Synthesis of Potential Cancer Therapeutic and Diagnostic Agents

By

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Thesis submitted in accordance with the conditions governing candidate for the degree of PHILOSOPHIAE DOCTOR (PhD)

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2011

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Dedicated to my beloved husband (Anuar Yacob),

my lovely children;

Farahan Hazeem Anuar and

Farahan Nafeesa Anuar,

and

my future baby..

Acknowledgements

In the name of Allah, the Most Gracious, the Most Merciful

Oh My Lord, the Almighty...
all the gratitude is dedicated to you, for granting me with **the best supervisor** I have ever imagine; **Dr Andrew Westwell**, who always there whenever I need him, who shared my laughs and most importantly, my tears, who suffered most due to my stupidity, but quickly regained my confidence with his endless support and guidance. Dear God, please fulfil all his wishes and make him the among the successful people in this field..and please extend your blessings to those who help me a lot throughout my work; to all the helpful **staff and students of the Welsh School of Pharmacy**, in particular **Dr Andrea Brancale** for his helpful assistance, to **Dr Claire Simons** for always being my 'silent motivator' and for her confidence towards my ability, for my **friends** and **fellow students** especially to Sis Nawal and the members of Lab 1.04 and 1.05 past and present for their constant support and guidance.

Thank you my Lord, for choosing me among those other intelligent people to receive scholarship to pursue my study here, and please keep my country Malaysia as a peaceful and prosperous country.

And most importantly, please grant your infinity blessing and love to my beloved husband, **Anuar Yacob** who have sacrifice everything for my favour, for my son **Hazeem** and my daughter **Nafeesa** who always being good, supportive and always pray for ummi's success, for **my mum** and **my late father** whose love and prayer brought me to where I am now, not to forget to all my **siblings**, **relatives**, **fellow friends** and **teachers/lecturers** who have encourage and supported me all the way until I am here today.

Dear God, all the gratitude is for you, for granted me with all the nice people and the beautiful things to make me where I am now. Thank you my Lord...

ABSTRACT

The need to find novel anticancer agents with better potency, efficacy and safety are highly demanded. Therefore, the first part of the study was aimed to synthesise new compounds based on stilbenes, indole-based isoxazoles and tricyclic anilides as potential antitumour agents, which later will be evaluated for their anticancer properties.

The syntheses of substituted stilbenes were achieved via catalyzed or uncatalyzed methods, yielding stilbene analogues in moderate to good yields. Preliminary antiproliferative studies on four cancer cell lines (prostate, non-small lung, colon and breast) demonstrated their antiproliferative potential in the micromolar range. Unfortunately, the stilbenes were unable to inhibit the Wnt-signaling pathway in colon cancer cells.

Next, the synthesis of indole-based isoxazole analogues was achieved via two different methods; affording the compounds in low to moderate yields. The compounds will later be tested for their anticancer properties.

The synthesis of 3289-8625 (tricyclic anilides) analogues, compounds which showed potent inhibitory activites on the PDZ domain of Dishevelled (PDZ-Dvl) as an important component in the Wnt signaling pathway was also carried out. The synthesis was achieved via various methods which gave rise to the formation of two analogues, which showed better binding affinities towards the PDZ-Dvl compared to the parent compounds.

Finally, the therapeutic potential of the stilbenes was expanded to the synthesis of stilbene-based analogues as novel positron emission tomography (PET) imaging probes especially for the detection of β-amyloid plaques in brain, which is a hallmark of Alzheimer's disease. The syntheses of stilbenes were sought using fluorine-19, using methods that can later be adapted for ^{18}F-PET radiochemistry. The syntheses of stilbenes attached to ^{19}F-linker were afforded in good yields. Stilbenes directly attached to potassium trifluoroborate were synthesised in moderate yield. Nevertheless, the attempt to synthesise stilbene derivatives attached to a potassium trifluoroborate linker using novel procedures failed.

Table of contents

ABBREVIATIONS

Aβ	β-amyloid
Anal. calcd:	Analytical calculated
APC	Adenomatous polyposis coli
Bcl-2	B-cell lymphoma-2
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic acid
Dvl	Dishevelled
EI	Electroionisation
ELISA	Enzyme-linked immunosorbent assay
ES	Electrospray
FDA	U.S. Food and Drug Administration
Frz	Frizzled
GMP	Good manufacturing practice
GSK3β	Glycogen synthase kinase 3 beta
HTS	High throughput screening
HWE	Horner-Wadsworth-Emmons reaction
IC_{50}	Half maximal inhibitory concentration
LEF	Lymphoid enhancer binding factor
LRP	Lipoprotein receptor related protein
M	Molarity
M^+	Molecular ion
MHz	Megahertz
mM	Milimolar
MRI	Magnetic resonance imaging spectroscopy
MS	Mass spectroscopy
MTT	[3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay
MW	Molecular weight
mz	Mass to charge ratio
N	Normality

NMR	Nuclear magnetic resonance
PET	Positron emission tomography
ppm	Parts per million
PTS	Polyoxyethanyl α-tocopheryl sebacate
RT	Room temperature
SPECT	Single photon emission computed tomography
TBAF	Tetra-*n*-butylammonium fluoride
TCF	T-cell-specific transcription factor
TLC	Thin layer chromatography
W	Watt

CHAPTER 1

INTRODUCTION

CHAPTER 1

INTRODUCTION

"During the past century, human life expectancy has increased significantly due to improved health care, including the availability of vaccines and new pharmaceutical drugs. With the ageing population on the rise, complex diseases such as Alzheimer's disease, Parkinson's disease and various types of cancers, combined with new diseases with debilitating consequences can reduce the general quality of life. This, together with the fact that the drug discovery and developmental process is competitive, lengthy, risky, and expensive with a high failure rate, posses challenging opportunities for the development of new drugs"
Chengalvala et al. 2005.

1.1 Cancer

Cancer is the name given to a range of specific diseases resulting from the body's own cells which have become aberrant growing out of control (Reichert and Wenger 2008). The hallmarks of cancer have been reviewed extensively by Hanahan and Weinberg (2000). The review highlights the six essential criteria that distinguished cancer from normal cells: "self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis". In other words, cancer - which consists of abnormal cells with diverse oncogenic mutations, are able to proliferate without control and metastasize to other tissues, which later can harm the life of the living creature (Doucas et al. 2005; Ashkenazi 2008).

Cancer is a major cause of morbidity in the UK. In 2007 alone, 297,991 people were diagnosed with cancer. The 20 most commonly diagnosed cancers in the UK is shown in figure 1.1 below (Office for National

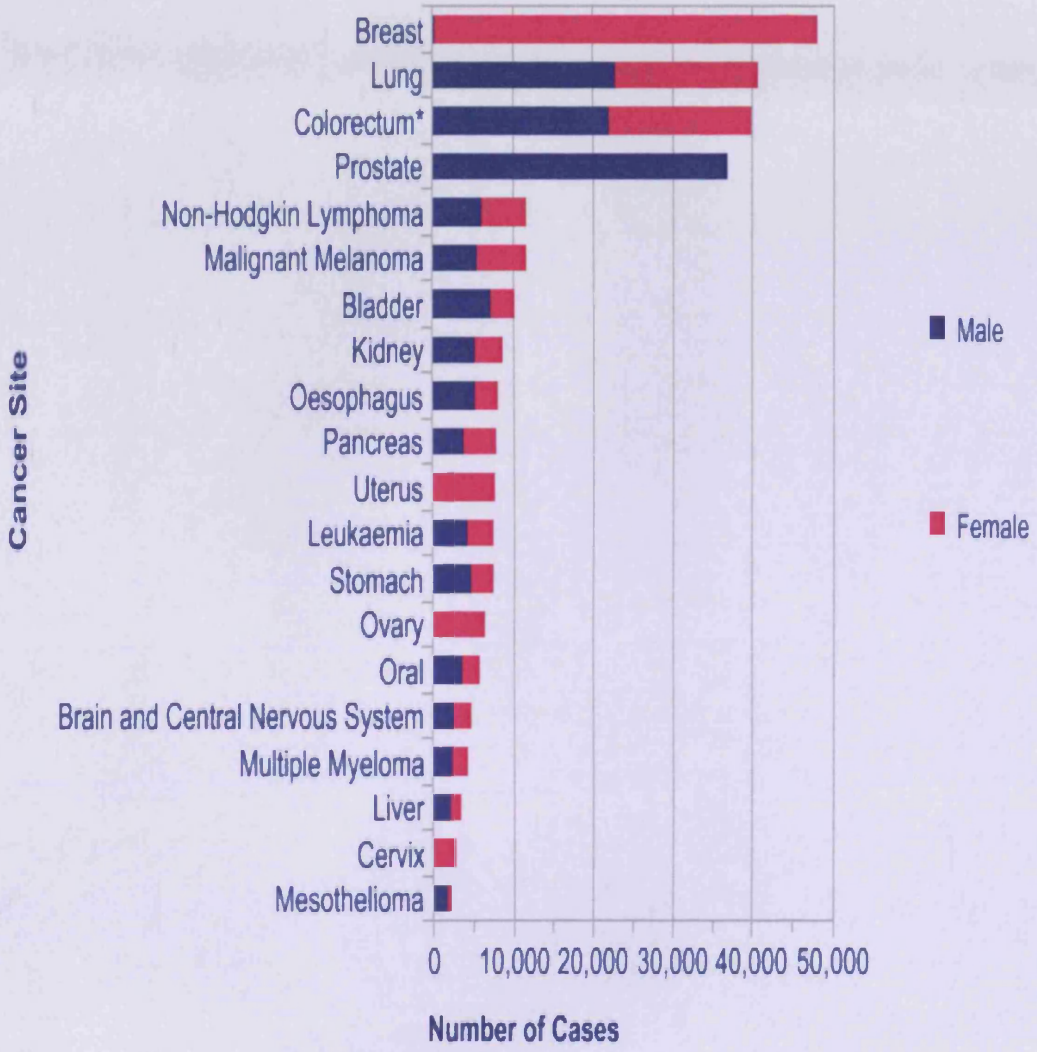


Figure 1.1: 20 most commonly diagnosed cancers (excluding non-melanoma skin cancer) in the UK and number of new cases (2007).

Statistics 2010; ISD online 2007). Cancer is also the second leading cause of death worldwide, where cancer has caused more death than heart disease for people under the age of 85. "In Europe, one of four deaths is attributable to cancer, and in those aged 45 to 64 that rate is almost double" (Albreht et al. 2008).

The 'War on Cancer' which commenced in 1970 was initiated to boost the anti-cancer drug programme. Since then, anticancer drug development is still considered slow which is reflected by the low number of anticancer drugs entering the clinics (Collins and Workman 2006; Reichert and Wenger 2008, de Bono and Ashworth 2010).

1.2 Cancer drug development

The need to distinguish cancerous cells (which need to be eradicated) from normal cells is the factor that makes cancer drug development very challenging (Reichert and Wenger 2008). Other than surgery, the major goal of killing cancer cells is achieved by various methods: chemotherapy (using anticancer drugs), radiation or immunotherapy; where induction of apoptosis is a prominent feature in killing the tumour cells. Despite aggressive therapies, resistance of many tumours to current treatment protocols still constitutes a major problem in cancer therapy (Fulda and Debatin 2006). Most conventional cancer therapies are only able to extent life expectancy of patients for perhaps a few years, despite the serious side effects that are caused upon treatment (Amin et al. 2009).

Natural products, without any doubt have contributed an important role as chemotherapy and chemopreventive agents in various cancer malignancies. Whether natural compounds which are directly extracted from living creatures or synthetic compounds derived from the natural original structure, they are now widely used as anticancer and chemopreventative agents. One commonly used example is Paclitaxel, a microtubule disruptor which is isolated from the bark of *Taxus brevifolia,* and has been used for treatment of various cancers including leukemia and breast cancer (Amin et al. 2009). Another example is resveratrol, which belongs to a class of polyphenolic compounds called stilbenes. Resveratrol is considered as a natural compound which can be developed into potential drugs due to its striking biological activity, despite its simple structure which allow various modifications on the stilbene ring (Pezzuto 2008). On the other hand, small molecules possess numerous advantages in the drug discovery process as

compared with peptides (such as higher metabolic stability and bioavailability) and even natural products (such as easier access to analogues with increased potency, easier synthesis, greater solubility, and support for structure-activity relationship studies). In addition, the methods for synthesising small molecule inhibitors are rapidly improving (Pagliaro et al. 2004).

Heterocycles are the most important chemical entities in drug discovery. They can be found in the vast majority of drugs either synthetic or natural product derived, which reflects their importance in drug design. They can imitate and stabilize the binding of various functional groups such as natural ligand or substrate (Broughton and Watson 2005). One common example is indole, which is considered as a privileged structure in drug design due to its abundance in biologically active natural or synthetic products (de Sa Alves et al. 2009).

Conventional cancer discovery processes usually involves the synergistic processes between chemical syntheses and biological evaluation. In recent years, the drug discovery processes have gradually shifted from "cytotoxic, non-specific chemotherapies" to more "personalized" ("molecularly targeted, rationally designed") medicine, where the agents target precisely on molecular pathology which is responsible for individual cancer progression (Collins and Workman 2006). As each cancer patient is not identical, this approach will allow a more personalized treatment and is anticipated to be a more reliable and effective for cancer treatment, which can cause less side-effects to the patient (de Bono and Ashworth 2010).

Traditionally, the classification of anticancer agents is based on the origin or mechanism of action of the drugs, for example as antitumour antibiotics (e.g. Doxorubicin), antimetabolites (e.g. 5-Fluorouracil), alkylating agents (e.g. Cisplatin) and plant alkaloids (e.g. Etoposide) (Figure 1.2). Nevertheless, the discovery of novel antitumour agents which exhibit various mechanism of actions in tumour cells due to their diverse "biochemical and pharmacological properties", resulted in the dilemma to accurately classify

the compounds based on the native classification method. This highlights the advancement that has been achieved in cancer drug development which makes it possible to develop drugs that showed multiple inhibitory activities in various cancer types (Carter et al. 1987; Schepartz 1995).

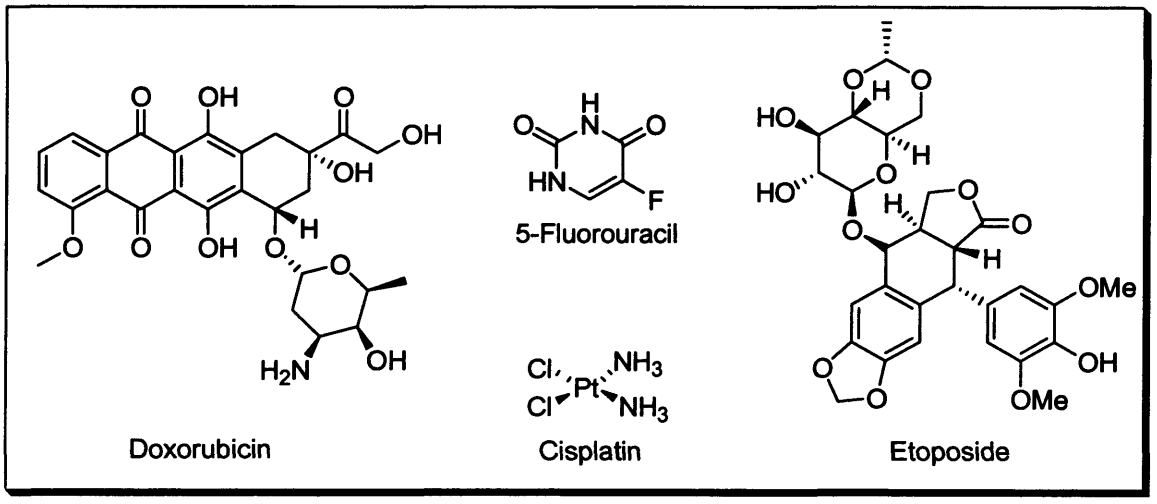
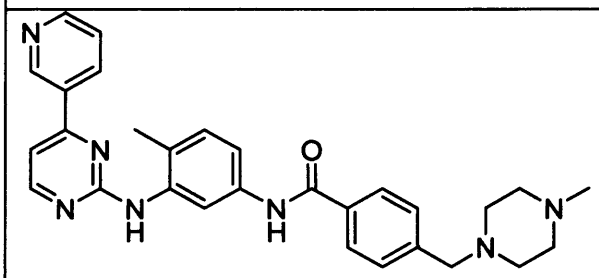
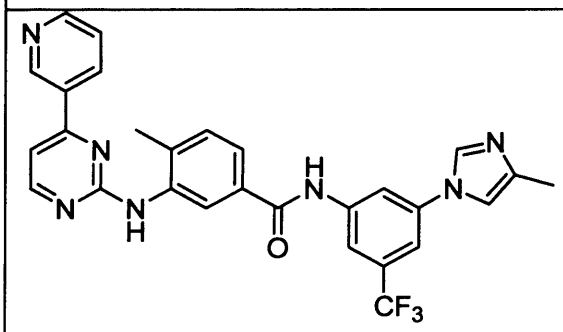
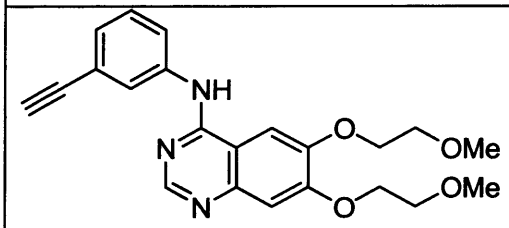
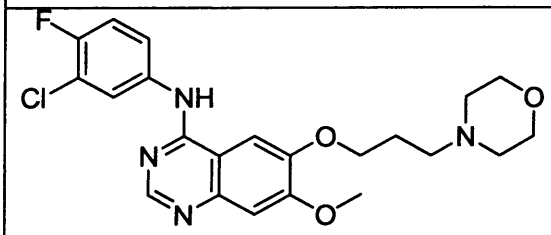
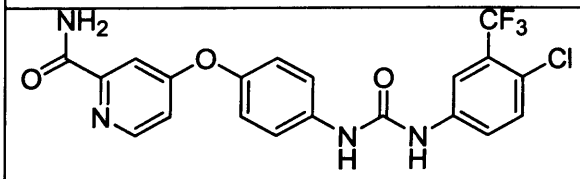
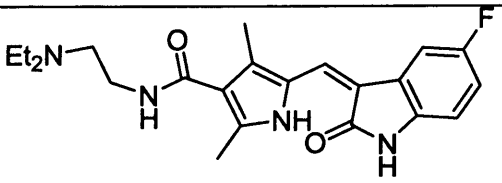
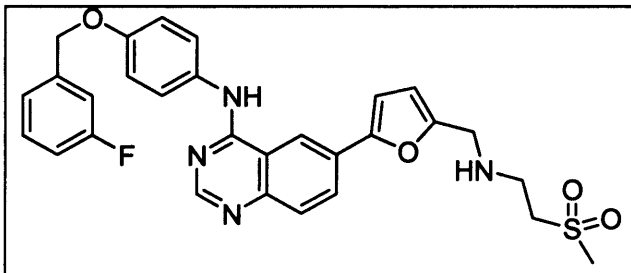
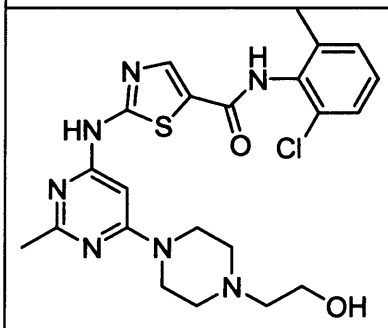


Figure 1.2: Examples of classic anticancer agents.

Protein kinase inhibitors have dominated cancer drug discovery over recent years. Protein kinases are enzymes that phosphorylate proteins, resulting in functional changes of target proteins (Gotlink and Verheul 2010). The ability of the small-molecule kinase inhibitors to selectively treat common malignancies such as breast and lung cancers have promoted the development of this series of inhibitors (Janne et al. 2009; Johnson 2009). However, their ability is limited to a subset of treated patients which limit its applicability in clinical settings (Janne et al. 2009). Tyrosine kinase inhibitors are probably the most well-studied protein kinase inhibitors, besides serine, threonine or lipid kinase inhibitors, due to the role of tyrosine kinases as important cellular signalling proteins which mediate various biological activities (Janne et al. 2009; Gotlink and Verheul 2010). Table 1.1 shows examples of small molecule kinase inhibitors already approved for the treatment of various cancers.

Table 1.1: Examples of clinically approved kinase inhibitors (Janne et al. 2009).

Structure	Name	Indication
	Imatinib (Gleevec; Novartis)	CML, GIST, HES
	Nilotinib (Tasigna; Novartis)	Imatinib-resistant CML
	Erlotinib (Tarceva; OSI Pharmaceuticals/ Genentech/Roche)	NSCLC, pancreas cancer
	Gefitinib (Iressa; AstraZeneca)	NSCLC
	Sorafenib (Nexavar; Bayer/Onyx)	Renal cancer
	Sunitinib (Sutent; Pfizer)	Renal cancer, imatinib-refractory GIST

	Lapatinib (Tykerb; GlaxoSmithKline)	Breast cancer
	Dasatinib (Sprycel; Bristol-Mayers Squibb).	Imatinib-resistant CML

CML=chronic myelogenous leukaemia; GIST=gastrointestinal stromal tumours; HES=hypereosinophilic syndrome; NSCLC=non-small cell lung cancer.

Another successful approach especially in the treatment of hormone related-cancers (such as breast cancer and prostate cancer) is antihormonal therapy. The therapy is based on the use of selective estrogen modulators (for breast cancer) and selective androgen modulators (for prostate cancer) to inhibit the hormone receptors on the hormone receptor-positive cancers (Rau et al. 2005; Jordan et al. 2008). The success of antihormonal therapy which could enhance the patient survival significantly had driven the progress of targeted therapy and individualized medicine strategies (Jordan 2009). Tamoxifen has become the most commonly used estrogen modulator, while Flutamide and Bicalutamide are potent anti-androgen agents (Figure 1.3) (Rau et al. 2005; Jordan 2009). Nevertheless, the treatment option is restricted to those patients with hormone receptor-positive cancers (not effective to patients with receptor-negative cancers), and the susceptibility to drug-resistance or relapse upon antihormonal treatment (Jordan et al. 2008; Jordan 2009) is problematic.

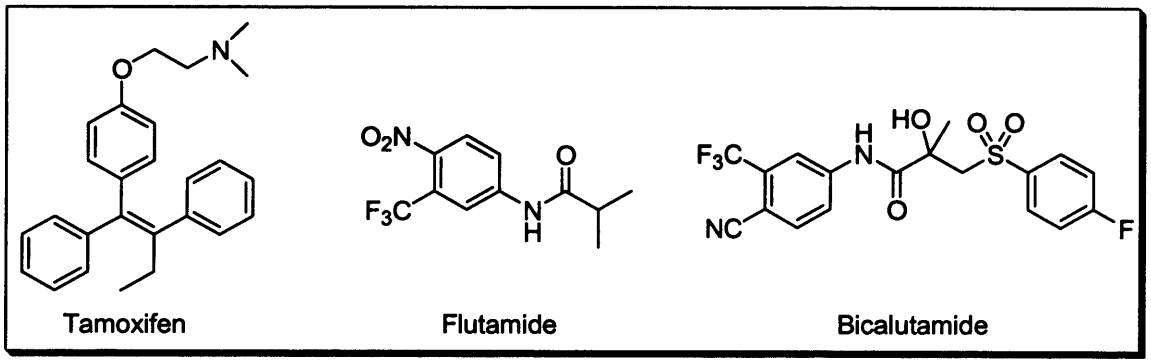


Figure 1.3: Structures of commonly used antihormonal agents.

1.3 Small molecule inhibitors targeting protein-protein interactions in cancer drug development

Interactions between specific pairs or groups of proteins play a major role in the regulation of various processes in the cell. Abnormal protein-protein interaction is closely related to the initiation of many human diseases, either through the loss of crucial protein interaction or through the formation of a protein complex at an unsuitable time or location (Ryan and Matthews 2005; White et al. 2008).

Typically, the development of therapeutic agents is mostly dedicated to the inhibition of a single protein, usually a defined ligand-binding site on an enzyme or receptor, which is amenable for drug design. Nevertheless, the increasing knowledge on the importance of protein-protein interactions which are abundant in nature and their crucial role to mediate various biological processes, the development of small molecule inhibitors specifically targeting protein-protein interactions is seen as an emerging and important modality in cancer drug discovery (Vincent et al. 2007; White et al. 2008). Protein-protein interactions represent an enormous and diverse group of targets for therapeutic intervention (Arkin 2005). In most cases, the existence of "hot spots" on the surface of the protein which are responsible for protein-protein binding, and the presence of small and narrow pockets within the protein which is amenable to be manipulated to design inhibitors for that particular protein, make protein-protein interactions an interesting drug target (Arkin and Wells 2004; White et al. 2008).

One important example in the search of inhibitors for protein-protein interaction is the inhibition of an intracellular receptor of the Wnt signalling pathway, the PDZ domain of Dishevelled. The inhibition of this protein is anticipated to subsequently inhibit the expression of β-catenin level which is responsible for gene transcription (Zhang et al. 2009b). The potency of inhibiting this protein interaction is highlighted by the number of small-molecule antagonists which have been designed to inhibit this particular protein (Wang et al. 2008).

1.4 Non-invasive imaging techniques in medicine

Non-invasive imaging techniques are of particular importance in the health sector with the main goals being diagnosis, disease monitoring and staging. In order to cater for the needs for various parameters in imaging, a number of methods have been developed including "X-ray diffraction and CT, optical (fluorescence) methods, MRI, single photon emission computed tomography (SPECT) and positron emission tomography (PET)". Of all the imaging methods, PET has become a popular method of choice for disease monitoring in drug discovery and development due to its high sensitivity and good resolution of radiolabelled compound *in vivo* (Daniels et al. 2010).

The advancement of PET imaging techniques for disease intervention emphasizes the needs for development of specific radiotracers. In view of this, radiolabelled compound attached to ^{18}F as a positron emitting isotope is of interest due to its convenient half-life (110 minutes) to allow sufficient synthesis of radiotracers to GMP standard, ample time for distribution and minimal radioactive exposure to the patient. Among the PET radiotracers which are currently in clinical trial are stilbene-based compounds, which found excellent applicability for the detection of amyloid plaques in the brain of Alzheimer's disease patients (Cai et al. 2008; Rowe et al. 2008; Daniels et al. 2010).

1.5 General aims and objectives

This first few part of the study aimed to synthesis a range of small molecules which are intended to be developed as new anticancer agents. For this purpose, three different approaches to synthesise small molecule inhibitors for cancer have been sought:

- In chapter 2, the study was dedicated to the synthesis of resveratrol-based stilbene analogues as potential anticancer agents. The synthesis of stilbenes is achieved using various methods ranging from catalysed, uncatalysed or using microwave irradiation. The synthesised stilbenes were later screened for their ability to inhibit cancer cell proliferation on a panel of cancer cell lines (by Mr Huw Mottram, Welsh School of Pharmacy). At the same time, the stilbenes were further evaluated for their ability to inhibit Wnt signaling pathway in colon cancer stem cells (in collaboration with Prof. J. P. Medema, Academic Medical Centre, Amsterdam, the Netherland).
- Chapter 3 was dedicated to the synthesis of indole-based isoxazole compounds which are anticipated to possess interesting antitumour activities. The syntheses of the compounds were achieved via two approaches: either via cyclization of alkynyl ketones with hydroxylamine, or via dipolar cycloaddition between nitrile oxides and terminal alkynes to afford the title compounds. The compounds are yet to be tested for their antiproliferative activity on cancer cell lines.
- Chapter 4 is intended for the synthesis of compound 3289-8625 derivatives (compound 6569188 and 7359885) which have been shown to target specifically on the PDZ domain of Dishevelled, one of the important protein-protein interactions in the Wnt signalling pathway. The synthesized compounds were later tested for their binding affinity to Dishevelled PDZ domain via NMR-based chemical perturbation experiments and fluorescence-binding affinity studies (by our project collaborator, Prof. Jie J. Zheng, St. Jude Children's Research Hospital, Memphis, USA).

The next part of the study was dedicated to establish methods for the synthesis of stilbene-based imaging agents specifically designed to act as PET imaging probes. This study was the extension of the initial study on the synthesis of stilbenes as potential anticancer agents. The aim is to synthesise two series of stilbene compounds; either attached to alkylfluoride chain or attached to a trifluoroborate moiety. The approach taken was to synthesis stilbenes attached to cold (^{19}F) stilbene first, which upon successful incorporation will be expanded to the synthesis of radiolabelled ^{18}F compounds in the future.

CHAPTER 2

STILBENE ANALOGUES AS POTENTIAL ANTICANCER AGENTS

CHAPTER 2

STILBENE ANALOGUES AS POTENTIAL ANTICANCER AGENTS

2.1 Resveratrol

Resveratrol (3,4',5-trihydroxy-*trans*-stilbene) (Figure 2.1) is a polyphenolic natural product, synthesised by a wide variety of plant species including grapes and peanuts during stressful situations and present at relatively high concentration in red wine (at concentrations between 5 to 13 μM) and grape juice. Its stilbene structure is related to the synthetic oestrogen diethylstilbestrol (Baur and Sinclair 2006; Zhang and Go 2007; Hope et al. 2008; Jiang 2008). Resveratrol is a classic example of a stilbene, which is referred to a family of compound which consists of the alkene ethene, surrounded by benzene rings. In nature, it is also found as a glycoside or methoxide (Regev-Shoshani et al. 2004).

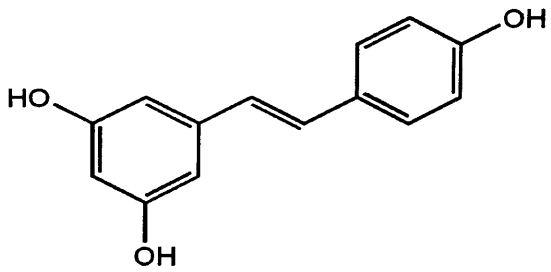


Figure 2.1: Structure of resveratrol

Resveratrol was first discovered in the 1940s, but it was not until 1992 when it attracted vast interest due to the finding that it was responsible for the cardioprotective constituent of red wine (Baur and Sinclair 2006). Therefore, is closely related to a phenomenon called "French Paradox", due to the fact that the French population have a relatively low incidence of coronary heart diseases despite their relatively high saturated fat diet (Simoni et al. 2006; Yoo et al. 2006).

Numerous biological activities have been ascribed to resveratrol, which may explain its anti-inflammatory, antioxidant, antimutagen, anticarcinogenic, anti-cancer, anti-fungal, antibacterial and cardioprotective properties (Baur and Sinclair 2006; Yoo et al. 2006; Zhang and Go 2007; Jiang 2008). Moderate consumption of red wine is also associated with a lower incidence of dementia and Alzheimer's disease, which is closely related to its neuroprotective property and therapeutic potential (Vingtdeux et al. 2008).

Unfortunately, the clinical use of resveratrol may be limited by its poor oral bioavailability. The bioavailability of resveratrol is almost zero due to its fast and extensive metabolism (Regev-Shoshani et al. 2004), as it is rapidly inactivated by phase II conjugation enzymes (Gao et al. 2006; Zhang and Go 2007). This resulted in resveratrol having a short initial half-life of around 8-14 minutes (Baur and Sinclair 2006). It has been found that resveratrol and its metabolites are lost mainly in the urine in the course of 72 hours (Walle et al. 2004). Upon absorption, resveratrol is metabolised in the liver by the phase-2 enzymes, to form water-soluble *trans*-resveratrol-3-*O*-glucuronide and the primary metabolite *trans*-resveratrol-3-*O*-sulfate, which indicated that most transformation occurred on the 3-OH position (Yu et al. 2002; Athar et al. 2007). These will make resveratrol substantially more hydrophilic, which results in decreased ability to enter cells. Albumin also has been found to bind to resveratrol to be distributed in the body only when the concentration is high (Jiang 2008). In conclusion, the available *in-vivo* studies indicate that resveratrol although absorbed to a high extent by the organism, has poor bioavailability and may be converted *in vivo* into compounds lacking anti-proliferative activity (Wenzel and Somoza 2005).

2.2 Resveratrol in cancer

Resveratrol has gained considerable attention because of its potential cancer chemopreventive and anticancer properties. Resveratrol had been shown to have potent effects on a number of cancer cells, with properties involving inhibition of initiation, promotion and progression (Walle et al. 2004; Yoo et al.

2006). Among its various actions, resveratrol has been demonstrated to inhibit cellular survival signalling. Resveratrol may interfere with apoptosis pathways both by directly triggering apoptosis-promoting signalling cascades and by blocking anti-apoptotic mechanisms. By blocking survival and anti-apoptotic pathways, resveratrol can sensitize cancer cells, which may result in synergistic antitumour activities (Fulda and Debatin 2006, Simoni et al. 2006; Chan et al. 2008). In colon cancer cells, resveratrol is capable of inducing apoptosis via the death receptor and mitochondrial pathways, where caspase-6 activation is among a prominent feature for apoptosis induction (Chan et al. 2008).

Resveratrol has been shown to suppress angiogenesis and metastasis, through modulation of multiple pathways involved in cell growth, apoptosis and inflammation (Tseng et al. 2004). The anti-carcinogenic effects of resveratrol appear to be closely associated with its antioxidant activity, and it has been shown to inhibit cyclooxygenase, hydroperoxidase, protein kinase C, Bcl-2 phosphorylation and nuclear factor kappa-B (NF-kB) among many others (Athar et al. 2007). Extensive studies have revealed multiple molecular targets of resveratrol, which affect cell growth, inflammation, apoptosis, angiogenesis, invasion and metastasis, whether through anti-oxidant properties, or through pro-oxidant effects which can cause oxidative DNA damage that may lead to cell cycle arrest or apoptosis, as reviewed by Athar et al. (2009).

2.3 Stilbene derivatives as promising scaffolds for anticancer agents

Although resveratrol clearly exerts an effective anticancer influence, previous studies have shown that the bioavailability of this compound is quite low (Regev-Shoshani et al. 2004; Baur and Sinclair 2006). Therefore, in order to develop a resveratrol-based anticancer drug with potent anti-tumourigenic activity and better bioavailability, the free phenolic group on the stilbene backbone could be blocked, leaving only those groups which contribute to antitumour activity, thus preventing the rapid biotransformation associated with resveratrol itself (Yoo et al. 2006). A study done by Heynekamp et al.

(2006) showed that resveratrol and its analogues induced markedly different apoptosis-inducing activities against sensitive and resistant leukaemia cells, suggesting that minor structural changes in these hydroxylated stilbenes have major effects on biological activity.

Other stilbene derivatives exhibit quite similar biological properties as reported for resveratrol (Gao et al. 2006; Simoni et al. 2006). The stilbene moiety is commonly encountered in natural products and many members are associated with therapeutically important pharmacological properties. Resveratrol is probably the best-known stilbenoid, although the *cis*-stilbene combretastatin A4 is a related natural product that has been extensively tested as an anticancer agent (de Lima et al. 2009).

Novel stilbenoids based on the resveratrol structure may provide a solution to the bioavailability limitations exert by resveratrol. The methoxylation of hydroxyl groups results in an increase in lipophilicity. These changes will influence bioavailability, susceptibility to metabolism and the pharmacological profile of the resulting analogues, as the methoxylated stilbenes are metabolised more slowly. Methoxylated analogues of resveratrol provide a useful starting point for the rational design of chemopreventive and chemotherapy agents with improved pharmacological properties (Zhang and Go 2007; Wilson et al. 2008), as shown by the 3,5-dimethoxy moiety which is frequently associated with noticeable biological activity. Methoxylated stilbenes also undergo different metabolic conversion and have higher bioavailability with respect to resveratrol (Sale et al. 2004). A case in point is 3,4,5,4'-tetramethoxy-*trans*-stilbene (DMU-212), a synthetic methoxylated analogue of resveratrol- which had an improved pharmacokinetic profile, selective growth inhibitory effects against cancer cells with almost no inhibitory effect on the growth of normal cells, and a unique mode of action compared to resveratrol (Simoni et al. 2006). A 3,4-dihydroxyl group is also important to enhance anticancer activity of *trans*-resveratrol analogues (Murias et al. 2004). Previous study also suggested that 2,3',4,5'-tetramethoxy-*trans*-stilbene other than DMU-212 are potent apoptosis-inducing agents with clinical potency. Another example is shown

by analogue 2,3',4,4',5'-pentamethoxy-*trans*-stilbene, which have been found to be a potent inducer of apoptosis in colon cancer cells via targeting microtubules (Li et al. 2009).

The introduction of fluorine atom into resveratrol analogues also has proven to improve the bioavailability of resveratrol. This growing interest is due to the unusual and unique chemical properties of fluorine as the most electronegative element in periodic table. The incorporation of fluorine into a drug increases its lipophilicity, thus enhancing its absorption into biological membranes (Alloatti et al. 2008). One example is by Moran et al. (2009) which reported the synthesis of fluorinated analogues of resveratrol still retaining the 3,4',5-substitution pattern. From their study, one fluorinated analogue, (*E*)-3,5-difluoro-4'-acetoxy stilbene showed better potency compared to the parent compound resveratrol and had a broad spectrum of anticancer activity when tested on a panel of 60 cancer cell lines by the US National Cancer Institute.

Another study by Minutolo et al. (2005) suggested that some naphthalene-based resveratrol analogues were able to induce apoptosis in MDA-MB-231 human breast cancer cell line. Further study of this series of compounds by replacing the aromatic core of the initial naphthalene-based resveratrol analogues with pseudo-heterocyclic (salicylaldoxime) or heterocyclic (benzofuran, quinoline or benzothiazole) scaffolds resulted in some compounds with comparable antiproliferative activity to the previously active naphthalene-based compounds on MDA-MB-231 cells (Bertini et al. 2010).

The simplicity of resveratrol, associated with its interesting anti-cancer property, offers promise for the rational design of new chemotherapeutic agents and efforts have recently been devoted regarding a detailed study on the structure-activity relationship (SAR) of this type of substituted stilbene derivatives (Simoni et al. 2006). Several targets have been proposed for resveratrol and other *trans*-stilbene polyphenols, including p53/Bax pathway,

CD95-CD95 ligand pathway, or the putative resveratrol-binding proteins (Gosslau et al. 2008).

2.4 Reported procedures for making stilbene derivatives.

A large number of stilbene derivatives have been isolated from various plant species. The naturally occurring stilbenoid compounds are attracting considerable attention because of their wide -range of biological properties and potential therapeutic values. As a result of their interesting activities and the simplicity of their structure, a large number of derivatives were synthesized using various synthetic procedures with the aim of extending the structure-activity relationship information of this class of molecule. Listed below are examples of synthetic methods reported for making stilbenes.

2.4.1 Ruthenium (Ru)-catalysed cross metathesis

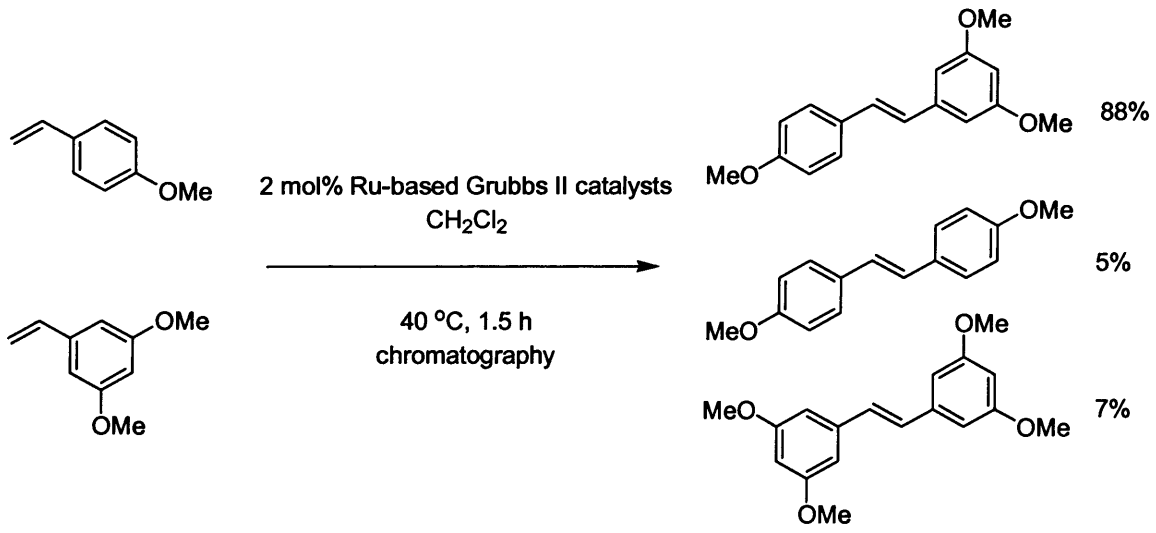


Figure 2.2: Alkene cross-metathesis.

Alkene cross-metathesis can provide convenient access to stilbenoid compounds, as reported by Valder et al. (2006) (Figure 2.2) and Ferre-Filmon et al. (2005). This Ru-catalysed cross-metathesis employing the Grubbs-II catalysts enables a straight forward synthetic access to a variety of unsymmetrical and symmetrical substituted *trans*-stilbenes. This protocol is

particularly attractive because "readily available starting materials (styrenes) are reliably converted into valuable products in a single step" (Valder et al. 2006) without even the need to protect sensitive hydroxyl functional groups (Ferre-Filmon et al. 2005). Although the reaction afforded only (*E*)-double bonds when styrenes were used as the substrates, it obviously lacks selectivity by giving a mixture of three different coupling products. This is probably due to their ability to either perform secondary metathesis on the newly formed C=C double bond of the stilbenoids compounds, or due to homocoupling. However, the selectivity issues in the formation of the coupling products can be improved by the use of one of the reaction partner in excess to the other (Ferre-Filmon et al. 2005).

2.4.2 The Wittig reaction

The Wittig reaction is the reaction of an α-phosphorous stabilised anion with a carbonyl derivative (Lawrence 1996). The Wittig reaction has been known for decades as a primary method for synthesising a stilbene block unit from two aromatic building blocks (Ferre-Filmon et al. 2005). The stilbene skeleton has been efficiently constructed by Wittig reaction between an aromatic aldehyde or ketone and an aromatic phosphonium ylide. A large number of starting materials (benzyl halides and aryl aldehydes) are commercially available (Husemoen et al. 2003). The reaction usually afforded the alkene in specific position and high yielding, and in many instances is stereoselective (Lawrence 2005). However, as the reactions are not catalytic, stereoselectivity issue still remain the main concern due to the formation of a mixture of *E* and *Z* alkenes, which is mainly due to the poor control over the configuration of the assembled C=C double bonds (Ferre-Filmon et al. 2005).

As showed by Roberti et al. (2003), preparation of stilbene derivatives was accomplished between the appropriate aromatic aldehydes and suitable aromatic ylides obtained from phosphonium salts (Figure 2.3). The reaction produced a mixture of *E* and *Z* isomers (7:3) which was purified and separated by flash chromatography. The same approach also utilized by Zhang and Go (2007), Alloatti et al. (2008) and Gosslau et al. (2008) to

synthesise their stilbenes. Unfortunately, other than selectivity issue between the formation of *E* and *Z* isomer, these method also suffered of low yield of *trans* product, and the production of triphenylphosphine oxide as a byproduct which needs to be purified by chromatography (Lion et al. 2005).

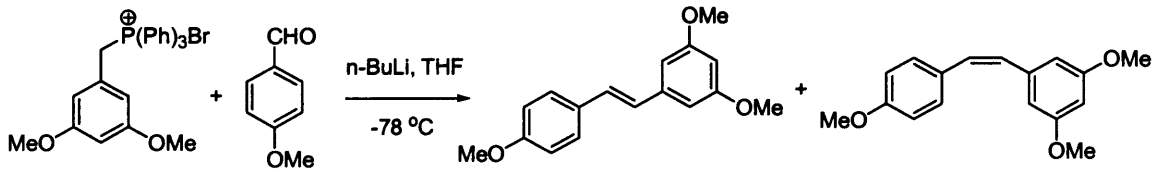


Figure 2.3: The Wittig reaction

2.4.3 Modified Wittig reaction (Horner-Wadsworth-Emmons reaction)

Figure 2.4: The HWE reaction

The coupling of phosphonic acid esters with carbonyl compounds, also referred to as the Horner-Wadsworth-Emmons (HWE) reaction, represents another powerful carbonyl olefination reaction (Figure 2.4). In contrast to the related Wittig-type reaction, the HWE reaction opens a convenient synthesis route for C=C double bonds, exhibiting *E*-configurations exclusively (Wadsworth and Emmons 1961). This methodology possessing the key advantages of both (*E*)-specificity and easy removal of the water-soluble dialkylphosphoric acid byproduct (Lion et al. 2005). As described by Lion et al. (2005), Heynekamp et al. (2006) and Li et al. (2006), this reaction involved the coupling of substituted benzylphosphonic acid diethyl esters with substituted benzaldehydes to afford exclusively *E*-stilbenes. This method was shown to give high overall yield and purity and without necessitating chromatographic purification at any stage.

In addition, Gester et al. (2007) also described the application of HWE reaction as novel labelling techniques in ^{18}F chemistry for the convenient synthesis of *E*-configured stilbenes, as outlined in figure 2.5 below. The procedure basically involved the reaction of substituted phosphonic acid esters with a readily available 4-(^{18}F)-fluorobenzaldehyde in the presence of potassium *tert*-butoxide in DMF at 60 °C for 15 min to give radiolabeled stilbene compounds. The procedure may be applied to the synthesis of polyphenolic compounds and aromatic amines bearing an *E*-configured stilbene backbone as pharmaceutically interesting compounds.

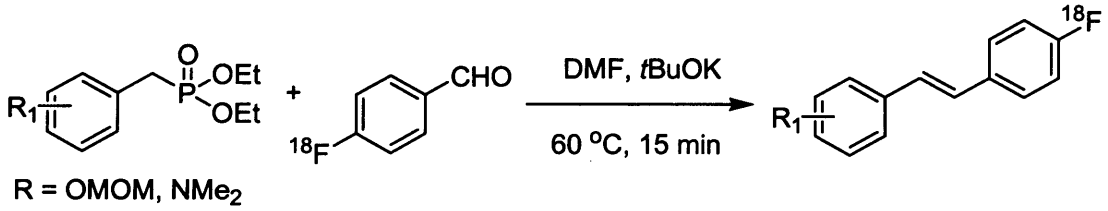


Figure 2.5: Synthesis of ^{18}F-labelled stilbenes via HWE reaction

2.4.4 Perkin reaction

The Perkin reaction is the pioneer method for the first chemical synthesis of resveratrol as reported by Spath and Kromp in 1941. This method was then re-investigated by Solladie et al. (2003) and employed by de Lima et al. (2009) for the synthesis of their stilbenes. This method first involved the condensation of substituted phenylacetic acid and substituted benzaldehyde (Figure 2.6). The decarboxylation was then carried out in the presence of copper chromite to afford the *cis-trans* mixture, where the *cis* isomer was always predominant. *Cis-trans* isomerisation of the mixtures in refluxing THF with phenyldisulfide led to the pure *trans* stilbenes in good yield. This method suffered from a long reaction time, tedious experimental work and harsh reaction conditions (Solladie et al. 2003). However, the need to use toxic quinoline-copper combination for decarboxylation of phenylacetic acid have represents a serious drawback especially when concerning the impact on the environment, which limits the synthetic utility of the Perkin type reaction to be further expanded for stilbene synthesis (Sinha et al. 2007)

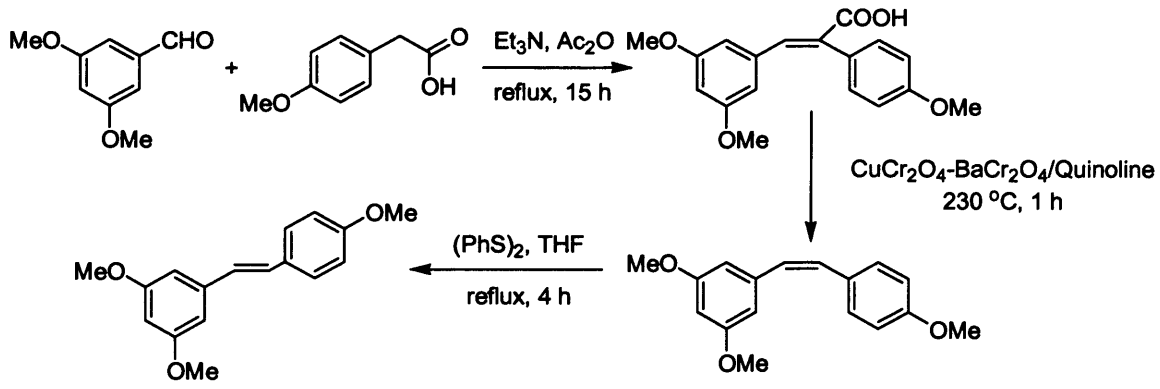


Figure 2.6: The Perkin reaction

2.4.5 Heck reaction (aryl halides as electrophiles)

The palladium-catalyzed C-C coupling between aryl halides or vinyl halides and activated alkenes (e.g. styrene, butyl acrylate) in the presence of a base is well-known now as a Heck reaction. "Industrial application of Heck reactions are rare because the reactivity of aryl halides decreases dramatically in order ArI>ArBr≥ArCl, which means that the cheap chlorides and even some bromides do not afford sufficiently high yields, turnover numbers and selectivity" (Ferre-Filmon et al. 2004). As exemplified by Guiso et al. (2001) and Farina et al. (2007) who used traditional Heck reactions for the synthesis of their stilbenes, the reactions were carried out by coupling the substituted styrene with substituted aryl halides in the presence of palladium (II) acetate, triphenyl phosphine as ligand, triethyl amine as base and acetonitrile as solvent to afford the stilbenes in moderate to good yields (Figure 2.7).

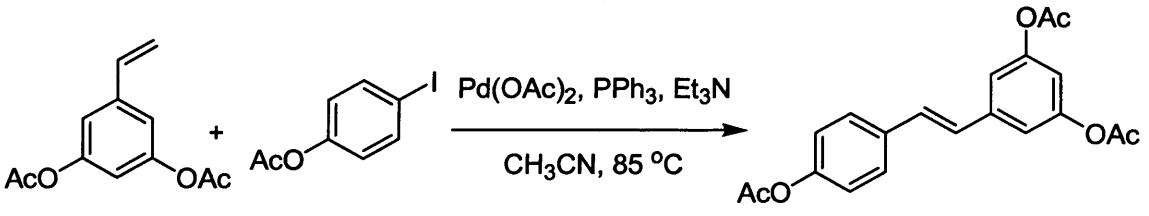


Figure 2.7: The Heck reaction with aryl halides

However, the traditional Heck reaction is often prone to unwanted oxidative degradation and other side reactions observed with phosphine ligands that are formed following a dissociation step in the catalytic cycle, hence highlighted the need for inert atmosphere conditions for efficient catalysis to occur. In certain condition, the addition of free ligand is needed to stabilise the palladium during the reaction, which later may afflict the final product purification (Eisnor et al. 2006). Therefore modified Heck reactions which use non-phosphine based complexes as catalyst precursors are more preferable. For example, nitrogen, oxygen and sulphur-containing palladacycles (Andrus and Song 2001) and palladium oxazoline complex (Eisnor et al. 2006) can provide excellent alternatives to their phosphorus-based counterparts.

2.4.6 Heck-Matsuda reaction (arydiazonium salt as electrophile)

This type of reaction comes from the synthetic advantages of using arenediazonium salts in Heck arylations. The reaction can be carried out under phosphine-free conditions, and are much easier than the traditional Heck protocol. Moreover, they are usually faster, less costly and greener. As reported by Moro et al. (2008), they managed to synthesise resveratrol, DMU-212 (3,4,5,4'-tetramethoxystilbene) and several analogues employing a very efficient palladium-catalyzed Heck-Matsuda arylation of styrenes with arenediazonium salts as the intermediates in a short, straightforward, regio- and stereoselective manner. The synthesis of resveratrol was achieved in 3 steps with an overall yield of 72% and DMU-212 was synthesised in a single step in 93% yield (Figure 2.8).

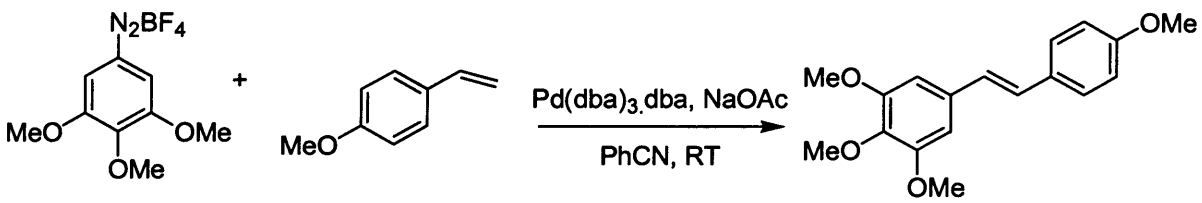


Figure 2.8: The Heck reaction with aryldiazonium salt

2.4.7 Heck reaction with ethene

2.4.7.1 Ethene gas as reagent

Ethene is the simplest form of acyclic alkene that can be found in nature. However, it has received relatively less attention in the Heck reaction (Kormos and Leadbeater 2008). One main reason which makes it less attractive to be used as a reagent in Heck reaction is that the difficulty in handling the ethene gas. "The over-reaction with the aryl halide coupling partner to give symmetrical stilbenes rather than monosubstituted alkene still remain as the major obstacle". Therefore, the amount of gas used in the reaction needs to be monitored precisely using a complex experimental setup which is usually costly and time consuming (Ferre-Filmon et al. 2004).

As shown in Figure 2.9 below, Kormos & Leadbeater (2008) have successfully designed strategies for the preparation of nonsymmetrical stilbenes using a one-pot two-step double Heck strategy. First they developed a protocol for the synthesis of styrene using ethene as the alkene coupling partner with aryl halide under controlled conditions featuring microwave irradiation. The stilbene-forming step involved the coupling of the second aryl halide partner to the crude styrene-containing reaction mixture to give the desired stilbene. Both steps involved the use of palladium as catalyst. DMF was used as solvent with a mixture of potassium carbonate and tributylamine as bases. All the reactions were done using microwave apparatus as it offers a convenient method for safely, easily and accurately loading vessels with gaseous reagents and monitoring the progress of reactions, yielding the desired stilbenes in low to moderate yield.

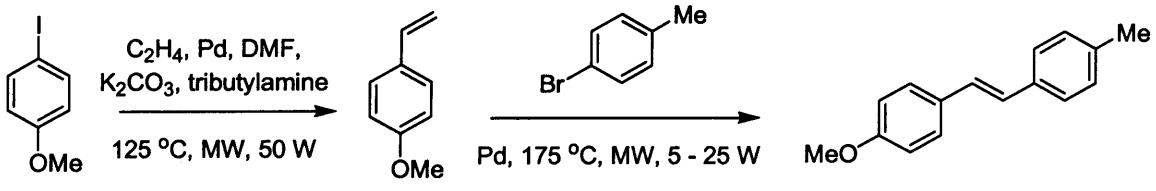


Figure 2.9: The Heck reaction with ethene gas

2.4.7.2 Substituted ethylenes as reagent

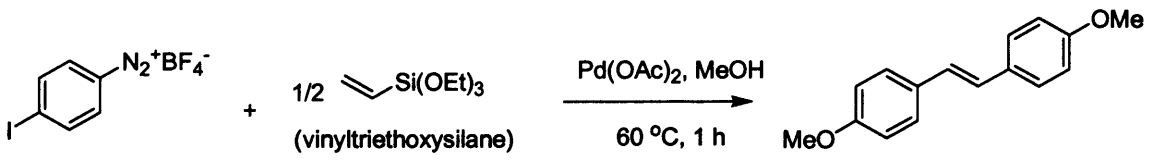


Figure 2.10: The Heck reaction with ethylene substitutes

As a way to counteract the problem of using ethene gas in stilbene synthesis, liquid vinyltrialkylsilane and vinyltrialkoxysilane were used as a more convenient approach to handle ethylene substitutes. For example, Sengupta et al. (1998) described a double Heck reaction of aryldiazonium salts with vinyltriethoxysilane which led to symmetrical *trans*-stilbene derivatives in moderate to good yields (Figure 2.10). The reaction used readily available starting material, simple operational procedure, fast reaction times, and afforded stilbene products in good yields under mild condition and produced regioisomerically pure products.

2.4.8 Decarbonylative Heck reaction

This method was first utilised by Andrus et al. (2003) and then further refined by Andrus and Liu (2006) for the synthesis of phytoalexin resveratrol analogues. This method involved the coupling of acid chloride derived from inexpensive substituted benzoic acids with substituted styrenes in the presence of palladium acetate, *N*-ethylmorpholine (NEM) as base and a carbene-type ligand. This approach takes only four steps and gives resveratrol in excellent 53% overall yield. The nature of the added base is critical for the success of the transformation, as shown by the use of NEM, a non-coordinating amine which proved optimal for the reaction.

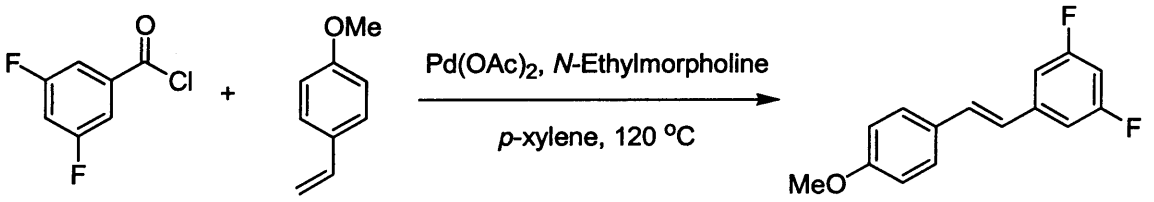


Figure 2.11: Decarbonylative Heck reaction

2.4.9 Suzuki reaction

The Suzuki reaction is another alternative to synthesise stilbenes and naphthalene-based stilbene analogues under palladium catalysis, as shown by Eddarir et al. (2001), Andrus & Song (2001) and Minutolo et al. (2005). The method usually involves the palladium cross coupling of boranes or boronic acids and esters with organic halides or diazonium salts. This method showed high selectivity and good susceptibility to different functional groups on either coupling partners. Other advantages include easy preparation of reagents which are facile to handle due to their insensitive nature towards water (Ferre-Filmon et al. 2004).

As shown in Figure 2.12 (Andrus and Song 2001), the cross coupling of styrylboronic acid and aryldiazonium salts with the presence of palladium (II) acetate can lead to formation of various stilbene derivatives. The reaction proceeded in the absence of any added base under mild conditions and with high yields (68-87%).

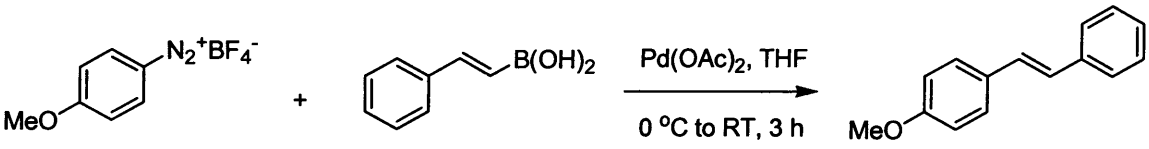


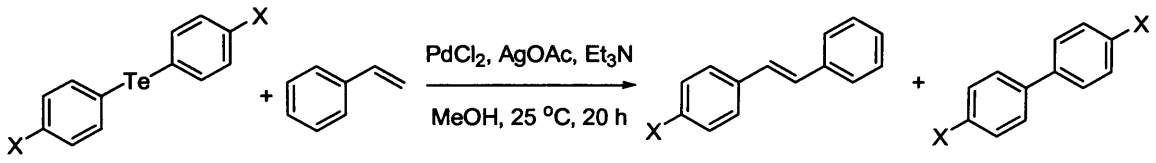
Figure 2.12: The Suzuki reaction

2.4.10 The Stille reaction

The Stille reaction is one of the most powerful tools in C-C coupling processes, which involves the "palladium catalysed coupling of organotin reagents with organic electrophiles such as aryl and vinyl halides or triflate" (Ferre-Filmon 2004). Organotin derivatives offered various advantages as a useful reagent in Stille reaction; "air stability, resistance to hydrolysis, easy handling and storage, and tolerance to a wide variety of functional groups". The C-C coupling reaction can be carried out under mild condition without

the need for any additives such as base, which is susceptible for various sensitive functional groups. This makes the reaction to have diverse functionality whether "in general organic synthesis, polymer functionalisation and particularly in natural product syntheses". Unfortunately, the formation of toxic tin byproducts which are harmful and can hamper later product purification is a major disadvantage of this procedure, which has driven many efforts to address this problem, mainly by the modification of the tin reagents (Carrera et al. 2008).

Nevertheless, the application of organostannanes intermediates in the syntheses of stilbene derivatives via Stille vinylation is extremely rare (Ferre-Filmon et al. 2004). One example described by Nishibayishi et al. (1996), involved the reaction of organic tellurides with various alkenes in the presence of palladium (II) salts to give corresponding substituted stilbenes (Figure 2.13).



X = H, Br, Me, OMe

Figure 2.13: The Stille reaction

2.4.11 The Negishi reaction

The new carbon-carbon double bond by the Negishi coupling reaction involved an organozinc compound, an organic halide and a nickel or palladium catalyst (King et al. 1977). Bosanac and Wilcox (2001) have showed that the Negishi reaction can be applied to the synthesis of stilbenes. In this palladium-catalysed reaction, an organozinc reagent was coupled with an organohalide to yield exclusively *Z*-isomers in 71% yield (Figure 2.14).

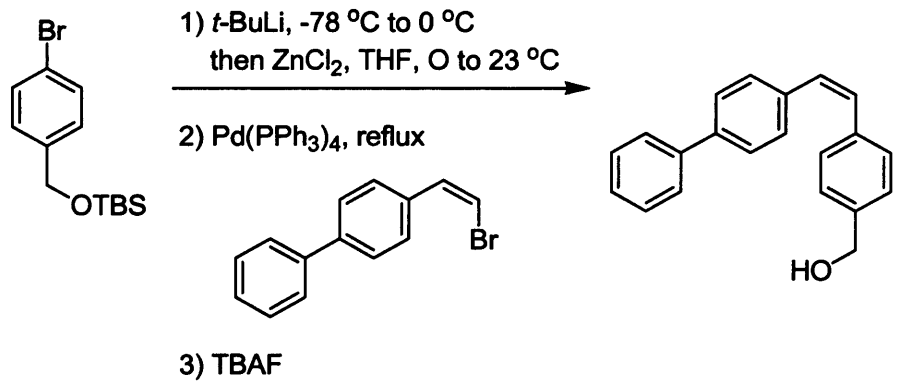


Figure 2.14: The Negishi reaction

2.4.11 The McMurry coupling

The McMurry reaction is an organic reaction in which two ketone or aldehyde groups are coupled and undergo reductive dimerisation to yield alkenes upon treatment with low-valent titanium reagents such as titanium(III) chloride and a reducing agent (McMurry and Fleming 1974). This carbonyl coupling reaction is able to provide symmetrical stilbenes from the corresponding aldehydes and ketones which can be manipulated to the synthesis of various symmetrical chemical entities (Ferre-Filmon et al. 2004). Ali et al. (1992), obtained symmetrical polyalkoxy- and polysilyloxystilbenes by reductive coupling of alkoxy and silyloxybenzaldehydes, respectively, with zinc and titanium tetrachlorides (Figure 2.15). All products isolated under these reaction conditions possessed a *trans* stilbene structural motif. Nevertheless, the *Z*-isomers can still be synthesised if the orientation of the two carbonyl moieties is controlled by geometric constraints (Ferre-Filmon et al. 2004).

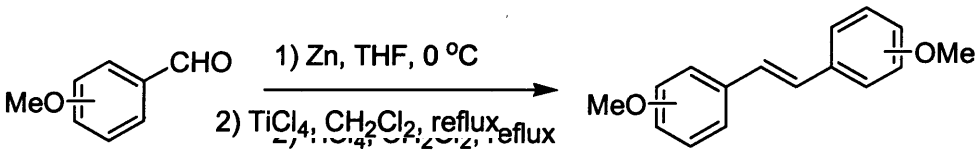


Figure 2.15: The McMurry reaction

2.5 Research objective

The aims of this study are to synthesise substituted stilbene analogues based on the resveratrol scaffold, and to evaluate their biological activities. The more lipophilic resveratrol derivatives are anticipated to have improved drug-like properties and potencies to be developed as potential anti-cancer agents. Substituted stilbene analogues were targeted to have better resistance towards metabolism and therefore increased bioavailability and drug-likeness.

The initial part of the study was to exploit traditional methods for parallel solution phase synthesis based on the reported Horner-Wadsworth-Emmons (HWE) reaction in order to synthesise a library of methoxylated, hydroxylated, fluorinated and pyridine-based stilbene analogues. The synthesis procedure was further expanded by using microwave irradiation and palladium-catalyzed based methods to compare various synthetic methods for stilbene synthesis. The synthesised stilbene analogues were further tested on a panel of cancer cell lines [prostate (PC3), non-small lung (A549), colon (Lovo) and breast cancer (MCF-7)] in order to evaluate their antiproliferative activity and to generate possible structure activity relationships. At the same time, the synthesised stilbenes were further evaluated for their ability to inhibit Wnt signaling pathway (discussed in chapter 4) in colon cancer stem cells, where the aberrant activation of this pathway is often implicated in driving the formation of various human cancers, particularly those of the digestive tract.

2.6 Synthesis of methoxylated, hydroxylated and fluorinated stilbene analogues via Horner-Wadsworth-Emmons (HWE) reaction.

There are a number of synthetic methods that had been developed towards the synthesis of stilbene analogues. Generally, the synthesis was achieved by combining two molecular fragments together via carbon-carbon double bond (C=C). The synthetic methods chosen will give exclusively *trans* isomers with no detectable *cis* isomers. Any methods that give mixtures of *E* and *Z* isomers which will necessitate chromatographic separation at the end

were not considered in this study. Therefore, as the initial part of the study an efficient method for the synthesis of *trans*-stilbenes via HWE reaction as described by Lion et al. (2005) was chosen. This method involves the reaction of a phosphonate ester with a benzaldehyde to produce predominantly *trans* alkenes as shown below (Figure 2.16).

Figure 2.16: The Horner-Wadsworth-Emmons reaction.

2.6.1 The Michaelis-Arbuzov rearrangement

The first step in the reaction is the formation of a phosphonate ester as an intermediate, via Michaelis-Arbuzov reaction. It is one of the most widely used and versatile methods for the synthesis of phosphonates (Bhattacharya and Thyagarajan 1981). The reaction for the formation of diethyl phosphonate ester is very straight-forward. In this case, the methoxybenzyl bromide is converted to the required substituted diethyl phosphonate esters by the reaction with an ester of trivalent phosphorus (e.g. triethyl phosphate). Figure 2.17 shows the reaction mechanism involved in the synthesis of the phosphonate esters.

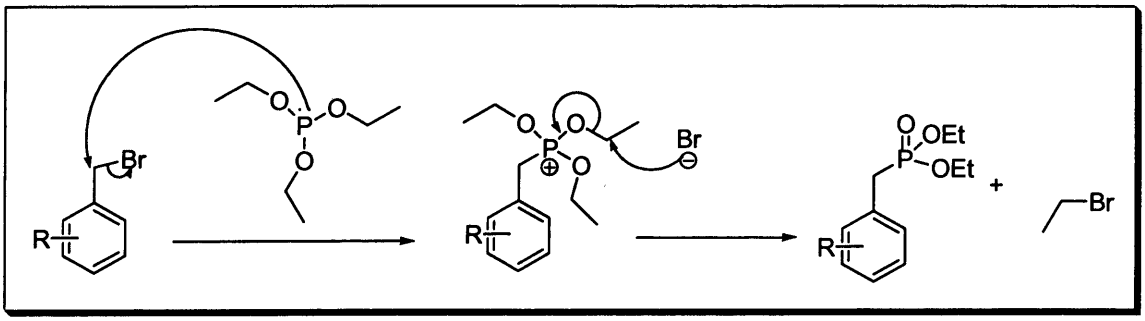


Figure 2.17: Synthesis of phosphonate esters: Michaelis-Arbuzov reaction

The overall reaction conditions were monitored by TLC. The phosphonate esters were subjected to further synthesis procedure for stilbene synthesis. The Michaelis-Arbuzov reaction (Figure 2.18) proved to

be an efficient method to synthesise phosphonate esters yielding the desired products in excellent yields (Table 2.1).

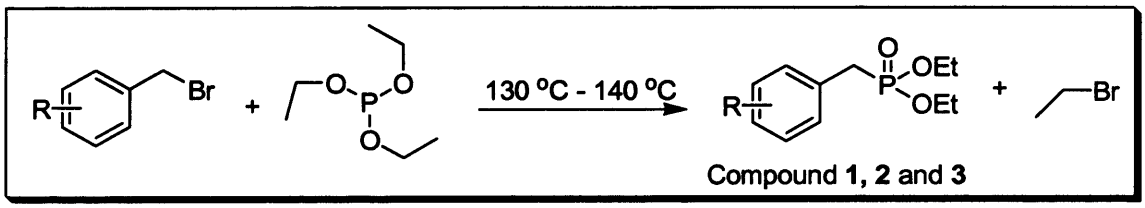


Figure 2.18: The Michaelis-Arbuzof reaction

Table 2.1: Summary of the synthesis of the phosphonate esters.

Compound no.	R	Yield (%)
1	4-OMe	96
2	3-OMe	99
3	3,5-diOMe	98

2.6.2 The Horner-Wadsworth-Emmons (HWE) reaction.

Horner-Wadsworth-Emmons olefination chemistry as described by Lion et al. (2005) was chosen as the main reaction procedure for the synthesis of substituted *trans*-stilbene to ensure regioselectivity. Reaction of substituted benzylphosphonic acid diethyl ester with substituted benzaldehydes in DMF using sodium methoxide as the base in the presence of 18-crown-6 at 120 °C afforded substituted stilbenes exclusively in the (*E*)-conformation. A slight excess of benzaldehyde was required to ensure complete reaction and the limiting factor is phosphonate ester. Excess benzaldehydes can easily be eliminated by using Girard's reagent [(carboxymethyl) trimethylammonnium chloride hydrazide), forming a weakly basic nitrogen nucleophile and acetic acid, giving water-soluble benzaldehyde hydrazide derivatives which can simply removed by a wash with brine. The diethylphosphoric acid byproduct is water soluble and can easily be removed. The reaction mechanism involved in the synthesis of stilbenes via HWE reaction is summarised in Figure 2.19.

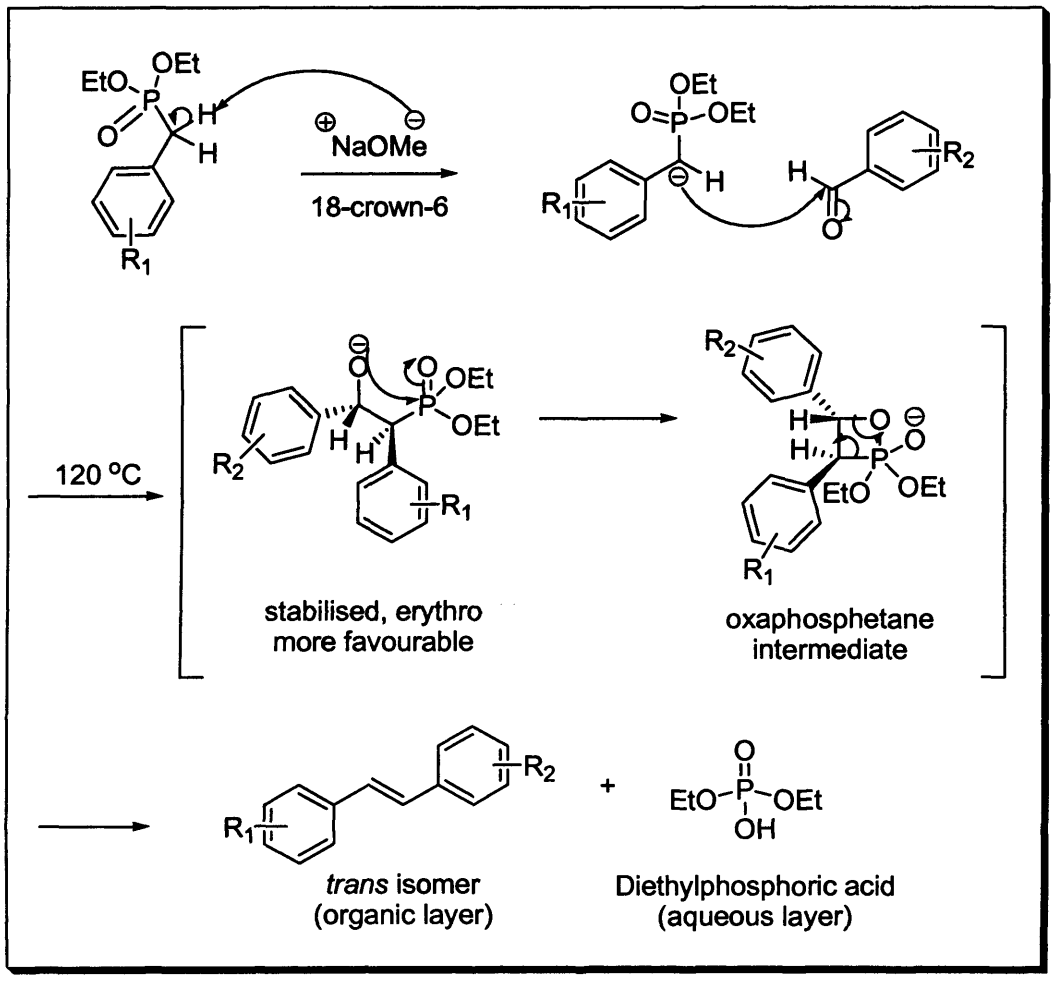


Figure 2.19: Reaction mechanism of the HWE reaction

Unlike the Wittig reaction which uses the phenylphosphonium intermediate, the HWE reaction uses phosphonate stabilised carbanions which will react with the carbonyl group of the benzaldehyde. Deprotonation of the phosphonate ester by a strong base such as sodium methoxide resulted in the generation of phosphonate ion. The carbon atom of the carbonyl group bears a slight positive charge resulting from the difference in electronegativity between the carbon and the oxygen group and therefore will become the target for the nucleophilic phosphonate ion to attack. This nucleophilic addition will form oxaphosphetane, an unstable ring structure which can undergo rapid elimination to afford the corresponding *trans*-alkenes on heating (Bruckner 2002).

18-crown-6 ether is used to improve the dissolution of sodium methoxide in DMF which results in the generation of greater nucleophilicity (Reichwein & Pagenkopf 2003). The *trans* product is favoured over the *cis* product as the *trans* pathway seems to be formed more quickly, and the *cis*-oxaphosphetane will be gradually converted to the *trans*-oxaphosphetane (Bruckner 2002). The products obtained by this method showed no detectable *Z* isomer by ^{1}H NMR analysis. This is confirmed by looking at the ethylenic protons which appeared as two distinct doublets with a coupling constant of 16-17 Hz (*cis* isomer would show a coupling constant of 9-10 Hz).

2.6.3 Synthesis of methoxylated stilbene analogues

To begin, methoxylated stilbene analogues were chosen first for synthesis, as the starting materials are readily available and there is no need to protect any functional group. The reaction started with the making of substituted methoxy benzyl phosphonate esters **(1)**, **(2)** or **(3)** (refer 2.6.1) which were then coupled with differently substituted methoxy benzaldehydes to afford the methoxylated stilbenes (Figure 2.20 and Table 2.2).

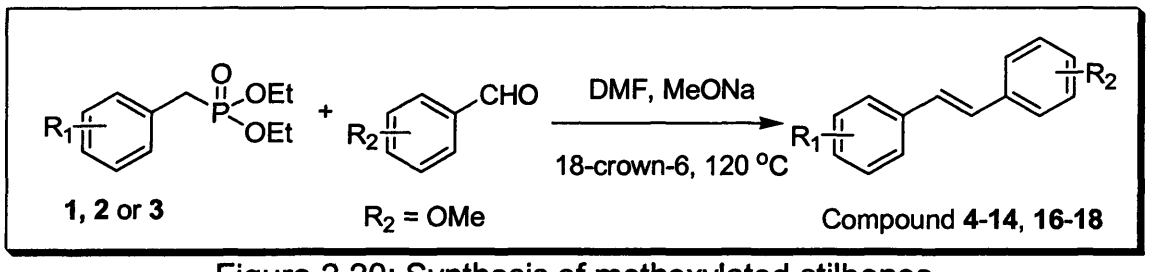


Figure 2.20: Synthesis of methoxylated stilbenes

Table 2.2: Summary of the synthesis of methoxylated stilbenes.

Compound no.	R_1	R_2	Yield (%)
4	4-OMe	4-OMe	91
5	3-OMe	4-OMe	84
6	3-OMe	3-OMe	57
7	3-OMe	3,5-diOMe	16
8	4-OMe	3.5-diOMe	18

9	4-OMe	3,4-diOMe	68
10	4-OMe	2,4-diOMe	57
11	4-OMe	2,5-diOMe	45
12	3,5-diOMe	3,4-diOMe	40
13	3,5-diOMe	2,4-diOMe	19
14	4-OMe	3,4,5-triOMe	21
16	3,5-diOMe	3,5-diOMe	18
`17	3,5-diOMe	2,5-diOMe	22
18	3,5-diOMe	3,4,5-triOMe	74

The attempt to synthesise compound **15** using compound **1** and 3-methoxy 4-hydroxy benzaldehyde, without protection of the hydroxyl group was unsuccessful, due to the nature of the unprotected hydroxyl group which was unstable under these reaction conditions as it can undergo other side reactions. This highlighted the need to find a suitable protecting group in order to synthesise stilbene analogues with hydroxylated substitution which should be stable under the reaction condition and can easily be cleaved without affecting the stilbene double bond. Further study by Paola Casule, an Erasmus student (February 2010 to July 2010) in our lab identified methoxyethoxymethyl ether (MEM) group as a suitable protecting group to synthesise hydroxylated stilbenes via HWE reaction, where deprotection method using pyridinium *p*-toluene sulfonate in refluxing methanol managed to give the desired hydroxylated compound without necessitating column chromatographic purification at any stage.

Traces of DMF can still be detected in most of the reaction products even after extraction with ether, which is largely immiscible with DMF. This made the purification steps harder, as the DMF cannot be easily eliminated even after prolonged evaporation due to its high boiling point. Nevertheless, keeping the impure compound which had undergone work-up at room temperature for several days increased the probability of the compounds to precipitate thus making the final purification step easier. The Girard's reagent was able to remove excess benzaldehyde from most of the compounds,

except for compounds **7**, **8**, **13**, **14**, **16** and **17** which could not be eliminated fully from the reaction mixture even after the addition of more Girard's reagent and prolonged stirring for more than 24 hours. These make it necessary to use column chromatography to separate the excess benzaldehydes, thus contributing to lowering the yield of the final compounds.

The overall yield obtained from the HWE reaction varied greatly between different substitutions. Formation of compounds **4**, **5** and **18** proceeded smoothly and gave clean precipitate after quenching with water with no need for further purification, giving the pure compounds in excellent yields. Compounds **6** and **9** were obtained in moderate yield after recrystallization using ethanol/ethanol-water. Compounds number **10**, **11**, **13** and **17** were prepared in low to moderate yield maybe due to the steric hindrance by the 2-methoxy substituents which prolonged the reaction times. Even after 24 hours of heating the starting material can still be detected and the reaction did not go to completion.

Compounds with a 3,5-dimethoxy substitution at the phosphonate ester were found to be harder to synthesize (compound **12**, **13**, **16** and **17**, with exception of compound **18**). A few attempts had to be made before obtaining the desired compounds. These compounds also suffered from the presence of unreacted phosphonate esters even after 24 hours of reaction and the need for column chromatography purification. It was also not surprising to get compound **14** in low yield (21%), due to the inductive effect of three methoxy groups as electron donating groups on the benzaldehyde ring and the *para*-methoxy group at the phosphonate ester ring which will make the reaction slower, thus contributing to lower yields.

The hardest compounds to make were compounds **7** and **8**. Attempts to synthesise the compounds using compound **3** as the intermediate and their 3-methoxy and 4-methoxy benzaldehydes as their counterpart failed to give the desired compounds. Nevertheless, when compound **1** and compound **2** were used as the intermediates, with 3,5-dimethoxy

benzaldehyde as their counterpart, the desired compounds **8** and **7**, respectively were obtained at low yields after purification using column chromatography to remove unreacted starting materials.

2.6.4 Synthesis of methoxylated stilbenes with other substitution (fluorine, bromine or nitro groups).

Following the successful approach to the synthesis of methoxylated analogues of stilbenes via HWE reaction, the same protocols were chosen to synthesise methoxylated stilbenes with other substitution patterns, where substitution with fluorine was chosen first. The same phosphonate esters were used (compound **1** or **2**) and 3- and 4-fluorobenzaldehyde as their counterpart (Figure 2.21). Using this procedure, a few stilbene analogues bearing methoxy and fluoro substitution have been successfully synthesized as outlined in table 2.3 below:

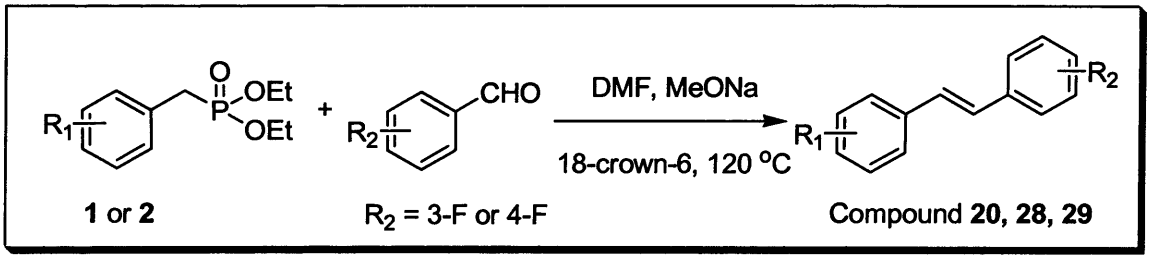


Figure 2.21: Synthesis of compound 20, 28 and 29.

Table 2.3: Summary of the synthesis of fluorinated stilbenes via HWE reaction.

Compound no.	R_1	R_2	Yield (%)
20	3-OMe	3-F	48
28	3-OMe	4-F	7
29	4-OMe	4-F	79

Compound **20** and **29** proceeded smoothly giving the compounds in moderate to good yields after recrystalisation using ethanol. Recrystalisation of compound **28** using ethanol and ethanol/water gave mixtures of 2 types of

crystals. The recrystallisation was then repeated using different solvent pairs: methanol-water and acetone-water, but both procedures still did not give any difference to the formed crystals. The next attempt involves the separation of the crystals using column chromatography (hexane:ethyl acetate = 5:1), where two separable crystals were obtained, the desired product in 7% yield and the unexpected product in around 8% yield. The yields of the two crystals were very low, maybe due to the loss of the compounds after several attempts of recrystalisation and separation by column chromatography.

The unexpected compound was then subjected to identification using NMR for proton, carbon and fluorine and mass spectrometry. The ^{1}H-NMR result showed two peaks at δ 3.88 and 3.86 ppm indicative of two methoxyl groups. The proton signal also lacking any indication of proton-to-fluorine coupling which usually showed as complex coupling, especially when the protons are adjacent to the expected fluorine atom. Although the decoupled fluorine NMR also showed a single peak at δ -114.11 ppm, the high noise to signal ratio suggested that the fluorine peak detected might just be a slight impurities which still remain within the product. The ethylenic bond with the coupling constant (*J* value) of 16.5 ppm can be detected from the proton NMR suggesting the formation of a *trans* stilbene isomer. The compound was subjected to further analysis with mass spectrometry, which indicated the rise of a major peak at *m/z* 240.10 which is different to the parent compound (compound **28** had calculated molecular weight of 228.28). At this point, the structure of the unexpected compound was expected to be a 3,4'-dimethoxystilbene (as previously synthesised compound **5**). This was proved to the fact that compound **5** and the unexpected compound both have identical ^{1}H- and ^{13}C-NMR, and identical molecular weight (compound **5** had calculated molecular weight of 240.12). Nevertheless, the unexpected compound is expected to be less pure indicated by a slight decrease in melting point compared to the purer compound **5** (compound **5** = 106-108 °C; unexpected compound = 101-104 °C).

The reason for this unusual observation is expected to be due to the displacement of the fluorine atom with methoxyl group from the base used

(sodium methoxide). This displacement is expected to take place before the formation of the stilbene, due to the electron withdrawing nature of the carbonyl group of the 4-fluorobenzaldehyde (Figure 2.22). The displacement of fluorine is harder to occur after the formation of stilbene due to the lack of electron withdrawing effect to drive the fluorine substitution. Since methoxy is a better nucleophile than fluorine, the formation of the unexpected compound is slightly higher (8%) compared to compound **28** (7%). The factor which determines this kind of condition to occur is unknown. Repeated attempt to synthesis compound **28** still resulted in the same observation.

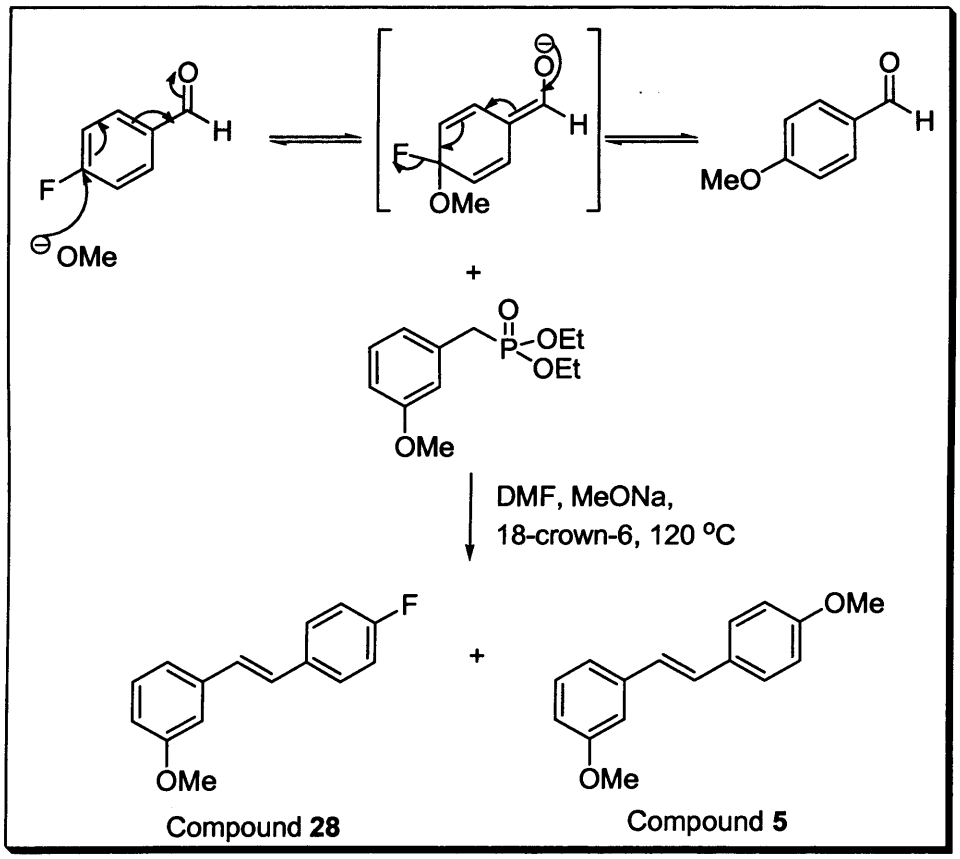


Figure 2.22: Attempt to synthesis compound **28** which gave rise to the formation of the unexpected compound **5**.

The next attempts were dedicated to the synthesis of other stilbene derivatives containing nitro and bromo substitution, in order to further expand the applicability of the HWE method (Figure 2.23). Four compounds have been synthesised as outlined in the table 2.4 below:

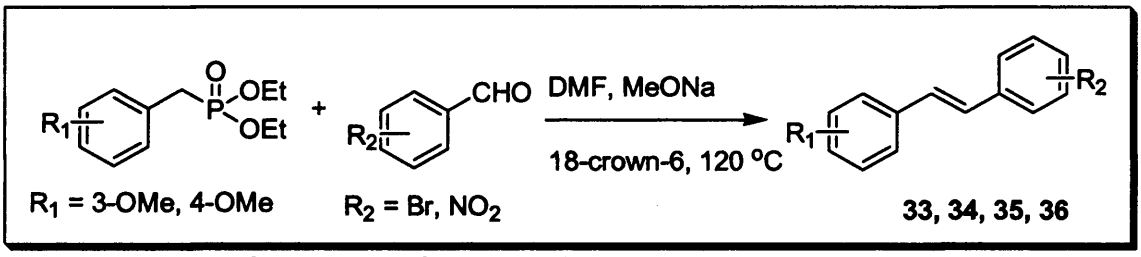


Figure 2.23: Synthesis of methoxylated stilbenes with bromo and nitro substitution.

Table 2.4: Summary of the synthesis of nitrated and brominated stilbene.

Compound no.	R_1	R_2	Yield (%)
33	3-OMe	4-NO_2	52
34	4-OMe	4- NO_2	41
35	3-OMe	4-Br	30
36	4-OMe	4-Br	69

These compounds suffered from incomplete reaction, with traces of the phosphonic acid diethyl esters still detected even after 30 hours of heating. Upon quenching with water, all the reactions formed precipitates which were collected and further recrystallised in hot ethanol to afford the pure title compounds in moderate yields.

The synthesis procedure was also expanded to test the applicability of the HWE reaction by making some styryl pyridines. The same approach was applied using 6-chloropyridine 3-carboxaldehyde with compound **1** or **2** as the starting materials (Figure 2.24).

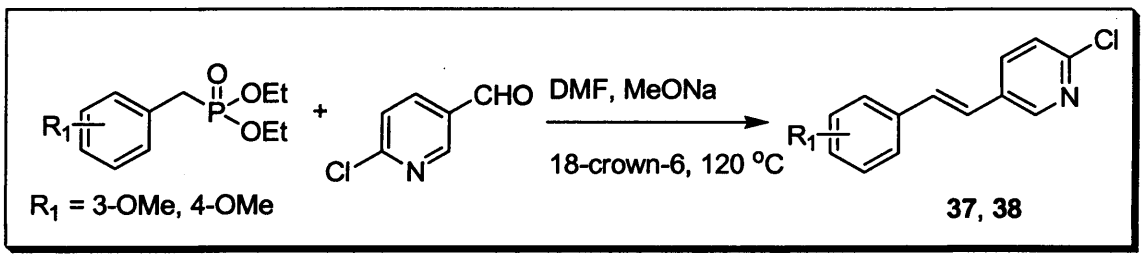


Figure 2.24: Synthesis of styrylpyridines.

Table 2.5: Synthesis of styrylpyridines.

Compound no.	R_1	R_2
37	3-OMe	6-chloropyridine
38	4-OMe	6-chloropyridine

The TLC suggested that the reactions did not go to completion, as the phosphonate esters can still be detected even after 48 hours of heating. Compound **37** formed oily residue after quenching with water, and the removal of excess of benzaldehydes via Girard's reagent was successful. The crude compound was obtained as a solid which was further purified by recrystallization in ethanol.

The synthesis of compound **38** was more straightforward as the reaction formed precipitate after quenching with water, which was further recrystallized in ethanol to afford white solid. But after recrystallization, the purity of both compounds were not satisfactory as indicated by the proton NMR. The number of proton under the peak at the aromatic region showed more than 9 expected protons. There was even an unidentified peak at around δ 5.43 ppm indicating the possibility of a side reaction, although carbon-carbon double bond peaks, indicating a *trans* stilbene (J = 16.5 Hz) were detected. The same case was seen in both compounds even after repeated attempts to recrystallise them. These findings suggested the possibility of side reactions that interfere with the formation of the desired compounds. This may be due to the lone pair of the pyridine ring which was able to further react and the harsh reaction condition that make the HWE procedure less suitable for the synthesis of styryl pyridine.

At this stage, HWE reaction was found as an efficient method for making methoxylated, fluorinated and other substituted stilbene analogues. The next effort was focused in making hydroxylated stilbenes by finding a suitable protecting group to protect the phenol, as the initial attempt to make hydroxylated stilbene without protection of the hydroxyl group was unsuccessful. A method by Sinha et al. (2007) describing the synthesis of

hydroxystilbene with *trans* selectivity under microwave irradiation without the need for protection of the hydroxyl group was found to be attractive. This method was chosen for the next stage of the synthesis due to its mild, simple and environmentally benign reaction condition.

2.7 Synthesis of hydroxylated stilbenes under microwave activation

According to Sinha et al. (2007), the synthesis of hydroxylated (*E*)-stilbenes via modified Perkin reaction between substituted benzaldehydes and phenylacetic acids bearing 4-hydroxy substitution at the aromatic ring, in the presence of piperidine-methylimidazole and polyethylene glycol under microwave irradiation is an unusual, mild but convenient one-pot two-step synthesis of hydroxylated stilbenes with *trans* selectivity. The developed protocol provides a green alternative to the classical Perkin reaction method employing a toxic decarboxylating agent in the form of quinoline-copper salt, and the requirement for harsh protection-deprotection steps. The microwaves can effectively bring out the simultaneous condensation-decarboxylation reaction, especially in view of the fact that all the intermediates proposed in the mechanism are more polar than the starting material. Figure 2.25 showed the hypothesised reaction mechanism involved in the formation of hydroxylated stilbenes under microwave irradiation.

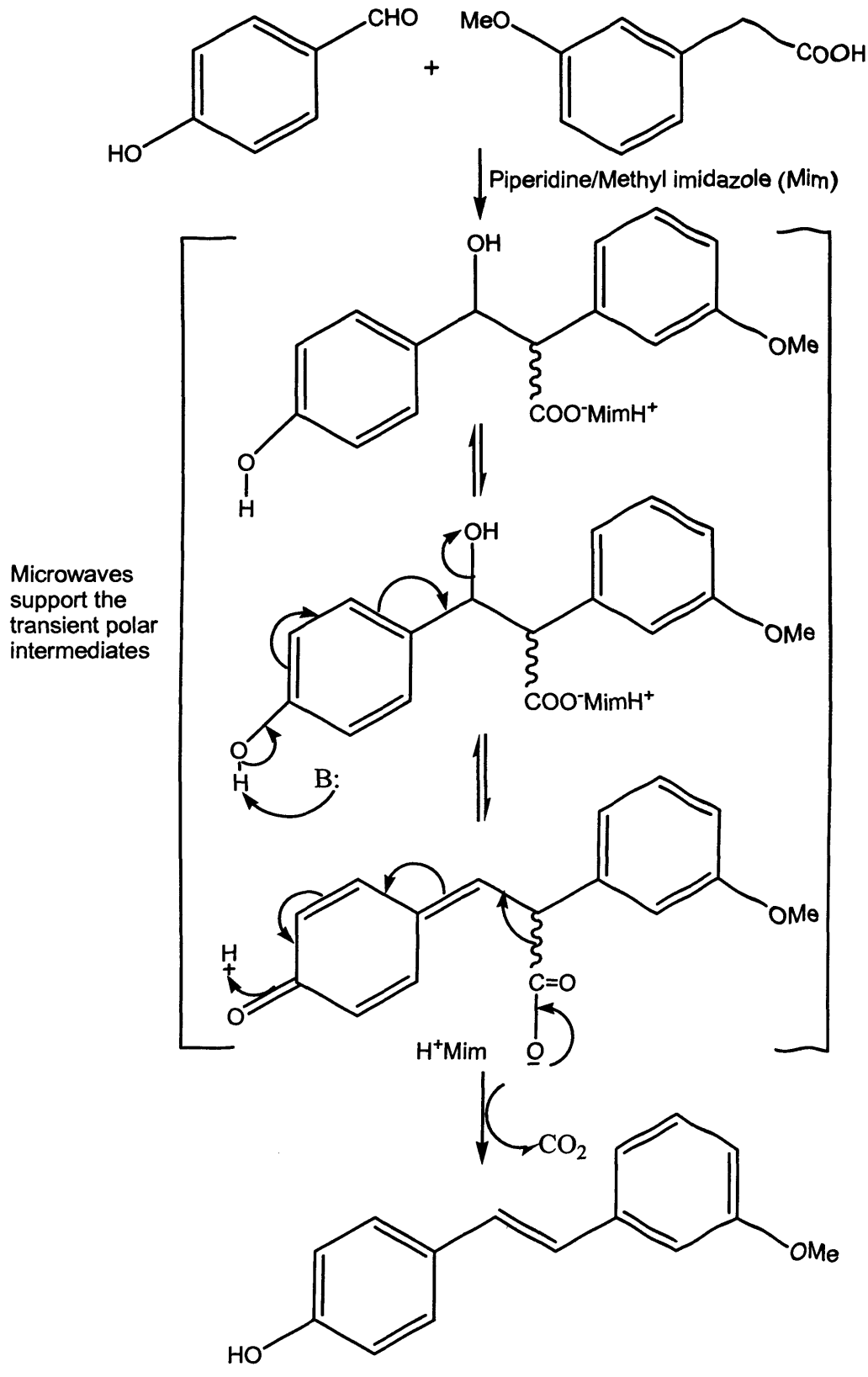


Figure 2.25: Hypothesis of the reaction mechanism involved in the synthesis of hydroxylated stilbenes under microwave irradiation (Sinha et al. 2007).

Synthesis of this series of compounds was started by the synthesis of compound **22** (3-methoxy 4'-hydroxy stilbene) by irradiating a mixture of 4-hydroxy benzaldehyde with 3-methoxy phenyl acetic acid in the presence of piperidine-methyl imidazole as the base and poly-ethylene glycol (PEG; average molecular weight: 200) as the solvent at 160 °C for 10 minutes (Figure 2.26). After acidification, extraction and column chromatography, the pure compound was obtained in 22% yield (as opposed to the 44% reported yield).

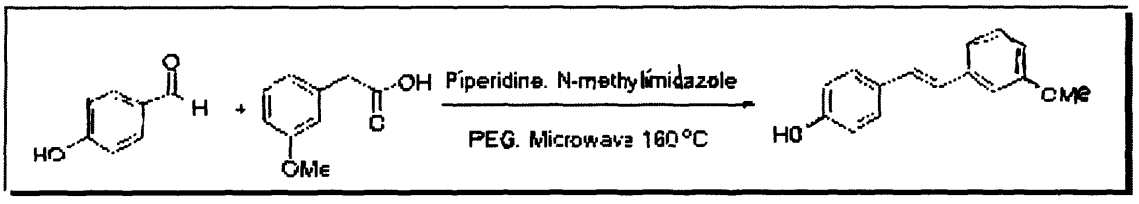


Figure 2.26: Synthesis of compound **22** via microwave irradiation

At this stage the reason for the lower yield was unknown. Nevertheless, further attempts to synthesise compounds **23** to **27** using the same method were carried out as outlined in table 2.6:

Table 2.6: Attempts to the synthesis of other hydroxylated stilbenes via microwave irradiation.

Compound no.	Substituted benzaldehyde	Substituted phenyl acetic acid
23	4-OH, 3-OMe	4-OH
24	3,4-diOMe	4-OH
25	3,5-diOMe	4-OH
26	4-OH, 3-OMe	3-OMe
27	3F	4-OH

All of the experiments were carried out under the same chemical condition as compound **22** and irradiated for 10 minutes. But after the work-up, none of the reaction mixture showed sign of desired compound formation even though the TLC showed the limiting reagent (substituted phenyl acetic acid)

were fully consumed. The TLC also showed the formation of many impurities indicating the possibility of side reactions. Purification by column chromatography only managed to get the starting benzaldehydes as the main product. The synthesis procedure was repeated by prolonging the reaction time from 10 to 20 minutes. The increased reaction time did not improve the result and only added to the formation of more impurities. At this stage, this synthetic procedure was considered as not efficient for the synthesis of hydroxylated stilbene with limited susceptibility to various ring substitution.

Nevertheless, in order to ascertain the specific role of microwave irradiation in this type of synthesis, the reaction of compound **22**, **26** and **27** were carried out using the same chemical condition but under conventional heating at reflux temperature (160 °C). After heating for 24 hours, the TLC showed spots of the unreacted starting material with many impurities and no product formation can be detected after the work-up. This finding confirmed the applicability of the said method for microwave irradiation, but not using conventional heating.

2.8 Synthesis of fluorinated stilbenes via palladium-catalyzed Heck reaction.

The Heck reaction is an established method for formation of the carbon-carbon bond. It is widely used in the synthesis of stilbenes because of its applicability, flexibility and adaptability. The synthesis of fluorinated stillbenes by using palladium-catalyzed Heck reaction was intended to compare its applicability and reproducibility with the HWE reaction for the synthesis of stilbene analogues.

2.8.1 Decarbonylative Heck reaction

The first approach chosen to synthesise fluorinated stilbenes via Heck reaction was by a method described by Andrus and Liu (2006) which was reported to be efficient in synthesising fluorinated stilbenes giving the

products in good overall yield (74% to 88%). The procedure involved the reaction of substituted benzoyl chloride and substituted styrene in the presence of palladium (II) acetate, *N*-ethyl morpholine as base in *p*-xylene at 120 °C as outlined in figure 2.27 below:

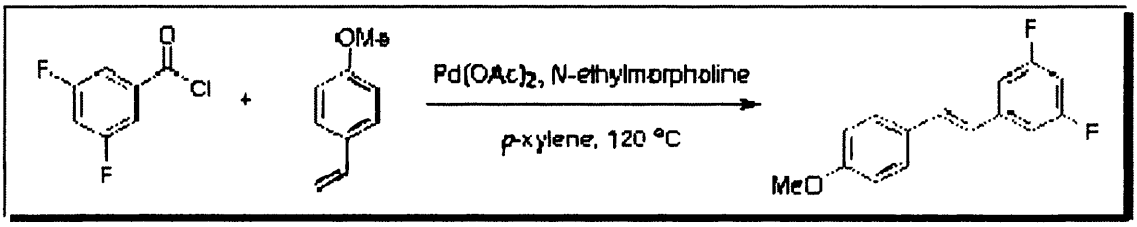


Figure 2.27: Decarbonylative Heck reaction

To begin the synthesis, 3,5-difluorobenzoyl chloride (1 equiv.) was reacted with 4-methoxy styrene (1 equiv.) under the reaction conditions described above. Even after heating for 48 hours, the reaction did not go to completion. However, the reaction was stopped and after the work-up with ethyl acetate and brine, the NMR showed no product formation. Although most of the xylene had been eliminated, some still remained and could be detected from ^{1}H-NMR. The TLC also showed the formation of many impurities. Then the reaction was repeated using the same procedure but under nitrogen, but the inert condition seemed not to give any difference in the reaction condition and the product formation. So, in our hands, this method was not successful to synthesise fluorinated stilbene analogues.

2.8.2 Heck-Matsuda coupling reaction

Next attention was focused on the synthesis of fluorinated stilbene analogues by using Heck-Matsuda reaction, which involved the Heck arylation of styrene with arenediazonium salt (Moro et al. 2008). This reaction can be carried out in the absence of ligand (phosphine-free condition) and reported to be much easier to handle, faster, less costly and greener than the traditional Heck protocol. The diazonium salt should be an efficient leaving group giving nitrogen gas as the by-product.

The reaction was started by preparing the diazonium salt following the method described by Doyle and Bryker (1979). Boron trifluoride diethyl etherate was added to *para*-methoxy aniline in cooled dry dichloromethane in the presence of *tert*-butyl nitrite. The resulting crystalline precipitate showed to be the pure compound with no need for further purification and giving the product in excellent yield (97% to 99%). The reaction involved is outlined in figure 2.28:

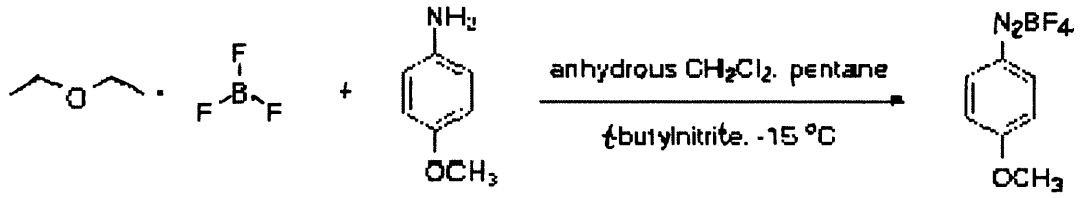


Figure 2.28: Synthesis of *para*-methoxy diazonium tetrafluoroborate salt

The afforded diazonium salt was used straight away in the next reaction. In order to test the applicability of this method, the synthesis of 4-fluoro 4'-methoxy stilbene (compound **29**) was chosen to compare the result of the same compound synthesized using HWE reaction. The synthesis of another analogue, 2-fluoro 4'-methoxy stilbene (compound **30**) was carried out to check the reproducibility of this synthetic procedure. The synthetic procedure is outlined in figure 2.29 below:

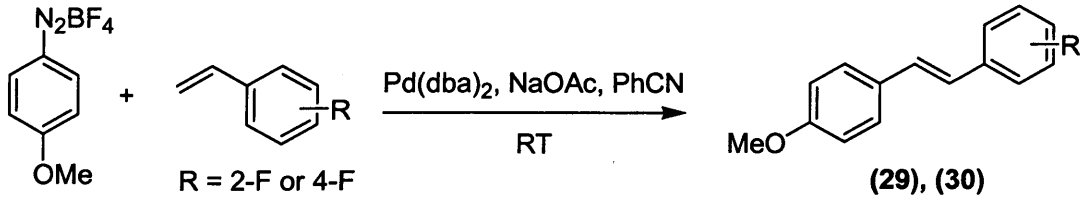


Figure 2.29: Heck-Matsuda reaction

The synthesis of compound **29** via this method was shown to be unsuccessful. The reaction was completed within 3 hours as determined by TLC, where the limiting reagent (4-fluoro styrene) can no longer be detected. Unfortunately, the work-up of the reaction was not as easy as expected. The

high boiling point solvent benzonitrile was very hard to evaporate *in vacuo*. The extraction of the reaction mixture using water and common organic solvents failed as the mixture formed an emulsion and was not immiscible with both water and common organic solvents. The crude product showed no methoxy peak and no fluorine peak which indicated the failure of the reaction. It was anticipated that the starting materials had undergone degradation during the reaction. Repetition of the same reaction under inert atmosphere did not improve the reaction outcome. The synthesis of compound **30** using the same approach, under normal condition and under nitrogen was also unsuccessful. No product formation can be detected even after 72 hours of reaction. The crude product only showed the possible formation of side product polystyrene (with 32 equivalent protons detected by ^{1}H-NMR at the aromatic region).

2.8.3 Heck reaction via palladium-oxazoline complex and Heck reaction in water as solvent.

The failure of the previous attempts prompted the need to find a more reliable Heck reaction procedure for stilbene synthesis. Therefore, another Heck reaction procedure was sought, this time using method described by Eisnor et al. (2006) which described the synthesis of stilbenes by a palladium (Pd)- oxazoline complex (compound **29a**). Unlike other palladium catalysts which are air sensitive and prone to further oxidation, necessitating inert atmosphere and in some cases necessary for addition of ligands which will hamper later product purification, this Pd-oxazoline complex can efficiently operate in open air and does not require additional ligand. The synthesis of compound **29a** is outlined in figure 2.30 below (Gossage et al. 2004):

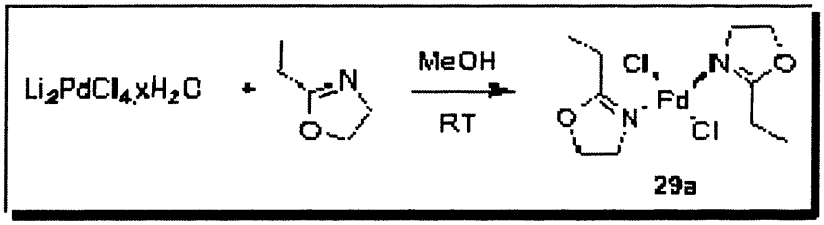


Figure 2.30: Synthesis of palladium-oxazoline complex (**29a**).

The synthesis of the complex **29a** involved the treatment of methanolic lithium tetrachloropallade (II) hydrate with 2-ethyl-2-oxazoline, which is a cheap and commercially available polymer precursor, giving the pure title compound in moderate yield (65% to 70%) without the need for further purification. The complex is reported to be stable in open air and can be stored at -20 °C until required for next synthesis, without losing its catalytic activity.

The next step was the synthesis of the fluorinated stilbene as described by Eisnor et al. (2006). The synthesis involved the addition of 1 equivalent of substituted aryl halides (in this case 4-fluoroiodobenzene), 1.2 equivalent of substituted styrene (e.g. 4-methoxy, 4-fluoro) in DMF, 1.2 equivalent sodium acetate as base and 10 mol% of the complex **29a**. The reaction procedure is outlined in Figure 2.31 below:

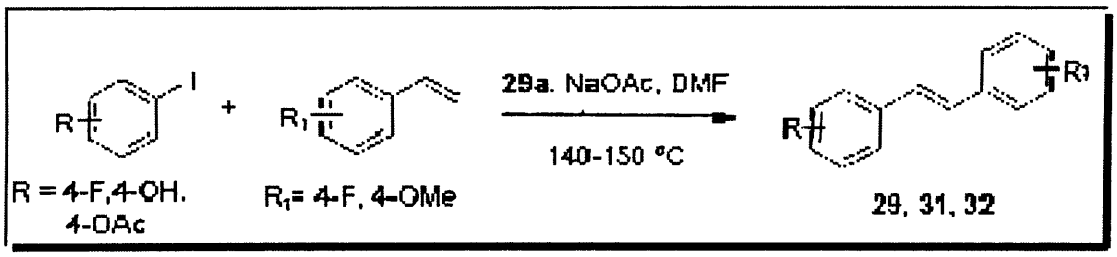


Figure 2.31: Synthesis of stilbenes via Heck reaction.

The synthesis was started by making compound **29** (4-fluoro 4'-methoxy stilbene). The reaction ran smoothly and completed within 5 hours. The reaction mixture was quenched with water to get precipitate which was then subjected to column chromatography to get the pure compound in 55% yield. The reduction of palladium-oxazoline complex loading from 10 to 2 mol% did not change the reaction time, but only reduced the yield to nearly quarter (14%) even though the TLC showed full consumption of the starting materials. In order to test the reproducibility of the method, the synthesis of compound **31** (4,4'-difluorostilbene) under the same reaction condition was carried out. The reaction also ran smoothly but needed longer reaction time

(48 hours) to afford the compound after column chromatography in 55% yield. At this stage, this method was considered to be reliable for stilbene synthesis.

Further studies were dedicated to the synthesis of hydroxylated stilbenes via this method. Therefore, the synthesis of compound **32** (4-fluoro 4'-hydroxystilbene) was carried out without any protection of the hydroxyl group by reacting 4-iodophenol with 4-fluorostyrene under the same reaction condition. The reaction suffered from incomplete consumption of starting materials even after 48 hours of heating, and following the work-up and column chromatography purification the compound was obtained in low yield (13%) with a slight trace of impurities. The attempt to recystalise the compound using ethanol/ethanol water failed to give a purer compound.

The finding highlighted the need to protect 4-iodophenol in order to increase the yield and to improve the purity of the hydroxylated stilbene. Acetoxy group was chosen as the protecting group due to its stability and extensive used as the protecting group for stilbene synthesis under Heck reaction [e.g. Moro et al. (2008), Farina et al. (2007), Andrus and Liu (2006)]. The protection of *para*-iodophenol was achieved following the procedure described by Adamski-Werner et al. (2004) as outlined in figure 2.32 below:

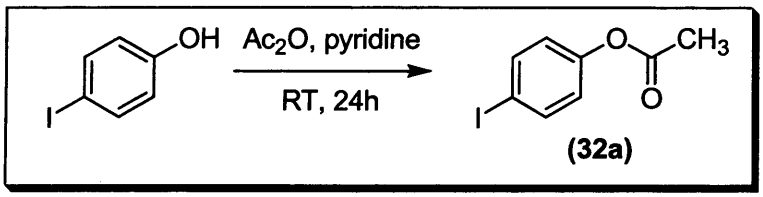


Figure 2.32: Protection of 4-iodophenol

The synthesis of acetic acid 4-iodophenol ester (compound **32a**) was achieved by addition of 4-iodophenol with acetic anhydride and pyridine at room temperature for 24 hours. The pure compound was afforded after column chromatography in good yield (88%). The protected iodophenol was then subjected to the synthesis of protected stilbene under the same reaction condition where the reaction completed within 24 hours. After the work-up and column chromatography, pure compound **32** was obtained in 87% yield

without the need for deprotection of the acetoxy group. In fact, the acetoxy group was readily cleaved during the reaction where the addition of deuterium oxide (deuterium exchange) in the NMR tube of compound **32** showed the disappearance of the OH peak at 9.5 ppm.

On the other hand, another effort to synthesise stilbene analogues via Heck reaction was reported by Lipshutz and Taft (2008) who described a Heck coupling reaction at room temperature in water as the only solvent. Attracted to the mild and environmentally attractive reaction condition, the synthesis of compound **29** was sought using the reported procedure. The reaction involved the coupling of 4-fluoroiodobenzene with 4-methoxy styrene in the presence of Johnson-Mattey catalyst [(dtbpf)$PdCl_2$], triethylamine as base and a nonionic, Vitamin E-based amphiphile 'PTS' (15 wt % in water) (Figure 2.33). The PTS (Figure 2.34) forms nanomicelles in water which allows cross-coupling to take place. The reaction was carried out for up to 30 hours at room temperature, but the traces of the limiting reagent (4-fluoroiodobenzene) can still be detected. Nevertheless, the reaction stopped and the product isolated by filtration through a pad of silica gel. The recrystallization in ethanol of the crude product afforded the pure compound in 35% yield.

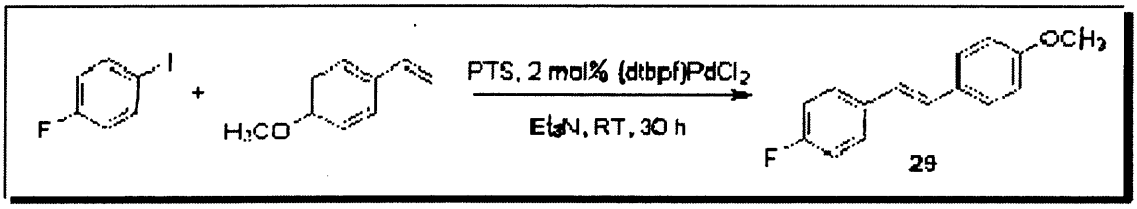


Figure 2.33: Heck coupling in PTS at RT

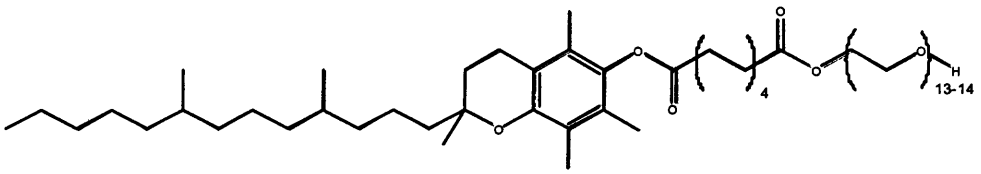


Figure 2.34: Structure of 'PTS'

The overall results for the synthesis of fluorinated stilbenes via Heck reaction are summarised in table 2.7 below:

Table 2.7: Summary of the synthesis of stilbenes via Heck coupling

Compound no	R	R_1	Method used	Yield (%)
29	4-F	4-OMe	10 mol% Pd-oxazoline	55
			2 mol% Pd-oxazoline	14
			In 'PTS' at RT	35
31	4-F	4-F	10 mol% Pd-oxazoline	55
32 (unprotected)	4-OH	4-F	10 mol% Pd-oxazoline	13
32 (protected) *(it was obtained as deprotected)*	**32a**	4-F	10 mol% Pd-oxazoline	87

In conclusion, Heck reaction promoted by catalyst **29a** is a reliable method for synthesising stilbenes with different substitution. The synthesis of compound **29** using the HWE and Heck reaction suggested that the HWE method is superior to the Heck reaction in terms of compound yields.

Following the success in synthesis of protected hydroxylated stilbene via Heck reaction, the same approach was applied to the synthesis of hydroxylated stilbene via HWE reaction. The synthesis of protected aldehyde was employed as described by Banerjee et al. (2006) (Figure 2.35). This method involved the protection of vanillin (3-methoxy-4-hydroxybenzaldehyde) with acetic anhydride in the presence of sodium hydroxide as base and in ice-cold water and ether as the solvents. The method proved to be very efficient, mild and afforded the protected vanillin as white solid in 92% yield.

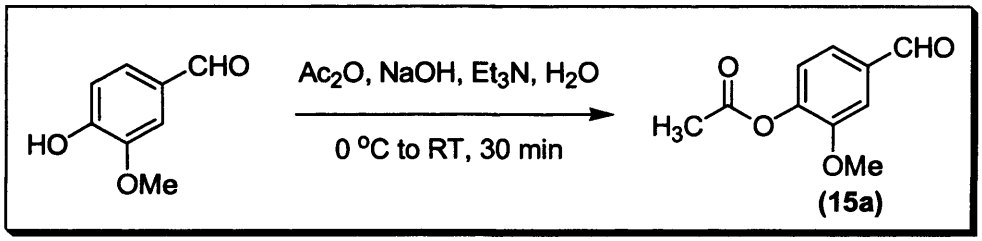


Figure 2.35: Synthesis of protected vanillin **(15a)**

The protected vanillin (4-formyl-2-methoxyphenyl acetate; compound **15a**) can be used straight away in the next reaction without the need for any purification, and was subjected to HWE reaction with compound **2** to make compound **15**. Upon heating of the reaction, the reaction mixture turned from light yellow to dark red. Following three hours of heating, the TLC showed the formation of many impurities with no fluorescence spot indicating the formation of stilbene. At this stage, this approach was considered to be unsuccessful. It was suggested that the acetoxy protecting group cannot survive the HWE reaction condition due to the strong base used which then caused the ester bond to break and released the free phenolic group to further react.

2.9 Biological study

2.9.1 Antiproliferative activity of stilbene analogues

A number of stilbene analogues based on the resveratrol pharmacophore which were synthesised using various methods were subjected to preliminary antiproliferative study, carried out in collaboration with the Tenovus Cancer Research Group, Welsh School of Pharmacy, Cardiff University by Mr Huw Mottram. The antitumour screening which involved four cancer cell lines, namely PC3 (prostate), A549 (non-small lung), Lovo (colon) and MCF-7 (breast), were able to provide useful knowledge on the ability of the stilbenes to inhibit cancer cell's growth and to induce cancer cell death over a range of compound concentrations. The MTT assay was used to detect the viability of the cells after incubation with the stilbenes which will later determine the cytotoxicity of the test compounds. The principal of the assay lies on the ability of the test compounds to induce the reduction of yellow MTT to a

water soluble purple formazan which is largely impermeable in living cells. The compounds were prepared as 100mM solutions in DMSO and stored at -20 °C until used. Decimal dilutions of compounds were prepared in cell growth medium immediately prior to each assay (PC3 and Lovo in DMEM +10% FCS, A549 and MCF7 in RPMI +10% heat inactivated FCS), with final concentrations of 0.1 – 100 μM per well. Eight treatment replicates were made for each compound concentration. Cells were seeded into 96-well microtiter plates at a density of $5x10^3$ (MCF-7 and Lovo) and $3x10^3$ cells per well (PC3 and A549), and allowed 24 hours to adhere. The viability of the cells is measured as the number of active cells after the treatment with the test compounds, and is proportional to the level of purple formazan where the colour can be quantified using a spectrophotometer. The results obtained were plotted as a dose-response curve using OriginPro® Software and IC_{50} values calculated. Table 2.8 summarizes the results of the MTT assay:

Table 2.8: IC_{50} values of four human cancer cell lines after incubation with selected stilbenes.

Compounds	IC_{50} (μM)			
	PC3	A549	Lovo	MCF-7
4	>100	>100	>100	>100
5	>100	>100	>100	>100
6	>100	>100	100	>100
7	>100	>100	100	>100
8	>100	>100	40	>100
9	40	40	5	40
10	>100	>100	60	100
11	>100	>100	>100	>100
12	50	100	50	50
13	50	>100	50	>100
14	50	>100	>100	0.1
16	>100	>100	>100	>100
17	>100	1	>100	>100
18	>100	>100	>100	100

20	>100	>100	100	>100
22	50	50	20	50
28	>100	>100	>100	>100
29	>100	>100	>100	>100
32 (1)	>100	>100	>100	>100
32 (2)	50	50	30	50

From the table, it can be concluded that some of the stilbenes may have the ability to inhibit the growth of certain cancer cell lines in the μM range. Unfortunately, no structure activity relationship can be generated from the results. The most striking results were shown by compound **14** which can inhibit the MCF-7 breast cancer cell line at less than 0.1 μM, and compound **17** which can inhibit the A549 cell line at 1 μM. Compound **9** also showed good antiproliferative activity on Lovo cell line at 5 μM. Figure 2.36 summaries the structures of compounds exhibiting the best antiproliferative potential from the assay. One common feature from the compounds which showed promising antiproliferative activity is the existence of at least one methoxyl group in both sides of the stilbene benzene rings. This is in accordance with the previous study which showed that the methoxyl group on the stilbene skeleton is responsible for the apoptosis (programmed cell death) inducing activity of the stilbenes on cancer cells (Li et al. 2009). Nevertheless, a more detailed and comprehensive study should be carried out in order to test the inhibitory activity of this compounds in a wider range of concentrations and to further test the cytotoxicity activity of the stilbenes whether via apoptosis- or necrosis-based cell death.

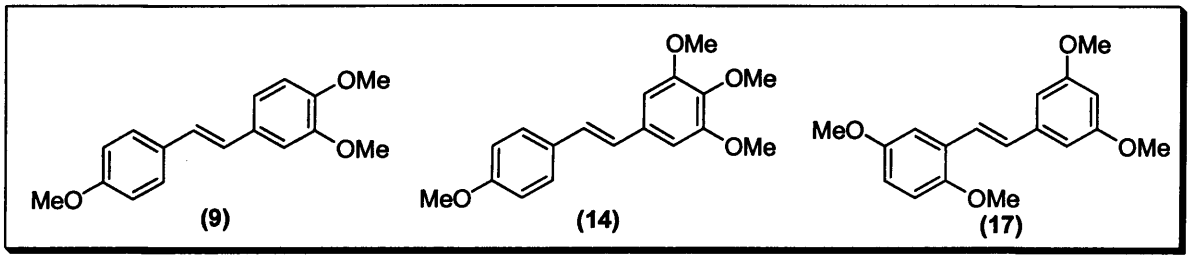


Figure 2.36: Structures of compounds showing the best antiproliferative activity from the MTT assay.

2.9.2 Testing of stilbene analogues in Wnt signaling assay

Resveratrol and other stilbene analogues have the ability to inhibit cancer cells growth through multiple pathways; one of them is by inhibiting the Wnt signalling pathway (discussed in chapter 4). A study by Hope et al. (2008) highlighted the ability of resveratrol to inhibit the Wnt signaling pathway in colon-derived (NCM460) and colon cancer (RKO) cell lines, both lacking intrinsic activation of Wnt pathway. They suggested that the Wnt inhibitory effect may be at least partially due to inhibition of β-catenin nuclear translocation. However, other anti-Wnt mechanisms must be operative, as Wnt inhibition was seen in NCM460 cells in the absence of demonstrable effects on β-catenin quantity or intracellular localization. Therefore, based on their findings, the next study was dedicated to test the ability of the synthesised substituted stilbenes to inhibit the Wnt signalling pathway. The study was done on colon cancer stem cells (CSC) that was lentivirally transduced with TOP-GFP [TCF (T-cell-specific transcription factor)/LEF (lymphoid enhancer binding factor) reporter driving expression of GFP (green fluorescence protein)] construct, with mutant APC (adenomatous polyposis coli) (Vermeulen et al. 2010). The principal of the assay relies on the use of the TCF/LEF reporter that directs the expression of enhanced GFP upon activation of the pathway, where a positive inhibitory activity of the stilbene analogues will be shown as the ability of the stilbenes to reduce the expression of the GFP construct on CSC after up to two days of incubation with the stilbene compounds. This kind of assay enables primary screening of the tested stilbenes for their ability to inhibit the Wnt signalling pathway, especially at the downstream level of β-catenin where the transcription of Wnt-responsive genes mediated by the complexation of β-catenin to TCF/LEF occurred (refer Figure 4.1). The compounds were tested in a broad range of concentration: 2 mM, 10 μM and 50 nM. This study was done in the laboratory of Prof. J. P. Medema, Academic Medical Centre, Amsterdam, the Netherlands.

Unfortunately, neither of the parent compound resveratrol nor the tested stilbene analogues (together with other series of compounds which

have been designed as inhibitors of Wnt signalling pathway, tested at the same time in the assay) showed significant ability to inhibit the GFP construct expression compared to control. Both the resveratrol and the stilbene analogues were not able to reduce the intensity of the GFP expression and were not able to reduce the number of cells upon treatment in CSC transduced cells. This might be due to the inability of the compound to inhibit the GFP expression at the downstream level of Wnt signalling pathway especially at the TCF/LEF transcription level. Nevertheless, this observation suggested that the stilbene analogues might be able to inhibit the Wnt signalling at other level of the pathway such as at the β-catenin level or at the upstream/receptor level of the Wnt pathway, thus highlighted the need for further testing of the compounds using a suitable assay protocol targeting various levels of Wnt signalling pathway.

CHAPTER 3

SYNTHESIS AND EVALUATION OF INDOLE-CONTAINING 3,5-DIARYL ISOXAZOLES AS POTENTIAL ANTITUMOUR AGENTS

CHAPTER 3

SYNTHESIS AND EVALUATION OF INDOLE-CONTAINING 3,5-DIARYL ISOXAZOLES AS POTENTIAL ANTITUMOUR AGENTS.

3.1 Introduction

3.1.1 Indole as a privileged structure in drug discovery

A number of oxygen and nitrogen heterocyclic derivatives have shown interesting biological activities and are of synthetic interest as they represent important classes of natural and non-natural products (Duchet et al. 2010). One of the most prominent structures found is the indole scaffold, which can be considered as a privileged structural motif found in various synthetic compounds and which can be found abundantly in natural products. There are a huge number of substituted indoles that have shown interesting pharmaceutical properties in a variety of therapeutic areas which explains the vast number of drugs containing the indole moiety, such as the analgesic Pravodoline, antiemetic Ramosetron and anti-inflammatory Indomethacin (Figure 3.1), among many others (Yeung et al. 2002; de Sa Alves et al. 2009). As further reviewed by de Sa Alves et al. (2009), the chemistry of indole synthesis have attracted many chemists due to the evidence that showed many alkaloids containing the indole nucleus, as well as the essential amino acid tryptophan and the discovery of plant hormones, which all possess striking importance especially in drug discovery.

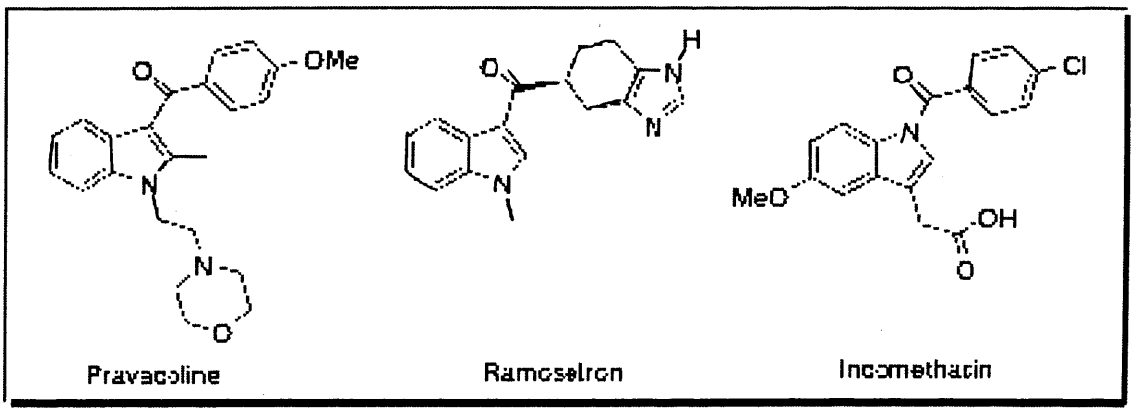


Figure 3.1: Example of indole-based drugs

3.1.2 3,5-Diaryl substituted azole as a promising scaffold for new anticancer agents

On the other hand, a number of 3,5-diaryl-substituted azoles have been reported as promising new antitumour agents. Among those, 3,5-diaryl-1,2,4-oxadiazoles have shown striking importance as promising pro-apoptotic and antitumour agents, being identified using caspase- and cell-based high-throughput screening assays, as exemplified by compound **1d** (Figure 3.2) (Jessen et al. 2005; Zhang et al. 2005). Compound **1d** was found to be able to trigger apoptosis, interestingly, through arrest at G_1 phase of the cell cycle. This distinguishes compound **1d** from other anticancer agents including commonly used vinca alkaloids and taxanes that causes cell cycle arrest at the M phase. Importantly, compound **1d** is able to selectively induce apoptosis only in certain cancer cell types, such as human breast cancer cells T47D and ZR75-1, and is also not active against primary normal cells such as HUVEC. This highlights its selectivity which can provide better toxicity profiles for this kind of apoptosis inducer.

Their data suggested that the 3,5-diaryl-1,2,4-oxadiazoles might be a new class of anticancer agents that are tumour-selective. Further extension of these work lead to another series of more water-soluble derivatives (compound **2a-b**), Figure 3.2) that showed better efficacy in cancer xenograft model *in vivo* (Kemnitzer et al. 2009). Further recent work done by Ziedan et al. (2010) demonstrated that a number of indole-based 3,5-diaryl substituted oxadiazoles (compound **3a-b**) have the ability to posses antiproliferative activity in the low micromolar IC_{50} range (using COLO 320 and MIA PaCa-2 human cancer cell lines) and were able to induce apoptosis in sensitive cell lines through caspase activation (Figure 3.2).

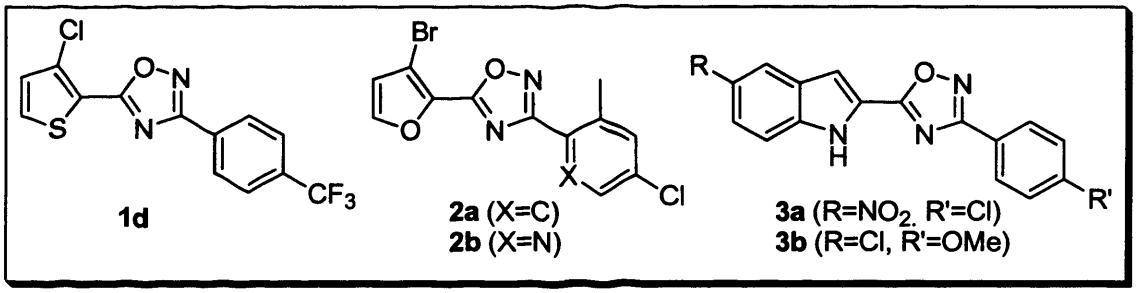


Figure 3.2: Structures of biologically active 3,5-diaryl-1,2,4-oxadiazoles

Interestingly, further recent work done by Ziedan (2010) suggested another series of indole-based 3,5-diaryl substituted isoxazoles, which is another subset of substituted azole compounds, showed interesting antiproliferative activity in two human cancer cell lines; namely MDA-MB-231 (breast cancer) and HeLa (cervical cancer) cell lines. Several compounds in this series possess low micromolar IC_{50} activities; compound **4a** was found to be the most active, although not as potent as the clinically used anticancer agent Doxorubicin, while 3 compounds (compound **5a, 6a** and **6b**) were also found to be active in both cancer cell lines (Figure 3.3).

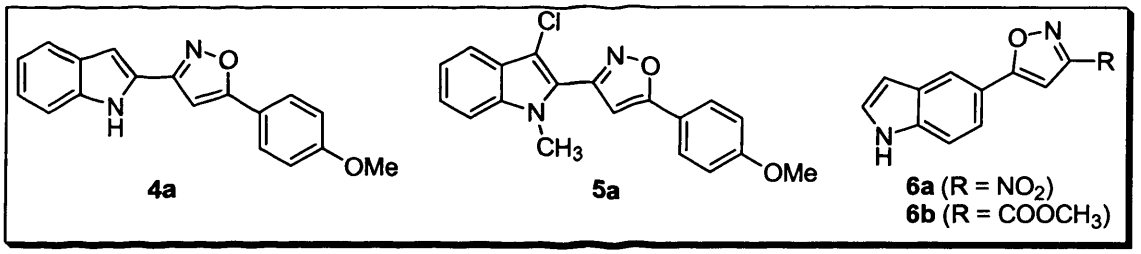


Figure 3.3: Structures of biologically active 3,5-diaryl substituted isoxazoles

3.1.3 Aims of study

In view of the potential of the indole-based 3,5-diaryl-1,2,4-oxadiazoles and 3,5-diaryl isoxazoles as promising antitumour agents, this study is aimed to extend the recent work to further synthesise indole-based 3,5-diaryl substituted isoxazoles, and later on to evaluate their antiproliferative activity. This work would provide a substantial comparison to previously described 3,5-diaryl oxadiazole and isoxazole compounds and so to generate consequential structure activity relationship from the afforded structures.

3.2 Synthesis of 3-(1*H*-indol-2-yl)-5-phenylisoxazoles and 3-(1-methyl-1*H*-indol-2-yl)-5-phenylisoxazoles via condensation or cyclisation of alkynyl ketones and hydroxylamine.

The first part of the study focused on the synthesis of a 3-indolylisoxazole series. The syntheses were achieved following three chemical steps:

i) Synthesis of acyl chloride from corresponding indole-2 carboxylic acid

ii) Synthesis of alkynyl ketones from the reaction between indol-2-carbonyl chloride and phenylacetylenes

iii) Cyclisation of alkynylketones with hydroxylamine to give 3,5-disubstituted isoxazoles.

The overall reaction is outlined in Figure 3.4 below:

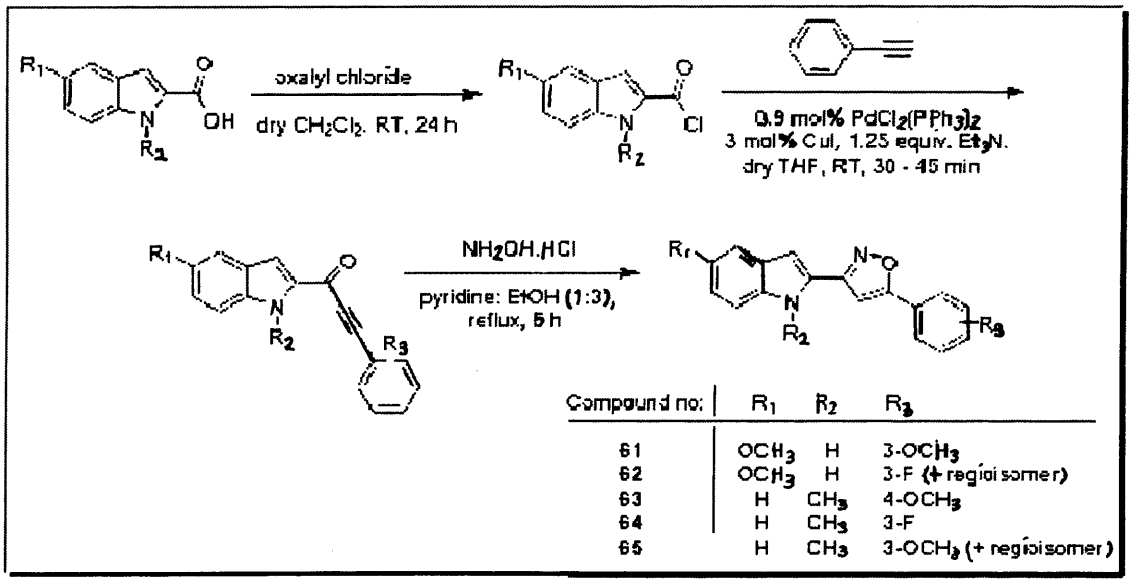


Figure 3.4: Overall reaction scheme for synthesis of 3-indolylisoxazoles

3.2.1 Synthesis of acid chlorides

To start the synthesis, different substituted indole carboxylic acids were used as the starting material in order to synthesise the corresponding acid chloride. This transformation will permit further coupling with acetylene in the next step. Although thionyl chloride whether neat or in non-polar solvents such as toluene is commonly used as the chlorinating agent, the need for reflux at

high temperature made it less attractive for this kind of reaction. Therefore, the milder and more selective oxalyl chloride was chosen. The reaction was done in dichloromethane (or tetrahydrofuran) at room temperature with stirring for 24 hours. *N,N*-dimethylformamide (DMF) was used as a catalyst to accelerate the reaction (Figure 3.5). The overall yield of the reaction was more than 96% and upon evaporation of the solvents, the crude compound was used straight away in the next reaction without further purification.

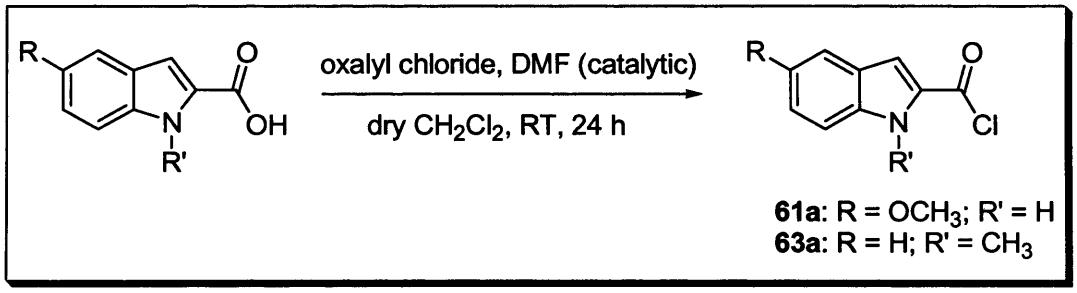


Figure 3.5: Synthesis of acid chloride

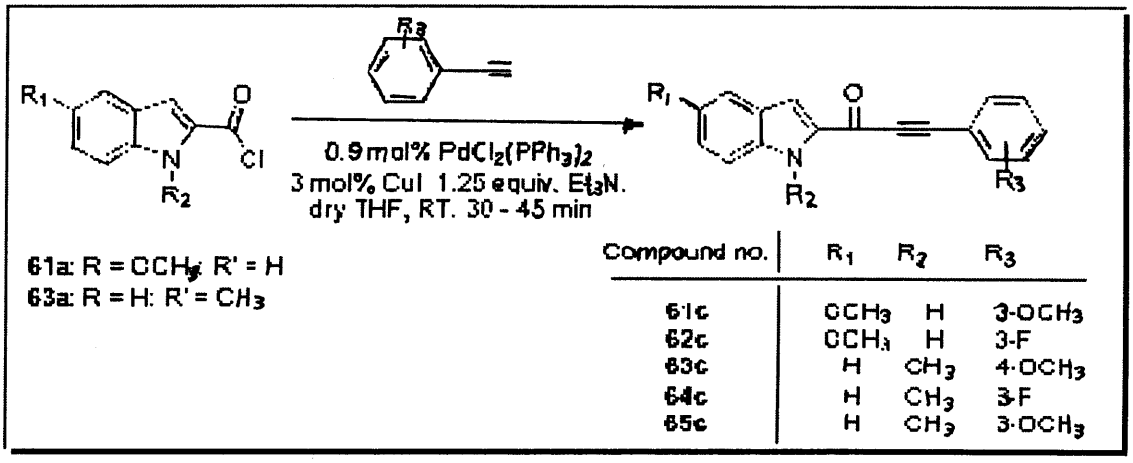
3.2.2 Synthesis of alkynyl ketones

There are a number of ways to synthesise alkynyl ketones that have been reported previously; these include reaction of acetylene with acid chloride (e.g. Bernini et al. 2009) and by reaction of acetylenic Grignard reagent intermediates with aldehydes followed by oxidation (Tangdenpaisal et al. 2009). Nevertheless, palladium and/or copper-catalysed cross couplings of terminal acetylenic derivatives with acid chlorides are usually considered as the method of choice. Increased functional group tolerance together with better yields makes the method attractive despite the instability of acid chlorides which needs to be considered.

Therefore, to proceed with the synthesis, the method described by Cox et al. (2005) was chosen for the alkylnyl ketones synthesis. The reaction involved the coupling between indole-2-carbonyl chloride and substituted phenylacetylenes using 0.9 mol% $PdCl_2(PPh_3)_2$, 3 mol% copper iodide and 1.25 mmol triethylamine as base in anhydrous tetrahydrofuran at room temperature, under nitrogen atmosphere. The reaction was very rapid, and

was complete after 30-45 minutes by TLC analysis. The isolated crude products needed further purification by column chromatography (Figure 3.6). Nevertheless, variable and inconsistent yields were achieved after every reaction. For example, 3-(3-methoxy-phenyl)-1-(1-methyl-1*H*-indol-2-yl)-propynone **(65c)** was achieved in 95% yield but the yield of 1-(5-methoxy-1*H*-indol-2-yl)-3-(3-methoxy-phenyl)-propynone **(61c)** was quite low (23%). The need for really anhydrous conditions resulted in variable yields of each alkylnyl ketone, where sometimes the major product was the Glaser oxidation adduct from self-condensation of the terminal alkyne.

One attempt to synthesise alkynylketones from 5-methoxy-1*H*-indole-2-carbonyl chloride **(61a)** and 4-methoxyphenyl-acetylene was unsuccessful, where only the product of Glaser dimerisation was afforded as the main product together with unreacted **61a**, even after repeated attempts under carefully controlled anhydrous conditions (Figure 3.7). Another attempt to react compound **61a** with 1-ethynyl-4-nitrobenzene was also unsuccessful (Figure 3.8). The reason for the selectivity which determined the successful coupling of compound **61a** to different kinds of phenyl acetylene is unknown. The structure of the alkynyl ketones was confirmed by the disappearance of the acetylenic proton in ^{1}H-NMR and the appearance of two quaternary alkynyl carbons C2 and C3 at around δ 85 to 92 ppm in ^{13}C-NMR.



Compound no.	R_1	R_2	R_3
61c	OCH_3	H	3-OCH_3
62c	OCH_3	H	3-F
63c	H	CH_3	4-OCH_3
64c	H	CH_3	3-F
65c	H	CH_3	3-OCH_3

Figure 3.6: Synthesis of alkynyl ketones **61c** to **65c**

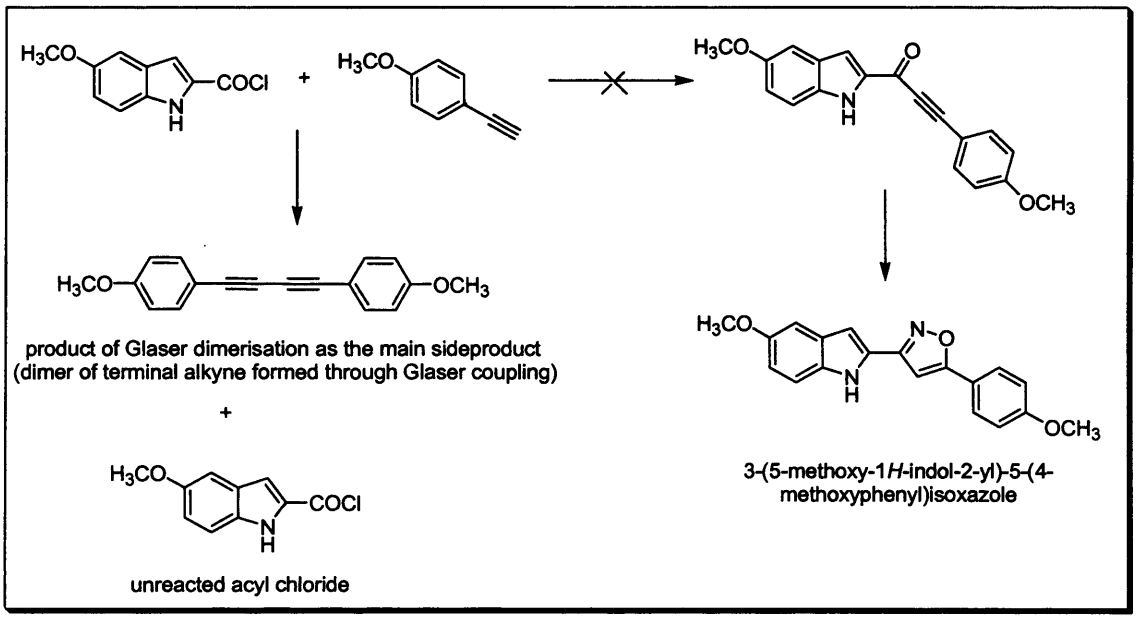


Figure 3.7: Attempt to synthesise 3-(5-methoxy-1*H*-indol-2-yl)-5-(4-methoxyphenyl)isoxazole.

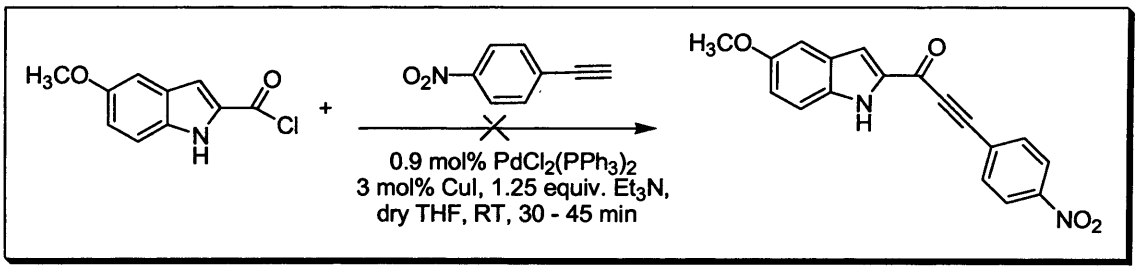


Figure 3.8: Unsuccessful coupling of compound **61a** to 1-ethynyl-4-nitrobenzene

3.2.3 Synthesis of 3,5-diaryl isoxazoles.

The final procedure to synthesise 3,5-diaryl isoxazoles was achieved using the method described by Johnston and Shotter (1968), which mentioned the formation of isoxazoles via cyclisation of alkynyl ketones. The method involved the formation of two intermediates: the first one is the formation of an oxime intermediate via condensation reaction between carbonyl group of alkynyl ketones and hydroxylamine, followed by the reaction with the alkyne moiety to give the desired product (Figure 3.9).

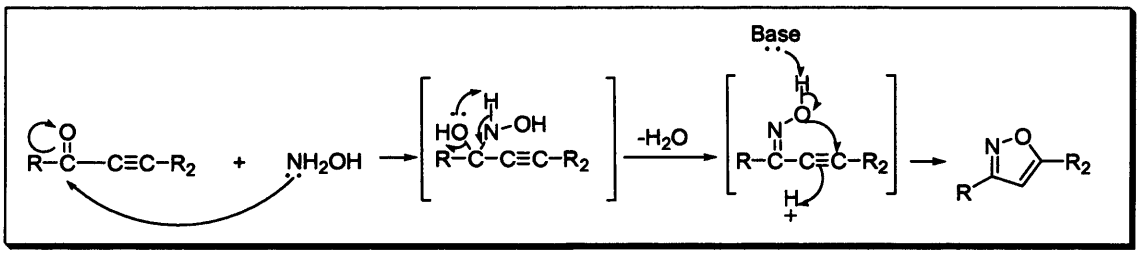


Figure 3.9: Reaction mechanism for the formation of isoxazoles.

This procedure was initially reported to give only one isomer. Nevertheless, Adlington et al. (2000) also mentioned that the formation of other regioisomer in a mixture with the initial isomer is possible (Figure 3.10) via an alternative mechanism pathway (Figure 3.11). To date, no papers have mentioned the criteria which determine the formation of a certain isomer; however, the formation of the initial isomer (A) is more favorable in most cases.

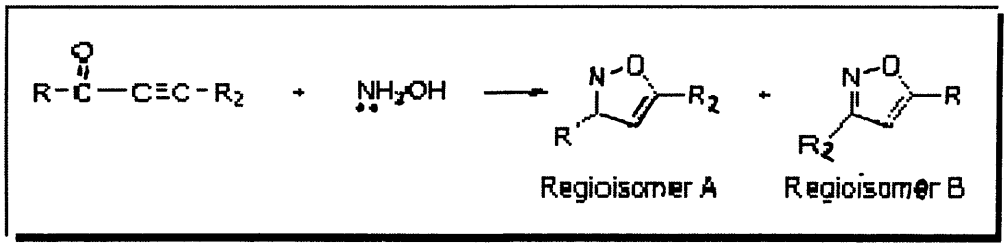


Figure 3.10: Formation of regioisomer A and B.

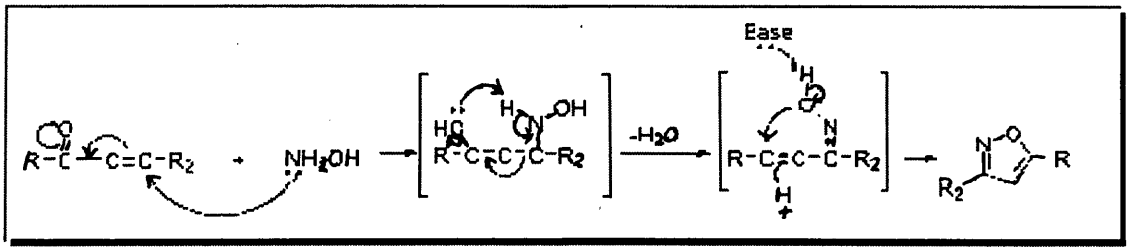


Figure 3.11: Alternative pathway for the formation of regioisomer B

For this reaction, hydroxylamine hydrochloride together with 1-(1H-indol-2-yl)-3-(substituted phenyl)-prop-2-yn-1-one or 1-(1-methyl-indol-2-yl)-3-(substituted phenyl)-prop-2-yn-1-one were dissolved in a mixture of ethanol/pyridine (3:1) and refluxed for 5 hours or until the completion of the reaction. After the work-up, the crude products were purified using column chromatography to afford the compound in a fairly low yield (from 56% to as

low as 2% for regioisomers). Out of 5 reactions, 2 of them gave formation of regioisomers: **62c** to form regioisomers **62** and **62b**, and **65c** to form regioisomers **65** and **65b**; both of them which needed further column chromatography separation (as shown in Figure 3.12 for the formation of regioisomers **62** and **62b**). Another 3 reactions gave only single isomer (isomer A – compounds **61**, **63** and **64**).

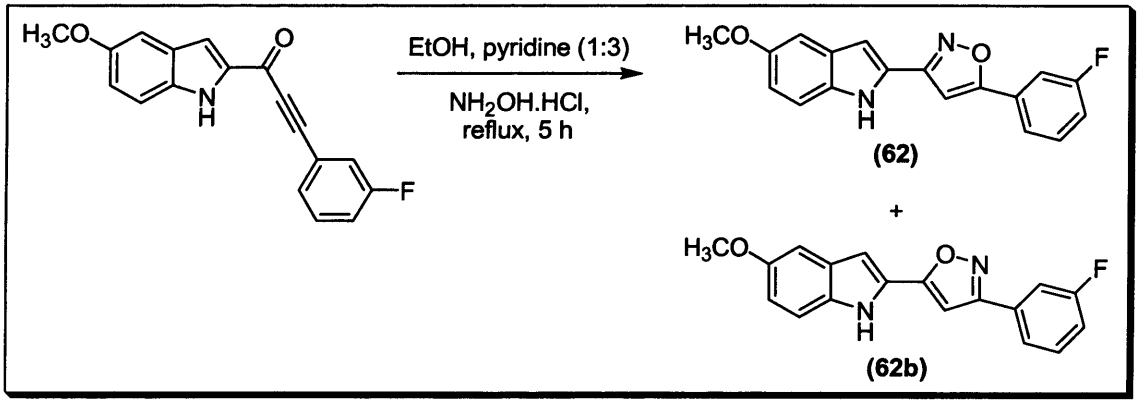


Figure 3.12: Synthesis of compound **62** which gave rise to regioisomer **62b**.

3.2.4 Determination of regioisomers

In order to distinguish the characteristics between different formed regioisomers, the method described by Stephens and Arafa (2006) was employed. The determination of the regioisomers can be achieved by either using ^{13}C-NMR spectra or by using mass spectrometry analysis. For 3,5-diphenyl isoxazoles, the chemical shift of C4 of isoxazoles can be calculated using the following equation:

$$\delta(C4) = 98.1 + 4(\alpha_{5Ph}) + 0.66\ (\alpha_{3Ph}) \quad (1)$$

where:

δ(C4): is the chemical shift of the C4 of isoxazole

98.1 : is the chemical shift of the C4 of unsubstituted 3,5-diphenylisoxazole (standard value)

α_{5Ph} : is the Hammet constant for the substitution on the 5-phenyl group

α_{3Ph} : is the Hammet constant for the substitution on the 3-phenyl group

If the equation is applied to compound **62** and **62b**, where the Hammet constant for 3-fluorophenyl is +0.337 (Hammet 1937), while indole as an electron donating group would have a negative value for Hammet constant, therefore:

Compound **62** : δ(C4) = 98.1 + 4(0.337) + 0.66 ([-]αindole)
Compound **62b**: δ(C4) = 98.1 + 4([-]αindole) + 0.66 (0.337)

It is clearly shown that compound **62** will have higher C4 value than compound **62b**. This showed that the compound which have a higher C4 value (δ 98.2 ppm) is compound **62** while another regioisomer with a lower C4 value (δ 97.07 ppm) is compound **62b**. But in contrast to previous discussion, compound **62b** was found to be more favourable in terms of formation than its regioisomer counterpart (compound **62**) which is indicated by a slightly higher yield obtained (5% and 2%, respectively). The very low yield obtained also resulted from uncyclised alkynylketones even after prolonged reaction. On the other hand, compound **65** and its regioisomer **65b** followed the usual pattern of regioisomer formation, where compound **65** has a much higher yield (20%) after column chromatography separation compared to their 5-indolylisoxazole counterpart (4% yield).

Another method that can be used to distinguish between different regioisomer is the fragmentation pattern from mass spectroscopy (electron impact).

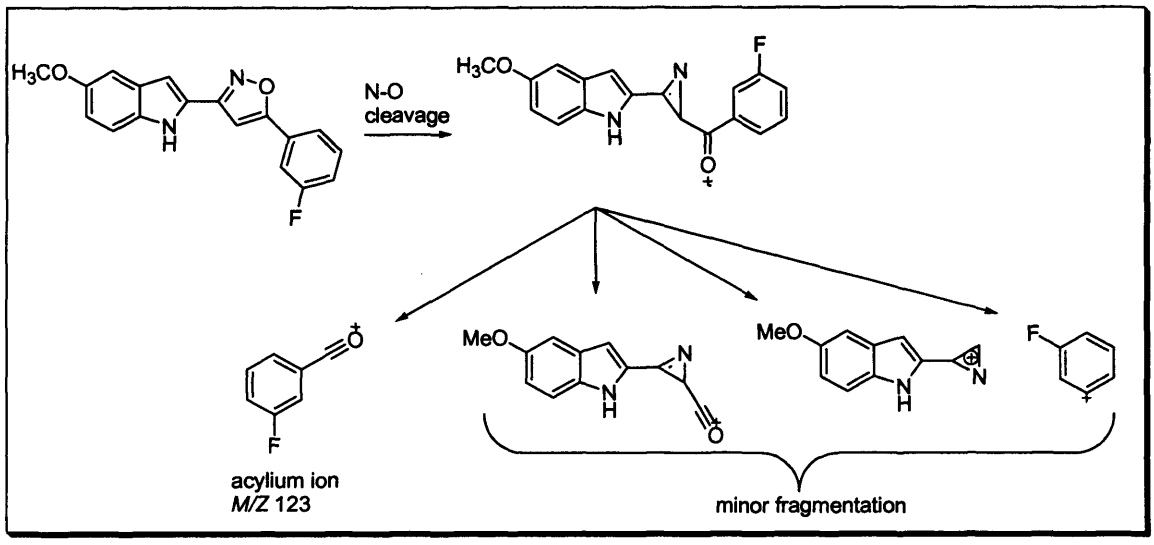


Figure 3.13: Fragmentation pattern of compound **62**

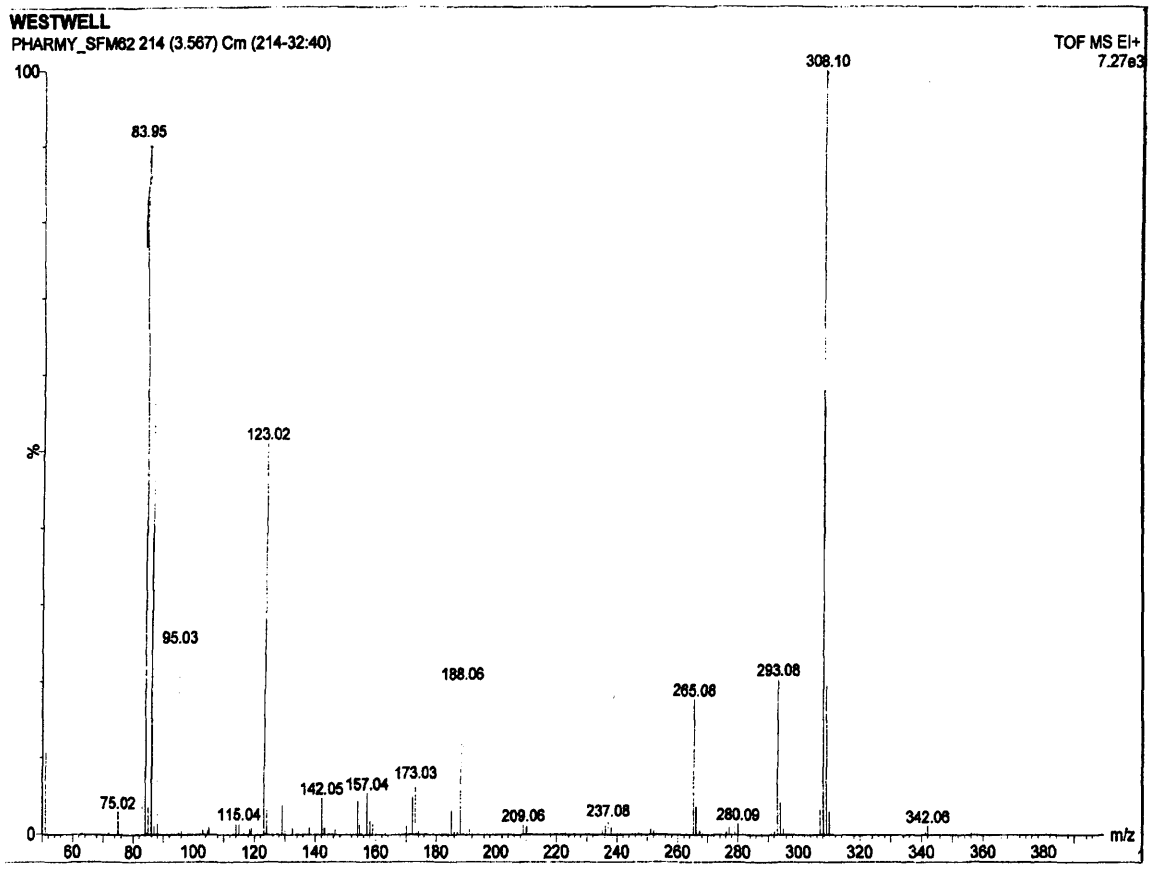


Figure 3.14: The mass spectrometry of compound **62**

The mass spectrometry of compound **62** showed a fragmentation of acylium ion signal at *m/z* 123 together with the signal of the isoxazoles (*m/z* 308), that correspond well with the expected fragmentation pattern.

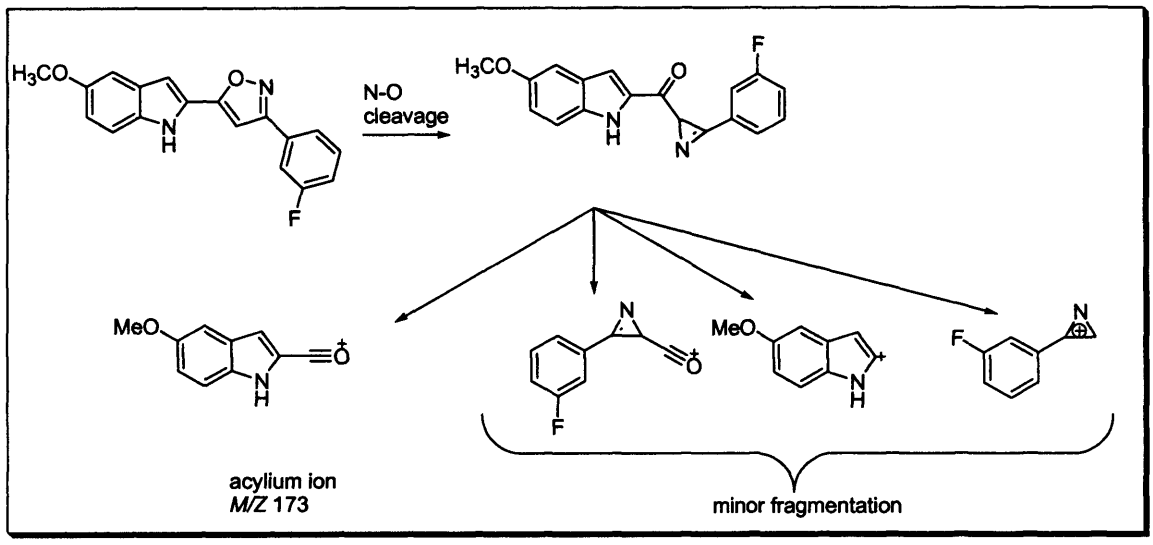


Figure 3.15: Fragmentation pattern of compound **62b**

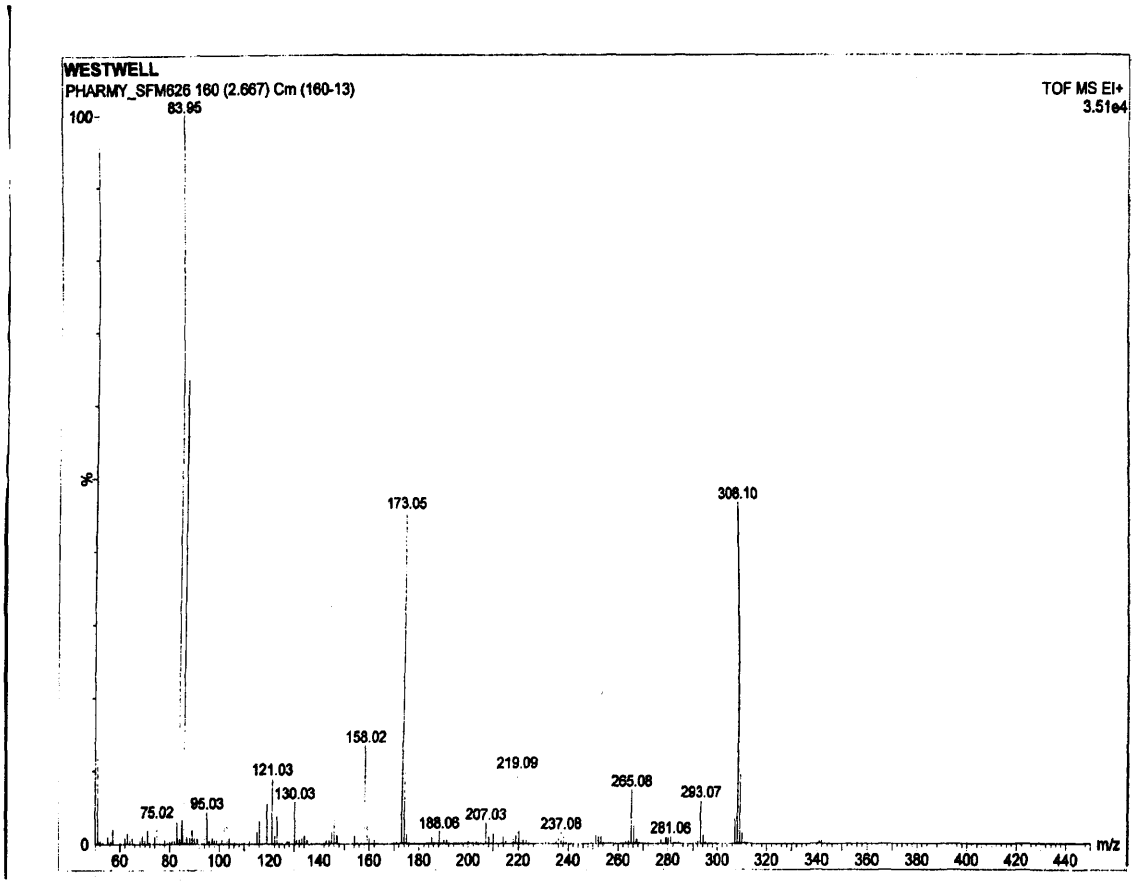


Figure 3.16: The mass spectrometry of compound **62b**.

In compound **62b** spectrum, the signal of the acylium ion was detected at *m/z* 173 together with the signal of the isoxazole at *m/z* 308, also agreeing well with the expected fragmentation pattern of 5-indolylisoxazoles.

In conclusion, verification of regioisomers formed was confirmed by careful characterisation of ^{13}C-NMR data and assignment of acylium ion of electron impact mass spectrometry fragmentation.

3.3 Synthesis of 5-(1-methyl-indol-2-yl)-3-substituted phenylisoxazoles via 1,3-dipolar cycloaddition

The first pathway to synthesise 5-indolylisoxazole compounds seemed quite complicated, especially due to the unspecific nature of the reaction to afford mixed regioisomers in certain compounds. Therefore, another method was sought which was expected to be more regioselective to produce 5-indolyl isoxazoles. The synthesis was chosen following the method as described by Lee (1982). The method basically involved the synthesis of isoxazoles by dipolar cycloaddition between aldoximes and terminal alkyenes using sodium hypochlorite. Specifically, for the synthesis of 5-(1-methyl-indol-2-yl)-3-substituted phenylisoxazoles the following methods were employed:

i) Formation of 2-ethynyl-1-methyl-1*H*-indole from 1-methyl-1*H*-indole-2-carbaldehyde
ii) Formation of aldoxime from the reaction of various carboxaldehydes and hydroxylamine hydrochloride.
iii) Formation of isoxazoles by dipolar cycloaddition between the aldoxime and terminal alkyne using sodium hypochlorite.

3.3.1 Synthesis of 2-ethynyl-1-methyl-indole

The synthesis of 2-ethynyl-1-methyl-indole (compound **66b**) was achieved following the method described by Roth et al. (2004). In this reaction, Bestmann-Ohira reagent (or dimethyl-1-diazo-2-oxopropylphosphonate) was formed from the reaction between *p*-toluenesulfonylazide (**66a**), dimethyl-2-oxopropylphosphonate and potassium carbonate, which in the presence of methanol leads to the in situ generation of dimethyl (diazomethyl)phosphonate. This latter reagent leads to the formation of the desired terminal alkyne, in this case 2-ethynyl-1-methyl-indole, from its

corresponding aldehyde (Figure 3.17). The mechanism of the reaction includes a Horner-Wadsworth-Emmons reaction, loss of nitrogen and rearrangement of the resulting alkenylidenecarbene into the alkyne. However, the reaction did not go to completion even after prolonged reaction time, thus affording the desired acetylene in moderate yield (55%). This is in line with the original paper, which reported that if the aldehydes bearing electron donating group was used, the reactions would not go to completion resulting in low yield, as was the case in the afforded compound.

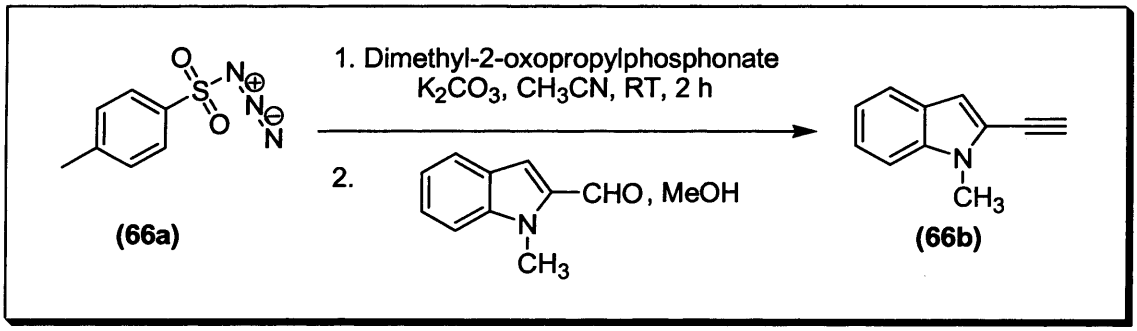


Figure 3.17: Synthesis of 2-ethynyl-1-methyl-indole **(66b)**

3.3.2 Synthesis of aldoximes

The next part of the synthesis was the formation of aldoximes. Aldoximes, are synthesised via a straightforward condensation reaction between aldehyde and hydroxylamine. The synthesis involved the reaction between substituted benzaldehydes with hydroxylamine hydrochloride in the presence of sodium carbonate as base in an aqueous methanolic solution ($MeOH/H_2O$=3:1) (Figure 3.18).

Upon the completion of the reaction, the aldoxime precipitates out of the reaction mixture, where the powder was collected by filtration and used without further purification in the next reaction. The pure products were afforded in good yields; 78% for compound **68b** and 86% for compound **69b**.

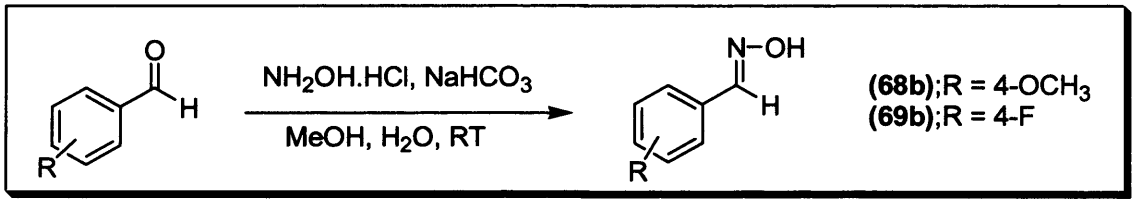


Figure 3.18: Synthesis of aldoximes **68b** and **69b**.

3.3.3 Synthesis of 5-(1-methyl-indol-2-yl)-3-phenylisoxazoles

The next part of the synthesis is the formation of 5-indolylisoxazoles. The reaction should be regioselective in order to avoid the problem of nonspecific regioisomer formation. The selected procedure followed the method by Lee (1982) involving dipolar cycloaddition reaction. For the reaction, terminal alkyne solution was dissolved in dichloromethane followed by addition with triethylamine as base and 2 equivalent of 13% sodium hypochlorite solution. Next, aldoxime solution in dichloromethane was added dropwise at 0 °C and the mixture was left to stir at room temperature under nitrogen atmosphere for 24 hours or until the completion of the reaction as indicated by TLC (Figure 3.19). Upon work up, the crude product was subjected to column chromatography purification to afford pure compound in low yield (23% for compound **66** and 29% for compound **67**).

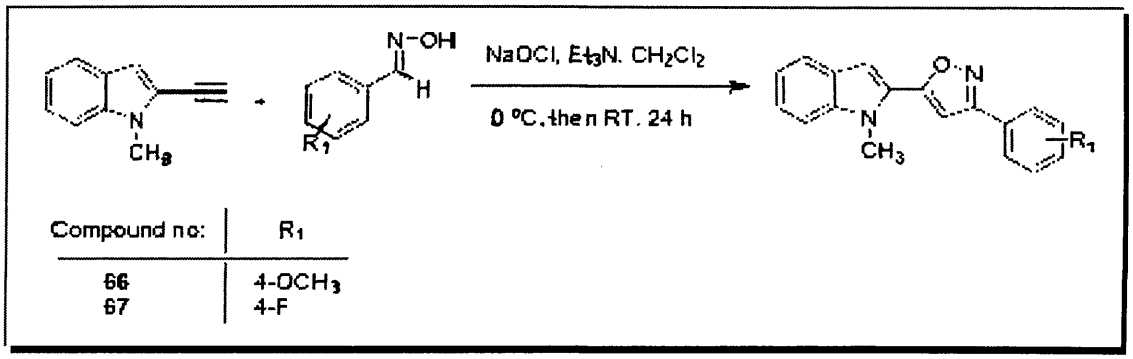


Figure 3.19: Synthesis of compound **66** and **67**.

The reaction mechanism follows the 1,3-dipolar cycloaddition of nitrile oxide (synthesized in situ during the reaction) with alkynes to afford 5-(1-methyl-1*H*-indol-2-yl)-5-(substituted phenyl)isoxazoles (Figure 3.20), without

formation of any regioisomer. This showed that this method was regioselective although the overall yield was low. Addition of catalyst is expected to give a better yield, as reported by Vieira et al. (2009) which showed the increase in yield of isoxazoles with the addition of copper(I) iodide. Compound **66** and **67** will provide useful structure activity relationship insight in comparison to their 3-indolylisoxazoles counterparts.

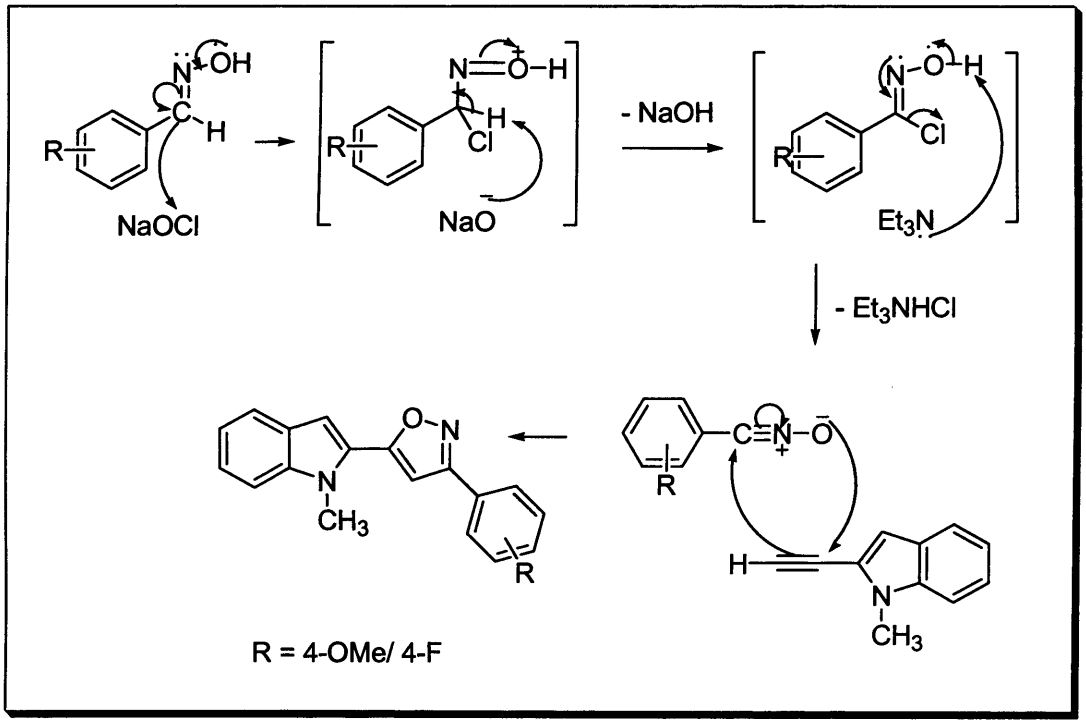


Figure 3.20: Reaction mechanism for the synthesis of isoxazoles via 1,3-dipolar cycloaddition.

3.4 Synthesis of 5-(1*H*-indol-5-yl)-3-substitued phenylisoxazoles via 1,3-dipolar cycloaddition

Following the success of the synthesis of 5-indolylisoxazoles giving specific isomers, another series of 5-indolyl isoxazoles based on 5-ethynylindoles moiety was sought to provide useful comparison for structure activity relationship study. The following procedure was employed to synthesise compounds of this series:

i) Synthesis of 5-ethynylindole from 5-iodoindole via Sonogashira coupling.

ii) Synthesis of substituted benzaldoximes from substituted benzaldehydes.

iii) Synthesis of isoxazoles via dipolar cycloaddition

3.4.1 Synthesis of 5-ethynylindole

The chosen method for the synthesis of 5-ethynylindole was by Sonogashira coupling using 5-iodoindole as the starting material. Following the procedure by Henon et al. (2006), to start the synthesis, 5-iodoindole was coupled with triethylsilylacetylene in the presence of triethylamine as base, $Pd(PPh_3)_4$ in combination with copper iodide as catalysts, in acetonitrile with heating at reflux for 5 hours to afford intermediate 5-triethylsilylethynylindole **[68a(i)]**. After the work-up, the reaction was used straight away in the next reaction without further purification.

The next step involved the removal of the triethylsilyl group from the intermediate. The chosen method was following the procedure described by Mathieu et al. (2006), where 1M tetrabutylammonium fluoride was used in tetrahydrofuran, and the reaction was left for 24 hours at room temperature for complete conversion to terminal alkyne, 5-ethynylindole **(68a)**. Following the work-up, the crude product needed more purification by column chromatography. The overall yield of the reaction over two steps was 69%.

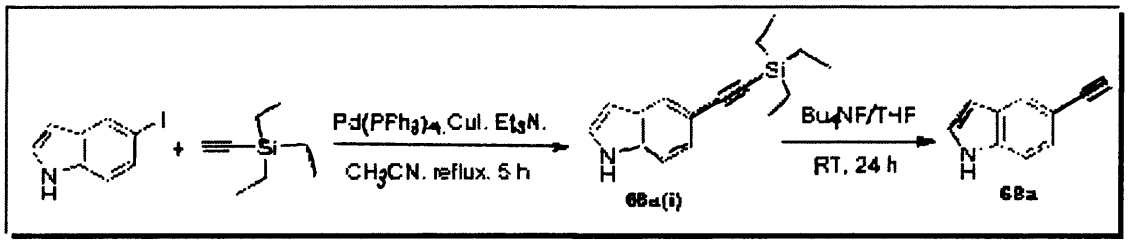


Figure 3.21: Synthesis of 5-ethynylindole **68a**

3.4.2 Synthesis of substituted benzaldoxime

The synthesis of substituted benzaldoxime was achieved by using the same procedure as described at 3.3.2. The same benzaldoximes from 3.3.2; compounds **68b** and **69b** were further used for this series of 5-indolylisoxazoles. Another oxime, 3,4,5-trimethoxybenzaldoxime (**70b**) was synthesised in excellent yield, 90%.

3.4.3 Synthesis of 5-(1*H*-indol-5-yl)-3-phenylisoxazoles

For these series of isoxazoles, the same procedure was employed as described in 3.3.3. The reaction mechanism involved 1,3-dipolar cycloaddition which started with the formation of nitrile oxides from substituted benzimidoyl chlorides intermediate which later underwent cycloaddition with 5-ethynylindole. The reactions however suffered from incomplete conversion of unreacted starting materials even after prolonged reaction times. Upon termination of the reactions, the afforded crude products needed further purification by column chromatography to afford the pure isoxazoles in low yield (7% yield for compound **68**, 36% for compound **69** and 29% for compound **70**).

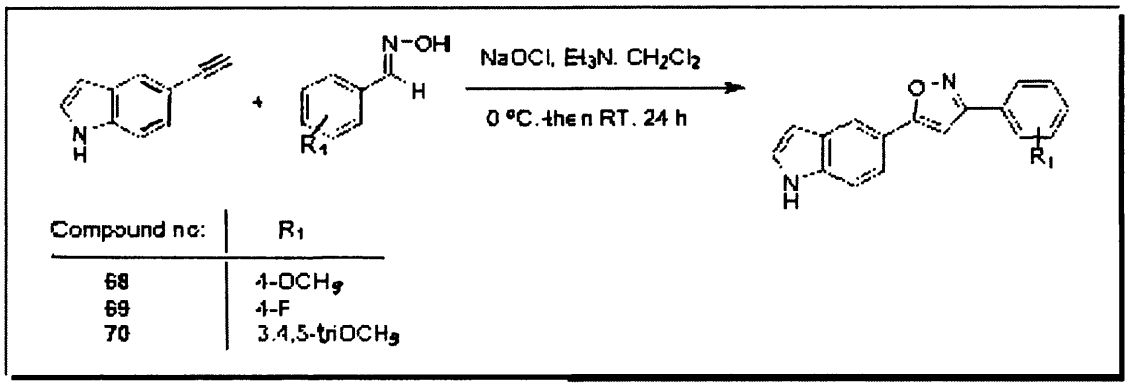


Figure 3.22: Synthesis of compound **68**, **69** and **70**.

3.5 Failed experiments:

3.5.1 Synthesis of indole-3-yl-isoxazole series via 1,3-dipolar cycloaddition using 1*H*-indole-3-carbaldehyde oxime

The synthesis of this series of isoxazoles was intended to provide useful structure activity relationship to previously synthesised indole-2-yl-isoxazoles series. The method of choice was via 1,3-dipolar cycloaddition due to its regioselectivity showed in the previous experiments. Unfortunately, the synthesis of this series of compounds was not as reproducible as expected. The first part of the synthesis involved the formation of compound **71a** (1*H*-indole-3-carbaldehyde oxime) followed by 1,3-dipolar cycloaddition with substituted phenylacetylene to afford the proposed compounds **71, 72, 73** and **74** (Figure 3.23).

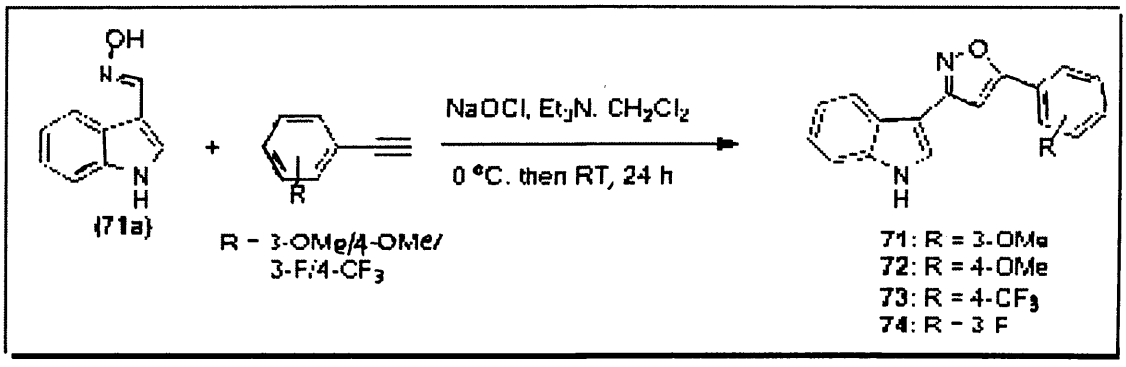


Figure 3.23: Proposed synthetic procedure for compounds **71, 72, 73** and **74**.

3.5.1.1 Synthesis of (*E*)-1*H*-indole-3-carbaldehyde oxime.

The synthesis of indole-3-oxime (**71a**) was achieved by the same reaction procedure described in 3.3.2 to synthesis benzaldoxime from indole-3-carboxaldehyde. The indol-3-oxime was afforded in good yield (73%) and in good purity with no need for further purification (Figure 3.24).

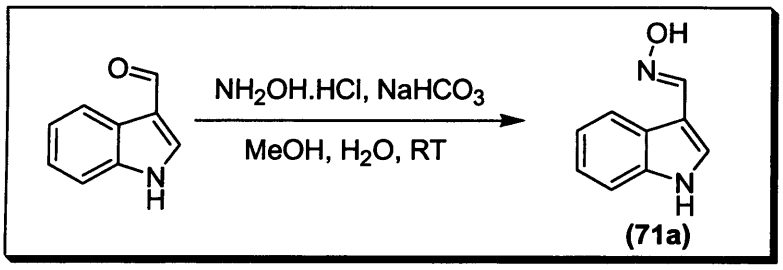


Figure 3.24: Synthesis of 1*H*-indole-3-carbaldehyde oxime (**71a**)

3.5.1.2 Synthesis of 3-(1*H*-indol-3-yl)-5-phenylisoxazoles

The next part of the synthesis involved the synthesis of isoxazoles via 1,3-dipolar cycloaddition with substituted phenyl acetylene (summarized in Figure 3.25). For the first attempt, 3-methoxyphenyl acetylene was used as the starting material for the first compound of this series. The same method was employed as was previously described (refer 3.3.3 and 3.4.2). Unfortunately, even after repeated attempts, the starting material had not reacted indicated by no product formation even after prolonged stirring. Addition of different substituted phenyl acetylene (either 4-methoxy or 3-fluoro substituted) did not give any difference in the observation. Further substitution of dichloromethane with different solvents [THF, DMF, ethyl acetate, dimethylsulfoxide (DMSO) or *tert*-butanol:THF (3:1)] also did not improve the outcome of the reaction.

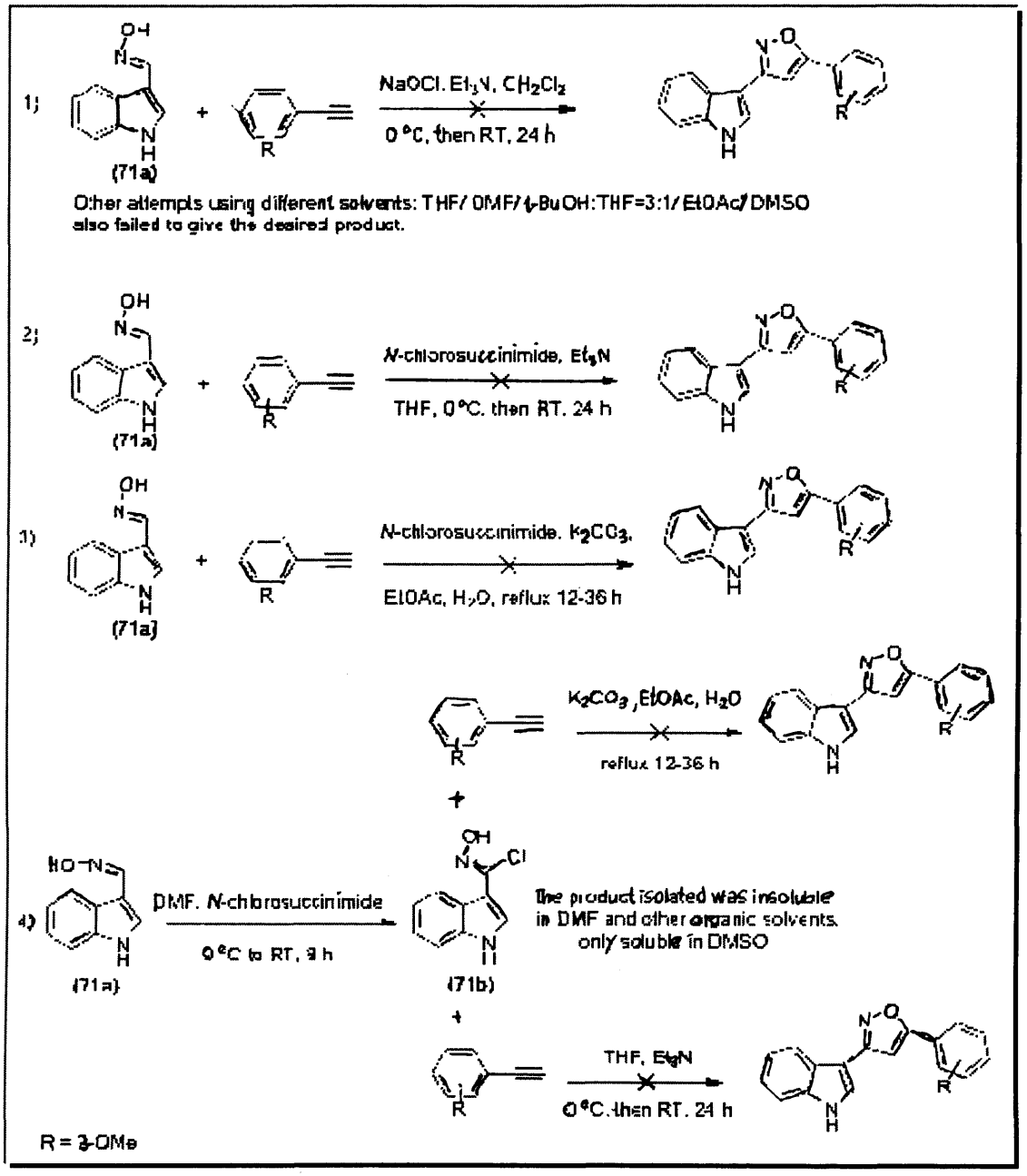


Figure 3.25: Summary for the attempted methods to synthesise indole-3-yl-isoxazole series

Nevertheless, one interesting observation was detected when the reaction was done in solvents other than dichloromethane and DMSO: the formation of an insoluble precipitate which was not the desired compound. It was predicted that the precipitate was an intermediate of the reaction, whose NMR analysis showed that the precipitate still retained the compound **71a** core structure, only missing one hydrogen peak at around δ 8.1 ppm which belongs to the proton of the oxime. This indicated that the precipitate was

probably *N*-hydroxy-1*H*-indole-3-carbimidoyl chloride (compound **71b**), which should be the first intermediate formed following the reaction with sodium hypochlorite before the attack of the base to form nitrile oxide species. Whereas in dichloromethane there was no reaction detected, the reaction in DMSO showed the possibility of nearly full consumption of compound **71a** to another compound/intermediate, but still without any desired product formation indicated by TLC and following NMR determination of precipitate formed upon quenching with water.

Another attempt was also sought by changing the reagent for chlorination of oxime from sodium hypochlorite to *N*-chlorosuccinimide in THF with triethylamine as base. The reaction was again found to be unsuccessful. A further attempt to change the base to potassium carbonate in ethyl acetate/water as solvent with heating at reflux for up to 36 hours also did not change the fate of the reaction. At this stage, the insolubility of the intermediate **71b** was seen as the biggest obstacle that made all the attempted reactions fail. Although the intermediate dissolved in DMSO, this was apparently not a suitable solvent for the reaction to occur.

In order to test this hypothesis, compound **71b** was independently synthesised using *N*-chlorosuccinimide in DMF from compound **71a**. After 3 hours of stirring at room temperature, compound **71b** precipitated out of the reaction mixture and was collected. The NMR of the collected precipitate, which only dissolved in DMSO, showed the same spectrum as was previously seen which gave enough evidence to support the initial hypothesis. The precipitate was used straight away in the next cycloaddition reaction in THF and triethylamine, but even after up to 48 hours of stirring no reaction occurred, most probably due to its insolubility in THF.

Another attempt was made to synthesise 3-ethynyl-1*H*-indole following the method in 3.3.1 as an alternative reagent to synthesise the regioisomer, 5-(1*H*-indol-3-yl)-3-phenylisoxazoles also failed to give the desired product, where no reaction occurred even after prolonged stirring for up to 48 hours (Figure 3.26). This highlights the failure of previously

successful method to synthesise indole-3-yl isoxazoles series, thus emphasising the need to find alternative pathways in order to synthesise this kind of isoxazole series.

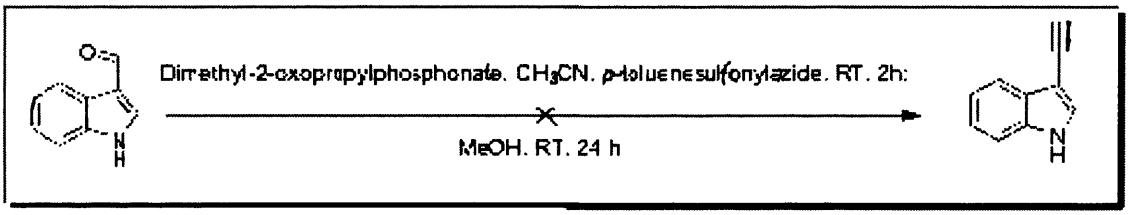


Figure 3.26: Attempt to synthesise 3-ethynyl-1*H*-indole.

3.5.2 Attempt to synthesis 3-(5-methoxy-1*H*-indol-2-yl)-5-(4-(trifluoromethyl)phenyl)isoxazole (and regioisomer).

Synthesis of this compound via the formation of alkyknyl ketone and cyclisation using hydroxylamine hydrochloride (pathway 1) suffered from low yields of alkynyl ketone intermediate [1-(5-methoxy-1*H*-indol-2-yl)-3-(4-(trifluoromethyl)phenyl)prop-2-yn-1-one; 12% yield] and product (together with the formation of regioisomer). Attempts to separate the isomers using column chromatography [hexane:ethyl acetate (10:1)] only resulted in separation of around 20% of the compound mixtures, where the rest remain as mixed isomers. The separated regioisomers were obtained in a very low yield and were very hard to purify due to the presence of lots of impurities (Figure 3.27).

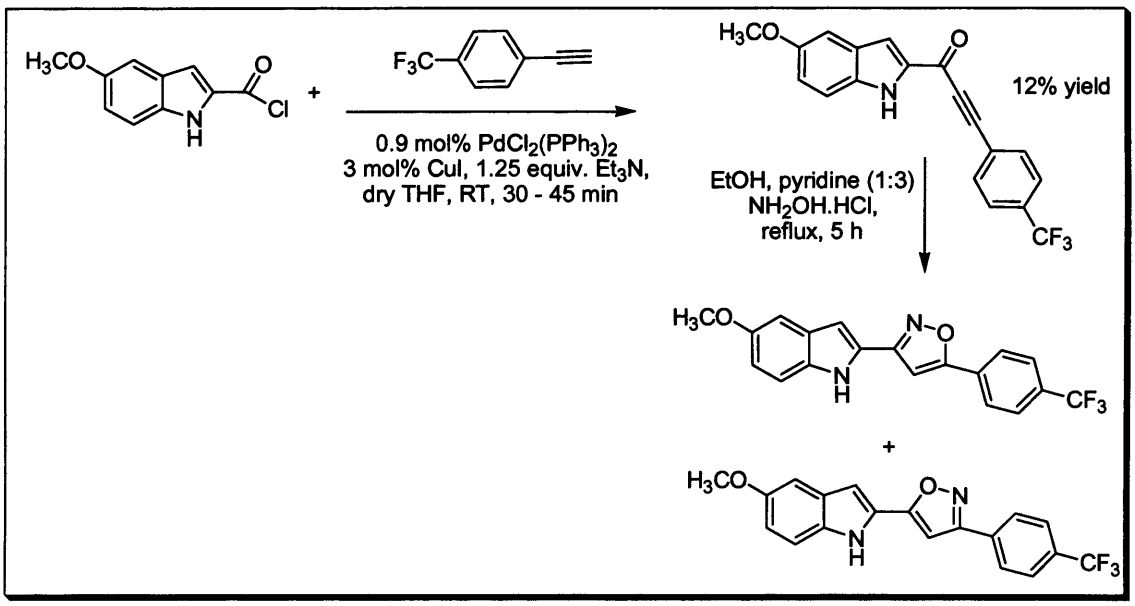


Figure 3.27: Attempt to synthesise 3-(5-methoxy-1*H*-indol-2-yl)-5-(4-(trifluoromethyl)phenyl)isoxazole (and regioisomer)

3.6 Future study: Biological evaluation.

The synthesised isoxazoles will be further evaluated for their biological activity in human cancer cell lines. The initial screening will involve the determination of their antiproliferative activity. The result is anticipated to generate useful structure-activity relationship for the identification of lead compounds which will provide further encouragement for the development of this series of compounds.

CHAPTER 4

SYNTHESIS AND EVALUATION OF SPECIFIC INHIBITORS TARGETING THE DISHEVELLED-PDZ DOMAIN OF THE WNT SIGNALLING PATHWAY

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SYNTHESIS AND EVALUATION OF SPECIFIC INHIBITORS TARGETING THE DISHEVELLED-PDZ DOMAIN OF THE WNT SIGNALLING PATHWAY

4.1 Introduction

4.1.1 Wnt signalling pathway

Wnts are secreted cysteine-rich glycoproteins that act as short-range ligands to locally activate receptor-mediated signalling pathways (Moon et al. 2004). The Wnt signalling pathway is one of the most evolutionary conserved biochemical signalling pathways. It is involved in a vast array of processes including embryonic development, adult tissue homeostasis (Staal and Clevers 2003; Bryja et al. 2007), cell tissue polarity, tissue patterning, control of cellular proliferation and development of neoplasia, in which cells respond to Wnts in a context-dependent manner through changes in survival and proliferation, cell fate and movement (Moon et al. 2004, Shan et al. 2005).

The pathway is initially activated by a Wnt ligand binding to a Frizzled (Frz) receptor and a low-density lipoprotein receptor related protein (LRP) 5 or LRP6, which subsequently transduces a signal through one of at least three distinct intracellular signalling pathways: the "Wnt/Ca^{2+}" pathway, the "Wnt/polarity" pathway (Horvath et al. 2007) and the classical and the best understood which is the highly conserved "Wnt/β-catenin" canonical pathway (Horvath et al. 2007; Leonard and Ettensohn 2007) (Figure 4.1). In all these pathways, the 7 transmembrane domain Frz proteins are bound by the secreted molecule Wnt and transduce the signal to the cytoplasmic protein Dishevelled (Dvl), the component at which these signalling pathways diverge (Wong et al. 2003; Moon et al. 2004).

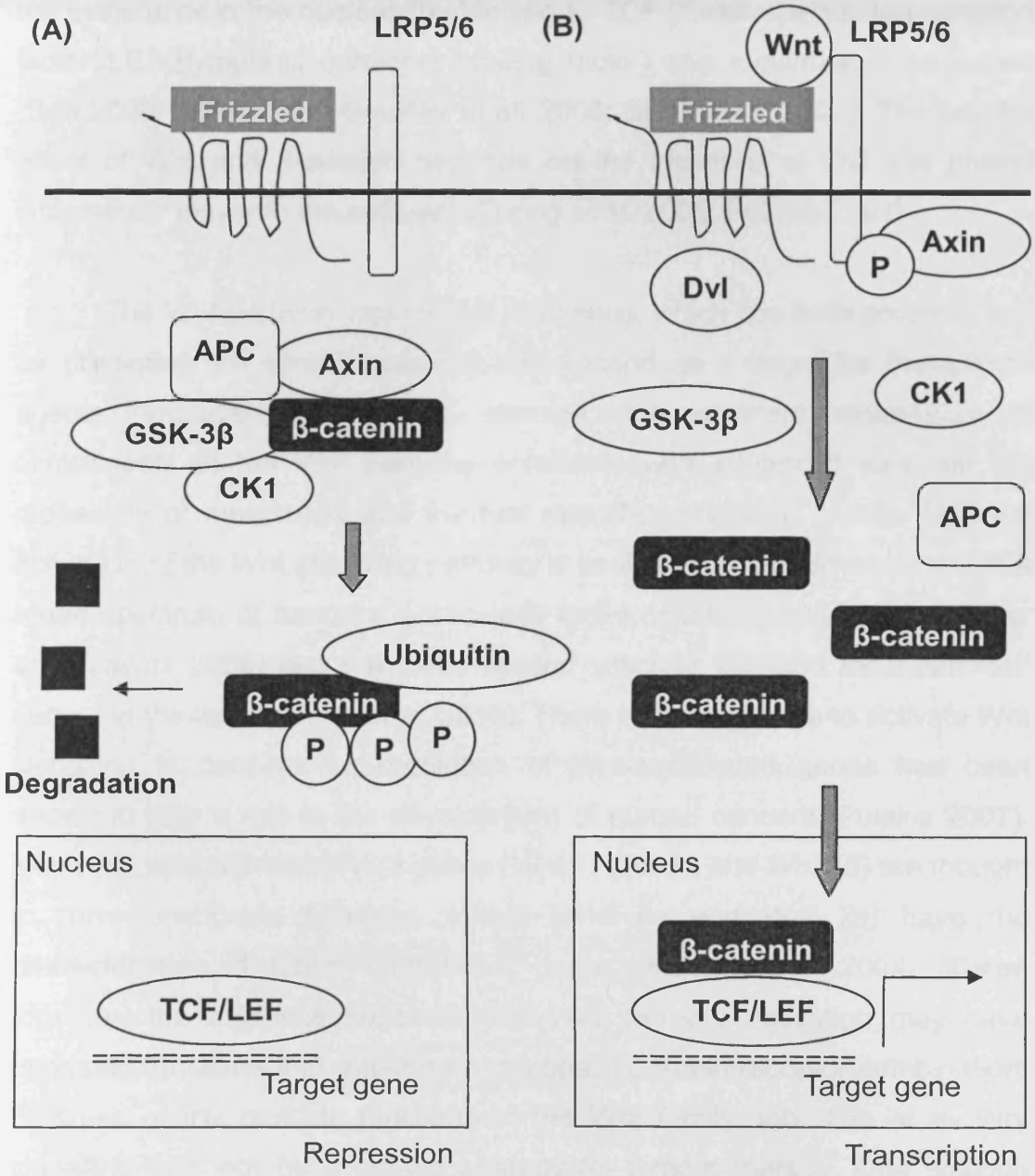


Figure 4.1: The Wnt signalling pathway (A: Wnt off; B: Wnt on)

The binding of Wnt ligand to Frz and LRP 5/6 will result in the recruitment of axin to the LRP proteins and the cytoplasmic protein Dvl to the Frz receptor by an unknown mechanism that may include phosphorylation (Sen 2005; Shan 2005; Smalley et al. 2005; Leonard and Ettensohn 2007; Polakis 2007). Upon Wnt stimulation, Dvl is phosphorylated and the degradation complex formed by Axin, APC (adenomatous polyposis coli), CK1 (casein kinase 1) and GSK3β (glycogen synthase kinase-3 beta) is inhibited leading to the stabilization of β-catenin. β-catenin can then act in

the cytosol, or in the nucleus, by binding to TCF (T-cell-specific transcription factor)/LEF (lymphoid enhancer binding factor) and regulating transcription (Sen 2005; Shan 2005; Smalley et al. 2005; Bryja et al. 2007). The cellular effect of Wnt and β-catenin depends on the functions of Dvl, the pivotal intracellular player in the pathway (Zhong et al. 2006; Polakis 2007).

The Wnt pathway has clinical relevance, which lies in its potential first for predicting the clinical outcome and second as a target for therapeutic agents. Importantly, in some cancers, the aberrant expression of components of the Wnt pathway correlates with advanced tumours, the probability of metastasis and survival rate (Doucas et al. 2005). Aberrant activation of the Wnt signalling pathway is implicated in the development of a broad spectrum of tumours, particularly those of the digestive tract (Barker and Clevers 2006) and colorectal cancer, which is the third most common cancer in the world (Chen et al. 2010). There are many ways to activate Wnt signalling in cancers. Up-regulation of Wnt-associated genes has been shown to play a role in the development of human cancers (Polakis 2007). However, although many Wnt genes (*Wnt 1, Wnt 3a and Wnt 16)* are thought to have oncogenic potential, others (*Wnt 5a and Wnt 7a)* have the characteristics of tumor suppressor genes (Moon et al. 2004). Taken together, the evidence suggests that Wnt pathway activation may have opposing functions that depend on the specific ligand/receptor combination. Because of the multiple functions of the Wnt family, inhibition of all Wnt signalling may not be a perfect strategy for tumour therapy. One solution would be to target only Wnt signalling molecules that contribute significantly to tumourigenesis (Fujii et al. 2007).

It appears that Wnt signalling is a key regulator in stem cell renewal (Wong et al. 2003). Wnt proteins are major components of mesenchymal stem cells, stromal cells, and a heterogenous population of fibroblast-like cells that support the growth, differentiation and survival of different cell lineages and cell types by providing cytokines/chemokines, adhesion molecules and extracellular matrix molecules (Moon et al. 2004). Targeted disruption of Wnt signalling by "loss-of-function mutations" is linked to

dramatic phenotypes, ranging from embryonic lethality to severe central nervous system, limb, lung and kidney defects (Sen 2005) and various human cancers (Barker and Clevers 2007). For example, eighty percent of colorectal cancers alone reveal activation of this pathway by either inactivation of the tumour-suppressor gene adenomatous polyposis coli (APC) or mutation of the proto-oncogene β-catenin (Herbs and Kolligs 2007).

Dvl was one of the first components of the Wnt signalling pathway to be defined; yet its biochemical function remains poorly understood. Dvl family members are 500 to 600 amino acids in length and have been found in animals ranging from Drosophila to humans (Leonard and Ettensohn 2007). Dvl family proteins (Dsh in *Drosophila;* XDsh in *Xenopous*; Dvl-1 to 3 in mammals) are key intracellular mediators of the Wnt signalling pathway, where the Wnt signal is passed from the membrane Wnt receptor Fz to Dvl, which then relays the signal to downstream components (Lee et al. 2009; Zhang et al. 2009b). In addition to playing a role in the canonical Wnt signalling pathway, Dvl proteins are also involved in the planar cell polarity (PCP) pathway and the calcium-signalling pathway (Wharton 2003; Smalley et al. 2005; Leonard and Ettensohn 2007). Dvl is hypothesised to serve as the branch point at which the two pathways diverge, making its regulation of particular importance (Leonard and Ettensohn 2007). Some ectopically expressed Frz family members can recruit Dvl to the plasma membrane, and RNA interference studies have shown that Dvl is required for the Frz activity in Frz/LRP receptor complex but not for LRP function. Dvl is required to recruit axin to the Frz/LRP complex in a signal dependent manner (Smalley et al. 2005). The regulation of Dvl on Wnt signalling pathway is crucial in normal development and its aberrant activation is implicated in many tumours (Zhang et al. 2009b)

All Dvl family members contain three highly conserved domains (Figure 4.2): an N-terminal DIX (for DIshevelled and aXin proteins) domain contains ~80 amino acids, a central PDZ (for Post Synaptic Density-95, Discs-large and Zonula occludens-1 proteins) domain contains ~90 amino acids, and a DEP (for Dishevelled, EGL-10 and Pleckstrin proteins) domain

as the most C-terminal conserved domain contains ~90 amino acids (Wharton 2003; Wong et al. 2003). The DIX domain is thought to be required for the canonical (β-catenin pathway), multimerisation and interaction with axin. The DEP domain is required for the PCP pathway signalling. The PDZ domain, on the other hand, may function in both pathways, by mediating the interaction of Dvl with a variety of binding partners, including the Frz receptor. PDZ domain plays an important role in both the canonical and non canonical Wnt pathways, and is involved in distinguishing between the two pathways (Wharton 2003; Wong et al. 2003; Smalley et al. 2005; Leonard and Ettensohn 2007). Wong et al. (2003) demonstrated that there is a direct interaction between Frz and Dvl. Specifically, the key interaction in the pathway is the direct recognition of PDZ domain of Dvl with the Frz conserved sequence (KTXXXW) that is C-terminal to the seventh transmembrane domain (Fujii et al. 2007; Lee et al. 2009; Zhang et al. 2009b). The interaction is essential in the transduction of the Wnt signal from Frz to the downstream components of the pathway. Thus, the findings shed light on the mechanism by which the receptor Frz communicates with the downstream component Dvl (Wang et al. 2008).

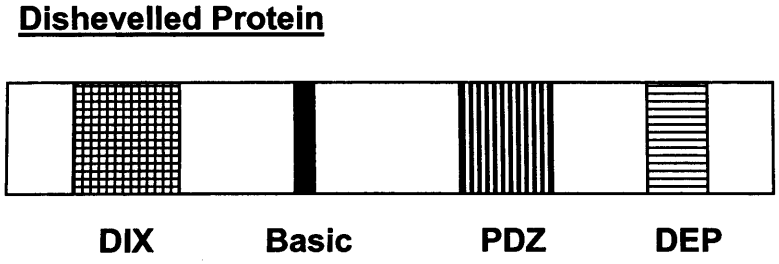


Figure 4.2: The Dishevelled Protein (adapted from Nusse 2008).

The special role of the Dvl PDZ domain in the Wnt pathway makes it an ideal pharmaceutical target (Fujii et al. 2007). The PDZ domain is a common protein-protein interaction module that are characterised by the ability to recognise the extreme COOH-terminus of important drug target proteins or internal peptides (Sheng and Sala 2001; Wang et al. 2008; Zhang et al. 2009b, Lee and Zheng 2010). PDZ domain mediates crucial protein-protein interactions that enforce localisation and organisation of proteins in a

variety of submembranous complexes associated with G protein-coupled receptors, cell signal mediators including ion channels, transmembrane receptors and regulatory enzymes (Harris and Lim 2001; Wang et al. 2008; Lee and Zheng 2010). Small molecule inhibitors of the PDZ domain in Dvl might be useful in dissecting molecular mechanisms and formulating pharmaceutical agents that target cancers or other diseases in which Wnt signalling is involved (Wang et al. 2008). A study carried out by Shan et al. (2005) showed that there is a direct interaction between Frz and Dvl and revealed a previously unknown connection between the membrane-bound receptor and downstream components of the Wnt signalling pathways. Therefore an inhibitor of the Dvl PDZ domain is likely to effectively block the Wnt signalling pathway at the Dvl level (Shan et al. 2005; Zhang et al. 2009b).

Frizzled-7 (Frz-7), which is 1 of 10 members of Frz family, is reported to be over expressed in colon cancer cells, as well as in other cancer types such as melanoma, lung cancer and lymphoblastic leukaemia (Ueno et al. 2009). In normal cells, its expression is very limited. Ueno et al. (2008) had revealed that the Frz 7 played a pivotal role as a receptor for canonical Wnt signalling pathway and may be important for the invasion and metastasis of cancer cells. Later, their data showed that Frz 7 may be a potential therapeutic target for cancers associated with aberrant activation of the Wnt signalling pathway. Since Frz 7 interacts directly with a PDZ protein interaction domain of the Dvl family, disrupting the Frz7-Dvl protein-protein interaction represents a promising strategy for cancer therapy (Fujii et al. 2007).

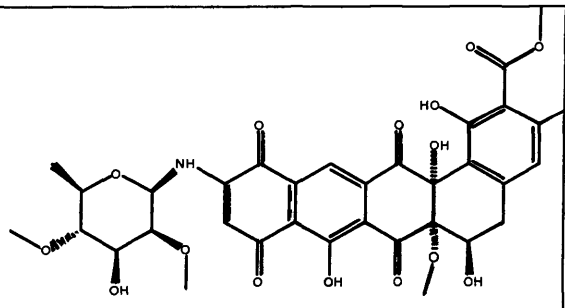
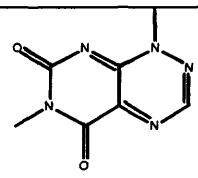
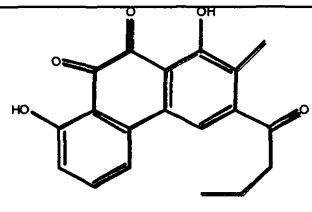
4.1.2 Small molecule inhibitors of the Wnt signalling pathway.

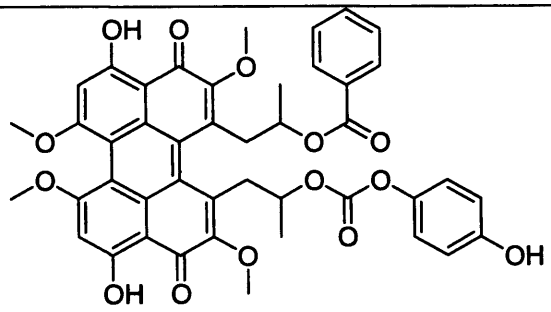
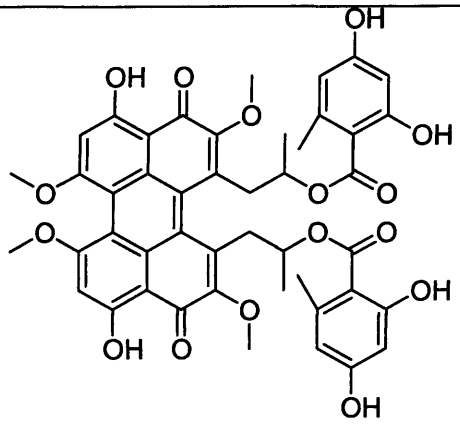
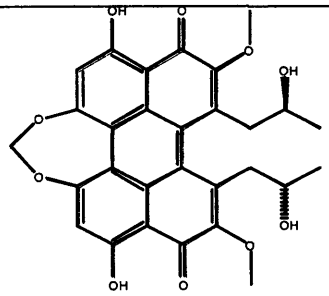
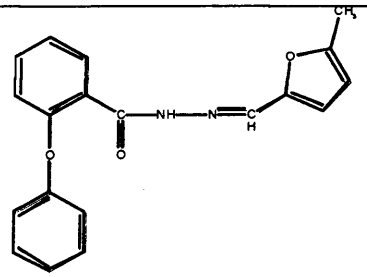
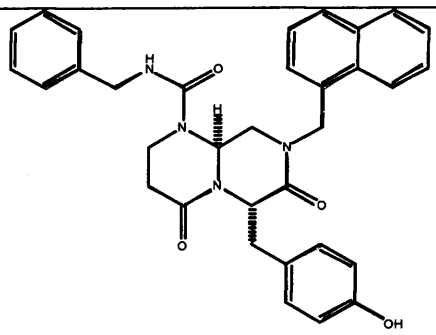
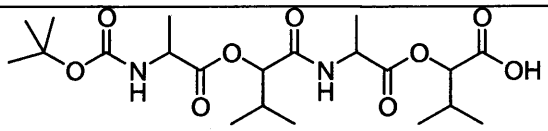
The development of therapeutics specifically targeting the aberrant Wnt pathway in cancer cells is still largely in its infancy. The discovery of the role of Wnt signalling pathway as one of the fundamental molecular mechanisms in colon cancer has stimulated the search for small molecule inhibitors of the Wnt pathway proteins. To date, despite the prevalence of colon cancer in the

adult population, there is no small molecule approved by the FDA for targeted therapy for colon cancers (Chen et al. 2010).

Several approaches are currently being explored in pursuit of these selective Wnt pathway inhibitors, such as antibody-based therapeutics (He et al. 2004) and viral-based therapies targeting Wnt-addicted cancers (Lipinski et al. 2004). However, a number of existing drugs, including NSAIDS (non-steroidal anti-inflammatory drugs) and Vitamin A/D derivatives also show promise in treating Wnt addicted cancers and have the advantage of already being in the clinical use for other disorders (Barker & Clevers 2006). Table 4.1 shows some examples of potential small molecule inhibitors of the Wnt signaling pathway.

Table 4.1: Small-molecule inhibitors of the Wnt signalling pathway (Barker and Clevers 2007).

Name	Structure	Screening method	IC_{50} (μM) *	Integration target
ZTM 000990		ELISA-based HTS of 7000 natural compounds	0.64	β-catenin-Tcf
PKF 118-310		ELISA-based HTS of 7000 natural compounds	0.8	β-catenin-Tcf
PKF 118-744		ELISA-based HTS of 7000 natural	2.4	β-catenin-Tcf

		compounds		
PKF 115-584		ELISA-based HTS of 7000 natural compounds	3.2	β-catenin-Tcf
PKF 222-815		ELISA-based HTS of 7000 natural compounds	4.1	β-catenin-Tcf
CGP 049090		ELISA-based HTS of 7000 natural compounds	8.7	β-catenin-Tcf
PNU-74654		*In silico* screen 0f 18000 synthetic compounds	ND	β-catenin-Tcf
ICG-001		Cell-based HTS of 5000 synthetic compounds	3.0	CBP-β-catenin
NSC 668036		*In silico* screen of	ND	Frizzled - dishevelled

		250000 drug-like compounds		

*Concentration required to reduce Tcf- β-catenin activity in 50% colon cancer cells. CBP, cAMP-responsive element binding protein (CREB) binding protein; ELISA, enzyme-linked immunosorbent assay; HTS, high-throughput screening; ND, not determined; Tcf, T-cell factor.

Fujii et al. (2007) have showed that inhibiting the PDZ domain of Dvl is a potential strategy for the treatment of Wnt-related cancer, as indicated by a small molecule inhibitor, FJ9, which is among the first non-peptide inhibitor to show therapeutic efficacy through disruption of Dishevelled-PDZ protein-protein interactions and currently a lead compound in drug development (Figure 4.3).

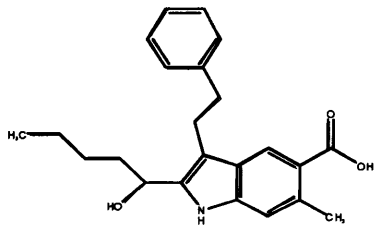


Figure 4.3: Structure of FJ9, a Frz-Dvl inhibitor (Fujii et al. 2007).

Further initiatives to manipulate the PDZ domain of Dvl for the development of novel therapeutic agents of Wnt signalling pathway was later reviewed by Wang et al. (2008), where a number of compounds that showed potent antagonistic activity on the PDZ domain are outlined below (Table 4.2).

Table 4.2: Antagonists of PDZ protein-protein interactions (Wang et al. 2008).

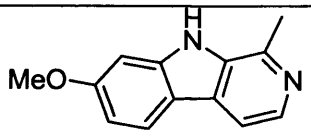
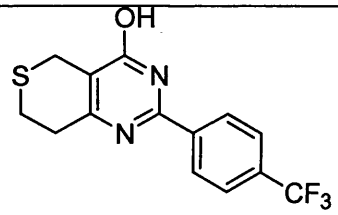
Target	Antagonist	Phase of development	Roles/Functions
PSD-95 PDZ2/NMDA or nNOS	NA-1	Phase 1 (Ending March 2007)	Treatment of ischemic brain damage; in animal toxicity studies, NA-1 showed safety and tolerability after intravenous administration.

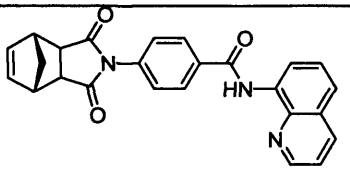
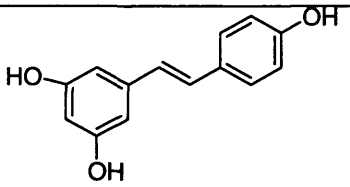
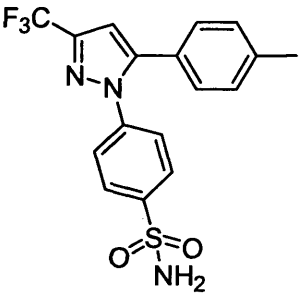
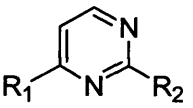
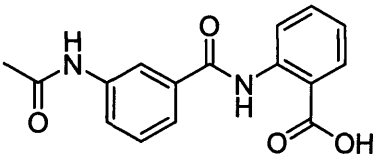
PSD-95 PDZ2/NMDA or nNOS	Flavonoids	Lead	Flavonoids bind to the PSD-95 PDZ2 domain.
Dishevelled PDZ/Frz7 Wnt receptor	Frz7 peptide (GSKTLQSWRRYH) Dapper peptide (SGSLKLMTTV)	Biological study	In *Xenopus* embryos, Frz7 (or Dapper) peptide attenuates Wnt3A-induced canonical Wnt signaling
Dishevelled PDZ/Frz7 Wnt receptor	NSC-668036	Biological study HIT	In *Xenopus* embryos, NSC-668036 inhibits the canonical Wnt signaling induced by Wnt-3A.
Dishevelled PDZ/Frz7 Wnt receptor	FJ9	Lead	Induction of apoptosis in human cancer cell lines and tumour growth inhibition in a mouse xenograft model.

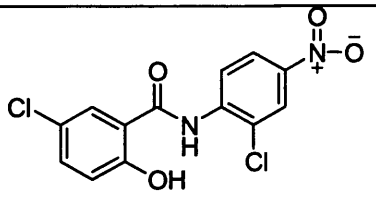
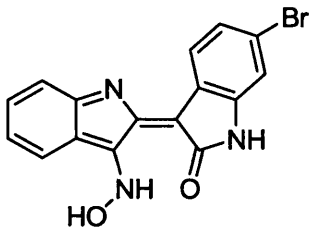
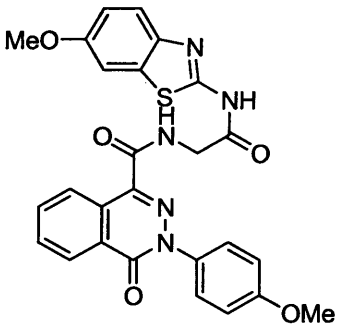
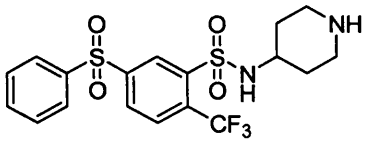
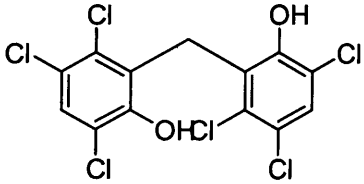
NMDA: *N*-methyl-D-aspartate; nNOS: neuronal nitric oxide synthase.

Recently, Chen et al. (2010) also had listed some of the more current efforts for anti-Wnt compounds, as shown in table 4.3 below:

Table 4.3: Recent Wnt signalling active compounds:

Target	**Agent**	**Activity**	**Structure**
Anti-diabetic	Thiazolidinediones, harmine	Wnt target genes	 Harmine
Axin	XAV939, IWR	Axin stabilisation, tankyrase1 and 2 inhibition.	 XAV939

		Blocks Axin degradation	 IWR-1
c-Met kinase Caspase activation	NSAIDS, celecoxib, resveratrol	Suppression of Tcf gene expression B-catenin localization	 Resveratrol  Celecoxib
DKK-1	(1-(4-(naphthalene-2-yl)pyrimidin-2-yl)piperidin-4-yl)methanamine (NPPM)	Dkk-1 inhibitor	 NPPM
Dishevelled	Niclosamide compound: 3289-8625, 3289-5066	Dishevelled downregulation. Dishevelled PDZ domain	 3289-5066 Structure of 3289-8925 is reported in Figure 4.7.

Frizzled	Niclosamide	Frizzled trafficking	 Niclosamide
GSK3β	6-bromoindirubin-3'-oxime (BIO)	GSK3 inhibitor, Wnt activator	 BIO
Porcupine	IWP	Inhibits porcupine activity	 IWP-1
Secreted Frizzled-related protein 1 (sFRP-1)	Diphenylsulfone sulphonamide	Inhibits human sFRP-1 activity	 Diphenylsulfone sulphonamide
Siah-1	Hexachlorophene	Siah-1 mediated β-catenin degradation	 Hexachlorophene

On the other hand, Ewan et al. (2010) have discovered potent small molecule inhibitors which showed good activity in their cell-based assay

(compound CCT031374, CCT036477 and CCT070535) (Figure 4.4). Their study is based on the ability of the tested compounds to inhibit their reporter cell lines that had an inactive basal TCF-reporter, which could be induced later through the activation of Dvl-estrogen receptor fusion protein following addition of estrogen. Compound CCT036477 showed the best activity *in vivo* which could present a useful template for further study of compounds targeting Wnt signalling pathway.

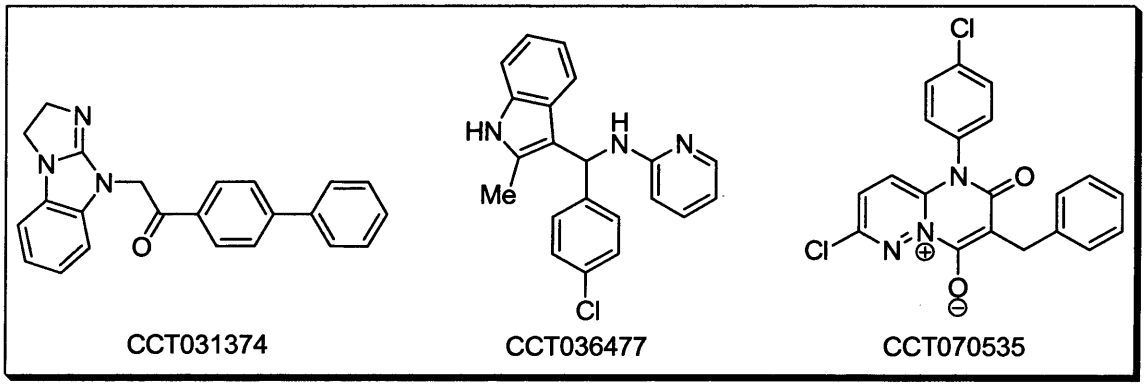


Figure 4.4: Structures of compounds CCT031374, CCT036477 and CCT070535.

The ability of NSAIDS to act as potential Wnt signalling inhibitors has gained much interest over the past few years. A quite recent study was done by Lee et al. (2009), who found sulindac (Clinoril) and its metabolite, sulindac sulfone (Figure 4.5) can interact with the PDZ domain of Dvl and suppresses Wnt3A induced β-catenin signalling at the Dvl level. Their data suggest that sulindac and its metabolite have a potential anticancer property not only by their ability to inhibit cyclooxygenase (COX) 1/2 but also by their ability to inhibit aberrant canonical Wnt signalling by suppression of the Dvl PDZ domain.

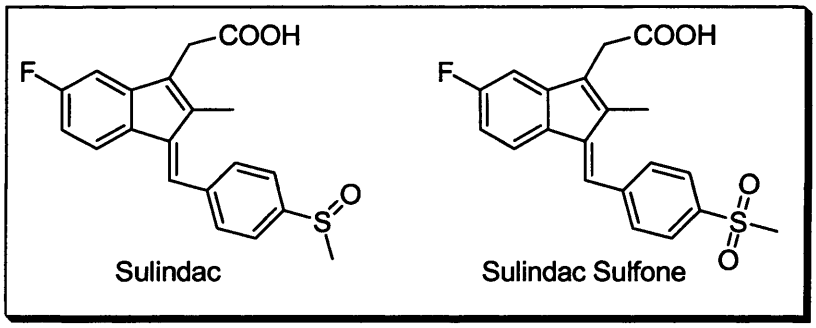


Figure 4.5: Structure of Sulindac and Sulindac Sulfone

The most recent studies in the area of Wnt signalling inhibitors suggested another two series of compounds with potent activities on the Wnt signalling pathway. Zhang et al. (2011) have reported a series of fluorinated *N,N*-dialkylaminostilbenes as potential Wnt inhibitors. In their study, compound **4r** (Figure 4.6) showed the best inhibitory activity at nanomolar level in the colorectal cancer cell line (LS174) especially at the transcription level downstream of β-catenin, and is able to inhibit the growth of human colorectal cancer cell xenografts in athymic nude mice. At the same time, Waaler et al. (2011) reported another two small molecules, namely JW67 and JW74 (Figure 4.6) which showed striking inhibitory activities as antagonists of the canonical Wnt signalling pathway specifically at the level of the destruction complex in colorectal cancer cell line SW480. The compounds which were identified from a high-throughput screening on reporter cell lines with TCF-responsive promoter were able to reduce the level of β-catenin with subsequent downregulation of Wnt target genes especially Axin2. Compound JW74 also showed good inhibitory activity *in vivo* by inhibiting the growth of tumour cells in a mouse xenograft model of colorectal cancer and inhibited tumour formation and growth in small intestine and colon of Apc^{Min} mice which is prone to develop polyposis and colon adenocarcinoma due to mutation in one allele of the Apc tumour suppressor gene.

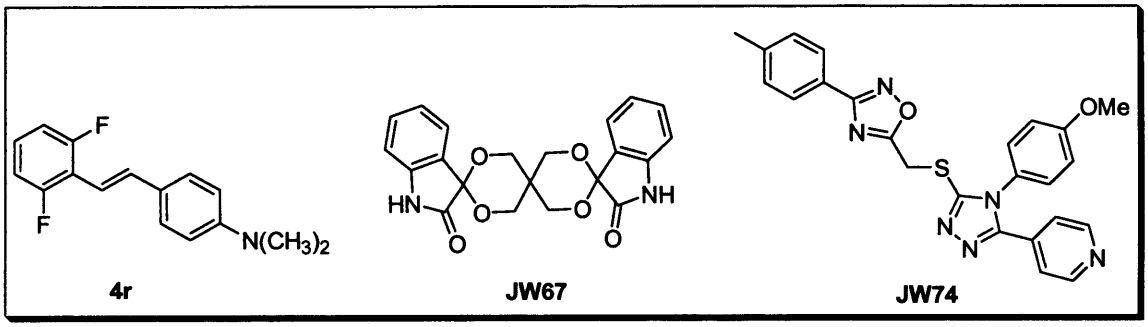


Figure 4.6: Structure of compounds **4r**, JW67 and JW74.

4.1.3 Derivatives of 3289-8625 as specific small molecule inhibitors of the PDZ domain of Dvl.

Inhibition of Wnt signalling pathway is one of the various approaches used nowadays for developing novel cancer drugs. Targeting the PDZ domain of Dishevelled (Dvl) is an attractive strategy due to the role of this domain to transduce Wnt signals from the membrane receptor Frizzled to downstream components. A previous study done by Grandy et al. (2009) has identified small drug-like molecule through structure-based ligand screening and NMR spectroscopy and showed the compound to interact at low micromolar affinity with the PDZ domain of Dvl. This compound (3289-8625) (Figure 4.7) was obtained from screening of commercially available databases for its binding to Dvl-PDZ domain. The compound was docked to the Dapper peptide binding site of Dvl PDZ domain (Protein Data Bank code: IL60) and was chosen as one of the potential hits. Further validation of the docking results using a ^{1}H-^{15}N correlation NMR study showed that compound 3289-8625 displayed the most significant chemical shift perturbation when titrated with ^{15}N-labelled Dvl-PDZ domain that indicated the strong binding affinity of the compound towards the protein. At the same time, a *Xenopus* testing system revealed that the compound could inhibit the Wnt signalling pathway by its permeability to the cell membrane, and ablity to retard the Wnt signalling pathway in the Hyaloid vessel system of the mouse eye that clearly showed the compound's ability to penetrate the membrane of vascular endolthelial cells *in vivo.* The compound is also able to suppress the growth of prostate cancer PC-3 cells. All this data highlights the promising therapeutic potential

of compound 3289-8625, which will provide a useful pharmacophore for further modification and structure-activity relationship study of future Wnt pathway inhibitors.

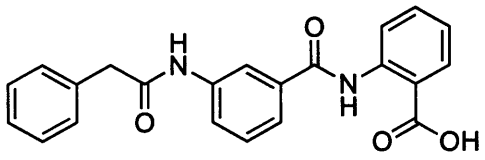
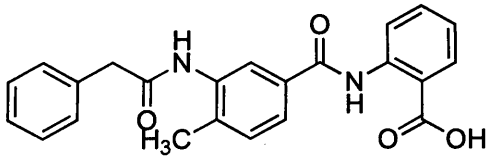
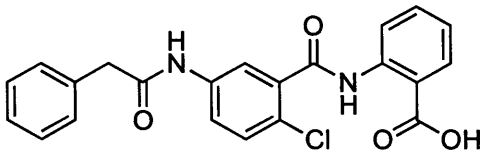


Figure 4.7: Structure of 3289-8625

Recent studies in Prof. Jie J. Zheng's laboratory, St Jude Children's Research Hospital, Memphis USA revealed that when the two derivatives of 3289-8625 (6569188 and 7359885) (Figure 4.8) were subjected to fluorescence spectroscopy measurement to measure their binding affinity towards the Dvl PDZ domain, the two derivatives showed better binding affinity towards the PDZ domain of Dvl compared to the parent compound (unpublished data) (Figure 4.9). This finding clearly provides a good basis for structure activity relationship studies to further improve the target compound specific to inhibit this particular protein.



6569188



7359885

Figure 4.8: Structures of compound 6569188 and 7359885.

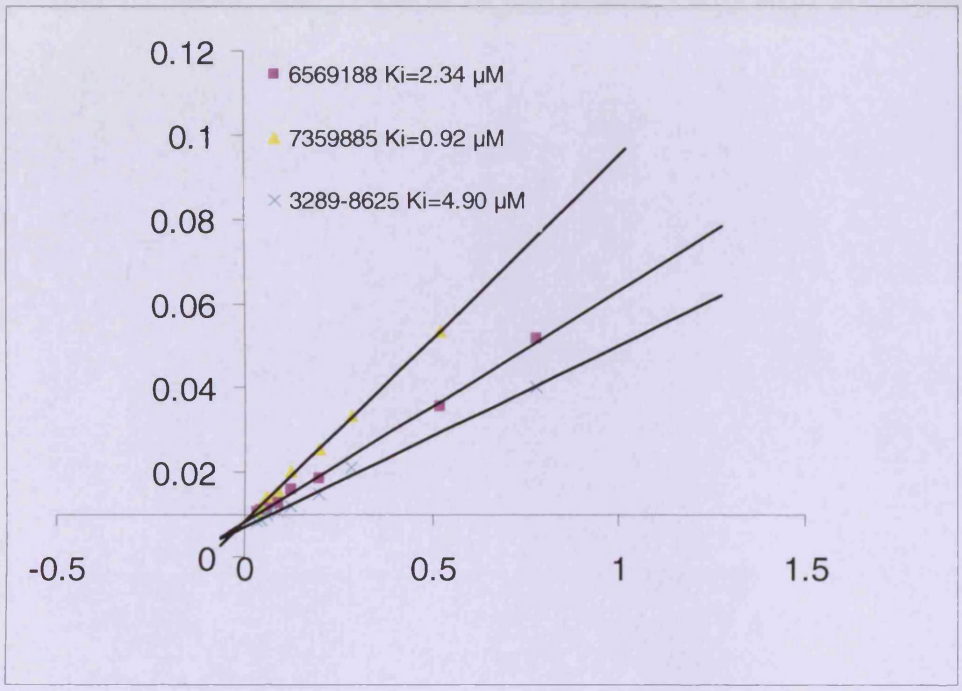


Figure 4.9: Binding study of compounds 3289-8625, 6569188 and 7359885 towards the Dvl PDZ domain characterised by fluoresence spectroscopy. Graph showing the changing of anisotropy of fluorescence label 2-((5(6)-tetramethylrhodamine)carboxyamino)ethyl methanethiosulfonate (TMR)-labelled PDZ domain with the increasing concentration of compounds 3289-8625, 6569188 and 7359885. (y axis= the fluorescence anisotropy; x axis= compounds concentrations)

4.1.4 Aim of study

The discovery of 3289-8625 as a potential inhibitor of the PDZ domain of Dvl opens opportunities to further manipulate structure activity relationship studies towards the development of more potent inhibitors of this series. Therefore, this study is aimed to synthesis analogues of 3289-8625, especially the one which will combine both compounds 6569188 and 7359885, and any possible analogues which arise during the synthesis, which is anticipated to have better affinity towards the Dvl-PDZ domain and to provide useful templates for further chemical optimisation. This work was carried out in collaboration with Prof. Jie J. Zheng (St. Judes Children's Research Hospital, Memphis), an expert in Wnt signalling who provided the

background work and the inspiration for the present studies through discussions with our group.

4.2 Synthesis of 3289-8625 analogues: first attempt.

4.2.1 Synthesis of the core benzoic acid derivative containing 5-amino-2-chloro-4-methyl substitution and possible analogues

The most crucial step in the synthesis of the target derivatives was the synthesis of the middle core benzene ring structure of the target compound, which consists of substitution with 5-amino, 2-chlorine and 4-methyl groups (compound **51** and **52**). The syntheses of the key compounds were achieved following three main steps as outlined in Figure 4.10 below:

i) Esterification of the benzoic acid using methanol
ii) reduction of methyl 2-methyl-5-nitrobenzoate to 5-amino-2-methyl-benzoic acid methyl ester
iii) chlorination of the benzoate with *N*-chlorosuccinimide (NCS)

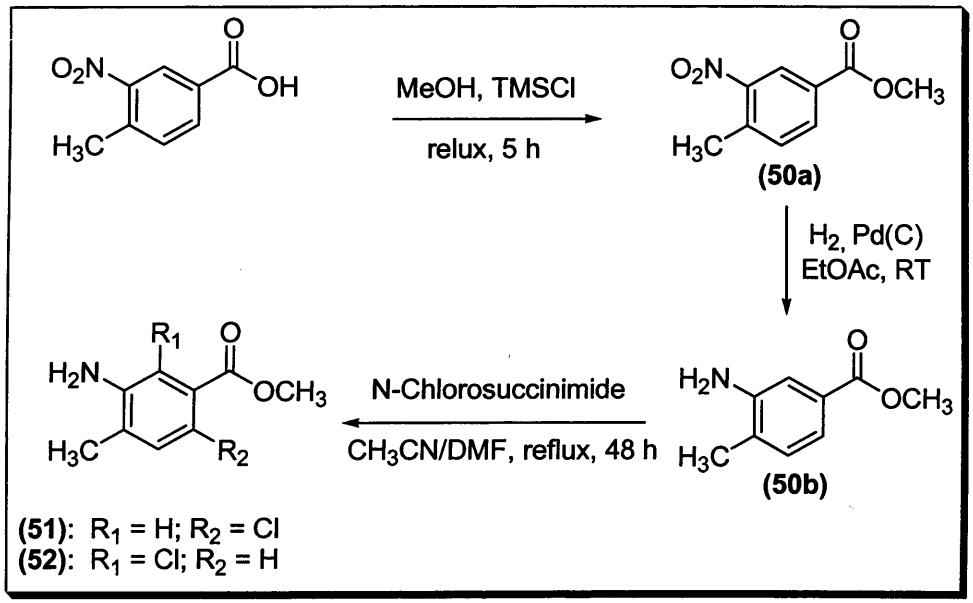


Figure 4.10: Synthesis of methyl 3-amino-4-methylbenzoate chlorinated analogues (compound **51** and **52**).

To start the synthesis, commercially available 4-methyl-3-nitro-benzoic acid was chosen as the starting material on a small scale (1g). The

aim was to synthesise the correct key compound first, which later can be expanded to synthesise of the desired compound on a larger scale.

The first step involved the esterification of 4-methyl-3-nitro-benzoic acid to methyl 4-methyl-3-nitrobenzoate (**50a**) in order to protect the hydroxyl group of the benzoic acid, using chlorotrimethylsilane (TMSCl) in methanol at reflux for 5 hours. After the workup, crude brown solid was formed. Purification of the product was achieved by using acid-base extraction to remove traces of unreacted benzoic acid gave the pure compound in good yield (71%). The appearance of a singlet on ^{1}H-NMR spectra at δ 3.98 ppm provides evidence for the formation of the methyl ester.

The next step involved the reduction of (**50a**) to 3-amino-4-methyl-benzoic acid methyl ester (**50b**) under hydrogen (atmospheric pressure) in the presence of palladium on carbon [Pd(C)] in ethyl acetate at RT. The reaction was carried out for 24 hours. After the workup, the purification of the crude mixture using column chromatography gave the pure compound **50b** in good yield (86%). The reaction using this method proved to give a clean reaction indicated by TLC with only a slight trace of unreacted starting materials detected. The target compound is evidenced by the appearance of a broad singlet at around δ 4.2 ppm for the amino group, and most importantly, by an up-field shift of all the aromatic protons (from δ 7.45-7.63 ppm of the nitro-precursor to δ 7.13-7.43 ppm of the amino product).

The third step that was the most important step involved the chlorination of the benzoate **50b** with *N*-chlorosuccinimide (NCS), which is a more convenient reagent for aromatic chlorination compared to chlorine or sulfuryl chloride. The first attempt involved the reaction of the benzoate **50b** with 1.5 equivalents of NCS in *N,N*-dimethylformamide (DMF) following the method described by Altenbach et al. (2004). After heating at reflux for up to 60 hours and following standard work-up of extraction with ethyl acetate and washing with brine, further purification using column chromatography only gave starting material as the main compound with only slight traces of compound **51** (methyl 5-amino-2-chloro-4-methyl benzoate) which cannot be

easily separated from the starting material, while compound **52** (methyl 3-amino-2-chloro-4-methyl benzoate) which was eluted first was afforded in 42% yield. This attempt highlights the failure of this method, where the desired compound **51** could not be isolated in pure form.

A further attempt was sought, this time by using the method described by Davis (2009) that uses acetonitrile as the solvent of choice. Compound **50b** was heated in acetonitrile at reflux using 1.05 equivalents of NCS for 48 hours, giving crude compound which needed further purification using column chromatography (hexane: ethyl acetate = 7:1). Compound **52** was eluted first in 43% yield, followed by mixtures of mostly compound **52** and a slight traces of starting material **50b** in around 32% yield. This was closely followed by the elution of unreacted starting material **50b** in 4% yield, before the elution of the last desired compound **51** in 20% yield. These entire compounds were confirmed by looking at ^{1}H-NMR. For compound **51**, the appearances of 2 distinct singlets at the aromatic region were evidence of the structure of the desired compound. Compound **52** showed two doublets with the coupling constant value (*J* value = 8.0 Hz) which clearly indicate the position of the protons which are *ortho* to each other.

The results clearly showed that the most favourable position for chlorination was the *ortho* position to amine and ester groups giving compound **52** in moderate yield. Nevertheless, the desired compound **51** was successfully obtained in low yield. At this stage, the chlorination method of **50b** using 1.05 equivalents NCS in acetonitrile was chosen as a suitable method for this reaction, giving compound **51** and **52** in low to moderate yield which will be used for further coupling reactions. The results of the chlorination procedure is summarised in Table 5.4.

Table 5.4: Summary of chlorination procedure of compound **50b** using NCS.

Reaction condition	Compounds afforded	Yield (%)
NCS (1.5 equivalent), DMF RT, 60 h	**52**	42
	Mostly starting material mixed with	56

	minor traces of **51**	
NCS (1.05 equivalent), MeCN, reflux, 48 h	**52**	43
	Mixtures of mostly compound (**52**) with minor traces of **50b**	32
	Unreacted starting material **(50b)**	4
	51	20

The next part of the synthesis was dedicated to the synthesis of desired compound **54** and its analogues **55** using the previously afforded compounds **51** and **52**. The main work involved the formation of amide bond between different benzene and aniline building blocks. The overall strategy for the synthesis of compounds **54** and **55** is outlined in Figure 4.11 below:

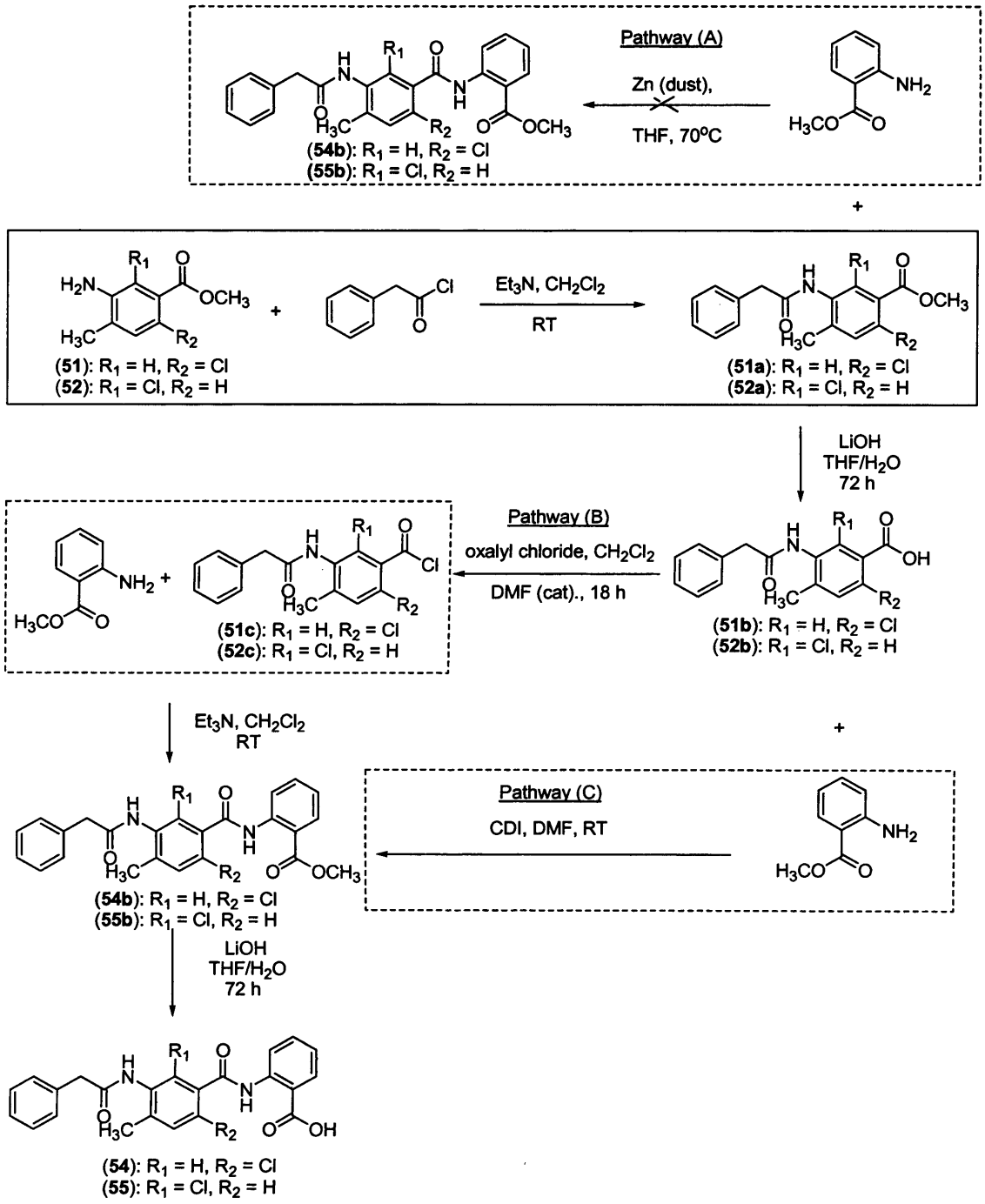


Figure 4.11: Strategy to synthesise compound **54** and **55**.

4.2.2 Synthesis of compound 51a (methyl 2-chloro-4-methyl-5-[2-phenylacetamido]benzoate and its analogues 52a via amide bond formation

The next step involved amide bond formation between the amino group of the benzoate and phenyl acetyl chloride as the benzene counterpart. The reaction is quite straightforward due to readily activated nature of the acyl chloride group on phenyl acetyl chloride which can be readily reacted with the amino group to form amide bond. Compounds **51** and **52** were subjected to reaction in triethylamine as the base and dichloromethane as the solvent at room temperature for 18 hours. Purification of compound **51b** was quite straightforward, as recrystallisation of the crude solid (in methanol/water) afforded the pure compound in 50% yield. Compound **52b** needed to be purified by column chromatography and was difficult to separate, as many spots of impurities/side products were seen under TLC. The crude compound needed further purification using column chromatography (dichloromethane: methanol). Further recrystallisation of the product in hexane:ethyl acetate afforded the pure compound **52b** in 33% yield. The overall reaction procedure manages to give compound **51b** and **52b** in moderate yield. This may be due to the base used (triethylamine) which could have unselective attack at the benzoate **51** and **52**, or the reaction of compound **51** and **52** with themselves which could raise the formation of side products.

4.2.3 Attempts to synthesise of compound 54 [2-(2-chloro-4-methyl-5-(2-phenylacetamido)benzamido)benzoic acid] and its analogues (55)

The next step involved the formation of another amide bond between the methyl ester (**51b** and **52b**) and methyl anthranilate. The reaction between the 'inactive' methyl ester with aniline was tested first before any attempt to 'activate' the methyl ester. There was only very few methods that reported the formation of amide bond via these pathway, among them are the use of zinc dust (Arora et al. 2005) and the formation of bislithium amide (Ooi et al. 1999). But as the reactivity of the bislithium amide is unknown, the method that involved the formation of amide bond between aniline and 'inactive' methyl ester in the presence of zinc (dust) at 70 °C was chosen as it was shown to be convenient and had great synthetic utility (Pathway A) (Figure

4.12). Unfortunately, even after 48 hours of heating, only starting material can be detected in the reaction mixture without any sign of product formation. At this stage, the method failed to give any product formation, which gave no option rather than to proceed with the activation of methyl ester.

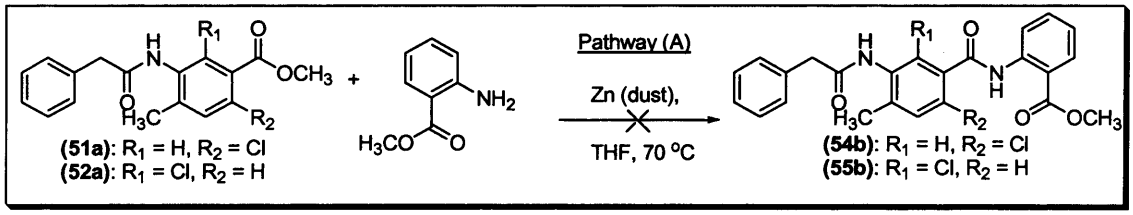


Figure 4.12: Attempts to synthesise **54b** and **55b** via pathway A.

A more straightforward approach was to hydrolyse the methyl ester group to carboxylic acid. A few approaches were tested using mild alkali/bases; (a) lithium hydroxide in methanol:water, (b) lithium hydroxide in THF:water and (c) sodium hydroxide in methanol:water but all showed only traces of more polar product formation even after 24 hours of stirring at RT. But by prolonging the reaction procedure of method (b) for up to 72 hours at RT finally showed the formation of the desired compound by TLC (Figure 4.13). This may be due to the methyl esters only dissolving in organic phase but not/partially dissolved in aqueous phase and that slowing down the formation of the benzoic acid. Upon completion of the reaction, the reaction was evaporated to remove methanol, followed by extraction of the aqueous layer with dichloromethane to remove possible unreacted methyl ester. Further acidification of the aqueous layer with 1N hydrochloric acid and extraction with dichloromethane, afforded the pure hydrolysed product in good yield (82% and 78% for compound **51b** and **52b** respectively). The formation of compound **51b** and **52b** was confirmed by ^{1}H-NMR, where the disappearance of methoxy peak and the rise of a single broad hydroxyl peak at around δ13.0 to 13.5 ppm are indicative of the correct compounds.

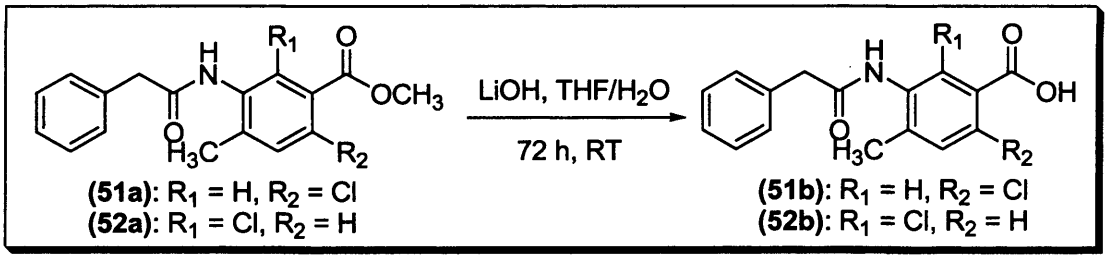


Figure 4.13: Hydrolysis procedure of compounds **51a** and **52a**.

The next step involved the formation of amide bond between compound **51b** and **52b** with methyl anthranilate (methyl 2-aminobenzoate). There are a number of strategies have been reported for amide bond formation, as extensively reviewed by Montalbetti and Falque (2005). The most popular and easiest one is the formation of acyl halides to activate the acid, or the use of coupling reagents such as carbonyl diimidazole (CDI) and carbodiimide. But since the aniline (methyl anthranilate) that is going to be used in the next step was predicted to be less reactive due to the presence of a methyl ester substitution at the *ortho* position of the aniline, the conversion of the benzoic acid to acyl chloride was chosen as the preferred method for coupling to amine. Methyl anthranilate was considered a low reactivity aniline due to the lone pair of the amino group which can be delocalized in the benzene ring, and most importantly, due to the existence of the electron withdrawing group at the ortho position of the amine, which can direct the lone pair of amine group towards itself, thus will dramatically reduce the nucleophilicity of the methyl anthranilate to efficiently react.

Due to restriction of the available amounts of compound **51b** (around 0.18 mmol/0.05 g) and **52b** (around 0.19 mmol/0.06 g), the next approach of amide bond formation via acyl chloride intermediate formation was tested first on compound **51b** (Pathway B) (Figure 4.14). The chlorinating agent chosen for this particular reaction is oxalyl chloride, where anhydrous tetrahydrofuran (THF) was used as the solvent under inert atmosphere together with a catalytic amount of DMF to accelerate the reaction. Thionyl chloride was not chosen for this reaction due to the need for heating which might be incompatible to other functionalities in **51b**, especially the previously formed amide bond. After 18 hours, the TLC of the reaction

indicated the formation of various spots, with possibly no detectable **51b** trace, thus indicated that the reaction might have gone to completion with the formation of side products. Nevertheless, the reaction was stopped and the solvent evaporated. The reaction mixture was used straight away in the next step, which involves the formation of amide bond. This time, potassium carbonate was chosen as base in anhydrous THF at RT. After 18 hours of stirring, the TLC again showed formation of various spots. As at this stage it was unknown whether the correct acyl chloride had been synthesised previously due to the uncertain ^{1}H-NMR of the acyl chloride, and the formation of the side products which might be due to the degradation of the starting material was already predicted. The use of a small quantity of starting material (0.18 mmol) for a reaction which might not run to completion and would lead to potential side products was also seen as a big challenge. An attempt to separate the pure compound in hexane:ethyl acetate was unsuccessful, as the characterisation of the afforded fractions were very hard to make and none of them indicated the possibility of the formation of compound **54b**. At this stage, the conclusion whether the method is applicable to synthesis of compound **54b** cannot be made, and the question whether the right acyl chloride intermediate have been synthesised also need to be considered thus highlighting the need to find better conditions to synthesise the acyl chloride intermediates.

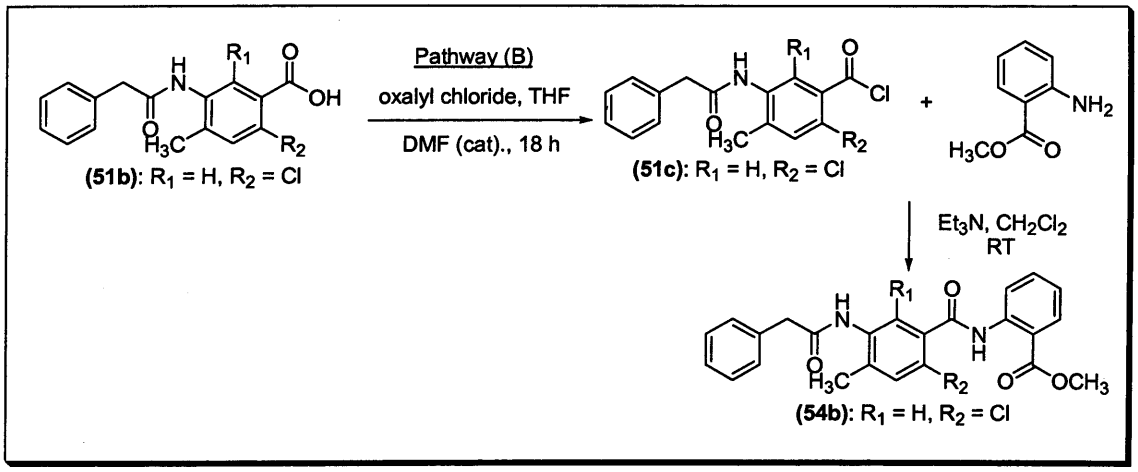


Figure 4.14: The formation of **54b** from **51b** via **51c** (Pathway B).

Therefore, due to the uncertainty of the methods in Pathways A and B, another method was sought which used carbonyl diimidazole (CDI) as a coupling reagent for compound **52b** with methyl anthranilate (Pathway C) (Figure 4.15). CDI was chosen as it allowed one-pot amide formation; without requiring the activation of any base. The reaction was done by simply adding CDI to compound **52b** in anhydrous DMF. The reaction was stirred for at least 1 hour, to allow the formation of acylimidazole intermediate, before the addition of amine. As expected, reaction with methyl anthranilate was quite slow due to the inactive nature of the aniline itself, and even at the termination of reaction at 96 hours, the starting material can still be detected in the TLC. The crude reaction mixture was further purified using column chromatography, and the target compound was obtained and confirmed by ^{1}H-NMR in less than 3 mg and in poor purity. At this stage, amide bond formation via Pathway C with methyl anthranilate was seen as less successful due to formation of compound **55b** in a very low yield (8% of impure compound) and poor purity, thus requiring further purification. At this stage, Pathway C was seen as a less attractive method for amide bond formation for this particular compound. This highlights the need to find a better method which can give a higher yield and better purity. Unfortunately, no starting material (compound **51b** and **52b**) was left to further test other method.

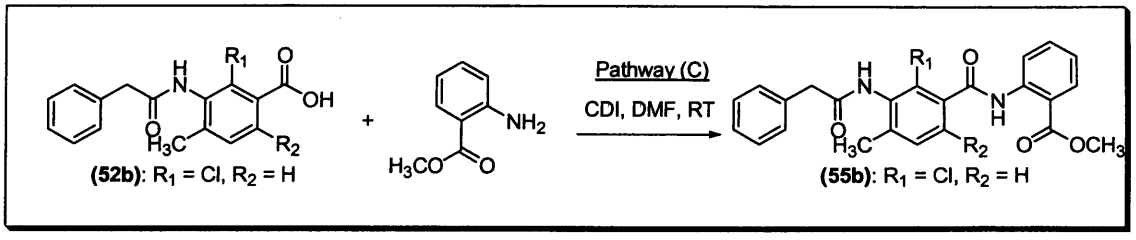


Figure 4.15: Amide bond formation via pathway C.

As a conclusion, the synthesis of all target compounds at this stage failed. It was anticipated that a larger quantity of starting materials and some modification of the synthesis strategy would improve the overall reaction procedure and accessibility of the final compounds.

4.3 Synthesis of 3289-8625 analogues: Second attempt.

The first attempt towards the synthesis of analogues 3289-8625 highlighted a few problems. The most obvious one is the problem with the reactivity of methyl anthranilate which requires good activating intermediates at the carboxylic acid counterpart to force the reaction to completion in a reasonable yield and good purity. At this stage, the formation of acyl chlorides intermediates was still seen as the best approach provided that the right acyl chloride is obtained. In view of this, the second attempt to synthesise these analogues was dedicated towards another pathway which involves the formation of the core benzene ring primarily attached with acyl chloride before being coupled with methyl anthranilate. This approach will provide greater possibility to synthesise the correct acyl chloride to ensure a more efficient method for amide bond formation with unreactive methyl anthranilate. The acyl chloride is also expected to be inert and stable enough to give a similar result as the methyl benzoate in the chlorination step using NCS. The synthesised amide bond between methyl anthranilate and the benzoyl chloride should also withstand the reduction reaction of nitro to amine using hydrogen gas and palladium on carbon as showed by Shen et al. (2007). The advantage of this approach would be to reduce the number of steps required to synthesise the final compound. The proposed reaction strategy is outlined in Figure 4.16 below:

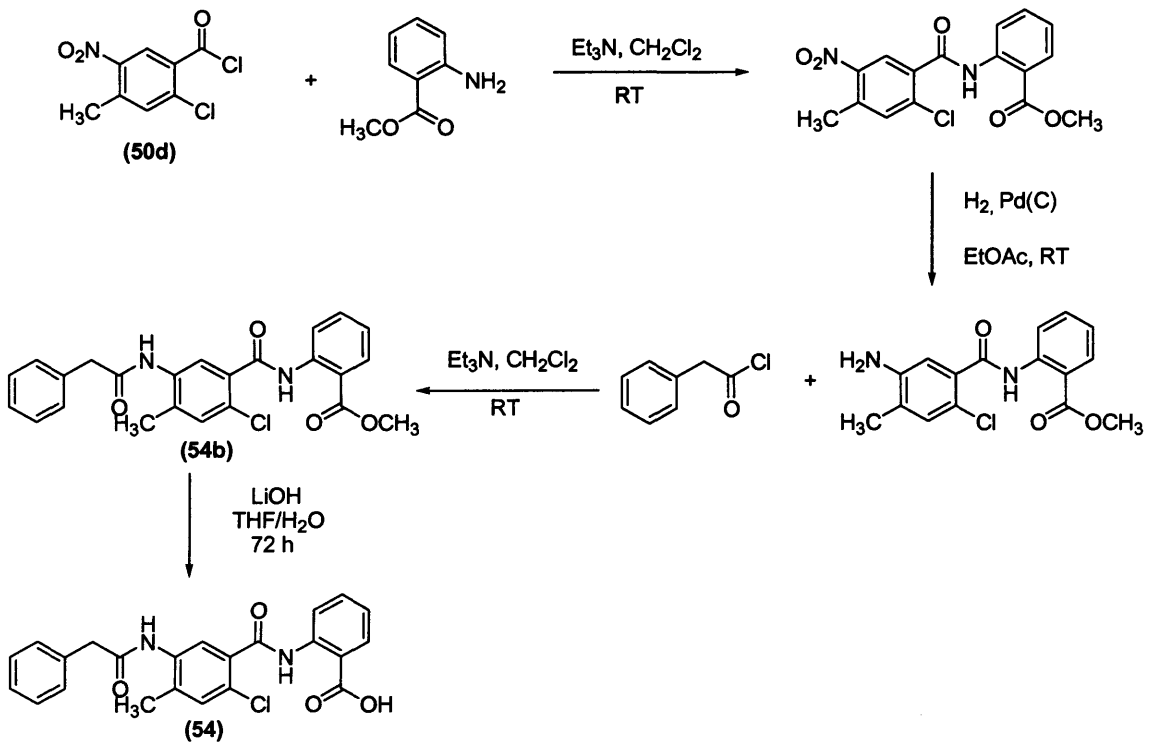


Figure 4.16: Proposed reaction procedure for second attempt

In order to achieve this, the starting material used (4-methyl-3-nitro-benzoic acid) would need to undergo acyl chloride formation first, using the same method to synthesis compound **51c** using oxalyl chloride in dichloromethane with the addition of catalytic DMF (Figure 4.17) to produce compound **50c** (4-methyl-3-nitro-benzoyl chloride. Compound **50c** was obtained in good yield (93%).

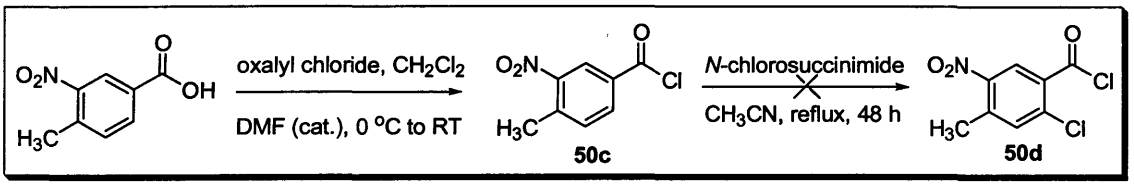


Figure 4.17: Attempt to synthesise compound **50d** via compound **50c**.

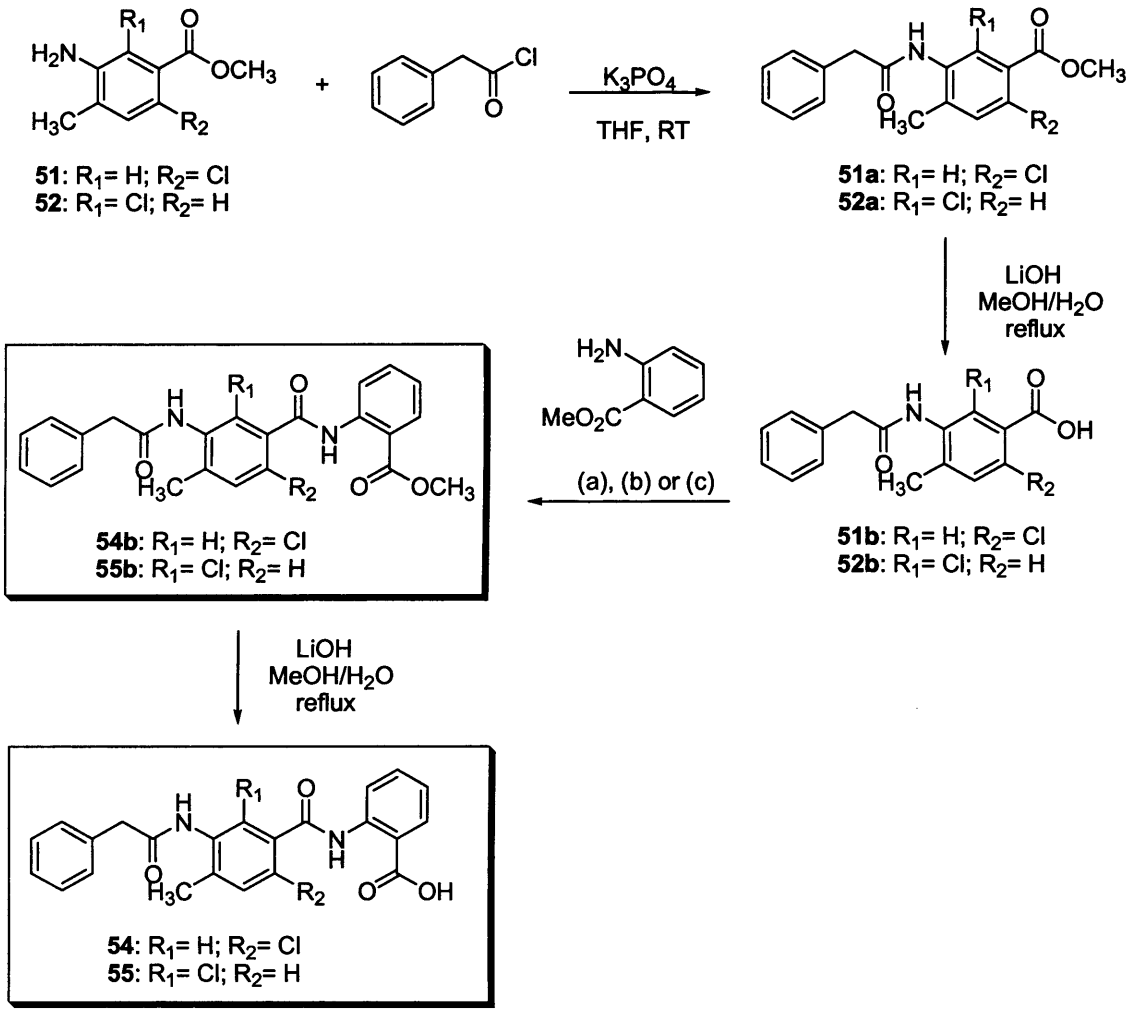
Compound **50c** was straight away subjected to the chlorination reaction using NCS in refluxing acetonitrile as previously mentioned for compound **51** and **52**. Unfortunately, after 48 hours of heating, no product formation can be detected by TLC. This indicated that no chlorination

reaction has occurred, where the benzoyl chloride might simply not react under these reaction conditions or might have undergone degradation due to its instability in the reaction condition. Increasing the load of the NCS used (from 1.05 to 2 equivalents) and prolonging the reaction time to 70 hours did not improve the reaction outcome.

This finding indicating the failure of the proposed method to give the desired compound thus highlights the need to find a better method to synthesise 3289-8625 analogues. This finding also suggested that the protection of benzoic acid starting material by esterification is still the best option which will allow further manipulation of reaction condition.

4.4 Synthesis of 3289-8625 analogues: Third attempt.

The third attempt of the synthesis of this analogues follow exactly the previous route described to synthesise compound **51** and **52** (in first attempt), with a few modifications made for the following amide bond reaction condition to improve the reaction outcomes. Compound **51** and **52** were synthesised first and afforded in a similar yield to previous attempts. This shows that the chlorination reaction is reproducible for this kind of compound. The overall proposed reaction conditions for the third attempt are outlined in Figure 4.18.



(a) (i) $(COCl)_2$, THF, DMF (cat.), RT; or (ii) $SOCl_2$, toluene, DMF (cat.), RT; or (iii) $SOCl_2$, reflux; or (iv) $(COCl)_2$, 1,2-dichloroethane, DMF (cat.), RT; then K_3PO_4, THF, RT
(b) PPh_3, DDQ, CH_2Cl_2, RT
(c) CH_3SO_2Cl, Et_3N, THF, RT

Figure 4.18: Proposed reaction condition for third attempt.

After the successful reaction to synthesise compounds **51** and **52**, both isomers were subjected to amide bond formation using a method described by Zhang et al. (2009), utilizing potassium phosphate (1.25 equivalents) as base in anhydrous THF under inert atmosphere. The method was described as simple, mild and highly efficient giving the desired compound in good to excellent yield. Potassium phosphate as a cheap and simple inorganic base is superior to other commonly used base, such as triethylamine and sodium hydroxide as it greatly minimizes unwanted side

reactions such as hydrolysis and racemisation which usually occur during amide formation with acyl chlorides. Both amide bond formations using compound **51** and **52** were completed within 18 hours of stirring at room temperature. This method proved to be mild, high yielding and showed only slight traces of impurities by TLC, which allowed the crude product **51a** and **52a** to be used straight away in the next hydrolysis reaction without the need for further purification.

The previous method described for hydrolysis of the methyl benzoate **51a** and **52a** involves the reaction using lithium hydroxide in methanol/water for 70 hours at room temperature. Nevertheless, some modification was made to the previously described method where this time heating at reflux is applied, where lithium hydroxide was still used as a mild base/alkali in methanol/water solution. Delightfully, the desired carboxylic acid was detected within 3 hours by TLC. It showed that the heat helped the dissolution of the methyl esters in the solvent system which helped to accelerate the overall reaction. Upon completion of the reaction, the solvent was evaporated to remove methanol, followed by extraction of the aqueous layer with dichloromethane to remove possible unreacted methyl ester. Further acidification of the aqueous layer with 1N hydrochloric acid and extraction with dichloromethane, the pure hydrolyse product was afforded in good yield (71% and 69% for compound **51b** and **52b** respectively). So far, the modifications applied to improve the reaction outcome of amide bond formation and hydrolysis were successful.

The last and the most crucial part of the third attempt is the formation of another amide bond between the methyl anthranilate and compound **51b** and **52b**. For the first approach, formation of acyl chloride intermediate was sought (Figure 4.19). For this purpose, four different reaction conditions were tested:

i) Oxalyl chloride in anhydrous THF with catalytic DMF at room temperature

ii) Thionyl chloride in anhydrous toluene with catalytic DMF at room temperature.

iii) Thionyl chloride at reflux

iv) Oxalyl chlorides in anhydrous 1,2-dichloroethane with catalytic DMF at room temperature

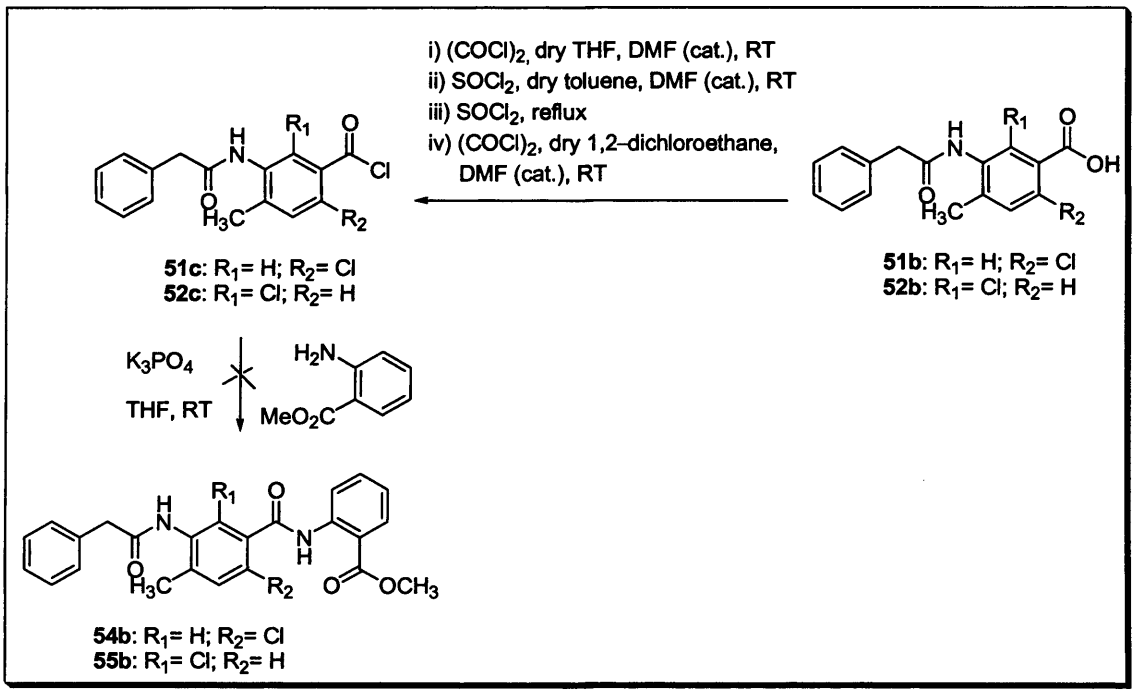


Figure 4.19: Attempts to synthesise compounds **54b** and **55b** via acyl chloride intermediate.

Although theoretically, all the methods should be able to generate the same acyl chlorides, the findings showed that the method (i) and (iii) failed to give the desired intermediates by ^{1}H-NMR determination and TLC. These may indicated that THF is a less effective solvent for this reaction and heating causes deleterious effect to the previously synthesized compound **51b** and **52b**. Where else, the use of toluene or 1,2-dichloroethane as solvents either with thionyl chlorides or oxalyl chlorides at room temperature gave better formation of acyl chlorides **51c** and **52c** with define and clearly interpreted ^{1}H-NMR spectra, as proved by the disappearance of the hydroxyl group of **51b** and **52b**. Compound **51c** and **52c** were used straight away in the next amide bond formation with potassium phosphate and THF at room temperature. Unfortunately, even after prolonged stirring for up to 50 hours no reaction had occurred. This indicated that the reaction had failed to give

any product, and formation of acyl chlorides intermediate was not sufficient to activate the methyl anthranilate for successful reaction.

Due to the failure of the acyl chlorides approaches to allow the amide bond formation with methyl anthranilate, another method was sought using triphenyl phosphine (PPh_3) and 2,3-dichloro-5,6-dicyanobenzoquinone (DDQ) which act as an excellent dehydration system due to their ability to eliminate water from the reacting molecule, in dichloromethane at room temperature, as described by Iranpoor et al. (2009) (Figure 4.20). The use of DDQ is preferred due to its high reactivity and good selectivity towards desired product. The method was claimed to be very rapid, neutral and mild without the need for any base, giving the amide bond in excellent yield. The method is said to be applicable to use in aromatic amine and aromatic or non-aromatic carboxylic acid. Nevertheless, when compound **52b** was subjected to reaction using this method, no product formation can be detected even after 48 hours of stirring at room temperature. This may be due to the less reactive nature of the methyl anthranilate which was very difficult to activate using this particular method.

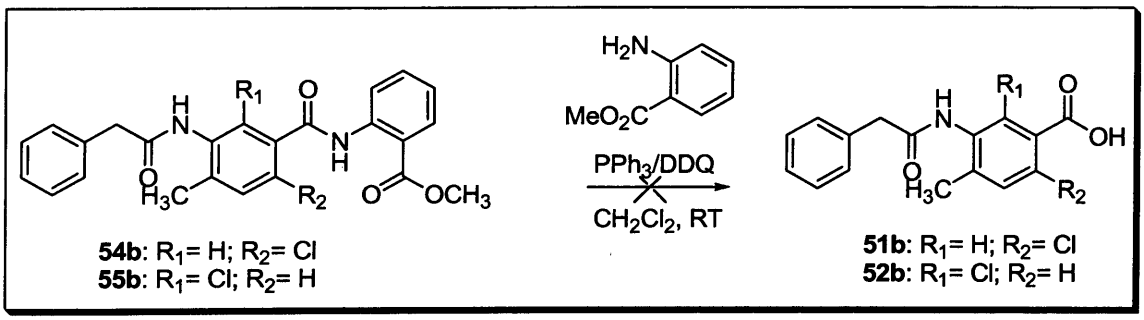


Figure 4.20: Attempt to synthesise compounds **54b** and **55b** via activation using PPh_3 and DDQ.

Following the failure of attempted methods either using the acyl chloride intermediate or using PPh_3 and DDQ to make the second amide bond formation, another method was sought, this time using a procedure described by Shen et al. (2007), which used 3 equivalents of

methanesulfonyl chloride (MsCl) as a reagent in the presence of triethylamine (3 equivalents) as base in anhydrous THF (Figure 4.21). Theoretically, the mesylate intermediate is commonly used to activate an alcohol instead of carboxylic acid. The proposed reaction mechanism for this particular reaction is by the production of mesylate intermediate which can act as a good leaving group for further coupling reaction to occur (Figure 4.22).

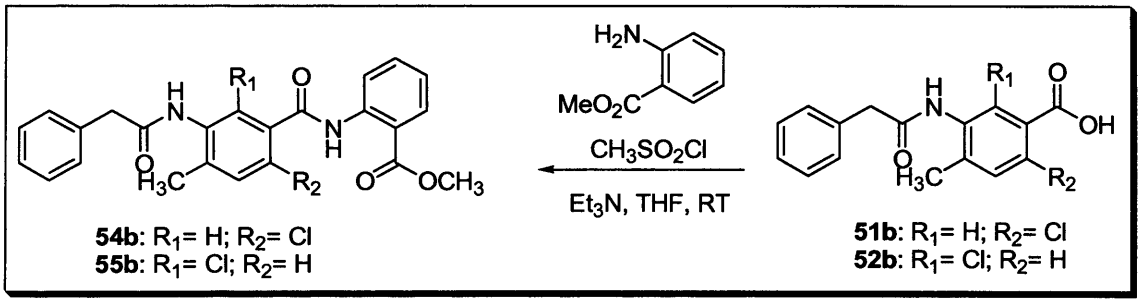


Figure 4.21: Synthesis of compounds **54b** and 55b using **MsCl**.

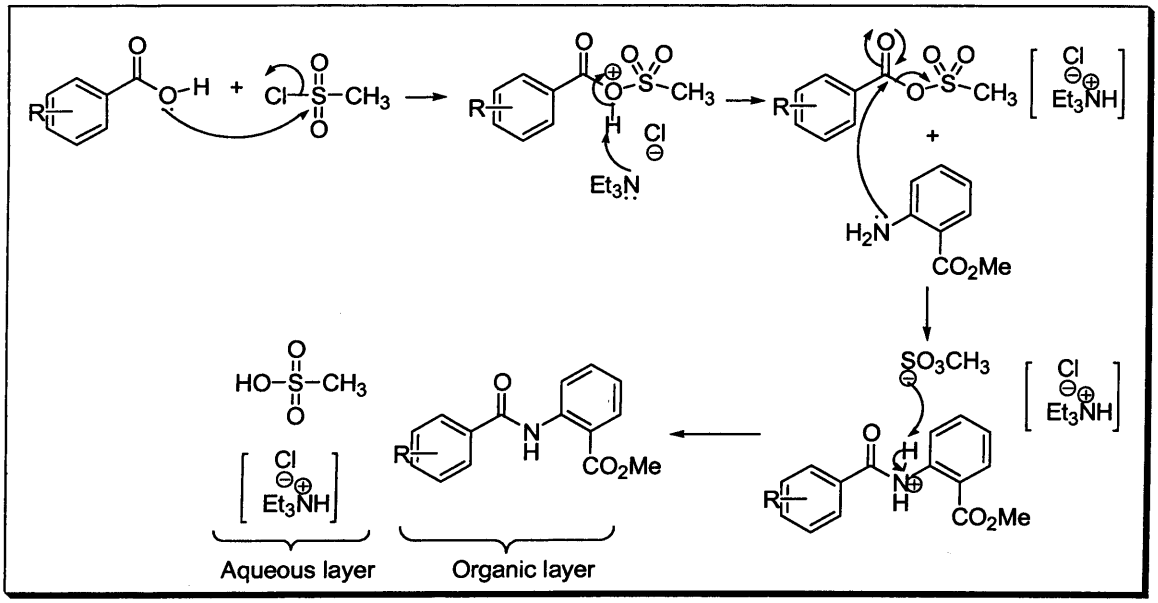


Figure 4.22: The proposed reaction mechanism for the formation of amide bond via the formation of mesylate intermediate from carboxylic acid.

Surprisingly, after 20 hours of stirring at room temperature for reactions containing compound **51b** and **52b**, TLC analysis showed the formation of an additional spot which might indicate the formation of the

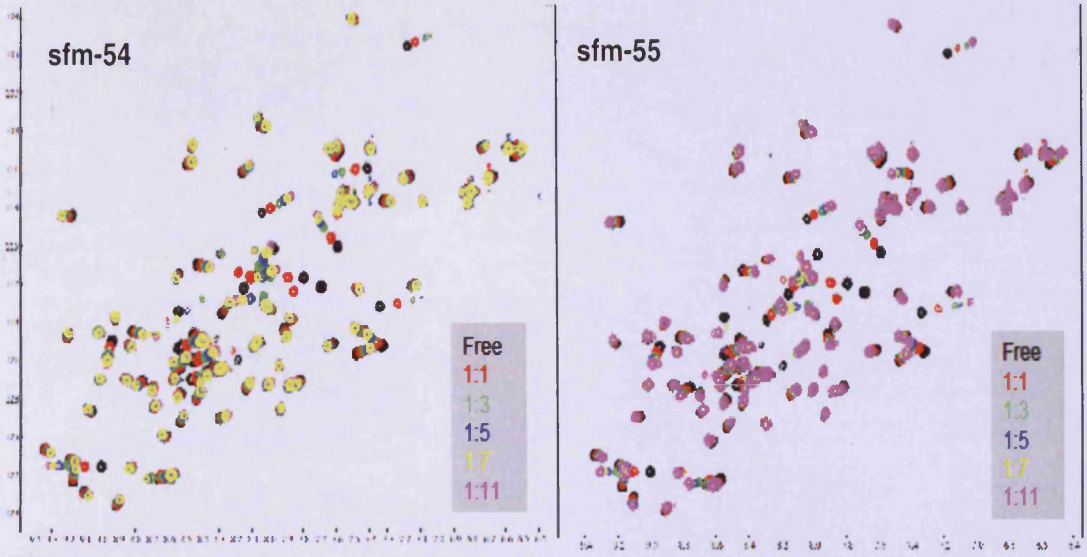
desired products, together with appreciable spots of starting materials which still can be detected. Prolonged stirring of the reaction mixtures for up to 72 hours did not improve the outcomes. Nevertheless, the reactions were stopped and upon aqueous work-up, the crude products still needed to undergo purification procedure using column chromatography in dichloromethane: methanol (98:2) to afford the desired products in good identical yield and purity (73% for both compounds **54b** and **55b** respectively). The findings indicated that the formation of mesylate intermediates were able to activate the less reactive methyl anthranilate which can drive the amide bond formation reaction to completion and in good yield.

The last step involved the hydrolysis reaction of compound **54b** and **55b** to afford the carboxylic acid derivatives of final compound **54** and **55**. As described earlier for the synthesis of compound **51b** and **52b**, both the methyl ester **54b** and **55b** were reacted with lithium hydroxide in refluxing methanol/water for 3 hours. Upon the work-up, the final compounds were afforded in good yields (72% for compound **54** and 74% for compound **55**). The afforded compounds were further evaluated for their binding affinity with the PDZ Dvl binding affinity using the NMR perturbation method.

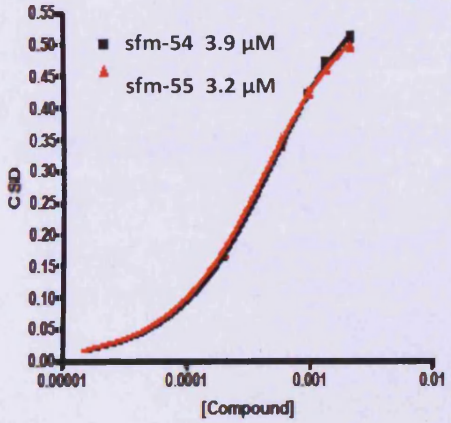
4.5 Characterization of binding and inhibitory ability of compounds 54b, 55b, 54 and 55 towards Dvl PDZ domain

The next part of the study was dedicated to test the ability of compounds **54** and **55** and their methyl ester counterparts (compound **54b** and **55b**) for their ability to bind to the PDZ domain of Dvl. The study was carried out by the group of Prof. Jie J. Zheng, St. Jude Children Research Hospital, Memphis, USA, as part of an ongoing research collaboration. The first part of the test was dedicated to evaluate the binding affinity of the compounds towards the Dvl PDZ domain by using NMR spectroscopy via a chemical shift perturbation experiment. The results of the experimants are shown in Figure 4.23. In order to do these, ^{15}N-labelled Dvl-PDZ domain (residues 247-341 of

mouse Dvl1) were used as ligand. Compound **54** and **55** were titrated individually in various concentrations into samples of the ligand and the



SFM-54 and SFM-55



Sfm-54b and sfm-55b is soluble in DMSO but cannot be dissolved into sample buffer

Figure 4.23: (Top) Interaction betweeen compound **54** and **55** with the Dvl PDZ domain. The data showed the ^{15}N-HSQC spectra of free PDZ domain (black)and PDZ domain with increasing concentrations of compound **54** and **55** (red, green, blue, yellow and pink, protein:ligands ratios of 1,3,5,7 and 11 equivalents); e.g. for red, PDZ domain with 1 equivalent of compound **54/55**. (Bottom) Graph showing the changing of fluorescence anisotropy of TMR-labelled Dvl PDZ domain with increasing concentration of compounds 54 and 55. (Y axis = the fluorescence anisotropy; X axis = compounds concentrations).

interactions were monitored by $^{1}H/^{15}N$ heteronuclear single-quantum coherence nuclear magnetic resonance ($^{1}H/^{15}N$-HSQC NMR). Unfortunately, compound **54b** and **55b** although soluble in DMSO, were not dissolved in the sample buffer which did not allow further testing. The binding of compound **54** and **55** to Dvl PDZ domain were verified using NMR spectroscopy. Their results were compared to a previous reported comprehensive study of compound 3289-8625 (Grandy et al. 2009). The chemical shift perturbation study of both compounds showed stronger binding affinity towards the Dvl PDZ domain compared to compound 3289-8625 or even with the naturally occurring membrane bound protein Dapper or Frizzled. This observation indicated the possibility of more potent Dvl PDZ inhibitory potential of compounds **54** and **55** to the parent compound 3289-8625.

To further confirmed the inhibitory potential of compound **54** and **55**, fluorescence anisotropy study to calculate the binding affinities of compound **54** and **55** to the Dvl PDZ domain were carried out. The study involved the titration of compound **54** and **55** into a solution of fluorescence label 2-((5(6)-tetramethylrhodamine)carboxyamino)ethyl methanethiosulfonate (TMR)-labelled PDZ domain. The determination of the binding affinity of the inhibitors **54** and **55** were done by measuring the anisotropy change due to ligand binding to the Dvl PDZ domain. The fluorescence based data showed that the measured binding affinity (K_d) of compound **54** was 3.9 μM while compound **55** exhibited an equivalent K_d value of 3.2 μM. The data generated from the study showed more than 3 folds better binding affinity results as compared to the parent compound 3289-8625 (K_d = 10.6±1.7 μM) and the naturally occurring Dvl PDZ inhibitor Dapper peptide (K_d = 11.6±1.4 μM) (Grandy et al. 2009).

The data generated from this study suggested that compound **54** and **55** might have better binding and inhibitory ability to Dvl PDZ domain compared to the parent compound 3289-8625 and Dapper peptide. The compounds can serve as a useful pharmacophore to study the structure activity relationship of this inhibitor series towards the Dvl PDZ domain. A

more detailed *in vivo* and *in vitro* study should also be carried out to further validate the anti-Wnt inhibitory activity of these particular compounds.

CHAPTER 5

TOWARDS THE SYNTHESIS OF *TRANS*-STILBENE ANALOGUES AS POTENTIAL PET RADIOTRACERS

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TOWARDS THE SYNTHESIS OF *TRANS*-STILBENE ANALOGUES AS POTENTIAL PET RADIOTRACERS

5.1 Introduction

5.1.1 Non-invasive techniques in Alzheimer's disease imaging.

Alzheimer's disease (AD) is a brain disorder associated with progressive memory loss and decrease of cognitive function (Zhang et al. 2005c). β-amyloid (Aβ) aggregation in brain is the prime suspect as the primary cause of AD. Histopathological studies done on post mortem analysis of AD sufferers revealed that extensive cortical amyloid-beta depositions were detected, although the exact role of amyloid plaques in the onset of dementia and AD is still controversial (Kung et al. 2010). To date, the only way to accurately diagnose AD is by microscopic detection of beta-amyloid plaques and neurofibrillary tangles at brain autopsy during *post mortem* (Pat et al. 2010, Wong et al. 2010). *In vivo* neuroimaging of Aβ plaque would be a non-invasive method for early diagnosis of AD and development of treatment strategies (Qu et al. 2007). Non-invasive techniques such as positron emission tomography (PET) and single photon emission computed tomography (SPECT) allow the detection of deposited Aβ and could enable the diagnosis of AD in its presymptomatic stages. Therefore, it is necessary to develop probes for PET and/or SPECT with a high affinity for Aβ plaques (Chen 2006) which may serve as suitable markers for monitoring the amyloid formation following the disease progression and for observing the outcomes of therapeutic intervention (Zhang et al. 2005c).

5.1.2 Development of ^{18}F-stilbenes as PET imaging agents for detecting Aβ plaques

During the last few years, there have been a number of stilbene derivatives that have been developed as potential tracers for detecting the amyloid

plaques (Zhang et al. 2005b; Zhang et al. 2005c; Zhang et al. 2007; Qu et al. 2007; Parhi et al. 2008; Hong et al. 2010). As reviewed by Kung et al. (2010), the rigid structure of stilbene, which has a highly conjugated aromatic system and is relatively planar, can bind specifically in between the β-sheets of the amyloid beta aggregates.

One stilbene-based radiotracer, ^{18}F-BAY94-9172, has shown good binding affinity to the Aβ plaques in patients and is able to discriminate between Alzheimer's disease and frontotemporal lobar degeneration in patients or healthy controls (Rowe et al. 2008). The agent had shown similar specificity and characteristics to the most specific and commonly used PET Aβ ligand, ^{11}C-PIB (Pittsburgh compound B; 2-[4'-(methylamino)phenyl]-6-hydrobenzothiazole) (Kung et al. 2010) (Figure 5.1). Both are considered as small planar molecules with extended aromatic rings and alkyl-amino substitution. ^{18}F-BAY94-9172, which is also known as Florbetaben and currently being developed by Bayer Healthcare Pharmaceutical Inc., have successfully completed phase II clinical trial with the ultimate goal of using PET imaging agent to correlate the Aβ-plaque burden in living human brain. An international Phase III clinical trial is currently underway (Pat et al. 2010). Another stilbene-based PET radiotracer, ^{18}F-AV-45 (Flobetapir F 18) (Figure 5.1) which is developed by Avid Radiopharmaceuticals, Inc. is currently in Phase III clinical trial with ultimate goal to image Aβ–plaque in living humans (Wong et al. 2010). The interim data showed that Flobetapir PET imaging results in patients nicely correlated with the levels of Aβ-plaque pathology later found in their brains at autopsy.

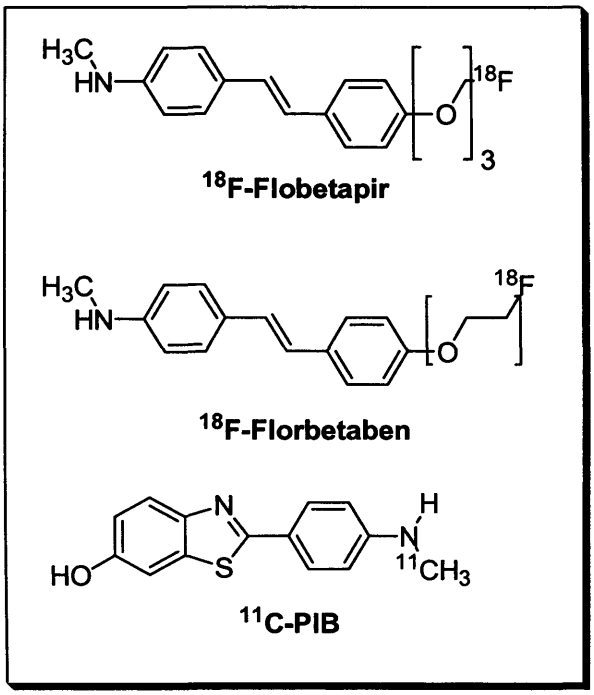


Figure 5.1: Chemical structures of ^{18}F-Flobetapir, ^{18}F-Florbetaben and ^{11}C-PIB.

5.1.3 Fluorine-18 as a facile source for the synthesis of PET imaging agents

There are a number of compounds that have been developed as PET and SPECT ligands targeting Aβ plaques, mainly using carbon-11 as their labelling precursor (Figure 5.2). Unfortunately, the 20 minutes radioactive decay half-life of ^{11}C represents major limitations on the use of ^{11}C-labelled compounds. The very short radioactive half-life needs an on-site cyclotron for the ^{11}C-labelled compound synthesis and therefore, reliable ^{11}C-radiochemists. This will restrict routine clinical use of the ^{11}C-labelled compounds and subsequently increase the cost of the studies (Rowe et al. 2008). Therefore, a tracer for imaging Aβ plaques that can be labeled with longer-lived isotopes is preferred. Table 5.1 showed the examples of various PET isotopes that have been developed for labeling molecules used for targeting the Aβ-plaques.

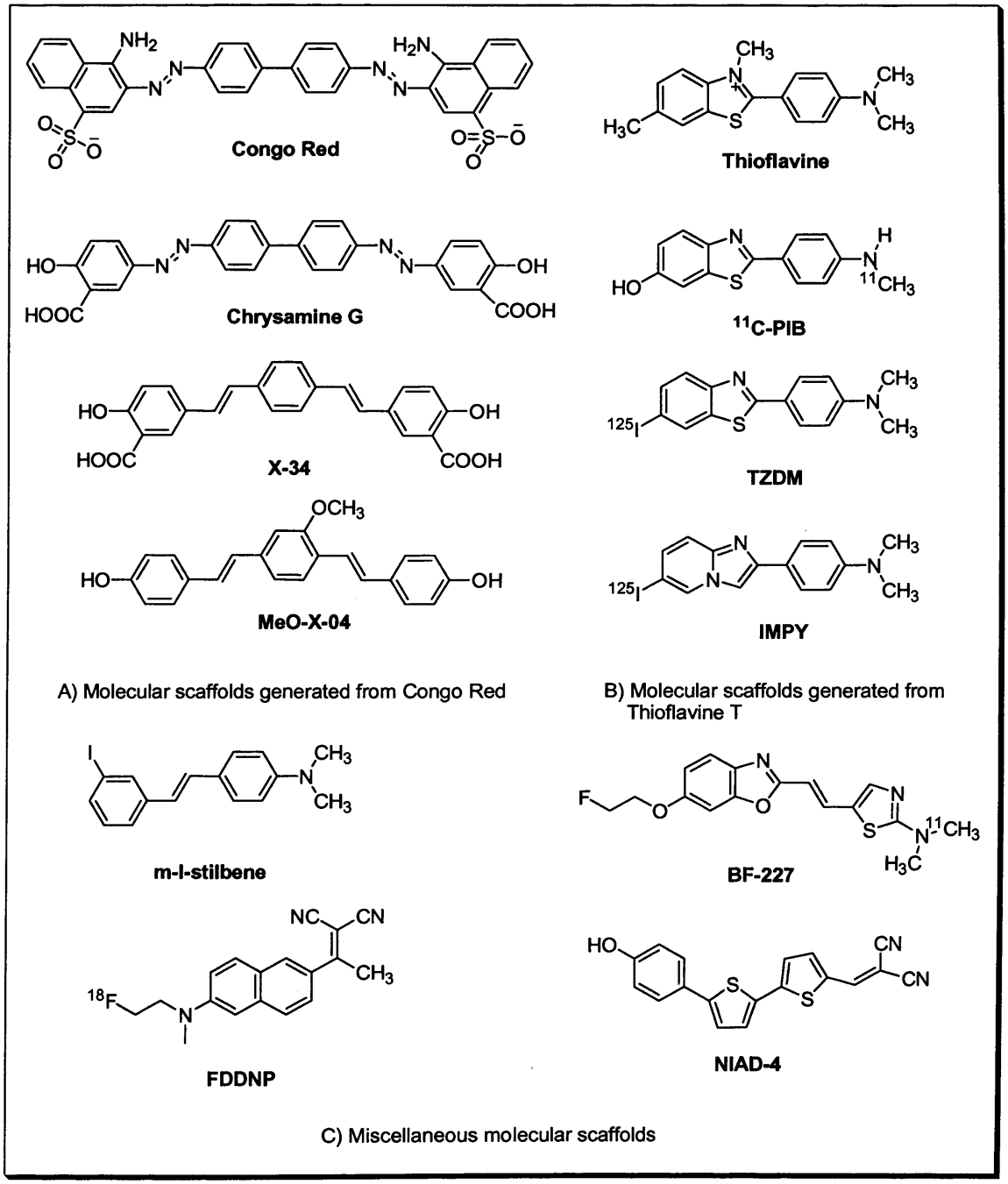


Figure 5.2: Examples of different amyloid imaging agents derived from various molecular scaffolds (taken from Nilsson 2009).

Table 5.1: PET isotopes for nuclear imaging.

Isotope	Half-life
^{124}I	4.17 days
^{86}Y	14.74 h

^{64}Cu	12.7 h
^{66}Ga	9.4 h
^{18}F	110 min
^{75}Br	96 min
^{68}Ga	68 min
^{11}C	20.39 min
^{13}N	10 min
^{15}O	2 min

Although metal-based positron isotopes such as ^{64}Cu, ^{66}Ga and ^{68}Ga have great potential in PET imaging, their incorporation into a labeled molecule can substantially change the pharmacological function (Daniels et al. 2010). Therefore, fluorine-18 has become the radioisotope of choice due to its various advantages compared to their metal-based counterparts. The 110 minutes half-life of ^{18}F allows centralized production and regional distribution of imaging agent for clinical use. Furthermore, the mode of decay of fluorine-18 is attractive for PET imaging (Zhang et al. 2007; Daniels et al. 2010). ^{18}F has a high percentage (96.7%) and relatively low energy (633 keV) of positron (β^+), resulting in PET images with best spatial resolution. Using ^{18}F can strike a balance between enough time to allow synthesis of the radiotracers and quality control measurements whilst limiting radioactive exposure for no longer than necessary to the patients. Other advantages of using ^{18}F are similar to the incorporation of fluorine into other pharmaceutical compounds. The fluorine atom is sterically similar to oxygen atom, and has a quite similar bond length to carbon, which makes the C-F bond likely to have similar binding affinities and ability to hydrogen bond compared to C-O bond (Daniels et al. 2010). Although the C-F bond is considered a strong bond, aliphatic C-F is often prone to enzymatic cleavage *in vivo.* The aryl C-F bond is generally stable *in vivo* but proved to be very challenging for its synthesis from ^{18}F-fluoride (Cai et al. 2008).

As described by Daniels et al. (2010), the traditional way of synthesizing ^{19}F-containing molecules often started with starting material that already contained fluorine atom that is then carried out through the reaction process to yield the final fluorinated compound. Unfortunately, this method is not suitable for the synthesis of ^{18}F molecules as the ^{18}F-introduction needs to be done in the final or penultimate stage of the synthesis in a fast and efficient manner to preserve the positron emitting property. Patient scanning needs to be carried out within 4-5 half-lives in order to generate meaningful data. Purification steps into injectable GMP standard also need to be done efficiently for patient administration. ^{18}F-fluoride is also a nucleophile with low reactivity that makes the ^{18}F incorporation into target molecules very challenging. All these factors from synthesis and purification highlight the challenges that are faced by the radiochemists in order to synthesise ^{18}F-containing compounds. At the same time, the incorporation of fluoride ion into a radiotracer/drug molecule is always challenging, "particularly when direct labeling of large, water soluble, polyfunctional and often temperature sensitive macromolecules" were considered (Ting et al. 2008a).

The importance of PET as a non-invasive imaging technique has gained much interest over other imaging technologies due to its sensitivity and selectivity. It is widely used as a drug development tool and in clinical research which has driven the need for increasing ^{18}F-labelled radiotracers. To date, ^{18}F-FDG (Figure 5.3) is the most commonly used PET-radiotracer in clinical settings as well as for oncology, cardiology and neurology diagnostic purposes (Cai et al. 2008; Peterson and Manning 2009). The principle of ^{18}F-FDG relies on its ability to detect cells that demonstrate an excess consumption of glucose, such as in the detection of hyper metabolic lesions or in the cells which are actively proliferating (Pery et al. 2010).

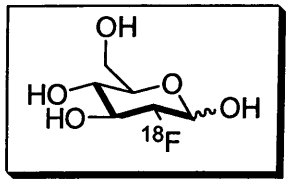


Figure 5.3: Structure of ^{18}F-FDG.

5.1.4 Aryltrifluoroborates in ^{18}F PET labeling

For decades, aryltrifluoroborates have received considerable attention in Suzuki-Miyaura cross-coupling reactions (Molander & Ham 2006). Recently, boronic acids derivatives, particularly aryltrifluoroborates have become an emerging important approach for ^{18}F-labeling mainly due to their "fluorophilicity for anionic [^{18}F]-fluoride to afford trifluoroborates with extraordinary chemoselectivity", which can be used as a captor of aqueous [^{18}F]-fluoride (Harwig et al. 2008). The aryltrifluoroborates are increasingly preferred due to their facile preparation in inexpensive aqueous potassium hydrogen fluoride (KHF_2) from their boronate ester or boronic acid equivalents, and they are stable to air and moisture. They can be readily isolated via precipitation from water and ether-miscible solvents (e.g. alcohols, acetone, acetonitrile). Many of these desirable characteristics have been attributed to the affirmed stability of the aryltrifluoroborates, which is highly dependent on the remarkable strength of the B-F bond (~140 kcal/mol) (Molander and Ham 2006; Ting et al. 2008a).

In order to be a reliable ^{18}F-radiolabel, the trifluoroborate needs to have an adequate stability and specific activity *in vivo*. A study carried out by Ting et al. (2008a) has provided useful insight on the advantages and potential of the trifluoroborates as a future labeling moiety. Their study validated their initial hypothesis that an aryl boronic acid/ester can be converted into an aryl trifluoroborate anion [ArB (^{18}F)(^{19}F)$_2$] in the presence of aqueous ($^{18/19}$ F)-fluoride, that showed the boronate ester intermediate can be an excellent aqueous ^{18}F-fluoride capture. This compound has sufficient specific activity that permits the first *in vivo* PET images of this compound, whilst demonstrating great stability and pharmacokinetic clearance in mice. Their study showed that the C-B bond and the B-F bond were stable *in vivo*, indicated by no detectable accumulation and leaching of free (^{18}F)-fluoride to the bone. In another toxicological and antinociceptive study by Oliveira et al. (2009), it has been shown for the first time that potassium thiophene 3-trifluoroborate administered orally only induced minimal toxicity and showed good antinociceptive effect in mice. Compared with commonly used

organoboron compounds, organotrifluoroborates have distinguishing features: greater nucleophilicity, ready accessibility, and remarkable stability in air and moisture (Fang et al. 2004; Molander et al. 2007a).

5.1.5 Incorporation of fluorine-18 into bioactive molecules

It is known the aromatic nucleophilic substitution (SnAr) is the most successful approach for introducing fluorine-18 at aryl carbon. However, the reaction required the aryl ring to possess a good leaving group and usually at least one electron withdrawing group at the *ortho* or *para* position (Cai et al. 2008). Although single step nucleophilic radiofluorination reaction with no carrier added (^{18}F)-fluoride is a preferred fluoridation reaction, the reaction is not always applicable, especially in the case of electron-rich aromatic compounds. Moreover, the typical procedure when utilizing (^{18}F)-fluoride ion as the labelling precursor needs harsh reaction condition, usually involving reactions in high temperatures and strongly basic conditions. These conditions are often not compatible with sensitive functional groups and may even lead to decomposition of sensitive compounds (Wuest 2007).

In order to overcome this problem, one of the ways used by the radiochemist that has becoming increasingly popular is via indirect labeling, that is to incorporate an alkyl group containing ^{18}F (^{18}F-fluoroalkyl reagent) that have been previously made, which act as a linker or prosthetic group into a drug or biomolecule at their existing sites such as hydroxyl (-OH), amine (-NH) or sulfhydryl (-C-SH or R-SH) groups of the parent compound to afford PET radiotracers. One of the most commonly used ^{18}F-fluoroalkyl reagent is the 2-[^{18}F]fluoroethyltosylate ([^{18}F]FETos) (e.g. Zuhayra et al. 2009; Lemaire et al. 2010). Nevertheless, this method requires the ^{18}F-fluoroalkyl reagent to be synthesised first before being incorporated into the molecule of choice which still requires more purification steps to obtain the final radiotracer. All these multiple steps can contribute to lowering of the overall radiochemical yield. Another better way is by using direct nucleophilic substitution, where the parent compound should undergo incorporation with

an alkyl group containing either sulfonates or halides leaving groups first before being subjected to fluoridation steps (e.g Wang et al. 2010).

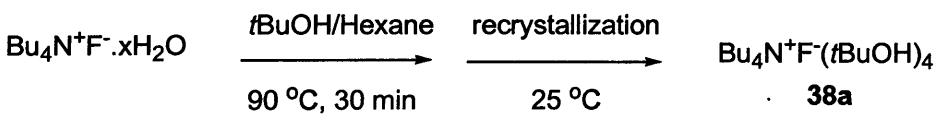
5.1.6 Aims of study

In light of the above discussion, direct nucleophilic substitution of an alkyl chain attached to the parent compound was chosen as the method of choice to synthesise cold stilbenes attached to a ^{19}F-linker using a hydroxylated stilbene as the parent compound, which later should be amenable to PET radiochemistry. Therefore, this study aimed to install an alkylfluoride chain (instead of a fluoride ion) and possibly a potassium alkyl trifluoroborate chain into hydroxylated stilbenes. This method might be amenable to any drug/imaging molecule bearing a hydroxyl and later on amino and sulfhydryl groups which have great potential to be developed into a PET radiotracer. Many of these compounds were neglected due to the lack of chemical synthesis regime which is suitable for their synthesis and which cannot be accomplished by any other typical fluoridation method. Stilbenes bearing a hydroxyl group on one aromatic ring were chosen as model compounds due to their accessibility in our lab and their striking potential as labelling moieties for Alzheimers Disease. This chapter will focus on the synthesis of 'cold' fluorinated trans-stilbene, which later should be applicable for translation to PET radiochemistry. To start the synthesis, an attempt to prepare tetrabutylammonium tetra(*tert*-butyl alcohol)-coordinated fluoride [TBAF(*t*BuOH)$_4$] was made, which has been reported to be a facile fluoride source. Next, the synthesis of stilbene containing alkylfluoride chain was attempted, from which we studied expansion to the synthesis of stilbene containing potassium alkyltyrifluoroborate chain using a novel method. At the same time, the synthesis of stilbenes attached directly to potassium trifluoroborates from their boronate esters counterparts were also explored.

5.2 Synthesis of Tetrabutylammonium Tetra(*tert*-Butyl Alcohol)-coordinated fluoride [TBAF(*t*BuOH)$_4$] as a facile fluoride source.

Fluoride ion proved to be more favorable for the synthesis of fluorinated compounds compared to fluorine because of its higher activity and considerable safety issues associated with handling fluorine. For clinical purposes, fluorine-18 is mainly produced from a cyclotron through a proton irradiation of ^{18}O-enriched water. But this method also showed to have many limitations as a truly naked fluoride ion is never obtained, as reviewed by Cai et al. (2008). Typical methods for the synthesis of fluorinated molecules are often based on the use of electrophilic reagents (e.g. xenon difluoride, fluorine gas or Selectflour) (Furuya et al. 2008). On the other hand, the field of fluorine-18 chemistry is highly focusing on the extension to new synthetic modalities achievable with ^{18}F fluoride ion as reagent (Cai et al. 2008).

Therefore, a robust and facile fluoride source is needed to ensure a reliable and reproducible nucleophilic fluoridation. In the search to find a good fluoride source, Kim et al. (2008) had introduced a new fluoride source, TBAF(*t*BuOH)$_4$ which was claimed to have good nucleophilicity, good solubility in organic solution, in dehydrated state for anhydrous reaction condition and low hygroscopicity for easy handling and for reduction of side reactions. The synthesis of TBAF(*t*BuOH)$_4$ (compound **38a**) is described below:



Commercially available TBAF hydrate was heated in *t*BuOH/hexane for 30 min at 90 °C and allowed to precipitate at RT. Compound **38a** was afforded as white crystalline solid in good yield.

Although the overall reaction was quite straightforward, the resulting crystals were not as good and reproducible as expected. The crystals were

soggy and hygroscopic with high tendency to melt especially when put under vacuum for drying or for weighting. The proton and carbon NMR showed the exact peak as had been reported. The only difference was at the *t*BuOH peak at around ~1.26-1.27 ppm where the area under the peak only represented 18 protons (the right compound should show 36 protons which represent four *t*BuOH atoms surrounding the fluoride ion source). The procedure was repeated and after seven attempts, the right compounds were obtained only once (during the fourth attempt) but still lacking the criteria of a flexible and stable fluoride source (due to its hygroscopic nature). Different approaches were tried to improve the resulting crystals, where the reactions were done in carefully controlled temperature and condition (the mixture's temperature must reach 25° C when the crystals were collected). Various times of drying the crystals under vacuum also gave different results at the *t*BuOH peak (the number of *t*BuOH protons were different between each batch-ranging from 18 to 32 protons). The overall yields also ranged from 66% to 100%.

Although the crystals were not consistent and not reproducible, it was decided to proceed to the next fluoridation using the afforded crystals, following the method described by Kim et al. (2008). So, the next important step for testing the fluoride source is to find a model compound (other than stilbene) with a good leaving group that is suitable for the fluoridation steps. Benzyl bromide was chosen as the first model compound as it is readily available. The fluoride sources that showed 36 and 27 protons at the *t*BuOH peak were chosen for fluoridation. The fluoridation steps were also carried out in two different solvents, in DMF or acetonitrile at 70 °C for comparison. The reaction condition is outlined in Figure 5.4 below:

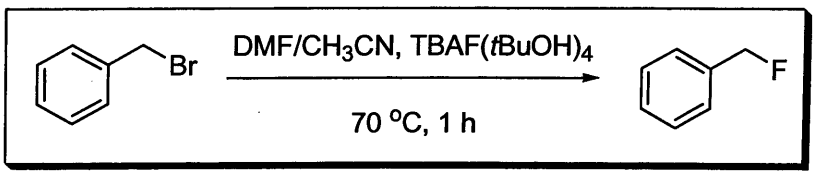


Figure 5.4: Fluoridation step

The reaction was carried out for up to five hours, but only the sign of starting material can be detected from the TLC. The reaction was further carried out overnight with addition of excess fluoride source, but the TLC still showed the unreacted starting material. The same results were seen in all different reaction conditions and fluoride sources. The observation showed that halide (in this case bromide) was not a good leaving group, and a better model compound with a better leaving group should be used. Therefore, mesylate and tosylate attached to benzyl moiety were chosen as the next model compounds to continue with fluoridation reaction.

4-methoxybenzyl mesylate (compound **38b**) was first chosen for the fluoridation step. The method described by Crossland and Servis (1970) was used as outlined below to synthesis the compound:

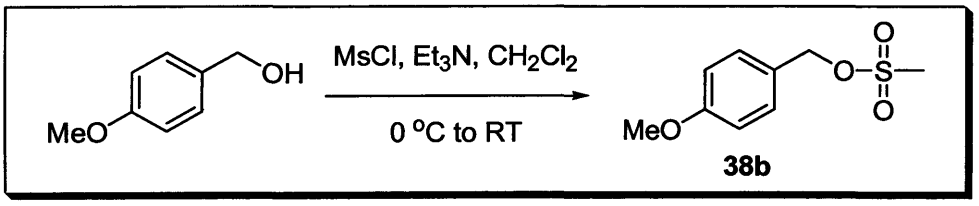


Figure 5.5: Synthesis of 4-methoxybenzyl mesylate **(38b)**

The reaction involved the synthesis of mesylate from *p*-methoxy benzyl alcohol in the presence of triethylamine as base and dichloromethane as solvent under inert atmosphere in ice-cold condition. The reaction was allowed to warm slowly to room temperature, followed by standard work-up using ether, water and 10% hydrochloric acid solution. Unfortunately, no product formation can be detected after the work-up even though the TLC of the crude reaction mixture showed the possibility of the product formation. The compound was predicted could not survive in the acidic work-up, so a modified version of the above procedure was followed which involved the work-up procedure in water and sodium bicarbonate, as reported by Lebegue et al. (2005) . After 24 h of stirring, the reaction was complete and compound **38b** was afforded as colourless oil in 78% yield.

The mesylate was then subjected to fluoridation using the same condition as the fluoridation for benzyl bromide. Unfortunately, no product formation can be detected even after two attempts; despite the observation that only traces of starting material can be detected at the termination of the reaction which strongly related to the possible degradation of compound **38b**. This is in accordance with the report by Bently et al. (1994), which mentioned 4-methoxybenzyl mesylate as a very reactive mesylate. Therefore, it was predicted that the mesylate can be easily decomposed during the fluoridation reaction due to its reactivity and unstability, which make it not suitable to test the fluoride source.

Therefore, another model compound, 4-methoxybenzyl tosylate (compound **38c**) was chosen which was predicted to have better stability to withstand the fluoridation step. This compound has been reported previously and has been successfully synthesized by Velusamy et al. (2004) from 4-methoxybenzyl alcohol catalysed by cobalt (II) chloride with *p*-toluensulfonic acid. Nevertheless, the synthesis of this tosylate was also not as easy as expected, with no or very small amount of product isolated. Listed below are the methods that had been used to synthesize the compound:

a) modification of the mesylate synthesis (Lebegue et al. 2005). The methanesulfonyl chloride was substituted with *p*-toluenesulfonyl chloride. No product formation can be detected after the work-up.
b) Using pyridine as base (Cho et al. 2003). The method involved the reaction of alcohol with *p*-toluenesulfonyl chloride in dichloromethane and pyridine as base at 0 °C, and then gradually warmed to RT (Figure 5.6). The method afforded the product after column chromatography in less than 1% yield.

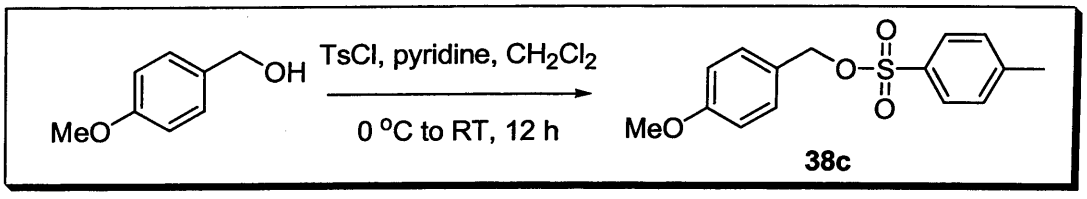


Figure 5.6: Synthesis of 4-methoxybenzyl tosylate **(38c)**.

After the unsuccessful attempts to synthesise compound **38c**, the issue about the stability of the tosylate was once again raised, until later a report by Kochi & Hammond (1953) that mentioned the fragility of the compound was noticed. The tosylate need very careful exclusion of moisture and rapid manipulation, and various attempts to recrystallize the compound in a variety of solvents were futile. The 4-methoxybenzyl tosylate was not well characterized because it is extremely reactive, as it will polymerizes slowly even at -60 °C, and very rapidly at RT. At this stage, the 4-methoxybenzyl alcohol was considered as an inappropriate starting material, and another alcohol (benzyl alcohol) was considered.

The next synthesis of benzyl tosylate **[38c(i)]** under a solvent free condition was chosen as described by Kazemi et al. (2007). The reaction involved the reaction of benzyl alcohol that was vigorously grinded with *p*-toluene sulfonyl chloride in the presence of dry potassium carbonate (Figure 5.7). After the work-up, traces of benzyl alcohol can still be detected. The crude oily compound was subjected to column chromatography (10 hexane: 1 ethyl acetate) to afford the pure compound **38c(i)** in 26% yield as white powder. It was believed that prolonging the grinding time of the starting materials could increase the yield of the benzyl tosylate significantly.

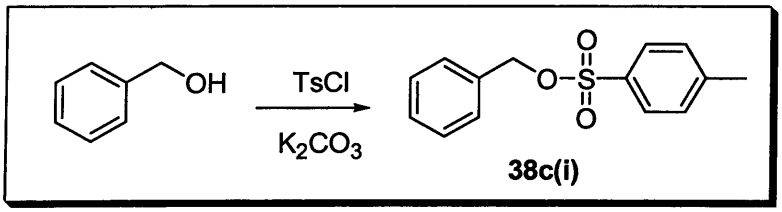


Figure 5.7: Synthesis of benzyl tosylate **[38c(i)]**

After the successful synthesis of the model compound, compound **38c(i)** was subjected to fluoridation as described in the previous fluoridation attempts using the afforded compound **38a** (TBAF (*t*BuOH)$_4$. After more than 1 hour, the TLC showed full consumption of the starting material in both reactions using DMF or acetonitrile. The TLC spot of the fluoridation reactions appeared lower than the starting material spot, which indicated the possibility of the desired more polar benzyl fluoride formation. The attempts

to purify the oily crude mixture using column chromatography in various eluents (hexane;ethyl acetate or dichloromethane;methanol) were not very successful as the truly pure benzyl fluoride was very hard to isolate. Nevertheless, the decoupled fluorine NMR of the purified mixture showed a single peak at -152.21 ppm which differs from the decoupled fluorine NMR peak of the TBAF(tBuOH)$_4$ (-155.68 ppm) that gave more confidence that the targeted benzyl fluoride had been successfully synthesised. But the formation of many impurities that were very hard to separate from the pure compound suggested that compound **38a** that had been synthesised was not as facile and as promising as has been claimed. In order to test the reliability of this reaction, the same fluoridation reaction was repeated but using the commercially available tetrabutylammonium fluoride trihydrate (TBAF). The TLC also showed full consumption of the starting material, not surprisingly with a quite similar TLC spots pattern as compared to compound **38a** and problems in purification indicating a relatively comparable reaction condition between the synthesised TBAF(tBuOH)$_4$ and commercially available TBAF. The decoupled fluorine NMR also showed a single peak at -152.23 ppm.

In conclusion, the commercially available TBAF was found to be a good fluoride source, and the synthesis of TBAF(tBuOH)$_4$ which has been claimed to be a better and more stable fluoride source showed no great differences in the overall reaction condition as compared to the commercially available TBAF for PET chemistry.

5.3 Synthesis of stilbene containing alkyl fluoride chain.

This part of the study focused on the synthesis of stilbenes attached to an alkyl fluoride chain. The aim was to synthesise the target compounds in a fast and efficient manner to get the fluorinated compounds in good yield. In this study, the alkyl chain was derived from 1,4-dibromobutane as it was the cheapest alkyl bromide in the market and due to its availability in the lab. The chosen method to synthesise the stilbene was based on the HWE reaction (chapter 2) that had been previously reported by Lion et al. (2005).

Previous study done by an Erasmus student (Paola Casule, February-July 2010) showed that the attempt to synthesis stilbene **39a** (*(E)*-1-(4-bromobutoxy)-4-(4-methoxystyryl)benzene) from 4-methoxybenzyl phosphonic acid diethyl ester (compound **1**) and 4-(4-bromobutoxy)benzaldehyde was unsuccessful (Figure 5.8). It was predicted that 1,4-dibromobutane and 4-hydroxybenzaldehyde was cleaved during the HWE reaction, leaving the free hydroxyl group to further react and gave unwanted side reactions. In order to avoid the same fate, for the next attempt two hydroxylated stilbenes which were previously synthesized in the lab were chosen for this study (compound **22** and **32**) (Figure 5.9).

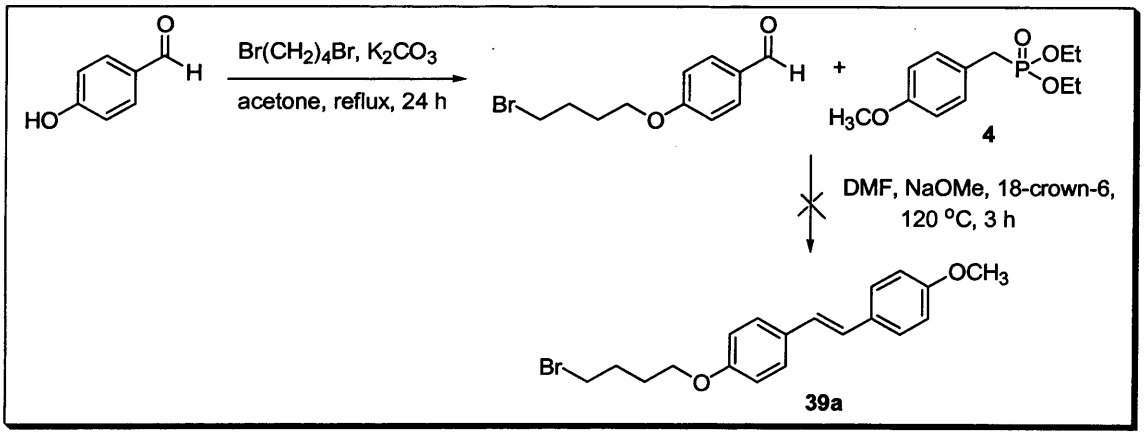


Figure 5.8: Previous study done by Paola Casule (February-July 2010)

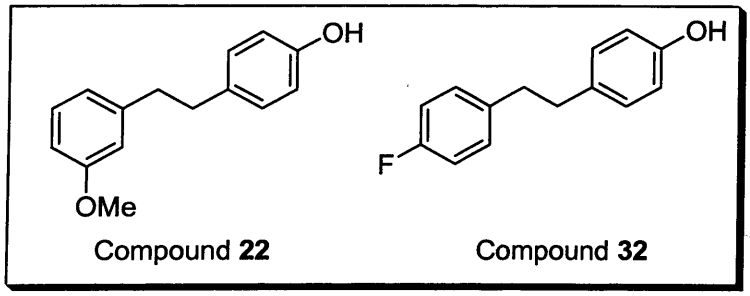


Figure 5.9: Compound **22** and **32**

The first step of the study involved the incorporation of a 1,4-dibromobutane at the hydroxyl group of compound **22** and **32**. The method involved the reaction of hydroxylated stilbene (1 equiv) with 1,4-dibromobutane (1.15 equiv) in acetone and 1.5 equivalent potassium carbonate as base, as shown in figure 5.10. The reaction needed purification

by column chromatography to remove unreacted starting materials and gave the desired product in good yield; compound **39a** (78%) and **39b** (75%).

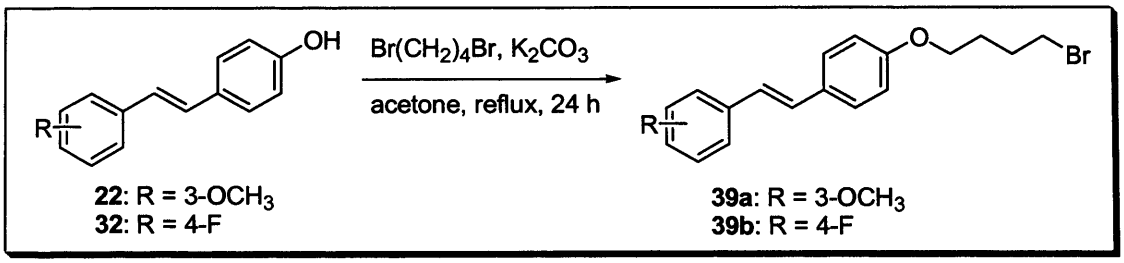


Figure 5.10: Alkylation procedure

The next crucial stage in this study is the fluoridation step. Previous study by Paola Casule also revealed that bromide is a less effective leaving group for the fluoridation reaction of the stilbene using tetrabutyl ammonium fluoride (TBAF) or cesium fluoride (CsF), whether heating in polar protic or polar aprotic solvents. Although the product fluoro compound was isolated, the reaction was low yielding and even after 24 hours of reaction lots of starting material remained unreacted (data not shown) (Figure 5.11). Therefore, it is important to find a better leaving group for an efficient fluoridation to occur.

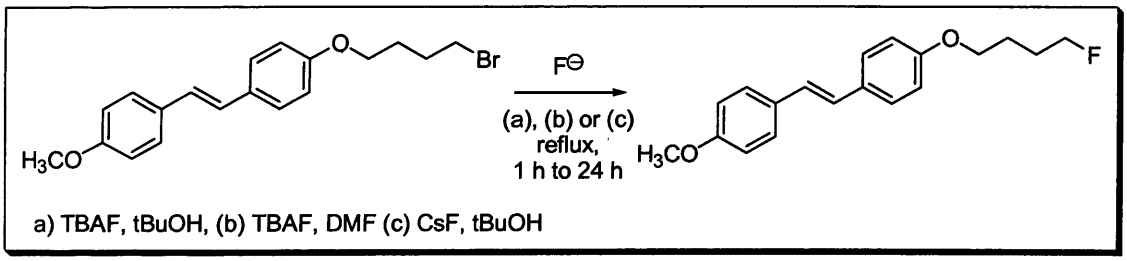


Figure 5.11: Less efficient fluoridation step using bromide as leaving group.

To improve the fluoridation step, mesylates were chosen as the leaving group of choice instead of halogens. The synthesis of mesylates was straightforward using silver methanesulfonate as the mesylate source in acetonitrile with overnight reflux (Figure 5.12). Silver is known to have strong affinity to couple with bromide and this helped the reaction to go to completion and good yields of mesylates **40a** and **40b** were obtained (78 and 75% yield, respectively). It is noteworthy that there is another method that

can be used in the future for the synthesis of compound attached to alkyl tosylates for efficient fluoridation by using commercially available ethylene glycol ditosylate derivatives in NaH and DMF with the hydroxylated stilbene (Wang et al. 2010).

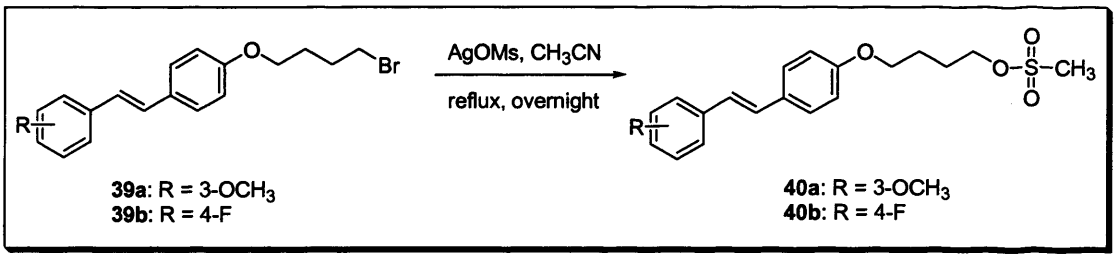


Figure 5.12: Synthesis of mesylates

The next crucial step was the fluoridation step of the stilbenes attached with alkyl mesylates chain using tetrabutylammonium fluoride (TBAF) or cesium fluoride (CsF) in *tert*-butanol (*t*-BuOH) or acetonitrile (CH_3CN). Stilbene **40a** was chosen as the model compound. As expected, the fluoridation step using TBAF in *t*-BuOH or CH_3CN showed nearly complete conversion of the mesylates to the possible product even after 30 minutes of reaction. After 1 hour, the reaction was terminated. Prior to the short column chromatography purification, reaction in *t*-BuOH gave 90% yield of pure compound **41** while reaction using CH_3CN afforded pure compound **41** in 86% yield. It was noted that the reaction in *t*-BuOH gave a cleaner reaction indicated by less side product formation compared to the reaction in CH_3CN as indicated by thin layer chromatography (TLC) analysis. The fluoridation of stilbene **40b** using TBAF in *t*-BuOH gave the pure fluorinated compound **42** in 81% yield. The study also showed that polar protic solvent is superior to the polar aprotic solvent for this kind of fluoridation reaction which gave less side product formation and better yield, as reported previously by Kim et al. (2006). The formation of the fluorinated compounds **41** and **42** were evidenced by the disappearance of methyl groups of the mesylates in ^{1}H and ^{13}C NMR and the appearance of single fluorine peak at δ -218.52 ppm for compound **41** and the presence of two fluorine peaks at δ -115.27 ppm and δ -218.57 ppm for compound **42** in decoupled ^{19}F-NMR.

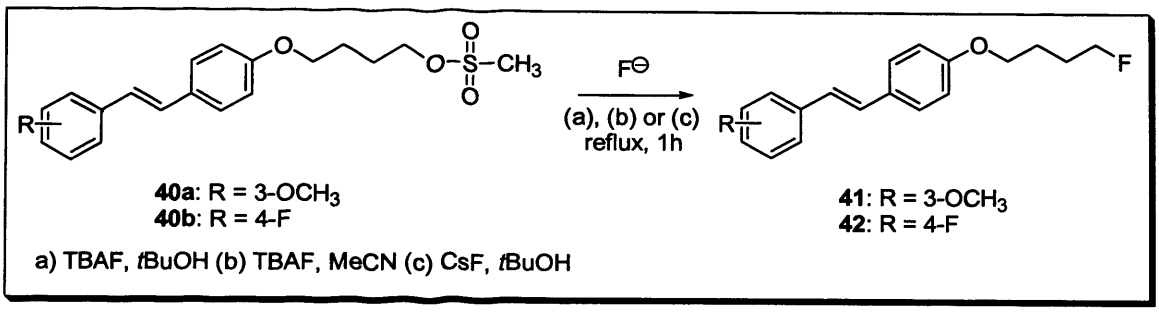


Figure 5.13: Fluoridation step

This study demonstrated that the synthesis of stilbenes containing alkyl fluoride chains can be achieved efficiently by using mesylates as the leaving group. TBAF also proved to be a good fluorinating agent in polar protic solvent such as *t*-BuOH. This fluoridation and alkylation method were anticipated to be extended to the synthesis of other compounds which may not be achievable with the typical fluoridation step for PET imaging especially in the case of electron-rich aromatic compounds, where single step nucleophilic fluoridation which need harsh reaction condition is preferred, provided that the compound should possess either a hydroxyl, amino or sulfyhydryl group in any part of the compound.

5.4 Potassium alkyltrifluoroborates as a new approach for labeling PET radiotracers.

The synthesis of compounds containing potassium trifluoroborate chain for PET imaging is still in its infancy. Nevertheless, this area of research have attracted much attention due to report by Ting et al. (2008a) which showed that ^{18}F-labelled aryltrifluoroborate radiotracers have good stability *in vivo* and are a promising modality for PET imaging. This method however needs the intermediate to possess a boronic ester substitution in order to capture the free fluoride for trifluoroborate synthesis. The synthesis, application and stability of the potassium organotrifluoroborates have been reviewed elsewhere (Darses and Genet 2008). To summarise, the synthesis of potassium organotrifluoroborates is usually achieved by using four different

methods (Figure 5.14): a) from isolated boronic acid (Molander and Biolatto 2003); (b) via transmetallation reaction (Molander and Ham 2006; Cho et al. 2009); c) via hydroboration (Petasis et al. 1997); and (d) via C-H bond activation (Murphy et al. 2007) with potassium hydrogen fluoride (KHF_2) as the main fluorinating agent. Unfortunately, these methods usually suffer from competitive binding with sensitive and existing functionalities in the substrate that can limit its usefulness (Molander and Figueroa 2006a; Darses and Genet 2008).

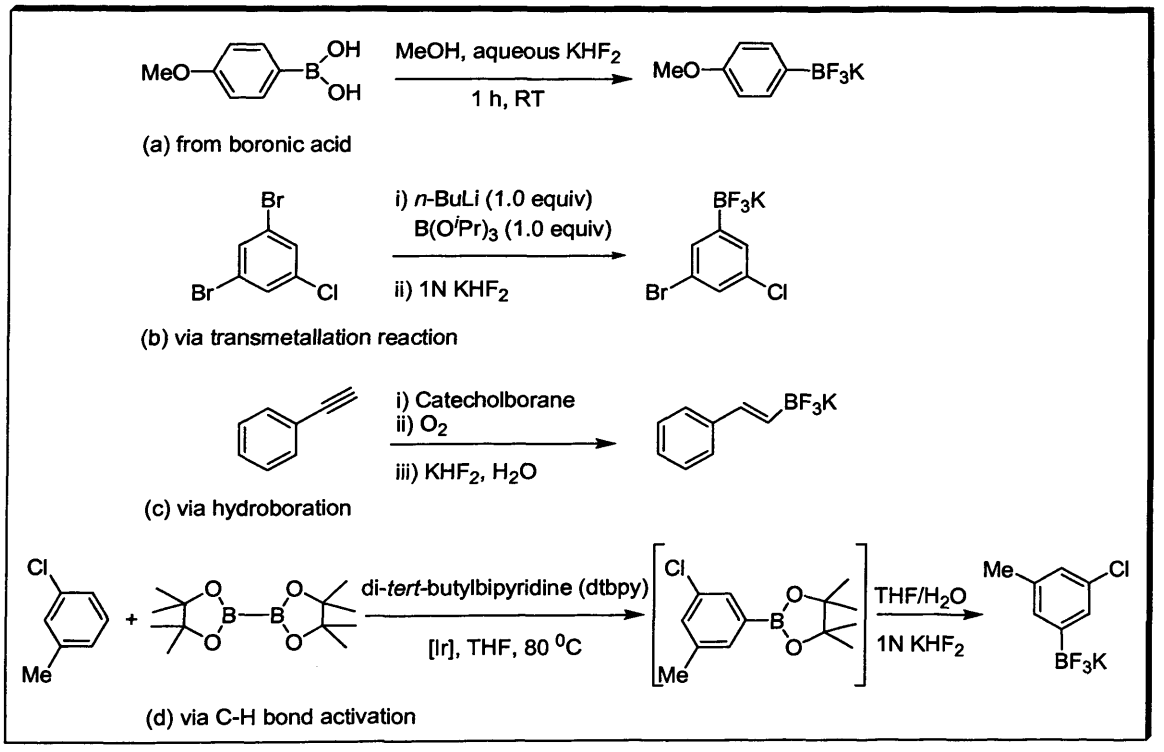


Figure 5.14: Methods to synthesise organotrifluoroborates

To date, there are no procedures that have been reported to synthesise compounds attached to alkyl trifluoroborates directly from its alkyl mesylates/triflates/halides (or any other leaving groups) counterparts. The existing methods only reported the synthesis of alkyl trifluoroborates compounds from alkyl chain that have been previously attached with boronic ester or trifluoroborates derivatives (e.g. Molander and Ham 2006; Molander et al. 2007b), or by synthesizing the Grignard reagent or organolithium intermediate first from alkyl halide which later will be reacted with borate

esters (e.g. trimethyl borate $B(OCH_3)_3$) to produce trifluoroborate upon quenching with KHF_2 (e.g. Molander and Figueroa 2006b).

Therefore in this study, an attempt was made to present a novel and a much simpler method for the synthesis of potassium alkyl trifluoroborates using alkyl mesylates as the intermediates and potassium tetrafluoroborates (KBF_4) as the trifluoroborate source. This approach, if successful was anticipated to have a wide applicability to a broad range of compound which may have great utility in PET imaging.

For this reaction, stilbene **40a** that have been synthesised previously was used as the intermediate and model compound. The compound was subjected to the synthesis using potassium tetrafluoroborate in different solvents (*t*-BuOH, CH_3CN, methanol/tetrahydrofuran or dimethylformamide (DMF)) at reflux (Figure 5.15). After 1 hour of heating, the reaction in *t*-BuOH and CH_3CN showed no sign of possible product formation. The same observation was seen even after 24 hour of prolonged reaction in these two particular solvents. However, the reaction in methanol/THF showed a slight possible product formation after 1 hour and a few additional side reaction spots detected by TLC. Even after prolonged reaction to 24 hours, the same observation was seen and the formation of a few other side reaction spots by TLC made this reaction look less attractive. Interestingly, the reaction in DMF showed a striking observation after 1 hour, indicated by the formation of possible product formation as the major constituent in the reaction. Prolonged reaction to 24 hours of heating did not give vast changes to the observation. Upon quenching with water, precipitate was collected and dried in air. Attempts to purify the crude compound by recrystallisation in acetone/ether or acetonitrile failed as the compound was found to be soluble in those solvents even at room temperature. The next attempt to purify in methanol/water managed to produce pure solid. Nevertheless, the NMR showed that the desired compound was not isolated. The product still retained the stilbene skeleton and the alkyl chain, but was missing the methyl peak of mesylate with no detectable fluorine peak from the decoupled

fluorine NMR. These highlight the failure of the attempted method to produce the desired potassium butyl trifluoroborates.

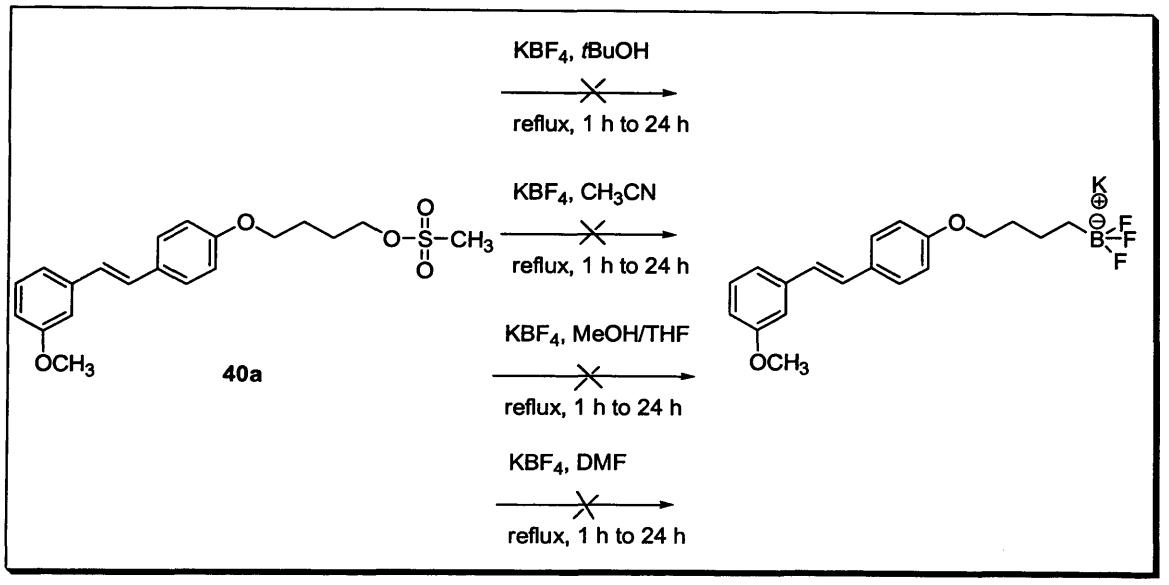


Figure 5.15: Attempts to synthesise potassium alkyl trifluoroborates.

Although this method looked attractive, repeated attempts of simple heating at reflux in DMF was not sufficient to synthesise the right compound, which emphasise the need to refine the attempted procedure until the correct compound is achievable. Other than refinement of the attempted procedure, future studies can also be dedicated to the synthesis of stilbenes attached to alkyl boronate esters intermediates via Grignard reagent (Molander and Figueroa 2006b; Gerbino et al. 2009), or by conversion of arenes to arylboronic acid by C-H activation chemistry (Murphy et al. 2007) which later will have better possibilities for transformation to trifluoroborate salt.

5.5 Synthesis of potassium (*E*)-stilbene trifluoroborate

This study was aimed to synthesise stilbene derivatives which are attached directly to a potassium trifluoroborate moiety. A few stilbenes that are attached directly to trifluoroborates have been prepared previously either using HWE or Wittig reactions (Molander and Figueroa 2006; Molander et al. 2007a). Nevertheless, all the compounds were made using potassium trifluoroborates substitution which have been synthesised previously from the

beginning of the reaction and were carried out through the multistep process to afford the potassium stilbene trifluoroborates, which is not compatible with future synthesis of ^{18}F derivatives. To date, there a no examples of Wittig or HWE reactions that have been reported using suitably functionalized boronic acid derivatives, most probably due to the acidic proton in these species. On the other hand, the Wittig and HWE reactions using boronate esters with stabilized ylides and phosphonates have been reported to a very limited extent (e.g. Lautens and Mancuso 2004; Gopalaratnam and Nelson 2006; Molander and Figueroa 2006a). The drawback of this method is that the diols such as catechol, pinacol and diethanolamine that is used to generate stable boronate esters add considerable expense to the overall process and must be separated from the final product (Molander and Ham 2006).

The first attempt made to synthesise stilbenes directly attached to potassium trifluoroborates was made following previously reported procedures (Molander and Ham 2006; Cho et al. 2009). The methods involved the formation of organolithium intermediates using triisopropyl borates and *n*-butyllithium in tetrahydrofuran (THF) at -78 °C for at least 1-3 hours followed by quenching with aqueous KHF_2 to afford the potassium stilbene trifluoroborates (Figure 5.16).

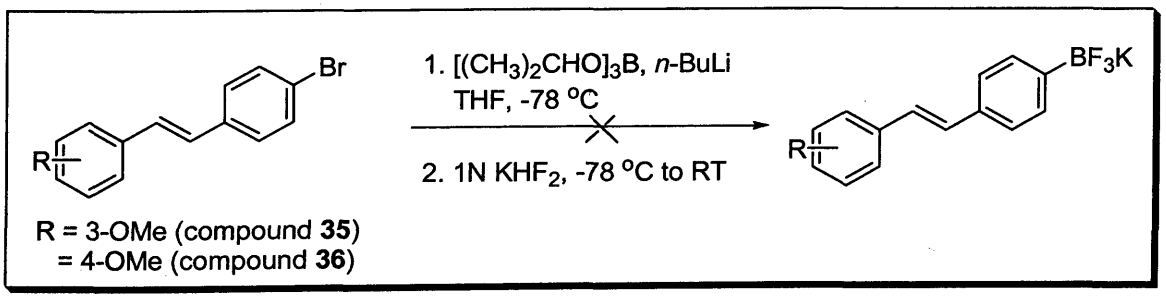


Figure 5.16: First attempt to synthesise potassium stilbene trifluoroborate

A few modifications have been attempted which involved the addition of borate ester during or after 1 hour of *n*-butyllitium addition, or even the addition of KHF_2 either at -78 °C or after the reaction being warmed to room temperature. None of the attempted methods succeeded although freshly purchased sources of *n*-butyllithium had been used. Attempts to modify the

method to synthesise boronic acid and boronic ester intermediates upon quenching the reaction with water and later on substitution with pinacol also failed to give stilbene boronate ester intermediates. It was anticipated that the use of new or different kind of borate ester (e.g. trimethyl borate) will improve the outcome of the reaction. Nevertheless, further attempt to synthesis of the compound via this method was not considered due to difficulty in getting the right compound. Therefore, another method was sought.

The next attempt was dedicated to the synthesis of stilbene boronate esters as intermediates which will later be transformed to potassium trifluoroborates upon quenching in aqueous KHF_2. The synthesis of stilbenes was achieved by using the method described by Lion et al. (2005) utilising the HWE coupling reaction. In the previous study done by Paola Casule (Erasmus student), 4-formylphenylboronic acid was transformed to potassium trifluoro(4-formylphenyl)borate first using KHF_2 as the fluorinating agent before being coupled to 4-methoxy phosphonic acid diethyl ester (compound **1**) to synthesise the stilbene attached to potassium trifluoroborates. Unfortunately, the reaction failed to give the desired product as the trifluoroborate was predicted to be cleaved during the harsh reaction condition leaving free boronic acid to further react (Figure 5.17).

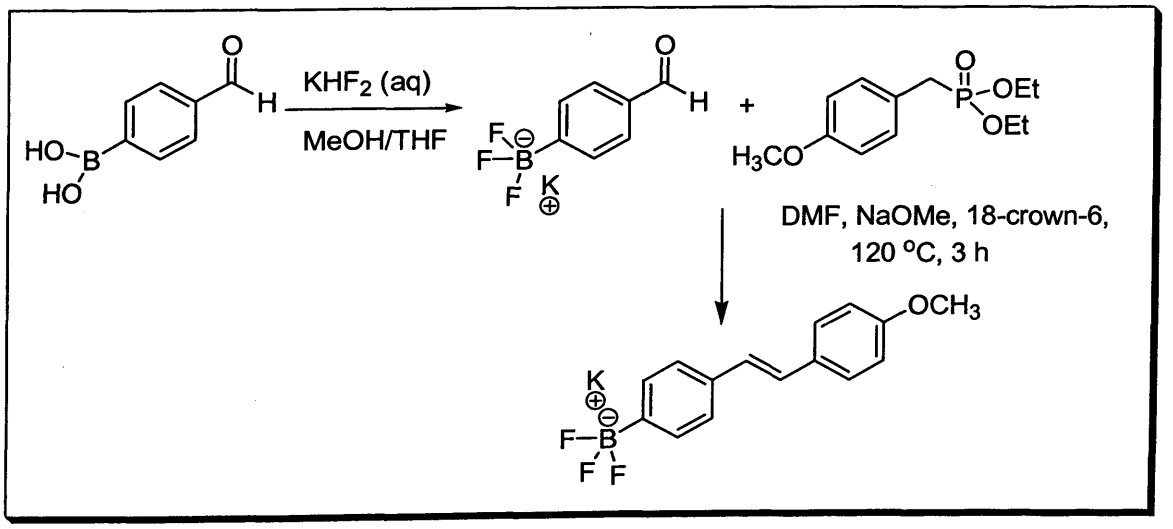


Figure 5.17: Previous attempt to synthesise potassium stilbene trifluoroborate (Paola Casule).

Therefore, in the next attempt (Figure 5.18), the boronic acid was protected first with pinacol to produce 4-(4,4,5,5,-tetramethyl-1,3,2-dioxaborolan-2-yl)benzaldehyde (compound **45a**) to make it more stable to withstand the harsh reaction conditions of the HWE reaction. The protection step was quite straightforward, which involved the reaction of the 4-formylphenylboronic acid with a pinacol in diethyl ether for 12h. The next step involved the reaction of compound **45a** with compound **2** (3-methoxybenzyl phosphonic acid diethyl ester) to afford a *trans*-stilbene (cvompound **45b**) in only 9% yield after column chromatography. The reaction suffered from unreacted starting material and limiting reagent even after prolonged heating (48 hours) which contributed to lowering the yield. The next crucial step was the fluoridation step with KHF_2 in methanol/tetrahydrofuran to make the trifluoroborate **45**. The reaction was achieved within 1 hour of the reaction time, where washing of the crude product with hot diethyl ether and water is sufficient to give pure compound **45** in 63% yield. It is anticipated that during the washing a small amount of product may have been washed away which can contribute to lowering the overall yield. It is also noted that signal of the carbon atom attached directly to boron atom was not observed due to the signal broadening of ^{11}B. The formation of compound **45** was proved by the appearance of a single fluorine peak at δ 34.68 ppm in decoupled fluorine NMR, together with the disappearance of methyl peak of the boronate ester in ^{1}H and ^{13}C-NMR.

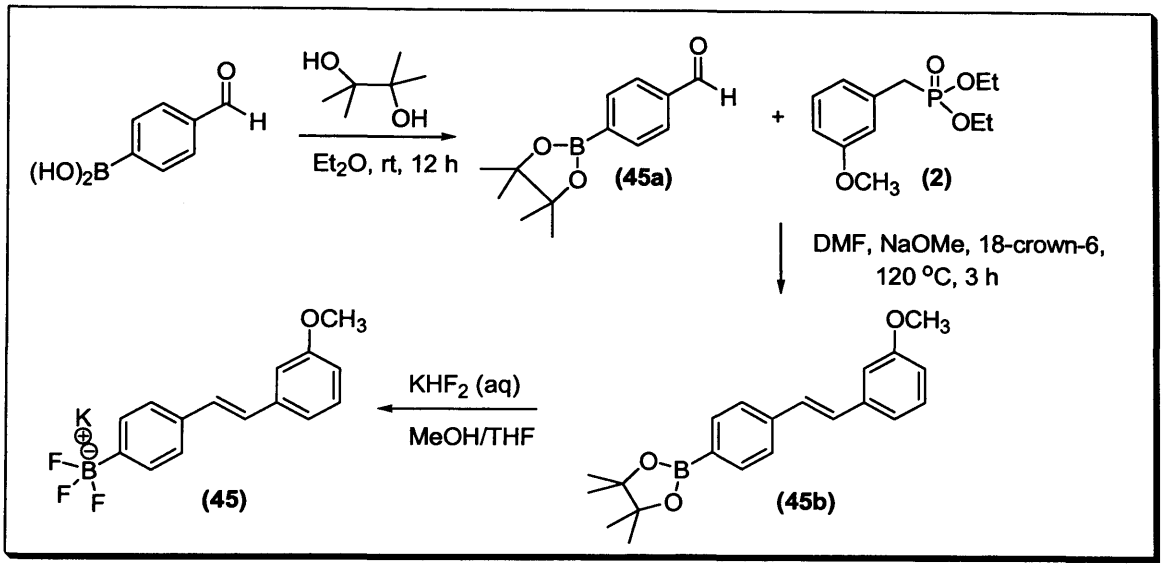


Figure 5.18: Overall reaction procedures to synthesise compound **45**.

The same derivatives containing *para*-methoxy group was also managed to be synthesised, but in a very low yield (less than 1%). The problem lies on the synthesis of 4-methoxy stilbene boronate ester which suffered from very slow reaction and unreacted starting material which gave a very low yield of impure compound (1%). Nevertheless, a single fluorine peak was observed after the fluoridation step which indicated that a trifluoroborate stilbene with 4-methoxy substitution had likely been successfully synthesised.

In conclusion, these results demonstrated that the synthesis of stilbene containing direct substitution with potassium trifluoroborate can be achieved from stilbene containing boronate ester, but suffered from very slow reaction and low yield. This lower reactivity results, in part, from the unfavorable hydrolysis of the pinacolboronates to boronic acids (Murphy et al. 2008). Therefore, a better method should be sought in order to synthesise stilbene containg boronate ester with better condition and improved yield, so it can be extended to ^{18}F-radiochemistry synthesis for the synthesis of radio labeled potassium stilbene trifluoroborates.

CHAPTER 6

EXPERIMENTAL

CHAPTER 6

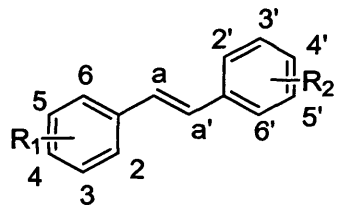
EXPERIMENTAL

6.1 CHEMISTRY

All chemicals obtained from commercial suppliers (e.g. Sigma Aldrich, Fisher Scientific) were used as received and were of analytical grade. The anhydrous solvents were purchased from Aldrich with subaseal stopper. Melting points were recorded on a Griffin Gallenkamp melting point apparatus and are uncorrected.

The ^{1}H, ^{13}C and ^{19}F NMR spectra were recorded on a Bruker Avance-500 spectrometer at 500, 125 and 471 MHz respectively, at 25°C. Chemical shifts (δ) are reported in parts per million (ppm). *J* values are reported in Hertz (Hz). Thin layer chromatography (TLC) was performed on 60 F_{254}, 0.2 mm thickness pre-coated aluminium sheets (Merck) and were visualized under both short and long wave ultraviolet light (254 nm and 366 nm) . Flash chromatography columns were performed using silica gel 60A (35-70 μm) from Fisher. A CEM Discover focused microwave (2450 MHz, 300 W) was used for the synthesis of hydroxylated stilbene analogues. High and low resolution mass spectroscopy using electron ionisation (EI) were run on a Waters GCT Premier, and using electrospray (ES) on a Waters LCT Premier XE. The mass spectrometries were performed as a service by School of Chemistry, Cardiff University. Elemental analysis (CHN) microanalysis was performed as a service by MEDAC Ltd, Surrey. Accurate mass (electrospray) spectrometry was performed by the EPSRC National Mass Spectrometry Service (Swansea, UK).

6.2 Synthesis of stilbene analogues as potential anticancer agents



Compound	R_1	R_2
4	4-OMe	4-OMe
5	3-OMe	4-OMe
6	3-OMe	3-OMe
7	3-OMe	3,5-diOMe
8	4-OMe	3.5-diOMe
9	4-OMe	3,4-diOMe
10	4-OMe	2,4-diOMe
11	4-OMe	2,5-diOMe
12	3,5-diOMe	3,4-diOMe
13	3,5-diOMe	2,4-diOMe
14	4-OMe	3,4,5-triOMe
16	3,5-diOMe	3,5-diOMe
`17	3,5-diOMe	2,5-diOMe
18	3,5-diOMe	3,4,5-triOMe
19	4-OMe	3-F
20	3-OMe	3-F
28	3-OMe	4-F
29	4-OMe	4-F
34	3-OMe	4-NO_2
35	4-OMe	4-NO_2
36	3-OMe	4-Br
37	4-OMe	4-Br

General method for the synthesis of substituted methoxybenzyl phosphonic acid diethyl esters

The substituted 4-methoxy, 3-methoxy or 3,5-dimethoxy benzyl bromide (40 mmol) was heated with excess of triethylphosphite (50 mmol) at 130°C to 140°C until the completion of the reaction. The remaining triethylphosphite was then removed by concentration of the solution *in vacuo* to afford phosphonate esters (as oils).

(4-Methoxybenzyl) phosphonic acid diethyl ester [1] (Lion et al. 2005).
Colourless oil. Yield = 96%.
^{1}H NMR ($CDCl_3$) δ 1.24 (t, *J* = 7.0 Hz, 6H, OCH_2CH_3), 3.08 (d, *J* = 21.0 Hz, 2H, PCH_2), 3.79 (s, 3H, OCH_3), 4.00 (m, 4H, OCH_2CH_3), 6.74 (d, *J* = 8.5 Hz, 2H, H3, H5), 7.21 (dd, *J* = 8.8, 2.5 Hz, 2H, H2, H6)

(3-Methoxybenzyl) phosphonic acid diethyl ester [2] (Murias et al. 2004)
Colourless oil. Yield = 99%.
^{1}H NMR ($CDCl_3$) δ 1.27 (t, *J* = 7.0 Hz, 6H, OCH_2CH_3), 3.15 (d, *J* = 21.5 Hz, 2H, PCH_2), 3.82 (s, 3H, OCH_3), 4.04 (m, 4H, OCH_2CH_3), 6.81 (d, *J* = 8.0 Hz, 1H, H4), 6.90 (d, *J* = 7.5 Hz, 2H, H2, H6), 7.24 (t, *J* = 7.8 Hz, 1H, H5)

(3, 5-dimethoxybenzyl) phosphonic acid diethyl ester [3] (Heynekamp et al. 2006).
Colourless oil. Yield = 98%.
^{1}H NMR ($CDCl_3$) δ 1.29 (t, *J* = 7.0 Hz, 6H, OCH_2CH_3), 3.12 (d, *J* = 22.0 Hz, 2H, PCH_2), 3.80 (s, 3H, OCH_3), 4.05 (m, 4H, OCH_2CH_3), 6.38 (q, *J* = 2.5 Hz, 1H, H4), 6.49 (t, *J* = 2.3 Hz, 2H, H2, H6)

6.2.1 Synthesis of (*E*)-methoxylated, fluorinated, brominated and nitrated stilbenes via Horner-Wardsworth-Emmons reaction

Substituted phosphonic acid diethyl esters (10 mmol) were dissolved in dry DMF (10 mL). Sodium methoxide (20 mmol) and 18-crown-6 (2 mmol) were added and the mixture were stirred at room temperature for 5 min. The

substituted methoxybenzaldehyde or fluorobenzaldehyde (15 mmol) dissolved in dry DMF (5 mL) was added dropwise at 0 °C. The mixture was stirred at room temperature for 1 h and then subsequently heated to 100 °C for 3-5 h, or until the completion of the reaction. The reaction was quenched by pouring into water (30 mL) with stirring. Reactions that gave solids were filtered and recrystallised from diluted ethanol. Reactions that gave oils were extracted with diethyl ether (3 x 20 mL). The ether was evaporated *in vacuo*, and the residues redissolved in dichloromethane (15 mL). Girard's reagent T (6 mmol) and acetic acid (60 mmol) were added, and the reaction stirred at room temperature for 2 h. After quenching with water (15 mL), the organic layers were collected, washed with brine (3 x 15 mL) and aqueous saturated sodium carbonate (3 x 15 mL), dried over magnesium sulfate and evaporated *in vacuo*. The residue which gave solids were purified by recrystallisation from ethanol/water, while the oily residues were purified by column chromatography (hexane/ethyl acetate) to afford pure substituted methoxylated or fluorinated stilbenes.

***(E)* - 4, 4' dimethoxystilbene [4]**

White solid. Yield = 91%. mp = 214-215 °C (lit: 214-215 °C [Zhang & Go 2007])

^{1}H NMR ($CDCl_3$) δ 3.85 (s, 6H, 2 x OCH_3), 6.92 (d, *J* = 9.0 Hz, 4H, H3, H5, H3', H5'), 6.96 (s, 2H, Hα, Hα'), 7.45 (d, *J* = 9.0 Hz, 4H, H2, H6, H2', H6')

***(E)* - 3, 4' dimethoxystilbene [5]**

White solid. Yield = 84%. 106-108 °C (lit: 106-109 °C [Zhang & Go 2007])

^{1}H NMR ($CDCl_3$) δ 3.86 (s, 3H, OCH_3), 3.88 (s, 3H, OCH_3), 6.82 (dd, *J* = 8.3, 2.5 Hz, 1H, H4), 6.93 (d, *J* = 5.0 Hz, 2H, H3', H5'), 6.98 (d, *J* = 16.5, 1H, Hα), 7.06 (s, 1H, H2), 7.09 (d, *J* = 16.5 Hz, 1H, Hα'), 7.12 (d, *J* = 8.0 Hz, 1H, H6), 7.29 (t, *J* = 7.5 Hz, 1H, H5), 7.48 (d, *J* = 9.0 Hz, 2H, H2', H6')

***(E)* - 3, 3' dimethoxystilbene [6]**

White crystals. Yield = 57%. mp = 92-93 °C (lit: 99-100 °C [Chen & Chen 2007]). Recystallised (EtOH/water).

^{1}H NMR ($CDCl_3$) δ 3.88 (s, 6H, 2 x OCH_3), 6.85 (dd, *J* = 8.0, 2.5 Hz, 2H, H4, H4'), 7.08 (s, 2H, H2, H2'), 7.10 (s, 2H, Hα, Hα'), 7.14 (d, *J* = 7.5 Hz, 2H, H6, H6'), 7.31 (d, *J* = 8.0 Hz, 2H, H5, H5')

***(E)* – 3, 3', 5 trimethoxystilbene [7]**

White solid. Yield = 16%. mp = 67-68 °C (lit: oil [Velder et al. 2006]). Column chromatography (hexane:ethyl acetate = 9:1).

^{1}H NMR ($CDCl_3$) δ 3.86 (s, 6H, OCH_3 x 2), 3.88 (s, 3H, OCH_3), 6.44 (t, *J* = 2.2 Hz, 1H, H4), 6.70 (d, *J* = 2.5 Hz, 2H, H2, H6), 6.86 (dd, *J* = 8.0, 2.5 Hz, 1H, H4'), 7.05 (d, *J* = 16.5 Hz, 1H, Hα), 7.09 (d, *J* = 16.5 Hz, 1H, H α'), 7.08 (m, 1H, H2'), 7.17 (d, *J* = 7.5 Hz, 1H, H6'), 7.30 (t, *J* = 8.0 Hz, 1H, H5')

***(E)* – 3, 4', 5 trimethoxystilbene [8]**

Tan-coloured crystals. Yield = 18%. mp = 54-55 °C (lit: 55-57 °C [Murias et al. 2004]). Column chromatography (hexane:ethyl acetate = 6:1).

^{1}H NMR ($CDCl_3$) δ 3.86 (s, 9H, 3 x OCH_3), 6.41 (t, *J* = 2.3 Hz, 1H, H4), 6.68 (d, *J* = 2.0 Hz, 2H, H2, H6), 6.926 (d, *J* = 9.0 Hz, 2H, H3', H5'), 6.93 (d, *J* = 16.0 Hz, 1H, Hα), 7.07 (d, *J* = 16.0 Hz, 1H, H α'), 7.47 (d, *J* = 8.5 Hz, 2H, H2', H6')

***(E)* – 3, 4, 4' trimethoxystilbene [9]**

White solid. Yield = 68%. mp = 132 °C (lit: 135 °C [Zhang & Go 2007]). Recrystallise (EtOH/water).

^{1}H NMR ($CDCl_3$) δ 3.86 (s, 3H, OCH_3), 3.93 (s, 3H, OCH_3), 3.95 (s, 3H, OCH_3), 6.88 (d, *J* = 8.0 Hz, 1H, H5), 6.92 (d, *J* = 9.0 Hz, 2H, H3', H5'), 6.96 (d, *J* = 16.5 Hz, 1H, H α), 7.00 (d, *J* = 16.5 Hz, 1H, Hα'), 7.05 (dd, *J* = 8.5, 2 Hz, 1H, H6), 7.08 (d, *J* = 5.0 Hz, 1H, H2), 7.46 (d, *J* = 9.0 Hz, 2H, H2', H6')

***(E)* – 2, 4, 4' trimethoxystilbene [10]**

Pale yellow solid. Yield = 57%. mp = 86-88 °C (lit: 86-88 °C [Zhang & Go 2007]). Recrystallise (EtOH/water).

^{1}H NMR ($CDCl_3$) δ 3.85 (s, 3H, OCH_3), 3.86 (s, 3H, OCH_3), 3.89 (s, 3H, OCH_3), 6.50 (d, *J* = 2.5 Hz, 1H, H3), 6.54 (dd, *J* = 8.5, 2.5 Hz, 1H, H5), 6.91

(d, *J* = 9.0 Hz, 2H, H3', H5') 6.98 (d, *J* = 16.0 Hz, 1H, Hα), 6.28 (d, *J* = 16.0 Hz, 1H, Hα'), 6.47 (d, *J* = 9.0 Hz, 2H, H2', H6'), 6.51 (d, *J* = 9.0 Hz, 1H, H6)

***(E)* – 2, 4', 5 trimethoxystilbene [11]**

Yellowish crystals. Yield = 45%. mp = 67-68 °C (lit: 67-68 °C [Heynekamp et al. 2006]). Recrystallise (EtOH/water).

^{1}H NMR ($CDCl_3$) δ 3.84 (s, 3H, OCH_3), 3.86 (s, 3H, OCH_3), 3.71 (s, 3H, OCH_3), 6.80 (dd, *J* = 8.5, 3 Hz, 1H, H4), 6.86 (d, *J* = 9.0 Hz, 1H, H5), 6.92 (d, *J* = 8.5 Hz, 2H, H3', H5'), 7.08 (d, *J* = 16.5 Hz, 1H, Hα), 7.17 (d, *J* = 3.0 Hz, 1H, H2), 7.35 (d, *J* = 16.5 Hz, 1H, Hα'), 7.51 (d, *J* = 9.0 Hz, 2H, H2', H6')

***(E)* – 3, 3', 4, 5' tetramethoxystilbene [12]**

White crystals. Yield = 40%. mp = 68=69 °C (lit: 67 °C [Murias et al. 2004]). Column chromatography (hexane:ethyl acetate = 6:1).

^{1}H NMR ($CDCl_3$) δ 3.86 (s, 6H, 2 x OCH_3), 3.93 (s, 3H, OCH_3), 3.97 (s, 3H, OCH_3), 6.41 (t, *J* = 2.3 Hz, 1H, H4'), 6.69 (d, *J* = 2.5 Hz, 2H, H2', H6'), 6.89 (d, *J* = 8.0 Hz, 1H, H5), 6.93 (d, *J* = 16.0 Hz, 1H, Hα), 7.06 (d, *J* = 16.0 Hz, 1H, Hα'), 7.08 (m, 2H, H2, H6)

***(E)* – 2, 3', 4, 5' tetramethoxystilbene [13]**

Colourless crystals. Yield = 19%. mp = 81-82 °C (lit: 81-82 °C [Li et al.2006]). Column chromatography (hexane: ethyl acetate = 6:1).

^{1}H NMR ($CDCl_3$) δ 3.86 (s, 6H, 2 x OCH_3), 3.86 (s, 3H, OCH_3), 3.90 (s, 3H, OCH_3), 6.40 (t, *J* = 2.0 Hz, 1H, H4'), 6.50 (d, *J* = 2.5 Hz, 1H, H3), 6.54 (dd, *J* = 8.5, 2.5 Hz, 1H, H5), 6.70 (d, *J* = 2.0 Hz, 2H, H2', H6'), 6.97 (d, *J* = 16.0 Hz, 1H, Hα), 7.39 (d, *J* = 16.0 Hz, 1H, Hα'), 7.52 (d, *J* = 8.5 Hz, 1H, H6)

***(E)* – 3, 4, 4', 5 tetramethoxystilbene [14]**

White crystals. Yield = 21%. mp = 157-158 °C (lit: 157-159 °C [Murias et al. 2004]). Column chromatography (hexane: ethyl acetate = 6:1).

^{1}H NMR ($CDCl_3$) δ 3.86 (s, 3H, OCH_3), 3.89 (s, 3H, OCH_3), 3.94 (s, 6H, 2 x OCH_3), 6.75 (s, 2H, H2, H6), 6.93 (d, *J* = 16.5 Hz, 1H, Hα), 6.93 (d, *J* = 2.0 Hz, 2H, H3', H5'), 7.00 (d, *J* = 16.5 Hz, 1H, Hα'), 7.47 (d, *J* = 9.0 Hz, 2H, H2', H6')

***(E)* – 3, 3', 5, 5' tetramethoxystilbene [16]**

White crystals. Yield = 18%. mp = 129-130 °C (lit: 129-135 °C [Murias et al. 2004]). Column chromatography (hexane: ethyl acetate = 5:1).

^{1}H NMR ($CDCl_3$) δ 3.86 (s, 12H, 4 x OCH_3), 6.43 (t, *J* = 2.3 Hz, 2H, H4, H4'), 6.70 (d, *J* = 2.0 Hz, 4H, H2, H6, H2', H6'), 7.04 (s, 2H, Hα, Hα')

***(E)* – 2, 3', 5, 5' tetramethoxystilbene [17]**

Milk white amorphous solid. Yield = 22%. mp = 54-55 °C (lit: 54-55 °C [Li et al. 2006]). Column chromatography (hexane: ethyl acetate = 6:1).

^{1}H NMR ($CDCl_3$) δ 3.85 (s, 3H, OCH_3), 3.86 (s, 6H, 2 x OCH_3), 3.87 (s, 3H, OCH_3), 6.42 (s, 1H, H4'), 6.73 (d, *J* = 2.0 Hz, 2H, H2', H6'), 6.83 (dd, *J* = 6.8, 1.6 Hz, 1H, H4), 6.87 (d, J = 9.0 Hz, 1H, H3), 7.06 (d, *J* = 16.5 Hz, 1H, Hα), 7.17 (d, *J* = 2.0 Hz, 1H, H6), 7.46 (d, *J* = 16.5 Hz, 1H, Hα')

***(E)* – 3, 3', 4, 5, 5' pentamethoxystilbene [18]**

White crystals. Yield = 74%. mp = 132 °C (lit: 136-138 °C [Murias et al. 2004]) Recrystallise (EtOH/water).

^{1}H NMR ($CDCl_3$) δ 3.86 (s, 6H, 2 x OCH_3), 3.90 (s, 3H, OCH_3), 3.94 (s, 6H, 2 x OCH_3), 6.42 (t, *J* = 2.5 Hz, 1H, H4'), 6.69 (d, *J* = 2.0 Hz, 2H, H2, H6), 6.76 (s, 2H, H2', H6'), 6.96 (d, *J* = 16.0 Hz, 1H, Hα), 7.04 (d, *J* = 16.0 Hz, 1H, Hα')

***(E)* – 3-fluoro-4'-methoxy stilbene [19]**

Colourless crystals. Yield = 1%. Mp = 108-110 °C (lit: 108-110 °C [Ager et al. 1972]). Recrystallise (EtOH/water).

^{1}H NMR ($CDCl_3$) δ 3.86 (s, 3H, OCH_3), 6.93 (d, *J* = 8.5 Hz, 1H, H6), 6.94 (d, *J* = 10.0 Hz, 1H, H4), 6.95 (d, *J* = 16.5 Hz, 1H, Hα), 7.07 (d, *J* = 16.5 Hz, 1H, Hα'), 7.21 (dt, *J* = 10.5, 2 Hz, 1H, H5), 7.26 (d, *J* = 8.0 Hz, 2H, H2,' H6'), 7.32 (m, 1H, H2), 7.48 (d, *J* = 8.5 Hz, 2H, H3', H5')

***(E)* – 3'-methoxy 3-fluoro stilbene [20]**

Colourless oil. Yield = 48%. (lit: Mitchell and Phillips 1975). Column chromatography (hexane: ethyl acetate = 9:1).

^{1}H NMR ($CDCl_3$) δ 3.89 (s, 3H, OCH_3), 6.88 (dd, *J* = 2.5, 8.5 Hz, 1H, H4'), 7.00 (t, *J*= 9.0 Hz, 1H, H5), 7.10 (m, 3H, Hα', H2', H6'), 7.15 (d, *J* = 16.0 Hz, 1H, Hα), 7.25 (d, *J* = 10.0 Hz, 1H, H4), 7.33 (m, 3H, H2, H6, H5').

***(E)* – 3'-methoxy 4-fluoro stilbene [28]**

White crystals. Yield = 7%. Mp = 45-46 °C (lit: 43-44 °C [Ager et al. 1972]). Column chromatography (hexane: ethyl acetate = 9:1).

^{1}H NMR ($CDCl_3$) δ 3.88 (s, 3H, OCH_3), 6.86 (dd, *J*= 2.5, 8.5 Hz, 1H, H4'), 7.03 (t, *J* = 16.0 Hz, 2H, Hα, Hα'), 7.10 (m, 4H, H2, H6, H2', H6'), 7.31 (t, *J* = 8.0 Hz, 1H, H5'), 7.50 (q, *J* = 5.0 Hz, 2H, H3,H5)

***(E)* – 4-fluoro 4'-methoxy stilbene [29]**

White solids. Yield = 79%. Mp = 146-147 °C (lit: 147-149 °C [Pews and Ojha 1969]). Recrystallise (EtOH/water).

^{1}H NMR ($CDCl_3$) δ 3.86 (s, 3H, OCH_3), 6.92 (d, *J* = 8.5 Hz, 2H, H3', H5'), 6.98 (d, *J* = 8.5 Hz, 2H, H2', H6'), 7.06 (t, *J* = 16.0 Hz, 2H, Hα, Hα'), 7.47 (m, 4H, H3, H5, H2, H6)

***(E)* – 4-nitro 3'-methoxy stilbene [33]**

Light orange solid. Yield = 52%. Mp = 78-79 °C (lit: 87-88 °C [Guesten & Salzwedel 1967]). Recrystallise (EtOH/water).

^{1}H NMR ($CDCl_3$) δ 3.89 (s, 3H, OCH_3), 6.92 (dd, *J* = 2.5, 8.5 Hz, 1H, H4'), 7.10 (t, *J* = 2.0 Hz, 1H, H2'), 7.16 (d, *J* = 16.5 Hz, 1H, Hα '), 7.17 (d, *J* = 8.0 Hz, 1H, H6'), 7.26 (d, 1H, *J* = 16.0 Hz, 1H, Hα), 7.34 (t, *J* = 8.0 Hz, 1H, H5'), 7.62 (d, *J* = 8.5 Hz, 2H, H2, H6), 8.24 (d, *J* = 9.0 Hz, 2H, H3, H5)

***(E)* – 4-nitro 4'-methoxy stilbene [34]**

Orange solid. Yield = 41%. Mp = 126-127 °C (lit: 133 °C [Guesten and Salzwedel 1967]). Recrystallise (EtOH/water).

^{1}H NMR ($CDCl_3$) δ 3.88 (s, 3H, OCH_3), 6.96 (d, *J* = 9.0 Hz, 2H, H3', H5'), 7.03 (d, *J* = 16.0 Hz, 1H, Hα'), 7.25 (d, *J* = 16.0 Hz, 1H, Hα), 7.52 (d, *J* = 9.0 Hz, 2H, H2', H6'), 7.62 (d, *J* = 9.0 Hz, H2, H6) 8.23 (d, *J* = 9.0 Hz, 2H, H3, H5).

***(E)* – 4-bromo 3'-methoxy stilbene [35]**

Yellow crystals. Yield = 30%. Mp = 80-81 °C [lit: Alowi et al. 2006]

Recrystallise (EtOH/water).

^{1}H NMR ($CDCl_3$) δ 3.88 (s, 3H, OCH_3), 6.86 (dd, *J* = 2, 8.5 Hz, 1H, H4'), 7.04 (d, *J* = 16.5 Hz, 1H, Hα), 7.06 (d, *J* = 3.0 Hz, 1H, H2'), 7.10 (d, *J* = 16.0 Hz, 1H, Hα'), 7.13 (d, *J* = 8.0 Hz, 1H, H6'), 7.31 (d, *J* = 8.0 Hz, 1H, H5') , 7.40 (d, *J* = 8.5 Hz, 2H, H2, H6), 7.50 (d, *J* = 8.5 Hz, 2H, H3, H5).

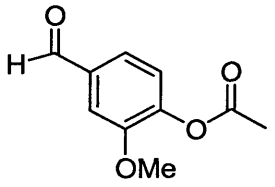
***(E)* – 4-bromo 4'-methoxy stilbene [36]**

Pale yellow solid. Yield = 69%. Mp = 181-182 °C (lit: 177-179 °C [Diana et al. 1978]). Recrystallise (EtOH/water).

^{1}H NMR ($CDCl_3$) δ 3.86 (s, 3H, OCH_3), 6.92 (d, *J* = 16.0 Hz, 1H, Hα), 6.93 (d, *J* = 8.0 Hz, 2H, H3', H5'), 7.07 (d, *J* = 16.0 Hz, 1H, Hα'), 7.37 (d, *J* = 8.0 Hz, 2H, H2', H6'), 7.48 (t, *J* = 8.0 Hz, 4H, H3,H5, H2, H6).

Synthesis of protected acetyl vanillin

A solution of acetic anhydride (10.9 mmol) in ether (7.6 mL) was added to a well stirred mixture of 3-methoxy 4-hydroxy benzaldehyde (vanillin) (10 mmol) and NaOH (10 mmol) in water (7.6 mL) in ice-cold condition. After the addition was complete the reaction mixture was stirred at RT for 30 min. The organic layer was separated and the aqueous layer was extracted with ether (2 x 10 mL). The combined organic extracts were washed with water, dried and evaporated to give the acetyl vanillin as white solid.



4-formyl-2-methoxyphenyl acetate [15a]

White powder. Yield = 92%. Mp = 75-76° C (lit: 75-76° C [Banerjee et al. 2006])

^{1}H NMR ($CDCl_3$) δ 2.37 (s, 3H, CH_3), 3.94 (s, 3H, OCH_3), 7.25 (d, *J* = 8.0 Hz, 1H, H5), 7.50 (d, *J* = 8.0 Hz, 1H, H6), 7.53 (s, H-3), 9.98 (s, CHO)

6.2.2 General method for the synthesis of 4-hydroxy 3'-methoxy (*E*)-stilbene.

A mixture of 4-hydroxy benzaldehydes (6.5 mmol), 3-methoxy phenylacetic acid (7.2 mmol), methylimidazole (9.8 mmol), piperidine (9.8 mmol) and polyethylene glycol (PEG - average molecular weight: 200) (4-5 mL) were added in a 50 mL round bottom flask. The flask was shaken well and irradiated under focused monomode microwave system (50 W, 160 °C) fitted with reflux condenser for 10 min. After the completion of the reaction, the reaction mixture was cooled and acidified with diluted 1M HCl (pH 5). Then the aqueous layer was extracted with ethyl acetate (2 x 20mL) and the organic layer was dried over magnesium sulphate to obtain crude product. It was further purified by column chromatography with a 1:5 mixture of ethyl acetate and hexane to give the pure hydroxylated stilbene analogues.

(E) – 3'-methoxy 4-hydroxy stilbene [22]

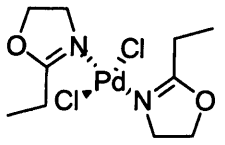
White solid. Yield = 25%. mp = 120-122 °C (lit: 132-134 °C [Sinha et al. 2007])

^{1}H NMR ($CDCl_3$) δ 3.88 (s, 3H, OCH_3), 6.83 (d, *J* = 2.5 Hz, 1H, H2'), 6.86 (d, *J* = 8.5 Hz, 2H, H3, H5), 6.97 (d, *J* = 16.5 Hz, 1H, Hα), 7.07 (d, *J* = 16.5 Hz, 1H, Hα'), 7.12 (d, *J* = 7.5 Hz, 2H, H4',H6'), 7.30 (t, *J* = 8 Hz, 1H, H5'), 7.43 (d, *J* = 8.5 Hz, 2H, H2, H6).

6.2.3 Synthesis of fluorinated stilbenes via Heck reaction:

Synthesis of palladium-oxazoline complex.

To a methanol solution of Li_2PdCl_4 (0.28M; 2.0 mL, 0.56 mmol) was added MeOH (7 mL) and 2-ethyl-2-oxazoline (1.0 mL; 9.9 mmol) at room temperature in air. The mixture was stirred for 3 h during which time an orange precipitate formed. This solid was then isolated by filtration and washed with MeOH (3 x 7 mL), petroleum ether (3 x 7mL) and diethyl ether (3 x 7 mL) and dried in air. The palladium-oxazoline complex was obtained as an orange solid.



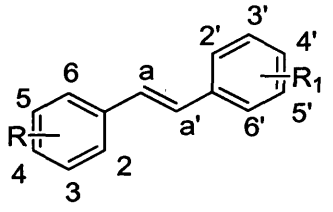
Palladium oxazoline complex (Pd-Ox) [29a]

Orange solid. Yield = 70%. Mp = 173-174 °C (lit: 178-179 °C [Gossage et al. 2004])

^{1}H NMR ($CDCl_3$) δ 1.42 (t, *J* = 8.0 Hz, 3H, CH_3), 3.02 (q, *J* = 8.0 Hz, *J* = 16.0 Hz, 2H, CH_2CH_3), 3.99 (t, *J* = 10.0 Hz, 2H, CH_2N), 4.45 (t, *J* = 10.0 Hz, 2H, CH_2O).

General procedure for the synthesis of substituted stilbenes via Heck reaction

Substituted iodobenzene (5 mmol), palladium-oxazoline complex (5 mol%), substituted styrene (5 mmol) and sodium acetate (6 mmol) were dissolved in DMF (5 mL). The mixture was heated between 140-150 °C until the completion of the reaction (5-48 h) monitored by TLC. The reaction mixture was cooled to RT, quenched with water and the precipitate was collected and allowed to dry. The precipitate was then purified by column chromatography (hexane: ethyl acetate = 5:1) to afford the pure compound in moderate yield.



Compound no.	R	R_1
29	4-F	4-OMe
31	4-F	4-F
32 (unprotected)	4-OH	4-F
32 (protected)	4-OAc	4-F

(E) – 4-fluoro 4'-methoxy stilbene [29]

White solids. Yield = 79%. Mp = 146-147°C (lit: 147-149°C [Pews and Ojha 1969])

^{1}H NMR ($CDCl_3$) δ 3.86 (s, 3H, OCH_3), 6.92 (d, *J* = 8.5 Hz, 2H, H3', H5'), 6.98 (d, *J* = 8.5 Hz, 2H, H2', H6'), 7.06 (d, *J* = 16.0 Hz, 1H, Hα), 7.09 (d, *J* = 16.0 Hz, 1H, Hα'), 7.47 (m, 4H, H3, H5, H2, H6)

(E) – 4, 4'-difluoro stilbene [31]

Light yellow crystals. Yield = 55%. Mp = 132-134°C (lit: 130°C [Ager et al. 1972])

^{1}H NMR ($CDCl_3$) δ 7.03 (s, 2H, Hα, Hα'), 7.08 (t, *J* = 9.0 Hz, 4H, H3, H5, H3', H5'), 7.49 (dd, *J* = 2.5, 9.0 Hz, 4H, H2, H6, H2', H6').

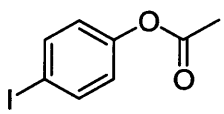
(E) – 4-fluoro 4'-hydroxy stilbene [32]

Yellowish solid. Yield = 87%. Mp = 179-180 °C (lit: 179-180 °C [Lion et al. 2005])

^{1}H NMR ($CDCl_3$) δ 6.77 (d, 2H, H2', H6', *J* = 9.0 Hz), 7.01 (d, *J* = 16.5 Hz, 1H, Hα'), 7.09 (d, *J* = 16.5 Hz, 1H, Hα), 7.18 (t, *J* = 9.0 Hz, 2H, H2, H6), 7.41 (d, *J* = 9.0 Hz, 2H, H3', H5'), 7.58 (m, 2H, H3, H5), 9.56 (s, 1H, OH)

Synthesis of protected iodophenol (acetic acid 4-iodophenol ester)

Acetic anhydride (4.3 mL, 45.47 mmol) was added to a solution of 4-iodophenol (1.0 g, 4.55 mmol) in pyridine (5 mL). After stirring for 24 h, the reaction was concentrated *in vacuo* and purified by flash chromatography (10 hexane: 1 ethyl acetate) to yield the protected iodophenol as a white solid.



Acetic-acid 4-iodophenol ester [32a]

White crystals. Yield = 88%. Mp = 46-47 °C (lit: 45-46 °C [Adamski-Werner et al. 2004])

^{1}H NMR ($CDCl_3$) δ 2.3 (s, 3H, CH_3), 6.89 (d, *J* = 9 Hz, 2H, H-2, H6), 7.71 (d, *J* = 9 Hz, 2H, H3, H5)

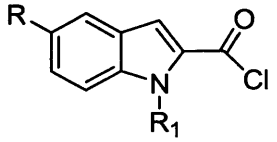
Method for synthesis of substituted stilbenes via Heck reaction in PTS

(dtbpf) $PdCl_2$ (0.01 mmol) and 4-fluoroiodobenzene (0.5 mmol) were added under inert atmosphere to a flask equipped with a large stir bar and a septum. Commercially available PTS solution (1.0 mL), triethylamine (1.5 mmol) and 4-methoxy styrene (1.0 mmol) were added via syringe. The heterogenous mixture was stirred vigorously at RT, becoming pseudo-homogenous after 20-40 minutes. After the completion of the reaction, the mixture was diluted with ethyl acetate (~1.5 mL) and filtered through a pad of silica gel using ethyl acetate as the eluent. The volatiles were removed *in vacuo.* The crude product was purified by recrystallisation in ethanol to afford compound **29** in moderate yield (35%) (NMR as previously described).

6.3 Synthesis of indole-containing 3,5-diarylisoxazoles as potential anticancer agents

6.3.1 Synthesis of 3-(1H-indol-2-yl)-5-phenylisoxazoles and 3-(1-methyl-1H-indol-2-yl)-5-phenylisoxazoles via condensation or cyclisation of alkynyl ketones and hydroxylamine

General procedure for the synthesis of acid chlorides



(61a) ; R = OMe; R1 = H

(63a) ; R = H; R1 = CH3

To a stirred suspension of indole-2-carboxylic acid (20 mmol) in dry dichloromethane (30 mL), oxalyl chloride (25 mmol) was added followed by a catalytic amount of DMF (1-2 drops). The mixture was stirred at room temperature for 24 hours. The resulting solution was dried *in vacuo* to afford the acid chlorides in excellent yield which were used straight away in the next reaction.

5-Methoxy-1*H*-indole-2-carbonyl chloride [61a]

Prepared according to the general procedure for the synthesis of acid chlorides, using 5-methoxy-1*H*-indole-2-carboxylic acid as the starting material.

Dark green solid. Yield: 97%; m.p: 186-188 °C [ref: Wahlstroem et al. 2004].

^{1}H NMR ($CDCl_3$) δ 3.76 (s, 3H, OCH_3), 6.89 (dd, *J* = 2.5, 9.0 Hz, 1H, H5), 6.99 (d, *J* = 1.5 Hz, 1H, H3), 7.10 (d, *J* = 1.0 Hz, 1H, H4), 7.32 (d, *J* = 9.0 Hz, 1H, H6), 11.95 (s, 1H, NH).

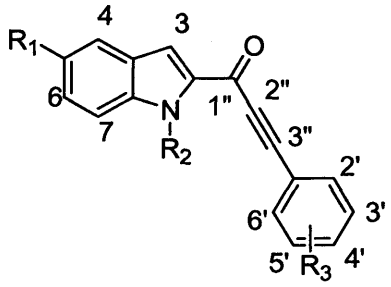
1-Methyl-1*H*-indole-2-carbonyl chloride [63a]

Prepared according to the general procedure for the synthesis of acid chlorides, using 1-methyl-1*H*-indole-2-carboxylic acid as the starting material.

Brown solid. Yield: 98%; m.p.: 123 °C [ref: Eggers et al. 2007].

^{1}H NMR ($CDCl_3$) δ 4.00 (s, 3H, CH_3), 7.11 (t, *J* =8.0 Hz, 1H, H5), 7.22 (s, 1H, H3), 7.32 (td, *J* = 1.0, 8.0 Hz, 1H, H6), 7.56 (d, *J* = 9.0 Hz, 1H, H4), 7.68 (d, *J* = 9.0 Hz, 1H, H7).

General procedure for preparation of 1-(1H-indol-2-yl)-3-(phenyl)-prop-2-yn-1-one and 1-(1-methyl-1H-indol-2-yl)-3-(phenyl)-prop-2-yn-1-one



Compound no:	R1	R2	R3
61c	OCH_3	H	3-OCH_3
62c	OCH_3	H	3-F
63c	H	CH_3	4-OCH_3
64c	H	CH_3	3-F
65c	H	CH_3	3-OCH_3

To a solution of indole-2-carbonylchloride (1.55 mmol) and terminal alkyne (1.1 mmol) in dry THF was added triethylamine (1.1 mmol), CuI (3 mol%) and $PdCl_2(PPh_3)_2$ (0.9 mol%). The mixture was stirred at room temperature under nitrogen atmosphere. The reaction was completed in 30 minutes to 1 hour by TLC analysis. The mixture was washed with water (5 mL) and extracted with ethyl acetate (5 mL x 3) to remove the amine hydrochloric acid salt. The organic layer was dried over $MgSO_4$ and evaporated *in vacuo*. The

resulting crude compound was purified by column chromatography (hexane: ethyl acetate = 4:1).

1-(5-methoxy-1*H*-indol-2-yl)-3-(3-methoxyphenyl)-prop-2-yn-1-one [61c]

Prepared according to general procedure starting with 5-methoxyindol-2-carbonylchloride and 3-ethynylanisole.

Green solid. Yield: 23%, m.p.: 147°C.

^{1}H NMR ($CDCl_3$) δ 3.88 (s, 3H, OCH_3), 3.89 (s, 3H, OCH_3), 7.06 (d, *J* = 2.0 Hz, 1H, H2'), 7.08 (d, *J* = 2.5 Hz, 1H, H3), 7.11 (dd, *J* = 2.0, 8.0 Hz, 1H, H4'), 7.15 (d, *J* = 2.5 Hz, 1H, H4), 7.23 (d, *J* = 9.0 Hz, 1H, H5'), 7.39 (d, *J* = 8.0 Hz, 1H, H5), 7.41 (d, *J* = 8.0 Hz, 1H, H6), 7.45 (dd, *J* = 2.0, 8.0 Hz, 1H, H6'), 8.97 (s, 1H, NH).

^{13}C NMR ($CDCl_3$) δ 55.45, 55.67 (OCH_3 x 2), 86.52, 91.61 (C2", C3"), 102.82, 113.03, 113.26, 117.51 (C3, C4, C6, C7), 118.96, 119.35, 127.57, 129.81 (C2', C4', C5', C6'), 121.15, 127.89, 133.73 (C2, C3a, C7a), 137.12 (C1'), 155.04 (C3'), 159.50 (C5), 168.50 (C1").

1-(5-methoxy-1*H*-indol-2-yl)-3-(3-fluorophenyl)-prop-2-yn-1-one [62c]

Prepared according to general procedure starting with 5-methoxy indol-2-carbonyl chloride and 3-fluorophenyl acetylene.

Light green solid. Yield: 33%, m.p.: 142 °C.

^{1}H NMR ($CDCl_3$) δ 3.89 (s, 3H, OCH_3), 7.11 (dd, *J* =2.5, 9.0 Hz, 1H, H6'), 7.14 (d, *J* = 2.5 Hz, 1H, H3), 7.23 (td, *J* =2.5, 9.0 Hz, 1H, H5'), 7.36 (d, *J* = 9.0 Hz, 1H, H5), 7.37-7.44 (m, 2H, H 2', H4'), 7.46 (d, *J* = 2.0 Hz, 1H, H4), 7.51 (d, *J* = 7.5 Hz, 1H, H6), 9.17 (s, 1H, NH)

^{13}C NMR ($CDCl_3$) δ 55.67 (OCH_3), 87.07, 89.63 (C2", C3"), 102.83, 113.20, 113.32, 130.40 (C3, C4, C6, C7), 118.08 (d, *J* = 21.4 Hz, C2'), 119.56 (d, *J* = 22.7 Hz, C4'), 122.0 (d, *J* = 10.0 Hz, C1'), 127.89 (C7a), 128.89 (d, *J* = 2.5 Hz, C5'), 130.46 (C6'), 133.75 (C3a), 136.96 (C2), 155.11 (C5), 161.35 (d, *J* = 249.5 Hz, C3'), 168.18 (C1").

1-(1-methyl-1*H*- indol-2-yl)-3-(4-methoxyphenyl)-prop-2-yn-1-one [63c]

Prepared according to general procedure starting with 1-methylindol-2-carbonyl chloride and 4-ethynylanisole.

White solid. Yield: 64%, m.p.: 112 °C.

^{1}H NMR ($CDCl_3$) δ 3.88 (s, 3H, OCH_3), 4.15 (s, 3H, CH_3), 7.97 (d, *J* = 9.0 Hz, 2H, H3', H5'), 7.20 (td, *J* = 2.0, 8.0 Hz, 1H, H5), 7.40 (td, *J* = 2.5, 8.0 Hz, 1H, H6), 7.46 (d, *J* = 9.0 Hz, 1H, H4), 7.66 (s, 1H, H3), 7.67 (d, *J* = 9.0 Hz, 2H, H2', H6'), 7.77 (d, *J* = 7.5 Hz, 1H, H7)

^{13}C NMR ($CDCl_3$) δ 32.08 (CH_3), 55.45 (OCH_3), 87.81, 90.75 (C2", C3"), 110.41 (C3', C5'), 114.40, 115.97, 120.97, 123.29, 126.67 (C3, C4, C5, C6, C7), 112.23, 126.01 (C3a, C7a), 134.90 (C2', C6'), 136.28 (C1'), 140.93 (C2), 161.52 (C4), 169.87 (C1").

1-(1-methyl-1*H*-indol-2-yl)-3-(3-fluorophenyl)-prop-2-yn-1-one [64c]

Prepared according to general procedure starting with 1-methylindol-2-carbonylchloride and 3-fluorophenyl acetylene.

Yellow solid. Yield: 64%, m.p.: 94 °C

^{1}H NMR ($CDCl_3$) δ 4.12 (s, 3H, CH_3), 7.19-7.23 (m, 2H, H2', H6'), 7.37 (dd, *J* = 2.5, 9.5 Hz, 1H, H4'), 7.39 (m, 1H, H5'), 7.42 (t, *J* = 8.0 Hz, 1H, H5), 7.45 (t, *J* = 8.0 Hz, 1H, H6), 7.50 (dd, *J* = 2.0, 8.0 Hz, 1H, H4), 7.65 (s, 1H, H3), 7.76 (dd, *J* = 2.0, 8.0 Hz, 1H, H7)

^{13}C NMR ($CDCl_3$) δ 31.62 (CH_3), 87.72, 88.28 (C2", C3"), 110.60, 116.68, 121.16, 123.20, 127.08 (C3, C4, C5, C6, C7), 117.85 (d, *J* = 20.2 Hz, C2'), 119.45 (d, *J* = 22.7 Hz, C4'), 128.82 (C6'), 130.41 (d, *J* = 8.8 Hz, C5'), 122.30, 125.99 (C3a, C7a), 135.93 (C1'), 141.13 (C2), 161.35 (d, *J* = 246.5 Hz, C3'), 169.20 (C1").

1-(1-methyl-1*H*-indol-2-yl)-3-(3-methoxyphenyl)-prop-2-yn-1-one [65c]

Prepared according to general procedure starting with 1-methylindol-2-carbonyl chloride and 3-ethynylanisole.

Yellow solid. Yield: 95%, m.p.: 75 °C

^{1}H NMR ($CDCl_3$) δ 3.87 (s, 3H, OCH_3), 4.13 (s, 3H, CH_3), 7.06 (dq, *J* = 1.5, 8.0 Hz, 1H, H4'), 7.21 (td, *J* = 2.0, 8.0 Hz, 1H, H5'), 7.23 (s, 1H, H2'), 7.33 (td, *J* = 2.0, 8.0 Hz, 1H, H5), 7.36 (d, *J* = 8.0 Hz, 1H, H4), 7.40 (dd, *J* = 2.0, 8.0

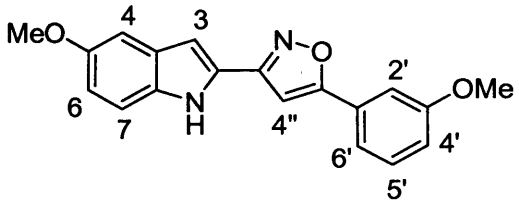
Hz, 1H, H6'), 7.46 (td, *J* = 2.0, 8.0 Hz, 1H, H6), 7.68 (s, 1H, H3), 7.76 (d, *J* = 8.0 Hz, 1H, H7)

^{13}C NMR ($CDCl_3$) δ 32.07 (CH_3), 55.44 (OCH_3), 87.71, 89.59 (C2", C3"), 110.45, 117.27, 117.51, 117.59, 121.20 (C3, C4, C5, C6, C7), 123.39, 125.46, 126.49, 127.73 (C2', C4', C5' C6'), 121.36, 126.01 (C3a, C7a), 136.11 (C1'), 141.05 (C2), 159.50 (C3'), 169.59 (C1").

General procedure for the synthesis of 3,5-diaryl isoxazoles (via condensation/cyclisation of alkynyl ketones and hydroxylamine)

To a stirred solution of 1-(1*H*-indol-2-yl)-3-(phenyl)-prop-2-yn-1-one and 1-(1-methyl-2-indol-2-yl)-3-(phenyl)-prop-2-yn-1-one (**61c** to **65c**) in ethanol (15 ml) and pyridine (5 ml) was added hydroxylamine hydrochloride (2.8 mmol). The mixture was heated under reflux for 5 hours and monitored by TLC. The reaction mixture was cooled and the solvent was evaporated to dryness. Water (10 mL) was added to the residue and extracted with ethyl acetate (10 mL x 3). The organic layer was dried and evaporated. The crude compound was further purified by column chromatography (hexane: ethyl acetate = 6:1) as eluent.

3-(5-Methoxy-1*H*-indol-2-yl)-5-(3-methoxyphenyl)isoxazole (61):



Prepared according to the general procedure starting with 1-(5-methoxy-1*H*-2-indol-2-yl)-3-(3-methoxyphenyl)-prop-2-yn-1-one **[61c]**.

Orange solid. Yield: 25%; m.p.: 202 °C

^{1}H NMR ($CDCl_3$) δ 3.90 (s, 3H, OCH_3), 3.91 (s, 3H, OCH_3), 6.80 (s, 1H, H4"), 6.97 (d, *J* = 2.0 Hz, 1H, H2'), 6.99 (d, *J* = 2.5 Hz, 1H, H3), 7.05 (dd, *J* = 2.0, 8.0 Hz, 1H, H4'), 7.12 (d, *J* = 2.5 Hz, 1H, H4), 7.35 (d, *J* = 9.0 Hz, 1H, H5'), 7.40 (d, *J* = 8.0 Hz, 1H, H6), 7.42 (d, *J* = 8.0 Hz, 1H, H7), 7.46 (dd, *J* = 2.0, 8.0 Hz, 1H, H6'), 8.76 (s, 1H, NH).

^{13}C NMR ($CDCl_3$) δ 55.43, 55.78 (OCH_3 x 2), 97.32 (C4"), 102.35, 103.31, 111.87, 112.32 (C3, C4, C6, C7), 115.18, 116.29, 119.41, 130.04 (C2', C4', C5', C6'), 125.57, 128.62 (C3a, C7a), 131.88 (C2), 132.11 (C1'), 154.90 (C3'), 160.03 (C5), 162.97 (C5"), 163.80 (C3").

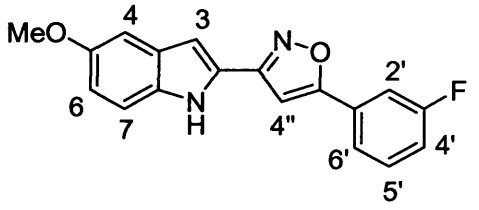
MS (EI positive ion) *m/z* 320.12 (M^+)

HRMS (ES) *m/z* = 321.1236 $[M+H]^+$; calcd: 320.1161.

3-(5-Methoxy-1*H*-indol-2-yl)-5-(3-fluorophenyl)isoxazole (62) and 5-(5-Methoxy-1*H*-indol-2-yl)-3-(3-fluorophenyl)isoxazole (62b):

Prepared according to the general procedure starting with 1-(5-methoxy-1*H*-2-indol-2-yl)-3-(3-fluorophenyl)-prop-2-yn-1-one **[62c]**. The first isomer **(62)** was eluted first followed by the second isomer **(62b)**, while a few fractions remain inseparable as a mixture of both isomers.

3-(5-Methoxy-1*H*-indol-2-yl)-5-(3-fluorophenyl)isoxazole (62)



Light yellow crystals. Yield: 2%; m.p.: 214 °C

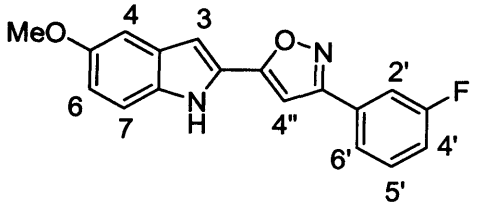
^{1}H NMR ($CDCl_3$) δ 3.90 (s, 3H, OCH_3), 6.88 (s, 1H, H4-isoxazole), 6.91 (d, *J* =2.5 Hz, 1H, H4), 6.99 (dd, *J* =2.5, 9.5 Hz, 1H, H4'), 7.14 (d, *J* = 2.0 Hz, 1H, H3), 7.20 (m, 1H, H2'), 7.38 (d, *J* = 9.0 Hz, 1H, H6), 7.51 (dd, *J* = 5.0, 9.5 Hz, 1H, H5'), 7.59 (d, *J* = 9.5 Hz, 1H, H6'), 7.67 (d, *J* = 8.0 Hz, 1H, H7), 8.94 (s, 1H, NH)

^{13}C NMR ($CDCl_3$) δ 55.82 (OCH_3), 98.19 (C4"), 102.44, 104.12, 112.19, 114.83 (C3, C4, C6, C7), 113.05 (d, *J* = 23.4 Hz, C2'), 117.30 (d, *J* = 21.8 Hz, C4'), 121.64 (C6'), 131.80 (d, *J* = 7.8 Hz, C5'), 126.77, 129.84 (C3a, C7a), 131.23 (C1'), 134.77 (C2), 151.32 (C5"), 155.17 (C5), 164.23 (d, *J* = 246.2 Hz, C3') 166.13 (C3").

MS (EI positive ion) *m/z* 308.10 (M^+)

HRMS (ES) *m/z* = 309.1034 $[M+H]^+$; calcd: 308.0961.

5-(5-Methoxy-1*H*-indol-2-yl)-3-(3-fluorophenyl)isoxazole (62b)



Yellow solid. Yield: 5%; m.p.: 117-118 °C

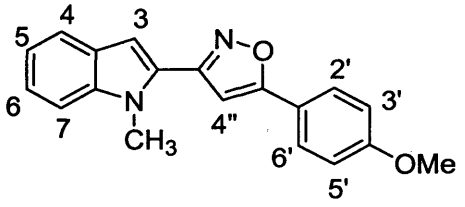
^{1}H NMR ($CDCl_3$) δ 3.90 (s, 3H, OCH_3), 6.79 (s, 1H, H4"), 6.99 (d, *J* = 2.0 Hz, 1H, H4), 7.00 (dd, *J* = 2.5, 9.0 Hz, 1H, H4'), 7.13 (d, *J* = 2.0 Hz, 1H, H3), 7.20 (m, 1H, H2'), 7.36 (d, *J* = 9.0 Hz, 1H, H6), 7.49 (dd, *J* = 5.0, 9.0 Hz, 1H, H5'), 7.60 (d, *J* = 9.5 Hz, 1H, H6'), 7.67 (d, *J* = 8.0 Hz, 1H, H7), 8.67 (s, 1H, NH)

^{13}C NMR ($CDCl_3$) δ 55.77 (OCH_3), 97.07 (C4"), 102.37, 103.51, 112.19, 115.37 (C3, C4, C6, C7), 114.01 (d, *J* = 23.9 Hz, C2'), 117.21 (d, *J* = 21.4 Hz, C4'), 122.61 (C6'), 130.60 (d, *J* = 8.8 Hz, C5'), 125.34, 128.60 (C3a, C7a), 130.67 (C1'), 132.12 (C2), 150.90 (C5"), 154.96 (C5), 162.08 (d, *J* = 245.7 Hz, C3'), 164.13 (C3").

MS (EI positive ion) *m/z* 308.10 (M^+)

HRMS (ES) *m/z* = 309.1031 $[M+H]^+$; calcd: 308.0961.

3-(1-methyl-1*H*-indol-2-yl)-5-(4-methoxyphenyl)isoxazole (63)



Prepared according to general procedure starting with 1-(1-methyl-1*H*- indol-2-yl)-3-(4-methoxyphenyl)-prop-2-yn-1-one **[63c]**

Yield: 56%; m.p.: 168 °C

^{1}H NMR ($CDCl_3$) δ 3.90 (s, 3H, OCH_3), 4.07 (s, 3H, CH_3), 6.79 (s, 1H, H4"), 7.03 (d, *J* = 9.0 Hz, 2H, H3', H5'), 7.05 (s, 1H, H3), 7.20 (t, *J* = 8.0 Hz, 1H, H5), 7.36 (t, *J* = 8.0 Hz, 1H, H6), 7.43 (d, *J* = 9.0 Hz, 1H, H4), 7.69 (d, *J* = 7.5 Hz, 1H, H7) , 7.86 (d, *J* = 9.0 Hz, 2H, H2', H6')

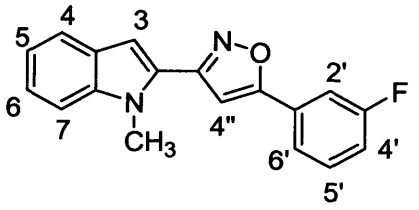
^{13}C NMR ($CDCl_3$) δ 31.83 (CH_3), 55.39 (OCH_3), 99.55 (C4"), 109.82 (C3', C5'), 104.69, 114.41, 120.51, 121.52, 123.65 (C3, C4, C5, C6, C7), 121.18,

127.25 (C3a, C7a), 128.28 (C2', C6'), 127.30 (C1'), 138.88 (C2), 161.16 (C4'), 162.27, 164.05 (C3", C5").

MS (EI positive ion) *m/z* 304.13 (M^+)

Anal. calcd for $C_{19}H_{16}N_2O_2$; C, 74.98; H, 5.30; N, 9.20; found C, 74.84; H, 5.29; N, 9.20.

3-(1-Methyl-1*H*-indol-2-yl)-5-(3-fluorophenyl)isoxazole (64)



Prepared according to general procedure starting with 1-(1-methyl-1*H*- indol-2-yl)-3-(3-fluorophenyl)-prop-2-yn-1-one **[64c]**

Yield: 18%; m.p.: 124 °C

^{1}H NMR ($CDCl_3$) δ 4.07 (s, 3H, CH_3), 6.83 (s, 1H, H4"), 7.08 (d, *J* = 2.0 Hz, 1H, H3), 7.19-7.23 (m, 2H, H2', H6'), 7.37 (td *J* = 2.5, 9.5 Hz, 1H, H5), 7.43 (d, *J* = 8.0 Hz, 1H, H7), 7.49 (q, *J* = 5.0, 9.5 Hz, 1H, H5'), 7.64 (dd, *J* = 2.5, 9.5 Hz, 1H, H4'), 7.68 (d, *J* = 8.0 Hz, 1H, H4), 7.72 (t, *J* = 8.0 Hz, 1H, H6)

^{13}C NMR ($CDCl_3$) δ 31.82 (CH_3), 99.54 (C4"), 105.01, 109.87, 120.62, 121.60, 122.61 (C3, C4, C5, C6, C7), 113.80 (d, *J* = 22.7 Hz, C2'), 117.02 (d, *J* = 21.4 Hz, C4'), 123.86 (C6'), 130.62 (d, *J* = 8.8 Hz, C5'), 127.20, 130.89 (C3a, C7a), 138.95 (C2), 161.73 (C5"), 162.08 (d, *J* = 248.2 Hz, C3'), 164.66 (C3").

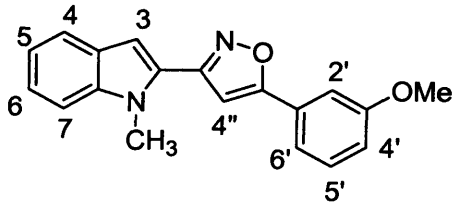
MS (EI positive ion) *m/z* 292.11 (M^+)

Anal. calcd for $C_{18}H_{13}N_2OF$; C, 73.96; H, 4.48; N, 9.58; found C, 73.93; H, 4.40; N, 9.58.

3-(1-Methyl-1*H*-indol-2-yl)-5-(3-methoxyphenyl)isoxazole (65) and 5-(1-Methyl-1*H*-indol-2-yl)-3-(3-methoxyphenyl)isoxazole (65b):

Prepared according to the general procedure starting with 1-(1-methyl-1*H*-2-indol-2-yl)-3-(3-methoxyphenyl)-prop-2-yn-1-one **[65c]**. The first isomer **(65b)** was eluted first followed by the second isomer **(65)**, while a few fractions remain inseparable as a mixture of both isomers.

3-(1-Methyl-1*H*-indol-2-yl)-5-(3-methoxyphenyl)isoxazole (65)



Yellow solid. Yield: 20%; m.p.: 73-74 °C

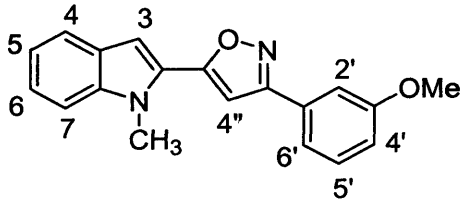
^{1}H NMR ($CDCl_3$) δ 3.92 (s, 3H, OCH_3), 4.04 (s, 3H, CH_3), 6.81 (s, 1H, H4"), 7.05 (dq, *J* = 1.5, 8.0 Hz, 1H, H4'), 7.07 (s, 1H, H2'), 7.21 (td, *J* = 2.0, 8.0 Hz, 1H, H5'), 7.35 (td, *J* = 2.0, 8.0 Hz, 1H, H5), 7.42 (d, *J* = 8.0 Hz, 1H, H4), 7.43 (dd, *J* = 2.0, 8.0 Hz, 1H, H6'), 7.45 (td, *J* = 2.0, 8.0 Hz, 1H, H6), 7.49 (s, 1H, H3), 7.72 (d, *J* = 8.0 Hz, 1H, H7)

^{13}C NMR ($CDCl_3$) δ 31.82 (CH_3), 55.44 (OCH_3), 99.81 (C4"), 104.83, 109.85, 111.88, 120.56, 121.55 (C3, C4, C5, C6, C7), 116.26, 119.14, 123.74, 130.05 (C2', C4', C5' C6'), 127.15, 127.24 (C3a, C7a), 130.06 (C1'), 138.91 (C2), 160.06 (C3'), 162.60, 164.30 (C3", C5").

MS (EI positive ion) *m/z* 304.12 (M^+)

Anal. calcd for $C_{19}H_{16}N_2O_2$; C, 74.98; H, 5.30; N, 9.20; found C, 74.91; H, 5.22; N, 9.23.

3-(1-Methyl-1*H*-indol-2-yl)-5-(3-methoxyphenyl)isoxazole (65b)



Light yellow solid. Yield: 4%; m.p.: 125 °C

^{1}H NMR ($CDCl_3$) δ 3.92 (s, 3H, OCH_3), 4.18 (s, 3H, CH_3), 6.86 (s, 1H, H4"), 6.98 (s, 1H, H2'), 7.04 (dq, *J* = 1.5, 8.0 Hz, 1H, H4'), 7.19 (td, *J* = 2.0, 8.0 Hz, 1H, H5'), 7.34 (td, *J* = 2.0, 8.0 Hz, 1H, H5), 7.42 (d, *J* = 8.0 Hz, 1H, H4), 7.43 (dd, *J* = 2.0, 8.0 Hz, 1H, H6'), 7.46 (s, 1H, H3), 7.47 (td, *J* = 2.0, 8.0 Hz, 1H, H6), 7.71 (d, *J* = 8.0 Hz, 1H, H7)

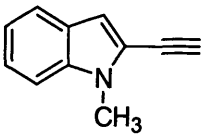
^{13}C NMR ($CDCl_3$) δ 32.44 (CH_3), 55.45 (OCH_3), 99.70 (C4"), 104.94, 109.93, 111.16, 120.19, 121.22 (C3, C4, C5, C6, C7), 116.34, 118.42, 123.29, 130.19 (C2', C4', C5' C6'), 127.32, 128.33 (C3a, C7a), 128.73 (C1'), 139.21 (C2), 157.42 (C3'), 160.05, 169.18 (C3", C5").

MS (EI positive ion) *m/z* 304.13 (M^+)

HRMS (ES) *m/z* = 305.1281 $[M+H]^+$; calcd: 304.1212.

6.3.2 Synthesis of 5-(1-methyl-indol-2-yl)-3-substituted phenylisoxazoles via 1,3-dipolar cycloaddition

Synthesis of 2-ethynyl-1-methyl-indole



(66b)

p-Toluenesulfonylazide: A stirring solution of sodium azide (0.11 mol) in 20 mL water was diluted with ethanol (40 mL) and a warm solution of *p*-toluenesulfonylchloride (190.65 mol) in 100 ml ethanol was added causing a separation of NaCl and a light brown mixture. After stirring for 3.5 hours, most of the solvent was evaporated and 20 mL water was added to the residue where 2 layers are formed. The oily layer is separated, dried ($MgSO_4$) and filtered to afford *p*-toluenesulfonylazide as colourless oil which solidifies at low temperatures (-20 °C) (yield: 89%).

Dimethyl-2-oxopropylphosphonate (1.2 mmol) was added to a suspension of K_2CO_3 (3 mmol) and *p*-toluenesulfonylazide (1.2 mmol) in acetonitrile (15 mL) and the mixture was stirred at room temperature. After 2 hours, 1-methyl indole-2-carboxaldehyde (1 mmol) dissolved in 2 mL methanol was added and the stirring was continued for 24 hours. The solvents were removed *in vacuo* and the resultant residue was extracted with ethyl acetate, dried using magnesium sulfate and concentrated *in vacuo.* Purification with column chromatography (dichloromethane: methanol = 9:1) afforded the pure compound in moderate yield.

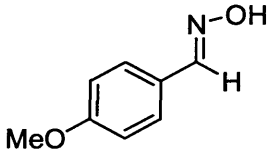
Orange solid. Yield: 44%; m.p.: 55 °C [lit: Banzies et al. 1986].

^{1}H NMR ($CDCl_3$) δ 3.54 (s, 1H, H-acetylene), 3.87 (s, 3H, CH_3), 6.92 (s, 1H, H3), 7.21 (qd, *J* = 2.0, 8.0 Hz, 1H, H5), 7.37 (d, *J* = 8.0 Hz, 1H, H4), 7.36 (qd, *J* = 1.0, 8 Hz, 1H, H6), 7.67 (d, *J* = 8.0 Hz, 1H, H7).

General procedure for synthesis of aldoximes

To a solution of hydroxylamine hydrochloride (7.4 mmol) in water (15 mL) was added sodium bicarbonate (11.2 mmol) portion wise at 0 °C and the mixtures were stirred for 30 min at room temperature. The aldehyde (6.2 mmol) dissolved in methanol (20 mL) was then added to the reaction mixture and the mixture was stirred for additional hour. Water was added to the reaction mixture and the precipitate was collected by filtration to give a pure product of aldoxime.

4-Methoxybenzaldoxime (68b)

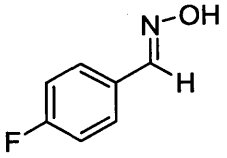


Prepared according to the general procedure for synthesis of aldoxime using 4-methoxybenzaldehyde as the starting material.

Light brown solid. Yield: 78%; m.p.: 55-57 °C [lit: m.p.: 63-66 °C (Owston et al. 2007)].

^{1}H NMR ($CDCl_3$) δ 3.86 (s, 3H, OCH_3), 6.94 (dd, *J* = 2.0, 9.0 Hz, 2H, H3, H5), 7.55 (dd, *J* = 2.5, 8.5 Hz, 2H, H2, H6), 8.13 (s, 1H, C<u>H</u>=N-OH).

4-Fluorobenzaldoxime (69b)



Prepared according the general procedure for synthesis of aldoxime using 4-fluorobenzaldehyde as the starting material.

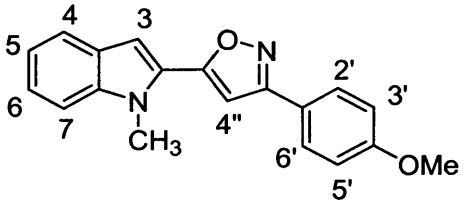
Brown solid. Yield: 86%; m.p.: 88-90°C [lit: m.p.: 115-116 °C (ref: Jain et al. 2005).

^{1}H NMR ($CDCl_3$) δ 2.0 (bs, 1H, CH=N-O<u>H</u>), 7.11 (t, *J* = 8.0 Hz, 2H, H2, H6), 7.59 (dd, *J* = 2.5, 8.5 Hz, 2H, H3, H5), 8.15 (s, 1H, C<u>H</u>=N-OH)

General procedure for the preparation of 3,5-diarylisoxazoles (via 1,3-dipolar cycloaddition)

To a solution of terminal alkyne (1.4 mmol) and triethylamine (0.14 mmol) in anhydrous dichloromethane was added under inert atmosphere a 13% aqueous solution of sodium hypochlorite NaOCl (2.6 mmol), and at 0 °C, a solution of oxime (1.4 mmol) in anhydrous dichloromethane was added dropwise over a period of 1 hour. The reaction was stirred at room temperature for 24 hours. After reaction completion as indicated by TLC, water was added and the mixture was extracted with dichloromethane. The organic layer was dried over $MgSO_4$, evaporated under reduced pressure and the residue was purified by column chromatography using hexane/ethyl acetate (5:1) as eluent.

5-(1-Methyl-1*H*-indol-2-yl)-3-(4-methoxyphenyl)isoxazole (66)



Light brown crystals. Yield: 23%; m.p.: 169 °C

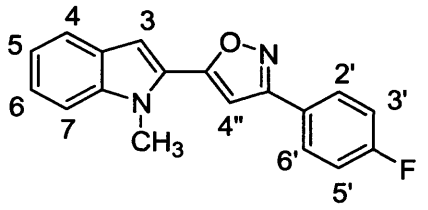
^{1}H NMR ($CDCl_3$) δ 3.91 (s, 3H, OCH_3), 4.06 (s, 3H, CH_3), 6.79 (s, 1H, H4"), 7.03 (dd, *J* = 1.5, 8.5 Hz, 2H, H3', H5'), 7.20 (t, *J* = 8.0 Hz, 1H, H5), 7.35 (t, *J* = 0.5, 8.0 Hz, 1H, H6), 7.42 (d, *J* = 8.5 Hz, 1H, H4), 7.70 (d, *J* = 8.0 Hz, 1H, H7), 7.86 (dd, *J* = 2.0, 8.0 Hz, 2H, H2', H6')

^{13}C NMR ($CDCl_3$) δ 31.84 (CH_3), 55.39 (OCH_3), 99.56 (C4"), 104.96, 109.82, 114.41, 120.51, 121.52 (C3, C4, C5, C6, C7), 121.33, 124.30 (C3a, C7a), 123.65, 128.28 (C2'/C6', C3'/C5'), 127.30 (C1'), 138.88 (C2), 161.16 (C4'), 162.27, 164.05 (C3", C5").

MS (EI positive ion) *m/z* 304.13 (M^+)

Anal. calcd for $C_{19}H_{16}N_2O_2$; C, 74.98; H, 5.30; N, 9.20; found C, 74.55; H, 5.27; N, 9.19.

5-(1-Methyl-1*H*-indol-2-yl)-3-(4-fluorophenyl)isoxazole (67)



Brown crystals. Yield: 29%; m.p.: 145 °C

^{1}H NMR ($CDCl_3$) δ 4.06 (s, 3H, CH_3), 6.80 (s, 1H, H4"), 7.07 (s, 1H, H3), 7.21 (t, *J* = 8.0 Hz, 1H, H5), 7.22 (q, *J* = 2.0, 9.0 Hz, 2H, H2', H6'), 7.36 (t, *J* = 1.0, 8.0 Hz, 1H, H6), 7.44 (d, *J* = 8.5 Hz, 1H, H4), 7.72 (d, *J* = 8.0 Hz, 1H, H7), 7.90 (q, *J* = 2.0, 9.0 Hz, 2H, H3', H5')

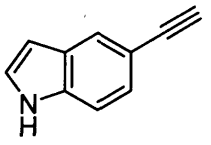
^{13}C NMR ($CDCl_3$) δ 31.84 (CH_3), 99.52 (C4"), 104.92, 109.85, 120.60, 121.57, 123.81 (C3, C4, C5, C6, C7), 116.22 (d, *J* = 22.7 Hz, C3', C5'), 125.08, 127.03 (C3a, C7a), 128.85 (d, *J* = 8.0 Hz, C2', C6'), 127.21 (C1'), 138.93 (C2), 161.75 (C5"), 162.94 (d, *J* = 250.7 Hz, C4'), 164.50 (C3").

MS (EI positive ion) *m/z* 292.11 (M^+)

Anal. calcd for $C_{18}H_{13}FN_2O$; C, 73.96; H, 4.48; N, 9.58; found C, 73.93; H, 4.50; N, 9.59.

6.3.3 Synthesis of 5-(1*H*-indol-5-yl)-3-phenylisoxazoles via 1,3-dipolar cycloaddition

Synthesis of 5-ethynylindole (68a)



A mixture of 5-iodoindole (0.82 mmol), triethylsilylacetylene (1.28 mmol), $Pd(PPh_3)_4$ (0.024 mmol), CuI (0.042 mmol) and triethylamine (0.23 mmoL) in acetonitrile (10 mL) was refluxed for 4 hours. After completion of reaction as indicated by TLC, water was added and the mixture was extracted with ethyl acetate, the organic layer was dried over magnesium sulfate and evaporated *in vacuo*. The residue containing 5-(2-triethylsilylethynyl)-indole **(68a-i)** was used in the next step without further purification.

The residue was dissolved in THF, 1 mmol of 1 M TBAF was added, and the mixture was stirred at room temperature for 24 hours. Water was added and the mixture was extracted with ethyl acetate. The organic layer was dried over magnesium sulfate and evaporated under reduced pressure. The residue was purified by column chromatography using petroleum ether:ethyl acetate (2:1) as eluent to afford compound 5-ethynyl-1*H*-indole **(68a)** in good purity.

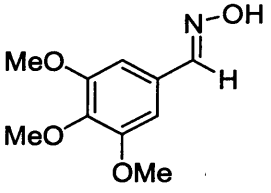
Brown solid. Yield: 61%; m.p.: 59-60 °C

^{1}H NMR ($CDCl_3$) δ 2.95 (s, 1H, H-acetylene), 6.48 (d, *J* = 8.0 Hz, 1H, H7), 7.16 (dd, *J* = 2.0, 8.0 Hz, 1H, H6), 7.29 (s, 2H, H 2, H3), 7.87 (s, 1H, H4), 8.23 (bs, 1H, NH).

^{13}C NMR ($CDCl_3$) δ 74.02 (CH of acetylene), 85.26 (C of acetylene), 101.25, 110.98, 122.93, 124.77, 126.90 (C2, C3, C4, C6, C7), 111.98, 126.87, 137.01 (C3a, C5, C7a).

Synthesis of substituted benzaldoxime

3,4,5-trimethoxybenzaldoxime (70b)



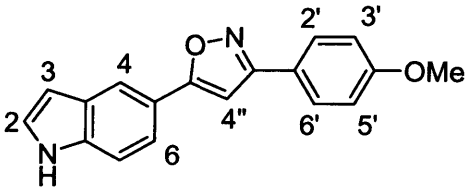
Prepared according the general procedure for synthesis of aldoxime using 3,4,5-trimethoxybenzaldehyde as the starting material.

White solid. Yield: 90%; m.p.: 88-90 °C [lit: m.p.: 95-97 °C (Simoni et al. 2006).

^{1}H NMR ($CDCl_3$) δ 3.90 (s, 9H, OCH_3), 6.84 (s, 2H, H2, H6), 8.08 (s, 1H, CH=N-OH).

Synthesis of 5-(1H-indol-5-yl)-3-phenylisoxazoles

5-(1H-indol-5-yl)-3-(4-meyhoxyphenyl)isoxazole (68)



Prepared according to the general procedure for synthesis of 3,5-diaryl isoxazoles (via 1,3-dipolar cycloaddition) using 5-ethynylindole **(68a)** and 4-methoxybenzaldoxime **(68b)** as the starting materials.

Brown solid. Yield: 7%; m.p.: 151-152 °C

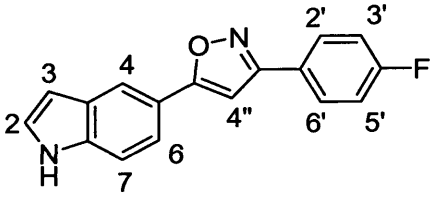
^{1}H NMR ($CDCl_3$) δ 3.90 (s, 3H, OCH_3), 6.67 (s, 1H, H2), 6.76 (s, 1H, H4"), 7.02 (d, *J* = 8.0 Hz, 2H, H3', H5'), 7.32 (s, 1H, H4), 7.50 (d, *J* = 8.0 Hz, 1H, H6), 7.70 (d, *J* = 8.0 Hz, 1H, H7), 7.85 (d, *J* = 8.0 Hz, 2H, H2', H6'), 8.19 (s, 1H, H3), 8.33 (s, 1H, NH).

^{13}C NMR ($CDCl_3$) δ 55.74 (OCH_3), 96.13 (C4"), 103.64, 112.06, 119.01, 120.05, 126.19 (C2, C3, C4, C6, C7), 114.68 (C3',C5'), 120.32 (C1'), 121.37, 129.70 (C3a, C7a), 128.47 (C2', C6'), 137.13 (C2), 161.43 (C4'), 162.86, 163.09 (C3", C5").

MS (EI positive ion) *m/z* 290.11 (M^+)

HRMS (ES) *m/z* = 291.1122 $[M+H]^+$; calcd: 290.1055.

5-(1H-indol-5-yl)-3-(4-fluoroyphenyl)isoxazole (69)



Prepared according to the general procedure for synthesis of 3,5-diaryl isoxazoles (via 1,3-dipolar cycloaddition) using 5-ethynylindole **(68a)** and 4-fluorobenzaldoxime **(69b)** as the starting materials.

Brown crystals. Yield: 36%; m.p.: 162-163 °C

^{1}H NMR ($CDCl_3$) δ 6.69 (s, 1H, H2), 6.77 (s, 1H, H4"), 7.09 (dd, *J* = 2.5, 9.5 Hz, 2H, H3', H5'), 7.32 (s, 1H, H4), 7.51 (d, *J* = 8.0 Hz, 1H, H7), 7.70 (d, *J* =

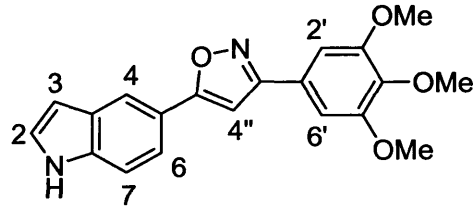
8.0 Hz, 1H, H6), 7.90 (dd, *J* = 2.5, 8.5 Hz, 2H, H2', H6'), 8.19 (s, 1H, H3), 8.34 (s, 1H, NH).

^{13}C NMR ($CDCl_3$) δ 95.86 (C4"), 103.63, 111.63, 119.00, 120.16, 125.59 (C2, C3, C4, C6, C7), 115.89 (d, *J* = 25.1 Hz, C3', C5'), 120.28 (C1'), 125.08, 128.06 (C3a, C7a), 128.72 (d, *J* = 10.2 Hz, C2', C6'), 136.75 (C2), 162.00 (C5"), 162.90 (d, *J* = 248.5 Hz, C4'), 169.03 (C3").

MS (EI positive ion) *m/z* 278.09 (M^+)

HRMS (ES) *m/z* = 279.0932 $[M+H]^+$; calcd: 278.0855.

5-(1H-indol-5-yl)-3-(3,4,5-trimethoxyphenyl)isoxazole (70)



Prepared according to the general procedure for synthesis of 3,5-diaryl isoxazoles (via 1,3-dipolar cycloaddition) using 5-ethynylindole **(68a)** and 3,4,5-trimethoxybenzaldoxime **(70b)** as the starting materials.

White solid. Yield: 29%; m.p.: 57-58 °C

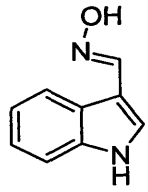
^{1}H NMR ($CDCl_3$) δ 3.94 (s, 3H, OCH_3), 3.95 (s, 6H, OCH_3 x 2), 6.66 (s, 1H, H2), 6.78 (s, 1H, H4"), 7.14 (s, 2H, H2', H6'), 7.30 (s, 1H, H4), 7.50 (d, *J* = 8.0 Hz, 1H, H6), 7.70 (d, *J* = 8.0 Hz, 1H, H7), 8.19 (s, 1H, H3), 8.77 (s, 1H, NH)

^{13}C NMR ($CDCl_3$) δ 56.31 (OCH_3 x 2), 60.93 (OCH_3), 96.01 (C4"), 103.47, 104.16, 111.67, 118.94, 120.07 (C2, C3, C4, C6, C7), 119.53 (C1'), 124.98, 128.07 (C3a, C7a), 125.71 (C2', C6'), 136.80 (C2), 139.54 (C3', C5'), 153.61 (C4'), 162.85, 172.17 (C3", C5").

MS (EI positive ion) *m/z* 350.13 (M^+)

HRMS (ES) *m/z* = 351.1343 $[M+H]^+$; calcd: 350.1267.

Synthesis of (E)-1H-indole-3-carbaldehyde oxime



Prepared according the general procedure for synthesis of aldoxime using indole-3-carboxaldehyde as the starting material.

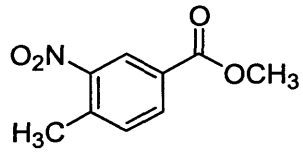
Yield: 73%, m.p.: 178-179 °C

^{1}H NMR (DMSO-d_6) δ 7.12 (t, *J* = 8.0 Hz, 1H, H5), 7.18 (t, *J* = 8.0 Hz, 1H, H6), 7.45 (d, *J* = 8.0 Hz, 1H, H4), 7.80 (s, 1H, NH), 7.86 (d, *J* = 8.0 Hz, 1H, H7), 8.25 (d, *J* = 1.5 Hz, 1H, H2), 11.19 (s, 1H, CH=N-OH), 11.57 (s, 1H, CH=N-OH).

^{13}C NMR (DMSO-d_6) δ 106.31 (C3), 111.73, 118.15, 119.86, 121.84, 130.43 (C2, C4, C5, C6, C7), 126.19, 134.90 (C3a, C7a), 138.35(-CH=N-OH).

6.4 Synthesis of specific inhibitors targeting the Dvl PDZ domain of Wnt signaling pathway: analogues of 3289-8625

Synthesis of methyl 3-nitro-4-methyl-benzoate (50a)

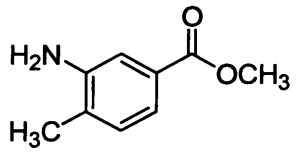


A mixture of 3-nitro-4-methyl-benzoic acid (0.1 mmol) and chlorotrimethylsilane (0.35 mmol) in methanol (0.5 mmol) was heated at reflux for 5 hours until the evolution of hydrogen chloride was no longer observed. Volatile product was removed under atmospheric pressure. The resulting brown solid were further purified by using acid base extraction, where the solid were diluted in ethyl acetate, and excess benzoic acid were washed away be extracting three times with sodium bicarbonate solution, washed with brine, dried over magnesium sulphate and evaporated to give the pure compound.

Light yellow solid. Yield: 71%; m.p.: 42-44 °C [lit: 49-50 °C (Noyce and Dolby 1961)].

^{1}H NMR ($CDCl_3$) δ 2.69 (s, 3H, CH_3), 3.98 (s, 3H, OCH_3), 7.45 (d, *J* = 8.0 Hz, 1H, H5), 8.16 (d, *J* = 8.0 Hz, 1H, H6), 8.63 (d, *J* = 1.5 Hz, 1H, H2).

Methyl 3-amino-4-methyl-benzoate (50b)



Pd(C) (10%) was added to a solution of compound **50a** (11 mmol) in ethanol (200 mL) at room temperature. The reaction mixture was stirred vigorously under an atmosphere of H_2 (balloon) for 23 h before being filtered through a plug of Celite and washed with ethanol (2 x 10 mL). The resulting filtrate was concentrated under vacuum before being purified by column chromatography (hexane: diethyl ether = 4:1).

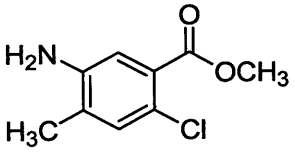
Yellow crystals. Yield: 86%; m.p.: 113-115 °C [lit: 114-115 °C (Noyce and Dolby 1961)].

^{1}H NMR ($CDCl_3$) δ 2.25 (s, 3H, CH_3), 3.90 (s, 3H, OCH_3), 4.19 (bs, 2H, NH_2), 7.13 (d, *J* = 8.0 Hz, 1H, H5), 7.40 (s, 1H, H2), 7.43 (d, *J* = 8.0 Hz, 1H, H6)

General procedure for the synthesis of Methyl 5-amino-2-chloro-4-methylbenzoate (51) and Methyl 3-amino-2-chloro-4-methylbenzoate (52).

A mixture of compound **50b** (16.9 mmol) and *N*-chlorosuccinimide (NCS) (1.15 equivalents) in acetonitrile (50 mL) was refluxed for 18 h, then poured into a mixture of 1g Na_2SO_3 dissolved in 200 mL water. The mixture was extracted twice with 50 mL portions of diethyl ether. The organic phases were collected, washed with water (50 mL) and brine (50 mL), dried over magnesium sulfate and evaporated *in vacuo.* Further purification need to be carried out to separate the formed isomers by column chromatography using hexane: ethyl acetate (6:1) as eluent, to afford compound **51** and **52** in low to moderate yield respectively.

Methyl 5-amino-2-chloro-4-methylbenzoate (51)



Prepared according to the general procedure to synthesis compound **51** and **52**. Compound **51** was eluted second in low yield.

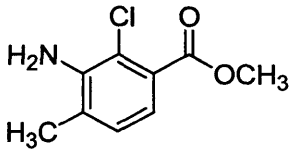
Dark brown oil. Yield: 20%; [lit: Berger et al. 2010].

^{1}H NMR ($CDCl_3$) δ 2.18 (s, 3H, CH_3), 3.75 (bs, 2H, NH_2), 3.91 (s, 3H, OCH_3), 7.14 (s, 1H, H 6), 7.18 (s, 1H, H3).

^{13}C NMR ($CDCl_3$) δ 17.20 (CH_3), 52.18 (OCH_3), 117.24 (C6), 122.40, 127.70, 127.79 (C2, C4, C5), 132.52 (C3), 143.18 (C1), 166.26 (C=O).

MS (EI positive ion) *m/z* 199.03 (M^+)

Methyl 3-amino-2-chloro-4-methylbenzoate (52)



Prepared according to the general procedure to synthesise compound **51** and **52**. Compound **52** was eluted first in moderate yield.

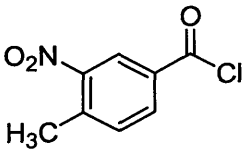
Orange oil. Yield: 43%; [ref: Mitani et al. 2006]

^{1}H NMR ($CDCl_3$) δ 2.27 (s, 3H, CH_3), 3.92 (s, 3H, OCH_3), 4.3 (bs, 2H, NH_2), 7.02 (d, J = 8.0 Hz, 1H, H5), 7.18 (d, J = 8.0 Hz, 1H, H6)

^{13}C NMR ($CDCl_3$) δ 18.33 (CH_3), 52.20 (OCH_3), 111.3, 126.94, 128.35 (C2, C3, C4), 120.08, 127.94 (C5, C6), 142.21 (C1), 166.71 (C=O).

MS (EI positive ion) *m/z* 199.03 (M^+)

3-nitro-4-methyl-benzoyl chloride (50c)



To a stirred suspension of 3-nitro-4-methyl-benzoic acid (20 mmol) in dry dichloromethane (30 mL), oxalyl chloride (25 mmol) was added followed by a catalytic amount of DMF (1-2 drops). The mixture was stirred at room temperature for 24 hours. The resulting solution was dried *in vacuo* to afford the acid chlorides in excellent yield which was used straight away in the next reaction.

Yield: 93%; pale green oil [ref: Desrousseaux et al. 2000]

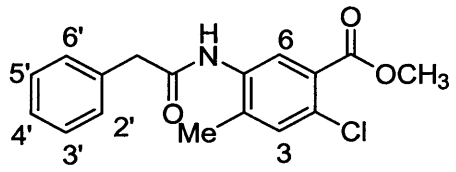
^{1}H NMR ($CDCl_3$) δ 2.69 (s, 3H, CH_3), 7.55 (d, *J* = 8.0 Hz, 1H, H5), 8.23 (d, *J* = 8.0 Hz, 1H, H6), 8.72 (d, *J* = 1.5 Hz, 1H, H2).

General procedure for the synthesis of chloro-substituted-4-methyl-5-(2-phenylacetamido)benzoic acid methyl ester (51a and 52a)

A solution of phenyl acetyl chloride (2 mmol) in anhydrous tetrahydrofuran (4 mL) was cooled to 0 °C under nitrogen atmosphere. Potassium phosphate (2.5 mmol) was added in one portion followed by the addition of compound **51** or **52** (2 mmol). The mixture was allowed to react for 18h at room temperature. The reaction was quenched with water (6 mL) and ethyl acetate (2 mL). The organic layer was evaporated *in vacuo* to obtained crude compound which can be further purified by recrystallisation in hot ethanol.

Methyl 2-chloro-4-methyl-5-(2-phenylacetamido)benzoate (51a)

Prepared according to the general procedure for the synthesis of amide bond starting with compound **51** and phenyl acetyl chloride.



Light yellow solid. Yield: 78%; m.p.: 152-153 °C

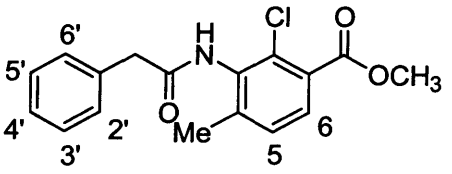
^{1}H NMR ($CDCl_3$) δ 1.93 (s, 3H, CH_3), 3.80 (s, 2H, CH_2), 3.91 (s, 3H, OCH_3), 6.97 (s, 1H, H3), 7.20 (s, 1H, H6), 7.32-7.47 (m, 5H, phenyl), 8.41 (s, 1H, NH).

^{13}C NMR ($CDCl_3$) δ 17.00 (CH_3), 44.71 (CH_2), 52.37 (OCH_3), 117.24, 124.94, 128.02, 129.64, 132.55 (Ar-CH), 127.89, 129.31, 134.19, 134.25, 138.64 (Ar-CH), 165.53, 169.10 (C=O x2).

MS (EI positive ion) *m/z* 317.08 (M^+)

Methyl 2-chloro-4-methyl-3-(2-phenylacetamido)benzoate (52a)

Prepared according to general procedure for the synthesis of amide bond starting with compound **52** and phenyl acetyl chloride.



Light yellow solid. Yield: 88%; m.p.: 122-123 °C

^{1}H NMR ($CDCl_3$) δ 2.25 (s, 3H, CH_3), 3.82 (s, 2H, CH_2), 3.90 (s, 3H, OCH_3), 7.02 (s, 1H, NH), 7.17 (d, *J* = 8.0 Hz, 1H, H5), 7.29-7.46 (m, 5H, phenyl), 7.65 (d, *J* = 8.0 Hz, 1H, H6)

^{13}C NMR ($CDCl_3$) δ 19.03 (CH_3), 44.02 (CH_2), 52.43 (OCH_3), 127.79, 128.64, 129.26, 129.53, 129.79 (Ar-CH), 128.35, 130.94, 133.91, 134.43, 141.99 (Ar-CH), 165.80, 169.44 (C=O x2).

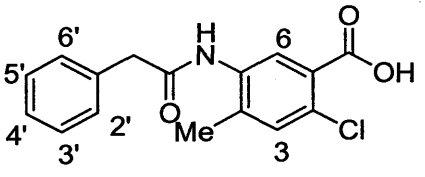
MS (EI positive ion) *m/z* 317.08 (M^+)

General procedure for the synthesis of chloro-substituted-4-methyl-5-(2-phenylacetamido)benzoic acid (51b and 52b).

Either of the methyl esters **51a** or **52a** (0.6 mmol) and lithium hydroxide (1 mmol) in methanol (10 mL) and water (6 mL) was allowed to stand at 50-60°C for 3 hours. The solution was concentrated to remove methanol and the aqueous layer was extracted with dichloromethane to remove unreacted esters. The aqueous layer was carefully acidified with 1N hydrochloric acid to pH 2-3 and extracted with dichloromethane. The organic layer was collected, dried over magnesium sulfate and evaporated to dryness to give the pure compound in good yield.

2-chloro-4-methyl-5-(2-phenylacetamido)benzoic acid (51b)

Prepared according to general procedure for the synthesis of benzoic acid from the corresponding **51 a**.



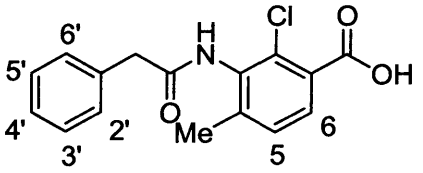
White solid. Yield: 71%; m.p.: 228 °C

^{1}H NMR (DMSO-d_6) δ 2.22 (s, 3H, CH_3), 3.75 (s, 2H, CH_2), 7.23-7.36 (m, 5H, phenyl), 7.42 (s, 1H, H3), 7.93 (s, 1H, H6), 9.64 (s, 1H, NH), 12.8-13.3 (bs, 1H, COOH).

^{13}C NMR (DMSO-d_6) δ 17.47 (CH_3), 42.58 (CH_2), 52.37 (OCH_3), 126.40, 127.02, 128.31, 129.08, 132.20 (Ar-CH), 127.89, 129.31, 134.19, 134.25, 138.64 (Ar-CH), 165.53, 169.10 (C=O x2).

2-chloro-4-methyl-3-(2-phenylacetamido)benzoic acid (52b)

Prepared according to general procedure for the synthesis of benzoic acid from the corresponding **52a**.



Light yellow solid. Yield: 69%; m.p.: 222 °C

^{1}H NMR (DMSO-d_6) δ 2.13 (s, 3H, CH_3), 3.70 (s, 2H, CH_2), 7.26-7.36 (m, 5H, phenyl), 7.38 (d, *J* = 8.0 Hz, 1H, H5), 7.57 (d, *J* = 8.0 Hz, 1H, H6), 9.83 (s, 1H, NH), 13.11-13.39 (bs, 1H, COOH).

^{13}C NMR (DMSO-d_6) δ 18.37 (CH_3), 42.71 (CH_2), 117.22, 126.50, 127.83, 128.23, 129.11 (Ar-CH), 125.90, 130.34, 134.92, 135.88, 141.15 (Ar-CH), 166.72, 169.04 (C=O x2).

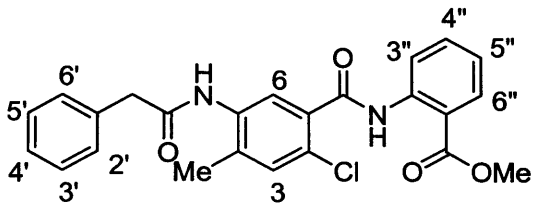
General procedure for the synthesis of Methyl 2-(chloro-substituted-4-methyl-5-(2-phenylacetamido)benzamido)benzoate

To a benzoic acid (compound **51b** or **52b**) (1.26 mmol) was added anhydrous THF (6 mL) under inert atmosphere, treated with triethylamine (3 equivalents) and then methanesulfonyl chloride (3 equivalents). To the reaction mixture was then added methyl anthranilate (1.26 mmol). The reaction was stirred at room temperature for 72 hours or until the completion of reaction as monitored by TLC. The reaction mixture was evaporated to remove THF, then redissolved in dichloromethane and partitioned between saturated $NaHCO_3$. The organic layer was collected, dried ($MgSO_4$) and evaporated *in vacuo* to give the crude compound. Further purification using

column chromatography (dichloromethane:methanol = 98:2) afforded the pure compound in moderate to good yield.

Methyl 2-(2-chloro-4-methyl-5-(2-phenylacetamido)benzamido)benzoate (54b)

Prepared according to the general procedure for the synthesis of amide bond formation starting with compound **51b** and methyl anthranilate.



Yellowish solid. Yield: 73%; m.p.: 110-112 °C

^{1}H NMR ($CDCl_3$) δ 1.94 (s, 3H, CH_3), 3.78 (s, 2H, CH_2), 3.90 (s, 3H, OCH_3), 7.142 (td, *J* = 1.0, 8.0 Hz, 1H, H4"), 7.16 (s, 1H, NH), 7.32-7.42 (m, 5H, H 2'-H5'), 7.45 (s, 1H, H3), 7.59, (td, *J* = 1.0, 8.0 Hz, 1H, H5"), 8.06 (dd, *J* = 1.5, 8.0 Hz, 1H, H6"), 8.11 (s, 1H, H6), 7.61 (d, *J* = 8.0 Hz, 1H, H3"), 11.84 (s, 1H, NH').

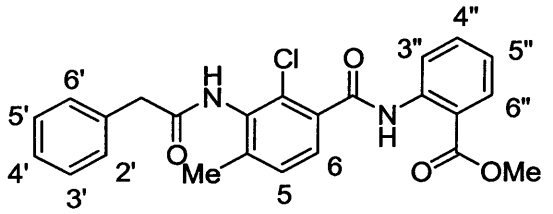
^{13}C NMR ($CDCl_3$) δ 17.07 (CH_3), 44.52 (CH_2), 52.46 (OCH_3), 120.60, 123.11, 123.15, 127.73, 129.24, 129.56, 130.95, 131.91, 134.80 (Ar-CH), 115.68, 126.49, 133.02, 134.11, 134.54, 134.63, 114.00 (Ar-CH), 165.33, 168.41, 169.35 (C=O x3).

MS (EI positive ion) *m/z* 436.12 (M^+)

HRMS(ES) *m/z* = 454.1528 $[M+NH_4]^+$; calcd: 436.1190.

Methyl 2-(2-chloro-4-methyl-3-(2-phenylacetamido)benzamido)benzoate (55b)

Prepared according to the general procedure for the synthesis of amide bond formation starting with compound **52b** and methyl anthranilate.



Light yellow solid. Yield: 73%; m.p.: 150-152 °C

^{1}H NMR ($CDCl_3$) δ 2.16 (s, 3H, CH_3), 3.72 (s, 2H, CH_2), 3.83 (s, 3H, OCH_3), 7.26 (td, *J* = 1.0, 8.0 Hz, 1H, H4"), 7.28 (d, *J* = 8.0 Hz, 1H, H5), 7.33-7.41 (m, 5H, phenyl), 7.50 (d, *J* = 8.0 Hz, 1H, H6), 7.69 (td, *J* = 1.0, 8.0 Hz, 1H, H5"), 7.98 (dd, *J* = 1.5, 8.0 Hz, 1H, H6"), 8.36 (d, *J* = 8.0 Hz, 1H, H3"), 9.90 (s, 1H, NH), 11.08 (s, 1H, NH').

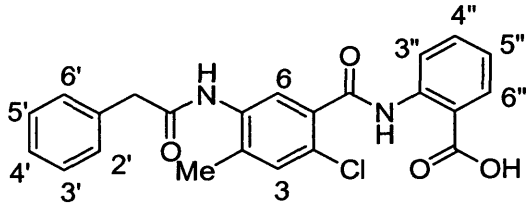
^{13}C NMR ($CDCl_3$) δ 19.18 (CH_3), 44.08 (CH_2), 52.45 (OCH_3), 120.61, 123.10, 126.99, 127.74, 129.23, 129.29, 129.64, 133.68, 134.71 (Ar-CH), 115.53, 128.60, 130.92, 134.51, 134.59, 140.42, 141.12 (Ar-CH), 165.16, 168.49, 169.30 (C=O x3).

MS (EI positive ion) *m/z* 436.12 (M^+)

Anal. calcd for $C_{24}H_{21}ClN_2O_4$; C, 65.98; H, 4.84; N, 6.41; found C, 65.77; H, 4.83; N, 6.54.

2-(2-chloro-4-methyl-5-(2-phenylacetamido)benzamido)benzoic acid (54)

Prepared according to general procedure for the synthesis of benzoic acid (chloro-substituted-4-methyl-5-(2-phenylacetamido)benzoic acid – compound **51b** or **52b**) from the corresponding compound **54b** and lithium hydroxide.



White solid. Yield: 72%; m.p.: 242 °C

^{1}H NMR (DMSO-d_6) δ 2.25 (s, 3H, CH_3), 3.73 (s, 2H, CH_2), 7.22 (td, *J* = 1.0, 8.0 Hz, 1H, H4"), 7.27-7.38 (m, 5H, phenyl), 7.48 (s, 1H, H3), 7.67, (td, *J* = 1.0, 8.0 Hz, 1H, H5"), 7.81 (s, 1H, H6), 8.04 (dd, *J* = 1.5, 8.0 Hz, 1H, H6"), 8.60 (d, *J* = 8.0 Hz, 1H, H3"), 9.68 (s, 1H, NH), 11.64 (s, 1H, NH'), 13.61-13.84 (bs, 1H, COOH).

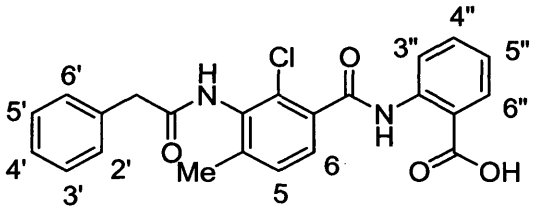
^{13}C NMR (DMSO-d_6) δ 17.47 (CH_3), 42.59 (CH_2), 120.04, 123.40, 124.88, 125.36, 128.30, 129.09, 131.19, 131.64, 134.21 (Ar-CH), 116.95, 126.53, 133.43, 135.70, 135.89, 135.93, 140.29 (Ar-CH), 164.13, 169.45, 169.46 (C=O x3).

MS (ES positive ion) *m/z* 423.11 (M^+H)

Anal. calcd for $C_{23}H_{19}ClN_2O_4$; C, 65.33; H, 4.53; N, 6.62; found C, 65.26; H, 4.53; N, 6.69.

2-(2-chloro-4-methyl-3-(2-phenylacetamido)benzamido)benzoic acid (55)

Prepared according to general procedure for the synthesis of benzoic acid (chloro-substituted-4-methyl-5-(2-phenylacetamido)benzoic acid – compound **51b** or **52b**) from the corresponding compound **55b** and lithium hydroxide.



Light yellow solid. Yield: 74%; m.p.: 208 °C

^{1}H NMR (DMSO-d_6) δ 2.17 (s, 3H, CH_3), 3.72 (s, 2H, CH_2), 7.23 (td, *J* = 1.0, 8.0 Hz, 1H, H4"), 7.28 (d, *J* = 8.0 Hz, 1H, H5), 7.33-7.41 (m, 5H, phenyl), 7.53 (d, *J* = 8.0 Hz, 1H, H6), 7.68 (td, *J* = 1.0, 8.0 Hz, 1H, H5"), 8.03 (dd, *J* = 1.5, 8.0 Hz, 1H, H6"), 8.61 (d, *J* = 8.0 Hz, 1H, H3"), 9.89 (s, 1H, NH), 11.60 (s, 1H, NH'), 13.6-13.9 (bs, 1H, COOH).

^{13}C NMR (DMSO-d_6) δ 18.31 (CH_3), 42.17 (CH_2), 120.05, 123.40, 126.51, 128.24, 128.99, 129.12, 129.37, 131.19, 134.79 (Ar-CH), 117.05, 128.76, 131.19, 134.13, 134.19, 140.35, 141.07 (Ar-CH), 164.47, 169.07, 169.45 (C=O x3).

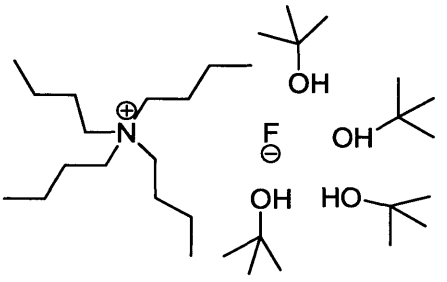
MS (ES positive ion) *m/z* 445.09 (M^+Na)

Anal. calcd for $C_{23}H_{19}ClN_2O_4$; C, 65.33; H, 4.53; N, 6.62; found C, 65.18; H, 4.48; N, 6.69.

6.5 Towards the synthesis of *trans*-stilbene analogues as potential PET radiotracers

Synthesis of Tetrabutylammonium tetra (*tert*-butyl alcohol)-coordinated fluoride (TBAF(*t*BuOH)$_4$ (38a).

Commercially available TBAF hydrate (1.0 g, 3.17 mmol) was added to *tert*-butanol (88 mL) and *n*-hexane (22 mL). The mixture was stirred for 30 minutes at 90 °C. During this time TBAF dissolved completely. The solution was cooled to room temperature, and a white crystalline solid precipitated. The crystalline solid was filtered and washed rapidly with 40 mL of 70% *t*BuOH/hexane. The filtrate was kept in vacuum for 15-20 minutes to remove residual solvent and TBAF(*t*BuOH)$_4$ was afforded as white crystalline solid.

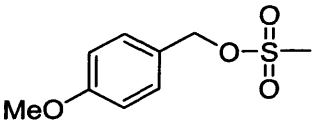


White crystalline solid. Yield = 88%. (Kim et al. 2008)

^{1}H NMR ($CDCl_3$) δ 1.01 (t, *J* = 7.5 Hz, 12H, CH_3x4), 1.27 (s, 36H), 1.46 (m, CH_2x4), 1.67 (m, CH_2x4), 3.36 (m, CH_2x4).

Synthesis of 4-methoxy benzyl mesylate (38b)

4-Methoxy benzyl alcohol (6 mmol, 1 equiv) was dissolved under inert atmosphere in dry dichloromethane (7 mL), followed by addition of triethylamine (12 mml, 2 equiv). The reaction mixture was cooled to 0 °C. Methanesulfonyl chloride (MsCl) (12 mmol, 2 equiv) in dry dichloromethane (3 mL) was added dropwise. After complete addition, the reaction mixture was allowed to slowly warm to RT. The reaction mixture was washed with water and saturated sodium carbonate. The organic layer was dried and concentrated in vacuo to afford yellowish oil which was used for fluoridation reaction without further purification.

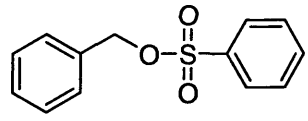


Yellowish oil. Yield = 78%. (Bentley et al. 1994)

^{1}H NMR ($CDCl_3$) δ 3.69 (s, 3H, CH_3), 3.84 (s, 3H, OCH_3), 4.60 (s, 2H, CH_2), 6.91 (d, *J* = 8.5 Hz, 2H, H-3, H-5), 7.34 (d, *J* = 8.5 Hz, 2H, H-2, H-6)

Synthesis of benzyl tosylate [38c(i)]

A mortar was charged with dry potassium carbonate (5 g), benzyl alcohol (10 mmol), *p*-toluenesulfonyl chloride (TsCl) (15 mmol) and grinded vigorously for 5 minutes. After the completion of tosylation, the remaining tosyl chloride was removed by addition of powdered potassium hydroxide (50 mmol) and vigorously grinded (2 minutes). Addition of a few drops of *t*-BuOH accelerate the disappearance of TsCl. The product was extracted by addition of ether (50 mL), filtered and finally by the evaporation of organic solvent. The purification of the oily crude product by column chromatography (10 hexane: 1 Et_2O) afforded the pure benzyl tosylate.



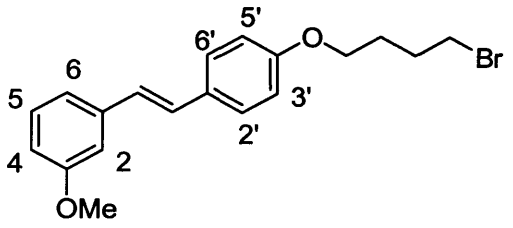
White powder. Yield = 26%. Mp = 56-57 °C (lit: 58.5-58.9 °C [Kochi and Hammond 1953)].

^{1}H NMR ($CDCl_3$) δ 5.08 (s, 2H, CH_2), 7.27 (m, 2H), 7.34 (m, 3H), 7.35-7.39 (m, 5H)

General procedure for the synthesis of (E)-1-(4-bromobutoxy)-4-(substituted-styryl)-benzene

To a solution of hydroxylated compound **22** or **32** (1 mmol) in acetone (30 mL), anhydrous K_2CO_3 (4 mmol) and 1,4-dibromobutane (1.15 mmol) were added and the mixture refluxed for 12 h. After the completion of the reaction, K_2CO_3 was removed by filtration and the solvent was evaporated under reduced pressure to give the crude product. This was further purified by column chromatography (hexane:ethyl acetate = 20:1) to afford the pure compound in good yield.

(*E*)-1-(4-bromobutoxy)-4-(3-methoxystyryl)benzene (39a)

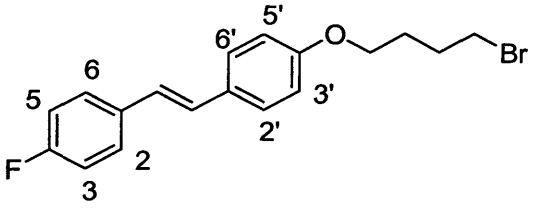


Light yellow solid. Yield: 78%; m.p.: 61-62 °C

^{1}H NMR ($CDCl_3$) δ 1.97-2.01 (m, 2H, CH_2), 2.09-2.14 (m, 2H, CH_2), 3.53 (t, *J* = 6.5 Hz, 2H, CH_2), 3.89 (s, 3H, OCH_3), 4.03 (t, *J* = 6.0 Hz, 2H, CH_2), 6.87 (dd, *J* = 2.0, 8.5 Hz, 1H, H4), 6.93 (d, *J* = 9.0 Hz, 2H, H3', H5'), 7.03 (d, *J* = 16.5 Hz, 1H, Hα), 7.11 (s, 1H, H2), 7.14 (d, *J* = 16.5 Hz, 1H, Hα'), 7.17 (d, *J* = 7.5 Hz, 1H, H6), 7.33 (t, *J* = 8.0 Hz, 1H, H5), 7.49 (d, *J* = 8.5 Hz, 2H, H2', H6').

^{13}C NMR ($CDCl_3$) δ 27.99, 29.87, 33.89, 67.28 (CH_2 x 4), 55.61 (OCH_3), 111.97 (Ar-CH/Cα), 113.28 (Ar-CH/Cα), 114.96 (C2', C6'), 119.28 (Ar-CH/Cα), 126.90 (Ar-CH/Cα), 127.86 (C3', C5'), 128.19 (Ar-CH/Cα), 128.90 (Ar-CH/Cα), 130.14 (C1), 139.19 (C1'), 158.72 (C4'), 159.99 (C3).

(*E*)-1-(4-bromobutoxy)-4-(4-fluorostyryl)benzene (39b)



White powder. Yield: 75%; m.p.: 109-110 °C

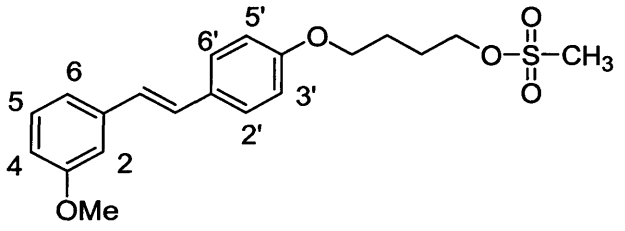
^{1}H NMR ($CDCl_3$) δ 1.97-2.01 (m, 2H, CH_2), 2.08-2.14 (m, 2H, CH_2), 3.52 (t, *J* = 6.5 Hz, 2H, CH_2), 4.04 (t, *J* = 6.5 Hz, 2H, CH_2), 6.91 (dd, *J* = 2.0, 8.5 Hz, 2H, H3', H5'), 6.97 (d, *J* = 16.5 Hz, 1H, Hα), 6.98 (d, *J* = 16.5 Hz, 1H, Hα'), 7.06 (td, *J* = 2.0, 8.5 Hz, 2H, H2, H6), 7.45 (dd, *J* = 2.0, 8.5 Hz, 2H, H2', H6'), 7.48 (t, *J* = 9.5 Hz, 2H, H3, H5).

^{13}C NMR ($CDCl_3$) δ 27.92, 29.49, 33.43, 66.93 (CH_2 x 4), 114.73 (C3', C5'), 115.05 (Cα'), 115.65 (d, *J* = 21.4 Hz, C3, C5), 125.47 (Cα), 127.66 (d, *J* = 7.6 Hz, C2, C6), 128.02 (C2', C6'), 130.08 (C1'), 133.86 (d, *J* = 2.5 Hz, C1), 158.64 (C4'), 161.15 (d, *J* = 246.9 Hz, C4).

General procedure for the synthesis of (E)-4-(4-(substituted styryl)phenoxy)butyl methanesulfonate

A solution of (*E*)-1-(4-bromobutoxy)-4-(substituted-styryl)-benzene (0.5 mmol) and silver methanesulfonate (AgOMs) (5 mmol) in anhydrous acetonitrile (10 mL) was refluxed overnight under argon. Upon completion of the reaction by TLC, the solvent was evaporated to dryness. The residue was redissolved in ethyl acetate and was filtered to remove the silver bromide side product. The resulting filtrate was evaporated *in vacuo* to afford the pure compound of substituted methanesulfonate **40a** or **40b** in good yield without the need for further purification.

(*E*)-4-(4-(3-methoxystyryl)phenoxy)butyl methanesulfonate (40a)

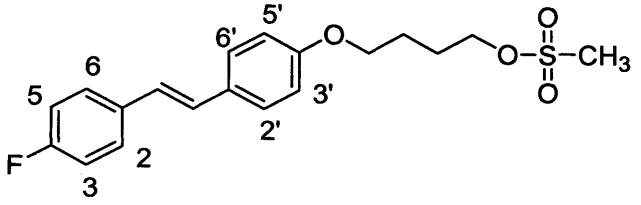


Light yellow solid. Yield: 98%; m.p.: 77-78 °C

^{1}H NMR ($CDCl_3$) δ 1.94-2.02 (m, 4H, CH_2 x 2), 3.04 (s, 3H, SO_2CH_3), 3.87 (s, 3H, OCH_3), 4.06 (t, *J* = 6.0 Hz, 2H, CH_2), 4.35 (t, *J* = 6.0 Hz, 2H, CH_2), 6.82 (dd, *J* = 2.0, 8.5 Hz, 1H, H4), 6.89 (d, *J* = 9.0 Hz, 2H, H3', H5'), 6.96 (d, *J* = 16.5 Hz, 1H, Hα), 7.05 (s, 1H, H2), 7.06 (d, *J* = 16.5 Hz, 1H, Hα'), 7.15 (d, *J* = 7.5 Hz, 1H, H6), 7.29 (t, *J* = 8.0 Hz, 1H, H5), 7.46 (d, *J* = 8.5 Hz, 2H, H2', H6').

^{13}C NMR ($CDCl_3$) δ 25.40, 26.14 (CH_2 x 2), 37.46 (SO_2CH_3), 55.25 (OCH_3), 67.00, 69.59 (CH_2 x 2), 111.54 (Ar-CH/Cα), 112.94 (Ar-CH/Cα), 114.69 (C2', C6'), 119.02 (Ar-CH/Cα), 126.63 (Ar-CH/Cα), 127.80 (C3', C5'), 128.48 (Ar-CH/Cα), 126.90 (Ar-CH/Cα), 130.23 (C1), 139.10 (C1'), 158.54 (C4'), 159.91 (C3).

(*E*)-4-(4-(4-fluorostyryl)phenoxy)butyl methanesulfonate (40b)



White powder. Yield: 96%; m.p.: 106 °C

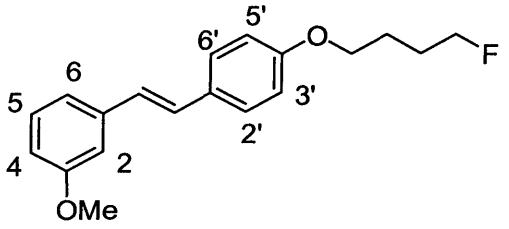
^{1}H NMR ($CDCl_3$) δ 1.94-2.02 (m, 4H, CH_2 x 2), 3.04 (s, 3H, SO_2CH_3), 4.06 (t, *J* = 6.5 Hz, 2H, CH_2), 4.35 (t, *J* = 6.5 Hz, 2H, CH_2), 6.91 (dd, *J* = 2.0, 8.5 Hz, 2H, H3', H5'), 6.97 (d, *J* = 16.5 Hz, 1H, Hα), 6.98 (d, *J* = 16.5 Hz, 1H, Hα'), 7.06 (t, *J* = 8.5 Hz, 2H, H2, H6), 7.44 (dd, *J* = 2.0, 8.5 Hz, 2H, H2', H6'), 7.48 (t, *J* = 9.5 Hz, 2H, H3, H5).

^{13}C NMR ($CDCl_3$) δ 25.40, 26.14 (CH_2 x 2), 37.43 (SO_2CH_3), 67.02, 69.65 (CH_2 x 2), 114.72 (C3', C5'), 115.04 (Cα'), 115.65 (d, *J* = 21.4 Hz, C3, C5), 125.51 (Cα), 127.70 (d, *J* = 7.6 Hz, C2, C6), 127.98 (C2', C6'), 130.15 (C1'), 133.84 (d, *J* = 2.5 Hz, C1), 158.54 (C4'), 161.14 (d, *J* = 246.9 Hz, C4).

General procedure for the synthesis of (E)-1-(4-(4-fluorobutoxy)styryl)-substituted benzene

TBAF (tetrabutylammonium fluoride) or CsF (cesium fluoride) (2 mmol) was added to a solution of a substrate ((*E*)-4-(4-(substituted styryl)phenoxy)butyl methanesulfonate) (**40a** or **40b**) (1 mmol) in acetonitrile or *tert*-butanol (4 mL). The mixture was stirred for 1h at 70 °C. The residue was dissolved in water (6 mL) and extracted from the aqueous phase with diethyl ether (6 mL x 3). The organic layer was dried and evaporated under reduced pressure. The residue was purified by short column chromatography (hexane:ethyl acetate = 6:1) to afford the desired compound **41** or **42** in good yield.

(*E*)-1-(4-(4-fluorobutoxy)styryl)-3-methoxybenzene (41)



Light yellow powder. Yield: 90%; m.p.: 56 °C

^{1}H NMR ($CDCl_3$) δ 1.92-2.00 (m, 4H, CH_2 x 2), 3.88 (s, 3H, OCH_3), 4.06 (t, *J* = 5.5 Hz, 2H, CH_2), 4.53 (t, *J* = 6.0 Hz, 2H, CH_2), 6.83 (dd, *J* = 2.0, 8.5 Hz, 1H, H4), 6.91 (d, *J* = 9.0 Hz, 2H, H3', H5'), 6.97 (d, *J* = 16.5 Hz, 1H, Hα), 7.06 (s, 1H, H2), 7.07 (d, *J* = 16.5 Hz, 1H, Hα'), 7.12 (d, *J* = 7.5 Hz, 1H, H6), 7.29 (t, *J* = 8.0 Hz, 1H, H5), 7.47 (d, *J* = 8.5 Hz, 2H, H2', H6').

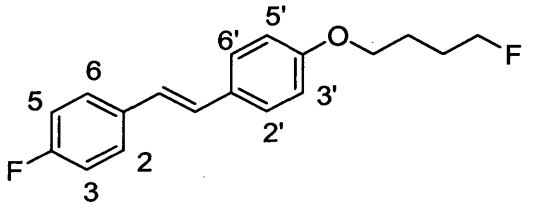
^{13}C NMR ($CDCl_3$) δ 25.32, 29.74 (CH_2 x 2), 55.26 (OCH_3), 67.31, 83.18 (CH_2 x 2), 111.48 (Ar-CH/Cα), 112.90 (Ar-CH/Cα), 114.68 (C2', C6'), 119.01 (Ar-CH/Cα), 126.48 (Ar-CH/Cα), 127.80 (C3', C5'), 128.54 (Ar-CH/Cα), 129.63 Ar-CH/Cα), 130.03 (C1), 139.13 (C1'), 158.70 (C4'), 159.89 (C3).

^{19}F NMR ($CDCl_3$) δ -218.52.

MS (EI positive ion) *m/z* 300.13 (M^+)

Anal. calcd for $C_{19}H_{21}FO_2$; C, 75.98; H, 7.05; found C, 75.91; H, 7.27.

(*E*)-1-fluoro-4-(4-(4-fluorobutoxy)styryl)benzene (42)



White powder. Yield: 81%; m.p.: 110-112 °C

^{1}H NMR ($CDCl_3$) δ 1.90-2.00 (m, 4H, CH_2 x 2), 4.06 (t, *J* = 5.5 Hz, 2H, CH_2), 4.53 (t, *J* = 5.5 Hz, 2H, CH_2), 6.93 (dd, *J* = 2.0, 8.5 Hz, 2H, H3', H5'), 6.97 (d, *J* = 16.5 Hz, 1H, Hα), 6.99 (d, *J* = 16.5 Hz, 1H, Hα'), 7.06 (t, *J* = 2.0, 8.5 Hz, 2H, H2', H6'), 7.45-7.50 (m, 4H, H2, H3, H5, H6).

^{13}C NMR ($CDCl_3$) δ 25.31, 27.60, 67.31, 84.48 (CH_2 x 4), 114.70 (C3', C5'), 115.49 (Cα'), 115.66 (d, *J* = 21.4 Hz, C3, C5), 125.39 (Cα), 127.65 (d, *J* = 7.6 Hz, C2, C6), 128.01 (C2', C6'), 130.93 (C1'), 133.84 (d, *J* = 2.5 Hz, C1), 158.67 (C4'), 161.12 (d, *J* = 246.9 Hz, C4).

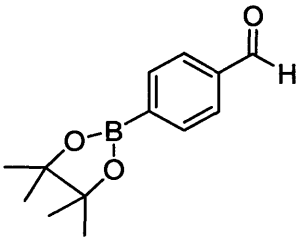
^{19}F NMR ($CDCl_3$) δ -115.27, -218.57.

MS (EI positive ion) *m/z* 288.14 (M^+)

Anal. calcd for $C_{18}H_{18}F_2O$; C, 74.98; H, 6.29; found C, 75.43; H, 6.66.

Synthesis of potassium (E)-stilbene trifluoroborate

Synthesis of 4-(4,4,5,5,-tetramethyl-1,3,2-dioxaborolan-2-yl)benzaldehyde (45a)

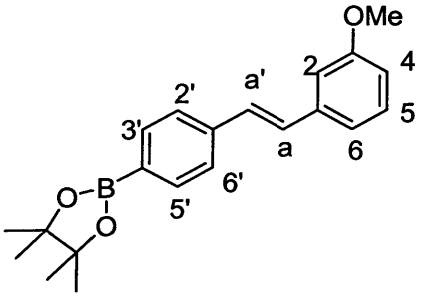


4-formylbenzeneboronic acid (33.35 mmol) and pinacol (33.35 mmol) were added to anhydrous diethyl ether (200 mL) and the mixture was stirred for 9 h at room temperature whereby the remaining solid dissolves. The solution was washed with water (3 x 30 mL) and dried over $MgSO_4$. Evaporation of the solvent under reduced pressure leads to a pure white to yellowish solid of **45a** which does not need further purification.

White solid. Yield: 90%; m.p.: 50 °C [lit: 51 °C (Oehlke et al. 2007)].

^{1}H NMR ($CDCl_3$) δ 1.39 (s, 12H, CH_3 x 4), 7.88 (d, *J* = 8.0 Hz, 2H, H3, H5), 7.98 (d, *J* = 8.0 Hz, 2H, H2, H6), 10.07 (s, 1H, CHO).

Synthesis of (*E*)-2-(4-(3-methoxyxtyryl)phenmyl-4,4,5,5,-tetramethyl-1,3,2-dioxaborolane (45b)



Synthesis of **45b** was achieved following the previous method for the synthesis of stilbenes via HWE method (6.2.2) where compound **45a** and compound **2** were used as the starting materials.

White solid. Yield: 9%; m.p.: 78 °C

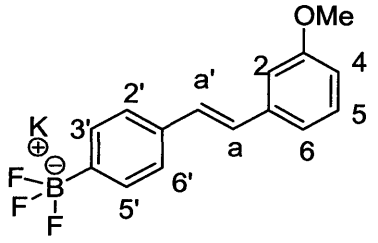
^{1}H NMR ($CDCl_3$) δ 1.40 (s, 12H, CH_3 x 4), 3.88 (s, 3H, OCH_3), 6.86 (dd, *J* = 2.0, 8.5 Hz, 1H, H4), 7.10 (s, 1H, H2), 7.13 (d, *J* = 16.5 Hz, 1H, Hα), 7.17 (d,

J = 7.5 Hz, 1H, H6), 7.18 (d, *J* = 16.5 Hz, 1H, Hα'), 7.31 (t, *J* = 8.0 Hz, 1H, H5), 7.57 (d, *J* = 8.0 Hz, 2H, H3', H5'), 7.85 (d, *J* = 8.0 Hz, 2H, H2', H6')

^{13}C NMR ($CDCl_3$) δ 24.91 (CH_3 x 4), 55.26 (OCH_3), 83.81 ($B(O)_2$-$\underline{C}_2$-$(CH_3)_4$), 111.81, 113.62 (Cα, Cα'), 119.36 (C2', C6'), 126.19 (C3', C5'), 128.96 (Ar-CH), 129.59 (Ar-CH),129.68 (Ar-CH), 135.52 (Ar-CH), 138.68 (C1'), 139.59 (C1), 159,95 (C3).

C4' attach to B could not be detected from the NMR spectra.

Synthesis of potassium (*E*)-trifluoro(4-(3-methoxystyryl)phenyl)borate (45)



Compound **45b** (2.32 mmol) was dissolved in a mixture of methanol (15 mL) and THF (10 mL) with stirring before addition of 4.5 M KHF_2 (aqueous) (20.7 mmol). The resulting mixture was stirred for 1 h before concentrating to dryness. The residue, a white solid was collected by filtration, washed several times with hot diethyl ether (total ~100 mL) followed by water (~50 mL) and dried to afford the pure trifluoroborate salt.

White solid. Yield: 63%; m.p.: >250 °C [lit: reported as a mixed of *cis* and *trans* isomers, m.p. (*E* + *Z*) = 236-243 °C (Molander et al. 2007)].

^{1}H NMR (Acetone-d_6) δ 3.85 (s, 3H, OCH_3), 6.81 (dd, *J* = 2.0, 8.5 Hz, 1H, H4), 7.09 (d, *J* = 16.5 Hz, 1H, Hα), 7.15 (d, *J* = 7.5 Hz, 1H, H6), 7.16 (s, 1H, H2), 7.19 (d, *J* = 16.5 Hz, 1H, Hα'), 7.26 (t, *J* = 8.0 Hz, 1H, H5), 7.34 (d, *J* = 8.0 Hz, 2H, H3', H5'), 7.51 (d, *J* = 8.0 Hz, 2H, H2', H6').

^{13}C NMR (Acetone-d_6) δ 55.46 (OCH_3), 112.09, 113.65 (Cα, Cα'), 119.63 (C2', C6'), 125.63 (C3', C5'), 126.65 (Ar-CH), 130.35 (Ar-CH),131.30 (Ar-CH), 132.93 (Ar-CH), 134.84 (C1'), 140.54 (C1), 161.06 (C3).

^{19}F NMR (Acetone-d_6) δ 34.68.

C4' attached to B could not be detected from the NMR spectra.

MS (ES negative ion) *m/z* 277.10 $(M\text{-}K^+)^-$,calcd for $C_{15}H_{13}BF_3O$ $(M\text{-}K^+)^-$ 277.1012

CHAPTER 7

GENERAL CONCLUSION AND FUTURE STUDIES

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7.1 General discussion

Cancer is becoming an exceedingly common disease. The area of targeted cancer drug discovery is becoming more complex, involving multi-disciplinary areas. The need to find potent anticancer agents which can counteract the current problem of resistance, efficacy and selectivity of the classical anticancer agents has long been desperately awaited. Unfortunately, the number of compounds able to pass the clinical trials and gain regulatory approval is still very low, due to the complexity of the disease itself which makes the cancer drug discovery processes very challenging. Many scientist and pharmaceutical companies have shifted their approach to the more targeted personalised therapies. Nevertheless, the area of high throughput screening to identify lead compounds for cancer therapy has yet still makes a significant contribution in cancer drug development.

Due to the increasing potential of natural products to be developed as new anticancer agents, the second chapter of this thesis is dedicated to the development of resveratrol-based stilbene analogues as new anticancer agents. Although most of the stilbenes synthesized in this chapter have been previously reported, the compounds are aimed to show novel therapeutic potential especially as new anti cancer agents. The simplicity of the stilbene scaffold makes it amenable to various modifications and substitutions in order to enhance its bioavailability, but still retaining the small size which is an important feature for good drug-like properties, making it potentially a better drug candidate compared to the parent compound resveratrol. Synthesis of methoxylated, fluorinated, brominated and nitrated stilbenes were conveniently achieved using HWE reaction, giving the compounds in variable yields and good purity. The synthesis was further expanded in order to compare various synthetic procedures for stilbene formation, using microwave activation via modified Perkin reaction and Heck coupling

reaction. By using microwave activation, synthesis of hydroxylated compound **22** was achieved without the need to protect the free hydroxyl group in low yield. Nevertheless, the procedure was lacking applicability to various substitution patterns, as indicated by the failure of all other attempts to synthesise different hydroxylated stilbenes. At the same time, Heck reaction via palladium –oxazoline complex proved to be a reliable method giving methoxylated, fluorinated and hydroxylated stilbenes in moderate to good yield. Synthesis of compound **29** via both HWE and Heck reaction methods showed that the HWE reaction is superior to the Heck reaction for stilbene synthesis in terms of overall product yield and cost effectiveness. The stilbenes were then subjected to screening on a panel of cancer cell lines, namely prostate (PC3), non-small lung (A549), colon (Lovo) and breast cancer (MCF-7), where some of them demonstrated inhibitory activity in the micromolar range. The best result is showed by compound **14** which can inhibit the MCF-7 breast cancer cell line at less than 0.1 uM, and compound **17** which can inhibit the A549 cell line at 1 uM. Unfortunately, no structure activity relationship can be generated from these results. But one common feature from the compounds which showed promising antiproliferative activity is the existence of methoxyl group on stilbene skeleton, which is in accordance to the previous study showing the importance of the methoxyl group on stilbene backbone to induce cancer cell death in cancer cell lines. These findings highlighted the potential of a few tested stilbene analogues to inhibit certain cancer cell types, although a more detailed and comprehensive study should be carried out to further validate their anticancer property. At the same time, the stilbene analogues were tested for their ability to inhibit the Wnt signaling pathway, which is one of a prominent signaling pathway which becomes aberrant in cancer. Unfortunately, none of the stilbene analogues were able to reduce the intensity of the GFP (green fluorescence protein) or to reduce the number of colon cancer stem cells (CSC) after treatment of the compounds in different concentrations, which suggested the inability of the compounds to inhibit the Wnt signaling pathway especially at the downstream level. Nevertheless, a more specific assay which could determine the ability of the stilbene analogues to inhibit the Wnt

signaling at different level is anticipated to give better insights of the ability of the synthesised stilbenes to act as novel Wnt inhibitors.

The next chapter was dedicated to the synthesis of indole-based 3,5-diaryl isoxazoles as novel anticancer agents. The study was carried out to extend the previous work on the synthesis of indole-based 3,5-diaryl oxadiazoles and isoxazoles, which posses low micromolar antiproliferative activity and apoptosis induction activity through caspase activation. The syntheses were achieved using two different pathways: either via cyclization of alkynyl ketones with hydroxylamine which suffered from unselective regioisomer formation, or via dipolar cycloaddition between nitrile oxides and terminal alkynes which gave selective regioisomers, to afford title compounds with different substitution patterns on the phenyl ring and indole ring in low to moderate yields. The compounds will later be tested on human cancer cell lines for their antiproliferative and anticancer properties in order to generate structure-activity relationship data for further development of this series of compounds.

The next study was aimed at the synthesis of 3289-8625 derivatives, especially the one which will combine structural features of both derivatives 6569188 and 7359885. All these derivatives had been previously reported to be potent inhibitors of Dishevelled (Dvl)-PDZ domain of the Wnt signalling pathway, and further synthesis of their analogues is anticipated to show better binding affinity towards the protein-protein interaction domain. The syntheses were achieved using various methods, which gave rise to two major analogues; compound **54** and **55**. The testing of the two analogues using NMR-based chemical shift perturbation assay and fluorescence-binding affinity study confirmed the ability of the analogues to bind more tightly towards the Dvl-PDZ domain, with more than three fold affinity compared to the parent compound and even to the naturally occurring membrane bound peptide Dapper. The findings highlighted the potency of compound **54** and **55** to be developed as potential Wnt signaling antagonists, where the expansion of 3289-8625 scaffolds through structure-activity

studies can open the possibilities for the development of more potent Wnt inhibitors.

On the other hand, the development of non-invasive imaging modalities especially PET for disease monitoring, staging or even in drug development is of increasing interest owing to the sensitivity and good resolution achieved by the imaging technique. This highlights the need to have reliable imaging probes specifically designed to the targeted site. The case of interest is the imaging of amyloid plaques which is a prominent feature in Alzheimer's disease. Stilbene-based compounds not only show promising potential as good anticancer agents, but also as novel PET imaging probes, as indicated by two stilbene-based compounds, Florbetaben and Flobetapir F 18, which are currently in the late phase clinical trial for non-invasive imaging of Alzheimer's disease. Therefore, this study is dedicated to the synthesis of 'cold' (^{19}F) *trans*-stilbenes, which later is anticipated to be applicable for translation to PET radiochemistry. Stilbenes attached to alkylfluoride chain has been successfully synthesised in good yields, purity and within a time-frame which is applicable to PET radiochemistry. Although the next attempt to synthesise stilbenes attached to potassium alkyltrifluoroborate chain failed, the synthesis of stilbene attached directly to potassium trifluoroborate moiety via HWE reaction managed to give the desired compound in moderate yield. Nevertheless, the compound suffered from low susceptibility to different substitution on the stilbene ring which limits its applicability for PET imaging.

7.2 Future studies

The works presented here can generate various opportunities for further studies. Further modification and more diverse substitution patterns of stilbene-based analogues, compound 3289-8625 derivatives and possibly indole-based 3,5-diaryl isoxazoles will generate more reliable structure activity relationship studies, which upon detailed biological evaluations are anticipated to generate potent lead compounds for anticancer drug development. On the other hand, the synthesis of stilbene attached to

alkylfluoride chain is amenable to expansion to radiochemistry synthesis using different substitution pattern and different length of alkyl chain which upon *in vivo* testing can predict its applicability for imaging of β-amyloid plaques. Nevertheless, the synthesis of stilbenes whether attached directly or indirectly via alkyl chain to potassium trifluoroborate should be refined before any attempt to forward the reaction to radiochemistry synthesis.

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