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Synthesis of a novel series of non-symmetrical bispyridinium compounds bearing a xylene linker and evaluation of their reactivation activity against tabun and paraoxon-inhibited acetylcholinesterase

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Abstract

Nine potential non-symmetrical xylene-bridged AChE reactivators were synthesized using modifications of currently known synthetic pathways. Their potency to reactivate AChE inhibited by the nerve agent tabun and the insecticide paraoxon together with nine symmetrical xylene-bridged compounds, was tested *in vitro*. Seven compounds were promising against paraoxon-inhibited AChE. Two compounds were found to be more potent against tabun-inhibited AChE than obidoxime at a concentration applicable *in vivo*.

Keywords: *acetylcholinesterase, reactivation, nerve agent, tabun, pesticide, paraoxon, oxime*

Introduction

Organophosphorus compounds (OP) are commonly known as nerve agents (soman, sarin, tabun, VX, etc.), pesticides (chlorpyrifos, paraoxon, diazinon, etc.), compounds used for industrial purposes (tributylphosphate) or as potential therapeutics (metrifonate) [1–4]. Their chemical structure is derived from phosphonic and phosphoric acid or their thio-analogues respectively [1–2]. They are able to irreversibly phosphonylate or phosphorylate the serine hydroxyl in the active site of acetylcholinesterase (AChE, EC 3.1.1.7) inhibiting the cleavage of the neurotransmitter acetylcholine which is essential to terminate cholinergic transmission (Scheme 1) [1–2]. The accumulated acetylcholine causes cholinergic overstimulation and subsequent cholinergic crisis which normally causes serious malfunction of the breathing centre in the medulla oblongata followed by death [2]. In the last decade, the risk of intoxication by these compounds has rapidly increased with growing agricultural production and with the threat of terrorist attacks [5–6].

Anticholinergic drugs such as atropine are used to counteract the effects of OP-inhibited enzyme at peripheral cholinergic receptors [7]. However, they are not able to restore natural enzyme activity. For this reason, AChE reactivators are used to cleave the covalent bond OP-enzyme by a reactive nucleophilic group and to restore the activity of AChE (Scheme 2) [2]. The commonly used reactivators are monoquaternary or bisquaternary compounds carrying the hydroxyiminomethyl (oxime) group as nucleophilic agent, e.g. pralidoxime – “the golden standard of AChE reactivators” (**1**, 2-hydroxyiminomethyl-1-methylpyridinium chloride), oxime HI-6

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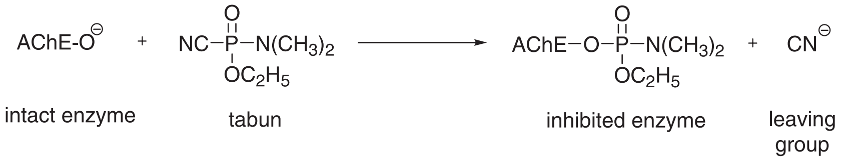


Scheme 1. AChE inhibited by nerve agent tabun.

(**2**, 1-(2-hydroxyiminomethylpyridinium)-3-(4-carbamoylpyridinium)-2-oxapropane dichloride), obidoxime (**3**, Toxogonine®, 1,3-bis(4-hydroxyiminomethyl- pyridinium)-2-oxapropane dichloride) (Figure 1) [8–11]. Nevertheless, every type of OP needs a specific structure for the AChE reactivator and there is not a broad spectrum reactivator after more than fifty years of investigations [12–15]. Therefore, the development and selection of new effective reactivators of AChE-like antidotes of OP are very important.

In this work, eighteen compounds (**4–21**) are described (Scheme 3). Our research was focused on finding more rigid bisquaternary structures than the commonly used reactivators possess (**2, 3**). In order to fix the conformation of the molecule, we decided to use a xylene linker connecting two pyridinium rings bearing oxime moieties non-symmetrically (nine novel compounds, **4–12**), in contrast with symmetrical ones (nine compounds prepared previously, **13–21**) [16]. The idea and design of an aromatic linker was used by comparison with inhibitors of AChE with a triazole linker prepared using click chemistry methods [17–18]. Moreover, each position of oxime on the pyridinium ring is able to reactivate another type of inhibitor. While position four is more suitable for tabun or pesticide-inhibited AChE, position two is effective against sarin, soman or VX-inhibited AChE [19–21]. Position three has usually low efficacy due to the dissociation constant at the pH of human blood [19]. Therefore, the combination of various positions should extend the reactivation to a broader spectrum of OP inhibitors. Both symmetrical and non-symmetrical compounds were tested *in vitro* on tabun (GA) and paraoxon-inhibited AChE.

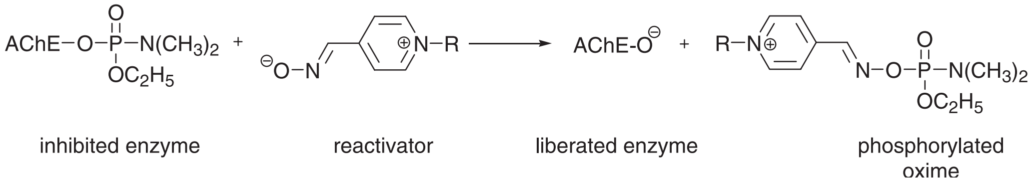
Materials and methods

Chemistry

The compounds were synthesized using conventional methods [15–16]. Initially, the hydroxyiminomethylpyridine was mixed in acetone with 5 equivalent excess of the corresponding dibromoxylene to afford the monoquaternary compounds (Scheme 4). The monoquaternary salt was easily purified by the recrystallization from acetonitrile, whereas the biproduct is almost insoluble. Secondly, a different hydroxyiminomethylpyridine was added in DMF to obtain the non-symmetrical bisquaternary salt, usually in satisfactory yield (27-91%).

Preparation of quaternary salts. (A) Preparation of monoquaternary salts – A solution of the hydroxyiminomethylpyridine (1.0 g, 8.2 mmol) and dibromoxylene (10.8 g, 40.9 mmol) in acetone (100 mL) was stirred at reflux. The reaction mixture was then cooled to room temperature; the crystalline crude product was collected by filtration, washed with acetone (3 × 20 mL) and recrystallized from acetonitrile (MeCN). (B) Preparation of bisquaternary salts – A solution of the monoquaternary salt (0.50 g, 1.3 mmol) and hydroxyiminomethylpyridine (0.30 g, 2.4 mmol) in DMF (10 mL) was stirred at 70–100°C. The reaction mixture was then cooled to room temperature and portioned with acetone (50 ml); the crystalline crude product was collected by filtration, washed with acetone (3 × 20 mL) and recrystalized from MeCN.

*2,3′-bis(hydroxyiminomethyl)-1,1′-(1,2-phenylenedimethyl)-bispyridinium dibromide (**4**)*. Prepared by method B *via* **22**. The reaction mixture was stirred at 70°C and stopped after 10 h. Yield 0.48 g (73%), TLC R_f 0.15, m.p. 215–216 °C. ^{1}H NMR (300 MHz, DMSO d_6): δ (ppm) 9.37 (s, 1H, PyrH), 9.18 (d, 1H, J = 6.0 Hz, PyrH), 9.06 (d, 1H, J = 6.0 Hz, PyrH), 8.84 (d, 1H, J = 8.1 Hz, PyrH), 8.76-8.64 (m, 2H, PyrH + -*CH*=NOH), 8.57 (d, 1H, J = 8.1 Hz, PyrH), 8.44 (s, 1H, -*CH*=NOH), 8.31-8.17 (m, 2H, PyrH), 7.56-7.27 (m, 3H, ArH), 6.61 (d, 1H, J = 7.4 Hz, ArH), 6.36 (s, 2H, -CH_2-), 6.22 (s, 2H, -CH_2-). ^{13}C NMR (75 MHz, DMSO d_6): δ



Scheme 2. Oxime induced reactivation of tabun-inhibited AChE.

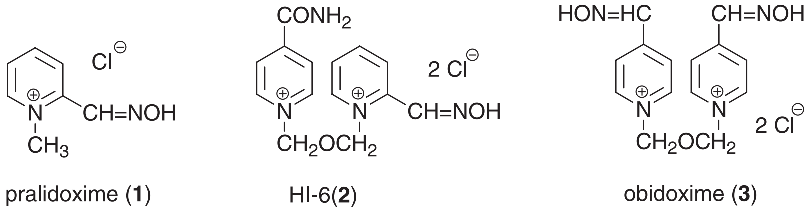
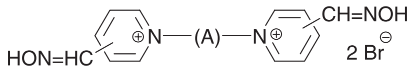


Figure 1. Examples of oxime reactivators currently used against OP intoxications.

(ppm) 147.77, 147.76, 147.73, 147.70, 146.34, 146.28, 143.30, 142.73, 141.28, 133.80, 133.78, 132.66, 131.40, 130.22, 128.59, 128.53, 126.27, 125.96, 60.41, 57.94. Analysis: calculated 47.27% C, 3.97% H, 11.02% N; found 47.07% C, 4.18% H, 11.04% N. ESI-MS: m/z 347.1 [M^{2+}-H^+] (calculated for [$C_{20}H_{20}N_4O_2^{2+}$-H^+] 347.16).

*2,4′-bis(hydroxyiminomethyl)-1,1′-(1,2-phenylenedimethyl)-bispyridinium dibromide (**5**)*. Prepared by method B *via* **23**. The reaction mixture was stirred at 70°C and stopped after 10 h. Yield 0.50 g (76%), TLC R_f 0.15, m.p. 205–206°C. ^{1}H NMR (300 MHz, DMSO d_6): δ (ppm) 9.15 (d, 2H, J = 6.3 Hz, PyrH), 9.05 (d, 1H, J = 6.3 Hz, PyrH), 8.76-8.64 (m, 2H, PyrH + -*CH*=NOH), 8.60-8.48 (m, 2H, PyrH + -*CH*=NOH), 8.33 (d, 2H, J = 6.3 Hz, PyrH), 8.26-8.19 (m, 1H, PyrH), 7.52-7.35 (m, 2H, ArH), 7.31 (d, 1H, J = 7.4 Hz, ArH), 6.61 (d, 1H, J = 7.4 Hz, ArH), 6.35 (s, 2H, -CH_2-), 6.16 (s, 2H, -CH_2-). ^{13}C NMR (75 MHz, DMSO d_6): δ (ppm) 149.00, 147.71, 146.26, 146.13, 145.42, 145.10, 141.32, 132.52, 131.72, 130.10, 129.70, 129.34, 128.15, 126.26, 126.04, 124.42, 59.68, 57.93. Analysis: calculated 47.27% C, 3.97% H, 11.02% N; found 46.53% C, 4.17% H, 10.79% N. ESI-MS: m/z 347.1 [M^{2+}-H^+] (calculated for [$C_{20}H_{20}N_4O_2^{2+}$-H]$^+$347.16).

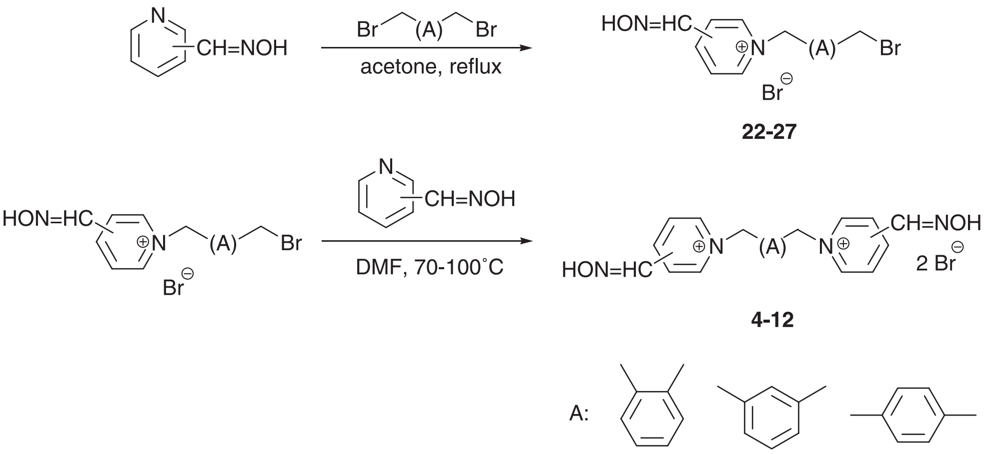


Compound	A	Oxime position
4	*o*-phenylene	2,3'-CH=NOH
5	*o*-phenylene	2,4'-CH=NOH
6	*o*-phenylene	3,4'-CH=NOH
7	*m*-phenylene	2,3'-CH=NOH
8	*m*-phenylene	2,4'-CH=NOH
9	*m*-phenylene	3,4'-CH=NOH
10	*p*-phenylene	2,3'-CH=NOH
11	*p*-phenylene	2,4'-CH=NOH
12	*p*-phenylene	3,4'-CH=NOH
13	*o*-phenylene	2,2'-CH=NOH
14	*o*-phenylene	3,3'-CH=NOH
15	*o*-phenylene	4,4'-CH=NOH
16	*m*-phenylene	2,2'-CH=NOH
17	*m*-phenylene	3,3'-CH=NOH
18	*m*-phenylene	4,4'-CH=NOH
19	*p*-phenylene	2,2'-CH=NOH
20	*p*-phenylene	3,3'-CH=NOH
21	*p*-phenylene	4,4'-CH=NOH

Scheme 3. Potential oxime reactivators tested against tabun- and paraoxon-inhibited AChE.

*3,4′-bis(hydroxyiminomethyl)-1,1′-(1,2-phenylenedimethyl)-bispyridinium dibromide (**6**)*. Prepared by method B *via* **23**. The reaction mixture was stirred at 100°C and stopped after 2 h. Yield 0.57 g (86%), TLC R_f 0.15, m.p. 224–226°C. ^{1}H NMR (300 MHz, DMSO d_6): δ (ppm) 9.31 (s, 1H, PyrH), 9.14-9.04 (m, 3H, PyrH), 8.81 (d, 1H, J = 8.1 Hz, PyrH), 8.49 (s, 1H, -*CH*=NOH), 8.40 (s, 1H, -*CH*=NOH), 8.33-8.19 (m, 3H, PyrH), 7.57-7.47 (dd, 2H, J = 3.3 Hz, ArH), 7.34-7.23 (m, 2H, ArH), 6.19 (d, 4H, J = 13.5 Hz, -CH_2-). ^{13}C NMR (75 MHz, DMSO d_6): δ (ppm) 149.01, 145.34, 145.05, 144.73, 143.23, 142.63, 142.18, 133.76, 132.81, 132.42, 130.14, 130.05, 129.81, 129.63, 128.55, 124.36, 60.38, 59.66. Analysis: calculated 47.27% C, 3.97% H, 11.02% N; found 46.07% C, 4.27% H, 10.93% N. ESI-MS: m/z 347.1 [M^{2+}-H^+] (calculated for [$C_{20}H_{20}N_4O_2^{2+}$-H^+] 347.16).

2,3′-bis(hydroxyiminomethyl)-1,1′-(1,3-phenylenedimethyl)-bispyridinium dibromide (7). Prepared by method B *via* **24**. The reaction mixture (MeCN-30mL/EtOH-10mL/DMF-8mL) was stirred at 70°C and stopped after 26 h. Yield 0.36 g (27%), TLC R_f 0.15, m.p. 199–201°C. ^{1}H NMR (300 MHz, DMSO d_6): δ (ppm) 9.47 (s, 1H, PyrH), 9.30 (d, 1H, J = 6.0 Hz, PyrH), 9.19 (d, 1H, J = 6.0 Hz, PyrH), 8.77 (d, 1H, J = 8.1 Hz, PyrH), 8.71-8.61 (m, 2H, PyrH + -*CH*=NOH), 8.48-8.35 (m, 2H, PyrH + -*CH*=NOH), 8.26-8.16 (m, 2H, PyrH), 7.64-7.45 (m, 3H, ArH), 7.29 (d, 1H, J = 7.4 Hz, ArH), 6.15 (s, 2H, -CH_2-), 5.93 (s, 2H, -CH_2-). ^{13}C NMR spectrum (75 MHz, DMSO d_6): δ (ppm) 147.09, 146.56, 146.02, 146.00, 144.34, 143.19, 142.40, 141.97, 141.35, 134.93, 134.89, 133.71, 130.03, 129.27, 128.46, 128.13, 127.92, 126.11, 62.78, 59.92. Analysis: calculated 47.27% C, 3.97% H, 11.02% N; found 47.10% C, 4.08% H, 10.98% N. ESI-MS: m/z 347.1 [M^{2+}-H^+] (calculated for [$C_{20}H_{20}N_4O_2^{2+}$-H^+] 347.16).



Scheme 4. Two step synthesis of non-symmetrical bisquaternary compounds.

*2,4′-bis(hydroxyiminomethyl)-1,1′-(1,3-phenylenedimethyl)-bispyridinium dibromide (**8**)*. Prepared by method B *via* **25**. The reaction mixture was stirred at 70°C and stopped after 8.5 h. Yield 0.43 g (65%), TLC R_f 0.15, m.p. 208–210°C. ^{1}H NMR (300 MHz, DMSO d_6): δ (ppm) 9.30 (d, 1H, J = 6.0 Hz, PyrH), 9.19 (d, 2H, J = 6.0 Hz, PyrH), 8.71-8.61 (m, 2H, PyrH + -*CH*=NOH), 8.48-8.39 (m, 2H, PyrH + -*CH*=NOH), 8.30-8.18 (m, 3H, PyrH), 7.60-7.45 (m, 3H, ArH), 7.29 (d, 1H, J = 7.4 Hz, ArH), 6.15 (s, 2H, -CH$_2$-), 5.88 (s, 2H, -CH$_2$-). ^{13}C NMR (75 MHz, DMSO d_6): δ (ppm) 148.75, 147.09, 146.56, 146.00, 145.04, 144.97, 141.38, 135.15, 134.88, 130.04, 129.18, 128.07, 127.93, 127.80, 126.14, 124.32, 62.05, 59.94. Analysis: calculated 47.27% C, 3.97% H, 11.02% N; found 47.06% C, 4.21% H, 11.17% N. ESI-MS: m/z 347.1 [M^{2+}-H$^+$] (calculated for [$C_{20}H_{20}N_4O_2^{2+}$-H$^+$] 347.16).

*3,4′-bis(hydroxyiminomethyl)-1,1′-(1,3-phenylenedimethyl)-bispyridinium dibromide (**9**)*. Prepared by method B *via* **25**. The reaction mixture was stirred at 70°C and stopped after 5 h. Yield 0.32 g (48%), TLC R_f 0.15, m.p. 200–202°C. ^{1}H NMR (300 MHz, DMSO d_6): δ (ppm) 9.51 (s, 1H, PyrH), 9.31-9.19 (m, 3H, PyrH), 8.78 (d, 1H, J = 8.1 Hz, PyrH), 8.46 (s, 1H, -*CH*=NOH), 8.41 (s, 1H, -*CH*=NOH), 8.31-8.16 (m, 3H, PyrH), 7.85 (s, 1H, ArH), 7.65-7.47 (m, 3H, ArH), 5.95 (d, 4H, J = 14.4 Hz, -CH$_2$-). ^{13}C NMR (75 MHz, DMSO d_6): δ (ppm) 147.10, 146.46, 145.98, 144.42, 144.41, 143.20, 142.39, 142.02, 141.34, 134.99, 134.67, 133.74, 129.57, 128.50, 128.04, 126.07, 62.65, 59.70. Analysis: calculated 47.27% C, 3.97% H, 11.02% N; found 46.45% C, 4.07% H, 10.79% N. ESI-MS: m/z 347.1 [M^{2+}-H$^+$] (calculated for [$C_{20}H_{20}N_4O_2^{2+}$-H$^+$] 347.16).

*2,3′-bis(hydroxyiminomethyl)-1,1′-(1,4-phenylenedimethyl)-bispyridinium dibromide (**10**)*. Prepared by method B *via* **26**. The reaction mixture was stirred at 100°C and stopped after 8 h. Yield 0.49 g (74%), TLC R_f 0.15, m.p. 228–230°C. ^{1}H NMR (300 MHz, DMSO d_6): δ (ppm) 9.48 (s, 1H, PyrH), 9.26 (dd, 2H, J = 6.2 Hz, PyrH), 8.80-8.70 (m, 2H, PyrH + -*CH*=NOH), 8.70-8.60 (m, 1H, PyrH), 8.43 (d, 1H, J = 8.2 Hz, PyrH), 8.36 (s, 1H, -*CH*=NOH), 8.26-8.16 (m, 2H, PyrH), 7.63 (d, 2H, J = 7.8 Hz, ArH), 7.35 (d, 2H, J = 7.8 Hz, ArH), 6.16 (s, 2H, -CH$_2$-), 5.95 (s, 2H, -CH$_2$-). ^{13}C NMR (75 MHz, DMSO d_6): δ (ppm) 147.10, 146.46, 145.98, 144.42, 144.41, 143.20, 142.39, 142.02, 141.34, 134.99, 134.67, 133.74, 129.57, 128.50, 128.04, 126.07, 62.65, 59.70. Analysis: calculated 47.27% C, 3.97% H, 11.02% N; found 47.22% C, 4.13% H, 10.86% N. ESI-MS: m/z 347.1 [M^{2+}-H$^+$] (calculated for [$C_{20}H_{20}N_4O_2^{2+}$-H$^+$] 347.16).

*2,4′-bis(hydroxyiminomethyl)-1,1′-(1,4-phenylenedimethyl)-bispyridinium dibromide (**11**)*. Prepared by method B *via* **27**. The reaction mixture was stirred at 100°C and stopped after 8 h. Yield 0.48 g (73%), TLC R_f 0.15, m.p. 239–241°C. ^{1}H NMR spectrum (300 MHz, DMSO d_6): δ (ppm) 9.29 (d, 1H, J = 6.0 Hz, PyrH), 9.21 (d, 2H, J = 6.0 Hz, PyrH), 8.73 (s, 1H, -*CH*=NOH), 8.70-8.60 (m, 1H, PyrH), 8.49-8.40 (m, 2H, PyrH + -*CH*=NOH), 8.32-8.17 (m, 3H, PyrH), 7.61 (d, 2H, J = 7.3 Hz, ArH), 7.35 (d, 2H, J = 7.3 Hz, ArH), 6.16 (s, 2H, -CH$_2$-), 5.89 (s, 2H, -CH$_2$-). ^{13}C NMR (75 MHz, DMSO d_6): δ (ppm) 148.75, 147.09, 146.46, 145.98, 145.02, 141.35, 134.92, 129.48, 128.06, 127.91, 126.07, 124.36, 61.91, 59.71. Analysis: calculated 47.27% C, 3.97% H, 11.02% N; found 46.98% C, 4.33% H, 10.76% N. ESI-MS: m/z 347.1 [M^{2+}-H$^+$] (calculated for [$C_{20}H_{20}N_4O_2^{2+}$-H$^+$] 347.16).

*3,4′-bis(hydroxyiminomethyl)-1,1′-(1,4-phenylenedimethyl)-bispyridinium dibromide (**12**)*. Prepared by method B *via* **27**. The reaction mixture was stirred at 100°C and stopped after 2 h. Yield 0.60 g (91%), TLC R_f 0.15, m.p. 264–265°C. ^{1}H NMR spectrum

(300 MHz, DMSO d_6): δ (ppm) 9.48 (s, 1H, PyrH), 9.29-9.17 (m, 3H, PyrH), 8.75 (d, 1H, J = 8.1 Hz, PyrH), 8.43 (s, 1H, -*CH*=NOH), 8.36 (s, 1H, -*CH*=NOH), 8.26 (d, 2H, J = 6.3 Hz, PyrH), 8.23-8.16 (m, 1H, PyrH), 7.72-7.61 (m, 4H, ArH), 5.93 (d, 4H, J = 15.8 Hz, $-CH_2-$). ^{13}C NMR (75 MHz, DMSO d_6): δ (ppm) 148.75, 145.03, 144.43, 143.20, 142.36, 142.01, 135.37, 135.07, 133.73, 129.66, 129.55, 128.50, 124.36, 62.70, 61.95. Analysis: calculated 47.27% C, 3.97% H, 11.02% N; found 47.12% C, 4.38% H, 10.91% N. ESI-MS: m/z 174.1 [M^{2+}] (calculated for [$C_{10}H_{10}N_2O^{2+}$] 174.08).

*1-(2-bromomethylbenzyl)-3-hydroxyiminomethylpyridinium bromide (**22**)*. Prepared by method A. The reaction mixture was stopped after 2 h. Yield 2.87 g (91%), TLC R_f 0.60, m.p. 189–190°C. ^1H NMR (300 MHz, DMSO d_6): δ (ppm) 9.30 (s, 1H, PyrH), 9.07 (d, 1H, J = 6.0 Hz, PyrH), 8.78 (d, 1H, J = 8.0 Hz, PyrH), 8.39 (s, 1H, -*CH*=NOH), 8.27-8.17 (m, 1H, PyrH), 7.60 (d, 1H, J = 7.0 Hz, ArH), 7.55-7.41 (m, 2H, ArH), 7.37 (d, 1H, J = 7.0 Hz, ArH), 6.12 (s, 2H, $-CH_2-$), 4.96 (s, 2H, $-CH_2-$). ^{13}C NMR (75 MHz, DMSO d_6): δ (ppm) 144.68, 143.25, 142.48, 142.18, 137.19, 133.58, 131.93, 131.60, 130.62, 130.11, 129.68, 128.38, 60.31, 31.70. Analysis: calculated 43.55% C, 3.65% H, 7.26% N; found 43.67% C, 3.91% H, 7.32% N. ESI-MS: m/z 305.0 [M^+] (calculated for [$C_{14}H_{14}BrN_2O^+$] 305.03).

*1-(2-bromomethylbenzyl)-4-hydroxyiminomethylpyridinium bromide (**23**)*. Prepared by method A. The reaction mixture was stopped after 2 h. Yield 3.10 g (98%), TLC R_f 0.60, m.p. 204–206°C. ^1H NMR (300 MHz, DMSO d_6): δ (ppm) 9.06 (d, 2H, J = 6.0 Hz, PyrH), 8.46 (s, 1H, -*CH*=NOH), 8.28 (d, 2H, J = 6.0 Hz, PyrH), 7.60 (d, 1H, J = 6.6 Hz, ArH), 7.53-7.38 (m, 2H, ArH), 7.31 (d, 1H, J = 6.6 Hz, ArH), 6.06 (s, 2H, $-CH_2-$), 4.95 (s, 2H, $-CH_2-$). ^{13}C NMR (75 MHz, DMSO d_6): δ (ppm) 148.87, 145.33, 145.06, 137.07, 132.26, 131.55, 130.34, 129.95, 129.64, 124.23, 59.55, 31.68. Analysis: calculated 43.55% C, 3.65% H, 7.26% N; found 43.46% C, 4.01% H, 7.25% N. ESI-MS: m/z 305.0 [M^+] (calculated for [$C_{14}H_{14}BrN_2O^+$] 305.03).

*1-(3-bromomethylbenzyl)-3-hydroxyiminomethylpyridinium bromide (**24**)*. Prepared by method A. The reaction mixture was stopped after 2 h. Yield 2.80 g (89%), TLC R_f 0.60, m.p. 210–211°C. ^1H NMR spectrum (300 MHz, DMSO d_6): δ (ppm) 9.45 (s, 1H, PyrH), 9.22 (d, 1H, J = 6.0 Hz, PyrH), 8.75 (d, 1H, J = 8.0 Hz, PyrH), 8.38 (s, 1H, -*CH*=NOH), 8.20 (dd, 1H, J = 6.5 Hz), 7.59-7.31 (m, 4H, ArH), 5.93 (s, 2H, $-CH_2-$), 4.49 (s, 2H, $-CH_2-$). ^{13}C NMR (75 MHz, DMSO d_6): δ (ppm) 144.38, 143.68, 143.28, 142.24, 141.99, 133.80, 133.70, 128.95, 128.50, 127.38, 127.23, 126.73, 63.44, 62.35. Analysis: calculated 43.55% C, 3.65% H, 7.26% N; found 43.61% C, 3.99% H, 7.27% N. ESI-MS: m/z 305.0 [M^+] (calculated for [$C_{14}H_{14}BrN_2O^+$] 305.03).

*1-(3-bromomethylbenzyl)-4-hydroxyiminomethylpyridinium bromide (**25**)*. Prepared by method A. The reaction mixture was stopped after 2 h. Yield 3.10 g (98%), TLC R_f 0.60, m.p. 157–158°C. ^1H NMR (300 MHz, DMSO d_6): δ (ppm) 9.18 (d, 2H, J = 6.0 Hz, PyrH), 8.43 (s, 1H, -*CH*=NOH), 8.27 (d, 2H, J = 6.0 Hz, PyrH), 7.61 (s, 1H, ArH), 7.53-7.38 (m, 3H, ArH), 5.87 (s, 2H, $-CH_2-$), 4.69 (s, 2H, $-CH_2-$). ^{13}C NMR (75 MHz, DMSO d_6): δ (ppm) 148.75, 145.03, 139.04, 134.73, 130.13, 129.55, 129.32, 128.60, 124.38, 62.24, 33.63. Analysis: calculated 43.55% C, 3.65% H, 7.26% N; found 43.57% C, 3.91% H, 7.31% N. ESI-MS: m/z 305.0 [M^+] (calculated for [$C_{14}H_{14}BrN_2O^+$] 305.03).

*1-(4-bromomethylbenzyl)-3-hydroxyiminomethylpyridinium bromide (**26**)*. Prepared by method A. The reaction mixture was stopped after 2 h. Yield 2.90 g (92%), TLC R_f 0.60, m.p. 189–190°C. ^1H NMR (300 MHz, DMSO d_6): δ (ppm) 9.44 (s, 1H, PyrH), 9.21 (d, 1H, J = 8.0 Hz, PyrH), 8.75 (d, 1H, J = 8.0 Hz, PyrH), 8.37 (s, 1H, -*CH*=NOH), 8.24-8.14 (m, 1H, PyrH), 7.61-7.48 (m, 4H, ArH), 5.93 (s, 2H, $-CH_2-$), 4.71 (s, 2H, $-CH_2-$). ^{13}C NMR (75 MHz, DMSO d_6): δ (ppm) 144.42, 143.24, 142.33, 141.96, 139.24, 133.99, 133.74, 129.98, 129.18, 128.49, 62.92, 33.54. Analysis: calculated 43.55% C, 3.65% H, 7.26% N; found 43.53% C, 3.95% H, 7.25% N. ESI-MS: m/z 305.0 [M^+] (calculated for [$C_{14}H_{14}BrN_2O^+$] 305.03).

*1-(4-bromomethylbenzyl)-4-hydroxyiminomethylpyridinium bromide (**27**)*. Prepared by method A. The reaction mixture was stopped after 2 h. Yield 3.05 g (97%), TLC R_f 0.60, m.p. 198–201°C. ^1H NMR (300 MHz, DMSO d_6): δ (ppm) 9.18 (d, 2H, J = 6.0 Hz, PyrH), 8.43 (s, 1H, -*CH*=NOH), 8.26 (d, 2H, J = 6.0 Hz, PyrH), 7.52 (d, 4H, J = 1.4 Hz, ArH), 5.86 (s, 2H, $-CH_2-$), 4.71 (s, 2H, $-CH_2-$). ^{13}C NMR (75 MHz, DMSO d_6): δ (ppm) 148.73, 145.03, 145.00, 139.16, 134.24, 129.98, 129.04, 124.36, 62.20, 33.54. Analysis: calculated 43.55% C, 3.65% H, 7.26% N; found 43.61% C, 3.94% H, 7.27% N. ESI-MS: m/z 305.0 [M^+] (calculated for [$C_{14}H_{14}BrN_2O^+$] 305.03).

Biochemistry

In vitro testing of synthesized oximes involved a standard collection of experimental procedures. The 10% rat brain homogenate was used as a source of

Table I. Reactivation potencies of tested oximes (%, mean value of three independent determinations) – time of inhibition – 30 min; time of reactivation by AChE reactivators – 10 min; pH 8; temperature 25°C.

Inhibitor	Reactivation (%)			
	Tabun		Paraoxon	
Reactivator/Concentration	10^{-3} M	10^{-5} M	10^{-3} M	10^{-5} M
pralidoxime (**1**)	4 ± 1	0	42 ± 1	0
HI-6 (**2**)	2 ± 1	4 ± 1	35 ± 2	0
obidoxime (**3**)	11 ± 0	0	76 ± 2	37 ± 2
4	0	0	0	0
5	0	0	0	0
6	0	0	0	0
7	0	0	0	49 ± 4
8	0	0	0	53 ± 3
9	0	0	0	0
10	0	0	0	46 ± 1
11	0	0	0	53 ± 6
12	0	0	0	0
13	0	6 ± 2	0	5 ± 0
14	0	0	3 ± 0	0
15	0	3 ± 1	44 ± 0	17 ± 0
16	0	13 ± 2	12 ± 0	65 ± 0
17	0	0	8 ± 0	6 ± 0
18	0	10 ± 1	7 ± 2	51 ± 1
19	0	19 ± 2	0	63 ± 0
20	0	3 ± 1	0	8 ± 0
21	0	7 ± 1	0	27 ± 1

AChE. The brain homogenate (0.5 mL) was mixed with 20 μL of an isopropanol solution of GA (*O*-ethyl-*N,N*-dimethylphosphoramidocyanidate, obtained from the Military facility Brno, 95% purity) or paraoxon (*O,O*-diethyl-*O*-(4-nitrophenyl)phosphate, analytical standard 99.2% from Sigma-Aldrich) and distilled water (0.5 mL) to achieved 95% inhibition of AChE. The mixture was incubated at 25°C for 30 min. 2.5 mL of a solution of sodium chloride (3 M) was added to the mixture and adjusted 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 (analyzed by potentiometric titration of decomposed acetylcholine iodide) was measured at pH 7.6 and temperature 25°C on an autotitrator RTS 822 (Radiometer, Denmark). The same procedure was repeated with enzyme further subjected to 10 min incubation with an aqueous solution of reactivator (0.2 mL - 10^{-3} M or 10^{-5} M), which replaced 0.2 mL of water. Activities of intact AChE (a_0), inhibited AChE (a_i) and reactivated AChE (a_r) were deduced from the rate of consumption of NaOH solution (0.01 M) with time. The percentage reactivation (%) was calculated from the measured data according to the formula:

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

The whole method is described in detail in the work of Kuca and Cabal [22]. Pralidoxime, HI-6 and obidoxime of HPLC purity, previously synthesized in our laboratory, were used as references. The obtained data are summarized in Table I.

Results and discussion

The reactivation potency of the tested compounds depends not only on the structure of the OP inhibitor [2,14–16] but also on the reactivator's structure. Moreover, a reactivator suitable for *in vivo* experiments should exceed 10% reactivation ability *in vitro* [2]. Therefore, it is extraordinarily difficult to reactivate AChE inhibited by the nerve agent GA [23–25]. The lone electron pair located on the amidic group makes nucleophilic attack almost impossible [23]. In addition, some conformational changes occur in the cavity of the GA-inhibited enzyme [25]. As can be seen from Table I, obidoxime (**3**) is able to satisfactorily reactivate GA-inhibited AChE at 10^{-3} M. However, the concentration 10^{-3} M is not attainable with *in vivo* experiments [26]. A concentration 10^{-5} M is more appropriate and attainable for human use [2]. Only two compounds with a xylene bridge (**16, 19**) have reactivation ability against GA at a concentration 10^{-5} M. None of the non-symmetrical compounds show sufficient reactivation ability against GA-inhibited AChE.

On the other hand, pesticides are known as weaker inhibitors of AChE compared to nerve agents [2,27]. In the past, the design of AChE reactivators was focused on preparation of potent compounds against nerve agents. Formerly, it was determined that commonly used reactivators are not suitable for pesticide intoxications [28–29]. In contrast to this, all reference compounds showed satisfactory ability in reactivation of paraoxon-inhibited AChE at concentration 10^{-3} M, especially obidoxime (**3**). None of eighteen new compounds was able to exceed obidoxime in potency and only one (**15**) gave a result comparable with that of reference substances at 10^{-3} M. At the 10^{-5} M, the situation was changed. Of the reference substances, only obidoxime showed ability to reactivate paraoxon-inhibited AChE. Moreover, seven xylene-linked compounds (**7-8, 10-11, 16, 18-19**) exceeded the potency of obidoxime at 10^{-5} M. In addition, there was the finding of this interesting phenomenon that these compounds showed higher reactivation ability at lower concentration (e.g. **7-8, 10-11, 16, 18-21**). This is probably caused by coincident reactivation and inhibition of the enzyme by the reactivator itself as was described earlier [15–16].

Consequently, we can recommend the structural factors appropriate for reactivation of paraoxon-inhibited AChE by novel compounds [14]. The oxime functional group breaks down the bond OP inhibitor-enzyme and is essential for activity of the reactivator [30–31]. The results confirm that the position of hydroxyiminomethyl groups influences the reactivation potency [30–33]. In this case, compounds bearing oxime groups in positions 3-4 (**6, 9, 12**) and 3-3 (**14, 17, 20**) were almost ineffective, compounds with oxime groups in positions 2-3 (**7, 10**), 2-4 (**8, 11**), 2-2 (**16, 19**) and 4-4 (**15, 18, 21**) showed satisfactory reactivation ability against paraoxon-inhibited AChE. Another important factor for reactivation potency is the structure of the connecting chain as was discussed previously [14–16]. For the xylene-linked compounds, the less sterically limited connecting bridge (*m*- and *p*phenylene; **7-8, 10-11, 16, 18-19**) seemed to be better than the *o*-phenylene chain (**4-5, 13-15**) for paraoxon-inhibited AChE. Some cation-π interaction probably occurs in the cavity of enzyme but more investigations are required to confirm this hypothesis. No less, the quaternary nitrogen is also important for the good affinity of reactivator to the enzyme [34–35]. Additionally, the previously obtained data for the pesticide chlorpyrifos confirm our results for paraoxon-inhibited AChE [16].

In conclusion, nine novel non-symmetrical reactivators with xylene linker were prepared in satisfactory yield and purity. Ability of these nine compounds together with nine symmetrical ones to reactivate GA and paraoxon-inhibited AChE was measured *in vitro.* Seven compounds were found to be promising against paraoxon-inhibited AChE at physiological concentration. Only two symmetrical reactivators exceed the potency of obidoxime against GA. Although the non-symmetrical compounds were less potent than the symmetrical ones, they confirm the dominance of the sterically less *m*- and *p*-xylene connecting chain in reactivation of paraoxon-inhibited AChE.

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