SYNTHESIS OF BLOOD-BRAIN BARRIER PERMEABLE AURONES AS MODULATORS OF MICROGLIA

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SYNTHESIS OF BLOOD-BRAIN BARRIER PERMEABLE AURONES AS MODULATORS OF MICROGLIA

by

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LIST OF ABBREVIATIONS

µg	Microgram
µM	Micromolar
^{13}C	Carbon-13
^{1}H	Proton
ACN	Acetonitrile
AD	Alzheimer's disease
ALS	Amyotrophic lateral sclerosis
BBB	Blood-brain barrier
cAMP	Cyclic adenosine monophosphate
CNS	Central Nervous System
CNS-	Low BBB permeation
CNS+	High BBB permeation
CNS+/-	Uncertain BBB permeation
CO_2	Carbon dioxide
DI	Deionized water
DMEM	Dulbecco's Modified Eagle's Medium
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
ESI+	Positive electrospray ionization
FBS	Fetal bovine serum
FDA	Food and Drug Administration
h	Hour
HCl	Hydrogen chloride
HD	Huntington's disease
HIV	Human immunodeficiency virus
HO-1	Heme oxygenase-1
HPLC	High Performance Liquid Chromatography
IGF-1	Insulin-like growth factor-1
IL-10	Interleukin-10

IL-1β	Interleukin-1 beta
IL-6	Interleukin-6
iNOS	Inducible nitric oxide
K_2CO_3	Potassium carbonate
KOH	Potassium hydroxide
L-NOHA	N omega-hydroxyl-L-arginine
LOD	Limit of Detection
LOQ	Limit of Quantitation
LPS	Lipopolysaccharide
M	Molarity
MeOH	Methanol
mg	Milligram
MHC	Major Histocompatibility Complex
mins	Minutes
mM	Millimolar
MS	Multiple sclerosis
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
Na_2SO_3	Sodium sulfite
NDs	Neurodegenerative diseases
NMR	Nuclear Magnetic Resonance
NO	Nitric oxide
Nrf2	Nuclear factor erythroid 2-related factor 2
NVU	Neurovascular unit
O.D.	Optical density
PAMPA	Parallel Artificial Membrane Permeability Assay
PBL	Porcine Brain Lipid
PBS	Phosphate buffer solution
PD	Parkinson’s disease
P_e	Effective permeability
pg	Picogram

PGE_2	Prostaglandin E_2
PI3K	Phosphatidylinositol 3-kinase
PVDF	Polyvinylidene fluoride
r^2	Correlation coefficients
ROS	Reactive oxygen species
rt	Room temperature
SAR	Structure-Activity Relationship
TGF-β	Tissue growth factor-beta
TNF-α	Tumour necrosis factor-alpha
t_R	Retention time
UV	Ultraviolet
VWD	Variable Wavelength Detector
$ZnCl_2$	Zinc chloride
δ	Delta

LIST OF APPENDICES

SINTESIS AURON TELAP RINTANGAN OTAK-DARAH SEBAGAI MODULATOR MIKROGLIA

ABSTRAK

Peradangan saraf umumnya merupakan salah satu ciri dimiliki pelbagai penyakit neurodegeneratif (NDs). Mikroglia memainkan peranan penting sebagai punca yang memburukkan lagi keadaan penyakit. Sulfuretin, suatu sebatian auron semula jadi, telah dilaporkan mempunyai sifat anti-radang yang kuat terhadap pengaktifan makrofaj dan mikroglia. Dalam kajian ini, satu siri sebatian auron yang menggabungkan amina dan kumpulan berfungsi lipofilik pada gelang A dan/atau gelang B telah disintesis bertujuan menambahbaik aktiviti sulfuretin ke arah menyasarkan mikroglia otak di samping mengatasi rintangan otak-darah (BBB). Kesan auron yang disintesis pada sel mikroglia tikus BV-2 yang dirangsang oleh lipopolisakarida (LPS) telah diuji dan modulasinya kepada fenotip M1 yang diaktifkan dan fenotip alternatif M2 yang diaktifkan dicirikan. Selain itu, kebolehtelapan BBB aurone telah ditentukan dengan menggunakan model PAMPA-BBB. Keputusan menunjukkan bahawa beberapa auron memaparkan aktiviti perencatan yang ketara terhadap paras nitrik oksida (NO) lebih daripada 50% pada 1 hingga 10 μM ($p < 0.01$ berbanding kawalan). Perencat yang berpotensi diwakili oleh sebatian auron dengan moieti yang besar dan planar pada gelang A (**3f**) atau pada gelang B (**1e** dan **1f**) dan mempunyai satu kumpulan piperidina pada gelang B (**1a**, **2a**, **2b**, dan **3f**). Auron aktif menghalang polarisasi mikroglia BV-2 ke arah keadaan M1 yang ditunjukkan oleh pengurangan penanda mikroglia M1 dalam rembesan IL-1β dan TNF-α daripada mikroglia yang diaktifkan LPS. Bagaimanapun, sebatian-sebatian berkenaan tidak mendorong mikroglia ke arah keadaan M2. Seterusnya, auron **2a**, **2b**, dan **1f** telah menunjukkan kebolehtelapan BBB pasif yang tinggi [P_e (10^{-6} cm/s) = 11.71 ± 2.05;

11.83 ± 2.92; 13.68 ± 2.32 masing-masing] dalam PAMPA mungkin disebabkan lipofilik optimumnya. Secara keseluruhan, kajian ini menunjukkan bahawa auron terpilih secara selektif berupaya menghalang polarisasi mikroglia kepada keadaan pro-radang terutamanya **2a**. Sebatian **2a** didapati tidak toksik terhadap sel, berketelapan BBB yang tinggi, serta poten mewakili sebatian peneraju baru dalam usaha pembangunan auron sebagai perencat mikroglia diaktifkan.

SYNTHESIS OF BLOOD-BRAIN BARRIER PERMEABLE AURONES AS MODULATORS OF MICROGLIA

ABSTRACT

Neuroinflammation is widely recognized as one of the characteristic hallmarks of many neurodegenerative diseases (NDs) and microglia have been implicated to play both causative and exacerbating roles. Sulfuretin, a naturally occurring aurone, has been reported to have potent anti-inflammation property against macrophage and microglia activation. In the present study, a series of aurones incorporating basic amines and lipophilic functionalities at ring A and/or ring B were synthesized to improve upon present sulfuretin activity towards targeting brain microglia while overcoming the blood-brain barrier. The effects of the synthesized aurones on LPS-stimulated BV-2 microglial cells were examined and their modulation to an activated M1 phenotype and alternatively activated M2 phenotype were characterized. Also, the BBB permeability of the aurones were determined using the parallel artificial membrane permeability assay for blood-brain barrier (PAMPA-BBB) model. The results revealed that several aurones showing significant inhibitory activities against nitric oxide (NO) levels of more than 50% at 1 to 10 μM ($p < 0.01$ versus control). The potent inhibitors were represented by aurones with bulky, planar moieties at ring A (**3f**) or at ring B (**1e** and **1f**) and having a pendant piperidine at ring B (**1a, 2a, 2b**, and **3f**). The active aurones inhibited the BV-2 microglia polarizing towards the M1 state were indicated by attenuation of M1 microglia markers IL-1β and TNF-α secretions in LPS-activated microglia. However, they did not induce the microglia towards the M2 state. Next, the aurones **2a**, **2b**, and **1f** showed high passive BBB permeability [P_e (10^{-6} cm/s) = 11.71 ± 2.05; 11.83 ± 2.92; 13.68 ± 2.32 respectively] in PAMPA possibly owing to their optimal lipophilicities. Taken altogether, this study indicates that the selected

aurones selectively inhibit the microglia polarization to a pro-inflammatory state, particularly **2a**. Compound **2a**, being non-cell toxic, BBB permeant and potent, represents a new lead for the development of aurones as inhibitors of activated microglia.

CHAPTER 1

INTRODUCTION

1.1 Neurodegeneration and neuroinflammation

Worldwide, neurodegenerative diseases (NDs) including Alzheimer's disease (AD), Parkinson's disease (PD), multiple sclerosis (MS), Huntington's disease (HD) and amyotrophic lateral sclerosis (ALS) have accounted for a large and increasing health burden. According to the non-profit group of Alzheimer's Disease International, AD is expected to reach 135 million cases globally by 2050, up from the present 44 million cases. Also, in accordance with the World Health Organization (WHO), NDs, particularly AD and other dementia will soon overtake cancer and ranked second leading cause of mortality after cardiovascular disease by 2040. The growing numbers and the rising burden of NDs are well recognized globally. Extensive research efforts across the academic, non-profit and industry sectors are therefore hard at work to halt or slow these neurodegenerative problems. Neurodegeneration, in general, is characterized by a slow and progressive loss of neuronal damage in specific regions of the brain and spinal cord. It represents the pathological condition of most NDs, commonly characterized by impaired cognitive and movement function. Particularly, increased oxidative stress, deposition of protein aggregates, neuroinflammation and mitochondrial dysfunction, induction of apoptosis and alteration of autophagy have been widely described as the main cellular and molecular events involved in the onset and development of NDs pathophysiology (Guzman-Martinez et al., 2019; Li et al., 2013; Sweeney et al., 2017).

Among the events, neuroinflammation, the innate immune response within the central nervous system (CNS), a common pathological hallmark of major NDs have been highlighted as the key driver (Muhammad et al., 2019). Inflammation is referred to an important biological response to harmful stimuli including autoimmune diseases, infectious agents, damaged cells and irritants (Schramm et al., 2018). This response is a defence mechanism that initially participates in homeostatic and neuroprotective processes by eliminating or inhibiting diverse pathogens, promote tissue repair and remove cellular debris. There are fundamentally two types of inflammation: acute and chronic. Acute inflammation comprises the immediate and rapid reaction to a harmful stimulus and it is essentially a defence reaction that paves the way for the restoration of the damaged site. In contrast, chronic inflammation is referred to a slow and persistent inflammation for a prolonged period (Streit et al., 2004). Long standing chronic neuroinflammatory responses, however, are detrimental as it would trigger a cascade of events including regeneration inhibition, leading to progressive neuronal damage and eventually causing neurodegeneration via sustained accumulation of neurotoxic pro-inflammatory mediators in the brain.

1.1.1 Pathogenesis of Neuroinflammation: Role of Microglia

Fundamentally, neuroinflammation is famously in relation with the involvement of glial cells, particularly microglia. Microglia makes up the innate immune system of central nervous system and play a critical role as one of the key cellular mediators of the inflammatory responses, thereby becoming the most integral part for any discussion on inflammation-mediated neurodegeneration (Glass et al., 2010; Liu et al., 2003). In depth, microglia are able to acquire different phenotypes, in order to mediate

multiple aspects of neuroinflammation, such as cytotoxicity, repair, regeneration, and immunosuppression. Traditionally, microglial polarization could be classified into two opposing states, the classical, proinflammatory M1 phenotype and the alternative, anti-inflammatory M2 phenotype depending on their activation status as shown in Figure 1.1 (Guo et al., 2022; Orihuela et al., 2016; Wang et al., 2021). Hence, it is vital to note that neuroinflammation, characterized by activation of glial cells could play a dual role in the CNS, bringing about either neurotoxic or neuroprotective effects. In response to any insult or CNS stimuli, resting microglia are activated and triggered to release various pro-inflammatory mediators, including reactive oxygen species (ROS), pro-inflammatory enzymes, and a number of cytokines and chemokines (e.g., interleukin (IL)-1β, IL-6, tumour necrosis factor-alpha (TNF-α) and others). Under normal physiological circumstances, the acute inflammatory response is short and brings about a return to normal homeostasis via mediation of anti-inflammatory cytokines and several pro-resolving lipid mediators once the trigger has been eliminated. The chronic neuroinflammation, characterized by dysregulation and overactivation of microglia, however, leads to neurotoxic consequences where decomposition and death of neurons and glial cells take place. In other words, the persistent and uncontrolled inflammatory cascades in the brain mediated by glial activation have consequently interfered with homeostatic integrity and resulted in pathological progression in NDs through the impact on neuronal plasticity and cognitive decline.

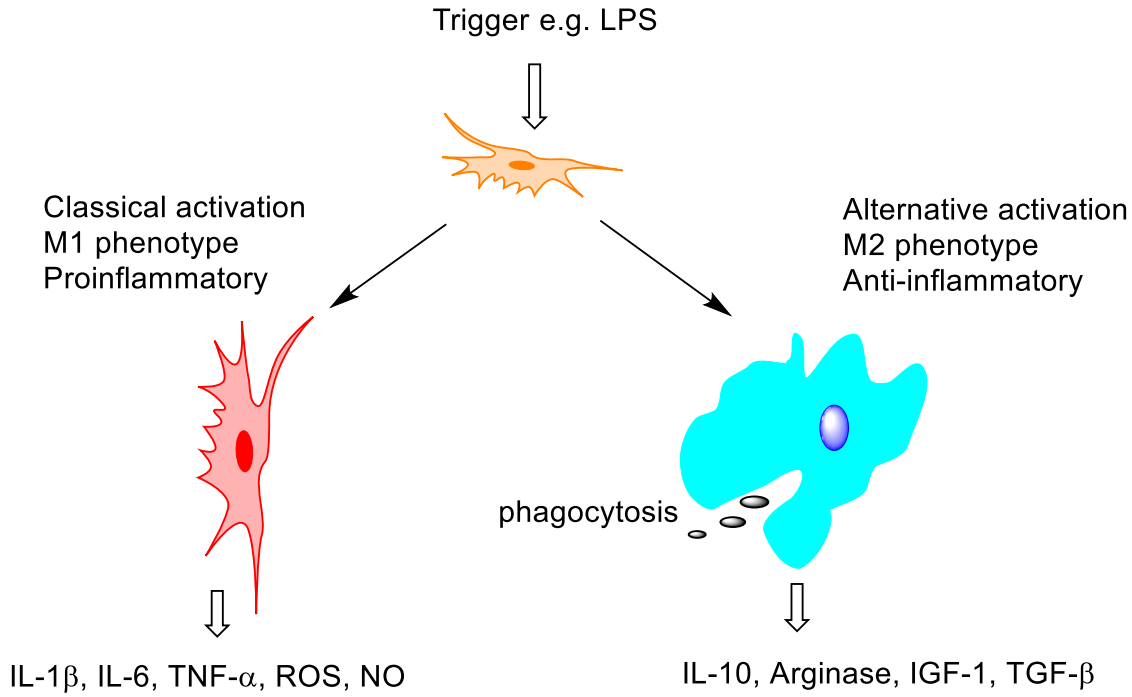


Figure 1.1 Activation of microglia into M1 and M2 phenotypes. LPS: Lipopolysaccharide, IL-1β: interleukin-1beta, IL-6: interleukin-6, IL-10: interleukin-10, TNF-α: tumour necrosis factor α, ROS: reactive oxygen species, NO: nitric oxide, IGF-1: insulin-like growth factor, TGF-β: tissue growth factor.

The research in this topic was initially motivated by several neuropathological findings in AD and PD brains. For instance, in brains of AD patients, reactive microglia were found to colocalize with neuritic plaques in the cortical region of AD brains (Haga et al., 1989; Itagaki et al., 1989; Rogers et al., 1988). Meanwhile, large numbers of reactive microglia expressing MHC II receptors were found in the substantia nigra, a pivotal region of PD pathogenesis where dopaminergic neurons were most prominently degenerating (McGeer et al., 1988). Since then, further extensive studies have established the association of microglial activation with the pathogenesis of multiple neurodegenerative disorders including ALS, MS, human immunodeficiency virus (HIV) acquired immunodeficiency syndrome dementia

complex and other related nervous system pathologies (Brown, 2001; Dickson et al., 1993; Raine, 1994).

1.1.2 Potential agents as inhibitors of microglia-mediated neuroinflammation

Given that the microglial polarization states have been concerned in relation with the pathogenesis of NDs, a list of agents with different targets had been identified as the potential approaches to deal with the microglial activation. For instance, the drugs of Candesartan cilexetil (Daniele et al., 2015; Liu et al., 2019) and rifampin and its autooxidation product rifampicin quinone (Acuña et al., 2019) has been identified as microglial inhibitor through regulation or modification of TLRs receptors on microglia. Drug targeting CB2 receptor such as β-caryphyllene (Javed et al., 2016; Ojha et al., 2016) and JWH133 (Chung et al., 2016) were also been mentioned to elicit significant neuroprotective effects against glial activation. Besides, bioactive flavonoids including Tanshinone I (Wang et al., 2015b) and α-asarone from Annonaceae and Araceae species (Kim et al., 2015) were cited to provide an alternative strategy to regulate microglia M1 activation via targeting JAK/STAT and NF-κB signalling pathways. Some other microglial regulating drugs such as apocynin, resveratrol and diphenyleneiodonium which suppress the microglial activation through targeting NADPH oxidase were also found as promising candidates for clinical trials in NDs' patients (Choi et al., 2012; Sharma and Nehru, 2016; Wang et al., 2014; Wang et al., 2015a; Zhang et al., 2010). Apart from these, other agents including minocycline (Du et al., 2022; Kobayashi et al., 2013; Wang et al., 2020), dextromethorphan (Cheng et al., 2015), etanercept (Wu et al., 2016), glatiramer acetate (Ratchford et al., 2012) and lenalidomide (Valera et al., 2015) are capable of modulating the inflammatory,

attenuating the production of pro-inflammatory cytokines from microglia thereby inhibiting their activation. Recent studies had also revealed a series of plant-derived bioactive compounds with excellent anti-inflammatory properties such as curcumin (Ghasemi et al., 2019), ginsenoside Rg1 (Heng et al., 2016; Zhou et al., 2015), piperine (Wang-Sheng et al., 2017), rosmarinic acid (Lv et al., 2019; Wei et al., 2018) to ameliorate microglia-mediated neuroinflammation. The discovery of these agents has been an imperative step forward into the medicinal field, but their potential applications in inhibiting the microglial activation is yet far-reaching. Therefore, further investigations into these agents are required in order to develop safe and effective treatments for diseases associated with microglial activation.

1.1.3 Current treatment modalities

Currently, the treatments for NDs mostly aim to provide symptomatic relief but are not effective in curtailing disease progression. These conventional treatments include dopaminergic treatments for PD and movement disorders, cholinesterase inhibitors for cognitive disorders, antipsychotic drugs for dementia, anti-inflammatory and analgesic for neuronal infections and pain (Chaudhuri and Schapira, 2009; Desai and Grossberg, 2005; Rees et al., 2011). The effects were consistent with the findings by Finkel (2004) and Lee et al. (2015), where among the few Food and Drug Administration (FDA) approved drugs, acetylcholinesterase inhibitors such as donepezil and rivastigmine, used for palliative treatments could only slow the course of AD but not for long term. Also, Bonuccelli et al. (2002) cited that dopamine agonists including pergolide and bromocriptine (Parlodel^{R}) were therapeutically effective, yet causing cardiovascular and endocrinological problems. Among several therapeutic strategies,

neuroimmunomodulatory therapies have been raised as one of the potential therapies in the management of disease progression. This approach is primarily emphasised more on pathogenesis of neuroinflammation mediated neurodegeneration. For instance, anti-inflammatory medications like minocycline, resveratrol, tanshinone and silymarin offer therapeutic promises against PD by inhibiting the NADPH oxidase and microglial activation and pro-inflammatory cytokine release (Hussain et al., 2018; Rees et al., 2011). Taken altogether, including the roles of microglia activation in implicating the neuroinflammation and neurodegeneration, from a therapeutic point of view, a compound that inhibits the microglia and modulates polarization towards neuroprotective M2 phenotype would be desirable.

1.2 Overview of Sulfuretin and its anti-inflammatory potentials

Flavonoids, a diverse group of polyphenols found in natural products have been valued for a long time in scientific community due to their wide spectrum of biological qualities particularly their anti-inflammatory capacities. From a drug discovery perspective, flavonoids are considered a privileged scaffold. Sulfuretin, a major aurone isolated from the heartwood of the Chinese lacquer tree (*Toxicodendron verniciflumm*) has demonstrated several pharmacological effects in recent reports. In general, sulfuretin is amorphous orange in colour and structurally contains the aurone scaffold with three hydroxyl groups at the C-6, C-3' and C-4' positions (Figure 1.2). Lee et al. (2012) and Song et al. (2010) had reported remarkable anti-inflammatory activity of sulfuretin in mice models of arthritis and allergic airway inflammation. Several studies had also emphasised the neuroprotective potential of sulfuretin where it had attenuated the lipopolysaccharide (LPS)-induced inflammatory secretions by

RAW264.7 murine macrophages (Shin et al., 2010) and BV-2 murine microglial cells (Cho et al., 2012; Hong et al., 2012; Seung-Hwan et al., 2014). The literature review shows that sulfuretin significantly suppressed the production of pro-inflammatory mediators including nitric oxide (NO), inducible nitric oxide synthase (iNOS), prostaglandin E_2 (PGE_2), cyclooxygenase-2 (COX-2), tumour necrosis factor-alpha (TNF-α) and interleukin-1beta (IL-1β). Furthermore, this aurone was also reported to show protective effects against neurotoxicity in an *in vitro* model of PD as well as improving AD by activating the nuclear factor erythroid 2-related factor 2 (Nrf2)/ heme oxygenase-1 (HO-1) and phosphatidylinositol 3-kinase (PI3K)/Akt signalling (Kwon et al., 2015; Kwon et al., 2014). On the basis of above studies, it is anticipated that sulfuretin might be promising therapeutic candidate against inflammation-mediated NDs.

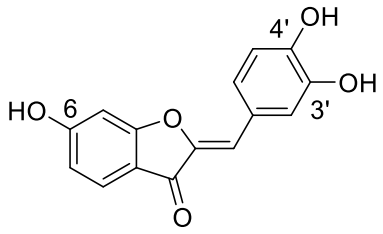


Figure 1.2 Structure of sulfuretin, the lead compound for the present study.

1.3 Problem statement and hypotheses

The purpose of the study is to further investigate on aurones as potential anti-inflammatory drug candidates, followed by fostering the development of novel synthetic analogues. In view of underrepresented in nature, aurones have been overlooked in comparison to other subclasses of flavonoids. However over last two decades, aurones have been increasingly receiving attention among the scientific community owing to their ability to modulate several biological pathways (Mazziotti et al., 2021).

In the present study, sulfuretin, one of the members of aurone, was chosen as a lead compound and its effective neuroprotective potential was explored. As described in the previous part, emerging evidence suggests the potential role of aurone sulfuretin against neurodegeneration due to its of anti-inflammatory and neuroprotective properties. However, the reports on the promising therapeutic effects of the sulfuretin remains scarce particularly with respect to microglial-mediated neuroinflammation. Moreover, despite this versatile display of anti-inflammatory action, the studies regarding the effective concentrations of sulfuretin in the microglial cells were limited and there is little cytotoxicity effect was shown at 10 μM in the preliminary work. Consequently, this makes up for the problem in determining a safe yet promising dosage in counteracting the inflammation. Also, there is a further challenge with the drug-like property of the compound. Even though the anti-inflammatory activity of sulfuretin is broadly examined experimentally, but there are limited studies regarding the drug-like property of the compound, particularly the in BBB permeability to the brain. Given its structure and using an online predictor (https://www.cbligand.org/BBB/predictor.php, (Liu et al., 2014)), sulfuretin was predicted to show poor permeation (Figure 1.3) across the blood-brain barrier (BBB)

which would greatly hinder its ability to target microglial cells within the CNS. Therefore, using sulfuretin as the lead, we aimed to improve upon sulfuretin's present microglia activity as well as its pharmacokinetic properties particularly its BBB permeability through the incorporation of basic amine moieties into the aurone scaffold, a strategy that has been gave fruitful results in previous medicinal chemistry projects (Liew et al., 2015; Liew et al., 2017). In depth, the versatility of aurone scaffold lies in the simplicity of their molecular and physiochemical descriptors including molecular mass, topological polar surface area and lipophilicity would provide a well-suited platform for which to introduce additional key functionalities while maintaining reasonable drug-likeness in compliance with the Lipinski rule-of-five (Lipinski et al., 1997).

Simultaneously, the common limitations of natural products such as poor availability, time-consuming and cost-ineffectiveness during isolation and purification could be alternatively resolved. Also, the development of novel synthetic compounds sharing the common motifs of sulfuretin lies in our objective in investigating the chemical agents able to target activated microglia. Current literatures (Kwon and Koh, 2020; Wang et al., 2021) had suggested that microglial activation is indeed pathogenesis of NDs and it was recognised as the viable therapeutic target of neuroinflammation-mediated neurodegeneration. Therefore, same goes to the analogous, their potential in regulating microglia M1/M2 polarization were explored meanwhile emphasizing their drug-like profiles particularly the across blood-brain barrier (BBB) simultaneously. The analysis of structure-activity relationships may also provide a useful insight in seeking a novel therapeutic approach against NDs, via determining the structural and functional peculiarities of the best performing derivative.

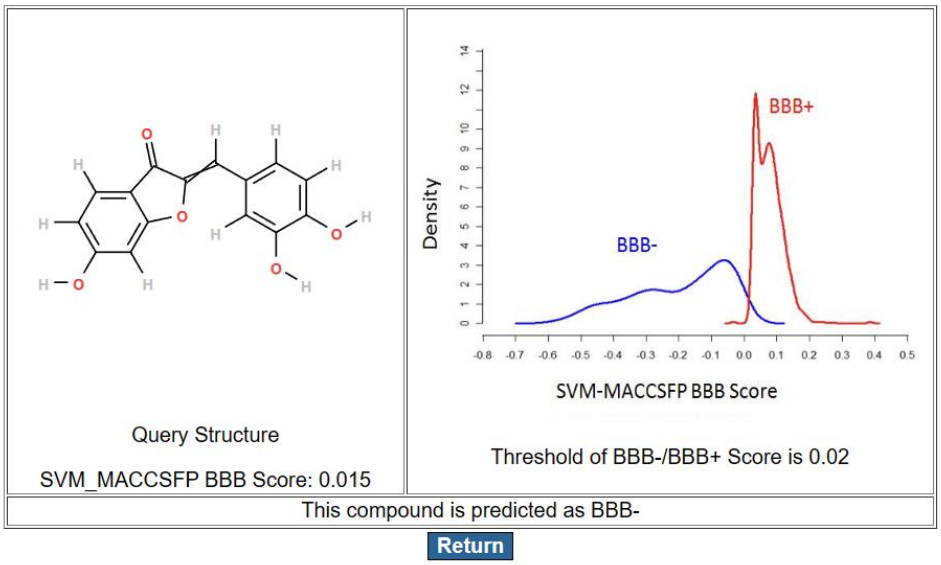


Figure 1.3 Prediction of sulfuretin's BBB permeability based on https://www.cbligand.org/BBB/predictor.php

1.4 Objectives of the study

To verify the aforementioned hypotheses, the specific objectives of the study are as follow:

(i) To synthesize a series of aurone derivatives using sulfuretin as lead.

(ii) To evaluate the ability of compounds to inhibit BV-2 microglial M1 polarization state.

(iii) To evaluate the ability of compounds to promote BV-2 microglial M2 anti-inflammatory state.

(iv) To evaluate the blood-brain barrier permeability of the active compounds using *in vitro* assay.

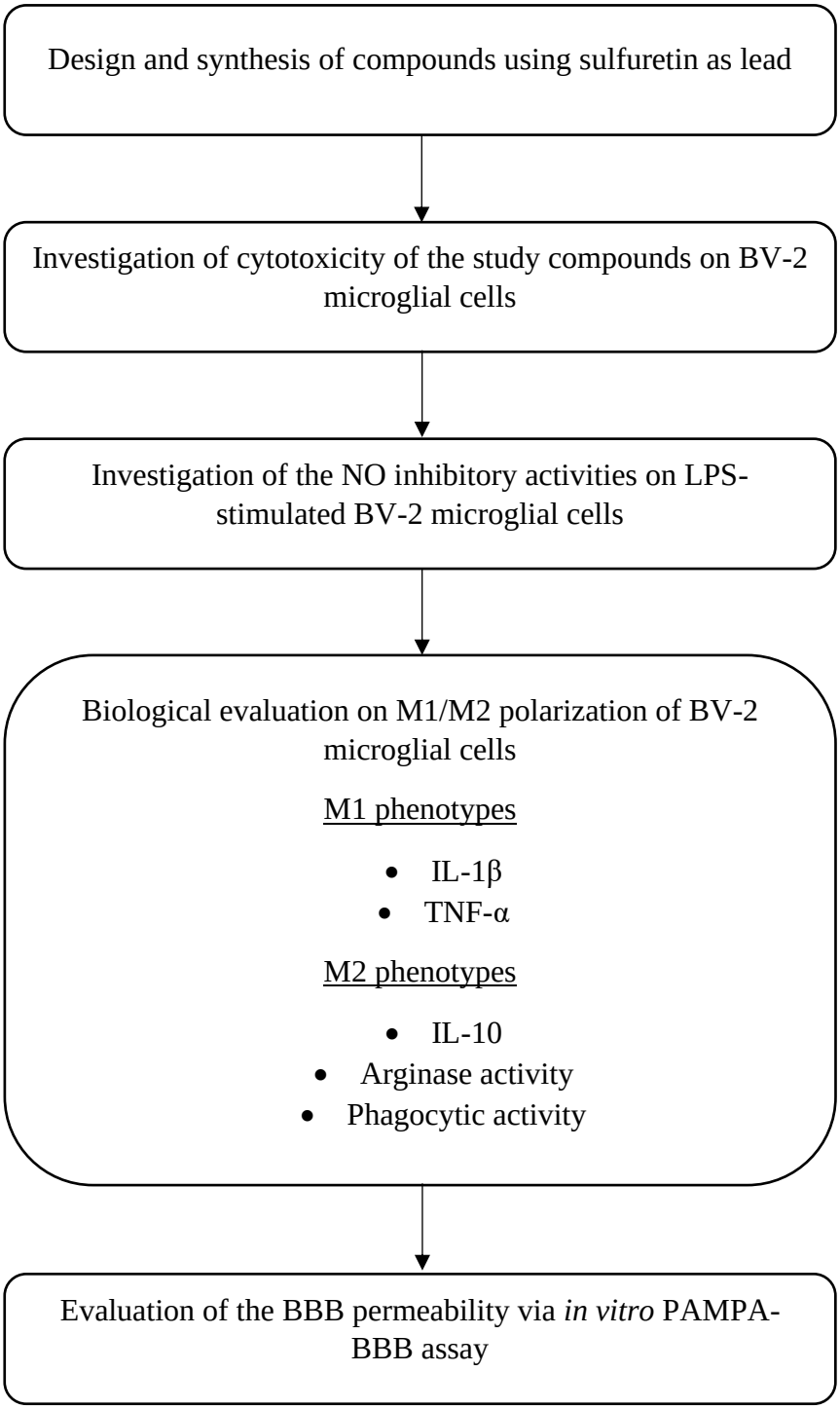


Figure 1.4 Flowchart of the experimental design employed in the present study.

CHAPTER 2

SYNTHESIS OF TARGET COMPOUNDS

2.1 Introduction

This chapter describes the synthesis of target compounds for the evaluation of potential neuroprotective properties based on their anti-inflammatory activity. These compounds were fundamentally divided into five groups, which possess the aurone scaffold with introduction of various functionalities at rings A and B using different chemical strategies. The functionalities included hydroxy, methoxy, aromatic rings, halogens, basic amines and lipophilic groups and the rationale underlying the design were discussed. Also, chemical considerations of their synthesis and the experimental methods are presented. The structures of the synthesized compounds were identified by proton-1 (^{1}H) and carbon-13 (^{13}C) nuclear magnetic resonance (NMR) spectroscopy.

2.2 Rationale of target compound design

Aurones are a minor subclass of the flavonoids of natural products and their skeleton, featuring a benzofuranone coupled to an aromatic ring via an exocyclic alkene has recently drawn a modest amount of synthetic interest. In general, the chemical structure of a target compounds influences the physiochemical properties and further determines the absorption, distribution, metabolism, excretion and toxicity (ADME/Tox) and ultimately the pharmacodynamic activity upon the target. Particularly, these functionalities are the key to establish specific interactions with the

target proteins via formation of hydrogen bonds or other weaker interactions, also, contribute to the metabolic stability and overall bioavailability, via stereoelectronic or electrostatic considerations (Ertl et al., 2020). Thereby, the concept of modification of a compound with varied functional groups has cast a new light in drug research and development strategy of medicinal chemistry in many guises. In the present study, a total of twenty-eight target compounds were synthesized and categorized into five groups based on the modification at ring A and B of the aurone scaffold, using sulfuretin as the lead compound (Table 2.1).

Group 1 are sulfuretin analogues with introduction of either hydroxyl or methoxy functionalities at the 6-position of benzofuranone. Hydroxyl functional group has been recognized as the most abundant functional group in all the drugs databases and widely accepted as the optimal substituent choice when modifying the structures of lead compounds (Mao et al., 2016). Particularly, advantages of hydroxyl group in an arene are reported in enhancing selectivity and decrease metabolism of the compound as it could influence the electron density of the phenyl ring through its ability to interact with aromatic electrons as well as improve water solubility. Meanwhile, methoxy group, a nucleophilic functional group, which can donate electrons into phenyl ring and exhibit the ability to form a covalent bond with a biological target. Addition of a simple methyl group could result in increasing selectivity of a drug for one biological target and prolong duration of action (Harrold and Zavod, 2018). Besides, the incorporation of these two functionalities allows the comparison with regards their lipophilicities and hydrogen donor and acceptor properties. As for compound **1e** and **1f**, both ring B were replaced with bulky aromatic functionalities, biphenyl and indole moieties respectively. The rationale underlying of this choice is because aromatic ring is popular for their good synthetic accessibility

and the regioselectivity of aromatic substitution is predictable (Dörwald, 2013). Also, the planar and hydrophobic structures of aromatic ring allow the formation of van der Waals and hydrophobic interactions with flat hydrophobic regions of the binding sites. Moreover, the nitrogen atom in the indole ring can serve as a hydrogen bond donor and therefore participate in hydrogen bonds (Harrold and Zavod, 2018).

Group 2 is similar with Group 1, but vary in bearing different pendant amine at ring B, limited to tertiary amines such as *N*,*N*-dimethyl amine, pyrrolidine and piperidine. **2a** and **2b** provided suitable comparisons with Group 1 compounds by which to explore the placement of amine moieties at the pendant benzylidene. According to Jampilek (2019), amine functionalities including nitrogen-containing heterocycles have increased their prevalence as biologically active chemical entities in medicinal chemistry owing to their basic and ionisable properties. Particularly, incorporation of an amine to a compound gave the competitive edges in facilitating the aqueous solubility, offering holistic lipophilicity and enhancing the binding to many drug targets including receptors and enzyme (Ertl et al., 2020).

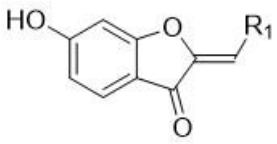
Next, Group 3 are fundamentally chloroaurones with a series of alkoxy derivatives were bonded at the 6-position of the ring A and a chlorine atom is placed at either 2- or 3- positions of ring B. Various alkoxy moieties were introduced into the system due to these functionalities being known for their modest polarity with improved solubility without compromising permeability (Ertl et al., 2020). Meanwhile, the placement of chlorine was on account of their properties of metabolically inert, lipophilic and well suited to improve the affinity of a lead to its target owing to the electron withdrawing properties (Dörwald, 2013). Additionally, the intermolecular interaction formed by chlorine are highly specific and are associated with lower

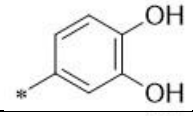
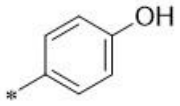
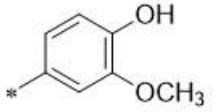
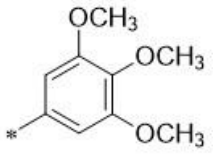
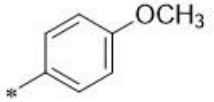
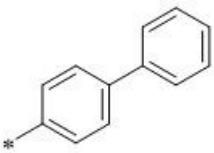
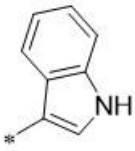
desolvation penalty than classical hydrogen bonds, hence, contribute to the stability of some ligand-protein complexes (Ertl et al., 2020).

Subsequently, Group 4 is mainly differed by the substitution of three methoxy groups at the *meta* and *para* position at benzylidene part of the system with other groups. In the Group 4, various amine moieties were introduced into at either the position 6 or position 7 of ring A. In order to further explore the benzylidene variations, Group 5, a series of aurone with piperidine-substituted benzofuranone was developed. For **5c** and **5d**, fluorine atoms were grafting into the ring B. In general, the fluorine functional group, was reported as the second most abundant functional group in CNS drugs, thereby, it is the optimal substituted choice when developing target compounds in the present study (Mao et al., 2016). The substitution of a hydrogen atom with a fluorine atom could additionally slightly improve the lipophilicity of a compound. Other than methoxy and halogens, ring B was also replaced with various substituents including bi-phenyl, naphthalene, and a piperidinyl-ethyl indole as seen in **5e**, **5f** and **5g** respectively to assess the potency of the target compounds.

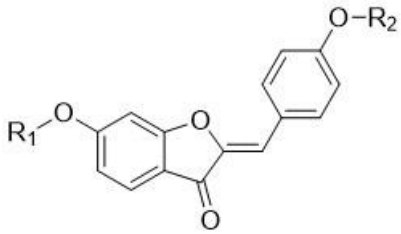
Table 2.1 Structures of target compounds

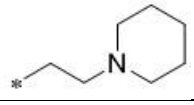
Group 1

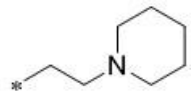
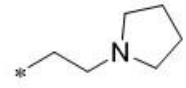
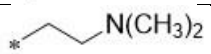


Compound	R_1	%Yield
Sulfuretin		35
1a		55
1b		33
1c		49
1d		68
1e		84
1f		55

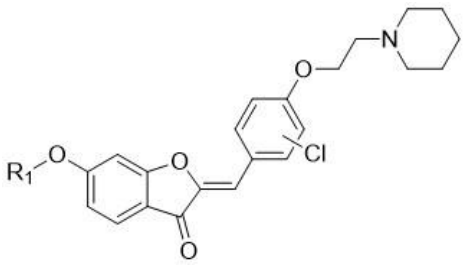
Group 2



Compound	R_1	R_2	%Yield
2a	H		25

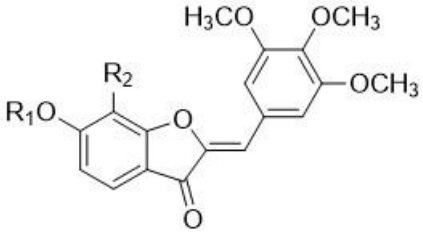
2b	CH_3		19
2c	CH_3		26
2d	CH_3		53
2e	CH_3		33

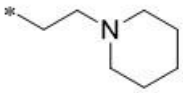
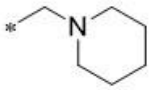
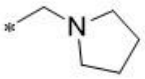
Group 3



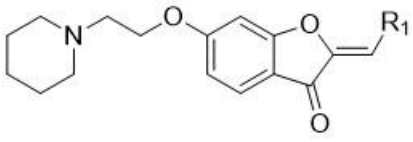
Compound	R_1	Cl Position	%Yield
3a	CH_3	2’	33
3b	CH_3	3’	38
3c	n-C_4H_9	3’	23
3d	$CH_2CH(CH_3)_2$	3’	22
3e	n-C_8H_{13}	3’	74
3f	Benzyl	3’	27

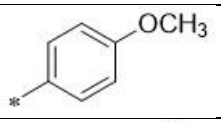
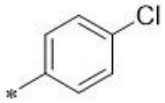
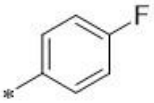
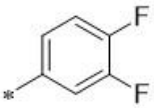
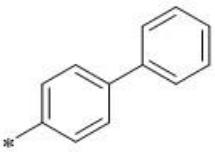
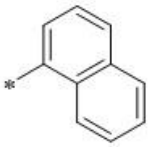
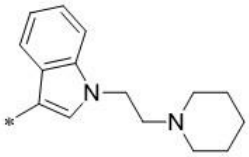
Group 4



Compound	R_1	R_2	%Yield
4a		H	69
4b	H		54
4c	H		57

Group 5



Compound	R_1	%Yield
5a		46
5b		27
5c		16
5d		17
5e		16
5f		33
5g		31

2.3 Chemical consideration

In the present study, a total of 28 aurone derivatives with different substituents in ring A and B were synthesized to explore the potential of the scaffold as a platform to design a blood-brain barrier (BBB) permeable agent as modulators of microglia. In general, most of the aurone derivatives were synthesized via aldol condensation where a functionalized or commercially obtained benzaldehyde with a corresponding benzofuran-3(*2H*)-one were involved in the reaction (Beney et al., 2001; Lee et al., 2010). 'Aldol' is the abbreviation of aldehyde and alcohol, and the condensation reaction could be either acid-catalysed or base-catalysed. In detail, in the presence of

acid/base catalysts, an enol (under acidic conditions) or enolate ion (under basic conditions) is formed by benzofuranone and subsequently reacted with corresponding benzaldehyde, accompanied by the dehydration to yield a desired aurone (Figure 2.1 and Figure 2.2).

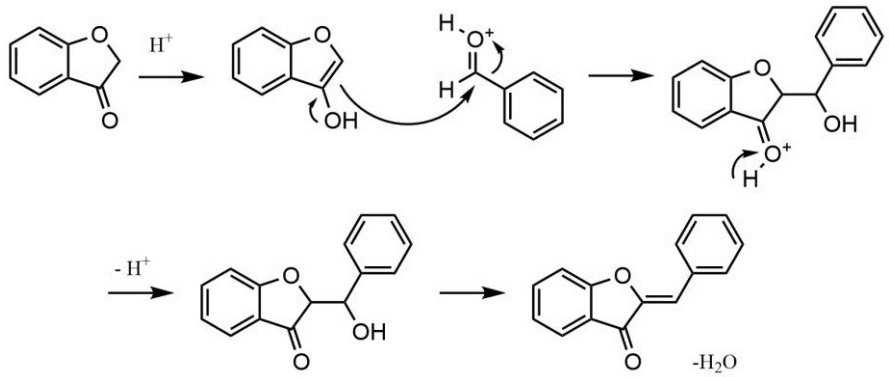


Figure 2.1 Preparation of aurones via acid-catalysed aldol condensation.

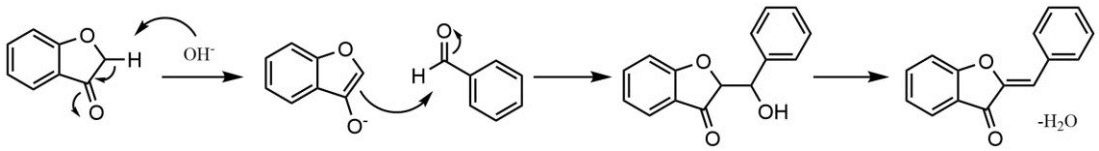
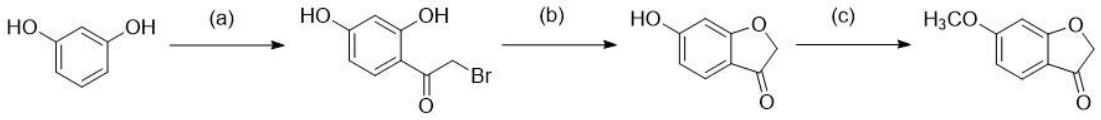


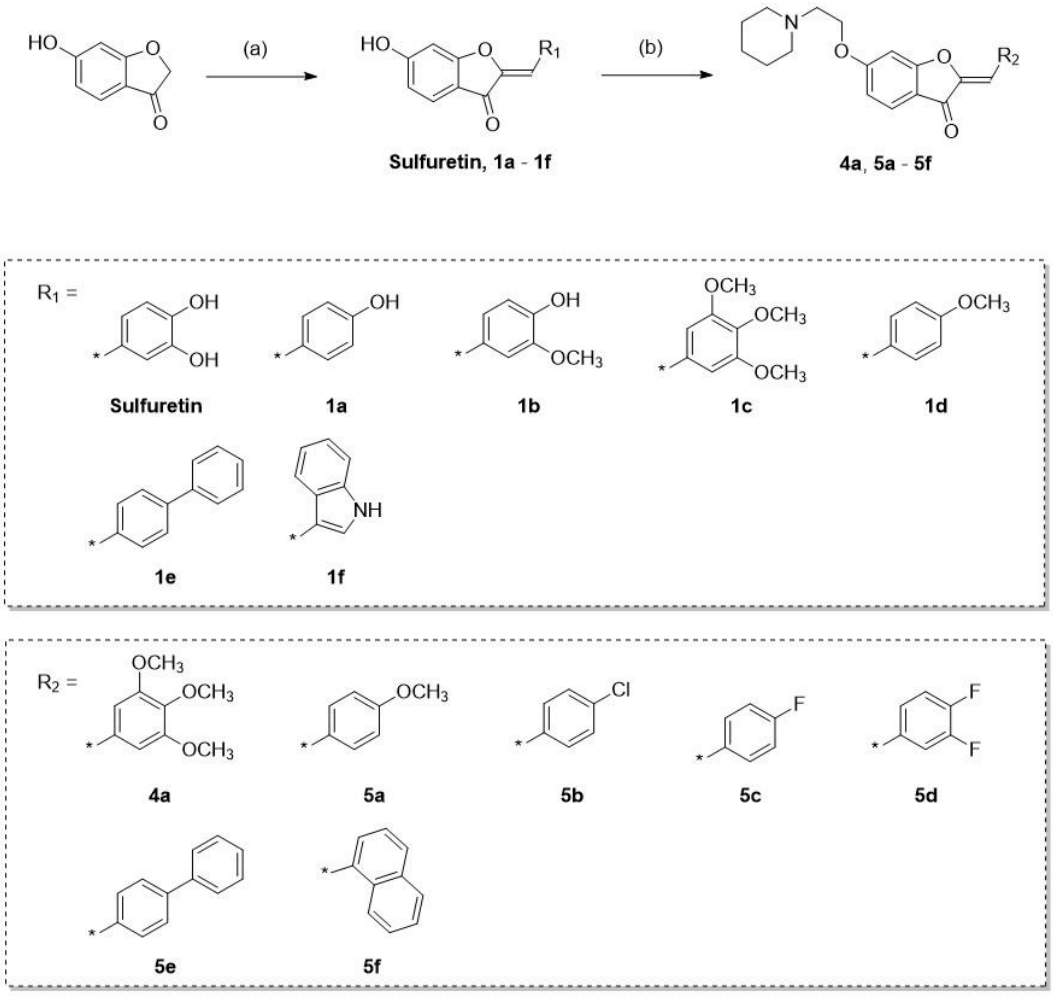
Figure 2.2 Preparation of aurones via base-catalysed aldol condensation.

The synthesis of the target compounds in the present study began with the construction of the 6- hydroxybenzofuran-3(*2H*)-one core following methods previously described (Haudecoeur et al., 2011). Resorcinol was acylated with bromoacetonitrile in an acid-catalysed Houben-Hoesch condition to obtain α-bromo-2,4-hydroxyacetophenone. Subsequent treatment of this intermediate with sodium acetate in methanol produced the benzofuranone in good yields (60 – 80%) (Scheme 2.1). 6-hydroxybenzofuranone was reacted with various benzaldehydes and indole 3-

carboxaldehyde via aldol condensation to yield the sulfuretin analogues of Group 1 (Scheme 2.2).



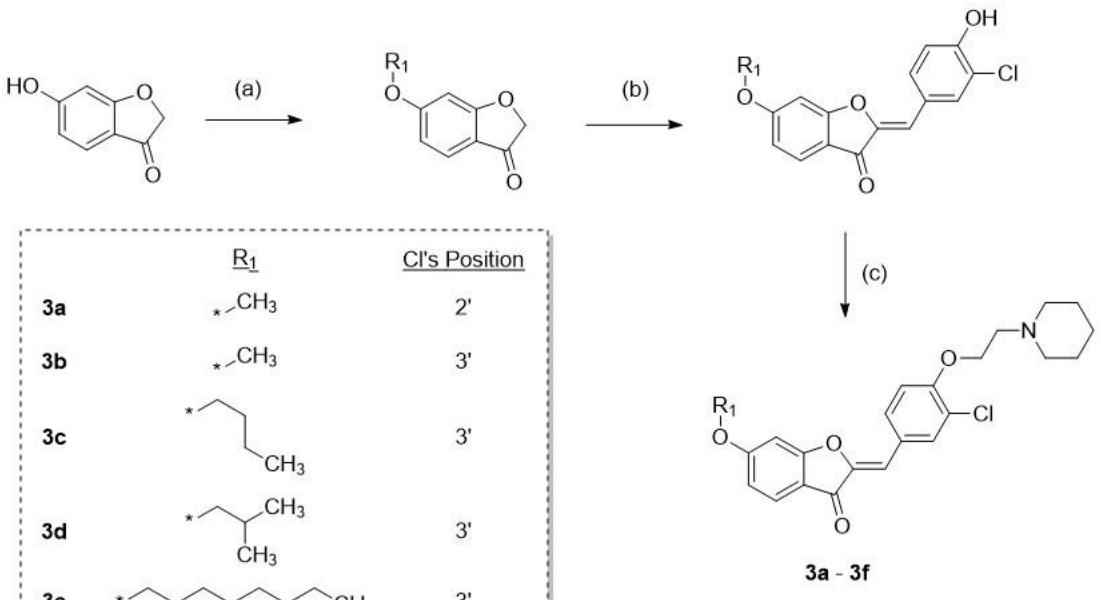
Scheme 2.1 Reagents and conditions: (a) Bromoacetonitrile, $ZnCl_2$, HCl, diethyl ether, 0°C, followed by HCl 1M, 100°C. (b) Sodium acetate, MeOH, 70°C, 2 h. (c) Dimethyl sulfate, K_2CO_3, acetone, rt, 2 h.



Scheme 2.2 Reagents and conditions: (a) Substituted benzaldehydes, 4-(2-(piperidin-1-yl)ethoxy)benzaldehyde, or indole 3-carboxaldehyde, KOH, MeOH,

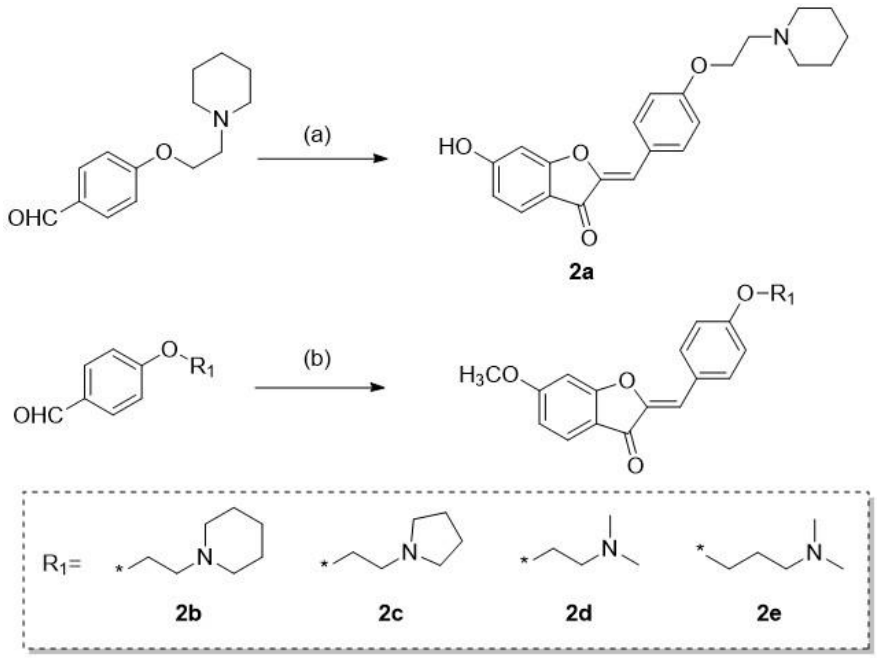
70°C, 2 h. (b) 1-(2-chloroethyl)piperidine hydrochloride, K_2CO_3, DMF, 80°C, 18 - 24 h.

Similarly, several alkylated and benzylated benzofuranone intermediates, prepared via base-catalysed alkylation or benzylation of 6-hydroxybenzofuranone, were coupled to 3'-chloro-4- hydroxybenzaldehyde via aldol condensation and the products subsequently subjected to base-catalysed alkylation with 1-(2-chloroethyl)piperidine hydrochloride to yield the Group 3 aurones (Scheme 2.3). A series of 4-O-functionalized benzaldehydes (*N*,*N*-dimethylaminoethyl, *N*,*N*-dimethylaminopropyl, piperidine ethyl and pyrrolidine ethyl) prepared by alkylation of 4-hydroxybenzaldehyde, were coupled to 6-methoxybenzofuranone and 6-hydroxybenzofuranone to give the amino-functionalized Group 2 aurones (Scheme 2.4). Grafting of a pendant piperidine moiety was achieved by potassium carbonate-catalysed alkylation to selected 6-hydroxyaurones of Group 1 to prepare aurones **4a** and **5a** – **5f** (Scheme 2.2).



	R_1	Cl's Position
3a	CH_3	2'
3b	CH_3	3'
3c	CH_3	3'
3d	CH_3, CH_3	3'
3e	CH_3	3'
3f		3'

Scheme 2.3 Reagents and conditions: (a) Dimethyl sulfate, benzyl chloride, *n*-butyl chloride, isobutyl chloride, or *n*-octyl chloride, K_2CO_3, DMF, 80°C. (b) 3-chloro-4-hydroxybenzaldehyde, conc. HCl, acetic acid, rt, 4 – 5 h. (c) 1-(2-chloroethyl)piperidine hydrochloride, K_2CO_3, DMF, 80°C, 18 - 36 h.



Scheme 2.4 Reagents and conditions: (a) 6-Hydroxybenzofuranone, KOH, MeOH, MeOH, 70°C, 2 h. (b) 6-Methoxybenzofuranone, KOH, MeOH, MeOH, 70°C, 2 h.

A similar reaction performed on aurone **1f** containing an indolyl methylene ring B however, produced an unexpected product **5g**, an aurone with two pendant piperidine moieties, the first being attached via O-ethyl linkage to ring A while the second to the indole nitrogen (Scheme 2.5). This indicated that the indole nitrogen was just as reactive as the phenolic OH as a nucleophile under the employed basic condition. Treatment of 6-hydroxy-3',4',5'-trimethoxyaurone **1c** with molar equivalents of formaldehyde and piperidine or pyrrolidine in ethanol via Mannich reaction produced aurones **4b** and **4c** in moderate yields (Scheme 2.6). Mannich reaction is classically an