University of Hradec Králové

Faculty of Science

Department of Chemistry

New hybrid acetylcholinesterase reactivators derived from 2-pyridinealdoxime and pyridostigmine.

Bachelor Thesis

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Declaration

„I declare that this Bachelor Thesis was composed by myself, that the work contained herein is my own except where explicitly stated otherwise in the text, and that this work has not been submitted for any other degree or professional qualification except as specified.“

Date: Signature:

Acknowledgement

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Anotace

Hlavním cílem této bakalářské práce je syntéza nových hybridních reaktivátorů acetylcholinesterasy na bázi 2-pyridinaldoximu a pyridostigminu a jejich následné biologické hodnocení *in vitro*. Reaktivace acetylcholinesterasy je klíčovým krokem v terapii otrav organofosforovými sloučeninami. Teoretická část této bakalářské práce obsahuje obecné informace o funkci acetylcholinesterasy v organismu, její inhibici a následné reaktivaci. Experimentální část by se dala rozdělit na dvě části – syntetickou a biologickou. Syntetická část popisuje přípravu uvedených sloučenin lišících se délkou spojovacího řetězce (linkeru) a funkčními skupinami, zatímco biologická část odhaluje, zda tyto sloučeniny jsou účinnými reaktivátory acetylcholinesterasy či nikoliv.

Klíčová slova

Acetylcholinesterasa, organofosforová sloučenina, reaktivátor, inhibice, reaktivace

Annotation

The main goal of this Bachelor Thesis is synthesis of novel hybrid acetylcholinesterase reactivators derived from 2-pyridinealdoxime and pyridostigmine as well as their *in vitro* evaluation. Acetylcholine reactivation is a crucial step in therapy of organophosphorous compound intoxication. The theoretical part of this Thesis includes general information about acetylcholinesterase function in a living organism, its inhibition and reactivation. The experimental part could be divided into two parts – synthetic and biological. The synthetic part describes preparation of mentioned compounds varying in the linker length and main functional groups, whereas the biological part reveals whether these compounds are potent reactivators or not.

Keywords

Acetylcholinesterase, organophosphorus compound, reactivator, inhibition, reactivation

List of abbreviations

*h*AChE	human acetylcholinesterase
ACh	acetylcholine
AChR	acetylcholine receptor
BBB	blood-brain barrier
*h*BChE	human butyrylcholinesterase
DMF	dimethylformamide
DMSO	dimethylsulfoxide
mAChR	muscarinic acetylcholine receptor
nAChR	nicotinic acetylcholine receptor
NA	nerve agent
OC	organophosphorus compound
TEA	triethylamine
THF	tetrahydrofuran
TLC	thin layer chromatography

Table of contents

1. THEORETICAL PART

1.1 INTRODUCTION

Organophosphorus compounds (OC) belong among the most toxic substances known nowadays. The toxicity of these agents is defined by irreversible inhibition of acetylcholinesterase (AChE), in particular by phosphorylation of serine hydroxyl group constituting the active site of the enzyme. Poisoning by such substances can lead to many health issues and possibly to death when not treated properly in time.

Nerve agents (NA), representing a significant group of OC, can be divided into two main classes. The former class of NA, designated as G-agents, includes sarin, soman, tabun, and cyclosarin (Fig.1). [1,2,3,4,5]

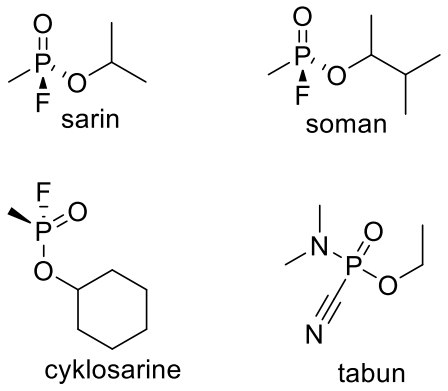


Figure 1. Chemical structure of G-agents

The second class of NA, is denoted as V-agents, contain substances such as VX, CVX (Chinese VX) and RVX (Russian analogue of VX) (Fig.2).

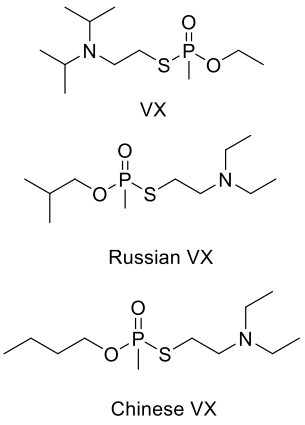


Figure 2. Chemical structures of V-agents

By comparing these two classes together, due to lower volatility and higher toxicity, V-agents are considered to be more toxic to human beings than G-agents. On the other hand, the risk associated mainly with the respiratory toxicity of G-agents should not be neglected.[6]

The former aim of organophosphorus research was the design of novel potent pesticides. However, shortly after the discovery of the toxicity of these compounds, such research redirected to the military sector, in particular to chemical warfare agents. Nerve agents were firstly synthesized by German chemist Gerhard Schrader in the 1930‘s under the Nazi regime. The first agent discovered was tabun (Fig.1), followed by sarin (Fig.1), soman (Fig.1) and finally cyclosarin (Fig.). Nevertheless, none of these substances was used during the World War II. The first documented use of NA was executed many years after their discovery, during the Gulf War in 1990 – 1991. In spite of the lack of bulletproof evidence, misuse of NA has been widely discussed over the last two decades during continuing military conflicts in the Middle East. Nowadays, OC mostly serve to socially acceptable purpose as pesticides, that protect crops from harmful factors enhancing thus the harvest production. Among frequently applied organophosphorus pesticides belong parathion, malathion, chlorpyrifos, diazinon or dichlorvos (Fig.3). However, organophosphorus pesticides still exert the same mode of actions as NA being thus very dangerous when ingested or manipulated inappropriately. [2,3,7]

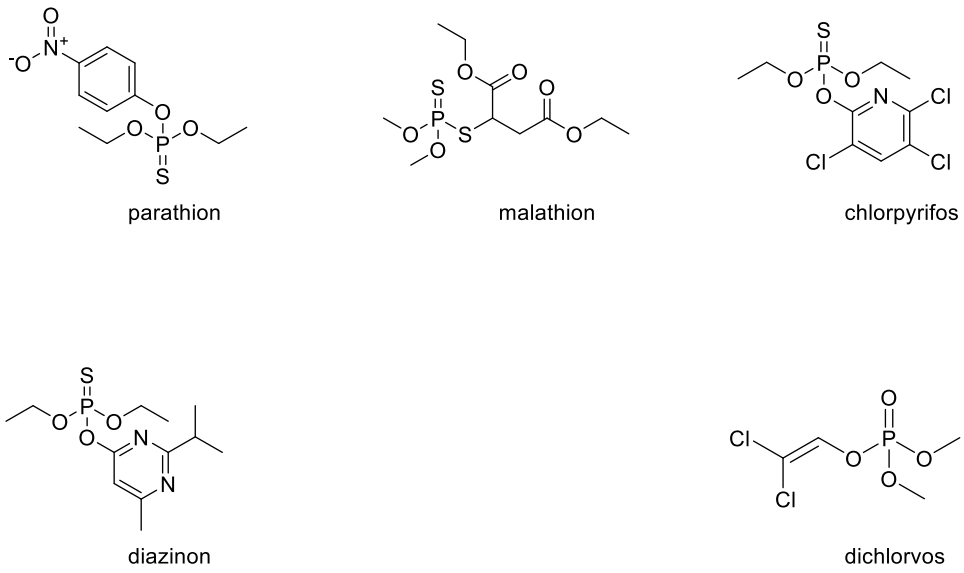


Figure 3. Chemical structures of frequently applied organophosphorous pesticides

1.2 CHEMICAL-PHYSICAL PROPERTIES OF NERVE AGENTS

Chemical-physical properties of G-agents and V-agents significantly differ from each other thus causing different toxic effect on target organisms.

G-agents:

Sarin (2-[fluoro(methyl)phosphoryl]oxypropane; GB; (Fig.1) is colourless liquid without any odour in a pure state or with very weak fruity odour. The molecular weight of sarin is 140.094 g/mol, the boiling point is 147°C, whereas the melting point is -57°C. Sarin is miscible with water, however, making it unstable in the presence of water. The density of sarin is 1.0887 g/mL at 25°C. Its partition coefficient (log $K_{o/w}$) is -1.4, making sarin rather hydrophilic than lipophilic. Vapour density is 4.86 (referred to air = 1), while volatility has been determined to be 22 000 mg/m^3 at 25°C. Sarin can be fully thermally decomposed after 2.5 hours at 150°C by forming a mixture of phosphorus compounds and propylene. Incurred fumes are considered very toxic.[8]

Soman (3-[fluoro(methyl)phosphoryl]oxy-2,2-dimethylbutane; GD; (Fig.1) is colourless liquid of fruity odour in a pure state or camphor-like odour if it contains impurities. Molecular weight of soman is 182.175 g/mol, the boiling point is 198°C and the melting point is -42°C. Comparing to sarin, soman is less miscible with water, being more miscible with non-polar solvents. The density of soman is 1.0222 g/mL at 25°C. Its partition coefficient is 1.78, pointing out to higher lipophilicity. Vapour density is 6.3, while volatility has been determined to be 3 900 mg/m^3 at 25°C. Thermally decomposed soman emits toxic phosphorus oxides.[9]

Tabun ([dimethylamino(ethoxy)phosphoryl]formonitrile; GA; (Fig.1) has been described as colourless to dark amber liquid with weak fruity odour in smaller concentrations and fish odour in larger concentrations. Molecular weight of tabun is 162.129 g/mol, the boiling point is 240°C and the melting point is -50°C. Tabun is soluble in water as well as in organic solvents. The density of tabun is 1.077 g/mL at 25°C. Its partition coefficient is 0.29, making tabun rather hydrophilic than lipophilic. Vapour density is 5.3, while volatility has been determined to be 490 mg/m^3 at 25°C. When being thermally decomposed, tabun emits toxic fumes of phosphorus oxides.[10]

Generally, using G-agents as chemical weapons, absorption by lungs is a typical route of administration.[8,9,10]

V-agents:

VX (*N*-[2-[ethoxy(methyl)phosphoryl]sulfanylethyl]-*N*-propan-2-ylpropan-2-amine; (Fig. 2) is colourless or amber coloured liquid with no odour. Molecular weight of VX is 267.368 g/mol, the boiling point is 298°C and the melting point is -51°C. VX is soluble in water as well as in organic solvents. The density of agent VX is 1.008 g/mL at 20°C. Vapour density is 9.2. Its partition coefficient is 2.09, making VX more lipophilic. Volatility has been determined to be 10.5 mg/m^3 at 25°C.[11]

Shortly after VX discovery, two structural isomers of it were developed during the Cold War, sharing similar toxic effects on living organisms as VX.

Russian VX (*N*,*N*-diethyl-2-[methyl(2-methylpropoxy)phosphoryl]sulfanylethanamine; VR, RVX; (Fig. 2) was developed by the former Soviet Union in 1963. Due to the lack of analytical data on VR, only predicted data are available. Molecular weight of VR is 267.37 g/mol, the boiling point is 329.06°C and the melting point is -55°C. Russian VX ought to be more soluble in water than VX. The density has been estimated to be 1.0 g/mL at 25°C. Its partition coefficient is supposed to be 2.14, making VR slightly more lipophilic. Appearance, as well as volatility, is considered to be similar to VX. Comparing VR and VX, VR has been recognized as more prone to decomposition than VX, probably due to the presence of diethylamino group instead of diisopropylamino group present in VX.[12]

Chinese VX (*O*-butyl *S*-[2-(diethylamino)ethyl] methylphosphothionate; CVX; (Fig.2) is a Chinese version imitating VX. Similarly as in the case of VR, only predicted data have been generated. From these data, the only accessible information about CVX is its molecular weight of 267.37 g/mol.[13]

Generally, using V-agents as chemical weapons, absorption by skin is a typical route of administration.

1.3 MODE OF ACTION OF ORGANOPHOSPHORUS COMPOUNDS

1.3.1 ACETYLCHOLINE

Acetylcholine (ACh; Fig 1.4) is a major transmitter liable for the process of neural transmission in the synaptic cleft. ACh is present in all autonomic ganglia, autonomically innervated organs, neuromuscular junctions, and multiple synapses of the central nervous system. Its physiological and pharmacological effects have been properly investigated and documented in many living species, including humans. Synthesis of ACh is a one-step process catalysed by the enzyme choline acetyltransferase from the choline and acetyl coenzyme A (acetyl-CoA). Synthesized ACh is secreted into the synaptic cleft where it binds to acetylcholine receptors (AChR). The interaction between AChR and ACh causes changes in receptor conformation, leading to the bilateral release of cations (K^+, Ca^{2+}), thus generating an electric potential. The electric potential is afterwards transduced to another neuron.[14]

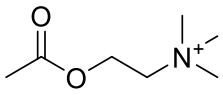


Figure 4. Acetylcholine molecule

1.3.2 ACETYLCHOLINE RECEPTORS

There are two types of AChR: muscarinic receptors and nicotinic receptors.

Nicotinic acetylcholine receptors (nAChRs) are ionotropic receptors found in autonomic ganglia, neuromuscular junctions, adrenal medulla and partially in the central nervous system. Nicotinic receptors gained their name because of their main agonist, nicotine (Fig.5), naturally occurring in tobacco plant - *Nicotiana tabacum*. The structure of nAChRs consists of five subunits symmetrically arranged around a central pore. Activation of nicotinic receptors leads to muscular fasciculation and tremors, enhances alertness, causes mydriasis and hypertension.[14,15,16]

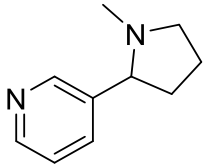


Figure 5. Chemical structure of nAChR agonist - nicotine

Muscarinic acetylcholine receptors (mAChRs) are receptors found in the central nervous system and organs innervated by cholinergic system. Muscarinic receptors were named after their agonist muskarine (Fig.6) – a mycotoxin naturally occurring in a mushroom *Amanita muscaria.* In contrast with nAChRs, mAChRs belong among the receptors bounded to G-protein, which initiates cascade of various events within the targeted cell. Activated mAChRs result in for example, bradycardia, salivation, and miosis.[17]

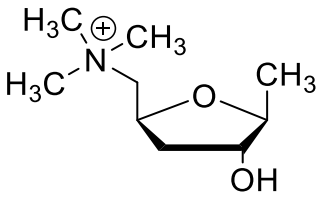


Figure 6. Chemical structure of mAChR agonist - muskarine

1.3.3 CHOLINESTERASES

The main function of cholinesterases is the hydrolysis of choline esters released into synaptic cleft and thereby terminating the intracellular communication pathway, in our case, cholinergic signalling pathway. There are two types of cholinesterases acetylcholinesterase (AChE, EC 3.1.1.7; Fig.7) and butyrylcholinesterase (BChE, EC 3.1.1.8), both belonging to hydrolases due to their biochemical properties. AChE and BChE can exist in various molecular forms, although AChE manifests in more varieties than BChE. The particular difference between these two enzymes is their tissue specificity. AChE is distributed in all cholinergic sites, red blood cells, and grey matter. In contrast, BChE can be found primarily in plasma, liver, intestines and white matter. The main biological role of AChE is to break down the molecules of ACh to acetate and choline, whereas BChE participates in detoxification of naturally occurring ester compounds, such as cocaine, physostigmine, however, it‘s physiological role remains still not completely clear.[5,14,18,19]

The active site of AChE consists of two parts – the peripheral anionic site and the esteratic site. In human beings, the esteratic site includes three amino acid residues – Ser203, Glu334 and His447, each located in the centre of a 20Å deep gorge of AChE, lined by aromatic residues. The peripheral anionic site is situated

in the rim of the gorge and contains mainly aromatic residues, such as tyrosine (Tyr72, Tyr124, Tyr341), tryptophane (Trp286) and aspartic acid (Asp74).[20,21]

Figure 7. Perspective view of folded AChE model

The first step of ACh hydrolysis affords an intermediate of ACh and the serine residue of AChE. Afterwards, free choline is released. Within the second step acetylated AChE is attacked by a molecule of water which facilitates acetic acid molecule formation. Finally, choline is reuptaken to the presynaptic neuron and participates in the synthesis of new ACh molecule. The whole process of ACh hydrolysis is illustrated in Figure 8.[22,23]

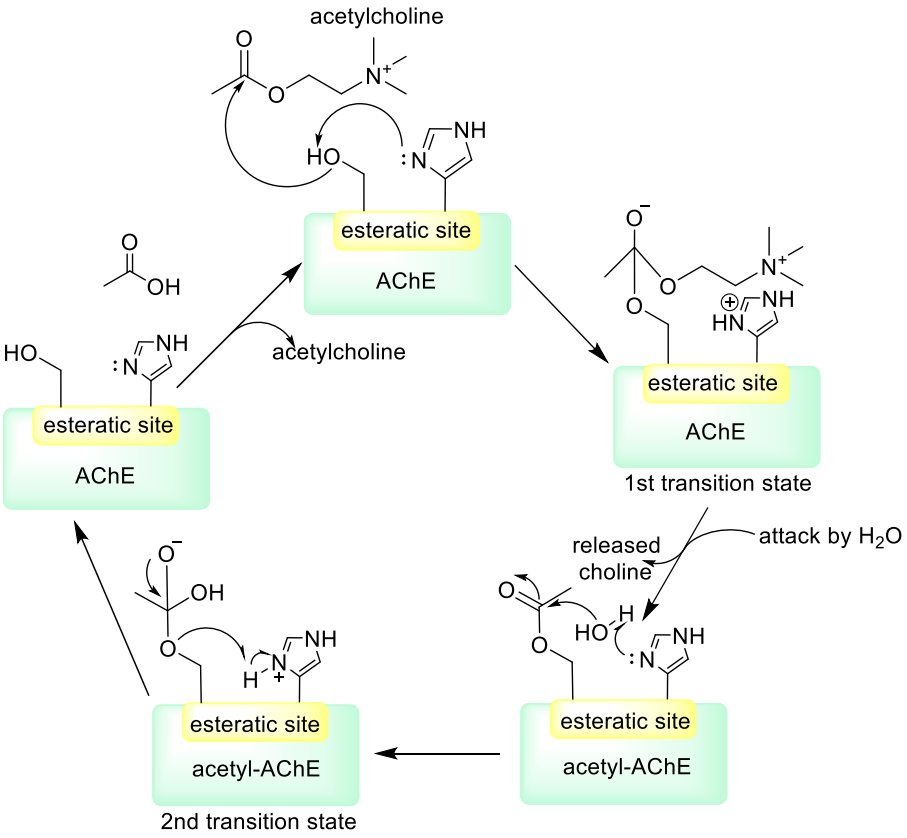


Figure 8. Mechanism of ACh hydrolysis by AChE

1.3.4 INHIBITION OF ACETYLCHOLINESTERASE

AChE can be inhibited by various chemical substances, such as OPs (synthetic, irreversible) or physostigmine (naturally occurring, reversible, Fig.9), causing the disruption of ChE catalytic activity and its biological function. According to the mode of action, AChE inhibitors can be divided into two main groups, reversible and irreversible.[20,24,25]

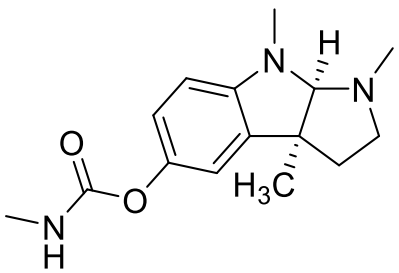


Figure 9. Molecule of physostigmine

Reversible inhibitors include various compounds with different functional groups and have been successfully applied as a treatment of multiple diseases such as Alzheimer's disease, myasthenia gravis, glaucoma and also as an antidote to anticholinergic overdose. [24,26,27]

Irreversible inhibition is mainly caused by OCs. The principal toxicological effect of OCs is irreversible phosphorylation of AChE that could lead to an acute cholinergic crisis. Likewise a natural substrate – ACh, OCs can be attacked by catalytic serine of AChE with an emergence of covalent bond between the hydroxym group of serine and phosphorus atom of OC. After enzyme phosphorylation, there are two scenario that can happen. Firstly, dephosphorylation may occur. Dephosphorylation of the enzyme is very slow (it can even last for days) and thus phosphorylated enzyme remains biologically inactive and cannot hydrolyse the neurotransmitter. Such situation leads to ACh aggregation in the synaptic cleft which may subsequently result in overstimulation of muscarinic and nicotinic ACh receptors and finally disruption of cholinergic neurotransmission. Most of the OCs display the inhibition mechanism similar to ACh hydrolysis. After OC enters the AChE catalytic active site, phosphorus atom of OC is nucleophilicly attacked by serine. This reaction results in formation of bipyramidal transition state which is followed by production of phosphorylated serine and release of halide ion. In the next step, histidine residue is incapable to activate water molecule which causes noticeably slow spontaneous hydrolysis.[28,29,30]

The second scenario that may occur is known as „ageing“. Mechanism of ageing is characterised by yielding phosphonate adducts yielded by dealkylation from previously formed intermediate (Fig. 10). Such phosphonate adduct is specified as „aged“ conjugate and may create the ionic bond with protonated histidine in the catalytic site of AChE. A newly formed ionic bond is highly stable and blocks the enzyme for desirable hydrolysis and reactivation by antidotes. Ageing half- time is a characteristic parameter of each organophosphorus compound, being very short for soman (2-4 minutes), 5 hours for sarin, 46 hours for tabun and almost 48 hours for VX.[30,31,32]

The possible fatality of OC poisoning is therefore also depending on the length of the ageing process.

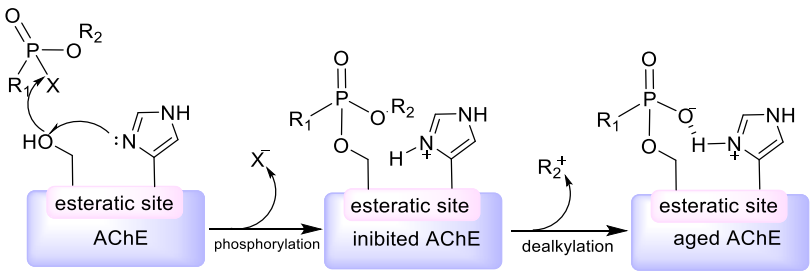


Figure 10. Inhibition and ageing proces of AChE caused by organophosphorus compounds

1.4 CLINICAL PRESENTATION OF NERVE AGENT POISONING

To understand the origin of clinical manifestations of NA poisoning it is essential to know the basic mechanism of its partial components such as - function of ACh as a neurotransmitter, binding of ACh to muscarinic or nicotine receptors and their activation, regulation of ACh via AChE, mode of action of nerve agent and possible use of AChE reactivators.

Clinical manifestation of NA poisonings can be divided into three main groups: muscarinic effects, nicotine effects, and central nervous system effects.[33]

Muscarinic effects are associated with the activation of muscarinic receptors by excessive ACh. Depending on a location of mAChRs, the symptoms of intoxication could be divided into several subgroups:

Ophthalmic – conjunctivitis, lacrimation, miosis, blurred vision, bad visual acuity, loss of dark adaptation and ocular pain exacerbated by accommodation.
Respiratory - watery rhinorrhea, stridor, wheezing, cough, increased bronchial secretion, chest tightness, dyspnoea, apnoea, and cyanosis.
Cardiovascular – bradyarrhythmia, prolongation of PR interval, atrioventricular blocks, and hypotension
Dermal – such as flushing and sweating
Gastrointestinal – salivation, nausea, vomiting, diarrhoea, abdominal pain and cramping, tenesmus
Genitourinary – incontinence and uncontrolled defecation
[3,33,34]

Nicotinic effects are analogously associated with the activation of nAChRs by acetylcholine.
Such effects may occur in various systems of the human body that are listed below:
Cardiovascular: tachycardia, hypertension
Striated muscle: fasciculations, twitching, cramping, weakness, paralysis
Metabolic: hyperglycemia, metabolic acidosis, ketosis, leukocytosis, and hypokalemia

Central nervous system effects, as the name suggests, are related to the central nervous system activity.
Such effects include: anxiety, restlessness, insomnia, depression, confusion, ataxia, convulsions, central respiratory centre depression, coma, areflexia and tremors.

The amount of NA used and the route of exposure are factors responsible for the seriousness of NA poisoning and its toxic effects on the organism.

There are two main routes of exposure to a NA:
1) droplets absorbed by skin tissue and
2) inhalation of NA vapours
[3,33,34]

1.5 THERAPY OF ORGANOPHOSPHATE POISONING

1.5.1 STANDARD THERAPY OF ORGANOPHOSPHATE POISONING

Targeted standard therapy of organophosphate poisoning includes three steps. Firstly, atropine (Fig. 11) has to be applied v. or i.m because of its parasympatholytic effects. The main function of atropine is to antagonize the OC's cholinergic effect on muscarinic receptors. To restrain the convulsions arisen from the poisoning, diazepam (Fig. 12) has to be applied intramuscularly. The administration of diazepam induces allosteric modification of GABA receptors causing higher activity of chloride channels and thus hyperpolarization of membranes and termination of the neural impulse. Finally,the last component of the standard therapy i represented by AChE reactivators that restore the activity of affected enzyme. [35,36,37,38]

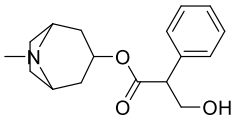


Figure 11. Chemical structure of atropine

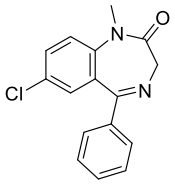


Figure 12. Molecule of diazepam

1.5.2 ACETYLCHOLINESTERASE REACTIVATORS

AChE activity can be restored in three possible ways – by synthesis of AChE *de novo*, by spontaneous reactivation or by induced reactivation. Synthesis of AChE *de novo* is a time-consuming process which usually lasts from fifty to one hundred days and thus it is unsuitable for immediate therapy. Another possibility is spontaneous reactivation has been described in Chapter 1.3.4 in detail. This process is still not very efficient due to it‘s duration time.[39]

The third way of AChE reactivation is the application of AChE reactivators – oximes. Such reactivator should be a potent nucleophile and also should be able to break the bond of covalently bonded inhibitor. Consequently, the enzyme obtains its normal biological function. This type of reactivation is denoted as induced reactivation.[40,41]

1.5.3 STRUCTURAL ASPECTS OF AChE REACTIVATORS

For successful reactivation proces four main characteristics are fundamental. The first requirement is the presence of quaternary nitrogen in the molecule of the reactivator. Bearing this quarternary nitrogen in the molecule enables the oxime to enter the active site of AChE. The number of such atoms determines whether the molecule is monoquarternary or bisquarternary. The second requirement is the length and the type of the linker between two nitrogen atoms. According to previous investigations, the optimal linker length for sarin, tabun, and VX inhibited AChE

was estimated to be three or four carbon atoms. On the other hand, reactivation of cyclosarine inhibited AChE with methoxime (having only one carbon atom linker) showed better effectiveness than reactivation with longer linker reactivators.[20,42,43]

The common sign of all reactivators is the presence of an oxime group (R- CH=NOH), whose nucleophilic properties enable the reactivation process. The last crucial requirement is the position of an oxime functional group within the aromatic system. The strongest reactivation effect show molecules where an oxime group in position 2 or 4, mainly because of its mesomeric effects. Aditionally, it was found, that presence of the second aldoxime functional group is not substantial for succesful reactivation, but it certainly enhances the affinity of the molecule to inhibited AChe.[44,30]

Another important aspect of potent AChE reactivators is dissociation constant - pK_a. For successful dephosphorylation and reactivation the enzyme, a disociated oximate anion is required. However, for proper penetration through biological barriers, especially across the blood-brain barrier (BBB) uncharged undisociated molecule is essential. For this reason, the pK_a value of reactivators should range from 7.04 to 8.2 when the percentage of oximate anion and its undisociated form is approximately fifty fifty.[30]

1.5.4 THE REACTIVATION PROCESS

The reactivation of inhibited enzyme is composed of two steps (Fig. 13). In the first step, the oximate anion attacks the phosphorus atom of the OC-enzyme complex by forming a reversible complex. In the second step, the oxime-phosphorus complex originates and the enzyme is reactivated. To this date, a variety of oximes have been designed and synthesized, but none of them is universal and effective in treating the poisoning by every OC. In addition to this fact, the successful reactivation is only possible when the OC-enzyme complex is not „aged“.[18,30,44,45]

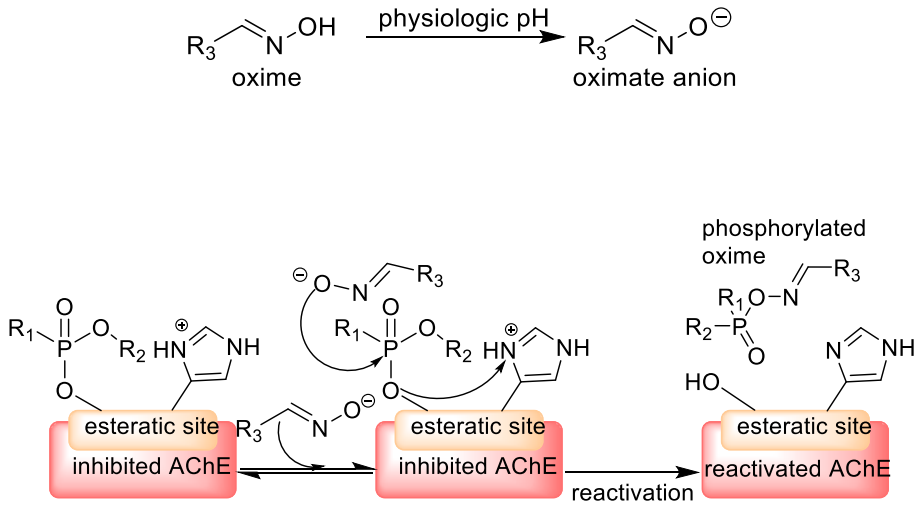


Figure 13. Reactivation process

1.5.5 COMMERCIALLY AVAILABLE REACTIVATORS

The most commonly used AChE reactivators nowadays are pralidoxime (2-PAM), methoxime, obidoxime, trimedoxime and HI-6. Structures of these compounds are listed below (Fig 14.). These reactivators were synthesized in order to serve as OC antidotes. Although these compounds are the only ones that are approved, they possess two main drawbacks, i.e. 1) lack of universality and 2) low ability to cross the blood-brain barrier. Therefore current research is targeted to create novel reactivators lacking those drawbacks.[46,47]

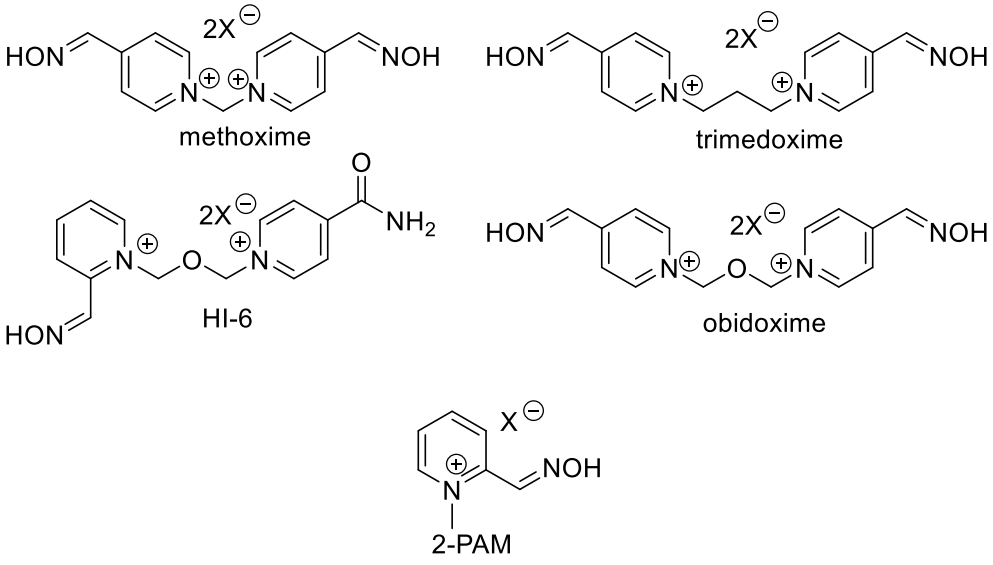


Figure 14. Comercially available AChE reactivators

The standard form of reactivators is water soluble salt (halide, alkylsulphate, etc.) that is usually applied as an intramuscular injection. To reach the effect, the concentration of the reactivator has to be around 4 μg/ml. The therapeutic dose is then determined to milligrams per kilogram. Common obidoxime dosage is 5mg/kg, that reaches its effect in 5-10 minutes and lasts for 120-180 minutes. Common pralidoxime dosage is 10 mg/kg that, reaches the effect in 5-10 minutes and lasts less than obidoxime, approximately 50-55 minutes. The standard dose of HI-6 is 200 or 500mg for i.m. administration and the desired plasma concentration is reached within 4-6 minutes. The effect lasts for 125 minutes for 200mg dosage and 250 minutes for 500mg. The oximes are excreted by urine by 99 % and the duration of excretion varies for each oxime.[48,49,50]

1.5.6 EFFECTIVITY OF REACTIVATORS

The effectivity of novel reactivator is generally dependent on affinity to inhibited AChE. The affinity is characterised by a dissociation constant of complex enzyme-reactivator K_{dis}. The K_{dis} constant is higher when the enzyme-reactivator affinity is lower and is dependent on multiple factors, such as structure and size of the molecule, it's functional groups, steric compatibility, electrostatic effects and hydrophobic interactions. [24,27,51]

The therapy by currently used oxime reactivators is still insufficient, lacking three main aspects. There is no universal reactivator to each type of OC. The next issue is that commercially available reactivators are quite hydrophobic thus these compounds do not provide sufficient concentration in the brain. For example, pralidoxime penetrates the blood-brain barrier only in 10 %, other bisquarternary reactivators only in 1-3%. The last significant drawback is that no available reactivator is able to reactivate aged enzyme. All these mentioned shortcomings, therefore, lead to further research in this field and synthesis of novel oximes lacking those drawbacks, which are also the main goal of this thesis.[24,27,30,43]

2. AIMS OF THE THESIS

Within this Bachelor Thesis several goals have been determined:

1) Synthesis of new hybrid acetylcholinesterase reactivators derived from 2-pyridinealdoxime and pyridostigmine.

2) Biological evaluation of newly synthesized compounds **6** – **13**. In our case, determination of inhibition potential and reactivation ability of the oximes had to be performed.

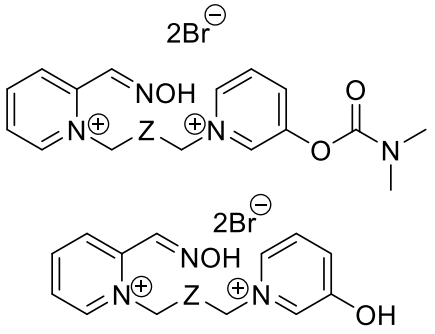
3) Based on obtained results, structure-activity relationship had to be deduced.

3. EXPERIMENTAL PART

3.1 DESIGN

The design of newly synthesized compounds was based on the structures of 2-pyridinealdoxime and pyridostigmine. 2-Pyridinealdoxime is a key reactivating moiety of two mostly used commercially available reactivators – HI-6 and pralidoxime. It´s significant reactivating properties could be explained by appropriate value of pK_a, since the percentage of occurance of oximate and oxime in such molecules is almost fifty fifty. For importance of pK_a value (see Chapter 1.5.3.) Pyridostigmine belongs among the representatives of prophylactic tools used in case of OC intoxications. The mode of action of pyridostigmine consists in occupation of the active site of AChE prior to intoxication. Thereafter, when an OC enters the body, it could not bind to the active site serine and thus could not evoke harmful toxic effects. Such action of pyridostigmine is mediated by the presence of carbamate moiety which reversibly carbamoylates the active site serine. Therefore, after some time such complex dissociates and the function of AChE is restored.[52]

Within our study, we have prepared two subgroups of compounds (Fig. 16). The first subfamily bears carbamate moiety. However, in particular, in this group an undesirable inhibiting activity of reactivators towards AChE could be observed. For this reason, we have decided to prepare similar subgroup, however, with the presence of hydroxyl group instead of carbamate to observe the effect of these two groups on AChE inhbition. Apart from the presence of hydroxy/carbamate group, newly prepared compounds differ in their linker length. For this purpose, we have decided to use from 3-carbon to 6-carbon linkers. Resulted structure-activity relationship study towards reactivation and inhibition is described further.



Z = 1-4 methylene groups

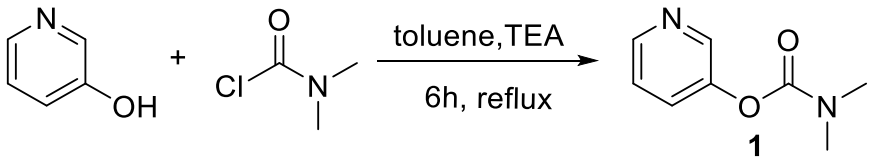
Figure 45. General structures of two subgroups of final compounds

3.2 GENERAL CHEMICAL METHODS

All the chemical reagents used were purchased from Sigma-Aldrich (Czech Republic). The course of the reactions was monitored by thin layer chromatography (TLC) on aluminium plates precoated with silica gel 60 F254 or cellulose (Merck, Czech Republic) and then visualized by UV 254. Mobile phases used for TLC were as follows: buthanol/H_2O/acetic acid (5:2:1) for cellulose and CH_2Cl_2/methanol/aqueous solution of NH_3 (33%) (9:1:0.2) for silica gel. Melting points were determined on a melting point apparatus M-565 (Büchi, Switzerland) and are uncorrected. Uncalibrated purity was ascertained by LC-UV using a reverse phase C18 chromatographic column. All the biologically tested compounds exhibited purity 95 – 99 % at a wavelength 254 nm. NMR spectra of target compounds were recorded on Varian S500 spectrometer (operating at 500 MHz for ^{1}H and 126 MHz for ^{13}C; Varian Comp. Palo Alto, USA). Chemical shifts are reported in parts per million (ppm). Spin multiplicities are given as s (singlet), br s (broad singlet), d (doublet), dd (doublet of doublets), t (triplet), or m (multiplet). The coupling constants (*J*) are reported in Hertz (Hz). Mass spectra (m/z) were determined by spectrometer Agilent 6470 (QQQ) (Santa Clara, USA).

3.3 SYNTHESIS OF INTERMEDIATES

PYRIDIN-3-YL DIMETHYLCARBAMATE (1)



1

Appearance: pale yellow oil

Molecular weight: 166.16

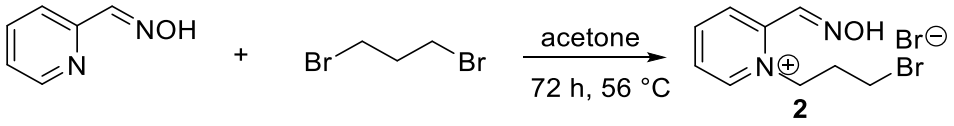
Procedure: Into a two-necked round bottom flask was added 3-hydroxypyridine (1.00 g, 10.52 mmol), triethylamine (1.77 mL, 12.70 mmol) and toluene (25 mL). Subsequently, dimethylcarbamyl chloride (1.77 mL, 19.22 mmol) was added dropwise. The mixture was refluxed for 6 hours under inert atmosphere. After cooling to room temperature, excessive toluene was removed. Finally, crude product was purified by colon chromatography using silicagel and ethyl acetate/methanol (9:1) as a mobile phase. Concentration in vacuo afforded key intermediate (1.71 g)

Yield: 98 %

^{1}H NMR (500 MHz, DMSO-d_6) δ 8.42 (dd, J = 4.7, 1.4 Hz, 1H), 8.40 (d, J = 2.7 Hz, 1H), 7.62 – 7.58 (m, 1H), 7.46 – 7.41 (m, 1H), 3.06 (s, 3H), 2.92 (s, 3H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 153.75, 148.16, 146.35, 143.69, 129.80, 124.26, 36.56, 36.31.

m/z: found for $[C_8H_{11}N_2O_2]^+$ 167.1; calculated for $[C_8H_{11}N_2O_2]^+$ 167.08

1-(3-BROMOPROPYL)-2-((HYDROXYIMINO)METHYL)PYRIDINIUM BROMIDE (2)



2

Appearance: gray solid

Molecular weight: 324.01

Melting point: 155 – 157°C

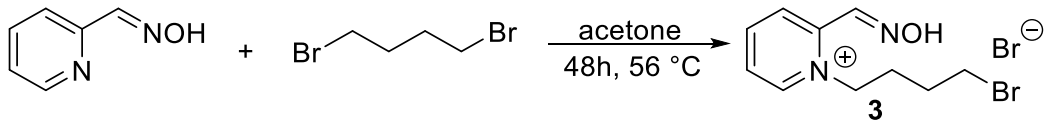
Procedure: 2-Pyridinealdoxime (10.00 g, 81.89 mmol) was dissolved in acetone (80 mL). Thereafter, 1,3-dibromopropane (41.56 mL, 409.43 mmol) was added. The mixture was stirred and refluxed for 72 hours at 56 °C under inert conditions. Subsequently, the mixture was filtered and washed with warm acetone (50 mL). The solid product (1.06 g) did not need any further purification.

Yield: 4 %

^{1}H NMR (500 MHz, DMSO-d_6) δ 13.15 (s, 1H), 9.09 (dd, J = 6.3, 1.4 Hz, 1H), 8.78 (s, 1H), 8.60 – 8.54 (m, 1H), 8.40 (dd, J = 8.2, 1.5 Hz, 1H), 8.14 – 8.08 (m, 1H), 4.89 – 4.77 (m, 2H), 3.64 (t, J = 6.6 Hz, 2H), 2.44 – 2.35 (m, 2H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 147.24, 146.34, 145.59, 141.60, 127.72, 126.13, 56.81, 33.10, 30.25.

m/z: found for $[C_9H_{12}BrN_2O]^+$ 245.3; calculated for $[C_9H_{12}BrN_2O]^+$ 245.01

1-(4-BROMBUTYL)-2-((HYDROXYIMINO)METHYL)PYRIDINIUM BROMIDE (3)



Appearance: gray solid

Molecular weight: 338.04

Melting point: 184 - 186°C

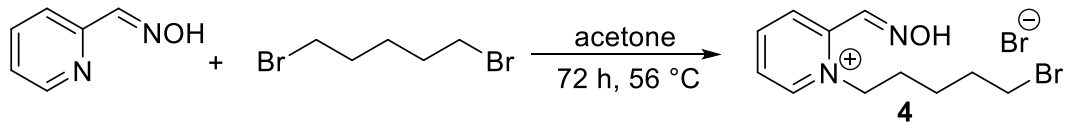
Procedure: 2-Pyridinealdoxime (10.00 g, 81.89 mmol) was dissolved in acetone (80 mL). Thereafter, 1,4-dibromobutane (48.89 mL, 409.43 mmol) was added. The mixture was stirred and refluxed for 48 hours at 56 °C under inert conditions. After cooling to room temperature, the mixture was partially dried out on the rotavapor and put into the fridge for 48 hours. Formed solid was filtered and washed with diethyl ether. Thin layer chromatography (TLC) showed the residues of 2-pyridinealdoxime. Therefore, tetrahydrofuran (THF) was added to remove those residues and the mixture was stirred for 10 minutes. Finally, the solid was dissolved in a small amount of acetonitrile and precipitated by diethyl ether to gain desired intermediate (1.38 g).

Yield: 5 %

^{1}H NMR (500 MHz, DMSO-d_6) δ 13.13 (s, 1H), 9.11 (dd, J = 6.3, 1.4 Hz, 1H), 8.81 (s, 1H), 8.59 – 8.52 (m, 1H), 8.40 (dd, J = 8.2, 1.5 Hz, 1H), 8.13 – 8.07 (m, 1H), 4.80 (t, J = 7.0 Hz, 2H), 3.57 (t, J = 6.1 Hz, 2H), 2.01 – 1.79 (m, 4H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 147.07, 146.08, 145.45, 141.62, 127.71, 126.05, 57.15, 34.28, 29.27, 28.83.

m/z: found for $[C_{10}H_{14}BrN_2O]^+$ 259.2; calculated for $[C_{10}H_{14}BrN_2O]^+$ 259.03

1-(5-BROMOPENTYL)-2-((HYDROXYIMINO)METHYL)PYRIDINIUM BROMIDE (4)



4

Appearance: white solid

Molecular weight: 352.07

Melting point: 121 – 123°C

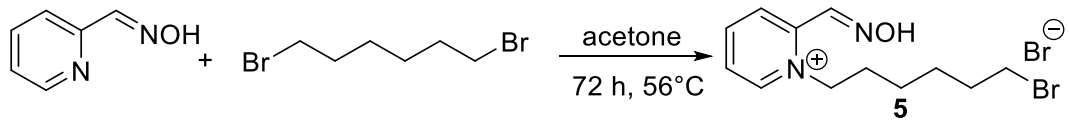
Procedure: 2-Pyridinealdoxime (10.00 g, 81.89 mmol) was dissolved in acetone (80 mL). Thereafter, 1,5-dibromopentane (55.77 mL, 409.43 mmol) was added. The mixture was stirred and refluxed for 72 hours at 56 °C under inert conditions. After cooling to room temperature, the flask with the reaction mixture was put into the fridge for 24 hours. Afterwards, it was filtered and washed with acetonitrile (40 mL) to remove the residues of 2-pyridinealdoxime. Obtained product was dried on the rotavapor and then put into the desiccator for further drying (1.33 g).

Yield: 5 %

^{1}H NMR (500 MHz, DMSO-d_6) δ 13.12 (s, 1H), 9.11 (dd, J = 6.2, 1.4 Hz, 1H), 8.80 (s, 1H), 8.60 – 8.52 (m, 1H), 8.40 (dd, J = 8.3, 1.5 Hz, 1H), 8.15 – 8.08 (m, 1H), 4.76 (t, J = 7.7 Hz, 2H), 3.53 (t, J = 6.6 Hz, 2H), 1.89 – 1.76 (m, 4H), 1.50 – 1.40 (m, 2H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 146.99, 146.16, 145.40, 141.66, 127.66, 126.06, 57.77, 34.98, 31.63, 29.59, 24.22.

m/z: found for $[C_{11}H_{16}BrN_2O]^+$ 273.3; calculated for $[C_{11}H_{16}BrN_2O]^+$ 273.04

1-(6-BROMOHEXYL)-2-((HYDROXYIMINO)METHYL)PYRIDINIUM BROMIDE (5)



Appearance: white solid

Molecular weight: 366.09

Melting point: 134 – 135°C

Procedure: 2-Pyridinealdoxime (10.00 g, 81.89 mmol) was dissolved in acetone (80 mL). Thereafter, 1,6-dibromohexane (62.98 mL, 409.43 mmol) was added. The mixture was stirred and refluxed for 72 hours at 56 °C under inert conditions. After cooling to room temperature, the flask with the reaction mixture was put into the fridge for 24 hours. Afterwards, it was filtered. The solid phase was suspended in acetone (60 mL) and the mixture was left to stir for 20 minutes. The suspension was filtered again. The solid phase was put into a round bottom flask and, afterwards, a diethyl ether (80 mL) was added into it. Formed mixture was stirred firmly for 10 minutes at room temperature. Finally, the product was filtered and washed with diethyl ether to remove the residues of 2-pyridinealdoxime and to obtain desired intermediate (1.50 g).

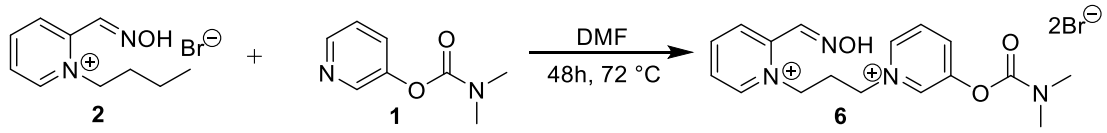
Yield: 5 %

^{1}H NMR (500 MHz, DMSO-d_6) δ 13.11 (s, 1H), 9.11 (dd, J = 6.3, 1.4 Hz, 1H), 8.79 (s, 1H), 8.59 – 8.50 (m, 1H), 8.39 (dd, J = 8.2, 1.5 Hz, 1H), 8.14 – 8.07 (m, 1H), 4.79 – 4.68 (m, 2H), 3.51 (t, J = 6.7 Hz, 2H), 1.86 – 1.72 (m, 4H), 1.46 – 1.29 (m, 4H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 146.95, 146.16, 145.37, 141.68, 127.66, 126.07, 57.95, 35.16, 32.06, 30.33, 27.08, 24.63.

m/z: found for $[C_{12}H_{18}BrN_2O]^+$ 287.3; calculated for $[C_{12}H_{18}BrN_2O]^+$ 287.06

3.4 SYNTHESIS OF THE FINAL PRODUCTS

1-(3-((DIMETHYLCARBAMOYL)OXY)PYRIDIN-1-IUM-1-YL)PROPYL)-2-((HYDROXYIMINO)METHYL)PYRIDIN-1-IUM BROMIDE (6)



Appearance: dark brown solid

Molecular weight: 490.19

Melting point: 46 – 47°C

Procedure: Intermediate **2** (0.50 g, 1.54 mmol), intermediate **1** (0.38 g, 2.31 mmol) and dimethylformamide (DMF, 10 mL) were purged into a round bottom flask. The mixture was stirred for 48 hours at 72°C. After cooling to room temeperature, acetone (100 mL) was added for precipitation, which lasted for 15 minutes, stirred continuously at room temperature. This step was repeated three times with a decantation step in between each step. Filtered solid was several times evaporated with toluene to remove the traces of DMF. Finally, due to the presence of impurities, small amount (3 mL) of acetonitrile was added and the suspension was stirred for 15 minutes at room temperature. Filtration of the solid gave the final product (70 mg).

Yield: 9 %

^{1}H NMR (500 MHz, DMSO-d_6) δ 13.23 (s, 1H), 9.28 – 9.25 (m, 1H), 9.08 (dd, *J* = 6.3, 1.4 Hz, 1H), 9.06 – 9.00 (m, 1H), 8.83 (s, 1H), 8.61 – 8.51 (m, 2H), 8.45 (dd, *J* = 8.2, 1.6 Hz, 1H), 8.29 – 8.22 (m, 1H), 8.16 – 8.11 (m, 1H), 4.86 (t, *J* = 7.6 Hz, 2H), 4.80 (t, *J* = 7.5 Hz, 2H), 3.10 (s, 3H), 2.97 (s, 3H), 2.62 – 2.53 (m, 2H). ^{13}C NMR (126 MHz, CD_3OD) δ 151.12, 145.90, 145.45, 141.40, 140.76, 139.40, 139.23, 129.83, 128.52, 128.18, 127.42, 126.82, 58.14, 54.98, 35.93, 35.59, 31.64.

m/z: found for $[C_{17}H_{22}N_4O_3]^{2+}$ 329.3; calculated for $[C_{17}H_{22}N_4O_3]^{2+}$ 329.17

2-((HYDROXYIMINO)METHYL)-1-(3-(3-HYDROXYPYRIDIN-1-IUM-1- YL)PROPYL)PYRIDIN-1-IUM BROMIDE (7)

Appearance: pale gray solid

Molecular weight: 419.12

Melting point: 131 – 133°C

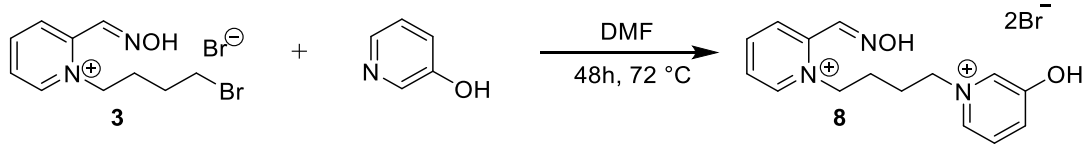
Procedure: 3-Hydroxypyridine (0.21 g, 2.21 mmol), intermediate **2** (0.50 g, 1.54 mmol) and DMF (10 mL) were purged into a round bottom flask. The mixture was stirred for 48 hours at 72°C. After cooling to room temeperature, acetone (100 mL) was added for precipitation, which lasted for 15 minutes, stirred continuously. This step was repeated three times with a decantation step in between each step. Filtered solid was several times evaporated with toluene to remove the traces of DMF. Thereafter, acetonitrile (100 mL) was added to the product and the mixture was stirred for 30 minutes at 80°C. Hot mixture was filtered and washed with a little amount of methanol. Filtered solid was, finally, suspended in acetone (100 mL) and stirred for another 24 hours to afford the final product (230 mg).

Yield: 36%

^{1}H NMR (500 MHz, DMSO-d_6) δ 13.18 (s, 1H), 11.93 (bs, 1H), 9.15 (d, J = 6.2 Hz, 1H), 8.84 (s, 1H), 8.67 (d, J = 2.3 Hz, 1H), 8.64 (d, J = 5.5 Hz, 1H), 8.60 – 8.53 (m, 1H), 8.43 (dd, J = 8.3, 1.5 Hz, 1H), 8.17 – 8.09 (m, 1H), 8.03 – 7.94 (m, 2H), 4.86 (t, J = 7.8 Hz, 2H), 4.76 (t, J = 7.6 Hz, 2H), 2.59 – 2.51 (m, 2H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 157.15, 147.40, 146.08, 145.62, 141.56, 135.92, 133.31, 131.76, 128.87, 127.71, 125.95, 57.58, 54.75, 31.74.

m/z: found for $[C_{14}H_{17}N_3O_2]^{2+}$ 258.4; calculated for $[C_{14}H_{17}N_3O_2]^{2+}$ 258.13

2-((HYDROXYIMINO)METHYL)-1-(4-(3-HYDROXYPYRIDIN-1-IUM-1- YL)BUTYL)PYRIDIN-1-IUM BROMIDE (8)



Appearance: brown solid

Molecular weight: 433.14

Melting point: 97 – 99°C

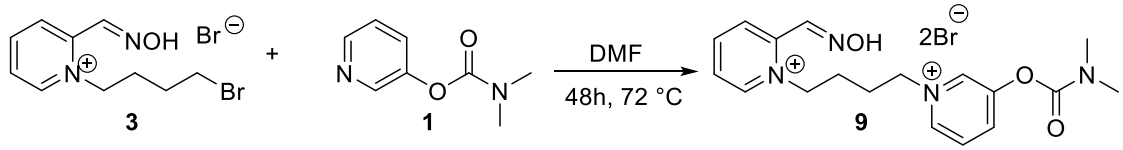
Procedure: 3-Hydroxypyridine (0.21 g, 2.22 mmol), intermediate **3** (0.50 g, 1.48 mmol) and DMF (10 mL) were purged into a round bottom flask. The mixture was stirred for 48 hours at 72°C. After cooling to room temeperature, acetone (100 mL) was added for precipitation, which lasted for 15 minutes, stirred continuously. This step was repeated three times with a decantation step in between each step. Filtered solid was several times evaporated with toluene to remove the traces of DMF. Crude product was suspended in acetone (80 mL) and stirred overnight at room temperature to gain the final product (224 mg).

Yield: 35 %

^{1}H NMR (500 MHz, DMSO-d_6) δ 13.14 (s, 1H), 11.87 (bs, 1H), 9.13 (dd, *J* = 6.4, 1.4 Hz, 1H), 8.79 (s, 1H), 8.65 (d, *J* = 1.8 Hz, 1H), 8.61 (d, *J* = 5.7 Hz, 1H), 8.59 – 8.51 (m, 1H), 8.41 (dd, *J* = 8.2, 1.6 Hz, 1H), 8.14 – 8.07 (m, 1H), 8.03 – 7.92 (m, 2H), 4.81 (t, *J* = 7.5 Hz, 2H), 4.62 (t, *J* = 7.2 Hz, 2H), 2.06 – 1.95 (m, 2H), 1.87 – 1.78 (m, 2H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 157.06, 147.04, 146.12, 145.45, 141.58, 135.83, 133.11, 131.54, 128.73, 127.67, 126.01, 59.98, 57.19, 27.34, 26.91.

m/z: found for $[C_{15}H_{19}N_3O_2]^{2+}$ 272.4; calculated for $[C_{15}H_{19}N_3O_2]^{2+}$ 272.15

1-(4-(3-((DIMETHYLCARBAMOYL)OXY)PYRIDIN-1-IUM-1-YL)BUTYL)-2-((HYDROXYIMINO)METHYL)PYRIDIN-1-IUM BROMIDE (9)



Appearance: gray solid

Molecular weight: 504.22

Melting point: 166 – 167°C

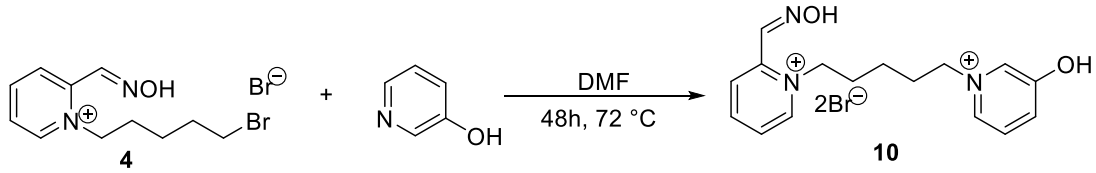
Procedure: Intermediate **3** (0.64 g, 1.89 mmol), intermediate **1** (0.47 g, 2.84 mmol) and dimethylformamide (DMF, 10 mL) were purged into a round bottom flask. The mixture was stirred for 48 hours at 72°C. After cooling to room temeperature, acetone (100 mL) was added for precipitation, which lasted for 15 minutes, stirred continuously at room temperature. This step was repeated three times with a decantation step in between each step. Filtration of the solid gave the final product (23 mg).

Yield: 2 %

^{1}H NMR (500 MHz, DMSO-d_6) δ 13.17 (s, 1H), 9.30 – 9.26 (m, 1H), 9.09 (dd, J = 6.4, 1.4 Hz, 1H), 9.03 (dd, J = 5.9, 1.6 Hz, 1H), 8.79 (s, 1H), 8.58 – 8.52 (m, 2H), 8.41 (dd, J = 8.2, 1.5 Hz, 1H), 8.26 – 8.19 (m, 1H), 8.14 – 8.08 (m, 1H), 4.79 (t, J = 7.6 Hz, 2H), 4.70 (t, J = 7.2 Hz, 2H), 3.10 (s, 3H), 2.97 (s, 3H), 2.09 – 2.00 (m, 2H), 1.90 – 1.79 (m, 2H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 152.22, 150.16, 147.06, 146.14, 145.46, 141.93, 141.57, 139.46, 139.24, 128.55, 127.68, 126.03, 60.39, 57.18, 36.82, 36.44, 27.25, 26.85.

m/z: found for $[C_{18}H_{24}N_4O_3]^{2+}$ 343.2; calculated for $[C_{18}H_{24}N_4O_3]^{2+}$ 343.18

2-((HYDROXYIMINO)METHYL)-1-(5-(3-HYDROXYPYRIDIN-1-IUM-1- YL)PENTYL)PYRIDIN-1-IUM BROMIDE (10)



Appearance: beige solid

Molecular weight: 447.16

Melting point: 111 – 113°C

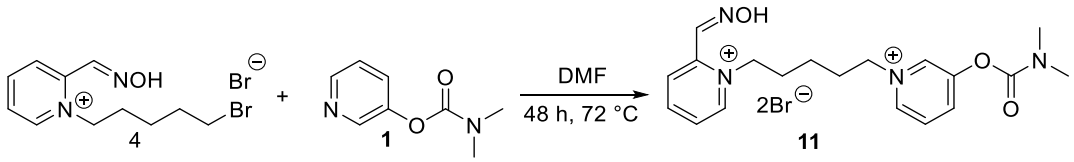
Procedure: 3-Hydroxypyridine (0.20 g, 2.13 mmol), intermediate **4** (0.50 g, 1.42 mmol) and DMF (10 mL) were purged into a round bottom flask. The mixture was stirred for 48 hours at 72°C. After cooling to room temeperature, acetone (100 mL) was added for precipitation, which lasted for 15 minutes, stirred continuously. This step was repeated three times with a decantation step in between each step. Filtered solid was several times evaporated with toluene to remove the traces of DMF. Crude product was suspended in acetone (80 mL) and stirred overnight at room temperature to gain the final product (195 mg).

Yield: 31 %

^{1}H NMR (500 MHz, DMSO-d_6) δ 13.13 (s, 1H), 11.86 (bs, 1H), 9.16 (dd, J = 6.4, 1.4 Hz, 1H), 8.79 (s, 1H), 8.70 – 8.66 (m, 1H), 8.63 (d, J = 5.7 Hz, 1H), 8.59 – 8.54 (m, 1H), 8.40 (dd, J = 8.2, 1.6 Hz, 1H), 8.15 – 8.08 (m, 1H), 8.02 – 7.93 (m, 2H), 4.76 (t, J = 7.7 Hz, 2H), 4.56 (t, J = 7.3 Hz, 2H), 2.01 – 1.89 (m, 2H), 1.89 – 1.79 (m, 2H), 1.44 – 1.26 (m, 2H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 157.05, 146.97, 146.17, 145.42, 141.62, 135.86, 133.12, 131.44, 128.70, 127.66, 126.03, 60.40, 57.58, 30.15, 29.79, 22.05.

m/z: found for $[C_{16}H_{21}N_3O_2]^{2+}$ 286.4; calculated for $[C_{16}H_{21}N_3O_2]^{2+}$ 286.16

1-(5-(3-((DIMETHYLCARBAMOYL)OXY)PYRIDIN-1-IUM-1-YL)PENTYL)-2-((HYDROXYIMINO)METHYL)PYRIDIN-1-IUM BROMIDE (11)



Appearance: dark yellow oil

Molecular weight: 518.29

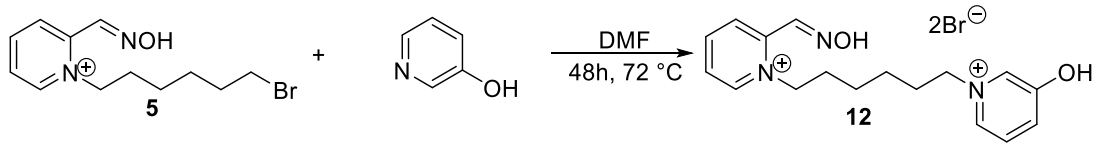
Procedure: Intermediate **4** (0.83 g, 2.36 mmol), intermediate **1** (0.59 g, 3.54 mmol) and dimethylformamide (DMF, 10 mL) were purged into a round bottom flask. The mixture was stirred for 48 hours at 72°C. After cooling to room temeperature, acetone (100 mL) was added for precipitation, which lasted for 15 minutes, stirred continuously at room temperature. This step was repeated three times with a decantation step in between each step. Filtered solid was several times evaporated with toluene to remove the traces of DMF. Due to the presence of impurities, crude product was suspended in acetone (80 mL) and stirred overnight at 56°C to gain the final product (65 mg).

Yield: 5 %

^{1}H NMR (500 MHz, DMSO-d_6) δ 13.25 (s, 1H), 9.20 – 9.16 (m, 1H), 9.01 (dd, *J* = 6.7, 1.5 Hz, 1H), 8.79 (dd, *J* = 5.7, 1.4 Hz, 1H), 8.74 (s, 1H), 8.18 – 8.12 (m, 2H), 8.01 (dd, *J* = 7.9, 1.6 Hz, 1H), 7.76 – 7.69 (m, 1H), 7.54 – 7.48 (m, 1H), 4.59 (t, *J* = 7.5 Hz, 2H), 4.33 (t, *J* = 7.4 Hz, 2H), 3.10 (s, 3H), 2.97 (s, 3H), 2.05 – 2.00 (m, 2H), 1.70 – 1.69 (m, 4H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 156.20, 154.86, 149.04, 147.12, 145.16, 140.93, 140.37, 138.76, 138.34, 125.44, 124.23, 124.03, 61.49, 55.78, 35.12, 35.10, 29.64, 27.55, 26.15.

m/z: found for $[C_{19}H_{26}N_4O_3]^{2+}$ 357.3; calculated for $[C_{19}H_{26}N_4O_3]^{2+}$ 357.20

2-((HYDROXYIMINO)METHYL)-1-(6-(3-HYDROXYPYRIDIN-1-IUM-1- YL)HEXYL)PYRIDIN-1-IUM BROMIDE (12)



Appearance: beige solid

Molecular weight: 461.19

Melting point: 125 – 127°C

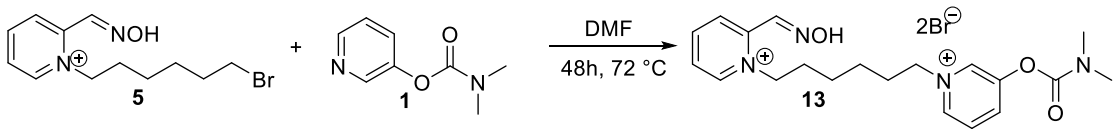
Procedure: 3-Hydroxypyridine (0.12 g, 1.23 mmol), intermediate **5** (0.30 g, 0.82 mmol) and DMF (10 mL) were purged into a round bottom flask. The mixture was stirred for 48 hours at 72°C. After cooling to room temeperature, acetone (100 mL) was added for precipitation, which lasted for 15 minutes, stirred continuously. This step was repeated three times with a decantation step in between each step. Filtered solid was several times evaporated with toluene to remove the traces of DMF and to obtain the final product (111 mg).

Yield: 29 %

^{1}H NMR (500 MHz, DMSO-d_6) δ 13.11 (s, 1H), 11.88 (bs, 1H), 9.15 (dd, J = 6.3, 1.4 Hz, 1H), 8.79 (s, 1H), 8.67 – 8.63 (m, 1H), 8.63 – 8.59 (m, 1H), 8.59 – 8.51 (m, 1H), 8.39 (dd, J = 8.2, 1.5 Hz, 1H), 8.16 – 8.07 (m, 1H), 8.00 – 7.87 (m, 2H), 4.76 (t, J = 7.7 Hz, 2H), 4.55 (t, J = 7.4 Hz, 2H), 1.92 – 1.83 (m, 2H), 1.83 – 1.74 (m, 2H), 1.41 – 1.34 (m, 2H), 1.33 – 1.24 (m, 2H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 157.25, 146.93, 146.15, 145.38, 141.65, 135.59, 133.07, 131.41, 128.64, 127.64, 126.05, 60.61, 57.84, 30.51, 30.20, 24.92, 24.86.

m/z: found for $[C_{17}H_{23}N_3O_2]^{2+}$ 300.3; calculated for $[C_{17}H_{23}N_3O_2]^{2+}$ 300.18

1-(6-(3-((DIMETHYLCARBAMOYL)OXY)PYRIDIN-1-IUM-1-YL)HEXYL)-2-((HYDROXYIMINO)METHYL)PYRIDIN-1-IUM BROMIDE (13)



Appearance: pale yellow oil

Molecular weight: 532.28

Procedure: Intermediate **5** (0.60 g, 1.64 mmol), intermediate **1** (0.41 g, 2.46 mmol) and dimethylformamide (DMF, 10 mL) were purged into a round bottom flask. The mixture was stirred for 48 hours at 72°C. After cooling to room temeperature, acetone (100 mL) was added for precipitation, which lasted for 15 minutes, stirred continuously at room temperature. This step was repeated three times with a decantation step in between each step. Filtered solid was several times evaporated with toluene to remove the traces of DMF. Due to the presence of impurities, crude product was suspended in acetone (80 mL) and stirred overnight at 56°C to gain the final product (40 mg).

Yield: 5 %

^{1}H NMR (500 MHz, DMSO-d_6) δ 13.20 (s, 1H), 9.30 – 9.22 (m, 1H), 9.15 – 9.08 (m, 1H), 9.03 (d, J = 6.0 Hz, 1H), 8.74 (s, 1H), 8.61 – 8.54 (m, 1H), 8.52 (dd, J = 8.4, 2.2 Hz, 1H), 8.48 (dd, J = 8.2, 1.7 Hz, 1H), 8.19 (dd, J = 8.7, 5.9 Hz, 1H), 8.11 – 8.05 (m, 1H), 4.85 (t, J = 7.8 Hz, 2H), 4.81 – 4.74 (m, 2H), 3.20 (s, 3H), 3.06 (s, 3H), 2.16 – 2.08 (m, 2H), 2.06 – 1.95 (m, 2H), 1.62 – 1.46 (m, 4H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 152.58, 150.92, 147.55, 145.84, 145.07, 141.23, 140.73, 139.36, 138.76, 128.36, 127.33, 126.45, 61.74, 58.45, 36.05, 35.73, 30.61, 30.16, 24.98, 24.94.

m/z: found for $[C_{20}H_{28}N_4O_3]^{2+}$ 371.4; calculated for $[C_{20}H_{28}N_4O_3]^{2+}$ 371.2

3.5 INHIBITORY ACTIVITY OF NEWLY SYNTHESIZED REACTIVATORS

To evaluate the inhibition constants of prepared oximes, Ellman's assay was used.[14,23,41] This spectrophotometric method was found to be effective even for low concentrations of enzyme.[17] Ellman's assay consists of two reactions, where in the first reaction cholinesterase (AChE or BChE) hydrolyzes the thioesther group in acetylthiocholine to an intermediate – thiocholine (Fig. 16). Within the second reaction, the thiocholine molecule reacts with 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) to form a yellow product 2-nitro-thiobenzoic acid (TNB) (Fig. 17). The intensity of the yellow colour corresponds to the amount of hydrolysed thiocholine. This method is not expensive or time consuming, making it suitable for laboratory use. Despite the advantages of this method, there are also few disadvantages, such as the sensitivity of DTNB to light exposure, reaction with sulfhydryl groups in biological samples and instability of reagent solution over longer time period.[57]

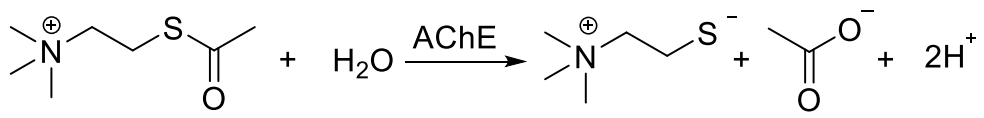


Figure 16. Acetylthiocholine hydrolysis

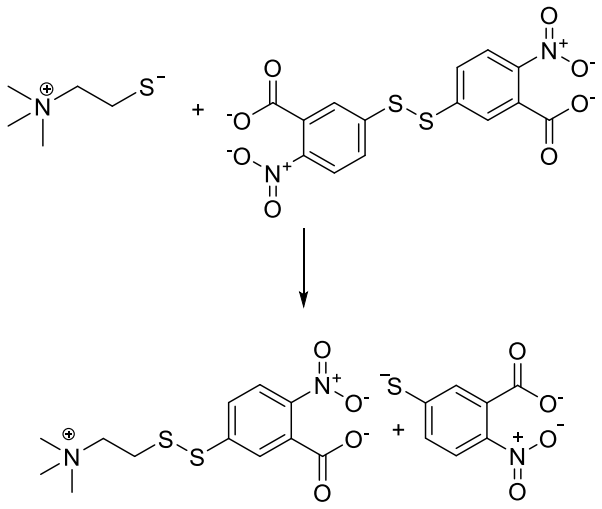


Figure 17. Formation of yellow coloured 5-thio-2-nitrobenzoic acid

3.5.1 MEASUREMENT OF THE INHIBITION CONSTANT:

The enzyme was diluted in 0.01% BSA buffer (for ATCh) or 0.1m phosphate buffer (for BTCh). The final content of the kyvette used for the measurement of the inhibition constant consisted of 0.3 mM of DTNB, 0.01% BSA buffer, 0.1 – 100 μM of tested oxime, diluted cholinesterase and 0.1 – 0.7 mM of ATCh. The experiment was carried out on the Tecan Infinite M200PRO plate reader. To determine oxime inhibition constant, oxime concentrations were prepared to gain 20 – 80% inhibition compared to the control activity (without the oxime). [53,54,55]

The inhibition constant (K_i; the concentration of an oxime which inhibits 50 % of the enzyme activity) was calculated from formulae as follows:

$$\%_{act} = \frac{I_{act} - NE_{act}}{\mathrm{K}} \,.\, 100$$

$$K_i = \frac{\%_{act} \,.\, [I]}{100 - \%_{act}}$$

where $\%_{act}$ is a percentage of enzyme residual activity, I_{act} corresponds to the measured absorbance in enzymatic reaction, while NE_{act} corresponds to the measured absorbance in non-enzymatic reaction, K_i is the inhibition constant and $[I]$ is the concentration of the inhibitor.

For the purpose of our study, three standard reactivators have been selected – pralidoxime, HI-6, and trimedoxime (Table 1). In Table 2 are listed the results obtained for our novel reactivators **6 - 13**.

Table 1. Inhibition constant (K_i) of three standard reactivators towards human AChE (*h*AChE) and human BChE (*h*BChE).

	***h*AChE**	***h*BChE**	**Ratio**
Compound	K_i **± SD** (μM)	K_i **± SD** (μM)	(*h*AChE/*h*BChE)
* **pralidoxime**	176.10 ± 10.33	392.20 ± 47.90	**0.45**
* **HI-6**	24.90 ± 1.407	215.20 ± 10.59	**0.12**
trimedoxime	** 180.00 ± 10.00	*** 190.00 ± 40.00	**0.94**

[53], SD – standard deviation

Table 2. Inhibition constant (K_i) of novel reactivators towards *h*AChE and *h*BChE.

	***h*AChE**	***h*BChE**	**Ratio**
Compound	**K_i ± SD** (μM)	**K_i ± SD** (μM)	(*h*AChE/*h*BChE)
6	3.25 ± 0.31	0.45 ± 0.08	**7.2**
7	6.81 ± 1.05	1.58 ± 0.24	**4.3**
8	14.18 ± 1.23	71.45 ± 23.14	**0.19**
9	4.42 ± 0.61	10.24 ± 0.76	**0.43**
10	13.79 ± 2.48	91.55 ± 12.78	**0.15**
11	1.32 ± 0.09	2.94 ± 1.45	**0.45**
12	2.97 ± 0.18	25.39 ± 1.69	**0.12**
13	0.32 ± 0.05	7.86 ± 1.03	**0.04**

3.6 MEASUREMENT OF THE REACTIVATION

For the measurement of reactivating capability of novel oximes, different organophosphorus inhibitors were used (sarin, VX, tabun, cyclosarin) in 10^{-4} M concentration. The rest of the content in the kyvette consisted of 0.3 mM of DTNB, 0.01% BSA buffer, 0.01 – 0.1 mM of tested oxime, diluted cholinesterase and 1 mM of acetylthiocholine. At the beginning of the experiment, recombinant human AChE and purified human plasma BChE were incubated with a certain OC up to 1 hour. The concentration of AChE in the reaction mixture was 0.016 μM. The attained inhibition of the enzymes by OC was 95 – 100 %. The inhibited enzyme was purified at a C18-E column (Phenomenex, Torrance, CA, USA), to remove the residues of free OC. Filtered enzyme was incubated with 0.1 mM of tested oxime and after certain period of time (2-240 minutes) an aliquot was taken and diluted in the kyvette with DTNB. The restored enzyme activity was measured by an addition of acetylthiocholine (1 mM) according to the Ellman's assay. The experiment was carried out on spectrophotometer CARY 300 with a temperature

control set at 25°C. The absorbance was measured at 412 nm in each case. The percentage of reactivation was calculated from the formula as follows:

$$\%react = \frac{A_{samp.}}{A_c}$$

where A_{samp} corresponds to the absorbance of reactivated enzyme in certain time and A_c is the absorbance of inhibited enzyme without oxime.

From the tested series, only compounds **7**, **8**, **10** and **12** were forwarded for evaluation of their reactivating properties, since substances **6**, **9**, **11** and **13** exerted high rate of inhibition of *h*ACHE with the percentage that increased with time. The results listed in Figure 19 sum up the reactivation properties of the AChE inhibited by various OCs after 30 minutes and reactivated by novel oximes.

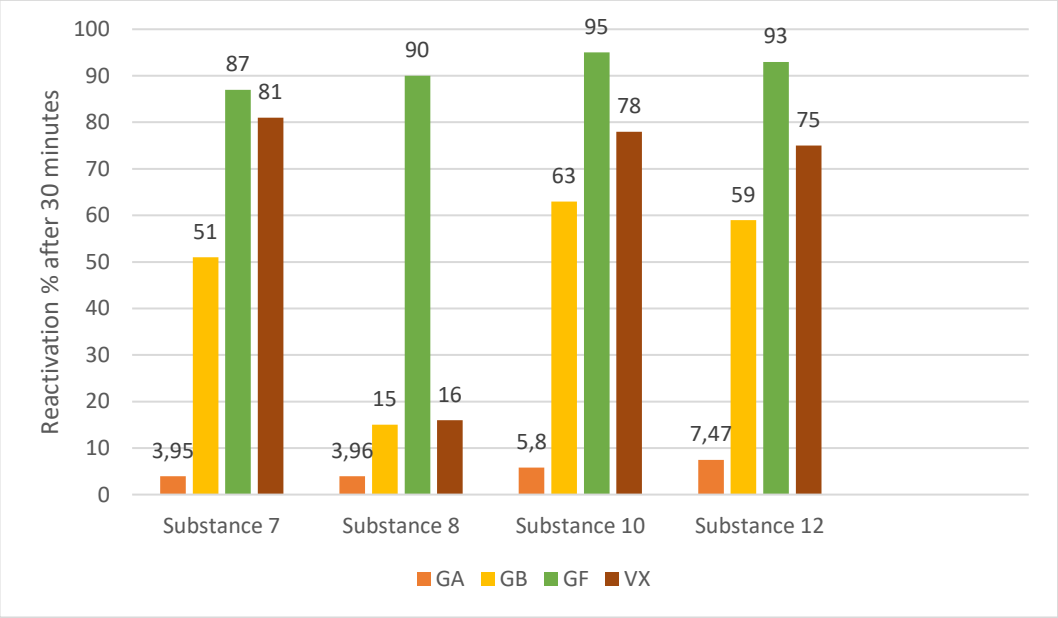


Figure 18. Screening of organophosphate inhibited human acetylcholinesterase reactivation by 0.01-0.1 mM oximes at 25°C.

4. DISCUSSION

The inhibition constants of novel reactivators were compared to those of standard AChE reactivators (pralidoxime, HI-6, trimedoxime)[56]. According to obtained results, it was determined, that novel oximes exert lower K_i than the reference substances. Comparing two subgroups, i.e. carbamate and hydroxyl, not surprisingly carbamate representatives showed stronger inhibition towards both enzymes than corresponding hydroxyl derivatives. Regarding selectivity towards AChE, compound **13** bearing carbamate moiety was the most selective one. The same substance was also the most potent inhibitor of the whole series. From the point of view of the linker length, the inhibitory potential of the carbamate subgroup increased with the tether length. However, the same lawfulness was not observed in hydroxyl subgroup.

In terms of reactivation, only compounds **7**, **8**, **10** and **12** were forwarded for evaluation of their reactivating properties, since substances **6**, **9**, **11** and **13** exerted high rate of inhibition of *h*ACHE with the percentage that increased with time. All tested compounds showed only weak reactivation of tabun-inhibited AChE. The activity of acetylcholinesterase inhibited by resting nerve agents, i.e. sarin, VX and cyclosarin, restored better by 51 – 95 %, apart from compound **8** that showed poor results towards sarin, VX and tabun.

Considering all the experiments, compound **10** should be highlighted as the best derivative of the series. Such compound showed not only low inhibitory potency towards AChE and BChE, but also it exerted the best results from the point of view of reactivation, since it reactivated AChE inhibited by sarin (63 %), cyclosarin (95 %) and VX (78 %). Therefore, it could be recommended for further *in vitro* and *in vivo* evaluation.

5. CONCLUSION

Within this Thesis, eight novel hybrid acetylcholinesterase reactivators were prepared. Obtained bisquaternary compounds differed in the linker length as well as in the presence/absence of carbamate moiety. Biological evaluation of novel compounds included two experiments – determination of the inhibition constant towards AChE and BChE and determination of reactivating cabability. From the point of view of inhibition, the subgroup containing carbamate scaffold proved to be stronger inhbitiors comparing to their analogues with hydroxyl group. All newly synthesized compounds turned out to possess higher inhibitory potential towards both cholinesterases than standard reactivators (pralidoxime, HI-6, trimedoxime). Due to weaker inhbition of AChE, only compounds bearing hydroxyl group were evaluated for their reactivation abilities. Almost all substances reactivated cyclosarin-inhibited AChE up to 90% in less than 30 minutes. Beside cyclosarin, the same compounds showed relatively good results towards sarin and agent VX. The poorest outcomes were obtained for tabun. Generally, compound **10** could be highlighted as the best derivative of the series exerting low inhibitory potential towards both cholinesterases and simultaneously the best reactivating properties. Therefore, this substance could be recommended for further evaluation *in vitro* and *in vivo*.

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