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FULL PAPER

Synthesis of a Novel Series of Structurally Different MB327 Derivatives and Their Affinity Characterization at the Nicotinic Acetylcholine Receptor

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Abstract: A novel series of 30 symmetric bispyridinium and related N-heteroaromatic bisquaternary salts with a propane-1,3-diyl linker was synthesized and characterized for their binding affinity at the MB327 binding site of nAChR (nicotinic acetylcholine receptor) from *Torpedo californica*. Compounds targeting this binding site are of particular interest for the research into new antidotes against organophosphate poisoning, as therapeutically active 4-*tert*-butyl substituted bispyridinium salt **MB327** was previously identified as nAChR resensitizer. Efficient access to the target compounds was provided by newly developed methods enabling N-alkylation of sterically hindered or electronically deactivated heterocycles exhibiting a wide variety of functional groups. Determination of binding affinities towards the MB327 binding site at the nAChR, using a recently developed mass spectrometry (MS) based Binding Assay, revealed that several compounds reached affinities similar to that of **MB327** (pK_i = 4.73 ± 0.03). Notably, newly prepared lipophilic 4-*tert*-butyl-3-phenyl substituted bispyridinium salt PTM0022 (**3h**), was found to have a significantly higher binding affinity with a pK_i value of 5.16 ± 0.07, thus representing a considerable progress towards the development of more potent nAChR resensitizers.

Introduction

The recent use of sarin as chemical weapon in Syria as well as the high number of fatal poisonings with organophosphorus pesticides underline the threat emanating from organophosphorus compounds (OPCs) to humanity.[1-3] Therefore, there is an urgent need for effective medical treatment of OPC intoxications. OPCs inactivate the enzyme acetylcholinesterase (AChE), which results in accumulation of acetylcholine (ACh) in the synaptic cleft of both muscarinic and nicotinic synapses. Whereas overstimulation of muscarinic receptors (mAChRs) can be antagonized by using atropine, administration of anticholinergics to counteract nAChR overstimulation is precluded by their therapeutic limitations.[4] Thus ongoing overstimulation by accumulated ACh forces the nAChRs into a desensitized state,[5,6] which is currently therapeutically inaccessible.[7] Resulting disturbance of neuromuscular transmission may cause paralysis of respiratory and other skeletal muscles as most severe and often lethal consequences of OPC poisoning.

Current medical countermeasures make use of oximes such as obidoxime or pralidoxime to reactivate the inhibited AChE, thus treating cholinergic crisis. However, in many cases oximes were shown to be ineffective. Fast dealkylation of the AChE-organophosphonate complex in case of soman intoxication or very slow reactivation rates of tabun-inhibited AChE for most commonly used oximes are only two of several examples for which the reactivation kinetic of this phenomenon has been well studied.[8,9] Hundreds of different oximes have been developed and tested over the last decades, without the successful identification of a universal reactivator of AChE.[10] In consequence, restoration of nAChR-mediated cholinergic signaling in the event of OPC poisoning remains a highly challenging task demanding for an alternative approach not exclusively based on AChE reactivation.

One very promising strategy might be the direct intervention at the nAChR to recover its activity from desensitization as recently demonstrated in measurements using a novel electrophysiological (SURFE^{2}R, surface electronic event reader) platform by Niessen et al.[11] These studies revealed that the 4-*tert*-butyl substituted bispyridinium compound **MB327** can resensitize the desensitized nAChR and restore channel function.[11,12] As previous investigations have already shown that **MB327** targets the orthosteric binding site with only weak affinity, functional recovery of the nAChR is suggested to be mediated rather by allosteric modulation.[13,14] In contrast to an oxime-based therapy aiming at reactivation of inhibited AChE, an allosterically mediated resensitization of the nAChR has the advantage to restore nAChR activity and thereby, preserving neuromuscular

function in any case of OP (organophosphorus) nerve agent poisoning.

The therapeutic effect of **MB327** against OPC poisoning has also been demonstrated in several *ex vivo*, *in vitro* as well as *in vivo* studies. In case of tabun-poisoned guinea pigs, for example, treatment with **MB327** significantly increased the survival rate as compared to the oxime HI-6, both administered together with physostigmine and hyoscine.[15] Furthermore, Seeger et al. found that **MB327** restores soman-impaired neuromuscular transmission in human- and rat-respiratory muscle preparations at concentrations of 100–200 µmol L^{-1}.[16] Unfortunately, **MB327** also acts as an AChE inhibitor at slightly higher concentrations (IC_{50} about 600 µmol L^{-1}) and antagonizes nicotinic currents at concentrations ≥ 100 µM,[17,18] possibly leading to adverse effects, if administered *in vivo*.[19] In consequence, the therapeutic window of **MB327** is too narrow for administration in case of OPC poisoning, hence more potent and more selective nAChR resensitizers have to be developed.

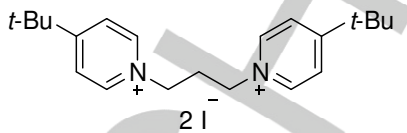
Though **MB327** has been studied extensively in the last years regarding its potential use in treatment regimens,[20-22] the targeted binding site at the nAChR was characterized only very recently.[23] Binding Assays using mass spectrometry for marker quantification (MS Binding Assays) enabling the affinity determination for ligands targeting the MB327 binding site have been established.[24] Based on these MS Binding Assays, we could characterize the affinity of **MB327** to the nAChR with a K_i value of 18.3 ± 2.6 µmol L^{-1}, which is in line with its potency determined in electrophysiological and pharmacological studies. For our MS Binding Assays, we use the nAChR from *Torpedo californica*, which shows a high degree of homology to the human muscle-type nAChR and represents the most common model system.[25,26]

In parallel to the development of [$^2H_6$]MB327 MS Binding Assays, our group identified two putative MB327 binding sites using *in silico* docking studies at the nAChR of *Torpedo marmorata* at 4°A resolution.[23] Respective sites are located inside the channel, one in the extracellular and one in the transmembrane domain. The developed MS Binding Assays allow to gain quantitative binding data for the nAChR for the very first time. The latter are an essential prerequisite for a detailed understanding of the binding interactions at a molecular level and for the development of valid structure-affinity relationships representing a key element in the search for new antidotes against OPC intoxication. Hence, we envisaged the synthesis and affinity characterization of a novel series of structurally different **MB327** analogues, which were expected to be of interest to gain first insights into structure-affinity relationships. Herein, we report the results of this endeavor.

Results and Discussion

Structure of Target Compounds

With **MB327** as lead structure, we focused on the investigation of symmetrical bispyridinium compounds with distinct structural modifications. The results of how these structural changes affect affinity to the MB327 binding site should provide information on the molecular interactions important for the binding event. **MB327** is a symmetric bispyridinium diiodide with a *tert*-butyl group in 4-position of each pyridinium ring and a propyl linker connecting both aromatic rings (Figure 1).



MB327

Figure 1. Structure of **MB327**.

Depending on the structural characteristics, the influence of which on the binding affinity should be studied, target compounds were divided into four different types (I–IV), comprising in total 30 structurally related compounds (Figure 2). Compounds of type I and II are bispyridinium salts substituted with either one lipophilic (Me, *i*-Pr, *t*-Bu) or one hydrophilic (OMe, NMe_2, *N*-methylpyrrolidine) substituent on each pyridinium ring. These moieties were introduced to investigate the influence of the substituent position at the ring system, the steric demand of the functional groups as well as hydrogen bridge interactions between polar moieties and the binding pocket on the binding affinity. Bispyridinium salts of type III were directly delineated from the lead structure **MB327** by introduction of an additional substituent and designed to investigate the resulting affinity. These compounds exhibit a 4-*tert*-butyl group as well as a second functional group (e.g., Ph, CN, OMe, COOEt, Cl) in 2- or 3-position of the pyridinium ring.

In consequence, from these compounds, substituent effects can be directly derived by comparing the binding affinity with that of **MB327**, which is devoid of any substituent in 2- or 3-position. To investigate the importance of π-π interactions with aromatic amino acid side chains of the nAChR during the binding event and to expand the structural diversity amongst the test compounds, pyridine was finally replaced by other heteroaromatic compounds such as isoquinoline, thiazole or 2-*tert*-butylpyrazine, thus resulting in new target structures of type IV.

Common to all newly synthesized compounds, however, is the propane-1,3-diyl (C3) linker, known from **MB327**. This linker was used for all test compounds, as **MB327** showed a significantly higher resensitization potency in SURFE^{2}R-based electrophysiological experiments as compared to structurally analogous compounds with a shorter i.e. an ethane-1,2-diyl (C2) or a longer i.e. a butane-1,4-diyl (C4) linker.[11]

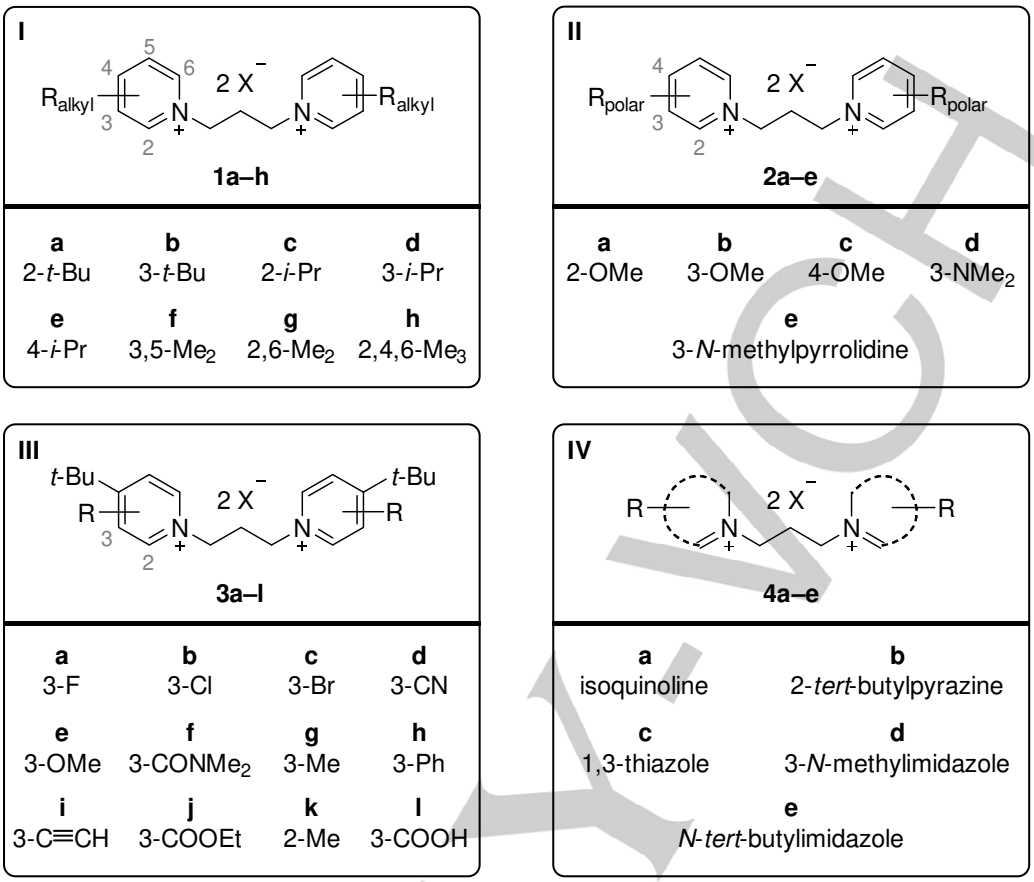


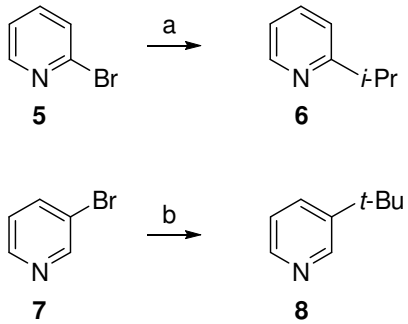
Figure 2. Structures of envisaged target compounds (type I–IV). X = Cl^-, I^-, OTf^-

Chemistry

As shown in Figure 2, all target compounds are symmetric bispyridinium salts or related from N-aromatic subunits derived salts with a propane-1,3-diyl linker. As such, these compounds are easily accessible in a two-step reaction sequence. In the first step, the required nitrogen heterocycles are synthesized which in the second step afford the target compounds upon N-alkylation. Of the 30 nitrogen heterocycles employed in the synthesis of the target compounds, 25 were either commercially available or synthesized according to literature procedures,[27-29] including the 3-substituted 4-*tert*-butylpyridine derivatives, the preparation of which was recently reported by us.[30] The remaining five pyridine derivatives namely, 4-(*tert*-butyl)-2-methylpyridine (**15**), 3-*tert*-butylpyridine (**8**), 2- (**6**), 3- (**11**), and 4-isopropylpyridine (**13**) were prepared as described below.

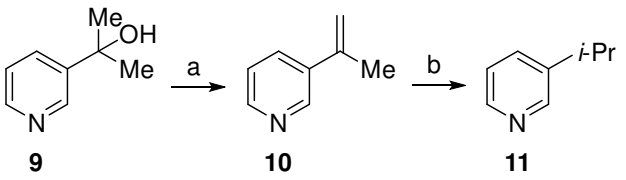
Preparation of alkyl substituted pyridine derivatives

2-Isopropylpyridine (**6**) as well as 3-*tert*-butylpyridine (**8**) were prepared from the corresponding bromopyridines **5** and **7** by cuprate mediated substitution reactions resulting in low to moderate yields of 19 and 43%, respectively, for the target compounds (Scheme 1).[27] The low yields are to be assigned to side reactions that led to pyridine as side product, which in each of the two cases was isolated in large amounts. These side reactions might result from a halogen metal exchange caused by the intermediate isopropyl cuprate and by a β-H elimination of the putative (*t*-Bu)$_2$Cu(III)pyridyl complex.[31] Interestingly, THF turned out to be far less favorable for the substitution with the *tert*-butyl cuprate, affording **8** in 12% only (43% in Et_2O). This is likely due to the more reactive cuprate oligomers, present in Et_2O, as compared to the less reactive monomers, predominant in THF, which is a specific feature of cyano cuprates found by Krause and Gschwind.[32] Besides, yields were also negatively affected by the remarkable high volatility of **6** and **8**.



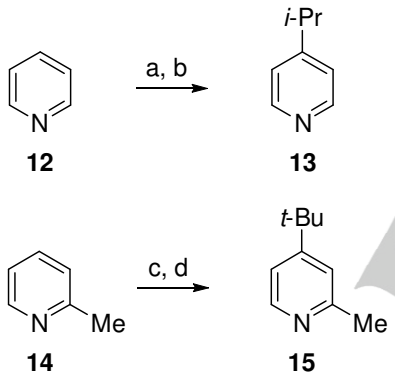
Scheme 1. Synthesis of alkyl pyridines **6** and **8**. *Reagents and conditions*: a) CuCN (4.0 eq.), *i*-PrMgCl (8.0 eq.), –78 °C for 2 h → RT for 15 h, THF, 19%; b) CuCN (4.0 eq.), *t*-BuMgCl (8.0 eq.), –78 °C for 2 h → RT for 15 h, Et_2O, 43% (12% yield in THF).

As 3-isopropylpyridine (**11**) was found to be not accessible by a cuprate mediated substitution of **7**, it was synthesized in two steps from tertiary alcohol **9**.[33] Acid catalyzed dehydration of **9** and subsequent hydrogenation of the resulting alkene **10** with Pd/C and H_2 afforded **11** in 51% yield (Scheme 2).



Scheme 2. Synthesis of **11**. *Reagents and conditions:* a) conc. AcOH / conc. H_2SO_4 (1:2.7), 150 °C, 30 min; b) Pd/C (5 mol%), H_2, MeOH, 16 h, 51% over both steps.

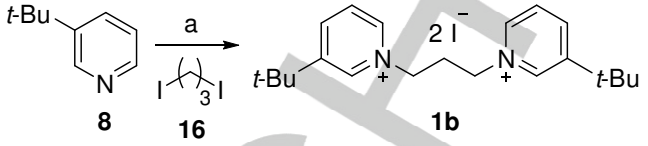
The synthesis of 4-isopropylpyridine (**13**) and 4-(*tert*-butyl)-2-methylpyridine (**15**) was accomplished in a two-step reaction starting from **12** and **14**, respectively (scheme 3). Treatment of **12** with AcCl and of **14** with PhOCOCl followed by an addition of the respective organocuprates to the thus formed *N*-acyliminium intermediates provided after oxidation **13** and **15** in 33 and 47% yield, respectively.



Scheme 3. Synthesis of **13** and **15**. *Reagents and conditions*: a) AcCl (1.0 eq.), THF, –78 °C, 75 min, then $(i\text{-Pr})_2CuCN(MgCl)_2$ (1.1 eq.) 80 min; b) DDQ (1.0 eq.), CH_2Cl_2, RT, 70 min, 33% over both steps; c) PhOCOCl (1.0 eq.), *t*-BuZnBr (1.1 eq.), CuCN (1.1 eq.), LiCl (2.2 eq.), –78 °C for 5 h → RT for 16 h, THF; d) S_8 (1.1 eq.), naphthalene (15 eq.), 200 °C, 20 min, mw: 300 W, 47% over both steps.

Preparation of the target compounds

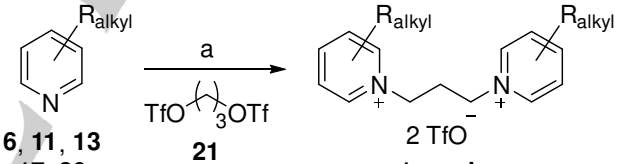
As mentioned above, the synthesis of symmetric bis(pyridinium) salts with a propane-1,3-diyl linker (Figure 2) is commonly accomplished by reaction of 1-3-diiodo- or 1,3-dibromopropane with an excess of the respective pyridine derivatives in DMF or MeCN at 70 °C.[34-36] However, when these reaction conditions were used for the synthesis of compounds of type I and II, in most cases, only a negligible conversion but substantive decomposition of reactants occurred. This is mainly attributed to the low reactivity of the alkyl halides, especially when sterically hindered 2-substituted pyridine derivatives were employed (**6**, **17**, **19**, **20**). Besides, in case of N-alkylated **22** and **23**, also demethylation reactions by iodide ions occurred. Notably, even for the reaction of 1,3-diiodopropane (**16**) with 2.5 equivalents of the pyridine derivative **8**, devoid of any substituents in 2- and 6-position and of any electron withdrawing groups, required 72 h at 70 °C in DMF for full conversion, affording **1b** in 79% yield (Scheme 4).



Scheme 4. Synthesis of **1b**. *Reagents and conditions*: 1,3-diiodopropane (**16**, 1.0 eq.), **8** (2.5 eq.), DMF, 70 °C, 72 h, 79%.

Hence, 1,3-diiodopropane (Scheme 4) did not seem suitable for an efficient preparation of the desired target compounds. This problem could, however, be overcome employing the far more reactive propane-1,3-diyl bis(trifluormethanesulfonate) (**21**). With bistriflate **21**, compounds of type I–III (**1a**, **c**–**h**, Table 1; **2a**–**e**, Table 2; **3a**–**l**, Table 3) were easily accessible in fair to excellent yields (47–99%) by alkylation of the corresponding heterocycles. Reactions were performed either at 50 °C in CH_2Cl_2 or, if necessary, at higher temperatures without a solvent. Resulting bis(trifluoromethanesulfonate) salts were finally purified by crystallization without any problems.

Table 1. Synthesis of bispyridinium compounds of type I (**1a**, **c**–**h**).

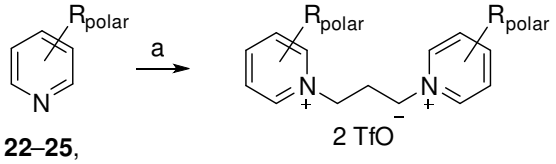


Entry	Starting Material		*T*	t	Product	Yield
	R_{alkyl}	No.	(°C)	(min)	**1**	(%)
1	2-*t*-Bu	**17**	85	300	**a**	73
2	2-*i*-Pr	**6**	50	5	**c**	77
3	3-*i*-Pr	**11**	50	2	**d**	88
4	4-*i*-Pr	**13**	50	60	**e**	81
5	3,5-Me_2	**18**	50	1	**f**	69
6	2,6-Me_2	**19**	70	1	**g**	75
7	2,4,6-Me_3	**20**	50	15	**h**	81

Reagents and conditions: a) pyridine derivatives (2.5 eq.), **21** (1.0 eq.), no solvent, *T*, *t*.

In contrast to the above-mentioned alkylation reaction with 1,3-diiodopropane (**16**), the high reactivity of bistriflate **21** allowed short reaction times (1–30 min in most cases) and gave also access to bispyridinium compounds with sterically demanding substituents in 2-position such as **1a**, **1c**, **1g**, **1h** (Table 1), and **3k** (Table 3).

Table 2. Synthesis of bispyridinium compounds of type II (**2a**–**e**).

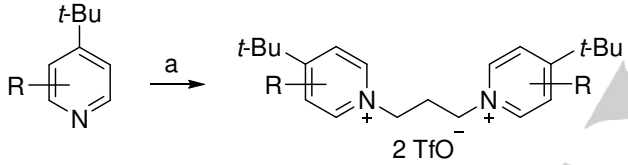


22–**25**, *rac*–**26** **2a**–**e**

Entry	Starting Material		*t*	Product	Yield
	R_{polar}	No.	(min)	**2**	(%)
1	2-OMe	**22**	1	**a**	67
2	3-OMe	**23**	1	**b**	83
3	4-OMe	**24**	1	**c**	84
4	3-NMe_2	**25**	10	**d**	47
5	3-*N*-methylpyrrolidine	***rac*-26**	30	**e**	72

Reagents and conditions: a) pyridine derivatives (2.5 eq.), **21** (1.0 eq.), 50 °C, *t*, entry 1-4: no solvent, entry 5: reaction in CH_2Cl_2.

Table 3. Synthesis of bispyridinium compounds of type III (**3a**–**l**).



27–**36**, **15** **3a**–**k**, **l**[a]

Entry	Starting Material		*t*	Product	Yield
	R	No.	(min)	**3**	(%)
1	3-F	**27**[b]	4	**a**	85
2	3-Cl	**28**	30	**b**	81
3	3-Br	**29**	30	**c**	99
4	3-CN	**30**	40	**d**	85
5	3-OMe	**31**	5	**e**	83
6	3-$CONMe_2$	**32**	30	**f**	77
7	3-Me	**33**	20	**g**	89
8	3-Ph	**34**	30	**h**	99
9	3-C≡CH	**35**	40	**i**	91
10	3-COOEt	**36**	30	**j**	98
11	2-Me	**15**	30	**k**	82
12[a]	3-COOEt	**3j**	16 h	**l**	66

Reagents and conditions: a) pyridine derivatives (2.5 eq.), **21** (1.0 eq.), CH_2Cl_2, 50 °C, *t*. [a] **3l** was synthesized from **3j** (1.0 eq.) in 66% yield by basic hydrolysis using aq. NaOH (0.1 M, 2.5 eq.) in H_2O/MeCN (5:1) at RT for 16 h. [b] 4-(*tert*-butyl)-3-fluoropyridine was released *in situ* from the corresponding hydro chloride **27**.

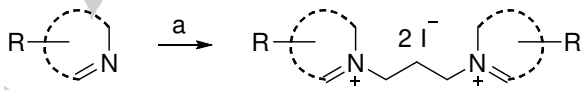
Despite the high reactivity of bistriflate **21**, a variety of functional groups, i.e. alkylarylethers (**2a**–**c**, **3e**), nitrile (**3d**), halide (**3a**–**c**), terminal alkyne (**3i**), ester (**3j**), amide (**3f**), and even secondary (**2d**) and tertiary amino substituents (**2e**) were well tolerated. Also, the carboxylic acid **3l** (Table 3, entry 12) could be synthesized by basic hydrolysis of the ester function of **3j** with aqueous NaOH. To this end, however, milder conditions had to be employed (hydroxide ion concentration of only 0.055 mol L^{-1}), to reduce a Zincke-König type cleavage to a minimum, thus affording **3l** in 66% yield after crystallization.

Although biological evaluation of synthesized bispyridinium salts **1a**–**h** (Table 1), **2a**–**e** (Table 2) and **3a**–**l** (Table 3) using our MS Binding Assays yielded reliable results, the triflate counterion was assumed to cause interferences in electrophysiological measurements on the SURFE^{2}R platform.[37] Thus, we decided to synthesize compounds of type IV with iodide instead of triflate counterions.

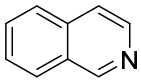
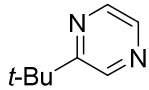
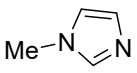
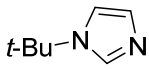
In consequence, compounds **4a**–**e** were synthesized by reaction of the respective N-heteroaromatics **37**–**41** with 1,3-diiodopropane (**16**) in MeCN under microwave heating to 90–120 °C. The yields were good to excellent (78–92%, Table 4). Under microwave conditions, higher reaction temperatures could be reached resulting in distinctly reduced reaction times. Besides, also side reactions appeared to be absent this time.

Solubility of compounds of type I–IV in water proved to be sufficient for biological testing and allowed the preparation of solutions with concentrations of at least 2.5 mM. Interestingly, a faster dissolution in water was observed for triflate salts of type I–III compared to iodide salts of type IV.

Table 4. Synthesis of non-bispyridinium compounds of type IV (**4a**–**e**).



37–**41** **4a**–**e**

Entry	Starting Material		*T*	t	Product	Yield
	$N_{het.}$	No.	(°C)	(h)	**4**	(%)
1		**37**	90	3	**a**	86
2		**38**	120	16	**b**	78
3		**39**	120	16	**c**	87
4		**40**	90	1	**d**	92
5		**41**	90	1	**e**	82

Reagents and conditions: a) $N_{het.}$ (2.5 eq.), **16** (1.0 eq.), MeCN, *T*, *t*, mw: 150W.

To evaluate the stability of synthesized target compounds in water, NMR measurements of a random selection of compounds (**1b**, **2a**, **2c**, **3d**, **3l)** after 12 h incubation in D_2O at room temperature were performed and provided no indications for hydrolysis.

Preparation of deuterated MB327 analogs for MS Binding Assays

For the recently established MS Binding Assays targeting the MB327 binding site at the *Torpedo*-nAChR, deuterated **MB327** analogs [$^2H_6$]MB327 (**46**) and [$^2H_{18}$]MB327 (**49**) were required as MS marker and internal standard, respectively. Their synthesis was accomplished as follows.

The deuterium labeled compound **46** was synthesized in four steps starting from malonic ester **42** in an overall yield of 18% and a deuteration degree of ≥ 99% (Scheme 5). At first, **42** was treated with D_2O and pyridine according to Cocker et al. affording the d_2-derivative **43** in 40% yield.[38] Reduction with $LiAlD_4$ in Et_2O and subsequent treatment of crude d_6-propanediol **44** with Tf_2O provided the corresponding bistriflate **45** in 58% yield over both steps. Reaction of **45** with 2.5 equivalents 4-*tert*-butylpyridine finally afforded **46** in a yield of 77%.

The synthesis of [$^2H_{18}$]MB327 (**49**) started from pyridine (**12**) and was performed in three steps, the total yield amounting to 25% and the deuteration degree to ≥ 99% (Scheme 6). In the first step, pyridine (**12**) was treated with AcCl to generate the corresponding *N*-acylpyridinium ion *in situ* which upon trapping with (*t*-Bu)$_2$CuCN(MgCl)$_2$ afforded **47** with good regioselectivity (79:21 for C4-isomer). Oxidation of 1,4-dihydropyridine **47** with DDQ led to d_9-labeled pyridine **48** in 31% yield which upon reaction of triflate **21** with 2.5 equivalents provided **49** in 81% yield.

Scheme 5. Synthesis of [$^2H_6$]MB327 (**46**). *Reagents and conditions*: a) pyridine (6.0 eq.), D_2O (12 eq.), RT, 20 h, 40%, 99.6% D; b) $LiAlD_4$ (3.0 eq.), Et_2O, 50 °C, 5.5 h; c) Tf_2O (2.0 eq.), pyridine (2.0 eq.), CH_2Cl_2, –78 °C → RT, 3 h, 58% over both steps; d) 4-*tert*-butylpyridine (2.5 eq.), CH_2Cl_2, 50 °C, 30 min, 77%, 99% D.

Scheme 6. Synthesis of [$^2H_{18}$]MB327 (**49**). Reagents and conditions: a) AcCl (1.0 eq.), THF, –78 °C, 60 min, then d_9-*t*-BuMgCl (2.2 eq.), CuCN (1.1 eq.), THF, –78 °C, 1.5 h; b) DDQ (1.0 eq.), CH_2Cl_2, RT, 1 h, 31% over both steps; c) **21** (0.4 eq.), 50 °C, 2.5 h, 81%, 99% D.

Biological evaluation

All target compounds synthesized in the context of this study were catalogued with a certain PTM number (Pharmacy and Toxicology Munich). Binding affinities towards the MB327 binding site at the *Torpedo*-nAChR were determined in MS Binding Assays using [$^2H_6$]MB327 (**46**) as marker (Table 5).[24]

With these MS Binding Assays, developed recently in our group, it was possible for the first time to characterize binding of **MB327** towards the nAChR (pK_i of 4.75 ± 0.07 for **MB327** in autocompetition experiments with [$^2H_6$]MB327 (**46**) as marker). According to the corresponding experimental protocol, aliquots of nAChR-enriched membranes, prepared from *Torpedo californica* electroplaque tissue and frozen in storage buffer (120 mmol L^{-1} NaCl, 5 mmol L^{-1} KCl, 8.05 mmol L^{-1} Na_2HPO_4 and 1.95 mmol L^{-1} NaH_2PO_4, pH 7.4) were thawed, directly diluted with incubation buffer and incubated in presence of the marker (and test compounds if required). Both buffers, storage and incubation buffer, are almost identical in this assay, except that the latter additionally contains 1 mmol L^{-1} $CaCl_2$. With the aim to simplify the developed MS Binding Assays, we examined the possibility to use only one buffer for the whole procedure, i.e. for the storage of *Torpedo* membrane preparations as well as for the incubation in the binding experiments described here. Since the storage buffer, differing from the incubation buffer by the lack of $CaCl_2$, was also identical to the incubation buffer that had been used in [^{3}H]epibatidine binding studies,[18,37] which were conducted recently for characterization of bispyridinium compounds at the

orthosteric binding site of the *Torpedo*-nAChR, it appeared obvious to choose this buffer also for incubation in [$^2H_6$]MB327 MS Binding Assays. Hence, saturation and autocompetition experiments were conducted exactly as previously described,[24] but employing the storage buffer also for incubation of binding samples. Under these marginally modified conditions, we found a K_d of 14.2 ± 1.4 µmol L^{-1} and a B_{max} of 263 ± 30 pmol [mg protein]$^{-1}$ (n = 3) in saturation experiments using [$^2H_6$]MB327 (**46**) as marker and a pK_i of 4.73 ± 0.03 (K_i = 18.7 ± 1.2 µmol L^{-1}, n = 3) for autocompetition experiments using [$^2H_6$]MB327 (**46**) as marker and native **MB327** as competitor (for further details see supplementary information). As there was no significant difference (verified by two-sided t-test) between the affinity constants, obtained in experiments with $CaCl_2$ as opposed to experiments without $CaCl_2$ in the incubation buffer,[24] we decided to use the $CaCl_2$ free storage buffer also as incubation buffer in all binding experiments.

Although this marginal modification of the sample matrix was not expected to affect the quantification of the MS marker, we still decided to again examine the parameters selectivity, linearity, lower limit of quantification (LLOQ), accuracy as well as precision of the LC-ESI-MS/MS quantification method according to the FDA's guidance on bioanalytical method validation.[39] In short, all requirements of the Center for Drug Evaluation and Research (CDER) guideline of the FDA regarding the above mentioned parameters were fulfilled (for further detail see supplementary information), clearly demonstrating LC-ESI-MS/MS quantification to be robust and reliable also under these binding conditions. This modified protocol for [$^2H_6$]MB327 MS Binding Assays (for more detail see experimental part) was finally used for all further determinations of binding affinities of target compounds.

Initially, the influence of the position of the *tert*-butyl substituent on the binding affinity was investigated by comparing the pK_i value of 2-*tert*-butyl and 3-*tert*-butyl substituted compounds **1a** (PTM0002) and **b** (PTM0001) (type I) with that of 4-*tert*-butyl substituted **MB327** (Table 5, entries 1–3). Interestingly, the difference between both 2- and 3-regioisomers was negligible, but binding affinity decreased when the *tert*-butyl group was moved from the 4- to the 3- or 2-position. Moreover, reduction of the steric demand of the lipophilic substituent by replacing the *tert*-butyl group in **MB327**, **1a** (PTM0002) and **1b** (PTM0001) by an isopropyl group (**1c**–**e**, PTM0003, PTM0013, PTM0014, entries 4–6) led to a distinct reduction of binding affinity, except for the 3-substituted compounds, where the difference was less pronounced. A decrease in binding affinity was also observed for the lutidine (**1f**, **1g**, PTM0005 and PTM0004, entries 7 and 8) and collidine derived bispyridinium salts (**1h**, PTM0006, entry 9), especially when the 4-position remained unsubstituted (**1f**, PTM0005: 3,5-Me_2, pK_i = 3.76 ± 0.14; **1g**, PTM0004: 2,6-Me_2, pK_i = 3.80 ± 0.04; **1h**, PTM0006: 2,4,6-Me_3, pK_i = 4.30 ± 0.09 as compared to **MB327**: pK_i = 4.73 ± 0.03; see Table 5 entries 1 and 7–9).

Comparing the affinities of the bispyridinium salts substituted with lipophilic (type I, Table 5, entries 1–9) with those substituted with polar residues of type II (Table 5, entries 10–14), it becomes obvious that the replacement of the alkyl substituents by an *N*-methylpyrrolidine moiety in 3-position (**2e**, PTM0027) or a polar methoxy group (**2a**–**c**, PTM0008–PTM0010) in 2-, 3- or 4-position has a negative influence on binding affinity. The determined pK_i values range from 3.87 ± 0.13 to 3.97 ± 0.04 and are thus at least about half a log unit lower than those found for the *tert*-butyl substituted compounds **1a** (PTM0002), **1b** (PTM0001) and **MB327** (pK_i = 4.43 ± 0.12 to 4.73 ± 0.03, Table 5, entries 1–3). In contrast, the 3-dimethylamino substituted bispyridinium salt **2d** (PTM0007) showed a higher pK_i value (4.78 ± 0.05, Table 5, entry 13) than the 3-*tert*-butyl substituted compound **1b** (PTM0001, pK_i = 4.44 ± 0.10, Table 5, entry 3). In conclusion, the NMe_2-group is suggested as potential substitute for the *tert*-butyl-substituent.

The binding affinities of type III compounds exhibiting a F, Cl, Br, CN, OMe, $CONMe_2$, Me, C≡CH or COOEt substituent in 3-position (Table 5, entries 15–21 and 23–25) in addition to the 4-*tert* butyl group in **MB327** varied from 4.00 ± 0.12 in case of 3-CN derivative **3d** (PTM0019, Table 5, entry 18) to 4.80 ± 0.08 for 3-alkyne substituted **3i** (PTM0025, Table 5, entry 23) without a clear tendency when regarding the nature of the substituent.

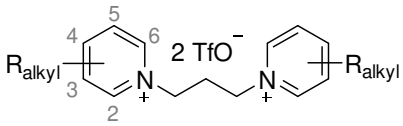
However, the results indicate that the 4-*tert*-butyl group in the bispyridinium salts has a beneficial effect on binding affinity. This becomes evident by comparing the pK_i value of 4-*tert*-butyl substituted **MB327** (pK_i = 4.73 ± 0.03, Table 5, entry 1) with those of 3-methoxy substituted **2b** (PTM0009, pK_i = 3.79 ± 0.09, Table 5, entry 11) and 4-*tert*-butyl-3-methoxy derivative **3e** (PTM0016, pK_i = 4.46 ± 0.07). Apparently, the addition of a *tert*-butyl group in 4-position increased the binding affinity of the 3-methoxy derivative **2b** (PTM0009) by 0.67 log units from 3.79 ± 0.09 to 4.46 ± 0.07 for **3e** (PTM0016, compare Table 5, entries 11 and 19).

In accordance with the negative effect of polar substituents on binding affinity as discussed above, zwitterionic carboxylic acid **3l** (PTM0028) exhibited the lowest binding affinity of all tested compounds with a pK_i value of 2.74 ± 0.13 (Table 5, entry 26). The highest binding affinity, on the other hand, displayed the lipophilic 3-phenyl substituted MB327 analogue **3h** (PTM0022) with a pK_i value of 5.16 ± 0.07 (Table 5, entry 22), which is about half a log unit higher than the pK_i value of **MB327** (Table 5, entry 1) and two and a half order of magnitude above the one found for carboxylic acid **3l** (PTM0028, Table 5, entry 26). The statistical significance of the higher affinity found for the phenyl substituted derivative **3h** (PTM0022) as compared to **MB327** was finally also verified by means of two-sided *t*-test (5% significance level). The two additional aromatic phenylpyridinium rings present in **3h** (PTM0022) are likely to be involved in lipophilic interactions with the target giving rise to an increase in affinity.

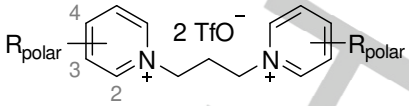
According to the pK_i values found for the non-bispyridinium compounds of type IV (**4a**–**e**, PTM0032–PTM0034, PTM0057, PTM0059, entries 27–31) none of the heterocyclic systems studied revealed a significantly higher binding affinity than **MB327**. Interestingly, electron poor 2-*tert*-butylpyrazinium salt **4b** (PTM0057, pK_i = 3.56 ± 0.09, Table 5, entry 28) showed a clearly lower pK_i value as compared to the structurally related but far less electron-deficient 3-*tert*-butylpyridinium salt **1b** (PTM0001, pK_i = 4.44 ± 0.10, Table 5, entry 3). Binding affinity of bisimidazolium salts decreased significantly upon transition from *N*-*tert*-butyl substituted **4e** (PTM0059, pK_i = 4.34 ± 0.13, Table 5, entry 31) to *N*-methyl substituted **4d** (PTM0034, pK_i = 3.55 ± 0.11, Table 5, entry 30). This indicates a general, beneficial effect of the *tert*-butyl group over smaller groups that had similarly been observed for the bispyridinium salts (compare e.g. **MB327** with **1e** (PTM0013), Table 5, entries 1 and 6). Though being unsubstituted and hence smaller than the *N*-methylimidazole derivative **4e** (PTM0059, Table 5, entry 31), the 1,3-thiazole derived compound **4c** (PTM0033) still had a reasonable affinity (pK_i 4.07 ± 0.13, Table 5, entry 29).

Table 5: Structures of synthesized target substances and binding affinities to the MB327 binding site.

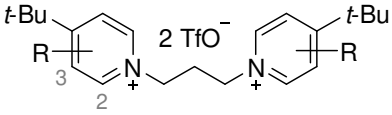
Type I: **1a–h**



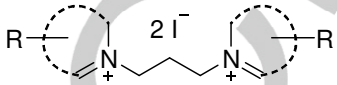
Type II: **2a–e**



Type III: **3a–l**



Type IV: **4a–e**



Entry	Compound	R_{alkyl} / R_{polar} / R / $N_{het.}$	pK_i[a]	PTM-Code
1	**MB327**	4-*t*-Bu	4.73 ± 0.03	
2	**1a**	2-*t*-Bu	4.43 ± 0.12	0002
3	**1b**	3-*t*-Bu	4.44 ± 0.10	0001
4	**1c**	2-*i*-Pr	3.83 ± 0.11*	0003
5	**1d**	3-*i*-Pr	4.18 ± 0.11	0014
6	**1e**	4-*i*-Pr	4.27 ± 0.11	0013
7	**1f**	3,5-Me_2	3.76 ± 0.14*	0005
8	**1g**	2,6-Me_2	3.80 ± 0.04*	0004
9	**1h**	2,4,6-Me_3	4.30 ± 0.09	0006
10	**2a**	2-OMe	3.87 ± 0.13*	0010
11	**2b**	3-OMe	3.79 ± 0.09*	0009
12	**2c**	4-OMe	3.97 ± 0.04*	0008
13	**2d**	3-NMe_2	4.78 ± 0.05	0007
14	**2e**	3-*N*-methylpyrrolidine	3.63 ± 0.13*	0027
15	**3a**	3-F	4.23 ± 0.05*	0021
16	**3b**	3-Cl	4.54 ± 0.09	0020
17	**3c**	3-Br	4.62 ± 0.08	0018
18	**3d**	3-CN	4.00 ± 0.12*	0019
19	**3e**	3-OMe	4.46 ± 0.07	0016
20	**3f**	3-$CONMe_2$	4.43 ± 0.10	0024
21	**3g**	3-Me	4.57 ± 0.08	0017
22	**3h**	3-Ph	5.16 ± 0.07*	0022
23	**3i**	3-C≡CH	4.80 ± 0.08	0025
24	**3j**	3-COOEt	4.50 ± 0.04	0015
25	**3k**	2-Me	4.31 ± 0.09	0023
26	**3l**	3-COOH	2.74 ± 0.13*	0028
27	**4a**	isoquinoline	4.77 ± 0.06	0032
28	**4b**	2-*tert*-butylpyrazine	3.56 ± 0.09*	0057
29	**4c**	1,3-thiazole	4.07 ± 0.13	0033
30	**4d**	*N*-methylimidazole	3.55 ± 0.11*	0034
31	**4e**	*N*-*tert*-butylimidazole	4.34 ± 0.13	0059

[a] Data are given as mean ± SEM of three independent experiments. pK_i values with a statistically significant difference towards MB327's binding affinity are marked by an asterisk (verified by means of two-sided *t*-test employing a 5% significance level).

Notably, bis(isoquinolinium) salt **4a** (PTM0032, pK_i = 4.77 ± 0.06), which is able to undergo more extensive lipophilic interactions, was found to have the highest binding affinity of all non-bispyridinium compounds, being in the same range as the binding affinity of **MB327**. Altogether, the substitution of bispyridinium compounds by addition of polar but uncharged functional groups generally led to a slight decrease in binding affinity, whereas the addition of a carboxylic acid function reduced binding affinity dramatically (**3l**, PTM0028, Table 5, entry 26). The sole exception is the 3-dimethylamino substituted bispyridinium compound **2d** (PTM0007, Table 5, entry 13), which shows a slightly increased binding affinity as compared to **MB327** (Table 5, entry 1).

Notably, more lipophilic 3-phenyl-4-*tert*-butyl substituted bispyridinium salt **3h** (PTM0022, pK_i = 5.16 ± 0.07, Table 5, entry 22) revealed the highest binding affinity of all bispyridinium salts tested so far, representing an important progress in development of new nAChR resensitizers with improved potency although its pharmacological effects remain to be determined. Very recently, Wein et al. described the putative binding site as composed of two subdomains, one of which is lined with more hydrophilic and the other with more lipophilic amino acids.[23] Accordingly, the design of non-symmetric compounds displaying two different polar termini, one with higher the other with lower polarity, could be a promising strategy for increasing the binding affinity.

Conclusions

In summary, a set of 30 symmetric bispyridinium and related salts with a propane-1,3-diyl linker were synthesized and characterized for their binding affinity towards the **MB327** binding site at the *Torpedo*-nAChR. Binding affinities were determined using our recently developed MS Binding Assays and discussed regarding **MB327**'s binding affinity, the most potent nAChR resensitizer so far, as reference. To the best of our knowledge, this represents the first study providing quantitative binding data for the **MB327** binding site, which, in turn, should be useful for further developments in structure and ligand-based design of nAChR resensitizers.

All target compounds were synthesized via N-alkylation of the respective aromatic N-heterocycles using either propane-1,3-ditriflate or propane-1,3-diiodide as alkylating agents. Optimized reaction conditions allowed very fast and clean reactions in the presence of a wide variety of functional groups. Being also well suited for the alkylation of less reactive nitrogen heterocycles, such as sterically hindered 2-*tert*-butylpyridine or electron deficient 2-*tert*-butylpyrazine, makes both methods highly valuable for organic synthesis.

Regarding structure-affinity relationships, the influence of various lipophilic and hydrophilic functional groups as well as of different heterocycles on the binding affinity of the bisquaternary salts was investigated. Notably, molecular structures allowing for additional lipophilic interactions were found to play a vital role in the binding event. In line with this observation, lipophilic 3-phenyl-4-*tert*-butyl substituted bispyridinium compound **3h** (PTM0022) was found to possess a significantly improved binding affinity as compared to **MB327**.

Further, differences in binding affinities were found between polar (OMe, NMe_2, COOEt, etc.) and apolar (C≡CH, Me, *i*-Pr, etc.) substituted compounds, generally revealing higher binding affinities for the less polar derivatives. Interestingly, NMe_2 was identified to be the only but notable exception, revealing better results than most apolar substituted compounds. In consequence, the role of hydrogen bridge acceptors on the binding affinity is worth to be investigated in more detail.

Finally, there is compelling evidence, that anionic functional groups have a distinct negative influence on the binding process, since the addition of two carboxylic acid functions to the structure of **MB327** resulted in a severe drop in binding affinity (**3l**, PTM0028). To get deeper insights into the interaction of bisquaternary salts with the MB327 binding site at the nAChR, further studies based on structurally more diverse non-symmetric bispyridinium compounds are under way.

Experimental Section

General Methods

Anhydrous reactions were carried out in vacuum-dried glassware under argon atmosphere. Microwave reactions were performed in sealed glass vials using a CEM Discover SP microwave synthesizer. THF, 1,4-dioxane and CH_2Cl_2 were distilled prior to use under nitrogen atmosphere and dried according to standard procedures.[40] Et_2O (technical grade) was purified by distillation and dried by refluxing for 6 h over sodium without benzophenone under nitrogen atmosphere before being distilled once again prior to use. All other chemicals were used as purchased from commercial sources and solvents were distilled before use. TLC was carried out using plates purchased from Merck (silica gel 60 F_{254} on aluminum sheet). Flash chromatography (FC) was performed using Merck silica gel 60 (40–63 µm mesh size) as stationary phase. Melting points were determined with a BÜCHI 510 melting point apparatus and are uncorrected. IR spectroscopy was performed using an FT-IR Spectrometer 1600 and Paragon 1000 (PerkinElmer); oils were measured as film and solid samples as KBr pellets. High-resolution (HR) mass spectrometry was performed on a Finnigan MAT 95 (EI), Finnigan LTQ FT (ESI) and Jeol JMS 700 MStation (FAB). ^{1}H and ^{13}C NMR spectra were recorded with a Bruker BioSpin Avance III HD 400 and 500 MHz (equipped with a Prodigy™ cryo probe) using TMS as internal standard and integrated with MestReNova (Version 10.0.2), Mestrelab Research S.L. 2015. The purity of the test compounds was determined by means of quantitative NMR using Sigma Aldrich TraceCERT® maleic acid or dimethylsulfone as internal calibrants.[41,42] The purity of all tested compounds was > 95%. See the Supporting Information for the characterization data of the described compounds.

The following compounds were prepared according to literature: Propane-1,3-diyl bis(trifluoromethanesulfonate) (**21**),[43,44] 2-*tert*-butylpyridine (**17**),[27] 2-(pyridin-3-yl)propan-2-ol (**9**),[33] *rac*-nicotine (***rac*-26**),[28] 1-(*tert*-butyl)-1*H*-imidazole (**41**)[29] and the 4-*tert*-butyl substituted pyridine derivatives 4-(*tert*-butyl)-3-methylpyridine (**33**), 4-(*tert*-butyl)-3-methoxypyridine (**31**), 4-(*tert*-butyl)-3-chloropyridine (**28**), 3-bromo-4-(*tert*-butyl)pyridine (**29**), 4-(*tert*-butyl)pyridine-3-carbonitrile (**30**), ethyl 4-(*tert*-butyl)pyridine-3-carboxylate (**36**), 4-(*tert*-butyl)-3-fluoropyridin-1-ium chloride (**27**), 4-(*tert*-butyl)-3-phenylpyridine (**34**), 4-(*tert*-butyl)-*N*,*N*-dimethylpyridine-3-carboxamide (**32**), and 4-(*tert*-butyl)-3-ethynylpyridine (**35**).[30]

General Procedures

Synthesis of symmetric bispyridinium compounds by N-alkylation with 21 (GP1): **21** (1.0 eq.) was carefully added to a solution of the corresponding pyridine derivative (2.5 eq.) in CH_2Cl_2 (0.8 mL/mmol) or to the neat pyridine derivative (2.5 eq.) at room temperature. The resulting mixture was stirred at 50 °C for the indicated period and residual CH_2Cl_2

was removed *in vacuo* if necessary. The residue was finally recrystallized from the given solvent.

Synthesis of symmetric bispyridinium compounds by N-alkylation with 16 (GP2): **16** (1.0 eq.) was added to a solution of the corresponding aromatic nitrogen heterocycle (2.5 eq.) in MeCN (0.8 mL/mmol) and the resulting mixture was heated to 90 °C (150 W) in a sealed microwave reactor for the indicated period. The solvent was removed *in vacuo* and the residue was recrystallized from the given solvent.

Synthesis of 2- and 3-alkylpyridines by cuprate mediated substitution reactions of 2- and 3-bromopyridines (GP3): The corresponding bromopyridine derivative (1.0 eq.) was added dropwise to a suspension of the respective dialkylcuprate (4.0 eq., prepared according to GP4) in THF or Et_2O at –78 °C. The reaction mixture was stirred for 2 h at –78 °C before being allowed to stir for additional 15 h at room temperature. The mixture was cooled to 0 °C and the reaction was quenched by addition of saturated aqueous NH_4Cl/conc. NH_3 (1:1, 8 mL/mmol). The mixture was filtrated over celite® and the residue was alternately washed with Et_2O (3 x 5 mL/mmol) and saturated aqueous NaCl (2 x 5 mL/mmol). The layers were separated, and the organic phase was washed with saturated aqueous NaCl solution (10 mL/mmol). The combined aqueous layers were extracted with Et_2O (3 x 10 mL/mmol) and the combined organic layers were dried over $MgSO_4$. The solvent was carefully removed under reduced pressure (40 °C bath temperature, 450 mbar) and the resulting crude product was purified in a first step by FC and subsequently by a vacuum distillation ($5 \cdot 10^{-2}$ mbar at 200 °C). The pure alkylpyridine was obtained as a colorless liquid.

Preparation of (*i*-Pr)$_2$CuCN(MgCl)$_2$, (*t*-Bu)$_2$CuCN(MgCl)$_2$ and (d_9-*t*-Bu)$_2$CuCN(MgCl)$_2$ (GP4): The corresponding Grignard reagent (solution in Et_2O or THF, 2.0 eq.) was added to a suspension of CuCN (1.0 eq.) in Et_2O or THF (2.0–4.5 mL/mmol) at –78 °C and the resulting mixture was stirred for 45 min at –78 °C, for 5 min at room temperature and again for 10 min at –78 °C.

Preparation of *t*-BuCu(CN)ZnBr·2LiCl: *t*-BuZnBr (0.5 M solution in THF, 11.0 mmol, 22.0 mL, 1.0 eq.) was added to a solution of CuCN (995 mg, 11.0 mmol, 1.0 eq.) and LiCl (933 mg, 22.0 mmol, 2.0 eq.) in THF (50 mL) at –10 °C and the resulting 0.15 M cuprate suspension was stirred for 10 min at 0 and –78 °C each.

Synthesized compounds

Pyridine derivatives: 3-*tert*-Butylpyridine (**8**, colorless liquid, 43%), 3-isopropylpyridine (**11**, colorless and highly volatile liquid, 51%), 2-isopropylpyridine (**6**, colorless liquid, 20%), 4-isopropylpyridine (**13**, clear and highly volatile liquid, 33%), 4-(*tert*-butyl)-2-methylpyridine (**15**, colorless liquid, 47%).

Compounds of type I (1a–h): 1,1'-(Propane-1,3-diyl)bis[2-(*tert*-butyl)pyridin-1-ium] bis(trifluoromethanesulfonate) (**1a**, colorless solid, 73%), 1,1'-(propane-1,3-diyl)bis[3-(*tert*-butyl)pyridin-1-ium] diiodide (**1b**, yellow crystals, 79%), 1,1'-(propane-1,3-diyl)bis(2-isopropylpyridin-1-ium) bis(trifluoromethanesulfonate) (**1c**, colorless solid, 77%), 1,1'-(propane-1,3-diyl)bis(3-isopropylpyridin-1-ium) bis(trifluoromethanesulfonate) (**1d**, colorless solid, 88%), 1,1'-(propane-1,3-diyl)bis(4-isopropylpyridin-1-ium) bis(trifluoromethanesulfonate) (**1e**, colorless solid, 81%), 1,1'-(propane-1,3-diyl)bis(3,5-dimethylpyridin-1-ium) bis(trifluoromethanesulfonate) (**1f**, colorless solid, 69%), 1,1'-(propane-1,3-diyl)bis(2,6-dimethylpyridin-1-ium) bis(trifluoromethanesulfonate) (**1g**, colorless solid, 75%), 1,1'-(propane-1,3-diyl)bis(2,4,6-trimethylpyridin-1-ium) bis(trifluoromethanesulfonate) (**1h**, colorless solid, 81%).

Compounds of type II (2a–e): 1,1'-(Propane-1,3-diyl)bis(2-methoxypyridin-1-ium) bis(trifluoromethanesulfonate) (**2a**, colorless solid, 67%), 1,1'-(propane-1,3-diyl)bis(3-methoxypyridin-1-ium) bis(trifluoromethanesulfonate) (**2b**, colorless solid, 83%), 1,1'-(propane-1,3-diyl)bis(4-methoxypyridin-1-ium) bis(trifluoromethanesulfonate) (**2c**, colorless solid, 84%), 1,1'-(propane-1,3-diyl)bis[3-(dimethylamino)pyridin-1-ium] bis(trifluoromethanesulfonate) (**2d**, yellowish solid, 47%), *rac*-{1,1'-(propane-1,3-diyl)bis[3-(1-methylpyrrolidin-2-yl)pyridin-1-ium]} bis(trifluoromethanesulfonate) (**2e**, yellowish solid, 72%).

Compounds of type III (3a–l): 1,1'-(Propane-1,3-diyl)bis[4-(*tert*-butyl)-3-fluoropyridin-1-ium] bis(trifluoromethanesulfonate) (**3a**, colorless solid, 85%), 1,1'-(propane-1,3-diyl)bis[4-(*tert*-butyl)-3-chloropyridin-1-ium] bis(trifluoromethanesulfonate) (**3b**, colorless solid, 81%), 1,1'-(propane-1,3-diyl)bis[3-bromo-4-(*tert*-butyl)pyridin-1-ium] bis(trifluoromethanesulfonate) (**3c**, colorless solid, 99%), 1,1'-(propane-1,3-diyl)bis[4-(*tert*-butyl)-3-cyanopyridin-1-ium] bis(trifluoromethanesulfonate) (**3d**, slightly beige solid, 85%), 1,1'-(propane-1,3-diyl)bis[4-(*tert*-butyl)-3-methoxypyridin-1-ium] bis(trifluoromethanesulfonate) (**3e**, colorless solid, 83%), 1,1'-(propane-1,3-diyl)bis[4-(*tert*-butyl)-3-(dimethylcarbamoyl)-pyridin-1-ium] bis(trifluoromethanesulfonate) (**3f**, colorless solid, 77%), 1,1'-(propane-1,3-diyl)bis[4-(*tert*-butyl)-3-methylpyridin-1-ium] bis(trifluoromethanesulfonate) (**3g**, colorless solid, 89%), 1,1'-(propane-1,3-diyl)bis[4-(*tert*-butyl)-3-phenylpyridin-1-ium] bis(trifluoromethanesulfonate) (**3h**, colorless solid, 99%), 1,1'-(propane-1,3-diyl)bis[4-(*tert*-butyl)-3-ethynylpyridin-1-ium] bis(trifluoromethanesulfonate) (**3i**, colorless solid, 91%), 1,1'-(propane-1,3-diyl)bis[4-(*tert*-butyl)-3-(ethoxycarbonyl)pyridin-1-ium] bis(trifluoromethanesulfonate) (**3j**, colorless solid, 98%), 1,1'-(propane-1,3-diyl)bis[4-(*tert*-butyl)-2-methylpyridin-1-ium] bis(trifluoromethanesulfonate) (**3k**, colorless solid, 82%), 4-(*tert*-butyl)-1-{3-[4-(*tert*-butyl)-3-carboxypyridin-1-ium-1-yl]propyl}-pyridin-1-ium-3-carboxylate trifluoromethanesulfonate (**3l**, colorless solid, 66%).

Compounds of type IV (4a–e): 2,2'-(Propane-1,3-diyl)bis(isoquinolin-2-ium) diiodide (**4a**, yellow solid, 86%), 1,1'-(propane-1,3-diyl)bis[3-(*tert*-butyl)pyrazin-1-ium] diiodide (**4b**, yellow powder, 78%), 3,3'-(propane-1,3-diyl)bis(thiazol-3-ium) diiodide (**4c**, beige solid, 87%), 3,3'-(propane-1,3-diyl)bis(1-methyl-1*H*-imidazol-3-ium) diiodide (**4d**, colorless solid, 92%), 3,3'-(propane-1,3-diyl)bis[1-(*tert*-butyl)-1*H*-imidazol-3-ium] diiodide (**4e**, colorless solid, 82%).

Deuterated compounds: 4-(2-[$^2H_3$]Methyl[1,1,1,3,3,3-$^2H_6$]propan-2-yl)pyridine (**48**, colorless liquid, 31%), 1,1'-(propane-1,3-diyl)bis[4-(2-[$^2H_3$]methyl[1,1,1,3,3,3-$^2H_6$]propan-2-yl)pyridin-1-ium] bis(trifluoromethanesulfonate) (**49**, colorless solid, 81%), diethyl [$^2H_2$]malonate (**43**, colorless liquid, 40%), [$^2H_6$]propane-1,3-diol (44) and [$^2H_6$]propane-1,3-diyl bis(trifluoromethanesulfonate) (**45**, grayish and turbid oil, 58%), 1,1'-([$^2H_6$]propane-1,3-diyl)bis[4-(*tert*-butyl)pyridin-1-ium] bis(trifluoromethanesulfonate) (**46**, colorless solid, 77%).

Biological Evaluation

General Procedure for [$^2H_6$]MB327 MS Binding Assays: Membranes from frozen *Torpedo californica* electroplaque tissue were prepared as previously reported and stored in storage buffer (120 mmol L^{-1} NaCl, 5 mmol L^{-1} KCl, 8.05 mmol L^{-1} Na_2HPO_4, 1.95 mmol L^{-1} NaH_2PO_4, pH 7.4).[24] Total protein concentration was determined by the bicinchoninic acid method using bovine serum albumin as standard.[45] All binding experiments were performed as described previously,[24] but in absence of $CaCl_2$ during incubation. For saturation and competition experiments, aliquots of the membrane preparation (60–120 µg protein per sample) were incubated with [$^2H_6$]MB327 (**46**) as marker (saturation: 2–160 µmol L^{-1}, competition: 10 µmol L^{-1}) in triplicates in storage buffer in a total volume of 125 µL for 2 h at 25 °C. In competition experiments the concentrations of the competitors ranged from 100 nmol L^{-1} to 1 mmol L^{-1}. After incubation, binding samples were processed exactly as previously described.[22] In saturation experiments, the maximum density of binding sites (B_{max}) and the equilibrium dissociation constant (K_d) were determined from saturation isotherms calculated for specific binding employing the "one-site – specific binding" regression tool (Prism v. 5.0, GraphPad Software, La Jolla, CA, USA). Competition curves were analyzed with the "one site–fit Ki" regression tool fixing top and bottom level of the sigmoidal competition curves to total binding (in absence of competitor, n = 3) and

nonspecific binding (determined by heat denaturation, n = 3). For statistical comparisons, data were examined by means of a *t*-test (two sided, α = 0.05). If not stated otherwise, results are given as means ± SEM.

Validation of the LC-ESI-MS/MS quantification method: In accordance with the FDA's guidance for bioanalytical method validation selectivity, lower limit of quantification (LLOQ), linearity, precision, and accuracy were investigated exactly in the same way as described for marker quantification in binding samples resulting from incubation in presence of 1 mmol L^{-1} $CaCl_2$.[24]

Supporting Information

Synthetic procedures and analytical characterization of all compounds as well as details to the improved MS Binding Assay protocol and validation data can be found within the supporting information.

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Keywords: Resensitizer • MS Binding Assay • Bispyridinium • Drug design • Nitrogen heterocycles

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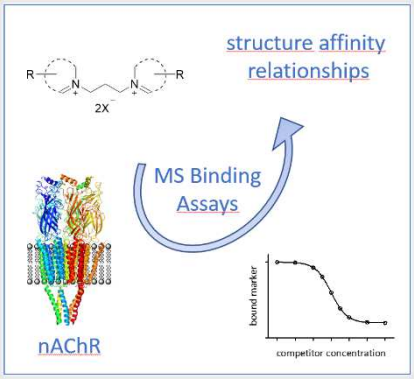

Toward potential antidotes against organophosphate poisoning: A series of new bisquaternary compounds was designed, synthesized and characterized for their binding affinity toward the nicotinic acetylcholine receptor (nAChR) using our recently developed MS Binding Assays. New binders and important structural motives were identified, thus laying the groundwork for structure affinity relationship guided drug design of potential nAChR resensitizers.