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The effect of absolute configuration on activity, subtype selectivity (M3/M2) of 3α-acyloxy-6β-acetoxyltropane derivatives as muscarinic M3 receptor antagonists

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ABSTRACT

Both enantiomers of 3α-acyloxy-6β-acetoxyltropane derivatives **1**–**4** were prepared respectively and underwent functional studies and radioreceptor binding assays. 6*S* Enantiomers showed obvious muscarinic M3, M2 antagonistic activity, while the 6*R* ones elicited little muscarinic activity by functional studies. Besides, the affinity of 6*S* enantiomers to muscarinic M3 receptors of rat submandibulary gland, M2 receptors of rat left atria was much larger than that of corresponding 6*R* enantiomers. All these pharmalogical results indicated 6*S* configuration was favorable for 3α-acyloxy-6β-acetoxyltropane derivatives to bind with muscarinic M3 or M2 receptors and elicited antagonistic activity. Furthermore, the muscarinic M3 activity and subtype selectivity (M3/M2) of 6*S* enantiomers could be improved by increasing the electron density of carbonyl oxygen or introducing methylene group between the carbonyl and phenyl ring in C-3α position. Understanding the effect of absolute configuration on activity, subtype selectivity (M3/M2) of 3α-acyloxy-6β-acetoxyltropane derivatives will provide the clues for designing muscarinic M3 antagonists with high activity and low side effects or toxicity.

1. Introduction

Applying muscarinic M3 receptor antagonists has become one of the most important pharmaceutical approaches in the treatment of respiratory and urinary tract disorders such as chronic obstructive pulmonary disease and urinary incontinence.[1–4] However, muscarinic M3 receptor antagonists with lower selectivity towards M2 receptors will exhibit undesirable side effects in clinical use.[5] So, it is necessary to evaluate not only activity but also subtype selectivity (M3/M2) for muscarinic M3 receptor antagonists. In our previous studies, many racemic 3α-acyloxy-6β-acetoxyltropane derivatives elicited antagonistic activity to muscarinic M3 receptors and moderate subtype selectivity (M3/M2),[6,7] and the 6β-acetoxyl was confirmed to be crucial for the muscarinic activity,[8,9] as well as subtype selectivity (M3/M2). Furthermore, the functional antagonistic activity of some (3*S*,6*S*)-3α-acyloxy-6β-acetoxyltropane derivatives[1] to muscarinic M3 receptors was larger than that of corresponding 6*R* enantiomers, suggesting that the absolute configuration of 3α-acyloxy-6β-acetoxyltropane derivatives might play an important role in muscarinic activity, as well as subtype selectivity (M3/M2). As in a chiral environment such as in vivo, two enantiomers demonstrate different chemical, biochemical, and pharmacologic behaviors, the investigation of single isomers rather than racemates is more favorable for improving activity and reducing side effects or toxicity.[10–12] Therefore, it is more significant to study the activity and subtype selectivity (M3/M2) for both enantiomers of 3α-acyloxy-6β-acetoxyltropane derivatives, if compared with their racemates. Besides, up to date, the examination of subtype selectivity (M3/M2) for chiral 3α-acyloxy-6β-acetoxyltropane derivatives through biological assays has not been reported.

In this paper, we prepared both enantiomers of **1** (3α-benzoyloxy-6β-acetoxytropane), **2** (3α-orthochlorobenzoyloxy-6β-acetoxytropane), **3** (3α-orthonitrobenzoyloxy-6β-acetoxytropane) and **4** (3α-phenylacetoxy-6β-acetoxytropane) respectively and determined each binding affinity of the eight enantiomers to muscarinic M3 receptors of rat submandibulary gland, M2 receptors of rat left atria. The in vitro functional activity of the four pairs of enantiomers on isolated guinea pig ilea (M3), left atria (M2) was also investigated, as evaluation of antagonistic effect on these models

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[1] As the absolute configuration of C_3, as well as C_1 and C_5 is in agreement with that of C_6 for discussed compounds in this article, the absolute configurations of their enantiomers are simply expressed as 6*S* or 6*R* for convenience in the following discussion.

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may provide a better indication of their potential activity. The structure–activity relationship (SAR) and structure–subtype selectivity (M3/M2) relationship (SSSR) were preliminarily discussed. Understanding the effect of absolute configuration on activity, subtype selectivity (M3/M2) will provide the clues for successive design of muscarinic M3 receptor antagonists with high activity and low side effects or toxicity.

2. Results and discussion

2.1. Chemistry

(−)-**5** ((−)-3α-Hydroxy-6β-acetoxytropane) and (+)-**5**, two enantiomers, were acquired by resoluting (±)-**5** with (+) or (−)-2,3-dibenzoyl tartaric acid in isopropanol. The absolute configuration of (−)-**5** and that of (+)-**5** were determined as (6*S*)-**5** and (6*R*)-**5**, respectively according to the relationship between the absolute configuration and optical activity of chiral tropane derivatives.[13] The resoluted (6*S*)-**5** and (6*R*)-**5** were examined to be optically pure by HPLC (Fig. 1). Carbonylating (6*S*)-**5** and (6*R*)-**5**, respectively gave four pairs of optically pure enantiomers of **1**–**4**, which are (6*S*)-**1**, (6*S*)-**2**, (6*S*)-**3**, (6*S*)-**4**, and (6*R*)-**1**, (6*R*)-**2**, (6*R*)-**3**, (6*R*)-**4** (Chart 1).

2.2. Pharmacology

2.2.1. Effects of carbachol on isolated guinea pig ilea (M3), left atria (M2)

Carbachol, a typical muscarinic agonist, could stimulate contraction of isolated ilea (M3) or induce bradycardia of isolated left atria (M2). Cumulative addition of carbachol produced a log concentration-dependent contractile response to isolated ilea (M3), while inhibiting-contractile response to isolated left atria (M2). The pEC_{50} (negative logarithm of the concentration of agonist causing a half-maximal response) values of carbachol for muscarinic M3, M2 receptors are 6.45 ± 0.22 and 6.31 ± 0.24, respectively. Four 6*S* enantiomers could inhibit carbachol-induced contraction on isolated ilea (M3) and carbachol-induced bradycardia on isolated left atrial (M2). But their corresponding 6*R* enantiomers could not do, indicating that 6*R* configuration was not favorable for **1**–**4** to elicit antagonistic activity to muscarinic M3 or M2 receptors. Like (6*S*)-**4** (Fig. 2), (6*S*)-**1**, (6*S*)-**2** and (6*S*)-**3** shifted the carbachol-induced response curves to the right with a parallel manner. The pA_2 values of four 6*S* enantiomers are 6.51 ± 0.52, 6.56 ± 0.34, 6.41 ± 0.44 and 6.91 ± 0.28 for ilea (M3), while 5.88 ± 0.39, 6.20 ± 0.35, 6.07 ± 0.40 and 5.96 ± 0.43 for left atria (M2), respectively (Table 1). The pA_2 value of each of 6*S* enantiomers for ilea (M3) is obviously larger than that for left atria (M2), indicating that the 6*S* enantiomers act as selective functional antagonists to the muscarinic M3 receptors.

2.2.2. [^{3}H]NMS binding to rat submandibulary glands (M3), left atria (M2)

[^{3}H]NMS (^{3}H-labelled *N*-methylscopolamine) binding studies were performed with a crude membrane fraction prepared from rat submandibulary glands (M3) or left atria (M2). Specific binding of [^{3}H]NMS to submandibulary glands (M3) or left atria (M2) was saturable. The dissociation equilibrium constant (K_d) and receptor density (B_{max}) are 0.58 ± 0.06 nM and 63.37 ± 3.94 fmol/mg protein (n = 3) for submandibulary glands (M3), while 0.30 ± 0.06 nM and 27.09 ± 2.29 fmol/mg protein (n = 3) for left atria (M2) respectively (Fig. 3).

The affinities of both enantiomers of **1**–**4** to submandibulary glands (M3), left atria (M2) were examined. The binding of [^{3}H]NMS to muscarinc M3 or M2 receptors decreased with increasing concentrations of 6*S* or 6*R* enantiomer of **1**–**4** in a log concentration-dependent manner (Fig. 4). As shown in Table 2, four 6*R* enantiomers displayed very weak binding affinity, among which the smallest K_i value is 66 μM for (6*R*)-**3** to muscarinc M3

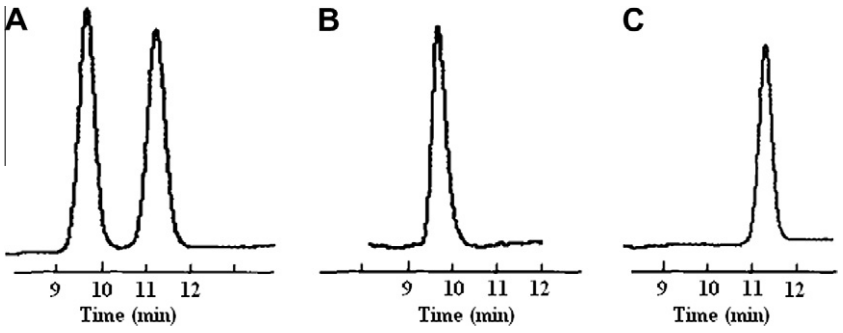

Figure 1. Dynamic chromatograms of (A) (±)-**5**, (B) (6*S*)-**5**,(C) (6*R*)-**5** on Chiralpak AD (150 × 4.6 mm I.D.) under normal phase conditions. Eluent: *n*-hexane–2-propanol–diethylamine 80:20:0.1 (v/v/v); flow rate: 0.7 mL/min; column temperature: 25 °C. The peak-time of (6*S*)-**5**, (6*R*)-**5** is 9.71, 11.29 min, respectively.

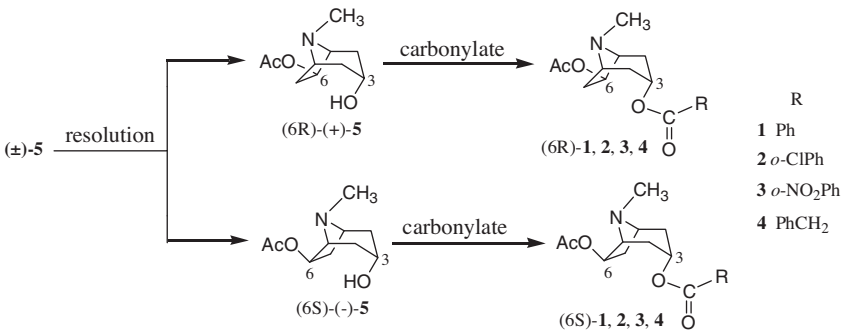

Chart 1. Synthesis of both enantiomers of **1**–**4**.

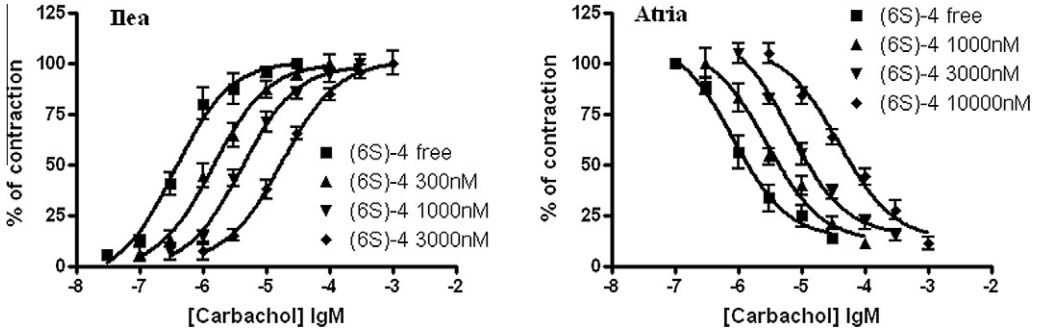

Figure 2. Effects of muscarinic agonist carbachol on ilea (M3), left atria (M2) with incubation of (6*S*)-**4** (n = 3–5).

Table 1
Results of both enantiomers of **1**–**4** to dynamic test on isolated guinea pig ilea (M3), left atria (M2) (n = 3–5)

Compd	Functional studies ($pA_2 \pm SE$)	
	Ilea (M3)[a]	Left atria (M2)[b]
(6*S*)-**1**	6.51 ± 0.52*	5.88 ± 0.39*
(6*R*)-**1**	—	—
(6*S*)-**2**	6.56 ± 0.34*	6.20 ± 0.35*
(6*R*)-**2**	—	—
(6*S*)-**3**	6.41 ± 0.44*	6.07 ± 0.40*
(6*R*)-**3**	—	—
(6*S*)-**4**	6.91 ± 0.28*	5.96 ± 0.43*
(6*R*)-**4**	—	—

pA_2: a logarithmic measure of the potency of the antagonist.
*P <0.05.
'—': Not detected.
[a] The pEC_{50} value of carbachol for ilea (M3) is 6.45 ± 0.22.
[b] The pEC_{50} value of carbachol for left atria (M2), 6.31 ± 0.24.

receptors. This finding could be used to explain that the antagonistic effect of 6*R* enantiomers on isolated ilea (M3) or left atria (M2) was not detected in above functional studies due to their weak binding affinity. Besides, the K_i(M2)/K_i(M3) values of the four 6*R* enantiomers are approximately equal to 1. So, it is less significant to discuss the SAR or SSSR for 6*R* enantiomers.

Four 6*S* enantiomers displayed obvious inhibition to the binding of [^{3}H]NMS with muscarinc M3 or M2 receptors, if compared to their corresponding 6*R* enantiomers. The binding affinity of 6*S* enantiomers is at least 18-fold that of corresponding 6*R* enantiomers for muscarinic M3 receptors ((6*S*)-**3**, K_i = 3.7 μM and (6*R*)-**3**, K_i = 66 μM), while 7-fold that of 6R ones for M2 receptors ((6*S*)-**4**, K_i = 12 μM and (6*R*)-**4**, K_i = 86 μM) (Table 2), indicating that 6*S* configuration was favorable for 3α-acyloxy-6β-acetoxyltropane derivatives to bind with muscarinic M3 or M2 receptors. The results are in good agreement with that concluded from the functional studies. Also, it can be found that for muscarinic M3

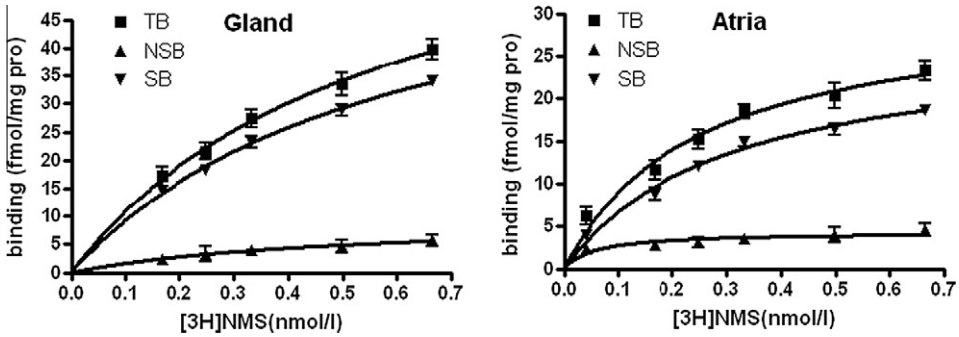

Figure 3. Saturation isotherms of [^{3}H]NMS binding to submandibulary glands (M3), left atria (M2). [^{3}H]NMS with increasing concentrations were incubated in rat submandibulary glands (M3), left atria (M2) and specific binding (SB) was defined as the difference between total binding (TB) and nonspecific binding (NSB) observed in the presence of 10 μM atropine. Points represent the mean ± SEM of three experiments each performed in duplicate. In some points, the error deviation is hidden inside the symbol.

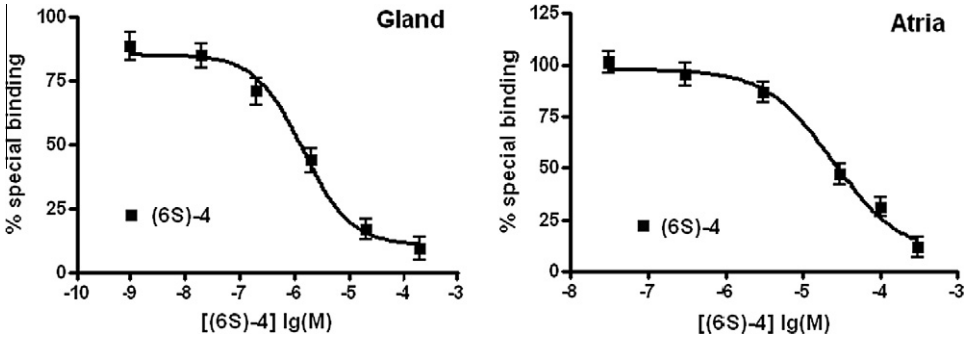

Figure 4. Specific binding of tropane derivatives as muscarinic antagonists competing against [^{3}H]NMS. Glandular (M3), left atria (M2) protein was incubated with 0.29 nM [^{3}H]NMS and increasing concentrations of (6*S*)-**4** as representative.

Table 2
Binding affinity of both enantiomers of **1**–**4** to rat submandibulary glands (M3), left atria (M2)

Compd	Binding affinity (K_i ± SE, μM)		Subtype selectivity (M3/M2) (K_i(M2)/K_i(M3))
	Submandibulary glands (M3)[a]	Left atria (M2)[b]	
(6*S*)-**1**	2.7 ± 0.51	10.7 ± 2.6	4.0
(6*R*)-**1**	71 ± 14	77 ± 14	1.1
(6*S*)-**2**	1.4 ± 0.28	8.8 ± 2.2	6.3
(6*R*)-**2**	83 ± 20	95 ± 24	1.1
(6*S*)-**3**	3.7 ± 0.63	9.8 ± 2.8	2.6
(6*R*)-**3**	66 ± 11	84 ± 17	1.3
(6*S*)-**4**	0.92 ± 0.21	12 ± 2.3	13.0
(6*R*)-**4**	95 ± 24	86 ± 20	0.90

[a] The K_d (dissociation constant), B_{max} (maximal number of binding sites) and Hill coefficient of [^{3}H]NMS are 0.58 nM, 63.37 (fmol/mg wet weight), 0.91 for submandibulary glands (M3).
[b] The K_d (dissociation constant), B_{max} (maximal number of binding sites) and Hill coefficient of [^{3}H]NMS are 0.30 nM, 27.09 (fmol/mg wet weight), 0.93 for left atria (M2).

receptors the stronger the binding affinity of 6*S* enantiomer is, the weaker that of corresponding 6*R* enantiomer be.

Among the four 6*S* enantiomers, (6*S*)-**4** elicited the strongest binding affinity to muscarinic M3 receptors (K_i = 0.92 μM), while the weakest binding affinity to M2 receptors (K_i = 12 μM), resulting in its largest subtype selectivity (M3/M2) (K_i(M2)/K_i(M3) = 13.0-fold). This finding suggested that introducing methylene group between the carbonyl and phenyl ring in C-3α position could improve not only muscarinic M3 activity but also subtype selectivity (M3/M2). (6*S*)-**2** displayed stronger inhibition to the binding of [^{3}H]NMS with muscarinc M3 receptors (K_i = 1.4 μM), and subtype selectivity (M3/M2) (K_i(M2)/K_i(M3) = 6.3-fold), if compared to (6*S*)-**1** (K_i = 2.7 μM), and (K_i(M2)/K_i(M3) = 4.0-fold). Introduction of 2-chloro substitution on the phenyl ring of C-3α increases the electron density of carbonyl oxygen via *p*–π conjugation, in which the Cl atom is regarded as electron-donating group. The C-3α carbonyl oxygen, an important pharmacophore atom and electron donor, realizes the H-bond with certain polar residue in the muscarinic receptors.[8] Increasing the electron density of carbonyl oxygen improved the binding affinity of (6*S*)-**2** for muscarinic M3 receptors rather than M2 receptors. So, the subtype selectivity (M3/M2) of (6*S*)-**2** compared to (6*S*)-**1** enhanced. (6*S*)-**3** elicited weaker inhibition to the binding of [^{3}H]NMS with muscarinic M3 receptors (K_i = 3.7 μM), as well as subtype selectivity (M3/M2) (K_i(M2)/K_i(M3) = 2.6-fold), if compared to (6*S*)-**1**. Nitro group is a strong electron-withdrawing group. Introduction of 2-nitro substitution on the phenyl ring of C-3α greatly reduces the electron density of carbonyl oxygen. This decreased more binding affinity of (6*S*)-**3** for muscarinic M3 receptors. In that case, the subtype selectivity (M3/M2) of (6*S*)-**3** compared to (6*S*)-**1** dropped.

3. Conclusions

The synthesis from two chiral starting materials (6*S*)-**5** and (6*R*)-**5** has afforded eight optically pure 3α-acyloxy-6β-acetoxyltropane derivatives with definite absolute configuration, (6*S*)-**1**, (6*S*)-**2**, (6*S*)-**3**, (6*S*)-**4**, and (6*R*)-**1**, (6*R*)-**2**, (6*R*)-**3**, (6*R*)-**4**. The eight enantiomeric-pure compounds were applied to study the muscarinic M3, M2 antagonistic activity and subtype selectivity (M3/M2) through functional studies in vitro and radioreceptor binding assays. The functional studies showed that 6*S* enantiomers elicited obvious antagonistic effects towards muscarinic M3 or M2 receptors, while the corresponding 6*R* enantiomers had little biological activities. The radioreceptor binding assays registered that the affinity of 6*S* enantiomers was much larger than that of corresponding 6*R* enantiomers. These findings indicated that 6*S* configuration was favorable for 3α-acyloxy-6β-acetoxyltropane derivatives to combine with muscarinic M3, as well as M2 receptors and elicited antagonistic activities. It was in good agreement with our former studies concerning the absolute configuration of active tropane derivatives.[14] Besides, the muscarinic M3 activity, as well as subtype selectivity (M3/M2) of 6*S* enantiomers could be improved by increasing the electron density of carbonyl oxygen or introducing methylene group between the carbonyl and phenyl ring in C-3α position. All these pharmalogical results implied that the absolute configuration of 3α-acyloxy-6β-acetoxyltropane derivatives played an important role in the muscarinic M3 antagonistic activity and subtype selectivity (M3/M2).

4. Experimental

4.1. Instruments and materials

Melting points were recorded on a WRS-1A melting point apparatus. NMR spectra were recorded in $CDCl_3$ on a Bruck AM-400 NMR spectrophotometer operating. Mass spectra (MS) were determined on an HP-5988 GC/MS spectrophotometer and infrared (IR) spectra on a Nicolet-Magna IR 750 spectrophotometer using KBr discs. HPLC enantioseparations were performed by using Chiralpak AD (150 × 4.6 mm I.D.) under normal phase conditions. Rotary power measurements were performed on a Perkin-Elmer 241MC polarimeter at 20 ± 2 °C. Reactions were monitored by TLC using silica gel type HSG (F254) of Qingdao Ocean Chemical Plant and visualized with iodine vapours or Dragendorff reagent. Column chromatography was performed on silica gel (Qingdao Ocean, 10–40 μM). Solvents for synthesis were redistilled. (±)-**5** was obtained from Hangzhou Minsheng Pharmaceutical Factory (China), and (+),(−)-2,3-dibenzoyl tartaric acids were purchased from Aldrich Chemical Inc. (USA). Tris-[hydroxymethyl] amino methane (Tris) was purchased from Toronto Research Chemicals (Canada). Carbachol and atropine were purchased from Sigma corporation (USA), and [^{3}H]NMS (spec. act. 43 Ci/mM) from Amersham Inc. (England). (6*S*)-**1**, (6*R*)-**1**, (6*S*)-**2**, (6*R*)-**2**, (6*S*)-**3**, (6*R*)-**3**, (6*S*)-**4** and (6*R*)-**4** were dissolved in distilled water as the test drugs and preserved under 4 °C for functional in vitro studies and radioreceptor binding assays.

4.2. Resolution of (±)-3α-hydroxy-6β-acetoxytropane (5)

7.524 g (37.8 mmol) (±)-**5** and 12.969 g (36.2 mmol) (+)-2,3-dibenzoyl-D-tartaric acid were dissolved in 60 ml isopropanol, placed at room temperature for 24 h. Precipitate was collected and recrystallized in absolute alcohol. 8.214 g of white needle crystals were obtained, mp 183–184 °C, $[\alpha]_D^{20}$ +63.6° (*c*, 1.03, H_2O). The salt was treated in usual manner to give the base 3.224 g (43%) (6*S*)-**5** as colourless oil, $[\alpha]_D^{20}$ −20.5° (*c*, 1.10, $CHCl_3$). The mother liquor was evaporated in vacuo. The residue was dissolved in water and adjusted with concentrated ammonium hydroxide to pH 9–10.

The solution was extracted with 30 ml CH_2Cl_2, and the organic layer was dried over sodium carbonate and evaporated to dryness. The residue 3.215 g with 6.530 g (18.2 mmol) (−)-2,3-dibenzoyl-L-tartaric acid in 25 ml isopropanol, treated as mentioned above, 9.269 g salt as white needle crystals were obtained, mp 180–181 °C, $[\alpha]_D^{20}$ −63.2° (*c*, 0.90, H_2O), followed by getting the colourless oil 3.062 g (41%), (6*R*)-**5**, $[\alpha]_D^{20}$ +19.7° (*c*, 0.94, $CHCl_3$).

4.3. General procedure for the preparation of enantiomers of 1–4

Equimolar quantity of (6*S*)-**5** or (6*R*)-**5** was dissolved in 2 ml CH_2Cl_2 with 0.1 ml pyridine. The 0.3–0.5 ml acid chloride was then added dropwise while stirring at room temperature during 4–12 h. The reaction liquor was evaporated in vacuo. The residue was dissolved in water and adjusted with concentrated ammonium hydroxide to pH 9–10. The solution was extracted with CH_2Cl_2 (5 times × 6 ml). The organic phase was dried over anhydrous sodium sulfate and evaporated to dryness. The crude product was purified by column chromatography over silica gel. CH_2Cl_2/CH_3OH (25:1) eluted the pure ester.

4.3.1. (6*S*),(6*R*)-3α-benzoyloxy-6β-acetoxytropane (1)

Pale yellow oil, (6*S*)-**1**, (254 mg, 61%), $[\alpha]_D^{20}$ +27.1° (*c*, 1.20, $CHCl_3$); (6*R*)-**1** (232 mg, 76%), $[\alpha]_D^{20}$ −26.7° (*c*, 1.13, $CHCl_3$). IR (KBr) cm^{-1}: 2950, 2809, 1718, 1602. EI-MS *m*/*z*: 303 (M^+, 12.54), 182, 122, 94, 82, 43. ^1H NMR ($CDCl_3$, 400 MHz) δ: 2.11 (s, 3H, CH_3CO), 2.73 (s, 3H, CH_3N), 1.30–2.52 (m, 6H, 2, 4, 7-H), 3.41–3.80 (m, 2H, 1, 5-H), 5.27–5.53 (m, 1H, 3-H), 5.70 (dd, *J* = 4.0, 8.0 Hz, 1H, 6-H),7.50–7.73 (m, 3H, Ph-H), 8.03–8.25 (m, 2H, Ph-H).

4.3.2. (6*S*),(6*R*)-3α-orthochlorobenzoyloxy-6β-acetoxytropane (2)

Pale yellow oil, (6*S*)-**2**, (195 mg, 65%), $[\alpha]_D^{20}$ +10.1° (*c*, 0.85, $CHCl_3$); (6*R*)-**2** (236 mg, 70%), $[\alpha]_D^{20}$ −9.8° (*c*, 0.93, $CHCl_3$). IR (KBr) cm^{-1}: 2939, 2857, 1732, 1592. EI-MS *m*/*z*: 337 (M^+, 11.67), 182, 139, 122, 94, 82, 43. ^1H NMR ($CDCl_3$, 400 MHz) δ: 1.70, 1.87 (d, *J* = 15.4 Hz, 2H, 2, 4-H), 2.00 (s, 3H, CH_3CO), 2.05–2.30 (m, 3H, 2, 4, 7-H), 2.49 (s, 3H, CH_3N), 2.56 (dd, *J* = 7.6, 15.0 Hz, 1H, 7-H), 3.18 (s, 1H, 5-H), 3.31 (m, 1H, 1-H), 5.27 (m, 1H, 3-H), 5.46 (dd, *J* = 3.0, 7.5 Hz, 1H, 6-H), 7.27–7.45 (m, 3H, Ph-H), 7.78–7.87 (m, 1H, Ph-H).

4.3.3. (6*S*),(6*R*)-3α-orthonitrobenzoyloxy-6β-acetoxytropane (3)

Pale yellow oil, (6*S*)-**3**, (228 mg, 68%), $[\alpha]_D^{20}$ +42.8° (*c*, 1.15, $CHCl_3$); (6*R*)-**3** (192 mg, 56%), $[\alpha]_D^{20}$ −40.7° (*c*, 1.10, $CHCl_3$). IR (KBr) cm^{-1}: 2938, 2857, 1732, 1609. EI-MS *m*/*z*: 348 (M^+, 9.27), 182, 122, 94, 82, 43. ^1H NMR ($CDCl_3$, 400 MHz) δ: 1.72–2.30 (m, 5H, 2, 4, 7-H), 2.01 (s, 3H, CH_3CO), 2.37 (dd, *J* = 7.7, 14.1 Hz, 1H, 7-H), 2.54 (s, 3H, CH_3N), 3.18 (s, 1H, 5-H), 3.35 (s, 1H, 1-H), 5.26–5.36 (m, 2H, 3, 6-H), 7.60–7.76 (m, 3H, Ph-H), 7.90–7.98 (m, 1H, Ph-H).

4.3.4. (6*S*),(6*R*)-phenylacetoxy-6β-acetoxytropane (4)

Pale yellow oil, (6*S*)-**4**, (155 mg, 64%), $[\alpha]_D^{20}$ +2.0° (*c*, 1.05, $CHCl_3$); (6*R*)-**4**, (139 mg, 57%), $[\alpha]_D^{20}$ −2.3° (*c*, 1.22, $CHCl_3$). IR (KBr) cm^{-1}: 2947, 2858, 1732, 1604. EI-MS *m*/*z*: 317 (M^+, 19.58), 182, 122, 94, 82, 43. ^1H NMR ($CDCl_3$, 400 MHz) δ: 1.64–2.15 (m, 6H, 2,4,7-H), 2.03 (s, 3H, CH_3CO), 2.44 (s, 3H, CH_3N), 3.08 (s, 1H, 5-H), 3.21 (m, 1H, 1-H), 3.61 (s, 2H, CH_2), 4.95 (m, 1H, 3-H), 5.21 (dd, *J* = 3.1, 7.6 Hz, 1H, 6-H), 7.20–7.36 (m, 5H, Ph-H).

4.4. Functional in vitro studies

4.4.1. Guinea pig ilea (M3)

Guinea pigs (220–260 g) of either sex provided by animal experimental center of Shanghai Jiao Tong University were killed by a blow to the head and exsanguinated. The ileal muscle was rapidly removed and gently cleaned of adhering connective tissue in a pre-warmed (37 °C) and oxygenated (95% O_2 + 5% CO_2) medium of the Kreb's solution of the following composition: NaCl 6.6 g, $CaCl_2$ 0.28 g, KCl 0.35 g, $MgSO_4{\cdot}7H_2O$ 0.294 g, KH_2PO_4 0.162 g, $NaHCO_3$ 2.1 g, glucose 2.0 g in 1000 ml distilled water. Strips of ileal muscle (1.5 cm) prepared were transferred to 10 ml organ baths with filled Kreb's solution and loaded with a tension of 1000 mg. The preparation was allowed to equilibrate for 30 min, changing the bath fluid every 15 min. Contractions were recorded isotonically with an electromechanical transducer connected to Bridge amplifier and Powerlab system recorder. Cumulative concentration–response curve was obtained for carbachol. The concentration of carbachol in the organ bath was increased approximately 3-fold at each step, with each addition being made only after the response to the previous addition had attained a maximal level and remained steady. The contractile response of ileal muscle for each dose of carbachol was expressed as a percentage of the maximal response in the control curve. The results were expressed in terms of EC_{50}, the concentration of agonist required to produce 50% of the maximum contraction. After stable concentration–response curves for carbachol were obtained, (6*S*)-**1**, (6*R*)-**1**, (6*S*)-**2**, (6*R*)-**2**, (6*S*)-**3**, (6*R*)-**3**, (6*S*)-**4** or (6*R*)-**4** was added and the tissue was stimulated cumulatively with carbachol as before. For the antagonistic test, three different concentrations of (6*S*)-**1**, (6*R*)-**1**, (6*S*)-**2**, (6*R*)-**2**, (6*S*)-**3**, (6*R*)-**3**, (6*S*)-**4** and (6*R*)-**4** were investigated.

4.4.2. Electrically stimulated guinea pig left atria (M2)

The heart was rapidly removed, and the right and left atria were separated. The left atria were amounted in Kreb's solution and loaded with a tension of 500 mg. After a period of stabilization of 45 min, tissues were electrically stimulated through platinum electrode by square-wave submaximal pulse (2 Hz, 6 ms, 10 V) and inotropic activity was recorded isometrically. The response of left atria for each dose of carbachol was expressed as a percentage of the contractile response before the agonist added. For the antagonistic test, two or three different concentrations of (6*S*)-**1**, (6*R*)-**1**, (6*S*)-**2**, (6*R*)-**2**, (6*S*)-**3**, (6*R*)-**3**, (6*S*)-**4** and (6*R*)-**4** were studied.

4.5. Radioreceptor binding assays

The binding of (6*S*)-**1**, (6*R*)-**1**, (6*S*)-**2**, (6*R*)-**2**, (6*S*)-**3**, (6*R*)-**3**, (6*S*)-**4** and (6*R*)-**4** to muscarinic M3 or M2 receptors was determined using rat submandibulary glands or left atria. A male SD rat (220–250 g) was killed by cervical dislocation. The submandibulary glands, left atria were removed, cleaned adhering tissue in ice-cold 50 mM Tris buffer (pH 7.4). Homogenisation of the submandibulary glands, left atria was carried out in 1 g:20 ml (w:v) volume ice-cold 0.32 M sucrose in Tris buffer using a Waring blender and further disrupted with an Ultraturrax Tissuemizer. The crude homogenate was centrifuged for 10 min at 1000*g* and the resulting supernatant was centrifuged for 30 min at 20000*g* to yield a membrane pellet. The pellet was resuspended in Tris buffer as a crude membrane fraction. All the procedures were performed at 4 °C. In the saturation binding assays, membranes (0.1 mg protein) were incubated vibrantly at 37 °C for 30 min in 0.080–1.52 nM [^{3}H]NMS with or without 10 μM atropine sulfate in a total volume of 0.4 ml. The reaction was terminated by rapid filtration through glass fiber filters, washed three times with ice-cold Tris buffer. The protein concentration was determined with the micro BCA kit (Pierce, Rockford, IL), using bovine serum albumin as the standard. For competition binding assays, membranes (0.1 mg protein) were incubated with 0.4 nM [^{3}H]NMS at 37 °C for 60 min and increasing concentrations of (6*S*)-**1**, (6*R*)-**1**, (6*S*)-**2**, (6*R*)-**2**, (6*S*)-**3**, (6*R*)-**3**, (6*S*)-**4** or (6*R*)-**4** in total volume of 0.4 ml. All the dilutions for the test compounds were made in Tris buffer. Assays were performed in duplicate.[15]

4.6. Statistics and data analysis

For the ilea or atria contraction assay, EC_{50} values and the slopes of the log concentration–response curves for carbachol were calculated by means of nonlinear curve fitting of sigmoidal dose–response logistic transformation using program GraphPad PRISM 4.0 (San Diego, CA, USA). The pA_2 values for (6*S*)-**1**, (6*S*)-**2**, (6*S*)-**3** and (6*S*)-**4** were determined according to Arunlakshana and Schild.[16] In saturation binding tests, nonlinear curve fitting was used to generate affinity (K_d) and capacity (B_{max}) values for [^{3}H]NMS. The displacement data were analyzed using the commercial software PRISM to obtain IC_{50} values for competing ligands. The affinity, expressed as K_i was calculated from IC_{50} value according to Cheng and Prusoff.[17] Data were expressed as mean ± SEM. The statistically significant differences were determined by Student's *t*-test and comparisons between means were made considered significant if $P < 0.05$.

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