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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

In vitro reactivation of organophosphorus (OP)-inhibited electric eel acetylcholinesterase by novel monoquaternary pyridinium oximes

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Abstract The synthesis of a series of monoquaternary pyridinium oximes bearing either a long-chain alkyloxymethyl or benzyloxymethyl side chain and the corresponding in vitro evaluation for the reactivation of electric eel acetylcholinesterase-inhibited organophosphorus inhibitors viz. sarin, DFP, and VX is reported. The data were compared with that of 2-PAM and obidoxime. Compounds bearing a benzyloxymethyl and 4-methylbenzyloxymethyl side chain showed better reactivation compared to obidoxime. However, none of the newly synthesized oximes bearing an aliphatic side chain could surpass the reactivation potential of 2-PAM. The p*K*a of the new oximes were determined and correlated with the observed reactivation potential.

Keywords Pyridinium oximes · Reactivators · 2-PAM · Acetylcholinesterase · Organophosphorus inhibitors

Abbreviations

AChE	Acetylcholinesterase
2-PAM	2-(hydroxyiminomethyl)-1-methylpyridinium chloride

Introduction

Acetylcholinesterase (AChE) terminates cholinergic neurotransmission by catalyzing the hydrolysis of the neurotransmitter acetylcholine (ACh) (Tougu, 2001). Hydrolysis of ACh is efficiently brought about by the serine hydroxyl moiety present at the bottom of the narrow 20 Å active site gorge of the enzyme AChE (Sussman *et al.*, 1991). AChE is the primary target of organophosphorus compounds (OPs) that irreversibly inhibit the enzyme (MacPhee-Quigly *et al.*, 1985). Some of these OPs have been developed in the past as chemical warfare nerve agents viz. sarin, soman, tabun, VX etc. Further, OP pesticides such as paraoxon, malathion, chlorpyriphos etc., are also used in the agriculture (Eyer, 2003). These OPs continue to be threat to the society due to their possible use during military conflicts or in terrorist acts (MacIlwain, 1993; Nagao *et al.*, 1997). The broad distribution of OPs causes several millions intoxication each year, of which some are fatal (Kwong, 2002; Gunnel and Eddleston, 2003).

OPs inhibit AChE by forming a stable alkyl phosphonate, phosphate or phosphoramidate conjugates that are covalently attached to the serine hydroxy group present in the active site (Bajgar, 2004). The function of the AChE can be restored by removal of the OP moiety from OP–AChE conjugate, a process known as reactivation. Strong nucleophiles such as oximes are reactivators of OP-inhibited AChE (Volans, 1996). The strength of the nucleophile, the orientation of the nucleophile with respect to the phosphate conjugated to the active center serine, and dealkylation of the OP–AChE conjugate (aging) are well known factors that affect the process of reactivation (Wong *et al.*, 2000). Based on these factors, various quaternary pyridinium oximes viz. 2-PAM, obidoxime, HI-6, and TMB-4 (Fig. 1) were subsequently developed and used as therapeutic agents in the treatment of OP intoxication.

However, none of the currently available oximes is able to reactivate inhibited AChE due to diversity in the

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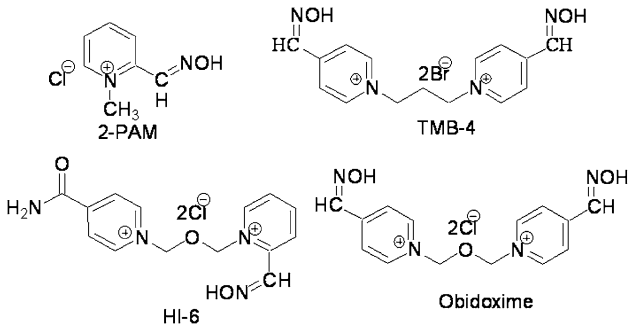


Fig. 1 Commonly used reactivators

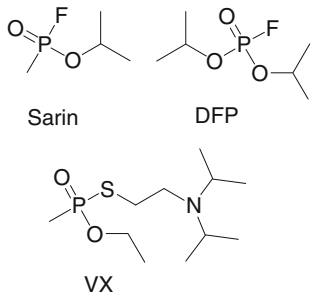


Fig. 2 Structure of OP inhibitors used in this study

structure of OP compounds (Bajgar, 1996). Therefore, an effective therapy by a single oxime to all the known nerve agents is still lacking.

The numerous new oximes have been synthesized and tested in the last decade (Musilek *et al.*, 2007; Bharate *et al.*, 2009; Jeong *et al.*, 2009; Worek *et al.*, 2007). Recently several bis-pyridinium oximes linked by variable lengths of alkylene and bis-methoxy alkane chain were reported as AChE reactivators (Pang *et al.*, 2003; Acharya *et al.*, 2009a, b; Musilek *et al.*, 2006). 2-PAM and 4-PAM analogs of quaternary monopyridinium oximes with varying side chain are also reported (Odzak *et al.*, 2007; Bharate *et al.*, 2009). Again, docking studies with these types of compounds with OP-inhibited AChE led to the assumption that the linker between pyridinium and aromatic ring should be longer to achieve better reactivation (Odzak *et al.*, 2007).

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 (Epstein *et al.*, 1978; Katrolia *et al.*, 1994). Therefore, in continuation of our work on antidotes against nerve agents (Acharya, *et al.*, 2008, 2009a, b, 2010), herein we report the synthesis of a new series of monoquaternary pyridinium oximes bearing either a long-chain alkyloxymethyl or a benzyloxymethyl side chain to enhance the lipid solubility and stability, which is found to be low with the currently available antidotes (Gray, 1984) as well as to allow the hydrophobic interactions with some of the aromatic residues that are present in the active site gorge of the AChE (Tougu, 2001). The synthesized oximes were evaluated for their in vitro reactivation potential against electric eel AChE inhibited by sarin, VX, and diisopropylfluoro phosphate (DFP) (Fig. 2).

Materials and methods

Chemicals

Chloromethyl ethers were prepared according to the known synthetic method (Farren *et al.*, 1925). Sarin, DFP, and VX were prepared in this laboratory with >98 % purity (GC and ^{31}P NMR). 2-PAM was prepared according to the method of Ginsburg and Wilson (1957). The synthesis of pyridinium oximes involved alkylations of 3-, and 4-pyridine aldoxime with chloromethoxy alkane, chloromethoxymethyl benzene, and 1-chloromethoxymethyl-4-methyl benzene (Scheme 1). The method afforded compounds **3a**–**3r** with good yields within 3–6 h (Table 1). 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 and characterized by their elemental analysis and spectral data.

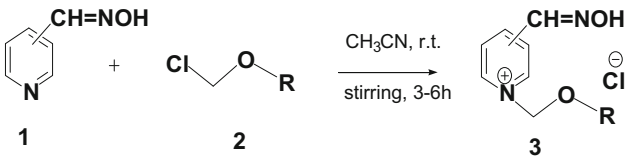
Chemistry

*General procedure for the synthesis of monoquaternary pyridinium oximes (**3a**–**3r**)*

Hydroxyiminomethyl pyridine (1.0 g, 8.2 mmol) in 50 mL dry acetonitrile was taken in a two-neck round bottom flask equipped with a calcium chloride guard tube, magnetic stirrer, and dropping funnel. To this was added chloromethoxy alkane, (10.0 mmol) slowly over 30 min with stirring at room temperature. It was then further stirred for 3–6 h at room temperature and monitored by TLC. The solid obtained was filtered off, washed repeatedly with hot dry acetone and recrystallized with dry methanol–acetone mixture to give pure product.

*Dodecyloxymethyl-4-(hydroxyiminomethyl)-pyridinium bromide (**3a**)*

FTIR (KBr cm^{-1}): 3423 (O–H str), 3011, 2920, 2853 (C–H str), 1646, 1467 (C–C aryl), 1301, 1159 (C–N str), 1111,



Scheme 1 Synthesis of quaternary monopyridinium oximes

Table 1 Physical data of monopyridinium oximes

Oxime	–CH=NOH	R	Time (h)	Yield[a] (%)	MP[b] (°C)	p*K*a
3a	4	$C_{12}H_{25}$	3	76	156–60	8.23 ± 0.04
3b	4	$C_{11}H_{23}$	3	70	206–10	8.20 ± 0.03
3c	4	$C_{10}H_{21}$	3	77	190–92	8.17 ± 0.03
3d	4	C_9H_{19}	4	78	186–90	8.15 ± 0.03
3e	4	C_8H_{17}	4	68	196–198	8.21 ± 0.05
3f	4	C_7H_{15}	5	62	198–200	8.22 ± 0.04
3g	4	C_6H_{13}	5	68	208–10	8.20 ± 0.05
3h	4	$–CH_2C_6H_5$	5	65	138–40	8.25 ± 0.03
3i	4	$–CH_2C_6H_4$-4-CH_3	6	57	140–42	8.23 ± 0.03
3j	3	$C_{12}H_{25}$	3	78	172–74	9.17 ± 0.04
3k	3	$C_{11}H_{23}$	3	70	174–76	9.23 ± 0.05
3l	3	$C_{10}H_{21}$	4	66	170–74	9.25 ± 0.05
3m	3	C_9H_{19}	5	60	158–60	9.41 ± 0.04
3n	3	C_8H_{17}	5	69	160–62	9.25 ± 0.05
3o	3	C_7H_{15}	5	51	152–54	9.24 ± 0.05
3p	3	C_6H_{13}	6	62	180–82	9.27 ± 0.05
3q	3	$–CH_2C_6H_5$	5	63	144–46	9.25 ± 0.04
3r	3	$–CH_2C_6H_4$-4-CH_3	6	66	144–48	9.14 ± 0.03
2-PAM						7.83 ± 0.04
Obidoxime						7.85 ± 0.04

[a] Isolated yields

[b] Uncorrected melting points

1018 (C–O–C str), 778 cm^{-1}–$(CH_2)_n$–; ^{1}H NMR (400 MHz, DMSO-d_6) δ 0.85 (t, $J = 8$ Hz, 3H, $–CH_3$), 1.21 (m, 18H, $–CH_2–$), 1.59 (m, 2H, $–CH_2–$), 3.56 (t, $J = 6$ Hz, 2H, OCH_2), 5.89 (s, 2H, NCH_2), 8.29 (d, $J = 8$ Hz, 2H, –Py), 8.47 (s, 1H, CH=N), 9.08 (d, $J = 8$ Hz, 2H, –Py), 12.98 (s, 1H, OH); ^{13}C NMR (100 MHz, DMSO-d_6) δ 13.92, 22.14, 25.24, 28.66, 28.73, 28.98, 29.03, 29.06, 31.33, 70.28, 88.23, 124.06, 143.68, 145.26, 150.04; Anal. Calcd for $C_{19}H_{33}ClN_2O_2$: C, 63.95; H, 9.25; N, 7.85. Found: C, 63.54; H, 9.37; N, 8.18; *ESI–MS: *m/z* 321.35 $[M]^+$ $[C_{19}H_{33}N_2O_2]^{+\cdot}$

*Undecyloxymethyl-4-(hydroxyiminomethyl)-pyridinium bromide (**3b**)*

FTIR (KBr cm^{-1}): 3416 (O–H str), 3010, 2922, 2852 (C–H str), 1644, 1463 (C–C aryl), 1302, 1169 (C–N str), 1111 (C–O–C str), 1007, 779 cm^{-1}–$(CH_2)_n$–; ^{1}H NMR (400 MHz, DMSO-d_6) δ 0.85 (t, $J = 8$ Hz, 3H, $–CH_3$), 1.22 (m, 16H, $–CH_2–$), 1.61 (m, 2H, $–CH_2–$), 3.57 (t, $J = 6$ Hz, 2H, OCH_2), 5.91 (s, 2H, NCH_2), 8.29 (d, $J = 8$ Hz, 2H, –Py), 8.47 (s, 1H, CH=N), 9.10 (d, $J = 8$ Hz, 2H, –Py), 13.00 (s, 1H, OH); ^{13}C NMR (100 MHz, DMSO-d_6) δ 13.94, 22.07, 25.18, 28.61, 28.67, 28.92, 28.96, 31.26, 70.17, 88.11, 123.97, 143.67, 145.16, 149.97; Anal. Calcd for $C_{18}H_{31}ClN_2O_2$: C, 63.06; H, 9.05; N, 8.17. Found: C, 63.47; H, 9.24; N, 7.91; *ESI–MS: *m/z* 307.25 $[M]^+$ $[C_{18}H_{31}N_2O_2]^{+\cdot}$

*Decyloxymethyl-4-(hydroxyiminomethyl)-pyridinium bromide (**3c**)*

FTIR (KBr cm^{-1}): 3429 (O–H str), 3009, 2921, 2853 (C–H str),1645, 1466 (C–C aryl), 1301, 1155 (C–N str), 1110, 1016 (C–O–C str), 778 cm^{-1}–$(CH_2)_n$–; ^{1}H NMR (400 MHz, DMSO-d_6) δ 0.84 (t, $J = 6$ Hz, 3H, $–CH_3$), 1.23 (m, 14H, $–CH_2–$), 1.52 (m, 2H, $–CH_2–$), 3.56 (t, $J = 6$ Hz, 2H, OCH_2), 5.90 (s, 2H, NCH_2), 8.29 (d, $J = 8$ Hz, 2H, –Py), 8.47 (s, 1H, CH=N), 9.09 (d, $J = 8$ Hz, 2H, –Py), 12.98 (s, 1H, OH); ^{13}C NMR (100 MHz, DMSO-d_6) δ 13.94, 22.06, 25.17, 28.60, 28.64, 28.91, 31.25, 70.15, 88.09, 123.96, 143.68, 145.14, 149.97; Anal. Calcd for $C_{17}H_{29}ClN_2O_2$: C, 62.10; H, 8.82; N, 8.52. Found: C, 62.33; H, 8.36; N, 8.23; *ESI–MS: *m/z* 293.28 $[M]^+$ $[C_{18}H_{31}N_2O_2]^{+\cdot}$

*Nonyloxymethyl-4-(hydroxyiminomethyl)-pyridinium bromide (**3d**)*

FTIR (KBr cm^{-1}): 3410 (O–H str), 3016, 2924, 2853 (C–H str), 1647, 1463 (C–C aryl), 1403, 1292 (C–N str), 1154, 1103 (C–O–C str), 999, 785 cm^{-1} –$(CH_2)_n$–; ^{1}H NMR

(400 MHz, DMSO-d_6) δ 0.84 (t, $J = 6$ Hz, 3H, $-CH_3$), 1.22 (m, 12H, $-CH_2-$), 1.50 (m, 2H, $-CH_2-$), 3.56 (t, $J = 6$ Hz, 2H, OCH_2), 5.90 (s, 2H, NCH_2), 8.30 (d, $J = 8$ Hz, 2H, –Py), 8.47 (s, 1H, CH=N), 9.11 (d, $J = 8$ Hz, 2H, –Py), 13.00 (s, 1H, OH); ^{13}C NMR (100 MHz, DMSO-d_6) δ 13.98, 22.11, 25.22, 25.66, 28.44, 28.81, 29.34, 31.29, 70.75, 88.81, 124.06, 143.68, 145.26, 150.04; Anal. Calcd for $C_{16}H_{27}ClN_2O_2$: C, 61.04; H, 8.58; N, 8.90. Found: C, 60.65; H, 8.97; N, 8.56; *ESI–MS: *m/z* 279.21 $[M]^+$ $[C_{16}H_{27}N_2O_2]^{+\cdot}$

*Octyloxymethyl-4-(hydroxyiminomethyl)-pyridinium bromide (**3e**)*

FTIR (KBr cm^{-1}): 3434 (O–H str), 3010, 2925, 2855(C–H str), 1644, 1463(C–C aryl), 1302(C–N str), 1161, 1108(C–O–C str), 1015, 779 cm^{-1}–$(CH_2)_n$–; ^{1}H NMR (400 MHz, DMSO-d_6) δ 0.84 (t, $J = 6$ Hz, 3H, $-CH_3$), 1.21 (m, 10H, $-CH_2-$), 1.52 (m, 2H, $-CH_2-$), 3.57 (t, $J = 6$ Hz, 2H, OCH_2), 5.91 (s, 2H, NCH_2), 8.30 (d, $J = 4$ Hz, 2H, –Py), 8.47 (s, 1H, CH=N), 9.10 (d, $J = 4$ Hz, 2H, –Py), 13.01 (s, 1H, OH); ^{13}C NMR (100 MHz, DMSO-d_6) δ 14.38, 22.50, 25.65, 29.03, 29.07, 31.64, 70.62, 88.55, 124.42, 144.16, 145.59, 150.45; Anal. Calcd for $C_{15}H_{25}ClN_2O_2$: C, 59.90; H, 8.31; N, 9.31. Found: C, 59.78; H, 8.66; N, 8.88; *ESI–MS: *m/z* 265.22 $[M]^+$ $[C_{15}H_{25}N_2O_2]$.

*Heptyloxymethyl-4-(hydroxyiminomethyl)-pyridinium bromide (**3f**)*

FTIR (KBr cm^{-1}): 3425 (O–H str), 3029, 2928, 2862(C–H str), 1641, 1458 (C–C aryl), 1301(C–N str), 1157, 1111(C–O–C str), 998, 796 cm^{-1}–$(CH_2)_n$–; ^{1}H NMR (400 MHz, DMSO-d_6) δ 0.84 (t, $J = 8$ Hz, 3H, $-CH_3$), 1.22 (m, 8H, $-CH_2-$), 1.52 (m, 2H, $-CH_2-$), 3.58 (t, $J = 6$ Hz, 2H, OCH_2), 5.93 (s, 2H, NCH_2), 8.30 (d, $J = 8$ Hz, 2H, –Py), 8.48 (s, 1H, CH=N), 9.13 (d, $J = 4$ Hz, 2H, –Py), 13.05 (s, 1H, OH); ^{13}C NMR (100 MHz, DMSO-d_6) δ 13.90, 22.03, 25.46, 25.67, 28.43, 29.22, 31.25, 70.40, 88.81, 123.27, 142.72, 145.59, 148.67; Anal. Calcd for $C_{14}H_{23}ClN_2O_2$: C, 58.63; H, 8.02; N, 9.77. Found: C, 58.48; H, 8.22; N, 9.54; *ESI–MS: *m/z* 251.23 $[M]^+$ $[C_{14}H_{23}N_2O_2]^{+\cdot}$

*Hexyloxymethyl-4-(hydroxyiminomethyl)-pyridinium bromide (**3g**)*

FTIR (KBr cm^{-1}): 3414 (O–H str), 3010, 2924, 2855(C–H str), 1645, 1463(C–C aryl), 1302(C–N str), 1158, 1108, 1015 (C–O–C str),933, 778 cm^{-1} –$(CH_2)_n$–; ^{1}H NMR (400 MHz, DMSO-d_6) δ 0.83 (t, $J = 6$ Hz, 3H, $-CH_3$), 1.24 (m, 6H, $-CH_2-$), 1.52 (m, 2H, $-CH_2-$), 3.58 (t, $J = 8$ Hz, 2H, OCH_2), 5.91 (s, 2H, NCH_2), 8.29 (d, $J = 8$ Hz, 2H, –Py), 8.47 (s, 1H, CH=N), 9.10 (d, $J = 8$ Hz, 2H, –Py), 13.00 (s, 1H, OH); ^{13}C NMR (100 MHz, DMSO-d_6) δ 13.94, 22.12, 25.33, 25.69, 28.51, 29.21, 31.29, 70.21, 88.95, 123.48, 142.61, 145.66, 148.42; Anal. Calcd for $C_{13}H_{21}ClN_2O_2$: C, 57.24; H, 7.70; N, 10.27. Found: C, 57.57; H, 7.88; N, 9.80; *ESI–MS: *m/z* 237.19 $[M]^+$ $[C_{13}H_{21}N_2O_2]^{+\cdot}$

*Benzyloxymethyl-4-(hydroxyiminomethyl)-pyridinium bromide (**3h**)*

FTIR (KBr cm^{-1}): 3436 (O–H str), 3012, 2939, 2822 (C–H str), 1637, 1596, 1459 (C–C aryl), 1293 (C–N str), 1159, 1108 (C–O–C str), 1006, 753 cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6) δ 4.72 (s, 2H, OCH_2), 6.04 (s, 2H, NCH_2), 7.33 (m, 5H, –Ph), 8.26 (d, $J = 8$ Hz, 2H, –Py), 8.46 (s, 1H, CH=N), 9.14 (d, $J = 8$ Hz, 2H, –Py), 13.05 (s, 1H, OH); ^{13}C NMR (100 MHz, DMSO-d_6) δ 72.04, 87.83, 122.22, 123.89, 128.07, 128.43, 136.15, 143.87, 145.10, 149.99; Anal. Calcd for $C_{14}H_{15}ClN_2O_2$: C, 60.32; H, 5.38; N, 10.05. Found: C, 60.44; H, 5.67; N, 9.80; *ESI–MS: *m/z* 243.25 $[M]^+$ $[C_{14}H_{15}N_2O_2]^{+\cdot}$

*(4-Methyl-benzyloxy)-4-(hydroxyiminomethyl)-pyridinium bromide (**3i**)*

FTIR (KBr cm^{-1}): 3427 (O–H str), 3018, 2947, 2832 (C–H str), 1642, 1607, 1458 (C–C aryl), 1304 (C–N str), 1154, 1099 (C–O–C str), 1011, 798 cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6) δ 2.28 (s, 3H, $-CH_3$) 4.67 (s, 2H, OCH_2), 6.02 (s, 2H, NCH_2), 7.18 (m, 4H, –Ph), 8.26 (d, $J = 8$ Hz, 2H, –Py), 8.47 (s, 1H, CH=N), 9.12 (d, $J = 8$ Hz, 2H, –Py), 13.04 (s, 1H, OH); ^{13}C NMR (100 MHz, DMSO-d_6) δ 21.21, 72.41, 88.21, 124.37, 128.07, 129.04, 129.44, 133.52, 144.26, 145.60, 150.43; Anal. Calcd for $C_{15}H_{17}ClN_2O_2$: C, 61.53; H, 5.81; N, 9.57. Found: C, 61.18; H, 6.28; N, 9.61; *ESI–MS: *m/z* 257.23 $[M]^+$ $[C_{15}H_{17}N_2O_2]^{+\cdot}$

*Dodecyloxymethyl-3-(hydroxyiminomethyl)-pyridinium bromide (**3j**)*

FTIR (KBr cm^{-1}): 3420 (O–H str), 3015, 2936, 2838 (C–H str), 1651, 1460(C–C aryl), 1410, 1299 (C–N str), 1152, 1106 (C–O–C str), 1009, 789 cm^{-1}–$(CH_2)_n$–; ^{1}H NMR (400 MHz, DMSO-d_6) δ 0.85 (t, $J = 6$ Hz, 3H, $-CH_3$), 1.23 (m, 18H, $-CH_2-$), 1.52 (m, 2H, $-CH_2-$), 3.58 (t, $J = 6$ Hz, 2H, OCH_2), 5.96 (s, 2H, NCH_2), 8.22 (m, 1H, –Py), 8.41 (s, 1H, CH=N), 8.81 (d, $J = 8$ Hz, 1H, –Py), 9.10 (d, $J = 4$ Hz, 1H, –Py), 9.30 (s, 1H, –Py), 12.32 (s, 1H, OH); ^{13}C NMR (100 MHz, DMSO-d_6) δ 13.92, 22.05, 25.16, 28.58, 28.66, 28.91, 28.98, 31.25, 70.40, 88.81,

128.11, 133.50, 140.81, 142.89, 143.29, 144.05; Anal. Calcd for $C_{19}H_{33}ClN_2O_2$: C, 63.95; H, 9.25; N, 7.85. Found: C, 63.60; H, 9.38; N, 8.14; *ESI–MS: *m/z* 321.31 $[M]^+$ $[C_{19}H_{33}N_2O_2]^{+\cdot}$

*Undecyloxymethyl-3-(hydroxyiminomethyl)-pyridinium bromide (**3k**)*

FTIR (KBr cm^{-1}): 3434 (O–H str), 3017, 2934, 2856(C–H str), 1644, 1456 (C–C aryl), 1290 (C–N str), 1159, 1108 (C–O–C str), 1002, 796 cm^{-1}–$(CH_2)_n$–; ^{1}H NMR (400 MHz, DMSO-d_6) δ 0.84 (t, $J = 6$ Hz, 3H, –CH_3), 1.23 (m, 16H, –CH_2–), 1.52 (m, 2H, –CH_2–), 3.59 (t, $J = 8$ Hz, 2H, OCH_2), 5.96 (s, 2H, NCH_2), 8.22 (m, 1H, –Py), 8.41 (s, 1H, CH=N), 8.81 (d, $J = 8$ Hz, 1H, –Py), 9.11 (d, $J = 8$ Hz, 1H, –Py), 9.31 (s, 1H, –Py), 12.34 (s, 1H, OH); ^{13}C NMR (100 MHz, DMSO-d_6) δ 13.94, 22.18, 25.29, 28.44, 28.71, 28.89, 28.92, 31.34, 70.51, 88.82, 126.36, 133.51, 140.49, 142.82, 143.48, 144.11; Anal. Calcd for $C_{18}H_{31}ClN_2O_2$: C, 63.06; H, 9.05; N, 8.17. Found; C, 63.31; H, 8.81; N, 8.29; *ESI–MS: *m/z* 307.29 $[M]^+$ $[C_{18}H_{31}N_2O_2]^{+\cdot}$

*Decyloxymethyl-3-(hydroxyiminomethyl)-pyridinium bromide (**3l**)*

FTIR (KBr cm^{-1}): 3443 (O–H str), 3012, 2929, 2858(C–H str), 1649, 1461(C–C aryl), 1302(C–N str), 1153, 1111(C–O–C str), 1019, 777 cm^{-1}–$(CH_2)_n$–; ^{1}H NMR (400 MHz, DMSO-d_6) δ 0.85 (t, $J = 6$ Hz, 3H, –CH_3), 1.23 (m, 14H, –CH_2–), 1.52 (m, 2H, –CH_2–), 3.59 (t, $J = 8$ Hz, 2H, OCH_2), 5.97 (s, 2H, NCH_2), 8.22 (m, 1H, –Py), 8.41 (s, 1H, CH=N), 8.81 (d, $J = 8$ Hz, 1H, –Py), 9.11–9.12 (d, $J = 4$ Hz, 1H, –Py), 9.32 (s, 1H, –Py), 12.35 (s, 1H, OH); ^{13}C NMR (100 MHz, DMSO-d_6) δ 13.92, 22.06, 25.17, 25.69, 28.61, 29.01, 29.21, 31.25, 70.37, 88.75, 126.49, 139.40, 142.36, 142.83, 143.59, 144.03; Anal. Calcd for $C_{17}H_{29}ClN_2O_2$: C, 62.10; H, 8.82; N, 8.52. Found: C, 62.36; H, 8.65; N, 8.22; *ESI–MS: *m/z* 293.27 $[M]^+$ $[C_{18}H_{31}N_2O_2]^{+\cdot}$

*Nonyloxymethyl-3-(hydroxyiminomethyl)-pyridinium bromide (**3m**)*

FTIR (KBr cm^{-1}): 3439 (O–H str), 3015, 2936, 2830 (C–H str), 1631, 1594, 1461(C–C aryl), 1297 (C–N str), 1155, 1106 (C–O–C str), 998, 768 cm^{-1}–$(CH_2)_n$–; ^{1}H NMR (400 MHz, DMSO-d_6) δ 0.84 (t, $J = 6$ Hz, 3H, –CH_3), 1.23 (m, 12H, –CH_2–), 1.52 (m, 2H, –CH_2–), 3.59 (t, $J = 8$ Hz, 2H, OCH_2), 5.95 (s, 2H, NCH_2), 8.22 (m, 1H, –Py), 8.41 (s, 1H, CH=N), 8.81 (d, $J = 8$ Hz, 1H, –Py), 9.08 (d, $J = 4$ Hz, 1H, –Py), 9.29 (s, 1H, –Py), 12.31 (s, 1H, OH); ^{13}C NMR (100 MHz, DMSO-d_6) δ 13.94, 22.08, 25.17, 25.71, 28.65, 29.22, 31.27, 70.41, 88.84, 125.94, 138.22, 142.91, 143.07, 143.33, 144.45; Anal. Calcd for $C_{16}H_{27}ClN_2O_2$: C, 61.04; H, 8.58; N, 8.90. Found: C, 61.39; H, 8.68; N, 8.76; *ESI–MS: *m/z* 279.23 $[M]^+$ $[C_{16}H_{27}N_2O_2]^{+\cdot}$

*Octyloxymethyl-3-(hydroxyiminomethyl)-pyridinium bromide (**3n**)*

FTIR (KBr cm^{-1}): 3427 (O–H str), 3009, 2941, 2838 (C–H str), 1645, 1609, 1463(C–C aryl), 1296(C–N str), 1159, 1098 (C–O–C str), 1008, 787 cm^{-1}–$(CH_2)_n$–; ^{1}H NMR (400 MHz, DMSO-d_6) δ 0.84 (t, $J = 8$ Hz, 3H, –CH_3), 1.23 (m, 10H, –CH_2–), 1.51 (m, 2H, –CH_2–), 3.59 (t, $J = 6$ Hz, 2H, OCH_2), 5.97 (s, 2H, NCH_2), 8.22 (m, 1H, –Py), 8.41 (s, 1H, CH=N), 8.81 (d, $J = 8$ Hz, 1H, –Py), 9.12 (d, $J = 8$ Hz, 1H, –Py), 9.32 (s, 1H, –Py), 12.35 (s, 1H, OH); ^{13}C NMR (100 MHz, DMSO-d_6) δ 13.90, 22.05, 25.50, 28.71, 29.20, 31.21, 66.92, 87.84, 126.81, 137.25, 142.40, 142.86, 143.34, 143.87; Anal. Calcd for $C_{15}H_{25}ClN_2O_2$: C, 59.90; H, 8.31; N, 9.31. Found: C, 59.68; H, 8.24; N, 9.59; *ESI–MS: *m/z* 265.19 $[M]^+$ $[C_{15}H_{25}N_2O_2]^{+\cdot}$

*Heptyloxymethyl-3-(hydroxyiminomethyl)-pyridinium bromide (**3o**)*

FTIR (KBr cm^{-1}): 3435 (O–H str), 3011, 2928, 2856 (C–H str), 1639, 1454 (C–C aryl), 1308, (C–N str), 1165, 1094 (C–O–C str), 998, 779 cm^{-1}–$(CH_2)_n$; ^{1}H NMR (400 MHz, DMSO-d_6) δ 0.83 (t, $J = 6$ Hz, 3H, –CH_3), 1.22 (m, 8H, –CH_2–), 1.52 (m, 2H, –CH_2–), 3.58 (t, $J = 6$ Hz, 2H, OCH_2), 5.98 (s, 2H, NCH_2), 8.21 (m, 1H, –Py), 8.41 (s, 1H, CH=N), 8.81 (d, $J = 8$ Hz, 1H, –Py), 9.09 (d, $J = 8$ Hz, 1H, –Py), 9.33 (s, 1H, –Py), 12.39 (s, 1H, OH; ^{13}C NMR (100 MHz, DMSO-d_6) δ 13.90, 22.03, 25.46, 25.67, 28.43, 29.22, 31.25, 66.94, 94.49, 126.72, 139.62, 141.23, 142.42, 143.12, 143.96; Anal. Calcd for $C_{14}H_{23}ClN_2O_2$: C, 58.63; H, 8.02; N, 9.77. Found: C, 58.71; H, 8.50; N, 9.48; *ESI–MS: *m/z* 251.27 $[M]^+$ $[C_{14}H_{23}N_2O_2]^{+\cdot}$

*Hexyloxymethyl-3-(hydroxyiminomethyl)-pyridinium bromide (**3p**)*

FTIR (KBr cm^{-1}): 3424 (O–H str), 3010, 2924, 2856(C–H str), 1645, 1459(C–C aryl), 1296(C–N str), 1158, 1110, 1015 (C–O–C str), 786 cm^{-1}–$(CH_2)_n$; ^{1}H NMR (400 MHz, DMSO-d_6) δ 0.83 (t, $J = 8$ Hz, 3H, –CH_3), 1.23 (m, 6H, –CH_2–), 1.51 (m, 2H, –CH_2–), 3.60 (t, $J = 6$ Hz, 2H, OCH_2), 5.98 (s, 2H, NCH_2), 8.22 (m, 1H, –Py), 8.41 (s, 1H, CH=N), 8.82 (d, $J = 8$ Hz, 1H, –Py), 9.13 (d, $J = 4$ Hz, 1H, –Py), 9.33 (s, 1H, –Py), 12.37 (s, 1H, OH); ^{13}C NMR (100 MHz, DMSO-d_6) δ 13.92, 22.12, 25.38, 25.61, 28.48,

29.52, 31.34, 67.22, 94.29, 126.77, 139.58, 141.14, 142.47, 143.22, 143.89; Anal. Calcd for $C_{13}H_{21}ClN_2O_2$: C, 57.24; H, 7.70; N, 10.27. Found: C, 57.33; H, 7.92; N, 10.02; *ESI–MS: *m/z* 237.25 $[M]^+$ $[C_{13}H_{21}N_2O_2]^{+\cdot}$

*Benzyloxymethyl-3-(hydroxyiminomethyl)-pyridinium bromide (**3q**)*

FTIR (KBr cm^{-1}): 3430 (O–H str), 3016, 2935, 2820(C–H str), 1635, 1598, 1463(C–C aryl), 1293(C–N str), 1160, 1108 (C–O–C str), 1007, 765 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6) δ 4.76 (s, 2H, OCH_2), 6.09 (s, 2H, NCH_2), 7.32 (m, 5H, –Ph), 8.19 (m, 1H, –Py), 8.39 (s, 1H, CH=N), 8.78 (d, $J = 8$ Hz, 1H, –Py), 9.13 (d, $J = 4$ Hz, 1H, –Py), 9.33 (s, 1H, –Py), 12.34 (s, 1H, OH); ^{13}C NMR (100 MHz, DMSO-d_6) δ 72.32, 88.61, 126.60, 127.70, 128.27, 128.48, 133.05, 133.40, 138.05, 142.86, 143.37, 144.01; Anal. Calcd for $C_{14}H_{15}ClN_2O_2$: C, 60.32; H, 5.38; N, 10.05. Found: C, 59.88; H, 5.47; N, 10.18; *ESI–MS: *m/z* 243.21 $[M]^+$ $[C_{14}H_{15}N_2O_2]^{+\cdot}$

*(4-Methyl-benzyloxy)-3-(hydroxyiminomethyl)-pyridinium bromide (**3r**)*

FTIR (KBr cm^{-1}): 3427 (O–H str), 3012, 2947, 2832 (C–H str), 1642, 1607, 1458 (C–C aryl), 1304 (C–N str), 1154, 1090 (C–O–C str), 1011, 787 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6) δ 2.22 (s, 3H, $–CH_3$) 4.59 (s, 2H, OCH_2), 6.06 (s, 2H, NCH_2), 7.17 (m, 4H, –Ph), 8.18 (m, 1H, –Py), 8.38 (s, 1H, CH=N), 8.78 (d, $J = 8$ Hz, 1H, –Py), 9.11 (d, $J = 4$ Hz, 1H, –Py), 9.29 (s, 1H, –Py), 12.34 (s, 1H, OH); ^{13}C NMR (100 MHz, DMSO-d_6) δ 20.72, 72.24, 88.47, 126.48, 127.58, 128.70, 128.94, 133.05, 133.40, 137.57, 142.70, 143.07, 143.11; Anal. Calcd for $C_{15}H_{17}ClN_2O_2$: C, 61.53; H, 5.81; N, 9.57. Found: C, 61.66; H, 5.94; N, 9.71; *ESI–MS: *m/z* 257.25 $[M]^+$ $[C_{15}H_{17}N_2O_2]^{+\cdot}$

Determination of acid dissociation constant (p*K*a)

The acid dissociation constants (p*K*a) of all the oximes were determined using the method of Albert and Sergeant (1971). 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. Oxime stock solutions (30–50 μL, 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 wave-length 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 p*K*a 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. Aqueous solutions of oximes (30–50 μL) 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 p*K*a 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 as p*K*a 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). The 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 U. 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 (Gomori, 1955). 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.

In vitro reactivation studies

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.* (1961). 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 U/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)

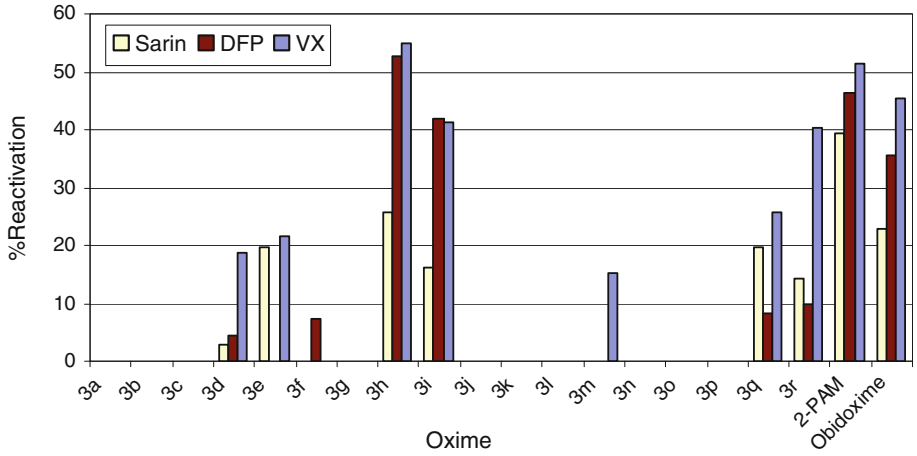


Fig. 3 Efficacy of tested oximes in reactivation of OP-inhibited AChE in comparison with 2-PAM and obidoxime. Source of enzyme: electric eel, inhibitor agent sarin, DFP, and VX; time of inhibition 15 min; time of reactivation 10 min; pH 8.0; temperature 37 °C. The values are average of three runs with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

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) to start reactivation. The final volume of the reactivation cocktail was 500 µL. The final concentration of OP inhibitor and oxime was diluted tenfold in the reactivation cocktail. After 10 min of reactivation the enzyme activity was assayed by Ellman's method (Fig. 3). An aliquot of reactivation cocktail (20 µL) 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 and 1.25 mM, respectively. The reactivation of inhibited enzyme was then studied at an interval of 10 min and followed up to 1 h (Fig. 4). Percentage reactivation was calculated using the following equation (Acharya *et al.*, 2008, 2009a, b, 2010)

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

Where E_o 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

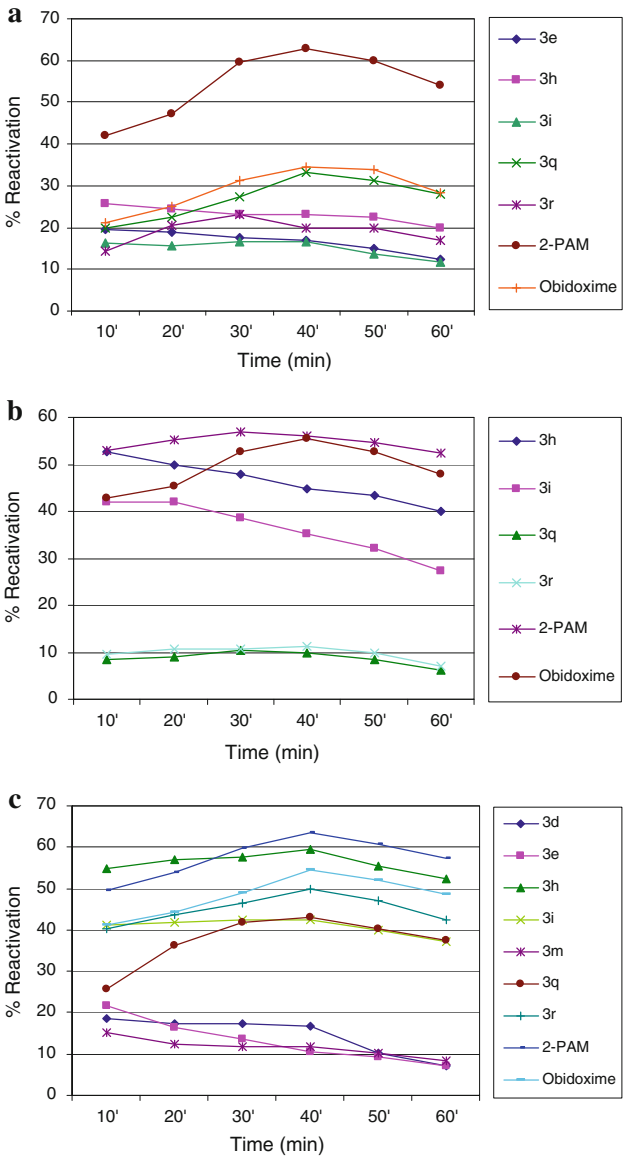


Fig. 4 Time-dependent reactivation profile **a** sarin, **b** DFP, and **c** VX of synthesized monopyridinium oximes

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 not oxime. Under these conditions spontaneous reactivation was found to be insignificant. All the values are corrected for their oxime-induced hydrolysis.

Results and discussion

The oximes studied in this investigation represent a homologous series that differ only in the length of the alkyl group in the alkoxymethyl and methyl group in the benzyloxymethyl side chain. With an increasing number of carbon atoms, the lipophilicity of the compound increases and the dissociation ability of the oxime group increases as well (Patocka and Bielavsky 1972). The in vitro reactivation data of sarin, DFP, and VX-inhibited electric eel AChE by monoquaternary pyridinium oximes bearing alkoxymethyl side chain at 10^{-3} M oxime concentration is presented in Fig. 3. Most of the oximes with aliphatic side chains showed either poor or insignificant reactivation. Oximes containing benzyloxymethyl side chain have showed increased reactivation for DFP- and VX-inhibited AChE. It is evident from these data that best reactivation was observed with the oxime 1-benzyloxymethyl-4-(hydroxyiminomethyl)-pyridinium chloride (**3h**) and 4-(hydroxyimino-methyl)-1-(4-methyl-benzyloxymethyl)-pyridinium chloride (**3i**) in case of DFP- and VX-inhibited AChE. The oximes, **3h** and **3i** respectively reactivated 52 and 42 % of DFP-inhibited AChE in comparison to respectively 46 and 35 % reactivation by 2-PAM and obidoxime at a concentration of 10^{-3} M after 10 min. Similarly, both these oximes (**3h** and **3i**) were also able to reactivate 54 and 41 % of the VX-inhibited AChE, respectively in comparison to 52 and 45 % respective reactivation by 2-PAM and obidoxime at the same oxime concentration and same interval of time. In case of sarin-inhibited AChE, none of these oximes could surpass the reactivation potential of 2-PAM. However, the oxime **3h** showed only 26 % reactivation of sarin-inhibited AChE which was at par with that of obidoxime.

The greater activity of some of the oximes warranted to study their time-dependent reactivation profile (Fig. 4). This is worth noticing that, in case of sarin- and DFP-inhibited AChE (Fig. 4a, b), the reactivation potential of most of the synthesized oximes either decreased gradually or remained constant over 30–40 min. However, the reactivation of VX-inhibited AChE (Fig. 4c) by the synthesized oximes increased gradually with time and reached maximum in 40 min. The oxime **3h** was able to reactivate 59 % the VX-inhibited AChE after 40 min as compared to 63 and 54 % reactivation observed by 2-PAM and obidoxime, respectively.

The reactivation of OP-inhibited AChE is also dependent on factors such as physicochemical properties of the reactivator including steric and electronic factors, lipophilic–hydrophilic balance, p*K*a etc. (Aldridge and Reiner, 1972). Since the oximate anion is involved in the nucleophilic attack during the reactivation of OP-inhibited AChE, and its concentration is pH dependent, therefore the p*K*a values should be taken into consideration while searching for new oxime reactivators (Aldridge and Reiner, 1972, Sinko *et al.*, 2006). In the series of pyridinium oximes, the optimum p*K*a value for the reactivator should be in the range of 7.6–8.0 (Hegedorn *et al.*, 1972; Eto, 1974). In this regard, the p*K*a values of the oximes were determined spectrophotometrically in the pH range of 6.04–10.37 (Fig. 5). Two characteristic maxima were obtained in UV in the region 200–600 nm. These maxima correspond to the absorption of different oxime ionized forms as a result

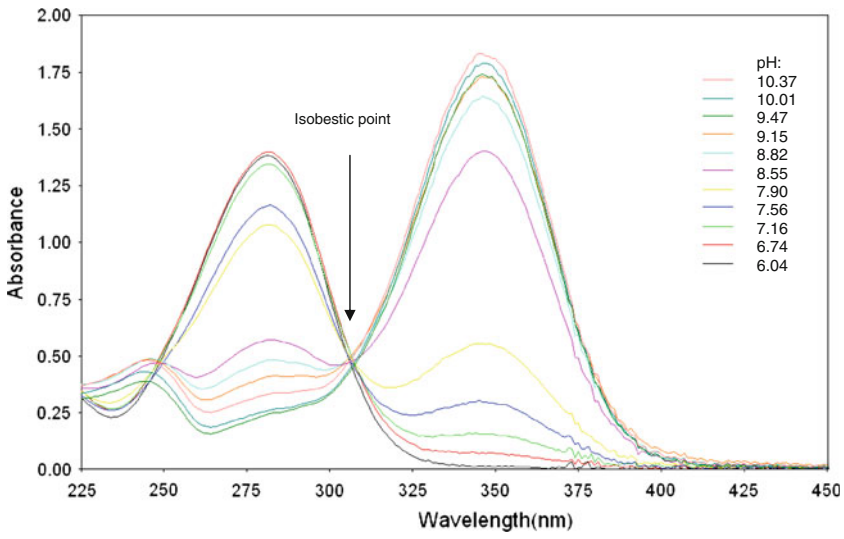


Fig. 5 Spectrophotometric determination of p*K*a of oxime **3a**

of $\pi \rightarrow \pi^*$ transitions within the aromatic system (Odzak *et al.*, 2007). The absorption around 280 and 345 nm were due to absorption of non-dissociated and dissociated form of 4-pyridinium oximes, respectively (Fig. 5). However, for 3-pyridinium oximes, the characteristic maxima were at around 250 and 290 nm for non-dissociated and dissociated form of the oxime group, respectively. The sharp isobestic point at 305–310 nm referred to the acid–base equilibrium for 4-pyridinium oximes. The p*K*a of bis-pyridinium oximes were found to be in the range of 8.15–9.41 (Table 1).

The reactivation of OP-inhibited AChE by quaternary pyridinium oximes depends on several factors such as structure of the inhibitor, structure of the reactivator, position of the oxime group on the pyridinium ring and in case of bis-pyridinium compounds, the nature of the connecting chain between the pyridinium rings (Kuca *et al.*, 2006). From the present study it is evident that the oximes **3h** and **3i** were found to be most active in reactivating DFP-inhibited AChE. The common structural feature of these oximes found most active in reactivating DFP-inhibited AChE is placement of oximino moiety on the position 4 of the pyridinium ring. This results correlates with many other studies (Kassa *et al.*, 2007). However, in case of VX-inhibited AChE, no such preference for the positioning of the oxime on the pyridinium 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 thereby leaving more free space for oxime access to the phosphorylated site with least steric hindrance compared to DFP or sarin (Maxwell *et al.*, 2008). This is manifested in the reactivation data that most of the oximes in this study have shown significant reactivation toward VX-inhibited AChE. It is further reported that aromatic side chains in the pyridinium rings are stabilized with the π–π interactions with the aryl residues present near the peripheral anionic site of the active site gorge of the AChE (Odzak *et al.*, 2007). Thus, an analogy of bis-pyridinium oximes with monopyridinium oximes **3h** and **3i** can be drawn, where benzyl or 4-methyl benzyl moiety resides at the peripheral anionic site. This localization is facilitated by π–π interactions leading to direct the pyridinium oxime function at phosphorylated site. Absence of such an interaction in aliphatic monooximes could be the reason of poor or no reactivation of inhibited AChE.

It is worth noticing that the p*K*a of bis-pyridinium oximes having oximino function at position 4 on the pyridinium ring were found to be in the lower the range (8.15–8.25) as compared to the oximes where oximino function is attached at position 3 on the pyridinium ring (9.14–9.41). This finding is in close agreement to our earlier reports (Acharya *et al.*, 2010). The high reactivation potential of oximes **3h** and **3i** for OP-inhibited AChE may be due to their low value of p*K*a which make the oximes to dissociate sufficiently to be active at the pH used for the study.

Conclusion

In conclusion, we have synthesized a new series of quaternary monopyridinium oximes and evaluated their in vitro reactivation efficacy against OP-inhibited AChE. The oximes with alkoxymethyl side chain were found to be poor reactivators of AChE. However, oximes with benzyloxymethyl and 4-methyl-benzyloxymethyl side chain have shown significant reactivation potential against OP-inhibited AChE. Based upon this study, pyridinium oximes with substituted aryloxymethyl side chain may be synthesized to study the effect of substitution in the aromatic ring on the reactivation potential of AChE inhibited by various OP inhibitors.

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