Search for the Pharmacophore of Bispyridinium-Type Allosteric Modulators of Muscarinic Receptors

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The bis(dichlorobenzyl) ether of the bispyridinium oxime TMB 4 stabilizes antagonist binding to M_2-cholinoceptors which is indicative of an allosteric action. More than 10 derivatives of the lead compound were synthesized to investigate structure–activity relationships. The allosteric potency of the compounds was indicated by the concentrations which retarded the rate of dissociation of [^{3}H]*N*-methylscopolamine from porcine cardiac cholinoceptors by a factor of 2 (EC_{50}). Compared with TMB 4, the bis(dichlorobenzyl) derivative **4a** displayed a more than 200-fold higher potency (EC_{50} = 4.7 μM). One of the dichlorobenzyl groups could be replaced by a methyl group without loss of activity (EC_{50} = 4.5 μM). Further shortening of this end of the molecule was accompanied by a moderate decline in potency to a minimum of EC_{50} = 26 μM. The second quaternary nitrogen was not a prerequisite for an allosteric activity. It is concluded that one half of the lead compound is pivotal for an interaction with the allosteric site of the M_2-cholinoceptor, whereas the opposite end of the molecule modulates the allosteric activity.

Introduction

Muscarinic acetylcholine receptors may be subject to allosteric modulation,[1,2] especially the M_2-subtype.[3] The allosteric effect is indicated by an altered dissociation of antagonist–receptor complexes in the presence of the allosteric compound. Numerous drugs from various pharmacological groups have been shown to affect M_2-cholinoceptors allosterically, e.g., gallamine,[4] verapamil,[5] and quinidine.[6] In particular, phthalimidopropyl-substituted hexamethonium derivatives[7,8] have to be pointed out: the allosteric potency is high, the combinations with atropine display an overadditive antimuscarinic action in isolated cardiac preparations,[9,10] and the combinations were found in animal experiments to have an overadditive antidote effect against organophosphorus poisoning.[11] More recently, bispyridinium compounds were described,[12,13] which are closely related to the bisquaternary hexamethonium compounds with respect to molecular shape and allosteric potency.[14]

The structural heterogeneity among the allosteric modulators raised the question of whether structural elements can be defined which govern the allosteric activity. This knowledge may help to gain insight into the molecular interaction between ligands and the receptor protein. However, structure–activity relationships have not been systematically analyzed so far. In the present investigation, it is attempted to unravel structure–activity relationships for the bispyridinium derivatives. A number of derivatives of the symmetrically built lead compound **4a** were synthesized. (This compound has been described in ref 12 and named DUO 3.[13]) In the derivatives, one half of the molecule was systematically shortened including the loss of one positive charge. Furthermore, the parent compound of **4a**, i.e., the unsubstituted bispyridinium oxime TMB 4 (Figure 1), was included in the investigation. In order to quantify the allosteric activity, the effect of the compounds on the dissociation of [^{3}H]NMS was measured in porcine cardiac membranes. As shown previously,[15] it seems justified to consider the results representative for cardiac M_2-receptors.

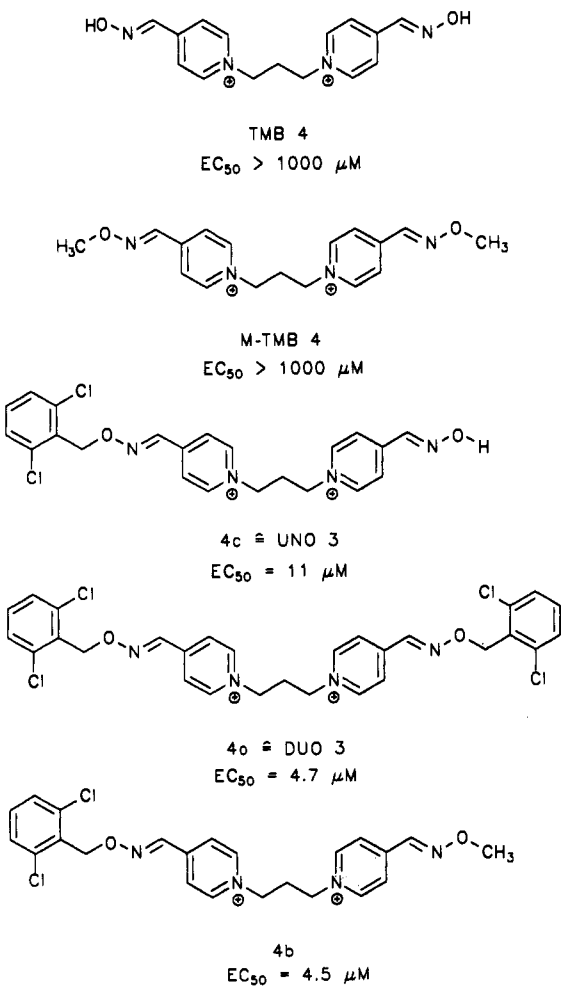

Figure 1. Uni- and bilateral substituted derivatives of the bispyridinium oxime TMB 4. Included are the EC_{50} values as a measure of the allosteric potency (EC_{50} = concentration reducing [^{3}H]*N*-methylscopolamine ([^{3}H]NMS) dissociation rate by 50%).

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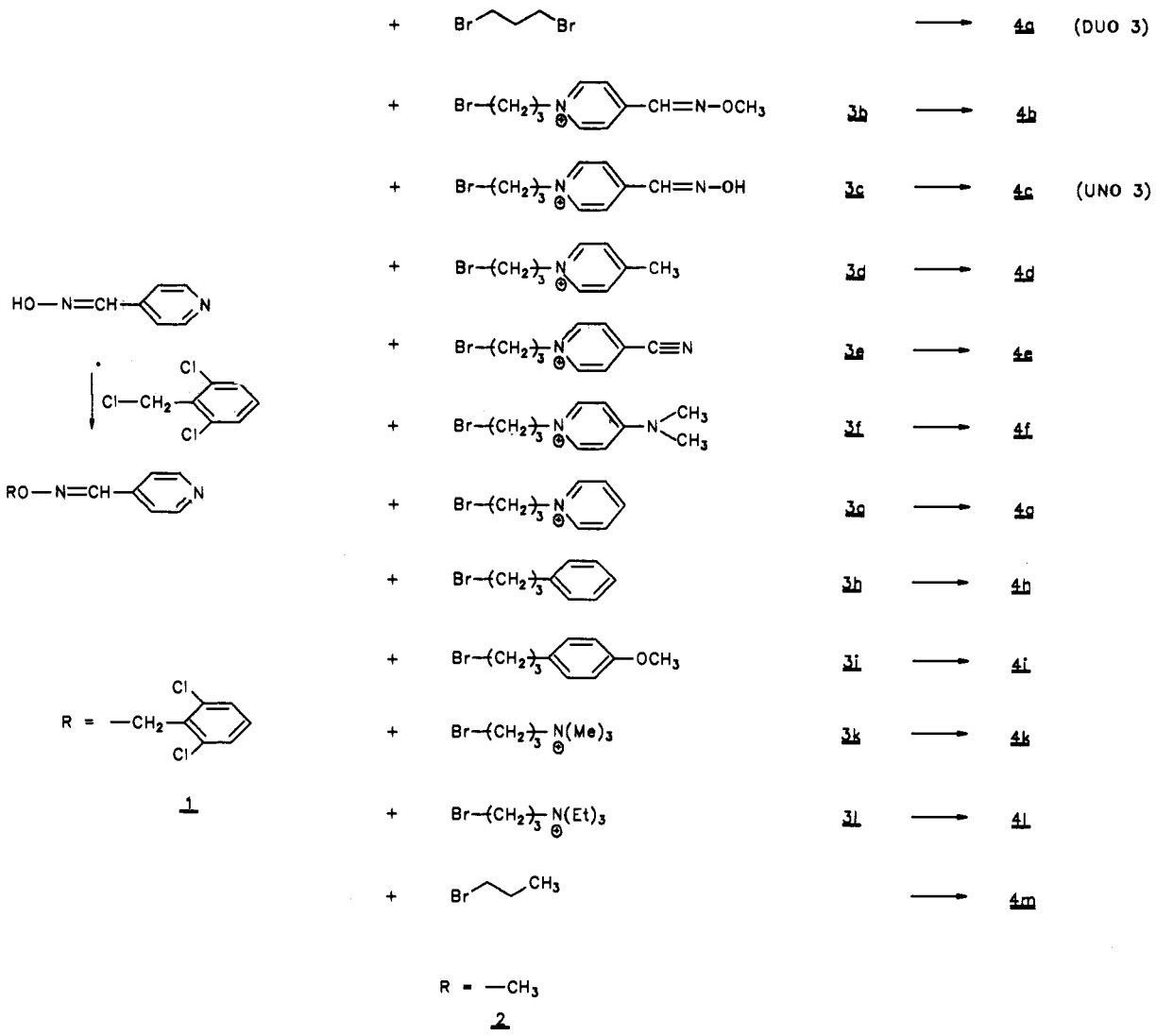
Scheme 1



Chemistry

The key compound 1 of all the syntheses is the 2,6-dichlorobenzyl ether of pyridine-4-carboxaldehyde (*E*)-oxime obtained by a Williamson analogue conversion of the oxime and dichlorobenzyl chloride in the presence of sodium methanolate. Alternatively, the ether is formed by phase-transfer catalysis with tetrabutylammonium chloride in $NaOH/CH_2Cl_2$. The corresponding methyloxime ether is synthesized in the same manner using methyl iodide. As illustrated in Scheme 1, the (bromopropyl)pyridinium salts **3b–g** were prepared by a general procedure: The pyridine derivatives were alkylated in the presence of a 2-fold excess of dibromopropane to avoid the formation of bis(pyridinium)propanes. 3-(*p*-methoxyphenyl)propyl bromide (**3i**) and (3-bromopropyl)-trimethylammonium iodide (**3k**) were obtained by substitution of the corresponding alcohol with bromide by treatment with hydrobromic acid/sulfuric acid. The analogue triethylammonium derivative **3l** is derived by alkylation of triethylamine with dibromopropane.

The symmetrical bispyridinium salt **4a** was obtained by reaction of 2 mol of 1 with 1 mol of dibromopropane. All other derivatives **4b–m** were prepared by a general procedure: The oxime ether 1 was refluxed with equimolar amounts of the corresponding bromopropyl derivatives **3b–l** and bromopropane, respectively, in acetonitrile.

Results

The procedure to test whether the test compounds affected the dissociation of [^{3}H]NMS from cardiac muscarinic receptors allosterically is illustrated by means of representative experimental data.

The time course of [^{3}H]NMS dissociation under control conditions and in the presence of the test compound **4b** is depicted in Figure 2. **4b** retarded [^{3}H]NMS dissociation concentration dependently. These experiments revealed that the dissociation could be described by a monoexponential function. Thus, the time course was characterized by the half-life time $t_{1/2}$ and the apparent rate constant of dissociation k_{-1}, respectively.

As can be taken from Figure 2, the residual binding at 4 min after starting the dissociation increased depending on the extent to which the dissociation rate was reduced. This fact was utilized in the "two-point kinetic experiments", in which the 4-min value of [^{3}H]NMS binding was recorded as an indicator of the dissociation rate. In Figure 3, residual binding is plotted versus the concentration of **4b** as well as of **4h,m**. At high concentrations, residual binding remained near the starting level (t = 0 min), indicating that [^{3}H]NMS dissociation was considerably retarded.

The course of dissociation was monophasic, permitting a calculation of the apparent rate of dissociation, k_{-1}, from

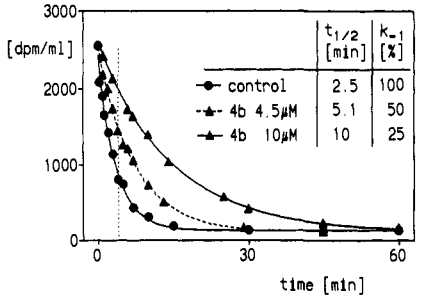


Figure 2. Dissociation of [^{3}H]NMS from porcine cardiac membranes under control conditions and in the presence of the indicated concentration of **4b** (representative experiments). Monoexponential curve fitting led to the half-life time $t_{1/2}$ of dissociation and the apparent dissociation rate constant k_{-1}, respectively. The stippled line indicates [^{3}H]NMS binding 4 min after starting the dissociation.

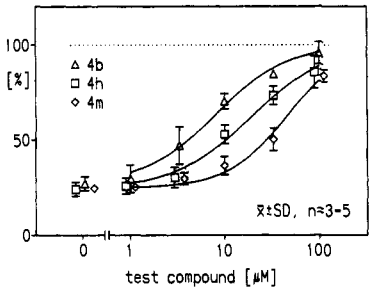


Figure 3. Residual specific [^{3}H]NMS binding 4 min after starting the dissociation of [^{3}H]NMS in the absence and in the presence of the compounds **4b,h,m** (two-point kinetic experiments). Indicated are means ± SD of n = 3–5 separate experiments.

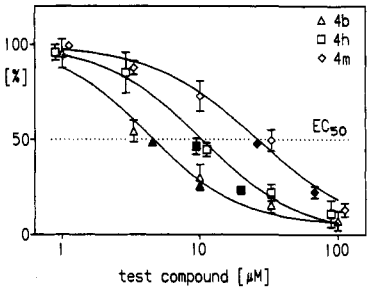
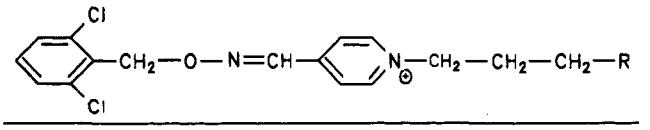


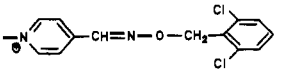
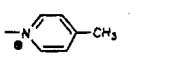
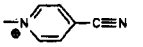
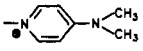
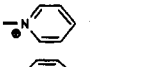
Figure 4. Effect of **4b,h,m** on the apparent dissociation rate constant, k_{-1}, expressed in percent of the control value: open symbols, data from two-point kinetic experiments; closed symbols, data from complete kinetic experiments. For details, see text. Sigmoid curves were fitted to the data from the two-point kinetic experiments by nonlinear regression analysis; the EC_{50} values and the slope factors of the concentration–effect curves are compiled in Table 1.

the starting level of [^{3}H]NMS binding (t = 0 min) and the residual binding (t = 4 min). Figure 4 depicts the concentration-dependent decline of k_{-1} as obtained by transforming the data displayed in Figure 3 (open symbols). Sigmoid curves were fitted to the data, which permitted the derivation of the concentration reducing dissociation rate by 50% (EC_{50}) and the slope factor n_H of the curve (Table 1). The results obtained in "complete kinetic experiments" (Figure 2) are included in Figure 4 (closed symbols); the data were in good agreement with the results of the two-point kinetic experiments, thus validating this procedure.

The effects of the other test compounds were investigated as outlined above. All compounds were capable of reducing the dissociation rate of [^{3}H]NMS to less than 10% of that of the control. However, the allosteric potency

Table 1. Structure, Hydrophobicity, and Allosteric Potency of Bispyridinium Compounds[a]



compd	R	log P	EC_{50} (μM)	n_H
4a		0.6	4.7 ± 0.3	1.7 ± 0.2
4b		−2.0	4.5 ± 1.0	1.2 ± 0.4
4c		−1.7	11 ± 0.6	1.3 ± 0.2
4d		−1.3	12 ± 0.5	1.1 ± 0.3
4e			17 ± 0.0	1.1 ± 0.1
4f		−1.5	4.2	1.3
4g			21 ± 0.4	1.2 ± 0.4
4h		0.5	9.4 ± 0.5	1.2 ± 0.4
4i		0.3	6	1.5
4k	$-N(Me)_3$	−1.7	23, 25	1.5, 1.3
4l	$-N(Et)_3$	−1.4	23, 22	0.9, 0.9
4m	—H	−0.6	26 ± 4	1.2 ± 0.2

[a] log P: log(octanol/buffer partition coefficients). EC_{50}: concentration reducing the [^{3}H]NMS dissociation rate by 50%. n_H: slope factors of the concentration–effect curves from which the EC_{50} values were derived (means ± SD, n = 3–5, unless otherwise indicated).

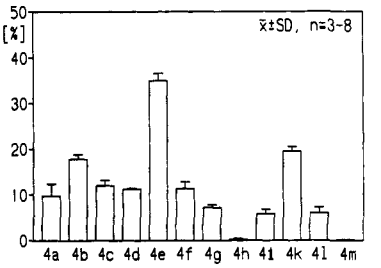


Figure 5. Specific [^{3}H]NMS binding in the presence of EC_{50} concentrations of the indicated test compounds. Indicated are means ± SD of n = 3–8 experiments.

of the compounds differed, as indicated by the EC_{50} values compiled in Table 1.

In order to check whether the compounds affected the equilibrium binding of [^{3}H]NMS, the binding was measured in the presence of the EC_{50} concentration for the retarding action of [^{3}H]NMS dissociation. As shown in Figure 5, all compounds inhibited [^{3}H]NMS binding but to a different extent.

Discussion

The tested compounds retarded radioligand dissociation which is, to date, the most reliable evidence for an allosteric action.[1] This action promotes receptor occupation by the radioligand. However, equilibrium binding of [^{3}H]NMS was reduced by the compounds (Figure 5), demonstrating an inhibitory action on radioligand association. At concentrations being equieffective with respect to the effect on the dissociation (i.e., EC_{50}), the compounds reduced [^{3}H]NMS binding to a different extent. Accordingly, the

ability to inhibit radioligand association does not parallel the allosteric action on radioligand dissociation. Obviously, the structure–activity relationships underlying these effects are deviating. This finding may be taken to support the hypothesis (for review, see ref 1) that allosteric modulators interact with two sites of the muscarinic receptor: a primary site which is also occupied by traditional antagonists such as atropine and a secondary site which mediates the allosteric effect. The present study focuses on the molecular properties governing the interaction with the allosteric site.

The comparison of the EC_{50} and log P values exhibited no correlation between these parameters (Table 1). Thus, it is unlikely that the allosteric effect results from a nonspecific intercalation of the compounds in hydrophobic domains of the receptor protein or in the phospholipid bilayer surrounding the receptor.

The parent compound of the bispyridinium derivatives, i.e., TMB 4, displayed a rather weak allosteric activity; at the highest applied concentration of 1000 μM, the dissociation of [^{3}H]NMS was only reduced to 60% of the control value (data not shown). This observation is in line with previous findings.[7] Unilateral introduction of a dichlorobenzyl substituent (**4c**, EC_{50} = 11 μM) increased the allosteric activity by a factor of about 100. A second dichlorobenzyl group at the opposite oxime moiety (**4a**) further augmented the potency, yet by a factor of 2 only. A similar observation was made previously in guinea pig cardiac membranes.[12] Remarkably, the second dichlorobenzyl group could be replaced by a methyl (**4b**) without loss of allosteric activity. As revealed by M-TMB 4 (Figure 1), a bilateral methyl substitution does not suffice for a high potency. In conclusion, a dichlorobenzyl substituent introduced in one of the oxime moieties of TMB 4 strongly increases the allosteric activity. Yet, it is unlikely that only half of the molecule interacts with the allosteric site of the receptor, since substitution of the opposite oxime moiety affected the allosteric potency.

The part of the molecule containing the opposite oxime moiety was subjected to extensive chemical modifications. Shortening of this site of the molecule was accompanied by a diminished allosteric activity (**4f** $\simeq$ **4b** > **4d** > **4g** $\simeq$ **4l** $\simeq$ **4k** $\simeq$ **4m**). **4m** revealed a 6-fold lower potency than **4f**. Nevertheless, **4m** retained a considerable potency although the second positive charge was removed. In fact, comparison of **4g** with **4h** demonstrates that the second quaternary ammonium is dispensible. In this context, it is remarkable that the modifications leading to shortening in **4f**,**b**,**d**,**g**,**l**,**k** are probably associated with an increased density of positive charge located at the nitrogen. Thus, both the diminution of the para substituent and the increase of the charge density over the nitrogen may contribute to the reduced allosteric potency found with these compounds. In fact, analysis of quantitative structure–activity relationships of the bispyridinium derivatives **4c–g** reveals a positive correlation between the EC_{50} values and the sterimol parameter B3 (see ref 16) which characterizes the width of the shape of the substituent (Figure 6).

With respect to the charge density over the nitrogen, the ability of **4c** to release a proton may be considered. **4c** is a weak acid with a pK_a = 8.4. At the pH of the incubation medium, 10% of **4c** is in the deprotonized state with the remaining electron pair capable of neutralizing the positive charge over the pyridinium nitrogen by mesomerism. At

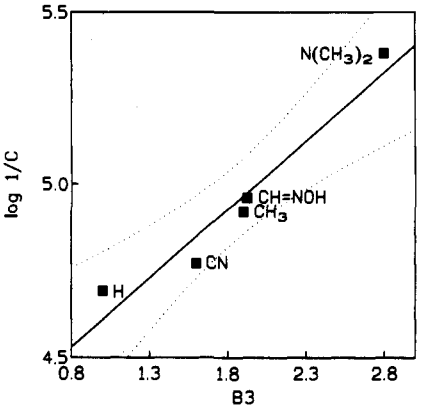


Figure 6. Correlation between the allosteric potency and the sterimol parameter B3. The regression analysis affords log 1/EC_{50} = 0.397(±0.19)B3 + 4.370(±0.64) (n = 5; r = 0.968; s = 0.078; F = 44.5). The 95% confidence interval is given (see stippled line in the diagram). B3 values: CH=NOH 1.92, CH_3 1.90, CN 1.60, $N(CH_3)_2$ 2.80, and H 1.00.

present, it cannot be excluded that the deprotonized form of **4c** mediates the allosteric action. If this were the case, the EC_{50} for the deprotonized form would amount to 1 μM (10% of the EC_{50} = 11 μM). Thus, even if the deprotonized form of **4c** were the active form, the hypothesis could be maintained that a reduced charge density over the nitrogen warrants a higher allosteric potency in this series of compounds.

In line with this, it cannot be decided whether the slightly higher potency of **4i** compared with **4h** is based on the elongation of the molecule or the increased electron density of the aromatic ring created by the +M substituent.

In conclusion, shortening of the lead compound **4a** including removal of half of the molecule together with the cationic nitrogen (**4m**) resulted in a loss of allosteric activity by a factor of 5. However, **4m** is still, by a factor of 40, more potent than the bisoxime TMB 4. Obviously, the unilateral introduction of an appropriate substituent such as dichlorobenzyl group plays a pivotal role for the interaction with the allosteric site, whereas the substituent at the opposite end of the molecule modulates the allosteric potency. Thus, future research will have to focus on the structure–activity relationships of the unilateral substituent dominating the allosteric potency.

Experimental Section

Melting points were determined with a Dr. Tottoli melting point apparatus (Büchi) and are not corrected. ^{1}H and ^{13}C NMR spectra were recorded on Varian XL 300 (^{1}H NMR, 299.956 MHz; ^{13}C NMR, 75 MHz) and Varian EM 360 (60 MHz) spectrometers. The centers of the peaks of $CDCl_3$ and DMSO-d_6 were used as internal references. Abbreviations for data quoted are d, doublet; t, triplet; q, quartet; and m, multiplet. Coupling constants are in hertz. IR spectra, recorded as KBr discs, were obtained using a Perkin-Elmer 298 spectrometer. UV/vis spectra were recorded on a Perkin-Elmer Lambda 2 UV/vis spectrometer and HR-FAB mass spectra on a Finnigan MAT 8430. HPLC was carried out on a Waters 600 E multisolvent delivery system equipped with a Waters 484 tunable absorbance detector, and a Hewlett-Packard HP 3394 A integrator, and a LiChrocart RP 18 column (7 μm; length, 12.5 cm) was used. Dry solvents were used throughout. Chemicals were of analytical grade and purchased from SIGMA, Deisenhofen, or Merck, Darmstadt.

All new derivatives were characterized by HR-FAB mass spectra: The isotope patterns of the molecular ions (negative and positive) correspond to the calculated patterns of these peaks. The purity of the derivatives was checked by HPLC. Carbon, hydrogen, and nitrogen elemental analyses were carried out in

Table 2. Physical Data of the Compounds 3

compd	yield (%)	mp (°C)	formula	molecular weight	recrystn solvent[a]
3b	32	134	$C_{10}H_{14}Br_2N_2O$	338.0	Eth/acetone/CH_2Cl_2
3c	73	178–181	$C_9H_{12}Br_2N_2O$	324.0	EtOH/MeOH
3d	50	41	$C_9H_{13}Br_2N$	295.0	EtOH/CH_2Cl_2 (1/8)/Eth
3e	80	167–168	$C_9H_{10}Br_2N_2$	306.0	EtOH/MeOH (5/1)
3f	88	130	$C_{10}H_{16}Br_2N_2$	324.1	EtOH/Eth
3g	52	118	$C_8H_{11}Br_2N$	281.0	EtOH/CH_2Cl_2 (5/1)/Eth

[a] The substances were recrystallized from ethanol (EtOH)/methanol (MeOH) or EtOH/methylene chloride mixtures. In some cases, a few drops of diethyl ether (Eth) were added till clouding.

Table 3. Spectroscopic Data of the Compounds 3

compd	IR (cm^{-1})	^1H NMR (60 MHz, DMSO-d_6, δ (ppm), J (Hz))
3b	1640, 1600, 1450, 940, 850, 610	2.20–2.60 (m, 2H, $-CH_2-$), 3.50 (t, 2H, CH_2Br), 4.60 (t, 2H, CH_2N), 5.50 (s, 3H, CH_3), 8.20 (d, 2H, pyr), 8.50 (s, 1H, CH=), 9.10 (d, 2H, pyr)
3c	3440, 1640, 1610, 1510, 1455, 630	2.51 (q, 2H, 7.4, $-CH_2-$), 3.60 (t, 2H, 7.4, CH_2Br), 4.75 (t, 2H, 7.4, CH_2N), 8.23 (d, 2H, 7.4, pyr), 8.45 (s, 1H, CH=), 9.12 (d, 2H, 7.4, pyr), 12.71 (s, 1H, NOH)
3d	1640, 1470, 840, 810, 700	2.20–2.70 (m, 2H, $-CH_2-$), 2.60 (s, 3H, CH_3), 3.60 (t, 2H, CH_2Br), 4.70 (t, 2H, CH_2N), 8.05 (d, 2H, pyr), 9.50 (d, 2H, pyr)
3e	1640, 1560, 1510, 1460, 850	2.3–2.9 (m, 2H, $-CH_2-$), 3.60 (t, 2H, CH_2Br), 4.93 (t, 2H, CH_2N), 8.80 (d, 2H, pyr), 9.66 (d, 2H, pyr)
3f	1640, 1560, 1170, 820	2.2–2.6 (m, 2H, $-CH_2-$), 2.25 (s, 6H, $N(CH_3)_2$), 3.55 (t, 2H, CH_2Br), 4.35 (t, 2H, CH_2N), 7.05 (d, 2H, pyr), 8.35 (d, 2H, pyr)
3g	1630, 1490, 770, 680	2.60 (q, 2H, $-CH_2-$), 3.60 (t, 2H, CH_2Br), 4.90 (t, 2H, CH_2N), 8.20 (m, 2H, pyr), 8.72 (t, 1H, pyr), 9.25 (d, 2H, pyr)

Table 4. Physical Data of the Compounds 4

compd	yield (%)	mp (°C)	formula	molecular weight	recrystn solvent[a]
4a	52	205	$C_{29}H_{26}Br_2Cl_4N_4O_2$	764.2	EtOH
4b	32	178	$C_{23}H_{24}Br_2Cl_2N_4O_2$	619.2	EtOH/CH_2Cl_2 (3/1)
4c	24	175–179	$C_{22}H_{22}Br_2Cl_2N_4O_2$	605.5	EtOH
4d	31	hygr[b]	$C_{22}H_{23}Br_2Cl_2N_3O$	576.2	EtOH/CH_2Cl_2 (2/1)/Eth
4e	76	223	$C_{22}H_{20}Br_2Cl_2N_4O$	587.1	EtOH/Eth
4f	31	109	$C_{23}H_{26}Br_2Cl_2N_4O$	605.1	EtOH/CH_2Cl_2 (2/5)/acetone
4g	33	110	$C_{21}H_{21}Br_2Cl_2N_3O$	562.1	EtOH/Eth
4h	60	130	$C_{22}H_{21}BrCl_2N_2O$	480.2	EtOH/CH_2Cl_2 (1/6)
4i	74	109	$C_{23}H_{23}BrCl_2N_2O_2$	510.3	EtOH/CH_2Cl_2 (1/5)
4k	26	145	$C_{18}H_{25}BrCl_2IN_3O$	589.1	EtOH/CH_2Cl_2 (5/3)
4l	49	199	$C_{22}H_{31}Br_2Cl_2N_3O$	584.2	EtOH/CH_2Cl_2
4m	30	179	$C_{18}H_{17}BrCl_2N_2O$	404.1	EtOH/CH_2Cl_2 (2/7)

[a] The substances were recrystallized from ethanol (EtOH)/methylene chloride mixture. In some cases, a few drops of diethyl ether (Eth) were added till clouding. [b] Compound is hygroscopic.

the analytical section of the Department of Chemistry, University of Bonn, and are within ±0.4% of the theoretical values.

Chemistry. Pyridine-4-carboxaldehyde (*E*)-*O*-(2,6-dichlorobenzyl)oxime (1). A mixture of pyridine-4-carboxaldehyde (*E*)-oxime (4.4 g, 36 mmol) and sodium methylate (36 mmol) was dissolved in methanol at 0 °C. After the mixture was warmed up to room temperature, 2,6-dichlorobenzyl chloride (7.04 g, 36 mmol) in methanol was added. The mixture was refluxed for 4 h, cooled to room temperature, and evaporated *in vacuo* and the oil purified by the means of column chromatography (silica gel; mobile phase: ethyl acetate/CH_2Cl_2 = 2/5). The crystalline residue was recrystallized from cyclohexane/ether (10/1) to give 1.0 g (50%) of colorless crystals: mp 83 °C; ^1H NMR (60 MHz, DMSO-d_6) δ 5.50 (s, 2H, OCH_2), 7.20–7.35 (m, 3H), 7.40 (d, 2H, 7, pyr), 8.00 (s, 1H, HC=N), 8.60 (d, 2H, 7, pyr); IR 1600, 1440 cm^{-1}.

Pyridine-4-carboxaldehyde (*E*)-Methyloxime (2). The procedure as described above using methyl iodide gave a colorless oil (40%): ^1H NMR (60 MHz, DMSO-d_6) δ 4.01 (s, 3H, CH_3), 7.50 (d, 2H, 7, pyr), 8.03 (s, 1H, =CH), 8.66 (d, 2H, 7, pyr); IR (KBr) 2915, 2810, 1595, 1050, 925, 820 cm^{-1}.

General Procedure for the Syntheses of Para-Substituted (Bromopropyl)pyridinium Bromides 3b–g. Dibromopropane (30 mmol) and 10 mmol of the various substituted pyridine derivatives were refluxed in acetonitrile (60 mL) for 4–90 h. The solvent was removed *in vacuo*. The remaining oil was crystallized in solvent mixtures. For analytical and spectroscopic data, see Tables 2 and 3.

3-(*p*-Methoxyphenyl)propyl Bromide (3i). To a solution of 3-(*p*-methoxyphenyl)-1-propanol (1.5 g, 9 mmol) in CH_2Cl_2 (25 mL) was added PBr_3 (0.82 g, 3 mmol) slowly at 0–5 °C. The mixture was allowed to stand for 16 h. After addition of water, the product was extracted by cyclohexane to give 1.4 g (68%) of a colorless oil: n^{20}_D = 1.5513; bp 108–110 °C (0.01 mmolHg); ^1H NMR (60 MHz, $CDCl_3$) δ 2.10 (q, 2H, $-CH_2-$), 2.70 (t, 2H, CH_2 atom), 3.35 (t, 2H, CH_2-Br), 3.80 (s, 3H, OCH_3), 7.10 (d, 2H, arom), 7.6 (d, 2H, arom).

(3-Bromopropyl)trimethylammonium Iodide (3k) was synthesized as described in ref 17.

(3-Bromopropyl)triethylammonium Bromide (3l). A mixture of triethylamine (5 g, 50 mmol) and dibromopropane (20 g, 0.1 mol) in acetonitrile (70 mL) was refluxed for 4 h. Crystallization was initiated by addition of a mixture of methanol/diethyl ether. The crystalline solid was filtered and recrystallized from ethanol/diethyl ether to give 13.8 g (93%) of 3l: mp 90–94 °C; ^1H NMR (60 MHz, DMSO-d_6) δ 1.26 (t, 9H, CH_3), 1.96–2.60 (m, 6H, $-CH_2-$), 2.9–3.8 (m, 10H); IR 2990, 1580, 1506, 1010, 795 cm^{-1}.

(*E*,*E*)-1,1′-(1,3-Propanediyl)bis[4-[[(2,6-dichlorobenzoxyl)imino]methyl]pyridinium] Dibromide (4a). A mixture of 1 (4.0 g, 14.2 mmol) and dibromopropane (1.4 g, 7.1 mmol) in acetonitrile (70 mL) was refluxed for 6 days. After cooling, the crystalline solid was filtered and recrystallized from ethanol to give 2.8 g (52%) of fine yellowish needles. For analytical and spectroscopic data, see Tables 4 and 5.

General Procedure for the Syntheses of Derivatives 4b–m. Equimolar amounts of pyridine-4-carboxaldehyde (*E*)-*O*-(2,6-dichlorobenzyl)oxime (1) and the corresponding bromopropyl derivatives 3 in acetonitrile (60 mL) were heated at reflux for 80 h. In the case of incomplete conversion, remaining 1 can be removed by addition of water, extraction with diethyl ether, and evaporation of water. Otherwise, after the mixture was cooled to room temperature, the solvent was removed *in vacuo* and the

Table 5. Spectroscopic Data of the Compounds 4

compd	IR (KBr, cm^{-1})	^{1}H NMR (300 MHz, DMSO-d_6, δ (ppm), J (Hz))[a]
4a	1640, 1600, 1435, 1000, 770	2.71 (q, 2H, $-CH_2-$), 4.79 (t, 4H, 7.2, NCH_2), 5.56 (s, 4H, OCH_2), 7.45 (m, 4H, 8.9, arom), 7.54 (m, 4H, 8.9, arom), 8.24 (dt, 4H, 6.9, 2.0, pyr), 8.26 (s, 2H, CH=), 9.22 (dt, 4H, 6.9, 2.0, pyr)
4b	1640, 1600, 1440, 950, 840, 770	2.64 (m, 2H, $-CH_2-$), 4.08 (s, 3H, CH_3), 4.71 (m, 4H, NCH_2), 5.58 (s, 2H, OCH_2), 7.43–7.53 (m, 3H, arom), 8.22 (d, 2H, 6.5, pyr), 8.28 (d, 2H, 6.5, pyr), 8.55 (s, 1H, CH=), 8.60 (s, 1H, CH=), 9.11 (m, 4H, pyr)
4c	3300 (br), 1640, 1595, 995, 790, 765	2.68 (q, 2H, 7.0, $-CH_2-$), 4.78 (m, 4H, 7.0, CH_2N), 5.56 (s, 2H, OCH_2), 7.45 (m, 1H, 8.1, arom), 7.54 (m, 2H, 8.1, arom), 8.24 (d, 2H, 6.9, pyr), 8.24 (d, 2H, 6.9, pyr), 8.27 (d, 2H, 6.9, pyr), 8.46 (s, 1H, CH=), 9.15 (d, 2H, 6.9, pyr), 9.22 (d, 2H, 6.9, pyr), 12.8 (s, 1H, NOH)
4d	hygr[b]	2.61 (s, 3H, CH_3), 2.65 (q, 2H, $-CH_2-$), 4.70 (t, 2H, 8.0, CH_2N), 4.75 (t, 2H, 8.0, CH_2N), 5.58 (s, 2H, OCH_2), 7.59 (m, 3H, arom), 8.01 (dt, 2H, 7.0, pyr), 8.22 (dt, 2H, 7.0, pyr), 8.60 (s, 1H, CH=), 9.00 (dt, 2H, 7.0, pyr), 9.17 (dt, 2H, 7.0, pyr)
4e	1640, 1600, 1460, 1430, 790, 770	2.68 (q, 2H, 7.2, $-CH_2-$), 4.74 (t, 2H, 7.2, CH_2N), 4.84 (t, 2H, 7.2, CH_2N), 5.57 (s, 2H, OCH_2), 7.42–7.56 (m, 3H, arom), 8.21 (d, 2H, 7.0, pyr), 8.58 (s, 1H, CH=), 8.73 (d, 2H, 7.0, pyr), 9.12 (d, 2H, 7.0, pyr), 9.43 (d, 2H, 7.0, pyr)
4f	1640, 1560, 1430, 1200, 995, 760	2.5 (m, 2H, $-CH_2-$), 3.18 (s, 6H, $N(CH_3)_2$), 4.29 (t, 2H, CH_2N), 4.67 (t, 2H, CH_2N), 5.58 (s, 2H, OCH_2), 7.43–7.57 (m, 2H, arom), 8.20 (d, 2H, 6.7, pyr), 8.29 (d, 2H, 6.7, pyr), 8.59 (s, 1H, CH=), 9.11 (d, 4H, 6.7, pyr)
4g	1640, 1440, 910, 780, 680	2.65 (q, 2H, 7.5, $-CH_2-$), 4.72 (t, 2H, 7.5, CH_2N), 4.74 (t, 2H, 7.5, CH_2N), 5.59 (s, 2H, OCH_2), 7.43–7.58 (m, 3H, arom), 8.17–8.20 (m, 2H, pyr), 8.23 (dt, 2H, 7.0, pyr), 8.60 (s, 1H, CH=), 8.64 (tt, 1H, 7.0, 1.5, pyr), 9.12 (dt, 2H, 7.0, pyr)
4h	1640, 1600, 1440, 1090, 910, 760, 680	2.24 (m, 2H, $-CH_2-$), 2.64 (m, 2H, CH_2 arom), 4.63 (t, 2H, CH_2N), 5.57 (s, 2H, OCH_2), 7.13–7.29 (m, 5H, arom), 7.43–7.57 (m, 3H, arom), 8.16 (dt, 2H, 7.0, pyr), 8.56 (s, 1H, CH=), 9.09 (dt, 2H, pyr)
4i	1640, 1600, 1515, 1440, 1090, 780	2.21 (q, 2H, $-CH_2-$), 2.60 (t, 2H, 7.1, CH_2 arom), 3.69 (s, 3H, OCH_3), 4.61 (t, 2H, 7.4, CH_2N), 5.57 (s, 2H, OCH_2), 6.82 (d, 2H, 8.6, arom), 7.11 (d, 2H, 8.6, arom), 7.45–7.57 (m, 3H, arom), 8.16 (d, 2H, 7.0, pyr), 8.56 (s, 1H, CH=), 9.09 (d, 2H, 7.0, pyr)
4k	1645, 1440, 1010, 970	2.36–2.52 (m, 2H, $-CH_2-$), 3.07 (s, 9H, $N(CH_3)_3$), 3.32–3.42 (m, 2H, CH_2N), 4.65 (t, 2H, CH_2N), 5.60 (s, 2H, OCH_2), 7.43–7.58 (m, 3H, arom), 8.24 (d, 2H, pyr), 8.60 (s, 1H, CH=), 9.10 (d, 2H, pyr)
4l	1640, 1465, 1435, 1000, 960, 780, 765	1.20, (t, 9H, 7.2, 3 × CH_3), 2.35–2.45 (m, 2H, $-CH_2-$), 3.13–3.32 (q, 8H, 4 × CH_2N), 4.69 (t, 2H, 7.4, CH_2N), 5.58 (s, 2H, OCH_2), 7.43–7.57 (m, 3H, arom), 8.24 (d, 2H, 6.8, pyr), 8.60 (s, 1H, CH=), 9.19 (d, 2H, 6.8, pyr)
4m	1640, 1600, 1440, 920, 840, 780	0.87 (t, 3H, 7.0, CH_3), 1.92 (m, 2H, $-CH_2-$), 4.53 (t, 2H, 7.0, CH_2N), 5.56 (s, 2H, OCH_2), 7.49 (m, 3H, arom), 8.19 (dt, 2H, 7.0, pyr), 8.57 (s, 1H, CH=), 9.10 (dt, 2H, 7.0, pyr)

[a] pyr is pyridine, and arom are aromatic hydrogens. [b] IR was not measurable.

obtained oil crystallized from ethanol/diethyl ether. Recrystallization from ethanol/methylene chloride (2/1 or 6/1) gives 4. For analytical and spectroscopic data, see Tables 4 and 5.

Determination of Lipophilicity. General Procedure: 5×10^{-5} M of the derivative was dissolved in Na_2HPO_4 buffer (pH 7.4, 0.078 mol) saturated with octanol and the absorbance measured at 290 nm. A 0.7-mL portion of this solution was shaken for 2 h with 0.7 mL of octanol saturated with the buffer. The solution was centrifuged, the water layer separated, and again the absorbance measured. From the difference of the absorbances, the octanol/buffer partition coefficient and the log P value were calculated.

Pharmacology. Radioligand Binding. Freshly excised hearts of domestic pigs were obtained from the local slaughterhouse. All preparation steps were carried out at a temperature of 4 °C. A 40-g specimen of ventricular tissue was minced and rinsed in 0.32 M sucrose solution to be cleaned of blood. The tissue was homogenized in 0.32 M sucrose (20 mL/g of wet weight) by means of a Waring Blendor (New Hartfort) and a Potter Elvejhem glass homogenizer with a motor-driven Teflon pestle. In order to remove detritus, the homogenate was centrifuged for 11 min at 300g (2000 rpm, Beckman rotor 35). The supernatant was centrifuged for 40 min at 80000g (32 000 rpm, Beckman rotor 35). The pellets were resuspended in 50 mM Tris-HCl buffer (pH 7.4). Aliquots of 1 mL were shock-frozen in liquid nitrogen and stored at −20 °C.

The binding assay has been described previously.[7,15] Binding of [^{3}H]NMS (0.2 nM, specific activity 85.1 Ci/mM) was measured in an incubation medium consisting of 3 mM $MgHPO_4$ and 50 mM Tris-HCl, pH 7.3 at 37 °C. Nonspecific binding of the radioligand was determined in the presence of 1 μM atropine and amounted to 2–5% of the total binding. After 120 min of incubation, 1-mL aliquots were drawn from the incubation medium and filtered under suction through glass fibre filters (Schleicher & Schüll, No. 6; Dassel, Germany). Immediately, the filters were washed twice with 5 mL of ice-cold distilled water. Membrane-bound radioactivity was determined by liquid scintillation counting (Ready Protein scintillation cocktail, LS 6000 counter, Beckman).

[^{3}H]NMS binding under control conditions was characterized by (means ± SD, n = 6) an equilibrium dissociation constant, K_D = 440 ± 70 pM, and a receptor concentration of B_{max} = 87 ± 13 fmol/mL membrane suspension. Equilibrium binding was attained 5 min after starting the incubation.

Radioligand dissociation was visualized after preincubating the membranes with [^{3}H]NMS for 30 min by adding 1 μM atropine to the assay. The test compounds were applied simultaneously with atropine. To determine the effect of the compounds on the dissociation of [^{3}H]NMS, two procedures were used. Complete kinetic experiments: 1-mL aliquots were taken from a 22.5-mL assay at various time intervals up to 2 h. The data were subjected to computer-aided nonlinear regression analysis, yielding the half-life time of dissociation $t_{1/2}$ and the apparent rate constant k_{-1}, respectively. Two-point kinetic experiments: [^{3}H]NMS binding at time t = 0 min and the residual binding at t = 4 min after addition of atropine were determined. After correction for the nonspecific binding, the two data points were used to calculate the apparent rate, k_{-1}, of the underlying monoexponential time course of dissociation. For each concentration, 1.5-mL samples were prepared in quadruplicate.

Sigmoid curves were obtained by nonlinear regression analysis based on a four-parameter logistic equation, which yielded the half-maximum concentration EC_{50} and the slope factor n_H (Hill coefficient). Curve fitting was carried out by means of the Inplot software (GraphPad, San Diego).

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