New Methods in Synthesis of Acetylcholinesterase Reactivators and Evaluation of their Potency to Reactivate Cyclosarin-Inhibited AChE

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Received 7 March 2005; Revised 30 June 200; Accepted 7 July 2005

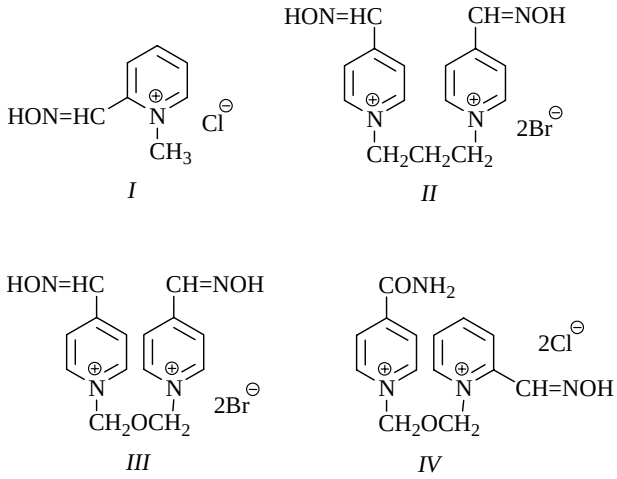
Nine potential AChE reactivators were synthesized using modification of currently known synthetic pathways. Their potency to reactivate AChE inhibited by cyclosarin nerve agent was tested *in vitro*. According to the previous results, 1,4-bis(2-hydroxyiminomethylpyridinium)butane dibromide seems to be the most potent AChE reactivator. The reactivation potency of these compounds depends on structural factors such as presence of quaternary nitrogens, length of the linking chain between both pyridinium rings, and position of the oxime moiety at the pyridinium ring.

Highly toxic organophosphorus compounds (OP) considered as chemical warfare agents (sarin, soman, tabun, cyclosarin or VX) belong to irreversible inhibitors of acetylcholinesterase (AChE; EC 3.1.1.7) [1, 2], an enzyme playing an important role in cholinergic transmission in the nervous system [3]. Therefore, its inhibition is life-limiting factor. Inhibitory effect is based on phosphorylation or phosphonylation of serine hydroxy group at the esteric site of the active center of the enzyme (Scheme 1) [4].

For the treatment of toxic effects of these agents, parasympatolytics such as atropine and OP-inhibited AChE reactivators are commonly used [5, 6]. Monoquaternary pralidoxime (*I*; 2-PAM, 2-hydroxyiminomethyl-1-methylpyridinium chloride) [5—7] or more extended bisquaternary compounds, such as trimedoxime (*II*; TMB-4, 1,3-bis(4-hydroxyiminomethylpyridinio-1-yl)propane dibromide) [8], obidoxime (*III*; toxogonine®, 1,3-bis(4-hydroxyiminomethylpyridinio-1-yl)-2-oxapropane dibromide) [9], and H-oxime HI-6 (*IV*; 1-(2-hydroxyiminomethylpyridinio-1-yl)-3-(4-carbamoylpyridinio-1-yl)-2-oxapropane dichloride)[10, 11] belong to the fundamental representatives of these aldoximes.

Our research was focused on finding of new efficient methods in synthesis of AChE reactivators *V*—*VII* in three series (see Schemes 2 and 3).

Afterwards, their ability to reactivate AChE inhibited by nerve agent cyclosarin (*O*-cyclohexylmethylfluorophosphonate), which is considered to be one of the most toxic nerve agents used for military purposes, was tested [12].



A new synthetic pathway was used for the preparation of monoquaternary pyridinium salts *Va*—*Vc* in refluxing acetone (Scheme 2). The purity of these is high (in the range from 98 to 99 %) and the yields are acceptable for laboratory preparation. Unfortunately, their resulting values are lower than in [13].

For bispyridinium salts *VIa*—*VIc*, *VIIa*—*VIIc* (Scheme 3), an older published system [14] was applied using DMF and differs in temperature (100 °C). Bet-

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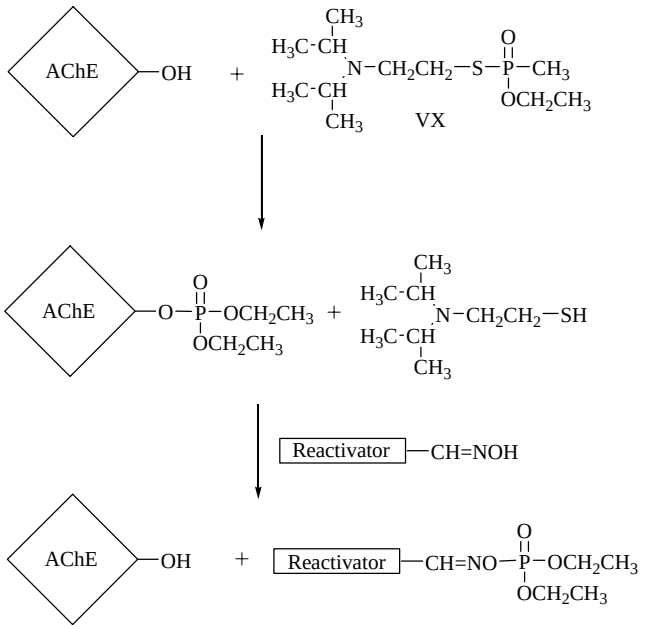
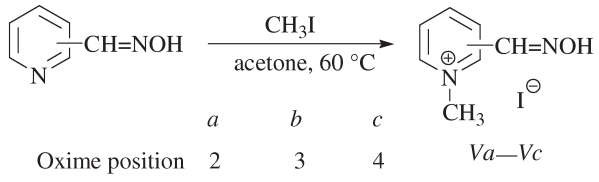


Scheme 1. Inhibition and reactivation of AChE.



Scheme 2. Synthesis of the monoquaternary oximes.

Table 1. Reactivation Potency/%[a] of Synthesized Oximes

Compound	$c/(10^{-5}$ mol dm$^{-3})$	$c/(10^{-3}$ mol dm$^{-3})$
Va	0	0
Vb	0	6
Vc	0	3
VIa	0	1
VIb	2	2
VIc	0	0
VIIa	8	7
VIIb	0	0
VIIc	0	2

a) Mean value of two or three independent determinations. Time of inhibition by cyclosarin – 30 min; time of reactivation by AChE reactivators – 10 min; pH 8; temperature 25 °C.

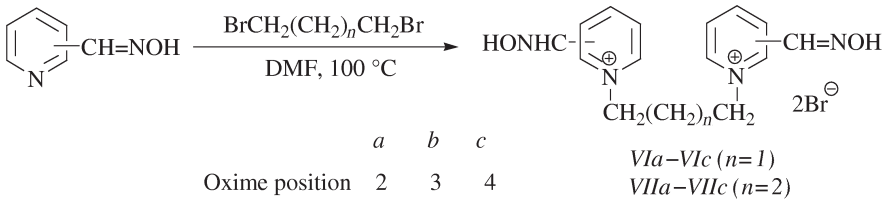
ter or comparable yields were obtained in shorter time period (*i.e. VIc* – 80 %, 6 h; *VIIc* – 80 %, 5 h) than in articles [7, 15] (*VIc* – 88 %, 48 h; *VIIc* – 81 %, 16 h), except the 2-hydroxyiminomethylpyridinium derivatives [15]. Previously not reported ^{13}C NMR and MS spectra were measured.

Reactivation potencies of all synthesized oximes are shown in Table 1. As it can be seen, bisquaternary oxime *VIIa* is the most potent AChE reactivator at the concentration 10^{-3} mol dm^{-3}. However, this concentration is too high and owing to this fact not applicable for human use [1, 16]. At the lower concentration 10^{-5} mol dm^{-3} (probably attainable concentration *in vivo*), only oxime *VIIa* achieved reactivation potency higher than 5 %. It is generally known that increasing of reactivation potency to 5—10 % is sufficient for survival [1]. On the other hand, currently there are many AChE reactivators able to better reactivate AChE inhibited by cyclosarin [5]. The reactivation insufficiency of monoquaternary oximes *Va*—*Vc* is probably caused by the absence of the second pyridinium ring.

This work confirms the fact that reactivation potency of AChE reactivators depends also on the position of oxime group [17]. Measured results, in the case of compound *VIIa*, partially confirm this rule. On the other hand, compounds *Va* and *VIa* are not as sufficient as it was supposed. Length of the connecting chain between both pyridinium rings is another important structural factor [14, 18]. In this case, as it can be seen, compound *VIIa* with the long four-carbon connecting chain seems to be the most potent AChE reactivator.

Although these compounds were not extraordinarily potent reactivators of AChE inhibited by cyclosarin, they could be effective for reactivation of AChE inhibited by other nerve agents or pesticides, because reactivation potency of AChE reactivators depends on the nerve agent used [5, 6, 16].

At the preparation of potential AChE reactivators we investigated structural factors such as presence of quaternary nitrogens, length of the linking chain between both pyridinium rings, and position of the functional oxime group at the pyridinium ring.



Scheme 3. Synthesis of the bisquaternary oximes.

EXPERIMENTAL

Solvents (acetone, diethyl ether, DMF) and reagents were purchased from Fluka and Aldrich and used without further purification. Reactions were monitored by TLC using DC-Alufolien Cellulose F (Merck, Germany) and elution by BuOH—CH_3COOH—H_2O (φ_r = 5 : 1 : 2), detection by solution of Dragendorf agent (solution containing 10 cm^3 of CH_3COOH, 50 cm^3 of H_2O, and 5 cm^3 of basic solution prepared by mixing of two fractions – fraction I: 850 mg of $Bi(NO_3)_3$, 40 cm^3 of H_2O, 10 cm^3 of CH_3COOH; fraction II: 8 g of KI, 20 cm^3 of H_2O). Temperature data were measured on Micro heating stage PHMK 05 (VEB Kombinat Nagema, Radebeul, Germany).

NMR spectra were generally recorded at Varian Gemini 300 instrument (^1H 300 MHz, ^{13}C 75 MHz, Palo Alto, CA, USA). In all cases the chemical shift values for ^1H spectra are reported in δ relative to residual $CHD_2SO_2CD_3$ (δ = 2.50), shift values for ^{13}C spectra are reported in δ relative to solvent peak of dimethyl sulfoxide-d_6 (δ = 39.43).

MS spectra were recorded using the combination of high-performance liquid chromatography and mass spectrometry. Liquid chromatograph HP1100 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 238.7 kPa. Drying gas temperature was 335 °C and flow 13 dm^3 min^{-1}.

Quaternary Salts

Two synthetic pathways were used for the preparation of mono- (method *A*) and bisquaternary (method *B*) aldoximes:

A. A solution of the hydroxyiminomethylpyridine (2.0 g; 16.4 mmol) and methyl iodide (1.6 cm^3, 24.6 mmol) in acetone (100 cm^3) was stirred (4—4.5 h) at 60 °C. The reaction mixture was cooled to room temperature; the crystalline crude product was collected by filtration and washed with ether (2 × 30 cm^3).

B. A solution of the hydroxyiminomethylpyridine (1 g; 8.2 mmol) and dibromopropane or dibromobutane (0.43 cm^3, 3.7 mmol) in DMF (10 cm^3) was stirred (4—6 h) at 100 °C. The reaction mixture was cooled to room temperature and portioned with acetone (30 cm^3); a precipitate was filtered and washed with acetone (2 × 20 cm^3).

1-Methyl-2-(hydroxyiminomethyl)pyridinium Iodide (Va)

Method A. Yield = 1.38 g (32 %) as yellow crystals, R_f = 0.6, m.p. = 231 °C (reported 224—225 °C [19]). ^1H NMR spectrum is consistent with literature data [13]. ^{13}C NMR spectrum is consistent with literature data of nitrate derivative [20]. ESI-MS: *m/z* 137.1 $[M]^+$ (for $[C_7H_9N_2O]^+$ calculated 137.07).

1-Methyl-3-(hydroxyiminomethyl)pyridinium Iodide (Vb)

Method A. Yield = 3.00 g (69 %) as yellow crystals, R_f = 0.6, m.p. = 154—157 °C (reported 154—155 °C [19]). ^1H NMR spectrum is consistent with literature data [13]. ^{13}C NMR spectrum (DMSO-d_6), δ: 145.06, 143.22, 143.10, 141.09, 132.86, 127.66, 48.17. ESI-MS: *m/z* 137.1 $[M]^+$ (for $[C_7H_9N_2O]^+$ calculated 137.07).

1-Methyl-4-(hydroxyiminomethyl)pyridinium Iodide (Vc)

Method A. Yield = 3.01 g (70 %) as yellow crystals, R_f = 0.6, m.p. = 178—180 °C (reported 181—183 °C [19]). ^1H NMR spectrum is consistent with literature data [13]. ^{13}C NMR spectrum is consistent with literature data [20]. ESI-MS: *m/z* 137.1 $[M]^+$ (for $[C_7H_9N_2O]^+$ calculated 137.07).

1,3-Bis(2-hydroxyiminomethylpyridinio-1-yl)propane Dibromide (VIa)

Compound was prepared in our laboratory earlier [14]. ^{13}C NMR spectrum is consistent with literature data of chloride derivative [15]. ESI-MS: *m/z* 285.1 $[M^{2+} - H^+]^+$ (for $[C_{15}H_{18}N_4O_2^{2+} - H^+]^+$ calculated 285.14).

1,3-Bis(3-hydroxyiminomethylpyridinio-1-yl)propane Dibromide (VIb)

Method B. Yield = 1.38 g (84 %) as a white powder, R_f = 0.15, m.p. = 231—233 °C (reported 230—231 °C [21]). ^{1}H NMR spectrum is consistent with literature data [21]. ^{13}C NMR spectrum (DMSO-d_6), δ: 144.52, 143.18, 142.70, 141.67, 133.37, 128.16, 57.60, 31.48. ESI-MS: *m/z* 285.1 $[M^{2+} - H^+]^+$ (for $[C_{15}H_{18}N_4O_2^{2+} - H^+]^+$ calculated 285.14).

1,3-Bis(4-hydroxyiminomethylpyridinio-1-yl)propane Dibromide (VIc)

Method B. Yield = 1.32 g (80 %) as a white powder, R_f = 0.15, m.p. = 233—235 °C (reported 238—241 °C [8]). ^{1}H NMR and ^{13}C NMR spectra are consistent with literature data [15]. ESI-MS: *m/z* 285.1 $[M^{2+} - H^+]^+$ (for $[C_{15}H_{18}N_4O_2^{2+} - H^+]^+$ calculated 285.14).

1,4-Bis(2-hydroxyiminomethylpyridinio-1-yl)-butane Dibromide (VIIa)

Compound was prepared in our laboratory earlier [14]. ^{13}C NMR spectrum is consistent with literature data of chloride derivative [15]. ESI-MS: *m/z* 299.1 $[M^{2+} - H^+]^+$ (for $[C_{16}H_{20}N_4O_2^{2+} - H^+]^{2+}$ calculated 299.16).

1,4-Bis(3-hydroxyiminomethylpyridinio-1-yl)-butane Dibromide (VIIb)

Method B. Yield = 1.47 g (87 %) as a white powder, R_f = 0.15, m.p. = 248—250 °C ([22]). ^{1}H NMR spectrum (DMSO-d_6), δ: 12.22 (s, 2H), 9.43 (s, 2H), 9.21 (d, 2H, J = 6.0 Hz), 8.77 (d, 2H, J = 8.0 Hz), 8.38 (s, 2H), 8.17—8.27 (m, 2H), 4.85 (t, 4H, J = 7.1 Hz), 2.62—2.82 (m, 2H). ^{13}C NMR spectrum (DMSO-d_6), δ: 144.41, 143.26, 142.53, 141.50, 133.39, 128.12, 60.03, 26.94. ESI-MS: *m/z* 299.1 $[M^{2+} - H^+]^+$ (for $[C_{16}H_{20}N_4O_2^{2+} - H^+]^{2+}$ calculated 299.16).

1,4-Bis(4-hydroxyiminomethylpyridinio-1-yl)-butane Dibromide (VIIc)

Method *B.* Yield = 1.36 g (80 %) as a white powder, R_f = 0.15, m.p. = 239—241 °C (reported 239—241 °C [8]). ^{1}H NMR and ^{13}C NMR spectra are consistent with literature data [15]. ESI-MS: *m/z* 299.1 $[M^{2+} - H^+]^+$ (for $[C_{16}H_{20}N_4O_2^{2+} - H^+]^{2+}$ calculated 299.16).

Reactivation Potency

In vitro testing of synthesized oximes involved a standard collection of experimental procedures. The whole method is in detail described in [16]. The reactivating efficacy of oximes was evaluated in 10 % rat brain homogenate that was incubated with cyclosarin (*O*-cyclohexylmethylfluorophosphonate) for 30 min and then the tested oxime of appropriate concentrations (10^{-4} mol dm^{-3} and 10^{-2} mol dm^{-3}) was added for 10 min. Afterwards, the activity of brain AChE was measured by the potentiostatic method with the usage of automatic titrator RTS 822 (Radiometer, Denmark). The data about initial rate of enzyme reaction with substrate made possible the calculation of the percentage of increase in the activity of reactivated enzyme in the reaction mixture.

Acknowledgements. The authors express their appreciation to Mrs. M. Hrabinová for her technical assistance. The study was supported by the Grant of the Grant Agency of Charles University No. 302/2005/B-CH/FaF and by the Grant of the Ministry of Defense No. ONVLAJEP 20031.

REFERENCES

1. Bajgar, J., *Adv. Clin. Chem. 38*, 151 (2004).
2. Marrs, T. C., *Pharmacol. Ther. 58*, 51 (1993).
3. Patočka, J., Kuča, K., and Jun, D., *Acta Med.* (Hradec Králové) *47*, 215 (2004).
4. Kuča, K., Bielavský, J., Cabal, J., and Kassa, J., *Bioorg. Med. Chem. Lett. 13*, 3545 (2003).
5. Kassa, J., *J. Toxicol., Clin. Toxicol. 40*, 803 (2002).
6. Kuča, K., Bielavský, J., Cabal, J., and Bielavská, M., *Tetrahedron Lett. 44*, 3123 (2003).
7. Wilson, I. B., Ginsburg, S., and Meilisch, E. K., *J. Am. Chem. Soc. 77*, 4286 (1955).
8. Poziomek, E. J., Hackley, B. E., and Steinberg, G. M., *J. Org. Chem. 23*, 714 (1958).
9. Krejčová, G. and Kassa, J., *Toxicology 185*, 129 (2003).
10. Rousseaux, C. G. and Gua, A. K., *Can. J. Physiol. Pharmacol. 67*, 1183 (1989).
11. Kassa, J., Cabal, J., Bajgar, J., and Szinicz, L., *ASA Newslett. 97-4*, 16 (1997).
12. Krejčová, G., Ševelová, L., and Kuča, K., *Zprav. Voj. Farm. 14*, 5 (2004).
13. Hampl, F., Mazač, J., Liška, F., Šrogl, J., Kábrt, L., and Suchánek, M., *Collect. Czech. Chem. Commun. 60*, 883 (1995).
14. Kuča, K., Cabal, J., Patočka, J., and Kassa, J., *Lett. Org. Chem. 1*, 84 (2004).
15. Pang, Y. P., Kollmeyer, T. M., Hong, F., Lee, J. C., Hammond, P. I., Haugabouk, S. P., and Brimijoin, S., *Chem. Biol. 10*, 491 (2003).
16. Kuča, K. and Kassa, J., *J. Enzyme Inhib. Med. Chem. 18*, 529 (2003).
17. Kuča, K. and Patočka, J., *J. Enzyme Inhib. Med. Chem. 19*, 39 (2004).
18. Kuča, K., Patočka, J., and Cabal, J., *J. Appl. Biomed. 1*, 207 (2003).
19. Ginsburg, S. and Wilson, I. B., *J. Am. Chem. Soc. 79*, 481 (1957).
20. Waysbort, D., Balderman, D., and Amitai, G., *Org. Magn. Reson. 16*, 7 (1981).
21. Sikder, A. K., Ghosh, A. K., and Jaiswal, D. K., *J. Pharm. Sci. 82*, 258 (1993).
22. Substance was found at CAS database without references.