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Design, synthesis and evaluation of new α-nucleophiles for the hydrolysis of organophosphorus nerve agents: application to the reactivation of phosphorylated acetylcholinesterase

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ABSTRACT

A series of new α-nucleophiles including oximes and amidoximes have been synthesized, and their ability to efficiently and selectively cleave the P–S bond of organophosphorus nerve agents has been evaluated. The relationship between the chemical structure of the α-nucleophiles and their reactivity towards PhX hydrolysis is reported. A significant effect induced by an *ortho*-hydroxyl group of aryl- and pyridyl-oximes, amidoximes on their organo-phosphono-thioase reactivity was discovered. The evaluation of the initial rates of PhX hydrolysis reaction in the presence of α-nucleophiles allowed the discovery of new uncharged molecules with increased reactivity compared to positively charged 2-pralidoxime used as antidote. The mechanism of their action was studied in details by kinetic analysis, HPLC and LC–MS methods. The most promising structures were then considered as potent in vitro re-activators of phosphylated acetylcholinesterase (AChE), which was confirmed by the preliminary results of AChE reactivation initially inhibited by VX.

1. Introduction

Chemical warfare agents (CWA) (sarin, soman, cyclosarin, tabun, methylphosphonothioate VX) as well as insecticides (paraoxon, parathion, tetraethyl pyrophosphate (TEPP)) are extremely toxic organophosphorus compounds (Fig. 1). Their mechanism of action consists of the irreversible inhibition of acetylcholinesterase (AChE).[1] Being phosphylating agents, these compounds react with a serine hydroxyl group in the active site of AChE, which leads to the irreversible inhibition. This event results in the accumulation of neurotransmitter acetylcholine at nerve synapses, which leads to nervous and respiratory failure and eventually can kill in minutes.[2] Due to the easy access to CWA and the similarity between their chemical precursors and commonly used pest-control agents, any efforts to control the proliferation of the chemical weapons were unsuccessful.[3] Therefore, the development of effective organophosphorus nerve agent (OPNAs) decontaminants and medical treatments of the OP poisoning as well as prevention of their toxicity remains a challenging problem.

α-Nucleophiles including H_2O_2, activated hydrogen peroxides,[4] oxidative chlorides[5] and peracids[6] are the most commonly used decontaminants of organophosphorus nerve agents. *ortho*-Iodosobenzoates and their derivatives have also been shown to effectively hydrolyze organophosphorus compounds, such as sarin, soman, tabun and paraoxon (but not VX).[7] An interesting approach consists in the use of inorganic substances for OP agents cleavage, for example, alumina supported fluoride reagents[8] and nanosized metal oxides particles.[9] Another approach to the destruction of chemical warfare agents and their poisoning treatment involves the use of enzymes, such as organophosphate hydrolases,[10] PTE–phosphotriesterase,[11] laccase[12] and chloroperoxidase (CPO).[13] New concept towards the safe organophosphorus chemical weapons neutralization consists of the use of catalytic antibodies,[14] which have been found to hydrolyze VX and PhX.[15] However, despite all the existing methods mentioned above the development of more effective tools for the detoxification of dangerous V-type OP nerve agents remains a challenging task.

Between all the CWA methylphosphonothioate VX exhibits the highest toxicity (LD_{50}=8 μg kg^{-1} (iv) and 28 μg kg^{-1} (percutaneous, rabbit)).[1] Moreover, VX is the most persistent

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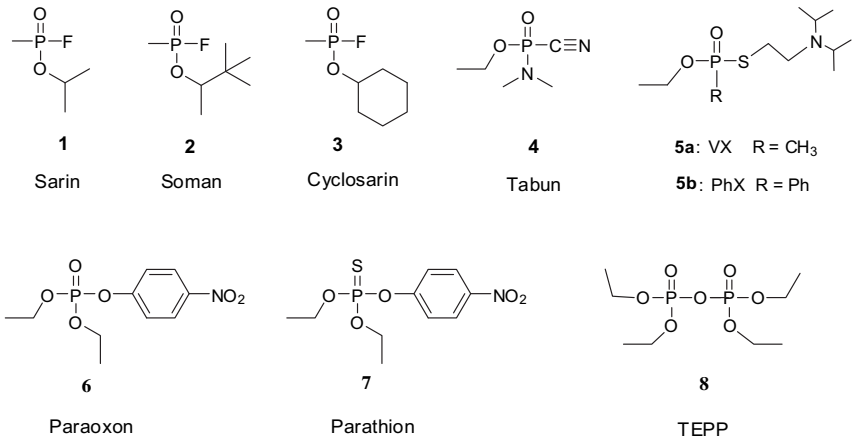


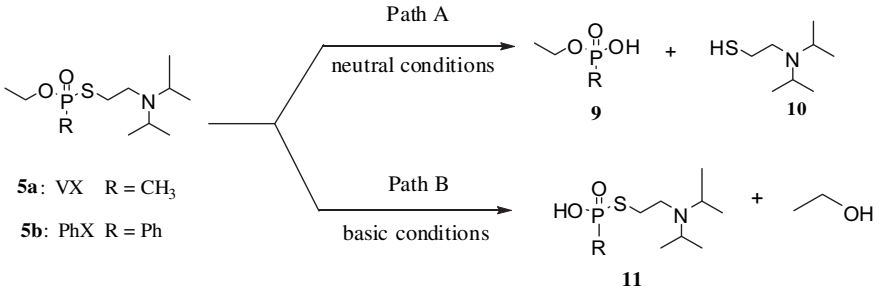
Fig. 1. Structures of organophosphorus CWA and insecticides.

organophosphorus agent due to its low volability and Henry's law constants.[16] Additionally, this compound is the most resistant to water hydrolysis,[17] which can be explained by its low hydrosolubilisation and the presence of a quite stable P–S bond. On the other hand, when VX hydrolysis is performed in neutral conditions the reaction is quite selective and leads to relatively nontoxic phosphonic acid **9**,[18] whereas in basic media, besides the main hydrolytic pathway A, pathway B competes resulting in formation of exceedingly toxic phosphothioate **11** (Scheme 1).[19] Therefore, the development of decontaminants able to selectively cleave the P–S bond of V-type of OPN agents is of paramount importance.

2. Results and discussions

2.1. Screening of the α-nucleophiles in PhX hydrolysis

Among the known α-nucleophiles, oximes were proved to be effective decontaminants of OPNAs.[22] The computational studies of the mechanism of the reaction of oximes with phosphonates[23] showed no possible formation of the phosphorane intermediate, which is important for the selective hydrolysis of V-type nerve agents. Moreover, oximes were shown to reactivate the phosphylated AChE.[24] As we showed recently through the use of a



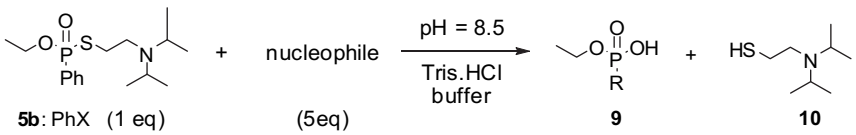
Scheme 1. Hydrolytic pathways of VX and PhX in different conditions.

It is interesting to note, that phenylphosphonothioate **5b** (PhX),[20] which is the aromatic derivative of VX **5a**, exhibits the same hydrolysis profile as VX, while its toxicity is lower and the inhibition of AChE in vitro is pseudo-irreversible.[15] For safety and regulatory reasons[21] we decided to study the hydrolysis of less toxic PhX.

The present work is focused on the development of new α-nucleophiles able at performing two complementary reactions: (1) the selective P–S bond cleavage of V-type organophosphorus nerve agents (OPNAs), to be used as decontaminants and (2) the reactivation of AChE inhibited by VX, to be used as curative treatment. In this article we describe the study of the relationship between the structure of the α-nucleophiles and their organo-phosphono-thioase activity, as well as our investigations on the mechanism of their reaction with PhX.

preliminary high-throughput screening assay, aryl- and pyridyl-amidoximes, and hydroxamic acids also exhibited quite promising organo-phosphono-thioase activity.[25]

In order to investigate the influence of the α-nucleophiles structure on their hydrolytic activity, libraries of known and original molecules, such as oximes, amidoximes and hydroxamic acids were evaluated in the hydrolysis of PhX. The screening test of the α-nucleophiles efficacy for this reaction was performed by means of HPLC monitoring the consumption of PhX **5b** during the hydrolysis. For all evaluated nucleophiles the reaction conditions were the following: 5 equiv of hydrolyzing agent were used in the presence of 1 equiv of PhX **5b** in 0.1 N Tris/HCl buffer at pH=8.5 and at 20–22 °C. In all the cases the reactions were performed during 2 h (Scheme 2).



Scheme 2. Screening test of the α-nucleophiles in PhX hydrolysis.

2.1.1. Aryl and pyridinaldoximes. 2-Pralidoxime **12** (2-PAM), which is known as antidote for organophosphorus pesticides was chosen as the reference compound.[26] Compound **12** hydrolyzes quantitatively PhX in our reaction conditions, demonstrating the high reactivity of positively charged pyridinium oxime (Table 1).

Table 1
Activity of benzaldoximes in PhX hydrolysis reaction

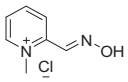
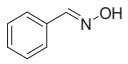
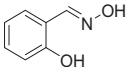
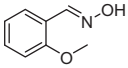
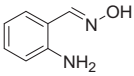
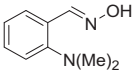
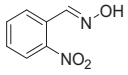
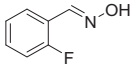
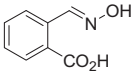
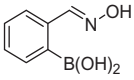
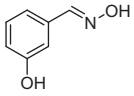
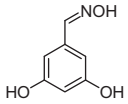
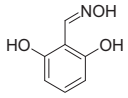
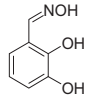
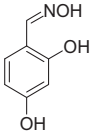
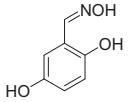
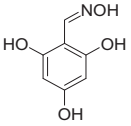
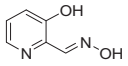
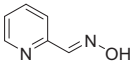
Structure		PhX hydrolyzed, %
12		100
13		11
14		60
15		3
16		2
17		3
18		15
19		5
20		42
21		7
22		13
23		11
24		61
25		73
26		41

Table 1 (*continued*)

Structure		PhX hydrolyzed, %
27		55
28		80
29		84
30		22

For uncharged *ortho* monosubstituted aryl oximes **13**–**22** bearing different electron-donating and electron-withdrawing groups, it was found that the electronic effects did not impact significantly the hydrolytic activity. For example, benzaldoximes **15**, **16** and **17** substituted in *ortho*-position with methoxy, amino and dimethylamino groups (donors by mesomeric effect) showed slightly lower hydrolytic activity towards PhX than nonsubstituted oxime **13** (Table 1). Aryl oximes **18** and **19** bearing a nitro or fluoro electron-withdrawing group in *ortho*-position (Table 1) as well as oxime **21** (Table 1) exhibited almost similar hydrolytic activity to oxime **13**. Very interestingly, benzaldoximes **14** and **20** (Table 1) having a hydroxyl or a carboxyl group in *ortho*-position showed enhanced activity towards hydrolysis of PhX as compared to **13**, which can be explained by the intramolecular hydrogen bonding between hydroxyl group and nitrogen atom of oxime.[27] This hydrogen bonding was shown by Shimizu to decrease the pK_a of oximes bearing *ortho*-hydroxyl group in comparison with nonsubstituted ones (for example, for **14** pK_a=9.3 compared to **13** with pK_a=10.9).[28] It is suggested that lower pK_a results in the increase of the oximate concentration leading to the observed enhanced hydrolytic activity.

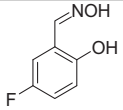
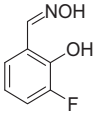
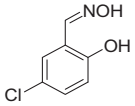
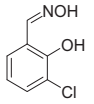
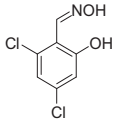
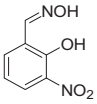
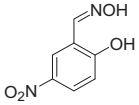
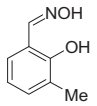
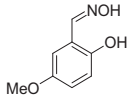
This fact is in accordance with PhX hydrolysis data obtained for compounds **22** and **23** bearing one or two hydroxyl group in *meta* position to the oxime function, and whose efficacies are in the same order of magnitude as nonsubstituted oxime **13** (Table 1). Identically, preventing the H-bonding by the substitution the *ortho*-hydroxyl functionality by a methoxy group in compound **15**, leads, as could be expected, to the inactivation of the nucleophile towards the hydrolysis of PhX (Table 1). The reactivity of other di- and trisubstituted *ortho*-hydroxyl aryl oxime was then envisioned. Compound **24** possessing two hydroxyl groups in *ortho*-position to the oxime function shows the same activity as nucleophile **14** bearing one hydroxyl group in the *ortho*-position (Table 1), this result suggests that the second hydroxyl function does not influence the oxime pK_a. Other *ortho*-hydroxyloximes **25**, **26** and **27** substituted with additional hydroxyl group in position 3, 4 and 5, respectively, exhibit quite comparable activity as oxime **14** (Table 1). Only in the case of compound **28** (Table 1) the significant increase of hydrolytic activity in comparison with oxime **14** was observed. This increased hydrolytic reactivity might be ascribed to the additional electronic donating effect of the third OH group in *para* position compared to **24** and **26**. Very interestingly, the same effect of *ortho*-hydroxyl group was also observed for uncharged pyridinealdoxime derivatives. Indeed, pyridinealdoxime **29** bearing OH function in *ortho*-position

exhibited significantly higher hydrolytic activity than non-substituted compound **30** (Table 1). It is interesting to note, that the pyridine ring contributes to decrease the oxime pK_a function of compounds **29** (pK_a=8.2±0.09, see ESD) and **30** (pK_a=9.85±0.05),[29] leading to increased reactivities compared to aryl oximes **14** and **13**, respectively. Having identified the positive *ortho*-hydroxyl effect of aryl- and pyridylbenzaldoximes on their hydrolytic activity towards PhX, we decided next to study the influence of the nature of the other substituents in the aromatic ring of *ortho*-hydroxybenzaldoximes.

Accordingly, *ortho*-hydroxylbenzaldoximes **31**–**39** containing electron-donating and electron-withdrawing groups were studied in order to evaluate their reactivity. It is assumed that the presence of electron-withdrawing groups in *ortho*- and *para*-positions to the hydroxyl function decreases the pK_a of the phenol facilitating H-bonding with the free electron pair of the nitrogen atom. These events result in increased oximate concentration leading to increased hydrolytic activities.

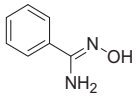
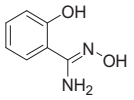
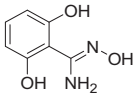
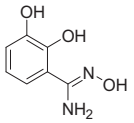
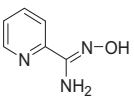
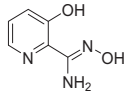
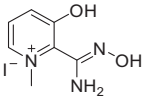
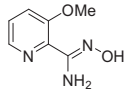
For example, benzaldoximes **31**–**35** (Table 2) substituted with halogen atoms showed slightly higher (**34** and **35**) to better (**31** and **33**) hydrolytic activities than nonsubstituted *ortho*-hydroxyloxime **14**. It is interesting to note that oximes bearing halogen atom in *para*-position position to the hydroxyl group (**31** and **33**) showed higher organo-phosphono-thioase activity than *ortho*-substituted ones **32** and **34**. The introduction of NO_2 group in *ortho*- or *para*-position to hydroxyl function for compounds **36** (pK_a=6.27±0.02, see ESD) and **37** (pK_a=6.7)[29] is supposed to significantly decrease their pK_a. Therefore, under the standard experimental conditions (pH=8.5) compounds **36** and **37** exist almost totally in the phenolate form. This situation is not optimum for the formation of hydrogen bond with the nitrogen atom of the oxime and, consequently, these molecules exhibit lower hydrolytic activity towards PhX (Table 2). In contrast, benzaldoximes **38** and **39** substituted in *ortho*- or *para*-position to the hydroxyl function with electron-donating methyl and methoxy groups correspondingly exhibited similar activities as nonsubstituted *ortho*-hydroxylbenzaldoxime **14** (Table 2).

Table 2
Activity of *ortho*-hydroxylbenzaldoximes in PhX hydrolysis

Structure		PhX hydrolyzed, %
31		83
32		60
33		77
34		66
35		63
36		22
37		16
38		55
39		60

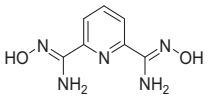
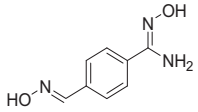
2.1.2. Aryl and pyridinamidoximes. As we reported recently, benzalamidoximes were found to have a quite promising organo-phosphono-thioase activity towards PhX.[25] Inspired by our results obtained for aryl- and pyridylaldoximes series we decided to evaluate the influence of *ortho*-hydroxyl group and the pyridine ring onto the hydrolytic activity of amidoximes. We discovered that, as observed for arylaldoxime **14** (Table 1), amidoxime **41** (Table 3, pK_a=8.88±0.6, see ESD) bearing hydroxyl group in *ortho*-position to the nucleophilic function, has a significantly higher hydrolytic

Table 3
Activity of amidoximes in PhX hydrolysis

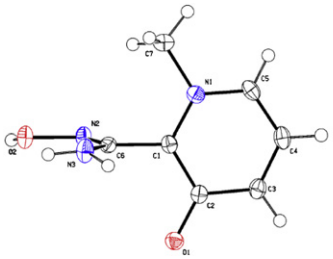
Structure		PhX hydrolyzed, %
40		25
41		91
42		100
43		100
44		25
45		100
46		9
47		29

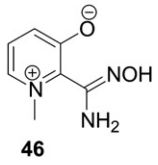
(*continued on next page*)

Table 3 (*continued*)

Structure		PhX hydrolyzed, %
48		15
49		18

activity towards PhX than nonsubstituted compound **40** (Table 3). Identically, *ortho*-hydroxylsubstituted amidoximes **42** and **43** (Table 3) bearing an additional hydroxyl group showed a dramatic increase of the organo-phosphono-thioase activity as compared to **40**. Indeed, due to intramolecular H-bonding between the amidoxime function and the OH group, a quantitative hydrolysis of PhX took place within 2 h of reaction for these compounds. At this stage of our study we have identified new uncharged aryl derivatives capable to hydrolyze efficiently and quantitatively PhX as does pyridinium 2-pralidoxime **12**, a known antidote. In connection with these results it appears that α-hydroxybenzalamidoximes have superior reactivity towards PhX than α-hydroxybenzaldoximes. We further discovered that α-hydroxyl effect is also effective for pyridylamidoxime derivative **45** (Table 3, 100% PhX hydrolysis) compared to **44** (Table 3, 25% PhX hydrolysis). Similarly to oxime functionality it is suggested that the increased reactivity observed for hydroxy aryl- and pyridyl-amidoxime is ascribed to the existence of an intramolecular hydrogen bonding between the hydroxyl group and the nitrogen atom of amidoxime group, which results in decrease of pK_a (for example, for **45** pK_a=7.96±0.1 compared to **44** with pK_a=12.06±0.03, see ESD). These hypotheses are in accordance with results obtained for compound **46**, which showed low activity towards PhX hydrolysis (Table 3). X-ray analysis of **46** revealed that the compound exists in zwitterion form in which the hydroxyl group is totally deprotonated, preventing any activation of the nucleophilic function by intramolecular H-bonding (see X-ray crystal structure in Fig. 2, ESD).





46

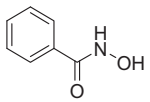
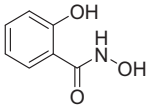
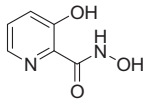
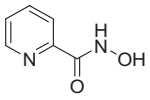
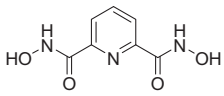
Fig. 2. X-ray structure of amidoxime **46**.

The low reactivity of amidoxime **47** (29% hydrolysis of PhX) bearing an *ortho*-methoxy group is also in agreement with our findings (Table 3). Compound **48** possessing two amidoxime functions and **49** bearing both amidoxime and oxime functions showed low activity, which proves the absence of any additive effect (Table 3). It was found that in contrast to oximes the pyridine ring does not affect significantly the organo-phosphono-thioase activity of amidoxime derivatives. For example, pyridylamidoxime **44** (Table 3) showed the same reactivity as arylamidoxime **40** (Table 3), but in the case of compound **45** the replacement of benzene ring with pyridine leads to the increase of the hydrolytic activity in comparison with **41**.

The comparison of PhX hydrolysis results obtained for aldoximes and amidoximes species allowed us to conclude that in all the cases amidoximes (**40**, **41**, **42** and **43**) exhibit higher activity than aldoximes (**13**, **14**, **24** and **25**) with a similar structure.

2.1.3. Hydroxamic acids. Finally, the reactivity of hydroxamic acid was also investigated. All the hydroxamic acids evaluated **50**–**54** showed good to excellent results for the hydrolysis of PhX. In contrast to the α-nucleophiles mentioned before (oximes and amidoximes) the presence of hydroxyl group in *ortho*-position to the hydroxamic function does not affect significantly the reactivity towards PhX hydrolysis, however an increase of reactivity has been observed for pyridyl hydroxamic acids. For example, compounds **50** and **51** exhibit a comparable hydrolytic activity for PhX (Table 4). However, for pyridyl derivative **52** the presence of the hydroxyl group in *ortho*-position to the hydroxamic function increases significantly the percentage of hydrolyzed PhX in comparison with compound **53** (Table 4). For compounds **51** and **52**, the activation mode of the nucleophilic function by the hydroxyl group is not completely understood yet and would need further studies.

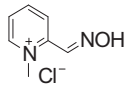
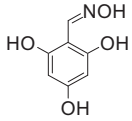
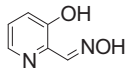
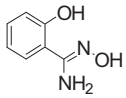
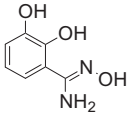
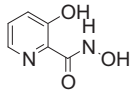
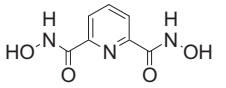
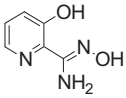
Table 4
Activity of hydroxamic acids in PhX hydrolysis

Structure		PhX hydrolyzed, %
50		85
51		83
52		100
53		75
54		100

2.1.4. Kinetics and mechanistic insights. The screening of oximes, amidoximes and hydroxamic acid towards selective PhX hydrolysis allowed us to identify new structurally simple uncharged molecules with high organo-phosphono-thioase activity. These molecules are able to quantitatively and selectively hydrolyze PhX in 2 h at room temperature, as the well known pralidoxime. We decided next to perform kinetic studies with the most reactive molecules identified.

The initial rates of PhX hydrolysis reaction for nucleophiles **12**, **28**, **29**, **41**, **43**, **45**, **52** and **54** selected by the preliminary screening were evaluated (see ESD). It was found, that amidoxime **43** shows the highest initial rate, molecules **41** and **52** have almost the same values as the reference compound 2-pralidoxime **12** (Table 5). It should be mentioned, that amidoxime **45** exhibited such a high PhX hydrolytic activity, that it was not possible to evaluate the initial rate for it in the standard conditions of the test (1.0 mM PhX and 5.0 mM nucleophile in mixture acetonitrile/0.1 M Tris/HCl buffer 11/89 at pH 8.5 and 25 °C).

Table 5
Initial rates of PhX hydrolysis

Entry	Structure	Initial rate, μmol/min
1	 12	30
2	 28	22
3	 29	19
4	 41	31.3
5	 43	38.1
6	 52	28.6
7	 54	14.7
8	 45	>40[a]

Reagents and conditions: 1.0 mM PhX and 5.0 mM nucleophile in mixture acetonitrile/0.1 M Tris HCl buffer 11/89 at pH 8.5 and 25° C.
[a] To high to be accurately measured.

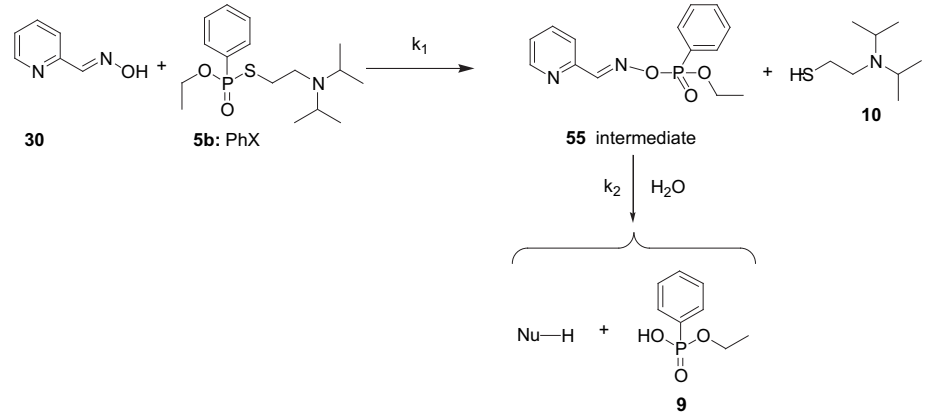
In order to understand the origin of this high hydrolytic activity, the kinetic profile of compound **45** was studied at different PhX/nucleophile ratios, temperature, pH and compared with reference compound pralidoxime **12** (see ESD). It was then found, that initial rate for amidoxime **45** is higher than for pralidoxime **12**. The different types of curves obtained for nucleophiles **45** and **12** evidence the different mechanisms of PhX hydrolysis. While the kinetic profile of pralidoxime **12** is more or less linear, the kinetic profile of amidoxime **45** shows a plateau reminiscent of a possible inhibition (see ESD). It was thus supposed, that, contrary to pralidoxime **12** for which the initial nucleophilic addition rate yielding the phosphoryloxime is predominant, reaction of amidoxime **45** with PhX includes two stages of kinetic significance: (i) formation of the intermediate phosphorylamidoxime and (ii) its hydrolysis (Scheme 3). Inhibition of nucleophile **45**, which was observed at 40 °C allowed us to assume that the rate of the intermediate formation rises more quickly than the rate of its hydrolysis. The decrease of the pH from 8.5 to 7.55 results in significant decrease of the initial rate of the reaction (Table 6). The kinetic study of PhX hydrolysis by amidoxime **45** also allowed us to conclude that this compound is not really catalytic, since there is no turnover.

In order to explain the results of the kinetic analysis obtained for amidoxime **45** the reaction of PhX with pyridinaldoxime **30** was studied by HPLC and LC–MS. The analysis of reaction mixture by LC–MS allowed to identify four peaks with $[M+H]^+$ 123, 187, 330 and 291, which correspond to pyridinaldoxime **30**, phosphonic acid **9**, PhX **5b**, and the intermediate phosphoryloxime **55**, respectively (Scheme 3).

The study of the reaction by HPLC proved the formation of the intermediate (phosphoryloxime **55**), which is quite stable and hydrolyzes slowly to phosphonic acid **9**. Thus, the process consists of two stages, which have different rates. In order to evaluate the stability of the intermediate the reaction was studied at different temperatures. It was found, that stability of phosphoryloxime decreases with elevation of the temperature. For example, at 20 °C phosphonic acid started to form after 8 h of reaction, and even at 40 °C the reaction was quite slow (5 h), which proves that pyridinaldoxime **30** does not exhibit any catalytic activity.

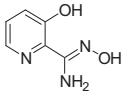
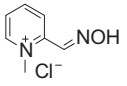
The reaction of amidoxime **45** with PhX (**45**/PhX=0.4/1) was further studied by LC–MS and ^{31}P NMR. Thus, in the LC–MS spectrum of the reaction mixture were detected the following compounds: PhX **5b**, phosphonic acid **9**, aminothiol **10**, Tris and intermediate phosphorylamidoxime **56**. The presence in LC–MS spectrum of peak with $[M+1]^+$=136, which was assigned to isoxasole structure **57** allowed to assume, that phosphorylamidoxime **56** in basic conditions undergoes intramolecular cyclization (Scheme 4). The results obtained are in good correlation with the ^{31}P NMR data. Thus, in the ^{31}P NMR spectrum of the reaction mixture peak at 49.1 corresponds to PhX, peak at 29.8 to phosphonic acid **9** and 15.1 to phosphorylamidoxime **56**.

The cyclization of phosphorylamidoxime **56** explains the absence of the catalytic activity of amidoxime **45**. This fact is in accordance with results obtained by Rebeck, which showed, that in basic conditions β-hydroxy oxime reacts with OP nerve agent with similar cyclization of the OP ester to the isoxasole,[30] and also with



Scheme 3.

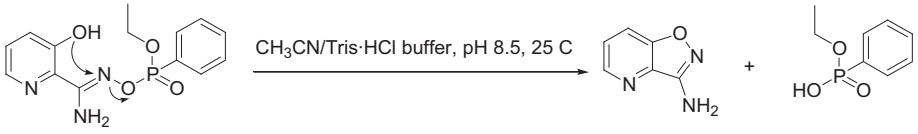
Table 6
Initial rates of PhX hydrolysis μmol/min

PhX/nucleophile ratio	pH 8.5, 25 °C			pH 8.5, 40 °C		pH 7.55, 25 °C
	1/1	1/0.4	1/0.2	1/0.4	1/0.2	1/0.2
 **45**	37.5	8	4.5	10.9	5.2	2.1
 **12**	4.4	2	0.7	5.3	2.7	0.2

This means that much higher concentrations of compounds **29** and **45** are required to reach the desirable reactivation of enzyme. Nevertheless, the results obtained for the reactivation of VX poisoned AChE are quite promising regarding that K_d of the nucleophiles can be further improved by structural modifications increasing the affinity of the nucleophiles to the inhibited enzyme.

3. Conclusions

In conclusion, the new uncharged nucleophiles, such as oximes and amidoximes show a comparable and in some cases increased reactivity towards PhX hydrolysis in comparison with 2-pralidoxime have been described. Among them 3-hydroxy-pyridylamidoxime was found to be the most reactive towards PhX hydrolysis. The



56 **57** **9**

Scheme 4. Intramolecular cyclization of phosphorylamidoxime.

the results we observed with akin β-hydroxy oximes.[24p,25] It should be mentioned, that amidoxime **45** due to the cyclization of the intermediate **56** possess the privilege of preventing the recapture phenomenon in comparison with oximes known antidotes against OP poisoning. Oximes or amidoximes that do not possess an OH group in the beta position, after reactivation of AChE form an ester with organophosphorus agents, which in its turn exhibit powerful AChE inhibition.[31]

2.2. Poisoned AChE reactivation

Considering that pralidoxime, HI-6 and other pyridiniumaldoximes are the best known poisoned AChE reactivators to date, we wondered whether the synthesized nucleophiles could also be used as poisoned AChE reactivators, which would make them valuable dual nerve agents poisoning countermeasures: (1) through the hydrolysis of the nerve agent and (2) as reactivator of the poisoned AChE. Therefore the ability of the most active α-nucleophiles **25**, **28**, **29**, **41** and **45** to reactivate the human AChE poisoned with VX was studied by spectrophotometry using Ellman's reagent and compared with pralidoxime **12**. It was found that compounds **25**, **28** and **41** do not show sufficient in vitro reactivation of AChE poisoned with VX (Table 7, entries 4–6). Whereas oxime **29** and amidoxime **45** (Table 7, entries 2 and 3) exhibited high reactivation, especially **29**, which reactivation rate constant (k_r) was higher than the reference compound pralidoxime **12**. However, these nucleophiles exhibited much lower affinity to the inhibited enzyme in comparison with **12**, this low affinity being due to their high dissociation constants (K_d).

Table 7
Specific reactivation rate constant at a given concentration of reactivator(k_{obs}), reactivation rate constant (k_r) and dissociation constant (K_d)[a]

Compound	Entry	k_{obs}, min^{-1}	k_r, min^{-1}	K_d, μM^{-1}
12	1	0.080 ± 0.005 at 1 mM	0.06 ± 0.01	215 ± 75
29	2	0.03 ± 0.01 at 1 mM	0.5 ± 0.1	$30 \pm 10\times10^3$
45	3	0.010 ± 0.002 at 10 mM	0.08 ± 0.1	$30 \pm 20\times10^3$
25	4	NA		
28	5	0.005 ± 0.001 at 10 mM		
41	6	0.004 ± 0.002 at 10 mM		

[a] $k_{obs} = \left(\frac{k_r}{1+\frac{K_d}{R}}\right)$, R—reactivator concentration.

kinetic analysis showed that 3-hydroxy-pyridyl-amidoxime reacts with PhX much faster than 2-pralidoxime but due to the isoxasole derivative formation this compound does not show any catalytic activity. The preliminary results of the VX poisoned AChE reactivation allowed to find very promising neutral α-nucleophiles with high reactivation rate constant. The synthesis and evaluation of the reactivators with improved affinity towards poisoned enzyme is in progress in our laboratories.

4. Experimental section

4.1. General materials and methods

The chemicals used in the synthesis were purchased from Aldrich, Acros, Alfa-Aesar or ABCR and used without further purification. The solution of PhX in CH_3CN (20 mg/mL) and the solutions of VX in isopropanol (1 mM and 5 mM) were stored at −20 °C. PhX,[20] phosphonic acid **9**,[32] **17**,[33] **21**,[34] **23**,[35] **29**,[25] **47**,[36] **52**[25] and **53**[25] were prepared as previously described. The following solvents were distilled from the indicated drying agents: CH_2Cl_2 (CaH_2), THF (Na), CH_3CN (CaH_2) or dried over 4 Å molecular sieves. Thin layer chromatographies (TLC) were carried out using Merck silica gel plates 0.25 nm (Kieselgel 60 F_{254}, 40–60 μm, 230–400 mesh ASTM). TLC plates were visualized using a combination of UV lamps (256, 365 nm), followed by detection with *p*-anisaldehyde, ceric ammonium molybdate or ninhydrin. Flash chromatographies were performed using silica gel Merck Kieselgel (40–60 μm, 230–400 mesh ASTM) according to a standard procedure. All aqueous buffers were prepared by using water purified with a Milli-Q system (purified to 18.2 MW cm^{-1}).

Tris/HCl buffer 0.1 N, 0.15 N NaCl, pH 8.5: [Tris]=0.1 N, [NaCl]=0.15 N, adjusted to pH 8.5 with concentrated HCl solution.

Tris/HCl buffer 0.1 N, 0.15 N NaCl, pH 7.55: [Tris]=0.1 N, [NaCl]=0.15 N, adjusted to pH 7.5 with concentrated HCl solution.

Tris/HCl buffer 0.05, pH 8: [Tris]=0.05 N, [NaCl]=0.15 N, adjusted to pH 8 with concentrated HCl solution.

Phosphate buffer 0.1 N pH 7: [$NaHPO_4$]=0.1 N, [Na_2PO_4]=0.1 N.

^1H NMR, ^{13}C NMR and ^{31}P NMR spectra were recorded on Brucker DPX200, DPX300, DPX400 and DPX500. Chemical shifts (δ) are quoted in parts per million and are calibrated relative to solvent

residual peaks. Multiplicities are reported as follows: s=singlet, d=doublet, t=triplet, m=multiplet, br=broad. Coupling constant are reported in Hertz. Mass spectra were recorded on an MS/MS high resolution Micromass ZABSpecTOF spectrometry. Infrared spectra of samples were measured using a Nicolet 380 FI IR Spectrometer from Thermo Electron Corporation as a CH_3Cl solution or solid on a diamond plate. UV-visible spectra were obtained on KRONTON Instruments UVIKON 943 and UVIKON 933. Absorption cells (Hellma 100–OS 10 mm).

4.2. General experimental procedure for oximes and amidoximes synthesis

Hydroxylamine hydrochloride (4 mmol) and Na_2CO_3 (2 mmol) were mixed together in water (3 mL) until the solid dissolves and the gas emission stops. This aqueous solution was added to the corresponding aldehyde or nitrile (1 mmol) in absolute ethanol (0.5 mL).The mixture was heated at 70 °C for 2–24 h. The reaction mixture was then allowed to cool down to room temperature. If the compound precipitated, the solid was filtered off, washed with cold water and dried under vacuum. Otherwise the reaction mixture was extracted with ethyl acetate and dried over Na_2SO_4. After removal of the solvent the compound was purified by column chromatography (cyclohexane/ethyl acetate) to afford the desired oxime or amidoxime.

*4.2.1. 2,6-Dihydroxybenzaldoxime (**24**).* Obtained with 45% yield from 2,6-dihydroxy-benzaldehyde[37] using the general procedure and purification by flash chromatography (cyclohexane/ethyl acetate 30/70). ^{1}H NMR (300 MHz, CD_3OD): δ=6.32 (d, 2H, J=8 Hz), 7.01 (t, 1H; J=8 Hz), 8.56 (s, 1H). ^{13}C NMR (75 MHz, CD_3OD): δ=106.62, 107.53, 132.20, 148.51, 158.91 ppm. IR (cm^{-1}): 3500–2500 (br), 1622, 1467, 1227. MS (ESI): m/z: calcd for [M]: 153.14; found: 154 $[M+H]^+$.

*4.2.2. 2,4,6-Trihydroxybenzaldoxime (**28**).* Obtained with yield 50% as orange powder after purification by column chromatography (cyclohexane/ethyl acetate=1/1). ^{1}H NMR (300 MHz, CD_3OD): δ=5.84 (s, 2H), 8.43 (s, 1H). ^{13}C NMR (125 MHz, CD_3OD): δ=95.47, 99.69, 107.18, 148.76, 160.15, 161.86 ppm. IR (cm^{-1}): 3364, 1507, 1015. MS (ESI): m/z: calcd for [M]: 169.13; found: 170 $[M+H]^+$.

*4.2.3. 4,6-Dichloro-2-hydroxybenzaldoxime (**35**).* Obtained as a white solid after filtration with yield 93%. ^{1}H NMR (400 MHz, CD_3OD): δ=6.91 (s, 1H), 7.02 (s, 1H), 8.62 ppm (s, 1H). ^{13}C NMR (100 MHz, CD_3OD): δ=115.01, 116.76, 121.46, 135.46, 136.98, 148.61, 160.54 ppm. IR (cm^{-1}): 3363, 2988, 1605, 1556, 1408. MS (ESI): m/z: calcd for [M]: 206.03; found 206 $[M]^+$.

*4.2.4. 3-Nitro-2-hydroxybenzaldoxime (**36**).* Obtained with yield 81% after filtration. ^{1}H NMR (400 MHz, CD_3OD): δ=7.04 (t, 1H, J=8 Hz), 7.83 (dd, 1H, J=8, 1.6 Hz), 8.00 (dd, 1H, J=8, 1.6 Hz), 8.41 ppm (s, 1H). ^{13}C NMR (75 MHz, CD_3OD): δ=120.42, 123.48, 126.93, 135.12, 137.83, 147.80, 152.90 ppm. IR (cm^{-1}): 3411, 2536, 1622, 1526, 739. MS (ESI): m/z: calcd for [M]: 182.13; found 183 $[M+H]^+$.

*4.2.5. 2,6-Dihydroxybenzamidoxime (**42**).* Obtained with yield 60% from 2,6-dihydroxybenzonitrile[38] using the general procedure and purification by flash chromatography (cyclohexane/ethyl acetate 40/60). ^{1}H NMR (300 MHz, CD_3OD): δ=6.33 (d, 2H, J=8 Hz), 6.98 ppm (m, 3H, J=8 Hz). ^{13}C NMR (75 MHz, CD_3OD): δ=108.5, 110.6, 132.9, 148.2, 158.4 ppm. IR (cm^{-1}): 3505, 3333, 1630, 1254. MS (ESI): m/z: calcd for [M]: 168.15; found: 169 $[M+H]^+$.

*4.2.6. 2,3-Dihydroxybenzamidoxime (**43**).* Obtained with yield 70% as whitish solid using the general procedure and purification by flash chromatography (cyclohexane/ethyl acetate 40/60). ^{1}H NMR (300 MHz, CD_3OD): δ=6.71 (t, 1H, J=8 Hz), 6.80 (dd, J=8, 1.5 Hz), 7.00 ppm (dd, 1H, J=8, 1.5 Hz). ^{13}C NMR (75 MHz, CD_3OD): δ=116.23, 117.05, 117.17, 119.53, 146.85, 146.97, 155.46 ppm. IR (cm^{-1}): 3354, 2487, 1622, 1583, 1462. MS (ESI): m/z: calcd for [M]: 168.15; found: 169 $[M+H]^+$.

*4.2.7. 2-Hydroxypyridylamidoxime (**45**).* Obtained with yield 82% as white needle like crystals after filtration. ^{1}H NMR (400 MHz, DMSO-d_6): δ=6.36 (br s, 2H), 7.29–7.34 (m, 2H, J=3.8 Hz), 8.11 (dd, 1H, J=3.8, 2.1 Hz), 10.25 (s, 1H), 12.06 (s, 1H). ^{13}C NMR (100 MHz, CD_3OD): δ=125.11, 126.17, 133.88, 140.39, 155.03, 155.67 ppm. IR (cm^{-1}): 3428, 2849, 1653, 1594, 1567, 1390, 939. MS (ESI): m/z: calcd for [M]: 153.14; found 154 $[M+H]^+$.

*4.2.8. N-Methyl-3-hydroxy-2-pyridylamidoxime iodide (**46**).* *N*-methyl-2-cyano-3-hydroxypyridine iodide (221 mg, 0.84 mmol) and hydroxylamine (50 wt % in H_2O, 5 equiv) in methanol were refluxed for 18 h. The reaction mixture was allowed to cool down at room temperature and was extracted with ethyl acetate, dried over Na_2SO_4. After purification by column chromatography (silica gel, DCM/MeOH=90/10) pale orange compound **46** was obtained with yield 45%.

^{1}H NMR (300 MHz, DMSO-d_6): δ=3.95 (s, 3H), 5.90 (br s, 2H), 6.95 (d, 1H, J=9 Hz), 7.20–7.25 (dd, 1H, J=9, 5 Hz), 7.34 (d, 1H, J=5 Hz), 9.66 (br s, 1H). ^{13}C NMR (125 MHz, DMSO-d_6): δ=45.57, 126.29, 133.85, 136.06, 146.23 ppm. MS (ESI): m/z: calcd for [M]: 167.17; found 168 $[M]^+$.

The final atomic coordinates and crystallographic data for molecule **46** have been deposited at the Cambridge Crystallographic Data Centre (12 Union Road, CB2 1EZ, UK, fax: +44 1223 336033; e-mail: deposit@ccdc.cam.ac.uk) and are available on request quoting the deposition number CCDC 818187.

*4.2.9. N′-Hydroxy-4-((hydroxyimino)methyl)benzimidamide (**49**).* Obtained with yield 60% from 4-cyanobenzaldehyde accordingly to the general procedure and purified by crystallization from CH_2Cl_2. ^{1}H NMR (300 MHz, DMSO-d_6): δ=5.82 (s, 2H), 7.57 (d, 2H, J=8 Hz), 7.69 (d, 2H, J=8 Hz), 8.14 (s, 1H), 9.71 (s, 1H), 11.28 ppm (s, 1H). ^{13}C NMR (75.5 MHz, DMSO-d_6): δ=125.61, 126.09, 133.43, 133.97, 147.74, 150.35 ppm. IR (cm^{-1}): 3333, 1648, 1435, 1289, 916. MS (ESI) m/z: calcd for [M]: 179.18; found: 180 $[M+H]^+$.

4.3. Hydrolysis of PhX in the presence of nucleophiles

High-performance liquid chromatography separations were performed on Schimadzu 10 AVP HPLC (column Agilent Zorbax SB-CN, 5 μm, 4.6×150 mm) with 0.012% (v/v) trifluoroacetic acid CH_3CN/0.008% (v/v) trifluoroacetic acid H_2O as the eluent [75% H_2O (2.5 min), linear gradient from 75% to 40% (2.5–5.00 min), 40% (5.00–10.00 min), linear gradient from 40% to 75% (10.00–11.00 min), 75% (11.00–15.00 min)] at a flow rate 1 mL/min. UV detection was achieved at 263 nm. The volume injected was 20 μL.

4.3.1. Screening tests. Samples preparation: Samples containing 1 mM of PhX (50 μL of 100 mM stock solution in CH_3CN) and nucleophile 5 mM were prepared in CH_3CN (500 μL) and Tris/HCl buffer (0.1 N pH 8.5, V_{TOT}=5 mL).

HPLC analysis: Each sample was analysed by HPLC at t=0 and t=2 h. The PhX peaks areas (rt≈8 min) were measured.

4.3.2. Kinetic studies. Samples preparation: Samples containing 1 mM of PhX (50 μL of 100 mM stock solution in CH_3CN) and nucleophile 5 mM, 1 mM, 0.4 mM or 0.2 mM were prepared in CH_3CN (500 μL) and Tris/HCl buffer (0.1 N pH 8.5 or 0.1 N pH 7.55,

V_{TOT}=5 mL).The samples were dispatched in HPLC samples and put into the HPLC auto-sampler.

For the experiment at 40 °C, the samples were maintained at 40 °C in an oil bath, and the injections were done manually every 15 min.

HPLC analysis: Each sample was analysed by HPLC every 15 min. The PhX peaks areas (rt≈8 min) were measured.

4.4. In vitro reactivation of VX-inhibited human AChE

Recombinant human AChE (hAChE) was produced and purified as previously described.[39] VX was from DGA maôtrise NRBC (Vert le Petit, France). All chemicals were from Sigma.

Stock solution of VX was 5 mM in isopropanol. The nerve agents were further diluted in MeOH to low μM concentrations. The inhibition of hAChE was performed in phosphate buffer (0.1 M, pH 7.0, 0.1% BSA) at 25 °C. An approximatively stoichiometric amount of VX was chosen to attain an inhibition plateau >90% in about 1 h while carefully avoiding 100% inhibition. Under these conditions, there is no inhibitor left that can affect the reactivation rate measurements and ageing of the conjugate is negligible (half-life is 7.9 days, data not shown).

OP-inhibited hAChE was incubated at 25 °C with different concentrations of reactivator at 25 °C in phosphate buffer (0.1 M, pH 7.0, 0.1% BSA). The final concentrations of compounds used for VX-hAChE reactivation were: 1, 3, 10 and 20 mM or 0.1, 0.5, 1 and 2 mM for 2-PAM. 50-μl aliquots were transferred to 1-mL cuvettes at time intervals ranging from 1 min to 10 min depending on the reactivation rate for measurement of hAChE activity using 0.75 mM propionylthiocholine in Ellman's buffer (phosphate 0.1 M, pH 7.0, 0.1% BSA, 0.5 mM DTNB, 25 °C).[40] The same procedure was applied to the control containing the uninhibited enzyme and reactivator. The enzyme activity in the control remained constant during the experiment. The percentage of reactivated enzyme ($\%E_{react}$) was calculated as the ratio of the recovered enzyme activity and activity in the control. The dissociation constant K_D of inhibited enzyme–reactivator complex (*E-PR*) and the reactivity rate constant k_r were calculated by non-linear fit using the standard concentration-dependent reactivation equation derived from the following scheme:

$$E\text{-}P + R \overset{K_D}{\rightleftharpoons} E\text{-}PR \overset{k_r}{\rightarrow} E + P - R$$

$$\%E_{react} = 100 \cdot \left(1 - e^{k_{obs} \cdot t}\right) \quad \text{and} \quad k_{obs} = \frac{k_r[R]}{K_D + [R]}$$

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tet.2011.05.130.

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