Chemico-Biological Interactions 187 (2010) 234–237

Contents lists available at ScienceDirect

Chemico-Biological Interactions

journal homepage: www.elsevier.com/locate/chembioint

Synthesis and evaluation of novel analogues of vitamin B_6 as reactivators of tabun and paraoxon inhibited acetylcholinesterase

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ARTICLE INFO

Article history:
Available online 6 February 2010

Keywords:
Acetylcholinesterase
Tabun
Paraoxon
Pyridoxal oxime
Reactivation

ABSTRACT

A series of novel pyridinium oximes was prepared by reactions of quaternization of pyridoxal oxime with substituted phenacyl bromides in acetone at room temperature. The structures of compounds were determined according to the data obtained by IR spectroscopy, mass spectrometry, ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy as well as by elemental analysis. We tested pyridoxal oxime (1) and five prepared oximes in 1 mM concentration as reactivators of human erythrocytes acetylcholinesterase (AChE) inhibited by organophosphorus compounds tabun and paraoxon: 1-phenacyl-3-hydroxy-4-hydroxyiminomethyl-5-hydroxymethyl-2-methylpyridinium bromide (2), 1-(4′-chlorophenacyl)-3-hydroxy-4-hydroxyiminomethyl-5-hydroxymethyl-2-methylpyridinium bromide (3), 1-(4′-fluorophenacyl)-3-hydroxy-4-hydroxyiminomethyl-5-hydroxymethyl-2-methylpyridinium bromide (4), 3-hydroxy-4-hydroxyiminomethyl-5-hydroxymethyl-2-methyl-1-(4′-methylphenacyl)pyridinium bromide (5), 3-hydroxy-4-hydroxyiminomethyl-5-hydroxymethyl-2-methyl-1-(4′-methoxyphenacyl)pyridinium bromide (6). However, tested oximes were not efficient in reactivation of either tabun or paraoxon inhibited AChE. The maximum restored enzyme activity in 24 h was below 25%. Therefore, this class of compounds cannot be considered as potential improvement in a search for new and more efficient antidotes against OP poisoning.

1. Introduction

The organophosphorus (OP) compounds are widely used in agriculture as insecticides (parathion, paraoxon, malathion), in industry and technology as softening agents, additives to lubricants, and in military technology as chemical warfare agents (sarin, soman, cyclosarin, tabun, VX agent) [1]. They are extremely potent inhibitors of the enzyme acetylcholinesterase (AChE; EC 3.1.1.7) that is responsible for the termination of the action of acetylcholine (ACh) at cholinergic synapses. Knowledge of the basic mechanism of toxicity of organophosphorus compounds allowed the development of pharmacologically based medical countermeasures. The current standard treatment for poisoning with highly toxic OPs usually consist of combined administration of anticholinergic drugs (preferably atropine), and an AChE reactivators (called oximes) [2,3]. The reactivation of inhibited AChE depends not only on the inhibitors used but also on the chemical structure of the reactivator [4]. Unfortunately, none of the currently used oximes is sufficiently effective against all AChE inhibitors, there is no single AChE reactivator having the ability to sufficiently reactivate inhibited enzyme regardless of the chemical structure of the inhibitor [5]. Besides acting as reactivators, oximes are also reversible inhibitors of AChE and therefore they could protect the catalytic site against phosphorylation due to a direct competition between the oxime and the phosphorylating agent [6–9].

Commonly used reactivators are characterised by the presence of several structural features: functional oxime group, quaternary nitrogen group and different length of linking chain between two pyridinium rings in the case of bispyridinium reactivators. In the present study, we have synthesized five new oximes, as derivatives of vitamin B_6, and tested them as reactivators of AChE inhibited by tabun or paraoxon.

2. Materials and methods

2.1. Preparation of the new oximes

Preparation of the new oximes 2–6 is shown in Scheme 1. Pyridoxal oxime 1 was prepared according to the described procedures [10]. Solvents and reagents were purchased from Fluka and Aldrich

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doi:10.1016/j.cbi.2010.02.004

and used without further purification. IR spectra were measured on Paragon 500 FT-IR spectrophotometer with KBr pellets. ^{1}H NMR and ^{13}C NMR spectra were measured on a Varian XL-GEM 300 spectrophotometer in DMSO-d_6 solutions and chemical shifts reported in δ values downfield from TMS as an internal standard. Mass spectra were recorded using API2000 LC/MS/MS System from Applied Biosystems/MDS SCIEX.

The synthesis of quaternary salts 2–6: pyridoxal oxime (1) (0.36 g; 2 mmol) was dissolved in acetone (300 mL) and stirred (20 min) at 50 °C. The reaction mixture was cooled to room temperature, and substituted phenacyl bromides (R = H, Cl, F, CH_3, OCH_3) were added (2 mmol). The reaction mixture was stirred for 24 h, and left in dark for 3 weeks at room temperature. The crystalline crude product was collected by filtration under reduced pressure and recrystallized. The purity of all compounds was determined by ^{1}H and ^{13}C NMR, MS and elemental analysis.

2.1.1. 1-Phenacyl-3-hydroxy-4-hydroxyiminomethyl-5-hydroxymethyl-2-methylpyridinium bromide (2)

M.p. after crystallization from methanol 250–251 °C. IR (KBr): 3276, 3096–2730, 1700, 1641–1449, 1228, 1084–978 cm^{-1}. ^{1}H NMR (DMSO-d_6) δ 13.00 (bs, 1H, NO*H*), 11.96 (bs, 1H, O*H*), 8.62 (s, 1H, H-6), 8.11 (d, J = 7.34 Hz, 2H, H-3″, H-5″), 8.10 (d, J = 7.26 Hz, 2H, H-2″, H-6″), 7.80 (s, 1H, H-4″), 6.64 (s, 2H, C*H*$_2$CO), 4.80 (m, 2H, *CH*$_2$OH), 3.43 (bs, 1H, CH$_2$*OH*), 1.91 (s, 3H, C*H*$_3$). ^{13}C NMR (DMSO-d_6) δ 190.70, 152.57, 145.56, 145.43, 137.33, 135.11, 129.05, 128.53, 128.15, 64.51, 58.52, 13.29. Anal. calc. for $C_{16}H_{17}N_2O_4Br$ (M = 381.22 g mol^{-1}): C 50.41, H 4.49, N 7.35, Br 20.96; found: C 50.58, H 4.33, N 7.48, Br 20.93%. MS m/z: 381.0 (83%); 299.3 (100%); 281.4 (52.45%); 269.2 (37.06%); 161.0 (47.55%); 106.0 (31.46%). Yield 0.29 g (38%).

2.1.2. 1-(4′-Chlorophenacyl)-3-hydroxy-4-hydroxyiminomethyl-5-hydroxymethyl-2-methylpyridinium bromide (3)

M.p. after crystallization from methanol 230–233 °C. IR (KBr): 3384, 3069–2984, 1688, 1641–1592, 1261, 1053–1002 cm^{-1}. ^{1}H NMR (DMSO-d_6) δ 13.01 (bs, 1H, NO*H*), 8.67 (bs, 1H, O*H*), 8.59 (s, 1H, H-6), 8.12 (d, J = 8.63 Hz, 2H, H-2″, H-6″), 7.77 (d, J = 8.61 Hz, 2H, H-3″, H-5″), 6.66 (s, 2H, C*H*$_2$CO), 5.84 (bs, 1H, CH$_2$*OH*), 4.81 (s, 2H, C*H*$_2$OH), 2.51 (s, 3H, C*H*$_3$). ^{13}C NMR (DMSO-d_6) δ 189.86, 152.56, 145.53, 145.49, 139.69, 137.32, 135.08, 130.46, 129.16, 128.18, 64.47, 58.51, 13.34. Anal. calc. for $C_{16}H_{16}N_2O_4BrCl$ (M = 415.66 g mol^{-1}): C 46.23, H 3.88; N 6.74; found: C 46.34, H 3.86, N 6.72%. MS m/z: 415.0 (54.16%), 335.2 (43.05%), 333.0 (100%), 302.8 (39.58%), 164.1 (36.11%). Yield 0.55 g (66%).

2.1.3. 1-(4′-Fluorophenacyl)-3-hydroxy-4-hydroxyiminomethyl-5-hydroxymethyl-2-methylpyridinium bromide (4)

M.p. after crystallization from ethyl-acetate 225–225.5 °C. IR (KBr): 3384, 3069–2923, 1688, 1641–1592, 1261, 1053–1002 cm^{-1}. ^{1}H NMR (DMSO-d_6) δ 11.45 (bs, 1H, NO*H*), 8.54 (bs, 1H, O*H*), 8.49 (s, 1H, H-6), 8.17 (d, J = 8.03 Hz, 2H, H-2″, H-6″), 7.47 (d, J = 8.05 Hz, 2H, H-3″, H-5″), 6.26 (s, 2H, C*H*$_2$CO), 5.38 (bs, 1H, CH$_2$*OH*), 4.61 (s, 2H, C*H*$_2$OH), 2.24 (s, 3H, C*H*$_3$). ^{13}C NMR (DMSO-d_6) δ 190.27, 165.61, 147.02, 144.94, 137.70, 130.73, 135.11, 132.28, 130.50, 125.79, 63.84, 60.42, 12.81. Anal. calc. for $C_{16}H_{16}BrFN_2O_4$ (M = 399.21 g mol^{-1}): C 48.14, H 4.04, N 7.02; found: C 47.53, H 4.22, N 6.95%. MS m/z: 399.2 (53.52%), 319.1 (9.86%), 317.25 (100%), 299.1 (30.28%), 287.3 (36.62%), 137.0 (32.39%), 133.0 (36.62%). Yield 0.23 g (36%).

2.1.4. 3-Hydroxy-4-hydroxyiminomethyl-5-hydroxymethyl-2-methyl-1-(4′-methylphenacyl) pyridinium bromide (5)

M.p. after crystallization from methanol 240–241.5 °C. IR (KBr): 3388, 3040–2861, 1686, 1643–1571, 1257, 1087–980 cm^{-1}. ^{1}H NMR (DMSO-d_6) δ 13.02 (bs, 1H, NO*H*), 8.66 (bs, 1H, O*H*), 8.59 (s, 1H, H-6), 8.01 (d, J = 8.13 Hz; 2H, H-3″, H-5″), 7.99 (d, J = 8.12 Hz, 2H, H-2″, H-6″), 6.60 (s, 2H, C*H*$_2$CO), 4.77 (s, 2H, *CH*$_2$OH), 3.44 (bs, 1H, CH$_2$*OH*), 2.51 (s, 3H, C*H*$_3$), 2.45 (s, 3H, C*H*$_3$). ^{13}C NMR (DMSO-d_6) δ 189.89, 152.54, 145.56, 145.51, 139.71, 137.35, 135.11, 132.28, 130.50, 128.18, 64.52, 58.53, 13.37. Anal. calc. for $C_{17}H_{19}N_2O_4Br$ (M = 395.2478 g mol^{-1}): C 51.66, H 4.85, N 7.09; found: C 51.65, H 4.83, N 7.27%. MS m/z: 395.2 (100%), 313.0 (44.37%), 295.5 (21.83%), 264.8 (22.54%), 162.1 (33.09%), 132.9 (22.54%). Yield 0.49 g (62%).

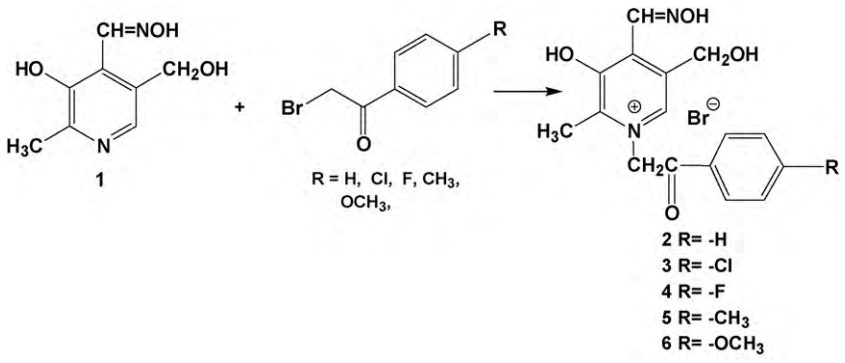
2.1.5. 3-Hydroxy-4-hydroxyiminomethyl-5-hydroxymethyl-2-methyl-1-(4′-methoxyphenacyl) pyridinium bromide (6)

M.p. after crystallization from ethyl-acetate 267–268 °C. IR (KBr): 3381, 3041–2839, 1677, 1641–1516, 1243, 1047–978 cm^{-1}. ^{1}H NMR (DMSO-d_6): 13.00 (bs, 1H, NO*H*), 12.01 (bs, 1H, O*H*), 8.66 (s, 1H, H-6), 8.58 (s, 2H, C*H*$_2$CO), 8.08 (d, J = 8.89 Hz; 2H, H-3″, H-5″), 7.19 (d, J = 8.94 Hz; 2H, H-2″, H-6″), 4.80 (m, 2H, *CH*$_2$OH), 3.90 (s, 3H, OC*H*$_3$), 3.44 (bs, 1H, CH$_2$*OH*), 2.50 (s, 3H, C*H*$_3$). ^{13}C NMR (DMSO-d_6): 189.89, 152.54, 145.56, 145.51, 139.71, 137.35, 135.11, 132.28, 130.50, 128.18, 64.52, 58.53, 13.37. Anal. calc. for $C_{17}H_{19}N_2O_5Br$ (M = 411.2472 gmol^{-1}): C 49.65, H 4.66, N 6.81, Br 19.43; found: C 49.79, H 4.46, N 6.83, Br 19.35%. MS m/z: 411.2 (96.5%); 331.0 (3.49%); 329.35 (100%); 311.0 (17.48%); 299.1 (42.65%); 134.0 (57.34%); 106.0 (60.84%). Yield 0.55 g (67%).

2.2. Reactivation of inhibited acetylcholinesterase

The reactivation efficacy of the pyridoxal oxime (1) and newly synthesized compounds 2–6 (cf. Scheme 1) were evaluated according to previously published procedure [11]. The source of AChE was native intact human erythrocytes; the final dilution during enzyme assay was 400-fold. All experiments were done in 0.1 M phosphate buffer, pH 7.4, at 25 °C and the substrate was acetylthiocholine, ATCh (1 mM final concentration). The enzyme activity was measured spectrophotometrically according to the Ellman procedure [12] with the thiol reagent DTNB (5,5′-dithiobis-2-nitrobenzoic acid), final concentration 0.3 mM. The increase in absorbance was read at 436 nm. All spectrophotometric measurements were performed on a CARY 300 spectrophotometer (Varian Inc., Australia).

The undiluted erythrocyte AChE was incubated with 5 μM tabun (*O*-ethyl-*N*,*N*-dimethylphosphoramidocyanidate) or 1 μM paraoxon (*O*,*O*-diethyl-*O*-(4-nitrophenyl)phosphate) up to 60 min, achieving 95–100% inhibition. The excess of organophosphorus compound was extracted with a 5-fold volume of hexane. Incubation mixture was diluted 10-times with 0.1 M phosphate buffer, pH 7.4, containing the oxime to start the reactivation. Final oxime concentrations used for the reactivation of inhibited AChE was 1 mM. After a given time of reactivation aliquots were diluted 40-times in buffer, DTNB and ATCh (acetylthiocholine iodide, 1.0 mM final concentration) were added, and the enzyme activity was measured. An equivalent sample of uninhibited enzyme was diluted to the same extent as the inhibited AChE, and control activity was measured in the presence of oxime at concentrations used for reactivation. Both activities of control and reactivation mixture were corrected for oxime-induced hydrolysis of ATCh. Under these conditions, the enzyme activity in the absence of the oxime was stable and no spontaneous reactivation of the phosphorylated enzyme took place.



Scheme 1. Synthesis of the oximes 2–6 from pyridoxal oxime (1).

Table 1
Reactivation of inhibited human erythrocyte AChE by pyridoxal oxime (1) and oximes 2–6.

Oxime (1 mM)	Tabun-inhibited AChE		Paraoxon-inhibited AChE	
	$React_{max}$ (%)	Time of $React_{max}$ (h)	$React_{max}$ (%)	Time of $React_{max}$ (h)
1	<10	23	<15	23
2	<10	23	15	23
3	<10	23	10	23
4	<10	23	20	23
5	<10	23	23	23
6	<10	23	20	23

3. Results and discussion

Pyridinium, imidazolium and quinuclidinium oximes are prepared in laboratories in Croatia since the middle of 1970s [5]. The majority of prepared compounds are mono- and bis-pyridinium oximes. Numerous attempts had been made to improve the antidotal properties of the conventional mono- and bis-pyridinium oximes by modifying their structure. Pyridoxal oxime, a derivative of B_6 vitamin, is suitable to be incorporated into a reactivator molecule and can be used for synthesis of compounds structurally similar to common antidotes. Very little data seem to be available on the reactivating property of this group of compounds. Milatović et al. [13] synthesized three novel compounds, derivatives of pyridoxal oxime, which combine the structural features of pyridoxal oxime and toxogonine, which were tested as reactivators of AChE inhibited by sarin, soman, tabun and VX. Their reactivating potency was compared with the reactivating potency of the conventional antidotes Toxogonin and PAM-2. They showed reactivating potency comparable with that of the conventional antidotes. This was a reason why we in the present study synthesized five new oximes derivatives of pyridoxal oxime and tested them as reactivators of tabun or paraoxon inhibited AChE.

A new synthetic pathway was used for the preparation of pyridoxal oxime salts 2–6 (Scheme 1). The purity of prepared compounds is high and the yields are acceptable for laboratory preparation.

Reactivation ability of tested oximes is summarized in Table 1. Newly synthesized oximes (2–6), as well as pyridoxal oxime (1), were not efficient in reactivation of either tabun or paraoxon inhibited AChE. The maximum restored enzyme activity in 24 h was below 25%. Although prepared oximes (2–6) and pyridoxal oxime (1) were weak reactivators they showed better reactivation of paraoxon inhibited AChE. Weak reactivating activity was also observed with similar compound derivatives of pyridoxal oxime, the reactivation was slow and not complete [13–15].

For pyridoxal oxime itself result of weak reactivation potency could be expected due to a lack of quaternary nitrogen in its structure. The most probable explanation for obtained low reactivation potencies of oximes 2–6 could be in a short linker between two aromatic rings which makes the structure less flexible and therefore inappropriate for accommodation within the active site, as it was shown with similar compounds [16]. Also, vicinal groups to oxime moiety could influence the p*K*a value of the oxime group lowering its nucleophilicity at the pH 7.4 and most certainly present steric hindrances that prevent efficient orientation of the oxime group towards the phosphorylated serine [9]. Therefore, this class of compounds cannot be considered as potential improvement in a search for new and more efficient antidotes against OP poisoning.

Conflicts of interest

None.

Acknowledgment

This work was supported by the Ministry of Science, Education and Sports of the Republic of Croatia (No. 022-0222148-2889; 291-0580000-0169, 073-0731674-0841).

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