SYNTHESIS, CHARACTERIZATION AND CHOLINESTERASE INHIBITORY ACTIVITY OF NOVEL PIPERIDONE GRAFTED SPIRO HETEROCYCLES

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SYNTHESIS, CHARACTERIZATION AND CHOLINESTERASE INHIBITORY ACTIVITY OF NOVEL PIPERIDONE GRAFTED SPIRO HETEROCYCLES

By

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to GOD for giving me LIFE,

to my Spiritual Master for
illuminating my way of LIFE,

to my parents and parents in-law,
for donating the LOVE in my LIFE,

and to my dear husband for
integrating my LIFE.

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LIST OF ABBREVIATION

Solvents

$CDCl_3$	Deuterated chloroform
DCM	Dichloromethane
DMSO	Dimethyl sulfoxide
DMSO	Deuterated dimethyl sulfoxide
MeOD	Deuterated methanol

Chemicals

AChE	Acetylcholinesterase
AChI	Acetylthiocholine iodide
Ala	Alanine
Asp	Asparagine
BChE	Butyrylcholinesterase
BChI	Butyrylthiocholine iodide
DTNB	Dithiobisnitrobenzoic acid
Gly	Glycine
His	Histidine
Leu	Leucine
Phe	Phenylalanine

Ser	Serine
*Tc*AChE	*Torpedo californica* acetylcholinesterase
Trp	Tryptophan
Tyr	Tyrosine
Val	Valine

Instruments and Techniques

^{1}HNMR	Proton Nuclear Magnetic Resonance
^{13}CNMR	Carbon Nuclear Magnetic Resonance
H,H-COSY	Proton-proton correlated spectroscopy
FTIR	Fourier Transforms Infrared spectroscopy
HMBC	Heteronuclear Multiple Bond Connectivity
HMQC	Heteronuclear Multiple Quantum Coherence
IR	Infrared
TLC	Thin Layer Chromatography
UV	Ultraviolet

Symbols

α	alpha
β	beta
δ	delta
μg	microgram

µL	microliter
µM	micromolar
1D	one dimensional
2D	two-dimensional
br.s	broad singlet
d	doublet
dd	doublet of doublets
IC_{50}	half maximal inhibitory concentration
J	coupling constant
m	multiplet
MHz	megahertz
mp	melting point
ppm	part per million
s	singlet
t	triplet

SINTESIS, PENCIRIAN DAN AKTIVITI PERENCATAN KOLINESTERASE BAGI SEBATIAN BARU HETROSIKLIK SPIRO BERGRAF PIPERIDON

ABSTRAK

Lima siri baru spiro-oksindola-pirrolizina dan hibrid pirolidina telah disintesis menggunakan tindak balas facil satu-pot tiga komponen linear atau kitaran asid α-amino, isatin dan pelbagai dipolarofil *N*-akriloil-bisarilidenapiperidin-4-on.

Tindak balas ini menghasilkan terbitan baru heterosiklik mono-spiro dan bis-spiro dengan menukar nisbah bahan pemula daripada 1:1:1 daripada 1:2:2 dipolarofil, asid α-amino dan isatin. Siklotambahan-spiro ini telah dicirikan dengan menggunakan analisis unsur, teknik spektroskopi IR, 1-D dan 2-D NMR serta data kristalografi X-Ray.

Selain itu, semua sebatian baru yang disintesis telah diuji aktiviti terhadap penyakit Alzheimer menggunakan asai kolorimetrik Ellman. Dalam asai ini, aktiviti perencat kolinesterase terhadap sebatian yang disintesis telah disaring secara *in vitro* menggunakan enzim asetilkolinesterase (AChE) daripada belut elektrik dan enzim butirilkolinesterase (BChE) daripada serum kuda, yang mempunyai peranan utama dalam manifestasi dan perkembangan penyakit Alzheimer.

Keputusan menunjukkan heterosiklik mono-spiro mempunyai aktiviti perencatan yang lebih baik terhadap kedua-dua enzim berbanding dengan heterosiklik bis-spiro.

Sebatian **9i**, **10g**, **12b**, **9e**, **10e** dan **12e** menunjukkan aktiviti perencatan tertinggi di dalam siri berkaitan setanding dengan aktiviti bagi dadah piawai, galantamina.

Kajian simulasi komputer permodelan molekul yang menggunakan struktur kristal Torpedo californica (*Tc*AChE) dan BChE (hBChE) manusia turut menunjukkan orientasi dan mekanisme interaksi pengikatan sebatian yang paling aktif di dalam tapak aktif gorge pada reseptor AChE dan BChE. Kajian permodelan ini memberikan keputusan yang bertepatan dengan yang diperoleh melalui kajian *in vitro*.

SYNTHESIS, CHARACTRIZATION AND CHOLINESTERASE INHIBITORY ACTIVITY OF NOVEL PIPERIDONE GRAFTED SPIRO HETEROCYCLES

ABSTRACT

Five new series of new spiro-oxindole-pyrrolizine and pyrrolidine hybrids were synthesized employing facile one-pot three-component reaction of linear or cyclic α-amino acids, isatin and various derivatives of *N*-acryloyl-bis arylidene piperidin-4-ones dipolarophiles.

These reactions afforded new mono-spiroheterocycles and bis-spiroheterocycles by changing the ratio of the starting materials from 1:1:1 to 1:2:2 of dipolarophiles, α-amino acid and isatin. These spiro-cycloadducts were further elucidated using elemental analysis, IR, 1-D and 2-D NMR spectroscopy techniques as well as X-Ray crystallographic data.

The newly synthesized compounds were also evaluated for their activity against Alzheimer's disease using Ellman's colorimetric assay. In this assay the cholinesterase inhibitory activity of the aforementioned compounds were screened *in vitro* against acetylcholinesterase enzyme (AChE) from *electric eel* and butyrylcholinesterase enzyme (BChE) from equine serum, which have the major roles in the manifestation and progression of Alzheimer's disease.

The results revealed that mono-spiroheterocycles displayed better inhibitory activity against both enzymes in comparison to bis-spiroheterocycles, therein compounds **9i**, **10g**, **12b**, **9e**, **10e** and **12e** showed the highest inhibitory activities among their relevant series comparable to the activity of the standard drug, galanthamine.

Novel computer based molecular modeling simulation studies using crystal structure of *Torpedo californica* AChE (*Tc*AChE) and human BChE (*h*BChE) were also employed to disclose the orientation and binding interaction mechanism of the active compounds inside the active site gorge of AChE and BChE receptors.

CHAPTER ONE

INTRODUCTION

1.1. 1, 3-Dipolar cycloaddition

1,3-Dipolar cycloaddition, also known as the Huisgen reaction, provides an efficient method for the synthesis of various types of five-membered heterocyclic compounds, such as pyrrolidines and pyrrolizines, owing to its high regio- and stereo-selectivity.[1] This type of reaction takes place between a 1,3-dipole (**1**) and a substituted alkene as dipolarophile (**2**), to form a five-membered heterocyclic ring (**3**) (Figure 1.1). While 1,2-disubstituted alkenes are incorporated into 1,3-dipolar cycloaddition reactions, new chiral centers can be formed in a stereospecific manner due to "syn" addition of dipole (**1**) to the double bond of dipolarophile (**2**). Depending on the structure of the dipole, this type of reaction can furnish complex heterocycles with up to four new contiguous chiral centers in a single step. [2-4]

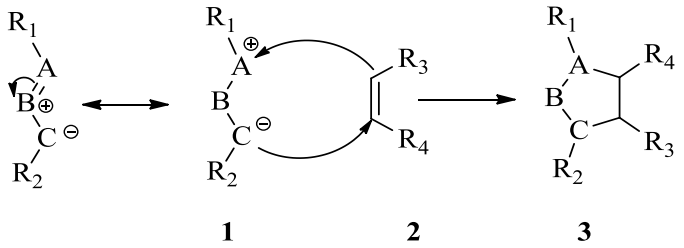


Figure 1.1: Schematic representation of 1,3-dipolar cycloaddition reactions

The asymmetric version of this reaction using either chiral dipolarophile or even chiral catalysts is relatively new methodology, which offers a powerful and reliable synthetic

methodology to access enantiomerically pure five-membered heterocyclic rings in regio- and stereo controlled fashion. [5, 6]

1.1.1. 1,3-Dipoles

The 1,3-dipole, bears a positive and a negative charge distributed over three atoms. The most common atoms incorporated in the 1,3-dipole are nitrogen, carbon, oxygen or sulfur. As depicted in Figure 1.2, 1,3-dipoles can be classified into nitrones (**4**), azomethine ylides (**5**), nitrile oxides (**6**), carbonyl ylides (**7**), azides (**8**), imines (**9**), diazoalkanes (**10**), and diazoacetates (**11**) which are widely used to afford variety of biologically active target heterocycles.[7]

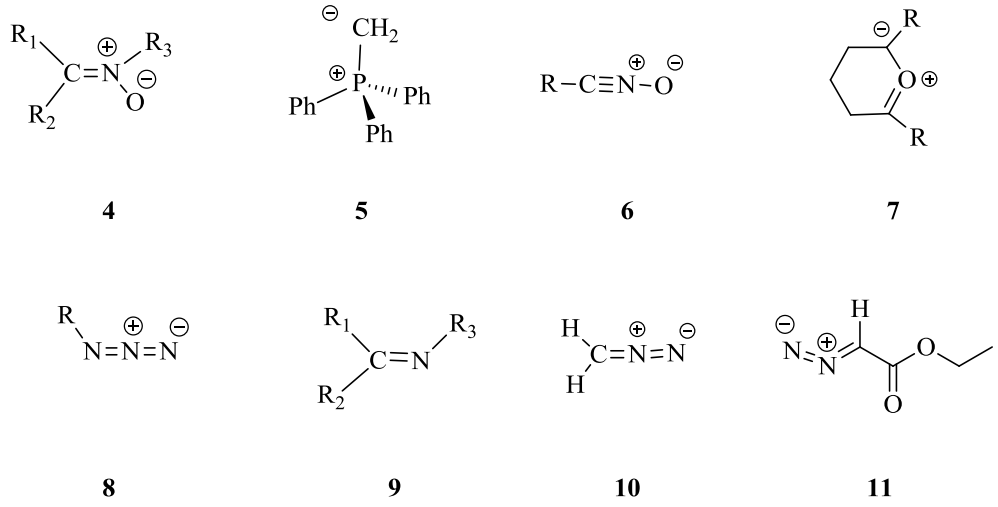


Figure 1.2: Structures of various 1,3-dipoles

1.1.2. Dipolarophiles

The dipolarophile is a reactive multiple bond system. α, β-unsaturated aldehydes and ketones (**12**), allylic alcohols and halides (**13**), vinylic ethers (**14**) and alkynes (**15**) are examples of dipolarophiles. It must be pointed out that dipolarophiles incorporating conjugated double bonds such as dipolarophile (**12**) can exist in two main conformations, *cis* and *trans,* which can have a major impact on the outcome of an asymmetric 1,3-dipolar cycloaddition reaction (Figure 1.3).

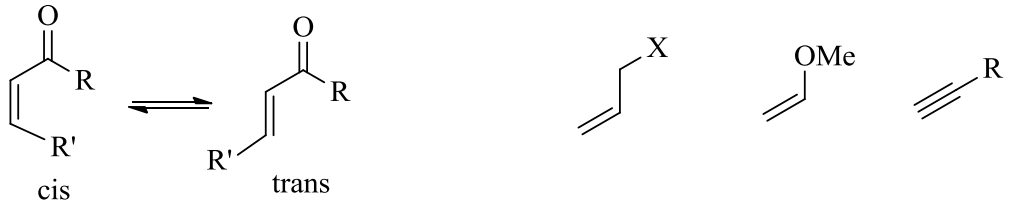


Figure 1.3: Structures of various dipolarophiles

1.2. Multi-component reaction of azomethine ylides

Multi-component reactions offer a wide range of possibilities for the efficient synthesis of highly complex molecules in a single operational step. These reactions eliminate the need for several workups and purification steps, enabling a great saving of both solvents and reagents. Multi-component reactions have attracted considerable attention since an initial report in 1850 by Strecker, who introduced a novel method for the synthesis of amino acids.[8] Passerini further discovered that isonitriles can participate in multicomponent reactions and this finding formed the basis of the well-known Ugi

reaction.[9] The development of new multi-component reactions often enables efficient and convenient entry into interesting structures.

The multi-component, 1,3-dipolar cycloaddition of azomethine ylides to an electron-deficient dipolarophile is an important method for the construction of nitrogen containing five-membered pyrrolidine and pyrrolizine heterocyclic rings in a single step. Pyrrolidines and pyrrolizines are among the major building blocks used for the synthesis of natural products and pharmacologically active heterocyclic compounds. [10, 11] Azomethine ylides are generated *in situ* from decarboxylative condensation of either aldehydes or ketones with α-amino acids under reflux. [12] As depicted in Figure 1.4, these dipolar molecules contain a negatively charged atom, usually a carbanion, directly attached to a nitrogen atom with positive charge, in which both atoms have full octets of electrons.[13, 14]

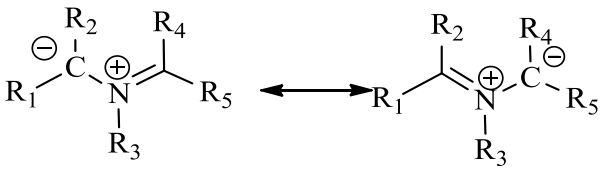


Figure 1.4: Schematic representation of azomethine ylides

1.3. Biological significance of spiro oxindoles, pyrrolidines and pyrrolizines

The spiro oxindoles can be obtained from the cycloaddition reaction of azomethine ylides generated *in situ* from isatin and α-amino acids, to dipolarophiles bearing exocyclic double bonds. This heterocyclic system is the core structure of many

pharmacological agents and natural alkaloids.[15] Spirotryprostatin A (**16**), a natural product isolated from the fermentation broth of *Aspergillus fumigatus*, has been identified as a novel inhibitor of microtubule assembly.[16] Spirotryprostatin B (**17**) inhibits the growth of human chronic myelogenous leukemia K562 cells and human promyelocytic leukemia HL-60 cells.[17] Pteropodine (**18**) and isopteropodine (**19**) have been shown to modulate the function of muscarinic and serotonin receptors.[18] Strychnofoline (**20**) belongs to a class of natural products isolated from the leaves of *Strychnos usambarensis*, which displays antimitotic activity against cultures of mouse melanoma and Ehrlich tumor cells (Figure 1.5).[19] A prominent structural feature of these and related spirotryprostatin alkaloids is the presence of a spiro-[pyrrolidin-3,3'-oxindole] nucleus.[20]

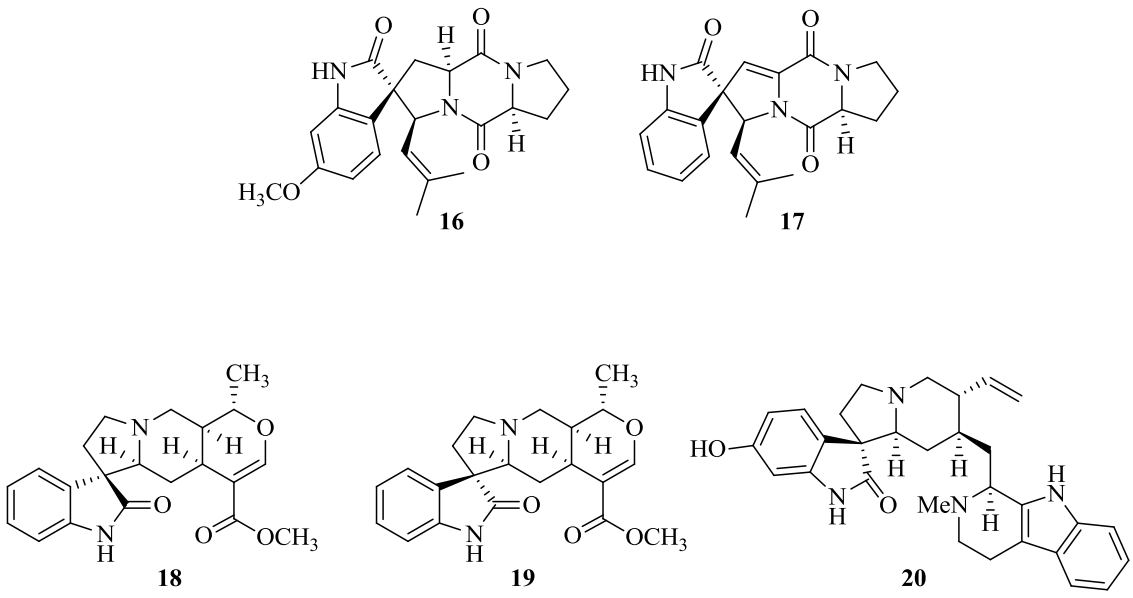


Figure 1.5: Structures of various spiro oxindoles

Spiro oxindole derivatives were also found to be potent aldose reductase inhibitors (ARIs), which help to treat and prevent diabetic complications arising from elevated

levels of sorbitol. Some spiro pyrrolidines are potential antileukemic and anticonvulsant agents and possess antiviral and local anesthetic activities. [21] It is also worth to note that several natural spiro oxindoles act as potent non-peptide inhibitor of the p53–MDM2 interaction. [22]

Kumar *et al*. reported novel three-component tandem reactions of cyclic mono ketones, isatin and sarcosine to form di-spiropyrrolidines, which represented pronounced biological activity.[23] Fokas *et al.*, has reported synthesis of a library of spiro [pyrrolidine-2,30-oxindole] compounds, which can be used as bioactive agents, bio-separation agents or pesticides.[24]

Pyrrolidine and pyrrolizine ring system are also found in a large number of biologically active natural and artificial compounds.[25] As shown in Figure 1.6, polyhydroxylated pyrrolidines (**21-25**) such as DMPD (**21**), Hyacinthacines (**22**), Alexine (**23**), Broussonetine C(**24**), Glycomimetics (**25**) have drawn considerable interest in recent years, primarily due to their ability to inhibit glycosidases.[26] Glycosidases are valuable therapeutic agents in treatment of many serious diseases like diabetes [27], cancer [28], and viral infections such as HIV.[29] Besides, novel synthetic pyrrolizines also showed potent anti-mycobacterial,[30] anti-inflammatory [31] and anti-tubercular activities.[30]

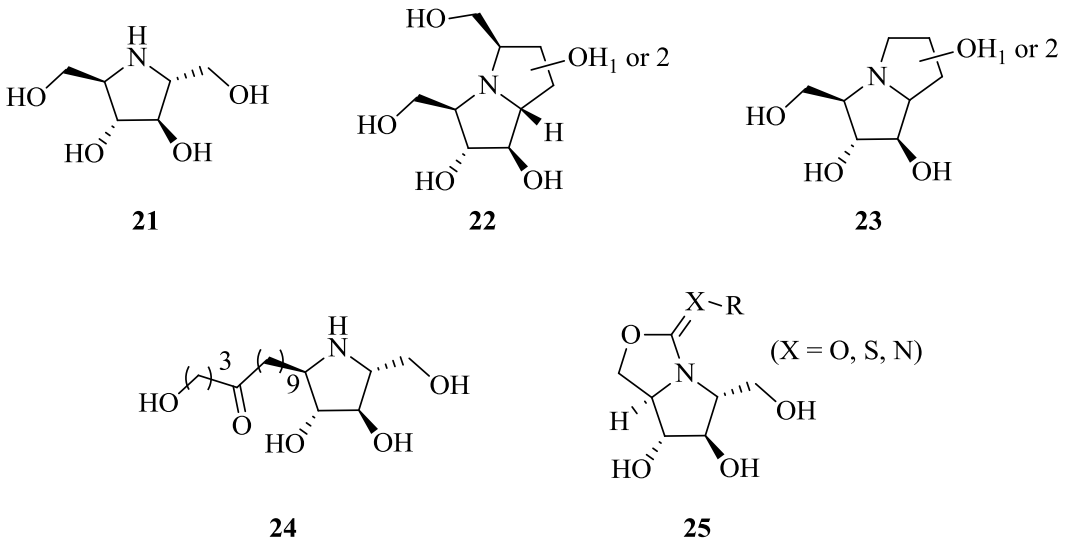


Figure 1.6: Structures of various biologically active pyrrolidines

1.4. Alzheimer's disease (AD)

Alzheimer's disease (AD) is the most common form of dementia, accounting for 50–60% of all the cases. The prevalence of dementia is lower than 1% in individuals aged 60–64 years, however displays an almost exponential increase with age, with 24% to 33% prevalence in individuals aged 85 years or more (Figure 1.7).[32]

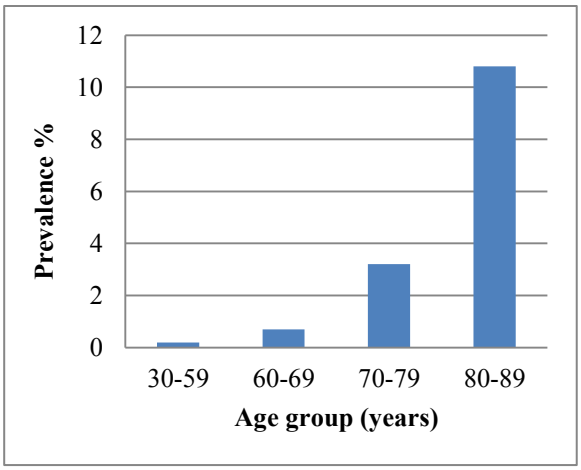


Figure 1.7: The diagram for representation of increase in prevalence of AD with age

1.4.1. Definition of Alzheimer's disease

AD is a progressive disease with debilitating consequences for the patients and their families.[33] Epidemiologically, AD is classified into early-onset familial AD (FAD) and late-onset sporadic AD, or SAD. FAD is associated with mutations in three genes coding for amyloid precursor protein (APP), presenilin-1 (PS-1) and presenilin-2 (PS-2), which are inherited. FAD most commonly occurs in patients between 40 and 65 years of age and is characterized by a rapid progression.[34] Late onset SAD accounts for the majority (95%) of AD cases in people above 65 years.

This neurodegenerative disorder typically begins with subtle and poorly recognized failure of memory that slowly becomes more severe and eventually incapacitating. Other common finding comprises progressive impairment of cognitive functions including memory loss, confusion, poor judgment, language disturbance, withdrawal and hallucinations. Death usually results from general inanition, malnutrition, and pneumonia. At advanced stages of the disease, AD patients also exhibit behavioral disturbances including agitation, irritability, anxiety, delusions and depression. The typical clinical duration of the disease is 8–10 years. [32, 35]

1.4.2. Major Alzheimer's disease risk factors

The major risk factors of AD are classified into: firstly, family history, in which individuals with brother or sister affected by AD are more likely to develop this disease.[36, 37]

Secondly, individuals with ε4 form of gene apolipoprotein E, (APOE- ε4), are at increased risk of developing AD.[38] APOE-ε4 is one of three common forms of the APOE gene, which provides the blueprint for a protein that carries cholesterol in the bloodstream. Everyone inherits one form of the APOE gene from each parent. Those who inherit one APOE-ε4 gene have increased risk of developing AD and of developing it at an earlier age than those who inherit the APOE-ε2 or APOE-ε3 forms of the APOE gene. Thirdly, cardiovascular disease risk factors such as physical inactivity, high cholesterol (especially in midlife), diabetes, smoking and obesity, are associated with a higher risk of developing AD and other dementias.[39-41] Then, social engagement such as remaining mentally and socially active and consuming a diet low in saturated fats and rich in vegetables, may support brain health and prevent developing AD [42, 43] and lastly, head trauma and Traumatic Brain Injury (TBI) are associated with an increased risk of AD and other dementias. Moderate head injuries are associated with twice the risk of developing AD compared with no head injuries, and severe head injuries are associated with 4.5 times the risk (Figure 1.8).[44]

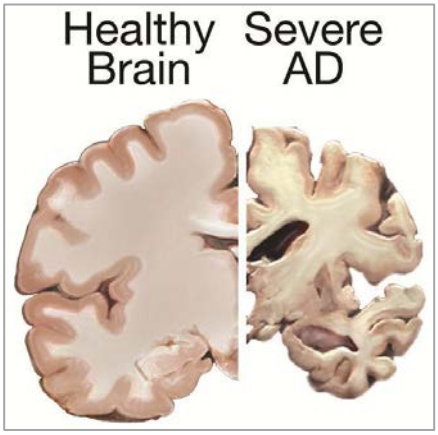


Figure 1.8: Schematic representation of healthy brain and severely affected AD brain [45]

1.4.3. Pathology of Alzheimer's disease

The pathology of AD has been studied intensely in the last 20 years. Animal models have provided valuable information to understand the mechanisms. Typical pathological phenomena are extracellular senile plaques, consisting amyloid beta peptides (Aβ), and intracellular neurofibrillary tangles composed of phosphorylated τau protein. Both Aβ peptides and τau proteins are normal cellular constituents, which in AD both take an abnormal fibrillary structure, associated with a poor solubility in aqueous media (Figure 1.9).

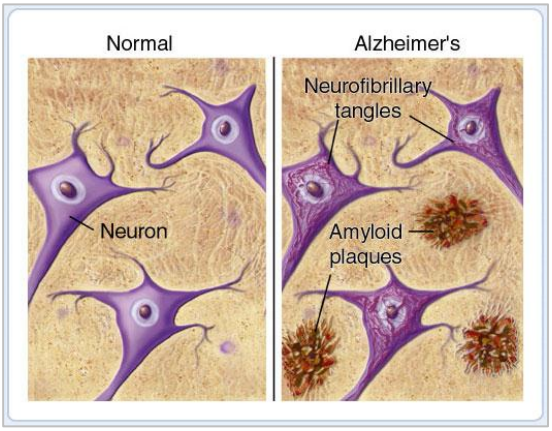


Figure 1.9: Amyloid plaques and neurofibrillary tangles in Alzheimer's disease [46]

Aforementioned extracellular senile plaques, physically damage the cholinergic neurons in the basal forebrain leading to deficiency in cholinergic neurotransmission in the hippocampus and cerebral cortex and contributes to the cognitive deficit of AD.[47, 48]

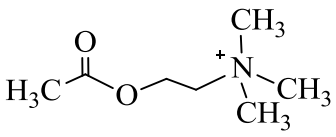
1.4.4. Cholinergic hypothesis

The cholinergic hypothesis was the first theory proposed to explain AD and has led to the development of the drugs currently approved to treat mild to moderate AD. Based on the finding, loss of cholinergic activity is commonly observed in the brains of AD

patients.[49] This hypothesis postulates that the cognitive impairments experienced by AD patients results from the deficiency in cholinergic neurotransmission and acetylcholine generation decline.[50]

1.4.5. Acetylcholine (ACh) and butrylylcholine (BCh)

Acetylcholine (ACh, **26**) is an organic, polyatomic cation that acts as a neurotransmitter in both the peripheral nervous system (PNS) and central nervous system (CNS) in many organisms including humans. It is an ester acid and possess the chemical formula of "$CH_3COO(CH_2)_2N^+(CH_3)_3$" with systematic name of "2-acetoxy-*N,N,N*-trimethylethan-aminium". In contrary to acetylcholine, butyrylcholine (**27**) is not a physiological substrate in human brain but a synthetic compound, which is used as a substrate to differentiate between the two types of cholinesterase enzymes (Figure 1.10).



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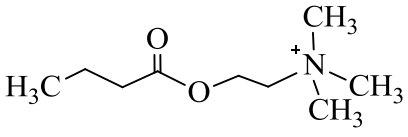

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Figure 1.10: Acetylcholine (**26**) and butrylylcholine (**27**)

The rational way to enhance the cholinergic neurotransmission is to inhibit the cholinesterase enzymes responsible for the metabolic breakdown of acetylcholine (ACh). Acetylcholinesterase inhibitors (AChEI) can enhance cholinergic neurotransmission by increasing acetylcholine (ACh) availability in the synaptic cleft.[51]

1.4.6. Cholinesterases

In vertebrates, there are two types of cholinesterase, acetylcholinesterase (AChE) and butyrylcholinesterase (BChE), which can be distinguished based on their substrate specificities, distribution in various tissues and sensitivity toward various inhibitors. AChE (EC 3.1.1.7) (Figure 1.11) is a serine hydrolase enzyme located in peripheral and/or central nervous cholinergic system.[52] This enzyme is a membrane-bound enzyme, which is mainly found in the brain, muscles, erythrocytes and cholinergic neurons. It is an essential enzyme in the human body to degrade acetylcholine and the terminate neurotransmission impulses.[53] Recent studies revealed that AChE could also play a key role in accelerating senile amyloid β-peptide (Aβ) plaques deposition.[54] AChE has high catalytic activity since each enzyme is able to degrade about 25,000 molecules of acetylcholine per second. [55]

Figure 1.11: Molecular representation of AChE enzyme [56]

AChE is the target of natural toxins such as alkaloids and polypeptide toxins, synthetic pesticides and warfare agents, as well as drugs designed to combat neuromuscular disorders, such as myasthenia gravis, glaucoma and Alzheimer's disease.[57]

Another cholinesterase, butyrylcholinesterase (BChE) (Figure 1.12), is also involved in metabolic degradation of acetylcholine and differs from AChE for tissue distribution and sensitivity to wide variety of substrates and inhibitors.[52]

The specific function of BChE is not clearly known but it is suggested to act as a scavenger of cholinergic toxins in addition to have an auxiliary role in synaptic neurotransmission. [58] This enzyme is found in the intestine, liver, kidney, heart, lung, and blood serum and plays a major role in the metabolism of ester containing compounds. BChE can displace AChE in acetylcholine degradation when it is inhibited or absent. Normally, in the healthy brain, AChE is predominant but in AD brain, BChE activity rises while AChE activity remains unchanged or diminished. [59] Many species such as human, horse, mice exhibit high BChE activity in their plasma, while rat has higher AChE activity than BChE in its plasma. AChE and BChE share 65% amino acid sequence homology. [60]

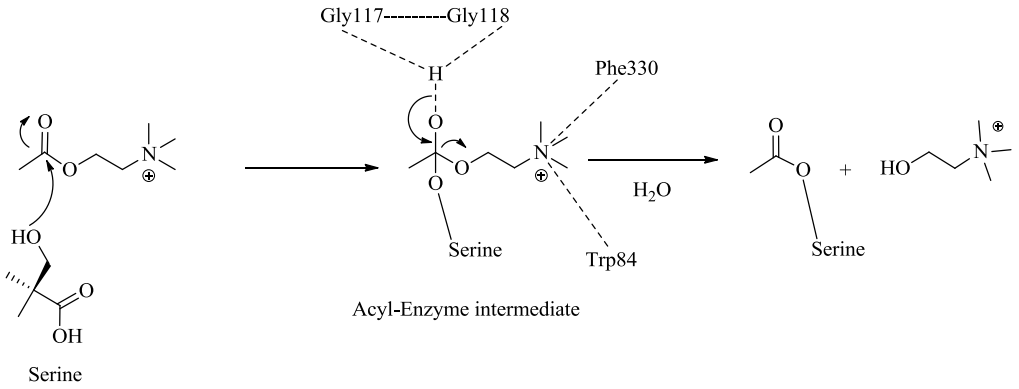
Figure 1.12: Molecular representation of BChE enzyme [61]

1.4.7. AChE and BChE structural specifications

The overall architecture of the AChE and BChE enzymes is quite similar. The active site is located at the bottom of a 20 Å deep cavity named as "aromatic gorge". Substrate and inhibitor guidance down the aromatic gorge is facilitated by hydrophobic interactions with aromatic residues lining the gorge wall such as phenylalanine (Phe), tryptophan (Trp) and tyrosine (Tyr).[62]

The overall structure of BChE is very similar to that of AChE. However, in the active site of BChE aromatic residues such as tryptophan (Trp) and phenylalanine (Phe) are replaced with hydrophobic ones such as leucine (Leu) and valine (Val), making BChE more appropriate to accommodate bulkier substrates and inhibitors. [63] AChE and BChE possess a catalytic triad (CT) composed of Ser200, His440 and Glu327 residues in *Tc*AChE as well as His438, Ser198 and Glu325 in *h*BChE enzyme.[64]

The mechanism of substrate binding to catalytic triad site plausibly is through nucleophilic attack of serine hydroxyl moiety to carbonyl group of the substrate to give an acyl-enzyme intermediate. Subsequently, a water molecule de-acylates serine residue by hydrolyzing its ester linkage to the substrate (Scheme 1.1).



Scheme 1.1: Schematic representation acetylcholine substrate hydrolysis in AChE catalytic triad

Four other binding sub-sites incorporate into proper orientation of the choline substrates in the catalytic cavity. Using *Tc*AChE residue numbering, these sub-sites are:

(i) A three-pronged oxyanion hole, formed by the backbone amides of Gly117, Gly118, and Ala201 that stabilizes the negative charge developed at the C=O moiety of the substrate in the acylation/de-acylation process. [65]

(ii) The aromatic rings of Trp84 and Phe330 that stabilize the quaternary ammonium function of the choline moiety through π-π interactions.[66]

(iii) A concave hydrophobic pocket (the acyl-binding pocket) consisting of residues Phe288 and Phe290 where are located in the so-called acyl loop in which the acetyl or propanoyl moiety of the substrate is bound (Figure 1.13). [67] and,

(iv) A peripheral anionic site composed of aromatic Tyr 70, Asp72, Tyr 121, Trp279 and Tyr334 residues to guide the substrate to catalytic triad.[65]

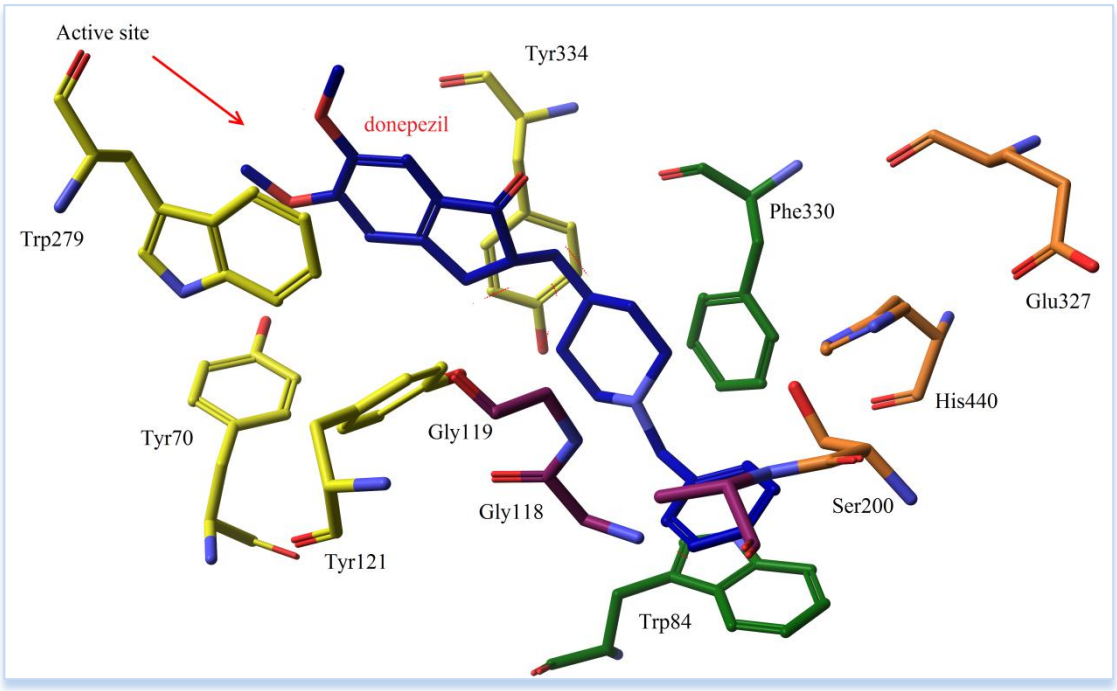


Figure 1.13: Peripheral anionic site (yellow), choline binding site (green), oxyanion hole (maroon) and catalytic triad (orange) residues of *Tc*AChE in complex with donepezil (blue) [56]

As mentioned earlier, most differences between BChE and AChE are confined to the residues lining the gorge wall in peripheral anionic site, where the aromatic residues are replaced with hydrophobic one. In addition, acyl-binding pocket residues in AChE, Phe288 and Phe290, are replaced by Leu286 and Val288, in BChE. These changes make

it possible for the binding of the bulkier substrates moiety in BChE in contrast to AChE (Figure 1.14). [63]

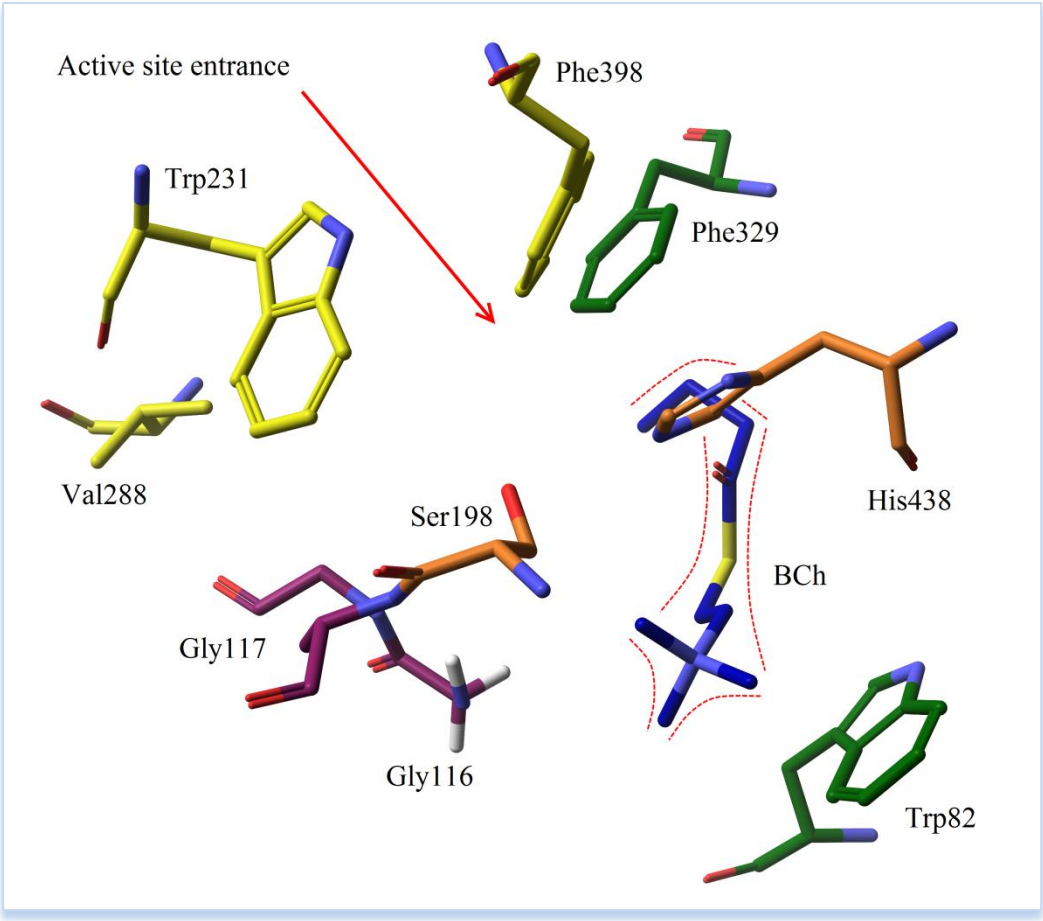


Figure 1.14: Peripheral anionic site (yellow), choline binding site (green), oxyanion hole (maroon) and catalytic triad (orange) residues of *h*BChE in complex with its BCh substrate (blue) [61]

1.4.8. Cholinesterase inhibitor drugs to treat AD

Drugs capable of inhibiting cholinesterase enzymes, plausibly affect central cholinergic function, by increasing level of ACh, thus improving cognition and even some of the behavioral problems experienced by AD patients. Marketed acetylcholinesterase inhibitors (AChEI's) (Figure 1.15) often used in combination with diverse conventional drugs such as antidepressants, antioxidants, and neuro-protectants. [68]

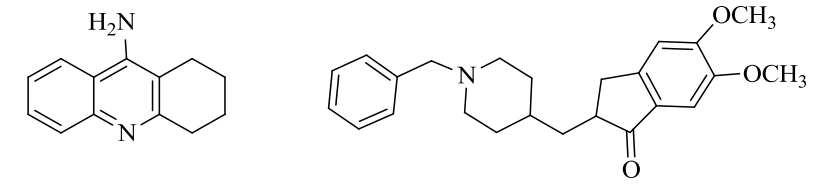
Typical analysis of new acetylcholinesterase inhibitors (AChEIs) are performed by assaying *in vitro* activity using Ellman's method. Based on this assay, the structure-activity relationship (SAR) is determined. [69] *Ex vivo* and *in vivo* as well as toxicology studies will be performed on the most active inhibitors at *in vitro* stage to estimate their bioavailability and pharmacodynamics profile.

AChE inhibitors inhibit AChE *via* two different mechanisms: firstly, competitive, reversible mechanism, by interacting with the active site of the enzyme through a network of polar and hydrophobic molecular interactions, and secondly, non-competitive, irreversible mechanism, through strong covalent binding to the active site of enzyme, thus destructing catalytic site of the enzyme. All the AD drugs are classified as reversible inhibitors whereas pesticides and nerve gases are belong to latter class.

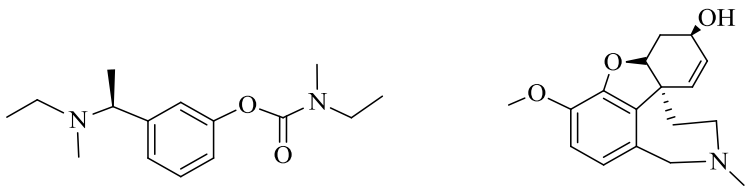
Many AChE inhibitors used clinically for the treatment of AD are alkaloidal natural products or natural product analogues, such as rivastigmine, galanthamine and huperzine A, along with many of the synthetic AChE inhibitors such as donepezil and tacrine. However, after a long period of using these AChE inhibitors to treat mild or moderate AD in clinical practice, a profile of adverse effects was gradually recognized, including increased rates of heart attack, acute liver toxicity, and hip fracture in older adults. Most of the efforts in AChE inhibitor discovery concentrated on structural modification of the existing drugs or a combination of active substructures, while the resulting compounds might suffer the risk of adverse effects due to the similarities in the scaffold. [68, 70]

Tacrine (**28**) was the first AChE inhibitor permitted by the FDA to enter the medical market although its adverse effects such as hepatotoxicity and gastrointestinal disorders, led to its early withdrawal from the market. Tacrine is cholinesterase inhibitor with high selectivity toward plasma BChE.[57]

Donepezil (**29**) the reversible, non-competitive acetylcholinesterase inhibitor (AChEI) was approved and became available for use in patients with mild to moderate AD in February 1997 and is now being prescribed worldwide.



28 **29**



30 **31**

Figure 1.15: Structures of the acetyl cholinesterase inhibitors as FDA approved Alzheimer's disease therapeutics.

Rivastigmine (**30**) is a reversible dual inhibitor of both AChE and BChE with a short plasma half-life but a relatively longer duration of ChE inhibition.[71] This inhibitor

displays specific activity for central AChE over peripheral AChE and currently being used for the treatment of AD and memory dysfunction. Recent investigations indicated that rivastigmine also produces significant beneficial effect in patients with vascular dementia or dementia associated with Parkinson's disease. [72]

Galanthamine (**31**) is an alkaloid extracted from several species of amaryllidaceae. It is a centrally acting, selective, competitive, and reversible AChEI, which in addition to inhibit AChE with a mild potency behaves as an allosteric potentiating ligand at nicotinic receptors contributing to neuroprotection. It has been shown that galanthamine maintains long-term cognitive function and delays emergence of behavioral symptoms. [73, 74] It is as effective as tacrine in the treatment of AD, but it has a lower toxicity and prevents neuronal damage generated by reactive oxygen species (ROS) and nitrogen species, such as hydrogen peroxide (H_2O_2) and nitric oxide (NO) *in vitro*. On the basis of these premises, novel classes of AChE inhibitors (AChEIs) targeting the peripheral site have emerged as promising disease-modifying anti-AD drug candidates.[75]

1.5. Molecular modeling simulation

Molecular modeling technology is extensively used in different stages of the drug discovery procedure to represent the binding orientation template of an active substrate/inhibitor into its relevant receptor and predict the binding affinity of newly design drugs to the receptor.[76]

Pioneering research works in the area of molecular docking date back into the early 1980s. However, it took at least a decade for this technology to become popular among computational chemists and pharmaceutical researchers.[77] Molecular docking procedure typically consists of two interrelated task: (i) to sample possible lowest energy conformational states of the protein-ligand complex and (ii) to calculate of the free energy of these complex to produce a score, which can be further correlated to biological activities or other function, the so-called scoring.[78]

Practically, identification of novel compound by using *in silico* methods is faster and economical in contrast to traditional approaches.[79] Among the most commonly used ligand screening tools, docking analysis have been used extensively to predict the binding orientation and affinities of many potent enzyme inhibitors as well as receptor antagonists. Many drugs developed partly by computer-aided drug design methods are in late-stage of clinical trials or have now reached the market.[80]

1.6. Problem statement

Nowadays, to use cholinesterase inhibitor drugs such as donepezil or galanthamine is the most practiced approach for symptomatic improvement of cognitive impairments in the individuals suffering from Alzheimer's disease. Despite the tremendous efforts in search of novel disease modifying agents working *via* β-amyloid or tau pathways, none is clinically available due to their adverse effects. Besides, limited number of potent cholinesterase inhibitor drugs, keeps the search for new inhibitors going worldwide. In the present study, novel piperidone grafted spiropyrrolizine and spiropyrrolidine derivatives were synthesized, thoroughly characterized and evaluated for their inhibitory activities against cholinesterase enzymes. In addition, the molecular interactions and orientation of most active inhibitors with cholinesterase enzymes were also studied.

1.7. Objectives

1. To synthesize a library of new biologically active spiro heterocycles employing eco-friendly, one-pot multi component reaction methodology

2. To elucidate and characterize the structure of newly synthesized compounds using elemental analysis as well as 1D and 2D NMR spectroscopy techniques

3. To evaluate the cholinesterase inhibitory activities of synthesized compounds against AChE and BChE enzymes using colorimetric Ellman's method

4. To perform the molecular modelling studies on the most active inhibitors to disclose the binding interaction and orientation inside their relevant enzymes

CHAPTER TWO

MATERIALS AND METHODS

2.1. Chemicals and solvents

The chemicals and solvents used in the synthesis and characterization of the synthesized compounds are as follows: acetic acid glacial, AR grade; benzaldehyde; 3-nitrobenzaldehyde, 99%; *o*-anisaldehyde, 98%; 2-fluorobenzaldehyde, 97%; 4-fluorobenzaldehyde, 98%; 2-chlorobenzaldehyde, 98.5%; 4-chlorobenzaldehyde, 98.5%; 2,4-dichlorobenzaldehyde, 98.5%; 2-hydroxy-5-nitro-benzaldehyde; 2-bromobenzaldehyde, 97%; 2-hydroxy-3-methoxybenzaldehyde; 1-naphtaldehyde, 95%; *o*-tolualdehyde, 98%; *p*-tolualdehyde, 99%; hydroxylamine hydrochloride; 1-(2-chloroethyl)pyrrolidine hydrochloride; 4-piperidinone monohydrate hydrochloride; sarcosine, 98%; 4-chloro-*N*-methyl-piperidine hydrochloride, 99%; L-phenylalanine; L-proline; piperidine, 99%; isatin; 5-chloroisatin, 95%; magnesium sulphate anhydrous ; acetone, AR grade; chloroform, AR grade; dichloromethane, AR grade; diethyl ether, AR grade; dimethyl sulfoxide-d_6; chloroform-d_1; methanol-d_4; petroleum ether, AR grade; ethyl acetate, AR grade; 2-methoxybennzaldehyde; pyridine; potassium bromide, FT-IR grade; TLC silica gel 60 F254, aluminum sheets, 20 cm × 20 cm.

2.2. General experimental methods

2.2.1. Monitoring of reactions

Silica gel-G plates (Merck) were used for TLC analysis employing a mixture of petroleum ether (60–80°C) and ethyl acetate as eluent. At initial steps of the reaction,