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Quaternary salts of 4,3′ and 4,4′ bis-pyridinium monooximes. Part 2: Synthesis and biological activity

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Abstract—In continuation of our investigations of unsymmetrical bisquaternary monooximes, we synthesized four new series of compounds bridged by hexyl, heptyl, octyl and nonyl groups. All eight monooximes viz., dibromides of 1-(4-hydroxyiminomethylpyridinium)6-(3/4-carbamoylpyridinium)hexane, 1-(4-hydroxyiminomethylpyridinium)-7-(3/4-carbamoylpyridinium)-heptane, 1-(4-hydroxyiminomethylpyridinium)-8-(3/4-carbamoylpyridinium)octane, 1-(4-hydroxyiminomethylpyridinium)-9-(3/4-carbamoylpyridinium)nonane as well as the corresponding bis-oximes were synthesized and characterized by spectral data. Their ability to reactivate tetraethylpyrophosphate (TEPP) inhibited mouse total brain cholinesterase was investigated and compared with the conventional oxime 2-pyridinealdoxime chloride (2-PAM). Mouse brain homogenate was used as the source of acetylcholinesterase. Among all the compounds, tested the compound with the hexylene bridge (**6b**) and a 3-carbamoyl group on the second pyridine ring was found to be the most active acetylcholinesterase reactivator (72%) which is greater than that of 2-PAM (56%). However, the activity was reversed; as the chain length increased from a heptylene to a nonylene bridge, they potentiated the inhibitory effect of TEPP rather than reactivation. It is interesting to note that compound **6b** with a carbamoyl group at the 3rd position of the pyridine ring showed dose dependent reactivation whereas the corresponding compound **6a** with the carbamoyl group present at the 4th position of the pyridine ring showed reactivation at lower concentration (30 μM) and potentiation of TEPP inhibition at higher concentrations (100 and 300 μM).

Deliberate self-poisoning with organophosphorus (OP) pesticides has become an increasingly common response to emotional distress in young adults, and it is now one of the most frequent reasons for emergency hospital admission in India.[1] On average, most patients will require 5–14 days of intensive care monitoring. Mortality has been reported as between 15% and 36%.[2,3] Mechanism of reactivation of TEPP inhibited cholinesterase by the oximes is presented in Scheme 1.[4,5] Furthermore, phosphorylated AChE can undergo a fairly rapid process of 'aging' (loss of one alkyl or alkoxy group) so that within hours of pesticide poisoning it becomes completely resistant to the reactivators. Although the highly toxic nature of OP compounds has been known for many years, there still exist serious limitations in the antidotal therapy available against poisoning by these compounds. Current medical protection against the toxicity of OP compounds consists of a regimen of anticholinergic drugs, such as atropine, that antagonizes the effects of accumulated acetylcholine and an AChE 'reactivator' that restores enzyme activity.

Pralidoxime is the oxime used worldwide, for the treatment of OP pesticide poisoning and available in three common forms: pralidoxime chloride (2-PAM, used world-wide), iodide (used in rural India) and mesylate (P2S, used in the UK).[3,6] There is considerable published data on the role and choice of available oximes (like 2-PAM, obidoxime, HI-6 and HLö-7) for the treatment of OP pesticide poisoning.[7–9] Earlier we reported the synthesis and biological activity of bis-pyridinium monooximes and found that compounds with a propyl or butyl bridge were potent reactivators of TEPP inhibited AChE in comparison to 2-PAM.[10] Propane and butane bridged bis-pyridinium monooximes were also tested on nerve agents (also known as chemical warfare agents such as sarin, tabun and soman, in mode of action they are related to OP pesticides but are highly toxic to mammals) and found more effective.[11,12]

Keywords: Organophosphate; Pesticide; Tetraethylpyrophosphate; Acetylcholinesterase; Pralidoxime; Bis-pyridinium monooximes.
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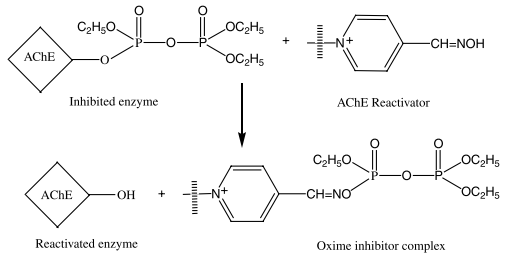
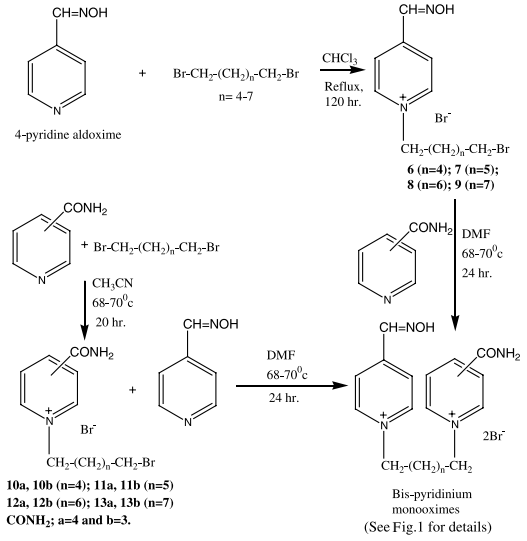


Scheme 1. Reactivation of acetylcholinesterase inhibited by tetraethylpyrophosphate.



Scheme 2. Synthesis of alkylene-linked bis-pyridinium monooximes.

Pang et al. also reported that 1,7-heptylene-bis-*N*,*N*′-syn-2-pyridiniumaldoxime is 100 times more potent than 2-PAM in reactivating hAChE poisoned by isofluorophate.[13] Hence, we continued our investigations, and synthesized bis-pyridinium monooximes with hexylene to nonylene bridges and evaluated their effectiveness for the reactivation of TEPP inhibited enzyme.

Anaesthetized animals were sacrificed by decapitation and exsanguinated; the mice brains were removed and used as a source of AChE after homogenization. Tetraethylpyrophosphate (TEPP), 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB), acetylthiocholine iodide (Asch), eserine, 4-pyridinealdoxime, 1,6-dibromohexane, 1,7-dibromoheptane, 1,8-dibromooctane, 1,9-dibromononane, isonicotinamide and nicotinamide were obtained from the Sigma-Aldrich chemicals private limited, Hyderabad, India. KH_2PO_4, NaOH and NaCl were purchased from Merck chemicals private limited, Hyderabad, India. 2-PAM was provided as a gift sample by the Troika Parenterals private limited, Ahmedabad, India.

Solvents were dried or distilled before use. Melting points were obtained on a Mel-temp apparatus (Shital Scientifics), Mumbai, India in open capillary tubes and are uncorrected. Infrared spectra (IR) were recorded with KBr pellet on a Perkin-Elmer BX Series, infrared spectrophotometer. ^{1}H NMR spectra were recorded on Avance-300 MHz spectrometer in DMSO-d_6.[16]

The general procedure for the synthesis of compounds were as follows: (Scheme 2). Selected physicochemical data of compounds with hexylene, heptylene, octylene and nonylene bridges are given in Table 1.

Procedure A

Step I: Preparation of oxime intermediate compounds. 4-Pyridinealdoxime and 1,6-dibromohexane, 1,7-dibromoheptane, 1,8-dibromooctane, or 1,9-dibromononane in 1:1.2 molar ratio were added to chloroform and heated at reflux with stirring for 120 h and then cooled to room temperature. The product was collected by filtration, washed with chloroform and crystallized from acetonitrile.

Step II: Preparation of bis-pyridinium monooximes. Intermediate compound **6**, **7**, **8**, or **9** and isonicotinamide or nicotinamide in 1:1.2 molar ratio were added to dimethylformamide (DMF). The reaction mixture was heated at 68–70 °C for 24 h and then cooled to room temperature. The product was collected by filtration and crystallized with absolute ethanol.

The title compounds were also prepared by an alternative method as follows:

Procedure B

Step I: Preparation of amide intermediate compounds. Isonicotinamide or nicotinamide and 1,6-dibromohexane, 1,7-dibromoheptane, 1,8-dibromooctane or 1,9-dibromononane in 1:1.1 molar ratio were added to acetonitrile and heated at 68–70 °C for 20 h. The product

Table 1. Physical data of compounds

Compound	Conditions of the reaction	Molecular formula	Mp[a] (°C)	Yield (%)
6a	DMF, 68–70 °C, 24 h	$C_{18}H_{24}N_4Br_2O_2$	248–250	80
6b	DMF, 68–70 °C, 24 h	$C_{18}H_{24}N_4Br_2O_2$	246–248	74
7a	DMF, 68–70 °C, 24 h	$C_{19}H_{26}N_4Br_2O_2$	242–244	56
7b	DMF, 68–70 °C, 24 h	$C_{19}H_{26}N_4Br_2O_2$	246–248	58
8a	DMF, 68–70 °C, 24 h	$C_{20}H_{28}N_4Br_2O_2$	252–254	67
8b	DMF, 68–70 °C, 24 h	$C_{20}H_{28}N_4Br_2O_2$	248–250	64
9a	DMF, 68–70 °C, 24 h	$C_{21}H_{30}N_4Br_2O_2$	238–240	68
9b	DMF, 68–70 °C, 24 h	$C_{21}H_{30}N_4Br_2O_2$	242–244	66

[a] Melting points are uncorrected.

was collected by filtration, washed with acetonitrile and crystallized using chloroform.

Step II: Preparation of bis-pyridinium monooximes. The isonicotinamide or nicotinamide intermediates (**10a**, **10b**, **11a**, **11b**, **12a**, **12b**, **13a**, or **13b**) and 4-pyridinealdoxime in 1:1.2 molar ratio were added to DMF. The reaction mixture was heated at 68–70 °C for 24 h. The product was collected by filtration and washed with acetonitrile. All compounds were purified by recrystallisation with DMF, and then dried under vacuum.

Compounds **6c**, **7c**, **8c** and **9c** were synthesized according to the methods of Wilson and Ginsburg[4] and Pang et al.[13] Compounds **6c**, **7c**, **8c** and **9c** are known in the literature.[13]

Brain AChE activity was determined by the colorimetric procedure described by Voss and Sachsse.[14] Physical data, TLC, IR and ^{1}H NMR spectra confirmed the structures and purity of the synthesized compounds. All bis-pyridinium monooxime products decomposed (>180 °C) before melting. All the synthesized compounds were evaluated for their in vitro reactivation capabilities of TEPP inhibited AChE enzyme by colorimetry. Compound **6b,** the most potent which, has an hexylene bridge with a carbamoyl group at the 3rd position, showed 72 ± 3.51 % reactivation compared to 56 ± 2.84 % exhibited by 2-PAM. Compound **6a** with a heptylene bridge and the carbamoyl group present at the 4th position showed inhibition of the enzyme instead of reactivation. The corresponding bis-oxime **6c** showed 23 ± 2.46 % reactivation (Fig. 2). Compounds **7a**, **7b** and **7c** exhibited significant reactivation of the enzyme (antidotal property) at low concentration (30 μM) but, at higher concentrations (100 and 300 μM), potentiated inhibition of the enzyme by TEPP. However, the bis-oxime **7c** reactivated AChE even at 100 μM concentration (14 ± 2.36%). Compounds **8a**, **8b**, **8c**, **9a**, **9b** and **9c** showed only inhibition of the enzyme at all concentrations tested (Fig. 3). Compounds **7a**, **7b** and **7c** have shown 15 ± 1.4%, 23 ± 2.6% and 39 ± 2.8% reactivation, respectively, at 30 μM concentration (Fig. 2). Compound **6b** (hexylene bridge) has excellent reactivation (20 ± 1.95%) at increased (10×) concentration of TEPP (1 μg), whereas 2-PAM had only 2 ± 0.6% reactivation. All other compounds did not show reactivation at this concentration of TEPP.

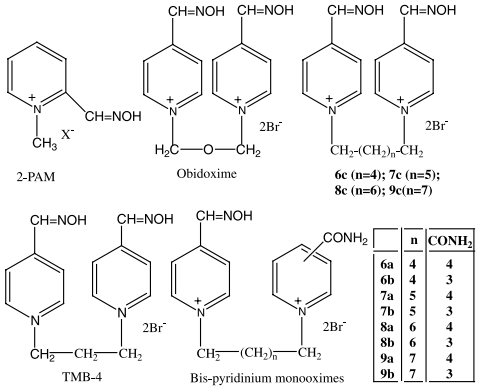


Figure 1. Structures of the standard oximes used for the treatment of OP pesticide poisoning as well as our newly synthesized bis-pyridinium monooximes.

All the conventional oximes reported to date differ from each other by the number of pyridinium rings present (mono-pyridnium vs bis-pyridinium oximes), the position of the oxime group on the pyridinium ring, and, in the case of bis-pyridinium oximes, by the chemical structure of the bridge between the pyridinium rings (Fig. 1). The bis-pyridinium monooximes differ from bis-pyridinium oximes both in the length of the side chain and the position of the substituent on the second pyridine ring. The bis-pyridinium monooxime **6b** showed higher reactivation (72%) than the conventional oxime 2-PAM (52%). It is interesting to note that changing the carbamoyl group from the 3rd to the 4th position (**6a**) resulted not only in loss of reactivating capacity but also a gain in inhibitory effect. However, the corresponding bis-oxime showed less potency than **6b** and 2-PAM (Fig. 2). Further increase in the chain length from a 6-carbon bridge (hexylene) to 7-carbon bridge (heptylene) resulted in a slight increase in activity at lower concentration (30 μM) but showed inhibition of the enzyme at higher concentrations (100 and 300 μM). However, the

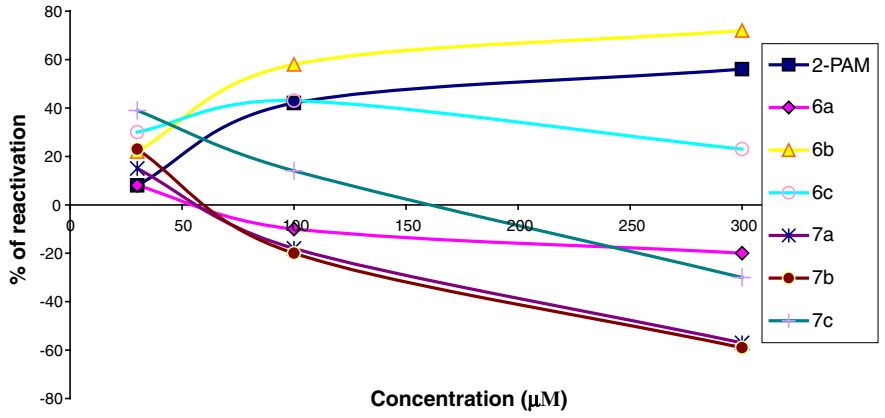


Figure 2. Reactivation of mouse brain AChE inhibited with TEPP by 2-PAM and test compounds.

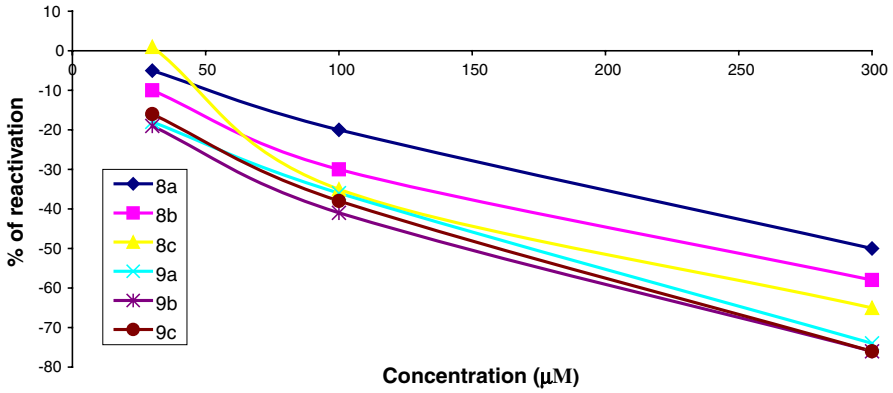


Figure 3. Reactivation of mouse brain AChE inhibited with TEPP by test compounds.

compounds with 8 (octylene), or 9-carbon (nonylene) bridges showed only inhibition of the enzyme instead of reactivation at all concentrations (Fig. 3). From our studies, it was observed that the optimal alkylene side chain length for reactivation of OP inhibited AChE in bis-pyridinium monooximes is from propyl to hexyl bridges with a carbamoyl group present at the 3rd position of the second pyridine ring.

Four new series of asymmetrically substituted 4,3′ and 4,4′ bis-pyridinium monooximes bridged by hexylene, heptylene, octylene and nonylene groups were prepared. Evaluation of these compounds as antidotes for anti-AChE intoxication in the mouse brain model revealed that their effectiveness depends significantly on the length of the side chain. The bis-pyridinium monooximes reactivate the inhibited enzyme faster than the mono-pyridinium and bis-pyridinium oximes. From our data, it can be concluded that propyl to hexyl bridge compounds with a carbamoyl group at the 3rd position of the second pyridine ring were potent reactivators of AChE inhibited with TEPP. Further studies are required to establish the mechanism by which a change in the position of carbamoyl group results in drastic changes in AChE reactivation.

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14. Voss, G.; Sachsse, K. *Toxicol. Appl. Pharmacol.* **1970**, *16*, 764, The mouse brain was removed, rinsed with cold saline, blotted dry, and weighed. It was homogenized under cold conditions in a sufficient amount of 0.9% NaCl to give a final solution of 100 mg brain tissue/mL of saline. The homogenate was then centrifuged at 3000 rpm for 10 min. The reactivating efficacy of oximes was evaluated by adding TEPP (100 ng) to the homogenate for 10 min followed by tested oximes for 10 min. The solution was incubated at 30 °C for 10 min after the addition of substrate (i.e., acetylthiocholine iodide). All assays were carried out in triplicate. Percentage reactivation was calculated with the following equation:[15] $\frac{E_r - E_i}{E_0 - E_i} \times 100$. In the above equation, E_0 is the control enzyme activity at 0 min, E_i is the inhibited enzyme activity, and E_r is the activity of inhibited enzyme after incubation with the oxime test compounds.
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16. The new compounds gave satisfactory ^{1}H NMR Spectra: Compound **6a:** 1.31m, 4H (CH_2–CH_2–*CH_2*– *CH_2*–CH_2–CH_2); 1.92 m, 4H (CH_2–*CH_2*–CH_2–CH_2–*CH_2*–CH_2); 4.62–4.67 m, 4H (*CH_2*–CH_2–CH_2– CH_2–CH_2–*CH_2*); 8.42–8.44 m, 4H (Py); 8.67–8.80 m, 3H (CO*NH_2*, *CH*=NOH); 9.26–9.27 m, 4H (Py).
Compound **6b**: 1.33 m, 4H (CH_2–CH_2–*CH_2*– *CH_2*–CH_2–CH_2); 1.91 m, 4H (CH_2–*CH_2*–CH_2–CH_2–*CH_2*–CH_2); 4.59–4.62 m, 4H (*CH_2*–CH_2–CH_2– CH_2–CH_2–*CH_2*); 8.39–8.44 m, 4H (Py); 8.58–8.72 m, 3H (CO*NH_2*, *CH*=NOH); 9.23–9.35 m, 4H (Py).

Compound **7b**: 1.29 m, 6H (CH_2–CH_2–*CH_2*–*CH_2*–*CH_2*–CH_2–CH_2); 1.88–1.90 m, 4H (CH_2–*CH_2*–CH_2–CH_2–CH_2–*CH_2*–CH_2); 4.54–4.59 m, 4H (*CH_2*–CH_2–CH_2–CH_2–CH_2–CH_2–*CH_2*); 8.18–8.23 m, 4H (Py); 8.42–8.67 m, 3H (CO*NH_2*, *CH*═NOH); 9.00–9.08 m, 4H (Py).

Compound **8a**: 1.26 m, 8H (CH_2–CH_2–*CH_2*– *CH_2*–*CH_2*–*CH_2*–CH_2–CH_2); 1.90 m, 4H (CH_2–*CH_2*–CH_2– CH_2–CH_2–CH_2– *CH_2*–CH_2); 4.59–4.64 m, 4H (*CH_2*–CH_2–CH_2– CH_2–CH_2–CH_2– CH_2–*CH_2*); 8.41–8.43 m, 4H (Py); 8.67 m, 3H (CO*NH_2*, *CH*═NOH); 9.22–9.24 m, 4H (Py).