Bisquaternary Ligands of the Common Allosteric Site of M_2 Acetylcholine Receptors: Search for the Minimum Essential Distances between the Pharmacophoric Elements

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Structurally diverse molecules, such as alcuronium, gallamine, and tubocurarine as well as W84 and WDUO, are known to interact allosterically with ligand binding to muscarinic M_2 acetylcholine receptors. Preliminary molecular modeling studies revealed two positive charges in the middle and two lateral aromatic areas to be essential elements of a high allosteric potency. To find out the optimum distances between these pharmacophoric elements, a systematic variation of the spacer in the series of W84, WDUO, and IWDUO compounds was performed. The allosteric reduction of the rate of dissociation of the antagonist [^{3}H]-*N*-methylscopolamine from porcine heart M_2 receptors served as a test system. The minimal essential distance between the positive charges was found to be 10 Å. The length of the peripheral spacers connecting the positive charge and the lateral aromatic moiety appears to depend on the chemical functionality; the peripheral spacers have to be long and flexible enough to position the aromatic skeletons in the spatial neighborhood of the alkane middle chain: in the case of an oxime ether containing peripheral spacer, six atoms are required, and in the case of an alkane chain, four carbon atoms are necessary to adopt the pharmacophoric S-shape conformation.

Introduction

The concept of allosteric modulation of proteins induced by an effector that interacts with a specific site topologically distinct from the substrate binding site is well-established for enzymes.[1] Recently, this phenomenon has also been discussed for nicotinic[2−4] and muscarinic acetylcholine receptors.[5−8] For the latter receptors, structurally heterogeneous molecules have been found to bind to ligand-occupied receptors and to diminish the rate of dissociation of the ligand. This stabilizing effect on ligand binding is induced by, e.g., alcuronium,[9] gallamine,[10] tubocurarine,[11] and newly synthesized bisquaternary molecules derived from hexamethonium[12,13] and the bispyridinium compound TMB-4,[14,15] respectively. The delay of ligand dissociation may result in a receptor subtype-specific elevation of ligand equilibrium binding which opens various therapeutic perspectives, such as for the therapy of dementia or pain.[7] Therefore, efforts are made to develop modulators with specificity and high affinity to ligand-occupied receptors. Often, muscarinic M_2 receptors occupied by the antagonist *N*-methylscopolamine (NMS) are applied as a model system. The molecular structure of the allosteric binding site is not yet known. To specify the location of the allosteric site, Jakubik and Tucek[16] pretreated membranes with dithiothreitol and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide and measured the effect of alcuronium on the [^{3}H]NMS binding. From the results they suggested that two acidic amino acids were responsible for the interaction between alcuronium and the muscarinic M_2 receptor, one located at the second extracellular loop and another more deeply inside the transmembranal region of the receptor protein. A more indirect approach to characterize the allosteric binding site is to look at the structures of the ligands which bind to this site. Tränkle et al.[17] reported a ranking of the potency of the structurally different ligands to interact with NMS-occupied receptors: alcuronium > W84 > gallamine > tubocurarine. Molecular modeling studies using these effectors resulted in a hypothesis of the pharmacophore consisting of two positively charged groups and two aromatic areas in a distinct spatial arrangement, which can be described as an S-shape (Figure 1).[18] This hypothesis could be confirmed by a 3D QSAR analysis in an extended series of compounds consisting of various alkane bisammonium and bispyridinium derivatives.[19] In this model the positive charges may interact with acidic amino acids of the receptor protein as proposed by Jakubik and Tucek,[16] and the aromatic rings may find hydrophobic spaces for interaction. According to this hypothesis derived from pharmacological studies and theoretical calculations, the allosteric potency of the modulators should be sensitively dependent on the distance between the centers of the positive charges. To test the hypothesis, we set out to synthesize derivatives of the W84-, IWDUO-, and WDUO-type with varying lengths of the interquaternary middle chain (*m*) (Scheme 1). All compounds contained the phthalimido moiety as bilateral aromatic substituents, which has previously been found to provide a high allosteric activity and which probably guides a specific orientation at NMS-occupied M_2 receptors.[20] For some of the compounds, i.e., W84-3/6, W84-3/7, and WDUO-

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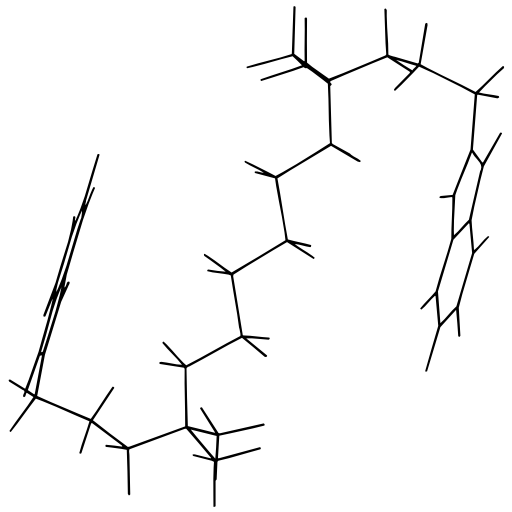
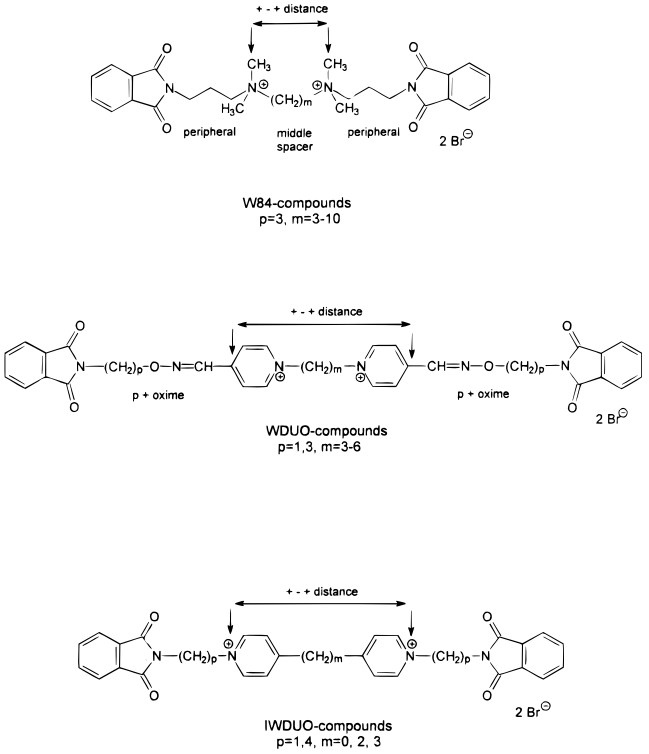


Figure 1. Postulated pharmacophoric conformation of W84.

Scheme 1. Schematic Structural Formulas of the Compounds Studied[a]

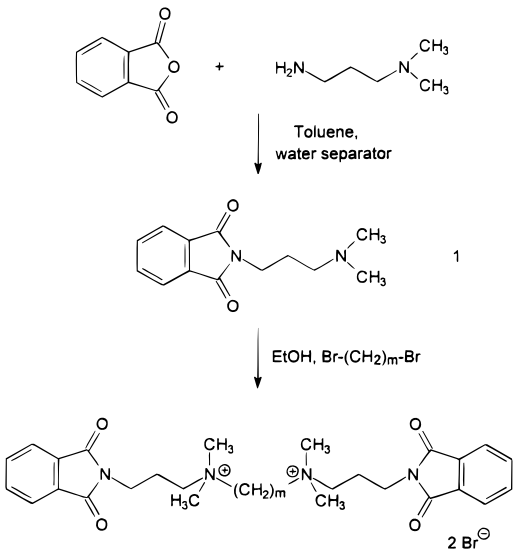


[a] *p* indicates the part varied in the peripheral spacer, *m* the middle spacer.

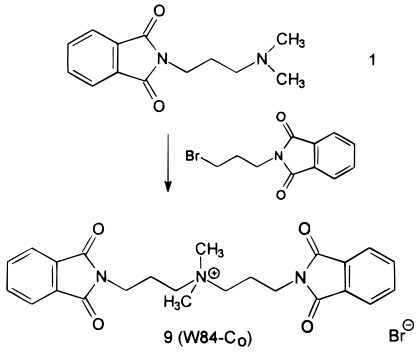
1/3, evidence has been reported[21−23] for an interaction with the common allosteric site of the M_2 receptor.[24] In porcine heart homogenates the potency of the compounds was measured to interact with [^{3}H]NMS-occupied M_2 receptors. Assuming an S-shaped conformation of the modulators to be a prerequisite for binding, the change in the length of the middle chain (*m*) will be accompanied by an alteration of the orientation of the lateral aromatic moieties. In addition, the position of the aromatic rings relative to the middle chain can be altered by changing the distance between the positive charges and the peripheral rings (*p*). To find to what extent these modifications are important, we tried to vary systematically both spacers *m* and *p* (Scheme 1).

Previous conformational studies utilizing molecular dynamics and scan options[19] revealed especially the alkane bisammonium compounds to be highly flexible. Thus, molecular modeling in the new series of compounds studied here will hardly lead to meaningful quantitative structure−activity relationships. However, to better understand the structural requirements for high allosteric potency, we performed selected modeling experiments utilizing the template approach which has already been proven worthwhile.[18,19]

Scheme 2. Synthesis Pathway of the W84 Compounds **2**−**9**



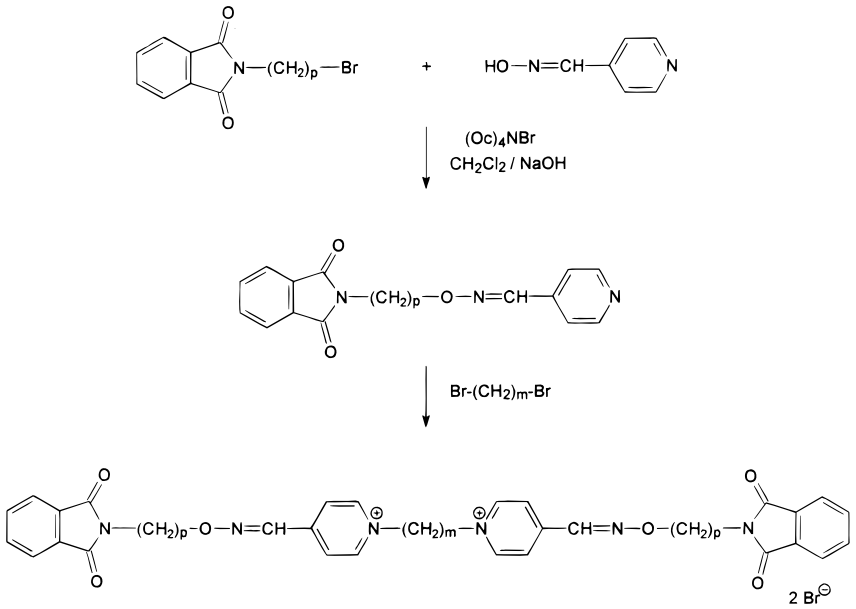
m	No.	Abbreviations
3	2	W84-3/3
4	3	W84-3/4
5	4	W84-3/5
6	5	W84-3/6
7	6	W84-3/7
8	7	W84-3/8
10	8	W84-3/10



Synthesis

W84 can be obtained by conversion of bis(dimethylamino)hexane with two molecules of phthalimidopropane bromide.[25] Owing to the nonavailability of bis(dimethylamino)alkanes of varying chain lengths, a two-step pathway has to be used for the other compounds (Scheme 2). Refluxing of phthalic acid anhydride in the presence of (dimethylamino)propylamine on a water separator gave the imide derivative **1**.[13] The connection

Scheme 3. Synthesis Pathway of the WDUO Compounds **10-16**



p	m	No.	Abbreviations
1	3	10	WDUO-1/3
1	4	11	WDUO-1/4
1	6	12	WDUO-1/6
3	3	13	WDUO-3/3
3	4	14	WDUO-3/4
3	5	15	WDUO-3/5
3	6	16	WDUO-3/6

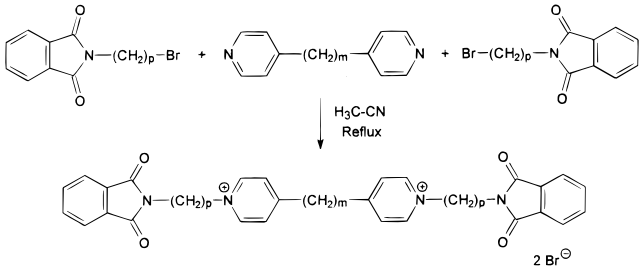
of two molecules of **1** could be achieved by refluxing the reaction mixture with dibromoalkanes in a polar solvent, e.g., acetone or ethanol, for at least 16 h. After cooling, the bisquaternary substances **2**−**8** crystallized from the reaction solution directly in rather high purity.

The derivative W84-3/0 (**9**), lacking any interquaternary chain, was synthesized by the N-alkylation of the *N*,*N*-dimethyl-*N*-(3-phthalimidopropyl)amine (**1**) with phthalimidopropane bromide.

The WDUO derivatives were synthesized in analogy to Bejeuhr et al.[12] (see Scheme 3): The oxime ether was obtained by a Williamson analogue conversion of pyridine-4-carboxaldehyde oxime and phthalimidoalkane bromide in the presence of the phase-transfer catalyst tetraoctylammonium bromide, aqueous sodium hydroxide solution, and dichloromethane. After purification by means of column chromatography, 2 mol of the ether was connected with the corresponding dibromoalkane by refluxing in acetonitrile. Whereas derivatives with an ethylene chain in the periphery and in the middle were not formed due to side reactions, WDUO-1/3, -1/4, and -1/6 (**10**−**12**) as well as WDUO-3/3, -3/4, -3/5, and -3/6 (**13**−**16**) could be isolated in rather high yields.

The bispyridinium compounds with structurally inverted pyridine rings (IWDUO) **17**−**23** were built up by N-alkylation of commercially available bispyridines of varying alkane chain with phthalimidoalkane bromides in acetonitrile (Scheme 4).

Scheme 4. Synthesis Pathway of the IWDUO Compounds **17-23**



p	m	No.	Compound
1	3	17	IWDUO-1/3
2	3	18	IWDUO-2/3
3	3	19	IWDUO-3/3
4	3	20	IWDUO-4/3
4	0	21	IWDUO-4/0
4	2	22	IWDUO-4/2
3	2	23	IWDUO-3/2

Pharmacology

In porcine cardiac M_2 receptors we measured the ability of the compounds to retard the dissociation of the radioligand [^{3}H]-*N*-methylscopolamine ([^{3}H]NMS; control $t_{1/2}$ = 2 min). The compounds reduced the apparent rate constant k_{-1} of [^{3}H]NMS dissociaton concentration dependently. All concentration−effect curves approached the zero level of k_{-1} at high concen-

Table 1. Pharmacological Data (EC_{50} values, Hill coefficient n_H, SE in parentheses) Distances between the Centers of the Positive Charges and the Total Length

no.	compd	EC_{50} (μM)	log-($1/EC_{50}$)	n_H	+−+ distance (Å)	total length (Å)
	W84-3/*m*					
2	W84-3/3	3.80	5.42 (±0.06)	0.90 (±0.10)	5.34	23.94
3	W84-3/4	2.69	5.59 (±0.04)	1.08 (±0.15)	6.59	25.25
4	W84-3/5	1.39	5.89 (±0.01)	1.00 (±0.02)	7.79	26.36
5	W84-3/6[a]	1.44	5.85 (±0.06)	0.95 (±0.10)	9.13	27.76
6	W84-3/7[a]	0.37	6.43 (±0.07)	0.90 (±0.06)	10.34	28.91
7	W84-3/8	0.57	6.30 (±0.02)	1.01 (±0.04)	11.66	30.30
8	W84-3/10	0.53	6.30 (±0.01)	0.94 (±0.03)	14.22	32.90
9	W84-3/0	8.82	5.06 (±0.03)	0.85 (±0.05)		
	WDUO-*p*+ oxime/*m*					
10	WDUO-1/3[a]	0.51	6.29 (±0.03)	1.21 (±0.10)	10.30	29.26
11	WDUO-1/4	1.90	5.72 (±0.04)	1.10 (±0.09)	11.93	31.97
12	WDUO-1/6	1.40	5.85 (±0.05)	1.10 (±0.12)	14.36	34.05
13	WDUO-3/3	0.52	6.28 (±0.06)	1.07 (±0.15)	10.14	33.74
14	WDUO-3/4	0.35	6.45 (±0.07)	1.06 (±0.07)	11.83	36.77
15	WDUO-3/5	0.51	6.30 (±0.05)	0.98 (±0.11)	12.68	36.61
16	WDUO-3/6	0.45	6.35 (±0.04)	1.11 (±0.10)	14.24	39.05
	IWUO-*p*/*m*					
17	IWDUO-1/3	50.8	4.29 (±0.05)	0.88 (±0.10)	10.43	20.86
18	IWDUO-2/3	3.49	5.46 (±0.03)	0.95 (±0.07)	10.29	25.41
19	IWDUO-3/3	4.87	5.31 (±0.02)	1.10 (±0.07)	10.36	25.25
20	IWDUO-4/3	0.63	6.20 (±0.03)	1.06 (±0.04)	10.07	31.02
21	IWDUO-4/0	4.65	5.33 (±0.03)	1.03 (±0.09)	7.21	28.05
22	IWDUO-4/2	0.47	6.33 (±0.06)	1.32 (±0.20)	9.45	30.53
23	IWDUO-3/2	2.34	5.63 (±0.05)	0.88 (±0.10)	9.42	26.62

[a] W84-3/6 reported in ref 25, there denoted as W84; W84-3/7 reported ref 29, there denoted as C7/3′-phth; WDUO-1/3.[12]

trations of the modulators. The drug concentration inducing a reduction of k_{-1} to 50% of the control value (EC_{50}) served as a measure of the allosteric potency (Table 1). The EC_{50} can be taken to reflect the concentration for half-maximum occupancy of the NMS−receptor complexes, i.e., the K_D value of alloster binding.[22]

Results and Discussion

The potency of all compounds to interact allosterically with [^{3}H]NMS-occupied receptors as well as distances between the positive charges and the total length of the molecules are compiled in Table 1; the stability of selected compounds is given in Table 2.

W84 has been found to hydrolyze in different buffered media, e.g., 3.6 mM $MgHPO_4$/50 mM Tris buffer, within a half-life of some 5.5 h at 37 °C.[26] To make sure that all compounds are stable over the course of the pharmacological testing (about 2 h), the hydrolysis of each derivative has been observed UV spectroscopically in this buffer at 20 °C for 24 h. As expected, the half-lives are found to be in the same range for all W84 derivatives. WDUO derivatives turned out to be rather stable: After a period of 24 h no decomposition was observed. Randomly tested IWDUO derivatives showed similar results. Thus, no hydrolysis problems will arise in the course of pharmacological testing.

Table 2. Half-Lives of Chemical Hydrolysis of the W84 Derivatives **2**−**8** in TRIS-Buffered Medium

no.	compd	$t_{1/2}$ (h)
2	W84-3/3	14.5
3	W84-3/4	14.0
4	W84-3/5	17.4
5	W84-3/6	18.0
6	W84-3/7	16.4
7	W84-3/8	35.2
8	W84-3/10	19.3

To check whether the allosteric potency is correlated to the overall lipophilicity of the W84 derivatives, the octanol/water partition coefficient was determined for most of the compounds using the standard procedure.[27,14] Due to dissolution problems of the WDUO derivatives, log k_w values were measured in case of WDUO-1/3, -1/4, and -1/6 (**11**−**13**) using RP-HPLC.[28] In both series of measurements the compounds exhibit a certain extent of hydrophilicity. Accordingly, no solubility problems were observed in the pharmacological experiments.

The log *P* values were determined in the series of W84 compounds; no correlation was found between the allosteric potency and the overall lipophilicity (data not shown). Therefore, no further experiments were carried out in this respect.

Concentration−effect curves for the allosteric delay of [^{3}H]NMS dissociation from muscarinic M_2 receptors are displayed in Figure 2. The EC_{50} concentrations retarding the rate of [^{3}H]NMS dissociation to one-half of the control value and the Hill coefficients characterizing the slope of the curves are included in Table 1. For all compounds the slope factors were not statistically different from unity which is compatible with the assumption that alloster binding to NMS-occupied receptors follows a simple adsorption isotherm with the EC_{50} reflecting the equilibrium dissociation constant K_D.

To inspect the data in more detail, the dependence of the allosteric potency of the W84 derivatives on the length of the interquaternary middle chain is displayed in Figure 3: With increasing length of the alkane chain, the potency is found to reach a maximum for the heptamethonium compound whose length is equivalent to about 10 Å. Molecules with a longer alkyl middle chain appear to be similarly potent. The heptamethonium derivative has already been extensively studied in different tissues by Mitchelson et al.[29]

The results described here resemble the findings of functional experiments performed by Ohnesorge;[30] the ability of W84 derivatives with varying lengths of the interquaternary chain (C_6 to C_9) was studied to antagonize the action of carbachol in isolated beating guinea pig atria. The optimum distance was found to be about eight methylene groups. The inhibition of agonist action

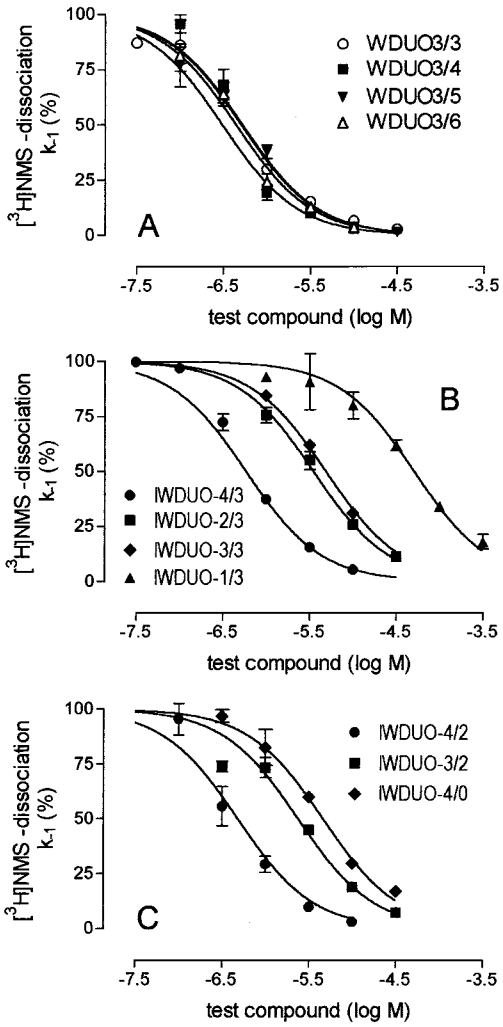


Figure 2. Effect of all compounds studied on the apparent rate constant k_{-1} of [^{3}H]NMS dissociation from muscarinic M_2 receptors, expressed as percentage of the control value. Indicated are mean values ± SE; $n = 1-4$.

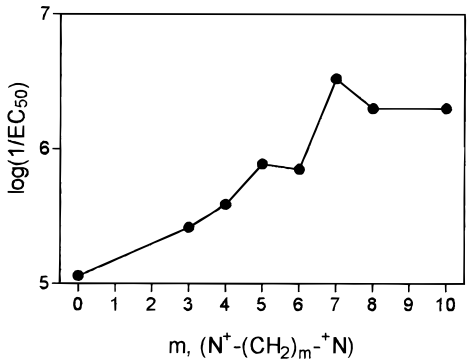


Figure 3. Dependence of the allosteric potency on the length of the middle chain (m) of all W84 compounds. The EC_{50} values were derived from concentration−effect curves based on repeated measurements at 4−6 concentrations for each compound.

under the applied conditions possibly reflects an interaction of the W84 derivatives with free muscarinic M_2 receptors.[31] The correspondence between the results of the functional experiments and our binding experiments at ligand-occupied receptors suggests similar structure−activity relationships for the interaction of W84 derivatives with free and NMS-occupied receptors.

The potency of the bispyridinium derivatives WDUO-1/3 and -3/3 is in the same range as that of W84-3/7, -3/8, and -3/10, respectively. Interestingly, an increase of the length of the interquaternary middle chain does not lead to a significant change in potency in both series. This may indicate that the optimum distance between the positive charges found in W84-3/7 is already realized in WDUO-1/3 and -3/3, respectively. Taking the nitrogens as centers of the positive charges for a comparison of the W84 and the WDUO series, the distances between the charges are seemingly different (hexane versus propane chain). However, semiempirical calculations in the analogous series of DUO derivatives carrying 2,6-dichlorobenzyl substituents instead of phthalimidomethyl groups revealed that the centers of positive charges are located over the carbon atoms in a *para* position to the pyridine nitrogens rather than over the nitrogens.[15] Thus, the distance between both *para* carbon atoms in the pyridinium rings of the WDUO derivatives has to be compared with the distance between the ammonium nitrogen atoms in the W84 series. For this purpose, the structures of W84-3/6, W84-3/7, and WDUO-1/3 were built up and computed according to ref 15. Comparing the so-obtained molecules in a fully extended conformation, we found the distance between the *para* carbon atoms of the pyridinium rings in WDUO-1/3 to be close to the distance between the charges in W84-3/7 (WDUO-1/3, 10.30 Å; W84-3/7, 10.34 Å) and larger than the distance found in the less potent W84-3/6 (9.13 Å). Thus, the 1,3-bispyridinium propane chain in the WDUO derivatives appears to be equivalent to the heptamethonium chain in the W84 derivatives.

Via comparing the corresponding pairs WDUO-1/4/WDUO-3/4 (**11**/**14**) and WDUO-1/6/WDUO-3/6 (**12**/**16**), the extension of the four-membered linker (p (=1) + oxime) between the center of positive charge and the phthalimido moiety (WDUO-1/m) to six members (WDUO-3/m) augmented the potency by a factor of almost 3. In contrast, the corresponding pair WDUO-1/3/WDUO-3/3 (**10**/**13**) shows identical allosteric potency. A reason for this seemingly contradictory behavior might be a complementary conformational change of both the peripheral chain and the middle chain. Whereas a molecule with a three-membered middle chain in combination with a four-membered group in the periphery (1 + oxime) exhibits high potency, an increase of middle linkers while keeping the peripheral chain constant seems to laterally dislocate the aromatic rings from their optimal conformation. If the peripheral chain is then elongated to a propylene + oxime linker, the shift of the lateral rings caused by the longer middle chain can be counterbalanced by the enhanced flexibility of the peripherical linker. In any case, the finding shows that the lateral skeletons contribute to high potency.

Since the chemical variation of both the interquaternary chain m and the linker between the positive charge and the aromatic skeleton p turned out to be difficult in the case of WDUO compounds, electrostatically comparable IWDUO compounds were synthesized with variations in both linkers. The variation of both linkers m and p caused a variation in potency over a range of 2 orders of magnitude in the IWDUO series. Again, the influence of the interquaternary linker m cannot be discussed without consideration of the influence of the

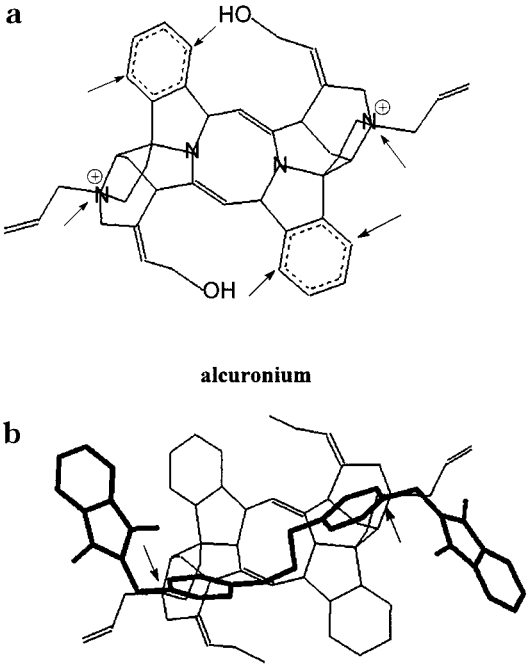


Figure 4. (a) Low-energy conformation of alcuronium; arrows indicate the atoms used for matching. (b) IWDUO-1/3 (bold) fitted onto alcuronium using the corresponding positively charged nitrogens as matching pairs (indicated by arrows).

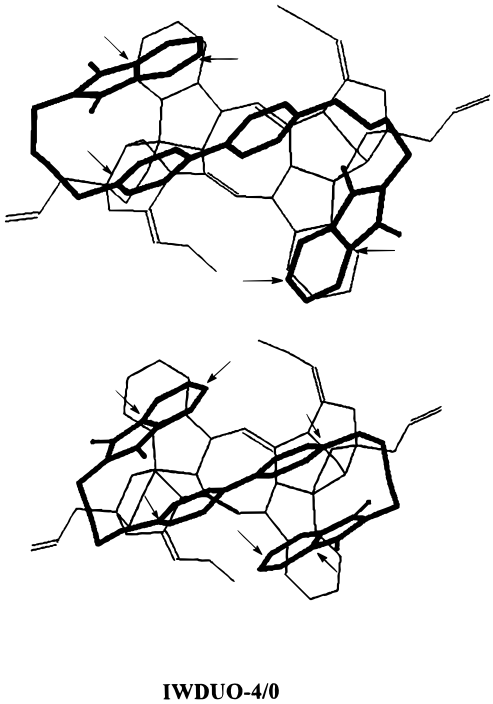


Figure 5. IWDUO-4/0 (bold) fitted onto alcuronium using one corresponding positively charged nitrogen and two aromatic carbon atoms at either ring (above) or a medium position of both positively charged nitrogens in IWDUO-4/0 (same distance to the positive charges of alcuronium) and two aromatic carbon atoms at either ring (below) as matching pairs.

lateral spacer *p* and vice versa. The elongation of the lateral linker ($p = 1-4$) with a simultaneously constant interquaternary distance ($m = 3$) led to a 70-fold amplification of the potency. The exceptionally low allosteric potency of IWDUO1/3 (**18**) can be explained by the inability of the molecule to adopt the S-shape conformation. Upon matching onto alcuronium the lateral phthalimido ring of IWDUO-1/3 cannot reach the aromatic indole area of the template alcuronium (see Figure 4). In turn, the elongation of the interquaternary linker ($m = 0-3$) at a steady lateral distance ($p = 4$) revealed an optimum of this spacer at $m = 2$ with a similar potency at $m = 3$. At present, it can be summarized that within the IWDUO series the optimal distance between the positively charged centers is an ethylene or propylene spacer combined with a butylene lateral chain. Thus, the results in the IDWUO series also support the hypothesis of an S-shape conformation.

In contrast to the WDUO compounds, semiempirical calculations in the IWDUO series[19] revealed the positive charges to be concentrated over the pyridinium nitrogens. The similarity of the molecular electrostatic potential in both series of molecules had a pharmacological equivalence: the sterically homologuous derivatives WDUO-1(+ oxime)/3 and IWDUO-4/3 (compare with Table 1) show almost the same allosteric potency.

Taken together, the results of the variation in length of the linkers *m* and *p* in all three series of compounds suggest that both positive charges have to be at least in a distance of 9.5−10 Å which is contained in alcuronium, WDUO-1/3, IWDUO-4/2 or -4/3, and W84-3/7. The peripheral linker between the center of positive charge and the phthalimide skeleton *p* is an additional determinant of the allosteric potency. It has to be long and flexible enough to enable the phthalimido moiety to adopt the right orientation in the proposed sandwichlike pharmacophoric conformation.[19] The optimum length depends on the chemical nature of the peripheral linker *p*, i.e., alkane or oxime moiety. In case of the sterically more restricted oxime linker, more alkane units than in case of an alkane linker have to be added in order to provide higher flexibility. If one of the linkers, either the peripheral or middle one, does not reach the minimum essential length, the compounds still show a remarkable, yet reduced, potency. For instance; IWDUO-4/0 (**21**) having a middle spacer of 7.21 Å instead of about 10 Å is only 10-fold less active than IWDUO-4/2; the positive charges are not in the right place, but the phthalimides can nevertheless reach the required aromatic position (see Figure 5). This parallels the results obtained with W84-3/4 and -3/5 which also have considerable potency although the middle chain is rather short. On the other hand, IWDUO-2/3 (**18**) and IWDUO-3/3 (**19**) show a comparable weak potency, although the middle chain has the optimal length, however, the lateral spacers are slightly too short to allow the phthalimides to properly reach the aromatic position. These comparisons demonstrate that the structure−activity relationships of the linkers *m* and *p* cannot be considered independent of each other. For a top potency, *all four* pharmacophoric elements must have the chance to occupy the demanded positions. For this, the spacers are of the right length and flexibility no matter which chemical nature they have.

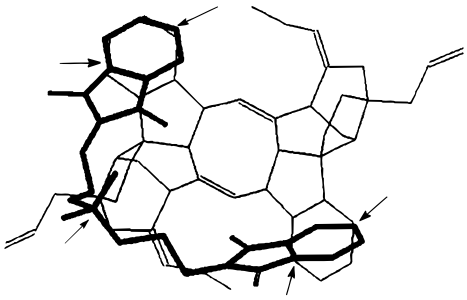
Removing one positive charge in the W84 series (**9**, W84-3/0) by replacement with the phthalimido group led to a similar decrease in potency. Herein, three of the hypothesized four pharmacophoric elements are able to adopt the right position, i.e., one positive charge and

Table 3. Analytical Data of All Compounds

no.	compd	formula (mol wt)	yield (%)	mp (°C)	IR (ν, cm^{-1})
2	W84-3/3	$C_{29}H_{38}N_4O_4Br_2$ (666.1)	38	129−130	3000, 2960, 1770, 1700, 1610, 1460, 720
3	W84-3/4	$C_{30}H_{40}N_4O_4Br_2$ (680.2)	51	214−215	2970, 1770, 1710, 1610, 1460, 1405, 725
4	W84-3/5	$C_{31}H_{42}N_4O_4Br_2$ (694.5)	83	231	3000, 2940, 1770, 1710, 1610, 1460, 1405, 725
7	W84-3/8	$C_{34}H_{48}N_4O_4Br_2$ (736.6)	41	260−261	3030, 2940, 1775, 1710, 1625, 1465, 1400, 725
8	W84-3/10	$C_{36}H_{52}N_4O_4Br_2$ (764.6)	45	240	3000, 2920, 1770, 1700, 1610, 1465, 1400, 715
9	W84-3/0	$C_{24}H_{26}N_3O_4Br$ (500.4)	30	252−253	2940, 1765, 1700 (br), 1605, 1400, 1375, 720
11	WDUO-1/4	$C_{34}H_{30}N_6O_6Br_2$ (778.5)	40	255[a]	1775, 1725, 1640, 1605, 1400, 1350, 725
12	WDUO-1/6	$C_{36}H_{34}N_6O_6Br_2$ (806.5)	36	236[b]	1775, 1725, 1600, 1410, 1400, 730
13	WDUO-3/3	$C_{37}H_{36}N_6O_6Br_2$ (820.4)	41	230	1775, 1700, 1640, 1600, 1400, 1390, 725
14	WDUO-3/4	$C_{38}H_{38}N_6O_6Br_2$ (834.6)	25	253	1770, 1700, 1645, 1600, 1400, 1380, 740
15	WDUO-3/5	$C_{39}H_{40}N_6O_6Br_2$ (848.6)	43	180	1770, 1710, 1640, 1595, 1400, 1040, 720
16	WDUO-3/6	$C_{40}H_{42}N_6O_6Br_2$ (862.6)	39	228	1770, 1710, 1640, 1595, 1390, 1040, 970, 725

no.	compd	formula (mol wt)	reaction time	yield (%)	mp (°C)	IR (ν, cm^{-1})
17	IWDUO-1/3	$C_{31}H_{26}N_4O_4Br_2$ (678.4)	21	50	240−241	1780(w), 1715, 1580, 1355, 960, 720
18	IWDUO-2/3	$C_{33}H_{30}N_4O_4Br_2$ (706.5)	26	32	254−256	1770(w), 1700(br), 1630, 1380(br), 1005, 720
19	IWDUO-3/3	$C_{35}H_{34}N_4O_4Br_2$ (734.5)	20	46	274	1770(w), 1700(br), 1635, 1380(br), 835, 720
20	IWDUO-4/3	$C_{37}H_{38}N_4O_4Br_2$ (762.5)	44	29	207−208	1770(w), 1700(br), 1635, 1370(br), 1040, 720
21	IWDUO-4/0	$C_{34}H_{32}N_4O_4Br_2$ (720.5)	28	15	218	1760(w), 1690(br), 1390, 810, 720
22	IWDUO-4/2	$C_{36}H_{36}N_4O_4Br_2$ (748.5)	28	19	299	1760(w), 1690(br), 1370, 840, 720
23	IWDUO-3/2	$C_{34}H_{32}N_4O_4Br_2$ (720.5)	24	30	282−284	1760(w), 1690(br), 1370, 715

[a] A second polymorph has been found characterized by mp 197 °C. [b] A second polymorph has been found characterized by mp 169 °C.



W84-3/0

Figure 6. W84-3/0 (bold) fitted onto alcuronium using one positively charged nitrogen and two aromatic carbon atoms at either ring as matching pairs.

two aromatic areas (see Figure 6). Moreover, cutting-off both aromatic rings at the ends of hexamethonium resulted in a complete loss of the allosteric activity.[13] Thus, it can be stated that solely the entire molecule is able to inhibit the [^{3}H]NMS dissociation effectively.

Summary

Ion−ion interactions play a decisive role in the mutual recognition between a ligand and the corresponding receptor because it is through their electrostatic fields that two species start to interact. Thus, the findings of this ligand-related study are in line with the already mentioned results of Jakubik and Tucek[16] who looked

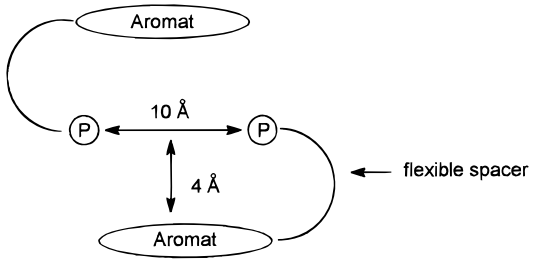


Figure 7. Pharmacophore model and distances between the essential elements (P = positively charged center).

from the receptor's point of view: Two negatively charged acidic amino acids at the extracellular site of the receptor protein are able to interact strongly with twice positively charged derivatives, if the distance between the charges in both the receptor and the molecules is almost the same. For further orientation of the W84 derivatives on the receptor, the heterocycles at both ends of the molecule are responsible. When the entire molecule consisting of two positively charged groups and two aromatic areas is able to adopt the active conformation, which is characterized by an S-shape (see Figure 7), the fit to the allosteric site is maximal.

Experimental Section

Chemical Synthesis. Melting points were determined with Dr. Tottoli's melting point apparatus (Büchi, Switzerland) and were not corrected. ^{1}H and ^{13}C NMR spectra were recorded

Table 4. ^{1}H NMR Data of Compounds **2**−**4** and **6**−**10** (δ (ppm), J (Hz, in parentheses), DMSO-d_6)[a]

no.	compd	phthalimide hydrogens	N_{phth}-CH_2	N_{phth}-CH_2-CH_2	CH_2-N^+	N^+-CH_3	N^+-CH_2	-CH_2
2	W84-3/3	7.75 (m)	3.79 (t, 6.5)	2.23 (m)	3.55 (m, 4.1)	3.19 (s)	3.48 (t)	
3	W84-3/4	7.78 (m)	3.76 (t, 6.4)	2.21 (quin)	3.43 (m)	3.13 (s)	3.43 (m)	1.88 (m)
4	W84-3/5	7.74 (m)	3.73 (t, 7.1)	2.19 (quin)	3.49 (m, 4.6)	3.15 (s)	3.38 (m, 4.6)	1.83 (m) 1.49 (m)
6	W84-3/7	7.78 (m)	3.75 (t, 6.9)	2.19 (quin)	3.44 (m, 4.1)	3.12 (s)	3.33 (m, 4.2)	1.72 (m) 1.36 (m)
7	W84-3/8	7.79 (m)	3.76 (t, 6.4)	2.18 (m)	3.42 (m, 4.1)	3.10 (s)	3.31 (m, 4.3)	1.70 (m) 1.30 (m)
8	W84-3/10	7.87 (m)	3.65 (t, 6.2)	2.04 (m)	3.34 (m)	2.99 (s)	3.24 (m)	1.60 (m) 1.22 (m)
9	W84-3/0	7.80 (m)	3.63 (t, 6.5)	2.02 (br)	3.25−3.40 (m)	3.02 (s)	3.25−3.40 (m)	

[a] Pyridine hydrogens: 9.22 (d, 2H, 6.7), 8.63 (t, 1H, 7.8), 8.18 (dd, 2H, 7.8, 6.7); 4.68 (t, 2H, 7.5, $CH_2N^+_{pyr}$).

on a Varian XL 300 instrument (^{1}H 299.956 and ^{13}C 75 MHz). Abbreviations for data quoted are s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet. ^{1}H and ^{13}C NMR assignments given for each compound were confirmed by randomly running HETCOR experiments. IR spectra, recorded as KBr disks, were obtained using a Perkin-Elmer 298 spectrometer, and UV/vis spectra were recorded on a Hewlett-Packard HP 8452A photodiode array spectrometer. Analyses indicated by the symbols of the elements were within ±0.4% of the theoretical value. Dry solvents were used throughout.

N,*N*-Dimethyl-*N*-(3-phthalimidopropyl)amine (**1**) was prepared according to ref 13.

General Procedure for Synthesis of *N*,*N*′-Bis(3-phthalimidopropyl)-*N*,*N*,*N*′,*N*′-tetramethyl-1,*x*-alkanediaminium Dibromides 2−4 and 6−8 (W84-3/3 to W84-3/10). 1 (2.32 g, 10 mmol) and 5 mmol of the corresponding dibromoalkane were dissolved in ethanol (100 mL) and refluxed for at least 16 h (TLC control on silica gel using methanol as mobile phase). After cooling, the mostly crystalline precipitate was collected and recrystallized from a solvent given in Table 3. For analytical data and IR data see Table 3, and for NMR data see Table 4.

***N*,*N*-Bis(3-phthalimidopropyl)-*N*,*N*′-dimethylaminium Bromide (9) (W84-3/0).** Phthalimidopropane bromide (1.74 g, 6.5 mmol) and **1** (1.5 g, 6.5 mmol) were heated under reflux in ethanol (50 mL) for 18 h. After cooling, the crystals were filtered and twice recrystallized from methanol. For analytical data and IR data see Table 3, and for ^{1}H NMR data see Table 4.

The 4,4′-bis(phthalimidomethoxyiminomethyl)-1,1′-alkane-1,*x*-diyl-bispyridinium dibromide **11** and **12** (**WDUO-1/4** and **-1/6)** as well as **13**−**16** (**WDUO-3/3**, **-3/4**, **-3/5**, and **-3/6)** were synthesized according to.ref 12. For analytical and IR data see Table 3; for ^{1}H NMR see Table 5.

WDUO-1/4: ^{13}C NMR (DMSO-d_6) δ 26.77 (−CH_2), 59.49 (N^+CH_2), 70.34 (N-CH_2), 123.59 (C-3phth), 124.67 (C-3pyr), 131.08 (C-2phth), 135.04 (C-4phth), 145.28 (C-2pyr), 146.48 (C-4pyr), 147.34 (N═CH), 166.66 (C═O).

WDUO-1/6: ^{1}H NMR (DMSO-d_6) δ 1.29 (m, 4H, −CH_2−), 1.84 (m, 4H, N^+-CH_2-CH_2), 4.58 (t, J = 7.3 Hz, 4H, N^+-CH_2), 5.73 (s, 4H, N-CH_2), 7.90 (m, 8H, H_{phth}), 8.16 (d, J = 6.9 Hz, 4H, 3-pyrH), 8.60 (s, 2H, N═CH), 9.13 (d, J = 6.9 Hz, 4H, 2-pyrH); ^{13}C NMR (DMSO-d_6) δ 24.53 (−CH_2−), 30. (−CH_2−), 60.22 (N^+CH_2), 70.28 (N-CH_2), 123.42 (C-3phth), 124.47 (C-3pyr), 130.94 (C-2phth), 134.85 (C-4phth), 145.01 (C-2pyr), 146.24 (C-4pyr), 147.17 (N═CH), 166.44 (C═O).

General Procedure for Synthesis of *N*,*N*′-Bis(3-phthalimidoalkyl)-1,1′-alkane-1,*x*-diylbispyridinium Dibromides 17−23 (IWDUO). Bispyridinium alkane (1 mol) and corresponding bromoalkylphthalimide (2 mol) were refluxed in acetonitrile for 19−46 h (see Table 2). After cooling to room temperature, a white precipitate was collected and recrystallized several times from EtOH or mixtures of EtOH/diethyl ether. For analytical data and IR data see Table 3, and for ^{1}H NMR data see Table 6.

Table 5. ^{1}H NMR Data of WDUO Compounds **11**−**17** (δ (ppm), J (Hz, in parentheses), DMSO-d_6)

no.	compd	Phth	N-CH_2	N═CH	3-Pyr	2-Pyr	N^+_{Py}-CH_2	-CH_2-	-CH_2
10	WDUO-1/3	7.94 (m)	5.75	8.62	8.20 (d, 6.7)	9.11 (d, 6.7)	4.70 (t, 7.3)	2.62 (m)	
11	WDUO-1/4	7.92 (m)	5.72	8.63	8.18 (d, 6.9)	9.20 (d, 6.9)	4.70 (m)	1.97 (m)	
12	WDUO-1/6	7.90 (m)	5.73	8.60	8.16 (d, 6.9)	9.13 (d, 6.9)	4.58 (t, 7.3)	1.89 (m)	1.29 (m)

no.	compd	Phth	N-CH_2	CH_2	CH_2	N═CH	*m*-Pyr	*o*-Pyr	N-CH_2	-CH_2	-CH_2
13	WDUO-3/3	7.77 (m)	4.82 (t)	2.18 (q)	4.45 (t)	8.48	8.25 (d)	9.17 (d)	4.83 (t)	2.71	
14	WDUO-3/4	7.82 (m)	4.33 (t)	2.05 (q)	3.75 (t)	8.47	8.24 (d)	9.16 (d)	4.68 (t)	1.98	-
15	WDUO-3/5	7.82 (m)	4.35 (t)	2.07 (q)	3.72 (t)	8.44	8.22 (d)	9.14 (d)	4.61 (t)	1.98	1.27 (q)
16	WDUO-3/6	7.81 (m)	4.34 (t)	2.05 (q)	3.71 (t)	8.48	8.19 (d)	9.16 (d)	4.62 (t)	1.97	1.33 (t)

Table 6. ^{1}H NMR Data of IWDUO Compounds (δ (ppm), DMSO-d_6)

no.	compd	Phth	CH_2	CH_2	CH_2	CH_2	o-Pyr	m-Pyr	CH_2	CH_2
17	IWDUO-1/3	7.9 (m)	6.30 (s)				8.99 (d)	8.12 (d)	3.01 (t)	2.08 (m)
18	IWDUO-2/3	7.8 (m)	4.17 (t)			4.84 (t)	9.12 (d)	8.05 (d)	2.94 (t)	2.08 (m)
19	IWDUO-3/3	7.7 (m)	3.77 (t)	2.52 (quin)		4.73 (t)	8.83 (d)	7.98 (d)	3.01 (t)	2.07 (quin)
20	IWDUO-4/3	7.8 (m)	3.60 (t)	1.62 (m)	1.94 (m)	4.61 (t)	9.02 (d)	8.07 (d)	2.95 (t)	2.10 (quin)
21	IWDUO-4/0	7.85 (m)	3.64 (t)	1.68 (m)	2.03 (m)	4.75 (t)	9.40 (d)	8.80 (d)		
22	IWDUO-4/2	7.84 (m)	3.61 (t)	1.61 (m)	1.93 (m)	4.59 (t)	9.03 (d)	8.13 (d)	3.32 (s)	
23	IWDUO-3/2	7.86 (m)	3.63 (t)	2.29 (m)		4.65 (t)	9.07 (d)	8.16 (d)	3.49 (s)[a]	

[a] Hidden by D_2O signal.

The octanol/water partition coefficients were determined according to refs 14 and 27; the log k_w values were measured according to ref 28.

Stability Determination. TRIS-HCl−Mg buffer: 3.6 mM $MgHPO_4$, 50 mM TRIS, pH adjusted by using HCl to pH 7.3, 500 mL of distilled water. A 10^{-4} M solution of each compound was prepared in TRIS buffer; the absorption spectra of these solutions were recorded between 220 and 800 nm every 15 min for a period of 24 h. The decrease in the absorption at the maximum wavelength at 300 nm was registered. A nonlinear regression analysis applying the InPlot software (GraphPad Software Inc., San Diego, CA) yielded the degradation constant k_{-1} and the half-life $t_{1/2}$ of the hydrolysis.

Molecular Modeling. All molecules were built up using the 2D-to-3D conversion program CORINA;[32] the so-obtained

geometries were optimized by means of force field calculation (MMX, PC-Model program package), and the lengths of the molecules were measured at the largest distances in completely extended molecules. Distribution of charges was computed using semiempirical methods (AM 1, MOPAC).[33]

The fitting procedure: The starting geometries of IWDUO-4/0, IWDUO-1/3, and W84-Co were obtained by means of molecular mechanics calculations (MM+ of HyperChem) with additional restraint forces. The atoms to be overlayed were tethered to the Cartesian coordinates of the corresponding alcuronium atoms. After geometry optimization all applied restraints were removed, and then MM+ calculations were repeated. The so-obtained structures were fitted onto alcuronium using the RMS Fit option of HyperChem 5.0 ChemPlus. In alcuronium both positively charged nitrogens and aromatic atoms in indole skeletons adjacent to the pyrrolidine ring were used as fitting atoms. The fitting atoms of IWDUO-4/0, IWDUO-1/3, and W84-3/0 are marked with arrows (see Figures 4−6).

Pharmacology. Preparation of Porcine Cardiac Membranes. The applied procedure has been described in detail recently.[14,17] Ventricular tissue (40 g) from porcine hearts was homogenized in sucrose solution (0.32 M, 20 mL/g original tissue weight) using a Waring Blendor (New Hartfort) and a Potter Elvejhem glass homogenizer. To separate crude material, the homogenate was centrifuged at 300*g* (2000 rpm in a Beckman rotor type 35) for 11 min. The supernatant was centrifuged at 80000*g* (32 000 rpm in the same rotor type) for 40 min. After the pellets were resuspended in a buffer containing 50 mM Tris-HCl, pH 7.4 (4 mL/g original tissue weight), 1-mL aliquots were drawn from the membrane suspension under continuous stirring. Reaction vials were filled with the aliquots, shock-frozen in liquid nitrogen, and stored at −80 °C.

[^{3}H]NMS Binding Assay. Binding of [^{3}H]NMS (specific activity 84.1 Ci/mmol) was determined as described previously.[14,17] Experiments were performed in an incubation medium consisting of 2.6 mM $MgHPO_4$, 45 mM Tris-HCl, pH 7.3 at 37 °C. Unspecific binding was assessed in the presence of 1 μM atropine and was less than 10% of total binding. Inhibition of [^{3}H]NMS (0.2 nM) by unlabeled NMS after 120 min served to characterize control binding: $pK_D = 9.22 \pm 0.18$ and $B_{max} = 32 \pm 8$ fmol/mL membrane suspension (means ± SEM, 5 experiments).

Dissociation of the radioligand was visualized by addition of 1 μM atropine after incubating 0.2 nM [^{3}H]NMS for a period of 30 min with the membranes. To determine the effects of the test compounds on the dissociation of [^{3}H]NMS, they were added simultaneously with atropine. Then, 1-mL aliquots were taken from the assay (∼20 mL) at various time intervals for up to 2 h. Membrane-bound radioactivity was separated by rapid filtration through glass fiber filters under suction (Schleicher & Schuell, No. 6; Dassel, Germany). Filters were washed twice with 5 mL of ice-cold distilled water. Filters were placed into scintillation vials, 5 mL of liquid scintillation cocktail was added (Ready Protein, Beckman), and the radioactivity was measured in a LS6000 counter (Beckman). Curve fitting through the data points applying a monoexponential decay function yielded the half-life from which the apparent rate constant k_{-1} was calculated. In some cases, the data were completed by twopoint kinetic experiments:[34] i.e., specific binding of [^{3}H]NMS was measured at $t = 0$ min, and then residual binding of [^{3}H]NMS was determined at $t = 4$ min after addition of atropine. To obtain for a given compound the concentration−effect curve, apparent rate constants of dissociation k_{-1} found in various experiments carried out on different days were compiled and a curve was fitted to the whole body of data using a four-parameter logistic function. To check whether the curve slope was statistically different from unity, the partial *F*-test was applied. A value of $p > 0.05$ was taken as a criterion for significance (Prim 2.01, GraphPad Software, San Diego, CA).

[^{3}H]NMS was purchased from DuPont-New England Nuclear (Bad Homburg, Germany). NMS and atropine were obtained from Sigma Chemical (Munich, Germany).

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