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Properly substituted 1,4-dioxane nucleus favours the selective M_3 muscarinic receptor activation

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ABSTRACT

Novel analogues of *cis*-*N*,*N*,*N*-trimethyl-(6-methyl-1,4-dioxan-2-yl)methanaminium iodide (**2a**) were synthesized by inserting methyl groups alternatively or simultaneously in positions 5 and 6 of the 1,4-dioxane nucleus in all combinations. Their biological profile was assessed by receptor binding assays at human muscarinic M_1–M_5 receptors stably expressed in CHO cells and by functional studies performed on classical isolated organ preparations, namely, rabbit electrically stimulated vas deferens, and guinea pig electrically stimulated left atrium, ileum, and lung strips. The results showed that the simultaneous presence of one methyl group in both positions 5 and 6 with a *trans* stereochemical relationship with each other (diastereomers **4** and **5**) or the geminal dimethylation in position 6 (compound **8**) favour the selective activation of M_3 receptors. Compounds **4**, **5**, and **8** might be valuable tools in the characterization of the M_3 receptor, as well as provide useful information for the design and development of novel selective M_3 antagonists.

1. Introduction

Muscarinic receptors belong to membrane-bound acethylcholine (ACh) receptors and are members of the family of heterotrimeric G protein-coupled receptors which, upon activation by ACh, initiate an intracellular chemical pathway to transduce, propagate, and amplify the received signals.[1] Their characteristic common structural elements are the seven transmembrane (7TM) helical segments. On the basis of genetic and pharmacological characterizations they have been classified into five subtypes, termed M_1–M_5.[2] Muscarinic receptors are widely distributed in both the central and the peripheral nervous systems, in parasympathetic innervated organs and in several non-innervated tissues, and play a critical role in a wide range of diseases, including cognitive function disorders[3] such as Alzheimer's disease,[4] schizophrenia,[5] and Parkinson's disease,[6] as well as visceral disorders[7] such as overactive bladder,[8] irritable bowel syndrome,[9] and chronic obstructive pulmonary disease.[10] Moreover, the muscarinic system regulates glucose-induced insulin secretion; in particular, functional studies have revealed that the M_3 subtype is involved in the insulinotropic effect of ACh.[11] Recently, it has been reported that ACh is synthesized and released in lung and other multiple cancers and acts as an autocrine growth factor, suggesting that this cholinergic autocrine loop may provide new significant therapeutic opportunities.[12]

Though several muscarinic agonists and antagonists have been synthesized, their therapeutic utility is limited by various side effects due to the lack of a marked subtype-discrimination.

The modest results obtained with muscarinic agonists might be due to the high sequence identity of the TM regions among the five human muscarinic receptor subtypes[13] and to the 'particularly sensitive' structure of the muscarinic agonists, whose small chemical modifications often cause a decrease in activity, albeit not a total loss. In both cases, the major problem has been the insufficient selectivity of the molecules studied to date. Therefore, the discovery of novel selective agonists might improve the characterization of muscarinic receptors and facilitate identification of therapeutic agents potentially useful in different pathological states.

Muscarine is the specific agonist for which muscarinic receptors have been named (Fig. 1). Other pentatomic cyclic compounds, such as *cis*-*N*,*N*,*N*-trimethyl-(2-methyl-1,3-dioxolan-4-yl)methanaminium iodide (**1a**), are potent non-selective muscarinic agonists.[14] Recently, we have reported that the enlargement of the 1,3-dioxolane nucleus of **1a** by inserting a methylene group between the 1-oxygen atom and the 2-carbon atom afforded the

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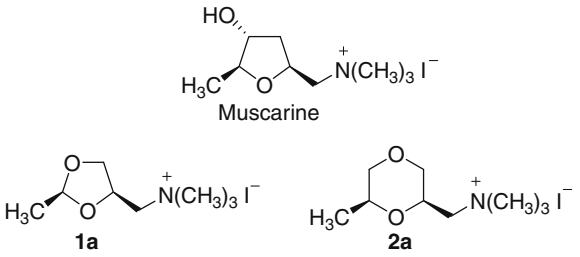


Figure 1. Chemical structures of muscarine and compounds **1a** and **2a**.

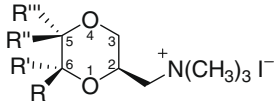
higher homologue *cis-N,N,N*-trimethyl-(6-methyl-1,4-dioxan-2-yl)methanaminium iodide (**2a**), which in functional assays showed the same cholinergic profile as **1a**.[15]

The hexacyclic 1,4-dioxane structure of compound **2a**, compared to the 1,3-dioxolane one of its lower homologue **1a**, has the advantage of offering the carbon 5 as an additional position where it is possible to introduce chemical functions potentially able to modulate the selectivity of the ligand. Therefore, in the present study, methyl groups have alternatively or simultaneously been inserted in positions 5 and 6 of the 1,4-dioxane nucleus in all combinations, in order to investigate their role on muscarinic potency and subtype selectivity (compounds **3**–**12**) (Fig. 2).

The muscarinic profile of the novel compounds has been evaluated through radioligand binding and functional assays.

2. Chemistry

Diastereomers **3a** and **3b** were prepared following the synthetic procedure reported in Scheme 1. The reaction of glycerine with 2-chloro-1,1-dimethoxypropane gave the corresponding diastereomeric mixture of [2-(1-chloro-ethyl)-[1,3]dioxolan-4-yl]-methanol (**13**)[16] (*cis*/*trans* ratio = 1:1), whose treatment with KOH afforded the corresponding 3,6,8-trioxa-bicyclo[3.2.1]octane **14**. The subsequent regiospecific opening[17] with $LiAlH_4$ and $AlCl_3$ yielded a mixture of (2*R**,5*R**)-(5-methyl-[1,4]dioxan-2-yl)-methanol and (2*R**,5*S**)-(5-methyl-[1,4]dioxan-2-yl)-methanol (**15a** and **15b**, respectively), whose diastereomers were separated by column chromatography. Finally, reaction of the alcohols **15a** and **15b** with tosyl chloride and subsequent amination with dimethylamine provided the corresponding amines **17a** and **17b**, which were treated with methyl iodide to give the methiodides **3a** and **3b**, respectively.



3a, R = R' = R''' = H, R'' = CH_3
3b, R = R' = R'' = H, R''' = CH_3
4, R = R''' = CH_3, R' = R'' = H
5, R = R''' = H, R' = R'' = CH_3
6, R = R'' = H, R' = R''' = CH_3
7, R = R'' = CH_3, R' = R''' = H
8, R = R' = CH_3, R'' = R''' = H
9, R = R' = H, R'' = R''' = CH_3
10a, R = R'' = R''' = CH_3, R' = H
10b, R = H, R' = R'' = R''' = CH_3
11a, R = R' = R'' = CH_3, R''' = H
11b, R = R' = R''' = CH_3, R'' = H
12, R = R' = R'' = R''' = CH_3

Figure 2. Chemical structures of compounds **3–12**.

The *trans* stereochemical relationship between the 2-side chain and the 5-methyl group of **3b** was assigned through single-crystal X-ray diffraction analysis (Fig. 3).

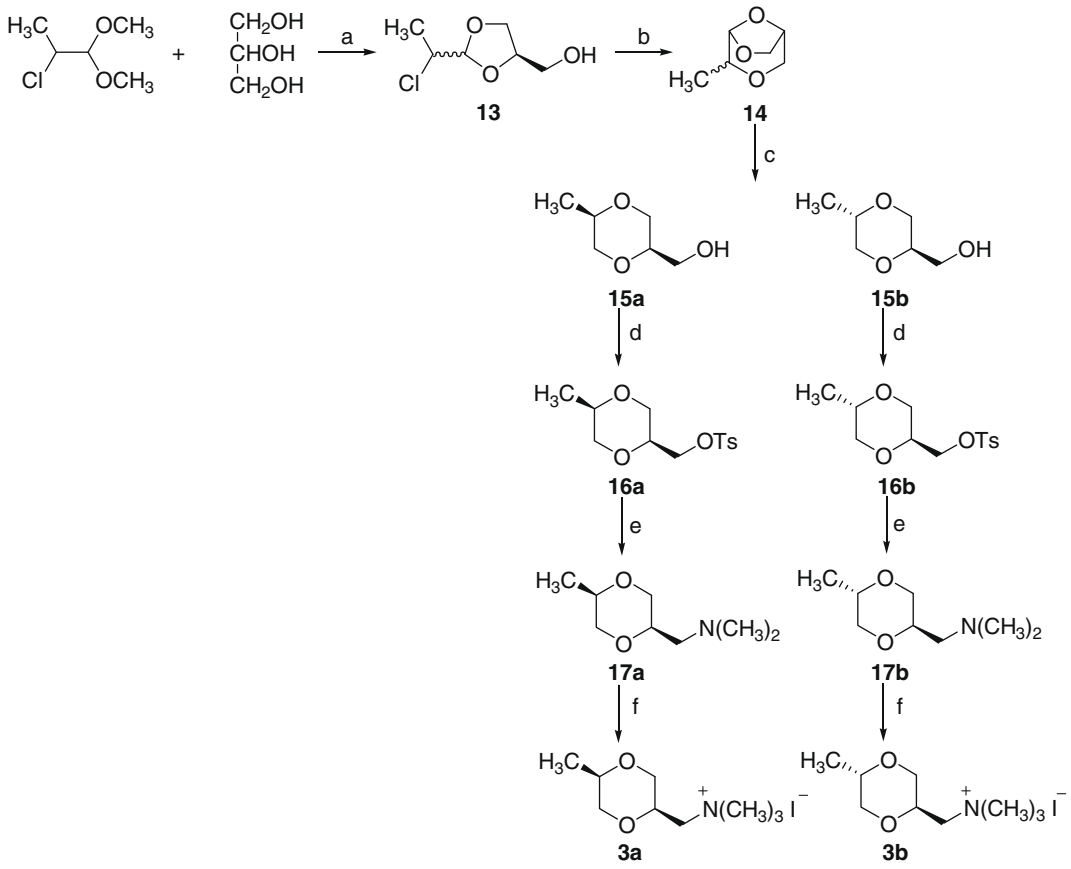
The synthetic procedure described for compounds **3a** and **3b**, through the opening of the intermediate 4,5-dimethyl-3,6,8-trioxabicyclo[3.2.1]octane,[18] proved poorly suited to synthesize the stereoisomers **4**–**7**, because it was very difficult to separate the mixture of diastereomers and to obtain sufficient amounts of each of them. Therefore, for compounds **4**–**7** we followed the same method we recently described for the synthesis of the enantiomers of **2a** and **2b** (Scheme 2).[19] Two parallel divergent synthetic procedures were carried out starting from (2*R**,3*S**)- and (2*R**,3*R**)-2,3-dimethyloxirane to prepare the pairs of diastereomers **4**/**5**, and **6**/**7**, respectively. The preparation of (2*R**,3*R**)- (**18a**) and (2*R**,3*S**)-3-allyloxy-butan-2-ol (**18b**) was readily accomplished in basic conditions by treating (2*R**,3*S**)- and (2*R**,3*R**)-2,3-dimethyl-oxirane, respectively, with Na and allyl alcohol. The structure of β-hydroxy allyl ethers **18a** and **18b** was assigned by the chemical shift of the OH proton observed in the relative ^{1}H NMR spectra.[20] The intermediate alcohol **18a** was subjected to oxymercuration–demercuration reaction with mercury(II) acetate followed by an aqueous solution of iodine and potassium iodide which stereoselectively afforded a mixture of the diastereomeric forms (2*S**,3*S**,5*S**)- and (2*S**,3*S**,5*R**)-5-iodomethyl-2,3-dimethyl-[1,4]-dioxane (**19a** and **19b**, respectively), which were separated by column chromatography. The amination of **19a** and **19b** with dimethylamine followed by treatment with an ethereal solution of methyl iodide yielded the methiodides **4** and **5**, respectively. Analogously, diastereomers **6** and **7** were obtained from (2*R**,3*S**)-3-allyloxy-butan-2-ol (**18b**).

The structure of the compounds **4**–**7** was determined at the level of the iododerivative intermediates by ^{1}H NMR spectral studies. It is known that in six-membered rings, the chemical shift difference between the axial and equatorial protons is of about 0.5 ppm;[21] the same behaviour occurs for axial and equatorial methyl groups.[22]

The stereoselective cyclization of **18a** produced only two (**19a** and **19b**, ratio 7:3) of the four 5-iodomethyl-2,3-dimethyl-[1,4]-dioxane diastereomers with a *trans* stereochemical relationship between the methyl groups in positions 2 and 3. From the ^{1}H NMR spectra of **19a** and **19b** it can be deduced that, due to their similar chemical shift (δ 1.08 ppm and 1.14 ppm), the two methyl groups are in *trans* equatorial position. In the ^{1}H NMR spectrum of **19a**, the precursor of **4**, the axial C6–H at δ 3.28 ppm shows two large coupling constants (J = 10.26 Hz and J = 11.11 Hz), one with the equatorial geminal proton at δ 3.98 ppm and the other with the axial C5–H at δ 3.64 ppm. Moreover, an evident nuclear Overhouse effect (NOE) between the axial protons C5–H at δ 3.64 ppm and C3–H at δ 3.34 ppm was observed, and the equatorial position of the two methyl groups in positions 2 and 3 was confirmed. In conclusion, all three substituents of compound **19a** are in equatorial position.

In the ^{1}H NMR spectrum of compound **19b**, the proton in position 5 is equatorial because none of the protons in position 6 shows a large coupling constant with it. Also the two methyl groups are equatorial because they show the same chemical shift values as in compound **19a** (δ 1.08 ppm and 1.14 ppm) and because an evident NOE effect between the axial protons C6–H at δ 3.80 ppm and C2–H at δ 3.20 ppm was observed. Therefore, the precursors of the two diastereomers **4** and **5** were assigned the structures reported in Figure 4.

By stereoselective cyclization of (2*R**,3*S**)-3-allyloxy-butan-2-ol (**18b**) only the two diastereomers **19c** and **19d** with a *cis* stereochemical relationship between the methyl groups in positions 2 and 3 were obtained. In this case, one methyl group is in axial position and the other is in equatorial position. In fact, in the ^{1}H NMR



Scheme 1. Reagents: (a) TsOH, toluene; (b) KOH powder; (c) $LiAlH_4$, $AlCl_3$, diethyl ether; (d) *p*-TsCl, pyridine; (e) Me_2NH, benzene; (f) CH_3I, diethyl ether.

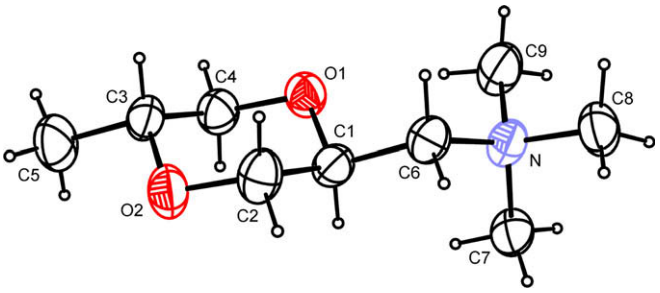


Figure 3. A view of the cation in the X-ray structure of **3b**; 30% probability ellipsoids are shown.

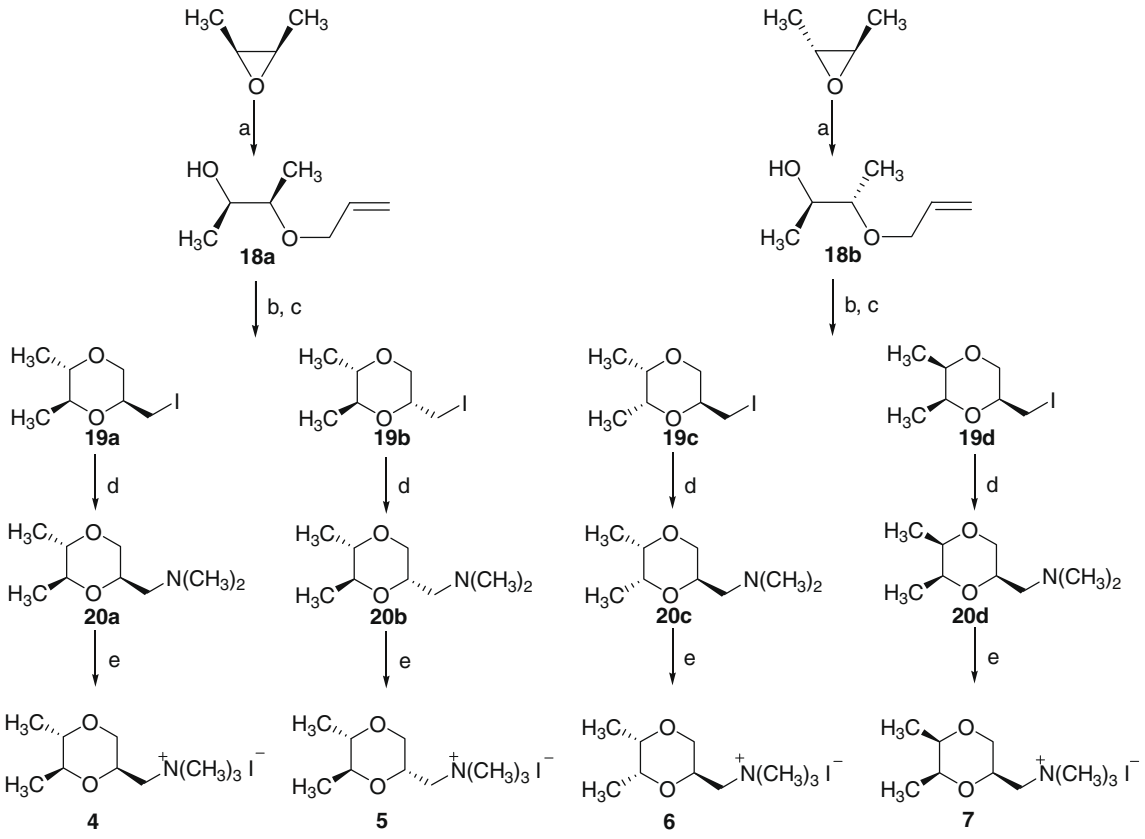
spectra, a difference of about 0.30 ppm in the chemical shift of the two methyl groups was observed. The *trans* stereochemical relationship between the chain in 5 and the methyl substituents in positions 2 and 3 of **19c** was confirmed by an evident NOE effect between the axial C5–H at δ 3.78 ppm and the 3-methyl protons at δ 1.29 ppm; no NOE effect was observed between the same protons of the diastereomer **19d**. Moreover, in compound **19d** an evident NOE effect between the axial proton C6–H at δ 3.47 ppm and the 2-methyl protons at δ 1.40 ppm was also observed. Therefore, the precursors of the two diastereomers **6** and **7** were assigned the structures reported in Figure 5.

Compounds **8**–**12** were obtained following the synthetic procedure shown in Scheme 3. The opening of 2,2-dimethyloxirane in the presence of Na afforded 1-allyloxy-2-methyl-propan-2-ol (**21**).[23] Intermediates **22**, **23**, and **25** were obtained by opening the appropriate oxirane in the presence of $HClO_4$. Treatment of 2-allyloxy-propionic acid methyl ester[24] with CH_3MgI furnished 3-allyloxy-2-methyl-butan-2-ol (**24**). The reaction of **21**–**25** with mercury(II) acetate followed by treatment with potassium iodide and iodine gave the intermediate iodo-derivatives **26**–**30**, whose amination with dimethylamine and subsequent treatment with methyl iodide afforded compounds **8**–**12**.

The structures of diastereomers **10a**, **10b**, **11a**, and **11b** were assigned by comparing their ^{1}H NMR spectra with those of the corresponding 6- and 5-methyl analogues **2a**, **2b** and **3a**, **3b**, respectively, whose structures were assigned by X-ray crystallography. Similarly to the case of the tertiary amines of compounds **2a** and **2b**,[15] the chemical shift value of the methylene group of the 2-side chain in the *cis* diastereomer **33a** (δ 2.14–2.43 ppm) is at higher fields than those of the *trans* diastereomer **33b** (δ 2.39–2.69 ppm). In the **11a**/**11b** pair, the chemical shift of the axial methyl group in position 5 of diastereomer **11a** (δ 1.21 ppm) is at lower fields than those of the equatorial methyl group of *trans* analogue **11b** (δ 0.99 ppm), analogously to the **3a**/**3b** pair (δ 1.10 and 0.99 ppm, respectively). Moreover, during the separation of the mixture of 6-methyl substituted stereoisomers by column chromatography, the *cis* diastereomers eluted first, whereas the opposite occurred for the mixture of 5-methyl substituted stereoisomers.

3. Results and discussion

The muscarinic pharmacological profile of all methiodides was evaluated by receptor binding assays following a previously described protocol.[25] (2-Hydroxyethyl)trimethylammonium chloride carbamate (carbachol) and 4-[*N*-(3-chlorophenyl)carbamoyloxy]-2-butynyltrimethylammonium chloride (McN-A-343) were used as reference compounds. [^{3}H]*N*-methylscopolamine ([^{3}H]NMS)



Scheme 2. Reagents: (a) Na, allyl alcohol; (b) $(CH_3COO)_2Hg$; (c) KI, I_2; (d) Me_2NH, benzene; (e) CH_3I, diethyl ether.

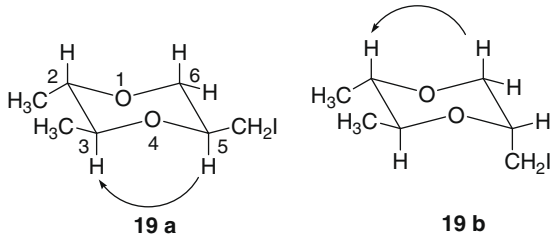


Figure 4. NOE correlations of compounds **19a** and **19b**.

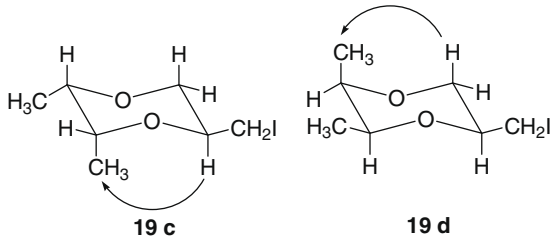


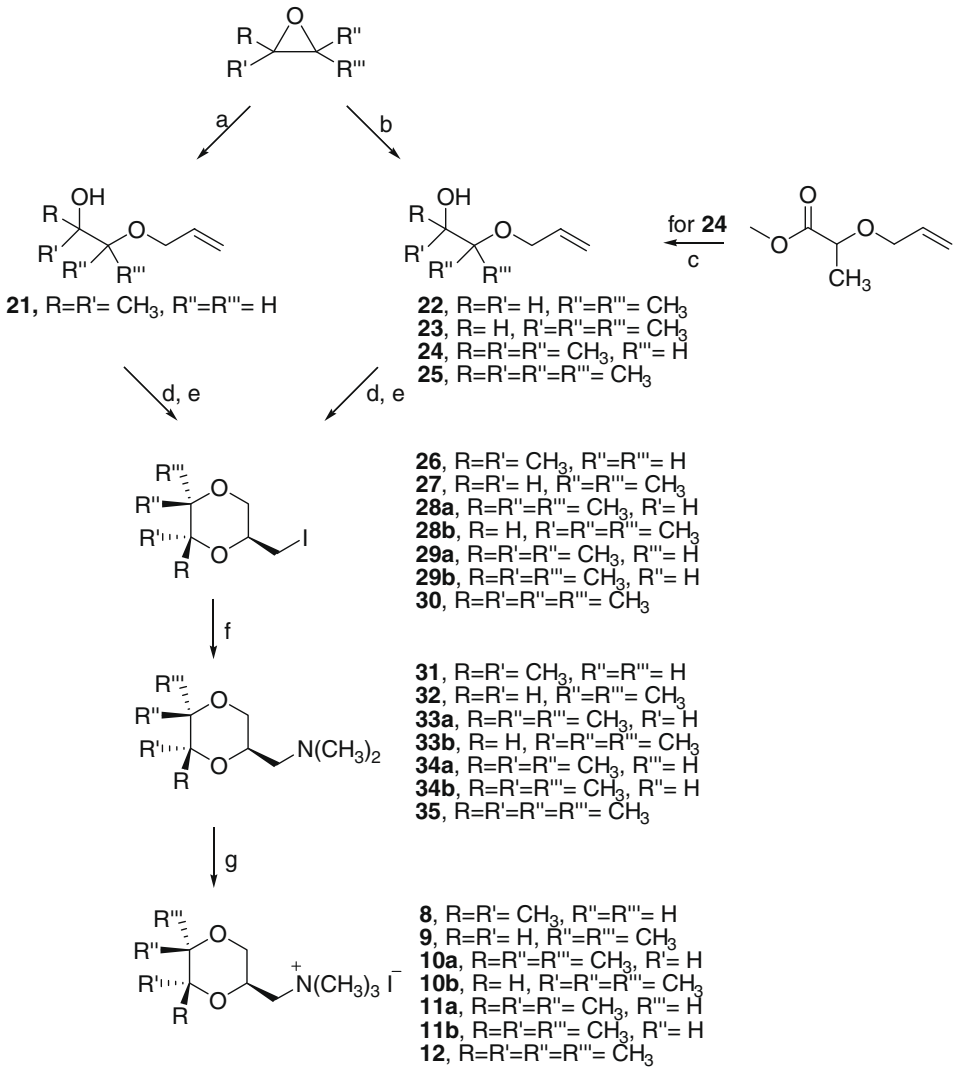
Figure 5. NOE correlations of compounds **19c** and **19d**.

was the radioligand used to label cloned human muscarinic hM_1–hM_5 receptors, expressed in Chinese hamster ovary (CHO) cells. Affinity values, expressed as pK_i, are reported in Table 1.

Moreover, the muscarinic activity of compounds **3–12** was evaluated by functional studies performed on classical isolated organ preparations, namely, rabbit electrically stimulated vas deferens,[26] and guinea pig electrically stimulated left atrium,[27] ileum,[28] and lung strips.[29] It is known that negative inotropic responses to muscarinic agonists in guinea pig atria are directly mediated by M_2 muscarinic receptors,[30] and that the contractions of isolated strips of ileum are primarily mediated by M_3 receptor activation.[31] Instead, the inhibition of electrically induced contractions of rabbit vas deferens has for long been considered an effect mediated by M_1-receptor subtypes,[26] whereas more recent studies attribute this effect to M_4 receptor stimulation.[32] Similarly, the validity of the guinea pig lung strips as an M_4 model[33] has been questioned.[34] Therefore, in the present paper we will use the terms vas deferens and lung muscarinic receptor models for the latter two preparations. The functional data obtained for the new compounds, reported in Table 2, are expressed as pD_2 ($-\log ED_{50}$, agonist potency), or as pK_B ($-\log K_B$, antagonist potency), and as α (intrinsic activity). Carbamyl-β-methylcholine chloride (bethanechol) and McN-A-343 were included in the study as reference compounds.

From an analysis of the binding data of compounds **3**–**12**, reported in Table 1, it can be observed that all tested compounds show low affinity values for all muscarinic subtypes. However, compounds **3a**, **5**, and **7** are particularly interesting, because, analogously to the leads **2a** and **2b**, they show significant selectivity for the M_2 subtype compared to the other muscarinic subtypes.

Based on our previous experience and on literature data,[19,35] which showed that poor binding affinity would not prevent good functional activity, we tested the compounds on functional models of muscarinic receptors, since discrepancies between binding studies and functional activity of agonists are quite common.[36] The apparent discrepancy between binding and functional data is accounted for by the species difference in receptor targets and by the type of information collected in each experimental model, that is, on the one hand, affinity data to human receptors, collected in binding assays, and, on the other, evaluation of the agonistic potency (which depends on the intrinsic activity of the compounds in addition to their affinity) in isolated animal tissues.



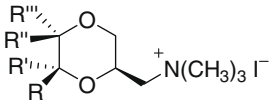
Scheme 3. Reagents: (a) Na, allyl alcohol; (b) $HClO_4$, allyl alcohol; (c) CH_3MgBr; (d) $(CH_3COO)_2Hg$; (e) KI, I_2; (f) Me_2NH, benzene; (g) CH_3I, diethyl ether.

An analysis of the functional data reported in Table 2 shows that, unlike the lead compounds **2a** and **2b**, none of the compounds tested on rabbit vas deferens activates the muscarinic receptors of this tissue. Concerning M_2 and M_3 subtypes and the guinea pig lung model, all the compounds behave as full or partial agonists with the exception of compound **10b**, which is unable to bind the muscarinic receptors at concentrations up to 30 μM, and of compound **11a**, which behaves as a weak antagonist at guinea pig lung muscarinic sites. The shift of the methyl group from position 6 to 5 of the 1,4-dioxane nucleus is detrimental to muscarinic activity: compounds **3a** and **3b** are less potent than the corresponding 6-methyl derivatives **2a** and **2b** at M_2 and M_3 receptors and less efficacious in the lung tissue. Moreover, unlike the **2a**/**2b** pair, where the *cis* diastereomer **2a** is more potent than the *trans* **2b**, the stereochemical relationship between the 2-side chain and the 5-methyl group does not influence the activity at any muscarinic preparation, the diastereomers **3a** and **3b** showing similar potency profiles.

The simultaneous presence of one methyl group in both positions 5 and 6 furnishes the diastereomers **4**–**7**. All four diastereomers show similar potency values at M_2, whereas the diastereomers **4** and **5**, with a *trans* stereochemical relationship between the methyl groups in positions 5 and 6, are more active at M_3 receptors than the diastereomers **6** and **7**, in which a *cis* stereochemical relationship exists between the same substituents. Interestingly, the two compounds **4** and **5**, which exhibit pD_2 values at M_3 receptors (6.95 and 6.70, respectively) significantly higher than those at M_2 ones (4.93 and 5.01, respectively) display good selectivity for the M_3 subtype (M_3/M_2 = 105 and 49, respectively). In addition, compounds **4** and **5**, showing respectively 74- and 141-fold lower potency in the guinea pig lung, prove to be selective for M_3 sites also in comparison with the muscarinic receptors of this tissue. On the contrary, no discrimination between M_2 and M_3 subtypes is observed in the case of compound **7**, while derivative **6** combines a full agonist activity at ileal tissue with only a weak efficacy at M_2 receptors. In the lung model, the diastereomers **4**, **5**, and **7** behave as partial agonists; diastereomer **6** can neither activate nor block the muscarinic receptors of this tissue at concentrations up to 10 μM.

Of note is the effect produced by the geminal dimethylation; in fact, the 5,5-dimethyl derivative **9** behaves as a weak partial agonist at both M_3 and lung muscarinic receptors (pD_2 = 4.91 and 4.85, respectively) and is inactive at the other subtypes, while, interestingly, the 6,6-dimethyl analogue **8** fully activates the M_3 subtype, being selective for this subtype with respect to M_2, lung and vas deferens muscarinic receptors. This observation indicates that, unlike the case of the dimethyl analogue of the dioxolane **1a**,[37] the presence of two methyl groups in position 6 is still well tolerated by the corresponding M_3 receptor site, compound **8**

Table 1
Affinity constants, expressed as pK_i[a] of compounds **2–12**, carbachol, and McN-A-343 for human cloned muscarinic receptors, expressed in CHO cells



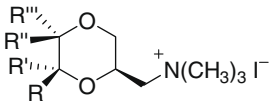
Compd	R	R′	R″	R‴	hM_1	hM_2	hM_3	hM_4	hM_5
					pK_i[a]				
2a	CH_3	H	H	H	4.52 ± 0.07	5.80 ± 0.10	5.12 ± 0.11	4.94 ± 0.10	5.11 ± 0.07
2b	H	CH_3	H	H	<4	5.31 ± 0.10	4.45 ± 0.09	4.06 ± 0.07	4.17 ± 0.06
3a	H	H	CH_3	H	<4	4.95 ± 0.10	4.18 ± 0.10	4.03 ± 0.05	4.03 ± 0.06
3b	H	H	H	CH_3	<4	4.76 ± 0.11	4.35 ± 0.12	<4	4.20 ± 0.08
4	CH_3	H	H	CH_3	4.61 ± 0.08	4.86 ± 0.11	4.96 ± 0.11	4.51 ± 0.08	4.81 ± 0.07
5	H	CH_3	CH_3	H	<4	4.78 ± 0.10	<4	<4	<4
6	H	CH_3	H	CH_3	<4	4.05 ± 0.10	<4	<4	<4
7	CH_3	H	CH_3	H	<4	4.70 ± 0.10	<4	<4	4.01 ± 0.08
8	CH_3	CH_3	H	H	4.32 ± 0.07	4.74 ± 0.11	4.67 ± 0.11	4.35 ± 0.07	4.55 ± 0.08
9	H	H	CH_3	CH_3	<4	4.02 ± 0.09	4.11 ± 0.11	<4	<4
10a	CH_3	H	CH_3	CH_3	4.17 ± 0.08	4.22 ± 0.08	4.49 ± 0.09	4.24 ± 0.10	4.28 ± 0.09
10b	H	CH_3	CH_3	CH_3	4.02 ± 0.08	4.14 ± 0.10	4.33 ± 0.08	4.06 ± 0.08	4.08 ± 0.07
11a	CH_3	CH_3	CH_3	H	4.70 ± 0.07	4.56 ± 0.09	4.79 ± 0.09	4.59 ± 0.09	4.49 ± 0.09
11b	CH_3	CH_3	H	CH_3	4.42 ± 0.07	4.47 ± 0.06	4.98 ± 0.08	4.52 ± 0.07	4.78 ± 0.06
12	CH_3	CH_3	CH_3	CH_3	<4	4.33 ± 0.08	4.39 ± 0.08	4.27 ± 0.07	4.18 ± 0.06
Carbachol					4.42 ± 0.09	5.91 ± 0.06	4.36 ± 0.10	5.20 ± 0.07	4.16 ± 0.07
McN-A-343					5.71 ± 0.09	5.49 ± 0.07	5.62 ± 0.09	5.04 ± 0.07	5.42 ± 0.06

[a] The values represent the arithmetic mean ± SEM of at least three experiments.

showing a pD_2 value (pD_2 = 6.94) of the same order of magnitude of compound **2a** (pD_2 = 7.34). Instead, this modification is detrimental for M_2 and the other two muscarinic receptor models, making compound **8** an interesting novel selective M_3 subtype agonist (M_3/vas deferens >275; M_3/M_2 = 81; M_3/lung = 123). The decline of muscarinic profile produced by 5,5-dimethyl substitution is not improved by the introduction of a methyl group in position 6. In fact, the functional responses at M_2 and M_3 receptors are only scarcely ameliorated when the methyl group is in *cis* stereochemical relationship with the 2-side chain (compound **10a**), and they are completely absent when the methyl group is in *trans* stereochemical relationship (compound **10b**).

When a third methyl group is inserted into position 5 of the 6,6-dimethyl substituted derivative, affording diastereomers **11a** and

Table 2
Potency values, expressed as pD_2 ($-\log ED_{50}$),[a] intrinsic activity (α)[b] and dissociation constant (pK_B)[c] of compounds **2–12**, and bethanechol in the isolated rabbit vas deferens, guinea pig left atrium (M_2), longitudinal ileum (M_3), and lung muscarinic receptors[d]



Compd	R	R′	R″	R‴	Rabbit vas deferens		Guinea pig atrium (M_2)		Guinea pig ileum (M_3)		Guinea pig lung	
					α	pD_2 (pK_B)	α	pD_2 (pK_B)	α	pD_2 (pK_B)	α	pD_2 (pK_B)
2a	CH_3	H	H	H	1.00 ± 0.01	5.65 ± 0.06	1.00 ± 0.01	7.57 ± 0.11	1.00 ± 0.01	7.34 ± 0.13	0.81 ± 0.07	5.90 ± 0.14
2b	H	CH_3	H	H	1.00 ± 0.01	5.08 ± 0.24	1.00 ± 0.01	6.66 ± 0.19	1.00 ± 0.01	6.02 ± 0.16	1.00 ± 0.01	6.01 ± 0.03
3a	H	H	CH_3	H		[g]	1.00 ± 0.01	5.44 ± 0.16	1.00 ± 0.01	5.71 ± 0.18	0.55 ± 0.26	5.13 ± 0.22
3b	H	H	H	CH_3		[g]	0.95 ± 0.06	5.75 ± 0.02	1.00 ± 0.01	5.55 ± 0.07	0.57 ± 0.07	5.08 ± 0.01
4	CH_3	H	H	CH_3		[f]	0.80 ± 0.08	4.93 ± 0.01	0.88 ± 0.02	6.95 ± 0.11	0.35 ± 0.15	5.08 ± 0.30
5	H	CH_3	CH_3	H		[e]	0.89 ± 0.02	5.01 ± 0.01	1.18 ± 0.23	6.70 ± 0.17	0.65 ± 0.15	4.59 ± 0.18
6	H	CH_3	H	CH_3		[f]	0.27 ± 0.09	5.04 ± 0.01	0.85 ± 0.11	4.91 ± 0.04		[g]
7	CH_3	H	CH_3	H		[e]	0.91 ± 0.01	5.18 ± 0.10	1.00 ± 0.01	5.27 ± 0.01	0.50 ± 0.10	5.00 ± 0.25
8	CH_3	CH_3	H	H		[f]	0.73 ± 0.01	5.03 ± 0.04	1.16 ± 0.05	6.94 ± 0.03	0.18±0.16	4.85 ± 0.30
9	H	H	CH_3	CH_3		[f]		[g]	0.68 ± 0.01	4.91 ± 0.02	0.22 ± 0.20	4.85 ± 0.30
10a	CH_3	H	CH_3	CH_3	0	(4.61 ± 0.20)	0.33 ± 0.07	4.92 ± 0.17	0.47 ± 0.14	5.12 ± 0.08		[g]
10b	H	CH_3	CH_3	CH_3		[f]		[f]		[f]		[g]
11a	CH_3	CH_3	CH_3	H		[f]		[g]	0.47 ± 0.11	5.25 ± 0.08	0	(5.15 ± 0.15)
11b	CH_3	CH_3	H	CH_3		[e]	0.46 ± 0.16	4.99 ± 0.01	0.66 ± 0.17	5.70 ± 0.04		[g]
12	CH_3	CH_3	CH_3	CH_3		[f]	0.71 ± 0.14	5.12 ± 0.01	0.93 ± 0.07	5.09 ± 0.16		[g]
McN-A-343					1.00 ± 0.01	6.40 ± 0.16						
Bethanechol							1.00 ± 0.01	5.88 ± 0.05	1.00 ± 0.01	6.16 ± 0.07	1.00 ± 0.01	5.01 ± 0.13

[a] pD_2 values are the negative logarithm of the agonist concentration that caused 50% of the maximum response attainable in that tissue.
[b] Intrinsic activity was measured by the ratio between the maximum response of the agonist and the maximum response of McN-A-343 at rabbit vas deferens, and bethanechol at guinea pig atrium, ileum and lung receptors.
[c] Dissociation constants were calculated according to Furchgott.[49]
[d] The results are the means ± SEM of four to six independent experiments.
[e] Not tested.
[f] This compound showed no agonist activity and, when tested as antagonist, proved inactive up to 30 μM.
[g] This compound showed no agonist activity and, when tested as antagonist, proved inactive up to 10 μM.

11b, the *cis* compound **11a** behaves as a partial agonist at M_3 receptor with a pD_2 value about 17-fold lower than that of the corresponding 6,6-dimethyl derivative **8**, and behaves as a weak antagonist on lung muscarinic receptors. No interaction with M_2 and vas deferens muscarinic receptors is observed. The *trans* isomer **11b** partially activates both M_2 and M_3 receptors and is inactive at the other tissues. Finally, the tetramethyl derivative **12**, as well, fails to activate vas deferens and lung muscarinic receptors, but behaves as a weak full and partial agonist at M_3 and M_2 receptors, respectively, with the same potency value (pD_2 = 5.09 and 5.12, respectively).

In conclusion, this study strengthens the effectiveness of the 1,4-dioxane nucleus as a suitable scaffold for designing active muscarinic agonists endowed, in some cases, with subtype-selectivity. It confirms the importance of the stereochemical relationship between the methyl substituent and the side chain, with the *cis* 6-methyl derivative **2a** adhering quite well to the rule of the fourth N-substituent governing the high efficacy of muscarinic agonists. Moreover, it highlights that the substituent in position 5 may be involved in the receptor interaction modulating the subtype-selectivity. In fact, among the polymethyl compounds, the presence of one methyl group in both positions 5 and 6 with a *trans* stereochemical relationship with each other (diastereomers **4** and **5**) favours the selective activation of the M_3 receptor subtype. Interestingly and surprisingly, the same subtype preference is produced by the geminal dimethylation in position 6 (compound **8**).

Finally, it is well known that the replacement of the methyl group of muscarinic agonists with bulkier groups modulates their profile from agonists to antagonists.[38] Therefore, compounds **4**, **5**, and **8** not only are valuable tools in the characterization of the M_3 receptor but also provide useful information for the design and development of novel selective M_3 antagonists.

4. Experimental

4.1. Chemistry

Melting points were taken in glass capillary tubes on a Büchi SMP-20 apparatus and are uncorrected. IR and ^{1}H NMR spectra were recorded on Perkin–Elmer 297 and Varian EM-390 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), d (doublet), t (triplet), q (quartet), or m (multiplet). Although the IR spectra data are not included because of the lack of unusual features, they were obtained for all compounds reported and were consistent with the assigned structures. The elemental compositions of the compounds were performed by the Microanalytical Laboratory of our department and agreed to within ±0.4% of the calculated value. When the elemental analysis was not included, crude compounds were used in the next step without further purification. Chromatographic separations were performed on silica gel columns (Kieselgel 40, 0.040–0.063 mm, Merck) by flash chromatography. Compounds were named following IUPAC rules as applied by Beilstein-Institut AutoNom (version 2.1), a software for systematic names in organic chemistry. Carbachol, McN-A-343, and bethanechol chloride were commercially available (Sigma).

4.1.1. (2*R**,4*R**)/(2*S**,4*R**)-[2-(1-Chloro-ethyl)-[1,3]dioxolan-4-yl]-methanol (13)

A solution of 2-chloro-1,1-dimethoxypropane (13.86 g, 100 mmol), propane-1,2,3-triol (12 g, 130 mmol), and *p*-toluenesulfonic acid (0.25 g, 1.5 mmol) in toluene (100 mL) was refluxed with vigorous stirring and in a Stark apparatus for 5 h. After cooling, the mixture was washed with K_2CO_3 saturated solution (50 mL) and dried over Na_2SO_4. Evaporation of the solvent gave a residue, which was purified by distillation under reduced pressure: bp 135 °C (10 mmHg). A mixture of two diastereomers was obtained (ratio 1:1, 12.5 g, 75% yield). ^{1}H NMR ($CDCl_3$): δ 1.45–1.50 (two d, 6, CH_3), 2.85 (br s, 2, OH), 3.60–5.15 (m, 14, CH, CH_2 and cycle).

4.1.2. (4*R**)/(4*S**)-4-Methyl-3,6,8-trioxa-bicyclo[3.2.1]octane (14)

A diastereomeric mixture of **13** (10.5 g; 63 mmol) and powdered KOH (5.3 g; 94 mmol) was heated until an exothermic reaction developed. After cooling, the residue was distilled under reduced pressure: bp 80 °C (25 mmHg). A mixture of two diastereomers **14** was obtained (5 g, 61% yield). ^{1}H NMR ($CDCl_3$): δ 1.10 (d, 3, CH_3), 1.29 (d, 3, CH_3), 3.39–5.15 (m, 14, cycle).

4.1.3. (2*R**,5*R**)-(5-Methyl-[1,4]dioxan-2-yl)-methanol (15a) and (2*R**,5*S**)-(5-methyl-[1,4]dioxan-2-yl)-methanol (15b)

A solution of $AlCl_3$ (5.1 g, 38 mmol) in Et_2O (30 mL) was added dropwise over 3 min to a stirred solution of **14** (5.0 g, 38 mmol) and $LiAlH_4$ (1.5 g, 39 mmol) in Et_2O (70 mL). The reaction mixture was refluxed vigorously until the starting material disappeared. Then it was cooled to 0 °C and quenched cautiously by the dropwise addition of Na_2SO_4 saturated solution. The solid was filtered off and the filtrate was dried over Na_2SO_4. Removal of the solvent gave an oil, which was purified by column chromatography eluting with cyclohexane/EtOAc (7:3). The *trans* isomer **15b** eluted first: 1.6 g (32% yield). ^{1}H NMR ($CDCl_3$): δ 1.08 (d, 3, CH_3), 2.21 (br s, 1, OH), 3.25–3.80 (m, 8, CH_2O and cycle). The second fraction was the *cis* isomer **15a**: 0.3 g (6% yield); ^{1}H NMR ($CDCl_3$): δ 1.20 (d, 3, CH_3), 1.92 (br s, 1, OH), 3.50–4.01 (m, 8, CH_2O and cycle).

4.1.4. Toluene-4-sulfonic acid (2*S**,5*R**)-5-methyl-[1,4]dioxan-2-ylmethyl ester (16a)

Tosyl chloride (0.75 g, 3.9 mmol) was added to a stirred solution of **15a** (0.35 g, 2.6 mmol) in pyridine (4 mL) at 0 °C over 30 min. After being stirred for 3 h at 0 °C, the mixture was left for 20 h at 4 °C in the freezer. Then it was poured into ice and concentrated HCl (4 mL) and extracted with $CHCl_3$. The organic layers were washed with HCl 2 N (15 mL), $NaHCO_3$ saturated solution (15 mL), and H_2O (15 mL) and then dried over Na_2SO_4. The solvent was concentrated in vacuo to give a residue, which was purified by column chromatography. Eluting with cyclohexane/EtOAc (9:1) afforded compound **16a**: 0.35 g (47% yield). ^{1}H NMR ($CDCl_3$): δ 1.10 (d, 3, CH_3), 2.43 (s, 3, $ArCH_3$), 3.21–4.41 (m, 8, CH_2O and cycle), 7.37 (d, 2, ArH), 7.82 (d, 2, ArH).

4.1.5. Toluene-4-sulfonic acid (2*S**,5*S**)-5-methyl-[1,4]dioxan-2-ylmethyl ester (16b)

This was prepared as described for compound **16a** starting from **15b**: 75% yield. ^{1}H NMR ($CDCl_3$): δ 1.05 (d, 3, CH_3), 2.46 (s, 3, $ArCH_3$), 3.19–4.00 (m, 8, CH_2O and cycle), 7.35 (d, 2, ArH), 7.79 (d, 2, ArH).

4.1.6. Dimethyl-((2*R**,5*R**)-5-methyl-[1,4]dioxan-2-ylmethyl)-amine (17a)

A solution of **16a** (0.58 g, 2 mmol) and dimethylamine (5 mL) in dry benzene (15 mL) was heated in a sealed tube for 60 h at 120 °C. After evaporation of the solvent, the residue was dissolved in $CHCl_3$, which was washed with NaOH 2 N and dried over Na_2SO_4. The solvent was concentrated in vacuo to give a residue, which was purified by column chromatography. Eluting with $CHCl_3$/CH_3OH (9:1) afforded **17a** as the free base: 0.3 g (94% yield). ^{1}H NMR ($CDCl_3$): δ 1.19 (d, 3, CH_3), 2.28 (m, 2, CH_2N), 2.30 (s, 6, $N(CH_3)_2$), 2.70–2.82 (m, 2, cycle), 3.48–3.79 (m, 4, cycle).

4.1.7. Dimethyl-((2*R**,5*S**)-5-methyl-[1,4]dioxan-2-ylmethyl)-amine (17b)

This was prepared as described for **17a** starting from **16b**: 76% yield. ^{1}H NMR ($CDCl_3$): δ 1.08 (d, 3, CH_3), 2.15 (m, 1, CH_2N), 2.28 (s, 6, $N(CH_3)_2$), 2.37 (m, 1, CH_2N), 3.31 (m, 2, cycle), 3.53–3.81 (m, 4, cycle).

4.1.8. (2*R**,5*R**)-*N*,*N*,*N*-Trimethyl-(5-methyl-1,4-dioxan-2-yl)methanaminium iodide (3a)

A solution of **17a** (0.3 g, 1.9 mmol) in Et_2O (10 mL) was treated with an excess of methyl iodide and left at rt in the dark for 24 h. The solid was filtered and recrystallized from EtOH; mp 236–237 °C. ^{1}H NMR (DMSO): δ 1.10 (d, 3, CH_3), 3.12 (s, 9, $N(CH_3)_3$), 3.35 (m, 2, CH_2N), 3.52–3.75 (m, 4, cycle), 3.98 (m, 1, cycle), 4.23 (m, 1, cycle). Anal. Calcd for $C_9H_{20}INO_2$: C, 35.89; H, 6.69; N, 4.65. Found: C, 35.92; H, 6.72; N, 4.58.

4.1.9. (2*R**,5*S**)-*N*,*N*,*N*-Trimethyl-(5-methyl-1,4-dioxan-2-yl)methanaminium iodide (3b)

This was prepared as described for **3a** starting from **17b**. The solid was filtered and recrystallized from EtOH; mp 273 °C. ^{1}H NMR (DMSO): δ 0.99 (d, 3, CH_3), 3.10 (s, 9, $N(CH_3)_3$), 3.25 (m, 2, CH_2N), 3.30 (m, 2, cycle), 3.51 (m, 1, cycle), 3.68 (m, 2, cycle), 4.10 (m, 1, cycle). Anal. Calcd for $C_9H_{20}INO_2$: C, 35.89; H, 6.69; N, 4.65. Found: C, 36.02; H, 6.93; N, 4.42.

4.1.10. (2*R**,3*R**)-3-Allyloxy-butan-2-ol (18a)

(2*R**,3*S**)-2,3-Dimethyl-oxirane (5.0 g, 69.3 mmol) was added dropwise to a stirred solution of freshly cut sodium (0.5 g, 21.7 mmol) in allyl alcohol (49 mL) at rt. After 1 h at rt the reaction mixture was refluxed for 3 h. Most of the unreacted allyl alcohol was then separated by distillation at atmospheric pressure. After cooling to rt 6 N aqueous sulphuric acid (5 mL) was added to the residual solution to neutralize the sodium alloxide, and solvent removal was continued to afford an oil which was distilled under reduced pressure: bp 65 °C (20 mmHg); 5.10 g (56% yield). ^{1}H NMR ($CDCl_3$): δ 1.07 (d, 3, CH_3), 1.15 (d, 3, CH_3), 2.68 (br s, 1, OH), 3.20 (m, 1, CH), 3.58 (m, 1, CH), 3.91 (m, 1, CH_2), 4.15 (m, 1, CH_2), 5.18–5.26 (m, 2, C=CH_2), 5.90 (m, 1, CH=C).

4.1.11. (2R*,3S*)-3-Allyloxy-butan-2-ol (18b)

This was prepared as described for (2*R**,3*R**)-3-(allyloxy)butan-2-ol starting from (2*R**,3*R**)-2,3-dimethyl-oxirane: bp 70 °C (20 mmHg); 37% yield. ^{1}H NMR ($CDCl_3$): δ 1.10 (d, 3, CH_3), 1.14 (d, 3, CH_3), 2.21 (br s, 1, OH), 3.43 (m, 1, CH), 3.89 (m, 1, CH), 4.03 (m, 2, OCH_2), 5.10–5.28 (m, 2, C=CH_2), 5.92 (m, 1, CH=C).

4.1.12. (2*S**,3*S**,5*S**)-5-Iodomethyl-2,3-dimethyl-[1,4]dioxane (19a) and (2*S**,3*S**,5*R**)-5-iodomethyl-2,3-dimethyl-[1,4]dioxane (19b)

A solution of mercury(II) acetate (12.1 g, 37.9 mmol) in H_2O (75 mL) and acetic acid (0.1 mL) was added to a stirred solution of **18a** (5.1 g, 39.2 mmol). The reaction mixture was heated to reflux for 45 min, then allowed to stand overnight at rt. After the reaction mixture was filtered a solution of KI (6.5 g, 39.2 mmol) in H_2O (40 mL) was added to the filtrate and a mixture of (2*S**,5*S**,6*S**)-/(2*R**,5*S**,6*S**)-((5,6-dimethyl-1,4-dioxan-2-yl)methyl)mercury(II) iodide separated as an oil, which was dissolved in $CHCl_3$ (25 mL). A solution of I_2 (7.5 g, 29.5 mmol) in $CHCl_3$ (170 mL) was added and the reaction mixture was heated to boiling and then allowed to stand at rt for 18 h. The organic phase was washed with 10% Na_2SO_3, 10% KI, and dried over Na_2SO_4. The evaporation of the solvent in vacuo afforded a residue. The mixture of diastereomers **19a** and **19b** (ratio 7:3) was separated by column chromatography eluting with petroleum ether/EtOAc (99.5:0.5). Compound **19a** eluted first: 2.0 g (20% yield). ^{1}H NMR ($CDCl_3$): δ 1.08 (d, J = 6.41 Hz, 3, CH_3), 1.14 (d, J = 5.98 Hz, 3, CH_3), 3.02 (dd, J = 6.41, 10.68 Hz, 1, CH_2I), 3.07 (dd, J = 5.98, 10.68 Hz, 1, CH_2I), 3.17 (dq, J = 6.41, 8.55 Hz, 1, 2-CH), 3.28 (dd, J = 10.26, 11.11 Hz, 1, 6-CH), 3.34 (dq, J = 5.98, 8.55 Hz, 1, 3-CH), 3.64 (m, 1, 5-CH), 3.98 (dd, J = 2.56, 11.11 Hz, 1, 6-CH). The second fraction was **19b**: 0.7 g (7% yield). ^{1}H NMR ($CDCl_3$): δ 1.08 (d, J = 6.41 Hz, 3, CH_3), 1.14 (d, J = 5.99 Hz, 3, CH_3), 3.20 (dq, J = 6.41, 8.97 Hz, 1, 2-CH), 3.48 (dq, J = 5.99, 8.97 Hz, 1, 3-CH), 3.56 (m, 2, CH_2I), 3.58 (d, J = 11.97 Hz, 1, 6-CH), 3.80 (dd, J = 0.85, 11.97 Hz, 1, 6-CH), 3.86 (m, 1, 5-CH).

4.1.13. (2*S**,3*R**,5*S**)-5-Iodomethyl-2,3-dimethyl-[1,4]dioxane (19c) and (2*R**,3*S**,5*S**)-5-iodomethyl-2,3-dimethyl-[1,4]dioxane (19d)

These compounds were prepared following the procedure described for **19a** and **19b** starting from **18b**. The mixture of diastereomers **19c** and **19d** (ratio 8:2) was separated by column chromatography eluting with petroleum ether/EtOAc (9.9:0.1). **19c** eluted first: (21% yield). ^{1}H NMR ($CDCl_3$): δ 1.02 (d, J = 6.84 Hz, 3, CH_3), 1.29 (d, J = 6.84 Hz, 3, CH_3), 3.02 (d, 2, CH_2I), 3.30 (dd, J = 10.25, 11.11 Hz, 1, 6-CH), 3.74 (m, 1, 3-CH), 3.78 (m, 1, 5-CH), 3.83 (m, 1, 2-CH), 3.95 (dd, J = 2.99, 11.54 Hz, 1, 6-CH). The second fraction was **19d**: 0.7 g (16% yield). ^{1}H NMR ($CDCl_3$): δ 1.10 (d, J = 6.84 Hz, 3, CH_3), 1.40 (d, J = 6.84 Hz, 3, CH_3), 3.07 (dd, J = 2.99, 5.55 Hz, 2, CH_2I), 3.47 (dd, J = 10.69, 10.90 Hz, 1, 6-CH), 3.55–3.66 (m, 3, cycle), 3.92 (m, 1, 3-CH).

4.1.14. ((2*R**,5*S**,6*S**)-5,6-Dimethyl-[1,4]dioxan-2-ylmethyl)-dimethyl-amine (20a)

A solution of **19a** (0.8 g, 3.1 mmol) and dimethylamine (10 mL) in dry benzene (30 mL) was heated in a sealed tube for 60 h at 120 °C. After evaporation of the solvent, the residue was dissolved in $CHCl_3$, which was washed with 2 N NaOH and dried over Na_2SO_4. The solvent was concentrated in vacuo to give a residue, which was purified by column chromatography. Eluting with $CHCl_3/CH_3OH$ (9.5:0.5) afforded **20a** as the free base: 0.4 g (74% yield). ^{1}H NMR ($CDCl_3$): δ 1.10 (d, 3, CH_3), 1.14 (d, 3, CH_3), 2.19 (dd, 1, CH_2N), 2.24 (s, 6, $N(CH_3)_2$), 2.37 (dd, 1, CH_2N), 3.17–3.39 (m, 3, cycle), 3.75–3.84 (m, 2, cycle).

4.1.15. ((2*S**,5*S**,6*S**)-5,6-Dimethyl-[1,4]dioxan-2-ylmethyl)-dimethyl-amine (20b)

This was prepared as described for **20a** starting from **19b**: 86% yield. ^{1}H NMR ($CDCl_3$): δ 1.07 (d, 3, CH_3), 1.12 (d, 3, CH_3), 2.24 (m, 2, CH_2N), 2.29 (s, 6, $N(CH_3)_2$), 2.63 (m, 1, cycle), 2.73 (m, 1, cycle), 3.23 (m, 1, cycle), 3.49 (m, 1, cycle), 3.72 (m, 1, cycle).

4.1.16. ((2*R**,5*S**,6*R**)-5,6-Dimethyl-[1,4]dioxan-2-ylmethyl)-dimethyl-amine (20c)

This was prepared as described for **20a** starting from **19c**: 82% yield. ^{1}H NMR ($CDCl_3$): δ 1.02 (d, 3, CH_3), 1.29 (d, 3, CH_3), 2.10 (m, 2, CH_2N), 2.24 (s, 6, $N(CH_3)_2$), 2.33 (m, 1, cycle), 3.31 (m, 1, cycle), 3.78 (m, 2, cycle), 3.95 (m, 1, cycle).

4.1.17. ((2*R**,5*R**,6*S**)-5,6-Dimethyl-[1,4]dioxan-2-ylmethyl)-dimethyl-amine (20d)

This was prepared as described for **20a** starting from **19d:** 86% yield. ^{1}H NMR ($CDCl_3$): δ 1.02 (d, 3, CH_3), 1.21 (d, 3, CH_3), 2.18 (m, 2, CH_2N), 2.25 (s, 6, $N(CH_3)_2$), 2.39 (m, 1, cycle), 3.46 (m, 1, cycle), 3.65 (m, 2, cycle), 3.90 (m, 1, cycle).

4.1.18. ((2*R**,5*S**,6*S**)-5,6-Dimethyl-1,4-dioxan-2-yl)-*N*,*N*,*N*-trimethylmethanaminium iodide (4)

This was prepared as described for **3b** starting from **20a**. The solid was filtered and recrystallized from 2-PrOH; mp 160–161 °C. ^{1}H NMR (DMSO): δ 0.98 (d, 3, CH_3), 1.08 (d, 3, CH_3), 3.05–3.25 (m, 11, CH_2N, $N(CH_3)_3$), 3.27–3.43 (m, 3, cycle), 3.65 (m, 1, cycle), 4.19 (m,

1, cycle). Anal. Calcd for $C_{10}H_{22}INO_2$: C, 38.11; H, 7.04; N, 4.44. Found: C, 38.01; H, 6.89; N, 4.60.

4.1.19. ((2*S**,5*S**,6*S**)-5,6-Dimethyl-1,4-dioxan-2-yl)-*N*,*N*,*N*-trimethylmethanaminium iodide (5)

This was prepared as described for **3b** starting from **20b**. The solid was filtered and recrystallized from EtOH; mp 214–216 °C. ^{1}H NMR (DMSO): δ 1.03 (d, 3, CH_3), 1.12 (d, 3, CH_3), 3.25 (m, 2, CH_2N), 3.57 (s, 9, $N(CH_3)_3$), 3.78–4.00 (m, 2, cycle), 4.30 (m, 2, cycle), 4.60 (m, 1, cycle). Anal. Calcd for $C_{10}H_{22}INO_2$: C, 38.11; H, 7.04; N, 4.44. Found: C, 38.22; H, 7.22; N, 4.61.

4.1.20. ((2*R**,5*S**,6*R**)-5,6-Dimethyl-1,4-dioxan-2-yl)-*N*,*N*,*N*-trimethylmethanaminium iodide (6)

This was prepared as described for **3b** starting from **20c**. The solid was filtered and recrystallized from EtOH; mp 183–184 °C. ^{1}H NMR (DMSO): δ 0.94 (d, 3, CH_3), 1.21 (d, 3, CH_3), 3.10 (s, 9, $N(CH_3)_3$), 3.20 (m, 2, CH_2N), 3.32 (m, 2, cycle), 3.65 (m, 1, cycle), 3.80 (m, 1, cycle), 4.29 (m, 1, cycle). Anal. Calcd for $C_{10}H_{22}INO_2$: C, 38.11; H, 7.04; N, 4.44. Found: C, 38.41; H, 7.01; N, 4.60.

4.1.21. ((2*R**,5*R**,6*S**)-5,6-Dimethyl-1,4-dioxan-2-yl)-*N*,*N*,*N*-trimethylmethanaminium iodide (7)

This was prepared as described for **3b** starting from **20d**. The solid was filtered and recrystallized from 2-PrOH; mp 190–191 °C. ^{1}H NMR (DMSO): δ 0.95 (d, 3, CH_3), 1.12 (d, 3, CH_3), 3.11 (s, 9, $N(CH_3)_3$), 3.33 (m, 2, CH_2N), 3.36 (m, 2, cycle), 3.70 (m, 1, cycle), 3.95 (m, 1, cycle), 4.20 (m, 1, cycle). Anal. Calcd for $C_{10}H_{22}INO_2$: C, 38.11; H, 7.04; N, 4.44. Found: C, 38.40; H, 7.28; N, 4.51.

4.1.22. 1-Allyloxy-2-methyl-propan-2-ol (21)

This was prepared as described for **18a** starting from 2,2-dimethyloxirane: 44% yield. ^{1}H NMR ($CDCl_3$): δ 1.21 (s, 6, CH_3), 2.22 (s, 1, OH), 3.28 (s, 2, CH_2O), 4.05 (m, 2, OCH_2), 5.18–5.36 (m, 2, C=CH_2), 5.90 (m, 1, CH=C).

4.1.23. 2-Allyloxy-2-methyl-propan-1-ol (22)

Perchloric acid (70%, 3.4 mL) was added to a stirred solution of 2,2-dimethyloxirane (5.0 g, 69.3 mmol) in allyl alcohol (40 mL) at 0 °C. After 0.5 h at room temperature the reaction mixture was poured in H_2O (75 mL) and extracted with Et_2O. The organic phase was washed with $NaHCO_3$ and dried over Na_2SO_4. Removal of dried solvents gave a residue, which was purified by column chromatography, eluting with cyclohexane/EtOAc (9.5:0.5): 3.0 g (33% yield). ^{1}H NMR ($CDCl_3$): δ 1.20 (s, 6, CH_3), 2.02 (t, 1, OH), 3.43 (d, 2, CH_2O), 3.98 (m, 2, OCH_2), 5.10–5.35 (m, 2, C=CH_2), 5.93 (m, 1, CH=C).

4.1.24. 3-Allyloxy-3-methyl-butan-2-ol (23)

This was prepared as described for **22** starting from 2,2,3-trimethyloxirane. After evaporation of the solvent, the residue was purified by column chromatography eluting with petroleum ether/EtOAc (9:1) (73% yield). ^{1}H NMR ($CDCl_3$): δ 1.15 (m, 9, CH_3), 2.60 (d, 1, OH), 3.63 (m, 1, CH), 3.98 (m, 2, OCH_2), 5.10–5.31 (m, 2, C=CH_2), 5.90 (m, 1, CH=C).

4.1.25. 3-Allyloxy-2-methyl-butan-2-ol (24)

A solution of 3 M CH_3MgI in Et_2O (13.9 mL, 41.7 mmol) in Et_2O (10 mL) was added dropwise to a stirred solution of 2-allyloxy-propionic acid methyl ester[24] (2.4 g, 16.6 mmol) in Et_2O (20 mL). After stirring at rt for 2 h the reaction mixture was cooled to 0 °C and quenched cautiously by the dropwise addition of Na_2SO_4 saturated solution. The solid was filtered off and the filtrate was dried over Na_2SO_4. Removal of the solvent afforded a residue, which was purified by column chromatography eluting with cyclohexane/EtOAc (99:1) to give an oil: 1.8 g (75% yield). ^{1}H NMR ($CDCl_3$): 1.12 (d, 3, CH_3), 1.15 (s, 3, CH_3), 1.18 (s, 3, CH_3), 2.50 (br s 1, OH), 3.23 (m, 1, CH), 3.88–4.21 (m, 2, OCH_2), 5.12–5.26 (m, 2, C=CH_2), 5.91 (m, 1, CH=C).

4.1.26. 3-Allyloxy-2,3-dimethyl-butan-2-ol (25)

This was prepared as described for **22** starting from 2,2,3,3-tetramethyloxirane.[39] After evaporation of the solvent, the residue was purified by column chromatography eluting with cyclohexane/EtOAc (9.2:0.8) 73% yield. ^{1}H NMR ($CDCl_3$): δ 1.20 (m, 12, CH_3), 2.52 (br s, 1, OH), 3.99 (m, 2, CH_2), 5.11–5.35 (m, 2, C=CH_2), 5.91 (m, 1, CH=C).

4.1.27. 6-Iodomethyl-2,2-dimethyl-[1,4]dioxane (26)

This was prepared as described for **19a** starting from **21**: 59% yield. ^{1}H NMR ($CDCl_3$): δ 1.18 (s, 3, CH_3), 1.32 (s, 3, CH_3), 3.02 (d, 2, CH_2I), 3.15 (dd, 2, cycle), 3.42 (d, 1, cycle), 3.91 (m, 1, cycle), 4.01 (dd, 1, cycle).

4.1.28. 5-Iodomethyl-2,2-dimethyl-[1,4]dioxane (27)

This was prepared as described for **19a** starting from **22**: 20% yield. ^{1}H NMR ($CDCl_3$): δ 1.12 (s, 3, CH_3), 1.32 (s, 3, CH_3), 3.14 (d, 2, CH_2I), 3.41–3.76 (m, 5, cycle).

4.1.29. (3*S**,5*S**)/(3*R**,5*S**)-5-Iodomethyl-2,2,3-trimethyl-[1,4]dioxane (28a and 28b)

This was prepared as described for **19a** starting from **23**. A mixture of the two diastereomers was obtained: 53% yield. ^{1}H NMR ($CDCl_3$): δ 1.12 (m, 18, CH_3), 3.11 (m, 4, CH_2I), 3.41–3.99 (m, 8, cycle).

4.1.30. (3*R**,6*S**)-6-Iodomethyl-2,2,3-trimethyl-[1,4]dioxane (29a) and (3*S**,6*S**)-6-iodomethyl-2,2,3-trimethyl-[1,4]dioxane (29b)

These were prepared as described for **19a** starting from **24**. The two diastereomers were separated by column chromatography eluting with cyclohexane/EtOAc (9.9:0.1). **29b** eluted first: 17% yield. ^{1}H NMR ($CDCl_3$): δ 1.02 (d, 3, CH_3), 1.11 (s, 3, CH_3), 1.21 (s, 3, CH_3), 3.00 (m, 2, CH_2I), 3.18–3.40 (m, 2, cycle), 3.88 (m, 1, cycle), 4.01 (m, 1, cycle). The second fraction was **29a**: 2% yield. ^{1}H NMR ($CDCl_3$): δ 1.03 (s, 3, CH_3), 1.20 (d, 3, CH_3), 1.28 (s, 3, CH_3), 3.11 (m, 2, CH_2I), 3.35–3.81 (m, 4, cycle).

4.1.31. 5-Iodomethyl-2,2,3,3-tetramethyl-[1,4]dioxane (30)

This was prepared as described for **19a** starting from **25**: 30% yield. ^{1}H NMR ($CDCl_3$): δ 1.12 (s, 6, CH_3), 1.39 (s, 6, CH_3), 3.09 (m, 2, CH_2I), 3.45–3.86 (m, 3, cycle).

4.1.32. (6,6-Dimethyl-[1,4]dioxan-2-ylmethyl)-dimethyl-amine (31)

This was prepared as described for **20a** starting from **26**: 94% yield. ^{1}H NMR ($CDCl_3$): δ 1.10 (s, 3, CH_3), 1.35 (s, 3, CH_3), 2.18 (m, 1, CH_2N), 2.24 (s, 6, $N(CH_3)_2$), 2.30 (m, 1, CH_2N), 3.20 (dd, 1, CH), 3.25 (dd, 1, cycle), 3.48 (d, 1, cycle), 3.80 (dd, 1, cycle), 4.00 (m, 1, cycle).

4.1.33. (5,5-Dimethyl-[1,4]dioxan-2-ylmethyl)-dimethyl-amine (32)

This was prepared as described for **20a** starting from **27**: 86% yield. ^{1}H NMR ($CDCl_3$): δ 1.08 (s, 3, CH_3), 1.30 (s, 3, CH_3), 2.15 (m, 1, CH_2N), 2.24 (s, 6, $N(CH_3)_2$), 2.43 (m, 1, CH_2N), 3.39 (d, 1, CH), 3.51–3.62 (m, 4, cycle).

4.1.34. Dimethyl-((2*R**,6*S**)-5,5,6-trimethyl-[1,4]dioxan-2-ylmethyl)-amine (33a) and dimethyl-((2*R**,6*R**)-5,5,6-trimethyl-[1,4]dioxan-2-ylmethyl)-amine (33b)

These were prepared as described for **20a** starting from a mixture of **28a** and **28b**. The two diastereomers were separated by column chromatography eluting with petroleum ether/EtOAc/CH_3OH/

NH_4OH (3:1.8:0.1:0.01). The *cis* diastereomer **33a** eluted first: 0.6 g (17% yield). ^1H NMR ($CDCl_3$): δ 1.06 (d, 3, CH_3), 1.14 (s, 3, CH_3), 1.36 (s, 3, CH_3), 2.14–2.43 (m, 2, CH_2N), 2.22 (s, 6, $N(CH_3)_2$), 3.50 (m, 3, cycle), 3.72 (m, 1, cycle). The second fraction was the *trans* diastereomer **33b**: 0.5 g (14% yield); ^1H NMR ($CDCl_3$): δ 1.16 (s, 3, CH_3), 1.19 (d, 3, CH_3), 1.27 (s, 3, CH_3), 2.31 (s, 6, $N(CH_3)_2$), 2.39–2.69 (m, 2, CH_2N), 3.49–3.91 (m, 4, cycle).

4.1.35. Dimethyl-((2*R**,5*R**)-5,6,6-trimethyl-[1,4]dioxan-2-ylmethyl)-amine (34a)

This was prepared as described for **20a** starting from **29a**: 97% yield. ^1H NMR ($CDCl_3$): δ 1.08 (s, 3, CH_3), 1.25 (d, 3, CH_3), 1.39 (s, 3, CH_3), 2.21–2.41 (m, 2, CH_2N), 2.22 (s, 6, $N(CH_3)_2$), 3.51 (m, 3, cycle), 3.99 (m, 1, cycle).

4.1.36. Dimethyl-((2*R**,5*S**)-5,6,6-trimethyl-[1,4]dioxan-2-ylmethyl)-amine (34b)

This was prepared as described for **20a** starting from **29b**: 91% yield. ^1H NMR ($CDCl_3$): δ 1.05 (d, 3, CH_3), 1.11 (s, 3, CH_3), 1.26 (s, 3, CH_3), 2.12 (m, 1, CH_2N), 2.22 (s, 6, $N(CH_3)_2$), 2.29 (m, 1, CH_2N), 3.25 (m, 2, cycle), 3.79 (m, 1, cycle), 3.95 (m, 1, cycle).

4.1.37. Dimethyl-(5,5,6,6-tetramethyl-[1,4]dioxan-2-ylmethyl)-amine (35)

This was prepared as described for **20a** starting from **30**: 83% yield. ^1H NMR ($CDCl_3$): δ 1.07 (s, 6, CH_3), 1.31 (s, 3, CH_3), 1.35 (s, 3, CH_3), 2.15 (m, 1, CH_2N), 2.22 (s, 6, $N(CH_3)_2$), 2.30 (m, 1, CH_2N), 3.50 (m, 2, cycle), 4.00 (m, 1, cycle).

4.1.38. (6,6-Dimethyl-1,4-dioxan-2-yl)-*N*,*N*,*N*-trimethylmethanaminium iodide (8)

This was prepared as described for **3b** starting from **31**. The solid was filtered and recrystallized from 2-PrOH; mp 180–181 °C. ^1H NMR (DMSO): δ 1.04 (s, 3, CH_3), 1.31 (s, 3, CH_3), 3.01 (m, 1, CH_2N), 3.09 (s, 9, $N(CH_3)_3$), 3.15 (m, 1, CH_2N), 3.30 (m, 2, cycle), 3.50 (d, 1, cycle), 3.65 (dd, 1, cycle), 4.35 (m, 1, cycle). Anal. Calcd for $C_{10}H_{22}INO_2$: C, 38.11; H, 7.04; N, 4.44. Found: C, 38.32; H, 7.22; N, 4.55.

4.1.39. (5,5-Dimethyl-1,4-dioxan-2-yl)-*N*,*N*,*N*-trimethylmethanaminium iodide (9)

This was prepared as described for **3b** starting from **32**. The solid was filtered and recrystallized from EtOH; mp 215–216 °C. ^1H NMR (DMSO): δ 1.05 (s, 3, CH_3), 1.32 (s, 3, CH_3), 3.11 (s, 9, $N(CH_3)_3$), 3.28–3.52 (m, 6, CH_2N, cycle), 4.05 (m, 1, cycle). Anal. Calcd for $C_{10}H_{22}INO_2$: C, 38.11; H, 7.04; N, 4.44. Found: C, 38.01; H, 6.92; N, 4.36.

4.1.40. *N*,*N*,*N*-Trimethyl((2*R**,6*S**)-5,5,6-trimethyl-1,4-dioxan-2-yl)methanaminium iodide (10a)

This was prepared as described for **3b** starting from **33a**. The solid was filtered and recrystallized from *i*-PrOH; mp 176–178 °C. ^1H NMR (DMSO): δ 0.98 (d, 3, CH_3), 1.04 (s, 3, CH_3), 1.15 (s, 3, CH_3), 3.08 (s, 9, $N(CH_3)_3$), 3.22–3.59 (m, 5, CH_2N, cycle), 4.16 (m, 1, cycle). Anal. Calcd for $C_{11}H_{24}INO_2$: C, 40.13; H, 7.35; N, 4.25. Found: C, 40.40; H, 7.09; N, 4.07.

4.1.41. *N*,*N*,*N*-Trimethyl((2*R**,6*R**)-5,5,6-trimethyl-1,4-dioxan-2-yl)methanaminium iodide (10b)

This was prepared as described for **3b** starting from **33b**. The solid was filtered and recrystallized from *i*-PrOH; mp 205–206 °C. ^1H NMR (DMSO): δ 0.99 (s, 3, CH_3), 1.07 (s, 3, CH_3), 1.16 (d, 3, CH_3), 3.12 (s, 9, $N(CH_3)_3$), 3.20–3.41 (m, 3, CH_2N, cycle), 3.88 (m, 1, cycle), 4.01 (m, 1, cycle), 4.31 (m, 1, cycle). Anal. Calcd for $C_{11}H_{24}INO_2$: C, 40.13; H, 7.35; N, 4.25. Found: C, 39.89; H, 7.48; N, 4.60.

4.1.42. *N*,*N*,*N*-Trimethyl((2*R**,5*R**)-5,6,6-trimethyl-1,4-dioxan-2-yl)methanaminium iodide (11a)

This was prepared as described for **3b** starting from **34a**. The solid was filtered and recrystallized from *i*-PrOH; mp 226–228 °C. ^1H NMR (DMSO): δ 1.02 (s, 3, CH_3), 1.21 (d, 3, CH_3), 1.40 (s, 3, CH_3), 3.09 (m, 11, CH_2N, $N(CH_3)_3$), 3.29–3.57 (m, 3, cycle), 4.29 (m, 1, cycle). Anal. Calcd for $C_{11}H_{24}INO_2$: C, 40.13; H, 7.35; N, 4.25. Found: C, 39.87; H, 7.02; N, 3.89.

4.1.43. *N*,*N*,*N*-Trimethyl((2*R**,5*S**)-5,6,6-trimethyl-1,4-dioxan-2-yl)methanaminium iodide (11b)

This was prepared as described for **3b** starting from **34b**. The solid was filtered and recrystallized from *i*-PrOH; mp 168–169 °C. ^1H NMR (DMSO): δ 0.99 (d, 3, CH_3), 1.04 (s, 3, CH_3), 1.22 (s, 3, CH_3), 3.09 (m, 12, CH_2N, $N(CH_3)_3$, cycle), 3.68 (m, 1, cycle), 4.29 (m, 1, cycle). Anal. Calcd for $C_{11}H_{24}INO_2$: C, 40.13; H, 7.35; N, 4.25. Found: C, 40.42; H, 7.59; N, 4.38.

4.1.44. *N*,*N*,*N*-Trimethyl(5,5,6,6-tetramethyl-1,4-dioxan-2-yl)methanaminium iodide (12)

This was prepared as described for **3b** starting from **35**. The solid was filtered and recrystallized from *i*-PrOH; mp 252–253 °C. ^1H NMR (DMSO): δ 0.99 (s, 3, CH_3), 1.00 (s, 3, CH_3), 1.23 (s, 3, CH_3), 1.36 (s, 3, CH_3), 3.10 (s, 9, $N(CH_3)_3$), 3.19–3.45 (m, 4, CH_2N, cycle), 4.29 (m, 1, cycle). Anal. Calcd for $C_{12}H_{26}INO_2$: C, 41.99; H, 7.63; N, 4.08. Found: C, 41.79; H, 7.41; N, 4.00.

4.2. X-ray crystal structure analysis of (2*R**,5*S**)-*N*,*N*,*N*-trimethyl-(5-methyl-1,4-dioxan-2-yl)methanaminium iodide (3b)

Crystals of **3b** were obtained from methanol/2-propanol/butanol solutions in 1:1:2 volume ratios. In spite of repeated attempts, only crystals of limited quality could be obtained. X-ray data collection was performed at room temperature with an Oxford Diffraction Xcalibur 3 CCD diffractometer and graphite-monochromated Mo Kα radiation (λ = 0.71069 Å). Intensity data were corrected for absorption by a multi-scan procedure.[40] The structure was solved by direct methods, with SIR-97,[41] and refined by full-matrix least-squares with SHELXL-97.[42] In the final refinement cycles all non-hydrogen atoms were refined anisotropically and hydrogens were in geometrically calculated positions, riding on the respective carrier atoms, each with an isotropic temperature factor linked to that of its carrier atom. The absolute structure could not be determined unambiguously,[43] presumably due to limited quality of the data and to the presence of a centrosymmetric pattern formed by the heavy iodine atoms; nevertheless, the aspects of interest for the present study are unambiguously clarified by the structural model. For graphics ORTEP-3 was employed.[44] Crystal data, data collection parameters and analysis statistics are summarised in Table 3, as Supplementary data, and values of bond distances and selected values of bond angles and torsion angles are listed in Table 4. Crystallographic data have been deposited with the Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK (Fax: +44 1223 336033; e-mail: deposit@ccdc.cam.ac.uk), and are available on request quoting the deposition number CCDC 734905.

4.3. Pharmacology

4.3.1. Binding studies

4.3.1.1. Cell culture and membrane preparation. Chinese hamster ovary cells stably expressing cDNA encoding human muscarinic hM_1–hM_5 receptors were generously provided by Professor R. Maggio (Department of Experimental Medicine, University of L'Aquila, Italy). Growth medium consisted in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Gibco,

Grand Iland, NY), 50 units/mL of penicillin G and 0.05 mg/mL streptomycin, 2 mM L-glutamine (Sigma Aldrich, Milano, Italy), non essential aminoacids solution 100× (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 phosphate buffer (25 mM sodium phosphate, 5 mM $MgCl_2$, pH 7.4), and homogenized for 30 s using an Ultra-Turrax (setting 3). The pellet was sedimented at 17,000*g* for 15 min at 4 °C, and the membranes were resuspended in the same buffer, rehomogenized with Ultra-Turrax, and stored at −80 °C.[45] An aliquot was taken for the assessment of protein content according to the method of Bradford[46] using the Bio-Rad protein assay reagent (Bio-Rad Laboratories, Munchen, West Germany) and bovine serum albumin was used as the standard.

4.3.1.2. Binding assays. Radioligand binding assays were 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 phosphate buffer containing 5 mM $MgCl_2$ at pH 7.4. Final membrane protein concentrations were 30 μg/mL (hM_1), 70 μg/mL (hM_2), 25 μg/mL (hM_3), 50 μg/mL (hM_4), and 25 μg/mL (hM_5).

In homologous competition curves, [^{3}H]NMS was present at 0.2 nM in wells containing increasing concentration of unlabeled NMS (0.03–1000 nM) and at 0.075–0.2 nM in wells without unlabeled ligand. In heterologous competition curves, a fixed concentration of the tracer (0.2 nM) was 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 air flow, 25 μL of scintillation liquid (Microscint-20, Perkin–Elmer Life Science, Boston, MA) was added, and the radioactivity was counted by a TopCount NXT microplate scintillation counter (Perkin–Elmer Life Science, Boston, MA). The binding data were analyzed by the weighted least-squares iterative curve fitting program LIGAND[47] to obtain the affinity constant (K_i) of the tested agents.

4.3.2. Functional studies with isolated organs

General considerations. Male guinea pigs (250–350 g) and New Zealand white rabbits (3.0–3.5 kg) (Morini, S. Polo, Italy) were used. The tissues for in vitro experiments were removed from animals fasted 24 h before the experiments and killed by CO_2 inhalation. Isolated preparations were set up according to the following techniques.

4.3.2.1. Electrically stimulated rabbit vas deferens. According to Eltze,[26] the prostatic portion of each vas deferens was mounted in a 10 mL organ bath, containing a modified Krebs solution (mM composition: NaCl 134, KCl 3.4, $CaCl_2$ 2.8, KH_2PO_4 1.3, $NaHCO_3$ 16, $MgSO_4$ 0.6, glucose 7.7) kept at 31 °C and bubbled with 95% O_2–5% CO_2. Yohimbine (1.0 μM) was present throughout the experiment to prevent prejunctional α_2-adrenoceptors stimulation. For isometric recordings, the tissues were left to equilibrate for 45 min under a passive load of 0.75 g before electrical field stimulation through platinum electrodes was applied by square-wave pulses (0.5 ms, 0.05 Hz, supramaximal intensity 450 mA; LACE Elettronica Mod. ES3, Ospedaletto PI, Italy).

4.3.2.2. Electrically stimulated guinea pig left atrium. The heart was rapidly dissected and right and left atria were separated. As described by Eglen,[27] the left atria were mounted in 20 mL organ baths under 0.5 g tension at 33 °C, immersed in a modified Krebs–Henseleit solution (mM composition: NaCl 118.9, KCl 4.6, $CaCl_2$ 2.5, KH_2PO_4 1.2, $NaHCO_3$ 25, $MgSO_4{\cdot}7H_2O$ 1.2, glucose 11.1) and gassed with a 95% O_2–5% CO_2 mixture. After a period of stabilization of 45 min, tissues were electrically stimulated through platinum electrodes by square-wave submaximal pulses (2 Hz, 5 ms, 5 V) and inotropic activity was recorded isometrically.

4.3.2.3. Guinea pig ileum. Ileal segments 2–3 cm long were set up under 1.0 g tension at 37 °C in 10 mL organ baths filled with Krebs–Henseleit solution (see above), bubbled with carbogen. Tissues were allowed to equilibrate for 45 min and afterwards contractile responses were isometrically recorded.[28]

4.3.2.4. Guinea pig lung strips. Strips of peripheral lung tissue (15 × 2 × 2 mm) were cut from the peripheral margin of a lower lobe and mounted under 0.3 g resting tension at 37 °C in 10 mL organ baths filled with Krebs–Henseleit solution (see above), bubbled with carbogen.[35] After a period of 45 min of equilibration, contractile responses were isometrically recorded.

4.3.2.5. Protocols. Agonist concentration–response curves were constructed in each tissue by cumulative application of concentrations of the test compounds.[48] The agonist potency was expressed as pD_2 ($-\text{Log } ED_{50}$) calculated by linear regression analysis using the least square method. Intrinsic activity (α) was calculated as a fraction of the maximal response to the reference full agonist, Bethanechol or McN-A-343. When the compounds were tested as antagonists, a concentration–response curve to the full agonist Bethanechol or McN-A-343 was repeated after 10 (ileal tissue), 30 or 60 min (lung strips) incubation with the test compounds (100 nM–30 μM). The potency of the compounds acting as antagonists was expressed as pK_B value according to Furchgott's method.[49]

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Supplementary data

Crystal data and structure refinement parameters for **3b**. Bond lengths (Å) and selected values of bond angles (°) and torsion angles (°) for **3b** are available. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2009.10.027.

References and notes

1. Ishii, M.; Kurachi, Y. *Curr. Pharm. Des.* **2006**, *12*, 3573.
2. Eglen, R. M. *Prog. Med. Chem.* **2005**, *43*, 105.
3. van der Zee, E. A.; Luiten, P. G. M. *Prog. Neurobiol.* **1999**, *58*, 409.
4. Clader, J. W.; Wang, Y. *Curr. Pharm. Des.* **2005**, *11*, 3353.
5. Dean, B.; Bymaster, F. P.; Scarr, E. *Curr. Mol. Med.* **2003**, *3*, 419.
6. Calabresi, P.; Centonze, D.; Gubellini, P.; Pisani, A.; Bernardi, G. *Trends Neurosci.* **2000**, *23*, 120.
7. Wallis, R. M.; Napier, C. M. *Life Sci.* **1999**, *64*, 395.
8. Hegde, S. S. *Br. J. Pharmacol.* **2006**, *147*, S80.
9. Dinan, T. G.; Clarke, G.; Quigley, E. M.; Scott, L. V.; Shanahan, F.; Cryan, J.; Cooney, J.; Keeling, P. W. *Am. J. Gastroenterol.* **2008**, *103*, 2570.
10. Gosens, R.; Zaagsma, J.; Meurs, H.; Halayko, A. J. *Respir. Res.* **2006**, *7*, 73.
11. Winzell, M. S.; Ahrén, B. *Pharmacol. Ther.* **2007**, *116*, 437.
12. Song, P.; Spindel, E. R. *J. Pharmacol. Sci.* **2008**, *106*, 180.
13. Pedretti, A.; Vistoli, G.; Marconi, C.; Testa, B. *Chem. Biodiv.* **2006**, *3*, 481.
14. Angeli, P. *Farmaco* **1995**, *50*, 565.
15. Piergentili, A.; Quaglia, W.; Giannella, M.; Del Bello, F.; Bruni, B.; Buccioni, M.; Carrieri, A.; Ciattini, S. *Bioorg. Med. Chem.* **2007**, *15*, 886.
16. Hallonquist, E. G.; Hibbert, H. *Can. J. Res.* **1933**, *8*, 129.
17. Duclos, R. I., Jr.; Makriyannis, A. *J. Org. Chem.* **1992**, *57*, 6156.

18. Fjeldskaar, I. R.; Grace, D.; Roemming, C.; Skatteboel, L. *Acta Chem. Scand. B* **1988**, *B42*, 280.
19. Piergentili, A.; Quaglia, W.; Giannella, M.; Del Bello, F.; Buccioni, M.; Nesi, M.; Matucci, R. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 614.
20. Landmann, B.; Hoffman, R. W. *Chem. Ber.* **1987**, *120*, 331.
21. Traficante, D. D.; Meadows, M. D. *Concepts Magn. Reson.* **1997**, *9*, 359.
22. Gelas, J.; Veyssières-Rambaud, S. *Carbohydr. Res.* **1974**, *37*, 293.
23. Beckwith, A. L. J.; Bowry, V. W. *J. Org. Chem.* **1988**, *53*, 1632.
24. Gilchrist, T. L.; Wasson, R. C.; King, F. D.; Wootton, G. *J. Chem. Soc., Perkin Trans. 1* **1987**, *11*, 2517.
25. Dei, S.; Angeli, P.; Bellucci, C.; Buccioni, M.; Gualtieri, F.; Marucci, G.; Manetti, D.; Matucci, R.; Romanelli, M. N.; Scapecchi, S.; Teodori, E. *Biochem. Pharmacol.* **2005**, *69*, 1637.
26. Eltze, M. *Eur. J. Pharmacol.* **1988**, *151*, 205.
27. Eglen, R. M.; Watson, N. *Pharmacol. Toxicol.* **1996**, *78*, 59.
28. Barocelli, E.; Ballabeni, V.; Bertoni, S.; Dallanoce, C.; De Amici, M.; De Micheli, M.; Impicciatore, M. *Life Sci.* **2000**, *67*, 717.
29. Roffel, A. F.; Elzinga, C. R. S.; Zaagsma, J. *Eur. J. Pharmacol.* **1993**, *250*, 267.
30. Eglen, R. M.; Montgomery, W. W.; Whiting, R. L. *J. Pharmacol. Exp. Ther.* **1988**, *247*, 911.
31. Ehlert, F. J.; Sawyer, G. W.; Esqueda, E. E. *Life Sci.* **1999**, *64*, 387.
32. Budriesi, R.; Cacciaguerra, S.; Di Toro, R.; Bolognesi, M. L.; Chiarini, A.; Minarini, A.; Rosini, M.; Spampinato, S.; Tumiatti, V.; Melchiorre, C. *Br. J. Pharmacol.* **2001**, *132*, 1009.
33. Haddad, E. B.; Trifilieff, A.; Landry, Y.; Gies, J. P. *Pulm. Pharmacol.* **1993**, *6*, 119.
34. Roffel, A. F.; Davids, J. H.; Elzinga, C. R. S.; Wolf, D.; Zaagsma, J.; Kilbinger, H. *Br. J. Pharmacol.* **1997**, *122*, 133.
35. Scapecchi, S.; Nesi, M.; Matucci, R.; Bellucci, C.; Buccioni, M.; Dei, S.; Guandalini, L.; Manetti, D.; Martini, E.; Marucci, G.; Romanelli, M. N.; Teodori, E.; Cirilli, R. *J. Med. Chem.* **2008**, *51*, 3905.
36. Scapecchi, S.; Matucci, R.; Bellucci, C.; Buccioni, M.; Dei, S.; Guandalini, L.; Martelli, C.; Manetti, D.; Martini, E.; Marucci, G.; Nesi, M.; Romanelli, M. N.; Teodori, E.; Gualtieri, F. *J. Med. Chem.* **2006**, *49*, 1925.
37. Chang, K. J.; Deth, R. C.; Triggle, D. J. *J. Med. Chem.* **1972**, *15*, 1409.
38. Dei, S.; Bellucci, C.; Buccioni, M.; Ferraroni, M.; Guandalini, L.; Manetti, D.; Marucci, G.; Matucci, R.; Nesi, M.; Romanelli, M. N.; Scapecchi, S.; Teodori, E. *J. Med. Chem.* **2007**, *50*, 1409.
39. Sawwan, N.; Greer, A. *J. Org. Chem.* **2006**, *71*, 5796.
40. *Oxford Diffraction, CrysAlisPro (Version 1.171.29.2)*. Oxford Diffraction Ltd: Abingdon, Oxforddhire, England, 2006.
41. Altomare, A.; Burla, M. C.; Camalli, M.; Cascarano, G. L.; Giacovazzo, C.; Guagliardi, A.; Moliterni, A. G. G.; Polidori, G.; Spagna, R. *J. Appl. Crystallogr.* **1999**, *32*, 115.
42. Sheldrick, G. M. *SHELXL97, Program for Crystal Structure Refinement*; University of Göttingen, Göttingen: Germany, 1986.
43. Flack, H. D.; Bernardinelli, G. *Acta Crystallogr., Sect. A* **1999**, *55*, 908.
44. Farrugia, L. J. *J. Appl. Crystallogr.* **1997**, *30*, 565.
45. Dörje, F.; Wess, J.; Lambrecht, G.; Tacke, R.; Mutschler, E.; Brann, M. R. *J. Pharmacol. Exp. Ther.* **1991**, *256*, 727.
46. Bradford, M. M. *Anal. Biochem.* **1976**, *72*, 248.
47. Munson, P. J.; Rodbard, D. *Anal. Biochem.* **1980**, *107*, 220.
48. van Rossum, J. M. *Arch. Int. Pharmacodyn. Ther.* **1963**, *143*, 299.
49. Furchgott, R. F.; Bursztyn, P. *Ann. N.Y. Acad. Sci.* **1967**, *144*, 882.