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1 ABBREVIATIONS

Acetyl CoA- acetyl coenzyme A

ACh- acetylcholine

AChE- acetylcholinesterase

BBB- blood brain barrier

CAT- choline acetyltransferase

CNS- central nervous system

DMSO- dimethyl sulfoxide

EtOH- ethanol

Li BH_4- lithium borohydride, reduction agent

MeOH- methanol

MS- mass spectroscopy

MS- EI- mass spectroscopy method, electron ionization (EI)

MS- ES- mass spectroscopy method, electrospray (ES)

$NaBH_4$- sodium borohydride, reduction agent

NMR- nuclear magnetic resonance **OP**- organophosphate

OPC- organophosphorous compound

PNS- peripheral nervous system

Pro- PAM- pralidoxime prodrug

2- PAM- pralidoxime

TLC- thin layer chromatography

TMB- 4- trimedoxime, an oxime

2 ABSTRACT

Acetylcholinesterase (AChE) is an enzyme crucial to normal nerve signaling because of its rapid hydrolysis of acetylcholine, a common neurotransmitter. Inhibition of this enzyme may cause bradycardia, hypotension and difficulty in breathing, and the outcome might be deadly. Nerve agents are a group of lipophilic and volatile organophosphorous AChE inhibitors. Because of their ability to penetrate unbroken skin and pass into the central nervous system (CNS), they are ideal for chemical warfare. A group of mono-and bispyridinium compounds called *oximes* are able to reactivate inhibited AChE, and there is great interest in synthesizing better and more effective oximes. To be able to reactivate inhibited AChE the pyridine nitrogen has to be positively charged, but due this positive charge the oximes have problems passing the blood- brain- barrier (BBB) and enter the CNS. As a consequence of this, nerve agent intoxication is difficult to treat. The solution to this problem may be to synthesize an oxime *prodrug*; in other words a compound that can freely pass the BBB (e. g has lipophilic properties) and enter the CNS, and once inside the CNS regain its active form. When a polar compound like an oxime has successfully entered the CNS, it is in reality trapped. Little progress has been made in regard to synthesizing an effective oxime prodrug since the pralidoxime prodrug was synthesized in 1975. New oximes have been introduced the last decade, but none of these have properties that allow a passing of the BBB to a satisfactory extent.

In this master thesis I have synthesized some known mono- and bispyridinium oximes according to literature protocol. Pralidoxime, a monopyridinium oxime and TMB- 4, a bispyridinium oxime, were successfully synthesized in high yields. TMB- 4 was synthesized via two different protocols, a direct route and a new, indirect route of synthesis. Potential oxime prodrugs were synthesized by reduction of mono- and bispyridinium oximes with $NaBH_4$. Oximes that resisted reduction by $NaBH_4$ were later attempted reduced using $LiBH_4$. Other oximes were synthesized using new synthetic methods developed by the author. A new monopyridinium oxime was synthesized and isolated, whereas synthesis of bispyridinium derivatives of TMB- 4 proved difficult.

3 INTRODUCTION

3.1 Acetylcholine- a neurotransmitter Synthesis, release and inactivation

Acetylcholine (ACh) is synthesized in the nerve terminus from choline, which is taken up into the nerve terminal by an active transport system. Free choline is acetylated by a cytosolic enzyme, *choline acetyltransferase*, (CAT), which transfers the acetyl group from acetyl coenzyme A (acetyl CoA). Most of the synthesized ACh is packed into vesicles with a concentration of approximately 100 mmol/L. Release happens through exocytosis, which is triggered by a calcium influx to the nerve terminus. After release, ACh diffuse across the synaptic cleft to reach receptors on the surface of the postsynaptic cell.

In fast cholinergic synapses, for example neuromuscular and ganglionic synapses, ACh is hydrolyzed very fast (within 1 ms) to inactive choline and acetate by *acetylcholinesterase* (AChE). AChE is an enzyme bound to the basal membrane of the nerve terminal. The basal membrane is located between the pre- and postsynaptic membranes. Some of the released ACh is actually hydrolyzed before being able to interact with postsynaptic receptors. ACh-molecules that reach a receptor remains bound to the receptor for, on average, 2 ms, and are quickly hydrolyzed after dissociation, preventing them from combining with a second receptor. The result is that transmitter action is very rapid and very brief, which is important for the initiation of fast muscular responses in synapses than may have to transmit high frequency signals.

3.1.1 Structure of acetylcholinesterase

Each molecule of AChE consists of six active sites, where each site has a central esteratic site and a peripheral anionic site (Nair, Hunter 2004). The esteratic site is composed of three amino acid residues (Ser, His and Glu). These residues are referred to as a catalytic triad. Together these residues perform the catalytic functions of the enzyme. The catalytic triad enables nucleophilic attack on ACh and subsequent hydrolysis. The other component of the active site is the anionic subsite. This term is in fact misleading since the site is actually uncharged and lipophilic (Harel *et al.* 1993; Sussman *et al.* 1991). The anionic site binds ACh, enabling the ester linkage of ACh to interact with the esteratic site of AChE. ACh is hydrolyzed and the acetyl group is transferred to the serine residue (Nair, Hunter 2004). A free choline molecule is released. The acetylated enzyme is hydrolyzed rapidly, forming free enzyme and acetic acid. Approximately 10000 molecules of ACh are hydrolyzed pr second in each of the six active sites on the enzyme (Nair, Hunter 2004). The structure of the active site with ACh attached is shown in Fig.1.

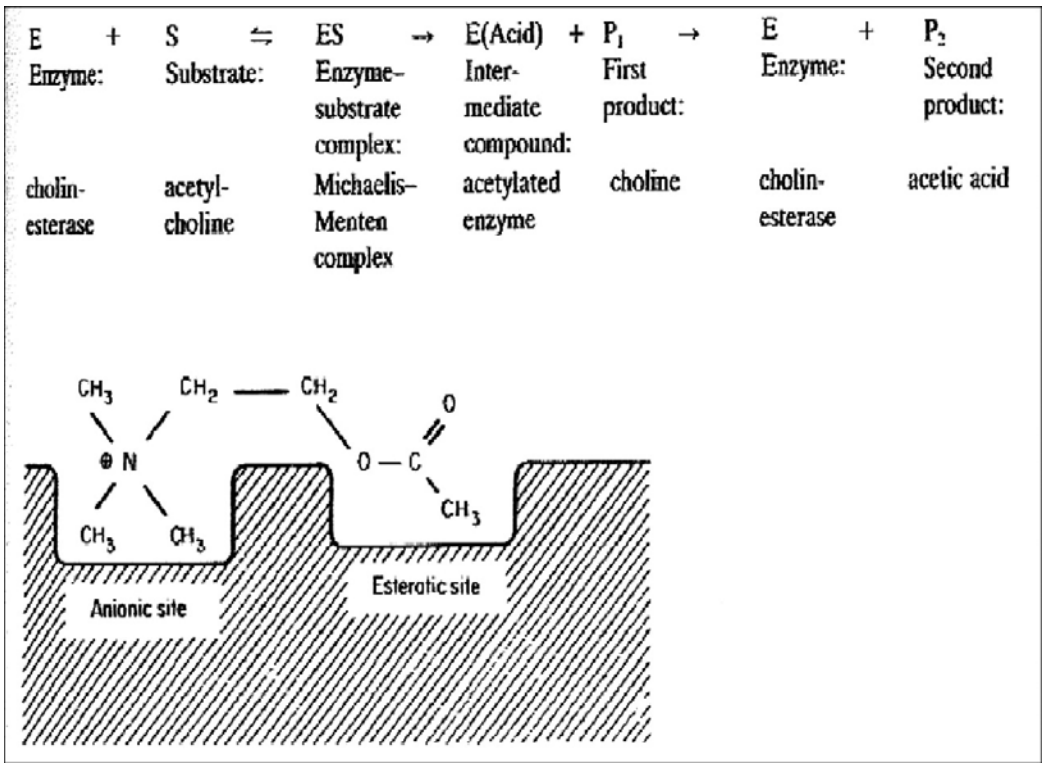


Fig.1: Active site of acetylcholine esterase with its substrate acetylcholine (Nair, Hunter 2004).

3.2 Anticholinesterases interfere with acetylcholine activity

Anticholinesterases inhibit AChE by binding reversibly or irreversibly to the esteratic site. With the esteratic site occupied, the enzyme is no longer able to hydrolyze its substrate ACh, and ACh is not inactivated after being released from the postsynaptic receptor. This means that one molecule of ACh can activate the same receptor several times, or activate several receptors. The consequence of AChE inhibition is a constant activation of postsynaptic receptors, which may lead to a depolarization block, a decrease in the postsynaptic cell's electrical excitability. The main reason for this loss of excitability is the inactivation of voltage- sensitive sodium channels, which are rendered unable to open in response to brief depolarizing stimuli. A depolarization block is associated with accumulation of ACh in plasma and other tissue fluids. This can cause life threatening physiological effects unless the inhibited AChE regains its normal function. A group of organophosphate anticholinesterases inhibit AChE by phosphorylating the esteratic site. Spontaneous hydrolysis of phosphorylated AChE is extremely slow, about 10^7 to 10^{12} – fold slower than deacetylation (Johnson *et al*. 2000). This causes exposure to anticholinesterases to be potentially life threatening.

3.3 Effects of anticholinesterases

Effects of anticholinesterases on autonomic cholinergic synapses mainly reflect increased ACh- activity at parasympathetic postganglionic synapses (Rang *et al.* 1999). This means increased secretions from salivary, lachrymal, bronchial and gastrointestinal glands, increased peristaltic activity, bronco constriction, bradycardia and hypotension, pupillary constriction and fall in oculary pressure. Large doses of anticholinesterases can stimulate and later block autonomic ganglia and cause complex autonomic effects. (Depolarization block, see chapter 3.2). Poisoning from contact with anticholinesterases may cause difficulty in breathing. An additional depolarization block may lead to a fatal outcome for the individual or animal in question. Effects on neuromuscular junction can be seen as initial muscle cramps and later paralysis due to depolarization block (Rang *et al.* 1999). Tertiary anticholinesterases compounds such as physostigmine and non-polar organophoshates can penetrate the blood-brain- barrier (BBB) freely and affect the CNS. Initially anticholinesterases cause an excitation of the CNS, often seen as convulsions. The excitation is followed by a subsequent depression which may lead to unconsciousness and respiratory failure (Rang *et al.* 1999), due to an inhibition of the respiratory centre in the medulla oblongata (Johnson *et al.* 2000). These

central effects are mainly the result of activation of muscarinic receptors, and are antagonized by atropine (Rang *et al.* 1999).

3.4 Different groups of anticholinesterases

Peripherally acting anticholinesterases fall into three main categories according to their way of interaction with the active site on the AChE- enzyme, which also determines the duration of action (Rang *et al.* 1999):

Short- acting anticholinesterases
Medium- duration anticholinesterases
Irreversible anticholinesterases

3.4.1 Short- acting anticholinesterases

Edrophonium is a quaternary ammonium compound that binds to the anionic site of the enzyme only. The ionic bond is readily reversible, and the drug action is very brief. Edrophonium is too short- acting for therapeutic use, and its main use is diagnosis of myasthenia gravis (Rang *et al.* 1999). In myasthenia gravis transmission fails because there are not enough ACh receptors, and anticholinesterases improve transmission because they increase the number of free ACh molecules. The chemical structure of edrophonium is shown in Fig. 2.

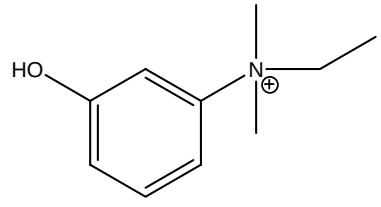


Fig. 2: Structure of edrophonium, a short- acting anticholinesterase (Rang et al. 1999).

3.4.2 Medium- duration anticholinesterases

Medium- duration anticholinesterases include neostigmine (used in combination with atropine to reverse non- depolarizing neuromuscular block), pyridostigmine (treatment of myasthenia gravis), and rivastigmine (treatment of dementia caused by Alzheimer's disease) (Felleskatalogen 2004). They are quaternary compounds, in contrast to physostigmine, a tertiary amine anticholinesterase found in the Calabar bean (used in combination with pilocarpine for treatment of glaucoma) (Samuelsson 1999). The medium- duration anticholinesterases all have strongly basic groups which bind to the anionic site of the AChE. They are however carbamyl esters, whereas ACh is an acetate ester. The carbamyl group is transferred to the serine –OH of the esteratic site in similar fashion to acetyl from ACh, but the carbamylated enzyme is much slower to hydrolyze. The hydrolysis can take several minutes, compared to microseconds for an acetylated enzyme (Rang *et al.* 1999). The slow recovery of the carbamylated enzyme means these drugs are quite long- lasting; AChE inhibited by rivastigmine regains its normal activity approximately 10 hours after administration of the drug, and pyridostigmine has a time of duration of 4-6 hours (Felleskatalogen 2004). Structures of the medium duration anticholinesterases mentioned above are found in Fig. 3.

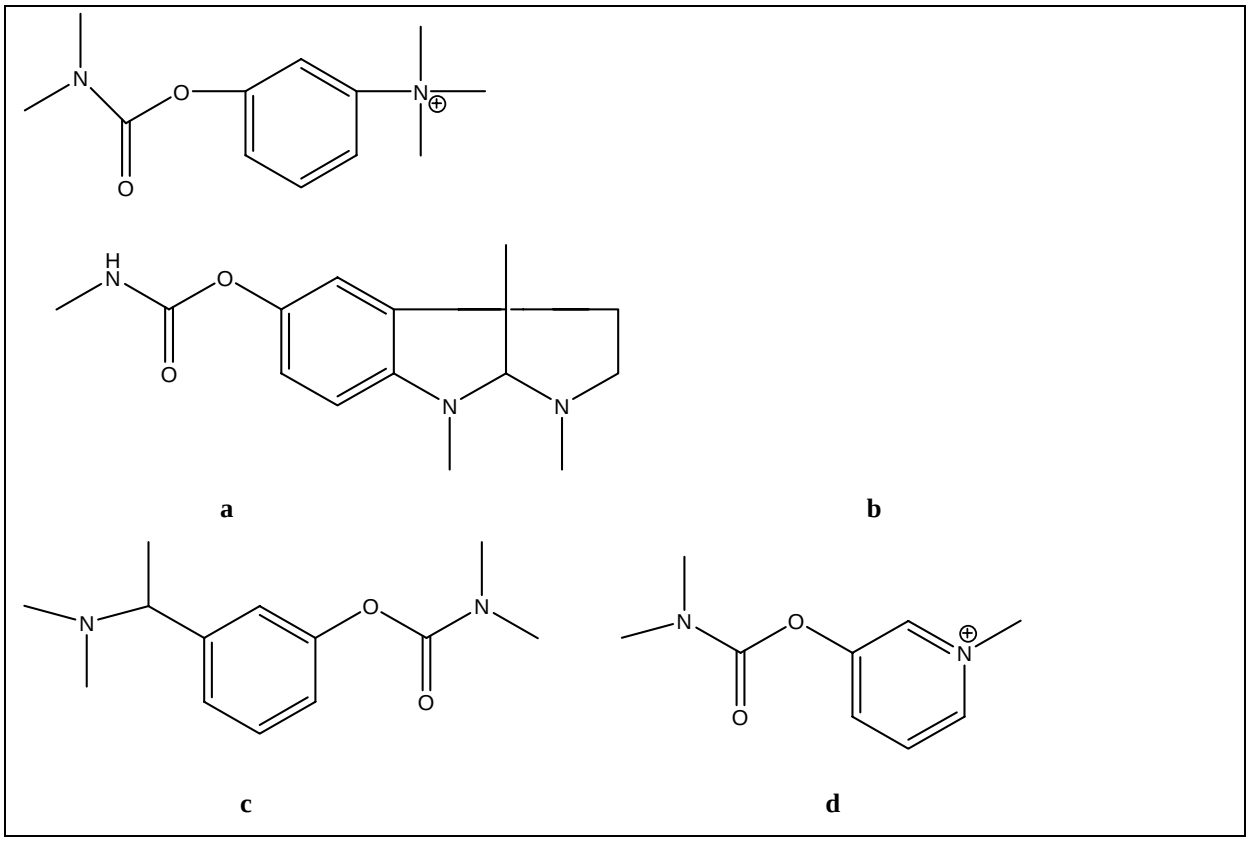


Fig. 3: Structure of the most important medium duration anticholinesterases
a) neostigmine
b) physostigmine
c) rivastigmine
d) pyridostigmine
(Rang *et al.* 1999; Goodman&Gilman's 2001)

3.4.3 Irreversible anticholinesterases

Organophosphorous compounds (OPCs) are extremely potent irreversible inhibitors of AChE. They have properties that make them useful in different areas. Organophosphates are used as insecticides, softening agents and additives to lubricants, and as nerve agents (Kassa 2002). Most of these compounds interact only with the esteratic site of AChE and have no cationic group (Rang *et al.* 1999). The general structure is shown in Fig.4. Irreversible anticholinesterases are pentavalent compounds containing a labile group such as fluoride or an organic group. After bonding to the esteratic site, the labile group is released (Rang *et al.* 1999). The residue of the molecule remains covalently attached to the enzyme's serine– OH-group, effectively blocking the esteratic site and hence inhibiting the normal function of the enzyme. The inactive phosphorylated enzyme is usually very stable. With some anticholinesterases virtually no hydrolysis occurs, and recovery of enzymatic activity depends on the synthesis of new enzyme molecules. This is a process that may take weeks (Rang *et al.* 1999).

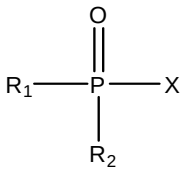


Fig. 4: General structure of organophosphorous compounds
R1 and R2 are normally simple alkyl- and aryl groups which both may be linked directly to the P atom (in phosphinates) or linked via -O- or -S- (in phosphates), or R1 may be bonded directly and R2 may be bonded via one of the groups mentioned above (in phosphonates). In phosphoroamidates, C is bonded to P through an amino group. The X- group is a leaving group (LG) and may be a substituted or branched aliphatic, aromatic or heterocyclic group, linked to –P -, –O- or –S-. The atom linked to P via a double bond may be S or O, and related compounds are called phosphorothioates and phosphates respectively. The P=O- analogue of a thioate ester is called an oxon, and this term is often incorporated in the trivial name, like parathion and paraoxon. (Johnson *et al.* 2000).

3.5 Nerve agents: Irreversible anticholinesterases

Nerve agents are related chemically to organophosphorous insecticides and have a similar mechanism of toxicity, but a much higher mammalian acute toxicity, particularly via the dermal route (Vale 2004). They are non- polar substances with high lipid solubility, and they are rapidly absorbed through mucous membranes, and even unbroken skin (Rang *et al.* 1999). The discovery of these properties lead to their use as chemical warfare agents, and they are referred to as nerve agents. The nerve agents acquired their name because they affect the transmission of nerve impulses in the nervous system (Vale 2004). The outdated term "nerve gas" is misleading, since all nerve agents in their pure state are colorless liquids. Their volatility varies widely; VX on one extreme is oil- like, whereas sarin, being the most volatile nerve agent, is approximately as volatile as water (http://www.nbcdefence.net/nore/nerve.htm; Holstege, Dobmeier 2005).

The nerve agent, either as a gas, aerosol or liquid, enters the body through inhalation or through the skin. Systemic poisoning may follow inhalation, ingestion or dermal exposure, though the onset of systemic toxicity is slower by the latter route (Vale 2004). The route for entering the body is of importance for the period of time required for the nerve agent to start having effect. The nerve agents have the quickest effect when absorbed through the respiratory system. This is due to the rapid diffusion into the blood circulation, and subsequent transport to target organs (http://www.acq.osd.mil/cp/cbdprimer_oct2001.pdf).

Exposure to high doses of a nerve agent will cause muscle paralysis of the respiratory muscles, and in addition the respiratory center of the CNS is affected (Johnson *et al.* 2000). The combination of these two is the direct cause of death. Consequently, death caused by a nerve agent is death by suffocation.

3.5.1 The dawn of a deadly weapon

Prior to and during World War 2, efforts were directed towards the development of chemical warfare agents. During his work with developing pesticides, a German chemist, Gerhard Schrader, discovered tabun by chance in 1936 (Holstege, Dobmeier 2005). Schrader first-handedly observed the effects of nerve agents on human beings in January 1937, when a drop of tabun spilled onto a lab bench. Within minutes Schrader and his laboratory assistant began to experience miosis, dizziness, and severe shortness of breath. It took them three weeks to fully recover (http://en.wikipedia.org/wiki/Nerve_gas). Tabun was the first of the substances later referred to as nerve agents.

The nerve agents are stable and easily dispersed, they have rapid effects both when absorbed through the skin and via respiration, and they are extremely toxic. Because of these properties, the nerve agents are considered among the most dangerous chemical warfare agents in the world. They are in fact classified as weapons of mass destruction by the United Nations according to UN Resolution 687 (http://en.wikipedia.org/wiki/Nerve_gas). The structures of the most important nerve agents are given in Fig.5.

Up to the end of the war, Schrader and his co-workers synthesized about 2 000 new organophosphorous compounds. Sarin was developed in 1938, and was named after the men who participated in synthesizing it: Schrader, Ambrose, Rudringer, and Vander Linde. Soman was the last nerve agent to be developed by the Germans in 1944 by Dr. Richard Kuhn (Holstege, Dobmeier 2005). The G agents, or German agents, tabun, sarin and soman are volatile, and both dermal and respiratory hazards (Vale 2004).

A British scientist synthesized an extremely toxic compound in 1952 while searching for a new pesticide to replace DDT (Holstege, Dobmeier 2005). This substance was forwarded to the US for production and was later coded VX. VX belong to a family of nerve agents called the V agents. The V agents are less volatile than the G-agents, and are primarily percutaneous contact hazards unless aerosolized (Vale 2004).

In the morning rush hour on March 20, 1995, a group of terrorists placed containers of sarin in carriages on three underground railway lines in open plastic bags, enabling the agent, which is liquid under temperate conditions, to evaporate (J. A. Vale 2004). Over 5,000 sought medical

attention, 984 of these were moderately poisoned and 54 were severely poisoned. 12 people died as a direct cause of the exposure to sarin (Okumura *et al.*1996, Sidell 1996). Two years later, The Chemical Weapons Convention forbid development, production, stockpiling and the use of chemical war agents (Johnson *et al.* 2002). There is however little reason to believe nerve agents won't be used again in future, being a very effective weapon for terrorists and in warfare.

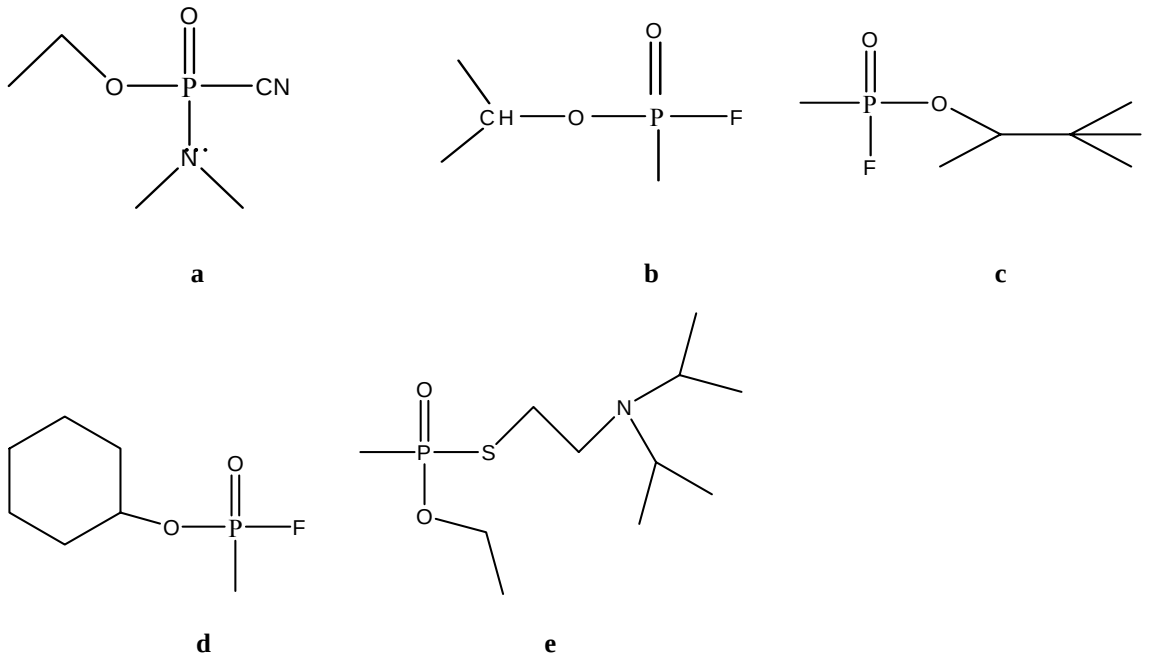


Fig. 5: The most common nerve agents are the following:
a) **Tabun**, O-ethyl dimethylamidophosphorylcyanide (GA) is the easiest to manufacture. Consequently, it is more likely that developing countries start their chemical warfare arsenal with this nerve agent whereas industrialized countries consider tabun to be out-of-date and of limited use.
b) **Sarin**, isopropyl methylphosphonofluoridate, (GB) is a volatile substance mainly taken up through inhalation.
c) **Soman,** pinacoyl methylphosphonofluoridate, (GD) and it is a moderately volatile substance which can be taken up by inhalation or skin contact.
d) **VX,** O-ethyl S-diisopropylaminomethyl methylphosphonothiolate, is a persistent substance which can remain on material, equipment and terrain for long periods of time. Uptake is mainly through the skin but also through inhalation of the substance as a gas or aerosol.
e) **Cyclosarin /GF agent**, cyclohexyl methylphosphonofluoridate, is a substance with low volatility which is taken up through skin contact and inhalation of the substance either as a gas or aerosol.

3.5.2 Biochemistry

The interaction between organophosphorous compounds (OPCs) and AChE all take place in the same manner, but the rate of each step depend on the structure of the OPC. In every case, the initial step of the inhibition occur when the OPC is in the oxon (P=O)-form. Pure thioates (P=S- compounds) are not significant inhibitors in their original form, but are metabolically activated to oxons *in vivo* (Johnson *et al.* 2000).

The interaction between OP- oxons and AChE include four stages (Johnson *et al.* 2000):
1. A serine group on AChE is phosphorylated.
2. A LG X leaves the complex. This leads to the formation of a relatively stabile covalent bond between the OPC and the enzyme, with a consequent inhibition of catalytic activity.
After inhibition, two reactions are possible:
3. Reactivation may happen spontaneously but slowly at a rate determined by the properties of the attached OPC and the enzyme. The rate might be changed by adding nucleophilic reagents such as oximes, which may accelerate the reactivation rate and thereby act as antidotes.
4. The AChE- OPC complex may go through an *ageing*. Ageing is a time dependent loss of the phosphorylated enzyme's ability to be reactivated. The ageing involves cleavage of one or more bonds in the R- O- P- chain with a subsequent loss of R, and formation of a charged monosubstituted phosphoric acid still attached to the enzyme. The reaction is called an ageing because it is usually a slow process, and the product, a covalent OPC- AChE complex, can no longer be reactivated by nucleophilic reactivating agents. Therapeutic oximes cannot break such a bond. The inhibition mechanism of AChE is shown in Fig. 6.

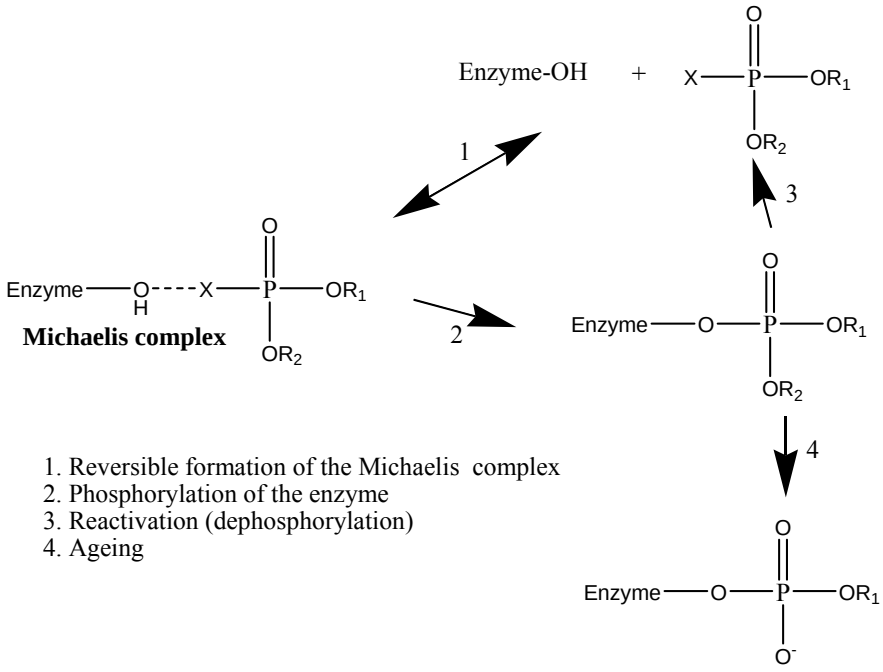


Fig. 6: AChE inhibition mechanism
Inhibition of AChE starts with the formation of the Michaelis complex in step 1. In step 2 the enzyme is phosphorylated and inactivated. The enzyme- OPC- complex may now spontaneously hydrolyze (step 3), rendering the enzyme reactivated, or the complex may go through an ageing (step 4). Once aged, reactivation is impossible (Johnson *et al.* 2000).

3.5.3 Ageing half- life

The ageing half- life of the nerve agent- AChE complex is an important factor when it comes to outcome of oxime treatment. If treatment is initiated too late, the oxime will not be able to reactivate inhibited AChE, and the result may be fatal.

The ageing half- life for three of the most dangerous nerve agents are (Opresko *et al.* 1998):

Tabun-AChE: 46 hours

Sarin- AChE: 5 hours

Soman- AChE: 1.3 minutes

3.6 Acetylcholinesterase reactivators

Compounds containing an oxime group (RCH= NOH) attached to a pyridine ring with a quaternary nitrogen are able to reactivate OPC- inhibited AChE by dephosphorylating the enzyme's active sites. The quaternary nitrogen atom is bonded to the anionic site, thereby placing the oxime group in the vicinity of the esteratic site. Several oximes are potentially effective in reactivating OPC- inhibited AChE. The basic structure for these compounds differs only by the number of pyridine rings (mono or bis) and by the position of the oxime group on the pyridine ring. The structure of the most important oximes can be found in Fig. 7. The more potent antimuscarinic bisquartarnary pyridine derivatives are those containing a hydrophobic substituent at position 3 or 4 in the pyridine ring (Kloog *et al.* 1986).

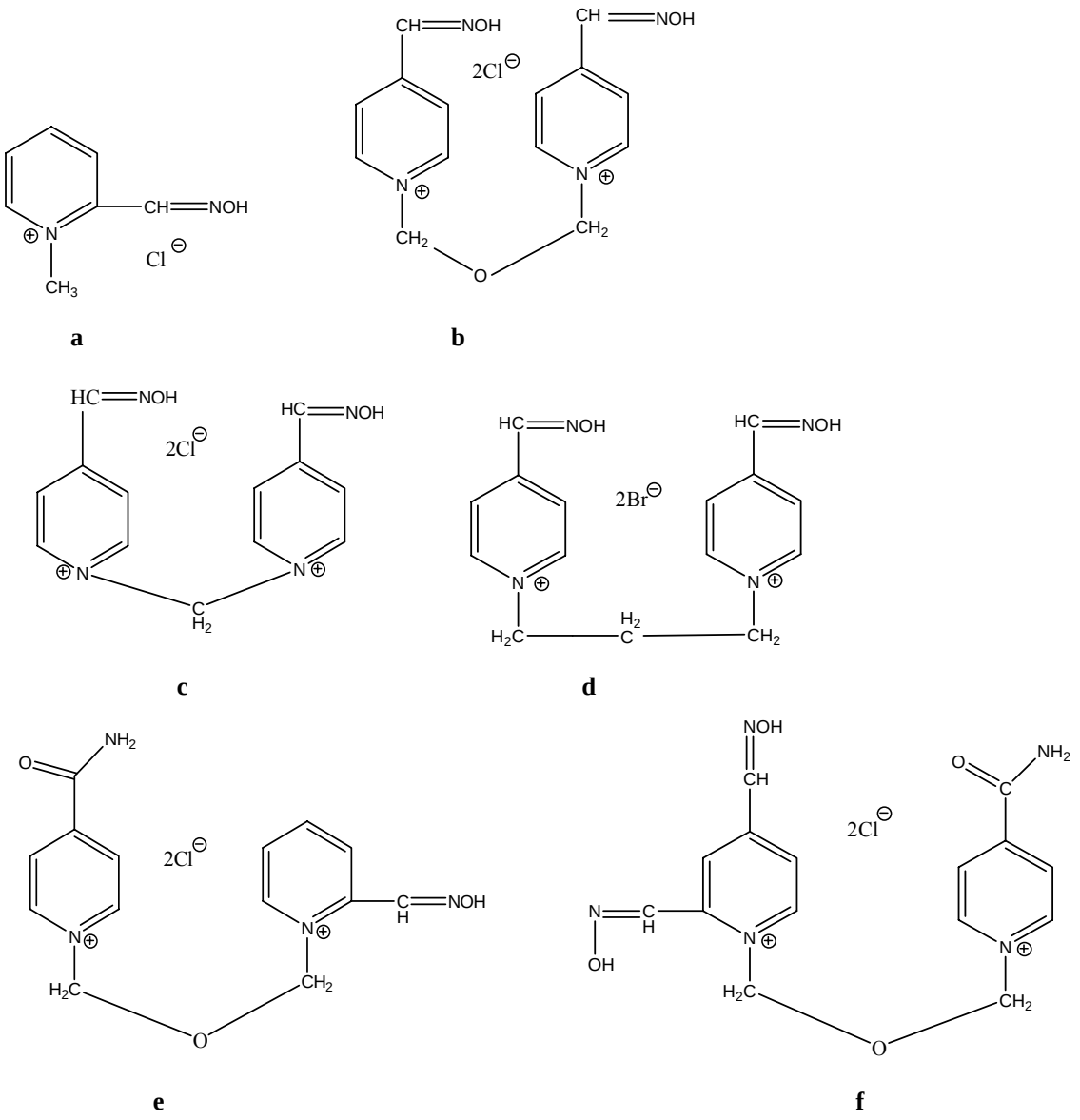


Fig. 7: The structures of the most common oximes
a) **Pralidoxime chloride**, 2-pyridinealdoxime-*N*-methyl chloride
b) **Obidoxime chloride**, bis-4-pyridinealdoxime-*N*-methylether dichloride
c) **Methoxime chloride**, *N*, *N*-methylen-4-pyridinealdoxime dichloride
d) **Trimedoxime bromide/ TMB-4**, 1, 1'- trimethylenebis- 4-(hydroxyiminomethyl) pyridine bromide
e) **HI-6 chloride**, 4- aminocarbonylpyridine- 1- methylenoxy- 2'-(hydroxyiminomethyl)- 1'-methylpyridine dichloride monohydrate
f) **HLø-7 chloride**, 1-[[[4-(aminocarbonyl) pyridine] methoksy] methyl] -2, 4-bis [(hydroxyimino) methyl] pyridine dichloride

3.6.1 Oxime deprotonation

To be pharmacologically active, oximes need to undergo a prior deprotonation. This deprotonation is shown in Fig. 8 (Brüggeman 1998).

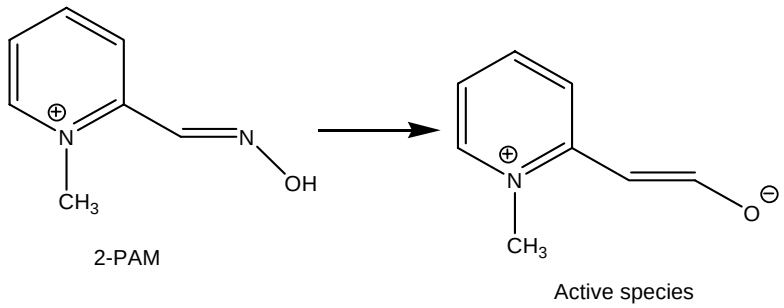


Fig. 8: Oxime deprotonation (Brüggeman 1998)
Oxime deprotonation is an essential step to render the oxime active and capable of reactivating OPC- inhibited AChE. The deprotonation causes a loss of a proton from the oxime OH- group, leaving the oxygen negatively charged.

3.6.2 Oxime reactivation mechanism of organophosphate- inhibited acetylcholinesterase

Oximes reactivate OPC- inhibited AChE through a nucleophilic attack on phosphorous. An oxime- phosphonate leaves the active site, and the regenerated esteratic site is subsequentially able to return to normal function, and bind and split its substrate ACh. The reactivation mechanism is shown in Fig. 9 below (Brüggeman 1998).

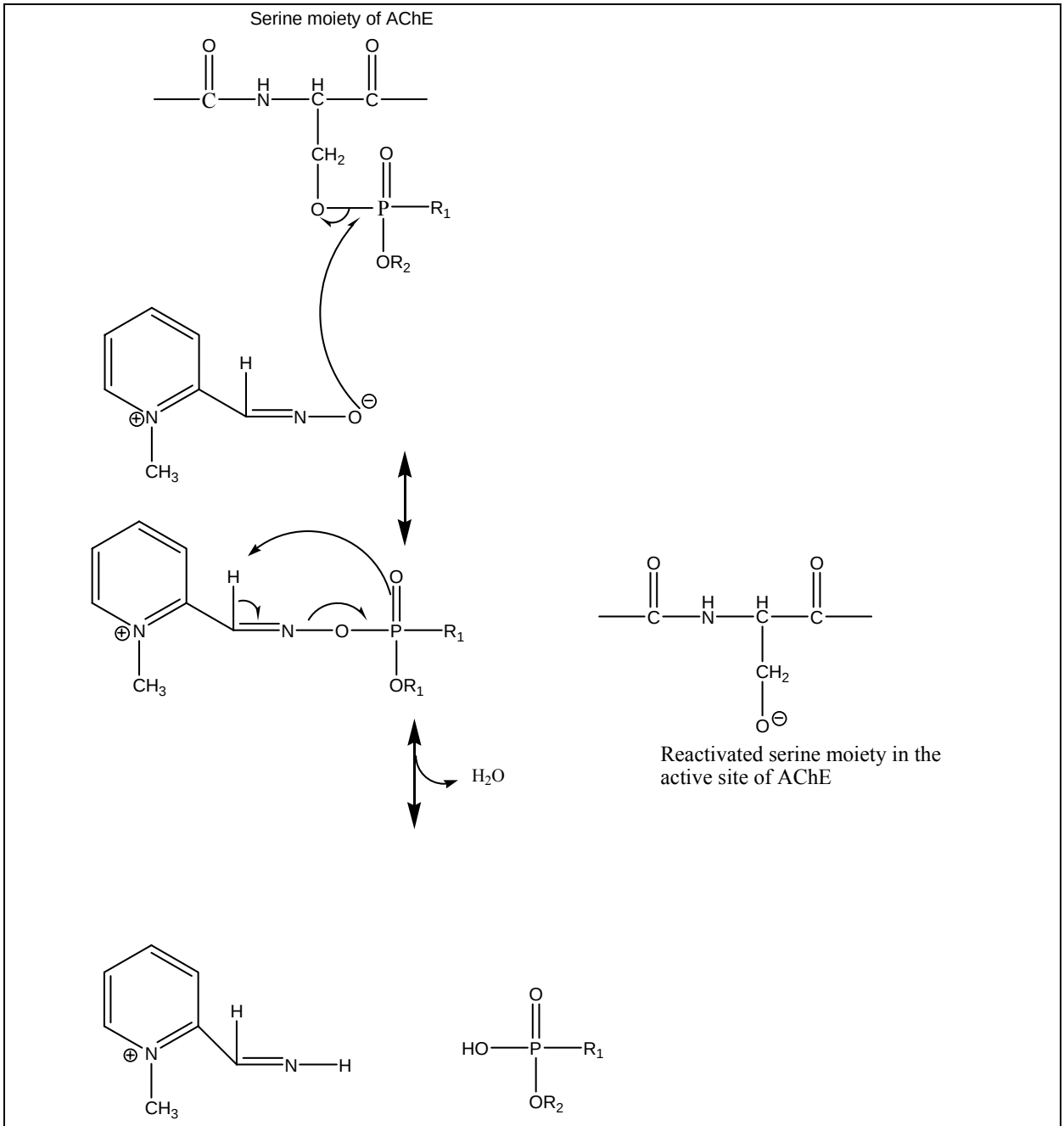


Fig. 9: Reactivation of OPC- inhibited AChE
The oxime group attacks phosphorous, and subsequentially removes the OPC from the active site of AChE. The active site (e.g. the serine moiety) is reactivated and the enzyme can return to normal function. The oxime- OPC complex goes through hydrolysis and is split in oxime, now inactive, and OPC (Brüggeman 1998).

3.6.3 Stability of oximes in solution

The stability of the oxime in aqueous solution is an important chemical property because of the necessity of storage in advance of possible clinical use. Pralidoxime, obidoxime and methoxime are all relatively stabile in water and can be stored in solution. Stability studies on obidoxime show that obidoxime ampoules can be stored for over 30 years at room temperature before the content decreases by 10% (Rubnov *et al.* 1999). HI-6 and HLø-7 on the other hand are unstable in aqueous solution and must be stored as lyophilized powder (Eyer *et al.* 1986). When these compounds are in solution, they decompose quickly under ambient or physiological conditions (pH 7.4 and 37º C). The degradation half- time of both these oximes in 1 mM solution have been found to be about 12 hours (Eyer *et al.* 1986; Eyer *et al.* 1989). This short degradation half- life is definitely one of the biggest problems associated with the use of these oximes. Therefore, dry/wet autoinjectors have been developed. For practical reasons, they contain both atropine and oxime, comprising the oxime as a powder which is dissolved in the atropine-containing solvent immediately before intramuscular injection. The autoinjectors are activated by breaking a membrane followed by shaking the device. The commercially available autoinjectors (Astra Tech AB, Sweden, and STI International, UK) are specified to deliver a dose of some 500 mg HI 6 dichloride and 2 mg atropine sulphate after a shaking time of 5 seconds (Thiermann *et al.* 1998).

3.6.4 Reactivation efficacy

Generally speaking, the reactivating efficiency of an oxime is determined by the nucleophilic strength of the oxime and its affinity for the OPC- inhibited enzyme. Oximes may differ in the position of the oxime group on the pyridine ring, but their reactivity is comparable because of the similar basic structure (Kassa 2002). The oximes affinity for the intact enzyme is characterized by the dissociation constant K_{dis} of the enzyme- oxime complex. A high value of K_{dis} means a decreased affinity of the oxime for the intact enzyme. The affinity of the OPC- inhibited enzyme is characterized by the dissociation constant of the inhibited enzyme-oxime complex K_R. An increase in K_R means a decrease in the affinity of the oxime for the OPC- inhibited enzyme. The affinity is determined by a number of physiochemical factors such as steric compatibility, electrostatic effects, hydrophobic interactions and the general shape and size of the reactivator, in addition to functional groups (Kassa 2002).
Pralidoxime and obidoxime have relatively low affinity for both active and phosphonylated AChE, while the affinity of the H- oximes is quite high. This corresponds to the reactivating

potency *in vitro*; pralidoxime and obidoxime are poor reactivators and the H- oximes are relatively good reactivators (Kassa, Cabal 1999 A; Kassa, Cabal 1999 B; Kassa, Cabal 1999 C).

Treatment of soman intoxication

Soman is one of the most treatment resistant war gases due to the presence of as slowly distributed soman depot in the poisoned individuals (Kassa 2002) and the extremely rapid ageing of soman- phosphonylated AChE (Opresko *et al.* 1998). In pyridostigmine and control rabbits intoxicated with soman and treated with oxime and atropine, HI- 6 was found to be three to five times more effective than pralidoxime (Koplovitz, Steward 1995). Pralidoxime, obidoxime, and methoxime were found to be virtually ineffective in reactivating soman-inhibited AChE in the peripheral nervous system (PNS) and in the CNS. Obidoxime may actually worsen the AChE- inhibition by soman (Kassa, Cabal 1999). The H-oximes have been found to be significantly more effective in reactivation of soman- inhibited AChE than the monopyridinium oximes, although the percentage reactivation within the CNS is unsatisfactory due to the H- oximes' poor ability to penetrate the BBB (Kassa 2002). The research data on the H- oximes are contradictory; J. Kassa have shown that the H- oximes cannot pass the BBB in sufficient concentration and exhibit their action within the CNS to a satisfactory extent (Kassa 2002), while other studies have shown that the H- oximes *can* in fact pass the BBB in sufficient concentration to produce biochemical and physiological effects in soman poisoning (Eyer *et al.*1992; Clement 1992; Kassa 1998; Koplovitz, Steward 1995).

Treatment of tabun intoxication

Bispyridinium oximes show different ability to reactivate tabun- inhibited AChE *in vitro,* whereas no monopyridinium oxime including pralidoxime is able to reactivate tabun-inhibited AChE (Cabal *et al.* 2004). Obidoxime, methoxime and HI-6 are poor AChE-reactivators; the percentage of AChE reactivation is lower than 10%. Pralidoxime has been found to be more effective against tabun poisoning than HI- 6, but the efficacy of HI- 6 increased three- fold when rabbits were pretreated with pyridostigmine before being treated with oxime and atropine (Koplovitz, Steward 1995). Trimedoxime (TMB-4) seems to be the most effective reactivator of tabun inhibited AChE; it has been found to be able to reactivate more than 40% of tabun-inhibited AChE at 1 mmol/l concentration *in vitro* (Cabal *et al.* 2004). The extremely poor effect by most oximes on tabun intoxicated individuals is caused

by a free electron pair located on the amidic nitrogen, making a nucleophilic attack by the oximes almost impossible (Dawson 1994; Koplovitz *et al.* 1995; Eto 1976; Cabal, Bajgar 1999).

Treatment of VX intoxication

VX has been reported to respond to all oxime treatment (Kassa 2002), and VX intoxication is therefore easy to treat compared to intoxication with the other nerve agents mentioned.

Treatment of sarin intoxication

The ability of pralidoxime, obidoxime and methoxime to reactivate sarin- inhibited AChE is relatively low. The H- oximes have proven to be very effective both peripherally and centrally, the latter in spite of the quaternary structure that should, at least to a certain extent, limit penetration of the BBB (Kassa 2002).

3.6.5 Therapeutic efficacy

Therapeutic efficacy of the oximes is normally measured by the protective ratio (PR) of the LD_{50} of the particular OPC in an animal being treated with an oxime, compared to the LD_{50} in unprotected animals. In most published experiments, a combination of atropine and oxime is used as an antidotal treatment since this is the treatment most likely to be used in for example combat. Therapeutic efficacy can also be measured by comparing the median effective dose (ED_{50}) that prevents death of rats after exposure to supralethal doses of tested OPCs, when the oximes are combined with the same dose of atropine.

ED_{50} data published show that pralidoxime in recommended doses alone is not able to prevent mortality in rats exposed to supralethal doses of any nerve agent tested (Kassa, Cabal 1999 A; Kassa, Cabal 1999 B, Kassa, Cabal 1999 C). Data show that obidoxime must be administered in doses higher than the recommended dose (approximately 2% of LD_{50}) to be effective, which gives a low safety ratio. The required dose of obidoxime is not sufficiently safe and may cause dangerous side effects. HI-6 and HLø-7 are effective in protecting rats poisoned with supralethal doses nerve agent, like soman, in doses comparable to recommended human therapeutic doses. The safety ratio of required doses of the H- oximes is very high, and HI-6 and HLø-7 are considered to be safe in use (Kassa 2002).

The therapeutic efficacy of the oximes seems to depend on many different factors, one of them being the particular nerve agent involved. Especially intoxications by soman and tabun are difficult to treat, whereas VX yields readily to common antidotal treatment. Other factors seem to be the administration route for both nerve agent and antidote, and timing. Treatment need to be initiated as soon as possible.

None of the oximes, including the H- oximes, can be regarded as a universally suitable reactivator (Worek *et al.*1997). Generally speaking, the H- oximes are promising antidotes because they are able to protect experimental animals from toxic effects, and improve the survival rate in animals intoxicated with lethal doses. The H- oximes are more effective in nerve agent intoxication than pralidoxime and obidoxime, especially when it comes to soman poisoning (Kassa 2002). Obidoxime has been found to be inferior to HI 6 against soman, sarin, cyclosarin and VX, and pralidoxime is generally less potent than the other oximes (Worek *ET al.*1997). However, pralidoxime and especially obidoxime are sufficiently effective in the treatment of OP- insecticide poisoning, since these insecticides are considerably less toxic than the nerve agents (Kassa, Bajgar 1996; Worek *et al.* 1996). Obidoxime has turned out to be the most potent and most efficacious oxime in reactivating AChE inhibited by various classes of OP insecticides and tabun (Worek *et al.*1997).

3.7 Clinical recommendations

It is important with immediate treatment after exposure to nerve agents. The first clinical signs of intoxication may not appear until about 50% of AChE is inhibited and some molecules of AChE will remain uninhibited for a long time (Johnson *et al.* 2000). The body has a huge surplus of AChE compared to what is needed to maintain normal body function, a kind of biochemical safety buffer. This means that initiation of treatment at the first sign of symptoms may be too late. It is important to notice that clinical signs of OPC intoxication become severe before all AChE is inhibited, perhaps at a 75-90% inhibition (Johnson *et al.* 2000). At this point of enzymatic inhibition, the body responds by increasing ACh levels, and the ACh will compete with the OP-oxons for the remaining uninhibited active sites on AChE (Johnson *et al.* 2000). When all AChE is inhibited, the patient will be dead.

There is limited experience with nerve agent intoxication in human, but it is generally accepted that clinically relevant amounts of nerve agent can be found in blood for a shorter period of time than the OP insecticides (Kassa 2002). The time of action for the nerve agents is in other words shorter than for the insecticides. Despite of the rapid disappearance from the blood, the toxic effects of the nerve agents may be prolonged because of the rapid ageing of the OPC- AChE- complex, especially regarding soman (Kassa 2002; Opresko *et al.* 1998). In absence of a clinical response after administration of an oxime, it is unlikely that treatment in excess of 24- 48 hours will lead to further reactivation of AChE (Kassa 2002).

Preferred treatment is a combination treatment of diazepam, oxime and atropine. These drugs should be administered as soon as possible after intoxication. In mild cases of intoxication, drying of bronchial secretions by administering atropine may be adequate therapy. This may be sufficient treatment until spontaneous reactivation of inhibited AChE or natural synthesis of new enzyme occurs (Johnson *et al.* 2000). Serious cases are treated with atropine, diazepam and oxime in combination with supportive ventilation, since AChE- inhibition affects the respiratory centre in the medulla oblongata (Johnson *et al.* 2000). In the case of a massive overdose, high concentrations of OPC may remain in the blood for days, causing a depot that slowly releases inhibiting oxon (Moretto, Johnson 1987). Oximes are cleared from the blood quite rapidly (Johnson 1975), and even if some inactivation has been achieved, another inhibition cycle and possible ageing of the inhibited AChE may follow. It is therefore important to achieve and maintain a sufficient blood concentration of the oxime, and not abort

treatment at an early point despite of immediate treatment response. It is recommended that the oxime should be administered for as long as atropine is indicated. For the majority of individuals this will be for less than 48 hours; the exception would be individuals exposed dermally to VX where a depot of VX might result in prolonged intoxication (Marrs 2004).

Diazepam

Diazepam is used as an anticonvulsant. Treatment with diazepam may be a valuable adjunct antidote in serious cases, even though the reasons for this are not clear. The structure is shown in Fig. 10. Diazepam appears to be more effective than other anticonvulsants such as the barbiturates, even though results from comparative studies have not been published. Diazepam has anti- GABA-ergic properties, which may cause diazepam to act as a specific antagonist in secondary GABA- ergic central pathways activated by ACh (Johnson *et al.* 2000). Diazepam may also counteract the unwanted CNS side effects of atropine (Kusic *et al.* 1991). If convulsions are not controlled by diazepam, severe rhabdomyolysis may be the result (Johnson *et al.* 2000).

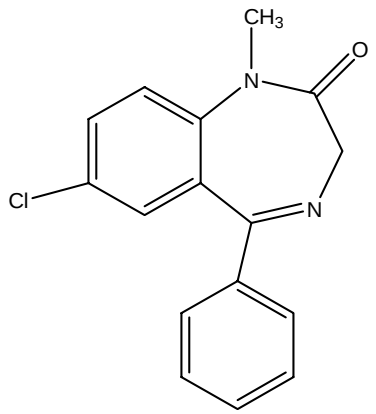


Fig. 10: Structure of diazepam, used as an anticonvulsant in the treatment of anticholinesterase poisoning.

Oximes

It has been found that bisquaternary pyridinium oximes inhibit K^+- evoked ACh release from rat brain stem slices (Kloog *et al.* 1986). Bisquaternary pyridinium oximes appear to behave as agonists at presynaptic muscarinic autoreceptors in the brain, thereby mimicking ACh's negative feedback on its own release. These oximes have also shown to possess antimuscarinic activity at postsynaptic receptors of guinea pig ileum. This suggests that bis-pyridinium oximes may act simultaneously as presynaptic agonists and postsynaptic

antagonists at cholinergic synapses (Kloog *et al.* 1986). Three oximes are available in autoinjectors for self- and buddy- administration: Pralidoxime as the methanesulphonate, methyl sulphate and chloride salts, obidoxime dichloride and HI-6 dichloride. Patients still showing symptoms following use of the auto injector need additional doses of oxime and atropine to reactivate inhibited enzyme and reduce cholinergic symptoms (Kassa 2002).

Atropine

Atropine is a physiological antidote and completely blocks the action of ACh at muscarinic receptors (Johnson *et al.* 2000).The structure of atropine is shown in Fig. 11. Atropine also reverses the parasympathetic overstimulation resulting from the inhibition of AChE. At the dose levels normally needed, atropine is also an antagonist at muscarinic receptors in the CNS, and may prevent convulsions and inhibition of the respiratory centre (Johnson *et al.* 2000). Atropine should be administered until the patient is fully atropinized; this will manifest itself with dry skin and sinus tachycardia (Vale 2004).

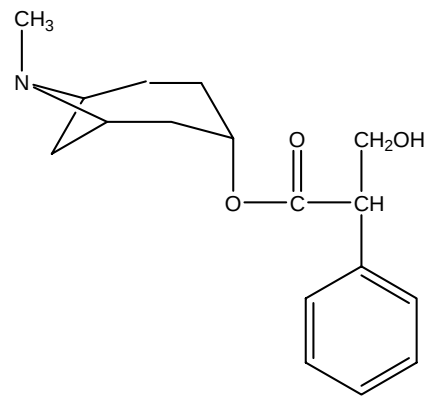


Fig.11: Structure of atropine, a physiological antidote that blocks the action of ACh. Atropine reverses the neuronal overstimulation caused by inhibition of AChE

3.7.1 Reaching and maintaining a therapeutic oxime blood concentration

There is still an ongoing debate as to what is the best way of reaching and maintaining a therapeutic oxime blood concentration. Bolus- dosing, and continuous intravenous infusion may both be an option.

Bolus dosing

Bolus-dosing schedules of the oximes currently available are based on data derived from human cases of OP insecticide poisoning. Under extreme conditions, for instance in battle, single bolus- doses given intramuscularly is probably the only practical solution. Bolus-dosing of the oxime by intravenous injection is by far more effective, but depending on the circumstances, intravenous bolus- dosing may not be possible.

Continuous intravenous infusion

An alternative method for administering therapeutic doses of an oxime is by continuous intravenous infusion. The threshold plasma concentration of oxime to counteract nerve agent poisoning in humans is presumed to be 4μg/ml (Sundwall 1961). Clinical studies indicate that both pralidoxime and obidoxime preferably should be given as continuous infusions after an initial bolus dose (Willems *et al.* 1992; Thiermann *et al.* 1997; Medicis *et al.* 1996). There is no reason to believe that these results cannot be extrapolated to include the newer H-oximes such as HI- 6 and HLø- 7.

When considering nerve agent intoxication in for instance battle, the patient would most likely be given an intramuscular bolus- dose initially, and not receive intravenous infusion until medical personnel arrived to the scene or he or she was moved to a hospital or a similar facility.

3.8 Oxime transport across the blood-brain-barrier; the use of prodrugs

Treatment of anticholinesterase poisoning with oximes is highly effective in the PNS, but fails to restore activity to a large fraction of the inhibited brain AChE (Ellyn, Wills 1968). The oximes are polar substances, a feature necessary for them to act as competitive agonists at the esteratic site of AChE. Unfortunately the oximes cannot readily pass the BBB because of their polar nature. OPCs in general and nerve agents in particular are small, lipophilic compounds that can freely pass the BBB. Since anticholinesterase intoxication causes a wide array of physiological effects due to inhibitory action within the CNS, it is desirable that the oxime is able to cross the BBB and reach the CNS. A solution to this problem may be to formulate a *prodrug* of the oxime. A prodrug is a compound that can freely pass the BBB and enter the CNS, and once inside the CNS regain its active form. A polar compound once inside the CNS is in reality trapped and unable to escape. Such a prodrug will in this context be lipophilic.

3.8.1 Synthesis of a pralidoxime prodrug

A reduced, lipophilic pralidoxime analogue, able to being oxidized to active pralidoxime *in vivo,* was synthesized in 1975 (Bodor *et al*. 1975). This compound is called pro- PAM (1-methyl-1, 6-dihydropyridine-2- carbaldoxime hydrochloride). The structure of pro- PAM is shown in Fig. 12. Pro- PAM is rapidly oxidized to pralidoxime *in vivo*. Pro- PAM is a tertiary amine at physiological pH and is therefore in principle able to cross the BBB and enter the CNS (Bodor *et al.* 1975). When inside the CNS, pro- PAM can be oxidized to pralidoxime, and once on its active form it can reactivate inhibited AChE in the CNS.

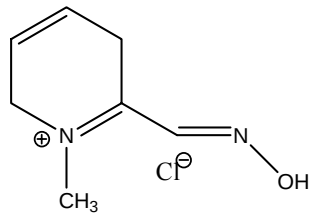


Fig.12: The pralidoxime prodrug pro- PAM. At physiological pH this compound exists as its free base, and it is significantly more lipophilic than pralidoxime. It can be quantitatively oxidized to pralidoxime, making it a pralidoxime prodrug (Bodor *et al.* 1975).

3.8.2 Problems associated with the pralidoxime prodrug

Despite of pro- PAM's lipophilic character, investigations have shown that pro- PAM does not have a better effect in the treatment of anticholinesterase intoxication than pralidoxime (Boscovic *et al.* 1980; Clement 1979; Heffron, Hobbiger 1980). Research shows that pro-PAM has limited effect when given subcutaneously. This is probably related to the short half-lives for formation ($t_{½}$ = 1 min) and elimination ($t_{½}$ ≈20 min) of the dihydropyridine prodrug in combination with slow transport from the site of injection to the brain (Kinley *et al.* 1982). Intravenous injection of an oxime is an effective administration route under certain clinical circumstances, but for emergency first- aid the intramuscular and subcutaneous routes is more practical. Under these conditions, it is unlikely that pro- PAM will be more effective in reactivating inhibited AChE in the CNS than pralidoxime, because of minor differences in the degree of reactivation of OPC- inhibited brain AChE (Kinley *et al.* 1982).

4 AIM OF THE STUDY

Little has happened the last decade in regard to synthesizing therapeutically active prodrugs of known pyridine oximes, and this is definitely an area of great interest in the future.
In my master thesis I have been interested in oximes, and potential prodrugs of these oximes.

In this master thesis, the following goals were set:

- Achieve an understanding of the biochemistry of anticholinesterases, anticholinesterase poisoning, oximes, and oxime facilitated AChE reactivation
- Synthesize a few well known mono- and bispyridinium oximes
- Synthesis of potential oxime prodrugs based on reduction of the pyridine ring system
- Development of improved synthetic methods for synthesis of mono- and bispyridinium oximes

If any compounds were to exhibit prodrug potential, these were to be tested against anticholinesterase poisoning by the Norwegian Defense Research Establishment (FFI)

5 RESULTS AND DISCUSSION

5.1 Synthesis

5.1.1 Pralidoxime/ 2- PAM in acetone

The synthesis of 2- PAM iodide was performed as described by Shek *et al.* 1975 with some modifications of the protocol. The procedure was originally meant for synthesis of [^{14}C] methyl labeled 2- PAM iodide, and the methyl iodide added to the solution was a mixture of ^{14}C methyl iodide and regular methyl iodide. In this synthesis the temperature was kept at approximately 50 ºC under normal pressure and the reaction mixture was allowed to react for 24 hours, thereby deviating from the original protocol where a pressure bottle was used, and the temperature was kept at 95ºC for 6 hours. Two separate experiments were completed. The yields were 44.2% and 54%, respectively. The difference in yield, though considerable, is probably due to the operator, since the molar ratio in the two experiments was equal. The structure was confirmed by ^{1}H- NMR- and MS- data for both experiments, and the structures of the two reaction product were identical. The high temperature in the reference article seems not to be essential for formation of the reaction product (2-PAM), at least not when the reaction time is increased. The reaction mechanism for the synthesis of 2- PAM is shown in Fig. 13 below.

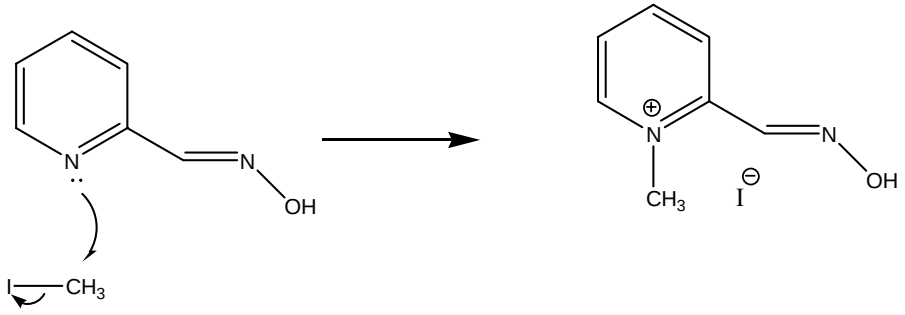


Fig. 13: Reaction mechanism of 2- PAM synthesis

5.1.2 2- PAM in absolute ethanol

2- PAM was also synthesized in ethanol according to Poziomek *et al.*1958. Addition of a methyl group in stead of larger groups like ethyl and allyl was not described in this article, but the procedure for ethyl addition was followed. The reaction time was reduced with 16 hours compared to the reference article method. A yellow precipitate fell out of the red- brown solution within a few hours. 2- PAM is highly soluble in ethanol and it was therefore necessary to add diethyl ether (or any another suitable non- polar solvent) to achieve complete precipitation.

The yield of 2- PAM after recrystallization and drying was 72.6%, somewhat high compared to the yield given by Poziomek *et al.*1958 where ethyl was added to the pyridinium nitrogen at a 34.8% yield. Even though not directly comparable, one would expect that addition of methyl to the pyridinium nitrogen would result in yields not too different from the yields for ethyl addition in the same location. This deviation may be caused by steric hindrance. Ethyl is a larger group than methyl, and in this case, where the oxime group is located at C- 2, there is a possibility that smaller groups like methyl may be added to the pyridine nitrogen at far higher yields than larger groups like ethyl and so on. Attempt to add 2- hydroxy- ethyl to the pyridine nitrogen resulted in a 9.9 % yield according to Poziomek *et al.* 1958, which may be consistent with the steric hindrance theory.

Oxime group alkylation

According to Poziomek *et al.* 1958, quaternization may sometimes be complicated by a side reaction involving alkylation of the oxime group. The alkylation mechanism is shown in Fig 14 (Poziomek *et al.* 1958).

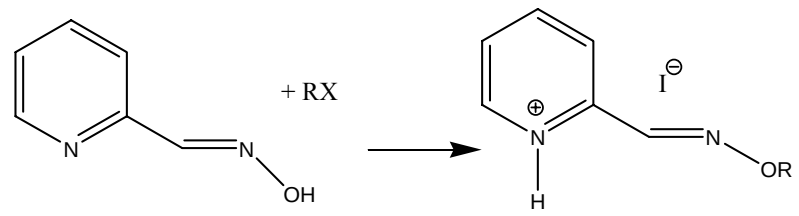


Fig. 14: Alkylation of the oxime group during quaternization (Poziomek *et al.* 1958)

The occurrence of this side reaction was reported to increase with increased steric hindrance at the site of the reaction e.g. the pyridine nitrogen. With highly hindered oximes such as

2, 6- diformylpyridine dioxime, the oxime ether was the sole reaction product. Separation of oxime ether hydrohalide and quaternary oximes can be accomplished by separation from neutral aqueous solution. At pH 6-7 the oxime ether and any unreacted pyridinealdoxime can be extracted from aqueous solution with chloroform ($CHCl_{3)}$, leaving the quaternary compound in the aqueous layer (Poziomek *et al.* 1958).

The yield from the 2- PAM synthesis in ethanol was 3.83 g, and the aqueous layer from the extraction yielded 3.59 g. One would assume the remaining 0.24 g would be found in the organic layer, which was not the case in this experiment. The organic yielded nothing, suggesting the following: a) no oxime ether was formed or b) the synthesis was complete, leaving no unreacted pyridine in solution. This is somewhat unlikely, and I assume the missing 0.24 g was lost during extraction and drying of the organic layer.

A ^{1}H- NMR specter of the product from the aqueous layer showed an extra peak at about 3.5 ppm. Formation of a hemihydrate during freeze drying was suspected. The ^{1}H- NMR specter was otherwise identical to the ^{1}H- NMR specter taken from 2- PAM synthesized in acetone. The compound was vacuum dried and a new ^{1}H- NMR-specter showed that the extra peak was no longer present, rendering two identical ^{1}H- NMR- spectra from two different synthesis protocols.

5.1.3 Reduction of 2- PAM to 1- methyl- 1, 2, 3, 6- tetrahydropyridine- 2- carbaldoxime with $NaBH_4$

The reduction was performed according to Bodor *et al.* 1975 with one exception. In the reference protocol, the temperature of the reaction mixture was first held at 20ºC during the addition of $NaBH_4$, and thereafter increased to 25 ºC. The reduction in this thesis was performed at a constant room temperature (approximately 20-25 ºC), since no apparent reason for not keeping a constant temperature was obvious. The reduction mechanism is shown in Fig. 15.

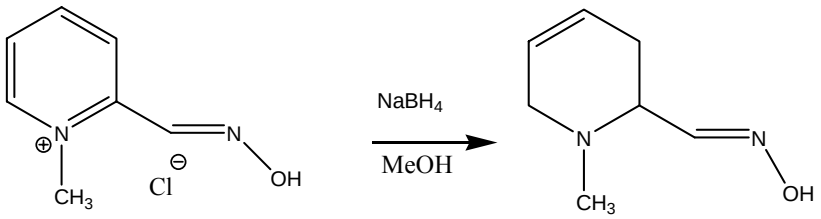
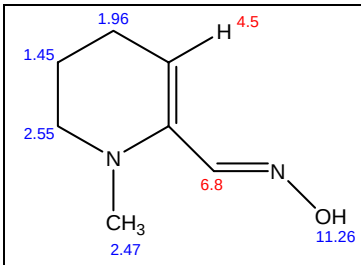


Fig. 15: 2- PAM reduction mechanism

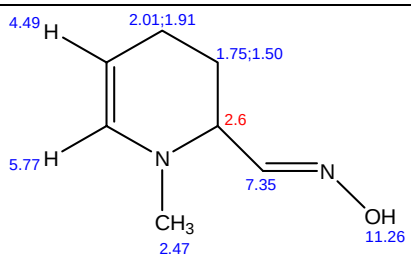
Theoretically, there are several possible structures for reduced 2- PAM. According to Bodor *et al.* 1975 however, the reaction product was invariably 1-methyl-1, 2, 3, 6-tetrahydropyridine-2-carbaldoxime.

MS- data show that only one double bond is present in the reaction product, meaning there are four possible structures of the reaction product. The structures are shown below in Fig. 16. ^{1}H- NMR estimates for the possible structures of the reaction product are shown below in Fig.16. They were obtained using ChemDraw Ultra ChemNMR. The ^{1}H- NMR shifts recorded for the reaction product after reduction of 2- PAM were δ 10.9 (s, OH), 7.4 (d), 5.7 (s), 3.3-2.7 (m), 2.3-2.1 (m). The recorded ^{1}H- NMR specter of the reaction product showed a dublet being emitted from the –CH = in the oxime group, indicating that 1 H is present at the adjacent pyridine ring C. This excludes compound 1 below as the reaction product, where a singlet would have been emitted. Compound 2 shows signals being emitted from H located on C- 3 at 1.5 or 1.75 ppm, and a signal from H located at C- 5 at 4.49 ppm. No such signals are present in the recorded ^{1}H- NMR specter, suggesting the reaction product is not compound 2.

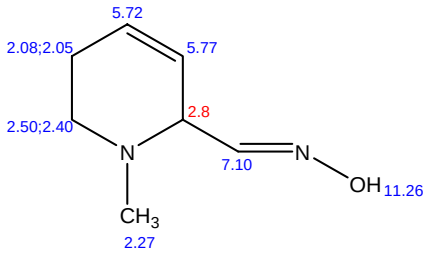
Compounds 3 and 4 show similar ppm- values and the estimated spectra shown below in Fig. 17 and 18 are very much alike. Judging from these ^{1}H- NMR spectra, both compounds are possible reaction products.



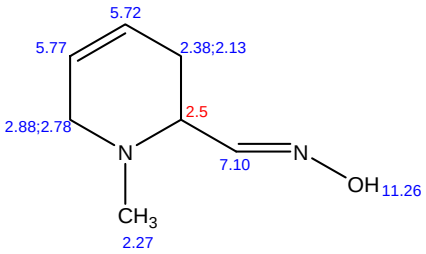
Compound 1



Compound 2



Compound 3



Compound 4

Fig. 16: Estimated ^{1}H- shifts for the possible structures of the reaction product after reduction of 2- PAM

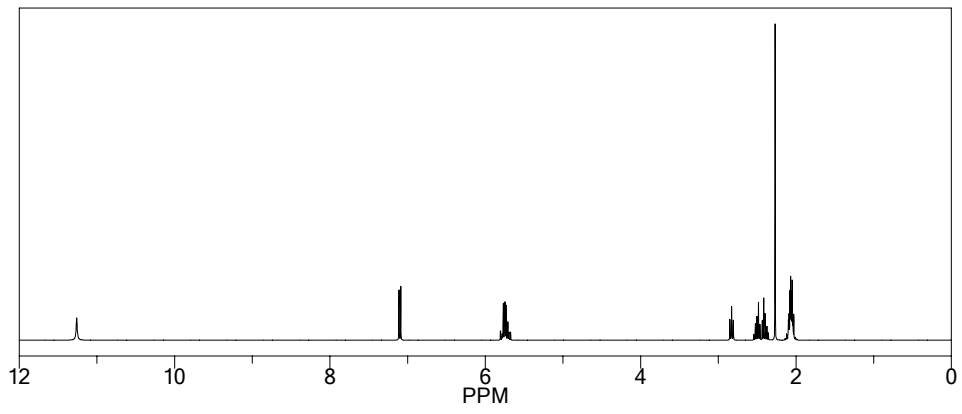


Fig. 17: Estimated ^{1}H- NMR specter for compound 3

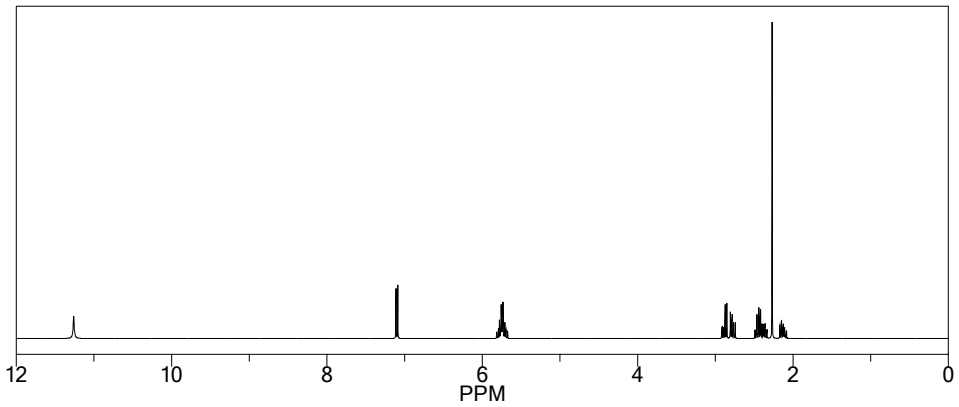


Fig. 18: Estimated ^{1}H- specter for compound 4

Estimated ^{13}C- NMR- shifts for compound 4 are shown below in Fig. 19, and the estimated ^{13}C- NMR specter is found in Fig. 20. The recorded ^{13}C- NMR specter of the reaction product from the 2- PAM reduction is identical to the estimated specter for compound 4. The shifts for the reaction product are 25, 45, 53, 60, 124, 127 and 150 ppm, which confirms that the reaction product after reduction of 2- PAM is compound 4.

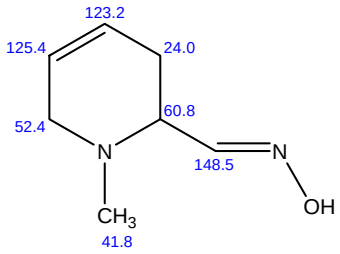


Fig. 19: Estimated ^{13}C- NMR- shifts for compound 4

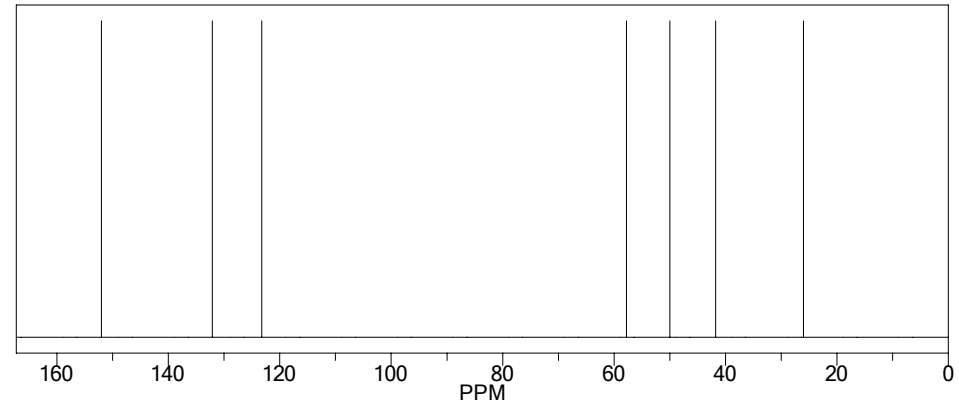


Fig. 20: Estimated ^{13}C- NMR specter for compound 3

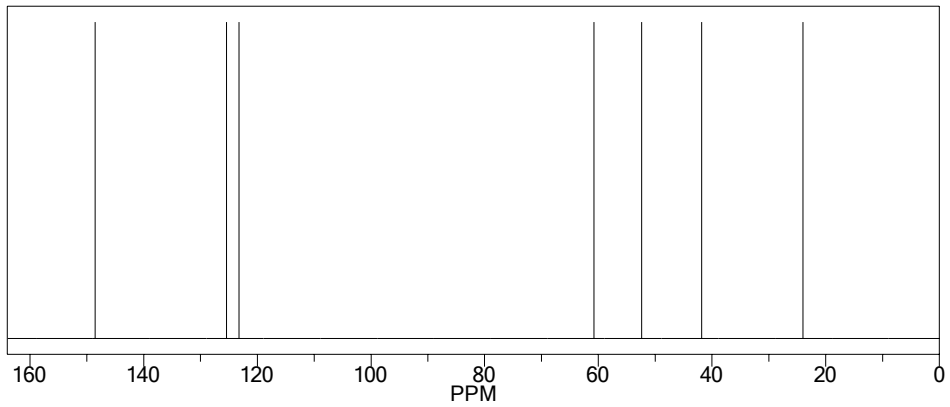


Fig. 21: Estimated ^{13}C- NMR specter for compound 4

The recorded melting point was slightly lower than the melting point given in Bodor *et al.* 1975. The ^{1}H- NMR data deviated slightly from what was given in the reference article considering the peaks upfield in the ^{1}H-NMR-spectre. According to Bodor *et al.* 1975 there should be a multiplet at 2.95-3.33 ppm (5 H) and a singlet at 2.30 ppm (3 H). The ^{1}H-NMR-spectre of 1-methyl-1, 2, 3, 6-tetrahydropyridine-2-carbaldoxime from reduction *7.2.*3 showed a multiplet at 3.3-2.8 ppm (3 H) and a multiplet at 2.3-2.1 ppm (5 H). ^{13}C- NMR- data show that the signal from the H located at C2 comes at 2.1 ppm, almost on top of the expected singlet at 2.3 ppm. Because of this, the total number of H at 2.3-2.1 ppm becomes 5 and not 3. Interestingly enough, the signal located at 7.4 ppm is a singlet and not a multiplet as expected. This is the signal emitted from the H located at the sp- hybridized C in the double bond. Due to the double bond, one would expect a splitting of the signal.

5.1.4 4- pyridinealdoxime-N-methyl iodide/ 4-PAM

The procedure to synthesize 4-PAM is identical to the method used in 2-PAM synthesis in acetone (Shek *et al.* 1975). The reaction mechanism for the synthesis is found in Fig. 22 below.

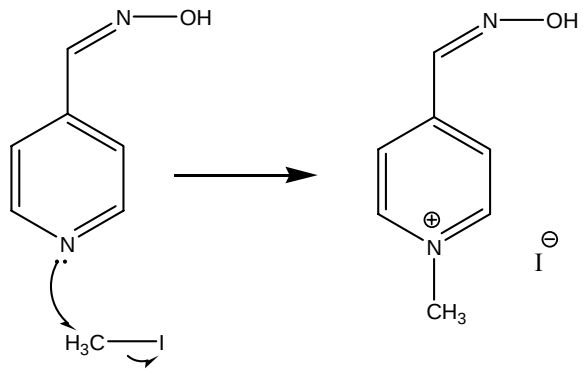


Fig. 22: 4- PAM synthesis reaction mechanism

Two experiments, identical in regard to molar ratio and procedure were completed. The yields were remarkably high compared to the 2- PAM synthesis. The yields were 84.7% and 95.8% respectively, almost a 100% increase compared to 2- PAM. In 2- PAM the oxime group is in ortho position to the pyridine nitrogen, whereas in 4- PAM the oxime group is in para position. This indicates that steric hindrance, or lack thereof, may be a factor of significant importance in pyridinium oxime synthesis. The oxime group, being a relatively electron rich group, seems to shield the pyridine nitrogen in the cases where the oxime group is found in ortho position. This is also consistent with the results of the work of E. J. Poziomek *et al.* 1958, where the para- oximes (e.g. 4- oximes) were synthesized in significantly higher yields than the ortho- oximes (e.g. 2- oximes).

5.1.5 Reduction of 4-PAM to 1- methyl- 1, 2, 3, 6- tetrahydropyridine- 4- carbaldoxime with $NaBH_4$

1- methyl- 1, 2, 3, 6- tetrahydropyridine- 4- carbaldoxime has been described earlier by Wells *et al.* 1967. A series of arecoline- like aldoximes were synthesized by $NaBH_4$ reduction of the corresponding pyridinealdoxime and evaluated as potential reactivators of organophosphate-inhibited AChE. Biological results showed that these aldoximes were less toxic than the quaternary aldoximes, but also much less effective as reactivator (Wells *et al.* 1967). Arecholine is a natural alkaloid related to muscarine and pilocarpine, and effects muscarinic and nicotinic receptors (Goodman & Gilman's 2001).

The reduction of 4- PAM was performed following the same protocol as for the 2-PAM reduction (Bodor *et al.* 1975). The 4- PAM reduction mechanism is found in Fig. 23.

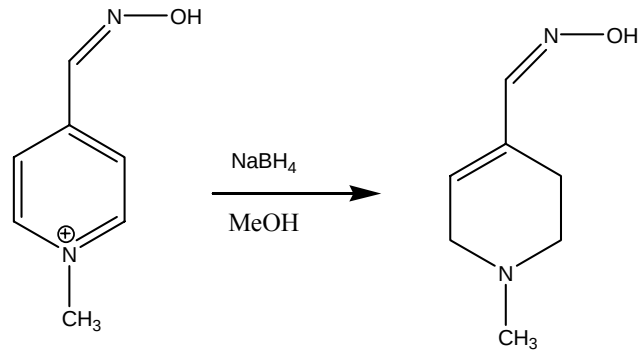
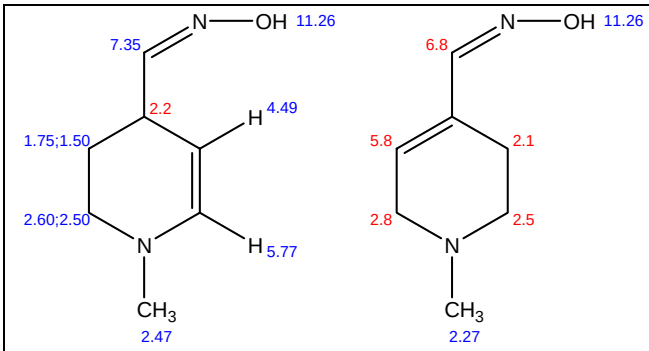


Fig. 23: 4- PAM reduction mechanism

MS- ES showed that the reaction product had only one double bond. Theoretically, there are two possible structures of the reaction product, the product shown in Fig. 23 above or a product where the double bond is located at C- 2. The estimated ^{1}H- NMR shifts for the two possible reaction products and their structures are shown in Fig. 24 below. The red numbers for compound 2 indicate uncertainty as to where the signals would appear in the specter. Compound 1 however emits signals at 1.5, 1.75, 4.49 and 5.77 ppm. These signals are absent in the ^{1}H- NMR specter of the actual reaction product, indicating that compound 2 reflects the structure of the 4- PAM reduction product. Interestingly enough, the signal located at 6.1 ppm is a singlet and not a multiplet as expected. This is the signal emitted from the H located at the sp- hybridized C in the double bond. Normally this signal would have been split, and the reason for this not being the case is unknown.



Compound 1 Compound 2

Fig. 24: Estimated 1H- NMR shifts for the two possible structures of the 4- PAM reduction product

The estimated ^{13}C- NMR shifts for compound 2 is shown in Fig. 25 below. The estimated ^{13}C- NMR specter for compound 2 is found in Fig. 26. The ^{13}C- NMR shifts recorded from the reaction product were 23, 48, 52, 55, 134, 138 and 150 ppm. This coincides with the estimated specter for compound 2. Since both ^{13}C- NMR and ^{1}H- NMR data suggest that the reaction product is compound 2, it is safe to conclude the reaction product is 1- methyl- 1, 2, 3, 6- tetrahydropyridine- 4-carbaldoxime.

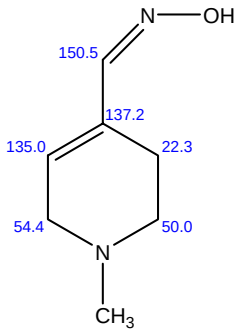


Fig. 25: Estimated ^{13}C- NMR shifts for compound 2

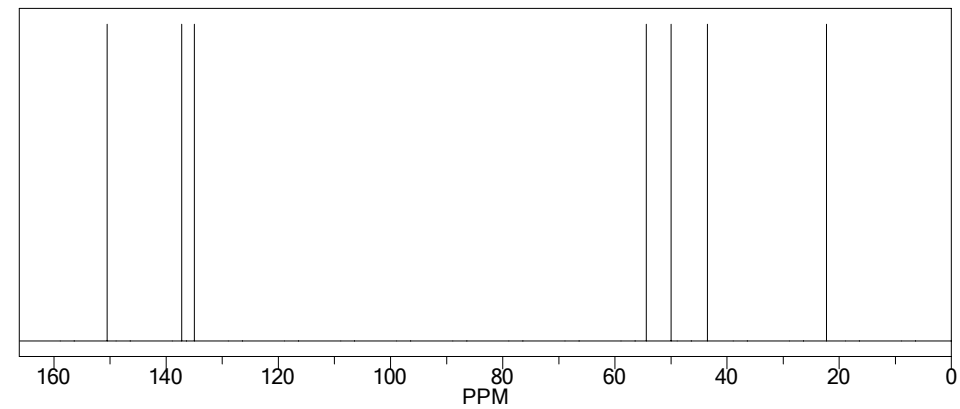


Fig. 26: Estimated ^{13}C- NMR specter for compound 2

4-PAM was reduced in two identical experiments. The only difference is that smaller amounts of toluene and *n*- hexane were used for recrystallization in the second reduction (reduction B). The yields were 42% for reduction A and 45.5% for reduction B. Interestingly; the crystals formed during recrystallization were not identical. Reduction A gave a fine powder with flat crystals, (from now on called crystal A), whereas reduction B gave a more rounded, almost granular looking crystal (crystal B). ^{1}H- NMR- spectra for the two crystals however, were identical, and MS- data showed the two crystals had identical masses. Thin layer chromatography (TLC) also showed that reduced 2-PAM and the two fractions of reduced 4-PAM had different properties when it came to hydrophilicity. Reduced 4- PAM seem to have a higher affinity for the silica than reduced 2- PAM, which suggests that reduced 4- PAM is more hydrophilic than reduced 2- PAM. There seems to be no difference in hydrophilicity between crystal A and crystal B from the two 4- PAM reductions. This supports the conclusion that the two crystals from the two reductions are in fact identical.

The properties of these two crystals differ slightly; crystal B was not completely soluble in DMSO- d_6 whereas crystal A was readily soluble. Considering the fact that DMSO- d_6 is an excellent solvent for a wide diversity of substances, it is hard to tell why not all of crystal B would dissolve. The insoluble substance is most likely an impurity. The reduction mixture contained a 5:1 $NaBH_4$/ 4-PAM ratio, which must have rendered most of added $NaBH_4$ unreacted. Excess $NaBH_4$ however is removed through the addition of water after removal of methanol on the rotavapor. Taking into consideration that ^{1}H- NMR, MS and TLC all indicate that the two crystals are in fact the same substance, there is reason to suspect that crystal B contains more impurities than crystal A. Recording of the melting pointy supports this theory: After melting both crystals changed color to brown, but crystal B assumed a much darker color, supporting the theory that crystal B contains more impurities than crystal A. The melting points for the two crystals were identical; 149-150º C, which suggest the two crystals are in fact the same compound.

5.1.6 TMB- 4

The method suggested by Poziomek *et al.* 1958 is a synthesis in absolute ethanol in a capped pressure bottle. Temperature was kept at 60ºC in the literature experiment. The reaction mechanism for the synthesis of TMB- 4 is shown in Fig. 27 below. Three attempts to synthesize TMB-4 were completed: one in ethanol as described by Poziomek *et al.* 1958, and two in methanol. All experiments were conducted in a normal round bottle flask at normal pressure and at a temperature a few degrees above the alcohol boiling point.

Synthesis in methanol yielded 48.8% and 60.5% respectively, whereas synthesis in ethanol gave a 72.8% yield. The low yield for the first methanol synthesis in probably due to the short reaction time (48 hours).The reaction time was increased in the second methanol synthesis to 72 hours and to 92 hours for the ethanol synthesis. There is reason to believe that synthesis in methanol would result in higher yields given the longer reaction time. The ^{1}H-NMR- spectra and MS- ES data for the three reaction products showed the same molecular structure and weight. The ^{1}H- NMR- spectra for the methanol synthesis reaction products are however showing some impurities in the area 3-4 ppm. This is not the case for the ethanol synthesis reaction product, where the specter was clean and with no peaks from impurities. According to this it seems that synthesis in ethanol gives a purer and cleaner reaction product.

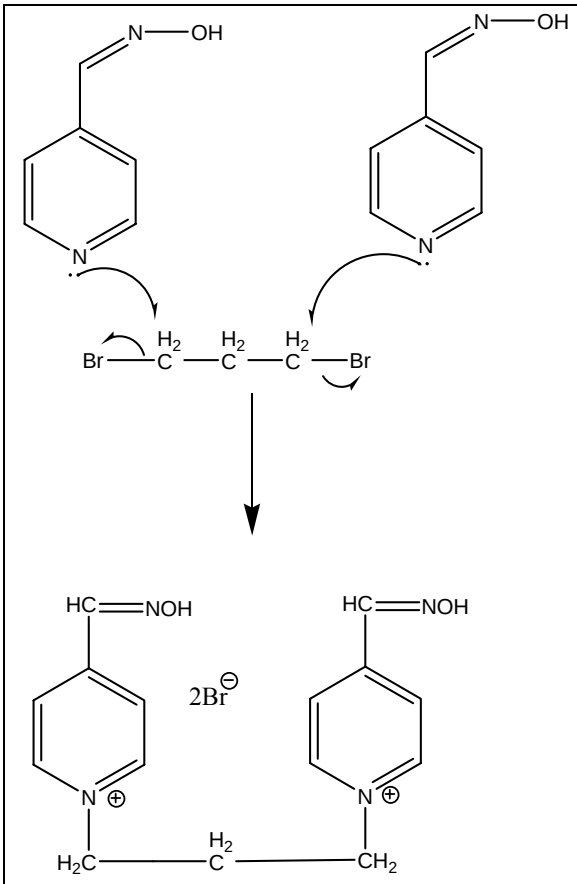


Fig. 27: TMB-4 reaction mechanism

5.1.7 4- pyridinealdoxime- N- propyl bromide

The synthesis of 4- pyridinealdoxime- *N*- propyl bromide was performed following a protocol extrapolated from the 2- PAM synthesis procedure suggested by Shek *et al.* 1975. The reaction mechanism is shown in Fig. 28.

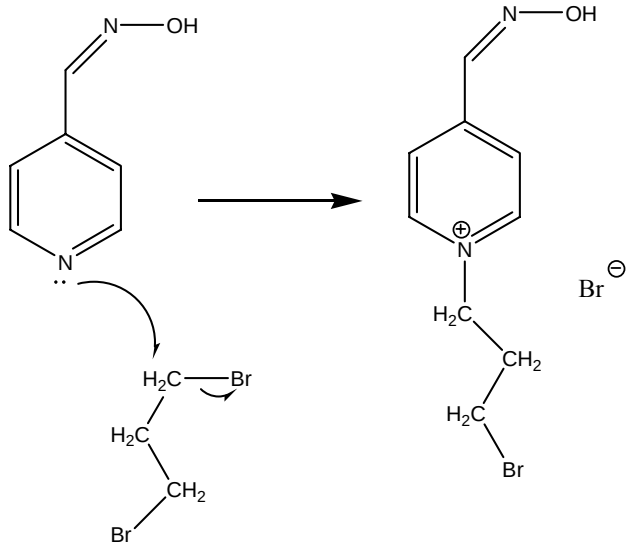


Fig. 28: 4- pyridinealdoxime- *N*- propyl bromide reaction mechanism

This procedure was initially an attempt to synthesize TMB-4 in acetone, but ^{1}H-NMR- data showed that the synthesis had not proceeded as planned. As soon as the propane bridge was added to one 4- pyridinealdoxime molecule, the product, 4- pyridinealdoxime- *N*-propyl bromide, got a positive charge and hence precipitated from the nonpolar solution. As soon as the pellet was formed, no further addition could occur. This was in other words a suitable method for isolating a reaction product. 4-pyridinealdoxime- *N*- propyl bromide was used further in a two- step synthesis of TMB-4.

5.1.8 TMB- 4 from 4- pyridinealdoxime and 4- pyridinealdoxime- N- propyl bromide. Synthesis via a two- step route

4- pyridinealdoxime- *N*- propyl bromide from the previous synthesis in acetone and 4- pyridinealdoxime was used in stead of 4- pyridinealdoxime and dibrompropane. Apart from this, the protocol was according to Poziomek *et al.* 1958. The reaction mechanism for the two- step synthesis is shown in Fig. 29.

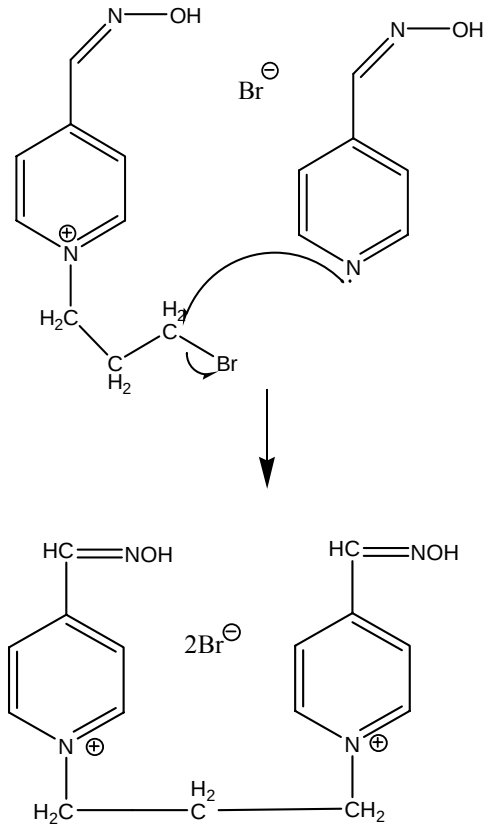


Fig. 29: TMB-4 reaction mechanism by a two- step route from 4- pyridinealdoxime and 4- pyridinealdoxime- *N*- propyl bromide

^{1}H-NMR- data and MS- data both confirmed that the reaction product was in fact TMB-4.

5.2 Attempted synthesis

5.2.1 2- pyridinealdoxime- N- propyl bromide in ethanol

2- pyridinealdoxime-*N*-propyl bromide was synthesized following the same procedure as developed for synthesis of 4- pyridinealdoxime- *N*- propyl bromide (Poziomek *et al.* 1958), apart from the oxime- halide ratio. In this synthesis the oxime- halide ratio was reduced from 1:5 to 3:5. The reaction mechanism is shown in Fig. 30.

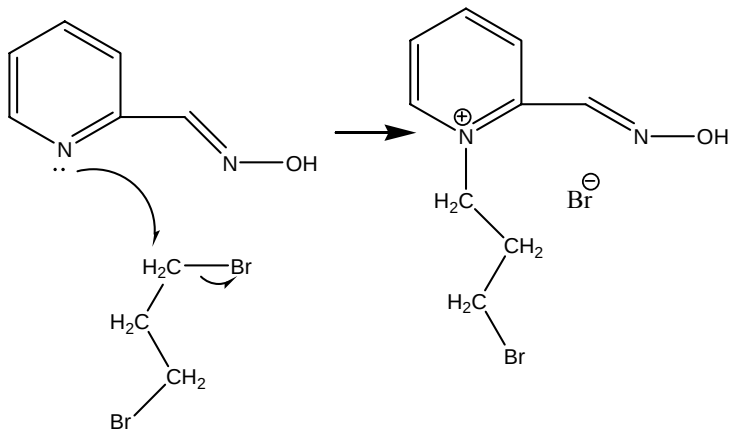


Fig. 30: Reaction mechanism of the synthesis of 2- pyridinealdoxime- *N*- propyl bromide

The yield of the reaction product, 46.4%, was quite high considering Poziomek *et al*. 1958, where attempts to add large a 2- hydroxyl ethyl group to the pyridine nitrogen in 2- oximes (e.g. the oxime group located next to the nitrogen) resulted in a 9.9% yield (Poziomek *et al.* 1958). The propyl bridge is a fairly bulky group, and steric hindrance was expected to be a factor of importance.

The ^{1}H- NMR specter of the reaction product after synthesis was difficult to interpret, but comparison with the ^{1}H- NMR spectra of 2- pyridinealdoxime and 2- PAM indicated that the synthesis was unsuccessful. MS- ES data however show that the reaction product contained a substance with a molecular weight identical to the weight of the 2- pyridinealdoxime- *N*- propyl cation. The rest of the reaction product was impurities, probably unreacted 2- pyridinealdoxime and dibromopropane. ^{1}H- NMR- data for this synthesis was difficult to interpret and the substance seems to be impure.

To isolate 2- pyridinealdoxime- *N*- propyl bromide**,** the reaction product was extracted with distilled water and chloroform. Knowing that unreacted 2- pyridine aldoxime is a lipophilic substance and that the wanted reaction product is polar, they could easily be separated. MS-ES data of the substance in the aqueous layer showed that the reaction product of interest was successfully isolated. ^{1}H- NMR- data on the other hand were hard to interpret. The ^{1}H-NMR specter for the aqueous layer suggested that no propyl chain had been added to the pyridine nitrogen, and the specter showed impurities. The same situation applied for the substance isolated in the organic layer. MS- data suggested this was 2- pyridinealdoxime, but the ^{1}H-NMR specter was inconclusive and did not support this conclusion. With this lack of data it was impossible to decide whether the isolated products were in fact 2-pyridinealdoxime-*N*-propyl bromide and 2-pyridinealdoxime.

5.2.2 2- pyridinealdoxime- N- propyl bromide in acetone

2- pyridinealdoxime- *N*- propyl bromide was attempted synthesized in acetone in a second experiment, following the protocol suggested by Shek *et al*. 1975. The reaction mechanism is identical to the mechanism performed in ethanol, and is found in Fig. 25.
The ^{1}H- NMR specter of the reaction product show unreacted 2- pyridine aldoxime and dibromopropane. A small amount of dibromopropane was added to a sample, and a new ^{1}H-NMR specter was recorded. The specter showed no new peaks, and the signals emitted from dibromopropane got stronger, whereas the peaks from 2- pyridine aldoxime were unaffected. If this synthesis had proceeded as planned, addition of dibromopropane would have yielded new peaks in the ^{1}H- NMR specter.

5.2.3 1- 1' - trimetylenebis- 2, 2- (hydroxyiminomethyl)- pyridine bromide

The reaction product from synthesis 6.*3.1*, 2- pyridinealdoxime- *N*- propyl bromide, was used in the synthesis of 1- 1'- trimetylenebis- 2, 2- (hydroxyiminomethyl)- pyridine bromide. The experimental protocol was derived from the protocol described by Poziomek *et al.* 1958. The reaction mechanism and structure of 1- 1'- trimetylenebis- 2, 2- (hydroxyiminomethyl) - pyridine bromide is shown in Fig. 31.

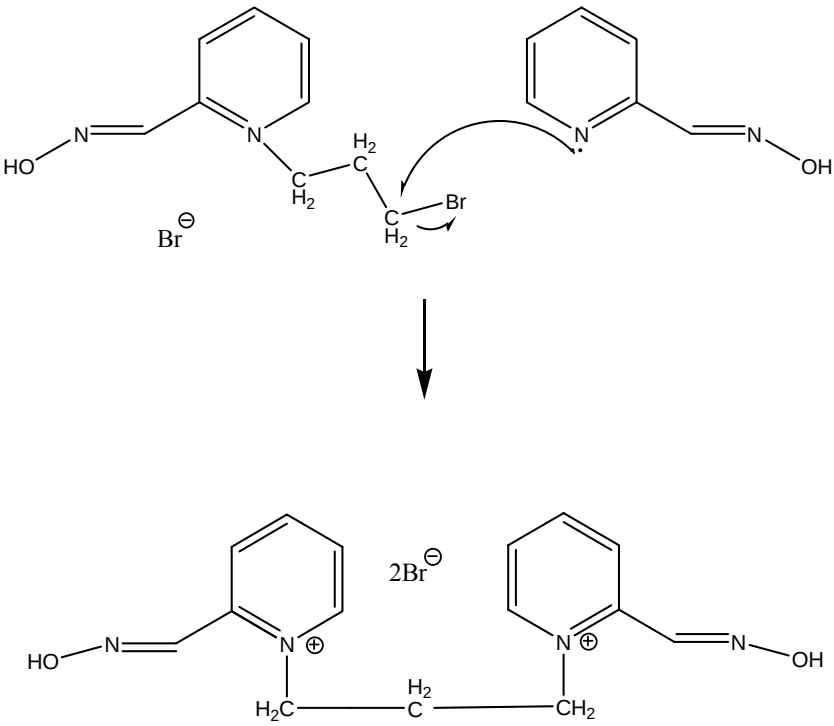


Fig. 31: Reaction mechanism and structure of 1-1'-trimetylenebis-2, 2- (hydroxyiminomethyl) - pyridine bromide

MS- ES and ^{1}H- NMR data showed unreacted 2- pyridinealdoxime, and the possible reaction product 2- pyridinealdoxime- *N*-propyl bromide was not visible in any of the spectra.

5.2.4 1- 1'- trimetylenebis- 2, 4- (hydroxyiminomethyl)- pyridine bromide

The reaction product from synthesis 6.*3 .1,* 2- pyridinealdoxime- *N*- propyl bromide, was used in the synthesis of 1-1'-trimetylenebis-2, 4- (hydroxyiminomethyl)-pyridine bromide.
The protocol was identical to the protocol used in the synthesis of 1-1'-trimetylenebis-2, 4-(hydroxyiminomethyl)-pyridine bromide, and was derived from the procedure described by Poziomek *et al.* 1958. The structure of the reaction product and the synthesis mechanism is shown in Fig. 32.

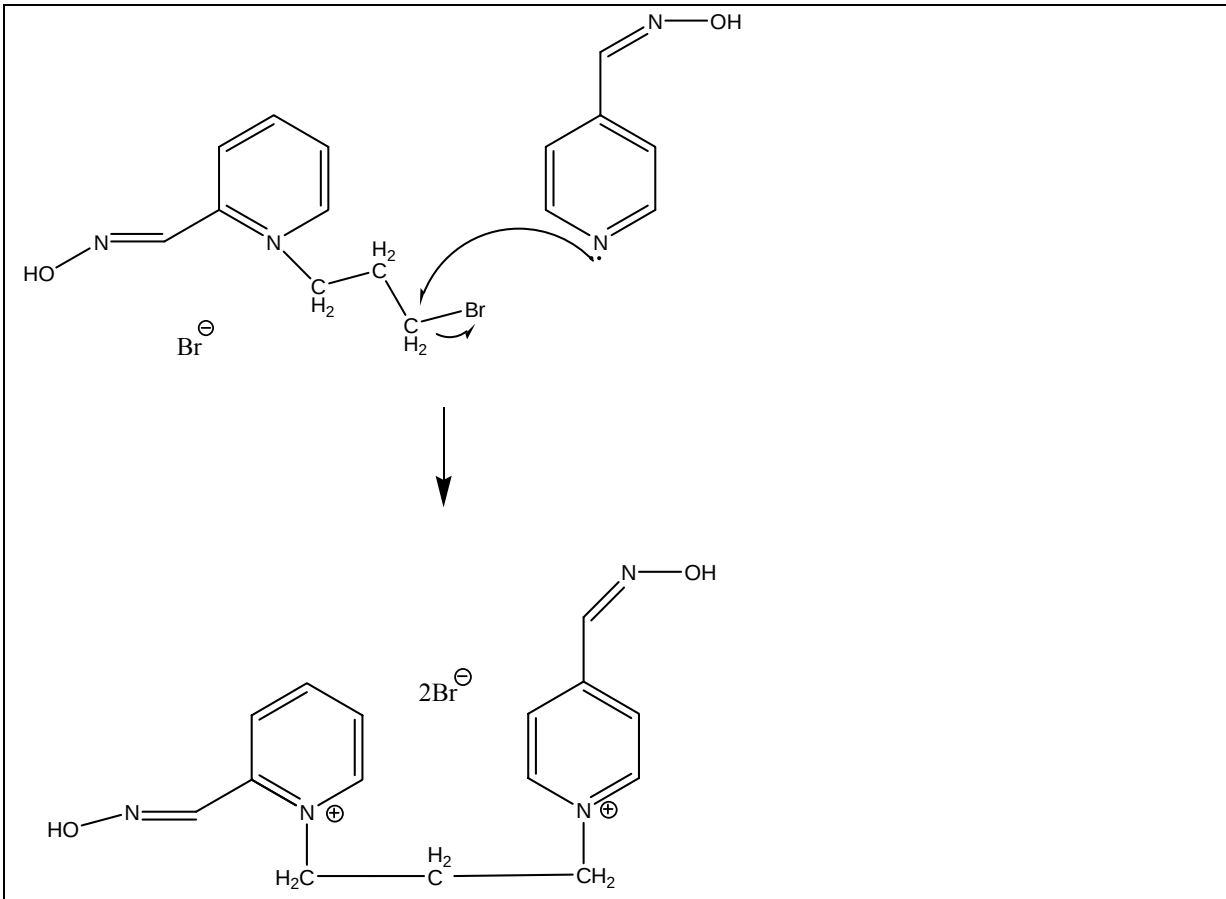


Fig. 32: Structure of 1-1'-trimetylenebis-2, 4-(hydroxyiminomethyl)-pyridine bromide

MS- and ^{1}H- NMR data showed unreacted 4- pyridinealdoxime. 2- pyridinealdoxime- *N*-propyl bromide was not to be found in any of the spectra.

5.2.5 Reduction of TMB- 4 with $NaBH_4$

In three separate experiments, TMB-4 was attempted reduced following the procedure suggested by Bodor *et al.* 1975. The intention was to remove one or more of the double bonds in the two pyridine rings. The reaction product with two reduced pyridine rings was expected to be found in the organic layer. A substance with soap- like properties was isolated from the interface between the etheral and the aqueous layers during extraction. The insolubility of the reaction product in either of the layers might indicate that only one of the pyridine rings was reduced. If this was the case, one ring would have become lipophilic, and the other unreduced ring would have stayed polar due to the positive charge on the pyridine nitrogen. In addition to being insoluble in water and diethyl ether, the substance was insoluble in a number of other solvents. However, there was a 10:1 ration of $NaBH_4$/ TMB-4. With this excess of reducing agent, one would expect that at least some of the reaction product had two reduced rings. Being insoluble in any solvent tested, it was impossible to record a ^{1}H- NMR specter, and MS- data show that the reduction failed. The identity of the reaction product was not clarified.

5.2.6 Reduction of TMB-4 with $LiBH_4$ in diethyl ether

Since TMB- 4 had proven to be at least partly resistant to reduction by $NaBH_4$, the experiment was repeated with $LiBH_4$, a more potent reducing agent. The reduction was performed in diethyl ether. TMB- 4, being a salt, is insoluble in ether, but $LiBH_4$ is most active in this solvent. The idea was that TMB-4 would dissolve as it got reduced by $LiBH_4$. No reduced TMB-4 or any other lipophilic substance was detected in the organic layer, and one could speculate that the insolubility of TMB- 4 in diethyl ether could not be overpowered by the reducing action of $LiBH_4$. The ^{1}H- NMR specter recorded from the substance isolated from the aqueous layer is very complex, and some signals from unreduced TMB- 4 is found in the complicated pattern. This, in addition to the fact that no compound of any kind was isolated from the organic layer, suggests that the reduction failed.

5.3 Conclusion

Pralidoxime was successfully synthesized and isolated, and reduced according to Bodor *et al.* 1975. The reaction product is in fact not pro- PAM, which is a dihydropyridine, but a tetrahydropyridine compound. The synthesis of pro- PAM is a four- step procedure, and the decision was made to focus on trying to synthesize new mono- and bispyridinium oximes instead of spending time synthesizing a well- documented and not very effective prodrug. 4-PAM (4-pyridinealdoxime-N-methyl iodide) was also synthesized and reduced.

TMB- 4 was successfully synthesized twice: In a one- step synthesis in alcohol and via a two-step synthesis where 4-pyridinealdoxime-N-propyl bromide was first synthesized in acetone, and thereafter allowed to react with 4- pyridinealdoxime in alcohol. This method is an excellent way of separating a reaction product, and can be used in manufacturing unsymmetrical bispyridinium oximes.

The reduction of TMB- 4 with $NaBH_4$ and $LiBH_4$ proved futile. The cause of this is unknown, but is probably related to the bispyridinium structure of the oxime. No prodrugs of oximes were successfully synthesized in this study. None of the 2- and 4- PAM reduction products are prodrugs, and no identifiable reaction product was isolated after the TMB- 4 reductions.

Attempts of synthesizing mono- and bispyridinium oximes with addition of propyl groups to 2- pyridinealdoxime failed; this is most likely due to steric hindrance and shielding of the pyridine nitrogen by the oxime group in 2- position.

To conclude, only some of the goals of this study were reached.

- Pralidoxime and TMB- 4 were successfully synthesized. Pralidoxime was reduced and the structure of the reaction product was confirmed as a tetrahydropyridine oxime described in literature.
- New methods were developed for synthesizing mono- and bispyridinium oximes such as pralidoxime and TMB- 4
- TMB- 4 resisted reduction when following the protocols described in Experimental.
- Synthesizing new 2, 2- and 2, 4- bispyridinium oximes proved difficult due to steric hindrance around the pyridine nitrogen in 2- pyridinealdoxime.
- No oxime prodrugs were successfully synthesized.

6 EXPERIMENTAL

6.1 Materials and methods

6.1.1 Reagents, solvents and solution

Reagents

1, 3- dibromopropane, 99%	Sigma Aldrich
Iodomethane (methyl iodide) 99.5%	Sigma Aldrich
Lithium borohydride 95%	Sigma Aldrich
4- pyridinealdoxime 98%	Sigma- Aldrich
Syn- 2- pyridinealdoxime 99+%	Sigma Aldrich
Sodium borohydride 95+%	Merck

Other reagents used in extraction and drying

Magnesium sulphate, anhydrous	Sigma- Aldrich
Sodium carbonate anhydrous powder	NMD

Solvents

Absolute ethanol	Arcus
Acetone puriss	Fluka
Methanol for liquid chromatography	Merck

Deuterated solvents

Acetonitrile- d_3, 99.8%	Cambridge Isotope Laboratory
Chloroform- d, 99.8% atom% D	Sigma- Aldrich
Deuterium oxide, 100.0% atom % D	Sigma- Aldrich
Methanol-d_4, 99.8%+ atom % D	Sigma- Aldrich
(Methyl sulfoxide)- d_6, “100” (min. 99.96 atom%D)	Sigma- Aldrich

Solvents for TLC, extraction and recrystallization

Acetonitrile for analysis	Merck
Diethyl ether for analysis	Riedel-de- Haën
Hexane HPLC grade	Rathburn Chemicals
Purified water	Elga PureLab Maxima HPLC (ionexchanged water HPLC grade)
Toluene	BDH laboratory supplies
Trichloromethane extra pure, stabilized with approx. 1% ethanol	Riedel- de- Haën

6.1.2 Methods

NMR analysis

^{1}H- NMR spectra were recorded on a BrukerAvanceDPX 200 instrument at 200 MHz.

^{13}C- NMR spectra were recorded on a BrukerAvanceDPX 300 instrument at 300 MHz. The values for the referance peaks are listed below (Williams, Fleming 1990).

Reference peaks:	$CDCl_3$: ^{1}H δ 7.25, ^{13}C δ 77.0
	DMSO-d_6: ^{1}H δ 2.50, ^{13}C δ 39.7
	CD_3OD: ^{1}H δ 3.34, ^{13}C δ 49.0

MS analysis

All MS- ES (electrospray) were recorded on a Waters/ Micromass Q- TOF2 instrument with either positive and negative ionization

MS- EI were recorded on a VG Prospec, Fisons Instruments

^{1}H- NMR estimations

^{1}H- NMR estimates were obtained using ChemDraw Ultra 9.0 ChemNMR

6.2 Synthesis

6.2.1 Pralidoxime/2- PAM in acetone

2- PAM was synthesized according to a method derived from the work of Shek *et al.* 1975. The structure of pralidoxime iodide is found in Fig. 33 below. 2- pyridinealdoxime (2.44 g, 0.020 mol) was dissolved in 35 ml of dry acetone in a 50 ml round bottle flask. The flask was placed on a silicone oil bath on a combined heater/ magnet stirrer. A magnet was added and the solution was stirred until all 2- pyridine aldoxime was dissolved. Methyl iodide (2.84 g, 0.020 mol) was added drop wise with a glass pipette. The mixture was stirred for 24 hours at a temperature of approximately 50°C with a reflux cooler-system attached. The yellow product was separated from the solution by filtration, washed with acetone and vacuum dried over night. The structure of the reaction product was verified by ^{1}H- NMR- and MS- ES analysis.

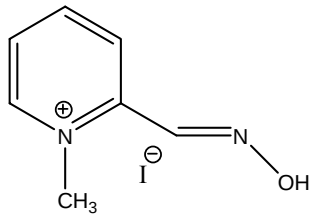


Fig. 33: Structure of pralidoxime iodide

Yield: 2.334 g (44.2%)

Characteristics: a fine, pale yellow powder

^{1}H- NMR (DMSO-d_6): δ 13 (s, OH), 9.01 (d), 8.66 (s), 8.57 (t), 8.39 (d), 8.1 (t)

MS- ES: Mw 137 g/mol (cation).Total Mw salt: 264 g/mol

6.2.2 Pralidoxime/2- PAM in absolute ethanol

2- PAM was synthesized following a method developed by Poziomek *et al.* 1957.
The structure of 2- PAM is found in Fig. 33 above. 2- pyridinealdoxime (2.44 g, 0.02 mol) was dissolved in 50 ml of absolute ethanol in a 100 ml round bottle flask. The flask was placed on a silicone oil bath on a combined heater/ magnet stirrer. A magnet was added and the solution was stirred until all 2- pyridinealdoxime was dissolved. Methyl iodide (5.66 g, 0.04 mol) was added drop wise with a glass pipette.
The mixture was stirred for 144 hours at a temperature of approximately 80°C with a reflux cooler-system attached. Diethyl ether was added to achieve complete precipitation, and the yellow reaction product was separated from the solution by filtration. The product was thereafter recrystallized from absolute ethanol and diethyl ether, and dried in a vacuum pump over night.

Yield: 3.83 g (72.6%)
Characteristics: A fine, pale yellow powder

Extraction

The reaction product was dissolved in 100 ml distilled water and the pH was adjusted to pH 6-7 by adding small amounts of $Ca(OH)_2$. The aqueous solution was extracted with 50 ml chloroform three times. The three organic layers were combined, dried over anhydrous $MgSO_4$, and the chloroform was evaporated on rotavapor. The aqueous layer was freeze dried over night and thereafter vacuum dried to remove any hemihydrates that may have been created during the freeze drying process. The structure of the product from the aqueous layer was confirmed by ^{1}H-NMR.

Yield organic layer: nothing
Yield aqueous layer: 3.59 g (64.1%)
Characteristics: a fine, pale yellow, fluffy powder.
^{1}H- NMR (DMSO-d_6): δ 13 (s, OH), 9.01 (d), 8.68 (s), 8.57 (t), 8.36 (d), 8.1 (t)

6.2.3 Reduction of 2- PAM to 1-methyl- 1, 2, 3, 6- tetrahydropyridine- 2- carbaldoxime with $NaBH_4$

2-PAM was reduced following a procedure developed by Bodor *et al.* 1975. The structure of the reduction product 1- methyl- 1, 2, 3, 6- tetrahydropyridine- 2- carbaldoxime is found in Fig. 34. 2- PAM from synthesis *6.2.1* (2.67 g, 0.01mol) was dissolved in 50 ml methanol and put on a magnet stirrer. $NaBH_4$ (1.96 g, 0.052 mol) was added in small portions while stirring at room temperature. After addition of $NaBH_4$, the reaction mixture was stirred for 45 minutes at room temperature. Most of the methanol was evaporated on rotavapor, and the residue was dissolved in 25 ml of distilled water. The aqueous solution was saturated with Na_2CO_3 and extracted with ether. The etheral phase was dried over anhydrous $MgSO_4$ over night, and the solvent was evaporated on rotavapor. The remaining solid was recrystallized from a mixture of toluene and *n*-hexane. The structure of the reaction product was confirmed by ^{13}C- NMR, ^{1}H- NMR and MS- ES data.

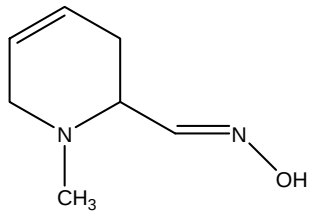


Fig. 34: Structure of 1- methyl- 1, 2, 3, 6- tetrahydropyridine- 2- carbaldoxime

Yield: 0.837 g (59.5%)

Characteristics: a pale peach- colored granular powder

^{1}H- NMR (DMSO-d_6): δ 10.9 (s, OH), 7.4 (d), 5.7 (s), 3.2-2.7 (m), 2.3-2.1 (m)

^{13}C- NMR (DMSO-d_6): 25, 45, 53, 60, 124, 127, 150 ppm

MS- ES: Mw 141 g/mol

Melting point: 104.4-106°C

6.2.4 4- pyridinealdoxime- N- methyl iodide/ 4- PAM

4- PAM was synthesized according to a method derived from the work of Shek *et al.* 1975. The structure of 4- PAM is shown in Fig. 35. 4- pyridinealdoxime (2.44 g, 0.020 mol) was dissolved in 35 ml of dry acetone in a 50 ml round bottle flask. The flask was placed on a silicone oil bath on a combined heater/ magnet stirrer. A magnet was added and the solution was stirred until all 4- pyridine aldoxime was dissolved. Methyl iodide (2.84 g, 0.020 mol) was added drop wise with a glass pipette. The mixture was stirred for 24 hours at a temperature of approximately 50°C with a reflux cooler-system attached. The yellow product was separated from the solution by filtration, washed with acetone and vacuum dried over night. The structure was confirmed by ^{13}C- NMR, in addition to confirmation by ^{1}H-NMR and MS- ES analysis.

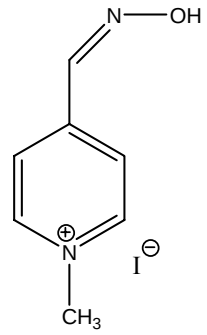


Fig. 35: Structure of 4- pyridinealdoxime- *N*- methyl iodide/ 4-PAM

Yield: 4.472 g (84.7%)

Characteristics: a fine, yellow powder, stronger color than 2- PAM

^{1}H-NMR (DMSO-d_6): δ 12.9 (s, OH), 9.1 (m), 8.6 (s), 8.3 (m), 4.5 (s)

MS- ES: Mw 137 g/mol (cation). Total Mw salt: 264 g/mol

6.2.5 Reduction of 4-PAM to 1- methyl-1, 2, 3, 6- tetrahydropyridine-4-carbaldoxime with $NaBH_4$

4- PAM was reduced following a procedure developed by Bodor *et al.* 1975. The structure of the reduction product is found in Fig. 36 below. 4- PAM from synthesis 7.*2.4* (2.65 g, 0.010 mol) was dissolved in 50 ml methanol and put on a magnet stirrer. $NaBH_4$ (1.95 g, 0.050 mol) was added in small portions while stirring at room temperature. After addition of $NaBH_4$, the solution was stirred for 45 minutes at room temperature. Most of the methanol was evaporated on rotavapor, and the residue was dissolved in 25 ml of distilled water. The aqueous solution was saturated with Na_2CO_3 and extracted with ether. The etheral layer was dried over anhydrous $MgSO_4$ over night, and the solvent was evaporated on rotavapor. The remaining solid was recrystallized from toluene and *n*- hexane. The structure was confirmed by ^{1}H-NMR, ^{13}C- NMR and MS- ES analysis.

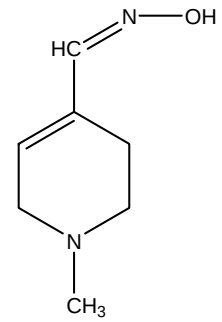


Fig. 36: Structure of 1- methyl- 1, 2, 3, 6- tetrahydropyridine- 4- carbaldoxime

Yield: 0.590 g (41.8%)

Characteristics: a pale yellow-white crystalline powder

^{1}H- NMR (DMSO-d_6): δ 10.9 (s, OH), 7.8 (s), 6.1 (s), 3.1 (s), 2.55 (m), 2.2 (s)

^{13C}- NMR (DMSO-d_6): 23, 48, 52, 55, 134, 138, 150 ppm.

MS- MS: Mw 141 g/mol

Melting point: 149- 150º C

6.2.6 1-1'- trimetylenebis- 4- (hydroxyiminomethyl)- pyridine bromide/ TMB- 4

TMB- 4 was synthesized following a method derived from the work of Poziomek *et al.* 1958. The structure of TMB- 4 is found in Fig. 7d. 4- pyridinealdoxime (3.70 g, 0.03 mol) was dissolved in 50 ml absolute ethanol in a 100 ml round bottle flask. The flask was placed on a silicone oil bath on a combined heater/ magnet stirrer. A magnet was added and the solution was stirred until all 4- pyridinealdoxime was dissolved. Dibromopropane (2.02 g, 0.010 mol) was added drop wise with a glass pipette. The mixture was stirred for 92 hours at a temperature of approximately 80°C with a reflux cooler-system attached. The reaction mixture was cooled to room temperature. Diethyl ether in sufficient amounts was added to achieve complete precipitation. The white reaction product was removed from the solution by filtration and vacuum dried over night. The structure of the reaction product TMB- 4 was confirmed by ^{1}H- NMR and MS- ES analysis.

Yield: 3.248 g (72.8%)
Characteristics: a fine, white powder
^{1}H- NMR (DMSO-d_6): δ 12.84 (s, OH), 9.2 (d), 8.5 (s), 8.3 (d), 4.8 (m), 2.7 (m)
MS- ES: Mw 285 g/mol (cation). Total Mw salt: 446 g/mol

6.2.7 4- pyridinealdoxime- N- propyl bromide

4- pyridinealdoxime- *N*- propyl bromide was synthesized according to a method derived from the work of Shek *et al.* 1975. The structure of the reaction product is found in Fig. 37 below. 4- pyridinealdoxime (2.44 g, 0.020 mol) was dissolved in 35 ml of dry acetone in a 50 ml round bottle flask. The flask was placed on a silicone oil bath on a combined heater/ magnet stirrer. A magnet was added and the solution was stirred until all 4- pyridinealdoxime was dissolved. Dibromopropane (2.02 g, 0.010 mol) was added drop wise with a glass pipette. The mixture was stirred for 24 hours at a temperature of approximately 50°C with a reflux cooler-system attached. The grey-white product was separated from the solution by filtration, washed with acetone and vacuum dried over night. The structure of 4- pyridinealdoxime- *N*- propyl bromide was confirmed by ^{1}H- NMR and MS- ES analysis.

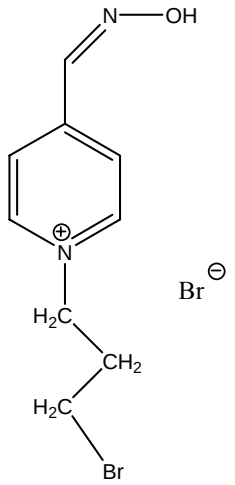


Fig. 37: Structure of 4- pyridinealdoxime- *N*- propyl bromide

Yield: 0.764 g (17.1%)

Characteristics: a fine, grey- white powder.

^{1}H- NMR (DMSO-d_6): δ 12.8 (s, OH), 9.1 (m), 8.5 (s), 8.2 (m), 4.6 (m), 3.65 (m), 2.4 (m)

MS- ES: Mw 243 g/mol (cation)

6.2.8 TMB- 4 from 4- pyridinealdoxime and 4- pyridinealdoxime- N- propyl bromide. Synthesis via a two- step route

TMB- 4 via this two-step route was synthesized following a method derived from the work of Poziomek *et al*. 1958 and Shek *et al.* 1975. The structure of TMB- 4 is shown in Fig. 7b.
4- pyridinealdoxime- *N*- propyl bromide from synthesis 6*.2.7* (0.4 g, 1.23 mmol) was dissolved in 35 ml of absolute ethanol in a 100 ml round bottle flask. The flask was placed on a silicone oil bath on a combined heater/ magnet stirrer. A magnet was added and the solution was stirred until all 4- pyridinealdoxime- *N*- propyl bromide was dissolved. 4- pyridinealdoxime (0.4 g, 3.3 mmol) was added. The mixture was stirred for 72 hours at a temperature of approximately 73°C with a reflux cooler-system attached. Diethyl ether was added to achieve complete precipitation. The white reaction product was removed from the solution by filtration, and vacuum dried over night. The structure of the reaction product TMB- 4 was confirmed by MS- ES and ^{1}H-NMR analysis

Yield: 0.183 g (33.4%)
Characteristics: a fine, off-white powder
^{1}H- NMR (DMSO-d_6): δ 12.8 (s, OH), 9.2 (d), 8.5 (s), 8.2 (d), 4.8 (m), 2.7 (m)
MS- ES: Mw 285 g/mol (cation). Total Mw salt: 446 g/mol

6.3 Attempted synthesis

Since this thesis has included extensive work on method development, not all experiments have yielded the desired results. A brief description of these experiments is given here. They are discussed further in chapter 5. The title reflects the wanted reaction product and not necessarily the actual reaction product, which sometimes could not be identified.

6.3.1 2- pyridinealdoxime- N- propyl bromide in ethanol

2- pyridineldoxime- *N*- propyl bromide synthesized following a method derived from the protocol described by Poziomek *et al*.1958. The structure of the attempted reaction product is shown in Fig. 38. 2- pyridinealdoxime (3.66 g, 0.03 mol) was dissolved in 50 ml of absolute ethanol in a round bottle flask. The flask was placed on a silicone oil bath on a combined heater/ magnet stirrer. A magnet was added and the solution was stirred until all 2- pyridinealdoxime was dissolved. Dibromopropane (10.10 g, 0.050 mol) was added drop wise with a glass pipette. The mixture was stirred for 48 hours at a temperature of approximately 85°C with a reflux cooler-system attached. Diethyl ether in sufficient amounts was added to achieve complete precipitation. The brown product was separated from the solution by filtration, washed with ether and vacuum dried over night.

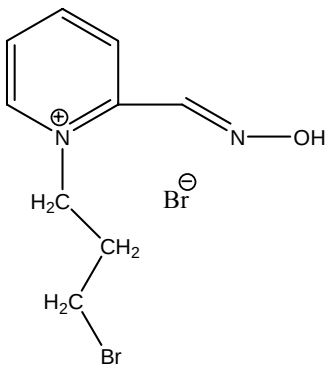


Fig. 38: Structure 2- pyridinealdoxime- *N*- propyl bromide

Yield: 4.5 g (46.4%)
Characteristics: a brown, coarse, “lumpy” powder
MS-ES: 60% Mw 243 g/mol (cation)
The ^{1}H- NMR specter was inconclusive

Extraction of the reaction product

3.0 g of the reaction product from the synthesis above was dissolved in 100 ml H_2O and extracted three times with 50 ml chloroform. The aqueous layer was freeze dried for two days and the organic layer was removed on rotavapor and thereafter vacuum dried.

Yield aqueous layer: 2.6 g
MS-ES aqueous layer: 100% Mw 243 g/mol (cation)
Yield organic layer: 0.089 g
MS-EI organic layer: Mw 122 g/mol
The ^{1}H- NMR spectra was inconclusive for the compounds isolated from both layers.

6.3.2 2- pyridinealdoxime- N- propyl bromide in acetone

2- pyridinealdoxime- *N*- propyl bromide was synthesized in a second experiment according to a protocol derived from the procedure described by Shek *et al.* 1975. The structure of the expected reaction product is shown in Fig. 38 above. 2- pyridinealdoxime (1.22 g, 0.010 mol) was dissolved in 35 ml of dry acetone in a 50 ml round bottle flask. The flask was placed on a silicone oil bath on a combined heater/ magnet stirrer. A magnet was added and the solution was stirred until all 2- pyridine aldoxime was dissolved. Dibromopropane (4.04 g, 0.020 mol) was added drop wise with a glass pipette. The mixture was stirred for 24 hours at a temperature of approximately 50°C with a reflux cooler-system attached. The ^{1}H- NMR specter recorded showed unreacted 2- pyridinealdoxime and dibromopropane.

6.3.3 1- 1'- trimetylenebis- 2, 2- (hydroxyiminomethyl)- pyridine bromide

1-1'- trimetylenebis- 2- (hydroxyiminomethyl)- pyridine bromide was attempted synthesized using a protocol derived from the method suggested by Poziomek *et al.* 1958. The structure of the attempted reaction product is found in Fig. 39. 2- pyridinealdoxime- N- propyl bromide (0.5 g, 1.5 mmol) from synthesis 6.*3.1* and 2- pyridinealdoxime (0.94 g, 7.7 mmol) was dissolved in 35 ml methanol. The flask was placed on a silicone oil bath on a combined heater/ magnet stirrer. The mixture was stirred for 96 hours at 68°C. The methanol was removed in rotavapor, and the product was dried in a vacuum pump over night.

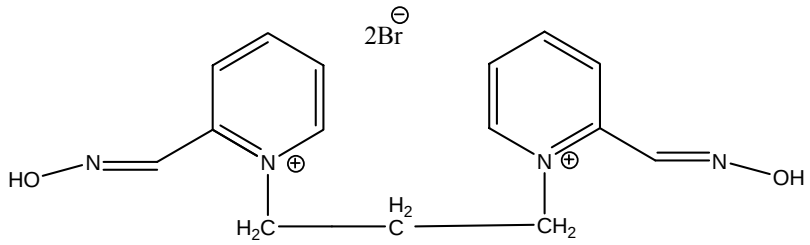


Fig. 39: Structure of 1- 1'- trimetylenebis- 2, 2 - (hydroxyiminomethyl)- pyridine bromide

Yield: 1.32 g

^{1}H- NMR and MS- data showed the synthesis was unsuccessful.

6.3.4 1- 1'- trimetylenebis- 2, 4- (hydroxyiminomethyl)- pyridine bromide

1- 1'- trimetylenebis- 2, 4- (hydroxyiminomethyl)- pyridine bromide was synthesized following the same procedure as synthesis 6.*3.3* (Poziomek *et al.* 1958). The structure of the attempted reaction product is shown in Fig. 40. 2- pyridinealdoxime- *N*- propyl bromide (0.5 g, 1.5 mmol) from synthesis 6.*3.1* and 4- pyridinealdoxime (0.94 g, 7.7 mmol) was dissolved in 35 ml methanol. The flask was placed on a silicone oil bath on a combined heater/ magnet stirrer. The mixture was stirred for 96 hours at 68°C. The methanol was removed in rotavapor, and the product was dried in a vacuum pump over night.

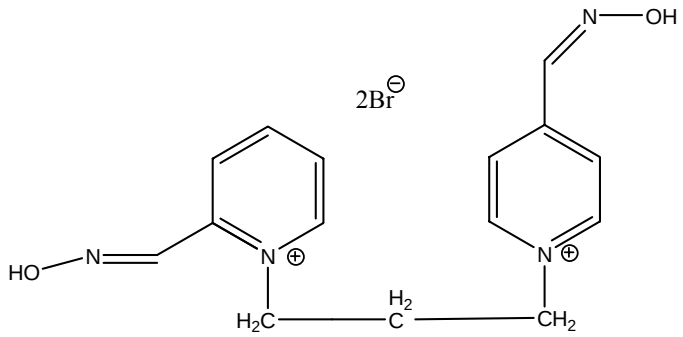


Fig. 40: Structure of 1- 1'- trimetylenebis- 2, 4- (hydroxyiminomethyl) - pyridine bromide

Yield: 1.25g

The MS- and ^{1}H- NMR data showed the synthesis was unsuccessful.

6.3.5 Reduction of TMB-4 with $NaBH_4$

The reduction of TMB-4 was performed according to the protocol described by Bodor *et al.* 1975. The structure of TMB- 4 is shown in Fig.7d. No structure of the attempted reduction product is shown, since it was impossible to tell which double bonds, if any, would be removed. TMB-4 (1.5 g, 3.36 mmol) was dissolved in 50 ml methanol. $NaBH_4$ (1.17g, 0.036 mol) was added in small portions while stirring at room temperature. After addition of $NaBH_4$, the solution was stirred for 45 minutes at room temperature. Most of the methanol was evaporated on rotavapor, and the residue was dissolved in 25 ml of distilled water. The aqueous solution was saturated with Na_2CO_3 and extracted with ether. A pale yellow substance with apparent amphiphilic properties was located at the interface between the two layers. This substance was removed from the solution with filtration and vacuum dried over night. The etheral layer was dried over anhydrous $MgSO_4$ over night and the ether was removed on rotavapor. The substance isolated from the interface between the two layers was insoluble in a number of solvents, such as dimethyl sulfoxide (DMSO), chloroform, acetonitrile, acetone, methanol and water, and it was therefore impossible to record a NMR-specter.

6.3.6 Reduction of TMB-4 with $LiBH_4$ in diethyl ether

TMB-4 was attempted reduced following the same procedure used in previous reductions (Bodor *et al.* 1975). Once again, no structure of a possible reaction product is shown, since there was no way of knowing what the actual structure would be if any double bonds were removed. TMB-4 (0.25 g, 0.56 mmol) was dissolved in 35 ml diethyl ether. $LiBH_4$ (0.024 g, 1.12 mmol) was added while stirring at room temperature. The solution was stirred for 72 hours at room temperature with a $CaCl_2$- tube attached. The reaction mixture was extracted with 3x50 ml distilled water. The etheral layer phase was dried over anhydrous $MgSO_4$ over night, and the solvent was evaporated on rotavapor. Nothing was isolated from the etheral layer. The aqueous layer was freeze dried for a sufficient period of time. A ^1H- NMR specter of the substance isolated from the aqueous layer showed the reduction failed.

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