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Design and synthesis of new bis-pyridinium oxime reactivators for acetylcholinesterase inhibited by organophosphorous nerve agents

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Abstract—New bis-pyridinium oxime reactivators connected with a $CH_2CH_2OCH_2CH_2$ linker between two pyridinium rings were designed and synthesized. In the test of their potency to reactivate AChE inhibited by cyclosarin, the bis-pyridinium oxime **6b** achieved reactivation potency higher than 10% at the lower concentration 10^{-4} M.

1. Introduction

Organophosphorus nerve agents such as tabun (GA), sarin (GB), soman (GD), cyclosarin (GF), and VX are compounds that exert their biological effects by inhibition of cholinesterases (ChE), in particular, acetylcholinesterase (AChE).[1] Thus, the inhibition of AChE increases the amount of acetylcholine (ACh) at central and peripheral sites of the nerve system. After the organophosphorus compounds attach to AChE to inhibit it, AChE may be spontaneously reactivated by hydrolytic cleavage of the organophosphoryl–AChE bond, or the dealkylation of the organophosphoryl–AChE complex may proceed. This is the process known as aging that makes reactivation of AChE activity by any current reactivator no longer possible.[2] Because the nerve agents differ in structures, their rates of spontaneous reactivation and aging differ. For example, the soman–enzyme complex does not spontaneously reactivate; the half-life for aging is about 2 min.[3] The half-life for aging of the sarin–enzyme complex is about 5 h, and a small percentage (5%) of the enzyme undergoes spontaneous reactivation.[4] Therefore, soman is probably the most dangerous organophosphorus agent among the known organophosphorus nerve agents. Cyclosarin is also a highly toxic nerve agent, which is resistant to conventional reactivators because of the bulky cyclohexyl moiety.[5]

In the middle of 1950s, Wilson[6] reported that hydroxylamine reactivated organophosphoryl-inhibited ChE faster than water did, and later reported that an oxime, 2-PAM, was far more effective than hydroxylamine in reactivating the enzyme.[7] After thorough study of many of these compounds, bis-pyridinium oximes such as TMB4,[8] Toxogonin,[9] and HI-6[10] have been developed and are used currently in many countries (Fig. 1). Among these oxime reactivators, HI-6 is the most effective against nerve agent-inhibited ChE. The structural characteristics of HI-6 compared with other pyridinium oximes can be described in several points; (1)

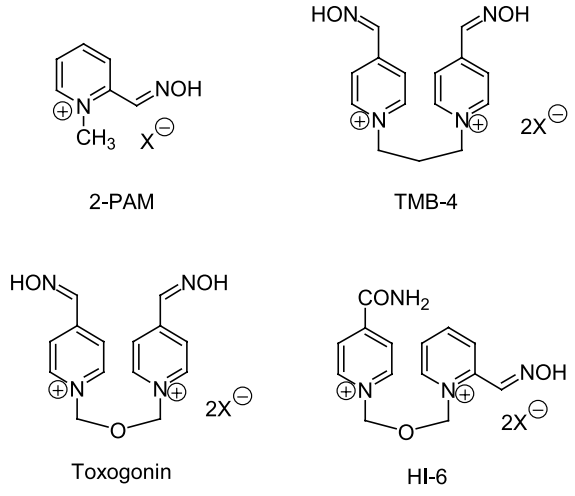

Figure 1. Structures of currently used AChE reactivators.

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bis-pyridinium oxime HI-6 versus mono-pyridinium oxime 2-PAM, (2) CH_2OCH_2 chain between pyridinium rings in HI-6 versus $CH_2CH_2CH_2$ chain in TMB4, (3) 4-carbamoylpyridinium in HI-6 versus 4-(hydroxyiminomethyl)pyridinium in Toxogonin. These speculations prompted us to design new oximes in which a longer ether linker was introduced to connect two pyridine rings. Moreover, a recent study suggested that the reactivation activity is dependent on the number of the methylene groups between the two quaternary nitrogens of bis-pyridinium oximes.[5,11] In order to develop new bis-pyridinium oxime reactivators for the *ChE* inhibited by organophosphorus nerve agents, we newly designed and synthesized bis-pyridinium oxime reactivators, involving a $CH_2CH_2OCH_2CH_2$ linker instead of a CH_2OCH_2 chain or polymethylene chains. Afterwards, we have tested their potency to reactivate AChE inhibited by suitable nerve agent representative, cyclosarin.

2. Chemistry

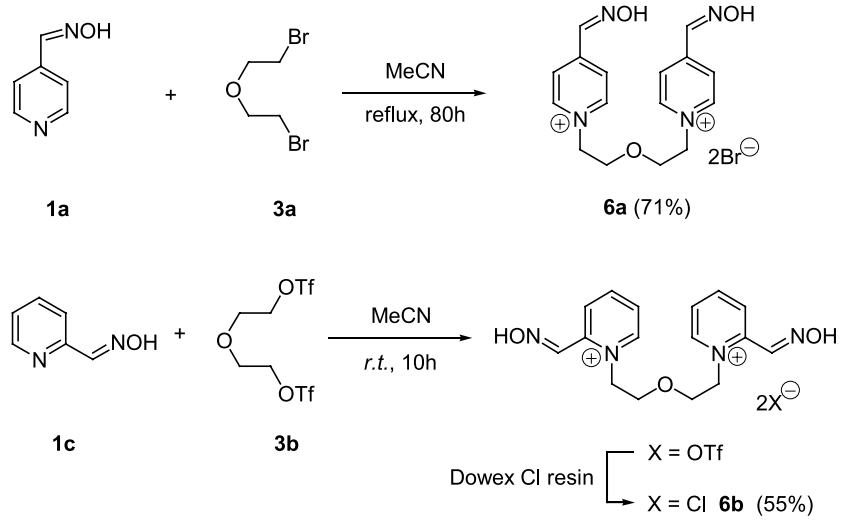
Bis-4-pyridiniumaldoxime **6a** connected with a $CH_2CH_2OCH_2CH_2$ linker between the two quaternary nitrogens was obtained from the alkylation of the corresponding aldoxime **1a** with 2-bromoethylether **3a** under the reflux condition of MeCN (Scheme 1). However, bis-2-pyridiniumaldoxime **6b** was not obtained by the above method, therefore bistriflate **3b** as an alkylating reagent was used in MeCN at room temperature. The resulting triflate salt was subjected to ion-exchange chromatography on Dowex–Cl resin to provide **6b**.[12] Next, we tried to prepare bis-pyridinium oximes from pyridiniumaldoxime and isonicotinamide, connected with a $CH_2CH_2OCH_2CH_2$ linker. There are two possible methods of synthesizing the bis-pyridinium oximes. According to previous reports,[13] the reaction of isonicotinamide **2** with bromide **3a** followed by treatment with aldoxime **1** should be a more efficient method than the reaction of aldoxime **1** with bromide **3a** followed by treatment with isonicotinamide **2**. Therefore, isonicotinamide **2** was treated with bromide **3a** in MeCN at 65–70 °C to give mono-pyridinium bromide **4** in 71% yield (Scheme 2). However, the reaction of isonicotinamide **2** and bromide **3a** in the reflux condition of MeCN provided bis-4-isonicotinamide **5** instead of mono-pyridinium bromide **4** as a major product, and 81% yield of **5** was obtained when 2 equiv of the isonicotinamide **2** was reacted with **3a** in MeCN for 80 h. Bromide **4** was heated with aldoxime **1a** in MeCN to give bis-pyridinium **6c** in 72% yield, and bis-pyridinium **6d** was obtained from bromide **4** and aldoxime **1b** in 76% yield. The newly synthesized bis-pyridinium oximes **6** involving a $CH_2CH_2OCH_2CH_2$ linker were identified by their ^1H NMR spectra.[14]

3. In vitro experiment

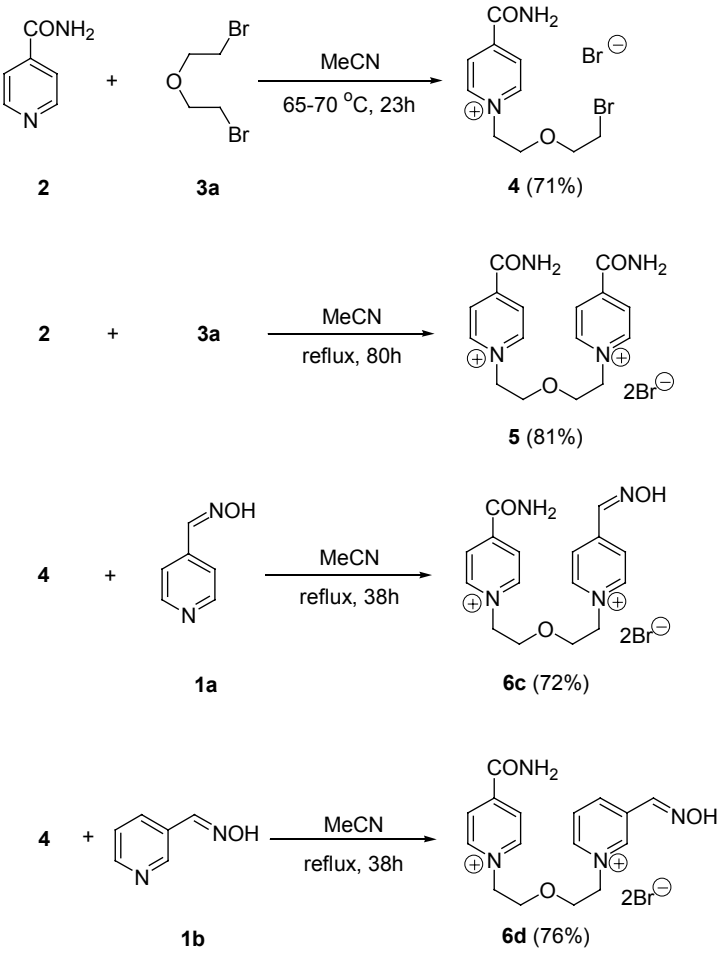
In vitro testing of synthesized oximes involved a standard collection of experimental procedures. The whole method is in detail described in the work of Kuca and Kassa.[15] The reactivating efficacy of oximes was evaluated in 10% rat brain homogenate that was incubated with cyclosarin (*O*-cyclohexylmethylfluorophosphonate) for 30 min and then, the tested oxime of appropriate concentrations (10^{-4} and 10^{-2} M) was added for 10 min. The activity of brain AChE was measured by 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 percentage of increase in the activity of reactivated enzyme in the reaction mixture.

4. Results and discussion

We have compared reactivation potency of newly synthesized oximes with two commercially available AChE reactivators—pralidoxime and obidoxime. As it is shown in Figure 2, both commercially available AChE reactivators are able to reactivate cyclosarin-inhibited



Scheme 1.



Scheme 2.

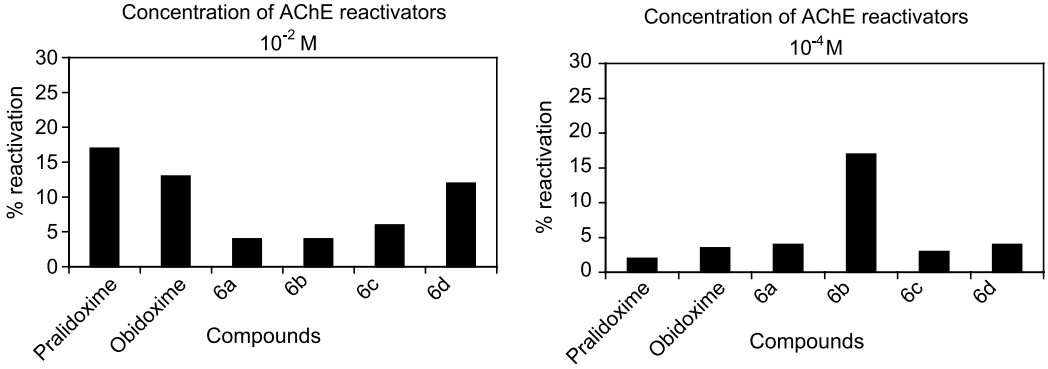


Figure 2. Reactivation potency of newly synthesized compounds **6a**–**d**.

AChE at high concentrations (10^{-2} M). Unfortunately, their potency at the concentration 10^{-4} M is low. Due to this fact, oxime **6b** is the most potent AChE reactivator from the group of tested oximes. On the other hand, currently there are many AChE reactivators better able to reactivate AChE inhibited by cyclosarin.[16] For example, HI-6 is currently the most potent cyclosarin-inhibited AChE reactivator. It is able to reactivate cyclosarin-inhibited AChE to 95% at the concentration 10^{-4} M. On the other hand, its reactivation potency at the concentration at the concentration 10^{-2} M is 6%. This fact is caused by the inhibition of AChE by high doses of HI-6.[17]

Our work confirms the fact that compounds with oxime group at the position 2 on the pyridinium ring are the most potent reactivators of cyclosarin-inhibited AChE.[18] 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.[16,19]

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14. Compound **6a**: mp 195–198 °C; ^{1}H NMR (500 MHz, D_2O): δ 4.03 (t, J = 5.0 Hz, 4H, $2CH_2$), 4.78 (t, J = 5.0 Hz, 4H, $2CH_2$), 8.11 (d, J = 6.8 Hz, 4H, ArH), 8.36 (s, 2H, N=CH), 8.71 (d, J = 6.8 Hz, 4H, ArH). Compound **6b**: mp 210–212 °C; ^{1}H NMR (500 MHz, D_2O): δ 4.02 (t, J = 4.9 Hz, 4H, $2CH_2$), 4.92 (t, J = 4.9 Hz, 4H, $2CH_2$), 8.02 (td, J = 6.9, 1.3 Hz, 2H, 2ArH), 8.40 (dd, J = 8.2, 1.1 Hz, 2H, ArH), 8.56 (t, J = 7.8 Hz, 2H, ArH), 8.64 (s, 2H, N=CH), 8.72 (d, J = 6.2 Hz, 2H, ArH). Compound **6c**: mp 224–227 °C; ^{1}H NMR (500 MHz, D_2O): δ 4.03 (t, J = 4.9 Hz, 2H, CH_2), 4.06 (t, J = 4.8 Hz, 2H, CH_2), 4.78 (t, J = 4.9 Hz, 2H, CH_2), 4.89 (t, J = 4.8 Hz, 2H, CH_2), 8.14 (d, J = 6.6 Hz, 2H, ArH), 8.31 (d, J = 6.5 Hz, 2H, ArH), 8.36 (s, 1H, N=CH), 8.73 (d, J = 6.7 Hz, 2H, ArH), 8.96 (d, J = 6.6 Hz, 2H, ArH). Compound **6d**: mp 185–187 °C; ^{1}H NMR (500 MHz, D_2O): δ 4.05–4.08 (m, 4H, $2CH_2$), 4.84–4.91 (m, 4H, $2CH_2$), 8.09 (dd, J = 8.0, 6.1 Hz, 1H, ArH), 8.32 (d, J = 6.9 Hz, 2H, ArH), 8.33 (s, 1H, N=CH), 8.71 (d, J = 8.0 Hz, 1H, ArH), 8.80 (d, J = 6.1 Hz, 1H, ArH), 8.97 (d, J = 6.9 Hz, 2H, ArH), 9.00 (s, 1H, ArH).
15. *Reactivation assay*: reactivation efficacies of the oximes were tested in vitro on the model of AChE inhibited by cyclosarin using standard reactivation test with electrometric instrumentation.[17] Rat brain homogenate (10%) was used as the suitable source of the enzyme. The homogenate (0.5 mL) was mixed with 0.5 mL of 0.01 μM cyclosarin in dry isopropanol and incubated for 30 min (25 °C). Then 2.5 mL of 3 M NaCl was added and supplied by distilled water to a volume of 23 mL. After that, 2 mL of 0.02 M acetylcholine bromide was added and enzyme activity was assayed titrimetrically at pH 8.0 and 25 °C on the Autotitrator RTS 822 (Radiometer, Denmark). The activities of intact (a_0) and GF-inhibited (a_i) AChE were determined. Afterwards, GF-inhibited AChE was incubated 10 min with solution of reactivator (10^{-4} and 10^{-2} M concentration), the activity of reactivated AChE (a_r) was obtained. The activity values a_0, a_i, and a_r were calculated from the slopes of the initial part of the titration curves. Each value represents arithmetic mean from three independent measurements.
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