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Search for dual function inhibitors for Alzheimer's disease: Synthesis and biological activity of acetylcholinesterase inhibitors of pyridinium-type and their Aβ fibril formation inhibition capacity

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Abstract—Alzheimer's disease (AD) represents the most common neurodegenerative disorder, which is expressed through decline of mental function. Current treatment approaches include acetylcholinesterase inhibitors and NMDA-receptor partial antagonists. The most explored recent approaches that are closely related to the pathogenesis of this disease based on formally articulated amyloid hypothesis are: Aβ fibril formation inhibitors, amyloid precursor protein, and secretase inhibitors. [Scarpini, E.; Scheltens, P.; Feldman, H. *Lancet Neurol.* **2003**, *2*, 539] In view of the development of new AChE inhibitors as drugs capable of reducing the symptoms of AD, the capacity of newly synthesized AChE inhibitors of pyridinium-type to inhibit the AChE was examined and compared to those of other inhibitors of this type presented earlier. [Kapková, P.; Stiefl, N.; Sürig, U.; Engels, B.; Baumann, K.; Holzgrabe, U. *Arch. Pharm. Pharm. Med. Chem.* **2003**, *336*, 523; Alptüzun, V.; Kapková, P.; Baumann, K.; Erciyas, E.; Holzgrabe, U. *J. Pharm. Pharmacol.* **2003**, *55*, 1397] Furthermore, the anti-Aβ fibril formation property of AChE inhibitors of pyridinium- and bispyridinium-type was evaluated to expand their activity profile and to reveal potential additive pharmacological effects which may reinforce their therapeutic application besides their capacity of increasing acetylcholine levels. Aβ fibril formation studies were performed by means of thioflavin T fluorescence assay.

1. Introduction

One of the most prominent features of Alzheimer's disease (AD) is a significant deficit in cholinergic transmission in certain brain areas.[1,4–7] It could be found that concentrations of acetylcholine (ACh) decrease by nearly 90% in patients with AD. The current focus of AD treatment is the use of agents that increase the availability of intrinsic ACh by inhibiting the enzyme acetylcholinesterase (AChE). This may restore the cholinergic function in the brain and significantly reduce the severity of dementia. Another key feature in pathology of the brain of patients with AD is the progressive deposition of the amyloid β peptide (Aβ) in fibrillar form.[8] The polymerization of normally soluble proteins or peptides in β-pleated sheet form yielding insoluble amyloid deposits is a key step of a variety of so-called amyloid diseases, for example, AD and prion diseases.[9] In AD, amyloid deposits in diffuse form or as plaques are composed of β-amyloid (Aβ), a 39- to 43-amino-acid peptide derived from a larger parent protein, known as amyloid precursor protein (APP).[10] Through end-specific monoclonal antibodies, it was found that plaques representing the initially deposited species were exclusively positive for Aβ42(43).[11] In vitro studies indicated that the longer form Aβ 1–42 has a higher propensity to form fibrils. Furthermore, the prevalence of the higher molecular weight species Aβ 1–42 has been reported to lead to extensive amyloid formation.[12] As with other amyloidogenic proteins the in vitro polymerization of Aβ into fibrils occurs spontaneously (spontaneous Aβ-fibrillogenesis), in case the protein is present at a certain critical concentration.[13] Fibrillar amyloid can be stained with certain dyes such as thioflavin T or Congo red.

Several substances of peptidic and non-peptidic origin with anti-amyloid potency were reported. Among the latter are endogenous compounds such as melatonin,

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rifampicin, benzofuran, and naphthylazo derivatives.[14–16] Other researchers have patented several isoindoline[17] and piperidine[18] derivatives inhibiting amyloid protein aggregation and deposition. The classical antibiotics, for example, tetracycline and doxycycline, have also been found to be useful anti-Aβ fibril formation inhibitors.[19]

On the other hand, studies appeared suggesting the role of AChE not only in the hydrolysis of the neurotransmitter ACh, but also in accelerating the aggregation of Aβ into amyloid fibrils (AChE-induced Aβ-fibrillogenesis).[20,21] Investigation of the active site of AChE allows the identification of one peripheral anionic site (PAS) and at least five major binding sites: the oxyanion hole, the esteratic site, the anionic substrate binding site, the active site-selective aromatic binding site, and the acyl binding site.[22] De Ferrari et al.[23] presented the involvement of PAS in interaction with amyloid β-peptide. Within this context, it is expected that a peripheral site blocker could prevent the Aβ peptide from interacting with AChE, and thus inhibits a potential contribution of AChE to the fibril formation process.

Consequently, inhibition of AChE (possibly PAS inhibition of AChE), reduction of Aβ production by blocking the APP cleavage enzymes, or inhibition of Aβ fibril formation are some of the current strategies for the development of therapeutic interventions in AD. Most Alzheimer's disease clinicians and researchers concede that there is no single treatment or therapeutical approach that will alleviate this condition, and that a multiple-functional approach or multipotent drugs will be required.[24]

In a previous study, we hypothesized the ditopic bisbenzylethers of bispyridinium-type like DUO3 to be advantageous.[2] Docking studies showed that DUO3 was too long to fit optimally into the AChE gorge. Shorter compounds[3] such as **3c** or **3b** represent a significantly higher activity than the ditopic compounds (Fig. 1). In this line, we now report on the AChE inhibition of derivatives of the pyridinium-type with different position of the nitrogen in order to change the electronic properties, and their ability to block Aβ fibril formation. Aβ fibril formation was studied by thioflavin T fluorescence assay, employing synthetic Aβ 1–42 and Aβ 1–40 peptide.

2. Results and discussion

Naphthyl, dichlorobenzyl, and phthalimidomethyl oxime ethers were synthesized as described in experimental part. DUO3 was synthesized according to Bejeuhr et al.[25]

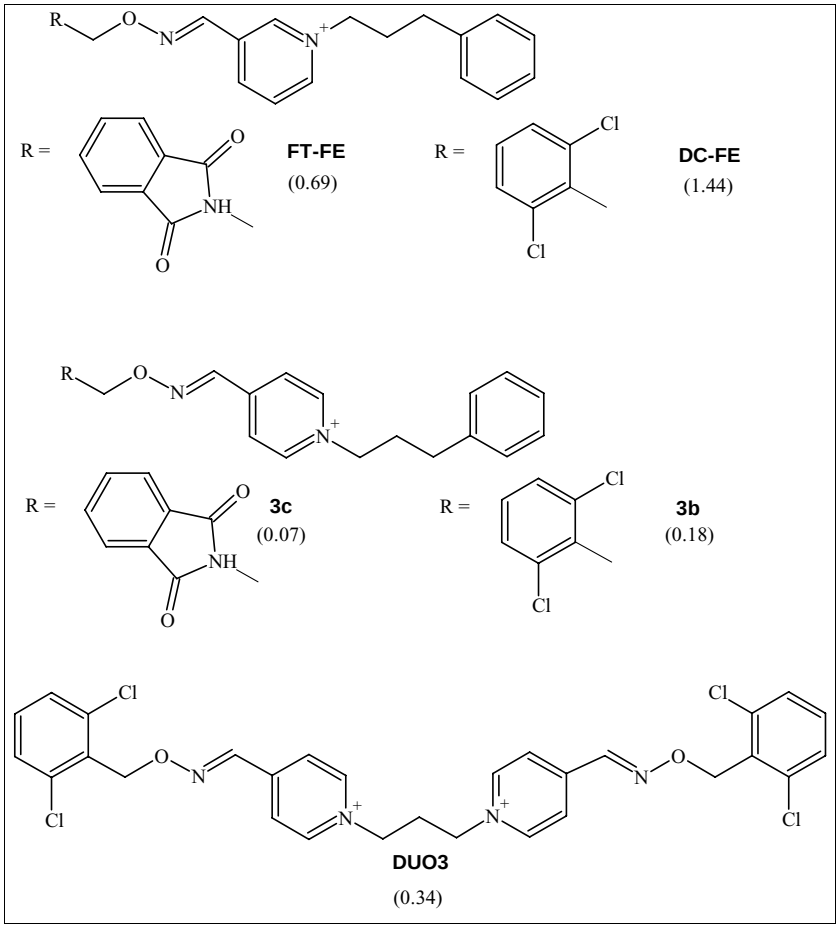


Figure 1. Structures of pyridinium- and bispyridinium-type compounds presented in this study; IC_{50} values [μM] in parentheses.

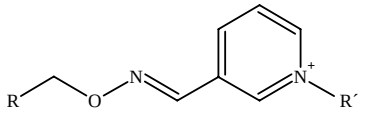
Since the position of nitrogen could affect the electronic properties of the pyridinium ring, the modification of the interaction of the entire molecule with enzyme by shifting the nitrogen from the 4 position as in **3b** and **3c**[3] into the 3 position is supposed. The new series of naphthyl (NF), and dichlorobenzyl (DC) and phthalimidomethyl (FT) oxime ether derivatives with the pyrdinium nitrogen in position 3 was evaluated for inhibition activity toward the acetylcholinesterase by means of Ellman's test.[26] Both consisted of pyridiniumpropyl-, phenylpropyl- and propane moieties at the opposite end of the molecule. The following structure–activity relationships are reported in Table 1.

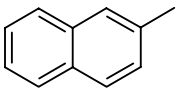
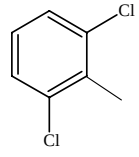
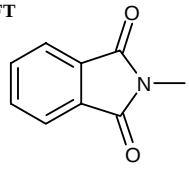
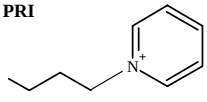
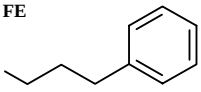
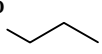
As in the previous study[3] phthalimido-phenyl substituted compound (FT-FE$_{IC_{50}}$ = 0.69 ± 0.04 μM) is the most active compound found in the 3-pyridinium series. However, its activity is not as high as that of corresponding 4-pyridinium derivative **3c** (IC_{50} = 0.073 ± 0.02 μM), and does not reach the inhibition range of tacrine (IC_{50} = 0.044 ± 0.04 μM),[2] one of the potent acetylcholinesterase inhibitors.

In general, it could be confirmed that the phenylpropyl substitution at the end of pyridinium moiety mediates the most active compounds of each of the series (NF-FE, DC-FE, and FT-FE). As already shown in previous studies the phenyl ring provides the chance to favorably interact with aromatic residues localized within the AChE gorge. In the phenylpropyl substituted series, the inhibitory activity of the phthalimide derivative FT-FE is followed by the dichlorobenzyl derivative DC-FE and naphthyl derivative NF-FE. In docking studies, the effect was explained by stronger interactions of the phthalimide group at the bottom of the AChE gorge in comparison to the chlorobenzyl group. Generally, π-π-stacking could be found between the phenyl ring of the phthalimido moiety and Trp^{84} of the AChE gorge, as well as between benzyl group of dichloro moiety and Trp^{84}. We assume that the stronger interactions of phthalimido group and the AChE gorge could be expected due to the presence of hydrogen bonds between the carbonyl groups of the phthalimido-moiety, and the serine and tyrosine residues (Ser$^{200/122}$ and Tyr$^{130/334}$).

Furthermore, comparing the three series of naphthyl (NF), dichlorobenzyl (DC), and phthalimid (FT) compounds, replacement of the phenyl ring with positively charged pyridinium ring resulted in an obvious loss in inhibitory activity toward AChE. This tendency was already observed also in a previous study of dichloro- and phthalimido 4-pyridinium derivatives.[3] The reduction of the molecule up to the propane moiety also decreased the AChE inhibition activity. The IC_{50}-values of propyl derivatives being in the range of 4.6–10.1 μM, between the inhibition concentrations of pyridiniumpropyl (25.8–29.7 μM) and phenylpropyl (0.69–1.68 μM) substituted pyridinium derivatives indicate the importance of the

Table 1. AChE inhibitory activity of NF, DC, and FT series of pyridinium compounds



R	NF 	DC 	FT 
R′		IC_{50}[a] [μM]	
PRI 	×	29.69 ± 5.80	25.81 ± 2.01
FE 	1.68 ± 0.12	1.44 ± 0.16	0.69 ± 0.04
PRO 	4.6 ± 0.56	10.10 ± 2.20	5.47 ± 1.24

The pharmacological data are means ± SEM, n = 3 experiments.
[a] IC_{50}, concentration inhibiting activity by 50%; × = was not available by synthesis.

phenylpropane moiety for a high AChE inhibition activity of these derivatives.

Four representative compounds from the pyridinium (**3b**, **3c**, and DC-FE) and bispyridinium (DUO3) class synthesized up to now in our research group were tested in a thioflavin T fluorescence assay for their ability to block the Aβ fibril formation (Table 2).

In Table 2, the inhibitory activity of selected compounds on Aβ fibril formation is shown in parallel to their activity in inhibiting acetylcholinesterase catalytic activity (potency expressed as IC_{50}). Compounds were tested at three different concentrations (c = 1 mM; 100 μM; 10 μM) in the fibril formation assay. The thioflavin T assay revealed the compounds **3b**, DUO3, and DC-FE to be active in inhibiting Aβ fibril formation, with DUO3 and **3b** being equipotent and DC-FE being somewhat weaker. Compound **3c** was found to be only marginally active. At concentrations equimolar to Aβ 1–40, fibril formation by DUO3 and **3b** was inhibited by 50%. Against Aβ 1–42, the compounds were distinctly weaker, requiring 3- to 30-fold excess of compound over peptide to see a similar inhibition of Aβ fibril formation. All fluorescence data were corrected for quenching by the test compounds. It cannot be completely ruled out that the compounds compete with thioflavin T itself. Therefore, at the end of the experiment, we tried to quantify the formed fibrillar Aβ by Congo red. However, for reasons unknown, all four substances interacted already with Congo red alone, causing a bathochromic shift in absorbance similar to the one seen with Congo red in the presence of fibrillar Aβ. Quantification of the amount of fibrils formed in the presence of the test compounds with this independent method was therefore not possible.

Amyloid formation is a nucleation-dependent process.[12] This process is characterized by two phases: a slow nucleation (the protein undergoes a series of association steps to form an ordered oligomeric nucleus) and growth phase (the nucleus rapidly grows to form larger polymers). In the steady state phase, the ordered aggregate and the monomer appear to be at equilibrium.[27,28] Based on the course of Aβ fibril formation curves of our compounds (not shown), it can be stated that DUO3 retards the nucleation phase of amyloidogenesis, whereas **3b** seems to be active in inhibition of fibril elongation/growth.

Even though the AChE inhibition activity of **3b** and DUO3 is lower than that of **3c**, the fact, that these compounds inhibit the spontaneous Aβ fibril formation makes them very interesting regarding their dual function—the inhibition of AChE and that of Aβ fibrillogenesis. The molecular basis of the anti-amyloidogenic activity of the pyridinium compounds likewise other above-mentioned compounds[14–19] is not understood; however, it may be related to the propensity of these drugs to bind to the fibrillogenic structure.

On the other hand, the research group of Inestrosa[23] came up with another aspect of Aβ fibril formation, which discusses the involvement of the peripheral site of the acetylcholinesterase in the Aβ aggregation process. Based on this assumption, AChE inhibitors, binding to this site of the enzyme, may attract attention and gain interest in the process of drug development for AD. As our aforementioned compounds show ditopic binding mode in the gorge of acetylcholinesterase, that is, they interact with both its catalytic and peripheral binding sides, it could be expected, that our pyridinium derivatives may not only be able to inhibit spontaneous Aβ fibril formation but also the AChE-induced Aβ-fibrillogenesis. Such as positive line-up of inhibitory capabilities makes this substance promising for potential use in Alzheimer's disease treatment and therapy.

3. Conclusion

In the present study, we presented new derivatives of the pyridinium type. They were tested for their inhibitory activity toward acetylcholinesterase and compared to those of other derivatives of this class synthesized earlier by our research group.[2,3] Another aim of this investigation was to assess the potential of our AChE inhibitors of the pyridinium-and bispyridinium-type to block the spontaneous Aβ-fibril formation. We found the pyridinium substance **3b** and the bispyridinium derivative DUO3 to possess interesting potencies. Apart from their

Table 2. Effect of pyridinium compounds on the formation of Aβ 1–42 and Aβ 1–40 fibrils. Monomeric Aβ was incubated at 37 °C for 5 days in the presence or absence of the test substances

	Control ± SEM	% Inhibition ± SEM			
		3b	**3c**	DUO3	DC-FE
Aβ 1–42	0 ± 7				
c = 1 mM		56 ± 2	13 ± 2	55 ± 2	42 ± 8
c = 100 μM		14 ± 2	0 ± 3	61 ± 5	21 ± 2
c = 10 μM		0 ± 3	0 ± 2	15 ± 2	1 ± 3
Aβ 1–40	0 ± 11				
c = 1 mM		74 ± 2	23 ± 9	92 ± 9	54 ± 5
c = 100 μM		60 ± 1	0 ± 7	55 ± 8	32 ± 11
c = 10 μM		0 ± 11	0 ± 11	0 ± 7	11 ± 12
Inhibition of AChE IC_{50} [μM]		0.18	0.07	0.34	1.44

The amount of Aβ fibrils formed during this period was quantified by thioflavin T fluorescence (indicated in % of the control ± SEM, n = 3). The fluorescence values were corrected for quenching by the substance alone.

inhibitory activity toward acetylcholinesterase, they also inhibited the self-assembly and formation of amyloid fibrils. The results indicate that drugs with this clinical profile may be potentially useful for Alzheimer's therapy. Optimizing potency and activity of such dually potent compounds which affect two different targets of AD is our future perspective.

4. Experimental

Melting points were determined with a Büchi 510 melting point apparatus (Büchi, Switzerland) and are not corrected. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AV 400 instrument (^{1}H 400.132 MHz; ^{13}C 100.613 MHz). Abbreviations for data quoted are: s, singlet; d, doublet; t, triplet; quin, quintet; dd, doublet of doublets; m, multiplet; br s, broad signal. The centers of the peaks of $CDCl_3$ and DMSO-d_6 were used as internal references. IR spectra of compounds were recorded as potassium bromide pellets on a Jasco FT/IR-400 spectrometer. Dry solvents were used throughout. The electrospray ionization (ESI) mass spectra were measured on an Agilent 1100 LC/MSD Trap. The conditions of the spray chamber were as follows: ion polarity, positive; drying gas temperature, 300 °C; nebulizer pressure, 10 psi; drying gas flow, 5.00 L min^{-1}. Reagents used for synthesis were purchased from Aldrich, Fluka, and Merck companies. Organic solvents were purchased from Merck Company. Thin-layer chromatographies were done on pre-coated silica gel 60 F_{254} plates (Merck). The spots were visualized with UV light or iodine. Column chromatography was performed on silica gel 60 30–70 mesh (Merck).

Naphthylmethyl, dichlorobenzyl, and phthalimidomethyl oxime ethers (NF, DC, and FE), and 1-(3-bromopropyl)pyridinium bromide were synthesized according to Botero Cid et al.[29] and Bejeuhr et al.[25]

4.1. 3-[([(Naphthyl-1-il)methoxy]imino)methyl]pyridine (NF)

Yield 16%, yellowish oil. IR (KBr) ν (cm^{-1}) 1642, 1438, 1018, 980, 746. ^{1}H NMR ($CDCl_3$): δ ppm 5.71 (2H, s, OCH_2), 7.25–7.27 (1H, m, H-5′), 7.43–7.56 (4H, m, H-2, H-3, H-6, H-7), 7.85 (2H, t, J = 8.9 Hz, H-4′, H-5′), 7.94 (1H, d, J = 7.8 Hz, H-8′) 8.11–8.14 (2H, m, H-4, N=CH), 8.57 (1H, br s, H-6), 8.71 (1H, br s, H-2).

4.2. 3-[([(2,6-Dichlorophenyl)methoxy]imino)methyl]pyridine (DC)

Yield 60%, mp 73 °C. IR (KBr) ν (cm^{-1}) 1604, 1438, 980, 746. ^{1}H NMR ($CDCl_3$): δ ppm 5.54 (2H, s, OCH_2), 7.25 (1H, dd, J = 8.8/8.3 Hz, H-4′), 7.34 (1H, dd, J = 4.8/7.9 Hz, H-5), 7.35–7.38 (2H, m, H-3′, H-5′), 7.99 (1H, dt, J = 7.9/1.7 Hz, H-4), 8.12 (1H, s, N=CH) 8.61 (1H, dd, J = 4.9/1.2, H-6), 8.75 (1H, d, J = 1.7 Hz, H-2).

4.3. 3-[([Phthalimidomethoxy]imino)methyl]pyridine (FT)

Yield 32%, mp 169 °C. IR (KBr) ν (cm^{-1}) 1776, 1722, 1608, 1435, 1067, 729, 713 ^{1}H NMR ($CDCl_3$): δ ppm 5.59 (2H, s, OCH_2), 7.42 (1H, dd, J = 5.2/8.0 Hz, H-5), 7.84–7.89 (2H, m, H-4′, H-5′), 7.90–7.95 (3H, m, H-3′, H-6′, H-4), 8.34 (1H, s, N=CH), 8.57 (1H, dd, J = 1.9/5.0 Hz H-6), 8.70 (1H, d, J = 1.9 Hz, H-2).

5. General procedure for synthesis of the final compounds

Corresponding dichlorobenzyl, naphthylmethyl or phthalimidomethyl oxime ethers and bromopropyl derivatives were heated in acetonitrile (60 ml) at reflux for 80 h. In case of incomplete conversion, remaining (NF, DC, and FE) could 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 obtained oil or solid was crystallized using the solvent mixtures of methanol/diethyl ether.

5.1. 1-(3-Phenylpropyl)-3-[([(naphthyl-1-il)methoxy]imino)-methyl]pyridinium bromide (NF-FE)

Yield 47%, mp 179 °C. IR (KBr) ν (cm^{-1}) 1637, 1605, 1461, 1166, 789. ^{1}H NMR (DMSO-d_6): δ ppm 2.28 (2H, quin, J = 7.4 Hz, N^+–CH_2– CH_2–CH_2-Ar), 2.67 (2H, t, J = 7.4 Hz, Ar-CH_2), 4.68 (2H, t, J = 7.4 Hz, N^+–CH_2), 5.78 (2H, s, OCH_2), 7.15–7.28 (5H, m, Ar-H), 7.55 (1H, dd, J = 8.1/7.2 Hz, H-3′), 7.60 (1H, dd, J = 3.9/1.4 Hz, H-2′), 7.65 (2H, t, J = 7.6 Hz, H-6′, H-7′), 7.98 (2H, t, J = 8.8 Hz, H-4′, H-5′), 8.14–8.17 (2H, m, H-5, H-8′), 8.49 (1H, s, N=CH), 8.69 (1H, d, J = 8.2 Hz, H-4), 9.09 (1H, d, J = 6.0 Hz, H-6), 9.27 (1H, br s, H-2).^{13}C NMR (DMSO-d_6): δ ppm 31.55, 31.75, 61.01, 74.94, 123.87, 125.37, 126.01, 126.11, 126.52, 127.67, 128.12, 128.18, 128.38, 128.52, 129.08, 131.23, 132.18, 133.29, 140.21, 141.79, 143.08, 144.39, 144.97. ESI-MS m/z: 381.5 (M^+).

5.2. 1-(Propyl)-3-[([(naphthyl-1-il)methoxy]imino)methyl]-pyridinium bromide (NF-PRO)

Yield 58%, mp 192 °C. IR (KBr) ν (cm^{-1}) 1635, 1602, 1471, 1159, 738. ^{1}H NMR (DMSO-d_6): δ ppm 0.90 (3H, t, J = 7.3 Hz, CH_3), 1.95 (2H, sextet, J = 7.3 Hz, N^+–CH_2–CH_2), 5.60 (2H, t, J = 7.4 Hz, N^+–CH_2), 5.78 (2H, s, OCH_2), 7.55 (1H, dd, J = 8.1/7.1 Hz, H-3′), 7.61 (1H, dd, J = 3.6/1.6 Hz, H-2′), 7.65 (2H, t, J = 7.6 Hz, H-6′, H-7′), 7.98 (2H, t, J = 8.9 Hz, H-4′, H-5′), 8.15–8.21 (2H, m, H-8′, H-5), 8.52 (1H, s, N=CH), 8.73 (1H, d, J = 8.2 Hz, H-4), 9.09 (1H, m, H-6), 9.29 (1H, br s, H-2). ^{13}C NMR ($CDCl_3$): δ ppm 10.18, 23.99, 62.42, 74.96, 123.86, 125.37, 126.01, 126.51, 127.67,128.16, 128.51, 129.08, 131.32, 132.24, 132.27, 133.29, 141.88, 142.94, 144.43, 144.93. ESI-MS m/z: 305.4 (M^+).

5.3. 1-[3-(Pyridinium-1-yl)propyl]-3-[([(2,6- dichlorophenyl)methoxy]imino)methyl]pyridinium dibromide (DC-PRI)

Yield 42%, mp 230 °C. IR (KBr) ν (cm^{-1}) 1633, 1486, 1015, 925, 777. ^{1}H NMR (DMSO-d_6): δ ppm 2.93 (2H, quin, J = 8.1 Hz, N^+–CH_2–CH_2), 5.03 (2H, t, J = 7.8 Hz, N^+–CH_2), 5.04 (2H, t,J = 7.9 Hz, N^+–CH_2), 5.58 (2H, s, OCH_2), 7.26 (1H, dd, J = 7.2/

8.8 Hz, H-4′), 7.36 (2H, d, J = 7.7 Hz, H-3′, H-5′), 8.03–8.10 (3H, m, H-3″, H-5″, H-5), 8.33 (1H, s, N=CH) 8.51 (1H, t, J = 7.8 Hz, H-4″), 8.76 (1H, d, J = 8.3 Hz, H-4), 9.40–9.44 (3H, m, H-6, H-2″, H-6″), 9.56 (1H, br s, H-2). ^{13}C NMR (DMSO-d_6): δ ppm 31.54, 57.39, 57.88, 70.10, 128.19, 128.36, 128.81, 131.14, 131.79, 132.09, 136.26, 142.20, 143.37, 144.86, 144.96, 145.33, 145.86. ESI-MS m/z: 200.9 (M^+).

5.4. 1-(3-Phenylpropyl)-3-[([(2,6-dichlorophenyl)methoxy]imino)methyl]pyridinium bromide (DC-FE)

Yield 60%, mp 120 °C. IR (KBr) ν (cm^{-1}) 1636, 1608, 1436, 1021, 948, 757. ^{1}H NMR (DMSO-d_6): δ ppm 2.44 (2H, quin, J = 7.4 Hz, N^+–CH_2–CH_2–CH_2-Ar), 2.81 (2H, t, J = 7.3 Hz, Ar-CH_2), 5.04 (2H, t, J = 7.3 Hz, N^+–CH_2), 5.56 (2H, s, OCH_2), 7.10–7.28 (6H, m, Ar-H, H-5), 7.36 (2H, d, J = 7.7 Hz, H-3′, H-5′), 8.01(1H, dd, J = 6.1/6.8 Hz, H-4′), 8.34 (1H, s, N=CH), 8.58 (1H, d, J = 8.2 Hz, H-4), 9.54 (1H, br s, H-2), 9.59 (1H, d, J = 6.0 Hz, H-6). ^{13}C NMR (DMSO-d_6): δ ppm 31.54, 31.72, 61.05, 71.04, 126.12, 128.17, 128.38, 128.79, 131.26, 131.74, 131.98, 132.05, 137.27, 140.21, 141.68, 143.24, 144.81, 145.14. ESI-MS m/z: 399.7 (M^+).

5.5. 1-(Propyl)-3-[([(2,6-dichlorophenyl)methoxy]imino)methyl]pyridinium bromide (DC-PRO)

Yield 42%, mp 219 °C. IR (KBr) ν (cm^{-1}) 1636, 1577, 1479, 1013, 986, 792. ^{1}H NMR (DMSO-d_6): δ ppm 1.02 (3H, t, J = 6.9 Hz, CH_3), 2.13 (2H, quin, J = 6.2 Hz, N^+–CH_2-CH_2), 5.14 (2H, m, N^+–CH_2), 5.56 (2H, s, OCH_2), 7.28 (1H, dd, J = 7.2/8.6 Hz, H-4′), 7.38 (2H, d, J = 7.8 Hz, H-3′, H-5′), 8.11 (1H, br s, H-5), 8.46 (1H, s, N=CH), 8.70 (1H, d, J = 7.5 Hz, H-4), 9.69 (1H, br s, H-6), 9.78 (1H, br s, H-2). ^{13}C NMR (DMSO-d_6): δ ppm 10.17, 23.94, 62.68, 71.36, 128.21, 128.80, 128.92, 131.63, 131.75, 132.05, 136.26, 144.83, 145.11, 145.24. ESI-MS m/z: 323.5 (M^+).

5.6. 1-[3-(Pyridinium-1-yl)propyl]-3-[([phthalimidomethoxy]imino)methyl]pyridinium dibromide (FT-PRI)

Yield 36%, mp 226–227 °C. IR (KBr) ν (cm^{-1}) 1775, 1720 1425, 1185, 775, 715. ^{1}H NMR (DMSO-d_6): δ ppm 2.68 (2H, quin, J = 7.2 Hz, Ar-CH_2-CH_2), 4.75 (2H, t, J = 7.2 Hz, N^+–CH_2), 4.77 (2H, t, J = 7.2 Hz, N^+–CH_2), 5.71 (2H, s, OCH_2), 7.91–7.94 (2H, m, H-4′, H-5′), 7.96–7.98 (2H, m, H-3′, H-6′), 8.19–8.27 (3H, m, H-3″, H-5″, H-5), 8.56 (1H, s, N=CH) 8.66 (1H, t, J = 8.0 Hz, H-4″), 8.70 (1H, d, J = 8.2 Hz, H-4), 9.15 (2H, d, J = 5.6 Hz, H-2″, H-6″), 9.17 (1H, d, J = 6.1 Hz, H-6), 9.29 (1H, br s, H-2). ^{13}C NMR (DMSO-d_6): δ ppm 31.47, 57.36, 57.89, 70.17, 123.69, 128.19, 128.37, 131.19, 131.82, 135.14, 142.16, 143.69,144.95, 145.66, 145.86,146.17, 166.84. ESI-MS m/z: 201.2 (M^+).

5.7. 1-(3-Phenylpropyl)-3-[([phthalimidomethoxy]imino)methyl]pyridinium bromide (FT-FE)

Yield 41%, mp 193–195 °C. IR (KBr) ν (cm^{-1}) 1777, 1722, 1465, 1020, 728, 713. ^{1}H NMR (DMSO-d_6): δ ppm 2.25 (2H, quin, J = 7.8 Hz, N^+–CH_2–CH_2–CH_2-Ar), 2.64 (2H, t, J = 7.0 Hz, Ar-CH_2), 4.64 (2H, t, J = 7.0 Hz, N^+–CH_2), 5.68 (2H, s, OCH_2), 7.14–7.26 (5H, m, Ar-H), 7.88–7.92 (2H, m, H-4′, H-5′), 7.93–7.95 (2H, m, H-3′, H-6′), 8.15 (1H, dd, J = 6.0/8.1 Hz, H-5), 8.46 (1H, s, N=CH), 8.61 (1H, d, J = 8.4 Hz, H-4), 9.08 (1H, d, J = 6.0 Hz, H-6) 9.16 (1H, br s, H-2). ^{13}C NMR (DMSO-d_6): δ ppm 31.51, 31.66, 61.06, 70.10, 123.67, 126.10, 128.17, 128.37, 131.20, 131.70, 135.11, 140.20, 141.64, 143.54, 145.48, 146.12, 166.84. ESI-MS m/z: 400.5 (M^+).

5.8. 1-(Propyl)-3-[([phthalimidomethoxy]imino)methyl]pyridinium bromide (FT-PRO)

Yield 58%, mp 225–228 °C. IR (KBr) ν (cm^{-1}) 1771, 1718, 1435, 1187, 728. ^{1}H NMR (DMSO-d_6): δ ppm 0.87 (3H, t,J = 7.4 Hz, CH_3), 1.92 (2H, sextet, J = 7.4 Hz, N^+–CH_2–CH_2), 4.57 (2H, t, J = 7.4 Hz, N^+–CH_2), 5.68 (2H, s, OCH_2), 7.88–7.91 (2H, m, H-4′, H-5′), 7.93–7.96 (2H, m, H-3′, H-6′), 8.19 (1H, dd, J = 6.0/8.0 Hz, H-5), 8.49 (1H, s, N=CH), 8.65 (1H, dt, J = 1.2/8.6 Hz, H-4), 9.10 (1H, d, J = 6.4 Hz, H-6), 9.20 (1H, br s, H-2). ^{13}C NMR (DMSO-d_6): δ ppm 10.15, 23.89, 62.47, 70.11, 123.68, 128.21, 131.21, 131.79, 135.11, 141.67, 143.47, 145.44, 146.13, 166.84. ESI-MS m/z: 324.5 (M^+).

5.9. Inhibition of AChE

The inhibitory potency against AChE was investigated by a slightly modified colorimetric method of Ellman et al.[26] Acetylcholinesterase (AChE)—E.C. 3.1.1.7 from Electric Eel was purchased from Sigma–Aldrich (Steinheim, Germany). 5,5′-Dithiobis-(2-nitrobenzoic acid), potassium dihydrogen phosphate, potassium hydroxide, sodium hydrogen carbonate, and acetylthiocholine iodide were obtained from Fluka (Buchs, Switzerland). Spectrophotometric measurements were performed on a Varian Cary 50 UV–Vis spectrophotometer. Details of the inhibition study are given in Kapková et al.[2]

5.10. Aβ

Aβ 1–42 and 1–40 used were obtained from Bachem Switzerland (purity > 96%, peptide content 81.4%). The peptides were stored at −20 °C, as recommended by the manufacturer. The Aβ peptide was routinely dissolved in hexafluoroisopropanol (HFIP) at 20 mg/ml. This solution was kept at room temperature for 1–2 h, until the peptide completely dissolved. The HFIP was removed under a stream of nitrogen until a clear film remained in the test tube. The residue was then dissolved in DMSO to obtain a 2 mM stock solution, which was subsequently stored frozen at −20 °C.

5.11. Thioflavin T fluorescence assay

Aβ fibril formation was assessed using the method described by LeVine.[30] Briefly, Aβ from the 2 mM peptide

stock solution in DMSO was diluted in 25 mM phosphate buffer containing 120 mM NaCl, 3 μM thioflavin T, and 0.02% sodium azide, final pH 7.4. The final DMSO concentration was always 10% v/v and experiments were routinely carried out at final peptide concentrations of 100 μM for Aβ 1–40 and 30 μM for Aβ 1–42. Incubations were performed at 37 °C on 96-well fluorescence microtiter plates. At regular time points, the fluorescence signal was measured (excitation wavelength 450 nm, emission wavelength 482 nm, and slit widths set to 5 nm) on a Perkin-Elmer LS50B fluorimeter, adapted for 96-well microtiter plates. Alternatively, Aβ was incubated as described, except without thioflavin T in the buffer, and small aliquots were removed at regular time intervals and mixed with a 10 mM phosphate buffer containing 6 μM thioflavin T, pH 6.0, and the fluorescence was measured as above. The morphology of the Aβ fibrils obtained under these conditions has been assessed in a previous study.[13,31] At the end of the assay, the amount of Aβ fibrils formed was furthermore quantified by Congo red binding, as described by Klunk et al.[32] Also, we tested whether the reduction of the thioflavin T fluorescence was not due to quenching by the test substance itself. For this purpose, the test compounds were added to Aβ fibrils labeled with thioflavin T and the fluorescence was measured immediately after.

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