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Synthesis of a novel series of bispyridinium compounds bearing a xylene linker and evaluation of their reactivation activity against chlorpyrifos-inhibited acetylcholinesterase

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Abstract

Nine potential AChE reactivators were synthesized using a modification of currently known synthetic pathways. Their potency to reactivate AChE inhibited by insecticide chlorpyrifos was tested *in vitro*. 2,2′-Bis(hydroxyiminomethyl)-1,1′-(1,4-phenylenedimethyl)-bispyridinium dibromide seems to be the most potent AChE reactivator. The reactivation potency of these compounds depends on structural factors such as length of the linking chain between both pyridinium rings and position of the oxime moiety on the pyridinium ring.

Keywords: *Acetylcholinesterase, reactivation, pesticide, chlorpyrifos, reactivator, oxime, inhibition, AChE*

Introduction

Highly toxic organophosphorus compounds (organophosphates, OP), for example the nerve agents (e.g. sarin, soman, tabun and VX) or insecticides (e.g. chlorpyrifos, parathion and paraoxon), inactivate the enzyme acetylcholinesterase (AChE, EC 3.1.1.7) by phosphorylation or phosphonylation of its active site [1–3]. The inhibition of AChE depends on the chemical structure of the inhibitors whereas reactivation of inhibited AChE depends not only on the inhibitors used but also on the chemical structure of the reactivator [4]. Pralidoxime (**1**; 2-PAM, 2-hydroxyiminomethyl-1-methylpyridinium chloride) is a commonly used monoquaternary AChE reactivator for the treatment of toxic effects of OP [5,6]. More extended bisquaternary compounds, such as trimedoxime (**2**; TMB-4, 1,3-bis(4-hydroxyiminomethylpyridinium)propane dibromide), obidoxime (**3**; Toxogonine®, 1,3-bis(4-hydroxyiminomethylpyridinium)-2-oxapropane dibromide), H-oxime HI-6 (**4**; 1-(2-hydroxyiminomethylpyridinium)-3-(4-carbamoylpyridinium)-2-oxapropane dichloride) and methoxime (**5**, bis(4-hydroxyiminomethylpyridinium)methane dichloride) are representatives of these aldoximes [7–11].

Due to the high variability of AChE inhibitors, there is no single AChE reactivator having the ability to sufficiently reactivate inhibited enzyme regardless of the chemical structure of the inhibitor. Therefore, many laboratories throughout the world have decided to synthesize new reactivators of OP-inhibited AChE, in order to reactivate OP-inhibited AChE regardless of the chemical structure of the organophosphorus inhibitor.

Our research was focused on finding more rigid symmetrical bisquaternary structures than commonly used reactivators have (**2, 3**). In order to fix the structure of the compounds, we decided to use a xylene linker

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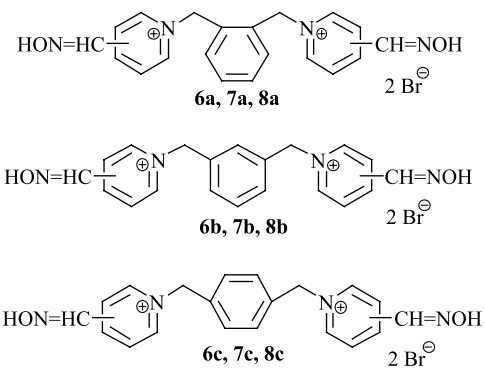


Compound	Oxime position
6a–c	2
7a–c	3
8a–c	4

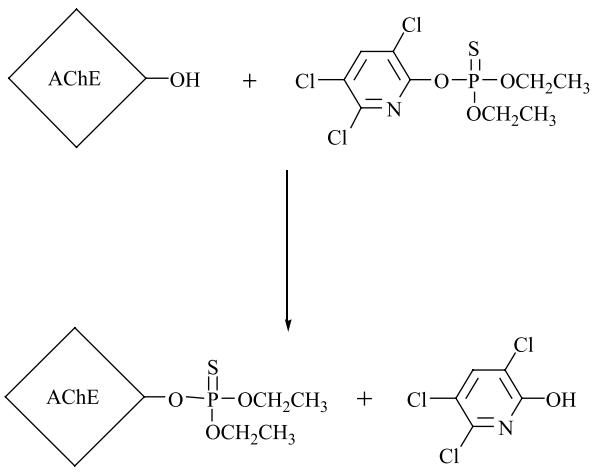
Scheme 1. Symmetrical bisquaternary xylene-linked compounds.

connecting two pyridinium rings bearing carbaldoxime moieties in various positions (Scheme 1). Afterwards, we tested their ability to reactivate AChE inhibited by chlorpyrifos (Scheme 2), which is commonly used as an insecticide for agricultural purposes.

Materials and methods

Chemistry

Solvents (acetone, DMF, ethanol) and reagents were purchased from Fluka and Sigma-Aldrich (Czech Republic) and used without further purification. Reactions were monitored by TLC using DC-Alufolien Cellulose F (Merck, Germany) and mobile phase BuOH-CH_3COOH-H_2O (5: 1: 2). For detection a solution of Dragendorff reagent (solution containing 10 ml CH_3COOH, 50 ml H_2O and 5 ml of basic solution prepared by mixing of two fractions – fraction I.: 850 mg $Bi(NO_3)_3$, 40 ml H_2O, 10 ml CH_3COOH; fraction II.: 8 g KI, 20 ml H_2O) was used. Melting points were measured on a micro heating stage PHMK 05 (VEB Kombinat Nagema, Radebeul, Germany) and are uncorrected.



Scheme 2. AChE inhibited by chlorpyrifos.

NMR spectra were generally recorded on a Varian Gemini 300 (^{1}H 300 MHz, ^{13}C 75 MHz, Palo Alto CA, USA). In all cases, the chemical shift values for ^{1}H spectra are reported in ppm (δ) relative to residual $CHD_2SO_2CD_3$ (δ 2.50) and shift values for ^{13}C spectra are reported in ppm (δ) relative to the solvent peak dimethylsulfoxide – d_6 δ 39.43. Signals are quoted as s (singlet), bs (broad singlet), d (doublet), t (triplet) and m (multiplet).

Mass spectra were recorded using a combination of high performance liquid chromatography and mass spectrometry. HP1100 HPLC system was obtained from Agilent Technologies (Waldbronn, Germany). It consisted of vacuum degasser G1322A, quaternary pump G1311A, autosampler G1313A and quadrupole mass spectrometer MSD1456 VL equipped with electrospray ionization source. Nitrogen for the mass spectrometer was supplied by a Whatman 75–720 nitrogen generator. Data were collected in positive ion mode with an ESI probe voltage of 4000 V. The pressure of the nebulizer gas was set up to 35 psig. Drying gas temperature was operated at 335°C and flow at 13 l/min.

Preparation of quaternary salts. Two synthetic pathways were used for preparation of the bisquaternary aldoximes:

A) A solution of the hydroxyiminomethylpyridine (1.0 g, 8.2 mmol) and dibromoxylene (0.97 g, 3.7 mmol) in DMF (10 mL) was stirred at 100°C. The reaction mixture was cooled to room temperature and mixted with acetone (30 mL); the crystalline crude product was collected by filtration, washed with acetone (2 × 20 mL) and recrystalized from acetonitrile.

B) A solution of the hydroxyiminomethylpyridine (1.0 g, 8.2 mmol) and dibromoxylene (0.97 g, 3.7 mmol) in acetonitrile (10 mL) was stirred at 80°C. The reaction mixture was cooled to room temperature and mixted with acetone (30 ml); the crystalline crude product was collected by filtration, washed with acetone (2 × 20 mL) and recrystalized from ethanol.

*2,2′-bis(hydroxyiminomethyl)-1,1′-(1,2-phenylene-dimethyl)-bispyridinium dibromide (**6a**)*. Prepared by method *A*. The reaction was stopped after 8.5 h. Yield 0.93 g (50%), TLC R_f 0.15, m.p. decomp. 170°C. ^{1}H NMR (300 MHz, DMSO d_6): δ (ppm) 9.07 (d, 2H, J = 6.32 Hz), 8.80 − 8.58 (m, 6H), 8.32 − 8.23 (m, 2H), 7.38 − 7.30 (m, 2H), 6.57 − 6.49 (m, 2H), 6.39 (s, 4H), 3.56 (bs, 2H). ^{13}C NMR (75 MHz, DMSO d_6): δ (ppm) 147.99,

146.21, 145.87, 141.16, 131.41, 129.09, 128.24, 125.76, 125.40, 57.68. Analysis: calculated 47.27% C, 3.97% H, 11.02% N; found 47.51% C, 4.13% H, 10.68% N. ESI-MS: m/z 347.1 $[M^{2+} - H^+]^+$ (calculated for $[C_{20}H_{20}N_4O_2^{2+} - H^+]^+$347.16).

*2,2′-bis(hydroxyiminomethyl)-1,1′-(1,3-phenylene-dimethyl)-bispyridinium dibromide (**6b**)*. Prepared by method *B*. The reaction was stopped after 5 h. Yield 0.39g (21%), TLC R_f 0.15, m.p. decomp. 199–201°C. ^{1}H NMR (300 MHz, DMSO d_6): δ (ppm) 9.20 (d, 2H, J = 6.0 Hz), 8.80 − 8.57 (m, 4H), 8.43 (d, 2H, J = 7.7 Hz), 8.29 − 8.13 (m, 2H), 7.55 − 7.40 (m, 1H), 7.28 (d, 2H, J = 7.7 Hz), 7.17 (s, 1H), 6.11 (s, 4H), 3.95 (bs, 2H). ^{13}C NMR (75 MHz, DMSO d_6): δ (ppm) 147.10, 146.32, 145.96, 141.21, 134.89, 130.04, 127.86, 127.76, 126.03, 59.76. Analysis: calculated 47.27% C, 3.97% H, 11.02% N; found 47.00% C, 4.10% H, 10.99% N. ESI-MS: m/z 347.1 $[M^{2+} - H^+]^+$ (calculated for $[C_{20}H_{20}N_4O_2^{2+} - H^+]^+$347.16).

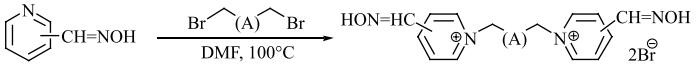
*2,2′-bis(hydroxyiminomethyl)-1,1′-(1,4-phenylene-dimethyl)-bispyridinium dibromide (**6c**)*. Prepared by method *A*. The reaction was stopped after 7 h. Yield 0.79 g (42%), TLC R_f 0.15, m.p. decomp. 240°C. ^{1}H NMR (300 MHz, DMSO d_6): δ (ppm) 9.26 (d, 2H, J = 6.3 Hz), 8.78 − 8.59 (m, 4H), 8.47 − 8,39 (m, 2H), 8.25 − 8.15 (m, 2H), 7.31 (s, 4H), 6.14 (s, 4H), 3.53 (bs, 2H). ^{13}C NMR (75 MHz, DMSO d_6): δ (ppm) 147.10, 146.46, 145.98, 141.30, 134.52, 127.97, 127.90, 126.04, 59.68. Analysis: calculated 47.27% C, 3.97% H, 11.02% N; found 47.16% C, 3.99% H, 11.44% N. ESI-MS: m/z 347.1 $[M^{2+} - H^+]^+$ (calculated for $[C_{20}H_{20}N_4O_2^{2+} - H^+]^+$347.16).

*3,3′-bis(hydroxyiminomethyl)-1,1′-(1,2-phenylene-dimethyl)-bispyridinium dibromide (**7a**)*. Prepared by method *A*. The reaction was stopped after 1.5 h. Yield 1.75 g (93%), TLC R_f 0.15, m.p. 223–226°C. ^{1}H NMR (300 MHz, DMSO d_6): δ (ppm) 9.33 (s, 2H), 9.13 (d, 2H, J = 5.8 Hz), 8.82 (d, 2H, J = 8.0 Hz), 8.40 (s, 2H), 8.28 − 8.20 (m, 2H), 7.57 − 7.49 (m, 2H), 7.35 − 7.27 (m, 2H), 6.23 (s, 4H), 3.39 (s, 2H). ^{13}C NMR (75 MHz, DMSO d_6): δ (ppm) 144.73, 143.22, 142.64, 142.19, 133.74, 132.52, 130.12, 129.78, 128.53. Analysis: calculated 47.27% C, 3.97% H, 11.02% N; found 47.52% C, 3.85% H, 11.09% N. ESI-MS: m/z 347.1 $[M^{2+} - H^+]^+$ (calculated for $[C_{20}H_{20}N_4\ O_2^{2+} - H^+]^+$347.16).

*3,3′-bis(hydroxyiminomethyl)-1,1′-(1,3-phenylene-dimethyl)-bispyridinium dibromide (**7b**)*. Prepared by method *A*. The reaction was stopped after 1.5 h. Yield 1.77 g (94%), TLC R_f 0.15, m.p. 238241°C. ^{1}H NMR (300 MHz, DMSO d_6): δ (ppm) 9.50 (s, 2H), 9.25 (d, 2H, J = 5.8 Hz), 8.78 (d, 2H, J = 8.0 Hz), 8.40 (s, 2H), 8.25 − 8.17 (m, 2H), 7.85 (s, 1H), 7.61 (d, 2H, J = 7.4 Hz), 7.57 − 7.49 (m, 1H), 5.96 (s, 4H), 3.38 (s, 2H). ^{13}C NMR (75 MHz, DMSO d_6): δ (ppm) 144.53, 143.17, 142.53, 141.90, 134.86, 133.69, 129.98, 129.69, 129.48, 128.46, 62.81. Analysis: calculated 47.27% C, 3.97% H, 11.02% N; found 47.36% C, 3.84% H, 11.08% N. ESI-MS: m/z 347.0 $[M^{2+} - H^+]^+$ (calculated for $[C_{20}H_{20}N_4O_2^{2+} - H^+]^+$347.16).

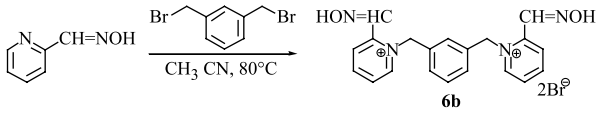
*3,3′-bis(hydroxyiminomethyl)-1,1′-(1,4-phenylen-dimethyl)-bispyridinium dibromide (**7c**)*. Prepared by method *A*. The reaction was stopped after 2 h. Yield 1.46 g (78%), TLC R_f 0.15, m.p. decomp. 162°C. ^{1}H NMR (300 MHz, DMSO d_6): δ (ppm) 9.47 (s, 2H), 9.23 (d, 2H, J = 6.3 Hz), 8.75 (d, 2H, J = 8.0 Hz), 8.36 (s, 2H), 8.24 − 8.15 (m, 2H), 7.67 (s, 4H), 5.95 (s, 4H), 3.38 (s, 2H). ^{13}C NMR (75 MHz, DMSO d_6): δ (ppm) 144.42, 143.21, 142.37, 142.01, 135.14, 133.74, 129.63, 128.50, 62.71; Analysis: calculated 47.27% C, 3.97% H, 11.02% N; found 47.26% C, 3.91% H, 10.92% N. ESI-MS: m/z 347.1 $[M^{2+} - H^+]^+$ (calculated for $[C_{20}H_{20}N_4O_2^{2+} - H^+]^+$347.16).

*4,4′-bis(hydroxyiminomethyl)-1,1′-(1,2-phenylene-dimethyl)-bispyridinium dibromide (**8a**)*. Prepared by method *A*. The reaction was stopped after 2.5 h. Yield 1.23 g (66%), TLC R_f 0.15, m.p. 243 − 244°C, reported 247–248°C, 238.3–239.1°C [12,14–15].



Compound	Oxime position	(A)
6a	2	o-phenylene
6c	2	p-phenylene
7a	3	o-phenylene
7b	3	m-phenylene
7c	3	p-phenylene
8a	4	o-phenylene
8b	4	m-phenylene
8c	4	p-phenylene

Scheme 3. Synthesis of symmetric bisquaternary compounds.



6b

Scheme 4. Synthesis of 2,2′-bis(hydroxyiminomethyl)-1,1′-(1,3-phenylenedimethyl)-bispyridinium dibromide.

^{1}H NMR (300 MHz, DMSO d_6): δ (ppm) 9.10 (d, 4H, J = 6.0 Hz), 8.49 (s, 2H), 8.30 (d, 4H, J = 5.8 Hz), 7.55 − 7.47 (m, 2H), 7.30 − 7.21 (m, 2H), 6.16 (s, 4H), 3.36 (s, 2H). ^{13}C NMR (75 MHz, DMSO d_6): δ (ppm) 149.28, 145.63, 145.33, 133.01, 130.30, 129.83, 124.66, 59.90. Analysis: calculated 47.27% C, 3.97% H, 11.02% N; found 47.18% C, 3.82% H, 11.16% N. ESI-MS: m/z 347.1 $[M^{2+} - H^+]^+$ (calculated for $[C_{20}H_{20}N_4O_2^{2+} - H^+]^+$347.16).

*4,4′-bis(hydroxyiminomethyl)-1,1′-(1,3-phenylenedimethyl)-bispyridinium dibromide (**8b**)*. Prepared by method *A*. The reaction was stopped after 1.5 h. Yield 1.36 g (73%), TLC R_f 0.15, m.p. 226–228°C, reported 225–226°C [12]. ^{1}H NMR (300 MHz, DMSO d_6): δ (ppm) 9.21 (d, 4H, J = 6.6 Hz), 8.45 (s, 2H), 8.28 (d, 4H, J = 6.6 Hz), 7.78 (s, 1H), 7.61 − 7.47 (m, 3H), 5.89 (s, 4H), 3.37 (s, 2H). ^{13}C NMR (75 MHz, DMSO d_6): δ (ppm) 148.75, 145.12, 145.05, 135.12, 130.01, 129.52, 129.24, 124.33, 62.13. Analysis: calculated 47.27% C, 3.97% H, 11.02% N; found 46.32% C, 3.97% H, 10.71% N. ESI-MS: m/z 347.1 $[M^{2+} - H^+]^+$ (calculated for $[C_{20}H_{20}N_4O_2^{2+} - H^+]^+$347.16).

*4,4′-bis(hydroxyiminomethyl)-1,1′-(1,4-phenylenedimethyl)-bispyridinium dibromide (**8c**)*. Prepared by method *A*. The reaction was stopped after 5 h. Yield 1.61 g (86%), TLC R_f 0.15, m.p. decomp. 285°C, reported decomp. 286°C, 290.5–291.5°C [12,14–15]. ^{1}H NMR (300 MHz, DMSO d_6): δ (ppm) 9.20 (d, 4H, J = 6.6 Hz), 8.42 (s, 2H), 8.25 (d, 4H, J = 6.6 Hz), 7.63 (s, 4H), 5.88 (s, 4H), 3.50 (bs, 2H). ^{13}C NMR (75 MHz, DMSO d_6): δ (ppm) 148.75, 145.03, 135.30, 129.55, 124.36, 61.98. Analysis: calculated 47.27% C, 3.97% H, 11.02% N; found 47.24% C, 4.10% H, 10.90% N. ESI-MS: m/z 347.1 $[M^{2+} - H^+]^+$ (calculated for $[C_{20}H_{20}N_4O_2^{2+} - H^+]^+$347.16).

Biochemistry

In vitro testing of the synthesized oximes involved a standard collection of experimental procedures. The 10% rat brain homogenate was used as a source of AChE. The brain homogenate (0.5 ml) was mixed with 29 μL of an isopropanol solution of chlorpyrifos (*O,O*-diethyl-*O*-(3,5,6-trichloro-2-pyridyl)-phosphorothioate, analytical standard 99.2% from Sigma-Aldrich to achieved 95% inhibition of AChE) and incubated at 25°C for 30 min. A solution of sodium chloride (2.5 mL 3 M) was added to the mixture and filled to a volume of 23 mL with distilled water. Finally, 2 mL of a solution of acetylcholine iodide (0.02 M) was added. The enzyme activity was measured at pH 8.0 and 25°C on an autotitrator RTS 822 (Radiometer, Denmark). Activities of intact AChE (a_0) and inhibited AChE (a_i) were deduced from the consumption of NaOH solution (0.01 M) with time. After the incubation with chlorpyrifos-inhibited AChE (30 min), the reactivator was added to the solution and the mixture was incubated for 10 min. Activity of the reactivated AChE (a_r) was also computed from the consumption of NaOH solution with time.

The percentage of reactivation (%) was calculated from the measured data according to:

$$x = \left(1 - \frac{a_r - a_i}{a_0 - a_i}\right) \cdot 100[\%] \qquad (1)$$

Table I. Reactivation potencies (%)[a] of tested oximes. Time of inhibition by chlorpyrifos –30 min; time of reactivation by AChE reactivators –10 min; pH 8; temperature 25°C.

	Compound	% (10^{-3} M)	% (10^{-5} M)
Synthesized oximes	**6a**	19	3
	6b	26	39
	6c	41	68
	7a	0	0
	7b	5	0
	7c	8	0
	8a	20	0
	8b	37	25
	8c	34	8
Reference Compounds	2-PAM (**1**)	38	4
	TMB-4 (**2**)	66	38
	obidoxime (**3**)	63	35
	HI-6 (**4**)	20	11
	MMC (**5**)	45	10

[a] Mean value of three independent determinations.

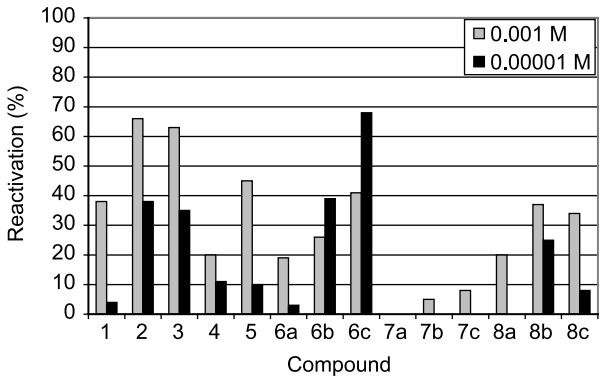


Figure 1. Efficacy of tested oximes in reactivation of chlorpyrifos-inhibited AChE.

Pralidoxime, methoxime, HI-6, obidoxime and trimedoxime of HPLC purity previously synthesized in our laboratory were used as references.The whole method is described in detail in the work of Kuca and Kassa [17].

Results and discussion

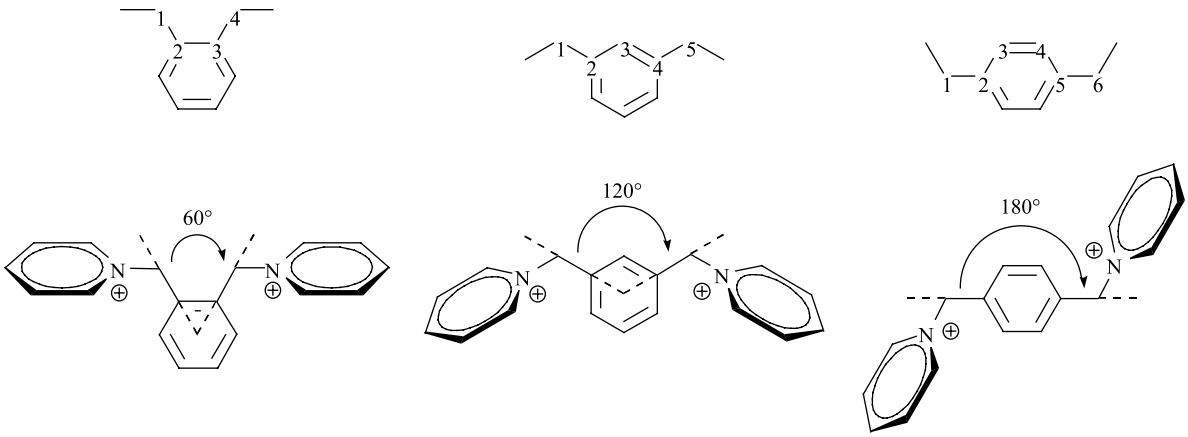
Previously published reaction conditions but differing in the temperature (100°C) used were applied to the syntheses of the required compounds (Scheme 3) [11]. The yields of four former known compounds (**6c, 8a**–**c**) are comparable with published data [12–15].

Unfortunately, this system was not applicable in the case of 2,2′-bis(hydroxyiminomethyl)-1,1′-(1,3-phenylenedimethyl)-bispyridinium dibromide (**6b**), where other conditions were used (Scheme 4) [16].

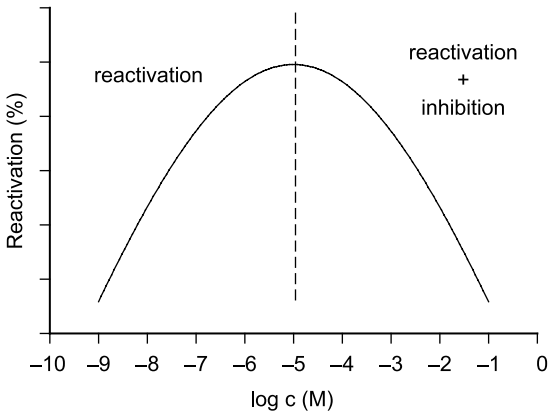
Reactivation potencies of all the synthesized oximes and reference compounds are shown in Table I and Figure 1.

The most potent reactivators of chlorpyrifos-inhibited AChE seem to be the reference compounds obidoxime (**3**) and TMB-4 (**2**) at concentrations of 10^{-3} M. However, new synthesized oximes (**6a**–**c, 8a**–**c**) showed satisfactory reactivation results, too. Unfortunately, concentration 10^{-3} M is not aplicable for further *in vivo* testing [17–21]. The concentration 10^{-5} M is more suitable from the point of view of the reactivator's toxic effect on the patient [17]. This means that the oxime **6c** surpasses all other *in vitro* tested compounds (68% reactivation) and is the most promising from these compounds tested *in vitro*. Oximes **6b**, **8b**, obidoxime (**3**) and trimedoxime (**2**) have also satisfactory reactivation ability at 10^{-5} M concentration.

The reactivation potency of the measured compounds depends on the structure of the OP inhibitor [17–18]. Consequently, we can recommend structural factors appropriate for reactivation of chlorpyrifos-inhibited AChE [17]. The oxime functional group breaks down the bond OP inhibitor-enzyme and is essential for the activity of the reactivator [21–23]. Our results confirm that the position of the hydroxyiminomethyl group influences the reactivation potency. While the substances bearing the oxime group in position-3 are practically ineffective (**7a**–**c**), substances bearing the oxime group in position 2 or 4 show promising results (**6a**–**c, 8a**–**c**) [22]. In comparison (**6a**–**c, 8a**–**b**), the length of the linking chain also influences the reactivation potency [4]. In our case, the length of the linker varies from four to six atoms between the pyridinium rings (Scheme 5). The most promising compound (**6c**) has a six atom-linker in contrast to the active reference substances trimedoxime (**2**) and obidoxime (**3**), which have only a three atom-bridge. Than important role is also played by plays also the "rigidity" of the linking chain. The only bonds accessible for free rotation in these molecules are the methylene junctions in contrast to free rotation in the molecules with a propane (**2**) or 2-oxapropane linking chain (**3, 4**). These short junctions cause the non-coplanarity of each pyridinium ring to the phenylene ring. Therefore, there could be



Scheme 5. Lengths and angles of linking chains in 6, 7 and 8.



Scheme 6. Bell-shaped curve of reactivation process.

determined angles between bonds outgoing from the phenylene ring (Scheme 5). From this point of view, the most promising are compounds with longer angles meaning a greater distance between the pyridinium rings (**6b**–**c, 8b**–**c**).

The higher reactivation activity of some compounds (**6b**–**c**) at the lower concentration is caused by their inhibition of intact AChE. The measurements were made for two concentrations and the whole concentration scale is bell-shaped (Scheme 6) [17]. The reactivation process is characterized in the increasing limb and the decreasing limb shows both reactivation and inhibition of liberated intact AChE by the reactivator itself. Every reactivator varies in the optimal concentration for reactivation and in the case of **6b**–**c** the optimum lies at lower concetrations.

Conclusion

Nine potential AChE reactivators have been synthesized using a modification of currently known synthetic pathways and also *in vitro* tested their potency to reactivate AChE inhibited by the insecticide chlorpyrifos. 2,2′-Bis(hydroxyiminomethyl)-1,1′-(1,4-phenylenedimethyl)-bispyridinium dibromide (**6c**) seems to be the most potent AChE reactivator. The reactivation potency of these compounds depends on structural factors such as length of the linking chain between both pyridinium rings and position of the functional oxime group on the pyridinium ring.

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