SYNTHESIS, BIOLOGICAL ACTIVITY AND MOLECULAR DOCKING OF IMIDAZOLE-PHENAZINE DERIVATIVES AS POTENTIAL INHIBITORS FOR NS2B-NS3 DENGUE PROTEASE

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by

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LIST OF SYMBOLS

%	Percentage
π	Pi
µg	Microgram
µg mL^{-1}	Microgram per milliliter
±	Plus-minus
>	More than
≤	Equal and less than
°C	Degree Celsius
µL	Microliter
µM	Micromolar
µmol	Micromole
Å	Angstrom
$Å^2$	Angstrom square
cm	Centimeter
cm^{-1}	Per centimeter
d	Doublet
dd	Doublet of doublet
g	Gram
GI	Gastrointestinal
h	Hour
J	Coupling constant
kcal mol^{-1}	Kilocalorie per mol
kg	Kilogram
L	Liter
log Kp	Log of permeability coefficient (Kp)
m	Multiplet
m/z	Mass-to-charge ratio
mg	Milligram
mg mL^{-1}	Milligram per milliliter
MHz	Mega Hertz

mL	Milliliter
mm	Millimeter
mM	Millimole
nm	Nanometer
ppm	Parts per million
rpm	Rotation per minute
s	Singlet
t	Triplet
v	Velocity of AMC produce
δ	Chemical shift
δ_C	Carbon chemical shift
δ_H	Proton chemical shift
λ	Wavelength
λ_{em}	Emission wavelength
λ_{ex}	Excitation wavelength

LIST OF ABBREVIATIONS

^{13}C-NMR	13 Carbon Nuclear Magnetic Resonance
1D-NMR	One Dimensional Nuclear Magnetic Resonance
^{1}H-NMR	Proton Nuclear Magnetic Resonance
2D	Two dimensions
2D-NMR	Two Dimensional Nuclear Magnetic Resonance
3D	Three dimensions
AMC	7-amino-4-methylcoumarin
ATR	Attenuated Total Reflection
BOC-Gly-Arg-Arg-MCA	*tert*-Butyloxycarbonylglycyl-*L*-arginyl-*L*-arginine-4-methylcoumaryl-7-amide
Bz-Nle-Lys-Arg-Arg-H	Peptidic inhibitor
BBB	Blood-brain barrier
C=O	Carbonyl
CADD	Computational-aided drug design
CCDC	Cambridge Crystallographic Data Centre
CC_{50}	Cytotoxic concentration 50%
CDC	Centers for Disease Control and Prevention
CDs	Communicable diseases
CH	Carbon-hydrogen bond
CH_2	Methylene
CH_3	Methyl
CNS	Central nervous system
COSY	Homonuclear correlation spectroscopy
CPU	Central processing unit
DCM	Dichloromethane
DENV	Dengue virus
DEPT	Distortionless Enhancement by Polarization Transfer
DF	Dengue fever
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
EDG	Electron donating group
ELISA	Enzyme-linked immunosorbent assay

et al.	And others
EWG	Electron withdrawing group
FEB	Free energy of binding
FT-IR	Fourier Transform Infrared
H_2O	Water
H_2SO_4	Sulphuric acid
HA	Hydrophobic: Alkyl
HMBC	Heteronuclear Multiple Bond Correlation
HRMS	High Resolution Mass Spectroscopy
HSQC	Heteronuclear Single Quantum Correlation
IC_{50}	Half-maximal inhibitory concentration
IPharm	Malaysian Institute of Pharmaceuticals and Nutraceuticals
IPS	Institute Postgraduate Study
IR	Infrared
K_i	Inhibitor constant
KKM	Kementerian Kesihatan Malaysia
MCA	7-methoxycoumarin-4-acetic acid
MeOH	Methanol
MS	Mass spectroscopy
NaOH	Sodium hydroxide
NCDs	Non-communicable diseases
NMR	Nuclear Magnetic Resonance
NPs	Natural products
NS	Non-structural
OH	Hydroxyl
PAINS	Pan-Assay INterference compoundS
PDB	Protein Data Bank
RdRp	RNA-dependent RNA polymerase
RFU	Relative Fluorescence Unit
RMSD	Root mean square deviation
RNA	Ribonucleic acid
SAR	Structure-activity relationship
SD	Standard deviation
SI	Selectivity index

sp.	Species
sp^2, sp^3	Hybrid orbitals
TLC	Thin layer chromatography
Tris-HCl	Trisaminomethane hydrochloride
UCSF	The University of California, San Francisco
UV	Ultraviolet
VDW	Van der Waals
WHO	World Health Organisation

LIST OF APPENDICES

SINTESIS, AKTIVITI BIOLOGI DAN PENDOKAN MOLEKUL TERBITAN IMIDAZOLA-FENAZIN SEBAGAI POTENSI PERENCAT BAGI PROTEASE DENGGI NS2B-NS3

ABSTRAK

Denggi adalah satu jangkitan virus bawaan nyamuk yang sangat serius, merupakan ancaman kesihatan global yang signifikan dan telah mengakibatkan kira-kira setengah juta kematian setiap tahun. Protease NS2B-NS3 memainkan peranan penting dalam replikasi dan pematangan virus. Dalam kajian ini, satu siri terbitan imidazol-fenazin telah disintesis melalui kondensasi terbitan benzaldehid dan 2,3-diaminofenazina, membentuk perantara asas Schiff, diikuti oleh pengoksidaan udara bagi mendorong siklodehidrogenasi *in situ*. Sebanyak lima belas sebatian berjaya disintesis dengan hasil antara 15% hingga 90%. Teknik spektroskopi, termasuk NMR 1D dan 2D, FT-IR, dan HRMS, telah digunakan untuk menjelaskan struktur sebatian ini. Sebatian-sebatian ini diuji untuk aktiviti biologi terhadap protease DENV2 NS2B-NS3 menggunakan ujian *in vitro* dengan substrat fluorogenik Boc-Gly-Arg-Arg-AMC. Enam terbitan (**64b**, **64e**, **64f**, **64g**, **64k**, dan **64m**) menunjukkan perencatan protease denggi yang lebih baik, dengan nilai IC_{50} antara 54.8 µM dan 92.7 µM, berbanding kuersetin (IC_{50} = 104.8 µM). Terbitan nitro, 2-(3-Hidroksi-4-nitrofenil)-1H-imidazo[4,5-b]fenazina (64f), adalah yang paling berkesan, dengan IC_{50} sebanyak 54.8 µM. Kajian *in silico* menggunakan AutoDock Vina turut menyokong keputusan eksperimen, menunjukkan sifat pengikatan yang lebih baik bagi enam terbitan aktif, dengan tenaga pengikatan bebas antara -8.03 hingga -8.50 kcal/mol, berbanding kuersetin (-7.20 kcal/mol). Penemuan ini mencadangkan bahawa sebatian yang disintesis mempunyai potensi sebagai agen anti-denggi.

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ABSTRACT

Dengue is a serious mosquito-borne viral infection that poses a significant global health threat, resulting in approximately half a million deaths annually. The NS2B-NS3 protease plays a crucial role in viral replication and maturation. In this study, a series of imidazole-phenazine derivatives were synthesised *via* condensation of benzaldehyde derivatives and 2,3-diaminophenazine, forming intermediate Schiff bases, followed by air oxidation to induce *in situ* cyclodehydrogenation. Fifteen compounds were successfully synthesised with yields ranging from 15% to 90%. Spectroscopic techniques, including 1D and 2D NMR, FT-IR, and HRMS, were used to elucidate their structures. The compounds were screened for biological activity against DENV2 NS2B-NS3 protease using an *in vitro* assay with the fluorogenic substrate Boc-Gly-Arg-Arg-AMC. Six derivatives (**64b**, **64e**, **64f**, **64g**, **64k**, and **64m**) showed better inhibition of dengue protease, with IC_{50} values between 54.8 µM and 92.7 µM, compared to quercetin (IC_{50} = 104.8 µM). The nitro derivative, 2-(3-Hydroxy-4-nitrophenyl)-1H-imidazo[4,5-b]phenazine (**64f**), was the most effective, with an IC_{50} of 54.8 µM. *In silico* studies using AutoDock Vina further supported the experimental results, revealing better binding affinities for the six active derivatives, with free binding energies ranging from -8.03 to -8.50 kcal/mol, compared to quercetin (-7.20 kcal/mol). These findings suggest that the synthesised compounds hold promise as potential anti-dengue agents.

CHAPTER 1

INTRODUCTION

1.1 Background of study

The strategic design and synthesis of small molecule inhibitors represents an impressive strategy to combat viral infections (Ha *et al*., 2020). By precisely targeting specific enzyme functions that are critical for viral replication, such inhibitors represent a promising avenue for therapeutic intervention. In the early stages of organic synthesis, conventional synthetic chemists pursued three main goals: 1) the elucidation of molecular structures, 2) the discovery of new compounds and 3) the deciphering of reaction mechanisms (Hudlicky *et al*., 2007). However, due to technological advances and the increasing importance of practical applications, these goals have evolved and merged into five main approaches in modern organic synthesis. These approaches include the integration of biological insights, medicinal chemistry principles, advances in materials science and environmental considerations alongside traditional synthesis endeavours (Hudlicky *et al*., 2007).

Initially, the proposed study focussed on the synthesis of benzylidene-phenazine derivatives, prompted by the theoretical insights of the *in silico* study on NS2B-NS3 protease by Maywan Hariono and colleagues (Salin *et al*., 2022). By leveraging the findings of Deng and colleagues in 2012, who identified compound **1** as an active against the NS2B-NS3 protease with an IC_{50} of 13.12 ± 1.03 μM through virtual screening and a hit compound ethyl 4-hydroxy-3,5-bis((2-(4-nitrophenyl)hydrazinylidene)-methyl)benzoate (**2**) with an IC_{50} of 14.58 μM *via* scaffold hopping, combined with *in vitro* assays against the DENV2 NS2B-NS3 protease. In addition, their further studies on a dimer (**3**) consisting of linked

N-heterocyclic compounds with a 3-nitrophenyl group resulted in an IC_{50} of 29.41 µM (Figure 1.1) (Deng *et al*., 2012).

From these compounds, the nitrophenyl, hydrazinylidene, and *N*-heterocyclic groups were identified as key structural elements responsible for inhibiting the protease, serving as pharmacophores. These findings guided the design of a structure incorporating these pharmacophores, while also considering feasibility for laboratory-scale synthesis. This led to the selection of benzylidene-phenazine derivatives (**4**) as candidates for further screening as DENV2 NS2B-NS3 protease inhibitors. The pharmacophore study suggested that two derivatives of benzylidene-phenazine **4A** and 4**B** (Figure 1.1) showed promise as DENV2 NS2B-NS3 inhibitors (Salin *et al*., 2022).

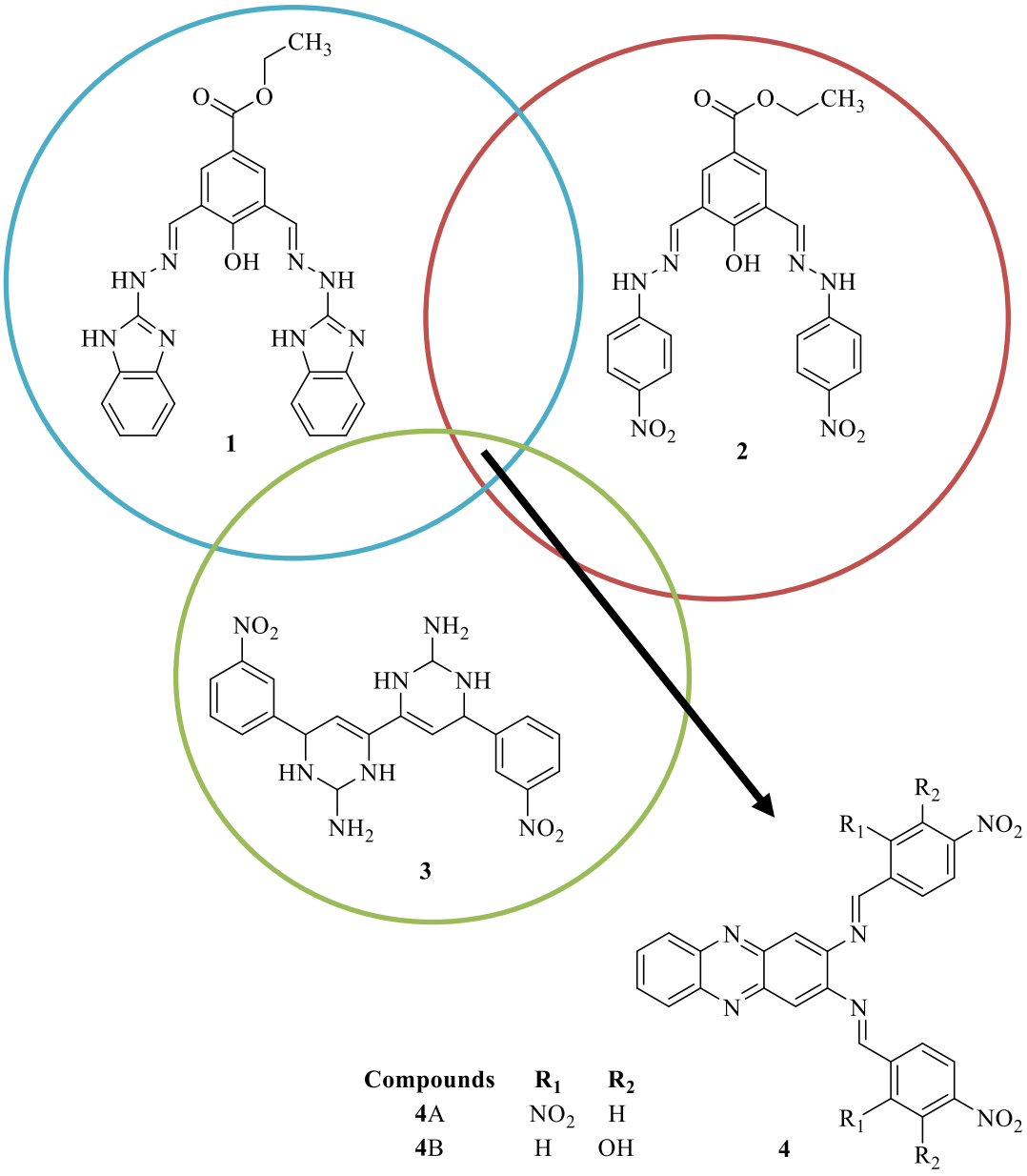
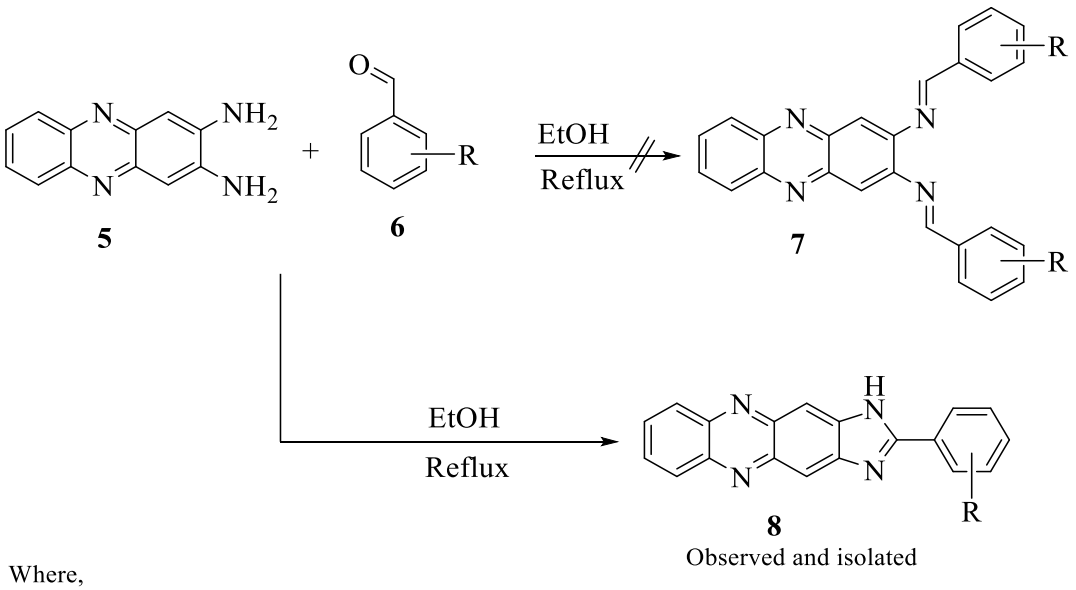


Figure 1.1 The 2D chemical structures of the lead compound **1** (IC_{50} = 13.1 µM), **2** (IC_{50} = 29.41 µM), **3** (IC_{50} = 14.58 µM), and **4** the suggested compound designed by extracting the common functional group in the previous known active compounds against DENV2 NS2B-NS3 protease (Salin *et al*. 2022).

Thus, the design was proposed to be synthesise *via* Hugo Schiff's method. It involves the reaction of aldehydes with a primary aromatic amine under various reaction conditions, often in the presence of different solvents resulting in the formation of called Schiff bases (Subasi, 2022). Methanol and ethanol are commonly

used solvents for the preparation of Schiff bases, either at room temperature or under reflux conditions (Shariff *et al*., 2022). In general, a Schiff base is the product formed by the condensation of primary amines with carbonyl compounds which is simpler and cost efficient (Kajal *et al*., 2013).

The fifteen analogues of benzylidene-phenazines derivatives (**7**) were synthesised according to the previous report on formation of Schiff bases (Arun *et al*., 2016). The preparation involves refluxing a mixture of 1 mmol of 2,3-diaminophenazine (**5**) and 2 mmol of nitrobenzaldehyde derivatives (**6**) in anhydrous ethanol at 80°C under atmospheric pressure. The mixture was expected to condense on both side of amine group of 2,3-diaminophenazine (**5**) forming (**7**) (Arun *et al*., 2016; Ezhumalai *et al*., 2018).



Scheme 1.1 Synthesis reaction between 2,3-diaminophenazine (**5**) and benzaldehyde **6**.

However, the experimental results were not in agreement with the theoretical predictions persuading further investigation to understand the formation of imidazole-phenazine (**8**) as a final compound. Thus, for this project the objective was changed to investigate the synthesis and biological evaluation of imidazole-phenazine (**8**) derivatives as potential inhibitors targeting the NS2B-NS3 dengue protease.

With diverse tools, chemists can tackle synthetic challenges in a broad spectrum of applications, from drug discovery to materials development and environmental remediation (Campos *et al*., 2019). In this study, we pursue the organic synthesis of different classes of *N*-heterocyclic compounds as a hybrid to evaluate them as DENV2 NS2B-NS3 protease inhibitors. The synthesis of imidazole-phenazine is a green chemistry-inspired approach using ethanol as a solvent without further catalysts and employing atmospheric oxygen as the main oxidising agent. At the same time, the synthesised compound possessed a high potential to mimic the biological activity of the reference compound.

1.2 General introduction

A disease is a condition that affects living organisms and is usually due to either an infection or a deterioration in health (Van Seventer *et al*., 2016). Diseases are usually categorised into communicable and non-communicable diseases (Ackland *et al*., 2003). Communicable diseases are contagious diseases that are transmitted from one person to another through various means such as direct contact, carriers, vectors or through the air (Van Seventer *et al*., 2016). Dengue infection, due to mosquito-borne pathogenic flaviviruses dengue virus (DENV) and spread to people by *Aedes albopictus* and *Aedes aegypiti* is one of the prime examples of communicable diseases

(Kuno *et al*., 1998). It poses a significant global health treat, especially in tropical and subtropical regions.

DENV are RNA viruses with positive-sense, single-stranded genomes. Their genetic material consists of seven non-structural proteins (NS) and three structural proteins (capsid, C; membrane, prM/M; and envelope, E), all of which were originally encoded as a single precursor polyprotein (Figure 1.1) (Maus *et al*., 2021). The four major dengue virus serotypes DENV1, DENV2, DENV3 and DENV4 were the most important re-emerging arboviruses in terms of geographical distribution and frequency of infection (Bharaj *et al*., 2008). Therefore, these different serotypes were thought to be the causative agents of dengue fever including dengue haemorrhagic fever, which is usually fatal in severe cases. Without appropriate treatment, severe dengue infection has a high morbidity and mortality rate (Wong *et al*., 2020).

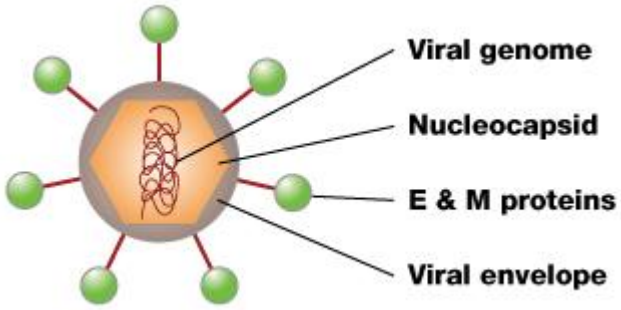


Figure 1.2 The molecular model of dengue virus (Nature Education, 2011)

Viral maturation involves cleavage of the polyprotein by the viral NS2B-NS3 protease complex and the host protease (Maus *et al*., 2021). The active site of the NS3 serine protease has a catalytic triad consisting of three amino acid residues, namely SER135, HIS51 and ASP75 (Zuo *et al*., 2009). However, optimal catalytic activity

requires NS2B as a cofactor (Abduraman *et al*., 2018). NS2B contributes to the activity of NS3 by facilitating membrane attachment via its hydrophobic domain, while its hydrophilic domain supports the activation of NS3 (Yap *et al*., 2017). Therefore, inhibitors targeting this complex are a focus in the development of dengue fever drugs, as they are effective as antiviral agents and reduce viral pathogenicity.

There is currently no specific antiviral treatment for dengue fever, making it imperative to research and development of potential anti-dengue inhibitors is essential. The development of potential anti-dengue inhibitors is a multidisciplinary field that combines virology, structural biology, drug design and immunology. Researchers are making significant strides in identifying promising candidates for dengue treatment, but further studies and clinical trials are needed to bring effective inhibitors to the market.

1.3 Problem statement

Dengue virus (DENV) infection leads to increasingly harmful dengue fever, dengue shock syndrome and fatal haemorrhagic complications (Rajapakse, 2011). The global incidence of dengue fever has grown significantly in the last few decades. The dengue cases have sharply climbed during last two decades and represents an immense public health challenge. The World Health Organisation stated that the quantity of instances that have been reported cases worldwide resurgence in 2023 with more than six million cases of dengue and over 6000 deaths attributed to the disease reported across 92 countries and territories (ECDC, 2024). This rebound is distinguished by a discernible rise in the quantity, intensity, and concurrent occurrence of several outbreaks that impact areas that were previously free of dengue disease (WHO, 2023).

Dengue fever is widespread worldwide, but there is no authorised medication to treat a such severe viral infection. The first dengue vaccine, Dengvaxia, is licenced in certain countries, but only for people without prior dengue infection, and its safety and efficacy in the elderly and young children remains uncertain (CDC 2024, Salje *et al*., 2021). Consequently, the demand for therapeutic drugs targeting DENV infection is increasing. This highlights the pressing need for the discovery and development of efficient drugs to combat dengue fever.

1.4 Research objectives

The overall objective of this research is to synthesise, characterise, and evaluate imidazole-phenazine derivatives for their structural, biological, and computational properties, with a focus on understanding their formation and inhibitory activity against the DENV2 NS2B-NS3 protease. Through experimental and *in silico* studies, the research aims to align practical findings with theoretical predictions and assess the therapeutic potential of these compounds.

1. To synthesise and characterise imidazole-phenazine compound using spectroscopic techniques.

2. To validate the DENV2 NS2B-NS3 protease inhibitory activity of the synthesised imidazole-phenazines derivatives.

3. To evaluate the *in silico* study of the synthesised compound using DENV2 NS2B-NS3 Wichapong model.

1.5 Significant of study

Recent advances in medicinal chemistry have driven the design and development of several heterocyclic scaffolds as potential antiviral drug candidates for dengue virus (DENV) suppression. In particular, previous research has highlighted the efficacy of compounds such as quinoline, piperidone, thioguanine and benzimidazole as DENV2 NS2B-NS3 protease inhibitors (Hariono *et al*., 2019). These compounds, characterised by an *N*-heterocyclic structure, offer promising scaffolds for the development of inhibitors.

Our focus lies on exploring hybrid compounds of phenazine and imidazole. Phenazine, a member of the *N*-heterocyclic compound family, is found in both terrestrial and marine microbes and has attracted attention due to its diverse biological activities, including antimicrobial and enzyme inhibitory properties (Spicer *et al*., 2000; Yan *et al*., 2021). Despite its potential, there remains a paucity of research on phenazine derivatives as the DENV2 NS2B-NS3 protease inhibitors, with only previous *in silico* studies indicating potential binding with the DENV2 NS2B-NS3 Wichapong model (Salin *et al*., 2022). Imidazole, another aromatic heterocyclic scaffold, is known for its versatile biological activities, including antiviral, anti-HIV and anticancer properties (Bhatnagar *et al*., 2011). In 2018, an imidazole complex was proposed as a potential agent against dengue fever, the efficacy of which is being investigated in further studies (Sucipto *et al*., 2018).

Previously, imidazole-phenazine derivatives have been identified for their anticancer (Labrecque *et al*., 2021), antiproliferative and tuberculostatic effects (Gobis *et al*., 2012). However, despite their potential, there are no studies on their efficacy as

DENV inhibitors. Our research addresses this gap by synthesising imidazole-phenazine derivatives, conducting biological evaluations and applying *in silico* studies targeting the DENV2 NS2B-NS3 protease. This study represents a novel investigation of the potential of this hybrid compound as an antiviral agent. The reactions involved only a one-pot reaction, which is simpler. In addition, the affordability of the starting materials increases feasibility. This would be an advantage for the scale-up process.

1.6 Scope of study

In this thesis, the synthesis of imidazole-phenazine compounds and their evaluation on biological activity in suppressing DENV2 NS2B-NS3 dengue protease are the main focus of interest. Chapter 1 provides an overview of the context and general approaches of the research. Chapter 2 provides insights into the literature sources that have informed our research concepts and framework, with a particular focus on *N*-heterocyclic compounds, imidazole and phenazine, and dengue virus type 2 as the primary area of investigation. Chapter 3 contains a thorough description of the procedures carried out in the project. In Chapter 4, we take an in-depth look at the research results, present our findings and discuss them in detail. Finally, Chapter 5 serves as the conclusion of this thesis and summarises the research results obtained in the respective projects as well as future recommendations.

CHAPTER 2

LITERATURE REVIEW

2.1 Overview

This section offers an overview of the relevant literature centering DENV2 NS2B-NS3, previous treatment methods, the general concept of protease inhibition and the use of previous *N*-heterocyclic compounds to inhibit dengue virus. It also delves into the existing research on phenazine and imidazole compounds and emphasises their potential as agents against dengue viruses. A few mechanisms of organic synthesis reactions of imidazole-phenazine hybrid compounds and their biological activity to date were also discussed. Finally, the general concept of molecular docking studies on DENV2 NS2B-NS3pro (Wichapong model) was discussed.

2.2 Dengue

Dengue is a disease known for centuries in different parts of the world, but its formal discovery and characterisation can be attributed to several individuals in different historical eras. The earliest recorded symptoms reminiscent of dengue were documented in a Chinese medical encyclopaedia in 992 AD, although the information may originally date from the Chin dynasty between 265–420 AD (Bock *et al*., 2006). This ancient reference called the illness 'water poison' and connected it to the insect that fly (Gubler, 1998). Epidemics with similar characteristics to dengue, including the course and pattern of spread, were observed as early as 1635 in the West Indies and 1699 in Central America (Gubler, 1998, Murray *et al*., 2013). A significant outbreak occurred in Philadelphia in 1780, and dengue epidemics continued to occur in the United States into the early 20th century, with the last outbreak recorded in New Orleans in 1945 (Gubler, 1997).

It was not until the end of the 19th and beginning of the 20th century that the viral cause of dengue fever and its transmission by mosquitoes were clearly identified (Dick *et al*., 2012). The identification of dengue viruses and the understanding of their role in causing the disease occurred in the mid-20th century (Dick *et al*., 2012). Albert Sabin and other researchers made significant contributions to the identification and characterisation of dengue viruses (Reich *et al*., 2013). In the 1950s, researchers identified four different serotypes of the dengue virus (DENV) which are referred as DENV1, DENV2, DENV3 and DENV4 (Henchal *et al*., 1990) (Figure 2.1). In October 2013, the fifth variant of dengue virus 5 (DENV5) was discovered. In contrast to the other four serotypes, which spread mainly *via* the human population, this serotype is associated with the sylvatic cycle. Genetic bottlenecks, natural selection, and genetic recombination are all possible contributing factors of the new serotype's emergence (Mustafa *et al*., 2015). Any serotype can cause dengue fever.

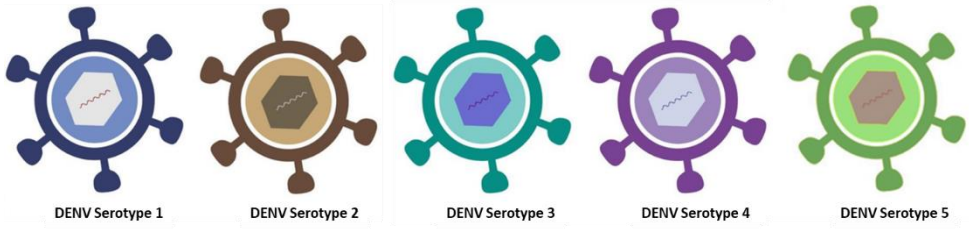


Figure 2.1 Dengue virus stereotypes: The dengue virus is roughly spherical (Zeyaullah *et al*., 2022)

Dengue is now known to be caused by five different serotypes of dengue viruses and is spread primarily by Aedes mosquitoes through a cycle involving transmission from humans to mosquitoes and back to humans (Figure 2.2). Female Aedes mosquitoes, which require blood for reproduction, transmit the virus mainly to humans (WHO, 2023). The mosquitoes become DENV vectors when they feed on an infected individual during the typical five-day viraemic period. After an extrinsic incubation

period of 8-12 days, facilitated by high temperatures, the virus migrates from the mosquito's intestinal tract into its salivary glands (Watts *et al*., 1987). If the mosquito feeds on another human after this time, DENV infection can occur. This process is aided by the mosquito's salivary protein, which prevents blood clotting and facilitates feeding (Schneider *et al*., 2004). In general, dengue virus (DENV) infections can take various forms, ranging from asymptomatic cases to mild, non-specific febrile illnesses to classic dengue fever or, in a minority of cases, severe disease. Severe disease typically results in plasma leakage and can also lead to significant haemorrhage. Both can lead to a reduction in intravascular volume, reduced organ perfusion and the risk of shock and mortality.

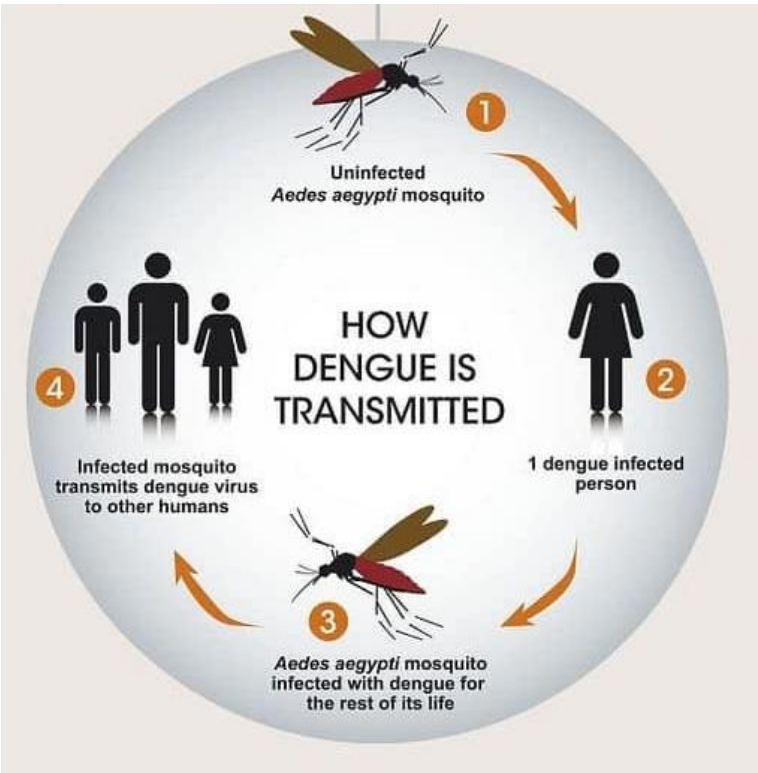


Figure 2.2 The transmission of dengue virus (Islam *et al*., 2021)

Research by Messina and colleagues in 2019 predicted minimal changes in the total global area threatened by dengue fever between 2015 and 2080. Climate change has led to rising temperatures, which exacerbate the dengue problem in endemic regions by accelerating viral amplification and increasing the survival rate of mosquitoes (Kamimurai *et al*., 2002). This has prolonged transmission times and increased the number of infected individuals, which has put a strain on public health in dengue-endemic countries. It is estimated that by 2080, an additional 2.25 billion individuals compared to 2015 will be at risk of dengue, putting nearly 6.1 billion people or 60% of the world's population at vulnerability overall (Messina *et al*., 2019). In addition, Aedes mosquitoes, especially *Aedes albopictus* and *Aedes aegypti,* serve as vectors for the transmission of DENV. They can multiply rapidly in various standing water sources such as forks in trees, tyres, flowerpots, bowls and plastic bottles or barrels. The larvae only require 7 to 10 days to complete their life cycle from egg to adult. (Getachew, 2015; CDC, 2022).

Nations situated in subtropical and tropical regions contend with DENV infections. Between 2023 and early December, over 5 million dengue cases and 5000 deaths were reported worldwide in 86 countries or territories. In Europe, autochthonous cases occurred in Italy (82), France (43) and Spain (3). Three places in France, Martinique, Guadeloupe and the Antilles entered an epidemic phase (phase 4) from 23 August 2023. At the beginning of November, Saint-Martin and Saint-Barthélemy entered epidemic phase 3, as dengue cases had been increasing since October 2023. From 7 December, dengue symptoms decreased in Guadeloupe and Martinique, while they increased in Saint-Martin and remained stable in Saint-Barthélemy. Réunion reported low activity, while French Guiana recorded stable high activity with 1948 cases

reported until 23 November 2023 (ECDC, 2023). Figure 2.3 summarises the regions that reported cases of dengue virus in 2023.

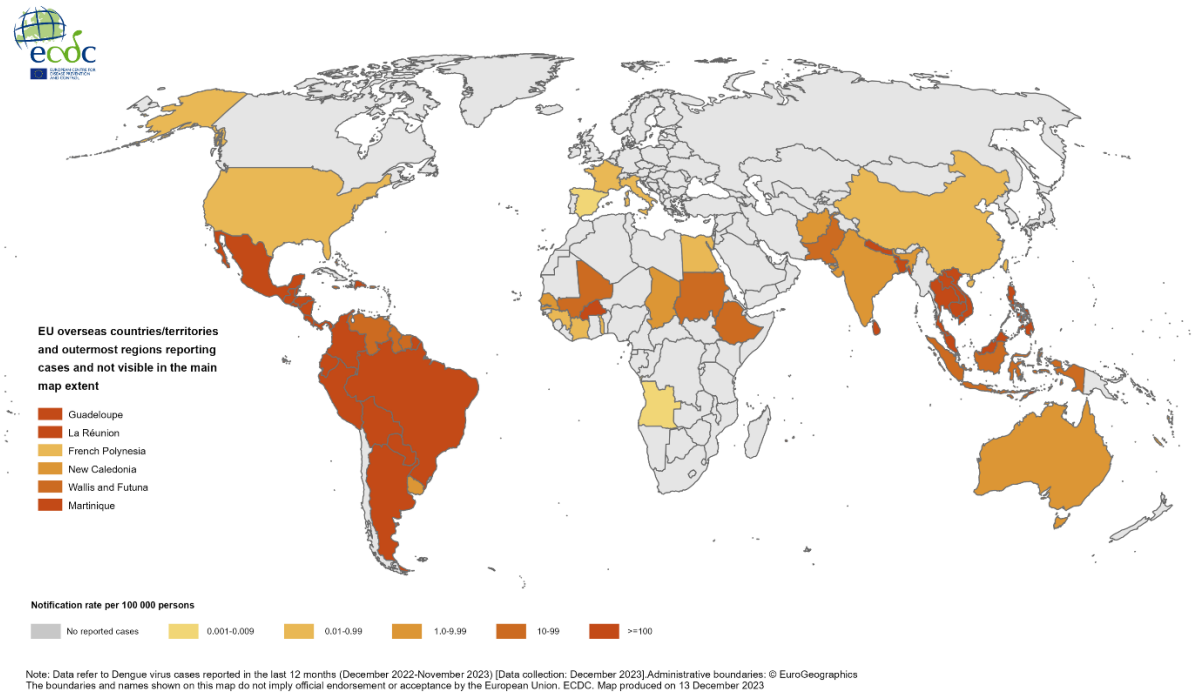


Figure 2.3 Dengue virus cases reported throughout the EU overseas countries and outermost region in end of 2022 until Nov 2023 (Data collection December 2023) (ECDC, 2023).

2.2.1 Dengue virus infection in Malaysia

By December 2023, the number of dengue cases in Malaysia increased significantly by 91.3 % compared to the previous year. The total number of cumulative cases has reached 111,417 with a sharp increase from the total 58,239 cases reported in 2022 (IFRC, 2023). In terms of dengue-related deaths, 84 deaths due to dengue-related complications were recorded, compared to 39 deaths in the same period in 2022 (MOH, 2023).

In 2023, daily dengue cases averaged over 300, indicating a significant outbreak. In the first three epidemiological weeks of 2024 alone, there was an increase of 13% with 3,971 cases reported compared to 3,525 cases in the previous week (Figure 2.4). The cumulative number of dengue cases by week 3 of 2024 reached 10,677, an increase of 51% compared to the same period in 2023 when there were 7,058 cases. Two dengue-related deaths were reported by week 3 of 2024, compared to three deaths in the same period in 2023 (MOH, 2024). There are currently 73 dengue hotspot areas nationwide, with the state of Selangor having the highest number with a total of 12619 cases up to February 2024 (CPRC, MOH).

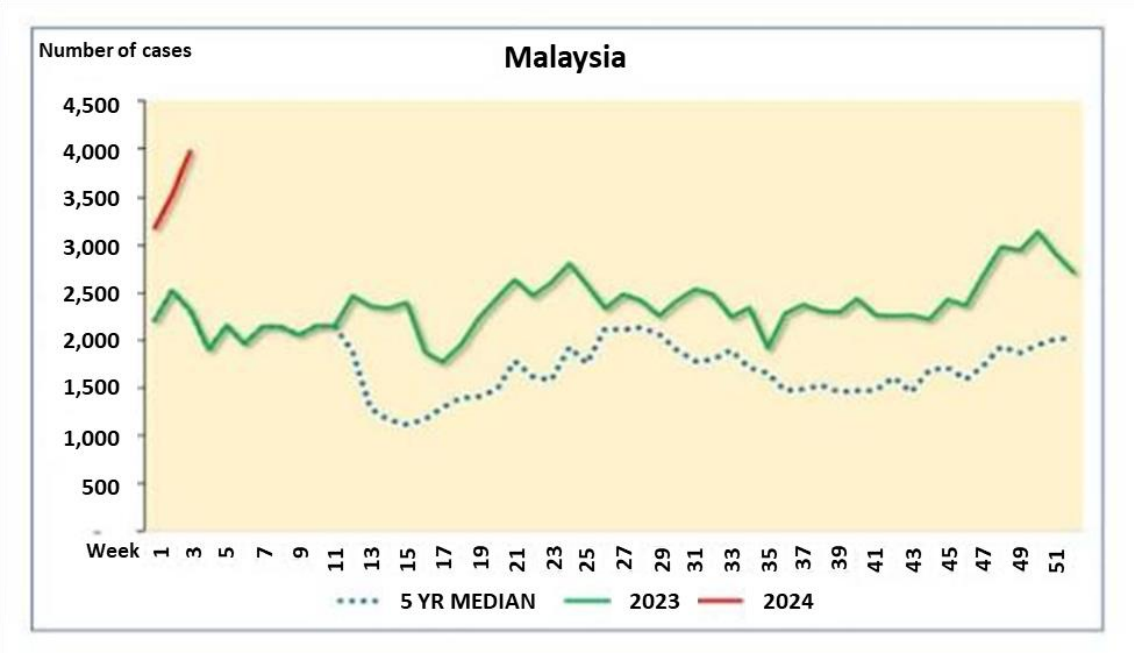


Figure 2.4 Dengue cases reported weekly from 2023, 2024 and median 2019-2023 in Malaysia (MOH, 2024).

According to the Ministry of Health (MOH), a surge in dengue fever cases is expected by the end of the year, following a trend that has been observed over the past ten years. Typically, these outbreaks occur about every four to five years and are often attributed to changes in the dominant serotypes in certain areas. The last peak was

recorded in 2019 (Salim *et al*., 2021). In addition, the Malaysian Meteorological Department (MetMalaysia) has predicted an increase in rainfall in the coming weeks given the beginning of monsoon season. These heavier rains could lead to an escalation in dengue cases from December 2023 to March 2024 as they create more breeding grounds for mosquitoes (Singh *et al*., 2022).

2.2.2 Dengue virus treatment

The critical phase of dengue fever occurs in the first 48-72 hours after infection, during which the patient's condition can suddenly deteriorate. If patients successfully survive this phase, the likelihood of a fatal outcome is significantly reduced as the immune system produces antibodies such as immunoglobulin proteins (IgG, IgM, IgA, IgE and IgD) to combat antigens (Dwek, 2009). B cells and T cells also contribute to antibody production in response to antigens. Typically, IgM antibodies appear about five days after the onset of symptoms during an infection and remain in the body for 2–3 months, occasionally longer (Kabir *et al*., 2021). Thus, recovered patients develop temporary immunity to the specific serotype that caused their infection, but remain susceptible to other serotypes of dengue virus. Hospitalisation becomes necessary if platelet levels fall and require supervision. (WHO, 2012, Uno *et al*., 2018).

The first authorised dengue vaccine to hit the market was Dengvaxia by Sanofi Pasteur in 2015. It represented a major advance in the fight against DENV infection and is now authorised in Latin American and a few Asian countries (Thomas *et al*., 2019). Clinical trials have demonstrated that the vaccine is effective against all four serotypes of dengue, with serotypes 3 and 4 showing the greatest efficiency (Thomas *et al*., 2019). These outcomes align with the Phase II experiment that was carried out in Thailand earlier (Sabchareon *et al*., 2012; Guy *et al*., 2017). However, the applicability of the

vaccine is limited to individuals without prior dengue infection and uncertainties remain regarding its safety and efficacy in the elderly and young children (WHO, 2018, Salje *et al*., 2021). In addition, shortcomings in ongoing studies have shown that the vaccine may increase the likelihood of certain recipients experiencing severe forms of DENV infection.

The search for effective drugs and vaccines against dengue therefore continues. Efforts to combat dengue virus type 2 (DENV2) have led researchers to investigate the potential of various synthetic compounds and natural products as a means of developing a cure. Both synthetic compounds (Tian *et al*., 2018) and natural products (Altamish *et al*., 2022) show promise as they can target specific aspects of the virus life cycle or immune response pathways. Researchers aim to identify compounds that can inhibit viral replication, prevent viral entry into host cells or modulate the host immune response to limit the severity of infection. This approach involves rigorous testing in preclinical models and clinical trials to assess efficacy, safety and potential side effects (Kok *et al*., 2023). Although the identification of compounds with optimal therapeutic properties remains a challenge, the search for novel synthetic compounds emphasises the urgency and commitment to finding a cure for DENV2 and alleviating the global burden of dengue fever.

In addition, the growing risk of dengue virus infection is directly linked to the increasing availability of virus sources, as humans play a central role as carriers and multipliers of the virus. To combat this, many efforts have been made to manage the environment and use pesticides for vector control with the aim of reducing human-mosquito contact. These strategies include the removal of mosquito breeding sites

through preventative fogging measures in areas where the breeding index of Aedes is particularly high to reduce mosquito populations and prevent transmission of the virus (MOH). Other individual measures include the installation of mosquito screens on windows and doors and the use of mosquito nets during sleep (Tayal *et al*., 2023; Mahmud *et al*., 2019).

2.2.3 Anti-dengue fluorogenic assay using DENV2 NS2B-NS3 protease

Proteases, referred to as biocatalysts, are essential for catalysing biological reactions in living things. In addition, they can be isolated from cells and subsequently used to facilitate numerous commercially important processes (Robinson, 2015). DENV falls into the category of enveloped viruses and is distinctive by a single-stranded RNA genome with positive polarity and a length of approximately 10.7 kilobases (Chung *et al*., 2010). The RNA genome is transcribed to produce a single polyprotein comprising three structural proteins, namely the pre-membrane (prM), the envelope (E) and the capsid (C), as well as seven non-structural proteins designated NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 (Abduraman *et al*., 2018).

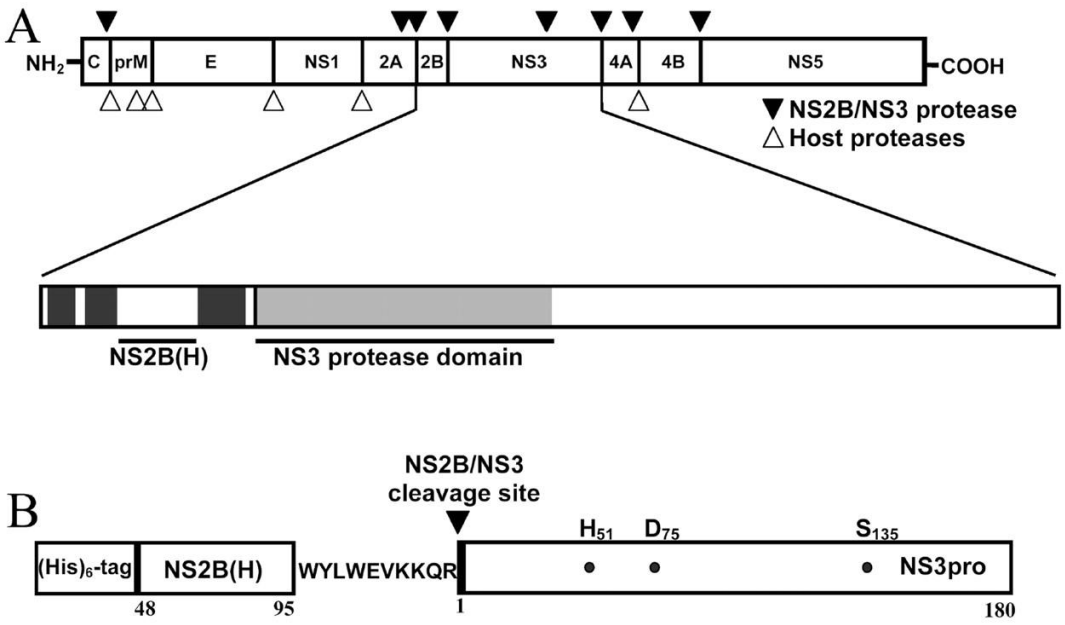


Figure 2.5 Organization of the dengue virus polyprotein and the NS2B-NS3pro construct (Niyomrattanakit *et al.*, 2004).

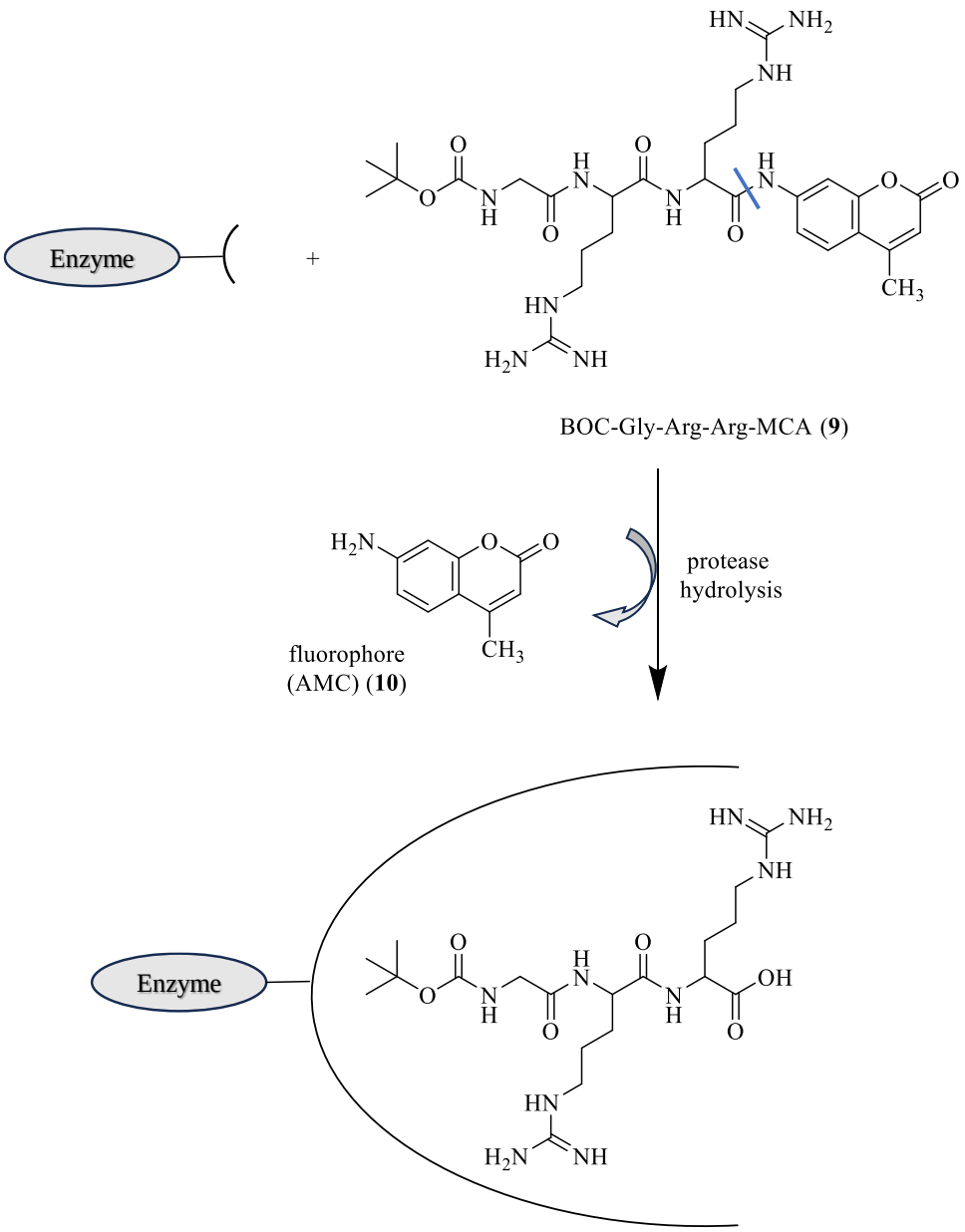
The NS2B-NS3 protease complex (Figure 2.5) in dengue is vital for viral replication and essential for cleaving the non-structural region of the four viral polyprotein specific link, namely NS2A-NS2B, NS2B-NS3, NS3-NS4A and NS4B-NS5 (Tan *et al*., 2018). The NS3 serine protease has one active site of the catalytic triad, which consists of the three amino acid residues ASP75, SER135 and HIS51 (Gibbs *et al*., 2018). However, to achieve optimal catalytic activity, NS2B serves as a cofactor for the NS3 serine protease (Chanprapaph *et al*., 2005). NS2B contributes to the function of NS3 through its hydrophobic region by assisting in membrane tethering during the cleavage process. At the same time, its hydrophilic region contributes to preserving and facilitating the activation of NS3 (Shiryaev *et al*., 2007). Consequently, this unique two-component system of NS2B-NS3 protease represents a significant target to develop most anti-dengue drugs. (Lescar *et al*., 2008). The protease has a strict selectivity for the reaction substrate to be used.

Fluorogenic substrates offer a highly convenient method as they allow continuous monitoring and can be utilized effectively at low concentration (Fields, 2001). There are three types of fluorogenic substrates used in enzymatic assays and they can be categorised into different groups based on their mechanism and properties (Knight, 1995, Ranganathan *et al*., 2018). These types include aromatic amines, contact quenched and resonance energy transfer quenched (Knight, 1995). A fluorogenic peptide substrate consists of three main components, i.e., a peptide sequence that the target protease recognises and cleaves selectively, a fluorophore bound to one end of the peptide sequence, and a quencher bound to the other end of the peptide sequence (More *et al*., 2021). In its quenched state, the fluorophore and the quencher are close to each other, which usually leads to quenching of the fluorescence. This means that the

substrate emits little to no fluorescence in this state because the energy transfer from the excited fluorophore to the quencher dampens the fluorescence signal (More *et al*., 2021).

When the fluorogenic peptide substrate is exposed to the specific protease, it theoretically recognised and cleaved at the peptide bond within the substrate sequence targeted by the protease. As the protease hydrolyses the peptide bond, it cleaves the substrate into two fragments. This process physically separates the fluorophore from the quencher and interrupts the energy transfer mechanism. As a result, the fluorophore emit fluorescence, which can be measured using a fluorescence spectrometer. By measuring the fluorescence intensity over time, the activity of the protease can be quantified. The rate of fluorescence increase is directly proportional to the activity of the protease and enables the determination of enzyme kinetics and the screening of protease inhibitors or activators (Maus *et al*., 2023).

Boc-Gly-Arg-Arg-MCA (**9**) falls under the first type of aromatic amines, as it contains the AMC fluorophore. Aminomethylcoumarin (AMC) or 7-amino-4-methylcoumarin (**10**) is an aromatic amine that changes its fluorescent properties when acylated by an amino acid. When the peptidase cleaves the amide bond into Boc-Gly-Arg-Arg-MCA, this leads to an increase in fluorescence emissions that can monitored at appropriate wavelengths (Maus *et al*., 2021, 2023). This change in fluorescence serves as a convenient and well-established method for studying the activity of proteases and proteolytic enzymes (Zimmerman *et al*., 1977). Scheme 2.1 summarises the cleavage of the amide bond in Boc-Gly-Arg-Arg-MCA by protease to generate the fluorophore AMC.

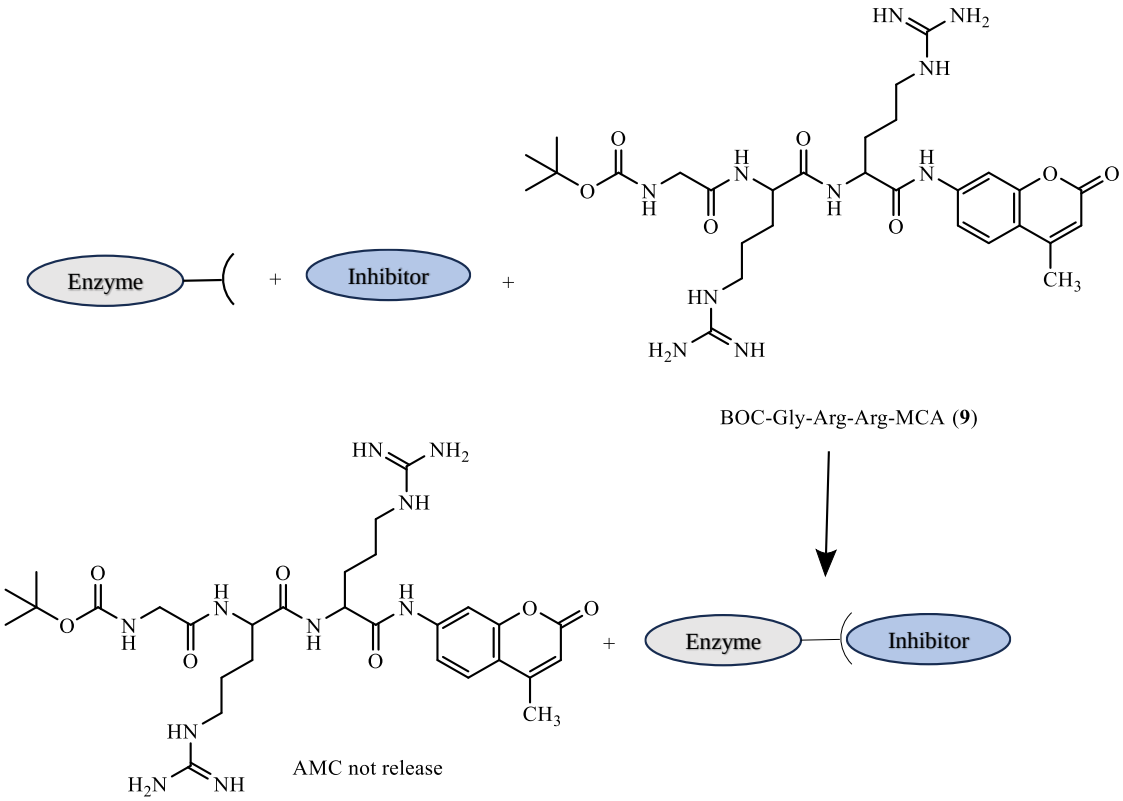


Scheme 2.1 The protease cleaves of amide bond in Boc-Gly-Arg-Arg-MCA, generating the fluorophore AMC (without the presence of inhibitors) (Poreba *et al*., 2015).

To date, many researchers have used the fluorogenic Boc-Gly-Arg-Arg-MCA to determine the enzyme activities of the DENV2 NS2B-NS3 protease (Yusof *et al*.,2000; Rothan *et al*., 2012; Sivasothy *et al*., 2021). The method has also been utilised to study the NS2B-NS3 protease inhibitory activities, in the presence of an inhibitor. The lock-and-key mechanism is a concept in enzymology that describes the specificity

of enzyme-substrate interactions (Damborsky *et al*., 2009). The role of the key in opening a lock is analogous to how two connected parts must align perfectly to function effectively. The fluorogenic substrate must fit exactly into the active centre of the DENV2 NS2B-NS3 enzyme for a reaction to release the AMC fluorophore to occur.

In the context of inhibition, if an inhibitor molecule binds to the enzyme's active site, it can block further binding of the substrate. Thus, in the presence of an inhibitor targeting the NS2B-NS3 protease, no AMC fluorophore is released because it has been interfered, preventing the fluorescent substrate from being bound and cleaved. In this way, the enzymatic reaction is inhibited. The percentage of inhibition can later be calculated by measuring the fluorescence signal of the enzyme activities with and without inhibitors (Carmona *et al*., 2009). Scheme 2.2 illustrates the mechanism of the protease assay in the presence of the inhibitor.



Scheme 2.2 Illustration of the protease assay mechanism in the presence of the inhibitor (Poreba *et al*., 2015).

2.3 *N*-heterocyclic compounds with anti-dengue properties

N-heterocyclic compound is an organic compound that has a heterocyclic ring structure with nitrogen (N) atoms as integral components (Ahmad *et al*., 2024). Commonly found in natural products, pharmaceuticals and materials, these compounds are of great importance due to their versatile properties and applications in multiples fields (Heravi & Zadsirjan, 2020). Since nitrogen serves as a common heteroatom, *N*-heterocyclic compounds exhibit various chemical and physical properties determined by their specific structural configurations and attached functional groups. Examples of *N*-heterocyclic compounds are pyridine, pyrrole, imidazole, quinoline and purine. They play a crucial part in biological processes for example, interaction with DNA and RNA structure (Özkay *et al*., 2010). In addition, these compounds are important for drug development as they target specific biological signalling pathways for therapeutic intervention (Barmade & Ghuge, 2018).

These *N*-heterocyclic structures have proven to be a good scaffold for the design and development of antiviral agents to combat dengue virus (DENV) (De *et al*., 2022). Recent developments in medicinal chemistry have already discovered several promising heterocyclic scaffolds: quinoline (**11**), piperidone (**12**), thioguanine (**13**) and quinoxaline (**14**) as inhibitors of DENV2 NS2B-NS3 protease (Figure 2.5) (Hariono *et al*., 2019).

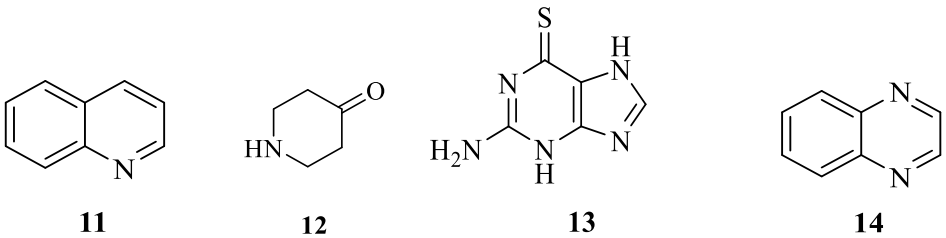


Figure 2.6 The scaffolds of previously discovered *N*-heterocyclic compounds as DENV2 NS2B-NS3 protease inhibitors.