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European Journal of Medicinal Chemistry 44 (2009) 1335–1340
EUROPEAN JOURNAL OF MEDICINAL CHEMISTRY
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Short communication

Synthesis and evaluation of novel bis-pyridinium oximes as reactivators of DFP-inhibited acetylcholinesterase

Jyotiranjan Acharya*, Arvind Kumar Gupta, Devendra Kumar Dubey, Syed Kalbey Raza

Process Technology Development Division, Defence Research and Development Establishment, Jhansi Road, Gwalior, Madhya Pradesh 474002, India

Received 10 October 2007; received in revised form 25 February 2008; accepted 26 February 2008
Available online 7 March 2008

Abstract

A series of novel bis-pyridinium oximes connected by bis-methoxymethyl benzene, 1,4-bis-methoxymethyl (*cis*)-but-2-ene and 1,4-bis-methoxymethyl but-2-yne linkers were synthesized and their in vitro reactivation efficacy was evaluated against diisopropyl phosphorofluoridate (DFP) inhibited acetylcholinesterase (AChE) and compared with the established antidote 2-PAM and obidoxime. However, the best reactivation was observed with the standard oxime 2-PAM. The reactivation efficacy of 1,3-dimethoxymethyl benzene bis-[4,4′-(hydroxyiminomethyl) pyridinium] dichloride (**3d**) and 1,4-dimethoxy but-2-ene bis-[4,4′-(hydroxyiminomethyl) pyridinium] dichloride (**3g**) was comparable with that of obidoxime, another standard antidote.

Keywords: Bis-pyridinium oximes; Reactivators; 2-PAM; Organophosphorus pesticides; Nerve agents; Acetylcholinesterase; DFP

1. Introduction

The extensive use of organophosphorus (OP) pesticides viz. diisopropyl phosphorofluoridate (DFP), diethyl *p*-nitrophenyl phosphorofluoridate (paraxon) for pest control causes huge loss to the civil society as is evident from the estimated 3 millions of intoxications resulting in 300 thousands fatalities per year [1]. Besides, huge stockpiles of highly toxic organophosphate-based chemical warfare agents (nerve agents, e.g., tabun and sarin) are still available (Fig. 1). In spite of worldwide efforts to prevent synthesis, storage and use of these compounds, the repeated use of chemical warfare agents during military conflicts [2] and terrorist attacks [3,4] indicates that they constitute a persistent threat for the civilization. The intoxication with nerve agents leads to inhibition of acetylcholinesterase by phosphorylation of its active-site serine residue [5]. The subsequent accumulation of the neurotransmitter acetylcholine and over-stimulation of cholinergic receptors result in a generalized cholinergic crisis including breakdown of neuromuscular function [6].

The standard treatment of nerve agent poisoning includes a muscarine antagonist, e.g. atropine, and a reactivator of OP-inhibited AChE [7]. At present oximes such as pralidoxime (2-PAM), TMB-4, HI-6 and obidoxime are available reactivators of OP-inhibited AChE (Fig. 2). These compounds are well tried as antidotes against OP poisoning but are considered to be rather ineffective against certain nerve agents [8,9]. AChE reactivators should be available as antidotes of poisoning by such compounds. One of the most widely used antidotes is 2-PAM. While 2-PAM is effective against sarin, DFP and tabun, its efficacy against other nerve agents is at best marginal [10]. On the other hand, HI-6 and related oximes though effective are unstable in aqueous solution. The efficacy of oximes depends on several factors including post-inhibitory reactions such as spontaneous dealkylation (aging) [11] spontaneous dephosphorylation (spontaneous reactivation) [12] as well as upon the chemical structure of the reactivators [13] and source of AChE [14]. In addition, these organophosphorus pesticides and nerve agents behave differently towards reactivation due to their broad structural variability [15]. Moreover

* Corresponding author. Tel.: +91 751 2340245; fax: +91 751 2341148.
E-mail address: jracharya@rediffmail.com (J. Acharya).

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doi:10.1016/j.ejmech.2008.02.029

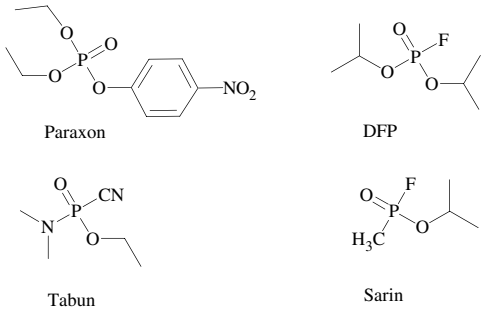


Fig. 1. Organophosphorus pesticides and nerve agents.

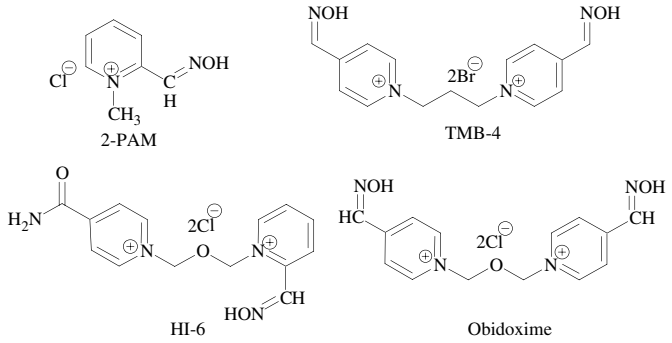


Fig. 2. Pyridinium oximes used as reactivators of OP-inhibited AChE.

their deleterious side effects often become difficult to counteract [16]. In order to overcome these limitations numerous new oximes have been synthesized and tested during last decades [6,12,17]. Furthermore an effective therapy by a single oxime to all the known nerve agents is still lacking [18].

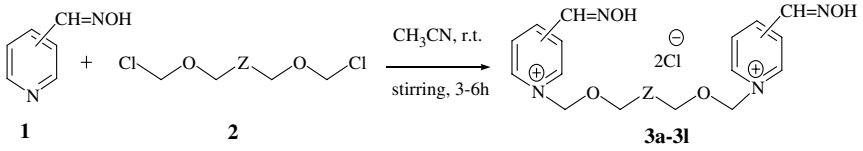
2. Chemistry

The crystal structure of AChE [19] provides templates for the detailed structural studies on ligand access to the active-site gorge of AChE and steric constraints within the gorge that governs the selectivity in oxime reactivation [20]. Recently several bis-pyridinium aldoximes linked by variable lengths of alkylene and bis-methoxy alkane chain were reported as AChE reactivators [21,22]. Recently, *N*-benzyl-4-(hydroxyiminomethyl) pyridinium bromide and its analogous were reported to be much more active in the reactivation of tabun-inhibited human erythrocyte AChE. Again the docking studies with these types of compounds with tabun-inhibited AChE led to the assumption that the linker between pyridinium and benzyl ring should be longer to achieve better reactivation [23]. Pang et al. designed alkylene linked bis-pyridinium oximes on the basis of QSAR studies involving 3D structure of isoflurophate inhibited human AChE (hAChE) and found that 1,7-heptylene-bis-*N*,*N*′-*syn*-2-pyridiniumaldoxime was 100 times more potent than 2-PAM in reactivating hAChE inhibited by isoflurophate [24]. In a very recent development, Musilek et al. reported the synthesis and evaluation of a new series of bis-pyridinium oximes bearing (*E*) and (*Z*)-but-2-ene linkers, which showed promising reactivation ability against chlorpyriphos and paraxon inhibited AChE [25,26]. Such oximes have shown promising reactivation profile against OP-inhibited AChE. In the past, reactive oxime moiety was incorporated into the variety of micelle forming molecules, resulting in significant enhancement of hydrolysis of OP compounds [27–29]. Reactivation potential of such oximes attracted our attention to further explore their efficacy by synthesizing quaternary bis-pyridinium oximes with different kinds of linkers. Keeping the above points in mind, we focused our attention on symmetrical bis-pyridinium oxime analogous of TMB-4 and obidoxime class of compounds having an aryl moiety or a carbon–carbon double or triple bond embedded in a diether linkage between two pyridinium rings. We thought that insertion of unsaturation or phenyl ring in the linker would probably, through π-stacking interactions with unsaturated motifs help orient the oxime molecule in the active-site gorge of AChE in such a way as to position the oxime group in reactive proximity of the OP-inhibited serine residue in the active site [30].

In continuation of our studies on antidotes for OP poisoning [31], herein, we report the synthesis and in vitro reactivation studies against DFP-inhibited eel AChE of a series of symmetrical bis-pyridinium oximes. The eel AChE was selected as target enzyme because in earlier work done in this laboratory [31,32], also the same source of AChE was used. Hence it was convenient for us to compare the reactivation profile of different oximes. By adopting a simple synthetic methodology we have synthesized a novel series of bis-pyridinium oximes. The reaction involved alkylations of pyridine aldoxime with isomeric bis-chloromethoxymethyl benzene, bis-chloromethoxy (*cis*)-but-2-ene and bis-chloromethoxy but-2-yne (Scheme 1). The method afforded compounds **3a–3l** with good yields within 3–6 h (Table 1). The compounds were characterized by their elemental analysis and IR, ^{1}H NMR and ^{13}C NMR spectral data (Tables 2 and 3).

3. Results and discussion

The quaternary pyridinium oxime reactivators reported to date differ from each other by the number of pyridinium rings (mono-pyridinium or bis-pyridinium), or the position of the



Scheme 1. Synthesis of bis-pyridinium oximes.

Table 1
Bis-pyridinium oximes **3a**–**3l**

Oxime (**3**)	−CH=NOH	*Z*	Time (h)	Yield[a] (%)	MP[b] (°C)
3a	4	*p*-C_6H_4	3	75	148–50
3b	3	*p*-C_6H_4	4	71	136–38
3c	2	*p*-C_6H_4	4	75	164–66
3d	4	*m*-C_6H_4	4	70	146–48
3e	3	*m*-C_6H_4	4	68	138–40
3f	2	*m*-C_6H_4	5	58	136–38
3g	4	(*cis*)-CH=CH−	5	68	152–54
3h	3	(*cis*)-CH=CH−	5	65	104–06
3i	2	(*cis*)-CH=CH−	6	57	178–80
3j	4	−C≡C−	3	78	176–78
3k	3	−C≡C−	3	70	118–20
3l	2	−C≡C−	5	66	142–44

[a] Isolated yields.
[b] Uncorrected melting points.

oxime group on the pyridinium ring. In the case of bis-pyridinium oximes, they differ by their chemical structure and length of the bridge between the pyridinium rings [21,22]. In this paper, we report the synthesis of symmetrical bis-pyridinium oxime analogs of TMB-4 and obidoxime class of compounds having an aryl moiety or a carbon–carbon double or triple bond embedded in a diether linkage between two pyridinium rings and evaluated their in vitro reactivation potential against DFP-inhibited electric eel AChE. The results were compared with the standard reactivators viz. 2-PAM and obidoxime. The in vitro reactivation profile of DFP-inhibited electric eel AChE by oximes is depicted in Fig. 3. It is evident from these data that the newly synthesized oximes showed significant reactivation of AChE inhibited by DFP. However, maximum reactivation was observed with the standard oxime 2-PAM at a concentration of 1×10^{-3} M (Fig. 3). Nevertheless the reactivation efficacy of new oximes was comparable with that of obidoxime, another standard antidote. The newly synthesized oximes **3d**, **3e**, **3g** and **3j**, respectively, exhibited 32%, 28%, 33% and 28% reactivation of DFP-inhibited AChE in comparison to 52% and 33% respective reactivation by 2-PAM and obidoxime at a concentration of 1×10^{-3} M. However, at a lower concentration of 1×10^{-4} M, the oxime **3d** was able to reactivate 28% of enzyme activity followed by obidoxime (26%) and 2-PAM (21%). The higher reactivation efficacy of **3d** for DFP-inhibited AChE at the lower concentration 10^{-4} M in comparison to 2-PAM and obidoxime showed that they can be promising candidates for in vivo reactivation as well [6]. The time dependent reactivation of DFP-inhibited AChE showed that the reactivation was complete within the 10 min.

It is worth noticing (Fig. 3) that **3e** is the only 3-substituted bis-pyridinium oxime that showed comparable reactivation with that of 4-substituted oximes (**3e** and **3d**, **3g**, **3j**). This particular oxime (**3e**) bears 1,3-dimethoxy linkage similar to one present in **3d**. It indicates that 1,3-dimethoxy linkage augments the reactivation potential of 3-pyridinium oximes too. However, it is difficult to unambiguously define the exact role of the linker in the reactivation of DFP-inhibited AChE. To further investigate the role of linkers on reactivation of bis-pyridinium oximes, we have studied the effect of a carbon–carbon double bond and triple bond in the linker of the bis-pyridinium oximes. The reactivation potential of two newly synthesized compounds (**3g**, **3j**) was found to be comparable with that of obidoxime. Since the reactivation efficacy of bis-pyridinium oximes is reported to be dependent on the length and stereochemistry of the linkers [18,20,26], our observations also point out that an optimal selection of linkers with appropriate disposition of oxime function (probably at 4-position) is necessary for obtaining the best reactivation. The detailed investigation with linkers of gradually varying lengths and stereochemistry is under way and shall be reported in due course of time.

In conclusion, we have synthesized a series of new bis-pyridinium oximes and evaluated their in vitro reactivation efficacy against DFP-inhibited AChE. Though the studied oximes did not provide highly promising reactivation against DFP-inhibited electric eel AChE, nevertheless before concluding their therapeutic potential, the reactivation of OP-inhibited mammalian AChE remains to be explored.

Table 2
Elemental analysis of compounds

Oxime	MF	MW	Calculated (%)			Observed[a] (%)		
			C	H	N	C	H	N
3a	$C_{22}H_{24}Cl_2N_4O_4$	479	55.11	5.01	11.69	55.32	4.92	11.43
3b	$C_{22}H_{24}Cl_2N_4O_4$	479	55.11	5.01	11.69	55.22	4.81	11.95
3c	$C_{22}H_{24}Cl_2N_4O_4$	479	55.11	5.01	11.69	54.76	5.34	11.78
3d	$C_{22}H_{24}Cl_2N_4O_4$	479	55.11	5.01	11.69	55.18	5.38	11.98
3e	$C_{22}H_{24}Cl_2N_4O_4$	479	55.11	5.01	11.69	55.46	4.87	11.31
3f	$C_{22}H_{24}Cl_2N_4O_4$	479	55.11	5.01	11.69	54.67	5.14	11.34
3g	$C_{18}H_{22}Cl_2N_4O_4$	429	50.34	5.12	13.05	50.02	5.41	12.71
3h	$C_{18}H_{22}Cl_2N_4O_4$	429	50.34	5.12	13.05	50.53	4.76	13.44
3i	$C_{18}H_{22}Cl_2N_4O_4$	429	50.34	5.12	13.05	50.55	5.27	13.38
3j	$C_{18}H_{20}Cl_2N_4O_4$	427	50.58	4.68	13.11	50.24	4.95	13.33
3k	$C_{18}H_{20}Cl_2N_4O_4$	427	50.58	4.68	13.11	50.51	4.88	12.86
3l	$C_{18}H_{20}Cl_2N_4O_4$	427	50.58	4.68	13.11	50.85	4.78	12.92

[a] Elemental analysis is within ±0.4% of the calculated value.

Table 3
Spectral data of oximes

Oxime	IR (KBr) ν_{max} (cm^{-1})	^{1}H NMR (400 MHz) δ, ppm (DMSO-d_6)	^{13}C NMR (100 MHz) δ, ppm (DMSO-d_6)
3a	3395, 3044, 2950, 1640, 1511, 1408, 1286, 1101, 1005, 840, 768	4.70 (s, 4H, OCH_2), 6.04 (s, 4H, NCH_2), 7.30 (s, 4H, −Ph), 8.26−8.28 (d, 4H, −Py), 8.47 (s, 2H, N═CH) 9.13−9.15 (d, 4H, −Py), 13.05 (s, 2H, OH)	71.67, 87.84, 123.99, 128.27, 136.24, 144.04, 145.20, 150.28
3b	3393, 3050, 2970, 1624, 1493, 1440, 1297, 1082, 1000, 852, 781	4.70 (s, 4H, OCH_2), 5.95 (s, 4H, NCH_2), 7.12 (s, 4H, −Ph), 7.95−7.99 (t, 2H, Py), 8.17 (s, 2H, N═CH), 8.58−8.60 (d, 2H, −Py), 8.80−8.82 (d, 2H, −Py), 8.89 (s, 2H, −Py), 12.56 (s, 2H, OH)	71.89, 88.65, 126.33, 128.28, 133.54, 136.24, 141.23, 143.05, 143.24, 143.41
3c	3420, 3045, 2978, 1603, 1520, 1447, 1285, 1096, 997, 850, 782	4.72 (s, 4H, OCH_2), 5.98 (s, 4H, NCH_2), 7.23 (s, 4H, −Ph), 7.73−7.76 (t, 2H, −Py), 8.05−8.07 (d, 2H, −Py), 8.25−8.29 (t, 2H, −Py), 8.34 (s, 2H, N═CH), 8.72−8.73 (d, 2H, −Py), 12.52 (s, 2H, OH)	71.15, 89.21, 126.10, 128.10, 134.69, 136.54, 141.39, 146.05, 146.60, 147.32
3d	3385, 3016, 2954, 1640, 1514, 1423, 1295, 1103, 1007, 847, 788	4.72 (s, 4H, OCH_2), 6.09 (s, 4H, NCH_2), 7.27 (s, 3H, −Ph), 7.31 (s, 1H, −Ph), 8.27−8.29 (d, 4H, −Py), 8.48 (s, 2H, N═CH) 9.19−9.21 (d, 4H, −Py), 13.09 (s, 2H, OH)	71.85, 87.93, 123.99, 127.77, 127.95, 128.69, 136.50, 144.04, 145.21, 150.05
3e	3396, 3035, 2966, 1622, 1487, 1442, 1301, 1081, 996, 850, 780	4.71 (s, 4H, OCH_2), 5.97 (s, 4H, NCH_2), 7.31 (s, 3H, −Ph), 7.35 (s, 1H, −Ph), 7.94−7.97 (t, 2H, −Py), 8.19 (s, 2H, N═CH), 8.55−8.57 (d, 2H, −Py), 8.77−8.79 (d, 2H, −Py), 8.94 (s, 2H, −Py), 12.62 (s, 2H, OH)	71.25, 88.23, 123.49, 127.34, 127.81, 128.24, 136.25, 144.16, 145.21, 146.10, 150.15
3f	3412, 3054, 2976, 1611, 1517, 1442, 1287, 1101, 1001, 854, 780	4.71 (s, 4H, OCH_2), 6.09 (s, 4H, NCH_2), 7.28 (s, 3H, −Ph), 7.34 (s, 1H, −Ph), 7.75−7.78 (t, 2H, −Py), 8.07−8.09 (d, 2H, −Py), 8.22−8.26 (t, 2H, −Py), 8.37 (s, 2H, N═CH), 8.75−8.77 (d, 2H, −Py), 12.68 (s, 2H, OH)	72.81, 90.45, 123.52, 125.85, 128.44, 129.29, 134.52, 141.31, 145.10, 146.41, 148.15
3g	3391, 3033, 2971, 1634, 1487, 1442, 1297, 1080, 1001, 855, 785	4.27−4.29 (d, 4H, OCH_2), 5.60−5.62 (t, 2H, −CH═), 5.98 (s, 4H, NCH_2), 8.31−8.33 (d, 4H, −Py), 8.43 (s, 2H, N═CH), 9.09−9.11 (d, 4H, −Py), 13.06 (s, 2H, OH)	66.11, 87.71, 124.26, 131.77, 145.16, 145.26, 150.12.
3h	3393, 3037, 2972, 1631, 1485, 1440, 1298, 1081, 996, 850, 780	4.29−4.31 (d, 4H, OCH_2), 5.65−5.67 (t, 2H, −CH═), 6.01 (s, 4H, NCH_2), 8.21−8.23 (t, 2H, −Py), 8.42 (s, 2H, N═CH), 8.81−8.83 (d, 2H, −Py), 9.16−9.17 (d, 2H, −Py), 9.36 (d, 2H, −Py), 12.37 (s, 2H, OH)	66.31, 88.42, 126.82, 128.22, 135.10, 141.34, 143.27, 143.44, 144.05
3i	3401, 3048, 2967, 1621, 1527, 1437, 1285, 1091, 992, 851, 782	4.22−4.24 (d, 4H, OCH_2), 5.71−5.73 (t, 2H, −CH═), 5.96 (s, 4H, NCH_2), 8.10−8.13 (t, 2H, −Py), 8.54−8.56 (d, 2H, −Py), 8.58−8.61 (t, 2H, −Py), 8.48 (s, 2H, N═CH), 9.08−9.10 (d, 2H, −Py), 12.61 (s, 2H, OH)	65.83, 87.67, 125.62, 129.24, 135.18, 142.23, 143.16, 143.54, 144.05
3j	3395, 3037, 2958, 2838, 2730, 1642, 1603, 1517, 1456, 1298, 1080, 1007, 843	4.45 (s, 4H, OCH_2), 5.98 (s, 4H, NCH_2), 8.30−8.32 (d, 4H, −Py), 8.48 (s, 2H, N═CH), 9.14−9.18 (d, 4H, −Py), 13.04 (s, 2H, OH)	57.88, 82.49, 87.12, 123.97, 144.28, 145.27, 150.29
3k	3393, 3032, 2966, 2829, 2733, 1641, 1609, 1526, 1454, 1302, 1086, 1010, 841	4.28 (s, 4H, OCH_2), 5.88 (s, 4H, NCH_2), 8.03−8.06 (t, 2H, −Py), 8.22 (s, 2H, N═CH), 8.62−8.64 (d, 2H, −Py), 9.01−9.03 (d, 2H, −Py), 9.22 (d, 2H, −Py), 12.23 (s, 2H, OH)	58.16, 82.63, 87.78, 128.20, 133.51, 141.44, 143.31, 143.57
3l	3381, 3035, 2961, 2840, 2731, 1638, 1598, 1520, 1465, 1296, 1081, 997, 837	4.40 (s, 4H, OCH_2), 6.16 (s, 4H, NCH_2), 8.12−8.15 (t, 2H, −Py), 8.50−8.52 (d, 2H, −Py), 8.57−8.60 (t, 2H, −Py), 8.46 (s, 2H, N═CH), 9.10−9.12 (d, 2H, −Py), 13.26 (s, 2H, OH)	58.86, 82.43, 87.18, 128.28, 134.56, 140.78, 143.11, 143.88

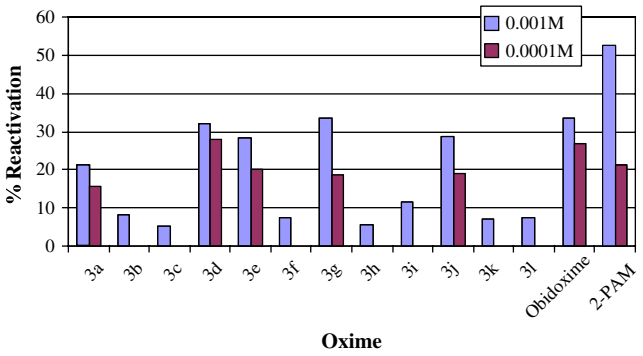


Fig. 3. Efficacy of tested oximes in reactivation of DFP-inhibited AChE in comparison with 2-PAM. Source of enzyme: electric eel; inhibitor agent: DFP; inhibitor concentration: 1.08×10^{-5} M; time of inhibition: 15 min; time of reactivation: 10 min; pH: 8.0; and temperature: 37 °C. The values are average of three runs with a maximum SD of ±2%.

4. Experimental section

4.1. Chemistry

Electric eel AChE (EC 3.1.1.7), 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), acetylthiocholine iodide, 2-, 3-, and 4-pyridine aldoxime and 1,4-benzenedimethanol, 1,3-benzenedimethanol, *cis*-1,4-but-2-ene diol, 1,4-but-2-yne diol and paraformaldehyde 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. Diisopropyl phosphorofluoridate (DFP) was prepared in this laboratory with >98% purity (GC and ^{31}P NMR). Bis-chloromethyl ethers were prepared according to the known synthetic method [33]. 2-PAM was prepared according to the method of Ginsburg and Wilson [34]. The bis-pyridinium oximes were synthesized, characterized by their IR, ^{1}H NMR and ^{13}C NMR spectral data (Table 2) and their purity was checked by melting point as well as on TLC [commercially available pre-coated cellulose on alumina sheets (E. Merck)] with 1-butanol:acetic acid:water (3:1:1) as solvent system.

4.2. General experimental procedure

4.2.1. Synthesis of quaternary bis-pyridinium oximes (*3a–3l*)

Pyridine aldoxime, 2.7 g (0.022 mol) 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 bis-chloromethoxy alkane (0.01 mol) 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 solids obtained were filtered off, washed repeatedly with hot dry acetone and recrystallized from dry methanol–acetone mixture. Their purity was checked on TLC (cellulose, DSO–Fluka) and characterized by spectral data. The physical parameters are given in Table 1.

4.3. In vitro reactivation studies

The in vitro reactivation of DFP-inhibited AChE using test oximes was carried out in triplicate in phosphate buffer (0.1 M, pH 8.0 at 37 °C) using the method of Ellman et al. [35]. 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) (352 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 DFP (1.08×10^{-2} M) in isopropanol was 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 DFP (1.08×10^{-4} 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. No further increase in the inhibition of enzyme activity was observed even after 1 h of the incubation with DFP at this concentration. It was then followed by addition of 50 μL of oxime test solution (1×10^{-2} M, 1×10^{-3} M) to start reactivation. The final volume of the reactivation cocktail was 500 μL. The final concentration of DFP was 1.08×10^{-5} 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. 3). Twenty microliters 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 1 h. Percentage reactivation was calculated using the following equation [32]:

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

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 as 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 DFP but no oxime. Under these conditions spontaneous reactivation was found to be insignificant. All the values are corrected for their oxime induced hydrolysis.

Acknowledgement

Authors thank Dr. R. Vijayraghavan, Director, and Prof. M.P. Kaushik, Associate Director, Defence Research and Development Establishment, Gwalior for their keen interest in this work. The authors are also thankful to Dr. B.K. Bhattacharya for his valuable suggestions.

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