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# Monoquaternary pyridinium salts with modified side chain—synthesis and evaluation on model of tabun- and paraoxon-inhibited acetylcholinesterase

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ABSTRACT

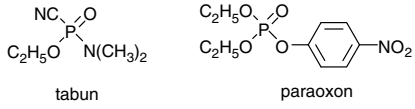
Acetylcholinesterase reactivators are crucial antidotes for the treatment of organophosphate intoxication. Eighteen monoquaternary reactivators of acetylcholinesterase with modified side chain were developed in an effort to extend the properties of pralidoxime. The known reactivators (pralidoxime, HI-6, obidoxime, trimedoxime, methoxime) and the prepared compounds were tested in vitro on a model of tabun- and paraoxon-inhibited AChE. Monoquaternary reactivators were not able to exceed the best known compounds for tabun poisoning, but some of them did show reactivation better or comparable with pralidoxime for paraoxon poisoning. However, extensive differences were found by a SAR study for various side chains on the non-oxime part of the reactivator molecule.



## 1. Introduction

An enzyme acetylcholinesterase (E.C. 3.1.1.7; AChE) plays the essential role in the human neuronal system degrading neurotransmitter acetylcholine within a synaptic cleft. There are many natural or artificial compounds that are able to inhibit AChE activity.[1] The competitive (reversible) AChE inhibitors are used for various purposes such as treatment of Alzheimer's disease or Myasthenia gravis.[2] On the other hand, the irreversible AChE inhibitors such as organophosphate inhibitors (OPI) belong to the most dangerous compounds. The OPI, including pesticides (e.g., parathion, paraoxon, diazinon), nerve agents (e.g., tabun, soman, sarin, VX) or industrial compounds (e.g., tributylphosphate, tri-*O*-cresylphosphate), covalently bind on the AChE active site (Ser200; wild-type *Torpedo californica*) and block its activity (Fig. 1).[3,4] Consequently, the acetylcholine cannot be degraded and it permanently stimulates the cholinergic receptors. The muscarinic (e.g., lacrimation, salivation, miosis) or nicotinic symptoms (e.g., muscle spasms) occur and the organism is endangered by failure of breath centre in central nervous system and subsequent death from suffocation.[4]

The treatment of OPI intoxication usually consists in prophylaxis, symptomatic and causal medication. The persons working or handling with OPI (e.g., farmers, soldiers) may be pre-treated with reversible AChE inhibitors (e.g., pyridostigmine) to block the OPI binding, with other esterases (e.g., butyrylcholinesterase) to scavenge the OPI or with oxime (e.g., HI-6) to reactivate OPI–AChE complex.[4] The symptomatic treatment counteracts the symptoms of intoxication using parasympatolytics such as atropine. The main causal treatment is focused on splitting the covalent bond between the AChE and OPI moiety.[5] The AChE reactivators such as pralidoxime (**1**; 2-hydroxyiminomethyl-1-methylpyridinium chloride), oxime HI-6 (**2**; 1-(2-hydroxyiminomethylpyridinium)-3-(4-carbamoylpyridinium)-2- oxapropane dichloride) obidoxime (**3**; Toxogonine®, 1,3-bis(4-hydroxyiminomethylpyridinium)-2-oxapropane dichloride), trimedoxime (**4**; 1,3-bis(4-hydroxyiminomethylpyridinium)-propane dibromide) or methoxime (**5**; 1,1-bis(4-hydroxyiminomethylpyridinium)-methane dichloride) are used in advantage of nucleophilic hydroxyiminomethyl (oxime) functional group, which is able to cleave the OPI moiety from the active site and restore AChE activity (Fig. 2).[6–8] Unfortunately, there is no sin-




tabun paraoxon

**Figure 1.** Examples of the organophosphorus acetylcholinesterase inhibitors.

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gle reactivator applicable for every type of OPI. Furthermore, the additional anticonvulsive treatment (e.g., diazepam) to counteract muscle seizures and respiratory support is necessary.[4]

After the inhibition of AChE, the process called aging takes place. Besides, the OPI moiety being covalently bound in the active site, some hydrogen bonds are changing and the negative charge occurs due to partial degradation of the OPI–AChE complex. Afterwards, the aged OPI–AChE complex cannot be reactivated by nucleophilic oxime.[9,10]

In this paper, we present detailed results on the design, synthesis and in vitro evaluation of several monoquaternary pyridinium reactivators. These reactivators extend the properties of pralidoxime against OPI poisoning.

## 2. Design and synthesis monoquaternary pyridinium reactivators

The monoquaternary pyridinium salts were developed in this paper. Some of them were also published previously.[11–13] Although the monoquaternary compounds are known as weaker AChE reactivators compared to the bisquaternary ones, many countries (including U.S.) are still equipped by the monoquaternary pralidoxime (**1**).[14,15] Namely, the pralidoxime (**1**) is often used against pesticide poisoning, although it and also bisquaternary reactivators (mostly developed against nerve agents) are known as not convenient for pesticides.[16,17]

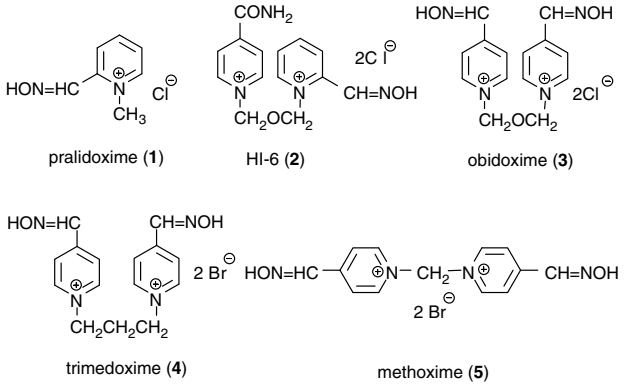
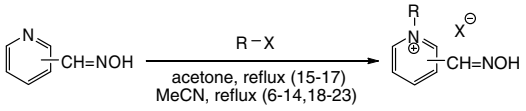



**Figure 2.** Commercial acetylcholinesterase reactivators.

The main aim of this paper was to extend the properties of pralidoxime (**1**) with its modification. Besides the essential oxime functional group, there is possibility to improve pralidoxime (**1**) or its analogues (**7–8**) via change of its side chain.[11] The various aliphatic and aromatic side chains were used in effort to improve its reactivation ability (Fig. 3). The side chains were used as improvement of lipophilicity of **1** (Fig. 3; **9–23**).[18] Notably, **1** was found to be more lipophilic compared to bisquaternary compounds and it is penetrating through blood–brain barrier in 10%.[19] Besides its low reactivation ability, it is still able to reactivate AChE in the brain tissue, where the bisquaternary compounds are only penetrating in maximum of 6%, but with better reactivation ability.[20]

Moreover, the allyl and aryl–alkyl side chains were designed not only for the lipophilicity increase, but they were chosen for the presence of π-electrons (Fig. 3; **12–23**) too.[21–24] The π-electrons are hoped to interact with the AChE active site via π–π or cation–π interactions. Furthermore, the various positions of oxime functional group on pyridinium ring were used in effort to determine the best structural features for reactivation of OP pesticides, where only few data were published.[25–27]

The monoquaternary reactivators (**6–23**) were prepared via standard synthetic strategy.[28,29] The hydroxyiminomethylpyridine (0.5 g; 4.1 mmol) was dissolved in acetone or MeCN (10 mL), and the corresponding alkylating agent (6.1 mmol) was added. The mixture was heated under reflux for several hours (6.5–48), terminated and allowed to cool at room temperature. The crystalline crude product was collected by filtration under reduced pressure and recrystallized from MeCN. The purity of all compounds was determined by NMR, ESI–MS and elemental analysis.




| Compound | R | X | Oxime position | Reaction time | Yield (%) |
|---|---|---|---|---|---|
| **6** | $CH_3$- | I | 2-CH=NOH | 6.5 | 69 |
| **7** | $CH_3$- | I | 3-CH=NOH | 6.5 | 94 |
| **8** | $CH_3$- | I | 4-CH=NOH | 6.5 | 83 |
| **9** | $CH_3(CH_2)_2$- | Br | 2-CH=NOH | 16 | 10 |
| **10** | $CH_3(CH_2)_2$- | Br | 3-CH=NOH | 8 | 45 |
| **11** | $CH_3(CH_2)_2$- | Br | 4-CH=NOH | 6.5 | 7 |
| **12** | $CH_2$=CH-$CH_2$- | Br | 2-CH=NOH | 10 | 70 |
| **13** | $CH_2$=CH-$CH_2$- | Br | 3-CH=NOH | 9 | 90 |
| **14** | $CH_2$=CH-$CH_2$- | Br | 4-CH=NOH | 8 | 77 |
| **15** | benzyl | Br | 2-CH=NOH | 14 | 56 |
| **16** | benzyl | Br | 3-CH=NOH | 7.5 | 43 |
| **17** | benzyl | Br | 4-CH=NOH | 7 | 61 |
| **18** | 2-phenylethyl | Br | 2-CH=NOH | 10 | 6 |
| **19** | 2-phenylethyl | Br | 3-CH=NOH | 10 | 72 |
| **20** | 2-phenylethyl | Br | 4-CH=NOH | 9 | 66 |
| **21** | 3-phenylpropyl | Br | 2-CH=NOH | 48 | 8 |
| **22** | 3-phenylpropyl | Br | 3-CH=NOH | 10 | 83 |
| **23** | 3-phenylpropyl | Br | 4-CH=NOH | 9 | 86 |

**Figure 3.** Monoquaternary compounds with modified side chain.

## 3. AChE reactivation results and SAR discussion

The prepared monoquaternary reactivators (**6–23**) and previously known compounds (**1–5**) were assayed for their reactivation potency using a rat brain homogenate inhibited by tabun (GA) and the pesticide paraoxon (POX).[30] The reactivation results are shown in Table 1.

A reactivation in vitro should exceed 10% to suggest a promising compound warranting further testing.[31] Noticeably, not all tested compounds were able to fulfil this criterion for GA-inhibited AChE. The commercially available reactivators pralidoxime (**1**), oxime HI-6 (**2**) and methoxime (**5**) are known to be not suitable for treatment of GA intoxication.[31–33] Although obidoxime (**3**) and trimedoxime (**4**) are the most potent commercially available compounds against GA, they have increased toxicity compared with **1–2** or **5**.[34] Since the maximal attainable plasma concentration in humans is $10^{-4}$ M, **3** is therefore the best compound amongst the previously known reactivators.[35] Additionally, the prepared compounds did not reach the in vitro limit for reactivation of GA-inhibited AChE with exception of **12**. Moreover, compound **12** had only borderline reactivation ability at concentration $10^{-3}$ M, which is not convenient for in vivo use.

The reactivation did change for POX-inhibited AChE. The commercial compounds **1–2** had half the reactivation capability when compared to **3** at $10^{-3}$ M and no reactivation at $10^{-5}$ M. Compound **5** showed comparable reactivation with **3** at $10^{-3}$ M; however, no reactivation at $10^{-5}$ M. Trimedoxime (**4**) resulted as the best at $10^{-5}$ M and consequently it was the best compound amongst the previously known reactivators against POX-inhibited AChE. The prepared compounds (**9**,**12**) showed promising reactivation at $10^{-3}$ M compared to pralidoxime (**1**) or its analogue (**6**), whereas they were less effective than commercial bisquaternary compounds (**3–4**). For concentration attainable in vivo ($10^{-5}$ M), the prepared compounds had minimal or no reactivation capability.

Besides the poor reactivation ability of prepared compounds compared to commercial reactivators, the interesting structure–activity dependence was found.[27] The main structural features, which influence the reactivation potency, are the oxime functional group (its position and amount), the connecting or side linker, amount of quaternary heteroaromatic rings and other functional groups on the second heteroaromatic ring for bisquaternary reactivators.[36,37] For GA-inhibited AChE, at least one oxime in position four on the heteroaromatic ring is necessary for substantial reactivation (**3–4**), whilst an oxime in position two has a low or no reactivation capability (**1–2**, **12**). Additionally, the optimal linker length suitable for GA intoxication varies from three to four carbon–carbon bonds (**3–4** compared to **5**). Two quaternary heteroaromatic rings are beneficial for reactivation of GA (**3–4** compared to **1**). Although the influence of other functional group is not apparent in this study, it was previously confirmed.[34]

For POX-inhibited AChE, at least one oxime in position four on the heteroaromatic ring was supposed to be necessary (**3–4**).[27,34] However, the oxime in position two was found to be effective amongst the prepared compounds in case of POX (**9**, **12**, **18**, **20**). This difference may be explained by structure of monoquaternary reactivators based on less bulky and less fixed molecule compared to bisquaternary ones. Furthermore, the connecting linker between two heteroaromatic rings with three to four carbon–carbon bonds showed the best results (**3–4**). Nevertheless, the side chain in monoquaternary compounds had extensive impact on the reactivation capability. Namely, the prolongation of aliphatic chain increased the reactivation ability (**9** compared to **1** or **6**). Interestingly, the use of π-electrons (allyl; **12** compared to **9**) slightly decreased the ability, whereas the benzyl showed complete loss of activity.[11] The 2-phenylethyl and 3-phenylpropyl decreased ability two times (**18**, **21** compared to **9**). The mentioned differences presumably consist in bulky side chain and distance of π-electrons from oxime functional group, which is influencing the interactions within the enzyme active site. Two quaternary heteroaromatic rings are beneficial for reactivation of POX (**3–4** compared to **1**), although one heteroaromatic ring with modified side chain may increase the reactivation ability (**9** compared to **1** or **6**), too. The influence of other functional group to the POX-inhibited AChE was previously confirmed.[27]

**Table 1**
Reactivation potencies of tested oximes (%, mean value of three independent determinations ±SD)

| Inhibitor | Reactivation ±SD (%) | | | |
|---|---|---|---|---|
| | 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**) | 37 ± 1 | 28 ± 2 | 76 ± 2 | 37 ± 2 |
| Trimedoxime (**4**) | 30 ± 0 | 0 | 46 ± 1 | 50 ± 4 |
| Methoxime (**5**) | 0 | 0 | 71 ± 3 | 0 |
| **6** | 4 ± 1 | 0 | 42 ± 1 | 0 |
| **7** | 0 | 0 | 1 ± 0 | 0 |
| **8** | 1 ± 0 | 1 ± 0 | 16 ± 0 | 3 ± 1 |
| **9** | 6 ± 1 | 0 | 52 ± 1 | 0 |
| **10** | 1 ± 0 | 1 ± 0 | 0 | 0 |
| **11** | 0 | 0 | 22 ± 0 | 0 |
| **12** | 9 ± 1 | 0 | 38 ± 0 | 0 |
| **13** | 1 ± 0 | 0 | 0 | 0 |
| **14** | 3 ± 0 | 0 | 12 ± 0 | 0 |
| **15** | 6 ± 0 | 0 | 0 | 0 |
| **16** | 0 | 2 ± 0 | 0 | 0 |
| **17** | 0 | 0 | 0 | 0 |
| **18** | 0 | 4 ± 0 | 16 ± 0 | 9 ± 0 |
| **19** | 0 | 0 | 7 ± 1 | 3 ± 0 |
| **20** | 4 ± 0 | 1 ± 0 | 2 ± 0 | 0 |
| **21** | 1 ± 0 | 2 ± 0 | 20 ± 1 | 5 ± 1 |
| **22** | 0 | 4 ± 0 | 0 | 4 ± 2 |
| **23** | 0 | 2 ± 0 | 7 ± 2 | 4 ± 1 |

## 4. Conclusions

The monoquaternary reactivators of AChE were developed in an effort to extend the properties of pralidoxime. Their reactivation was tested on the model of tabun- and paraoxon-inhibited AChE in vitro. Although the monoquaternary compounds were weak reactivators of tabun compared to known oximes (trimedoxime, obidoxime), two of them showed reactivation of paraoxon better or comparable to pralidoxime. The SAR results showed interesting differences between reactivation of tabun and paraoxon. Notably, the oxime functional group in position two was found to be effective in case of paraoxon poisoning. In addition, the modified side chain had extensive impact on reactivation capability of monoquaternary compounds. These preliminary in vitro findings suggest an apparent influence of weak interactions within the AChE active site for the main reactivation process and suggest further necessary studies (lipophilicity, X-ray, docking studies).

## 5. Experimental

### 5.1. Chemical preparation

Solvents (acetone, DMF, MeCN) 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, detection by 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 A: 850 mg

$Bi(NO_3)_3$, 40 mL $H_2O$, 10 mL $CH_3COOH$; fraction B: 8 g KI, 20 mL $H_2O$). Melting points were measured on micro heating stage PHMK 05 (VEB Kombinat Nagema, Radebeul, Germany) and were uncorrected.

NMR spectra were generally recorded at Varian Gemini 300 ($^1H$ 300 MHz, $^{13}C$ 75 MHz, Palo Alto CA, USA). In all cases, the chemical shift values for $^1H$ spectra are reported in ppm ($\delta$) relative to residual $CHD_2SO_2CD_3$ ($\delta$ 2.50) or $D_2O$ ($\delta$ 4.79), shift values for $^{13}C$ spectra are reported in ppm ($\delta$) relative to solvent peak dimethylsulfoxide-$d_6$ $\delta$ 39.43. Signals are quoted as s (singlet), d (doublet), t (triplet) and m (multiplet).

Mass spectra were recorded using 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 mass spectrometer was supplied by Whatman 75-720 nitrogen generator. Data were collected in positive ion mode with an ESI probe voltage of 4000 V. The pressure of nebulizer gas was set up to 35 psig. Drying gas temperature was operated at 335 °C and flow at 13 L/min.

### 5.2. Prepared monoquaternary salts

#### 5.2.1. 2-Hydroxyiminomethyl-1-methylpyridinium-iodide (6)

TLC $R_f$ 0.37. M.p. 233–236 °C. $^1H$ NMR, $^{13}C$ NMR and ESI–MS spectra are consistent with the literature data.[26] EA: calculated 31.84% C, 3.44% H, 10.61% N; found 31.97% C, 3.49%H, 10.78% N.

#### 5.2.2. 3-Hydroxyiminomethyl-1-methylpyridinium-iodide (7)

TLC $R_f$ 0.36. M.p. 133–137 °C. $^1H$ NMR, $^{13}C$ NMR and ESI–MS spectra are consistent with the literature data.[26] EA: calculated 31.84% C, 3.44% H, 10.61% N; found 31.24% C, 3.61%H, 10.76% N.

#### 5.2.3. 4-Hydroxyiminomethyl-1-methylpyridinium-iodide (8)

TLC $R_f$ 0.41. M.p. 178–181 °C. $^1H$ NMR, $^{13}C$ NMR and ESI–MS spectra are consistent with the literature data.[26] EA: calculated 31.84% C, 3.44% H, 10.61% N; found 31.89% C, 3.41%H, 10.59% N.

#### 5.2.4. 2-Hydroxyiminomethyl-1-propylpyridinium-bromide (9)

TLC $R_f$ 0.56. M.p. 184–190 °C. $^1H$ NMR (300 MHz, DMSO $d_6$): $\delta$ 9.07 (d, 1H, $J$ = 6.2 Hz, H-6), 8.81 (s, 1H, —*CH*═NOH), 8.61–8.53 (m, 1H, H-5), 8.41 (d, 1H, $J$ = 8.2 Hz, H-3), 8.16–8.07 (m, 1H, H-4), 4.71 (t, 2H, $J$ = 7.3 Hz, N–*CH*$_2$–), 1.90–1.73 (m, 2H, –*CH*$_2$–CH$_3$), 0.92 (t, 3H, $J$ = 7.3 Hz, –CH$_2$–*CH*$_3$). $^{13}C$ NMR (75 MHz, DMSO $d_6$): $\delta$ 146.70, 145.83, 145.11, 141.42, 127.35, 125.75, 59.01, 23.74, 10.16. EA: calculated 44.10% C, 5.35% H, 11.43% N; found 44.51% C, 5.37%H, 11.60% N. ESI–MS: $m/z$ 165.0 $[M]^+$ (calculated for $[C_9H_{13}N_2O]^+$ 165.10).

#### 5.2.5. 3-Hydroxyiminomethyl-1-propylpyridinium-bromide (10)

TLC $R_f$ 0.53. M.p. 193–196 °C. $^1H$ NMR (300 MHz, DMSO $d_6$): $\delta$ 9.28 (s, 1H, H-2), 9.07 (d, 1H, $J$ = 6.2 Hz, H-6), 8.73 (d, 1H, $J$ = 8.2 Hz, H-4), 8.35 (s, 1H, —*CH*═NOH), 8.22–8.14 (m, 1H, H-5), 4.60 (t, 2H, $J$ = 7.3 Hz, N–*CH*$_2$–), 2.04–1.87 (m, 2H, –*CH*$_2$–CH$_3$), 0.89 (t, 3H, $J$ = 7.3 Hz, –CH$_2$–*CH*$_3$). $^{13}C$ NMR (75 MHz, DMSO $d_6$): $\delta$ 144.26, 143.20, 142.38, 141.32, 133.32, 128.01, 62.25, 23.94, 10.14. EA: calculated 44.10% C, 5.35% H, 11.43% N; found 44.01% C, 4.97%H, 11.79% N. ESI–MS: $m/z$ 165.0 $[M]^+$ (calculated for $[C_9H_{13}N_2O]^+$ 165.10).

#### 5.2.6. 4-Hydroxyiminomethyl-1-propylpyridinium-bromide (11)

TLC $R_f$ 0.57. M.p. 135–137 °C. $^1H$ NMR (300 MHz, DMSO $d_6$): $\delta$ 9.04 (d, 2H, $J$ = 6.2 Hz, H-2,6), 8.44 (s, 1H, —*CH*═NOH), 8.24 (d, 2H, $J$ = 6.2 Hz, H-3,5), 4.54 (t, 2H, $J$ = 7.3 Hz, N–*CH*$_2$–), 2.00–1.85 (m, 2H, –*CH*$_2$–CH$_3$), 0.88 (t, 3H, $J$ = 7.3 Hz, –CH$_2$–*CH*$_3$). $^{13}C$ NMR (75 MHz, DMSO $d_6$): $\delta$ 150.05, 146.54, 123.96, 120.50, 61.53, 23.95, 10.15. EA: calculated 44.10% C, 5.35% H, 11.43% N; found 44.31% C, 4.73%H, 11.59% N. ESI–MS: $m/z$ 165.0 $[M–H^+]^{2+}$ (calculated for $[C_9H_{13}N_2O]^+$ 165.10).

#### 5.2.7. 1-Allyl-2-hydroxyiminomethylpyridinium-bromide (12)

TLC $R_f$ 0.43. M.p. 185–188 °C. $^1H$ NMR (300 MHz, DMSO $d_6$): $\delta$ 9.11 (d, 1H, $J$ = 6.2 Hz, H-6), 8.67 (s, 1H, —*CH*═NOH), 8.65–8.57 (m, 1H, H-5), 8.44 (d, 1H, $J$ = 8.2 Hz, H-3), 6.20-6.04 (m, 1H, –*CH*═CH$_2$), 5.50 (d, 2H, $J$ = 5.0 Hz, N–*CH*$_2$–), 5.38 (d, 1H, $J$ = 10.5 Hz, –CH═*CH*$_2$), 5.12 (d, 1H, $J$ = 17.0 Hz, –CH═*CH*$_2$). $^{13}C$ NMR (75 MHz, DMSO $d_6$): $\delta$ 147.04, 145.98, 145.62, 141.39, 131.72, 127.70, 125.54, 119.67, 59.47. EA: calculated 44.47% C, 4.56% H, 11.52% N; found 43.68% C, 4.72%H, 11.44% N. ESI–MS: $m/z$ 163.1 $[M]^+$ (calculated for $[C_9H_{11}N_2O]^+$ 163.09).

#### 5.2.8. 1-Allyl-3-hydroxyiminomethylpyridinium-bromide (13)

TLC $R_f$ 0.44. M.p. 163–165 °C. $^1H$ NMR (300 MHz, DMSO $d_6$): $\delta$ 9.29 (s, 1H, H-2), 9.07 (d, 1H, $J$ = 6.2 Hz, H-6), 8.77 (d, 1H, $J$ = 8.2 Hz, H-4), 8.39 (s, 1H, —*CH*═NOH), 8.25–8.17 (m, 1H, H-5), 6.27–6.11 (m, 1H, –*CH*═CH$_2$), 5.53–5.41 (m, 2H, N–*CH*$_2$–), 5.35 (d, 2H, $J$ = 6.15). $^{13}C$ NMR (75 MHz, DMSO $d_6$): $\delta$ 144.33, 143.19, 142.28, 141.77, 133.43, 131.40, 128.19, 122.20, 62.50. EA: calculated 44.47% C, 4.56% H, 11.52% N; found 44.54% C, 4.81%H, 11.80% N. ESI–MS: $m/z$ 163.1 $[M]^+$ (calculated for $[C_9H_{11}N_2O]^+$ 163.09).

#### 5.2.9. 1-Allyl-4-hydroxyiminomethylpyridinium-bromide (14)

TLC $R_f$ 0.49. M.p. 189–190 °C. $^1H$ NMR (300 MHz, DMSO $d_6$): $\delta$ 9.05 (d, 2H, $J$ = 6.0 Hz, H-2,6), 8.46 (s, 1H, —*CH*═NOH), 8.27 (d, 2H, $J$ = 6.0 Hz, H-3,5), 6.28–6.03 (m, 1H, CH$_2$═*CH*–CH$_2$–), 5.53–5.19 (m, 4H, *CH*$_2$═CH–*CH*$_2$–). $^{13}C$ NMR (75 MHz, DMSO $d_6$): $\delta$ 148.57, 145.01, 144.94, 131.59, 124.07, 121.77, 61.72. EA: calculated 44.47% C, 4.56% H, 11.52% N; found 44.22% C, 4.81% H, 11.76% N. ESI–MS: $m/z$ 163.1 $[M]^+$ (calculated for $[C_9H_{11}N_2O]^+$ 163.09).

#### 5.2.10. 1-Benzyl-2-hydroxyiminomethylpyridinium-bromide (15)

TLC $R_f$ 0.66. M.p. 217–219 °C. $^1H$ NMR (300 MHz, DMSO $d_6$): $\delta$ 9.29 (d, 1H, $J$ = 6.0 Hz, H-6), 8.75 (s, 1H, —*CH*═NOH), 8.70–8.60 (m, 1H, H-5), 8.43 (d, 1H, $J$ = 8.1 Hz, H-3), 8.26–8.17 (m, 1H, H-4), 7.48-7.33 (m, 3H, Ph), 7.31–7.21 (m, 2H, Ph), 6.15 (s, 2H, –*CH*$_2$–). $^{13}C$ NMR (75 MHz, DMSO $d_6$): $\delta$ 147.09, 146.37, 145.88, 141.36, 133.83, 129.12, 128.79, 127.82, 127.21, 126.00, 60.21. EA: calculated 53.26% C, 4.47% H, 9.56% N; found 53.07% C, 4.51% H, 9.69% N. ESI–MS: $m/z$ 213.1 $[M]^+$ (calculated for $[C_{13}H_{13}N_2O]^+$ 213.10).

#### 5.2.11. 1-Benzyl-3-hydroxyiminomethylpyridinium-bromide (16)

TLC $R_f$ 0.67. M.p. 170–173 °C. $^1H$ NMR (300 MHz, DMSO $d_6$): $\delta$ 9.46 (s, 1H, H-2), 9.24 (d, 1H, $J$ = 6.0 Hz, H-6), 8.75 (d, 1H, $J$ = 8.1 Hz, H-4), 8.38 (s, 1H, —*CH*═NOH), 8.25–8.16 (m, 1H, H-5), 7.65–7.55 (m, 2H, Ph), 7.50–7.38 (m, 3H, Ph), 5.94 (s, 2H, –*CH*$_2$–). $^{13}C$ NMR (75 MHz, DMSO $d_6$): $\delta$ 144.34, 143.19, 142.21, 141.91, 133.96, 133.68, 129.33, 129.12, 128.87, 128.43, 63.28. EA: calculated 53.26% C, 4.47 % H, 9.56% N; found 53.22% C, 4.50% H, 9.67% N. ESI–MS: $m/z$ 213.1 $[M]^+$ (calculated for $[C_{13}H_{13}N_2O]^+$ 213.10).

#### 5.2.12. 1-Benzyl-4-hydroxyiminomethylpyridinium-bromide (17)

TLC $R_f$ 0.71. M.p. 191–193 °C. $^1H$ NMR (300 MHz, DMSO $d_6$): $\delta$ 9.20 (d, 2H, $J$ = 6.0 Hz, H-2,6), 8.43 (s, 1H, —*CH*═NOH), 8.26 (d,

2H, $J$ = 6.0 Hz, H-3,5), 7.61–7.51 (m, 2H, Ph), 7.50–7.38 (m, 3H, Ph), 5.88 (s, 2H, –$CH_2$–). $^{13}$C NMR (75 MHz, DMSO $d_6$): $\delta$ 148.69, 145.01, 144.94, 134.22, 129.25, 129.13, 128.74, 124.32, 62.58. EA: calculated 53.26% C, 4.47% H, 9.56% N; found 52.70% C, 4.86% H, 9.21% N. ESI–MS: $m/z$ 213.1 $[M]^+$ (calculated for $[C_{13}H_{13}N_2O]^+$ 213.10).

### 5.2.13. 1-(2-Phenylethyl)-2-hydroxyiminomethylpyridinium-bromide (18)

TLC $R_f$ 0.66. M.p. 164–166 °C. $^1$H NMR (300 MHz, DMSO $d_6$): $\delta$ 8.90 (d, 1H, $J$ = 6.0 Hz, H-6), 8.73 (s, 1H, —$CH$═NOH), 8.59–8.48 (m, 1H, H-5), 8.36 (d, 1H, $J$ = 8.1 Hz, H-3), 8.10–7.96 (m, 1H, H-4), 7.39–7.10 (m, 5H, Ph), 5.04 (t, 2H, $J$ = 7.3 Hz, N–$CH_2$–), 3.17 (t, 2H, $J$ = 7.3 Hz, Ph–$CH_2$–). $^{13}$C NMR (75 MHz, DMSO $d_6$): $\delta$ 146.82, 145.95, 145.28, 141.37, 135.69, 128.91, 128.52, 127.12, 125.82, 58.46. EA: calculated 54.74% C, 4.92% H, 9.12% N; found 54.14% C, 4.98% H, 9.21% N. ESI–MS: $m/z$ 227.1 $[M]^+$ (calculated for $[C_{14}H_{15}N_2O]^+$ 227.12).

### 5.2.14. 1-(2-Phenylethyl)-3-hydroxyiminomethylpyridinium-bromide (19)

TLC $R_f$ 0.63. M.p. 215–216 °C. $^1$H NMR (300 MHz, DMSO $d_6$): $\delta$ 9.30 (s, 1H, H-2), 9.04 (d, 1H, $J$ = 6.2 Hz, H-6), 8.71 (d, 1H, $J$ = 8.2 Hz, H-4), 8.32 (s, 1H, —$CH$═NOH), 8.17–8.09 (m, 1H, H-5), 7.35–7.20 (m, 5H, Ph), 4.91 (t, 2H, $J$ = 7.3 Hz, N–$CH_2$–), 3.30 (t, 2H, $J$ = 7.3 Hz, Ph–$CH_2$–). $^{13}$C NMR (75 MHz, DMSO $d_6$): $\delta$ 144.28, 143.11, 142.39, 141.55, 136.00, 133.16, 128.84, 128.53, 127.83, 127.00, 61.62, 36.26. EA: calculated 54.74% C, 4.92% H, 9.12% N; found 55.30% C, 5.19% H, 9.44% N. ESI–MS: $m/z$ 227.1 $[M]^+$ (calculated for $[C_{14}H_{15}N_2O]^+$ 227.12).

### 5.2.15. 1-(2-Phenylethyl)-4-hydroxyiminomethylpyridinium-bromide (20)

TLC $R_f$ 0.67. M.p. 220–222 °C. $^1$H NMR (300 MHz, DMSO $d_6$): $\delta$ 9.03 (d, 2H, $J$ = 6.2 Hz, H-2,6), 8.42 (s, 1H, —$CH$═NOH), 8.20 (d, 2H, $J$ = 6.2 Hz, H-3,5), 7.34–7.20 (m, 5H, Ph), 4.86 (t, 2H, $J$ = 7.3 Hz, N–$CH_2$–), 3.27 (t, 2H, $J$ = 7.3 Hz, Ph–$CH_2$–). $^{13}$C NMR (75 MHz, DMSO $d_6$): $\delta$ 148.33, 144.98, 144.90, 136.05, 128.82, 128.53, 126.98, 123.74, 60.88, 36.28. EA: calculated 54.74% C, 4.92% H, 9.12% N; found 54.71% C, 5.19% H, 9.33% N. ESI–MS: $m/z$ 227.1 $[M]^+$ (calculated for $[C_{14}H_{15}N_2O]^+$ 227.12).

### 5.2.16. 1-(3-Phenylpropyl)-2-hydroxyiminomethylpyridinium-bromide (21)

TLC $R_f$ 0.73. M.p. 129–131 °C. $^1$H NMR (300 MHz, DMSO $d_6$): $\delta$ 9.10 (d, 1H, $J$ = 6.0 Hz, H-6), 8.76 (s, 1H, —$CH$═NOH), 8.59–8.50 (m, 1H, H-5), 8.38 (d, 1H, $J$ = 8.1 Hz, H-3), 8.13–8.04 (m, 1H, H-4), 7.33–7.15 (m, 5H, Ph), 4.78 (t, 2H, $J$ = 7.3 Hz, N–$CH_2$–), 2.71 (t, 2H, $J$ = 7.3 Hz, N–$CH_2$–$CH_2$–), 2.20–2.04 (m, 2H, Ph–$CH_2$–). $^{13}$C NMR (75 MHz, DMSO $d_6$): $\delta$ 146.76, 145.92, 145.08, 141.39, 140.27, 128.35, 128.11, 127.37, 126.07, 125.73, 57.59, 31.77, 31.35. EA: calculated 56.09% C, 5.33% H, 8.72% N; found 55.84% C, 5.43% H, 8.83% N. ESI–MS: $m/z$ 241.1 $[M]^+$ (calculated for $[C_{15}H_{17}N_2O]^+$ 241.13).

### 5.2.17. 1-(3-Phenylpropyl)-3-hydroxyiminomethylpyridinium-bromide (22)

TLC $R_f$ 0.71. M.p. 143–145 °C. $^1$H NMR (300 MHz, DMSO $d_6$): $\delta$ 9.33 (s, 1H, H-2), 9.13 (d, 1H, $J$ = 6.0 Hz, H-6), 8.72 (d, 1H, $J$ = 8.1 Hz, H-4), 8.35 (s, 1H, —$CH$═NOH), 8.19–8.11 (m, 1H, H-5), 7.32–7.13 (m, 5H, Ph), 4.71 (t, 2H, $J$ = 7.3 Hz, N–$CH_2$–), 2.67 (t, 2H, $J$ = 7.3 Hz, N–$CH_2$–$CH_2$–), 2.37–2.21 (m, 2H, Ph–$CH_2$–). $^{13}$C NMR (75 MHz, DMSO $d_6$): $\delta$ 144.37, 143.18, 142.60, 141.18, 140.21, 133.28, 128.30, 128.13, 127.96, 126.02, 60.79, 31.82, 31.49. EA: calculated 56.09% C, 5.33% H, 8.72% N; found 54.52% C, 4.98% H, 8.52% N. ESI–MS: $m/z$ 241.1 $[M]^+$ (calculated for $[C_{15}H_{17}N_2O]^+$ 241.13).

### 5.2.18. 1-(3-Phenylpropyl)-4-hydroxyiminomethylpyridinium-bromide (23)

TLC $R_f$ 0.73. M.p. 161–163 °C. $^1$H NMR (300 MHz, DMSO $d_6$): $\delta$ 9.10 (d, 2H, $J$ = 6.0 Hz, H-2,6), 8.45 (s, 1H, —$CH$═NOH), 8.22 (d, 1H, $J$ = 6.0 Hz, H-3,5), 7.32–7.12 (m, 5H, Ph), 4.65 (t, 2H, $J$ = 7.3 Hz, N–$CH_2$–), 2.66 (t, 2H, $J$ = 7.3 Hz, N–$CH_2$–$CH_2$–), 2.33-2.17 (m, 2H, Ph–$CH_2$–). $^{13}$C NMR (75 MHz, DMSO $d_6$): $\delta$ 148.22, 145.00, 144.95, 140.22, 128.32, 128.14, 126.01, 123.90, 60.02, 31.88, 31.47. EA: calculated 56.09% C, 5.33% H, 8.72% N; found 56.66% C, 5.94% H, 9.04% N. ESI–MS: $m/z$ 241.1 $[M]^+$ (calculated for $[C_{15}H_{17}N_2O]^+$ 241.13).

## 5.3. In vitro assay

In vitro testing of reactivators has been described in detail.[28] Briefly, the 10% rat brain homogenate in distilled water was used as a source of AChE. The brain homogenate (0.5 mL) was mixed with 20 μL of isopropanol solution of tabun (tabun, *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). The mixture was incubated at 25 °C for 30 min to achieve 95% inhibition of AChE. The mixture was filled in assay vessel to the volume 23 mL with distilled water and sodium chloride (3 M; 2.5 mL) was added. Finally, 2 mL of acetylcholine iodide (0.02 M; substrate for enzymatic reaction) was added. The enzyme activity (analyzed by potentiometric titration of decomposed acetylcholine iodide) was measured at pH 7.6 and 25 °C using an autotitrator Titrando 842 (Metrohm, Switzerland).

The same procedure was repeated with rat brain homogenate, which was 30 min incubated with tabun or paraoxon and further treated for 10 min with an aqueous solution of reactivator (0.2 mL – replacing same amount of distilled water). Activities of intact AChE ($a_0$), inhibited AChE ($a_i$) and reactivated AChE ($a_r$) were deduced from the amount of NaOH solution (0.01 M) versus time; NaOH reacted with acetic acid released from decomposed acetylcholine iodide. The percentage of reactivation (%) was calculated from the measured data according to the formula

$$x = \left(1 - \frac{a_0 - a_r}{a_0 - a_i}\right) \times 100 \ (\%).$$

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