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New oxime reactivators connected with $CH_2O(CH_2)_nOCH_2$ linker and their reactivation potency for organophosphorus agents-inhibited acetylcholinesterase

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Abstract—New bis-pyridinium oxime reactivators **6** with $CH_2O(CH_2)_2OCH_2$ and $CH_2O(CH_2)_4OCH_2$ linkers between the two pyridinium rings were designed and synthesized. In the in vitro test of their potency to reactivate AChE inhibited by organophosphorus agents at 5×10^{-3} M concentration, the reactivation ability of 1,2-dimethoxy-ethylene-bis-*N*,*N*′-4-pyridiumaldoxime dichloride (**6a**) was 63% for housefly (HF) AChE inhibited by diisopropyl fluorophosphates (DFP), 51% for bovine red blood cell (RBC) AChE inhibited by DFP, 67% for HF-AChE inhibited by paraoxon, and 81% for RBC-AChE inhibited by paraoxon. Except in the case of DFP-inhibited HF AChE test of 2-PAM, the activities of **6a** are much higher than the activities of 2-PAM and HI-6 which are AChE reactivators currently in use.

1. Introduction

Exposure to the organophosphorus nerve agents such as sarin, soman, cyclosarin, and VX causes acute intoxication. High doses of organophosphorus nerve agents cause convulsions and paralysis of the respiratory muscles. Several other organophosphorus agents such as parathion, malathion, and diazinon have also been developed, and used as insecticides because of their low volatility and stability in aqueous solution (Fig. 1).[1] It is well known that these organophosphorus agents exert their biological effects by inhibiting AChE, where the serine residue of the active site can attack the phosphorous atom of the organophosphorus agents to form a strong P–O bond (Scheme 1).[2] The inhibition of acetylcholinesterase (AChE) increases the amount of acetylcholine (ACh) at central and peripheral sites of the nervous system. The organophosphorus agents-inhibited AChE can be reactivated by introducing nucleophiles such as pyridinium oximes.[3] This oxime-induced reactivation is the primary therapeutic approach to organophosphorus poisoning. The search for more effective pyridinium oxime reactivators is a focus of research program aimed at treating poisoning due to organophosphorus insecticides and chemical warfare agents. The most commonly used mono-pyridinium oxime is 2-PAM, and the bis-pyridinium oximes are currently being investigated as oxime reactivators. After thorough study of many of these oximes, bis-pyridinium oximes such as TMB4, Toxogonin, and HI-6 have been developed and are used currently in many countries (Fig. 2).[4] Among these pyridinium oxime reactivators, TMB4 has a $CH_2CH_2CH_2$ linker between two pyridinium rings, whereas Toxogonin and HI-6 have a CH_2OCH_2 linker. Currently a number of new bis-pyridinium oxime reactivators are being designed and synthesized.[5] Among these oxime reactivators, several bis-pyridinium oximes linked by a variable-length alkylene chain were designed by using a ligand docking program and the X-ray crystal structure of AChE.[6] This work led to the identification of two potential binding sites for bifunctional AChE inhibitors: a high-affinity tryptophan residue at position 84, deep in the catalytic gorge (catalytic binding site), and a low-affinity tryptophan residue at position 279, near the AChE surface (peripheral binding site). The importance of these dual-site binding properties was applied to the design of bis-pyridinium oximes to develop potent reactivator

Keywords: Organophosphorus agents; Bis-pyridinium oxime reactivators; Acetylcholinesterase; Linker; Pesticide.
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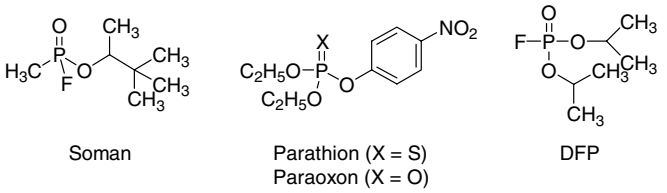
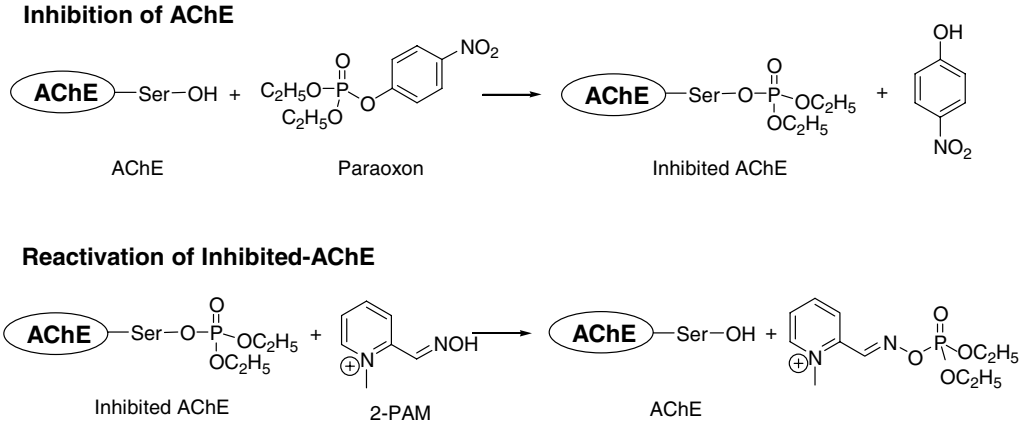


Figure 1. Structures of organophosphorus agents.



Scheme 1. Inhibition of AChE by paraoxon and its reactivation by 2-PAM.

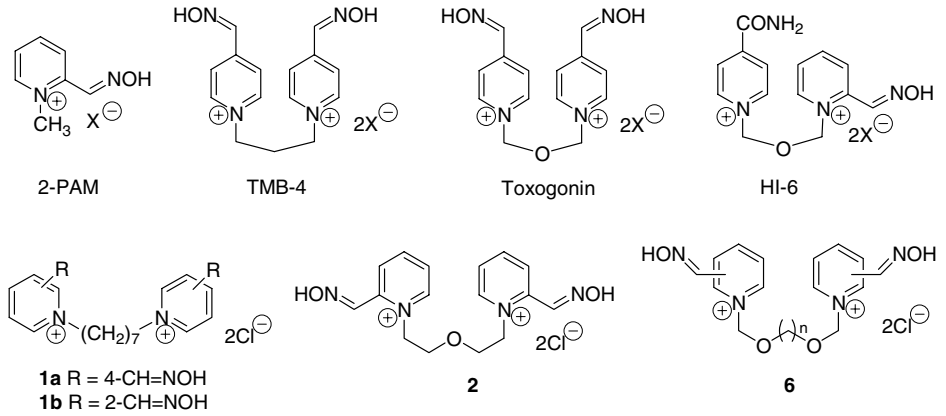


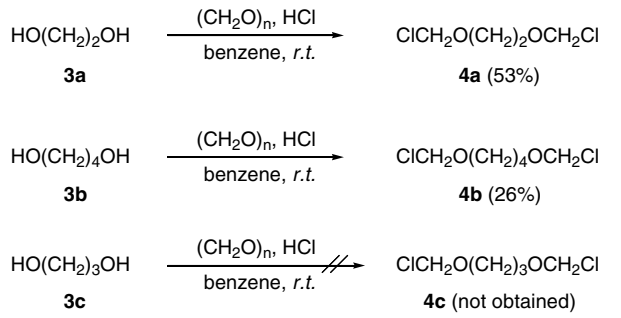
Figure 2. Structures of AChE reactivators.

for AChE inhibited by organophosphorus agents. For example, Pang and coworkers developed a dimeric oxime **1**, 1,7-heptylene-bis-*N*,*N*′-pyridiniumaldoxime dichloride. The oxime **1** is 100 times more potent than 2-PAM in reactivating hAChE after exposure to echothiophate or isoflurophate.[7]

In the continuation of our efforts to develop new oxime reactivators, we have designed and evaluated new substances that differ from the currently used oximes in the linker between the two pyridinium rings.[8] We were especially interested in incorporation of an oxygen atom in the spacer connecting the two pyridinium rings, because oxime reactivators with linkers of oxygen atom and methylene such as Toxogonin and HI-6 show strong reactivation activity. These speculations prompted us to design new oximes in which a longer ether linker was introduced to connect the two pyridine rings. Previously we reported the reactivation property of bis-pyridinium oxime reactivators **2** containing a $CH_2CH_2OCH_2CH_2$ linker between the two quaternary nitrogens. In this report, the two pyridine rings of bis-pyridiniumaldoximes were connected with $CH_2O(CH_2)_nOCH_2$ linkers between the two quaternary nitrogens (Scheme 2).

2. Chemistry

The required synthons 1,2-bis-chloromethoxyethane **4a** and 1,4-bis-chloromethoxybutane **4b** were obtained from the corresponding ethylene glycol and 1,4-butanediol by reaction with two equivalent of paraformaldehyde in benzene treated with dry HCl gas for 10 h at room temperature. Vacuum distillation of the reaction



Scheme 2. Synthesis of bis-chloromethoxyalkanes.

Table 1. Efficacy of tested oximes in reactivation of DFP-inhibited AChE

Compound	AChE reactivation	
	Mean	SE
6a	62.7	2.2
6b	65.3	2.71
6c	47	1.81
6d	58.1	2.18
1a	14.1	0.74
1b	19	0.86
2-PAM	75.1	1.63
HI-6	6.6	0.39

Table 2. Reactivation potency of oximes for DEP- and paraoxon-inhibited AChE

Compound	Inhibition by DFP		Inhibition by paraoxon			
	RBC		HF		RBC	
	Mean	SE	Mean	SE	Mean	SE
6a	51	2.25	67.4	1.27	80.7	3.80
6b	15.1	0.44	73.7	0.93	31.6	2.53
6d	51	1.93	7.5	0.15	12.8	1.12
2-PAM	35.3	0.77	41.4	0.32	60.2	2.75
HI-6	21	3.12	8.3	0.09	52.2	3.43

mixtures provided **4a** (53%) and **4b** (26%), respectively. The similar reaction of propanediol failed to give the desired 1,3-bis-chloromethoxypropane **4c** due to the possible formation of 1,3-dioxane. The targeted bis-pyridinium oximes having a $CH_2O(CH_2)_nOCH_2$ linker between the two quaternary nitrogens (**6a**, **6b**) were obtained by the alkylation of the pyridine aldoximes (**5a**, **5b**) with **4a** in acetonitrile at 45 °C for 20 h. Similarly the other oximes (**6c**, **6d**) were prepared by the reaction of **5a**, **5c** with **4b** in DMF at r.t. for 24 h. The structures of the newly synthesized compounds were verified by ^1H NMR spectra and mass spectra (Tables 1 and 2).

3. Materials and methods

3.1. Materials

All new pyridinium oximes and HI-6 were prepared in our laboratory, and 2-PAM was purchased from Sigma–Aldrich. The dimeric oxime **1a** and **1b** were prepared by the reported method.[7] DFP and paraoxon are commercially available from Fluka and Sigma–Aldrich, respectively. AChEs from two different species used in this experiment: extract from housefly head was received from Central Research Center, National Agricultural Cooperative Federation, Korea, and bovine red blood cell (RBC) AChE was purchased from Sigma–Aldrich (Scheme 3).

3.2. In vitro determination of AChE activity

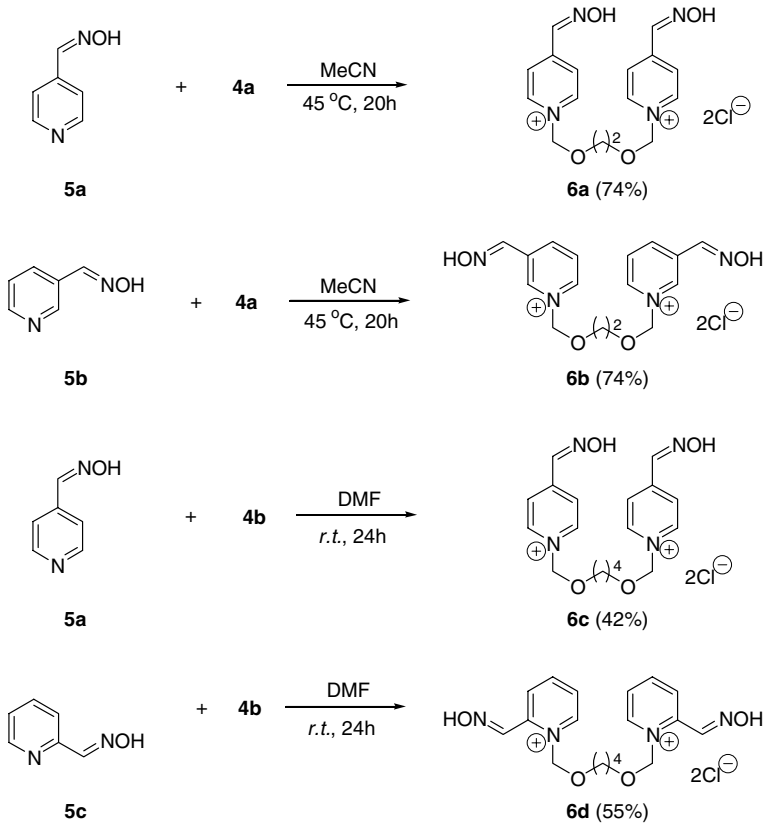
The enzyme activity was measured in a 96-well Microplate using a microplate reader (Benchmark Microplate Reader, Bio-Rad) at 415 nm and 37 °C with acetylthiocholine (1 mM) as substrate and DTNB (1 mM) as chromogen in 0.05 M Tris–HCl buffer, pH 7.8.[9] Total reaction volume was adjusted to 250 μL with slight modification from Ellman's AChE assay method. The enzyme concentrations of both AChEs in 250 μL of final reacting solutions were 0.05 mg/ml for HF AChE and 0.02 U/mL for RBC AChE, respectively. For RBC AChE, 1% of Triton X-100 (Sigma–Aldrich) was added in the Tris–HCl buffer to preserve enzyme activity. AChE activity was measured with the change of optical density per minute (ΔOD/min), percentage reactivation of AChE activity was calculated by comparison with the AChE activity without inhibitor showing 0.2 ΔOD/min.

3.3. AChE inhibition and reactivation

DFP and paraoxon were used as organophosphorus inhibitors and the AChE reactivating capability of 2-PAM and HI-6 were examined against OP-inhibited HF and RBC AChE, respectively. AChE was inhibited with the minimum quantity of inhibitor necessary to inactivate 99% of activity for 10 min at room temperature. The concentration of DFP was 12.5 μM for HF AChE and 25 μM for RBC AChE, respectively, and 20 μM of paraoxon for both AChEs. To remove excess inhibitor once reaction with enzyme was complete, the enzyme solution was mixed with twofold volume of hexane and vigorously shaken with vortex mixer for 1 min. The aqueous phase was separated by centrifugation at 3000*g* for 10 min at 3 °C.[10] The solution containing phosphorylated AChE was incubated with various concentration of 2-PAM or HI-6 for various reactivation periods. To remove small molecules such as reactivator and phosphorylated oxime, the reactivating mixture was filtered through a micro spin-column packed with Sephadex-G50 (Bio-Rad) in a centrifuge at 300*g* for 1 min at 3 °C.[11] The AChE activity of the filtrate was measured in a 96-well microplate, and the percentage reactivation of AChE activity was calculated as previously described.

3.4. AChE reactivation with newly synthesized oxime compounds

The reactivating ability of the newly synthesized oxime compounds was evaluated against DFP or paraoxon-inhibited HF or RBC AChE, respectively. The inhibited AChE was extracted with hexane, and then the aqueous



Scheme 3. Synthesis of new bis-pyridinium oximes.

phase was reacted with 5 mM of each oxime compound for 30 min for DFP-inhibited AChE and for 1 h for paraoxon-inhibited AChE, respectively. The percentage reactivation of AChE was calculated as previously described.

4. Results and discussion

It has been previously described that three molecular forms of AChEs are generated after the post-transcriptional process of alternative splicing from the same genetic origin in most animal species.[12] Each type of AChE exists in multiple forms, multimeric for nerve and muscle, dimeric for red blood cell, and monomeric for embryonic and tumor cells, respectively, and their structural differences appear only in the C-terminal extension with 40 residue peptides in contrast to the well-preserved functional subsites such as catalytic triad, acyl pocket, and hydrophobic subsite.[13] House fly brain AChE and bovine red blood cell AChE were selected for this study as alternative forms of multimeric and dimeric AChEs, respectively. AChEs from Dipteran insects, such as house fly and fruit fly have been well characterized and conveniently used for in vitro AChE inhibition experiments.[14] Bovine red blood cell AChE has been also frequently used for the inhibition assay because of its simple preparation procedure compare to other sources.[11,15] DFP has been chosen for this study because of its structural similarity to nerve gas. Parathion is inactive toward AChE in vitro, but its metabolite paraoxon is active. The sulfur-for-oxygen substitution is carried out in the liver by the mixed-function oxidases.[16] This reaction is also carried out in the insects, typically more efficiently.[17] These two organophosphorus compounds have been used as representatives for organophosphorus AChE inhibitor by many researchers.[18]

We have compared the reactivation potency of the oxime **6** with two currently used AChE reactivators (2-PAM, HI-6) and with the previously reported 1,7-heptylene-bis-*N*,*N*′-pyridiniumaldoximes (**1a**, **1b**).[7] As it is shown in Figure 3, the reactivation test was carried out for DFP-inhibited housefly (HF). The bis-pyridinium oximes **6** connected with either $CH_2O(CH_2)_2OCH_2$ linker or $CH_2O(CH_2)_4OCH_2$ linker between the two quaternary nitrogens showed strong potency at 5×10^{-3} M concentration. Moreover, these new oximes **6** showed much stronger activity compared with **1a** and **1b**. The major difference in structure between **6** and **1** is the presence of oxygen atoms in the linker between two pyridinium rings in the case of **6**. The oximes **6a**, **6b**, and **6d** displayed reactivation potency of 63%, 65%, and 58%, respectively, and therefore these oximes were selected for further evaluation. Figure 4 shows the reactivation potency of these oximes (**6a**, **6b** and **6d**) for RBC-AChE inhibited by DFP, HF-AChE inhibited by paraoxon, and RBC-AChE inhibited by paraoxon, respectively. The oximes **6a** and **6b** which have two methylene groups between two oxygen atoms are more potent than the oxime **6d** which has four methylene groups between

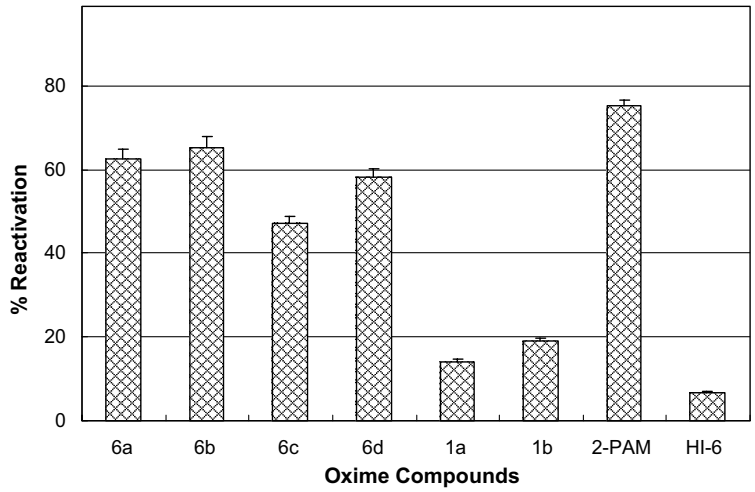


Figure 3. Reactivation potency of tested oximes for DFP-inhibited HF AChE.

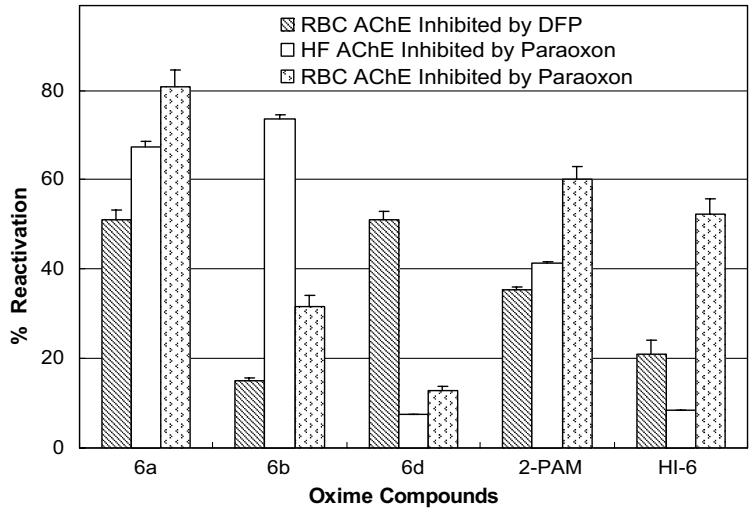


Figure 4. Reactivation potency of oximes for DFP- and paraoxon-inhibited AChE.

two oxygen atoms. Thus the length of the linker between two pyridinium rings is also important to the reactivation potency in bis-pyridinium oximes. In comparison of **6a** with **6b**, the oxime **6a** which has para-substituted oxime group is more potent than **6b** having the oxime group at the meta-position of pyridinium ring, and this result correlates with many other studies.[5,8] The reactivation potency of **6a** was compared with the potency of 2-PAM and HI-6. 2-PAM is quite potent for HF-AChE inhibited by DFP and RBC-AChE inhibited by paraoxon, whereas HI-6 shows very weak potency in all tests. The oxime **6a** was more potent than 2-PAM, and was very potent for HF-AChE inhibited and RBC-AChE inhibited by paraoxon.

5. Experimental

5.1. General

All reactions were carried out under N_2 atmosphere unless otherwise noted. Acetonitrile was distilled over CaH_2 prior to use. Organic extracts or filtrates were washed with brine, dried over anhydrous Na_2SO_4, and concentrated *in vacuo*. Flash chromatography was performed with Merck-EM Type 60 (230-400 mesh) silica gel. ^{1}H NMR spectra were measured on Bruker Win 200 MHz spectrometers. Mass spectrometric data (API-ES, Atmospheric pressure ionization electron spray) were determined by use of Hewlett-Packard 1100 Series Liquid Chromatograph/Mass Spectrometer electron impact (EIMS) method are reported as m/z (relative intensity). Melting points are uncorrected.

5.1.1. 1,2-Bis-chloromethoxy-ethane (4a). A mixture of ethylene glycol (5.00 g, 80.6 mmol) and powdered calcium chloride (5.0 g) in dried benzene (70 mL) was treated with paraformaldehyde (7.26 g, 161 mmol). HCl gas was obtained in situ from H_2SO_4 (22 mL) and NaCl (47 g), and HCl gas was bubbled through the reaction mixture over 10 h. The reaction mixture was filtered and the filtrate was purified by distillation to give **4a** as colorless liquid (6.75 g, 53%). ^{1}H NMR (200 MHz, $CDCl_3$) δ 3.90 (s, 4H, $2OCH_2$), 5.53 (s, 4H, $2ClCH_2$); ^{13}C NMR (50 MHz, $CDCl_3$) δ 68.5, 82.9.

5.1.2. 1,4-Bis-chloromethoxy-butane (4b). A mixture of 1,4-butane-diol (2.00 g, 22.2 mmol) and powdered calcium chloride (2.0 g) in dried benzene (40 mL) was treated with paraformaldehyde (1.33 g, 44.4 mmol). HCl gas was obtained in situ from H_2SO_4 (6 mL) and NaCl (13 g), and HCl gas was bubbled through the reaction mixture over 10 h. The reaction mixture was filtered and the filtrate was purified by distillation to give **4b** as colorless liquid (1.10 g, 26%). ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.68–1.74 (m, 4H, $2CH_2$), 3.68–3.74 (m, 4H, $2OCH_2$), 5.50 (s, 4H, $2OCH_2Cl$); ^{13}C NMR (50 MHz, $CDCl_3$) δ 25.5, 70.0, 83.2.

5.1.3. 1,2-Dimethoxy-ethylene-bis-*N*,*N*′-4-pyridiumaldoxime dichloride (6a). A mixture of **4a** (0.10 g, 0.63 mmol) and 4-pyridinealdoxime (0.23 g, 1.89 mmol) in anhydrous acetonitrile (10 mL) was heated at 45 °C for 20 h. The resulting white precipitate was collected by filtration and washed with acetonitrile followed by ethanol to give **6a** (0.17 g, 74%).

Mp 162–165 °C; ^{1}H NMR (200 MHz, D_2O) δ 3.94 (s, 4H, OCH_2), 5.91 (s, 4H, NCH_2), 8.27 (d, J = 6.6 Hz, 4H, ArH), 8.40 (s, 2H, N=CH), 8.92 (d, J = 6.8 Hz, 4H, ArH); ^{13}C (50 MHz, D_2O) δ 69.4, 88.1, 124.3, 142.6, 145.8, 150.0; API-ES: *m*/*z* 332.1 [M-2Cl].

5.2. 1,2-Dimethoxy-ethylene-bis-*N*,*N*′-3-pyridiumaldoxime dichloride (6b)

A mixture of **4a** (0.10 g, 0.63 mmol) and 3-pyridinealdoxime (0.23 g, 1.89 mmol) in anhydrous acetonitrile (10 mL) was heated at 45 °C for 20 h. The resulting white precipitate was collected by filtration and washed with acetonitrile followed by ethanol to give **6b** (0.17 g, 74%). mp 155–157 °C; ^{1}H NMR (200 MHz, D_2O) δ 3.98 (s, 4H, OCH_2), 5.99 (s, 4H, NCH_2), 8.16–8.23 (m, 2H, ArH), 8.40 (s, 2H, N=CH), 8.83 (d, J = 8.2 Hz, 2H, ArH), 8.96 (d, J = 5.8 Hz, 2H, ArH), 9.21 (s, 2H, ArH; ^{13}C (50 MHz, D_2O) δ 69.5, 88.8, 127.9, 133.2, 140.1, 142.1, 143.8, 144.3; API-ES: *m*/*z* 332.1 [M-2Cl].

5.3. 1,2-Dimethyl-propylene-bis-*N*,*N*′-4-pyridiumaldoxime dichloride (6c)

A solution of 4-pyridine aldoxime (1.31 g, 10.7 mmol) in DMF (20 mL) was treated with **4b** (1.00 g, 11.4 mmol) and the mixture was stirred at rt for 27 h. The obtained precipitate was washed with acetone and ethyl acetate to give **6d** (2.32 g, 95%) as a white solid. mp 128–130 °C; ^{1}H NMR (200 MHz, D_2O) δ 1.95–2.01 (m, 4H, CH_2CH_2), 3.75–3.81 (m, 4H, OCH_2), 5.88 (s, 4H, NCH_2O), 8.26–8.29 (m, 4H, ArH), 8.41 (s, 2H, N=CH), 8.90–8.94 (m, 4H, ArH); ^{13}C (50 MHz, D_2O) δ 27.7, 67.1, 88.1, 124.3, 142.5, 145.8, 149.9.

API-ES: *m*/*z* 387.2 [M-CNOH].

5.4. 1,2-Dimethyl-propylene-bis-*N*,*N*′-2-pyridiumaldoxime dichloride (6d)

A solution of 2-pyridine aldoxime (1.31 g, 10.7 mmol) in DMF (20 mL) was treated with **4b** (1.00 g, 11.4 mmol) and the mixture was stirred at rt for 27 h. The obtained precipitate was washed with acetone and ethyl acetate to give **6d** (1.06 g, 55%) as a white solid. m.p. 118–120 °C; ^{1}H NMR (200 MHz, D_2O) δ 1.54–1.68 (m, 4H, CH_2CH_2), 3.57–3.74 (m, 4H, OCH_2), 6.07 (s, 4H, NCH_2O), 8.06–8.14 (m, 2H, ArH), 8.49–8.53 (m, 2H, ArH), 8.60–8.64 (m, 2H, ArH), 8.71 (s, 2H, N=CH), 8.94–8.97 (m, 2H, ArH); ^{13}C (50 MHz, D_2O) δ 27.6, 73.0, 90.5, 129.5, 130.2, 144.6, 146.9, 149.1, 149.8; API-ES: *m*/*z* 359.0 [M-2Cl].

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