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The absolute configuration plays an important role in muscarinic activity of BGT-A and its analogs

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ABSTRACT

Both enantiomers of 2, 3, and 4, three bioactive analogs of muscarinic agonist BGT-A were prepared respectively and underwent functional studies and radioreceptor binding assays. 6*S* enantiomers of 2, 3, and 4 showed obvious muscarinic activity, while 6*R* ones elicited little muscarinic activity by functional studies. Besides, the affinity of 6*S* enantiomers of 2, 3, and 4 was greatly larger than that of their 6*R* enantiomers respectively. All these pharmalogical results indicated the 6*S* configuration was beneficial for the active BGT-A analogs to bind with the muscarinic receptors. The finding was in good agreement with our previous SAR study to BGT-A and its active analogs by computational approach. The understanding to the relationship between muscarinic activity and absolute configuration will provide the basis for successive screening of BGT-A analogs as effective muscarinic agonists or antagonists in clinical use.

1. Introduction

Muscarinic drugs have potential for therapeutic use in several pathological states.[1,2] As a consequence, the muscarinic compounds have been studied widely in the past decades and the study of the relationship between muscarinic activity and absolute configuration of them has been interested by medicinal chemists.[3,4] Baogongteng A ([2*S*,6*S*]-2α-hydroxy-6β-acetoxynortropane, (6*S*)-BGT-A)[‡] (**1**) (Fig. 1) was the first naturally occurring tropane alkaloid as muscarinic acetycholine receptor (mAChR) agonist isolated from the stem of Chinese medicinal plant Baogongteng (*Erycibe obtusifolia* Benth) and was used as a myotic agent to treat glaucoma in clinics.[5] However, the limited amounts of (6*S*)-BGT-A available from the natural resources and the poor yield of total synthesis of (±)-BGT-A make it necessary to find an alternative of (6*S*)-BGT-A through bioactive screening of BGT-A analogs.[6–10] Certain (±)-3-substituted-6β-acetoxyl tropane analogs of BGT-A were found to be mAChR agonist or antagonist. **2** ((±)-3α-parachlorobenzoyloxy-6β-acetoxytropane) and **3** ((±)-3α-paranitrobenzoyloxy-6β-acetoxytropane) have higher antagonistic activity, while **4** ((±)-3α-benzenesulfonyloxy-6β-acetoxytropane) (Fig. 1) agonistic activity. The structure-activity relationship (SAR) study indicated that 6β-acetoxyl and the absolute configuration of carbon 6 of these analogs were crucial for the mAChR activity, due to the fact that 6β-hydroxy analog of BGT-A was inactive[11,12] and the value of the agonistic activity of synthetic (±)-BGT-A was nearly half as that of the natural (6*S*)-BGT-A,[13,14] and the analyzing result acquired by calculating the structural parameters of some active analogs revealing that their mAChR activities being connected with the 6*S* configuration.[15] The understanding of the relationship between mAChR activity and absolute configuration of BGT-A analogs will provide the basis for successive screening of effective hypotoxic mAChR agonists or antagonists in clinical use. However, the knowledge of the relationship between the absolute configuration and mAChR activity has not been further identified through biological assay to the enantiomers of bioactive BGT-A analogs. In this paper, we prepared both enantiomers of **2, 3** and **4** respectively, and determined each binding affinity of the six enantiomers to mAChR of the rat submandibulary gland. The in vitro functional activity of the three pairs of enantiomers on isolated iris muscle was also investigated, as evaluation of the activity on this model may provide a better indication of their potential activity. Both enantiomers of **2, 3** were tested for the muscarinic activity as potential antagonists, while, both enantiomers of **4** as agonists. Besides, the mydriatic or myotic effect of the enantiomers of **2, 3, 4** was tested in vivo.

2. Results and discussion

2.1. Chemistry

(−)-**5** ((−)-3α-hydroxy-6β-acetoxytropane) and (+)-**5**, two enantiomers, were acquired by resoluting (±)-**5** with (+) or (−)-

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‡ As the absolute configuration of C2 or C3 is in agreement with that of C6 for discussed compounds in this article, the absolute configurations of their enantiomers are simply expressed as 6S or 6R for convenience in the following discussion.

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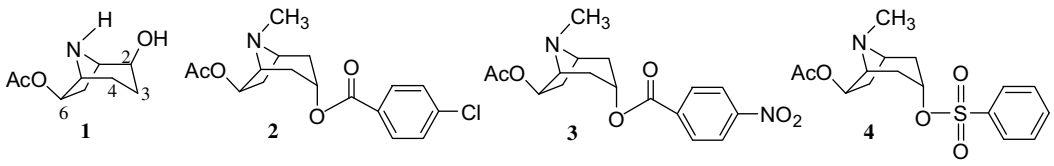

Figure 1. Structures of **1**–**4**.

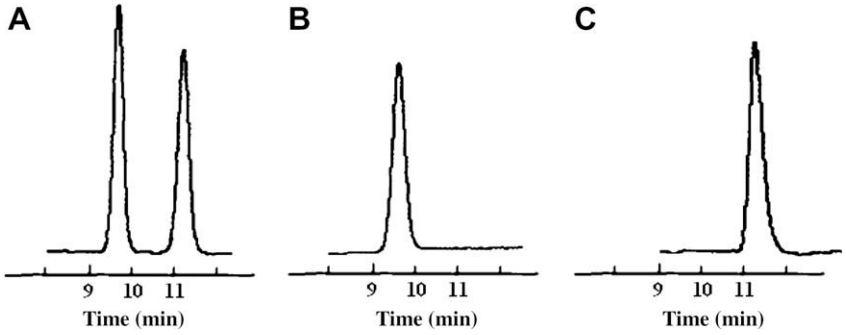

Figure 2. Dynamic chromatograms of A. enantiomers of **5**. B. (6*S*)-**5**. C. (6*R*)-**5** on Chiralpak AD (150 × 4.6 mm I.D.) under normal phase conditions. Eluent: *n*-hexane/2-propanol/diethylamine 80:20:0.1 (v/v/v); flow rate: 4.7 ml/min; column temperature: 25 °C. The peak-time of (6*S*)-**5**, (6*R*)-**5** are 9.69 min, 11.28 min, respectively.

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 the chiral tropane compounds.[16] The resoluted (6*S*)-**5** and (6*R*)-**5** were examined to be optically pure by HPLC (Fig. 2). Carbonylating or sulfonylating (6*S*)-**5** and (6*R*)-**5** respectively gave three pairs of optically pure enantiomers of **2, 3, 4**, (6*S*)-**2**, (6*S*)-**3**, (6*S*)-**4**, (6*R*)-**2**, (6*R*)-**3**, and (6*R*)-**4** (Chart 1).

2.2. Pharmacology

2.2.1. Effect of carbachol, (6*S*)-4 and (6*R*)-4 on the contraction of isolated iris muscle

(6*S*)-**4** like carbachol, a typical muscarinic agonist, could stimulate the contraction of isolated iris muscle, whereas, (6*R*)-**4** could not, indicating that 6*S* configuration was crucial for **4** to elicit muscarinic agonistic activity. Cumulative addition of mAChR agonists carbachol or (6*S*)-**4** to isolated iris muscle produced a log concentration-dependent contractile response (Fig. 3). The pEC_{50} and E_{max} values for carbachol and (6*S*)-**4** are shown in Table 1. (6*S*)-**4** produces 87% maximum contraction compared to that of carbachol, and the potency of (6*S*)-**4** (pEC_{50} = 6.06) is lower than that of carbachol (pEC_{50} = 6.57).

2.2.2. Effect of carbachol on the contraction of isolated iris muscle under pre-incubation of (6*S*)-2, (6*R*)-2, (6*S*)-3, (6*R*)-3

(6*S*)-**2** and (6*S*)-**3** could inhibit the contraction of isolated iris muscle induced by carbachol, but their enantiomers (6*R*)-**2**, (6*R*)-**3** could not do, indicating that 6*R* configuration was not benefit for **2**, **3** to elicit antagonistic activity to mAChRs. (6*S*)-**2**, (6*S*)-**3**, shifted the carbachol-induced response curves to the right with a parallel manner (Fig. 4), generating pA_2 values of 5.86 ± 0.24 and 6.11 ± 0.17 against carbachol, respectively (Table 1).

2.2.3. [^{3}H]NMS binding to submandibulary glands

[^{3}H]NMS (^{3}H-labelled *N*-methylscopolamine) binding studies were performed with a crude membrane fraction prepared from the submandibulary glands. Specific binding of [^{3}H]NMS to mAChRs of submandibulary glands was saturable. The dissociation equilibrium constant (K_d) and receptor density (B_{max}) were 0.71 ± 0.20 nM and 70.50 ± 12.20 fmol/mg protein (n = 3), respectively (Fig. 5).

The binding affinities of (6*S*)-**2**, (6*R*)-**2**, (6*S*)-**3**, (6*R*)-**3**, (6*S*)-**4**, and (6*R*)-**4** were examined. As shown in Figure 6, [^{3}H]NMS binding to mAChRs was strongly inhibited by (6*S*)-**2**, (6*S*)-**3**, (6*S*)-**4** and weakly inhibited by (6*R*)-**2**, (6*R*)-**3** in a log concentration-dependent manner, but could not be inhibited by (6*R*)-**4**. The binding affinity of (6*S*)-**4** is the largest (K_i, 0.04 μM), and the binding affinity of (6*S*)-**2** (K_i, 9 μM), (6*S*)-**3** (K_i, 3.7 μM) are 8-fold, 120-fold as that

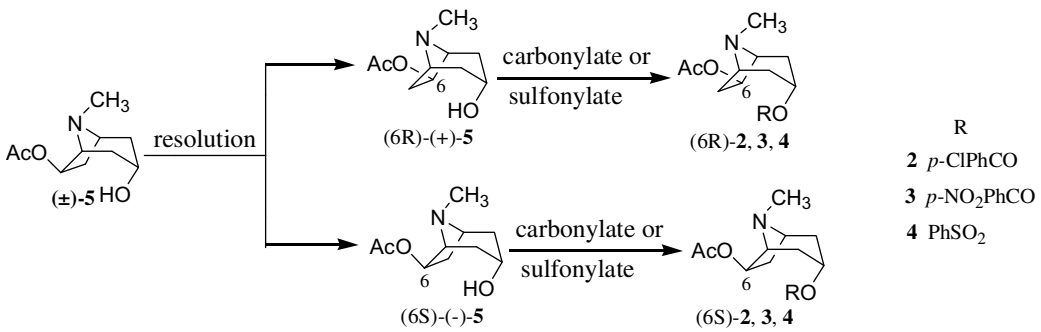

Chart 1. Synthesis of the enantiomers of **2**, **3**, and **4**.

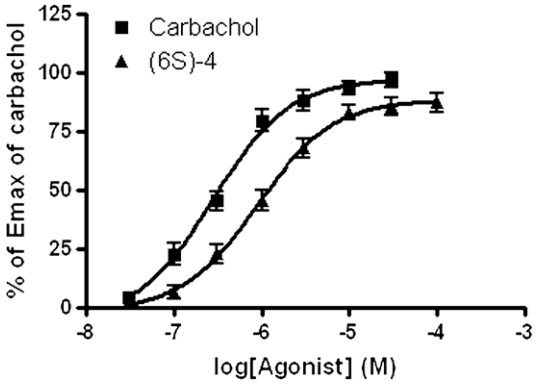

Figure 3. Effects of mAChR agonists carbachol and (6*S*)-**4** on the contractile response of isolated guinea-pig iris muscle. The contractile response of iris muscle to each dose of the agonists is expressed as percentage of that induced by 10^{-4} M carbachol. The data points represent mean ± SEM (n = 3–5).

Table 1
Results of (6*S*)-**2**, (6*R*)-**2**, (6*S*)-**3**, (6*R*)-**3**, (6*S*)-**4**, and (6*R*)-**4** to dynamic test on mAChRs of the isolated guinea-pig iris muscle (n = 3–5).

Compound	pEC_{50}	E_{max} %	pA_2
Carbachol	6.57 ± 0.08*	100 ± 5.67*	—
(6*S*)-**2**		—	5.86 ± 0.24*
(6*R*)-**2**	—	—	—
(6*S*)-**3**	—	—	6.11 ± 0.17*
(6*R*)-**3**	—	—	—
(6*S*)-**4**	6.06 ± 0.07*	87.10 ± 3.8*	—
(6*R*)-**4**	—	—	—

pEC_{50}: Negative logarithm of the concentration of agonists causing a half-maximal response.
E_{max}: maximal response, expressed relative to that of carbachol.
pA_2: a logarithmic measure of the potency of the antagonist. *$P < 0.05$.
"—": not detected.

of (6*R*)-**2** (K_i, 73 μM), (6*R*)-**3** (K_i, 450 μM) respectively (Table 2), indicating that 6*S* configuration was benefit for **2**, **3**, and **4** to combine with mAChRs. The affinity of (6*S*)-**3** is great than that of (6*S*)-**2**, while (6*R*)-**3** less than (6*R*)-**2**, suggesting that the stronger the binding of 6*S* enantiomer of active BGT-A analog to mAChRs is, the weaker the binding for its corresponding 6*R* enantiomer be.

2.2.3.1. Mydriatic effects of(6*S*), (6*R*)-2, (6*S*), or (6R)-3 and myotic effects of (6*S*) or (6*R*)-4 on conscious guinea-pig in vivo. The mean mydriatic or myotic percentages caused by both enantiomers of **2**, 3, and **4** in different groups were recorded in Table 3. Obvious mydriatic effect of (6*S*)-**2**, **3** (mydriatic percentage > 30%, $P < 0.05$) and myotic effect of (6*S*)-**4** (myotic percentage < −40%, $P < 0.05$) in test groups were observed, compared to 3.7% mydriatic percentage caused by the tests of control to the right pupil as reference. By comparison, (6*R*)-**2**, **3**, **4** elicited little influence on the rabbit's pupil (changing percentage in the scale of <±5%). These results indicated (6*S*)-**2**, **3** had muscarinic antagonistic activity, while (6*S*)-**4** agonistic activity, and (6*R*)-**2**, **3**, **4** showed little mAChR activity.

3. Conclusions

The synthesis from two chiral starting materials (6*S*)-**5**, (6*R*)-**5** has afforded six optically pure BGT-A analogs, (6*S*)-**2**, (6*S*)-**3**, (6*S*)-**4**, (6*R*)-**2**, (6*R*)-**3**, and (6*R*)-**4** with definite absolute configurations. The six enantiomeric-pure compounds were applied to study the relationship between the absolute configuration and mAChR activities through functional studies in vitro, in vivo and radioreceptor binding assays. The results of functional studies showed the 6*S* enantiomers of **2, 3**, and **4** elicited obvious muscarinic agonistic or antagonistic effect, while their 6*R* enantiomers showed little biological effect, indicating 6*S* configuration was benefit for BGT-A analogs to elicit muscarinic activity. It was in good agreement with that of our recent studies concerning the absolute configuration of active BGT-A analogs.[17] The radioreceptor binding assays registered that affinity of 6*S* enantiomers of **2, 3**, and **4** was greatly larger than that of their 6*R* enantiomers, and the larger the affinity of 6*S* enantiomer of active BGT-A analog is, the smaller the affinity for its corresponding 6*R* enantiomer be. All these pharmalogical results imply that the absolute configuration of BGT-A analogs plays an important role in evaluating their mAChR activities, and provide the basis for the screening of effective muscarinic agonists or antagonists in clinical use.

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 vapors 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

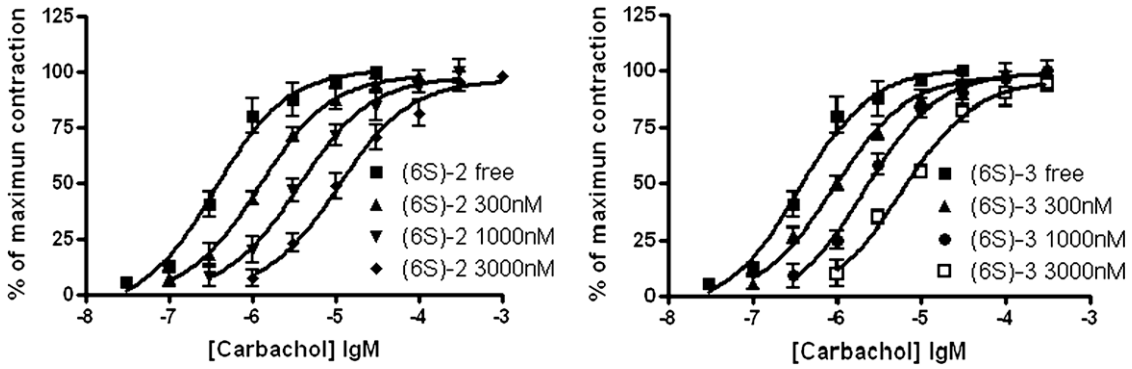

Figure 4. Effects of muscarinic agonist carbachol on iris contraction (n = 3–5).

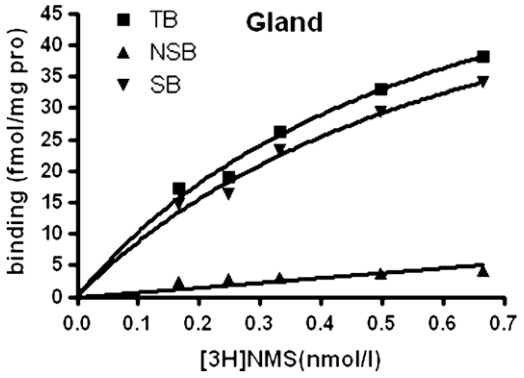

Figure 5. Saturation isotherms of [^{3}H]NMS binding to submandibulary glands. [^{3}H]NMS with increasing concentrations were incubated in rat submandibulary glands and specific binding (SB) was defined as the difference between total binding (TB) and nonspecific binding (NSB) observed in the presence of 10 μM atropine. Points represent mean ± SEM of three experiments each performed in duplicate. In some points, the error deviation is hidden inside the symbol. (n = 3–5).

from Toronto Research Chemicals (Canada). Carbachol (79H0110) and atropine (69H0545) were purchased from Sigma corporation (USA.), and [^{3}H]NMS (spec. act. 43 Ci/mM) from Amersham Inc. (England). (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)

6.270 g (31.5 mmol) (±)-**5** and 10.991 g (30.7 mmol) (+)-2,3-dibenzoyl-D-tartaric acid were dissolved in 48 ml isopropanol, placed at room temperature for 24 h. Precipitate was collected and recrystallized in absolute alcohol. 7.725 g of white needle crystals were obtained, mp.183-184 °C, $[\alpha]_D^{20}$ +63.8° (*c*, 0.95, H_2O). The salt was treated in usual manner to give the base 2.633 g (42%) (6*S*)-**5** as colorless oil, $[\alpha]_D^{20}$ −20.3° (*c*, 1.02, $CHCl_3$). 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.

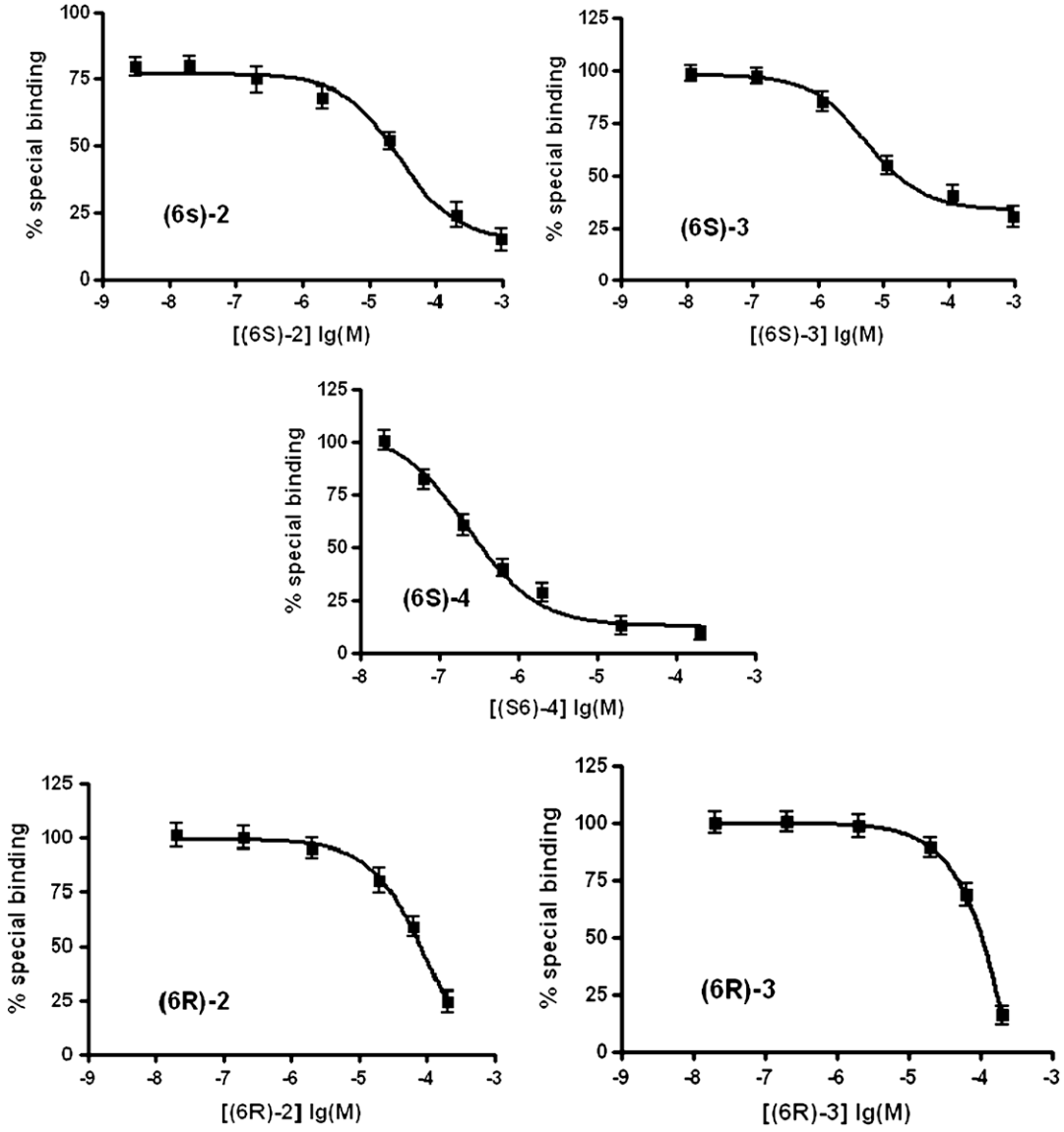

Figure 6. Specific binding of BGT-A analogs as muscarinic ligands competing against [^{3}H]NMS. Glandular protein was incubated with 0.33 nM [^{3}H]NMS and increasing concentrations of (6*S*)-**2**, (6*S*)-**3**, (6*S*)-**4**, (6*R*)-**2**, or (6*R*)-**3**.

Table 2
Binding affinities for rat submandibulary gland of (6*S*)-**2**, (6*R*)-**2**, (6*S*)-**3**, (6*R*)-**3**, (6*S*)-**4**, or (6*R*)-**4**.

Compound	Submandibulary glands[a] K_i (μM)
(6*S*)-**2**	9.0 ± 1.1
(6*R*)-**2**	73 ± 22
(6*S*)-**3**	3.70 ± 0.51
(6*R*)-**3**	450 ± 34
(6*S*)-**4**	0.040 ± 0.003
(6*R*)-**4**	—

[a] The K_d (dissociation constant), B_{max} (maximal number of binding sites) and Hill coefficient of H-labelled *N*-methylscopolamine are: 0.71 nM, 70.5 (fmol/mg wet weight), 0.92. "—": not detected.

Table 3
Mydriatic or myotic effect caused by (6*S*)-**2**, (6*R*)-**2**, (6*S*)-**3**, (6*R*)-**3**, (6*S*)-**4**, or (6*R*)-**4**.

Compound	Percentage of the pupil diameter changing (%)
Control	2.7 ± 0.5*
(6*S*)-**2**	32.2 ± 1.9*
(6*R*)-**2**	3.0 ± 1.5
(6*S*)-**3**	35.1 ± 2.1*
(6*R*)-**3**	3.7 ± 1.3
(6*S*)-**4**	−44.0 ± 3.1*
(6*R*)-**4**	−4.7 ± 2.2

The positive values represent mydriatic effect, while the negative myotic effect. The left pupil diameters (mm) of the rabbits were measured under constant light, and readings were taken before and at 10 min after dropping 0.05 ml agent, while the right for the control as reference. *$P < 0.05$.

The residue 2.980 g with 5.442 g (15.2 mmol) (−)-2,3-dibenzoyl-L-tartaric acid in 20 ml isopropanol, treated as mentioned above, 7.022 g (40%) salt as white needle crystals were obtained, mp 180–181 °C, $[\alpha]_D^{20}$ −63.2° (*c*, 0.95, H_2O), followed by getting the colorless oil 2.320 g (37%), (6*R*)-**5**, $[\alpha]_D^{20}$ +19.9° (*c*, 1.02, $CHCl_3$).

4.3. General procedure for the preparation of enantiomers of 2, 3, and 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× 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. Dichloromethane/methanol (25:1) eluted the pure ester.

4.3.1. (6*S*), (6*R*)-3α-Parachlorobenzoyloxy-6β-acetoxytropane (2)

Pale yellow oil, (6*S*)-**2**, (191 mg, 65%), $[\alpha]_D^{20}$ +20.2° (*c*, 0.40, $CHCl_3$); (6*R*)-**2** (231 mg, 71%), $[\alpha]_D^{20}$ −19.8° (*c*, 0.53, $CHCl_3$). IR (KBr) cm^{-1}: 2939, 2857, 1732, 1592. EI-MS *m*/*z*: 337 (M^+), 182, 122, 94, 82, 43. ^1H NMR ($CDCl_3$, 400 MHz) δ: 1.98 (s, 3H, CH_3CO), 2.56 (s, 3H, CH_3N), 1.18–2.77 (m, 6H, 2, 4, 7-H), 3.2–3.6 (m, 2H, 1, 5-H), 5.1–5.4 (m, 1H, 3-H), 5.53 (dd, 1H, *J* = 4.0, 8.0 Hz, 6-H), 7.38 (d, 2H, *J* = 14 Hz, Ph-H), 7.92 (d, 2H, *J* = 14 Hz, Ph-H).

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

Pale yellow oil, (6*S*)-**3**, (268 mg, 51%), $[\alpha]_D^{20}$ +30.9° (*c*, 1.05, $CHCl_3$); (6*R*)-**3** (202 mg, 66%), $[\alpha]_D^{20}$ −30.7° (c, 1.15, $CHCl_3$). IR (KBr) cm^{-1}: 2938, 2857, 1732, 1609. EI-MS *m*/*z*: 348 (M^+, 8.07), 182, 149, 122, 94, 82, 43. ^1H NMR ($CDCl_3$, 400 MHz) δ: 2.34 (s, 3H, CH_3CO), 2.82 (s, 3H, CH_3N), 1.54–3.3 (m, 6H, 2, 4, 7-H), 3.40–3.80 (m, 2H, 1, 5-H), 5.40–5.90 (m, 2H, 3, 6-H), 8.20–8.60 (m, 4H, Ph-H).

4.3.3. (6*S*), (6*R*)-3α-Benzenesulfonyloxy-6β-acetoxytropane (4)

Pale yellow oil, (6*S*)-**4**, (168 mg, 35%), $[\alpha]_D^{20}$ −6.1°(c, 1.60, $CHCl_3$); (6*R*)-**4**, (155 mg, 27%), $[\alpha]_D^{20}$ +5.9° (c, 1.35, $CHCl_3$). IR (KBr) cm^{-1}: 2948, 2858, 1733, 1586. EI-MS *m*/*z*: 339 (M^+, 8.56), 182, 122, 94, 82, 43. ^1H NMR ($CDCl_3$, 400 MHz) δ: 1.76–2.60 (m, 6H, 2,4,7-H), 2.04 (s, 3H, CH_3CO), 2.54 (s, 3H, CH_3N), 3.17 (s, 1H, 5-H), 3.42 (m, 1H, 1-H), 4.80 (m, 1H, 3-H), 5.45 (dd, *J* = 3.0, 6.4 Hz, 1H, 6-H), 7.34–7.70 (m, 3H, Ph-H), 7.86–7.95 (m, 2H, Ph-H).

4.4. Functional in vitro studies

(6*S*)-**4** and (6*R*)-**4** were tested for stimulating the contraction of isolated iris muscle as agonistic activity, while (6*S*)-**2**, (6*R*)-**2**, (6*S*)-**3**, and (6*R*)-**3** for inhibiting the carbachol-induced contraction of isolated iris muscle as antagonistic activity. Guinea-pigs (250–350 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 longitudinal muscle was rapidly removed and gently cleaned of adhering connective tissue in a prewarmed (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 longitudinal muscle (1.5 cm) prepared were transferred to 10 ml organ baths and loaded with a tension of 500 mg. The preparation was allowed to equilibrate for 30 min, changing the bath fluid every 10 min. Contractions were recorded isotonically with an electromechanical transducer connected to Bridge amplifier and Powerlab system recorder. Cumulative concentration–response curves were obtained for carbachol, (6*S*)-**4**, (6*R*)-**4**. The concentration of agonist 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 responses of iris muscle to each dose of the muscarinic agonists were expressed as percentages of the maximum effect induced by carbachol. For the antagonistic test, after stable concentration-response curves for carbachol was obtained, (6*S*)-**2**, (6*R*)-**2**, (6*S*)-**3**, or (6*R*)-**3** was added and the tissue was stimulated cumulatively with carbachol as before. Three different concentrations of (6*S*)-**2**, (6*R*)-**2**, (6*S*)-**3**, or (6*R*)-**3** were investigated.

4.5. Radioreceptor binding assays

The binding of (6*S*)-**2**, (6*R*)-**2**, (6*S*)-**3**, (6*R*)-**3**, (6*S*)-**4**, or (6*R*)-**4** to mAChR was determined using the submandibulary glands of the rat. A male SD rat (220–240 g) was killed by cervical dislocation. The submandibulary glands were removed, cleaned adhering tissue in ice-cold 50 mM Tris buffer (pH 7.4). Homogenisation of the submandibulary glands 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 20,000*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 assay, membranes (0.1 mg protein) were incubated vibrantly at 37 °C for 30 min in 0.078–1.55 nM [^3H]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 stan-

dard. For competition binding assays, iris muscle membranes (0.1 mg protein) were incubated with 0.4 nM [^{3}H]NMS at 37 °C for 60 min with increasing concentrations of (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.[18]

4.6. Functional in vivo studies

0.10–0.15 ml 1 M chloric acid was added respectively to the solutions of 0.02 M of (6*S*)-**2**, (6*R*)-**2**, (6*S*)-**3**, (6*R*)-**3**, (6*S*)-**4**, or (6*R*)-**4** to pH 6, which were used as test drugs. Twenty health rabbits (2.5–3.0 kg) of either sex provided by animal experimental center of Shanghai Jiao Tong University were randomly divided into four groups, five in each group. The left pupil diameters of the rabbits were measured under constant light, and readings were taken before and at 10 min after 0.05 ml of agent dropping, while the right for the control as reference. Mydriatic or myotic effects were expressed as the percentage of diameter changing value after application of agent to the diameter before application of agent.

4.7. Statistics and data analysis

For the iris contraction assay, EC_{50} values (concentration of agonists causing a half-maximal response) and the slopes of the log concentration–response curves for carbachol, (6*S*)-**4**, were calculated by means of nonlinear curve fitting of sigmoidal dose–response logistic transformation using program GraphPad PRISM 4.0 (San Diego, CA, USA). pA_2 values for (6*S*)-**2** and (6*S*)-**3** were determined according to Arunlakshana and Schild.[19] 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 analysed using the commercial software PRISM to obtain IC_{50} values for competing ligands. Affinities, expressed as K_i were calculated from IC_{50} values according to Cheng and Prusoff.[20] Data were expressed as means ± 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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