SYNTHESIS AND CHARACTERISATION OF NEW DISPIRO PYRROLIDINES AS CHOLINESTERASE INHIBITORS

NADIA BINTI MOHAMED YUSOFF

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by

NADIA BINTI MOHAMED YUSOFF

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DCE	1,2-Dichloroethane
[bmim]Br	1-Butyl-3-methylimidazolum bromide
ACh	Acetylcholine
AChI	Acetylcholine iodide
AChE	Acetylcholinesterase enzyme
AD	Alzheimer's disease
Å	Angstrom unit
ATR	Attenuated Total Reflection
β	Beta; second letter Greek alphabet
BCh	Butyrylcholine
BChE	Butyrylcholinesterase enzyme
BChI	Butyrylcholinesterase iodide
^{13}C	Carbon Nuclear Magnetic Resonance
CHN	Carbon, hydrogen and nitrogen (Elemental analysis)
CT	Catalytic triad
^{1}H-^{1}H COSY	Correlation Spectroscopy
J	Coupling constant
˚C	Degree Celsius
δ	Delta, chemical shift scale
DMSO-d_6	Deuterated dimethyl sulfoxide
DMSO	Dimethyl sulfoxide
DEPT	Distortionless enhancement by polarization transfer
DTNB	Dithiobisnitrobenzoic acid

d	Doublet
dd	Doublet of doublet
EtOH	Ethanol
EtOAc	Ethyl acetate
FTIR	Fourier Transform Infrared
IC_{50}	Half-maximal inhibitory concentration
HMBC	Heteronuclear multiple bond correlation
HSQC	Heteronuclear single quantum coherence
His	Histidine
*h*AChE	Human acetylcholinesterase enzyme
*h*BChE	Human butyrylcholinesterase enzyme
HIV	Human Immunodeficiency Viruses
IR	Infra-red
Leu	Leucine
V_{max}	Maximum velocity
MHz	MegaHertz
m.p.	Melting point
K_m	Michaelis-Menten
MCR	Multicomponent reaction
m	Multiplet
MTB	Mycobacterium Tuberculosis
nm	Nanometre
NMR	Nuclear Magnetic Resonance spectroscopy
1D-NMR	One-Dimensional Magnetic Resonance
OD	Optical detection

%	Percent
Phe	Phenylaniline
^{1}H	Proton Nuclear Magnetic Resonance
s	Singlet
NaOH	Sodium hydroxide
SE	Standard error
THF	Tetrahydrofuran
TMS	Tetramethylsilane
TLC	Thin-layer chromatography
Typ	Tryptophan
2D-NMR	Two-Dimensional Magnetic Resonance
Tyr	Tyrosine
UV	Ultra-violet
Val	Valine

SINTESIS DAN PENCIRIAN TERBITAN BAHARU DISPIRO PIROLIDINA SEBAGAI PERENCAT KOLINESTERASE

ABSTRAK

Sebatian spiro terkenal dengan pelbagai aktiviti biologinya dan telah menjadi tumpuan yang penting kepada para penyelidik untuk mengkaji dan menghasilkannya secara sintetik. Penyakit Alzheimer dikenali sebagai penyakit kemerosotan neuron dimana kadar bilangan kes dari seluruh dunia dilaporkan meningkat pada setiap tahun. Rawatan sedia ada hanya mampu melambatkan simptom, ini mendorong kepada pembangunan ubat yang lebih berkesan secara berterusan dalam mensasarkan perencatan aktiviti enzim kolinesterase. Dalam kajian ini, tiga sebatian dienon, 3,5-bis(arilidin)-1-feniletil-4-piperidon **(19-21)** lima belas sebatian heterosiklik spiro baharu (**22-24**) yang dikenali sebagai dispiro pirolidina telah berjaya disintesiskan dengan purata hasil yang baik iaitu 85.4-94.6 and 81.3-98.1 %. Diantara tiga sebatian dienon yang telah disintesis, hanya 3,5-bis(4-metilbenzilidin)-1-feniletil-4-piperidon **(20)** merupakan sebatian baharu**.** Sebatian dispiro pirolidina **22(a-e)**, **23(a-e)** and **24(a-e)** disintesiskan menggunakan penambahan siklo [3+2] di antara 3,5-bis(arilidin)-1-feniletil-4-piperidon **(22-24)** dengan azometin ilid yang telah dihasilkan secara *in situ* melalui reaksi N-metilglicin dengan isatin yang bersesuaian. Sebatian unsur ini telah dicirikan dengan IR, ^{1}H NMR, ^{13}C NMR, dan DEPT135. Beberapa sebatian yang terpilih dianalisis dengan lebih lanjut menggunakan NMR 2D HSQC dan HMBC. Sebatian tersebut telah diuji aktiviti biologinya secara *in vitro* terhadap asetilkolinesterase daripada belut elektrik dan butirilcholinesterase daripada serum kuda menggunakan kaedah ujian Ellman. Semua sebatian yang telah disintesis

menunjukkan perencatan aktiviti terhadap butirilcholinesterase enzim yang lebih baik berbanding asetilkolinesterase enzim. Sebatian **22b** telah menunjukkan potensinya sebagai calon perencat kolinesterase yang baharu dengan peratusan perencatan aktiviti yang sewajarnya dengan IC_{50} 12.78 ± 1.52 μM terhadap enzim butirilkolinesterase. Kajian kinetik bagi sebatian **22b** terhadap butirilkolinesterase mencadangkan ianya mod perencatan campuran.

SYNTHESIS AND CHARACTERISATION OF NEW DISPIRO PYRROLIDINES AS CHOLINESTERASE INHIBITORS

ABSTRACT

Spiro compounds are widely known for their diverse biological activities and become an important target by researchers to study and to produce synthetically. Alzheimer's disease known as a neurodegenerative disorder with its number of cases reported increases every year worldwide. With available treatments that are only able to slow down the symptoms, there is an urgent need to continue developing more efficient drugs in targeting the cholinesterase enzymes activities inhibition. In this study, three dienones derivatives, 3,5-bis(arylidene)-1-phenylethyl-4-piperidone **(19-21)** and fifteen new spiro heterocycles are known as dispiro pyrrolidines (**22-24**) were successfully synthesised afforded in good yield with a range of 85.4-94.6 and 81.3-98.1 %, respectively. Among the three synthesised dienones, only 3,5-bis(4-methylbenzylidene)-1-phenylethyl-4-piperidone **(20)** compound is new. Dispiro pyrrolidine compounds **22(a-e)**, **23(a-e)** and **24(a-e)** were synthesised via [3+2]-cycloaddition of 3,5-bis(arylidene)-1-phenylethyl-4-piperidone **(19-21)** with azomethine ylide generated *in situ* from N-methylglycine and appropriate isatin. The synthesised compounds were characterised by IR, ^{1}H NMR, ^{13}C NMR, and DEPT135. Several selected compounds were analysed further with 2D NMR of HSQC and HMBC. These compounds were assayed *in vitro* for their biological activity against acetylcholinesterase from electric eel and butyrylcholinesterase from equine serum using Ellman's assay. The results showed that the synthesised compounds exhibited better inhibitory activity against butyrylcholinesterase enzyme

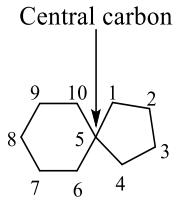
than the acetylcholinesterase enzyme. Compound **22b** showed its potential as a new candidate for cholinesterase inhibitor by exhibited an appropriate inhibitory activity with IC_{50} of 12.78 ± 1.52 μM against butyrylcholinesterase enzyme. The kinetic study of compound **22b** against butyrylcholinesterase suggested that it was a mixed-mode inhibition.

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Spiro compounds represent an important class of diverse natural occurring compound found in plants and animals (Smith & Baxendale, 2015). Since natural products are known as a valuable source of traditional medicine, there is a growing interest in biological activity study of spiro compounds and their synthetic derivatives. The structure of the spiro compound consists of cyclic structure fused at the central carbon, for example, spiro[4.5]decane (**1**). The interesting conformation and structure of spiro scaffold and their effect on the biological system made it prevalent as a template in drug discovery (Zheng, Tice, & Singh, 2014). First spirocyclic compound termed as spirocyclane was created in 1900 by von Baeyer described as two cyclic hydrocarbons connected by a single carbon.



Spiro[4.5]decane **(1)**

Heterocyclic compounds are an organic compound commonly found consist of 5 to 7-membered ring system which comprise more than half of all the known organic compounds (Gupta, 2015). A heterocyclic compound such as oxindole, consist of bicyclic structure, six-membered of a benzene ring fused to five-membered rings containing nitrogen. Among a large variety of nitrogen-containing heterocycles compounds, heterocycle such as oxindole with spiro system is of interest, many of which exhibited promising and diversified biological activities, such anti-

Alzheimer's disease (Rudrangi *et al*., 2011; Kia *et al*., 2013). The widely know biological potency of spiro compound and oxindole lead researchers to investigate and constructed heterocyclic scaffolds such as spiro heterocyclic compound comprising oxindole and pyrrolidine moieties (Sheat, Hussein, & Abood, 2018; Dandia *et al*., 2017; Haddad *et al*., 2015).

One of the main methods for the construction of highly functionalized heterocyclic scaffolds has been reported using 1,3-cycloaddition reaction. The reaction involved the addition of 1,3-dipole and azomethine ylide to unsaturated double or triple bonds. The azomethine ylide mostly generated *in situ* due to their high reactivity. The reactions are regio and stereoselective, construct two carbon-carbon bonds simultaneously forming a complex ring system (Karlsson & Högberg, 2016; Wei *et al*., 2013).

1.2 Problem statements

Alzheimer's disease (AD) is a chronic and irreversible degenerative brain disease known as a common cause of dementia. According to Alzheimer's Disease International 2015, the number of people with dementia are 47 million worldwide and estimated to increase more than 131 million by 2050 (Prince *et al*., 2016). In Malaysia, the occurrences of dementia are expected at 0.126% and 0.454% in 2020 and 2050, respectively (Rees *et al.*, 2016; Tey *et al*., 2016). AD causes a progressive cognitive malfunction, functional disability and mood deterioration during mid- to late-adult life. The cause of AD is believed due to the presence of neurofibrillary tangles and the accumulation of extracellular β-amyloid (Aβ) plaque in the brain (A. Kumar, Singh, & Ekavali, 2015). Currently, the progression of the symptom of AD

reported can be slow with the existing drugs as no cure available (Casey, Antimisiaris, & O'Brien, 2010).

In the development of more effective drug in inhibiting the cholinesterase enzymes activities for the treatment of AD, spiro compounds were reported to display a significant cholinesterase inhibitory activity and have became one of the potential candidates as AD drugs. Inspired by previously reported of biological potency of spiro compound, this research work focused on the synthesis of new spiropyrrolidine derivatives by 1,3-dipolar cycloaddition reaction and its biological evaluation as anti-Alzheimer's disease agent.

1.3 Objectives

The main objectives of this study are:

1) To synthesise and characterise a new series of spiropyrrolidine compounds by different spectroscopic techniques (IR, 1D and 2D NMR) and elemental analysis.
2) To study *in vitro* enzyme inhibitory activities of synthesised spiropyrrolidine compounds against cholinesterase enzymes.

1.4 Scope of the study

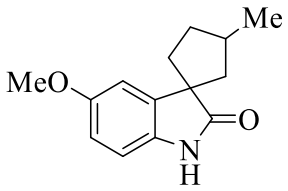
The chemical structures of synthesised compounds were elucidated and confirmed using the available instruments such as FT-IR and NMR spectrometers at School of Chemical Sciences, USM, Penang campus. However, the cholinesterase inhibition assays on the synthesised compounds were carried out in the laboratory of Associate Professor Dr Vikneswaran Murugaiyah at School of Pharmaceutical Sciences, USM, Penang campus.

CHAPTER 2

LITERATURE REVIEW

2.1 Spiro compound

Spiro compounds consist of two or more cyclic structures fused at a central carbon by sharing one atom known as the spiroatom. According to Saraswat *et al*., (2016), the first spiran was created in 1900 by von Baeyer describing a bicyclic hydrocarbon connected by a single central carbon. The isolated spiro compounds from natural products ranging from plants to animals have important applications in medicinal chemistry (Molvi *et al*., 2015). A spiro containing nitrogen atom has unique conformational features which include spiro-linked carbon and such structure has been found to play a significant role in biological systems and exhibits significant bioactivity.

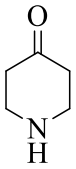


Horsfiline **(2)**

Horsfiline **(2)**, an unsubstituted spirooxindole core was reported to have been isolated from *Horsfieldia superba* tree by Bodo and coworker in 1991 (Galliford & Scheidt, 2007). The spirooxindole structure consists of spiro ring fused at 3-position of the oxindole core. A wide range of pharmacological activities exhibited by spirooxindole include as cholinesterase inhibitory (Basiri *et al*., 2017), antimicrobial (Dandia *et al*., 2017; Kathirvelan *et al*., 2015), antibacterial and antifungal (Turan-Zitouni *et al*., 2005; Raj *et al*., 2003). Due to those reasons, growing effort has been

made to synthesise target spirooxindole compounds. Moreover, in recent years, the number of publication on the synthesis and biological evaluation of synthetic spiro compounds were increasing to find a more pharmaceutically active molecule (Zheng, Tice, & Singh, 2014; Sheat, Hussein, & Abood, 2018)

2.2 Piperidone compound



Piperidinone **(3)**

Piperidone **(3)** compounds are a class of chemical compounds sharing a piperidine skeleton and known for their varied biological activities such as antioxidant, antimicrobial, antitumor and anti-HIV (Kumar & Govindappa, 2012). Piperidone and its derivatives were used in the construction of larger molecules with varied biological properties (Dimmock *et al*., 2001). In this study, three series of 3,5-bis(arylidene)-phenethylpiperidine-4-one were prepared by aldol reaction according to the method described by Ali *et al*., (2013). 3,5-Bis(arylidene)-phenethylpiperidine-4-one then underwent 1,3-dipolar cycloaddition reaction with appropriate isatin and N-methylglycine to obtain a spiro heterocyclic compounds comprising of oxindole and pyrrolidine. The spiro compounds were then evaluated for their potential as cholinesterase inhibitors. The cholinesterase inhibitory activity of spiro compounds was studied against acetylcholinesterase (AChE) from *Electrophorus electricus* (electric eel) and butyrylcholinesterase (BChE) from equine serum.

2.3 1,3-Dipolar cycloaddition reaction

1,3-Dipolar cycloaddition, also known as the Huisgen cycloaddition, is a classical tandem reaction. It is a powerful tool reported use in the construction of various type of nitrogen contained five-membered heterocyclic compounds such as pyrrolidine or spirooxindole. The reaction involves a dipolarophile and 1,3-dipole molecule. The reaction is known to be regioselective and chemoselective where it only occurred to available C=C bond and not at C=O functional group. Dipolarophiles compounds are usually alkenes or alkynes while 1,3-dipoles is a species represented by closed-shell all-octet valence structure contains four electrons where central atom must have a pair of an electron to stabilise the species by dispelling of positive charge (Ess & Houk, 2008). Figure 2.1 shows the reaction of the 1,3-dipole molecule **(i)** with dipolarophiles **(ii)** lead to five-membered heterocyclic ring **(iii)** (Ajay Kumar, 2013; Firestone, 1968).

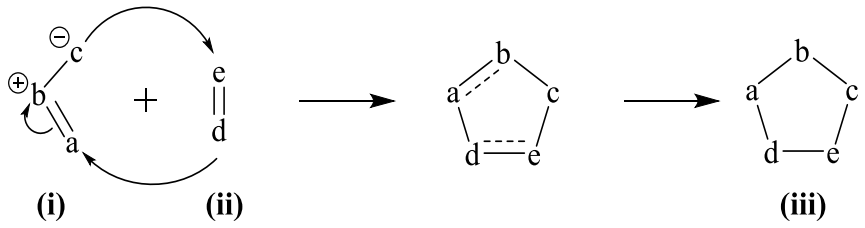


Figure 2.1: Formation of the five-membered heterocyclic ring from 1,3-dipolar cycloaddition (Ajay Kumar, 2013)

2.4 Heterocyclic compounds in Drugs Discovery

Heterocyclic compounds are cyclic compounds containing at least one atom other than carbon such as nitrogen, oxygen and sulphur in a ring formation and classified as aliphatic and aromatic heterocyclic compounds (Gupta, 2015).

Pyrrolidine, a natural alkaloid compound consist of a bicyclic structure of six-membered of benzene ring fused to five-membered ring containing nitrogen, reported

to have significant biological activities such as anticancer, antimicrobial and antifungal (Lotfy *et al*., 2017; Hamzah, Shaameri, & Goksu, 2013; Raj *et al.*, 2003). Due to these properties, pyrrolidine compounds and their N-substituted analogue become a precursor in the construction of other biological importance compounds (Al-hamdany, Dabbagh, & Shareef, 2012).

Thus, numerous studies conducted on spiro-heterocycles containing pyrrolidine and indole due to their interesting conformational features implications for biological systems, which are motifs in many pharmacologically relevant alkaloids.

2.5 Spiropyrrolidine

Spiropyrrolidine compounds were reported exhibiting highly pronounced biological activities, such as antimicrobial, antitumor and antibiotic properties (Galliford & Scheidt, 2007). Some spiropyrrolidines are identified as potential antileukemic and anti-convulsant agent and possess antiviral and local aesthetic activities (Li *et al*., 2008). Recently, growing efforts have been made in synthesise and characterised synthetic derivatives of spiropyrrolidine comprising oxindole. Several spiropyrrolidine compounds reported possessing very promising biological activities (Saraswat *et al*., 2016).

2.5.1 Synthesis of new spiropyrrolidine derivatives

Several methods used in the synthesis of new spiro compound with oxindole, and pyrrolidine moiety reported in the literature. The method involved commonly 1,3-dipolar cycloaddition reaction.

A series of novel dimethoxyindanone embedded spiropyrrolidine synthesised by Almansour *et al*., (2015) in ionic liquid, 1-butyl-3-methylimidazolium bromide

([bmim]Br) via three-component [3+2]-cycloaddition reaction (Figure 2.2). The intermediate compounds, 2-arylmethylidene-5,6-dimethoxy-2,3-dihydro-1H-inden-1-ones (**5)** prepared by condensing 5,6-dimethoxy-1-indanone **(4)** with appropriate aromatic aldehydes in dilute methanolic sodium hydroxide. Compound 2-arylmethylidene-5,6-dimethoxy-2,3-dihydro-1H-inden-1-ones (**5)** undergoes 1,3-dipolar cycloaddition reaction with azomethine ylide generated *in situ* from isatin **(6),** and phenylaniline **(7)** afforded 82-92% yield of the functionalized spiropyrrolidines **(8)** in 1 hour. The study showed the yield for the compound with the electron-donating group, **8a** was higher than compound **8b** that contained electron-withdrawing group, with a percentage yield of 92 and 90%, respectively. It suggested the presence of electron-deficient [$bmim^+$] hydrogen atom help in furnishing azomethine ylide and spiropyrrolidine **(8)**. The reaction starts with imidazole ring hydrogen atom of [$bmim^+$] form a hydrogen bond with carbonyl function of isatin **(6)** to facilitate the attack of phenylaniline **(7)** lone pair on isatin forming azomethine ylide *via* decarboxylative condensation of sarcosine and isatin. The hydrogen bond formed between the hydrogen atom of [$bmim^+$] with 2-arylmethylidene-5,6-dimethoxy-2,3-dihydro-1H-inden-1-ones (**5)** carbonyl was suggested help provide an easy addition of azomethine ylide to the more electron-deficient β-carbon affording spiropyrrolidine **(8)** regio- and stereoselectively (Almansour *et al*., 2015).

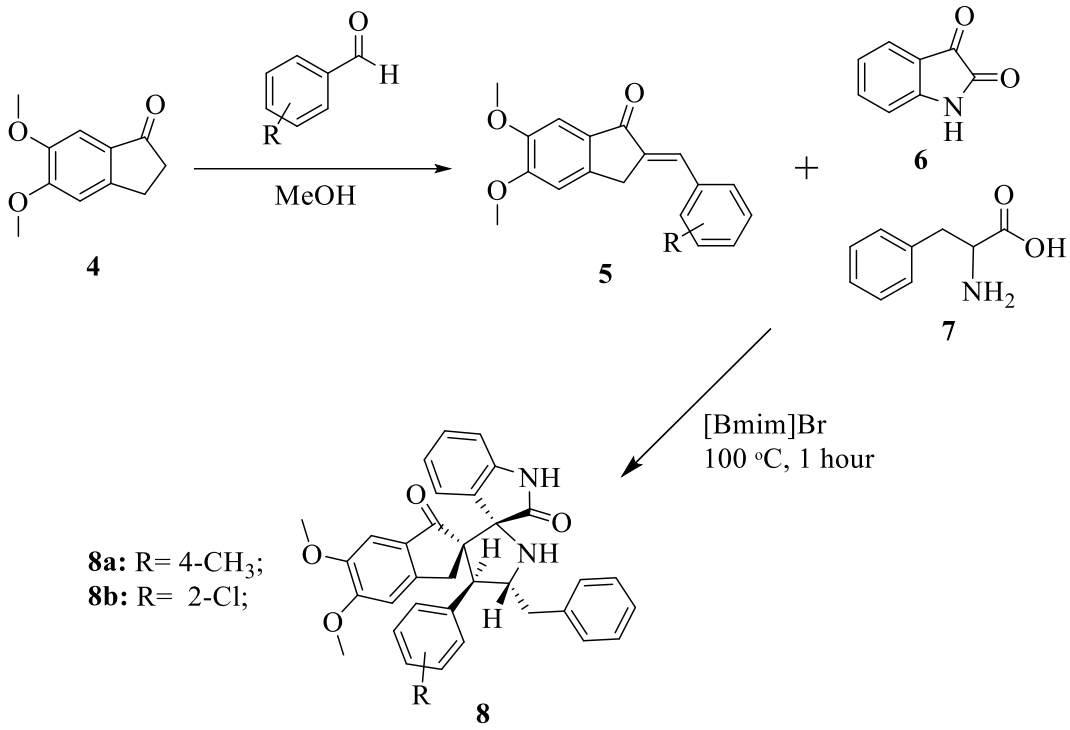
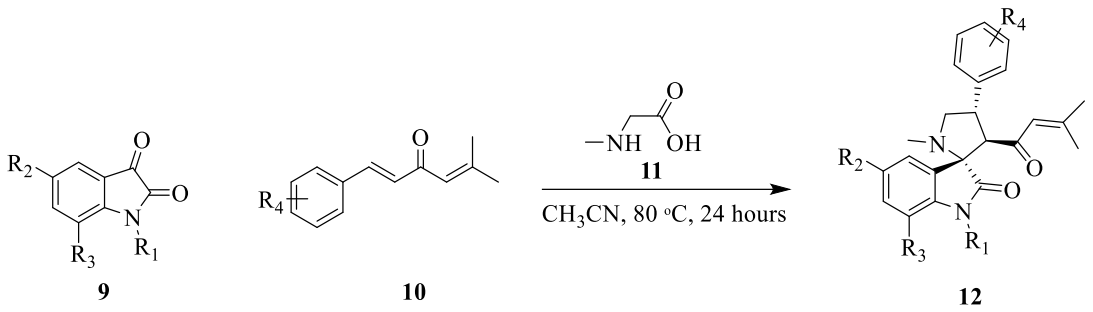


Figure 2.2: Synthesis of novel dimethoxyindanone embedded spiropyrrolidine (Almansour *et al*., 2015)

Liu and co-worker (2016) constructed a novel spiropyrrolidine oxindole fused with a turmerone motif (Figure 2.3) through a multicomponent 1,3-cycloaddition reaction between dienones **(10)** with azomethine ylides in acetonitrile (CH_3CN), 1,2-dichloroethane (DCE), ethyl acetate (EtOAc), ethanol (EtOH), tetrahydrofuran (THF), water, and toluene. The azomethine ylides were generated thermally *in situ* from sarcosine **(11)** and isatin derivatives **(9)**. The optimal condition of reaction produced the highest yield of compounds (86%) using CH_3CN as reaction solvent at 80 ˚C for 24 hours. The percentage yields of compound **12** with presence of electron-withdrawing substituent, bromo (-Br), chloro (-Cl) and fluoro (-F) on the isatin derivatives **(9)** and dienones **(10)** gives higher yields of products up to 93% compared to electron-donating substituent phenyl ($-C_6H_5$), methyl ($-CH_3$) and methoxy ($-OCH_3$). The highest yield obtained for the compound with electron-withdrawing substituent was compound **12a** with 93% while for the compound with

electron-donating substituent was compound **12b** was 88%. Liu *et al*., (2016) suggested the presence of electron-withdrawing group substituent in dienones **(10)** could increase the electron density of aromatic ring by making aromatic ring become partially positive. Therefore, the rate of reaction accelerated and the yield of products increased (Liu *et al*., 2016).



12a: R_1= CH_3, R_2= H, R_3= H, R_4= 4-BrC_6H_4;
12b: R_1= Ph, R_2= H, R_3= H, R_4= 4-MeC_6H_4;

Figure 2.3: Synthesis of a novel spiropyrrolidine oxindole fused with a turmerone motif (Liu *et al*., 2016)

Hassaneen *et al*., (2017) synthesised a new derivatives spirooxindole-spiropiperidinone-pyrrolidines (**14)** (Figure 2.4) through 1,3-dipolar cycloaddition reaction with ethyl 3,5-bis[phenylmethylidene]-4-oxopiperidine-N-carboxylate (**13)** by multicomponent reaction (MCR) in six different solvent (acetonitrile, ethanol, methanol, tetrahydrofuran (THF), toluene, and water) at different temperatures for around 2 hours. Azomethine ylide was generated *in situ* from the reaction of 1H-indole-2,3-dione (isatin) **(6)** with N-methylglycine (sarcosine) **(11)**. The highest yield of dispiro-oxindolopyrrolidines, **14a** obtained at temperature 60 ˚C were from the reaction in methanol, 92% and followed by 85% yield in ethanol. No reaction occurred in water due to the poor solubility of sarcosine (**11)** and isatin **(6)** in it. The study showed the rate of reaction increase with a rise of temperature. The increase of carbonyl stretching band at 1685 cm^{-1} in the IR spectrum of **14a** from the normal

value of **13a** indicated the loss of conjugation carbon double bond (C=C) from one side of **13a**. The bands for carbonyl group of oxindole and ester moiety exhibited at 1718 and 1710 cm^{-1}, respectively.

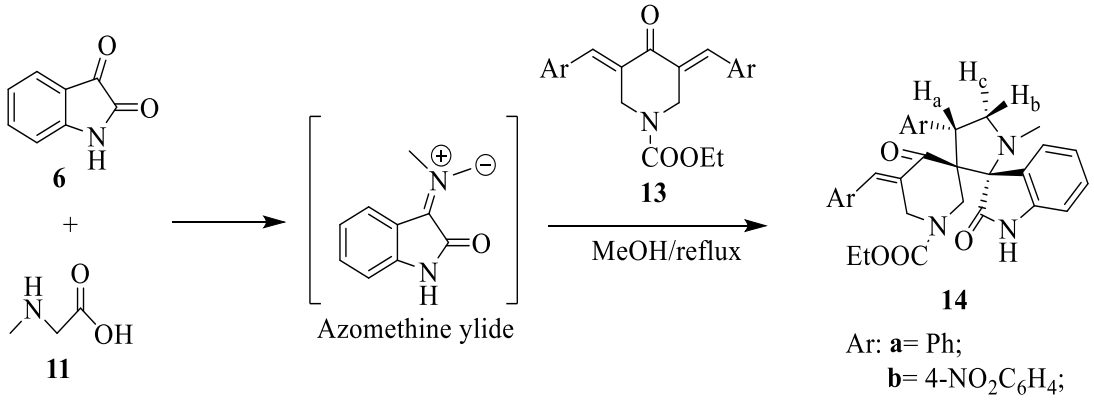


Figure 2.4: Synthesis of a new derivatives spirooxindole-spiropiperidinone-pyrrolidines (Hassaneen *et al*., 2017)

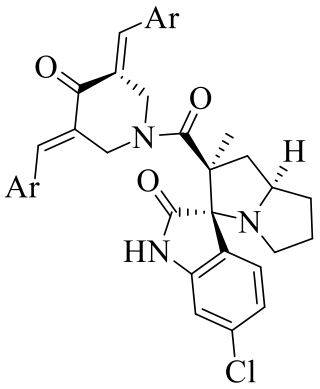
2.6 Biological activities of spiropyrrolidine

The natural and synthetic spiropyrrolidine derivatives were reported exhibiting highly pronounced biological activities, such as antimicrobial, antitumor and antibiotic properties (Galliford & Scheidt, 2007). Some spiropyrrolidines identified as potential cholinesterase inhibitor agent. Thus, spiropyrrolidine encourage the derivation and screening of their analogues as new therapeutic agents.

2.6.1 Cholinesterase inhibitory activity

Novel hybrid spiro heterocycles comprising pyrrolizine, spirooxindole and piperidine moieties synthesised by Kia *et al*., (2013) displayed significant cholinesterase inhibitory activity. The synthesised spiropyrrolizine compounds, **15** were reported having a single diastereomer despite the presence of three stereocenters (Figure 2.5). Among the hybrid spiro heterocyclic compounds, compound **15g** and **15e** against both acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) showed

maximum inhibitory activity with IC_{50} values of 3.33 and 3.13 μM, respectively. Docking simulation was done for both **15g** and **15e** to investigate their interaction with the active site of cholinesterase enzymes. The studies reported that the location of the inhibitor's binding site affects by the size of the inhibitor. The most active AChE inhibitor, compound **15g** was suggested act only as AChE peripheral anionic binding site inhibitor through a strong hydrogen bonding attraction and mild hydrophobic with the residues such as Tyr 334, Trp 279 and Tyr 70. This is due to narrow active-site gorge of AChE receptor that prevents compound **15g** from entering it. The most active BChE inhibitor, compounds **15e** was suggested act as BChE choline binding site inhibitor. The choline site inhibitor located in the active site of BChE is comprised of TRp 82 and Phe 329 residues able to form a strong π-π stacking interaction and hydrophobic interaction with compound **15e**, respectively. The size of the BChE active site is larger than AChE and thus able to accommodate the large size of compound **15e** into it (Kia *et al*., 2013).



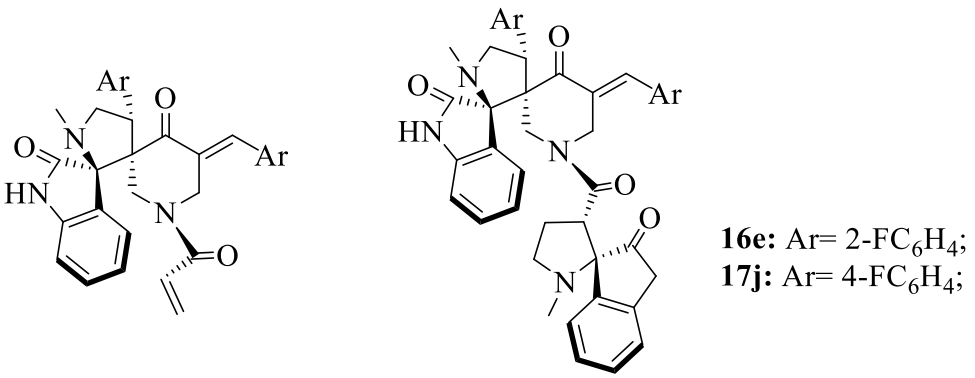
15

Ar: **15e**= 2-FC_6H_4;
15g= 2,4-$Cl_2C_6H_3$;

Figure 2.5: Spiropyrrolizines derivatives (**15**) (Kia *et al*., 2013)

In 2014, a mono- and bis-spiropyrrolidines were synthesised and studies by Kia and co-workers (Figure 2.6). The biological activities of compounds were evaluated for their *in vitro* activity against cholinesterase enzymes with galantamine as the reference standard. The IC_{50} of galantamine against AChE and BChE are 2.09 and

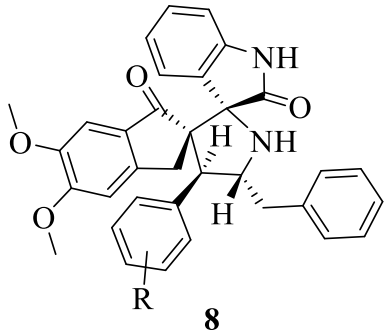
19.34, respectively. Both compounds in series of mono-spiropyrrolidines **(16)** and bis-spiropyrrolidines **(17)** were reported showed a better inhibition against both enzymes, especially toward AChE with IC_{50} value less than 10µM. The presence of substituents at ortho position of the phenyl ring in **16** and **17** exhibited better inhibitory activities than para substitution derivatives against AChE. Compound **16e** and **17j** were found to be the most potent compound and higher inhibition than galantamine targeting BChE and AChE with IC_{50} value of 3.21 and 2.35 µM, respectively. Molecular docking studies showed compound **17** with more aromatic cores than compound **16** formed more interaction with AChE. These due to aromatic side chains residues such as phenylanaline (Phe) and tyrosine (Tyr) presence in AChE active site formed an extensive hydrophobic interaction of π-π and σ-π with compound **16**. While for compound **17**, the docking showed it to be preferable binds at peripheral anionic site (PAS) through hydrogen bonding interaction with Tyr 334 and hydrophobic interactions with Tyr 279, Tyr 70 and Tyr 121. This binding blocked the entry of the enzyme active site channel and prevented substrate insertion and accommodation into it. The result of kinetic finding for compound **17j** indicated it is competitive AChE inhibitor agreed with docking analysis. Moderate inhibition of compounds **16** and **17** were suggested due to the presence of aliphatic side-chain residue at the gorge wall of BChE active site such leucine (Leu) and valine (Val) that cause it interaction to be unfavourable with both series of compounds (Kia *et al*., 2014).



16 **17**

Figure 2.6: Mono- **(16)** and bis-spiropyrrolidine **(17)** compounds against AChE and BChE (Kia *et al*., 2014)

Inhibitors of cholinesterase enzymes activities of synthesised novel dimethoxyindanone embedded spiropyrrolidine (Figure 2.7) were evaluated by Almansour *et al*., (2015). The presence of electron-withdrawing moiety in the series displayed better inhibitory potencies towards both cholinesterase enzymes than electron-donating moiety. Compound **8f** was reported exhibited highest inhibitory activities against AChE with IC_{50} of 1.57 µM. The presence of electronic withdrawing nature of the nitro group in compound **8f** cause it to strongly binds at AChE active site and inhibited choline substrate hydrolysis. The molecular docking simulation of compound **8f** showed its hydrophobic interaction with AChE aromatic side chain of the peripheral anionic site (PAS) such as Trp 279, Tyr 70, Tyr 334 and Phe 331. Compound **8h** with methoxy moiety also reported having good activity against both AChE and BChE with IC_{50} of 3.67 and 14.69 µM.

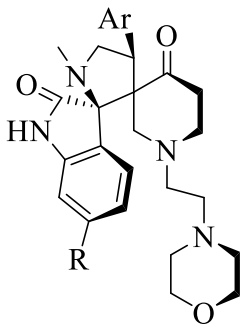


8

8f: R= 3-O_2N;
8h: R= 4-OCH_3;

Figure 2.7: Synthesis of novel dimethoxyindanone embedded spiropyrrolidine **(8)** (Almansour *et al*., 2015)

Basiri *et al.*, (2017) reported that most of synthesised spiropyrrolidine against acetylcholinesterase enzyme (AChE) and butyrylcholinesterase enzyme (BChE) displayed good potency than standard drug, Galantamine. Compound **18k** showed the highest inhibition against BChE with IC_{50} values of 7.21 µM while compound **18l** showed the highest inhibition against AChE with IC_{50} values of 1.37 µM (Figure 2.8). From the study, the presence of both nitro moiety on oxindole moiety (R) and chloro atom on the aromatic ring (Ar) give a positive impact on inhibition of both AChE and BChE. The substitution of chloro atom on an aromatic ring was suggested increase the electron density of aromatic and enhanced interaction of inhibitor with AChE active site's amino acids. The presence of multiple aromatic core or electronically rich aromatic cores leads to efficient binding through π-π stacking or other polar interactions with aromatic binding site of AChE. The studies also reported that the lower value of IC_{50} value obtained on BChE inhibitions than AChE inhibitions was due to BChE contained less aromatic than AChE. Thus, the effect of BChE inhibition was less evident (Basiri *et al*., 2017).



18k: Ar= p-ClC_6H_5, R= Cl;
18l : Ar= p-ClC_6H_5, R= NO_2;

18

Figure 2.8: Piperidone grafted spiropyrrolidines **(18)** against AChE and BChE (Basiri *et al*., 2017)

2.7 Alzheimer's disease

2.7.1 Overview

Alzheimer disease (AD) is a multifarious neurodegenerative disorder and known as the most common form of dementia worldwide. The symptom caused by AD was reported discovered by German psychiatrist and neuropathologist Dr Alois Alzheimer of AD in 1907 (Heneka & Obanion, 2007). According to Ito *et al*., (2010), Alzheimer's disease is a chronic and irreversible disease that affects daily activities. The symptoms are manifested by progressive deterioration of cognitive dysfunction, intellectual decline, memory loss and personality change It is the most prevalent form of dementia with up to 80% cases in the old population. Based on World Alzheimer report 2017, the number of people living with dementia was estimated to be 47 million and rise to 131 million by 2050 (Prince *et al*., 2016).

Researches showed that woman are more affected by AD with 16% at the age of (older than) 71 and above compared to 11% of men. People with higher education years were reported to have a lower risk for AD (Ito *et al.*, 2010).

2.7.2 Pathophysiology of Alzheimer's disease

Alzheimer's disease neuropathology is characterised by two distinct deformities, the presence of neurofibrillary tangles and the accumulation of extracellular β-amyloid (Aβ) plaque in the brain. The neurofibrillary tangles composed of hyper-phosphorylated tau protein are found inside the neurons and the β-amyloid plaques are found in the neocortex of the brain. The increased number of tangles and plaques cause the neuron to gradually lose their ability to communicate and consequently die, resulting in an overall shrinkage of brain tissue. The death of neuron especially in the hippocampus restricts the ability to form new memories (El Khoury & Luster, 2008; Heneka & Obanion, 2007; Huang & Mucke, 2012; Rees *et al*., 2016). The cause of AD is still debatable, and several hypotheses have been put forward to explain this disorder such as amyloid, tau, and cholinergic hypotheses (Casey *et al*., 2010).

The amyloid cascade hypothesis was suggested by Glenner and Wong in 1984 state the abnormal accumulation of hydrophobic amyloid-β plaques outside the neuron in basal forebrain is the major cause of AD. The uncontrollable cleavage of amyloid precursor protein (APP) results in aggregation and formation of insoluble plaques, which trigger a cascade change inside the brain, leading to neuronal death and thus causing AD (Huang & Mucke, 2012). However, Terry *et al*., (1991) suggested the hypothesis of plaques load may not correlate well with degree of dementia in humans as studies showed no plaques were founded in most of severely impaired memory AD patient at post-mortem analysis (Anand *et al*., 2014; Huang & Mucke, 2012; Korolev, 2014; A. Kumar *et al*., 2015).

Tau protein is a microtubule-associated protein responsible in stabilizing microtubule. It can be found in neuronal axon microtubule network and is the main component of insoluble neurofibrillary tangles. Tau hypothesis postulates that the

hyperphosphorylated of tau protein due to AD progression disrupt the protein ability in bind and stabilise microtubules. Thus, it reduced axonal transport which leads to neurodegeneration and cell death (Anand *et al.*, 2014; Huang & Mucke, 2012; Ito *et al.*, 2010; Korolev, 2014; A. Kumar *et al.*, 2015).

The cholinergic hypothesis suggested dysfunction of acetylcholine containing neurons in the brain contributes substantially to cognitive function impairment and dementia in those with advanced age and Alzheimer's disease (AD) (Anand *et al*., 2014; Auld, Kornecook, Bastianetto, & Quirion, 2002; Barnes, Dickerson, Frost, Jiskoot, & Wolk, 2015; Heneka & Obanion, 2007; Mckhann *et al*., 2011; Nelson & Tabet, 2015).

The progression of the disease can be divided into three stages of mild Alzheimer's disease (early-stage), moderate Alzheimer's disease (middle-stage) and severe Alzheimer's disease (late-stage) (McKhanna *et al*., 2012).

2.8 Cholinesterase enzymes

Cholinesterase is a family of enzymes present in the central nervous system and responsible catalyse the hydrolysis of the neurotransmitter acetylcholine (ACh) into choline and acetic acid restoring cholinergic transmission inside the brain to its resting state after activation for the proper functioning of the human nervous system. A human brain contains two types of cholinesterase enzyme hydrolyse and regulate acetylcholine in vertebrates are acetylcholinesterase enzyme (AChE, EC 3.1.1.7) and butyrylcholinesterase enzyme (BChE, EC 3.1.1.8) (Pohanka, 2011).

The acetylcholinesterase enzyme mainly found abundant in the brain, muscles, erythrocyte and cholinergic neuron where it plays a vital role in impulse cholinergic

neurotransmission at the cholinergic synapse (Patocka, Kuca, & Jun, 2004). The plasma cholinesterase enzyme known as butyrylcholinesterase enzyme can be found in most tissue including plasma, liver, heart kidney, lung and intestine. Though the exact physiological function of BChE is unclear, it can hydrolyse ester-containing drugs and act as a backup for acetylcholinesterase and scavenge poisons that might inhibit cholinesterase activity (Ekim, 2003).

Five commercially approved drugs for AD treatment by US Food and Drug Administration (FDA) are rivastigmine, galantamine, tacrine, donepezil and memantine. Rivastigmine, galantamine and donepezil reported effective only for treatment at early stage of AD and memantine only effective for later stage of the disease. Tacrine can cause liver damage thus it rarely prescribes for treatment (Watkins *et al.,* 1994).

Most of the available treatment focus on improving the cognitive function of AD patient by elevating the level of acetylcholine (ACh) in the brain *via* the inhibition of cholinesterase enzyme activity. However, the treatments only able to delays the progression of symptom associated with Alzheimer's disease and not completely cure the disease. Some people were reported to have no response to the treatment. Therefore, it has led to a great interest in developing new spiropyrrolidine analogues that can compete with the available treatments in suppressing the cholinesterase enzyme activity as promising AD treatment.

CHAPTER 3

MATERIALS AND METHODS

3.1 Chemicals and solvents

The chemicals and solvents used in the synthesis and characterization of synthesised compounds are as follows: 1-phenylethyl-4-piperidone 98% (Sigma-Aldrich, USA), Benzaldehyde 99% (Sigma-Aldrich, USA), 4-Methoxybenzaldehyde 98% (Sigma-Aldrich, USA), 4-Methylbenzaldehyde 97% (Sigma-Aldrich, USA), Isatin 98% (Acros Organic), 5-Bromoisatin 98% (Acros Organics), 5-Chloroisatin 95% (Acros Organics), 5-Fluoroisatin 98% (Sigma-Aldrich, USA), 5-Nitroisatin 97% (Acros Organics), Sarcosine 98% (Acros Organics); Acetone, AR Grade (QReC); Ethanol, AR Grade (QReC); Methanol, AR Grade (QReC); Hexane AR Grade (QReC); Methyl sulfoxide-d6-deuteration degree for NMR (Acros Organics). All chemicals and solvents were of reagent grade. All solvents were used without further purification.

3.2 General experimental methods

3.2.1 Reaction monitoring

The progress of reactions was monitor using thin-layer chromatography (TLC). An alumina-backed silica gel plates (Merck 60 F_{254}) was used to spot the reaction mixture and starting material. Due to different polarity of compounds, the solvent system used was acetone: hexane with two different ratios of 3:7 and 1:9. The optimum completion time of reactions was determined by the appearance of product

and disappearance of reactant spots under UV radiation lamp at wavelength 258 nm respectively.

3.2.2 Recrystallization

Single solvent and multi-solvent system were employed for the purification of crude products. The solid product was dissolved in a solvent depending upon their solubility and heated on a hot plate until the sample was completely dissolved. The solution was filtered and concentrated by heating, until 1/2-1/3 of its original volume before allowing it cooling slowly at room temperature to form a crystal. The crystallized sample was finally filtered by filter paper.

3.3 Characterisation of compounds

3.3.1 Melting point

The melting point of the synthesised compounds was measured using open capillary tubes by Stuart Scientific SMPI melting point apparatus in the temperature ranging from 25 to 350 ˚C at School of Chemical Sciences. Recorded temperatures are uncorrected.

3.3.2 Fourier Transform Infrared (FT-IR) spectroscopy

The Fourier transform infrared (FT-IR) spectra of the intermediates and final compounds were recorded using Perkin Elmer 2000 FT-IR at the School of Chemical Sciences, USM. All sample were analysed in the range 4000-600 cm^{-1}.

3.3.3 Nuclear Magnetic Resonance (NMR) spectroscopy

The 1D (^{1}H, ^{13}C) and 2D (^{1}H,^{1}H-COSY, HSQC and HMBC) NMR spectra were recorded on a Bruker (Avance) 500 MHz NMR instrument using TMS as an internal standard. Chemical shifts are given in parts per million (δ-scale) and the coupling constants are given in Hertz (Hz). The analysed samples were prepared by dissolving approximately 20 mg of solid sample with 2 mL deuterated dimethylsulfoxide (DMSO-d_6) in NMR tube. The spectrum data were plotted in the graph of intensity versus frequency using standard Bruker software.

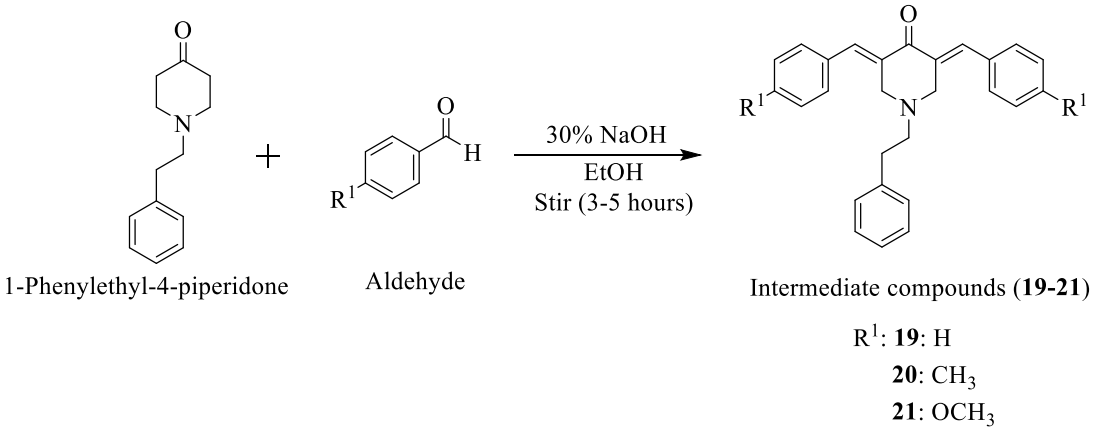
3.4 General procedure for the synthesis of 3,5-bis(arylidene)-1-phenylethyl-4-piperidinone (19-21)

Following the procedure reported in the literature by Ali *et al*., (2014), 1-phenylethyl-4-piperidone (1 mmol) and appropriate aryl aldehyde (2 mmol) was dissolved in ethanol (10 mL) and sodium hydroxide (30%, 2-3 mL). The reaction mixture was stirred for 2-6 hours at room temperature and completion of the reaction was checked using TLC. The mixture was then poured into crushed ice and neutralised with hydrochloric acid. The precipitate solid obtained was filtered and washed with water to afford the product which was purified by recrystallisation. The plausible mechanism of reaction for intermediate derivatives **(19-21)** was outlined in Figure 4.1.

The reaction sequence is outlined in **Scheme 3.1** and the 3,5-bis(arylidene)-1-phenylethyl-4-piperidinone analogue **(19-21)** described in this study are shown in Table 3.1.

Table 3.1: Molecular formula and molecular weight of 3,5-bis(arylidene)-1-phenylethyl-4-piperidinone analogues **(19-21)**

Compound	Ar	Molecular Weight
19	C_6H_5	383.54 $gmol^{-1}$
20	$(4\text{-}CH_3)C_6H_5$	411.54 $gmol^{-1}$
21	$(4\text{-}OCH_3)C_6H_5$	443.59 $gmol^{-1}$



Scheme 3.1: Steps for synthesis of (3*E*, 5*E*)-3,5-bis(arylidene)-1-phenylethyl-4-piperidinone **(19-21)**

3.4.1 (3*E*, 5*E*)-3,5-dibenzylidene-1-phenylethyl-4-piperidinone (19)

Bright yellow solid (2.12 g, 94.6%), m.p. 167-170 ˚C, Lit. m.p. 165 ˚C (Ali *et al*., 2013). FTIR (ATR, cm^{-1}): 1669 (C=O), 1608 (C=C), 3026 (C-H), 1181 (C-N). ^{1}H NMR (500 MHz, DMSO-d6): δ 2.70 (2H, t, 8-CH_2, *J*= 7.00, 8.00 Hz), 2.81 (2H, t, 7-CH_2, *J*= 8.00, 7.00 Hz,), 3.88 (4H, s, 2-CH_2, 6-CH_2), 7.14-7.23 (5H, m, H-10, H-11, H-12, H-13, H-14), 7.42-7.51 (10H, m, H-17, H-18, H-19, H-20, H-21), 7.58 (2H, s, H-15). ^{13}C (125 MHz, DMSO-d6): δ 33.31(C-8), 54.49 (C-2, 6), 58.30 (C-7), 126.32 (C-12), 128.64 (C-10, 14), 129.03 (C-11, 13), 129.23 (C-18, 20), 129.70 (C-19), 130.95 (C-17, 21), 134.28 (C-3, 5), 135.09 (C-16), 135.27 (C-15), 140.50 (C-9), 187.43 (C-4).

3.4.2 (3*E*, 5*E*)-3,5-bis(4-methylbenzylidene)-1-phenylethyl-4-piperidinone (20)

Yellow solid (2.19 g, 90.1%), m.p. 118-121 ˚C, FTIR (ATR, cm^{-1}): 1668 (C=O), 1606 (C=C), 3024 (C-H), 1174 (C-N). ^{1}H NMR (500 MHz, DMSO-d6): δ 2.36 (6H, s, 22-CH_3), 2.71 (2H, t, *J*= 7.00, 8.00 Hz, 8-CH_2), 2.81 (2H, t, *J*=8.00, 7.00 Hz, 7-CH_2), 3.86 (4H, s, 2-CH_2, 6-CH_2), 7.14-7.24 (5H, m, H-10, H-11, H-12, H-13, H-14), 7.29 (4H, d, H-18, H-20, *J*= 8.00 Hz), 7.40 (4H, d, H-17, H-21, *J*= 8.50 Hz), 7.58 (2H, s, H-15). ^{13}C (125 MHz, DMSO-d6): δ 21.46 (C-22), 33.33 (C-8), 54.59 (C-2, 6), 58.42 (C-7), 126.32 (C-12), 128.65 (C-10, 14), 129.04 (C-11, 13), 129.88 (C-18, 20), 131.03 (C-17, 21), 132.35 (C-3, 5), 133.50 (C-16), 135.19 (C-15), 139.59 (C-19), 140.54 (C-9), 187.28 (C-4).

3.4.3 (3*E*, 5*E*)-3,5-bis(4-methoxybenzylidene)-1-phenylethyl-4-piperidinone (21)

Yellow solid (1.13 g, 85.4%), m.p. 143-146 ˚C, Lit. mp 147 ˚C (Ali *et al*., 2013). FTIR (ATR, cm^{-1}): 1667 (C=O), 1596 (C=C), 3023 (C-H), 1166 (C-N); ^{1}H NMR (500 MHz, DMSO-d6): δ 2.73 (2H, t, 8-CH_2, *J*= 7.00, 8.00 Hz), 2.82 (2H, t, 7-CH_2, *J*= 8.00, 6.50 Hz), 3.82 (6H, s, 22-OCH_3), 3.84 (4H, s, 2-CH_2, 6-CH_2), 7.04 (4H, d, 18-CH, 20-CH, *J*= 9.00 Hz), 7.15-7.25 (5H, m, 10-CH, 11-CH, 12-CH, 13-CH, 14-CH), 7.45 (4H, d, 17-CH, 21-CH, *J*= 8.50 Hz), 7.56 (2H, s, H-15). ^{13}C (125 MHz, DMSO-d6): δ 33.33 (C-8), 54.65 (C-2, 6), 55.77 (C-22), 58.52 (C-7), 113.65, 114.79 (C-18, 20), 126.33 (C-12), 127.72 (C-3, 5), 128.66 (C-10, 14), 129.04 (C-11, 13), 132.21 (C-16), 132.92 (C-17, 21), 134.88 (C-15), 140.59 (C-9), 160.51 (C-19), 187.07 (C-4).