind with good affinity to the five human muscarinic receptors, without notable differences between quaternary salts (**10**–**15**) and tertiary bases (**4**–**9**); a fairly similar situation is found for antagonist potency in functional models. Therefore, as happened in our preceding works,[13–16] our chemical manipulation induced activity also in the tertiary bases, which do not present a permanent charge and can be useful to treat CNS disorders. From the same table it can be seen that, as already verified for the parent molecules,[16] the agreement between affinity and functional activity is in general good for the well-characterized M_2 (guinea pig atrium) and M_3 (guinea pig ileum) models, while there is no correlation between functional activity on guinea pig lung preparation (a putative M_4 model)[19] and the binding data on hm4 as well as on the other human cloned subtypes, reinforcing the doubts regarding the validity of the model.[20] Also the rabbit vas deferens preparation has been questioned as M_1 model[18]; therefore, only a selected set of derivatives were tested by this preparation.

Unfortunately, contrary to our expectations and unlike what happened with the agonists of the same series,[13–15] insertion of a further stereogenic center on the oxathiolane moiety did not improve the subtype selectivity of the antagonists: none of the new compounds displays a selectivity of at least one order of magnitude for one subtype to the relative exclusion of all others. The

Table 1. Binding parameters and functional activities of derivatives **4**–**15**

Compound		Binding affinities ($pK_i \pm SE$)[a]					Functional activities ($pK_b \pm SE$)[b]			
Compound	Stereochemistry	hm1	hm2	hm3	hm4	hm5	Rabbit vas deferens	Guinea pig atrium (M_2)	Guinea pig ileum (M_3)	Guinea pig lung
(−)-**4**	2*R*, 3′*R*,5′*R*	7.13 ± 0.08	6.64 ± 0.11	6.29 ± 0.12	6.29 ± 0.07	6.45 ± 0.07	n.t.	7.27 ± 0.14	6.95 ± 0.02	5.75 ± 0.05
(+)-**4**	2*S*,3′*S*,5′*S*	6.96 ± 0.08	6.25 ± 0.13	6.33 ± 0.11	5.99 ± 0.06	6.29 ± 0.06	n.t.	6.00 ± 0.04	6.55 ± 0.23	5.29 ± 0.19
(+)-**5**	2*R*,3′*S*,5′*R*	6.96 ± 0.07	7.40 ± 0.09	6.55 ± 0.11	6.50 ± 0.07	6.47 ± 0.07	7.76 ± 0.07	7.10 ± 0.20	7.17 ± 0.06	5.63 ± 0.23
(−)-**5**	2*S*,3′*R*,5′*S*	6.84 ± 0.07	6.66 ± 0.10	6.52 ± 0.11	6.39 ± 0.06	6.64 ± 0.05	n.t.	6.42 ± 0.14	6.99 ± 0.06	<5
(−)-**6**	2*R*,2′*S*,3′*R*,5′*R*	7.10 ± 0.07	6.97 ± 0.10	6.71 ± 0.11	6.45 ± 0.07	6.56 ± 0.06	7.18 ± 0.19	7.34 ± 0.05	7.39 ± 0.14	7.24 ± 0.14
(+)-**6**	2*S*,2′*R*,3′*S*,5′*S*	6.81 ± 0.08	5.99 ± 0.11	6.41 ± 0.11	6.35 ± 0.07	6.28 ± 0.07	n.t.	5.48 ± 0.02	6.43 ± 0.09	<5
(−)-**7**	2*R*,2′*S*,3′*S*,5′*R*	7.85 ± 0.07	8.38 ± 0.09	7.37 ± 0.11	7.37 ± 0.07	7.23 ± 0.06	9.27 ± 0.15	9.37 ± 0.15	8.71 ± 0.21	6.92 ± 0.15
(+)-**7**	2*S*,2′*R*,3′*R*,5′*S*	8.00 ± 0.07	7.19 ± 0.11	7.74 ± 0.10	7.48 ± 0.06	7.60 ± 0.08	8.56 ± 0.10	7.56 ± 0.01	7.96 ± 0.05	5.20 ± 0.14
(+)-**8**	2*R*,2′*R*,3′*R*,5′*R*	7.42 ± 0.08	7.18 ± 0.09	7.62 ± 0.11	7.03 ± 0.08	7.52 ± 0.07	7.68 ± 0.12	7.05 ± 0.22	7.71 ± 0.07	<5
(−)-**8**	2*S*,2′*S*,3′*S*,5′*S*	6.27 ± 0.08	5.84 ± 0.10	6.16 ± 0.10	5.82 ± 0.07	6.12 ± 0.06	n.t.	5.71 ± 0.03	5.95 ± 0.08	5.19 ± 0.13
(+)-**9**	2*R*,2′*R*,3′*S*,5′*R*	6.97 ± 0.07	7.42 ± 0.09	6.70 ± 0.10	6.59 ± 0.07	6.54 ± 0.06	n.t.	7.72 ± 0.07	7.59 ± 0.08	6.16 ± 0.15
(−)-**9**	2*S*,2′*S*,3′*R*,5′*S*	7.03 ± 0.08	6.52 ± 0.09	6.46 ± 0.10	6.30 ± 0.07	6.42 ± 0.07	n.t.	6.59 ± 0.07	7.10 ± 0.03	5.13 ± 0.08
(−)-**10**	2*R*,3′*R*,5′*R*	7.27 ± 0.09	7.16 ± 0.10	6.58 ± 0.14	6.55 ± 0.08	6.74 ± 0.07	n.t.	7.39 ± 0.01	7.50 ± 0.08	6.53 ± 0.05
(+)-**10**	2*S*,3′*S*,5′*S*	7.53 ± 0.07	6.85 ± 0.10	6.68 ± 0.09	6.68 ± 0.05	7.04 ± 0.07	7.55 ± 0.13	7.20 ± 0.20	6.95 ± 0.13	5.54 ± 0.20
(+)-**11**	2*R*,3′*S*,5′*R*	6.49 ± 0.06	6.71 ± 0.11	6.38 ± 0.10	5.93 ± 0.06	6.09 ± 0.07	7.21 ± 0.09	7.00 ± 0.01	7.62 ± 0.06	5.25 ± 0.04
(−)-**11**	2*S*,3′*R*,5′*S*	7.80 ± 0.07	7.38 ± 0.10	7.29 ± 0.07	6.98 ± 0.06	7.52 ± 0.07	7.61 ± 0.16	6.93 ± 0.01	7.89 ± 0.02	<5
(−)-**12**	2*R*,2′*S*,3′*R*,5′*R*	7.25 ± 0.07	7.07 ± 0.10	6.54 ± 0.12	6.50 ± 0.06	6.71 ± 0.06	7.92 ± 0.07	7.91 ± 0.05	7.11 ± 0.09	7.45 ± 0.16
(+)-**12**	2*S*,2′*R*,3′*S*,5′*S*	7.92 ± 0.08	7.15 ± 0.09	7.57 ± 0.10	7.23 ± 0.06	7.63 ± 0.06	n.t.	6.57 ± 0.04	7.03 ± 0.19	5.41 ± 0.14
(−)-**13**	2*R*,2′*S*,3′*S*,5′*R*	6.93 ± 0.06	7.02 ± 0.09	6.49 ± 0.14	6.33 ± 0.06	6.52 ± 0.06	n.t.	7.51 ± 0.05	7.22 ± 0.05	6.06 ± 0.06
(+)-**13**	2*S*,2′*R*,3′*R*,5′*S*	8.52 ± 0.07	7.75 ± 0.10	8.38 ± 0.11	7.80 ± 0.06	8.52 ± 0.09	9.01 ± 0.24	7.81 ± 0.11	8.67 ± 0.06	6.37 ± 0.14
(+)-**14**	2*R*,2′*R*,3′*R*,5′*R*	8.16 ± 0.07	7.67 ± 0.11	8.16 ± 0.11	7.72 ± 0.11	7.85 ± 0.07	8.86 ± 0.16	7.98 ± 0.17	9.00 ± 0.20	7.59 ± 0.14
(−)-**14**	2*S*,2′*S*,3′*S*,5′*S*	6.81 ± 0.07	6.26 ± 0.09	6.49 ± 0.11	6.22 ± 0.05	6.48 ± 0.06	n.t.	6.03 ± 0.15	6.68 ± 0.05	5.24 ± 0.02
(+)-**15**	2*R*,2′*R*,3′*S*,5′*R*	7.03 ± 0.06	7.11 ± 0.08	6.79 ± 0.12	6.52 ± 0.07	6.65 ± 0.08	n.t.	6.97 ± 0.04	7.10 ± 0.01	6.48 ± 0.12
(−)-**15**	2*S*,2′*S*,3′*R*,5′*S*	7.71 ± 0.07	7.24 ± 0.10	7.11 ± 0.08	6.95 ± 0.06	7.38 ± 0.07	8.18 ± 0.13	6.92 ± 0.15	7.90 ± 0.20	5.64 ± 0.06
B		7.66 ± 0.07	7.13 ± 0.09	7.46 ± 0.10	7.02 ± 0.07	6.85 ± 0.09	n.t.	7.52 ± 0.07[c]	7.64 ± 0.21[c]	5.24 ± 0.15
NMS[d]		9.49 ± 0.06	9.75 ± 0.10	9.87 ± 0.10	9.85 ± 0.07	9.68 ± 0.05	n.t.	9.33 ± 0.03*	9.21 ± 0.07*	8.19 ± 0.06*

n.t., not tested.

[a] Binding parameters of muscarinic antagonists at five human muscarinic receptor subtypes. The affinity estimates (as pK_i) were derived from both [^{3}H]NMS homologous and heterologous competition curves, and represent the means (±SEM) of at least three experiments.

[b] pK_b values in functional tests ± SEM; $n = 3$. The results labelled with a star (*) represent pA_2 values.

[c] In Ref. 12, pA_2 values for guinea pig atrium (7.69) and guinea pig ileum (7.84) were reported.

[d] *N*-Methylscopolamine.

reasons for this disappointing result are not easily explained. It can be speculated that the region of the receptor binding site, where the bulky, lipophilic groups of the antagonists bind,[23,24] is not sufficiently differentiated in the five subtypes. In light of these results, a further extension of the length of the lipophilic groups in position 2′ to explore the nearby space in the receptor subtypes appears to be worthwhile, as suggested by one of the referees of our previous paper.[16] Enantioselectivity is generally modest (Eudismic Index, EI, defined as pK_i (eutomer) − pK_i (distomer), generally well below 1); as a matter of fact, the role played by stereochemistry on affinity and subtype selectivity, appears to be complex, as already verified for the non-oxidized oxathiolanes.[16] Hopefully, it could be clarified using molecular modeling and eudismic analysis[25] approaches, now that the series of 1,3-oxathiolane and 1,3-oxathiolane-3-sulfoxides agonists and antagonists has been completed.

Even if the main goal of our research was unsuccessful, a few molecules displaying an interesting pharmacological profile have been identified. The enantiomeric couple of tertiary amines **7** shows interesting affinity both in binding and in functional assays. In particular, isomer (−)-**7** shows good binding to hm2 subtype (pK_i = 8.38) and noteworthy potency in functional tests (pK_b = 9.27 on rabbit vas deferens; pK_b = 9.37 on guinea pig atrium; pK_b = 8.71 on guinea pig ileum). Moreover, the same compound presents also a low hm2/hm3 selectivity in binding tests, that is maintained in functional experiments; this makes it an interesting pharmacological tool since, being a tertiary amine, it will probably be able to reach the CNS. As far as quaternary derivatives are concerned, an interesting profile is presented by compound (+)-**14**, that shows good values both in affinity and in activity, with a low selectivity for hm1 and hm3 with respect to hm2 (threefold in both cases) that is increased in functional assays (M_1/M_2 = 8; M_3/M_2 = 10). The enantiomeric couple **14** presents also the highest enantioselectivity, both in binding (EI = 1.35, 1.41, 1.67, 1.50, 1.37, respectively) and functional studies (EI = 1.95 and 2.32 for M_2 and M_3 subtypes, respectively).

In conclusion, the new series of synthesized antagonists shows good affinity for all human cloned muscarinic receptors. Unfortunately, despite the presence of three or four stereogenic centers, subtype selectivity remains poor. However, the tertiary amine (−)-**7** shows very high affinity in binding tests, and noteworthy potency in functional tests; moreover, lacking the permanent charge on nitrogen atom, it may represent an interesting tool to study CNS muscarinic receptors.

5. Experimental

5.1. Chemistry

All melting points were taken on a Büchi apparatus and are uncorrected. Infrared spectra were recorded with a Perkin-Elmer Spectrum RX I FT-IR spectrophotometer in Nujol mull for solids and neat for liquids. NMR spectra were recorded on a Bruker Avance 400 spectrometer (400 MHz for ^{1}H NMR, 100 MHz for ^{13}C), and chromatographic separations were performed on a silica gel column by gravity chromatography (Kieselgel 40, 0.063–0.200 mm; Merck) or flash chromatography (Kieselgel 40, 0.040–0.063 mm; Merck). The IR spectra data are consistent with the assigned structures. Yields are given after purification, unless otherwise stated. Where analyses are indicated by symbols, the analytical results are within ±0.4% of the theoretical values. Optical rotation was measured at a concentration of 1 g/100 mL (c = 1), unless otherwise stated, with a Perkin-Elmer polarimeter (accuracy ±0.002°). When reactions were performed in anhydrous conditions, the mixtures were maintained under nitrogen.

Compounds were named following IUPAC rules as applied by Beilstein-Institut AutoNom 2000 4.01.305, a software for systematic names in organic chemistry.

5.1.1. (2*S*,3′*S*,5′*S*)-1-Methyl-2-(2,2-diphenyl-1,3-oxathiolan-5-yl)pyrrolidine 3-oxide (2*S*,3′*S*,5′*S*)-(+)-4 and (2*S*,3′*R*,5′*S*)-1-methyl-2-(2,2-diphenyl-1,3-oxathiolan-5-yl)pyrrolidine 3-oxide (2*S*,3′*R*,5′*S*)-(−)-5. A solution of 1 equiv of (2*S*,5′*S*)-(−)-**1**[16] in CH_3COOH was added to 3.6 equiv of H_2O_2 30%. After 2 h at rt the mixture was made alkaline with NaOH 3 N, extracted with CH_2Cl_2 and dried over Na_2SO_4. Filtration and evaporation afforded a mixture of two diastereomeric sulfoxides in 15:85 ratio (calculated from ^{1}H NMR). The obtained oily mixture was separated by flash chromatography, yielding the title compounds.

Starting from (2*R*,5′*R*)-(+)-**1**,[16] in the same way, (2*R*,3′*R*,5′*R*)-1-methyl-2-(2,2-diphenyl-1,3-oxathiolan-5-yl)pyrrolidine 3-oxide (2*R*,3′*R*,5′*R*)-(−)-**4** and (2*R*,3′*S*,5′*R*)-1-methyl-2-(2,2-diphenyl-1,3-oxathiolan-5-yl)pyrrolidine 3-oxide (2*R*,3′*S*,5′*R*)-(+)-**5** were obtained.

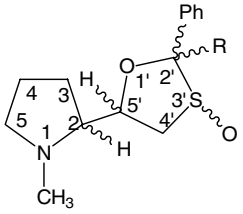
The chemical, physical, and spectroscopic characteristics of the four isomers are reported in Tables 2 and 3. (2*S*,3′*S*,5′*S*)-(+)-**4** was transformed in the corresponding oxalate by treatment with oxalic acid (1 equiv) in ethyl acetate.

5.1.2. (2*S*,2′*R*,3′*S*,5′*S*)-1-Methyl-2-(2-cyclohexyl-2-phenyl-1,3-oxathiolan-5-yl)pyrrolidine 3-oxide (2*S*,2′*R*,3′*S*,5′*S*)-(+)-6 and (2*S*,2′*R*,3′*R*,5′*S*)-1-methyl-2-(2-cyclohexyl-2-phenyl-1,3-oxathiolan-5-yl)pyrrolidine 3-oxide (2*S*,2′*R*,3′*R*,5′*S*)-(+)-7. Using the same procedure described above, and starting from (2*S*,2′*R*,5′*S*)-(+)-**2**,[16] compounds (+)-**6** and (+)-**7** were obtained in a 20:80 ratio.

Starting from (2*R*,2′*S*,5′*R*)-(−)-**2**,[16] in the same way, (2*R*,2′*S*,3′*R*,5′*R*)-1-methyl-2-(2-cyclohexyl-2-phenyl-1,3-oxathiolan-5-yl)pyrrolidine 3-oxide (2*R*,2′*S*,3′*R*,5′*R*)-(−)-**6** and (2*R*,2′*S*,3′*S*,5′*R*)-1-methyl-2-(2-cyclohexyl-2-phenyl-1,3-oxathiolan-5-yl)pyrrolidine 3-oxide (2*R*,2′*S*,3′*S*,5′*R*)-(−)-**7** were obtained.

The chemical, physical, and spectroscopic characteristics of the four isomers are reported in Tables 2 and 3.

Table 2. Chemical and physical characteristics of derivatives **4–9**



Compound	R	Stereochemistry	$[\alpha]_{20}^{D}$ (CH_2Cl_2)	Eluent[a]	Isomers order of elution and ratio	Analysis
(+)-**4**	Phenyl	2*S*,3′*S*,5′*S*	+112.8	A	(+)-**4**/(−)-**5** 15:85	$C_{20}H_{23}NO_2S$
(−)-**5**	Phenyl	2*S*,3′*R*,5′*S*	−148.2			$C_{20}H_{23}NO_2S$
(−)-**4**	Phenyl	2*R*,3′*R*,5′*R*	−112.8	A	(−)-**4**/(+)-**5** 15:85	$C_{20}H_{23}NO_2S$
(+)-**5**	Phenyl	2*R*,3′*S*,5′*R*	+148.2			$C_{20}H_{23}NO_2S$
(+)-**6**	Cyclohexyl	2*S*,2′*R*,3′*S*,5′*S*	+140.0	B	(+)-**6**/(+)-**7** 20:80	$C_{20}H_{29}NO_2S$
(+)-**7**	Cyclohexyl	2*S*,2′*R*,3′*R*,5′*S*	+67.8			$C_{20}H_{29}NO_2S$
(−)-**6**	Cyclohexyl	2*R*,2′*S*,3′*R*,5′*R*	−140.0	B	(−)-**6**/(−)-**7** 20:80	$C_{20}H_{29}NO_2S$
(−)-**7**	Cyclohexyl	2*R*,2′*S*,3′*S*,5′*R*	−67.8			$C_{20}H_{29}NO_2S$
(−)-**8**	Cyclohexyl	2*S*,2′*S*,3′*S*,5′*S*	−82.0	B	(−)-**8**/(−)-**9** 65:35	$C_{20}H_{29}NO_2S$
(−)-**9**	Cyclohexyl	2*S*,2′*S*,3′*R*,5′*S*	−119.0			$C_{20}H_{29}NO_2S$
(+)-**8**	Cyclohexyl	2*R*,2′*R*,3′*R*,5′*R*	+82.0	B	(+)-**8**/(+)-**9** 65:35	$C_{20}H_{29}NO_2S$
(+)-**9**	Cyclohexyl	2*R*,2′*R*,3′*S*,5′*R*	+119.0			$C_{20}H_{29}NO_2S$

[a] A, abs EtOH/NH_4OH/CH_2Cl_2/diethyl ether/petroleum ether (225:12.4:540:540:1350); B, abs EtOH/NH_4OH/CH_2Cl_2/diethyl ether/petroleum ether (180:9.9:360:360:900).

5.1.3. (2*S*,2′*S*,3′*S*,5′S)-1-Methyl-2-(2-cyclohexyl-2-phenyl-1,3-oxathiolan-5-yl)pyrrolidine 3-oxide (2*S*,2′*S*,3′*S*,5′*S*)-(−)-8 and (2*S*,2′*S*,3′*R*,5′*S*)-1-methyl-2-(2-cyclohexyl-2-phenyl-1,3-oxathiolan-5-yl)pyrrolidine 3-oxide (2*S*,2′*S*,3′*R*,5′*S*)-(−)-9. Using the same procedure described above, and starting from (2*S*,2′*S*,5′*S*)-(−)-**3**,[16] compounds (−)-**8** and (−)-**9** were obtained in a 65:35 ratio.

Starting from (2*R*,2′*R*,5′*R*)-(+)-**3**,[16] in the same way, (2*R*,2′*R*,3′*R*,5′*R*)-1-Methyl-2-(2-cyclohexyl-2-phenyl-1,3-oxathiolan-5-yl)pyrrolidine 3-oxide (2*R*,2′*R*,3′*R*,5′*R*)-(+)-**8** and (2*R*,2′*R*,3′*S*,5′*R*)-1-methyl-2-(2-cyclohexyl-2-phenyl-1,3-oxathiolan-5-yl)pyrrolidine 3-oxide (2*R*,2′*R*,3′*S*,5′*R*)-(+)-**9** were obtained.

The chemical, physical, and spectroscopic characteristics of the four isomers are reported in Tables 2 and 3.

5.2. General procedure for the synthesis of dimethyl pyrrolidinium iodides 10–15

An anhydrous diethyl ether solution of the suitable sulfoxides **4–9** was treated with an excess of methyl iodide and kept for 1 night at rt in the dark. The obtained solid was filtered, dried under vacuum and recrystallized from absolute ethanol/diethyl ether.

The chemical and physical characteristics, and the ^1H NMR spectra of the four diphenyl isomers **10** and **11** and of the eight cyclohexylphenyl derivatives (**12–15**) are reported in Tables 4 and 5.

5.3. Crystal structure determination and refinement collection of (+)-4 oxalate

Diffraction data were collected at 100 K using an Oxford Diffraction Xcalibur3 diffractometer equipped with a CCD area detector and graphite monochromated Mo-Kα radiation ($\lambda = 0.71069$). The crystal data and some details of the data collection and structure refinement are summarized in Table 6; more details are reported in the Supplementary Data. Data collection was performed using a ω scan with the CrysAlis CCD program.[26] Data reduction was carried out and the absorption correction applied with the CrysAlis Red program.[27] The crystal structure was solved by direct methods using the SIR97 program,[28] which gave the position of most of the non-hydrogen atoms. The remaining atoms were identified by successive Fourier difference syntheses. One independent compound molecule and one oxalate molecule were found in the asymmetric unit. Refinement was carried out on F^2 by full-matrix least square techniques, using the SHELX97 program package.[29] Hydrogen atoms were added in the model constrained to idealized positions and refined using a riding model with riding isotropic displacement parameters. All the non-hydrogen atoms were refined anisotropically, with the exception of atom C2 that was modeled in two different position. Crystallographic data have been deposited with the Cambridge Crystallographic Data Center as supplementary publication with number CCDC 659667. Copies of the data can be obtained, free of charge, on application to CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (fax: +44 (0)1223 336033 or e-mail: deposit@ccdc.cam.ac.uk).

5.4. Pharmacology

5.4.1. Binding studies

5.4.1.1. Cell culture and membrane preparation. Chinese hamster ovary cells, stably expressing cDNA encoding human muscarinic m1-m5 receptors, were grown in Dulbecco' modified Eagle's medium supplemented with 10% fetal calf serum (Gibco, Grand Iland, NY), 50 U/mL of penicillin G, 0.05 mg/mL of streptomycin, 2 mM of L-glutamine (Sigma–Aldrich, Milano,

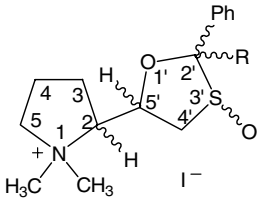
Table 3. ^{1}H NMR and ^{13}C NMR of the isomers of *N*-methyl derivatives **4–9**

Compound	^{1}H NMR ($CDCl_3$) δ (ppm)	^{13}C NMR ($CDCl_3$) δ (ppm)
(+)-**4**	1.80–1.94 (m, 3H), 1.95–2.10 (m, 1H) (CH_2CH_2); 2.32–2.40 (m, 1H, C*H*H–N); 2.45 (s, 3H, N–CH_3); 2.74–2.79 (m, 1H, CH–N); 2.92 (dd, *J* = 12.8 Hz, *J* = 11.2 Hz, 1H, C*H*HS); 3.09–3.17 (m, 1H, C*H*HN); 3.20 (dd, *J* = 12.7 Hz, *J* = 3.6 Hz, 1H, C*H*HS); 5.06–5.15 (m, 1H, CH–O); 7.32–7.44 (m, 6H), 7.49–7.59 (m, 4H) (aromatics)	23.52, 28.16 (CH_2–CH_2); 42.65 (N–CH_3); 53.80 (CH_2–S); 58.09 (CH_2–N); 66.97 (CH–N); 84.98 (CH–O); 109.05 (S–C–O); 126.77, 127.91, 128.08, 128.57, 129.17, 130.11 (CH aromatics); 135.84, 140.08 (C aromatics)
(−)-**4**	1.80–1.94 (m, 3H), 1.95–2.10 (m, 1H) (CH_2CH_2); 2.32–2.40 (m, 1H, C*H*H–N); 2.45 (s, 3H, N–CH_3); 2.74–2.79 (m, 1H, CH–N); 2.92 (dd, *J* = 12.8 Hz, *J* = 11.2 Hz, 1H, C*H*HS); 3.09–3.17 (m, 1H, C*H*HN); 3.20 (dd, *J* = 12.7 Hz, *J* = 3.6 Hz, 1H, C*H*HS); 5.06–5.15 (m, 1H, CH–O); 7.32–7.44 (m, 6H), 7.49–7.59 (m, 4H) (aromatics)	23.52, 28.16 (CH_2–CH_2); 42.65 (N–CH_3); 53.80 (CH_2–S); 58.09 (CH_2–N); 66.97 (CH–N); 84.98 (CH–O); 109.05 (S–C–O); 126.77, 127.91, 128.08, 128.57 129.17, 130.11 (CH aromatics); 135.84, 140.08 (C aromatics)
(−)-**5**	1.77–1.94 (m, 2H), 2.02–2.18 (m, 2H) (CH_2–CH_2); 2.33–2.43 (m, 1H, C*H*HN); 2.50 (s, 3H, N–CH_3); 2.77–2.84 (m, 1H, CH–N); 3.06 (dd, *J* = 14.0 Hz, *J* = 6.8 Hz, 1H, C*H*HS); 3.11–3.21 (m, 1H, C*H*H–N); 3.26 (dd, *J* = 14.0 Hz, *J* = 8.0 Hz, 1H, C*H*H–S); 4.17–4.28 (m, 1H, CH–O); 7.30–7.41 (m, 3H), 7.41–7.51 (m, 5H), 7.57–7.64 (m, 2H) (aromatics)	23.50, 27.40 (CH_2–CH_2); 42.10 (N–CH_3); 55.77 (CH_2–S); 58.02 (CH_2–N); 67.61 (CH–N); 80.41 (CH–O); 109.08 (S–C–O); 126.93, 127.61, 127.93, 127.97, 128.06, 128.28, 128.87, 129.06, 130.04, 132.41 (CH aromatics); 135.68, 136.71 (C aromatics)
(+)-**5**	1.77–1.94 (m, 2H), 2.02–2.18 (m, 2H) (CH_2–CH_2); 2.33–2.43 (m, 1H, C*H*HN); 2.50 (s, 3H, N–CH_3); 2.77–2.84 (m, 1H, CH–N); 3.06 (dd, *J* = 14.0 Hz, *J* = 6.8 Hz, 1H, C*H*HS); 3.11–3.21 (m, 1H, C*H*H–N); 3.26 (dd, *J* = 14.0 Hz, *J* = 8.0 Hz, 1H, C*H*H–S); 4.17–4.28 (m, 1H, CH–O); 7.30–7.41 (m, 3H), 7.41–7.51 (m, 5H), 7.57–7.64 (m, 2H) (aromatics)	23.50, 27.40 (CH_2–CH_2); 42.10 (N–CH_3); 55.77 (CH_2–S); 58.02 (CH_2–N); 67.61 (CH–N); 80.41 (CH–O); 109.08 (S–C–O); 126.93, 127.61, 127.93, 127.97, 128.06, 128.28, 128.87, 129.06, 130.04, 132.41 (CH aromatics); 135.68, 136.71 (C aromatics)
(+)-**6**	1.07–1.38 (m, 7H), 1.62–1.70 (m, 2H) (cyclohexane); 1.72–1.87 (m, 4H, CH*H*–CH_2 pyrrolidine + CH*H* cyclohexane); 1.94–2.10 (m, 1H, CH*H* pyrrolidine); 2.11–2.22 (m, 1H, CH cyclohexane); 2.32–2.40 (m, 1H, C*H*H–N); 2.53 (s, 3H, N–CH_3); 2.92 (dd, *J* = 12.8 Hz, *J* = 3.2 Hz, 1H, C*H*H–S); 2.91–3.02 (m, 2H, CH–N + C*H*HS); 3.12–3.20 (m, 1H, C*H*H–N); 4.67–4.74 (m, 1H, CH–O); 7.25–7.42 (m, 5H, aromatics)	23.37 (CH_2 pyrrolidine); 26.08, 26.26, 26.34 (CH_2 cyclohexane); 27.11, 27.29 (CH_2 pyrrolidine + CH_2 cyclohexane); 28.48 (CH_2 cyclohexane); 42.48 (N–CH_3); 47.16 (CH cyclohexane); 54.03 (CH_2–S); 58.05 (CH_2–N); 65.97 (CH–N); 82.14 (CH–O); 113.67 (S–C–O); 127.61, 127.90, 127.99, 128.24, 128.57 (CH aromatics); 134.70 (C aromatic)
(−)-**6**	1.07–1.38 (m, 7H), 1.62–1.70 (m, 2H) (cyclohexane); 1.72–1.87 (m, 4H, CH*H*–CH_2 pyrrolidine + CH*H* cyclohexane); 1.94–2.10 (m, 1H, CH*H* pyrrolidine); 2.11–2.22 (m, 1H, CH cyclohexane); 2.32–2.40 (m, 1H, C*H*H–N); 2.53 (s, 3H, N–CH_3); 2.92 (dd, *J* = 12.8 Hz, *J* = 3.2 Hz, 1H, C*H*H–S); 2.91–3.02 (m, 2H, CH–N + C*H*HS); 3.12–3.20 (m, 1H, C*H*H–N); 4.67–4.74 (m, 1H, CH–O); 7.25–7.42 (m, 5H, aromatics)	23.37 (CH_2 pyrrolidine); 26.08, 26.26, 26.34 (CH_2 cyclohexane); 27.11, 27.29 (CH_2 pyrrolidine + CH_2 cyclohexane); 28.48 (CH_2 cyclohexane); 42.48 (N–CH_3); 47.16 (CH cyclohexane); 54.03 (CH_2–S); 58.05 (CH_2–N); 65.97 (CH–N); 82.14 (CH–O); 113.67 (S–C–O); 127.61, 127.90, 127.99, 128.24, 128.57 (CH aromatics); 134.70 (C aromatic)
(+)-**7**	0.78–0.95 (m, 3H), 1.30–1.41 (m, 2H), 1.55–1.72 (m, 3H) (CH_2 cyclohexane); 1.73–1.88 (m, 2H, CH_2 pyrrolidine); 1.88–2.10 (m, 4H, CH_2 pyrrolidine + CH_2 cyclohexane); 2.28–2.40 (m, 1H, CH cyclohexane); 2.40–2.48 (m, 1H, C*H*HN); 2.52 (s, 3H, N–CH_3); 2.81–2.92 (m, 2H, CH*H*–S + CH–N); 2.90–2.98 (m, 1H, C*H*H–S); 3.10–3.18 (m, 1H, CH*H*–N); 4.11–4.19 (m, CH–O); 7.25–7.42 (m, 5H, aromatics)	23.17, 25.90, 26.08, 27.00, 27.37, 29.40, 29.50 (CH_2 pyrrolidine + CH_2 cyclohexane); 41.08 (CH cyclohexane); 42.22 (N–CH_3); 53.40 (CH_2–S); 57.76 (CH_2–N); 67.96 (CH–N); 80.82 (CH–O); 111.53 (S–C–O); 127.13, 128.28, 128.58 (CH aromatics); 133.75 (C aromatic)
(−)-**7**	0.78–0.95 (m, 3H), 1.30–1.41 (m, 2H), 1.55–1.72 (m, 3H) (CH_2 cyclohexane); 1.73–1.88 (m, 2H, CH_2 pyrrolidine); 1.88–2.10 (m, 4H, CH_2 pyrrolidine + CH_2 cyclohexane); 2.28–2.40 (m, 1H, CH cyclohexane); 2.40–2.48 (m, 1H, C*H*HN); 2.52 (s, 3H, N–CH_3); 2.81–2.92 (m, 2H, CH*H*–S + CH–N); 2.90–2.98 (m, 1H, C*H*H–S); 3.10–3.18 (m, 1H, CH*H*–N); 4.11–4.19 (m, CH–O); 7.25–7.42 (m, 5H, aromatics)	23.17, 25.90, 26.08, 27.00, 27.37, 29.40, 29.50 (CH_2 pyrrolidine + CH_2 cyclohexane); 41.08 (CH cyclohexane); 42.22 (N–CH_3); 53.40 (CH_2–S); 57.76 (CH_2–N); 67.96 (CH–N); 80.82 (CH–O); 111.53 (S–C–O); 127.13, 128.28, 128.58 (CH aromatics); 133.75 (C aromatic)
(−)-**8**	0.70–0.82 (m, 1H), 0.82–0.98 (m, 2H), 1.15–1.24 (m, 1H), 1.24–1.42 (m, 2H), 1.53–1.62 (m, 1H), 1.62–1.74 (m, 2H) (CH_2 cyclohexane); 1.74–1.86 (m, 2H, CH_2 pyrrolidine); 1.86–1.96 (m, 1H, C*H*H cyclohexane); 1.97–2.06 (m, 3H, CH_2 pyrrolidine + CH cyclohexane); 2.23–2.31 (m, 1H, C*H*H–S); 2.31–2.44 (m, 4H, N–CH_3 + CH*H*–N); 2.56–2.63 (m, 1H, CH–N); 3.03–3.12 (m, 1H, CH*H*–N); 3.12–3.21 (m, 1H, CH*H*–S); 4.83–4.94 (m, 1H, CH–O); 7.29–7.41 (m, 5H, aromatics)	23.44, 26.04, 26.19, 27.12, 28.58, 30.15 (CH_2 cyclohexane + CH_2 pyrrolidine); 41.43 (CH cyclohexane); 42.29 (N–CH_3); 52.10 (CH_2–S); 57.69 (CH_2–N); 67.91 (CH–N); 83.56 (CH–O); 111.30 (S–C–O); 127.15, 127.82, 128.21, 128.46, 128.68 (CH aromatics); 137.30 (C aromatic)

Table 3 (*continued*)

Compound	^{1}H NMR ($CDCl_3$) δ (ppm)	^{13}C NMR ($CDCl_3$) δ (ppm)
(+)-**8**	0.70–0.82 (m, 1H), 0.82–0.98 (m, 2H), 1.15–1.24 (m, 1H), 1.24–1.42 (m, 2H), 1.53–1.62 (m, 1H), 1.62–1.74 (m, 2H) (CH_2 cyclohexane); 1.74–1.86 (m, 2H, CH_2 pyrrolidine); 1.86–1.96 (m, 1H, C*H*H cyclohexane); 1.97–2.06 (m, 3H, CH_2 pyrrolidine + CH cyclohexane); 2.23–2.31 (m, 1H, C*H*H–S); 2.31–2.44 (m, 4H, N–CH_3 + CH*H*–N); 2.56–2.63 (m, 1H, CH–N); 3.03–3.12 (m, 1H, CH*H*–N); 3.12–3.21 (m, 1H, CH*H*–S); 4.83–4.94 (m, 1H, CH–O); 7.29–7.41 (m, 5H, aromatics)	23.44, 26.04, 26.19, 27.12, 28.58, 30.15 (CH_2 cyclohexane + CH_2 pyrrolidine); 41.43 (CH cyclohexane); 42.29 (N–CH_3); 52.10 (CH_2–S); 57.69 (CH_2–N); 67.91 (CH–N); 83.56 (CH–O); 111.30 (S–C–O) 127.15, 127.82, 128.21, 128.46, 128.68 (CH aromatics); 137.30 (C aromatic)
(−)-**9**	0.83–1.08 (m, 3H), 1.12–1.28 (m, 3H), 1.49–1.70 (m, 3H), 1.70–1.92 (m, 4H) (cyclohexane + CH_2 pyrrolidine); 1.92–2.10 (m, 2H, CH_2 pyrrolidine); 2.36–2.45 (m, 1H, CH*H*–N); 2.47 (s, 3H, N–CH_3); 2.82–2.88 (m, 1H, CH–N); 3.06 (dd, J = 13.6 Hz, J = 7.2 Hz, 1H, CH*H*–S); 3.13–3.18 (m, 1H, CH*H*–N); 3.68 (dd, J = 14.0 Hz, J = 5.8 Hz, 1H, CH*H*–S); 4.35–4.41 (m, 1H, CH–O); 7.29–7.46 (m, 5H, aromatics)	23.40, 25.96, 26.16, 26.32, 26.46, 28.36, 29.59 (CH_2 cyclohexane + CH_2 pyrrolidine); 42.61, 42.49 (N–CH_3 + CH cyclohexane); 55.42 (CH_2–S); 58.01 (CH_2–N); 67.93 (CH–N); 81.54 (CH–O); 112.19 (S–C–O); 127.22, 127.61, 127.96, 128.27, 128.80 (CH aromatics); 134.05 (C aromatic)
(+)-**9**	0.83–1.08 (m, 3H), 1.12–1.28 (m, 3H), 1.49–1.70 (m, 3H), 1.70–1.92 (m, 4H) (cyclohexane + CH_2 pyrrolidine); 1.92–2.10 (m, 2H, CH_2 pyrrolidine); 2.36–2.45 (m, 1H, CH*H*–N); 2.47 (s, 3H, N–CH_3); 2.82–2.88 (m, 1H, CH–N); 3.06 (dd, J = 13.6 Hz, J = 7.2 Hz, 1H, CH*H*–S); 3.13–3.18 (m, 1H, CH*H*–N); 3.68 (dd, J = 14.0 Hz, J = 5.8 Hz, 1H, CH*H*–S); 4.35–4.41 (m, 1H, CH–O); 7.29–7.46 (m, 5H, aromatics)	23.40, 25.96, 26.16, 26.32, 26.46, 28.36, 29.59 (CH_2 cyclohexane + CH_2 pyrrolidine); 42.61, 42.49 (N–CH_3 + CH cyclohexane); 55.42 (CH_2–S); 58.01 (CH_2–N); 67.93 (CH–N); 81.54 (CH–O); 112.19 (S–C–O); 127.22, 127.61, 127.96, 128.27, 128.80 (CH aromatics); 134.05 (C aromatic)

Table 4. Chemical and physical characteristics of derivatives **10–15**



Compound	R	Stereochemistry	$[\alpha]_{20}^{D}$ (CH_2Cl_2)	Mp[a] (°C)	Analysis
(+)-**10**	Phenyl	2*S*,3′*S*,5′*S*	+78.3[b]	223–225	$C_{21}H_{26}INO_2S$
(−)-**10**	Phenyl	2*R*,3′*R*,5′*R*	−78.3[b]	223–225	$C_{21}H_{26}INO_2S$
(−)-**11**	Phenyl	2*S*,3′*R*,5′*S*	−72.2	242–243	$C_{21}H_{26}INO_2S$
(+)-**11**	Phenyl	2*R*,3′*S*,5′*R*	+72.2	242–243	$C_{21}H_{26}INO_2S$
(+)-**12**	Cyclohexyl	2*S*,2′*R*,3′*S*,5′*S*	+93.5	135–136 (d)	$C_{21}H_{32}INO_2S$
(−)-**12**	Cyclohexyl	2*R*,2′*S*,3′*R*,5′*R*	−93.5	135–136 (d)	$C_{21}H_{32}INO_2S$
(+)-**13**	Cyclohexyl	2*S*,2′*R*,3′*R*,5′*S*	+43.1	209–210[c]	$C_{21}H_{32}INO_2S$
(−)-**13**	Cyclohexyl	2*R*,2′*S*,3′*S*,5′*R*	−43.1	209–210[c]	$C_{21}H_{32}INO_2S$
(−)-**14**	Cyclohexyl	2*S*,2′*S*,3′*S*,5′*S*	−45.0	178–180	$C_{21}H_{32}INO_2S$
(+)-**14**	Cyclohexyl	2*R*,2′*R*,3′*R*,5′*R*	+45.0	178–180	$C_{21}H_{32}INO_2S$
(−)-**15**	Cyclohexyl	2*S*,2′*S*,3′*R*,5′*S*	−61.1	127–128 (d)	$C_{21}H_{32}INO_2S$
(+)-**15**	Cyclohexyl	2*R*,2′*R*,3′*S*,5′*R*	+61.1	127–128 (d)	$C_{21}H_{32}INO_2S$

[a] From abs EtOH/anhyd diethyl ether.
[b] In MeOH.
[c] From ethyl acetate.

Italy) and non-essential amino acids (Sigma–Aldrich, Milano, Italy) and 500 μg/mL of geneticin (Gibco, Grand Iland, NY) in a humidified atmosphere consisting of 5% CO_2 and 95% air.

Confluent CHO cell lines were scraped, washed with buffer (25 mM sodium phosphate containing 5 mM $MgCl_2$ at pH 7.4), and homogenized for 30 s using an Ultra-Turrax (setting 3). The pellet was sedimented 17,000*g* for 15 min at 4 °C and the membranes were re-suspended in the same buffer, re-homogenized with Ultra-Turrax and stored at −80 °C.[30] An aliquot was taken for the assessment of protein content according to the method of Bradford[31] using the Bio-Rad protein assay reagent (Bio-Rad Laboratories, München, Germany) and bovine serum albumin was used as the standard.

5.4.1.2. Binding assay. The radioligand binding assay was run in polypropylene 96-well plates (Sarstedt, Verona, Italy) and performed for 120 min at room temperature in a final volume of 0.25 mL in 25 mM sodium

Table 5. ^{1}H NMR of the isomers of dimethyl pyrrolidinium iodides **10–15**

Compound	^{1}H NMR ($CDCl_3$) δ (ppm)
(+)-**10**	2.11–2.29 (m, 4H, CH_2–CH_2); 2.99 (dd, J = 13.2 Hz, J = 12.4 Hz, 1H, C*H*H–S); 3.36 (s, 3H, N–CH_3); 3.37 (s, 3 H, N–CH_3); 3.63–3.70 (m, 1H, C*H*H–S); 3.70–3.76 (m, 1H, C*H*H–N); 3.78–3.86 (m, 1H, CH*H*–N); 4.16–4.24 (m, 1H, CH–N); 5.73–5.82 (m, 1H, CH–O); 7.31–7.61 (m, 10H, aromatics)[a]
(−)-**10**	2.11–2.29 (m, 4H, CH_2-CH_2); 2.99 (dd, J = 13.2 Hz, J = 12.4 Hz, 1H, C*H*H–S); 3.36 (s, 3H, N–CH_3); 3.37 (s, 3H, N–CH_3); 3.63–3.70 (m, 1H, C*H*H–S); 3.70–3.76 (m, 1H, C*H*H–N); 3.78–3.86 (m, 1H, CH*H*–N); 4.16–4.24 (m, 1H, CH–N); 5.73–5.82 (m, 1H, CH–O); 7.31–7.61 (m, 10H, aromatics)[a]
(−)-**11**	2.26–2.44 (m, 2H), 2.44–2.61 (m, 2H) (CH_2–CH_2); 2.88–2.99 (m, 1H, C*H*H–N); 3.04 (dd, J = 15.2 Hz, J = 5.2 Hz, 1H, C*H*H–S); 3.48 (s, 3H, N–CH_3); 3.50 (s, 3H, N–CH_3); 3.86–4.01 (m, 2H, C*H*H–S + C*H*H–N); 4.78–4.94 (m, 2H, CH–N + CH–O); 7.31–7.52 (m, 10H, aromatics)
(+)-**11**	2.26–2.44 (m, 2H), 2.44–2.61 (m, 2H) (CH_2–CH_2); 2.88–2.99 (m, 1H, C*H*H–N); 3.04 (dd, J = 15.2 Hz, J = 5.2 Hz, 1H, C*H*H–S); 3.48 (s, 3H, N–CH_3); 3.50 (s, 3H, N–CH_3); 3.86–4.01 (m, 2H, C*H*H–S + C*H*H–N); 4.78–4.94 (m, 2H, CH–N + CH–O); 7.31–7.52 (m, 10H, aromatics)
(+)-**12**	0.91–1.44 (m, 6H), 1.53–2.06 (m, 6H); 2.06–2.54 (m, 3H) (CH_2 cyclohexane + CH_2 pyrrolidine); 2.72–2.81 (m, 1H, C*H*H–S); 3.23 (s, 3H, N–CH_3); 3.35–3.52 (m, 4H, N–CH_3 + CH*H*–S); 3.78–3.91 (m, 1H, CH*H*–N); 3.92–4.03 (m, 1H, CH*H*–N); 4.76–4.88 (m, 1H, CH–N); 5.03–5.11 (m, 1H, CH–O); 7.11–7.18 (m, 2H), 7.28–7.45 (m, 3H) (aromatics)
(−)-**12**	0.91–1.44 (m, 6H), 1.53–2.06 (m, 6H); 2.06–2.54 (m, 3H) (CH_2 cyclohexane + CH_2 pyrrolidine); 2.72–2.81 (m, 1H, C*H*H–S); 3.23 (s, 3H, N–CH_3); 3.35–3.52 (m, 4H, N–CH_3 + CH*H*–S); 3.78–3.91 (m, 1H, CH*H*–N); 3.92–4.03 (m, 1H, CH*H*–N); 4.76–4.88 (m, 1H, CH–N); 5.03–5.11 (m, 1H, CH–O); 7.11–7.18 (m, 2H), 7.28–7.45 (m, 3H) (aromatics)
(+)-**13**	0.76–0.92 (m, 2H), 1.21–1.34 (m, 1H), 1.49–1.90 (m, 7H) (CH_2 cyclohexane); 2.14–2.39 (m, 4H, CH cyclohexane + C*H*H–CH_2 pyrrolidine); 2.75 (dd, J = 15.2 Hz, J = 4.2 Hz, 1H, C*H*H–S); 2.81–2.92 (m, 1H, C*H*H pyrrolidine); 3.03–3.10 (m, 1H, C*H*H–S); 3.47 (s, 3H, N–CH_3), 3.49 (s, 3H, N–CH_3), 3.67–3.75 (m, 1H, C*H*H–N); 3.81–3.88 (m, 1H, CH*H*–N); 4.75–4.83 (m, 1H, CH–O); 4.87–4.93 (m, 1H, CH–N); 7.11–7.18 (m, 2H), 7.35–7.41 (m, 3H) (aromatics)
(−)-**13**	0.76–0.92 (m, 2H), 1.21–1.34 (m, 1H), 1.49–1.90 (m, 7H) (CH_2 cyclohexane); 2.14–2.39 (m, 4H, CH cyclohexane + C*H*H–CH_2 pyrrolidine); 2.75 (dd, J = 15.2 Hz, J = 4.2 Hz, 1H, C*H*H–S); 2.81–2.92 (m, 1H, C*H*H pyrrolidine); 3.03–3.10 (m, 1H, C*H*H–S); 3.47 (s, 3H, N–CH_3), 3.49 (s, 3H, N–CH_3), 3.67–3.75 (m, 1H, C*H*H–N); 3.81–3.88 (m, 1H, CH*H*–N); 4.75–4.83 (m, 1H, CH–O); 4.87–4.93 (m, 1H, CH–N); 7.11–7.18 (m, 2H), 7.35–7.41 (m, 3H) (aromatics)
(−)-**14**	0.76–0.98 (m, 3H), 1.23–1.42 (m, 3H), 1.56–1.90 (m, 5H), 2.18–2.44 (m, 4H) (cyclohexane + CH_2 pyrrolidine); 3.16–3.22 (m,1H, CH*H*–N); 3.42 (s, 3H, N–CH_3); 3.49–3.57 (m, 1H, CH*H*–S); 3.61 (s, 3H, N–CH_3); 3.95–4.05 (m, 1H, CH*H*–N); 4.05–4.16 (m, 1H, CH*H*–S); 4.86–4.96 (m, 1H, CH–N); 5.51–5.58 (m, 1H, CH–O); 7.23–7.52 (m, 5H, aromatics)
(+)-**14**	0.76–0.98 (m, 3H), 1.23–1.42 (m, 3H), 1.56–1.90 (m, 5H), 2.18–2.44 (m, 4H) (cyclohexane + CH_2 pyrrolidine); 3.16–3.22 (m,1H, CH*H*–N); 3.42 (s, 3H, N–CH_3); 3.49–3.57 (m, 1H, CH*H*–S); 3.61 (s, 3H, N–CH_3); 3.95–4.05 (m, 1H, CH*H*–N); 4.05–4.16 (m, 1H, CH*H*–S); 4.86–4.96 (m, 1H, CH–N); 5.51–5.58 (m, 1H, CH–O); 7.23–7.52 (m, 5H, aromatics)
(−)-**15**	0.78–1.36 (m, 4H), 1.55–1.93 (m, 7H), 2.13–2.28 (m, 2H), 2.28–2.44 (m, 1H), 2.66–2.78 (m, 1H), (cyclohexane + CH_2–CH_2 pyrrolidine); 2.95 (dd, J = 15.2 Hz, J = 5.0 Hz, 1H, C*H*H–S); 3.60 (s, 3H, N–CH_3); 3.62 (s, 3H, N–CH_3); 3.76–3.87 (m, 1H,CH*H*–N); 3.88–3.99 (m, 1H, CH*H*–N); 4.20 (dd, J = 15.2 Hz, J = 9.6 Hz, 1H, CH*H*-S); 4.75–4.82 (m, 1H, CH–N); 5.50–5.61 (m, 1H, CH–O); 7.22–7.50 (m, 5H, aromatics)
(+)-**15**	0.78–1.36 (m, 4H), 1.55–1.93 (m, 7H), 2.13–2.28 (m, 2H), 2.28–2.44 (m, 1H), 2.66–2.78 (m, 1H), (cyclohexane + CH_2–CH_2 pyrrolidine); 2.95 (dd, J = 15.2 Hz, J = 5.0 Hz, 1H, C*H*H–S); 3.60 (s, 3H, N–CH_3); 3.62 (s, 3H, N–CH_3); 3.76–3.87 (m, 1H,CH*H*–N); 3.88–3.99 (m, 1H, CH*H*–N); 4.20 (dd, J = 15.2 Hz, J = 9.6 Hz, 1H, CH*H*–S); 4.75–4.82 (m, 1H, CH–N); 5.50–5.61 (m, 1H, CH–O); 7.22–7.50 (m, 5H, aromatics)

[a] In CD_3OD.

phosphate buffer containing 5 mM $MgCl_2$ at pH 7.4. Final membrane protein concentrations were 30 μg/mL (m1), 70 μg/mL (m2), 25 μg/mL (m3), 50 μg/mL (m4), and 25 μg/mL (m5).

In homologous competition curves [^{3}H]NMS was present at 0.2 nM in tubes containing increasing concentration of unlabeled NMS (0.03–1000 nM) and at 0.075–0.2 nM in tubes without unlabeled ligand. In heterologous competition curves, fixed concentrations of the tracer (0.2 nM) were displaced by increasing concentrations of several unlabeled ligands (0.01–1000 μM); all measurements were obtained in duplicate. At the end of the binding reaction, free radioligand was separated from bound ligand by rapid filtration through UniFilter GF/C plates (Perkin-Elmer Life Science, Boston, MA) using a FilterMate Cell Harvester (Perkin-Elmer Life Science, Boston, MA); after filtration, the filters were washed several times with ice cold buffer and allowed to dry overnight at room temperature under a flow of air, added of 25 μL of scintillation liquid (Microscint-20, Perkin-Elmer Life Science, Boston, MA) and counted by TopCount NXT Microplate Scintillation Counter (Perkin-Elmer Life Science, Boston, MA). The binding data were analyzed by the weighted least-squares iterative curve fitting LIGAND[32] to obtain the affinity constant (K_i) and the binding capacity (B_{max}).

5.4.2. Functional studies. 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. Dose–response curves were constructed by cumulative addition of the reference agonist. The concentration of agonist in the organ bath was increased approximately threefold at each step, with each addition being made only after the response to the previous addition

Table 6. Crystal data and structure refinement for (+)-**4** oxalate

Formula	$C_{22}H_{25}NO_6$ S
Formula weight	431.5
Space group	$P2_12_12_1$
Unit cell dimensions	
a (Å)	8.3784(11)
b (Å)	10.2172(9)
c (Å)	24.425(3)
Volume (Å ^{3})	2090.8(4)
Z	4
Density (calcd) (g/cm^3)	1.371
Abs coeff, (mm^{-1})	0.194
Radiation	MoKα
Temperature (K)	100
Theta range for data collection (°)	4.58–28.55
Reflns collected	12825
No. of unique reflns	4577
Completeness (%)	89.2
Value of R_{int}	0.0330
Absolute structure parameter	0.08(11)
Final R indices [$I > 2$sigma(I)]	R_1 = 0.0567, wR_2 = 0.1427
R indices (all data)	R_1 = 0.0742, wR_2 = 0.1567
Largest diff. peak and hole, e Å $^{-3}$	0.588 and −0.298

had attained a maximal level and remained steady. Following 30 min of washing, tissues were incubated with the antagonist for 60 min, and a new dose–response curve to the agonist was obtained. Responses were expressed as a percentage of the maximal response obtained in the control curve. Contractions were recorded by means of a force displacement transducer connected to the MacLab system PowerLab/800. In addition parallel experiments in which tissues did not receive any antagonist were run in order to check any variation in sensitivity. In all cases, parallel experiments in which tissues received only the reference agonist were run in order to check any variation in sensitivity.

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

5.4.2.1. Guinea pig ileum. This preparation was set up according to Bolognesi et al.[33] Two-centimeter-long portions of terminal ileum were taken at about 5 cm from the ileum–cecum junction. The tissue was cleaned and the ileum longitudinal muscle was separated from the underlying circular muscle that was mounted in PSS of the following composition (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) and glucose (11.7), at 37 °C. Tension changes were recorded isotonically. Tissues were equilibrated for 30 min, and dose–response curves to arecaidine propargyl ester (APE) were obtained at 30 min intervals, the first one being discarded and the second one being taken as the control.

5.4.2.2. Guinea pig stimulated left atria. This preparation was set up according to Leung and Mitchelson.[34] 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 APE was constructed.

5.4.2.3. Guinea pig lung strips. This preparation was set up according to Scapecchi et al.[17] The lungs were rapidly removed and strips of peripheral lung tissue were cut either from the body of a lower lobe with the longitudinal axis of the strip parallel to the bronchus or from the peripheral margin of the lobe. The preparations were mounted, with a preload of 0.3 g, in PSS with the following composition (mM): NaCl (118.78), KCl (4.32), $CaCl_2.2H_2O$ (2.52), $MgSO_4.7H_2O$ (1.18), KH_2PO_4 (1.28), $NaHCO_3$ (25), glucose (5.55). Contractions were recorded isotonically at 37 °C after tissues were equilibrated for 1 h, then two cumulative dose–response curves to APE (0.01, 0.1, 1, 10, 100 μM) were obtained at 45-min intervals, the first one being discarded and the second one being taken as the control.

5.4.2.4. Rabbit stimulated vas deferens. This preparation was set up according to Eltze.[35] Vasa deferentia were carefully dissected free of the 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); 10^{-6} M yohimbine, and 10^{-8} M tripitramine were included to block alpha$_2$-adrenoreceptors and M_2 muscarinic receptors, respectively. The solution was maintained at 30 °C and tissues were stimulated through platinum electrodes by square-wave pulses (0.1 ms, 2 Hz, 10–15 V). Contractions were measured isometrically after tissues were equilibrated for 1 h, then a cumulative dose–response curve to pCl-McN-A-343 was constructed.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2008.04.013.

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