SYNTHESIS, CHARACTERIZATION AND *IN SILICO* STUDIES OF PIPERIDONE DERIVATIVES: DENGUE PROTEASE INHIBITORY STUDIES ON SELECTED COMPOUNDS

By

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Nor Hashima Binti Idris

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LIST OF ABBREVATION

Solvents

$CDCl_3$	Deuterated chloroform
$CHCl_3$	Chloroform
DMSO	Dimethyl sulfoxide
DMSO-d_6	Deuterated dimethyl sulfoxide
EtOH	Ethanol

Chemicals

Tris-HCl	Tris(hydroxymethyl)aminomethane-HCl buffer
Ser	Serine
Asp	Aspartic
Asn	Asparagine
Thr	Theonine
Gly	Glycine
Pro	Proline
Phe	Phenylalanine
Tyr	Tyrosine
Val	Valine
HCl	Hydrochloric acid

DENV-1	Dengue Virus Type 1
DENV-2	Dengue Virus Type 2
DENV-3	Dengue Virus Type 3
DENV-4	Dengue Virus Type 4
DENV-5	Dengue Virus Type 5
DENV	Dengue Virus
DHF	Dengue hemorrhagic
NS2B	Non-structural 2B
NS3	Non-structural 3

Instruments and Techniques

^{1}H NMR	Proton Nuclear Magnetic Resonance
^{13}C NMR	Carbon Nuclear Magnetic Resonance
^{1}H-^{1}H-COSY	Correlated spectroscopy
DEPT	Distortionless enhancement by polarization transfer
HMBC	Heteronuclear Multiple Bond Connectivity
HSQC	Heteronuclear Single Quantum Coherence
FTIR	Fourier Transforms Infrared spectroscopy
UV	Ultraviolet
TLC	Thin Layer Chromatography

TMS	Tetramethylsilane

Symbols

δ	delta
µg	microgram
µL	microliter
µM	micromolar
1D	One dimensional
2D	Two dimensional
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
PDB	Protein data bank
q	quartet
s	singlet
t	triplet

LIST OF CONFERENCE

1. Idris, N., Osman, H. (2015). Piperidone Derivative: Synthesis and characterization. 5th International Conference for Young Chemist (ICYC), University Sains Malaysia, 5th-7th August 2015 at City Bayview Hotel, Georgetown, Penang, Malaysia.

SINTESIS, PENCIRIAN DAN KAJIAN *IN SILIKO* BAGI TERBITAN PIPERIDON: KAJIAN PERENCAT DENGGI PROTEASE TERHADAP SEBATIAN TERPILIH

ABSTRAK

Komponen yang aktif daripada segi biologi **26**(**a-j**) and **27**(**a-j**) yang mempunyai terbitan piperidone telah disintesis dengan mengabungkan terbitan asetofenon dengan pelbagai terbitan 3,5-bis(arylidenepiperidin)-4-on dengan kehadiran K_2CO_3 sebagai pemangkin dibawah tindak balas penggantian nukleofilik. Sebatian yang disintesis diperolehi dengan purata hasil 58.00-89.90 % dan dicirikan secara menyeluruh dengan IR, ^{1}H NMR, ^{13}C NMR, DEPT dan analisis unsur. Struktur sebatian selanjutnya dikenal pasti dengan teknik spektroskopi 2D NMR (COSY, HSQC dan HMBC) dan juga kristalografi sinar X. Sebatian yang disintesis disaring secara maya menggunakan struktur model homologi Wilchapong melalui kajian pendokan molekul dengan menggunakan AutoDock 4.2.5 untuk mendedahkan mekanisme interaksi ikatan dan orientasi sebatian terhadap protease DENV2 NS2B-NS3. Sebatian **26h** dan **27h** diperhatikan mempunyai ikatan tertinggi terhadap protease disamping mempunyai interaksi dengan asid amino penting iaitu His51 dan Ser135 dalam kawasan triad pemangkin masing-masing dengan nilai -11.06 kcal/mol dan -11.36 kcal/mol. Sebatian selebihnya mempunyai tenaga ikatan yang baik berbanding dengan struktur hablur model homologi. Sebatian **26h** dan **27h** yang terpilih melalui ujian aktiviti perencat terhadap enzim protease NS2B-NS3 denggi-2 menggunakan substrat peptida Boc-Gly-Arg-Arg-MCA. Kedua-dua sebatian yang mempunyai *p*-NO_2 pada gelang aromatik menunjukkan aktiviti perencatan yang

tinggi terhadap protease dengan nilai IC_{50} masing-masing ialah 15.13 µM ±1.05 dan 16.14 µM ±1.23.

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ABSTRACT

New biologically active compounds **26**(**a-j**) and **27**(**a-j**) comprising piperidone derivatives were synthesized by incorporating acetophenone derivatives with varies of 3,5-bis(arylidenepiperidine)-4-one derivatives in the presence of K_2CO_3 as catalyst under nucleophilic substitution reaction. The synthesized compounds were obtained in the range of 58.0-89.9 % yields and were fully characterized by IR, ^{1}H NMR, ^{13}C NMR, DEPT and elemental analysis. The structures of some compounds were further confirmed by 2D NMR (COSY, HSQC and HMBC) spectroscopic techniques as well as X-ray crystallography. The synthesized compounds were virtually screened using Wilchapong homology model structure through molecular docking study by AutoDock 4.2.5 to disclose the binding interaction mechanism and orientation of the compounds towards DENV2 NS2B-NS3 protease. Compounds **26h** and **27h** were observed to have the highest binding affinity towards the protease while having interaction with the essential amino acid residues, His51 and Ser135 in the catalytic triad with -11.06 kcal/mol and -11.36 kcal/mol, respectively. The rest of the compounds exhibit good energy binding compared to the crystal structure of the model. The selected compounds, **26h** and **27h** then underwent inhibition activity assays toward DENV2 NS2B-NS3 protease by using fluorogenic substrate Boc-Gly-Arg-Arg-MCA. Both compounds which bearing *p*-NO_2 at the aromatic ring showed high inhibition activity against the protease with IC_{50} value of 15.13 µM ±1.05 and 16.14 µM ±1.23, respectively.

CHAPTER 1

INTRODUCTION

The synthesis and chemistry of heterocyclic compounds have been recognized as an important branch of organic chemistry for years due to the attractiveness of their structural diversity and remarkable ability to serve as active pharmacophores and biomimetics. Heterocyclic compounds are the rings that possess at least one different atom other than carbon atom, such as oxygen, nitrogen or sulfur (Figure 1.1).

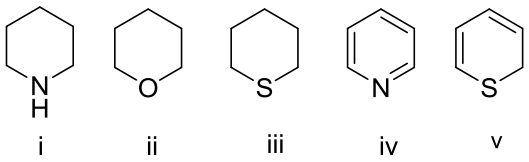


Figure 1.1: Heterocyclic compounds: (i) piperidine, (ii) oxane, (iii) thiane, (iv) pyridine and (v) thiopyran.

The potential applications of the heterocyclic compounds have been studied, these including in many fields, their utility in fundamental and theoretical studies and the possibility for the preparation of virtually unlimited number of novel heterocycles with diverse properties lead to a multitude of studies on the synthesis of such compounds. Nitrogen heterocycles and heterocyclic ring system having piperidin-4-one nucleus have received a great deal of attention in the literature and present years due to their role as active pharmacophores of historical significance.

Piperidone is a family of organic chemicals characterized by a 6-membered ring, one carbon substituted with nitrogen and a double-bonded oxygen atom.

Piperidones are named by the location of the nitrogen or amine group and the carbonyl group (ketone) on the ring. It is used in pharmaceutical companies and chemical manufacturers as starting material having anti-microbial activity (Soundarrajan, Saraswathi, and Asiyaparvin, 2011). Compounds having piperidone are associated with diverse pharmacological properties such as anti-cancer, anti-microbial, anti-convulsant, anti-viral, anti-HlV, anti-fungal and anti-mycobacterial (Nagender *et al*., 2014; Reddy *et al*., 2013). Previous studies reported that there are peptide that can contribute to the dengue virus inhibitory , from the literature, this is not a stable peptide, on this note, newly piperidone derivatives compounds were synthesized with an aim to obtain compounds that could provide an inhibitory activity towards current fatal disease, dengue virus. Currently there is no vaccine or effective anti-viral therapy available in market for the prevention or treatment of the dengue fever.

Dengue is a disease caused by the infection of the dengue virus and it has become a worldwide health issues and it cases have been reported increasing yearly (Schwartz *et al*., 2015). According to the World Health Organization (WHO), it is estimated that 3900 million people, in 128 countries, are at risk of infection with dengue viruses (WHO 2015). Dengue virus (DENV) is belongs to *Flavivirade* family which is transmitted to human by the bites of infected female mosquito namely *Aedes aegypti* and *Aedes albopictus*. There are five types of dengue virus (DENV): DENV-1, DENV-2, DENV-3, DENV-4 and DENV-5 (Mustafa *et al*., 2015), which each of them can cause dengue disease and among them, DENV type 2 is being the most prevalent virus in Malaysia (Liu *et al*., 2014). DENV can cause severe epidemic disease

ranging from the mild dengue fever (DF) to the life-threatening dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) (Amorim *et al*., 2014).

DENV contains an approximately 11 kb positive-strand RNA genome that is transcribed as a single polyprotein which consists of three structural proteins, the capsid (C), precursor/mature membrane protein (prM/M) and the envelope (E) form the virion; the seven non structural proteins (NS1, NS2A, NS2B, NS3, NS4, NS4B and NS5) (Zhou *et al*., 2013). In the search for new drugs against this infection, the NS2B-NS3 protease of the dengue virus was investigated as molecular target. NS2B is a cofactor of the NS3 serine protease, which is crucial in the virus replication process in mammalian host cell (Sousa *et al*., 2015). NS2B-NS3 protease is a serine protease, which utilizes a combination of mechanism that is common to enzyme catalysis. It plays an important role in the viral replication as it is required for processing of the polyprotein prior to the assembly of the viral replicase complex (Tomlinson *et al*., 2011; Lai *et al*., 2013; Liu *et al*., 2014) . Three conserved amino acid residues namely serine 135 (Ser135), histidine 51 (His51) and aspartic 75 (Asp75) formed the catalytic triad, the active site of NS2B-NS3 protease (Idrees and Ashfaq, 2014). Hence, NS2B-NS3 protease is a potential target for the development of therapeutics against the dengue virus (Yildiz *et al*., 2013). Bioactivities interactions of the synthesized compounds towards DENV2 NS2B-NS3 protease can be predicted by docking studies.

Computational docking plays an important role in the discovery of potential ligands that can fit at the binding site of a target biomacromolecule (Kitchen *et al*., 2004). Molecular docking process involves two basic steps: prediction

of the ligand conformation include its position and orientation within the active sites and assessment of the binding affinity (Novikov and Chilov, 2009). The conformations of the ligand in the active site of the protease are ranked based on their scoring function or free energy of binding (FEB) which is an important criteria in the virtual screening and lead optimization of ligands for drug discovery. The free energy of binding, ΔG can be calculated based on equation as follows:

$$\Delta G=\left(V^{L\text{-}L}_{bound}- V^{L\text{-}L}_{unbound}\right) + \left(V^{P\text{-}P}_{bound}-V^{P\text{-}P}_{unbound}\right) + \left(V^{P\text{-}L}_{bound}-V^{P\text{-}L}_{unbound}+\Delta S_{conf}\right)$$

Where P refers to the protein, L refers to the ligand, V are the pair-wise evaluations and ΔS_{conf} denotes the loss of conformational entropy upon binding (Huey *et al*., 2007).

AutoDock is one of the popular molecular docking softwares that can be used to predict the interaction between ligands and the biomacromolecular targets. AutoDock combines two methods: rapid grid-based energy evaluation and efficient search of the torsional freedom to achieve minimum interaction energy between the ligand and the target protein by exploring all available degree of freedom for the system. In addition, it is capable to find the suitable inhibitor in a short time (Morris and Huey, 2009). The combination of molecular modeling and bioassays studies become a useful approach for further drug development. In this study, *in silico* study has been performed as a proof of concept with the main aim to have correlation with the *in vitro* anti-dengue activity result.

Problem statement

Currently, Dengue virus (DENV) is an important human arthropod-borne virus with a major impact on public health. Despite the enormous disease burden and health care's core associated with DENV infections, there is currently no licensed vaccines or specific treatment available. Hence, there is an urgent need for the researchers to search and develop the vaccines and anti-dengue drugs for the treatment of dengue. There have been many discoveries of potential anti-dengue synthetic compounds using many approaches such as *in silico* screening from compounds libraries, high throughput *in vitro* screening and structure-based drug design. From the literature review, peptide can contribute to the virus inhibitory, but it is not a stable structure. In the present study, new piperidone and its potential derivatives were synthesized due to their biological activities reported in many research studies, and the selected compounds are predicted for their molecular interaction studies towards DENV-2 NS2B-NS3 protease active site. The selected potentials compounds were further analyzed and evaluated for enzyme inhibitory activities in the search for the new DENV2 NS2B-NS3 protease inhibitors.

Objectives

The aims of the present study are as follow:

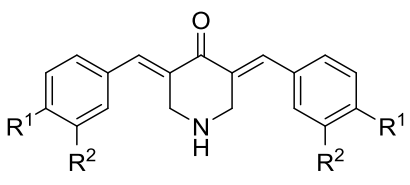
1. To synthesize new piperidone derivatives (20 compounds).
2. To purify, characterize and elucidate the structures of synthesized compounds using NMR spectroscopy (1D-2D NMR), FT-IR and elemental analysis.
3. To investigate the binding interaction of the most active inhibitors inside the active site of DENV2 NS2B-NS3 protease by molecular docking analysis.
4. To evaluate the anti-dengue activities of the selected synthesized compounds against DENV2 NS2B-NS3 protease.

CHAPTER 2

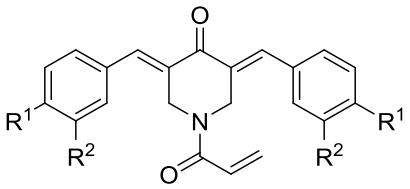
LITERATURE REVIEW

2.1 Previous studies on piperidone

Piperidone and its derivatives were found to show quite diverse biological activities such as anti-viral and anti-microbial (Reddy *et al*., 2013). There is special interest in studying the biological role of the nitrogen and the substituents in 4-piperidone series such as 3,5-bis(benzylidene)-4-piperidone, which showed good biological activities (Dimmock *et al*., 2001). In the research, a series of 3,5-bis(benzylidene)-4-piperidone (**1**) and related *N*-acryloyl analogues (**2**) were prepared as the candidates of cytotoxic agents and were studied against murine P388 and L1210 neoplasm that have been reported to be predictors of clinically useful anti-cancer drugs (Suffnes *et al*., 1979). The results discovered that, the average IC_{50} for both analogues **1** and **2** were 44.0 and 1.77 µM, respectively, revealing 25 fold increases in cytotoxicity for analogue **2** that afford the theory of cytotoxicity mentioned in (Dimmock *et al*., 1993).



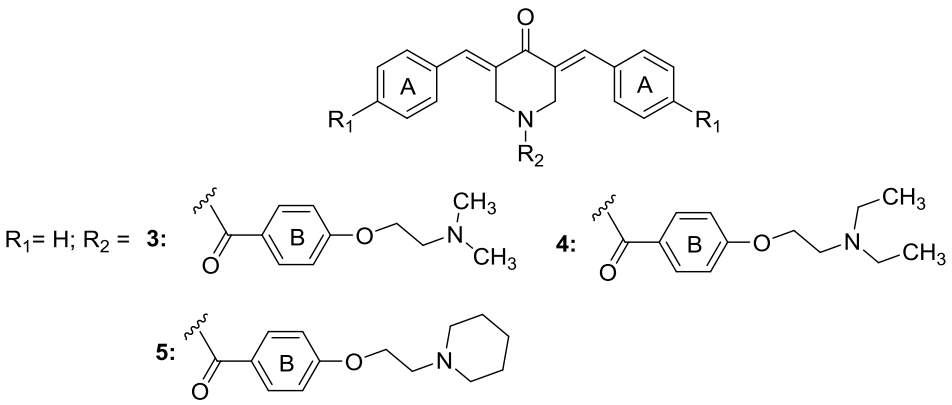
1



2

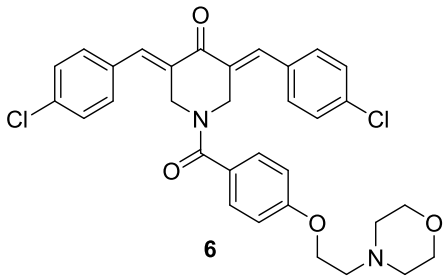
a: $R^1=R^2=H$; **b**: $R^1=Cl, R^2=H$
c: $R^1=R^2=Cl$; **d:** $R^1=F, R^2=H$
e: $R^1=NO_2, R^2=H$;
f: $R^1=OCH_3, R^2=H$

Compound **1** were used as lead molecule for the development candidates for anti-mycobacterial agents that showed excellent potential towards *M.tuberculosis* (Umarshankar *et al*., 2008), it was also reported to completely inhibited the microorganism growth at concentration of 6.25 µg/mL (Jha *et al*., 2006). Modifications of the compounds were done after considering the van de Waals interactions between aryl B and a possible complementary area on binding site. All synthesized compounds were evaluated initially against *M. tuberculosis* H37Rv (ATCC 27294) using concentration of 12.5 µg/mL and 6.25 µg/mL. The research uncovered that the expanding of hydrophilic-hydrophobic methoxy substituent in ring B lead to the 1-[4-(2-aminoeth-oxy)phenylcarbonyl]-3,5-bis(benzylidene)-4-piperidones (**3-5**) resulted in 98, 99 and 100 % inhibition at 6.25 µg/mL, respectively, which shown as a new cluster of anti-mycobacterial (Das *et al*., 2008).



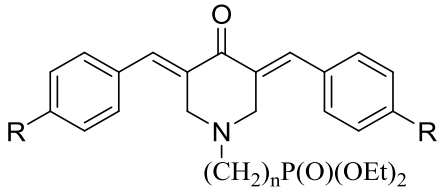
In 2013, R.S.P Singh and co-workers have prepared almost the same structure moieties and screened against the drug-sensitive D6 and the C235 drug-resistant strains of *Plasmodium falciparum* using the SYBR Green-I based fluorescence assay, the results showed that the compound (**6**) demonstrated potent anti-malarial properties with IC_{50} values of 0.60 and 1.97 µM against the drug sensitive D6 strain and the C235 drug-resistant strain of *Plasmodium falciparum*. This compound concentrates in red blood cells, permeates across CACO-2 cells and lowers

glutathione concentrations in erythrocytes. These data revealed compound **6** to be a promising lead compound in the search for novel anti-malarial agents (R. S. P. Singh *et al*., 2013).



6

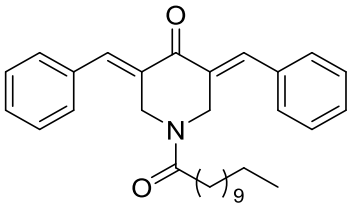
Makarov (2009) observed that some representative of 3,5-bis(benzylidene)-piperidine-4-one derivatives from the previous research (Szakács *et al*., 2004) demonstrate fluorescence properties. In the case of cytotoxic material, natural fluorescence could be very helpful in tracking targeted organelle in the cell. Cytotoxicity activity increase with the increases of electron withdrawing properties of the phenyl ring substituents of piperidones and their derivatives. Combination of fluorescent and anti-tumor activity properties makes these types of compounds attractive candidates for drugs. In this research, they have developed effective synthetic approaches to ω-aminophosphonates bearing piperidone and differing in alkylene chain length. Cytotoxic properties studies revealed that a number of phosphonates in the 3,5-bis(benzylidene)piperidin-4-ones series **7** have IC_{50} values in the low micromolecular range towards four human carcinoma cell lines. Thus, it concludes that the cytotoxicity increases with the increase of electron withdrawing properties of the substituted R and donor amino groups result in increased fluorescence emission (Makarov *et al*., 2009).



7

n=1: R= F (7b); NO_2 (7c);
n=2: R= F (7e); NO_2 (7f);
n=3: R= F (7h); NO_2 (7i);
n=4: R= NO_2 (7k)

Most recently, Potter and co-workers (2015) was actively working on design and develop a fatty acid conjugated of the established pharmacophore 3,5-bis(benzylidene)-4-piperidone as the therapeutic agents. All the synthesized compounds were evaluated for cytostatic against murine L1210 leukemia cells while selected compounds from cytostatic result were investigated for their topoisomeras IIα inhibitory activity. The study indicated that *N*-subtituted 3,5-bis(benzylidene)-4-piperidone **8** emerged as one of the most potent catalytic inhibitor of topoisomerase IIα in vitro. This research shows that the compound was effectively non-toxic anti-cancer with strong anti-oxidant ability which may act as an auxiliary therapy in cancer (Potter *et al*., 2015).

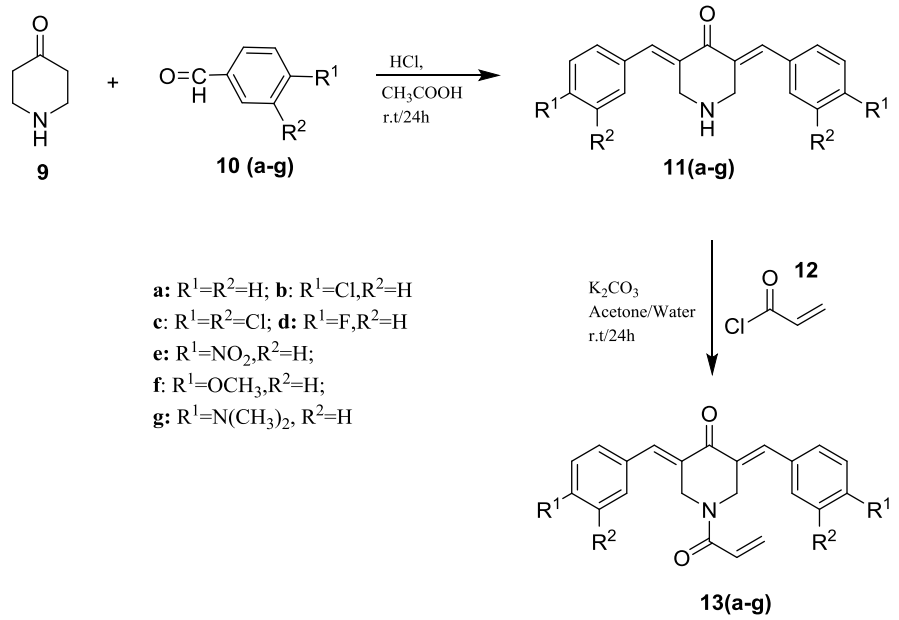


8

2.2 Method of the synthesis of piperidone

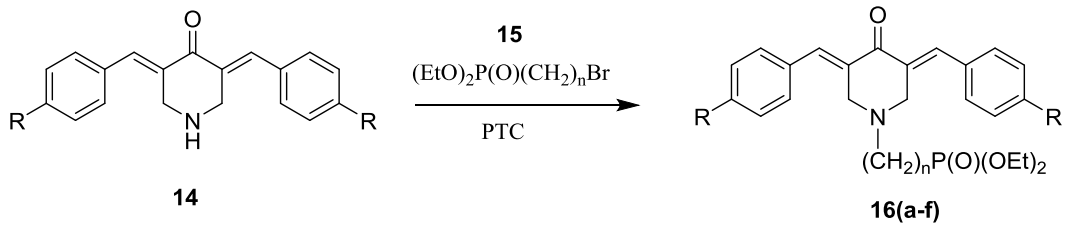
Several methods were reported in the literature for introducing varies ring and group in the core structure of 3,5-bis(benzylidene)piperidin-4-one. J.R. Dimmock and co-

workers were the first synthetic chemist to prepare 3,5-bis(benzylidene)piperidin-4-one for the past years since 1990 by Claisen-Schmidt condensation of 4-piperidone of hydrochloric with a series of aromatic aldehyde **10**(**a-g**) in the presence of HCl in acetic acid. Then, the study proceed to the acylation of 3,5-bis(benzylidene)piperidin-4-one **11**(**a-g**) with acryloyl chloride (**12**) in the presence of potassium carbonate furnished 1-acryloyl-3,5-bis(benzylidene)piperidin-4-one **13**(**a-g**) (Scheme 2.1). This method involves simple work-up and usually the result is a single product (Dimmock *et al*., 2001).



Scheme 2.1: The synthesis of derivatives of 1-acryloyl-3,5-bis(benzylidene)-4-piperidone **13**(**a-g**).

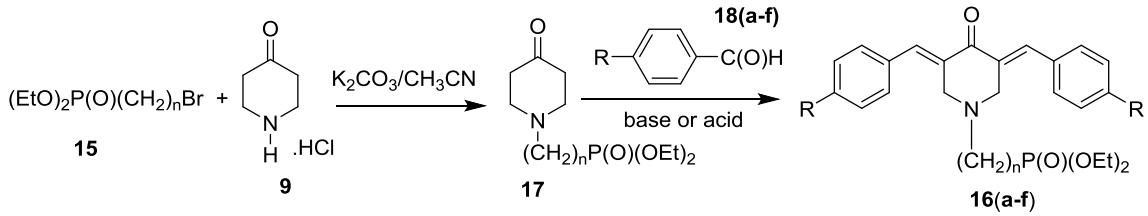
Makarov (2009) has introduced two possible routes to afford *N*-(ω-phosphorylalkyl)-3,5-bis(benzylidene)-piperidin-4-one **16**(**a-f**). The first path was alkylation of parent NH-3,5-bis(benzylidene)-piperidin-4-one (**14**) by phosphorylated halogen (**15**) as outline in Scheme 2.2.



n=1 : R= $N(CH_3)_2$, 14(a) : F, 14(b) : NO_2, 14(c)
n=2 : R= $N(CH_3)_2$, 14(d) : F, 14(e) : NO_2, 14(f)

Scheme 2.2: Synthetic approach to *N*-phosphorylalkylated 3,5-bis(benzylidene)-4-piperidone.

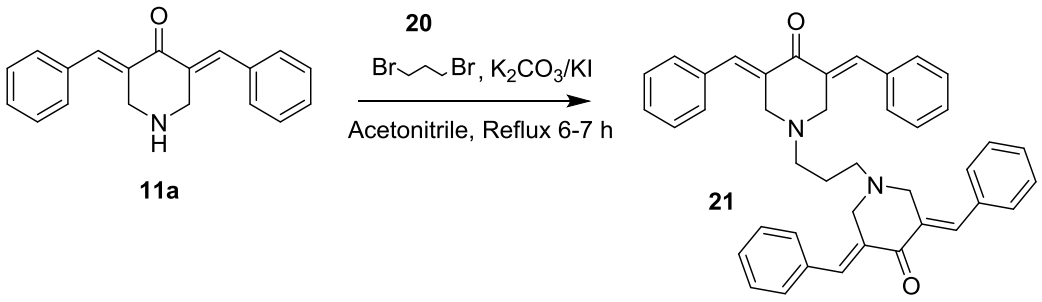
Aza Michael reaction was also used as another route to synthesis of β-aminophosphonates bearing the piperidone (**17**), then condensation of phosphorylated piperidone with substituted benzaldehydes under either base or acid condition affording the target *N*-phosphorylalkyl substituted 3,5-bis(benzylidene)piperidin-4-one **16**(**a**-**f**) (Scheme 2.3) (Makarov *et al*., 2009).



n=1 : R= $N(CH_3)_2$, 14(a) : F, 14(b) : NO_2, 14(c)
n=2 : R= $N(CH_3)_2$, 14(d) : F, 14(e) : NO_2, 14(f)

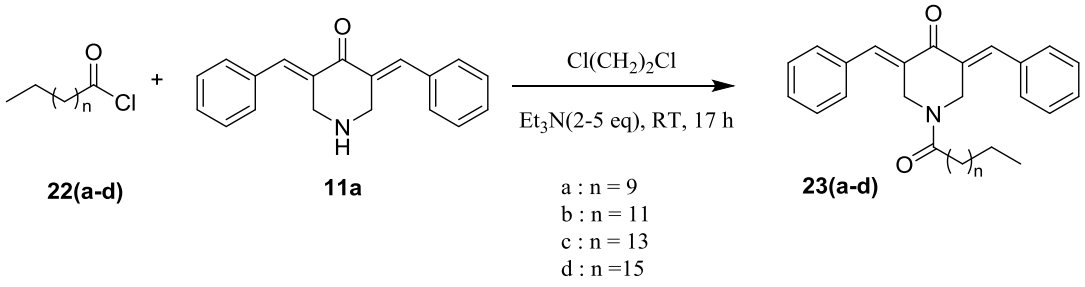
Scheme 2.3: Synthesis of phosphonates.

A base catalysed condensation reaction of two mol equivalents of 3,5-bis(benzylidene)-4-piperidones (**11a**) with one mol equivalent of 1,3-dibromopropane (**20**) heated at reflux temperature for 6-7 hours was used to synthesize the dimer (**21**) as depicted in Scheme 2.4 (S. Das *et al*., 2013).



Scheme 2.4: Synthesis of the dimer (**21**) by base catalysed condensation.

Recently, Potter and co-workers (2015) employed one-pot synthesis by adding the prepared acid chloride dropwise into the mixture of appropriate 3,5-bis(benzylidene)piperidin-4-one and triethyl amine. The reaction was stirred at room temperature overnight as illustrated in Scheme 2.5.



Scheme 2.5: Synthetic strategy to synthesize 3,5-bis(benzylidene)piperidin-4-one fatty acid amides.

In the present study, the synthesis of *N*-subtituted-3,5-bis(benzylidene)piperidin-4-one with the phenacyl bromide was followed the methodology used in Scheme 2.1.

2.3 Docking for drugs discovery

Dengue virus is belongs to the family *Flaviviridae* and is a vital emerging pathogen in the development of vaccines and antiviral therapy. Tomlinson and co-workers (2009) identified the novel dengue virus (Type 2) protease inhibitors by targeting NS3 viral protease that required for virus replication. While other researchers have identified protease inhibitors either using high-throughput screening (HTS) method or using bioassays method by identifying the compounds that mimic the peptide substrate (Leung *et al*., 2001; Yin *et al*., 2006a,b). In the first method, they used virtual screening to predict which chemical compounds are commercially available in the library that could inhibit the dengue virus protease by using two previously reported dengue virus type 2 (DENV2) protease crystal structure, the NS3 protease domain alone, PDB (ID: 1BEF) (H. M. K. Murthy, Clum, and Padmanabhan, 1999) and the NS3 protease domain complex with the Mung-Bean Bowman-Birk inhibitor, PDB (ID: 1DF9) (K. Murthy *et al*., 2000). The EUDOC program performed the virtual screening and the compound having lowest energy score was then tested for solubility and protease inhibition activity. *In vitro* assays resulted in this study indicated more than half of the tested compounds showed reduced *in vitro* NS2B-NS3 activity (Figure 2.1). This strategy gives a logical progression for early stage drug discovery that can be used to successfully identify a potential candidate for antiviral drugs (Tomlinson *et al*., 2009).

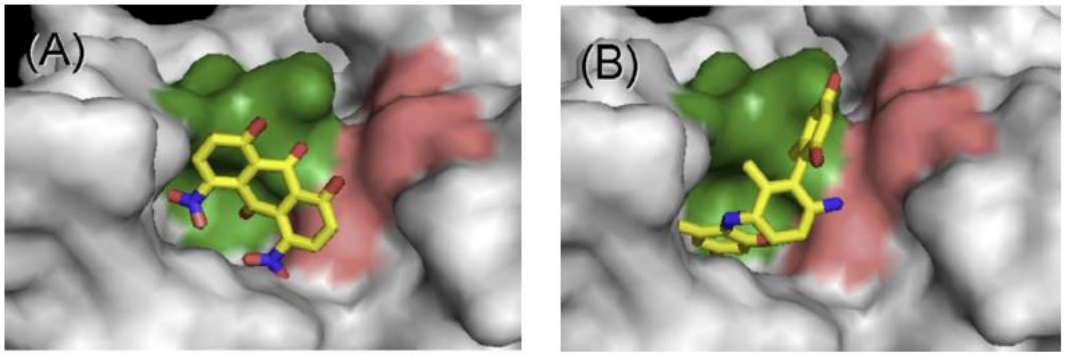


Figure 2.1: EUDOC-predicted models of bound conformations of compounds that demonstrated dengue antiviral activity in cell culture. (A) ARDP0006, EC_{50} = 4.2±1.9 and (B) ARDP0009, EC_{50} =35±8.0 were predicted to interact with active site (pink) and P1 pocket (green) residues of the DEN2V protease. Picture was generated from (Tomlinson *et al*., 2009).

In another study, a group of researcher has explored the structure-based molecular docking of cyclic peptide as dengue virus inhibitor. Tambunan and co-workers (2010) found that disulphide cyclic peptide could be a potential inhibitor against NS2B-NS3 complex to inhibit DENV infection. In their research, seven cyclopentapeptide inhibitors involving S-S disulfide bridge designed against DENV2 NS2B-NS3 protease. All peptide were then analyzed for its enzyme-inhibitor binding interaction by docking study. The enzyme structure used in this study was available in the protein data bank (PDB) (ID: 2FOM). The docking was performed using AutoDock 4.0 software which was reported to be the most popular docking program (Hetényi and van der Spoel, 2002) . The seven cyclopentapeptides proposed are suitable for design of drug because of their high activity and specificity despite the low solubility. The proposed cyclopentapeptide structures exhibited as the promising peptide inhibitor with lowest free energy binding of -8.39 kcal/mol and small inhibition constant, Ki of 0.71 µM. It indicated that the ligands were favorable towards binding site compared to the standard inhibitor (Ki=5.80 µM) (Figure 2.2) (Tambunan and Alamudi 2010).

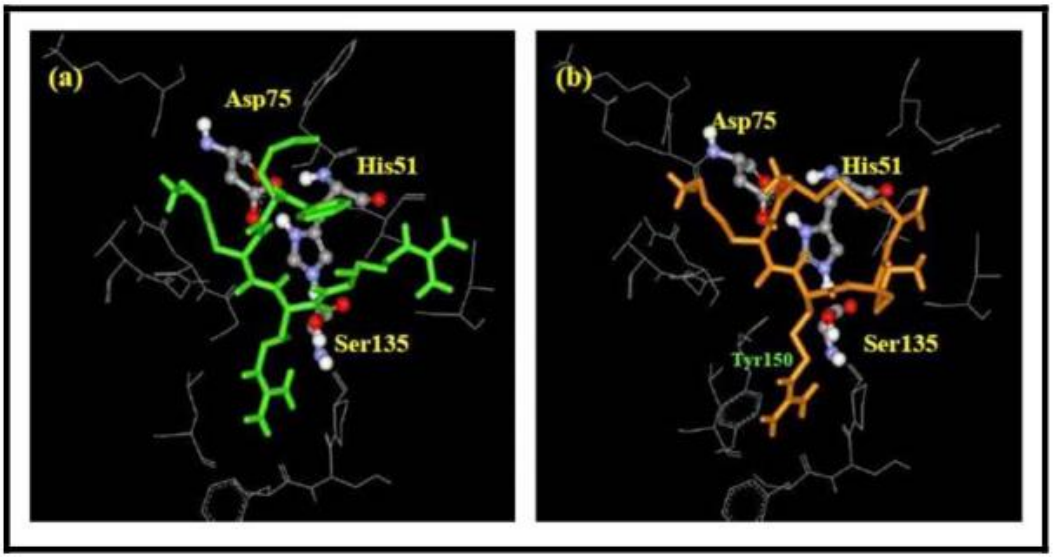


Figure 2.2: View of ligand conformation at the binding site. (a) Standard ligand, (b) cyclopeptide ligand. Contact residues of the enzyme that interact with the ligands are shown as grey sticks. Catalytic triads are shown as balls and sticks. Picture was generated from (Tambunan and Alamudi, 2010).

Continuation from their previous study, a cyclic peptide with a positively charged amino acid was designed as DENV NS2B-NS3 protease, using computational docking techniques. The protein structure was retrieved from PDB (ID: 2FOM) and the peptide were docked using MOE (Molecular Operating Environment) software package. The study reported that peptide (Cys-NRK-Cys) was successfully blocked NS2B-NS3 protease and may serve as an important drug candidate while, peptides (Cys-RKG-Cys), (Cys-KRR-Cys), and (Cys-GNRK-Cys) had potential interaction with the active site (Idrees and Ashfaq, 2014) (Table 2.1). Thus, the study concluded that these peptides could serve as important inhibitors to inhibit the viral replication and need further *in vitro* investigation to confirm their efficiency.

Peptide	S score	RMSD	Interacting Residue (Hydrogen bonding)	Close contact residue
Cys-RKG-Cys	-10.10698	3.335608	Arg54, Asp75, Glu101, Ser135, Asn152, Gly153	Trp50. His51, Leu128, Pro132, Tyr150
Cys-NRK-Cys	-11.12951	2.531304	His51, Asp75, Glu101, Asp129, Phe130, Ser135, Tyr150, Asn152, Gly153, Tyr161	Leu128, Pro132
Cys-RGK-Cys	-7.159595	2.026983	Phe130, Asn152, Gly153	His51, Asp75, Leu128, Pro132, Ser135, Tyr150, Gly151
Cys-KRR-Cys	-8.459515	2.113997	Trp50, Ser135, Gly151, Gly153	His51, Arg54, Asp75, Tyr150, Asn152, Tyr161
Cys-GRKR-Cys	-8.357669	2.004721	Leu128, Phe130, Gly153	His51, Ser127, Asp129, Ser131, Pro132, Ser135, Tyr150, Gly151, Tyr161
Cys-GNRK-Cys	-7.681766	1.846913	Phe130, Ser135, Gly151, Asn152, Gly153	His51, Asp75, Leu128, Pro132, Tyr150, Tyr161
Cys-KKRR-Cys	-8.353346	2.254074	Arg54, Leu128, Phe130, Asn152, Gly153	His51, Pro132, Ser135, Gly151, Val154, Tyr161

Table 2.1: Peptide interaction with DENV NS2B-NS3. Table was adapted from (Idrees and Ashfaq, 2014)

Most recent study by Yotmanee (2015) revealed that previous crystal structure of DENV2 NS3 using PDB (ID: 1BEF) and (ID: 1DF9) cannot confirm the analysis of the interaction between potential inhibitor and the complete NS2B-NS3 protein due to the missing cofactor NS2B in these crystal structures. Although the crystal structure of the DENV2 NS2B-NS3 complex (ID: 2FOM) was available, the information on the substrate/inhibitor binding was not inferable (Yotmanee *et al*.,

2015). Thus, homology modeling for DENV2 was prepared by Wichapong and co-workers (2010) due to the lacking of 3D structure of the inhibitor-bound form of DENV NS2B-NS3 protease. It was built based on the available data that recommended that the West Nile Virus (WNV) protease should be close to the active inhibitor complex form of DENV2 NS2B/NS3 protease. The X-ray structure of the related WNV NS2B-NS3 protease complexed with the peptidic inhibitor benzoyl-norleucine (P4)-lysine (P3)-arginine (P2)-arginine (P1)-aldehyde (Bz-Nle-Arg-Arg-H) (2FP7) was recently published (Figure 2.3) (Wichapong *et al*., 2010)

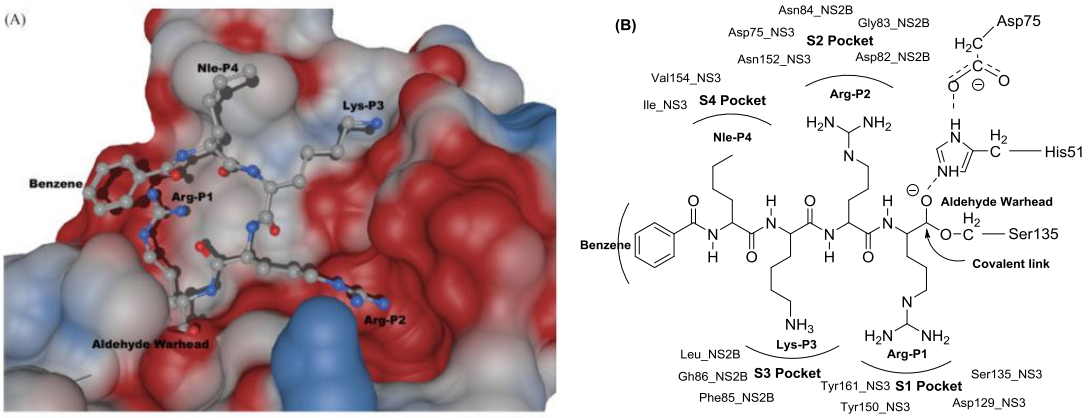


Figure 2.3: (A) Interaction of the tetrapeptidic inhibitor with the WNV NS2B-NS3 protease (taken from the crystal 2FP7). (B) Schematic representation of the interaction of the inhibitor with the substrate pocket of WNV NS2B-NS3 protease. Diagram was generated from (Wichapong *et al*., 2010).

Thus, the researchers introduced the DENV protease homology models and WNV protease crystal structure by superimposing them on their backbone atom of NS2B-NS3 protease (Figure 2.4). The analysis revealed that the homology model proposed by Wichapong showed high structural similarity as indicated by low RMSD value (0.06 Å). This model is the first completed structure of the protease including the complete cofactor NS2B in the productive form, thus it could be applied for target-based design and screening of small molecule inhibitors (Wichapong *et al*., 2010).

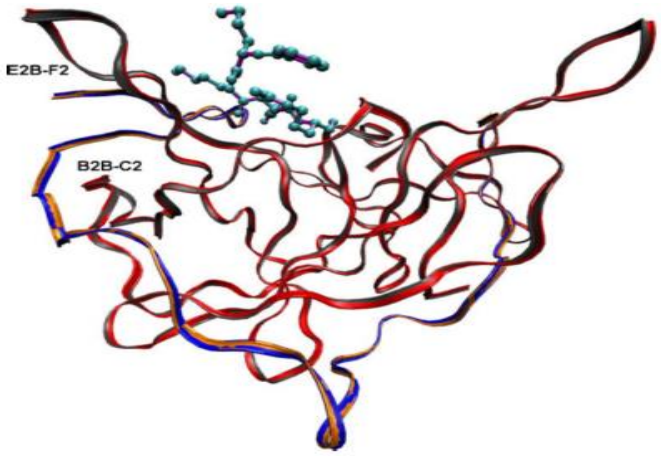


Figure 2.4: Superimposition of WNV and DENV. The individual structure is colored as follows; WNV and 2FP7: NS2B blue, NS3 red, inhibitor cyan; DV-2: NS2B orange, NS3 gray, inhibitor magenta. Picture was generated from (Wichapong *et al*., 2010).

CHAPTER 3

MATERIALS AND METHODS

3.1 Chemicals and Materials

3.1.1 Chemicals and Solvents

The chemicals and solvents used in the synthesis and characterization of the synthesized compounds are as follows: 2-Bromo-4'-methoxyacetophenone 97% (Sigma-Aldrich,USA), 2-Bromo-4'-floroacetophenone 97% (Sigma-Aldrich,USA), Potassium Carbonate (Merck, Germany), Magnesium sulphate anhydrous (Merck, Germany); Acetone, AR grade (QReC); Chloroform, AR grade (QReC); Dichloromethane, AR grade (QReC); Diethyl ether, AR grade (QReC); Ethanol, AR Grade (QReC); Ethyl-acetate, AR Grade(QReC); Dimethylsulfoxide-d_6-deuteration degree for NMR (Merck, Germany); Chloroform-D_1-deuteration degree for NMR spectroscopy (Merck, Germany); Methanol, AR grade (QReC); n-hexane, AR grade (QReC); Petroleum ether AR grade (QReC); Potassium bromide, FT-IR grade (Sigma-Aldrich,USA). All chemicals and solvents were of reagent grade and were used without further purification.

3.1.2 Materials

The following are the list of materials used for the extraction, separation and purification of all synthesized compounds; TLC Silica gel 60 F254, aluminiums sheets, 20 cm x 20 cm (Merck, Germany), and Silica gel 60, for column chromatography, (0.040-0.063 mm), (230-400 mesh ASTM) (Merck, Germany).

3.2 Characterization of Compounds

All the synthesized compounds were characterized by their melting points and different spectroscopic techniques; Infrared (IR), 1D and 2D Nuclear Magnetic Resonance (NMR) spectroscopy, elemental analysis and X-Ray Crystallography analysis was performed when applicable.

3.2.1 Melting point

The melting point of all synthesized compounds were measured using open capillary tubes by Stuart Scientific SMPI melting point apparatus in the temperature ranging from 25 to 350 ^{o}C at the School of Chemical Sciences, USM.

3.2.2 Infrared (IR) Spectroscopy

The Fourier transform infrared (FT-IR) spectra were recorded on a Perkin-Elmer 2000 FT-IR at the School of Chemical Sciences, USM. All samples were prepared as potassium bromide (KBr) disc and analyzed in the range of 4000-650 cm^{-1}. FT-IR is a simple and rapid analysis that can be used to identify the functional groups of the compounds.

3.2.3 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 by Bruker (Avance) 500 MHz NMR instrument using TMS as an internal standard at School of Chemical Sciences, USM. Standard Bruker software was used throughout for plotting. The samples were prepared by dissolving the solid sample (~20.00 mg) into the solvent (2 mL) using either deuterated dimethylsulfoxide (DMSO-d_6) or deuterated chloroform ($CDCl_3$-d_1) which was then transferred into an

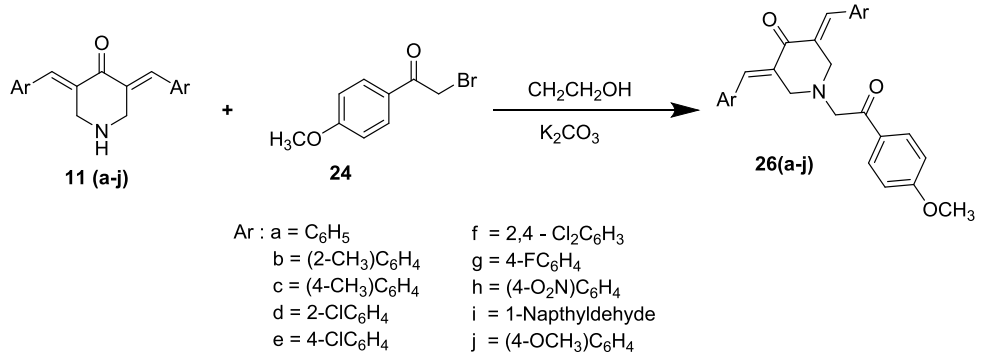
NMR tube and analyzed using the instrument. Chemical shift is given in parts per million (δ- scale) and the coupling constants are given in Hertz (Hz). The spectrum data is usually plotted in graph of intensity versus frequency.

3.2.4 CHN Elemental analysis

CHN analysis is the analytical technique to estimate the percentage of each element of carbon (C), hydrogen (H) and nitrogen (N) in a compound. Elemental analysis was performed on a Perkin Elmer 2400 Series II, Elemental CHN analyzer at School of Chemical Sciences, USM and the experimental values were within ± 0.4% of the theoretical values.

3.3 General Procedure for the synthesis of 3,5-bis((*E*)-benzylidene)-1-(2-(4-methoxyphenyl)-2-oxoethyl) piperidin-4-one and its derivatives. 26(a-j)

A mixture of the starting materials, 3,5-bis((*E*)-benzylidene)piperidin-4-one **11**(**a-j**) (0.73 mmol) and K_2CO_3 (1.45 mmol) was stirred in ethanol (10 mL) at room temperature. A 2-bromo-4-methoxy-acetophenone (**24**) (1.45 mmol) was added dropwise to the mixture while stirring. The ethanol is used as reaction medium instead of methanol due to its solubility with the starting materials. The reaction mixture was left overnight, resulting in the formation of a yellow colored solid. After completion of the reaction as evident by TLC of ethylacetate:petroleum ether with ratio 2:8, the reaction mixture was poured into the ice, the precipitate formed, filtered and washed with water to afford **26**(**a-j**). The steps to synthesize all the targeted compounds are shown in the Scheme 3.1. (Dimmock *et al*., 2001)



Scheme 3.1 : Steps to synthesize piperidone derivatives **26**(**a-j**).

3.3.1 Synthesis of 3,5-bis((*E*)-benzylidene)-1-(2-(4-methoxyphenyl)-2-oxoethyl) piperidin-4-one (26a)

Yellow solid, (0.27 g, 87.38 %), mp 115-117 ^{o}C, IR KBr (cm^{-1}): 2941 (Csp^{2}H), 1682 (C=O), 1602 (C=C), 1175 (C-N); Anal. Calcd. For $C_{28}H_{25}NO_3$: C, 79.41; H, 5.95; N, 3.31, Found: C, 78.83; H, 5.59; N, 3.09. ^{1}H NMR (500 MHz, $CDCl_3$): δ 3.84 (3H, s, H-15), 4.00 (2H, s, H-7) , 4.10 (4H, s, H-2, H-6), 6.87 (2H, d, H-11, H-13, *J*=8.50 Hz), 7.39-7.40 (10H, m, H-2', H-3', H-4', H-5', H-6'), 7.90 (2H, d, H-10, H-14, *J*=9.00 Hz). ^{13}C NMR (125 MHz, $CDCl_3$): δ 54.55 (C-2, C-6), 55.48 (C-15), 62.32 (C-7), 113.75 (C-11, C-13), 128.62 (C-4'), 128.73 (C-9), 129.06 (C-2', C-6'), 130.43 (C-3', C-5'), 130.46 (C-14, C-10), 132.98 (C-1'), 135.14 (C-5, C-3), 136.81 (C-arylidene), 163.73 (C-12), 187.26 (C-4), 195.01 (C-8).

3.3.2 1-(2-(4-methoxyphenyl)-2-oxoethyl)-3,5-bis((*E*)-2-methylbenzylidene) piperidin-4-one (26b)

Orange solid, (0.21 g, 70.50 %), mp 118-120 ^{o}C, IR KBr (cm^{-1}): 2931 (Csp^{2}H), 1673 (C=O), 1597 (C=C), 1257 (C-N); Anal. Calcd. For $C_{30}H_{29}NO_3$: C, 79.80; H, 6.47; N, 3.10, Found: C, 79.55; H, 6.44; N, 2.89. ^{1}H NMR (500 MHz, $CDCl_3$): δ 2.35 (6H, s, H-7'), 3.84 (3H, s, H-15) 3.91 (4H, s, H-2, H-6), 3.92 (2H, s, H-7), 6.86 (2H, d, H-13, H-11, *J*=8.50 Hz), 7.22-7.27 (8H, m, H-3', H-4', H-5', H-6'), 7.84 (2H, d, H-14, H-10, *J*=9.00 Hz), 8.00 (2H, s, H-arylidene). ^{13}C NMR (125 MHz, $CDCl_3$): δ 20.04 (C-7'), 54.42 (C-2, C-6), 55.46 (C-15), 62.28 (C-7), 113.69 (C-11, C-13), 125.63 (C-5'), 128.76 (C-9), 128.87 (C-6'), 128.91 (C-3'), 130.31 (C-4'), 130.35 (C-10, C-14), 133.12 (C-2'), 134.19 (C-1'), 136.28 (C-arylidene), 138.16 (C-3, C-5), 163.64 (C-12), 194.88 (C-4), 207.02 (C-8).

3.3.3 1-(2(-4-methoxyphenyl)-2-oxoethyl)-3,5-bis((*E*)-4-methylbenzylidene) piperidin-4-one(26c)

Yellow solid, (0.20 g, 67.10 %), mp 130-132 ^{o}C, IR KBr (cm^{-1}): 2966 (Csp^{2}H), 1688 (C=O), 1605 (C=C), 1190 (C-N); Anal. Calcd. For $C_{30}H_{29}NO_3$: C, 79.80; H, 6.47; N, 3.10, Found: C, 79.60; H, 6.43; N, 2.95. ^{1}H NMR (500 MHz, $CDCl_3$): δ 2.37 (6H, s, H-7'), 3.83 (3H, s, H-15), 3.99 (2H, s, H-7), 4.09 (4H, s, H-2, H-6), 6.86 (2H, d, H-11, H-13, *J*=9.00 Hz), 7.20 (4H, d, H-3', H-5', *J*=8.00 Hz), 7.30 (4H, t, H-2', H-6', *J*=8.00 Hz), 7.81 (2H, s, H-arylidene), 7.91 (2H, d, H-10, H-14, *J*=9.00 Hz). ^{13}C NMR (125 MHz, $CDCl_3$): δ 21.42 (C-7'), 54.66 (C-2, C-6), 55.46 (C-15), 62.37 (C-7), 113.72 (C-11, C-13), 128.76 (C-9), 129.37 (C-2', C-6'), 130.49 (C-3', C-5'), 130.55 (C-10, C-14), 132.22 (C-1'), 132.37 (C-4'), 136.80 (C-arylidene), 139.40 (C-3, C-5), 163.70 (C-12), 195.03 (C-4), 207.01 (C-8).