TRUXILLIC ACID DERIVATIVES, NEUROMUSCULAR BLOCKING AGENTS WITH VERY HIGH AFFINITY FOR THE ALLOSTERIC BINDING SITE OF MUSCARINIC ACETYLCHOLINE RECEPTORS

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Bis-quaternary salts of 3-piperidinopropyl esters of α-truxillic acid were synthesized in order to study their allosteric action on muscarinic acetylcholine receptor. Using radioligand binding studies, it has been demonstrated that most of prepared compounds bind with high affinity to the allosteric binding site of M_2 muscarinic receptor subtype (K_d values in the range 1–10 nM). Bulky substitution of the quaternary ammonium center led to effective positive modulators of [^{3}H]*N*-methylscopolamine binding to M_2 receptors. Due to its high allosteric potency, the structure of phenacyl derivative seems to be the most promising candidate for future design of photoaffinity probes or radiolabelled ligands for mapping the allosteric binding site.

Key words: Allosteric binding; Cooperativity; Muscarinic receptors; Nicotinic receptors; Truxillic acid; Quaternary ammonium salts; Cyclobutanes.

Muscarinic acetylcholine receptors (mAChR) belong to a superfamily of G-protein coupled receptors[1] (GPCR) whose function is to transduce chemical signals across cell membranes. Binding of the neurotransmitter to the GPCR leads to the association with a G-protein. The G-proteins either stimulate or inhibit the production of a second messenger and thus the initial signal is transduced to some effector system[2]. In the muscarinic receptors, the extracellular chemical signal is mediated by a molecule of acetylcholine. There are five subtypes of muscarinic receptors which have been identified in cloning studies as distinct molecular entities[3]. All subtypes exhibit strong conservation of sequence in the regions that are considered to bind "orthosteric" ligands (*e.g.*, acetylcholine, atropine-like drugs, *R*-(–)-3- qui-

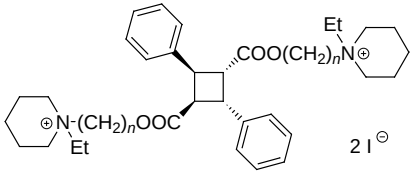
nuclidinyl benzilate). As a consequence, attempts to discover subtype-specific ligands have failed. Sufficiently selective ligand with affinity for one of the subtypes at least 100-fold higher than affinity for the other subtypes is not available.

In addition to the binding site for orthosteric ligands, muscarinic receptors also contain an allosteric binding site[4]. It was formerly predicted on the basis of kinetic studies that this site may be located close to and just extracellular to the "classical" binding site[5]. Some site-directed mutagenesis studies brought an experimental support of that prediction[6]. However, little is known about the molecular nature of the site and there is no unambiguous evidence of the crucial importance of any amino acid residue in the receptor for an interaction with the allosteric ligands. Allosterically acting drugs represent a novel approach for affecting muscarinic receptors[7]. The interactions follow the allosteric ternary complex model. Recently, some allosteric ligands have been considered to increase the affinity of acetylcholine for some of the five subtypes of muscarinic receptors[8,9].

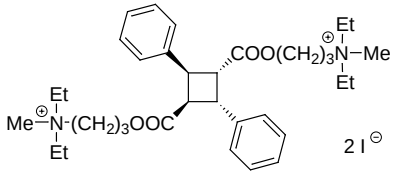
Search for suitable allosteric modulators is highly demanded to gain insight into the mechanism of ligand–receptor interaction, to determine the location of binding sites for different types of ligands, and to correlate between ligand structure and their affinity to particular receptor subtypes. Currently, a radioligand is not commercially available to label an allosteric recognition site and thus, direct competition experiments with unlabelled modulators are not possible. Discovery of convenient ligands for better understanding of the phenomena can thus be regarded as a crucial step towards future development of allosteric drugs[10]. Known allosteric modulators were found mostly among neuromuscular blockers and acetylcholinesterase inhibitors and reactivators. They exhibit wide structural diversity, which raises the question whether structural elements or physico-chemical properties which govern the allosteric potency can be defined[11–13]. Perhaps the only common structural feature is the presence of quaternary or protonated tertiary nitrogen. Gallamine[14], *d*-tubocurarine[15], tacrine[16] and obidoxime[17] are examples of the substances which have been found to decrease the binding of radiolabelled muscarinic antagonists (negative cooperativity). Alcuronium[18], strychnine and eburnamonine[19] belong to the less numerous group of positive allosteric modulators.

The present study focused on bis-quaternary salts of α-truxillic (2α,4β-diphenylcyclobutane-1α,3β-dicarboxylic) acid[20,21]. Among these bis-quaternary structures, anatruxonium (**1**) and cyclobutonium (**3**) were recognized as non-depolarizing myorelaxants. The fact that their therapeutic values were reduced by reported significant side effects on the cardiac

muscle, led us to the presumption that similar derivatives could behave as allosteric modulators of cardiac M_2 receptor subtype. Preliminary experiments with authentic samples of compounds **1**–**3** showed that the 3-piperidinopropyl derivative **1** acts as high-affinity negative allosteric modulator. We aimed therefore to prepare set of yet undescribed bis-quaternary analogues of **1**. The compounds were examined in radioligand binding studies with [^{3}H]*N*-methylscopolamine at cardiac muscarinic M_2 receptors. In a parallel examination, change in original neuromuscular potency in connection with particular structural changes was assessed by inhibition of evoked contraction of nervus phrenicus-diaphragm preparation.



1, *n* = 3 Anatruxonium
2, *n* = 4 Truxillonium



3, Cyclobutonium

EXPERIMENTAL

Melting points were determined on a Kofler block and are uncorrected. Infrared spectra were recorded on a Perkin–Elmer PE 580 spectrometer (wavenumbers in cm^{-1}). ^{1}H NMR spectra were taken on a Varian UNITY-200 (200 MHz, FT mode) at 23 °C. Chemical shifts are given in ppm (δ-scale), coupling constants (*J*) and width of symmetrical multiplets (*W*) in Hz. FAB mass spectra were recorded on a VG Analytical ZAB-EQ spectrometer. Thin-layer chromatography was performed on precoated aluminium sheets Aluminium oxide 60 F_{254} neutral, type E (Merck). For preparative flash column chromatography aluminium oxide according to Brockmann, type II neutral (Reanal, Hungary) was used. 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), gallamine triethiodide and atropine sulfate were purchased from Sigma–Aldrich Co Ltd., (–)-[*N*-methyl-^{3}H]scopolamine methyl chloride ([^{3}H]NMS) was obtained from Amersham, U.K. Bis(3-piperidinopropyl) α-truxillate (**4**) was prepared according to Arendaruk[20].

(2α,4β-Diphenylcyclobutane-1α,3β-diyl)dimethyl Bis(3-piperidinopropionate) Dihydrochloride (**5**)

Method A. DCC (4.3 g, 20.8 mmol) was added to a stirred mixture of 2α,4β-diphenylcyclobutane-1α,3β-dimethanol[22] (2.24 g, 8.3 mmol), 3-piperidinopropionic acid (2.8 g, 17.8 mmol) and 4-methylbenzenesulfonic acid monohydrate (80 mg, 0.4 mmol) in dry pyridine (40 ml). The mixture was stirred at room temperature for 8 h and then allowed to stand for another 16 h. Pyridine was removed *in vacuo*, the residue treated with 5% sulfuric acid (200 ml), filtered, the filtrate treated with concentrated ammonia (25 ml) and extracted with ether. The etheral extract was washed with water and dried over anhydrous sodium sulfate. After

evaporation of the solvent, the crude product was chromatographed on a column of aluminium oxide in chloroform–ethyl acetate–triethylamine (1 : 1 : 0.06) yielding free base of **5** as colorless oil (3.54 g). This was dissolved in dichloromethane (100 ml) and saturated with dry gaseous hydrogen chloride. The solvent was removed and the residue crystallized from methanol–acetone to give 3.5 g (67%) of the dihydrochloride **5**, m.p. 205–206 °C. ^{1}H NMR (D_2O): 7.48 m, 10 H (arom. H, *W* = 48); 4.10 m, 4 H (2 × CH_2O, *W* = 38); 3.78 dd, 2 H (H-2, H-4, $J_{2,1}$ = 11, $J_{2,3}$ = 7); 3.78 m, 2 H (H-1, H-3, *W* = 24); 3.28 br and 2.76 br, 8 H (4 × CH_2N); 3.04 t, 4 H (2 × CH_2O, *J* = 7); 2.54 t, 4 H (2 × CH_2N, *J* = 7); 1.96–1.30 m, 12 H (6 × $CH_2C\mathbf{H}_2CH_2$). IR (KBr): 2 631, 2 612, 2 576, 2 528, 2 479, 2 464, 2 403, 2 279 (NH^+); 1 740, 1 732 (C=O); 1 256, 1 221 (C–O); 3 056, 3 032 (C–H, arom.); 1 601, 1 497, 1 453 (arom. ring). MS, *m/z* (%): 583 (6, M – Cl); 547 (100, M – Cl – HCl); 462 (6); 408 (7); 274 (16, $(M - 2\ Cl)^{2+}$). For $C_{34}H_{48}Cl_2N_2O_4$ (619.7) calculated: 65.90% C, 7.81% H, 11.44% Cl, 4.52% N; found: 65.73% C, 7.69% H, 11.27% Cl, 4.39% N.

Method B. To finely powdered 3-piperidinopropionic acid hydrochloride (4.33 g, 22.4 mmol), one drop of DMF and thionyl chloride (16 ml, 223 mmol) were added. After stirring for 30 min at 50 °C, the mixture was coevaporated three times with dry toluene and the residue suspended in dry chloroform (50 ml). 2α,4β-Diphenylcyclobutane-1α,3β-dimethanol (3 g, 11.2 mmol) in chloroform (50 ml) and *N*,*N*-diisopropylethylamine (4 ml, 22.9 mmol) were added at 0 °C. After stirring for 1 h at room temperature the solvent was evaporated and the residue treated with 5% sulfuric acid. The acidic extract was washed with ether, decolorized by addition of charcoal, filtered and basified (to pH ≈ 9) by addition of concentrated ammonia under cooling. Resulting oily base was taken into etheral solution and after drying and removal of the solvent converted to hydrochloride **5** (6.1 g, 88% yield) by the same procedure as described above.

Bis(3-piperidinopropyl) 2α,4β-Diphenylcyclobutane-1α,3β-dicarboxylate Dihydrobromide (**6**)

Methanolic solution of hydrogen bromide was added to a solution of amine **4** (1.0 g, 1.8 mmol) in methanol (10 ml). Evaporation to dryness and recrystallization from methanol yielded 1.12 g (86%) of dihydrobromide **6**, m.p. 218–220 °C. ^{1}H NMR (D_2O): 7.45 m, 10 H (arom. H, *W* = 38); 4.54 dd, 2 H (H-2, H-4, $J_{2,1}$ = 11, $J_{2,3}$ = 7); 4.13 dd, 2 H (H-1, H-3, $J_{1,2}$ = 11, $J_{1,4}$ = 7); 3.87 m, 4 H (2 × CH_2O, *W* = 40); 3.36 br and 2.79 br, 8 H (4 × CH_2N); 2.65 t, 4 H (2 × CH_2N, *J* ≈ 8); 2.04–1.45 m, 16 H (8 × $CH_2C\mathbf{H}_2CH_2$). IR (KBr): 2 623, 2 531, 1 425 (NH^+); 1 727 (C=O); 1 177, 1 056 (C–O); 3 061, 3 032 (C–H, arom.); 1 604, 1 586, 1 497, 1 455, 1 001 (arom. ring); 2 944, 1 475, 1 464 (CH_2). MS, *m/z* (%): 627 (11, M – Br); 547 (100, M – Br – HBr); 436 (23); 422 (5); 377 (6); 274 (53, $(M - 2\ Br)^{2+}$). For $C_{34}H_{48}Br_2N_2O_4$ (708.6) calculated: 57.63% C, 6.83% H, 22.55% Br, 3.95% N; found: 57.21% C, 6.77% H, 22.28% Br, 3.87% N.

N,*N*′-[(2α,4β-Diphenylcyclobutane-1α,3β-diyl) Bis(carbonyloxypropane-3,1-diyl)]-bis(*N*-ethylpiperidinium) Dibromide (**7**)

A mixture of ethyl bromide (9 ml) and amine **4** (1.0 g, 1.8 mmol) was refluxed for 8 h. The solid residue obtained by evaporation of the excess of the reagent was crystallized from a mixture of ethanol and ethyl acetate to give the salt **7** (1.09 g, 78%), m.p. 200–204 °C, dec. ^{1}H NMR (D_2O): 7.47 s, 10 H (arom. H, *W* = 31); 4.55 dd, 2 H (H-2, H-4, $J_{2,1}$ = 11, $J_{2,3}$ = 7);

4.12 dd, 2 H (H-1, H-3, $J_{1,2}$ = 11, $J_{1,4}$ = 7); 3.85 m, 4 H (2 × CH_2O, *W* = 46); 3.27 q, 8 H (2 × CH_2CH_3, *J* = 7); 3.20 m, 8 H (4 × CH_2N, *W* = 48); 2.83 m, 4 H (2 × CH_2N, *W* = 60); 1.93–1.58 m, 16 H (8 × $CH_2CH_2CH_2$); 1.19 t, 6 H (2 × CH_2CH_3, *J* = 7). IR (KBr): 1 714 (C=O); 1 198, 1 056 (C–O); 3 057, 3 025, 3 003 (C-H, arom.); 1 603, 1 584, 1 497, 1 453, 1 008 (arom. ring); 2 945, 2 864, 1 484, 1 474 (CH_2); 2 970, 2 889 (CH_3). MS, *m/z* (%): 685 (66, M – Br); 639 (25); 603 (9, M – Br – HBr); 575 (100, M – Br – C_2H_5Br); 490 (27); 464 (46); 450 (19); 302, $(M - 2\,Br)^{2+}$). For $C_{38}H_{56}Br_2N_2O_4$ (764.7) calculated: 59.69% C, 7.38% H, 20.90% Br, 3.66% N; found: 59.78% C, 7.21% H, 20.73% Br, 3.49% N.

General Procedure for Preparation of Quaternary Salts **8**–**13**

Amine **4** (1.0 g, 1.8 mmol) in dry methanol (15 ml) was refluxed with corresponding alkyl bromide (4.0 mmol) under an atmosphere of argon for 5 h. Removal of the volatiles and crystallization from appropriate solvents yielded the quaternary ammonium salts as white solids.

N,N′-[(2α,4β-Diphenylcyclobutane-1α,3β-diyl) bis(carbonyloxypropane-3,1-diyl)]-bis(*N*-allylpiperidinium) dibromide (**8**). Yield 1.26 g (87%), m.p. 147–155 °C, dec. (ethanol–ethyl acetate). ^{1}H NMR (D_2O): 7.45 s, 10 H (arom. H, *W* = 32); 5.93–5.60 m, 6 H (2 × CH_2=CH); 4.53 dd, 2 H (H-2, H-4, $J_{2,1}$ = 11, $J_{2,3}$ = 7); 4.10 dd, 2 H (H-1, H-3, $J_{1,2}$ = 11, $J_{1,4}$ = 7); 3.92–3.47 m, 4 H (2 × CH_2O); 3.82 d, 2 H (2 × CH_2=CH–C**H**$_2$); 3.30–3.10 m, 8 H (4 × CH_2N); 2.79 m, 4 H (2 × CH_2N, *W* = 60); 1.92–1.58 m, 16 H (8 × $CH_2CH_2CH_2$). IR (KBr): 1 724 (C=O); 1 191, 1 057 (C–O); 3 057, 3 028, 2 999 (C–H, arom.); 1 603, 1 584, 1 497, 1 455, 1 006 (arom. ring); 1 439, 998, 908, (CH_2=CH); 2 945, 2 868, 1 479, 1 473 (CH_2). MS, *m/z* (%): 707 (21, M – Br); 627 (10, M – Br – HBr); 587 (100, M – Br – C_3H_5Br); 502 (15); 462 (17); 314 (95, $(M - 2\,Br)^{2+}$). For $C_{40}H_{56}Br_2N_2O_4$ (788.7) calculated: 60.92% C, 7.16% H, 20.26% Br, 3.55% N; found: 60.04% C, 7.06% H, 19.87% Br, 3.41% N.

N,N′-[(2α,4β-Diphenylcyclobutane-1α,3β-diyl) bis(carbonyloxypropane-3,1-diyl)]-bis(*N*-benzylpiperidinium) dibromide (**9**). Yield 1.30 g (80%), m.p. 146–149 °C (water–acetone). ^{1}H NMR (DMSO-d_6): 7.56 m, 10 H (2 × benzyl, *W* = 21); 7.21 m, 10 H (2 × phenyl, *W* = 18); 4.35 dd, 2 H (H-2, H-4, $J_{2,1}$ = 11, $J_{2,3}$ = 7); 3.92 dd, 2 H (H-1, H-3, $J_{1,2}$ = 11, $J_{1,4}$ = 7); 3.58–2.62 m, 16 H (2 × CH_2O, 6 × CH_2N); 1.98–1.41 m, 16 H (8 × $CH_2CH_2CH_2$). IR (KBr): 1 726 (C=O); 1 187, 1 180, 1 057 (C–O); 3 057, 3 030 (C–H, arom.); 1 603, 1 583, 1 497, 1 453, 1 002 (arom. ring); 2 945, 1 473 (CH_2). MS, *m/z* (%): 807 (11, M – Br); 764 (7); 727 (7, M – Br – HBr); 637 (100, M – Br – C_7H_7Br); 545 (6); 512 (7); 364, (30, $(M - 2\,Br)^{2+}$). For $C_{48}H_{60}Br_2N_2O_4$ (888.8) calculated: 64.86% C, 6.80% H, 17.98% Br, 3.15% N; found: 64.69% C, 6.67% H, 17.66% Br, 2.98% N.

N,N′-[(2α,4β-Diphenylcyclobutane-1α,3β-diyl) bis(carbonyloxypropane-3,1-diyl)]-bis(*N*-phenacylpiperidinium) dibromide (**10**). Yield 1.55 g (89%), m.p. 217–222 °C, dec. (ethanol–ethyl acetate). ^{1}H NMR (DMSO-d_6): 8.08–7.58 m, 10 H (2 × phenacyl); 7.28 m, 10 H (2 × phenyl, *W* = 40); 5.29 s, 4 H (2 × $COCH_2$); 4.33 dd, 2 H (H-2, H-4, $J_{2,1}$ = 10.5, $J_{2,3}$ = 7.0); 3.87 dd, 2 H (H-1, H-3, $J_{1,2}$ = 10.5, $J_{1,4}$ = 7.0); 3.80–3.40 m, 16 H (2 × CH_2O, 6 × CH_2N); 1.96–1.55 m, 16 H (8 × $CH_2CH_2CH_2$). IR (KBr): 1 727 (C=O, ester); 1 698 (C=O); 1 189, 1 231, 1 226, 1 054 (C–O); 3 085, 3 061, 3 030 (C–H, arom.); 1 597, 1 582, 1 496, 1 450, 1 003 (arom. ring); 2 937, 2 871, 1 477, 1 466 (CH_2). MS, *m/z* (%): 863 (27, M – Br); 845 (5); 783 (42, M – Br – HBr); 665 (100, M – Br – C_8H_7BrO); 581 (18); 554 (37); 540 (7); 695 (6); 392 (48, $(M - 2\,Br)^{2+}$). For $C_{50}H_60Br_2N_2O_6$ (944.8) calculated: 63.56% C, 6.40% H, 16.91% Br, 2.96% N; found: 63.69% C, 6.34% H, 16.98% Br, 2.94% N.

N,*N*′-[(2α,4β-Diphenylcyclobutane-1α,3β-diyl) bis(carbonyloxypropane-3,1-diyl)]-bis{*N*-[2-(2-naphthyl)-2-oxoethyl]piperidinium} dibromide (**11**). Yield 1.75 g (92%), m.p. 211–215 °C, dec. (methanol–acetone). ^{1}H NMR (DMSO-d_6): 8.85 s, 2 H (2 × H_1-naphthyl); 8.09 m, 8 H (naphthyl, *W* = 53); 7.73 m, 4 H (naphthyl, *W* = 28); 7.22 m, 10 H (2 × phenyl, *W* = 28); 5.42 s, 4 H (2 × $COCH_2$); 4.32 dd, 2 H (H-2, H-4, $J_{2,1}$ = 10.5, $J_{2,3}$ = 7.0); 3.84 dd, 2 H (H-1, H-3, $J_{1,2}$ = 10.5, $J_{1,4}$ = 7.0); 3.80–3.42 m, 16 H (2 × CH_2O, 6 × CH_2N); 2.06–1.54 m, 16 H (8 × $CH_2C\mathbf{H}_2CH_2$). IR (KBr): 1 719 (C=O, ester); 1 686 (C=O); 1 191, 1 185, 1 049 (C–O); 3 052, 3 031, 3 011 (C–H, arom.); 1 623, 1 594, 1 570, 1 508, 1 493, 1 452 (arom. ring). MS, *m/z* (%): 963 (30, M – Br); 883 (25, M – Br – HBr); 715 (100, M – Br – $C_{12}H_9BrO$); 630 (16); 590 (11); 545 (17); 442 (60, $(M - 2\,Br)^{2+}$). For $C_{58}H_{64}Br_2N_2O_6$ (1 045.0) calculated: 66.67% C, 6.17% H, 15.29% Br, 2.68% N; found: 66.10% C, 6.05% H, 15.52% Br, 2.63% N.

N,*N*′-[(2α,4β-Diphenylcyclobutane-1α,3β-diyl) bis(carbonyloxypropane-3,1-diyl)]-bis[*N*-(4-benzyloxybenzyl)piperidinium] dibromide (**12**). Yield 1.77 g (88%), m.p. 208–209 °C, dec. (ethanol–ethyl acetate). ^{1}H NMR (DMSO-d_6): 7.52–7.11 m, 28 H (aromatic H); 5.20 s, 4 H (2 × benzylic CH_2); 4.44 m, 4 H (2 × benzylic CH_2); 4.38 dd, 2 H (H-2, H-4, $J_{2,1}$ = 11, $J_{2,3}$ = 7); 3.96 dd, 2 H (H-1, H-3, $J_{1,2}$ = 11, $J_{1,4}$ = 7); 3.94–3.44 m, 4 H (2 × CH_2O); 3.20 m, 8 H (*W* = 40, CH_2N); 2.69 m, 4 H (*W* = 36, CH_2N); 1.96–1.40 m, 16 H (8 × $CH_2C\mathbf{H}_2CH_2$). IR (KBr): 1 731, 1 717, 1 701 (C=O); 1 254, 1 224, 1 192, 1 183 (C–O); 3 057, 3 030 (C–H, arom.); 1 610, 1 582, 1 514, 1 497, 1 453 (arom. ring). MS, *m/z* (%): 1 019 (10, M – Br); 975 (23); 939 (4, M – Br – HBr); 743 (100, M – Br – $C_{12}H_{13}BrO$); 659 (7); 617 (5); 547 (28); 470 (47, $(M - 2\,Br)^{2+}$). For $C_{62}H_{72}Br_2N_2O_6$ (1 101.1) calculated: 67.63% C, 6.59% H, 14.51% Br, 2.54% N; found: 67.38% C, 6.52% H, 14.39% Br, 2.52% N.

N,*N*′-[(2α,4β-Diphenylcyclobutane-1α,3β-diyl) bis(carbonyloxypropane-3,1-diyl)]-bis{*N*-[2-(4-azidophenyl)-2-oxoethyl]piperidinium} dibromide (**13**). Yield 1.28 g (68%), decomposes above 160 °C (ethanol). ^{1}H NMR (DMSO-d_6): 8.18 d, 4 H (2 × phenacyl, *J* = 8.5); 7.43 d, 4 H (2 × phenacyl, *J* = 8.8); 7.38 m, 10 H (2 × phenyl, *W* = 15); 5.34 s, 4 H (2 × $COCH_2$); 4.42 dd, 2 H (H-2, H-4, $J_{2,1}$ = 11, $J_{2,3}$ = 7); 3.95 dd, 2 H (H-1, H-3, $J_{1,2}$ = 11, $J_{1,4}$ = 7); 3.89–3.46 m, 16 H (2 × CH_2O, 6 × CH_2N); 2.11–1.62 m, 16 H (8 × $CH_2C\mathbf{H}_2CH_2$). IR (KBr): 2 132 (N_3); 1 728 (C=O, ester); 1 685 (C=O); 1 283, 1 239, 1 180 (C–O); 3 029 (C–H, arom.); 1 597, 1 574, 1 506, 1 496, 1 455, 1 419 (arom. ring). MS, *m/z* (%): 945 (47, M – Br); 917 (9, M – Br – N_2); 901 (20); 865 (62, M – Br – HBr); 839 (13); 809 (6); 706 (100, M – Br – $C_8H_6BrN_3O$); 680 (35); 622 (18); 595 (44); 545 (96); 433 (58, $(M - 2\,Br)^{2+}$); 403 (40, $(M - 2\,Br - 2\,N_2)^{2+}$). For $C_{50}H_{58}Br_2N_8O_6$ (1 026.9) calculated: 58.48% C, 5.69% H, 15.56% Br, 10.91% N; found: 58.03% C, 5.84% H, 15.8% Br, 10.42% N.

N,*N*′-[(2α,4β-Diphenylcyclobutane-1α,3β-diyl) Bis(carbonyloxypropane-3,1-diyl)]-bis[*N*-(2-cyclohehylethyl)piperidinium] Dibromide (**14**)

Amine **4** (300 mg, 0.55 mmol) was stirred with 2-cyclohexylethyl bromide (1.0 ml) for 2 h at 50 °C, then for additional 3 h at 100 °C. Cooled reaction mixture was diluted with toluene, resulting precipitate filtered off, washed with several portions of toluene and crystallized from acetone–methanol to give 338 mg (66%) of **14**, m.p. 232–233 °C. ^{1}H NMR (DMSO-d_6): 7.39 m, 10 H (2 × phenyl, *W* = 40); 4.42 dd, 2 H (H-2, H-4, $J_{2,1}$ = 10.5, $J_{2,3}$ = 7.0); 3.97 dd, 2 H (H-1, H-3, $J_{1,2}$ = 10.5, $J_{1,4}$ = 7.0); 3.67 m, 4 H (2 × CH_2O, *W* = 84); 3.30–2.92 m, 16 H (8 × CH_2N); 1.84–0.81 m, 42 H (20 × CH_2, 2 × CH). IR (KBr): 1 725 (C=O); 1 258, 1 231, 1 186, 1 180 (C–O); 1 603, 1 495, 1 448 (arom. ring). MS, *m/z* (%): 847 (53, M – Br); 801 (5); 767 (8, M – Br – HBr); 657 (51, M – Br – $C_8H_{13}Br$); 572 (24); 532 (19);

487 (10); 384 (100, $(M - 2\,Br)^{2+}$). For $C_{50}H_{76}Br_2N_2O_4$ (929.0) calculated: 64.65% C, 8.25% H, 17.20% Br, 3.02% N; found: 64.42% C, 8.08% H, 17.10% Br, 3.25% N.

Reaction of **4** with Phenethyl Iodide

Solution of amine **4** (1.0 g, 1.8 mmol) and (2-iodoethyl)benzene (930 mg, 4.0 mmol) in chloroform (5 ml) in a sealed tube was heated to 40 °C for 5 days. The solvent was removed and the residue crystallized from a mixture of methanol and acetone (1 : 2) to yield 992 mg of crystalline material which was identified as dihydroiodide **15**, m.p. 248–250 °C. ^{1}H NMR (DMSO-d_6): 8.92 br, 2 H (2 × NH); 7.49 m, 10 H (arom. H, W = 38); 4.51 dd, 2 H (H-2, H-4, $J_{2,1}$ = 11, $J_{2,3}$ = 7); 4.06 dd, 2 H (H-1, H-3, $J_{1,2}$ = 11, $J_{1,4}$ = 7); 3.87 m, 4 H (2 × CH_2O, W = 74); 3.34 br and 2.84 br, 12 H (6 × CH_2N); 2.01–1.43 m, 16 H (8 × $CH_2C\mathbf{H}_2CH_2$). IR (KBr): 2 710, 2 683, 2 675, 2 637, 2 578, 2 538 (NH^+); 1 729, 1 681 (C=O); 1 263, 1 186, 1 176 (C–O); 3 027 (C–H, arom.); 1 602, 1 496, 1 452 (arom. ring). MS, *m/z* (%): 675 (12, M – I); 547 (100, M – I – HI); 422 (3); 378 (8); 274 (30, $(M - 2\,I)^{2+}$). For $C_{34}H_{48}I_2N_2O_4$ (802.6) calculated: 50.88% C, 6.03% H, 31.62% I, 3.49% N; found: 51.11% C, 6.27% H, 30.37% I, 3.41% N.

Radioligand Binding Assays

Tissue preparation. Experiments were performed on homogenates of heart atria taken from male Wistar rats (200–230 g body weight). Tissue was homogenized with Polytron homogenizer, in 10-fold volume of ice-cold medium. The homogenization and incubation medium consisted of 100 mM NaCl and 20 mM HEPES, pH 7.4. The homogenates were diluted with the homogenization medium to 2-fold volume and centrifuged at –4 °C for 15 min at 700 g, the pellets were discarded and supernatants were divided into the Eppendorf tubes and kept frozen at –80 °C until the day of experiment.

Incubations. Incubations were performed at 25 °C in a total volume of 1.6 ml and lasted 3 h unless indicated otherwise. The concentration of [^{3}H]NMS was 200 pM, close to half of the apparent dissociation constant K_{RL} for complex [^{3}H]NMS–receptor (K_{RL} = 436 pM, under the condition used). Tissue concentration was 2 mg (original wet weight) per tube. Non-specific radioligand binding was defined using atropine (5 μM). Incubations were terminated by an addition of 3 ml of ice-cold water and subsequent vacuum filtration on Whatman GF/B glass fiber filters (presoaked in water for 3 h), with two washes of filters with 3 ml of water. Radioactivity was determined by scintillation counting.

Data analysis. Data treatment was performed as described in refs[5,23]. Data were analyzed with nonlinear regression analysis using SigmaPlot 4.0 (SPSS, Erkrath, Germany). Unless stated otherwise, data are presented as mean ± standard error of the mean (S.E.M.).

Symbols R, L and A denote the receptor, the orthosteric ligand [^{3}H]NMS and the allosteric ligand, respectively. K_{RL}, K_{AR} denote the apparent dissociation constants of the correspondent complexes. The cooperativity coefficient α expresses the mutual potency of the allosteric and orthosteric ligands to induce by receptor occupation a shift of each others binding affinity[24]. Values of $\alpha < 1$ suggest positive cooperativity, values of $\alpha > 1$ suggest negative cooperativity.

Nervus Phrenicus-Diaphragm Preparation

Wistar female rats weighing 200–300 g were used for experiments. Left phrenic nerve-hemidiaphragm preparation was incubated in thermostated organ bath (37 °C, volume

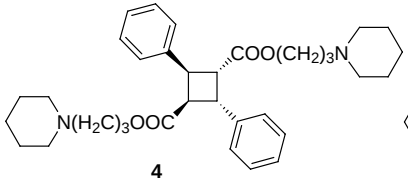
10 ml), in Tyrode's solution of the following composition (in mM) NaCl 137.0; KCl 2.7; $CaCl_2$ 1.8; $MgCl_2$ 1.0; NaH_2PO_4 0.4; $NaHCO_3$ 11.9; glucose 11.2; choline 0.001; gassed intensively with O_2–CO_2 and attached to a tensometer under a tension of 2 g in an electrode holder which allowed indirect stimulation of the striated muscle by a nerve electrode (Grass stimulator, pulse duration 0.2 ms, 8 V, 0.2 Hz or 50 Hz). The phrenic nerve was stimulated continuously at 0.2 Hz. After equilibration and consolidation of baseline (during approximately 10–30 min, with the exchange of medium every 10 min) the experimental cycles started. The medium was exchanged and the preparation stimulated continuously for a given period (for 15 or 30 min in these experiments). At the end of this cycle, a train of tetanic stimulation (50 Hz for 3 s) was applied. The medium was then exchanged and the cycle repeated. Antagonists were applied in cumulatively increased concentration. The response of the tensometer was electronically recorded by a computerised A/D converter. In some experiments, recovery of twitches after washout of the drug was recorded. The tetanic fading was used to measure the effect of an antagonist. Spontaneous decrease of the tetanic contractions during time was recorded in a separate experiment and the responses from antagonist-affected experiments were corrected by data calculated from the linear regression. EC_{50} value for a given substance was calculated from a dose-response plot of tetanus fading *versus* log concentration of the drug fitted by non-linear regression to a sigmoidal dose-response equation using a program Prism (GraphPad Software, Inc.).

RESULTS AND DISCUSSION

We found out that anatruxonium (**1**) slows down the dissociation rate of tritiated muscarinic antagonist, [^{3}H]NMS, from M_2 receptor subtype. Its affinity is superior to many of yet known allosteric modulators. Since three-membered aliphatic spacer of the molecule seems to be optimum for its interaction with allosteric site, we decided initially to check whether protonated tertiary amino group would retain some of the activity of the bis-quaternary substance. Diamine **4** was prepared by condensation of α-truxillic acid dichloride with 3-piperidinopropanol according to Arendaruk[20]. Dihydrobromide **6** was used for the bioassay. Preparation of isomeric diester **5** by coupling of 2α,4β-diphenylcyclobutane-1α,3β-dimethanol[22] with 3-piperidinopropionic acid is rather less trivial. Activation of the acid is complicated due to virtual insolubility of its protonated form in most organic solvents and due to reverse Michael reaction which takes place under basic conditions. We exploited two methods: DCC-mediated coupling in pyridine and activation in neat reaction with excess of thionyl chloride.

Bis-quaternary ammonium bromides **7**–**14** were prepared by standard alkylation reaction of the diamine **4** with corresponding alkyl bromides. Reactive bromides were used in a slight stoichiometric excess and the reactions were completed within 5 h reflux of methanolic solution of both components. Ethyl- and cyclohexylethyl bromides were used in a large ex-

cess as reaction media. As shown below, introduction of benzyl- and phenacyl groups led to substances with most convenient binding properties. An attempt to prepare a phenethyl analogue thus followed. 2-Haloethylbenzenes are reported to undergo substitution reaction with 1,4-diazabicyclo[2.2.2]octane at 54 °C without observable formation of styrene[25]. However, in all analogous experiments with our substrate **4**, elimination reaction prevailed over substitution, yielding only ammonium halides **6** and **15**. The synthesized compounds were characterized by their ^{1}H NMR, IR and FAB mass spectra.



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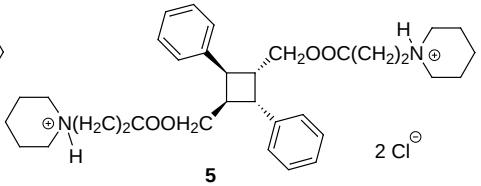

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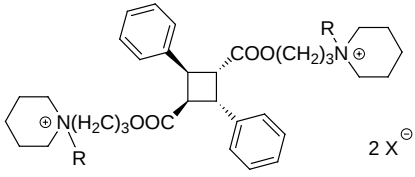
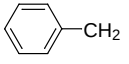
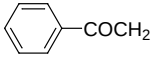
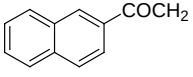
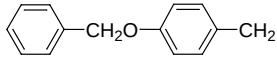
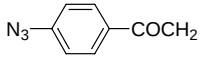
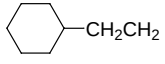


	R	X
6	H	Br
7	CH_3CH_2	Br
8	CH_2=$CHCH_2$	Br
9		Br
10		Br
11		Br

	R	X
12		Br
13		Br
14		Br
15	H	I

All anatruxonium analogues examined were found to be purely allosteric modulators of [^{3}H]NMS binding. It has been demonstrated that simple modification, such as quaternization, of parent compound **6** led to a number of compounds with different effects on [^{3}H]NMS binding (Table I). We

found out that benzyl, phenacyl and (2-naphthyl)acetyl groups led to the positive modulatory effects, although ethyl and allyl groups afforded modulators with the negative effect. The effects of negative and positive modulators are demonstrated in Figs 1 and 2. Ammonium salts **5** and **6** exhibited negative modulatory action as well. Interestingly, in comparison with compound **6** the isomeric ester functionality of **5** caused a substantial decrease in affinity for receptor, while the nature of interaction did not change.

A 2-cyclohexylethyl substitution was used in order to test the working hypothesis that the presence of a benzene ring in the substituent is necessary for positive cooperativity with NMS. Somewhat surprisingly, the absence of aromatic ring did not exclude the ability of compound **14** to increase an affinity of [^{3}H]NMS for receptor. Moreover, the affinity of **14** for unliganded receptor was found to be the highest among the positive modulators. The presence of benzene ring should not therefore be regarded as a crucial structural feature of positive allosteric modulators of [^{3}H]NMS binding to M_2 receptors. It appears that the volume of chosen *N*-substituents

TABLE I
Quantitative parameters of the binding and action of allosteric modulators

Compound	pK_{AR} ± S.E.M., M (number of experiments)	α
Gallamine	6.830 ± 0.031 (4)	21.40
1	8.234 ± 0.193 (9)	21.25
3	7.602 ± 0.180 (5)	11.08
5	6.504 ± 0.152 (3)	23.30
6	7.644 ± 0.168 (4)	18.57
8[a]	7.725 ± 0.435 (3)	18.91
9	7.253 ± 0.085 (3)	0.322
10	6.873 ± 0.141 (4)	0.158
11	6.991 ± 0.104 (3)	0.171
13[b]	7.186 ± 0.199 (4)	0.211
14	7.609 ± 0.162 (3)	0.515

[a] Compound strongly hygroscopic. [b] Data obtained under conditions of low ionic strenght (20 mM HEPES + 10 mM NaCl), apparent dissociation constant for [^{3}H]NMS was K_{RL} = 145 pM.

may play important role in tuning the nature of the cooperative interaction at muscarinic receptors.

We examined some of these compounds in a functional assay on nicotinic acetylcholine receptors. Inhibition of evoked contraction of nervus phrenicus-diaphragm preparation was used in the arrangement described by Wessler[26]. Data in Table II imply, in contrast to situation on muscarinic receptors, the simple inverse relation between the volume of *N*-substituents and potency of compounds (expressed in terms of EC_{50}). An explanation offers that direct steric hindrance to interaction of quaternary nitrogens with receptor results in a decrease of the affinity of bulky-substituted compounds for nicotinic receptors.

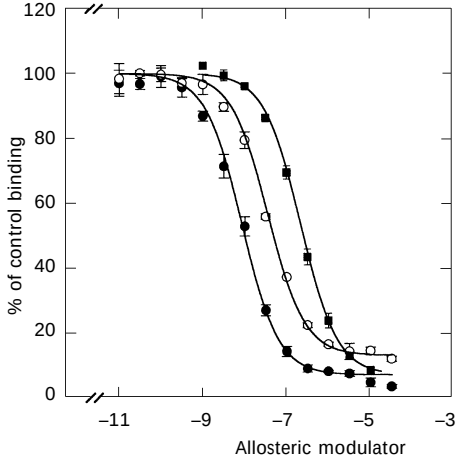


FIG. 1

Allosteric inhibition of the specific binding of [^{3}H]*N*-methylscopolamine to rat atrial membranes induced by 1 (●), 3 (○) and the prototypic allosteric inhibitor gallamine (■). Abscissa: $\log_{10}$ of the molar concentration of the drug. Ordinate: [^{3}H]*N*-methylscopolamine binding in the presence of the drug, expressed as per cent of the binding in the absence of the drug. Full lines represent the best fits of data to Eq. 6 in the study of Ehlert[24]. Points are means ± S.E.M. of nine (**1**), four (**3**) and four (gallamine) experiments with incubations performed in duplicate

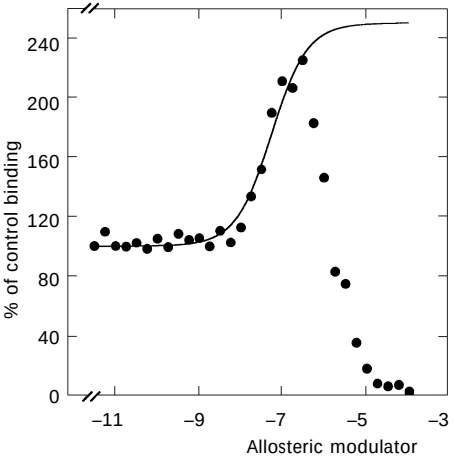


FIG. 2

Changes in the specific binding of [^{3}H]*N*-methylscopolamine to rat atrial membranes induced by **10** (●). Abscissa: $\log_{10}$ of the molar concentration of the drug. Ordinate: [^{3}H]*N*-methylscopolamine binding in the presence of the drug, expressed as per cent of the binding in the absence of the drug. Points are means of four experiments with incubation performed in duplicate. Full line represents the best fit of data to Eq. 6 in the study of Ehlert[24] within the concentration range of $10^{-11.5}$ to $10^{-6.5}$ M, where an equilibrium was reached[5]

A relatively direct approach to elucidate the allosteric binding site at the molecular level might be to synthesize a reactive ligand that covalently binds to this site. Thus, amino acid residues would be identified to which this ligand binds[27]. It is well known that aryl azides after irradiation generate highly reactive intermediates. Hence, with respect to commercial availability of 2-(4-azidophenyl)-2-oxoethyl bromide, we decided primarily to prepare the reactive analogue of positive allosteric modulator **10**. The new compound retained the feature of positive allosteric modulator of [^{3}H]NMS binding to M_2 receptors, although the low-ionic-strength incubation medium had to be used to overcome the decrease in solubility. The pK_{AR} (7.186) for this bifunctional photoreactive probe **13** was obtained from experiments without irradiation. Consequently, a direct comparison of the binding parameters of reactive probe **13** with those of template compound **10** is not available. Nevertheless, **13** appears to be a promising prototype of photoaffinity probe. Considering mutual positive cooperativity ($\alpha = 0.211$), the affinity of **13** for the receptor occupied by [^{3}H]NMS increases approximately five times relative to the unliganded receptor.

In conclusion, the results of this research demonstrate that relatively small structural modification of parent structure can significantly affect the mechanism of action of an allosteric modulator. We assume that the achieved results, though indirectly, support the concept that both positive and negative muscarinic allosteric modulators share identical or overlapping binding sites.

TABLE II
Inhibitory effects of muscarinic allosteric modulators on evoked concentration of nervus phrenicus-diaphragm

Compound	pEC50 ± S.E.M.	Relative potency
d-Tubocurarine	6.876 ± 0.036	1.000
1	6.659 ± 0.024	0.607
8	6.088 ± 0.015	0.163
9	5.983 ± 0.008	0.128
10	5.467 ± 0.019	0.039
11	4.149 ± 0.055	0.002

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