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Synthesis and in vitro evaluation of bis-quaternary 2-(hydroxyimino)-*N*-(pyridin-3-yl)acetamide derivatives as reactivators against sarin and VX inhibited human acetylcholinesterase (*h*AChE)

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ABSTRACT

A series of bis-quaternary pyridinium derivatives **3a**–**3i** of 2-(hydroxyimino)-*N*-(pyridin-3-yl)acetamide (**2**) have been synthesized. The synthesized pyridinium compounds have an amide group in conjugation to the oxime moiety. These compounds were evaluated in vitro for their reactivation efficacy against organophosphorus (OP) nerve agents (NAs) (sarin and VX) inhibited human erythrocyte ghost acetylcholinesterase (*h*AChE) and compared with the reactivation efficacy of 2-PAM and obidoxime. The pK_a values of the synthesized compounds were found closer to the pK_a values of 2- and 4-pyridinium oxime reactivators such as 2-PAM and obidoxime. Some of the compounds have shown better reactivation efficacy than 2-PAM, and obidoxime against sarin and VX inhibited AChE.

1. Introduction

The use of the organophosphorus (OP) compounds as pesticides (parathion, malathion, chlorpyriphos) have been restricted in many countries but still are in use in the developing countries owing to their effectiveness and low cost.[1] Unfortunately, some of these OP compounds were evolved as chemical warfare (CW) agents in the form of nerve agents (NA) such as sarin, soman, tabun and VX (Fig. 1).[2] With growing threats of recent terrorist activities worldwide, one should not deny the possibility of the repeated use of NAs on a mass scale in the future. Further, the decontamination and/or destruction of large stockpile of NAs envisage a serious threat to human health. In this regard, the development of effective medical treatment regimen of OP poisoning has attracted the attention of many researchers.

Acetylcholinesterase (AChE) is the key enzyme responsible for the hydrolysis of the neurotransmitter, acetylcholine (ACh). Intoxication by the OP compounds inhibit the enzyme AChE irreversibly by forming the covalent bond between serine residue of the active site of AChE and phosphorus atom of the OP inhibitor.[3] The inhibition of AChE leads to the overstimulation of the cholinergic receptor in the synapse which finally results in the breakdown of neuromuscular function. The inhibited AChE can be reactivated by the dephosphorylation of the serine residue by a nucleophile such as pyridinium oxime. At present, the standard treatment for OP poisoning involves the use of a muscarinic receptor antagonist such as atropine, an oxime as reactivator of inhibited AChE, and an anticonvulsant agent such as diazepam.[4] The commonly used reactivators are quaternary mono- or bis-pyridinium compounds carrying the hydroxyiminomethyl (oxime) group as nucleophilic agent. Oximes viz. 2-hydroxyiminomethyl-1-methylpyridinium chloride (2-PAM), 1,3-bis-(4-hydroxyiminomethylpyridino-1-yl) propane dibromide (TMB-4), 1-(2-hydroxyimino-methylpyridino-1-yl)-3-(4-carbamoylpyridino-1-yl)-2-oxapropane dichloride (HI-6) and 1,3-bis-(4-hydroxyiminomethylpyridino-1-yl)-2-oxapropane dichloride (obidoxime) are commercially available oxime reactivators used in the treatment of OP poisoning (Fig. 2).[5] The binding interaction of the OP compounds with the enzyme AChE differs owing to their diverse structural features which results in the variation of the reactivation efficacy of 2-PAM and other oximes.[6] Each type of OP needs the AChE reactivator having specific structure and

Abbreviations: AChE, acetylcholinesterase; 2-PAM, 2-(hydroxyiminomethyl)-1-methylpyridinium chloride; OP, organophosphorus; NA, nerve agent.

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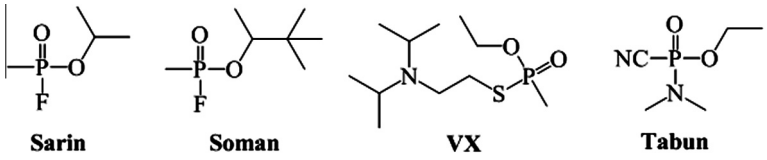


Figure 1. Structure of the organophosphorus nerve agents.

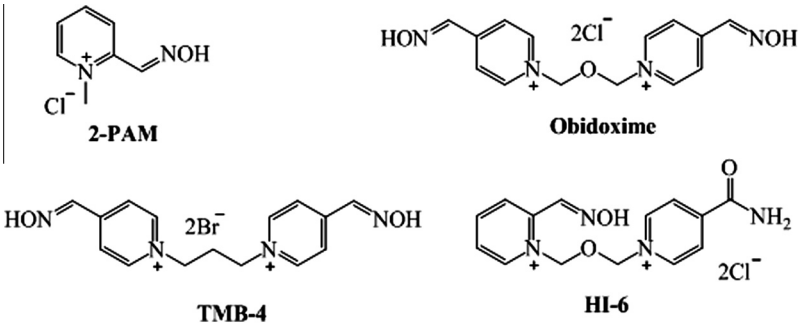


Figure 2. Structure of the pyridinium oxime reactivators.

hence a broad spectrum reactivator is lacking till date. Therefore, the development of universal and effective oxime reactivators of AChE in the treatment of OP poisoning is very important. To overcome these difficulties, a large number of oxime reactivators were synthesized and screened for the search of an effective and universal AChE reactivator during last several decades.[7]

2. Result and discussion

2.1. Chemical structures of the studied oximes

In continuation to our study on antidotes against OP intoxication,[8] herein we report the synthesis of bis-quaternary derivatives of 2-(hydroxyimino)-*N*-(pyridin-3-yl)acetamide (**3a**–**3i**). The amide group (–NHCO–) has both the features of a hydrogen bond donor (–NH–) and hydrogen bond acceptor (>C=O), which may result in better binding interactions with the enzyme AChE. This kind of binding interaction may be responsible for the reduced K_D (dissociation constant) value of some of the oximes (**3a**–**3i**) in comparison to 2-PAM and obidoxime (Fig. 3).

Previously, we have reported the effect of various types of linkers on the reactivation efficacy of the bis-quaternary pyridinium oximes.[8] The intermediate 2-(hydroxyimino)-*N*-(pyridin-3-yl)acetamide (**2**) was prepared from 3-aminopyridine (**1**), chloral hydrate and hydroxylamine hydrochloride in water. The coupling of oxime **2** with various terminal dibromo aliphatic and xylene linkers yielded the desired bis-quaternary products (**3a**–**3i**) (Scheme 1).

The attempted synthesis of corresponding 2-(hydroxyimino)-*N*-(pyridin-2-yl)acetamide and 2-(hydroxyimino)-*N*-(pyridin-4-yl)acetamide using 2-aminopyridine and 4-aminopyridine, respectively, resulted only trace amount of conversions. This may be due to the less nucleophilic $-NH_2$ group in conjugation to the ring nitrogen in 2- and 4-aminopyridine.

2.2. In vitro reactivation kinetics of the synthesized oximes against NA-inhibited *h*AChE

The reactivation kinetic parameters (rate constants and dissociation constant) depend on the source of enzyme, experimental buffer pH, temperature and the experimental protocols. The synthesized compounds (**3a**–**3i**) were assayed for their in vitro

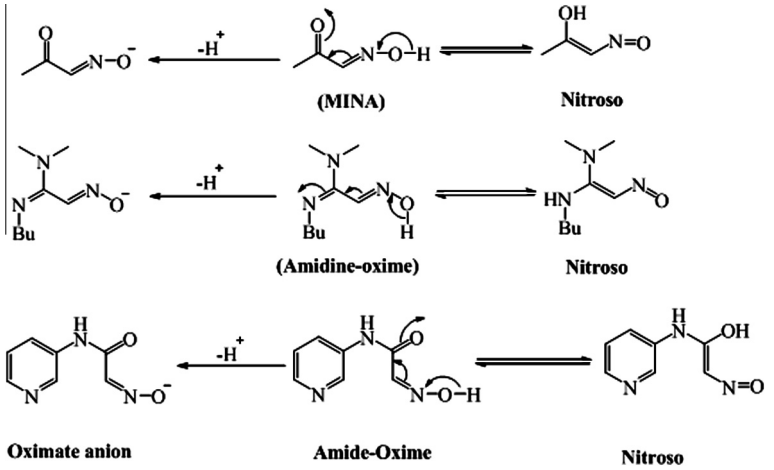
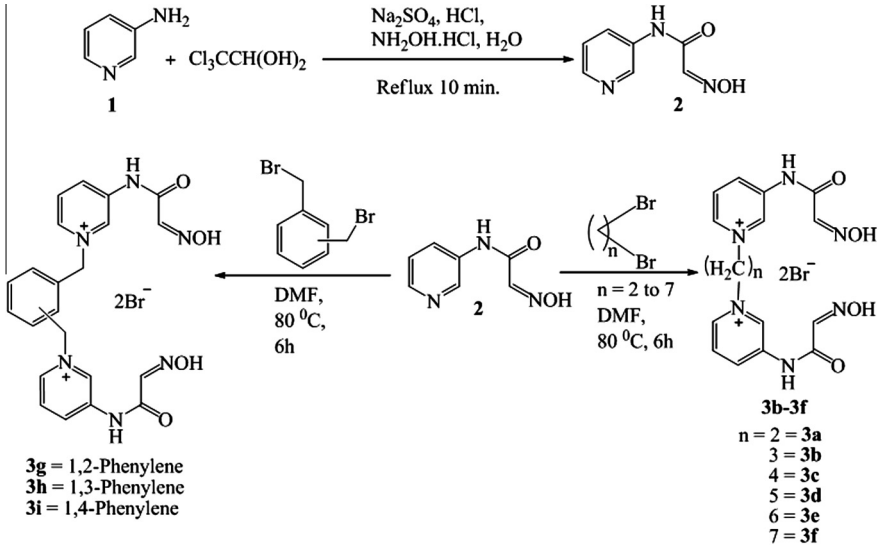


Figure 3. Conjugation of oxime group with amidine, carbonyl and amide group.



Scheme 1. Synthesis of 2-(hydroxyimino)-*N*-(pyridin-3-yl)acetamide (**2**) and corresponding mono and bis-quaternary pyridinium compounds (**3a**–**3i**).

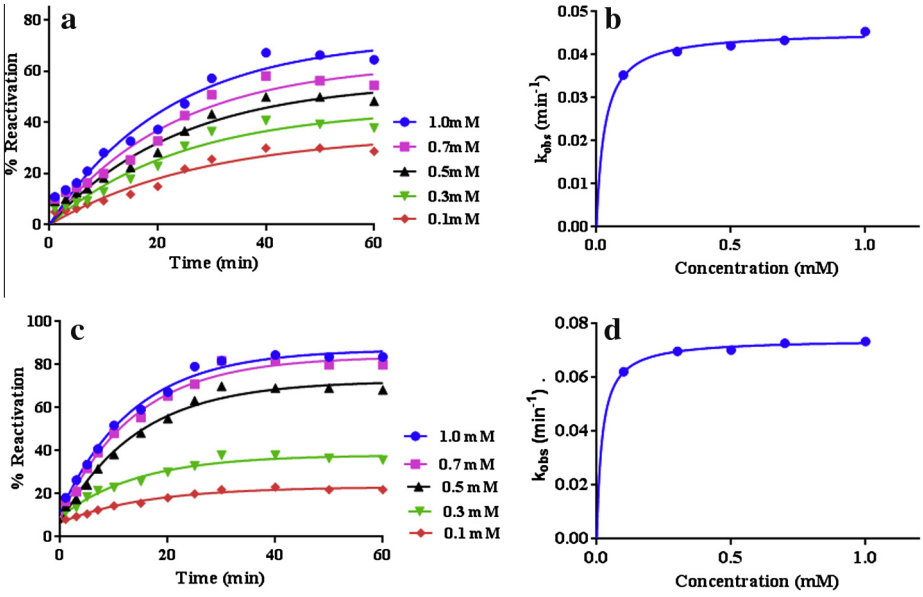


Figure 4. Time and concentration dependent reactivation profile of oxime **3d** against sarin (a) and VX (c) inhibited *h*AChE; plot of k_{obs} versus [**3d**] (mM) for sarin (b) and VX (d) inhibited *h*AChE.

reactivation potency at five different concentrations (0.1–1 mM) using erythrocyte ghost *h*AChE inhibited by nerve agents: sarin and VX (Fig. 4). Reactivation study was monitored till one hour at different time intervals and kinetic data were generated (Fig. 5 and Table 1).

The OP inhibitors were chosen on the basis of their structural divergence feature. The in vitro reactivation data were compared with those of 2-PAM and obidoxime. The affinity of the reactivator towards the OP-inhibited enzyme is expressed in terms of dissociation constant (K_D μM). The values of the K_D for the oximes **3a**–**3i** (Table 1) against sarin inhibited AChE varies largely between 20 and 57 μM, except the oxime **3f** (84.29 μM). As compared to 2-PAM (K_D 25.72 μM) and obidoxime (K_D 21.48 μM), both the oximes **3g** (K_D 20.82 μM) and **3i** (K_D 8.55 μM) showed better affinity towards sarin inhibited AChE. In case of VX–AChE conjugate, oximes **3d**, **3e**, **3i** showed better affinity towards phosphorylated enzyme as compared to 2-PAM and obidoxime.

The efficiency of the oxime for the de-phosphorylation of the phosphoryl residue from the OP-inhibited AChE is expressed in terms of reactivity constant (k_r min^{-1}). The rate constant k_r for the oximes **3a**–**3i** towards the sarin and VX inhibited AChE were found in the range of 0.045–0.0.11 and 0.048–0.23 min^{-1}, respectively, (Table 1). The second order rate constant k_{r2} gives the specific reactivity of the oxime and it depends on K_D and k_r. In comparison to the k_{r2} of 2-PAM (2.23 $mM^{-1}min^{-1}$) and obidoxime (8.03 $mM^{-1}min^{-1}$), the k_{r2} of the oximes **3a**–**3i** ranges between 1.1 and 10.85 $mM^{-1}min^{-1}$, for the sarin inhibited AChE. Similarly, in case of VX-inhibited AChE the k_{r2} of the obidoxime (8.31 mM^{-1} min^{-1}) is more than all oximes **3a**–**3i** (1.95–5.04 $mM^{-1}min^{-1}$) (Table 1).

In addition to the kinetic parameters, the inhibition potential of the oximes (IC_{50}) towards the enzyme is also the important factor. Therefore the inhibition potential (IC_{50}) values of the oximes **3a**–**3i** were also determined and found in the range of 0.93–2.53 mM. The synthesized oximes (**3a**–**3i**) can act as good reactivator if they have

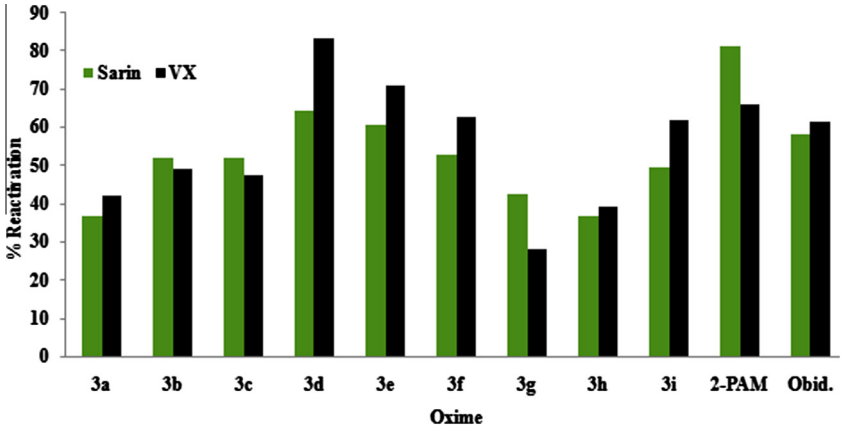


Figure 5. Reactivation efficacy (%) of the oximes **3a–3i** in comparison to 2-PAM and obidoxime at 60 min against sarin and VX inhibited *h*AChE (1.0 mM concentration of oximes, 0.1 M phosphate buffer, pH 7.4 and temperature 37 °C).

Table 1
In vitro reactivation kinetic constants and IC_{50} values for the oximes against NA-inhibited *h*AChE

Oxime	K_D μM (±SE)		k_r min^{-1} (±SE)		k_{r2} ($mM^{-1}min^{-1}$)		IC_{50} (mM)
	Sarin	VX	Sarin	VX	Sarin	VX	
3a	42.86 (4.6)	48.66 (8.2)	0.05 (0.0007)	0.20 (0.0048)	1.25	4.17	2.53
3b	57.96 (10.5)	88.86 (12.7)	0.06 (0.0018)	0.23 (0.0065)	1.10	2.62	1.07
3c	52.32 (9.7)	86.12 (3.3)	0.08 (0.0002)	0.23 (0.0017)	1.46	2.63	1.06
3d	30.23 (4.7)	19.71 (2.1)	0.05 (0.0007)	0.07 (0.0006)	1.50	3.76	1.79
3e	24.39 (2.9)	16.86 (2.0)	0.05 (0.0005)	0.07 (0.0005)	2.16	3.97	1.43
3f	84.29 (13.2)	65.82 (5.9)	0.12 (0.0036)	0.13 (0.0019)	1.42	1.95	0.93
3g	20.82 (1.8)	22.57 (3.0)	0.05 (0.0003)	0.05 (0.0008)	2.24	3.47	1.7
3h	48.38 (4.0)	54.93 (9.7)	0.10 (0.0012)	0.12 (0.0032)	2.16	2.17	1.67
3i	8.55 (2.0)	17.46 (1.5)	0.09 (0.0027)	0.09 (0.0005)	10.85	5.04	2.1
2-PAM	25.72 (2.1)	31.39 (3.9)	0.06 (0.0004)	0.11 (0.0014)	2.23	3.59	1.19
Obid.	21.48 (3.0)	21.86 (1.6)	0.17 (0.0019)	0.18 (0.0011)	8.03	8.31	0.87

Table 2
Physical and elemental analysis data of the compounds **3a–3i**

Entry	Oxime	Yield (%)	MP (°C)	pK_a	C	H	N	C	H	N
					Calculated (%)			Found (%)		
2	**3a**	65	200–202	8.14 ± 0.03	37.09	3.50	16.22	36.99	3.55	16.18
3	**3b**	60	238–240	8.17 ± 0.04	38.37	3.79	15.79	38.33	3.81	15.82
4	**3c**	63	240–242	8.16 ± 0.03	39.58	4.06	15.39	39.51	4.14	15.46
5	**3d**	65	216–218	8.14 ± 0.05	40.73	4.32	15.00	40.76	4.41	14.91
6	**3e**	62	241–243	8.25 ± 0.05	41.83	4.56	14.63	41.78	4.61	14.61
7	**3f**	58	201–203	8.22 ± 0.04	42.87	4.80	14.29	42.81	4.91	14.21
8	**3g**	62	244–246	8.10 ± 0.05	44.46	3.73	14.14	44.39	3.81	14.16
9	**3h**	65	256–258	8.23 ± 0.03	44.46	3.73	14.14	44.39	3.77	14.02
10	**3i**	67	243–245	7.95 ± 0.04	44.46	3.73	14.14	44.41	3.66	14.05

considerable nucleophilicity which can be determined on the basis of their acid dissociation constant (pK_a). The pK_a of the oximes **3a–3i** were found in the range of 7.95–8.2 (Table 2). Now, the comparison of the kinetic data, pK_a and IC_{50} values can lead to the meaningful conclusion for the oximes **3a–3i** as reactivators against OP-inhibited AChE.

3. Discussion

The reactivation of the OP-inhibited AChE depends on several factors including structure of the reactivator,[9] structure of the inhibitor (OP), source of the enzyme,[10] on post-inhibitory reactions such as spontaneous dealkylation (aging)[11] and spontaneous dephosphorylation (spontaneous reactivation) of the OP–AChE complex.[12] The oxime moiety present in the pyridinium reactivators acts as strong nucleophile and effectively breaks the O—P bond in the OP–AChE conjugate during the reactivation process. It is well known that the substituents having negative inductive/resonance effect in conjugation to the oxime group enhances its ionization tendency into oximate anion (better nucleophile) which in turn increases the reactivation efficacy of the oxime reactivator.[13] The oxime group of the monoisonitrosoacetone (MINA) is in conjugation with carbonyl group and therefore was found to show good reactivation efficacy against OP inhibited AChE.[14] Recently amidine–oximes[15a] and amide conjugated oximes[15b,c] were reported as reactivators against OP-inhibited AChE. The electron withdrawing functional groups such as carbonyl, amidine and amide in conjugation to the oxime group can assist the dissociation of oxime group into more active oximate anion thereby increasing their reactivation efficacy (Fig. 3).

The structures of OP inhibitors, VX and sarin are different; therefore variation in the reactivation of OP-inhibited AChE by

the reactivators was expected. The steric influence that differs in the structures of VX and sarin might contribute to the variation towards in vitro oxime reactivation which can be explained on the basis of molecular volumes of these OP agents. Inhibition of AChE by OP NAs leads to the cleavage of the leaving group to form alkylmethylphosphonate–AChE conjugates. The differences in the molecular volume of sarin and VX arise mainly because of the *O*-alkyl substituents. Therefore the 20 Å deep active site gorge of AChE will be largely occupied by sarin followed by VX inhibited AChE.[16] The compound **3g**–**3i** have the aromatic xylene linker (π-electrons) which can interact well with the AChE active site residues via π–π or cation–π interactions. This may be the major reason that amongst the synthesized compounds, the compound **3i** showed far better reactivation efficacy than 2-PAM and obidoxime against sarin and VX inhibition. As compared to *ortho* and *meta*-xylene linkers (compound **3g** and **3h**, respectively, Scheme 1), the *para*-xylene linker (**3i**) disposed the two oxime groups far apart from each other. This kind of orientation of the oxime **3i** may allow one of the two pyridinium rings to reside on the rim, and other pyridinium ring penetrates into gorge of the enzyme where phosphorylation of active site of enzyme takes place.[8b,18b] This kind of the interaction of aromatic xylene linker might be responsible for the lower value of the rate constant K_D for the oximes **3g** and **3i** than other synthesized oximes, toward sarin and VX-inhibited AChE. Similarly the AChE reactivators and/or inhibitor having conformationaly flexible longer aliphatic chain (six to seven carbon chain) might orient well within the range of peripheral to catalytic site of enzyme AChE.[17] This might be the plausible reason that the oximes **3d** and **3e** showed better affinity (less K_D value) toward sarin and VX inhibited AChE, in comparison to 2-PAM and obidoxime.

The reactivation efficacy of the pyridinium oxime reactivators depend on certain structural and physicochemical parameters such as position of the oxime group in the pyridinium ring, steric factors, length and nature of the connecting chain, and most importantly, its acid dissociation constant (pK_a) value.[18] The extent of dissociation of the oxime group into the more nucleophilic oximate anion can be attributed from its pK_a value. All the synthesized compounds (**3a**–**3i**) have 3-substituted pyridinium ring and their pK_a value were found in the range of 7.95–8.25 (Table 2 and Fig. 6).

The pK_a values of the compounds (**3a**–**3i**) were found closer to those of 2- and 4-pyridinium oxime reactivators (pK_a 7.8–8.5). Generally it is observed that the 3-pyridinium oxime reactivators have $pK_a \geqslant 9.0$ which reflect their lesser tendencies to dissociate into oximate anion.[19] This might have resulted in the lower reactivation efficacy of the 3-pyridinium oxime reactivators as compared to their 2- and 4-isomers. However, in the synthesized compounds (**3a**–**3i**), the oxime group is not directly attached to 3-position of the pyridinium ring but is attached to the side chain at 3-position. Hence the resonance effect of the pyridinium ring may not affect directly to the nucleophilicity of 3-substituted side chain oxime group which in fact is reflected in their lower pK_a values (pK_a 7.95–8.25). In addition, the conjugation of the amide group with oxime group may assist the dissociation of the compounds **3a**–**3i** which might be responsible for their lower pK_a value as compared to 3-pyridinium oximes. The pK_a of the oximes may affect the values of kinetic rate constant (k_r and k_{r2}). The oxime **3i** has the least pK_a value (7.95) among the oximes **3a**–**3i**, which might be one of the reasons responsible for the higher second order rate constant (k_{r2}) against sarin and VX inhibited AChE.

The IC_{50} values of the oximes **3a**–**3i** (Fig. 7) showed that **3a**, **3d**, **3e**, **3g**, **3h** and **3i** were less inhibitors as compared to 2-PAM and obidoxime (Table 1). Among the synthesized oximes (**3a**–**3i**), the oxime **3f** has least IC_{50} value (0.93 mM) followed by oxime **3b** (1.07 mM) and **3c** (1.06 mM). Therefore these three oximes (**3b**, **3c** and **3f**) inhibit the enzyme AChE relatively more as compared to other oximes, which might be one of the plausible reasons responsible for their less reactivation efficacy. Overall, the oximes **3a**, **3d**, **3e**, **3g**, and **3i** were found to show relatively low inhibition and better reactivation efficacy against VX-inhibited AChE while oxime **3i** showed better reactivation against sarin-inhibited AChE.

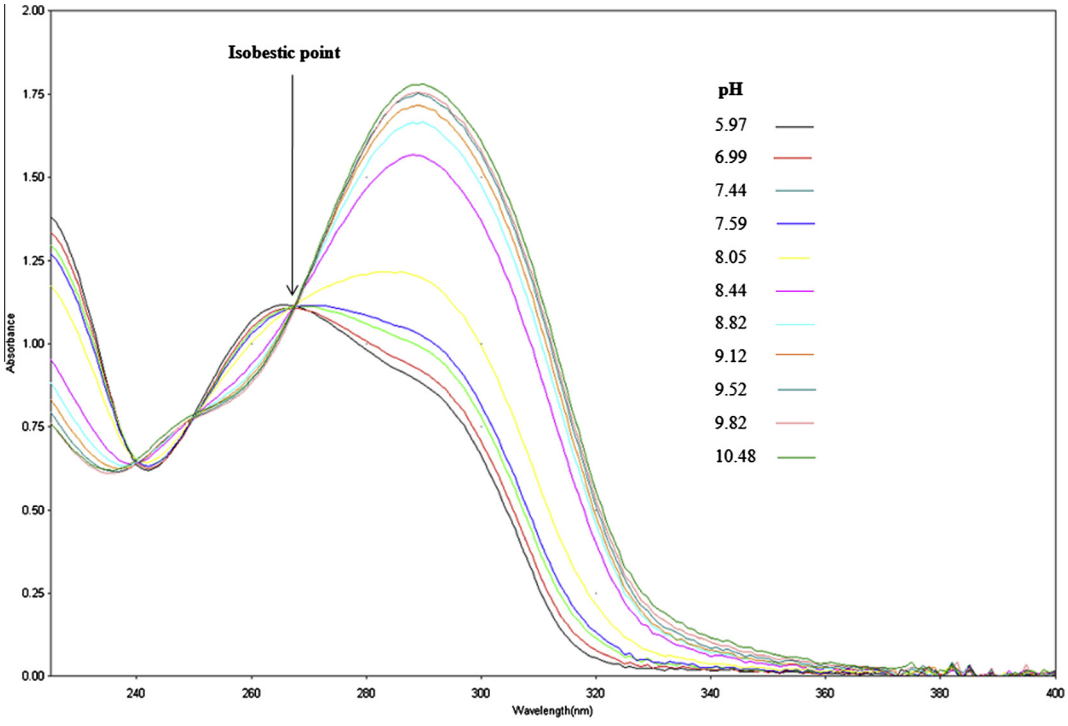


Figure 6. Spectrophotometric determination of pK_a of the oxime **3d**.

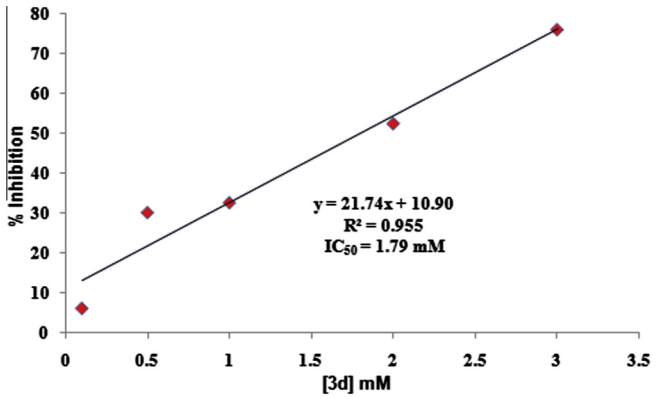


Figure 7. IC_{50} of the oxime **3d**.

4. Conclusions

We have synthesized the series of bis-quaternary pyridinium acetamide oxime reactivators (**3a**–**3i**) from 2-(hydroxyimino)-*N*-(pyridin-3-yl)acetamide (**2**). These are the pyridinium reactivators having amide group in conjugation to the oxime functionality. The synthesized compounds were evaluated in vitro for their reactivation efficacy against sarin and VX inhibited *h*AChE. Based upon this study, **3a**, **3d**, **3e**, **3g**, and **3i** may provide a useful therapeutic potential for the reactivation of AChE inhibited by OP NAs. The detailed study of antidotal efficacy including in vivo reactivation against sarin and VX will be reported in due course of time.

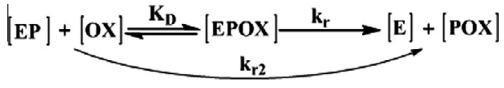
5. Experimental sections

5.1. Materials

Acetylthiocholineiodide (ATCh), 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), 3-aminopyridine, chloral hydrate, hydroxylamine hydrochloride, aliphatic linkers and α,α′-dibromoxylene, potassium dihydrogenphosphate, dipotassium-hydrogen phosphate, trizma-base and trizma-HCl were purchased from Sigma–Aldrich, USA and used without further purification. Glycine was obtained from E. Merck (India) and used without further purification. Anhydrous sodium sulfate was purchased from Qualigens, India. Solvents (DMF, acetone, and methanol) were purchased from S.D. Fine Chemicals (India) and dried before use. VX and sarin 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.[20] Obidoxime was synthesized using reported methods.[21] The bis-quaternary pyridinium salts were synthesized, characterized (I.R., ^{1}H and ^{13}C NMR spectral data) and their purity was checked by commercially available pre-coated cellulose on alumina sheets TLC plates (E. Merck) with 1-butanol/acetic acid/water (3:1:1) as solvent system (Table 2). Hemoglobin free human erythrocyte ghost enzyme acetylcholinesterase (*h*AChE) was prepared using the reported protocol.[22]

5.2. Enzyme assays

The activity of *h*AChE was measured spectrophotometrically (Carry 100 UV–Visible spectrophotometer) at 37 °C, using modified Ellman assay.[23] ATCh (0.45 mM) as substrate and DTNB (0.3 mM) as chromogen were used in 0.1 M phosphate buffer (pH 7.4). Cuvette contained total 3.0 mL volume of assay mixture.



Scheme 2. Oxime induced reactivation of OP-inhibited AChE.

5.3. Enzyme inhibition by NA

A freshly prepared stock solution of OP inhibitor (sarin, 1.4×10^{-2} M and VX, 1.33×10^{-2} M) was in isopropanol and stored under refrigeration. It was then diluted appropriately with triple distilled water just before use. Around 300 μL of enzyme suspension (0.1 M phosphate buffer, pH 7.4) was incubated by 5–10 μL of NA (sarin, 1.4×10^{-6} M and VX, 3.32×10^{-7} M) at room temperature for 10–15 min. AChE activity was measured to determine the extent of inhibition (95–98%). Excess of NA was removed by the extraction of NA inhibited enzyme mixture with tenfold volume of *n*-hexane. The mixture of NA inhibited enzyme was stored at −60 °C and consumed on the same day.

5.4. Reactivation kinetics of NA-inhibited *h*AChE by oxime

The NA inhibited *h*AChE suspension (20 μL) was transferred to the cuvette containing phosphate buffer (0.1 M, pH 7.4), DTNB and an oxime (0.1–1 mM). AChE activity was measured after different time intervals at 37 °C by adding ATCh. Spontaneous reactivation of the inhibited AChE was assayed using the same protocol, the reaction mixture contained enzyme and OP but no oxime. Under these conditions spontaneous reactivation was found to be insignificant. All the values were corrected for their oxime induced hydrolysis. The IC_{50} values of the oximes were determined by using the same protocol, for which oxime induced inhibition of enzyme was studied at different concentration.

The process of oxime induced reactivation of the NA-inhibited AChE can be represented by Scheme 2.

[EP] is the phosphylated enzyme, [OX] is the reactivator, [EPOX] is the Michaelis-type complex between phosphylated-AChE and oxime reactivator, [E] is the reactivated enzyme, [POX] is the phosphylated oxime, K_D is the dissociation constant and is inversely proportional to the affinity of reactivator towards the phosphylated enzyme [EP], k_r is the rate constant for the displacement of phosphoryl residue from [EPOX] by oxime and it expresses the reactivation efficiency of the reactivator.

For the reactivation process where complete reactivation occurs with $[OX] >> [EP]_0$ then pseudo first order rate equation[24] can be derived;

$$K_obs = \frac{k_r[OX]}{K_D + [OX]} \quad (1)$$

where k_{obs} is the observed first-order rate constant of the reactivation at any given oxime concentration.

The value of k_{obs} is not proportional to the oxime concentration but underlies a saturation kinetics.[25] The rate constants k_r and K_D follow Michaelis–Menten kinetics.[26] The second order reactivation rate constant k_{r2} is the ratio of k_r and K_D;

$$k_{r2} = \frac{k_r}{K_D} \quad (2)$$

All the kinetic rate constants were calculated by processing the experimental data with nonlinear regression analysis using curve-fitting programs provided by Prism™ Vers. 6.0 (GraphPad software, San Diego, USA).

5.5. Determination of acid dissociation constant (pK_a)

The acid dissociation constants (pK_a) of all the compounds (**3a**–**3i**) were determined spectrophotometrically using the method of

Albert and Sergeant.[27] The method is based on the direct determination of the ratio of molecular species (protonated) to the 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 the interest would be present wholly in either form. 30–50 μL of oxime stock solutions (5×10^{-3} M) were diluted to 3 mL in a cuvette containing either 0.1 M hydrochloric acid or 0.1 M sodium hydroxide solution and the absorption spectra of the oxime in acid or alkali were measured over the wavelength range of 200–600 nm with a reference to blank solution at 25 ± 1 °C. The spectra, thus obtained in acid or alkali, were of protonated (D_m) and deprotonated (D_i) molecules. Eleven different pH values, ranging from 5.97 to 10.48 were selected to determine the pK_a of oximes. For this, appropriate buffers consisting of phosphate (pH 5.97–8.05), tris (pH 8.44–9.12) and glycine–NaOH (pH 9.52–10.48), were used to determine the dissociation constants of oximes. 30–50 μL of aqueous solutions of oximes was 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 pK_a were obtained using Eq. 3;

$$pK_a = pH + \log[(D_i - D)/(D - D_m)] \quad (3)$$

where, D_m, and D_i, correspond to the optical density of protonated and deprotonated forms of the compounds (**3a**–**3i**), and D is the optical density in the buffer. The average value of the eleven measurements was considered as the pK_a of the compound with respect to oximino functionality. Thermospectronic Unicam 300 UV–Visible double beam spectrophotometer with quartz cells of 10 mm were 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 the buffers were determined using a Mettler-Toledo Seven Easy pH meter equipped with Inlab® Expert Pro glass electrode with an accuracy of ±0.01 units. The pH meter was calibrated at 25 °C using the two point calibration method with commercially available Mettler-Toledo standard buffer solutions pH 7.00 and 9.21.

Reagents: Freshly prepared standard solutions of oximes (5×10^{-3} M) in distilled water were used as stock solutions. Buffer solutions of appropriate pH were prepared according to the reported method.[28] Solutions of the oximes in 0.1 M hydrochloric acid and 0.1 M sodium hydroxide were used for determining the analytical wavelength of the undissociated and dissociated forms respectively.

5.6. Synthetic procedure

5.6.1. Synthesis of 2-(hydroxyimino)-*N*-(pyridin-3-yl)acetamide (2)

In a 50 mL round bottom flask, 3-aminopyridine (2.82 g, 30 mM) was dissolved in a mixture of 10 mL water and 2.5 mL conc. hydrochloric acid at room temperature. In another 250 mL round bottom flask, chloral hydrate (4.98 g, 30.12 mM) was dissolved in 70 mL of water and sodium sulfate (50 g, 352.01 mM) was added to it. The reaction mixture was stirred at room temperature. Previously prepared solution of 3-aminopyridine was added to the reaction mixture of chloral hydrate followed by addition of hydroxylamine hydrochloride (6.6 g, 95 mM). The reaction mixture was then refluxed on an oil bath and stirred for 10 min. It was then brought to the room temperature and neutralized by adding 28–30% aqueous ammonia solution till the product, 2-(hydroxyimino)-*N*-(pyridin-3-yl)acetamide was precipitated out. Tan colored solid product was filtered off, washed repeatedly by cold water and dried under vacuum (3.21 g; yield: 65%; mp: 186–188 °C). Purity of the product was checked by pre-coated silica on alumina sheets TLC plates (Merck), (ethyl acetate/hexane 8:2, R_f = 0.31).

5.6.2. Synthesis of 1,1′-(pentane-1,5-diyl)bis(3-(2-(hydroxyimino)acetamido)pyridinium) dibromide (3d)

In a typical experimental procedure, 2-(hydroxyimino)-*N*-(pyridin-3-yl)acetamide (1.66 g, 10.1 mM) and 1,5-dibromo pentane (1.14 g, 5 mM) were dissolved in 15 mL dry DMF in a 50 mL round bottom flask fitted with a water condenser and calcium chloride guard tube. It was then stirred at 80 °C for 6 h and monitored by TLC. The reaction mixture was then brought to room temperature, cooled in an ice bath and the brown colored solid appeared was then filtered off and washed repeatedly by hot dry methanol followed by hot dry acetone to give the desired product 1,1′-(pentane-1,5-diyl)bis(3-(2-(hydroxyimino)acetamido)pyridinium) dibromide (**3d**). Yield: 3.65 g (65%); mp: 216–218 °C. All other products were synthesized using the same protocol.

5.6.3. Characterization of the synthesized compounds 3a–3i

Purity of the synthesized pyridinium compounds were checked by thin-layer chromatography (TLC, cellulose, Merck) with 1-butanol/acetic acid/water (3:1:1) as mobile phase. Melting points were determined with open capillary tube on a Gallenkamp (variable heater) melting point apparatus and were uncorrected. The structure of the synthesized compounds was confirmed by their elemental analysis and spectral data. Elemental analyses were conducted on an ELEMENTAR, vario MICRO cube, Universal micro analyzer and were within ±0.4% of the calculated values (Table 2). Infra-red (I.R.) spectra was obtained as KBr discs on a Bruker TENSOR-27 FTIR spectrometer. ^{1}H NMR (DMSO-d_6) spectra were recorded on Bruker Avance 400 spectrometer at 400 MHz using tetramethylsilane as internal standard and expressed in the δ (ppm) values. The –OH and –NH protons appeared as a singlet in the range of δ 12.00 and 11.00, respectively, and were exchangeable with D_2O. ^{13}C NMR (DMSO-d_6) chemical shifts values were obtained using the same instrument at 100 MHz (Supplementary data).

Spectroscopic characterization of the synthesized compounds:

Compound ***2****:* Tan colored power. IR (KBr) ν_{max} (cm^{-1}) 3499, 3397, 3258, 3064, 1826, 1689, 1563, 1482, 993, 799, 645; ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.34 (m, 1H, Ar-H), 7.65 (s, 1H, –CH=NOH), 8.08 (1H, Ar-H), 8.28 (m, 1H, Ar-H), 8.83 (d, J = 2.4 Hz, 1H, Ar-H), 10.38 (s, –NH), 12.28 (s, –OH); ^{13}C NMR (100 MHz, DMSO-d_6) δ 123.63, 127.06, 135.12, 141.51, 143.72, 144.65, 160.85; ESI-MS: m/z 166.0837 [M$^+$] (calcd for $C_7H_7N_3O_2$ 165.15).

Compound ***3a****:* Brown powder. IR (KBr) ν_{max} (cm^{-1}) 3403, 3077, 2970, 1710, 1589, 1564, 992, 825, 676; ^{1}H NMR (400 MHz, DMSO-d_6) δ 5.27 (s, 4H, –CH$_2$), 7.72 (s, 2H, Ar-H), 8.13 (m, 2H, Ar-H), 8.60 (d, J = 8.4 Hz, 2H, Ar-H), 8.75 (d, J = 6.4 Hz, 2H, Ar-H), 9.48 (s, 1H, Ar-H), 9.58 (s, 1H, –CH=NOH), 11.18 (s, 1H, –NH), 11.23 (s, 1H, –NH), 12.57 (s, 1H, –OH), 12.58 (s, 1H, –OH); ^{13}C NMR (100 MHz, DMSO-d_6) δ 60.12, 128.03, 128.33, 135.69, 135.88, 136.06, 136.63, 137.65, 138.55, 138.77, 140.26, 142.84, 142.94, 161.20, 161.49; ESI-MS: m/z 192.3547 (calcd for $C_9H_{10}N_3O_2^+$ 192.19).

Compound ***3b****:* Light brown powder. IR (KBr) ν_{max} (cm^{-1}) 3425, 3076, 2982, 1701, 1611, 1563, 986, 823, 695; ^{1}H NMR (400 MHz, DMSO-d_6) δ 2.6 (m, 2H, –CH$_2$–), 4.74 (m, 4H, –CH$_2$–), 7.73 (s, 2H, Ar-H), 8.13 (m, 2H, Ar-H), 8.61 (d, J = 9.2 Hz, 2H, Ar-H), 8.82 (d, J = 5.6 Hz, 2H, Ar-H), 9.52 (s, 2H, –CH=NOH), 11.21 (s, 2H, –NH), 12.57 (s, 2H, –OH); ^{13}C NMR (100 MHz, DMSO-d_6) δ 31.82, 58.14, 128.21, 135.06, 135.54, 138.60, 139.76, 142.95, 161.38; ESI-MS: m/z 372.4724 (calcd for $C_{17}H_{20}N_6O_4^{2+}$ 372.38).

Compound ***3c****:* Light brown powder. IR (KBr) ν_{max} (cm^{-1}) 3455, 3086, 2976, 1701, 1614, 1565, 993, 830, 713; ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.96 (m, 4H, –CH$_2$–), 4.69 (m, 4H, –CH$_2$–), 7.72 (s, 2H, Ar-H), 8.11 (m, 2H, Ar-H), 8.61 (d, J = 9.6 Hz, 2H, Ar-H), 8.83 (d, J = 6.0 Hz, 2H, Ar-H), 9.49 (s, 2H, –CH=NOH), 11.22 (s, 2H, –NH), 12.58 (s, 2H, –OH); ^{13}C NMR (100 MHz, DMSO-d_6) δ 27.11,

60.44, 128.15, 134.96, 135.44, 138.52, 139.67, 142.99, 161.43; ESI-MS: *m/z* 386.5322 (calcd for $C_{18}H_{22}N_6O_4^{2+}$ 386.40).

Compound ***3d****:* Brown powder. IR (KBr) ν_{max} (cm^{-1}) 3445, 3063, 2977, 1704, 1632, 1561, 1000, 829, 683; ^1H NMR (400 MHz, DMSO-d_6) δ 1.13 (m, 2H, $-CH_2-$), 1.93 (m, 4H, $-CH_2-$), 4.62 (m, 4H, $-CH_2-$), 7.74 (s, 2H, Ar-H), 8.11 (m, 2H, Ar-H), 8.61 (d, J = 8.4 Hz, 2H, Ar-H), 8.85 (d, J = 6.0 Hz, 2H, Ar-H), 9.48 (s, 2H, –CH=NOH), 11.22 (s, 2H, –NH), 12.58 (s, 2H, –OH); ^{13}C NMR (100 MHz, DMSO-d_6) δ 21.72, 29.83, 60.82, 128.06, 134.84, 135.46, 138.43, 139.63, 142.94, 161.37; ESI-MS: *m/z* 400.6017 (calcd for $C_{19}H_{24}N_6O_4^{2+}$ 400.43).

Compound ***3e****:* Light brown powder. IR (KBr) ν_{max} (cm^{-1}) 3392, 3067, 2866, 1707, 1639, 1564, 995, 826, 682; ^1H NMR (400 MHz, DMSO-d_6) δ 1.33 (m, 4H, $-CH_2-$), 1.90 (m, 4H, $-CH_2-$), 4.62 (m, 4H, $-CH_2-$), 7.74 (s, 2H, –CH=NO–), 8.10 (m, 2H, Ar-H), 8.62 (d, J = 8.4 Hz, 2H, Ar-H), 8.85 (d, J = 6.0 Hz, 2H, Ar-H), 9.47 (s, 2H, –CH=NOH), 11.21 (s, 2H, –NH), 12.57 (s, 2H, –OH); ^{13}C NMR (100 MHz, DMSO-d_6) δ 24.57, 30.24, 61.05, 128.04, 134.75, 135.42, 138.39, 139.60, 142.91, 161.34; ESI-MS: *m/z* 414.2576 (calcd for $C_{20}H_{26}N_6O_4^{2+}$ 414.46).

Compound ***3f****:* Tan powder. IR (KBr) ν_{max} (cm^{-1}) 3436, 3059, 2978, 1706, 1657, 1557, 989, 809, 679; ^1H NMR (400 MHz, DMSO-d_6) δ 1.28 (m, 6H, $-CH_2-$), 1.88 (m, 4H, $-CH_2-$), 4.61 (m, 4H, $-CH_2-$), 7.72 (s, 2H, Ar-H), 8.10 (m, 2H, Ar-H), 8.62 (d, J = 9.6 Hz, 2H, Ar-H), 8.84 (d, J = 6.0 Hz, 2H, Ar-H), 9.46 (s, 2H, –CH=NOH), 11.20 (s, 2H, –NH), 12.57 (s, 2H, –OH); ^{13}C NMR (100 MHz, DMSO-d_6) δ 25.44, 28.08, 30.89, 31.18, 61.65, 128.52, 135.27, 135.89, 138.90, 140.03, 143.44, 161.87; ESI-MS: *m/z* 428.3424 (calcd for $C_{21}H_{28}N_6O_4^{2+}$ 428.48).

Compound ***3g****:* Off white powder. IR (KBr) ν_{max} (cm^{-1}) 3140, 3065, 2975, 1702, 1590, 1559, 990, 809; ^1H NMR (400 MHz, DMSO-d_6) δ 6.15 (s, 4H, benzylic $-CH_2$), 7.36 (m, 2H, Ar-H), 7.57 (m, 2H, Ar-H), 7.69 (s, 2H, Ar-H), 8.13 (m, 2H, Ar-H), 8.70 (d, J = 9.2 Hz, 2H, Ar-H), 8.83 (d, J = 6.0 Hz, 2H, Ar-H), 9.37 (s, 2H, –CH=NOH), 11.25 (s, 2H, –NH), 12.55 (s, 2H, –OH); ^{13}C NMR (100 MHz, DMSO-d_6) δ 60.69, 128.45, 130.43, 130.52, 132.42, 135.09, 135.24, 138.86, 139.85, 142.94, 161.46; ESI-MS: *m/z* 433.3020 (calcd for $C_{22}H_{22}N_6O_4^{2+}$ 434.45).

Compound ***3h****:* Light brown powder. IR (KBr) ν_{max} (cm^{-1}) 3428, 3083, 2973, 1701, 1588, 1556, 1502, 995, 807, 736, 673; ^1H NMR (400 MHz, DMSO-d_6) δ 5.94 (s, 4H, benzylic $-CH_2$), 7.57 (m, 3H, Ar-H), 7.69 (m, 3H, Ar-H), 8.14 (m, 2H, Ar-H), 8.66 (m, 2H, Ar-H), 8.97 (d, J = 6.0 Hz, 2H, Ar-H), 9.53 (s, 2H, Ar-H), 11.25 (s, 2H, –NH), 12.56 (s, 2H, –OH); ^{13}C NMR (100 MHz, DMSO-d_6) δ 63.25, 128.42, 129.27, 129.83, 130.15, 134.90, 135.23, 135.30, 138.74, 139.80, 142.96, 161.45; ESI-MS: *m/z* 434.3869 (calcd for $C_{22}H_{22}N_6O_4^{2+}$ 434.45).

Compound ***3i****:* Light brown powder. IR (KBr) ν_{max} (cm^{-1}) 3424, 3115, 2972, 1704, 1589, 1561, 998, 812, 730, 673; ^1H NMR (400 MHz, DMSO-d_6) δ 5.91 (s, 4H, benzylic $-CH_2$), 7.60 (m, 4H, Ar-H), 7.68 (s, 2H, Ar-H), 8.12 (m, 2H, Ar-H), 8.63 (d, J = 8.8 Hz, 2H, Ar-H), 8.95 (d, J = 6.0 Hz, 2H, Ar-H), 9.55 (s, 2H, –CH=NOH), 11.18 (s, 2H, –NH), 12.56 (s, 2H, –OH); ^{13}C NMR (100 MHz, DMSO-d_6) δ 63.08, 128.45, 129.62, 135.14, 135.28, 138.76, 139.78, 142.97, 161.45; ESI-MS: *m/z* 433.3914 (calcd for $C_{22}H_{22}N_6O_4^{2+}$ 434.45).

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2014.03.023.

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