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Bioorganic & Medicinal Chemistry Letters
Bioorganic & Medicinal Chemistry Letters 17 (2007) 3172–3176

Novel series of bispyridinium compounds bearing a (*Z*)-but-2-ene linker—Synthesis and evaluation of their reactivation activity against tabun and paraoxon-inhibited acetylcholinesterase

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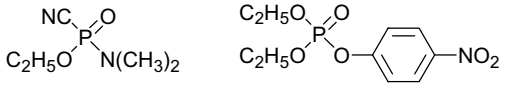
Received 19 February 2007; revised 7 March 2007; accepted 9 March 2007
Available online 13 March 2007

Abstract—Six novel AChE reactivators with a (*Z*)-but-2-ene linker were synthesized using the known synthetic pathways. Their ability to reactivate AChE, which had been previously inhibited by nerve agent tabun or pesticide paraoxon, was tested in vitro and compared to pralidoxime, HI-6, obidoxime, and K075. The novel synthesized compounds were found to be ineffective against GA-inhibited AChE but the ability of (*Z*)-1,4-bis(4-hydroxyiminomethylpyridinium)-but-2-ene dibromide to reactivate paraoxon-inhibited AChE was comparable with that of oxime K075. Notably, the oxime group in position four substantially increased the ability of the novel compounds to reactivate paraoxon-inhibited AChE.

Acetylcholinesterase (AChE, EC 3.1.1.7) is a well-known enzyme studied for various reasons, for example, Alzheimer's disease, Parkinson disease, an eco-toxicology marker.[1–3] The enzyme occurs throughout invertebrates and vertebrates species.[4,5] Many inhibitors of AChE exist both in natural and artificial compounds.[5,6] The organophosphorus inhibitors of AChE (OPI) are some of the oldest artificial inhibitors synthesized (Fig. 1), for example the first members of this group were synthesized as military nerve agents (NA; e.g., sarin, soman, tabun).[5] Afterwards many similar compounds with decreased toxicity were prepared. These are currently used in agricultural production as pesticides (e.g., parathion, chlorpyrifos, diazinon) or for industrial purposes as softening agents and flame retardants.[7] Therapeutically, metrifonate was also proved for treatment of Alzheimer's disease.[8]

All OPI irreversibly inhibit AChE through binding to a serine hydroxyl within the active site of the enzyme. Subsequently, the AChE is not able to fulfill its physiological role in cholinergic transmission and so leads to over-stimulation by acetylcholine with the resultant possibility of respiratory failure and death.[5]

The AChE reactivators (e.g., pralidoxime, obidoxime, HI-6; Fig. 2) in combination with atropine have been used to counteract the poisonous effects of OPI. The reactivator is able to cleave the covalent bond between the OPI and AChE, restoring the activity of the enzyme. This reactivation process consists of an attack of the nucleophilic oxime group (in the form of oximate anion) on the covalent bond. However, there is no reactivator able to coun-



tabun paraoxon

Figure 1. Examples of organophosphorus inhibitors of acetylcholinesterase.

Keywords: Acetylcholinesterase; Reactivation; Nerve agent; Tabun; Pesticide; Paraoxon; Reactivator; Oxime.
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doi:10.1016/j.bmcl.2007.03.025

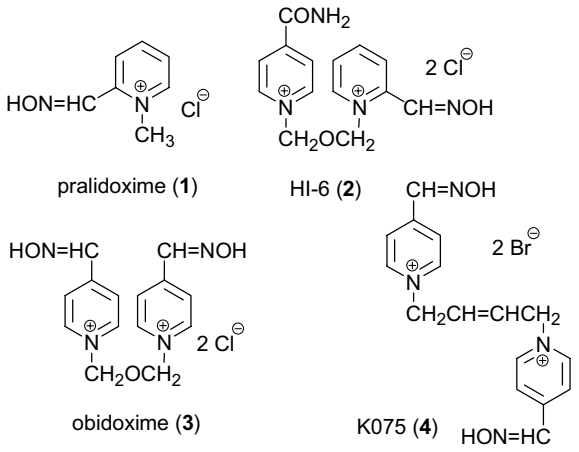
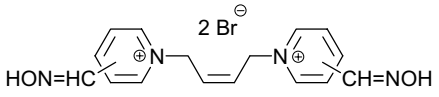


Figure 2. Examples of AChE reactivators used and tested.

teract the full spectrum of OPI.[9] Moreover, the covalent bond present between OPI and the enzyme is very stable and is subjected to intramolecular modifications called 'aging'.[10] For example, the nerve agent tabun (GA; *O*-ethyl-*N*,*N*-dimethylphosphoramidocyanidate) undergoes one of the fastest aging processes that makes GA-inhibited AChE almost impossible for reactivation.[11] Ekström et al. described this modification by changes within the cavity of GA-inhibited AChE, especially at the hydrogen bonds of His447.[12,13] Additionally, the conformational change of Pro338 partially closes the narrow AChE cleft. Consequently the phosphoramidoyl group of GA is replaced by a molecule of water and the rest of GA molecule is coordinated in the enzyme's cavity.



Compound	Oxime position
5	2,2′-CH=NOH
6	3,3′-CH=NOH
7	4,4′-CH=NOH
8	2,3′-CH=NOH
9	2,4′-CH=NOH
10	3,4′-CH=NOH

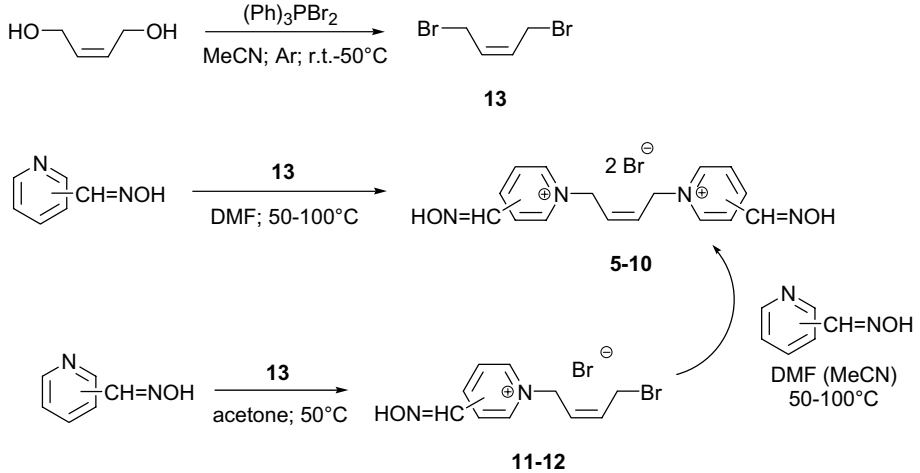
Figure 3. Six oxime reactivators tested against tabun and paraoxon-inhibited AChE.

The reactivators of AChE bearing a (*E*)-but-2-ene linker have been published previously.[14,15] Some of these reactivators (e.g., K075; Fig. 2) showed very promising activity in the reactivation of GA in vitro and so were subjected to further in vivo testing.[16] Owing to the in vitro results, the idea to modify the (*E*)-formation of the double bond for related (*Z*)-but-2-ene linker was used. Consequently, six novel reactivators (**5**–**10**) were prepared in an appropriate yield and purity (Fig. 3). At first, a novel synthetic approach for preparation of (*Z*)-1,4-dibromobut-2-ene (**13**) from a corresponding diol was used.[17] Second, monoquaternary substances (**11**–**12**) were synthesized in the presence of excess alkylating agent. Finally, the bisquaternary compounds were produced (**5**–**10**) (Scheme 1).[18] Their reactivation activity was measured using a model of GA and paraoxon-inhibited rat brain AChE.

In vitro testing of synthesized oximes involved standard experimental procedures and is described in full by the work of Kuca and Cabal.[19] Briefly, a 10% rat brain homogenate (the source of AChE) in water was inhibited by GA or paraoxon. After 30 min of incubation with the OPI moiety, this achieved 95% inhibition of AChE. Next the reactivator was added to the solution for a further 10 min. Activities of intact AChE (a_0), inhibited AChE (a_i), and reactivated AChE (a_r) were deduced from the rate of consumption of a NaOH solution (0.01 M). The percentage of reactivation (%) was calculated from the measured data according to the formula:

$$x = \left(1 - \frac{a_0 - a_r}{a_0 - a_i}\right) \cdot 100[\%]$$

Pralidoxime, HI-6, obidoxime, and K075 (**1**–**4**) of HPLC purity were synthesized in our laboratory and used as references. Collected data are summarized in Table 1.



Scheme 1. Preparation of bisquaternary substances with (*Z*)-but-2-ene linker.

Table 1. Reactivation potencies of tested oximes (%, mean value of three independent determinations)—time of inhibition—30 min; time of reactivation by AChE reactivators—10 min; pH 7.6; temperature 25 °C

Inhibitor	Reactivation (%)			
	tabun		paraoxon	
Reactivator/concentration	10^{-3} M	10^{-5} M	10^{-3} M	10^{-5} M
Pralidoxime (**1**)	4 ± 1	0	42 ± 1	0
HI-6 (**2**)	2 ± 1	4 ± 1	35 ± 2	0
Obidoxime (**3**)	11 ± 0	0	76 ± 2	37 ± 2
K075 (**4**)	16 ± 1	23 ± 1	60 ± 1	46 ± 2
5	2 ± 0	0	26 ± 1	13 ± 0
6	0	0	5 ± 1	8 ± 1
7	0	0	40 ± 3	46 ± 1
8	0	0	0	14 ± 0
9	0	1 ± 0	19 ± 1	29 ± 0
10	0	5 ± 0	3 ± 0	18 ± 1

The required reactivation potency able to counteract the effect of OPI in vivo should exceed 10% of reactivated AChE in vitro.[5] In the case of NA, there are only a few reactivators able to fulfill this criterion.[11] Our results for GA-inhibited AChE confirmed this. Only two known substances (**3**, **4**) were able to exceed 10% capability. However, a concentration of 10^{-3} M is not suitable for human use due to the noxious effects of the reactivator itself.[20] For this reason, only K075 (**4**) at a concentration of 10^{-5} M was chosen for further testing. Surprisingly, six of the novel substances showed no reactivation ability, not even compound **7** which differed from K075 only in the shape of the double bond in the connecting linker.

The reactivation ability of oximes for the paraoxon-inhibited AChE was different. First, the reactivators developed for NA are not suitable for treatment of OP pesticides (**1**–**2**) at concentrations applicable for human use with exception of obidoxime (**3**).[21] However, the higher toxicity of obidoxime in comparison to HI-6 is well known.[22] All of our novel reactivators showed some reactivation ability at a concentration of 10^{-5} M which is more appropriate for human use. Although the concentration is lower, some of them (**6**–**10**) had higher reactivation ability in contrast with concentration 10^{-3} M. This phenomenon can be explained as inhibition of AChE by the reactivator itself, as was previously published.[23] One novel compound (**7**) showed the most promising result and was able to extend the reactivation potency of K075 at the concentration 10^{-5} M.

Additionally, the structural factors necessary for reactivation of GA and paraoxon-inhibited AChE were determined. The main structural features which influence the reactivation potency are the oxime functional group (its position and amount), the quaternary heteroaromatic ring, and the connecting linker for bisquaternary reactivators.[24] As previously mentioned, the GA-inhibited AChE is subject to intramolecular changes which partially close the cavity of AChE. For this reason, only compounds with compatible shapes and relatively small sizes can effectively counteract the exposure of GA.[25] By these means, compounds with an oxime moiety at position four of the heteroaromatic ring are preferred (**3**–**4**). Nevertheless, our results confirmed that other features are also necessary for the reactivation process. While oxime K075 with (*E*)-but-2-ene linker was promising in in vitro tests, the novel reactivator **7** with (*Z*)-but-2-ene linker was ineffective. This observation could be plausibly explained by the distinct molecular shapes arising from differences in the connecting chain (Fig. 4). The interaction with the GA-inhibited AChE is unknown and will be further investigated.

In contrast, the reactivation efficacy of paraoxon-inhibited AChE for K075 and the novel compound **7** was comparable. Generally, the OP pesticides were weaker inhibitors of AChE and moreover, the structural changes in the enzyme's cavity do not occur.[26] The novel compounds showed increasing efficacy for paraoxon-inhibited AChE at a concentration of 10^{-5} M, influenced by the position of the oxime moiety: position 3,3′ < position 2,2′ < position 2,3′ < position 3,4′ < position 2,4′ < position 4,4′. Remarkably, the oxime group in position four on heteroaromatic ring increases the ability of compound to reactivate paraoxon-inhibited AChE.[27,28] In addition, the novel reactivators contain two quaternary nitrogens, which are considered to have better affinity for the cavity of AChE.[29–32] The influence of (*E*)- or (*Z*)-but-2-ene linker will also be further investigated.

In conclusion, six novel reactivators of AChE with (*Z*)-but-2-ene linker were prepared in satisfactory yield and purity. The ability of these six compounds together with pralidoxime, HI-6, obidoxime, and K075 to reactivate GA and paraoxon-inhibited AChE was measured in vitro. Only K075 exceeded the potency of obidoxime against GA. Compounds bearing an oxime group at position four were found to be promising against

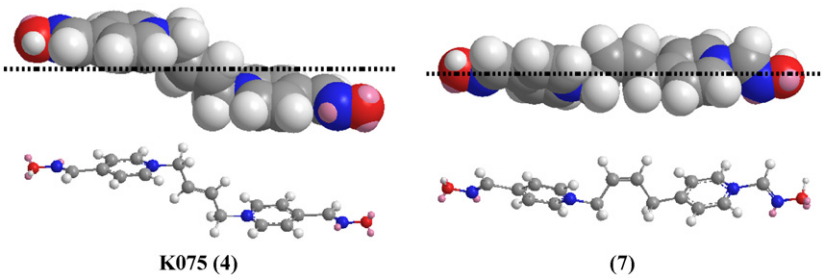


Figure 4. Different spatial orientation of (*E*)- and (*Z*)-1,4-bis(4-hydroxyiminomethylpyridinium)-but-2-ene dibromide (from Chem 3D, version 9.0; CambridgeSoft Corp.).

paraoxon-inhibited AChE at a concentration applicable for use in vivo. Although the novel compounds were not effective against GA-inhibited AChE, they showed promising results in reactivation of paraoxon- inhibited AChE.

Acknowledgments

The authors express their appreciation to Mrs. M. Hrabinova and Ms. Petra Hanusova for their technical assistance. The work was supported by the grant of Grant Agency of Charles University No. 302/2005/B-CH/FaF and by the grant of Ministry of Defence of Czech Republic No. FVZ0000501.

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17. Preparation of the connecting chain: Triphenylphosphine dibromide (20.12 g, 47.7 mmol) was suspended in anhydrous MeCN (50 ml) under argon atmosphere. (*Z*)-but-2-en-1,4-diol (1.87 ml, 22.7 mmol) was added in anhydrous MeCN (20 ml) and mixture was stirred at 50 °C for 3 h. The reaction mixture was evaporated under reduced pressure, extracted with ether (6× 50 ml). Flash chromatography of evaporated extract (100% hexane to hexane–EtOAc 7:3) was used to obtain (*Z*)-1,4-dibromobut-2-ene (**13**) as pale yellow oil (7.46 g, 77%). ^{1}H NMR spectrum (300 MHz, $CDCl_3$): δ (ppm) 5.92–5.85 (m, 2H, –CH=CH–), 4.01 (AB system, 4H, $J_{AB} = J_{BA} = 2.1$ Hz). ^{13}C NMR spectrum (75 MHz, $CDCl_3$): δ (ppm) 129.74, 24.52.
18. Preparation of quaternary salts: (A) Preparation of symmetrical salts—A solution of the hydroxyiminomethylpyridine (0.5 g, 4.1 mmol) and (*Z*)-1,4-dibromobut-2-ene (0.39 g, 1.8 mmol) in DMF (10 ml) was stirred at 50–100 °C. The reaction mixture was cooled to the room temperature and portioned with acetone (50 ml). The crystalline crude product was collected by filtration, washed with acetone (3× 20 ml), and recrystallized from MeCN (**5**–**7**). (B) Preparation of non-symmetrical salts—A solution of the hydroxyiminomethylpyridine (1.5 g, 12.3 mmol) and (*Z*)-1,4-dibromobut-2-ene (7.88 g, 36.8 mmol) in acetone (30 ml) was stirred at 50 °C. The reaction mixture was cooled to the room temperature. The crystalline crude product was collected by filtration, washed with acetone (3× 20 ml), and recrystallized from MeCN (**11**–**12**). A solution of the monoquaternary salt (0.50 g, 1.5 mmol) and corresponding hydroxyiminomethylpyridine (0.27 g, 2.2 mmol) in DMF (10 ml) or MeCN (50 ml) was stirred at 50–100 °C. The reaction mixture was cooled to the room temperature and portioned with acetone (50 ml); the crystalline crude product was collected by filtration, washed with acetone (3× 20 ml), and recrystallized from MeCN (**8**–**10**).
(*Z*)-1,4-bis(2-hydroxyiminomethylpyridinium)-but-2-ene dibromide (**5**). Prepared by method A. The reaction mixture was stirred at 50 °C and stopped after 8 h. Yield 0.24 g (29%), mp 219–221 °C. ^{1}H NMR spectrum (300 MHz, D_2O): δ (ppm) 8.95 (d, 2H, J = 6.0 Hz, PyrH), 8.72 (s, 2H, –*CH*=NOH), 8.68–8.55 (m, 2H, PyrH), 8.45 (d, 2H, J = 7.8 Hz, PyrH), 8.22–8.04 (m, 2H, PyrH), 6.22–6.03 (m, 2H, –CH=), 5.71 (d, 4H, J = 4.1 Hz, –CH_2–). ^{13}C NMR spectrum (75 MHz, D_2O): δ (ppm) 146.40, 145.76, 145.21, 141.68, 127.77, 126.99, 55.58. EA: calcd 41.95% C, 3.96% H, 12.23% N; found 41.37% C, 4.14% H, 11.78% N. ESI-MS: *m*/*z* 149.1 $[M]^{2+}$ (calcd for $[C_8H_9N_2O]^{2+}$ 149.17).
(*Z*)-1,4-bis(3-hydroxyiminomethylpyridinium)-but-2-ene dibromide (**6**). Prepared by method A. The reaction mixture was stirred at 100 °C and stopped after 2 h. Yield 0.75 g (89%), mp 230–232 °C. ^{1}H NMR spectrum (300 MHz, D_2O): δ (ppm) 9.18 (s, 2H, PyrH), 8.96 (d, 2H, J = 6.0 Hz, PyrH), 8.80 (d, 2H, J = 8.0 Hz, PyrH), 8.41 (s, 2H, –*CH*=NOH), 8.23–8.13 (m, 2H, PyrH), 6.34–6.23 (m, 2H, –CH=), 5.65 (d, 4H, J = 4.1 Hz, –CH_2–). ^{13}C NMR spectrum (75 MHz, D_2O): δ (ppm) 144.23, 143.89, 142.51, 141.98, 133.37, 128.08, 127.91,57.74. EA: calcd 41.95% C, 3.96% H, 12.23% N; found 41.58% C, 4.12% H, 12.00% N. ESI-MS: *m*/*z* 149.1 $[M]^{2+}$ (calcd for $[C_8H_9N_2O]^{2+}$ 149.17).
(*Z*)-1,4-bis(4-hydroxyiminomethylpyridinium)-but-2-ene dibromide (**7**). Prepared by method A. The reaction mixture was stirred at 100 °C and stopped after 2 h. Yield 0.69 g (82%), mp 230–232 °C. ^{1}H NMR spectrum (300 MHz, D_2O): δ (ppm) 8.90 (d, 4H, J = 6.0 Hz, PyrH), 8.41 (s, 2H, –*CH*=NOH), 8.26 (d, 4H, J = 6.0 Hz, PyrH), 6.34–6.20 (m, 2H, –CH=), 5.56 (d, 4H, J = 4.1 Hz, –CH_2–). ^{13}C NMR spectrum (75 MHz, D_2O): δ (ppm) 148.77, 145.74, 144.12, 127.96, 124.56, 57.00. EA: calcd 41.95% C, 3.96% H, 12.23% N; found 39.66% C, 4.55% H, 11.41% N. ESI-MS: *m*/*z* 149.1 $[M]^{2+}$ (calcd for $[C_8H_9N_2O]^{2+}$ 149.17).
(*Z*)-1-(2-Hydroxyiminomethylpyridinium)-4-(3-hydroxyiminomethylpyridinium)-but-2-ene dibromide (**8**). Prepared by method B via (**11**). The reaction mixture was stirred at 50 °C and stopped after 6 h. Yield 0.44 g (65%), mp 194–195 °C. ^{1}H NMR spectrum (300 MHz, D_2O): δ (ppm) 9.14 (s, 1 H, PyrH), 9.00–8.89 (m, 2H, PyrH), 8.79 (d, 1H, J = 8.2 Hz, PyrH), 8.70 (s, 1H, –*CH*=NOH), 8.65–8.56 (m, 1H, PyrH), 8.47–8.37 (m, 2H, PyrH+ –*CH*=NOH), 8.20-8-06 (m, 2H, PyrH), 6.27–6.08 (m, 2H, –CH=), 5.73 (d, 2H, J = 4.5 Hz, –CH_2–), 5.60 (d, 2H, J = 5.1 Hz, –CH_2–). ^{13}C NMR spectrum (75 MHz, D_2O): δ (ppm) 146.27, 145.68, 145.26, 144.12, 142.39, 141.59, 133.29, 128.24, 128.00, 127.67, 127.16, 126.43, 57.65, 55.56. EA: calcd 41.95% C, 3.96% H, 12.23% N; found 40.77% C, 4.21% H, 11.61% N. ESI-MS: *m*/*z* 149.1 $[M]^{2+}$ (calcd for $[C_8H_9N_2O]^{2+}$ 149.17).
(*Z*)-1-(2-Hydroxyiminomethylpyridinium)-4-(4-hydroxyiminomethylpyridinium)-but-2-ene dibromide (**9**). Pre-

pared by method B via (**11**). The reaction mixture was stirred at 50 °C and stopped after 5 h. Yield 0.43 g (63%), mp 181–183 °C. ^{1}H NMR spectrum (300 MHz, D_2O): δ (ppm) 8.95 (d, 1H, J = 6.0 Hz, PyrH), 8.88 (d, 2H, J = 6.0 Hz, PyrH), 8.69 (s, 1H, –*CH*═NOH), 8.64–8.54 (m, 1H, PyrH), 8.47–8.37 (m, 2H, PyrH+ –*CH*═NOH), 8.26 (d, 2H, J = 6.0 Hz, PyrH), 8.15–8.05 (m, 1H, PyrH), 6.25–6.07 (m, 2H, –CH═), 5.71 (d,2H, J = 4.5 Hz, –CH_2–), 5.54 (d, 2H, J = 5.1 Hz, –CH_2–). ^{13}C NMR spectrum (75 MHz, D_2O): δ (ppm) 148.66, 146.28, 145.67, 145.63, 145.24, 144.00, 141.59, 128.02, 127.67, 127.16, 126.69, 124.48, 56.96, 55.54. EA: calcd 41.95% C, 3.96% H, 12.23% N; found 41.51% C, 4.13% H, 11.89% N. ESI-MS: m/z 149.1 $[M]^{2+}$ (calcd for $[C_8H_9N_2O]^{2+}$ 149.17).

(*Z*)-1-(3-Hydroxyiminomethylpyridinium)-4-(4-hydroxyiminomethylpyridinium)-but-2-ene dibromide (**10**). Prepared by method B via (**12**). The reaction mixture was stirred at 100 °C and stopped after 1.5 h. Yield 0.44 g (65%), mp 197–199 °C. ^{1}H NMR spectrum (300 MHz, D_2O): δ (ppm) 9.17 (s, 1H, PyrH), 8.98–8.88 (m, 3H, PyrH+ –*CH*═NOH), 8.79 (d, 1H, J = 8.1 Hz), 8.48–8.37 (m, 2H, PyrH+ –*CH*═NOH), 8.27 (d, 2H, J = 6.0 Hz, PyrH), 8.21-8.13 (m, 1H, PyrH), 6.37–6.22 (m, 2H, –CH═), 5.63 (d, 2H, J = 4.1 Hz, –CH_2–), 5.58 (d, 2H, J = 4.1 Hz, –CH_2–). ^{13}C NMR spectrum (75 MHz, D_2O): δ (ppm) 148.75, 145.74, 144.25, 144.12, 142.52, 133.36, 128.12, 128.07, 127.71, 124.56, 57.71. EA: calcd 41.95% C, 3.96% H, 12.23% N; found 38.85% C, 4.26% H, 9.98% N. ESI-MS: m/z 149.1 $[M]^{2+}$ (calcd for $[C_8H_9N_2O]^{2+}$ 149.17).

(*Z*)-1-(4-Bromobut-2-enyl)-2-hydroxyiminomethylpyridinium bromide (**11**). Prepared by method B. The reaction mixture was stopped after 4 h. Yield 2.05 g (50%), mp 140–142 °C. ^{1}H NMR spectrum (300 MHz, D_2O): δ (ppm) 8.87 (d, 1H, J = 6.0 Hz, PyrH), 8.68 (s, 1H, –*CH*═NOH), 8.65–8.52 (m, 1H, PyrH), 8.41 (d, 1H, J = 7.7 Hz, PyrH), 8.15–8.01 (m, 1H, PyrH), 6.35–6.20 (m, 1H, –CH═), 5.87–5.75 (m, 1H, –CH═), 5.55 (d, 2H, J = 6.9 Hz, –CH_2–N), 4.22 (d, 2H, J = 8.5 Hz, –CH_2–Br). ^{13}C NMR spectrum (75 MHz, D_2O): δ (ppm) 145.43, 141.53, 132.13, 127.78, 126.71, 124.24, 54.91, 54.89. ESI-MS: m/z 255.0 $[M]^+$ (calcd for $[C_{10}H_{12}BrN_2O]^+$ 255.01).

(*Z*)-1-(4-Bromobut-2-enyl)-4-hydroxyiminomethylpyridinium bromide (**12**) Prepared by method B. The reaction mixture was stopped after 2 h. Yield 3.55 g (86%), mp 175–177 °C. ^{1}H NMR spectrum (300 MHz, D_2O): δ (ppm) 8.82 (d, 2H,J = 6.0 Hz, PyrH), 8.38 (s, 1H, –*CH*═NOH), 8.22 (d, 2H, J = 6.0 Hz, PyrH), 6.42–6.28 (m, 1H, –CH═), 5.96–5.83 (m, 1H, –CH═), 5.40 (d, 2H, J = 7.1 Hz, –CH_2–N), 4.24 (d, 2H, J = 8.5 Hz, –CH_2–Br). ^{13}C NMR spectrum (75 MHz, D_2O): δ (ppm) 148.47, 145.75, 143.99, 133.50, 124.44, 124.09, 56.28. EA: calcd 35.74% C, 3.60% H, 8.34% N; found 36.09% C, 3.74% H, 8.39% N. ESI-MS: m/z 255.0 $[M]^+$ (calcd for $[C_{10}H_{12}BrN_2O]^+$ 255.01).

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