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Short communication

In-vitro regeneration of sarin inhibited electric eel acetylcholinesterase by bis-pyridinium oximes bearing xylene linker

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Abstract

A series of bis-pyridinium oximes connected by xylene linker were synthesized and their in-vitro reactivation potential was evaluated against acetylcholinesterase (AChE) inhibited by nerve agent, sarin. Among the synthesized compounds, α,α′xylene-bis-[3,3′-(hydroxyiminomethyl) pyridinium] dibromide (**3b**) was found to be most potent reactivator for AChE inhibited by sarin. The oxime **3b** exhibits 34% regeneration of inhibited AChE, in comparison to 20 and 15% regeneration by 2-PAM and obidoxime, respectively, at a concentration of 10^{-4} M within 10 min.

Keywords: Bis-pyridinium oximes; Reactivators; 2-PAM; Organophosphorus nerve agents; Sarin; Acetylcholinesterase; Xylene linkers

1. Introduction

Some of the most deadly compounds ever known to man were developed in the past for use as chemical warfare agents [1]. Phosphate esters and related Phosphorous (V) compounds viz. isopropyl methylphosphonofluoridate (sarin), pinacolyl methylphosphonofluoridate (soman), *O*-ethyl,*N*,*N*-dimethyl-phosphoramidocyanidate (tabun) and *O*-ethyl-*S*-(2-diisopropy-laminoethyl) methylphosphonothioate (VX) are classified as nerve agents. In spite of world wide efforts to prevent synthesis, storage and use of these compounds, the repeated use of chemical warfare agents during military conflicts [2] and terrorist attacks [3] displays that they constitute a persistent threat for the population.

Hence, efforts are on to develop an effective medical treatment regimen of OP poisoning. The intoxication with nerve agents leads to inhibition of acetylcholinesterase by phosphorylation of their active site serine residue [4]. The subsequent accumulation of the neurotransmitter acetylcholine and over-stimulation of cholinergic receptors results in a generalized cholinergic crisis including breakdown of neuromuscular function.

The standard treatment of nerve agent poisoning includes a muscarine antagonist, e.g., atropine, and a reactivator of OP-inhibited AChE [5]. The presently available reactivators are oximes such as pralidoxime (2-PAM) and obidoxime (Fig. 1). These compounds are well tried as antidotes against OP poisoning but are considered to be rather ineffective against certain nerve agents [6,7]. Numerous new oximes have been synthesized and tested in the last decades [8–10]. The efficacy of oximes depends on post-inhibitory reactions such as spontaneous dealkylation (aging) [11] and spontaneous dephosphorylation (spontaneous reactivation) [12] of the OP-AChE complex. However, these organophosphorous nerve agents behave differently towards reactivation due to their broad structure variability and most often their deleterious side effects become difficult to counteract [13]. Furthermore, an effective therapy by a single oxime to all the known nerve agents is still lacking.

Abbreviations: AChE,; acetylcholinesterase; 2-PAM,; 2-(hydroxyimino-methyl)-1-methylpyridinium chloride.

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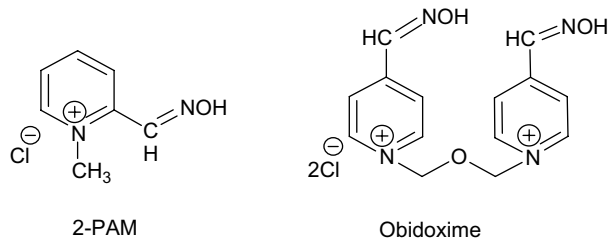


Fig. 1. Oximes used as reactivatores of inhibited AChE.

2. Chemistry

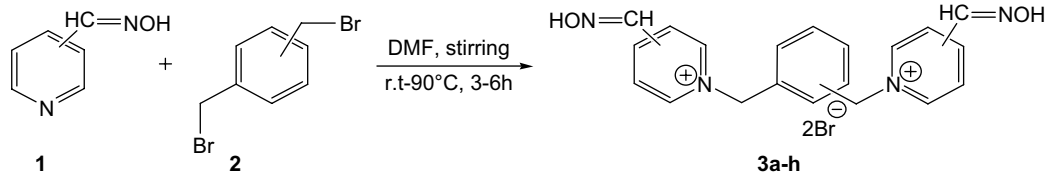
Recently, several bis-pyridinium aldoximes linked by a variable length alkylene chain were reported as AChE reactivators [14,15]. Some of such oximes have shown promising reactivation profile against OP-inhibited AChE. Reactivation potential of such oximes attracted our attention to further explore the synthesis of bis-pyridinium oximes with different kind of linkers. In the past, reactive oxime moiety has been incorporated into the varieties of micelle forming molecules, resulting in significant enhancement of hydrolysis of OP compounds [16,17]. In this context, we focused our attention on symmetrical bis-pyridinium oximes analogous of TMB-4 and obidoxime class of compounds having a xylene linker between two pyridinium rings with an aim to enhance the lipid solubility and stability, which is found to be low with the currently available antidotes [18] as well as to allow the hydrophobic interactions with some of the aromatic residues that are present in the active site gorge of the enzyme acetylcholinesterase [19].

Herein, we report the synthesis and in-vitro reactivation studies of a series of pyridinium oximes linked with a xylene bridge. By adopting a simple and mild synthetic method [20], we have synthesized a series of bis-pyridinium oximes. The reaction involved alkylations of isomeric pyridinealdoxime (**1**) with α,α' dibromoxylene (**2**) (Scheme 1). The method afforded the desired products (**3a–h**) with good yields within 3–6 h (Table 1). Purity of the synthesized compounds was checked by thin-layer chromatography (TLC, cellulose, DSO, Fluka) with 1-butanol:acetic acid:water (3:1:1) as mobile phase. Melting points were determined using IA9200 Electrothermal, U.K., melting point apparatus and are uncorrected. Elemental analysis was conducted on a NOD 1106 Carlo Erba Instrumentazione analyser and is within ±0.4% of the calculated values (Table 1).

The structure of the compounds was confirmed by their spectral data. Infra-red (IR) spectra was obtained as KBr discs on a Bruker TENSOR-27 FTIR spectrophotometer. IR absorption in the range of 3393–3342 cm^{-1} was due to oximino OH group. The other characteristic absorptions were at 3025–3035 cm^{-1} for C–H str (aryl) and at 2988–2965 cm^{-1} for C–H str (aliphatic). The characteristic absorptions for oximes were quite distinct and appeared at 1642–1600 cm^{-1} and 1010–990 cm^{-1}. ^1H NMR (DMSO-d_6) spectra was recorded on Bruker Avance 400 spectrometer at 400 MHz using tetramethylsilane as internal standard and expressed in the δ (ppm) values. The OH protons appeared as a singlet in the range of δ 12.41–13.01 and were exchangeable with D_2O. Signals at δ 5.9–6.0 were due to N–CH_2 protons. Pyridine and N=CH protons appear in the range of δ 8.2–9.1. ^{13}C NMR (DMSO-d_6) chemical shifts values were obtained using the same instrument at 100 MHz (Table 2). The methylene carbon attached to pyridinium nitrogen (N–CH_2) appeared in the regions of δ 57.8–62.9. Aromatic carbons of phenyl ring absorbed in the range of δ124.5–138.5 and carbons on pyridinum rings appeared in the range of 129.2–148.9 δ. The oximino carbon (CH=N) appeared at δ 144.50–150.8. The detailed spectral data are presented in Table 2.

3. Results and discussion

The in-vitro reactivation of sarin inhibited AChE by bis-pyridinium oximes bearing xylene bridge at three different concentrations (10^{-4} M, 5×10^{-5} M and 10^{-5} M) is depicted in Fig. 2. It is evident from these data that best reactivation was observed with *N*,*N*′-*p*-xylene-bis[(3,3′-hydroxyiminomethyl)-pyridinium] dibromide (**3b**) and *N*,*N*′-*m*-xylene-bis[(3,3′-hydroxyiminomethyl)-pyridinium] dibromide (**3e**). The oximes, **3b** and **3e**, respectively, reactivated 35 and 25% of sarin inhibited eel AChE in comparison to, respectively, 21 and 15% reactivation by 2-PAM and obidoxime at the concentration 10^{-4} M after 10 min. It may also be noted that the reactivation potential of two other oximes of the series i.e., *N*,*N*′-*p*-xylene-bis[(2,2′-hydroxyiminomethyl)-pyridinium] dibromide (**3c**) and *N*,*N*′-*o*-xylene-bis[(3-hydroxyiminomethyl)-pyridinium] dibromide (**3g**) was found to be at par with that of 2-PAM. At the lower concentration of 10^{-5} M (probably attainable concentration *in vivo*), the oximes **3b** and **3e**, respectively, exhibited reactivation potency of 20 and 10% in comparison to 7% reactivation exhibited by 2-PAM within 10 min. It is generally known that increasing of reactivation potency to 5–10% is sufficient for survival [9]. This suggests that these oximes may be applicable for *in vivo* experiments in animal models.



Scheme 1. Synthesis of bis-pyridinium compounds.

Table 1
Physical data of compounds

Oxime	−CH═NOH	Xylene	Reaction temperature (°C)	Time (h)	Yield[a] (%)	M.P[b] (°C)	Calculated (%)			Found[c] (%)		
							C	H	N	C	H	N
3a	4	*para*	Ambient	3	82	268–270	47.24	3.93	11.02	47.52	4.11	10.81
3b	3	*para*	Ambient	4	80	258–260	47.24	3.93	11.02	46.91	4.25	11.34
3c	2	*para*	Ambient	4	70	205–207	47.24	3.93	11.02	46.95	4.15	11.24
3d	4	*meta*	Ambient	3	81	240–242	47.24	3.93	11.02	47.61	3.78	10.71
3e	3	*meta*	50	5	81	234–236	47.24	3.93	11.02	47.32	3.67	11.35
3f	4	*ortho*	70	4	78	238–240	47.24	3.93	11.02	47.48	4.11	10.95
3g	3	*ortho*	70	6	72	208–10	47.24	3.93	11.02	46.87	4.09	11.37
3h	2	*ortho*	90	6	55	202–204	47.24	3.93	11.02	47.11	4.05	11.25

[a] Isolated yield.
[b] The melting points are uncorrected.
[c] Elemental analyses (C, H, N) were within ±0.4% of the calculated values.

All the conventional oximes reported to date differ from each other by the number of pyridinium rings (mono-pyridinium or bis-pyridinium), the position of the oxime group on the pyridinium ring, and, in the case of bis-pyridinium oximes, by the chemical structure and length of the bridge between the pyridinium rings [16,17]. That is why the compounds differing in these features were synthesized and evaluated. It is worth noting that these oximes showed better reactivation of inhibited enzyme even in comparison to established antidote 2-PAM. On changing the position of oxime functionality from 3 to 4 i.e., the 4-isomers of bis-pyridinium oximes (**3a**, **3d**, & **3f**) decreased the reactivation potential. Similarly by changing the oxime group from position 3 to 2 (**3c** & **3h**), reactivation efficiency was found to be at par with 2-PAM. Further, from the study of the effect of different isomers of xylene bridge (Fig. 2), it was observed that the oximes connected by *p*-xylene linkers showed the highest reactivation followed by the oximes having *m*- and *o*-xylene linkers (**3b**, **3e**, & **3g**). It has been found that the best in-vitro reactivation of sarin inhibited AChE of these bis-pyridinium oximes is observed with the placement of oxime functionality at position 3 in the pyridinium ring with a *p*-xylene linker. Although the synthesized compounds did not differ in the number of bonds between two pyridinium rings yet the disposition of alkyl residues at 1,4 (*para*-) and 1,3 (*meta*-) positions of the xylene linkage does make the two oxime functions to remain at two different locations, which is responsible for the difference in the reactivation profile of two compounds. Thus the 1,4-isomer (**3b**) does make the oxime functions to fall farther apart than that of 1,3-isomer (**3e**). The maximum reactivation activity of **3b** is well in agreement with 20 Å active site gorge of AChE [21,22], where one of the two pryridinium rings might reside at the rim, and other pyridinium ring penetrates into gorge of the enzyme where phosphorylation of active site of enzyme takes place.

In conclusion, we have synthesized series of bis-pyridinium oximes and evaluated their in-vitro reactivation efficacy against sarin inhibited AChE. Based upon this study, **3b** may provide a useful therapeutic potential for the reactivation of AChE inhibited by sarin. The detailed study of antidotal efficacy including *in vivo* reactivation against sarin and other nerve agents is under progress and will be reported in due course of time.

4. Experimental section

4.1. Chemistry

Electric eel AChE (EC.3.1.1.7), 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), acetylthiocholineiodide, 2-, 3-, & 4-pyridinealdoxime and α,α′ dibromoxylenes were purchased from Sigma-Aldrich, USA and used without further purification. Potassium dihydrogenphosphate and dipotassium hydrogenphosphate were obtained from E. Merck (India) and used without further purification. Solvents (DMF, acetonitrile, acetone, methanol) were purchased from SD Fine Chemicals (India) and dried before use. Nerve agent sarin was prepared in this laboratory with >98% purity (GC and ^{31}P NMR). 2-PAM was prepared according to the reported method [23]. The bis-pyridinium oximes were synthesized and characterized by their IR, ^{1}H NMR & ^{13}C NMR spectral data (Table 2) Purity of the synthesized oximes was checked by TLC (cellulose, DSO, Fluka) with 1-butanol:acetic acid:water (3:1:1) as solvent system.

4.2. General synthetic procedure

In a typical experimental procedure, a mixture of 4-pyridinealdoxime (1.34 g, 11.00 mmol) and α,α′ dibromo-*p*-xylene (1.32 g, 5.00 mmol) in 25 mL dry DMF was stirred at room temperature for 3 h. The solids appeared were filtered off and washed repeatedly with hot dry acetone. Re-crystallization from methanol–acetonitrile or methanol–acetone mixture gave the pure compound (**3a**). Yield: 2.10 g (82%). All other compounds were synthesized as the per reaction conditions shown in Table 1.

4.3. In-vitro reactivation studies

The in-vitro reactivation of sarin inhibited AChE using test oximes were carried out in triplicate in phosphate buffer (pH 8.0 at 37 °C, 0.1 M) using the method of Ellman et al. [24].

Table 2
Spectral data of compounds

Oxime	IR (KBr) $\nu_{max}(cm^{-1})$	^1H NMR (400 MHz) δ/ppm, (DMSO-d_6)	^{13}C NMR (100 MHz) δ/ppm, (DMSO-d_6)
3a	3393, 3031, 2966, 1602, 1435, 1295,1152, 1004, 760	5.87 (s, 4H, NCH_2), 7.63(s, 4H, −Ph) 8.25−8.27 (d, 4H, −Py), 8.43 (s, 2H, CH=N) 9.17−9.18 (d, 4H, −Py), 12.26 (s, 2H, OH)	62.12, 124.50, 127.18, 128.81, 129.73, 135.46, 145.20, 148.92, 150.80.
3b	3368, 3029, 2988, 1625, 1430, 1290, 1153, 990, 761	5.95 (s, 4H, NCH_2), 7.67 (s, 4H, −Ph), 8.18−8.21 (t, 2H, −Py), 8.36 (s, 2H, N=CH), 8.74−8.76 (d, 2H, −Py), 9.21−9.23 (d, 2H, −Py), 9.46 (s, 2H, −Py), 12.26 (s, 2H, OH)	62.84, 128.63, 129.71, 129.77, 133.89, 135.25, 142.14, 142.51, 143.32, 144.56.
3c	3378, 3028, 2967, 1656, 1431, 1312, 1008, 764	6.07 (s, 4H, NCH_2), 7.29 (s, 4H, -Ph), 8.14−8.17 (t, 2H,- Py), 8.43−8.45 (d, 2H, -Py), 8.58−8.62 (t, 2H, −Py) 8.66 (s, 2H, N = CH), 9.14−9.16 (d, 2H, −Py), 12.78(s, 2H, OH)	60.15, 126.10, 127.91, 128.10, 134.69, 136.54, 141.39, 146.05, 146.60, 147.32.
3d	3385, 3025, 2966, 1635, 1426, 1292, 1005, 756	5.92 (s, 4H, NCH_2), 7.94 (s, 3H, −Ph), 7.70 (s, 1H, −Ph), 8.26−8.28 (d, 4H, −y), 8.45 (s, 2H, N=CH) 9.14−9.15 (d, 4H, −Py) 12.88 (s, 2H, OH)	62.31, 124.48, 129.35, 129.62, 130.16, 135.23, 145.16, 148.88
3e	3342, 3032, 2971, 1629, 1435, 1290, 992, 767	δ 5.92 (s, 4H, NCH_2), 7.57 (s, 3H, −Ph), 7.3 (s, 1H, -Ph), 8.18−8.21 (t, 2H, −Py), 8.37 (s, 2H, N=CH), 8.75−8.77 (d, 2H, −Py), 9.14−9.16 (d, 2H, −Py), 9.40 (s, 2H, −Py), 12.27(s, 2H, OH)	62.96, 128.63, 129.61, 129.83, 130.15, 133.84, 135.02, 142.06, 142.68, 143.32, 144.67.
3f	3390, 3035, 2968, 1631, 1425, 1308, 1012, 760	6.09 (s, 4H, NCH_2), 7.24−7.26 (t, 2H, −Ph), 7.50−7.53 (t, 2H, −Ph), 8.28−8.30 (d, 4H, −Py), 8.48 (s, 2H, N=CH), 9.03−9.05 (d, 4H, −Py), 12.92 (s, 2H, OH)	60.19, 124.93, 130.12, 130.60, 133.30, 145.63, 145.87, 149.54
3g	3388, 3030, 2967, 1615, 1442, 1298, 992, 768	6.17 (s, 4H, NCH_2), 7.32−7.34 (t, 2H, −Ph), 7.56−7.58 (t, 2H, −Ph), 8.24−8.28 (t, 2H, −Py), 8.43 (s, 2H, N=CH), 8.83−8.85 (d, 2H, −Py), 9.07−9.08 (d, 2H, −Py), 9.29 (s, 2H, −Py), 12.32 (s, 2H, OH)	60.52, 128.65, 129.94, 130.27, 132.65, 133.89, 142.32, 142.79, 143.34, 144.86.
3h	3377, 3031, 2965, 1635, 1440, 1305, 998, 762	6.26 (s, 4H, NCH_2), 6.56−6.58 (t, 2H, −Ph), 7.35−7.37 (t, 2H, −Ph), 8.23−8.27 (t, 2H, −Py), 8.60−8.62 (d, 2H, −Py), 8.65 (s, 2H, N=CH), 8.71−8.74 (t, 2H, −Py), 8.93−8.95 (d, 2H, −Py), 12.35 (s, 2H, OH)	57.81, 125.52, 125.85, 128.44, 129.29, 131.52, 141.31, 146.10, 146.41, 148.15.

Values depicted in figures are average of triplicate runs with a maximum relative standard deviation of ±2%. AChE stock solution (stock A) was prepared in phosphate buffer (pH 7.6, 0.1 M) (360 units/0.5 mL). An aliquot of stock A was then diluted 50 times with phosphate buffer to give stock B. A freshly prepared stock solution of sarin (1.4×10^{-2} M) was in isopropanol and stored under refrigeration. It was then diluted appropriately with triple distilled water just before use. All oxime stock solutions were prepared in triple distilled water. DTNB stock solution (10 mM) was prepared in phosphate buffer (pH 7.6, 0.1 M). The substrate stock (acetylthiocholine iodide, 75 mM) was prepared in distilled water. The incubation mixture was prepared by the addition of 50 μL of sarin (1.4×10^{-6} M) to a mixture of 50 μL enzyme (stock B) in 350 μL phosphate buffer (pH 8.0, 0.1 M). The mixture was allowed to stand for 15 min at ambient temperature to give 96 ± 1% inhibition of enzyme activity. It was then followed by addition of 50 μL of oximes test solution (10^{-3} M, 5×10^{-4} M & 10^{-4} M) to start reactivation. The final volume of the reactivation cocktail was 500 μL. Thus, reactivation cocktail indeed composed of 0.08 M phosphate buffer at a pH between 7.6 and 8.0. The final concentration of sarin was 1.4×10^{-7} M and oxime was diluted 10 fold in the reactivation cocktail. After 10 min of reactivation, the enzyme activity was assayed by Ellman's method (Fig. 2). Twenty micro litres of reactivation cocktail was transferred to a cuvette containing 50 μL DTNB in phosphate buffer (pH 8.0, 0.1 M). The enzyme activity was then assayed by addition of 50 μL of substrate to the cuvette against a blank containing reactivation cocktail without substrate. The final volume of the assay mixture was adjusted to 3 mL and final concentration of DTNB and substrate was 0.16 mM and 1.25 mM, respectively. The reactivation of inhibited enzyme was then studied at an interval of 10 min and followed up

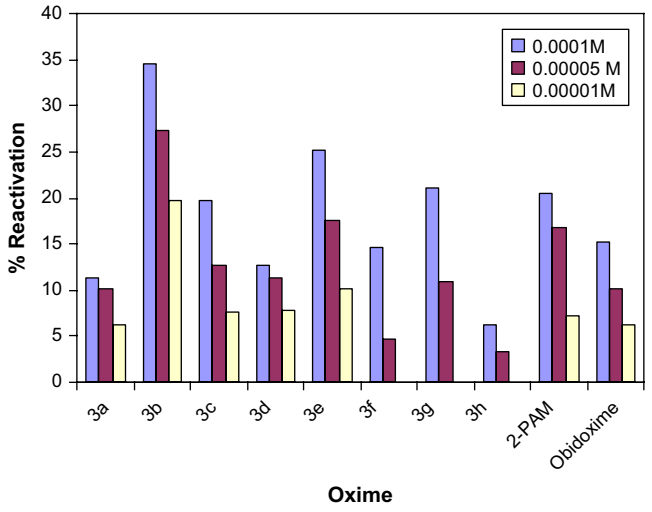


Fig. 2. Efficacy of tested oximes in reactivation of sarin inhibited AChE in comparison with 2-PAM. Source of enzyme: electric eel, inhibitor agent sarin, time of inhibition 15 min; time of reactivation 10 min; pH 8.0 & temperature 37 °C. The values are average of three runs with a maximum S.D. of ±2%.

to 1 h. Percentage reactivation was calculated using the following equation:

$$\%\ \text{Reaction} = (E_r - E_i / E_0 - E_i) \times 100$$

Where E_0 is the control enzyme activity at 0 min (without inhibitor and oxime), E_i is the inhibited enzyme activity (without oxime) determined in the similar manner described above and E_r is the activity of reactivated enzyme after incubation with the oxime test compounds. Spontaneous reactivation of inhibited AChE was assayed using the same protocol, the reaction mixture contained enzyme and sarin but no oxime. Under these conditions spontaneous reactivation was found to be insignificant. All the values are corrected for their oxime induced hydrolysis.

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