SYNTHESIS, CHARACTERIZATION, CONFORMATIONAL STUDY AND ANTIBACTERIAL ACTIVITY OF *N*-ACYLHYDRAZONES

By

Khong Pek Yao

A Final Year Project submitted to the Department of Chemical Science,

Faculty of Science,

Universiti Tunku Abdul Rahman,

In partial fulfilment of requirement for the degree of Bachelor of Science (Honours) Chemistry

May 2023

ABSTRACT

SYNTHESIS, CHARACTERIZATION, CONFORMATIONAL STUDY AND ANTIBACTERIAL ACTIVITY OF *N*-ACYLHYDRAZONES

KHONG PEK YAO

Researchers have put so much effort in *N*-acylhydrazones recently due to their biological activities such as antibacterial, antitumor, anticancer, antioxidant and anti-inflammatory. A series of new *N*-acylhydrazone derivatives **SB 1- SB 10** were synthesized in yields ranging from 52 to 78% through the reactions of carboxylic acid hydrazide with a variety of substituted benzaldehydes. The structures of the synthesized compounds were confirmed by IR, ^{1}H- and ^{13}C-NMR, DEPT, NOE, HMQC and HMBC spectral data. From the relative configuration of imine double bond and the conformation of CONH bond of synthesized compounds, the C=N double bonds of *N*-acylhydrazones showed (*E*)-configuration, while the NMR data of all synthesized *N*-acylhydrazones indicated the existence of *cis*-/*trans*- conformers for each compound in DMSO-d_6. In *N*-acylhydrazones **SB 1 – SB 5**, the signals of the *cis*- and *trans*-amide conformers coalesced as the temperature increased between 298 and 373 K. The synthesized compounds were evaluated for their antibacterial activities against three microbial strains which were two Gram-positive bacteria (*Bacillus subtilis* and *Staphylococcus aureus*) and one Gram-negative

bacteria (*Pseudomonas aeruginosa*). The result suggested that **SB 1 - SB 9** exhibited poor antibacterial

activities against all tested bacteria strain. Particularly, compound **SB 10** (2-OH, $4NEt_2$) showed moderate antibacterial activity against Gram-negative bacteria *Pseudomonas aeruginosa* at MIC value of 62.5 µg/mL.

ABSTRAK

SINTESIS, KARAKTERISASI, KAJIAN KONFORMASI DAN AKTIVITI ANTIBAKTERIA *N*-ACYLHYDRAZONE

KHONG PEK YAO

Penyelidik telah melakukan banyak usaha dalam *N*-acylhydrazones baru-baru ini kerana aktiviti biologi mereka seperti antibakteria, antitumor, antikanser, antioksidan dan anti-radang. Satu siri terbitan *N*-acylhydrazone baharu SB 1-SB 10 telah disintesis dalam hasil antara 52 hingga 78% melalui tindak balas asid karboksilik hidrazida dengan pelbagai benzaldehid tersubstitusi. Struktur sebatian yang disintesis telah disahkan oleh data spektrum IR, ^{1}H- dan ^{13}C-NMR, DEPT, NOE, HMQC dan HMBC. Daripada konfigurasi relatif ikatan berganda imina dan pengukuhan ikatan CONH bagi sebatian tersintesis, ikatan berganda C=N bagi *N*-acylhydrazones menunjukkan konfigurasi (E), manakala data NMR bagi semua *N*-acylhydrazones tersintesis menunjukkan kewujudan *cis*-/*trans*- conformers untuk setiap sebatian dalam DMSO-d_6. Dalam *N*-acylhydrazones **SB 1 – SB 5**, isyarat konformer *cis*- dan *trans*-amide bergabung apabila suhu meningkat antara 298 dan 373 K. Sebatian yang disintesis dinilai untuk aktiviti antibakteria terhadap tiga strain mikrob iaitu dua bakteria Gram-positif (*Bacillus subtilis* dan *Staphylococcus aureus*) dan satu bakteria Gram-negatif (*Pseudomonas aeruginosa*). Hasilnya mencadangkan bahawa **SB 1 - SB 9** mempamerkan aktiviti antibakteria yang

lemah terhadap semua strain bakteria yang diuji. Khususnya, kompaun **SB 10** (2-OH, $4NEt_2$) menunjukkan aktiviti antibakteria sederhana terhadap bakteria Gram-negatif *Pseudomonas aeruginosa* pada nilai MIC 62.5 µg/mL.

ACKNOWLEDGEMENT

I would like to appreciate my supervisor Dr. Sim Kooi Mow, co-supervisor Dr. Teo Kah Cheng for guiding me through my Final Year Project. I have gained a lot of experience and knowledge of Chemistry and Microbiology from both of them. Besides that, I would also like to appreciate all the lab staffs for providing helps for me during my Final Year Project. This project can be completed successfully due to their willingness and kindness to impart their knowledge and helps for me.

Moreover, I would like to appreciate my family members who support me all the time. My family members gave me a lot of spiritual support among these three years.

Lastly, I would like to appreciate my lab mate Tan Yi Ying who providing a lot of help for me throughout my Final Year Project.

DECLARATION

I hereby declare that the project report is based on my original work except for quotations, and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UTAR or other institutions.

Name: Khong Pek Yao

Date: 25/4/2023

APPROVAL SHEET

This project report entitled "**SYNTHESIS, CHARACTERIZATION, CONFORMATIONAL STUDY AND ANTIBACTERIAL ACTIVITY OF *N*-ACYLHYDRAZONES**" was prepared by KHONG PEK YAO and submitted as partial fulfillment of the requirements for the degree of Bachelor of Science (Honours) Chemistry at Universiti Tunku Abdul Rahman.

Approved by,

Supervisor

KM SIM

_____ Date : 25/4/2023

(DR SIM KOOI MOW)

Associate Professor

Department of Chemical Science

Faculty of Science

Universiti Tunku Abdul Rahman

FACULTY OF SCIENCE

UNIVERSITI TUNKU ABDUL RAHMAN

Date: 25/4/2023

PERMISSION SHEET

It is hereby certified that **KHONG PEK YAO** (ID No: 20ADB01392) has completed this final year project entitled "**SYNTHESIS, CHARACTERIZATION, CONFORMATIONAL STUDY AND ANTIBACTERIAL ACTIVITY OF *N*-ACYLHYDRAZONES**" under the supervision of DR. SIM KOOI MOW from the Department of Chemical Science, Faculty of Science.

I hereby give permission to the University to upload the softcopy of my final year project in pdf format into the UTAR Institutional Repository, which may be made accessible to the UTAR community and public.

Yours truly,

(KHONG PEK YAO)

TABLE OF CONTENTS

CHAPTER

LIST OF TABLES

LIST OF FIGURES

LIST OF SCHEMES

LIST OF SYMBOLS/ABBREVIATIONS

δ	Chemical shift
δ_C	Chemical shift of carbon
δ_H	Chemical shift of proton
mmol	Millimole
g mol^{-1}	Molecular weight
µg mL^{-1}	Microgram per millimetre
mM	Millimolar
kJ mol^{-1}	Kilojoule per mole
T_c	Coalescence temperature
Δv	Peak separation
k_c	Rate constant of *cis/trans* equilibrium
$\Delta G^{\neq}$	Energy of rotational barrier
Hz	Hertz
nm	Wavelength
J	Coupling constant
s	Singlet
d	Doublet
dd	Doublet of doublets
t	Triplet
m	Multiplet
c	*cis*
t	*trans*

LIST OF SYMBOLS/ABBREVIATIONS

SB	Schiff base
NAH	*N*-acylhydrazone
R_f	Retention factor
1D-NMR	One dimensional Nuclear Magnetic Resonance
2D-NMR	Two dimensional Nuclear Magnetic Resonance
DEPT	Distortionless Enhancement by Polarization Transfer
HMQC	Heteronuclear Multiple Quantum Correlation
HMBC	Heteronuclear Multiple Bond Correlation
NOE	Nuclear Overhauser Effect
FT-IR	Fourier Transform Infrared Spectroscopy
TLC	Thin Layer Chromatography
MIC	Minimum Inhibitory Concentration
MH	Mueller-Hinton

CHAPTER 1

INTRODUCTION

1.1 Indole

Indole, an organic heterocyclic molecule has a benzene ring attached to a pyrrole ring. It has the molecular formula C_8H_7N and a molecular weight of 117.15 g/mol. Indole is a colourless to pale yellow crystalline solid that has a distinct odor, which is often described as a pungent floral scent. Indole is widely distributed in nature and is found in a variety of plant and animal sources, including coal tar, animal feces and certain vegetables such as broccoli, cauliflower and cabbage. It is also a component of tryptophan, an essential amino acid that is found in many proteins. It also has biological activity which shown to have a variety of physiological effects in humans, including acting as a neurotransmitter and modulating immune responses.

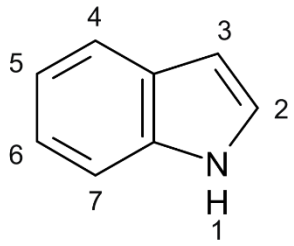


Figure 1.1: Chemical Structure of indole

1.1.1 Application of indole

Indoles are widely studied in the field of biological chemistry, as they offer numerous biological properties such as antitumor, antioxidant, antibacterial, anticancer and anti-inflammatory. They are used as active ingredients in the development of drugs for the treatment of various diseases, including cancer, depression and infectious diseases. Indole is an important building block in organic synthesis, and it is used as a precursor for the synthesis of a wide range of compounds, including pharmaceuticals, agrochemicals and dyes.

1.1.1.1 Antibacterial activity

With an indole-core structure, CZ74 exhibits strong antibacterial effect against several tested Gram-positive bacteria, including methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus* (Yuan et al., 2019).

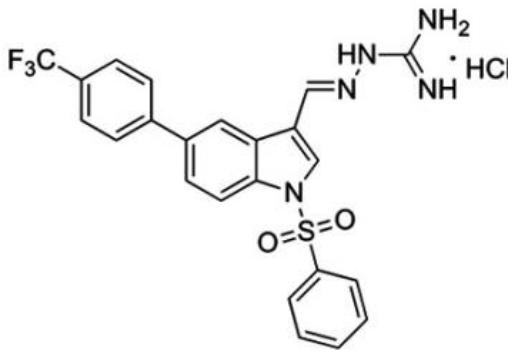


Figure 1.2: Chemical structure of CZ74

1.1.1.2 Anti-inflammatory and analgesic activities

According to Özdemir et al. (2015), they have successfully synthesized the following indole complexes, **Complex (21)** and **Complex (22)** which were found to show great anti-inflammatory activity.

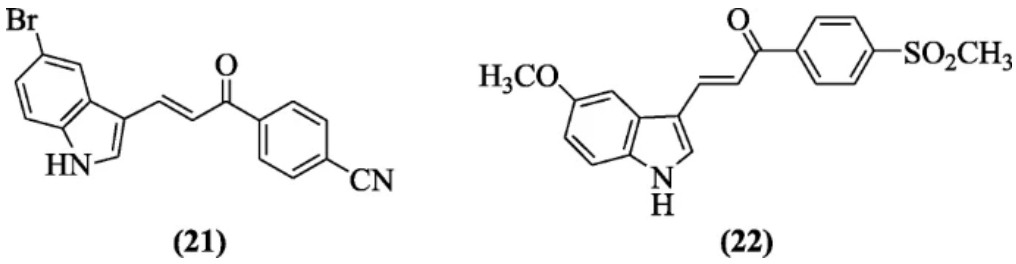


Figure 1.3: Chemical structure of 3-(5-Bromo-1H-indol-3-yl)-1-(4-cyanophenyl)prop-2-en-1-one (21) and compound 3-(5-methoxy-1H-indol-3-yl)-1-(4-(methylsulfonyl)phenyl)prop-2-en-1-one (22)

1.1.1.3 Anticancer activity

(*E*)-1-((1-(4-chlorobenzyl)-1*H*-indol-3-yl) methylene)-4-(6-((dimethylamino) methyl) benzo[*d*]thiazol-2-yl) semicarbazide has shown excellent antitumor activity against breast cancer cells MDA-MB-231, lung cells (H460), and colon cells (HT29) in MTT assay with positive controls as PAC-1 and oncrasin-1 (Ma et al., 2015).

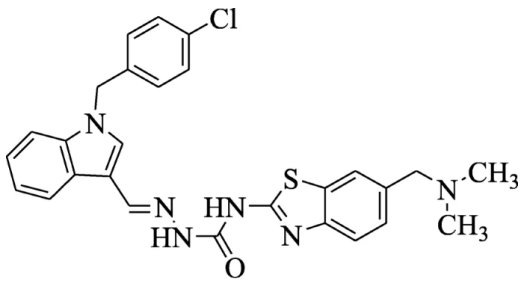


Figure 1.4: (E)-1-((1-(4-chlorobenzyl)-1*H*-indol-3-yl) methylene)-4-(6-((dimethylamino) methyl) benzo[d]thiazol-2-yl) semicarbazide

1.2 Hydrazide

Hydrazides are a group of organic compounds that consist of the hydrazide functional group (-$CONHNH_2$). In this functional group, a carbonyl group (C=O) not only connected to a nitrogen atom, but also connected to a hydrogen atom and an amino group (-NH_2). A carboxylic acid can be used to produce hydrazides by reacting with hydrazine or a hydrazine derivative, such as hydrazine hydrate or phenylhydrazine. During this reaction, the carbonyl group of the carboxylic acid is converted into the hydrazide functional group.

1.2.1 Application of Hydrazide

Previous studies have shown that hydrazides have great biological, pharmaceutical and industrial applications, including those for antibacterial

agents, medicines, herbicides, antimalarial, antimycobacterial, anticonvulsant, anti-inflammatory, antidepressant, anticancer and dyes (Mali et al., 2021).

1.2.1.1 Antibacterial activity

Nineteen new compounds of hydrazide-hydrazones of 5-nitrofuran-2-carboxylic acid were synthesized. The research showed that the substitution of alkyl chains showed greater antibacterial activity against Gram-negative bacteria than the hydrazones with aryl substituents (Popiołek et al., 2019).

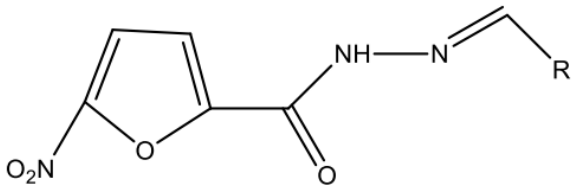
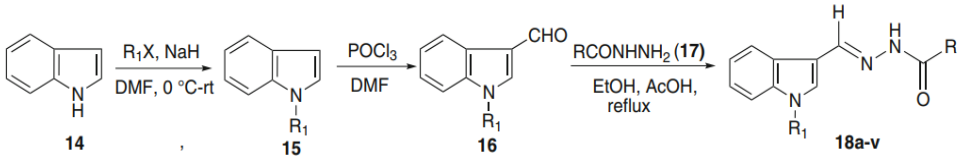


Figure 1.5: 5-nitrofuran-2-carboxylic acid hydrazide-hydrazones derivatives

1.2.1.2 Anticancer activity

The *in vitro* antiproliferative effects of a range of *N*'-((1-(substituted)-1*H*-indol-3-yl)methylene)hydrazides against various cancer cell lines were investigated. Twenty-two substances were synthesized, and several of the analogues showed activity against breast and prostate cancer cells (Sundaree et al., 2016).



Scheme 1.1: Synthesis of a series of indolyl hydrazide-hydrazones 18a-v

1.3 Schiff base

German chemist Hugo Schiff reported the final products of the interaction between primary amines and carbonyl compounds for the first time in 1864, and his name is where the term "Schiff base" originates. According to the IUPAC's suggestions, Schiff bases are classified as chemical substances (imines) with a hydrocarbonyl group attached to the nitrogen atom, $R_2C = NR^3$ ($R^3 \neq H$) . They are often seen as being equivalent to azomethines (Raczuk et al., 2022).

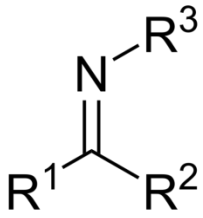
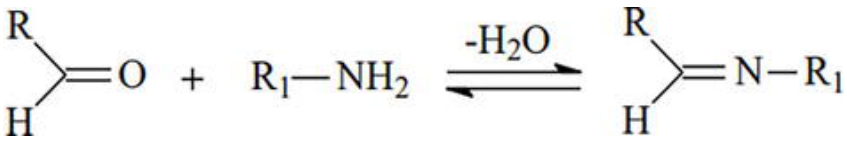


Figure 1.6: Chemical structure of Schiff base

The reaction that forms a Schiff base is reversible. One mole of water is produced as a result of the reaction, and the water in the surroundings leads the reaction to move to the left. Therefore, the reaction is typically conducted in solvents where water may be distilled out of the surroundings and create an

azeotrope. Hydrolysis will not take place if the reaction is carried out using amines that have an electronegative atom with unpaired electrons in the nitrogen atom, once the process is completed, and Schiff bases can be isolated with great efficiency (Tuna Subasi, 2022).



Scheme 1.2: Formation of Schiff base

1.3.1 Application of Schiff base

Schiff bases (SBs) are generally utilized in many fields of science, including analytical, inorganic and organic chemistry. They are also recommended for use in solar energy applications. They are utilized as catalysts, dyes and polymer stabilisers. The broad range of pharmacological and biological functions, such as antimalarial, analgesic, anti-inflammatory, antiviral, antibacterial, and antifungal actions, additionally highlight the need for SB synthesis (Tuna Subasi, 2022).

1.3.1.1 Antibacterial activity

The antibacterial efficacy of SBs generated from 5-aminopyrazoles, specifically 5-(benzylideneamino)-3-(4-methoxyphenylamino)-*N*-phenyl-1*H*-pyrazole-4-carboxamides, was demonstrated against multidrug resistant bacteria (MDRB). Citrofloxacin was less effective than compounds 1-3 against Gram-positive bacteria *Staphylococcus epidermidis*. Additionally, compounds 2 and 3 demonstrated the same MIC values as ciprofloxacin and were also effective against Gram-negative *Acinetobacter baumanni* (Ceramella et al., 2022).

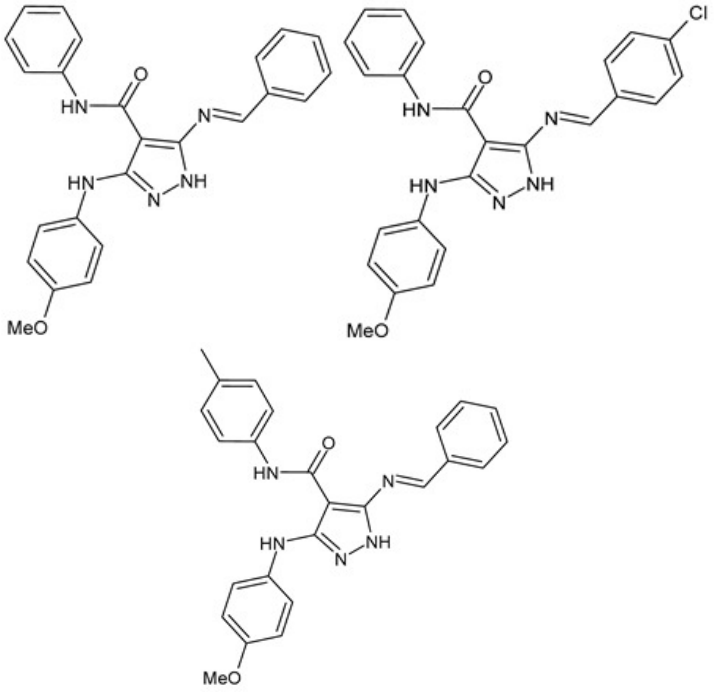


Figure 1.7: Chemical structure of Compounds 1-3

1.3.1.2 Anticancer activity

Schiff base ligand L [1(pyridine-2-ylimino methyl) naphthalene-2-ol] was synthesized and performed good anticancer activity against breast and lung

cancer cell lines MCF-7 and H-460 (Sadia et al., 2021). According to research on the growth inhibition carried out by Schiff bases, the amine group in the molecules of these substances is the one that causes the inhibitory effect on cell lines (Yıldırım et al., 2014).

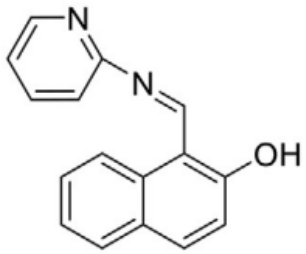


Figure 1.8: Chemical structure of Schiff base ligand L

1.4 Hydrazone

Hydrazones are produced when hydrazine (N_2H_4) or its derivatives combine with a carbonyl component, such as an aldehyde or a ketone. Hydrazones are the final products from the condensation reaction between a carbonyl molecule and hydrazine or its derivatives. The existence of a $C=N-NH_2$ functional group, in which the nitrogen atoms are joined by a double bond, is what distinguishes hydrazones from other organic compounds. Because of their unique chemical and physical characteristics, hydrazones can be used for a broad range of applications, including as ligands in coordination chemistry, intermediates in organic synthesis, and reagents in analytical chemistry.

It is commonly known that *N*-acylhydrazones have four possible different configurations of (*E*/*Z*)-configurational isomers which they may exist in relative to the C=N double bonds and (*E′*/*Z′*)-rotamers coming from the inversion of amide bonds. However, due to the steric hindrance in relation to the *N*-acylhydrazones moiety, these *N*-acylhydrazones exist mostly or c completely in the (*E*)-configuration, and the *E*/*Z* isomerization was not seen (Gu et al., 2012).

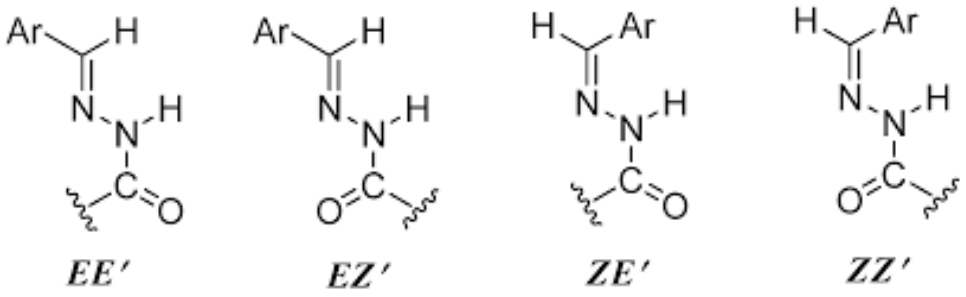


Figure 1.9: Four possible forms of *N*-acylhydrazone derivatives

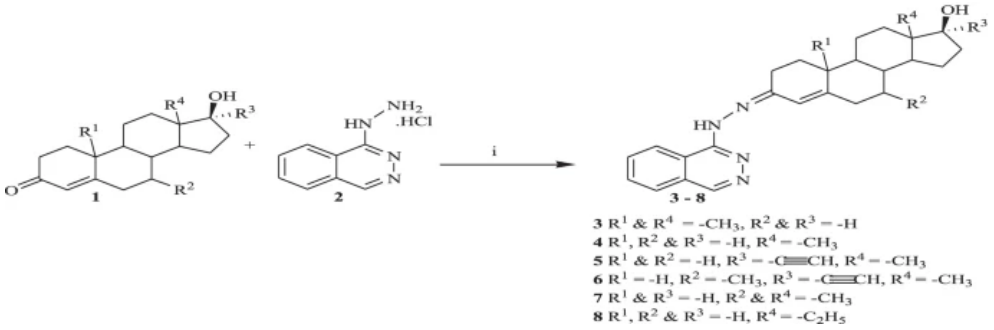
1.4.1 Application of Hydrazone

The chemical properties of hydrazones make them useful in drug development. For example, the presence of the hydrazone functional group can act as a pharmacophore, which is a specific structural feature responsible for a drug's biological activity. Hydrazones have been used in the development of drugs for a variety of medical diseases. For example, hydrazone derivatives have been developed as antimalarial drugs by targeting the heme detoxification pathway of the malaria parasite. Additionally, hydrazones have been studied

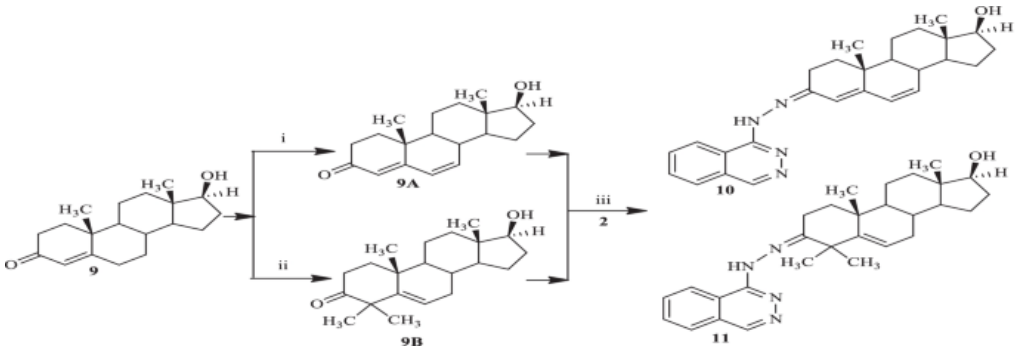
for their potential activity as antitumor agents, as they have been found to induce apoptosis in cancer cells.

1.4.1.1 Antimicrobial activity

The broth microdilution method was utilized to study the antibacterial effect of steroidal hydrazone. Among the substances studied, compounds 6, 7 and 10) had the best antibacterial action since they each had an ethinyl at position 17 and a methyl at position 7 respectively. Due to the compounds' extra -C=C- in the structure, compound 10 exhibited remarkable antibacterial activity. As a result, the development of a new antibacterial agent was suggested using these substituted steroidal skeletons (Mistry and Singh, 2022).



Scheme 1.3: Synthesis of a series of steroidal hydrazone (compound 6,7)



Scheme 1.4: Synthesis of a series of steroidal hydrazone (compound 10)

1.4.1.2 Anticancer activity

6-bromo/6-chloro-2-methyl-quinolin-4-yl-hydrazines as the precursor, a series of quinoline hydrazone analogues based on the physiologically active heterocycle quinoline were synthesized. When the powerful chemical 18j was contrasted with the therapeutically effective anticancer drugs bendamustine and chlorambucil, it showed that quinolyl hydrazones have potential as anticancer agents (Katariya, Shah and Reddy, 2020). The **X** is Cl or Br, $\mathbf{R^1}$ is -OC_6H_{13}, $\mathbf{R^2}$ = -H, $\mathbf{R^3}$ = -H.

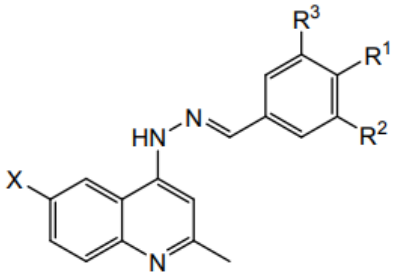
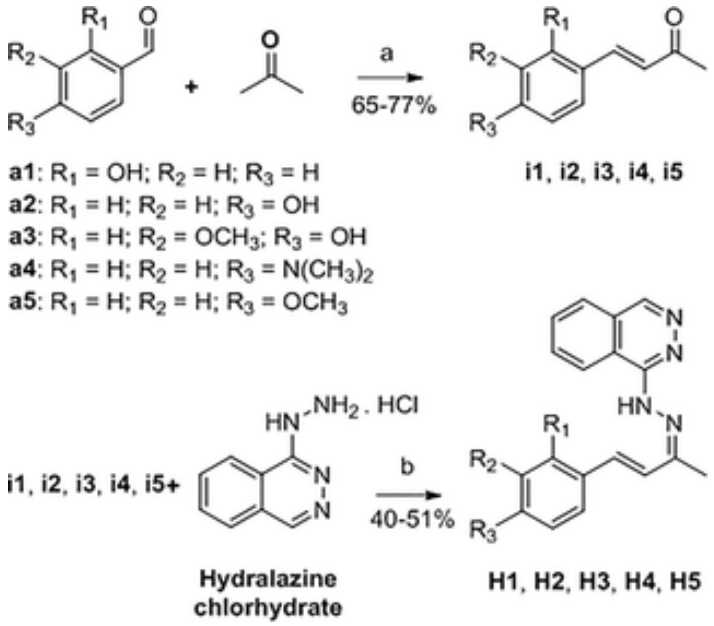


Figure 1.10: Structure of Compound 18j

1.4.1.3 Anti-inflammatory activity

With the use of molecular modification techniques, five hydrazone derivatives (H1, H2, H3, H4, and H5) with the general structure $R_1R_2C = NNR_3R_4$ were created. H5 has the ability to inhibit the COX-2 enzyme, which reduces the metabolism of arachidonic acid and the formation of prostaglandins, an essential inflammatory mediator. In conclusion, H5 is a candidate for a new multi-target oral anti-inflammatory drug because it showed relevant antinociceptive and anti-inflammatory potential and had many sites of action (Medeiros et al., 2021).



Scheme 1.5: Synthetic route for hydrazone derivatives H1-H5.

1.5 Antibacterial activity

The term "antibacterial activity" describes a substance's ability to inhibit or kill bacteria. This is crucial for a number of reasons, including the development of medicines to treat bacterial diseases. Several techniques, including as agar diffusion assays and broth dilution assays, can be used to determine the antibacterial activity. These assays evaluate a drug's ability to suppress or kill a panel of bacterial strains by exposing it to the substance. The chemical composition, concentration, and the type of bacteria being tested are only a few of the variables that can influence a substance's antibacterial efficacy. It is crucial to identify the underlying causes of this problem and seek solutions in order to both reduce it and enhance the efficiency of infection therapies due to the increased prevalence of unsuccessful infection treatments and bacterial resistance to antibiotics (Kowalska-Krochmal and Dudek-Wicher, 2021).

1.5.1 Determination of minimum inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) is the lowest concentration of a particular antibacterial agent that can inhibit the visible growth of bacteria. This information is important for determining the appropriate dosage of an antibacterial agent to use in treating a particular infection. It is commonly used in microbiology to determine the effectiveness of an antibacterial agent against a specific pathogen. The MIC value can vary depending on the bacteria being

tested, the type of antibacterial agent used, and the environment. MIC testing is typically performed by exposing the bacteria to different concentrations of the antibacterial agent in a liquid or solid medium and observing whether growth occurs (Kowalska-Krochmal and Dudek-Wicher, 2021).

1.6 Objective of the research

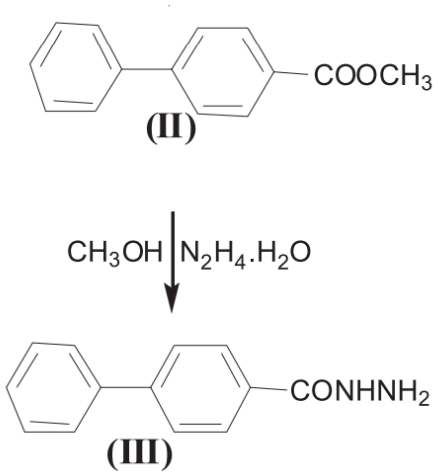
1. To synthesize a carboxylic acid hydrazide and a series of *N*-acylhydrazones derivatives.
2. To characterize the structure of carboxylic hydrazide and *N*-acylhydrazones by using different spectroscopic techniques such as FT-IR, NOE experiments, 1D- and 2D- NMR (^{1}H NMR, ^{13}C NMR, DEPT, HMQC and HMBC).
3. To evaluate the antibacterial activity of the synthesized *N*-acylhydrazones by using broth microdilution method.

CHAPTER 2

LITERATURE REVIEW

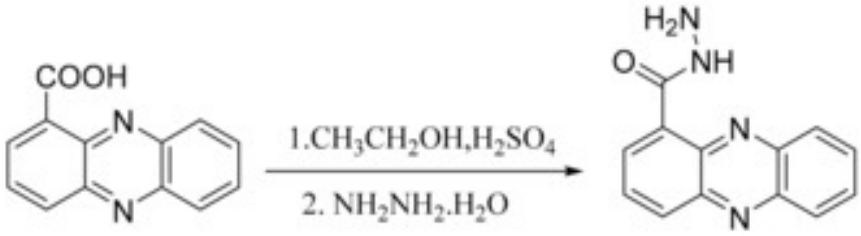
2.1 Synthesis of carboxylic acid hydrazide

According to Shamroukh (2013), biphenyl-4-carboxylic acid hydrazide can be produced by reacting the 0.2 M biphenyl-4-carboxylic acid methyl ester with excess amount of hydrazine hydrate (0.30 M) in 250 mL of ethanol, then the reagent mixture was subjected to reflux for 3 hours and cooled. Then, the crude product was separated by filtration and crystallized by using ethanol. The percentage yield was 86.79%.



Scheme 2.1: Synthesis of biphenyl-4-carboxylic acid hydrazide (Shamroukh et al., 2013)

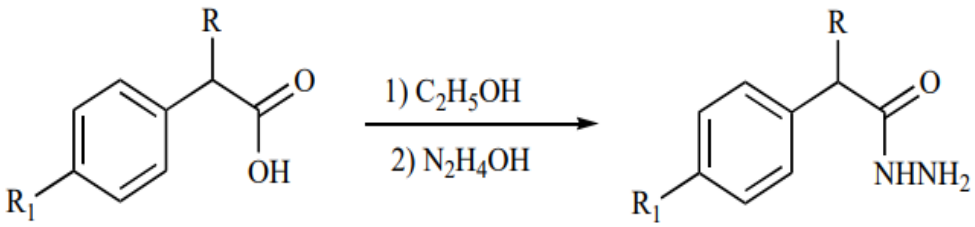
According to Wu S. et al. (2021), 100 mL of anhydrous ethanol were gently mixed with 1 mL of concentrated sulfuric acid in an ice bath, cooled down to room temperature and then 10 mmol of phenazine-1-carboxylic acid (PCA) was added. The reagent mixture was refluxed for 21 hours. The mixture was subsequently cooled to room temperature. A 50 mL portion of the concentrated solution was heated before being added to 200 mL of cold water. Saturated potash was used to adjust the pH to pH = 10, after which dichloromethane extraction (60 mL x 4) was carried out. A black solution resulted from collecting and combining the organic phase, drying it with anhydrous sodium sulphate, filtering it, and then rotary evaporating it to remove the dichloromethane. The dark solution was converted into phenazine-1-hydrazine by adding 40 mL anhydrous ethanol and 5 mL hydrazine hydrate, and the reagent mixture was subjected refluxed for eight hours. After cooled down, the crude products were separated out and filtered and washed with anhydrous ethanol for three times.



Scheme 2.2: Synthesis of Phenazine-1-hydrazine (Wu et al., 2021)

According to Narang, Narasimhan and Sharma (2012), the 2-(4-isobutylphenyl) propanoic acid react with 4-methylthiophenyl acetic acid, this

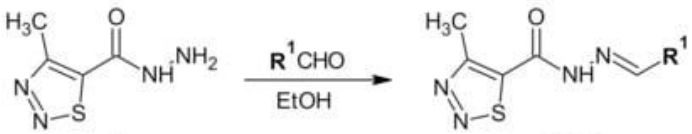
process is called esterification. Then, the crude ester reacts with hydrazine hydrate in absolute alcohol, the final product is acid hydrazides.



Scheme 2.3: Synthesis of acid hydrazides (Narang, Narasimhan and Sharma, 2012)

2.2 Synthesis of *N*-acylhydrazones

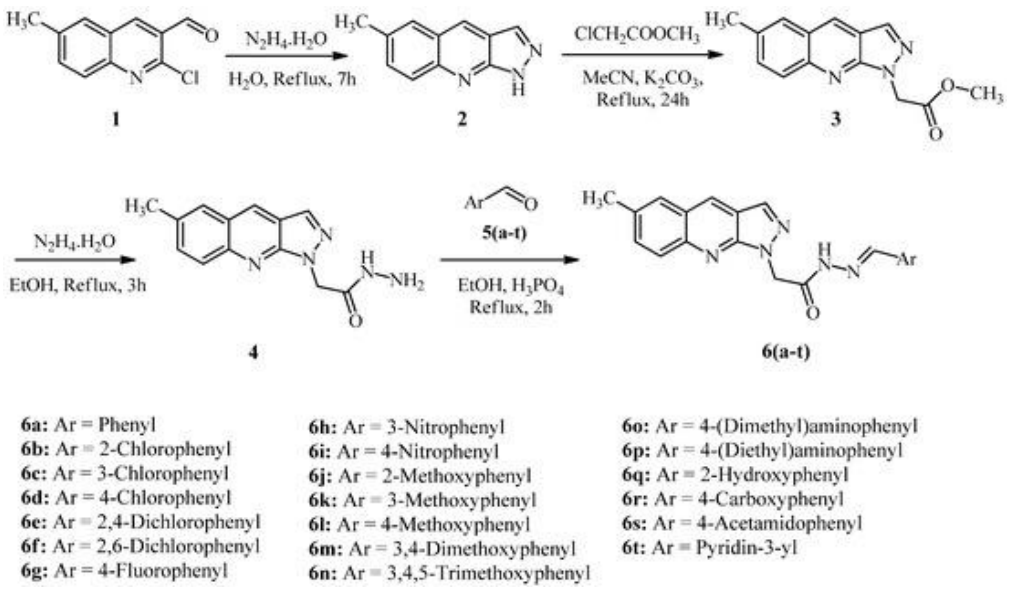
A series of new hydrazones derivatives (2-16) of 4-methyl-1,2,3-thiadiazole-5-carboxylic acid hydrazide were synthesized by reacting 0.01 mole of hydrazide and 0.01 mol of different substituted aldehyde dissolved in 15 mL of ethanol (96%) in a round-bottomed flask The reagent mixture was subjected to reflux for three hours. Then, it was allowed to cool down to room temperature and placed in a refrigerator for twenty-four hours. The precipitate was filtered off and recrystallized from ethanol. Most of the compound synthesized (2-16) were obtained in a good yield (57%-98%) (Paruch et al., 2021).



Compound No.	R¹	Compound No	R¹
2	2-ClC_6H_4	10	3-I-4-OH-5-$OCH_3C_6H_2$
3	3-ClC_6H_4	11	2-Cl-6-$NO_2C_6H_3$
4	4-ClC_6H_4	12	2,3-di$OCH_3C_6H_3$
5	2-FC_6H_4	13	2,4-di$OCH_3C_6H_3$
6	3-FC_6H_4	14	3,4-di$OCH_3C_6H_3$
7	4-FC_6H_4	15	5-nitrofuran-2-yl
8	3-OC_2H_5-4-OHC_6H_3	16	1*H*-pyrrol-2-yl
9	2-Br-6-OHC_6H_3		

Scheme 2.4: Synthesis scheme of a series of new hydrazone derivatives of 4-methyl-1,2,3-thiadiazole-5-carboxylic acid hydrazide

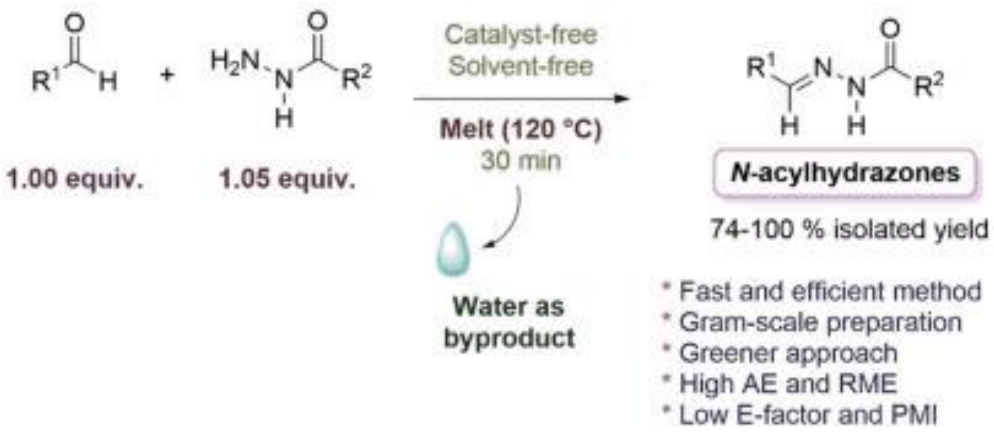
According to Munir et al. (2021), after cyclocondensation with hydrazine monohydrate, complex (2) was produced by the substitution of the quinoline ring with the pyrazole scaffold using complex (1) as a precursor. Under SN_2 conditions, complex (2) was reacted with methyl chloroacetate to produce complex (3). The synthetic ester (3) was then undergo hydrazinolysis to produce complex (4), by condensation in acid with a number of aromatic aldehydes 5(a-t) to produce *N*-acylhydrazones 6(a-t) in 82-99% yield. The progress of the reaction was then monitored by Thin Layer Chromatography and the chemical structures of the synthesized compounds 6(a–t) were identified by using different techniques such as FT-IR, ^{1}H NMR and ^{13}C NMR spectroscopies and mass spectrometry.



6a: Ar = Phenyl
6b: Ar = 2-Chlorophenyl
6c: Ar = 3-Chlorophenyl
6d: Ar = 4-Chlorophenyl
6e: Ar = 2,4-Dichlorophenyl
6f: Ar = 2,6-Dichlorophenyl
6g: Ar = 4-Fluorophenyl
6h: Ar = 3-Nitrophenyl
6i: Ar = 4-Nitrophenyl
6j: Ar = 2-Methoxyphenyl
6k: Ar = 3-Methoxyphenyl
6l: Ar = 4-Methoxyphenyl
6m: Ar = 3,4-Dimethoxyphenyl
6n: Ar = 3,4,5-Trimethoxyphenyl
6o: Ar = 4-(Dimethyl)aminophenyl
6p: Ar = 4-(Diethyl)aminophenyl
6q: Ar = 2-Hydroxyphenyl
6r: Ar = 4-Carboxyphenyl
6s: Ar = 4-Acetamidophenyl
6t: Ar = Pyridin-3-yl

Scheme 2.5: Synthesis of 6-methyl-1H-pyrazolo[3,4-b]quinoline substituted N-acylhydrazones **6**(**a**–**t**) (Munir et al., 2021)

According to Mayurachayakul (2022), they have successfully synthesized a series of *N*-acylhydrazones via a solid-state melt reaction under catalyst- and solvent-free conditions. This method was considered as a green synthesis since the reaction was in high atom economy (94.69–94.90%) and reaction mass efficiency (84.27–87.33%). The reactions of 2-furoic hydrazide, 2-thiophenecarboxylic acid hydrazide, and substituted alkyl/aryl acylhydrazides with aromatic aldehydes reacting at 120 °C in only 30 minutes and produced the desired products in excellent isolated yields (74-100%) without the need for purification using column chromatography.



Scheme 2.6: Synthesis of *N*-acylhydrazones (Mayurachayakul et al., 2022)

2.3 Conformational study of *N*-acylhydrazones

2.3.1 Determination of the relative configuration of the *N*-acylhydrazones

The *N*-acylhydrazone below was synthesized and showed the duplication of the hydrogen signals in the ^{1}H NMR spectrum. This situation could be due to the existence of the *E* or *Z* isomers of the C=N at the imine group (Lopes et al., 2013).

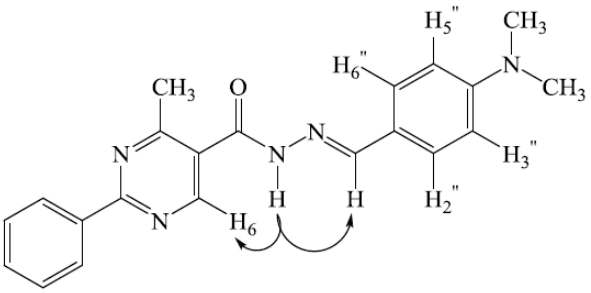


Figure 2.1: *N*-acylhydrazone synthesized

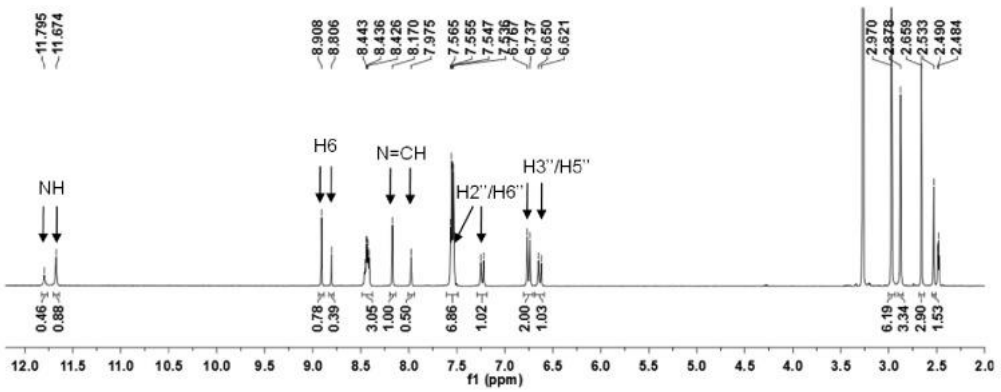


Figure 2.2: ^{1}H NMR spectrum of synthesized *N*-acylhydrazone

Differential Nuclear Overhauser Effect (differential NOE) can be carried out to evaluate the spatial proximity of ^{1}H-^{1}H. The NH of the amide was chosen as the hydrogen atom to be subjected to irradiation. The singlet that shifted less was chosen and more abundant at 11.67 ppm out of the two singlets associated to the NH hydrogen that were present. Even though just one amide signal at 11.67 ppm was selected for irradiation in the NOE experiments, the amide hydrogen at 11.79 ppm displayed the same irradiated signal phase. Furthermore, the NOE for both the H_6 and N=CH signals was positive with values of 11% and 15%, respectively. Due to the angle and distance, it was not predicted that NH irradiation of the *Z* isomer would result in a positive NOE on N=CH.

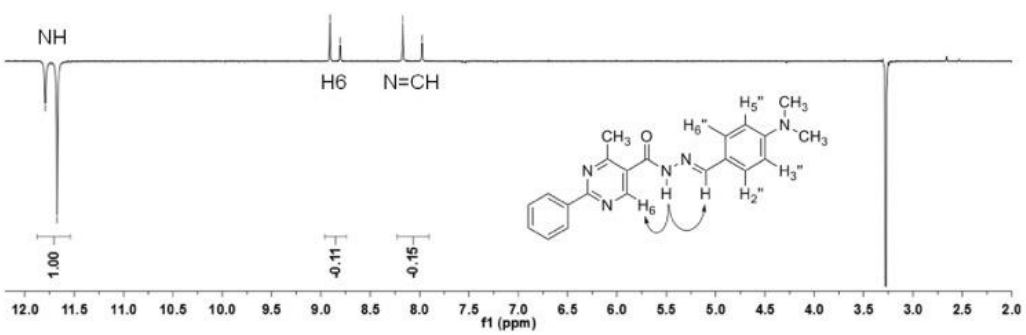


Figure 2.3: Differential NOE spectrum for irradiation of NH of synthesized *N*-acylhydrazone

2.3.2 CO-NH rotational barrier

There are lots of research showed that the presence of the *cis*/*trans*-amide conformers is based on the CO-NH bond rotation. Moreover, the *E*/*Z* isomers is based on the C=N bond of imine group in the *N*-acylhydrazones.

According to Hamzi, Barhoumi-Slimi and Abidi (2016), due to the steric repulsion, the $Z_{C=N}$ isomer of *N*-acylhydrazones is disfavored to form. Hence, the compound 1-6 were in the $E_{C=N}$ configuration as two *cis*/*trans*-amide conformers. The NH-stretching vibration (νNH) for *cis*-amide was around 3200 cm^{-1} whereas the νNH for *trans*-amide was above 3200 cm^{-1} with a band at 1540 cm^{-1}, this result might be the characteristic of the *N*-acylhydrazone moiety.

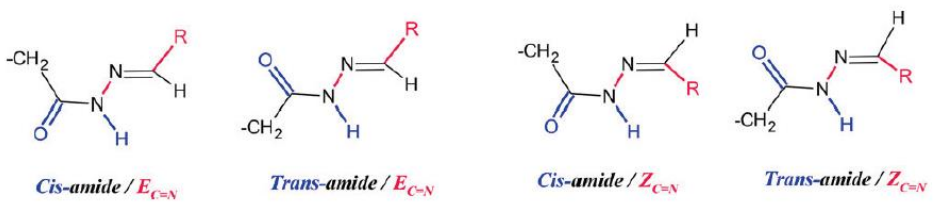


Figure 2.4: A variety of conformations and configurations exist in hydrazone derivatives (Hamzi, Barhoumi-Slimi and Abidi, 2016)

Dynamic NMR studies can be conducted to determine the energy barriers of the rotation and evaluate rate constants of the *cis*/*trans*-amide equilibrium after detecting the two rotamers around the CO-NH bond in the ^{1}H NMR spectra at room temperature. The coalescence-temperature method was carried out in DMSO-d_6 for the synthesized *N*-acylhydrazone. The Gibbs free energies of activation ($\Delta G_1^{\#}$) was determined by using Eyring equation (Hamzi, Barhoumi-Slimi and Abidi, 2016).

Eyring equation $\Delta G_1^{\#} = 19.14T_c\,(10.32 + \log T_c/k_1) \times 10^{-3}$

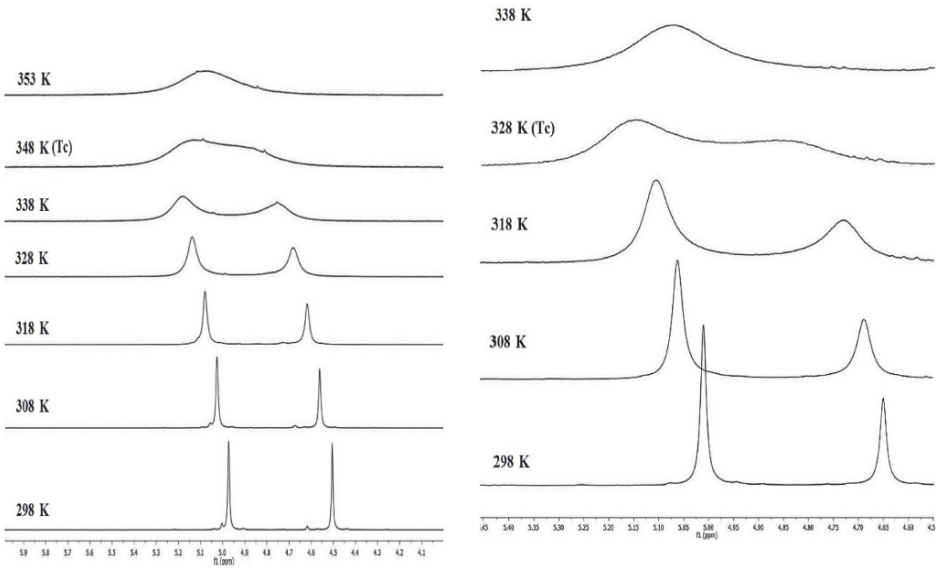
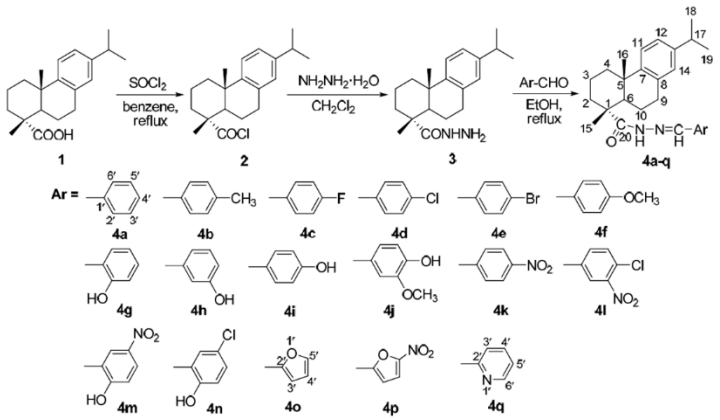


Figure 2.5: OCH_2 region of ^{1}H NMR spectra of the synthesized *N*-acylhydrazone (Figure 7) in DMSO-d_6 at variable temperatures

2.4 Antibacterial Activity

The *N*-acylhydrazone moiety was discovered as a key pharmacophore structure in pharmaceutical research in recent years. According to Gu et al. (2012), a series of new *N*-acylhydrazone derivatives were synthesized by react dehydroabietic acid hydrazide with a variety of substituted arylaldehydes. Then, the antibacterial activity of all the new *N*-acylhydrazone derivatives was

evaluated against the gram-positive bacteria *Staphylococcus aureus* and *Bacillus subtilis* as well as the Gram-negative bacteria *Escherichia coli* and *Pseudomonas fluorescens*.



Scheme 2.7: Synthesis of *N*-acylhydrazone derivatives (4a–q) from dehydroabietic acid

The results showed that those compounds with electron-withdrawing substituents such as halo and nitro in the aromatic ring showed greater antibacterial activities than those with electron-donating groups such as methyl and methoxy. Furthermore, it indicated that the introduction of the halogen atom at 4'-position generally increased antibacterial activity, particularly F (compound 4c) and Cl (compound 4d) atoms, and compound 4n with 3'-Cl substituents also showed marked antibacterial activity (Gu et al., 2012).

2.4.1 Determination of minimum inhibitory concentration (MIC)

Referring to scheme 2.7, with MIC values (1.9 µg/mL), which are comparable to those of amikacin, compound 4p demonstrated the strongest antibacterial activity against *Staphylococcus aureus* and *Bacillus subtilis* among them.

Strong inhibitions (3.9–7.8 µg/mL) were also displayed by compounds 4l, 4n, and 4p against the Gram-negative bacteria of *Escherichia coli* and *Pseudomonas fluorescens*. Additionally, compounds 3 and 4a, 4e, 4g, and 4h had shown moderate activity (15.6-31.2 µg/mL) against at least one strain of the four bacteria, whereas compounds 4b, 4f, 4i, 4j, 4o, and 4q only mildly inhibited the four strains, which have the MIC value at 62.5-100 µg/mL (Gu et al., 2012).

CHAPTER 3

MATERIALS AND METHODOLOGY

3.1 Chemicals used

Table 3.1: Chemicals used in the synthesis of indole ester

Chemical name Molecular formula	**Molecular weight, g mol^{-1}**	**State**	**Manufacturer Country**
Acetic acid CH_3COOH	60.05	Liquid	Merck, Germany
Absolute ethanol C_2H_5OH	46.07	Liquid	Fisher Scientific, Malaysia
Concentrated Hydrochloric acid HCl	36.46	Liquid	Fisher Scientific, UK
Ethyl acetate $C_4H_8O_2$	88.11	Liquid	LAB-SCAN, Ireland
Sodium sulphate anhydrous Na_2SO_4	142.04	Solid	Merck, Germany

Table 3.2: Chemicals used in the synthesis of carboxylic acid hydrazide

Chemical name Molecular formula	**Molecular weight, g mol^{-1}**	**State**	**Manufacturer Country**
Hydrazine hydrate $NH_2NH_2{\cdot}H_2O$	50.06	Liquid	Merck, Germany
Absolute ethanol C_2H_5OH	46.07	Liquid	Fisher Scientific, Malaysia

Ethyl acetate $C_4H_8O_2$	88.11	Liquid	LAB-SCAN, Ireland

Table 3.3: Chemicals used in the synthesis of *N*-acylhydrazones

Chemical name Molecular formula	**Molecular weight, g mol^{-1}**	**State**	**Manufacturer Country**
4-Methoxybenzaldehyde $C_8H_8O_2$	136.15	Liquid	Fisher Scientific, UK
2-Methoxybenzaldehyde $C_8H_8O_2$	136.15	Solid + Liquid	Merck, Germany
4-Bromobenzaldehyde C_7H_5BrO	185.02	Solid	Merck, Germany
2-Bromobenzaldehyde C_7H_5BrO	185.02	Liquid	Merck, Germany
4-Fluorobenzaldehyde C_7H_5FO	124.11	Liquid	Merck, Germany
2-Fluorobenzaldehyde C_7H_5FO	124.11	Liquid	Fisher Scientific, UK
3,4-Dimethoxybenzaldehyde $C_9H_{10}O_3$	166.17	Solid	Merck, Germany
4-Nitrobenzaldehyde $C_7H_5NO_3$	151.12	Solid	Merck, Germany
2-Nitrobenzaldehyde $C_7H_5NO_3$	151.12	Solid	Merck, Germany
4-diethylamino-2-hydroxybenzaldehyde $C_{11}H_{15}NO_2$	193.24	Solid	Merck, Germany
Tartaric acid $C_4H_6O_6$	150.09	Solid	Fisher Scientific, UK
Absolute ethanol C_2H_5OH	46.07	Liquid	Fisher Scientific, Malaysia

Table 3.4: Chemicals used in Thin Layer Chromatography

Chemical name **Molecular formula**	**Molecular weight, g mol^{-1}**	**State**	**Manufacturer** **Country**
Chloroform $CHCl_3$	119.38	Liquid	Merck, Germany
Ethyl acetate $C_4H_8O_2$	88.11	Liquid	LAB-SCAN, Ireland
n-Hexane C_6H_{14}	86.18	Liquid	Merck, Germany
Ethanol C_2H_5OH	46.07	Liquid	Fisher Scientific, Malaysia

Table 3.5: Chemicals used in recrystallization

Chemical name **Molecular formula**	**Molecular weight, g mol^{-1}**	**State**	**Manufacturer** **Country**
Ethanol C_2H_5OH	46.07	Liquid	Fisher Scientific, Malaysia

Table 3.6: Chemicals used in FT-IR and NMR spectroscopies

Chemical name **Molecular formula**	**Molecular weight, g mol^{-1}**	**State**	**Manufacturer** **Country**
Dimethyl sulfoxide-d_6 C_2D_6OS	84.17	Liquid	Fisher Scientific, UK
Potassium Bromide KBr	119.00	Solid	Sigma-Aldrich USA

Table 3.7: Chemicals used in antibacterial activity analysis

Chemical name **Molecular formula**	**Molecular weight, g mol^{-1}**	**State**	**Manufacturer** **Country**
Ethanol C_2H_5OH	46.07	Liquid	Rinting Scientific, Malaysia
p-iodonitrotetrazolium chloride $C_{19}H_{13}C_lIN_5O_2$	505.7	Solid	MP Biomedical, Germany
Streptomycin sulfate,	1457.7	Solid	Merck,

Streptomyces sp.			Germany

3.2 Instrumentation

Table 3.8: Instruments used in the project

Instrumentation	Manufacturer, Model
Nuclear Magnetic Resonance Spectrometer	JEOL, JNM-ECX400
FT-IR Spectrometer	Perkin Elmer, Spectrum RX1
Melting point apparatus	Stuart, SMP10
Sonicator	Elmasonic, S 100 H
Rotary Evaporator	BUCHI, Rotavapor R-200 Heating Bath B-491
Lamina air flow cabinet	ESCO, USA
Incubator	Memmert, Germany
UV spectrophotometer	Thermo scientific, Germany

3.3 Experimental procedure

3.3.1 Synthesis of ester

A mixture of 50 mmol and 65 mmol were weighed into a 250 mL round bottom flask. Then, 70 mL of acid was used as a solvent to dissolve the mixture. Small amount of boiling chips and a magnetic stir bar were placed into the round bottom flask and the mixture was subjected to reflux in an oil bath at 70ºC for about 7 hours. Thin Layer Chromatography was used to monitor the reaction process in order to determine the reaction progress. After the reaction, the reagent mixture was cooled to room temperature and poured into a beaker containing 300 mL of ice-distilled water and extracted three times using 30 mL of ethyl acetate. With the aid of the dehydrating agent

sodium sulphate anhydrous, the organic layer was extracted and dried. A rotary evaporator was then used to concentrate the organic layer.

3.3.2 Synthesis of carboxylic acid hydrazide

A mixture of 50 mmol ester and 150 mmol 99 % hydrazine hydrate (7.28 mL) were weighed into a 250 mL round bottom flask. The mixture was dissolved in 25 mL of absolute ethanol. Small amount of boiling chips and a magnetic stir bar were placed into the round bottom flask and the mixture was subjected to reflux in an oil bath at 80ºC for about 6 hours. Thin Layer Chromatography was used to monitor the reaction process in order to determine the reaction progress. After the reaction, the reagent mixture was cooled to room temperature and poured into a beaker containing 300 mL of ice-distilled water and extracted three times using 30 mL of ethyl acetate. With the aid of the dehydrating agent sodium sulphate anhydrous, the organic layer was extracted and dried. A rotary evaporator was then used to concentrate the organic layer. The crude product formed was purified by recrystallization in 95 % hot ethanol (Mirfazli et al., 2014).

3.3.3 Synthesis of *N*-acylhydrazones SB 1-SB10

A series of *N*-acylhydrazones were synthesized by refluxing the carboxylic hydrazide with various benzaldehydes listed in **Table 3.9**.

Table 3.9: Various benzaldehydes used in the synthesis of *N*-acylhydrazones

Benzaldehyde	**Molecular weight, g mol^{-1}**	**2 mmol**	
		Mass (g)	**Volume (µL)**
4-Methoxybenzaldehyde	136.15	-	244
2-Methoxybenzaldehyde	136.15	0.2723	-
4-Bromobenzaldehyde	185.02	0.3700	-
2-Bromobenzaldehyde	185.02	-	234
4-Fluorobenzaldehyde	124.11	-	215
2-Fluorobenzaldehyde	124.11	-	222
3,4-Dimethoxybenzaldehyde	166.17	0.3323	-
4-Nitrobenzaldehyde	151.12	0.3022	-
2-Nitrobenzaldehyde	151.12	0.3022	-
4-diethylamino-2-hydroxy-benzaldehyde	193.24	0.3865	-

Mass of reagent used, g = Number of mole (mol) × Molecular weight (gmol $^{-1}$)

Volume of reagent used, mL = Mass of reagent used (g) / Density of reagent (gmL^{-1})

Equimolar (2.0 mmol) of carboxylic acid hydrazide and benzaldehydes were added into a 50 mL round bottom flask. The mixture was dissolved in 15 mL of absolute ethanol. Small amount of boiling chips and a magnetic stir bar

were placed into the round bottom flask and the mixture was subjected to reflux in an oil bath at 80ºC for about 8 hours. Thin Layer Chromatography was used to monitor the reaction process in order to determine the reaction progress. Once the reaction is completed, the reaction mixture was allowed to cool down to room temperature and poured into a beaker containing about 10 g of crushed ice. The crude product formed was filtered and washed with cold distilled water in order to remove the residue of catalyst. The crude product was recrystallized by using hot ethanol.

3.3.4 Purification by recrystallization

The crude product was purified by recrystallization by using minimum amount of hot ethanol that could fully dissolve the crude product. Then, the solution was undergone a hot filtration to remove insoluble substance such as filter paper fibre, dust and some residues. The filtration apparatus was preheated to avoid the crystallization of product from the solution during the filtration process which might cause product loss and eventually resulted in low percentage of yield. Then, the filtrate was collected into a conical flask filled with small amount of boiling ethanol. This is to ensure that the vapour produced from the hot ethanol would keep the filtration apparatus warm and hence avoid crystallization of the product. A quick filtration is important in order to prevent the solution from cooling and product could retain on the cotton wool. Therefore, rinsing of glass rod used and the cotton wool was necessary at the end of filtration with small amount of hot ethanol.

The filtrate collected in the conical was heated to saturation by 1/3 of its original volume. The saturated solution was allowed to cool to room temperature and allowed crystallization. The crystallized product was then washed with minimum amount of cold ethanol and dried in oven.

3.4 Characterization

3.4.1 Thin Layer Chromatography (TLC)

TLC technique was used to determine the purity of the product as well as to monitor the process of reaction. Small amount of the reactant mixture or product (~ 0.5 mL) was transferred into a clean sample vial and was diluted with minimum amount of ethanol followed by chloroform. Adequate amount of the samples was spotted on the baseline drawn on the TLC plate using a capillary tube. The spots should at least 0.5 cm away from one another to prevent occurrence of overlapping.

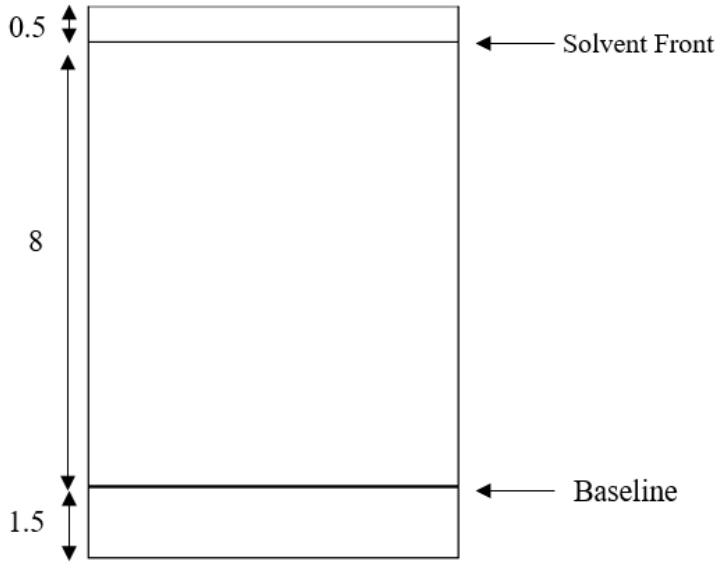


Figure 3.1: Outline of a TLC Chromatogram

The TLC developing solvent was a mixture of hexane and ethyl acetate (1:1). When the solvent front has moved within approximately 0.5 cm of the top of the TLC plate, the plate was removed from the developed chamber. The

mobile phase was allowed to evaporate, the result was then observed under the ultraviolet lamp with short wavelength (254 nm). The spots on the plate were marked with pencil. The retention factor, Rf for each spot were calculated.

$$R_f = \frac{D}{L}$$

D = Distance travelled by sample from the baseline

L = Distance travelled by solvent from the baseline

3.4.2 Melting point

Melting point of all synthesized compounds were measured to determine the purity of the synthesized compound. A sharp melting point can be obtained if the compound is pure whereas a lower and broader melting point if the compound is impure.

A very little amount of the synthesized compound was inserted into a capillary. The capillary tube was then placed into a melting point apparatus with temperature set around 200ºC. The melting point range of all synthesized compounds were recorded from the temperature at which the synthesized compound started to melt as the melting of the synthesized compound can occur over a range of temperatures. Determination of melting point of all synthesized compounds were repeated twice in order to obtain a consistent result.

3.4.3 Fourier Transform Infrared (FTIR) Spectroscopy

Infrared spectroscopy is a fundamental technique that allow us to identify the functional groups present in the molecules (precursor and *N*-acylhydrazones). By looking at the specific peaks occurred in the IR spectrum, the important functional group can be identified. The vibration of particular chemical bonds or functional groups inside the sample generates the IR peaks. IR spectrum databases, such as the NIST Chemistry WebBook, can be used to determine the functional group in charge of the IR peak. Standard IR spectra for common functional groups can be found in IR spectral databases. By comparing the frequency and shape of the sample's IR peak to the standard spectra, the presence of the functional group in the sample can be determined.

Since it is transparent in the infrared region of the electromagnetic spectrum and has a low refractive index, potassium bromide (KBr) is a frequently used substance in infrared spectroscopy. This property makes it the ideal substance for making IR sample pellets. A little portion of the sample is mixed with KBr powder to generate an IR sample, which is then compressed into a thin pellet by using a hydraulic press. The resulting pellet allows the IR beam pass through and interact with the sample molecules since it is transparent to IR radiation.The KBr must be kept in the oven to prevent water from the air from contaminating the salt which would cause IR interference.

3.4.4 Nuclear Magnetic Resonance (NMR) Spectroscopy

NMR spectroscopy is a useful and reliable analytical method used to identify the chemical composition and characteristics of molecules. It is based on the principle of nuclear magnetic resonance, a phenomenon that some atomic nuclei displayed in the presence of a magnetic field. In NMR spectroscopy, a sample is exposed to electromagnetic radiation, often in the radiofrequency range, while being held in a high magnetic field. Atomic nuclei in the sample absorb the radiation energy and shift from a lower energy state to a higher energy state. NMR gives information about the number of magnetically distinct atoms of the type being studied such as proton NMR (^{1}H NMR) and carbon-13 NMR (^{13}C NMR). Apart from ^{1}H and ^{13}C NMR, DEPT (Distortionless Enhancement by Polarization Transfer), HMQC (Heteronuclear Multiple Quantum Coherence) and HMBC (Heteronuclear Multiple Bond Coherence) can be obtained from NMR spectrometer. By analyzing these spectra, the structure of each synthesized compound hence can be identified.

For the sample preparation, about 20 mg of solid product was weighed in a clean sample vial and was dissolved with minimum amount of DMSO-d_6. The dissolved sample solution was then transferred into an NMR tube to a height of 4 cm. The tube was labelled and finally ready for analysis.

3.5 Conformational study of *N*-acylhydrazones

3.5.1 Determination of the relative configuration of the imine double bond

As mentioned earlier, *N*-acylhydrazones may exist in four possible forms, which were *cis/trans* conformers at the CO-NH bond and *E/Z* geometrical isomers at the imine double bond (Hamzi, Barhoumi-Slimi and Abidi, 2016). Differential Nuclear Overhauser Effect (differential NOE) experiments can be conducted to distinguish the protons by their spatial location within a molecule. The proton of the amide NH was selected for irradiation and due to the presence of two singlet signals in the spectrum, the signal at the up field region was selected. Hence, any nearby protons such as N=CH that interact with it will experience an enhancement in signal intensity and from the *E/Z* isomer of the compounds can be determined (Lopes et al., 2013). The presence of doubled signals in ^{1}H-NMR spectra which were attributed to the presence of *cis/trans*-amide conformers of *N*-acylhydrazones and the ratio of *cis/trans*-amide conformers were determined from the integrations of the NH signal in the ^{1}H-NMR spectra (Patorski, Wyrzykiewicz and Bartkowiak, 2013).

3.5.2 Rotational barriers around the CO-NH bond for *N*-acylhydrazones

The energy of the rotational barriers or the Gibbs free energy of activation, $\Delta G^{\neq}$ and the rate constant of *cis/trans* equilibrium, *k*c were calculated from the dynamic NMR experiments in DMSO-d_6 for *N*-acylhydrazones by using

coalescence-temperature method. The temperature of NMR spectrometer was increased within the range of 293.15 K - 393.15 K which then led to coalescence of the signals of *cis/trans*-amide conformers. The rate constant of *cis/trans* equilibrium, *k*c was calculated using the equation $kc = (\pi \times \Delta\upsilon)/\sqrt{2}$ where $\Delta\upsilon$ is the peak separation in hertz between the two singlets. However, the energy of the rotational barrier was calculated according to the Eyring equation, $\Delta G_{\neq}$ (J mol^{-1}) = 19.14 *Tc* (10.32 + log T_c/k_c) (Hamzi, Barhoumi-Slimi and Abidi, 2016).

3.6 Antibacterial evaluation

N-acylhydrazones synthesized were evaluated for the antibacterial activity by using broth microdilution method against two Gram-positive bacteria: *Bacillus subtilis* (ATCC 13061), *Staphylococcus aureus* (ATCC 6538) and one Gram-negative bacteria: *Pseudomonas aeruginosa* (ATCC 27853). All strains of each bacteria were bought from American Type Culture Collection (ATCC). Minimum inhibitory concentration (MIC) of the synthesized compounds were recorded accordingly.

3.6.1 Preparation of antibacterial assay

3.6.1.1 Preparation of media

The Mueller-Hinton (MH) agar and broth were prepared by dissolving 15 g and 1.68 g respectively in 400 mL and 80 mL of distilled water. The media

were then autoclaved and the agar media was poured into some petri dishes and allowed it to cool until solidified then sealed the lid with parafilm for prevention of contamination purposes. Meanwhile, the broth was sealed with parafilm and stored at ambient temperature.

3.6.1.2 Preparation of the bacterial glycerol stock solution

Each of the bacteria was streaked onto MH agar from 50 % glycerol stock solution and incubated at 37 ºC overnight in an incubator. The bacteria colonies that freshly grown on the *MH* agar plates were used to prepare the bacteria suspension.

3.6.1.3 Preparation of standard drug

Streptomycin was used as positive control. 100 μg/mL of streptomycin solution was prepared by dissolving 1 mg of streptomycin in 10 mL of sterile distilled water. The solution kept in a 4°C chiller when not used.

3.6.1.4 Preparation of *N*-acylhydrazone compounds

One mg/mL of *N*-acylhydrazones was prepared by dissolving 2 mg of *N*-acylhydrazone in 2 mL of dimethyl sulfoxide (DMSO) into each labelled sample vial. The solution was mixed well to ensure the compound is dissolved completely.

3.6.1.5 Preparation of *p*-iodonitrotetrazolium chloride (INT)

p-iodonitrotetrazolium chloride (INT) acted as an indicator of the bacteria growth. 0.4 mg/mL of INT indicator was prepared by dissolving 2 mg of *p*-iodonitrotetrazolium chloride in 5 mL of sterile distilled water and a few drops of concentrated ethanol into a centrifuge tube and wrapped it with aluminium foil because INT indicator is sensitive to light. The solution was kept in the 4°C chiller when not used.

3.6.1.6 Preparation of bacteria suspension

Five mL and 4.950 mL of sterile *MH* broth was added in two separate centrifuge tubes. Bacteria colonies from the cultured plates were inoculated into the tube containing 5 mL of sterile *Mueller Hinton* broth by using inoculating loop. The mixture was vortexed to mix well followed by transferring approximate 1 mL of the bacteria suspension into the cuvette for absorbance measurement. The sterile *MH* broth was used as blank and the absorbance was measured at 625 nm. The absorbance obtained should be within the range of 0.08-0.10 (turbidity equivalent to 1×10^8 colony forming per unit) according to the CLSI standard (Yong et al., 2018).

Once the desired absorbance was obtained, 50 µL of the bacteria suspension was transferred into the tube containing 4.950 mL of sterile broth resulting 1 x 10^6 cfu/mL bacteria suspension .

3.6.2 Determination of minimum inhibitory concentration (MIC) by using broth microdilution method

Broth microdilution method is used in this study to determine the minimum inhibitory concentration, value of the synthesized compounds against the chosen bacterial strains. All the procedure was done by using 96-well plate in laminar flow to avoid contamination. Two 96-well plates were assayed for a total of ten *N*-acylhydrazone compounds for each bacteria. Four compounds and positive control were tested at the first plate and the remaining six compounds were tested at the second plate.

100 µL of sterile Mueller-Hinton broth were filled at the four corners of each plate as sterility control. Column 2 for the first plate was assigned for positive control where 50 µL of 100 µg/mL streptomycin was diluted serially into each well containing 50 µL of sterile broth and followed by the addition of 50 µL of bacteria suspension giving the final concentration ranging from 0.195 to 25 µg/mL. Columns 4,6,8 and 10 for the first plate and columns 3,5,7,9,10 and 11 for the second plate were assigned for the tested *N*-acylhydrazone compounds where serial dilution was done by diluting serially 50 µL of each compound into corresponding well containing 50 µL of sterile broth and again followed by the addition of 50 µL of bacteria suspension giving the final concentration ranging from 1.95 to 250 µg/mL. Column 12 (row E was assigned for the growth control where 50 µL of bacteria suspension was added into each well containing 50 µL sterile broth. After all the procedure was done, the plates were sealed with parafilm and incubated overnight at 37 ℃ incubator.

Twenty μL of INT solution (0.4 μg/mL) was added into each well and incubated in a dark place for 15 minutes after overnight incubation. The growth of the bacteria was observed by the colour change due to the presence of INT solution. The formation of reddish pink indicated the presence of bacteria activity whereas colourless indicated the inhibition of bacteria growth. Triplicate assays were done to obtain consistent and accurate results.

3.7 Calculation

Determination of mass of materials that required in the synthesis.

Mass (g) = number of moles (mol) x molecular weight (g mol^{-1})

Determination of volume of materials that required in the synthesis.

$$\text{Volume (mL)} = \frac{Mass\ (g)}{Density\ of\ the\ materials\ (g\ mL^{-1})}$$

Determination of percentage yield for the synthesized products.

$$\text{Percentage yield (\%)} = \frac{Experimental\ mass\ of\ product}{Theoretical\ mass\ of\ product} \times 100\%$$

Determination of the retardation factor value, R_f

$$R_f = \frac{Distance\ travelled\ by\ sample\ from\ the\ baseline}{Distance\ travelled\ by\ solvent\ from\ the\ baseline}$$

Conversion of μg/mL to mM (μmol/mL).

$$\text{mM} = \frac{\mu g/mL}{Molecular\ weight} \times 1000$$

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Synthesis of ester

4.1.1 Proposed mechanism for the synthesis of ester

The ester was synthesized which involved the condensation of with under acidic condition. In this reaction, hydrazone was formed when the carbonyl carbon of was attacked by the nucleophile. Upon heating under acidic condition, tautomerization process was occurred and the hydrazone was then converted to enehydrazine. Finally, the desired ester was obtained upon imine exchange with the elimination of ammonia as by-product.

4.2 Synthesis of carboxylic acid hydrazide

4.2.1 Proposed mechanism for the synthesis of carboxylic acid hydrazide

The carboxylic acid hydrazide was obtained by nucleophilic acyl substitution reaction of ester with hydrazine hydrate in absolute ethanol at 80°C. The percentage yield of the carboxylic acid hydrazide was 46%. The nucleophilic acyl substitution reaction begins with attack by the lone pair electron of nitrogen of hydrazine hydrate on the carbonyl group of ester to give a tetrahedral intermediate. Expulsion of ethoxide ion, followed by a fast proton transfer gives the carboxylic acid hydrazide and ethanol as side-product.

4.2.2 Structure elucidation of carboxylic acid hydrazide

Carboxylic acid hydrazide was acted as the key precursor for the synthesis of *N*-acylhydrazones. It is a light brown solid with melting point range of 222-224 °C. Thin Layer Chromatography analysis was carried out to determine the purity of the precursor, the solvent system prepared was hexane : ethyl acetate (1:1). Single spot was observed on the TLC plate and the R_f value was 0.56. The carboxylic acid hydrazide was further characterized with various spectroscopic techniques such as FT-IR, ^{1}H NMR, ^{13}C NMR, DEPT, HMBC and HMQC.

Table 4.1: Summary of the physical properties of carboxylic acid hydrazide

Physical Appearance	Light brown solid
Melting point (°C)	222-224
R_f value	0.26
Percentage yield (%)	46

The IR spectrum of N-H stretch in carboxylic acid hydrazide is indicated by the two significant intensity absorption bands at 3300 cm^{-1} and 3200 cm^{-1} . The structure is composed of an aromatic ring and sp^3 carbon, as indicated by the other two medium intensity peaks at 3088 cm^{-1} and 2982 cm^{-1}, which stand in for aromatic C-H stretch and aliphatic C-H stretch, respectively. The medium intensity absorption bands at 1685 cm^{-1} indicate the structure contains an amide carbonyl (C=O) functional group. More absorption bands, like those at 1089 cm^{-1} (the C-N stretch) and 859 cm^{-1}, have been observed in the lower field of the IR spectrum.

Table 4.2: Summary of FT-IR spectral data of 2-(5-bromo-2-methyl-1*H*-indol-3-yl) acetohydrazide

Functional group	**Wavenumber (cm^{-1})**
NH_2/N-H stretch	3300/3200
Aromatic C-H stretch	3053
sp^3 C-H stretch	2952

C=O stretch	1685
C=C stretch	1664
C-N stretch	1008

The ^{1}H NMR spectra of the carboxylic acid hydrazide are shown in **Figures 4.3** and **4.4**. The two singlet signals at δ 10.86 and δ 9.82 were belonged to the NH and hydrazide NH, respectively. A 2H broad singlet at δ 5.15 indicated the presence of the amino group of hydrazide. The aromatic protons of benzene ring were observed at δ 7.86 (d, *J* = 1.8 Hz), 7.72 (dd, *J* = 7.9, 1.8 Hz) and 7.34 (d, *J* = 7.9 Hz). The two singlet signals at δ 3.59 and δ 2.52 were belonged to the methylene and methyl protons, respectively. From the **Figure 4.5**, the signals of NH, amino group of hydrazide and hydrazide NH were disappeared after the addition of D_2O.

The ^{13}C NMR spectrum of carboxylic acid hydrazide (**Figure 4.6**) showed the presence of 11 separate carbon resonances. One of the carbon signals in δ 180.5 appeared at the downfield region due to the carbonyl amine carbon while two carbon signals in δ 13.9 and δ 39.9 at the upfield region were due to the methylene and methyl carbon. The electronegative oxygen atom in the carbonyl group, which pulls electron density away from the carbon atom through the pi bond, produces the deshielding effect. As a result, the carbon atom gains partial positive charge that makes it more magnetic field-sensitive. The remaining carbon signals between δ 115.3 to δ 145.5 were due to the aromatic carbons.

A kind of two-dimensional nuclear magnetic resonance (NMR) spectroscopy called HMQC (Heteronuclear Multiple Quantum Coherence) is used to correlate signals from two different kinds of nuclei in a molecule. The usage of HMQC is particularly focused on the correlation of proton (^{1}H) signals with signals from other nuclei, such as carbon (^{13}C) or nitrogen (^{15}N). There was a total of five HMQC correlations between δ_H 7.76/δ_C 170.8, δ_H 7.44/δ_C 142.7, δ_H 7.12/δ_C 132.8, δ_H 3.49/δ_C 39.9, and δ_H 2.62/δ_C 21.9 based on the HMQC spectrum in **Appendix A2**. N-H and NH_2 hydrogen atoms do not exhibit any HMQC correlation in the HMQC spectrum since they are not related to any carbon atoms.

HMBC (Heteronuclear Multiple Bond Correlation) is a kind of two-dimensional nuclear magnetic resonance (NMR) spectroscopy that is used to correlate signals from protons (^{1}H) with signals from other nuclei, such as carbon (^{13}C) or nitrogen (^{15}N). In contrast to the HMQC experiment, the HMBC experiment uses long-range couplings between the two nuclei, typically through multiple bonds. Hence, HMBC can be used to identify the quaternary carbon in the ^{13}C NMR spectrum.

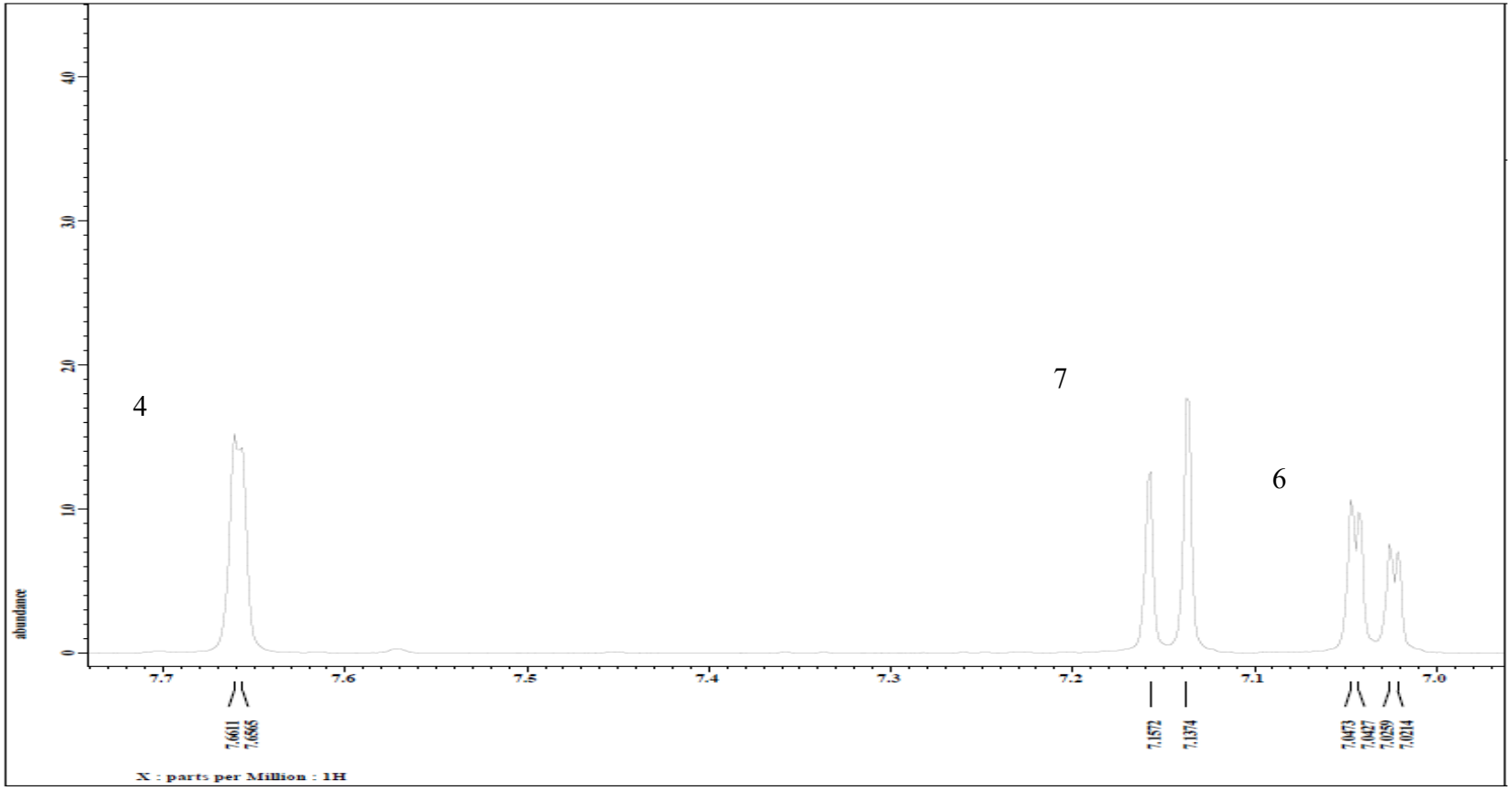


Figure 4.5: ^{1}H NMR spectrum of carboxylic acid hydrazide after the addition of D_2O

4.3 Synthesis of *N*-acylhydrazones SB 1-SB 10

4.3.1 Proposed mechanism for the synthesis of *N*-acylhydrazones SB 1-SB 10

The *N*-acylhydrazones **SB 1-SB 10** can be synthesized by reacting the key precursor carboxylic hydrazide with a series of aromatic aldehydes via acid-catalyzed condensation. First, the amino group of carboxylic hydrazide acts as a nucleophile, attacking the carbonyl group (C=O) of benzaldehydes to yield an alkoxide ion intermediate. Next, the alkoxide ion was converted into carbinolamine through the proton transfer from nitrogen to oxygen. Oxygen atom of the carbinolamine was then undergone protonation with acid and converted the hydroxyl group –OH to a water molecule which is a better leaving group. Finally, *N*-acylhydrazones were formed with the loss of a proton from nitrogen atom. acid was regenerated at the end of the reaction.

4.3.2 Synthesis and characterization of *N*-acylhydrazones SB 1-SB 10

The condensation reaction of carboxylic acid hydrazide with a series of substituted benzaldehydes in the presence of acid resulted in the synthesis of ten new *N*-acylhydrazones, labelled as **SB 1- SB 10**. *N*-acylhydrazones **SB 1-SB 10** were produced in yields ranging from 58 to 88%. Higher yields were found for *N*-acylhydrazones with nitro groups (**SB 8** and **SB 9**) or methoxy groups (**SB 1**, **SB 2**, and **SB 7**). However, when the halogen or hydroxyl group

was substituted (**SB 3**, **SB 4**, **SB 5**, **SB 6** and **SB 10**), a poor yield of *N*-acylhydrazones was obtained. The *N*-acylhydrazones were either light brown solid or dark brown solid, with melting points between 212 °C and 253 °C. The purity of the synthesized compounds was determined through TLC analysis by using the solvent system of hexane:ethyl acetate (1:1) with a single spot observed, which produced an R_f value ranging from 0.23 to 0.73. **Table 4.4** summarized the physical properties of *N*-acylhydrazones **SB 1 - SB 10**. Through the FT-IR, ^{1}H NMR, ^{13}C NMR, DEPT, HMQC, and HMBC spectral data, all the synthesized *N*-acylhydrazones were identified.

Figure 4.7: Chemical structure and numbering of *N*-acylhydrazones **SB 1- SB 10**

SB	R_1	R_2	R_3
1	H	H	OCH_3
2	OCH_3	H	H
3	H	H	Br
4	Br	H	H
5	H	H	F
6	F	H	H
7	H	OCH_3	OCH_3
8	H	H	NO_2
9	NO_2	H	H
10	OH	H	$N(CH_2CH_3)_2$

Table 4.4: Summary of physical properties of *N*-acylhydrazones **SB 1-SB 10**

Compound	Physical appearance	Percentage yield (%)	R_f value (EA : Hexane = 1:1)
SB 1 (4-OMe)	Light Brown Solid	64%	0.30
SB 2 (2-OMe)	Light Brown Solid	76%	0.54
SB 3 (4-Br)	Light Brown Solid	53%	0.56
SB 4 (2-Br)	Dark-Brown Solid	68%	0.63
SB 5 (4-F)	Light Brown Solid	54%	0.56
SB 6 (2-F)	Dark-Brown Solid	62%	0.60
SB 7 (3,4-OMe_2)	Dark-Brown Solid	74%	0.30
SB 8 (4-NO_2)	Light Brown Solid	78%	0.49
SB 9 (2-NO_2)	Light Brown Solid	72%	0.61
SB 10 (2-OH,4NEt_2)	Light Brown Solid	52%	0.49

4.4.3 IR characterization of *N*-acylhydrazones SB 1 – SB 10

The IR spectra of the *N*-acylhydrazones **SB 1–SB 10** (**Figure 4.8** and **Appendices B1–B9**) showed the presence of C=N and C=C functional groups at 1635–1523 cm^{-1} as well as N-H absorption bands at 3432–3194 cm^{-1}. A medium intensity peak for the aromatic C-H stretch was observed at 3047 cm^{-1}, while the aliphatic C-H stretch was observed at 2904 cm^{-1}, indicating that the structure consists of an aromatic ring and sp^3 carbon. The strong intensity absorption band at 1673-1651 cm^{-1} indicates the existence of an amide carbonyl (C=O) functional group in the structure. Around 659-562 cm^{-1}, the C-Br stretching band was observed. In compounds **SB 8** and **SB 9**, the nitro group may be observed to have two separate bands at 1518-1338 cm^{-1} and 1367-1344 cm^{-1}. The presence of C-O-C stretch was observed at 1269-1250 cm^{-1}. The presence of C-F stretch was observed at 1235-1192 cm^{-1}. Lastly, the presence of -OH stretch was observed at 3331 cm^{-1}. The summary of the FTIR spectral data of *N*-acylhydrazones **SB 1- SB 10** is tabulated in **Table 4.5**.

Table 4.5: Summary of FTIR spectral data of N-acylhydrazones SB 1- SB 10

Compound	N-H	Aromatic C-H	sp^3 C-H	C=O	Imine C=N	Aromatic C=C	C-O-C	C-F	NO_2	O-H
SB1 (4-OMe)	3289	3047	2904	1654	1603	1569	1251	-	-	-
SB2 (2-OMe)	3432	3051	2953	1667	1602	1557	1250	-	-	-
SB3 (4-Br)	3290	3058	2898	1655	1604	1560	-	-	-	-
SB4 (2-Br)	3412	3057	2905	1654	1590	1533	-	-	-	-
SB5 (4-F)	3412	3050	2914	1659	1604	1560	-	1235	-	-
SB6 (2-F)	3415	3054	2903	1674	1614	1548	-	1192	-	-
SB7 (3,4-OMe_2)	3286	3047	2905	1656	1599	1473	1269	-	-	-
SB8 (4-NO_2)	3395	3080	2957	1670	1583	1583	-	-	1518,1338	-
SB9 (2-NO_2)	3301	3054	2903	1651	1523	1523	-	-	1523, 1344	-
SB10 (2-OH, 4-NEt_2)	3331	2974	2926	1673	1635	1601	-	-	-	3331

4.3.4 NMR structural characterization of *N*-acylhydrazones SB 1 – SB 10

4.3.4.1 ^{1}H NMR structural characterization of *N*-acylhydrazones SB 1 – SB 5

A total of ten *N*-acylhydrazones (**SB 1 – SB 10**) were synthesized, however only five of them (**SB 1 – SB 5**) were fully characterized and interpreted by 1D-NMR (^{1}H NMR, ^{13}C NMR, NOE and DEPT) and 2D-NMR (HMBC and HMQC) spectral data, while **SB 6 – SB 10** could not be analyzed due to the breakdown of the NMR spectrometer.

Compound **SB 1**(4'-OCH_3) was chosen as representative compound for the discussion of structure interpretation of the synthesized *N*-acylhydrazones (**SB 1 – SB 5**). The structure of compound **SB 1** (4'-OCH_3) is shown in **Figure 4.9**. The structure of the synthesized *N*-acylhydrazones can be confirmed by comparing the ^{1}H NMR spectra of the key precursor and *N*-acylhydrazones. The signal of the primary amine at δ 4.35 was absent in the ^{1}H NMR spectra of the *N*-acylhydrazones. Furthermore, the presence of three sets of 1H singlet signals at δ3.74-3.75, δ7.91-8.15 and δ11.10-11.35 were due to the methoxy protons, imine protons and CO-NH, respectively.

The ^{1}H NMR spectral data of the *N*-acylhydrazones **SB 1 – SB 5** are summarized **Table 4.6**. The ^{1}H NMR spectra of compound **SB 2 – SB 5** were displayed in **Appendices C1 – C4**.

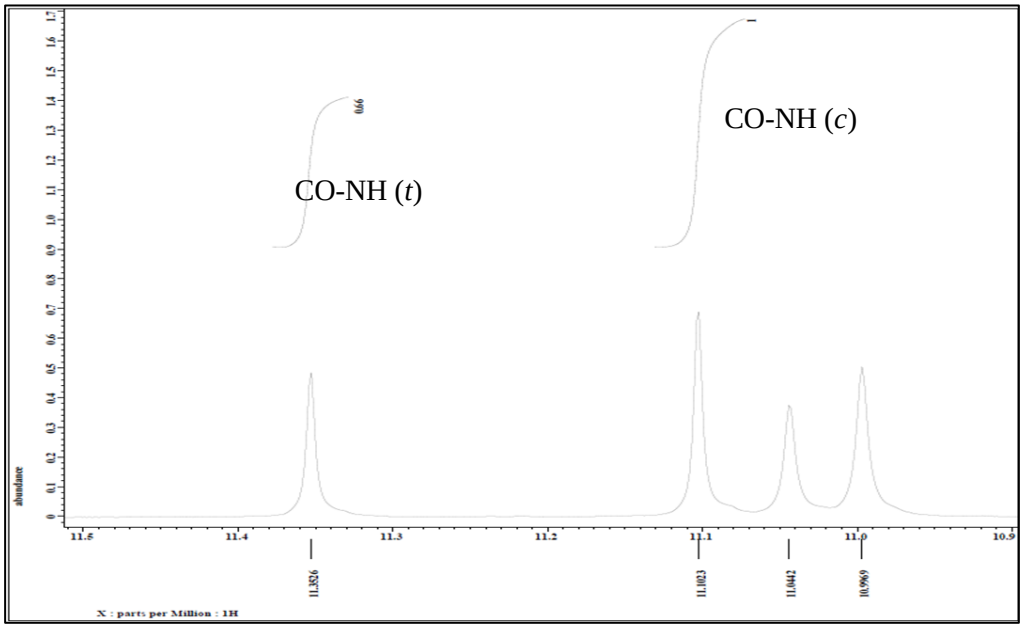


Figure 4.13: Integration of CO-NH of *N*-acylhydrazone **SB1** (4'-OCH_3) (% *cis*/*trans*-amide

4.3.4.2 ^{13}C NMR, DEPT and 2D-NMR structural characterization of *N*-acylhydrazones SB 1 – SB 5

The structure of *N*-acylhydrazones **SB 1 – SB 5** can be double confirmed by the ^{13}C NMR spectra (**Figures 4.14 & 4.15 and Appendices D1-D4**). The structural characterization of *N*-acylhydrazones **SB 6 – SB 10** could not be determined due to the breakdown of the NMR spectrometer. In the ^{13}C NMR spectra, the presence of imine carbon N=CH was observed at δ 143.4-146.8 and carbonyl amine CONH was appeared at δ 11.10-11.35.

Compound **SB 1**(**4'-OCH$_3$**) was chosen as representative compound for the discussion of structure interpretation of the synthesized *N*-acylhydrazones (**SB 1 – SB 5**). In the ^{13}C NMR spectra of each compound, there were paired peaks can be observed which indicated the presence of *cis* and *trans* conformers. In **Figures 4.14** and **4.15**, the *trans* conformer was responsible for the carbon resonance at δ 167.4, while the *cis* conformer was responsible for the carbonyl carbon signal with a greater intensity at δ 172.8. The summary of the ^{13}C NMR spectral data of *N*-acylhydrazones **SB 1 - SB 5** is tabulated in **Table 4.7**.

Eight quaternary carbons were observed in the ^{13}C NMR spectra of compound **SB 1** based on the DEPT spectrum as shown in **Figure 4.15**. **Appendices E1 – E4** displayed the DEPT spectra of *N*-acylhydrazones **SB 2 – SB 5**.

Figure 4.17 showed the HMQC spectrum of N-acylhydrazone **SB 1**. HMBC spectrum of the *N*-acylhydrazone **SB 1** (**Figures 4.18** – **4.20**) showed the HMBC correlations from the CONH proton to carbonyl amide and imine carbon. However, the imine proton showed HMBC correlations with C-1', indicating the bonding of the phenyl ring to the imine carbon..**Appendices F1-4** was the HMQC spectrum of **SB 2 – 5** while **Appendices G1 – G4** were the HMBC spectrum of **SB 2- 5**.

4.3.5 Conformational study of *N*-acylhydrazones SB 1 – SB 5

4.3.5.1 Determination of the relative configuration of imine double bond and the conformation of CONH bond

The *N*-acylhydrazones can exist as *E*/*Z* geometrical isomers in DMSO-d_6 solution due to rotation along C=N–NH bond as shown in **Figure 4.21**. The *E*/*Z* geometrical isomers of the imine double bond were determined by carrying out differential Nuclear Overhauser Effect (NOE) in DMSO-d_6 for each of the synthesized *N*-acylhydrazones (**SB 1-SB 5**). The conformational study of *N*-acylhydrazones **SB 6 – SB 10** could not be determined due to the breakdown of NMR. **Figures 4.22**, **4.23** and **Appendices I1–I6** show the NOE spectra of *N*-acylhydrazones **SB 1 - SB 5** respectively. *N*-acylhydrazone **SB 1(4'-OCH$_3$)** was chosen as representative compound for the discussion of conformational study of the synthesized *N*-acylhydrazones (**SB 1 – SB 5**).

The integration region of the CO-NH signal in the ^{1}H NMR spectra of *N*-acylhydrazones **SB 1 – SB 5** was analyzed to determine the ratios of the *cis*/*trans*-amide conformers. The integrations and the percentage of *cis*/*trans*-amide of the *N*-acylhydrazones **SB 1–SB 5** are shown in **Figure 4.24**, **Appendices H1–H4**, and **Table 4.9**, respectively. The intensity of the ^{1}H and ^{13}C NMR signals of the *cis* conformer, which are stronger than those of the *trans* conformer, can be used as further support for the predominance of the *cis*-amide conformation in *N*-acylhydrazones **SB 1 - SB 5**.

Table 4.8: Summary of percentage of isomer for *N*-acylhydrazones **SB 1- SB 5** in DMSO-d_6

Compound	**Isomer (%)**	
	E	***Z***
SB 1 (4-OMe)	100	0
SB 2 (2-OMe)	100	0
SB3 (4-Br)	100	0
SB 4 (2-Br)	100	0
SB 5 (4-F)	100	0
SB 6 (2-F)	Not Determined	Not Determined
SB 7 (3,4-OCH_3)	Not Determined	Not Determined
SB 8 (4-NO_2)	Not Determined	Not Determined
SB 9 (2-NO_2)	Not Determined	Not Determined
SB 10 (2-OH, 4-NEt_2)	Not Determined	Not Determined

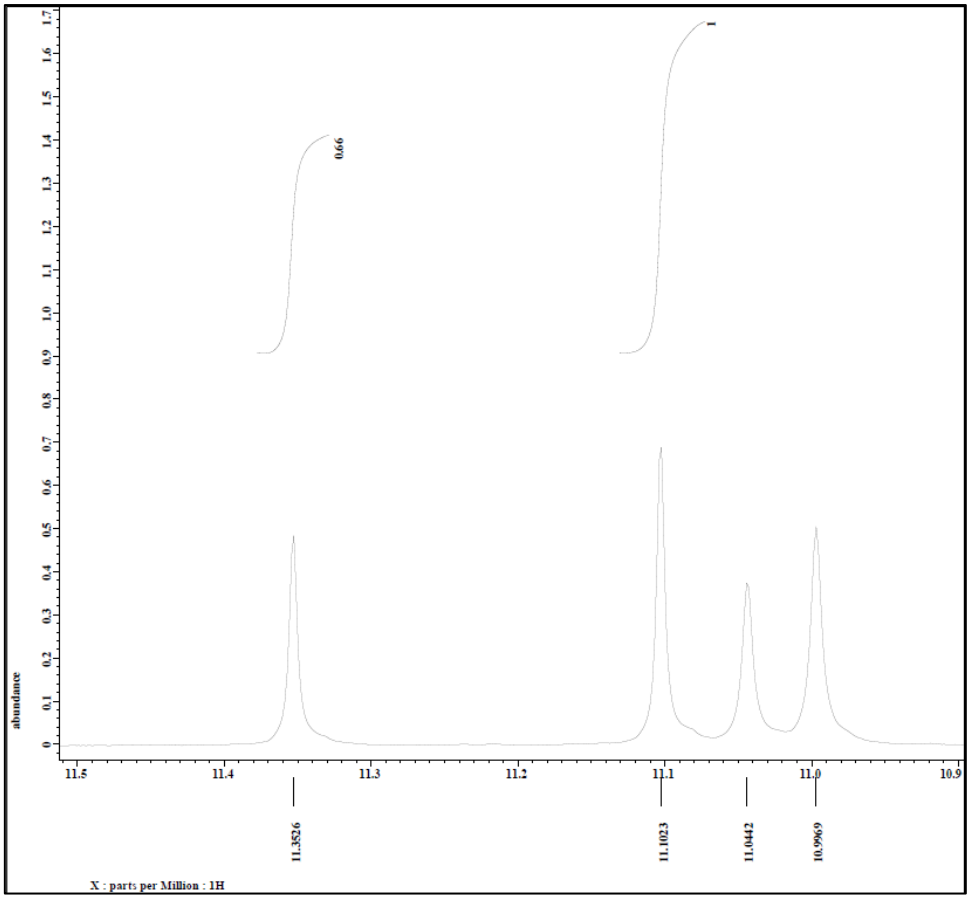


Figure 4.24: The integration of CONH of *N*-acylhydrazone **SB 1** (**4'-OCH3**)

4.3.5.2 Rotational barriers around the CONH bond for *N*-acylhydrazones SB 1 – SB 5

To determine the energy barriers of the rotation of *cis*/*trans* rotamers for *N*-acylhydrazones and the rate constant of the *cis*/*trans* equilibrium, dynamic NMR studies were carried out. **Figure 4.25** shows the ^{1}H NMR spectra of the *N*-acylhydrazone **SB 1** at various temperatures. **Appendices J1 – J3** shows ^{1}H NMR spectra of the *N*-acylhydrazones **SB 2 – SB 5** at various temperatures. The *N*-acylhydrazones **SB 1 – SB 5** have been analyzed using the coalescence-temperature technique in DMSO. The rate constants, k_c, in the

N-acylhydrazones **SB 1 – SB 5** were presented in **Table 4.10** were derived using the formula (k_c = (π x υ) / $2^{1/2}$) at the coalescence temperature (T_c). Spectra collected at temperatures below coalescence were used to determine the peak separation ($\Delta\upsilon$) between the two signals (Hamzi, Barhoumi-Slimi and Abidi, 2016).

When the temperature reached 100 ^{o}C, the *cis-trans* exchange process is sufficiently quick to become undetectable by NMR, resulting in a single broad signal. A complete coalescence of both *cis* and *trans* signals was observed for all groups at this temperature. The only signal that was visible was the average of the *cis* and *trans* conformers.

4.4 Antibacterial activity of N-acylhydrazones SB 1 – SB 10

4.4.1 Determination of minimum inhibition concentration (MIC)

All the synthesized *N*-acylhydrazones **SB 1 – SB 10** were used to evaluate their antibacterial activity against two Gram-positive bacteria (*Bacillus subtilis* and *Staphylococcus aureus*) and one Gram-negative bacteria (*Pseudomonas aeruginosa*) by using broth microdilution method. MIC value (µg/mL) is defined as the lowest concentration of the antibacterial agent needed to inhibit the visible growth of the bacteria tested. Streptomycin was utilized as a positive control. **Table 4.12** was the result of the MIC value of the synthesized *N*-acylhydrazones **SB 1 – SB 10**, and **Figure 4.26 to Figure 4.28** were the 96-well plate of each bacteria tested in *N*-acylhydrazones **SB 1 – SB 10**.

Based on the result collected, *N*-acylhydrazones **SB 1 – SB 9** showed poor antibacterial activity against *Bacillus subtilis*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* at MIC value of 250 µg/mL and 125 µg/mL respectively as compared to the positive control Streptomycin. Among them, *N*-acylhydrazone **SB 10** (2-OH, $4NEt_2$) showed moderate antibacterial activity against Gram-negative bacteria *Pseudomonas aeruginosa* at MIC value of 62.5 µg/mL. The moderate antibacterial activity of *N*-acylhydrazone **SB 10** (2-OH, $4NEt_2$) was due to the presence of the hydroxyl group at the *ortho* position of the phenyl ring.

Structure-activity relationships (SAR) evaluations led researchers to the conclusion that different aromatic ring substitutions significantly changed the antibacterial activity of the synthesized compounds. In comparison to compounds with electron-donating groups (methyl and methoxy), those with electron-withdrawing substituents in the aromatic ring (halo and nitro) demonstrated greater antibacterial activity. According to the results of the antibacterial assay from Gu W. et al. (2012), adding a halogen atom to the 4th position often improved antibacterial activity, especially that of the F and Cl atoms. Previous research found that adding halogen atoms to pharmacophore structures was frequently advantageous for antibacterial activity. However, *N*-acylhydrazones **SB 3** (4-Br), **SB 4** (2-Br), **SB 5** (4-F), **SB 6** (2-F), **SB 8** (4-NO_2) and **SB 9** (2-NO_2) did not show good antibacterial activity against the bacteria tested. Hence, the antibacterial activity can be concluded that must base on the characteristics of the *N*-acylhydrazones moiety since the structure of the synthesized *N*-acylhydrazones were different with the synthesized *N*-acylhydrazones in literature review.

CHAPTER 5

5.1 Conclusion

This study successfully synthesized carboxylic acid hydrazide, a key precursor for the synthesis of *N*-acylhydrazones, with a 46% yield. The condensation reaction of carboxylic acid hydrazide with a series of substituted benzaldehydes resulted in the synthesis of ten *N*-acylhydrazones, **SB 1 - SB 10**, with a yield which ranged from 55% to 88%. FT-IR, ^{1}H NMR, ^{13}C NMR, DEPT, NOE, HMQC and HMBC were used to characterize the structures of carboxylic acid hydrzide and *N*-acylhydrazones.

N-acylhydrazones **SB 1 – SB 9** showed poor antibacterial activity against *Bacillus subtilis*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* at MICvalue of 250 µg/mL and 125 µg/mL respectively as compared to the positive control streptomycin. Among them, *N*-acylhydrazone **SB 10** (2-OH, 4NEt$_2$) showed moderate antibacterial activity against Gram-negative bacteria *Pseudomonas aeruginosa* at MIC value of 62.5 µg/mL. The moderate antibacterial activity of *N*-acylhydrazone **SB 10** (2-OH, 4NEt$_2$) was due to the presence of the hydroxyl group at the ortho position of the phenyl ring.

5.2 Future studies

Different biological activities such as antifungal, antioxidant and antitumor can be further studied on the synthesized *N*-acylhydrazones **SB 1 – SB 10**. The compound synthesized can be analyzed by using Mass Spectrometry to determine the mass-to-charge ratio (m/z) and the exact molecular weight of the compounds. CHN analyser can be used to determine the carbon, hydrogen and nitrogen elemental content in the compounds.

REFERENCES

Balouiri M, Sadiki M, Ibnsouda SK., 2016. Methods for *in vitro* evaluating antimicrobial activity: A review. *Journal of Pharmaceutical Analysis,* 6(2), pp. 71-79.

Ceramella, J. et al., 2022. A Review on the Antimicrobial Activity of Schiff Bases: Data Collection and Recent Studies. *Antibiotics,* 11(2), p.191.

Gu W. et al., 2012. Synthesis and Antibacterial Evaluation of New *N*-acylhydrazone Derivatives from Dehydroabietic Acid. *Molecules*, 17(4), pp. 4634–4650.

Hamzi, I., Barhoumi-Slimi, T. M. and Abidi, R., 2016. Synthesis, characterization, and conformational study of acylhydrazones of α,β-unsaturated aldehydes: Synthesis, characterization, and conformational study of acylhydrazones of α,β-unsaturated aldehydes. *Heteroatom Chemistry*, 27(3), pp. 139–148.

Katariya, K.D., Shah, S.R. and Reddy, D., 2020. Anticancer, antimicrobial activities of quinoline based hydrazone analogues: Synthesis, characterization and molecular docking. *Bioorganic Chemistry*, 94, p.103406.

Kowalska-Krochmal, B. and Dudek-Wicher, R., 2021. The Minimum Inhibitory Concentration of Antibiotics: Methods, Interpretation, Clinical Relevance. *Pathogens*, 10(2), p.165.

Kuodis, Z., Rutavičius, A., Matijoška, A. and Eicher-Lorka, O., 2007. Synthesis and isomerism of hydrazones of 2-(5-thioxo-4,5-dihydro-1,3,4-thiadiazol-2-ylthio)acetohydrazide. *Open Chemistry*, Vol. 5 (Issue 4), pp. 996-1006.

Lopes, A. B. et al., 2013. Characterization of amide bond conformers for a novel heterocyclic template of *N*-acylhydrazone derivatives. *Molecules*, 18(10), pp. 11683–11704.

Ma, J., et al., 2015. Design, synthesis, biological evaluation and preliminary mechanism study of novel benzothiazole derivatives bearing indole-based moiety as potent antitumor agents. *European Journal of Medicinal Chemistry*, 96, pp.173–186.

Mali, S.N., Thorat, B.R., Gupta, D.R. and Pandey, A., 2021. Mini-Review of the Importance of Hydrazides and Their Derivatives—Synthesis and Biological Activity. *Engineering Proceedings*, 11(1), p. 21.

Mayurachayakul, P. et al., 2022. Catalyst- and solvent-free synthesis of *N* -acylhydrazones via solid - state melt reaction. *Asian Journal of Organic Chemistry*, 11(5). p. e202200117.

Medeiros, M.A.M.B. et al., 2021. Antinociceptive and anti-inflammatory effects of hydrazone derivatives and their possible mechanism of action in mice. *PLOS ONE*, 16(11), p.e0258094.

Mirfazli, S. S. et al., 2014. *N*-Substituted indole carbohydrazide derivatives: synthesis and evaluation of their antiplatelet aggregation activity. *Daru Journal of Faculty of Pharmacy*, 22(1), p. 65.

Mistry, S. and Singh, A.K., 2022. Synthesis and *in vitro* antimicrobial activity of new steroidal hydrazone derivatives. *Future Journal of Pharmaceutical Sciences*, 8(7), pp. 1-10.

Munir, R. et al., 2021. Synthesis of novel *N*-acylhydrazones and their C-N/N-N bond conformational characterization by NMR spectroscopy. *Molecules*, 26(16), pp. 4908-4925.

Narang, R., Narasimhan, B. and Sharma, S., 2012. A review on biological activities and chemical synthesis of hydrazide derivatives. *Current Medicinal Chemistry*, 19(4), pp. 569–612.

Özdemir, A. et al., 2015. Synthesis and evaluation of new indole-based chalcones as potential antiinflammatory agents. *European Journal of Medicinal Chemistry*, 89, pp.304-309.

Paruch, K. et al., 2021. Novel Derivatives of 4-Methyl-1,2,3-Thiadiazole-5-carboxylic acid hydrazide: synthesis, lipophilicity, and *in vitro* antimicrobial activity screening. *Applied Sciences*, 11(3), pp.1180-1191.

Popiołek Ł. et al., 2020. Synthesis of promising antimicrobial agents: hydrazide-hydrazones of 5-nitrofuran-2-carboxylic acid. *Chemical Biology & Drug Design*, 95(2), pp. 260–269.

Raczuk, E. et al., 2022. Different Schiff Bases—Structure, importance and classification. *Molecules*, 27(3), pp.787-810.

Sadia, M. et al., 2021. Schiff base ligand L synthesis and its evaluation as anticancer and antidepressant agent. *Journal of King Saud University - Science*, 33(2), pp.101331-101336.

Shamroukh, A.H. et al., 2013. Studies on the reactivity of amino-1-(6-phenyl-pyridazin-3-yl)-1*H*-pyrazole-4-carboxylic acid hydrazide towards some reagents for biological evaluation. *Journal of Heterocyclic Chemistry*, 51(4), pp.899-905.

Sundaree, S. et al., 2016. Synthesis and anticancer activity study of indolyl hydrazide–hydrazones. *Medicinal Chemistry Research*, 25(5), pp.941-950.

Wu, S. et al., 2021. Synthesis, crystal structure and bioactivity of phenazine-1-carboxylic acylhydrazone derivatives. *Molecules*, 26(17), pp. 5320-5331.

Yong, Y.Y. et al., 2018. Effect of refrigerated storage on betacyanin composition, antibacterial activity of red pitahaya (*Hylocereus polyrhizus*) and cytotoxicity evaluation of betacyanin rich extract on normal human cell lines. *LWT - Food Science and Technology*, 91, pp. 491–497.

Yuan, W. et al., 2019. Indole-core-based novel antibacterial agent targeting FtsZ. *Infection and Drug Resistance*, 12, pp. 2283–22.