SYNTHESIS, CHARACTERIZATION AND BIOLOGICAL STUDIES ON SYMMETRICAL 2,7-DISUBSTITUTED 9,9'-DIHEXYLFLUORENE DERIVATIVES WITH AMINE AND ETHER BRIDGING GROUPS

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by

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LIST OF ABBREVIATIONS

%	Percentage
μgmL^{-1}	Microgram per Milliliter
^{13}C NMR	Carbon Nuclear Magnetic Resonance
^{1}H NMR	Proton Nuclear Magnetic Resonance
$CDCL_3$	Deuterated Chloroform
CFU	Colony Forming Units
CO_2	Carbon Dioxide
COSY	Correlation Spectroscopy
d	Doublet
DEPT	Distortionless Enhancement by Polarization Transfer
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DPPH	2, 2-Diphenyl-1- picrylhydrazyl
EDG	Electron Donating Group
EWG	Electron Withdrawing Group
FBS	Fetal Bovine Serum
FDA	Food and Drugs Administration
FT-IR	Fourier-Transform Infrared
FT-NMR	Fourier Transform Nuclear Magnetic Resonance
HEK293	Human Embryonic Kidney 293
HeLa	Human Epithelial Carcinoma Cell Line
HMBC	Heteronuclar Multiple Bond Correlation

HMQC	Heteronuclear Multiple Quantum Correlation
HT-29	Human Colorectal Adenocarcinoma Cell Line
Hz	Hertz
IC_{50}	The Half Maximal Inhibitory Concentration
J	Coupling Constant
K_2CO_3	Potassium Carbonate
KI	Potassium Iodide
kPa	Kilopascal
M	Molar
m	Multiplet
MCF-7	Michigan Cancer Foundation-7 (breast adenocarcinoma cells)
MHA	Mueller-Hinton Agar
MHz	Megahertz
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
NCI	National Cancer Institute
nm	Nanometer
ºC	Degree Celcius
PBS	Phosphate Buffered Saline
ppm	Part per million
q	Quartet
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
s	Singlet
SDA	Sbouraud Dextrose Agar
t	Triplet

UV	Ultraviolet
Vero	Verda Rino (Green African monkey kidney cells)
WHO	The World Health Organization
δ	Chemical Shift
λ	Lambda (wavelength)
π	Pi
σ	Sigma

SINTESIS, PENCIRIAN DAN KAJIAN BIOLOGI PADA TERBITAN SIMETRI 2,7-DIPENUKARGANTIAN 9,9'-DIIHEKSILFLUORENA YANG MENGANDUNGI KUMPULAN TITIAN AMINA DAN ETER

ABSTRAK

Tiga terbitan fluorena baru yang dinamakan 1,1'-(9,9-diheksil-9H-fluorena-2,7-diyl)bis(*N*,*N*-bis(piridina-2-metil)metanmina (**FP1**), 1,1'-(9,9-diheksil-9H-fluorena-2,7-diyl)bis(*N*,*N*-bis(benzo[d]thiazol-2-metil)metanmina (**FT2**) dan 8,8'-(((9,9-diheksil-9H-fluorena-2,7-diyl)bis(metilena))bis(oxi))diquinolina (**FQ3**) telah berjaya disintesis dengan hasil yang memuaskan iaitu 60 %, 66 % dan 63 %, masing-masing. Struktur molekul bagi kesemua sebatian yang disintesis telah dijelaskan melalui spektroskopi FT-IR, ^{1}H NMR, ^{13}C NMR, DEPT, ^{1}H-^{1}H COSY, ^{1}H-^{13}C HMQC, ^{1}H-^{13}C HMBC. Ciri pendarfluor bagi kesemua sebatian telah dikaji melalui UV-Vis dan spektroskopi pendarfluor. Kajian biologi termasuk aktiviti *in-vitro* sitotoksik, antimikrob dan antioksidan bagi kesemua sebatian telah disiasat. Aktiviti *in-vitro* sitotoksik bagi kesemua sebatian telah diuji terhadap sel kanser serviks manusia (HeLa), dengan menggunakan 5-fluororacil sebagai ubat standad (IC_{50} = 27.82 µg/mL). **FP1** menunjukkan aktiviti sitotoksik dengan menghasilkan nilai IC_{50} bersamaan 28.58 ± 0.05 µg/mL manakala **FT2** dan **FQ3** menunjukkan tiada aktiviti untuk sel kanser HeLa dengan nilai IC_{50} didapati bersamaan 141.13 ± 0.03 µg/mL dan 223.81 ± 0.02 µg/mL, masing-masing. Aktiviti antioksidan ditunjukkan melalui aktiviti penangkap radikal DPPH dimana kesemua sebatian didapati mempamerkan aktiviti antioksidan yang rendah. Sementara itu, kajian aktiviti antimikrob bagi kesemua sebatian yang disintesis dikendalikan melalui kaedah penyerapan disk yang

dijalankan ke atas *Staphylococcus aureus, Bacillus subtilis, Escherichia coli, Candida albicans* dan *Saccharomyces cerevisiae*. Kloramfenikol digunakan sebagai kawalan positif dan zon perencatan direkodkan dalam milimeter. **FP1** dan **FT2** didapati menunjukkan aktiviti antimikrob yang baik terhadap semua mikroorganisma yang diuji manakala **FQ3** menunjukkan tiada aktiviti kecuali pada kepekatan 10 mg/mL, dimana ia menunjukkan aktiviti sederhana ke atas yis *Saccharomyces cerevisiae*.

SYNTHESIS, CHARACTERIZATION AND BIOLOGICAL STUDIES ON SYMMETRICAL 2,7-DISUBSTITUTED 9,9'-DIHEXYLFLUORENE DERIVATIVES WITH AMINE AND ETHER BRIDGING GROUPS

ABSTRACT

Three new fluorene derivatives namely, 1,1'-(9,9-dihexyl-9H-fluorene-2,7-diyl)bis(*N*,*N*-bis(pyridine-2-ylmethyl)methanamine (**FP1**), 1,1'-(9,9-dihexyl-9H-fluorene-2,7-diyl)bis(*N*,*N*-bis(benzo[d]thiazol-2-ylmethyl)methanamine (**FT2**) and 8,8'-(((9,9-dihexyl-9H-fluorene-2,7-diyl)bis(methylene))bis(oxy))diquinoline (**FQ3**) have been successfully synthesized in moderate yield 60 %, 66 % and 63 %, respectively. The molecular structure of all the synthesized compounds were elucidated via FT-IR, ^{1}H NMR, ^{13}C NMR, DEPT, ^{1}H-^{1}H COSY, ^{1}H-^{13}C HMQC, ^{1}H-^{13}C HMBC spectroscopy. Fluorescence properties of all the final compounds were examined by UV-Vis and fluorescence. Biological studies including *in-vitro* cytotoxicity, antimicrobial and antioxidant activities of the title compounds were investigated. The *in-vitro* cytotoxic activity of the title compounds was evaluated against human cervical (HeLa) cancer cell line, with 5-fluororacil used as standard drug (IC_{50} = 27.82 µg/mL). **FP1** demonstrated cytotoxic activity with IC_{50} value 28.58 ± 0.05 µg/mL while **FT2** and **FQ3** showed no activity for HeLa cell as the IC_{50} values were determined to be 141.13 ± 0.03 µg/mL and 223.81 ± 0.02 µg/mL, respectively. Antioxidant activity of the target compounds was demonstrated by DPPH radical scavenging activity where the title compounds were found to exhibit very low scavenging activity. Meanwhile, Antimicrobial test of all the title compounds was conducted by disc diffusion assay performed on *Staphylococcus*

aureus, Bacillus subtilis, Escherichia coli, Candida albicans and *Saccharomyces cerevisiae*. Chloramphenicol was used as positive control and the inhibitory zones were recorded in millimeters. **FP1** and **FT2** were found to have good antimicrobial activity against the tested microorganisms while **FQ3** showed no activity except for concentration at 10 mg/mL, where it shows moderate activity on the yeast *Saccharomyces cerevisiae*.

CHAPTER 1

INTRODUCTION

1.1 Fluorene

The hydrocarbon fluorene has been known for more than seven decades. It was first discovered by a French chemist, Marcellin Berthelot when he carried out a research on the pyrogenetic hydrocarbons at the College de France in 1867 (Rieveschl and Ray, 1938). Fluorene was isolated from anthracene oil fraction of coal tar which boiled between 270 ºC and 310 °C (Hartnell, 1979). It was then recrystallized from boiling alcohol to afford white fluorescent laminae which melted at 113 °C. Berthelot was impressed by its beautiful fluorescence and named it as “Fluorene”. Its odor was described as being “insipid, sweetish, and at the same time distressing to breathe”. In the early of 20th century, the amount of investigation on fluorene remains quite scanty as compared to other hydrocarbons such as anthracene or phenanthrene.

Fluorene ($C_{13}H_{10}$) is a polycyclic aromatic hydrocarbon that has three rings covalently bonded together (Figure 1.1). The term 'polycyclic' refers to the multiple rings involved, 'aromatic' denotes the presence of benzene rings, and 'hydrocarbon' shows that the molecule contains only carbon and hydrogen atoms.

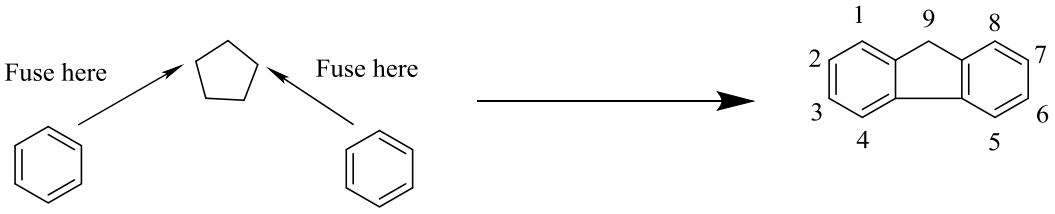


Figure 1.1 Chemical structure and atomic numbering of fluorene.

Since seven decades ago, a considerable amount of studies on fluorene had been carried out by X-ray crystallography. The planarity of the molecule is the most significant point of difference in the structure analyzed. The molecule is of interest from a biological point of view because of its relation to the carcinogenic dibenzofluorenes, and it is crucial to know whether these compounds have planar molecules similar to the carcinogenic compounds based on 1,2-benzanthracene. The first X-ray crystal structure investigation of fluorene was reported by Hengstenberg *et al.* in I929, followed by other researchers in the following years before it arrived at the conclusion that the molecule is planar (Burns and Iball, 1955). The methylene bridge in the fluorene enhanced the planarity of the two phenyl rings. As such, it increased the overlap of the orbitals and the degree of conjugation of the aromatic system (Abbel, 2008). Hence, fluorene absorbs at longer wavelengths than other compounds with closely related structure.

The classical method for the synthesis of fluorene is intramolecular Friedel−Crafts alkylation (Figure 1.2) promoted by Brønsted or Lewis acids (Xu *et al*., 2015). Friedel–Crafts alkylations are among the most powerful C–C bond-forming processes for the synthesis of functionalized aromatic compounds (Sarkar *et al*., 2012). Intramolecular reactions normally occur much more easily as compared to intermolecular reaction.

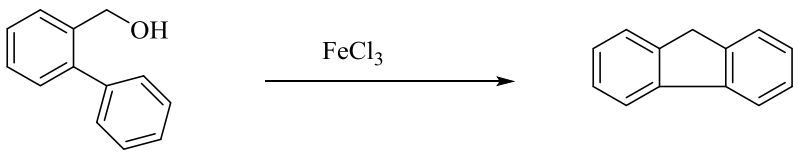


Figure 1.2 Intramolecular Friedel-Crafts alkylation for the synthesis of fluorene.

In recent years, transition-metal-mediated cyclization for the synthesis of fluorene (Figure 1.3) have been extensively investigated as a promising route utilizing a C-H bond activation strategy (Liu *et al*., 2010; Morimoto *et al*., 2012; Hwang *et al*., 2009). It is apparent that the key to accessing fluorene derivatives is the construction of carbon−carbon bonds. The cleavage of carbon-carbon σ-bonds by transition-metal complexes and their use for chemical transformation would provide a conceptually new strategy in organic synthesis.

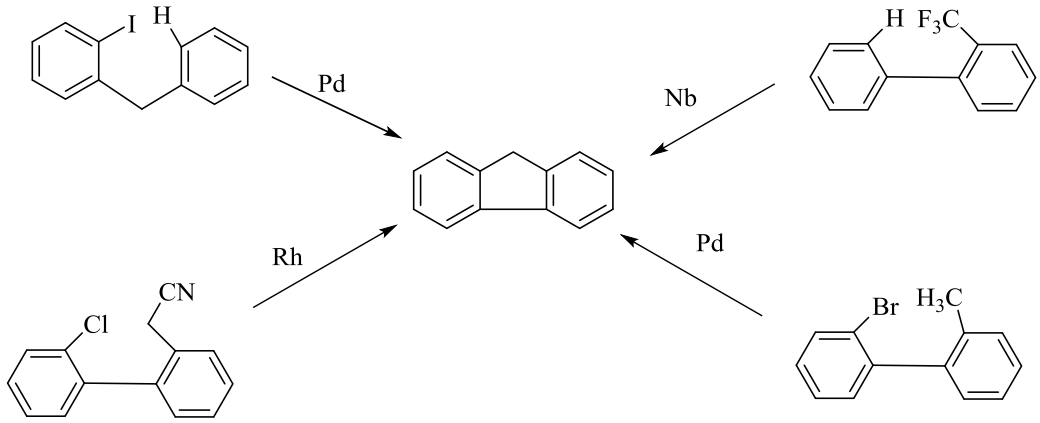


Figure 1.3 Precedent metal-mediated routes to a fluorene skeleton. (Campeau *et al*., 2006; Fuchibe and Akiyama, 2006; Tobisu *et al*., 2008; Dong and Hu, 2006)

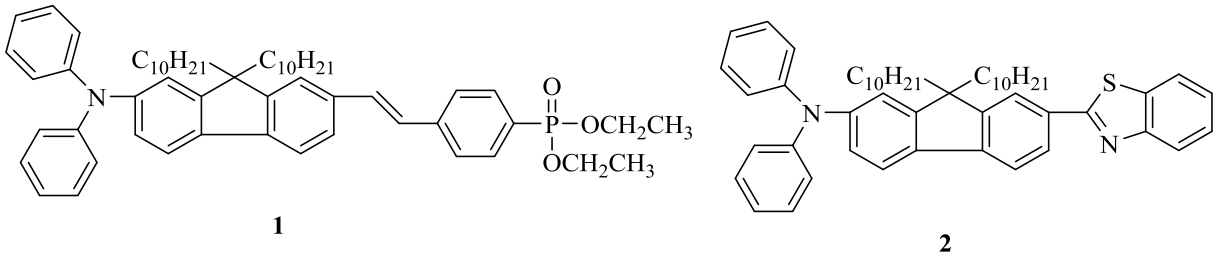
However, previous studies had shown that some of these methods have many limitations and disadvantages including the reactions were inclined to backward, non-availability of the substrates, drastic reaction conditions, uses of toxic and expensive chemicals as well as production of large amounts of waste, which limit their applications in industry. Therefore, the simple, straightforward, high yielding, practical, and environmentally friendly method for the synthesis of substituted fluorene derivatives are still in high demand and continuously developed by many researchers.

1.1.1 Fluorene Derivatives

An extensive research on fluorene to explore its potential applications in various areas had been carried out. The alteration or introduction of some fragments to the fluorene may result in enhancing its application in different areas. Fluorene derivatives received a huge attention due to their luminescent and electroluminescent properties, caused by the inter- and intramolecular charge distribution. In the industrial sector, fluorene could be a good candidate for blue light-emitting and hole-transporting materials, 'naked eye' sensors for sensing various metal ions and as efficient multifunctional chemosensor-filtering devices (Li *et al*., 2004; Hung *et al*., 2016). Besides, fluorene had also been regarded as potential candidate for semiconducting applications as well as essential agent in solar cells (Hayashi *et al*., 2009; Chandrasekharam *et al*., 2011). A simple, low-cost and efficient fine chemical is among the important criteria needed for industrial use. On the other hand, the development of the production in drugs and pharmaceuticals has brought the fluorene compound to the vast attention of the researchers from all over the world. This can be exemplified through the testing of fluorene compounds with various alteration and substitution that showed numerous biological applications such as interaction of DNA with fluorene, antibacterial, anticancer and antiproliferative.

Although fluorene contains benzene rings which are known to be highly carcinogenic to human, but many established drugs for the treatment of various diseases are prepared from benzene-containing compounds. Many reports had claimed that alteration of the structure of polycyclic aromatic hydrocarbons could mitigate their deleterious effects, emphasizing their interaction with specific cell organelles to evoke specific cytotoxic reactions (Banik *et al*., 2010). As the result,

many fluorene, carbazoles, anthracenes, and related structures are in current clinical use.

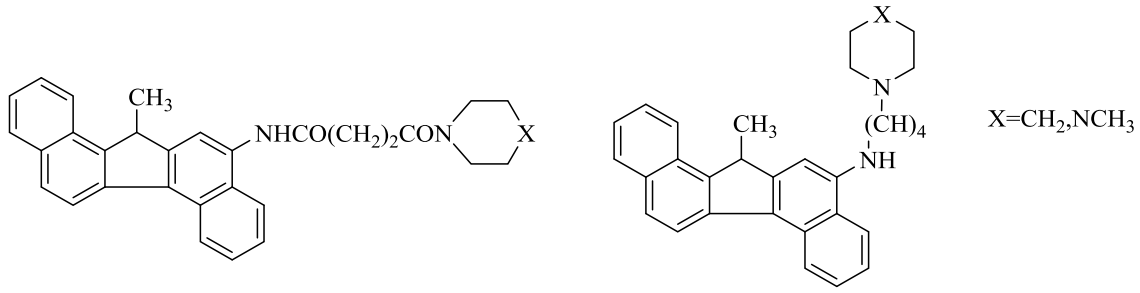


1 **2**

Figure 1.4 Fluorene dyes **1**-**2** with substitution at 9-position and 2,7-position demonstrate interaction with DNA. (Przonska *et al*., 2004)

Facile modification of fluorene at the 9-position allows for the introduction of alkyl moiety or other functional groups into the fluorene as well as to induce steric hindrance which helps in improving its optical characteristic (Goodman *et al*., 2005; Sannasi *et al*., 2015). Studies on fluorene dyes **1**-**2** in Figure 1.4 as conducted by Przonska *et al*. with the substitution at 9-position and 2,7-position showed the breakage of the helix structure of both normal and tumor DNAs as well as the partial denaturation with the luminescence evidence upon injection of the dyes.

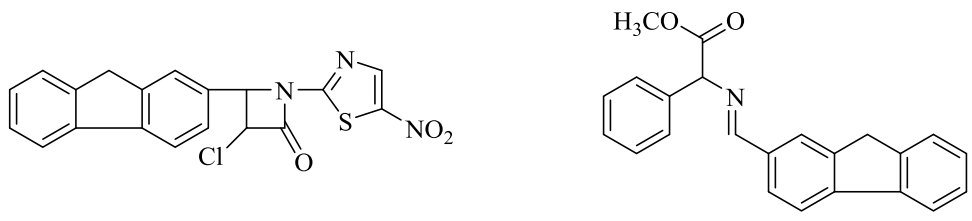
Studies of some fluorene derivatives with various substitution positions that exhibit positive cytotoxic activities had well been reported. In 2009, Banik *et al*. synthesized a 4-carbon side chain with a heterocyclic base at the end of the aromatic ring in fluorene through nitrogen (Figure 1.5) and evaluation of its cytotoxicity against a number of tumor cell lines. Besides, Marinova *et al*. has reported the studies on the synthesis, cytotoxicity and other biological tests of fluorene derivative wherein the cell viability decreased significantly after being treated with the compounds (Marinova *et al*., 2013; Marinova *et al*., 2016). This observation indicated that the compounds could behave as potential anticancer agents.



3 **4**

Figure 1.5 Dibenzofluorene derivatives exhibit cytotoxic activity.

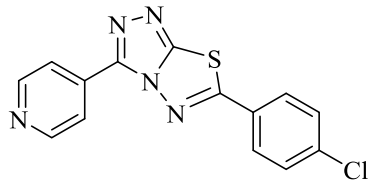
Beside cytotoxic activity, antimicrobial and antioxidant activities are among the preferred biological tests of fluorene compounds. Ahmad *et al*. synthesized some heterocyclic fluorene compounds derived from Schiff base (Figure 1.6 **5**) and evaluated their activity *in vitro* growth inhibitory against a standard strain of pathogenic microorganism including Gram–positive bacteria and Gram-negative bacteria. The result showed that the studied compounds effectively inhibit the growth of bacteria (Ahmad *et al*., 2015). The research on fluorene derivatives that had been conducted by Yorur-Gorecy *et al*. and Thirunarayanan in 2016 and 2017, respectively, showed good free radical scavenging activity (Figure 1.6 **6**) by inhibiting DPPH radical in a concentration-dependent manner (Yorur-Goreci *et al*., 2016; Thirunarayanan, 2017).



5 **6**

Figure 1.6 Fluorene derivatives which contains electron donating functional moiety substituted at 7-position **5**. Fluorene derivative substituted at 2-position **6**.

Compounds introduced to fluorene also played an important role in enhancing the effectiveness of the fluorene derivatives toward its biological properties. Heterocyclic compounds itself have always been on the forefront of attention because of their numerous uses in pharmaceutical applications (Kaur, 2015). Typical hetero atoms include nitrogen, oxygen, and sulfur. Nitrogen, oxygen and sulfur-containing heterocycles have a special interest because they constitute an important class of natural and non-natural products, many of which exhibit useful biological activities. However, nitrogen-containing heterocycle is the most preferable and its unique structures had led to several applications in different areas (Sondhi *et al*., 2005; Bhuiyan *et al*., 2006). Besides, small-ring nitrogen and sulfur-containing heterocycles (Figure 1.7) had also been investigated for a long time owing to their synthetic diversity and therapeutic relevance. Structure **7** in Figure 1.7 displays the chemical representation of synthesized nitrogen heterocycle with proven antiproliferative activity, with superior selectivity for gastric cancer cell lines whilst structure **8** in Figure 1.7 shows chemical representation of synthesized benzimidazole hybrid heterocycles with superior selectivity for leukemia cell lines. Food and Drugs Administration (FDA) databases had revealed the structural significance of nitrogen-based and sulfur-based heterocyclic compounds in the drug design and engineering of pharmaceuticals with nearly 60% of unique small-molecule drugs containing a nitrogen heterocycle (Martins, 2015).



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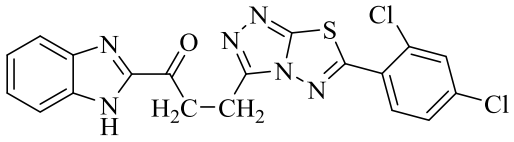

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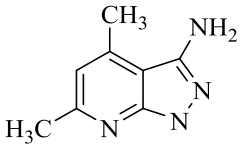
Figure 1.7 Synthetized triazolo[1,3,4]thiadiazole derivative, 6-(4-chlorophenyl)-3-(pyridin-4-yl)[1,2,4]triazolo[3,4-b] **7** (Kamel and Abdo, 2014). Synthetized benzimidazole hybrid heterocycles **8** (Husain *et al*., 2013).

Among all the heterocycles, pyridine, thiazole and quinoline have attracted interest as these heterocycles possess tremendous applications in medicinal field. Pyridine is a basic heterocyclic organic compound with the chemical formula C_5H_5N and has a conjugated system of six π-electrons resembling benzene, that are delocalized over the heterocyclic ring. Nitrogen-containing six-membered aromatic pyridine and its derivatives (Figure 1.8) play a vital role in the field of heterocyclic chemistry and at the same time are claimed to be widely used for many applications in medicinal science such as anticancer, antimicrobial and antidiabetic (Altaf *et al*., 2015). Similar to the pyridine, quinoline is also a nitrogen-based heterocycle that received a broad attention from researchers in different field of studies (Figure 1.9). The ring system of quinoline exists in alkaloids, therapeutics and synthetic analogues with exciting biological activities such as anticancer, anti-bacterial as well as antioxidant activities (Miri et al., 2011; Marella *et al*., 2013; Orhan Puskullu *et al*., 2013).



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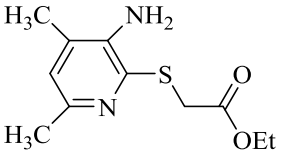

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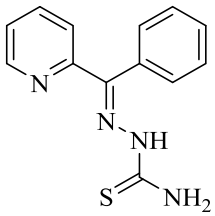
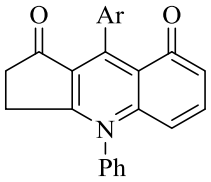


11

Figure 1.8 4,6-dimethyl-1l2-pyrazolo[5,4-b]pyridin-3-amine **9**, ethyl 2-((3-amino-4,6-dimethylpyridin-2-yl)thio)acetate **10** and 2-(phenyl(pyridin-2-yl)methylene) hydrazine1-carbothioamide **11**.



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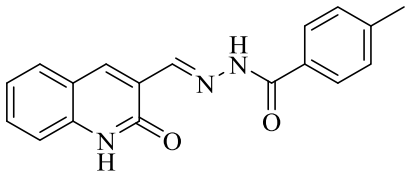
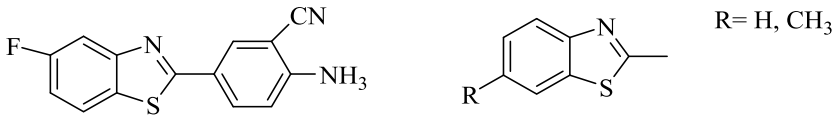


13

Figure 1.9 Cyclopenta[*b*]quinoline-1,8-dione derivatives **12**. 2-Oxo-1,2-dihydro quinoline-3-carbaldehyde (4'-methylbenzoyl) hydrazine **13**.

On the other hand, benzothiazole is a heterocycle containing both nitrogen and sulfur. It has been identified to play an important role in medical chemistry. Its derivatives are associated with a wide range of biological properties such as antimicrobial, antimalarial, anticancer, hypertension as well as in the application related to the drug development for the treatment of allergies (Figure 1.10) (Cabrera-Pérez *et al*., 2016; Sharma *et al*., 2013).



14 **15**

Figure 1.10 Representative examples of antitumor **14** and antioxidant compounds **15** containing benzothiazole as basic unit.

In view of the importance of fluorene and heterocyclic compounds primarily in biological applications such as anticancer, antimicrobial and antioxidant, the synthesis and engineering on the new compounds of 9,9'-dihexylfluorene with heterocyclic substituents at 2,7-substituted position has continued to be explored by the researchers.

1.2 Biological Activities

1.2.1 Cytotoxicity Activity

Cytotoxicity test is one of the biological screening tests that uses tissue cells *in vitro* to observe the cell growth, reproduction and morphological effects by medical devices (Li *et al*., 2015). Cytotoxic is defined as toxic or detrimental to cells. The prefix cyto- connotes a cell, origins from the Greek word kytos meaning hollow, as a cell or container. Toxic is from the Greek toxikon which means poison. Besides *in vivo*, *in vitro* test is the alternative method into non-clinical safety testing that economize time and costs, as well as to protect animals. Currently, animal tests are mandatory for the evaluation of acute toxicity of chemicals and new drugs. The replacement of *in vivo* test by alternative *in vitro* assays would offer the opportunity to screen and access numerous compounds or extracts at the same time, to predict

acute oral toxicity and thus increase drug development processes as well as to show a proactive pursuit of ethical and animal welfare issues.

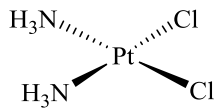
Cancer is a life threatening disease and remains a tremendous health problem all over the world. According to The World Health Organization (WHO), it is estimated that there were 7.6 million deaths due to cancer in 2008 and this number is likely to rise to 13.1 million deaths by the year 2030 (Farooqui *et al*., 2013). Cervical cancer has been reported as one of the common cancers that could lead to the death of Malaysian female cancer patients and the number of cervical cancer is increasing gradually (Othman *et al*., 2009). Cancer cells differ from normal cells in many ways that allow them to grow out of control and become invasive. The significant difference is that cancer cells are less specialized than normal cells. Normal cells mature into very distinct cell types with specific functions while cancer cells do not. In addition, cancer cells are able to ignore signals that normally tell cells to stop dividing or that begin a process known as programmed cell death, or apoptosis, which the body uses to get rid of unneeded cells. To be specific, apoptosis is a process by which cells are systematically destroyed and removed without elicits inflammatory response. Cells undergo orderly typical morphological changes such as membrane blebbing, shrinkage, chromatin condensation and phagocytosis of apoptosis bodies (Manahan, 2002). Cancer drugs act upon rapidly dividing cancer cells and destroy them. There are many kinds of anticancer drugs discovered such as alkylating agents, anti-metabolites, anti-microtubule agents, topoisomerase inhibitors and cytotoxic agents (Swift and Golsteyn, 2014; Mihlon *et al*., 2010).

In vitro cytotoxicity screenings provide important preliminary data to confirm compounds synthesized with potential anticancer properties (Ping, 2014). The IC_{50} value shows the inhibition concentration at which only 50% of the cells are viable.

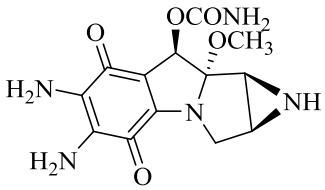
The parameters measured were according to the National Cancer Institute (NCI) guidelines in which IC_{50}< 30 μgmL^{-1} is considered active (Aini *et al*., 2008). Cell-based assays using established cell lines are commonly employed in which the toxic effects of compounds can be evaluated. Some of the common cell lines used in the cytotoxicity study including Vero (green African monkey kidney cells), MCF-7 (breast adenocarcinoma cells), HT-29 (colon adenocarcinoma cells), HEK293 (human embryonic kidney-derived epithelial cells) and HeLa (cervical adenocarcinoma cells). In this study, HeLa was chosen as cell lines for *in vitro* cytotoxicity screening of all compounds synthesized. HeLa cells were the first line of human cells to survive *in vitro* (in a test tube) grown by a researcher named Dr. George Gey in 1951 (Lucey *et al*., 2009). After more than 50 years, there are now billions of HeLa cells in laboratories all over the world. It's the most commonly used cell line known to be extremely resilient.

The development of potent and effective novel antineoplastic drugs is one of the most intensely persuaded goals of contemporary medicinal chemistry. The discovery of new synthetic compounds shows the importance of an extensive cooperation between industry and cancer institute (Schwartsmann *et al*.,1988). Synthetic compounds are usually obtained from pharmaceutical industries and academic institutions. Cisplatin, or *cis*-diamminedichloroplatinum(II) (Figure 1.11 **16**), that serve as alkylating agent, is one of the examples of a successful synthesized compound widely used as anticancer drugs. It is one of the most active anticancer agents that have ever been introduced for clinical use (Ellahioui *et al*., 2017). Most of the anticancer drugs that serve as alkylating agent are simple in structure. Smaller molecule makes it easier to bind with the double helix of DNA. For cytotoxic drug, mitomycin C (Figure 1.11 **17**) is among the examples of commercial cytotoxic drugs

classes available in market. Mitomycin C drugs act upon rapidly dividing cancer cells by selectively inhibit the synthesis of deoxyribonucleic acid (DNA).



16



17

Figure 1.11 Cisplatin **16** and Mitomycin C (MC) **17**.

However, the effectiveness of many existing cytotoxic drugs, sometimes known as antineoplastic drugs is limited by their toxicity to normal rapidly growing cells or limited by the identification of unique biochemical aspects of malignancies that could be exploited to selectively target tumor cells. It has been reported that over 600,000 compounds screened with cytotoxicity by then, less than 40 agents were routinely used in the clinic (Ajit, 2009). Compounds synthesized must be selectively toxic on cancer cells, which mean toxic to cancer cells but non-toxic to normal cells.

1.2.2 Antioxidant Activity

Antioxidants are substances or chemicals that interact with and neutralize free radicals, thus preventing them from causing damage to cells. Free radical is an atom or molecule that bears an unpaired electron and is extremely reactive, capable of engaging in rapid change reaction that destabilize other molecules and generate many more free radicals. Having an unpaired electron makes the molecule highly unstable and in order to become more stable, free radicals attack other molecule to form pairs with other electron and left that molecule as free radicals. Excessive free radicals such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) may be

harmful as they can initiate biomolecular oxidations which lead to cell injury and death, and create oxidative stress (Chew and Lim, 2018). As the result, it will lead to numerous diseases and disorders such as cancer, aging, arteriosclerosis and arthritis. Some free radicals are generated in the human body when oxidation occurs during aerobic respiration while some other free radicals are derived from external sources such as tobacco smoke, ultra-violet light, ionizing radiation and environmental pollutants.

ROS is a type of free radicals that contain the oxygen element. It is the most common type of free radicals produced in living tissue. Examples of ROS include superoxide ($O_2•^-$), peroxyl (ROO•), hydrogen peroxide (H_2O_2), alkoxyl (RO•) and hydroxyl ion (OH^-). ROS are the contributors of oxidative stress when present at high level of concentration in which it become toxic and disrupt the antioxidant defense system of the body as well as oxidize nucleic acids, proteins, lipids or DNA that result in initiating degenerative diseases (Darkwah *et al*., 2018). The increased production of ROS is considered a universal feature of stress conditions where oxidative stress can be defined as "state in which oxidation exceeds the antioxidant systems in the body secondary to a loss of the balance between them" (Yoshikawa and Naito, 2002). Examples of some ROS are listed in Table 1.1 below.

Table 1.1 Common Reactive Oxygen Species (ROS)

$HO_2•$	Hydroperoxyl
$CO_3•$	Carbonate
HOBr	Hypobromous acid
$ONOO^-$	Peroxynitrite
NO•	Nitric oxide
CIO^-	Hypochlorite ion

Antioxidants are the main defense mechanism in the body acting as free radical scavengers. Antioxidants can be grouped into three classes depending on its abilities in human body (Mehta and Gowder, 2015). The first class of antioxidants is the work done by the enzymes to control initial free radical production. For example, superoxide, hydrogen peroxide, and hydroxyl radicals are formed upon aerobic respiration. Enzymes such as catalase, dismutase and glutathione peroxidases will take action on those radicals either to decrease their formation or to remove them. These enzymes require metal cofactor to function. Secondly, we need antioxidants intake as the antioxidants produced naturally by the body are inadequate to neutralize all of the free radicals in the body. These antioxidants intake are important in human body as they are capable of ending the chain reaction of free radicals through proton donation. The third class of antioxidant is replenishers. The source of protons comes from structures that can readily donate a proton while remaining stable so as not to become a free radical. Examples of replenishers include carotenoids, flavonoids coenzyme Q and glutathione.

In this study, antioxidant test of all the synthesized compounds were conducted by DPPH scavenging activity method. This method was introduced in 1958 by Marsden Blois who was working at Stanford University. DPPH scavenging method become popular in antioxidant studies as it is a rapid, simple, highly sensitive and inexpensive method to measure antioxidant capacity by using the free radical, 2,2-Diphenyl-1- picrylhydrazyl (DPPH) (Shekhar and Anju, 2014). Figure 1.12 below shows DPPH upon accepting electron from antioxidant and reduced to a stable molecule. DPPH is a purple chromagen stable free radical that accepts an electron or hydrogen radical to become a stable diamagnetic molecule. DPPH scavenging activity assay is based on the theory that a hydrogen donor is an antioxidant. In this

assay, DPPH is reduced by antioxidant/reducing compounds to the corresponding pale yellow hydrazine (Boligon *et al*., 2014). The free radical DPPH with an odd electron gives a maximum absorption at 517 nm (purple colour). The purple colour turns to yellow followed by the formation of DPPH upon absorption of hydrogen from an antioxidant. This reaction is stoichiometric with respect to the number of hydrogen atoms absorbed. Therefore, the antioxidant effect can be easily evaluated by following the decrease of UV absorption at 517 nm.

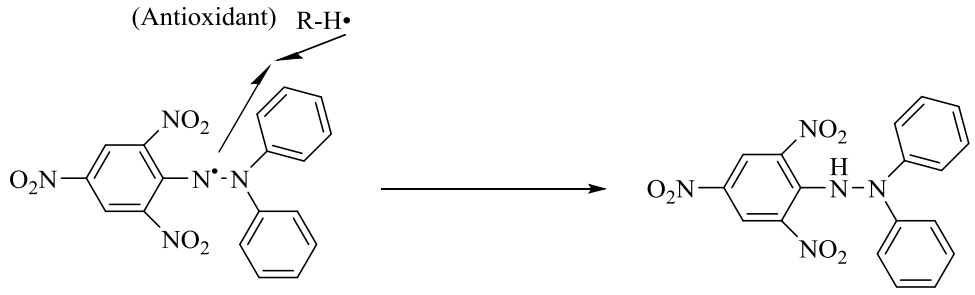


Figure 1.12 Structure of DPPH and reduced DPPH.

1.2.3 Antimicrobial Activity

Antimicrobial can be defined as any substance of natural, synthetic or semisynthetic that kills or inhibits the growth of microorganisms but causes little or no damage to the host. The history of antimicrobial discoveries improved before the 20th century. Louis Pasteur, a French chemist and microbiologist, proved the "Germ Theory", which proposed that certain diseases are caused by specific microbes (Pouyan, 2014). This theory was suggested long before the discovery of bacteria, but did not receive general acceptance. In 1864, Pasteur demonstrated that microorganisms arise from living "germs" rather than from nonliving matter. From the studies, Pasteur made a conclusion that there are a great variety of microorganisms, each capable of reproducing its own kind. According to this theory,

different diseases are caused by different type of microorganisms. In other words, microorganisms are responsible for a variety of infectious diseases that had been afflicting mankind from ancient days.

One of the important medical treatments is antimicrobial chemotherapy. A German scientist named Paul Ehrlich was the first to investigate antibacterial dyes (Sumthong and Verpoorte, 2007). After a few failures, Ehrlich came up with his 606^{th} preparation of an arsphenamine compound in 1910, to be the first chemical compound in the world shown to cure a human disease. The compound later named as salvarsan, a remedy for syphilis. In 1928, Alexander Fleming discovered an antibiotic named penicillin which established into the clinical used in 1940s. Antibiotic is described as compounds isolated from one living organism which could kill or inhibit the growth of other organisms. All antibiotics are antimicrobials, but not all antimicrobials are antibiotics. From his study, Fleming found that a fungus from the *Penicillium* genus, which is *Penicillium* mold inhibited the growth of *Staphylococcus aureus* bacteria in a petri dish. This finding lead to the discovery that microorganism would produce substances that could inhibit the growth of other microorganisms. Penicillin is known as a miracle drug in terms of safety and efficacy which led to the golden age era of antimicrobial chemotherapy. Realizing the importance of antimicrobial therapeutic in medication, a number of subsequent antimicrobial discoveries by many other researchers continue to develop. Figure 1.13 below shows the evolution of penicillin years by years.

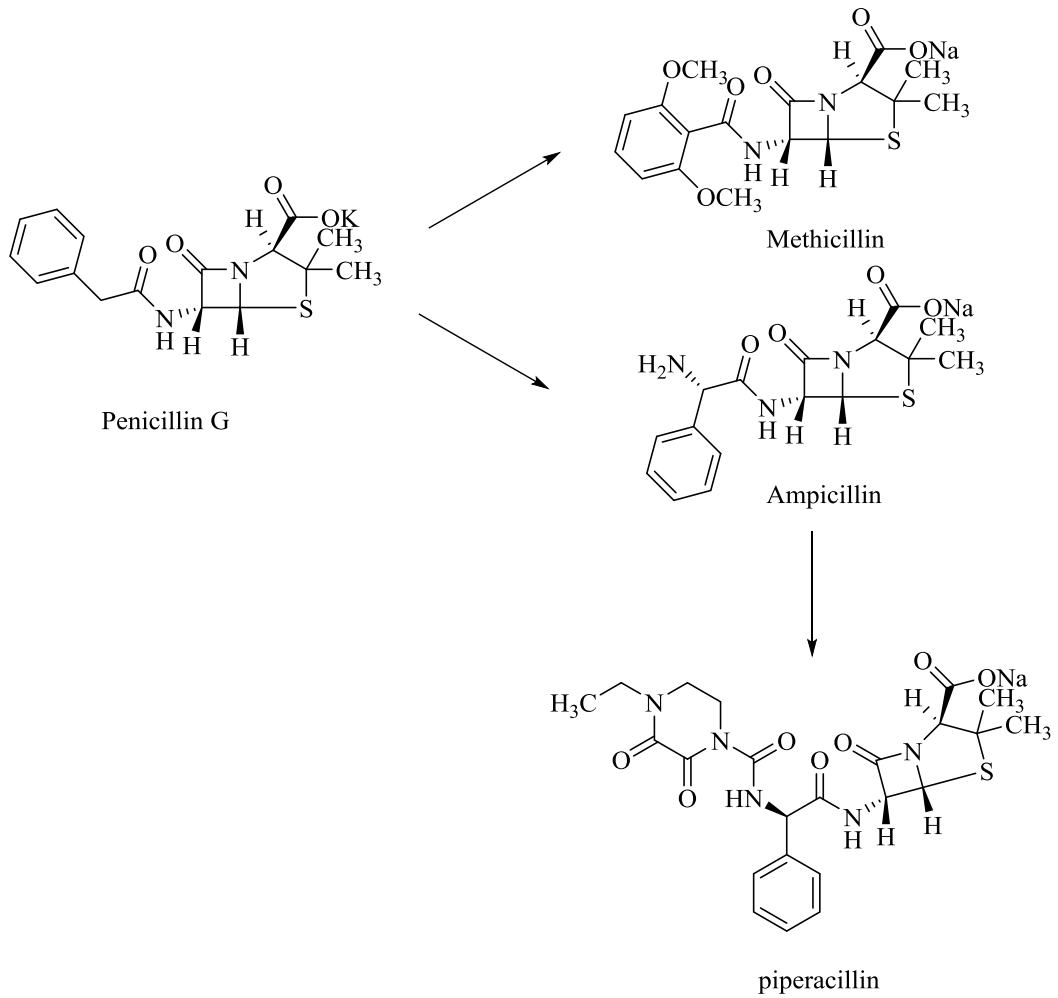
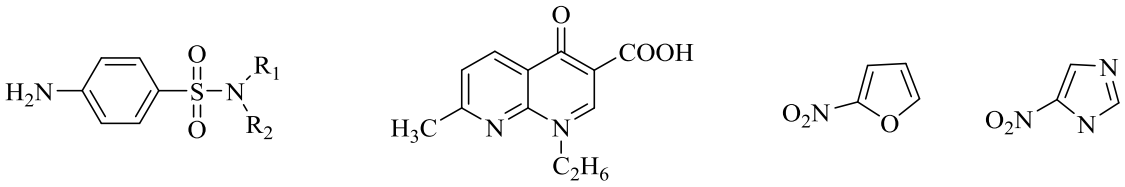


Figure 1.13 Evolution of penicillin. (Saga and Yamaguchi, 2009)

There are several synthetic antimicrobial agents that were synthesized based on model compounds sulfonamides, quinolones and nitroheterocyclic (nitrofurans, metronidazole) compounds (Figure 1.14). Sulfonamide was first discovered in 1935 by Gerhard Domagk (Yousef *et al*., 2018). Sulfonamide is a synthetic red dye popularly known by its trade name of Prontosil (Yousef *et al*., 2018). This discovery resulted in the effectiveness in treating killer diseases such as meningitis, child bed fever and pneumonia. Since then, sulfonamide derivatives are extremely useful and synthesized until today (Yousef *et al*., 2018). On the other hand, quinolones are a group of synthetic antibacterial agents derived from nalidixic acid. Nalidixic acid was accidently discovered in 1962 as a byproduct from chloroquine, an antimalarial

agent. Realizing the importance of quinolone, research and development of its derivatives continuously develop. Nitroheterocyclic compounds such as nitrofurans and nitroimidazoles played an important role to treat bacterial infection. The nitrofurans are derivatives of 5-nitro-2-furaldehyde, that show antimicrobial activity only when the nitro group is located at the 5-position. Metronidazole is a nitroimidazole structure containing nitro group. The nitro group is reduced to NH_2 after entry to bacterial cell which makes concentration gradient for more metronidazole molecules to flow inside the bacteria. The aromatic amine can attack DNA and cause bacterial death (Reeves, 2012).



18 **19** **20**

Figure 1.14 Antibacterial sulfonamide's common core structure **18**. Nalidixic acid **19**. Nitroheterocyclic. (nitrofurans and metronidazole) **20**.

Bacteria evolve in some way that reduces or eliminates the effectiveness of drugs or chemicals designed to cure infections, thus become resistant. WHO define antimicrobial resistance as microorganisms such as bacteria, viruses, fungi and parasites develop the ability to defeat the effect of the drugs designed to kill them. There are two types of antimicrobial resistance which are intrinsic resistance and adaptive or acquired resistance (Molchanova *et al*., 2017). Intrinsic resistance is the ability of the pathogen to resist the antibacterial treatment due to inherent structural or functional properties. Meanwhile, the ability of the bacteria to adapt to non-lethal conditions by rapidly altering their transcriptomes in response to a stressful

environment is best described for adaptive resistance. As such, a continuous development of antimicrobial agents is crucial in order to sustain the effectiveness of the antimicrobial agents and save lives. However, researchers encounter several problems that lead to the slow development of new antimicrobial agent such as time consuming and the high cost. On an average, research and development of anti-infective drugs takes around 15-20 years, and can cost more than $1000 million (Rai *et al*., 2013). Synthesis of newer antimicrobial compound have to be discovered and introduced, not only based on model compounds of sulfonamides, quinolones and nitroheterocyclic (nitrofurans, metronidazole) compounds, but variety of chemical classes of compounds.

1.3 Problem Statement

There has been vast research conducted on fluorene to explore its potential application in biological area including cytotoxicity, antioxidant and antimicrobial activities. However, the synthesis and investigation on biological application of fluorene derivatives with symmetrical heterocyclic substitution at 2,7-position remain quite scanty. This research aims to introduce heterocyclic pyridine, benzothiazole and quinoline, respectively with amine and ether as bridging groups to the core system of fluorene derivative at symmetrical 2,7-position. In addition, the introduction of these heterocyclic are expected to enhance the compounds to be more potent cytotoxicity, antioxidant and antimicrobial agents.

1.4 Research Objectives

The overall objectives of this study are shown as follow:

i. To synthesize a series of new symmetrical 2,7-disubstituted 9,9'-dihexylfluorene derivatives with amine and ether bridging groups.

ii. To elucidate the structure of all synthesized compounds using FT-IR and NMR as well as their fluorescence properties using UV-Vis and fluorescence spectroscopy.

iii. To investigate the cytotoxicity of all synthesized compounds based on MTT assay against HeLa cell line. To study antioxidant activity of all synthesized compounds by DPPH scavenging activity assay and antimicrobial activity by disc diffusion assay against two Gram-Positive bacteria (*Staphylococcus aureus* and *Bacillus subtilis*), one Gram-Negative bacteria (*Escherichia coli*) and two yeasts (*Candida albicans* and *Saccharomyces cerevisiae*).

1.5 Scope of the Studies

The focuses of this study are to synthesize new fluorene derivatives with heterocyclic substituents at 2,7-position via amine and ether bridging groups, respectively and to investigate their potential anticancer, antioxidant and antimicrobial applications. The first and second fluorene derivatives are substituted with heterocyclic pyridine and benzothiazole, respectively via amine bridging group. The third fluorene derivative is substituted with heterocyclic quinoline with ether bridging group. All of the synthesized compounds are characterized by using nuclear magnetic resonance (NMR), infrared spectroscopy (IR), UV-Visible spectrophotometer and fluorescence spectroscopy. NMR spectroscopy is one of the

principle techniques used to obtain structural information by determining the chemical shifts in ^{1}H and ^{13}C nuclei of the synthesized compounds based on 1D and 2D-NMR experiments. IR is used for identifying the main functional groups in the compounds. UV-Vis is performed to identify the absorption maxima (λ_{max}) in the UV spectra of all synthesized compounds before fluorescence is carried out to confirm that the synthesized compounds exhibit fluorescence.

Potential biological application including cytotoxicity, antioxidant and antimicrobial were investigated on all the synthesized compounds. *In vitro* cytotoxicity test is carried out as preliminary data to confirm compounds synthesized with potential anticancer properties with IC_{50} value showing the inhibition concentration at which only 50% of the cells are viable. Antioxidant test is conducted by DPPH scavenging activity assay to investigate the antioxidant activity of all the synthesized compounds. Antimicrobial test is conducted by disc diffusion assay on several microorganism including Gram-Positive bacteria, Gram-Negative bacteria and yeasts to explore the antimicrobial activity of all synthesized compounds.

CHAPTER 2

EXPERIMENTAL

2.1 Chemicals

The chemicals reagent used for the synthesis of all intermediates and final compounds are given in Table 2.1.

Table 2.1 Chemicals, manufacturer name and the percentage purity (%)

Chemicals	Manufacturer	Percentage Purity (%)
8-hydroxyquinoline	Across Organics, Belgium	99.0
Potassium Iodide	Fisher Scientific, UK	>99.0
Potassium Carbonate	Quality Reagent Chemicals, New Zealand	99.0
2,7-Bis(bromomethyl)-9,9-dihexyl-9H-fluorene	Sigma Aldrich, USA	97.0
Bis(2-pyridylmethyl)amine	Tokyo Chemical Industry, Japan	>98.0
2-aminobenzenethiol	Tokyo Chemical Industry, Japan	>95.0
Iminodiacetontrile	Tokyo Chemical Industry, Japan	>98.0
Quinoline	Tokyo Chemical Industry, Japan	>98.0