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Synthesis and in vitro evaluation of xylene linked carbamoyl bis-pyridinium monooximes as reactivators of organophosphorus (OP) inhibited electric eel acetylcholinesterase (AChE)

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ABSTRACT

A series of carbamoyl bis-pyridinium monooximes linked with xylene linker were synthesized and their in-vitro reactivation potential was evaluated against acetylcholinesterase (AChE) inhibited by organophosphorus inhibitors (OP) such as sarin, DFP and VX and the data were compared with reactivation obtained with 2-PAM and obidoxime. Amongst the synthesized compounds, 3-carbamoyl-2′hydroxyiminomethyl-1-1′-(1,4-phenylenedimethyl)-bispyridinium dibromide (**5e**) 3-carbamoyl-2′hydroxyiminomethy l-1-1′-(1,3-phenylenedimethyl)-bispyridinium dibromide (**5k**) and 4-carbamoyl-2′hydroxyiminomethyl-1-1′-(1,3-phenylenedimethyl)-bispyridinium dibromide (**5l**) were found to be the most potent reactivators for electric eel AChE inhibited by sarin and DFP. However, in case of VX inhibited AChE, none of the synthesized oximes could surpass the reactivation potential of 2-PAM and obidoxime. The pKa values of all the oximes were determined and correlated with their observed reactivation potential.

1. Introduction

Quaternary mono- and bis-pyridinium oximes are important therapeutic agents used as reactivators of acetylcholinesterase (AChE) in the treatment of poisoning resulting from the exposure of organophosphorus inhibitors (OP) [1]. Some of these OPs have been synthesized and used as warfare agents (nerve agents e.g., sarin, soman, tabun and VX) in the past (Fig. 1) [2,3]. They have also been used as pesticides (e.g., parathion, malathion, chlorpyriphos) resulting in severe occupational hazards [4]. The use of nerve agents during war and acts of terrorism has emphasized the development of 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 [5]. The subsequent accumulation of the neurotransmitter acetylcholine and overstimulation of cholinergic receptors results in a generalized cholinergic crisis including breakdown of neuromuscular function [6].

The reactivation of OP-inhibited AChE can be achieved effectively by the nucleophillic attack of a strong nucleophile on the P-atom which breaks the phosphorus-AChE bond in the phosphorylated AChE. Quaternary pyridinium oximes such as 2-pyridine aldoxime methochloride (2-PAM), trimedoxime (TMB-4), obidoxime and HI-6 (Fig. 2) are currently used as reactivators in the treatment of OP poisoning [7]. However, these OP nerve agents behave differently towards reactivation due to their broad structure variability and most often their deleterious side effects become difficult to counteract [8]. Furthermore an effective therapy by a single oxime to all the known nerve agents is still lacking.

2. Chemistry

Several new oximes have been synthesized and tested in the last decades [9–11]. The reactivation of OP-inhibited AChE depends on several factors including structure of the reactivator [12], structure of the inhibitor (OP), source of the enzyme AChE [13] as well as on post-inhibitory reactions such as spontaneous dealkylation (aging) [14] and spontaneous dephosphorylation (spontaneous reactivation) of the OP-AChE complex [15].

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]. Recently, *N*-benzyl-4-(hydroxyiminomethyl) pyridinium bromide and its analogous were reported to be more active in the reactivation of tabun-inhibited human erythrocyte AChE. Again the docking studies with these types of compounds led to the assumption that the linker

Abbreviations: AChE, Acetylcholinesterase; 2-PAM, 2-(hydroxyiminomethyl)-1-methylpyridinium chloride; DFP, diisopropyl flurophosphate.

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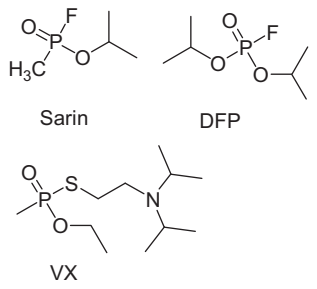


Fig. 1. Structure of OP inhibitors used in this study.

between pyridinium and benzyl ring should be long enough to achieve desired reactivation [18]. Xylene linked bis-pyridinium oximes have gained considerable interest in the recent past owing to their improved reactivation potential for OP inhibited AChE [19]. Further, structure-activity relationship (SAR) and docking studies with xylene linked pyridinium oximes revealed π-π and cation-π interactions within the enzyme active-sites [20]. Previously, we have also reported symmetrical bis-pyridinim oximes linked by a xylene, methoxyalkane and methoxymethyl benzene as AChE reactivators [21–23]. Some of these oximes have shown promising reactivation potential against OP inhibited AChE. Reactivation potential of such oximes attracted our attention to further explore xylene linked pyridinium oximes. In this context we focused our attention on carbamoyl bis-pyridinium monooximes 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 [24] as well as to allow the hydrophobic interactions with some of the aromatic residues that remain in the active site gorge of the enzyme acetylcholinesterase [25]. Therefore, in continuation of our work on antidotes against nerve agents, herein we report the synthesis and in-vitro evaluation of a series of carbamoyl bis-pyridinium monooximes linked by xylene bridge as reactivators of electric eel AChE inhibited by sarin, VX and a model OP inhibitor diisopropyl flurophosphate (DFP).

By adopting a simple and mild two-step synthetic protocol, we have synthesized a series of carbamoyl bis-pyridinium monooximes. The intermediate mono quaternary pyridinium monooximes (**3a**–**3i**) were prepared according to the reported method [19], by the reaction of isomeric pyridine aldoxime (**1a**–**1c**) with α,α′ dibromoxylene (**2a**–**2c**) (Scheme 1). The subsequent condensation of intermediate **3** with 3- and 4-carbamoylpyridine (**4a** and **4b)** gave desired products (**5a**–**5r)** with good yields within 3–6 h (Table 1).

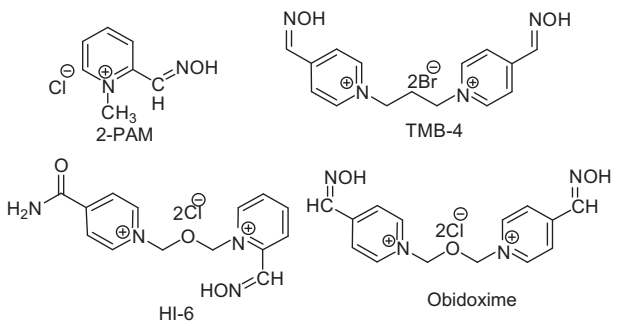


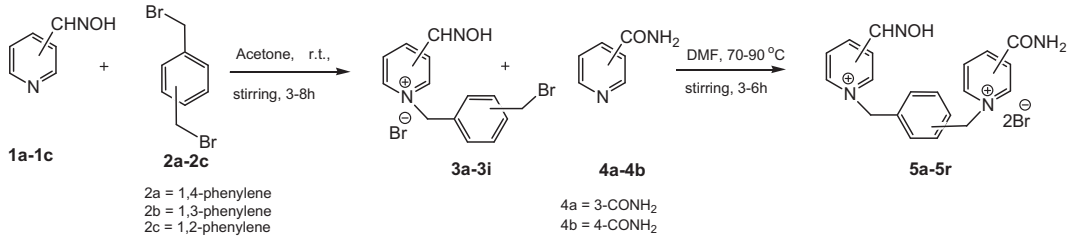
Fig. 2. Commonly used reactivators.

3. Results and discussion

The in-vitro reactivation data of OP (sarin, DFP and VX) inhibited electric eel AChE by carbamoyl bis-pyridinium monooximes bearing xylene bridge at two distinct concentrations (10^{-3} M & 10^{-4} M) is presented in Fig. 3. It is evident from these data that best reactivation was observed with 3-carbamoyl-2′hydroxyimi nomethyl-1-1′-(1,4-phenylenedimethyl)-bispyridinium dibromide (**5e**) 3-carbamoyl-2′hydroxyiminomethyl-1-1′-(1,3-phenylenedim ethyl)-bispyridinium dibromide (**5k**) and 4-carbamoyl-2′hydro xyiminomethyl-1-1′-(1,3-phenylenedimethyl)-bispyridinium dibro mide (**5l**) in case of sarin (Fig. 3a) and DFP (Fig. 3b) inhibited electric eel AChE. The oximes, **5e, 5k** and **5l** respectively reactivated 47%, 48% and 45% of sarin inhibited AChE in comparison to respectively 42% and 21% reactivation by 2-PAM and obidoxime at the concentration 10^{-3} M after 10 min. However at a lower oxime concentration (10^{-4} M), the reactivation potential of oxime **5l** only was found to be greater than that of obidoxime and 2-PAM in reactivating sarin inhibited AChE. In case of DFP inhibited AChE, the oximes **5e**, **5k** and **5l** were able to reactivate 54%, 54% and 48% respectively in comparison to 51% and 43% reactivation by 2-PAM and obidoxime at the concentration 10^{-3} M after 10 min. It may also be noted that at lower oxime concentration, oxime **5k** was found to be more active than 2-PAM and obidoxime in reactivating DFP inhibited AChE. Furthermore, the reactivation potential of other two oximes i.e., **5e** and **5l** was at par with 2-PAM and more than that of obidoxime. It is worth noticing that in case of VX inhibited AChE (Fig. 3c), synthesized oximes such as **5b, 5c, 5d, 5j, 5k** and **5l** have shown significant reactivation potential. However, none of these could surpass the reactivation potential of 2-PAM. The best reactivation was observed with 2-PAM (52%) followed by **5b** (43%) at 10^{-3} M after 10 min. The oximes **5d** and **5j** reactivated 40% of the VX inhibited AChE which was at par with that of obidoxime at the same concentration. However at lower concentration, oxime **5d** showed highest reactivation ability followed by 2-PAM, obidoxime and **5j**.

The time dependent reactivation profile of some selected oximes indicated that for most of the oximes, reactivation increased with time and reached maximum in 30–40 min. The oxime **5k** was able to reactivate 83% followed by **5e** (75%) and **5l** (73%) of the sarin inhibited AChE after 40 min as compared to 63% and 34% reactivation observed by 2-PAM and obidoxime respectively. However in case of DFP inhibited AChE, oxime **5k** was able to reactivate 81% followed by 56% reactivation each by 2-PAM and **5e**, obidoxime (54%) and **5l** (41%). It may also be noted that the reactivation potential of oxime **5l** in reactivating DFP inhibited AChE decreased gradually with time. Maximum reactivation potential of 2-PAM and obidoxime in reactivating VX inhibited AChE was 63% and 54% respectively after 40 min as compared to 45% reactivation by oxime **5j** in 30 min. The OP inhibitors used in this study have aging half-life of more than an hour [26]. Thus no appreciable amount of aged enzyme was present during the course of reactivation studies. Therefore, the observed time-dependent reactivation of inhibited AChE is of non-aged enzyme only and obtained plateau of reactivation vs time indicates maximum possible reactivation achievable with lowest time.

The pKa values should be taken into consideration while searching for new oxime reactivators [27] essentially due to the fact that deprotonated oxime group is involved in the nucleophilic attack during reactivation and its concentration is pH dependant. Therefore the pKa values of the oximes were determined spectrophotometrically in the pH range of 6.04–10.37 (Fig. 4). In the series of pyridinium oximes, the optimum pKa value for the reactivator should be in the range of 7.6–8.0 [28,29]. Two characteristic maxima were obtained in UV in the region 200–600 nm. These



Scheme 1. Synthesis of carbamoyl bis-pyridinium monooximes.

maxima correspond to the absorption of different oxime ionized forms as a result of $\pi \rightarrow \pi^*$ transitions within the aromatic system [18]. The first absorption around 280 nm was a result of absorption of non-dissociated oxime group and the maximum above 340 nm was a result of the absorption of dissociated oxime group (Fig. 4). The sharp isobestic point at 305–308 nm referred to the acid–base equilibrium.

From the present study it is evident that the oximes **5e, 5k** and **5l** were found to be most active in reactivating both sarin and DFP inhibited AChE. The common structural feature of the oximes found most active in reactivating DFP and sarin inhibited AChE is that the oximino moiety is on the position 2 of the pyridinium ring with placement of the amide function at position 3- or 4-. The best activity of **5e, 5k** and **5l** may be attributed to the optimal length of the reactivator imparted by position of amide and oxime functionality. This optimal length positions amide at the periphery of the 20 Å gorge of AChE [25] and extends oxime function to the active site via xylene linker through the gorge. There are two factors which determine the distance between oxime and amide. First is the positioning of the amide function in the pyridinium ring, and second is the linkage of xylene (1, 4- or 1, 3-). Close examination of **5e, 5k** and **5l** reveals that the xylene linkage of 1, 4- or 1, 3- and position of amide at 3- (if the xylene linkage is 1, 4- or 1, 3-) or 4- (if the xylene linkage is 1,3-) provides appropriate length between amide and oxime functions. While in case of other oximes connected by 1, 2- or 1, 4- xylene linkers with corresponding placement of amide function at position 4- in the pyridinium ring, alters the required optimal length. This could be the plausible explanation for observed activity of **5e, 5k** and **5l** over other oximes. This is in close agreement with reported study by Musilek et al. [30], where oxime group in position 2 or 3 have shown increased reactivation towards tabun inhibited AChE, as compared to similar compounds with oxime in position 4. However in case of VX inhibited AChE, no such preference for the position of the oxime on the pyridinium ring with respect to reactivation was observed. This may be attributed to the fact that, in case of VX inhibited AChE, the active site gorge is least occupied (due to the cleavage of bulkier *N, N*-diisopropyl-S-ethyl leaving group) thereby leaving more free space for oxime access to the phosphorylated site with least steric hindrance compared to DFP or sarin [31]. This is manifested in the reactivation data that most of the oximes in this study have shown significant reactivation towards VX inhibited AChE irrespective of the position of the oxime moiety on the pyridinium ring, the nature of the linker and position of the carbamoyl moiety.

The dephosphorylation of phosphorylated enzyme is also dependent on factors such as physicochemical properties of the reactivator including steric and electronic factors, lipophilic-hydrophilic balance and pKa [21]. The pKa of bis-pyridinium oximes were found to be in the range of 7.69–8.79 (Table 1). It is worth noticing that the pKa of bis-pyridinium oximes having oximino function at position 2 or 4 on the pyridinium ring were found to be in the lower range (7.69–8.15) as compared to the oximes where oximino function is attached at position 3 on the pyridinium ring (8.59–8.79). This difference in the pKa values may be attributed to the resonating structures in which the isomer with 2 and 4

Table 1
Physical and elemental analysis data of carbamoyl bis-pyridinium oximes.

Oxime	−CH = NOH	Xylene	$-CONH_2$	Temp. (°C)	Time (h)	Yield[a] (%)	M.P[b](°C)	pKa	C	H	N	C	H	N
									Calculated (%)			Found (%)		
5a	4	1,4-	3-	75	3	82	272–74	8.15 ± 0.05	47.24	3.93	11.02	46.95	4.15	11.24
5b	4	1,4-	4-	70	3	75	278–80	8.09 ± 0.04	47.24	3.93	11.02	47.17	4.16	11.41
5c	3	1,4-	3-	80	4	71	248–50	8.79 ± 0.05	47.24	3.93	11.02	47.61	3.78	10.71
5d	3	1,4-	4-	70	4	65	270–72	8.77 ± 0.05	47.24	3.93	11.02	47.54	4.21	11.04
5e	2	1,4-	3-	80	5	56	234–36	7.72 ± 0.03	47.24	3.93	11.02	46.87	4.09	11.37
5f	2	1,4-	4-	80	5	58	238–40	7.76 ± 0.03	47.24	3.93	11.02	46.97	3.95	10.84
5g	4	1,3-	3-	75	6	72	224–26	7.95 ± 0.03	47.24	3.93	11.02	47.45	4.14	11.24
5h	4	1,3-	4-	75	6	78	198–200	8.01 ± 0.04	47.24	3.93	11.02	46.91	3.77	11.27
5i	3	1,3-	3-	75	8	77	140–44	8.76 ± 0.05	47.24	3.93	11.02	47.48	4.11	10.95
5j	3	1,3-	4-	70	7	80	230–32	8.75 ± 0.05	47.24	3.93	11.02	46.89	3.76	11.32
5k	2	1,3-	3-	80	8	62	248–50	7.75 ± 0.03	47.24	3.93	11.02	46.97	4.10	11.23
5l	2	1,3-	4-	80	8	66	299–201	7.69 ± 0.03	47.24	3.93	11.02	47.32	3.67	11.35
5m	4	1,2-	3-	75	5	67	230–34	7.85 ± 0.03	47.24	3.93	11.02	47.41	4.02	10.88
5n	4	1,2-	4-	75	4	62	240–42	7.94 ± 0.03	47.24	3.93	11.02	46.91	4.25	11.34
5o	3	1,2-	3-	80	6	55	230–32	8.69 ± 0.05	47.24	3.93	11.02	47.27	3.75	11.18
5p	3	1,2-	4-	80	6	48	190–92	8.59 ± 0.05	47.24	3.93	11.02	47.52	4.11	10.81
5q	2	1,2-	3-	90	6	46	222–24	7.79 ± 0.04	47.24	3.93	11.02	46.92	3.98	11.12
5r	2	1,2-	4-	90	7	48	188–90	7.77 ± 0.03	47.24	3.93	11.02	47.11	4.05	11.25
Obidoxime	4	–	–	–	–	–	–	7.83 ± 0.04	–	–	–	–	–	–
2-PAM	2	–	–	–	–	–	–	7.85 ± 0.04	–	–	–	–	–	–

[a] Isolated yield.
[b] The melting points are uncorrected.

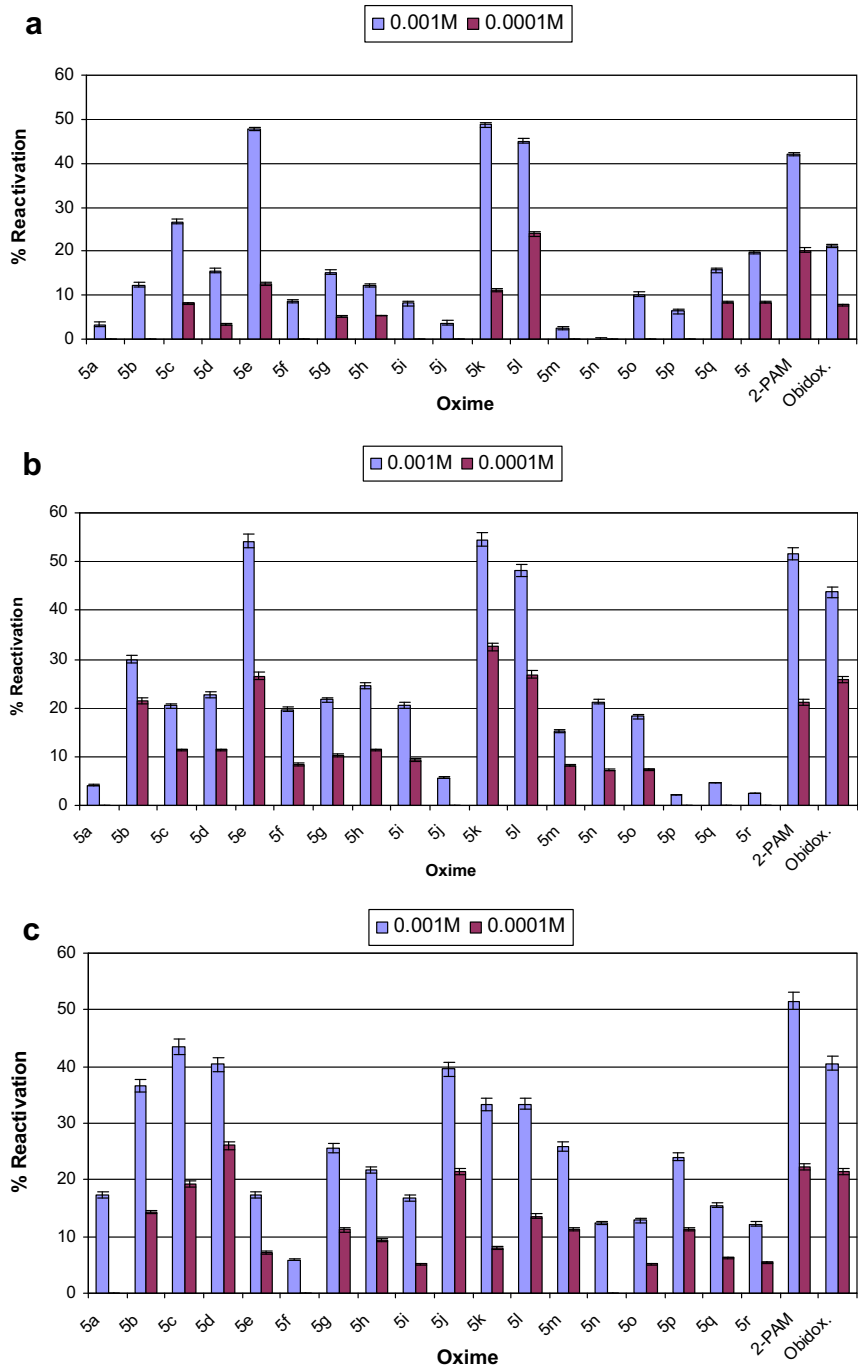


Fig. 3. Efficacy of tested oximes in reactivation of OP-inhibited AChE in comparison with 2-PAM and obidoxime. Source of enzyme: electric eel, inhibitor agent (a) sarin, (b) DFP and (c) VX; time of inhibition 15 min; time of reactivation 10 min; pH 8.0 & Temperature 37 °C. The values are average of three runs with a maximum S.D. of ±2.5%.

position passes through a resonating structure where the positive charge of the pyridinium ring resides on the carbon at 2 and 4 position. This makes the oxime function to realize more electron withdrawing effect as compared to 3-isomer which is manifested in lower pKa of 2, 4 isomers [32]. The high reactivation potency of oximes **5e, 5k** and **5l** for AChE inhibited by sarin or DFP at pH 8.0 may be due to their low value of pKa (7.69–7.75) which is closer to the physiological pH and make the oximes to dissociate sufficiently to be active at the pH under study.

In conclusion, we have synthesized series of carbamoyl bis-pyridinium monooximes and evaluated their in-vitro reactivation efficacy against OP inhibited AChE. Based upon this study, **5e, 5k** and **5l** may provide a useful therapeutic potential for the reactivation of AChE inhibited by sarin and DFP. The detailed study of antidotal efficacy including *in-vivo* reactivation against sarin and DFP and determination of kinetic rate constant with human AChE 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,

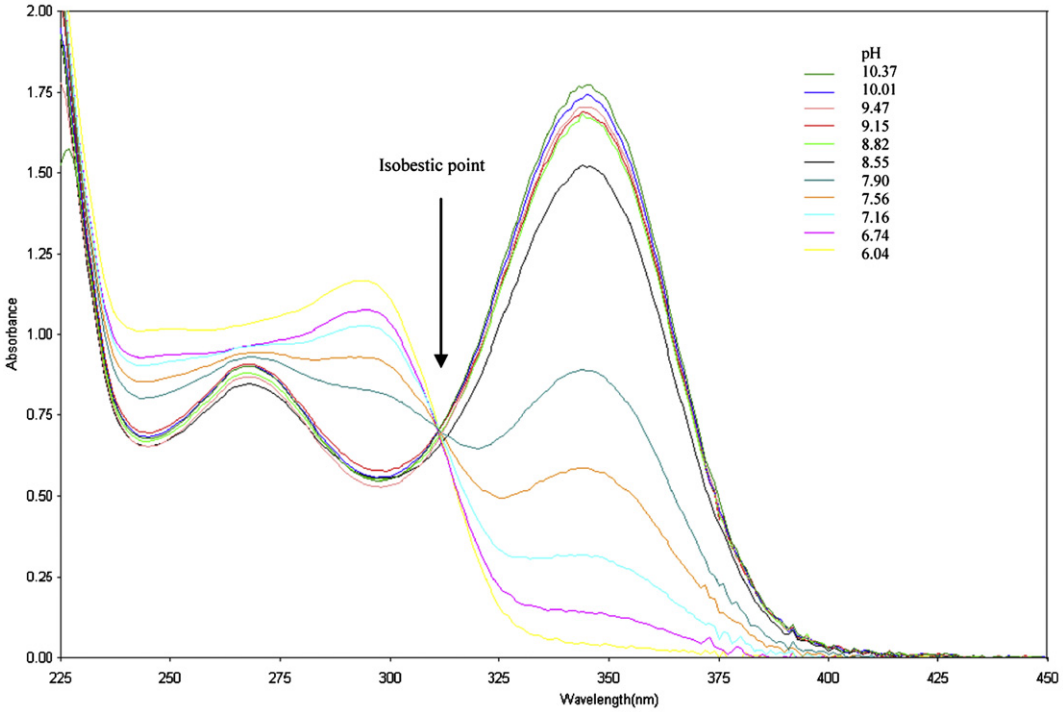


Fig. 4. Spectrophotometric determination of pKa of oxime **5f**.

nicotinamide, isonicotinamide, α,α′ dibromoxylene, Potassium dihydrogenphosphate, dipotassium-hydrogen phosphate trizma-base and trizma-HCl were purchased from Sigma–Aldrich, USA and used without further purification. Glycine were obtained from E. Merck (India) and used without further purification. Solvents (DMF, acetonitrile, acetone, methanol) were purchased from S.D. Fine Chemicals (India) and dried before use. VX, sarin and DFP was prepared in this laboratory with >98% purity (GC and ^{31}P NMR). 2-PAM was prepared according to the method of Ginsburg and Wilson [33] The bis-pyridinium oximes and obidoxime were synthesized, characterized by their I.R., ^{1}H NMR & ^{13}C NMR spectral data (Table 2) and their purity was checked by commercially available pre-coated cellulose on alumina sheets TLC plates (E. Merck) with 1-butanol: acetic acid: water (3:1:1) as solvent system.

4.2. Synthetic procedure

4.2.1. Synthesis of mono-pyridinium oxime intermediate

In a typical experimental procedure a solution of 4-pyridinealdoxime (2.44 g, 20.00 mmol) in 50 mL dry acetone was added slowly to a stirred solution of α,α′ dibromo-p-xylene (13.2 g, 50.00 mmol) in 100 mL dry acetone at room temperature. The mixture was stirred at r.t. for 4 h. The solids appeared were filtered off and washed repeatedly with hot dry acetone to give the intermediate 1-(4-bromomethyl benzyl)-4-(hydroxyimino methyl)-pyridinium bromide. The compound thus obtained was sufficiently pure for use in the next step. m.p. 194–96 °C. Yield: 5.65 g (73%). All other intermediate pyridinium monooximes were prepared in a similar manner.

4.2.2. Synthesis of Carbamoyl bis-pyridinium monooximes

3-carbamoyl-4′-hydroxyiminomethyl-1,1′-(1,4-phenylenedimethyl)-bispyridinium dibromide (**5a**). In a typical experimental procedure, a mixture of 1-(4-bromomethyl benzyl)-4-(hydroxyimino methyl)-bispyridinium bromide (**3a**) (1.54 g, 4.0 mmol) and nicotinamide (0.61 g, 5.0 mmol) in 20 mL dry DMF was stirred at 70–75 °C 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 (**5a**). Yield: 1.66 g (82%). All other compounds were synthesized as per the reaction conditions shown in Table 1.

4.2.3. Spectral characterization of Carbamoyl bis-pyridinium monooximes

Purity of the synthesized compounds were checked by thin-layer chromatography (TLC, cellulose, DS-O, Fluka) with 1-butanol, acetic acid, water (3:1:1) as mobile phase. Melting points were determined using melting point apparatus IA9200 Electrothermal, U.K., and are uncorrected. The structure of the compounds was confirmed by their elemental analysis and spectral data. Elemental analyses was conducted on an ELEMENTAR, vario MICRO cube, Universal micro analyzer and were within ±0.4% of the calculated values (Table 1). Infra-red (I.R.) spectra was obtained as KBr discs on a Bruker TENSOR-27 FTIR spectrophotometer (Table 2). 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} C–H str (aryl) and at 2988–2965 cm^{-1} for C–H str (aliphatic). The absorptions in the range of 1678–1709 cm^{-1} is due to amide carbonyl group. The characteristic absorptions for oximes were quite distinct and appeared at 1642-1600 cm^{-1} and 1010-990 cm^{-1} ^{1}H NMR (DMSO-d_6) spectra were 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 12.24–13.10 and were exchangeable with D_2O. Signals at δ 5.85–6.19 were due to N–CH_2 protons. The aryl protons appeared in the range of δ 7.23–8.27. Pyridine and N=CH protons appeared in the range of δ 8.17–9.92. ^{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)

Table 2
Spectral data of carbamoyl bis-pyridinium oximes.

Oxime	I.R. (KBr) $\gamma_{max}(cm^{-1})$	^1H NMR(400 MHz) δ, ppm, DMSO-d_6)	^{13}C NMR (100 MHz) δ, ppm, DMSO-d_6)
5a	3279, 3143, 3028, 2946, 1703, 1646, 1505, 1434, 1396, 1278, 1262, 1004, 775, 667	5.87 (s, 2H, NCH_2), 5.95 (s, 2H, NCH_2), 7.61–7.69(m, 4H, -Ph), 8.20 (s, 1H, NH_2), 8.24–8.25 (d, 2H, -Py), 8.27–8.31 (t, 1H, -Py), 8.41 (s, 1H, CH═N), 8.64 (s,1H, NH_2), 8.97–8.99 (d,1H), 9.17–9.18 (d, 2H, -Py), 9.34–9.35 (d, 1H, -Py), 9.69 (s, 1H, -Py), 12.87 (s, 1H, OH)	62.12, 62.95, 124.41, 128.29, 128.41, 129.71, 129.97, 134.15, 135.03, 135.45, 144.09, 145.33, 146.11, 148.89
5b	3361, 3169, 3118, 3038, 2997, 1669, 1623, 1570, 1454, 1430, 1399, 1279, 1129, 995, 777, 754	5.85 (s, 2H, NCH_2), 5.93 (s, 2H, NCH_2), 7.60–7.63 (m, 4H, -Ph) 8.24–8.25 (d, 2H, -Py), 8.29 (s, 1H, NH_2), 8.40 (s, 1H, CH═N), 8.44–8.46 (d,2H), 8.67 (s,1H, NH_2), 9.15–9.16 (d, 2H, -Py), 9.38–9.39 (d, 2H, -Py), 12.87 (s, 1H, OH)	61.18, 62.82, 123.11, 126.22, 128.98, 129.61, 134.77, 135.85, 144.37, 145.42, 148.07, 148.93
5c	3270, 3148, 3025, 2955, 1696, 1638, 1514, 1441, 1403, 1282, 1275, 1011, 768, 671	5.95 (s, 2H, NCH_2), 5.98 (s, 2H, NCH_2), 7.66–7.71 (m, 4H, -Ph), 8.17–8.22 (t, 1H, -Py), 8.28–8.32 (m, 2H, -Py), 8.36 (s, 1H, CH═N), 8.66 (s, 1H, NH_2), 8.73–8.75 (d, 1H, -Py), 8.99–9.01 (d, 1H, -Py), 9.23–9.24 (d, 1H, -Py), 9.38–9.39 (d, 1H, -Py), 9.47 (s, 1H, $-NH_2$), 9.72 (s, 1H, -Py), 12.27 (s, 1H, OH)	64.31, 64.55, 128.63, 129.96, 130.19, 130.51, 133.95, 134.22, 134.33, 142.99, 144.46, 144.73, 144.85
5d	3355, 3144, 3108, 3037, 2988, 1657, 1631, 1560, 1459, 1404, 1260, 1123, 631	5.93 (s, 2H, NCH_2), 5.95 (s, 2H, NCH_2), 7.66 (s, 4H, -Ph), 8.17–8.21 (t, 1H, -Py), 8.30 (s, 1H, NH_2), 8.35 (s, 1H, CH═N), 8.45–8.47 (d, 1H), 8.70 (s, 1H, NH_2), 8.73–8.75 (d, 1H, -Py), 9.19–9.21 (d, 2H, -Py), 9.41 (s, 1H, -Py), 9.43–9.45 (d, 2H, -Py), 12.28 (s, 1H, OH)	62.51, 63.32, 125.11, 128.28, 129.49, 133.25, 134.89, 141.66, 142. 69, 143.55, 145.35, 146.10, 148.88
5e	3350, 3291,3148, 3025, 2937, 1696, 1646, 1583, 1508, 1442, 1393, 1188, 672	5.95 (s, 2H, NCH_2), 6.12 (s, 2H, NCH_2), 7.65 (s, 4H, -Ph), 8.20–8.22 (m, 2H, -Py), 8.25 (s, 1H, NH_2), 8.44–8.46 (d, 1H, -Py), 8.67–8.71 (m, 3H, -Py), 8.98–9.01 (d, 1H, -Py), 9.23–9.24 (d, 1H, -Py), 9.39–9.41 (d, 1H, -Py), 9.65 (s,1H, NH_2), 13.10 (s, 1H, OH).	60.32, 62.14, 125.63, 128.52, 129.11, 129.96, 130.36, 130.89, 134.02, 134.57, 142.01, 143.40, 144.08, 146.11, 146.53, 147.38, 147.74, 148.58
5f	3357, 3150,3115, 3031, 2994, 1678, 1622, 1568, 1455, 1399, 1264, 1131, 621	5.92 (s, 2H, NCH_2), 6.10 (s, 2H, NCH_2), 7.67 (s, 4H, -Ph), 7.98 (s, 1H, NH_2), 8.18–8.21 (t, 1H, -Py), 8.34 (s, 1H, NH_2), 8.38 (s, 1H, CH═N), 8.43–8.47 (m, 2H, -Py), 8.63–8.69 (m, 3H), 9.20–9.21 (d, 1H, -Py), 9.33–9.35 (m, 1H, -Py), 13.07 (s, 1H, OH).	59.82, 61.21, 126.43, 127.51, 128.17, 129.36, 130.09, 133.91, 135.27, 135.84, 141.08, 145.50, 146.18, 146.72, 147. 53, 148.10
5g	3266, 3135, 3034, 2943, 1697, 1649, 1500, 1441, 1390, 1282, 1266, 1001, 776, 661	5.86 (s, 2H, NCH_2), 5.95 (s, 2H, NCH_2), 7.52–7.59 (m, 3H, -Ph), 7.76 (s, 1H, -Ph), 8.21 (s, 1H, NH_2), 8.25–8.30 (m, 3H, -Py), 8.44 (s, 1H, CH═N), 8.64 (s, 1H, NH_2), 8.99–9.01 (d, 1H, -Py), 9.14–9.16 (d, 2H, -Py), 9.31–9.32 (d, 1H, -Py), 9.64 (s,1H, -Py), 12.89 (s, 1H, OH)	62.05, 62.76, 123.84, 127.63, 129.14, 129.75, 130.41, 130.93, 134.16, 134.88, 135.79, 144.14, 145. 21, 145.79, 146.20, 147.01, 148.94
5h	3246, 3124, 3049, 2955, 1705, 1641, 1507, 1435, 1388, 1298, 1279, 998, 772, 669	5.86 (s, 2H, NCH_2), 5.94 (s, 2H, NCH_2), 7.52–7.59 (m, 3H, -Ph), 7.75 (s, 1H, -Ph), 8.26–8.27 (d, 2H, -Py), 8.31 (s, 1H, NH_2), 8.44 (s, 1H, CH═N), 8.47–8.48 (d, 2H, -Py), 8.71 (s,1H, NH_2), 9.15–9.17 (d, 2H, -Py), 9.38–9.40 (d, 2H, -Py), 12.89 (s, 1H, OH)	62.25, 62.77, 124.35, 126.21, 128.95, 129.88, 130.69, 135.70, 136.42, 144.91, 146.65, 147.90, 148.33
5i	3231, 3130, 3029, 2948, 1699, 1652, 1505, 1448, 1397, 1287, 1268, 1006, 770, 667	5.97 (s, 2H, NCH_2), 6.00 (s, 2H, NCH_2), 7.50–7.54 (m, 2H, -Ph), 7.71 (s, 1H, -Ph), 8.19 (s, 1H, -Ph), 8.22–8.24 (t, 1H, -Py), 8.28–8.32 (m, 2H, -Py), 8.39 (s, 1H, CH═N), 8.66 (s, 1H, NH_2), 8.76–8.78 (d, 1H, -Py), 9.01–9.03 (d, 1H, -Py), 9.24–9.25 (d, 1H, -Py), 9.40–9.41 (d, 1H, -Py), 9.49 (s, 1H, $-NH_2$), 9.73 (s, 1H, -Py), 12.24 (s, 1H, OH)	62.91, 63.17, 127.89, 128.55, 129.17, 129.54, 130.61, 134.58, 135.00, 135.88, 142.65, 143.25, 143.96, 144.46, 144.98, 146.27, 147.11
5j	3228, 3137, 3065, 2943, 1709, 1654, 1496, 1450, 1392, 1280, 1263, 1005, 772, 669	5.92 (s, 2H, NCH_2), 5.94 (s, 2H, NCH_2), 7.51–7.59 (m, 3H, -Ph), 7.76 (s, 1H, -Ph), 8.18–8.21 (m, 1H, -Py), 8.28 (s, 1H, NH_2), 8.38 (s, 1H, CH═N), 8.46–8.48 (d, 2H, -Py), 8.69 s,1H, NH_2), 8.75–8.77 (d, 1H, -Py), 9.14–9.16 (d, 1H, -Py), 9.37–9.41 (m, 3H, -Py), 12.26 (s, 1H, OH)	62.75, 63.01, 125.56, 128.88, 129.31, 129.62, 130.20, 133.48, 134.85, 142.08, 142.82, 143. 59, 144.61, 146.04, 148.92
5k	3223, 3110, 3071, 3005, 2940, 1682, 1633, 1573, 1513, 1458, 1257, 1182, 1135, 1006, 771, 676	5.92 (s, 2H, NCH_2), 6.10 (s, 2H, NCH_2), 7.29–7.31 (d, 1H, -Ph), 7.48–7.52 (m, 2H, -Ph), 7.57–7.59 (d, 1H, -Ph), 8.19–8.22 (m, 2H, -Py), 8.27 (s,1H, NH_2), 8.41–8.43 (d, 1H, -Py), 8.61–8.66 (m, 3H), 8.98–9.01 (d, 1H, -Py), 9.20–9.21 (d, 1H, -Py), 9.26–9.28 (d, 1H, -Py), 9.65 (s,1H, NH_2), 13.04 (s, 1H, OH).	62.11, 62.93, 125.81, 127.55, 127.90, 129.17, 129.45, 129.73, 130.04, 134.28, 135.16, 141.11, 144.02, 144.86, 145.44, 146.37, 146.91, 147.48
5l	3298, 3137, 3039, 2969, 1685, 1658, 1573, 1515, 1459, 1385, 1311, 1170, 998, 780, 727	5.91 (s, 2H, NCH_2), 6.09 (s, 2H, NCH_2), 7.27–7.28 (d, 1H, -Ph), 7.48–7.57 (m, 3H, -Ph), 7.94 (s, 1H, NH_2), 8.19–8.23 (t, 1H, -Py), 8.30 (s, 1H, NH_2), 8.42–8.47 (m, 3H), 8.63–8.69 (m, 3H), 9.20–9.21 (d, 1H, -Py), 9.33–9.35 (m, 1H, -Py), 13.07 (s, 1H, OH).	61.91, 63.08, 125.82, 127.45, 127.66, 128.71, 129.55, 130.50, 135.17, 142.03, 145.46, 145.83, 146.19, 146.72, 147.87, 148.26
5m	3404, 3379, 3141, 3104, 3058, 2981, 1691, 1618, 1565, 1458, 1393, 1275, 1129, 875, 729	6.09 (s, 2H, NCH_2), 6.17 (s, 2H, NCH_2), 7.25–7.26 (d, 2H, -Ph), 7.51–7.53 (d, 2H, -Ph), 8.23 (s, 1H, NH_2), 8.28–8.29 (d, 1H, -Py), 8.32–8.35 (m, 2H, -Py), 8.48 (s, 1H, NH_2), 8.65 (s, 1H, CH═N), 9.04–9.05 (m, 3H, -Py), 9.18–9.20 (d, 1H, -Py), 9.53 (s, 1H, -Py), 12.93 (s, 1H, OH).	59.34, 59.79, 125.08, 128.43, 129.31, 129.78, 132.17, 132.76, 134.25, 144.01, 145. 19, 145.34, 145.70, 146.50, 148.85
5n	3398, 3366, 3154, 3109, 3034, 2983, 1690, 1634, 1571, 1455, 1411, 1286, 1137, 874, 724	6.08 (s, 2H, NCH_2), 6.15 (s, 2H, NCH_2), 7.23–7.27 (m, 2H, -Ph), 7.51–7.53 (m, 2H, -Ph), 7.94 (s, 1H, NH_2), 8.28–8.31 (m, 2H, -Py), 8.47 (s, 1H, CH═N), 8.48–8.50 (d, 2H, -Py), 8.73 (s, 1H, NH_2), 9.02–9.03 (d, 2H, -Py), 9.24–9.26 (d, 2H, -Py), 12.92 (s, 1H, OH)	59.75, 60.27, 124.421, 126.29, 129.12, 129.65, 130.12, 132.50, 132.87, 145.08, 145.20, 145.64, 146.13, 148.77, 149.14

(*continued on next page*)

Table 2 (*continued*)

Oxime	I.R. (KBr) $\gamma_{max}(cm^{-1})$	^1H NMR(400 MHz) δ, ppm, DMSO-d_6)	^{13}C NMR (100 MHz) δ, ppm, DMSO-d_6)
5o	3436, 3391, 3134, 3110, 3041, 2987, 1674, 1632, 1576, 1459, 1409, 1271, 1140, 861, 720	6.15 (s, 2H, NCH_2), 6.18 (s, 2H, NCH_2), 7.25–7.32 (m, 2H, -Ph), 8.20–8.24 (m, 2H, -Ph), 8.32–8.35 (m, 2H, -Py), 8.39 (s, 1H, NH_2), 8.64(s, 1H, CH=N), 8.79–8.81 (d, 1H, -Py), 9.04–9.06 (d, 2H, -Py), 9.18–9.20 (d, 2H, -Py), 9.26 (s, 1H, NH_2), 9.53 (s, 1H, -Py), 12.27 (s, 1H, OH)	61.59, 61.79, 128.87, 128.95, 129.01, 130.77, 131.65, 131.83, 134.17, 134.28, 142.54, 143.46, 144.51, 144.62, 145.05, 145.79, 146.49, 146.80
5p	3430, 3356, 3161, 3109, 3051, 2978, 1682, 1634, 1576, 1459, 1398, 1274, 1128, 874, 729, 609	6.14 (s, 2H, NCH_2), 6.15 (s, 2H, NCH_2), 7.25–7.29 (m, 2H, -Ph), 7.95 (s, 1H, -Ph), 8.21–8.23 (m, 1H, -Ph), 8.32 (s, 1H, NH_2), 8.39 (s, 1H, CH=N), 8.64 (s, 1H, NH_2),8.79–8.81 (d, 2H), 9.04–9.06 (d, 2H), 9.18–9.20 (d, 2H), 9.26 (s, 1H, -Py), 9.53 (s, 1H, -Py),12.27 (s, 1H, OH)	59.46, 59.98, 125.77, 128.38, 129.20, 130.55, 132.23, 132.35, 134.02, 141.91, 142.34, 143.12, 144.55, 146.01, 148.24
5q	3384, 3166, 3072, 3032, 2937, 1709, 1671, 1644, 1583, 1440, 1400, 1191, 1140, 735, 663	6.17 (s, 2H, NCH_2), 6.19 (s, 2H, NCH_2), 7.26–7.27 (m, 2H, -Ph), 7.51–7.53 (m, 2H, -Ph), 8.22–8.24 (m, 2H, -Py), 8.29 (s, 1H, NH_2), 8.41–8.43 (d, 1H, -Py), 8.62–8.67 (m, 3H, -Py), 9.01–9.03 (d, 1H, -Py), 9.26–9.27 (d, 1H, -Py), 9.41–9.44 (d, 1H, -Py), 9.61 (s, 1H, NH_2), 13.01 (s, 1H, OH).	58.09, 59.85, 126.42, 126.88, 128.24, 128.75, 129.18, 129.46, 129.94, 131.10, 132.31, 133.62, 141.30, 143.96, 145.15, 146.05, 146.34, 146.88, 148.08
5r	3428, 3375, 3145, 3113, 3042, 2993, 1672, 1629, 1565, 1451, 1403, 1279, 1132, 870, 721, 604	6.12 (s, 2H, NCH_2), 6.14 (s, 2H, NCH_2), 7.23–7.24 (m, 2H, -Ph), 7.55–7.57 (m, 2H, -Ph), 8.18–8.21 (t, 1H, -Py), 8.33 (s, 1H, NH_2), 8.39 (s, 1H, CH=N), 8.43–8.47 (m, 2H, -Py), 8.63–8.67 (m, 3H), 9.23–9.24 (d, 1H, -Py), 9.31–9.33 (m, 2H, -Py), 13.08 (s, 1H, OH).	58.14, 59.78, 125.59, 125.91, 128.04, 129.49, 129.75, 130.22, 131.30, 132.48, 142.09, 146.33, 147.81, 148.36

appeared in the regions of δ 57.8–62.9. Aromatic carbons of phenyl ring absorbed in the range of δ 124.5–138.5 and carbons on pyridinum rings appeared in the range of δ 129.2–148.9. The oximino carbon (CH=N) appeared at δ 144.50–150.8. The detailed spectral data is presented in Table 2.

4.3. In-vitro reactivation studies

The in-vitro reactivation of OP (sarin, DFP and VX)-inhibited AChE using test oximes were carried out in triplicate in phosphate buffer (0.1 M, pH 8.0 at 37 °C) using the method of Ellman et al. [34]. Values depicted in figures are average of triplicate runs with maximum relative standard deviation of ±2.5%. AChE stock solution (stock A) was prepared in phosphate buffer (pH 7.6, 0.1 M) (332 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 OP inhibitor (sarin, 1.4×10^{-2} M; DFP 1.08×10^{-2} M and VX, 1.33×10^{-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 OP (sarin, 1.4×10^{-6} M; DFP 1.08×10^{-4} M and VX, 3.32×10^{-7} 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 96–98% inhibition of enzyme activity. It was then followed by addition of 50 μL of oximes test solution (10^{-2} M & 10^{-3} M) to start reactivation. The final volume of the reactivation cocktail was 500 μL. The final concentration of OP inhibitor 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. 3). 20 μL 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 to 60 min. Percentage reactivation was calculated using the following equation [16–18];

$$\% \text{ Reactivation} = (E_r - E_i / E_o - E_i) \times 100$$

Where $\mathbf{E_o}$ is the control enzyme activity at 0 min (without inhibitor and oxime), $\mathbf{E_i}$ is the inhibited enzyme activity (without oxime) determined in the similar manner described above and $\mathbf{E_r}$ 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 OP but no oxime. Under these conditions spontaneous reactivation was found to be insignificant. All the values are corrected for their oxime induced hydrolysis.

Caution! Sarin, VX and DFP are highly toxic compounds and should be handled by trained personals only with proper protection using efficient fume hood. Great caution should be exercised especially while preparing their dilute solutions and residues containing these agents after each experiment must be properly destroyed by using 20% alkali solution.

4.4. Determination of acid dissociation constant (pKa)

The acid dissociation constants (pKa) of all the oximes were determined using the method of Albert and Sergeant [35]. The method is based on direct determination of the ratio of molecular species (protonated) to dissociated (deprotonated) species in a series of non-absorbing buffer solutions. For this purpose, the spectra of molecular species were obtained first in buffer solution of particular pH in which compounds of interest would be present wholly in either form. 30–50 μL of oxime stock solutions (5×10^{-3} M) were diluted to 3 mL in a cuvette containing either 0.1 M hydrochloric acid or 0.1 M sodium hydroxide solution and the absorption spectra of oxime in acid or alkali were measured over the wavelength range of 200–600 nm with a reference to blank solution at 25 ± 1 °C. The spectra, thus obtained in acid or alkali, were of protonated (D_m) and deprotonated (D_i) molecules. Eleven different pH values, ranging from 6.04 to 10.37 were selected to determine the pKa of oximes. For this, appropriate buffers consisting of phosphate (6.04–7.90), tris (8.55–9.15) and glycine-NaOH (9.47–10.37), were used to determine the dissociation constants of oximes. 30–50 μL of aqueous solutions of oximes were diluted to 3 mL in each buffer and optical densities were determined at analytical wave lengths using buffer blank at 25 ± 1 °C. A set of 11 values of pKa were obtained using Eq.(1)

$$pKa = pH + \log[(D_i - D)/(D - D_m)] \quad (1)$$

where, D_m, and D_i, correspond to the optical density of protonated and deprotonated forms of the oxime, and D is the optical density in the buffer.

The average value of the 11 measurements was considered the pKa of the compound with respect to oximino functionality. Thermospectronic Unicam 300 UV–Visible double beam spectrophotometer with quartz cells of 10 mm was used for spectrometric analysis. The quartz cells were attached to a thermostatic water bath (Julabo) for maintaining the constant temperature (25 ± 1.0 °C). pH values of buffers were determined using a Mettler-Toledo SevenEasy pH meter equipped with Inlab® Expert Pro glass electrode with an accuracy of ±0.01 units. The pH meter was calibrated at 25 °C using the two point calibration method with commercially available Mettler-Toledo standard buffer solutions pH 7.00 and 9.21.

Reagents - Freshly prepared standard solutions of oximes (5×10^{-3} M) in distilled water were used as stock solutions. Buffer solutions of appropriate pH were prepared according to the reported method [36]. Solutions of oximes in 0.1 M hydrochloric acid and 0.1 M sodium hydroxide were used for determining the analytical wavelength of undissociated and dissociated forms respectively.

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