Design, Synthesis, and Action of Oxotremorine-Related Hybrid-Type Allosteric Modulators of Muscarinic Acetylcholine Receptors

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A novel series of muscarinic receptor ligands of the hexamethonio-type was prepared which contained, on one side, the phthalimidopropane or 1,8-naphthalimido-2,2-dimethylpropane moiety typical for subtype selective allosteric antagonists and, on the other, the acetylenic fragment typical for the nonselective orthosteric muscarinic agonists oxotremorine, oxotremorine-M, and related muscarinic agonists. Binding experiments in M_2 receptors using [^{3}H]*N*-methylscopolamine as an orthosteric probe proved an allosteric action of both groups of hybrids, **7a**−**10a** and **8b**−**10b**. The difference in activity between **a**-group and **b**-group hybrids corresponded with the activity difference between the allosteric parent compounds. In M_1−M_3 muscarinic isolated organ preparations, most of the hybrids behaved as subtype selective antagonists. [^{35}S]GTPγS binding assays using human M_2 receptors overexpressed in CHO cells revealed that a weak intrinsic efficacy was preserved in **8b**−**10b**. Thus, attaching muscarinic allosteric antagonist moieties to orthosteric muscarinic agonists may lead to hybrid compounds in which functions of both components are mixed.

Introduction

Molecular cloning studies revealed the existence of five (M_1−M_5) molecularly distinct muscarinic acetylcholine receptors (mAChRs), which belong to the superfamily of rhodopsin-like G protein-coupled receptors (GPCRs). The odd-numbered receptors (M_1, M_3, and M_5) are preferentially linked to G proteins of the G_q class, whereas the even-numbered receptors (M_2 and M_4) are primarily coupled to G proteins of the G_i family.[1,2] Given the significant amino acid sequence identity among the five receptors at the acetylcholine (orthosteric) binding site,[3] a high degree of mAChR subtype selectivity is hardly achieved for muscarinic agonists.[4] In addition, since a variety of tissues and cells express multiple mAChRs, the physiological and pathophysiological roles of the muscarinic cholinergic system are far from being completely clarified. To this end, a promising approach is represented by the use of gene targeting techniques, with the generation of mutant mouse strains deficient in one or two mAChR subtypes.[5,6]

Moreover, ligands can interact with a binding site on the receptor complex that is distinct from the orthosteric site. This "allosteric site", whose existence has been demonstrated for different GPCRs,[7] is likely to be less conserved than the orthosteric ligand binding site, thus representing a potential target in the search for new ligands with a more pronounced receptor subtype selectivity. As far as muscarinic receptors are taken into account, allosteric modulators act at a site apart from the "common ligand binding site" of the receptor protein. The detection and quantitation of allosteric interactions of ligands with mAChRs have been the subject of detailed investigations.[8−12]

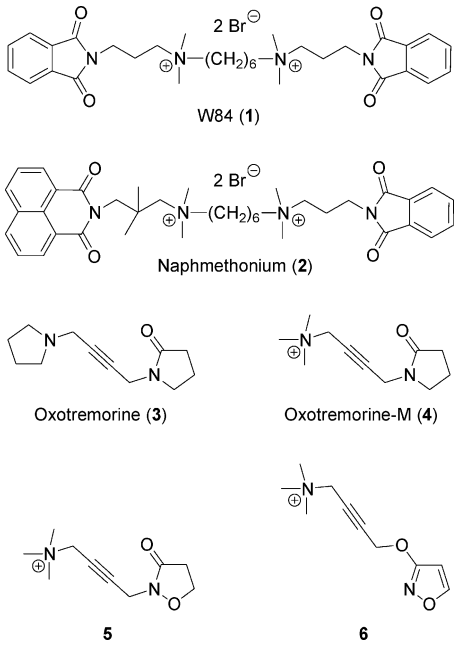

Figure 1. Molecular structure of the reference compounds in this study.

Indeed, allosteric modulators are able to enhance the binding of the orthosteric ligands denoted as positive cooperativity, or to diminish the ligand binding called negative cooperativity, or to leave the binding unaffected known as neutral cooperativity.[13] Receptor mutagenesis and molecular modeling based on the crystal structure of bovine rhodopsin yielded a three-dimensional model of allosteric/orthosteric M_2 receptor sites. Allosteric agents bind in the receptor's extracellular loop region close to the orthosteric binding cavity site that is lined by the transmembrane helices.[14,15] Among the compounds prepared and studied by some of us, symmetrical W84[16,17] and nonsymmetrical naphmethonium[18,19] (compounds **1** and **2**, Figure 1) are two

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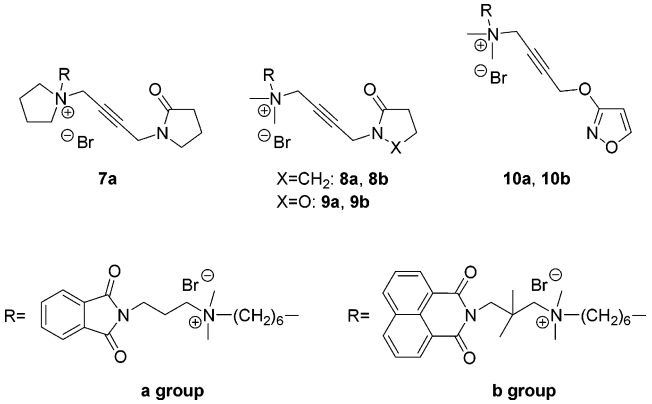


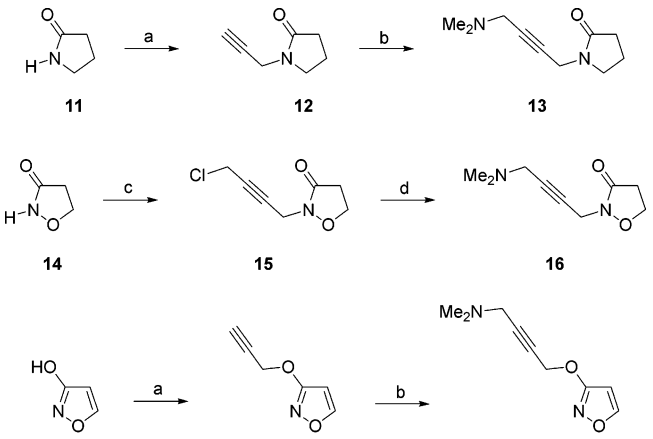
Figure 2. Target hexamethonio-type hybrid derivatives.

archetypal derivatives which gave a major contribution to the assessment of the structure/activity relationships of bis(ammonio)alkane-type allosteric modulators.[13] With regard to receptor function bis(ammonio)alkane-type agents behave as antagonists.[20,21] Worth mentioning, replacement of the phthalimido moiety of **1** with the 1,8-naphthalimido residue of **2** coupled with dimethylation of the lateral propyl chain brought about a 25−50-fold increase of allosteric potency, which was shifted into the nanomolar concentration range.[18] For such a pronounced increase in potency it is a prerequisite that both, ring and side chain modifications, are carried out on the same side of the molecule, thus yielding a dominant side with regard to allosteric potency.[19]

On the other hand, we had previously prepared and studied a series of analogues of the potent yet nonselective muscarinic agonists oxotremorine and oxotremorine-M (compounds **3** and **4**, Figure 1).[22−25] Among the investigated compounds, the quaternary salts **5** and **6** (Figure 1), in which the nature of the heterocyclic ring and the attachment point of the acetylenic side chain were changed, retained the overall pharmacological profile of the parent compounds at M_1, M_2, and M_3 receptor subtypes.[22,23] We wondered what pharmacological profile allosteric/orthosteric hybrid compounds would have that contain on the one side half of an allosteric modulator and on the other side an orthosteric agonist. Therefore, we designed a group of novel hybrid hexamethonio-type bisquaternary derivatives by incorporating the skeleton of oxotremorine and oxotremorine-M and their related analogues into the structure of the allosteric modulators W84 (derivatives **7a**−**10a**, Figure 2) and naphmethonium (derivatives **8b**−**10b**, Figure 2). We aimed at evaluating to what extent the introduction of an allosteric moiety could affect the binding topology of the orthosteric agonists and their capability of receptor activation. This paper reports the synthesis of compounds **7**−**10** and the pharmacological proof of their allosteric interaction with M_2 muscarinic receptors. In addition, target compounds were tested in functional in vitro assays at muscarinic M_1−M_3 receptor subtypes.

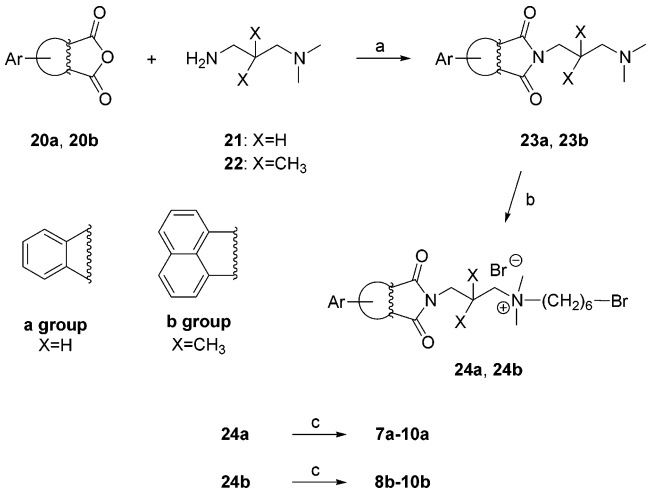
Chemistry. The synthesis of target compounds was accomplished along the reaction sequences depicted in Schemes 1 and 2. Commercially available 2-pyrrolidinone **11** was reacted with propargyl mesylate[26] in a THF/KOH suspension containing tetrabutylammonium iodide.[27] The resulting *N*-propargyl-2-pyrrolidinone **12** was subjected to a CuCl-catalyzed Mannich reaction with dimethylamine and paraformaldehyde,[27,28] to afford the corresponding tertiary base **13** (Scheme 1).[29] In a similar way, known 3-hydroxyisoxazole **17**[30] was transformed into the corresponding propargyloxy intermediate **18** and then into the related dimethylamino derivative **19** (Scheme 1).

Scheme 1[a]



[a] (a) Propargyl mesylate, KOH/THF, $(n\text{-Bu})_4N^+I^-$; (b) Me_2NH/(HCHO)n/CuCl/HOAc/dioxane; (c) 1,4-dichloro-2-butyne, K_2CO_3/acetone; (d) Me_2NH/CH_3OH.

Scheme 2[a]



[a] (a) Refluxing toluene; (b) $Br(CH_2)_6Br$ (excess); (c) refluxing CH_3CN/base (oxotremorine, or **13**, or **16**, or **19**).

Conversely, due to a ring opening during the Mannich reaction step, preparation of the tertiary base **16** was achieved through the intermediacy of the *N*-2-butynyl chloride **15**, in turn obtained by treating isoxazolidin-3-one **14** with 1,4-dichloro-2-butyne in a suspension of potassium carbonate in refluxing acetone (Scheme 1).[25]

The anhydrides **20a** and **20b** were refluxed in toluene with an equimolar amount of 1,3-diamino-N^1,N^1-dimethylpropane **21** or 1,3-diamino-N^1,N^1-2,2-tetramethylpropane **22**, respectively, using a water separator (Scheme 2). Alkylation of the resulting phthalimidopropylamine **23a** and 1,8-naphthalimidopropylamine **23b** with a huge excess of 1,6-dibromohexane[18,31] afforded monocharged intermediates **24a** and **24b**, which were refluxed in acetonitrile with an equimolar amount of oxotremorine or each of the tertiary bases **13**, **16**, and **19**, thereby affording the desired hexamethonio-type hybrid compounds **7a**−**10a** and **8b**−**10b** (Scheme 2). Compound **7b** was impossible to isolate in sufficient yield and purity.

Results and Discussion

Compounds **7**−**10** were assayed in binding experiments performed in homogenates of porcine heart ventricles containing muscarinic receptors of the M_2 subtype. The orthosteric

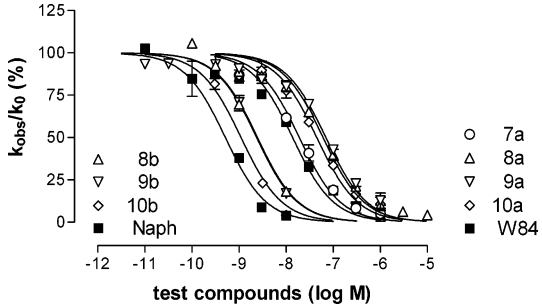


Figure 3. Allosteric inhibition by the indicated test compounds of the dissociation of [^{3}H]NMS from porcine heart M_2 receptors. *Ordinate:* Apparent rate constant of [^{3}H]NMS dissociation in the presence of test compound k_{obs} relative to the respective control value k_0. *Abscissa:* log-[drug concentration]. Indicated are mean values and standard errors of three independent experiments with triplicated values. Nonlinear regression analysis based on a four parameter logistic function with the upper and lower plateaus fixed at 100% and 0% respectively, and slope factors of unity.

antagonist [^{3}H]*N*-methylscopolamine ([^{3}H]NMS) was used as a probe to measure the interaction of the compounds under study with the allosteric site of the M_2 receptors. The inhibitory effect of the test compounds on the dissociation of [^{3}H]NMS from porcine heart receptors is reported in Figure 3, where the results obtained with the parent compounds W84 and naphmethonium, respectively, have been included for comparison. The curves reflect ternary complex formation, i.e., the binding of the test compounds to M_2 receptors whose orthosteric site is occupied by [^{3}H]NMS.[32,33]

Inspection of the two sets of curves points out that the overall affinity profile of phthalimido-alkyl hybrids **7a**−**10a** parallels that of their reference alloster W84, and, likewise, the affinities of 1,8-naphthalimido-alkyl hybrids **8b**−**10b** are comparable to that of the structurally related naphmethonium. Moreover, comparison of the two sets of curves makes clear that the affinity of the 1,8-naphthalimido-alkyl **b** group hybrids is roughly by about 1.5 orders of magnitude higher than the affinity of their related phthalimido-alkyl **a** group analogues (Table 1). Therefore, the difference in affinity between naphmethonium and W84 appears to be transferred into their related new derivatives, and this evidence suggests that the novel hybrids and their parent compounds share a similar mode of interaction with the allosteric site of M_2 muscarinic receptors whose orthosteric site is blocked by NMS. Moreover, we investigated the effect of the novel hybrid derivatives on the equilibrium binding of [^{3}H]NMS to porcine heart M_2 receptors. In Table 1, the [^{3}H]NMS binding values are reported for the presence of concentrations of derivatives **7**−**10** (and reference compounds W84 and naphmethonium) which are equieffective to reduce [^{3}H]NMS dissociation to 25% of the control value. Under these experimental conditions, which aim to get insight into the affinity of test compounds for the free M_2 receptors, a general trend was observed, since derivatives **7a**−**10a** showed a depressing action on [^{3}H]NMS binding (negative cooperativity with the radioligand) whereas the corresponding **b**-group compounds hardly diminished [^{3}H]NMS binding (almost neutral cooperativity). These findings suggest that the **a**-group compounds have a higher affinity for free relative to [^{3}H]NMS-occupied M_2 receptors, whereas the affinities of the **b**-group compounds are similar for both states of receptor occupancy. Thus, the difference existing between the parent compounds W84 and naphmethonium in this regard is also encountered with their related hybrid compounds.

The question arises whether the agonist moiety of the hybrids contribute to binding affinity. Previous work carried out under the same assay conditions[34] showed that truncation of W84 by leaving off a phthalimidomethyl-moiety on one side of the molecule reduced the binding affinity for the NMS-occupied M_2 receptor by 1.3 log units (EC_{50diss} = 6.57). The EC_{50diss} values of the W84 derived hybrids presented here (**7a**−**10a**, Table 1) exceed the EC_{50diss} of the "truncated W84" by 0.5 to 1.1 log units. This observation strongly suggests that the agonist-derived acetylenic moiety of the hybrid compounds is involved in the binding to the M_2 receptors whose orthosteric site is blocked by NMS.

Next, we intended to evaluate the functional characteristics of the novel hybrid compounds at muscarinic receptor subtypes. The parent compound W84 is known to act as an antagonist at muscarinic receptors[20] and an allosteric antagonist action toward acetylcholine has also been observed with naphmethonium.[35]

Functional assays were carried out using isolated organ preparations. The rabbit vas deferens was taken as an M_1 model,[36,37] but it should be mentioned that there is still some controversy in how far M_4 receptors are involved, see for example ref 4. The guinea pig left atrium was used to measure M_2 receptor mediated actions, and guinea pig ileum and urinary bladder served as M_3 models. In these assays, compounds **7a**−**10a** generally behaved as antagonists.

Since the allosteric properties were already demonstrated in the radioligand binding experiments, we did not extend the applied concentrations (<100 μM) into the region where the unsurmountable allosteric antagonism is expected to occur (e.g. >1000 μM for W84[20]). The hybrids displayed 3−10-fold higher affinity at M_2 relative to M_1 receptors and a lower affinity at ileal or bladder M_3 receptors (Table 2). This rank order (M_2 > M_1 > M_3) parallels that previously seen with W84.[20] As a consequence, the results on the W84-related hybrids indicate that the nonselective muscarinic activity of orthosteric agonists (oxotremorine, oxotremorine-M, and related compounds **5** and **6**) is switched into a subtype selective inhibitory activity. The weak partial agonist activity of **8a** and **9a** to stimulate M_3 ileal receptors, however, suggests that hybrid formation does not necessarily extinguish intrinsic efficacy completely. On the whole, the M_2-preferring derivatives **7a**−**10a** shared the antagonistic profile and selectivity observed for their parent allosteric agent W84.

Hybrids **8b**−**10b**, containing the 1,8-naphthalimido residue of naphmethonium, all antagonized the M_2-mediated negative inotropic effect. Worth noting, the oxyisoxazole hybrid **10b**, which was the most potent M_2 antagonist among the compounds under investigation, displayed an agonistic action of low potency but rather good efficacy at the M_1 receptor subtype. Derivative **9b** behaved essentially as a muscarinic antagonist with almost equal affinity at M_1 and M_2 and clearly lower affinity at M_3. Compound **8b** showed a mixed M_2 antagonist/M_3 partial agonist profile, with an almost negligible affinity but good efficacy at M_1 receptor subtypes. It is remarkable that **8b**, for yet unknown reasons, shows different responses and affinities in the ileum and the urinary bladder, although both preparations are thought to reveal M_3 receptor mediated actions. However, it has to be taken into consideration that partial agonists endowed with low intrinsic efficacy can exhibit an opposite behavior, agonism or antagonism, in tissues expressing the same receptor system but different efficiency of the stimulus-response coupling such as ileum (high signal amplification) and urinary bladder (low signal amplification).[38] Furthermore, it should be mentioned that the potencies or affinities, respectively, of the oxotremorineM/

Table 1. Allosteric Action of the Indicated Test Compounds

	W84 and "**a-group**"		naphmethonium and "**b-group**"	
test compound	pEC_{50diss}[a]	[^{3}H]NMS-bdg. at EC_{25diss} [%][b]	pEC_{50diss}	[^{3}H]NMS-bdg. at EC_{25diss} [%]
W84	7.87 ± 0.03	71 ± 2		
naphmethonium			9.29 ± 0.12	90 ± 5
7	7.67 ± 0.07	54 ± 2		
8	7.22 ± 0.03	53 ± 2	8.61 ± 0.07	100 ± 17
9	7.08 ± 0.06	45 ± 2	8.63 ± 0.06	79 ± 15
10	7.30 ± 0.09	45 ± 2	8.96 ± 0.07	112 ± 15

[a] pEC_{50diss}: minus log concentration of test compound for a half-maximum allosteric inhibition of [^{3}H]NMS dissociation from porcine heart M_2 receptors. [b] [^{3}H]NMS-binding at EC_{25diss}: [^{3}H]NMS equilibrium binding to the M_2 receptors in the presence of test compound at a concentration that reduced [^{3}H]NMS dissociation by 75% down to a level of 25%. Indicated are mean values and standard errors of three experiments with triplicated and quadruplicated values, respectively.

Table 2. In Vitro Functional Activity of Compounds **7a**−**10a** and **8b**−**10b** at Muscarinic Receptor Subtypes in Rabbit Vas Deferens (M_1), Guinea Pig Left Atrium (M_2), Guinea Pig Ileum (M_3), and Urinary Bladder (M_3)

	M_1: rabbit vas deferens			M_2: guinea pig left atrium			M_3: guinea pig ileum			M_3: guinea pig urinary bladder		
compd	pEC_{50}[a]	i.a.[b]	pK_B[c]	pEC_{50}[a]	i.a.[b]	pK_B[c]	pEC_{50}[a]	i.a.[b]	pK_B[c]	pEC_{50}[a]	i.a.[b]	pK_B[c]
7a		0	6.20 ± 0.13		0	6.85 ± 0.09		0	5.53 ± 0.05		0	5.29 ± 0.06
8a		0	5.33 ± 0.02		0	6.31 ± 0.24		0.29 ± 0.06	4.57 ± 0.03		0	4.1 ± 0.03
9a		0	5.75 ± 0.13		0	6.13 ± 0.15		0.34 ± 0.15	4.35 ± 0.05[d]		−	−
10a		0	5.60 ± 0.14		0	6.61 ± 0.07		0	4.78 ± 0.06		−	−
8b	4.44 ± 0.21	0.94 ± 0.03	n.d.		0	6.80 ± 0.12	6.25 ± 0.07	0.45 ± 0.05	6.45 ± 0.13[d]		0	5.15 ± 0.12
9b		0.28 ± 0.15	6.77 ± 0.03		0	6.47 ± 0.17		0	5.26 ± 0.13		0	4.89 ± 0.04
10b	5.05 ± 0.14	0.99 ± 0.004	n.d.		0	7.02 ± 0.14	< 4	0	6.00 ± 0.14		0	4.91 ± 0.21

[a] pEC_{50} ± SE values are the negative logarithm of the agonist concentration that caused 50% of the maximum response. [b] i.a. = intrinsic activity (α) measured by the ratio between the maximum response of the compound and the maximum response of the reference agonist. [c] Apparent pK_B values ± SE were calculated according to Furchgott [ref 43]. [d] pK_D values ± SE were calculated according to McKay [ref 42]; n.d. = not determinable. Reference agonists used were McN-A-343 (M_1) and bethanechol (M_2, M_3).

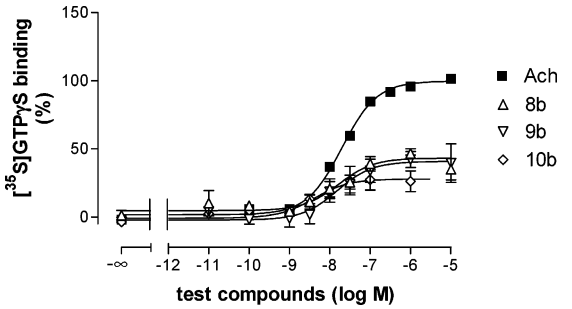


Figure 4. Effects of acetylcholine and the indicated test compounds on [^{35}S]GTPγS binding to membranes of CHO cells expressing muscarinic M2 receptors. *Ordinate:* [^{35}S]GTPγS binding as a percentage of the acetylcholine induced maximum increase (100%) over the baseline level of binding (0%). *Abscissa:* log[drug concentration]. Indicated are mean values and standard errors of 2−14 experiments with quadruplicated values. Error bars are not shown when they do not exceed the symbols. Nonlinear regression analysis based on a four parameter logistic function with slope factors of unity.

naphmethonium-hybrid **8b** are clearly lower than the potencies observed previously for oxotremorine M in the vas deferens, the left atrium and the ileum.[22]

To find out whether no sign of intrinsic activity in organ bath experiments has to be interpreted as a complete lack of capability to activate M_2 receptors, the actions of **8b**−**10b** on the binding of [^{35}S]GTPγS were measured in cell membranes from CHO cells stably transfected with the human M_2 gene (Figure 4). Derivatives **8b**−**10b** revealed an intrinsic efficacy which was, however, considerably lower than that of acetylcholine. Probably, the detection of a weak intrinsic efficacy has become possible because the assay uses membranes from cells that overexpress M_2 receptors so that small receptor signals are amplified. In any case, these findings strongly suggest that hybrid formation does not fully abolish intrinsic efficacy even in M_2 receptors.

Conclusion

In conclusion, a novel class of muscarinic allosteric agents is presented that was designed to contain a fragment typical for muscarinic allosteric antagonists and a residue representing an orthosteric muscarinic agonist. The pharmacological profile of the target compounds essentially reflected the behavior of their respective allosteric parent compounds. However, as hybrid formation did not fully abolish agonist intrinsic efficacy, appropriate structural variations of the hybrid compounds might eventually lead to subtype selective muscarinic agonists.

Experimental Section

Material and Methods. Oxotremorine and 2-pyrrolidinone were purchased from Aldrich. Dimethylamines **13**[29] and **16**[22] were prepared according to procedures reported in the literature. On the contrary, known tertiary base **19**[22] was prepared by an alternative method to avoid the use of the irritant 1,4-dichloro-2-butyne. Monoquaternary bromides **24a**,**b** as well as model compounds **1** and **2** were synthesized as previously described (refs 39 and 18). W84 dibromide and naphmethonium dibromide can be purchased from Axxora Biochemicals (Deutschland). Melting points were determined on a model B 540 Büchi or a Sanyo Gallenkamp melting point apparatus (Sanyo Gallenkamp, UK) and are uncorrected. ^{1}H NMR and ^{13}C NMR spectra were recorded with a Varian Mercury 300 (^{1}H, 300.063; ^{13}C, 75.451 MHz) or a Bruker AV 400 spectrometer (^{1}H, 400.132 MHz; ^{13}C, 100.613 MHz). Chemical shifts (δ) are expressed in ppm and coupling constants (J) in Hertz. Abbreviations for data quoted are: s, singlet; d, doublet; t, triplet; m, multiplet; br, broad. The center of the peak of DMSO-d_6 was used as internal reference. TLC analyses were performed on commercial silica gel 60 F_{254} aluminum sheets; spots were further evidenced by spraying with a dilute alkaline potassium permanganate solution. Microanalyses (C, H, N) of new compounds agreed with the theoretical value within ±0.4%.

Synthesis of 3-[4-(Dimethylamino)-2-butynyl)oxyisoxazole 19. A. A solution of 3-hydroxyisoxazole[30] (2.00 g, 23.51 mmol) and propargyl mesylate[26] (2.21 g, 47.02 mmol) in THF (30 mL) was dropped into a stirred and ice-cooled suspension of powdered KOH

(1.58 g, 28.21 mmol) and tetrabutylammonium iodide (1.74 g, 4.7 mmol) in THF (50 mL). The reaction was stirred at rt for about 4 h and subsequently concentrated at reduced pressure. Water (30 mL) was added, and the crude mixture was repeatedly extracted with diethyl ether (3 × 25 mL). The pooled organic extracts were dried over anhydrous Na_2SO_4 and concentrated under vacuum; the desired intermediate isoxazolyl ether (2.17 g, 75% yield) was obtained after purification on a silica gel column chromatography (eluent: 10% ethyl acetate/petroleum ether). 3-(2-Propynyl)oxyisoxazole **18**: colorless liquid, bp 90−95 °C/0.2 mmHg; R_f 0.57 (eluent: 20% ethyl acetate/petroleum ether). ^{1}H NMR ($CDCl_3$): 2.58 (t, 1H, H−C≡, J = 1.9), 4.85 (d, 2H, ≡CCH_2O, J = 1.9), 5.97 (d, 1H, H-4, J = 1.9), 8.12 (d, 1H, H-5, J = 1.9). Anal. $C_6H_5NO_2$ (C, H, N).

B. A sealed metal container was charged with dioxane (10 mL), paraformaldehyde (235 mg, 8.30 mmol), CuCl (63 mg, 0.64 mmol), glacial acetic acid (252 mg, 4.20 mmol), and dimethylamine (630 μL, 9.50 mmol). The mixture was stirred for 0.5 h at rt, and then a solution of **18** (1 g, 8.12 mmol) in dioxane (5 mL) was added. The reaction was kept under stirring overnight at rt, and then the solvent was eliminated under reduced pressure and 2 N HCl (15 mL) was added. After extraction with diethyl ether (3 × 10 mL), the residual aqueous phase was made basic with solid K_2CO_3 and then extracted with ethyl acetate (3 × 10 mL). After the usual workup, the crude tertiary base was submitted to silica gel column chromatography (eluent: 5% methanol/dichloromethane) and then to distillation under vacuum (1.010 g, 69% yield). The physical and analytical data for 3-[4-(dimethylamino)-2-butynyl)oxyisoxazole **19** matched those previously reported.[22]

General Procedure for the Synthesis of Bis(ammonio)hexanes 7a−10a and 8b−10b. Equimolar amounts of oxotremorine **3** (or dimethylamines **13**, **16**, and **19**) and monoquaternary bromide **24a** (or **24b**) were dissolved in acetonitrile (30 mL/mmol) and refluxed for 2−4 days. After the reaction was completed (TLC monitoring, eluent: CH_3OH/0.2 M aqueous NH_4NO_3 3:2), about one-half of the solvent was evaporated and the colorless solids obtained were collected by filtration and crystallized from 2-propanol/methanol. Crystalline precipitates were filtered, washed with diethyl ether, and dried in vacuo to give the desired salts in 35 to 68% yield.

2-{3-[1-(6-{1-[4-(2-Oxopyrrolidin-1-yl)but-2-ynyl]-pyrrolidinium}hexyl)-1,1-dimethylammonio]propyl}isoindoline-1,3-dione Dibromide 7a. Mp 179 °C (from 2-propanol/methanol 3:2). Anal. $C_{31}H_{46}Br_2N_4O_3$ (C, H, N).

2-{3-[1-(6-{1,1-Dimethyl-1-[4-(2-oxopyrrolidin-1-yl)but-2-ynyl]ammonium}hexyl)-1,1-dimethylammonio]propyl}isoindoline-1,3-dione Dibromide 8a. Mp 206 °C (from 2-propanol/methanol 3:2). Anal. $C_{29}H_{44}Br_2N_4O_3$ (C, H, N).

2-{3-[1-(6-{1,1-Dimethyl-1-[4-(2-oxopyrrolidin-1-yl)but-2-ynyl]ammonium}hexyl)-1,1-dimethylammonio]-2,2-dimethylpropyl}benzo[*de*]isoquinoline-1,3-dione Dibromide 8b. Mp 192 °C (from 2-propanol/methanol 4:1). Anal. $C_{35}H_{50}Br_2N_4O_3$ (C, H, N).

2-{3-[1-(6-{1,1-Dimethyl-1-[4-(3-oxoisoxazolidin-2-yl)but-2-ynyl]ammonium}hexyl)-1,1-dimethylammonio]propyl}isoindoline-1,3-dione Dibromide 9a. Mp 197 °C (from 2-propanol/methanol 7:3). Anal. $C_{28}H_{42}Br_2N_4O_4$ (C, H, N).

2-{3-[1-(6-{1,1-Dimethyl-1-[4-(3-oxoisoxazolidin-2-yl)but-2-ynyl]ammonium}hexyl)-1,1-dimethylammonio]-2,2-dimethylpropyl}benzo[*de*]isoquinoline-1,3-dione Dibromide 9b. Mp 184 °C (from 2-propanol/methanol 3: 2). Anal. $C_{34}H_{48}Br_2N_4O_4$ (C, H, N).

2-{3-[1-(6-{1,1-Dimethyl-1-[4-(isoxazol-3-yloxy)but-2-ynyl]-ammonium}hexyl)-1,1-dimethylammonio]propyl}isoindoline-1,3-dione Dibromide 10a. Mp 202 °C (from 2-propanol/methanol 4:1) Anal. $C_{28}H_{40}Br_2N_4O_4$ (C, H, N).

2-{3-[1-(6-{1,1-Dimethyl-1-[4-(isoxazol-3-yloxy)but-2-ynyl]-ammonium}hexyl)-1,1-dimethylammonio]-2,2-dimethylpropyl}-benzo[*de*]isoquinoline-1,3-dione Dibromide 10b. Mp 210 °C (from 2-propanol/methanol 4:1). Anal. $C_{34}H_{46}Br_2N_4O_4$ (C, H, N).

Radioligand Binding Assays. The binding of [^{3}H]*N*-methylscopolamine ([^{3}H]NMS) (0.2 nM; specific activity 70−83.5 Ci/mmol; Perkin-Elmer Life Sciences, Boston, MA) to porcine heart homogenates was measured as described previously.[40] Measurements were carried out in a buffer composed of 4 mM Na_2HPO_4 and 1 mM KH_2PO_4 (pH 7.4) at 23 °C. Nonspecific [^{3}H]NMS binding was determined in the presence of 10^{-6} M atropine and was less than 5% of the total binding. Membranes were separated by vacuum filtration through glass fiber filters (Schleicher and Schüll, No. 6; Dassel, Germany) and membrane-bound radioactivity was determined by liquid scintillation counting. The pK_D of NMS binding was 9.98 ± 0.06 (mean ± SEM, n = 4). [^{3}H]NMS dissociation is monophasic; its $t_{1/2,control}$ amounted to 5.2 ± 0.1 min; mean ± SEM, n = 60). For studying test compound effects on [^{3}H]NMS dissociation, cardiac membranes were prelabeled with [^{3}H]NMS (0.2 nM) for 30 min, and radioligand dissociation was then revealed by the addition of 1 μM atropine, either in the absence or in the presence of allosteric modulator. Measurements were performed as two-point kinetic experiments[41] with specific [^{3}H]NMS binding being recorded at t = 0 and t = 10 min.

The action of the test compounds on [^{3}H]NMS equilibrium binding was screened using the equieffective concentration $EC_{0.25,diss}$, i.e., the concentration of test compound that reduces [^{3}H]NMS dissociation to 25% of the control. The appropriate time of incubation was determined according to Lazareno and Birdsall[42] (eq 31 therein) with five dissociation half-lives being used to obtain equilibrium binding conditions. Equilibrium binding data (from three independent experiments with quadruplicated values) in the presence of allosteric modulator were expressed in percent relative to the control binding in the absence of modulator, which was set as 100%. Nonlinear regression analysis of data and curve plotting was carried out using the software Prism (version 3.02, GraphPAD Software, San Diego, CA).

Studies with Isolated Organs. The experiments were performed applying experimental procedures supervised and approved by the "Ministero della Salute" and associated guidelines in the European Communities Council Directive of November 24, 1986 (86/609/ECC). Male New Zealand white rabbits (3.0−3.5 kg) and male guinea pigs (300−400 g) were fasted 18 h before the experiments and killed by CO_2 inhalation. Rabbit vas deferens, guinea pig left atrium, terminal ileum, and urinary bladder were rapidly excised and set up as previously described in detail.[22,23] Briefly, the prostatic portion of each vas deferens, suspended under 0.75 g load in a modified Krebs solution at 31 °C (according to Eltze[36]), and guinea pig left atrium, mounted under 0.5 g load in Krebs−Henseleit solution at 32 °C bubbled with 95% O_2−5% CO_2, were electrically stimulated through platinum electrodes (0.05 Hz, 0.5 ms, supramaximal voltage for rabbit vas deferens and 2 Hz, 5 ms, 5V for guinea pig atrium). Guinea pig terminal ileum and longitudinal strips of urinary bladder were suspended in isolated organ baths containing Krebs−Henseleit solution at 37 °C bubbled with 95% O_2−5% CO_2 and kept respectively at 37 °C under a tension of 1 g or at 35 °C under 2 g preload. Changes in mechanical activity of the tissues were recorded isometrically after a 30 min equilibration period. Cumulative concentration−response curves to the compounds under study or to the conventional M_1 (McN-A-343 = 4-[*N*-(3-chlorophenyl)carbamoyloxy]-2-butynyltrimethylammonium chloride) and M_2/M_3 (Bethanechol) full agonists were obtained by increasing the concentration of the drugs by 0.3 log units (in the range 1 nM to 100 μM) according to Van Rossum's method.[43]

The apparent potency of the agonists was expressed as their pEC_{50} value (−log of the molar concentration which gives 50% of the maximum response) calculated by linear regression analysis of the concentration−response curves using the least-squares method. Intrinsic activity (α) was determined by comparing the maximum response to the test compounds with that to the conventional full agonist. Apparent affinity pK_D of partial agonists (α = 0.3−0.4) was estimated through McKay's method,[44] by comparing their concentration−response curves with that of the full agonist bethanechol. Compounds devoid of intrinsic activity (α < 0.3) were studied as antagonists: concentration−response curves to McN-A-343 or bethanechol were constructed in the absence and in the presence of the test compounds (1−100 μM) left in contact with

the tissues for 30 min. The antagonistic potency (pK_B) was calculated according to Furchgott's method.[45] Data are presented as mean ± SEM ($n = 6-8$ independent experiments).

[^{35}S]GTP Binding Assay. Cell culture and [^{35}S]GTPγS binding experiments were carried out as described previously.[46] In short, Chinese hamster ovary (CHO) cells stably transfected with the human M_2 receptor gene (kindly donated by Prof. Dr. G. Lambrecht, Department of Pharmacology, Biocenter Niederursel, University of Frankfurt/Main, Germany) were cultured under the conditions described before and treated for 24 h before harvesting with 5 mM sodium butyrate in order to boost receptor expression. After harvesting the cells, a membrane suspension was prepared for use in the [^{35}S]GTPγS binding experiments (0.07 nM [^{35}S]GTPγS, 10 μM GDP, 10 mM HEPES, 100 mM NaCl, 10 mM $MgCl_2$, pH 7.4; test compounds at the indicated concentrations). After an incubation period of 60 min at 30 °C, the incubation medium was filtered through glass fiber filters and filter bound radioactivity was measured by scintillation counting. As determined in homologous competition experiments with [^{3}H]NMS, the density of M_2 receptors amounted to about 6 pmol/mg membrane protein.

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Supporting Information Available: Microanalyses and NMR data are available free of charge via the Internet at http://pubs.acs.org.

References

(1) Felder, C. C.; Bymaster, F. P.; Ward, J.; DeLapp, N. Therapeutic opportunities for muscarinic receptors in the central nervous system. *J. Med. Chem.* **2000**, *43*, 4333−4353, and references therein.
(2) Eglen, R. M.; Choppin, A.; Watson, N. Therapeutic opportunities from muscarinic receptor research. *Trends Pharmacol. Sci.* **2001**, *22*, 409−414, and references therein.
(3) Hulme, E. C.; Birdsall, N. J. M.; Buckley, N. J. Muscarinic receptor subtypes. *Annu. Rev. Pharmacol. Toxicol.* **1990**, *30*, 633−673.
(4) Caulfield, M. P.; Birdsall, N. J. M. International Union of Pharmacology. XVII. Classification of muscarinic acetylcholine receptors. *Pharmacol. Rev.* **1998**, *50*, 279−290.
(5) Wess, J. Novel insights into muscarinic acetylcholine receptor function using gene targeting technology. *Trends Pharmacol. Sci.* **2003**, *24*, 414−420.
(6) Wess, J.; Duttaroy, A.; Gomeza, J.; Zhang, W.; Yamada, M.; Felder, C. C.; Bernardini, N.; Reeh, P. W. Muscarinic receptor subtypes mediating central and peripheral antinociception studied with muscarinic receptor knockout mice: a review. *Life Sci.* **2003**, *72*, 2047−2054.
(7) Christopoulos, A. Allosteric binding sites on cell-surface receptors: novel targets for drug discovery. *Nature Rev. Drug Discovery* **2002**, *1*, 198−210, and references therein.
(8) Leppik, R. A.; Miller, R. C.; Eck, M.; Paquet, J. L. Role of acidic amino acids in the allosteric modulation by gallamine and antagonist binding at the M_2 muscarinic acetylcholine receptors. *Mol. Pharmacol.* **1994**, *45*, 983−990.
(9) Tuček, S.; Proška, J. Allosteric modulation of muscarinic acetylcholine receptors. *Trends Pharmacol. Sci.* **1995**, *16*, 205−212.
(10) Ellis, J. Allosteric binding site on muscarinic receptors. *Drug. Dev. Res.* **1997**, *40*, 193−204.
(11) Holzgrabe, U.; Mohr, K. Allosteric modulators of ligand binding to muscarinic acetylcholine receptors. *Drug Discovery Today* **1998**, *3*, 214−222.
(12) Birdsall, N. J. M.; Lazareno, S. Allosterism at muscarinic receptors: ligands and mechanisms. *Mini Rev. Med. Chem.* **2005**, *5*, 523−543.
(13) Mohr, K.; Tränkle, C.; Holzgrabe, U. Structure/activity-relationships of M_2 muscarinic allosteric modulators. *Recept. Channels* **2003**, *9*, 229−240.
(14) Voigtländer, U.; Jöhren, K.; Mohr, M.; Raasch, A.; Tränkle, C.; Buller, S.; Ellis, J.; Höltje, H.-D.; Mohr, K. Allosteric site on muscarinic acetylcholine receptors: identification of two amino acids in the muscarinic M_2 receptor that account entirely for the M_2/M_5 subtype selectivities of some structurally diverse allosteric ligands in *N*-methylscopolamine-occupied receptors. *Mol. Pharmacol.* **2003**, *64*, 21−31.
(15) Huang, X. P.; Prilla, S.; Mohr, K.; Ellis, J. Critical amino acid residues of the common allosteric site on the M_2 muscarinic acetylcholine receptor: more similarities than differences between the structurally divergent agents gallamine and bis(ammonio)alkane-type W84. *Mol. Pharmacol.* **2005**, *68*, 769−778.
(16) Jepsen, K.; Lüllmann, H.; Mohr, K.; Pfeffer, J. Allosteric stabilization of ^{3}H-*N*-methylscopolamine binding in guinea-pig myocardium by an antidote against organophosphorus intoxication. *J. Pharmacol. Toxicol.* **1988**, *63*, 163−168.
(17) Tränkle, C.; Mies-Klomfass, E.; Botero Cid, H. M.; Holzgrabe, U.; Mohr, K. Identification of a [^{3}H] ligand for the common allosteric site of muscarinic acetylcholine M_2 receptors. *Mol. Pharmacol.* **1998**, *54*, 139−145.
(18) Muth, M.; Bender, W.; Scharfenstein, O.; Holzgrabe, U.; Balatkova, E.; Tränkle, C.; Mohr, K. Systematic development of high affinity bis(ammonio)alkane-type allosteric enhancers of muscarinic ligand binding. *J. Med. Chem.* **2003**, *46*, 1031−1040.
(19) Muth, M.; Sennwitz, M.; Mohr, K.; Holzgrabe, U. Muscarinic allosteric enhancers of ligand binding: pivotal pharmacophoric elements in hexamethonio-type agents. *J. Med. Chem.* **2005**, *48*, 2212−2217.
(20) Tränkle, C.; Andresen, I.; Lambrecht, G.; Mohr, K. M_2 receptor binding of the selective antagonist AF-DX 384: possible involvement of the common allosteric site. *Mol. Pharmacol.* **1998**, *53*, 304−312.
(21) Lüllmann, H.; Ohnesorge, F. K.; Schauwecker, G. C.; Wassermann, O. Inhibition of the actions of carbachol and DFP on guinea pig isolated atria by alkane-bis-ammonium compounds. *Eur. J. Pharmacol.* **1969**, *6*, 241−247.
(22) Dallanoce, C.; Conti, P.; De Amici, M.; De Micheli, C.; Barocelli, E.; Chiavarini, M.; Ballabeni, V.; Bertoni, S.; Impicciatore, M. Synthesis and functional characterization of novel derivatives related to oxotremorine and oxotremorine-M. *Bioorg. Med. Chem.* **1999**, *7*, 1539−1547.
(23) Barocelli, E.; Ballabeni, V.; Bertoni, S.; Dallanoce, C.; De Amici, M.; De Micheli, C.; Impicciatore, M. New analogues of oxotremorine and oxotremorine-M: estimation of their in vitro affinity and efficacy at muscarinic receptor subtypes. *Life Sci.* **2000**, *67*, 717−723.
(24) Barocelli, E.; Ballabeni, V.; Bertoni, S.; De Amici, M.; Impicciatore, M. Evidence for specific analgesic activity of muscarinic agonists selected among a new series of acetylenic derivatives. *Life Sci.* **2001**, *68*, 1775−1785.
(25) De Amici, M.; Conti, P.; Fasoli, E.; Barocelli, E.; Ballabeni, V.; Bertoni, S.; Impicciatore, M.; Roth, B. L.; Ernsberger, P.; De Micheli, C. Synthesis and in vitro pharmacology of novel heterocyclic muscarinic ligands. *Il Farmaco* **2003**, *58*, 739−748.
(26) Masuyama, Y.; Watabe, A.; Ito, A.; Kurusu, Y. Carbonyl propargylation by 1-substituted prop-2-ynyl mesylates and carbonyl allenylation by 3-substituted prop-2-ynyl mesylates with tin (II) iodide and tetrabutylammonium iodide. *Chem. Commun.* **2000**, 2009−2010.
(27) Nilsson, B. M.; Vargas, H. M.; Hacksell, U. Urea and 2-imidazolone derivatives of the muscarinic agents oxotremorine and *N*-methyl-*N*-(1-methyl-4-pyrrolidino-2-butynyl)acetamide. *J. Med. Chem.* **1992**, *35*, 3270−3279.
(28) Bebbington, A.; Shakeshaft, D. An improved synthesis of oxotremorine. *J. Med. Chem.* **1965**, *8*, 274−275.
(29) Garvey, D. S.; Wasicak, J. T.; Chung, J. Y.-L.; Shue, Y.-K.; Carrera, G. M.; May, P. D.; McKinney, M. M.; Anderson, D.; Cadman, E.; Vella-Rountree, L.; Nadzan, A. M.; Williams, M. Synthesis and in vitro characterization of novel amino terminally modified oxotremorine derivatives for brain muscarinic receptors. *J. Med. Chem.* **1992**, *35*, 1550−1557, and references therein.
(30) Iwai, I.; Nakamura, N. Studies on acetylenic compounds. XLIV. Synthesis of 3-aminoisoxazoles and 3-hydroxyisoxazoles (3-isoxazolones). *Chem. Pharm. Bull.* **1966**, *14*, 1277−1286.
(31) Bender, W.; Staudt, M.; Mohr, K.; Holzgrabe, U. Probing the size of a hydrophobic binding pocket within the allosteric site of muscarinic acetylcholine M_2-receptors. *Life Sci.* **2000**, *66*, 1675−1682.
(32) Ehlert, F. J. Estimation of the affinities of allosteric ligands using radioligand binding and pharmacological null methods. *Mol. Pharmacol.* **1988**, *33*, 187−194.
(33) Stockton, J. M.; Birdsall, N. J. M.; Burgen, A. S. V.; Hulme, E. C. Modification of the binding properties of the muscarinic receptors by gallamine. *Mol. Pharmacol.* **1983**, *23*, 551−557.

(34) Gilsbach, R.; Grossmüller, M.; Alptuzun, V.; Erciyas, E.; Tränkle, C.; Holzgrabe, U.; Mohr, K. Cooperative interactions at M_2 muscarinic acetylcholine receptors: structure/activity relationships in stepwise shortened bispyridinium- and bis(ammonio)alkane-type allosteric modulators. *Neurochem. Res.* **2003**, *28*, 667−673.
(35) Schmalenbach, C.; Muth, M.; Tränkle, C.; Holzgrabe, U.; Mohr, K. Effect of the muscarinic alloster naphmethonium on acetylcholine induced G protein activation. *Book of Abstracts*, Annual Meeting of the German Pharmaceutical Society, Würzburg, P P15, page 104, 2003.
(36) Eltze, M. Muscarinic M_1 and M_2 receptors mediating opposite effects on neuromuscular transmission in rabbit vas deferens. *Eur. J. Pharmacol.* **1988**, *151*, 205−221.
(37) Harvey, A. L.; Kornisiuk, E.; Bradley, K. N.; Cervenansky, C.; Durán, R.; Sánchez, G.; Jerusalinsky, D. Effects of muscarinic toxins MT1 and MT2 from green mamba on different muscarinic cholinoceptors. *Neurochem. Res.* **2002**, *27*, 1543−1554.
(38) Ringdahl, B. Selectivity of partial agonists related to oxotremorine based on differences in muscarinic receptor reserve between the guinea pig ileum and urinary bladder. *Mol. Pharmacol.* **1987**, *31*, 351−356.
(39) Nassif-Makki, T.; Tränkle, C.; Bejeuhr, G.; Cambareri, A.; Pfletschinger, C.; Kostenis, E.; Mohr, K.; Holzgrabe, U. Bisquaternary ligands of the common allosteric site of M_2 acetylcholine receptors: Search for the minimum essential distances between the pharmacophoric elements. *J. Med. Chem.* **1999**, *42*, 849−858.
(40) Zlotos, D. P.; Buller, S.; Stiefl, N.; Baumann, K.; Mohr, K. Probing the pharmacophore for allosteric ligands of muscarinic M_2 receptors: SAR and QSAR studies in a series of bisquaternary salts of caracurine V and related ring systems. *J. Med. Chem.* **2004**, *47*, 3561−3571.
(41) Kostenis, E.; Mohr, K. Two-point kinetic experiments to quantify allosteric effects on radioligand dissociation. *Trends Pharmacol Sci.* **1996**, *17*, 280−283.
(42) Lazareno, S.; Birdsall, N. J. M. Detection, quantitation, and verification of allosteric interactions of agents with labeled and unlabeled ligands at G-protein-coupled receptors: interactions of strychnine and acetylcholine at muscarinic receptors. *Mol. Pharmacol*. **1995**, *48*, 362−378.
(43) Van Rossum, J. M. Cumulative dose−response curves. II. Technique for the making of dose−response curves in isolated organs and the evaluation of drug parameters. *Arch. Int. Pharmacodyn*. **1963**, *143*, 299−330.
(44) McKay, D. A. General analysis of the receptor-drug interaction. *Br. J. Pharmacol.* **1966**, *26*, 9−16.
(45) Furchgott, R. F. The classification of adrenoceptors (adrenergic receptors). An evaluation from the standpoint of receptor theory. *Handbook of Experimental Pharmacology*; Blanschko, H., Muscholl, E., Eds.; Springer: New York, 1972; Issue 33, pp 283−335.
(46) Zahn, K.; Eckstein, N.; Tränkle, C.; Sadee, W.; Mohr, K. Allosteric modulation of muscarinic receptor signaling: alcuronium-induced conversion of pilocarpine from an agonist into an antagonist. *J. Pharmacol. Exp. Ther.* **2002**, *2*, 720−728. bladder. *Mol. Pharmacol.* **1987**, *31*, 351−356.

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