Synthesis, Characterization and In-Vitro Reactivation Potency of a Series of Substituted Keto and Aldoximes as Acetylcholinesterase Reactivators

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A thesis presented as partial fulfilment of the requirements for the degree of
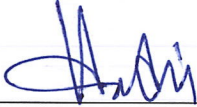
Master of Science in Chemistry

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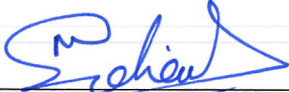
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National University of Sciences & Technology

MS THESIS WORK

We hereby recommend that the dissertation prepared under our supervision by: Aiemen Shahzadi, Regn No. 0000033116 Titled: Synthesis, Characterization and in Vitro Reactivation Potency of a Series of Substituted Keto and Aldoximes as Acetylcholinesterase Reactivators be accepted in partial fulfillment of the requirements for the award of **MS** degree.

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Dedication

I would like to dedicate it to my parents for their unconditional love and support and to my dearest Siblings.

Acknowledgements

Table of Contents

List of Figures

List of Tables

List of Abbreviations

Abbreviations	Explanation
DMF	Dimethyl formamide
FTIR	Fourier transform infrared spectroscopy
kg	Kilogram
ml	Millilitre
mM	Millimolar
MeCN	Acetonitrile
NMR	Nuclear magnetic resonance
TLC	Thin layer chromatography
UV-Vis	Ultraviolet-visible

Abstract

Acetylcholinesterase enzyme (AChE) is rendered inactive by the phosphorylation or phosphorylation of its active site by very hazardous organophosphorus substances, such as nerve agents or organophosphate pesticides. Inhibition of AChE results in an increased amount of acetylcholine in the central and peripheral nervous system which causes excess release of nerve impulses and then uncontrolled actions occur known as Cholinergic Syndrome. We have prepared oximes for the reactivation of such inhibited enzymes. Oximes act as nucleophiles and reactivate enzymes by attacking the phosphoryl group of organophosphates that have inhibited the active site of AChE. Primarily, these aldoximes are represented by mono-quaternary pralidoxime or more extended bis-quaternary compounds like trimedoxime, obidoxime, and H-oxime i.e. HI 6. The inhibition of acetylcholinesterase is contingent of structure of the inhibitors, whereas reactivation of inhibited acetylcholinesterase depends on both the chemical structure of reactivator and inhibitors utilized. No single AChE reactivator can sufficiently reactivate an inhibited enzyme due to the enormous diversity of AChE inhibitors, regardless of the inhibitor's chemical structure. Our study aimed to discover more new reactivators against acetylcholinesterase inhibition activity. For this more bis-oxime structures than those seen in routinely utilized reactivators. Mono-oximes of 4-acetylpyridine, 4-formylpyridine, indole-3-carboxaldehyde, and vanillin have been prepared. Mono-quaternary and bis-quaternary salts of 4-acetylpyridine oxime have also been synthesized by using 1,3-dibromopropane. Some other symmetrical and non-symmetrical bis-oximes were also prepared in this regard. All prepared samples have been characterized by FTIR, ^{1}H NMR, CHN and UV-Vis analysis. Reactivation activity will be estimated in vitro acetyl cholinesterase inhibition activity in human blood and plasma.

1   Chapter 1

1.1   Introduction

Organophosphates, which inhibit serine esterase and protease, are widely used as insecticides, pesticides, softeners, and lubricants. Some are also categorized as chemical warfare weapons. These compounds cause acute toxicity by inhibiting acetylcholinesterase (AChE), an enzyme that transforms the neurotransmitter acetylcholine (ACh). Sarin (GB) and VX were utilized in terrorist attacks in Japan, indicating the harm these compounds offer. Esterase enzymes are blocked by covalently interacting with the active center serine (by phosphorylation or phosphonylation), resulting in "endogenous acetylcholine poisoning". Acetylcholine accumulates as a result of cholinesterase inhibition, stimulating nicotinergic receptors in the adrenal medulla to produce an initial sympathomimetic response. This is followed by an extended parasympathomimetic response triggered by muscarinic receptor activation. Medication that is suitable must be used to regulate both responses. Furthermore, acetylcholine overflows at neuromuscular synapses due to organophosphates and organophosphonates results in a depolarising block that necessitates artificial breathing. Moreover, seizures are produced clinically when central cholinergic receptors are triggered. The standard therapy for OP ester intoxication involves co-administration of an AChE 'reactivator' to restore enzyme function and atropine to counteract the effects of accumulating acetylcholine. Unlike organophosphonates, intoxications with organophosphate insecticides cause phosphorylated AChE, which is readily reactivated by commercial oximes such as obidoxime and other pralidoxime salts. In the last forty years, this fact has led to the synthesis of hundreds of oximes with various structural changes.

1.2 Cholinesterase

Cholinesterase refers to a group of enzymes that aid in the breakdown of acetylcholine (ACh), into acetic acid and choline. This activity is necessary for a neurotransmitter to come back to its dormant condition after being activated. There are two categories involved:

1. Acetylcholinesterase (AChE), also known as acetylcholine acetylhydrolase, is present in various kinds of conducting tissue. These include muscle and nerve tissues, both in the central nervous system (CNS) and peripheral nervous system (PMS), as well as in sensory and motor fibers. It is found across muscarinic/nicotinic and noncholinergic fibers. The level of acetylcholinesterase activity is greater in motor neurons compared to sensory neurons. Acetylcholinesterase is present in membranes of red blood cells, where it serves as the Yt blood group antigen. This enzyme is present in several molecular forms that have comparable catalytic capabilities but vary in their oligomeric assembly and method of attachment to the cell surface. Within the mammalian brain, the predominant form of acetylcholinesterase is a tetrameric structure known as G4, with a minor proportion of a monomeric G1 (4S) form [1].

2. Pseudocholinesterase (BuChE) also referred to as butyrylcholinesterase or acylcholine acylhydrolase, is predominantly located in liver. In contrast to acetylcholinesterase (AChE), butyrylcholinesterase (BuChE) hydrolyzes butyrylcholine at a faster rate than the acetylcholine (ACh) [2].

1.3 Acetylcholinesterase

Acetylcholinesterase enzyme is a highly efficient enzyme found in the nervous system. It is primarily present at cholinergic synapses and myoneural synapses. Its main function is to quickly break down the neurotransmitter i.e. acetylcholine (ACh) into choline and acetate. This

process is crucial for cholinergic transmission in the nervous system. The name acetylcholinesterase was coined in 1949 by Nachmansohn and Augustintion refer to a particular type of cholinesterase enzyme that has the ability to break down acetylcholine more rapidly than other esterases. In 1964, the enzymology committee proposed the term "Acetylcholinesterase" to designate a cholinesterase that is both genuine and particular. The enzyme is distributed in both central and peripheral nerve tissues of many vertebrates, showing a significant degree of diversity. It has been observed that it is limited to glial cells and non-neuronal tissues as well. The six different molecular forms and structural dynamics of the enzyme show molecular variation, which enhances its ability to bind and interact with different ligands. Furthermore, AChE is recognized to have other non-traditional activities that are apart from its catalytic activity, specifically the hydrolysis of Ach. The diverse roles of AChE, both classical and non-classical, demonstrate its widespread presence in both neuronal and non-neuronal tissues, highlighting its adequacy.

Hence, the extensive dispersion and many functions, molecular configurations, and structural dynamics of AChE establish it as a versatile enzyme, necessitating a comprehensive understanding. These findings will aid in the expansion of targeted medications to treat different neurodegenerative diseases linked to this enzyme [3].

1.3.1   Molecular configuration of acetylcholinesterase enzyme

The monomer of AChE has a molar mass of approximately 60,000. It is a spheroid molecule with dimensions of about 45 x 60 x 65 A°. The monomer is composed of a core mixed beta sheet with 12 strands, encircled by fourteen alpha helices (106). Numerous important regions of the protein have been discovered through research: the catalytic active site, which is made up of two subsites; the aromatic gorge, in which the catalytic active site is situated; and the

peripheral anionic site, which is distinct from the catalytic active site and helps to confirm the residues that are within the aromatic gorge and its active site [4]. Figure 1 demonstrates the 3D structure of human acetylcholinesterase [5].
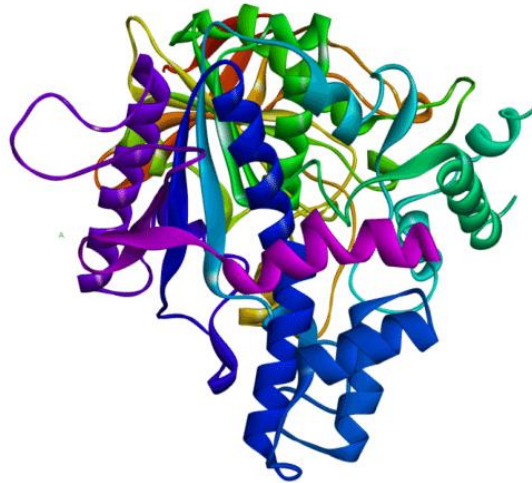
Fig. 1: 3D Structure of human acetylcholinesterase.

1.3.2 Active site of AChE

The molecule possesses an ellipsoidal morphology, measuring around 45 Å in length, 60 Å in width, and 65 Å in height. The monomer of the enzyme is a protein with an α/β structure, comprising 12 strands and a mixed beta sheet core, entangled by 14 alpha helices. The structure's most notable characteristic is a profound and slender ravine, approximately 20 Å in length, which extends halfway into the enzyme and expands at its foundation [6].

 The acetylcholinesterase's active site is positioned 4 Å from the molecule's bottom and contains two subdomains - the "anionic" subdomain and the "esteratic" subdomain, which correspond to active site machinery and choline binding pocket, respectively. The anionic site, which lacks an electric charge and is attracted to lipids, adheres to the positively charged pyridinium amine of the choline component of ACh.

It also binds to competitive inhibitors like as edrophonium and N-methylacridinium, which have quaternary ligands. Additionally, it binds to quaternary oximes, which are capable of effectively reactivating AChE that has been blocked by organophosphates. The 14 amino acids present in the aromatic gorge exhibit a remarkable degree of conservation among many taxa. Tryptophan 84 is an essential aromatic amino acid, and when it is replaced with alanine, there is a significant drop-in enzyme activity, specifically by a factor of 3000. Catalytic triad assembly is made up of three amino acid i.e. serine 200, glutamate 327 and histidine 440, unites the esteratic subsite with other serine hydrolases in its ability to hydrolyze ACh into acetate and choline (Fig. 2) [2].
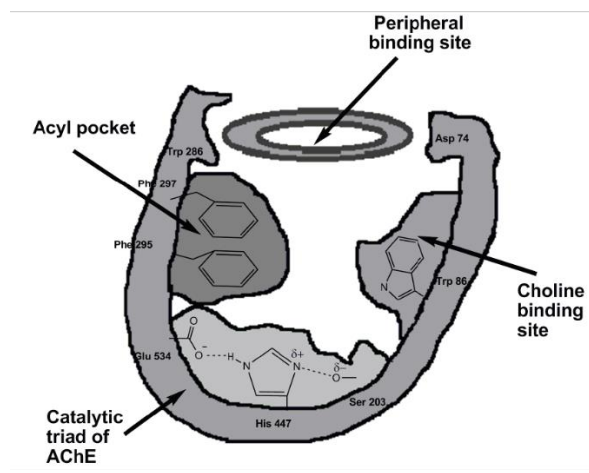
Fig. 2: Schematic representation of AChE binding sites.

1.3.3 Acetylcholine as neurotransmitter

Initially, acetylcholine was the first neurotransmitter that was discovered. All autonomic ganglia, several autonomically innervated organs and some synapses in CNS use acetylcholine as a neurotransmitter. Acetylcholine is a cholinergic transmitter in the autonomic nervous system that functions in the adrenal medulla, preganglionic sympathetic, and parasympathetic neurons. It also functions as a neurotransmitter in every organ that the parasympathetic nervous

system innervates. The sympathetic autonomic nervous system's neurotransmitter for the piloerector muscle and sweat glands is acetylcholine (ACh). In the peripheral nervous system, acetylcholine (ACh) functions as the neurotransmitter at the neuromuscular junction, which joins the skeletal muscle and motor nerve. The central nervous system's interneurons contain the majority of ACh, while a few major cholinergic pathways are distinguished by their lengthy axons [7].

The natural enzyme choline acetyltransferase facilitates a one-step process that produces acetylcholine (ACh). An enzyme that indicates cholinergic neuronal activity is present. A small amount of acetylcholine (ACh) is also found in the cytoplasm, but the majority of acetylcholine in nerve terminals is kept in translucent pouch measuring around 100 nm in size. An energy-dependent pump allows ACh to be absorbed into storage vesicles, where it acidifies the vesicle [7].

Neurotransmission involves the release of ACh from nerve tissue into synaptic cleft, where it adheres to ACh receptors on the post synaptic membrane. This process transmits signal from the nerve. Acetylcholinesterase (AChE), which is also found on the post synaptic membrane, ends the process of signal transmission by breaking down acetylcholine by hydrolysis. With the aid of choline acetyltransferase, presynaptic neuron reabsorbs choline that is released during the breakdown of ACh and uses it to combine with acetyl-CoA to form the neurotransmitter (Fig.3) [2].
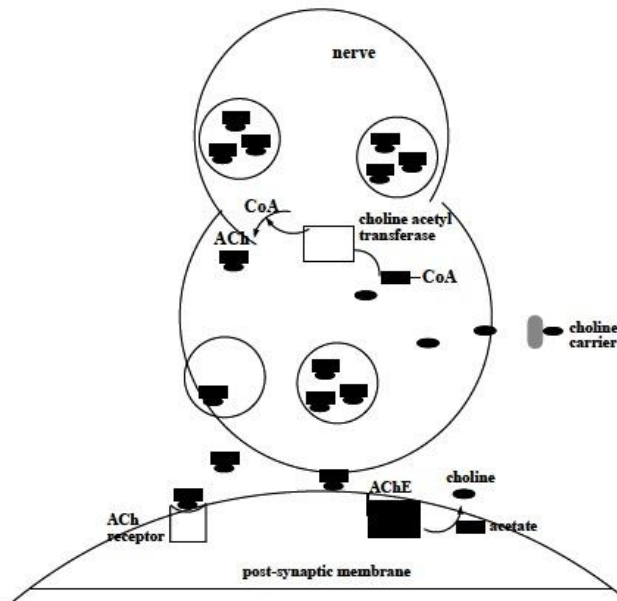
Fig. 3: Mechanism of acetylcholinesterase action in neurotransmission.

1.3.4 Hydrolysis of acetylcholine

The ester bond between acyl group and choline in the substrate is the target of a nucleophilic attack by the OH group of the serine on a carbon atom i.e. positively charged. This results in temporary formation of covalent bond between the enzyme and substrate. The imidazole nitrogen of histidine compound can temporarily create a hydrogen bond with the OH group of serine, which enhances nucleophilic process. The acyl group's transition from serine hydroxyl to water phase can be accelerated by the imidazole group. The enzyme that has been altered with an acyl group then undergoes a rapid breakdown that yields acetic acid, choline and the restoration of the enzyme's active site. The route might be likened to a pipeline, where substrate enters at one end and products release at other end by conformational changes, facilitated by electrostatic and hydrophobic forces (Fig. 4). Acetylcholine is a cholinergic transmitter that is widely present in synapses throughout the neurological systems of mammals. On the membrane of nervous tissues, acetylcholinesterase (AChE) is found, especially at cholinergic synaptic

connections and the endoplasmic reticulum. These membranes are typically involved in the conduction of nerve impulses. Acetylcholinesterase (AChE) is also present in erythrocytes [4].
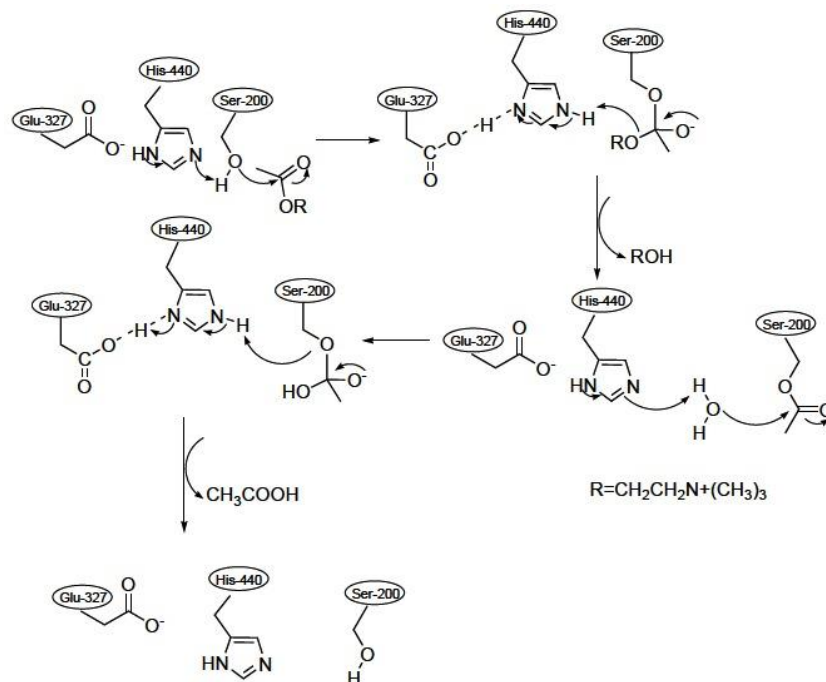
Fig. 4: Mechanism of ACh hydrolysis catalyzed by AChE.

The carboxyl ester undergoes enzymatic cleavage, resulting in the formation of an acyl enzyme complex and released choline. Next, the acyl enzyme is subjected to nucleophilic attack by water molecule, with assistance from histidine 440 group. Acetic acid is released as a result of this cleavage, and free enzyme is restored (Fig. 5) [7].
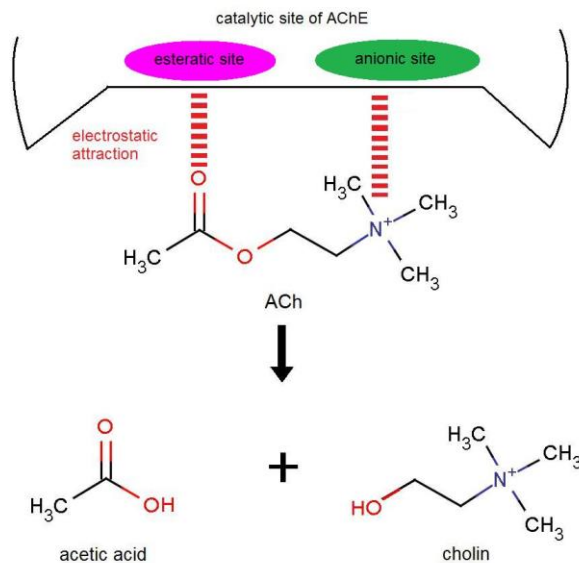
Fig. 5: Hydrolyses of acetylcholine.

1.4 Acetylcholinesterase inhibitors

Anti-cholinesterases, or AChE inhibitors, work by stopping the enzyme cholinesterase from breaking down ACh, which increases the neurotransmitter's effects' duration and concentration. Based on how they work, acetylcholinesterase inhibitors can be separated into two classes: irreversible and reversible. Reversible inhibitors, whether noncompetitive and competitive, are mostly used in medicine, but irreversible AChE activity modulators are linked to negative consequences [7].

1.4.1 Reversible acetylcholinesterase inhibitors

Reversible anticholinesterases are crucial for pharmacologically manipulating the activity of enzymes. These inhibitors are made up of compounds with a variety of functional groups, including quaternary, carbamate and tertiary ammonium groups. Several disorders, such as Alzheimer's disease, glaucoma and as a reactivator to anticholinergic overdose, have been

diagnosed and treated with them. Some reversible acetylcholinesterase inhibitors are shown in Fig. 6.
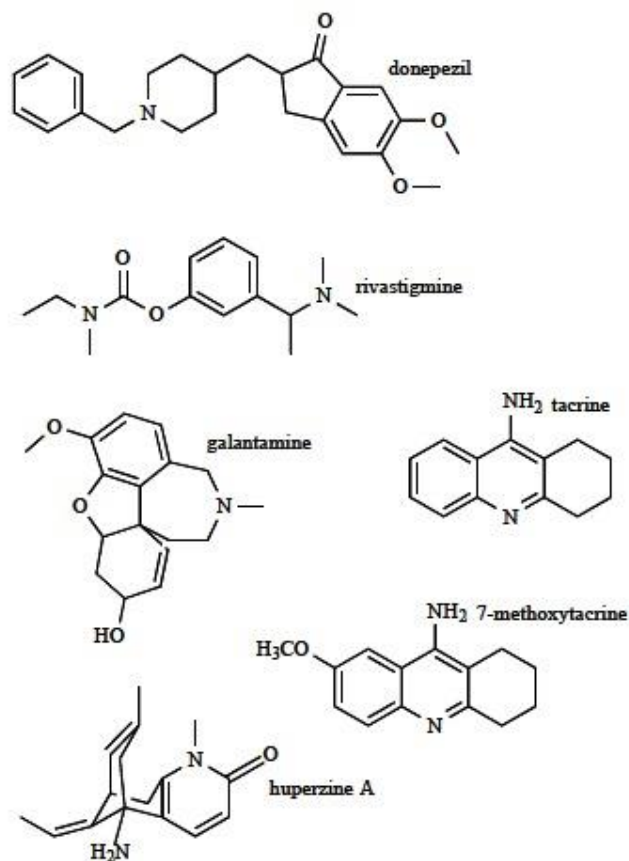
Fig. 6: Reversible acetylcholinesterase inhibitors.

Donepezil

It is a specific and reversible inhibitor of acetylcholinesterase that attaches to peripheral anionic site. By suppressing the production of amyloid plaque, this not only reduces symptoms in management of Alzheimer's disease but this inhibitor also has a preventive effect [8, 9].

Pfizer and Eisai are two pharmaceutical companies which manufacture Aricept. The medication is offered in the form of dissolving tablets and oral solution. It has a 100% oral bioavailability, meaning it is easily absorbed by the body and may effectively pass the blood

brain barrier (BBB). Additionally, donepezil is slowly eliminated from the body. Due to its approximate half-life of 70 hours, this medication can be administered once daily. The medication is offered in dose levels of 5 and 10 mg. Usually, a 5 mg daily dosage is started during treatment, and after a few weeks, it is increased to 10 mg. The dosage should be taken once daily, with a maximum of 23 mg. Additionally, studies conducted recently suggest that donepezil can boost speaking in kids with autism. However, at this time, its application in the treatment of other diseases like schizophrenia, vascular memory loss and Lewy body dementia is not licensed [10, 11].

Rivastigmine

This compound is a potent, slowly reversible carbamate inhibitor that hinders the function of cholinesterase by attaching to the esteratic region of the enzyme's active site. Rivastigmine inhibits both butyrylcholinesterase (BuChE) and acetylcholinesterase (AChE), unlike donepezil which only selectively inhibits AChE. The drug is taken orally in the form of liquid preparations or capsules, and the body absorbs it well. When consumed in a dose of 3 mg, its bioavailability is about 40%. It has few interactions with other drugs and is eliminated in the urine. Over the course of several weeks, the dosage of the medication progressively increased from 1.5mg taken 2 times in a day to 6 mg taken 2 times daily. Every two to four weeks, the dosage is raised by 3mg each day. In addition to being used to cure Alzheimer's disease (AD), rivastigmine is also efficient in treating Parkinson's disease dementia and Lewy bodies memory loss [12, 13].

Galantamine

The Galanthus Woronowii plant yields an alkaloid called Razadyne Nivalin, which is used to treat severe to mild forms of Alzheimer's disease (AD). This substance inhibits AChE in a

highly selective and competitive manner. It is instantaneously effective and readily eliminated. It engages in interactions with the aromatic gorge and the anionic subsite [14, 15].

Furthermore, the drug modifies the activation of nicotinic cholinergic receptors by acting as an allosteric ligand on them. Compared to acetylcholine (ACh) and cholinergic agonists, it binds to distinct sites on the nicotinic receptor. Its special ability to make nicotinic receptors more active (sensitized) in the presence of ACh [16, 17].

Carbamates

Carbamates are a class of chemical compounds that are produced from carbamic acid (NH2COOH). Fig. 7 illustrates the molecular configuration of physiologically active carbamates. In this configuration, X can represent either oxygen or sulfur, specifically in the case of thiocarbamates. R1 and R2 often denote organic or alkyl substituents, although they can also represent hydrogen. On the other hand, R3 is primarily an organic substituent, but occasionally it can represent a metal. These reversible AChE inhibitors have multiple applications. They are used as therapeutic drugs in human medicine for conditions like glaucoma, Parkinson's disease and Lewy bodies. They are also used in veterinary medicine as parasiticides and insecticides. They are also employed as a precaution against organophosphorus compound (OP) toxicity [18].
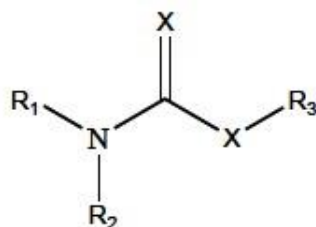
Fig. 7: Chemical structure of physiologically active carbamates.

In agriculture, carbamate chemicals are used as herbicides, insecticides, and fungicides. They belong to the second category of pesticides that impede cholinesterase enzymes. The insecticidal activity is exerted by carbamates that have a methyl group and hydrogen in the positions of R2 and R1, as depicted in the picture above. Desmedipham, methiocarb, carbofuran, pirimicarb and fenoxycarb are examples of carbamate insecticides (Fig. 8). Their ability to inhibit acetylcholinesterase (AChE) reversibly is what gives them their insecticidal effects. Compared to organophosphate (OP) insecticides, which induce serious cholinergic poisoning and irreversibly block acetylcholinesterase (AChE), carbamates are thought to be safer [19, 20].

Studies have indicated that stress can accelerate the absorption of carbamates into central nervous system (CNS), but in a healthy person, blood-brain barrier is not allowed to penetrate [21].
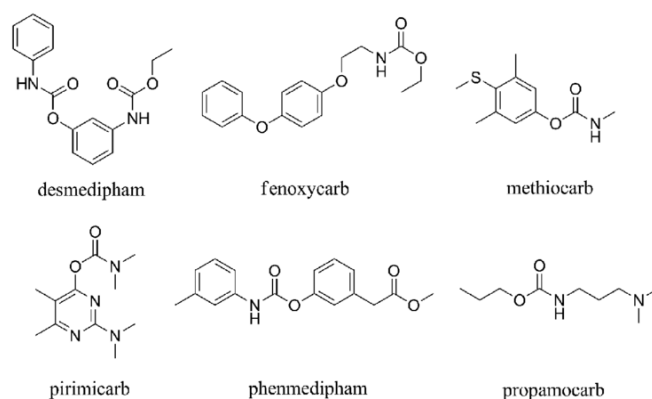
Fig. 8: Selected carbamate insecticides.

1.4.2   Irreversible inhibitors for acetylcholinesterase: organophosphorus chemicals

Phosphinic, phosphonic, phosphoric, or phosphoramidic acid are components of OPs, which are esters or thiols (Fig. 9).
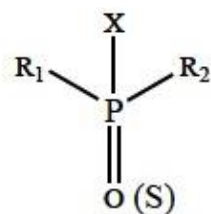
Fig. 9: OPs' general structural formula.

The phosphorus atom gets attached to R1 and R2, which are alkyl or aryl groups, either directly (forming phosphinates) or indirectly (forming phosphates or phosphothioates) via connecting via a sulfur or oxygen atom. Sometimes, R1 and phosphorus atom are directly coupled, whereas R2 and the oxygen or sulfur atom are joined, forming thiophosphonates or phosphonates. In molecules of phosphoramidates, the atom connected to phosphorus is either sulfur or oxygen, and at least 1 group is -NH2 (mono-substituted, unsubstituted, or bi-substituted). The leaving group attaches itself to phosphorus atom via a sulfur or oxygen atom, and it can be a part of several aliphatic, halogen, heterocyclic groups or aromatic. When the OP is broken down by phosphotriesterases or comes into touch with protein targets, the "X i.e. leaving group" is released from the phosphorus atom. In the medical and agricultural domains, "organophosphates" refer to class of pesticides and nerve agents that efficiently block acetylcholinesterase action [22, 23].

1.5   Sources of organophosphate exposure

Exposure to organophosphates is a significant concern for public health due to its impact on health, disease rates, healthcare, and overall safety in relation to toxicity. In 2000, the American Association of Poison Control Centers' Toxic Exposure Surveillance System recorded 10,073 instances of adults being exposed to organophosphate pesticides in the US. Out of the total number of exposures, 9609 were accidental and 276 were deliberate. Among these cases, 2720

14

were attended to at healthcare institutions. Exposure to organophosphates can be classified into two categories: (a) occupational exposure and (b) environmental or nonoccupational exposure. Unintentional, accidental, or deliberate poisoning can happen in both work-related and non-work-related situations involving exposure to organophosphates. Homicidal poisoning includes both instances of self-inflicted poisoning with suicidal intent and deliberate poisoning of one person by another. Utilizing organophosphates in chemical warfare constitutes an act of terrorism, involving deliberate poisoning intended to harm a significant number of people [24].

1.5.1   Organophosphorus insecticides

OPs have been widely employed as broad-spectrum insecticides for more than half a century, effectively managing various insect populations in both agricultural and domestic settings. Large-scale production of OP pesticides commenced following World War II, with parathion being one of the initial products introduced to the market, followed by azinphos-methyl and malathion. Frequently employed insecticides with high efficacy include chlorpyrifos, ethyl parathion, dimethoate, malathion, methyl parathion, and phosalone (Fig.10) [25, 26]. During the 1970s, organochlorine insecticides like heptachlor, DDT and dieldrin were prohibited due to their long-lasting nature and tendency to build up in the environment. They were substituted with more easily degradable organophosphates (OPs).
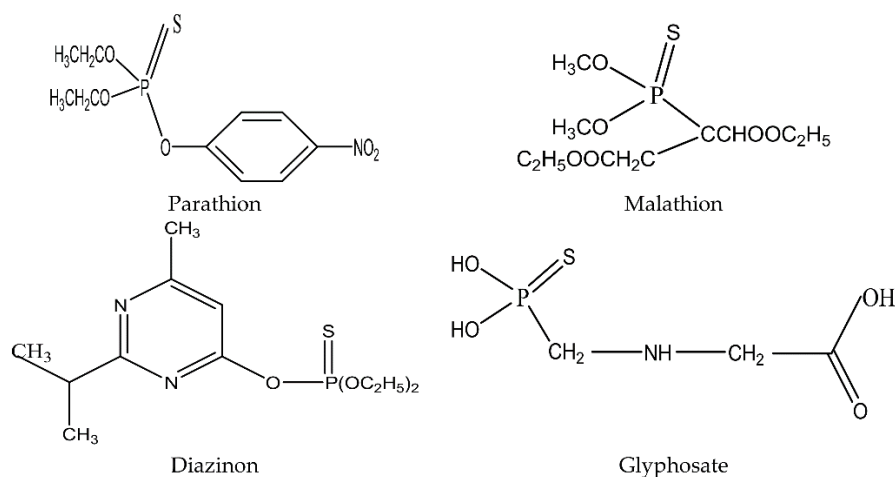
Fig. 10: Selected OP insecticides.

Despite the rapid degradation of OPs insecticides, which made them a desirable substitute for organochloride pesticides, they have higher levels of acute toxicity. This poses a potential danger to anybody who may come into contact with significant quantities of these pesticides, such as workers involved in their production and administration. Organophosphates (OPs) are a prevalent source of poisoning on a global scale, often stemming from their use in agriculture, intentional self-harm, or unintentional contact. Organophosphate pesticides can be taken through several ways, including as inhalation, ingestion, and cutaneous absorption [27].

The Oxo versions of OP pesticides exhibit similar levels of toxicity towards both warm-blooded and cold-blooded creatures. Conversely, mixed function oxidases transform thio forms into oxo forms. The activation happens in creatures with cold blood, while dealkylation into non-toxic chemicals is the more common process in warm-blooded organisms. As a result, many compounds of strong pesticides have been synthesized to minimize their toxicity towards warm-blooded species while retaining their toxicity against insects, thus increasing their selectivity. The insecticides that are both efficient and often used, and relatively safe for warm-blooded organisms are parathion, malathion, diazinon and glyphosate (Fig. 10) [28].

1.5.2 Organophosphates as nerve agents/gases

Organophosphorus pesticides are compounds that consist of esters of phosphonic, thiols, amides, phosphoric, phosphinic or thiophosphoric acids. These compounds also have two extra organic side chains, which can be either phenoxy, cyanide, or thiocyanate groups [29]. Certain OPCs are classified as phosphonothioates (S-substituted) and phosphonofluoridates, which are nerve poisons generally referred to as nerve agents [18].

These chemical warfare agents can be divided into 4 types:

- The Germans invented the G-series agents, which include cyclosarin (GF), tabun (GA), sarin (GB) and soman (GD).

- The V-series agents, which are characterized by the letter V for venomous, include Chinese VX and Russian VX in addition to VE, VG, VM, and VX [30].

- The features of the G and V series are combined in the GV series. The substance GV, 2-dimethylaminoethyl-(dimethylamido)-fluorophosphate, is an illustration of this. The G-series chemicals are generally less hazardous than the V series ones [31].

- Novichok-5, Novichok-7, A230, A232, A234, and substance-33 are among the chemicals of the Novichok series [32].
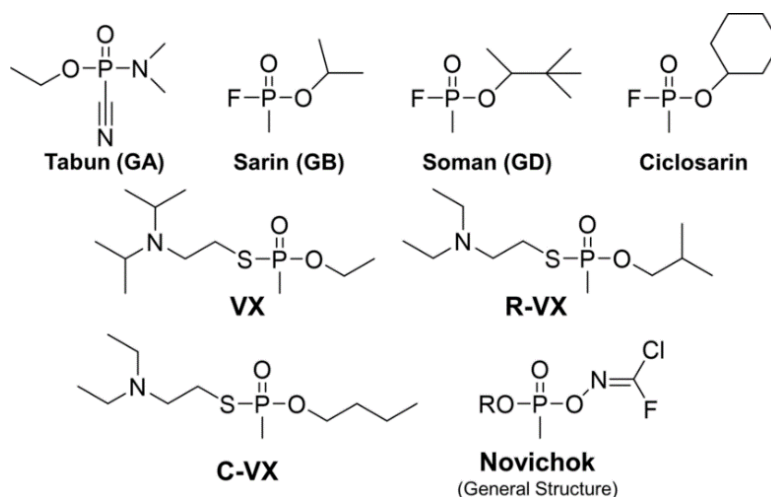
Fig. 11: Chemical Structures of Chemical Warfare Agents.

Acute poisoning caused by a nerve agent results in the constriction of pupils, defecation, involuntary urine and ultimately death due to the loss of control over respiratory muscles, leading to asphyxiation. Certain nerve agents have the ability to easily turn into vapor or aerosol and the main way they enter the body is through the respiratory system. Chemical warfare agents can also penetrate skin, necessitating the use of a complete respirator and body suit for individuals exposed to these agents. Furthermore, nerve agents have a prolonged and cumulative impact, meaning that their effects are intensified with repeated exposures. Individuals who survive nerve agent poisoning typically experience persistent neurological harm, which can result in ongoing psychological consequences.

1.5.3 Organophosphate-inhibited acetylcholinesterase

At extremely low concentrations, the most hazardous organophosphorus chemicals (OPs) act as powerful and long-lasting anticholinesterases, inhibiting the neuronal target enzyme acetylcholinesterase (AChE). Cholinergic pathways are extensively present in both the peripheral and central nervous systems of vertebrates, and they perform numerous vital functions necessary for life and normal physiological processes. Because of its quick turnover

rate, AChE is essential for the nervous system's function in both vertebrates and invertebrates, limiting the duration of the impact of the transmitter acetylcholine. The original purpose of the OP anticholinesterases was to serve as insecticides. However, they were later adapted for use as chemical warfare weapons during World War II [33].
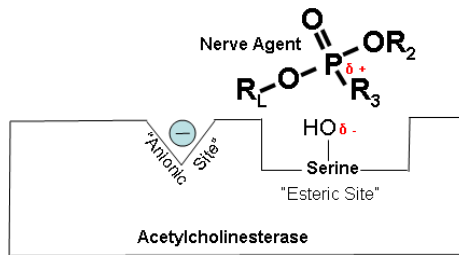
Fig. 12: Partially positively charged phosphorus is attracted to partially negatively charged serine.
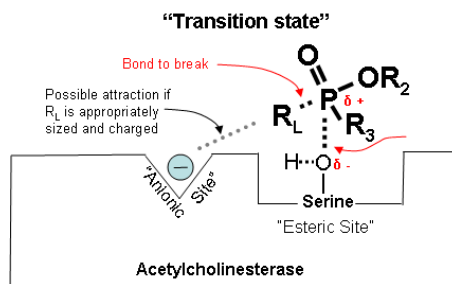
Fig. 13: The transition state illustrates the specific bonds that are broken and formed during a chemical reaction.
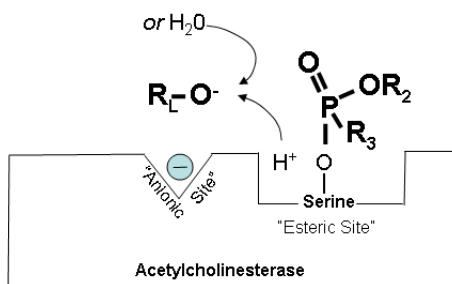
Fig. 14: A cholinesterase inhibitor links to acetylcholinesterase and inhibits the binding of acetylcholine.

1.5.4 Effects of blocked acetylcholine breakdown

Inhibiting the enzyme acetylcholinesterase causes increased levels of acetylcholine in the nervous system, causing disruptions in several normal physiological functions. These disruptions can manifest as severe symptoms of poisoning that can be life-threatening (Fig. 15). The cholinergic syndrome manifests in several toxic reactions including tremors, nausea, excessive salivation, tearing, urine, vomiting, bowel movements, constriction of the iris, convulsions, difficulty breathing, and eventually respiratory failure in cases of lethal poisoning. Respiratory failure mostly results from heightened activity in the peripheral nervous system, specifically an overactive cholinergic system. This leads to constriction of the bronchioles, excessive secretion in the bronchioles, and spasms in the respiratory muscles. Additionally, the suppression of the brain's respiratory control center also plays a role in contributing to respiratory failure [34].

Hypercholinergic activity in the brain not only affects respiratory rhythm but also causes excessive stimulation of postsynaptic nicotine and muscarinic receptors. This can lead to seizures, which, if left untreated, trigger the release of excitatory amino acids. This release then causes an excessive influx of calcium and stimulates N-methyl-D-aspartate (NMDA) receptors, resulting in neuropathology [35].
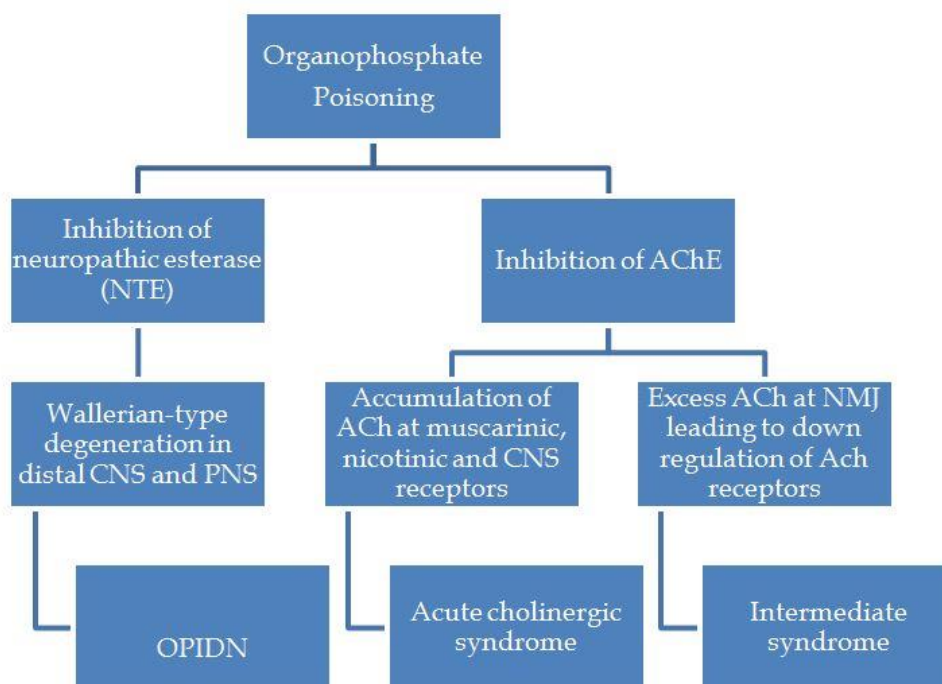
Fig. 15: Classification of organophosphate poisoning.

Acute cholinergic syndrome

The symptoms and signs of serious poisoning caused by anticholinesterase medicines can be predicted using the action of their biochemical mechanism, which is clearly related to acetylcholinesterase activity. These poisonings can have three different effects: central, nicotinic and muscarinic symptoms and signs. The period of effects is mostly determined by the compound's properties, such as liposolubility, durability of the OPC-AChE complex, and reactivatability in the presence of cholinesterase reactivators such as pyridinium oximes. It is important to note that only the organophosphorus compounds (OPCs) with a P=O bond, known as direct inhibitors, effectively inhibit acetylcholinesterase (AChE). To display AChE inhibitory actions and consequent toxicity, OPCs with a P=S group, also known as secondary inhibitors, must be metabolically converted into matching oxons with a P=O group [36].

21

The onset of symptoms of toxicity caused by inhibitors is rapid and occurs either during or immediately after exposure. On the other hand, the signs of poisoning caused by indirect inhibitors have a slower onset and last for a longer duration, even continuing for several days after exposure has ceased [37].

The individual's medical history, the conditions of the exposure, and the appearance of clinical symptoms suggestive of poisoning are used to make the simple clinical diagnosis (Fig. 16) [9].

The levels of activity of acetylcholinesterase (AChE) in plasma or red blood cells might be used to confirm the diagnosis. These enzymes' activity is acknowledged as biomarkers for determining OPC and carbamate exposure and/or toxicity. The AChE enzyme at the junctions of the target organs is the same as the one present in red blood cells. It is thought that this enzyme's levels reflect the impact on the intended organs. As a result, RBC AChE is thought to be a biological marker for these compounds' poisoning. However, it is crucial to remember that the above presumption is only valid in the event that the inhibitor is equally available to synapses and blood. It is difficult to ascertain whether inhibition of AChE in RBC and nervous system are related because blood is more obtainable than brain tissue. Thus, in comparison to the decrease in brain, decrease of AChE in RBC might be heightened [38].

Furthermore, the brain's acetylcholinesterase is replenished by de novo synthesis at a faster rate compared to red blood cells [37].
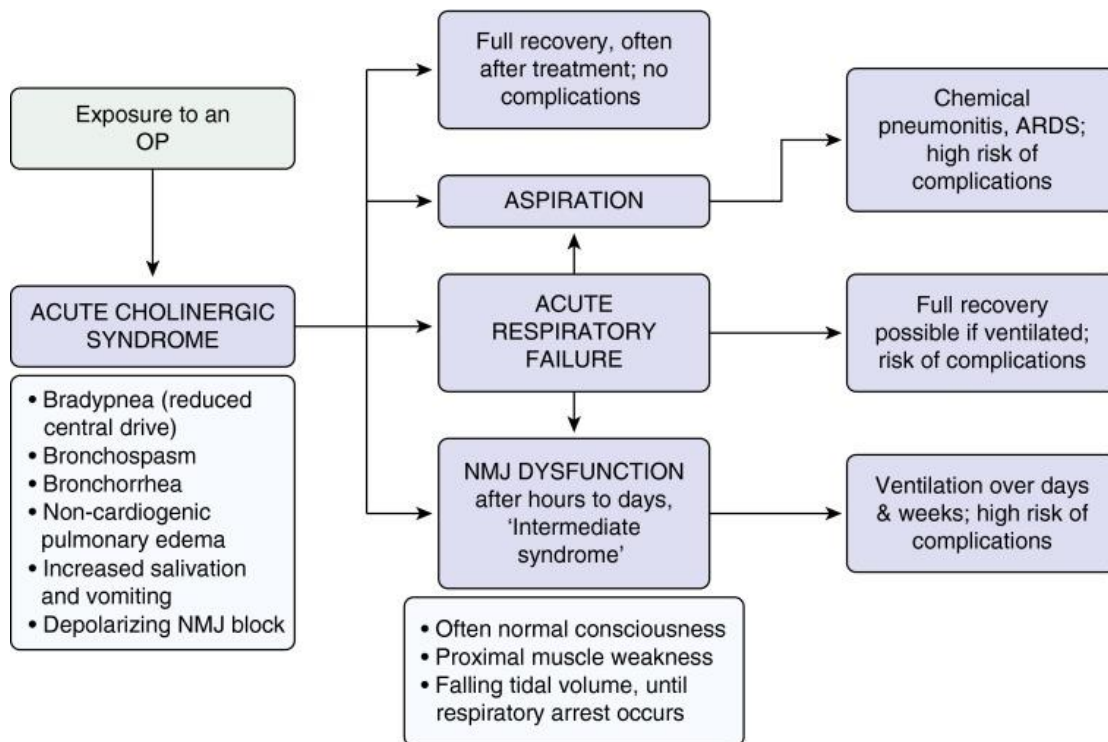
Fig. 16: Secondary toxicity of the respiratory system caused by organophosphorus (OP) poisoning.

Intermediate syndrome

In 1987 Senanayake and Karalliede documented a disorder that occurred after the treatment of cholinergic symptoms of organophosphate toxicity. Due to the occurrence of the condition prior to the manifestation of the long-term consequences of organophosphates (OPs), it was labelled as the intermediate syndrome. Since 1987, there have been other recorded cases of this illness, such as the one documented by Karademir et al. (1990). The syndrome is characterized by the onset of paralysis in the proximal limbs approximately l~ days after intoxication. The progress of the condition is unaffected by the use of atropine or oximes. Due to the impact on the respiratory muscles, breathing assistance is required [39].

Organophosphate myopathy

Several years ago, it was revealed that anticholinesterase organophosphates (OPs) can cause myopathy. Subsequently, several types of chemical compounds, such as insecticides (Systox) and chemical warfare agents like tabun, soman and sarin, a derivative of an insecticide called paraoxon, the medication like echothiophate have been demonstrated to have the ability to cause myopathic alterations in laboratory animals [40].

Following a single dosage exposure, degenerative alterations seem to initiate in the area of the motor endplate, characterized by an initial increase in eosinophils and swelling of the sarcoplasm. This leads to nuclear pyknosis. Typically, a tiny portion of fibers are impacted and problem is mostly resolved within 10-14 days [41].

At the ultrastructural level, there is excessive contraction and severe damage to the structural units of muscle fibers, known as sarcomeres, resulting in the loss of Z and A bands. There is a strong possibility that the accumulation of acetylcholine is directly connected to the development of myopathy. Additionally, it has been shown through histochemical analysis that there is an influx of calcium in the early stages of the myopathy [39].

Cardiotoxicity

Acute cholinergic syndrome is linked to several alterations in heart function. OP poisoning has been linked to cardiac arrhythmias, such as torsade des pointes, which can occur even after recovering from the acute illness. Moreover, both animal studies and autopsies of human poisonings resulting in death have revealed histologic indications of damage to the heart muscle [42].

Delayed neuropathy caused by organophosphates

It appears that the anticholinesterase properties of organophosphates are not the cause of organophosphate-induced delayed neuropathy. Most pesticide organophosphates currently available in Western countries are unable to cause this disease. In addition, several organophosphates (OPs), such as TOCP, exhibit limited or no anticholinesterase effects yet possess strong neuropathic properties. Considerable deliberation has been devoted to determining if the potent anticholinesterase nerve agents could potentially lead to the development of OPIDN [43].

Delayed neuropathy has not been commonly reported in experimental trials. Therapeutic advancements have raised concerns about the possibility of patients surviving extremely fatal dosages of organophosphate nerve agents, only to later develop this syndrome. The formation of OPIDN syndrome seems to occur through the process of phosphorylation and subsequent ageing of a protein termed as neuropathy target esterase in neurons. Given this premise, it is not surprising that the nerve agents have a low potential for causing OPIDN, as the quantities needed to inhibit AChE are far lower than those needed to inhibit NTE [44].

Several organophosphates (OPs) that were investigated as pesticides have been discovered to have neurotoxic effects. These include mipafox, as well as tri-S-alkyl defoliants known as DEF and Merphos. Leptophos has currently become a cause for concern, while there are suspicions surrounding EPN, cyanophenfos, and trichloronat. Nevertheless, the regulatory authorities' mandate to assess OP pesticides using the "hen test" has led to the discontinuation of most chemicals having neuropathic properties. Utilizing an NTE assay is a valuable indicator for OPIDN, both during hen test and in clinical settings for humans. Suppression of lymphocytic

NTE has been found to be indicative of the occurrence of OPIDN, even though the relationship between brain and NTE activity in hens is weak, save for a brief period after poisoning [45].

1.6 Nerve agent antidotes

Antidotes can help reduce the impact of nerve agent exposure. At the moment, atropine and a phosphorylated AChE reactivator are prescribed together to treat nerve agent toxicity [46].

As an adversary to muscarinic receptors, atropine has long been known to lessen the effects of excess acetylcholine in the intercellular gap between nerve cells. Atropine lessens secretions, prevents smooth muscle spasms and contractions, and helps to get neurotransmission back to

normal. Nevertheless, AChE activity and its physiological role cannot be restored by atropine. This level of efficacy is exclusive to oxime reactivators. The primary mechanism underlying oximes' effectiveness in treating organophosphate poisoning is the activation of acetylcholinesterase (AChE), which is suppressed by organophosphates [47].

1.6.1 Pyridinium oximes

The concept that specific chemicals could significantly enhance the natural process of phosphorylated AChE regeneration is associated with Wilson's investigations using choline and hydroxylamine. The presence of hydroxylamine indicated the need to search for compounds with enhanced nucleophilic characteristics, while the presence of choline suggested that the presence of quaternary nitrogen might potentially enhance the nucleophilic attack [48]. The quest for such compounds' potential as therapeutics led to Wilson and Ginsburg's development of pyridine-2-aldoxime methiodide (2-PAM) [49].

Currently, there are hundreds of chemicals identified that are referred to as reactivators of phosphorylated AChE. 2–PAM has emerged as an unofficial benchmark for evaluating the

effectiveness of other reactivators. Aldoximes generated from pyridine are the most well-known reactivators, and several of them have been used in human medicine as antidotes for organophosphate poisoning. The primary types of dialdoximes include bis-quaternary trimedoxime and obidoxime, while the bis-quaternary monoaldoximes include asoxime chloride (HI-6) and Hlö7. The chemical structures of a few phosphorylated acetylcholinesterase reactivators are shown in Fig. 17. Combined with a reactivator (R), the phosphorylated enzyme (EP) forms a transient complex (EPR). The restoration of the enzyme (E) and the creation of phosphorylated oxime (PR) are the results of this chemical reaction. They themselves function as strong anticholinesterases; nevertheless, they are naturally unstable and degrade in a matter of minutes or hours [50].
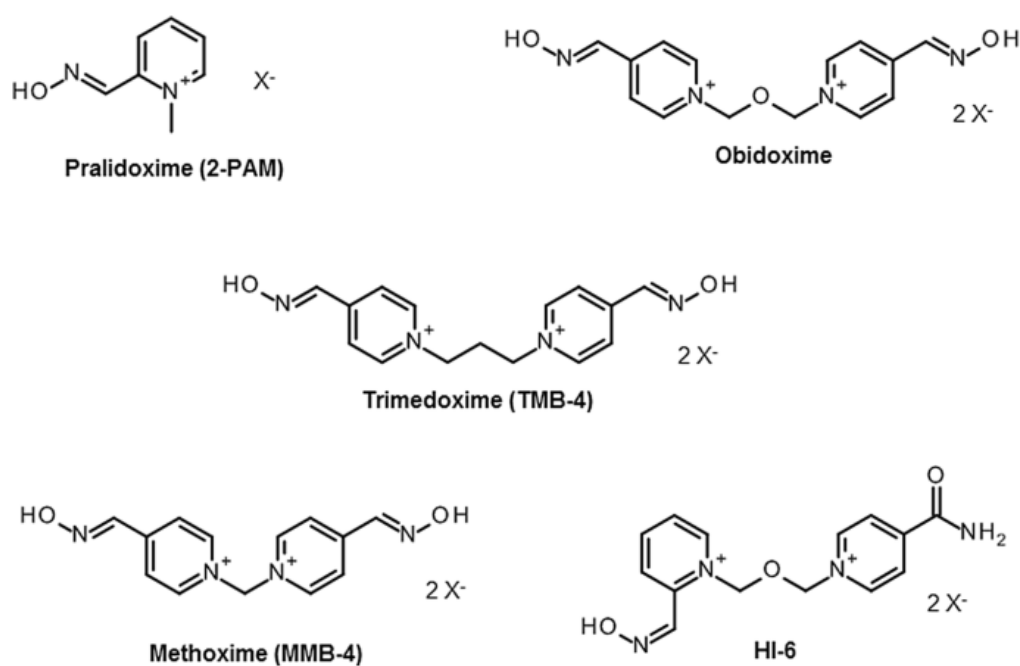
Fig. 17: Chemical structures of some pyridine oxime reactivators.

1.6.2 Mechanisms of action of pyridinium oximes

Oximes are principally responsible for reactivating phosphorylated cholinesterases, which is why they are considered to have strong antidotal activity. Oximes have an outstanding affinity for the enzyme and exhibit potent nucleophilic properties, they are able to remove phosphoryl group from enzyme and restore the function of phosphorylated cholinesterases. During the reactivation process, the enzyme undergoes dephosphorylation, resulting in the formation of active enzyme and phosphorylated oximes. This leads to restoration of enzyme activity. Reactivation can happen only if ester substituents on the phosphorus have not undergone hydrolysis, meaning that the phosphylated enzyme has not aged. Oxime phosphonates released during the reactivation process have the potential to be highly effective inhibitors of cholinesterases. This could result in the reactivated enzyme being inhibited again (Fig. 18).
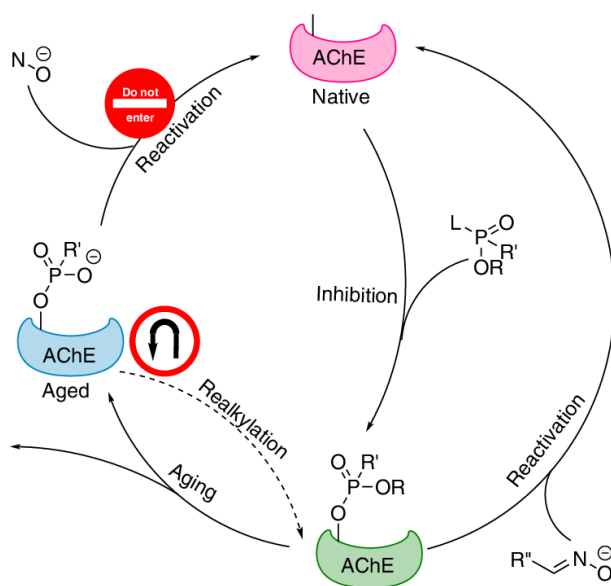
Fig. 18: Scheme depicts the mechanisms of inhibition, reactivation, ageing, and realkylation of AChE. "L" represents the leaving group that is expelled during inhibition. Realkylation can restore the aged acetylcholinesterase (AChE) to its inhibited, but unaged, condition, hence promoting reactivation by the use of oximes.

Oximes function as reversible inhibitors by binding to AChE and creating complexes with enzymes in either the active site, the allosteric site, or both. This binding prevents AChE from phosphorylation. The reversible inhibitor gives protection by directly competing with organophosphorus chemical for adhering to the active site. The allosteric site is bound by a reversible inhibitor, resulting in the indirect safeguarding of the active site. This phenomenon has been extensively established through in vitro research. However, its evaluation in experimental toxicity has been limited thus far. Historically, it has been believed that oximes, in addition to their role in reactivating AChE in cases of organophosphate poisoning, may also exhibit direct pharmacological effects. This was corroborated by observations indicating that in certain investigations, the therapeutic effects of oximes could not be solely accounted for by the reactivation of acetylcholinesterase (AChE) [51-53].

In experiments with adult rhesus macaques [54] or marmoset monkeys [55], The injection of HI-6 in combination with atropine and diazepam did not substantially reactivate soman-inhibited cholinesterase activity. This shows that HI-6's improved protective impact could be due to a mechanism other than cholinesterase reactivation. HI-6 may affect the GABA-ergic neurotransmission in the central nervous system (CNS) [56].

1.6.3 Treatment of OPC poisoning with pyridinium oximes

Out of the several categories of oximes that have been studied, the ones that have been used in clinical settings can be categorized into two groups: bispyridinium and monopyridinium oximes. Pralidoxime (PAM-2) is the only monopyridinium oxime in use right now. The most significant bispyridinium oximes are obidoxime, trimedoxime (TMB-4), HI-6 and HLö-7 [57].

1.6.4 Pralidoxime (PAM-2)

In 1955, pralidoxime was manufactured in the United States [58]. The four salts, namely methylsulphate, chloride (PAM-2 Cl), mesylate (P2S) and methiodide were examined and implemented in practical applications. PAM-2 demonstrates high efficacy in reactivating Acetylcholinesterase (AChE) that has been blocked by sarin or VX [59-61]. However, it has not been successful in reactivating the enzyme poisoned by tabun or soman [62, 63].
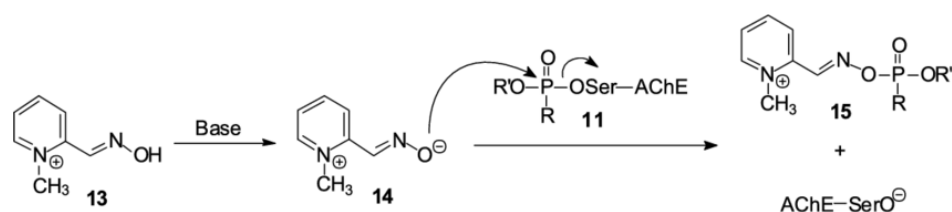
Fig. 19: Phosphorylated acetylcholinesterase reactivation using pralidoxime.

The ability of pralidoxime, a quaternary pyridinium salt, to enter central nervous system and blood brain barrier is limited. Pro-2-PAM was created as a pro-drug of PAM-2 that can efficiently enter the CNS in order to get around this restriction. Contrary to predictions, experimental trials revealed that it was even less effective than PAM-2 at preventing paraoxon-induced poisoning [64]. However, it seems that in cases of OPC poisoning, when administered alongside atropine, PAM-2 has the tendency to cross the BBB at elevated levels [65].

1.6.5 Trimedoxime (TMB-4)

Trimedoxime was manufactured in the United States in 1957 and received patent protection in 1967 [66]. It is significant bis-pyridinium oxime with a propylene bridge present among two pyridinium rings.
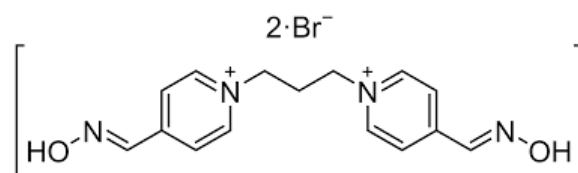
Fig. 20: Structure of Trimedoxime.

Experimental evidence demonstrated that TMB-4 exhibits more effectiveness in reactivating the AChE enzyme blocked by DFP compared to PAM-2. Additionally, TMB-4 is a superior reactivator than LüH-6 when dealing with tabun-inhibited enzyme [67]. The first oxime to demonstrate effectiveness in treating animals poisoned with tabun was trimedoxime. It has the potential to safeguard animals exposed to sarin or VX poisoning, however it does not provide protection for animals affected by soman intoxication [68, 69]. Nevertheless, TMB-4 exhibited the highest level of toxicity within the group of four compounds known as the "great four" [70].

1.6.6 Obidoxime (LüH-6)

Obidoxime chlorine was called for A. Lüttringhaus and I. Hagedorn, who manufactured it in Germany and applied it in 1964 in medicine [71]. The newly developed oxime demonstrated prompt and substantial efficacy as an antidote for poisonings caused by organophosphorus compounds (OPCs) [72].
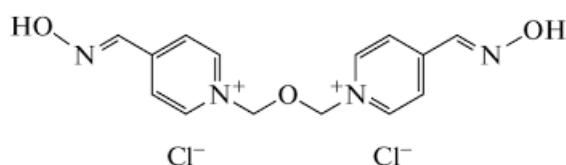
Fig. 21: Structure of obidoxime.

LüH-6, when administered alongside atropine, effectively safeguarded experimental animals from tabun, sarin, and VX poisoning due to its strong AChE reactivator properties [62, 73]. LüH-6 has demonstrated greater effectiveness as an antidote for tabun poisoning as compared to trimedoxime. Obidoxime shown ineffectiveness in treating intoxication caused by soman in primates, guinea pigs, and mice similar to PAM-2 and TMB-4. In contrast to TMB-4, LüH-6 can provide a partial resistance against soman when paired with atropine in guinea pigs that have already received pyridostigmine treatment. While TMB-4 is more toxic, this oxime still has the potential to cause liver damage [74].

1.6.7   HI-6

The first oxime that may partially rescue mice poisoned with nerve toxin and reactivate soman-inhibited AChE was synthesized in 1966. It was given the code classification HI-6 by I. Hagedorn and K. Schoene [75].
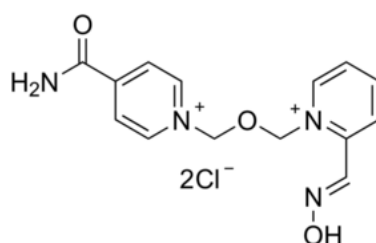
Fig. 22: Structure of HI-6.

Soon later, Ilse Hagedorn synthesized a similar compound and dubbed it HI-6, using her initials [76]. Research has demonstrated that HI-6 has more potency compared to LüH-6 and HS-6 in safeguarding several kinds of rodents against soman, sarin, and particularly VX-induced poisoning. However, HI-6 has the limitation of being unable to restore the activity of tabun-inhibited AChE [77]. Consequently, it was demonstrated to be ineffective as the sole oxime in countering tabun toxicity. HI-6 can, however, offer a same degree of protection against

soman and tabun poisoning in primates, according to further research. In fact, oxime can keep rats safe from many fatal doses of tabun when given in sufficiently significant dosages. When compared to other oximes, HI-6's level of toxicity is actually the lowest. HI-6's LD50 in rats is 781 mg/kg, or 2071 µmol/kg [68, 78].

The original HI-6 dichloride salt's pharmacokinetics were identical to those of HI-6 dimethanesulphonate, according to a recent study done on anaesthetized pigs. In the same study, the HI-6 salts rescued guinea pigs from poisoning by five times the fatal dose (LD50) of cyclosarin or soman when injected in conjunction with atropine [79].

1.6.8 HLö-7

The fourth and most recent significant compound known as "Hagedorn oxime" is HLö-7. It was synthesized in Freiburg, Germany, in 1986 and named after Ilse Hagedorn and Marianne Loffler.
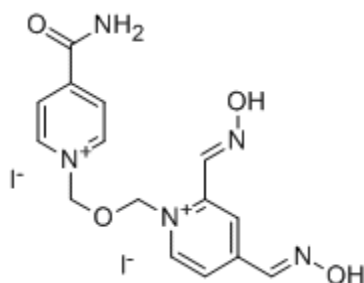
Fig. 23: Structure of HLö-7.

When any of the four main nerve agents block acetylcholinesterase (AChE), the new oxime efficiently recovers its function [80-82], alongside the enzyme that cyclosarin has blocked. Furthermore, compared to HLö-7, pralidoxime, LüH-6 and asoxime chloride shown higher efficacy in restoring the reduced transmission produced by in vitro superfusing cyclosarin, sarin, soman, or tabun into the myoneural junction preparation.

A study reveals that after tabun, sarin, soman, and cyclosarin reduced AChE activity in the diaphragm of mice, HLö-7 successfully restored it. While HI-6 and HLö-7 both have the capacity to reverse the effects of OP-induced hypothermia, suggesting that they can enter through the blood brain barrier (BBB) and enter the central nervous system [83] when administered with atropine, HLö-7 was shown to be 2.5 times more toxic than HI-6. HLö-7 had comparable cardiovascular tolerance to HI-6, however it was still inferior, particularly in anaesthetized guinea-pigs [84].

HLö-7 showed superior efficacy compared to HI-6 in countering tabun and VX poisoning but was found to be less efficient in treating sarin, soman, and cyclosarin intoxication. HLö-7 shown superior efficacy in protecting guineapigs against tabun poisoning compared to HI-6. However, HI-6 only showed a marginal advantage over HLö-7 in soman poisoning [85, 86].

1.7   Structure-activity relationship for pyridinium oximes

The activity relationship of pyridinium oximes with their structures is influenced by three key parameters that determine the ability of acetylcholinesterase reactivators towards blocked AChE:

The existence of quaternary nitrogen in the reactivator is the main factor. Furthermore, compared to mono-pyridinium compounds, bis-pyridinium oximes—which have 2 quaternary nitrogens—show greater attraction and more efficient reactivation of phosphorylated acetylcholinesterase.

The efficacy of bispyridinium nitrogens is influenced by the length of the connecting linker. Bispyridinium oximes now in use contain linkers such as $-CH_2-O-CH_2-$ or $-CH_2-CH_2-CH_2-$. It is generally accepted that the pyridinium ring(s) should have 1 or more oxime molecules at

34

positions 2 or 4. Positions 2 and 4 are considered more favorable than position 3, likely because of the variations in pKa [87-89]. Most of the pyridinium oximes that were synthesized and investigated for over twenty years met this requirement.

- **Quaternary nitrogen present in the structure of the reactivator**

It is commonly known that the choline element's charged quaternary group in acetylcholine, in addition to other quaternary binders such as pyridinium AChE reactivators, create a bond with the anionic center of acetylcholinesterase. Because of the quaternary nitrogen in the AChE reactivator's structure, oximes have a higher tendency for both inhibited and functioning acetylcholinesterase. In fact, this structural feature makes it possible for the AChE reactivator molecule to enter the enzyme cavity [90, 91].

Conversely, there is ongoing debate on the ability of quaternary charged chemicals, particularly AChE reactivators, to move through the blood brain barrier (BBB). Kassa and Bajgar determined the in vivo reactivation of brain AChE blocked by chemical warfare agents. Sakurada et al. have confirmed that oximes have a limited tendency to cross the blood brain barrier, with a penetration constant of approximately 10% [92]. Typically, AChE reactivators have one or two quaternary nitrogens in their chemical structure (pralidoxime and HI-6, respectively). According to our research, compared to monoquaternary oximes, bis-quaternary oximes had an elevated affinity for both healthy and phosphorylated AChE as shown in table below [93-95].

Table 1: K_{DIS} - Reactivation affinity of HI-6 and pralidoxime towards active acetylcholinesterase and K_R-G_B, K_R-G_F, K_R-V_X, K_R-G_A – affinity towards AChE inhibited by sarin, cyclosarin, VX and tabun respectively.

AChE Reactivator	K_{DIS} [μM]	K_R-G_B [μM]	K_R-G_F [μM]	K_R-V_X [μM]	K_R-G_A [μM]
HI-6	24	9	12	130	6
Pralidoxime	210	354	12000	127	575

- **Length and stiffness of the chain connecting pyridinium rings**

Length of connecting linker among both quaternary pyridinium rings affects the capability of bis-quaternary pyridinium reactivators to restart nerve agent-inhibited AChE. The nerve agent used for inhibition affects the length of the linking chain in the n-methylene linkage [96, 97]. It has been shown that three or four methylene groups are the ideal length of the connecting chain needed to reactivate sarin, tabun, or VX-inhibited AChE. Nevertheless, the most potent reactivator of OP-inhibited AChE appears to be methoxime, which possesses one methylene group [93].

Tragically, that requirement is only met in the case of n-methylene linkage chains. The aforementioned requirements are not met by compounds that include sulfur, oxygen, or other components embedded into the connecting chain that differ from the methylene group [98-100].

The differences that are seen can be explained by the existence of free electrons in chain and the interactions that occur between reactivator molecule and the active site of the enzyme. One main structural feature that affects the reactivation of enzyme is the "rigidity" of the chain. The pyridinium rings limited spatial orientation within the enzyme cavity is caused by the connecting chain's rigidity. To explore the influence of stiffness on reactivation efficiency, compounds with varying degrees of stiffness in the linking chain were synthesized. The connecting chain had groups of xylene, alkyne, and alkene. The efficiency of AChE reactivators rapidly decreases when the stiffness of the linking chain is added (Fig. 24) [101, 102].
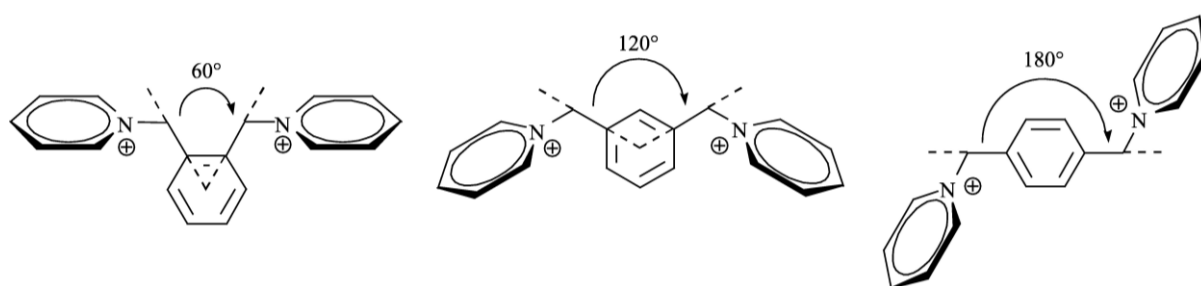
Fig. 24: Angles between bonds outgoing from phenylene ring.

The results indicated above were not achieved in the case of xylene linkers. Nevertheless, there are other significant factors that might be taken into consideration. The influence is determined by the angles present in the construction of the connection chain. The only bonds that can rotate freely in these molecules are the methylene junctions, unlike the molecules with a propane or butane connecting chain where rotation is also free. The inflexible xylene connections result in the non-coplanar arrangement of each pyridinium ring with the phenylene ring. Thus, it is possible to ascertain the angles formed by the bonds emanating from the phenylene ring. From this perspective, the most favorable compounds are those with larger angles, indicating a greater distance between the pyridinium rings [101].

- **The existence of the oxime group inside the oxime structure**

37

The oxime group's presence in the antidote's molecule is another important structural component. In the past, various nucleophilic agents such as geminal diols, hydroxamic acids, ketoximes and hydroxyiminoacetone were used to break the bond between the inhibitor and an enzyme. Since the aldoxime group is currently being used to its fullest capacity, it is included in all recently synthesized acetylcholinesterase reactivators [103, 104].

The oximate anion (-CH=NO-) is created when the oxime group in the environment of the human body separates. Then, this anion of oxime functions as a nucleophile, severing link among the inhibitor's phosphorus atom and enzyme [102].
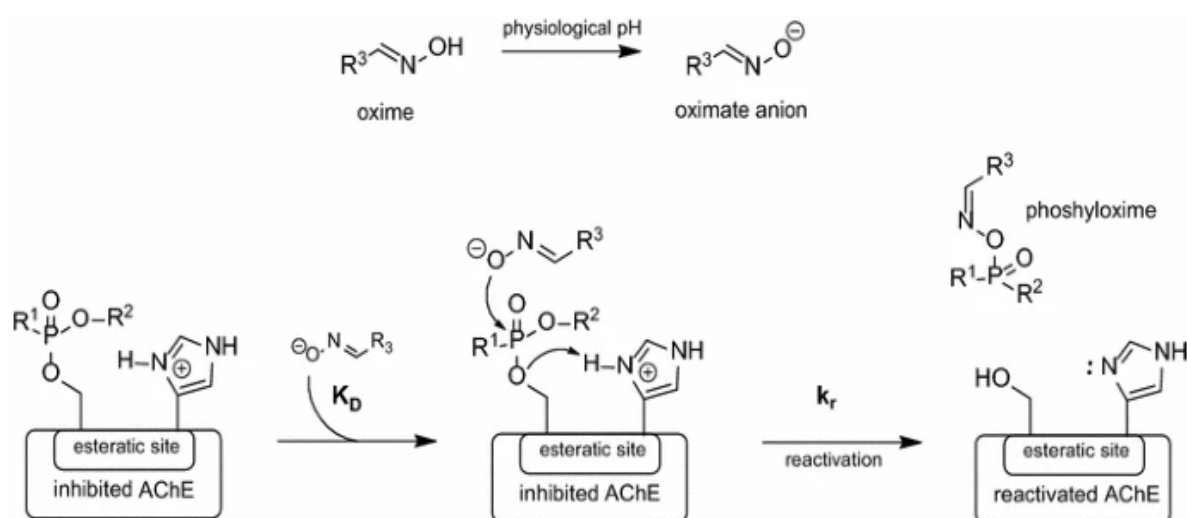
Fig. 25: Using oximate anion to restart OP-inhibited AChE results in the reactivation of free enzyme and phoshyloxime.

- **Oxime group's position**

An additional noteworthy structural element is also connected to the oxime group. The quaternary pyridinium ring is where the oxime group is found. Depending on where the oxime group is located, the reactivation efficacy of the oximes varies considerably. There is no general rule that says all oximes at a given location may make all inhibitions caused by nerve agents work again. Reactivators with an oxime group in regions 2 to 4 are usually more potent than those with an oxime group in position three. The difference in pKa values between the oximes at regions two and four and those at position 3 is responsible for this fact [100].

There are certain connections between the position of the oxime molecule and the nerve agent, even if it is not possible to define a universal rule for the oxime group's location in all nerve agents. This suggests that the particular nerve agent affects both the oxime group's position and the length of the chain that connects the quaternary pyridinium rings. For example, reactivators with an oxime in the second position are more successful in reactivating cyclosarin-inhibited AChE [103].

However, reactivators containing an oxime group at the fourth position of the pyridinium ring are now regarded as the most effective for reactivating tabun-inhibited AChE. Furthermore, poisonings caused by pesticides can be effectively treated with AChE reactivators that include an oxime group at position 4. Both reactivators (having oxime groups at positions 2 and 4) are particularly successful at reactivating inhibited AChE because of the high reactivability of VX and Sarin-inhibited AChE [105, 106].

- **The number of oxygen groups in the molecule of the reactivator**

The sixth structural element covered in this article is the reactivator, which has five oxime groups overall. One, two, three, and four oxime groups may be dispersed throughout one, two, or even three pyridinium rings in a single molecule of AChE reactivator. The number of oxime groups in a potential AChE reactivator does not increase its reactivation potency. The bigger size of acetylcholinesterase reactivator molecule may be connected to this phenomenon. The second oxime group is not necessary because the first oxime group (which has the lowest pKa) plays a major role in the reactivation process [107, 108].

1.8 Objectives

Objectives of my research work were preparation, characterization and in-vitro reactivation potency of aldoximes and ketoximes for AChE reactivation.

- Synthesis of new combinations of keto and aldoximes

- Detailed analysis and characterization of synthesized products

- In vitro potency of synthesized products as AChE reactivators

2   Chapter 2

2.1   Literature Review

Aardema, H et al. reviewed organophosphate insecticides poisoning in different cases. Organophosphate pesticide self-poisoning is a significant global health issue. Organophosphorus poisoning is defined by severe cholinergic crisis due to poisoning of acetylcholinesterase. Additional presentations include the intermediate neurotoxic syndrome and delayed polyneuropathy. Organophosphorus poisoning is less common in the Western world since there is a decrease in the availability of organophosphate pesticides. This can make it more challenging to identify and treat this specific type of poisoning. Recognition is crucial, given that pesticide exposure is linked to a significant mortality rate. Patients get normal resuscitation therapy, which consists of the administration of atropine to counteract the muscarinic effects. Despite the lack of evidence-based support, the World Health Organization has currently advised the use of oximes, while awaiting additional clinical research. With the expectation of gaining further understanding about the most effective approach to treating pesticide poisoning and the control of access to these extremely poisonous substances, it is anticipated that a significant reduction in fatalities can be achieved in the coming years [109].

Minton, N A, and V S Murray studied OP poisoning, deactivation and reactivation of acetylcholinesterase enzyme. Organophosphate chemicals are commonly employed as insecticides to manage insect vectors. These drugs are not optimal since they do not possess selectivity for target vectors and have resulted in significant toxicity and mortality in both people and domestic animals. Their hazardous properties have been acknowledged since the 1930s, coinciding with their development as chemical warfare agents. The mechanism by which organophosphates work has been thoroughly studied. The toxic effects that occur as a

result of inhibiting cholinesterase activity, leading to the buildup of acetylcholine at nerve endings, have been well understood. This understanding has been crucial in developing targeted antidote treatments using atropine and oximes. Nevertheless, the definitive identification of the most appropriate oxime for reactivating cholinesterases has not yet been determined conclusively, despite the widespread recommendation of pralidoxime. Further investigation is necessary to examine chronic toxicity, specifically the neuropathic consequences, as it significantly contributes to long-term illness in cases of severe acute or chronic exposure. Efforts should be made to prevent the exposure to potentially harmful organophosphates, especially among workers in sectors involved in making or using these compounds, as well as among vulnerable groups such as elders and children. Government authorities should be urged to regulate the licensing, manufacturing, storage, importation, methods of usage and distribution, food contamination, and disposal of organophosphate products [110].

Smulders, Chantal J G M et al. studied effects of OP insecticides on central nervous system. Long-term and short-term exposure to organophosphate (OP) insecticides might result in lasting neurological and neurobehavioral consequences that cannot be solely ascribed to acetylcholinesterase suppression. There is a suggestion that other proteins in the brain are involved. The researchers looked at how often used organophosphate pesticides affected rat nicotinic acetylcholine receptors (nAChRs), which are made in oocytes of Xenopus laevis. For this experiment, the two-electrode voltage clamp technique was used. At micromolar doses, a number of organophosphate (OP) insecticides, including parathion-ethyl, chlorpyrifos, and disulfoton, were able to efficiently suppress the ion current produced by acetylcholine (ACh). As the agonist ACh concentration rose, the inhibitory impact was stronger. It was demonstrated that certain OPs are more effective at inhibiting nAChRs than AChE when the potency of nAChR inhibition was compared to that of AChE inhibition. The inhibitory effects on nicotinic

acetylcholine receptors can be properly explained by a sequential two-step mechanism. This mechanism involves the quick and reversible binding of organophosphates (OP) to a distinct binding site, which leads to inhibition of the receptor. Subsequently, the receptor either stabilizes in a blocked state or undergoes desensitization. According to research, OPs interact with neuronal alpha-4 beta-2 nicotinic receptors to directly decrease the agonist-induced response. This implies that specific OP pesticides can also affect neuronal alpha4beta2 nAChRs [111].

Lorke, D. E., et al. synthesized 8 new pyridinium oximes and compared their efficacy with pralidoxime and obidoxime. Experimental K-oximes have been synthesized to find more effective reactivators of acetylcholinesterase phosphorylated by organophosphorus chemicals. These oximes have demonstrated promising in vitro efficacy. Nevertheless, the primary reason for oxime toxicity and the factor that limits the dosage is the inhibition of AChE by oximes, as measured by their inherent IC50. In order to evaluate the effectiveness of K-oxime in living organisms, the level of protection against death caused by diisopropylfluorophosphate (DFP) was measured using Cox survival analysis. This measurement was then compared to the level of protection provided by oximes that are currently available for clinical use. Oximes were administered at a dosage that was equitoxic, meaning it was half of the LD01. K-27 offered the best protection, lowering the relative risk of mortality (RR) to 16% of the control RR (P < 0.05). With the exception of obidoxime, K-53, and K-75, this level of protection was statistically significantly superior (P < 0.05) to all other oximes examined. When compared to K-113 (RR = 0.73) and 2-PAM (RR = 0.62), the efficacy of LüH-6 i.e. obidoxime (RR = 0.19), K-48 (RR = 0.28), K-74 (RR = 0.38), K-53 (RR = 0.22), and K-75 (RR = 0.29) were remarkably higher. K-108 and K-107 did not demonstrate any discernible shielding properties. Compared to the other oximes that were examined, Lethal Dose 50% (LD50) data show that K-113, K-

108, and K-107 are much more hazardous in living beings despite exhibiting strong inhibition of AChE in laboratory experiments. Because of this, the safe dosage range for these oximes is limited, and it is insufficient to prevent DFP-related death. In the future, in vivo LD50 assessments may be replaced with dosage estimations based on in vitro IC50 results, reducing the requirement for animals [112].

Jokanović, Milan, and Milos P Stojiljković reviewed the methods by which organophosphorus chemicals interact with cholinesterases and discussed the clinical symptoms of acute poisoning. The mechanisms of action of the pyridinium oximes pralidoxime, trimedoxime, obidoxime, HI-6, and HLö-7 were covered in this book as far as current understanding goes. These oximes are used to treat poisoning from organophosphorus compounds by acting as cholinesterase reactivators. Furthermore, they examined the original research on the efficacy of these oximes in treating poisoning brought on by nerve agents used during the war, including VX, sarin, tabun, soman, and cyclosarin, as well as OP insecticides. Finally, they looked at the standards for selecting oximes for use in auto-injectors in emergency scenarios and for potential development as antidotes for organophosphorus compound poisoning [37].

Sikder, Arun K, et al. synthesized two novel sets of 3,3′-bis-pyridinium mono-oximes, which have different substituents on either side. These compounds are connected by either an oxopropane or propane group. The compounds were thoroughly analyzed using spectral data and their acid dissociation constants (pKa) were determined. The researchers evaluated and compared the effectiveness of these compounds in reactivating electric eel acetylcholinesterase (AChE) that had been inhibited by diisopropylfluorophosphate, both in laboratory experiments and in protecting mice from diisopropylfluorophosphate intoxication. Next, the outcomes were contrasted with trimedoxime and 2-pyridine-aldoxime methiodide findings. The drugs' capacity to inhibit AChE in vitro was also evaluated. The compounds containing the

oxopropane linkage exhibited greater inhibitory potency and lower reactivation ability compared to the comparable derivatives containing propane. There was no noticeable link found between pKa, oxime inhibition of AChE, reactivation of inhibited AChE, and protection index. Modifying substituents in pyridine rings or adjusting connecting groups between pyridine rings did not enhance the effectiveness of antidotal treatment when compared to trimedoxime and 2-pyridine-aldoxime methiodide [113].

Schoene, K et al. examined that how well a number of pyridinium salts protected female mice from soman poisoning. The lethal effect of LD95 of soman was reduced to the 50% mortality level by administering 130 moles/kg of the most powerful compound. When tested in a lab environment, the same compounds that demonstrated protective properties in living organisms were also able to protect acetylcholinesterase (AChE) against soman. By contrasting the degrees of AChE inhibition in the presence and absence of 1 mM pyridinium salt, the protective efficacy was evaluated. The chemicals with the highest potency decreased the inhibition rate to nearly a fraction of its initial value. Additionally, these chemicals' effects on AChE's degradation of acetylcholine were investigated. The antagonistic inhibition constants of the pyridinium salts and the efficacy of protection in a lab setting are clearly and significantly correlated [51].

Hamilton, M G, and P M Lundy studied that, when used together with atropine, the bis-pyridinium oxime HI-6 was discovered to provide substantial defense against repeated exposure to lethal doses of the organophosphorus chemicals soman and tabun. Administering HI-6 along with atropine and diazepam to adult rhesus macaques resulted in the protection of three out of four animals from the deadly effects of 5 times the fatal dose of soman, and three out of 4 animals from 5 times the lethal dose 50% of tabun. Nevertheless, all three mice exposed to five times the lethal dosage of soman died when toxogonin was administered in place of HI-

6 in the therapeutic combination. Rats given HI-6 demonstrated protective ratios against soman and tabun of 3.5 and 2, respectively. These values ranged from 4 to 6 for both agents in guinea pigs. HI-6's ability to reactivate acetylcholinesterase (AChE) in tissue from tabun-poisoned rats or in primate plasma after soman or tabun exposure was not supported by any evidence. The results demonstrate the significant therapeutic benefit of HI-6 in primates, highlighting its particular efficacy against tabun and raising the possibility that HI-6 functions partly independently of AChE reactivation [54].

L W Harris and D L Stitcher examined that rats who were given a dose of 15 mg/kg were divided into 3 groups using VX and atropine treatment. Each group received a different treatment: normal saline at a dose of 30 mg/kg intravenously, pralidoxime at dosage of 30 mg/kg iv, and HS-6 at dosage of 30 mg/kg iv. One hour after the therapy was administered, the subjects were beheaded, and the activity of cholinesterase was measured in their diaphragm, blood and brain tissue. Both pralidoxime and asoxime chloride significantly restored the activity of VX-inhibited blood and diaphragm cholinesterase. Neither oxime appreciably revived Brain ChE activity. The efficacy of all these oximes in reactivating VX-inhibited ChE in living organisms provides a rationale for the high success of standard atropine/oxime therapy in treating VX poisoning [114].

Kuca, Kamil et al. describes the synthesis of a novel bis-quaternary reactivator for acetylcholinesterase-1, which is inhibited by tabun. 1-(4-hydroxyiminomethylpyridinium) and 4-(4-carbamoylpyridinium) butane dibromide make up the reactivator. The effectiveness of this oxime's reactivation is being contrasted with that of the reactivators that are currently in use, specifically pralidoxime, obidoxime, and HI-6. The concentration-reactivation relationship is shown in the article, showing that Compound 1 can successfully revive tabun-inhibited AChE in vitro at concentrations between 10^{-5} and 10^{-4} M. The remaining oximes that were

investigated showed little to no tendency to reactivate AChE that had been blocked by organophosphate tabun. In the end, we have succeeded in producing a very potent substance that can undo the tabun-induced inhibition of AChE-1. 1-(4-hydroxyiminomethylpyridinium)-4-(4-carbamoylpyridinium)-butane dibromide is the name given to this chemical. The potency of reactivating tabun-inhibited AChE was investigated using an in vitro method. It was demonstrated to be comparable to the reactivation potency of obidoxime and greater than the reactivation efficacy of HI-6and pralidoxime [115].

Musilek, Kamil et al. synthesized three AChE reactivators with an asymmetrical structure, containing a cyano-moiety and a propane linker, by modifying existing synthetic methods. They were tested in vitro to see if they could reactivate AChE, which had been inhibited by the insecticide paraoxon and the nerve agent tabun. The outcomes were then contrasted with pralidoxime, KO27, obidoxime, HI-6, and K048's capacity for reactivation. The results suggest that three medications may be useful in the fight against OP insecticide-inhibited AChE. Better results were attained with bis-quaternary compounds—that is, those with at least 1 oxime group in the 4th position. Not a single one of the substances examined could successfully restore OP-inhibited AChE at a level appropriate for in vivo studies [116].

Musilek, Kamil et al. synthesized six new AChE reactivators utilizing established synthetic methods, using a (Z)-but-2-ene linker. They were tested in vitro to see if they could recover the activity of acetylcholinesterase (AChE), which had been inhibited by the insecticide paraoxon or the nerve agent tabun. The outcomes were then contrasted with those of K075; pralidoxime; HI-6; and obidoxime. It was found that the newly synthesized compounds had no effect on GA-inhibited AChE. On the other hand, oxime K075 and (Z)-1,4-bis(4-hydroxyiminomethylpyridinium)-but-2-ene dibromide shown comparable capacities to reactivate OP-inhibited AChE. The ability of the novel compounds to recover the activity of

paraoxon-inhibited AChE was greatly increased by the inclusion of the oxime group at position four [117].

Kuca, Kamil et al. synthesized cholinesterase reactivators, K075 and K07, and their ability to reactivate acetylcholinesterase blocked by tabun in the rat brain was investigated in a controlled laboratory setting. When comparing the effectiveness, it was found that routinely used oximes (pralidoxime, obidoxime, and HI-6) had very little ability to reactivate. However, oximes K074, K075, and trimedoxime demonstrated satisfactory effectiveness. Additionally, compared to trimedoxime (10^{-3} and 10^{-2} M), K-oximes showed a minimal concentration (10^{-4} and 10^{-3} M) for recovering tabun-inhibited AChE. As of right now, tabun-inhibited AChE is thought to be most effectively reactivated by K-oximes [118].

Malinak, David et al. created a set of isoquinolinium-5-carbaldoximes, both symmetric and asymmetric, with the express purpose of reactivating cholinesterase. The potential of the novel compounds to inhibit either butyrylcholinesterase (BChE) or acetylcholinesterase (AChE) was evaluated. In contrast, only weak inhibitors were selected for additional tests including the reactivation of AChE or BChE blocked by sarin, VX, or paraoxon. The majority of the novel compounds demonstrated considerable inhibition of both enzymes. All of the investigated organophosphates' ability to reactivate acetylcholinesterase (AChE) was found to be negligible in comparison to obidoxime's capacity. Notably, at human-tolerable doses, it was found that two compounds could, more efficiently than obidoxime, restore the activity of BChE that has been inhibited by sarin or VX. When compared to obidoxime, one medication showed better reactivation of BChE-inhibited NEMP (a VX surrogate). Molecular docking investigations, which offer insights for developing highly potent BChE reactivators in the future, further supported the in vitro findings [119].

3 Chapter 3

3.1 Experimental

3.1.1 Chemicals

Following are the reagents that are used during synthesis of desired compounds:

Hydroxylamine hydrochloride (100g), 4-acetylpyridine (25g), 4-formylpyridine (5g), Indole-3-carboxaldehyde (10g), 4-chloromethylbenzaldehyde (1g), 1,3-dibromopropane, Sodium hydroxide.

3.1.2 Solvents

The solvents that were used are of analytical grade and used without any other purification such as ethanol, methanol, ethyl acetate, acetone, n-hexane, chloroform, pyridine, acetonitrile.

3.1.3 Instrumentation

Electronic analytical balance ATY224 was used for weighing the chemicals. The progress of reaction was monitored at regular intervals by taking Thin Layer Chromatography (TLC) that was later analyzed under UV lamp. To evaporate excess amount of solvents from reaction mixtures the rotary evaporator R-210 was used. To find out the melting points of synthesized compounds Melting point apparatus SMP10 was used. By using FT-IR ATR model ALPHA 20488 functional groups present in compounds were detected. For elemental analysis CHN CE-440 elemental analyzer used.

3.1.4 Solution preparations

3.3.1 Preparation of 20% NaOH

At room temperature, dissolved 20g of NaOH in small amount of distilled water in 100ml measuring flask. Then filled the flask up to mark with distilled water.

3.2 Synthesis of oximes

Equimolar ratios of hydroxylamine hydrochloride and aldehyde/ketone were dissolved in ethanol in round bottom flask. A few drops of 20% NaOH solution were added to neutralize the reaction mixture. A magnetic stirrer was added to RBF and reaction mixture was refluxed on oil bath. Reaction was monitored by TLC at regular intervals. On the completion of reaction excess solvent was removed by rotary evaporator. 5ml of water was added to reaction mixture and stirred on ice bath until precipitates form. Filtered the precipitates and washed with suitable solvent. Dried the precipitates in vacuum oven under reduced pressure. If products were impure than they were recrystallized by different solvents according to suitability like ethanol, DCM, n-hexane, acetone etc. Pure products are collected after drying and kept in glass vials.
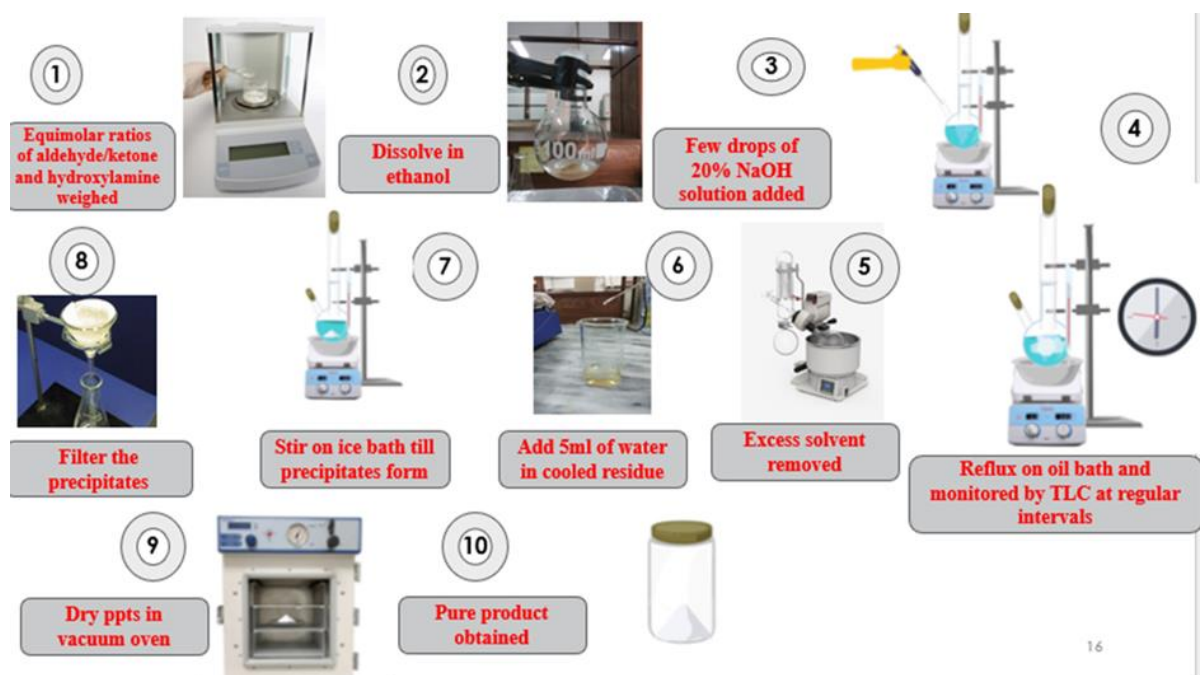
Fig. 26: General procedure for oxime synthesis.

3.2.1 Synthesis of 1-Pyridin-4-yl-ethanone oxime (4-Acetylpyridine oxime/4-APO)

Organic Synthesis approach

0.5g of hydroxylamine hydrochloride was dissolved in 1ml of water in round bottom flask and 670µL of 4-acetylpyridine was added in the same flask. A few drops of 20% NaOH solution was added in the reaction mixture until solution neutralized. Reaction mixture was stirred at 0-5˚C for 1 hour. White colored precipitates were formed and collected by filtration. Precipitates were washed with 500ml cold water and dried at 40˚C in vacuum oven under reduced pressure. Dried precipitates were recrystallized in ethanol.
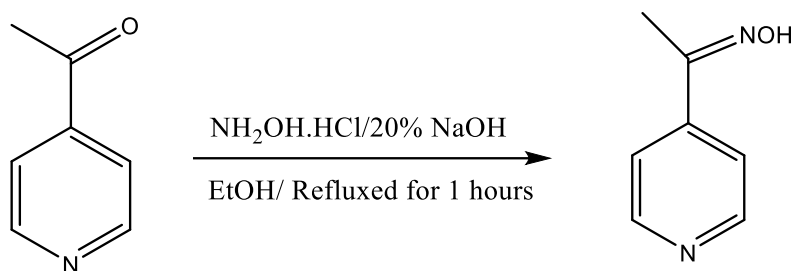
Fig. 27: Synthesis of 4-acetylpyridine oxime.

3.2.2 Synthesis of 4-pyridinecarboxaldehyde oxime (4-Formylpyridine oxime/4-FPO)

1g hydroxylamine hydrochloride and 1g of 4-Formylpyridine was dissolved in 10ml ethanol in round bottom flask. Drops of 20% NaOH solution were added until reaction mixture was neutralized (monitored with pH paper). Reaction mixture was refluxed at 70-80°C in an oil bath for almost 20 hours (reaction was monitored by TLC). After the completion of reaction excess ethanol was removed by rotary evaporator. 10ml of water was added to the cooled residue and stirred the solution on ice bath until oxime crystallizes. Precipitates were formed and collected by filtration. Precipitates were washed with 500ml cold water and dried at 40°C in vacuum oven under reduced pressure. Dried precipitates were recrystallized in water.
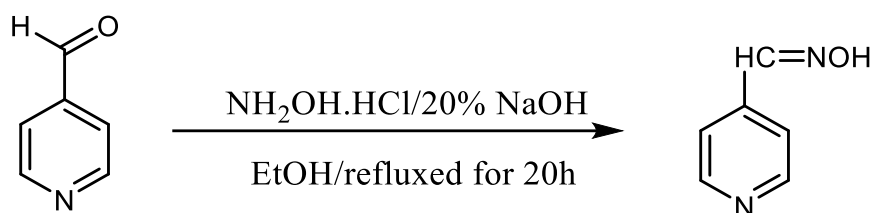
Fig. 28: Synthesis of 4-formylpyridine oxime.

3.2.3 Synthesis of (Z)-N-Hydroxy-1-(1H-indole-3-yl) methanimine (1H-Indole-3-carboxaldehyde oxime/I3CO)

0.5g of NH$_2$OH.HCL and 0.5g of 1H-indole-3-carboxaldehyde were dissolved in 10ml ethanol in a round bottom flask. An equimolar amount of NaOH solution was added to completely neutralize the reaction mixture. Reaction mixture was refluxed on oil bath for 2 hours. After the completion of reaction excess ethanol was removed by rotary evaporator. 10ml of water was added to the cooled residue and stirred the solution on ice bath until oxime crystallizes. Precipitates were formed and collected by filtration. Precipitates were washed with cold water and dried at 40˚C in vacuum oven under reduced pressure. Dried precipitates were recrystallized in ethanol.
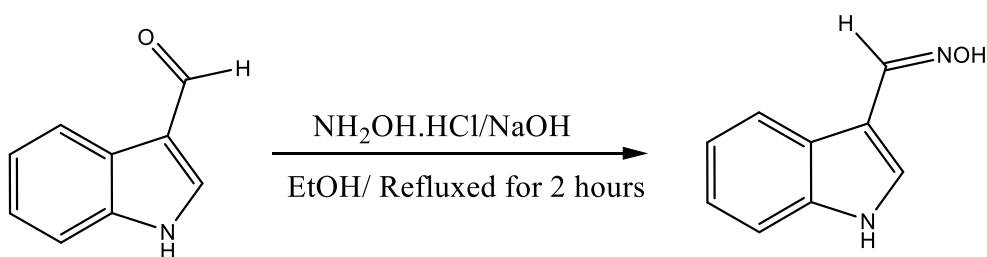
Fig. 29: Synthesis of 1H-indole-3-carboxaldehyde oxime.

3.2.4 Synthesis of Vanillin Oxime

0.5g hydroxylamine hydrochloride and 0.27g of sodium hydroxide was dissolved in 4ml ethanol in round bottom flask to neutralize the solution. 1.05g of vanillin was added and mixture was refluxed at 70-80˚C for 3 hours. A rotary evaporator was used to reduce the volume of mixture. The resulting solution was allowed to cool at room temperature. Crude crystals formed were filtered and washed with cold water. Crystals were recrystallized from ethanol.
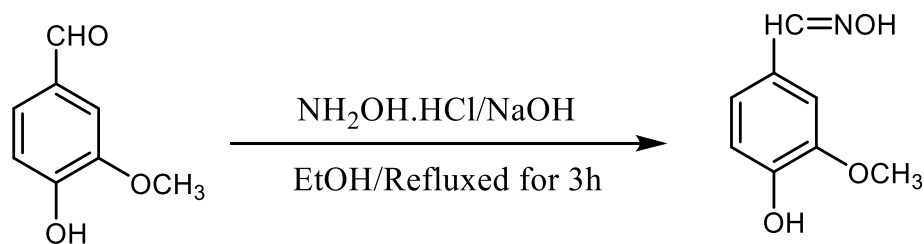
Fig. 30: Synthesis of vanillin oxime.

3.3 General procedure for synthesis of mono and bis-quaternary salts

1:5mmol of prepared aldoximes/ketoximes and 1,3-dibromopropane was dissolved in acetone in a round bottom flask. Reaction mixture was refluxed at 57-60°C in an oil bath and reaction progress was monitored by using TLC. Reaction mixture was cooled at room temperature after the completion of reaction. Product was collected by filtration, washed with acetone and recrystallized with MeCN. Mono and Bis Quaternary oximes were separated by recrystallizing the product in MeCN.
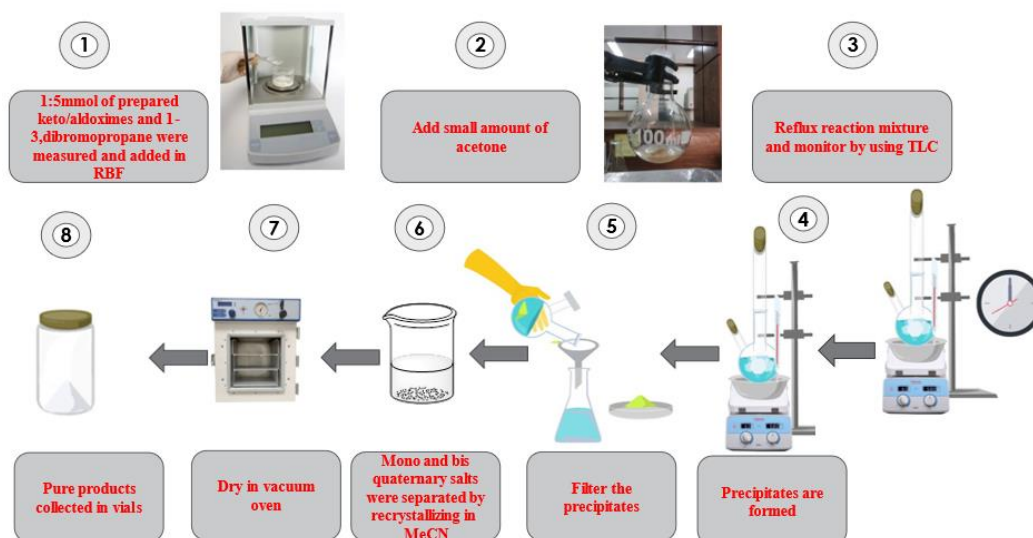
Fig. 31: General Procedure for quaternary oxime synthesis.

54

3.3.1 Synthesis of Mono and Bis quaternary salts 4-Acetylpyridine oxime

0.5g (4mmol) of 4-Acetylpyridine oxime, 2.08ml (20mmol) of 1,3-dibromopropane and 3.73ml of acetone were added in round bottom flask. Reaction mixture was refluxed at 57-60°C for 20 hours. Reaction progress was monitored by using TLC plates. Cooled reaction mixture at room temperature after the completion of reaction. Product was collected by filtration, washed with acetone and recrystallized with MeCN. Mono and Bis Quaternary oximes were separated by recrystallizing the product in MeCN.
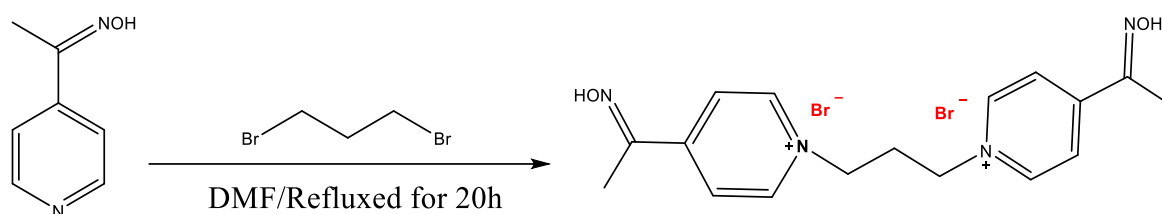
Fig. 32: Synthesis of bis-quaternary salt of 4-APO.

3.3.2 Synthesis of 4-((3-(hydroxyimino) methyl)-1H-indol-1-yl) methyl) benzaldehyde oxime [I3CO+4CMB]

0.100g indole-3-carboxaldehyde, 0.170g (1.7 eq) 1,4-chloromethylbenzaldehyde, 0.2g K_2CO_3, 3ml acetonitrile and 70µl DMF were added to round bottom flask. Reaction mixture was heated to reflux at 82-84°C for 12h. After the reaction completion, the resultant mixture was cooled to room temperature and filtered. Solution was dried over anhydrous sodium sulfate and solvents were evaporated under reduced pressure. The resultant residue was recrystallized using ethanol-water mixture. Resultant product was reacted with 0.16g hydroxylamine hydrochloride and 0.16g NaOH at 0°C in ethanol to get oxime.
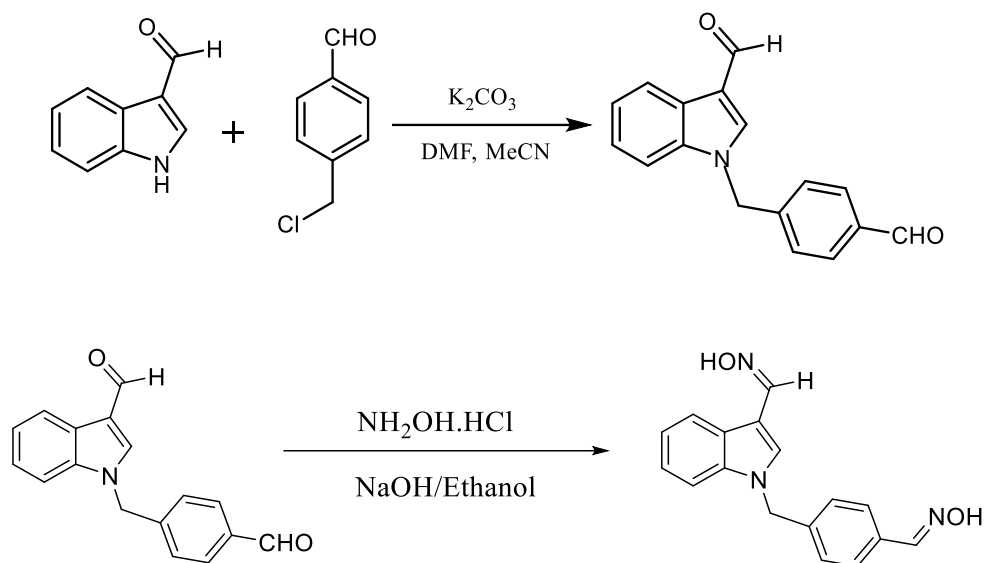
Fig. 33: Synthesis of bis-oxime from I3CO and 4CMBA.

3.3.3 Synthesis of (E)-1-(4-formylbenzyl)-4-((hydroxyimino) methyl) pyridin-1-ium (4-FPO+4CMBA)

0.100g 4-formylpyridine oxime, 0.170g (1.7 eq) 1,4-chloromethylbenzaldehyde, 0.2g K_2CO_3, 3ml acetonitrile and 70μl DMF were added to round bottom flask. Reaction mixture was heated to reflux at 82-84˚C for 12h. After the reaction completion, the resultant mixture was cooled to room temperature and filtered. Solution was dried over anhydrous sodium sulfate and solvents were evaporated under reduced pressure. The resultant residue was recrystallized using ethanol-water mixture.
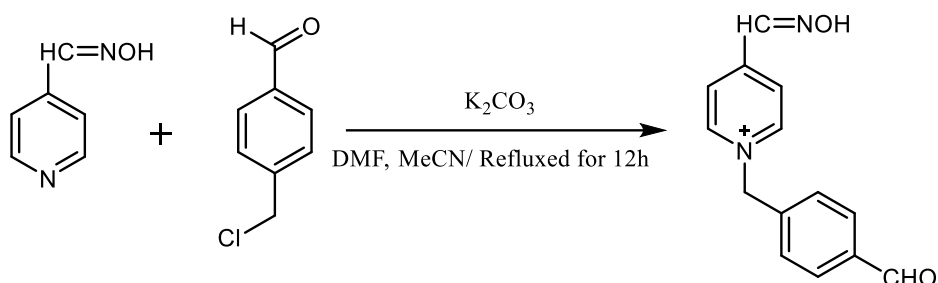
Fig. 34: Synthesis of Mono-oxime salt from 4-FPO and 4CMBA.

56

4   Chapter 4

4.1   Results and Discussion

4.1.1   Characterization of 1-Pyridin-4-yl-ethanone oxime (4-Acetylpyridine oxime/4-APO)

In a 1:1 molar ratio, 4-acetylpyridine oxime was reacted with hydroxylamine hydrochloride. TLC that was taken and observed under a UV light showed that reaction completed in 1 hour. R_f values for reactants and products were different. Different physical and spectroscopic methods were used to analyze synthesized oxime, and it was also submitted to FT-IR, ^{1}H-NMR, and CHN analysis. The melting points, which ranged from 156 to 158°C for the synthesized oxime, was measured. All synthetic substances have purity-indicating sharp melting points. Calculations were also made about the yield and molecular weight of the oxime. The yield of oxime that was synthesized was good. Oximes color varies basically from white to off-white. The physical data is given in the table below.

Table 2: Physical data of synthesized oxime 4-APO.

Compound	Molecular Weight (g/mol)	Physical appearance	Color	Melting Point($^\circ$C)	Yield (%)
4-APO	136.15	Crystals	White	158	93

The FTIR analysis of 4-APO revealed a broad band around 3000-3300cm-1, which corresponds to OH of oxime group and indicates formation of oxime. Due to the intermolecular hydrogen bond weakening, IR band shifting towards lower frequency, the intermolecular hydrogen linked O-H group displayed a weak and broad IR band. The IR band at 1610cm-1 indicates the C=N bond stretch and N-O bond displayed its IR at 921cm-1.
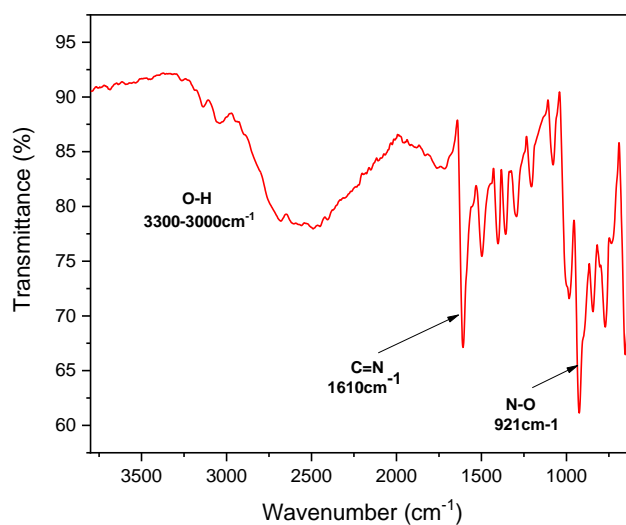
Fig. 35: FTIR spectrum of 4-APO.

The results of the CHN analysis of synthesised compound closely matched the percentage composition estimations that had been established theoretically.

Table 3: CHN Analysis of 4-APO.

Molecular Formula	Calculated			Found		
$C_7H_8N_2O$	C%	H%	N%	C%	H%	N%
	61.75	5.92	20.58	61.69	5.80	20.55

H-NMR of 4-APO was performed be 400-MHz spectrometer in DMSO as a solvent. The structure has 4 different types of protons. Ha appears as a doublet at the chemical shift of 8.62ppm, due to meta coupling of aromatic neighboring proton. Hb also appears as a doublet at the chemical shift of 7.32ppm, Hd of oxime appears as a singlet at 7.49ppm and 3 protons Hc of acetyl group appear as singlet at 2.13ppm.

Protons	Shift (ppm)	Splitting
Ha	8.62	d
Hb	7.32	d
Hd	7.49	s
Hc	2.13	s
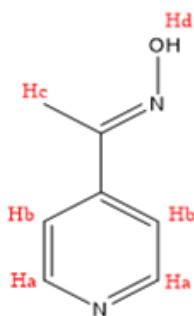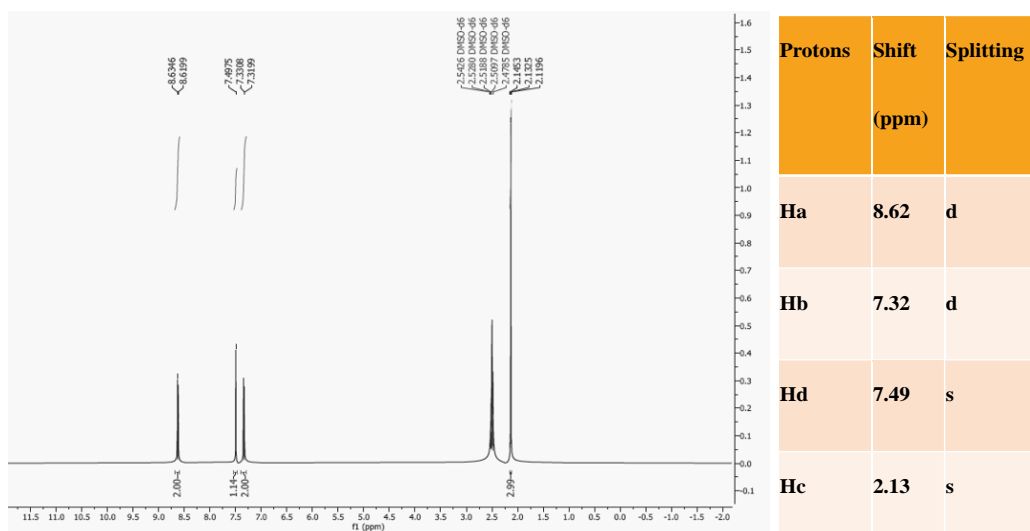
Fig. 36: ^{1}H-NMR of 4-APO.

4.1.2 Characterization of 4-pyridinecarboxaldehyde oxime (4-Formylpyridine oxime/4-FPO)

4-formylpyridine oxime was prepared by taking 4-formylpyridine in a 1:1 molar ratio with hydroxylamine hydrochloride. TLC at set intervals enabled the monitoring of reaction progress. Product R_f values and reactant R_f values differed. Synthesized oxime was examined physically and spectroscopically in addition to being subjected to FT-IR, H-NMR and CHN analyses. The melting point of the synthetic compound, which ranged from 131 to 133ºC, was measured. The yield and molecular weight of the generated molecule was also calculated. Its yield was 85%. The table below contains the physical information about oxime.

Table 4: Physical data of synthesized oxime 4-FPO.

Compound	Molecular weight (g/mol)	Physical appearance	Color	Melting point(˚C)	Yield (%)
4-FPO	122.13	Crystal	Off-white	131-133	85

The FTIR spectrum of 4-FPO revealed a broad band around 3100-3300cm^{-1}, which corresponds to OH of oxime group and indicates formation of oxime.

The IR band at 16O6cm^{-1} indicates the characteristic C=N bond stretch and N-O bond displayed its IR at 920cm^{-1}.

Fig. 37: FTIR spectrum of 4-FPO.

The percentage composition estimates that had been established theoretically closely matched the results of the CHN analysis of the synthesized product.

Table 5: CHN Analysis of 4-FPO.

Molecular formula	Calculated			Found		
	C%	H%	N%	C%	H%	N%
$C_6H_6N_2O$	59.01	4.95	22.94	58.98	4.91	22.89

H-NMR of 4FPO was performed be 400-MHz spectrometer in DMSO as a solvent. The structure has 4 different types of protons. Ha appears as a doublet at the chemical shift of 8.61ppm, due to coupling of aromatic neighboring proton. Hb also appears as a doublet at the chemical shift of 7.33ppm, Hd of oxime appears as a singlet at 8.67ppm and 3 protons Hc of aldehyde group appear as singlet at 6.19ppm.
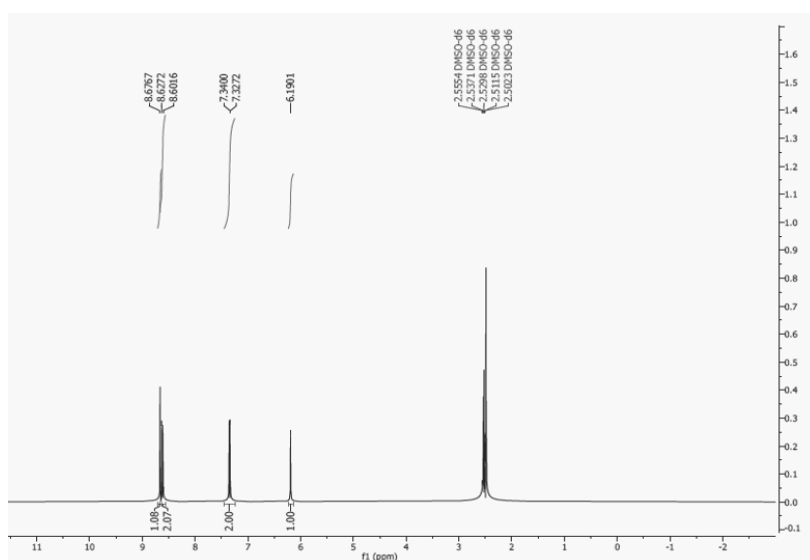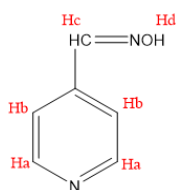
Protons	Shift (ppm)	Splitting
Ha	8.61	d
Hb	7.33	d
Hd	8.67	s
Hc	6.19	s

Fig. 38: ^{1}H-NMR of 4-FPO.

62

4.1.3 Characterization of (Z)-N-Hydroxy-1-(1H-indole-3-yl) methanimine (1H-Indole-3-carboxaldehyde oxime/I3CO)

Indole-3-carboxaldehyde oxime was prepared by taking indole-3-carboxaldehyde with hydroxylamine hydrochloride in ethanol. TLC at set intervals enabled the monitoring of reaction progress. Product R_f values and reactant R_f values differed. Synthesized oxime was examined physically and spectroscopically in addition to being subjected to FT-IR and CHN analyses. The melting point of the synthetic compound, which ranged from 179 to 181ºC, was measured. The yield and molecular weight of the generated molecule was also calculated. Its yield was 91%. The table below contains the physical information about oxime.

Table 6: Physical data of synthesized oxime I3CO.

Compound	Molecular weight(g/mol)	Physical appearance	Color	Melting point(˚C)	Yield (%)
I3CO	160.18	crystal	Light brown	179-181	91%

The FTIR spectrum of I3CO revealed a broad band around 3200-3400cm^{-1}, which corresponds to OH of oxime group and indicates formation of oxime. The IR band at 1615cm^{-1} indicates the characteristic C=N bond stretch and N-O bond displayed its IR at 921cm^{-1}.

Fig. 39: FTIR spectrum of I3CO.

The percentage content estimates that had been established theoretically closely matched the results of the CHN analysis of the synthesized product.

Table 7: CHN Analysis of I3CO.

Molecular Formula	Calculated			Found		
$C_9H_8N_2O$	C%	H%	N%	C%	H%	N%
	67.49	5.03	17.49	67.46	5.01	17.45

4.1.4 Characterization of Vanillin Oxime

Vanillin oxime was prepared by taking Vanillin with hydroxylamine hydrochloride in ethanol. TLC at set intervals enabled the monitoring of reaction progress. Product R_f values and reactant R_f values differed. Synthesized oxime was examined physically and spectroscopically in

64

addition to being subjected to FT-IR, H-NMR and CHN analyses. The melting point of the synthetic compound, which ranged from 117 to 118°C, was measured. The yield and molecular weight of the generated molecule was also calculated. Its yield was 89%. The table below contains physical information about oxime.

Table 8: Physical data of synthesized vanillin Oxime.

Compound	Molecular weight(g/mol)	Physical appearance	Color	Melting point(°C)	Yield (%)
Vanillin oxime	167.16	crystal	Off white	118	89

The FTIR spectrum of vanillin oxime revealed a broad band around 3350-3480cm^{-1}, which corresponds to OH of oxime group and indicates formation of oxime.

The IR band at 1620cm^{-1} indicates the characteristic C=N bond stretch and N-O bond displayed its IR at 945cm^{-1}.
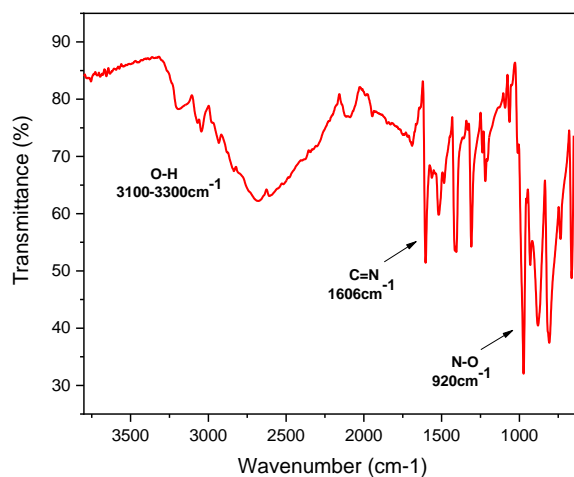
Fig. 40: FTIR spectrum of vanillin oxime.

The percentage composition estimates that had been established theoretically closely matched the results of the CHN analysis of the synthesized product.

Table 9: CHN analysis of vanillin oxime.

Molecular Formula	Calculated			Found		
$C_8H_9NO_3$	C%	H%	N%	C%	H%	N%
	57.48	5.43	8.38	57.46	5.40	8.35

H-NMR of vanillin oxime was performed be 400-MHz spectrometer in DMSO as a solvent. The structure has 7 different types of protons. Ha appears as a singlet at the chemical shift of 8.975ppm. Hb appears as a singlet at the chemical shift of 8.2775ppm, Hd of oxime appears as doublet at 7.13ppm and He also appears as doublet at 6.78ppm. Hc of OH, Hf and Hg appear as singlets at 7.063ppm, 6.160ppm and 3.86ppm respectively.
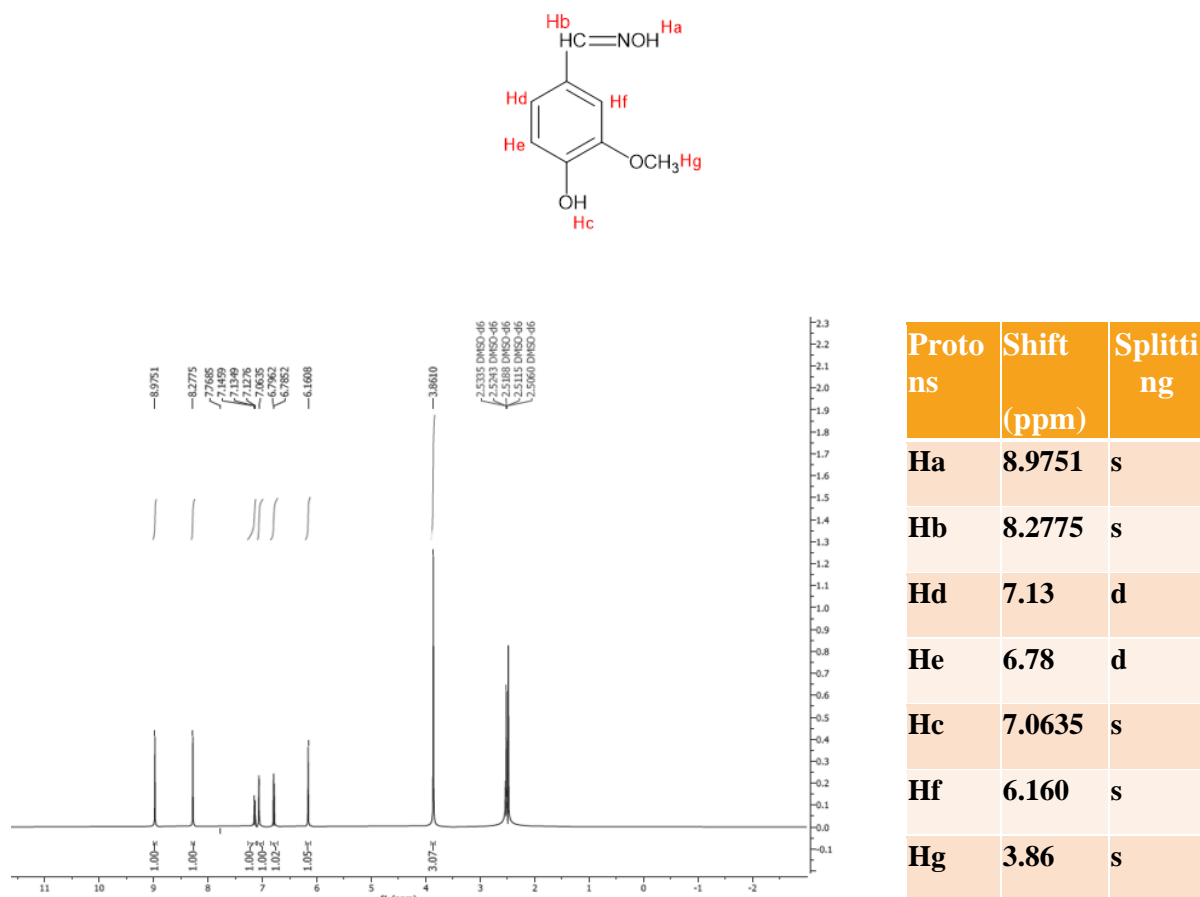
Fig. 41: ^{1}H-NMR of vanillin oxime.

Protons	Shift (ppm)	Splitting
Ha	8.9751	s
Hb	8.2775	s
Hd	7.13	d
He	6.78	d
Hc	7.0635	s
Hf	6.160	s
Hg	3.86	s

4.1.5 Characterization of 1,1'-(propane-1,3-diyl) bis(4-((E)-1-(hydroxyimino) ethyl) pyridin-1-ium)

(Bis-quaternary salt of 4-Acetylpyridine Oxime)

Bis-quaternary salt of 4-APO was prepared by taking 4-acetylpyridine oxime with 1,3-dibromopropane. TLC at set intervals enabled the monitoring of reaction progress. Product R_f values and reactant R_f values differed. Synthesized oxime was examined physically and spectroscopically in addition to being subjected to FT-IR and CHN analyses. The melting point of the synthetic compound, which ranged from 218 to 219ºC, was measured. The yield and

67

molecular weight of the generated molecule was also calculated. Its yield was 87%. The table

below contains physical information about oxime.

Table 10: Physical data of synthesized bis-quaternary 4-APO.

Compound	Molecular weight(g/mol)	Physical appearance	Color	Melting point(°C)	Yield (%)
Bis-4APO	314.39	Crystal	White	219	87

The FTIR spectrum of bis-quaternary salt of 4-APO revealed a broad band at 3408cm^{-1}, which

corresponds to OH of oxime group and indicates formation of oxime salt.

The IR band at 1634cm^{-1} indicates the characteristic C=N bond stretch and N-O bond displayed
its IR at 997cm^{-1}.

Fig. 42: FTIR spectrum of bis-quaternary 4-APO.
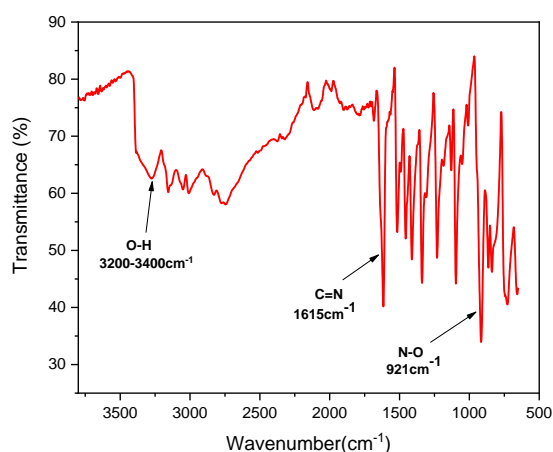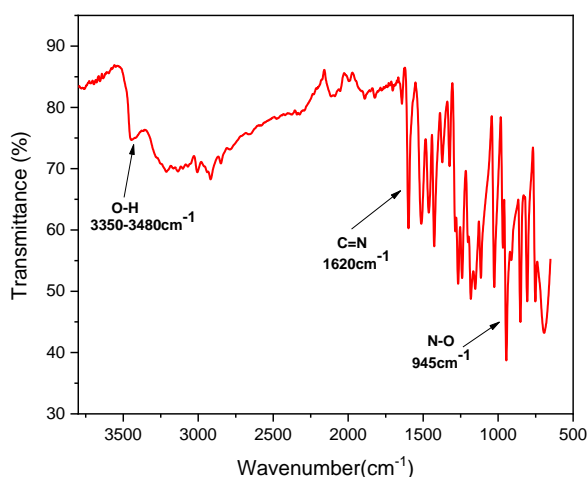
The percentage content estimates that had been established theoretically closely matched the results of the CHN analysis of the synthesized compound.

Table 11: CHN analysis of bis-4APO.

Molecular Formula	Calculated			Found		
$C_{17}H_{22}N_4O_2^{+2}$	C%	H%	N%	C%	H%	N%
	64.95	7.05	17.82	64.91	7.02	17.76

4.1.6 Characterization of 4-((3-(hydroxyimino) methyl)-1H-indol-1-yl) methyl benzaldehyde oxime [I3CO+4CMB]

This bis-oxime was prepared by taking indole-3-carboxaldehyde and 4-chloromethylbenzaldehyde. TLC at set intervals enabled the monitoring of reaction progress. Product R_f values and reactant R_f values differed. Synthesized oxime was examined physically and spectroscopically in addition to being subjected to FT-IR and CHN analyses. The melting point of the synthetic compound, which ranged from 161 to 162°C, was measured. The yield and molecular weight of the generated molecule was also calculated. Its yield was 78%. The table below contains physical information about oxime.

Table 12: Physical data of synthesized oxime (I3CO+4CMB).

Compound	Molecular weight(g/mol)	Physical appearance	Color	Melting point(˚C)	Yield (%)
I3CO+4CMB	293.33	crystal	brown	162	78

The FTIR spectrum of bis-oxime revealed a broad band at $3352cm^{-1}$, which corresponds to OH of oxime group. The IR band at $1619cm^{-1}$ indicates the characteristic C=N bond stretch and N-O bond displayed its IR at $939cm^{-1}$.
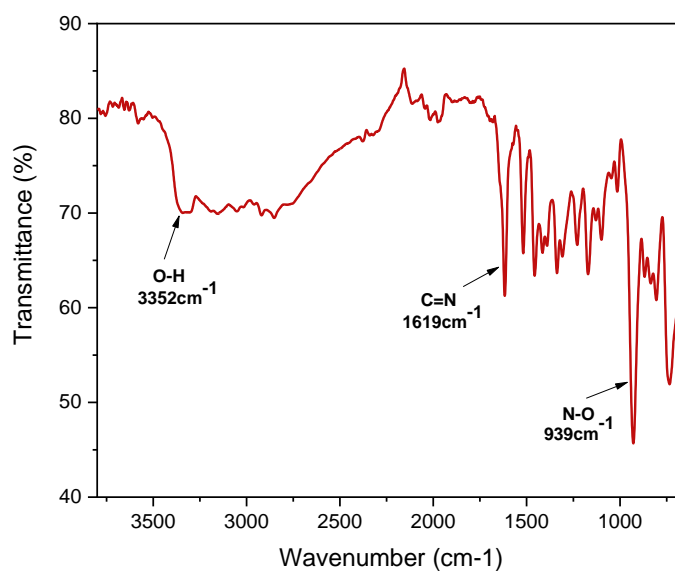
Fig. 43: FTIR spectrum of I3CO+4CMB.

The percentage content demonstrates that had been established theoretically closely matched the results of the CHN analysis of the synthesized product.

Table 13: CHN analysis of I3CO+4CMBA.

Molecular Formula	Calculated			Found		
$C_{17}H_{15}N_3O_2$	C%	H%	N%	C%	H%	N%
	69.61	5.15	14.33	69.56	5.09	14.29

4.1.7 Synthesis of (E)-1-(4-formylbenzyl)-4-((hydroxyimino)methyl) pyridin-1-ium (4-FPO+4CMB)

This mono-oxime salt was prepared by taking already prepared 4-formylpyridine oxime and 4-chloromethylbenzaldehyde. TLC at set intervals enabled the monitoring of reaction progress. Product R_f values and reactant R_f values differed. Synthesized oxime was examined physically and spectroscopically in addition to being subjected to FT-IR and CHN analyses. The melting point of the synthetic compound, which ranged from 131 to 132°C, was measured. The yield and molecular weight of the generated molecule was also calculated. Its yield was 82%. The table below contains physical information about oxime.

Table 14: Physical data of synthesized oxime (4FPO+4CMB).

Compound	Molecular weight(g/mol)	Physical appearance	Color	Melting point(˚C)	Yield (%)
4FPO+4CMB	241.27	crystal	Off white	132	82

The FTIR spectrum of mono-oxime salt revealed a broad band at 3355cm^{-1}, which corresponds to OH of oxime group. The IR band at 1617cm^{-1} indicates the characteristic C=N bond stretch and N-O bond displayed its IR at 946cm^{-1}.
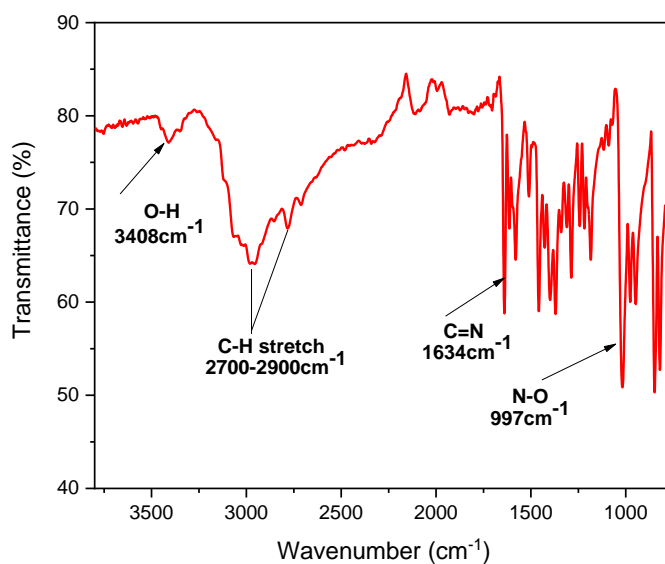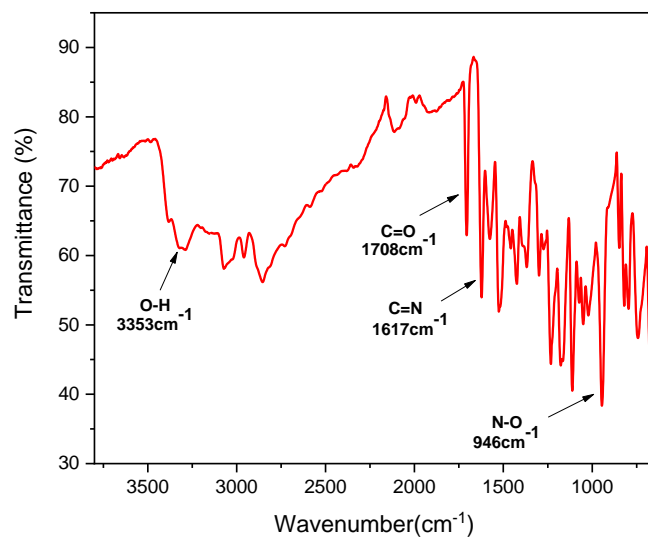

Fig. 44: FTIR spectrum of 4FPO+4CMB.

The percentage content estimates that had been established theoretically closely matched the results of the CHN analysis of the synthesized product.

Table 15: CHN analysis of 4FPO+4CMBA.

Molecular Formula	Calculated			Found		
$C_{14}H_{13}N_2O_2^+$	C%	H%	N%	C%	H%	N%
	69.70	5.43	11.61	69.66	5.40	11.58

5   Conclusion

7 different oximes named as 4-acetylpyridine oxime, 4-formylpyridine oxime, indole-3-carboxaldehyde oxime, vanillin oxime, bis-quaternary salt of 4-acetylpyridine oxime, bis-oxime 13CO+4CMBA and mono-oxime salt of 4-FPO+4CMBA were prepared. Out of seven oximes 3 oximes (Bis-4APO, 13CO+4CMBA and 4FPO+4CMBA) are novel compounds while 3 oximes (4-APO, I3CO and VO) have novel application i.e. reactivation of inhibited acetylcholinesterase enzyme. CHN analysis, FTIR spectroscopy and UV-VIS spectroscopy indicates the formation of desired products and NMR gives the confirmation of prepared compounds. Samples for application (in-vitro reactivation potency if these oximes for AChE reactivation) have been submitted in December 2023 but could not get results till this date.

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