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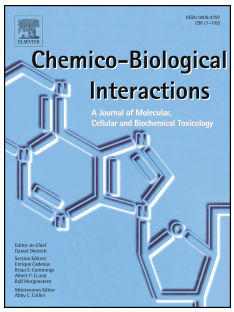

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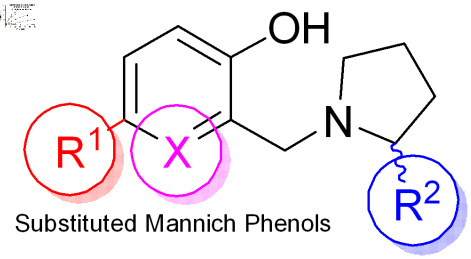
Credit Author Statement

De Koning: Conceptualization, Methodology, Validation, Formal analysis, Writing - Original Draft, Visualization, Supervision, Project administration; **Horn**: Methodology, Validation, Formal analysis, Investigation, Writing - Review & Editing, Visualization; **Worek:** Writing - Review & Editing, Visualization**;** Supervision, Project administration; **van Grol:** Methodology, Validation, Formal analysis, Investigation.



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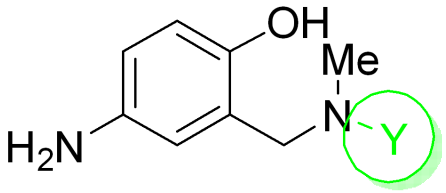
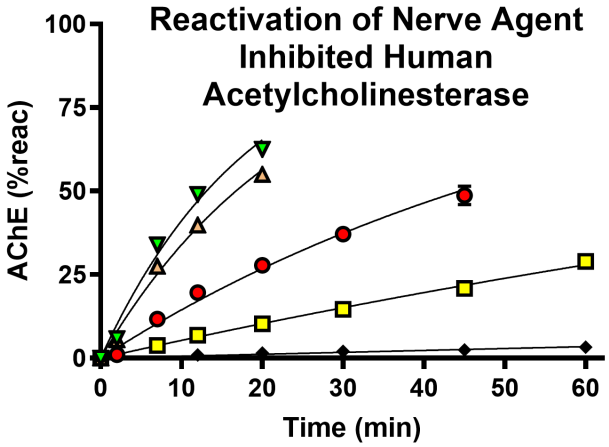


Mannich Phenol Dimers



Synthesis and in Vitro Evaluation of Novel Non-Oximes for the Reactivation of Nerve Agent Inhibited Human Acetylcholinesterase

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Abstract

Since several decades oximes have been used as part of treatment of nerve agent intoxication with the aim to restore the biological function of the enzyme acetylcholinesterase after its covalent inhibition by organophosphorus compounds such as pesticides and nerve agents. Recent findings have illustrated that, besides oximes, certain Mannich phenols can reactivate the inhibited enzyme very effectively, and may therefore represent an attractive complementary class of reactivators. In this paper we further probe the effect of structural variation on the in vitro efficacy of Mannich phenol based reactivators. Thus, we present the synthesis of 14 compounds that are close variants of the previously reported 4-amino-2-(1-pyrrolidinylmethyl)-phenol, a very effective non-oxime reactivator, and 3 dimeric Mannich phenols. All compounds were assessed for their ability to reactivate human acetylcholinesterase inhibited by the nerve agents VX, tabun, sarin, cyclosarin and paraoxon in vitro. It was confirmed that the potency of the compounds is highly sensitive to small structural changes, leading to diminished reactivation potency in many cases. However, the presence of 4-substituted alkylamine substituents (as exemplified with the 4-benzylamine-variant) was tolerated. More surprisingly, the dimeric compounds demonstrated non-typical behavior and displayed some reactivation potency as well. Both findings may open up new avenues for designing more effective non-oxime reactivators.

Keywords

Acetylcholinesterase, non-oxime, nerve agents, reactivation

1. Introduction

Nerve agents and a number of pesticides belong to a family of organophosphorus (OP) compounds that exert their toxicity through the covalent inhibition of the enzyme acetylcholinesterase (AChE).[1,2] Examples of OP's are the commonly used pesticides (e.g., chlorpyrifos, malathion, paraoxon), but also nerve agents (e.g., VX, soman, sarin and tabun). AChE is present on synaptic neurons, and catalyzes the hydrolysis of the neurotransmitter acetylcholine (ACh) in the synaptic clefts.[2,3] Inhibition of the enzyme by OP's occurs through the formation of a covalent bond between the active site serine-203 residue and the OP, resulting in the permanent phosphylation. This blockage leads to accumulation of ACh, over-stimulation of the ACh receptors, which in turn leads to severe symptoms and death.[4] Part of the treatment of OP poisoning involves the administration of compounds that contain a nucleophilic oxime functionality, which reactivates the enzyme by attack of the oximate anion on the phosphorus atom of the blocking phosphyl residue.[5,6] The most well-known examples of these reactivators of OP-inhibited AChE are the clinically employed HI-6, Obidoxime and 2-PAM (Figure 1), that were all developed in the fifties/sixties of the last century. From a structural point of view, all of these compounds have a pyridinium aldoxime group in common. The charged pyridinium moiety lowers the pKa of the oxime, necessary for the generation of a sufficient fraction of reactive oximate anions under physiological conditions, and contributes to the requisite affinity of the compound for the inhibited enzyme. The presence of charge(s), however, also results in limited brain accessibility of the oximes.[7] Another limitation is the lack of broad-spectrum activity: not one oxime is capable

of reactivating AChE inhibited by one of the many OP's. Hence, a tremendous effort has been devoted over the last six decades to research focusing on the design, synthesis and evaluation of novel compounds with the aim to improve brain permeability and increased spectrum activity.[8–11] The design of preferably low molecular weight compounds that lack charge (or possess overall neutrality, such as zwitterionic compounds), but have sufficient affinity for the enzyme and are at the same time reactive enough (appropriate pKa) for the intended phosphyl displacement reaction, is still an enormous challenge. While a great variety of chemical structures have been reported over the years, the use of an oxime functionality as the nucleophilic moiety has been virtually ubiquitous. Despite all of these efforts, none of these compounds has reached the clinic to date. Clearly, a complementary design strategy leading to fundamentally different compounds would be highly welcome.

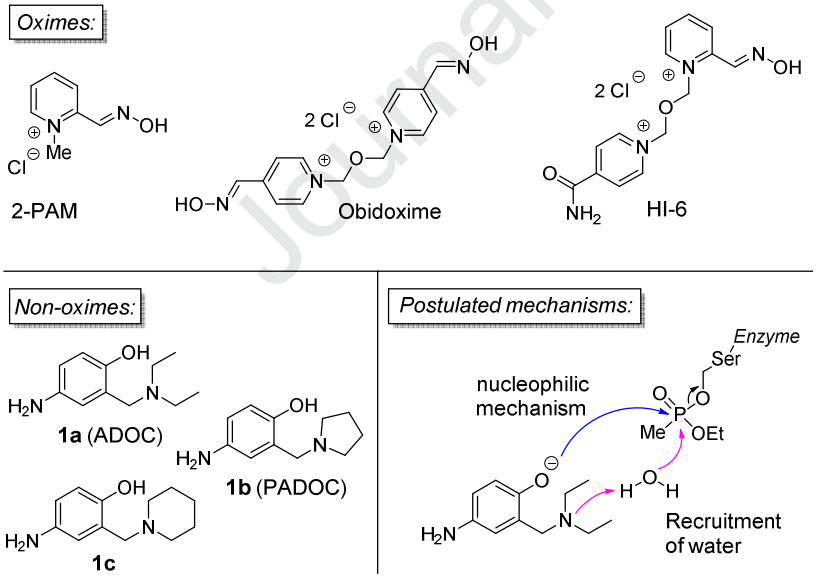

Figure 1. Structures of currently employed oximes and recently discovered non-oximes for the reactivation of nerve agent inhibited acetylcholinesterase. While the oximes work via direct nucleophilic attack on the phosphyl moiety the mechanism of reactivation by non-oximes is still unclear.

Several years ago, Katz reported that a Mannich phenol (ADOC, **1a** in Figure 1) was capable of reactivating OP-inhibited AChE in vitro.[12] Although the reactivation rates were far inferior to those of the current oximes, the discovery of a reactivator that lacked the oxime group (hence the expression 'non-oxime') was surprising and appealing, as this discovery pointed at the possibility of developing reactivators that were fundamentally different from current reactivator design. Moreover, the absence of charge, its relatively low molecular weight and its short route of synthesis are equally attractive features from a medicinal chemistry point of view.[13] The mechanism of reactivation of OP-inhibited AChE by ADOC remains to be unraveled, but both a nucleophilic mechanism (arguably through the phenolate) as well as an acid-base mechanism (generation of reactive hydroxyls by reactivator-mediated deprotonation of water) have been hypothesized (Figure 1).[14,15] Not long afterwards, Cadieux presented a number of ADOC derivatives in which the chemical space around ADOC was explored, by varying number, type and bulk of substituents and substituent positions, and their effect on the reactivation potency.[16] Unfortunately, none of these new derivatives showed any potency as a reactivator. We have recently reported a systematic investigation that focused solely on minor structural variations around the benzylic amine group.[17] Thus, a set of aliphatic, cyclic and unsaturated benzylic amine derivatives of ADOC were prepared and tested in vitro. Although most of the structures had no significant potency as a reactivator, we were happy to find that the ring-closed derivative of ADOC (**1b** or PADOC in Figure 1) was at least ten times more potent than ADOC in the reactivation of human AChE (hAChE), inhibited by various OP's.[18] Moreover, for VX and paraoxon (POX) inhibited hAChE its potency was equal or superior to that of HI-6, one of the best reactivators know to date. Another intriguing finding was that the potency of these non-oximes was very strongly influenced by small structural changes, thwarting analyses of structure-activity relationships. For instance, expansion of the pyrrolidine ring in PADOC

with just a single methylene group (to give **1c** in Figure 1) resulted in a dramatic decrease of reactivation potency. Determination of the reactivation parameters k_r (reactivity rate constant) and K_D (apparent dissociation constant) revealed that the reactivation potency of PADOC mainly stems from a high affinity for the inhibited enzyme. In line with the previous work, we here report 17 novel, non-oxime compounds (Figure 2) and their in vitro biological evaluation.

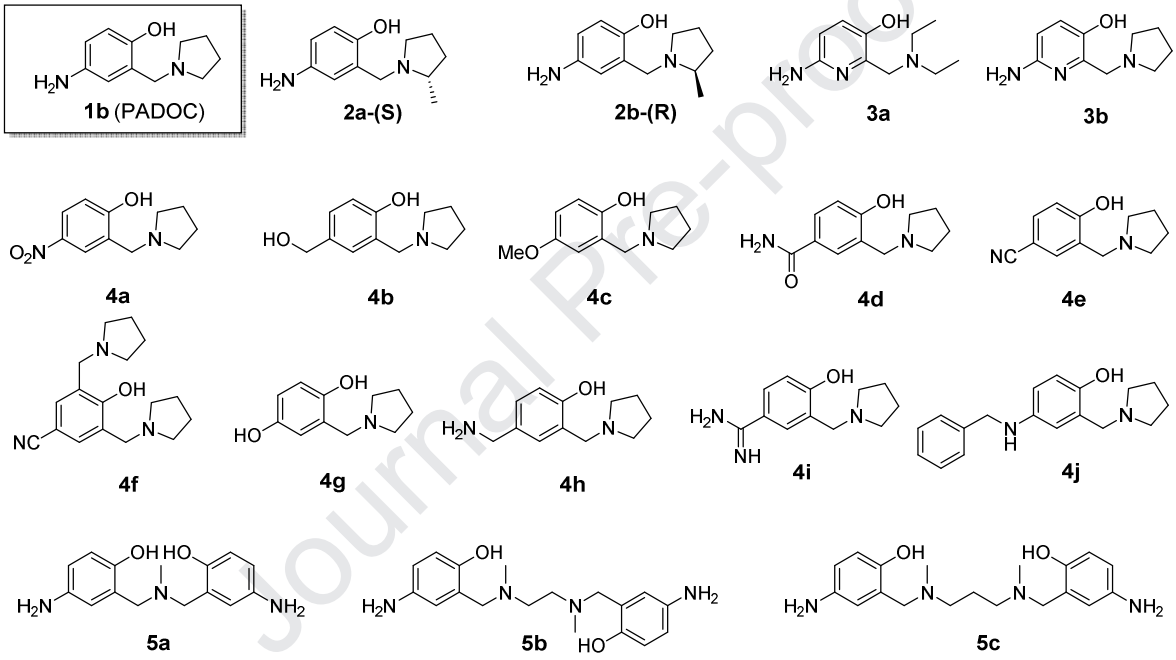

Figure 2. Non-oxime compounds synthesized in this study

Compounds **2** and **3** are close variants of the current lead compound **1b** and were selected on the basis of results previously reported by Hadad and co-workers. They found that 2-methylpyrrolidine variants of structurally related compounds, designed for re-alkylation of aged OP-inhibited AChE, exhibited an increased affinity for AChE. These compounds lacked

the 4-amine in **1**, and featured a pyridine instead of a benzene moiety.[10] In an analogous fashion, the pyridine feature was adopted in compounds **3**.

Compounds **4** represent structures in which the substituent at the 4-position of **1b** was varied. These substituents featured electron withdrawing groups, electron donating groups and moieties that may be able to exert further interaction (e.g., H-bonding) with enzyme residues.

Finally, we synthesized three dimeric compounds, in which two Mannich phenol units were connected through spacers of varying length. This design was inspired by the suggestion that interaction of two ADOC molecules to the enzyme at the same time may be required for efficient reactivation.[12] The chemical linkage of these molecules could possibly result in more efficient binding of the two non-oxime moieties compared to 2 separate molecules. In the absence of in silico models the design of compounds **5** was merely based on synthetic accessibility.

2. Materials and Methods

The synthetic procedures for all compounds are provided in this section. General information (chemicals, equipment, suppliers, etc) are provided in the supporting information. The biological experiments (determination of IC_{50}, reactivation screening, and reactivation kinetics) were carried out as previously described.[18] Brief descriptions are provided below, while details are provided in the supporting information.*2.1 2-Step synthetic procedure compound **2a/2b***

4-acetamidophenol (1.8g, 11.9mmol; 1.2eq) and paraformaldehyde (0.3g, 10.2 mmol; 1eq) were dissolved in ethanol (20 ml). (R)-(-)-2-methylpyrrolidine (or the S-isomer, 0.84ml, 7.7 mmol; 0.76 eq), was added and the mixture was refluxed at 80^{o}C for 24h. After concentration in vacuo, the residue was dissolved in MeOH (2 ml) and applied on a silica gel column,

which was subsequently eluted with a gradiënt of 0-10% MeOH in EtOAc. Yield 0.33g; 1.33mmol (17%). ^{1}H-NMR (400MHz, MeOH-d4) δ 7.29(s, CH); 7.22(d, CH); 6.69(d, CH); 4.22(d, CH_2); 3.44(dt, CH_2); 2.67(q, CH_2); 2.37(q, CH_2); 2.10(s, CH_3); 1.81(p, CH_2); 1.54(m, CH_2); 1.24(d, CH_2). ^{13}C-NMR (101MHz, MeOH-d4) δ 169.90(C=O); 154,20(C-OH); 129.93(CH); 122,62(C); 121.20(C); 120,91(CH); 115.13(CH); 59,98(CH_2); 55,56(CH_3); 53,45(CH_2); 32,33(CH_2); 17,63(CH3). $[M+H]^+$: 248 Da.

The product from the previous step (0.33g, 1.33mmol; 1eq) was dissolved in 10% HCl (10 ml). After 6h reflux at 110^{o}C, the mixture was concentrated and coevaporated with acetonitrile (2-3x). Yield: 0.38g; 1.52mmol; 19.8% (2 steps). ^{1}H NMR (400 MHz, Methanol-d4) δ 7.55 (s, 1H), 7.39 (d, J = 8.7 Hz, 1H), 7.09 (d, J = 8.7 Hz, 1H), 4.62 (d, J = 12.9 Hz, 1H), 4.24 (d, J = 13.0 Hz, 1H), 3.72 – 3.61 (m, 1H), 3.52 – 3.44 (m, 1H), 3.35 (d, J = 16.1 Hz, 7H), 2.39 (dd, J = 12.9, 5.0 Hz, 1H), 2.14 (dd, J = 15.2, 6.4 Hz, 1H), 2.02 (s, 1H), 1.81 (dd, J = 13.0, 8.7 Hz, 1H), 1.50 (d, J = 6.4 Hz, 3H). ^{13}C NMR (Methanol-d4, 101 MHz): δ (ppm) 156.85, 127.04, 126.05, 122.05, 118.54, 116.44, 64.66, 53.91, 51.25, 31.23, 21.05, 15.30. $[M+H]^+$: 206 Da.

*2.2 2-Step synthetic procedure compound **3a***

5-hydroxy-2-pivalamidopyridine (180 mg, 927 µmol; 1 eq) was dissolved in ethanol (5 ml). Formaldehyde solution (37% in water, 64 µl (0.93 eq)) and diethylamine (89 µl, 0.93 eq) were added. The mixture was refluxed for 240h at 95 °C. After concentration in vacuo, the mixture was purified by automated column chromatography (Biotage Isolera 4, using a gradient of 0-90 % EtOAc in heptane, containing 3% triethylamine). Yield: 80 mg, 285 µmol (33%) ^{1}H NMR (400 MHz, Methanol-d4) δ 7.77 (d, J = 8.8 Hz, 1H), 7.12 (d, J = 8.7 Hz, 1H), 3.91 (s, 2H), 2.71 (q, J = 7.2 Hz, 4H), 1.31 (s, 9H), 1.15 (t, J = 7.2 Hz, 6H). ^{13}C NMR (101

MHz, Methanol-d4) δ 177.77, 151.94, 142.55, 140.52, 124.98, 115.18, 57.77, 46.90, 39.10, 26.36, 10.15.

3-hydroxy-6-pivalamido-2-(diethylaminomethyl)pyridine was dissolved in 10% HCl and refluxed for 96h at 110 °C. Progress was monitored by LC-MS. After complete conversion the mixture was concentrated in vacuo and co-evaporated with acetonitrile (2-3 x). Quantitative yield. ^{1}H NMR (400 MHz, Deuterium Oxide) δ 7.63 (d, J = 9.5 Hz, 1H), 7.03 (d, J = 9.5 Hz, 1H), 4.36 (s, 2H), 3.24 (q, J = 7.3 Hz, 4H), 1.28 (t, J = 7.3 Hz, 6H). ^{13}C NMR (101 MHz, Deuterium Oxide) δ 149.11, 146.01, 135.28, 122.38, 117.28, 48.70, 48.14, 8.11.

*2.3 2-Step synthetic procedure compound **3b***

5-hydroxy-2-pivalamidopyridine (61 mg, 312 µmol, 1 eq) was dissolved in ethanol (5 ml). Paraformaldehyde (9 mg, 300 µmol, 0.96 eq) and pyrrolidine 23.4 µl (280 µmol, 0.90 eq) were added. The mixture was refluxed for 168h at 95 °C and concentrated in vacuo. The product was purified by automated column chromatography using a gradiënt of 0-90 % EtOAc in heptane, containing 3% Triethylamine. Yield: 54 mg, 195 µmol (70%). ^{1}H NMR (400 MHz, Methanol-d4) δ 7.76 (d, J = 8.8 Hz, 1H), 7.15 (d, J = 8.8 Hz, 1H), 3.96 (s, 2H), 2.82 – 2.72 (m, 4H), 1.97 – 1.85 (m, 4H), 1.31 (s, 9H). ^{13}C NMR (101 MHz, Methanol-d4) δ 177.87, 151.62, 142.31, 140.61, 124.95, 115.34, 58.45, 53.37, 39.09, 26.30, 23.12.

3-hydroxy-6-pivalamido-2-(pyrrolidinomethyl)pyridine was dissolved in 10% HCl and refluxed for 96h at 110 °C. Progress was monitored by LC-MS. After complete conversion the mixture was concentrated in vacuo and co-evaporated with acetonitrile (2-3 x). Quantitative yield. ^{1}H NMR (400 MHz, Methanol-d4) δ 7.74 (d, J = 9.5 Hz, 1H), 7.16 (d, J = 9.4 Hz, 1H), 4.59 (s, 2H), 3.54 (m, 4H), 2.17 (m, 4H). ^{13}C NMR (101 MHz, Methanol-d4) δ 146.14, 135.07, 117.09, 54.35, 49.36, 22.57.

*2.4 Synthetic procedure compound **4a***

Nitrophenol (5,28g, 37mmol, 1.2eq) and paraformaldehyde (0.95g, 31.5mmol, 1eq) were dissolved in THF. Pyrrolidine (2.5ml, 31.5mmol; 1eq) was added and the mixture was refluxed at 70^{o}C for 3h. After concentration purification was accomplished using column chromatography (0-15% MeOH in EtOAc). Yield 3.91g, 17.6mmol; (56%). ^{1}H NMR (400 MHz, DMSO-d6) δ 8.04 (s, 1H), 7.96 (d, J = 9.1 Hz, 1H), 6.57 (d, J = 9.1 Hz, 1H), 3.96 (s, 3H), 2.85 (s, 4H), 1.85 (s, 4H). ^{13}C NMR(DMSO, 101 MHz): δ (ppm) 170.54, 135.22, 126.43, 126.33, 122.62, 117.41, 56.03, 53.33, 23.51. $[M+H]^{+}$: 222 Da.

*2.5 Synthetic procedure compound **4b***

4-hydroxybenzylalcohol (2.48g, 20mmol; 1.2eq) and paraformaldehyde (0.53g, 16.6mmol; 1eq) were dissolved in MeOH (13.3ml) MeOH. Pyrrolidine (1.39ml, 16.6mmol; 1eq) was added and the mixture was refluxed for 3h at 70 ^{o}C. After concentration the mixture was purified by column chromatography (0-15% MeOH in EtOAc). Yield: 2.84g; 13.7mmol; (82%). ^{1}H NMR (400 MHz, DMSO-d6) δ 7.03 (s, 2H), 6.75 – 6.61 (m, 1H), 4.37 (s, 2H), 3.73 (s, 2H), 2.54 (s, 4H), 1.76 (s, 4H). ^{13}C NMR (DMSO, 101 MHz): δ (ppm) 156.31, 132.97, 127.64, 127.06, 123.20, 115.24, 63.30, 57.24, 53.53, 23.69. $[M+H]^{+}$: 207 Da.

*2.6 Synthetic procedure compound **4c***

4-methoxyphenol (2.48g, 20mmol; 1.2eq) and paraformaldehyde (0.60g, 16.6mmol; 1eq) were dissolved in toluene (13.3 mL). Pyrrolidine (3.9ml, 16.6mmol; 1eq) was added. After refluxing (19h at 110 ^{o}C) and concentration the product was purified by column

chromatography (20-0% pentaan in EtOAc. Yield: 2.6g; 12.6mmol; (76%). ^{1}H NMR (400 MHz, DMSO-d6) δ 6.68 (s, 1H), 6.66 (d, J = 2.9 Hz, 1H), 6.63 (d, J = 9.1 Hz, 1H), 3.69 (s, 2H), 3.66 (s, 3H), 2.51 (s, 8H), 1.75 (s, 4H). ^{13}C NMR(DMSO, 101 MHz): δ (ppm) 152.28, 151.03, 124.56, 116.00, 114.56, 113.39, 57.03, 55.74, 53.56, 23.67. $[M+H]^{+}$: 207.

*2.7 Synthetic procedure compound **4d***

4-hydroxybenzamide (1.74g, 12.7mmol; 1,2eq) and paraformaldehyde (0.30g, 10.0mmol; 1eq) were dissolved in MeOH (8mL). Pyrrolidine (0.83ml, 10mmol; 1eq) was added and the mixture was refluxed for 16h at 70 ^{o}C. The mixture was concentrated in vacuo and purified by column chromatography, using a gradient of 0-15% MeOH in EtOAc. Yield 1.64g; 7.45mmol (60%). ^{1}H NMR (400 MHz, Methanol-d4) δ 7.71 (d, J = 8.5 Hz, 1H), 7.66 (s, 1H), 6.76 (d, J = 8.4 Hz, 1H), 3.96 (s, 2H), 3.37 (s, 2H), 3.33 (s, 5H), 2.80 (s, 4H), 1.93 (s, 4H). ^{13}C NMR(Methanol-d4, 101 MHz): δ (ppm) 170.94, 163.19, 128.75, 128.63, 122.58, 121.74, 115.66, 57.13, 52.91, 23.06. $[M+H]^{+}$: 220 Da.

*2.8 Synthetic procedure compound **4e/4f***

4-cyanophenol (3.60g, 30mmol, 1.2eq) and paraformaldehyde (0. 79g, 25mmol, 1eq) were dissolved in toluene (20 mL). Pyrrolidine (2.08ml, 25mmol; 1eq) was added and the mixture was refluxed for 3h at 110 ^{o}C. After completion of the reaction the mixture was concentrated in vacuo and purified using column chromatography with an eluent of 0-15% MeOH in EtOAc. Yield (4e): 3.11g; 15.4mmol (62%). ^{1}H NMR (400 MHz, DMSO-d6) δ 7.54 (d, J = 6.6 Hz, 2H), 6.84 (d, J = 9.0 Hz, 1H), 3.78 (s, 2H), 2.57 (s, 4H), 2.51 (s, 4H), 1.77 (s, 4H). ^{13}C NMR(DMSO, 101 MHz): δ (ppm) 162.46, 133.12, 125.35, 120.14, 116.87, 100.44, 55.70, 53.43, 23.65. $[M+H]^{+}$: 202 Da.

2,6-(dipyrrolidinomethyl)-4-cyanophenol (**4f**) was isolated as well from the column purification. ^{1}H NMR (400 MHz, DMSO-d6) δ 7.45 (s, 2H), 5.67 (s, 4H), 3.72 (s, 4H), 2.54 (s, 9H), 1.74 (s, 8H). ^{13}C NMR(DMSO, 101 MHz): δ (ppm) 161.94, 131.81, 125.39, 120.34, 99.46, 55.08, 53.51, 23.67. $[M+H]^{+}$: 285 Da.

*2.9 Synthetic procedure compound **4g***

2,5-dihydroxybenzaldehyde (2.09g, 15mmol; 1eq) was dissolved in 1,2-dichloroethane (39.5ml) and stirred at room temperature for 5 minutes. Next, pyrrolidine (1.02ml, 18mmol; 1.2eq) and acetic acid (0.857ml, 15mmol; 1eq) were added. The mixture was cooled to 0 ^{o}C and sodium triacetoxyborohydride (4.45g, 21.0mmol; 1.4eq) was added. The ice bath was removed and the mixture was stirred for 16h. The solids were filtered and dissolved in water. Then 10% HCl was added until pH 2. Dichloromethane was added and the organic layer was separated from the water layer. The water fraction was brought to pH 10 using 5M NaOH solution, followed by extraction with EtOAc (2x). The combined EtOAc layers were dried with magnesium sulfate, and filtered. After concentration (in vacuo) the product was obtained. Yield: 1.99g, 10.3mmol (69%). ^{1}H NMR (400 MHz, DMSO-d6) δ 6.51 (s, 3H), 3.63 (s, 2H), 2.51 (s, 5H), 1.74 (s, 4H). ^{13}C NMR(DMSO, 101 MHz): δ (ppm) 149.91, 149.64, 124.31, 115.98, 115.61, 114.66, 57.09, 53.55, 23.66. $[M+H]^{+}$: 193 Da.

*2.10 Synthetic procedure compound **4h***

$LiAlH_4$ (0.35g. 9mmol, 6eq) was dissolved in THF (2 ml). To this solution was carefully added a solution of 2-(pyrrolidinomethyl)-4-cyanophenol 4e (0.32g, 1.5mmol,1eq) in THF (2 ml). After complete addition, the mixture was refluxed for 24h at 70^{o}C. The mixture was cooled to 0oC and water (6ml) was added. Next, 4M NaOH (3 ml) was added as well and the

resulting mixture was stirred for 15min at room temperature. Next, $MgSO_4$ was added and the mixture was stirred for another 15min at room temperature. The solids were filtered and washed with MeOH. The combined filtrates were concentrated in vacuo. Purification was accomplished by silica gel column chromatography using a gradient of 0-15% MeOH in EtOAc in the presence of 1% ammoniumhydroxide. Yield: 0.3g, 1.45mmol (97%). ^{1}H NMR (400 MHz, Methanol-d4) δ 7.10 (d, J = 8.2 Hz, 1H), 7.04 (s, 1H), 6.71 (d, J = 8.0 Hz, 1H), 3.82 (s, 2H), 3.68 (s, 2H), 2.65 (s, 4H), 1.87 (s, 4H). ^{13}C NMR (Methanol-d4, 101 MHz): δ (ppm) 156.44, 132.65, 127.64, 127.35, 122.68, 115.22, 57.31, 53.01, 44.83, 23.15. $[M+H]^+$: 206 Da.

*2.11 2-Step synthetic procedure compound **4i***

2-(pyrrolidinomethyl)-4-cyanophenol **4e** (0.17g, 0.86 mmol) was dissolved in a cooled (ice bath) solution of HCl in MeOH (3M, 2 ml). Acetylchloride (3.12ml, 43.7mmol, 51.4eq) was added. After 15 min of stirring the ice bath was removed and the mixture was refluxed for 24h at 75 ^{o}C. After cooling the precipitate was collected by filtration and washed with EtOAc. Yield: 0.18g, 0.79mmol (92.8%). ^{1}H NMR (400 MHz, Methanol-d4) δ 8.04 (s, 1H), 7.89 (d, J = 8.4 Hz, 1H), 7.14 – 6.96 (m, 1H), 4.44 (s, 2H), 3.55 (s, 2H), 2.19 (s, 2H), 2.06 (s, 2H). ^{13}C NMR (Methanol-d4, 101 MHz): δ (ppm) 170.01, 159.55, 132.21, 131.14, 125.11, 117.19, 115.02, 53.72, 53.20, 22.59. $[M+H]^+$: 235 Da.

The product from the previous step (0.1g, 0.42mmol, 1eq) was dissolved in ammonia (7N, 3 ml) and stirred for 24h at room temperature. Acetonitrile was added and the mixture was concentrated in vacuo. The addition of acetonitrile and concentration was repeated until dryness. ^{1}H NMR (400 MHz, DMSO-d6) δ 9.15 (d, J = 60.2 Hz, 4H), 8.14 (s, 1H), 7.77 (d, J = 8.6 Hz, 1H), 7.20 (d, J = 8.6 Hz, 1H), 4.26 (s, 2H), 3.18 (s, 4H), 1.93 (s, 4H). ^{13}C NMR

(DMSO, 101 MHz): δ (ppm) 165.29, 161.98, 133.65, 131.15, 119.07, 118.12, 116.29, 53.29, 52.05, 23.14. $[M+H]^+$: 219 Da.

*2.12 4-Step synthetic procedure compound **4j***

4-aminophenol (1.891g, 17.3mmol; 1,15eq) was dissolved in MeOH (20ml). After addition of benzaldehyde (1.528ml, 15.0mmol, 1eq) the mixture was refluxed for 20min at 90 °C. After cooling till about 40 °C, $NaBH_3CN$ (1.3g) was added and the mixture was refluxed for another 2h. After completion of the reaction, the mixture was concentrated in vacuo, and the product was isolated by column chromatography, using a gradient of 10-100 % EtOAc in toluene. Yield: 2.7g, 13.6mmol (90.2%). ^{1}H NMR (400 MHz, Acetone-d6) δ 7.40 (d, J = 7.6 Hz, 2H), 7.31 (t, J = 7.5 Hz, 2H), 7.22 (t, J = 7.3 Hz, 1H), 6.63 (d, J = 8.8 Hz, 2H), 6.55 (d, J = 8.8 Hz, 2H), 4.28 (s, 2H). ^{13}C NMR (101 MHz, Acetone-d6) δ 148.94, 142.18, 140.85, 128.21, 127.30, 126.55, 115.65, 113.94, 48.40.

4-(benzylamino)phenol (179mg, 898 µmol, 1 eq) and di-tert-butyl dicarbonate (239mg, 1.1 mmol, 1.2 eq) were added in water (4ml). The mixture was stirred vigorously. After the release of gas ceased (approximately 30 min) the mixture was concentrated in vacuo. Yield: 269mg, 898µmol (quant). ^{1}H NMR (400 MHz, Acetone-d6) δ 7.20 – 7.07 (m, 5H), 6.83 (d, J = 7.8 Hz, 2H), 6.60 (d, J = 8.7 Hz, 2H), 4.66 (s, 2H), 1.27 (s, 9H). ^{13}C NMR (101 MHz, Acetone-d6) δ 155.37, 139.09, 128.19, 127.64, 126.87, 121.22, 114.98, 79.17, 53.59, 27.57.

N-Boc-4-(N-benzyl)aminophenol (218mg, 728µmol, 1.3 eq) was dissolved in MeOH (20 ml). Pyrrolidine (47µl, 558µmol, 1.0 eq) and 37%-formaldehyde (42 µl, 564µmol, 1.01 eq) were added. The mixture was refluxed for 72h at 75°C. The mixture was concentrated in vacuo, and the title compound was purified by column chromatography, using a gradient of 0-10 % MeOH in EtOAc. Yield: 66mg, 173µmol (31% yield). ^{1}H NMR (400 MHz, Methanol-d4) δ

7.23 – 7.02 (m, 5H), 6.69 (d, J = 33.1 Hz, 2H), 6.53 (d, J = 8.6 Hz, 1H), 4.62 (s, 2H), 3.58 (s, 2H), 2.45 (s, 4H), 1.69 (s, 4H), 1.30 (s, 9H). ^{13}C NMR (101 MHz, Methanol-d4) δ 155.98, 155.72, 138.37, 133.36, 128.01, 127.68, 127.58, 127.07, 126.88, 122.85, 115.31, 80.19, 56.75, 53.79, 52.94, 27.29, 23.08.

4-(*N*-benzyl-*N*-Boc)-amino-2-(pyrrolidinomethyl)phenol (66 mg, 0.17 mmol) was dissolved in a solution of HCl in MeOH (1M). The mixture was refluxed for 5h at 85°C. After completion (LC-MS) the mixture was concentrated in vacuo and coevaporated 2-3 times with MeOH to yield the title compound (quantitative yield). ^{1}H NMR (400 MHz, Methanol-d4) δ 7.45 (s, 1H), 7.38 – 7.29 (m, 5H), 7.25 (d, J = 8.7 Hz, 1H), 6.93 (d, J = 8.7 Hz, 1H), 4.49 (s, 2H), 4.28 (s, 2H), 3.36 (s, 2H), 3.08 (s, 2H), 2.01 (d, J = 50.1 Hz, 4H). ^{13}C NMR (101 MHz, Methanol-d4) δ 157.25, 130.52, 130.26, 129.43, 128.79, 127.10, 126.22, 126.08, 118.75, 116.34, 55.61, 53.71, 52.53, 22.52.

*2.13 General 2-step procedure for the synthesis of compounds **5a-c***

4-*N*-Acetamidophenol (0.75g, 5mmol) was dissolved in ethanol (20 ml). Paraformaldehyde (0.15g, 5mmol) and the requisite diamine (2.5 mmol) were added. The mixture was refluxed for 20h and concentrated. The residue was purified by silica gel column chromatography using a gradient of 5-15% MeOH in EtOAc. The product was dissolved in 10% HCl and refluxed for 5h. Upon completion (monitoring by LC-MS) acetonitrile was added and the mixture was concentrated. The residue was coevaporated several times with acetonitrile to yield the product.

5a: ^{1}H NMR (400 MHz, Deuterium Oxide) δ 7.29 (d, J = 7.6 Hz, 4H), 7.02 – 6.89 (m, 2H), 4.47 – 4.20 (m, 4H), 2.79 (s, 3H). ^{13}C NMR (101 MHz, Deuterium Oxide) δ 155.98, 126.60, 126.33, 122.05, 117.89, 116.70, 55.86, 40.81. $[M+H]^{+}$: 273.88.

5b: ^{1}H NMR (400 MHz, Methanol-d4) δ 7.66 (d, J = 2.7 Hz, 2H), 7.44 (dd, J = 8.7, 2.7 Hz, 2H), 7.14 (d, J = 8.8 Hz, 2H), 4.56 (d, J = 39.6 Hz, 4H), 3.91 (d, J = 18.7 Hz, 4H), 2.96 (s, 6H). ^{13}C NMR (101 MHz, Methanol-d4) δ 157.11, 127.59, 126.54, 122.18, 116.88, 116.66, 54.63 (d, J = 7.9 Hz), 49.91, 39.73. $[M+H]^{+}$: 331.06.

5c: ^{1}H NMR (400 MHz, Methanol-d4) δ 7.62 (d, J = 2.7 Hz, 2H), 7.43 (dd, J = 8.7, 2.7 Hz, 2H), 7.13 (d, J = 8.7 Hz, 2H), 4.49 (d, J = 67.5 Hz, 4H), 3.38 (s, 4H), 2.92 (s, 6H), 2.58 – 2.39 (m, 2H). ^{13}C NMR (101 MHz, Methanol-d4) δ 157.06, 127.44, 126.30, 122.16, 117.39, 116.52, 54.09, 52.75, 39.52, 19.27. $[M+H]^{+}$: 345.15.

2.14 IC_{50} determination

Native hAChE was incubated with several concentrations (1 -1000 µM) of non-oxime and the enzyme activity was measured using the Ellman assay. Measurements were conducted in duplicate. The IC_{50} values were calculated from semi-logarithmic plots of the compound concentration versus the hAChE activity.

2.15 Enzyme inhibition

Human erythrocyte (ghosts) were incubated with an inhibitor (paraxon, or one of the nerve agents) to achieve ≥95% of inhibition. Both the inhibited and the control samples were dialyzed overnight at 4^{o}C to remove residual inhibitor. Aliquots of inhibited enzyme were stored -80 ^{o}C.

2.16 Reactivation of OP-inhibited hAChE

OP-inhibited hAChE was incubated with several concentrations of non-oxime. The fraction of reactivated enzyme (with respect to the 100% control) was determined by taking aliquots at several time points, during a total incubation time of 60 minutes, and measure enzyme activity using the Ellman assay. Experiments were carried out in duplicate.

3. Results and Discussion

3.1. Chemistry

The synthesis routes of all compounds are depicted in Figure 3. Synthesis of methylpyrrolidine derivatives **2** (Panel A) was accomplished by treating the commercially available compound **6a** with formaldehyde and the requisite pyrrolidine (**7b** or **7c**).[19] After purification, the resulting intermediate was subjected to acid to remove the acetyl group, which gave the target compounds as the ammonium salts. In a similar sequence of reactions pyridine derivatives **3a** and **3b** were prepared (Panel E). In these cases, the commercially available *N*-(4-Hydroxyphenyl) Pivalamide **6b** was used as the starting compound. Compounds **4a-4e** (Panel B) were all accessible via Betti reactions (using formaldehyde and **7a**) on their respective 4-substituted phenol precursors (**6c-g**). The conversion of cyanide **6g** to **4e** gave a double-substituted byproduct (**4f**) that was also isolated. Compound **4e** also served as an intermediate for further derivatization (Panel D). Thus, **4e** was reduced to give aminomethyl-substituted compound **4h**, and converted to **4i** in a two-step process via **8**. Compound **4g** was (Panel C) accessible via reductive amination of dihydroxybenzaldehyde **6h** and pyrrolidine (**7a**), under the influence of $NaBH(OAc)_3$. The synthesis of *N*-Benzyl substituted compound **4j** (Panel F) proved to be more challenging. The first step entailed reductive amination of benzaldehyde with *p*-aminophenol (**6h**) giving **9**. This compound proved to be unstable, so it was used directly in subsequent reactions after its purification by

column chromatography. Direct conversion of **9** to target compound **4j** was not productive as the benzylic amine in **9** competed with pyrrolidine **7a** in the Mannich reaction. Therefore, the amine in **9** was protected with a benzyloxycarbonyl (Boc) group. Installation of the Boc group was initially attempted using Boc_2O in dichloromethane or THF, in the absence and in the presence of triethylamine. This reaction suffered from a preference for the formation of the *tert*-butoxycarbonate (Boc protection of the phenol group in **9**), leading to a mixture of *O*-*tert*-butyloxycarbonyl-4-benzylaminophenol and *O,N-di-(tert*-butyloxycarbonyl)-4-benzylaminophenol as the major products. However, a selective and quantitative conversion of **9** into the desired compound **10** could be accomplished by treatment of **9** with Boc_2O in water, with vigorous stirring.[20,21] The subsequent Betti reaction was carried out in refluxing MeOH, instead of refluxing EtOH, as the latter led to premature loss of the Boc group (thermal deprotection). Finally, Mannich product **11** was treated with HCl in MeOH to furnish the desired compound **4j**. The dimeric compounds **5a-c** (Panel G) were synthesized from **6a** using a Betti reaction in the presence of methylamine (→**5a**), or in the presence of *N*,*N*'-dimethylethylenediamine (→**5b**) or in the presence of *N*,*N*'-dimethyl-1,3-propanediamine (→ **5c**). All compounds were satisfactorily characterized with ^{1}H-NMR ,^{13}C-NMR and mass spectrometry.

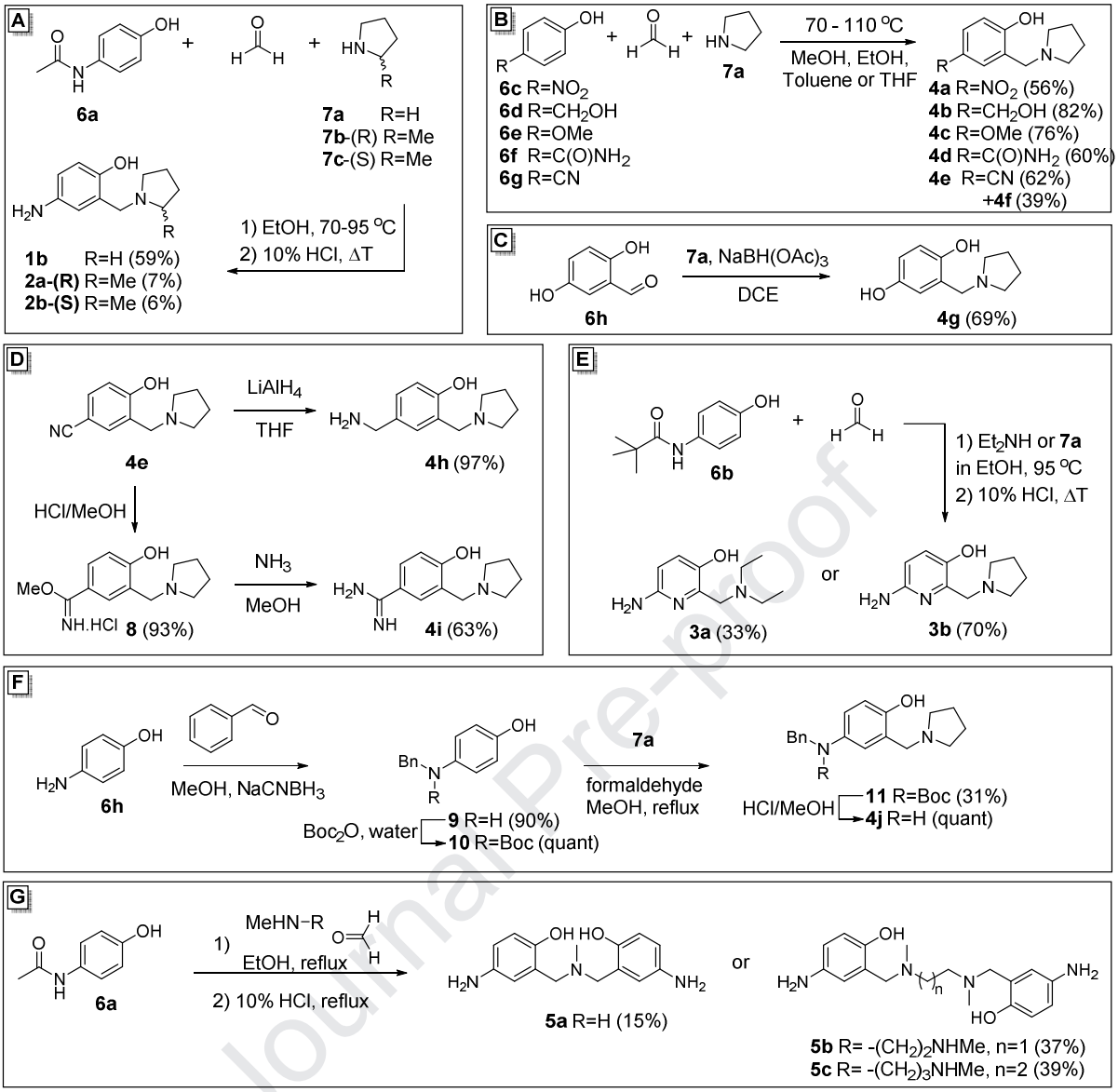

Figure 3. Synthesis routes for the various compounds

3.2. In vitro biological evaluation

The 50% inhibitory concentration (IC_{50}) was measured by exposing human erythrocyte AChE to various concentrations (1-1000 µM) of all compounds and recording the enzyme activity using the well-known Ellman assay.[22,23] Details are provided in SI, paragraph 2.6. The results are summarized in Table 1.

Table 1. The inhibition of native human AChE by non-oxime compounds was tested with 11 concentrations (1-1000 µM) in duplicate. Data are given as mean ± SD.

Compound	***IC_{50} (µM)***	***Compound***	***IC_{50} (µM)***
1b	6.3 ± 0.0 [17]	**4e**	>1000
2a	9.21 ± 0.03	**4f**	>1000
2b	42.7 ± 0.8	**4g**	19.9 ± 0.5
3a	973 ± 27	**4h**	348 ± 9
3b	135 ± 4.5	**4i**	>1000
4a	300.5 ± 14.9	**4j**	196 ± 5.3
4b	>1000	**5a**	>1000
4c	>1000	**5b**	838 ± 76
4d	>1000	**5c**	368 ± 5.1

Next we used an in vitro reactivation assay to assess the ability of all compounds to reactivate OP-inhibited hAChE.[24] To this end, human erythrocyte AChE (ghosts) was inhibited with VX, paraoxon-ethyl (PXE), tabun (GA), cyclosarin (GF) and sarin (GB) and subjected to 3-5 concentrations (10-1000 µM) of each reactivator. The enzymatic activity was measured at various time points within 60 minutes. The enzyme activity at each time point was corrected for the blank (no reactivator) and expressed as the percentage reactivation with respect to the 100% control. All experiments were carried out in duplicate. The reactivation curves of selected compounds are provided in the supporting information. Table 2 shows the percentage reactivation achieved after 60 min incubation with 10, 100 and 1000 µM

concentrations of each reactivator / OP-hAChE combination. The potencies of compounds **3a**, **4b** and **4c** were negligible and were therefore omitted from Table 2. None of the compounds reactivated GA-inhibited hAChE and therefore the column for GA is lacking in Table 2 as well. The red/italic values in Table 2 were expected to be higher (judging from reactivation percentages at lower concentrations), and were most probably attenuated by reversible inhibition or denaturation of the enzyme at higher reactivator concentrations (vide infra). Note, that cells in Table 2 were left blank when reactivation was less than 5%. Consequently, Table 2 allows for a quick but rough review of the performance of the reactivators, because compounds with a higher number of reactivation percentages (especially at lower concentrations) have, in general, a higher potency.

Table 2. Reactivation-%, after 60 min. incubation with 10, 100 and 1000 µM concentrations of reactivator of OP-inhibited hAChE

Compound	***Concentration (µM)***	***PXE***	***VX***	***GF***	***GB***
	1000	*<5%*	*<5%*	*<5%*	*<5%*
2a	100	*<5%*	38	11	22
	10	7	13		6
	1000	*21*	*16*	10	*6*
2b	100	53	62		34
	10	34	10		21
	1000	8	36	29	26
3b	100			6	

	10				
	1000			5	
4a	100				
	10				
	1000				7
4d	100				
	10				
	1000	9	5		6
4e	100				
	10				
	1000	10			
4f	100				
	10				
	1000	7	28		20
4g	100		16		10
	10				5
	1000	8	21		16
4h	100				
	10				
	1000	10			7
4i	100				
	10				
	1000	22	69	26	42
4j	100	8	52	19	10
	10				

	1000	33	73	6	12
5a	100		34	6	
	10				
	1000	13	48		17
5b	100	6	25	9	
	10		18	6	
	1000	16	26	6	6
5c	100	6		9	
	10	7		8	

Judging from the amount of numbers and their values in the corresponding columns, GF-hAChE was the most difficult to reactivate, while VX-hAChE was the easiest to reactivate. It is also evident that compounds **2**, **4j** and **5** were the most active from this series. A selection of the reactivation curves of those compounds are displayed in Figure 4, and from some of these compounds the reactivation parameters k_r (reactivation rate) and K_D (dissociation constant) were determined (Table 3). The parameters for **1b**, the most potent non-oxime reported to date, were added for comparison in Table 3. Compound **2b** appreciably reactivated VX-, PXE- and GB-inhibited hAChE. At 500 and 1000 µM concentrations the reactivation of OP-hAChE by **2a** and **2b** was attenuated, after an initial steep increase in enzyme activity, and followed by a progressing decline in activity over time. This

phenomenon is in line with previous observations for a number of PADOC derivatives[17], and was postulated to be due to a combination of reversible inhibition (compare IC_{50}'s in Table 1) and by denaturation or covalent modification[17] of the liberated AChE by the reactivator at higher reactivator concentrations (500 and 1000 µM). The possibility of re-inhibition of the enzyme by a putative OP-inhibitor adduct formed during reactivation (see the nucleophilic reactivation mechanism in Figure 1), cannot be excluded but was deemed less likely, as a synthetic PADOC-OP conjugate was not an inhibitor of hAChE and the decline in enzyme activity was also observed with native AChE incubated with **1a** or **1b** [17]. The previously observed sensitivity towards small structural changes was corroborated by the nearly 5-fold lower IC_{50} of **2a** compared to its isomer **2b,** as well as the approximately 50-fold loss of potency toward VX-AChE reactivation when compared to **1b,** which lacks the methyl substituent on the pyrrolidine ring. The pyridine-pyrrolidine compound **3b** was not very potent, but it was more potent than the diethyl substituted variant **3a**, which corresponds to the higher potency achieved by the same substitution in ADOC.[17]

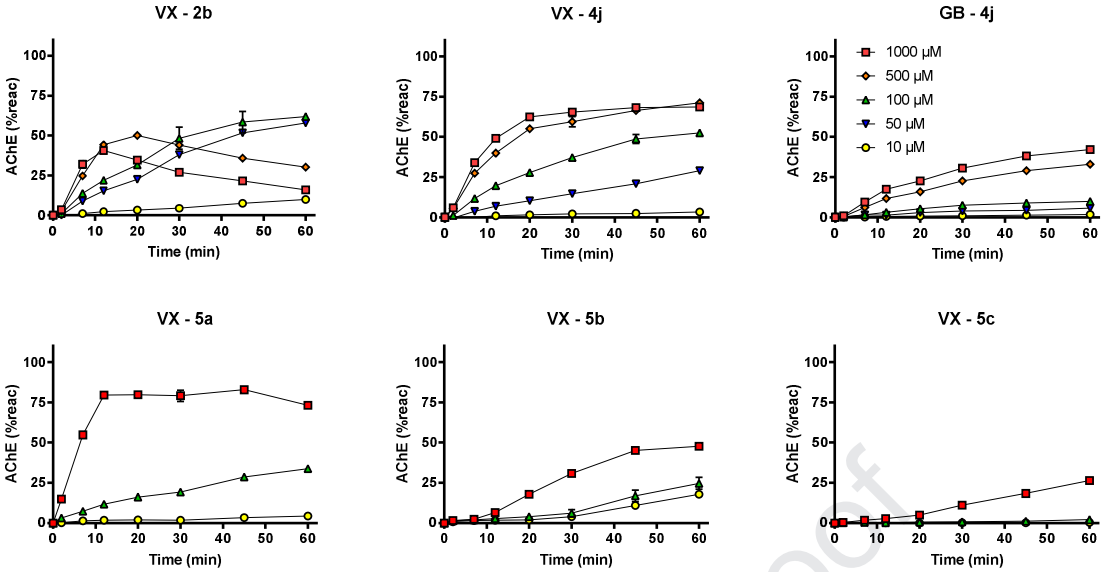

Figure 4. Selection of reactivation curves (all other curves are provided in the supporting information).

From the 4-substituted compounds **4j** had a mediocre and relatively broad spectrum activity (except for tabun) and it was the only compound from the 4-substituted series that displayed some potency, suggesting that a 4-N-alkyl substituent may be better tolerated in these designs than all other substitutions. The latter was confirmed by Cadieux and co-workers who reported that the 4-N-ethyl-derivative of **1a** (Figure 1), had a higher potency than **1a** itself (but lower than **1b**).[15] Another interesting observation was that no decline in enzymatic activity over time occurred with this compound when incubated with the enzyme at high concentrations. Whether this resulted from the structure (4-N-alkyl substituent) or simply because of a moderate affinity (IC_{50} 196 µM) of the compound for the enzyme remains to be investigated.

In light of the rather strong response of the reactivation potency towards small structural changes in the compounds reported here and in previous accounts [16,17], the observed potency of compounds **5** was rather surprising. Whereas in most cases the presence of substituents to the benzylic amine, in particular bulky ones[16,17], leads to a diminished potency, compounds **5** were actually capable of reactivating VX-inhibited hAChE. Moreover, compound **5a**, having the bulk more closely located around the central benzylic amine is more active than **5b** and **5c**, that have their substituents at more remote distances, respectively. Another peculiar observation was that the affinity for the native enzyme (IC_{50}) decreased going from **5a** to **5c** (>1000, 838 and 368 μM, respectively), while their potencies respectively decreased. Although the IC_{50} value cannot be used to predict the K_D or the overall potency, it is not uncommon that compounds that exhibit a low IC_{50} have a lower K_D as well, which increases their chances of exhibiting some reactivation potency (e.g., compare **3a**/**3b, 4b-f** (IC_{50}>1000 and no or low potency), or **1a/1b** [17]). In the case of compounds **5** this 'trend' is reversed.

Table 3. Kinetic parameters for the reactivation of OP-inhibited hAChE for some compounds and OPs.

Compound	***Agent***	***k_r (min^{-1})***	***K_D (μM)***	***kr_2 ($min^{-1}mM^{-1}$)***
1b	VX	0.93 ± 0.00	45 ± 1	20.67 ± 0.10 (ref. 18)
2a	VX	0.02 ± 0.00	41 ± 1	0.46 ± 0.02 (this work)
2b	VX	0.07 ± 0.01	209 ± 55	0.35 ± 0.06 (this work)
4j	GB	0.04 ± 0.00	1164 ± 129	0.03 ± 0.00 (this work)
4j	VX	0.09 ± 0.00	516 ± 11	0.17 ± 0.01 (this work)

4. Conclusion

The goal of this work was to further explore the chemical space for Mannich phenol based non-oximes in relation to their reactivation potency of nerve agent inhibited hAChE. To this end two isomers of 2-methylpyrrolidine derivatives of **1b** (→**2a/2b**), two pyridine derivatives of **1a** and **1b** (→**3a/3b**), nine 4-substituted derivatives of **1b** (→**4a-j**) and three dimeric Mannich phenols (**5a-c**) were synthesized. These 17 compounds were subjected to in vitro assays to determine their inhibitory potencies (IC_{50}) towards native hAChE and their reactivation potencies towards a number of OP-inhibited hAChE. Although none of the compounds had a potency that exceeded that of the lead non-oxime **1b**, some interesting features were revealed, that may guide future design of novel non-oximes. For instance, the 4-*N*-benzylamine substituted compound **4j** showed reasonable and relatively broad spectrum reactivation potency, without denaturation of the enzyme, and this result motivates us to further explore other *N*-alkylamine substituents at the 4-position. The Mannich phenol dimers **5**, in particular **5a**, also showed some potency for reactivation. These compounds are trend breaking, considering that the introduction of bulky substituents on the Mannich phenol system usually leads to diminished reactivation potency. Secondly, the correlation of higher potency with lower IC_{50} is usually not observed. Future quantum mechanics calculations and molecular modeling (QM/MM) may provide clues to explain these observations and aid the design of future dimers.

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Highlights

- Synthesis of new Mannich phenol (non-oxime) derivatives
- New 4-N-alkylamine derivatives of PADOC show reactivation potential
- Mannich phenol dimers show surprising reactivation potential
- The results point at potential new avenues for design of non-oximes

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: