Muscarinic Allosteric Enhancers of Ligand Binding: Pivotal Pharmacophoric Elements in Hexamethonio-Type Agents

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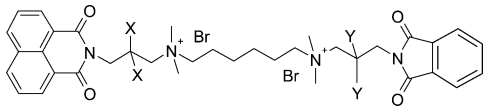
Bisphthalimidopropyl-substituted hexamethonio compounds have been established as allosteric modulators of ligand binding to muscarinic acetylcholine receptors. Enhancers of ligand binding are of special interest. This study aimed to unravel the structural elements inducing positive cooperativity with the binding of an antagonist. [^{3}H]-*N*-methylscopolamine binding to muscarinic M_2 receptors was measured in porcine heart homogenates. Dimethylation, but not monomethylation, of the lateral propyl chain in combination with an affinity increasing aromatic imide moiety, such as a 5-methylphthalimide and naphthalimide, on the same side of the molecule shifts the cooperativity toward positive values, resulting in enhancers of antagonist binding. Thus, lateral side chain dimethylation is a pivotal pharmacophoric element for positive cooperativity in hexamethonio-type muscarinic allosteric agents.

Introduction

The concept of allosteric modulators stabilizing the binding of orthosteric ligands, i.e., conventional agonists and antagonists, is well-established for muscarinic receptors.[1] Especially in the series of hexamethonio derivatives, quantitative structure−activity relationships were derived for the allosteric inhibition of dissociation of the antagonist *N*-methylscopolamine (NMS) from muscarinic receptors.[2] Initially, attention was drawn to this class of compounds because of their supraadditive protective action against organophosphorus poisoning in combination with the conventional muscarinic antagonist atropine.[3] Subsequently, an allosteric interaction with muscarinic receptors was demonstrated. The M_2 receptor subtype has a high sensitivity to allosteric modulation. The pharmacophore of these modulators consists of a hexamethonio middle chain linked on each side through lateral propyl chains to aromatic moieties that contain an imide function.[2,4] Such compounds may inhibit the dissociation of NMS in the nanomolar range of concentration, but they do not necessarily enhance the equilibrium binding of the orthoster NMS. An increase of NMS binding by an allosteric modulator is designated as positive cooperativity; neutral and negative cooperativity mean that binding is unchanged or reduced, respectively. Recently, the first compounds of the hexamethonio-type revealing positive cooperativity with NMS were described that are characterized by a double methylation of the lateral propyl chains.[5] The findings with the compounds **1a**−**d** displayed in Table 1 clearly demonstrate that dimethylation on the side of an affinity-enhancing imide moiety, i.e., naphthalimide instead of the monoaromatic phthalimide, induces positive cooperativity. Positive cooperativity is indicated in the table by a positive value of pα, which is the minus log value of the cooperativity factor α. pα = 0 and pα < 0 designate neutral or negative cooperativity, respectively. Equilibrium binding of the orthosteric NMS is enhanced when the allosteric modulator has higher affinity for NMS-occupied receptors (reflected by pEC_{50diss}, where EC_{50diss} is the concentration for a half-maximum inhibition of NMS-dissociation) than for NMS-free receptors (pK_A). Methylation of the propyl chain next to the naphthalimide (**1b**) induced positive cooperativity and increased binding affinities considerably. In contrast, methylation on the opposite side adjacent to the phthalimido group (**1c**) shifted the cooperativity to neutrality only and moderately increased the binding affinity for NMS-occupied receptors (pEC_{50diss}). Methylation at both sites (**1d**) resulted in positive cooperativity, but the affinities were less increased than in **1b**. Thus, with respect to the impact of structural modifications on the activity, the naphthalimide-containing side of the molecule appeared to be superior to the phthalimide-containing side and may be named the "dominant side" in this regard. Similar observations were made when comparing methylation in the neighborhood of 5-methylphthalimide with phthalimide.[6] Thus, 5-methylphthalimide is also capable of defining a dominant side with regard to the consequences of propyl chain methylation.

Table 1. Inhibition of the Dissociation (pEC_{50diss}), Affinity for the Free Receptor (pK_A), and Cooperativity (pα) of Compounds **1a**−**d** (data taken from ref 6)



entry	X	Y	pEC_{50diss}	pK_A	pα
1a	H	H	6.94 ± 0.30 *n* = 6	7.24 ± 0.13 *n* = 4	−0.20 ± 0.05 *n* = 4
1b	Me	H	8.36 ± 0.09 *n* = 11	8.29 ± 0.18 *n* = 8	0.37 ± 0.08 *n* = 8
1c	H	Me	7.28 ± 0.08 *n* = 4	7.28 ± 0.002 *n* = 3	−0.01 ± 0.004 *n* = 3
1d	Me	Me	7.73 ± 0.09 *n* = 5	7.41 ± 0.09 *n* = 5	0.56 ± 0.08 *n* = 5

The purpose of the present investigation was to answer the following questions: first, is it necessary to

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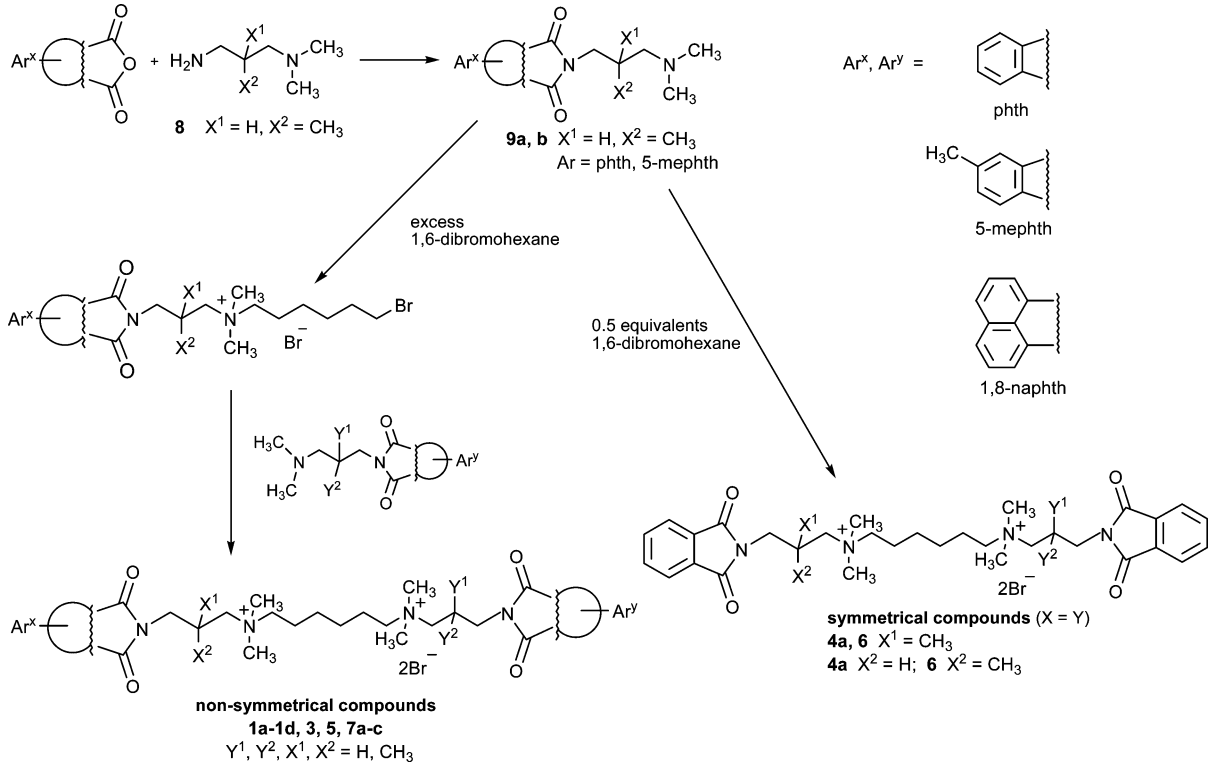
Scheme 1

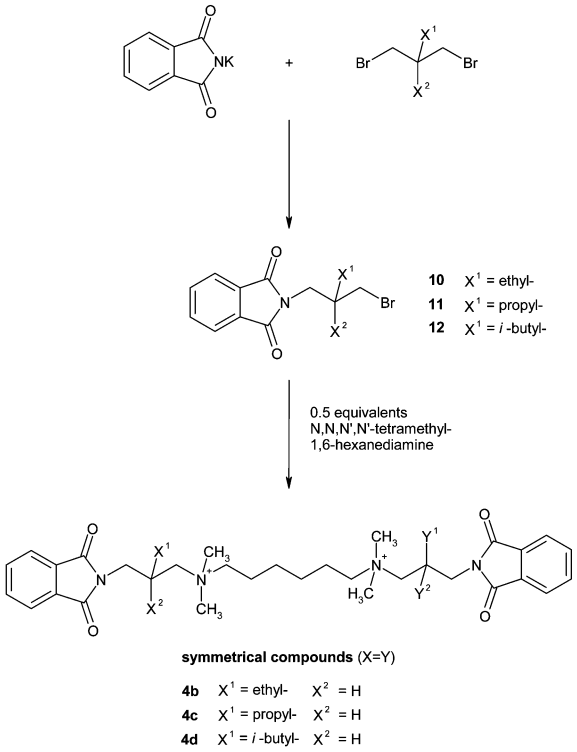


have a double methylation on one side or would monomethylation be sufficient? Therefore, we synthesized compounds **3** and **5**. Second, is a double methylation at one side as good as a monomethylation on both propyl chains (cf. compounds **4a** and **5**)? Third, is propyl chain methylation involved in defining a "dominant side"? Therefore, we choose compounds for propyl chain methylation that do not contain an aromatic moiety already defining a dominant side, i.e., we used diphthalimide agents. Fourth, are the consequences of mono/dimethylation maintained when one phthalimide is substituted with an affinity-increasing lateral ring system, i.e., 5-methylphthalimide (cf. **7a**−**c**)? Fifth, what is the effect of increasing the size of the propyl chain substituent from methyl to isobutyl on the cooperativity of the related hexamethonio compound (cf. compounds **4a**−**d**)? Answering these questions may result in a better insight into the pharmacophoric elements that are pivotal for the enhancement of antagonist binding by hexamethonio-type muscarinic allosteric agents.

Chemistry

The synthesis pathways are summarized in Schemes 1 and 2. Compound **1a** was synthesized according to ref 7, compounds **1b**−**d** according to 6, compounds **5** and **6** according to ref 5, and compounds **7a** and **7c** according to ref 6. To achieve the monoalkylated compounds **3** and **4a**, 3-(*N*,*N*-dimethylamino)-2-methylpropanenitrile was synthesized according to ref 8. The nitrile group was reduced to N^1,N^1,2-trimethylpropane-1,3-diamine **8** following a standard procedure with lithium aluminum hydride. The phthalimidopropylamines **9a** and **9b** were obtained by refluxing equimolar amounts of the corresponding phthalic acid anhydrides, *N*,*N*,2-trimethylpropane-1,3-diamine **8** and a catalytic amount of *p*-toluenesulfonic acid in toluene using a water separator.

Scheme 2



Conversion of two molecules of **9a** with 1,6-dibromohexane in acetonitrile gave the symmetrical compound **4a**. To obtain the nonsymmetrical compounds **3** and **7b**, *N*-(3-dimethylaminopropyl)phthalimide had to be alkylated by using an excess of 1,6-dibromohexane according to ref 5. Equimolar amounts of **9a** or **9b** and the alkylated phthalimidopropylamine derivative were subsequently refluxed in acetonitrile for 4 days to give compounds **3** and **7b**, respectively (see Scheme 1).

For the synthesis of the alkylated symmetrical W84 derivatives **4b**−**d** carrying ethyl-, propyl- or isobutyl groups, another synthetic pathway was developed (Scheme 2). Diethyl malonate was alkylated using sodium ethanolate and the corresponding bromoalkane, the ester groups were reduced by means of lithium aluminum hydride to give the corresponding propane-1,3-diols, and the hydroxyl groups of the diols were replaced with the bromides by phosphorus tribromide to achieve the 1,3-dibromopropane derivatives (compare to refs 9−11). These 1,3-dibromopropane derivatives were converted with potassium phthalimide in DMF to the corresponding *N*-(3-bromopropyl)phthalimides **10**−**12**. Due to sterical overcrowding in the propyl chain, the corresponding dialkylated *N*-(3-bromopropyl)phthalimides with two alkyl groups larger than methyl groups were not obtainable by the latter reaction. In the last step two molecules of **10**−**12** were connected with *N*,*N*,*N*′,*N*′-tetramethyl-1,6-hexanediamine in acetonitrile to give the symmetrical hexamethonio derivatives **4b**−**d**. The corresponding nonsymmetrical compounds of **4** carrying an alkyl group only at one propyl chain cannot be obtained, because it was neither possible to alkylate bis(dimethylamino)hexane with **10**, **11**, or **12** only at one side nor to follow the sequence in Scheme 1 according to the nonavailability of the corresponding compound **9** (see above).

The identities were established by 1D and 2D ^{1}H NMR and ^{13}C NMR spectroscopic experiments (see Experimental Section). Even though compounds **4a**−**d** contain two stereogenic centers giving rise to three isomers, two enantiomers and one meso form, the NMR spectra show only one set of signals, indicating that either the enantiomers or the meso form is present.

Pharmacology

Radioligand binding experiments were carried out with domestic pig heart ventricle homogenates containing muscarinic acetylcholine receptors of the M_2 subtype. The applied buffer (3 mM $MgHPO_4$, 50 mM TrisHCl, 37 °C, pH 7.3) yields allosteric activities that are similar to those found under organ bath conditions.[12,13] The orthosteric antagonist [^{3}H]-*N*-methylscopolamine ([^{3}H]NMS) was used as a probe to measure the interaction of the test compounds with the allosteric site of the M_2 receptors. Radioligand dissociation experiments revealed the potency of the test compounds to delay [^{3}H]NMS-dissociation ($EC_{50,diss}$). The minus log value $pEC_{50,diss}$ reflects the affinity of the allosteric agent for the [^{3}H]NMS-occupied receptor. On the basis of the results of the kinetic experiments, it was possible to compute the incubation time required for equilibrium binding of [^{3}H]NMS in the presence of the allosteric compound. Equilibrium binding experiments revealed the affinity of the allosteric agent for the unoccupied receptor (pK_A; K_A is the equilibrium dissociation constant of allosteric agent binding) as well as the cooperativity factor α.[14] α is the factor by which the alloster binding constant is changed when the agent is bound at NMS-occupied receptors instead of NMS-free receptors. According to the ternary model of allosteric interactions,[14,15] the orthosteric agent is subjected to the same change when it is bound to alloster-occupied receptors instead of alloster-free receptors.

Results and Discussion

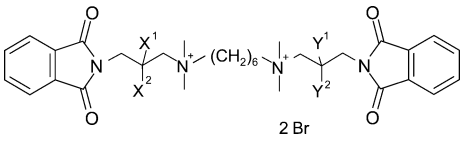
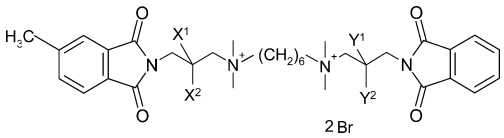
The affinity of compounds **3**−**7** to the free and NMS occupied receptor was found to be in the micromolar to higher nanomolar range (cf. pEC_{50diss} and pK_A, Table 2). Monomethylation of one propyl chain (cf. **3** versus **2**) hardly affected the affinity for NMS-occupied receptors and for unoccupied receptors; consequently negative cooperativity remained unchanged. When a second methyl group was introduced, its effect depended on the position relative to the first methyl group. If the second methylation was carried out on the opposite propyl chain (**4a** vs **3**), cooperativity was not affected. However, when the second methyl group was introduced at the position of the first methyl residue (cf. **5**), negative cooperativity was diminished to neutral cooperativity and binding affinities (pEC_{50diss}, pK_A) were elevated by about 1 order of magnitude compared with **2.** Thus, with respect to the impact of introducing a second methyl on cooperativity and affinity, we would conclude that the first methyl group defines a "dominant side" on the molecule. Remarkably, a double methylation at both sides shifted the cooperativity back to negative values (**6**).

In the 5-methylphthalimide-containing agents (Table 2, section c), monomethylation of the adjacent propyl chain (**7b**) slightly affected affinity values and negative cooperativity was weakened. Yet, only dimethylation of the propyl chain (**7c**) induced the major increase of affinities and switched cooperativity from negative to positive. Thus, irrespective of phthalimide or 5-methyl-phthalimide and the starting levels of binding affinity, only *di*methylation of the propyl chain is sufficient for a major change in binding affinities and cooperativities.

To get insight into whether an alkyl chain larger than methyl might improve the allosteric effectiveness, the series of compounds shown in Table 2 (section b) was synthesized. As already mentioned in the chemistry paragraph, the monoalkylation with ethyl, propyl, and isobutyl on *one* side turned out to be hardly possible. Therefore, monoalkylation was introduced on both propyl chains. Propyl and isobutyl increased the level of binding affinities, but only propyl weakened negative cooperativity. Having in mind that double methylation on both sides (**6**) enhances negative cooperativity compared with double methylation on one side only (**5**), it will be interesting to find out whether substitution on only *one* of the lateral propyl chains with propyl might even induce positive cooperativity. Furthermore, due to the two stereogenic centers in the monoalkylated compounds resulting in three isomers, a mixture of compounds was tested. Thus, it will be interesting to check the stereoselectivity of the allosteric actions. This study should be carried out in the high-affinity bis-naphthalimide compounds, and the synthesis of such compounds is in progress.

It is noteworthy that the stability of the mono- and dialkylated imide compounds, which tend to hydrolyze in the buffer system used for pharmacological evaluation,[5,16] was enhanced with the increase of the steric bulk (for half-lives, see the Supporting Information). As expected, the lipophilicity also increased with the increasing degree of alkylation. Both properties are advantageous for potential therapeutic applica-

Table 2. Inhibition of the Dissociation (pEC_{50diss}), Affinity for the Free Receptor (pK_A), and Cooperativity (pα) of Compounds **2**–**7**

entry	X^1	X^2	Y^1	Y^2	pEC_{50diss}	pK_A	pα
							
					a. Effect of Methylation		
2 (W84)	H	H	H	H	5.87 ± 0.06 $n = 4$	6.19 ± 0.13 $n = 4$	−0.47 ± 0.06 $n = 4$
3	Me	H	H	H	6.05 ± 0.03 $n = 3$	5.87 ± 0.05 $n = 3$	−0.33 ± 0.02 $n = 3$
4a	Me	H	Me	H	6.08 ± 0.02 $n = 3$	6.23 ± 0.09 $n = 3$	−0.33 ± 0.03 $n = 3$
5	Me	Me	H	H	6.87 ± 0.04 $n = 3$	6.90 ± 0.03 $n = 3$	−0.04 ± 0.03 $n = 3$
6	Me	Me	Me	Me	6.75 ± 0.04 $n = 3$	7.08 ± 0.09 $n = 3$	-0.21 ± 0.02 $n = 3$
					b. Effect of Alkyl Chain Length		
2 (W84)	H	H	H	H	5.87 ± 0.06 $n = 4$	6.19 ± 0.13 $n = 4$	−0.47 ± 0.06 $n = 4$
4a	Me	H	Me	H	6.08 ± 0.02 $n = 3$	6.23 ± 0.09 $n = 3$	−0.33 ± 0.03 $n = 3$
4b	Et	H	Et	H	6.14 ± 0.07 $n = 3$	6.43 ± 0.05 $n = 3$	−0.35 ± 0.01 $n = 3$
4c	Pr	H	Pr	H	6.89 ± 0.03 $n = 4$	6.83 ± 0.03 $n = 3$	−0.07 ± 0.03 $n = 3$
4d	i-Bu	H	i-Bu	H	6.81 ± 0.04 $n = 4$	7.05 ± 0.04 $n = 3$	−0.28 ± 0.02 $n = 3$
							
					c. Effect of Methylation of 5-Methylphthalimides		
7a[a]	H	H	H	H	6.49 ± 0.04 $n = 3$	6.73 ± 0.08 $n = 3$	−0.31 ± 0.02 $n = 3$
7b	Me	H	H	H	6.55 ± 0.02 $n = 3$	6.51 ± 0.02 $n = 3$	−0.06 ± 0.02 $n = 3$
7c[a]	Me	Me	H	H	7.26 ± 0.02 $n = 3$	7.26 ± 0.03 $n = 3$	0.19 ± 0.02 $n = 3$

[a] Reference 6.

tions, such as the management of organophosphorus poisoning.

Conclusion

By variation of the degree and size of alkylation of the middle position of the lateral propyl chains, the phthalimidopropyl-hexamethonio compounds could be shifted from negative to neutral cooperativity with an orthosteric muscarinic antagonist. Methylation of the lateral propyl chain in combination with an affinity augmenting imide moiety, such as a 5-methylphthalimide and naphthalimide, on the same side of the molecule shifts the cooperativity toward positive values, yielding enhancers of antagonist binding. Thus, lateral side chain dimethylation is a key structural element for positive cooperativity in hexamethonio-type muscarinic allosteric agents.

Experimental Section

For analytical instruments and chemicals, see ref 6. W84 was synthesized according to ref 16 and **1**, **5**, and **6** according to refs 5, 6, and 7, respectively.

General Procedure for the Synthesis of 2-(3-Dimethylamino-2-methylpropyl)isoindoline-1,3-dione (9a) and 2-(3-Dimethylamino-2-methylpropyl)-5-methylisoindoline-1,3-dione (9b). A mixture of phthalic acid anhydride and 5-methylphthalic acid anhydride (40 mmol), respectively, N^1,N^1,2-trimethylpropane-1,3-diamine **7** (4.6 g, 40 mmol), and a catalytic amount of *p*-toluenesulfonic acid was refluxed in toluene (100 mL) using a water separator. After a reaction time of 4 h, the solvent was evaporated. The oily residues were purified by means of column chromatography (silica gel, eluent CH_2Cl_2:MeOH = 1:1). The obtained oils crystallized after a few hours at room temperature. **9a**: yield 64%, mp 62 °C; ^{1}H NMR (δ(ppm), $CDCl_3$) 0.95 ($CHCH_3$, d, *J* 6.3), 2.28 ($N(CH_3)_2$, s), 2.32–2.39 ($CH_2N(CH_3)_2$, $CHCH_3$, m), 3.45–3.79 ($N_{phth}CH_2$, m), 7.71, 7.81 (H_{ar}, m)). **9b**: yield, 75%; mp 52 °C; ^{1}H NMR (δ(ppm), $CDCl_3$) 0.92 ($CHCH_3$, d, *J* 6.3), 2.25 ($N(CH_3)_2$, s), 2.32–2.39 ($CH_2N(CH_3)_2$, $CHCH_3$, m), 2.48 (phth-CH_3, s), 3.44–3.75 ($N_{phth}CH_2$, m), 7.47 (H_{ar}, d, *J* 7.6), 7.60 (H_{ar}, s), 7.68 (H_{ar}, d, *J* 7.6).

General Procedure for the Synthesis of 2-{3-[1-(6-{1,1-Dimethyl-1-[3-(1,3-dioxo-1,3-dihydroisoindol-2-yl)propyl]-ammonio}hexyl)-1,1-dimethylammonio]-2-methylpropyl}-isoindoline-1,3-dione Dibromide (3) and 2-{3-[1-(6-{1,1-Dimethyl-1-[3-(1,3-dioxo-1,3-dihydroisoindol-2-yl)-propyl]ammonio}hexyl)-1,1-dimethylammonio]-2-methylpropyl}-5-methylisoindoline-1,3-dione Dibromide (7b). Equimolar amounts (2 mmol) of the phthalimidopropylamines **9a** or **9b** and (6-bromohexyl)dimethyl-[3-(1,3-dioxo-1,3-dihydroisoindol-2-yl)propyl]ammonium bromide[5] (0.95 g) were dissolved in acetonitrile (50 mL), and a catalytic amount of KI and K_2CO_3 (1:1) was added. The reaction solution was refluxed for 4 days. After the reaction was completed (TLC monitoring: silica gel, eluent CH_3OH:0.2 M NH_4NO_3 solution (aq) = 3:2) about one-half of the solvent was evaporated and the solution was cooled to 4 °C for several days. The obtained white

precipitates were collected by filtration and washed several times with small amounts of acetonitrile and with pentane and dried in vacuo. **3**: yield 49%; mp 242 °C; ^{1}H NMR (δ(ppm), DMSO-d_6) 1.07 (CHCH_3, d, J 6.6), 1.27 ($N^+(CH_2)_2CH_2$, br), 1.66 ($N^+CH_2CH_2$, br), 2.05 ($N_{Phth}CH_2CH_2$, br), 2.50 ($N_{Phth}CH_2CH$, br), 3.01, 3.03 ($N^+(CH_3)_2$, s), 3.27 (N^+CH_2, br), 3.37 (CH_2N^+, br), 3.49−3.62 ($N_{Phth}CH_2$, m), 3.66 ($N_{Phth}CH_2$, t, J 6.2), 7.87 (H_{ar}, m). **7b**: yield 44%; mp 221 °C; ^{1}H NMR (δ(ppm), DMSO-d_6) 1.06 (CHCH_3, d, J 6.6), 1.30 ($N^+(CH_2)_2CH_2$, br), 1.68 ($N^+CH_2CH_2$, br), 2.06 ($N_{Phth}CH_2CH_2$, br), 2.50 ($N_{Phth}CH_2CH$, br), 2.51 (phth-CH_3, s), 3.04, 3.11 ($N^+(CH_3)_2$, s), 3.30 (N^+CH_2, br), 3.38 (CH_2N^+, br), 3.48−3.62 ($N_{Phth}CH_2$, m), 3.66 ($N_{Phth}CH_2$, t, J 6.2), 7.67 (H_{ar}, d, J 7.6), 7.71 (H_{ar}, s), 7.77 (H_{ar}, d, J 7.6), 7.89 (H_{ar}, m).

Synthesis of 2-{3-[1-(6-{1,1-Dimethyl-1-[3-(1,3-dioxo-1,3-dihydroisoindol-2-yl)-2-methylpropyl]ammonio}-hexyl)-1,1-dimethylammonio]-2-methylpropyl}isoindoline-1,3-dione Dibromide (4a). Two equivalents of the phthalimidopropylamine **9a** (2 mmol) and 1 equiv of 1,6-dibromohexane (1 mmol) were dissolved in acetonitrile (50 mL), and a catalytic amount of KI and K_2CO_3 (1:1) was added. The reaction solution was refluxed for 4 d. After the reaction was completed (TLC monitoring: silica gel, eluent CH_3OH:0.2 M NH_4NO_3 solution (aq) = 3:2), the solution was allowed to cool to room temperature and a white precipitate (yield 65%) was collected by filtration. It was washed several times with acetonitrile and pentane and dried in vacuo: mp 227 °C; ^{1}H NMR (δ(ppm), DMSO-d_6) 1.06 (CHCH_3, d, J 6.6), 1.26 ($N^+(CH_2)_2CH_2$, br), 1.68 ($N^+CH_2CH_2$, br), 2.51 ($N_{Phth}CH_2CH$, br), 3.08 ($N^+(CH_3)_2$, s), 3.28 (N^+CH_2, br), 3.33 (CH_2N^+, br), 3.49−3.62 ($N_{Phth}CH_2$, m), 7.87 (CH_{ar}, m).

General Procedure for the Synthesis of 2-(2-(Bromomethyl)butyl)isoindoline-1,3-dione (10), 2-(2-(Bromomethyl)pentyl)isoindoline-1,3-dione (11), and 2-(2-(Bromomethyl)-4-methylpentyl)isoindoline-1,3-dione (12). A mixture of potassium phthalimide (6 mmol) and the corresponding alkylated 1,3-dibromopropane derivative (18 mmol) in 30 mL of *N*,*N*-dimethylformamide was heated at 80 °C for 7 h. After the reaction was completed, the precipitated potassium bromide was removed from the solution and the solvent was evaporated under reduced pressure. The liquid residues were dissolved in diethyl ether and extracted with water. The organic layers were dried over Na_2SO_4, and the solvent was evaporated. The resulting liquids were purified by means of column chromatography (silica gel, eluent CH_2Cl_2). Colorless oils were obtained that crystallized after several days at 4 °C. **10**: yield 41%; mp 42 °C; ^{1}H NMR (δ(ppm), $CDCl_3$) 0.98 (CH_3, t, J 7.5), 1.42−1.58 (CHCH_2CH$_3$, m), 2.17 (CH_2CHCH_2, br), 3.44, 3.49 (CH_2Br, dd, J 4.8, 10.6), 3.70 (NCH_2, dd, J 6.6, 13.9), 3.79 (NCH_2, dd, J 7.3, 13.9), 7.74, 7.84 (CH_{ar}, m). **11**: yield 46%; mp 53 °C; ^{1}H NMR (δ(ppm), $CDCl_3$) 0.93 (CH_3, t, J 7.5), 1.32−1.57 ($CH_2CH_2CH_3$, m), 2.24 (CH_2CHCH_2, br), 3.42, 3.47 (CH_2Br, dd, J 4.7, 10.5), 3.69 (NCH_2, dd, J 6.4, 13.9), 3.75 (NCH_2, dd, J 7.1, 13.9), 7.74, 7.84 (CH_{ar}, m). **12**: yield 46%; mp 61 °C; ^{1}H NMR (δ(ppm), $CDCl_3$) 0.93 (CH_3, dd, J 6.5, 25.5), 1.15−1.22 (CHCH_2CH, m), 1.42−1.49 (CHCH_2CH, m), 1.68−1.78 (CH(CH_3)$_2$, m), 2.31 (CH_2CHCH_2, br), 3.40, 3.45 (CH_2Br, dd, J 4.7, 10.5), 3.67 (NCH_2, dd, J 5.9, 13.9), 3.75 (NCH_2, dd, J 8.0, 13.9), 7.74, 7.84 (CH_{ar}, m).

General Procedure for the Synthesis of 2-{3-[1-(6-{1,1-Dimethyl-1-[3-(1,3-dioxo-1,3-dihydroisoindol-2-yl)-2-ethylpropyl]ammonio}hexyl)-1,1-dimethylammonio]-2-ethylpropyl}isoindoline-1,3-dione Dibromide (4b), 2-{3-[1-(6-{1,1-Dimethyl-1-[3-(1,3-dioxo-1,3-dihydroisoindol-2-yl)-2-propylpropyl]ammonio}hexyl)-1,1-dimethylammonio]-2-propylpropyl}isoindoline-1,3-dione Dibromide (4c), and 2-{3-[1-(6-{1,1-Dimethyl-1-[3-(1,3-dioxo-1,3-dihydroisoindol-2-yl)-2-isobutylpropyl]ammonio}hexyl)-1,1-dimethylammonio]-2-isobutylpropyl}isoindoline-1, 3-dione Dibromide (4d). Two equivalents of the corresponding *N*-(3-bromopropyl)phthtalimides **10**−**12** (2 mmol) and 1 equiv of *N*,*N*,*N*′,*N*′-tetramethyl-1,6-hexanediamine (1 mmol) were dissolved in acetonitrile (50 mL), and a catalytic amount of KI and K_2CO_3 (1:1) was added. The reaction solution was refluxed for 3−7 days. After the reaction was completed (TLC monitoring: silica gel, eluent CH_3OH:0.2 M NH_4NO_3 solution (aq) = 3:2) about one-half of the solvent was evaporated and the solution was cooled to 4 °C for several days. The obtained white precipitates were collected by filtration and washed several times with small amounts of acetonitrile and with pentane and dried in vacuo. **4b**: yield 34%; mp 186 °C; ^{1}H NMR (δ(ppm), DMSO-d_6) 0.95 (CH_2CH_3, d, J 7.3), 1.30 ($N^+(CH_2)_2CH_2$, br), 1.34 (CHCH_2, br), 1.68 ($N^+CH_2CH_2$, br), 2.33 ($N_{Phth}CH_2CH$, br), 3.09 ($N^+(CH_3)_2$, s), 3.31 (N^+CH_2, br), 3.38 (CH_2N^+, br), 3.55−3.68 ($N_{Phth}CH_2$, m), 7.88 (CH_{ar}, m). **4c**: yield 51%; mp 71 °C; ^{1}H NMR (δ(ppm), DMSO-d_6) 0.85 (CH_2CH_3, t, J 6.7), 1.29 ($N^+(CH_2)_2CH_2$, br), 1.40 ($CH_2CH_2CH_3$, br), 1.48 (CHCH_2, br), 1.72 ($N^+CH_2CH_2$, br), 2.35 ($N_{Phth}CH_2CH$, br), 3.10, 3.11 ($N^+(CH_3)_2$, s), 3.32 (N^+CH_2, br), 3.39 (CH_2N^+, br), 3.56−3.67 ($N_{Phth}CH_2$, m), 7.88 (CH_{ar}, m). **4d**: yield 65%; mp 77 °C; ^{1}H NMR (δ(ppm), DMSO-d_6) 0.82, 0.91 ($CH_2CH(CH_3)_2$, d, J 6.5), 1.25 (CHCH_2, br), 1.27 ($N^+(CH_2)_2CH_2$, br), 1.69 ($N^+CH_2CH_2$, br), 1.75 ($CH_2CH(CH_3)_2$, m), 2.35 ($N_{Phth}CH_2CH$, br), 3.08, 3.09 ($N^+(CH_3)_2$, s), 3.31 (N^+CH_2, br), 3.40 (CH_2N^+, br), 3.61 ($N_{Phth}CH_2$, br), 7.88 (CH_{ar}, m).

Determination of the Lipophilicity. The test and reference substances (2-phenylethylamine, 2-phenylethanol, benzene, *N*,*N*′-dimethylaniline, chlorobenzene, toluene, ethylbenzene, cumene, biphenyl, and anthracene), respectively, were dissolved in methanol (4 μg/mL). Using a RP column (LiChroCart 125-4 HPLC cartridge; LiChrospher 100, RP 18, 5 μm, end-capped, Merck) and a mobile phase composed of methanol/phosphate buffer pH 7.4 = 70:30 (0.02% *N*,*N*-dimethylamine added), the retention times were determined and converted to k' values. $k' = (t_R - t_0)/t_0$ with t_R = retention time of the test substance and t_0 = hold-up time. The log k' values of the reference substances were correlated with the log P values reported in ref 17. The calibration curve was established (lipophilicity: $y = 2.4103x + 1.4865$; $R^2 = 0.9778$) and the log P values of the test substances calculated. The log P values are summarized in Table 3 (Supporting Information).

Determination of Stability in Buffer Solution. For the determination of the stability the compounds were dissolved in Mg/Tris buffer, pH 7.3 (20 μg/mL). By means of UV spectroscopy the absorption maxima of the test compounds were determined. The absorption values in the maximum wavelengths were observed over a period of 24 h. For compounds that exhibited an exponential decrease of absorption, the half-life periods ($t_{1/2}$) were determined and are summarized in Table 3 (Supporting Information).

Pharmacology. Preparation of porcine heart homogenates has been described previously.[18,19] Experiments were carried out at 37 °C using a buffer consisting of 3 mM $MgHPO_4$ and 50 mM Tris-HCl at pH 7.3. [^{3}H]-*N*-methylscopolamine ([^{3}H]NMS, PerkinElmer Life Sciences, Boston, MA) with a specific activity of 81.0−83.5 Ci/mmol was used as radioligand at a concentration of 0.2 nM. To determine nonspecific binding, 1 μM atropine was added. [^{3}H]NMS binding affinity under control conditions amounted to pK_D = 9.37 (9.33, 9.41; two homogenates, three experiments each). Rapid vacuum filtration (glass filters No. 6; Schleicher and Schüll, Dassel, Germany) was used to separate the membranes. After washing the filters twice with 5 mL of distilled water of 0 °C, 5 mL of scintillation fluid (Ready protein, Beckman, Palo Alto, CA) was added to measure radioactivity (Beckman LS 6000 counter).

To measure the effects of the test compounds on the dissociation of [^{3}H]NMS, "two-point kinetic experiments" were carried out (cf. ref 20). Therefore, membranes were preincubated for 30 min with [^{3}H]NMS to attain equilibrium binding. [^{3}H]NMS-dissociation was revealed after addition of 1 μM atropine either alone or in combination with the test compound. Specific [^{3}H]NMS binding at $t = 0$ and 10 was measured to characterize the monoexponential time course of [^{3}H]NMS dissociation. The obtained apparent rate constant of dissociation k_{-1} relative to the rate constant of dissociation of the control served to achieve concentration−effect curves for the

allosteric delay of [^{3}H]NMS dissociation. The half- life of dissociation amounted to $t_{1/2} = 2.8 \pm 0.1$ min (mean value ± SEM, $n = 41$). The obtained values were plotted over the log drug concentration. Curve fitting was based on a four parameter logistic function (GraphPad Prism Version 3.00; GraphPad, San Diego, CA). A partial *F*-test ($p < 0.05$ taken to indicate a significant difference) proved that the curves could be fitted with slope factors of unity.

To check whether a test compound is positive, neutral, or negative cooperative with [^{3}H]NMS, equilibrium binding experiments were carried out. The incubation time needed for obtaining [^{3}H]NMS equilibrium binding in the presence of an allosteric agent was computed based on eq 31 in the study of Lazareno and Birdsall.[21] Increasing concentrations of the test compounds were incubated with membranes and 0.2 nM [^{3}H]NMS. Data analysis was performed by nonlinear regression analysis based on the ternary complex model of allosteric interactions (eq 3 in ref 13).

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Supporting Information Available: Stability and lipophilicity data as well as combustion data. This material is available free of charge via the Internet at http://pubs.acs.org.

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