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Preparation and biological effects of pure stereoisomeric novel soft anticholinergics

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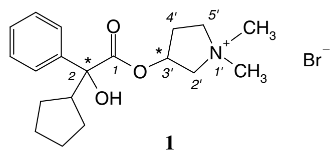
A series of pure stereoisomeric soft glycopyrrolate analogues **3**, **4** and **5** was synthesized using chiral intermediates and by careful separation of the stereoisomers formed during the last quaternization step of the synthesis. The stereochemistry of the products was elucidated using various 1D and 2D NMR techniques. Anticholinergic activity of the new compounds was determined by receptor bindig studies and performing tests on isolated organs and by *in vivo* tests. Receptor binding revealed that in the higher alkyl ester series the (2*R*, 1′*R*, 3′*R*) and the (2*R*, 1′*S*, 3′*S*) isomers were the compounds showing the highest receptor affinity furthermore it demonstrated the confines of the length of the alkyl chain. *In vitro* isolated organ experiments correlated well with the receptor binding results, and *in vivo* investigations indicated the soft character of the compounds.

1. Introduction

The therapeutic application of anticholinergic agents in the treatment e.g. of peptic ulcers, chronic obstructive pulmonary disease, Parkinson's disease or as midriatic agents or antiperspirants is often limited by various unwanted side effects such as tachycardia, palpitation, dry mouth, impaired vision or headache, to mention only a few such symptoms (Ji et al. 2002; Huang et al. 2003). This is why a continuous need exists to find new, safer anticholinergics with less systemic side effects.

Soft analogues of these drugs have long attracted considerable attention (see e.g. Bodor et al. 1980) and several derivatives of different anticholinergic drugs have been synthesized and tested for activity (Huang et al. 2003). One of the most effective anticholinergic compounds is glycopyrrolate [3-(2-cyclopentyl-2-phenyl-2-hydroxyacetoxy)-1,1-dimethylpyrrolidinium bromide, **1**] containing a quaternary *N*-atom, the charge of which prevents its crossing through lipid membranes and therefore, compared to e.g atropine, glycopyrrolate has reduced CNS-related side effects. The molecule contains two chiral centers: at position 2 of the acyl group and at position 3′ of the pyrrolidinyloxy moiety and hence this compound can exist in the form of the four stereoisomers 2*R*, 3′*R*, 2*R*, 3′*S*, 2*S*, 3′*R*, 2*S*, 3′*S*. The marketed drug is a mixture of stereoisomers (Draffan et al. 2003) while the (2*R*, 3′*R*) form is known as ritropirronium bromide (Armstrong et al. 1986).



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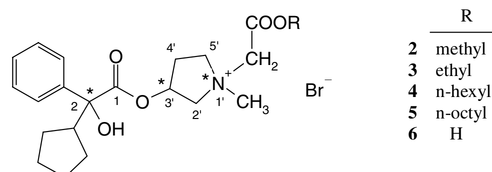
Soft analogues of glycopyrrolate such as compounds of formulas **2**, **3** and **6** containing three chiral centers (in addition to the two centers in the parent molecule the quaternary nitrogen bearing two different substituents is also asymmetric) have also been described (Ji et al. 2000, 2002) and were shown to possess the expected soft character. This was reflected by the relatively short duration of action and low systemic side effects. In addition, it was shown that enzymatic hydrolysis of the esters **2** and **3** yielded the corresponding zwitterionic acid **6** which was much less active in rats and was rapidly eliminated (Wu et al. 2005; Mori et al. 2006). The above known soft analogues, were either mixtures of all possible eight stereoisomers or mixtures of four stereoisomers containing the (*R*)-form of the cyclopentylmandeloyl unit while the remaining two chiral centers were in racemic forms.



	R
2	methyl
3	ethyl
4	n-hexyl
5	n-octyl
6	H

Considering the favorable biological test results of the above known compounds the question emerged whether any of the pure stereoisomers could have any advantage over the others or not. Therefore the aim of the present work was to synthesize and test pure stereoisomers of the soft glycopyrrolate analogues **3**, **4** and **5** and at the same time to study the influence of higher alkyl groups as R upon the extent and time course of anticholinergic activity. Thus, our primary target molecules were the pure stereoisomers of the hexyl esters **4** and octyl esters **5** with the

proviso that the configuration of C-2 in the cyclopentylmandeloyl unit was fixed as (*R*) since literature data showed the (*R*)-cyclopentylmandeloyl derivatives to be more active anticholinergics than the (*S*)-counterparts (Atkinson et al. 1977). This meant that only two chiral centers (N-1′ and C-3′ in the pyrrolidinyloxy moiety) and consequently only four stereoisomers had to be taken into consideration. In addition, for comparison analogous pure stereoisomers of the ethyl esters **3** were also synthesized.

2. Investigations, results and discussion

2.1. Chemistry

2.1.1. Synthesis of the pure stereoisomers

The target compounds were prepared by quaternization of the key intermediates **10** (see Scheme) with the bromoacetates **11**, **12** and **13**, respectively, wherein **10** was used either as a mixture of the (2*R*, 3′*S*) and (2*R*, 3′*R*) diastereomers (*Method A*) or as the individual diastereomers (*Method B*).

The diastereomeric mixture of compound **10** was prepared first by the known transesterification of (*R*)-methyl cyclopentylmandelate (**8**) with racemic 1-methyl-3-pyrrolidinol [(*R,S*)-(**9**)] (Franko and Lunsford 1960). Later it was found that much higher yields could be reached by our new version, i.e. by direct coupling of (*R*)-cyclopentylmandelic acid (**7**) with (*R,S*)-(**9**) under Mitsunobu conditions (Mitsunobu 1981). On the other hand, the individual diastereomers of **10** were obtained via two different routes. Thus, transesterification of (*R*)-methyl cyclopentylmandelate (**8**) with (*S*)-1-methyl-3-pyrrolidinol [(*S*)-(**9**)] as above proceeded with retention of configuration at C-3′ and yielded (2*R*, 3′*S*)-**10**. On the other hand, direct coupling of (R)-cyclopentylmandelic acid (**7**) with the same (*S*)-(**9**) under Mitsunobu conditions (with inversion of configuration at C-3′) led to (2*R*, 3′*R*)-**10**.

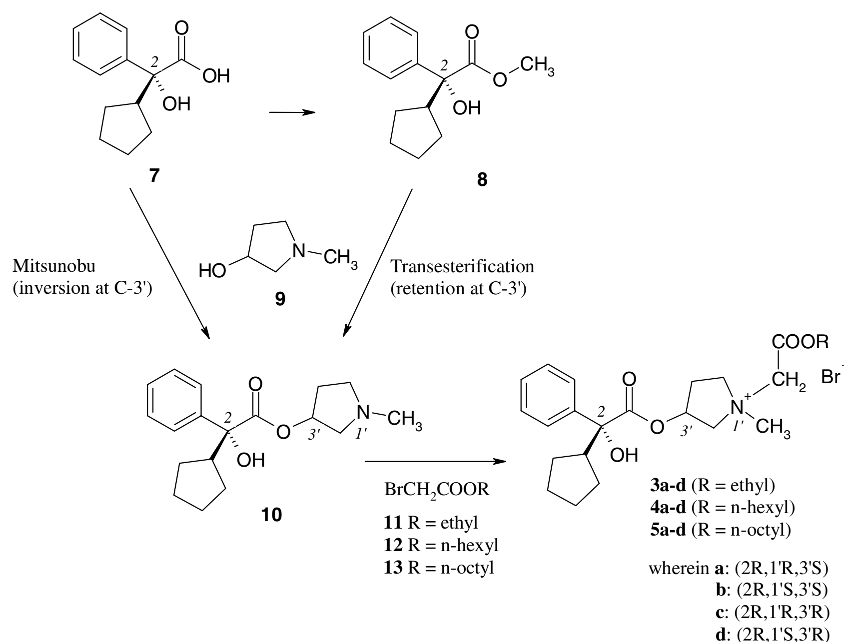
Next, in *Method A* quaternization of the mixture of (2*R*, 3′*S*)-**10** and (2*R*, 3′*R*)-**10** with the bromoacetates **12** and **13**, respectively, giving rise to the formation of a new chiral center at N-1′, led to mixtures of the four stereoisomeric target compounds **4a–d** and **5a–d**, respectively. The individual stereoisomers could be separated only partially by a combination of various chromatographic and crystallization methods (see Experimental).

Isolation of the pure stereoisomers was simpler in the other approach: in *Method B* quaternization of (2*R*, 3′*S*)-**10** with the bromoacetates **11** and **12**, respectively, as above afforded only two stereoisomers, i.e. the (2*R*, 1′*R*, 3′*S*) and the (2*R*, 1′*S*, 3′*S*) versions both of compounds **3** and **4**, respectively, and separation of these components meant less difficulty. On the other hand, in full analogy of the above-said, the final quaternization starting with (2*R*, 3′*R*)-**10** resulted in the formation of the other pair of isomers, i.e. the (2*R*, 1′*R*, 3′*R*) and the (2*R*, 1′*S*, 3′*R*) versions of the final products **3** and **4**, respectively. Finally, from among the twelve target compounds (**3a–d**, **4a–d** and **5a–d**) two pairs of compounds, i.e. **3a** + **3b**, further **5a** + **5d** were obtained as inseparable mixtures while all other stereoisomers could be isolated in pure state.

2.1.2. Structure elucidation and assignment of stereochemistry

The structures and stereochemistry of the new target compounds were elucidated by detailed NMR studies and purity of the samples was confirmed by HPLC. The assignment of the individual stereoisomers is illustrated below (Fig. 1) on the example of the four isomeric hexyl esters **4a–d**. A complete ^{1}H and ^{13}C NMR signal assigment was achieved by applying ^{1}H, ^{13}C, DEPT, and two-dimensional ^{1}H,^{1}H-COSY, ^{1}H,^{1}H-TOCSY and ^{1}H,^{13}C-HSQC correlation experiments. The characteristic ^{1}H and ^{13}C chemicals shifts are compiled in Table 1. Due to the high similarity of the chemical shifts of the isomers **4a–d** a simple differentation of the structures was not possible, only the $^{+}NCH_3$ and $^{+}NCH_2COOR$ chemical shifts exhibited characteristic differences. To reveal the stereochemistry we ran one-dimensional selective NOESY, two-dimen-

Scheme



sional ROESY and NOESY spectra affording evidences of interprotonic distances less than 5 Å. As an illustrative example the ROESY spectrum of compound **4d** (Fig. 2) is shown below. The double arrows in Fig. 1 denote the detected relevant NOE $^1H/^1H$ steric proximities.

Selective irradiation of the $^+NCH_3$ signal in **4b** resulted in NOE intensity enhancement at the H_{cis}-4′ and at the *ortho* hydrogen signals, which unambiguously proved the 1′*S* configuration and at the same time, the depicted preferred conformation of the *O*-acyl moiety. Irradiation of the NCH_3 signal in **4a** marked out only the hydrogen atom H_{trans}-2′, located on the same side of the pyrrolidine ring. In case of compound **4d** the appearance of a strong $^+NCH_3$/H-3′ cross peak in the ROESY spectrum (Fig. 2) gave evidence of the 1′*S* configuration, whereas the H-3′/H_{ortho} response revealed the conformation of the *O*-acyl group.

In compound **4c** due to the unfavourable signal overlapping (e.g. $^+NCH_3$ and H-4′$_{trans}$) the two-dimensional measurement does not work. Here we used again the one-dimensional selective NOESY. Irradiating the H_{ortho} hydrogen atoms a small, but significant NOE was observed at the $^+NCH_3$ signal, which is in accordance with the depicted configuration and conformation.

Due to the pseudorotation of the pyrrolidine ring and the high flexibility of the compounds **4a–d** conformational averaged structures are expected. Despite of this, the anomalous upfield shift of the $^+NH_3$ signals (3.24 and 3.03 ppm) can be explained by the well known anisotropic shielding effect of the aromatic ring, wherein the hydrogen atoms located above the plane of the aromatic ring show smaller chemical shifts. The smaller chemical shifts of the NCH_2 (cis) hydrogens in **4a** (4.74; 4.86) and **4d** (4.52; 4.69) are in accord with the relative steric arrangement. Preference of the conformations of compounds **4a–d**, where the aromatic ring is oriented towards the nitrogen atom is in accord with the stabilisation of the positive charge on the nitrogen by the π-system of phenyl group.

Table 1: Characteristic ^1H and ^{13}C chemical shifts of isomers 4a–d in $CDCl_3$

	4a 2*R*, 1′*R*, 3′*S*		**4b** 2*R*, 1′*S*, 3′*S*		**4c** 2*R*, 1′*R*, 3′*R*		**4d** 2*R*, 1′*S*, 3′*R*	
	^1H	^{13}C	^1H	^{13}C	^1H	^{13}C	^1H	^{13}C
1	–	174.5	–	174.6	–	174.6	–	174.5
2	–	79.8	–	79.7	–	79.4	–	79.8
2′$_{cis}$	4.36	70.3	4.16	68.9	3.91	70.0	4.20	70.5
2′$_{trans}$	4.46		4.68		4.55		4.38	
3′	5.52	73.1	5.57	73.4	5.55	73.3	5.53	73.1
4′$_{cis}$	2.06	31.5	1.96	30.1	2.24	29.6	2.25	30.1
4′$_{trans}$	2.79		2.93		3.05		2.88	
5′$_{cis}$	4.08	65.0	4.03	65.1	4.17	65.1	4.20	65.3
5′$_{trans}$	4.18		4.39		4.44		4.34	
NCH_{3cis}	–		**3.24**	50.7	**3.03**	50.6	–	
NCH_{3trans}	**3.69**	51.9	–		–		**3.69**	51.9
NCH_{2cis}	**4.74; 4.86**	62.8	–		–		**4.52 4.69**	62.9
NCH_{2trans}	–		**5.16; 5.20**	63.8	**5.08; 5.19**	63.7	–	
Ph_{ipso}		141.4		141.1		140.8		141.2
Ph_{ortho}	7.59	126.1	7.57	126.0	7.58	126.0	7.59	126.0
Ph_{meta}	7.34	128.5	7.36	128.6	7.37	128.6	7.36	128.6
Ph_{para}	7.27	128.2	7.30	128.2	7.27	128.0	7.27	128.0
HC–C-2	2.87	46.8	2.96	46.1	2.93	45.6	2.89	46.8

2.2. Biology: evaluation of the anticholinergic activity

2.2.1. Receptor binding

Evaluation of the affinity of the soft glycopyrrolate analogues **3a–d**, **4a–d** and **5a–d** for muscarinic receptors was done using [^{3}H]QNB as ligand and rat cortical membrane preparation as a source of the receptor. The affinity (summarized in Table 2) of these compounds for the muscarinic receptors (mainly M_1 in this preparation) was found one or two orders of magnitude lower than those of the reference compounds glycopyrrolate ($K_i = 0.8$ nM) and atropine ($K_i = 1.9$ nM) but our compounds were still strong antagonists of the muscarinic receptor. The nature of the interaction was characterized by the steep Hill slope, the value of which was close to unity indicating the antagonistic action. The difference between the effects of the pure stereoisomers was seen most clearly within the hexyl series as in this case all the four possible stereoisomers were isolated in pure state. The compounds **4b** (2*R*, 1′*S*, 3′*S*) and **4c** (2*R*, 1′*R*, 3′*R*) were equally, approximately four-fold, more active than **4a** (2*R*, 1′*R*, 3′*S*) or **4d** (2*R*, 1′*S*, 3′*R*). The same tendency was clear in case of the less active octyl series (**5b, 5c**), even though the other two isomers (**5a** + **5d**) were tested as a mixture. The above (2*R*, 1′*S*, 3′*S*) and (2*R*, 1′*R*, 3′*R*) compounds contain the

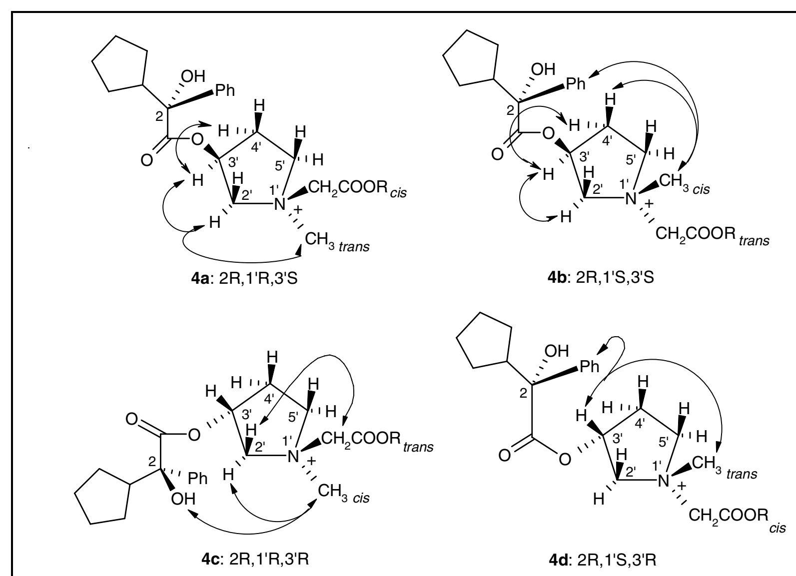


Fig. 1:
Stereochemistry of isomers **4a–d**

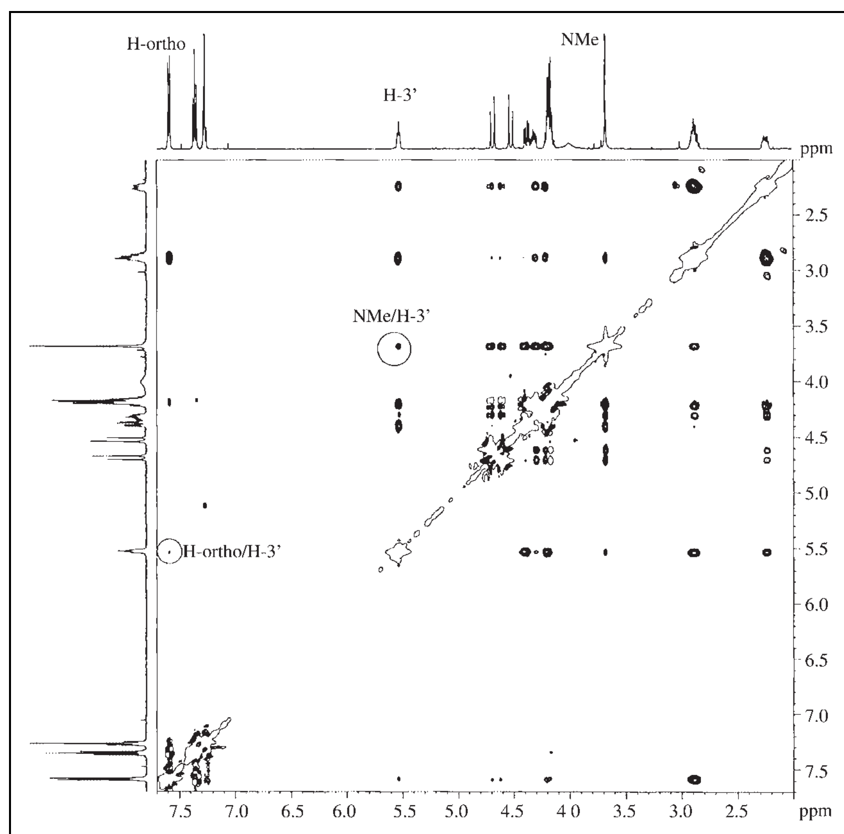


Fig 2:
Two-dimensional ROESY spectrum of compound **4d**

Table 2: Receptor binding strength of the glycopyrrolate analogues

Compound	Ki (nM), Average ± SD	Hill slope
3a + **3b**	65 ± 8	−0.96 ± 0.09
3c	16 ± 1	−0.93 ± 0.08
3d	16 ± 1	−1.07 ± 0.02
4a	67 ± 9	−1.22 ± 0.08
4b	13 ± 1	−1.19 ± 0.05
4c	15 ± 1	−1.24 ± 0.04
4d	58 ± 5	−1.15 ± 0.05
5a + **5d**	303 ± 7	−1.20 ± 0.08
5b	60 ± 5	−1.22 ± 0.11
5c	68 ± 6	−1.26 ± 0.06

Table 3: pA_2 values in two types of isolated organ experiments*

Antagonist	Trachea		Ileum	
	pA_2	Slope ± S.E.	pA_2	Slope ± S.E.
Atropine	8.85	0.98 ± 0.02[a]	8.52	0.96 ± 0.30[a]
Glycopyrrolate	9.43	1.53 ± 0.07[a]	9.66	1.03 ± 0.37[a]
3c	8.12	1.54 ± 0.18[a]	8.48	0.76 ± 0.08[a]
4b	8.21	1.14 ± 0.10[a]	8.14	1.31 ± 0.67[a]
5a + **5d**	7.23	0.79 ± 0.07	6.64	1.03 ± 0.37[a]

* data are presented of mean estimates in tissue samples from four animals
[a] deviation from unity is not significant (P > 0.05)

larger quaternizing group (CH_2COOR) in *trans* position to the cyclopentylmandeloyloxy moiety and this fact suggests that the sterically less crowded nature of these isomers may contribute to the higher receptor affinity, in contrast with the sterically more crowded *cis* isomers.
On the other hand, in the ethyl ester series the isomers **3c** (2*R*, 1′*R*, 3′*R*) and **3d** (2*R*, 1′*S*, 3′*R*), i.e. the compounds wherein the cyclopentylmandeloyloxy moiety is attached to the pyrrolidine ring in α-position have higher affinity indicating that in this case the steric position of the smaller CH_2COOEt group has less influence upon receptor affinity. The effect of the length of the alkyl chain in the ester group upon the receptor binding seemed to be negligible up to 6 carbon moiety but when the longer chain was used this already affected receptor binding (compare the whole hexyl and octyl series).

2.2.2. *Ex vivo experiments with isolated organs*

Determination of the pA_2 values in guinea pig trachea and ileum assay resulted in the expected results. In line with the receptor binding experiments in both of the isolated organ preparations atropine and glycopyrrolate were more active (Table 3) than our selected glycopyrrolate analogues chosen to represent compounds with markedly different receptor affinities. The ethyl and hexyl side chain containing compounds (**3c** and **4b**) were practically equally effective while the octyl chain containing esters showed weaker activity.

2.2.3. *In vivo experiments*

Carbachol induced bradycardia in the rat: The bradycardia protective effect of the selected new compounds was comparable both to their receptor binding affinity and their activity in the isolated organ experiments. In line with this their *in vivo* activity (Fig. 3) was lower than that of glycopyrrolate (GP) but what is more important their duration of action was notably shorter than that of the parent compound indicating their potential soft character.

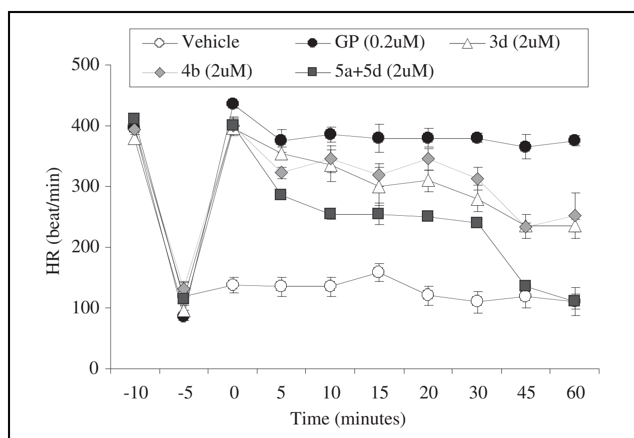


Fig. 3: Protective effect of different anticholinergics on carbachol induced bradycardia in anesthetized rats (mean ± SD; n = 3–5)

3. Experimental

3.1. Chemistry

Melting points were determined on a Boetius microscope and are uncorrected. Purity of the compounds was tested on TLC plates (silica gel, Merck). The spots were visualized under UV light and/or by exposure to iodine vapours. NMR spectra were recorded in $CDCl_3$, DMSO-d_6 or CD_3OD solutions using a Bruker Avance 500 spectrometer, operating at 500/125 MHz ($^1H/^{13}C$). Chemical shifts are given on the δ-scale and were referenced to TMS. Pulse programs for the 1D and 2D NMR experiments were taken from the Bruker software library. For structure elucidation and NMR signal assignment ^1H, ^{13}C, DEPT-135, selective 1D-NOESY, $^1H,^1H$-COSY, $^1H,^1H$-TOCSY, $^1H,^{13}C$-HSQC, $^1H,^{13}C$-HMBC, $^1H,^1H$-ROESY and $^1H,^1H$-NOESY spectra were recorded.
Analytical HPLC of compounds **3**, **4** and **5** was performed using a Waters (Milford, MA) HPLC system consisting of a model 510 isocratic pump working at 1 ml/min flow rate, a WISP programmable autoinjector with 10 µl injection volume and a model 486 single channel variable wavelength UV detector with 220 nm preset wavelength. The applied HPLC stationary phase was a Prontosil 120 C18 AQ 5 µm column with 150*4 mm geometry. Column temperature: 40 °C. The optimal mobile phase was a mixture of 30 mM ammonium acetate/MeOH/acetonitrile, in a ratio of 55/17.5/27.5 (v/v/v) for compounds **3**, in a ratio of 34/16/55 (v/v/v) for compounds **4** and in a ratio of 18/20/60 (v/v/v) for compounds **5**. The enantiomeric purity of 2-cyclopentylmandelic acid (**7**) was determined by chiral ligand exchange chromatography on a Nucleosil Chiral-1 5 µm, 250*4 mm chiral HPLC column. The mobile phase was 0.5 mM $CuSO_4$/acetonitrile 97/3 (v/v), flow rate: 1 ml/min, column temperature: 60 °C, detection wavelength: 220 nm. The retention time of the individual enantiomers was 13.5 min (R) and 15.5 min (S), respectively. The observed selectivity was 1.18.
(*R*)-2-Cyclopentylmandelic acid [(*R*)-(**7**)] was obtained by resolution of the racemic acid with (−)-cinchonidine (Mitsuya et al. 1999; Tsuchiya J et al. 1996; Mase T et al. 1996), (*R*)-methyl 2-cyclopentylmandelate [(*R*)-(**8**)] was prepared as described (Franko and Lunsford 1960) while (*R,S*)- and (*S*)-1-methyl-3-pyrrolidinol, [(*R,S*)- and (*S*)-(**9**)], were synthesized in two steps starting with (*R,S*)- and (*S*)-malic acid, respectively, as described (Sleevi et al. 1991). Ethyl bromoacetate (**11**) was purchased from Aldrich while the homologous n-hexyl (**12**) and n-octyl bromoacetates (**13**) were prepared by reaction of the corresponding alcohol with bromoacetyl bromide as described (Kronenthal, Schipper 1976). Found values of elemental analyses agreed with calculated vaues within the range of ± 1%.

3.1.1. Preparation of the quaternized target compounds **3**, **4** *and* **5**

Method A: A mixture of (2*R*, 3′*R*)-**10** and (2*R*, 3′*S*)-**10** (1.0 mM), together with the alkylating agent **12** or **13** (2.0 mM) in acetonitrile (12 ml) was stirred for 2 h at room temperature. After completion of the reaction the solvent was evaporated and the products were isolated as given below in the description of the individual compounds.
Method B: A mixture of (2*R*, 3′*R*)-**10** or (2*R*, 3′*S*)-**10** (0.3 mM) and the alkylating agent **11** or **12** (0.6 mM) in acetonitrile (5 ml) was allowed to react and the crude product was isolated as described under Method A above.

3.1.1.1. (2*R*,1′*R*,3′*S*)-3-(2-Cyclopentyl-2-phenyl-2-hydroxyacetoxy)-1-(ethoxycarbonyloxymethyl)-1-methylpyrrolidinium bromide (**3a**) and (2*R*, 1′*S*, 3′*S*)- 3-(2-cyclopentyl-2-phenyl-2-hydroxyacetoxy)-1-(ethoxycarbonyloxymethyl)-1-methylpyrrolidinium bromide (**3b**)

Method B above was followed starting with (2*R*, 3′*S*)-**10**. The crude product was purified by silica gel chromatography eluting with chloroform − methanol 9 : 1 and **3a** and **3b** were isolated as an inseparable mixture. Yield: 54%, mp. 163 °C, ratio **3a/3b** (^1H NMR): 4 : 1.
3a ^1H NMR ($CDCl_3$) δ 3.68 (3 H, s, NCH_3), 4.78 (1 H, d, NCH_2), 4.89 (1 H, d, NCH_2), 5.55 (1 H, m, H-3′); **3b** ^1H NMR ($CDCl_3$) δ 3.27 (3 H, s, NCH_3), 5.26 (1 H, d, NCH_2), 5.30 (1 H, d, NCH_2), 5.51 (1 H, m, H-3′).

3.1.1.2. (2*R*, 1′*R*, 3′*R*)-3-(2-Cyclopentyl-2-phenyl-2-hydroxyacetoxy)-1-(ethoxycarbonyloxymethyl)-1-methylpyrrolidinium bromide (**3c**) and (2*R*, 1′*S*,3′*R*)-3-(2-cyclopentyl-2-phenyl-2-hydroxyacetoxy)-1-(ethoxycarbonyloxymethyl)-1-methylpyrrolidinium bromide (**3d**)

Method B above was followed starting with (2*R*, 3′*R*)-**10** and the products **3c** and **3d** were separated by silica gel chromatography of the crude product eluting with chloroform − methanol 9 : 1.
3c: yield: 19%, mp. 98 °C, purity (HPLC): 93%. ^1H NMR ($CDCl_3$) δ 1.29 (3 H, t, $\underline{CH_3}CH_2O$), 2.24 (1 H, m, H_c-4′), 2.94 (1 H, m, $\underline{HC}$-C-2), 3.02 (3 H, s, $\overline{NCH_3}$), 3.07 (1 H, m, H_t-4′), 3.88 (1 H, m, H_c-2′), 4.13 (1 H, m, H_c-5′), 4.23 (2 H, q, $CH_3\underline{CH_2}O$), 4.46 (1 H, m, H_t-5′), 4.56 (1 H, m, H_t-2′), 5.10 (1 H, d, NCH_2), 5.22 (1 H, d, NCH_2), 5.56 (1 H, m, H-3′), 7.28 (1 H, t, Ph_p), 7.37 (2 H, t, Ph_m), 7.58 (2 H, d, Ph_o).
3d: yield: 28%, mp. 70 °C, purity (HPLC): 96%. ^1H NMR ($CDCl_3$) δ 1.33 (3 H, t, $\underline{CH_3}CH_2O$), 2.24 (1 H, m, H_c-4′), 3.66 (3 H, s, NCH_3), 4.65 (1 H, d, NCH_2), 4.74 (1 H, d, NCH_2), 5.54 (1 H, m, H-3′), 7.27 (1 H, t, Ph_p), 7.35 (2 H, t, Ph_m), 7.59 (2 H, d, Ph_o).

3.1.1.3. (2*R*, 1′*R*, 3′*S*)-3-(2-Cyclopentyl-2-phenyl-2-hydroxyacetoxy)-1-(*n*-hexyloxycarbonyloxymethyl)-1-methylpyrrolidinium bromide (**4a**) and (2*R*, 1′*S*, 3′*S*)-3-(2-cyclopentyl-2-phenyl-2-hydroxyacetoxy)-1-(n-hexyloxycarbonyloxymethyl)-1-methylpyrrolidinium bromide (**4b**)

Method B above was followed starting with (2*R*, 3′*S*)-**10** and the products **4a** and **4b** were separated by silica gel chromatography of the crude product eluting with chloroform − methanol 9 : 1.
4a: yield: 18%, mp. 146 °C, purity (HPLC): 93%.
4b: yield: 23%, mp. 125–128 °C, purity (HPLC): 96%.
For NMR data of **4a–b** see under 2.1.2. above.

3.1.1.4. (2*R*, 1′*R*, 3′*R*)-3-(2-Cyclopentyl-2-phenyl-2-hydroxyacetoxy)-1-(*n*-hexyloxycarbonyloxymethyl)-1-methylpyrrolidinium bromide (**4c**) and (2*R*, 1′*S*, 3′*R*)-3-(2-cyclopentyl-2-phenyl-2-hydroxyacetoxy)-1-(*n*-hexyloxycarbonyloxymethyl)-1-methylpyrrolidinium bromide (**4d**)

Method B above was followed starting with (2*R*, 3′*R*)-**10** and the products **4c** and **4d** were separated by silica gel chromatography of the crude product eluting with chloroform − methanol 9 : 1.
4c: yield: 20%, mp. 138 °C, purity (HPLC): 98%.
4d: yield: 55%, mp. 116 °C, purity (HPLC): 95%.
For NMR data of **4c–d** see under 2.1.2. above.
As an alternative, upon preparing compounds **4a–d** following Method A the products **4b** and **4c** could be isolated in pure state as described below while **4a** and **4d** were obtained in the form of an inseparable mixture. Thus, the crude product was triturated with ethyl acetate to give the mixture **4a** + **4d** as a solid, yield: 42%. The mother liquor was concentrated to dryness and the residue was purified by column chromatography on silica gel eluting with chloroform − methanol 9 : 1. Subsequently compounds **4b** (yield: 12%) and **4c** (yield: 6%) were separated by preparative thin layer chromatography developing with chloroform − methanol 9 : 1.

3.1.1.5. (2*R*, 1′*R*, 3′*S*)-3-(2-Cyclopentyl-2-phenyl-2-hydroxyacetoxy)-1-(*n*-octyloxycarbonyloxymethyl)-1-methylpyrrolidinium bromide (**5a**), (2*R*, 1′*S*, 3′*S*)-3-(2-cyclopentyl-2-phenyl-2-hydroxyacetoxy)-1-(n-octyloxycarbonyloxymethyl)-1-methylpyrrolidinium bromide (**5b**), (2*R*, 1′*R*, 3′*R*)-3-(2-cyclopentyl-2-phenyl-2-hydroxyacetoxy)-1-(n-octyloxycarbonyloxymethyl)-1-methylpyrrolidinium bromide (**5c**) and (2*R*, 1′*S*, 3′*R*)-3-(2-cyclopentyl-2-phenyl-2-hydroxyacetoxy)-1-(*n*-octyloxycarbonyloxymethyl)-1-methylpyrrolidinium bromide (**5d**)

By following Method A above and purification of the crude product by silica gel column chromatography eluting with chloroform − methanol 9 : 1 the compounds **5a** and **5d** were obtained in the form of a mixture inseparable by TLC and HPLC. On the other hand, **5b** and **5c** could be separated by a final preparative TLC, developing with chloroform − methanol 9 : 1.
Mixture of **5a** and **5d**: yield: 47%, ratio **5a/5d** (^1H NMR): 1 : 1.
5a ^1H NMR ($CDCl_3$) δ 2.10 (1 H, m, H_c-4′), 3.64 or 3.67 (3 H, s, NCH_3), 4.71 (1 H, d, NCH_2), 4.81 (1 H, d, NCH_2); **5d** ^1H NMR ($CDCl_3$) δ 2.28 (1 H, m, H_c-4′), 3.64 or 3.67 (3 H, s, NCH_3), 4.54 (1 H, d, NCH_2), 4.67 (1 H, d, NCH_2).

5b: yield: 10%, mp. 30 °C, purity (HPLC): 86%. ^{1}H NMR ($CDCl_3$) δ 0.90 (3 H, t, $\underline{CH_3}CH_2$), 2.00 (1 H, m, H_c-4′), 2.93 (1 H, m, $\underline{HC}$-C-2 and 1 H, m, H_t-4′), 3.27 (3 H, s, NCH_3), 4.10 (1 H, m, H_c-5′), 4.18 (2 H, t, $CH_3\underline{CH_2}$), 4.18 (1 H, m, H_c-2′), 4.29 (1 H, m, H_t-5′), 4.58 (1 H, m, H_t-2′), 5.16 (2 H, s, br, NCH_2), 5.56 (1 H, m, H-3′), 7.27 (1 H, t, Ph_p), 7.35 (2 H, t, Ph_m), 7.57 (2 H, d, Ph_o).
5c: yield: 7.5%, purity (HPLC): 93%. ^{1}H NMR ($CDCl_3$) δ 0.89 (3 H, t, $\underline{CH_3}CH_2$), 2.28 (1 H, m, H_c-4′), 2.93 (1 H, m, $\underline{HC}$−C-2), 2.53 (3 H, s, NCH_3), 3.05 (1 H, m, H_t-4′), 3.86 (1 H, m, H_c-2′), 4.12 (1 H, m, H_c-5′) 4.16 (2 H, t, $CH_3\underline{CH_2}$), 4.30 (1 H, m, H_t-5′), 4.50 (1 H, m, H_t-2′), 4.97 (1 H, d, NCH_2), 5.10 (1 H, d, NCH_2), 5.58 (1 H, m, H-3′), 7.27 (1 H, t, Ph_p), 7.38 (2 H, t, Ph_m), 7.59 (2 H, d, Ph_o).

3.1.2. *Mixture of (2R, 3′R)- and (2R, 3′S)-3-(2-Cyclopentyl-2-phenyl-2-hydroxyacetoxy)-1-methylpyrrolidine* [*(2R, 3′R)*-**10** *and (2R, 3′S)*-**10**]

A solution of diisopropyl azodicarboxylate (1.5 mM) in tetrahydrofuran (1 ml) was added dropwise to a mixture of (*R*)-**7** (1.5 mM), (*R,S*)-**9** (1.64 mM) and triphenylphosphine (1.5 mM) in tetrahydrofuran (4 ml) at room temperature. The reaction mixture was stirred at room temperature for 2 h and the solvent was removed *in vacuo*. The residue was suspended in ethyl acetate and extracted with 1 N hydrochloric acid. The aqueous solution was made alkaline with 5 N aqueous sodium hydroxide, followed by extraction with ether. The organic layer was dried over magnesium sulphate and concentrated in vacuo giving the title compound as a colourless oil. Yield: 87%, purity (HPLC): 97% (total area of two unresolved peaks).
1 : 1 mixture of (2*R*, 3′*R*)-**10** and (2*R*, 3′*S*)-**10:** ^{1}H NMR ($CDCl_3$) δ 2.35 and 2.39 (3 H, s, NCH_3), 2.56 and 2.68 (1 H, m, H_c-2′), 2.94 (1 H, m, $\underline{HC}$−C-2), 3.75 (1 H, s, $\underline{HO}$−C-2), 5.24 (1 H, m, H-3′), 7.27 (1 H, t, Ph_p), 7.36 (2 H, t, Ph_m), 7.67 (2 H, d, Ph_o).

3.1.2.1. (2*R*, 3′*S*)-3-(2-Cyclopentyl-2-phenyl-2-hydroxyacetoxy)-1-methylpyr-rolidine (2*R*, 3′*S*)-**10**

The methyl ester (*R*)-(**8**) was submitted to transesterification with (*S*)-**9** according to Franko and Lunsford (1960). Yield: 28%, purity (HPLC): 97%.
^{1}H NMR ($CDCl_3$) δ 2.39 (3 H, s, NCH_3), 2.68 (1 H, m, H_c-2′), 2.94 (1 H, m, $\underline{HC}$−C-2), 3.79 (1 H, s, br, $\underline{HO}$−C-2), 5.24 (1 H, m, H-3′), 7.27 (1 H, t, Ph_p), 7.35 (2 H, t, Ph_m), 7.67 (2 H, d, Ph_o).

3.1.2.2. (2*R*, 3′*R*)-3-(2-Cyclopentyl-2-phenyl-2-hydroxyacetoxy)-1-methylpyrrolidine (2*R*, 3′*R*)-**10**

(*R*)-(**7**) was coupled with (*S*)-**9** under Mitsunobu conditions as described above under 3.1.2. Yield: 49%, purity (HPLC): 95%. ^{1}H NMR ($CDCl_3$) δ 2.34 (3 H, s, NCH_3), 2.93 (1 H, m, $\underline{HC}$−C-2), 3.78 (1 H, m, $\underline{HO}$−C-2), 5.23 (1 H, m, H-3′), 7.27 (1 H, t, Ph_p), 7.34 (2 H, t, Ph_m), 7.67 (2 H, d, Ph_o).

3.2. *Biology: test methods*

3.2.1. *Receptor binding assay*

The binding of [^{3}H]quinuclidinyl benzylate ([^{3}H]QNB; Amersham, 42.0 Ci/mmol) to muscarinic receptors was measured according to the method of Yamamura and Snyder (1974), with some modifications, as described previously by Barlocco et al. (1997). Briefly, male Sprague-Dawley rats (180–220 g) were decapitated and cerebral cortices removed, discarding the white matter. Pooled tissue was homogenized in 10 volumes of ice-cold 50 mM Tris-HCl buffer (pH 7.4) by a motor driven glass homogenizer. The homogenate was centrifuged at 4 °C and 30,000 × g for 10 min. The pellet was washed twice with the same buffer by resuspension, followed by centrifugation at 4 °C and 30,000 × g for 10 min. The final pellet was resuspended in 10 volumes of Tris-HCl buffer and stored at −20 °C before use. Protein content was determined by the method of Bradford (1976) using bovine serum albumin as the standard.
In all binding experiments membranes (0.25 mg/ml protein), radioligand and competing drugs were incubated in a final volume of 1 ml of 50 mM Tris-HCl buffer (pH 7.4) for 60 min at 25 °C. For saturation studies membranes were incubated with 0.01–2 nM [^{3}H]QNB. In competition experiments the final concentration of the radioligand was 0.2 nM and competing drugs were given in eight concentrations. Non-specific binding was determined with 1 μM atropine. The incubation was terminated by rapid vacuum filtration over Whatman GF/B filters using a Brandel Cell Harvester. Samples were washed immediately with 3 × 4 ml ice-cold Tris-HCl buffer and placed in 6 ml scintillation fluid. Radioactivity was estimated by liquid scintillation counting.
Data are the mean ± S.E.M. of at least three experiments run in duplicate. GraphPad Prism 3.0 (GraphPad Software Inc., San Diego, CA, USA) was used to perform linear and non-linear regression analysis of the data. Saturation binding parameters (K_d and B_{max}) were determined by linear regression analysis of the transformed saturation binding data. Competition binding isotherms were analyzed by non-linear regression to derive estimates of the IC_{50} values and Hills slopes. IC_{50} values were converted to K_i values according to the equation of Cheng and Prusoff (1973).

3.2.2. *Guinea pig trachea and ileum assay (pA$_2$ value determination)*

Tracheal preparations were made as described previously in details by Preuss and Goldie (1999). Briefly: tracheas were isolated from male Dunkin-Hartley guinea pigs (280–300 g) and the ring preparations (2–3 mm in width) were suspended under 500 mg resting tension in an organ bath containing Krebs' bicarbonate buffer aerated with 95% O_2/5% CO_2. Changes in isometric tension were measured by a force displacement transducer (Experimetria, Budapest, Hungary) coupled to a Watanabe recorder. Cumulative concentration-effect curves were constructed to carbachol in the absence or presence of the antagonists. In each animal two preparations were used as time control (i.e., repeated carbachol curves in the absence of any antagonist) the remaining two preparations were used to test responses in the presence of two different concentrations of the antagonist.
Antagonists were added to the organ bath 30 min prior to commencement of the agonist concentration versus effect curves. Schild plots were constructed for the antagonists against carbachol and pA$_2$ values as well as slope estimates were obtained.
The ileum longitudinal muscle strips with adhering myenteric plexus were also prepared from male guinea-pigs (200–400 g). A segment of small intestine (8–10 cm) 10 cm proximal to the ileo-coecal valve was dissected. The longitudinal muscle strip was obtained by mounting segments of the whole ilea on a 1 ml pipette and gently tearing away the outer longitudinal muscle layer with a cotton swab. Longitudinal muscle strips were cut into 3–4 cm pieces. The strips were mounted in an organ bath containing Tyrode solution at 37 °C under a resting tension of 500 mg. Contractions were recorded isometrically with the same strain gauge system as above and registered on the Watanabe type polygraph. The tissues were left to equilibrate for 30 min. Dose-response curves to the agonist were constructed by addition of acetylcholine in increasing concentrations. The doses were given at 10 min intervals with 1 min contact time. After a 40 min equilibration period, the preparations were incubated with the antagonist for 20 min, and a second concentration-response curve to acetylcholine was constructed. The agonist (Ach) was non-cumulatively added at 10 min intervals (concentration range: 10^{-10} to 10^{-5} M). Antagonists were applied in the concentration range of 10^{-8} to 10^{-5} M, depending on the individual test compound.
Responses were measured as changes in isometric tension and calculated as a percentage of the maximum response attained in the initial concentration-response curve. Determination of antagonist potencies was done by constructing Schild double logarithmic plot of log (DR-1) versus -log M concentrations of the antagonist, and the slope of the plot was computed. If the slopes of the plots were not significantly different from unity, the interaction was accepted as competitive in nature, and antagonist potencies were expressed as pA$_2$ values (Arunlakshana and Schild 1959). If the slopes of the plots were significantly different from unity, the method of Ariens and Van Rossum (1957) was used to determine pD′ values for characterization of non-competitive antagonism. Statistical significance was assessed by ANOVA followed by Dunnett test.

3.2.3. *Antagonistic effect on carbachol induced bracycardia*

The experimental procedure described previously in details by Juhasz et al. (1998) was followed. Male Sprague-Dawley rats, weighing 300–350 g were anesthetized with sodium pentobarbital (50 mg/kg i.p.). Baseline electrocardiography (ECG) recordings were performed after 15 min stabilization periods. Needle electrodes were inserted subcutaneously into the limbs of the anesthetized rats and were joined to a Watanabe recorder. Recording of the heart rate (1/min) was taken before, during and after the administration of any of the compounds until basic ECG parameters returned to baseline at a paper speed of 25 mm/s. All drugs were administered by direct injection into the jugular vein. Anticholinergic drugs were administered in the approximate pharmacodynamic equivalent doses (0.2, 2.0 umol/kg) at time 0, while carbachol (5–8 ug/kg) was injected at −5, 5, 10, 15, 20, 30, 45, 60 min.

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