Cardiff University
Welsh School of Pharmacy

The Design, Synthesis and Biological Evaluation of Novel Antitumour Compounds

A thesis submitted to the faculty for degree of
PHILOSOPHIAE DOCTOR

By:
Hachemi Kadri

Supervisor:
Dr. Andrew Westwell

August 2010

Declaration

This work has not previously been accepted in substance for any degree and is not concurrently submitted in candidature for any degree.

Signed (candidate) Date 25/08/2010

STATEMENT 1

This thesis is being submitted in partial fulfillment of the requirements for the degree of PhD

Signed (candidate) Date 25/08/2010

STATEMENT 2

This thesis is the result of my own independent work/investigation, except where otherwise stated.
Other sources are acknowledged by explicit references.

Signed (candidate) Date 25/08/2010

STATEMENT 3

I hereby give consent for my thesis, if accepted, to be available for photocopying and for inter-library loan, and for the title and summary to be made available to outside organisations.

Signed (candidate) Date 25/08/2010

Acknowledgements

- I would like to express my deep and sincere gratitude to my supervisor, Doctor Andrew Westwell, Welsh School of Pharmacy, Cardiff University. His wide knowledge, understanding, encouraging and personal guidance have provided a good basis for the present thesis.

- The members of the Westwell group as well as the other medicinal chemistry groups and all the staff in the Welsh School of Pharmacy have contributed immensely to my personal and professional time at Cardiff. The Westwell group has been a source of friendships as well as good advice and collaboration. Other past and present group members that I have had the pleasure to work with or alongside.

- I would also like to thank all the members of the Broadway pharmacy, Walsall, whom I have had a great time working with while writing up this work.

- The financial support of the Algerian government and the British Association for Cancer Research (BACR) is gratefully acknowledged.

- Lastly and most importantly, I would like to thank my family especially my parents for all their encouragement and support in all my pursuits.

Publications

1. **Kadri. H.**; Dale, T. C.; Ewan, K.; B. R.; Westwell, A. D. The design, synthesis and antitumour evaluation of novel small molecule inhibitors of the Dishevelled PDZ domain. *Eur. J. Cancer.* **2008**, *6*, S 436.

2. Aiello, S.; Wells, G.; Stone, E. L.; **Kadri, H.**; Bazzi, R.; Bell, D. R.; Stevens, M. F. G.; Matthews, C. S.; Bradshaw, T. D.; Westwell, A. D. Synthesis and biological properties of benzothiazole, benzoxazole, and chromen-4-one analogues of the potent antitumor agent 2-(3,4-dimethoxyphenyl)-5-fluorobenzothiazole (PMX 610, NSC 721648). *J. Med. Chem.* **2008**, 51, 5135-5139.

3. **Kadri, H.**; Matthews, C. S.; Bradshaw, T. D.; Stevens, M. F. G, Westwell, A. D. Synthesis and antitumour evaluation of novel 2-phenylbenzimidazoles. *J. Enz. Inhib. Med. Chem.* **2008**, 23, 641-647.

4. Ziedan, N. I.; **Kadri, H.**; Westwell, A. D. The development of pro-apoptotic cancer therapeutics. *Mini-Rev. Med. Chem.* **2008**, 8, 711-718.

Contents

1. Chapter One

Introduction

2. Chapter Two

The synthesis and antitumour evaluation of (substituted) 2-phenylbenzimidazoles

3. Chapter Three

The synthesis and antitumour evaluation of (substituted) 2-phenylindoles

4. Chapter Four

The synthesis and antitumour evaluation of N-substituted benzimidazole and indole analogues of PMX610

5. Chapter Five

6. Chapter Six

Introduction

7. Chapter Seven

The design of Dvl-Fz protein-protein interaction small molecule inhibitors

8. Chapter Eight

The synthesis and biological evaluation of the designed Dvl PDZ inhibitors

9. Chapter Nine

The design and synthesis of analogues for the identified hit compound

10. Chapter Ten

Abbreviations

aFGF: Acidic Fibroblast Growth Factor

AhR: Aryl Hydrocarbon Receptor

APC: Adenomatosis Polyposis Coli

BRCA1: Breast Cancer Gene 1

BRCA2: Breast Cancer Gene 2

β-TRCP: β-Transducin Repeat Containing Protein

CADD: Computer Assisted Drug Design

CDK: Cyclin-dependent Kinase

CDKI: Cyclin-dependent Kinase Inhibitor

ChemDiv: Chemical Diversity

CI: Chemical ionization

CML: Chronic Myelogenous Leukemia

DBU: 1,8-Diazabicyclo[5.4.0]undec-7-ene

DCC: Dicyclohexylcarbodiimide

DCM: Dichloromethane.

DMAC: Dimethylacetamide

DMC: Dimethylcarbonate

DMF: Dimethylformamide

DMSO: Dimethylsulfoxide

DNA: Deoxyribonucleic Acid

Dvl: Dishevelled

EBV: Epstein Barr Virus

EDCI: 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide

EGFR: Epidermal Growth Factor Receptor

EWG: Electron-Withdrawing Group

Fz: Frizzled

GI_{50}: Median growth inhibition concentration

PPA: Polyphosphoric Acid

Rac1: Ras-related C3 Botulinum Toxin Substrate 1

Rb: Retinoblastoma Protein

RHOA: RAS Homologue Gene-Family Member A

RMSD: Root Mean Square Deviation

RT: Room Temperature

SFRP: Soluble Wnt Inhibitory Proteins

S_N2: Bimolecular Nucleophilic Substitution

SVL: Scientific Vector Language

TBAF: Tetrabutylammonium Fluoride

TCF: T-Cell Factor

TEA: Triethylamine

TGF-α: Transforming Growth Factor Alpha

TGF-β: Transforming Growth Factor Beta

TNF-α: Tumour Necrosis Factor Alpha

THF: Tetrahydrofuran

TLC: Thin Layer Chromatography

UV: Ultraviolet

VEGF: Vascular Endothelial Growth Factor

VS: Virtual Screening

WHO: World Health Organization

Abstract

Cancer is a leading cause of death worldwide. Chemotherapy is the main approach used currently for the treatment and management of this disease especially disseminated malignant tumours. Traditional anticancer drugs have a limited selectivity as they target mainly DNA synthesis or cell proliferation leading to severe side effects. However, the newer anti-cancer drugs are more selective in targeting certain aberrant signalling pathways in cancer cells.

In the first part of this work, a series of benzimidazole and indole analogues of a potent antitumour benzothiazole lead compound were synthesised to enhance its pharmaceutical properties. Their antitumour activity was tested against different human cancer cell lines. In most cases, these analogues did not display any significant activity except for some of the N-substituted ones with *p*-toluenesulfonyl group, which yielded submicromolar GI_{50} values.

The second part of the work focuses on the design and synthesis of antitumour agents targeting the Wnt signalling pathway. The aberrant activation of this signalling pathway has been implicated in a wide spectrum of cancers. Disheveled PDZ domain is an essential protein-protein interaction mediator in the Wnt signalling pathway. Novel antitumour agents were designed using molecular modelling approaches to selectively inhibit the PDZ domain and hence abolish tumouregenic effects associated with the aberrant activation of Wnt signalling. Biochemical binding assays showed that one of the hits obtained through high-throughput docking binds specifically to the Disheveled PDZ domain. Structural optimisation studies of this hit were also carried out in order to improve activity.

Part 1

The synthesis and biological evaluation of novel benzimidazole and indole based antitumour compounds

1. INTRODUCTION

1.1. Cancer

Cancer is a group of diseases characterised by unregulated growth and abnormal division of cells, which attain the ability to invade adjacent tissues or spread from their site of origin to other sites and anatomical parts in the body.[1] Cancer is considered a leading cause of death worldwide. According to the World Health Organization (WHO), cancer accounted for 7.6 million (13%) of all deaths worldwide in 2005.[2] Moreover, these figures are expected to continue rising to reach an estimated 9 million (2015) and 11.4 million (2030) cancer-related deaths.[2] It is also expected that cancer rates could increase from 10 million new cases globally in 2000 to 15 million new cases in 2020.[2]While various factors may well contribute to this estimated sharp increase in new cases, the worldwide steadily ageing population is recognised as the chief factor.[3]

The tissue of origin confers cancer distinct characteristics and features and therefore, over one hundred cancer types have been classified which can be divided into the following main categories.

Carcinomas: this class originates from epithelial cells and represents the most widespread group of cancers (85%) including the common forms of breast, lung, prostate and colon cancer.

Sarcomas: these type of cancers are derived from the connective tissue cells which originate from embryonic mesoderm such as bone, cartilage, fat, muscle and blood vessels.

Lymphomas: cancers of the lymphatic system, which often arise in the lymph nodes.

Leukaemia: cancer of the blood or bone marrow.

Blastoma: these cancers are most common in children and are derived from primitive or incompletely differentiated cells. Most common examples in this class include nephroblastoma (kidneys), medulloblastoma (cerebellum) and retinoblastoma (retina).

Germ cell tumours: tumours derived from germ cells and the most common cancers of this group are ovarian and testicular cancers.

1.2. Causes and risk factors

The massive research endeavour in the pathology and molecular biology of cancer over the past few decades has established that this disease develops due to abnormalities at the genetic level with mutations or alterations of mainly two gene classes; oncogenes and tumour suppressor genes.[4] Alterations to DNA sequences of these two gene classes trigger a rather complicated series of events at the cellular level leading to the multistage formation of tumour cells. These alterations can occur as a result of a combination of genetic factors and external factors which can be divided into three main categories including; physical, chemical and biological carcinogens (cancer causing substances or agents).

1.2.1. Physical carcinogens: examples include ultraviolet (UV) and ionizing radiation, which can damage cellular DNA causing formation of tumour cells. The primary source for UV radiation is sunlight and prolonged exposure is associated with increased risk of developing melanoma and skin cancers. Ionizing radiation particles (α and β particles) as well as X-ray and γ-rays are known to cause DNA fragmentation through the formation of free radicals.[5]

1.2.2. Chemical carcinogens: examples include but not limited to; asbestos, components of tobacco smoke and aflatoxins.[6-8] While prolonged exposure to the microscopic fibers of the insulating material asbestos is linked particularly to mesothelioma, tobacco smoking is a known cause of many forms of cancer and mainly lung cancer (90% of cases). Aflatoxins are potent carcinogens, which are produced by many fungus species and can gain entry to the body through contaminated food. Once ingested, these carcinogens can get metabolized to the highly reactive epoxide intermediates, which can cause DNA damage and ultimately lead to a tumour.

1.2.3. Biological carcinogens: examples include certain bacterial and viral infections, which are estimated to account for up to 20% of all cancers.[9]For bacterial infections, the most prominent example is the finding that chronic infection with *Helicobactor pylori is*

the main leading cause for developing gastric cancers.[10] On the other hand, viruses are linked to a variety of human malignancies including the role of Epstein Barr virus (EBV) in Burkitt's lymphoma, the human papillomavirus (cervical carcinoma) and hepatitis B and hepatitis C (liver cancer).[9]

Nevertheless, the aforementioned causes do not represent by any means an exhaustive list; indeed, only a selection are presented and more causes and risk factors are still being identified by research studies. In most cases, it is the combination between these factors that lead to developing cancer, as it is a multifactorial disease. It is also worthy to note that while most cancers are generally not inherited, an increased predisposition to certain types of cancer have been linked to inheritance of some genes. For instance, inherited mutations in the genes BRCA1 and BRCA2 have been closely associated with breast and ovarian cancers.[11]

1.3. Hallmarks of cancer

The underlying cellular and molecular routes involved in the carcinogenesis process differ between cancer types but the end result is the same since all cancers share certain characteristics and features. In 2000, Hanahan and Weinberg defined and described six common hallmarks for most cancers: self-sufficiency in growth signals, insensitivity to anti-growth signals, apoptosis (programmed cell death) evasion, limitless replicative potential, sustained angiogenesis and metastasis (Figure 1).[12]

1.3.1.Self-sufficiency in growth signals: normal cells require mitogenic growth signals, which are transmitted into the cell from their normal tissue microenvironment by transmembrane receptors to move from a quiescent state into the active proliferative state. On the other hand, tumour cells develop the ability to generate their own growth signals. Three main strategies are used by tumour cells to acquire growth signal autonomy. These strategies involve the overexpression of extracellular growth factor (GF) receptors, alterations in components of the downstream cytoplasmic circuitry and

alterations of extracellular matrix receptors (integrin). For instance, HER2/neureceptor is upregulated in stomach and mammary carcinomas.[13]Oncogenes activate cellular proliferation, leading to unregulated cell growth and differentiation. Most of these oncogenes are derived from a non-mutant version of genes known as proto-oncogenes, which are usually involved in normal cellular growth. Examples of oncogenes include ras, which is known to be mutated in up to 15% of all cancer, myc and abl.[14]

1.3.2. Insensitivity to growth inhibitory signals: normal cells also require anti-growth signals, which operate to maintain cellular quiescence and tissue homeostasis. These anti-growth signals can block cell proliferation by forcing cells into the quiescent state (G_0 of cell cycle) or by inducing their terminal differentiation to enter a postmitotic state where they are no longer able to re-enter the cell cycle. Cancer may arise from inactivation or malfunction of genes that normally suppress cell proliferation. This inhibitory signalling is mediated by ligands acting on cellular receptors such as transforming growth factor beta (TGF-β), and its receptor (TGF-β receptor) to transduce signals to the nucleus via secondary messengers.[15] These pathways involve mainly proteins such as retinoblastoma protein (Rb), cyclins, cyclin-dependent kinases (CDK) and their inhibitors (CDKi) to finely control the cell cycle clock.[15] Aberrant activation of these inhibitory pathways is associated with tumourgenesis. For instance, loss of Rb or members of the CDKi family and overexpression of certain cyclins and CDK have been implicated in a range of tumours.[16]

1.3.3. Evading apoptosis: the number of cells is maintained relatively constant through means of cell death and division. It follows that there is a continuous process of assessing the balance of anti-apoptotic(survival) and pro-apoptotic (death) signals received by cells.In normal cells, cell cycle arrestoccurs as a result of DNA damage to enable the potential repair. However, once damage exceeds the cell repair capacity, the balance of anti- and pro-apoptotic signals is lost and cells undergo programmed cell death (apoptosis) to avoid the risk that mutations are carried through the replicative process. Therefore, apoptosis represents a very powerful barrier to the development of cancer.

Nonetheless, cancer cells can sustain DNA damage by directing the balance of intracellular pro-apoptotic and anti-apoptotic molecules in favour of inhibition of apoptosis. Indeed, the tumour suppressor gene p53 and the oncogene bcl-2 which are two of the best characterised cancer-associated genes are crucially involved in apoptosis.[17,18]

1.3.4. Limitless replicative potential: normal cells have a limited and a finite number of cell divisions, which can they undergo before they enter a replicative senescence. This is a period of permanent growth arrest. The process is driven by the inability of cells to fully replicate the ends of their chromosomes (the telomeres) at each division and as subsequently, telomeres get progressively shorter. On the other hand, cancer cells acquire the ability to maintain the length of their telomeres mostly through upregulation of the enzyme complex telomerase. Telomerase consists of two main components; an RNA template (hTR) and a reverse transcriptase (hTERT).[19-21] This latter uses the RNA template as a guide in the resynthesis of the telomere DNA sequence which enables tumours to rebuild any lost telomere parts occurring at each round of cell division.

1.3.5. Sustained angiogenesis: in normal tissue, the process of angiogenesis which is the growth of new blood vessels is well controlled by a balance between the positive pro-angiogenic and the negative anti-angiogenic signals. Examples of pro-angiogenic signals include vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), acidic fibroblast growth factor (aFGF), transforming growth factors alpha and beta (TGF-α, TGF-β), platelet-derived growth factor (PDGF) and tumour necrosis factor alpha (TNF-α).[22,23] On the other hand, anti-angiogenic signals include angiostatin, endostatin, interleukins (IL-1β, IL-12, IL-18) and anti-thrombin III.[22,23]Solid tumours acquire the ability to grow new blood vessels to ensure a sustained supply of oxygen and nutrients by subverting the controlled balance between the positive and negative angiogenic signals. It follows therefore that the production of pro-angiogenic proteins is upregulated while the anti-angiogenic proteins production is down-regulated by cancer cells.

1.3.6. Tissue invasion and metastasis: this is the process by which cancer spreads from its primary site where it first originated to distant parts of the body.[24] It is very common in the late stages of the disease and accounts for 90% of cancer deaths. Metastasis involves a series of biological processes, which include the following:

- Detachment from immediate surrounding tissue at the local site mediated by the enzymatic digestion of the extracellular matrix.
- Specific directional motility and entry to blood or lymphatic vessels.
- Survival in the circulation until arrival at the metastatic site that may be chosen on the basis of provision of a favourable supply of appropriate growth factors.
- Adherence to the blood vessels endothelium at its destination and extravasation from the vessel.
- Proliferation and invasion of the new location.
- Angiogenesis initiation to ensure growth.

Research studies have established that metastasis is not a random process but rather driven by expression of specific chemokine receptors by tumour cells that allow them to find a suitable environment for colony establishment.[25]

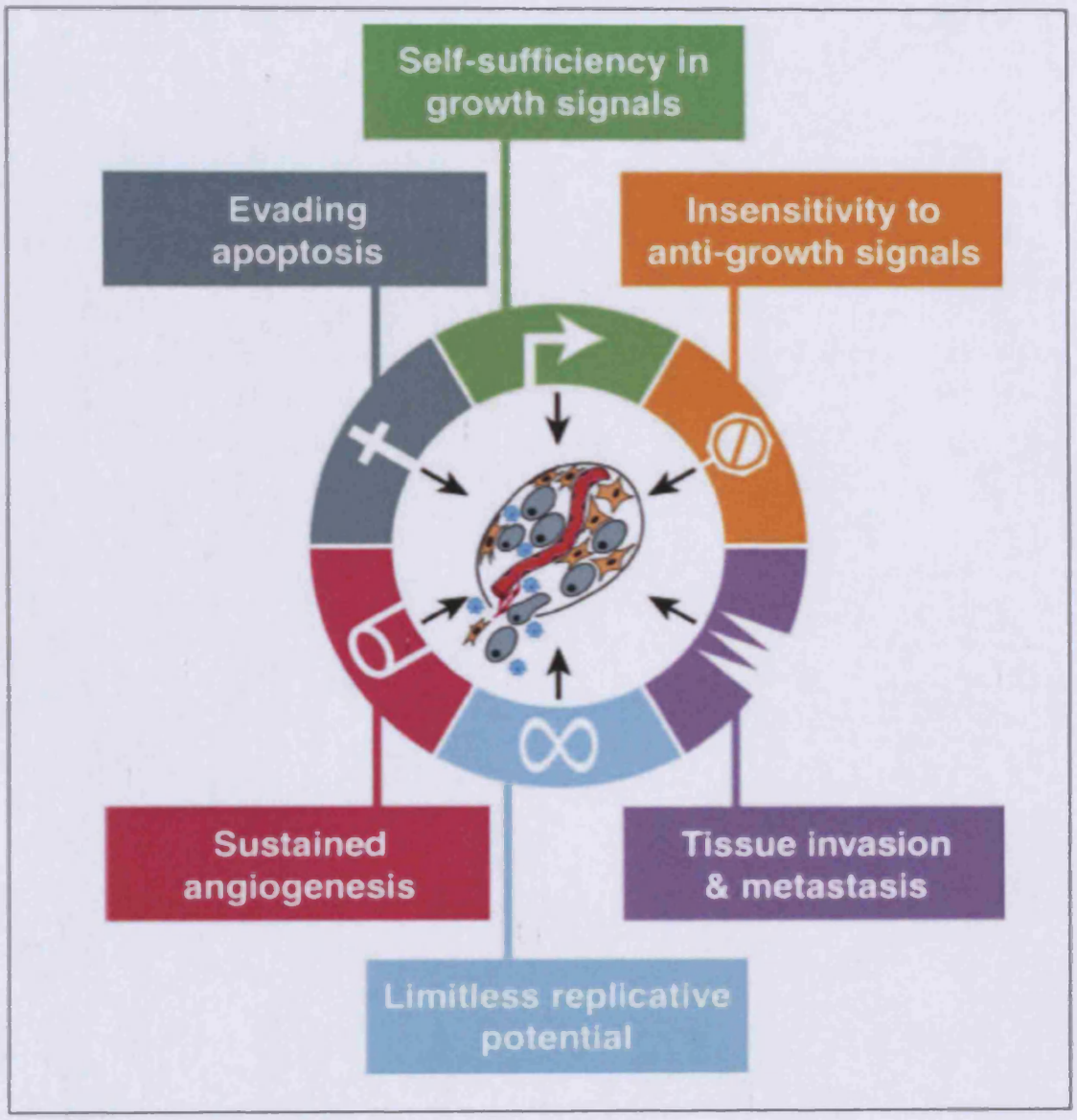


Figure 1: The six hallmarks of cancer (adapted from reference 12).

1.4. Current treatment for cancer

There are several approaches for cancer treatment and management, which include mainly surgery, radiotherapy, photodynamic therapy and chemotherapy. It is the case that most often more than one approach is used depending on the nature and stage of the

cancer. The treatment also depends on the patient's general health condition, age and lifestyle.

1.4.1. Surgery: is the first modality used successfully in the treatment of cancer especially by removal of some common solid tumors. However, it is usually used in combination with chemotherapy and/or radiotherapy. The administration of chemotherapy and radiotherapy can be carried out either before surgery to shrink the tumour which facilitates its removal or most commonly after to ensure absence of any metastases or local infiltration. A large area of the surrounding normal tissue is also often removed during the process with the aim of achieving total eradication of cancer cells.

1.4.2. Radiotherapy: it is estimated that around 40% of cancer patients will receive radiotherapy at some point either as a form of treatment or to manage symptoms.[26] The principal goal of this approach is to locally deliver a tumouricidal dose of ionizing radiation in the form of X-ray or γ-rays to the cancer site without affecting the surrounding healthy tissue.[27] The success of radiotherapy depends on the difference in radiosensitivity between tumour and normal tissue. Radiotherapy is usually categorized into three main types depending on method of delivery and position of radiation source: external beam radiotherapy (teletherapy, radiation source is outside the body), internal beam therapy (brachytherapy, radioactive source is placed in site of treatment) and systemic radioisotope therapy (radioisotopes administered by infusion or given orally). In this latter category, targeting can be achieved by attaching the radioisotope to an antibody or by exploiting the chemical properties of the isotope to achieve specific absorption at a given target site such as the use of iodine-131 (^{131}I) in the treatment of thyroid cancer.

In terms of side effects, radiotherapy is associated with acute complications which are often limited to the area being treated but systemic ones may occur which include fatigue, skin reactions, GI toxicity, oropharyngeal mucositis and myelosuppression. Long-term complications may occur months or years after treatment as a result of damage to blood vessels and connective tissue, which include an increased risk of developing both secondary leukaemia and solid tumours.

1.4.3. Photodynamic therapy (PDT): PDT centers around the use of a radiation light of an appropriate wavelength to induce formation of cytotoxic free radicals in the targeted cancer cells.[28] As well as a radiation source, PDT requires the use of a photosensitiser, which is selectively administered to the target tissue. A photosensitiser is a chemical compound (e.g. Photofrin), which can move from its ground state to an excited singlet state upon exposure to a light of a specific wavelength leading to the formation of reactive oxygen species. These free radicals undergo a series of rapid reactions with nearby biomolecules leading to damage and ultimately destruction of tumour cells through apoptosis and necrosis. Massive research efforts are currently undergoing in this area of cancer treatment especially in applying nanotechnology to photosensitisers.[29]

1.4.4. Chemotherapy: systemic chemotherapy is the main approach used currently for the treatment of disseminated malignant tumours. Traditional chemotherapy drugs can be grouped into alkylating agents, antimetabolites, anthracyclines, plant alkaloids and topisomerase inhibitors. While these classes of drugs affect mainly cell division or DNA synthesis, newer anti-cancer drugs are more selective in targeting certain aberrantly activated pathways in cancer cells. Examples of some current and prospective modalities of cancer treatments are listed in Table 1.

Category	Function	Examples
Antimetabolites	Interfere with intermediary metabolism of proliferating cells	Methotrexate, 5-Fluorouracil
Monoclonal Antibodies	Target cancer cells that express specific antigen	Herceptin, Zevalin
Mitosis Inhibitors	Target microtubules and proteins required in cell division	Taxol
Steroid hormones	Block steroid and hormone-dependent growth of certain tumours	Tamoxifen, Flutamide
Alkylating / cross-linking agents	Causes death of growing cells by damaging DNA	Endoxan, Cisplatin,Cyclophosphamide
Signal-transduction agents	Modulate communications between cells	Gleevec, Tarceva, Iressa
Angiogenesis inhibitors	Block blood vessel formation to tumour	Avastin
Histone-deacetylase inhibitors	Disrupts transcription of genes	SAHA
Telemorase inhibitors	Affect telomere maintenance required for tumour growth	BIBR1532
Antitumour antibiotics	Bind DNA to prevent DNA and/or RNA tsynthesis	Doxorubicin, Etoposide

Table 1: Examples of some current and prospective modalities of cancer chemotherapy treatment (modified from reference 30).

1.5. Cancer and drug discovery

The main focus of cancer drug discovery has traditionally been on targeting cell division and DNA synthesis leading to the identification of different anti-cancer drugs such as antimetabolites, alkylating, cross-linking and antimitotic agents. While such drugs have been proven to be effective, their selectivity for tumour cells over normal cells is limited leading to severe side effects.

Driven by the massive advances of cancer molecular and cellular biology, cancer drug discovery has become dominated by the target-driven approach in both academia and industry.[31]It is argued that drugs discovered through this approach would achieve more selectivity to the molecular target and hence overcome the limitations experienced with most conventional chemotherapies which are based on DNA targeting and their immense adverse effects. Typically, in this approach, the identification and validation of a substantial target, which plays a major role in the formation of tumour is the first and most important step. The screening of small compound libraries comes next in order; a process that can be achieved through high throughout screening tools. Then, the identified hit molecule can be developed to a drug-like lead compound which may also require further optimisation to achieve some desired pharmaceutical properties before becoming a clinical drug candidate. Undoubtedly, the development of imatinib (Gleevec, Figure 2) which is used mainly for the treatment of chronic myelogenous leukemia (CML) through this approach and its success pushed more research groups to adopt this methodology of drug discovery.[32]

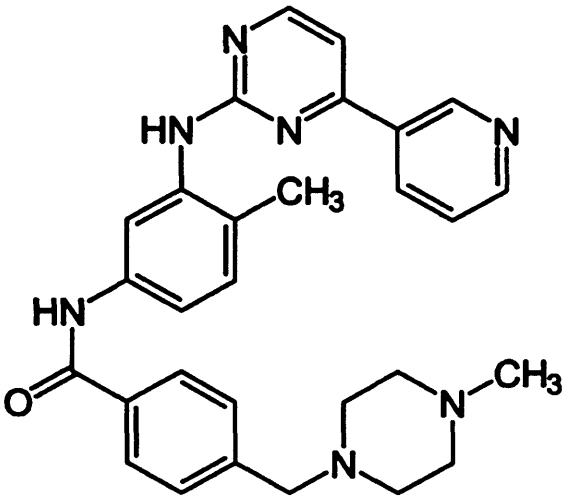


Figure 2: Chemical structure of imatinib (Gleevec)

On the other hand, chemistry-driven drug discovery which can be considered as conducting the drug discovery process in a reverse manner is emerging as another approach in cancer drug discovery.[33]Unlike the targeted approach, small molecules not targets are the main focus and potential compound leads are selected based on their induction of particular phenotypic responses in cellular environment. These phenotypic changes can be analysed by screening tools such as the NCI-60 panel. The approach is best illustrated by the discovery of the novel antitumour agent Phortress.

1.5.1. *Phortress: A novel, potent and selective antitumour agent.*

Phortress is an experimental antitumour agent with potent and selective activity against human-derived carcinomas of breast, ovarian and renal origin. It is currently in Phase 1 clinical trials in the Northern Institute for Cancer Research at Newcastle University. The discovery of this clinical antitumour agent is the result of iterative interactions between medicinal chemistry and pharmacology research groups.

The start of the Phortress development was based on the observation that the compound 2-(4-aminophenyl)benzothiazole (CJM 126, Figure 3) inhibited growth in the human derived breast cancer cell line MCF-7 at submicromolar concentrations. Subsequent structural optimisation studies led to the identification of the compound DF203 (2-(4-amino-3-methylphenyl)benzothiazole) with a remarkable differential activity *in vitro* and *in vivo* against a spectrum of tumour types including breast, ovarian, renal and colon cancers despite its simple structure.[34] However, DF203 exhibited a unique biphasic dose-response relationship in sensitive cell lines. At low nanomolar concentration of this compound, cell growth inhibition occurs but this was followed by a second growth phase in that a proliferative response was noticed at higher (low micromolar) concentrations. It was found that at higher concentrations, DF203 metabolism led to the production of a hydroxylated derivative (2-(4-amino-3-methylphenyl)-6-hydroxybenzothiazole), which inactivated the bio-activating enzyme, cytochrome P450 (CYP1A1).[35,36] In order to overcome this deactivating metabolism, fluorine substituents were introduced to the benzothiazole ring leading to the optimised compound (5F203 as shown in Figure 3).[35,36] However, one major limitation to the application of 5F203 in the clinic was its inherent lipophilicity and poor aqueous solubility. Therefore, to enhance pharmaceutical properties of 5F203, its lysyl amide prodrug (Phortress) was prepared in which activity and selectivity are retained.[37]

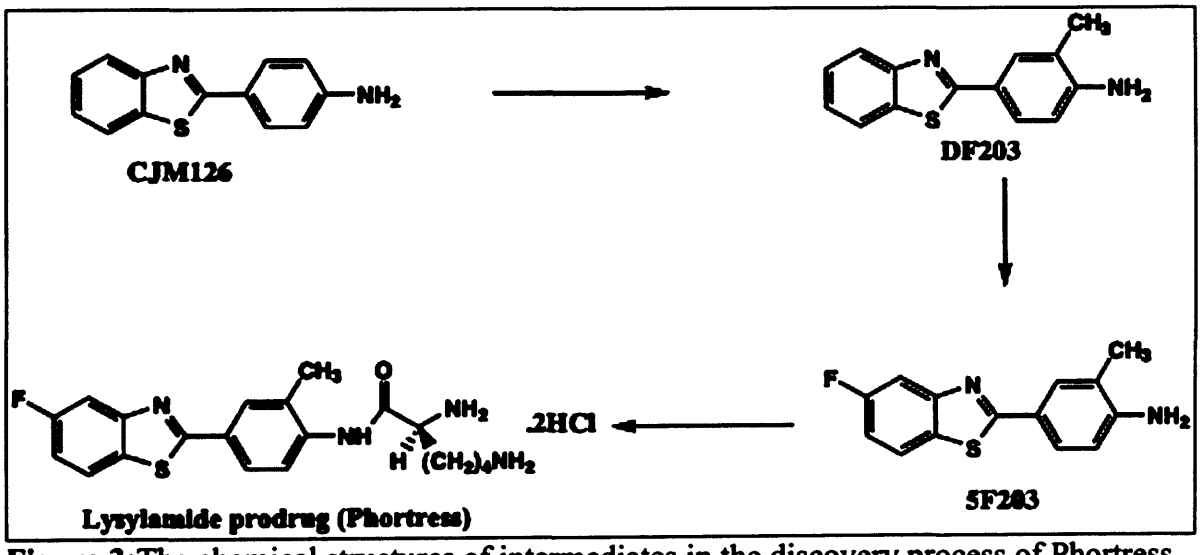


Figure 3: The chemical structures of intermediates in the discovery process of Phortress.

Phortress is readily absorbed and hydrolysed to 5F203. Mechanistic studies have established now that the mechanism of action of this compound lies in its interaction with the arylhydrocarbon receptor (AhR) to selectively induce expression of the cytochrome P450 CYP1A1 in breast and ovarian cancer cell lines (Figure 4).[34]

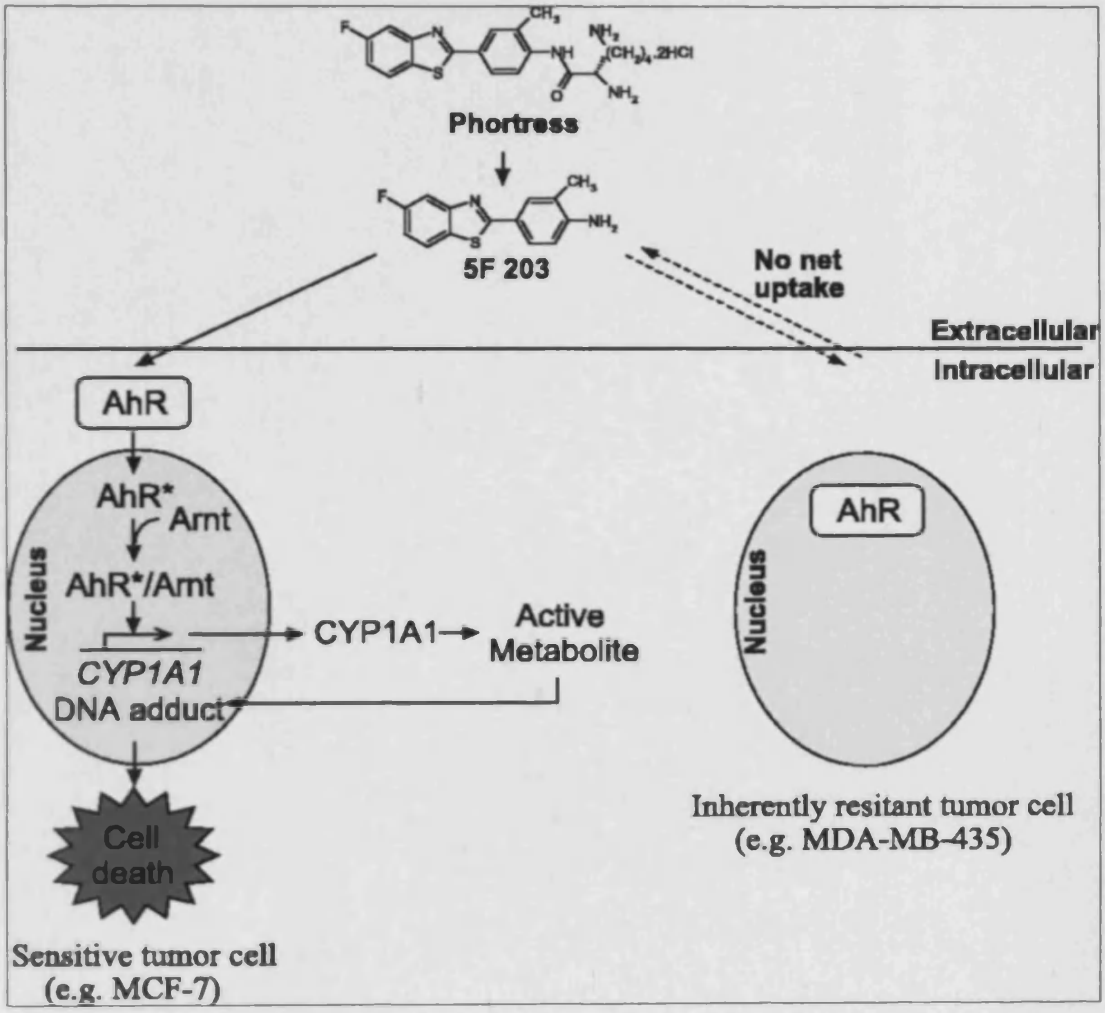


Figure 4: the proposed mechanism of action of Phortress (adapted from reference 34).

Moreover, as a continuation to exploring this chemistry-driven approach, a closely related benzothiazole based analogue; 2-(3,4-dimethoxyphenyl)-5-fluorobenzothiazole (GW 610, Figure 5) otherwise known as PMX610 (being developed in Pharminox laboratories; a UK based pharmaceutical company) has been found to have potent (sub-

nanomolar GI_{50}) and selective in vitro antitumor properties in colon and non-small cell lung cancer cell line.[38,39] Despite its structural similarities to 5F203, this new benzothiazole antitumour agent (PMX 610) is proven to have a different mechanism of action though it still not well understood as yet.[39]

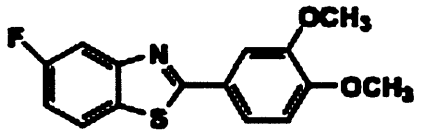


Figure 5: The chemical structures of PMX610.

1.6. Aims and objectives

PMX610 has a very potent antitumour activity with profound selectivity in colon and non-small cell lung cancer cell lines but *in vivo* studies of this compound using standard aqueous formulations were compromised by its high lipophilicity (logP 4.2). Therefore, this project aims to explore and evaluate the antitumour activity of other benzothiazole-related heterocycle analogues namely benzimidazole and indole congeners which are likely to have superior pharmaceutical properties. Promising results were obtained when this hypothesis was tested on another structurally-related heterocycle the benzoxazole ring by our group and more details of this work have already been published (reference 40).

The benzimidazole and indole nucleus provide a versatile scaffold in medicinal chemistry for the design of new therapeutic agents, and a number of substituted benzimidazoles possessing a range of biological activities have been reported.[41] Amongst the range of biological activities ascribed to benzimidazole derivatives have been antitumour, antiparasitic, antiviral and antimicrobial activity.[41] Of particular interest have been

reports of antitumour benzimidazoles with activity against cancer drug targets of current interest, as exemplified for example by a benzimidazole-based inhibitor of polo-like kinase 1,2-arylbenzimidazole-4-carboxamides as PARP-1 inhibitors and the thioredoxin-inhibitory antitumour quinols including benzimidazole derivatives.[42-44]On the other hand, anti-cancer agents containing the indole nucleus have been widely reported especially in the area of tubulin polymerisation inhibiton.[45]

Initial work will focus on optimising conditions for the synthesis of 2-substituted benzimidazole and indole congeners of PMX610 and a particular consideration will be drawn toward fluorinated ones. Once a robust synthetic route is established, small libraries with focused analogues will be prepared. Then, depending on the biological properties, the work may be extended to generate larger libraries with a wide variety of substituents.

References

1. Pecorino, L. Molecular biology of cancer: mechanisms, targets and therapeutics. *Oxford Press.***2005**,1-20.
2. World Health Organisation (WHO) statistics. http://www.who.int/cancer/en/
3. Weinberg, R. A. The biology of cancer. *Garland Science,* **2007,** 1-14.
4. Stanley, L. A. Molecular aspects of chemical carcinogenesis: the roles of oncogenes and tumour suppressor genes. *Toxicology***1995**, 96, 173-194.
5. Little, J. B. Radiation carcinogenesis. *Carcinogenesis***2000,** 21, 397-404.
6. Kamp, D. W. Asbestos-induced lung diseases: an update. *Trans. Res.* **2009,** 153, 143-152.
7. Wild, C. P.; Turner, P. C. The toxicology of aflatoxins as a basis for public health decisions. *Mutagenesis***2002,** 17, 471-481.
8. Saffiotti, U. J. Identification and definition of chemical carcinogens: review of criteria and research needs. *J.Toxicol. Environ. Health.***1980,** 6, 1029-1057.
9. Pagano, J. S.; Blaser, M.; Buendia, M-A.; Damania, B.; Khalili, K.; Raab-Traub, N.; Roizman, B. Infectious agents and cancer: criteria for causal relation. *Semin. Canc. Biol.* **2004,** 14, 453-471.
10. Dooley, C. P. Helicobacter pylori: review of research findings. *Ailment. Pharmacol. Ther.* **1991,** 129-143.
11. Ramus, S. J.; Gayther, S. A. The contribution of BRCA1 and BRCA2 to ovarian cancer. *Molecular Oncology***2009**,3, 138-150.
12. Hanahan, D.; Weinberg, R.A. The hallmarks of cancer. *Cell***2000,** 100, 57–70.
13. Slamon, D. J.; Clark, G. M.; Wong, S. G.; Levin, W. J.; Ullrich, A.; McGuire, W. L. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neuoncogene. *Science***1987,** 235, 177–182.
14. Malumbres, M.; Barbacid, M. RAS oncogenes: the first 30 years. *Nat. Rev. Cancer.***2003,** 3, 459-465.
15. Hinds, P, W.; Weinberg, R. A. Tumour suppressor genes. *Curr. Opin. Genet. Dev.*

1994, 4,135-141.

16. Burkhart, D. L.; Sage, J. Cellular mechanisms of tumour suppression by the retinoblastoma gene. *Nat. Rev. Cancer.***2008,** 8, 671-682.
17. Cory, S.; Adams, J. M. The Bcl 2 family: regulators of the cellular life-or-death switch.*Nat. Rev. Cancer.* **2002,** 2, 647-656.
18. Vousden, K.; Lu, X. Live or let die: The cell response to p 53. *Nat. Rev. Cancer.***2002,** 2, 594-604.
19. Reddel, R. R. The role of senescence and immortalization in carcinogenesis. *Carcinogenesis.* **2000,** 21, 477-48.
20. Blasco, M. A. Mammalian telomeres and telomerase: why they matter for cancer and aging. *Eur. J. Cell. Biol.* 2003, 82, 441-446.
21. Bachand, F.; Ibtissem, T.; Autexier, C. Human telomerase RNA-protein interactions. *Nucleic. Acids. Res.***2001,** 29, 3385-3393.
22. Kerbel, R. S. Tumor angiogenesis. *N. Engl. J. Med.***2008,** 358, 2039-2049.
23. Zerbini, G.; Lorenzi, M.; Palini, A.; Kerbel, R. S. Tumor Angiogenesis. *N. Engl. J. Med.***2008,** 359, 763-764.
24. Yilmaz, M.;Christofori, G.; Lehembre, F. Distinct mechanisms of tumor invasion and metastasis. *Trends. Mol. Med.***2007,** 13, 535-541.
25. Muller, A.; Homey, B.; Soto, H.; Ge, N.; Catron, D.; Buchanan, M. Eet al. Involvement of chemokine receptors in breast cancer metastasis.*Nature***2001,**410, 50-56.
26. Cancer Research UK (http://info.cancerresearchuk.org/)
27. Delaney, G.; Jacob, S.; Featherstone, C.; Barton, M. The role of radiotherapy in cancer treatment: estimating optimal utilization from a review of evidence-based clinical guidelines. *Cancer***2005,** 104, 1129-37.
28. Brown, S.; Brown, E. A.; Walker, I. The present and future role of photodynamic therapy in cancer treatment. *Lancet. Oncol.***2004,** 5, 497-508.
29. Wang, X.; Yang, L.; Chen, Z. G.; Shin, D. M. Application of nanotechnology in cancer therapy and imaging. *CA.Cancer. J. Clin.***2008,** 58, 97–110.
30. Atkins, J. H.; Gershell, L. J. Selective anticancer drugs. *Nat. Rev. Drug. Disc.***2002,**1. 491–492.

31. Eliseev, A.V. Target driven chemistry in drug discovery. *Drug.Discov. Today* **2004**,9, 348–349.

32. Druker, B. J. STI571 (Gleevec) as a paradigm for cancer therapy. *Trends Mol. Med.* **2002**, 8, 14-18.

33. Westwell, A.D; Stevens, M.F.G. Hitting the chemotherapy jackpot: strategy, productivity and chemistry. *Drug. Discov. Today***2004**, 9, 625–627.

34. Bradshaw, T. D.; Stevens, M.F.G.; Westwell, A. D. The discovery of the potent and selective antitumour agent 2-(4-amino-3-methylphenyl)benzothiazole (DF 203) and related compounds. *Cur. Med. Chem.***2001**, 8, 203-210.

35. Brantley, E.; Trapani, V.; Alley, M. C.; Hose, C. D.; Bradshaw, T. D.; Stevens, M. F. G.;Sausville, E. A.; Stinson, S. F. Fluorinated 2-(4-amino-3-methylphenyl)benzothiazoles induce CYP1A1 expression, become metabolized, and bind to macromolecules in sensitive human cancer cells. *Drug. Metab. Disp.***2004**, 32, 1392-1401.

36. Chua, M-S.; Kashiyama, E.; Bradshaw, T. D.; Stinson, S. F.; Brantley, E.; Sausville, E. A.; Stevens, M. F. G. Role of CYP1A1 in modulation of antitumor properties of the novel agent 2-(4-amino-3-methylphenyl)benzothiazole (DF 203, NSC 674495) in human breast cancer cells. *Cancer. Res.***2000**, 60, 5196-5203.

37. Bradshaw, T. D.; Westwell, A. D. The development of the antitumour benzothiazole prodrug, Phortress, as a clinical candidate. *Cur. Med. Chem.***2004**, 11, 1241-1253.

38. Pharminox Ltd (*www.pharminox.com*).

39. Mortimer, C. G.; Wells, G.; Crochard, J-P.; Stone, E. L.; Bradshaw, T. D.; Stevens, M. F. G.; Westwell, A. D. Antitumor benzothiazoles. 26. 2-(3,4-Dimethoxyphenyl)-5-fluorobenzothiazole (GW 610, NSC 721648), a simple fluorinated 2-arylbenzothiazole, shows potent and selective inhibitory activity against lung, colon, and breast cancer cell lines. *J. Med. Chem.* **2006**, 49,179-185.

40. Aiello, S.; Wells, G.; Stone, E. L.; Kadri, H.; Bazzi, R.; Bell, D. R.; Stevens, M. F. G.; Matthews, C. S.; Bradshaw, T. D.; Westwell, A. D.Synthesis and biological properties of benzothiazole, benzoxazole, and chromen-4-one analogues of the potent antitumor agent 2-(3,4-dimethoxyphenyl)-5-fluorobenzothiazole (PMX

610, NSC 721648). *J. Med. Chem.***2008**, *51*, 5135-5139.

41. Boiani, M.; Gonzalez, M. Imidazole and benzimidazole derivatives as chemotherapeutic agents. *Mini. Rev. Med. Chem.***2005**, 5, 409-424.
42. Lansing, T. J; McConnell, R.T.; Duckett, D.R.; Spehar, G.M.; Knick, V.B.; Hassler,D. F.; Noro, N.; Furuta, M.; Emmitte, K.A.; Gilmer, T. M.; Mook, R.A.Jr.; Cheung, M. In vitro biological activity of a novel small-molecule inhibitor of polo-like kinase 1. *Mol. Cancer. Therap.***2007**, 6, 450-459.
43. White, A.W.; Curtin, N. J.; Eastman, B.W.; Golding. B. T.; Hostomsky, Z.; Kyle, S.; Li, J.; Maegley, K.A.; Skalitzky, D.J.; Webber, S. E.; Yu, X.H.; Griffin, R.J. Potentiation of cytotoxic drug activity in human tumour cell lines, by amino-substituted 2-arylbenzimidazole-4-carboxamide PARP-1 inhibitors. *Bioorg. Med. Chem. Lett.***2004**, 14, 2433-2437.
44. Berry, J. M.; Bradshaw, T. D.; Fichtner, I.; Ren, R.; Schwalbe, C. H.; Wells, G.; Chew, E. H.; Stevens, M. F .G.; Westwell, A. D. Quinols as novel therapeutic agents. 2,4-(1-Arylsulfonylindol-2-yl)-4-hydroxycyclohexa-2,5-dien-1-ones and related agents as potent and selective antitumor agents. *J. Med. Chem.***2005**. 48, 639-644.
45. Brancale, A.; Silvestri, R. Indole, a core nucleus for potent inhibitors of tubulin polymirization. *Med. Res. Rev.* **2007**, 27, 209-238

2. The synthesis and antitumour evaluation of (substituted) 2-phenylbenzimidazoles

2.1. Chemistry

(Substituted) 2-phenylbenzimidazoles are most commonly synthesised via two main approaches as illustrated in Figure 1. One involves a condensation-dehydration of *o*-phenylenediamines with carboxylic acids or their derivatives (nitriles, imidates or orthoesters) while the other consists of a condensation with aldehydes under oxidative conditions.[1] In most cases, the first approach requires harsh conditions such as the use of strong acidic medium at elevated temperatures.[2] In addition to the relatively milder conditions, the use of the second approach has the advantage of having a wide range of differently substituted aldehydes commercially available.[3] Therefore, this latter approach was our first choice for the preparation of the 2-phenylbenzimidazole based analogues of PMX610.

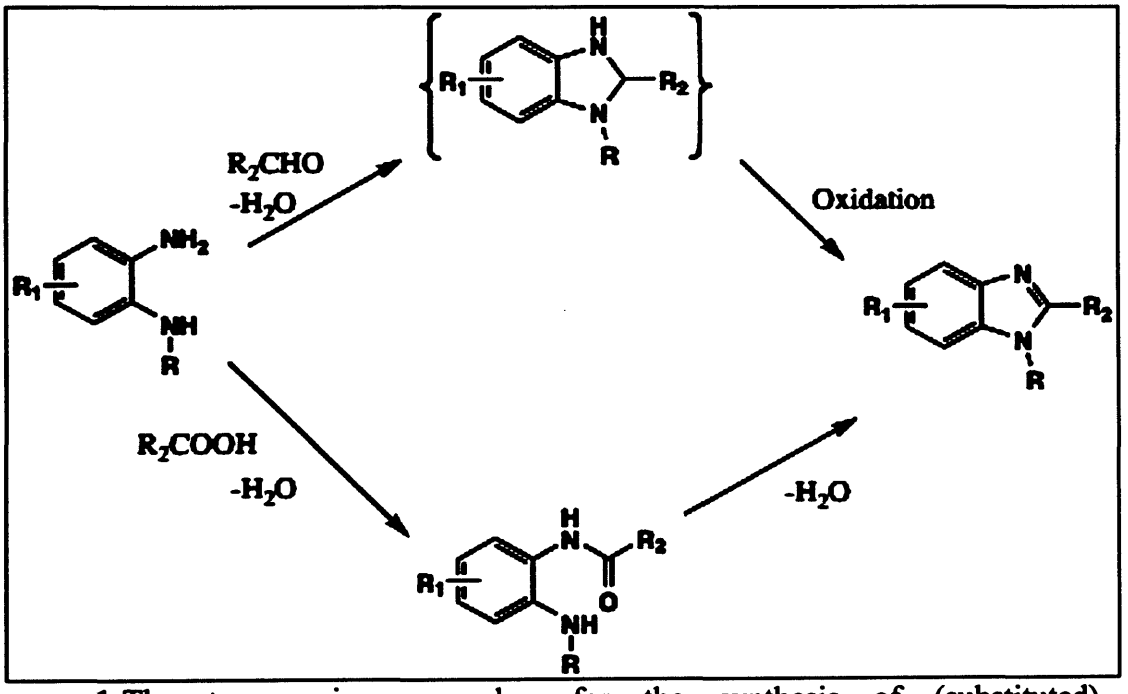
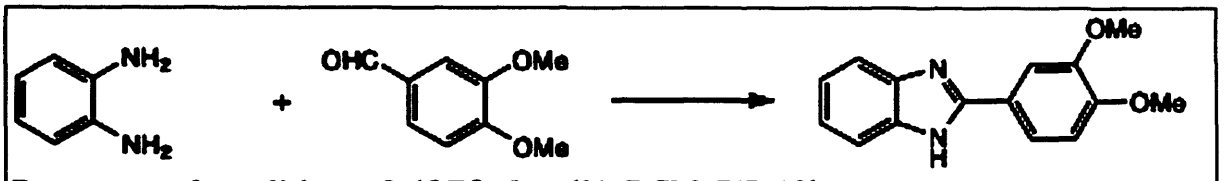


Figure 1: The two main approaches for the synthesis of (substituted) 2-phenylbenzimidazoles.

2.1.1. The synthesis of 2-phenylbenzimidazoles

2.1.1.1. The use of indium triflate $In(OTf)_3$:

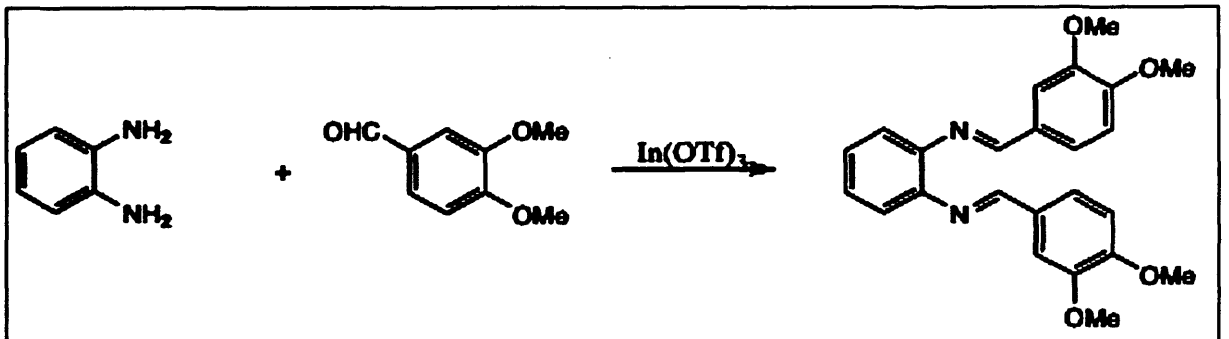
The use of the mild Lewis acid $In(OTf)_3$hasrecently been reported for the convenient synthesis of 2-arylbenzimidazoles in a one-pot procedure (Scheme 1).[4] It is also worthwhile to note that another triflate agent ytterbium triflate($Yb(OTf)_3$)was also reported previously for the synthesis of 2-arylbenzimidazole under similar conditions.[5]



Reagents and conditions: $In(OTf)_3$ 5 mol%, DCM, RT, 12hr.

Scheme 1

This synthetic route involves the condensation of o-phenylenediamines and aldehydes in the presence of catalytic amounts of the commercially available $In(OTf)_3$ (5mol%) at room temperature. However, when attempting this procedure, a mixture of several components was obtained with the diamine compound (N,N-Bis-(3,4-dimethoxy-benzylidene)-benzene-1,2-diamine) being the major isolated one as illustrated below (Scheme 2).



Reagents and conditions:$In(OTf)_3$ 5mol%, DCM, RT, 12hr.

Scheme 2

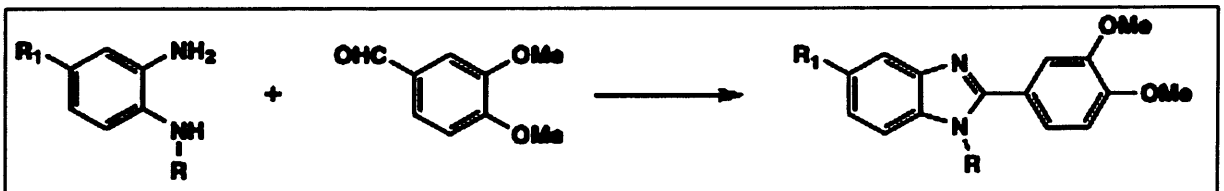
This particular side reaction has been previously reported to occur during the synthesis of benzimidazoles through the condensation of o-phenylenediamines and aldehydes.[6,7]It follows that each amino group of the phenylenediamine molecule reacts with a molecule of the aldehyde leading to the formation of this diamine.Therefore, it was anticipated that by maintaining a minimum concentration of the aldehyde in the reaction mixture

(dropwise addition), the formation of this byproductwould be prevented or limited. However, even with this modified protocol, there were no significant improvements in the overall yield and hence, efforts were turned into exploring alternative synthetic methods with different reagents.

2.1.1.2. Using sodium metabisulfite ($Na_2S_2O_5$):

There are several reported examples of the use of inorganic oxidizing reagents in the preparation of 2-arylbenzimidazoles which include, $NaHSO_3$,[8] Oxone®[9] and $Na_2S_2O_5$[10]. Of these reagents, 2-arylbenzimidazoles synthesis using $Na_2S_2O_5$ proceeds at relatively mild conditions with good yields.[11] Moreover, this reaction is amenable to microwave-assisted synthesis which usually affords excellent product yields and also meets with our research group interests in exploring the use of microwave-assisted synthesis for improving chemical reactions.

The preparation of 2-arylbenzimidazoles with this synthetic method involves the use of the inorganic oxidative reagent $Na_2S_2O_5$ to promote the condensation of o-phenylenediamine and substituted aldehydes in refluxing DMF (Scheme 3). Compared to the previous procedure (use of $In(OTf)_3$), the preparation of 2-(3,4-dimethoxyphenyl)-*1H*-benzimidazole using $Na_2S_2O_5$ (Scheme 3) afforded relatively good yields (> 55%) of the desired compound. Moreover, once the reaction is completed (TLC monitoring), the compound can be precipitated out by the addition of H_2O. The precipitated product is of high purity and any further purification desired can mostly be achieved by a simple recrystallisation from aqueous methanol.



R: H, Me
R_1: H, F

Reagents andConditions: $Na_2S_2O_5$, DMF, Reflux, 2.5 hr.

Scheme 3

Because of the mild conditions, ease of synthesis and purification as well as the overall good yields obtained, this synthetic method was used for the preparation of several substituted 2-phenylbenzimidazole based analogues to PMX610 as shown in Figure 2.

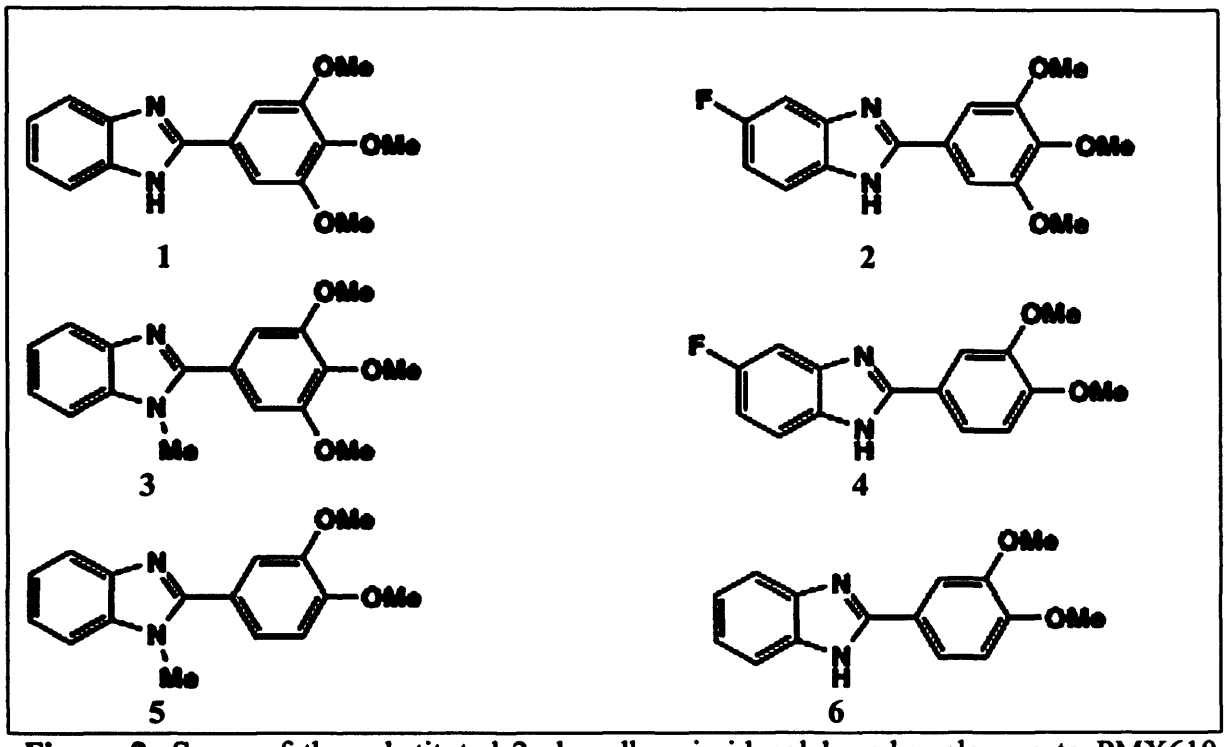


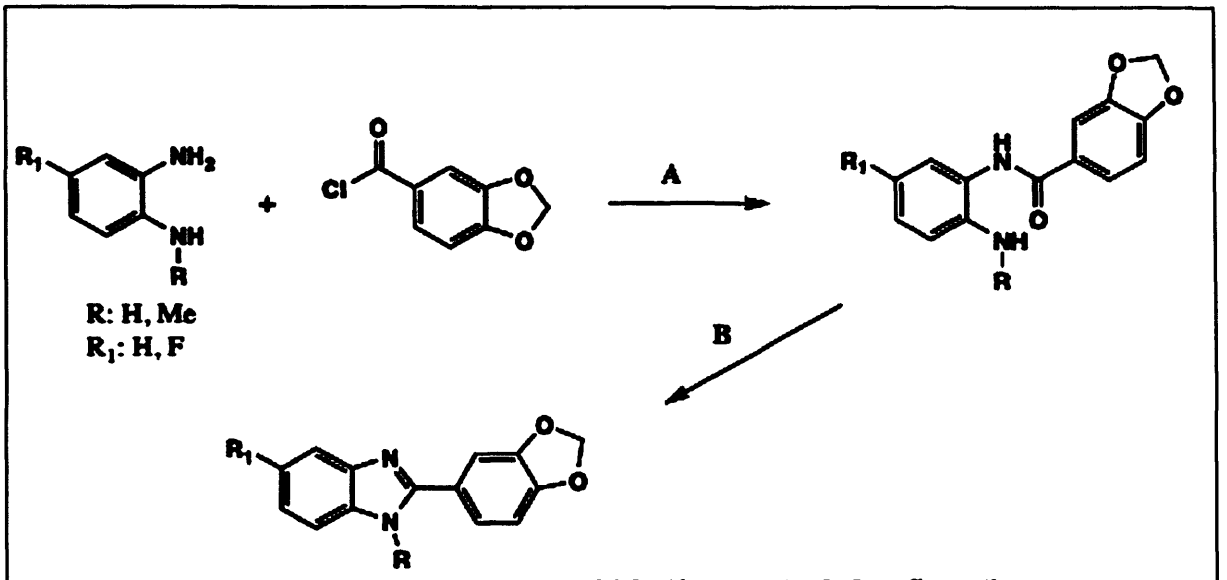
Figure 2: Some of the substituted 2-phenylbenzimidazolebased analogues to PMX610 synthesised using $Na_2S_2O_5$

2.1.1.3. Using piperonyloyl chloride *as a starting material:*

The preparation of compounds such as 2-(3,4-methylenedioxyphenyl)-1*H*-benzimidazole is important to further unravel the importance of the methoxy groups for the biological efficacy of these seriesof benzimidazoles. However, due to difficulties in obtaining piperonyl aldehyde commercially (certificate from the Home Office is required to permit its purchase), an alternative synthetic procedure using piperonyloyl chloride as a starting material was used.[12]

The preparation of 2-(3,4-methylenedioxyphenyl)-1*H*-benzimidazole from phenylenediamine and piperonyloyl chloride involves two consecutive synthetic steps

(Scheme 4).It follows that an amide intermediate is formed first from the condensation of the aniline and acyl chloride. This intermediate has to be heated under reflux in acetic acid for prolonged periods to generate the benzimidazole in its oxidized form.



Reagents and conditions: (A) TEA, THF, O°C, 1hr. (B) AcOH, reflux, 7hr.

Scheme 4

Nevertheless, the amide intermediate once isolated by the evaporation of the solvent (THF) *in vacuo,* was used directly in the following step without any purification. However, a column chromatography was necessary to obtain the final product (substituted) (2-(3,4-methylenedioxyphenyl)benzimidazole) in analytically pure form. Despite the harsh conditions employed and the need for column chromatography compared to the previous synthetic route (synthesis of 2-phenylbenzimidazoles using $Na_2S_2O_5$), the yields obtained were comparable (50-60%). The 2-phenylbenzimidazoles prepared through this approach are shown in Figure 3.

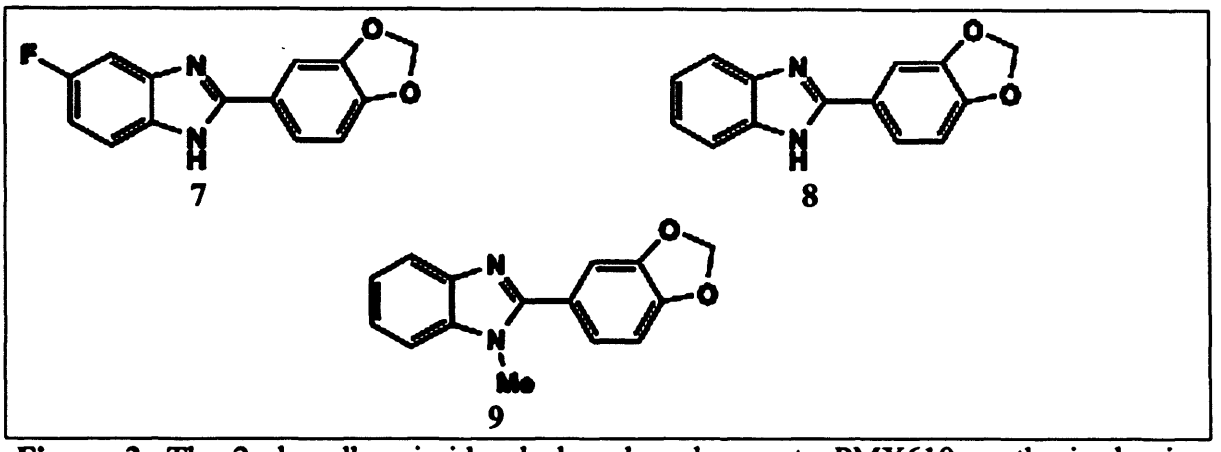


Figure 3: The 2-phenylbenzimidazole based analogues to PMX610 synthesised using piperonyloyl chloride as a starting material.

2.1.1.4. The isolation of 2-(3,4-dimethoxyphenyl)-5-fluoro-1*H*-benzimidazole *(4)isomers:*

The fluorinated 2-phenylbenzimidazole products (**2, 4, 7**), resulting from the reaction of 4-fluro-1,2-benzenediamine, although represented here as 5-fluorobenzimidazole products, are actually a rapidly equilibrating mixture of the formal 5-and 6-substituted products due to tautomerism of the benzimidazole N-proton (Figure 4).

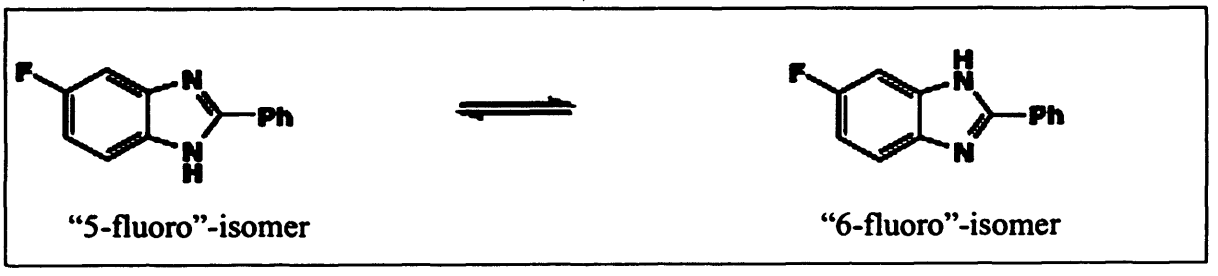
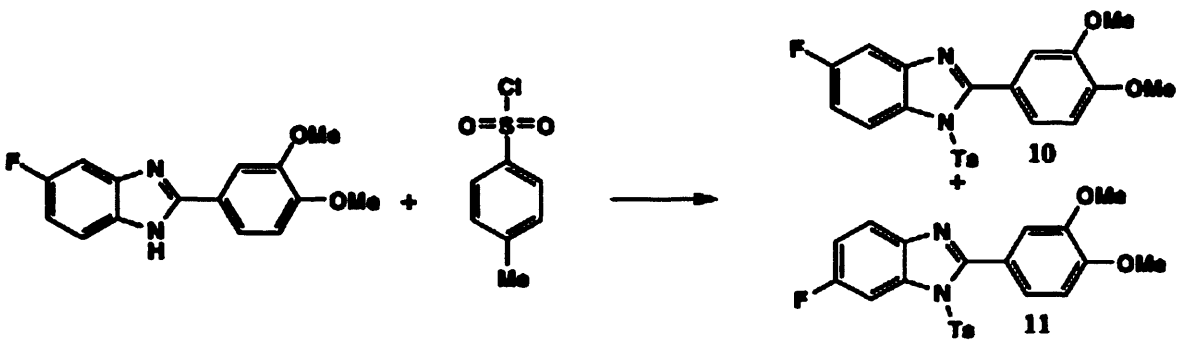


Figure 4: N-1 / N-3 proton tautomerisation of the fluorinated 2-phenylbenzimidazole products.

To investigate this observation further, it was anticipated that the deprotonation and substitution of the benzimidazole N-proton would prevent this rapid tautomerism and lead to aseparable mixture of 5- and 6-substituted products. Hence, the fluorinated 2-phenylbenzimidazole product (**4**) was treated with p-toluenesulfonyl chloride in pyridine, leading to the formation of a mixture of products, which were separated using column

chromatography to give the pure N-substituted 5-fluoro- and 6-fluoro-benzimidazole products **10** and **11** respectively (Scheme 5).



Reagents and conditions: p-toluenesulfonoylchloride, pyridine, RT, 16hr.

Scheme 5

For the characterisation of the individual isomeric products, NOESY (2D-NMR) experiment was performed. It uses the dipolar interaction of spins (the nuclear Overhauser effect, NOE) to establish correlation of protons.[13] The correlation between two protons depends on the distance between them. Hence, the cross-peaks in the resulting 2D spectrum connect resonance from protons that are close in space.

In this example, characterisation was carried out by observing the NOE between tosyl proton(H-6'') and the benzimidazole proton (H-7), allowing the H-7 proton to be characterised and identified for each particular fluorinated isomer on the basis of its ^{1}H NMR splitting pattern (Figure 5). The fluorine position is then defined by measuring coupling constants (*J*Hz) since fluorine-hydrogen (F-H) coupling is larger than hydrogen-hydrogen (H-H) coupling. Therefore, for the first isomer 2-(3,4-dimethoxyphenyl)-5-fluoro-N-tosylbenzimidazole (**10**), H-7 appears as a doublet of doublet with a meta coupling constant of 5.0 Hz; a typical ^3J (meta coupling) between H and F (Figure 5, Figure 6). NMR spectra of2-(3,4-dimethoxyphenyl)-5-fluoro-1-tosylbenzimidazole and 2-(3,4-dimethoxyphenyl)-6-fluoro-1-methylbenzimidazole are represented in Figure 6 and Figure 7 respectively.

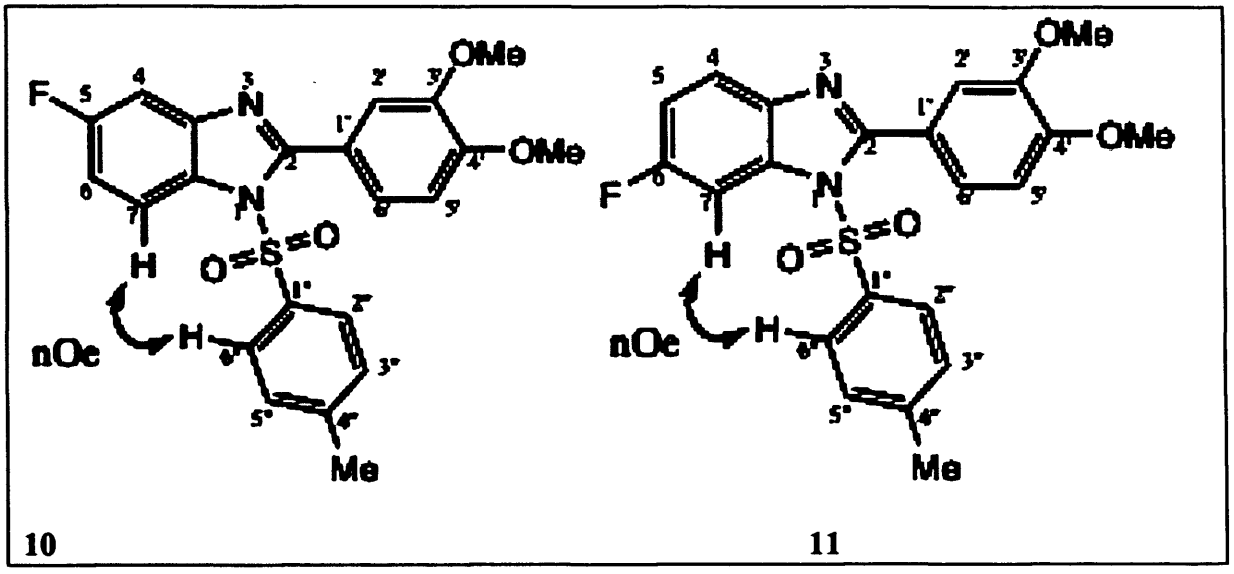


Figure 5: N-1 / N-3 proton tautomerisation and nOe effects in products **10**and **11**.

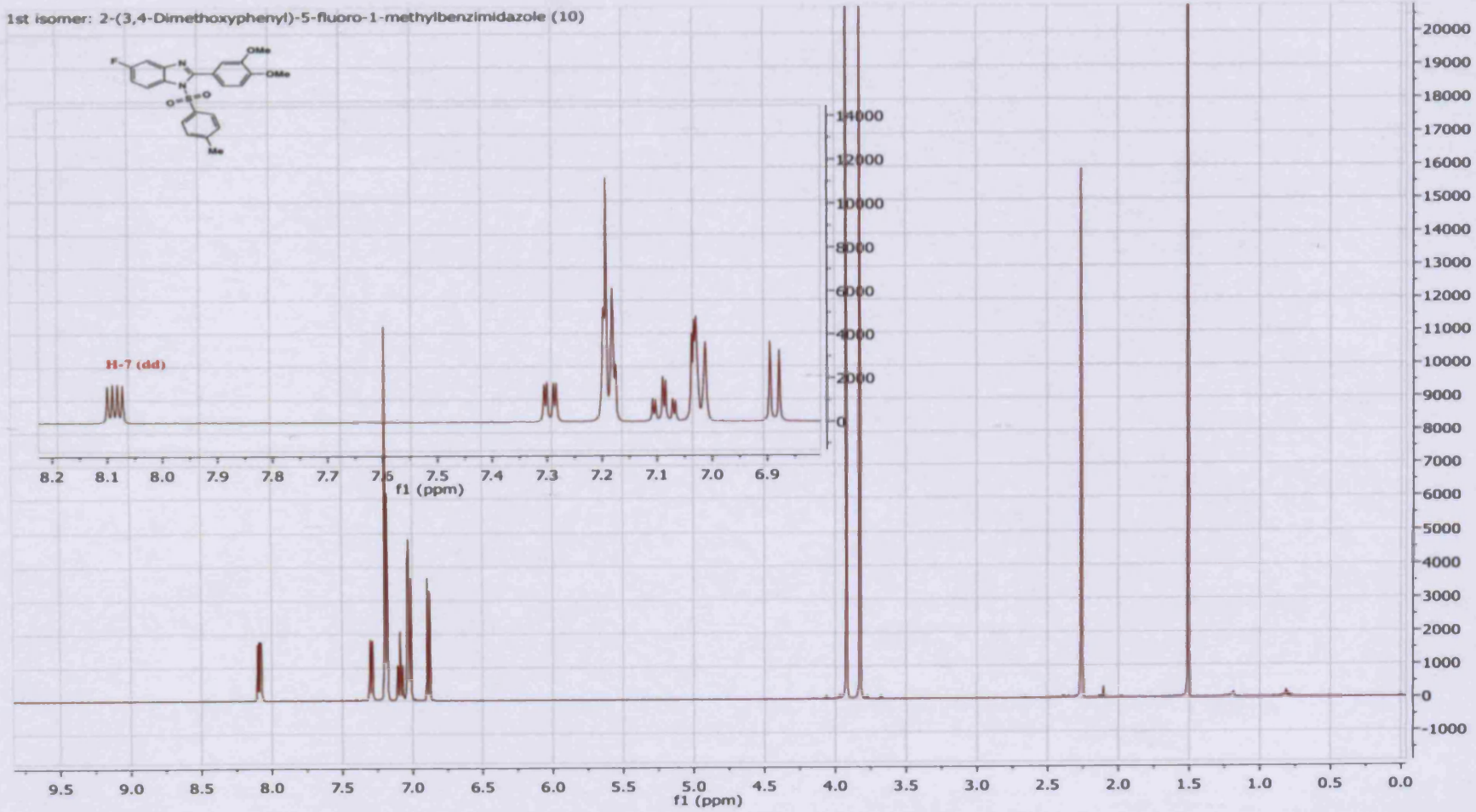


Figure 6:NMR of2-(3,4-Dimethoxyphenyl)-5-fluoro-1-methylbenzimidazole

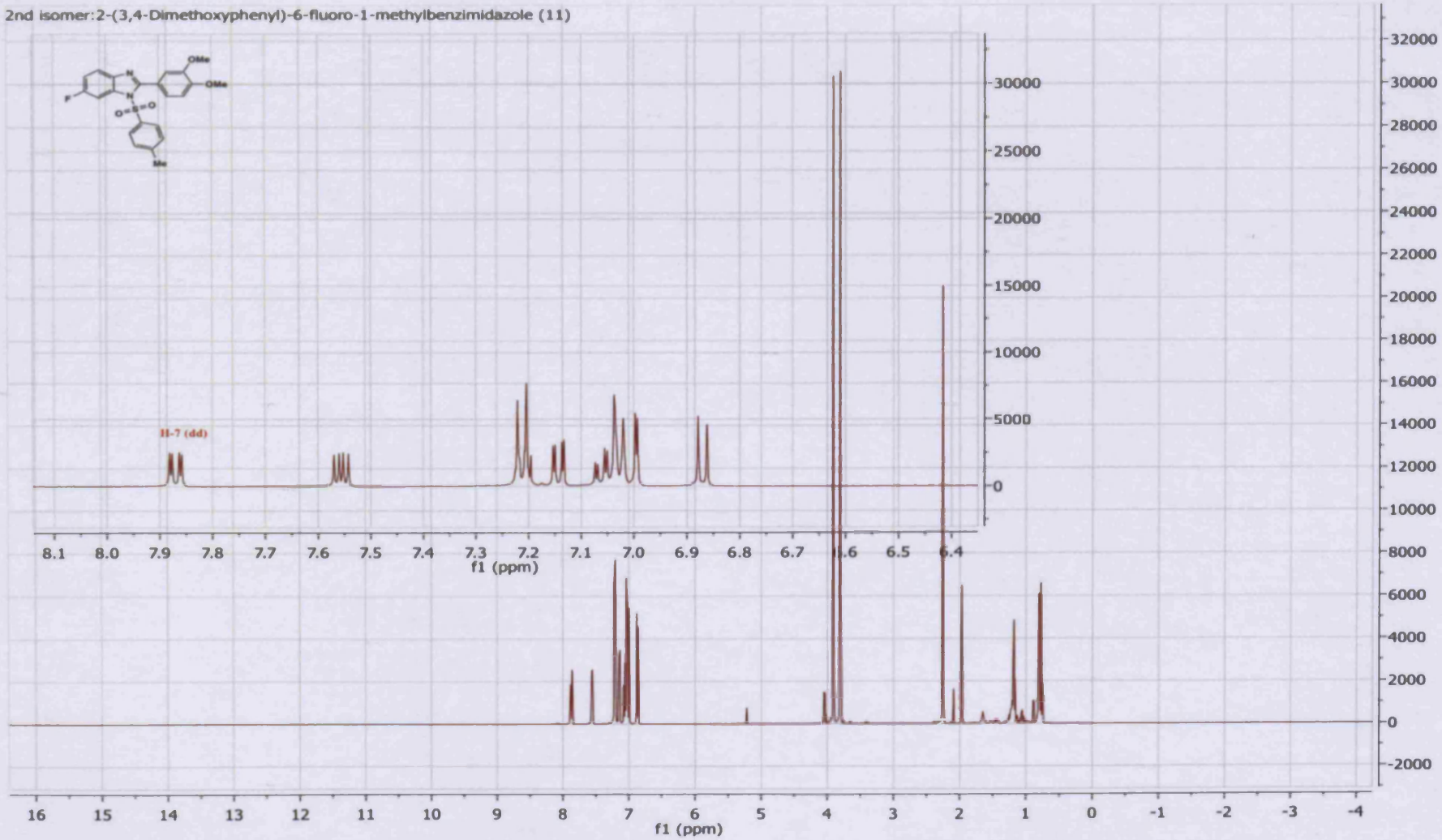


Figure 7:NMR of2-(3,4-dimethoxyphenyl)-**6**-fluoro-1-toslbenzimidazole

2.2. Biology

The new benzimidazole compounds were tested for in vitro antitumour activity in the human breast carcinoma cell lines MCF-7 (ER +ve) and MDA 468 (ER -ve) using the well-established MTT assay.[14]

The biological test assays for these series of PMX610 benzimidazole based analogues were carried out in the laboratories of our collaborators at the University of Nottingham under the supervision of Dr Tracey Bradshaw, who was involved in the earliest development of PMX610. The following is a summary of the protocol followed to conduct the MTT assays.

2.2.1. Protocol for MTT assays: Compounds were prepared as 10 mM top stocks, dissolved in DMSO, and stored at 4 °C, protected from light for a maximum period of 4 weeks. Human derived cell lines (MCF-7 (ER+), MDA 468 (ER-) breast carcinoma) were routinely cultivated at 37 °C in an atmosphere of 5% CO_2 in RPMI 1640 medium supplemented with 2 mM L-glutamine and 10% fetal calf serum and subcultured twice weekly to maintain continuous logarithmic growth. Cells were seeded into 96-well microtiter plates at a density of 5 x 10^3 per well and allowed 24 h to adhere before drugs were introduced (final concentration 0.1 nM - 100 mM, n = 8). Serial drug dilutions were prepared in medium immediately prior to each assay. At the time of drug addition and following 72 hours exposure, 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan (MTT) was added to each well (final concentration 400 mg/mL). Incubation at 37 °C for 4 h allowed reduction of MTT by viable cells to an insoluble formazan product. Well contents were aspirated and formazan solubilized by addition of DMSO:glycine buffer (pH 10.5) (4:1). Absorbance was read on an Anthos Labtec systems plate reader at 550 nm as a measure of cell viability; thus cell growth or drug toxicity was determined.

2.2.2.Results:

Table 1 lists the results (GI_{50} values) of the antitumour evaluation studies and includes the data previously obtained using the same method for PMX610.[14]

Compound	Mean GI_{50} / μM MCF-7	Mean GI_{50} / μM MDA 468
PMX 610	**<0.0001**	**<0.0001**
6	59.8	>100
4	85.7 (15.6)	94.4 (6.9)
5	90.3 (8.5)	84.2 (19.8)
1	50.8 (0.2)	43.6 (7.9)
2	53.4 (4.5)	39.8 (3.8)
3	>100	>100
7	70.6 (4.4)	43.2 (0.5)
8	49.4 (8.4)	26.3 (21.4)
9	72.4 (2.5)	79.3 (16.2)
10	**6.82 (0.07)**	**5.19 (1.09)**
11	**6.40 (0.28)**	**4.55 (0.40)**

Table 1. *In vitro* antitumour activity of the synthesised benzimidazoles against the MCF-7 and MDA 468 breast cancer cell lines (standard deviations in brackets).

The MCF-7 and MDA 468 cell lines were chosen here since they were previously shown to be exquisitely sensitive to the lead benzothiazole PMX 610. Inspection of Table 1 reveals that the new 2-phenylbenzimidazole derivatives prepared in this study do not display antitumour activity at the sub-nanomolar GI_{50} level seen for PMX 610, for the two breast cancer cell lines examined. In most cases, growth inhibitory values were in the micromolar range. This point is illustrated by comparison of the lead benzothiazole (PMX 610; MCF-7 GI_{50} = <0.0001 μM) with its direct benzimidazole analogue (4,MCF-7 GI_{50} = 85.7 μM). Notable observations were that the presence of a fluorine atom in the benzimidazole ring did not lead to substantial enhancement of antitumour activity

(compare **4** versus **6**, or **2** versus **1** for example). Also noteworthy was that the N-methylated compounds **5**, **3** and **9** were essentially inactive with GI_{50}values > 50 μM.

The most potent and interesting compounds from this new series were the two isomeric5-fluoro- and 6-fluoro-benzimidazoles (**10** and **11**) where the benzimidazole nitrogen bears an electron-withdrawing group (*p*-toluenesulfonyl). These two compounds displayed an antitumour activity in the low micromolar range in the cell lines used. In these cases low micromolar GI_{50} values were obtained, suggesting that there is space for further drug design and potential for the installation of other electron-withdrawing groups in this position in order to further optimise activity.

References

1. Grimmett, M. R. Imidazole and benzimidazole synthesis. *Academic Press.***1997,** 75- 92.
2. Thomas, J. B.; Fall,M.J.; Cooper, J. B; Burgess, J. P.; Caroll, F. I. Rapid in-plate generation of benzimidazole libraries and amide formation using EEDQ*Tetrahedron. Lett.***1997**,38, 5099-5012.
3. Huang, W.; Scarborough, R. M. A new "traceless" solid-phase synthesis strategy: Synthesis of a benzimidazole library.*Tetrahedron. Lett,* **1999,** 40, 2665-2668.
4. Trivedi, R.; De, S. K.; Gibbs, R. A. A convenient one-pot synthesis of 2-substituted benzimidazoles. *J. Mol. Cat A: Chemical***2006,** 245, 8-11.
5. Curini, M.; Epifano, F.; Montanari, F.; Rosati, O.; Taccone, S. Ytterbium triflate promoted synthesis of benzimidazole derivatives. *Synlett.***2004,** 1832-1834.
6. Smith, J. G.; Ho, I. Organic redox reactions during the interaction of o-phenylenediamine with benzaldehyde.*Tetrahedron Lett.* **1971,** 12, 3541-3544.
7. Ben-Alloum, A.; Bakkas, S.; Soufiaoui, M. Benzimidazoles: Oxydation hétérocyclisante par le nitrobenzène ou le diméthylsulfoxyde sur silice et sous irradiation micro-ondes ou ultra-violet.*Tetrahedron Lett.***1998,** 39, 4481-4483.
8. Weidner-Wells et al. Amidino benzimidazole inhibitors of bacterial two-component systems.*J. Bioorg. Med. Chem. Lett.***2001**,11,1545-1548.
9. Baulieau, P. L.; Hache, B.; von Moos, E. A practical Oxone®-mediated, high-throughput, solution-phase synthesis of benzimidazoles from 1,2-phenylenediamines and aldehydes and its application to preparative scale synthesis. *Synthesis,* **2003**,11, 1683-1692.
10. Lombardy,R. L.; Tanious, F.A.; Ramachandran, K.; Tidwell, R. R.; Wilson,W.D. Synthesis and DNA interactions of benzimidazole dications which have activity against Opportunistic Infections. *J. Med. Chem.* **1996**,39,1452-1462.
11. Navarrete-Vázquez, G.; Moreno-Diaz, H.; Aguirre-Crespo, F.; León-Rivera, I.; Villalobos-Molina, R.; Muñoz-Muñiz, O.; Estrada-Soto, S. Design, microwave-assisted synthesis, and spasmolytic activity of 2-(alkyloxyaryl)-1H-benzimidazole

derivatives as constrained stilbene bioisosteres. *Bioorg. Med. Chem. Lett.***2006**, 16, 4169-4173.

12. Wolkenberg, S.E et al. Identification of potent agonists of photoreceptor-specific nuclear receptor (NR2E3) and preparation of a radioligand. *Bioorg. Med. Chem. Lett.* **2006**, 16, 5001-5004.
13. Williams, D. H.; Flemin, I. Spectroscopic methods in organic chemistry, 5th edition, McGraw Hill, **1995**, 119-127.
14. Mortimer, C. G.; Wells, G.; Crochard, J-P.; Stone, E, L.; Bradshaw, T.D.; Stevens, M.F.G.; Westwell, A.D. Antitumor benzothiazoles. 26. 2-(3,4-dimethoxyphenyl)-5-fluoro benzothiazole (GW 610, NSC 721648), a simple fluorinated 2-aryl benzothiazole, shows potent and selective inhibitory activity against lung, colon, and breast cancer cell lines. *J. Med. Chem.***2006**, 49:179-185.

3. The synthesis and antitumour evaluation of (substituted) 2-phenylindoles

3.1.Chemistry

The synthesis of indoles has been a major area of focus in organic synthesis for the past decades due to their structural diversity and biological activity.[1] Thus, numerous procedures and well-established synthetic routes have been devised for the formation of the indole ring. Examples of the well-known classical methodologies include the Fischer indole synthesis, the Bischler indole synthesis, the Gassman synthesis from N-halo-anilines, the Madelung cyclisation of N-acyl-o-toluidines and the Batcho-Leimgruber indolesynthesis from *o*-nitrotoluenes and dimethylformamide acetals.[2] Moreover, novel methodologies are still emerging to overcome limitations of these classical procedures and to enable a wide range of substitution patterns of the indole nucleus.

Nevertheless, in recent years, the use of transition metals in the synthesis of indoles has become very popular because such catalysed reactions are generally tolerant to various chemical functionalities compared to the classical methodologies.[3] Of particular interest are the palladium or copper complex-catalysed reactions of 2-haloanilines and terminal alkynes for the synthesis of 2-subsituted indoles since these starting materials are commercially available or readily accessed through well-established synthetic routes.

3.1.1. The synthesis of substituted 2-phenylindoles

3.1.1.1. The use of palladium-catalysed reactions of o-iodoanilines and terminal alkynes

Initial efforts focused on establishing a suitable synthetic route toward substituted 2-phenyl indoles via the palladium-catalysed reactions of o-haloanilines and terminal alkynes. Therefore, our first objective was to ensure access to these starting materials either commercially or via robust synthetic procedures.

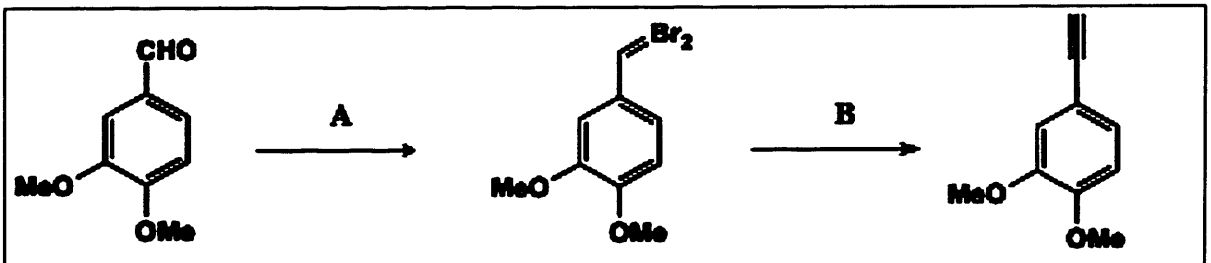
A. Access to terminal alkynes

The terminal alkyne; 3,4-dimethoxyphenylethyne is not commercially available and hence, it was essential to explore a reliable procedure for its preparation which can also be extended to the preparation of any other required terminal alkynes.

Terminal alkynes can be prepared via different synthetic routes from various substrates. Amongst the reported procedures, the one based on the use of aldehydes was considered the most suitable since several aldehydes bearing different substituents with desired substitution pattern have already been used in the synthesis of the benzimidazole series (Chapter 2) and were still available in good quantities.

A. 1. The preparation of 3,4-dimethoxyphenylethyne through Corey-Fuchs reaction

The synthesis of terminal alkynes from aldehydes using the Corey-Fuchs reaction proceeds over two steps with a relatively good yield (>65%) (Scheme 1).[4] In the first step, the reaction of carbon tetrabromide (CBr_4) with triphenylphosphine (Ph_3P) leads to the in situ generation of a highly reactive ylide which quickly undergoes a Wittig reaction with 3,4-dimethoxybenzaldehyde forming the intermediate (2,2-dibromo-1-(3,4-dimethoxyphenyl)ethene). This intermediate is isolated and purified before using it in the next step where it undergoes lithium-halogen exchange with butyllithium (n-BuLi) followed by alpha-elimination of the other bromide to form olefinic carbene that rapidly rearranges to the more stable form 3,4-dimethoxyphenylethyne (13).



13

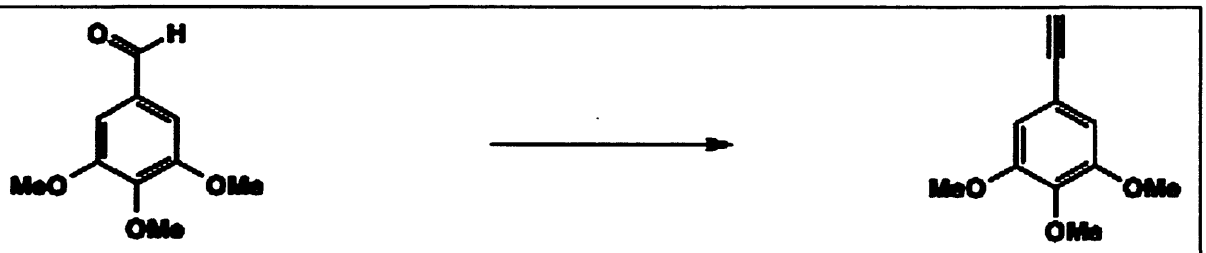
Reagents andconditions:A; CBr_4, Ph_3P, DCM, RT, 1 hr.**B;** n-BuLi, THF,-78°C, 1 hr, H_2O.

Scheme 1

Overall, while this synthetic route affords relatively good yields (> 60%) of the desired terminal alkynes from its corresponding aldehydes, laborious work is needed for each step and more importantly the harsh conditions required (mainly the use of the strong base n-BuLi) may compromise the procedure's compatibility with some aldehydes bearing base sensitive functional groups.

A. 2. The preparation of 3, 4, 5-trimethoxyphenylethyne in a one-pot procedure

Recently, an alternative one-pot procedure was reported for the synthesis of terminal alkynes from aldehydes as illustrated in Scheme 2.[5]In this reaction, dimethyl-2-oxopropylphosphonate reacts with p-toluenesulfonylazideto form dimethyldiazo-oxopropylphosphonate; otherwise known as Bestmann's reagent,which in the presence of potassium carbonate leads to the in situgeneration of dimethyl (diazomethyl)phosphonate. This latter reagent catalyses the formation of the desired terminal alkyne (3, 4, 5-trimethoxyphenylethyne in this case(14).



14

Reagents and conditions: Dimethyl-2-oxopropylphosphonate,p-toluenesulfonylazide, CH_3CN, RT, 2 h; MeOH, RT, 24 h.

Scheme 2

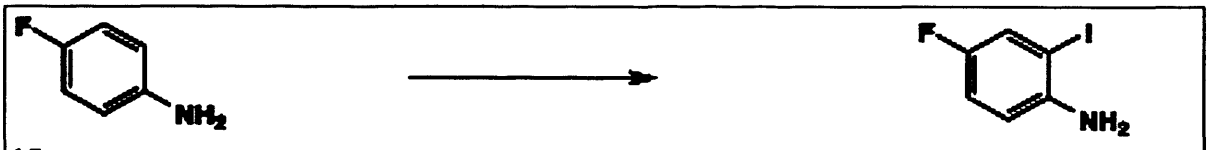
This procedure enables the easy preparation of the desired terminal alkynes in one step under mild conditions, which makes it more compatible with a wide range of functional groups compared to the previous synthetic route (Scheme 1). However, as described in the original paper, once aldehydes bearing electron donating groups are used, reactions do not go to completion resulting in low yields which was the case in this example (40%).[6]Attempts to improve the yield by employing higher concentrations of the reagent (up to 3 eq) and running the reaction for longer times resulted in no major improvements.

B. Preparation of o-iodoanilines

One of the major limitations of the metal catalysed reaction between the *o*-haloanilines and terminal alkynes is that it fails to give good yields of the desired indoles when 2-bromo- or 2-chloroanilines are employed and requires the use of iodoanilines which are most often not commercially available.[7]

B. 1. Synthesis of 4-fluoro-2- iodoaniline

2-Iodoanilines can be obtained via a selective *o*-iodination of the readily available anilines. Scheme 3 illustrates the first attempt to synthesise 4-fluoro-2- iodoaniline(**15**).



15

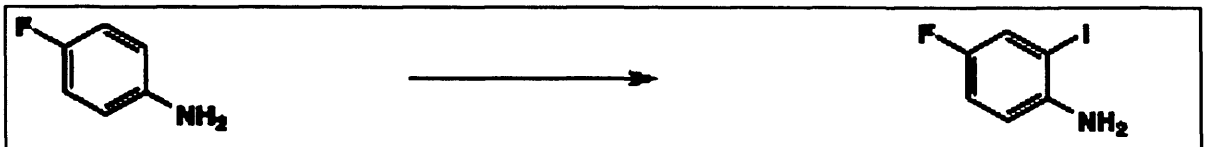
Reagents and conditions: $NaIO_4$, KI, NaCl, AcOH:H_2O (9:1), RT, 6 hr.

Scheme 3

This procedure was recently reported to achieve the selective monoiodination of aromatic compounds using a reagent system consisting of $NaIO_4$/KI/NaCl in aqueous acetic acid in excellent yields.[8] It is thought that the reaction proceeds via in situ generation of iodine monochloride which acts as the electrophile for the iodination.[8] However, when employing this procedure for the iodination of 4-fluoroaniline,very low yields of the desired product were obtained because ofdifficulties in its purification asseveral other byproducts were formed. Therefore, it was important to explore an alternative iodination methodology with better yields and fewer byproducts.

In a new attempt for the synthesis of 4-fluoro-2-iodoaniline, a procedure which is based on the use of molecular iodine (I_2) in an aqueous media was chosen (Scheme 4).[9]The iodination reaction proceeds efficiently to form the product; 4-fluoro-2-iodoaniline,which

wasobtained in good yields (64%) with high purity via an extraction with diethyl ether and subsequentremoval of the solvent.

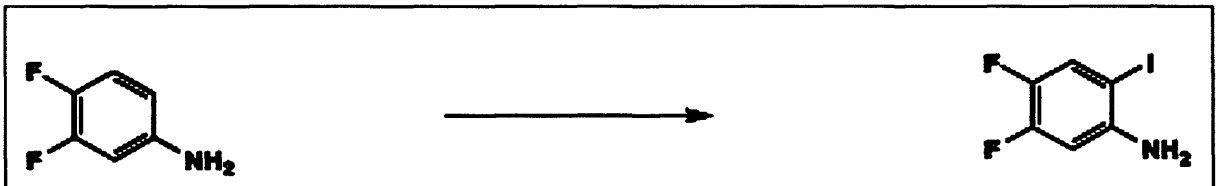


15

Reagents and conditions: $NaHCO_3$, H_2O, RT, 3 hr.

Scheme 4

This procedure was also used for the preparation of 4,5-difluoro-2-iodoaniline(**16**)in a good yield (69%) as shown in Scheme 5.

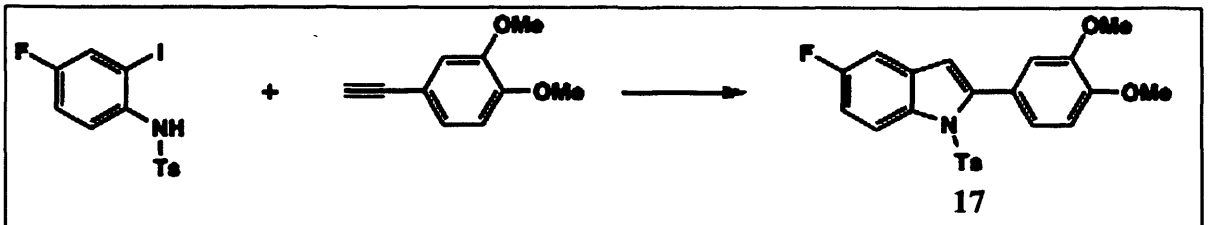


16

Reagents and conditions:I_2, $NaHCO_3$, H_2O, RT, 3 hr.

Scheme 5

Generally speaking, the palladium-catalysed annulation of o-iodoanilines with alkynes usually proceeds via aSonogashira coupling to form an alkynylaniline intermediate, which is isolated and subsequently treated with a strong base to promote its cyclisation to the corresponding indole. This methodology was significantly improved by Yamanaka et al who reported that treatment of terminal alkynes with *o*-iodo-*N*-mesylanilides under Sonogashira conditions could directly afford indole products in a single step through a coupling-cyclisation process using catalytic amounts of both palladium and copper.[10] Scheme 6 illustrates the preparation of 5-fluoro-3,4-dimethoxyphenyl-N-tosyl-indole (**17**)using this procedure.



Reagents and conditions: $PdCl_2(PPh_3)_2$, CuI, TEA, THF,reflux, 6 hr.

Scheme 6

Following this procedure, the indole product was obtained but only in low yields (25%) since some other byproducts were also isolated. The byproducts, which were successfully characterised include the uncyclised form of the indole product and the dimer of the terminal alkyne formed through Glaser coupling; a side reaction which is often encountered in the presence of copper (I) (Figure 1). It follows therefore that halting these side reactions would significantly improve the yield of the desired indoles.

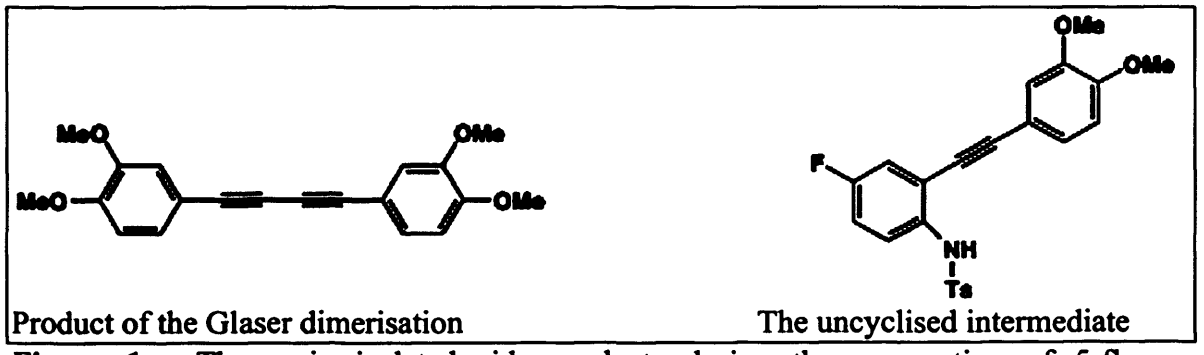
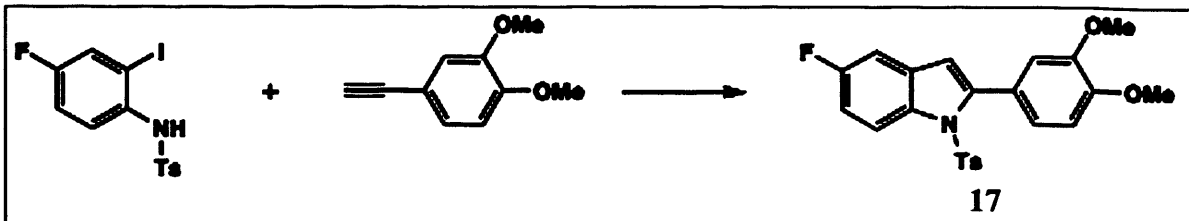


Figure 1: The main isolated side products during the preparation of 5-fluoro-3,4dimethoxyphenyl-N-tosyl-indole.

Recently, a copper-free modified protocol of this procedure via palladium catalyzed tandem Sonogashira coupling 5-endo-dig cyclisation at room temperature under ultrasonic irradiation was reported for the synthesis of 2-substituted indoles.[11] It was therefore anticipated, greater chemoselectivity would be achieved by using this copper-free procedure since the dimerisation of terminal alkyne molecules (Glaser reaction) is minimised and this would ultimately improve the overall yield of indole product.

Following the modified protocol (Scheme 7), it was noticed that the dimerisation of the terminal alkyne was reduced as it was expected. However, this was at the expense of the cyclisation of the alkynylaniline intermediate, since uncyclisedalkynyl aniline was the major isolated compound. This in turn signifies the importance of copper for the cyclisation step as Yamanaka et al mentioned in the original paper, that both palladium and copper are important for the indole ring formation although the exact role of each is not determined.[10]

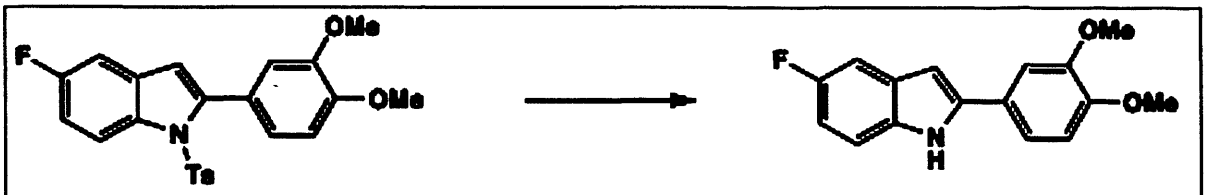


Reagents and conditions:2 mol% $Pd(OAc)_2$, Bu_4NOAc, CH_3CN, sonication, 6 hr.

Scheme 7

Nevertheless, as a further attempt, it was thought that the dropwise addition of the terminal alkyne to the reaction mixture (would minimise the formation of the alkyne dimer since at low alkyne concentration, Sonogashira coupling and subsequent cyclisation would be favored over Glaser coupling. Employing this modified procedure, the yield improved to 35%.

Another limitation that remained with this procedure is that the obtained indole product is still bearing the tosyl group at position 1. Therefore, the next step was to work out an easy procedure for the removal of this group. This was achieved by refluxing the product in THF with tetrabutylammonium fluoride (TBAF) as illustrated in scheme 8.[12]The NH-free indole (5-fluoro-3,4-dimethoxyphenyl-NH-indole, **18**) was obtained in good yields (over 80%) after purification with column chromatography.



18

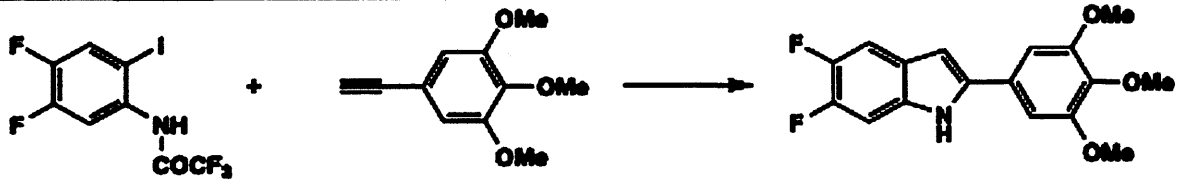
Reagents and conditions: TBAF, THF, Reflux, 70° C, 6 hr.

Scheme 8

Overall, relatively moderate yields are obtained in the synthesis of 2-substituted indoles via this optimised one-pot palladium-catalysed annulation of o-iodoanilines with terminal alkynes. However, several operative steps are required in this synthetic route including the preparation of both starting materialsand removal of tosyl group at the end of the reaction. Therefore, it was imperative to explore other synthetic routes, which enable the fast preparation of the desired 2-substituted indole analogues.

3.1.1.2. The use of domino copper-catalysedcoupling-cyclisation of o-iodoanilines with terminal alkynes

Cacchi and coworkers reported that the copper complex([Cu(phen)(PPh_3)$_2$]-NO_3) can be efficiently used to catalyse the preparation of 2-aryl-NH-indoles from the iodotrifluoroacetanilide and terminal alkynesbearing both electron-donating and electron-withdrawing groups (EWG) in a one-pot procedure through a domino copper-catalysedcoupling-cyclisation reaction.[13] Scheme 9 shows the synthesis of 4,5-difluoro-2(3,4,5-trimethoxyphenyl)indole employing this procedure.



19

Reagents and conditions: [Cu(phen)(PPh_3)$_2$]-NO_3, K_3PO_4, toluene, reflux, 12hr.

Scheme 9

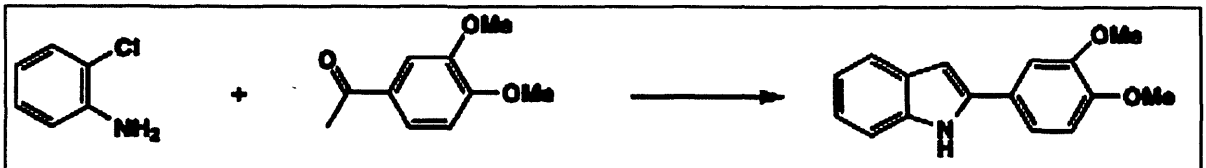
Reaction of 3,4-difluoro-2-iodotrifluoroacetanilide with 3,4,5-trimethoxyphenylethyne in the presence of 10 mol % $[Cu(phen)(PPh_3)_2]$-NO_3afforded 4,5-difluoro-2-(3,4,5-trimethoxyphenyl)-NH-indolein 30 % yield. It is worthwhile to note that the uncyclised intermediate is noticed while the dimerisation of the alkyne was very low since it was added dropwise.

Nevertheless, this procedure gives comparable yields to the previous one starting from the same substrates with the main advantage being the formation of the NH free indole products at the end of the reaction. However, this synthetic route still requires prior optimisation of the starting materials and the synthesis of the copper reagent [Cu (phen)$(PPh_3)_2$]-NO_3(for a detailed synthesis of this reagent,refer to Experimental section; Chapter 5).

3.1.1.3. The use of the intramolecular Heck cyclisation of 2-chloroanilines and acetophenones

Nazare et al reported a mild, one-step palladium catalysed synthesis of 2-substituted indoles from 2-chloroanilines and acetophenones.[14] The main advantage of this procedure as compared to the previous ones is that both substrates can be obtained from commercial sources with a wide range of substituents overcoming the limitations of the previously attempted procedures.

The procedureis based on the use of the commercially available catalyst $[Pd(tBu_3P)_2]$ with a base (K_3PO_4)and $MgSO_4$ to catalyse the coupling reaction of 2-chloroanilines and acetophenonesin dimethylacetamide (DMAC) at 140°C in a pressure tube under inert gas conditions. It is also reported that the addition of low amounts of acetic acid is necessary for a highly selective reaction toward the formation of the desired indole product otherwise various byproducts are formed.[14] However, whenattempting the synthesis of 2-(3,4-dimethoxyphenyl)NH-indole using this procedure (Scheme 10), many side products were detected and the 3,4-(dimethoxyphenyl)-NH-indole **(20)** was isolated in very low yield (15%).



20

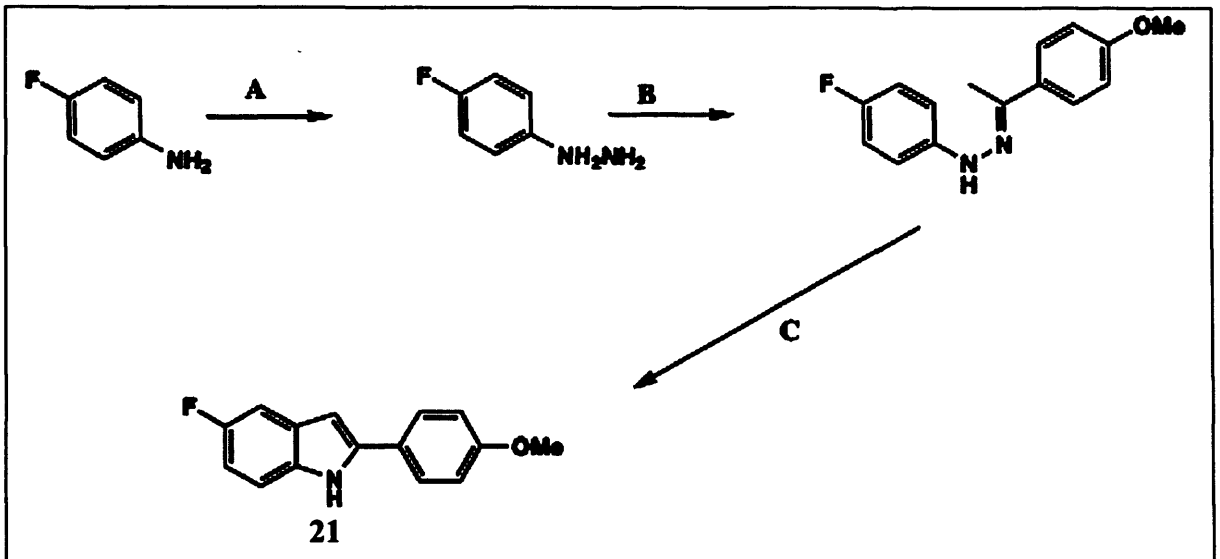
Reagents and conditions: [Pd(tBu3P)2], K_3PO_4, $MgSO_4$, DMAC, 145 °C , 24hr.

Scheme 10

At first it was thought that the low yield of this particular reaction was due to the electron-donating effect of the methoxy substituents of the 3,4-dimethoxyacetophenone since EWG substrates are generally preferred for the Heck coupling. However, no major improvements in the yield were noticed when using an acetophenone bearing electron-withdrawing group (p-NO_2 acetophenone, yield 19% only). A better explanation for the low yields of the isolated products lies in the instability of the palladium reagent (Pd $(tBu_3P)_2$). This limitation can be overcome by using this reagent freshly prepared prior to performing indole synthesis. However, this is not a convenient approach for the preparation of a library of indoles.

3.1.1.4. The use of Fischer indole synthesis

The Fischer indoles synthesis is a well-established classical synthetic route for the synthesis of indoles, which is commonly used in industry for the synthesis of different indole based drugs. It consists of the acid catalysed reaction of phenylhydrazines and acetophenones, which are both available commercially.[15] Phenylhydrazines can also be easily prepared by the reduction of the appropriate aryl diazonium salts, which in turn are prepared from their corresponding anilines. The preparation of 5-difluro-2(3,4-dimethoxyphenyl)indole(**21**)isrepresented in Scheme 11 as an example ofFischer indole synthesis. Generally, a phenylhydrazine is heated under reflux with an acetophenone to give a phenylhydrazone intermediate. After the removal of solvent *in vacuo*, polyphosphoric acid (PPA) is added to the residue obtained and the mixture is heated to 120°C. The reaction progress is monitored by TLC and can be completed within 30 mins-4 hrs with acetophenones bearing electron-donating groups reacting more rapidly.



Reagents and conditions: (A)$SnCl_2$, $NaNO_2$, HCl, H_2O,RT, 4hr.(B) p-methoxy-acetophenone, EtOH, 80°C, reflux, 1hr.(C) PPA, 125°C. 30 mins.

Scheme 11

The crude product precipitates once the reaction mixture contents were poured into cold water and subsequently the product was purified by column chromatography. It was also noticed that better yields were obtained when acetophenones bearing electron-donating groups were employed.

Overall, using the Fischer indole synthesis, a small library of substituted 2-phenylindole analogues was generated based on the substitution pattern of PMX610 as shown in Figure 2.On another note, the p-aminoacetophenone was first protected with a tosyl group prior to performing the synthesis of the desired indoles using this substrate. Interestingly, this protecting group was lost during the synthesis process to generate the unprotected 2-(p-aminophenyl)-1*H*-indole (**21**).

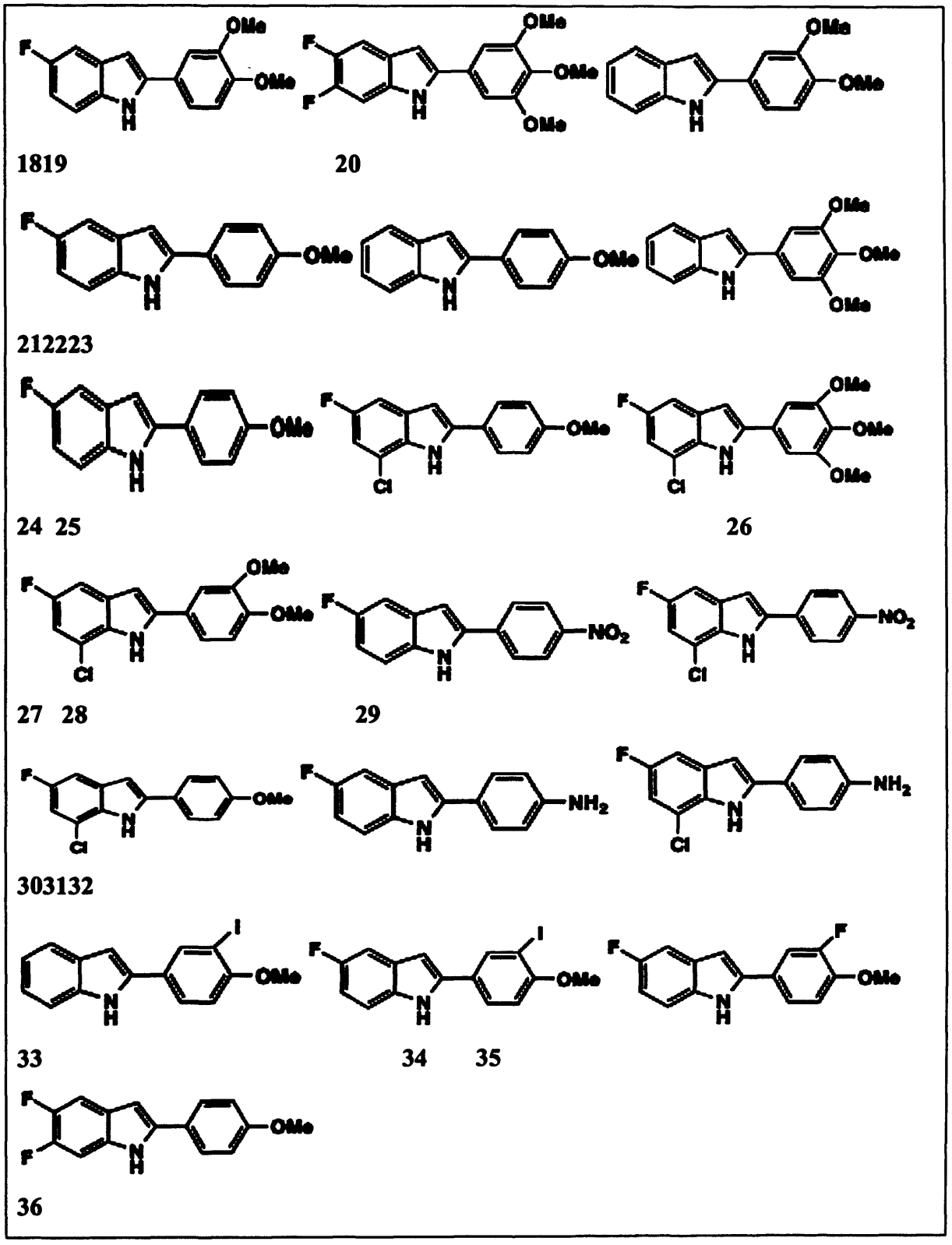


Figure 2: A library of 2-phenylindoles prepared via the Fischer indole synthesis.

3.2. Biology

The synthesised indole compounds (Figure 2) were sent this time to our collaborators in the Tenovus centre (Welsh School of Pharmacy, Cardiff University) where they were tested against the human breast carcinoma MCF-7 (ER +ve), non-small lung cancer A549, prostate cancer PC3 and colorectal cancer LOVO cell lines using MTT assays. These indole compounds were tested for in vitro antitumour activity against these four cell lines compared to the benzimidazole analogues which were tested only in the MCF-7 and MDA 468 cell lines since these two cell lines were previously shown to be exquisitely sensitive to the lead benzothiazole PMX 610 as outlined in Chapter 2. Nevertheless, **Table 1** lists the results (IC_{50} values) obtained for the indoles in the four cell lines.

The data obtained clearly reveals that the new 2-phenylindole derivatives prepared do not display a potent antitumour activity at the sub-nanomolar level (GI_{50} level seen for PMX 610) as was the case with the benzimidazoles (Chapter 2). In most cases, the obtained values were in the micromolar range and some of these compounds can essentially be considered inactive (IC_{50}>100μM) or have no significant activity that may be considered for further developments. For example, the IC_{50} values obtained for the compound **18** which is the direct indole analogue of PMX610 were >100μM in the four cell lines used.

The only case, which can be considered as an exception, was the indole **17** (2-(4,5-dimethoxyphenyl)-5-fluoro-N-tosyl-indole), which exerted a sub-micromolar activity in the four cell lines. Most intriguingly is the observation that this indole bears the tosyl group, which was the case with the most active compounds in the benzimidazole series (benzimidazole isomers (**10, 11**)). It can be said that the presence of the tosyl group in both series improves the antitumour activity despite the fact that this submicromolar activity is still far less potent than the lead compoundPMX610.

Compound	MCF7	A549	PC3	LOVO
20	50μM	>100μM	>100μM	50μM
18	>100μM	>100μM	>100μM	>100μM
36	>100μM	90μM	>100μM	9μM
22	>100μM	>100μM	>100μM	20μM
23	>100μM	>100μM	100μM	80μM
21	>100μM	>100μM	>100μM	>100μM
24	>100μM	>100μM	>100μM	>100μM
25	60μM	70μM	40μM	30μM
26	60μM	50μM	50μM	40μM
27	50μM	50μM	50μM	50μM
29	>100μM	90μM	>100μM	>100μM
31	60μM	40μM	80μM	50μM
35	>100μM	50μM	>100μM	>100μM
17	**0.8μM**	**0.9μM**	**0.7μM**	**0.6μM**

Table 1: The IC_{50}values for the synthesised PMX 610 based indole analogues in MCF-7 (ER +ve), A549, PC3 and LOVO cell lines.

References

1. Gribble, G.W. Recent developments in indole ring synthesis: methodology andapplications. *J. Chem. Soc.Perkin. Trans. 1*, **2000**, 1045-1075.
2. Humphrey, G. R.; Kuethe, J. T. Practical methodologies for the synthesis of indoles. *Chem. Rev.***2006**, 106, 2875-2911.
3. Cacchi, S.; Fabrizi, G. Synthesis and functionalization of indoles through palladium-catalyzed reactions. *Chem. Rev.***2005**, 105, 2873-2920.
4. Pagliai, F.; Pirali, T.; Del Grosso, E.; Di Brisco, R.; Tron, G.C.; Sorba; G.; Genazzani, A.A. Rapid synthesis of triazole-modified resveratrol analogues via click chemistry. *J. Med. Chem,***2006**, 49, 467-470.
5. Roth, G.; Liepold, G.; Müller, S.; Bestmann, H. J. Further improvements of the synthesis of alkynes from aldehydes. *Synthesis*, **2004**, 59-62.
6. Müller, S.; Liepold, G.; Roth, G.; Bestmann, H. J. An improved one-pot procedure for the synthesis of alkynes from aldehydes. *Synlett,***1996**, 521-522.
7. Suzuki, N.; Yasaki, S; Yasuhara,A., Sakamoto, T. Convenient indole synthesis from 2-iodoanilines and terminal alkynes by the sequential sonogashira reaction and the cyclization reaction promoted by tetrabutylammonium fluoride (TBAF).*Chem. Pharm. Bull.***2003**, 51, 1170-1173.
8. Emmanuvel, L.; Shukla, R. K.; Sudalai, A.; Gurunath, S.; Sivaram, S.NaIO4/KI/NaCl:a new reagent system for iodination of activated aromatics through in situ generation of iodine monochloride. *Tetrahedron Lett.***2006**, 47, 4793-4796.
9. Xiao, W. J.; Alper, H.Regioselective carbonylative heteroannulation of *o*-iodothiophenols with allenes and carbon monoxide catalyzed by a palladium complex: a novel and efficient access tothiochroman-4-one derivatives.*J. Org. Chem.***1999**,64, 9646-9652.
10. Sakamoto, T.; Kondo, Y.; Iwashita, S.; Nagano, T.; Yamanaka, H. Condensed heteroaromatic ring systems. XIII. One-step synthesis of 2-substituted 1-methylsulfonylindoles from N-(2-Halophenyl)methanesulfonamides.*Chem. Pharm. Bull.* **1988**, *36*, 1305-1308.

11. Palimkar, S, S.; Kumar, H. P.; Lahoti, R.J.; Srinivasan, K.V.Ligand-, copper-, and amine-free one-pot synthesis of 2-substituted indoles via Sonogashira coupling 5-endo-dig cyclisation. *Tetrahedron***2006**, 62, 5109-5115.

12. Zhang, H. C.; Ye, H.; Moretto, A. F.; Brumfield, K.; Maryanoff, B. E. Facile solid-phase construction of indole derivatives based on a traceless, activatingsulfonyl linker.*Org. Lett.***2000**, 2, 89-92.

13. Cacchi, S.; Fabrizi, G.; Parisi, L. M. 2-Aryl and 2-heteroaryl indoles from 1-alkynes and o-iodotrifluoroacetanilide through a domino copper-catalyzedcoupling-cyclization process. *Org. Lett.* **2003**, 5, 3843-3846.

14. Nazare, M.; Schneider, S.;Lindenschmidt, A.; Will, D.W.A flexible, palladium-catalyzed indole andazaindole synthesis by direct annulation ofchloroanilines and chloroaminopyridines withketones. *Angew. Chem. Int. Ed.***2004**, 43, 4526 –4528.

15. Robinson, B. The Fischer indole synthesis. *Chem. Rev.***1963**, 63, 373-401.

4. The synthesis and antitumour evaluation of N-substituted benzimidazole and indole analogues of PMX610

The *in vitro* cell line data obtained for the synthesised benzimidazole and indole based analogues of PMX-610 shows clearly that the N-substituted compounds (bearing the electron withdrawing group; tolyl group) display more significant antitumour activity than the non-substituted ones. In the cell linesused, low micromolar GI_{50}antitumour activity values were found for the 5- and 6-fluoro 2-(3,4-dimethoxyphenyl)-N-tosylbenzimidazole isomers **(10,11)** while (3,4-dimethoxyphenyl)-5-fluoro-1-tosylindole **(17)** exerted even more potent activity in the sub-micromolar range. Therefore, a small library of N-substituted benzimidazoles and indoles was prepared to further investigate this observation and study the effects of N-substitution on the antitumour activity. The chosen substituents were the other sulfonyl chlorides namely methylsulfonyl and benzenesulfonyl chloride as well as the methyl group because of their varying size and electron donating or withdrawing properties.

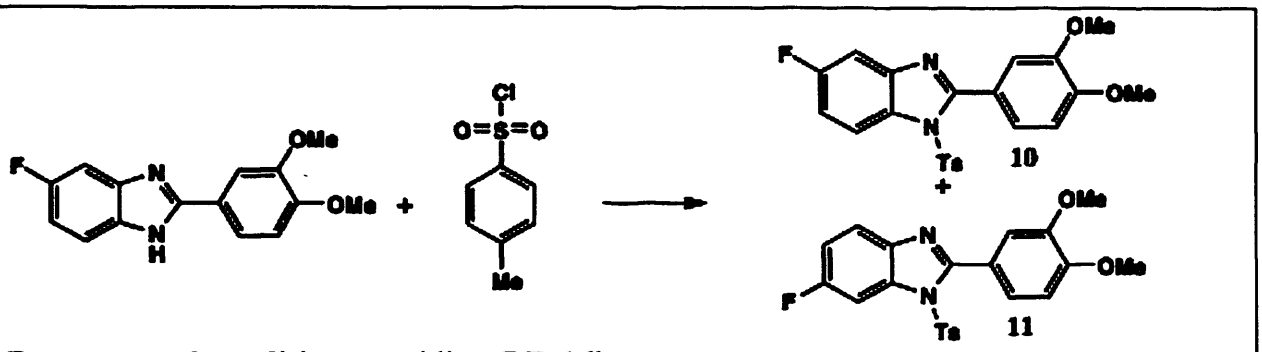
4.1. Chemistry

The chosen substituents can be introduced through substitution reactions (S_N2) using a base to deprotonate the indole or benzimidazole N-proton (NH) followed by the nitrogen lone pair nucleophilic attack on theelectrophile. While $S_N2$reactions can be accomplished under different conditions, the main focus was directed to finding a procedure, which allows access to good yields with relative ease of synthesis and purification.

4.1.1. The synthesis of N-substituted benzimidazoles:

4.I 1.1. The use of pyridine as a base:

As outlined in Chapter 2 for the synthesis of the benzimidazoles **(10, 11)**, the reaction proceeds under inert conditions using pyridine (solvent and a base) at room temperature overnight to produce these two isomers (Scheme 1) which are then isolated and separated using column chromatography.[1]



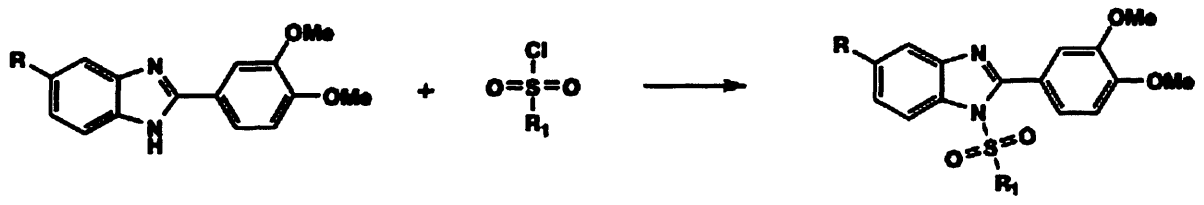
Reagents and conditions: pyridine, RT, 16hr.

Scheme 1

The main limitation of this synthetic route is the poor yield obtained (20%). This is more complicated by the fact that a relatively large amount of the crude product is required sincethe resulting isomers have very close *Rf* (retardation factor) values causing difficulties in their separation using traditional column chromatography procedures. Therefore, it was necessary to investigate an alternative procedure with a better yield.

4.1.1.2. The use of NaH as a base:

This synthetic route is based on the use of NaH as a base in DMF as solvent under inert gas conditions at room temperature as illustrated in Scheme 2. More than two-fold yield improvement (up to 60%) was achieved by employing these reagents and conditions. This can be explained by the use of thestrong base (NaH), which enables the effective deprotonation of the mildly acidic benzimidazole proton.



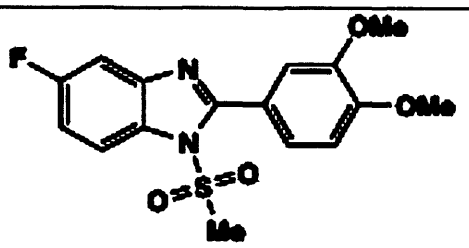
R: H, F

R_1: Me, Ph, 4-MePh.

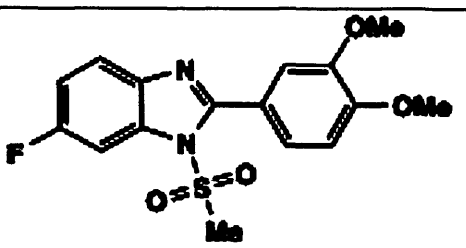
Reagents and conditions: NaH, DMF, RT.

Scheme 2

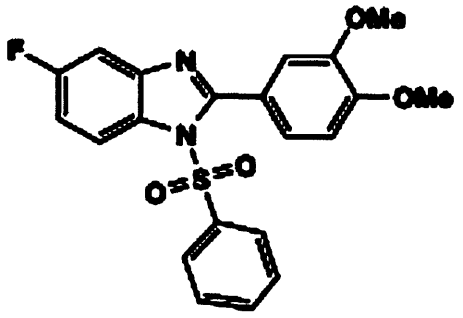
The use of this procedure led to the preparation of the following derivatives (Figure 1).



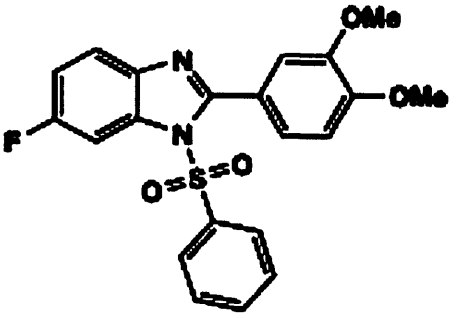
37



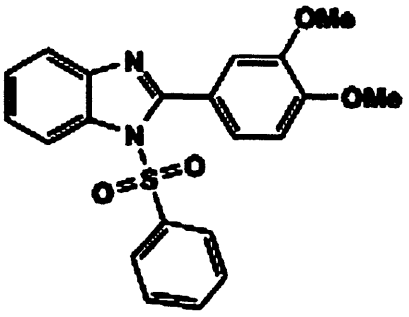
38



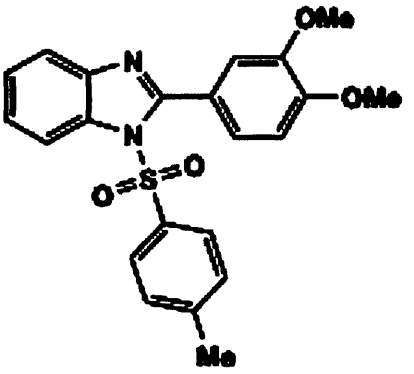
39



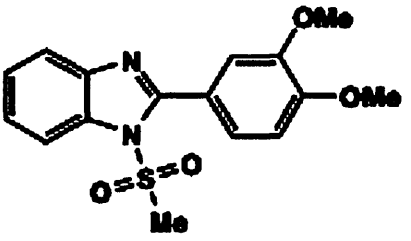
40



42



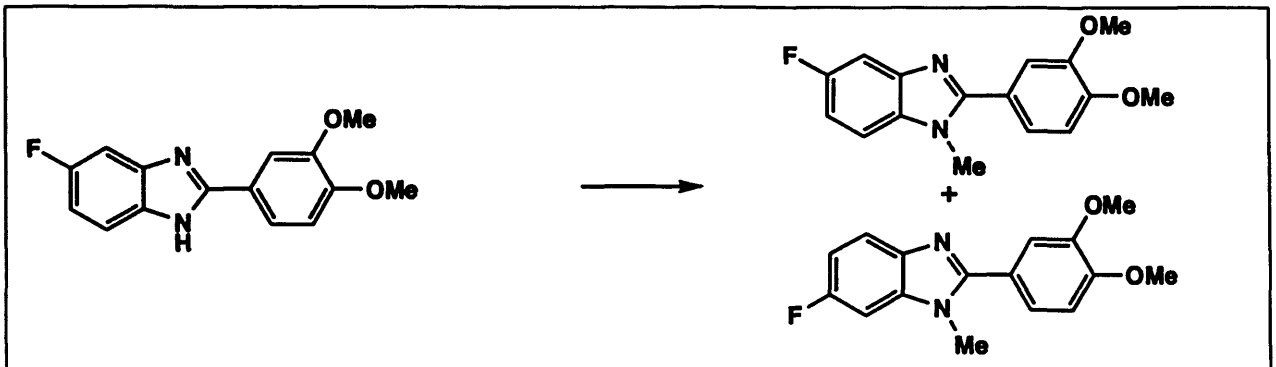
43



41

Figure 1: The synthesised N-substituted benzimidazoles

In addition to the synthesis of N-sulfonyl substituted benzimidazoles, 5-and 6-fluoro-3,4-(dimethoxyphenyl)-N-Me-benzimidazole isomers (Figure 2) were synthesised using this procedure (Scheme 3).



Reagents and conditions: NaH, DMF, MeI, RT.

Scheme 3

On the other hand, the non-fluorinated counterpart 3,4-(dimethoxyphenyl)-*N*-Me-benzimidazole (3)was previously prepared (Chapter 2) directly in a one-step from N-methylphenylenediamine and 3,4-dimethoxybenzaldehyde since both substrates are commercially available.

50 51

Figure 2: The chemical structures of 5-and 6-fluoro-3, 4-(dimethoxyphenyl)-*N*-Me-benzimidazole isomers

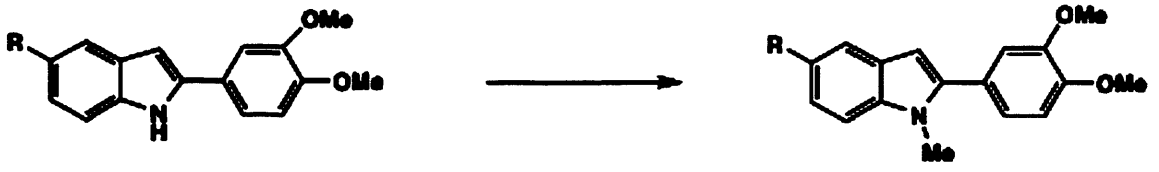
Nevertheless, the characterisation of the different synthesized isomers was accomplished by the use of ^{1}HNMR and NOESY experiments as described in detail in Chapter 2.(Refer also to the experimental section, Chapter 5).

4.1.2. The synthesis of N-substituted indoles

5-Fluoro- (3,4-dimethoxyphenyl)-1-tosyl-indole(**17**)was prepared via the use of Pd-catalysed coupling reaction as outlined in Chapter 3. However, for the synthesis of N-substituted indoles, the use of S_N2 conditions (NaH in DMF) was first attempted since the non-substituted indoles (**18, 20**) were already made in sufficient quantities using Fischer indole synthesis and this synthetic route was proved highly effective in terms of the obtained yields and ease of synthesis for the preparation of the N-substituted benzimidazoles above.

4.1.2.1. The use of NaH as a base:

The procedure described above for the synthesis of N-substituted benzimidazoles was applied for the preparation of the desired indoles under similar conditions. This synthetic method was indeed effective in the preparation of the fluorinated and non-fluorinated-2-(3,4-dimethoxyphenyl)-N-*Me*-indoles (Scheme 4) in good yields (52-65%).



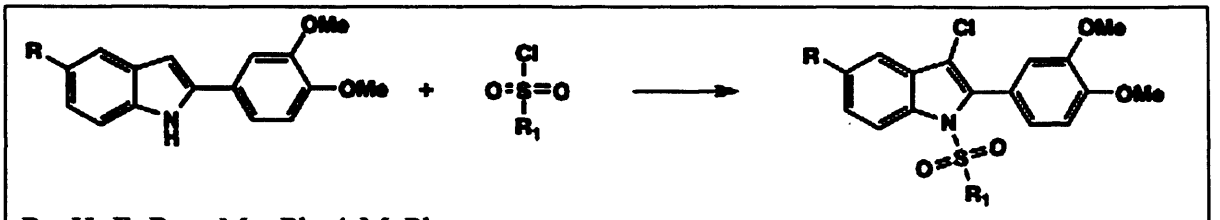
R = H; **53**
R = F; **52**

Reagents and conditions: NaH, MeI, DMF, RT, overnight.

Scheme 4

On the other hand, when applied to the sulfonyl chlorides, it led to the formation of a mixture of different components including the desired N-substituted indoles. Of these components, the major byproduct was isolated and the NMR characterisation revealed that it is the 3-chloro-N-arylsulfonylindoleas illustrated in Scheme 5. These findings are consistent with previous reports which showed that the N-arylsulfonylation of 2-arylindoles with arylsylfonyl halides is accompanied by 3-halogenation with the 3-halogenated products being the major products.[2] Moreover, it was noted that this 3-

halogenation is more likely to occur with electron rich 2-substituents.[2]It follows therefore, that the presence of the electron donating groups (dimethoxy) of the indole contribute to the formation of side products.



R =H, F; R_1 = Me, Ph, 4-MePh.

Reagents and conditions: NaH, DMF, RT.

Scheme 5

The following 3-halogenated products were obtained as shown in Figure 2.

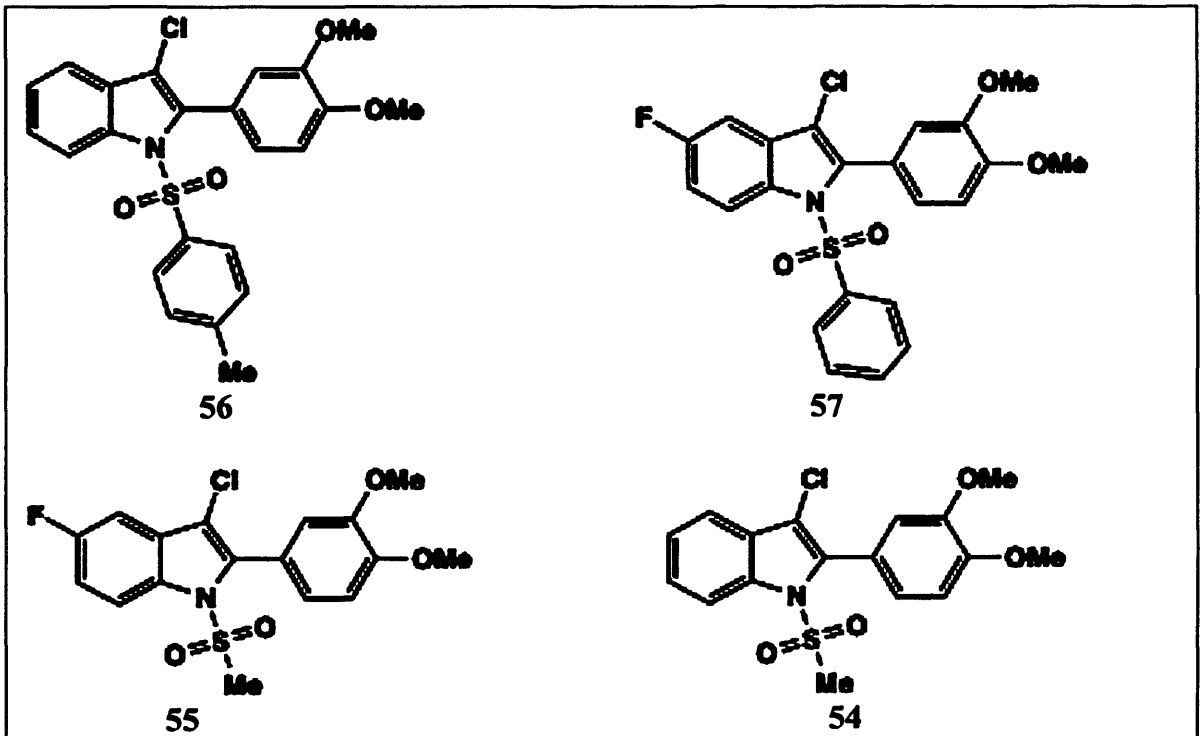
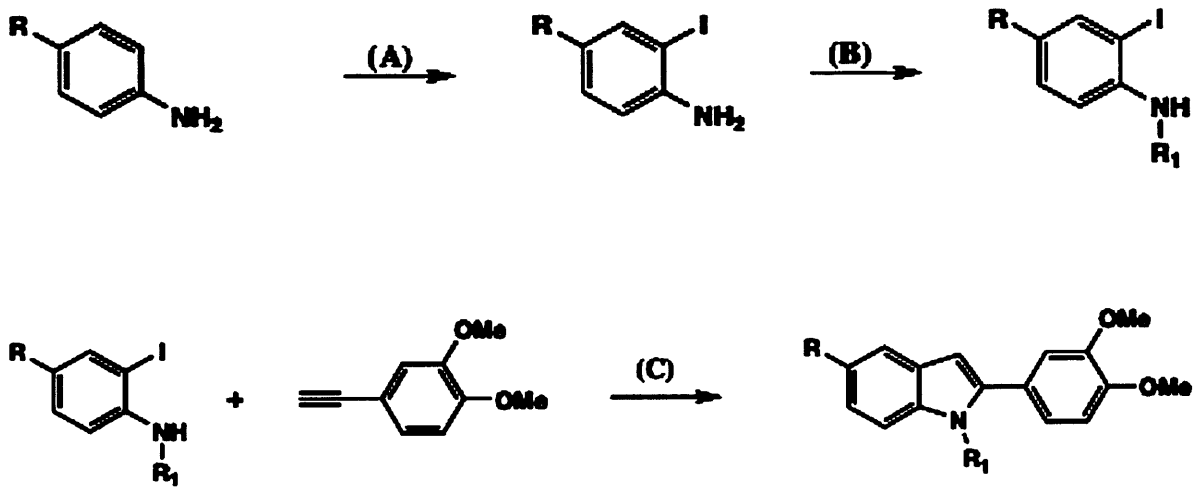


Figure 2: Chemical structures of3-chloro-N-substituted indole side products.

The next alternative option was to employ the Pd-catalysed since the reaction conditions required have already been established before in the synthesis of HK32 (Chapter 3).

4.1.2.2. The use of Pd -catalysed reactions

The palladium-catalysed synthetic route enables access to substituted 2-phenyl indoles via the Sonogashira coupling reactions of o-haloanilines and terminal alkynes and subsequent cyclisation to form the indole nucleus.[3,4] The required terminal alkyne (3,4-dimethoxyphenylethyne) was already prepared in a good quantity in our previous attempts for the synthesis of indole **(17)**. In this synthetic route, a selective o-iodination is accomplished by the use of molecular iodine in aqueous media in the presence of sodium hydrogen carbonate ($NaHCO_3$), the desired sulfonyl substituent was introduced to the iodinated aniline forming the sulfonamide.[5] This sulfonamide is then reacted with the terminal alkyne under Sonogashira conditions followed by one-pot base mediated cyclisation into the desired N-substituted indole.



R = H, F

R1 = SO_2Me, SO_2Ph, SO_2PhMe

	R	R_1	
a:	H	SO_2Me	(58)
b:	H	SO_2Ph	(60)
c:	H	SO_2PhMe	(61)
d:	F	SO_2Me	(59)
e:	F	SO_2Ph	(60)
f:	F	SO_2PhMe	(17)

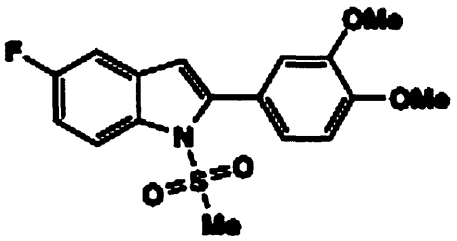
Reagents and conditions: (A) $NaHCO_3$, H_2O, RT, 3 hr.

(B) R_1SO_2Cl, Pyridine, DCM, RT, 18hr.

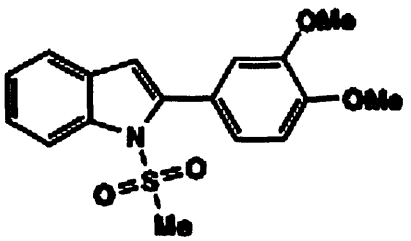
(C) $PdCl_2(PPh_3)_2$, CuI, TEA, THF,Reflux, 6 hr.

Scheme 5

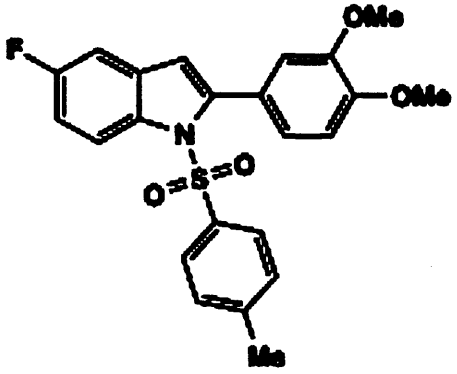
The use of this synthetic route enabled the synthesis of the following differently N-substituted indoles in average yields (45-62 %) as shown below in Figure 3.



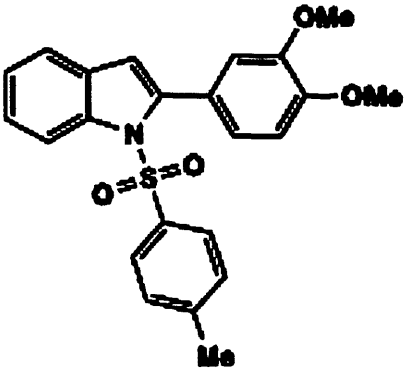
59



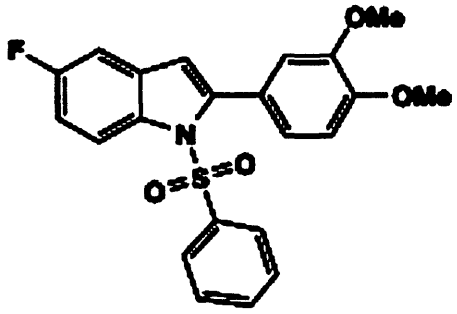
58



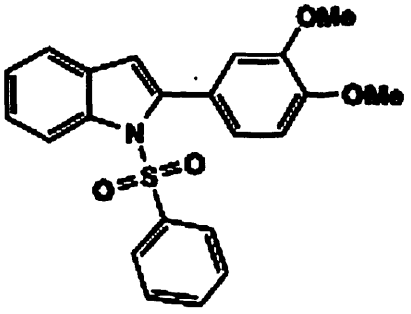
17



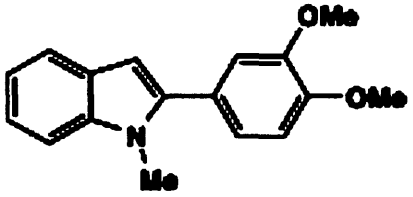
61



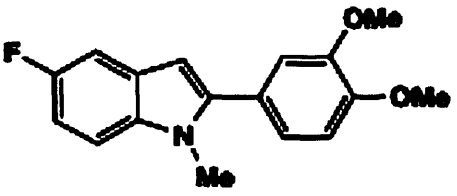
62



60



53



52

Figure 3: N-substituted indoles synthesised using Pd catalysed coupling reactions.

4.2.Biology

The new series of N-substituted benzimidazole and indole compounds as well as some of the side products (3-chloro-N-substitutedindoles) were tested for antitumour activity in the four different cell lines; MCF7, A549, LOVO and PC3 by our collaborators in Tenovus Centre, Welsh School of Pharmacy. The obtained results for the N-substituted benzimidazoles and N-substituted indoles are represented in Table 1 and Table2 respectively.

Compound	A549	LOVO	PC3	MCF7
41	>100	50	50	50
42	>100	>100	50	70
43	**10**	**5**	**5**	**5**
50	>100	>100	>100	>100
51	>100	>100	50	>100
37	>100	>100	>100	>100
38	>100	>100	>100	>100
39	>100	50	50	50
40	>100	50	50	50

Table 1: *In vitro* antitumour activity (IC_{50}) of the N-substituted benzimidazole analogues against cancer cell lines (MCF7, A549, LOVO and PC3).

Compound	A549	LOVO	PC3	MCF7
17	**0.9**	**0.6**	**0.7**	**0.8**
58	>100	100	50	50
59	>100	>100	>100	>100
62	50	50	50	50
60	>100	50	50	>100
52	>100	50	50	>100
53	>100	50	50	50
61	**5**	**0.5**	**0.5**	**0.5**
55	>100	>100	100	90
56	**5**	**0.5**	**0.5**	**5**
57	>100	50	>100	90
54	>100	>100	>100	>100

Table2: *In vitro* antitumour activity (IC_{50}) of the N-substituted indoles against cancer cell lines (MCF7, A549, LOVO and PC3).

Inspection of the antitumour activity data in Table 1 and Table 2 reveals that the prepared N-substituted benzimidazole and indole analogues do not exert antitumour activity at the sub-nanomolar GI_{50} level seen for PMX610.[6]

Table 1 shows clearly that in most cases, the benzimidazole compounds were devoid of any significant antitumour inhibitory activity (IC_{50}> 50 μM). The only exception is 2-(3,4-dimethoxyphenyl)-N-tosylbenzimidazole **(43)** which, showed low micromolar values in the four cell lines. Notably, this benzimidazole shows similar activity to its fluorinated analogues **(10 and 11)** where these compounds also bear the electron-withdrawing group, tosyl as N substituent.

From Table 2, a similar observation can be made whereby most of the prepared N-substituted indoles show no significant activity except for **61** and **56**. These compounds are also the non-fluorinated analogues of indole**17**, which bear the tosyl substituent. This suggests that the presence of fluorine on the ring does not necessarily improve its antitumour activity compared to the benzothiazoles where the presence of fluorine was essential. The presence of chlorine at position 3 **(56)** reduces the antitumour inhibitory effects by about ten fold (compare to **(61)**, from sub-micromolar to low micro-molar) at MCF-7 while maintaining the same level of inhibitory effects at the other cell lines.

Overall, it seems that the antitumour effect is linked to the tosyl group since even the use of the very closely related phenylsulfonyl groups did not have much effect (compare **17**and**62** for indole series, and**1, 11** and**39, 40** for benzimidazole series).

References

1. Kadri, H.; Matthews, C. S.; Bradshaw, T. D.; Stevens, M. F. G; Westwell, A. D. Synthesis and antitumour evaluation of novel 2-phenylbenzimidazoles. *J.Enz.Inhib. Med. Chem.* **2008,** 23-641-647.
2. Dalton, L.; Humphrey, G. L.; Cooper, M, M.; Joule, J. A. Indole β-nucleophilic substitution. Part 7. β-Halogenation of indoles. Attempted intramolecular β-nucleophilic substitution of α-aryl indoles. *J. Chem. Soc. Perkin. Trans.* **1983**,2417-2422.
3. Suzuki, N.; Yasaki, S; Yasuhara,A., Sakamoto, T. Convenient indole synthesis from 2-iodoanilines and terminal alkynes by the sequential sonogashira reaction and the cyclization reaction promoted by tetrabutylammonium fluoride (TBAF).*Chem. Pharm. Bull.***2003.** 51, 1170-1173.
4. Sakamoto, T.; Kondo, Y.; Iwashita, S.; Nagano, T.; Yamanaka, H. Condensed heteroaromatic ring systems. XIII. One-step synthesis of 2-substituted 1-methylsulfonylindoles from N-(2-halophenyl)methanesulfonamides.*Chem. Pharm. Bull.* **1988,** *36,* 1305-1308.
5. Xiao, W. J.; Alper, H.Regioselectivecarbonylativeheteroannulation of o-iodothiophenols with allenes and carbon monoxide catalyzed by a palladium complex: a novel and efficient access tothiochroman-4-one derivatives. *J. Org. Chem.***1999**,64, 9646-9652.
6. Mortimer, C. G.; Wells, G.; Crochard, J-P, Stone, E, L.; Bradshaw, T.D.; Stevens, M.F.G.; Westwell, A.D. Antitumor benzothiazoles. 26. 2-(3,4 fluorinated 2 aryl benzothiazole shows potent and selective inhibitory activity against lung, colon, and breast cancer cell lines. *J. Med. Chem.***2006,** 49, 179-185.

5. EXPERIMENTAL

5.1. General experimental details

Melting points were measured on a Griffin apparatus and are uncorrected. All commercially available starting materials used were purchased from Aldrich without further purification. All glassware was oven dried at 60°C for several hours or overnight.

Chromatography

Thin Layer Chromatography (TLC) was performed using precoated, aluminum backed silica gel plates (TLC Silica gel 60 F_{254}-Merck) and an ultra-violet (UV) lamp was used for visualisation (254nm). For column chromatography, silica gel (Silica 60A particle size 35-70 micron, Fisher Scientific) was used.

NMR spectroscopy

^{1}H, and ^{13}C NMR were recorded on a Bruker Avance 500 spectrometer with operating frequencies of 500, and 125 MHz respectively. The following abbreviations are used in the assignment of NMR signals: s (singlet), d (doublet), t (triplet), m (multiplet). *J* coupling constants were calculated on the Topspin program and are reported Hertz (Hz).

Mass Spectroscopy

Mass spectra were determined under electrospray conditions on a Bruker MicroTOF LC instrument at the Welsh School of Pharmacy, Cardiff, or at the Chemistry Department, Cardiff University.

CHN Analysis

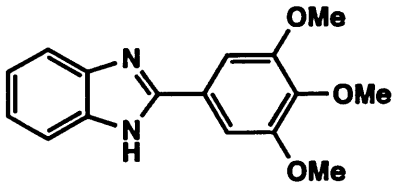
CHN analysis was carried out by MEDAC. The data were recorded on a Carlo-Erba EA 1108 element analyser, PC based data system, Eager 200 for Windows and a Sartorius Ultra Micro balance 4504MP8.

5.2. Synthesised compounds

5.2.1. Substituted 2-phenylbenzimidazoles:

General method for the synthesis of 2-phenylbenzimidazoles:[1] Sodium metabisulfite (0.60g, 3.16 mmol) was added to a stirring mixture of (substituted) 1,2-phenylenediamine (3.12 mmol) and substituted benzaldehyde (3.16 mmol) in DMF (10 mL), and the reaction mixture was heated under reflux for two hours. After completion of the reaction (monitored by TLC), the mixture was allowed to cool to room temperature. Addition of water (approximately 20 mL) caused precipitation of a pale yellow solid, which was collected by vacuum filtration and dried. The crude product was further purified by recrystallisation (aqueous methanol) or column chromatography (40% Hexane/ 60% EtOAc) to give the pure substituted 2-arylbenzimidazole as a white solid in yields 46-63%. The following compounds were prepared using this procedure.

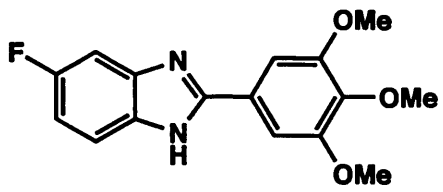
2-(3,4,5-Trimethoxyphenyl)-1*H*-benzimidazole (1)[2]



$C_{16}H_{16}N_2O_3$, MW: 284.31

Prepared from 1,2-phenylenediamine and 3,4,5-trimethoxybenzaldehyde (46% yield). **m.p.** 254–255 °C (**Lit** 259.9-262.1°C)[2]. **^{1}H NMR (DMSO-d_6):** δ 3.74 (3H, s, OMe), 3.91 (6H, s, 2 x OMe), 7.20 (2H, dd, *J* 3.0, 5.5 Hz, H-4, H-7), 7.53 (2H, s, H-2', H-6'), 7.60 (2H, m, H-5, H-6), 12.83 (1H, bs, NH). **CHN Anal.** Calcd for C, 67.59; H, 5.67; N, 9.85. Found, C 67.65, H, 5.71; N, 9.73%.

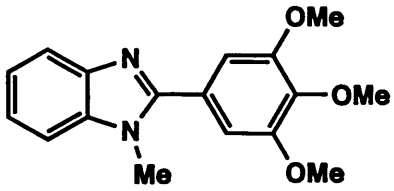
2-(3,4,5-Trimethoxyphenyl)-5-fluoro-1*H*-benzimidazole (2)



$C_{16}H_{15}FN_2O_3$, MW: 302.30

Prepared from 4-fluoro-1,2-phenylenediamine and 3,4,5-trimethoxybenzaldehyde (57% yield). **m.p.** 243-245 °C; **^{1}H NMR (DMSO-d_6):** δ 3.74 (3H, s, OMe), 3.91 (6H, s, 2 x OMe), 7.06 (1H, dt, *J* 2.5, 9.5 Hz, H-6), 7.40 (1H, d, *J* 9.0 Hz, Ar-H), 7.51 (2H, s, H-2', H-6'), 7.59 (1H, m, Ar-H), 12.83 (1H, bs, NH). **CHN Anal.** Calcd for C, 63.57; H, 5.00; N, 9.26. Found, C 63.51, H, 5.03; N, 9.14%.

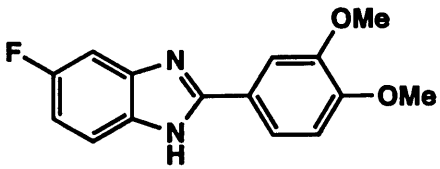
2-(3,4,5-Trimethoxyphenyl)-1-methyl-benzimidazole (3)



$C_{17}H_{18}N_2O_3$, MW: 298.34

Prepared from *N*-methyl-1,2-phenylenediamine and 3,4,5-trimethoxybenzaldehyde (59% yield). **m.p.** 114-116 °C, **^{1}H NMR ($CDCl_3$):** δ 3.89 (3H, s, NMe), 3.93 (3H, s, 4'-OMe), 3.94 (6H, s, 2 x OMe), 6.96 (2H, s, H-2', H-6'), 7.31 (2H, m, Ar-H), 7.39 (1H, m, Ar-H), 7.82 (1H, m, Ar-H). **CHN Anal.** Calcd for C, 68.44; H, 6.08; N, 9.39. Found: C, 68.85; H, 6.19; N, 9.48%.

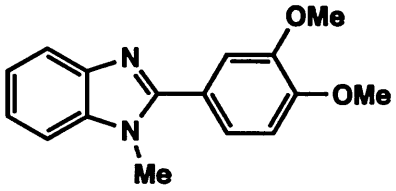
2-(3,4-Dimethoxyphenyl)-5-fluoro-1*H*-benzimidazole (4)



$C_{15}H_{13}FN_2O_2$, MW: 272.27

Prepared from 4-fluoro-1,2-phenylenediamine and 3,4-dimethoxybenzaldehyde (52% yield). **m.p.** 212-214°C, **^{1}H NMR (DMSO-d_6):** δ 3.83 (3H, s, OMe), 3.88 (3H, s, OMe), 7.03 (1H, dt, *J* 2.5, 10.5 Hz, H-6), 7.13 (1H, d, *J* 8.5 Hz, H-5'), 7.36 (1H, m, Ar-H), 7.55 (1H, m, Ar-H), 7.73 (1H, dd, *J* 1.5, 8.5 Hz, H-6'), 7.75 (1H, d, *J* 1.5 Hz, H-2'), 12.78 (1H, bs, NH). **CHN Anal.** Calcd for C, 66.17; H, 4.81; N, 10.28. Found: C, 66.00; H, 4.86; N, 10.08%.

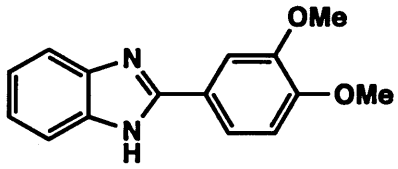
2-(3,4-Dimethoxyphenyl)-1-methylbenzimidazole (5)[3]



$C_{16}H_{16}N_2O_2$, MW: 268.31

Prepared from *N*-methyl-1,2-phenylenediamine and 3,4-dimethoxybenzaldehyde (61% yield); **m.p.** 107-109 °C; **^{1}H NMR ($CDCl_3$):** δ 3.85 (3H, s, NMe), 3.95 (3H, s, OMe), 3.96 (3H, s, OMe), 6.98 (1H, d, *J* 8.0 Hz, H-5'), 7.24 (1H, dd, *J* 2.0, 8.5 Hz, H-6'), 7.30 (2H, m, Ar-H), 7.36 (2H, m, Ar-H), 7.79 (1H, m, Ar-H). **CHN Anal.** Calcd for C, 71.62; H, 6.01; N, 10.44. Found: C 71.85; H, 6.02; N, 10.36%

2-(3, 4-Dimethoxyphenyl)-1*H*-benzimidazole (6)



$C_{15}H_{14}N_2O_2$, MW: 254.28

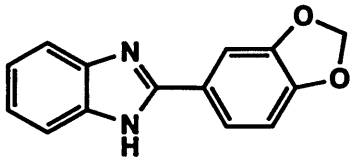
Prepared from 1,2-phenylenediamine and 3,4-dimethoxybenzaldehyde (55% yield). **m.p.** 231-233 °C (**Lit** 227-228°C)[3]; **^{1}H NMR (DMSO-d_6):** δ 3.84 (3H, s, OMe), 3.88 (3H, s, OMe), 7.13 (1H, d, *J* 8.5 Hz, H-5'), 7.18 (2H, dt, *J* 3.7, 7.0 Hz, H-5, H-6), 7.57 (2H, dd, *J* 3.5, 5.0 Hz, H-4, H-7), 7.76 (1H, dd, *J* 2.0, 8.5 Hz, H-6'), 7.78 (1H, d, *J* 2.0 Hz, H-2'),

12.84 (1H, bs, NH). **CHN Anal.** Calcd for C, 70.85; H, 5.55; N, 11.01. Found: C, 70.56; H, 5.62; N, 10.75 %.

General method for the synthesis of 2-(3,4-methylenedioxyphenyl)benzimidazoles.

Triethylamine (5.80 mL, 4 mmol) was added to a solution of (substituted)-1,2-phenylenediamine (0.29 g, 2.7 mmol) and piperonyloyl chloride (0.5 g, 2.7 mmol) in THF (10 mL) at 0°C with stirring. The reaction mixture was stirred at 0 °C for around one hour (until reaction was complete by TLC), then the solution was concentrated *in vacuo*. The resulting residue was further heated under reflux in acetic acid (100 °C) for 12 hours. The solution was then neutralised (aqueous sodium hydroxide), and the product extracted using ethyl acetate (2 x 20 mL), then dried ($MgSO_4$) and concentrated *in vacuo*. Purification by flash column chromatography (60% CH_2Cl_2-40% EtOAc) afforded the title compound as a white solid. This procedure was used in the preparation of the following compounds.

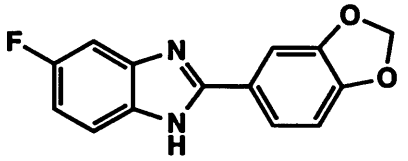
2-(3,4-Methylenedioxyphenyl)-1*H*-benzimidazole (7) [4]



$C_{14}H_{10}N_2O_2$, MW: 238.24

Prepared from 1,2-phenylenediamine and piperonyloyl chloride (46% yield). **m.p.** 242-244°C (**Lit** 245-247°C)[4] ; **^{1}H NMR (DMSO-d_6):** δ 6.13 (2H, s, OCH_2), 7.10 (1H, d, *J* 8.8 Hz, H-5'), 7.18 (2H, m, Ar-H), 7.56 (1H, m, Ar-H) 7.65 (1H, m, Ar-H), 7.69 (1H, d, *J* 1.5 Hz, H-2'), 7.72 (1H, dd, *J* 1.5, 8.5 Hz, H-6'), 12.79 (1H, bs, NH). **CHN Anal.** Calcd for C, 70.58; H, 4.23; N, 11.75. Found: C, 70.31; H, 4.21; N, 11.66%.

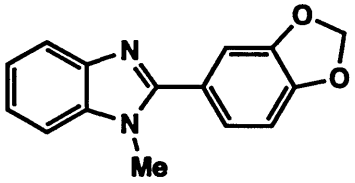
2-(3,4-Methylenedioxyphenyl)-5-fluoro-1*H*-benzimidazole (8).



$C_{14}H_9FN_2O_2$, MW: 256.23

Prepared from 4-fluoro-1,2-phenylenediamine and piperonyloyl chloride (46% yield), **m.p.** 205-207 °C; **^{1}H NMR (DMSO-d_6):** δ 6.13 (2H, s, OCH_2), 7.04 (1H, dt, *J* 2.5, 10.0 Hz, H-6), 7.10 (1H, d, *J* 8.0 Hz, H-5'), 7.36 (1H, m, Ar-H), 7.55 (1H, m, Ar-H) 7.66 (1H, d, *J* 2.0 Hz, H-2'), 7.70 (1H, dd, *J* 2.0, 8.0 Hz, H-6'), 12.86 (1H, bs, NH). **CHN Anal.** Calcd for C, 65.62; H, 3.54; N, 10.93. Found: C, 65.38; H, 3.56; N, 10.74%.

2-(3,4-Methylenedioxyphenyl)-1-methyl-benzimidazole (9) [5]



$C_{15}H_{12}N_2O_2$, MW: 252.27

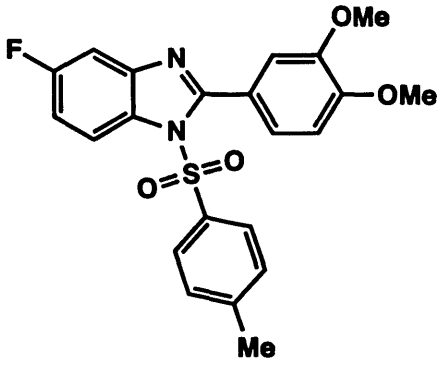
From *N*-methyl-1,2-phenylenediamine and piperonyloyl chloride (59% yield). **m.p.** 149-151 °C **(Lit** 157-158°C);[5] **^{1}H NMR ($CDCl_3$):** δ 3.84 (3H, s, NCH_3), 6.05 (2H, s, OCH_2), 6.94 (1H, d, *J* 8.0 Hz, H-5'), 7.23 (2H, m, Ar-H), 7.29 (2H, m, Ar-H) 7.36 (1H, m, Ar-H), 7.77 (1H, m, Ar-H). **CHN Anal.** Calcd for C, 71.42; H, 4.79; N, 11.10. Found: C, 71.02; H, 4.75; N, 11.00%.

Preparation of 2-(3,4-dimethoxyphenyl)- 5-fluoro-1-tosyl-benzimidazole and 2-(3,4-dimethoxyphenyl)- 6-fluoro-1-tosyl-benzimidazole.

To a stirring solution of **(4)** (0.73 mmol) in pyridine (10mL), p-toluenesulfonylchloride (1.1mmol) was added in small portions under nitrogen and left stirring overnight. The reaction mixture was then diluted with H_2O and extracted with DCM (3 x 20 mL), dried

over $MgSO_4$ and concentrated *in vacuo*. The use of column chromatography (80% DCM/ 20% EtOAc) enabled the isolation of the 2 isomers **(10 & 11)**.

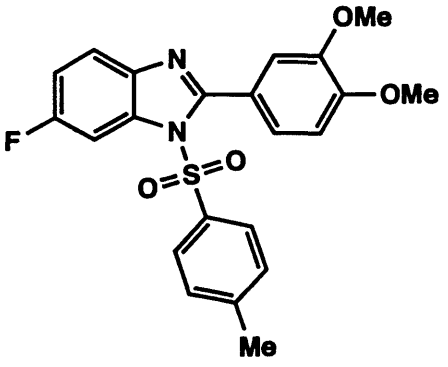
2-(3,4-Dimethoxyphenyl)-5-fluoro-1-tosylbenzimidazole (10)



$C_{22}H_{19}FN_2O_4S$, MW: 426.46

(21% yield). **m.p.**123-125°C, **^{1}H NMR ($CDCl_3$):** δ 2.31 (3H, s, Me), 3.81 (3H, s, OMe), 3.91 (3H, s, OMe), 6.88 (1H, d, *J* 8.0 Hz, H-5'), 7.01 (2H, d, *J* 8.5 Hz, H-3'', H-5''), 7.03 (1H, d, *J* 2.0 Hz, H-2'), 7.08 (1H, dt, *J* 2.5 Hz, 9.0 Hz, H-6), 7.17 (1H, dd, *J* 2.0 Hz, 8.0 Hz, H-6'), 7.18 (2H, d, *J* 8.5 Hz, H-2'', H-6''), 7.29 (1H, dd, *J* 2.5 Hz, 9.5Hz, H-4), 8.08 (1H, dd, *J* 5.0 Hz, 9.5 Hz, H-7). **CHN Anal.** Calcd for C, 61.96; H, 4.49; N, 6.57. Found: C, 61.64; H, 4.46; N, 6.38%.

2-(3,4-Dimethoxyphenyl)-6-fluoro-1-tosylbenzimidazole (11)

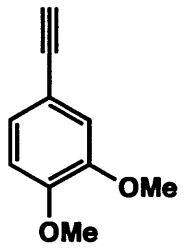


$C_{22}H_{19}FN_2O_4S$, MW: 426.46

(16% yield); **m.p.**129-131°C. **^{1}H NMR ($CDCl_3$):** δ 2.31 (3H, s, Me), 3.81 (3H, s, OMe), 3.91 (3H, s, OMe), 6.87 (1H, d, *J* 8.5 Hz, H-5'), 7.00 (1H, d, *J* 2.0 Hz, H-2'), 7.04 (2H, d, *J* 8.5 Hz, H-3'', H-5''), 7.07 (1H, dt, *J* 2.5 Hz, 9.0Hz, H-5), 7.15 (1H, dd, *J* 2.0 Hz, 8.5Hz, H-6'), 7.22 (2H, d, *J* 8.5 Hz, H-2'', H-6''), 7.56 (1H, dd, *J* 5.0 Hz, 9.5Hz, H-4), 7.88 (1H, dd, *J* 2.5 Hz, 9.5 Hz, H-7). **CHN Anal.** Calcd for C, 61.96; H, 4.49; N, 6.57. Found: C, 61.65; H, 4.48; N, 6.28%.

5.2.2. Substituted 2-phenyl indoles

3, 4-Dimethoxyphenylethyne (13)[6]

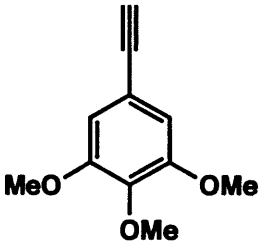


To a well stirred solution of carbon tetrabromide (3 g, 9 mmol) in dry DCM (20mL) at 0 °C, triphenylphosphine (4.5g, 18mmol) was added followed by 3,4-dimethoxybenzaldehyde (1.5g, 9mmol). The resultant mixture was stirred for 1 hour at room temperature, washed with water, dried over $MgSO_4$, filtered and concentrated *in vacuo* to give orange residue. Purification by column chromatography eluting with DCM afforded 2,2,dibromo-1-(3,4-dimethoxyphenyl)ethene as a pale yellow oil (74% yield). **^{1}H NMR ($CDCl_3$):** δ 3.87 (6H, s, 2 x OMe), 6.78 (1H, d, *J* 8.0 Hz, H-5'), 7.05 (1H, dd, *J* 2.5 Hz, 8.0 Hz, H-6'), 7.17 (1H, d, *J* 2.5 Hz, H-2'), 7.36 (1H, s, H-1).

2,2-Dibromo-1-(3,4-dimethoxyphenyl)ethene (2 g, 6.3mmol) was dissolved in dry THF (25mL) and the solution cooled to -78°C under inert gas conditions. *n*-BuLi (1.6M) was added to the stirred solution dropwise over a period of 30 minutes and the mixture was allowed to warm to room temperature and stirred further for 1hour. The reaction was quenched with saturated aqueous NH_4Cl, extracted with Et_2O, dried over $MgSO_4$, filtered and evaporated. The resultant residue was purified by column chromatography (eluting

with DCM) to afford the titled compound as a white solid (79% yield). **^{1}H NMR ($CDCl_3$):** δ 2.94 (1H, s, H-2), 3.81 (6H, s, 2 x OMe), 6.71 (1H, d, *J* 8.0 Hz, H-5'), 6.89 (1H, d, *J* 2.0 Hz, H-2'), 7.02 (1H, dd, *J* 8.0 Hz, 2.0 Hz, H-6').

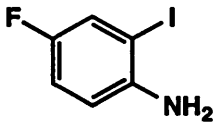
3, 4, 5-Trimethoxyphenylethyne (14)[7]



p-Toluenesulfonylazide: a stirring solution of NaN_3 (7.15g ,0.11mol) in H_2O (20 mL) was diluted with ethanol (40mL) and a warm solution of *p*-toluenesulfonyl chloride (19.05g, 190.65mmol) in ethanol (100mL) is added causing a separation of NaCl and the mixture takes light brown color. After stirring for 3.5 hours, most of the solvent is evaporated and H_2O (20 mL) is added to the residue where 2 layers are formed. The oily layer is separated, dried ($MgSO_4$) and filtered to afford *p-toluenesulfonylazide* as a colourless oil which solidifies at low temperatures (89% yield).

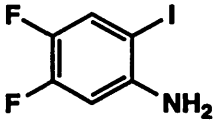
Dimethyl-2-oxopropylphosphonate (0.19g, 1.2 mmol) was added to a suspension of K_2CO_3 (0.41g, 3mmol) and *p*-toluenesulfonylazide (0.23g, 1.2mmol) in acetonitrile (15mL) and the mixture was stirred at room temperature. After 2 hours, 3,4,5-trimethoxybenzaldehyde (0.19 g, 1 mmol) dissolved in methanol (2mL) is added and stirring continued for 24 hours. The solvents were removed *in vacuo* and the resultant residue was extracted with EtOAc, dried ($MgSO_4$) and concentrated *in vacuo.* Purification with column chromatography (DCM) afforded pure 3,4,5-trimethoxyphenylethyne as a white solid (32% yield). **^{1}H NMR ($CDCl_3$):** δ 2.71 (1H, s, H-2), 3.79 (3H, s, OMe), 3.82 (6H, s, 2 x OMe), 6.68 (2H, s, H-2', H-6').

4-Fluoro-2-iodoaniline (15)[8]



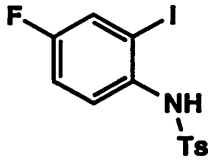
4-Fluoroaniline (1.12g, 10mmol) was placed in a beaker, $NaHCO_3$ (1.26g, 15mmol) and H_2O (20 mL) were added and the mixture was cooled down to 0 °C using ice bath. Powdered iodine (2.53g, 10 mmol) was then added in small portions at 5-minute intervals and the mixture stirred for further 3 hours at room temperature. Once the reaction is completed (TLC monitoring), $Na_2S_2O_3$ was added to the reaction mixture and the organic phase was extracted with Et_2O, dried over $MgSO_4$ and concentrated *in vacuo* to give the title compound (64% yield). **^{1}H NMR ($CDCl_3$):** δ 3.92 (2H, bs, NH_2), 6.72 (1H, m, Ar-H), 6.95 (1H, m, Ar-H), 7.41(1H, m, Ar-H).

4, 5-Difluoro-2-iodoaniline (16)[8]



Prepared from 3, 4-difluoroaniline (1.5g, 11.62mmol) by the same procedure described for the synthesis of 4-fluoro-2-iodoaniline **(15)** (68% yield). **^{1}H NMR ($CDCl_3$):** δ 4.05 (2H, bs, NH_2), 6.51(1H, m, Ar-H), 7.38(1H, m, Ar-H).

N-(4-Fluoro-2-iodophenyl)-4-methylbenzenesulfonamide

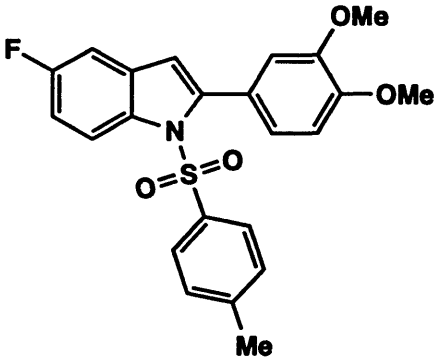


To a stirring solution of 4-fluoro-2-iodoaniline (0.76g, 3.2mmol), pyridine (0.39 mL, 4.8 mmol) in dry DCM (15 mL) under nitrogen gas was added p-toluenesulfonylchloride (4.8mmol in 10 mL DCM) dropwise at 0 °C. The mixture was then left to warm to room

temperature and stirred for further 18 hours. The reaction was quenched with 6N NaOH (100mL) and H_2O (100 mL) was added to dissolve the resultant anion. The layers were separated and the aqueous layer washed with DCM, cooled to 0 °C and acidified (0.2M HCl) causing the title compound to precipitate as white solid (74% yield). **^{1}H NMR ($CDCl_3$):** δ 2.48 (3H, s, Me), 6.61 (1H, s, NH), 7.07 (1H, m, Ar-H), 7.24 (2H,d, *J* 8.0 Hz, H-3', H-5'), 7.41 (1H, dd, *J* 3.0 Hz, 7.5 Hz, Ar-H), 7.61 (2H,_d, *J* 8.0 Hz, H-2', H-6'), 7.67 (1H,m, Ar-H).

5.2.2.1. The palladium-catalysed reaction between *o*-iodoanilines and terminal alkynes

2-(4,5-Dimethoxyphenyl)-5-fluoro-N-tosyl-indole (17) [9]



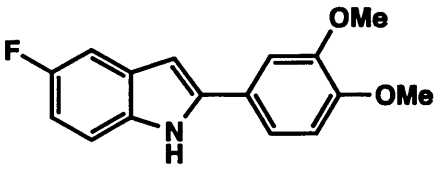
$C_{23}H_{20}FNO_4S$, MW: 425.47

A mixture of N-(4-fluro-2-iodophenyl)-4-methylbenzenesulfonamide (0.29g, 1mmol), 4,5-dimethoxyphenylethyne (0.58g, 1.5mmol), $PdCl_2(PPh_3)_2$ (35mg, 5mol%), CuI (19mg, 10mol%) and TEA (3mL) in THF (10mL) was heated under reflux for 5 hours. After the removal of solvent, the residue was diluted with H_2O and extracted with AcOEt, dried over $MgSO_4$ and concentrated *in vacuo*. Purification with column chromatography (70% ethyl acetate/ 30% hexane) afforded the title compound as a white solid (35% yield). **^{1}H NMR ($CDCl_3$):** δ 2.22 (3H, s, Me), 3.82 (3H, s, OMe), 3.88 (3H, s, OMe), 6.38 (1H, s, H-3), 6.82 (2H, m, Ar-H), 6.92-7.00 (4H, m, Ar-H), 7.16 (3H, m, Ar-H), 8.19 (1H, dd, *J* 4.5 Hz, 9.0 Hz, H-7). **ESI MS** m/z 448.08 [M+Na$^+$]. **CHN Anal.** Calcd for C, 64.93; H, 4.74; N, 3.29. Found: C, 64.64; H, 4.58; N, 3.80%.

2-(4,5-Dimethoxyphenyl)-5-fluoro-1-tosyl-indole (17) synthesis (Copper-free procedure)[10]

To a mixture of N-(4-fluoro-2-iodophenyl)-4-methylbenzenesulfonamide (390 mg, 1 mmol), $Pd(OAc)_2$ (2 mmol %), and Bu_4NOAc (2.5 mmol) in dry acetonitrile (8mL) under nitrogen atmosphere was added 3,4-dimethoxyphenylethyne (178.2mg, 1.1 mmol) dropwise. The reaction mixture was placed in a sonicator and stirred for 6 hours with the progress of the reaction monitored by TLC. After completion of the reaction, acetonitrile was evaporated, diluted with water, and extracted with ethyl acetate. The organic layer was dried over $MgSO_4$, filtered, concentrated *in vacuo* and the resultant residue was purified by column chromatography (70% ethyl acetate/ 30% hexane) as eluent to afford the titled compound (21% yield).

2-(4,5-Dimethoxyphenyl)-5-fluoro-*1H*-indole (18)



$C_{16}H_{14}FNO_2$, MW: 271.29

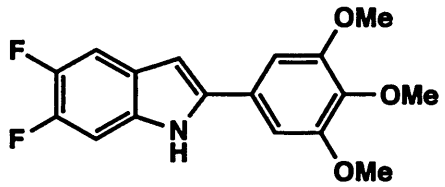
To a solution of 2-(4,5-dimethoxyphenyl)-5-fluoro-1-tosyl-indole (100mg, 0.24 mmol) in THF (15mL) was added, TBAF (0.18 g, 0.7mmol), under nitrogen, and the mixture was heated under reflux (80 °C) for 6 hours. After the removal of solvent, the residue was diluted with H_2O and extracted with AcOEt, dried over $MgSO_4$ and concentrated *in vacuo*. Purification with column chromatography afforded the title compound (82% yield). **^{1}H NMR (DMSO-d_6):** δ 3.80 (3H, s, OMe), 3.87 (3H, s, OMe), 6.80 (1H, s, H-3), 6.90 (1H, dt, *J* 2.5 Hz, 9.0 Hz, H-6), 7.04 (1H, d, *J* 8.5 Hz, H-5'), 7.25 (1H, dd, *J* 2.5 Hz, 10.0 Hz, H-4), 7.36 (1H, dd, 5.0 Hz, 9.0 Hz, H-7), 7.38 (1H, dd, *J* 2.0 Hz, 8.5 Hz, H-6'),7.45 (1H, d, *J* 2.0 Hz, H-2'), 11.51 (1H, s, NH). **^{13}C NMR (DMSO-d_6):** δ 55.55, 55.56, 97.81, 104.22, 108.87, 109.06, 111.86, 117.86, 124.74, 125.30, 129.05, 12 9.09, 133.54, 139.93, 148.67, 156.21, 158.04. **ESI MS** m/z 272.10 [M+H$^+$]. **CHN Anal.** Calcd for C, 70.84; H, 5.20; N, 5.16. Found: C, 70.82; H, 5.10; N, 5.17%.

5.2. 2. 2. The use of domino copper-catalysed synthesis of 2-substituted indoles: [11]

Preparation of [Cu(phen)(PPh$_3$)]NO$_3$:

Nitratobis(triphenylphosphine)copper(I): triphenylphosphine (24.22 g, 92.34 mmol) was slowly added to a boiling methanol (100mL). After the complete dissolution of triphenylphosphine, [Cu(NO$_3$)$_2$.2.5H$_2$O] (7.16 g, 30.78 mmol) was added in small portions leading to the formation of a white precipitate. The mixture was stirred for 30 minutes, after which, was cooled to room temperature. Then, it was filtered and the white residue was washed repeatedly with ethanol and diethyl ether. The resultant white solid was dried to give Cu(PPh$_3$)NO (56% yield). **m.p.** 241-242°C (**Lit** 238-240°C) [12]
Nitratobis(triphenylphosphine)copper(I) (977 mg, 1.50 mmol) was dissolved in chloroform (20 mL). After complete dissolution, triphenylphosphine (393 mg, 1.50 mmol), followed by 1,10-phenanthroline (270 mg, 1.50 mmol) were added which caused the solution to immediately take yellow colour. After stirring for 30 mins at RT, the solvent was removed *in vacuo* to give a [Cu(phen)(PPh$_3$)]NO$_3$ as a yellow solid. (65% yield). **m.p.** 198-199 C (**Lit** 202-204°C) [12]

5, 6-Difluoro-2-(3,4,5-trimethoxyphenyl)- 1*H*-indole (19)

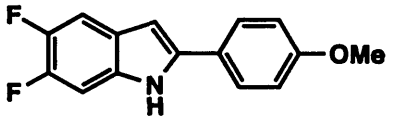


$C_{17}H_{15}F_2NO_3$, MW: 319.30

To a solution of 4,5-difluoro-iodotrifluoroacetanilide (1 mmol) in dry toluene (6mL), [Cu (phen)(PPh$_3$)]NO$_3$ and K$_3$PO$_4$ were added under nitrogen atmosphere and heated to reflux (110°C). 3,4,5-Trimethoxyphenylethyne (1.5mmol) was added dropwise over a period of 1 hr. After completion of the reaction (TLC monitoring), the reaction mixture was cooled, extracted with EtOAc, dried over MgSO$_4$ and concentrated *in vacuo*. The obtained residue was purified by column chromatography (Et$_2$O- Hexane) to afford the title

compound (30% yield). **^{1}H NMR (DMSO-d_6):** δ 3.70 (3H, s, OMe), 3.88 (6H, s, 2 x OMe), 6.93 (1H, s, H-3), 7.15 (2H, s, H-2', H-6'), 7.34 (1H, dd, *J* 8.0 Hz, 11.5 Hz, H-4), 7.50 (1H, dd, *J* 8.0 Hz, 11.5 Hz, H-7), 11.56 (1H, s, NH). **ESI MS** m/z 320.10 [M+H$^+$].

2-(4-Methoxyphenyl)- 5, 6-difluoro-1*H*-indole (36)

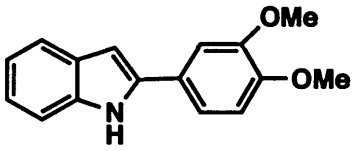


$C_{15}H_{11}F_2NO$, MW: 259,25

Prepared from 3,4-difluoroaniline and p-methoxyphenylethyne using the same procedure described above for the synthesis of **(19)** (34% yield), **^{1}H NMR (DMSO-d_6):** δ 3.81(3H, s, OMe), 6.75 (1H, s, H-3), 7.04 (2H, d, *J* 7.0 Hz, H-3', H-5'), 7.31 (1H, dd, *J* 7.0 Hz, 11.0 Hz, H-4), 7.46 (1H, dd, *J* 8.0 Hz, 11.5 Hz, H-7), 7.76 (2H, d, *J* 7.0 Hz , H-2', H-6'), 11.61 (1H, s, NH).

5.2.2.3. The intramolecular Heck annulation between chloroanilines and acetophenones.

2-(3,4-Dimethoxyphenyl)-*1H*-indole (20)[13]



$C_{16}H_{15}NO_2$, MW: 253.30

2-Chloroaniline (265 mg, 2mmol), 3,4-dimethoxyacetophenone (1.08g, 6mmol), acetic acid (0.15mL) and $MgSO_4$ (120 mg, 1mmol) were suspended in dimethylacetamide (12 mL) in a pressure tube. Nitrogen was bubbled through the solution for 10 minutes. K_3PO_4 (550 mg, 1.3mmol) and [Pd(t$Bu_3P)_2$] (120 mg, 0.1mmol) were added to the solution, and

nitrogen was bubbled through the mixture for an additional 5 minutes. The reaction mixture was heated to 145°C for 24 hours. After cooling to room temperature the reaction mixture was filtered, H_2O (30mL) was added to the filtrate and the mixture was extracted with ethyl acetate. The combined organic layers were dried over $MgSO_4$ and the solvents were removed in vacuo. The residue was purified by column chromatography (EtOAc/hexane) to afford (73.9mg, 15% yield) of the title product. **m.p.** 190-191°C **(Lit** 190°C).[14] **^{1}H NMR (DMSO-d_6):** δ 3.80 (3H, s, OMe), 3.87 (3H, s, OMe), 6.81 (1H, s, H-3), 6.98 (1H, t, *J* 8.0 Hz, Ar-H), 7.06 (2H, m, Ar-H), 7.40 (2H, m, Ar-H), 7.45 (1H, d, *J* 1.5 Hz, H- 2'), 7.50 (1H, d, *J* 8.0 Hz, H- 5'), 11.39 (1H, bs, NH). **^{13}C NMR(DMSO-d_6):** δ 55.57, 55.64, 97.60, 108.99, 110.98, 112.18, 117.49, 119.18, 119.62, 121.04, 125.12, 128.78, 136.86, 137.94, 148.43, 149.02. **ESI MS** m/z 254.11 [M+H$^+$].

5.2.2.4. The use of Fischer indole synthesis.[15]

A: Phenylhydrazines preparation

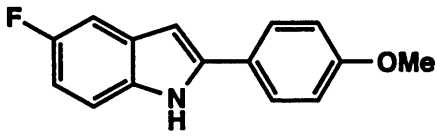
A solution of sodium nitrite (1.9g, 28.7 mmol) was added dropwise to a stirring solution of the substituted aniline (24.8mmol) in 6M aqueous hydrochloric acid (10mL) at O°C and the resultant yellow mixture was stirred for 2 hours. To this mixture, a solution of stannous chloride (33g, 0.174 mol) in concentrated HCl (30 mL) was added dropwise over 2 hours. After 1 hour of stirring, the mixture was poured into aqueous NaOH (10M) at O°C causing a precipitation of a solid which was suspended in H_2O and extracted with Et_2O. After drying over $MgSO_4$, filtration and evaporation of the solvent, the phenylhydrazine was obtained.

B: General method for the Fischer indole synthesis of 2-phenylindoles

Equivalent amounts of substituted phenylhydrazines (3.5mmol) and substituted acetophenones (3.5 mmol) were mixed in EtOH (20mL). A few drops of glacial AcOH were added and the solution was heated under reflux at 80°C for 1hr. The solvents were evaporated to give a solid, which was added to PPA (30mL), slowly heated to 120°C and kept at this temperature until the reaction is complete (TLC monitoring). A solid was

formed once the mixture is poured into a beaker containing cold water. This residue is collected, dried and purified using column chromatography (DCM/EtOAc) to give the pure corresponding indole (yield 18%-72%). The following compounds were prepared using this method.

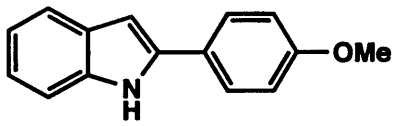
2-(4-Methoxyphenyl)- 5-fluoro-*1H*-indole (21)



$C_{15}H_{12}FNO$, MW: 241.26

Prepared from 4-fluorophenylhydrazine and p-methoxyacetophenone (42% yield). **^{1}H NMR (DMSO-d_6):** δ 3.81 (3H, s, OMe), 6.75 (1H, s, H-3), 6.89 (1H, dt, *J* 2.5 Hz, 9.0 Hz, H-6), 7.04 (2H, d, *J* 7.5 Hz, H-3',H-5'), 7.24 (1H, dd, *J* 2.5 Hz, 8.0 Hz, H-4), 7.34 (1H, dd, *J* 4.5Hz, 9.0 Hz, H-7), 7.79 (2H, dd, 7.5 Hz, H-2',H-6'), 11,49(1H, s, NH).

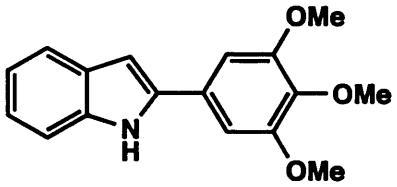
2-(4-Methoxyphenyl)-*1H*-indole (22)



$C_{15}H_{13}NO$, MW: 223.27

Prepared from phenylhydrazine and p-methoxyacetophenone (72% yield). **m.p.** 224-226 °C (**Lit.** 228-230 °C).[16] **^{1}H NMR (DMSO-d_6):** δ 3.81 (3H, s, OMe), 6.76 (1H, s, H-3), 6.97 (1H, dt, *J* 1.0 Hz, 8.0 Hz, Ar-H), 7.04 (2H, d, *J* 8.0 Hz, H-3', H-5'), 7.06 (1H, dd, *J* 1.5 Hz, 7.0 Hz, Ar-H), 7.38 (1H, d, *J* 8.0 Hz, Ar-H), 7.49 (1H, d, *J* 7.5 Hz, Ar-H), 7.80 (2H, d, *J* 8.0 Hz. H-2', H-6'), 11.39 (1H, bs, NH). **^{13}C NMR (DMSO-d_6):** δ 55.18, 97.30, 111.02, 114.32, 114.32, 119.18, 119.61, 120.99, 124.88, 126.33, 126.33, 128.80, 136.90, 137.74, 158.76.

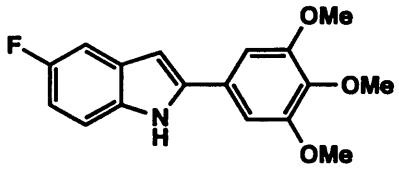
2-(3,4,5-Trimethoxyphenyl)-*1H*-indole (23)



$C_{17}H_{17}NO_3$, MW: 283.32

Prepared from phenylhydrazine and 3,4,5-trimethoxyacetophenone (73% yield). **m.p.** 190-191 °C. **^{1}H NMR (DMSO-d_6):** δ 3.72 (3H, s, OMe), 3.90 (6H, s, 2 x OMe), 6.93 (1H, s, H-3), 7.02 (1H, dt, *J* 1.5 Hz, 8.0 Hz, Ar-H), 7.11 (1H, dt, *J* 1.5 Hz, 8.0 Hz, Ar-H), 7.20 (2H, s, H-2', H-6'), 7.43 (1H, dd, *J* 1.5 Hz, 8.0 Hz, Ar-H), 7.51(1H, d, *J* 2.5 Hz, Ar-H), 11.39 (1H, s, NH). **^{13}C NMR (DMSO-d_6):** δ 56.03, 56.13, 60.09, 98.63, 102.96, 111.06, 119.28, 119.85, 120.12, 121.41, 127.81, 128.64, 136.93, 137.09, 137.79, 153.25, 153.25. **ESI MS** m/z 284.12 [M+H$^+$].

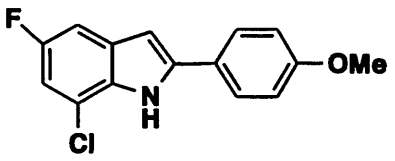
2-(3,4,5-Trimethoxyphenyl)-5-fluoro-*1H*-indole (24)



$C_{17}H_{16}FNO_3$, MW: 301.31

Prepared from 4-fluorophenylhydrazine and 3,4,5-trimethoxyacetophenone (43% yield). **m.p.** 210-211 °C. **^{1}H NMR (DMSO-d_6):** δ 3.70 (3H, s, OMe), 3.88 (6H, s, 2 x OMe), 6.91 (1H, s, 3-H), 6.94 (1H, dt, *J* 2.5 Hz, 9.0 Hz, H-6), 7.17 (2H, s, H-2', H-6'), 7.26 (1H, dd, *J* 2.5 Hz, 10.0 Hz, H-4), 7.38 (1H, dd, *J* 4.5 Hz, 8.5 Hz, H-7), 11.56 (1H, s, NH). **ESI MS** m/z 302.17 [M+H$^+$]. **CHN Anal.** Calcd for C, 67.76; H, 5.35; N, 4.65. Found: C, 67.16; H, 5.29; N, 5.10%.

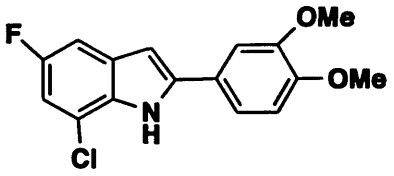
2-(4-Methoxyphenyl)- 7-chloro-5-fluoro-1*H*-indole (25)



$C_{15}H_{11}ClFNO$, MW: 275.71

Prepared from 2-chloro-4-fluorophenylhydrazine and p-methoxyacetophenone (38% yield). **^{1}H NMR (DMSO-d_6):** δ 3.82 (3H, s, OMe), 6.86 (1H, s, H-3), 7.05 (2H, d, *J* 7.0 Hz, H-3', H-5'), 7.10 (1H, dd, *J* 2.0 Hz, 9.0 Hz, H-4), 7.29 (1H, dd, *J* 2.5 Hz, 9.5 Hz, H-6), 7.91 (2H, d, *J* 7.0 Hz, H-2', H-6'), 11.52 (1H, s, NH). **ESI MS** m/z 276.05 [M+H$^+$]. **CHN Anal.** Calcd for C, 65.35; H, 4.02; N, 5.08. Found: C, 64.76; H, 4.11; N, 5.20%.

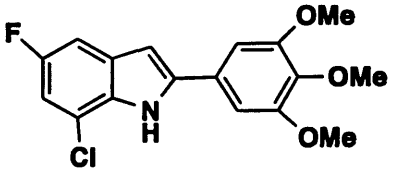
2-(3,4-Dimethoxyphenyl)-7-chloro-5-fluoro-1*H*-indole (26)



$C_{16}H_{13}ClFNO_2$, MW: 305.73

Prepared from 2-chloro-4-fluoro-phenylhydrazine and 3,4-dimethoxyacetophenone (31% yield) **^{1}H NMR (DMSO-d_6):** δ 3.81 (3H, s, OMe), 3.89 (3H, s, OMe), 6.92 (1H, s, H-3), 7.04 (1H, d, *J* 8.5 Hz, H-5'), 7.11 (1H, dd, *J* 2.5 Hz, 9.5 Hz, H-4), 7.29 (1H, dd, *J* 2.5 Hz, 9.5 Hz, H-6), 7.53 (1H, dd, *J* 2.0 Hz, 8.5 Hz, H-6'), 7.57 (1H, d, *J* 2.0 Hz, H-2'), 11.55 (1H, s, NH). **ESI MS** m/z 306.07 [M+H$^+$].

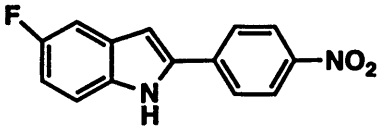
2-(3, 4, 5-Trimethoxyphenyl)-7-chloro-5-fluoro-1*H*-indole (27)



$C_{17}H_{15}ClFNO_3$, MW: 335.76

Prepared from 2-chloro-4-fluoro-phenylhydrazine and 3,4,5-trimethoxyacetophenone (41% yield). **^{1}H NMR (DMSO-d_6):** δ 3.71 (3H, s, OMe), 3.89 (6H, s, 2 x OMe), 7.04 (1H, s, H-3), 7.15 (1H, dd, *J* 2.5 Hz, 10.0 Hz, H-4), 7.29 (2H, s, H-2', H-6'), 7.26 (1H, dd, *J* 2.5 Hz, 10.0 Hz, H-6), 11.56 (1H, s, NH). **ESI MS** m/z 336.07 [M+H$^+$].

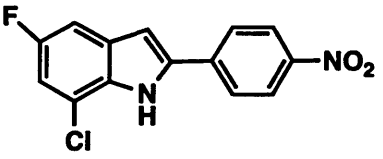
2-(4-Nitrophenyl)-5-fluoro-1*H*-indole (28)



$C_{14}H_9FN_2O_2$, MW: 256.23

Prepared from 2-chloro-4-fluoro-phenylhydrazine and p-nitroacetophenone (22% yield). **^{1}H NMR (DMSO-d_6):** δ 7.08 (1H, dt, *J* 2.5 Hz, 9.0 Hz, H-6), 7.23 (1H, s, H-3), 7.41(1H, dd, *J* 2.5Hz, 10.0 Hz, H-4), 7.49 (1H, dd, *J* 5.0 Hz, 9.0 Hz, H-7), 8.16 (2H, d, *J* 8.0 Hz, H-2', H-6'), 8.37 (2H, d, *J* 8.0 Hz, H-3',H-5'), 11.99 (1H, s, NH).

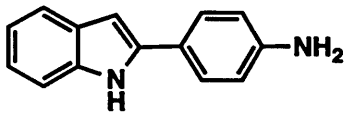
2-(4-Nitrophenyl)- 7-chloro-5-fluoro-1*H*-indole (29)



$C_{14}H_8ClFN_2O_2$, MW: 290.68

Prepared from 2-chloro-4-fluorophenylhydrazine and p-nitroacetophenone (19% yield). **^{1}H NMR (DMSO-d_6):** δ 7.29 (1H, s, H-3), 7.31 (1H, dd, *J* 9.5 Hz, 2.5 Hz, H-4), 7.45 (1H, dd, *J* 2.5 Hz, 9.0 Hz, H-6), 8.31 (2H, d, 8.5 Hz, H-2', H-6'), 8.36 (2H, dd, *J* 2.5 Hz, 8.5 Hz, H-3', H-5'), 11.98 (1H, s, NH).

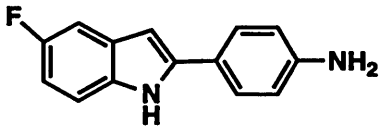
2-(4-Aminophenyl)-1*H*-indole (30)



$C_{14}H_{12}N_2$, MW: 208.26

Prepared from phenylhydrazine and p-aminoacetophenone (24% yield). **^{1}H NMR (DMSO-d_6):** δ 5.27 (2H, s, NH_2), 6.57 (1H, s, H-3), 6.64 (2H, d, *J* 8.5 Hz, H-3', H-5'), 6.93 (1H, dt, *J* 1.5 Hz, 8.0 Hz, H-6), 7.01 (1H, dt, *J* 1.5Hz, 8.5 Hz, Ar-H), 7.31 (1H, d, *J* 8.0 Hz, Ar-H), 7.43 (1H, d, *J* 7.5 Hz, Ar-H), 7.54 (2H, d, *J* 8.5Hz, H-2', H-6'), 11.26 (1H, s, NH). **^{13}C NMR (DMSO-d_6):** δ 95.42, 110.71, 113.94, 113.94, 118.92, 119.10, 120.24, 126.06, 126.06, 129.45, 130.51, 136.63, 139.15, 148.73.

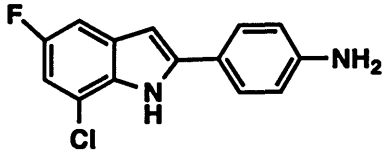
2-(4-Aminophenyl)-5-fluoro-1*H*-indole (31)



$C_{14}H_1FN_2$, MW: 226.25

Prepared from 4-fluoro-phenylhydrazine and p-aminoacetophenone (21% yield). **^{1}H NMR (DMSO-d_6):** δ 5.38 (2H, s, NH_2), 6.57 (1H, s, H-3), 6.64 (2H, d, *J* 7.0 Hz, H-3', H-5'), 6.82 (1H, dt, *J* 2.5 Hz, 10.5Hz, H-6), 7.16 (1H, dd, *J* 2.5 Hz, 10.0 Hz, H-4), 7.28 (2H, dd, *J* 5.0 Hz, 9.0 Hz, H-7), 7.51 (2H, d, *J* 7.0 Hz, H-2', H-6'), 11.27 (1H, s, NH). **ESI MS** m/z 227.10 [M+H$^+$].

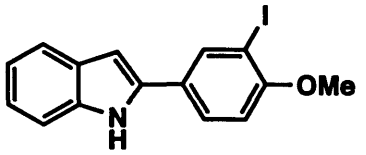
2-(4-Aminophenyl)-7-chloro-5-fluoro-*1H*-indole (32)



$C_{14}H_{10}ClFN_2$, MW: 260.69

Prepared from 2-chloro-4-fluoro-phenylhydrazine and p-aminoacetophenone (24% yield). **^{1}H NMR (DMSO-d_6):** δ 5.38 (2H, s, NH_2), 6.64 (2H, d, *J* 7.0 Hz, H-3', H-5'), 6.68 (1H, s, H-3), 7.02 (1H, dd, *J* 2.5 Hz, 9.5 Hz, H-4), 7.21 (1H, dd, *J* 2.5 Hz, 9.5 Hz, H-6), 7.64 (2H, d, *J* 7.0 Hz, H-2', H-6'), 11.27 (1H, s, NH).

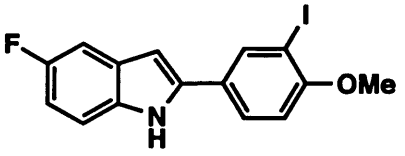
2-(3-Iodo-4-methoxyphenyl)-*1H*-indole (33)



$C_{15}H_{12}INO$, MW: 349.17

Prepared from phenylhydrazine and 3-iodo-4-methoxyacetophenone (44% yield). **^{1}H NMR (DMSO-d_6):** δ 3.88 (3H, s, OMe), 6.82 (1H, s, H-3), 7.00-7.11 (3H, m, Ar-H), 7.37 (1H, d, *J* 8.5 Hz, Ar-H), 7.49 (1H, d, *J* 8.0 Hz, H-5'), 7.87 (1H, dd, *J* 2.5 Hz, 8.0 Hz, H-6'), 8.28 (1H, d, *J* 2.5 Hz, H-2'), 11.46 (1H, s, NH). **^{13}C NMR (DMSO-d_6):** δ 56.52, 86.76, 98.01, 111.12, 111.89, 119.34, 119.80, 121.33, 126.50, 126.91, 128.60, 135.18, 136.03, 137.00, 157.19.

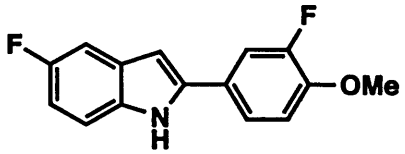
2-(3-Iodo-4-methoxyphenyl)-5-fluoro-1*H*-indole (34)



$C_{15}H_{11}FINO$, MW: 367.16

Prepared from 4-fluoro-phenylhydrazine and 3-iodo-4-methoxyacetophenone (39% yield). **^{1}H NMR (DMSO-d_6):** δ 3.88 (3H, s, OMe), 6.82 (1H, s, H-3), 6.91 (1H, td, *J* 2.5Hz, 9.0 Hz, H-6), 7.11(1H, d, 8.5 Hz, H-5'), 7.25 (1H, dd, *J* 2.5Hz, 9.5 Hz, H-4), 7.35 (1H, dd, *J* 4.5 Hz, 9.0 Hz, H-7), 7.85 (1H, dd, *J* 2.5 Hz, 8.5 Hz, H-6'), 8.28 (1H, d, *J* 2.5 Hz, H-2'), 11.46 (1H, s, NH).

2-(3-Fluoro-4-methoxyphenyl)-5-fluoro-*1H*-indole (35)



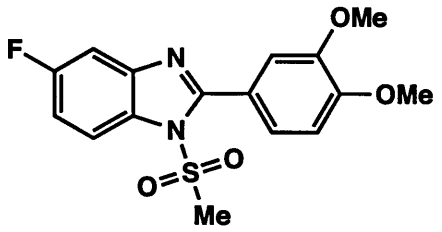
$C_{15}H_{11}F_2NO$, MW: 259.25

Prepared from 4-fluorophenylhydrazine and 3-fluoro-4-methoxyacetophenone (53% yield). **^{1}H NMR (DMSO-d_6):** δ 3.89 (3H, s, OMe), 6.84 (1H, s, H-3), 6.92 (1H, dt, *J* 2.5 Hz, 9.0 Hz, H-6), 7.26 (2H, m, H-Ar), 7.35 (1H, dd, *J* 4.5 Hz, 9.0 Hz, H-7), 7.64 (1H, dd, *J* 2.0 Hz, 8.5 Hz, Ar-H), 7.72 (1H, dd, *J* 2.5 Hz, 8.0 Hz, H-6'), 11.56 (1H, s, NH). **ESI MS** m/z 260.08 [M+H$^+$]. **CHN Anal.** Calcd for C, 69.49; H, 4.28, N, 5.40. Found, C 69.37, H, 4.29; N, 5.51%.

5.2.3. N-substituted benzimidazoles:

General method for the synthesis of N-substituted 2-phenylbenzimidazoles: NaH (53mg, 2.2 mmol) was added to a solution of the 2-phenylbenzimidazole (0.75 mmol) in DMF (10mL) at 0°C under nitrogen gas and stirred for 0.5 hour. Iodomethane or the sulfonyl chloride (1.5mmol) was then added and the solution left to be stirred at room temperature for 12 hours. The reaction mixture was diluted with H_2O (30 mL) and extracted with DCM (3 x 30mL). The organic phases were collected, dried over $MgSO_4$ and concentrated *in vacuo* to give a solid residue, which was purified by column chromatography (70%EtOAc/30% Hexane) to provide the desired N-substituted benzimidazole. The following compounds were prepared using this procedure.

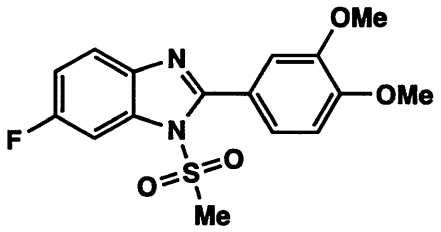
2-(3,4-Dimethoxyphenyl)-5-fluoro-1-methylsulfonylbenzimidazole (37)



$C_{16}H_{15}FN_2O_4S$, MW: 350.36

Prepared from **(4)** and methanesulfonyl chloride (48 % yield), **^{1}H NMR ($CDCl_3$):** δ 2.96 (3H, s, Me), 3.97 (3H, s, OMe), 3.98 (3H, s, OMe), 7.01(1H, d, *J* 8.5 Hz, H-5'), 7.18 (1H, dt, *J* 2.5 Hz, 9.0 Hz, H-6), 7.36 (1H, d, *J* 2.5 Hz, H-2'), 7.42 (1H, dd, *J* 2.0, 8.5 Hz, H-6'), 7.53 (1H, dd, *J* 2.5, 9.5 Hz, H-4), 8.02(1H, dd, *J* 4.5Hz, 9.0 Hz, H-7). **CHN Anal.** Calcd for C 54.85; H, 4.32; N, 7.99. Found: C, 54.52; H, 4.28; N, 8.22%.

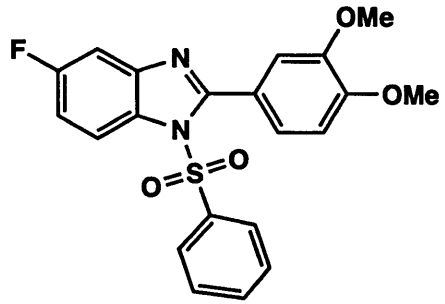
2-(3,4-Dimethoxyphenyl)-6-fluoro-1-methylsulfonylbenzimidazole (38)



$C_{16}H_{15}FN_2O_4S$, MW: 350.36

Prepared from **(4)** and methanesulfonyl chloride (39% yield), **^{1}H NMR ($CDCl_3$):** δ 2.98 (3H, s, Me), δ 3.97 (3H, s, OMe), 3.99 (3H, s, OMe), 6.99 (1H, d, *J* 8.0 Hz, H-5'), 7.21 (1H, dt, *J* 2.5 Hz, 9.0 Hz, H-5), 7.35 (1H, d, *J* 2.0 Hz, H-2'), 7.41 (1H, dd, *J* 2.0, 8.0 Hz, H-6'), 7.78(2H, m, H-4, H-7). Calcd for C 54.85; H, 4.32; N, 7.99. Found: C, 54.32; H, 4.18; N, 8.02 %.

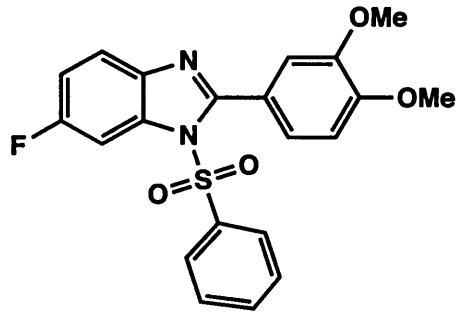
2-(3,4-Dimethoxyphenyl)-5-fluoro-1-phenylsulfonylbenzimidazole (39)



$C_{21}H_{17}FN_2O_4S$, MW: 412.43

Prepared from **(4)** and phenylsulfonyl chloride (35% yield), **^{1}H NMR ($CDCl_3$):** δ 3.88 (3H, s, OMe), 3.98 (3H, s, OMe), 6.94 (1H, d, *J* 8.0 Hz, H-5'), 7.13 (1H, d, *J* 2.0 Hz, H-2'), 7.18 (1H, dt, *J* 9.0 Hz, 2.5 Hz, H-6), 7.22 (1H, dd, *J* 2.0 Hz, 8.5 Hz, H-6'), 7.28-7.53 (5H, m, H-Ar), 8.17 (1H, dd, *J* 9.0, 4.5 Hz, H-7). **CHN Anal.** Calcd for C, 61.16; H, 4.15; N, 6.79. Found: C, 60.95; H, 3.99; N, 6.65%.

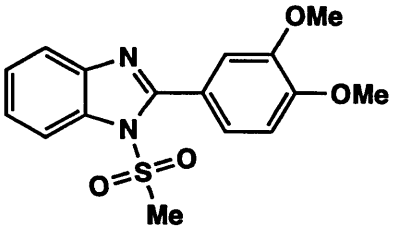
2-(3,4-Dimethoxyphenyl)-6-fluoro-1-phenylsulfonylbenzimidazole (40)



$C_{21}H_{17}FN_2O_4S$, MW: 412.43

Prepared from **(4)** and phenylsulfonyl chloride (39 % yield), **^{1}H NMR ($CDCl_3$):** δ 3.88 (3H, s, OMe), 3.99 (3H, s, OMe), 6.95 (1H, d, *J* 8.5 Hz, H-5'), 7.08 (1H, d, *J* 2.0 Hz, H-2'), 7.15 (1H, dt, *J* 9.0 Hz, 2.5 Hz, H-5), 7.20 (1H, dd, *J* 2.0 Hz, 8.5 Hz, H-6'), 7.32-7.55 (5H, m, H-Ar), 7.65 (1H, dd, *J* 5.0 Hz, 9.0 Hz, H-4), 7.98 (1H, dd, *J* 9.0 Hz, 2.5 Hz, H-7). **CHN Anal.** Calcd for C, 61.16; H, 4.15; N, 6.79. Found: C, 61.10; H, 3.98; N, 6.65%.

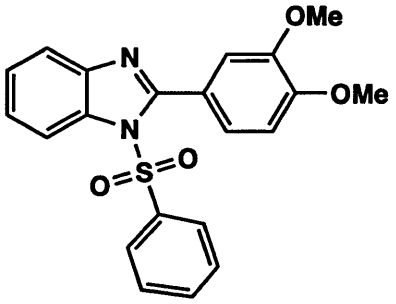
2-(3, 4-Dimethoxyphenyl)-1-methylsulfonylbenzimidazole (41)



$C_{16}H_{16}N_2O_4S$, MW: 332.37

Prepared from **(6)** and methylsulfonyl chloride (60% yield), **^{1}H NMR ($CDCl_3$):** δ 2.97 (3H, s, Me), 3.96 (3H, s, OMe), 3.98 (3H, s, OMe), 7.01 (1H, d, *J* 8.5 Hz, H-5'), 7.34-7.57 (4H, m, H -Ar), 7.83 (1H, dd, *J* 2.0 Hz, 8.5 Hz, H-6'), 8.04 (1H, d, *J* 2.0 Hz, H-2'). **^{13}C NMR ($CDCl_3$):** δ 41.61, 55.98, 56.09, 110.36, 113.94, 114.73, 120.43, 121.63, 124.2 3, 125.55, 125.58, 133.86, 142.47, 148.36, 151.34, 153.70. **CHN Anal.** Calcd for C, 57.82; H, 4.85; N, 8.42. Found: C, 57.70; H, 4.76; N, 8.32%.

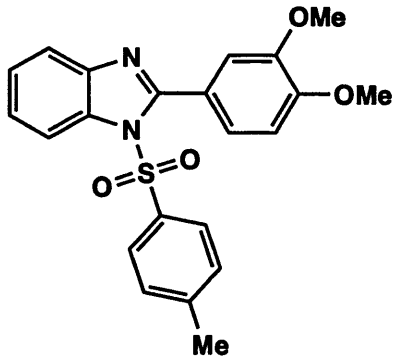
2-(3,4-Dimethoxyphenyl)-N-phenylsulfonylbenzimidazole (42)



$C_{21}H_{18}N_2O_4S$, MW: 394.44

Prepared from **(6)** and phenylsulfonyl chloride (55% yield), **^{1}H NMR ($CDCl_3$):** δ 3.90 (3H, s, OMe), 4.00 (3H, s, OMe), 6.96 (1H, d, *J* 8.0 Hz, H-5'), 7.12 (1H, d, *J* 2.0 Hz, H-2'), 7.25 (1H, dd, *J* 8.5, 2.0 Hz, H-6'), 7.28-7.53 (7H, m, Ar-H), 7.73 (1H, d, *J* 8.0 Hz, Ar-H), 8.24 (1H, d, *J* 8.0 Hz, Ar-H), **^{13}CNMR ($CDCl_3$):** δ 56.01, 56.01, 110.17, 113.95, 115.40, 120.21, 121.99, 124.46, 125.40, 125.42, 126.95, 126.95, 129.00, 129.00, 134.13, 134.27, 138.00, 142.53, 148.11, 151,14, 154.12.

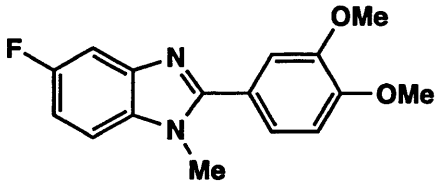
2-(3,4-Dimethoxyphenyl)-1-tosylbenzimidazole (43)



$C_{22}H_{20}N_2O_4S$, MW: 408.47

Prepared from **(6)** and p-toluene sulfonyl chloride (42% yield), **^{1}H NMR (DMSO):** δ 2.30 (3H, s, Me), 3.77 (3H, s, OMe), 3.88 (3H, s, OMe), 7.10 (1H, d, *J* 8.5 Hz, H-5'), 7.19 (2H, m, Ar-H), 7.30 (2H, d, *J* 8.5 Hz, H-3'', H-5''), 7.39 (2H, d, *J* 8.5 Hz, H-2'', H-6''), 7.47 (2H, m, Ar-H), 7.70 (1H, d, *J* 8.0 Hz, H-5'), 8.08 (1H, d, *J* 8.0 Hz, Ar-H). **^{13}H NMR (DMSO):** δ 21.01, 55.58, 55.62, 110.70, 113.93, 114.05, 114.87, 118.91, 119.98, 123.88, 125.37, 125.42, 126.51, 126.51, 130.12, 130.12, 134.80, 135.52, 141.51, 146.02, 153.30, 154.12.

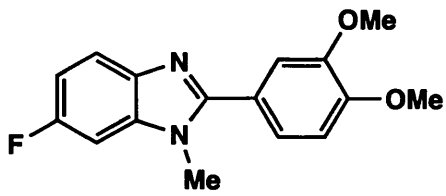
2-(3,4-Dimethoxyphenyl)-5-fluoro-N-methylbenzimidazole (50)



$C_{16}H_{15}FN_2O_2$, MW: 286.30

Prepared from **(4)** and iodomethane (67% yield), **^{1}H NMR ($CDCl_3$):** δ 3.72 (3H, s, Me), 3.85 (3H, s, OMe), 3.88 (3H, s, OMe), 6.92 (2H, m, Ar-H), 7.13 (2H, m, Ar-H), 7.28 (1H, d, *J* 2.0 Hz, H-2'), 7.40 (1H, dd, *J* 9.5 Hz, 2.5 Hz, H-4). **CHN Anal.** Calcd for C, 67.12; H, 5.28; N, 9.78. Found: C, 66.93; H, 5.36; N, 9.72%.

2-(3,4-Dimethoxyphenyl)-6-fluoro-1-methylbenzimidazole (51)



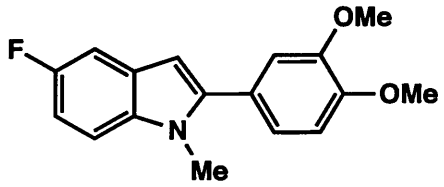
$C_{16}H_{15}FN_2O_2$, MW: 286.30

Prepared from **(4)** and iodomethane (54% yield), **^{1}H NMR ($CDCl_3$):** δ 3.78 (3H, s, Me), 3.82 (3H, s, OMe), 3.89 (3H, s, OMe), 6.92 (1H, d, *J* 8.5 Hz, H-5'), 7.02 (2H, m, Ar-H), 7.18 (1H, dd, *J* 8.5 Hz, 2.0 Hz, H-6'), 7.29 (1H, d, *J* 2.0 Hz, H-2'), 7.68 (1H, dd, *J* 2.5 Hz, 9.5 Hz, H-7). **CHN Anal.** Calcd for C, 67.12; H, 5.28; N, 9.78. Found: C, 66.99; H, 5.26; N, 9.80%.

5.2.4. N-substituted phenyl indoles:

Compounds **(52)** and **(53)** were prepared using the procedure described above for the synthesis of N-substituted benzimidazoles.

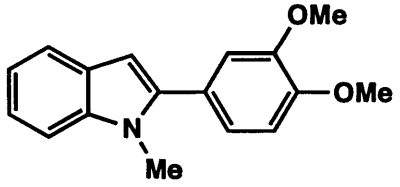
2-(3, 4-Dimethoxyphenyl)- 5-fluoro-1-methyl-indole (52)



$C_{17}H_{16}FNO_2$, MW: 285.31

Prepared from **(18)** and iodomethane (51% yield), **^{1}H NMR (DMSO-d_6):** δ 3.74 (3H, s, NMe), 3.80 (3H, s, OMe), 3.85 (3H, s, OMe), 6.52 (1H, s, H-3), 7.01 (1H, dt, *J* 2.5 Hz, 9.5 Hz, H-6), 7.12 (3H, m, H-2', H-5', H-6'), 7.30 (1H, dd, *J* 2.5 Hz, 10.0 Hz, H-4), 7.49 (1H, dd, *J* 4.5 Hz, 9.0 Hz, H-7). **CHN Anal.** Calcd for C, 71.56; H, 5.65; N, 4.91. Found: C, 71.94; H, 5.87; N, 4.62%.

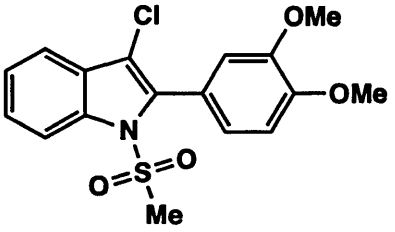
2-(3,4-Dimethoxyphenyl)-1-methylindole (53)



$C_{17}H_{17}NO_2$, MW: 267.32

Prepared from **(20)** and iodomethane (66% yield), **^{1}H NMR (DMSO-d$_6$):** δ 3.73 (3H, s, NMe), 3.80 (3H, s, OMe), 3.85 (3H, s, OMe), 6.55 (1H, s, H-3), 7.01-7.22 (5H, m, Ar-H), 7.46 (1H, d, *J* 2.0 Hz, H-2'), 7.58 (1H, d, 8.5 Hz, H-5'). **^{13}C NMR (DMSO-*d*$_6$):** δ 31.11, 56.02, 56.08, 101.14, 109.53, 111.17, 112.83, 119.85, 120.31, 121.52, 121.97, 1 25.58, 127.97, 138.19, 141.52, 149.05. 150.03.

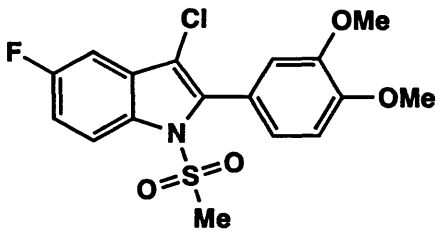
2-(3, 4-Dimethoxyphenyl)-3-chloro-1-methylsulfonyl-indole (54)



$C_{17}H_{16}ClNO_4S$, MW: 365.83

From **(20)** and methylsulfonyl chloride as a side product (28% yield). **^{1}H NMR (CDCl$_3$):** δ 2.85 (3H, s, Me), 3.93 (3H, s, OMe), 3.98 (3H, s, OMe), 7.00 (1H, d, *J* 8.0 Hz, H- 5'), 7.10 (1H, d, *J* 2.0 Hz, H-2'), 7.14 (1H, dd, *J* 8.0, 2.0 Hz, H-6'), 7.49-7.52 (2H, m, H-Ar), 8.22 (1H, d, *J* 7.5 Hz, 2.0 Hz, Ar-H), 8.32 (1H, d, *J* 8.0, 2.0 Hz, Ar-H). **^{13}C NMR (DMSO-*d*$_6$):** δ 42.19, 55.81, 56.21, 107.11, 110.12, 114.63, 114.84, 116.18, 119.06, 124. 61, 126.54, 131.50, 136.18, 145.00, 149.91,151.80, 157.65.

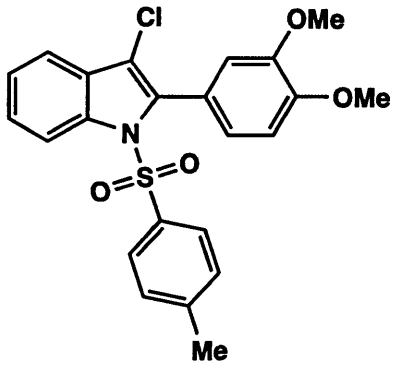
2-(3, 4-Dimethoxyphenyl)-3-chloro-5-fluoro-1-methylsulfonyl-indole (55)



$C_{17}H_{15}ClFNO_4S$, MW: 383.82.

From **(20)** and methylsulfonyl chloride as a side product (50% yield), **^{1}H NMR (DMSO-d_6):** δ 3.03 (3H, s, Me), 3.74 (3H, s, OMe), 3.84 (3H, s, OMe), 7.05 (1H, dd, *J* 2.5 Hz, 8.5 Hz, H-6'), 7.14 (1H, m, Ar-H), 7.23 (1H, m, Ar-H), 7.42 (1H, m, Ar-H), 7.83 (1H, m, Ar-H), 8.12(1H, dd, *J* 4.5 Hz, 9.0 Hz, H-7).

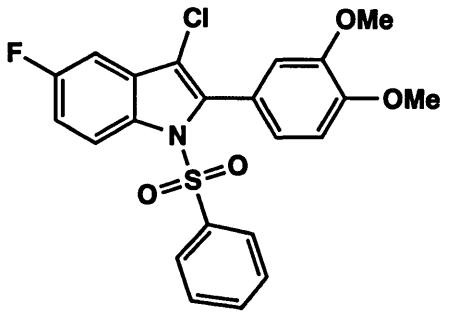
2-(4,5-Dimethoxyphenyl)-3-chloro-1-methylsulfonylindole (56)



$C_{23}H_{20}ClNO_4S$, MW: 441.9

From **(18)** and p-toluenesulfonyl chloride as a side product (57% yield), **^{1}H NMR ($CDCl_3$):** δ 2.46 (3H, s, Me), 3.92 (3H, s, OMe), 3.99 (3H, s, OMe), 6.99 (1H, d, *J* 2.0 Hz, H-2'), 6.98 (1H, d, *J* 8.0 Hz, H-5'), 7.04 (1H, dd, *J* 8.0, 2.0 Hz, H-6'), 7.09 (2H, d, *J* 8.0 Hz, H-3", H-5"), 7.30 (2H, d, *J* 8.0 Hz, H-2", H-6"), 7.41-7.47 (2H, m, Ar-H), 7.53 (1H, d, *J* 8.0 Hz, Ar-H), 8.38 (1H, d, *J* 8.5 Hz, Ar-H). **^{13}C NMR ($CDCl_3$):** δ 21.59, 55.87, 55.96, 105.03, 109.99, 114.85, 116.51, 118.61, 121.38, 124.40, 124.68, 126.02, 126.96, 126.96, 128.53, 129.35, 129.35, 134.71, 135.83, 136.23, 144.94, 147.93, 149.81.

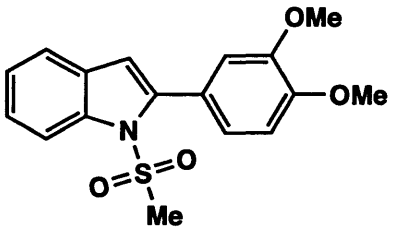
2-(3, 4-Dimethoxyphenyl)- 3-chloro-5-fluoro-1-phenylsulfonyl-indole (57)



$C_{22}H_{17}ClFNO_4S$, MW: 445.89

Prepared from **(20)** and phenylsulfonyl chloride as a side product (55% yield), **^{1}H NMR (DMSO-d_6):** δ 3.76 (3H, s, OMe), 3.87(3H, s, OMe), 6.95 (1H, d, *J* 2.5 Hz, H-2'), 7.01 (1H, dd, *J* 8.0 Hz, 2.0 Hz, H-6'), 7.10 (1H, d, *J* 8.0 Hz, H-5'), 7.35-7.65 (7H, m, H-Ar), 8.24 (1H, dd, *J* 9.0 Hz, 4.5 Hz, H-7).

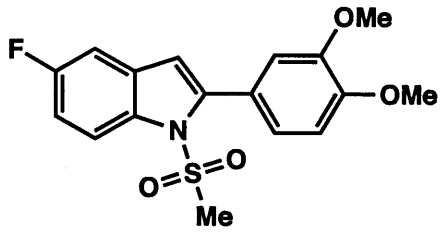
2-(3, 4-Dimethoxyphenyl)-1-methylsulfonylindole (58)



$C_{17}H_{17}NO_4S$, MW: 331.39

Prepared from **(18)** and methylsulfonyl chloride (52% yield) **^{1}H NMR ($CDCl_3$):** δ 2.75 (3H, s, Me), 3.92 (3H, s, OMe), 3.96 (3H, s, OMe), 6.71 (1H, s, H-3), 6.94 (1H, d, *J* 8.0 Hz, H-5'), 7.28 (2H, m, Ar-H), 7.39 (2H, m, Ar-H), 7.60 (1H, dd, *J* 8.0 Hz, 2.0 Hz, H-6'), 8.16 (1H, d, *J* 8.0 Hz, Ar-H). **^{13}C NMR ($CDCl_3$):** δ 39.31, 55.87, 56.02, 110.29, 112.59, 114.08, 115.97, 120.83, 122.49, 124.54, 124.94, 129.68, 131.96, 138.03, 141.96, 148.11, 149.79.

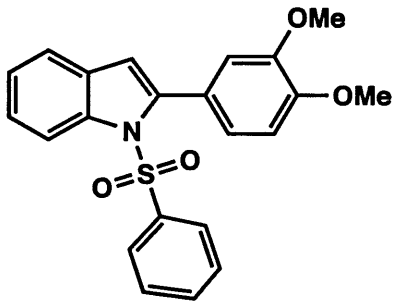
2-(3,4-Dimethoxyphenyl)- 5-fluoro-1-methylsulfonyl-indole (59)



$C_{17}H_{16}FNO_4S$, MW: 349.38

Prepared from **(20)** and methylsulfonyl chloride (59% yield), **^{1}H NMR (DMSO-d_6):** δ 3.08 (3H, s, Me), 3.78 (3H, s, OMe), 3.82 (3H, s, OMe), 6.82 (1H, s, H-3), 7.02 (dd, *J* 2.5 Hz, 8.0 Hz, H-6'), 7.11-7.26 (3H, m, Ar-H), 7.46 (1H, d, *J* 8.5 Hz, H-5'), 7.97 (1H, dd, *J* 4.5 Hz, 9.0 Hz, H-7).

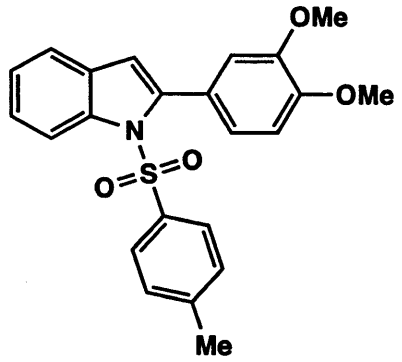
2-(3, 4-Dimethoxyphenyl)-1-phenylsulfonylindole (60)



$C_{22}H_{19}NO_4S$, MW: 393.46

Prepared from **(18)** and phenylsulfonyl chloride (47% yield), **^{1}H NMR ($CDCl_3$):** δ 3.91 (3H, s, OMe), 3.97 (3H, s, OMe), 6.53 (1H, s, H-3), 6.92 (1H, d, *J* 8.5 Hz, H-5'), 7.01-7.03 (2H, m, Ar-H),7.22-7.46 (8H,m, Ar-H), 8.35 (1H, d, *J* 8.5 Hz, Ar-H).**^{13}CNMR ($CDCl_3$):** δ 55.92, 55.99, 110.17, 113,03, 114.26, 116.68, 120.98, 122.87,124,39,124.72, 124.75, 126.78, 126.78, 128.98, 128.98, 130.51, 133.48, 137.76, 138.29, 142.00, 147.91, 149.65.

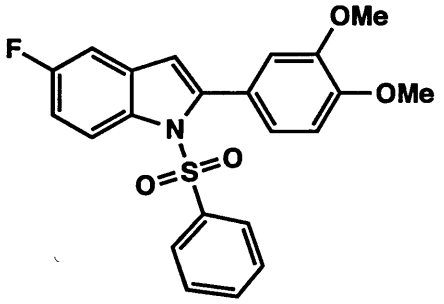
2-(3,4-Dimethoxyphenyl)-1-tosylindole (61)



$C_{23}H_{21}NO_4S$, MW: 407.48

Prepared from **(18)** and p-toluenesulfonyl chloride (52% yield), **^{1}H NMR (DMSO-d_6):** δ 2.26 (3H, s, Me), 3.79 (3H, s, OMe), 3.84 (3H, s, OMe), 6.74 (1H, s, H-3), 7.04-7.10 (3H, m, Ar-H), 7.22-7.38 (6H, m, Ar-H), 7.50 (1H, d, *J* 8.0 Hz, Ar-H), 8.15(1H, d, *J* 8.5 Hz, H-Ar). **^{13}C NMR (DMSO-d_6):** δ 20.92, 55.54, 55.54, 110.85, 113.24, 114.01, 116.08, 120.81, 122.54, 124.29, 124.53, 124.68, 126.26, 126.26, 129.60, 129.60, 130.36, 133.60, 137.50, 141.86, 144.97, 147.62, 149.32.

2-(3, 4-Dimethoxyphenyl)- 5-fluoro-1-phenylsulfonylindole (62)



$C_{22}H_{18}FNO_4S$, MW: 411.45

Prepared from **(20)** and phenylsulfonyl chloride (40% yield), **^{1}H NMR (DMSO-d_6):** δ 3.79 (3H, s, OMe), 3.85(3H, s, OMe), 6.75 (1H, s, H-3), 7.04-7.05 (3H, m, Ar-H), 7.22 (1H, dt, *J* 2.5 Hz, 9.5 Hz, H-6), 7.32-7.49 (6H, m, Ar-H), 8.14 (1H, dd, *J* 9.0, 4.5 Hz, H-7). **CHN Anal.** Calcd for C, 64.22; H, 4.41; N, 3.40. Found: C, 63.91; H, 4.51; N, 3.37%.

References

1. Navarrete-Vázquez, G.; Moreno-Diaz, H.; Aguirre-Crespo, F.; León-Rivera, I.; Villalobos-Molina, R.; Muñoz-Muñiz, O.; Estrada-Soto, S. Design, microwave-assisted synthesis and spasmolytic activity of 2-(alkyloxyaryl)-1H-benzimidazole derivatives as constrained stilbene bioisosteres. *Bioorg. Med. Chem. Lett.* **2006**, 16, 4169-4173.
2. Navarrete-Vazquez, G.; Moreno-Diaz, H.; Estrada-Soto, S.; Mariana, T. P.; León-Rivera, I.; Tlahuext, H.; Muñoz-Muñiz, O.; Torres-Gómez, H. Microwave assisted one-pot synthesis of 2-(substituted phenyl)-1H-benzimidazole derivatives. *Synth. Comm.* **2007**. 48, 2815-2825.
3. Chakrabarty, M.; Karmakar, S.; Mukherji, A.; Arima, S.; Harigaya, Y. Application of sulfamic acid as an eco-friendly catalyst in an expedient synthesis of benzimidazoles. *Heterocycles.* **2006**, 68, 967-974.
4. Das, B.; Kanth, B. S.; Reddy, K. R.; Kumar, A. S. Sulfonic acid functionalized silica as an efficient heterogeneous recyclable catalyst for one-pot synthesis of 2-substituted benzimidazoles. *J. Heter. Chem.* **2009,** 1499-1502.
5. Chakrabarty, M.; Mukherji, A.; Mukherjee, R.; Arima, S.; Harigaya, Y. A Keggin heteropoly acid as an efficient catalyst for an expeditious, one-pot synthesis of 1-methyl-2-(hetero)arylbenzimidazoles. *Tetrahedron. Lett.* **2007,** 30-5239-5242.
6. Pagliai, F.; Pirali, T.; Del Grosso, E.; Di Brisco, R.; Tron, G. C.; Sorba; G.; Genazzani, A. A. Rapid synthesis of triazole-modified resveratrol analogues via click chemistry. *J. Med. Chem,* **2006**, 49, 467-470.
7. Roth, G.; Liepold, G.; Müller, S.; Bestmann, H. J. Further improvements of the synthesis of alkynes from aldehydes. *Synthesis*, **2004,** 59-62.
8. Emmanuvel, L.; Shukla, R. K.; Sudalai, A.; Gurunath, S.; Sivaram, S. NaIO4/KI/NaCl: a new reagent system for iodination of activated aromatics through in situ generation of iodine monochloride. *Tetrahedron. Lett.* **2006**, 47, 4793-4796.

9. Sakamoto, T.; Kondo, Y.; Iwashita, S.; Nagano, T.; Yamanaka, H. Condensed heteroaromatic ring systems. XIII. One-step synthesis of 2-substituted 1-methylsulfonylindoles from N-(2-Halophenyl)methanesulfonamides. *Chem. Pharm. Bull.* **1988**, *36*, 1305-1308.
10. Palimkar, S, S.; Kumar, H. P.; Lahoti, R. J.; Srinivasan, K. V. Ligand-, copper-, and amine-free one-pot synthesis of 2-substituted indoles via Sonogashira coupling 5-endo-dig cyclisation. *Tetrahedron* **2006**, 62, 5109-5115
11. Cacchi, S.; Fabrizi, G.; Parisi, L. M. 2-Aryl and 2-heteroaryl indoles from 1-alkynes and o-iodotrifluoroacetanilide through a domino copper-catalyzed. coupling-cyclization process. *Org. Lett.* **2003**, 5, 3843-3846.
12. Bates, C. G.; Saejueng, P.; Murphy, J. M.; Venkataraman, D. Synthesis of 2-arylbenzo[b]furans via copper(I)-catalyzed coupling of o-iodophenols and aryl acetylenes. *Org. Lett.* **2002**, *4*, 4727-4729.
13. Nazare, M.; Schneider, S.; Lindenschmidt, A.; Will, D. W. A flexible, palladium-catalyzed indole and azaindole synthesis by direct annulation of chloroanilines and chloroaminopyridines with ketones. *Angew. Chem. Int. Ed.* **2004**, 43, 4526-4528.
14. Bruce, J. M. Heterocyclic compounds of nitrogen. Part 111. The synthesis of some 2-indolylbenzoquinones. *J. Chem. Soc.* **1960**, 360-365.
15. Robinson, B. The Fischer indole synthesis. *Chem. Rev.* **1963**, 63, 373-401.
16. Le Corre, M.; Hercouet, A.; Le Baron, H. New synthesis of indoles from o-acylaminobenzyltriphenylphosphonium salts. *J. Chem. Soc, Chem. Commun.* **1981**, 1, 14-15.

Part 2

The design and synthesis of novel small molecule inhibitors of the *Wnt signalling pathway* for use as selective antitumour agents

6. INTRODUCTION

6.1. Protein-protein interactions as a target in cancer drug discovery

The last decade was the genomics era with massive quantities of data generated in this field, especially through the successful decoding of the human genome.[1] However, this scientific endeavour has recently shifted toward the field of proteomics where the main focus is changed from looking for which genes encode which proteins into how proteins interact with each other. Due to their vast structural diversity, proteins often undergo a complex network of interactions to exert several effects including the fine control of protein localisation, substrate-processing activity and their tagging for destruction and recycling. Moreover, these protein-protein interactions play a major role in the well-tuned signal transduction between cells caused by neighbouring environmental stimuli leading to the appropriate set of responses.

There is currently growing interest in targeting the different protein-protein interactions for therapeutic purposes. Because of their desired pharmaceutical properties in terms of convenience of administration through the oral route, it is ideally hoped to be able to have small drug-like molecules to target specific protein-protein interactions. However, this task is very challenging since the interface area between the interacting proteins is often very large (1,500-3,000Å^2) compared to typically less than a third of that in the case of well-studied protein-small molecule interactions (300-1000 Å^2).[2,3] Moreover, these surfaces of interactions are generally flat lacking grooves or pockets where small molecules usually bind.[4] However, there is much evidence indicating that only a small subset of the interface residues contribute most of the free energy of binding leading to what are termed as "hot spots". [5,6] These hot spots are usually found at the centre of contact interfaces and they constitute less than half of the interaction interface. Therefore, efforts can be directed into designing inhibitory molecules that target these relatively small areas but this in itself poses another challenge, which lies in the ability to identify these hot spots.

Hanahan and Weinberg have defined and described six hallmarks (aquired capabalities) for most cancers which have been descrbied in detail previously (Chapter 1).[7] It is

believed that cancer cells have the ability to develop their own growth signalling pathways and evade inhibitory signals and apoptosis. They also acquire the ability of unlimited replicative potential, angiogenesis and metastasis. Table 1 lists some potential protein-protein targets for each of these hallmarks which can be explored as potential target pathways for developing and designing novel treatments.[7, 8]

6.1.1. The human protein double minute (HDM2) binders: *an example of successful targeting of protein-protein interactions*

Despite the fact that targeting protein-protein interactions is undoubtedly a very challenging task, several small-molecule inhibitors with significant potency to disrupt such interactions have been identified. One of the most interesting and best-characterised examples in the area of oncology is the successful targeting of the p53-HDM2 interactions although an inhibitor in this area has yet to reach clinical trials.[9]

The tumour suppressor protein p53 otherwise known as the guardian of the genome plays an essential role in the DNA-damage response. It is estimated that more than 50% of cancers have a malfunctioning p53.[10] One of the key functions of p53 is inducing apoptosis (programmed cell death) as a result of genomic stress and the complete mechanism involved is still being investigated. Under normal conditions, cellular levels of p53 are very low due to p53-HDM2 protein-protein interactions whereby HDM2 catalyses the binding of ubiquitination to p53 resulting in its proteosomal degradation. Therefore, it follows that the protection of p53 from this proteosomal degradation process by disrupting the p53-HDM2 interactions leads to an increased p53 activity, which in

Acquired capability	Example of mechanism	Protein–protein target
Self-sufficiency in growth signals	Overexpression of growth-factor receptors (e.g. Her2/*neu*)	ESX–Sur-2, Bcl-2 family
Insensitivity to anti-growth signals	Overexpression of *c-myc*, inactivation of retinoblastoma protein	β-catenin–T cell factor
Evading programmed cell death (apoptosis)	Inactivation of p53, overexpression of anti-apoptotic proteins	Bcl-2 family, MDM2–p53, XIAP–caspase
Limitless replicative potential	Activation of telomerase	-
Sustained angiogenesis	Induction of vascular endothelial growth factor	-
Tissue invasion and metastasis	Alteration of cell adhesion and motility	Rac–Tiam1

Table 1: Hallmarks of cancer and protein-protein targets (adapted from reference 8).

turn is associated with an antitumour activity. Investigation of the p53-HDM2 interface revealed that only three amino acid residues of p53 (Leu26, Trp23 and Phe19) contribute most of this interaction in the hydrophobic binding interface (hot-spot area of 300 A^{o2}).[11] In a search for a small molecule inhibitor of the p53-HDM2, high-throughput screening (HTS) led to the discovery of a series of tetra-substituted dihydroimidazoles which become known as Nutlins (research was carried out in Nutley-New Jersey, Hoffmann-la Roche laboratories). Structural optimisation of these molecules led to the identification of an even more potent inhibitor NUTLIN 3 (Figure 1) which disrupts the p53-MDM2 (the mouse homologue of HDM2) complexes with an IC_{50} of 90 nM.[12] It was also found that Nutlin-3 has a potent p53-blocking activity *in vitro* against human tumour xenografts *in vivo*.[12]

In a competing project at Johnson & Johnson, a series of benzodiazepinediones were identified as HDM-2 binders as a result of parallel screening of a library of 338,000 compounds using thermostability assays.[13] Further structural optimisation studies on this compound series led to the identification of a benzodiazepinedione (Figure 1) with high binding affinity to HDM-2 (K_d = 67 nM, IC_{50} = 420 nM).[14] In cells overproducing HDM2, these benzodiazepinediones were found to block p53-MDM2 complexes and promote their rapid dissociation.[15]

Most intriguing was the finding that the discovered small-molecule HDM2 binders (Nutlins and benzodiazepinediones) with remarkable differences in their structural scaffolds have a similar binding mode. X-ray crystallography studies showed that Nutlins and benzodiazepinediones disrupt the HDM2-p53 interactions by mimicking the key p53 amino residues in the interaction hot spot.[12,13] These compounds bind to the same binding region of p53 in the HDM2 and their aromatic or aliphatic moieties extend into HDM2 pockets which bind the key residues of p53 (Leu26, Trp23 and Phe19).

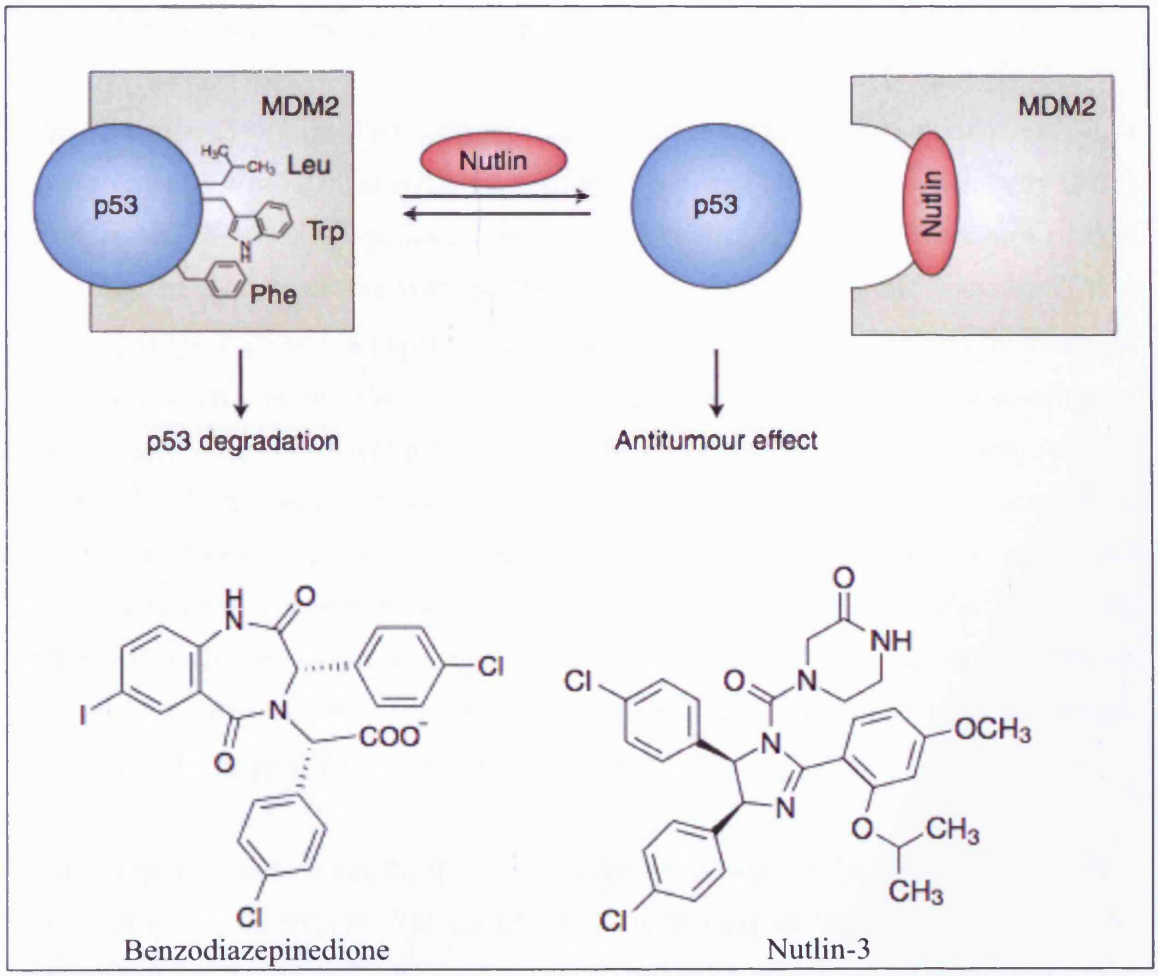


Figure 1. An illustrative diagram of p53-MDM2 protein-protein interaction and its disruption by a Nutlin inhibitor (adapted from reference 9). The chemical structures of Nutlin-3 and one of the benzodiazepinedione compound series are also shown.

6.2. The Wnt signalling pathway

The current understanding of the Wnt-dependent signaling pathways is mainly derived from several studies using various model organisms including, *Xenopus*, *Drosophila melanogaster*, *Caenorhabditis elegans* and mammals. These studies have established that Wnt-dependent signaling pathways consist of a complex network of proteins, which play

a major role in controlling many events during embryonic development and regulating cell proliferation, morphology, motility as well as cell fate.[16]

The Wnt-dependent signaling pathways consist of three different molecular pathways downstream of Wnt/Fz interaction; the canonical pathway, the planar cell polarity (PCP) pathway and the Wnt/Ca^{2+} pathway. Most of the published research in the area of Wnt signaling has focused on the Wnt/β-catenin pathway referred to as the "canonical" Wnt pathway (Figure 2), which plays a key role in regulating cell proliferation by means of gene expression control. The other two pathways, the Wnt/Ca^{2+} and Wnt/polarity are known as the "non-canonical pathways" and their components have only been recently identified.[16, 17] It is believed that the Wnt/Ca^{2+} signalling leads to transient increases in cytoplasmic free calcium which results in the activation of protein kinase C (PKC) and Ca^{2+} -calmodulin dependent protein kinase II.[18] On the other hand, the Planar Cell Polarity signalling regulates remodeling of the cytoskeleton and changes in cell adhesion and motility. This effect is mediated via the activation of the small GTPases RHOA (RAS homologue gene-family member A) and RAC1.[19]

The discrimination between the three Wnt pathways is made at the receptor level. While the common initial step in Wnt signalling is the binding of Wnt ligand to the seven-transmembrane Frizzled (Fz) receptor, signal transduction downstream thereafter differ in each of these three pathways depending mostly on which Wnt ligand and Fz receptor are activated. Wnt/Fz mediated interactions are associated with a wide range of responses as 19 different human Wnt factors and 10 different transmembrane receptors of the Frizzled receptor family have been identified so far.[16]

The Wnt/β-catenin pathway, which controls the cytosolic concentration of the oncoprotein β-catenin has been extensively studied in various vertebrate and invertebrate model systems. The activation of this canonical pathway and signal transduction require the hetero-dimerisation of Fz and the co-receptor LRP5 or LRP 6 (low-density lipoprotein receptor related protein).[20] A detailed representation of the several events, which take place during Wnt/β-catenin pathway activation is represented in Figure 2.

In the absence of Wnt ligand, APC, Axin and GSK-3 normally function as part of a β-catenin turnover complex, which targets β-catenin for ubiquitin-mediated proteosomal degradation. It follows that β-catenin is captured by APC and Axin within this destruction complex leading to the phosphorylation of a set of conserved serine and threonine residues sequentially in the amino terminus by the kinases CK1-α and GSK-3β.[21] The phosphorylated β-catenin binds to a component of an E3 ubiquitin ligase complex; β-TRCP (β-Transducin Repeat Containing Protein) leading to its ubiquitinylation and subsequently is directed into the proteosomal degradation machinery. As a consequence, cytosolic β-catenin levels are maintained at low levels allowing the TCF/LeF (T-cell factor/ Lymphoid enhancer) proteins to interact with other co-repressors and bind to Wnt target genes to block their transcription.

On the other hand, the binding of a Wnt ligand to a Fz receptor and its LRP 5 or LRP 6 c-receptor, induces the formation of Fz-Dvl complex which facilitates the relocation of axin to the cell membrane causing the inactivation and dissociation of β-catenin turnover complex. The unphosphorylated β-catenin escapes ubiquitinylation leading to its cytosolic accumulation and subsequent entry to the nucleus. Once in the nucleus, β-catenin activates Wnt target gene transcription by associating with nuclear TCF/LeF transcription factors. It is worthy to note that many of the transcriptional targets of the Wnt pathway (e.g. c-myc, cyclin D1 and survivin) are also oncogenes and may in part explain the tumour-promoting consequences of mutations to Wnt signalling components.

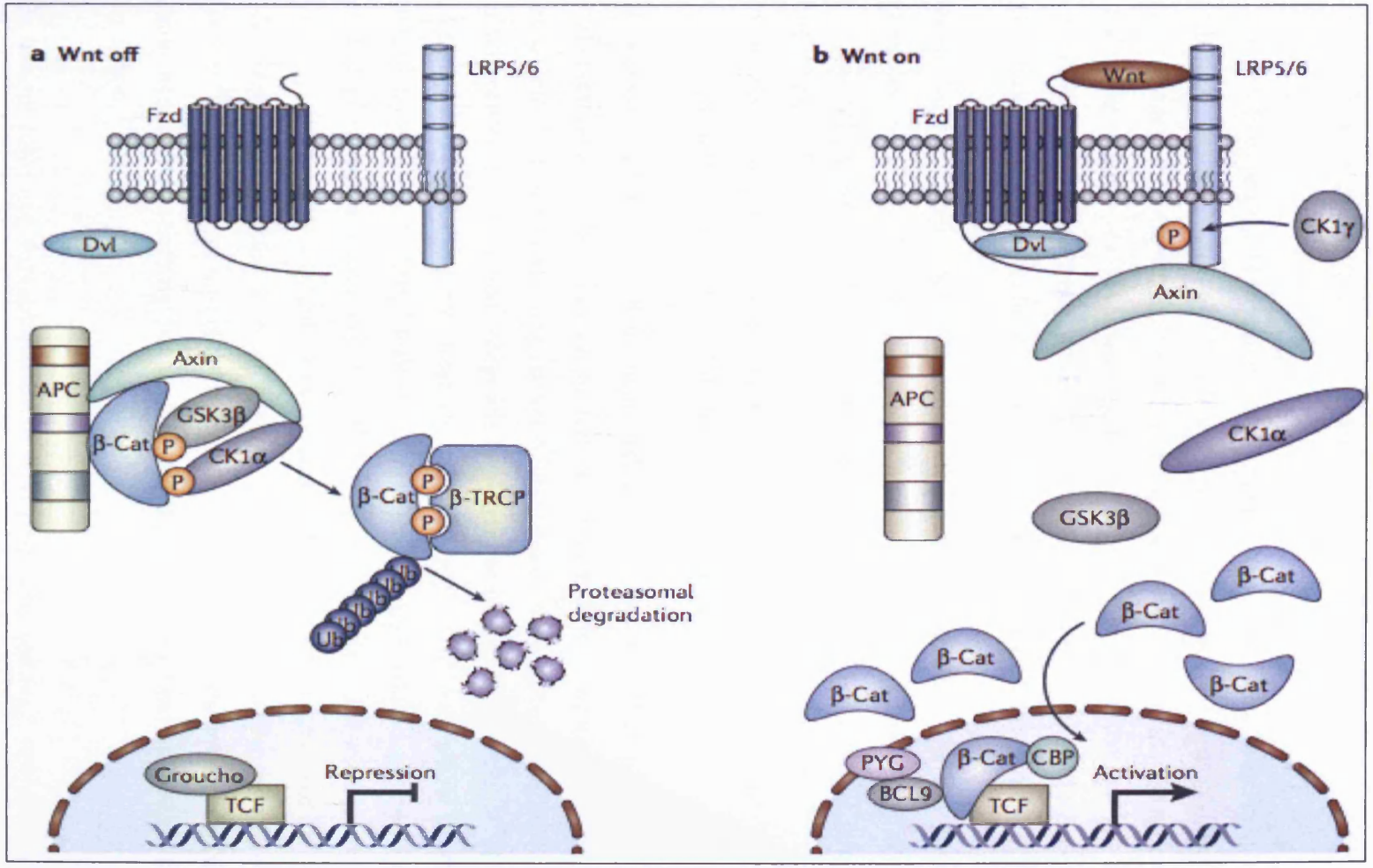


Figure 2: An overview of the Wnt signaling pathway (reproduced from reference 8). In the absence of active Wnt (left), β-catenin is degraded and Tcf/Lef transcription factors act as repressor. On the other hand, when a Wnt signal is present (right), β-catenin accumulates in the cytoplasm, enters the nucleus, and activates transcription together with Tcf/Lef transcription factors.

6.3. Wnt signaling pathway and cancer

The link between the Wnt pathway and cancer dates back to the initial discovery of this signalling pathway when the first Wnt (Wnt-1) was found as a protooncogene activated by the integration of the mouse mammary tumour virus in murine breast cancer.[22] The following studies in this area and the subsequent identification of downstream components of this pathway have all come to confirm and provide further evidence for the role of Wnt signalling in oncogenesis and cancer development (Table 2).[7, 16,17]

Multiple mechanisms are involved in the aberrant activation of the Wnt signalling pathway in cancer (Figure 3). Oncogenic mutations to the intracellular Wnt signalling components, β-catenin, APC and Axin are found in a large number of human tumours including colon cancers, hepatocellular carcinomas and melanomas.[16,17] Oncogenic mutations that prevent β-catenin turnover lead to the activation of TCF-dependent transcription in the absence of Wnt ligands.

Alternative mechanisms that inappropriately activate the Wnt pathway include the overexpression of Wnt ligands or loss of soluble Wnt antagonists.[23] The creation of autocrine Wnt-secretion / Wnt response loops have been shown to stimulate tumour cell proliferation and to prevent apoptosis in a number of human cancers.[24-27] Reduction of Wnt-induced signalling by treatment of cells with Wnt antibodies or soluble Wnt inhibitory proteins (SFRP) blocked cell proliferation and induced apoptosis in head and neck and colorectal cancer cell lines.[28] Additional yet-to-be identified mechanisms have been hypothesised to account for the presence of stabilised β-catenin and the activation of TCF-dependent transcription in several tumour types including breast and prostate cancer.[29,30] In several tissues Wnt ligands maintain the undifferentiated state of stem cells, raising the possibility that deregulated signalling may reactivate stem cell pathways in cancer.[29,30]

In tumour cell lines, the replacement of wild type APC and Axin reduced levels of TCF-dependent transcription and reversed a range of oncogenic readouts leading to increased

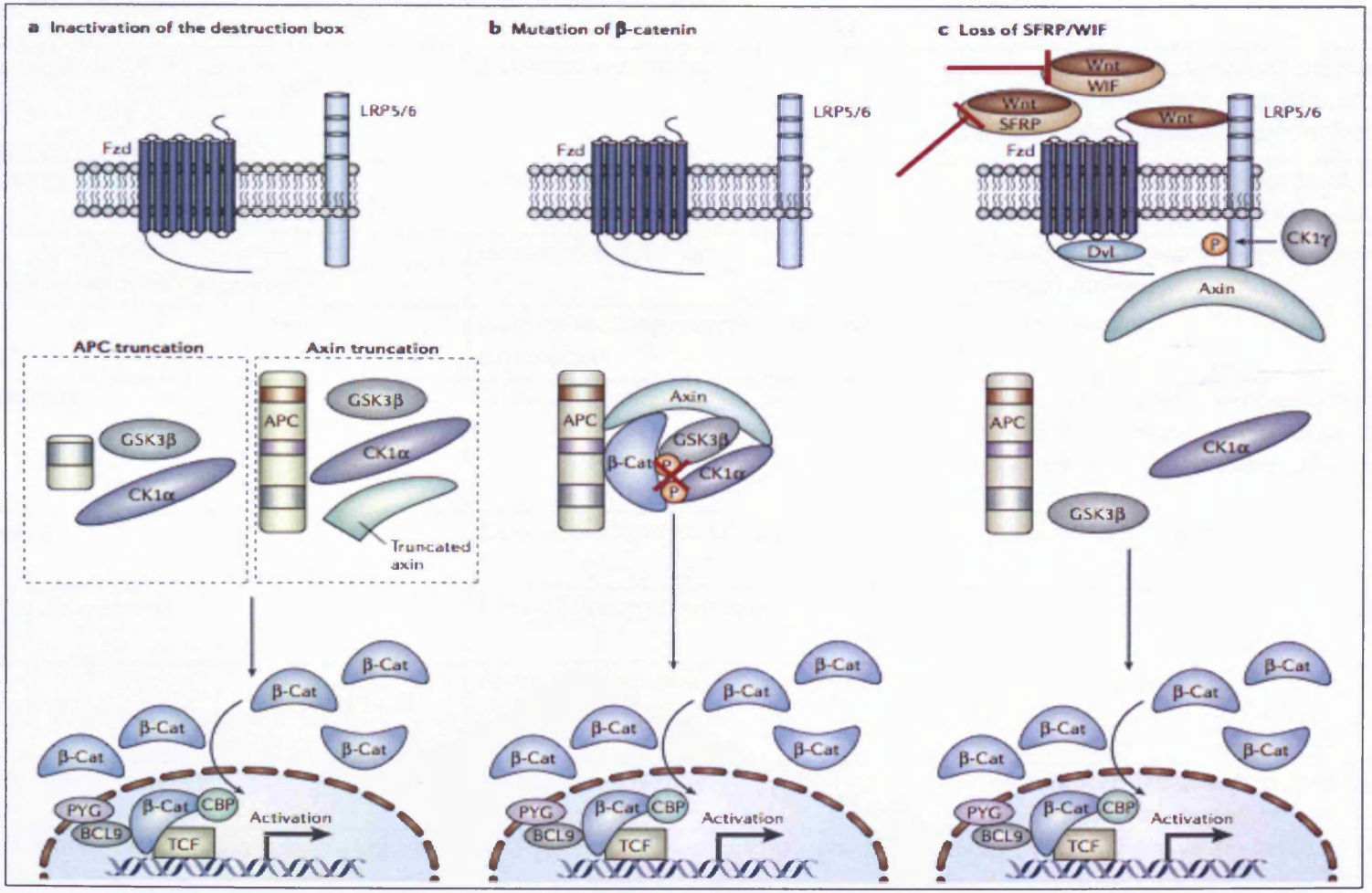


Figure 3: An illustrative depiction of some routes to aberrant activation in Wnt signaling in cancer cells (reproduced from reference 7).

Pathway component	Observed alterations	Disease
Wnt ligands	Increased expression	Colon cancer; breast cancer; melanoma; head & neck cancer, non-small-cell lung cancer; gastric cancer; mesothelioma
Frizzled receptors	Increased expression	Colon cancer; breast cancer; head & neck cancer; gastric cancer; synovial sarcomas
Dishevelled family members	Increased expression	Mesothelioma; non-small-cell lung cancer; cervical cancer
APC	Loss-of-function mutations/reduced expression	Colon cancer
β-catenin	Gain-of-function mutations	Colon cancer; gastric cancer; hepatocellular cancer, hepatoblastoma; Wilm's tumour; endometrial ovarian cancer; adrenocortical tumours
Axin 1	Loss-of-function mutations	Hepatocellular cancer
Axin 2/Conductin	Loss-of-function mutations	Colon cancer; hepatocellular cancer
SFRP family members	Reduced expression	Colon cancer; breast cance; gastric cancer; mesothelioma; non-small-cell lung cancer; Barrett's oesophagus; leukaemia
WIF family members	Reduced expression	Colon cancer; breast cancer; prostate cancer; lung cancer; bladder cancer

Table 2: The different cancers linked to the aberrant activation of Wnt signalling pathway (adapted from reference 8).

apoptosis.[31] In addition, expression of dominant negative forms of TCF in colon cancer cells blocked cell proliferation and induced enterocytic differentiation, regardless of the presence of additional (e.g. p53) mutations.[31] These studies support the idea that tumours require ongoing TCF-dependent transcription and identify Wnt/TCF-dependent signalling as a molecular therapeutic target.

6.4. The Frizzled-Dishevelled (Fz-Dvl) protein-protein interaction as a target for small synthetic anti-cancer molecules.

The aberrant activation of the Wnt signalling pathways have been implicated in different types of cancer as well as several other diseases. Therefore, many components of this signaling pathway have attracted interest as exciting targets for drug discovery research efforts from both academia and industry. Frizzled-Dishevelled (Fz-Dvl) interactions play a central role in relaying the Wnt signal to downstream components. Therefore, disrupting this protein-protein interaction could block signal transduction at the Dvl level and interfere with Wnt signaling events that contribute to disease states.

Dvl proteins (~ 700 amino acid residues) are essential components in Wnt signalling pathways and play a key role in tumourgenesis as described above.[32] There are three homologues, Dvl 1, Dvl 2 and Dvl 3, which were identified in mammalians. These Dvl proteins are highly conserved throughout the animal kingdom. The Dvl protein consists of three domains; N-terminal DIX domain, a central PDZ (Post-synaptic density-95/Discs large/Zonula occludens-1) domain and a C-terminal DEP domain. Protein-protein interactions involving Dvl protein are mediated by the PDZ domain, which therefore plays a major role in the Wnt signalling pathway. Studies have established that the Dvl PDZ domain directly interacts with a conserved sequence (KTXXXW) of Fz, which begins two amino acids after the seventh transmembrane domain.[33]

Compounds that target the Dvl PDZ domain would be expected to act only against tumours that are dependent on Wnt ligands or Dvl overexpression. However, autocrine Wnt signalling was shown to be required for cell proliferation in colorectal cancer cells with mutant β-catenin and APC.[24,34,35] This suggests that constitutive Wnt signalling may

be required to complement APC / β-catenin mutations during the progression of colorectal cancers and that the targeting of receptor-proximal signalling may have therapeutic potential in many genetic backgrounds. Taken together, the available evidence suggests that targeting the Dvl PDZ domain in cancer cells will suppress their growth and trigger apoptosis through up-regulation of caspase activity.

Indeed, Fujii and co-workers targeted this Dvl-Fz protein-protein interaction and they were able to show that an indole-2-carbinol based compound (FJ9, Figure 4) displaced the Fz C-terminus from the Dvl PDZ domain, resulting in the induction of apoptosis in tumour cell lines and inhibition of tumour growth in xenograft models.[36] Further studies by the same group indicate that the indole-2-amide scaffold bearing a small hydrophobic group on the 2-amide (Figure 4) can be used to antagonize the Dvl PDZ domain, down-regulating Tcf activation of transcription and inducing caspase activation and subsequent apoptosis.[37] Notably caspase activation was not observed in *hTERT*-immortalised normal human foreskin fibroblasts, suggesting that down-regulation of Tcf-induced transcription may induce apoptosis selectively in cancer cells.[37,38]

Using receptor-based virtual screening of the NCI (National Cancer Institute) library, Shan and co-workers identified a Dvl PDZ domain peptidomimetic inhibitor (NSC668036, Figure 4). NSC668036 was found to act as a Wnt antagonist with a binding affinity of 237 μM to inhibit signalling induced by Wnt3A in Xenopus.[39,40,41] The same group has also recently reported a new small-molecule inhibitor (3289-8625, Figure 4) of Dvl PDZ with a low micromolar affinity (K_d = 10.6 μM) using the same approach (structure-based virtual screening) but extending it to include the Chemical Diversity Inc. (ChemDiv, San Diego, CA) library.[42] This compound was shown to effectively block Wnt signalling in both in vitro and in vivo using a Xenopus testing system and prostate cancer PC-3 cells.[42]

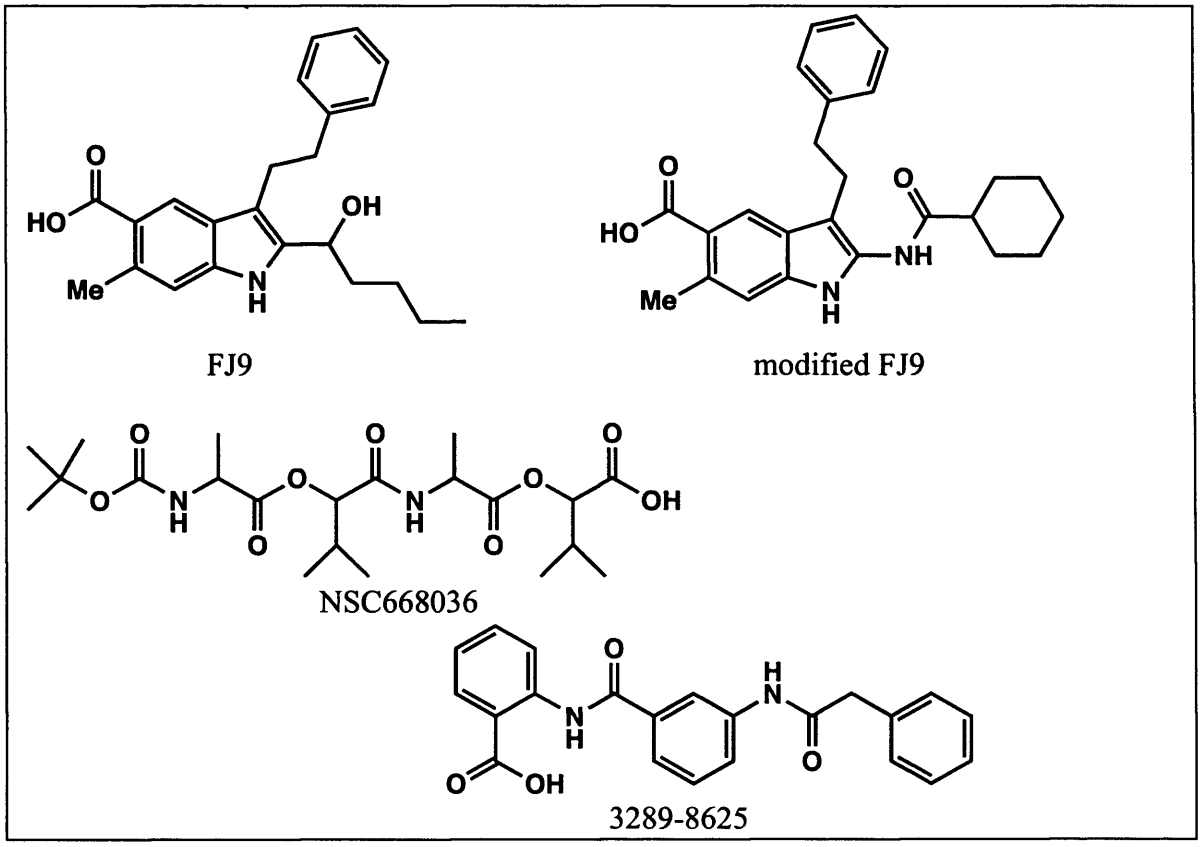


Figure 4: Chemical structures of small-molecule inhibitors of the Fz-Dvl protein-protein interactions.

6.5. Aims and objectives

Recent progress and success in the development of inhibitors of protein-protein interactions has opened the door and paved the way for developing drugs with novel and selective mechanisms. Our understanding of how the deregulation of Wnt signaling activity occurs has made major advances in the past few years providing a solid platform from which promising drug discovery and development programs targeting this pathway in cancers can be launched.

As described above, the PDZ domain of the intracellular Dvl PDZ protein binds directly to the transmembrane Fz protein to relay Wnt signals to downstream components of the beta-catenin degradation complex. Therefore, it is anticipated that an inhibitor of the Dvl PDZ domain would effectively block oncogenic Wnt signalling as exemplified by the

initial success of the identified inhibitors (Figure 4). Moreover, both NSC668036 and FJ9 provide a structural basis for the rational design of high-affinity inhibitors of the Dvl- Fz interactions.

The aim of this project is to combine the robust molecular modelling tools and protein-protein interaction assays for the identification of novel small molecule inhibitors of the Dvl PDZ domain. The work would be based on exploiting the available crystal structure of the Dvl PDZ domain to rationalize the inhibitory activity of the reported Dvl-Fz7 interaction inhibitors and closely related anlogues.[2,3] Having established the main molecular contacts and key residues mediating Dvl-Fz7 interaction, virtual compound library screening combined with structure-activity studies will be performed to successfully select promising candidate molecules. Biochemical binding assays and mechanistic studies could then determine the ability of the identified molecules to alter cell proliferation or induce apoptosis by blocking the Wnt signalling in cancer cells.

References

1. International Human Genome Sequencing Consortium. Finishing the euchromatic sequence of the human genome. *Nature* **2004,** 431, 931–45.
2. Jones, S.; Thornton, J. M. Principles of protein–protein interactions. *Proc. Natl Acad. Sci.* **1996,** 93, 13–20.
3. Cheng, A. C.; Coleman, R. G.; Smyth, K. T.; Cao, Q.; Soulard, P.; Caffrey, D. R.; Salzberg, A. C. ; Huang, E. S. Structure-based maximal affinity model predicts small-molecule druggability. *Nat. Biotechnol.***2007,** 25, 71–75.
4. Hopkins, A. L.; Groom, C. R. The druggable genome. *Nature Rev. Drug. Discov.* **2002,** 1, 727–730.
5. Clackson, T.; Wells, J. A. A hot spot of binding energy in a hormone-receptor interface. *Science* **1995,** 267, 383–386.
6. Moreira, I. S.; Fernandes, P. A.; Ramos, M. J. Hot spots: a review of the protein–protein interface determinant amino-acid residues. *Proteins.* **2007,** 68, 803–812.
7. Hanahan, D.; Weinberg, R. A. The hallmarks of cancer. *Cell,* **2000,** 100, 57–70.
8. Clevers, H.; Barker, N. Mining the Wnt pathway for cancer therapeutics. *Nat. Rev. Drug. Discov.* **2006,** 5, 997–1014.
9. White, A. W.; Westwell, A. D.; Brahemi, G. Protein-protein interactions as targets for small-molecule therapeutics in cancer. *Exp. Rev. Mol. Med.* **2008,** 10, 1-14.
10. Levin, A. J. p53, the cellular gatekeeper for growth and division. *Cell.* **1997,** 88, 323-331.
11. Kussie, P. H.; Gorina, S.; Marechal, V.; Elenbaas, B.; Moreau, J.; Levine, A. J.; Pavletich, N. P. Crystal structure of the MDM2 oncoprotein bound to thetransactivation domain of the p53 tumor suppressor. Science. **1996,** 274, 948-953.
12. Vassilev, L. T.; et al. In vivo activation of the p53 pathway by small-molecule antagonists of MDM2. *Science.* **2004,** 303, 844–848.

13. Grasberger, B. L. et al. Discovery and cocrystal structure of benzodiazepinedione HDM2 antagonists that activate p53 in cells. *J. Med. Chem.* **2005,** 48, 909–912.
14. Parks, D. J. et al. 1,4-Benzodiazepine-2,5-diones as small molecule antagonists of the HDM2–p53 interaction: discovery and SAR. Bioorg. Med. Chem. Lett. **2005**, 15, 765–770.
15. Koblish, H. K. et al. Benzodiazepinedione inhibitors of the Hdm2:p53 complex suppress human tumor cell proliferation in vitro and sensitize tumors to doxorubicin in vivo. Mol. Cancer Ther. **2006**, 5, 160–169.
16. Janssens, N.; Janicot, M.; Perera, T. The Wnt-dependent signaling pathways as target in oncology drug discovery. *Invest. New. Drugs.* **2006,** 24, 263–280.
17. Dev, K. K. Making protein interactions druggable: targeting PDZ domains. *Nat. Rev. Drug Discov.* **2004,** 1047-1056.
18. Kuhl, M.; Sheldahl, L.C.; Malbon, C. C.; Moon, R. T. Ca (2+)/calmodulin-dependent protein kinase II is stimulated by Wnt and Frizzled homologs and promotes ventral cell fates in Xenopus. *J Biol Chem.* **2000**, 275:12701–12711.
19. Habas, R.; Dawid, I. B.; He, X. Coactivation of Rac and Rho by Wnt/Frizzled signaling is required for vertebrate gastrulation. *Genes Dev.* **2003**,17:295–309.
20. Wehrli, M.; Dougan, S. T.; Caldwell, K.; O'Keefe, L.; Schwartz, S.; Vaizel-Ohayon, D.; Schejter, E.; Tomlinson, A.; DiNardo, S. Arrow encodes an LDL-receptor-related protein essential for Wingless signalling. *Nature.* **2000**, 407:527–530.
21. Ding, Y.; Dale, T. Wnt signal transduction: kinase cogs in a nano- machine? *Trends Biochem Sci* . **2002**, 27:327–329.
22. Nusse, R.; Varmus, H.E. Many tumors induced by the mouse mammary tumor virus contain a provirus integrated in the same region of the host genome. *Cell.* **1982**, 31, 99-10.
23. Mazieres, J.; He, B.; You, L.; Xu, Z.; Jablons, D.M. Wnt signaling in lung cancer. *Cancer letters. 2005,* 222, 1-10.
24. Bafico, A.; Liu, G.; Goldin, L.; Harris, V.; Aaronson, S. A. An autocrine mechanism for constitutive Wnt pathway activation in human cancer cells. *Cancer. Cell,* **2004,** 6, 497-506.

25. Mazieres, J.; You, L.; He, B.; Xu, Z.; Lee, A.Y.; Mikami, I.; McCormick, F.; Jablons, D. M. Inhibition of Wnt16 in human acute lymphoblastoid leukemia cells containing the t(1;19) translocation induces apoptosis. *Oncogene* **2005**, 24, 5396-5400.

26. You, L.; He, B.; Uematsu, K.; Xu, Z.; Mazieres, J.; Lee, A.; McCormick, F.; Jablons, D. M. Inhibition of Wnt-1 signaling induces apoptosis in beta-catenin-deficient mesothelioma cells. *Cancer research* **2004,** *64*, 3474-3478.

27. You, L.; He, B.; Xu, Z.; Uematsu, K.; Mazieres, J.; Mikami, I.; Reguart, N.; Moody, T. W.; Kitajewski, J.; McCormick, F. *et al.* Inhibition of Wnt-2-mediated signaling induces programmed cell death in non-small-cell lung cancer cells. *Oncogene* **2004,** 23, 6170-6174.

28. Rhee, C. S.; Sen, M.; Lu, D.; Wu, C.; Leoni, L.; Rubin, J.; Corr, M.; Carson, D. A. Wnt and frizzled receptors as potential targets for immunotherapy in head and neck squamous cell carcinomas. *Oncogene* **2002,** 21, 6598-6605.

29. Sato, N.; Meijer, L.; Skaltsounis, L.; Greengard, P.; Brivanlou, A. H. Maintenance of pluripotency in human and mouse embryonic stem cells through activation of Wnt signaling by a pharmacological GSK-3-specific inhibitor. *Nat Med.* ***2004,*** 10, 55-63.

30. Willert, K.; Brown, J. D.; Danenberg, E.; Duncan, A. W.; Weissman, I. L.; Reya, T.; Yates, J. R.; Nusse, R. Wnt proteins are lipid-modified and can act as stem cell growth factors. *Nature* **2003,** *423*, 409-414.

31. van de Wetering, M.; Sancho, E.; Verweij, C.; de Lau, W.; Oving, I.; Hurlstone, A.; van der Horn, K.; Batlle, E.; Coudreuse, D.; Haramis, A.P. *et al.* The beta-Catenin/TCF-4 complex imposes a crypt progenitor phenotype on colorectal cancer cells. *Cell* **2002** 111, 241-250.

32. Malbon, C. C.; Wang, H. Y. Dishevelled: A mobile scaffold catalyzing development. Curr. *Top. Dev. Biol.* **2006,** 72, 153-166.

33. Wong, H-C.; Bourdelas, A.; Krauss, A. Lee, H. J.; Shao, Y.; Mlodzik, M.; Shi, D. L.; Zheng, J. Direct binding of the PDZ domain of Dishevelled to a conserved internal sequence in the C-terminal region of Frizzled. *Mol. Cell.* **2003**, 12, 1251-1260.

34. He, B.; Reguart, N.; You, L.; Mazieres, J.; Xu, Z.; Lee, A. Y.; Mikami, I.; McCormick, F.; Jablons, D. M. Blockade of Wnt-1 signaling induces apoptosis in human colorectal cancer cells containing downstream mutations. *Oncogene.* ***2005,*** 24, 3054-3058.

35. Suzuki, H *et al.* Epigenetic inactivation of SFRP genes allows constitutive WNT signaling in colorectal cancer. *Nat Genet.* **2004,** 36, 417-422.

36. Fujii, N.; You, L.; Xu, Z.; Uematsu, K.; Shan, J.; He, B.; Mikami, I.; Edmondson, L. R.; Neale, G.; Zheng, J.; Guy, R.K.; Jablons, D.M. An antagonist of dishevelled protein-protein interaction suppresses β-catenin-dependent tumor cell growth. *Cancer Res.* **2007,** 67, 573-579.

37. Mahindroo, N.; Punchihewa, C.; Bail, A. M.; Fujii, N. Indole-2-amide based biochemical antagonist of Dishevelled PDZ domain interaction down-regulates Dishevelled-driven Tcf transcriptional activity. *Bioorg. Med. Chem. Lett.* **2008,** 18, 946-949.

38. You, L.; Xu, Z.; Punchihewa, C.; Jablons, D. M.; Fujii, N. Evaluation of a chemical library of small-molecule Dishevelled antagonists that suppress tumor growth by down-regulating Tcf-mediated transcription. *Mol. Cancer. Ther.* **2008.** 1633-1638.

39. Shan, J.; Shi, D. L.; Wang, J.; Zheng, J. Identification of a specific inhibitor of the Dishevelled PDZ domain. *Biochem.* **2005,** 44, 15495-15503.

40. Shan, J.; Zheng, J. J. Optimizing Dvl PDZ domain inhibitor by exploring chemical space. *J. Comput. Aided. Mol. Des.* **2009,** 23: 37-47.

41. Wang, N. X.; Lee, H. J.; Zheng, J. J. Therapeutic use of PDZ protein-protein interaction antagonism. *Drug. News. Perspect.* **2008,** 21, 137-142.

42. Grandy, D.; Shan, J.; Zhang, X.; Rao, S.; Akunuru, S.; Li, H.; Zheng, Y.; Alpatov, I.; Zhang, X. A.; Lang, R.; Shi, D. L.; Zheng, J. J. Discovery and characterization of a small molecule inhibitor of the PDZ domain of Dishevelled. *J. Biol. Chem.* **2009,** 284, 16256-16263.

7. The design of Dvl-Fz protein-protein interaction small molecule inhibitors

7.1. Computer-assisted drug design studies

Molecular modelling can be defined as the science or art of representing molecular structures numerically using computational chemistry programs through the application of quantum and classical physics.[1] These programs enable the simulation of the behaviour, geometrics, energy, bulk, electronic, spectroscopic and the other properties of molecules.[1] Nevertheless, experimental data as well as the chemist's expertise and intuition are essential in guiding the molecular modelling work and interpreting the obtained data. The field of study, which is concerned with the application and use of these molecular modelling programs in research and drug development has become known as computer assisted drug design (CADD).

Over the past few years, there have been massive advances in the development of tools and products dedicated to computer assisted drug design. Of such computational products, the programs which were mainly used in this project include; Molecular Operating Environment (MOE, 2008.10),[2] SYBYL 7.0 (Tripos SYBYL, 7.0)[3] and LigandScout (Inte:Ligand).[4] These modelling programs allow the use of several approaches for the design of novel small molecule inhibitors of the disheveled PDZ domain.

7.1.1. The use of structure-based virtual screening

Virtual screening has experienced a growing interest in its application in the drug discovery process over the recent years. It is considered as a complementary approach to high throughput screening (HTS) which when combined with robust structural biology techniques, has the potential to enhance the drug discovery process. This computational methodology encompasses the rapid *in silico* assessment and evaluation of large virtual libraries of chemical structures in order to identify those potential structures with high affinity for binding to a given target.[5] There are two main categories of this technique namely ligand-based and structure-based virtual screening.

A. Ligand-based virtual screening:

Ligand-based virtual screening focuses on building a pharmacophore model based on a set of structurally diverse ligands which are proven to bind to a certain receptor or target. Then, candidate ligands can be screened against this generated pharmacophore model in order to determine their compatibility and hence their potential binding.[6]

B. Structure-based virtual screening:

Structure-based virtual screening estimates the affinity of binding of candidate compounds to a protein target by docking these compounds into a pharmacophore model, which is derived from the available target structure.[7]

Fortuitously, the crystal structure of the Dvl PDZ domain bound to its ligand peptide Dapper is available in the Protein Data Bank (PDB: 1L6O). [8, 9, 10] This makes structure-based virtual ligand screening the more appropriate choice to identify potential ligands with the ability to inhibit the Fz-Dvl protein-protein interactions. Moreover, this approach was successfully implemented by Shan et al in the identification of a specific PDZ domain antagonist despite its low binding affinity (Ki= 237 μM). [11]

7.1.1.1. Structure-based virtual screening of potential Dvl PDZ domain ligands

Generally speaking, a structure-based virtual screening process consists of several steps as illustrated in Figure 1. The success of the process depends on the careful and thorough implementation of various computational techniques in each step.

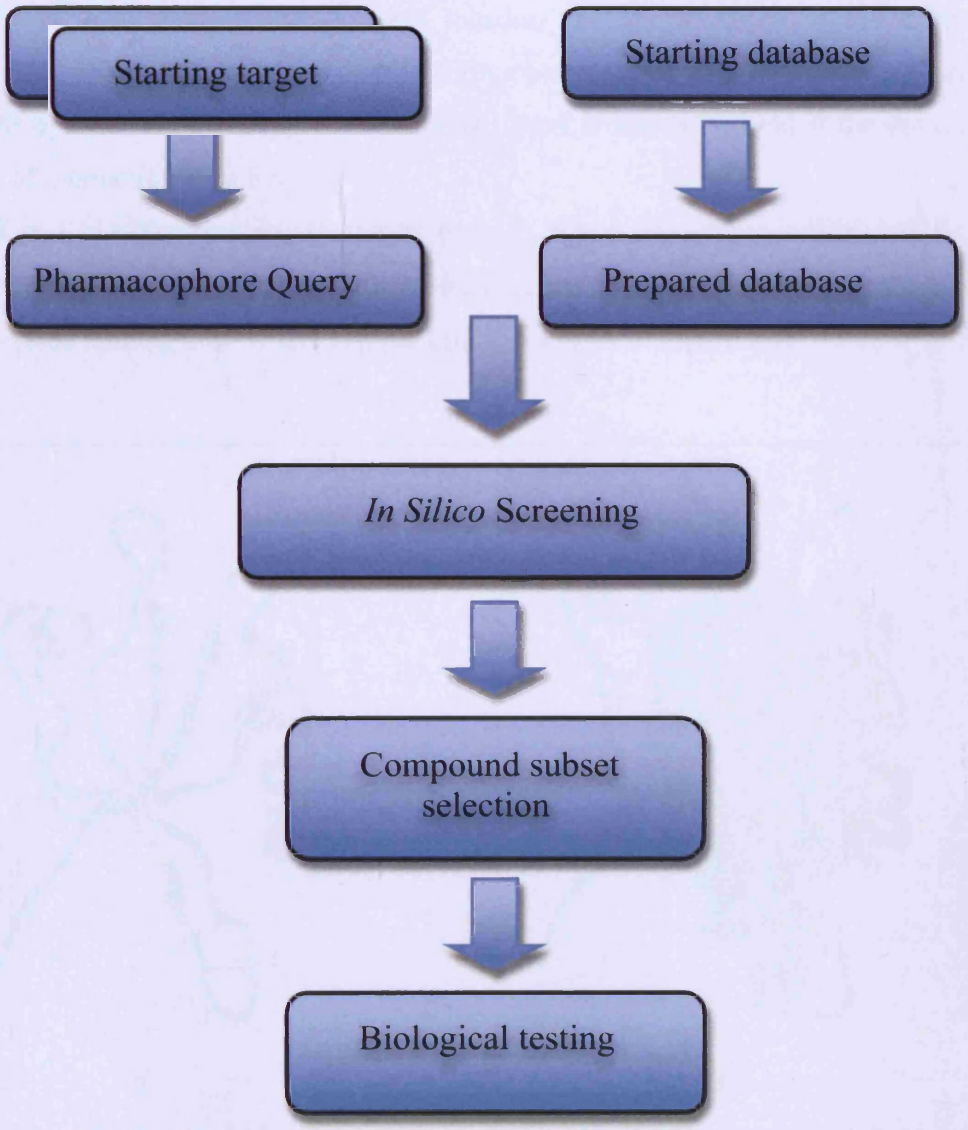


Figure 1: A description of the workflow of the different steps involved in structure-based virtual screening process (modified from reference 5)

A. Database and target preparation:

A.1. Target preparation: The crystal structure of Dvl PDZ bound to its natural peptide (8 amino acid residues) ligand dapper was retrieved as PDB file from the protein data bank (PDB entry 1L6O). [9,10] The active site, which is the set of residues that mediate the binding interactions with Dapper was identified using "Site Finder" function in MOE.

Site function is a feature, which allows location and identification of the different hydrophobic cavities in a target, which can form a potential binding site. This was further confirmed by the fact that Dapper as a natural ligand is known to bind at the same Dvl PDZ site of interaction with Fz. [10]

Dvl PDZ is a modular protein interaction domain, which has two α-helices and six β-sheets (Figure 2-A). One of the α helices and β-sheets together with the preceding loop form a peptide binding cleft where Dapper binds as shown in Figure 2-B

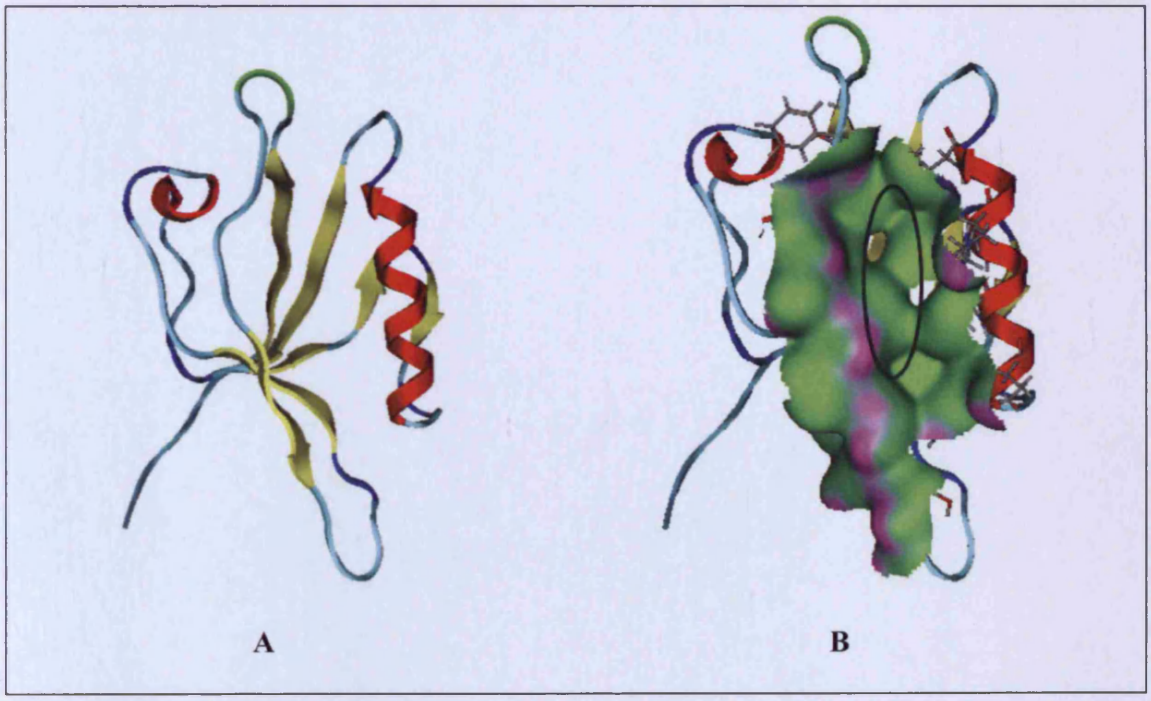


Figure 2: A: Ribbon diagram of the PDZ domain structure (α-helices are shown in red while β-sheets are shown in yellow). **B:** The binding site (active site) of the Fz7 peptide represented using MOE "Site finder" feature.

A.2. Pharmacophore query design: A pharmacophore is the collection or set of steric and electronic features of specific functional groups and their spatial placement, which are required to elicit a specific biological response. [12]

The main interactions between Dvl PDZ and Dapper (Figure 3) were analyzed in terms of the main residues contributing to these interactions.

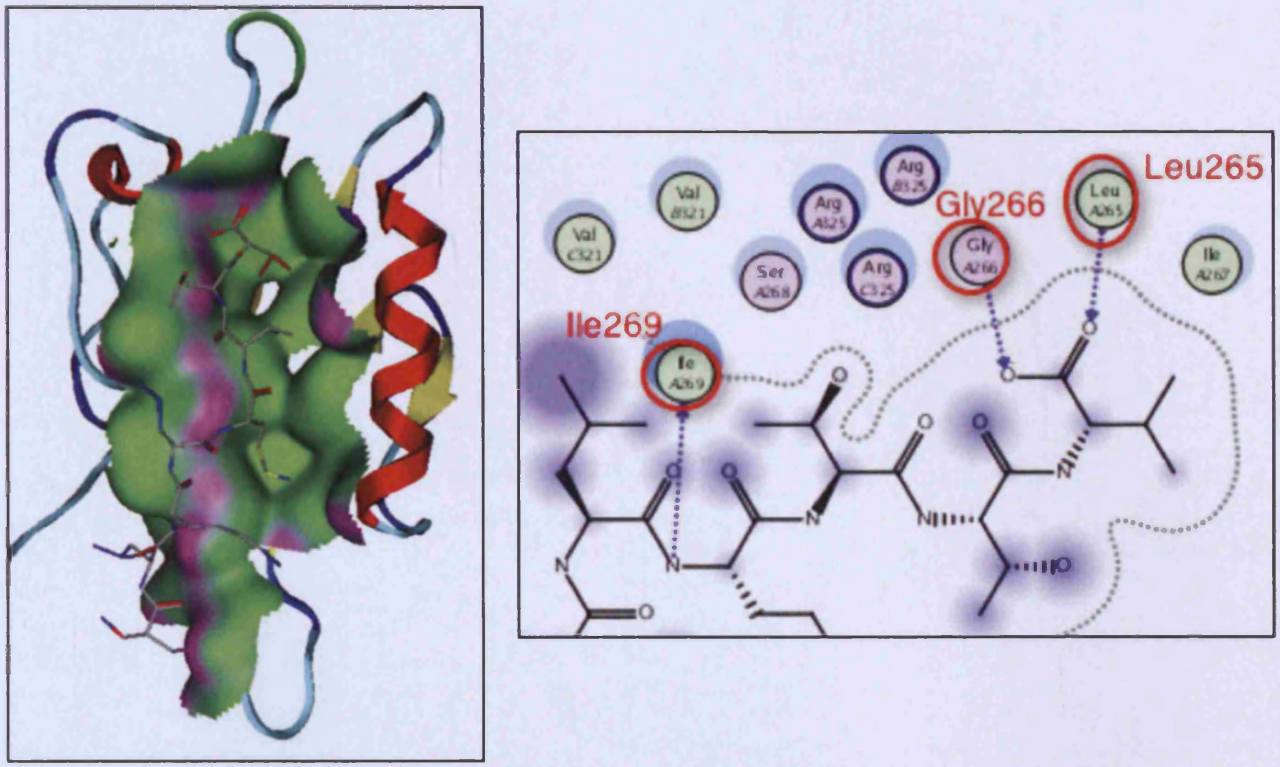


Figure 3: The complex structure of Dvl PDZ domain bound to its natural ligand Dapper is shown on the left panel while the right one shows a 2D depiction of the main hydrogen bonds mediating this interaction.

The analysis of Dvl PDZ-Dapper complex structure reveals that their interaction is mainly mediated by the hydrogen bonds formed with residues Leu 265, Gly 266, Ile 267 and Ile 269 in the β-B sheet.

A pharmacophore query was built using "pharmacophore query" which is a function in MOE which enables derivation of a pharmacophore from complex structures. The query generated consists of 5 main features as illustrated in Figure 4.

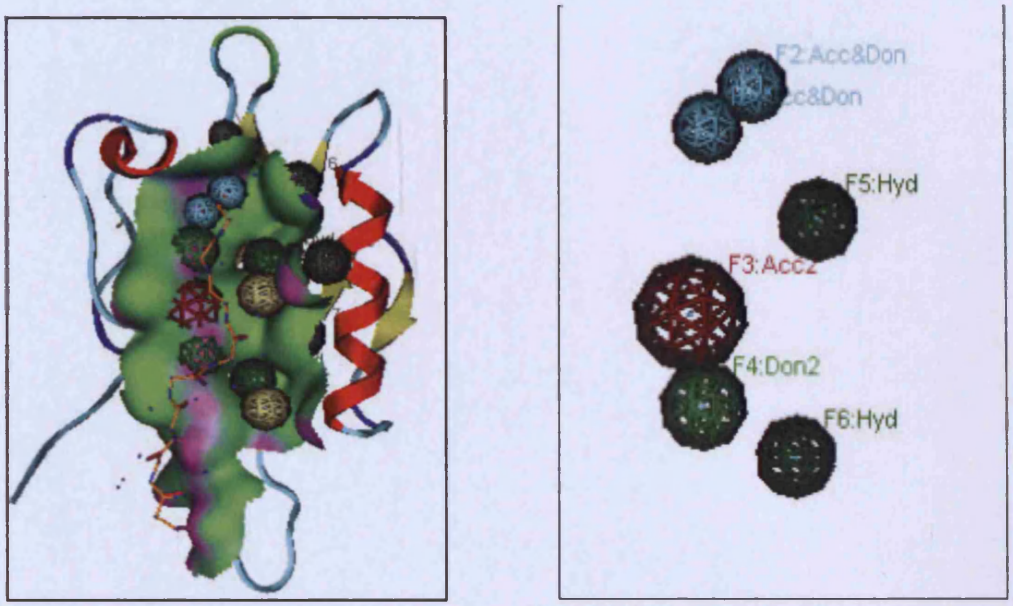


Figure 4: The generated pharmacophore query from the Dvl PDZ-Dapper complex using MOE. **F1, F2**: (Cyan colour) Acc & Don (acceptor and donor) correspond to the carboxylic acid moiety; **F3**, **F4**: Acc, Don respectively which correspond to the amide bond linking threonine and methionine. The black spheres (mainly in the panel on the left side) represent the exclusion volume to prevent steric clash. The yellow spheres represent the inclusion volume, which represent deeper hydrophobic cavities in the binding cleft where ligand occupation enhances interaction with the Dvl PDZ domain.

Having successfully identified the active site, which mediates Dvl-Fz protein-protein interactions and derived a pharmacophore query, the next step was to prepare a database of virtual compounds to run a virtual screening assay.

B. Database preparation: Virtual compound libraries are a collection of chemical compounds, which are readily accessible synthetically or commercially in sufficient quantities to run the required biological assays. Examples of such libraries include but are not limited to the Zinc database (> 4,500,000 molecule),[13] the NCI library (450,000 molecule),[14] Enamine (20,000 molecule),[15] and Maybridge (56,000 molecule)[16] . These databases are freely available in ready-to-dock, 3D format and were successfully downloaded.

Nevertheless, it is most important to prepare the chosen libraries before the screening process. By preparing, it is meant to have virtual compounds with drug-likeness properties via the use of physical and chemical filters. Drug-likeness is most often evaluated by the Lipinski's rule of five, which is one of the most frequently used empirical rules. [17] This rule describes molecular properties and pharmacokinetics, which account for ADME (absorption, distribution, metabolism and excretion). In fact, drug-likeness is usually considered before the construction of these virtual compound libraries.

Nevertheless, out of the above downloaded libraries, the lead-like Zinc subset library database has the largest number of compounds (1,000,000 compound). This library subset was downloaded as an SD file. Then, it was imported to the MOE *database viewer* module whereby it was prepared by removing any multiple fragment entries, deprotonating strong acids and protonating strong bases and accounting for Lipinski's rule filters. Then, they were energy minimized. The 3D conformations of these compounds were generated to account for the flexibility and rotations around bonds.

C. *In Silico* screening: The next step in the structure-based virtual screening is to run the virtual compounds against the pharmacophore generated. The "Pharmacophore query" module in MOE allows executing this process with flexible and variable criteria in that it allows a partial search where only some of the features are considered. It follows that the more restrictions accounted for, the fewer hit compounds will be generated. For instance, when the search process was applied to account for the five features mentioned above, no hits were obtained. Therefore, the screening was run again including 4 features only with F1, F2 and F4 as essential. This led to the generation of a small database of compounds, which fulfill the set criteria, and the compounds generated are represented in Figure 5.

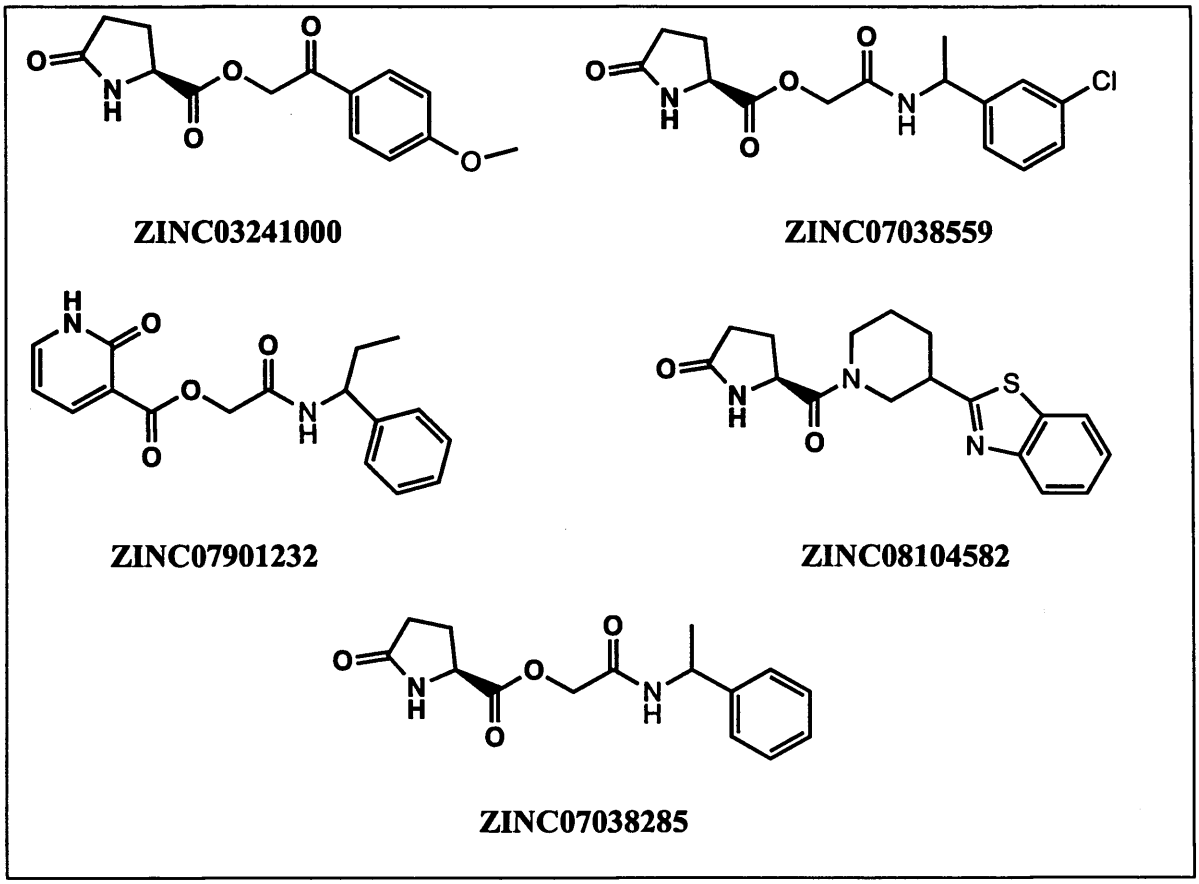


Figure 5: The main potential hits obtained from structure-based virtual screening process using Zinc lead-like database.

7. 1. 1. 2. Pharmacophore query based on using LigandScout:

LigandScout: is a molecular modelling program which allows creation of 3D pharmacophores from protein-ligand complex structures which can be exported to MOE to be used for virtual screening process.[18, 19] Moreover, there are many examples of the successful application of this strategy.[20,21] The pharmacophore derived is represented in Figure 6.

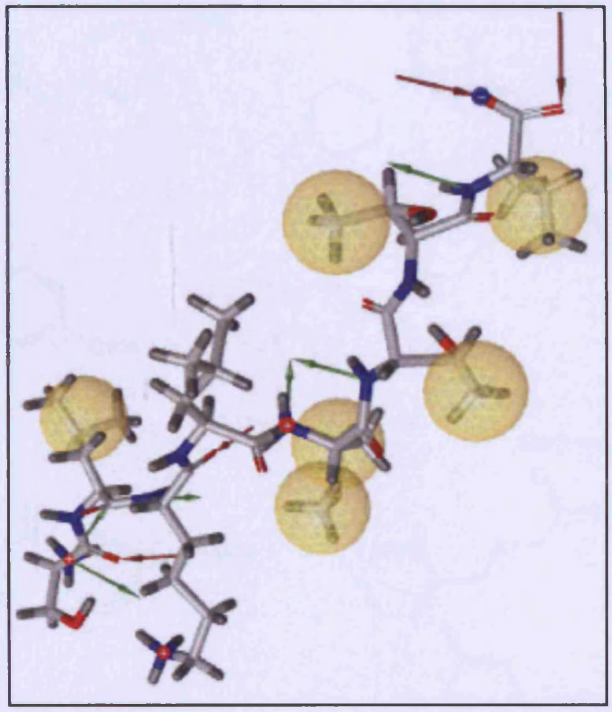


Figure 6: Dvl PDZ-Dapper complex structure based pharmacophore using LigandScout. The hydrogen bond acceptors (red arrows), hydrophobic interactions (yellow spheres), hydrogen bond donors (green arrows) are indicated.

From Figure 6, it appears that the LigandScout derived pharmacophore accounts for more features than the previous one. However, it is important to mention that only the terminal 4 residues (Met-Thr-Thr-Val) of Dapper (8 amino acid residues) are known to be involved in the interaction with Dvl PDZ.[8,10] On the other hand, even when only considering these 4 amino acid residues, the newly derived pharmacophore still accounts for more hydrophobic interactions.

Nevertheless, using this pharmacophore the same structure-based virtual screening process described above was implemented. The only modification was that the database of compounds used was a combination of the Zinc-lead like subset, NCI database, Maybridge and Enamine databases. The 8 potential hits obtained using this modified protocol are shown in Figure 7.

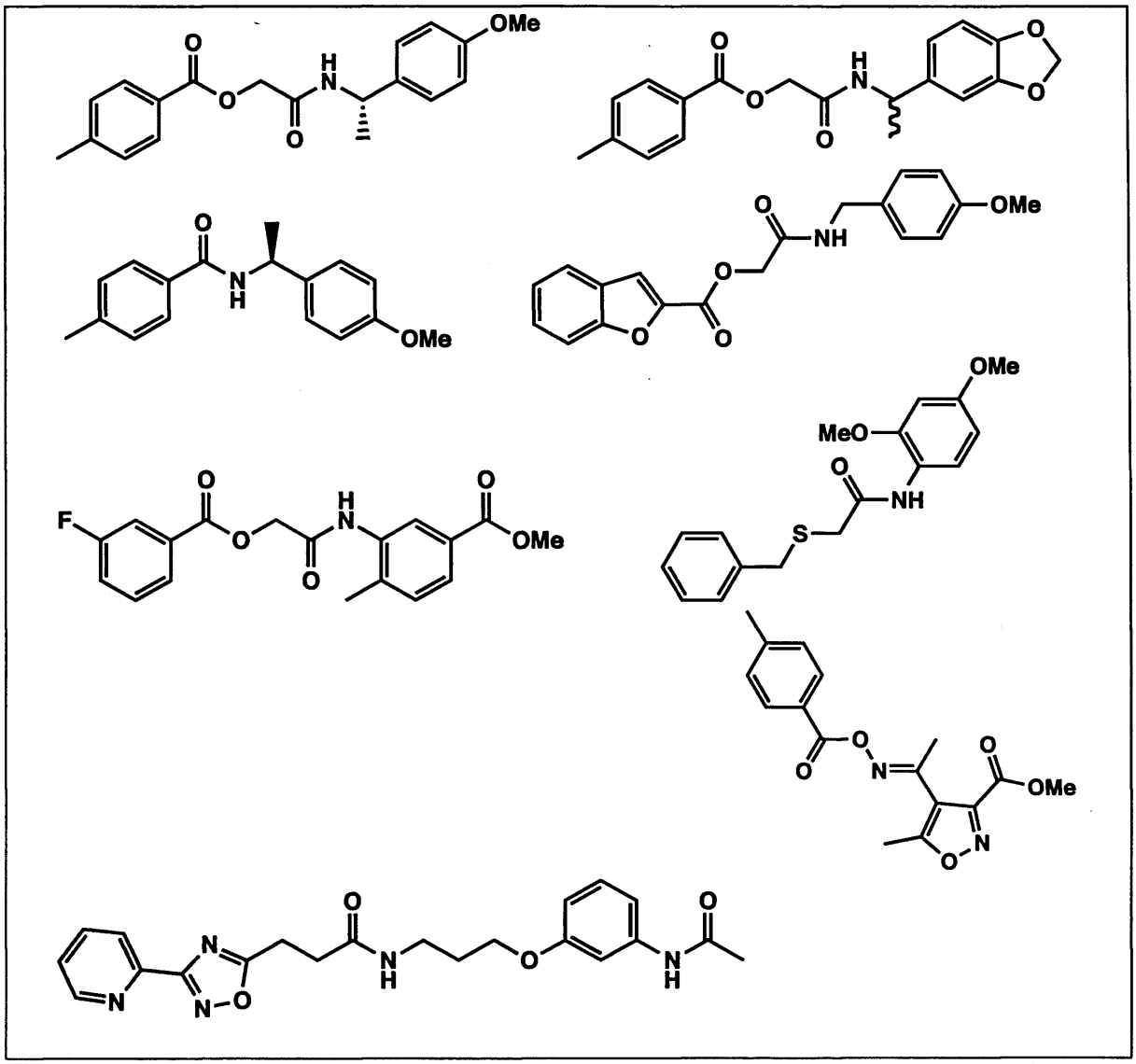


Figure 7: The main potential hits obtained from the modified structure-based virtual screening protocol.

7.1.2. Similarity search

Fujii and co-workers reported the indole carbinol (FJ9) as the first small molecule inhibitor of Dvl PDZ.[22] The same group later went on to report a derivative for this structure which has even more potent activity (Figure 8).[23]

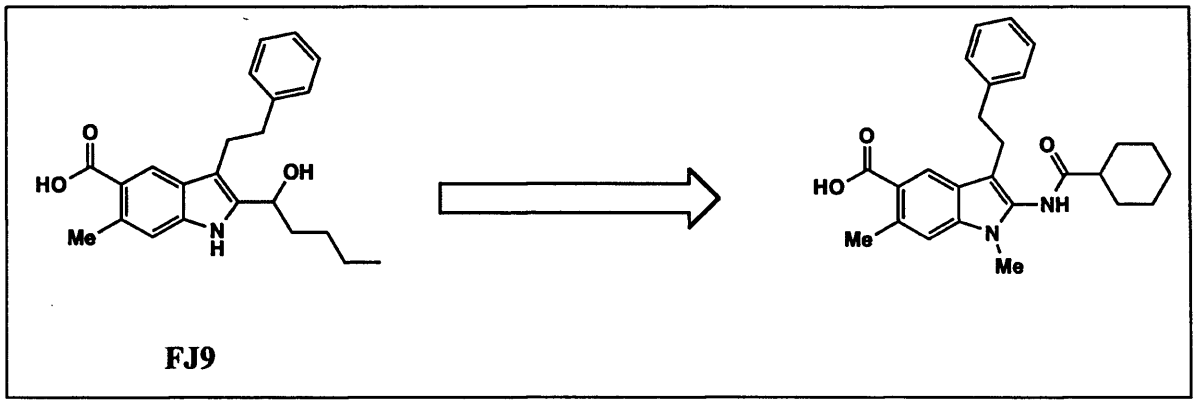


Figure 8: The indole carbinol (FJ9) and its derivative as known Dvl PDZ inhibitors.

The "Similarity Search" function in MOE enables a search in a virtual database for molecules, which are structurally similar to a query compound. It follows that a fingerprinted database and a query molecule are required in order to run a similarity search.

A. ***Query molecule:*** the modified structure of FJ09 was used as the query molecule since it was reported to have more potent inhibition of the Dvl-Fz protein-protein interaction.[23]

B. ***Fingerprinted database:*** a molecular database for which fingerprints already have been pre-calculated for all of the molecule entries. Molecular fingerprints otherwise known as structural keys or signatures represent a set of the core features derived from the structure of a molecule. It follows therefore that molecules can be compared by measuring and defining the extent to which their calculated set of features may match or overlap. Once fingerprints are derived for molecular database, a metric is necessary to compare these fingerprints with the ones of the query model. In MOE, *Tanimoto Coefficient (TC)* is the most common and widely used similarity metric. TC can be considered as the number of common features between the pair of compounds divided by the number of total features of both fingerprints. Therefore, TC can take a value between 1 and 0 ranging from most to least similar and is calculated by comparing the fingerprints between any pair of molecules.

There are several fingerprint systems implemented in MOE, which can be used. Of these systems, the fingerprint system "FP:MACCS" which contains a total of 166 structural keys corresponding to various groups present in organic compounds was chosen. The first step was to calculate the FP:MACCS for the previously prepared ZINC lead-like database as well as for the modified structure of FJ09. TC ≥ 0.85 which is the default criterion (in MOE) for considering two molecules to be similar was applied. However, when running a similarity search with this default criterion no hits were obtained. Therefore, it was necessary to reduce the TC threshold but no hits were obtained until TC was set as 0.5.

Nevertheless, the similarity search approach was stopped and not investigated any further as having hits with 50% similarity to the active compound is not sufficient since the active compound has low micromolar activity in the first place. However, it is important to note that this approach could have been improved by employing a bigger and more diverse molecular database or by considering a different set of fingerprints.

7.1.3. The use of high-throughput docking:

Docking is the computational simulation process to predict the preferred orientation of a ligand candidate when bound to its target.[24] Because of the flexibility of the ligand and target, the docking process aims to achieve an optimized best-fit conformation of the complex with minimum overall energy of the system. The strength and affinity of binding between the ligand and its target is often predicted in terms of scoring functions which mainly account for the number of favorable intermolecular interactions in terms of hydrogen bonds and hydrophobic interactions.[25]

7.1.3.1. High-throughput docking using GFscore:

GFscore is a general nonlinear consensus scoring function for high-throughput docking reported by Betzi et al recently.[26] This methodology extends the principle of consensus scoring in a non-linear neural network manner by combining the five scoring functions (FlexX Score, G_Score, D_Score, ChemScore, PMF Score) found in the Cscore package

from Tripos inc. It follows that a neural network has been trained on the scoring results to combine the 5 scoring functions and generate this Generalist Function score, GFscore. This was achieved by selecting a diverse database (1420 compounds of the NCI Diversity database) and a set of diverse protein-ligand complexes to train this neural network. Among the 200 protein-ligand dataset complexes, FlexX was able to generate 78 complexes with an accurate RMSD (< 2Å).[26] GFscore is reported to distinguish true negatives from false negatives in a given compound database and eliminate up to 75% of molecules with a confidence rate of 90%.[26] This in turn enriches the list of potential hits to be investigated for biological activity by considering only 25% of molecules.

This methodology was successfully applied by the same group to identify a small molecule inhibitor of the HIV-1 Nef protein-protein interactions with a submicromolar activity (K_d= 0.3 μM).[27]

The following steps were followed in the application of GFscore:

1. The FlexX/Cscore docking experiment needs to be run on the diversity database used to train the GFscore neural network. Therefore, the NCI diverse database was first downloaded from the GFscore server's website (http://gfscore.cnrs-mrs.fr/) and then docked using FlexX in the Dvl PDZ domain.
2. The obtained docking results were exported in ASCII file format using the comma separator.
3. Virtual screening experiment was performed on the Zinc lead-like database and the results were exported similarly (ASCII file).
4. The two exported files were uploaded to the GFscore calculation webpage mentioned above and a list of potential hits was obtained.

Having followed this process, a list of potential PDZ inhibitors was obtained. The next step was to further filter these hits. Therefore, a pharmacophore model was generated (MOE). This pharmacophore model was superimposed with the docking database pose (same 3D coordinates) during the MOE pharmacophore search using the option "use absolute position". This query enabled the selection of fewer compounds. This number

was further filtered by visual inspection of the chemical and geometrical properties of the ligand pose to give the potential hits, which were put forward for synthesis (Figure 9).

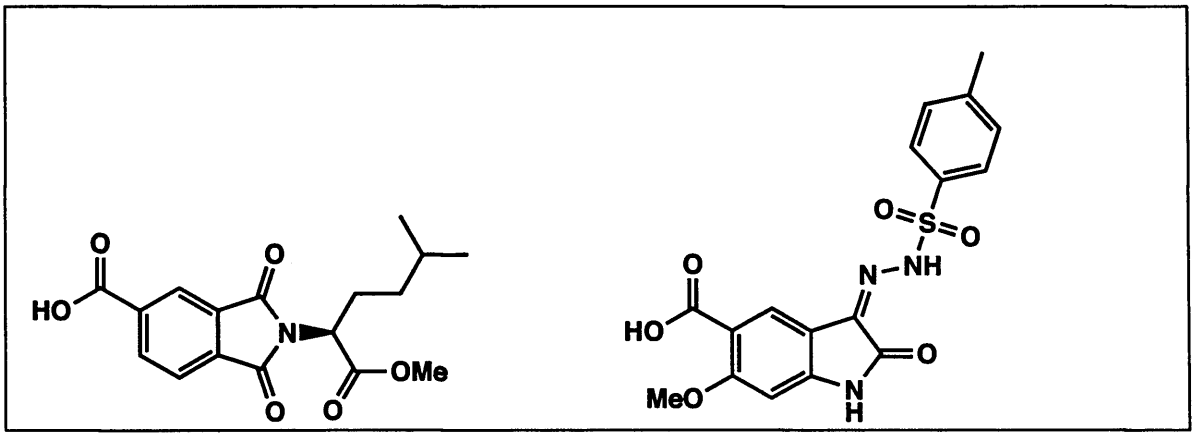


Figure 9: The 2 main structural scafolds obtined from the Gfscore high-throughput docking studies.

7.1.3.2. High-Throughput docking using Plants:

PLANTS (Protein-Ligand ANT System) is a docking algorithm based on ant colony optimisation (ACO), which is a class of stochastic optimisation algorithms.[28] During the docking process, an artificial ant colony is employed to identify ligand conformations with the lowest energy of binding to the target active site. The concept of this docking algorithm stems from the behaviour of real ants in finding the shortest path to food sources from their colony whereby they release pheromones to mark the paths they follow during their search. It follows therefore that high levels of pheromones determine path choice. Similarly, ACO algorithms imitate this behaviour by associating numerical values with each possible solution.[28]

PLANTS encompasses two empirical scoring functions; $PLANTS_{PLP}$ and $PLANTS_{CHEMPLP}$.[29] While the former accounts mainly for the steric complementarity between the ligand and the protein target, the latter accounts for the hydrogen bonding formed between the ligand-target complex.[29]

The ChemBridge lead-like database was chosen for high throughput docking with PLANTS as any potential hits can easily be purchased in sufficient quantities to run biological assays. The ChemBridge lead-like library is freely available for download from the website of ChemBridge corporation after simple registration procedure.[30] This database contains more than 450,000 compounds with diverse structures. Database preparation and pre-processing was performed as described above for the other databases used previously. Energy minimization and conformational analysis were performed using the implemented MMFF94X forcefield in MOE.

This database of compounds was docked using PLANTS V-1.06 in the PDZ domain active site identified earlier with the docking site limited to a 12Å radius sphere centered in this active site. The number of conformations was set to no more than 10 for each ligand.

The obtained docking results were saved as mol2 file type and then exported to MOE *Database Viewer* to enable better visual analysis of the docked confirmations in the PDZ domain. The docked compound candidates were ranked using PLANTS in-built scoring functions ($PLANTS_{PLP}$ and $PLANTS_{CHEMPLP}$).

The top-ranked ligand confirmations were then visually inspected using MOE. A particular interest was drawn into piperazine (HTS00568) as it adopts a conformation, which resides deep into the PDZ binding site as shown in Figure 10. The interactions of this ligand with PDZ domain are also depicted in 2D format.

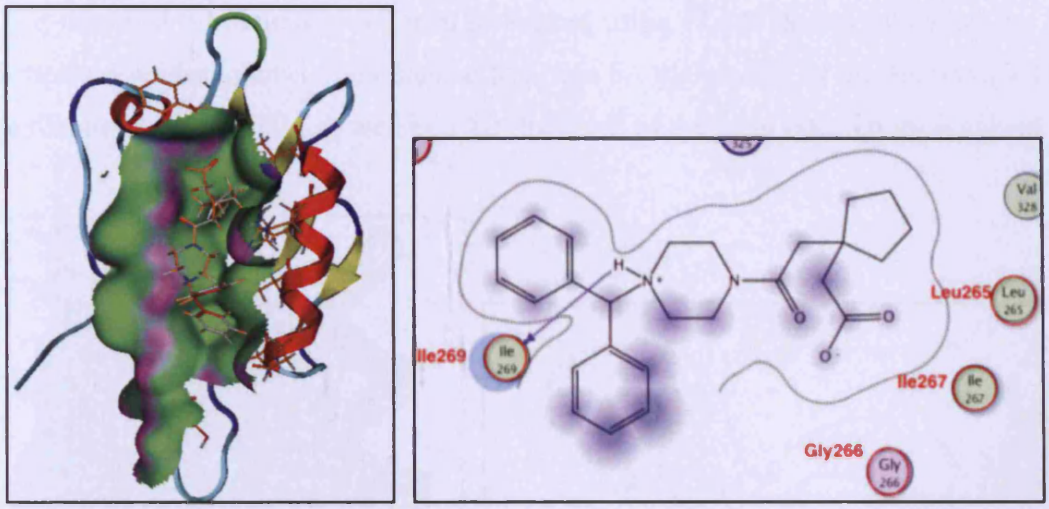
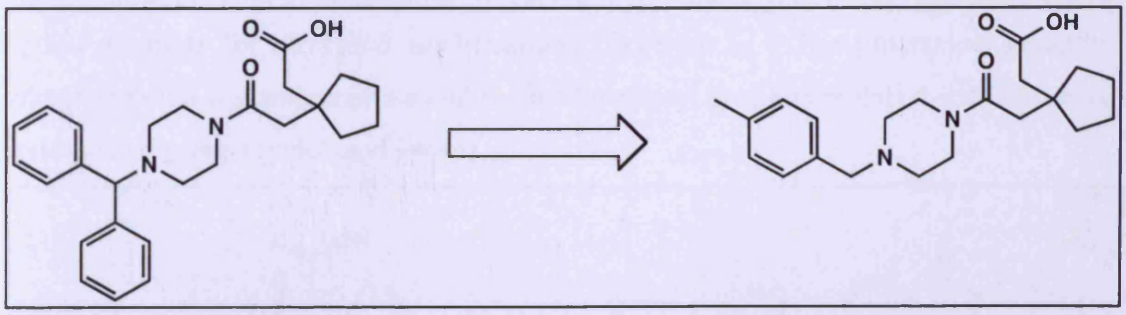


Figure 10. The conformation adopted by the piperazine (HTS00568) when docked into the active site of Dvl PDZ domain using PLANTS is shown in the panel on the left. The panel on the right represents a 2D depiction of the main hydrogen bonds mediating this interaction.

This hit confirmation adopts a favorable binding position with the medium size hydrophobic cyclopentane group residing deep into the hydrophobic cleft forming favorable hydrophobic interactions. This hit also shows hydrogen bonding with the side chain of the amino acid Ile 269 (note it is one of the hydrogen bonds that the N of dapper forms with Ile 269). On the other hand, one of the phenyl groups (hydrophobic) is exposed to the water surface (hydrophilic) leading to unfavorable interactions. Therefore, the structure of this hit was modified by removing one of the phenyl rings and introducing a methyl group to further enhance hydrophobic interactions (Scheme 1).



Scheme 1

The modified hit structure was then re-docked using PLANTS and following the same process described above. The obtained structure for the binding of this hit with Dvl PDZ is illustrated in Figure 11 as well as a 2D depiction of the main interactions involved.

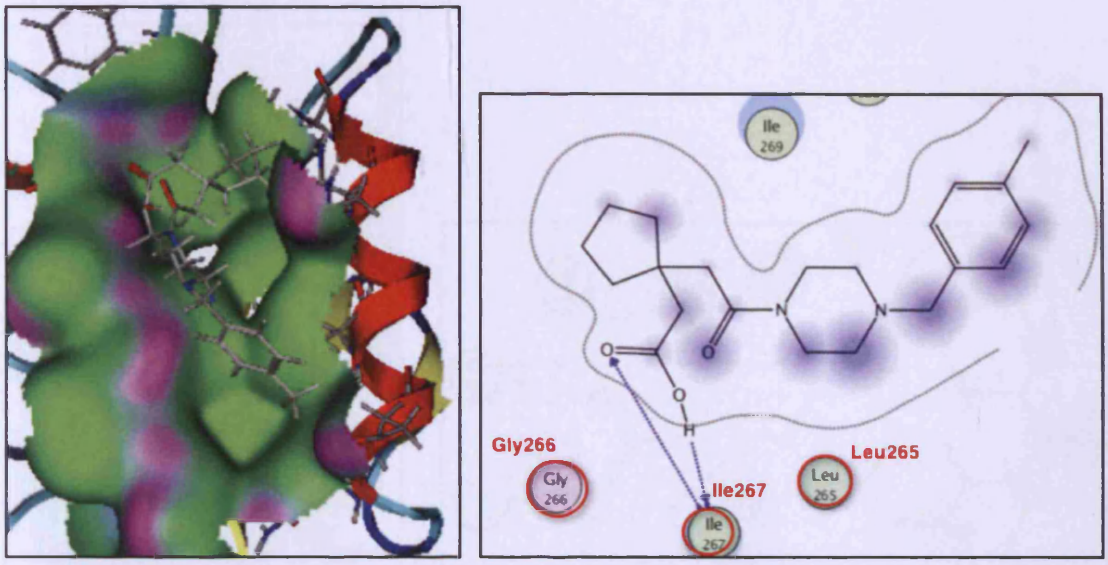
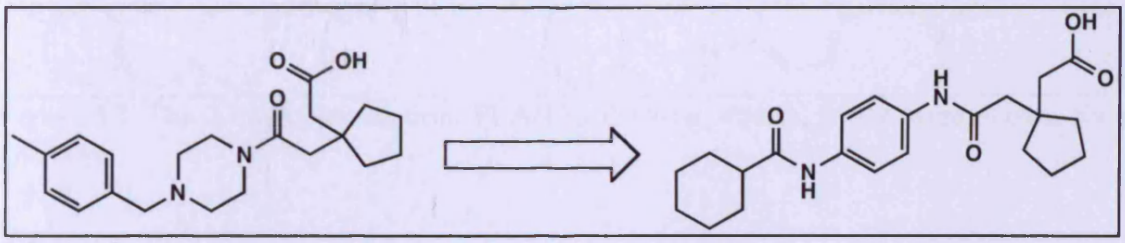


Figure 11. The conformation adopted by the modified structure of the piperazine (HTS00568) when docked into the active site of Dvl PDZ domain using PLANTS is shown in the panel on the left. The panel on the right represents a 2D depiction of the main hydrogen bonds mediating this interaction (Ile 267).

Indeed, the new piperazine compound adopts a more favorable binding mode when docked into Dvl PDZ domain. The carboxylic acid group of this compound is in closer proximity to Ile 267 leading to the formation of hydrogen bonding.

The promising results of the docking of the modified structure of HTS00568 encouraged further attempts for structural modifications (Scheme 2). The piperazine group is substituted with a *p*-aminoaniline and the methylphenyl group is replaced with the more hydrophobic group (cyclohexyl group).



Scheme 2.

The new structural modifications resulted in better hydrogen bonding interactions of the carboxyl group with Ile 267, Gly 266 and Leu 265 while still adopting an extended binding pose along the active site as shown in Figure 12.

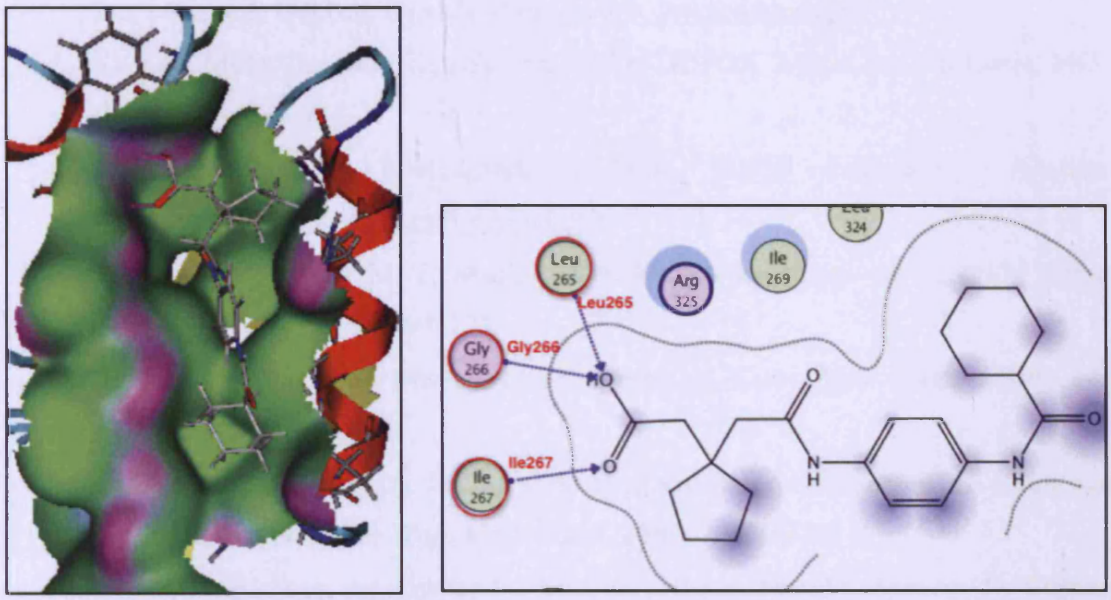


Figure 12: The binding mode of the modified structure as a result of PLANTS docking into the Dvl PDZ domain shown in the panel on the left whereas the panel on the right represents a 2D depiction of the main hydrogen bonds mediating this interaction.

Nevertheless, the following two hits (Figure 13) were selected for synthesis and subsequently for biological testing as potential Dvl PDZ inhibitors.

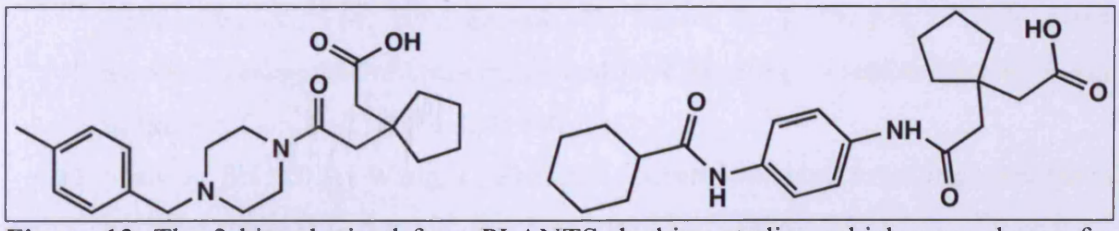


Figure 13. The 2 hits obtained from PLANTS docking studies, which were chosen for synthesis.

References

1. Leach, A. R. Introduction to computational chemistry. *Longman.* **1996.**
2. Molecular Operating Environment (MOE 2008.10). Chemical Computing Group, Inc. Montreal, Quebec, Canada. (http://www.chemcomp.com/)
3. SYBYL Molecular Modeling System. (7.1); TRIPOS, Assoc. Inc.: St-Louis, MO. (http://tripos.com/)
4. LigandScout 2.0. Inte:Ligand. A-2344 Maria Enzersdorf, Austria. (http://www.inteligand.com/ligandscout/)
5. Walters, W. P.; Stahl, M. T.; Murko, M. A. Virtual screening – an overview. *Drug Discov. Today* **1998**, 3, 160–178.
6. Sun, H. Pharmacophore-based virtual screening. *Curr. Med. Chem.* **2008**, 15, 1018-1024.
7. Cavasotto, C. N.; Orry, A. J. Ligand docking and structure-based virtual screening in drug discovery. *Curr. Top. Med. Chem.* **2007**, 7, 1006-1014.
8. Doyle, D. A.; Lee, A.; Lewis, J. ; Kim, E. ; Sheng, M. ; Mackinnon, R. Crystal structures of a complexed and peptide-free membrane protein-binding domain: molecular basis of peptide recognition by PDZ. *Cell.* **1996**, 85, 1067-1076.
9. Wong, H-C.; Bourdelas, A.; Krauss, A. Lee, H. J.; Shao, Y.; Mlodzik, M.; Shi, D. L.; Zheng, J. Direct binding of the PDZ domain of Dishevelled to a conserved internal sequence in the C-terminal region of Frizzled. *Mol. Cell.* **2003**, 12, 1251-1260.
10. Cheyette, B. N.; Waxman, J. S. ; Miller, J. R. Takemaru, K.; Sheldahl, L. C.; Khlebtsova, N.; Fox, E.P.; Earnest, T.; Moon, R. T; Dapper, a Dishevelled-associated antagonist of beta-catenin and JNK signaling, is required for notochord formation. *Dev. Cell.* **2002**, 2, 449-461.
11. Shan, J.; Shi, D. L.; Wang, J.; Zheng, J. Identification of a specific inhibitor of the dishevelled PDZ domain. *Biochem.* **2005**, 44, 15495-15503.
12. Guner, O. F. History and evolution of the pharmacophore concept in computer-aided drug design. *Curr. Top. Med. Chem.* **2002**, 2, 1321–1332.

13. Irwin, J. J.; Shoichet, B. K. ZINC-a free database of commercially available compounds for virtual screening. *J. Chem. Inf. Model.* **2005**,45, 177–182.

14. The NCI library (http://129.43.27.140/ncidb2) (downloaded February 2008).

15. Enamine database (http://www.enamine.net/index.php) (downloaded February 2008).

16. Maybridge database (http://www.maybridge.com/default.aspx)(downloaded February 2008).

17. Lipinski, C. A.; Lombardo, F.; Dominy, B. W.; Feeney, P. J. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Adv. Drug. Del. Rev.* **1997,** 23: 3–25

18. Wolber, G.; Langer, T. LigandScout: 3-D Pharmacophores derived from protein-bound ligands and their use as virtual screening filters. *J. Chem. Inf. Model.* **2005,** 45, 160-169.

19. Wolber, G.; Dornhofer, A. A.; Langer, T. Efficient overlay of small organic molecules using 3D pharmacophores *J. Comput. Aided Mol. Des.* **2007**, 20, 773-788.

20. Krovat, E. M.; Fruhwirth, K. H.; Langer, T. Pharmacophore identification, *in Silico* screening, and virtual library design for inhibitors of the human factor Xa. *J. Chem. Inf. Model.* **2005**, 45, 146-159.

21. Barreca, M. L.; De Luca, L.; Iraci, N.; Rao, A.; Ferro, S.; Maga, G.; Chimirri, A. Structure-based pharmacophore identification of new Chemical scaffolds as non-nucleoside reverse transcriptase inhibitors. *J. Chem. Inf. Model.* **2007**, 557-562.

22. Fujii, N.; You, L.; Xu, Z.; Uematsu, K.; Shan, J.; He, B.; Mikami, I.; Edmondson, L.R.; Neale, G.; Zheng, J.; Guy, R.K.; Jablons, D.M. An antagonist of dishevelled protein-protein interaction suppresses β-catenin-dependent tumor cell growth. *Cancer Res.* **2007**, 67, 573-579.

23. Mahindroo, N.; Punchihewa, C.; Bail, A. M.; Fujii, N. Indole-2-amide based biochemical antagonist of Dishevelled PDZ domain interaction down-regulates Dishevelled-driven Tcf transcriptional activity. *Bioorg. Med. Chem. Lett.* **2008,** 18, 946-949.

24. Moitessier, N.; Englebienne, P.; Lee. D.; Lawandi, J.; Corbeil, R. C. Towards the development of universal, fast and highly accurate docking/scoring methods: a long way to go. *BJP.* **2008**, 153, S7-S26.

25. Bortolato, A.; Moro, S. Designing a ligand for pharmaceutical purposes. *Expert Opinion on Drug Disc*, **2008**, 3, 579-590.

26. Betzi, S.; Suhre, K.; Chetrit, B. ; Guerlesquin, F.; Morelli, X. *J. Chem. Inf. Model.* **2006**, 46, 1704-1712.

27. Betzi, S.; Restouin, A.; Opi, S.; Arold, S. T.; Parrot, I.; Guerlesquin, F.; Morelli, X.; Collette, Y.; Protein-protein interaction inhibition (2P2I) combining high throughput and virtual screening: Application to the HIV-1 Nef protein. *Proc. Natnl. Acad. Sci.* **2007**, 104, 19256-19261.

28. Korb, O.; Stützle, T.; Exner, T. E. An Ant Colony Optimization approach to flexible protein-ligand docking. *Swarm. Intelligence* **2007**, 1, 115-134.

29. Korb, O.; Stützle, T.; Exner, T. E. Empirical scoring functions for advanced protein-ligand docking with PLANTS. *J. Chem. Inf. Model.* **2009**, 49, 84-96.

30. ChemBridge database (http://www.chembridge.com/data.html) (downloaded June 2008).

8.The synthesis and biological evaluation of the designed Dvl PDZ domain inhibitors

8.1.Chemistry

Initial efforts focused on establishing suitable synthetic routes toward the preparation of the hits, which were obtained from the performed molecular modelling studies (Chapter 7). These efforts were also directed into ensuring access to the required starting materials either commercially or through robust synthetic procedures with good yields.

8.1.1. Synthesis of structure-based virtual screening hits:

The following 2 hits (Figure 1) represent the main structural scaffolds obtained by the virtual screening approach as outlined in Chapter 7.

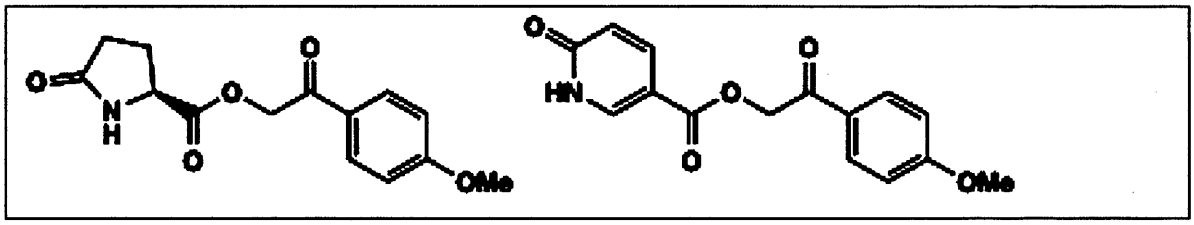
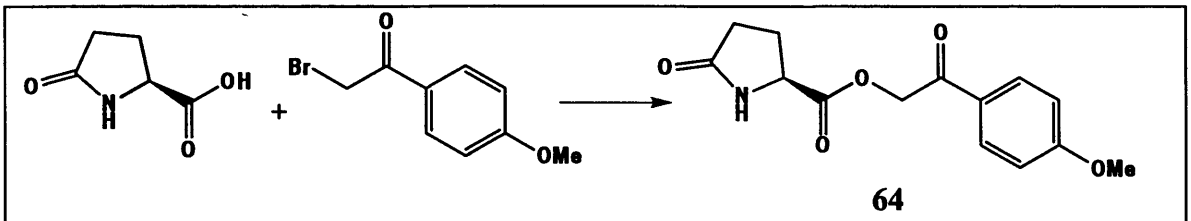


Figure 1: The 2 potential hits obtained via structure-based virtual screening.

8.1.1.1. Synthesis of *(S)*-2-(4-methoxyphenyl)-2-oxoethyl-5-oxopyrrolidine-2-carboxy late (64)

The synthesis of *S*-2-(4-methoxyphenyl)-2-oxoethyl 5-oxopyrrolidine-2-carboxylate **(64)** was achieved by heating under reflux a mixture of *S*-2-pyrrilidonecarboxylic acid and 2-bromo-4'-methoxyacetophenone in acetone. Both of these substrates were commercially available. The reaction took a few hours to give the desired product as shown is Scheme 1. The use of column chromatography enabled isolation of **(64)** in analytically pure form and relatively good yield (60%).

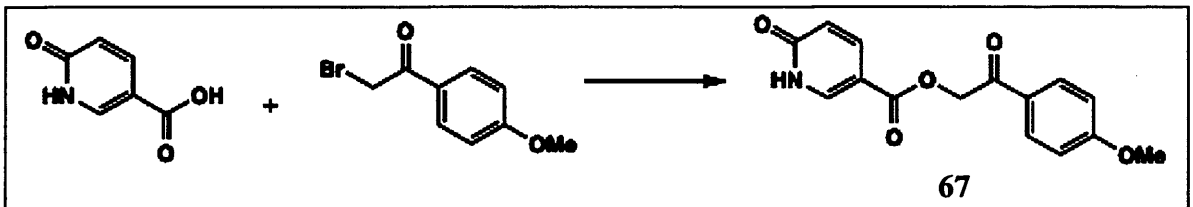


Reagents and conditions:acetone, reflux, 3hrs.

Scheme 1

8.1.1.2. Synthesis of 2-(4-methoxyphenyl)-2-oxoethyl-6-oxo-1,6-dihydropyridine-3-carboxylate (67)

The same procedure was employed for the synthesis of 2-(4-methoxyphenyl)-2-oxoethyl 6-oxo-1,6-dihydropyridine-3-carboxylate(**67**) from 6-oxo-1,6-dihydropyridine-3-carboxylic acidand 2-bromo-4'-methoxyacetophenone (Scheme 2) in a comparable yield (64%).



Reagents and conditions: Acetone, reflux, 3hrs.

Scheme 2

Subsequently, because of the ease of synthesis and mild conditions, this synthetic procedure was further employed for the preparation of other structurally related analogues starting with differently substituted acids and bromoacetophenones as shown below in Figure 2. The newly synthesised compounds included the (*R*) isomer of (*S*)-2-(4-methoxyphenyl)-2-oxoethyl-5-oxopyrrolidine-2-carboxylate **(65)**. Overall, relatively good yields (50%-70%) were obtained for the preparation of these products.

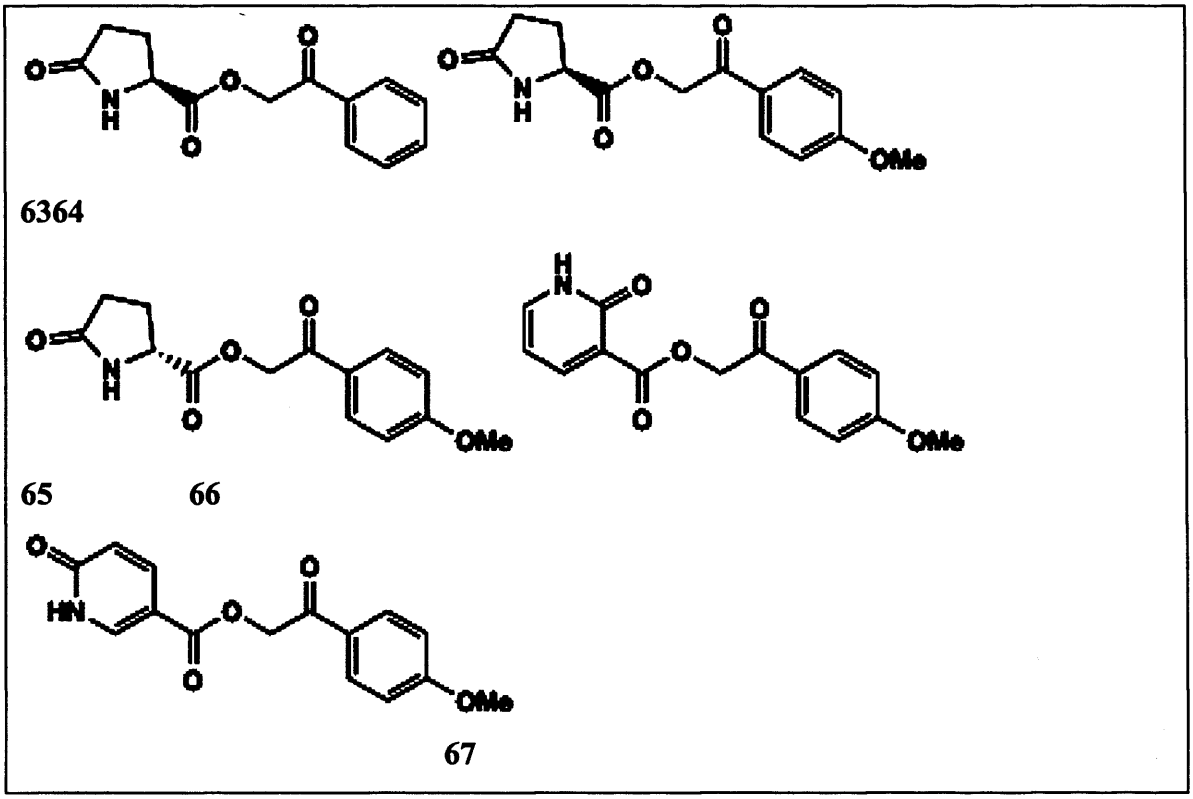


Figure 2. The synthesised derivatives of the 2 potential hits obtained from the structure-based virtual screening.

8.1.2. The synthesis of structure-based virtual screening hits (LigandScout approach):

The modified structure-based virtual screening approach led to the identification of the following hits as potential Dvl PDZ inhibitors (Figure 3).

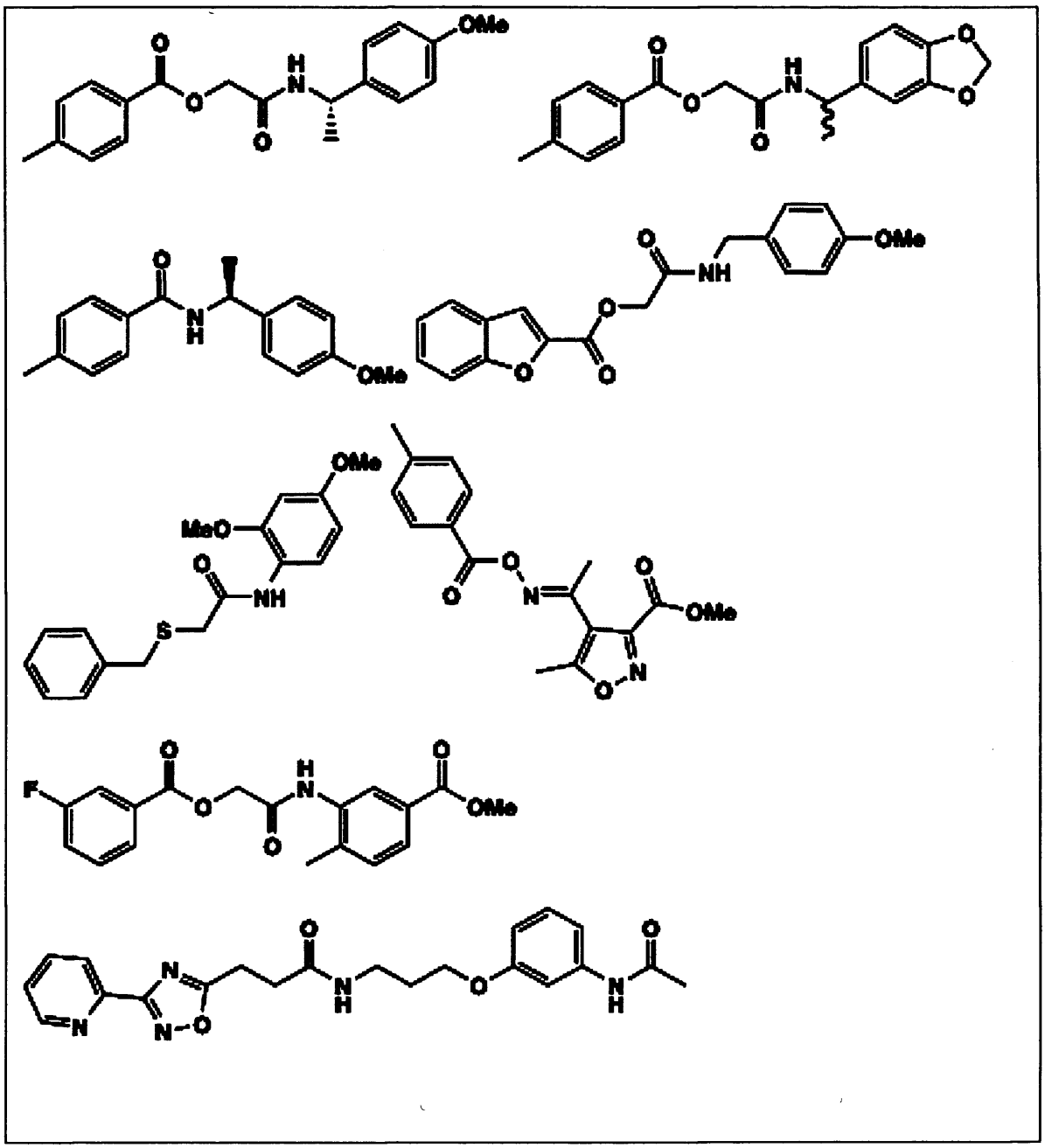
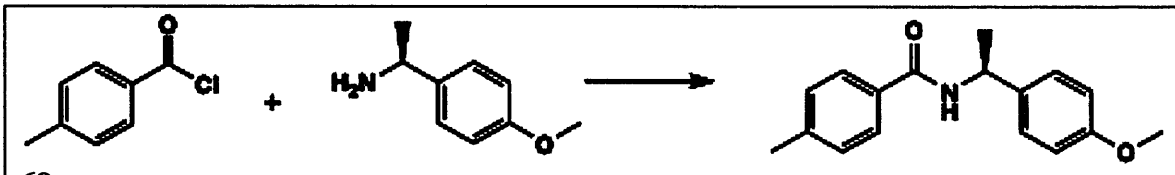


Figure 3. The structures of the potential hits obtained via structure-based virtual screening using LigandScout.

8.1.2.1. Synthesis of (*S*)-N(1-(4-methoxyphenyl)ethyl)-4-methylbenzamide (68)

The synthesis of **(68)** was easily achieved through the substitution reaction between the two commercially available substrates; p-toluoyl chloride and 4-methoxy-α-methylbenzylamine. The p-toluoyl chloride is added dropwise to a stirring mixture of the

amine and TEA in DCM at 0°C. Once the p-toluoyl chloride is fully added, the reaction is left stirring at room temperature as shown in Scheme 3 to afford compound **(68)**in excellent yield (85%).



68

Reagents and conditions: TEA, 0°C, 1hr; RT, 12 hrs.

Scheme 3.

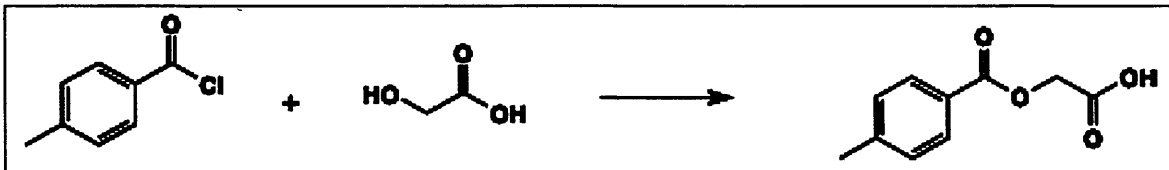
8.1.2.2. Synthesis of (*S*)-2-(1-(4-methoxyphenyl)ethylamino)-2-oxoethyl-4-methylbenzoate (70)

This compound **(70)** was prepared from 4-methoxy-α-methylbenzylamine and 2-(4-methylbenzoyloxy)acetic acid. While the former substrate is commercially available, the latter had to be prepared.

8.1.2.2.1. Synthesis of 2-(4-methylbenzoyloxy)acetic acid (69)

1st attempt: for the synthesis of 2-(4-methylbenzoyloxy)acetic acid, p-toluoyl chloride was added dropwise to a stirring solution of glycolic acid in DCM in the presence of slightly access of TEA (Scheme 4-A). The yield obtained was relatively low (<20%) and the reaction was taking a long time. Given that this particular type of reaction was going to be used for the preparation of other starting materials required in the synthesis of some of the obtained hits, it was essential to explore an alternative procedure with better yields.

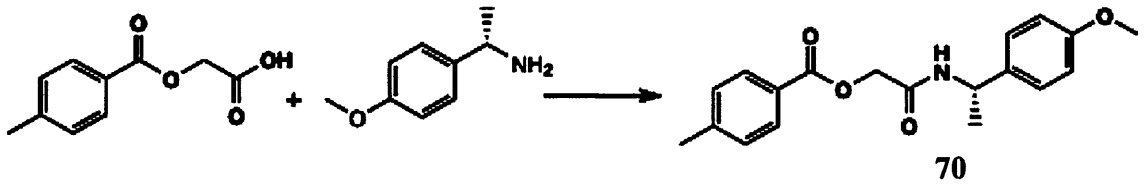
2nd attempt: the same reaction was repeated but this time by stirring the components in pyridine overnight. This led to the formation of the desired product in relatively good yield (60%) (Scheme 4-B).



Reagents and conditions: A: TEA, DCM, 24 hrs (15% yield) or
B: Pyridine, 12 hrs (60% yield).

Scheme 4

The compound **(70)**was then synthesised from the coupling of 4-methoxy-α-methylbenzylamine and the prepared compound **(69)**using the coupling agent carbonyldiimidazole (CDI)as represented below (Scheme 5). It follows that CDI reacts first with the carboxylic acid 2-(4-methylbenzoyloxy) acetic acid and activates it by making it more susceptible to the nucleophilic attack of the amine (4-methoxy-α-methylbenzylamine). One of the main advantages of using this procedure is that the final product can be isolated by precipitating from ice-cold water. This precipitate can be sufficiently purified by a thorough washing with water.



Reagents and conditions: CDI, DMF, 0°C, 1 hr; rt, 12 hrs.

Scheme 5

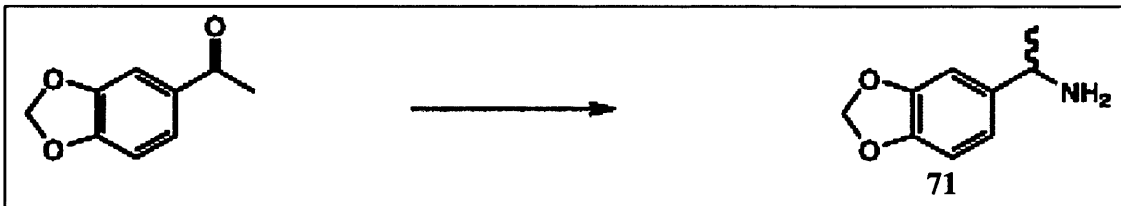
8.1.2.3. Synthesis of 2-(1-(benzo[*d*][1,3]dioxol-5-yl)ethylamino)-2-oxoethyl-4-methyl benzoate (72).

This compound was prepared from 2-(4-methylbenzoyloxy)acetic acid and 1-(benzo[*d*][1,3]dioxol-5-yl)ethaneamine following the same synthetic procedure described for the previous hit (S-2-(1-(4-methoxyphenyl)ethylamino)-2-oxoethyl-4-methylbenzoate). 2-(4-Methylbenzoyloxy)-acetic acid was already prepared from p-toluoyl chloride as described above while 1-(benzo[*d*][1,3]dioxol-5-yl)ethanamine had to be synthesised.

8.1.2.3.1. Synthesis of 1-(benzo[d][1,3]dioxol-5-yl)ethanamine (71)

1-(benzo[*d*][1,3]dioxol-5-yl)ethanamine is not commercially available and had to be prepared. Therefore, efforts were focused on deriving this desired amine from the commercially available ketone (1-(benzo[d][1,3]dioxol-5-yl)ethanone) through reductive amination reaction which involves the conversion of the ketone carbonyl group into the amino functionality.

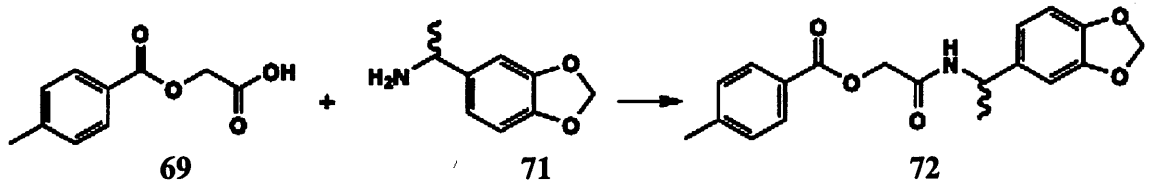
For the reductive amination reaction, treatment of ketones with ammonia in ethanol and titanium(IV) isopropoxide, followed by in situ reduction with sodium borohydride was reported to give their primary amine derivatives in excellent yields in a one-pot procedure.[1] This procedure was successfully applied to the synthesis of the racemic product(**71**)and afforded a relatively moderate yield (50%) of product.(Scheme 6).



Reagents and conditions: NH_3. EtOH, $Ti(O^iPr)_4$, RT, 6hr; $NaBH_4$, RT, 3hr.

Scheme 6

Having prepared the required starting substrates, 2-(1-(benzo[*d*][1,3]dioxol-5-yl)ethylamino)-2-oxoethyl-4-methylbenzoate **(72)** was prepared following the procedure described for **(70)** as outlined in Scheme 7.



Reagents and conditions: CDI, DMF, 0°C, 1 hr; RT, 16 hr.

Scheme 7

8.1.2.4. Synthesis of 2-(4-methoxybenzylamino)-2-oxoethylbenzofuran-2-carboxylate (75)

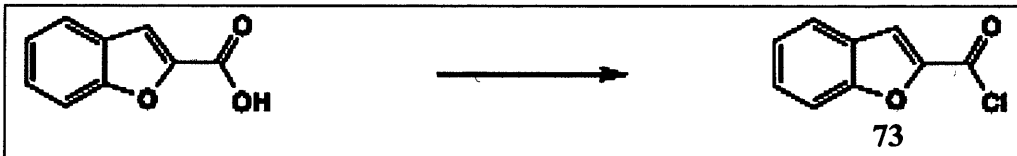
Compound **(75)** was synthesised using benzofuran-2-carbonyloxy-2-acetic acid**(74)** and 4-methoxybenzylamine. However, the acid had first to be prepared from benzofuran-2-carbonyl chloride**(73)**.

8.1.2.4.1. Synthesis of benzofuran-2-carbonyl chloride (73):

It was required to convert the carboxylic acid (benzofuran-2-carboxylic acid) into its acyl chloride derivative as the acyl chloride is more readily reactive in substitution reactions (Scheme 8).

1st attempt: thionyl chloride ($SOCl_2$) is widely used to convert carboxylic acids into acyl chlorides. It is a preferred reagent since the formed side products namely HCl and $SO_2$are gaseous and hence they are easily removed from the reaction media. Thus, benzofuran-carboxylic acid was dissolved in thionyl chloride and heated under reflux overnight leading to the formation of benzofuran-2-carbonyl chloride but the yield was not satisfactory (25%).

2nd attempt: this procedure was slightly modified by adding a drop of DMF (catalyst) and the reaction proceeds smoothly giving high yields (70%) within 5 hours.



73

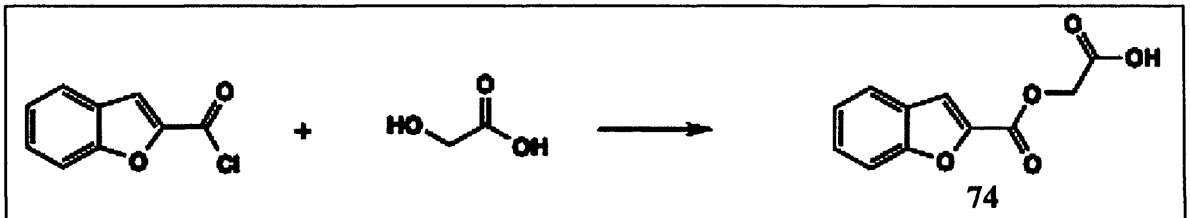
Reagents and conditions: A: $SOCl_2$, Reflux, 12 hrs (yield, 25%)
B: $SOCl_2$, DMF (1drop), reflux, 5hrs(yield, 70%)

Scheme 8

8.1.2.4.2.Synthesis of benzofuran-2-carbonyloxy-2-acetic acid (74):

This reaction is similar to the synthesis of **(69)**which is described above and therefore, the same procedure was employed whereby benzofuran-2-carbonyl chloride and glycolic

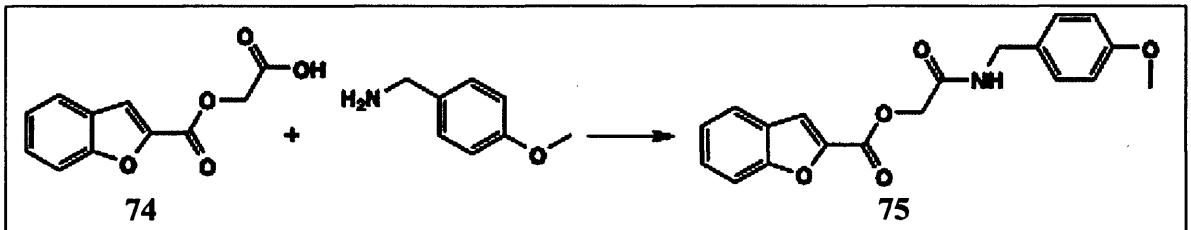
acid were stirred overnight in pyridine giving relatively good yield of product (60%) (Scheme 9).



Reagents and conditions s: Pyridine, 12 hrs.

Scheme 9

In the final step for the preparation of **(75)**, the previously used procedure for the preparation of**(70)** was employed (Scheme 10).

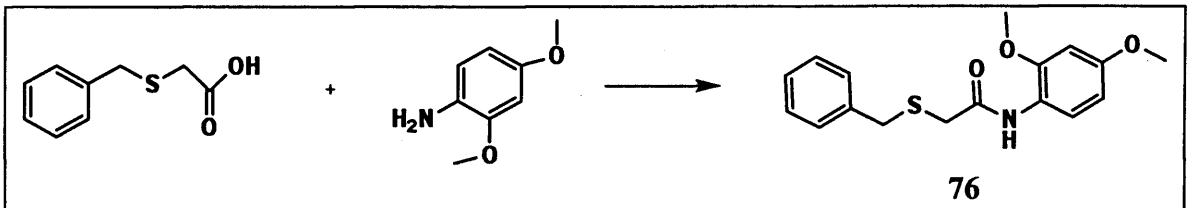


Reagents and conditions: CDI, DMF, 0°C, 1 hr; rt, 12 hr.

Scheme 10

8.1.2.5. Synthesis of 2-(benzylthio)-*N*-(2,4-dimethoxyphenyl)acetamide (76)

The synthesis of **(76)** was easily achieved using the commercially available *S*-benzylthioglycolic acid and 2,4-dimethoxyaniline following the procedure described for preparation of **(70)**in an excellent yield (83%)as outlined in Scheme 11.

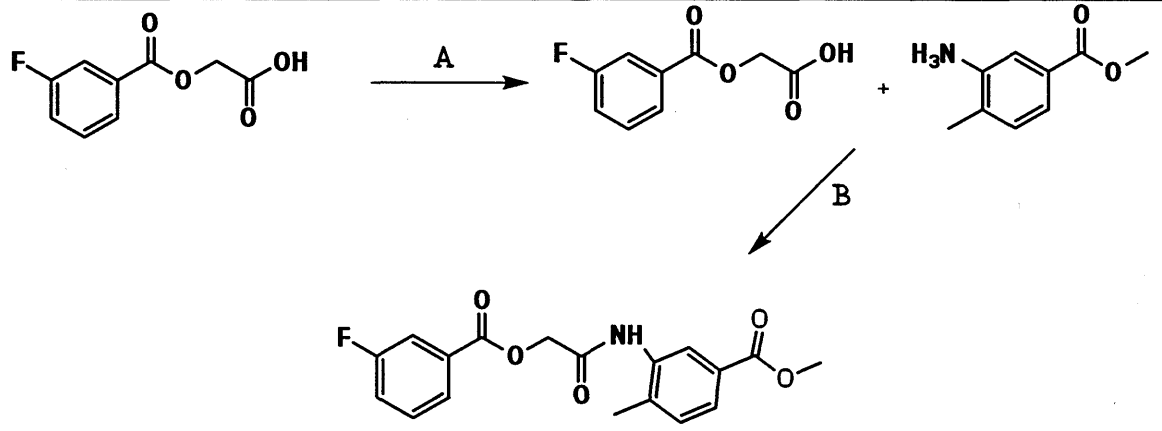


Reagents and conditions: CDI, DMF, 0°C, 1 hr; RT, 12 hr.

Scheme 11

8.1.2.6. Synthesis of methyl 3-(2-(3-fluorobenzoyloxy)acetamido)-4-methylbenzoate (77)

This product was prepared from 2-(3-fluorobenzoyloxy) acetic acid and methyl-3-amino-4-methylbenzoate as described for **(70)** (Scheme 12) in a relatively good yield (66%). The substrate (2-(3-fluorobenzoyloxy)acetic acid) was prepared from 3-fluorobenzoylchloride and glycolic acid (detailed synthesis in Chapter 10).



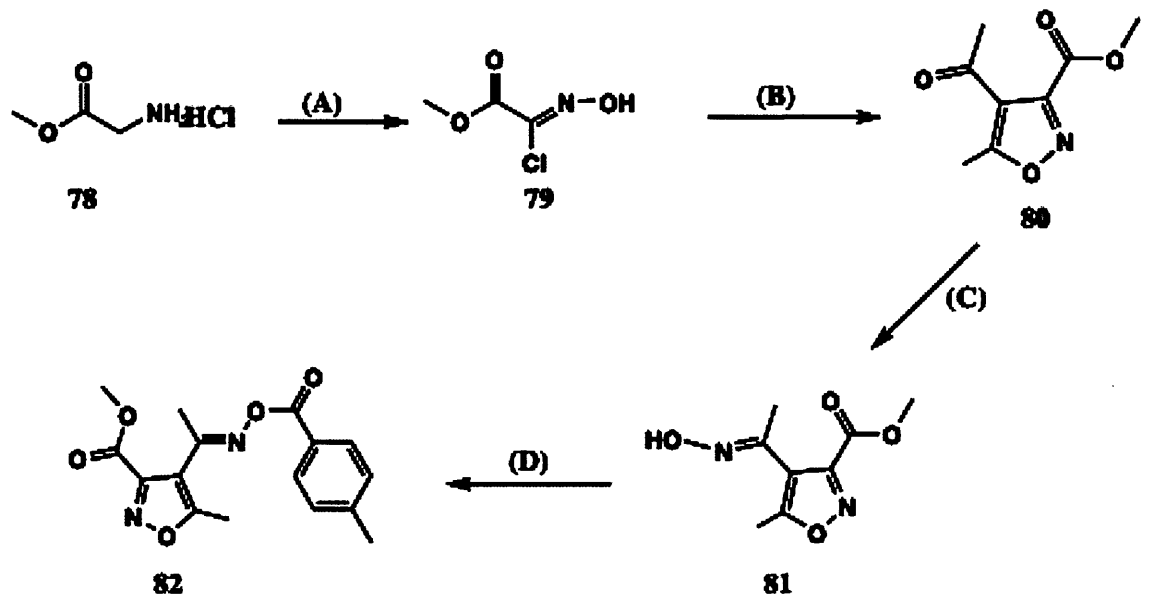
77

Reagents and conditions: A: Pyridine, ON, **B:**CDI, DMF, 0°C, 1 hr; rt, ON.

Scheme 12

8.1.2.7. Synthesis of methyl 5-methyl-4-(1-(4-methylbenzoyloxyimino)ethyl)isoxazole -3-carboxylate (82)

The synthesis of methyl 5-methyl-3- (1-(4-methylbenzoyloxyimino)ethyl)isoxazole-4-carboxylate **(82)** was achieved in a four-step process as represented in the following scheme (Scheme 13). It started with the synthesis of methyl 2-chloro-2-(hydroxyimino)acetate**(79)** which was cyclised to methyl 4-acetyl-5-methylisoxazole-3-carboxylate**(80)**and then reacted with hydroxylamine to give the corresponding oxime**(81)**. This latter was reacted with p-toluoyl chloride to provide the desired compound (48%).



Reagents and conditions: A: 2eq HCl, -5°C; 2eq aq $NaNO_2$; 0°C, 12 hrs.
B :Acetylacetone,NaOEt, EtOH, 2hrs.
C: $NH_2OH.HCl$, TEA, 1hr; EtOH, 80 °C.
D: p-toluoyl chloride,TEA, 0°C-1hr; RT, 12 hrs.

Scheme 13

8.1.2.7.1. *Synthesis of methyl 2-chloro-2- (hydroxyimino) acetate (79)*

(79) was prepared from the consecutive diazotization and reduction of the commercially available glycine methyl ester hydrochloride in acidic media using sodium nitrate[2] (Scheme 13-A).

8.1.2.7.2. *Synthesis of methyl 4-acetyl-5-methylisoxazole-3-carboxylate (80)*

1st attempt: methyl 4-acetyl-5-methylisoxazole-3-carboxylate was reported to be easily accessed from the one-step condensation of nitro malonyl and acetyl acetone at high temperature (170 °C) in high yields.[3] Moreover, the products were reported to be isolated in sufficient purity via distillation. However, when these conditions were attempted, poor yields were obtained and laborious purification was needed since different side products were formed.

2ndattempt: the synthesis of methyl 4-acetyl-5-methylisoxazole-3-carboxylate was successfully achieved from the previously prepared chloro-hydroxyliminoacetate and acetylacetone using conditions reported by Giovannoni and co-workers[4] (scheme 13-B).

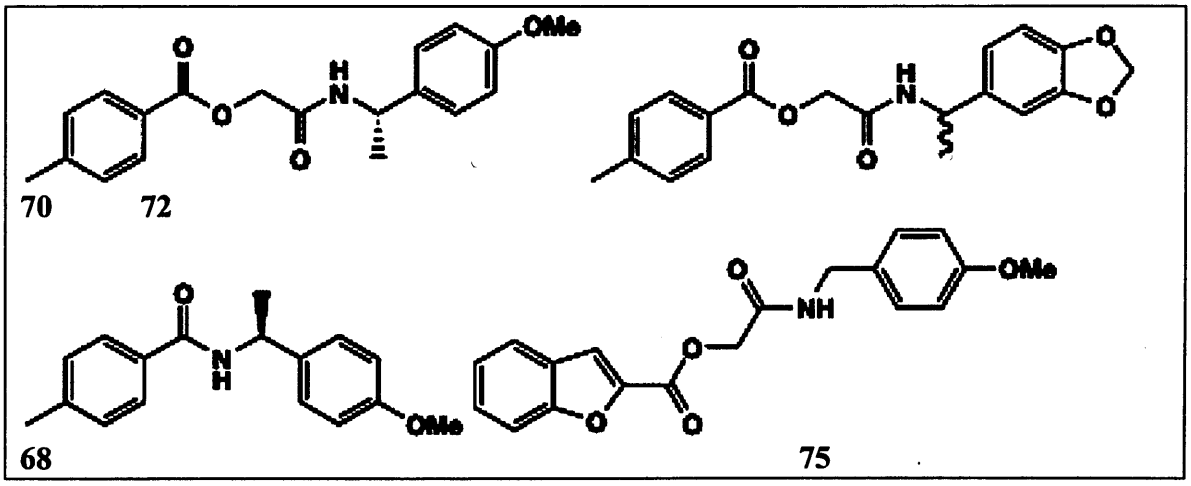
8.1.2.7.3. *Synthesis of methyl 4-(1-(hydroxyimino)ethyl-5-methylisoxazole-3-carboxylate (81)*

The synthesis of the oxime **(81)** was achieved in a straightforward method by treating compound **(80)** with hydroxylamine hydrochloride in a refluxing ethanol in the presence of TEA for a couple of hours (scheme 13-C).

8.1.2.7.4.*Synthesis of methyl 5-methyl-4- (1-(4methylbenzoyloxyimino)ethyl)isoxazole -3-carboxylate (82)*

This final step encompasses the acylation reaction between the previously prepared intermediate; **(81)** and p-toluoyl chloride (yield 48%). (Scheme 13-D).

Overall, 8 hits were successfully prepared in pure form and good quantities to conduct the necessary biological assays (Figure 4). Of these hits, compound **(83)** was commercially obtained. A 5 mg of this compound was purchased from Maybridge screening library as it was anticipated that any attempts for its preparation in the lab would be labor intensive and time consuming since several synthetic steps are required.



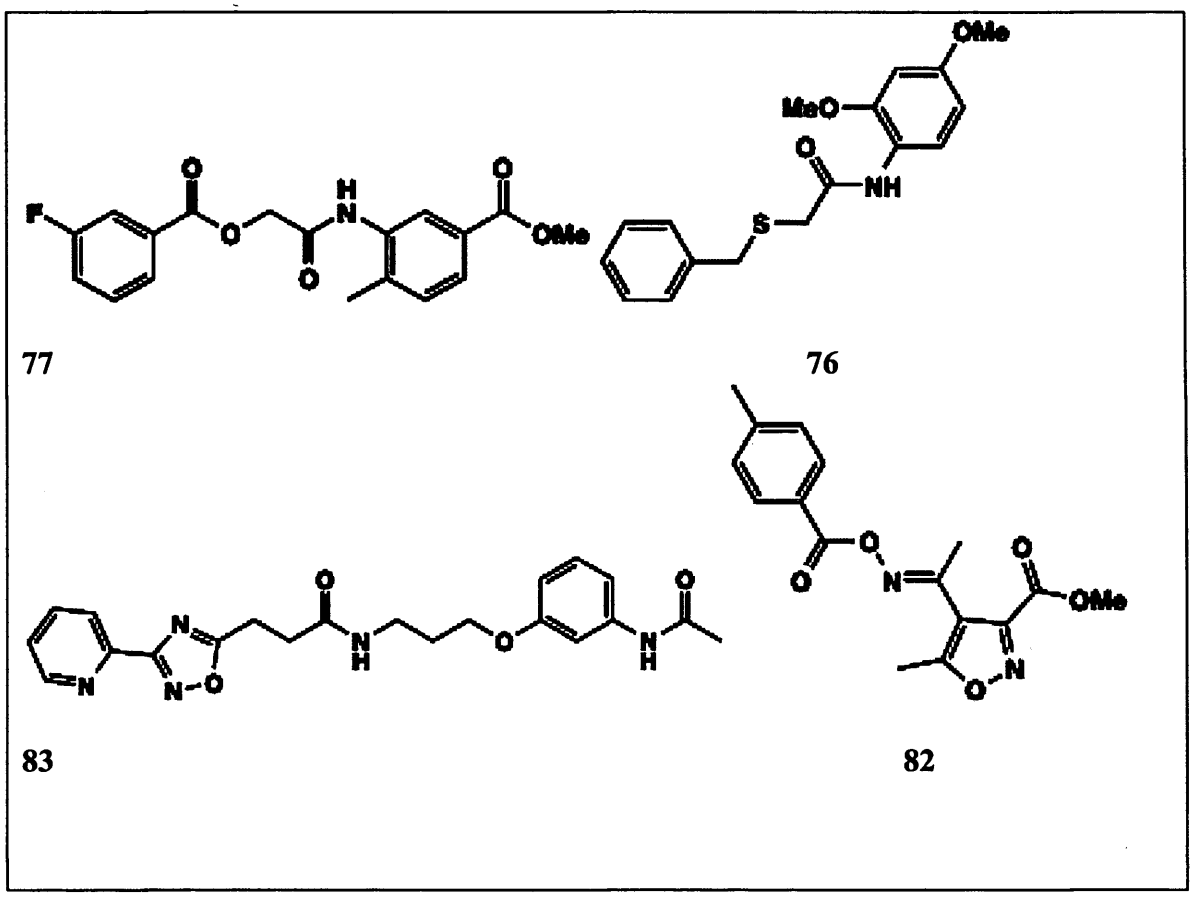


Figure 4: The structures of the synthesised potential hits obtained from the structure-based virtual screening studies (modified approach).

8.1.3.Synthesis of high throughput docking hits:

8.1.3.1. GFscore hits: The following 2 hits (Figure 5) represent the main structural scaffolds obtained from the molecular modelling studies using GFscore approach. While the synthesis of the first hit (isoindoline derivative) proved to be straightforward in one step from commercially available substrates, the second hit (isatin derivative) required a multi-step synthesis.

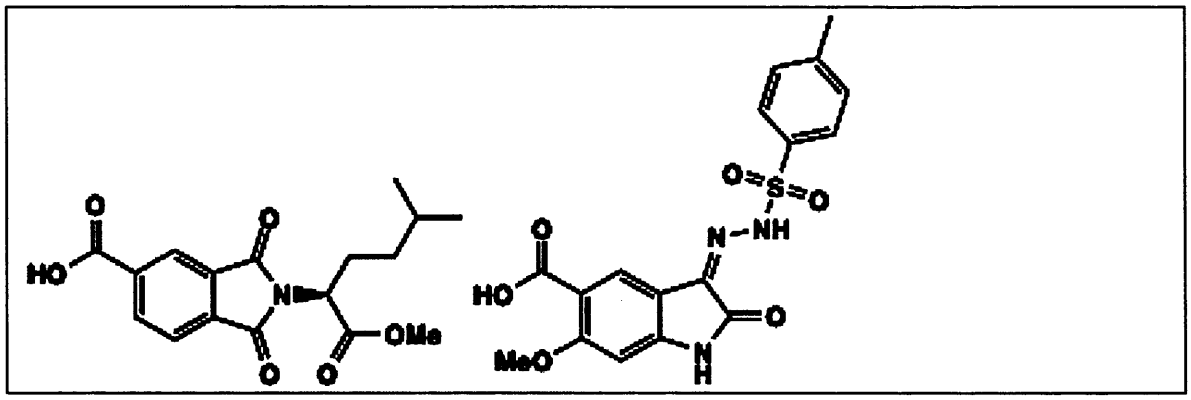
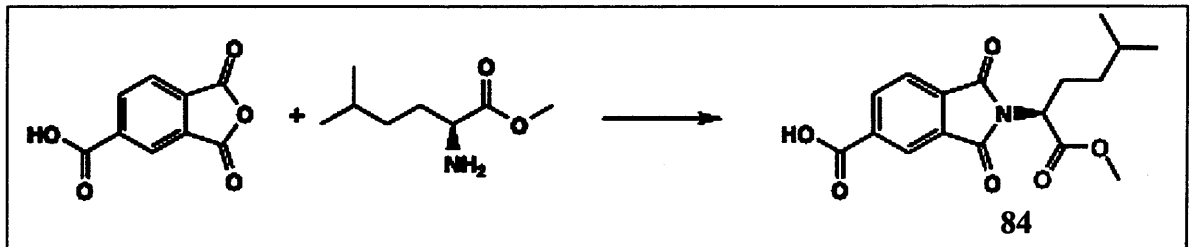


Figure 5: the hits obtained using GFscore approach.

8.1.3.1.1. Synthesis of methyl *(S)*-2-(1-methoxy-5-methyl-1-oxahexan-2-yl)-1,3-dioxo-isoindoline-5-carboxylic acid (84)

1st attempt: for the synthesis of **(84)**, 1,2,4-benzenetricarbocyclic acid anhydride and L-leucine methyl ester hydrochloride were heated under reflux in acetic acid overnight.[5] This procedure led to the formation of the desired product but in a very low yield (<15%) and thus a different synthetic route was investigated.

2nd attempt: it was reported that when using pyridine instead, the reaction gives better yields and proceeds smoothly (Scheme 17).[6] The same starting materials were employed and the use of pyridine in this case led to the formation of the desired product**(84)** in a very good yield (> 75%). Moreover, it was possible to purify the product through a straightforward column chromatography.



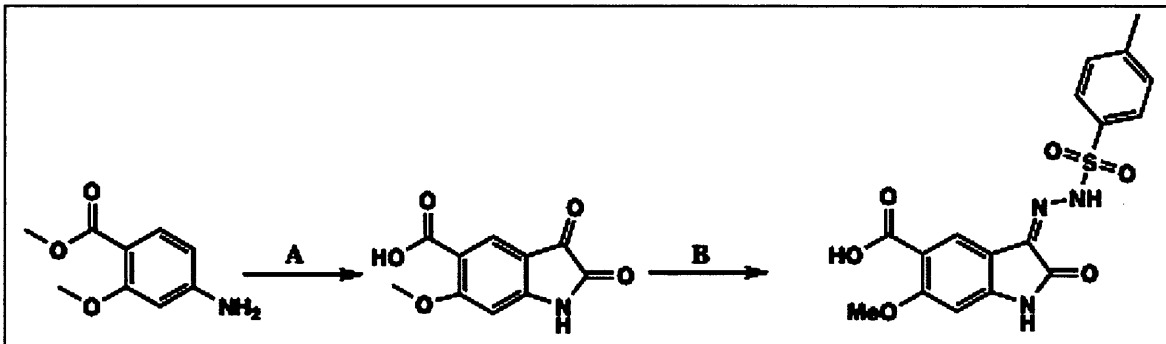
84

Reagents and conditions: Pyridine, Reflux, 12 hr.

Scheme 17

8.1.3.1.2. Synthesis of 6-methoxy-2-oxo-3-(2-tosylhydrazono)indoline-5-carboxylic acid (86)

The preparation of **(86)** was achieved over 2 main steps (Scheme 19).[7] The first step included the preparationof 6-methoxy-2,3-dioxoindoline-5-carboxylic acid (isatin) from methyl-4-amino-2-methoxybenzoate. This known as Sandmeyer isonitrosoacetanilide isatin synthesis, which involves cyclyzing the condensation product of the aniline, chloral hydrate and hydroxylamine in sulfuric acid at 90°C (yield 70%). This was accompanied by the hydrolysis of the methyl-ester to its corresponding carboxylic acid. In the second step, the isatin intermediate was reacted with p-toluenesulfonylhydrazide in a refluxing ethanol for 3 hours to give the title compound (40%).[8]



Reagents and conditions: A: $C_2H_3Cl_3O_2$, $NH_2OH.HCl$, sodium sulfate, aq HCl, 55 °C, 12 hrs; H_2SO_4, 90°C, 45mins.
B: EtOH, reflux, 3hrs.

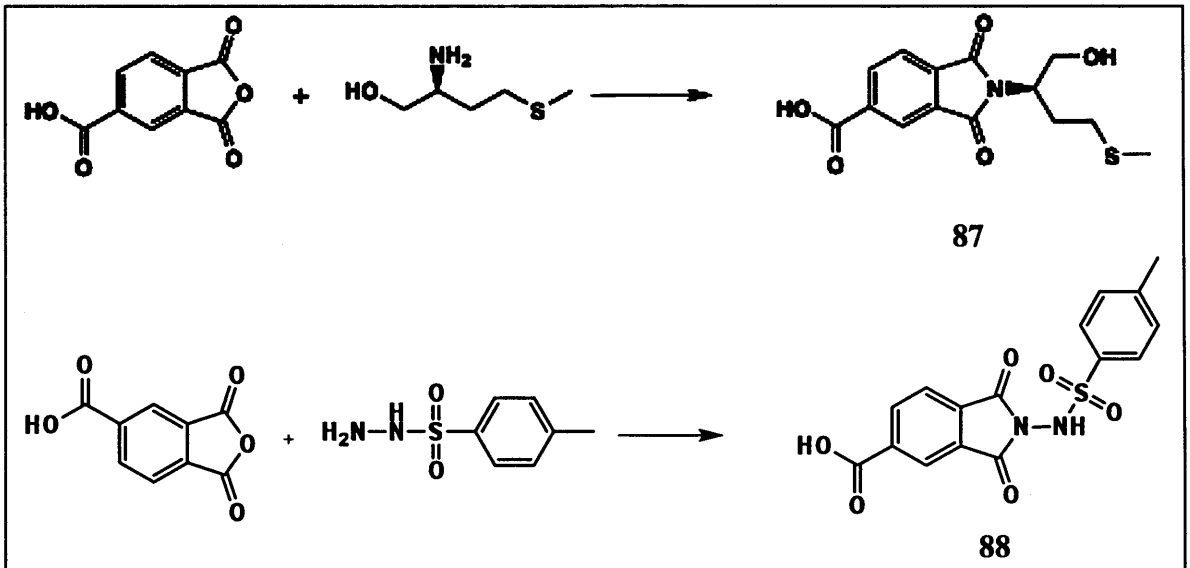
Scheme 19

Using these established synthetic routes, a further 2 indoline derivatives and 1 more isatin derivative were prepared.

8.1.3.1.3. Synthesis of methyl (S)-2-(1-hydroxy-4-methylthio)butan-2-yl)-1,3-dioxoindoline-5-carboxylic acid (87) and methyl 2-(4-methylphenylsulfonamido)-1, 3-dioxoisoindoline-5-carboxylic acid (88)

Both of these compounds were prepared as described above for**(84)**and as shown in Scheme 20.**(87)**was prepared from 1,2,4-benzenetricarbocyclic acid anhydride and *S*-

methioninol while **(88)** was prepared from 1,2,4-benzenetricarbocyclic acid anhydride and p-toluenesulfonylhydrazide.

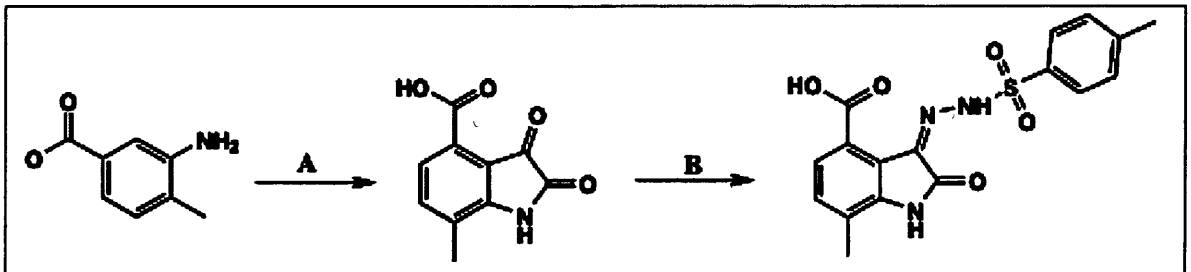


Reagents and conditions: Pyridine, Reflux, ON.

Scheme 20

8.1.3.1.4. Synthesis of 7-methyl-2-oxo-3-(2-tosylhydrazono)indoline-4-carboxylic acid (90)

Compound**(90)** was prepared from 7-methyl-2,3-dioxoindoline-4-carboxylic acid and p-toluenesulfonylhydrazide following the procedure described for the synthesis of **(86)** (yield 24 %) as represented in the following scheme (scheme 21).



89 **90**

Reagents and conditions: A: $C_2H_3Cl_3O_2$, $NH_2OH.HCl$, sodium sulfate, aq HCl, 55 °C, 12 hrs; H_2SO_4, 90°C, 45mins.
B: EtOH, reflux, 3hrs.

Scheme 21

Overall, the main compounds prepared based on GFscore modelling studies are represented in Figure 5.

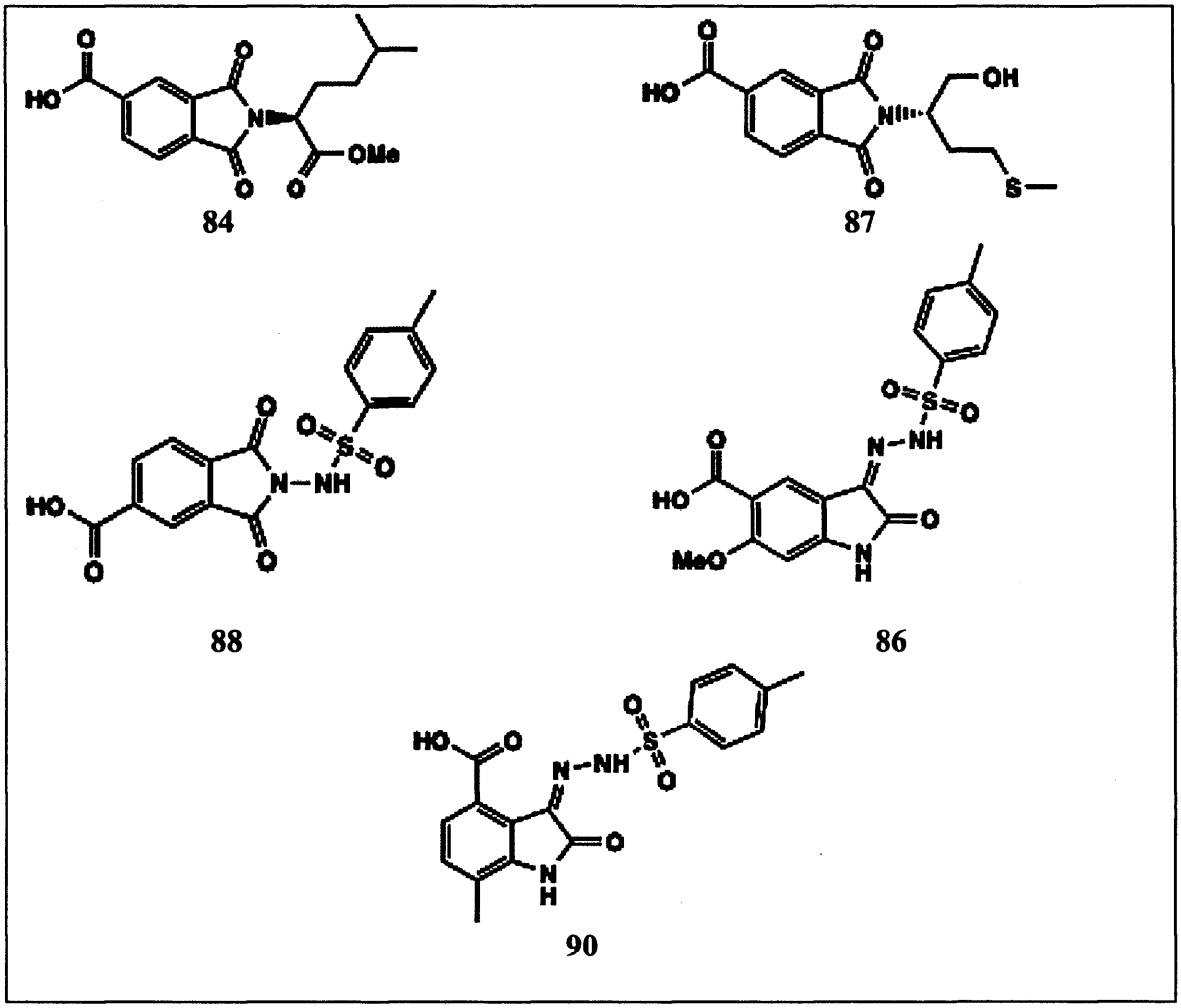


Figure 5: The compounds, which were synthesised based on GFscore modelling studies.

8.1.3.2. *PLANTS hits:*

The following 2 potential hits (Figure 6) were derived from high- throughput docking using PLANTS.Generally speaking, it can be said that these 2 hits have relatively similar structures and hence, efforts were focused on devising a synthetic route, which enables access to both structures in good yields.

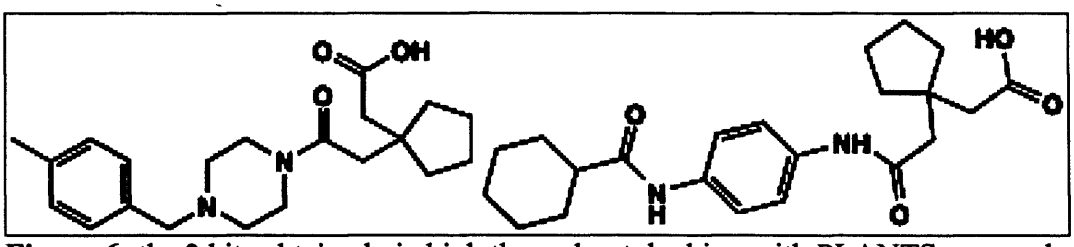
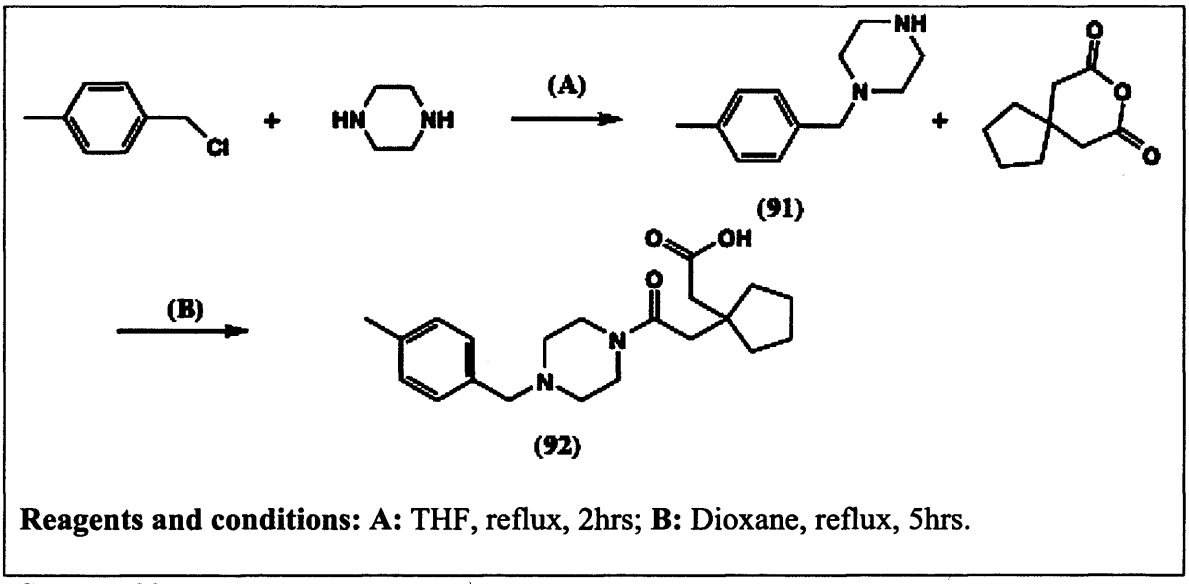


Figure 6: the 2 hits obtained via high throughput docking with PLANTS approach.

8.1.3.2.1. Synthesis of 2-(1-(2-(4-(4-methylbenzyl)piperazin-1-yl)-2oxoethyl)cyclo pentyl)acetic acid

Compound(**92**)was synthesised by preparingp-methylbenzylpiperazine(**91**)first and then reacting it with 3,3-tetramethyleneglutaric anhydride as illustrated in scheme 22.



Reagents and conditions: A: THF, reflux, 2hrs; **B:** Dioxane, reflux, 5hrs.

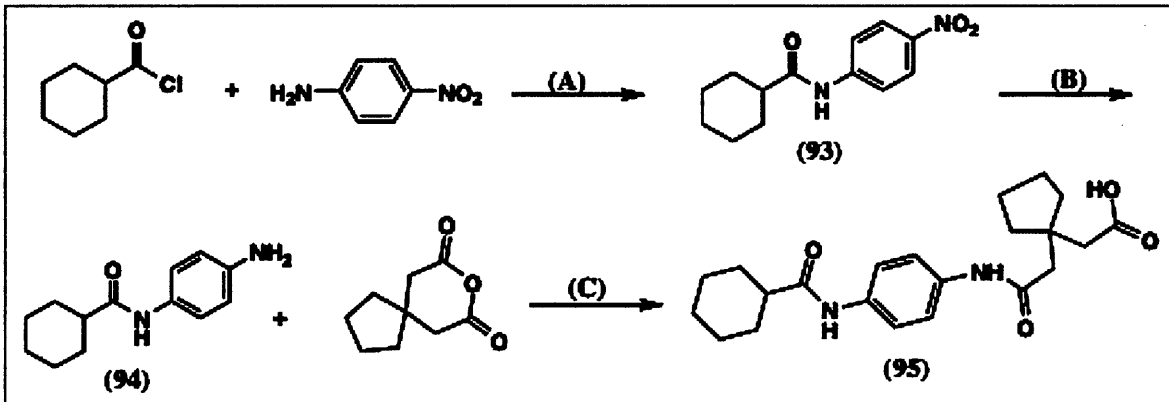
Scheme 22

p-Methylbenzylpiperazine(**91**) was prepared by reacting p-methylbenzylchloride with an excess of piperazine (2eq) (Scheme 22-A). The piperazine is first dissolved in THF and the temperature is reduced to 0°C. Methylbenzylchloride was then added and the solution was then heated under reflux for 2 hrs leading to the formation of (**91**), which was obtained in a pure form and good yield (79%) using column chromatography.

The synthesised piperazine **(91)**was then heated under reflux with the commercially available anhydride (3,3-tetramethyleneglutaric anhydride) in dioxane. The electron lone pair of the piperazine attacks the anhydride at one of the carbonyls leading to the opening of the ring and a free carboxylic acid. The crude product obtained was further purified using column chromatography using a mixture of hexane and ethylacetate (1:9) as eluent to give the desired compound (yield 66%)

8.1.3.2.2. Synthesis of 2-(1-(2-(4-(cyclohexanecarboxamido)phenylamino)-2-oxoethyl)cyclopentyl)acetic acid (95)

As mentioned before, the structure of this hit is mostly similar to the previously prepared compound **(92)** and therefore, a similar synthetic route was followed (Scheme 23).



Reagents and conditions: **A:** DMF, reflux, 115°C.

B: Fe, AcOH:EtOH, H_2O,reflux, 40 mins.

C: Dioxane, reflux, 5hrs.

Scheme 23

The synthesis consists of three main steps. It starts by reacting the *p*-nitrophenylaniline with cyclohexanecarbonyl chloride in refluxing DMF under inert gas conditions to obtain N-(4-nitrophenyl)cyclohexanecarboxamide in excellent yield (82%). This nitro compound was subsequently reduced to its amino counterpart (N-(4-aminophenyl)cyclohexanecarboxamide). There are many reported reagents and methods

for the reduction of nitro compounds. The most common ones are the catalytic hydrogenation over Pd/C or Raney nickel and the use of LAH, $SnCl_2$ or metal iron (Fe).[9,10] For this synthesis, the use of Fe in acidic media was chosen as the procedure requires mild conditions and highly tolerable to wide range of functional groups. It follows that N-(4-nitrophenyl)cyclohexanecarboxamide was heated under reflux in a mixture of EtOH:acetic acid (1 : 1) in the presence of excess amounts of Fe for 40 minutes (TLC monitored) to give N-(4-aminophenyl)cyclohexanecarboxamide in good yield (75%).

The third and final step was the formation of the amide bond as it was the case with the previous compound **(92)** by heating under reflux a mixture of N-(4-aminophenyl)cyclohexanecarboxamide with 3,3-tetramethyleneglutaricanhydride in dioxane to obtain the crude **(95)**.This latter was further purified using column chromatography (yield 54%).

8.2. Biology

8.2.1. Biochemical Binding Assays

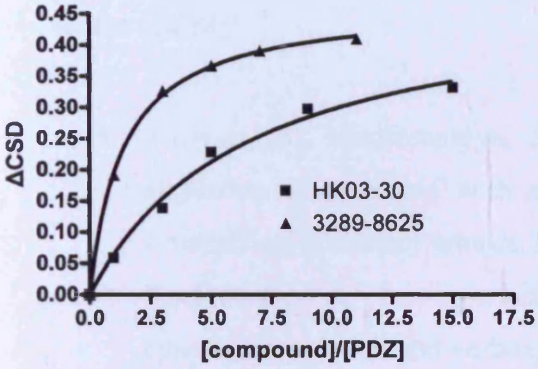
The biochemical binding assays were carried out in the laboratories of our collaborators in St. Jude Children's Research Hospital under the supervision of Prof Jie Zheng. They use NMR based experiments (^{15}N HSQC chemical-shift perturbation) to derive binding affinities (K_D) of PDZ ligands. A detailed description of the protocol followed is reported in previous publications by this research group.[11,12]

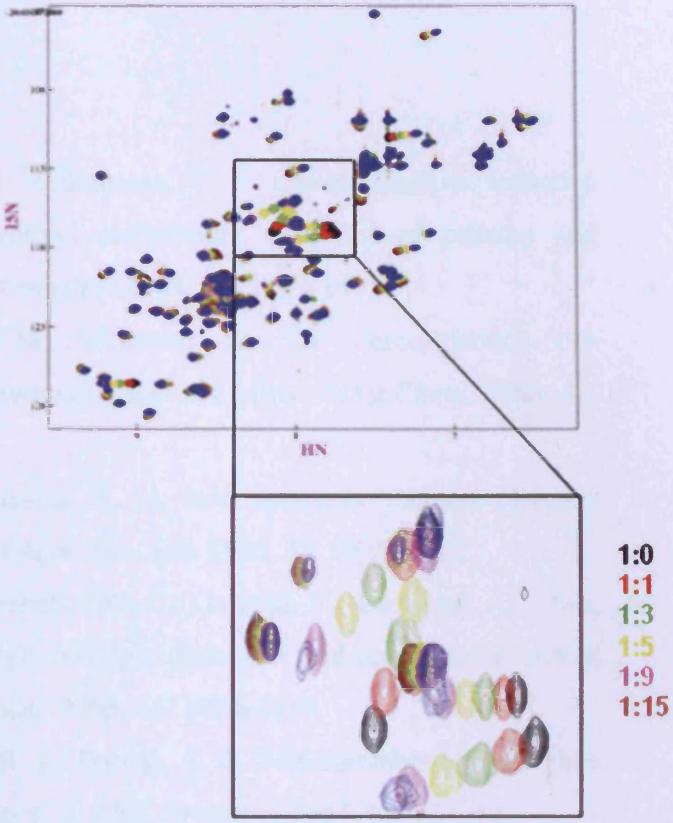
Compounds **(82)** and **(90)**, which showed promising activity in cell based assays as well as compounds **(92)**and **(95)**(good docking results) were chosen for testing using these assays.

8.2.2. Results

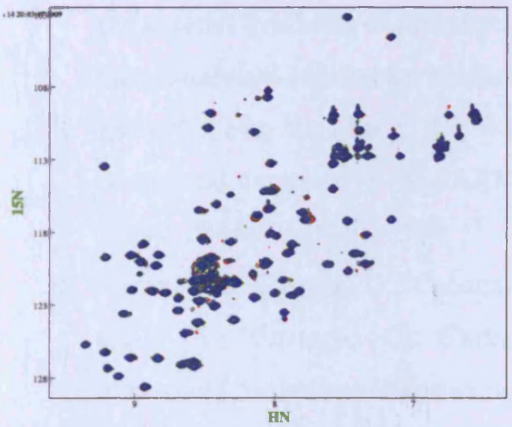
The main findings obtained from the NMR based experiments are illustrated in Figure 8.

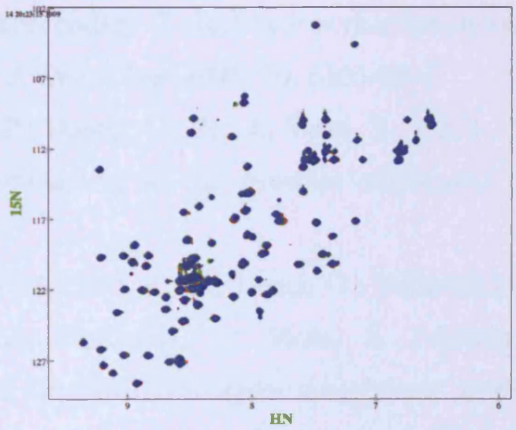
- **(82)** was not tested due to solubility problem (not soluble in buffer or in 20%DMSO).
- Both compounds **(92)**and **(90)** showed no obvious binding to the Dvl PDZ domain in the ^{1}H-^{15}N HSQC titration experiments.
- The hit compound **(95)** showed specific binding to the peptide-binding site of the Dvl PDZ domain with binding affinity in the low micromolar range as it is the case with compound **(3289-8625),** which is the most potent Dvl PDZ inhibitor reported so far. [13]





PDZ+(95)





PDZ+(92) **PDZ+(90)**

Figure 8: ^{15}N-HSQCspectra PDZ domain titrated with compounds **(90), (92)** and **(95)**. Spectra are colored by the protein to ligand ratios: black (1:0), red (1:1), green (1:3),yellow (1:5), cyan (1:9) and magneta (1:15).

References

1. Miriyala, B.; Bhattacharyya, S.; Williamson, J. S. Chemoselective reductive alkylation of ammonia with carbonyl compounds: synthesis of primary and symmetrical secondary amines.*Tetrahedron***2004**, 60, 1463-1471.
2. Kozikowski, A, P.; Adamcz, M. Methoads for the stereoselective cis-cyanohydroxylation and carboxyhydroxylation of olefins. *J.Org.Chem.* **1983,** 48, 366-372.
3. Shimizu, T.; Hayashi, Y.; Termaura, K. A new synthetic method of alkyl carnonocyanidate *N*-oxides. *Bull. Chem. Soc. Jpn.* **1985**. 58, 2519-2522.
4. Giovannoni, M,P.; Vergelli, C.; Ghelardini, C.; Galeotti, N.; Bartolini, A.; Piaz, V. D. [(3-Chlorophenyl)piperazinylpropyl]pyridazinones and analogues as potent antinociceptive agents. *J. Med. Chem.* **2003,** 46, 1055-1059.
5. Deka, K.; Barooah, N.; Sarma, R. J.; Baruah, J. B. Self-assembled carboxylate complexes of zinc, nickel and copper. *J. Mol. Structure*, **2007.** 827, 44-49.
6. Maillard, L. T.; Benohoud, M.; Durand, P.; Badet, B. A new supported reagent for the parallel synthesis of primary and secondary O-alkyl hydroxylamines through a base-catalyzed Mitsunobu reaction. *J. Org. Chem.***2005,** 70, 6303-6312.
7. Zhou, L.; Liu, Y.; Zhang, W.; Wei, P.; Huang, C.; Pei, J.; Yuan, Y.; Lai. L. Isatin compound as noncovalent SARS coronavirus 3C-like protease inhibitors.*J. Med. Chem.* **2006**, 49, 3440 – 344.
8. Varano, F.; Catarzi, D.; Colotta, V.; Calabri, F. R.; Lenzi, O.; Filacchioni, G.; Galli, A.; Costagli, C.; Carlà, V.; Deflorian, F.; Moro, S. 1-Substituted pyrazolo[1,5-c]quinazolines as novel Gly/NMDA receptor antagonists: synthesis, biological evaluation and molecular modeling study. *Bioorg. Med. Chem.***2005,** 13, 5536-5549.
9. Tafesh, A. M.; Weiguny, J. Review of the selective catalytic reduction of aromatic nitro compounds into aromatic amines, isocyanates, carbamates, and ureas using CO. *Chem. Rev.***1996**, 96, 2035–2052.
10. Fox, B. A.; Threlfall, T. L.2,3-Diaminopyridine. *Org. Synth.***1973**. Coll. Vol. 346.

11. Shan, J.; Shi, D. L.; Wang, J.; Zheng, J. Identification of a specific inhibitor of the dishevelled PDZ domain. *Biochem.* **2005**, 44, 15495-15503.

12. Shan, J.; Zheng, J. Optimizing Dvl PDZ domain inhibitor by exploring chemical space. *J. Comput. Aided. Mol. Des.***2009**, 23, 37-47.

13. Grandy, D.; Shan, J.; Zhang, X.; Rao, S.; Akunuru, S.; Li, H.; Zheng, Y.; Alpatov, I.; Zhang, X. A.; Lang, R.; Shi, D.L.; Zheng, J. J. Discovery and characterization of a small molecule inhibitor of the PDZ domain of Dishevelled. *J. Biol. Chem.***2009.** 284:16256-63,

9. The design and synthesis of analogues for the hit compound (95)

The biochemical binding assays showed that one of the potential hits obtained from molecular modelling studies namely 2-(1-(2-(4-(cyclohexanecarboxamido)phenylamino)-2-oxoethyl)cyclopentyl)acetic acid **(95)** (Figure 1) binds to the Dvl PDZ domain. Therefore, the next step was to implement a molecular dynamic (MD) simulation to explore the mode of binding of this compound, which in turn enables the synthesis of structural analogues with better binding affinity.

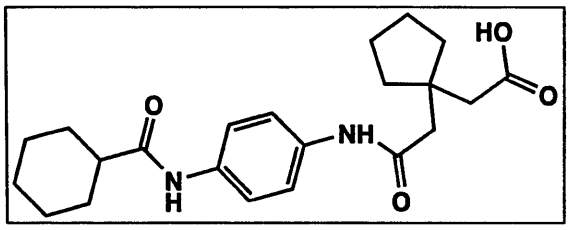


Figure 1: the chemical structure of **(95)**

9.1. Molecular modelling studies

Molecular dynamics (MD) is a sub-discipline of molecular modelling, which is centered on computationally simulating the motion of atoms, and molecules.[1,2] MD is widely used in different fields and frequently in the study of proteins and biomolecules. During MD simulation, molecules and atoms are allowed to interact for a period of time in an effort to generate a conformational search in space, or trajectory, under specified thermodynamic conditions such as constant temperature or constant pressure. This trajectory is important for two main reasons. The first is the fact that it allows calculation of the thermodynamic properties of a system by providing configurational and momentum information for each atom. The second reason is that it represents an exploration of the conformation space available to a particular system.

Short-running MD studies of the complex between the Dvl PDZ domain and **(95)** were performed using MOE to further investigate their interaction.[3] The starting structures of

ligand-protein complexes were prepared using the output from the PLANTS docking studies. The partial charges were calculated and the energy of the molecular system was minimized to RMS gradient of 0.1. Using the dynamics panel, constant volume (NVT) ensemble simulations were carried out.

Figure 2 shows the conformation of the complex ((**95**) -Dvl PDZ) with the lowest free energy of binding.

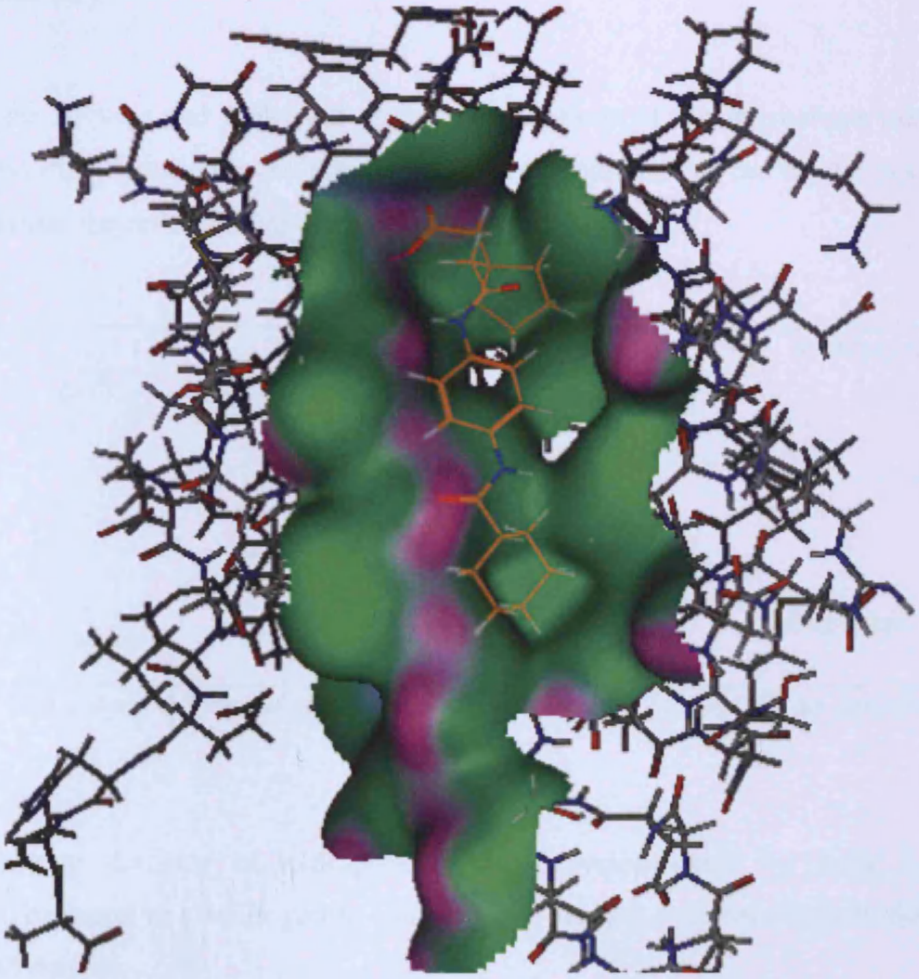


Figure 2: the conformation of the complex (Dvl PDZ- ligand 95) with the lowest free energy of binding from the performed MD simulations.

The main finding of this short-running MD simulation is that **(95)** adopts an extended binding pose along the active site of the Dvl PDZ domain, which supports the docking results, obtained by PLANTS. The carboxylic acid group of **(95)** is in close proximity with residues Ile 267, Gly 266 and Leu 265. On the other hand, while the cyclopentane part of **(95)** structure resides deeply in a hydrophobic cavity of the active site, the cyclohexane part is more exposed to the solvent.

9.2. Chemistry

Based on the docking and molecular dynamic simulations of the interactions between **(95)** and Dvl PDZ domain, several potential structural modifications can be introduced to further optimize the activity of this ligand as illustrated in Figure 3.

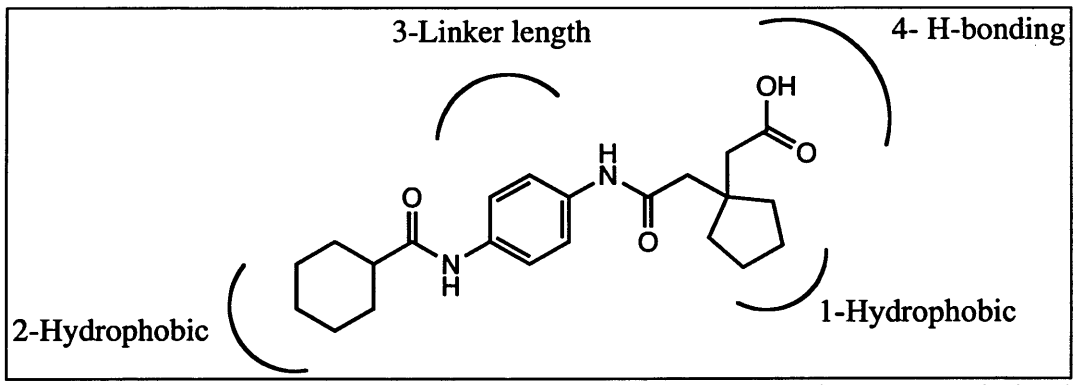
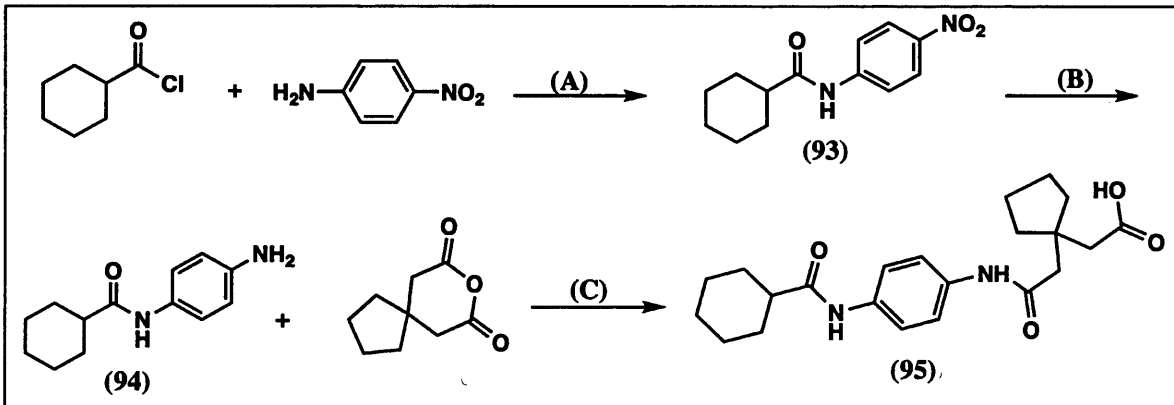


Figure 3: The potential areas of structural modifications to ligand **(95)** to optimize its activity.

1. Changing the size of hydrophobic group (cyclopentane) by using bigger (cyclohexane) or smaller groups (dimethyl) to study the effects on the binding to PDZ domain.
2. Changing the size of hydrophobic group (cyclohexane) by incorporating bigger (methylbenzene) or smaller (methyl) hydrophobic groups.

3. *The linker group:* the aniline group acts as a linker group connecting both hydrophobic parts (cyclopentane side and cyclohexane) of the structure. Hence, any change in this linker will force the molecule into adopting a different binding mode. The main aim of varying this group is to achieve another hydrogen bonding with residue Ile269 of the PDZ domain as seen for Dapper bound Dvl PDZ (refer to Figure 3, Chapter 7).
4. *Ester derivatives:* the docking as well as MD studies showed that the carboxylic acid moiety is essential for mediating the interaction between the Dvl PDZ and ligand **(95)**. Nevertheless, by masking the carboxylic acid moiety of the compound, the ester derivatives are more likely to be taken into cells by crossing membranes.

The preparation of these novel analogues with the desired structural modifications can mainly be achieved using the same synthetic steps followed in the synthesis of **(95)** as represented in Scheme 1. It is worthwhile to mention that this synthesis was discussed in detail in the previous chapter (Chapter 8).



Reagents and conditions: **A:** DMF, reflux, 115 °C.

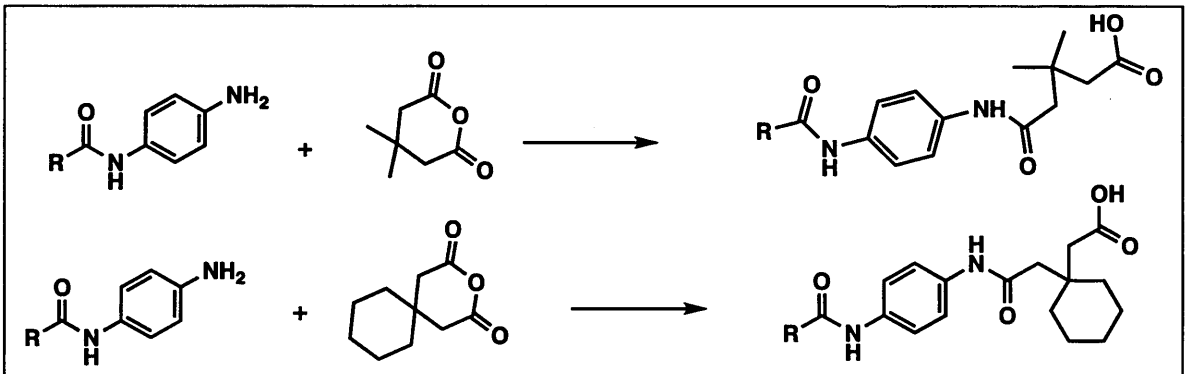
B: Fe, AcOH:EtOH, H_2O, reflux, 40 mins.

C: Dioxane, reflux, 5hrs.

Scheme 1

Nevertheless, key changes to this synthetic route in the preparation of **(95)** analogues will be discussed for each of the potential four areas of structural modifications highlighted above.

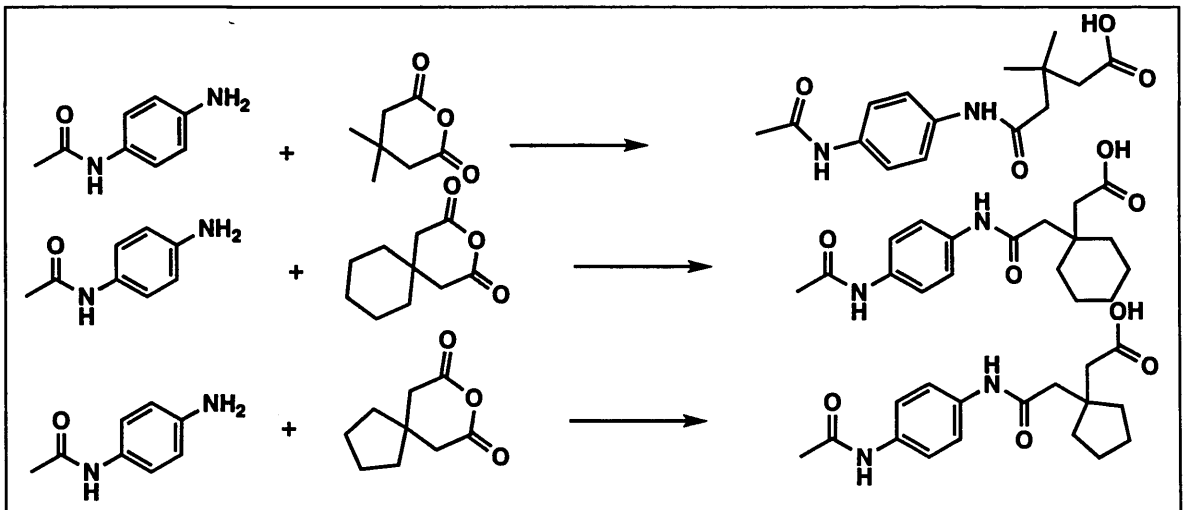
1: The hydrophobic moiety (cyclopentane): The main modifications to this part of the structure involved the use of dimethyl or the cyclohexane groups as replacement for cyclopentane. These two groups were mainly introduced by the use of the appropriate anhydride; 3,3-dimethylglutaric anhydride or cyclohexylglutaric anhydride (3-oxaspiro[5,5]undecane-2,4-dione) which when reacted with the corresponding aniline leads to the opening of the cyclic anhydride ring and subsequently formation of the desired product as shown in Scheme 2. The overall yields were comparable to those obtained for **(95)** (yield 52 - 61 %). The chemical structures of compounds prepared using this procedure are shown in Figure 4).



Reagents and conditions: Dioxane, Reflux, 5hrs.

Scheme 2

2: The hydrophobic moiety (cyclohexane): The use of smaller hydrophobic group in the form of methyl can be easily achieved by the use of acetyl chloride instead of cyclohexanecarbonyl chloride used in the synthesis of **(95)**. However, it was more cost and time effective to purchase *N-(*4-aminophenyl)acetamide and react it directly with the anhydride to give the final product in a one step process (Scheme 3). This resulted in excellent yields (> 80 %) as this procedure overcomes the need for the formation of a nitro substrate and its subsequent reduction.

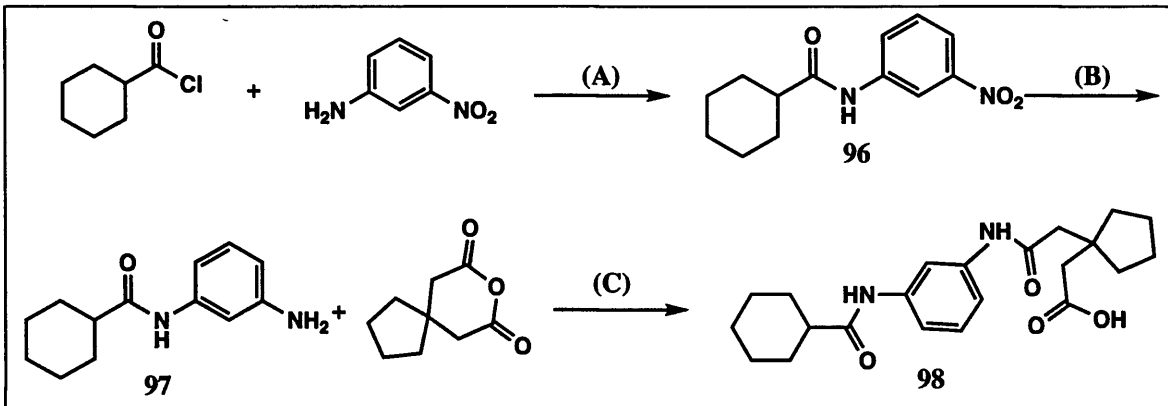


Reagents and conditions: Dioxane, Reflux, 5hrs.

Scheme 3

In addition to the use of methyl group, a methylbenzene (toluene) moiety was successfully introduced to the structure as a replacement for the cyclohexane. This work will be discussed within the next category of structural modifications, which is concerned with changes of linker group.

3: ***The linker group (benzene-1,4-diamine):*** The first change was simply to use the meta derivative of the diamine (benzene-1,4-diamine) linker. Hence, the same synthetic procedure was repeated with the only difference being the use of 3-nitroaniline as a starting substrate. For instance, the preparation of the structural isomer of **(95)** is shown in Scheme 4.



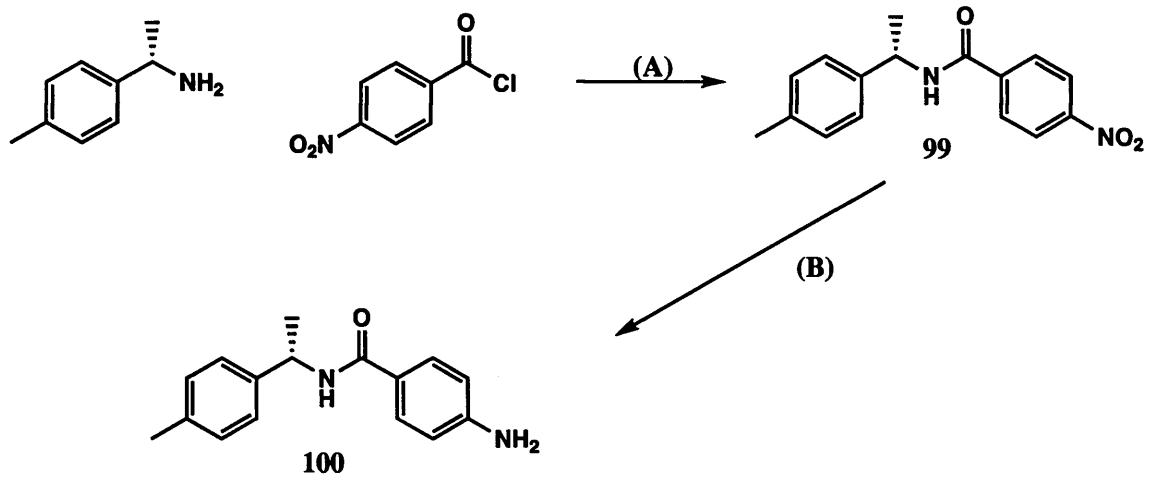
Reagents and conditions: **A:** DMF, reflux, 115 °C.

B: Fe, AcOH:EtOH, H_2O, reflux, 40 mins.

C: Dioxane, reflux, 5hrs.

Scheme 4

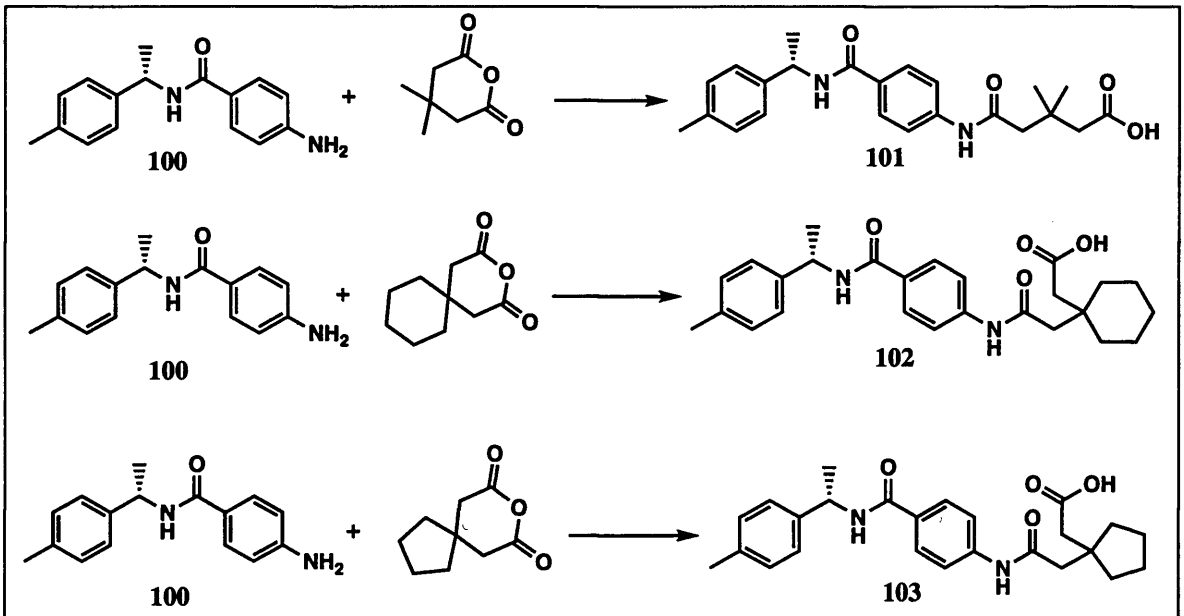
The other modification to the linking group was to use p-nitrobenzoylchloride instead of benzene-1,4-diamine. The two linking groups are similar in that they have the nitro group which has to be reduced before undergoing a condensation reaction with the anhydride. The only difference is that p-nitrobenzoylchloride acts initially as an electrophile because of the presence of the highly reactive carbonyl chloride. This allows the use of different nucleophiles to introduce a hydrophobic moiety replacement to the cyclohexane (second area of modifications). For instance, the preparation of (*S*)-4-amino-*N*-(1-*p*-tolylethyl)benzamide is illustrated in Scheme 5. The same reagents and conditions used in the synthesis of **(95)** are applied in this procedure. It follows that *p*-tolylethanamine is reacted with a slight excess of *p*-nitrobenzoylchloride to give a nitro intermediate which is subsequently reduced to the corresponding amine (*S*)-4-amino-*N*-(1-*p*-tolylethyl)benzamide)This latter was then reacted with different glutaric anhydrides to give novel **(95)** derivatives as shown in Scheme 6.



Reagents and conditions: A: DMF, reflux, 115 °C.

B: Fe, AcOH:EtOH, H_2O, reflux, 40 mins.

Scheme 5



Reagents and conditions: Dioxane, reflux, 5hrs.

Scheme 6

Overall, considering these three potential areas of structural modifications, the following (**95**) analogues were successfully prepared (yield 50-70%) (Figure 4).

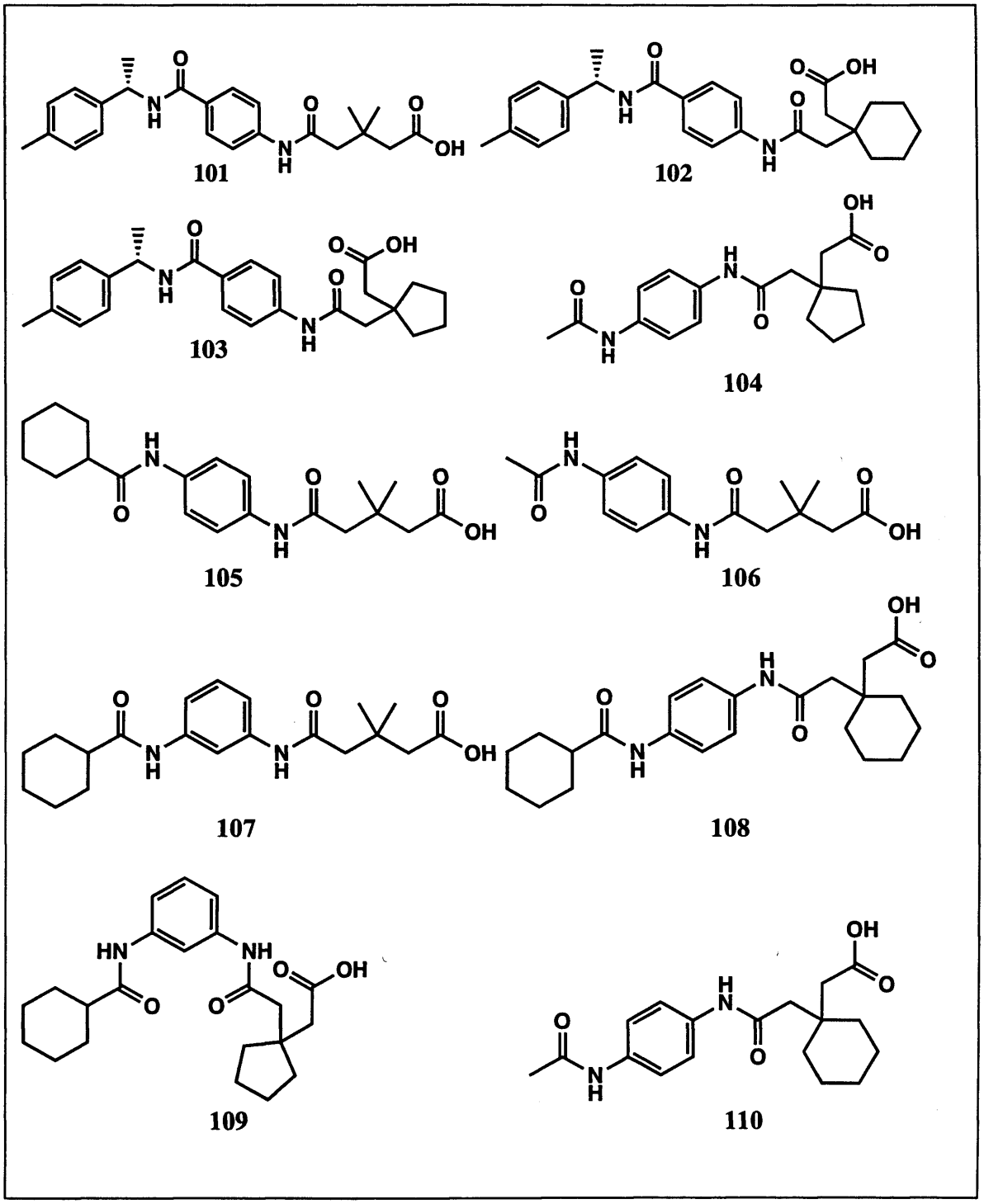


Figure 4: The synthesised novel analogues of **(95)**.

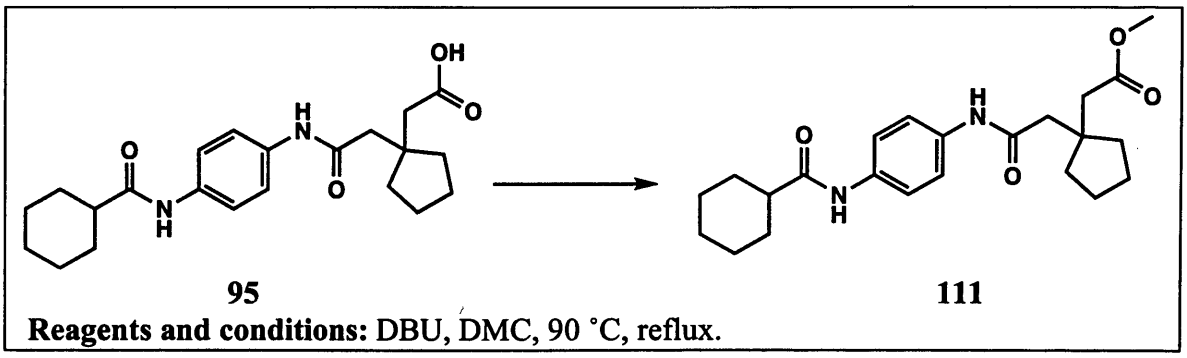
4. The synthesis of ester derivatives

The simplest ester derivatives of these compounds are their methylester counterparts. These ester derivatives can be obtained through esterification of the already prepared carboxylic acid compounds (Figure 2). There are many esterification methods using different reagents and under different conditions described in literature.

4.1. The synthesis of methyl 2-(1-(2-(4-(cyclohexanecarboxamido)phenylamino)-2-oxoethyl)cyclopentyl)acetate:

1st attempt: 1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU) was reported as an effective nucleophilic catalyst for carboxylic acid esterification with dimethyl carbonate (DMC).[4] Excellent yields (> 90%) were reported for various carboxylic acids to be obtained from a simple aqueous work-up and acid washings. It is thought that that DBU reacts with DMC forming an unstable carbamate, which acts as a highly effective methylating agent for the carboxylic acid.

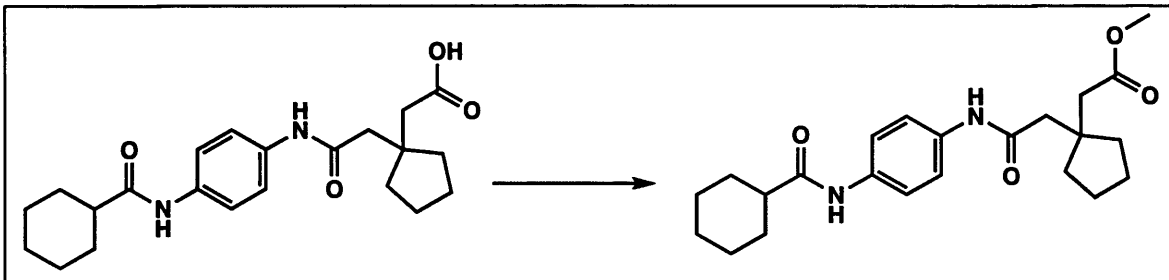
The procedure involves the addition of 1 equivalent of DBU to 10% solution of **(95)** in DMC and heating under reflux for 8 hours (Scheme 7). Upon completion (TLC monitored), the reaction was cooled to room temperature and diluted with EtOAc and H_2O. The organic layer was then washed with H_2O, twice with 2M HCl and twice with $NaHCO_3$ and twice with H_2O once more. Then, it was dried over $MgSO_4$, filtered and concentrated *in vacuo*. The crude product showed a mixture of components and needed further purification in form of column chromatography but the overall yield was poor (< 15%).



Reagents and conditions: DBU, DMC, 90 °C, reflux.

Scheme 7

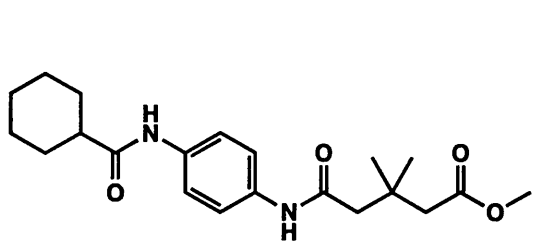
2nd attempt: this time, the carboxylic acid was heated under reflux in methanol in the presence of trimethylsilyl chloride (Scheme 8).[5] After the reaction is completed (TLC monitored), the solvent was removed *in vacuo* giving the crude product in sufficient purity and good yields (78 %).

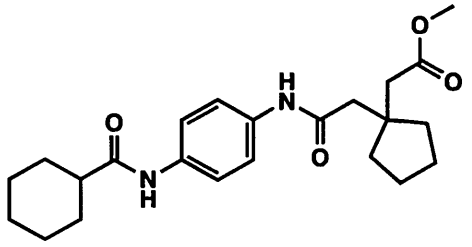


Reagents and conditions: TMSCl, MeOH, 69°C, reflux.

Scheme 8

This synthetic route was then used for the preparation of other methylester derivatives as shown in Figure 5.





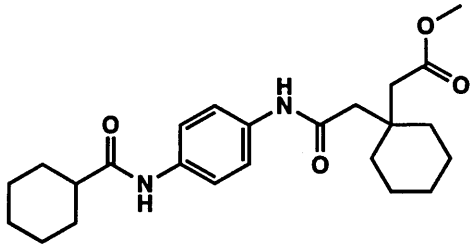


Figure 5: The chemical structures of the prepared methylester derivatives.

9.3. Biology

The new series of the synthesised **(95)** analogues were sent to our collaborators in the Netherlands under the supervision of Prof Jan Paul Medema (University of Amsterdam) to study their inhibition of the Wnt signaling pathway using specific cell lines developed for this purpose. Based on the cell line data, the most promising compounds will be sent for biochemical binding assays (Prof J Zheng, St. Jude Hospital, University of Tennessee).

References

1. Leach, A. R. Molecular modelling: principles and applications. *Pearson Education, 2nd Edition.* **2001.**
2. Leach, A. R. Introduction to computational chemistry. *Longman.* **1996.**
3. Molecular Operating Environment (MOE 2008.10). Chemical Computing Group, Inc. Montreal, Quebec, Canada. (http://www.chemcomp.com/)
4. Shieh, W. C.; Dell, S.; Repič, O. Nucleophilic catalysis with 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) for the esterification of carboxylic acids with dimethyl carbonate. *J. Org. Chem.* **2002,** 67, 2188-219.
5. Li, J.; Sha, Y. A convenient synthesis of amino acid methyl esters. *Molecules* **2008,** 13, 1111-1119.

10. EXPERIMENTAL

10.1. General experimental details

Melting points were measured on a Griffin apparatus and are uncorrected. All commercially available starting materials used were purchased from Aldrich without further purification. All glassware was oven dried at 60°C for several hours or overnight.

Chromatography

Thin Layer Chromatography (TLC) was performed using precoated, aluminum backed silica gel plates (TLC Silica gel 60 F_{254}-Merck) and an ultra-violet (UV) lamp was used for visualisation (254nm). For column chromatography, silica gel (Silica 60A particle size 35-70 micron, Fisher Scientific) was used.

NMR spectroscopy

^{1}H, and ^{13}C NMR were recorded on a Bruker Avance 500 spectrometer with operating frequencies of 500, and 125 MHz respectively. The following abbreviations are used in the assignment of NMR signals: s (singlet), d (doublet), t (triplet), m (multiplet). *J* coupling constants were calculated on the Topspin program and are reported Hertz (Hz).

Mass Spectroscopy

Mass spectra were determined under electroscopy conditions on a Bruker MicroTOF LC instrument at the Welsh School of Pharmacy, Cardiff, or at the Chemistry Department, Cardiff University.

CHN Analysis

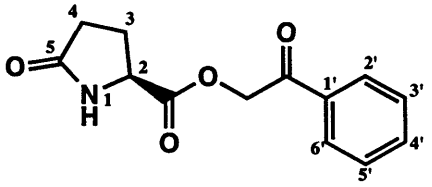
CHN analysis was carried out by MEDAC. The data were recorded on a Carlo-Erba EA 1108 element analyser, PC based data system, Eager 200 for Windows and a Sartorius Ultra Micro balance 4504MP8.

10.2. Synthesised compounds:

General procedure I:

A mixture of pyrrolidinonecarboxylic acid or hydroxynicotinic acid (3.88 mmol), (substituted) 2-bromoacetophenone (3,88 mmol) and TEA (1.1mL) in acetone (20 mL) was heated under reflux for 3 hours. After completion of the reaction (monitored by TLC), the solvent was evaporated and the residue was extracted using EtOAc, dried over $MgSO_4$ and concentrated in vacuo. The obtained residue was purified by column chromatography (90% EtOAc/10% Hexane) to give the title compound as a white solid. The following compounds were prepared using this procedure.

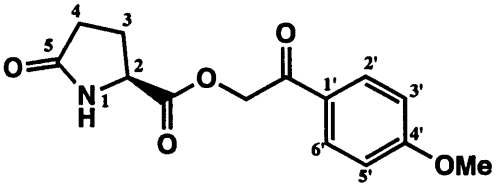
(*S*)-2-Oxo-2-phenylethyl-5-oxopyrrolidine-2-carboxylate (63)



$C_{13}H_{13}NO_4$, MW: 247.25

Prepared from (*S*)-2-pyrrolidinonecarboxylic acid and 2-bromoacetophenone (yield 63%). **^{1}H NMR ($CDCl_3$):** δ 2.41-2.53 (4H, m, H-3, 4), 4.45 (1H, m, H-2), 5.43 (2H, s, CH_2), 6.23 (1H, bs, NH), 7.52 (2H, m, Ar-H), 7.64 (1H, m, Ar-H), 7.91 (2H, m, Ar-H). **^{13}C NMR ($CDCl_3$):** δ 25.11, 29.17, 55.24, 66.65, 127.73, 127.73, 128.99, 128.99, 133.84, 134.19, 171.80, 177.94, 191.25. **ESI MS** m/z 270.06 [M+Na$^+$].

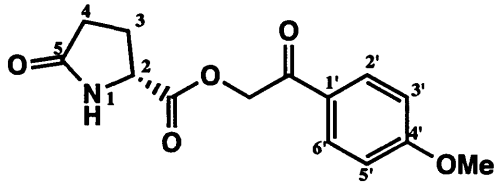
(*S*)-2-(4-Methoxyphenyl)-2-oxoethyl-5-oxopyrrolidine-2-carboxylate (64)



$C_{14}H_{15}NO_5$, MW: 277.27

Prepared from (*S*)-2-pyrrolidinonecarboxylic acid and 2-bromo-4'-methoxyacetophenone (yield 61%). **^{1}H NMR ($CDCl_3$):** δ 2.41-2.53 (4H, m, H-3, 4), 3.91 (3H, s, OCH_3), 4.46 (1H, m, H-2), 5.42 (2H, s, CH_2), 6.14 (1H, bs, N-H), 6,98 (2H, d, *J* 9.0 Hz, H-3', 5'), 7.90 (2H, d, *J* 9.0 Hz, H-2', 6'). **^{13}CNMR($CDCl_3$):**δ 25.10, 28.98, 55.17, 55.57, 66.30, 114.21 114.21,126.82, 130.04, 130.04, 164.32, 172.12, 177.96, 189.70. **ESI MS** m/z 300.06 [M+Na$^+$].

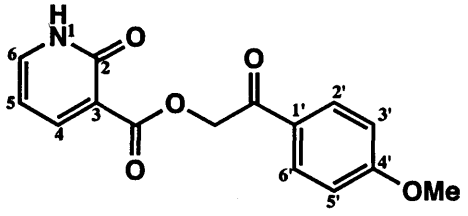
(*R*)-2-(4-Methoxyphenyl)-2-oxoethyl-5-oxopyrrolidine-2-carboxylate (65)



$C_{14}H_{15}NO_5$, MW: 277.27

Prepared from (*R*)-2-pyrrolidinonecarboxylic acid and 2-bromo-4'-methoxyacetophenone (yield 67 %). **^{1}H NMR ($CDCl_3$):** δ 2.41-2.53 (4H, m, H-3, 4), 3.91 (3H, s, OCH_3), 4.45 (1H, m, H-2), 5.42 (2H, s, CH_2), 6.14 (1H, bs, N-H), 6,98 (2H, d, *J* 9.0 Hz, H-3', 5'), 7.90 (2H, d, *J* 9.0 Hz, H-2', 6'). **^{13}C NMR ($CDCl_3$):** δ 25.11, 29.02, 55.23, 55.56, 66.29, 114.20, 114.20,126.83,130.04, 130.04, 164.30, 171.85, 177.92, 189.64. **ESI MS** m/z 300.06 [M+Na$^+$].

2-(4-Methoxyphenyl)-2-oxoethyl-2-oxo-1,2-dihydropridine-3-carboxylate (66)

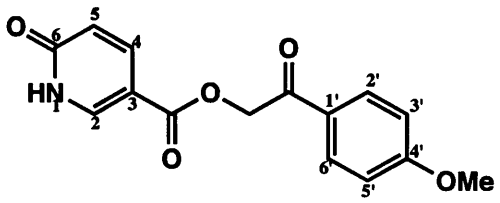


$C_{15}H_{13}NO_5$, MW: 287.27

Prepared from 2-hydroxynicotinic acid and 2-bromo-4'-methoxyacetophenone (yield 52 %). **^{1}H NMR (DMSO-*d*$_6$):** δ 3.89 (3H, s, OCH_3), 5.74 (2H, s, CH_2), 6.81 (1H, t, *J* 7.0 Hz, H-5), 7.14 (2H, d, *J* 9.0 Hz, H-3', 5'), 8.07 (2H, d, *J* 9.0 Hz, H-2', 6'), 8.20 (1H, dd, *J* 2.0

Hz, 7.5 Hz, H-6), 8.47 (1H, *J* 2.0 Hz, 7.5 Hz, H-4). 14.21 (1H, s, N-H). **^{13}C NMR (DMSO-d_6):** δ 55.70, 55.76, 108.26, 114.28, 114.28, 116.40, 126.95, 130.51, 130.51, 145.93, 146.02, 163.42, 163.97, 164.59, 189.93. **ESI MS** m/z 300.06 [M+H$^+$].

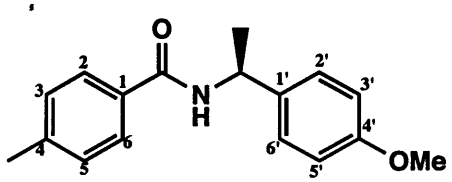
2-(4-Methoxyphenyl)-2-oxoethyl-6-oxo-1,6-dihydropridine-3-carboxylate (67)



$C_{15}H_{13}NO_5$, MW: 287.27

Prepared from 6-hydroxynicotinic acid and 2-bromo-4'-methoxyacetophenone (yield 64 %). **^{1}H NMR (DMSO-d_6):** δ 3.89 (3H, s, OCH_3), 5.74 (2H, s, CH_2), 6.81 (1H, t, *J* 7.0 Hz, Ar-H), 7.14 (2H, d, *J* 8.5 Hz, H-3', 5'), 8.07 (2H, d, *J* 8.5 Hz, H-2', 6'), 8.20 (1H, dd, *J* 2.0 Hz, 6.5 Hz, Ar-H), 8.47 (1H, dd, *J* 2.0 Hz, 7.5Hz, Ar-H), 14.19 (1H, s, N-H). **^{13}C NMR (DMSO-d_6):** δ 55.68, 55.74, 108.25, 114.28, 114.28, 116.41, 126.95, 130.50, 130.50, 145.93, 146.00, 163.42, 163.98, 164.59, 189.92. **ESI MS** m/z 300.06 [M+H$^+$].

(S)-*N*-(1-4-Methoxyphenyl)ethyl)-4-methylbenzamide (68):



$C_{17}H_{19}NO_2$, MW: 269.34

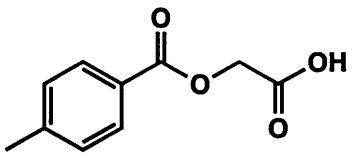
p-Toluoyl chloride (0.3mL, 2.2mmol) was added dropwise to a stirring solution of 4-methoxy-α-methylbenzylamine (0.29mL, 2mmol) and TEA (0.42mL, 3mmol) in 15 mL DCM at 0°C under nitrogen. The reaction was then stirred further at room temperature over night. Upon the completion of the reaction, the solvent was evaporated and the residue was extracted using ethyl acetate, dried over $MgSO_4$ and concentrated *in vacuo*. The obtained residue was further purified with column chromatography (EtOAc-Hexane)

(yield 83 %). **^{1}H NMR ($CDCl_3$):** δ 1.61 (3H, s, CH_3), 2.41 (3H, s, CH_3), 3.82 (3H, s, OCH_3), 5.31 (1H, m, H-1"), 6.21 (1H, bs, NH), 6.91(2H, d, *J* 7.0 Hz, H-3', 5'), 7.23 (2H, d, *J* 8.0 Hz, H-2', 6'), 7.35 (2H, d, *J* 7.0 Hz, H-3, 5). 7.67 (2H, d, 8.5 Hz, H-2, 6). **^{13}C NMR ($CDCl_3$):** δ 21.41, 21,61, 48.56, 55.32, 114.10, 114.10, 126.88, 126.88, 127.48, 127.48, 129.18, 129.18, 133.00, 140.96, 141.08,158.43, 167.10. **ESI MS** m/z 292.12 [M+Na$^+$].

General procedure II:

To a stirring solution of glycolic acid (7.5 mmol) in pyridine (6 mL), p-toluoyl chloride (6.50 mmol) is added dropwise and stirred over night. Upon completion of the reaction (TLC monitored), the solvent was evaporated and the resultant residue was extracted using ethyl acetate (3 x 30mL), dried over $MgSO_4$ and concentrated *in vacuo*. The obtained residue was purified by column chromatography.

2-(4-Methylbenzoyloxy) acetic acid (69)



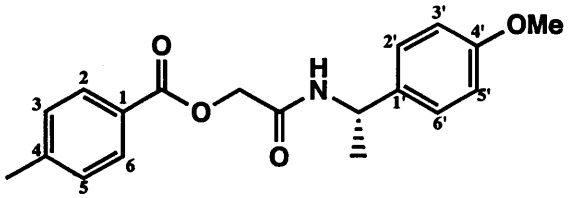
Prepared from p-toluoyl chloride and glycolic acid using general procedure II (yield 59 %). **^{1}H NMR (DMSO-d_6):** δ 2.37 (3H, s, CH_3), 4.78 (2H, s, CH_2), 7.37 (2H, d, *J* 9.0 Hz, H-3, 5), 7.96 (2H, d, *J* 9.0 Hz, H-2, 6), 12.83 (1H, s, OH).

General procedure III:[1]

A mixture of the acetic acid (1mmol) and carbonyl diimidazole (1mmol, 1eq) in DMF (5mL) was stirred at 0 °C for 1 hour. (Substituted) amine (1mmol, 1eq) was dissolved in DMF (2 mL) and added. The reaction was then stirred at 0 °C for another 1 hour, allowed to warm to room temperature to be kept stirring overnight. Once the reaction is completed (TLC monitored), the mixture was poured into a beaker containing ice-cold water. This immediately led to the formation of a precipitate, which was collected by

filtration and washed thoroughly with water to yield the desired product. No further purification was required.

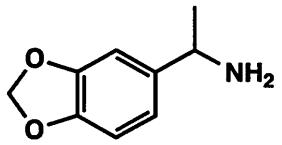
(*S*)-2-(1-(4-Methoxyphenyl)ethylamino)-2-oxoethyl-4-methylbenzoate (70)



$C_{19}H_{22}NO_4$, MW: 327.37

Prepared from 2-(4-methylbenzoyloxy)acetic acid and 4-methoxy-α-methylbenzylamine using general procedure III (65 % yield). **^{1}H NMR ($CDCl_3$):** δ 1.53 (3H, m, CH_3), 2.37 (3H, s, CH_3), 3.81 (3H, s, CH_3O), 4.81 (2H, s, CH_2), 5.21 (1H, m, H-1''), 6.32 (1H, bs, NH), 6.89 (2H, d, *J* 7.0 Hz, H-3', 5'), 7.27 (4H, m, H-3, 5, H-2', 6'), 7.94 (2H, d, *J* 7.0 Hz, H-2, 6). **^{13}C NMR ($CDCl_3$):** δ 21.65, 21.71, 47.96, 55.30, 63.38, 114.13, 114.13, 126.25, 127.29, 127.29, 129.40, 129.40, 129.76, 129.76, 134.71, 144.59, 158.99, 165.27, 166.29. **ESI MS** m/z 350.12 [M+Na$^+$].

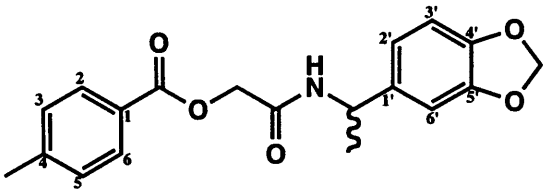
1-(Benzo[d][1,3]dioxol-5-yl)ethanamine (71)[2]



A mixture of the ketone (10 mmol), titanium(IV) isopropoxide (6.0 mL, 20 mmol) and ammonia in absolute ethanol(2 M, 25 mL, 50 mmol) was stirred under nitrogen in a well sealed flask at room temperature for 6 hrs. Sodium borohydride (0.6 g, 15 mmol) was then added and the resulting mixture was stirred at room temperature for an additional 3 h. The reaction was then quenched by pouring it into ammonium hydroxide (2 M, 25 mL), the resulting precipitate was filtered off, and washed with ethyl acetate (25 mL). The organic layer was separated and the remaining aqueous layer was extracted with ethyl acetate (25 mL). The combined organic solution was next extracted with hydrochloric acid (1 M, 30 mL) to separate the neutral materials. The acidic aqueous

extracts were washed with ethyl acetate (50 mL), then treated with aqueous sodium hydroxide (2 M) to a basic pH (10–12), and extracted with ethyl acetate (50 mL×3). The combined organic extracts were washed with brine (50 mL), dried ($MgSO_4$), and concentrated in vacuo to afford the title compound (yield 49 %). **^{1}H NMR ($CDCl_3$):** δ 1.43 (3H, m, CH_3), 5.08 (1H, m, H-1'), 6.01 (2H, s, O-CH_2-O), 6.40 (2H, bs, NH_2), 6.94-7.35 (3H, m, Ar-H).

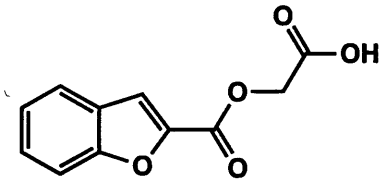
2-(1-(Benzo[d][1,3]dioxol-5-yl)ethylamino)-2-oxoethyl-4-methylbenzoate (72)



$C_{19}H_{19}NO_5$, MW: 341.36

Prepared from 2-(4-methylbenzoyloxy)-acetic acid and **(71)** using general procedure III (yield 57 %). **^{1}H NMR ($CDCl_3$):** δ 1.52 (3H, m, CH_3), 2.45 (3H, s, CH_3), 4.81 (2H, s, CH_2), 5.15 (1H, m, H-1"), 5.96 (2H, s, O-CH_2-O), 6.28 (1H, bs, NH), 6.78 (2H, m, H-Ar), 7.29 (2H, *J* 8.0 Hz, H-3, 5), 7.96 (2H, *J* 8.0 Hz, H-2, 6), 8.02 (1H,m, H-Ar). **^{13}C NMR ($CDCl_3$):** δ 21.72, 21.83, 48.37, 63.37, 106.78, 108.18, 108.38, 119.31, 129.21, 129.42, 129.42, 129.78, 129.78, 130.23, 144.26, 144.63, 147.94, 166.34, 171.11.

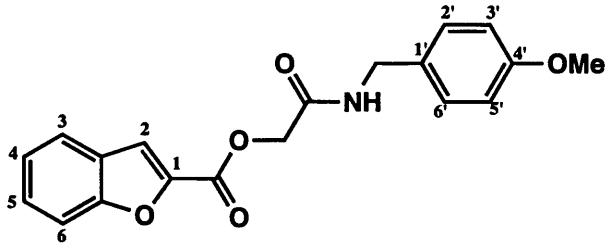
Benzofuran-2-carbonyloxy-2-acetic acid (74)



Benzofuran-carboxylic acid was dissolved in 5 ml thionyl chloride and a drop of DMF was added and the reaction mixture was heated under reflux for 5 hours. Once the reaction is completed (TLC monitored). The solvents were removed *in vacuo*. The resultant residue was used with glycolic acid as described above for the synthesis of 2-(4-

methylbenzoyloxy) acetic acid (yield 57 %). **^{1}H NMR (DMSO-d_6):** δ 5.01 (2H, s, CH_2), 7.05 (1H, m, H-2), 7.42-7.51 (3H, m, Ar-H), 7.78 (1H, m, Ar-H), 13.10 (1H, bs, COOH).

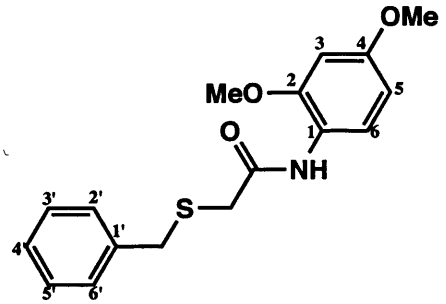
2-(4-Methoxybenzylamino)-2-oxoethylbenzofuran-2-carboxylate (75)



$C_{19}H_{17}NO_5$, MW: 339.34

Prepared from benzofuran-2-carbonyloxy-2-acetic acid and 4-methoxybenzylamine using general procedure III (yield 56 %). **^{1}H NMR ($CDCl_3$):** δ 3.81 (3H, s, OCH_3), 4.56 (2H, s, CH_2), 4.94 (2H, s, CH_2), 6.51 (1H, bs, NH), 6.87 (2H, *J* 8.0 Hz, H-3', 5'), 6.91 (1H, m, Ar-H), 7.37 (1H, m, Ar-H), 7.51 (1H, m, Ar-H), 7.57 (2H, *J* 8.0 Hz, H-2', 6'), 7.73 (1H, m, Ar-H), **^{13}C NMR ($CDCl_3$):** δ 43.16, 55.27, 63.42, 112.42, 113.25, 113.25, 115.45, 119.88, 123.03, 124.12, 124.12, 127.08, 128.30, 129.89, 143.10, 153.95, 158.63, 159.20, 167.82. **ESI MS** m/z 362.08 [M+Na$^+$].

Synthesis of 2-(benzylthio)-*N*-(2,4-dimethoxyphenyl)acetamide (76)

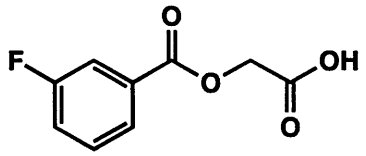


$C_{17}H_{19}NO_3S$, MW: 317.40

This compound was prepared from benzylthioglycolic acid and 2,4-dimethoxyaniline using general procedure III (yield 82 %). **^{1}H NMR ($CDCl_3$):** δ 3.30 (2H, s, CH_2), 3.82 (2H, s, CH_2), 3.83 (3H, s, OCH_3), 3.91 (3H, s, OCH_3), 6.51 (2H, m, Ar-H), 6.91(2H, d, 7.0 Hz, Ar-H), 7.25-7.34 (5H, d, 8.0 Hz, Ar-H), 8.20 (1H, d, 8.5 Hz, Ar-H), 8.96 (1H, bs,

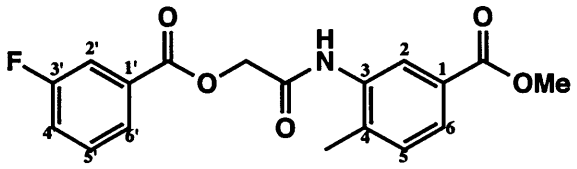
NH), **^{13}C NMR ($CDCl_3$):** δ 36.36, 37.00, 55.57, 55.78, 98.69, 103.76, 120.71, 127.42, 128.65, 128.65, 129.08, 129.08, 129.10, 138.12, 149,78, 156.73, 168.55. **ESI MS** m/z 340.08 [M+Na$^+$].

2-(3-Fluorobenzoyloxy)acetic acid.



Prepared from 3-fluorobenzoylchloride and glycolic acid using general procedure II (yield 52 %). **^{1}H NMR (DMSO-d_6):** δ 5.08 (2H, s, CH_2), 7.48 (1H, m, Ar-H), 7.57 (1H, m, Ar-H), 7.66 (1H, m, Ar-H), 7.78 (1H, m, Ar-H), 13.23 (1H, bs, COOH).

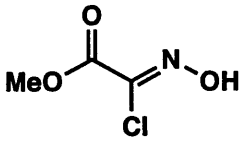
Methyl 3-(2-(3-fluorobenzoyloxy) acetamido)-4-methylbenzoate (77)



$C_{18}H_{16}FNO_5$, MW: 345.32

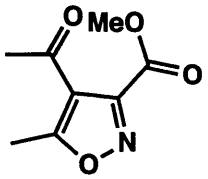
Prepared from 2-(3-fluorobenzoyloxy)acetic acid and methyl-3-amino-4-methylbenzoate using general procedure III (yield 66 %). **^{1}H NMR ($CDCl_3$):** δ 2.32 (3H, m, CH_3), 3.92 (3H, s, OCH_3), 5.03 (2H, s, CH_2), 7.28 (1H, d, *J* 8.0 Hz, Ar-H), 7.37 (1H, m, Ar-H), 7.52 (1H, m, Ar-H), 7.82 (3H, m, Ar-H), 7.94 (1H, d, *J* 8.0 Hz, Ar-H), 8.51 (1H, bs, NH). **ESI MS** m/z 368.09 [M+Na$^+$].

Methyl 2-chloro-2-(hydroxyimino)acetate (79)[3]



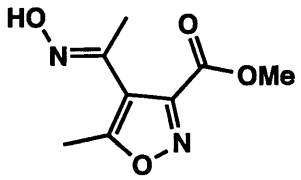
Glycine methyl ester hydrochloride (5g, 0.08 mol) was dissolved in H_2O (15 mL) and HCl (4mL) was added. The mixture was cooled to -5 °C and a solution of $NaNO_2$ (1eq) in H_2O (10mL) was added dropwise. A second equivalent of HCl and $NaNO_2$ were added inthe same way. The mixture was left in the fridge over night leading to the formation of a precipitate, which was collected. **^{13}C NMR ($CDCl_3$):** δ 54.11, 132.63, 159.22.

Methyl 3-acetyl-5-methylisoxazole-4-carboxylate (80)[4]



To a cooled (5 °C) and stirred solution of sodium ethoxide (2.42mL, 3M), a solution of acetyl acetone (0.74mL, 7.27mmol) was slowly added under nitrogen. A solution of 2-chloro-hydroxyliminoacetate in dry ethanol (10mL) was then added dropwise and the mixture was stirred for 2 hours. The mixture was neutralized with 6N HCl, extracted with ether, dried ($MgSO_4$) and concentrated in vacuo. The resultant residue was purified by column chromatography (hexane, ethyl acetate) to yield the titled compound (59 %). **^{1}H NMR (DMSO-d_6):** δ 2.43 (3H, s, CH_3), 2.68 (3H, s, CH_3), 3.91 (3H, s, OCH_3)

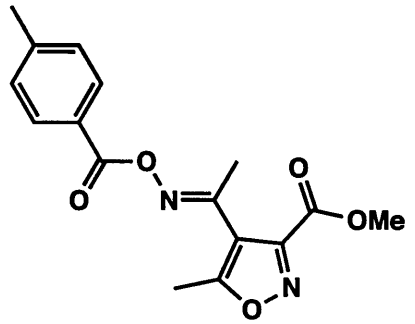
Methyl 3- (1-(hydroxyimino) ethyl-5-methylisoxazole-4-carboxylate (81)



Triethylamine (6mL) was added to a mixture of methyl 3-acetyl-5-methylisoxazole-4-carboxylate and hydroxylamine hydrochloride (0.3g, 4.36mmol) in ethanol (15mL) and heated under reflux for 3 hours. Once the reaction is completed (TLC monitored), the mixture was evaporated, and the obtained residue was extracted with ether, dried

($MgSO_4$) and the evaporation of the solvent produces the desired product (77 %) in sufficient purity. **^{1}H NMR (DMSO-d_6):** δ 2.47 (3H, s, CH_3), 2.59 (3H, s, CH_3), 3.84 (3H, s, OCH_3), 11.40 (1H, s, OH).

Methyl 5-methyl-3- (1-(4-methylbenzoyloxyimino) ethyl) isoxazole-4-carboxylate (82)



$C_{16}H_{16}NO_5$, MW: 316.31

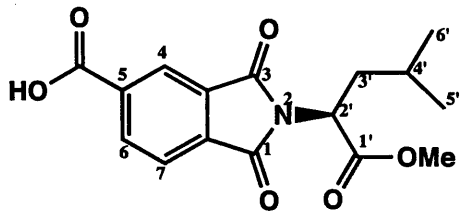
p-Toluoyl chloride (1.5 mmol) was added dropwise to a stirring solution of methyl 3- (1-(hydroxyimino)ethyl-5-methylisoxazole-4-carboxylate (1 mmol) and pyridine in DCM (15 mL) at 0 °C. The reaction was allowed to warm to room temperature and stirred over night. Upon the completion of the reaction, the solvent was evaporated and the resultant residue was extracted with ethyl acetate, dried over $MgSO_4$ and concentrated in vacuo. The obtained compound was further purified with column chromatography (EtOAc-Hexane) (yield 46 %). **^{1}H NMR (DMSO-d_6):** δ 2.41 (3H, s, CH_3), 2.43 (3H, s, CH_3), 2.53 (3H, s, CH_3), 3.95 (3H, s, OCH_3), 7.42 (2H, d, *J* 8.0 Hz, H-3, 5), 8.02 (2H, d, *J* 8.0 Hz, H-2, 6). **^{13}C NMR (DMSO-d_6):** δ 11.75, 17.04, 21.22, 53.14, 125.36, 129.37, 129.37, 129.60, 129.60, 144.41, 157.51, 165.20, 166.80, 167.74, 169.55, 173.07. **ESI MS** m/z 339.11 [M+Na$^+$]. **CHN Anal.** Calcd for C, 60.76; H, 5.10; N, 8.85. Found: C 60.54; H, 5.24; N, 8.79 %.

General procedure IV:[5]

To a stirring solution of a (substituted) amine (10mmol) in pyridine (10mL), 1,2,4-benzenetricarbocyclic acid anhydride (10mmol, 1eq) was added and the reaction mixture

was heated under reflux over night. Upon the completion of the reaction, the solvent was evaporated and the residue was extracted using ethyl acetate, dried over $MgSO_4$ and concentrated *in vacuo*. The obtained compound was further purified with column chromatography (EtOAc-Hexane).

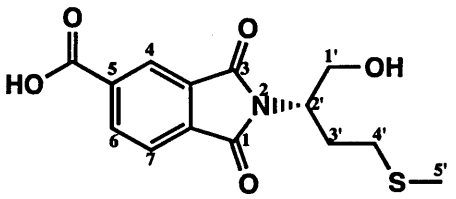
Methyl (*S*)-2-(1-methoxy-5-methyl-1-oxahexan-2-yl)1,3-dioxoisoindoline-5carboxylic acid (84)



$C_{16}H_{17}NO_6$, MW: 319.31

Prepared from L-leucine methyl ester hydrochloride and 1,2,4-benzenetricarbocyclic acid anhydride using general procedure IV (yield 80 %). **^{1}H NMR (DMSO-d_6):** δ 0.88 (6H, m, 2 x CH_3), 1.51 (1H, m, H-4'), 1.88 (1H, m, H-3'), 2.12 (1H, m, H-3'), 3.65 (3H, s, OCH_3), 4.95 (1H, m, H-2'), 8.04 (1H, dd, *J* 8.0 Hz, 0.5 Hz, H-7), 8.28 (1H, dd, *J* 2.0, 0.5 Hz, H-4), 8.41 (1H, dd, *J* 8.0, 1.5 Hz, H-6), 12.59 (1H, bs, COOH). **^{13}C NMR (DMSO-d_6):** δ 20.80, 22.90, 24.30, 36.73, 50.09, 52.63, 123.53, 123.92, 131.35, 134.11, 135.74, 136.79, 165.61, 166.43, 166.47, 169.53.

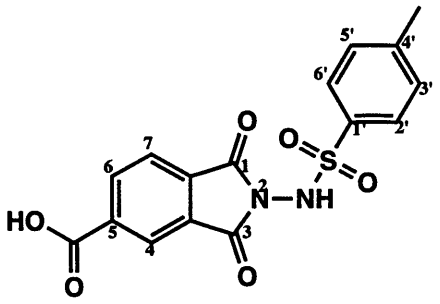
Methyl *(S)*-2-(1-hydroxy-4-methylthio)butan-2-yl)-1,3 dioxoisoindoline-5-carboxylic acid (87)



$C_{14}H_{15}NO_5S$, MW: 309.34

Prepared from *S*-methioninol and 1,2,4-benzenetricarbocyclic acid anhydride using general procedure IV (yield 52 %). **^{1}H NMR (DMSO-d_6):** δ 1.99 (1H), 2.02 (3H, s, CH_3), 2.09 (1H, m), 2.43 (2H, m), 2.45 (3H, s, CH_3), 3.61 (1H, m), 3.83 (1H, t, *J* 8.0 Hz), 4.30 (1H, m), 7.96 (1H, dd, *J* 8.0, 0.5 Hz, H-7), 8.21 (1H, d, *J* 2.5, 0.5 Hz, H-4), 8.36 (1H, dd, *J* 8.0, 2.5 Hz, H-6), 13.65 (1H, bs, COOH). **^{13}C NMR (DMSO-d_6):** δ 14.55, 27.24, 30.16, 53.38, 60.62,122.86, 123.26, 132.01, 134.88, 135.13, 136.10, 165.84, 166.43, 167.56.

Methyl 2-(4-methylphenylsulfonamido)-1,3-dioxoisoindoline-5-carboxylic acid (88)



$C_{16}H_{12}N_2O_6S$, MW: 360.34

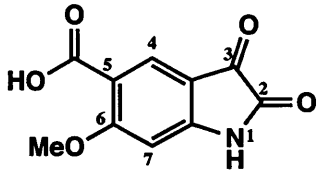
Prepared from p-toluenesulfonylhydrazide and 1,2,4-benzenetricarbocyclic acid anhydride using general procedure IV (yield 86%). **^{1}H NMR (DMSO-d_6):** δ 2.48 (3H, s, CH_3), 7.39 (2H, d, *J* 8.0 Hz, H-3', 5'), 7.74 (2H, d, *J* 8.0 Hz, H-2', 6'), 8.03 (1H, dd, *J* 8.0 Hz, 0.5 Hz, H-7), 8.24 (1H, dd, *J* 2.0 Hz, 0.5 Hz, H-4), 8.40 (1H, dd, *J* 8.0 Hz, 1.5 Hz, H-6), 11.05 (1H, bs, NH), 13.82 (1H, bs, COOH). **^{13}C NMR (DMSO-d_6):** δ 21.06, 123.73, 124.24, 127.44, 127.44, 127.56, 129.55, 129.55, 132.35, 135.91, 136.91, 136.93, 143.86, 163.71, 163.76, 165.54. **ESI MS** m/z 361.04 [M+H$^+$].

General procedure V:[6]

Chloral hydrate (1.47g, 8.88mmol) was added to a 250mL round-bottomed flask containing a suspension of hydroxylamine hydrochloride (1.85g, 26.6mmol), sodium sulfate (8.4g, 59mmol), and methoxybenzoate (17.4mmol) in water (50 mL) and 2M

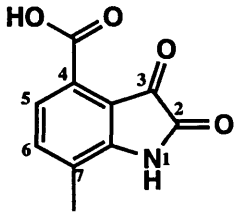
aqueous HCl (2.5mL). The mixture was then heated to 55 °C overnight with stirring. After cooling to room temperature, a precipitate is formed which was collected by filtration and washed thoroughly with water and left to be air-dried. This intermediate was then added in small portions to a 100 mL flask containing concentrated sulfuric acid (5 mL), which had been heated to 70 °C. The resulting deep red solution was heated to 90°C for 40 minutes and then cooled to room temperature. After that, the mixture was added rapidly to a vigorously stirred mixture of ice water (50 mL) and ethyl acetate (20 mL). The organic phase was separated and the aqueous phase was further extracted thoroughly with ethyl acetate the combined organic phases were dried over $MgSO_4$, concentrated *in vacuo* to give the desired isatin with accepted purity to pursue the next step.

6-Methoxy-2,3-dioxoindoline-5-carboxylic acid (85)



Prepared from methyl-4-amino-2-methoxybenzoate using general procedure V (yield 70 %). **^{1}H NMR (DMSO-d_6):** δ 3.91 (3H, s, OCH_3), 6.56 (1H, s, H-7), 7.76 (1H, s, H-4), 11.43 (1H, s, NH), 12.56 (1H, bs, COOH).

7-Methyl-2, 3-dioxoindoline-4-carboxylic acid (89)

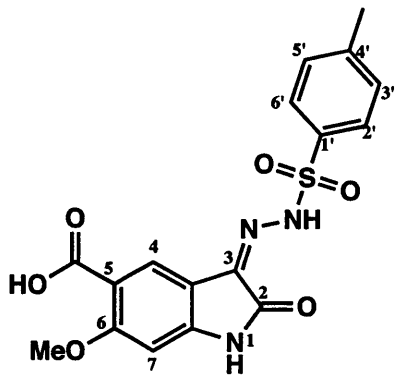


Prepared from methyl-3-amino-4-methylbenzoate using general procedure V (yield 54 %). **^{1}H NMR (DMSO-d_6):** δ 2.22 (3H, s, CH_3), 7.14 (1H, d, *J* 8.0 Hz, H-6*)*, 7.37 (1H, d, *J* 8.0 Hz, H-5*)*, 11.35 (1H, s, NH), 13.10 (1H, bs, COOH).

General procedure VI:[7]

The prepared isatin using general procedure V (0.65mmol) and p-toluenesulfonylhydrazide (0.65mmol, 1eq) were added to a 10 mL round bottom flask containing ethanol (20 mL) and the reaction mixture was heated under reflux for 3 hours. The solution was left to cool to room temperature leading to the formation of the desired compound as a yellow precipitate, which was collected by vacuum filtration and dried.

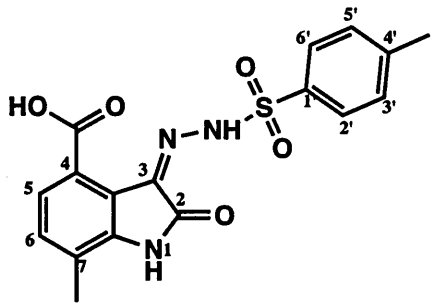
6-Methoxy-2-oxo-3-(2-tosylhydrazono) indoline-5-carboxylic acid (86)



$C_{17}H_{15}N_3O_6S$, MW: 389.38

Prepared from **(85)** and p-toluenesulfonylhydrazide using general procedure VI (36 %). **^{1}H NMR (DMSO-d_6):** δ 2.38 (3H, s, CH_3), 3.86 (3H, s, OCH_3), 6.58 (1H, s, H-7), 7.45 (2H, d, *J* 8.0 Hz, H-3',5'), 7.73 (1H, s, H-4), 7.87 (2H, d, *J* 8.0 Hz, H-2',6'), 11.41 (1H, s, NH), 12.35 (1H, bs, NH), 12.53 (1H, bs, COOH). **^{13}C NMR (DMSO-d_6):** δ 21.02, 56.23, 95.68, 110.98, 113,35, 114.48, 123.88, 127.65, 127.65, 128.89, 128.89, 135.06, 144.37, 147.12, 162.40, 162.55, 166.10. **ESI MS** m/z 390.06 [M+H$^+$].

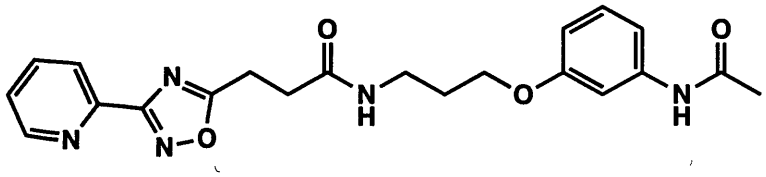
7-Methyl-2-oxo-3-(2-tosylhydrazono)indoline-4-carboxylic acid (90)



$C_{17}H_{15}N_3O_5S$, MW: 373.38

Prepared from **(89)** and p-toluenesulfonylhydrazide using general procedure VI (yield 24 %). **^{1}H NMR (DMSO-d_6):** δ 2.22 (3H, s, CH_3), 2.39 (3H, s, CH_3), 7.12 (1H, d, *J* 8.0 Hz, H-5), 7.25 (1H, d, *J* 8.0 Hz, H-6), 7.44 (2H, d, *J* 8.0 Hz, H-3',5'), 7.83 (2H, d, *J* 8.0 Hz, H-2',6'), 11.44 (1H, s, NH), 12.82 (1H, s, NH), 13.20 (1H, bs, COOH). **^{13}C NMR (DMSO-d_6):** δ 16.02, 20.99, 121.73, 122.40, 122.93, 123.41, 124.77, 127.71, 127.71, 129.69, 129.69, 132.46, 137.60, 141.24, 144.53, 161.99, 167.14. **ESI MS** m/z 374.05 [M+H$^+$].

N-(3-(3-Acetamidophenoxy)propyl)-3-(3-pyridin-2-yl)-1,2,4-oxadiazol-5 yl)propanamide (83)



Purchased from Maybridge corporation Ltd. **^{13}C NMR (DMSO-d_6):** δ 21.97, 24.03, 28.84, 31.06, 35.53, 60.19, 64.96, 69.79, 72.31, 105.44, 108.77, 11.23, 12 3.15, 125.94, 129.33, 137.59, 140.41, 145.81, 150.23, 158.73, 167.51, 168.24, 169.90, 18 0.28.

The following general procedure were used in the synthesis of (95) and its analogues.

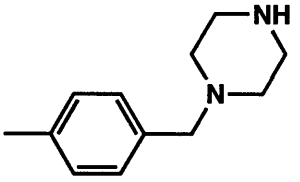
General procedure VII: to a stirring mixture of nitroaniline (10mmol) in DMF (10 mL) at room temperature, an acyl chloride (10mmol) was added under inert gas conditions. The reaction was then heated to 110 °C and left stirring until completion (5-6 hrs) (TLC monitored). Once completed, the mixture was poured into ice cold H_2O (20 mL) and the product precipitates. The precipitate was further washed with cold H_2O, then collected by vacuum filtration and dried.

General procedure VIII:[8] a mixture of the nitro compound (0.05 mol), reduced iron (30g), EtOH (40mL), H_2O (10mL) and HCl (10M, 0.5mL) was heated for 40 minutes. The iron is removed by filtration through a celite and washed with hot EtOH (3X 10mL). The filtrate and washings are collected and evaporated to dryness to give a crude product. Purification was accomplished by recrystallisation from methanol.

General procedure IX: the reduced nitro compound (2 mmol) was added to an equivalent amount of the glutaric anhydride (2 mmol) in refluxing (90 °C) dioxane (20mL) and the reaction mixture was kept stirring for 5-7 hours. After completion of the reaction (monitored by TLC), the solvent was evaporated and the residue was extracted using EtOAc, dried over $MgSO_4$ and concentrated *in vacuo*. The obtained residue was purified by column chromatography (85% EtOAc/15% Hexane) to give the title compound as a white solid.

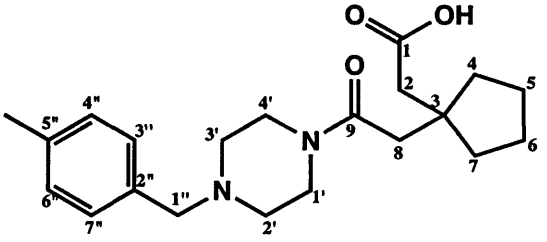
General procedure X:[9] Chlorotrimethylsilane (0.3 mol) was added slowly to a stirring mixture of the obtained acid (0.1mol) in methanol (10 mL) and the resulting mixture was heated under reflux for 2-3 hours. After completion of the reaction (monitored by TLC), the reaction mixture was concentrated on a rotary evaporator to give the ester product. Purification was accomplished by a straightforward column chromatography (yield 60-72%).

1-(4-Methylbenzyl)piperazine (91)



(91) was prepared from p-methylbenzylchloride and piperazine. The piperazine (140 mg, 1 mmol) is first dissolved in THF (20 mL) and the temperature is reduced to 0°C. Methylbenzylchloride (173mg, 2mmol) was added and the reaction mixture was then heated under reflux for 2 hrs (TLC monitoring). The crude product was isolated and further purified using column chromatography eluting with DCM to afford **(91)** in a good yield (79%). **^{1}H NMR (DMSO-d_6):** δ 2.20- 2.42 (8H, s, H-1, 2, 3, 4), 2.56 (3H, s, CH_3), 3.02 (1H, bs, NH), 3.40 (2H, s, CH_2), 7.05 (2H, d, *J* 8.5 Hz, H-3', 5'), 7.15 (2H, d, *J* 8.5 Hz, H-2', 6').

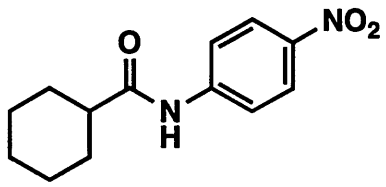
2-(1-(2-(4-(4-Methylbenzyl)piperazin-1-yl)-2-oxoethyl)cyclopentyl)acetic acid (92)



$C_{21}H_{30}N_2O_3$, MW: 358.47

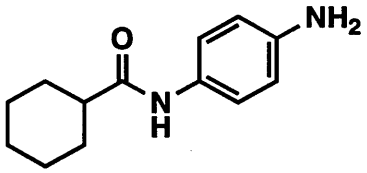
Prepared from **(91)** and 3,3-tetramethyleneglutaric anhydride using general procedure IX (yield 66 %). **^{1}H NMR (DMSO-d_6):** δ 1.53-1.55 (8H, m, H-4, 5, 6, 7), 2.26- 2.31 (11 H, m, H-1', 2', 3', 4', CH_3), 2.45 (2H, s, CH_2), 2.51 (2H, s, CH_2), 7.12 (2H, d, *J* 8.0 Hz, H-4", 6"), 7.18 (2H, d, *J* 8.0 Hz, H-3", 7"), 12.10 (1H, bs, COOH). **^{13}C NMR (DMSO-d_6):** δ 20.69, 23.51, 37.46, 38.72, 39.03, 40.09, 40.08, 41.70, 43.01,45.31, 52.37, 52.77, 61.59 , 128.74, 128.74, 128.83, 128.83, 134.70, 135.98,169.54, 173.48. **CHN Anal.** Calcd for C, 70.36; H, 8.43; N, 7.81. Found: C 70.26; H, 8.48; N, 7.79 %.

N-(4-Nitrophenyl)cyclohexanecarboxamide (93)



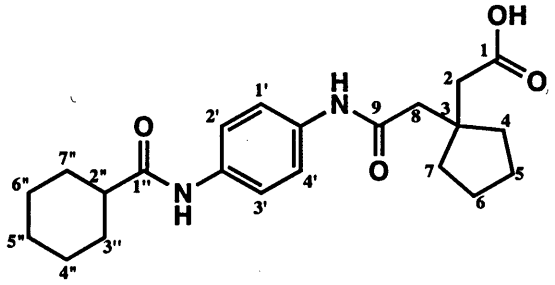
Prepared from 4-nitroaniline and cyclohexanecarbonyl chloride using general procedure VII (yield 82%). **^{1}H NMR (DMSO-d_6):** δ 1.08-1.70 (10H, m, H-2-6), 2.45 (1H, m, H-1), 7.34 (2H, d, *J* 8.0 Hz, H-2', 6'), 8.29 (2H, d, *J* 8.0 Hz, H-3', 5'), 10.45 (1H, s, NH).

N-(4-aminophenyl)cyclohexanecarboxamide (94)[10]



Prepared from **(93)** using general procedure VIII (yield 75%). **^{1}H NMR (DMSO-d_6):** δ 1.08-1.70 (10H, m, H-2-6), 2.31 (1H, m, H-1), 4.80 (2H, s, NH_2), 6.50 (2H, d, *J* 8.0 Hz, H-3', 5'), 7.34 (2H, d, *J* 8.0 Hz, H-2', 6'), 9.35 (1H, s, NH).

2-(1-(2-(4-(Cyclohexanecarboxamido)phenylamino)-2-oxoethyl)cyclopentyl)acetic acid (95)

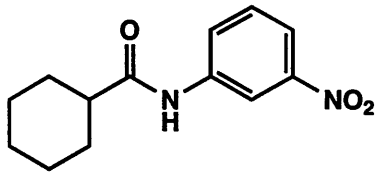


$C_{22}H_{30}N_2O_4$, MW: 386.48

Prepared from **(94)** and 3,3-tetramethyleneglutaric anhydride using general procedure IX (yield 54 %). **m.p.** 204-206 °C, **^{1}H NMR (DMSO-d_6):** δ 1.16-1.40 (6H, m, H-4", 5", 6"),

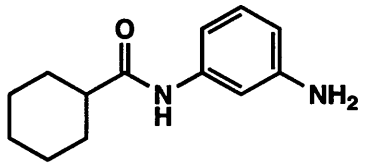
1.54- 1.75 (12H, m, H-4, 5, 6, 7, 3", 7"), 2.31 (1H, m, H-2"), 2.48 (4H, s, 2 x CH_2), 7.45-7.51 (4H, m, H-1', 2', 3',4'), 9.68 (1H, s, NH), 9.74 (1H, s, NH), 12.03 (1H, bs, COOH). **^{13}C NMR (DMSO-d_6):** δ 23.66, 23.66, 25.22, 25.22, 25.39, 29.13, 29.13, 37.15, 37.16, 42.15,43.04, 43.76, 44.74, 119.34, 119.34, 119.52, 119.52, 134.30,134.81, 169.80, 173.38 173.89. **CHN Anal.** Calcd for C, 68.37; H, 7.82; N, 7.24. Found: C 68.19; H, 7.91; N, 7.22%.

***N*-(3-Nitrophenyl)cyclohexanecarboxamide (96)**



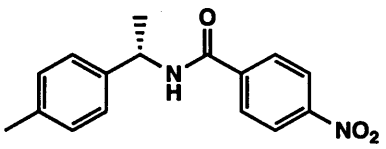
Prepared from 3-nitroaniline and cyclohexanecarbonyl chloride using general procedure VII (yield 86%). **^{1}H NMR (DMSO-d_6):** δ 1.20-2.02 (10H, m, H-2-6), 2.47 (1H, m, H-1), 7.14 (1H, m, Ar-H), 7.51 (1H, m, Ar-H), 8.36 (1H, m, Ar-H), 8.72 (1H, m, Ar-H), 10.49 (1H, s, NH).

***N*-(3-Aminophenyl)cyclohexanecarboxamide (97)** [10]



Prepared from **(96)** using general procedure VIII (yield 75%). **^{1}H NMR (DMSO-d_6):** δ 1.20-1.98 (10H, m, H-2-6), 2.45 (1H, m, H-1), 5.12 (2H, s, NH_2), 6.22 (1H, t, *J* 8.0 Hz, Ar-H), 6.75 (1H, d, *J* 8.0 Hz, Ar-H), 6.80-6.93(2H, m, *J* 8.0 Hz, Ar-H), 9.49 (1H, s, NH).

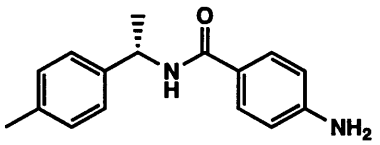
(*S*)-4-Nitro-*N*-(1-*p*-tolylethyl)benzamide (99)



Prepared from *(S)*-1-*p*-tolylethanamine and 4-nitrobenzoylchloride using general procedure VII (yield 73%). **^{1}H NMR (DMSO-d_6):** δ 1.52 (3H, s, CH_3), 2.25 (3H, s,

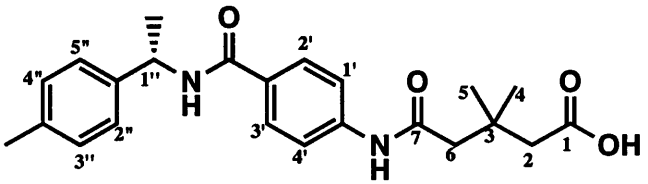
CH_3), 5.11 (1H, m, H-1''), 7.17 (2 H, d, *J* 8.0 Hz, H-3'', 4''), 7.25 (2 H, d, *J* 8.0 Hz, H-2'', 5''), 8.16 (2 H, d, *J* 8.0 Hz, H-2', 3'), 8.35 (2 H, m, H-1', 4'), 9.10 (1H, s , NH).

(*S*)-4-Amino-*N*-(1-*p*-tolylethyl)benzamide (100)



Prepared from **(99)** using general procedure VIII (yield 68%). **^{1}H NMR (DMSO-d_6):** δ 1.38 (3H, s, CH_3), 2.22 (3H, s, CH_3), 5.06 (1H, m, H-1''), 5.55 (2H, bs, NH_2), 6.51 (2 H, d, *J* 8.0 Hz, H-1', 4'), 7.06 (2 H, d, *J* 8.0 Hz, H-3'', 4''), 7.25 (2 H, d, *J* 8.0 Hz, H-2', 3'), 7.53 (2 H, m, H-2'', 5''), 8.21 (1H, s, NH).

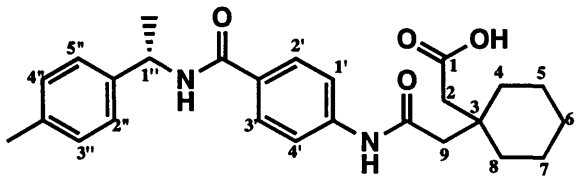
(*S*)-3,3-Dimethyl-5-oxo-5-(4-(1-*p*-tolylethylcarbamoyl)phenylamino)pentanoic acid (101)



$C_{23}H_{28}N_2O_4$, MW: 396.48

Prepared from **(100)** and 3,3-diamethylglutaric anhydride using general procedure IX (yield 53%). **^{1}H NMR (DMSO-d_6):** δ 1.10 (6H, s, 2 x CH_3), 1.44 (3H, m, CH_3), 2.26 (3H, s, CH_3), 2.38 (4H, s, 2 x CH_2), 5.13 (1H, m, 1"), 7.12 (2H, d, *J* 8.0 Hz, H-3", 4"), 7.26 (2H, d, *J* 8.0 Hz, H-2", 5"), 7.67 (2H, d, *J* 8.5 Hz, H-1', 4'), 7.84 (2H, d, *J* 8.5 Hz, H-2', 3'), 8.60 (1H, d, *J* 8.0 Hz, NH), 10.05 (1H, s, NH), 11.95 (1H, bs, COOH).

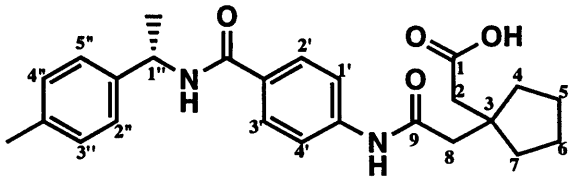
(*S*)-2-(1-(2-Oxo-2-(4-(1-*p*-tolylethylcarbamoyl)phenylamino)ethyl)cyclohexyl)acetic acid (102)



$C_{26}H_{32}N_2O_4$, MW: 436.54

Prepared from **(94)** and 3-oxaspiro[5.5]undecane-2,4-dione using general procedure IX (yield 58%). **^{1}H NMR (DMSO-d_6):** δ 1.34-1.49 (13 H, m, H-4, 5, 6, 7, 8, CH_3), 2.26 (3H, s, CH_3), 2.50 (4 H, s, 2 x CH_2), 5.13 (1H, m, 1"), 7.11 (2H, d, *J* 8.0 Hz, H-3", 4"), 7.26 (2H, d, *J* 8.0 Hz, H-2", 5"), 7.67 (2H, d, *J* 8.5 Hz, H-1', 4'), 7.83 (2H, d, *J* 8.5 Hz, H-2', 3'), 8.59 (1H, d, *J* 8.0 Hz, NH), 10.06 (1H, s, NH), 11.99 (1H, bs, COOH). **^{13}C NMR (DMSOd_6):**δ 20.57, 21.03, 22.23, 23.65, 23.68, 37.22, 40.00, 41.99, 42.18, 43.02, 43.85, 47.99, 118.16, 118.16, 125.95, 128.10, 128.10, 128.68, 128.68, 128.92, 135.48, 141.98, 1 42.11, 166.15, 171.05, 174.31.

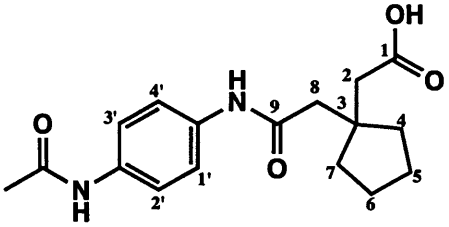
***(S)*-2-(1-(2-Oxo-2-(4-(1-*p*-tolylethylcarbamoyl)phenylamino)ethyl)cyclopentyl)acetic acid (103)**



$C_{25}H_{30}N_2O_4$, MW: 422.52

Prepared from **(100)** and 3,3-tetramethyleneglutaric anhydride using general procedure IX (yield 48%). **^{1}H NMR (DMSO-d_6):** δ 1.14-1.63 (9H, m, H-4, 5, 6, 7, CH_3), 2.26 (3H, s, CH_3), 2.48 (4 H, s, 2 x CH_2), 5.12 (1H, m, 1"), 7.12 (2H d, *J* 8.0 Hz, H-3", 4"), 7.26 (2H, d, *J* 8.0 Hz, H-2", 5"), 7.65 (2H, d, *J* 8.5 Hz, H-1', 4'), 7.82 (2H, d, *J* 8.5 Hz, H-2', 3'), 8.58 (1H, d, *J* 8.0 Hz, NH), 10.03 (1H, s, NH), 11.98 (1H, bs, COOH).**^{13}C NMR (DMSOd_6):**δ 20.57, 22.21, 23.61, 23.64, 37.20, 40.01,41.99, 42.16, 42.98, 43.84, 47.97, 118.15, 118.15, 125.96, 128.10, 128.10, 128.67, 128.67, 128.82, 135.48, 141.66, 141.98, 164.85, 170.50, 173.38.

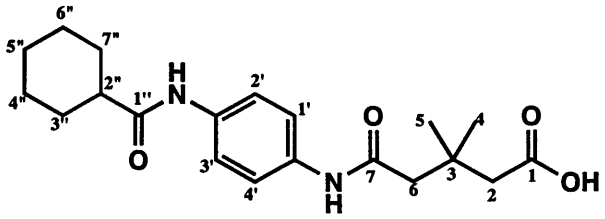
2-(1-(2-(4-Acetamidophenylamino)-2-oxoethyl)cyclopentyl)acetic acid (104)



$C_{17}H_{22}N_2O_4$, MW: 358.47

Prepared from *N*-(4-aminophenyl)acetamide and 3,3-tetramethyleneglutaric anhydride using general procedure IX (yield 54 %). **m.p.** 198-199°C, **^{1}H NMR (DMSO-d_6):** δ 1.57-1.63 (8H, m, H-4, 5, 6, 7), 2.01 (3H, s, CH_3), 2.48 (4H, s, 2 x CH_2), 7.47 (4H,s , H-1',2', 3',4'), 9.75 (1H, s, NH), 9.83 (1H, s, NH), 11.99 (1H, bs, COOH). **^{13}C NMR (DMSOd_6):** δ 23.65, 23.65, 23.82, 37.15, 42.14, 43.05, 43.75, 66.32, 119.28, 119.28, 119.59, 119.59, 134.40, 34.64, 67.88, 169.83, 173.39. **CHN Anal.** Calcd for C, 64.13; H, 6.96; N, 8.79. Found: C 64.28; H, 7.03; N, 8.89 %

5-(4-(Cyclohexanecarboxamido)phenylamino)-3,3-dimethyl-5-oxopentanoic acid (105)

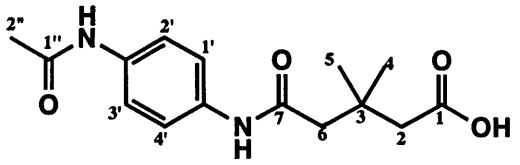


$C_{20}H_{28}N_2O_4$, MW: 360.45

Prepared from **(94)** and 3,3-dimethylglutaric anhydride using general procedure IX (yield 65%). **m.p.** 184-186°C, **^{1}H NMR (DMSO-d_6):** δ 1.08 (6H, s, 2 x CH_3), 1.09- 1.40 (6H, m, H-4", 5", 6"), 1.65-1.78 (4H, m, H-3", 7"), 2.29 (1H, m, H-2"), 2.33 (4H, s, 2 x CH_2), 7.45-7.52 (4H, m, H-1',2', 3',4'), 9.69 (1H, s, NH), 9.75 (1H, s, NH), 11.99 (1H, bs, COOH). **^{13}CNMR (DMSO-d_6):** δ 25.22, 25.22, 25.38, 27.23, 27.23, 29.13, 29.13, 32.53,

44.74, 45.17, 47.14, 119.35, 119.35, 119.65, 119.65, 134.24,134.86, 169.40, 172.99, 173. 90. **CHN Anal.** Calcd for C, 66.64; H, 7.83; N, 7.77. Found: C 66.38; H, 7.76; N, 7.84%.

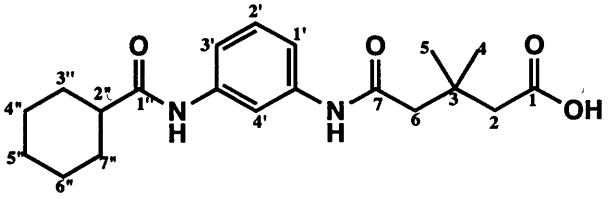
5-(4-Acetamidophenylamino)-3,3-dimethyl-5-oxopentanoic acid (106)



$C_{15}H_{20}N_2O_4$, MW: 292.33

Prepared from *N*-(4-aminophenyl)acetamide and 3,3-dimethylglutaric anhydride using general procedure IX (yield 66%). **m.p.** 187-188 °C, **^{1}H NMR (DMSO-d_6):** δ 1.10 (6H, s, 2 x CH_3), 2.12 (3H, s, CH_3), 2.37 (4H, s, 2 x CH_2), 7.45 (4H, s, H-1',2', 3',4'), 9.76 (1H, s, NH), 9.81 (1H, s, NH), 11.99 (1H, bs, COOH). **^{13}C NMR (DMSO-d_6):** δ 23.83, 23.28, 27.23, 32.54, 45.19, 47.16, 119.28, 119.28, 119.62, 119.62, 134.35,1 34.70, 167.86, 169.42, 172.99. **CHN Anal.** Calcd for C, 61.63; H, 6.90; N, 9.58. Found: C 61.38; H, 6.90; N, 9.57 %.

5-(3-(Cyclohexanecarboxamido)phenylamino)-3,3-dimethyl-5-oxopentanoic acid (107)

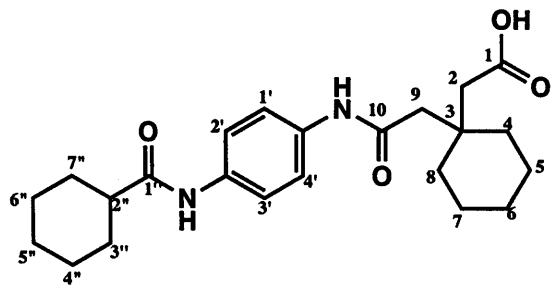


$C_{20}H_{28}N_2O_4$, MW: 360.45

Prepared from **(97)** and 3,3-dimethylglutaric anhydride using general procedure IX (yield 71%). **m.p.** 113-115 °C, **^{1}H NMR (DMSO-d_6):** δ 1.09 (6H, s, 2 x CH_3), 1.19-1.41 (6H, m, H-4", 5", 6"), 1.63-1.79 (4H, m, H-3", 7"), 2.34 (1H, m, H-2"), 2.36 (4H, s, 2 x CH_2), 7.16 (1H, m, Ar-H), 7.21 (1H, m, Ar-H), 7.29 (1H, d, *J* 8.0 Hz, Ar-H), 7.96 (1H, m, H-

4'), 9.76 (1H, s, NH), 9.81 (1H, s, NH), 12.05 (1H, bs, COOH). **^{13}C NMR (DMSO-d_6):** δ 22.02, 25.20, 25.38, 27.21, 29.10, 29.10, 32.55,44.75, 45.14, 47.19, 110.31, 113.94, 114.03, 128.56, 128.56, 139.26, 139.68, 169.72, 173.01, 174.24.

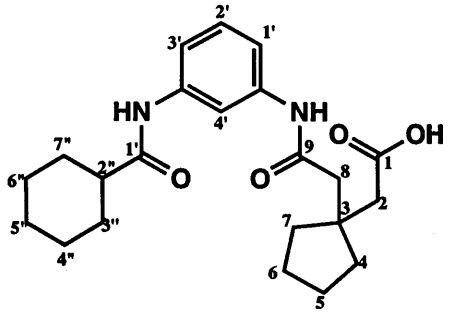
2-(1-(2-(4-(Cyclohexanecarboxamido)phenylamino)-2-oxoethyl)cyclohexyl)acetic acid (108)



$C_{23}H_{32}N_2O_4$, MW: 360.45

Prepared from **(94)** and 3-oxaspiro[5.5]undecane-2,4-dione using general procedure IX (yield 54%). **m.p.** 221-223°C, **^{1}H NMR (DMSO-d_6):** δ 1.07-1.51 (16H, m, H-4, 5, 6, 7, 8, 4", 5",6"), 1.63-1.79 (4H, m, H-3", 7"), 2.26 (1H, m, H-2"), 2.47 (4H, m, 2 x CH_2), 7.49 (4H, m, H-1', 2', 3',4'), 9.68 (1H, s, NH), 9.75 (1H, s, NH), 11.98 (1H, bs, COOH). **^{13}C NMR (DMSOd_6):** δ 21.05, 25.22, 25.22, 25.39, 25.57, 29.13, 35.07, 35.18, 40.00, 41 .12, 43.03, 44.74, 56.00, 119.34, 119.34, 119.54, 119.54, 134.24, 134.86, 169.60, 173.24, 173.89. **CHN Anal.** Calcd for C, 68.97; H, 8.05; N, 6.99. Found: C 68.56; H, 7.97; N, 7.06 %.

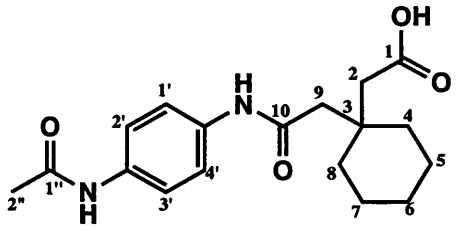
2-(1-(2-(3-(Cyclohexanecarboxamido)phenylamino)-2-oxoethyl)cyclopentyl)acetic acid (109)



$C_{22}H_{30}N_2O_4$, MW: 386.48

Prepared from **(97)** and 3,3-tetramethyleneglutaric anhydride using general procedure IX (yield 43%). **m.p.** 142-143°C, **^{1}H NMR (DMSO-d_6):** δ 1.25-1.41 (6H, m, H-4", 5", 6"), 1.55- 1.79 (12H, m, H-4, 5, 6, 7, 3", 7"), 2.50 (1H, m, H-2"), 2.51 (4H, m, 2 x CH_2), 7.16 (1H, t, *J* 8.0 Hz, Ar-H), 7.19 (1H, m, Ar-H), 7.28 (1H, d, *J* 8.0 Hz, Ar-H), 7.95 (1H, m, H-4'), 9.76 (1H, s, NH), 9.80 (1H, s, NH), 12.01 (1H, bs, COOH). **^{13}C NMR (DMSO-d_6):** δ 23.60, 23.66, 25.20, 25.38, 29.10, 37.15, 37.14, 40.00, 42.10, 43.04, 43.77, 44.75,1 10.28, 113.90, 113.97, 128.56, 128.56, 139.31, 139.67, 170.12, 173.39, 174.23.

2-(1-(2-(4-acetamidophenylamino)-2-oxoethyl)cyclohexyl)acetic acid (110)



$C_{18}H_{24}N_2O_4$, MW: 332.39

Prepared from *N*-(4-aminophenyl)acetamide and 3-oxaspiro[5.5]undecane-2,4-dione using general procedure IX (yield 72%). **m.p.** 213-215 °C, **^{1}H NMR (DMSO-d_6):** δ 1.36-1.49 (10H, m, H-4, 5, 6, 7, 8), 2.01 (3H, s, CH_3), 2.51 (4H, s, 2 x CH_2), 7.48 (4H,s , H-1',2', 3',4'), 9.76 (1H, s, NH), 9.82 (1H, s, NH), 11.96 (1H, bs, COOH).**^{13}CNMR (DMSO-d_6):** δ 18.51, 21.05, 21.05, 23.82, 35.08, 35.18, 41.12, 43.04, 56.00, 119.28, 119. .28, 119.60, 119.60, 134.34, 134.70, 167.86, 169.62, 173.24. **CHN Anal.** Calcd for C, 65.04; H, 7.28; N, 8.42. Found: C 64.85; H, 7.35; N, 8.46 % .

References

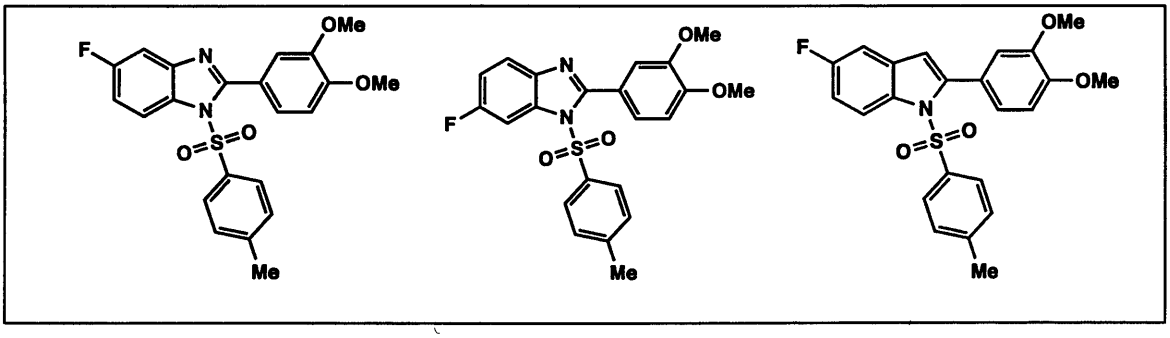
1. Paul, R.; Anderson, G. W. N,N'-carbonyldiimidazole, a new peptide forming reagent". *J. Am. Chem. Soc.* **1960,** 82, 4596–4600.
2. Miriyala, B.; Bhattacharyya, S.; Williamson, J. S. Chemoselective reductive alkylation of ammonia with carbonyl compounds: synthesis of primary and symmetrical secondary amines. *Tetrahedron* **2004,** 60, 1463-1471.
3. Kozikowski, A, P.; Adamcz, M. Methoads for the stereoselective cis-cyanohydroxylation and carboxyhydroxylation of olefins. *J. Org. Chem.* **1983,** 48, 366-372.
4. Giovannoni, M, P.; Vergelli, C.; Ghelardini, C.; Galeotti, N.; Bartolini, A.; Piaz, V. D. [(3-Chlorophenyl)piperazinylpropyl]pyridazinones and analogues as potent antinociceptive agents. *J. Med. Chem.* **2003,** 46, 1055-1059.
5. Maillard, L. T.; Benohoud, M.; Durand, P.; Badet, B. A new supported reagent for the parallel synthesis of primary and secondary O-alkyl hydroxylamines through a base-catalyzed Mitsunobu reaction. *J. Org. Chem.* **2005,** 70, 6303-6312.
6. Zhou, L.; Liu, Y.; Zhang, W.; Wei, P.; Huang, C.; Pei, J.; Yuan, Y.; Lai. L. Isatin compound as noncovalent SARS coronavirus 3C-like protease inhibitors. *J. Med. Chem.* **2006,** 49, 3440 – 344.
7. Varano, F.; Catarzi, D.; Colotta, V.; Calabri, F. R.; Lenzi, O.; Filacchioni, G.; Galli, A.; Costagli, C.; Carlà, V.; Deflorian, F.; Moro, S. 1-Substituted pyrazolo[1,5-c]quinazolines as novel Gly/NMDA receptor antagonists: synthesis, biological evaluation and molecular modeling study. *Bioorg. Med. Chem.* **2005,** 13, 5536-5549.
8. Fox, B. A.; Threlfall, T. L. 2,3-Diaminopyridine. *Org. Synth.* **1973.** Coll. Vol. 346.
9. Li, J.; Sha, Y. A convenient synthesis of amino acid methyl esters. *Molecules* **2008,** 13, 1111-1119.
10. Mohmeyer, N.; Schmidt, H-W. Synthesis and structure-property relationships of amphilic organogelators. *Chem. Eur. J.* **2007,** 13, 4499-4509.

Conclusion

This project was divided into two main parts and the findings can be briefly summarised as the following:

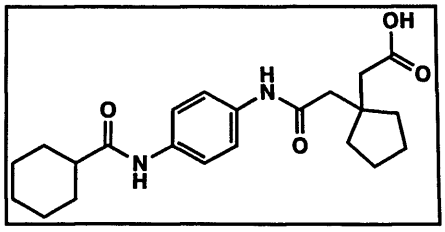
Part I: *The synthesis and biological evaluation of novel benzimidazole and indole based antitumour compounds*

- A new series of (substituted) 2-phenylbenzimidazols and indoles bearing oxygenated substituents on the phenyl ring has successfully been synthesised.
- The new compounds were less active than the former benzothiazole series when tested *in vitro* against four human cancer cell lines.
- Incorporation of sulfonyl substituents enhanced the activity of these series.
- The most active compounds in the N-substituted benzimidazole and indole series (shown below) exhibit low and sub-micromolar GI_{50} values respectively.



Part II: *The design and synthesis of novel small molecule inhibitors of the Dishevelled PDZ domain for use as selective antitumour agents*

- Different molecular modelling studies were performed and the potential hits were successfully synthesised.
- Biochemical binding assays shows that one of the hit compounds binds to Dvl PDZ.



- Further structural optimisation studies of the identified hit compound were carried out based on molecular dynamic simulations to identify more potent inhibitors and the biological testing are underway.