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Stereoisomers of N-substituted soft anticholinergics and their zwitterionic metabolite based on glycopyrrolate – syntheses and pharmacological evaluations

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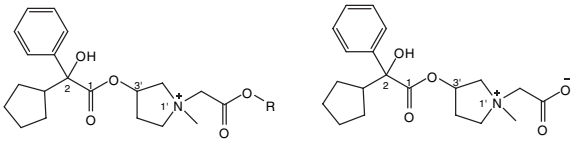
Purpose. In this study, isomers of two N-substituted soft anticholinergics based on glycopyrrolate, SGM (PcPOAGP_NA.Me) and SGE (PcPOAGP_NA.Et) [3′-(2-cyclopentyl-2-phenyl-2-hydroxyacetoxy)-1′-methyl-1′-alkoxycarbonylpyrrolidinium bromide] and their zwitterionic metabolite, SGa (PcPOAGP_NA.H) [3′-(2-cyclopentyl-2-phenyl-2-hydroxyacetoxy)-1′-methyl-1′-carboxymethylpyrrolidinium inner salt] were synthesized and their pharmacological activities were evaluated *in vitro* and *in vivo*. *Methods*. The isomers of SGM and SGE were synthesized with both optically pure methyl-cyclopentylmandelate and 3-hydroxy-*N*-methylpyrrolidine. Trans-esterification followed by quarternization with alkyl bromoacetate gave four isomers of SGM or SGE with the nitrogen chiral center unresolved (2*R*3′*S*-SGM, 2*R*3′*R*-SGM, 2*S*3′*S*-SGM, 2*S*3′*R*-SGM or 2*R*3′*S*-SGE, 2*R*3′*R*-SGE, 2*S*3′*S*-SGE, 2*S*3′*R*-SGE). The hydrolysis of these four isomers followed by HPLC separation resulted in eight fully resolved isomers of SGa (2*R*3′*R*1′*R*, 2*R*3′*S*1′*R*, 2*R*3′*R*1′*S*, 2*R*3′*S*1′*S*, 2*S*3′*R*1′R, 2*S*3′*S*1′*R*, 2*S*3′*R*1′*S*, and 2*S*3′*S*1′*S*). Pharmacological activities were assessed by using *in vitro* receptor-binding assay and guinea pig ileum pA_2-assay, and by evaluating the *in vivo* rabbit mydriatic effects. Results were compared to those obtained with conventional anticholinergic agents, such as glycopyrrolate, *N*-meythylscopolamine, and tropicamide, as well as those obtained with previously prepared racemic mixtures and *2R* isomers. *Results*. Receptor binding pK_i values at cloned human muscarinic receptors (M_1–M_4 subtypes) were in the 6.0–9.5 range for the newly synthesized SGM and SGE isomers, and in the 5.0–8.6 range for the SGa isomers. In all cases, 2*R* isomers were significantly more active than 2*S* isomers (27 to 447 times for SGM isomers, and 6 to 4467 times for SGa isomers). Among the four SGM isomers with unresolved 1′ (N) chiral center, the 3′*R* isomers were more active than the corresponding 3′*S* isomers (1.5–12.9 times), whereas, among the SGa isomers, the 3′*S* isomers were not always more active than the corresponding 3′*R* isomers indicating that activity determined based on configuration at chiral center 3′ is significantly affected by the configuration of the other two chiral centers, 2 and 1′. Among the completely resolved eight SGa isomers (all three chiral centers resolved), 1′*S* isomers were always more active than the corresponding 1′*R* isomers (1.8–22.4 times). Results also indicate that some isomers showed good M_3/M_2 muscarinic-receptor subtype-selectivity (about 3–5 times), and 2*R* and 3′*S* were the determining configurations for this property. Guinea pig ileum assays and rabbit mydriasis tests on SGa isomers further confirmed the stereospecificity. In rabbit eyes, some 2*R*-SGa isomers showed mydriatic potencies similar to glycopyrrolate and exceeded tropicamide, but their mydriatic effects lasted considerably shorter, and they did not induce dilation of the pupil in the contralateral, water-treated eye. These results indicate that these compounds are locally active, but safe and have a low potential to cause systemic side effects. The pharmacological potency of the eight SGa isomers was estimated as $2R3'S1'S \approx 2R3'R1'S \approx 2R3'S1'R > 2R3'R1'R > 2S3'R1'S > 2S3'S1'S \approx 2S3'R1'R > 2S3'S1'R$ ($p < 0.05$). *Conclusions.* The stereospecificity and M_3/M_2 muscarinic-receptor subtype-selectivity of soft anticholinergics, SGM, SGE, and SGa have been demonstrated. In agreement with previous results, the potential for their effective and safe use has been confirmed.

1. Introduction

Stereospecificity is known to be important at muscarinic receptors (Barlow et al. 1973; Pauling and Datta 1980). We have previously synthesized and studied the pharmacological potencies, pharmacokinetic characteristics and QSAR of various soft analogs of glycopyrrolate (Ji et al. 2000, 2002, 2005; Wu et al. 2005; Mori et al. 2006). Among them, SGM (PcPOAGP_NA.Me) and SGE (PcPOA*GP*_NA.Et), 3′-(2-cyclopentyl-2-phenyl-2-hydroxyacetoxy)-1′-methyl-1′-alkoxycarbonylpyrrolidinium bromide, and their common zwitterionic metabolite, SGa (PcPOA*GP*_NA.H), 3′-(2-cyclopentyl-2-phenyl-2-hydroxyacetoxy)-1′-methyl-1′-carboxymethylpyrrolidinium inner salt showed promising activity and safety in animal studies (Wu et al. 2005; Ji et al. 2005; Mori et al. 2006; Buchwald and Bodor 2006). These compounds indeed exhibited stereospecificity toward muscarinic receptors, and the anticholinergic activity has been improved with the 2*R* configuration. In addition, SGa also showed a moderate M_3/M_2 muscarinic-receptor subtype-selectivity that indicates a reduced systemic cardiac side effect (Wu et al. 2005). However, these soft anticholinergic structures possess three chiral centers so that up to eight different isomers are possible, e.g. 2*R*3′*R*1′*R*, 2*R*3′*S*1′*R*, 2*R*3′*R*1′*S*, 2*R*3′*S*1′*S*, 2*S*3′*R*1′*R*, 2*S*3′*S*1′*R*, 2*S*3′*R*1′*S*, and 2*S*3′*S*1′*S*. Previous investigations using only one resolved chiral center (2*R* or 2*S*) found that 2*R* enantiomers (a mixture of four diastereoisomers 2*R*3′*R*1′*R*, 2*R*3′*S*1′*R*, 2*R*3′*R*1′*S*, and 2*R*3′*S*1′*S*) were more active than 2*S* enantiomers (a mixture of 2*S*3′*R*1′*R*, 2*S*3′*S*1′*R*, 2*S*3′*R*1′*S*, and 2*S*3′*S*1′*S*). Here, further investigations in the stereospecificity of these soft glycopyrrolates are reported for five partially-resolved soft anticholinergics ester isomers (2*R*3′*S*-SGM, 2*R*3′*R*-SGM, 2*S*3′*S*-SGM, 2*S*3′*R*-SGM and 2*R*3′*S*-SGE) and eight fully resolved acidic metabolite SGa isomers. Compounds were systematically synthesized, and isomers were separated. Relative pharmacological activities and M_3/M_2 muscarinic-receptor subtype-selectivities were investigated by using *in vitro* receptor-binding assay, *in vitro* guinea pig ileum pA_2-assay, and *in vivo* mydriatic effect studies in rabbits.



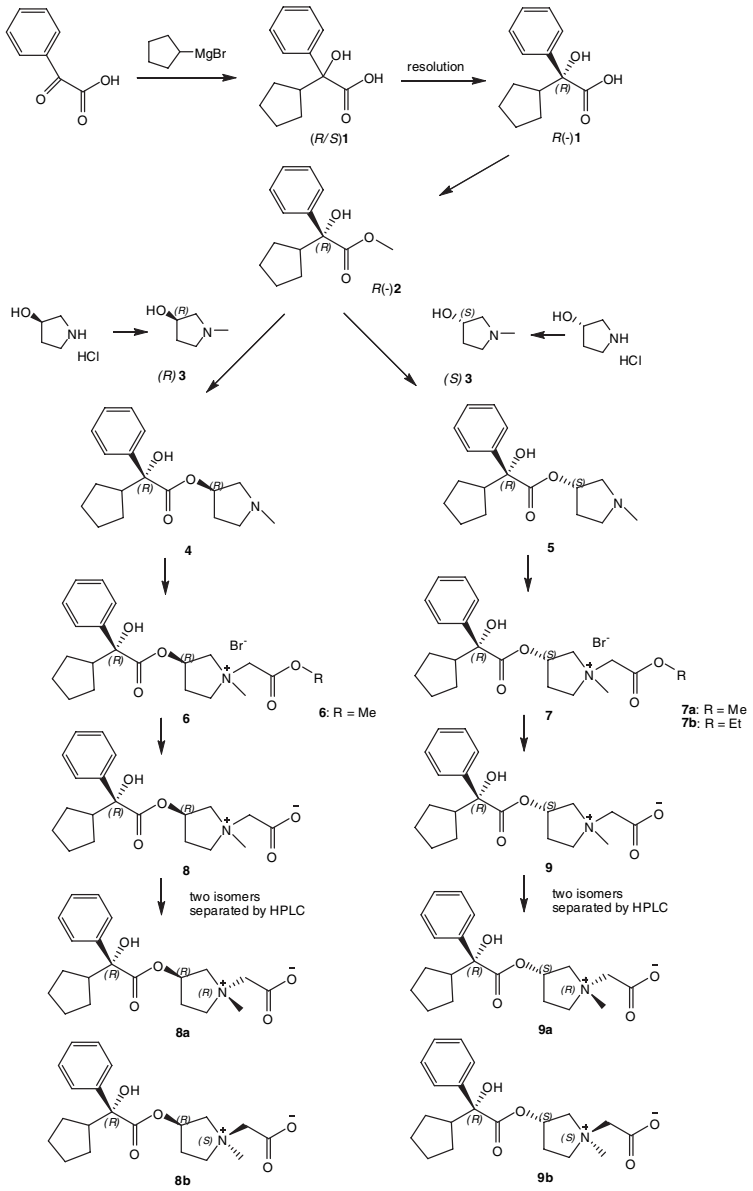
2. Investigations, results and discussion

2.1. Synthesis

Five soft anticholinergic ester (SGM and SGE) isomers and eight zwitterionic metabolite (SGa) isomers have been newly synthesized. The 2*R* diastereoisomers (2*R*3′*R*-SGM, 2*R*3′*S*-SGM, 2*R*3′*S*-SGE, 2*R*3′*R*1′*R*-SGa, 2*R*3′*R*1′*S*-SGa, 2*R*3′*S*1′*R*-SGa, and 2*R*3′*S*1′*S*-SGa) were obtained by the synthetic pathways reported previously (Ji et al. 2005) with a minor modification. As shown in Scheme 1, first, the racemic cyclopentylmandelic acid **1** was synthesized with cyclopentylmagnesium bromide and benzoylformic acid. This racemic acid was resolved by repeated crystallization of the salts produced between this acid and (−)-strychnine (March 1992; Pavia et al. 1988). The left rotatory (−22.5°) optically pure free acid *R*(−)**1** was recovered by basification of the salts with sodium hydroxide solution followed by acidification with hydrochloric acid. Methylation of *R*(−)**1** with methyl iodide and potassium carbonate in DMF at room temperature yields methyl 2*R*(−)cyclopentylmandelate, *R*(−)**2**. Transesterfication of *R*(−)**2** with *R*-3-hydroxy-*N*-methylpyrrolidine, (*R*)-**3** (made from *R*-3-hydroxypyrrolidine with paraformaldehyde and formic acid), gave (3′*R*)-*N*-methyl-3′-pyrrolidinyl-2*R*-cyclopentylmandelate **4**; or with *S*-3-hydroxy-*N*-methylpyrrolidine (*S*)-**3** (made from *S*-3-hydroxypyrrolidine with paraformaldehyde and formic acid), gave (3′*S*)-*N*-methyl-3′-pyrrolidinyl-2*R*-cyclopentyl mandelate **5**. Quarternization of **4** and **5** with methyl or ethyl bromoacetate in acetonitrile gave **6** (2*R*3′*R*-SGM), **7a** (2*R*3′*S*-SGM), and **7b** (2*R*3′*S*-SGE). Each of these has two diastereoisomers due to the 1′ nitrogen chiral center with an *R*:*S* ratio of 2:1 as shown by 1H NMR spectra. Hydrolysis of **6** and **7a** gave their zwitterionic inner salts **8** and **9**. Each zwitterionic salts also possesses two diastereoisomers with a ratio of 2:1 that could be separated by HPLC to give zwitterionic isomers **8a**, **8b**, **9a**, and **9b.** From ^{1}H NMR, **8a**, **8b**, as well as **9a**, **9b** were evidenced to be pairs of diastereoisomers based on chiral nitrogen. To identify the absolute configuration of these isomers, **8b** was dissolved in $CDCl_3$ for further investigation using nuclear Overhauser effect (NOE). The 2D ^{1}H−^{1}H NOESY spectrum indicated the methyl group on the nitrogen to be on the same side as the hydrogen at 3′-position of the pyrrolidinium ring. Accordingly, the configuration of the nitrogen should be *S*, and the absolute stereochemistry of **8b** 1′(*S*), 3′(*R*)-[2(*R*)-cyclopentyl-2-phenyl-2-hydroxyacetoxy]-1′-methyl-1′-carboxymethylpyrrolidinium inner salt (2*R*3′*R*1′*S*-SGa). Therefore, **8a** is 1′(*R*), 3′(*R*)-[2(*R*)-cyclopentyl-2-phenyl-2-hydroxyacetoxy]-1′-methyl-1′-carboxymethylpyrrolidinium inner salt (2*R*3′*R*1′*R*-SGa); **9a** is 1′(*R*), 3′(*S*)-[2(*R*)-cyclopentyl-2-phenyl-2-hydroxyacetoxy]-1′-methyl-1′-carboxymethylpyrrolidinium inner salt (2*R*3′*S*1′*R*-SGa); and **9b** is 1′(*S*),3′(*S*)-[2(*R*)-cyclopentyl-2-phenyl-2-hydroxyacetoxy]-1′-methyl-1′-carboxymethylpyrrolidinium inner salt (2*R*3′*S*1′*S*-SGa).

Grover and coworkers reported the highly stereoselective synthesis of (*S*)-cyclopentyl-mandelic acid in five steps starting with (*S*)-mandelic acid (Grover et al. 2000). We modified their procedure and obtained pure *S*(+)-cyclopentylmandelic acid in three steps with good yield. As displayed in Scheme 2, reaction of *S*(+)-mandelic acid with pivaldehyde in the presence of trifluoromethanesulfonic acid as catalyst gave the product of *cis*-(2*S*,5*S*)-2-(*tert*-butyl)-5-phenyl-1,3-dioxolan-4-one, **10,** in about 90% yield. At −78 °C, deprotonation of **10** with lithium *bis*(trimethylsilyl)amide followed by adding cyclopentyl bromide generated *cis*-(2*S*,5*S*)-2-(*tert*-butyl)-5-cyclopentyl-5-phenyl-1,3-dioxolan-4-one, **11**. Base hydrolysis of **11** with potassium hydroxide, followed by acidification with hydrochloric acid provided the expected (*S*)-(+)-cyclopentylmandelic acid **12**. After this step, the same procedures as for **8a**, **8b, 9a**, and **9b** including methylation, esterification, quaternization, and hydrolyses were followed to give the final four zwitterionic isomers, 1′(*R*),3′(*R*)-[2(*S*)-cyclopentyl-2-phenyl-2-hydroxyacetoxy]-1′-methyl-1′-carboxymethylpyrrolidinium inner salt **18a** (2*S*3′*R*1′*R*-SGa), 1′(*S*),3′(*R*)-[2(*S*)-cyclopentyl-2-phenyl-2-hydroxyacetoxy]-1′-methyl-1′-carboxymethylpyrrolidinium inner salt **18b** (2*S*3′*R*1′*S*-SGa), 1′(*R*),3′(*S*)-[2(*S*)-cyclopentyl-2-phenyl-2-hydroxyacetoxy]-1′-methyl-1′-carboxymethylpyrrolidinium inner salt **19a** (2*S*3′*S*1′*R*-SGa), and 1′(*S*),3′(*S*)-[2(*S*)-cyclopentyl-2-phenyl-2-hydroxyacetoxy]-1′-methyl-1′-carboxymethylpyrrolidinium inner salt **19b** (2*S*3′*S*1′*S*-SGa). They were also characterized by NMR.

Scheme 1

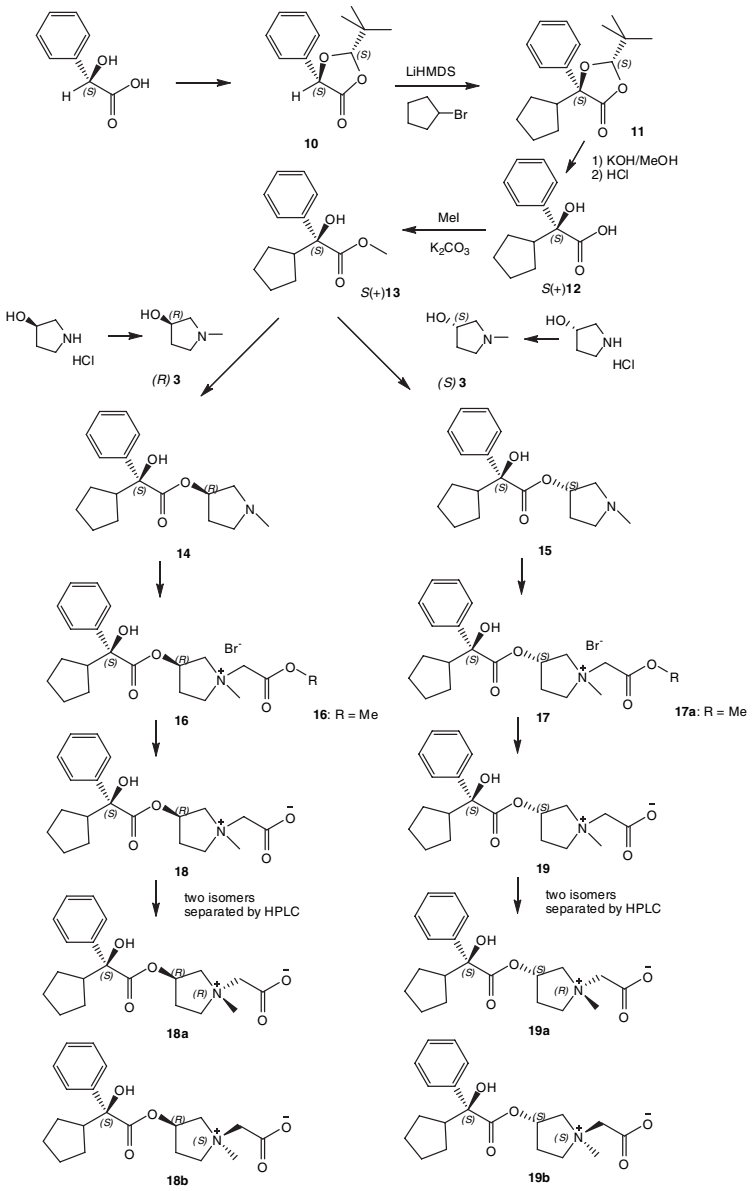


2.2. Receptor binding studies

The receptor binding affinities of soft analogs, pK_i, determined by radioligand binding assays using human cloned muscarinic receptor subtypes, M_1–M_4, are presented in Table 1. The pK_i of newly synthesized isomers were compared with that of the previously reported racemic and *2R* isomeric parent soft drugs (the methyl ester 2*R*-SGM and the ethyl ester 2*R*-SGE), racemic and *2R* isomeric SGa (the zwitterionic metabolite) as well as those of glycopyrrolate and *N*-methylscopolamine (Wu et al. 2005). The pK_i of the racemic forms, (±)SGM and (±)SGE, showed lower receptor binding affinities than their corresponding 2*R* isomers (7.8–8.3 vs. 8.7–9.5), confirming that stereospecificity is important at these receptors. The potencies of these 2*R* isomers are similar to those of glycopyrrolate (8.7–9.9) and *N*-methylscopolamine (9.2–9.9). Resolution of the 2 and 3′ chiral centers of (±)SGM resulted in four stereoisomers, 2*R*3′*R*-SGM, 2*R*3′*S*-SGM, 2*S*3′*R*-SGM, and 2*S*3′*S*-SGM with pK_i values of 9.0–9.5, 7.9–8.9, 7.0–7.6, and 6.0–6.5, respectively. These values indicate that among SGM isomers, not only are the 2*R* isomers more potent than the corresponding 2*S* isomers, but also the 3′*R* isomers are more potent than the corresponding 3′*S* isomers. 2*R*3′*S*-SGE showed a pK_i value of 8.2–8.7, similar to that of 2*R*3′*S*-SGM. The same table also shows the calculated M_3/M_2 muscarinic-receptor subtype-selectivities. Contrary to the previously reported 2*R*-SGM and 2*R*-SGE that show no M_3/M_2 subtype selectivity, 2*R*3′*S*-SGM and 2*R*3′*S*-SGE show significantly increased M_3/M_2 muscarinic-receptor subtype-selectivity ($p < 0.01$, *t*-test assuming equal variances). The M_3 affinity was $5.0_{\pm 1.1}$ fold higher than M_2 affinity for 2*R*3′*S*-SGM, and $2.8_{\pm 0.8}$ fold higher for 2*R*3′*S*-SGE indicating that the configuration of the 3′ chiral center may play an important role in the safety profile of these soft anticholinergics.

The receptor-binding pK_i of racemic (±)SGa and isomeric 2*R*-SGa from previous studies are also shown in Table 1. In agreement with soft drug design principles stating that the acidic moiety formed by hydrolysis of the parent ester should inactivate the drug, SGa compounds were found considerably less active than their corresponding parent es-

Scheme 2



ters; for example, pK_i of 5.5–6.4 for (±)SGa vs. 7.8–8.3 for (±)SGM and 7.3–7.9 for (±)SGE, as well as 7.5–8.2 for 2*R*-SGa vs. 8.9–9.5 for 2*R*-SGM and 8.7–8.9 for 2*R*-SGE (Ji et al. 2005; Wu et al. 2005). As discussed previously, the zwitterionic SGa retains some activity because the electronic distribution in its structure somewhat resembles those of the corresponding neutral, active anticholinergics (Wu et al. 2005). To obtain a better picture of the role of stereospecificity/stereoselectivity in this type of anticholinergics, SGa was chosen as a model compound in the present investigation. On one hand, because SGa, either in its fully racemic or 2*R* form, was very soluble and stable in aqueous solutions (buffer or biological media, pH 6–8), and, on the other hand, because 2*R*-SGa has been found topically active (e.g., in rabbit eyes) and could be excreted unchanged, rapidly through urine ($t_{1/2}$ 10–15 min after i.v. in rats) (Wu et al. 2005). As shown in Table 1, the pK_i of the completely resolved eight isomers of SGa, 2*R*3′*R*1′*R*-SGa, 2*R*3′*R*1′*S*-SGa, 2*R*3′*S*1′*R*-SGa, 2*R*3′*S*1′*S*-SGa, 2*S*3′*R*1′*R*-SGa, 2*S*3′*R*1′*S*-SGa, 2*S*3′*S*1′*R*-SGa, and 2*S*3′*S*1′*S*-SGa, cover a relatively wide range from 4.5 to 8.6. In all cases, the 2*R* isomers are more potent than the corresponding 2*S* isomers, and the 1′*S* isomers are more potent than the corresponding 1′*R* isomers. The relative potencies of the 3′*R* and 3′*S* isomers varied depending on the configuration of the 2 chiral center; e.g., 2*R*3′*S*1′*R* > 2*R*3′*R*1′*R* and 2*R*3′*S*1′*S* > 2*R*3′*R*1′*S*; but 2*S*3′*R*1′*R* > 2*S*3′*S*1′*R* and 2*S*3′*R*1′*S* > 2*S*3′*S*1′*S*. Also, similar to SGM isomers, among 2*R* isomers of SGa, the 2*R*3′*S* isomers (2*R*3′*S*1′*R* and 2*R*3′*S*1′*S*) showed highest M_3/M_2 muscarinic-receptor subtype-selectivities (5.2–5.5 fold higher) followed by the 2*R*3′*R* isomers (2*R*3′*R*1′*R* and 2*R*3′*R*1′*S*, 3.3–3.5 fold higher). The 2*S* isomers did not show any M_3/M_2 selectivity. Thus, the importance of the configuration at the 2 and 3′ chiral centers (2*R*3′*S*) on the M_3/M_2 selectivity of this type of anticholinergics has been demonstrated.

To compare stereoselectivity (fold increase in binding) at each chiral center, the ratios of binding activities of each corresponding paired isomers have been calculated; results are shown in Table 2. These are relative potencies calculated from the receptor binding affinity pK_i data of

Table 1: Receptor binding affinities, M_3/M_2 selectivities, and pA_2 values

Compd.	Subtypes of cloned muscarinic receptors[a]				Selectivity[b]	pA_2^c
	M_1	M_2	M_3	M_4	M_3/M_2	
(±) SGM[d]	7.91 ± 0.05 (1.02 ± 0.12)	7.79 ± 0.11 (1.25 ± 0.08)	7.80 ± 0.10 (1.17 ± 0.18)	8.29 ± 0.19 (1.12 ± 0.05)	1.0 ± 0.0	7.90 ± 0.13
(±) SGE[d]	7.51 ± 0.17 (0.91 ± 0.09)	7.32 ± 0.07 (1.23 ± 0.06)	7.54 ± 0.15 (1.18 ± 0.08)	7.94 ± 0.09 (1.18 ± 0.09)	1.8 ± 0.7	7.36 ± 0.34
(±) SGa[d]	6.19 ± 0.06 (1.11 ± 0.06)	5.48 ± 0.13 (1.02 ± 0.20)	5.84 ± 0.07 (1.01 ± 0.07)	6.44 ± 0.06 (0.84 ± 0.06)	2.4 ± 0.7	6.42 ± 0.30
2*R*-SGM[e]	8.89 ± 0.04 (0.83 ± 0.11)	8.87 ± 0.05 (1.10 ± 0.11)	9.00 ± 0.06 (0.83 ± 0.01)	9.52 ± 0.01 (0.83 ± 0.01)	1.4 ± 0.2	8.31 ± 0.05
2*R*-SGE[e]	8.67 ± 0.16 (0.86 ± 0.08)	8.84 ± 0.34 (0.92 ± 0.01)	8.74 ± 0.02 (1.09 ± 0.15)	8.85 ± 0.13 (0.89 ± 0.02)	1.1 ± 1.1	8.55 ± 0.16
2*R*-SGa[e]	8.11 ± 0.16 (1.12 ± 0.25)	7.48 ± 0.12 (0.95 ± 0.11)	8.12 ± 0.10 (0.80 ± 0.01)	8.23 ± 0.12 (1.02 ± 0.10)	4.4 ± 0.3	7.20 ± 0.19
2*R*3′*R*-SGM[f]	8.99 ± 0.04 (1.19 ± 0.12)	9.01 ± 0.06 (1.03 ± 0.09)	9.06 ± 0.14 (1.03 ± 0.18)	9.45 ± 0.01 (1.52 ± 0.66)	1.1 ± 0.1	–
2*R*3′*S*-SGM[f]	8.50 ± 0.03 (1.30 ± 0.20)	7.90 ± 0.04 (1.07 ± 0.17)	8.60 ± 0.09 (1.04 ± 0.27)	8.87 ± 0.09 (1.08 ± 0.01)	5.0 ± 1.1	–[h]
2*S*3′*R*-SGM[f]	7.23 ± 0.01 (0.98 ± 0.06)	7.22 ± 0.03 (1.09 ± 0.18)	6.99 ± 0.08 (1.15 ± 0.13)	7.57 ± 0.01 (1.11 ± 0.03)	0.6 ± 0.1	–
2*S*3′*S*-SGM[f]	6.40 ± 0.05 (0.92 ± 0.09)	6.47 ± 0.08 (0.99 ± 0.16)	5.95 ± 0.02 (1.06 ± 0.03)	6.39 ± 0.01 (1.44 ± 0.75)	0.3 ± 0.0	–
2*R*3′*S*-SGE[f]	8.68 ± 0.11 (1.21 ± 0.33)	8.21 ± 0.10 (1.27 ± 0.11)	8.64 ± 0.07 (1.33 ± 0.16)	8.71 ± 0.38 (1.15 ± 0.03)	2.8 ± 0.8	–
2*R*3′*R*1′*R*-SGa[g]	7.04 ± 0.09 (0.97 ± 0.13)	6.43 ± 0.07 (0.85 ± 0.21)	6.95 ± 0.04 (1.06 ± 0.04)	7.00 ± 0.05 (0.93 ± 0.01)	3.5 ± 0.2	6.32 ± 0.23
2*R*3′*R*1′*S*-SGa[g]	8.13 ± 0.06 (1.25 ± 0.01)	7.63 ± 0.02 (0.82 ± 0.15)	8.15 ± 0.02 (0.84 ± 0.17)	8.33 ± 0.04 (1.00 ± 0.06)	3.3 ± 0.0	7.45 ± 0.21
2*R*3′*S*1′*R*-SGa[g]	7.98 ± 0.01 (1.02 ± 0.03)	7.39 ± 0.09 (0.80 ± 0.22)	8.04 ± 0.01 (0.96 ± 0.03)	8.15 ± 0.06 (1.01 ± 0.06)	5.2 ± 0.7	7.33 ± 0.28
2*R*3′*S*1′*S*-SGa[g]	8.32 ± 0.04 (1.01 ± 0.01)	7.64 ± 0.01 (1.00 ± 0.04)	8.46 ± 0.12 (0.80 ± 0.21)	8.56 ± 0.07 (0.86 ± 0.06)	5.5 ± 1.1	7.15 ± 0.12
2*S*3′*R*1′*R*-SGa[g]	5.87 ± 0.04 (1.06 ± 0.05)	5.65 ± 0.06 (1.24 ± 0.07)	5.54 ± 0.16 (1.02 ± 0.12)	5.79 ± 0.12 (0.88 ± 0.04)	0.8 ± 0.1	5.14 ± 0.38
2*S*3′*R*1′*S*-SGa[g]	6.67 ± 0.06 (1.08 ± 0.03)	6.35 ± 0.01 (1.01 ± 0.01)	6.22 ± 0.05 (1.04 ± 0.08)	6.47 ± 0.01 (1.30 ± 0.28)	0.7 ± 0.0	5.69 ± 0.13
2*S*3′*S*1′*R*-SGa[g]	<4.5 –	<4.5 –	<4.5 –	<4.5 –	–	<4
2*S*3′*S*1′*S*-SGa[g]	5.84 ± 0.06 (1.13 ± 0.10)	5.61 ± 0.00 (1.20 ± 0.02)	5.61 ± 0.09 (1.03 ± 0.01)	5.85 ± 0.05 (0.95 ± 0.18)	1.0 ± 0.2	5.03 ± 0.26
Glycopyrrolate	9.76 ± 0.05 (1.37 ± 0.20)	9.19 ± 0.18 (0.99 ± 0.11)	8.73 ± 0.05 (1.14 ± 0.25)	9.90 ± 0.08 (1.02 ± 0.01)	0.4 ± 0.2	8.57 ± 0.12
Scopolamine methyl bromide	9.69 ± 0.01 (0.92 ± 0.10)	9.18 ± 0.21 (1.02 ± 0.02)	9.29 ± 0.12 (1.07 ± 0.01)	9.92 ± 0.21 (0.90 ± 0.04)	1.3 ± 0.4	9.16 ± 0.19

[a] Receptor binding at cloned human muscarinic receptors (M_1–M_4 subtypes); pK_i data represent mean ± SD of 3 experiments, and the numbers in parentheses denote Hill slopes. [b] M_3/M_2 affinity ratio (times) [c] pA_2 values were determined on 4–6 ileum strips obtained from different animals, and data represent mean ± SD. [d] Racemic forms. [e] Isomers based on the chiral center **2**. [f] Isomers based on the chiral centers **2 & 3.** [g] Isomers based on the chiral centers **2, 3, & 1**. [h] Data not available or not detectable

Table 1. The difference is significant between 2*R* and 2*S* isomers, being 27 to 447 times higher for SGM isomers and 6 to 4467 times higher for SGa isomers. The 3′*R* isomers of SGM (with unresolved 1′ chiral center, 2*R*3′*R*-SGM and 2*S*3′*R*-SGM) are more active (1.5–12.9 fold) than their corresponding 3′*S* isomers (2*R*3′*S*-SGM and 2*S*3′*S*-SGM). However, in SGa, the 3′*S* isomers were not always more active than the corresponding 3′*R* isomers, e.g., in 2*R* isomers, 3′*S* > 3′*R* (2*R*3′*S*1′*R* > 2*R*3′*R*1′*R* and 2*R*3′*S*1′*S* > 2*R*3′*R*1′*S*); but in 2*S* isomers, 3′*R* > 3′*S* (2*S*3′*R*1′*R* > 2*S*3′*S*1′*R* and 2*S*3′*R*1′*S* > 2*S*3′*S*1′*S*). Also, there are more significant differences between 2*R*3′*S*1′*R* and 2*R*3′*R*1′*R* than between 2*R*3′*S*1′*S* and 2*R*3′*R*1′*S* (8.7–14.1 fold vs. 1.0–2.0 fold) and between 2*S*3′*R*1′*R* and 2*S*3′*S*1′*R* than between 2*S*3′*R*1′*S* and 2*S*3′*S*1′*S* (11.0–23.4 fold vs. 4.1–6.8 fold). These results indicate that the activity based on chiral center 3′ can be affected by the configuration of the other two chiral centers, 2 and 1′. A comparison of all eight SGa isomers (all three chiral centers resolved) clearly indicates that 1′*S* isomers are more active than the corresponding 1′*R* isomers (1.8–22.4 fold). In all cases, Hill coefficients (*n*) were not very different from unity indicating that, in general, drug-receptor interactions obeyed the law of action and binding took place at only one site (Hulme et al. 1978).

2.3. pA_2 Studies

The pA_2 values determined from guinea pig ileum contraction assays, which represent the negative logarithm of the molar concentration of the antagonist that produces a two-fold shift to the right in an agonist's concentration-response curve, are a classical functional study of anticholinergic affinity (at M_3 muscarinic receptors) (Cheng and Prusoff 1973). For the soft anticholinergics of the present study, the pA_2 values obtained from ileum longitudinal contractions by using carbachol as agonists with the method of van Rossum (1963) are presented in Table 1. The pA_2 values are in general, comparable to the pK_i values obtained in the M_3 receptor binding studies. The pA_2 va-

Table 2: Comparative stereoselectivities[a]

Compd.	Subtypes of cloned muscarinic receptors[b]				Description[f]
	M_1	M_2	M_3	M_4	
SGM					
2*R*3′*S*/2*S*3′*S*[c]	125.9	26.9	446.7	302.0	*2R > 2S*
2*R*3′*R*/2*S*3′*R*[c]	57.5	61.7	117.5	75.9	
2*R*3′*R*/2*R*3′*S*[d]	3.1	12.9	2.9	3.8	*3R > 3S*
2*S*3′*R*/2*S*3′*S*[d]	6.8	5.6	11.0	1.5	
SGa					
2*R*3′*R*1′*R*/2*S*3′*R*1′*R*[c]	14.8	6.0	25.7	16.2	*2R >>2S*
2*R*3′*R*1′*S*/2*S*3′*R*1′*S*[c]	28.8	19.1	85.1	72.4	
2*R*3′*S*1′*R*/2*S*3′*S*1′*R*[c]	3020.0	776.2	3467.4	4466.8	
2*R*3′*S*1′*S*/2*S*3′*S*1′*S*[c]	302.0	107.2	707.9	512.9	
2*R*3′*S*1′*R*/2*R*3′*R*1′*R*[d]	8.7	9.1	12.3	14.1	*3S > 3R*
2*R*3′*S*1′*S*/2*R*3′*R*1′*S*[d]	1.5	1.0	2.0	1.7	
2*S*3′*R*1′*R*/2*S*3′*S*1′*R*[d]	23.4	14.1	11.0	19.5	*3R > 3S*
2*S*3′*R*1′*S*/2*S*3′*S*1’*S*[d]	6.8	5.5	4.1	4.2	
2*R*3′*R*1′*S*/2*R*3′*R*1′*R*[e]	12.3	15.8	15.8	21.4	*1R < 1S*
2*R*3′*S*1′*S*/2*R*3′*S*1′*R*[e]	2.2	1.8	2.6	2.6	
2*S*3′*R*1′*S*/2*S*3′*R*1′*R*[e]	6.3	5.0	5.2	4.8	
2*S*3′*S*1′*S*/2*S*3′*S*1′*R*[e]	21.9	12.9	12.9	22.4	

[a] Affinity ratio (times) between each two isomers based on each of the three different chiral centers. [b] Receptor binding at cloned human muscarinic receptors (M_1–M_4 subtypes) [c] Affinity ratio based on the chiral center **2**. [d] Affinity ratio based on the chiral center **3**. [e] Affinity ratio based on the chiral center **1**. [f] Concluded stereoselectivities

lues of newly developed SGa isomers significantly differed between 2*R* and 2*S* configulations (6.32 to 7.45 and <4 to 5.69, respectively, $p < 0.01$, t-test assuming equal variances). Similar to the previously reported 2*R* isomers (*2R-SGa*), the pA_2 values of the completely resolved 2*R* zwitterionic isomers (2*R*3′*R*1′*R*-SGa, 2*R*3′*R*1′*S*-SGa, 2*R*3′*S*1′*R*-SGa, and 2*R*3′*S*1′*S*-SGa) are one- to two-fold less (indicating one to two order of magnitude less active) than those of the corresponding 2*R* ethyl and methyl parent ester soft drugs (Ji et al. 2005). The retained moderate activity of some zwitterionic metabolite isomers is probably due to the spatial and electronic structural resemblance to the neutral, active anticholinergics (Wu et al. 2005). Among active 2*R* isomers, except for 2*R*3′*R*1′*R*-SGa that showed a lower activity (6.32), all others showed a similar, moderate activity (about 7.15 to 7.45).

2.4. Mydriatic activities

The mydriatic effects of the fully resolved eight SGa isomers were compared to those of (±)SGa, 2*R*-SGa, glycopyrrolate, and tropicamide *in vivo* in rabbits. Following a 100 µl topical administration, mydriatic responses were recorded at appropriate time-intervals as percent changes in pupil size. Maximum responses (R_{max}, % change in pupil size at 30 min to 1 h after administration) and areas under the response–time curve (AUC^{eff}_{0-168h}) are shown in Table 3. Results indicate that similar to the *in vitro* studies, 2*R* isomers are much more potent than 2*S* isomers (even if the 2*S* dose was increased to 0.4%), and that 2*R*3′*R*1′*R*-SGa is less potent than the other three 2*R* isomers. Figure 1 shows the activity-time profiles of four 2*R* and one 1′*S* SGa isomers (the most active *S* isomer) at 0.1% concentration. The pupil-dilating potency of the three most potent 2*R* isomers at a dose of 0.1% is similar to that of 0.05 to 0.1% glycopyrrolate and 0.5% tropicamide; however, the corresponding durations of action are much shorter than that of the “hard” glycopyrrolate (AUC^{eff} values of 200–300 vs. 2500, respectively), and somewhat shorter than that of tropicamide. The activities of 2*R*3′*R*1′*S*-SGa (the most active SGa isomer) and glycopyrrolate lasted for 10 h and 144 h, respectively, as shown in Fig. 2. These results indicate that good pharmacological effect can be achieved by some 2*R*-SGa isomers, and that these isomers can be rapidly eliminated from the body. Furthermore, the active 2*R*-SGa isomers did not cause any observable irritation reactions, such as eye-closing, lacrimation, mucous discharge, as well as change in the intraocular pressure during topical applications. Unlike other conventional anticholinergics (Hammer et al. 1991; Kumar et al. 1993), these 2*R*-SGa isomers did not induce dilation of the pupil in the contralateral (water-treated) eyes indicating no or low systemic side-effects. Therefore, these compounds are safe, promising short acting anticholinergics with the possibility of largely reduced unwanted side effects.

Table 3: Maximum response (R_{max}, maximum % change in pupil size) and area under the response-time curve (AUC_{eff}) after topical administration (0.1 mL)[a]

Compd.	Conc. (%)	R_{max} (%)	AUC^{eff}_{0-168h}
(±) SGa[b]	0.01	1.85 ± 2.14	0.7 ± 0.9
	1	45.37 ± 8.19	119 ± 34
2*R*-SGa[b]	0.01	31.00 ± 7.14	73 ± 24
	0.1	50.34 ± 7.92	182 ± 40
2*R*3′*R*1′*R*-SGa	0.1	24.40 ± 8.33	89 ± 50
2*R*3′*R*1′*S*-SGa	0.1	51.79 ± 16.62	308 ± 106
2*R*3′*S*1′*R*-SGa	0.1	43.90 ± 7.63	216 ± 29
2*R*3′*S*1′*S*-SGa	0.1	47.32 ± 19.64	274 ± 134
2*S*3′*R*1′*R*-SGa	0.1	0.00 ± 0.00	0 ± 0
	0.4	7.44 ± 0.60	11 ± 1
2*S*3′*R*1′*S*-SGa	0.1	3.87 ± 4.49	13 ± 15
	0.4	14.88 ± 1.19	37 ± 3
2*S*3′*S*1′*R*-SGa	0.1	0.00 ± 0.00	0 ± 0
	0.4	0.00 ± 0.00	0 ± 0
2*S*3′*S*1′*S*-SGa	0.1	3.87 ± 4.49	13 ± 15
	0.4	11.01 ± 3.81	28 ± 2
Glycopyrrolate[b]	0.05	48.73 ± 12.66	2476 ± 847
	0.1	52.95 ± 10.93	3732 ± 866
Tropicamide[b]	0.5	44.64 ± 11.17	451 ± 121

[a] Data represent mean ± SD of four trials.
[b] Data adapted from previous reports (Wu et al. 2005)

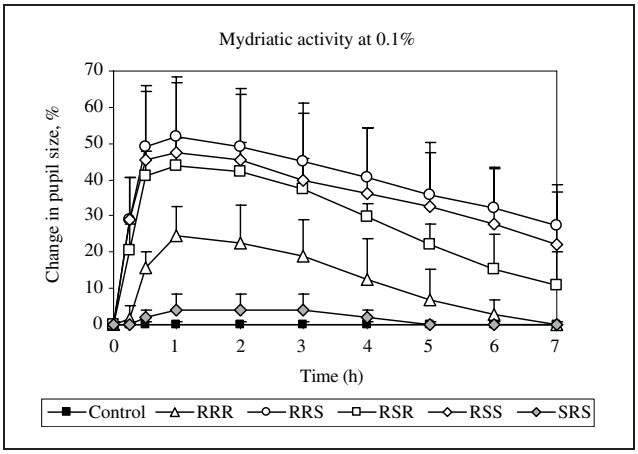


Fig. 1: Mydriatic response after topical administration of 0.1% SGa isomers (2*R*3′*R*1′*R*, 2*R*3′*R*1′*S*, 2*R*3′*S*1′*R*, 2*R*3′*S*1′*S*, 2*S*3′*R*1′*S*) in rabbits

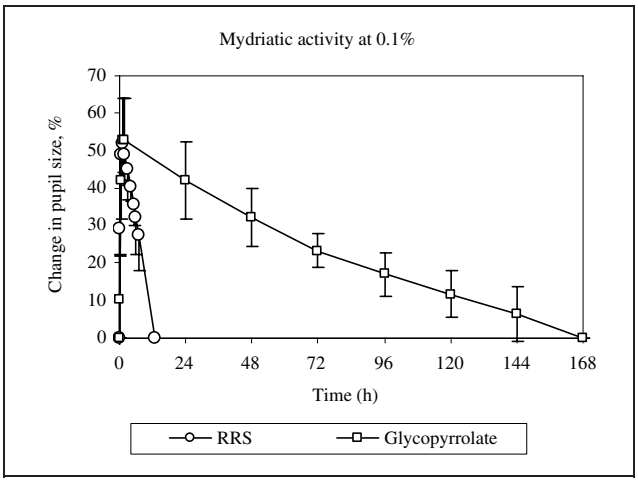


Fig. 2: Mydriatic response after topical administration of 2*R*3′*R*1′*S*-SGa or glycopyrrolate at a dose of 0.1% in rabbits

2.5. Conclusion

Isomers of *N*-substituted soft anticholinergics based on glycopyrrolate, SGM, and SGE, as well as their zwitterionic metabolite, SGa, have been synthesized and isolated. Their pharmacological activities were evaluated *in vitro* and *in vivo*. Receptor binding (pK_i) results indicate that stereo-specificity and stereo-selectivity are important in these anticholinergics. These compounds contain three chiral centers within their structures, and the most significant improvement of receptor binding activity was observed in 2*R* isomers, followed by that in 1′*S* isomers. The activities of 3′*R* and 3′*S* isomers could be affected by the configurations of the other two chiral centers. The most significant improvement of M_3/M_2 muscarinic-receptor subtype-selectivity was observed for the 2*R*3′*S* configurations followed by 2*R*3′*R*. The configuration of chiral center 1′ showed no effect on M_3/M_2 muscarinic-receptor subtype-selectivity. Results obtained from guinea pig ileum assays (pA_2) and rabbit mydriasis test on SGa isomers confirmed the stereo-specificity of these anticholinergics. The pharmacological potency of all possible eight SGa isomers can be summarized as 2*R*3′*S*1′*S* ≈ 2*R*3′*R*1′*S* ≈ 2*R*3′*S*1′*R* > 2*R*3′*R*1′*R* > 2*S*3′*R*1′*S* > 2*S*3′*S*1′*S* ≈ 2*S*3′*R*1′*R* > 2*S*3′*S*1′*R* (Student's t-test, $p < 0.05$). When administered topically (0.1%) in rabbit eyes, some 2*R*-SGa isomers (2*R*3′*S*1′*S*, 2*R*3′*R*1′*S*, and 2*R*3′*S*1′*R*) showed mydriatic potencies similar to that of glycopyrrolate and tropicamide; however, their mydriatic effects lasted considerably shorter, and they did not induce dilation of the pupil in the contralateral, water-treated eyes indicating that they are locally active, but safe with a low potential to cause systemic side effects. Hence, these studies proved utility and safety of these glycopyrrolate-based anticholinergics.

3. Experimental

3.1. Materials

Glycopyrrolate (glycopyrronium bromide) was kindly provided by Boehringer Ingelheim Chemicals, Inc. Carbamylcholine bromide (carbachol), atropine methylbromide (atropine MeBr), and scopolamine methylbromide (scopolamine MeBr) were obtained from Sigma Chemicals Co. (St. Louis, MO), and tropicamide (1%) was obtained from Bausch & Lomb Pharmaceutical (Tampa, FL). N-[^{3}H]-Methyl-scopolamine (NMS) was obtained from Amersham Biosciences UK Limited (Buckinghamshire, UK). Cloned human muscarinic receptor subtypes M_1–M_4 were obtained from Applied Cell Science Inc. (Rockville, MD). Scintiverse BD was from Fisher Scientific Co. (Pittsburgh, PA). (*R*)-3-hydroxy pyrrolidine hydrochloride and (*S*)-3-hydroxy pyrrolidine hydrochloride were from Astatech Inc. (Monmouth Junction, NJ). *N*-[^{3}H]-Methyl-scopolamine (NMS) was from Amersham Biosciences UK Limited (Buckinghamshire, UK). Cloned human muscarinic receptor subtypes M_1–M_4 were from Applied Cell Science Inc. (Rockville, MD). Scintiverse BD was from Fisher Scientific Co. (Pittsburgh, PA). Other chemicals used for synthesis were reagent or HPLC grade, and were obtained from Aldrich (Milwaukee, WI) and Fisher Scientific Co. Melting points were taken on Fisher-Johns melting apparatus. NMR spectra were recorded on Bruker Advance 300, 400 and 500 MHz NMR spectrometers, and are reported in ppm relative to TMS. NOESY was performed using 2D NMR spectrometer, Mercury-300BB. Animal studies were performed in accordance with the Guide for the Care and Use of Laboratory Animals adopted by the National Institute of Health, USA. Institutional animal care and use committee (IACUC) approval was obtained prior to the initiation of this research and during its execution.

3.2. Synthesis of 2R-isomers

3.2.1. Racemic cyclopentylmandelic acid, ***1***

Cyclopentylmagnesium bromide ether solution (100 ml, 2 M; 0.2 mol) was added drop-wise to benzoylformic acid (15 g, 0.1 mol) in 330 ml of anhydrous ethyl ether at 0 °C. The mixture was stirred at 0 °C for 30 min and at room temperature for 24 h. The reaction mixture was treated with 1 N HCl, and the aqueous solution was extracted with ether. The combined ether solution was treated with K_2CO_3 solution. The potassium carbonate solution was acidified with HCl and extracted with ether twice. The ether solution was dried with anhydrous sodium sulfate and evaporated to give a crude product. The crude product was washed with water to get pure racemic cyclopentylmandelic acid **1** (8.0 g, 36.4%). Needle-like crystal, m.p.: 153–154 °C. ^{1}H NMR ($CDCl_3$, 300 MHz): 1.28–1.39, 1.42–1.50, 1.51–1.61, 1.63–1.72 [8 H, m, $(CH_2)_4$], 2.93 [1 H, p, CHC(OH)], 7.26–7.30, 7.33–7.36, 7.65–7.67(5 H, m, Ph) ppm.

3.2.2. Resolution of racemic cyclopentylmandelic acid, R(−) ***1***

(−)-Strychnine (11.4 g) in 100 ml of methanol (suspension) was added to racemic cyclopentylmandelic acid **1** (7.5 g) in methanol (20 ml) at room temperature. The reaction solution was let to stand for overnight. The crystales were filtered and crystallized again with hot methanol. The second crop of crystales was collected by filtration and treated with sodium hydroxide solution. The basic solution was extracted with methylene chloride twice (methylene chloride solution discarded), and then acidified with hydrochloric acid to recover the resolved cyclopentylmandelic acid. To this resolved acid (20.6 mg in 0.1 ml of ethyl acetate), 13 µl of (+)-α-phenylethylamine was added. The precipitate formed was washed with hexane three times and dried under vacuum. The precipitate was identified by NMR as optically pure cyclopentylmandelic acid, R(−)**1**, (2.5 g, 33.3%). M.p.: 121–122 °C. $[\alpha]_D^{25^\circ} = -22.5^\circ$ (c = 1 g/100 ml, $CHCl_3$). ^{1}H NMR($CDCl_3$, 300 MHz): 1.28–1.39, 1.42–1.50, 1.51–1.61, 1.64–1.73 [8 H, m, $(CH_2)_4$], 2.93 [1 H, p, C*H*C(OH)], 7.25–7.28, 7.32–7.35, 7.64–7.65(5 H, m, Ph) ppm.

*3.2.3. Methyl (−)-cyclopentylmandelate, R(−)****2***

To a mixture of (−)-cyclopentylmandelic acid, *R*(−)**1**, (1.83 g, 8.3 mmol) and potassium carbonate (2.87 g, 21 mmol) in DMF (21 ml), methyl iodide (3.53 g, 25 mmol) was added at room temperature. The mixture was stirred at room temperature for 2 h, and then poured into water and extracted with hex-

ane three times. Evaporation of dried hexane extract gave a crude product. Flash chromatography of the crude product on silica gel with 1.5 : 1 hexane : methylene chloride gave pure product *R*(−)**2** (1.90 g, 95%). ^{1}H NMR ($CDCl_3$, 300 MHz): 1.32–1.36, 1.43–1.61 [8 H, m, $(CH_2)_4$], 2.90 [1 H, p, C*H*C(OH)], 3.71 (1 H, s, OH), 3.79 (3 H, s, CH_3), 7.25–7.28, 7.31–7.35, 7.63–7.65 (5 H, m, Ph) ppm.

*3.2.4. (R)-3-Hydroxy-N-Methyl pyrrolidine, (R)**3***

In a 100 ml flask, 4 g (*R*)-3-hydroxy pyrrolidine hydrochloride salt, 50 ml THF and 1.3 g NaOH were added and stirred for 20 min. Then, 1.1 g paraformaldehyde and 4.8 g formic acid (90%) were added. The mixture was heated (60 °C) and stirred for reflux for 2 h until all solid disappeared. The mixture was cooled down to 0 °C, added with 6.5 ml of 10 N NaOH solution (pH about 10), and extracted twice by ethyl ether (50 ml). The combined organic layer was dried over Na_2SO_4. Evaporation of the dried organic layer gave a yellowish, oily product of (*R*)**3** (3.0 g, 92%). ^{1}H NMR ($CDCl_3$, 300 MHz): 1.65–1.75 (m, 1 H), 2.15–2.36 (m, 2 H), 2.33 (s, 3 H), 2.55–2.59 (m, 2 H), 2.76–2.85 (m, 1 H), 4.30–4.40 (m, 1 H), 4.8–5.10 (brs, 1 H). ^{13}C NMR ($CDCl_3$, 300 MHz): 35.4, 41.9, 54.7, 64.9, 70.9.

*3.2.5. (S)-3-Hydroxy-N-methyl pyrrolidine, (S)**3***

Synthesis of (*S*)**3** was the same as for (*R*)**3**, except the starting material was (*S*)-3-hydroxy pyrrolidine hydrochloride salt. The resulted product (*S*)**3** (3.10 g, 95%) was also an oil. ^{1}H NMR ($CDCl_3$, 300 MHz): 1.50–1.60 (m, 1 H), 2.05–2.30 (m, 2 H), 2.28 (s, 3 H), 2.40–2.50 (m, 2 H), 2.70–2.80 (m, 1 H), 4.25–4.30 (m, 1 H), 4.80 (brs, 1 H). ^{13}C NMR ($CDCl_3$, 300 MHz): 35.4, 41.9, 54.7, 64.9, 70.9.

*3.2.6. 3′(R)-N-Methyl-3-pyrrolidinyl-2(R)-cyclopentylmandelate, **4***

A solution of *R*(−)**2** (0.7 g, 3 mmol) and (*R*)**3** (0.7 g, 7 mmol) in 40 ml of toluene was heated until 20 ml of toluene had distilled. Approximately 0.003 g of sodium was added, and the solution was stirred and heated for 2 h as the distillation was continued. More toluene was added at such a rate as to keep the reaction volume constant. Additional sodium was added at the end of an hour. The solution was then cooled and extracted with 3N HCl. The acid extract was made alkaline with concentrated NaOH and extracted three times with ether. Removal of dried ether solution gave a crude oil. Flash chromatography of the crude product on silica gel with 8 : 1 of EtOAc and EtOH gave an oil product of **4** (0.4 g, 44%). ^{1}H NMR ($CDCl_3$, 300 MHz): 1.28–1.37, 1.51–1.70, 1.83–1.90[8 H, m, $(CH_2)_4$], 2.27–2.40 (m, 3 H), 2.52–2.55 (m, 1 H), 2.64–2.72(m, 1 H), 2.74–2.81(m, 1 H), 2.33(3 H, s, NCH_3), 2.93[1 H, p, C*H*C(OH)], 3.85(1 H, bs, OH), 5.22(m, 1 H), 7.24–7.27, 7.31–7.35, 7.64–7.66(5 H, m, Ph)ppm.

*3.2.7. 3′(S)-N-Methyl-3-pyrrolidinyl-2(R)-cyclopentylmandelate, **5***

Synthesis of **5** was the same as for **4**, except *(S)***3** was used instead of *(R)***3**. The resulted product **5** (0.35 g, 39%) was also an oil. ^{1}H NMR ($CDCl_3$, 400 MHz): 1.28–1.37, 1.51–1.70, 1.75–1.82[8 H, m, $(CH_2)_4$], 2.15–2.22 (m, 1 H), 2.30–2.40 (m, 2 H), 2.65–2.70 (m, 1 H), 2.70–2.82(m, 2 H), 2.35(3 H, s, NCH_3), 2.95[1 H, p, C*H*C(OH)], 3.82(1 H, bs, OH), 5.22(m, 1 H), 7.24–7.27, 7.31–7.35, 7.64–7.66(5 H, m, Ph)ppm.

*3.2.8. 3′(R)-[2(R)-Cyclopentylphenylhydroxyacetoxy]-1′-methyl-1′-methoxycarbonylpyrrolidinium bromide, **6** (2R3′R-SGM)*

To compound **4** (0.3 g, 0.98 mmol) in 12 ml of dry acetonitrile, methyl bromoacetate (0.5 g, 3.2 mmol) was added at room temperature. The mixture was stirred for 6 h. Evaporation of acetonitrile gave a crude product. The crude product was dissolved in a small volume of methylene chloride and then poured into 50 ml of dry ethyl ether to precipitate. This step was repeated three times to obtain the pure product **6** (0.3 g, 70 %) as a white powder that was a mixture of two diastereoisomers in a NMR-estimated ratio of 2 : 1. ^{1}H NMR ($CDCl_3$, 400 MHz): 1.30–1.37, 1.41–1.50, 1.55–1.70[8 H, m, $(CH_2)_4$], 2.10–2.27(m, 1 H), 2.79–2.95 (m, 2 H), 3.05, 3.60 (2s, total 3 H, N-CH3), 3.75, 3.79 (2s, total 3 H, O-Me), 3.95–4.40 (m, 4 H), 4.68, 5.16 (2AB, total 2 H, N-CH2-COOMe), 5.52–5.58(m, 1 H), 7.23–7.29, 7.31–7.38, 7.56–7.60 (5 H, m, Ph)ppm.

*3.2.9. 3′(S)-[2(R)-Cyclopentylphenylhydroxyacetoxy]-1′-methyl-1′-methoxycarbonylpyrrolidinium bromide, **7a** (2R3′S-SGM)*

To compound **5** (0.16 g, 0.52 mmol) in 8 ml of dry acetonitrile, methyl bromoacetate (0.3 g, 1.9 mmol) was added at room temperature. Following the same procedure for **6**, the pure product **7a** (0.16 g, 80 %) was obtained. **7a** was also a white powder and a mixture of two diastereoisomers in a NMR-estimated ratio of 2 : 1. ^{1}H NMR ($CDCl_3$, 400 MHz): 1.30–1.70[8 H, m, $(CH_2)_4$], 1.95–2.00, 2.10–2.20(m, 1 H), 2.75–2.95 (m, 2 H), 3.30, 3.70 (2 s, total 3 H, N-CH3), 3.78, 3.82 (2 s, total 3 H, O-Me), 4.00–4.42 (m, 4 H), 4.90, 5.38 (2AB, total 2 H, N-CH2-COOMe), 5.52–5.58(m, 1 H), 7.23–7.29, 7.31–7.38, 7.56–7.60(5 H, m, Ph)ppm.

*3.2.10. 3′(S)-[2(R)-Cyclopentylphenylhydroxyacetoxy]-1′-methyl-1′-ethoxycarbonylpyrrolidinium bromide, **7b** (2R3′S-SGE)*

To compound **5** (0.16 g, 0.52 mmol) in 10 ml of dry acetonitrile, ethyl bromoacetate (0.32 g, 1.9 mmol) was added at room temperature. The mixture was stirred for 22 h, and the removal of acetonitrile gave a crude product. The crude product was dissolved in small volume of ethylene chloride, and then poured into a 50 ml of dry ethyl ether to get precipitate. This procedure was repeated three times, and the pure product **7b** (0.16 g, 80%) was obtained. **7** was also a white powder and a mixture of two diastereoisomers in a NMR-estimated ratio of 2 : 1. ^{1}H NMR ($CDCl_3$, 400 MHz): 1.32, 1.35(2t, 3 H, CH_3CH_2), 1.40–1.50, 1.53–1.63, 1.65–1.80[8 H, m, $(CH_2)_4$], 1.93–2.11 (m 2 H), 2.80–2.96 M, 2 H), 3.30, 3.70 (2s, 3 H, N-CH3), 4.10–4.60 (m, 6 H), 4.79, 5.30(2 H, 2set of dd, CH_2CO_2), 5.53(1 H, m), 7.24–7.29, 7.31–7.38, 7.56–7.60(5 H, m, Ph)ppm.

3.3. Hydrolysis of esters

Compounds **6** and **7a** (were mixed with equimolar ratios of 0.1 N NaOH. The mixtures were stirred at room temperature for 3 h to obtain the corresponding racemic zwitterionic products, **8** and **9** in aqueous solution (colorless, pH about 6.5).

3.4. HPLC separation for 8a, 8b, and 9a, 9b

The solutions of **8** and **9** each contained two isomers, **8a**, **8b** and **9a**, **9b**, respectively, at a ratio of 2 : 1 that could be separated by HPLC. The HPLC system consisted of a Spectra Physics (San Jose, CA) SP 8810 isocratic pump, a SP 8450 UV/Vis detector (wavelength set to 230 nm), a SP 4290 integrator, and a Supelco Discovery RP Amide C16 column. The mobile phase consisted of acetonitrile and water at a ratio of 30 : 70. With 100 µl injection at a flow rate of 1 ml/min, the retention times were 7.2 min for **8a** and **9a**, and 8.5 min for **8b** and **9b**. The effluence corresponding to each isomer was collected, and the solvent was evaporated to obtain the final zwitterionic isomers, **8a**, **8b**, and **9a**, **9b** as follows:

1′(R),3′(R)-[2(R)-Cyclopentyl-2-phenyl-2-hydroxyacetoxy]-1′-methyl-1′-carboxymethyl pyrrolidinium inner salt, **8a** *(2R3′R1′R-SGa)*: white powder ^{1}H NMR ($CDCl_3$, 300 MHz): 1.30–1.65 (m, 8 H), 2.02–2.12 (m, 1 H), 2.20–2.60 (brs, 1 H), 2.60–2.80 (m, 1 H), 2.82–2.92 (m, 1 H), 3.30 (s, 3 H), 3.55–3.65 (m, 1 H), 3.72–3.82 (m, 1 H), 3.90–4.05(m, 2 H), 4.10–4.15 (m, 1 H), 5.38–5.45 (m, 1 H), 7.15–7.20 (m, 1 H), 7.32–7.38 (m, 2 H), 7.55–7.62 (m, 2 H).

1′(S),3′(R)-[2(R)-Cyclopentyl-2-phenyl-2-hydroxyacetoxy]-1′-methyl-1′-carboxymethyl pyrrolidinium inner salt, **8b** *(2R3′R1′S-SGa)*: ^{1}H NMR ($CDCl_3$, 300 MHz): 1.30–1.75 (m, 8 H), 2.02–2.10 (m, 1 H), 2.10–2.40 (brs, 2 H), 2.70–2.80 (m, 1 H), 2.80–2.90 (m, 1 H), 2.95 (s, 3 H), 3.55–3.65 (m, 2 H), 3.85–4.0 (m, 3 H), 4.05–4.10 (m, 1 H), 5.38–5.45(m, 1 H), 7.15–7.20 (m, 1 H), 7.25–7.30 (m, 2 H), 7.50–7.60 (m, 2 H).

1′(R),3′(S)-[2(R)-cyclopentyl-2-phenyl-2-hydroxyacetoxy]-1′-methyl-1′-carboxymethyl pyrrolidinium inner salt, **9a** *(2R3′S1′R-SGa)*: white powder, ^{1}H NMR ($CDCl_3$, 500 MHz): 1.30–1.65 (m, 8 H), 2.02–2.12 (m, 1 H), 2.50–2.60 (m, 1 H), 2.78–2.88 (m, 1 H), 3.25 (s, 3 H), 3.65–4.05 (m, 4 H), 4.15–4.30 (brs, 2 H), 5.30–5.40 (m, 1 H), 7.13–7.23 (m, 1 H), 7.26–7.32 (m, 2 H), 7.55–7.60 (m, 2 H).

1′(S),3′(S)-[2(R)-cyclopentyl-2-phenyl-2-hydroxyacetoxy]-1′-methyl-1′-carboxymethyl pyrrolidinium inner salt, **9b** *(2R3′S1′S-SGa)*: white powder, ^{1}H NMR ($CDCl_3$, 500 MHz): 1.30–1.70 (m, 8 H), 1.90–1.98 (m, 1 H), 2.65–2.70 (m, 1 H), 2.85–2.90(m, 1 H), 3.15 (s, 3 H), 3.65–3.90 (m, 4 H), 4.05–4.10 (M, 1 H), 4.15–4.22 (brs, 1 H), 5.35–5.42 (m, 1 H), 7.18–7.23 (m, 1 H), 7.27–7.32 (m, 2 H), 7.53–7.58 (m, 2 H).

3.5. Determination of absolute configurations

Nuclear Overhauser effect (NOE) has been used to identify the absolute configuration of product **8b**. The compound was dissolved in $CDCl_3$, and the 2D ^{1}H-^{1}H NOESY spectrum was taken by Mercury-300BB.

3.6. Synthesis of 2S-isomers

*3.6.1. Cis-(2S, 5S)-2-(tert-butyl)-5-phenyl-1,3-dioxolan-4-one, **10***

S(+)-Mandelic acid in hexane suspension (50 g, 328 mmol) was mixed with pivaldehyde (42.7 ml, 396 mmol) then trifluoromethanesulfonic acid (1.23 ml, 14 mmol) at room temperature. The mixture was warmed to 36 °C, and the reaction was followed by TLC for 5 h until no starting material could be detected. The mixture was then cooled to room temperature and 8% aqueous $NaHCO_3$ was added. The water layer was removed and the organic layer was dried over Na_2SO_4. After filtration and removing the solvent, 62.17g of the crude product was obtained. Recrystallization of the crude product gave 44.71g of pure *cis*-(2*S*, 5*S*)-2-(*tert*-butyl)-5-phenyl-1,3-dioxolan-4-one in 88% yield as a needle-like crystal. ^{1}H NMR ($CDCl_3$, 300 MHz): 1.08 (s. 9 H), 5.24 (s, 1 H), 5.33 (s, 1 H), 7.40–7.46 (m, 5 H)ppm. ^{13}C NMR ($CDCl_3$, 300 MHz): 23.6, 34.4, 77.0, 109.3, 127.0, 128.7, 129.2, 133.4, 147.2.

3.6.2. Cis-(2S, 5S)-2-(tert-butyl)-5-phenyl-5-cyclopentyl-1,3-dioxolan-4-one, ***11***

At −78 °C, a lithium bis-(trimethylsilyl)amide in hexane solution (120 ml, 120 mmol, 1.0 M in hexane) was added to compound **10** (25g, 113.5 mmol, dissolved in 100 ml of dried THF), stirred for 1 h, followed by addition of cyclopentyl bromide (25 g, 168 mmol). This reaction was kept at −78 °C for 4 h, then slowly warmed up to room temperature and continued for overnight. The completion of the reaction was followed by TLC. With stirring, a solution of 10% of NH_4Cl (25 ml) was added to the mixture. Then, the mixture was poured into a separation funnel containing 10% NH_4Cl solution (200 ml). The aqueous layer was discarded, and the organic layer was dried over Na_2SO_4. The solvent was removed to give a crude product, which was then re-crystallized in hexane to give a pure product, **11** (20.36 g, yield 63%, white crystal). ^{1}H NMR ($CDCl_3$, 300 MHz): 1.15 (s, 9 H), 1.55–1.95 (m, 8 H), 2.74 (m, 1 H), 5.62 (s, 1 H), 7.44–7.56 (m, 3 H), 7.88–7.91 (n, 2 H) ppm. ^{13}C NMR ($CDCl_3$, 300 MHz): 23.5, 24.5, 25.3, 26.6, 35.6, 50.9, 83.2, 110.6, 124.9, 127.5, 127.9, 138.9, 173.7.

3.6.3. S(+)-Cyclopentylmandelic acid, ***12***

To a solution of *cis*-(2*S*, 5*S*)-2-(*tert*-butyl)-5-cyclopentyl-5-phenyl-1,3-dioxolan-4-one (14.35 g, 50 mmol) in 100 ml methanol and 50 ml water, 15 g of KOH was added slowly. The mixture was stirred and heated (65 °C) to reflux for 3–4 h, then cooled down to the room temperature, and methanol was removed. To the aqueous solution, 100 ml of ethyl acetate was added then acidified to pH 1 with 3N HCl. The mixture was poured to a separation funnel, and the organic layer was separated. The aqueous layer was extracted two times with ethyl acetate (50 ml). The combined organic layers were dried over Na_2SO_4, filtered, and the solvent was removed to provide 13.44 g of yellowish crude product, which was re-crystallized to give a pure product of *S*(+)-cyclopentylmandelic acid, **12** (6.89 g, yield 62%, white crystal). ^{1}H NMR ($CDCl_3$, 300 MHz): 1.28–1.75 (m, 8 H), 2.94 (m, 1 H), 7.24–7.34 (m, 3 H), 7.62–7.68 (m, 2 H). ^{13}C NMR ($CDCl_3$, 300 MHz): 25.9, 26.3, 26.4, 26.9, 47.1, 79.2, 125.8, 127.7, 128.2, 140.8, 180.9.

3.6.4. Methyl S(+)-cyclopentylmandelate, ***13***

S(+)-Cyclopentylmandelic acid, **12** (5.5 g, 25 mmol), and potassium carbonate (8.61 g, 63 mmol) in DMF (60 ml) solution was mixed with methyl iodide (10.6 g, 75 mmol). The mixture was stirred at room temperature for 3 h, poured to water, and extracted with hexane for three times. Evaporation of dried hexane extract gave a pure product of *S*(+)-cyclopentylmandelate, **13** (5.85 g, 100 %, clear oil). ^{1}H NMR ($CDCl_3$, 300 MHz): 1.32–1.61 [8 H, m, $(CH_2)_4$], 2.90 [1 H, p, C*H*C(OH)], 3.76 (s, 3 H), 3.78 (s, 1 H), 7.25–7.35 (m, 3H), 7.63–7.65 (m, 2 H). ^{13}C NMR ($CDCl_3$, 300 MHz): 25.9, 26.2, 26.3, 26.8, 47.1, 53.2, 79.1, 125.8, 127.3, 128.0, 141.6, 176.0.

3.6.5. (3′R)-N-Methyl-3′-pyrrolidinyl-(S)-cyclopentylmandelate, ***14***

In a 250 ml 3-neck flask equipped with Dean-Stark condenser, the mixture of methyl *S*(+)-cyclopentylmandelate, **13** (2 g, 8.8 mmol), (*R*)-3-hydroxy-*N*-methyl pyrrolidine, *(R)*-**3** (2 g, 20 mmol), and 100 ml of heptane was stirred and heated (110 °C) until 20 ml of heptane had been distilled. The temperature was reduced to 25 °C, and approximately 0.003 g of sodium was added. The mixture was stirred and heated to 110 °C again for 3 h as the distillation was continued. An additional piece of sodium (0.002 g) was added at the 1 h point. More heptane was added at such a rate as to keep the reaction volume constant. The mixture was cooled to 0 °C, mixed with 5 ml of water, and the organic layer was separated. The organic layer was extracted with 3N HCl. The acid extract was made alkaline (pH 10) with 5N NaOH and extracted three times with ether. Removal of dried ether solution (over Na_2SO_4) gave a clear, oily product **14** (1.6 g, 61.5%).^{1}H NMR ($CDCl_3$, 300 MHz): 1.28–1.80 [m, 9 H], 2.15–2.25 (m, 1 H), 2.30–2.40 (m, 1 H), 2.37 (s, 3 H),2.65–2.80 (m, 3 H), 2.90–3.00 (m, 1 H), 3.85 (1 H, brs, OH), 5.22 (m, 1 H), 7.20–7.35 (m, 3 H), 7.64–7.70 (m, 2 H). ^{13}C NMR ($CDCl_3$, 300 MHz):26.0, 26.4, 26.5, 26.7, 32.1, 42.0, 47.1, 54.8, 62.0, 76.5, 79.1, 125.8, 127.3, 128.0, 141.7, 175.3.

3.6.6. (3′S)-N-Methyl-3′-pyrrolidinyl-(S)-cyclopentylmandelate, ***15***

Following the same procedure as for **14**, except (*S*)-**3** was used instead of (*R*)-**3**, a clear, oily product of **15** (2.33 g, 89.6%) was obtained. ^{1}H NMR ($CDCl_3$, 300 MHz): 1.24–1.70 (m, 9 H), 1.80–1.88 (m, 1 H), 2.25–2.40 (m, 2 H), 2.35 (s, 3 H), 2.55–2.70 (m, 2 H), 2.75–2.82 (m, 1 H), 2.90–3.00 (m, 1 H), 3.95 (1 H, bs, OH), 5.22 (m, 1 H), 7.24–7.40 (m, 2 H), 7.64–7.69 (m, 5 H). ^{13}C NMR ($CDCl_3$, 300 MHz): 26.0, 26.3, 26.4, 26.7, 32.6, 42.0, 47.1, 54.9, 61.6, 76.4, 79.2, 125.8, 127.3, 128.0, 141.7, 175.2.

3.6.7. 3′(R)-[S-Cyclopentylphenylhydroxyacetoxy]-1′-methyl-1′-methoxycarbonylpyrrolidinium bromide, ***16*** *(2S3′R-SGM)*

To compound **14** (0.6 g, 1.96 mmol) in 30 ml of dry acetonitrile, methyl bromoacetate (1.0 g, 6.4 mmol) was added at room temperature. The mixture was stirred for 3 h. Evaporation of acetonitrile gave a crude product. The crude product was dissolved in a small volume of methylene chloride and poured into 100 ml of dry ethyl ether to obtain a precipitate. This procedure was repeated three times and gave the product **16** (0.81 g, 89 %, white powder). ^{1}H NMR ($CDCl_3$, 300 MHz): 1.30–1.70 (m, 8 H), 1.82–1.95 (brs, 1 H), 2.10–2.20 (m, 1 H), 2.75–2.90 (m, 2 H), 3.25, 3.60 (2s, total 3 H, N-CH3), 3.75, 3.79 (2s, total 3 H, O-Me), 4.10–4.60 (m, 4 H), 4.92, 5.35 (2AB, total 2 H, N-CH2-COOMe), 5.52–5.58 (m, 1 H), 7.23–7.38 (m, 3 H), 7.56–7.60 (m, 2H). ^{13}C NMR ($CDCl_3$, 300 MHz): 25.8, 25.9; 26.3, 26.4; 26.4, 26.5; 27.0, 27.0; 29.8, 30.1; 45.9, 46.8; 50.2, 51.4; 53.2, 53.2; 62.2, 63.2; 64.6, 64.7; 69.6, 69.7; 72.8, 73.1; 79.4, 79.6; 125.7, 125.7; 127.6, 127.9; 128.2, 128.4; 141.0, 141.2; 165.3, 165.5; 173.9, 174.2.

3.6.8. 3′(S)-[S-Cyclopentylphenylhydroxyacetoxy]-1′-methyl-1′-methoxycarbonylpyrrolidinium bromide, ***17*** *(2S3′S-SGM)*

Following the same procedure as for **16**, except compound **15** was used instead of compound **14**, the product of **17** (0.8 g, 88 %, white powder) was obtained. ^{1}H NMR ($CDCl_3$, 300 MHz): 1.30–1.75 (m, 8H), 1.80–1.90 (brs, 1H), 2.15–2.30 (m, 1H), 2.78–2.95 (m, 2H), 3.10, 3.65 (2s, total 3H, N-CH3), 3.75, 3.78 (2s, total 3H, O-Me), 4.15–4.52 (m, 4H), 4.85, 5.38 (2AB, total 2H, N-CH2-COOMe), 5.50–5.58 (m, 1H), 7.23–7.38 (m, 3H), 7.56–7.66 (m, 2H). ^{13}C NMR ($CDCl_3$, 300 MHz): 25.8, 25.9; 26.2, 26.3; 26.3, 26.4; 26.8, 26.9; 29.4, 29.6; 45.6, 46.9; 50.1, 51.4; 53.1, 53.1; 62.2, 63.3; 64.8, 64.8; 69.5, 69.8; 72.8, 73.2; 79.4, 79.6; 125.6, 125.9; 127.6, 127.9; 128.2, 128.4; 140.7, 141.1; 165.2, 165.5; 173.9, 174.2.

3.7. Hydrolysis of esters and HPLC separations

The procedures used for obtaining the 2*S*-isomers **18a**, **18b**, **19a** and **19b** (white powder) were the same as for 2*R*-isomers **8a**, **8b**, **9a** and **9b**.

1′(R),3′(R)-[2(S)-Cyclopentyl-2-phenyl-2-hydroxyacetoxy]-1′-methyl-1′-carboxymethyl pyrrolidinium inner salt, **18a** *(2S3′R1′R-SGa)*: ^{1}H NMR ($CDCl_3$, 300 MHz): 1.30–1.65 (m, 8 H), 2.02–2.45 (m, 2 H), 2.82–2.90 (m, 1 H), 3.10–3.18 (m, 1 H), 3.25 (s, 3 H), 3.50–4.05 (m, 6 H), 5.34–5.40 (m, 1 H), 7.23–7.38 (m, 3 H), 7.50–7.68 (m, 2 H).

1′(S),3′(R)-[2(S)-Cyclopentyl-2-phenyl-2-hydroxyacetoxy]-1′-methyl-1′-carboxymethyl pyrrolidinium inner salt, **18b** *(2S3′R1′S-SGa)*: ^{1}H NMR ($CDCl_3$, 300 MHz): 1.45–1.85 (m, 9 H), 2.05–2.15 (m, 1 H), 2.80–2.90 (m, 1 H), 3.00–3.10 (m, 1 H), 3.35 (s, 3 H), 3.70–3.80 (m, 1 H), 3.90–4.10 (m, 4 H), 4.22–4.35 (m, 1 H), 5.50–5.60 (m, 1 H), 7.36–7.55 (m, 3 H), 7.72–7.80 (m, 2 H).

1′(R),3′(S)-[2(S)-Cyclopentyl-2-phenyl-2-hydroxyacetoxy]-1′-methyl-1′-carboxymethyl pyrrolidinium inner salt, **19a** *(2S3′S1′R-SGa)*: ^{1}H NMR ($CDCl_3$, 300 MHz): 1.20–1.65 (m, 8 H), 1.95–2.10 (m, 1 H), 2.20 (brs, 1 H), 2.40–2.50 (m, 1 H), 2.78–2.90 (m, 1 H), 3.15 (s, 3 H), 3.70–3.90 (m, 2 H), 3.96–4.20 (m, 4 H), 5.38–5.50 (m, 1 H), 7.20–7.38 (m, 3 H), 7.55–7.65 (m, 2 H).

1′(S),3′(S)-[2(S)-cyclopentyl-2-phenyl-2-hydroxyacetoxy]-1′-methyl-1′-carboxymethyl pyrrolidinium inner salt, **19b** *(2S3′S1′S-SGa)*: ^{1}H NMR ($CDCl_3$, 300 MHz): 1.35–1.70 (m, 8 H), 2.00–2.15 (m, 1 H), 2.70–2.90 (m, 2 H), 3.00 (s, 3 H), 3.42 (brs, 1 H), 3.58–3.68 (m, 2 H), 3.80–3.95 (m, 3 H), 4.08–4.18 (m, 1 H), 5.38–5.48 (m, 1 H), 7.20–7.40 (m, 3 H), 7.55–7.62 (m, 2 H).

3.8. Receptor binding affinity

Receptor binding studies on soft anticholinergics isomers and their zwitterionic metabolite isomers, as well as glycopyrrolate, and *N*-methylscopolamine were performed with *N*-[^{3}H]-methyl-scopolamine (NMS) in assay buffer (phosphate-buffered saline, PBS, without Ca^{++} or Mg^{++}, pH 7.4), following the protocol from Applied Cell Science Inc. (Rockville, MD). A 10 mM NaF solution was added to the buffer as an esterase inhibitor. The assay mixture (0.2 ml) contained 20 µl diluted receptor membranes (receptor proteins: M_1, 38 µg/ml; M_2, 55 µg/ml; M_3, 27 µg/ml; M_4, 84 µg/ml). The final concentration of NMS for the binding studies was 0.5 nM. Specific binding was defined as the difference in [^{3}H]NMS binding in the absence and presence of 5 µM atropine for M_1 and M_2 or 1 µM atropine for M_3 and M_4. Incubation was carried out at room temperature for 2 h. The assay was terminated by filtration through a Whatman GF/C filter (presoaked overnight with 0.5% polyethyleneimine). The filter was then washed six times with 1 ml ice cold buffer (50 mM Tris-HCl, pH 7.8, 0.9% NaCl), transferred to vials, and 5 ml of Scintiverse was added. Detection was performed on a Packard 31800 liquid scintillation analyzer (Packard Instrument Inc., Downer Grove, IL). Data obtained from the binding experiments were fitted to the equation %[^{3}H] NMS bound = 100 − $[100x^n/k/(1 + x^n/k)]$, to obtain the Hill coefficient n, and then to the equation %[^{3}H] NMS bound = 100 − $[100x^n/IC_{50}/(1 + x^n/IC_{50})]$, to obtain the IC_{50} values (x being the concentration of the tested compound). Based on the method of Cheng and Prusoff (Cheng and Prusoff 1973), K_i was derived from the equation $K_i = IC_{50}/(1 + L/K_d)$, where L is the concentration of the radioligand. IC_{50} represents the concentration of the drug causing 50% inhibition of specific radioligand binding, and K_d represents the dissociation constant of the radioligand receptor complex. Data were analyzed

by a non-linear least-square curve-fitting procedure using Scientist software (MicroMath Inc., Salt Lake City, UT).

3.9. Determination of pA_2 values

Male guinea pigs weighing about 400 g were obtained from Harlan Inc. (Indianapolis, IN) and fasted overnight. Animals were sacrificed by decapitation, and the ileum (the region of 5 cm upward of the cecum) was isolated and removed. The ileum was cut into 2.5 cm pieces and suspended in an organ bath containing 30 ml of mixture of Tyrode's solution and 0.1 mM hexamethonium bromide. The organ bath was constantly aerated with oxygen and kept at 37 °C. One end of the ileum strip was attached to a fixed support at the bottom of the organ bath, and the other end to an isometric force transducer (Model TRN001, Kent Scientific Corp., Conn.) operated at 2–10 g range. The ileum strip was kept at a 2 g tension, and carbachol was used as antagonist. The ileum contracted cumulatively upon the addition of consecutive doses of carbachol (10–20 µl of 2×10^{-4} – 2×10^{-3} M in water solution). Contractions were recorded on a physiograph (Kipp & Zonen Flarbed Recorder, Holland). After the maximum response was achieved, the ileum was washed three times, and a fresh Tyrode's solution containing an appropriate concentration of the antagonist (anticholinergic compound tested) was replaced. An equilibration time of 10 min was allowed for the antagonists before the addition of carbachol. In each experiment, 5 to 6 different concentrations were used, and a Schild plot was used to obtain the pA_2 values. Four trials were performed for each antagonist.

3.10. In vivo mydriatic studies

The mydriatic effects of eight completely resolved SGa isomers were compared to those of glycopyrrolate, tropicamide, (±)SGa, and *2R*-SGa in rabbit eyes. Four healthy, male New-Zealand white rabbits weighing about 3.5 kg were used. A 100 µl of compound in water solution (pH 6.5) at various concentrations were administered in the eyes. Compound solutions were applied to one eye, and water was applied to the other eye that served as control. Experiments were carried out in a light- and temperature-controlled room. At appropriate time intervals, the pupil diameters of both eyes were recorded. Percent difference in pupil diameters between each time-point and zero time-point were calculated for both treated and control eyes and reported as mydriatic responses. Control eye dilations were monitored to determine whether systemic absorption had occurred or not. The area under the mydriatic response-time curve (AUC^{eff}) was calculated by the trapezoidal rule, and it was used to compare the activity and duration of action of the tested compounds.

3.11. Statistical analysis

Receptor binding affinities and pA_2 values were compared using Student's t-tests. Mydriatic activities (maximum response R_{max}% and area under the effect curves AUC_{eff}) were compared using ANOVA. A significance level of $p < 0.05$ was used in all cases.

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