Synthesis and evaluation of inhibitors against vitamin D₃ and all-trans retinoic acid metabolising enzymes as potential therapy for androgen-independent prostate cancer

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A thesis submitted in accordance with the conditions governing candidates for the degree of Philosophiae Doctor (PhD)

2005

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献给我致爱的父母亲—抗癌的勇士

To my mother and father for their enormous courage
and strength in fighting cancer
Acknowledgements

I am grateful to many people whom I have benefited from and who have contributed in different ways to the progress and development of this thesis. The person whom I am indebted to is my great supervisor, Dr. Claire Simons, who has always been very encouraging and supportive throughout my thesis, and has given me all the opportunities to experience and to explore in this stimulating and interesting research. Secondly, I would like to thank the Tenovus Cancer Research cell culture group, Dr. Bronwen A.J. Evans, Mrs. Carole Elford, Dr. Moray J. Campbell, Dr. Marc Le Borgne, Dr. Michael P. Coogan and Dr. Liling Ooi, for their valuable guidance and collaborations. Thirdly, I would like to thank all my friends, colleagues and the staff members in the Redwood building, for the pleasant company and interesting thoughts and ideas. I would like to extend my sincere gratitude to Dr. Kenneth T. Wann for his valuable support from the beginning of my stay in UK (eight years in total). I would also like to acknowledge the ORS Awards Scheme for a United Kingdom Scholarship. Finally, and most important, I wish to thank my mother and father, to whom this thesis is dedicated. Their constant love, inspiration and encouragement are immeasurable. Their strong will power in fighting and surviving cancer has always given me the motivation and enthusiasm in wanting to find a cure for cancer.
Abstract

The majority of prostate cancer patients demonstrate good initial responses to surgical castration and/or hormonal therapy. Unfortunately, hormonal therapy is not capable of producing durable responses in the majority of the patients who subsequently develop androgen-independent prostate cancer (AIPC). New effective therapies are needed in the management of AIPC patients.

One potential therapeutic strategy is to employ a differentiating agent to suppress prostate cancer cell proliferation. 1α,25-Dihydroxyvitamin D₃ (1α,25-(OH)₂-D₃) and all-trans retinoic acid (ATRA) have differentiating and anti-proliferative effects on prostate cancer cells. However, the use of 1α,25-(OH)₂-D₃ and ATRA is limited by the induction of the cytochrome P450 enzymes. The P450 enzymes that are responsible for the catabolism of 1α,25-(OH)₂-D₃ and ATRA are cytochrome 24 (CYP24) and 26 (CYP26) respectively. ATRA is also metabolised by other P450 4-hydroxylase enzymes in the liver. Therefore, a drug which can prolong the action of 1α,25-(OH)₂-D₃ or ATRA by inhibiting the P450 enzymes could have potential use in the treatment of AIPC.

Three series of novel compounds were synthesised in an attempt to probe for the key binding residues in the enzymes. The preparation of the rat kidney mitochondria and rat liver microsome enzymes were described herein to study the inhibition of the 25-hydroxyvitamin D₃ and ATRA metabolism respectively using the synthesised compounds. In addition, cancer cell-lines (MCF-7 and DU-145) were also used to study the inhibition of the 25-hydroxyvitamin D₃ and ATRA metabolism using the synthesised compounds. Reverse-transcriptase-polymerase chain reaction (RT-PCR) analysis was carried out to demonstrate the presence of CYP24 and CYP26 mRNA in the cancer cell-lines. Some of the synthesised compounds described herein show improved activities compared with ketoconazole and/or liarozole in these in vitro assays. Molecular docking studies using the homology model of CYP26 was carried out to investigate the binding of the substrate, ATRA and the synthesised compounds at the active site.

Furthermore, the anti-proliferative effects of some synthesised compounds, both alone and in combination with 1α,25-(OH)₂-D₃ in human prostate cancer cells (DU-145 and PC-3) were examined. The effects of ketoconazole and one of the synthesised compounds were further investigated to examine their effects, both alone and in combination with 1α,25-(OH)₂-D₃ on the vitamin D₃ target genes in DU-145 and PC-3 by real-time quantitative RT-PCR.
List of conference abstracts and papers

The following titles have been presented either as a conference abstract or paper. The full conference abstracts (1 – 5) and papers (6 – 8) can be seen in Appendix 2.

1. Design and synthesis of benzofuran derivatives as CYP24 inhibitors for androgen-independent prostate cancer.
   
   *Poster presentation. European Conference of the Pharmaceutical Chemistry Group of the Atlantic Arc (GP2A), October 2003, La Rochelle, France.*

2. Design and synthesis of P450 enzyme inhibitors as differentiating agent for androgen-independent prostate cancer.
   
   *Oral presentation. International Symposium of Medicinal Chemistry, August 2004, Copenhagen, Denmark.*


4. Synthesis and evaluation of retinoic acid metabolism blocking agents (RAMBAs) as indirect differentiating agents for cancer therapeutics.
   
   *Oral presentation. American Chemical Society national meeting, March 2005, San Diego, USA.*

5. Design and assessment of novel inhibitors of CYP24 to enhance VDR signalling in androgen-independent prostate cancer cells.
   
   *Poster presentation. NCRI (National Cancer Research Institute) Conference, October 2005, Birmingham, UK.*


## Contents

Title i
Declaration ii
Dedication iii
Acknowledgements iv
Abstract v
List of conference abstracts and publications vi
Contents vii
Abbreviations xii

### Chapter 1 Introduction

1.1 Prostate cancer and treatment 2
   1.1.1 Incidence 2
   1.1.2 The prostate gland 2
   1.1.3 Risk factors of prostate cancer 3
       1.1.3.1 Age 3
       1.1.3.2 Race 3
       1.1.3.3 Diet 3
       1.1.3.4 Family history and genetic factors 4
       1.1.3.5 Hormones 5
   1.1.4 Diagnosis and screening of prostate cancer 5
       1.1.4.1 PSA test 5
       1.1.4.2 Digital reaction examination (DRE) 6
       1.1.4.3 Transrectal ultrasound (TRUS) 6
   1.1.5 Staging of prostate cancer 6
       1.1.5.1 Pathologic evaluation 6
       1.1.5.2 Clinical evaluation 7
   1.1.6 Treatment and management of prostate cancer 8
       1.1.6.1 Radical prostatectomy 9
       1.1.6.2 Radiation therapy 9
       1.1.6.3 Hormone therapy 10
       1.1.6.4 Chemotherapy 13
       1.1.6.5 Watchful waiting 14
       1.1.6.6 New therapy 14

1.2 Androgens and prostate cancer 15
   1.2.1 Biosynthesis of steroid hormones 15
   1.2.2 Biosynthesis and regulation of androgens 17
   1.2.3 Androgen receptor 19
   1.2.4 Prostate cancer aetiology 20
   1.2.5 The aetiolooy of androgen-independent prostate cancer 20
   1.2.6 Conclusions 21
1.3 **Vitamin D₃**

1.3.1 **The vitamin D₃ endocrine system**
1.3.1.1 Metabolism and production of vitamin D₃ metabolites
1.3.1.2 Tissue distribution of vitamin D₃ hydroxylases and regulation of vitamin D₃
1.3.1.3 Role of vitamin D₃ in homeostasis

1.3.2 **The chemistry of the vitamin D₃**

1.3.3 **Therapeutic uses of vitamin D₃ analogues**

1.4 **The retinoids**

1.4.1 **Biosynthesis, metabolism and regulation of the retinoids**
1.4.2 **The chemistry of the retinoids**
1.4.3 **Therapeutic uses of the retinoids**

1.5 **The nuclear receptor superfamily**

1.5.1 **Vitamin D₃ receptor, Retinoic acid receptor and Retinoid X receptor**

1.6 **The role of vitamin D₃ and retinoids in prostate cancer**

1.6.1 **Anti-proliferative effects of 1α,25-(OH)₂-D₃ and retinoids in prostate cancer cells**
1.6.2 **Apoptotic effects of 1α,25-(OH)₂-D₃ and retinoids in prostate cancer cells**
1.6.3 **Pro-differentiation effects of 1α,25-(OH)₂-D₃ and retinoids in prostate cancer cells**
1.6.4 **Synergistic effects of 1α,25-(OH)₂-D₃ and retinoids in prostate cancer cells**
1.6.5 **Clinical studies of vitamin D₃ and retinoids in prostate cancer**

1.7 **The properties of the vitamin D₃ and retinoic acid hydroxylase enzymes**

1.7.1 **General features of the hydroxylase enzymes**
1.7.2 **Vitamin D₃ hydroxylases**
1.7.3 **Inhibitors of vitamin D₃ hydroxylase enzymes**
1.7.3.1 Vitamin D₃ hydroxylase enzymes inhibitors and prostate cancer
1.7.4 **Retinoic acid hydroxylase enzymes**
1.7.5 **Inhibitors of ATRA hydroxylase enzymes or RAMBAs**
1.7.5.1 RAMBAs and prostate cancer

**Chapter 2** **Aims of the investigation**
Chapter 3 Synthesis of 1- and 4-[(benzo[b]furan-2-yl)phenylmethyl]triazoles derivatives

3.1 Synthesis of 1- and 4- [(benzo[b]furan-2-yl)-phenylmethyl]-triazoles
  3.1.1 Synthesis of the phenacetyl bromide 56
  3.1.2 Synthesis of the substituted (benzo[b]furan-2-yl)-phenylmethanones 58
  3.1.3 Synthesis of the biphenyl ring 59
  3.1.4 Synthesis of the benzo[b]furan-2-yl(4-substituted phenyl)methanol 60
  3.1.5 Synthesis of the substituted 1- and 4-[(benzo[b]furan-2-yl)phenylmethyl]triazoles 61

3.2 General material and method 64

3.3 Experimental results for the synthesis of substituted 1- and 4-[(benzo[b]furan-2-yl)phenylmethyl]triazoles 65

Chapter 4 Synthesis of benzo[b]furan-2-carboxamido ethyl-imidazole and -1,2,4-triazole

4.1 Benzo[b]furan-2-carboxamido ethyl-imidazole and -1,2,4-triazole derivatives 79
  4.1.1 Synthesis of substituted ethyl benzo[b]furan-2-carboxylate 79
  4.1.2 Synthesis of substituted benzo[b]furan-2-carboxylic acid 81
  4.1.3 Synthesis of the substituted 2-amino-1-phenyl-ethanol 82
  4.1.4 Synthesis of the substituted N2-(2-hydroxy-2-phenylethyl)-benzo[b]furan-2-carboxamide 83
  4.1.5 Synthesis of the substituted 2-benzo[b]furan-2-yl-5-phenyl-4,5-dihydro-1,3-oxazole 84
  4.1.6 Synthesis of the substituted N2-[2-phenyl-2-(1H-1-imidazoyl)ethyl]-benzo[b]furan-2-carboxamide 87
  4.1.7 Synthesis of the substituted N2-[2-phenyl-2-(1H-1,2,4-triazol-1-yl)ethyl]-benzo[b]furan-2-carboxamide and the substituted N2-[2-phenyl-2-(4H-1,2,4-triazol-4-yl)ethyl]-benzo[b]furan-2-carboxamide 88

4.2 Experimental results for the synthesis of benzo[b]furan-2-carboxamido ethyl-imidazole and -1,2,4-triazole derivatives 91

Chapter 5 Synthesis of 6-substituted-2-(phenylmethylene)-3,4-dihydronaphthalene-1-one (tetralone) derivatives

5.1 Synthesis of 6-substituted-2-(phenylmethylene)-3,4-dihydronaphthalene-1-one derivatives 123

5.2 Experimental results for the synthesis of 6-substituted-2-(phenylmethylene)-3,4-dihydronaphthalene-1-one derivatives 133
Chapter 6  Inhibition of vitamin D₃ and all-trans retinoic acid metabolism in rat kidney mitochondria and rat liver microsomes

6.1 Vitamin D₃ metabolism study  163
6.2 All-trans retinoic acid metabolism study  164
6.3 Aims and objectives  164
6.4 Materials and equipments  165
6.5 Pharmacological preparation of the rat kidney mitochondria and rat liver microsome
   6.5.1 Method for the preparation of the rat kidney mitochondria  167
   6.5.2 Method for the preparation of the rat liver microsome  168
6.6 General assay for the inhibition studies of vitamin D₃ in rat kidney mitochondria and all-trans retinoic acid in rat liver microsome
   6.6.1 Set up of the high performance liquid chromatography (HPLC)  169
6.7 Biological results and discussions  170
   6.7.1 Metabolism of vitamin D₃ in rat kidney mitochondria  170
   6.7.2 Metabolism of all-trans retinoic acid in rat liver microsome  176
6.8 General conclusions  178

Chapter 7  In vitro cell culture studies of vitamin D₃ and all-trans retinoic acid metabolism

7.1 Aims and objectives  182
7.2 Overview  183
   7.2.1 Tissue culture  183
   7.2.2 Cell-lines used  184
   7.2.3 Methods of tissue culture  184
      8.2.3.1 Cells passaging  184
      8.2.3.2 Setting up of cells for vitamin D₃ and all-trans retinoic acid metabolism studies  185
7.3 General assay for metabolism of vitamin D₃ and all-trans retinoic acid in cell culture
   7.3.1 Methods  185
7.4 Biological results and discussions  187
   7.4.1 Metabolism of vitamin D₃ in MCF-7 and DU-145 cell-lines  187
   7.4.2 Metabolism of all-trans retinoic acid in MCF-7 cell-line  189
      7.4.2.1 Inhibition of all-trans retinoic acid metabolism in MCF-7 cell-line  190
      7.4.2.2 Molecular docking  197
Chapter 8  Further studies and investigations

8.1  Introduction of RT-PCR and real-time quantitative RT-PCR  206
8.2  RT-PCR analysis  211
8.3  Real-time quantitative RT-PCR analysis (qPCR)  213
  8.3.1  Results and discussions of the qPCR analysis  216
    8.3.1.1  Regulation of CYP24 mRNA in DU-145 and PC-3  217
    8.3.1.2  Regulation of p21\textsuperscript{waf1/cip1} mRNA in DU-145 and PC-3  218
    8.3.1.3  Regulation of GADD45\textalpha mRNA in DU-145 and PC-3  220
    8.3.1.4  Summary of the qPCR results  221
8.4  Cell proliferation and cell viability assay (MTT assay)  222
  8.4.1  Methods for MTT assay  222
  8.4.2  Results and discussions from the MTT assay  223
8.5  General conclusions  227
8.6  Antifungal and antileishmanial evaluations  227

Chapter 9  References  231

Appendix 1  X-ray crystal data
Appendix 2  Conference abstracts and publications
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Distribution</th>
</tr>
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<tbody>
<tr>
<td>25-(OH)_3-D_3</td>
<td>25-Hydroxyvitamin D_3</td>
</tr>
<tr>
<td>1α,25-(OH)_2-D_3</td>
<td>1α,25-Dihydroxyvitamin D_3 (Calcitriol)</td>
</tr>
<tr>
<td>24,25-(OH)_2-D_3</td>
<td>24,25-Dihydroxyvitamin D_3</td>
</tr>
<tr>
<td>1α,24, 25-(OH)_3-D_3</td>
<td>1α,24,25-Trihydroxyvitamin D_3</td>
</tr>
<tr>
<td>ACTH</td>
<td>Andrenocorticotrophic hormone</td>
</tr>
<tr>
<td>AIPC</td>
<td>Androgen-independent prostate cancer [synonym: hormone-refractory prostate cancer (HRPC)]</td>
</tr>
<tr>
<td>AR</td>
<td>Androgen receptor</td>
</tr>
<tr>
<td>ARE</td>
<td>Androgen response element</td>
</tr>
<tr>
<td>ATRA</td>
<td>All-trans retinoic acid</td>
</tr>
<tr>
<td>BPH</td>
<td>Benign prostatic hyperplasia</td>
</tr>
<tr>
<td>CDI</td>
<td>1,1'-Carbonyldiimidazole</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxyribose nucleic acid</td>
</tr>
<tr>
<td>CRH</td>
<td>Corticotrophin-releasing hormone</td>
</tr>
<tr>
<td>CSCC</td>
<td>Cholesterol side-chain cleavage</td>
</tr>
<tr>
<td>CYP1α</td>
<td>Cytochrome P450 1α-hydroxylase</td>
</tr>
<tr>
<td>CYP24</td>
<td>Cytochrome P450 24-hydroxylase</td>
</tr>
<tr>
<td>DCM</td>
<td>Dichloromethane</td>
</tr>
<tr>
<td>DES</td>
<td>Diethylstibestrol</td>
</tr>
<tr>
<td>DHEA</td>
<td>Dehydroepiandrosterone</td>
</tr>
<tr>
<td>DHT</td>
<td>Dihydrotestosterone</td>
</tr>
<tr>
<td>DMF</td>
<td>Dimethyl formamide</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DRE</td>
<td>Digital rectal examination</td>
</tr>
<tr>
<td>EI</td>
<td>Electron ionisation</td>
</tr>
<tr>
<td>ES</td>
<td>Electron spray</td>
</tr>
<tr>
<td>EtOAc</td>
<td>Ethyl acetate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>EtOH</td>
<td>Ethanol</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal bovine serum</td>
</tr>
<tr>
<td>FCS</td>
<td>Foetal calf serum</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and drug administration</td>
</tr>
<tr>
<td>FSH</td>
<td>Follicle-stimulating hormone</td>
</tr>
<tr>
<td>GADD45α</td>
<td>Growth-arrest and DNA-damage gene</td>
</tr>
<tr>
<td>GH</td>
<td>Growth hormone</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>HMBC</td>
<td>Heteronuclear multiple bond coherence</td>
</tr>
<tr>
<td>HRMS</td>
<td>High resolution mass spectrum</td>
</tr>
<tr>
<td>HRPC</td>
<td>Hormone-refractory prostate cancer [synonym: androgen-independent prostate cancer (AIPC)]</td>
</tr>
<tr>
<td>HSD</td>
<td>Hydroxysteroid dehydrogenase</td>
</tr>
<tr>
<td>HSQC</td>
<td>Heteronuclear single quantum coherence</td>
</tr>
<tr>
<td>LH</td>
<td>Luteinising hormone</td>
</tr>
<tr>
<td>LHRH</td>
<td>Luteinising hormone-releasing hormone</td>
</tr>
<tr>
<td>MeOH</td>
<td>Methanol</td>
</tr>
<tr>
<td>MOE</td>
<td>Molecular operating environment</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide-adenine dinucleotide phosphate (reduced form)</td>
</tr>
<tr>
<td>n.m.r.</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffer saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PDB</td>
<td>Protein data bank</td>
</tr>
<tr>
<td>PSA</td>
<td>Prostatic specific antigen</td>
</tr>
<tr>
<td>PTH</td>
<td>Parathyroid hormone</td>
</tr>
<tr>
<td>qPCR</td>
<td>Real-time quantitative RT-PCR</td>
</tr>
<tr>
<td>RA</td>
<td>Retinoic acid</td>
</tr>
<tr>
<td>RAMBA</td>
<td>Retinoic acid metabolism blocking agent</td>
</tr>
<tr>
<td>r.t.</td>
<td>Room temperature</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse transcriptase</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>S-FCS</td>
<td>Steroid-depleted foetal calf serum</td>
</tr>
<tr>
<td>THF</td>
<td>Tetrahydrofuran</td>
</tr>
<tr>
<td>t.l.c.</td>
<td>Thin layer chromatography</td>
</tr>
<tr>
<td>TNM</td>
<td>Tumour node metastases</td>
</tr>
<tr>
<td>TRUS</td>
<td>Transrectal ultrasound</td>
</tr>
<tr>
<td>VDR</td>
<td>Vitamin D receptor</td>
</tr>
<tr>
<td>VDRE</td>
<td>Vitamin D response element</td>
</tr>
<tr>
<td>v/v</td>
<td>volume/volume</td>
</tr>
<tr>
<td>wRPMI</td>
<td>Phenol-red free RPMI media</td>
</tr>
</tbody>
</table>
CHAPTER 1

Introduction
1. Introduction

1.1 Prostate cancer and treatment

1.1.1 Incidence

Prostate cancer, is the most common cancer among males in the USA, with an estimated 232,090 new cases and 30,350 deaths for 2005 alone, i.e. 1 in 6 men (Jemal et al., 2005). Prostate cancer was the sixth most common cancer in the world (Parkin, 2004) and third most common cancer in men in Europe (Bhandari et al., 2005), with some 156,000 new cases in 1995 (Bray et al., 2002).

1.1.2 The prostate gland

The prostate gland is an organ situated just below the bladder and in front of the rectum (Figure 1.1.1). It plays a role in producing the fluid that transports semen during ejaculation. The size and shape of the prostate gland are variable, but it is usually about 2 – 3 inches in diameter and the weight of a normal adult prostate is approximately 20 g (Griffin, 2000).

The prostate gland is composed of tall columnar secretory epithelial cells and stromal cells. The epithelial and stromal cells interact closely to maintain the normal function of the prostate gland (DeMarzo et al., 2003).

![Figure 1.1.1. The location of the prostate gland in males (Christie Hospital NHS Trust, 2003).]
1.1.3 Risk factors of prostate cancer

1.1.3.1 Age

Prostate cancer is a disease associated with increasing age, with 78 % cases are in men aged 65 years or above (Parkin, 2004). Autopsy studies reveal that the majority of men over 80 years old have areas of malignant tissue in their prostate glands; most die with it, not of it (Selley et al., 1997c).

1.1.3.2 Race

Prostate cancer incidence and mortality rates vary widely between ethnic groups (Sasagawa and Nakada, 2001). Both incidence and mortality data reveal that the incidence of clinical prostate cancer is low in Asian men and higher in Scandinavian men (Boring et al., 1992). Incidence rates vary 120-fold between Chinese men and African-American men living in San Francisco (Pienta and Esper, 1993). It is unclear if this is based on genetic or environmental factors; however, migration studies demonstrate that men tend to take on the risk for their host countries. An example of this is, Japanese-Americans show higher mortality rates when compared with the mortality rates in Japan (Cook et al., 1999). The possible explanation for differences in the incidence and mortality of prostate cancer is the dietary differences between ethnicities (Muir et al., 1991; Yu et al., 1991).

1.1.3.3 Diet

There has been considerable interest in evaluating the link between diet and prostate cancer, particularly dietary fat and vitamins.

Dietary fat is most frequently associated with prostate cancer. Rose and colleagues have shown a positive correlation between the mortality rate for prostate cancer and animal fat consumption by country (Rose et al., 1986). Several other studies also confirmed this relationship between high dietary fat and increased prostate cancer risk (Heshmat et al., 1985; Mettlin et al., 1989; Mills et al., 1989; Slattery et al., 1990; Snowdon et al., 1984).

The n-3 and n-6 polyunsaturated fatty acids have been shown in laboratory studies to have beneficial and risk factors, respectively, for several kinds of malignancy including prostate (Bagga et al., 1997; Gann et al., 1994; Harvei et al., 1997). Epidemiological data reported by Terry’s group indicated that fish fat consumption lowers the risk of prostate cancer (Terry et al., 2001). Persons who consume fish (including salmon, herring and mackerel which contain a high amount of n-3 fatty acid)
had a lower relative risk of prostate cancer either diagnosed or cancer death compared with persons who consumed less fish (Terry et al., 2001). The n-3 fatty acid is thought to influence cellular proliferation, the immune system and the potential of a tumour to invade locally and metastasise (Karmali, 1987).

Vitamins and supplement which have been shown by epidemiology or laboratory study to have protective effects on prostate cancer are as following and have been reviewed in detail by few authors (Heshmat et al., 1985; Willis and Wians Jr., 2003).

- Vitamin A (Cohen et al., 2000; Giovannucci, 1999)
- Vitamin E and D (Heinonen et al., 1998; Klein, 2005; Schwartz and Hulka, 1990)
- Phytoestrogens (Adlercreutz, 2002; Jacobsen et al., 1998; Klein, 2005; Shirai et al., 2002; Wang et al., 2002).

The role of vitamin A and vitamin D3 in prostate cancer will be discussed in section 1.6 of this chapter.

1.1.3.4 Family history and genetic factors

Several studies reveal a positive association between family history and prostate cancer risk (Goldgar et al., 1994; Steinberg et al., 1990). The risk for the first degree relative of a man diagnosed with prostate cancer is 2 - 3 fold compared with the risk of a man with no family history (Steinberg et al., 1990).

Researchers at the National Center for Human Genome Research, The Johns Hopkins University and Umeå University, Sweden, have identified the location of the first major gene that predisposes men to prostate cancer. They reported the localisation of a major susceptibility locus for prostate cancer (HPC1) to chromosome 1 (band q24-25). This international study involved 91 families with at least three first degree relatives with prostate cancer (Smith et al., 1996).

The two main genetic studies which demonstrated the gene that are associated with the risk and progression of prostate cancer are:

- Vitamin D receptor gene polymorphism (Habuchi et al., 2000; Ingles et al., 1997; Taylor et al., 1996). Two separate epidemiology studies involved white and black men in the United States (Ingles et al., 1997) and the Japanese group (Habuchi et al., 2000) revealed that inherited polymorphisms in the VDR gene may be linked with prostate cancer risk.
• The polymorphic CAG repeat sequence in the androgen receptor gene. A few research groups have found that men with short CAG repeat lengths had a higher risk of prostate cancer (Giovannucci et al., 1997; Ingles et al., 1997; Irvine et al., 1995). Coetzee and Ross postulated that a short CAG repeat lengths is associated with a higher level of androgen receptor transactivation function, therefore possibly resulting in a higher risk of prostate cancer (Coetzee and Ross, 1994).

1.1.3.5 Hormones
Steroid hormones play important role in the growth of the normal prostate epithelium and in the development of prostatic cancer. This is widely supported by the fact that: (1) prostate cancer does not occur in eunuchs and the incidence is very low in castrated men (Pienta and Esper, 1993); (2) the incidence of microscopic, latent prostate cancer show a very close correlation with increasing age (Wilding, 1995); (3), orchiectomy and anti-androgen therapy play a vital role in the first-line treatment of prostate cancer (McLeod, 2003).

1.1.4 Diagnosis and screening of prostate cancer
Early diagnosis of prostate cancer could provide improved prognostic factors and could lower the mortality and morbidity rate (Selley et al., 1997c). Patients with prostate cancer will complain of having nocturia, increase in urination frequency and urgency, which is a result of urinary tract obstruction due to the enlarged prostate gland (Selley et al., 1997a). Patients can be asymptomatic during the early stage, therefore screening for prostate cancer by the Prostate-Specific Antigen (PSA) testing is very important.

The first stage of diagnosis of prostate cancer is to measure serum PSA level and to carry out a Digital Rectal Examination (DRE). If there are suspicious findings from either of these tests, transrectal ultrasound technology will be used to allow histological confirmation of the presence of prostate cancer (Selley et al., 1997a).

1.1.4.1 PSA test
PSA is a glycoprotein which is secreted by prostate epithelium (Garnick, 1993). PSA is found mainly in the semen, and a small amount is present in the blood serum. An immuno-assay method is used to measure the PSA level (Garnick, 1993). Most men have levels under 4 ng/mL in the blood (Garnick, 1993). The PSA level is
raised after ejaculation, after prostate biopsy, surgery, prostatitis and also in patients with benign prostatic hyperplasia and prostate cancer (Frankel et al., 2003). Therefore, PSA measurement should always be carried out before prostate biopsy (Garnick, 1993).

Although PSA testing is not specific for prostate cancer, it is a quick and easy diagnostic tool for screening and staging of prostate cancer (Frankel et al., 2003). Moreover, PSA testing is important in patient undergoing prostate cancer treatment, as the patient’s PSA level can be an indicator as to how well the treatment is working for the patient (Selley et al., 1997c).

1.1.4.2 Digital rectal examination (DRE)

DRE is a common diagnostic and screening test for prostate cancer. During the examination, the doctor inserts the finger into the rectum to examine whether there is the presence of an abnormal mass in the prostate gland (Selley et al., 1997a). Similarly to PSA testing, DRE is also used for screening, diagnostic and clinical staging of prostate cancer.

1.1.4.3 Transrectal ultrasound (TRUS)

This is used in the diagnosis and clinical staging of prostate cancer. The doctor will use transrectal ultrasound (TRUS) to guide the biopsy needle through the wall of the rectum and into several areas of the prostate gland to remove samples of prostate tissues. Samples obtained allow accurate histologic grades of the sample neoplasm by the pathologist (Selley et al., 1997a).

1.1.5 Staging of prostate cancer

1.1.5.1 Pathologic evaluation

Pathologists grade the prostate cancers according to the Gleason system. The pathologist determines the differentiation of the tumour cells (Selley et al., 1997b). Gleason grade ranging from 1 to 5 divides the prostate cancer into 5 different histologic patterns. Gleason grade 1 represent well-differentiated prostate cancer (i.e. least aggressive), whereas Gleason grade 5 represent poorly-differentiated or anaplastic lesion (i.e. most aggressive tumour cells). A grade is assigned in two different areas that best describe the cancerous cells/tissues. These two grades are added together to give the Gleason score, ranging from 2 to 10. Scores of below 7 tend to have better prognosis (Selley et al., 1997b).
1.1.5.2 Clinical evaluation

The most commonly used staging system for prostate cancer is called the TNM System of the American Joint Committee on Cancer (AJCC) (Selly et al., 1997b). The TNM system applies to both clinical as well as pathological staging (see Box 1). This system describes the extent of the primary tumour (T stage), whether or not the cancer has spread to nearby lymph nodes (N stage) and the absence or presence of distant metastasis (M stage) (Selly et al., 1997b). Once this is determined, this is then combined with the Gleason score to give the stage grouping (Box 1), ranging from Stage 0 to Stage IV.

**Box 1.1.** The staging system for prostate cancer: the TNM system of the American Joint Committee on Cancer (AJCC). Permission granted from AJCC (American Joint Committee on Cancer, 1992).

**Primary Tumour (T)**

<table>
<thead>
<tr>
<th>Code</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>TX</td>
<td>Primary tumour cannot be assessed</td>
</tr>
<tr>
<td>T0</td>
<td>No evidence of primary tumour</td>
</tr>
<tr>
<td>T1</td>
<td>Clinically inapparent tumour not palpable or visible by imaging</td>
</tr>
<tr>
<td>T1a</td>
<td>Tumour incidental histologic finding in 5% or less of tissue resected</td>
</tr>
<tr>
<td>T1b</td>
<td>Tumour incidental histologic finding in more than 5% of tissue resected</td>
</tr>
<tr>
<td>T1c</td>
<td>Tumour identified by needle biopsy (e.g., because of elevated PSA)</td>
</tr>
<tr>
<td>T2</td>
<td>Tumour confined within the prostate*</td>
</tr>
<tr>
<td>T2a</td>
<td>Tumour involves half of a lobe or less</td>
</tr>
<tr>
<td>T2b</td>
<td>Tumour involves more than half of a lobe, but not both lobes</td>
</tr>
<tr>
<td>T2c</td>
<td>Tumour involves both lobes</td>
</tr>
<tr>
<td>T3</td>
<td>Tumour extends through the prostatic capsule**</td>
</tr>
<tr>
<td>T4</td>
<td>Tumour is fixed or invades adjacent structures other than the seminal vesicles</td>
</tr>
<tr>
<td>T4a</td>
<td>Tumour invades any of: bladder neck, external sphincter, or rectum</td>
</tr>
<tr>
<td>T4b</td>
<td>Tumour invades levator muscles and/or fixed to the pelvic wall</td>
</tr>
</tbody>
</table>

*Note: Tumour found in one or both lobes by needle biopsy, but not palpable or visible by imaging, is classified as T1c.

**Note: Invasion into the prostatic apex or into (but not beyond) the prostatic capsule is not classified as T3, but as T2.

**Regional Lymph Nodes (N)**

<table>
<thead>
<tr>
<th>Code</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>NX</td>
<td>Regional lymph nodes cannot be assessed</td>
</tr>
<tr>
<td>N0</td>
<td>No regional lymph node metastasis</td>
</tr>
<tr>
<td>N1</td>
<td>Metastasis in a single lymph node, 2 cm or less in greatest dimension</td>
</tr>
<tr>
<td>N2</td>
<td>Metastasis in a single lymph node, more than 2 cm but not more than 5 cm in greatest dimension; or multiple lymph node metastases, none more than 5 cm in greatest dimension</td>
</tr>
<tr>
<td>N3</td>
<td>Metastasis in lymph node more than 5 cm in greatest dimension</td>
</tr>
</tbody>
</table>
### Distant Metastasis (M)

<table>
<thead>
<tr>
<th>MX</th>
<th>Presence of distant metastasis cannot be assessed</th>
</tr>
</thead>
<tbody>
<tr>
<td>M0</td>
<td>No distant metastasis</td>
</tr>
<tr>
<td>M1</td>
<td>Distant metastasis</td>
</tr>
<tr>
<td>M1a</td>
<td>Nonregional lymph node(s)</td>
</tr>
<tr>
<td>M1b</td>
<td>Bone(s)</td>
</tr>
<tr>
<td>M1c</td>
<td>Other site(s)</td>
</tr>
</tbody>
</table>

### Histopathologic Grade (G)

<table>
<thead>
<tr>
<th>GX</th>
<th>Grade cannot be assessed</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1</td>
<td>Well differentiated (slight anaplasia)</td>
</tr>
<tr>
<td>G2</td>
<td>Moderately differentiated (moderate anaplasia)</td>
</tr>
<tr>
<td>G3-4</td>
<td>Poorly differentiated or undifferentiated (marked anaplasia)</td>
</tr>
</tbody>
</table>

### Stage Grouping

<table>
<thead>
<tr>
<th>Stage</th>
<th>T1a</th>
<th>N0</th>
<th>M0</th>
<th>G1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage 0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage I</td>
<td>T1a</td>
<td>N0</td>
<td>M0</td>
<td>G2, G3-4</td>
</tr>
<tr>
<td></td>
<td>T1b</td>
<td>N0</td>
<td>M0</td>
<td>Any G</td>
</tr>
<tr>
<td></td>
<td>T1c</td>
<td>N0</td>
<td>M0</td>
<td>Any G</td>
</tr>
<tr>
<td></td>
<td>T1</td>
<td>N0</td>
<td>M0</td>
<td>Any G</td>
</tr>
<tr>
<td>Stage II</td>
<td>T2</td>
<td>N0</td>
<td>M0</td>
<td>Any G</td>
</tr>
<tr>
<td>Stage III</td>
<td>T3</td>
<td>N0</td>
<td>M0</td>
<td>Any G</td>
</tr>
<tr>
<td>Stage IV</td>
<td>T4</td>
<td>N0</td>
<td>M0</td>
<td>Any G</td>
</tr>
<tr>
<td></td>
<td>Any T</td>
<td>N1</td>
<td>M0</td>
<td>Any G</td>
</tr>
<tr>
<td></td>
<td>Any T</td>
<td>N2</td>
<td>M0</td>
<td>Any G</td>
</tr>
<tr>
<td></td>
<td>Any T</td>
<td>N3</td>
<td>M0</td>
<td>Any G</td>
</tr>
<tr>
<td></td>
<td>Any T</td>
<td>Any N</td>
<td>M1</td>
<td>Any G</td>
</tr>
</tbody>
</table>

### 1.1.6 Treatment and management of prostate cancer

The clinical management and treatment depend on the prostate cancer disease, patients’ life expectancy and patients’ health conditions. The National Comprehensive Cancer Network has developed detailed cancer treatment guidelines for prostate cancer health professionals (Scherr et al., 2003). Recent reviews on the management and treatment of prostate cancer according to stages has been published by various authors (Garnick, 1993; Jani and Hellman, 2003; Selley et al., 1997a). The potential benefits and risks of the treatment options should always be considered. The five main types of treatments are described below.
1.1.6.1 Radical prostatectomy

Prostatectomy involves surgical removal of the entire prostate gland and some of the surrounding tissue. During the surgical procedure, the surgeon can assess the pathological disease of the patient, i.e. to assess the extent to which the cancer has spread. This could guide the physicians decision whether to give any other adjuvant therapy to the patients (Ohori et al., 1995). Radical retropubic prostatectomy, radical perinial prostatectomy and radical laproscopic prostatectomy are the three different types of radical prostatectomy. Radical retropubic prostatectomy involves skin incision in the lower abdomen to allow the removal of the whole prostate gland and the seminal vesicles. Complications associated with radical retropubic prostatectomy are intraoperative bleeding and postoperative complication, for examples, urinary incontinence and impotence (Catalona et al., 1999; Lepor et al., 2001; Stanford et al., 2000). Radical perinial prostatectomy and radical laproscopic prostatectomy have not achieved widespread use due to a lack of evidence of the long term efficacy.

1.1.6.2 Radiation therapy

Radiation therapy is used to kill cancer cells by high energy particles can be divided into two main types, namely the external-beam radiotherapy and brachytherapy.

External-beam radiation therapy

The use of external-beam radiation therapy has been studied and carried out extensively for early-stage and locally advanced prostate cancer. External-beam radiation therapy has undergone a technological revolution and, has shown promising success rate and reduction of side effects (Hanlon and Hanks, 2000; Shipley et al., 1999; Zagars et al., 1997; Zietman et al., 1995). The two newer form of external-beam radiation therapy, namely the three-dimensional conformal radiation therapy (3DCRT) and the intensity-modulate radiation therapy (IMRT) minimizes the dose of radiation reaching normal tissues while delivering high dose to the cancer cells to minimize complications (Pollack et al., 2000; Zelefsky et al., 2001).

Brachytherapy

Brachytherapy involves the use of small radioactive pellets that are implanted directly into the prostate gland. Transrectal ultrasound, CT scans or MRI is used to guide the placement of the radioactive material. The use of this treatment for early-stage prostate cancer is positive and its use can achieve disease-free survival comparable with
those of radical prostatectomy and external-beam radiotherapy (Jani and Hellman, 2003). The two main types of brachytherapy are the \textit{low-dose rate} using low energy radioactive sources (Iodine-125 or Palladium-103) and the \textit{high-dose rate} using high dose radioactive source (Iridium-192) (Vicini et al., 1999). Brachytherapy provides a more localised dose distribution compared with external-beam radiation therapy (Vicini et al., 1999). In the external-beam radiation therapy, the beam needs to transverse across normal tissues, such as, the bladder and rectum in order to reach the prostate. As a result, brachytherapy provides a lower incidence of rectal and neurovascular side-effects (Merrick et al., 2000; Merrick et al., 2001; Potters et al., 2001).

Overall, radiation therapy is a non-invasive method, with no anaesthesia risk. It is suitable in patients who would not be able to tolerate prostatectomy (Jani and Hellman, 2003).

Although the results were positive in the use of external-beam radiation therapy (Koper et al., 1999) and brachytherapy (Martinez et al., 2001; Vicini et al., 1999), there is a lack of randomized trials comparing radiotherapy with surgery for prostate cancer (Fletcher and Theodorescu, 2005).

1.1.6.3 Hormone therapy

Surgery and radiation therapy is the mainstay of treatment for early-stage prostate cancer. Whereas, treatment for locally or advanced disease rely on hormonal therapies to suppress the testosterone production (Scherr et al., 2003). The available hormonal therapies could be divided into 4 groups, which will be discussed in detail here.

\textit{Oestrogen therapy}

Oestrogen therapy in the form of diethylstilbestrol (DES) (Figure 1.1.2) was the earliest form of treatment for advanced prostate cancer in the 1980s (Scherr et al., 2002). DES is the first non-steroidal compound with potent oestrogenic activity (Dodds et al., 1938).

![Figure 1.1.2. The chemical structure of \textit{trans}-diethylstilbestrol.](image-url)
DES has an effect on suppressing the production of testosterone by suppression of pituitary luteinising hormone (LH) secretion from the pituitary gland (Malkowicz, 2001). Moreover, DES can increase the level of sex-hormone-binding globulin (SHBG), increase pituitary prolactin secretion and decrease testosterone production in the testes (Malkowicz, 2001). DES also gives a direct cytotoxic effect in prostate cancer in vitro through cell cycle control and apoptosis mechanism (Robertson et al., 1996).

The use of DES has fallen out of favour due to the unfavourable side-effects (Zagars et al., 1988). The most complicated side-effects are cardiovascular side-effects, for example, myocardial infarction and deep vein thrombosis (Malkowicz, 2001); gynaecomastia, nipple tenderness and breast pain (Stege, 2000). Unlike other hormonal therapy, DES offers additional benefits besides suppressing testosterone production, i.e., it does not cause bone loss and therefore has no potential of leading to osteoporosis (Scherr et al., 2002).

**Luteinising Hormone-Releasing Hormone (LHRH) Agonists**

Luteinising Hormone-Releasing Hormone (LHRH) was first isolated by Schally’s group (Schally et al., 2000) from porcine hypothalamic extracts. They demonstrated that this decapeptide (Figure 1.1.3) is responsible for the release of luteinising hormone (LH).

It was found that substitution of the amino acids in the basic LHRH structure, resulted in LHRH analogues with potent agonist activity. Paradoxically, it was found that these LHRH agonists decrease, rather than increase both the LH and testosterone levels and that they were very effective in reducing the serum testosterone levels (McLeod, 2003). The mode of action of LHRH agonist is to downregulate the LHRH receptors at the pituitary level, thus decreasing the release of testosterone (Limonta et al., 2001). A recent review anticipated that LHRH may exert direct anti-proliferative and apoptotic effects in prostate cancer cells (Kraus et al., 2005).

The use of LHRH analogues could initially lead to transient elevations in testosterone and dihydrotestosterone levels (Schellhammer, 2001) which is known as “tumour flare”. This manifestation of tumour flare may be prevented by 14 to 28 days administration of an antiandrogen drug, such as bicalutamide (Anon, 2005; Dalesio et al., 2000).
LHRH analogues have an increasing role in locally advanced and metastatic prostate cancer (Scherr et al., 2003). There are 4 different LHRH agonist currently available in United Kingdom for the treatment of prostate cancer (Anon, 2005). Common side-effects from LHRH agonist are hot flushes, loss of libido, weight gain, gynaecomastia and osteoporosis (Schally et al., 2000; Seidenfeld et al., 2000).

![Chemical structure of LHRH analogues](image)

Glutamic Acid — Histidine — Tryptophan — Serine — Tyrosine — Glycine — Leucine — Arginine — Proline — Glycine

P* = Amino acid-associated protein

**Figure 1.1.3.** The 10 amino acid sequence of LHRH (Ojeda and McCann, 2000).

**Anti-androgen therapy**

Anti-androgen drugs, for example, flutamide, bicalutamide (Casodex®) and cyproterone acetate (Figure 1.1.4), are used in patients with locally advanced disease (Kolvenbag et al., 2001). The function of anti-androgens is to block the androgen receptor and thus prevent the natural androgen substrate from binding to the androgen receptor. Androgen plays an important role in the pathology of prostate cancer by inducing growth of the prostate cancer cells (Debes and Tindall, 2002).

Monotherapy with 150 mg bicalutamide (Casodex®) has shown an equivalent effect to castration in patients with non-metastatic disease (Abrahamsson, 2001; Iversen et al., 2000; Kolvenbag et al., 2001). However, anti-androgens are not recommended in conjunction with surgical castration (Eisenberger et al., 1998). The side-effects profile for bicalutamide is better in terms of quality-of-life related to sexual interest and physical capacity (Kolvenbag et al., 2001; Seidenfeld et al., 2000).
**Figure 1.1.4.** The chemical structure of the anti-androgen drugs used in locally advanced prostate cancer.

**Combined androgen blockade**

This therapy involves the combination of an anti-androgen with an LHRH agonist to block androgen synthesis as well as preventing the binding of circulating androgen to the receptors. The use of LHRH analogues alone cannot block the androgen produced by the adrenal gland as the androgen produced from the adrenal gland is controlled by andrenocorticotropic hormone (ACTH). Therefore the use of the combined androgen blockade is to stop the action of the adrenal androgens (Chamberlain et al., 1997a; Dalesio et al., 2000).

**1.1.6.4 Chemotherapy**

Although more than 85% of patients with metastatic disease will respond to hormonal therapy, unfortunately, the prostate tumours eventually develop into androgen-independent type. One of the options for these patients with androgen-independent prostate cancer (AIPC) is to use cytotoxic therapy (Berry, 2005; Oh and Kandoff, 1998). The use of mitoxantrone (similar structure to the anthracycline, **Figure 1.15**) plus low dose of prednisolone was approved by Food and Drug Administration (FDA) for patients with AIPC in the 1990s (Moore et al., 1994). It exerts its cytotoxic effect through intercalating between adjacent base pairs of DNA (Skladanowski and Konopa, 2000).

Taxanes, paclitaxel, docetaxel and estramustine were also initiated to test their feasibility and therapeutic potential (Wit de, 2005). In estramustine, oestradiol-17β-phosphate is linked to a nitrogen mustard via a carbamate ester linkage (**Figure 1.1.5**). It
was designed for the purpose of releasing the mustard to the cancer cells, which acts as an alkylating agent, by cleavage of the linkage. However, it was found in *in vivo* and *in vitro* experiments that the mechanism involves the inhibition of microtubule function and mitosis (Hartley-Asp, 1984; Moraga *et al.*, 1992).

Recently two independent randomized studies have demonstrated that docetaxel-based chemotherapy (*i.e* docetaxel plus prednisolone or docetaxel plus estramustine) improves the overall survival of patients with AIPC (Petrylak *et al.*, 2004; Tannock *et al.*, 2004).

![Chemical structures](image)

**Figure 1.1.5.** The chemical structure of the commonly used cytotoxic agents for AIPC.

### 1.1.6.5 Watchful waiting

This form of management only applies to asymptomatic patients with localised prostate cancer. As the growth of prostate cancer is often very slow, watchful waiting may be the form of prostate cancer management, and more preferable if the patient’s life expectancy is less than 10 years. Patients will have regular check-ups which involve PSA testing and DRE (Chamberlain *et al.*, 1997b).

### 1.1.6.6 New therapy

There are a few promising research areas being investigated as alternative treatments for patients who have developed resistance to the standard androgen-ablation therapy. The American Cancer Society (http://www.cancer.org) has a very useful website to inform patients and health professionals regarding new clinical trials and new
treatments available. Some new approaches and novel agents targeting proteins involved in angiogenesis or cell cycle pathway and other small molecules that target specific signaling pathways have been discussed and reviewed in recent articles (Bhandari et al., 2005; Harris and Reese, 2001; Morris and Scher, 2002; van der Poel, 2004).

1.2 Androgens and prostate cancer

1.2.1 Biosynthesis of steroid hormones

The adrenal cortex, testis and ovary are the major tissues involve in the biosynthesis of steroid hormones. There are three major categories of steroid hormones (Figure 1.2.2):

- **Mineralocorticoids** – These are important for maintaining the body’s sodium and potassium content. Synthesis mainly occurs in the mitochondria and endoplasmic reticulum of the adrenal cortex.

- **Glucocorticoids** – These are important for maintaining the body’s carbohydrate reserves. Synthesis mainly occurs in the mitochondria and endoplasmic reticulum of the adrenal cortex.

- **Androgens** – e.g. testosterone and oestrogens, are responsible for the development of male and female secondary sex characteristics respectively. Synthesis mainly occurs in the testis (for testosterone) and ovary (for oestrogens).

Steroid hormones are synthesised via the steroidogenic pathways from the steroidal precursor cholesterol (Figure 1.2.1). Biosynthesis of the various steroid hormones involves P450 isozymes that are localised in steroidogenic tissues. P450 isozymes catalyse the oxygenation of carbons at position 3, 11, 17, 18 and 21, as depicted in Figure 1.2.2.

![Figure 1.2.1. Chemical structure of cholesterol and the steroid numbering system.](image)
Figure 1.22. Biosynthesis of the micronutrients, glucocorticoids, and sex hormones (i.e., androgens and estrogens).

17β-HSD = 17β-hydroxysteroid dehydrogenase.

Notes: P450 C21 = cholesterol side-chain cleavage enzyme; 3β-HSD = 3β-hydroxysteroid dehydrogenase.

Acetate $\rightarrow$ Cholesterol

Sex Hormones

Glucocorticoids

Micronutrients
1.2.2 Biosynthesis and regulation of androgens

Androgens play an important role during: (1) male sexual differentiation; (2) development and maintenance of the male secondary characteristics and (3) spermatogenesis (Mooradian et al., 1987). The testes produce 90 – 95 % of circulating androgens (Preslock, 1980).

Let’s first look at the pathway of androgen production and the example shown here will involve the prostate gland as the target tissue (Figure 1.2.3).

**Figure 1.2.3.** The regulation of androgen synthesis involving the prostate gland. The broken line indicates negative feedback loop. Abbreviations: LHRH = Luteinising hormone-releasing hormone; CRH = Corticotrophin-releasing hormone; ACTH = Adrenocorticotropic hormone; LH = Luteinising hormones; FSH = Follicle-stimulating hormone; GH = Growth hormone; DHEA = Dehydroepiandrosterone.
The diagram has been adapted from Galbraith and Duchesne (Galbraith and Duchesne, 1997).
Referring to the numbering in **Figure 1.2.3:**

1. The hypothalamus in the brain produces LHRH and secretes these hormones into the hypophyseal portal circulation to reach the anterior pituitary (Kraus *et al.*, 2005).

2. FSH and LH released from the pituitary gland stimulate Leydig cells to secrete testosterone. Secretion of these pituitary hormones is controlled by the negative feedback loop (depicted as broken lines in the figure) (Goodman, 1994b).

3. The hypothalamus produces and secretes CRH which then stimulate the release of ACTH from the pituitary gland (Goodman, 1994a).

4. ACTH then stimulates the adrenal gland, which covers the superior surface of the kidney, to produce three different kinds of corticosteroid hormones, namely aldosterone, hydrocortisone and adrenal androgens. The adrenal androgens include: androstenedione, DHEA and DHEA sulphate. Secretion of these corticosteroid hormones is also controlled by the negative feedback loop.

5. The testosterone which is released from the Leydig cells of the testes will be taken up by the prostate gland epithelium. In the prostate cells, testosterone is converted to 5α-dihydrotestosterone (5α-DHT), by 5α-reductase. 5α-DHT is the most active androgen, with a higher affinity for the androgen receptor than does testosterone (Berrevoets *et al.*, 2002).

6. The adrenal androgens released from adrenal gland will also have an effect on the growth of the prostate cells. DHEA can be converted into androstenedione and then to testosterone by two non-cytochrome P450 enzymes 3β-hydroxysteroid dehydrogenase (3β-HSD) and 17β-hydroxysteroid dehydrogenase (17β-HSD) respectively (also **Figure 1.2.2**). The process described above can either occur in the plasma or in the prostate cell itself, as the prostate cell is able to take up and metabolise the adrenal androgens (Harper *et al.*, 1974).

7. GH and prolactin are hormones that are synthesised and stored in the pituitary gland. The production of androgens from the testis and adrenal gland can be influenced by these two hormones. Prolactin can act with LH to stimulate testosterone secretion from the testes and can increase the adrenal androgen production (Galbraith and Duchesne, 1997).
1.2.3 Androgen receptor

Testosterone circulating in the blood is largely bound to albumin and sex-hormone-binding globulin (SHBG), and small fractions dissolved freely in the serum. The freely dissolved testosterone enters the target cells (e.g. prostate cells) where it either directly, or after conversion to 5α-DHT, binds to the androgen receptor (Figure 1.2.4). The androgen receptor (AR) is a member of the nuclear receptor superfamily (Griffin, 2000; Mangelsdorf et al., 1995). Vitamin D3 and retinoic acid receptors are also members of this nuclear receptor superfamily, these receptors will be discussed in section 1.5 in more detail.

ARs are found in the nucleus in the unbound state. The binding of androgen (testosterone or 5α-DHT) to the AR binding domain induces the formation of an AR homodimer complex. The DNA binding domain (DBD) of the receptor then binds to the androgen-responsive elements (AREs) in the promoter regions of target genes (Debes and Tindall, 2002). This will then allow transcription of target genes, such as genes that are involved in the control and regulation of prostate cell growth.

ARs are present in many parts of the body, however, the male accessory organs, e.g. prostate, genital skin and seminal vesicles have a higher concentration.

![Diagram of Androgen Receptor](image)

**Figure 1.2.4.** The mode of action of androgen in the androgen-responsive cells, e.g. prostate cell. Abbreviations: SHBG = Sex hormone binding globulin; AR = Androgen receptor; DBD = DNA binding domain; 5α-DHT = 5α-dihydrotestosterone.
1.2.4 Prostate cancer aetiology

Many risk factors are associated to the growth of prostate cancer cells, as discussed in section 1.1.3.

Huggins and Hodges (Huggins et al., 1941) discovered that androgens are required for the development, growth and maintenance of both the normal and carcinoma prostate (Montie and Pienta, 1994). Androgen regulates the development of prostate cancer through interaction with the androgen receptor. Changes to the androgen receptor is one of the main factor that contribute to the aetiology of prostate cancer (Debes and Tindall, 2002).

Genetic changes to the somatic and inherited genes have been associated with the molecular changes towards prostate cancer. The changes or mutation in certain genes e.g. tumour suppressor genes, could result in changes towards the rate of apoptosis, cell differentiation and cell proliferation (DeMarzo et al., 2003).

Tissue growth factors play an important role in maintaining the growth and development of prostate cancer cells, namely, epidermal growth factor (EGF), insulin-like growth factor (IGF), transforming growth factor (TGF) and fibroblast growth factor (FGF). The functions of each growth factor will not be discussed in this thesis, however, it is important to note that these growth factors are implicated in the pathogenesis of many cancers including prostate cancer (Dowling and Risbridger, 2000; Russell et al., 1998).

1.2.5 The aetiology of androgen-independent prostate cancer

During the long term androgen deprivation, the tumour cells lose the capacity to undergo apoptosis and do not depend on androgen for growth and survival (Brinkmann, 2001; Feldman and Feldman, 2001). At this stage, patient is said to have androgen-independent prostate cancer (AIPC), i.e. progression of prostate cancer despite continued androgen ablation. AIPC is also called, hormone-refractory prostate cancer (HRPC). Patients with AIPC will not respond to the hormonal therapy, and this is manifested by the increasing PSA levels, worsening of symptoms and progressive disease on imaging studies (Bhandari et al., 2005). The possible mechanisms by which AIPC occurs were reviewed by Feldman’s group (Feldman and Feldman, 2001) and Galbraith and Duchesne (Galbraith and Duchesne, 1997) and are summarised below:
• **Mutations to the androgen receptor gene**
  Mutation to the androgen receptor gene could allow the androgen receptors to continue to stimulate the growth of prostate cancer cells in the absence of androgen binding to the receptor.

• **Altered expression of the androgen receptor**
  The levels of androgen receptor expression are high in AIPC patients as detected by semi-quantitative polymerase chain reaction (PCR) analysis and by immunohistochemistry.

• **Overamplification of the androgen receptor gene**
  This may occur in patients treated with androgen ablation therapy. This overamplification potentially allows the prostate cancer cells to be more sensitive even at low concentrations of androgen.

• **Mutations in oncogenes or tumour suppressor genes**
  Experiments using prostate cancer cell-lines and primary cultures have shown that the cells can become androgen-independent by activation of proto-oncogenes, *e.g.* Ras; or inactivation of tumour suppressor genes, *e.g.* Rbl; or overexpression of anti-apoptotic gene, *e.g.* bcl-2.

• **Growth factors**
  The growth factors, *e.g.* the epidermal growth factor (EGF), insulin-like growth factor (IGF), transforming growth factors (TGF) and fibroblast growth factor (FGF) are up-regulated in AIPC. In the androgen deprived environment, as a result of the androgen ablation therapy, the prostate cancer cells depend on the growth factors to grow. In this androgen deprived environment, clonal selection of androgen-independent cells from a heterogenous population of androgen-dependent and androgen-independent cells could occur.

1.2.6 **Conclusions**
  So far, we have looked at the epidemiology, risk factors, treatment, aetiology of prostate cancer. In the next few chapters, the role of the vitamin D₃ and the retinoids, in the differentiation and proliferation of prostate cancer cells, will be discussed. The biosynthesis of vitamin D₃ and retinoids, and especially the enzymes that metabolise retinoids and vitamin D₃, will be discussed in further details in section 1.7.
1.3 Vitamin D₃

Vitamin D₃ metabolites are involved in a wide array of biological responses, including calcium homeostasis, immunology, cell differentiation and regulation of gene transcription (Bouillon et al., 1995; Ylikomi et al., 2002). The principal mediator in this host of cellular processes, is the hormone 1α,25-dihydroxyvitamin D₃ (1α,25-(OH)₂-D₃), also known as calcitriol. The chemistry, pharmacology and clinical implications of 1α,25-(OH)₂-D₃ will be discussed in this section.

1.3.1 The vitamin D₃ endocrine system

1.3.1.1 Metabolism and production of vitamin D₃ metabolites

In the presence of ultraviolet light, 7-dehydrocholesterol is catalysed into previtamin D₃ in the human skin. This precursor is rapidly transformed by a rearrangement of double bonds to form vitamin D₃ (Figure 1.3.1).

The next step that occurs in the liver, involves hydroxylation of vitamin D₃ at carbon 25 to give 25-hydroxyvitamin D₃ (25-(OH)-D₃) by vitamin D₃-25-hydroxylase (CYP27A1). Further processing occurs in the kidney which involves the enzyme 25-hydroxyvitamin D₃-1α-hydroxylase (CYP1α, also known as CYP27B1) that introduces a hydroxyl group at the α-position of carbon 1 of the A ring to produce 1α,25-dihydroxyvitamin D₃ (1α,25-(OH)₂-D₃) which is the hormonally active metabolite. The enzyme 25-hydroxyvitamin D₃-24-hydroxylase (CYP24) in the kidney, is involved in the catabolism of 25-(OH)-D₃ and 1α,25-(OH)₂-D₃ to 24,25-dihydroxyvitamin D₃ (24,25-(OH)₂-D₃) and 1α,24,25-dihydroxyvitamin D₃ (1α,24,25-(OH)₂-D₃) respectively (Figure 1.3.1).

The degradation of 1α,25-(OH)₂-D₃ to form calcitriolic acid, involves several steps catalysed by the CYP24 enzyme via the C-24 oxidation pathway (Figure 1.3.1).

The three vitamin D₃ hydroxylases mentioned above have been isolated and cloned. Based on their sequence-alignment (Chen et al., 1993; Guo et al., 1993; Monkawa et al., 1997), they were found to contain heme-binding and functional domains typical of cytochrome P450 heme protein enzyme (Ghazarian et al., 1974; Ghazarian et al., 1973; Jones et al., 1998).
Figure 1.3.1. The biosynthesis and metabolism of vitamin D₃.

1.3.1.2 Tissue distribution of vitamin D₃ hydroxylases and regulation of vitamin D₃

It was established by radiolabeled vitamin D₃ that the major tissue for expression of CYP27A1 is the liver (Ponchon et al., 1969). There has been some controversy over whether this enzyme is found in the mitochondrial or microsomal fractions of the liver (Jones et al., 1998; Omdahl et al., 2002).

Kidney is the major endocrine organ for 1α,25-(OH)₂-D₃ synthesis and it is the preferred site for CYP1α and CYP24 expression. It is now recognized that many tissues in the body express CYP1α, including prostate, colon, skin and osteoblast, in order to synthesise 1α,25-(OH)₂-D₃ locally (Schwartz et al., 1998; Tangpricha et al., 2001). CYP1α is expressed in both the proximal and distal convoluted tubules and the enzyme is attached to the inner mitochondrial membrane of the kidney (Omdahl et al., 2003). Similarly, it was original thought that CYP24 was located exclusively in the kidney to metabolise 25-(OH)-D₃ to 24,25-(OH)₂-D₃. Many metabolites of 1α,25-(OH)₂-D₃ were later isolated from plasma including 1α,24,25-(OH)₂-D₃ (Holick et al., 1973). Subsequently, it was shown that 24-hydroxylase is omnipresent as it is expressed in many target tissues expressing vitamin D₃ receptors, including kidney, intestine,
fibroblast, cartilage and many more (Gamblin et al., 1985; Lou et al., 2003; Omdahl et al., 1972).

The synthesis of 25-(OH)-D$_3$ by the liver appears to be loosely regulated, whereas, the synthesis and degradation of 1α,25-(OH)$_2$-D$_3$ are tightly regulated by specific cytochrome P450 enzymes, i.e. CYP1α and CYP24 (Omdahl et al., 2003) (Figure 1.3.2).

1α,25-(OH)$_2$-D$_3$, like other steroid hormones, is a negative feedback inhibitor. High plasma level of 1α,25-(OH)$_2$-D$_3$ exerts via its short feedback loop to suppress CYP1α. The parathyroid gland is a site of high localisation of 1α,25-(OH)$_2$-D$_3$ (Hughes and Haussler, 1978). 1α,25-(OH)$_2$-D$_3$ reduces parathyroid gland proliferation and parathyroid hormone (PTH) production by suppressing transcription of the parathyroid gene (Jones et al., 1998).

Moreover, 1α,25-(OH)$_2$-D$_3$ stimulates 24-hydroxylation to cause catabolism of 1α,25-(OH)$_2$-D$_3$ through a vitamin D$_3$ receptor (VDR)-dependent mechanism. This phenomenon predominates in the kidney but also occurs in all 1α,25-(OH)$_2$-D$_3$ target cells (Haussler et al., 1998).

Figure 1.3.2. The regulation of the 1α,25-(OH)$_2$-D$_3$. 
1.3.1.3 **Role of vitamin D₃ in homeostasis**

The classic role of 1α,25-(OH)₂-D₃ is to maintain normal development of the skeleton and maintenance of calcium homeostasis. Since the discovery of the physiological action of vitamin D₃ in preventing bony diseases like rickets and osteomalacia (DeLuca, 1988), it became clear that the role of 1α,25-(OH)₂-D₃ is to increase plasma calcium and phosphorus to mineralize skeleton to prevent bone diseases. The 1α,25-(OH)₂-D₃ hormone does this by (Ettinger and DeLuca, 1996; Reichel et al., 1989) (Figure 1.3.3):

- Stimulating intestinal transport of calcium and phosphorus
- Reabsorption of calcium in the renal distal tubule
- Increasing bone resorption (i.e. mobilisation of bone calcium)

![Diagram of vitamin D₃ homeostasis](image)

**Figure 1.3.3.** The role of 1α,25-(OH)₂-D₃ in homeostasis.

Besides the classical role of 1α,25-(OH)₂-D₃ in homeostasis, the role of 1α,25-(OH)₂-D₃ in restoring the normal balance of proliferation and differentiation of prostate cancer cells will be discussed in section 1.6.
1.3.2 The chemistry of the vitamin D₃

Many synthetic routes are carried out to synthesise 1α,25-(OH)₂-D₃ and its analogues. The Hoffman-La Roche group (Baggiolini et al., 1986; Baggioni et al., 1982) was the first to achieve total synthesis of 1α,25-(OH)₂-D₃. It gives flexibility for producing side chain modifications and other analogues based on this method. However, the disadvantage is that it takes 5 steps to produce the A-ring fragment, phosphine oxide, (I) and 9 steps to produce the C and D ring fragment, trimethylsilyl ether, (II) (Figure 1.3.4). The final steps involves a Horner-Wittig reaction between the phosphine oxide (I) and the CD-ring ketone (II) [Figure 1.3.4] followed by deprotection to produce 1α,25-(OH)₂-D₃.

The more popular approaches employed in industry involve the preparation of crystalline 1α,25(OH)₂D₃ from 25-hydroxycholesterol through an efficient 8-step synthesis (Barton et al., 1973).

There are many other methods being employed to synthesise 1α,25-(OH)₂-D₃. There are a few good reviews describing the chemical synthesis of vitamin D₃ compounds in greater detail (Barton et al., 1973; Dai and Posner, 1994; Lythgoe, 1980).

![Figure 1.3.4](image)

1α,25-dihydroxyvitamin D₃

Figure 1.3.4. Horner-Wittig reaction between the phosphine oxide (I) and the trimethylsilyl ether (II).

1.3.3 Therapeutic uses of vitamin D₃ analogues

Hundreds of vitamin D₃ analogues have been synthesised and biological activity has been evaluated (Bouillon et al., 1995; Jones et al., 1998). These analogues show alterations to the A, B and/or CD rings of 1α,25-(OH)₂-D₃. These vitamin D₃ analogues have been used clinically to target diseases such as bone disease (osteoporosis), skin disease (psoriasis) and as hormonal therapy (hypoparathyroidism).
One of the 1α,25-(OH)₂-D₃ analogues that is in clinical use for the local treatment of psoriatic plaques is Calcipotriol (Dovonex®), formulated as a topical treatment (Figure 1.3.5).

Vitamin D₃ compounds are formulated either on their own or with calcium carbonate, used as a supplement product for patients with vitamin D₃ deficiency due to intestinal malabsorption or chronic liver disease (Anon, 2005). The products ergocalciferol (vitamin D₂), alfalcacidol (1α-hydroxyvitamin D₃), calcitriol (1α,25-dihydroxyvitamin D₃), colecalciferol (vitamin D₃) and dihydrotachysterol (Figure 1.3.5) are listed in the British National Formulary 2004 (Anon, 2005). Calcitriol is also licensed for the management of postmenopausal osteoporosis.

![Chemical structures of vitamin D analogues](image)

**Figure 1.3.5.** Chemical structures of vitamin D analogues in clinical use.
1.4 The retinoids

Similarly to the active metabolite of vitamin D₃, 1α,25-(OH)₂-D₃, retinoic acid also plays an important role in development, differentiation and homeostasis in our body (Chambon, 1996). In this section, the chemistry, biology and role of retinoic acid will be discussed.

1.4.1 Biosynthesis, metabolism and regulation of the retinoids

The term retinoids refers to the natural forms and the many synthetic analogues of retinol (also known as vitamin A), retinal and retinoic acid (RA). The main dietary sources of vitamin A are provitamin A carotenoids from vegetables (carrots, spinach and broccoli); preformed retinyl esters and retinol from animal origin (liver and fish oil) (Blomhoff et al., 1992). Provitamin A carotenoids are chemically converted into retinol in the liver and intestine by oxidative cleavage of carotenoids (Blander and Olson, 1994). Retinol is then bound to the cellular retinol binding protein (CRBP). The resulting CRBP-retinol complex serves as a substrate for two different microsomal enzymes:

- **Lecithin:retinol acyl transferase (LRAT)**, which catalyse the esterification of retinol to retinyl esters. Retinyl ester is the storage form of retinol in many tissues e.g. small intestine, liver, skin, eye, testis etc. (Blomhoff et al., 1992; Napoli, 1996) (Figure 1.4.1).

- **Retinol dehydrogenase**, which catalyses the oxidation of retinol to retinaldehyde.
  This is the rate limiting step in the oxidative formation of RA (Blaner and Olson, 1994; Blomhoff et al., 1992; Chen et al., 2000b; Napoli, 1996) (Figure 1.4.1).

The metabolism of retinaldehyde to all-trans retinoic acid (ATRA) is mediated mainly by dehydrogenase but it can also be metabolised by cytochrome P450 enzymes, namely the CYPs 1A1, 1A2 and 3A4 in human (Zhang et al., 2000). Metabolites of ATRA generated in vivo include 13-cis-RA (Tang and Russell, 1990), 9-cis-RA (Heyman et al., 1992), 4-hydroxy-RA, 4-oxo-RA and other polar metabolites (Figure 1.4.1). There are many different cytochrome P450 isoforms (section 1.7.4) that are able to metabolise RA to inactive metabolites, namely, 4-hydroxy-RA, 4-oxo-RA and the subsequent polar inactive metabolites. Robert et al. (Roberts et al., 1979) reported that the formation of these metabolites in the hamster intestine and liver microsome homogenates required NADPH and oxygen and was strongly inhibited by carbon monoxide. Moreover, Van Wauwe et al. also showed that liarozole, which is a known
P450 inhibitor, enhanced endogenous RA plasma concentrations (Van Wauwe et al., 1992).

![Image of retinoic acid metabolism]

**Figure 1.4.1.** Biosynthesis and metabolism of all-trans retinoic acid.

There are several proteins which play key roles in ATRA homeostasis [reviewed in Ross et al. (Ross, 2003; Ross et al., 2001)]:

- **The cellular retinol binding protein** (CRBP), facilitates retinol uptake, and allows retinol to bind to lecithin:retinol acyl transferase (LRAT) for storage as retinyl ester (Ong, 1994). **[Figure 1.4.2, (1)]**

- **Lecithin:retinol acyl transferase** (LRAT) has a crucial role in diverting retinol away from oxidative activation, therefore preventing ATRA biosynthesis. ATRA can cause a strong induction of LRAT and hence a reduction in the conversion of retinol to ATRA (Kurlandsky et al., 1996). **[Figure 1.4.2, (2)]**

- **The cellular retinoic acid binding protein** (CRABP) which facilitates the retinoic acid uptake, and allows ATRA bind to cytochrome P450 hydroxylase for inactivation (Fiorella and Napoli, 1991). Thus, the binding of ATRA to CRABP decreases the elimination half-life of RA. **[Figure 1.4.2, (3)]**
• Cytochrome P450RAI (Retinoic Acid Inducible) (designated as CYP26) appears to be the novel cytochrome P450 hydroxylase enzyme expressed in numerous tissues, and is rapidly induced by ATRA (White et al., 1997). [Figure 1.4.2, (4)]

CYP26A1 has been cloned from zebra fish (White et al., 1996), mouse (Abu-Abed et al., 1998) and man (White et al., 1997). The hydroxylation of ATRA by CYP26 produces polar metabolites, *i.e.* hydroxyl or keto metabolites of ATRA, which are less bioactive than ATRA. After the discovery of P450RAI-1 (CYP26A1) by the White group (White et al., 1997), they identified another two members of CYP26, namely the P450RAI-2 (CYP26B1) and P450RAI-3 (CYP26C1) (Taimi et al., 2004). ATRA is the preferred substrate for all the three members of CYP26, except for CYP26C1 which can metabolise both ATRA and 9-cis RA (Taimi et al., 2004).

The recent information and advances of retinoid biosynthesis, transport and regulation are well reviewed in book and journal articles (Blaner and Olson, 1994; Blomhoff et al., 1992; Napoli, 1996; Ross et al., 2001).

![Figure 1.4.2.Regulation of retinoic acid homeostasis. LRAT and CYP26 play a crucial role in the down-regulation of ATRA biosynthesis.](image)

### 1.4.2 The chemistry of the retinoids

Well over 1000 new retinoids have been synthesised, and they have been studied in *in vitro* and *in vivo* assays. These are summarised in the edited book by Sporn *et al.* (Dawson and Hobbs, 1994). The purpose of the wide range of synthetic retinoid analogues is to provide reference samples for metabolic studies and samples for biological investigation. Secondly, it is to develop retinoids with reduced toxicity, while
maintaining or enhancing activity as chemotherapeutic agents (Ralhan and Kaur, 2003), i.e. to improve the therapeutic index of a retinoid compound.

Rosenberger's group at the Hoffmann-La Roche first reported the total synthesis of 4-hydroxy- and 4-keto-retinoic acid (Rosenberger, 1982). Since then, many analogues have been synthesised by modification of the ring, the lipophilic chain and modification of the ring and chain. A few exciting results have been noticed by these modifications producing novel synthesised retinoids (Figure 1.4.3), for examples:

- Modification of the lipophilic chain of ATRA: e.g. Polyenyl benzoic acids (1) – this benzoic acid-terminated retinoid had high activity in cell differentiation assays, reported by Sporn and Newton's group (Dawson and Hobbs, 1994).

- Modification of the lipophilic chain and the aromatic ring of ATRA: e.g. stilbenecarboxylic acids (2) and aryl naphthalenylcarboxylic acid (3) – these synthetic classes of retinoids were first reported by the Loeliger group (Dawson and Hobbs, 1994; Gollnick et al., 1990).

- Totally new series of synthetic retinoids – compounds (4 and 5), have been shown to have the biological activity of ATRA. The chalcone class of retinoids (4) and retinoids having an amide spacer (5) were prepared by Shudo and co-workers (Kagechika et al., 1988; Kagechika et al., 1989). These compounds have been reported to differentiate the human myeloid leukaemia cell line (HL-60) (Dawson and Hobbs, 1994).

![Figure 1.4.3. Examples of retinoid analogues with modified lipophilic chain and/or aromatic ring of the ATRA (1–3) and totally new series of retinoids (4 and 5) with the biological activity of ATRA.](image)

31
1.4.3 Therapeutic uses of the retinoids

Tretinoin (all-trans retinoic acid) became the first synthetic retinoid. Out of the many synthesised retinoids, there are only three retinoids that have become prescription-only medicine in the United Kingdom: Tretinoin, Isotretinoin and Acitretin (Anon, 2005). This is summarised in Figure 1.4.4 and Table 1.4.1.

Strong evidence exists between retinoic acid and cell differentiation and antiproliferation as demonstrated in both normal and malignant cells as reviewed in a few articles (Hill and Grubbs, 1992; Lotan, 1980). Moreover, synthetic retinoids have been shown to be useful and effective in the prevention of carcinogenesis in laboratory animals as supported by a substantial body of literature (Hill and Grubbs, 1992; Lotan, 1980; Moon et al., 1994). Promising results have been demonstrated in the treatment of acute promyelocytic leukaemia (APL) with ATRA (Chen et al., 1991; Huang et al., 1988). ATRA is licensed for use in patients previously untreated for APL, as well as those who have relapsed after standard chemotherapy or who are refractory to standard chemotherapy (Anon, 2005).

The use of these synthetic retinoids in dermatology has been beneficial and was reviewed by G.L. Peck (Peck, 1982; Peck and DiGiovanna, 1994). The drug of choice for severe cystic acne is isotretinoin, whereas, acitretin is the drug of choice for the treatment of psoriasis and related disorders of keratinisation (Gollnick et al., 1990; Goodman, 1984).

![Chemical structures of synthetic retinoids in clinical use.](image)

Figure 1.4.4. Chemical structures of synthetic retinoids in clinical use.
Table 1.4.1. Therapeutic application of some of the retinoids which are currently used clinically.

<table>
<thead>
<tr>
<th>Retinoid</th>
<th>Brand name</th>
<th>Dosage form</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tretinoin (All-trans Retinoic Acid)</td>
<td>Vesanoid®</td>
<td>capsule</td>
<td>Acute promyelocytic leukaemia</td>
</tr>
<tr>
<td></td>
<td>Retin-A®</td>
<td>cream and gel</td>
<td>Acne</td>
</tr>
<tr>
<td>Isotretinoin (13-cis Retinoic Acid)</td>
<td>Roaccutane®</td>
<td>capsule</td>
<td>Acne</td>
</tr>
<tr>
<td></td>
<td>Isotrex®</td>
<td>gel</td>
<td>Acne</td>
</tr>
<tr>
<td>Acitretin (metabolite of Eretinate)</td>
<td>Neotigason®</td>
<td>capsule</td>
<td>Severe extensive psoriasis</td>
</tr>
</tbody>
</table>

1.5 The nuclear receptor superfamily

Steroids, thyroid hormones, RA and 1α,25-(OH)₂-D₃ are lipophilic compounds that are able to permeate the plasma membrane and nuclear membrane to interact with its own receptor in the nucleus. These intracellular nuclear receptors for these lipophilic ligands function as a direct regulator of gene transcription (Evans, 1988; Lodish et al., 2003; Mangelsdorf et al., 1995). The nuclear receptors for RA and 1α,25-(OH)₂-D₃ will be discussed in this section.

1.5.1 Vitamin D₃ receptor (VDR), Retinoic acid receptor (RAR) and Retinoid X receptor (RXR)

The advance in molecular biology has led to the identification of the amino acid sequence and cloning of the nuclear receptors.

- Petkovitch’s group and Giguere’s group independently isolated the human retinoic acid receptor (RAR) in 1987 (Giguere et al., 1987; Petkovich et al., 1987).
- Baker et al. successfully cloned and isolated the human vitamin D₃ receptor (VDR) in 1988 (Baker et al., 1988).
- Mangelsdorf et al. identified retinoid X receptor (RXR) in 1990 (Mangelsdorf et al., 1990). 9-cis RA was later identified as the ligand for RXR (Rowe, 1997).

RAR, RXR and VDR are members of the nuclear receptor superfamily and they all share a common domain structure (Mangelsdorf et al., 1995) [Figure 1.5.1].
Androgen receptor, as discussed in section 1.2.3, is also a member of the nuclear receptor superfamily. These nuclear receptors are characterised by:

- ligand-binding domain (LBD), which is the binding recognition site for the ligand, i.e. retinoic acid or 1α,25-(OH)₂-D₃.
- DNA-binding domain (DBP), which targets the receptor to specific DNA sequences known as the nuclear receptor response element.

![Diagram of a nuclear receptor domain structure]

**Figure 1.5.1.** The common domain structure of the nuclear receptor.

RXR is identified as a cofactor, as it is required by several members of the nuclear receptor superfamily, e.g. VDR and RAR, for transcriptional activation. RXR forms a heterodimer with VDR or RAR, and in the presence of the ligand, i.e. 1α,25-(OH)₂-D₃, ATRA or 9-cis RA, transcription is then initiated through specific response elements, i.e. VDRE or RARE (**Figure 1.5.2 and Figure 1.5.3, A**) (Allenby et al., 1993; Jones et al., 1998). RXR can also form a homodimer (i.e. RXR/RXR) on interaction with 9-cis RA with RXR (Mangelsdorf and Evans, 1995) and interact with the retinoid X response element (RXRE) to initiate transcription of target genes (**Figure 1.5.3, B**).

ATRA which is bound to CRABP, is either, hydroxylated by cytochrome P-450 to 4-hydroxy-RA, which has weak biological activity, or translocated to the nucleus where it can bind to its nuclear receptor, RAR (**Figure 1.5.3**). The retinoic acid receptors (RARs) and retinoid X receptors (RXRs) are each composed of three subtypes (α, β and γ). The RAR family (RAR α, β and γ) is activated by both ATRA and by 9-cis RA (Mangelsdorf et al., 1995), whereas, the RXR family (RXR α, β and γ) is activated only by 9-cis RA (Heyman et al., 1992).
Figure 1.5.2. Vitamin D₃ receptor (VDR) forms a heterodimer with retinoid X receptor (RXR) in the nucleus. The DNA binding domain (DBD) bind to the specific DNA sequence known as the response element (VDRE).

Figure 1.5.3. The binding of ATRA or 9-cis RA to the RAR (A) or 9-cis RA to the RXR (B) nuclear receptors in the nucleus. The DNA binding domain (DBD) bind to the specific DNA sequence known as the response element (RARE or RXRE).

VDR, RXR and RAR have been identified in many cells, including:

- Keratinocytes (Fisher and Voorhees, 1996)
- Colon (Giuliano et al., 1991; Sonneveld et al., 1998)
- Breast (Koike et al., 1997; Sonneveld et al., 1998)
• Prostate (Blutt et al., 1997; Campbell et al., 1998; Kivineva et al., 1998; Peehl et al., 1994; Richter et al., 2002)

The presence of these receptors in various tissues allow 1α,25-(OH)2-D3 and RA to directly regulate gene expression by binding to its nuclear receptors within the target cells. The transcription of the genes have many effects on a variety of biological processes in the cells, including homeostasis, proliferation, differentiation and apoptosis (Bouillon et al., 1995; Gudas et al., 1994). The role of 1α,25-(OH)2-D3 and RA in cell differentiation and cell anti-proliferation will be discussed in the following section 1.6.

1.6 The role of vitamin D3 and retinoids in prostate cancer

Epidemiologic studies have indicated a strong link between incidence of prostate cancer and vitamin D3 and RA. Examples of such studies are as follows:

• Low level of circulating 1α,25-(OH)2-D3 which was co-related with the risk of prostate cancer (Schwartz and Hulka, 1990).

• An inverse correlation between mortality due to prostate cancer and exposure to ultraviolet light, which is the principal source of vitamin D3 (Hanchette and Schwartz, 1992; John et al., 2004).

• A positive association between latitude and prostate cancer mortality (Luscombe et al., 2001).

• Vitamin A intake is inversely related to the risk of prostate cancer (Kolonel et al., 1987; Pasquali et al., 1996).

1α,25-(OH)2-D3 and retinoids (which include natural and synthetic RA analogues) have been widely investigated in vitro (Zhao et al., 1999) and in vivo (Culine et al., 1999; Osborne et al., 1995) in controlling prostate cancer progression. The advance in genetic screening methods allowed researchers to use cDNA microarray technology to understand and to investigate the regulation of gene expression by 1α,25-(OH)2-D3 and retinoids (Peehl et al., 2004; van der Spek et al., 2003; White, 2004).

1.6.1 Anti-proliferative effects of 1α,25-(OH)2-D3 and retinoids in prostate cancer cells

1α,25-(OH)2-D3 and RA play crucial roles in the prostate cancer cell cycle which results in anti-proliferation of the prostate cancer cells. Proliferating cells continuously undergo the process of the cell cycle as illustrated in Figure 1.6.1. Whereas, non-proliferating cells will leave the cell cycle in G1 phase and will enter the
resting/quiescent phase. There are many types of proteins/genes that help control cell
growth and proliferation, e.g. tumour suppressor gene (e.g. p53, Rb gene), anti-apoptotic
protein (e.g. bcl-2) and cyclin-dependent kinases (CDK).

![Cell Cycle Diagram]

**Figure 1.6.1.** The cell cycle of the proliferating cells. The gene products/proteins that
are involved in apoptosis and cell-cycle arrest (G₀ phase) are shown here.

It has been shown by a few research groups that 1α,25-(OH)₂-D₃ and its
analogs inhibit the growth (anti-proliferation effect) of normal prostatic epithelial
cells, primary cultures of prostate cancer cells, and many different types of prostate
cancer cell lines, by:

- Increasing the expression of CDK inhibitor p21waf1/cip1 (Campbell et al., 1997;
  Johnson et al., 2002; Yang and Burnstein, 2003) and p27kip1 (Campbell et al.,
  1997). p21waf1/cip1 and p27kip1 belong to the VDR target genes, therefore, 1α,25-
  (OH)₂-D₃ exerts its anti-proliferative effects on the cancer cells by binding to the
  VDR resulting in transcription of the p21waf1/cip1 and p27kip1 genes (Freedman,
  1999).
- Decreasing CDK2 activity leading to a decrease in phosphorylation of
  retinoblastoma protein (Rb) resulting in the prostate cancer cell to arrest in the
  G₀/G₁ phase (Zhuang and Burnstein, 1998).
The anti-proliferative effects of retinoids have been studied on normal prostatic epithelial cells (Peehl et al., 1993), primary cultures of prostate cancer cells (Igawa et al., 1994; Peehl et al., 1993), prostate cancer cell lines (de Vos et al., 1997; Fong et al., 1993; Koshiuka et al., 2000; Peehl et al., 1993) and in rat primary and metastatic prostate carcinoma (Pollard et al., 1991; Slawin et al., 1993). Liang et al. observed the cell growth inhibition and S-phase arrest by a novel synthetic retinoid, CD437, in LNCaP and PC-3 cells, was associated with upregulation of p21^waf1/cip1^ mRNA levels (Liang et al., 1999).

1.6.2 Apoptotic effects of 1α,25-(OH)₂-D₃ and retinoids in prostate cancer cells

1α,25-(OH)₂-D₃ was found to induce apoptosis in prostate cancer cell lines by down-regulation of the VDR target genes, bcl-2 and bcl-Xₐ, the two anti-apoptotic proteins (Blutt et al., 2000a; Guzey et al., 2002).

The apoptotic effect of retinoids, for example, 4-HPR (Figure 1.4.5), have been examined in androgen-dependent and androgen-independent prostate cancer cell lines by some groups (Gao et al., 1999; Liang et al., 1999; Shen et al., 1999; Sun et al., 1999). The apoptotic effect of 4-HPR in LNCaP cells, is due to the decrease in bcl-2 expression and increase of bax gene expression, these effects are associated with apoptosis induction (Shen et al., 1999).

1.6.3 Pro-differentiation effects of 1α,25-(OH)₂-D₃ and retinoids in prostate cancer cells

The effect of 1α,25-(OH)₂-D₃ on differentiation of prostate cancer cell lines has been shown, by up-regulating the androgen receptor and increasing the secretion of prostate-specific antigen (PSA) in LNCaP cells (Zhao and Feldman, 2001; Zhao et al., 1999). The increasing PSA expression by 1α,25-(OH)₂-D₃ may be considered differentiation marker for epithelial prostate cells (Krishnan et al., 2003).

The effect of RA in differentiation of prostate cancer tissues has been examined by Kelly et al. (Kelly et al., 2000). The group investigated the histological changes of the tumour biopsy specimens of androgen-independent patients who were treated with ATRA. The increase in prostate-specific membrane antigen (PMSA) expression after treatment with ATRA indicated the tumour cells changed from metastatic to a high-grade phenotype.
1.6.4 Synergistic effects of 1α,25-(OH)₂-D₃ and retinoids in prostate cancer cells

Since the discovery that vitamin D₃ receptor (VDR) acts as a heterodimer with RXR (Mangelsdorf et al., 1995), it was suggested that there may be functional interactions between 1α,25-(OH)₂-D₃ and RA. Not surprisingly, there have been accumulated evidences that these two agents act synergistically or have additive differentiating and anti-proliferative effects in several cell types (James et al., 1995; Miyaura et al., 1985) including prostate cancer cell-lines (Campbell et al., 1998; Peehl et al., 1995).

1α,25-(OH)₂-D₃ and 9-cis RA has been found to act synergistically by:

- Inhibiting the growth of prostate cancer cells causing accumulation of cells in G₁ phase (Blutt et al., 1997; Elstner et al., 1999)
- Inducing androgen receptor in LNCaP prostate cancer cells (Zhao et al., 1999)

Despite these positive results in prostate cancer cell-lines, there are no studies showing synergistic or beneficial effects using both 1α,25-(OH)₂-D₃ and RA in vivo.

1.6.5 Clinical studies of vitamin D₃ and retinoids in prostate cancer

Besides the current clinical use of vitamin D₃ analogues mentioned in section 1.3.3, a number of research groups are synthesising and investigating vitamin D₃ analogues for the desirable anti-proliferative and pro-differentiating activities in prostate (Blutt et al., 2000b; Campbell et al., 1997; Chen et al., 2000c), colon (Cross et al., 1991), and other cancer (Prudencio et al., 2001). One of the vitamin D₃ analogues, EB1089 (Figure 1.6.2), has been extensively studied in various cancer cell-lines (Akutsu et al., 2001; Prudencio et al., 2001).

However, in order to develop vitamin D₃ analogues as effective chemotherapeutic agents, these analogues should possess desirable anti-proliferative and pro-differentiating activities rather than undesirable calcemic activity (Campbell and Koeffler, 1997). Many different types of non- or less- calcemic vitamin D analogues have been investigated for their effects on prostate cancer cell proliferation and differentiation in vitro. Examples of these are (Figure 1.6.2, i – iv):

- 19-nor-hexafluoride vitamin D₃ (i) (Campbell et al., 1997)
- 20-cyclopropyl-vitamin D₃ (ii) analogues (Koike et al., 1999)
- 19-nor-1α,25-dihydroxyvitamin D₂ (iii) (Chen et al., 2000c)
- 5,6-trans-16-ene vitamin D₃ (iv) analogues (Hisatake et al., 1999)
To date there have been two clinical trials of using calcitriol (1α,25-(OH)2-D3) for human prostate cancer. A small trial in 14 men with AIPC failed to show an objective response in any patient, but in two there were declines in PSA levels of 25% and 45% (Osborne et al., 1995). In another study (Gross et al., 1998), 7 patients with early recurrent prostate cancer after radical prostatectomy or radiotherapy were treated with calcitriol, all patients showed a statistically significant decline in the rate of PSA increase. In both of these studies doses of calcitriol were limited by hypercalcemia and hypercalciuria. A recent review by Trump et al. (Trump et al., 2004) discussed the use of combination therapy of 1α,25-(OH)2-D3 and other chemotherapeutic agent(s), e.g. dexamethasone, carboplatin or taxanes in AIPC. Intermittent use of 1α,25-(OH)2-D3 to reduce toxicity has showed positive results in Phase I and II clinical studies for AIPC (Beer, 2003; Trump et al., 2004).

Phase I and II study have been carried out to evaluate the efficacy of the vitamin D3, 1α-hydroxyvitamin D2 (1α-OH-D2) [Figure 1.6.2, (v)], in patients with AIPC (Liu et al., 2002; Liu et al., 2003). Although the results were encouraging, further investigations are necessary to elucidate the mechanism and effect of 1α-OH-D2.

The Phase II study of ATRA have demonstrated mild to moderate efficacy in AIPC (Culine et al., 1999; Trump et al., 1997), despite the anti-proliferative, pro-differentiative and apoptotic effects shown in vitro. Culine et al. (Culine et al., 1999) reported that the use of ATRA has minimal activity in AIPC. However, it is not known whether this moderate efficacy of RA is due to toxicity or rapid metabolism of RA in the body which results in under dosing.

The RA analogue, N-(4-hydroxyphenyl)-retinamide (4-HPR), also known as fenretinide (Figure 1.6.2, (vi)), has been studied for its effect in malignant (DU-145, PC-3) (Igawa et al., 1994; Sharp et al., 2001) and non-malignant (RWPE and WPE) prostate cancer cell lines (Sharp et al., 2001). 4-HPR has also demonstrated a benefit in both prevention and progression in prostate cancer rat models (Pienta et al., 1993; Pollard et al., 1991). However, the use of 4-HPR in clinical trials to treat prostate cancer has shown limited effect.
Figure 1.6.2. Vitamin D\textsubscript{2} and D\textsubscript{3} analogues and retinoid with promising in vitro activity or in clinical trials.

1.7 The properties of the vitamin D\textsubscript{3} and retinoic acid hydroxylase enzymes

1.7.1 General features of the hydroxylase enzymes

The hydroxylase enzymes that are involved in the metabolism of 25-hydroxyvitamin D\textsubscript{3} (CYP1\textalpha{} and CYP24) and retinoic acid (CYP26 and other non-specific cytochrome P450 4-hydroxylases present in the liver) are members of the cytochrome P450 superfamily (Leo and Lieber, 1985; Marill \textit{et al}., 2000; Omdahl \textit{et al}., 2002).

CYP1\textalpha{} and CYP24 are located in the inner mitochondrial membrane to receive NADPH-reducing equivalents, which are supplied via NADPH to a flavoprotein (ferredoxin reductase, \textbf{FR}) then to an iron-sulphur protein (ferredoxin, \textbf{FDX}) then to the cytochrome P450 monooxygenase, which catalyses the hydroxylation by utilizing one oxygen atom from O\textsubscript{2} to form hydroxylated vitamin D\textsubscript{3} (D-OH) and water (Figure 1.7.1) (Jones \textit{et al}., 1998; Omdahl \textit{et al}., 2002).

In contrast, CYP26 and other non-specific cytochrome P450 4-hydroxylases present in the liver, which are microsomal P450 enzymes, are located in the membrane of the endoplasmic reticulum of a cell. FAD/FMN-dependent NADPH-cytochrome
P450 oxidoreductase serves as the redox partner for this microsomal P450 enzyme. FAD serves as an electron acceptor from NADPH, and FMN interacts with and reduces the P450 enzymes (Lewis and Hlavica, 2000; Sevríukova et al., 1999) (Figure 1.7.2).

In both situations, a molecule of oxygen is split during the catalytic reactions with one atom used in the hydroxylation step and another atom reduced to water. Hence these P450 enzymes belong to the class of mixed-function oxidase enzymes. The general reaction of this P450 mediated monooxygenation can be represented as follows (Lewis, 2001):

\[
\begin{align*}
R-H + O_2 & \xrightarrow{P450} R-OH + H_2O \\
2H^+ + 2e^- &
\end{align*}
\]

\[
R = 1\alpha,25-(OH)_2D_3 \text{ or Retinoic acid}
\]

Notes

\[
\begin{align*}
D &= 25-(OH)-D_3 \\
FR &= \text{Ferredoxin-reductase (flavoprotein)} \\
FDX &= \text{Ferredoxin} \\
\text{red} &= \text{reduced form} \\
\text{NADPH} &= \text{Nicotinamide-adenine dinucleotide phosphate (reduced form)} \\
\text{P450} &= \text{cytochrome P450 enzyme located in the inner membrane of the mitochondrial}
\end{align*}
\]

Figure 1.7.1. The enzyme involved in hydroxylation of 25-(OH)-D3 (CYP1α and CYP24) is a mitochondrial cytochrome P450 that requires molecular oxygen, NADPH, and two redox carrier proteins, namely, the ferredoxin-reductase (FR) and an iron-sulphur protein redoxin (ferredoxin, FDX).
The heme protein has a porphyrin ring structure (Figure 1.7.3) and sits in the interior of the P450 enzyme. The iron atom of the heme protein is involved in the enzyme catalytic reaction (Figure 1.7.4). The sulphur atom of the P450 enzyme cysteine residue is ligated to the iron of the heme protein. Whereas, the dioxygen (O₂) is bound to the sixth coordination site of the heme iron (Lewis, 2001) during the P450 catalytic cycle (Figure 1.7.4).

Notes
RA = Retinoic acid  FAD = Flavin Adenine dinucleotide
-red = reduced form  FMN = Flavin Mononucleotide
NADPH = Nicotinamide-adenine dinucleotide phosphate (reduced form)
P450 = cytochrome P450 enzyme located in the endoplasmic reticulum of a cell

Figure 1.7.2. The enzyme involved in hydroxylation of retinoic acid is a cytochrome P450 that requires molecular oxygen, NADPH, and one redox partner, namely the FAD/FMN cofactor.
On addition of carbon monoxide (CO) to the P450 enzyme, it forms a complex which gives a major absorption band at about 450 nm wavelength, hence the name P450.

CYP1α, CYP24 and CYP26 can be found in other tissues, e.g. skin, colon, breast and prostate as well as in the kidney and liver as reviewed by Gudas et al. (Gudas et al., 1994) and Bouillon et al. (Bouillon et al., 1995).

![Figure 1.7.4](image-url) This catalytic cycle of P450 has been adapted from Lewis (Lewis, 2001). The binding of a substrate (RH), such as 1α,25-(OH)₂-D₃ in the case of CYP24 or ATRA in the case of CYP26, brings about the displacement of the bound water molecule (H₂O) present in the heme. The substrate becomes oxygenated via the activation of molecular O₂ mediated by P450 enzyme. The two electrons (e⁻) are provided by the redox carrier protein and the two hydrogen (H⁺) from NADPH.

### 1.7.2 Vitamin D₃ hydroxylases

The important role of vitamin D₃ in many physiological and pathological processes (Bouillon et al., 1995; Ettinger and DeLuca, 1996; Jones et al., 1998) has attracted many researchers in developing new drugs for targeting the key enzymes in the synthesis or metabolism of the active vitamin D₃ hormone, 1α,25-(OH)₂-D₃:-

- 25-Hydroxyvitamin D-1α-hydroxylase (CYP1α or CYP27B1) – the key enzymes in the synthesis of the biological active metabolite, 1α,25-(OH)₂-D₃.
- 24-Hydroxylases (CYP24) – a multicatalytic enzyme, that causes side-chain oxidative cleavage of 25-(OH)₂-D₃ resulting in calcitriol acid formation. CYP24

44
is a VDR target gene, *i.e.* binding of 1α,25-(OH)₂-D₃ to the VDR result in CYP24 gene transcription.

Please note that the term vitamin D₃ hydroxylase enzymes refer to both CYP1α and CYP24. Although human CYP1α and CYP24 enzymes have been purified and cloned (Monkawa *et al.*, 1997; Ohyama *et al.*, 1991; Ohyama and Okuda, 1991), the structural information from X-ray crystallography or NMR analysis is still missing.

### 1.7.3 Inhibitors of vitamin D₃ hydroxylase enzymes

Various azole compounds *e.g.* ketoconazole and liarozole (*Figure 1.7.5*), have been shown to inhibit cytochrome P450 vitamin D₃ metabolising enzymes. Theazole compounds bind directly to the prosthetic heme iron via a lone pair of electrons from the heterocyclic nitrogen and through interaction with other sites in the binding pockets (Schuster *et al.*, 2003). The use of these inhibitors have been shown to slow down the metabolism and depletion of the active vitamin D₃ hormone, 1α,25-(OH)₂-D₃ (Ly *et al.*, 1999; Zhao *et al.*, 1996).

**SDZ 89-443** and **(R)-VID400** (*Figure 1.7.5*) have been identified as potent CYP24 inhibitors and also more selective for CYP24 compared with CYP1α (Schuster and Egger, 1997; Schuster *et al.*, 2001a). These selective inhibitors of CYP24 are used by the Schuster group to study vitamin D₃ metabolism in human keratinocytes.

Posner’s group have synthesised potent 1α,25-(OH)₂-D₃ analogous with selective CYP24 inhibition properties (Posner *et al.*, 2004). One of the 1α,25-(OH)₂-D₃ analogous, CTA018, which is co-developed with Cytochroma Inc., showed dual mechanism of action, *i.e.* with enhanced anti-proliferative and pro-differentiating activities and also potent inhibition of CYP24.
Chapter 1

\[ \text{Figure 1.7.5. Azoole compounds which show inhibition of CYP24.} \]

1.7.3.1 **Vitamin D₃ hydroxylase enzymes inhibitors and prostate cancer**

1α,25-(OH)₂-D₃ has an effect on modulating the growth and differentiation of prostate cancer cells, and it has been postulated from *in vitro* and *in vivo* studies that inhibitors of vitamin D₃ metabolising enzymes together with 1α,25-(OH)₂-D₃ can be useful agents for the treatment of AIPC (Peehl et al., 2001; Peehl et al., 2002). For example, Ly and co-workers (Ly et al., 1999) demonstrated that liarozole could enhance the half-life of 1α,25-(OH)₂-D₃ and up-regulate the vitamin D₃ receptor in androgen-independent DU-145 cell line. Liarozole, is also currently used in Phase II trials for AIPC (Seidmon et al., 1995). There are also promising results from preclinical studies carried out in prostate cancer cells to study the combination of ketoconazole with 1α,25(OH)₂-D₃ (Peehl et al., 2001; Peehl et al., 2002).

Inhibition of vitamin D₃ metabolising enzymes (ideally selective CYP24 inhibitor) could be a promising combination therapy together with 1α,25-(OH)₂-D₃ for AIPC and in other cancers by sustaining the level of 1α,25-(OH)₂-D₃ and in the mean time, the dose of 1α,25-(OH)₂-D₃ could be reduced thus reducing the side-effects of 1α,25-(OH)₂-D₃. The combination could potentially slow the growth of the prostate cancer cells and restore normal responses to hormonal signaling (Zhao and Feldman, 2001).
1.7.4 Retinoic acid hydroxylase enzymes

In human, in addition to the already mentioned CYP26 (Ray et al., 1997; White et al., 1997), there are several other microsomal cytochrome P450 enzymes that have been identified in the metabolism of ATRA, i.e., CYPs 2C8 (Leo et al., 1989; Nadin and Murray, 1999), 2C9, 3A4 and 3A7 (Chen et al., 2000a; Marill et al., 2000; McSorley and Daly, 2000). As mentioned in section 1.4.1, CYP26 appears to be specific for 4-hydroxylation of ATRA, as it only recognises ATRA as its substrate, and the expression of CYP26 is induced by ATRA in vitro and in vivo.

1.7.5 Inhibitors of ATRA hydroxylase enzymes or RAMBAs

Due to the rapid metabolism of retinoic acid in cells, ATRA shows a decrease in plasma concentrations after repeated dosage (Muindi et al., 1994). Many research groups are searching for inhibitors of these cytochrome P450s that mediate the metabolism of RA, i.e. retinoic acid metabolism blocking agents (RAMBAs). The use of RAMBAs should delay in vivo ATRA metabolism thus resulting in increased endogenous levels (Njar, 2002). Liarozole and ketoconazole are capable of inhibiting the CYP-dependent metabolism of RA by hamster (Njar et al., 2000; Van Wauwe et al., 1990) and rat (Kirby et al., 2003) liver microsomes respectively. Moreover, it has been shown that liarozole with ATRA potentiates the anti-proliferative effect in in vitro tumour cell lines (Djikman et al., 1994; Wouters et al., 1992). In vivo studies using liarozole has also been studied, showing enhanced plasma levels of ATRA following oral dosing of liarozole in rat (Van Wauwe et al., 1992).

Njar's group have identified azoly1 retinoid compounds (Figure 1.7.6) which showed interesting activities against RA metabolism enzyme(s) in hamster liver microsomes (IC₅₀ = 0.68 – 1.6 µM) (Njar et al., 2000) and in breast and prostate cancer cell lines, which demonstrated enhanced anti-proliferative action compared with ATRA and liarozole (Njar, 2002; Patel et al., 2004).

The selective and potent CYP26 inhibitors, discovered by Janssen Pharmaceutical, are the 2-benzothiazolamine compounds, R115866 and R110610 (Figure 1.7.6) (Aelterman et al., 2001; Stoppie et al., 2000; Van heusden et al., 2002). In an in vivo study, R115866 was able to enhance endogenous RA levels and also to mimic the effects of RA (Stoppie et al., 2000). More recently, OSI Pharmaceuticals Inc. reported potent and selective CYP26 inhibitors, with a naphthyl-based backbone structure, as shown in Figure 1.7.6 (Mulvihill et al., 2005). The most active compound
showing \( IC_{50} = 3.3 \text{ nM} \) in the assay performed using microsomal preparations from T47D breast cancer cells induced to express CYP26 enzyme (Mulvihill et al., 2005).

![Chemical structures](image)

**Figure 1.7.6.** Examples of some Retinoic Acid Metabolism Blocking Agents (RAMBAs).

### 1.7.5.1 RAMBAs and prostate cancer

The rationale behind the use of ATRA with RAMBAs in the treatment of acute promyelocytic leukemia (APL) was that the remission duration for patients treated with ATRA was very brief (Chen et al., 1991; Miller et al., 1994). *In vivo* studies have shown that the use of a RAMBAs administered with retinoic acid, *e.g.* ketoconazole or liarozole, increased plasma half-life of retinoic acid and enhanced endogenous retinoic acid plasma levels in rats (Van Wauwe et al., 1990; Van Wauwe et al., 1992). In androgen-dependent and androgen-independent prostate cancer mouse models (R3327 Dunning prostate adenocarcinomas), liarozole, was shown to contribute to its antitumoural effect (De Coster et al., 1992; Dijkman et al., 1994). Although the combination therapy using liarozole or ketoconazole with ATRA was not used in prostate cancer, the use of these compounds by themselves has had some success in clinical trials, *e.g.* ketoconazole is currently used as second-line treatment in AIPC (Harris and Reese, 2001), whereas, liarozole is in phase II trials for AIPC (Seidmon et al., 1995).

In order to achieve sufficient and non-toxic levels of ATRA in target tissues, the use of a RAMBA could be a new strategy. This allows ATRA to play its role as a differentiation agent at lower doses to achieve effectiveness. The selective CYP26 inhibitor, R115866 and R116010 (Figure 1.7.6), have demonstrated strong anti-tumour
effects in androgen-independent rat prostate adenocarcinoma and in oestrogen-independent mouse mammary tumours (Njar, 2002; Stoppie et al., 2000; Van heusden et al., 2002), this suggests that CYP26 inhibitor can be a potential candidate for the treatment of prostate and breast cancer.
CHAPTER 2

Aims of the investigation
2. Aims of the investigation

The aims of the investigation were:

1. To synthesise organic compounds inhibiting the vitamin D₃ and ATRA metabolising enzymes.
2. To study vitamin D₃ and ATRA metabolism in rat kidney mitochondrial and rat liver microsomes respectively.
3. To study vitamin D₃ and ATRA metabolism in cancer cell-lines (MCF-7 and DU-145).
4. To study the inhibition of vitamin D₃ and ATRA metabolism in these *in vitro* assays 2 and 3 above using the synthesised compounds.

Since the X-ray crystal structures of CYP24 and CYP26 are unknown, the design of organic compounds targeting these enzymes is based on intuition. Judging from the structures of (Figure 2.1):

- inhibitors of vitamin D₃ and ATRA metabolising enzymes (liarozole and ketoconazole) and the more selective inhibitors for CYP24 (*SDZ 89-443* and *VID 400*); and
- organic compounds synthesised by our group which affect activity of the cytochrome P450 enzymes involved in hormone biosynthesis (Le Lain *et al.*, 2002; Vinh *et al.*, 1999; Vinh *et al.*, 2001) or retinoic acid catabolism (Greer *et al.*, 2003; Kirby *et al.*, 2003; Kirby *et al.*, 2002); as a result, three series of compounds were proposed. The methods will be described in Chapter 3 – 5.

**Series I** compounds are based on the 1-(benzofuran-2-yl)phenylmethyl back bone structure described by Vinh *et al.* (Vinh *et al.*, 1999; Vinh *et al.*, 2001) as selective P450 aromatase inhibitors. A lipophilic alkyl chain or phenyl ring attached to the compound as shown in the diagram below may mimic the lipophilic chain structure of ATRA and 1α,25-(OH)₂-D₃.

![Diagram](image)

**Series I**
1- and 4- [Benzofuran-2-yl]phenylmethyl|triazole derivatives
Figure 2.1. Chemical structures of ATRA, 1α,25-(OH)₂D₃, known inhibitors of CYP2A4 and CYP2D6 and some chemical structures synthesized by our group.
In **Series II**, the amide bond was chosen to link the benzofuran ring with the phenyl methyl ring, as shown below. This mimics the selective inhibitors of CYP24, SDZ 89-443 and VID 400 described by Schuster et al. (Schuster et al., 2001a). In addition, our group has also synthesised some 1,2-diphenylpropane compounds (Greer et al., 2003) with an amide bond linkage, which showed comparable inhibition results against retinoic acid metabolising enzymes with ketoconazole.

![Diagram of Series II with amide bond and hydrophobic groups]

**Benzofuran-2-carboxamido ethyl-imidazole and -1,2,4-triazole derivatives**

Dietary and epidemiological factors have been implicated in the low rates of breast and prostate cancer in China and Japan (Sasagawa and Nakada, 2001). The population in China and Japan consume a diet rich in soy beans, which are rich in isoflavonoids. Isoflavanoids belong to a group of phytoestrogens and the major constituents of isoflavonoids are genistein and daidzein (**Figure 2.2**).

![Structures of Genistein and Daidzein]

**Genistein**  
**Daidzein**

**Figure 2.2.** The structure of genistein and daidzein.

It has been shown in our own laboratory, that isoflavones and flavones (Le Lain et al., 2001); tetralones (Kirby et al., 2003; Smith et al., 2001); and coumarin (Le Lain et al., 2002) are known to affect the activity of cytochrome P450 enzymes involved in hormone biosynthesis. In view of these facts, it would be interesting to
investigate the inhibition of these compounds against the vitamin D$_3$ and ATRA metabolising enzyme.

Since it was shown by Kirby et al. (Kirby et al., 2003) that 2-(4-aminophenylmethyl)-6-hydroxy-3,4-dihyronaphthalene-1-one (Figure 2.1) showed comparable results with ketoconazole in the inhibition of RA metabolising enzymes, a series of tetralone compounds was synthesized (Series III). Hydrophobic and/or hydrophilic substituents were chosen to probe the active site of vitamin D$_3$ and ATRA metabolising enzymes.

![Series III](image)

6-Substituted-2-(phenylmethylene)-3,4-dihyronaphthalene-1-one derivatives
CHAPTER 3

Synthesis of 1- and 4- [(benzo[b]furan-2-yl)phenylmethyl]triazoles derivatives
3.1 Synthesis of 1- and 4-[(benzo[b]furan-2-yl)phenylmethyl]triazoles

The synthesis of the substituted 1- and 4-[(benzo[b]furan-2-yl)phenylmethyl]triazoles was carried out according to a sequence of 5 steps as outlined in Scheme 3.1. The reactions shown in Scheme 3.1 involve a modification of the procedure described by Pestellini and co-workers (Pestellini et al., 1987).

3.1.1 Synthesis of the phenacyl bromide

A wide range of brominated reagents, for example, AlCl₃/Br₂ and phenyltrimethylammonium tribromide (PTT), could be used to brominate aldehydes and ketones in the α-position. 4'-Ethylacetophenone (1) was reacted with bromine in the presence of aluminium chloride at 0 °C to give 2-bromo-1-(4-ethyl-phenyl)-ethanone (2) (Scheme 3.1). AlCl₃ acts as a Lewis acid and catalyst to promote bromination. The reaction was stirred for 3.5 h. The low temperature and high dilution with the solvent (THF) may slow down the reaction, to prevent the formation of the dibrominated acetophenone, 2,2-dibromo-1-(4-ethyl-phenyl)-ethanone. Bromine should be added dropwise over a 20 minute period to minimise the formation of 2,2-dibromo-1-(4-ethyl-phenyl)-ethanone.

The presence of the 2-bromo-1-(4-ethyl-phenyl)-ethanone was confirmed by ¹H n.m.r. by the disappearance of the CH₃ singlet at 2.6 ppm which was replaced by a CH₂ singlet observed at 4.4 ppm.
Scheme 3.1. General reaction scheme for the synthesis of substituted 1- and 4-benzozol[2,3-c][phenyl]imidazole derivatives. Reagents and conditions: (a) Bz₂Cl₂, THF, 0°C, 3.5 h. (b) 0°C, 2.5 h, i.e. N₃ in DME, 80°C. (c) (ii) H₂O₂, 4°C, 6 h. (d) (ii) Ph₃P=CH₂, PdCl₂(dpdpf), 4°C, 6 h. (e) (ii) NaBH₄, 1,4-dioxane, 6 h. (f) (ii) 1,2,4-triazole, H₂O₂, room temperature, 1 h. (g) 10°C, K₂CO₃, room temperature, 4 - 6 days.
3.1.2 Synthesis of the substituted (benzo[b]furan-2-yl)-phenyl-methanones

The synthesis of the substituted (benzo[b]furan-2-yl)-phenyl-methanones was achieved by the Rap-Stöermer reaction (Stöermer, 1900). This reaction (Scheme 3.2) involves two bases, sodium hydride and sodium methoxide.

In step 1, sodium hydride (NaH), a strong base, forms a sodium salt of salicylaldehyde with concurrent liberation of hydrogen gas. The resulting phenoxy anion, which acts as a nucleophile, attacks the α-carbon of the phenacyl bromide (2 or 3) with the loss of the bromine atom.

In step 2, sodium methoxide (NaOCH₃), a base, removes the proton in the α-carbon position, resulting in the formation of the carbanion. This carbanion attacks the aldehyde group of the salicylaldehyde, resulting in the formation of a cyclic ring. Protonation then occurs to form the intermediate, finally a molecule of water is lost to form the substituted (benzofuran-2-yl)-phenyl-methanones (5 or 6).

The crude product was recrystallised from methanol to give a reasonable yield for product 5 (34 %) and good yield for product 6 (92 %). The presence of impurities in the synthesis of compound 5 made recrystallisation to be difficult and thus a low yield was achieved.
Scheme 3.2. The formation of substituted (benzo[b]furan-2-yl)-phenyl-methanones by the Rap-Stöermer reaction. **Step 1** – NaH, DMF, 80 °C, 2.5 h. **Step 2** – NaOCH₃, 80 °C, 1.5 h.

### 3.1.3 Synthesis of the biphenyl ring

The synthesis of the biphenyls (8 and 10) was performed by the Suzuki reaction using the commercially available phenylboronic acids (7 or 9) [Scheme 3.4]. Miyaura et al. (Miyaura et al., 1981) reported the cross-coupling reaction of phenylboronic acid (i) with aryl bromide (ii) in the presence of palladium catalysts and base to provide the corresponding biaryl (iii) in good yields (Scheme 3.3). After the reaction was complete, 30 % H₂O₂, as an oxidising agent, was added to remove the residual phenyl boronic acid. The Suzuki reaction is tolerant to a wide variety of functional groups, and thus various substituted aromatic rings can be used. It is one of the direct methods for the formation of carbon-carbon bonds. Moreover, arylboronic acid is easy to handle as it is stable at high temperature.
Scheme 3.3. The Suzuki reaction – cross-coupling reaction of phenylboronic acid (i) with aryl bromide (ii) using palladium catalyst (Pd(PPh$_3$)$_4$) and Na$_2$CO$_3$ base to form biaryls (iii).

Phenylboronic acid (7) and 4-chloro-phenylboronic acid (9) were reacted with benzo[b]furan-2-yl-(4-bromo-phenyl)-methanone (6) to form (8) and (10) respectively (Scheme 3.4). The Suzuki reaction gives reasonably good yields of products 8 (58 %) and 10 (73 %) after purification by column chromatography and/or recrystallisation.

Scheme 3.4. The synthesis of benzo[b]furan-2-yl-biphenyl-4-yl-methanone (8) and benzofuran-2-yl-(4'-chloro-biphenyl-4-yl)-methanone (10) using Suzuki reaction. Reaction conditions: Pd(PPh$_3$)$_4$, Na$_2$CO$_3$ (aq.), toluene, 100 °C, then H$_2$O$_2$, r.t., 1 h.

3.1.4 Synthesis of the benzo[b]furan-2-yl(4-substituted phenyl)methanol

The ketone compounds (5, 8 and 10) can be reduced to the secondary alcohols (11, 12 and 13 respectively) by the action of the metal hydride NaBH$_4$. NaBH$_4$ is a mild reducing agent, it reduces ketones rapidly at room temperature in 1,4-dioxane solvent. The mechanism of this reaction involved nucleophilic addition (Scheme 3.5). The pair of electrons from the boron metal was transferred to the hydrogen to form hydride ion, H', which then acts as a nucleophile to attack the carbonyl carbon of the ketone compound.
Scheme 3.5. The synthesis of the secondary alcohol compounds (11, 12 and 13). 4 moles of the α-ketone compounds (5, 8 or 10) reacted with 1 mole of NaBH₄. Reaction conditions: NaBH₄, 1,4-dioxane, r.t., 8 h.

The reduction of the ketone compounds to the secondary alcohol compounds (11 – 13) using NaBH₄ gave very good yield (75 %, 83 % and 90 % respectively). Moreover, no further recrystallisation or column chromatography was required prior the next reaction step.

3.1.5 Synthesis of the substituted 1- and 4-[(benzo[b]furan-2-yl)phenylmethyl]triazoles

Drabel and Regel in 1975 patented a superior method (Draber and Regel, 1975) by reacting thionyl-imidazole with a substituted carbinol (Scheme 3.6) to form N-(1,1,1-trisubstituted)-methylazoles. The synthesis of the triazole compounds (14 – 19) involved the reaction of the carbinol compounds (11 – 13) with the in situ prepared di-triazole-sulphoxide (Scheme 3.7).

Scheme 3.7. The in situ prepared di-triazole-sulphoxide. Reaction conditions: 4:1 = 1,2,4-triazole:SOCl₂, acetonitrile, 10 °C, 1 h.

The mechanism of the synthesis of the triazole compounds (14 – 19) involved two nucleophilic substitution reactions (Scheme 3.8). The carbinol compounds (11 – 13) reacted with (I) or (II) to give the products (14 – 19) after undergoing a second nucleophilic substitution (Scheme 3.8).

Scheme 3.8. The mechanism of the synthesis of substituted 1- and 4-[[benzo[b]furan-2-yl]phenylmethyl]triazoles (14 – 19). Reaction conditions: 1,2,4-triazole, SOCl₂, acetonitrile, 10 °C, 1h then add K₂CO₃, carbinol compound (11, 12 or 13), r.t., 4 – 6 days.

The above reaction took 4 – 6 days to go to completion (Table 3.1). The reaction was purified by flash column chromatography using two different systems, i.e. petroleum ether/ethyl acetate and dichloromethane/methanol. The substituted 1-
[(benzo[b]furan-2-yl)phenylmethyl]triazoles (14, 16 and 18) elute out before the 4-[(benzo[b]furan-2-yl)phenylmethyl]triazoles (15, 17 and 19). The substituted 4-[(benzo[b]furan-2-yl)phenylmethyl]triazoles gave a lower yield compared with the substituted 1-[(benzofuran-2-yl)phenylmethyl]triazoles (Table 3.1). This shows that the nucleophile (II) is more nucleophilic compared with the nucleophile (III) to attack the carbon atom of the intermediate (I) (Scheme 3.8).

The products were confirmed by $^1$H n.m.r. after purification by flash column chromatograph. The $^1$H n.m.r. showed the disappearance of the OH group at approximately 2.7 – 2.9 ppm and the presence of the two singlet protons (H-3‴ and H-5‴) of the triazoles compounds, 14, 16 and 18, at approximately 8.1 and 8.2 ppm. The two protons (H-2‴ and H-5‴) of the triazoles compounds, 15, 17 and 19 appeared as a singlet at 8.3 ppm. These two protons are symmetrical and thus show only one singlet peak with two proton integration.

Table 3.1. Summary of the synthesis of the substituted 1- and 4- [(benzo[b]furan-2-yl)phenylmethyl]triazoles.

<table>
<thead>
<tr>
<th>Product</th>
<th>Total reaction (days)</th>
<th>Yield (%)</th>
<th>Ratio</th>
<th>Melting point (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td>4</td>
<td>67</td>
<td>5</td>
<td>Semi-solid at r.t.</td>
</tr>
<tr>
<td>15</td>
<td>4</td>
<td>13</td>
<td>1</td>
<td>126 -128</td>
</tr>
<tr>
<td>16</td>
<td>6</td>
<td>53</td>
<td>9</td>
<td>130 – 132</td>
</tr>
<tr>
<td>17</td>
<td>6</td>
<td>6</td>
<td>1</td>
<td>56 - 58</td>
</tr>
<tr>
<td>18</td>
<td>6</td>
<td>67</td>
<td>5</td>
<td>34 – 36</td>
</tr>
<tr>
<td>19</td>
<td>6</td>
<td>13</td>
<td>1</td>
<td>68 - 70</td>
</tr>
</tbody>
</table>
3.2 General material and method

All reactions were carried out under an atmosphere of nitrogen when necessary. $^1$H and $^{13}$C n.m.r. spectra were recorded on a Bruker Advance DPX300 spectrometer at 300 MHz and 75 MHz respectively. The n.m.r. solvent was CDCl$_3$ for all cases unless mentioned otherwise. Each resonance signal was reported according to the following convention:

- chemical shifts are given in parts per million (ppm) relative to the internal standard tetramethyl silane (Me$_4$Si).
- coupling constants [J in hertz (Hz)].
- multiplicity are denoted as s (singlet), d (doublet), t (triplet), m (multiplet) or combinations thereof.

Infra red (IR) spectra were recorded on a Perkin-Elmer 1600 (FTIR) spectrophotometer as either films or sodium chloride discs or as solids via a diffuse reflectance accessory using a potassium bromide (KBr) disc. Low resolution mass spectra ES (Electrospray) were recorded on Fisions VG Platform Electrospray Mass Spectrometer. Mass spectra were determined under EI (Electron Impact) or CI (Chemical Ionisation) conditions at the EPSRC National Mass Spectrometry Service Centre, University of Wales, Swansea. Accurate mass measurements were also performed at the EPSRC National Mass Spectrometry Service Centre. Microanalysis data were performed by Medac Ltd., Brunel Science Centre, Surrey.

For column chromatography, a glass column was slurry packed in the appropriate eluent with silica gel (Fluka Kieselgel 60). Flash column chromatography was performed with the aid of a pump. Analytical thin layer chromatography (t.l.c.) was carried out on pre-coated silica plates (Merck Kieselgel 60 F$_{254}$) with visualisation via UV light (254 nm) and/or vanillin stain.

Vanillin stain was prepared from the following ingredients:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vanillin</td>
<td>3 g</td>
</tr>
<tr>
<td>Absolute ethanol</td>
<td>250 mL</td>
</tr>
<tr>
<td>Concentrated H$_2$SO$_4$</td>
<td>5 mL</td>
</tr>
</tbody>
</table>

Melting points were determined using a Gallenkamp melting point apparatus and were uncorrected. All reagents and solvents employed were of general purpose or analytical grade and purchased from Sigma-Aldrich Chemical Company, Fluka or
Agros Chemicals. Solvents were appropriately dried over molecular sieves (3Å) or by distillation when necessary.

3.3 Experimental results for the synthesis of substituted 1- and 4-[(benzo[b]furan-2-yl)phenylmethyl]triazales

The numbering of compounds for n.m.r. characterisation is as follows:

All n.m.r. characterisations were made by comparison with previous n.m.r. spectra of the appropriate structure class and or predictions from ACD/HNMR and ACD/CNMR
2-Bromo-1-(4-ethyl-phenyl)-ethanone (2) (Mason, 2000)

\[(\text{C}_{10}\text{H}_{11}\text{BrO}, \text{MW: 227.100})\]

\[
\begin{align*}
\text{O} & \quad \text{Br}_2, \text{AlCl}_3, \text{THF} \\
\text{H}_3\text{C} & \quad 0 \, ^\circ \text{C}, 3.5 \, \text{hours} \\
\text{O} & \quad \text{Br—CH}_2
\end{align*}
\]

To a cooled solution (0 °C) of 4'-ethyl acetophenone (1) (2.52 mL, 16.87 mmol) in anhydrous THF (20 mL) was added a catalytic amount of aluminium chloride (0.23 g, 1.7 mmol). Bromine (0.87 mL, 16.87 mmol) was added dropwise to the solution (to give an orange solution) over a period of 20 minutes and the reaction stirred for a further 3.5 hours until the orange solution faded to a yellow colouration. The reaction was then concentrated under reduced pressure. The resulting residue was extracted with CH\(_2\)Cl\(_2\) (150 mL) and water (3 x 100 mL). The organic layer was then filtered and reduced in vacuo to give a dark green solution. Purification by flash column chromatography (petroleum ether – ethyl acetate 100:0 v/v increasing to 97.5:2.5 v/v) gave 2-bromo-1-(4-ethyl-phenyl)-ethanone (2) as a dark brown liquid. Yield: 3.56 g (93 %), t. l. c. system: petroleum ether – ethyl acetate 4:1 v/v, R\(_f\): 0.53, stain positive.

\(^1\)H n.m.r. \(\delta\) 7.91 (m, 2H, H-2 and H-6), 7.30 (m, 2H, H-3 and H-5), 4.43 (s, 2H, CH\(_2\)-Br), 2.70 (q, \(J = 7.6\) Hz, 2H, CH\(_2\)), 1.26 (t, \(J = 7.6\) Hz, 3H, CH\(_3\))

\(^{13}\)C n.m.r. \(\delta\) 191.40 (C=O), 151.54 (C, C-4), 132.08 (C, C-1), 129.64 (2 x CH, CH-2 and CH-6), 128.93 (2 x CH, CH-3 and CH-5), 31.48 (CH\(_2\), CH\(_2\)-Br), 29.46 (CH\(_2\), CH\(_2\)-CH\(_3\)), 15.54 (CH\(_3\), CH\(_2\)-CH\(_3\)).
Synthesis of benzo[b]furan-2-yl-phenyl-methanone derivatives (compound 5 and 6)

**Benzo[b]furan-2-yl(4-ethylphenyl)methanone (5)**

(C_{17}H_{14}O_{2}, MW: 250.296)

\[
\begin{align*}
(2) & \quad R = \text{CH}_2\text{CH}_3 \\
(3) & \quad R = \text{Br}
\end{align*}
\]

Salicylaldehyde (4) (1.68 mL, 15.74 mmol) in anhydrous DMF (2.0 mL), was added dropwise to a stirred solution of sodium hydride (60 % in mineral oil, 0.70 g, 17.31 mmol) in anhydrous DMF (8.0 mL). Hydrogen gas was liberated to give a bright yellow solution of the sodium salt. 2-Bromo-1-(4-ethyl-phenyl)-ethanone (2) (3.56 g, 15.74 mmol) in anhydrous DMF (10 mL) was then added dropwise and the resulting solution was stirred at 80 °C for 2.5 hours. Then sodium methoxide (0.26 g, 4.72 mmol) was added and the solution heated for another 1.5 hours at 80 °C. The solvent was then evaporated to about 1/3 its volume to give a brown syrup. The crude product was extracted with CH_2Cl_2 (200 mL) and water (3 x 100 mL). The organic layer was dried with MgSO_4, filtered and reduced in vacuo to give a yellow oily residue. The oily residue was recrystallised with methanol to yield the brown solid, benzo[b]furan-2-yl-(4-ethyl-phenyl)-methanone (5). Yield: 1.32 g (34 %), t. l. c. system: petroleum ether – ethyl acetate 5:2 v/v, R_f: 0.43, stained negative. Melting point 60 – 62 °C. Microanalysis: Calculated C = 81.58 %, H = 5.64 %; Found C = 81.44 %, H = 5.84 %.

^{1}H n.m.r. δ 8.05 (d, J = 8.1 Hz, 2H, Ar), 7.78 (d, J = 7.7 Hz, 1H, Ar), 7.70 (d, J = 7.9, 1H, Ar), 7.55 (m, 2H, Ar), 7.37 (m, 3H, Ar), 2.81 (q, J = 7.6 Hz, 2H, CH_2), 1.36 (t, J = 7.6 Hz, 3H, CH_3).

^{13}C n.m.r. δ 184.56 (C=O), 156.35 (C, C-7a), 152.83 (C, C-4'), 150.43 (C, C-1'), 135.22 (C, C-2), 130.19 (2 x CH, CH-2' and CH-6'), 128.66 (CH, CH-6), 128.52 (2 x CH, CH-3' and CH-5'), 127.48 (C, C-3a), 124.36 (CH, CH-5), 123.70 (CH, CH-4),
116.62 (CH, CH-7), 112.98 (CH, CH-3), 29.46 (CH₂, CH₂-CH₃), 15.71 (CH₃, CH₂-CH₃).
I.R. (KBr diffusion): 1643.8 cm⁻¹ (C=O).

The following analogue of compound 5 was prepared using the same general method detailed above.

**Benzo[b]furan-2-yl(4-bromophenyl)methanone (6)**

(C₁₃H₉BrO₂, MW: 301.138)

![Diagram of Benzo[b]furan-2-yl(4-bromophenyl)methanone (6)]

With benzo[b]furan-2-yl-(4-bromophenyl)methanone (6), a yellow residue was obtained. Recrystallisation with methanol gave a light yellow solid.

Yield: 5.0 g (92 %), t. l. c. system: petroleum ether – ethyl acetate 4:1 v/v, R₉: 0.55, stain negative. Melting point 155-157 °C [literature: 155 – 157 °C] (Buu-Hoi et al., 1957).

¹H n.m.r. δ 8.00 (dd, J = 1.6, 6.8 Hz, 2H, Ar), 7.74 (m, 4H, Ar), 7.57 (m, 2H, Ar), 7.40 (m, 1H, H-3).

¹³C n.m.r. δ 183.57 (C=O), 156.45 (C, C-7a), 152.44 (C, C-2), 136.26 (C, C-1’), 132.32 (2 x CH, CH-3’ and CH-5’), 131.46 (2 x CH, CH-2’ and CH-3’), 129.04 (CH, CH-6), 128.54 (C, C-4’), 127.32 (C, C-3a), 124.57 (CH, CH-5), 123.82 (CH, CH-4), 117.01 (CH, CH-7), 113.01 (CH, CH-3).
Synthesis of benzo[b]furan-2-yl-biphenyl-4-yl-methanone derivatives (compound 8 and 10)

Benzo[b]furan-2-yl-biphenyl-4-yl-methanone (8)

(C_{21}H_{14}O_2, MW: 298.340)

\[
\begin{align*}
\text{(6)} & \quad \text{(7) } R = H \\
\text{(9) } R = \text{Cl} \\
\text{(8) } R = H \\
\text{(10) } R = \text{Cl}
\end{align*}
\]

2M aqueous Na_2CO_3 (11.65 mL) was added to a solution of benzo[b]furan-2-yl(4-bromophenyl)methanone (6) (1.0 g, 3.32 mmol) in toluene (20 mL). The mixture was bubbled with nitrogen for one minute and then Pd(PPh_3)_4 (0.20 g, 0.166 mmol) was added to the mixture. Phenylboronic acid (0.81 g, 6.64 mmol) in ethanol (5 mL) was added to the above mixture and the reaction was refluxed at 100 °C for 4 hours. After the reaction was complete, the residual borane was oxidised by the addition of H_2O_2 (30 %, 2.5 mL) at room temperature for 1 hour. The crude product was extracted with CH_2Cl_2 (100 mL) and water (3 x 100 mL). The organic layer was dried with MgSO_4, filtered and reduced in vacuo to give a light yellow oily residue. Purification by flash column chromatography (petroleum ether – ethyl acetate 95:5 v/v increasing to 80:20 v/v) gave a mixture of the starting material and the product. The mixture was recrystallised with petroleum ether to yield light yellow fine crystals, benzo[b]furan-2-yl-biphenyl-4-yl-methanone (8). Yield: 0.57 g (58 %), t. l. c. system: petroleum ether – ethyl acetate 1:1 v/v, R_f: 0.63, stain negative. Melting point 150 – 152 °C [literature: 153 – 155 °C (Pestellini et al., 1984)].

^1H n.m.r. δ 8.21 (dd, J = 1.6, 8.2 Hz, 2H, Ar), 7.82 (m, 3H, Ar), 7.73 (m, 3H, Ar), 7.65 (s, 1H, H-3), 7.46 (m, 5H, Ar).

^13C n.m.r. δ 184.33 (C=O), 156.43 (C, C-7a), 152.80 (C, C-4’), 146.16 (C, C-1’’), 140.29 (C, C-1’), 136.31 (C, C-2), 130.58 (2 x CH, CH-2’ and CH-6’), 129.46 (2 x CH, CH-3’’ and CH-5’’), 128.83 (2 x CH, CH-3’ and CH-5’), 128.76 (CH, CH-4’’), 69
127.77 (2 x CH, CH-2'' and CH-6''), 127.66 (CH, CH-6), 127.48 (C, C-3a), 124.46 (CH, CH-5), 123.78 (CH, CH-4), 116.83 (CF, CH-7), 113.03 (CH, CH-3).

I.R. (KBr diffusion): 1641 cm⁻¹ (C=O) [Literature 1640 cm⁻¹] (Pestellini et al., 1984).

The following analogue of compound 8 was prepared using the same general method detailed above.

**Benzo[b]furan-2-yl(4'-chlorobiphenyl-4-yl)methanone (10)**

(C₂₁H₁₃ClO₂, MW: 332.785)

![Chemical structure](image)

With benzo[b]furan-2-yl(4'-chlorobiphenyl-4-yl)methanone (10), a yellow oily residue was obtained. Recrystallisation with petroleum ether yield light yellow solid.

Yield: 1.10 g (73 %), t. l. c. system: petroleum ether – ethyl acetate 4:1 v/v, Rf: 0.50, stain negative. Microanalysis: Calculated C = 75.79 %, H = 3.94 %; Found C = 76.14 %, H = 4.00 %. Melting point: 154 – 156 °C.

¹H n.m.r. δ 8.25 (dd, J = 1.7, 6.6 Hz, 2H, Ar), 7.81 (m, 4H, Ar), 7.69 (m, 2H, Ar), 7.52 (m, 5H, Ar).

¹³C n.m.r. δ 184.18 (C=O), 156.44 (C, C-7a), 152.75 (C, C-4''), 144.82 (C, C-1''), 138.71 (C, C-2), 136.57 (C, C-1''), 134.96 (C, C-4''), 130.67 (2 x CH, CH-2' and CH-6'), 129.64 (2 x CH, CH-3'' and CH-5''), 128.89 (2 x CH, CH-3' and CH-5''), 128.65 (CH, CH-6), 127.49 (2 x CH, CH-2'' and CH-6''), 127.44 (C, C-3a), 124.49 (CH, CH-5), 123.79 (CH, CH-4), 116.87 (CH, CH-7), 113.02 (CH, CH-3).

I.R. (KBr diffusion): 1644.9 cm⁻¹ (C=O).
**Synthesis of benzo[b]furan-2-yl-phenyl-methanol derivatives** (compound 11 – 13)

**Benzo[b]furan-2-yl(4-ethylphenyl)methanol (11)**

(C_{17}H_{18}O_{2}, MW: 252.311)

![Chemical structure](image)

(5) R = CH_{2}CH_{3}  
(8) R = phenyl  
(10) R = 4'-Cl-phenyl  
(11) R = CH_{2}CH_{3}  
(12) R = phenyl  
(13) R = 4'-Cl-phenyl

Benzo[b]furan-2-yl-(4-ethylphenyl)methanone (5) (1.2 g, 4.80 mmol) was dissolved in anhydrous 1,4-dioxane (40 mL). Sodium borohydride (0.18 g, 4.80 mmol) was then added and the mixture stirred under nitrogen at room temperature for 8 hours. The solvent was evaporated *in vacuo* and 1M aqueous hydrochloric acid (25 mL) was added to the residue in order to quench any excess reducing agent. The oil that separated was extracted with diethyl ether (2 x 150 mL), washed with water (3 x 100 mL), then the organic layer was dried with MgSO_{4}, filtered and reduced *in vacuo* to give a white solid, benzo[b]furan-2-yl(4-ethylphenyl)methanol (11). Yield: 1.0 g (83 %), t. l. c. system: petroleum ether – ethyl acetate 3:2 v/v, R_{f}: 0.64, stain positive. LRMS (El^+): m/z: 252.1 (M^+). Microanalysis: Calculated C = 80.93 %, H = 6.39 %; Found C = 80.73 %, H = 6.41 %. Melting point: 45 – 47 °C.

^{1}H n.m.r. δ 7.60 (m, 1H, Ar), 7.50 (m, 3H, Ar), 7.32 (m, 4H, Ar), 6.61 (s, 1H, H-3), 5.96 (d, J = 4.6 Hz, 1H, CH-OH), 2.91 (d, J = 4.6 Hz, 1H, OH), 2.76 (q, J = 7.6 Hz, 2H, CH_{2}), 1.34 (t, J = 7.6 Hz, 3H, CH_{3}).

^{13}C n.m.r. δ 160.31 (C, C-2), 159.21 (C, C-7a), 155.54 (C, C-4'), 144.97 (C, C-1'), 138.12 (C, C-3a), 128.58 (2 x CH, CH-3' and CH-5'), 127.34 (2 x CH, CH-2' and CH-6'), 124.67 (CH, CH-6), 123.25 (CH, CH-5), 121.58 (CH, CH-4), 111.81 (CH, CH-7), 104.35 (CH, CH-3), 71.02 (CH-OH), 29.08 (CH_{2}, CH_{2}-CH_{3}), 16.05 (CH_{3}, CH_{2}-CH_{3}).
The following analogues of compound 11 were prepared using the same general method detailed above.

**Benzo[b]furan-2-yl-biphenyl-4-yl-methanol (12)**

\[ \text{(C}_{21}\text{H}_{16}\text{O}_2, \text{MW: 300.355)} \]

![Diagram](image)

With benzo[b]furan-2-yl-biphenyl-4-yl-methanol (12), a white solid was obtained. Yield: 0.51 g (90 %), t. l. c. system: petroleum ether – ethyl acetate 1:1 v/v, \( R_F: 0.63 \), stain positive. LRMS (El) \( m/z: 300.1 \text{ (M^+)} \). Melting point: 121 – 123 °C [literature: 123 – 125 °C] (Pestellini et al., 1984).

\[ ^1\text{H n.m.r.} \delta 7.63 \text{ (m, 6H, Ar)}, 7.46 \text{ (m, 4H, Ar)}, 7.30 \text{ (m, 3H, Ar)}, 6.64 \text{ (d, J = 0.6 Hz, 1H, H-3)}, 6.04 \text{ (d, J = 3.1 Hz, 1H, CH-OH)}, 2.73, \text{ (d, J = 3.9 Hz, 1H, OH)}. \]

\[ ^{13}\text{C n.m.r.} \delta 158.84 \text{ (C, C-2)}, 155.57 \text{ (C, C-7a)}, 141.76 \text{ (C, C-1’)}, 141.10 \text{ (C, C-1’’)}, 139.69 \text{ (C, C-4’)}, 129.28 \text{ (2 x CH, CH-3’’ and CH-5’’)}, 128.47 \text{ (C, C3a-)}, 127.92 \text{ (2 x CH, CH-2’ and CH-6’)}, 127.83 \text{ (2 x CH, CH-3’ and CH-5’)}, 127.72 \text{ (2 x CH, CH-2’’ and CH-6’’)}, 127.60 \text{ (CH, CH-4’’)}, 124.82 \text{ (CH, CH-6)}, 123.34 \text{ (CH, CH-5)}, 121.64 \text{ (CH, CH-4)}, 111.83 \text{ (CH, CH-7)}, 104.57 \text{ (CH, CH-3)}, 70.91 \text{ (CH-OH)}. \]

I.R. (KBr diffusion): 3271.8 cm\(^{-1}\) (Broad, OH).

**Benzo[b]furan-2-yl(4’-chlorobiphenyl-4-yl)methanol (13)**

\[ \text{(C}_{21}\text{H}_{15}\text{ClO}_2, \text{MW: 334.800)} \]

![Diagram](image)

With benzo[b]furan-2-yl-(4’-chlorobiphenyl-4-yl)methanol (13), a white solid was obtained. Yield: 0.60 g (75 %), t. l. c. system: petroleum ether – ethyl acetate 1:1 v/v,
R<sub>f</sub>: 0.60, stain positive. Microanalysis: Calculated C = 75.20 %, H = 4.45 %; Found C = 75.34 %, H = 4.52 %. Melting point: 76 – 78 °C.

<sup>1</sup>H n.m.r. δ 7.54 (m, 7H, Ar), 7.44 (m, 3H, Ar), 7.25 (m, 2H, Ar), 6.59 (s, 1H, H-3), 6.00 (d, J = 4.5 Hz, 1H, CH-OH), 2.65 (d, J = 4.6 Hz, 1H, OH).

<sup>13</sup>C n.m.r. δ 158.69 (C, C-2), 155.55 (C, C-7a), 140.48 (C, C-1'), 140.31 (C, C-4'), 139.51 (C, C-1''), 134.02 (C, C-4''), 129.42 (2 x CH, CH-3' and CH-5''), 128.81 (2 x CH, CH-2' and CH-6''), 128.41 (C, C-3a), 127.80 (2 x CH, CH-2' and CH-6'), 127.64 (2 x CH, CH-3' and CH-5'), 124.87 (CH, CH-6), 123.36 (CH, CH-5), 121.65 (CH, CH-4), 111.82 (CH, CH-7), 104.59 (CH, CH-3), 70.82 (CH-OH).

I.R. (KBr diffusion): 3317.8 cm<sup>-1</sup> (Broad, OH).

**Synthesis of 1-[benzo[b]furan-2-yl-phenylmethyl]-1H-[1,2,4]triazole derivatives** (compound 14, 16 and 18) and **4-[benzo[b]furan-2-yl-phenylmethyl]-4H-[1,2,4]triazole derivatives** (compound 15, 17 and 19)

1-[Benzo[b]furan-2-yl-(4-ethylphenyl)methyl]-1H-[1,2,4]triazole (14) and 4-[Benzo[b]furan-2-yl-(4-ethylphenyl)methyl]-4H-[1,2,4]triazole (15)

\[\text{C}_{19}\text{H}_{17}\text{N}_3\text{O}, \text{MW: 303.362}\]
Thionyl chloride (0.47 g, 3.96 mmol) in anhydrous acetonitrile (10.0 mL) was added dropwise to a stirred solution of 1,2,4-triazole (1.09 g, 15.85 mmol) in anhydrous acetonitrile (10.0 mL) at a temperature of 10 °C. The white suspension formed was stirred for 1 h at 10 °C. A solution of benzo[b]furan-2-yl-(4-ethylphenyl)methanol (11) (1.0 g, 3.96 mmol) in anhydrous acetonitrile (10.0 mL) was added to the mixture followed by activated potassium carbonate (1.10 g, 7.93 mmol). The suspension was stirred under nitrogen at room temperature for 4 days. The resulting suspension was filtered and the filtrate was evaporated in vacuo to yield a light brown oil. The oil was extracted with CH₂Cl₂ (150 mL) and water (3 x 100 mL). The organic layer was dried with MgSO₄, filtered and reduced in vacuo to give a yellow oil. Purification by flash column chromatography (petroleum ether – ethyl acetate 90:10 v/v increasing to 70:30 v/v) gave 1-[benzo[b]furan-2-yl-(4-ethylphenyl)methyl]-1H-[1,2,4]triazole (14) (0.80 g, 67 %) as a yellow syrup. T. l. c. system: petroleum ether – ethyl acetate 1:1 v/v, Rf: 0.42, stain positive. HRMS (EI⁺) m/z Calculated for C₁₀H₁₇N₃O (M+H)⁺ 304.1444; Found 304.1442. The flash column chromatography was continued (dichloromethane – methanol 100:0 v/v increasing to 99:1 v/v) to give 4-[benzo[b]furan-2-yl-(4-ethylphenyl)methyl]-4H-[1,2,4]triazole (15) (0.16 g, 13 %) as a white solid. T. l. c. system: dichloromethane – methanol 9:1 v/v, Rf: 0.31, stain positive. Microanalysis: Calculated C = 75.23 %, H = 5.65 %, N = 13.84 %; Found C = 75.19 %, H = 5.74 %, N = 13.61 %. Melting point: 126 – 128 °C.

N.M.R. data for 1-[benzo[b]furan-2-yl-(4-ethylphenyl)methyl]-1H-[1,2,4]triazole (14)

¹H n.m.r. δ 8.19 (s, 1H, H-3′′′), 8.09 (s, 1H, H-5′′′), 7.58 (d, J = 8.4 Hz, 1H, Ar), 7.51 (d, J = 9.3 Hz, 1H, Ar), 7.39 - 7.27 (m, 6H, Ar), 6.89 (s, 1H, H-3), 6.63 (s, 1H, H-1), 2.73 (q, J = 7.6 Hz, 2H, CH₂), 1.30 (t, J = 7.6 Hz, 3H, CH₃).

¹³C n.m.r. δ 155.75 (C, C-2), 153.342 (C, C-7a), 152.65 (CH, CH-5′′′), 145.91 (C, C-4′), 143.67 (CH, CH-3′′′), 133.20 (C, C-1′), 129.06 (2 x CH, CH-2′ and CH-6′), 128.16 (2 x CH, CH-3′ and CH-5′), 127.96 (C, C-3a), 125.59 (CH, CH-6), 123.73 (CH, CH-5), 121.93 (CH, CH-4), 111.97 (CH, CH-7), 108.06 (CH, CH-3), 62.51 (CH, CH-1), 28.99 (CH₂), 15.85 (CH₃).

N.M.R. data for 4-[benzo[b]furan-2-yl-(4-ethylphenyl)methyl]-4H-[1,2,4]triazole (15)

¹H n.m.r. δ 8.25 (s, 2H, triazole H-2′′ and H-5′′′), 7.57 (d, J = 6.8 Hz, 1H, Ar), 7.46 (d, J = 8.4 Hz, 1H, Ar), 7.37 - 7.19 (m, 6H, Ar), 6.75 (s, 1H, H-3), 6.62 (s, 1H, H-1), 2.70 (q, J = 7.6 Hz, 2H, CH₂), 1.26 (t, J = 7.6 Hz, 3H, CH₃).
^{13}C n.m.r. δ 155.76 (C, C-2), 152.90 (C, C-7a), 146.26 (C, C-4'), 142.95 (2 x CH, CH-2''' and CH-5'''''), 132.98 (C, C-1'), 129.27 (2 x CH, CH-2' and CH-6''), 127.89 (2 x CH, CH-3' and CH-5''), 127.67 (C, C-3a), 125.91 (CH, CH-6), 123.94 (CH, CH-5), 122.01 (CH, CH-4), 112.01 (CH, CH-7), 108.04 (CH, CH-3), 58.28 (CH, CH-1), 28.95 (CH<sub>2</sub>), 15.83 (CH<sub>3</sub>).

The following analogues of compound 14 and 15 were prepared using the same general method detailed above.

1-(Benzol[b]furan-2-yl-biphenyl-4-yl-methyl)-1H-[1,2,4]triazole (16) and 4-(Benzol[b]furan-2-yl-biphenyl-4-yl-methyl)-4H-[1,2,4]triazole (17)

(C<sub>23</sub>H<sub>17</sub>N<sub>3</sub>O, MW: 351.406)

With 1-(benzo[b]furan-2-yl-biphenyl-4-yl-methyl)-1H-[1,2,4]triazole (16), a white solid was obtained after purification by flash column chromatography (petroleum ether – ethyl acetate 90:10 v/v increasing to 65:35 v/v). Yield: 0.34 g (53 %), t. l. c. system: petroleum ether – ethyl acetate 1:1 v/v, R<sub>F</sub>: 0.41, stain positive.

Microanalysis: Calculated C = 78.61 %, H = 4.88 %, N = 11.95 %; Found C = 78.38 %, H = 4.74 %, N = 11.89 %. Melting point: 130 – 132 °C.

The flash column chromatography was continued (dichloromethane – methanol 100:0 v/v increasing to 99:1 v/v) to give 4-(benzo[b]furan-2-yl-biphenyl-4-yl-methyl)-4H-[1,2,4]triazole (17). Yield: 0.04 g (6.25 %) as a yellow light solid. T. l. c. system: dichloromethane – methanol 9:1 v/v, R<sub>F</sub>: 0.31, stain positive. Microanalysis (C<sub>23</sub>H<sub>17</sub>N<sub>3</sub>O.0.35H<sub>2</sub>O): Calculated C = 77.29 %, H = 4.98 %, N = 11.81 %; Found C = 77.11 %, H = 4.63 %, N = 11.84 %. Melting point: 56 - 58 °C.

N.M.R. data for 1-(benzo[b]furan-2-yl-biphenyl-4-yl-methyl)-1H-[1,2,4]triazole (16)

<sup>1</sup>H n.m.r. δ 8.24 (s, 1H, H-3''''), 8.12 (s, 1H, H-5'''''), 7.68 (d, J = 8.3 Hz, 2H, Ar), 7.63 (m, 3H, Ar), 7.53 (m, 3H, Ar), 7.45 – 7.31 (m, 5H, Ar), 6.96 (s, 1H, H-3), 6.69 (s, 1H, H-1).
\[ ^{13}\text{C} \text{n.m.r. } \delta \ 155.80 \ (C, C-2), \ 152.99 \ (C, C-7a), \ 152.78 \ (CH, CH-5''), \ 143.76 \ (CH, CH-3''), \ 142.61 \ (C, C-1''), \ 140.55 \ (C, C-1'), \ 134.92 \ (C, C-4'), \ 129.36 \ (2 \times CH, CH-2' \text{ and CH-6'}), \ 128.58 \ (2 \times CH, CH-3''' \text{ and CH-5'''}, \ 128.27 \ (2 \times CH, CH-2''' \text{ and CH-6'''}), \ 128.23 \ (2 \times CH, CH-3' \text{ and CH-5'}), \ 127.92 \ (C, C-3a), \ 127.60 \ (CH, CH-4'''), \ 125.75 \ (CH, CH-6), \ 123.83 \ (CH, CH-5), \ 122.02 \ (CH, CH-4), \ 112.02 \ (CH, CH-7), \ 108.34 \ (CH, CH-3), \ 62.41 \ (CH, CH-1). \\
N.M.R. data for 4-(benzo[b]furan-2-yl-biphenyl-4-yl-methyl)-4\text{H}-[1,2,4]triazole (17)

\[ ^{1}\text{H} \text{n.m.r. } \delta \ 8.41 \ (s, 2H, triazole H-2''' \text{ and H-5'''}), \ 7.79 \ (d, J = 8.4 \text{ Hz}, 2H, Ar), \ 7.72 \ (m, 3H, Ar), \ 7.61 \ (m, 3H, Ar), \ 7.58 - 7.40 \ (m, 5H, Ar), \ 6.87 \ (s, 1H, H-3), \ 6.79 \ (s, 1H, H-1). \\
^{13}\text{C} \text{n.m.r. } \delta \ 155.85 \ (C, C-2), \ 152.44 \ (C, C-7a), \ 143.02 \ (C, C-1''), \ 142.93 \ (2 \times CH-2''' \text{ and CH-5'''}), \ 140.23 \ (C, C-1'), \ 134.50 \ (C, C-4'), \ 129.42 \ (2 \times CH, CH-2' \text{ and CH-6'}), \ 128.51 \ (2 \times CH, CH-3''' \text{ and CH-5'''}, \ 128.41 \ (2 \times CH, CH-2''' \text{ and CH-6'''}), \ 128.31 \ (CH, CH-4'''), \ 127.59 \ (2 \times CH, CH-3' \text{ and CH-5'} \text{ and C, C-3a),} \ 126.12 \ (CH, CH-6), \ 124.07 \ (CH, CH-5), \ 122.09 \ (CH, CH-4), \ 112.10 \ (CH, CH-7), \ 108.34 \ (CH, CH-3), \ 58.33 \ (CH, CH-1).

1-[Benzo[b]furan-2-yl-(4'-chloro-biphenyl-4-yl)-methyl]-1H-[1,2,4]triazole (18) and 4-[Benzo[b]furan-2-yl-(4'chloro-biphenyl-4-yl)-methyl]-4\text{H}-[1,2,4]triazole (19)

\[(\text{C}_{23}\text{H}_{16}\text{ClN}_{3}\text{O}, \text{MW: 385.852})

\[\text{(18)} \quad \text{(19)}

With 1-[benzo[b]furan-2-yl-(4'-chlorobiphenyl-4-yl)methyl]-1H-[1,2,4]triazole (18), a white solid was obtained after purification by flash column chromatography (petroleum ether – ethyl acetate 90:10 v/v increasing to 65:35 v/v). Yield: 0.38 g (67 %), t. l. c. system: petroleum ether – ethyl acetate 1:1 v/v, \( R_f \): 0.26, stain positive. Microanalysis: Theory C = 71.60 %, H = 4.18 %, N = 10.89 %; Found C = 71.45 %, H = 4.10 %, N = 10.62 %. Melting point: 34 – 36 °C.
Chapter 3

The flash column chromatography was continued (dichloromethane – methanol 100:0 v/v increasing to 99:1 v/v) to give 4-[benzo[b]furan-2-yl-(4’chlorobiphenyl-4-yl)methyl]-4H-[1,2,4]triazole (19) (0.075 g, 13 %) as a light yellow solid. T. l. c. system: dichloromethane – methanol 9:1 v/v, R_F: 0.50, stain positive. Microanalysis (C_{23}H_{16}ClN_3O_2H_2O): Theory C = 71.07 %, H = 4.25 %, N = 10.81 %; Found C = 71.05 %, H = 4.09 %, N = 10.75 %. Melting point: 68 – 70 °C.

N.M.R. data for 1-[benzo[b]furan-2-yl-(4’-chlorobiphenyl-4-yl)methyl]-1H-[1,2,4]triazole (18)

^1^H n.m.r. δ 8.24 (s, 1H, H-3”’), 8.11 (s, 1H, H-5”’), 7.64 (d, J = 8.3 Hz, 2H, Ar), 7.60 – 7.52 (m, 3H, Ar), 7.49 – 7.29 (m, 7H, Ar), 6.95 (s, 1H, H-3), 6.69 (s, 1H, H-1).

^1^C n.m.r. δ 155.80 (C, C-2), 152.80 (CH, CH-5”’ and C, C-7a), 143.74 (CH, CH-3”’), 141.36 (C, C-1’), 138.97 (C, C-1”’), 135.32 (C, C-4’), 134.38 (C, C-4’’), 129.52 (2 x CH, CH-2’’ and CH-6’’), 128.83 (2 x CH, CH-3”’ and CH-5’’), 128.67 (2 x CH, CH-2”’ and CH-6”’), 128.10 (2 x CH, CH-3’ and CH-5’), 127.87 (C, C-3a), 125.80 (CH, CH-6), 123.86 (CH, CH-5, 122.02 (CH, CH-4), 112.02 (CH, CH-7), 108.40 (CH, CH-3), 62.35 (CH, CH-1).

N.M.R. data for 4-[benzo[b]furan-2-yl-(4’chlorobiphenyl-4-yl)methyl]-4H-[1,2,4]triazole (19)

^1^H n.m.r. δ 8.31 (s, 2H, triazole H-2”’ and H-5”’), 7.65 (d, J = 8.3 Hz, 2H, Ar), 7.61 – 7.43 (m, 6H, Ar), 7.41 – 7.31 (m, 4H, Ar), 6.79 (s, 1H, H-3), 6.70 (s, 1H, H-1).

^1^C n.m.r. δ 155.85 (C, C-2), 152.29 (C, C-7a), 142.89 (2 x CH, CH-2”’ and CH-5”’), 141.75 (C, C-1’), 138.66 (C, C-1”’), 134.93 (C, C-4’), 134.58 (C, C-4’’), 129.59 (2 x CH, CH-2’’ and CH-6’’), 128.82 (2 x CH, CH-3”’ and CH-5’’), 128.42 (2 x CH, CH-2”’ and CH-6”’), 128.35 (2 x CH, CH-3’ and CH-5’), 127.56 (C, C-3a), 126.16 (CH, CH-6), 124.10 (CH, CH-5), 122.10 (CH, CH-4), 112.10 (CH, CH-7), 108.37 (CH, CH-3), 58.26 (CH, CH-1).
CHAPTER 4

Synthesis of benzo[b]furan-2-carboxamido ethyl-imidazole and -1,2,4-triazole derivatives
4.1 Synthesis of benzo[b]furan-2-carboxamido ethyl-imidazole and -1,2,4-triazole derivatives

The synthesis of N2-[2-phenyl-2-(1H-imidazolyl)ethyl]-benzo[b]furan-2-carboxamide derivatives (51 - 55), N2-[2-phenyl-2-(1H-1,2,4-triazol-1-yl)ethyl]-benzo[b]furan-2-carboxamide derivatives (56, 58, 60, 62 and 64), and N2-[2-phenyl-2-(4H-1,2,4-triazol-4-yl)ethyl]-benzo[b]furan-2-carboxamide derivatives (57, 58, 61, 63 and 65) involved a 5 step reaction as outlined in Scheme 4.1. It involved a modification of the procedure described by Schuster and co-workers (Schuster and Egger, 1997) and Moenius and co-workers (Moenius et al., 1999).

4.1.1 Synthesis of substituted ethyl benzo[b]furan-2-carboxylate

The method described in section 3.1.2 was applied to the synthesis of ethyl benzo[b]furan-2-carboxylate (21), however, the reaction did no go to completion (Method 1, Scheme 4.2). Purification by flash column chromatography isolated the intermediate compound 22. The $^1$H n.m.r. showed one extra singlet peak at 4.78 ppm which referred to two H atoms of the α-CH$_2$. A singlet peak at 10.58 ppm referred to the H atom of the aldehyde group. This confirmed that the formation of the benzofuran ring had not occurred. The method used by Suzuki’s group (Suzuki et al., 1983) was tried in the synthesis of the substituted ethyl benzo[b]furan-2-carboxylate (Method 2, Scheme 4.2). The formation of the products 21, 32 and 34 was successful using the base, K$_2$CO$_3$, as described by Suzuki’s group.

After purification by flash chromatography or recrystallisation with petroleum ether, the products were confirmed by $^1$H n.m.r. by the presence of the H-3 as a doublet at approximately 7.5 ppm (Scheme 4.2) and the presence of the C=O in the carbon n.m.r. at approximately 156 to 157 ppm. Overall, this method gave moderate yields (22 % - 53 %).
Scheme 4.1. General reaction scheme for the synthesis of N2-[2-phenyl-1H]-imidazol-2-yl-carboxamide derivatives (55), N2-[2-phenyl-2H]-imidazol-2-yl-[1H]-imidazol-2-yl-carboxamide derivatives (56) and 2-[N2-[2-phenyl-1H]-imidazol-2-yl]-carboxamide derivatives (57).
Scheme 4.2. The synthesis of substituted ethyl benzo[b]furan-2-carboxylate (21, 24 and 26). Reaction conditions, **Method 1**: (i) NaH, DMF, 80 °C, 2.5 h, (ii) NaOMe, 80 °C, 1.5 h. Reaction conditions, **Method 2**: (i) K₂CO₃, DMF, 130 °C, 6 - 7 h. **Method 1** failed to synthesise product 21 and thus **method 2** was used in the synthesis of products 21, 24 and 26.

4.1.2 Synthesis of substituted benzo[b]furan-2-carboxylic acid

The synthesis of the substituted benzo[b]furan-2-carboxylic acid involved base hydrolysis of the substituted ethyl benzo[b]furan-2-carboxylate (Scheme 4.3). The substituted benzo[b]furan-2-carboxylic acid compounds 27 – 29 were confirmed by the disappearance of the CH₂ and CH₃ peaks in the ¹H n.m.r. and ¹³C n.m.r. The alkaline hydrolysis of the benzo[b]furan-2-carboxylic acid ethyl ester compound produced good yields (88 % – 98 %).

Scheme 4.3. Synthesis of the substituted benzo[b]furan-2-carboxylic acid (27, 28 and 29). Reaction conditions: 2M NaOH (aq.), CH₃OH, r.t., 20 min., 85 - 98 %.
4.1.3 Synthesis of the substituted 2-amino-1-phenyl-ethanol

The synthesis of the substituted 2-amino-1-phenyl-ethanol derivatives (35 and 36) involved two steps as shown in Scheme 4.4 (a) and (b). The first step (Scheme 4.4 (a)) involved the base catalysed (NaOH) condensation of benzaldehydes, 30 and 33, with nitromethane in ice-cooled methanol to form the corresponding nitro-alcohol 32 and 34 (Langer et al., 2001). Overall, the condensation of benzaldehyde with nitromethane gave moderate yields (54 % - 74 % respectively) after purification by flash column chromatography.

![Scheme 4.4](image)

**Scheme 4.4.** The synthesis of substituted 2-amino-1-phenyl-ethanol (35 and 36). (a) nitromethane, 10 M NaOH (aq.), acetic acid (aq.) 2 % v/v, methanol, 5 – 10 °C, 8 h. (b) Raney nickel, formic acid (aq.) 50 % v/v, 40 psi H₂, room temperature, 24 h.

The next step (Scheme 4.4 (b)) involved the reduction of the nitro compound (32 and 34) to the corresponding amine compound (35 and 36). The most frequently employed method for nitro reduction to form a primary amine is catalytic hydrogenation, for example, palladium catalyst with H₂ gas (Pd/H₂). However, the use of Pd/H₂ in the reduction of 1-(4-fluorophenyl)-2-nitro-1-ethanol (32) and 1-(4-chlorophenyl)-2-nitro-1-ethanol (34) could result in the cleavage of the halogen atom in 32 and 34. Raney nickel was used as a catalyst in the hydrogenation of the nitro compound 32 and 34, as this method is more selective (i.e. occurs without dehalogenation) (Gowda et al., 2000; Langer et al., 2001) compared with Pd/H₂ catalytic hydrogenation.

The reaction went to completion after 24 hours and the products 35 and 36 were purified by flash column chromatography using a dichloromethane-methanol-triethylamine eluent system. 5 % of triethylamine was used in the mobile phase system to neutralize the acid in the silica gel as compound 35 and 36 are sensitive to acid.

The purified products were confirmed by ¹H, ¹³C n.m.r. and compared with the literature (Cho et al., 2002). Referring to Figure 4.5, the H atoms of the CH₂, Hₐ and Hₐ, are coupled to each other with the coupling constant, Jₐₐ = 12.8 Hz; and they are
each coupled to $H_X$, with different coupling constants, one large ($J_{BX} = 7.5$ Hz) and one small ($J_{AX} = 4.4$ Hz). This is denoted as the ABX system, which involved a partially strongly coupled 3-spin system.

Overall the reduction of the nitro compound 32 and 34 with Raney nickel hydrogenation gave reasonable yields (44% - 46% respectively).

![Diagram of the ABX system](image)

**Figure 4.5.** The ABX system of the substituted 2-amino-1-phenyl-ethanol (35 and 36). Assignment of the peaks for $H_A$, $H_B$ and $H_X$ in the $^1$H n.m.r. spectra. The $^1$H n.m.r. spectrum of compound 35 is shown here.

### 4.1.4 Synthesis of the substituted N2-(2-hydroxy-2-phenylethyl)-benzo[b]furan-2-carboxamide

The synthesis of the substituted N2-(2-hydroxy-2-phenylethyl)-benzo[b]furan-2-carboxamide (39 – 43) was carried out using the reagent 1,1'-carbonyldiimidazole (CDI) (37) with the corresponding substituted 2-amino-1-phenyl-ethanol (35, 36 or 38) (Scheme 4.6). CDI is used as a coupling reagent in the synthesis of the amide compound (39 – 43). The product was recrystallised from ethanol and ice water to give good purity and yield (72% - 88%).

83
Scheme 4.6. The synthesis of substituted benzofuran-2-carboxylic acid (2-hydroxy-2-phenylethyl)-amide (39 – 43). Reaction conditions: (a) 1,1'-carbonyldiimidazole (37), DMF, r.t., 1h. (b) substituted 2-amino-1-phenyl-ethanol (35, 36 or 38), r.t., 12 h., recrystallisation, 72 - 88 %.

4.1.5 Synthesis of the substituted 2-benzo[b]furan-2-yl-5-phenyl-4,5-dihydro-1,3-oxazole

This synthesis involved formation of an oxazole ring structure in which the oxazole compounds (45 - 49) were prepared by the reaction of the amide-containing compound (39 – 43) with methanesulphonic anhydride (44) and a base (triethylamine) (Gant and Meyers, 1994; Sund et al., 1987) [Scheme 4.7].

The mechanism of this reaction involved nucleophilic attack by the hydroxyl group at the sulphur atom of methanesulphonic anhydride (44) to form the intermediate (44a). The activation of the carboxyl-oxygen as a nucleophilic centre will then occur as a result of the abstraction of the amido proton by the base, triethylamine (Sund et al., 1987) (Scheme 4.7).
Scheme 4.7. Synthesis of the substituted 2-benzo[b]furan-2-yl-5-phenyl-4,5-dihydro-1,3-oxazole (45 – 49). Reaction conditions: (CH$_3$SO)$_2$O, NEt$_3$, 0 °C, 24 h, column chromatograph or recrystallisation, 54 - 67%.

The presence of the 4,5-dihydro-1,3-oxazole ring structure in the compound (45 – 49) was illustrated on the $^1$H n.m.r. as an ABX system (Figure 4.8). Figure 4.8 shows the signals of the three carbon-bound protons of the 4,5-dihydro-1,3-oxazole compound (45 – 49). The geminal pair, H$_A$ and H$_B$, are diastereotopic; they are coupled to each other with the largest of the three coupling constants (~15 Hz) and they are each coupled to H$_X$, with different coupling constants, one large (~10 Hz) and one small (~8 Hz). The H$_X$ signal (~5.8 ppm) in compound (45 – 49) is downfield from the H$_A$ and H$_B$ signals (~4.5 and 4.0 ppm).
Figure 4.8. The signals of the three protons of the 4,5-dihydro-1,3-oxazole compound (45 – 49). The $^1$H n.m.r. spectrum of compound 45 is shown here.

Overall, the synthesis of the oxazole compounds (45 – 49) gave moderate yield after purification by column chromatography or recrystallisation from ethanol (Table 4.1). The synthesis of compound 47 is very low, this may due to the presence of the electron withdrawing nitro group which discouraged the ring closure reaction.

Table 4.1. Summary of the synthesis of 2-(substituted-benzofuran-2-yl)-5-phenyl-4,5-dihydro-1,3-oxazole.

<table>
<thead>
<tr>
<th>Product</th>
<th>Total reaction hours</th>
<th>Yield (%)</th>
<th>Melting point ($^\circ$ C)</th>
<th>Purification methods</th>
</tr>
</thead>
<tbody>
<tr>
<td>45</td>
<td>24</td>
<td>53</td>
<td>79 – 81</td>
<td>Recrystallisation</td>
</tr>
<tr>
<td>46</td>
<td>24</td>
<td>67</td>
<td>116 – 118</td>
<td>Column chromatography</td>
</tr>
<tr>
<td>47</td>
<td>24</td>
<td>16</td>
<td>159 – 161</td>
<td>Column chromatography</td>
</tr>
<tr>
<td>48</td>
<td>24</td>
<td>66</td>
<td>88 – 90</td>
<td>Recrystallisation</td>
</tr>
<tr>
<td>49</td>
<td>24</td>
<td>65</td>
<td>103 – 105</td>
<td>Recrystallisation</td>
</tr>
</tbody>
</table>
4.1.6 Synthesis of the substituted $N2$-[2-phenyl-2-(1H-1-imidazoyl)ethyl]-benzo[b]furan-2-carboxamide

In this last step of the reaction scheme, heating of the oxazole compound (45 – 49) in the presence of imidazole opened the oxazole ring by nucleophilic displacement (Scheme 4.9) (Wehrmeister, 1963).

A reasonable yield of compounds 51 to 55 was produced after purification by column chromatography and/or recrystallisation from ethanol/water (2:1 v/v) (Table 4.2).

Scheme 4.9. The synthesis of the substituted $N2$-[2-phenyl-2-(1H-imidazoyl)ethyl]-benzo[b]furan-2-carboxamide (51 – 55). Reaction conditions: imidazole, isopropyl acetate, 125 °C, 24 h, recrystallisation and/or column chromatography, 28 – 72 %.

Table 4.2. The summary of the synthesis of the substituted $N2$-[2-phenyl-2-(1H-imidazoyl)ethyl]-benzo[b]furan-2-carboxamide (51 – 55).

<table>
<thead>
<tr>
<th>Product</th>
<th>Total reaction hours</th>
<th>Yield (%)</th>
<th>Melting point (°C)</th>
<th>Purification methods</th>
</tr>
</thead>
<tbody>
<tr>
<td>51</td>
<td>24</td>
<td>28</td>
<td>180 – 182</td>
<td>Recrystallisation</td>
</tr>
<tr>
<td>52</td>
<td>24</td>
<td>66</td>
<td>110 – 112</td>
<td>Recrystallisation</td>
</tr>
<tr>
<td>53</td>
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<td>204 – 206</td>
<td>Column chromatography</td>
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<tr>
<td>54</td>
<td>24</td>
<td>73</td>
<td>198 – 200</td>
<td>Recrystallisation</td>
</tr>
<tr>
<td>55</td>
<td>24</td>
<td>72</td>
<td>76 – 78</td>
<td>Recrystallisation</td>
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</tbody>
</table>
4.1.7 Synthesis of the substituted N2-[2-phenyl-2-(1H-1,2,4-triazol-1-yl)ethyl]-benzo[b]furan-2-carboxamide and the substituted N2-[2-phenyl-2-(4H-1,2,4-triazol-4-yl)ethyl]-benzo[b]furan-2-carboxamide

The synthesis of the substituted N2-[2-phenyl-2-(1H-1,2,4-triazol-1-yl)ethyl]-benzo[b]furan-2-carboxamide Scheme 4.10 (i) and the substituted N2-[2-phenyl-2-(4H-1,2,4-triazol-4-yl)ethyl]-benzo[b]furan-2-carboxamide Scheme 4.10 (ii) was carried out in one reaction.

Scheme 4.10. The synthesis of the substituted N2-[2-phenyl-2-(1H-1,2,4-triazol-1-yl)ethyl]-benzo[b]furan-2-carboxamide (56, 58, 60, 62 and 64) (i) and the substituted N2-[2-phenyl-2-(4H-1,2,4-triazol-4-yl)ethyl]-benzo[b]furan-2-carboxamide (57, 59, 61, 63 and 65) (ii). Reaction conditions: 1,2,4-triazole, isopropyl acetate, 130 °C, 28 – 36 h.

The mixture of the substituted N2-[2-phenyl-2-(1H-1,2,4-triazol-1-yl)ethyl]-benzo[b]furan-2-carboxamide (56, 58, 60, 62 and 64) and the substituted N2-[2-phenyl-2-(4H-1,2,4-triazol-4-yl)ethyl]-benzo[b]furan-2-carboxamide (57, 59, 61, 63 and 65) was first recrystallised from toluene and then from methanol/ethanol or with ethanol/water to yield substituted N2-[2-phenyl-2-(4H-1,2,4-triazol-4-yl)ethyl]-benzo[b]furan-2-carboxamide (57, 59, 61, 63 and 65). The filtrate from the recrystallisation was purified by flash column chromatography to give the substituted N2-[2-phenyl-2-(1H-1,2,4-triazol-1-yl)ethyl]-benzo[b]furan-2-carboxamide (56, 58,
60, 62 and 64) [Table 4.3]. The very low yield of 60 and 61 is due to difficulty in purification of these products.

Table 4.3. The summary of the synthesis of the substituted N2-[2-phenyl-2-(1H-1,2,4-triazol-1-yl)ethyl]-benzo[b]furan-2-carboxamide (56, 58, 60, 62 and 64) and synthesis of the substituted N2-[2-phenyl-2-(4H-1,2,4-triazol-4-yl)ethyl]-benzo[b]furan-2-carboxamide (57, 59, 61, 63 and 65).

<table>
<thead>
<tr>
<th>Reactants</th>
<th>Products</th>
<th>Reaction temperature (°C)/Reaction time (hours)</th>
<th>Yield (%)</th>
<th>Ratio</th>
<th>Melting Point (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>45 and 1,2,4-triazole</td>
<td>56</td>
<td>130/30</td>
<td>32</td>
<td>3.5</td>
<td>126 – 128</td>
</tr>
<tr>
<td></td>
<td>57</td>
<td></td>
<td>9</td>
<td>1.0</td>
<td>276 – 278</td>
</tr>
<tr>
<td>46 and 1,2,4-triazole</td>
<td>58</td>
<td>130/28</td>
<td>33</td>
<td>2.3</td>
<td>164 – 166</td>
</tr>
<tr>
<td></td>
<td>59</td>
<td></td>
<td>14</td>
<td>1.0</td>
<td>218 – 220</td>
</tr>
<tr>
<td>47 and 1,2,4-triazole</td>
<td>60</td>
<td>130/36</td>
<td>6</td>
<td>1.5</td>
<td>60 – 62</td>
</tr>
<tr>
<td></td>
<td>61</td>
<td></td>
<td>4</td>
<td>1.0</td>
<td>125 – 127</td>
</tr>
<tr>
<td>48 and 1,2,4-triazole</td>
<td>62</td>
<td>130/30</td>
<td>22</td>
<td>1.8</td>
<td>174 – 176</td>
</tr>
<tr>
<td></td>
<td>63</td>
<td></td>
<td>12</td>
<td>1.0</td>
<td>252 – 254</td>
</tr>
<tr>
<td>49 and 1,2,4-triazole</td>
<td>64</td>
<td>130/30</td>
<td>28</td>
<td>2.3</td>
<td>200 – 202</td>
</tr>
<tr>
<td></td>
<td>65</td>
<td></td>
<td>12</td>
<td>1.0</td>
<td>221 – 223</td>
</tr>
</tbody>
</table>

The synthesis of compound 56 was tried using the method described in section 3.1.5, however this reaction was not successful (Scheme 4.11). A product with a higher R_f value compared with the starting material (39) was isolated by column chromatography. The 1H n.m.r. showed absence of the OH peak and the 13C n.m.r. gave the same number of carbon atoms as compound 39. The Electron Spray (ES^+) showed a molecular weight peak of 322.1. This could be the formation of N2-(2-chloro-2-phenylethyl)benzofuran-2-carboxamide (39a, 66 % yield) which gives a peak at 322.1 [M+Na] (Scheme 4.11) and microanalysis of compound 39a confirmed the structure of 39a. Microanalysis (C_{17}H_{14}ClNO_{2}): Calculated C = 68.12 %, H = 4.71

89
% N = 4.67 %; Found C = 68.00 %, H = 4.69 %, N = 4.69 %. The unreacted SOCl₂ in the mixture could have reacted with compound 39 to form the product 39a.

\[
\begin{align*}
\text{N} & \quad \text{Cl-SOCl} \\
\text{N} & \quad \text{Acetonitrile} \\
\text{N} & \quad 0 \degree \text{C, 1 hour} \\
\text{N} & \quad \text{Prepared in situ}
\end{align*}
\]

**Scheme 4.11.** Formation of compound 39a from compound 39 using the method described in section 3.1.5. Reaction conditions: 1,2,4-triazole, SOCl₂, acetonitrile, 10 \degree \text{C}, 1h then add K₂CO₃, compound 39, r.t., 24 h.
4.2 Experimental results for the synthesis of benzofuran-2-carboxamido ethyl-imidazole and -1,2,4-triazole derivatives

The numbering of compounds for n.m.r. characterisation is as follows:

All n.m.r. characterisations were made by comparison with previous n.m.r. spectra of the appropriate structure class and or predictions from ACD/HNMR and ACD/CNMR (Advanced Chemistry Development Inc., Version 2.51, 1997) and ChemDraw Ultra™ 7.0 (CambridgeSoft).
Synthesis of ethyl benzo[b]furan-2-carboxylate derivatives (compounds 21, 24 and 26)

Ethyl benzo[b]furan-2-carboxylate (21) (Suzuki et al., 1983)

(C11H10O3, MW: 190.197)

![Chemical Reaction](image)

Activated potassium carbonate (16.55 g, 119.75 mmol) was added to a stirred solution of salicylaldehyde (4) (7.31 g, 59.88 mmol) in anhydrous DMF (15 mL). Ethyl bromoacetate (20) (10.0 g, 59.88 mmol) in anhydrous DMF (20 mL) was then added to the above mixture and the reaction was stirred at 130 °C for 6 hours. The solvent was then evaporated to about 1/3 its volume to give a brown syrup. The crude product was extracted with CH2Cl2 (200 mL) and water (3 x 100 mL). The organic layer was dried with MgSO4, filtered and reduced in vacuo to give a yellow residue. The residue was purified by flash column chromatography (petroleum ether – ethyl acetate 100:0 v/v increasing to 95:5 v/v) to give ethyl benzo[b]furan-2-carboxylate (21) as a yellow solid. Yield: 6.0 g (53 %), t. l. c. system: petroleum ether – ethyl acetate 3:1 v/v, Rf: 0.71, stain negative. Melting point: 40 – 42 °C. [Suzuki et al., 1983 did not purify this compound prior next reaction, therefore, literature melting point is not available.]

1 H n.m.r. δ 7.70 (ddd, J = 0.5, 1.2, 8.4 Hz, 1H, H-4), 7.62 (dd, J = 0.8, 8.4 Hz, 1H, H-7), 7.55 (d, J = 0.9 Hz, 1H, H-3), 7.45 (m, 1H, H-6), 7.33 (m, 1H, H-5), 4.48 (q, 2H, J = 7.1 Hz, CH3), 1.50 (t, 3H, J = 7.1 Hz, CH3).

13 C n.m.r. δ 159.95 (C, C-7a), 156.09 (C=O), 146.13 (C, C-2), 127.95 (CH, CH-6), 127.37 (C, C-3a), 124.15 (CH, CH-5), 123.19 (CH, CH-4), 114.15 (CH, CH-3), 112.72 (CH, CH-7), 61.87 (CH2), 14.73 (CH3).
The following analogues of compound 21 were prepared using the same general method detailed above.

**Ethyl 6-methoxybenzo[b]furan-2-carboxylate (24)**

(C$_{12}$H$_{12}$O$_4$, MW: 220.223)

![Chemical structure of 24](image)

With ethyl 6-methoxybenzo[b]furan-2-carboxylate (24), yellow residue was obtained. Purification by flash column chromatography gave brown solid.

Yield: 6.6 g (53 %), t. l. c. system: petroleum ether – ethyl acetate 3:1 v/v, R$_f$: 0.55, stain positive. Microanalysis: Calculated C = 65.45 %, H = 5.49 %; Found C = 65.32 %, H = 5.53 %. Melting point: 66 - 68 °C.

1 H n.m.r. δ 7.63 (d, J = 8.7 Hz, 1H, H-4), 7.57 (d, J = 0.9 Hz, 1H, H-3), 7.17 (d, J = 1.8 Hz, 1H, H-7), 7.03 (dd, J = 2.2, 8.7 Hz, 1H, H-5), 4.53 (q, J = 7.1 Hz, 2H, CH$_2$), 3.97 (s, 3H, OCH$_3$), 1.52 (t, J = 7.1 Hz, 3H, CH$_3$).

13 C n.m.r. δ 160.93 (C, C-7a), 160.01 (C-6), 157.53 (C, C=O), 145.41 (C, C-2), 123.42 (CH, CH-4), 120.67 (C, C-3a), 114.45 (CH, CH-3), 114.39 (CH, CH-5), 96.16 (CH, CH-7), 61.67 (CH$_2$), 56.09 (CH$_3$, -OCH$_3$), 14.79 (CH$_3$).

I.R. (KBr diffusion): 1714 cm$^{-1}$ (ester).

**Ethyl 5-nitrobenzo[b]furan-2-carboxylate (26)**

(C$_{11}$H$_{9}$NO$_3$, MW: 235.194)

![Chemical structure of 26](image)

With ethyl 5-nitrobenzo[b]furan-2-carboxylate (26), a brown residue was obtained. Purification by flash column chromatography gave yellow solid.

Yield: 3.1 g (22 %), t. l. c. system: petroleum ether – ethyl acetate 4:1 v/v, R$_f$: 0.60, stain negative. Microanalysis: Calculated C = 56.17 %, H = 3.86 %, N = 5.95 %; Found C = 56.38 %, H = 3.90 %, N = 5.88 %. Melting point: 139 - 141 °C.
\[ ^1 \text{H n.m.r. } \delta \ 8.69 \ (d, J = 2.3 \text{ Hz, H-4}), \ 8.42 \ (dd, J = 2.3, 9.1 \text{ Hz, H-6}), \ 7.75 \ (d, J = 9.1 \text{ Hz, H-7}), \ 7.70 \ (s, 1H, H-3), \ 4.53 \ (q, 2H, J = 7.1 \text{ Hz, CH}_2), \ 1.50 \ (t, 3H, J = 7.1 \text{ Hz, CH}_3). \]

\[ ^{13} \text{C n.m.r. } \delta \ 159.04 \ (C, \ C-7a), \ 158.42 \ (C=O), \ 149.14 \ (C, \ C-2), \ 145.23 \ (C, \ C-5), \ 127.69 \ (C, \ C-3a), \ 123.34 \ (CH, \ CH-6), \ 119.89 \ (CH, \ CH-4), \ 114.32 \ (CH, \ CH-3), \ 113.39 \ (CH, \ CH-7), \ 62.56 \ (CH_2), \ 14.69 \ (CH_3). \]

I.R. (KBr diffusion): 1720 cm\(^{-1}\) (ester).

**Synthesis of ethyl benzo[b]furan-2-carboxylic acid derivatives (compounds 27 - 29)**

**Benzo[b]furan-2-carboxylic acid** (27) (Suzuki et al., 1983)

\[(C_9H_6O_3, \text{ MW: 162.143}) \]

\[
\begin{array}{c}
\text{R}_1 \quad \text{R}_2 \\
\end{array}
\]

\[
\begin{array}{c}
\text{2M NaOH, Methanol} \\
\text{Room temperature, 20 min} \\
\end{array}
\]

\[
\begin{array}{c}
\text{2M NaOH, Methanol} \\
\text{Room temperature, 20 min} \\
\end{array}
\]

\[
\begin{array}{c}
\text{R}_1 = \text{R}_2 = \text{H} \\
\text{R}_1 = \text{OCH}_3; \text{R}_2 = \text{H} \\
\text{R}_1 = \text{H}; \text{R}_2 = \text{NO}_2 \\
\end{array}
\]

A solution of ethyl benzo[b]furan-2-carboxylate (21) (6 g, 31.55 mmol) in methanol (100 mL) was treated with 2M aqueous sodium hydroxide (50 mL) and warmed gently on a steam bath. Progress of the reaction was monitored by observing the disappearance of ethyl benzo[b]furan-2-carboxylate by t. l. c. After hydrolysis was complete (ca. 30 min.), the reaction solution was cooled and acidified to pH 1 by the dropwise addition of concentrated HCl. The solution was then extracted with diethyl ether (3 x 150 mL) and the organic layer dried with MgSO\(_4\), filtered and reduced *in vacuo* to give a crude yellow solid. The crude yellow solid was washed with water, collected and dried under vacuum with phosphorus pentoxide to give pure benzo[b]furan-2-carboxylic acid (27). Yield: 5.0 g (98%), t. l. c. system: petroleum ether – ethyl acetate 1:1 v/v, \( R_F \): 0.0, stain negative. Melting point: 190 – 192 °C

[1] H n.m.r. (Acetone-d\(_6\) \( \delta \ 7.73 \) (split d, J = 0.7, 7.9 Hz, H-4), 7.58 (split d, J = 1.7, 7.6, 1H, H-7), 7.57 (d, J = 0.8 Hz, H-3), 7.49 (m, 1H, H-6), 7.32 (m, 1H, H-5).

[13] C n.m.r. \( \delta \ 159.04 \) (C, C-7a), 158.42 (C=O), 149.14 (C, C-2), 145.23 (C, C-5), 127.69 (C, C-3a), 123.34 (CH, CH-6), 119.89 (CH, CH-4), 114.32 (CH, CH-3), 113.39 (CH, CH-7), 62.56 (CH\(_2\)), 14.69 (CH\(_3\)).
$^{13}$C n.m.r. (Acetone-$d_6$) $\delta$ 162.94 (C=O), 157.53 (C, C-7a), 147.78 (C, C-2), 129.15 (CH, CH-6), 128.88 (C, C-3a), 125.31 (CH, CH-5), 124.45 (CH, CH-4), 115.28 (CH, CH-3), 113.35 (CH, CH-7).

I.R. (KBr diffusion): 1670 and 940 cm$^{-1}$ (-COOH). [Literature found: 1680 and 943 cm$^{-1}$ (Suzuki et al., 1983)].

The following analogues of compound 27 were prepared using the same general method detailed above.

**6-Methoxybenzo[b]furan-2-carboxylic acid (28)**

(C$_{10}$H$_8$O$_4$, MW: 192.169)

![Methoxybenzo[b]furan-2-carboxylic acid](image)

With 6-methoxybenzo[b]furan-2-carboxylic acid (33), a yellow solid was obtained after drying under vacuum at 80 °C.

Yield: 5.4 g (88 %), t. l. c. system: petroleum ether – ethyl acetate 1:1 v/v, $R_F$: 0.0, stain positive. Microanalysis: Calculated C = 62.50 %, H = 4.20 %; Found C = 62.49 %, H = 4.19 %. Melting point: 194 – 196 °C.

$^1$H n.m.r. (DMSO-$d_6$) $\delta$ 13.36 (s, 1H, COOH), 7.67 (d, $J = 8.7$ Hz, 1H, H-4), 7.61 (s, 1H, H-3), 7.31 (s, 1H, H-7), 6.99 (split d, $J = 2.1$, 8.6 Hz, H-5), 3.85 (s, 3H, OCH$_3$).

$^{13}$C n.m.r. (DMSO-$d_6$) $\delta$ 160.41 (C=O and C, C-7a), 156.85 (C, C-6), 145.64 (C, C-2), 123.70 (CH, CH-4), 120.32 (C, C3a), 114.17 (CH, CH-3), 114.13 (CH, CH-5), 96.22 (CH, CH-7), 56.08 (CH$_3$, -OCH$_3$).

I.R. (KBr diffusion): 1689 and 930 cm$^{-1}$ (-COOH).

**5-Nitrobenzo[b]furan-2-carboxylic acid (29)**

(C$_9$H$_5$NO$_3$, MW: 207.140)

![Nitrobenzo[b]furan-2-carboxylic acid](image)

With 5-nitrobenzo[b]furan-2-carboxylic acid (29), yellow solid was obtained after dried under vacuum at 80 °C.
Yield: 2.5 g (95 %), t. l. c. system: petroleum ether – ethyl acetate 1:1 v/v, R<sub>f</sub>: 0.0, stain positive. Microanalysis: Calculated C = 52.19 %, H = 2.43 %, N = 6.76 %; Found C = 52.30 %, H = 2.43 %, N = 6.68 %. Melting point: 268 – 270 °C.

1 H n.m.r. (DMSO-d<sub>6</sub>) δ 14.00 (s, 1H, COOH), 8.75 (d, J = 2.2 Hz, 1H, H-4), 8.36 (dd, J = 2.2, 9.2 Hz, 1H, H-4), 7.97 (d, J = 9.2 Hz, 1H, H-7), 7.85 (s, 1H, H-3).

13 C n.m.r. (DMSO-d<sub>6</sub>) δ 159.79 (C, C-7a), 157.90 (C=O), 149.40 (C, C-5), 144.54 (C, C-2), 127.77 (C, C-3a), 123.06 (CH, CH-6), 120.18 (CH, CH-4), 114.49 (CH, CH-3), 113.58 (CH, CH-7).

1-(4-Fluorophenyl)-2-nitro-1-ethanol (32)

(C<sub>8</sub>H<sub>6</sub>FN<sub>3</sub>O, MW: 185.153)

\[
\text{HO} \quad \begin{array}{c}
\text{F} \\
\text{H} \\
\end{array} \quad \text{CH}_3 \\
\text{O} \quad \text{N} \\
\begin{array}{c}
\text{F} \\
\text{H} \\
\end{array}
\]

\[
\begin{array}{c}
\text{Acetic acid 2 %v/v, methanol} \\
0°C, 8 h \\
\end{array}
\]

\[
\text{HO} \quad \begin{array}{c}
\text{F} \\
\text{H} \\
\text{CH}_3 \\
\text{O} \quad \text{N} \\
\begin{array}{c}
\text{F} \\
\text{H} \\
\end{array}
\]

Nitromethane (31) (9.84 g, 161.15 mmol) and aqueous NaOH (10M, 80.57 mmol) were added to a solution of 4-fluorobenzaldehyde (30) (10.0 g, 80.57 mmol) in methanol (150 mL). The resulting solution was stirred at 0 °C for 8 h. Aqueous acetic acid (2 % v/v, 80.57 mmol) was added to the above reaction mixture and was stirred at room temperature for another 30 min. The methanol was removed under vacuum and the aqueous phase extracted with CH<sub>2</sub>Cl<sub>2</sub> (2 x 150 mL). The combined organic layers were washed with brine (2 x 100 mL), dried with MgSO<sub>4</sub>, filtered and reduced in vacuo to give a brown residue. The residue was purified by flash column chromatography (petroleum ether – ethyl acetate 100:0 v/v increasing to 85:15 v/v) to give 1-(4-fluorophenyl)-2-nitro-1-ethanol (32) as a brown syrup. Yield: 8.0 g (54 %), t. l. c. system: petroleum ether – ethyl acetate 4:1 v/v, R<sub>f</sub>: 0.33, stain positive.

1 H n.m.r. δ 7.44 (m, 2H, H-2 and H-6), 7.15 (m, 2H, H-3 and H-5), 5.51 (m, 1H, CH-OH), 4.64 (dd, J = 9.3, 13.3 Hz, 1H, CHHNO<sub>2</sub>), 4.54 (dd, J = 3.4, 13.3 Hz, 1H, CHHNO<sub>2</sub>), 3.10 (d, J = 3.4 Hz, 1H, OH). [Literature 1 H n.m.r. from Watanabe et al. (Watanabe et al., 2002)].

13 C n.m.r. δ 164.98 and 161.70 (C, C-4), 134.40 and 134.36 (C, C-1), 128.28 and 128.17 (CH, CH-2 and CH-6), 116.58 and 116.29 (CH, CH-3 and CH-5), 81.56 (CH<sub>2</sub>), 70.77 (CH-OH).
1-(4-Chlorophenyl)-2-nitro-1-ethanol (34)

(C₈H₅ClNO₃, MW: 201.608)

\[
\begin{align*}
\text{(33)} & \quad \text{Acetic acid 2 %v/v, methanol} \\
& \quad 0^\circ \text{C, 8 h} \\
\end{align*}
\]

Nitromethane (17.37 g, 284.56 mmol) and aqueous NaOH (10M, 142.28 mmol) were added to a solution of 4-chlorobenzaldehyde (20.0 g, 142.28 mmol) in methanol (150 mL). The resulting solution was stirred at 0 °C for 8 h. Aqueous acetic acid (2 % v/v, 142.28 mmol) was added to the above reaction mixture and was stirred at room temperature for another 30 min. The methanol was removed under vacuum and the aqueous phase extracted with CH₂Cl₂ (2 x 150 mL). The combined organic layers were washed with brine (2 x 100 mL), dried with MgSO₄, filtered and reduced \textit{in vacuo} to give a brown residue. The residue was purified by flash column chromatography (petroleum ether - ethyl acetate 100:0 v/v increasing to 85:15 v/v) to give 1-(4-chlorophenyl)-2-nitro-1-ethanol as a yellow syrup. Yield: 21.23 g (74 %), t. l. c. system: petroleum ether - ethyl acetate 4:1 v/v, Rₜ: 0.34, stain positive.

\[\text{H n.m.r.} \delta 7.50 - 7.42 \text{ (m, 4H, Ar), 5.48 (m, 1H, CH-OH), 4.68 (dd, J = 9.3, 13.3 Hz, 1H, CHHNO₂), 4.59 (dd, J = 3.6, 13.3 Hz, 1H, CHHNO₂), 3.31 (d, J = 3.6 Hz, 1H, OH). [Literature H n.m.r. from Choudary et al. (Choudary et al., 2001)].}\]

\[\text{C n.m.r.} \delta 137.05 \text{ (C, C-1), 135.22 (C, C-4), 129.64 (2 x CH, CH-2 and CH-6), 127.80 (2 x CH, CH-3 and CH-5), 81.43 (CH₂), 70.74 (CH-OH).}\]

2-Amino-1-(4-fluorophenyl)-1-ethanol (35)

(C₈H₁₀FNO, MW: 155.171)

\[
\begin{align*}
\text{(32)} & \quad \text{Formic acid 50 %v/v, Raney nickel, H₂} \\
& \quad \text{room temperature, 24 h} \\
\end{align*}
\]

Raney nickel (50 % slurry in H₂O, 6 mL) was added to a solution of 1-(4-fluorophenyl)-2-nitro-1-ethanol (32) (7.0 g, 37.83 mmol) in methanol (100 mL) and aqueous formic acid (50 % v/v, 8 mL). The reaction flask was then degassed and
purged with hydrogen. The reaction was carried out under 40 psi H$_2$ atmosphere using a Paar hydrogenator. The reaction flask was shaken at room temperature for 24 hours until all starting material had been consumed. After removal of hydrogen the reaction mixture was filtered, and the methanol was removed *in vacuo*. The aqueous residue was made alkaline with NH$_4$OH (28 %) and extracted with ethyl acetate (2 x 100 mL). The organic layer was then washed with brine (2 x 50 mL), dried with MgSO$_4$, filtered and reduced *in vacuo* to give 2-amino-1-(4-fluorophenyl)-1-ethanol (35) as a green residue. The residue was purified by flash column chromatography (dichloromethane – methanol - triethylamine 100:0:0.05 v/v increasing to 95:5:0.05 v/v) to give the product as a yellow solid. Yield: 2.60 g (44 %), t. l. c. system: dichloromethane – methanol 9:1 v/v, R$_F$: 0.08, stain positive. Melting point: 59 - 61 °C [Literature: 63 – 65 °C (Cho et al., 2002)].

$^1$H n.m.r. (DMSO-$d_6$) δ 7.37 (m, 2H, Ar), 7.14 (m, 2H, Ar), 4.87 (dd, J = 4.5, 7.3 Hz, 1H, CH-OH), 2.76 (dd, J = 4.3, 12.8 Hz, 1H, CH$_2$NH$_2$), 2.63 (dd, J = 7.6, 12.8 Hz, 1H, CH$_3$NH$_2$), 2.18 (brs, 3H, NH$_2$ and OH).

$^{13}$C n.m.r. (DMSO-$d_6$) δ 163.09 and 159.89 (C, C-4), 140.97 and 140.93 (C, C-1), 128.18 and 128.08 (CH, CH-2 and CH-6), 115.07 and 114.79 (CH, CH-3 and CH-5), 74.18 (CH-OH), 50.52 (CH$_2$).

I.R. (KBr diffusion): 3352, 2998, 2886, 1609, 1499 cm$^{-1}$. [Literature: 3357, 2998, 2878, 1603, 1501 cm$^{-1}$ (Cho et al., 2002)].

**2-Amino-1-(4-chlorophenyl)-1-ethanol (36)**

(C$_8$H$_{10}$ClNO, MW: 171.626)

![Chemical structure of 2-Amino-1-(4-chlorophenyl)-1-ethanol](image)

Raney nickel (50 % slurry in H$_2$O, 8 mL) was added to a solution of 1-(4-chlorophenyl)-2-nitro-1-ethanol (34) (13.84 g, 68.65 mmol) in methanol (100 mL) and aqueous formic acid (50 % v/v, 10 mL). The reaction flask was then degassed and purged with hydrogen. The reaction was carried out under 40 psi H$_2$ atmosphere using a Paar hydrogenator. The reaction flask was shaken at room temperature for 24 hours
until all starting material had been consumed. After removal of hydrogen the reaction mixture was filtered, and the methanol was removed in vacuo. The aqueous residue was made alkaline with NH$_4$OH (28%) and extracted with ethyl acetate (2 x 100 mL). The organic layer was then washed with brine (2 x 50 mL), dried with MgSO$_4$, filtered and reduced in vacuo to give a brown residue. The residue was purified by flash column chromatography (dichloromethane - methanol - triethylamine 100:0:0.05 v/v increasing to 95:5:0.05 v/v) to give 2-amino-1-(4-chlorophenyl)-1-ethanol (36) as a white solid. Yield: 5.40 g (46%), t. l. c. system: dichloromethane - methanol 9:1 v/v, R$_f$: 0.10, stain positive. Melting point: 93 - 95 °C [Literature: 95 – 97 °C (Cho et al., 2002)].

$^1$H n.m.r. (DMSO-$d_6$) δ 7.41 (m, 4H, Ar), 4.51 (dd, J = 4.4, 7.4 Hz, 1H, CH-OH), 2.71 (dd, J = 4.4, 12.9 Hz, 1H, CH$_2$NH$_2$), 2.61 (dd, J = 7.5, 12.9 Hz, 1H, CH$_2$NH$_2$), 2.20 (brs, 3H, NH$_2$ and OH).

$^{13}$C n.m.r. (DMSO-$d_6$) δ 143.83 (C, C-1), 131.50 (C, C-4), 128.20 (2 x CH, CH-2 and CH-6), 128.16 (2 x CH, CH-3 and CH-5), 74.19 (CH-OH), 50.46 (CH$_2$).

I.R. (KBr diffusion): 3348, 3236, 3104, 2975, 2834, 1611, 1493 cm$^{-1}$. [Literature: 3357, 3214, 3104, 2964, 2853, 1595, 1491 cm$^{-1}$ (Cho et al., 2002)].

**Synthesis of N2-(2-hydroxy-2-phenylethyl)benzo[b]furan-2-carboxamide derivatives** (compounds 39 – 43)

**N2-(2-Hydroxy-2-phenylethyl)benzo[b]furan-2-carboxamide** (39)

(C$_{17}$H$_{15}$NO$_3$, MW: 281.309)

\[\text{R}_1 = \text{R}_2 = \text{H} \]
\[\text{R}_1 = \text{OCH}_3; \text{R}_2 = \text{H} \]
\[\text{R}_1 = \text{H}; \text{R}_2 = \text{NO}_2 \]

To a suspension of benzo[b]furan-2-carboxylic acid (27) (4.50 g, 27.75 mmol) in anhydrous DMF (30 mL) was added of 1,1’-carboxyl-diimidazole (4.50 g, 27.75
mmol) and the reaction stirred at room temperature for 1 hr. The reaction was cooled to 0 °C and subsequently combined with a solution of 2-amino-1-phenyl-ethanol (3.81 g, 27.75 mmol) in DMF (10 mL). The mixture was stirred at room temperature for 8 h. After the reaction was complete, an aliquot amount of ice was poured into the flask to precipitate out the product which was then filtered and washed with 10 mL of ethanol to give the white solid, N-(2-hydroxy-2-phenylethyl)benzo[b]furan-2-carboxamide (39). The product was then collected and dried under vacuum with phosphorus pentoxide. Yield: 5.63 g (72 %), t. l. c. system: petroleum ether – ethyl acetate 3:2 v/v, \( R_f \): 0.45, stain positive. Microanalysis: Theory C = 72.58 %, H = 5.37 %, N = 4.98 %; Found C = 72.44 %, H = 5.32 %, N = 4.88 %. Melting point: 140 - 142 °C.

\(^1\)H n.m.r. (DMSO-\(d_6\)) \( \delta \) 8.67 (t, \( J = 5.6 \) Hz, 1H, NH), 7.78 (d, \( J = 7.7 \) Hz, 1H, Ar), 7.67 (d, \( J = 8.3 \) Hz, 1H, Ar), 7.56 (s, 1H, H-3), 7.47 (m, 1H, Ar), 7.41-7.24 (m, 5H, Ar), 5.60 (d, \( J = 4.4 \) Hz, 1H, OH), 4.83 (m, 1H, H-1), 3.58-3.34 (m, 2H, CH\(_2\)).

\(^{13}\)C n.m.r. (DMSO-\(d_6\)) \( \delta \) 158.53 (C=O), 154.54 (C, C-7a), 149.51 (C, C-2), 143.89 (C, C-1'), 128.41 (2 x CH, CH-3' and CH-5'), 127.53 (C, C-3a), 127.45 (CH, CH-4'), 127.10 (CH, CH-6), 126.35 (2 x CH, CH-2' and CH-6'), 124.02 (CH, CH-5), 123.08 (CH, CH-4), 112.12 (CH, CH-7), 109.66 (CH, CH-3), 71.28 (CH, CH1-OH), 47.35 (CH\(_2\)).

The following analogues of compound 39 were prepared using the same general method detailed above.

**N2-(2-Hydroxy-2-phenylethyl)-6-methoxybenzo[b]furan-2-carboxamide (40)**

(C\(_{18}\)H\(_{17}\)NO\(_4\), MW: 311.335)

![N2-(2-Hydroxy-2-phenylethyl)-6-methoxybenzo[b]furan-2-carboxamide (40)](image)

With N2-(2-hydroxy-2-phenylethyl)-6-methoxybenzo[b]furan-2-carboxamide (40), a light brown powder was obtained after drying under vacuum at 80 °C.

Yield: 6.38 g (88 %), t. l. c. system: petroleum ether – ethyl acetate 3:2 v/v, \( R_f \): 0.40, stain positive. Microanalysis: Theory C = 69.44 %, H = 5.50 %, N = 4.50 %; Found C = 69.47 %, H = 5.46 %, N = 4.37 %. Melting point: 147 - 149 °C.
\(^1\) H n.m.r. (DMSO-\(d_6\)) \(\delta\) 8.45 (t, \(J = 5.7\) Hz, 1H, NH), 7.62 (d, \(J = 8.7\) Hz, 1H, Ar), 7.46 (d, \(J = 0.8\) Hz, 1H, H-3), 7.39-7.30 (m, 4H, Ar), 7.27-7.20 (m, 2H, Ar), 6.95 (dd, \(J = 2.2, 8.6\) Hz, 1H, Ar), 5.58 (d, \(J = 4.4\) Hz, 1H, OH), 4.79 (m, 1H, H-1), 3.82 (s, 3H, OCH\(_3\)), 3.55-3.30 (m, 2H, CH\(_2\)).

\(^{13}\) C n.m.r. (DMSO-\(d_6\)) \(\delta\) 159.77 (C, C-6), 158.62 (C=O), 155.89 (C, C-7a), 148.73 (C, C-2), 143.91 (C, C-1'), 128.44 (2 x CH, CH-3' and CH-5'), 127.47 (CH, CH-4'), 126.37 (2 x CH, CH-2' and CH-6'), 123.34 (CH, CH-4), 120.65 (C, C-3a), 113.54 (CH, CH-5), 109.83 (CH, CH-3), 96.25 (CH, CH-7), 71.46 (CH, CH1-OH), 56.05 (OCH\(_3\)), 47.29 (CH\(_2\)).

**N2-(2-Hydroxy-2-phenylethyl)-5-nitrobenzo[b]furan-2-carboxamide (41)**

\(\text{C}_{17}\text{H}_{18}\text{N}_2\text{O}_5, \text{MW: 326.306}\)

With N2-(2-hydroxy-2-phenylethyl)-5-nitrobenzo[b]furan-2-carboxamide (41), a light yellow solid was obtained after drying under vacuum at 80 °C.

Yield: 3.47 g (88 %), t. l. c. system: petroleum ether – ethyl acetate 3:2 v/v, \(R_f\): 0.59, stain positive. Microanalysis: Theory C = 62.57 %, H = 4.32 %, N = 8.58 %; Found C = 62.42 %, H = 4.21 %, N = 8.59 %. Melting point: 174 - 176 °C.

\(^1\) H n.m.r. (DMSO-\(d_6\)) \(\delta\) 8.92 (t, \(J = 5.6\) Hz, 1H, NH), 8.80 (d, \(J = 2.1\) Hz, 1H, H-4), 8.35 (dd, \(J = 2.0, 9.1\) Hz, 1H, H-6), 7.93 (d, \(J = 9.1\) Hz, 1H, H-7), 7.79 (s, 1H, H-3), 7.44-7.26 (m, 5H, Ar), 5.62 (d, \(J = 2.9\) Hz, 1H, OH), 4.85 (m, 1H, H-1), 3.59-3.37 (m, 2H, CH\(_2\)).

\(^{13}\) C n.m.r. (DMSO-\(d_6\)) \(\delta\) 157.80 (C, C=O), 157.23 (C, C-7a), 152.26 (C, C-2), 144.51 (C, C-5), 143.81 (C, C-1'), 128.46 (2 x CH, CH-3' and CH-5'), 128.14 (C, C-3a), 127.51 (CH, CH-4'), 126.37 (2 x CH, CH-2' and CH-6'), 122.44 (CH, CH-4), 119.87 (CH, CH-6), 113.21 (CH, CH-7), 110.42 (CH, CH-3), 71.31 (CH, CH1-OH), 47.47 (CH\(_2\)).
N2-[2-(4-Fluorophenyl)-2-hydroxyethyl]benzo[b]furan-2-carboxamide (42)

(C_{17}H_{14}FNO_{3}, MW: 299.300)

With N2-[2-(4-fluorophenyl)-2-hydroxyethyl]benzo[b]furan-2-carboxamide (42), a white solid was obtained after drying under vacuum at 80 °C.

Yield: 3.60 g (85 %), t. l. c. system: petroleum ether – ethyl acetate 3:2 v/v, R_{f}: 0.41, stain positive. Microanalysis: Theory C = 68.22 %, H = 4.71 %, N = 4.68 %; Found C = 68.27 %, H = 4.67 %, N = 4.66 %. Melting point: 172 - 174 °C.

1 H n.m.r. (DMSO-\textit{d}_{6}) \delta 8.69 (t, J = 5.7 Hz, 1H, NH), 7.79 (d, J = 7.7 Hz, 1H, Ar), 7.68 (d, J = 8.3 Hz, 1H, Ar), 7.58 (s, 1H, Ar), 7.51 - 7.27 (m, 4H, Ar), 7.18 (t, J = 8.9 Hz, 2H, Ar), 5.68 (d, J = 4.5 Hz, 1H, OH), 4.85 (m, 1H, CH-OH), 3.57-3.37 (m, 2H, CH_{2}).

13 C n.m.r. (DMSO-\textit{d}_{6}) \delta 163.31 (C=O), 160.11 and 158.57 (C, C-4'), 154.56 (C, C-7a), 149.48 (C, C-2), 140.06 and 140.02 (C, C-1'), 128.37 and 128.26 (2 x CH, CH-2' and CH-6'), 127.54 (C, C-3a), 127.14 (CH, CH-6), 124.05 (CH, CH-5), 123.10 (CH, CH-4), 115.28 and 114.99 (2 CH, CH-3' and CH-5'), 112.15 (CH, CH-3), 109.74 (CH, CH-7), 70.78 (CH, CH1-OH), 47.24 (CH_{2}).

N2-[2-(4-Chlorophenyl)-2-hydroxyethyl]benzo[b]furan-2-carboxamide (43)

(C_{17}H_{14}ClNO_{3}, MW: 315.754)

With N2-[2-(4-chlorophenyl)-2-hydroxyethyl]benzo[b]furan-2-carboxamide (43), a white solid was obtained after drying under vacuum at 80 °C.

Yield: 3.25 g (84 %), t. l. c. system: petroleum ether – ethyl acetate 3:2 v/v, R_{f}: 0.47, stain positive. Microanalysis: Theory C = 64.67 %, H = 4.47 %, N = 4.43 %; Found C = 64.58 %, H = 4.51 %, N = 4.44 %. Melting point: 189 - 191 °C.
1 H n.m.r. (DMSO-d$_6$) 8.69 (t, J = 5.7 Hz, 1H, NH), 7.79 (d, J = 7.8 Hz, 1H, Ar), 7.68 (d, J = 8.3 Hz, 1H, Ar), 7.57 (s, 1H, Ar), 7.51–7.33 (m, 6H, Ar), 5.71 (d, J = 4.5 Hz, 1H, OH), 4.84 (ddd, J = 4.9, 7.2, 12.1 Hz, 1H, CH-OH), 3.56-3.37 (m, 2H, CH$_2$).

13 C n.m.r. (DMSO-d$_6$) 158.56 (C=O), 154.56 (C, C-7a), 149.45 (C, C-2), 142.88 (C, C-1'), 128.39 (2 x CH, CH-3' and CH-5'), 128.30 (2 x CH, CH-2' and CH-6'), 127.53 (C, C-3a), 127.15 (CH, CH-6), 124.05 (CH, CH-5), 123.11 (CH, CH-4), 112.15 (CH, CH-7), 109.75 (CH, CH-3), 70.75 (CH, CH$_1$-OH), 47.11 (CH$_2$).

**Synthesis of 2-benzo[b]furan-2-yl-5-phenyl-4,5-dihydro-1,3-oxazole derivatives** (compounds 45 – 49)

**2-Benzofuran-2-yl-5-phenyl-4,5-dihydro-1,3-oxazole** (45)

(C$_{17}$H$_{13}$NO$_2$, MW: 263.294)

![Chemical Structure of 2-Benzofuran-2-yl-5-phenyl-4,5-dihydro-1,3-oxazole (45)]

1) Methanesulphonic anhydride, Triethylamine, THF, 0 °C, 24 hours
2) NH$_3$ (aq.) 28%, room temp, 15 minutes

To a solution of N-(2-hydroxy-2-phenylethyl)benzo[b]furan-2-carboxamide (39) (2.0 g, 7.11 mmol) in anhydrous THF (15 mL) was added methanesulphonic anhydride (1.86 g, 10.66 mmol) dissolved in anhydrous THF (5 mL). The homogenous mixture was stirred at 0 °C for 15 minutes, then triethylamine (3.0 mL, 21.33 mmol) was added dropwise to the above mixture. After keeping the homogenous mixture in the fridge at 0 °C for 24 hours the reaction went to completion. The mixture was quenched by the addition of aqueous ammonia solution (28 %, 1 mL). After stirring at room temperature for 15 minutes the mixture was concentrated *in vacuo* and finally...
distributed between ethyl acetate (150 mL) and aqueous NaHCO₃ solution (2 x 100 mL). After repeated extraction the organic phases were dried and evaporated in vacuo. Purification of the resulting residue by recrystallisation from ethanol yielded 2-benzo[b]furan-2-yl-5-phenyl-4,5-dihydro-1,3-oxazole (45) (1.0 g, 54 %) as a yellow solid, t. l. c. system: petroleum ether – ethyl acetate 1:1 v/v, RF: 0.57, stain positive. LRMS (ES⁺) m/z: 286.1 (M+Na)⁺. Microanalysis (C₁₇H₁₃NO₂.0.1H₂O): Calculated C = 77.02 %, H = 5.02 %, N = 5.28 %; Found C = 77.05 %, H = 4.95 %, N = 5.25 %.

Melting point: 79 – 81 °C.

¹H n.m.r. δ 7.71 (d, J = 7.8 Hz, 1H, Ar), 7.45 (d, J = 8.4 Hz, 1H, Ar), 7.49-7.32 (m, 8H, Ar), 5.75 (dd, J₅‴.₄‴a = 8.1 Hz, J₅‴.₄‴b = 10.0 Hz, 1H, CH₅–CH₄‴.₄‴b), 4.60 (dd, J₄‴.₅‴b = 10.1 Hz, J₄‴.₄‴a = 15.1 Hz, 1H, CH₅–CH₄‴.₄‴aCH₄‴b), 4.14 (dd, J₄‴.₅‴b = 8.0 Hz, J₄‴.₄‴b = 15.1 Hz, 1H, CH₅–CH₄‴.₄‴b).

¹³C n.m.r. δ 157.28 (C, C-2‴″), 156.15 (C, C-7a), 144.59 (C, C-2), 140.69 (C, C-1‴), 129.33 (2 x CH, CH-3‴ and CH-5‴), 129.00 (CH, CH-4‴), 127.75 (C, C-3a), 127.15 (CH, CH-6), 126.32 (2 x CH, CH-2‴ and CH-6‴), 124.04 (CH, CH-5), 122.68 (CH, CH-4), 112.52 (CH, CH-7), 111.19 (CH, CH-3), 82.08 (CH-CH-5‴″), 63.60 (CH₂).

The following analogues of compound 45 were prepared using the same general method detailed above.

2-(6-Methoxybenzo[b]furan-2-yl)-5-phenyl-4,5-dihydro-1,3-oxazole (46)
(C₁₈H₁₅NO₃, MW: 293.320)

![Structure](image)

With 2-(6-methoxybenzo[b]furan-2-yl)-5-phenyl-4,5-dihydro-1,3-oxazole (46), a light yellow solid was obtained after purification by flash column chromatography (petroleum ether – ethyl acetate 90:10 v/v increasing to 50:50 v/v).

Yield: 3.17 g (67 %). t. l. c. system: petroleum ether – ethyl acetate 3:2 v/v, RF: 0.30, stain positive. Microanalysis: Calculated C = 73.71 %, H = 5.15 %, N = 4.77 %; Found C = 73.52 %, H = 5.03 %, N = 4.72 %. Melting point: 116 – 118 °C.
1 H n.m.r. δ 7.65 (d, J = 8.7 Hz, 1H, Ar), 7.50 (d, J = 0.5 Hz, 1H, Ar), 7.47-7.33 (m, 6H, Ar), 7.00 (d, J = 2.2, 8.7 Hz, 1H, Ar), 5.82 (dd, J₅''ₜ,₄''ₘ = 7.5 Hz, J₅''ₜ,₄''ₜ = 10.0 Hz, 1H, CH₅''ₜ-CH₄''ₕCH₄''ₘₘ; 4.49 (dd, J₄''ₘₗ,₄''ₜ = 10.0 Hz, J₄''ₘₗ,₄''ₗ = 15.1 Hz, 1H, CH₅''ₜ-CH₄''ₕCH₄''ₘₘ; 3.89 (dd, J₄''ₗₚ,₄''ₗ = 7.5 Hz, J₄''ₗₚ,₄''ₗ = 15.1 Hz, 1H, CH₅''ₜ-CH₄''ₕCH₄''ₘₘ; 3.86 (s, 3H, OCH₃).

C n.m.r. δ 159.90 (C, C-6), 156.71 (C, C-2''), 155.67 (C, C-7a), 143.35 (C, C-2), 141.07 (C, C-1'), 129.15 (2 x CH, CH-3' and CH-5'), 128.65 (CH, CH-4'), 126.15 (2 x CH-2' and CH-6'), 123.14 (CH, CH-4), 120.48 (C, C-3a), 113.81 (CH, CH-5), 111.35 (CH, CH-3), 96.26 (CH, CH-7), 80.64 (CH- CH-5''), 62.93 (CH₂), 56.08 (CH₃).

2-(5-Nitrobenzo[b]furan-2-yl)-5-phenyl-4,5-dihydro-1,3-oxazole (47)

(C₁₇H₁₂N₂O₄, MW: 308.291)

With 2-(5-nitrobenzo[b]furan-2-yl)-5-phenyl-4,5-dihydro-1,3-oxazole (47), a white solid was obtained after purification by flash column chromatography (petroleum ether – ethyl acetate 90:10 v/v increasing to 60:40 v/v).

Yield: 0.50 g (16 %), t. l. c. system: petroleum ether – ethyl acetate 3:2 v/v, Rf: 0.30, stain positive. Microanalysis: Calculated C = 66.23 %, H = 3.92 %, N = 9.08 %; Found C = 66.09 %, H = 3.89 %, N = 9.06 %. Melting point: 159 – 161 °C.

1 H n.m.r. (DMSO-d₆) δ 8.75 (d, J = 2.3 Hz, H-4), 8.36 (dd, J = 2.4, 9.1 Hz, H-6), 7.99 (d, J = 9.1 Hz, 1H, H-7), 7.79 (s, 1H, H-3), 7.48 – 7.36 (m, 5H, Ar), 5.89 (dd, J₅''ₜ,₄''ₘ = 7.8 Hz, J₅''ₜ,₄''ₜ = 10.0 Hz, 1H, CH₅''ₜ-CH₄''ₕCH₄''ₘₘ; 4.55 (dd, J₄''ₘₗ,₄''ₜ = 10.0 Hz, J₄''ₘₗ,₄''ₗ = 15.5 Hz, 1H, CH₅''ₜ-CH₄''ₕCH₄''ₘₘ; 3.96 (dd, J₄''ₗₚ,₄''ₗ = 7.8 Hz, J₄''ₗₚ,₄''ₗ = 15.5 Hz, 1H, CH₅''ₜ-CH₄''ₕCH₄''ₘₘ).

13 C n.m.r. (DMSO-d₆) δ 157.94 (C, C-2''), 155.03 (C, C-7a), 147.08 (C, C-5), 144.64 (C, C-1'), 140.64 (C, C-2), 129.20 (2 x CH, CH-2' and CH-6'), 128.83 (CH, CH-4'), 128.04 (C, C-3a), 126.30 (2 x CH, CH-3' and CH-5'), 122.62 (CH, CH-6), 119.59
(CH, CH-4), 113.27 (CH, CH-3), 111.96 (CH, CH-7), 81.24 (CH, CH-1), 62.94 (CH₂).

**2-Benzof[b]furan-2-yl-5-(4-fluorophenyl)-4,5-dihydro-1,3-oxazole (48)**

(C₁₇H₁₂FNO₂, MW: 281.285)

![Chemical Structure](48)

With 2-benzo[b]furan-2-yl-5-(4-fluorophenyl)-4,5-dihydro-1,3-oxazole (48), a yellow solid was obtained after recrystallisation from ethanol.

Yield: 2.0 g (66 %), t. l. c. system: petroleum ether – ethyl acetate 3:2 v/v; Rf: 0.74, stain positive. Microanalysis: Calculated C = 72.59 %, H = 4.30 %, N = 4.98 %; Found C = 72.40 %, H = 4.28 %, N = 4.90 %. Melting point: 88 - 90 °C.

¹ H n.m.r. (DMSO-d₆) δ 7.78 (d, J = 7.8 Hz, 1H, Ar), 7.72 (d, J = 8.3 Hz, 1H, Ar), 7.59 (s, 1H, Ar), 7.52 – 7.45 (m, 3H, Ar), 7.39 – 7.33 (m, 1H, Ar), 7.27 (t, J = 8.9 Hz, 2H), 5.86 (dd, J₅‴,₄‴⁻a = 7.8 Hz, J₅‴,₄‴⁻b = 9.9 Hz, 1H, CH₅‴·CH₄‴⁻aCH₄‴⁻b), 4.50 (dd, J₄‴⁻b,₅‴ = 9.9 Hz, J₄‴⁻b,₄‴⁻a = 15.3 Hz, 1H, CH₅‴·CH₄‴⁻aCH₄‴⁻b), 3.92 (dd, J₄‴⁻a,₅‴ = 7.8 Hz, J₄‴⁻a,₄‴⁻b = 15.3 Hz, 1H, CH₅‴·CH₄‴⁻aCH₄‴⁻b).

¹³ C n.m.r. (DMSO-d₆) δ 163.98 (C, C-4‴), 160.74 (C, C-2‴), 155.57 (C, C-4‴), 155.29 (C, C-7a), 144.20 (C, C-2), 137.15 (C, C-2‴), 137.11 (C, C-1‴), 128.59 (2 x CH, CH-3‴ and CH-5‴), 128.48 (2 x CH, CH-2‴ and CH-6‴), 127.37 (C, C-3a), 127.28 (CH, CH-6), 124.21 (CH, CH-5), 122.94 (CH, CH-4), 116.15 (2 x CH, CH-3‴ and CH-5‴), 115.86 (2 x CH-3‴ and CH-5‴), 112.13 (CH, CH-7), 111.26 (CH, CH-2), 80.22 (CH, CH-5‴), 62.86 (CH₂).
2-Benzof[b]furan-2-yl-5-(4-chlorophenyl)-4,5-dihydro-1,3-oxazole (49)

(C_{17}H_{12}ClNO_{2}, MW: 297.740)

With 2-benzo[b]furan-2-yl-5-(4-chlorophenyl)-4,5-dihydro-1,3-oxazole (49), a light yellow solid was obtained by recrystallisation from ethanol.

Yield: 1.85 g (65 %), t. l. c. system: petroleum ether – ethyl acetate 3:2 v/v, R_f: 0.62, stain positive. Microanalysis: Calculated C = 68.58 %, H = 4.06 %, N = 4.70 %; Found C = 68.56 %, H = 3.97 %, N = 4.72 %. Melting point: 103 - 105 °C.

1^H n.m.r. (DMSO-d_6) δ 7.70 (d, J = 7.7 Hz, 1H, Ar), 7.74 (d, J = 8.3 Hz, 1H, Ar), 7.62 (s, 1H, Ar), 7.54 – 7.36 (m, 6H, Ar), 5.88 (dd, J_5",4"_a = 7.5 Hz, J_5",4"_b = 10.0 Hz, 1H, CH_5"-CH_4"-aCH_4"-b), 4.53 (dd, J_4"_b,5"_a = 10.0 Hz, J_4"_b,4"_a = 15.3 Hz, 1H, CH_5"-CH_4"-aCH_4"-b), 3.92 (dd, J_4"_a,5"_a = 7.5 Hz, J_4"_a,4"_b = 15.3 Hz, 1H, CH_5"-CH_4"-aCH_4"-b).

13^C n.m.r. (DMSO-d_6) δ 155.57 (C, C-2"'), 155.30 (C, C-7a), 144.11 (C, C-2), 139.96 (C, C-1"'), 133.27 (C, C-4"'), 129.17 (2 x CH, CH-3' and CH-5"'), 128.10 (2 x CH-2' and CH-6"'), 127.36 (C, C-3a), 127.32 (CH, CH-6), 124.23 (CH, CH-5), 122.96 (CH, CH-4), 112.14 (CH, CH-7), 111.34 (CH, CH-3), 80.03 (CH, CH-5""), 62.91 (CH_2).
Synthesis of \( \text{N2-(2-phenyl-2-\((1H-1\text{-imidazoyl})\text{ethyl})\text{-benzo[b]furan-2-carboxamide derivatives} \) (compounds 51 – 55)\}

\( \text{N2-(2-Phenyl-2-\((1H-1\text{-imidazoyl})\text{ethyl})\text{-benzo[b]furan-2-carboxamide} \) (51)\}

\[(\text{C}_{20}\text{H}_{17}\text{N}_{3}\text{O}_2, \text{MW: 331.372)}\]

\[
\begin{align*}
\text{R}_1 & = \text{R}_2 = \text{R}_3 = \text{H} \\
\text{R}_1 & = \text{OCH}_3; \text{R}_2 = \text{R}_3 = \text{H} \\
\text{R}_1 & = \text{R}_3 = \text{H}; \text{R}_2 = \text{NO}_2 \\
\text{R}_1 & = \text{R}_2 = \text{H}; \text{R}_3 = \text{F} \\
\text{R}_1 & = \text{R}_2 = \text{H}; \text{R}_3 = \text{Cl}
\end{align*}
\]

A mixture of 2-benzo[b]furan-2-yl-5-phenyl-4,5-dihydro-1,3-oxazole (45) (0.33 g, 1.25 mmol) and imidazole (1.71 g, 25.07 mmol) dissolved in isopropyl acetate (3 mL) was heated at 125 °C for 24 hours. After completion of the reaction the mixture was partitioned between water (100 mL) and ethyl acetate (150 mL). The organic layer was washed three times with water (3 x 100 mL), dried over magnesium sulphate and concentrated in vacuo. The residue was recrystallised from ethanol to yield the white solid, \( \text{N2-(2-phenyl-2-\((1H-1\text{-imidazoyl})\text{-ethyl})\text{-benzo[b]furan-2-carboxamide} \) (51).\)

Yield: 0.114 g (28 %), t. l. c. system: dichloromethane – methanol 9:1 v/v, Rf: 0.74, stain negative. LRMS (ES\(^{+}\)) m/z: 332.13 (M+H\(^{+}\)). Microanalysis (C\(_{20}\)H\(_{17}\)N\(_{3}\)O\(_2\).0.4H\(_2\)O): Calculated: C = 71.00 %, H = 5.30 %; N = 12.42 %. Found: C = 71.08 %, H = 5.40 %, N = 12.21 %. Melting point: 180 – 182 °C.

\(^1\)H n.m.r. (DMSO-\(d_6\)) \(\delta\) 9.04 (t, J = 5.4 Hz, 1H, NH), 7.90 (s, 1H, H-2'), 7.79 (d, J = 7.7 Hz, 1H, Ar), 7.66 (d, J = 8.3 Hz, 1H, Ar), 7.56 (s, 1H, H-3), 7.52-7.33 (m, 8H, Ar and imidazole), 6.95 (s, 1H, H-imidazole), 5.75 (dd, J = 5.6, 9.3 Hz, 1H, H-1), 4.23-3.98 (m, 2H, CH\(_2\)).

\(^{13}\)C n.m.r. (DMSO-\(d_6\)) \(\delta\) 158.75 (C=O), 154.54 (C, C-7a), 149.00 (C, C-2), 139.52 (CH, CH-2''), 137.14 (C, C-1'), 129.11 (2 x CH, CH-3' and CH-5'), 128.92 (CH,
CH-6), 128.49 (CH, CH-5’’), 127.40 (C, C-3a), 127.31 (CH, CH-4’), 127.17 (2 x CH, CH2’ and CH-6’), 124.11 (CH, CH-5), 123.16 (CH, CH-4), 118.73 (CH, CH-4’’), 112.11 (CH, CH-7), 110.20 (CH, CH-3), 59.83 (CH, CH-1), 43.24 (CH2).

N2-[2-Phenyl-2-(1H-1-imidazoyl)ethyl]-6-methoxybenzo[b]furan-2-carboxamide (52)

(C21H19N3O3, MW: 361.398)

With N2-[2-phenyl-2-(1H-1-imidazoyl)ethyl]-6-methoxybenzo[b]furan-2-carboxamide (52), a brown solid was obtained after recrystallisation from ethanol/water (2:1 v/v).

Yield: 0.41 g (66 %), t. l. c. system: dichloromethane – methanol 9:1 v/v, Rf: 0.63, stain negative. Microanalysis (C21H19N3O3.0.1H2O): Calculated: C = 69.45 %, H = 5.33 %; N = 11.60 %; Found: C = 69.43 %, H = 5.41 %, N = 11.54 %. Melting point: 110 – 112 °C.

1H n.m.r. (DMSO-d6) δ 8.83 (t, J = 5.5 Hz, 1H, NH), 7.87 (s, 1H, H-2’’), 7.62 (d, J = 8.6 Hz, 1H, Ar), 7.45 (s, 1H, H-3), 7.38 - 7.29 (m, 6H, Ar and imidazole), 7.16 (d, J = 1.8 Hz, 1H), 6.96 (dd, J = 1.8, 8.7 Hz, 1H, Ar), 6.92 (s, 1H, H-imidazole), 5.70 (dd, J = 5.7, 9.4 Hz, 1H, H-1), 4.17 - 3.94 (m, 2H, CH2), 3.82 (s, 3H, CH3).

13C n.m.r. (DMSO-d6) δ 158.75 (C=O), 154.54 (C, C-7a), 149.00 (C, C-2), 139.52 (CH, CH-2’’), 137.14 (C, C-1’), 129.11 (2 x CH, CH-3’ and CH-5’), 128.92 (CH, CH-6), 128.49 (CH, CH-5’’), 127.40 (C, C-3a), 127.31 (CH, CH-4’), 127.17 (2 x CH, CH2’ and CH-6’), 124.11 (CH, CH-5), 123.16 (CH, CH-4), 118.73 (CH, CH-4’’), 112.11 (CH, CH-7), 110.20 (CH, CH-3), 59.83 (CH, CH-1), 43.24 (CH2).
**N2-[2-Phenyl-2-(1H-1-imidazolyl)ethyl]-5-nitrobenzo[b]furan-2-carboxamide (53)**

(C_{20}H_{16}N_{4}O_{4}, MW: 376.369)

With **N2-[2-phenyl-2-(1H-1-imidazolyl)ethyl]-5-nitrobenzo[b]furan-2-carboxamide (53)**, a brown solid was obtained after recrystallisation from ethanol/water (2:1 v/v). Further purification by flash column chromatography (dichloromethane – methanol 100:0 v/v increasing to 95:5 v/v) gave white solid.

Yield: 80 mg (33 %), t. l. c. system: dichloromethane – methanol 9:1 v/v, R_{f}: 0.23, stain negative. Microanalysis (C_{20}H_{16}N_{4}O_{4}.0.3H_{2}O): Calculated C = 62.92 %, H = 4.38 %, N = 14.68 %; Found C = 62.72 %, H = 4.13 %, N = 14.50 %. Melting point: 204 - 206 °C.

1 H n.m.r. (DMSO-\textit{d}_6) δ 9.23 (t, J = 5.6 Hz, 1H, NH), 8.79 (d, J = 2.4 Hz, 1H, H-4), 8.33 (dd, J = 2.4, 9.1 Hz, 1H, H-6), 7.90 (d, J = 8.6 Hz, 2H, Ar), 7.75 (s, 1H, H-2'''), 7.42 - 7.33 (m, 6H, Ar), 6.93 (s, 1H, H-3), 5.71 (dd, J = 5.6, 9.3 Hz, 1H, H-1), 4.22 - 3.98 (m, 2H, CH_{2}).

13 C n.m.r. (DMSO-\textit{d}_6) δ 158.05 (C=O), 157.24 (C, C-7a), 151.68 (C, C-2), 144.56 (C, C-1'), 139.43 (C, C-5), 137.15 (CH, CH-2'''), 129.14 (2 x CH, CH-3' and CH-5'), 128.96 (2 x CH, CH-2' and CH-6'), 128.54 (CH, CH-4'), 128.02 (C, C-3a), 127.12 (CH, CH-4'''), 122.62 (CH, CH-6) 119.98 (CH, CH-4), 118.72 (CH, CH-5''), 113.26 (CH, CH-3), 110.96 (CH, CH-7), 59.77 (CH, CH-1), 43.35 (CH_{2}).
\textbf{N2-[2-(4-Fluorophenyl)-2-(1H-1-imidazoyl)ethyl]-benzo[b]furan-2-carboxamide (54)}

(C\textsubscript{20}H\textsubscript{18}FN\textsubscript{3}O\textsubscript{2}, MW: 349.363)

With \textbf{N2-[2-(4-fluorophenyl)-2-(1H-1-imidazoyl)ethyl]-benzo[b]furan-2-carboxamide (54)} a brown solid was obtained after recrystallisation from ethanol/water (2:1 v/v).

Yield: 0.64 g (73 %), t. l. c. system: dichloromethane – methanol 9:1 v/v, R\textsubscript{f}: 0.38, stain negative. Microanalysis: Calculated: C = 68.76 %, H = 4.62 %; N = 12.02 %; Found: C = 68.56 %, H = 4.62 %, N = 12.11 %. Melting point: 198 – 200 °C.

\textsuperscript{1}H n.m.r. (DMSO-\textit{d}_6) \delta 9.00 (t, J = 5.5 Hz, 1H, NH), 7.88 (s, 1H, H-2'''), 7.78 (d, J = 7.7 Hz, 1H, Ar), 7.54 (s, 1H, H-3), 7.65 (d, J = 8.3 Hz, 1H, Ar), 7.48 (m, 3H, Ar), 7.35 (m, 2H, Ar), 7.23 (t, J = 8.8 Hz, 2H, Ar), 6.93 (s, 1H, H-4'''), 5.73 (dd, J = 6.2, 8.9 Hz, 1H, H-1), 4.18 – 3.96 (m, 2H, CH\textsubscript{2}).

\textsuperscript{13}C n.m.r. (DMSO-\textit{d}_6) \delta 163.77 (C=O), 160.53 (C, C-4'), 158.77 (C, C-4'), 154.55 (C, C-7a), 148.99 (C, C-2), 137.09 (CH, CH-2'''), 135.76 (C, C-1'), 135.53 (C, C-1'), 129.52 (CH, CH-2' or CH-6'), 129.41 (CH, CH-2' or CH-6'), 129.03 (CH, CH-5'''), 127.40 (C, C-3a), 127.33 (CH, CH-6), 124.13 (CH, CH-5), 123.19 (CH, CH-4'''), 118.63 (CH, CH-4), 116.05 (CH, CH-3' or CH-5'), 115.76 (CH, CH-3' or CH-5'), 112.13 (CH, CH-3), 110.25 (CH, CH-7), 59.08 (CH, CH-1), 43.20 (CH\textsubscript{2}).
N2-[2-(4-Chlorophenyl)-2-(1H-1-imidazoyl)ethyl]-benzo[b]furan-2-carboxamide (55)

\[
\text{C}_{20}\text{H}_{16}\text{ClN}_{3}\text{O}_2, \text{MW: 365.818}
\]

With N2-[2-(4-chlorophenyl)-2-(1H-1-imidazoyl)ethyl]-benzo[b]furan-2-carboxamide (55), a brown residue was obtained after recrystallisation from ethanol/water (2:1 v/v). Further purification by flash column chromatography (dichloromethane – methanol 100:0 v/v increasing to 96:4 v/v) gave a light yellow solid.

Yield: 0.44 g (72 %), t. l. c. system: petroleum ether – ethyl acetate 3:2 v/v, \( R_f \): 0.16, stain negative. Microanalysis (C\(_{20}\)H\(_{16}\)ClN\(_3\)O\(_2\).0.2H\(_2\)O): Calculated: C = 65.03 %, H = 4.47 %; N = 11.37%; Found: C = 64.93 %, H = 4.35 %, N = 11.36 %. Melting point: 76 - 78 °C.

\(^1\)H n.m.r. (DMSO-\(d_6\)) \( \delta \) 9.00 (t, \( J = 5.4 \) Hz, 1H, NH), 7.88 (s, 1H, H-2'''), 7.76 (d, \( J = 7.7 \) Hz, 1H, Ar), 7.63 (d, \( J = 8.3 \) Hz, 1H, Ar), 7.53 (s, 1H), 7.49 - 7.31 (m, 7H, Ar), 6.91 (s, 1H, ), 5.73 (dd, \( J = 6.2, 8.8 \) Hz, 1H, H-1), 4.16 - 3.95 (m, 2H, CH\(_2\)).

\(^{13}\)C n.m.r. (DMSO-\(d_6\)) 158.82 (C=O), 154.56 (C, C-7a), 148.98 (C, C-2), 138.48 (C, C-1), 137.19 (CH, CH-2'''), 133.20 (C, C-4''), 129.20 (2 x CH, CH-2'' and CH-6''), 129.08 (2 x CH, CH-3' and CH-5''), 127.41 (C, C-3a), 127.32 (CH, CH-5'''), 124.11 (CH, CH-6), 123.17 (2 x CH, CH-5 and CH-4''''), 118.71 (CH, CH-4), 112.12 (CH, CH-7), 110.30 (CH, CH-3), 59.13 (CH, CH-1), 43.10 (CH\(_2\)).
Synthesis of *N*2-[2-phenyl-2-(1*H*-1,2,4-triazol-1-yl)ethyl]-benzo*b*furan-2-carboxamide derivatives (compounds 56, 58, 60, 62 and 64) and *N*2-[2-phenyl-2-(4*H*-1,2,4-triazol-4-yl)ethyl]-benzo*b*furan-2-carboxamide derivatives (57, 59, 61, 63 and 65)

*N*2-[2-Phenyl-2-(1*H*-1,2,4-triazol-1-yl)ethyl]-benzo*b*furan-2-carboxamide (56) and *N*2-[2-Phenyl-2-(4*H*-1,2,4-triazol-4-yl)ethyl]-benzo*b*furan-2-carboxamide (57)

(C_{19}H_{16}N_{4}O_{2}, MW: 332.360)

\[
\begin{align*}
\text{(45)} & \quad R_1 = R_2 = R_3 = H \\
\text{(46)} & \quad R_1 = \text{OCH}_3, R_2 = R_3 = H \\
\text{(47)} & \quad R_1 = R_2 = H; R_2 = \text{NO}_2 \\
\text{(48)} & \quad R_1 = R_2 = H; R_3 = \text{F} \\
\text{(49)} & \quad R_1 = R_2 = H; R_3 = \text{Cl}
\end{align*}
\]

A mixture of 2-benzo*b*furan-2-yl-5-phenyl-4,5-dihydro-1,3-oxazole (45) (0.40 g, 1.52 mmol) and imidazole (2.10 g, 30.38 mmol) dissolved in isopropyl acetate (5 mL) was heated at 128 °C for 30 hours. After completion, the reaction the mixture was partitioned between water (100 mL) and ethyl acetate (150 mL). The organic layer was washed three times with water (3 x 100 mL), dried over magnesium sulphate and concentrated in vacuo. The residue was recrystallised from toluene to yield the light yellow solid which contained a mixture of two compounds as observed by t.l.c. system: petroleum ether – ethyl acetate 2:3 v/v, R_f: 0.18, stain negative and R_f: 0.0, stain negative. A second recrystallisation was carried out with methanol to yield the white solid *N*2-[2-phenyl-2-(4*H*-1,2,4-triazol-4-yl)ethyl]-benzo*b*furan-2-carboxamide (57). Yield: 0.055 g (9 %), t. l. c. system: dichloromethane – methanol 9:1 v/v, R_f: 0.50, stain negative. Microanalysis (C_{19}H_{16}N_{4}O_{2}): Calculated: C = 68.66
% H = 4.85 %; N = 16.85 %; Found: C = 68.53 %, H = 4.93 %, N = 16.65 %.
Melting point: 276 – 278 °C.

The filtrate from the second recrystallisation was purified by flash column
chromatography (petroleum ether – ethyl acetate 90:10 v/v increasing to 25:75 v/v) to
give \( N2-[2\text{-phenyl}-2-(1H-1,2,4-triazol-1-yl)ethyl]-benzo[b]furan-2-carboxamide (56) \)
(0.20 g, 32 %) as a white solid. T. l. c. system: petroleum ether – ethyl acetate 2:3 v/v,
R\(_F\): 0.18, stain negative. HRMS (ES\(^+\)) calculated for C\(_{19}\)H\(_{16}\)N\(_4\)O\(_2\) (M+H\(^+\)) 333.1346;
Found 333.1346. Melting point: 126 – 128 °C.

N.M.R. data for \( N2-[2\text{-phenyl}-2-(1H-1,2,4-triazol-1-yl)ethyl]-benzo[b]furan-2-carboxamide (56) \)

\(^1\)H n.m.r. (DMSO-\(d_6\)) \( \delta \) 9.00 (t, J = 5.5 Hz, 1H, NH), 8.80 (s, 1H, H-3'''), 8.08 (s, 1H, H-5''), 7.78 (d, J = 7.8 Hz, 1H, Ar), 7.65 (d, J = 8.3 Hz, 1H, Ar), 7.55 (s, 1H, H-3), 7.51-7.33 (m, 7H, Ar), 5.99 (dd, J = 5.5, 9.1 Hz, 1H, H-1), 4.26-4.02 (m, 2H, CH\(_2\)).

\(^13\)C n.m.r. (DMSO-\(d_6\)) \( \delta \) 158.84 (C=O), 154.55 (C, C-7a), 152.05 (CH, CH-5'''), 148.94 (C, C-2), 144.48 (C, CH-3'''), 138.14 (C, C-1'''), 129.04 (2 x CH, CH-3' and
CH-5'), 128.69 (CH, CH-6), 127.57 (2 x CH, CH-2' and CH-6'), 127.38 (C, C-3a), 127.30 (CH, CH-4''), 124.10 (CH, CH-5), 123.16 (CH, CH-4), 112.13 (CH, CH-7), 110.17 (CH, CH-3), 62.10 (CH, CH-1), 43.25 (CH\(_2\)).

N.M.R. data for \( N2-[2\text{-phenyl}-2-(4H-1,2,4-triazol-4-yl)ethyl]-benzo[b]furan-2-carboxamide (57) \)

\(^1\)H n.m.r. (DMSO-\(d_6\)) \( \delta \) 9.10 (t, J = 5.6 Hz, 1H, NH), 8.79 (s, 2H, triazole H-2'' and
H-5''), 7.78 (d, J = 7.4 Hz, 1H, Ar), 7.65 (dd, J = 0.7, 8.3 Hz, 1H, Ar), 7.54 (d, J = 0.8 Hz, 1H, H-3), 7.51-7.32 (m, 7H, Ar), 5.81 (dd, J = 5.1, 9.7 Hz, 1H, H-1), 4.25 - 3.98 (m, 2H, CH\(_2\)).

\(^13\)C n.m.r. (DMSO-\(d_6\)) \( \delta \) 157.36 (C=O), 153.09 (C, C-7a), 147.41 (C, C-2), 142.88 (2 x CH, CH-2'' and
CH-5'''), 141.40 (C, C-1'''), 137.00 (C, C-3a), 129.33 (2 x CH, CH-3' and
CH-5'), 128.58 (CH, CH-6), 127.58 (2 x CH, CH-2' and CH-6'), 127.29 (CH, CH-4''), 124.16 (CH, CH-5), 123.24 (CH, CH-4), 112.15 (CH, CH-7), 110.37 (CH, CH-3), 58.85 (CH, CH-1), 43.15 (CH\(_2\)).
The following analogues of compound 56 and 57 were prepared using the same general method detailed above.

\[ \text{N2-[2-Phenyl-2-(1H-1,2,4-triazol-1-yl)ethyl]-6-methoxybenzo[b]furan-2-carboxamide (58)} \]

and

\[ \text{N2-[2-phenyl-2-(4H-1,2,4-triazol-4-yl)ethyl]-6-methoxybenzo[b]furan-2-carboxamide (59)} \]

\[ \text{(C}_{20}\text{H}_{18}\text{N}_{4}\text{O}_{3}, \text{MW: 362.386)} \]

A mixture of 2-(6-methoxybenzo[b]furan-2-yl)-5-phenyl-4,5-dihydro-oxazole (46) (1.0 g, 3.41 mmol) and triazole (4.71 g, 68.18 mmol) dissolved in isopropyl acetate (5 mL) was heated at 130 °C for 30 hours. After completion of the reaction the mixture was partitioned between water (100 mL) and ethyl acetate (150 mL). The organic layer was washed three times with water (3 x 100 mL), dried over magnesium sulphate and concentrated \textit{in vacuo}. The residue was recrystallised from toluene and then a second recrystallisation from ethanol to yield the light brown solid, \text{N2-[2-phenyl-2-(4H-1,2,4-triazol-4-yl)ethyl]-6-methoxybenzo[b]furan-2-carboxamide (59).}

Yield: 0.18 g (14 %), t. l. c. system: dichloromethane – methanol 9:1 v/v, R\text{f}: 0.40, stain positive. Microanalysis (C\textsubscript{20}H\textsubscript{18}N\textsubscript{4}O\textsubscript{3}): Calculated: C = 66.29 %, H = 5.01 %; N = 15.45 %; Found: C = 66.54 %, H = 5.14 %, N = 15.45 %. Melting point: 218 – 220 °C.

The filtrate from the recrystallisation was purified by flash column chromatography (petroleum ether – ethyl acetate 90:10 v/v increasing to 20:80 v/v) to give \text{N2-[2-phenyl-2-(1H-1,2,4-triazol-1-yl)ethyl]-6-methoxybenzo[b]furan-2-carboxamide (58)} (0.41 g, 33 %) as a light yellow solid. T. l. c. system: petroleum ether – ethyl acetate 1:3 v/v, R\text{f}: 0.21, stain positive. Microanalysis (C\textsubscript{20}H\textsubscript{18}N\textsubscript{4}O\textsubscript{3}.0.1H\textsubscript{2}O): Calculated: C = 65.96 %, H = 5.04 %; N = 15.38 %; Found: C = 65.96 %, H = 5.02 %, N = 15.12 %. Melting point: 164 – 166 °C.
N.M.R. data for N2-[2-phenyl-2-(1H,1.2.4-triazol-1-yl)ethyl]-6-methoxybenzo[b]furan-2-carboxamide (58)

$^1$H n.m.r. (DMSO-$d_6$) δ 8.84 (t, J = 5.5 Hz, 1H, NH), 8.81 (s, 1H, H-3'), 8.09 (s, 1H, H-5''), 7.68 (d, J = 8.6 Hz, 1H, Ar), 7.50-7.37 (m, 6H, Ar), 7.22 (d, J = 1.7 Hz, 1H, Ar), 7.00 (dd, J = 1.7, 8.6 Hz, 1H, Ar), 5.99 (dd, J = 5.5, 9.0 Hz, 1H, H-1'), 4.25-4.02 (m, 2H, CH$_2$), 3.88 (s, 3H, CH$_3$).

$^{13}$C n.m.r. (DMSO-$d_6$) δ 161.08 (C=O), 160.08 (C, C-7a), 157.08 (C, C-6), 153.24 (CH, CH-5''), 149.36 (C, C-2), 145.67 (CH, CH-3''), 139.37 (C, C-1'), 130.24 (2 x CH, CH-3' and CH-5''), 129.87 (CH, CH-4'), 128.74 (2 x CH, CH-2' and CH-6'), 124.62 (CH, CH-4), 121.69 (C, C-3a), 114.82 (CH, CH-3), 111.49 (CH, CH-5), 97.40 (CH, CH-7), 63.33 (CH, CH-1), 57.24 (CH$_3$), 44.40 (CH$_2$).

N.M.R. data for N2-[2-phenyl-2-(4H,1,2,4-triazol-4-yl)ethyl]-6-methoxybenzo[b]furan-2-carboxamide (59)

$^1$H n.m.r. (DMSO-$d_6$) δ 8.93 (t, J = 5.6 Hz, 1H, NH), 8.79 (s, 2H, triazole H-2'' and H-5''), 7.64 (d, J = 8.7 Hz, 1H, Ar), 7.47 - 7.36 (m, 5H, Ar), 7.18 (d, J = 1.7 Hz, 1H, H-3), 6.97 (dd, J = 2.2, 8.7 Hz, 1H, Ar), 5.80 (dd, J = 5.2, 9.7 Hz, 1H, H-1'), 4.23 - 3.97 (m, 2H, CH$_2$), 3.84 (s, 3H, CH$_3$).

$^{13}$C n.m.r. (DMSO-$d_6$) δ 158.70 (C=O), 157.68 (C, C-7a), 154.68 (C, C-6), 146.90 (C, C-2), 141.64 (2 x CH, CH-2'' and CH-5''), 137.27 (C, C-1'), 129.32 (2 x CH, CH-3' and CH-5''), 128.70 (CH, CH-4'), 127.28 (2 x CH, CH-2' and CH-6'), 123.48 (CH, CH-4), 119.29 (C, C-3a), 112.41 (CH, CH-3), 109.09 (CH, CH-5), 94.99 (CH, CH-7), 57.69 (CH, CH-1), 54.83 (CH$_3$), 41.90 (CH$_2$).
**N2-[2-Phenyl-2-(1H-1,2,4-triazol-1-yl)ethyl]-5-nitrobenzo[b]furan-2-carboxamide** (60) and **N2-[2-phenyl-2-(4H-1,2,4-triazol-4-yl)ethyl]-5-nitrobenzo[b]furan-2-carboxamide** (61)

(C19H15N3O4, MW: 377.357)

A mixture of 2-(5-nitrobenzo[b]furan-2-yl)-5-phenyl-4,5-dihydro-1,3-oxazole (47) (0.20 g, 0.65 mmol) and triazole (0.90 g, 12.97 mmol) dissolved in isopropyl acetate (3 mL) was heated at 130 °C for 36 hours. After completion of the reaction the mixture was partitioned between water (100 mL) and ethyl acetate (150 mL). The organic layer was washed three times with water (3 x 100 mL), dried over magnesium sulphate and concentrated in vacuo. The residue was recrystallised from toluene and then a second recrystallisation from ethanol to yield white solid, **N2-[2-phenyl-2-(4H-1,2,4-triazol-4-yl)ethyl]-5-nitrobenzo[b]furan-2-carboxamide** (61). Yield: 9 mg (4 %), t. l. c. system: dichloromethane – methanol 9:1 v/v, Rf: 0.23, stain positive. Melting point: 125 – 127 °C. [Booked for HRMS – Nov 2003]

The filtrate from the recrystallisation was purified by flash column chromatography (petroleum ether – ethyl acetate 90:10 v/v increasing to 20:80 v/v) to give crude **N2-[2-phenyl-2-(1H-1,2,4-triazol-1-yl)ethyl]-5-nitrobenzo[b]furan-2-carboxamide** as a yellow solid. The crude solid was further purified again by flash column chromatography (dichloromethane – methanol 100:0 v/v increasing to 95:5 v/v) to give a pure **N2-[2-phenyl-2-(1H-1,2,4-triazol-1-yl)ethyl]-5-nitrobenzo[b]furan-2-carboxamide** (60) (15 mg, 6 %) as a light yellow solid. T. l. c. system: petroleum ether – ethyl acetate 1:3 v/v, Rf: 0.21, stain positive. Melting point: 60 – 62 °C. HRMS (El⁺) m/z Calculated for C19H13N3O4 (M⁺) 377.1119; Found 377.1122.

**N.M.R. data for N2-[2-phenyl-2-(1H-1,2,4-triazol-1-yl)ethyl]-5-nitrobenzo[b]furan-2-carboxamide** (60)

1H n.m.r. (DMSO-d6) δ 9.23 (t, J = 5.5 Hz, 1H, NH), 8.83 (d, J = 2.4 Hz, 1H, H-4), 8.82 (s, 1H, H-5’’), 8.38 (dd, J = 2.5, 9.2 Hz, 1H, H-6), 8.10 (s, 1H, H-3’’), 7.95 (d, J
= 9.2 Hz, 1H, H-7), 7.78 (s, 1H, H-3), 7.52 – 7.36 (m, 5H, Ar), 5.99 (dd, J = 5.5, 9.1 Hz, 1H, H-1), 4.29 – 4.05 (m, 2H, CH₂).

¹³C n.m.r. (DMSO-d₆) δ 158.13 (C=O), 157.25 (C, C-7a), 152.07 (CH, CH-5'''), 151.62 (C, C-2), 144.56 (C, C-5), 144.52 (CH, CH-3'''), 138.07 (C, C-1'), 129.07 (2 x CH, CH-2' and CH-6'), 128.74 (CH, CH-4'), 128.00 (C, C-3a), 127.57 (2 x CH, CH-3' and CH-5'), 122.62 (CH, CH-6), 119.99 (CH, CH-4), 113.27 (CH, CH-7), 110.93 (CH, CH-3), 62.01 (CH, CH-1), 43.36 (CH₂).

N.M.R. data for N₂-[2-phenyl-2-(4H-1,2,4-triazol-4-yl)ethyl]-5-nitrobenzo[b]furan-2-carboxamide (61)

¹H n.m.r. (DMSO-d₆) δ 9.29 (t, J = 5.5 Hz, 1H, NH), 8.80 (s, 3H, triazole H-2''' and H-5''', and H-4), 8.34 (dd, J = 2.4, 9.1 Hz, 1H, H-6), 7.91 (d, J = 9.1 Hz, 1H, H-7), 7.76 (s, 1H, H-3), 7.48 – 7.37 (m, 5H, Ar), 5.81 (dd, J = 5.1, 9.7 Hz, 1H, H-1), 4.27 – 4.01 (m, 2H, CH₂).

¹³C n.m.r. (DMSO-d₆) δ 158.15 (C=O), 157.26 (C, C-7a), 151.56 (C, C-2), 144.57 (C, C-5), 142.85 (2 x CH, CH-2''' and CH-5'''), 138.36 (C, C-1'), 129.33 (2 x CH-2' and CH-6'), 128.92 (CH, CH-4'), 128.01 (C, C-3a), 127.30 (2 x CH, CH-3' and CH-5'), 122.67 (CH, CH-6), 120.02 (CH, CH-4), 113.27 (CH, CH-7), 111.09 (CH, CH-3), 58.97 (CH, CH-1), 43.27 (CH₂).

N₂-[2-(4-Fluorophenyl)-2-(1H-1,2,4-triazol-1-yl)ethyl]benzo[b]furan-2-carboxamide (62) and N₂-[2-(4-Fluorophenyl)-2-(4H-1,2,4-triazol-4-yl)ethyl]benzo[b]furan-2-carboxamide (63)

(C₁₉H₁₅FN₄O₂, MW: 350.351)

A mixture of 2-benzo[b]furan-2-yl-5-(4-fluorophenyl)-4,5-dihydro-1,3-oxazole (48) (1.10 g, 3.91 mmol) and triazole (5.40 g, 78.21 mmol) dissolved in isopropyl acetate (5 mL) was heated at 130 °C for 30 hours. After completion of the reaction the mixture was partitioned between water (100 mL) and ethyl acetate (150 mL). The organic layer was partitioned twice with water (2 × 100 mL), dried over magnesium sulphate and concentrated in vacuo. The residue was recrystallised from ethanol/water
(2:1) to yield a mixture of N2-[(4-fluorophenyl)-2-(1H-1,2,4-triazol-1-yl)ethyl]benzofuran-2-carboxamide (62) and N2-[2-(4-fluorophenyl)-2-(4H-1,2,4-triazol-4-yl)ethyl]benzofuran-2-carboxamide (63). A second recrystallisation from dichloromethane/methanol (1:1) to yield N2-[(4-fluorophenyl)-2-(4H-1,2,4-triazol-4-yl)ethyl]benzofuran-2-carboxamide (63) as a white solid (0.16 g, 12%). T. l. c. system: dichloromethane – methanol 9:1 v/v, Rf: 0.29, stain positive. Microanalysis (C_{19}H_{13}FN_{4}O_{2}·0.1H_{2}O): Calculated: C = 64.80 %, H = 4.35 %; N = 15.91 %; Found: C = 64.80 %, H = 4.29 %, N = 15.87 %. Melting point: 252 – 254 °C.

The residue from the second recrystallisation was purified by flash column chromatography (petroleum ether – ethyl acetate 90:10 v/v increasing to 30:70 v/v) to give N2-[(4-fluorophenyl)-2-(1H-1,2,4-triazol-1-yl)ethyl]benzofuran-2-carboxamide (62) (0.31 g, 22%) as a white solid. T. l. c. system: dichloromethane – methanol 9:1 v/v, Rf: 0.48, stain positive. Microanalysis (C_{19}H_{13}FN_{4}O_{2}·0.5H_{2}O): Calculated: C = 63.50 %, H = 4.48 %; N = 15.59 %; Found: C = 63.78 %, H = 4.33 %, N = 15.29 %. Melting point: 174 – 176 °C.

N.M.R. data for N2-[(4-fluorophenyl)-2-(1H-1,2,4-triazol-1-yl)ethyl]benzofuran-2-carboxamide (62)

^1^H n.m.r. (DMSO-d_6) δ 8.99 (t, J = 5.6 Hz, 1H, NH), 8.78 (s, 1H, H-3'''), 8.07 (s, 1H, H-5''), 7.73 (d, J = 7.7 Hz, 1H, Ar), 7.60 (d, J = 8.3 Hz, 1H, Ar), 7.52 (m, 3H, Ar), 7.42 (m, 1H, Ar), 7.29 (m, 1H, Ar), 7.20 (t, J = 8.8 Hz, 2H), 5.99 (dd, J = 5.8, 8.7 Hz, 1H), 4.22 – 3.99 (m, 2H, CH_2).

^13^C n.m.r. (DMSO-d_6) δ 163.92 (C=O), 160.68 (C, C-4'), 158.89 (C, C-4''), 154.57 (C, C-7a), 152.18 (CH, CH-5'''), 148.92 (C, C-2), 144.51 (CH, CH-3'''), 134.33 (C, C-1''), 134.29 (C, C-1'''), 129.97 (CH, CH-2' or CH-6''), 129.85 (CH, CH-2' or CH-6'), 127.39 (C, C-3a), 127.30 (CH, CH-6), 124.09 (CH, CH-5), 123.16 (CH, CH-4), 115.99 (CH, CH-3' or CH-5''), 115.72 (CH, CH-3' or CH-5''), 112.12 (CH, CH-3), 110.25 (CH, CH-7), 61.32 (CH, CH-1), 43.28 (CH_2).

N.M.R. data for N2-[(4-fluorophenyl)-2-(4H-1,2,4-triazol-4-yl)ethyl]benzofuran-2-carboxamide (63)

^1^H n.m.r. (DMSO-d_6) δ 9.11 (t, J = 5.4 Hz, 1H, NH), 8.80 (s, 2H, triazole H-2''' and H-5'''), 7.78 (d, J = 7.6 Hz, 1H, Ar), 7.64 (d, J = 8.4 Hz, 1H, Ar), 7.57 - 7.46 (m, 4H, Ar), 7.37 – 7.31 (m, 1H, Ar), 7.27 (t, J = 8.8 Hz, 2H, Ar), 5.83 (dd, J = 5.4, 9.5 Hz, 1H, CH-1), 4.22 – 4.15 (m, 2H, CH_2).
13C n.m.r. (DMSO-<sup>d6</sup>) δ: 163.97 (C=O), 160.72 (C, C-4'), 158.85 (C, C-4'), 154.56 (C, C-7a), 148.84 (C, C-2), 142.78 (2 x CH, CH-2'' and CH-5''), 134.70 (C, C-1''), 134.66 (C, C-1'), 129.73 (CH, CH-2' or CH-6'), 129.62 (CH, CH-2' or CH-6'), 127.40 (CH, CH-6), 127.38 (C, C-3a), 124.16 (CH, CH-5), 123.22 (CH, CH-4), 116.29 (CH, CH-3' or CH-5'), 116.00 (CH, CH-3' or CH-5'), 112.14 (CH, CH-3), 110.38 (CH, CH-7), 58.08 (CH, CH-1), 43.07 (CH<sub>2</sub>).

N2-[2-(4-Chlorophenyl)-2-(1H-1,2,4-triazol-1-yl)ethyl]benzo[b]furan-2-carboxamide and (64) N2-[2-(4-chlorophenyl)-2-(4H-1,2,4-triazol-4-yl)ethyl]benzo[b]furan-2-carboxamide (65)

(C<sub>19</sub>H<sub>15</sub>ClN<sub>4</sub>O<sub>2</sub>, MW: 366.805)

![Structural formulas](64) ![Structural formulas](65)

A mixture of 2-benzo[b]furan-2-yl-5-(4-chlorophenyl)-4,5-dihydro-1,3-oxazole (49) (1.0 g, 3.37 mmol) and triazole (4.65 g, 67.33 mmol) dissolved in isopropyl acetate (5 mL) was heated at 130 °C for 30 hours. After completion of the reaction the mixture was partitioned between water (100 mL) and ethyl acetate (150 mL). The organic layer was washed two times with water (2 x 100 mL), dried over magnesium sulphate and concentrated in vacuo. The residue was recrystallised from ethanol/water (2:1) to yield a mixture of N2-[2-(4-chlorophenyl)-2-(1H-1,2,4-triazol-1-yl)ethyl]benzo[b]furan-2-carboxamide (64) and N2-[2-(4-chlorophenyl)-2-(4H-1,2,4-triazol-4-yl)ethyl]benzo[b]furan-2-carboxamide (65). The mixture was purified by flash column chromatography (petroleum ether-ethyl acetate 80:20 v/v increasing to 30:70 v/v) to give N2-[2-(4-chlorophenyl)-2-(1H-1,2,4-triazol-1-yl)ethyl]benzo[b]furan-2-carboxamide (64) as a white solid (0.35 g, 28%). T. l. c. system: dichloromethane – methanol 9.5:0.5 v/v, R<sub>f</sub>: 0.45, stain negative. Microanalysis (C<sub>19</sub>H<sub>15</sub>ClN<sub>4</sub>O<sub>2</sub>): Calculated: C = 62.22 %, H = 4.12 %; N = 15.27 %; Found: C = 62.22 %, H = 4.17 %, N = 15.26 %. Melting point: 200 – 202 °C. The flash column chromatography was continued (dichloromethane – methanol 100:0 v/v increasing to 95:5 v/v) to give N2-[2-(4-chlorophenyl)-2-(4H-1,2,4-triazol-4-yl)ethyl]benzo[b]furan-2-carboxamide (65) (0.15 g, 12 %) as white solid. T. l. c.
system: dichloromethane – methanol 9.5:0.5 v/v, Rf: 0.28, stain negative.
Microanalysis C\textsubscript{19}H\textsubscript{15}ClN\textsubscript{4}O\textsubscript{2}.0.3H\textsubscript{2}O: Calculated C = 61.42 %, H = 4.08 %, N = 14.98 %; Found C = 61.31 %, H = 4.22 %, N = 15.05 %. Melting point: 221 – 223 °C.
N.M.R. data for N2-[2-(4-chlorophenyl)-2-(1H-1,2,4-triazol-1-yl)ethyl]benzo[\textit{b}]furan-2-carboxamide (64)

\(^1\)H n.m.r. (DMSO-\textit{d}_6) \(\delta\) 9.00 (t, J = 5.3 Hz, 1H, NH), 8.79 (s, 1H, H-3\'\'), 8.09 (s, 1H, H-5\''\'), 7.78 (d, J = 7.7 Hz, 1H, Ar), 7.65 (d, J = 8.3 Hz, 1H, Ar), 7.54 (s, 1H, H-3), 7.55 – 7.45 (m, 5H, Ar), 7.34 (t, J = 7.5 Hz, 1H, Ar), 5.99 (dd, J = 6.1, 8.3 Hz, 1H), 4.21 – 4.01 (m, 2H, CH\(_2\)).

\(^13\)C n.m.r. (DMSO-\textit{d}_6) \(\delta\) 158.85 (C=O), 154.57 (C, C-7a), 152.22 (CH, CH-5\''\'), 148.89 (C, C-2), 144.60 (CH, CH-3\'\'), 137.04 (C, C1'), 133.42 (C, C-4'), 129.62 (2 x CH, CH-2' and CH6'), 129.05 (2 x CH, CH-3' and CH-5''), 127.39 (C, C-3a), 127.34 (CH, CH-6), 124.13 (CH, CH-5), 123.19 (CH, CH-4), 112.15 (CH, CH-7), 110.25 (CH, CH-3), 61.27 (CH, CH-1), 43.14 (CH\(_2\)).

N.M.R. data for N2-[2-(4-chlorophenyl)-2-(4H-1,2,4-triazol-4-yl)ethyl]benzo[\textit{b}]furan-2-carboxamide (65)

\(^1\)H n.m.r. (DMSO-\textit{d}_6) \(\delta\) 9.15 (t, J = 5.4 Hz, 1H, NH), 8.84 (s, 2H, triazole H-2'' and H-5'''), 7.80 (d, J = 7.7 Hz, 1H, Ar), 7.67 (d, J = 8.3 Hz, 1H, Ar), 7.57 (s, 1H, H-3), 7.56 - 7.47 (m, 5H, Ar), 7.36 (t, J = 7.5 Hz, 1H, Ar), 5.87 (dd, J = 5.6, 9.3 Hz, 1H, CH-1), 4.26 – 4.03 (m, 2H, CH\(_2\)).

\(^13\)C n.m.r. (DMSO-\textit{d}_6) \(\delta\) 158.87 (C=O), 154.57 (C, C-7a), 148.84 (C, C-3a), 142.84 (2 x CH, CH-2'' and CH-5'''), 137.37 (C, C-1'), 133.59 (C, C-4'), 129.36 (2 x CH, CH-2' and CH-6'), 129.29 (2 x CH, CH-3' and CH-5''), 127.38 (C, C-3a and CH, 6), 124.15 (CH, CH-5), 123.22 (CH, CH-4), 112.14 (CH, CH-7), 110.41 (CH, CH-3), 58.09 (CH, CH-1), 42.98 (CH\(_2\)).
CHAPTER 5

Synthesis of 6-substituted-2-(phenylmethylene)-3,4-dihydonaphthalene-1-one (tetralone) derivatives
5.1 Synthesis of 6-substituted-2-(phenylmethylene)-3,4-dihydronaphthalene-1-one (tetralone) derivatives

All the synthesis of 6-substituted-2-(phenylmethylene)-3,4-dihydronaphthalene-1-one (tetralone) derivatives were performed by the general methods outlined in Scheme 5.1. (a), (b) and (e). The synthesis was based on the procedure described by our group (Kirby et al., 2003; Maharlouie, 1996).

One of the methods for the preparation of 2-(phenylmethylene)-3,4-dihydronaphthalene-1-one derivatives is by direct condensation of tetralone and the appropriate benzaldehyde in 4 % ethanolic KOH. This general method is successfully used in the absence of the hydroxyl group on tetralone or benzaldehyde. The presence of hydroxyl group will form a salt complex with the 4 % ethanolic KOH. To overcome this problem, one of the solutions is to protect the hydroxyl group using a protecting group that will be stable under the alkaline conditions of this condensation reaction.
Scheme 5.1 (b). Synthesis of 6-substituted-2-(phenylmethylene)-3,4-dihydronaphthalene-1-one derivatives. Structures of the compounds and their corresponding numbers synthesised are listed in the scheme. Reagents and conditions: (I) 48 % HBr (aq.), reflux, 8 h. (II) 3,4-dihydro-2H-pyranyl, PTSA, acetic anhydride, diethyl ether, 2.5 h. (III) Corresponding substituted benzaldehyde, 4 % ethanolic KOH, 2 – 72 h. (IV) (i) Phenylboronic acid, Pd(PPh₃)₄, toluene, 100 °C, 5 h (ii) H₂O₂, room temperature, 1 h. (V) 2 M HCl (aq.), ethyl acetate/2-butanol (1/1 v/v), reflux, 1 h. (VI) Pd/C 10 %, methanol, 1 h.
In order to synthesise the 6-hydroxy-2-(phenylmethylene)-3,4-dihydonaphthalene-1-one derivatives, the commercially available 6-methoxy tetralone (66) was demethylated by refluxing in 48 % aqueous HBr. This reaction did not go to completion even after refluxing at 100 °C for 10 hours. The dark brown residue was then recrystallised from water. The desired product, 6-hydroxy tetralone (67) crystallised out in cold water to give a 36 % yield.

3,4-Dihydropyran is a useful reagent for the protection of primary, secondary, and tertiary alcohols to give tetrahydropyranyl ethers (69) which are stable to basic media (Bolitt et al., 1988; Smith and March, 2001). Para-toluensulphonic acid (PTSA) was used as an acid catalyst with an excess of 3,4-dihydropyran. It was found that the addition of a few drops of acetic anhydride to the mixture increased the yield possibly by preventing the hydrolysis reaction which can occur in the presence of water (Maharlouie, 1996). The yield reported here is 61 % after recrystallisation with ethanol.

Rapson and Shuttleworth reported the condensation of aromatic aldehydes with various cyclic ketone including tetralone in 4 % ethanolic KOH (Rapson and Shuttleworth, 1940). The mechanism is illustrated in Scheme 5.2. In this base-catalysed condensation between two carbonyl compounds, the hydroxide ion (from KOH) abstracts a proton from the α-carbon of the tetralone to from the carbanion (Morrison and Boyd, 1987). The carbanion, which is the nucleophilic species, attacks the electrophilic carbonyl carbon atom of the benzaldehyde to gives the unstable alkoxide. The alkoxide abstracts a hydrogen ion from water to form the intermediary aldol. The elimination of water from the aldol will form a carbon-carbon double bond which is conjugated to the aromatic ring, giving stability to the molecule (Maharlouie, 1996). The product will precipitate out so that it can be filtered and recrystallised before the next step. Some of the reactions required longer reaction times (12 – 72 h) until the product precipitated out (yield 25 % - 87 %). Some reactions required the product to be purified by flash column chromatography and hence the yield was lower.

The tetrahydropyranyl ether (69) underwent mild acid-catalysed hydrolysis (Smith and March, 2001a) to give the corresponding alcohol compound in good yield (90 % – 99 %). 2-Butanone was used as a co-solvent to facilitate the dispersion of the hydrochloric acid in an ethyl acetate solution (Maharlouie, 1996).
The scheme describes the formation of the 2-(Phenylmethylene)-3,4-dihydronaphthalene-1-one through a series of chemical reactions involving nucleophilic addition, dehydration, and condensation.

**Scheme 5.2.** General mechanism for the synthesis of 2-phenylmethylene-3,4-dihydronaphthalene-1-one. The scheme is modified from Maharlouie (Maharlouie, 1996).

Hassner and co-workers (Hassner et al., 1958) reported the use of concentrated sulphuric acid in the condensation of benzaldehyde with tetralone to form the 2-phenylmethylene-3,4-dihydronaphthalene-1-one. This method (Yoshihama et al., 1999) was employed in the synthesis of 2-[1-(4-hydroxyphenyl)methylidene]-6-methoxy-3,4-dihydronaphthalene-1-one (92) which only involved a one step synthesis (Scheme 5.3). Whereas, the base-catalysed condensation involved three steps: firstly to protect the 4-hydroxy-benzaldehyde with 3,4-dihydropyran, then the base catalysed condensation with 4% ethanol KBr and finally deprotection of the pyran protecting group to form 92 (Scheme 5.1 (c)).

**Scheme 5.3.** The use of concentrated hydrochloric acid in methanol in the synthesis of 2-[1-(4-hydroxyphenyl)methylidene]-6-methoxy-3,4-dihydronaphthalene-1-one.
Chapter 5

The synthesis of the saturated tetralone involved the catalytic hydrogenation of the C=C bond in the 2-phenylmethylene-3,4-dihyronaphthalen-1-one derivatives in methanol using H₂ and 10 % palladium on charcoal as catalyst at room temperature for 1 h. When the reaction was left under H₂ for 2 h at room temperature, the carbonyl (92) was reduced to methylene (97) in 63 % yield (Scheme 5.4). The ¹H n.m.r. showed the two H atoms of the CH₂ as a doublet at 2.61 ppm and the ¹³C n.m.r. showed an extra CH₂ peak at 35.6 ppm. It has been reported by Augustine (Augustine, 1965) that hydrogenolysis of the aryl ketone to the methylene by palladium catalyst at room temperature for 1 – 2 h. Therefore in order to prevent the carbonyl group of the tetralone compound being reduced to methylene, the reaction was stopped after 1 h.

Generally, the yield of the saturated tetralone derivative was quite good (57 – 83 %) after trituration with acetone. There were a few compounds where the yields were as low as 6 %, this was due to the higher polarity of the product that made the purification by flash column chromatography difficult.

![Chemical structure](image)

**Scheme 5.4.** The reduction of the C=C bond and carbonyl to methylene by 10 % palladium on charcoal with H₂ at room temperature for 2 h.

An unexpected result was observed in the ¹H n.m.r. spectra for the compounds 79, 83 and 93 (Figure 5.5). There was one singlet peak corresponding to 4 H atoms at around 2.5 ppm in all these three compounds (Figure 5.7). These four H atoms corresponded to the hydrogen atoms of carbon 3 and 4 of the tetralone. However, on the DEPT-¹³C n.m.r., the two CH₂ at carbon 3 and 4 appeared as two separate CH₂ peaks. Normally, these H atoms are expected to appear as two separate multiplet peaks at around 3.0 ppm and 2.5 ppm in the ¹H n.m.r. From these n.m.r. data, we could possibly ask two questions, firstly, is it an accidental degeneracy that these four H atoms give similar ppm; secondly, is the 2-bromobenzaldehyde attached to the β-carbon instead of the α-carbon of the tetralone?

127
To find out the answers, the structure of compound 83 was determined using a 400 MHz NMR (kindly carried out by Dr. M.P. Coogan of the Chemistry Department of Cardiff University) to carry out $^1$H n.m.r., HSQC (Heteronuclear Single Quantum Coherence) and HMBC (Heteronuclear Multiple Bond Coherence).

![Chemical Structure]

**Figure 5.5.** Compounds 79, 83 and 93 produced unexpected results on $^1$H n.m.r.

The 400 MHz $^1$H n.m.r. (Figure 5.7) showed that the base of the broad singlet at 2.95 ppm widened out and showed distinct shoulders, *i.e.* approximately 10 Hz at each side of the singlet. This broad shoulder showed that the four H atoms at carbon 3 and 4 co-incidentally have similar ppm values.

A HSQC experiment is used to correlate the chemical shift of the protons with the chemical shift of the directly bonded carbon (Reynolds and Enriquez, 2001). This experiment utilises one-bond coupling. Only the directly bonded hydrogen and carbons will give cross peaks, and it will look like a series of concentric ellipses on the spectra. The quaternary carbons are not seen in the spectra. The HSQC spectrum (Figure 5.8) showed that the two CH$_2$ peaks at 27 ppm and 29 ppm ($^{13}$C n.m.r., Y-axis) corresponded to the broad singlet peak at 2.95 ppm ($^1$H n.m.r., X-axis).

A HMBC experiment is used to determine the multiple-bond couplings over two or three bonds (Reynolds and Enriquez, 2001). The cross peaks shown on the spectrum (Figure 5.9) are between protons and carbons that are two or three bonds away. The results from HMBC (Figure 5.9) highlighted a few major points:-

- Both CH$_2$ at 27 and 29 ppm correlated the four H atoms of the broad singlet.
- The CH$_2$ at 27 ppm correlated to the H atom of the alkene (H-9) (Figure 5.5).
- The CH$_2$ at 29 ppm correlated to the H-5 (6.6 ppm).
- If the 2-bromobenzaldehyde was attached to the $\beta$-carbon instead of $\alpha$-carbon of the tetralone, the two CH$_2$ should be correlated to H-9. Therefore, it is
conclusive that the product is 2-[1-(2-bromophenyl)methylidene]-6-methoxy-3,4-dihyronaphthalene-1-one (83).

- The H atom of the alkene (H-9, 7.8 ppm) correlated to the C=O (187 ppm). In this case, the carbonyl is 3 bonds away from H-9. If the 2-bromobenzaldehyde is attached to the β-carbon of the tetralone, the carbonyl will be 4 bonds away from the H-9, and it will not be observed in the HMBC.

A crystal structure of 2-[1-(2-bromophenyl)methylidene]-6-methoxy-3,4-dihyronaphthalene-1-one (83) was obtained to confirm the structure (Figure 5.6). Full crystal data for compound 83 can be seen in Appendix 1. Therefore, we could conclude that the four H atoms from the two CH₂ at carbon 3 and 4 appear as one broad singlet peak is due to accidental degeneracy.

**Figure 5.6.** The crystal structure of compound 83.
Figure 5.7. $^1$H n.m.r. (400 MHz) for compound 83.
Figure 5.8. HSQC spectrum for compound 83.
Figure 5.9: HMBE spectrum for compound 83.

Chapter 5
5.2 Experimental results for the synthesis of 6-substituted-2-(phenylmethylene)-3,4-dihyronaphthalene-1-one derivatives

The numbering of compounds for n.m.r. characterisation is as follows:

![Chemical structures]

6-Hydroxy-3,4-dihydro-2H-naphthalen-1-one (67) (Kirby *et al.*, 2003)

(C_{10}H_{10}O_{2}, MW: 162.19)

\[
\begin{align*}
\text{H}_2\text{C} & \quad \text{O} \\
\text{66} & \quad \xrightarrow{48 \% \text{ HBr (aq.)}} \\
\text{Refluxed, 8 hours} & \quad \text{O} \\
\text{67}
\end{align*}
\]

A solution of 6-methoxy-3,4-dihydro-2H-naphthalen-1-one (66) (20.0 g, 123.31 mmol) in 48 % HBr was heated under reflux for 8 hours. The aqueous layer was then evaporated to about 1/3 its volume. The precipitate formed was then recrystallised from water to give light brown solid, 6-hydroxy-3,4-dihydro-2H-naphthalen-1-one (67). Yield: 6.56 g (36 %), t.l.c. system petroleum ether – ethyl acetate 3:1 v/v, R_f: 0.32, stain positive. Melting point: 154 – 156 °C [literature: 156 – 157 °C] (Kirby *et al.*, 2003).

\[^1\text{H n.m.r. (DMSO-}\delta) \delta 10.25 (s, br, 1H, OH), 7.73 (d, J = 8.5 Hz, 1H, H-8), 6.70 (d, J = 8.5 Hz, 1H, H-7), 6.64 (s, 1H, H-5), 2.81 (t, J = 5.9 Hz, 2H, -CH_2-CH_2-CH_2-C=O), 2.47 \]
(t, J = 6.2 Hz, 2H, –CH₂-CH₂-CH₂-CH₂-C=O), 1.97 (quintet, J = 6.1 Hz, 2H, –CH₂-CH₂-
CH₂-CH₂-C=O).

¹³C n.m.r. (DMSO-d₆) δ 198.14 (C=O), 162.29 (C, C-6), 148.21 (C, C-8a), 129.55 (CH,
CH-8), 124.72 (C, C-4a), 114.64 (CH, CH-5), 114.61 (CH, CH-7), 38.56 (CH₂, –CH₂-
CH₂-CH₂-C=O), 29.40 (CH₂, –CH₂-CH₂-CH₂-C=O), 23.15 (CH₂, –CH₂-CH₂-CH₂-C=O).

6-(Tetrahydro-pyran-2-yloxy)-3,4-dihydro-2H-naphthalen-1-one (69) (Kirby et al.,
2003)

(C₁₅H₁₈O₃, MW: 246.30)

A mixture of 6-hydroxy-3,4-dihydro-2H-naphthalen-1-one (67) (6.0 g, 36.99 mmol),
3,4-dihydro-2H-pyran (68) (7.47 g, 88.78 mmol), acetic anhydride (0.5 mL) and p-
toluensulfonic acid (60 mg) in diethyl ether (150 mL) was stirred at room temperature
for 2.5 hours. The resulting brown suspension turned into a clear brown solution on
stirring at room temperature. The organic layer was then washed with 2 % KOH (2 × 50
mL) and water (2 × 50 mL), dried with MgSO₄, filtered and reduced in vacuo to give a
brown residue. The residue was recrystallised from ethanol to give, off-white solid, 6-
tetrahydro-pyran-2-yloxy)-3,4-dihydro-2H-naphthalen-1-one (69). Yield: 5.56 g (61
%), t.l.c. system petroleum ether – ethyl acetate 3:1 v/v, Rᵢ: 0.60, stain positive.
Melting point: 88 – 90 °C [literature: 90 – 92 °C] (Kirby et al., 2003).

¹H n.m.r. δ 7.98 (d, J = 8.7 Hz, 1H, H-8), 6.94 (dd, J = 2.2, 8.7 Hz, 1H, H-7), 6.88 (s,
1H, H-5), 5.50 (t, J = 2.8 Hz, 1H, CH-2”), 3.83 (m, 1H, H₆-H₆”), 3.61 (m, 1H, H₆-H₆-
”), 2.91 (t, J = 6.0 Hz, 2H, –CH₂-CH₂-CH₂-C=O), 2.58 (t, J = 6.2 Hz, 2H, –CH₂-CH₂-
CH₂-C=O), 2.09 (quintet, J = 6.3 Hz, 2H, –CH₂-CH₂-CH₂-C=O), 1.98 (m, 1H, H₆-H₆-
”), 1.86 (quintet, J = 3.2 Hz, 2H, CH₂-5”), 1.79 – 1.57 (m, 3H, CH₂-4” and H₆-H₆-
”).
\[ ^{13}\text{C n.m.r.} \delta 197.48 (\text{C} = \text{O}), \ 161.43 (\text{C}, \text{C-6}), \ 147.32 (\text{C}, \text{C-8}), \ 129.93 (\text{CH}, \text{CH}-8), \ 127.24 (\text{C}, \text{C-4}), \ 115.47 (\text{CH}, \text{CH}-5), \ 115.34 (\text{CH}, \text{CH}-7), \ 96.29 (\text{CH}, \text{CH}-2''), \ 62.37 (\text{CH}_2, \text{CH}_2-6''), \ 39.31 (\text{CH}_2, -\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{C} = \text{O}), \ 30.49 (2 \times \text{CH}_2, -\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{C} = \text{O} \text{ and } \text{CH}_2-3''), \ 25.47 (\text{CH}_2, \text{CH}_2-5''), \ 23.78 (\text{CH}_2, -\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{C} = \text{O}), \ 18.86 (\text{CH}_2, \text{CH}_2-4''). \]

4-(Tetrahydro-pyran-2-yl oxy)-benzaldehyde (71)

\[
\begin{align*}
\text{H} & \quad \text{OH} \\
\text{70} & \quad + \quad \text{68} \\
\text{PTSA, acetic anhydride} & \quad \xrightarrow{\text{Room temperature, 2 hours}} \\
\end{align*}
\]

A mixture of 4-hydroxy-benzaldehyde (70) (5.0 g, 40.94 mmol), 3,4-dihydro-2H-pyran (68) (8.27 g, 98.26 mmol), acetic anhydride (1 mL) and p-toluensulfonic acid (50 mg) in diethyl ether (150 mL) was stirred at room temperature for 2 hours. The resulting light brown suspension turned into a clear brown solution on stirring at room temperature. The organic layer was then washed with 2% KOH (2 × 100 mL) and water (2 × 100 mL), dried with MgSO₄, filtered and reduced in vacuo to give as a yellow residue. Purification by flash column chromatography (petroleum ether – ethyl acetate 100:0 v/v increasing to 87.5:12.5 v/v) gave 4-(tetrahydro-pyran-2-yl oxy)-benzaldehyde (71) as colourless syrup. Yield: 6.37 g (76%), t.l.c. system petroleum ether – ethyl acetate 3:1 v/v, Rₐ: 0.55, stain positive. LRMS (ES⁺) m/z Calculated for C₁₂H₁₄O₃ [M+Na]⁺ 230.1; Found 229.1.

\[ ^{1}\text{H n.m.r.} \delta 9.86 (\text{s, 1H, O=CH}), \ 7.79 (\text{dt, J = 1.9 Hz, 8.7 Hz, 2H, H-2' and H-6'}), \ 7.12 (\text{dt, J = 1.8, 8.7 Hz, 2H, H-3' and H-5'}), \ 5.51 (\text{t, J = 2.9 Hz, 1H, CH-2''}), \ 3.81 (\text{m, 1H, CH₃}_6-6''), \ 3.59 (\text{m, 1H, CH₃}_6-6'''), \ 2.00 – 1.89 (\text{m, 1H, CH₃}_6-4'''), \ 1.85 (\text{m, 2H, CH₃}_6-4'' and CH₃}_6-3'''), \ 1.75 – 1.53 (\text{m, 3H, CH₃}_6-3''' and CH₂-5'''). \]
\(^{13}\)C n.m.r. \(\delta\) 191.36 (C=O), 162.57 (C, C-4'), 132.25 (2 x CH, CH-2' and CH-6'), 130.88 (C, C-1), 116.92 (2 x CH, CH-3' and CH-5'), 96.54 (CH, CH-2''), 62.49 (CH\(_2\), CH\(_2\)-6''), 30.47 (CH\(_2\), CH\(_2\)-3''), 25.44 (CH\(_2\), CH\(_2\)-5''), 18.84 (CH\(_2\), CH\(_2\)-4'').

**Synthesis of 2-benzylidene-3,4-dihydro-2H-naphthalen-1-one (unsaturated tetralone) derivatives** (compound 78 – 96)

2-(4-Bromo-benzylidene)-6-(tetrahydro-pyran-2-vloxy)-3,4-dihydro-2H-naphthalen-1-one (78)

\((C_{22}H_{21}O_{2}Br, MW: 413.30)\)

```
\begin{align*}
\text{O} & \text{O} \\
\text{H} & \text{Br}
\end{align*}
```

\(4 \text{ % KOH/EtOH}

Room temperature, 1 hour

\(\text{O} \text{O}

5' 4'

3' 6'

78

A mixture of the 6-(tetrahydro-pyran-2-vloxy)-3,4-dihydro-2H-naphthalen-1-one (69) (5.0 g, 21.43 mmol) and 4-bromobenzaldehyde (72) (3.97 g, 21.43 mmol) in 4 % ethanolic KOH was stirred at room temperature for 1 h. The resulting precipitate was collected, washed with water and finally recrystallised from ethanol to give 2-(4-bromobenzylidene)-6-(tetrahydro-pyran-2-vloxy)-3,4-dihydro-2H-naphthalen-1-one (78) as white solid. Yield: 4.55 g (51 %), t.l.c. system petroleum ether – ethyl acetate 3:1 v/v, \(R_F\): 0.61, stain positive. Microanalysis \((C_{22}H_{21}BrO_{3}0.5H_2O)\): Calculated C = 62.57 %, H = 5.25 %; Found C = 62.84 %, H = 5.02 %. Melting point: 165 - 167 °C.

\(^1\)H n.m.r. \(\delta\) 8.06 (d, \(J = 8.7\) Hz, 1H, H-8), 7.70 (s, 1H, –CH\(_2\)-CH\(_2\)-C=CH–Phenyl), 7.49 (d, \(J = 8.4\) Hz, 2H, H-3' and H-5'), 7.25 (d, \(J = 8.4\) Hz, 2H, H-2' and H-6'), 6.97 (dd, \(J = 2.4, 8.7\) Hz, 1H, H-7), 6.84 (d, \(J = 2.2\) Hz, 1H, H-5), 5.50 (t, \(J = 2.9\) Hz, 1H, CH-2''), 3.83 (m, 1H, H\(_{a6}\)-6''), 3.60 (m, 1H, H\(_{a6}\)-6''), 3.02 (m, 2H, –CH\(_2\)-CH\(_2\)-C=CH–Phenyl), 2.88 (m, 2H, –CH\(_2\)-CH\(_2\)-C=CH–Phenyl), 1.96 (m, 1H, H\(_{a6}\)- 3''), 1.84 (quintet, \(J = 3.5\) Hz, 2H, CH\(_2\)-5''), 1.71 – 1.55 (m, 3H, CH\(_2\)-4'' and H\(_{a6}\)-3'').

\(^{13}\)C n.m.r. \(\delta\) 186.98 (C=O), 161.62 (C, C-6), 145.95 (C, C-4a), 136.72 (C, C-2), 135.34 (C, C-1'), 135.01 (CH, –CH\(_2\)-CH\(_2\)-C=CH–Phenyl), 132.05 (2 x CH, CH-3' and CH-5'), 131.75 (2 x CH, CH-2' and CH-6'), 131.05 (CH, CH-8), 127.85 (C, C-8a), 122.93 (C,
C-4’), 116.01 (CH, CH-5), 114.93 (CH, CH-7), 96.38 (CH, CH-2’’), 62.48 (CH₂, CH₂-6’’), 30.53 (CH₂, CH₂-3’’), 29.58 (CH₃, -CH₂-C=CH₂-C=O), 27.66 (CH₂, CH₂-5’’), 25.50 (CH₂, -CH₂-C=CH₂-C=O), 18.89 (CH₃, CH₂-4’’).

The following derivatives of 2-benzyldiene-3,4-dihydro-2H-naphthalen-1-one (unsaturated tetralone) (79 – 87) were prepared using the same general method detailed above.

2-[1-(2-bromophenyl)methylidene]-6-(tetrahydro-pyran-2-yloxy)-3,4-dihydro-2H-naphthalen-1-one (79)

(C₂₂H₃BrO₃, MW: 413.304)

With 2-[1-(2-bromophenyl)methylidene]-6-(tetrahydro-pyran-2-yloxy)-3,4-dihydro-2H-naphthalene-1-one (79), a white solid was obtained. Yield: 7.60 g (87 %), t.l.c. system petroleum ether – ethyl acetate 3:1 v/v, Rₑ: 0.66, stain positive. Microanalysis (C₂₂H₁₉BrO₃.0.5H₂O): Calculated C = 62.57 %, H = 5.25 %; Found C = 62.32 %, H = 4.98 %. Melting point 76 – 78 °C.

¹H n.m.r. δ 8.19 (d, J = 8.1 Hz, 1H, H-8), 7.85 (s, 1H, -CH₂-Ch₂-C=CH-Phenyl), 7.70 (d, J = 8.0 Hz, 1H, CH-3’’), 7.28 (m, 2H, phenyl), 7.27 (m, 2H, phenyl), 7.07 (dd, 1H, J = 2.4, 8.7 Hz, H-7), 6.94 (d, J = 2.3 Hz, H-5), 5.59 (t, J = 2.8 Hz, CH-2’’), 3.92 (m, 1H, CH₃H₂-6’’), 3.69 (m, 1H, CH₃H₂-6’’), 2.98 (s, br, 4H, –CH₂-Ch₂-C=CH-Phenyl and –CH₂-Ch₂-C=CH-Phenyl), 2.12 – 1.98 (m, 1H, CH₂H₂-3’’), 1.85 (m, 2H, CH₂-5’’), 1.75 – 1.53 (m, 3H, CH₃H₃-3’’ and CH₂-4’’).

¹³C n.m.r. δ 186.98 (C=O), 161.65 (C, C-6), 146.27 (C, C-4a), 137.54 (C, -CH₂-Ch₂-C=CH-Phenyl), 136.96 (C, C-1’), 135.39 (CH, -CH₂-Ch₂-C=CH-Phenyl), 133.33 (CH, CH-3’’), 131.05 (CH, CH-4’’), 130.88 (CH, CH-6’’), 129.99 (CH, CH-8), 127.84 (C, C-8a), 127.40 (CH, CH-5’’), 125.31 (C, C-2’), 116.01 (CH, CH-7), 115.00 (CH, CH-5), 96.36 (CH, CH-2’’), 62.47 (CH₂, CH₂-6’’), 30.53 (CH₂, CH₂-3’’), 29.84 (CH₃, -CH₂-Ch₂-C=CH-Phenyl), 27.78 (CH₂, -CH₂-Ch₂-C=CH-Phenyl), 25.50 (CH₂, CH₂-5’’), and 18.88 (CH₃, CH₂-4’’).
Reaction was stirred at room temperature for 12 h. With 6-(tetrahydro-pyran-2-yloxy)-
2-{1-[4-(tetrahydro-pyran-2-yloxy)phenyl]methyldiene}-3,4-dihydro-2H-naphthalen-1-one (80), a colourless syrup was obtained after purification by column chromatography
with petroleum ether – ethyl acetate 100:0 v/v increasing to 87:13 v/v. Yield: 1.00 g (41
%), t.l.c. system petroleum ether – ethyl acetate 4:1 v/v, Rf: 0.51, stain positive. HRMS
(ES') m/z Calculated for C27H30O5 [M+H]+ 435.2166; Found 435.2165.

1H n.m.r. δ 8.06 (d, J = 8.7 Hz, 1H, H-8), 7.77 (s, 1H, -CH2-CH2-C=CH-Phenyl), 7.36 (d, J = 8.7 Hz, 2H, H-3’ and H-5’), 7.05 (d, J = 8.7 Hz, 2H, H-2’ and H-6’), 6.96 (dd, J = 2.3, 8.7 Hz, 1H, H-7), 6.84 (d, J = 2.3 Hz, 1H, H-5), 5.49 (t, J = 2.9 Hz, 1H, CH-2’’),
5.44 (t, J = 3.1 Hz, 1H, CH-2’’’), 3.85 (m, 2H, H3Hb-6’’ and H3Hb-6’’’), 3.59 (m, 2H,
H4Hb-6’’’ and H4Hb-6’’’’), 3.08 (m, 2H, -CH2-CH2-C=CH-Phenyl), 2.86 (m, 2H, -CH2-
CH2-C=CH-Phenyl), 1.95 (m, 2H, H3Hb-3’’’ and H4Hb-3’’’’), 1.84 (m, 4H, CH2-5’’’ and
CH2-5’’’’), 1.73 – 1.55 (m, 6H, CH2-4’’, H3Hb-3’’, CH2-4’’’ and H4Hb-3’’’’).

13C n.m.r. δ 187.30 (C-O), 161.38 (C, C-6), 157.70 (C, C-4’’), 145.89 (C, C-4a), 136.42
(CH, -CH2-CH2-C=CH-Phenyl), 134.40 (C, C-2), 131.92 (2 x CH, CH-2’ and CH-6’),
130.91 (CH, CH-8), 129.78 (C, C-8a), 128.17 (C, C-1’), 116.71 (2 x CH, CH-3’ and
CH-5’), 115.83 (CH, CH-7), 114.86 (CH, CH-5), 96.66 (CH, CH-2’’), 96.37 (CH, CH-
2’’’), 62.50 (CH2, CH2-6’’), 62.47 (CH2, CH2-6’’’), 30.69 (CH2, CH2-3’’), 30.60 (CH2,
CH2-3’’’), 29.63 (CH2, -CH2-CH2-C=CH-Phenyl), 27.69 (CH2, -CH2-CH2-C=CH-
Phenyl), 25.59 (CH2, CH2-5’’), 25.52 (CH2, CH2-5’’’), 19.12 (CH2, CH2-4’’), 18.92
(CH2, CH2-4’’’).
6-Methoxy-2-{1-[4-(tetrahydro-pyran-2-yloxy)phenyl]methylidene}-3,4-dihydro-2\textit{H}-naphthalen-1-one (81)

(C_{23}H_{24}O_{4}, MW: 364.434)

\begin{center}
\text{66} + \text{HOC}_{\text{31}} \rightarrow \text{81}
\end{center}

Reaction was stirred at room temperature for 12 h. With 6-methoxy-2-{1-[4-(tetrahydro-pyran-2-yloxy)phenyl]methylidene}-3,4-dihydro-2\textit{H}-naphthalen-1-one (81), a light brown solid was obtained. Yield: 3.00 g (42 %), t.l.c. system petroleum ether – ethyl acetate 3:1 v/v, \textit{Rf}: 0.47, stain positive. Microanalysis (C_{23}H_{24}O_{4}): Calculated C = 75.80 %, H = 6.64 %; Found C = 75.63 %, H = 6.67 %. Melting point 100 – 102 °C.

\textsuperscript{1}H n.m.r. \delta 8.15 (d, J = 8.7 Hz, 1H, H-8), 7.85 (s, 1H, \text{–CH}_2-\text{CH}_2-C=CH–Phenyl), 7.44 (d, J = 8.7 Hz, 2H, H-3' and H-5'), 7.14 (d, J = 8.7 Hz, 2H, H-2' and H-6'), 6.92 (dd, J = 2.5, 8.7 Hz, 1H, H-7), 6.75 (d, J = 2.4 Hz, 1H, H-5), 5.52 (t, J = 3.0 Hz, 1H, \text{CH}-2''), 3.97 (m, 1H, H_{a}H_{b}-6''), 3.91 (s, 3H, \text{–O-C}_3H_3), 3.77 (m, 1H, H_{a}H_{b}-6''), 3.16 (m, 2H, \text{–CH}_2-\text{CH}_2-C=CH–Phenyl), 2.95 (m, 2H, \text{–CH}_2-\text{CH}_2-C=CH–Phenyl), 2.05 (m, 1H, H_{a}H_{b}-3'''), 1.93 (m, 2H, CH_{2}-5''), 1.81 – 1.63 (m, 3H, H_{a}H_{b}-3''' and CH_{2}-4''').

\textsuperscript{13}C n.m.r. \delta 187.22 (C=O), 163.88 (C, C-6), 157.71 (C, C-4'), 146.02 (C, C-4a), 136.42 (CH, \text{–CH}_2-\text{CH}_2-C=CH–Phenyl), 134.34 (C, C-2), 131.92 (2 x CH, \text{CH}-2' and \text{CH}-6'), 131.10 (CH, \text{CH}-8), 129.76 (C, C-8a), 127.62 (C, C-1'), 116.72 (2 x CH, \text{CH}-3' and \text{CH}-5'), 113.67 (CH, \text{CH}-7), 112.65 (CH, \text{CH}-5), 96.65 (CH, \text{CH}-2'''), 62.51 (CH_2, \text{CH}_2-6''), 55.87 (CH_3, \text{–O-C}_3H_3), 30.69 (CH_2, \text{CH}_2-3''), 29.67 (CH_2, \text{–CH}_2-\text{CH}_2-C=CH–Phenyl), 27.68 (CH_2, \text{–CH}_2-\text{CH}_2-C=CH–Phenyl), 25.59 (CH_2, \text{CH}_2-5''), 19.12 (CH_2, CH_2-4'').
2-[1-(4-Bromophenyl)methylidene]-6-methoxy-3,4-dihydro-2H-naphthalen-1-one (82)

(C_{18}H_{13}BrO_{2}, MW: 343.214)

![Chemical structure diagram]

With 2-[1-(4-bromophenyl)methylidene]-6-methoxy-3,4-dihydro-2H-naphthalen-1-one (82), a light yellow solid was obtained. Yield: 2.5 g (63 %), t.l.c. system petroleum ether – ethyl acetate 3:1 v/v, R_{F}: 0.6, stain positive. Microanalysis (C_{18}H_{13}BrO_{2}): Calculated C = 62.99 %, H = 4.40 %; Found C = 62.79 %, H = 4.54 %. Melting point 126 – 128 °C.

^{1}H n.m.r. δ 8.13 (d, J = 8.7 Hz, 1 H, H-8), 7.76 (s, 1H, -CH_{2}-CH_{2}-C=CH–Phenyl), 7.56 (d, J = 8.4 Hz, 2H, H-3’ and H-5’), 7.31 (d, J = 8.3 Hz, 2H, H-2’ and H-6’), 6.90 (dd, J = 2.3, 8.7 Hz, 1H, H-7), 6.73 (d, J = 2.1 Hz, 1H, H-5), 3.89 (s, 3H, -O-CH_{3}), 3.08 (m, 2H, -CH_{2}-CH_{2}-C=CH–Phenyl), 2.94 (m, 2H, -CH_{2}-CH_{2}-C=CH–Phenyl).

^{13}C n.m.r. δ 186.47 (C=O), 163.70 (C, C-6), 145.66 (C, C-4a), 136.25 (C, C-2), 134.92 (CH, -CH_{2}-CH_{2}-C=CH–Phenyl), 134.92 (C, C-1’), 131.64 (2 x CH, CH-2’ and CH-6’), 131.33 (2 x CH, CH-3’ and CH-5’), 130.83 (CH, CH-8), 126.91 (C, C-8a), 122.52 (C, C-4’), 113.45 (CH, CH-7), 112.32 (CH, CH-5), 55.49 (CH_{3}, -O-CH_{3}), 29.20 (CH_{2}, -CH_{2}-CH_{2}-C=CH–Phenyl), 27.23 (CH_{2}, -CH_{2}-CH_{2}-C=CH–Phenyl).
2-[1-(2-Bromophenyl)methylidene]-6-methoxy-3,4-dihydro-2H-naphthalen-1-one (83)

(C₁₈H₁₅BrO₂, MW: 343.214)

![Chemical structure](image)

With 2-[1-(2-bromophenyl)methylidene]-6-methoxy-3,4-dihydro-2H-naphthalen-1-one (83), a white crystal was obtained. Yield: 3.22 g (68%), t.l.c. system petroleum ether – ethyl acetate 3:1 v/v, R₇: 0.62, stain positive. Microanalysis (C₁₈H₁₅BrO₂): Calculated C = 62.99%, H = 4.40%; Found C = 63.19%, H = 4.41%. Melting point 78 – 80 °C.

¹H n.m.r. δ 8.19 (d, J = 8.7 Hz, 1H, H-8), 7.85 (s, 1H, –CH₂-CH₂-C=CH–Phenyl), 7.69 (d, J = 7.9, 1H, H-3’’), 7.37 (m, 2H, Ar), 7.25 (m, 1H, Ar), 6.93 (dd, J = 2.4, 8.7 Hz, 1H, H-7), 6.75 (d, J = 2.3 Hz, 1H, H-5), 3.92 (s, 3H, -O-CH₃), 2.92 (s, br, 4H, –CH₂-CH₂-C=CH–Phenyl and –CH₂-CH₂-C=CH–Phenyl).

¹³C n.m.r. δ 186.87 (C=O), 164.15 (C, C-6), 146.40 (C, C-4a), 137.47 (C, C-2), 136.94 (C, C-1’’), 135.38 (CH, –CH₂-CH₂-C=CH–Phenyl), 133.34 (CH, CH-3’’), 131.23 (CH, CH-6’’), 130.88 (CH, CH-4’’), 130.02 (CH, CH-8), 127.42 (CH, CH-5’’), 127.30 (C, C-8a), 125.31 (C, C-2’’), 113.91 (CH, CH-7), 112.78 (CH, CH-5), 55.92 (CH₃, -O-CH₃), 29.86 (CH₂, –CH₂-CH₂-C=CH–Phenyl), 27.77 (CH₂, –CH₂-CH₂-C=CH–Phenyl).
2-{1-[2,5-Di(trifluoromethyl)phenyl]methylidene}-6-methoxy-3,4-dihydro-2H-naphthalen-1-one (84)

(C\textsubscript{2}\textsubscript{0}H\textsubscript{14}F\textsubscript{6}O\textsubscript{2}, MW: 400.314)

\[
\begin{array}{c}
\text{H}_{3}\text{C-O} \\
\textbf{66}
\end{array}
\quad + 
\begin{array}{c}
\text{H} \\
\text{O} \\
\text{CF}_3
\end{array}
\begin{array}{c}
\text{H}_{3}\text{C-O} \\
\textbf{74}
\end{array}
\xrightarrow{\text{4\% KOH/EtOH}}
\begin{array}{c}
\text{H}_{3}\text{C-O} \\
\textbf{84}
\end{array}
\]

4 % KOH/EtOH
Room temperature, 1 hour

With 2-{1-[2,5-di(trifluoromethyl)phenyl]methylidene}-6-methoxy-3,4-dihydro-2H-naphthalen-1-one (84), a white solid was obtained. Yield: 0.40 g (24 %), t.l.c. system petroleum ether – ethyl acetate 3:1 v/v, R\textsubscript{F}: 0.69, stain positive. Melting point: 98 – 100 °C. Microanalysis (C\textsubscript{2}\textsubscript{0}H\textsubscript{14}F\textsubscript{6}O\textsubscript{2}): Calculated C = 60.01 %, H = 3.52 %, Found C = 59.93 %, H = 3.48 %.

\textsuperscript{1}H n.m.r. \( \delta \) 8.22 (d, J = 8.8 Hz, 1H, H-8), 7.96 (d, J = 8.3 Hz, 2H, H-3' and H-6'), 7.81 (d, J = 8.2 Hz, 1H, H-4'), 7.67 (s, 1H, -CH\textsubscript{2}-CH\textsubscript{2}-C=CH–Phenyl), 6.99 (d, J = 2.5, 8.8 Hz, 1H, H-7), 6.79 (d, J = 2.4 H, 1H, H -5), 3.97 (s, 3H, -O-CH\textsubscript{3}), 3.01 (m, 2H, -CH\textsubscript{2}-CH\textsubscript{2}-C=CH–Phenyl), 2.92 (m, 2H, -CH\textsubscript{2}-CH\textsubscript{2}-C=CH–Phenyl).

\textsuperscript{13}C n.m.r. \( \delta \) 186.12 (C=O), 164.39 (C, C-6), 146.35 (C, C-4a), 139.94 (C, C-1'), 136.81 (C, C-2), 134.35, 132.68 (C, C-2'), 131.37 (CH, CH-8), 130.84 (CH, -CH\textsubscript{2}-CH\textsubscript{2}-C=CH–Phenyl), 127.83, 127.78 (CH, CH-6'), 127.42, 127.21 (CH, CH-4'), 126.94 (C, C-5'), 125.43 (C, C-8a), 125.26, 125.22 (CH, CH-3'), 121.80 (C, CF\textsubscript{3}), 114.10 (CH, CH-7), 112.85 (CH, CH-5), 55.92 (CH\textsubscript{3}), 29.69 (CH\textsubscript{2}, -CH\textsubscript{2}-CH\textsubscript{3}H\textsubscript{B}-CH\textsubscript{x}(C=O)), 27.80 (CH\textsubscript{2}, -CH\textsubscript{2}-CH\textsubscript{3}H\textsubscript{B}-CH\textsubscript{x}(C=O)).

\textsuperscript{19}F n.m.r. \( \delta \) -61.72, -63.59 ppm.
6-Methoxy-2-[1-(2-methylphenyl)methylidene]-3,4-dihydro-2H-naphthalen-1-one (85)

(C_{19}H_{18}O_2, MW: 278.345)

\[
\text{H}_3\text{C}-\text{O} + \text{H} - \text{O} - \text{CH}_3 \\
\text{66} \quad \text{75} \\
4\% \text{ KOH/EtOH} \\
\text{Room temperature, 72 hours} \\
\text{H}_3\text{C}-\text{O} \quad \text{85}
\]

Reaction was stirred at room temperature for 72 h. The brown residue obtained was then purified by column chromatography with petroleum ether – ethyl acetate 100:0 v/v increasing to 87:13 v/v to give 6-methoxy-2-[1-(2-methylphenyl)methylidene]-3,4-dihydro-2H-naphthalen-1-one (85) as a yellow solid. Yield: 1.57 g (71%), t.l.c. system petroleum ether – ethyl acetate 3:1 v/v, \( R_f \): 0.65, stain positive. Microanalysis (C_{19}H_{18}O_2): Calculated C = 81.99%, H = 6.52%; Found C = 81.77%, H = 6.55%.

\(^1\)H n.m.r. \( \delta \) 8.21 (d, \( J = 8.7 \) Hz, 1H, H-8), 7.94 (s, 1H, –CH2-CH2-C=CH–Phenyl), 7.30 (m, 4H, Ar), 6.95 (dd, \( J = 2.4, 8.7 \) Hz, 1H, H-7), 6.76 (d, \( J = 2.2 \) Hz, 1H, H-5), 3.93 (s, 3H, –O-CH3), 2.98 (m, 4H, –CH2-CH2-C=CH–Phenyl and –CH2-CH2-C=CH–Phenyl), 2.40 (s, 3H, CH3).

\(^{13}\)C n.m.r. \( \delta \) 187.30 (C=O), 164.05 (C, C-6), 146.45 (C, C-4a), 138.16 (C, C-2'), 136.51 (C, C-2), 135.66 (C, C-1'), 135.45 (CH, –CH2-CH2-C=CH–Phenyl), 131.18 (CH, CH-3'), 130.63 (CH, CH-6'), 129.33 (CH, CH-4'), 128.76 (CH, CH-8), 127.48 (C, C-8a), 125.90 (CH, CH-5'), 113.82 (CH, CH-5), 112.77 (CH, CH-7), 55.89 (CH3, –O-CH3), 30.07 (CH2, –CH2-CH2-C=CH–Phenyl), 27.74 (CH2, –CH2-CH2-C=CH–Phenyl), 20.50 (CH3).
2-[1-[4-(Dimethylamino)phenyl]methylidene]-6-methoxy-3,4-dihydro-2H-naphthalen-1-one (86)

(C_{20}H_{21}NO_{2}, MW: 307.386)

\[
\begin{array}{c}
\text{H}_2\text{C}-\text{O} & \text{+} & \text{H}-\text{O} \\
\text{66} & \text{76} \\
\end{array}
\]

\[
\text{4\% KOH/EtOH} \quad \text{Room temperature, 24 hours} \quad \text{H}_2\text{C}-\text{O} \\
\]

\[
\text{86} \\
\]

Reaction was stirred at room temperature for 24 h. With 2-[1-[4-(dimethylamino)phenyl]methylidene]-6-methoxy-3,4-dihydro-2H-naphthalen-1-one (86), an orange crystal was obtained. Yield: 0.38 g (7%), t.l.c. system petroleum ether – ethyl acetate 3:1 v/v, R_f: 0.32, stain positive. Melting point: 146 - 148 °C.

Microanalysis (C_{20}H_{21}NO_{2}): Calculated C = 78.15 %, H = 6.89 %, N = 4.55 %; Found C = 78.26 %, H = 6.95 %, N = 4.53 %.

\(^1\)H n.m.r. \(\delta\) 8.16 (d, J = 8.7 Hz, 1H, H-8), 7.89 (s, 1H, -CH\textsubscript{2}-CH\textsubscript{2}-C=CH-Phenyl), 7.47 (d, J = 8.8 Hz, 2H, H-3' and H-5'), 6.92 (dd, J = 2.5, 8.7 Hz, 1H, H-7), 6.77 (m, 3H, H-2', H-6' and H-5), 3.91 (s, 3H, -O-CH\textsubscript{3}), 3.21 (t, J = 6.2 Hz, 2H, -CH\textsubscript{2}-CH\textsubscript{2}-C=CH-Phenyl), 2.95 (t, J = 6.2 Hz, 2H, -CH\textsubscript{2}-CH\textsubscript{2}-C=CH-Phenyl).

\(^13\)C n.m.r. \(\delta\) 187.18 (C=O), 163.65 (C, C-6), 150.90 (C, C-4'), 145.86 (C, C-4a), 137.60 (CH, -CH\textsubscript{2}-CH\textsubscript{2}-C=CH-Phenyl), 132.50 (2 x CH, CH-2' and CH-4'), 131.66 (C, C-2), 130.91 (CH, CH-8), 127.94 (C, C-8a), 124.17 (C, C-1'), 113.52 (CH, CH-7), 112.57 (CH, CH-5), 112.08 (2 x CH, CH-3' and CH-5'), 55.85 (CH\textsubscript{3}, -O-CH\textsubscript{3}), 40.60 (2 x CH\textsubscript{3}, -N(CH\textsubscript{3})CH\textsubscript{3}), 29.62 (CH\textsubscript{2}, -CH\textsubscript{2}-CH\textsubscript{2}-C=CH-Phenyl), 27.82 (CH\textsubscript{2}, -CH\textsubscript{2}-CH\textsubscript{2}-C=CH-Phenyl).
2-{1-[4-(Dimethylamino)phenyl][methylidene]-6-(tetrahydro-pyran-2-yloxy)-3,4-dihydro-2H-naphthalen-1-one (87)

\(\text{C}_{24}\text{H}_{27}\text{NO}_3, \text{MW: 377.476}\)

\[
\begin{align*}
\text{69} & \quad + \quad \text{76} \\
\text{4% KOH/EtOH} & \quad \text{Room temperature, 72 hours} \\
\text{5} & \quad \text{4} \\
\text{6} & \quad \text{3} \\
\text{87} & \quad \text{N} \quad \text{CH}_3
\end{align*}
\]

Reaction was stirred at room temperature for 72 h. The brown residue obtained was purified by column chromatography with dichloromethane – methanol 100:0 v/v increasing to 99:1 v/v to give \(2-{1-[4-(\text{dimethylamino})\text{phenyl}][\text{methylidene}]-6-(\text{tetrahydro-pyran-2-yloxy})-3,4\text{-dihydro-2H-naphthalen-1-one (87)}\) as a orange solid. Yield: 1.72 g (35%), t.l.c. system petroleum ether – ethyl acetate 3:1 v/v, \(R_F: 0.59\), stain positive. Microanalysis (\(\text{C}_{24}\text{H}_{27}\text{NO}_3\)): Calculated C = 76.36 %, H = 7.21 %, N = 3.71 %; Found C = 76.12 %, H = 7.23 %, N = 3.57 %.

\(^1\text{H n.m.r.}\ \delta \ 8.14 \ (d, J = 8.7 \text{ Hz}, 1\text{H}, \text{H-8}), \ 7.88 \ (s, 1\text{H}, -\text{CH}_2-\text{CH}_2-\text{C}=\text{CH}-\text{Phenyl}), \ 7.47 \ (d, J = 8.8 \text{ Hz}, 2\text{H}, \text{H-3’} \text{ and H-5’}), \ 7.05 \ (dd, J = 2.4, 8.7 \text{ Hz}, 1\text{H}, \text{H-7}), \ 6.93 \ (d, J = 2.3 \text{ Hz}, 1\text{H}, \text{H-5}), \ 6.77 \ (d, J = 8.8 \text{ Hz}, 2\text{H}, \text{H-2’} \text{ and H-6’}), \ 5.58 \ (t, J = 3.0 \text{ Hz}, 1\text{H}, \text{CH-2’’}),\ 3.94 \ (m, 1\text{H}, H_{a}, H_{b}-6’’), \ 3.69 \ (m, 1\text{H}, H_{a}, H_{a}H_{b}-6’’), \ 3.21 \ (m, 2\text{H}, -\text{CH}_2-\text{CH}_2-\text{C}=\text{CH}-\text{Phenyl}), \ 3.08 \ (s, 6\text{H}, -\text{N} (\text{CH}_3)\text{CH}_3), \ 2.95 \ (m, 2\text{H}, -\text{CH}_2-\text{CH}_2-\text{C}=\text{CH}-\text{Phenyl}), \ 2.06 \ (m, 1\text{H}, H_{a}H_{b}-3’’), \ 1.94 \ (m, 2\text{H}, -\text{CH}_2-\text{CH}_2-\text{C}=\text{CH}-\text{Phenyl}), \ 1.82 - 1.65 \ (m, 3\text{H}, \text{CH}_2-4’’ \text{ and H}_{a}H_{b}-3’’)).

\(^13\text{C n.m.r.}\ \delta \ 187.28 \ (\text{C}=\text{O}), \ 161.13 \ (C, \text{C-6}), \ 150.89 \ (C, \text{C-4’’}), \ 145.70 \ (C, \text{C-4a}), \ 137.59 \ (\text{CH}, -\text{CH}_2-\text{CH}_2-\text{C}=\text{CH}-\text{Phenyl}), \ 132.36 \ (2 \times \text{CH}, \text{CH-3’} \text{ and CH-5’}), \ 131.77 \ (C, \text{C-2}), \ 130.76 \ (\text{CH}, \text{CH-8}), \ 128.52 \ (C, \text{C-8a}), \ 124.24 \ (C, \text{C-1’}), \ 115.68 \ (\text{CH}, \text{CH-7}), \ 114.78 \ (\text{CH}, \text{CH-5}), \ 112.08 \ (2 \times \text{CH}, \text{CH-2’} \text{ and CH-6’}), \ 96.38 \ (\text{CH}, \text{CH-2’’}), \ 62.48 \ (\text{CH}_2, \text{CH}_2-6’’), \ 40.62 \ (2 \times \text{CH}_3, -\text{N} (\text{CH}_3)\text{CH}_3), \ 30.59 \ (\text{CH}_2, \text{CH}_2-3’’), \ 29.61 \ (\text{CH}_2, -\text{CH}_2-\text{CH}_2-\text{C}=\text{CH}-\text{Phenyl}), \ 27.83 \ (\text{CH}_2, -\text{CH}_2-\text{CH}_2-\text{C}=\text{CH}-\text{Phenyl}), \ 25.55 \ (\text{CH}_2, \text{CH}_2-5’’), \ 18.96 \ (\text{CH}_2, \text{CH}_2-4’’).
**2-Biphenyl-4-ylmethylen-6-(tetrahydro-pyran-2-yloxy)-3,4-dihydro-2H-naphthalen-1-one (89)**

(C₈H₂₂O₃, MW: 410.50)

![Chemical Structure](image)

1. Pd(PPh₃)₄, toluene, 80 °C, 5 hours
2. H₂O₂, room temperature, 1 hour

2M aqueous Na₂CO₃ (8.50 mL) was added to a solution of 2-(4-bromo-benzylidene)-6-(tetrahydro-pyran-2-yloxy)-3,4-dihydro-2H-naphthalen-1-one (78) (1.0 g, 2.42 mmol) in toluene (20 mL). The mixture was bubbled with nitrogen for one minute and then Pd(PPh₃)₄ (0.14 g, 0.12 mmol) was added to the mixture. Phenylboronic acid (88) (0.59 g, 4.84 mmol) in ethanol (5 mL) was added to the above mixture and the reaction was refluxed at 100 °C for 5 hours. After the reaction was complete, the residual borane was oxidised by the addition of H₂O₂ (30 %, 2.5 mL) at room temperature for 1 hour. The crude product was extracted with CH₂Cl₂ (100 mL) and water (3 x 100 mL). The organic layer was dried with MgSO₄, filtered and reduced in vacuo to give a light yellow oily residue. Purification by flash column chromatography (dichloromethane – methanol 100:0 v/v increasing to 99:1 v/v) gave a yellow residue. The residue was recrystallised with methanol to yield light yellow solid, 2-biphenyl-4-ylmethylen-6-(tetrahydropyran-2-yloxy)-3,4-dihydro-2H-naphthalen-1-one (89). Yield: 0.50 g (50 %), t. l. c. system: petroleum ether – ethyl acetate 3:1 v/v, Rₚ: 0.62, stain positive. Microanalysis (C₈H₂₂O₃): Calculated C = 81.92 %, H = 6.38 %; Found C = 81.95 %, H = 6.37 %. Melting point 116 – 118 °C.

¹H n.m.r. δ 8.17 (d, J = 8.7 Hz, 1H, H-8), 7.94 (s, 1H, –CH₂-CH₂-C=CH–Phenyl), 7.71 (m, 3H, Ar), 7.55 (m, 3H, Ar), 7.43 (m, 1H, Ar), 7.30 (m, 1H, Ar), 7.08 (dd, J = 2.4, 8.7 Hz, 1H, H-7), 6.96 (d, J = 2.4 Hz, 1H, H-5), 6.94 (m, 1H, Ar), 5.61 (t, J = 2.9 Hz, 1H, CH-2”), 3.94 (m, 1H, H₆H₆-6”), 3.70 (m, 1H, H- H₆H₆-6”), 3.23 (m, 2H, –CH₂-CH₂-C=CH–Phenyl), 3.00 (m, 2H, –CH₂-CH₂-C=CH–Phenyl), 2.08 (m, 1H, H₆H₆- 3”), 1.95 (m, 2H, CH₂-5”), 1.84 – 1.66 (m, 3H, CH₂-4” and H₆H₆- 3”).
13C n.m.r. δ 187.23 (C=O), 161.53 (C, C-6), 146.01 (C, C-4a), 141.61 (C, –CH2-CH2-C=CH–Phenyl), 140.88 (C, C-1''), 136.12 (C, C-4'), 136.07 (CH, –CH2-CH2-C=CH–Phenyl), 135.46 (C, C-1'), 131.03 (CH, CH-4''), 130.85 (2 x CH, Ar), 130.07 (2 x CH, Ar), 129.31 (CH, CH-8), 128.06 (CH, C-8a), 127.50 (4 x CH, Ar), 115.74 (CH, CH-7), 114.91 (CH, CH-5), 96.39 (CH, CH-2''), 62.49 (CH2, CH2-6''), 30.55 (CH2, CH2-3''), 29.69 (CH2, –CH2-CH2-C=CH–Phenyl), 27.81 (CH2, –CH2-CH2-C=CH–Phenyl), 25.52 (CH2, CH2-5''), 18.90 (CH2, CH2-4'').

2-Biphenyl-2-ylmethylene-6-(tetrahydro-pyran-2-yloxy)-3,4-dihydro-2H-naphthalen-1-one (90)

(C28H26O3, MW: 410.504)

\[
\begin{align*}
\text{79} & \quad \text{88} \\
\text{1. Pd(PPh3)4, toluene, 80 °C, 5 hours} \\
\text{2. H}_2\text{O}_2, \text{ room temperature, 1 hour} \\
\text{90}
\end{align*}
\]

2-biphenyl-2-ylmethylene-6-(tetrahydro-pyran-2-yloxy)-3,4-dihydro-2H-naphthalen-1-one (90) was prepared using the same method detailed above (compound 89). A light yellow solid was obtained. Yield: 1.30 g (87 %), t. l. c. system: petroleum ether – ethyl acetate 3:1 v/v, Rf: 0.64, stain positive. Microanalysis (C28H26O3.0.2H2O): Calculated C = 81.21 %, H = 6.43 %; Found C = 81.22 %, H = 6.37 %. Melting point 158 – 160 °C.

1H n.m.r. δ 8.15 (d, J = 8.7 Hz, 1H, H-8), 7.79 (s, 1H, –CH2-CH2-C=CH–Phenyl), 7.54 – 7.32 (m, 9H, Ar), 7.06 (dd, J = 2.2, 8.7 Hz, 1H, H-5), 6.94 (d, J = 2.2 Hz, 1H, H-7), 5.59 (t, J = 2.8 Hz, 1H, CH-2''), 3.93 (m, 1H, CHaHb-6''), 3.70 (m, 1H, CHaHb-6''), 2.97 (m, 2H, –CH2-CH2-C=CH–Phenyl), 2.88 (m, 2H, –CH2-CH2-C=CH–Phenyl), 2.13 – 2.02 (m, 1H, CHaHb-3''), 1.93 (m, 2H, CH2-5''), 1.84 – 1.65 (m, 3H, CHaHb-3'' and CH2-4'').

13C n.m.r. δ 187.08 (C=O), 161.51 (C, C-6), 146.28 (CH, Ar), 142.85 (C, C-2''), 141.05 (C, C-1''), 136.68 (CH, –CH2-CH2-C=CH–Phenyl), 136.20 (C, C-4a), 134.69 (C, C-1'), 130.97 (CH, Ar), 130.60 (C, C-2), 130.22 (CH, Ar), 130.04 (CH, CH-Ar), 129.94 (2 x CH, CH-2'' and CH-6'''), 128.93 (CH, Ar), 128.67 (2 x CH, CH-3''' and CH-5'''), 128.32 (CH, Ar), 127.98 (C, C-8a), 127.80 (CH, CH-8), 127.35 (CH, CH-3'), 115.93
(CH, CH-5), 114.99 (CH, CH-7), 96.37 (CH, CH-2’), 62.47 (CH₂, CH₂-6’’), 30.56 (CH₂, CH₂-3’’), 29.78 (CH, -CH₂CH₂C=CH-Phenyl), 27.86 (CH₂, -CH₂CH₂C=CH-Phenyl), 25.54 (CH₂, CH₂-5’’), 18.92 (CH₂, CH₂-4’’).

2-[1-(4-Hydroxyphenyl)methylidene]-6-methoxy-3,4-dihydro-2H-naphthalen-1-one (91)

(C₁₈H₁₆O₃, MW: 280.318)

**Method 1**

Aqueous hydrochloric acid (2 M, 15 mL) was added to a solution of 6-methoxy-2-{1-[4-(tetrahydro-pyranyl-2-yloxy)phenyl]methylidene}-3,4-dihydro-2H-naphthalen-1-one (81) (2 g, 5.49 mmol), in 2-butanol-ethyl acetate (1/1 v/v, 40 mL), and the mixture stirred at 100 °C for 1 h. The yellow solution was evaporated in vacuo and the resulting yellow solid was washed with water-methanol (2/1 v/v, 40 mL) to give 2-[1-(4-hydroxyphenyl)methylidene]-6-methoxy-3,4-dihydro-2H-naphthalen-1-one (91), as yellow powder. Yield: 1.39 g (90 %), t. l. c. system: petroleum ether – ethyl acetate 3:1 v/v, Rf: 0.22, stain positive. Microanalysis (C₁₈H₁₆O₃.0.5H₂O): Calculated C = 74.55 %, H = 5.66 %; Found C = 74.72 %, H = 5.92 %.

¹H n.m.r. (DMSO-d₆) δ 9.92 (s, br, 1H, OH), 7.92 (d, J = 8.6 Hz, 1H, H-8), 7.62 (s, 1H, -CH₂CH₂C=CH-Phenyl), 7.40 (d, J = 8.4 Hz, 2H, Ar), 6.93 (m, 2H, Ar), 6.86 (d, J = 8.3 Hz, 2H, H-7 and H-5), 3.85 (s, 3H, CH₃), 3.06 (t, J = 6.0 Hz, 2H, -CH₂CH₂C=CH-Phenyl), 2.90 (t, J = 3.0 Hz, 2H, -CH₂CH₂C=CH-Phenyl).

¹³C n.m.r. (DMSO-d₆) δ 185.72 (C=O), 163.43 (C, C-6), 158.58 (C, C-4’), 146.06 (C, C-4a), 135.89 (CH, -CH₂CH₂C=CH-Phenyl), 132.87 (C, C-2), 132.32 (2 x CH, CH-2’ and CH-6’), 130.13 (CH, CH-8), 126.88 (C, C-8a), 126.57 (C, C-1’), 115.84 (2 x CH, CH-3’ and CH-5’), 113.93 (CH, CH-7), 112.57 (CH, CH-5), 55.86 (CH₃, -O-CH₃), 28.59 (CH₂, -CH₂CH₂C=CH-Phenyl), 27.09 (CH₂, -CH₂CH₂C=CH-Phenyl).
Method 2 (Yoshihama et al., 1999)

\[
\begin{align*}
\text{H}_2\text{C}-\text{O} & \quad + \quad \text{H} \quad \text{O} \\
66 & \quad 77 \\
\text{50:70 v/v HCl conc : MeOH} & \\
\text{100 °C, 2.5 hours} & \quad \rightarrow \\
\text{H}_2\text{C}-\text{O} & \quad \text{OH} \\
92
\end{align*}
\]

After a mixture of concentrated hydrochloric acid (50 mL) and methanol (70 mL) were added to a mixture of 6-methoxy-3,4-dihydro-2H-naphthalen-1-one (66) (1.50 g, 8.51 mmol) and 4-hydroxybenzaldehyde (77) (1.10 g, 9.00 mmol), the mixture was refluxed for 2.5 hours and cooled to room temperature. 100 mL of water was added and allowed to stand for one hour. The precipitated solid was filtered. The solid was then recrystallised from acetone to yield 2-[1-(4-hydroxyphenyl)methylene]-6-methoxy-3,4-dihydro-2H-naphthalen-1-one (91) as yellow solid. Yield: 0.63 g (26 %). t. l. c. system: petroleum ether – ethyl acetate 3:1 v/v, Rf: 0.22, stain positive.

\(^1\text{H} \) n.m.r. data same as above.

The following derivatives of 2-benzylidene-3,4-dihydro-2H-naphthalen-1-one (unsaturated tetralone) (92 - 96) were prepared using the same general method detailed in method 1 for compound 91.

6-Hydroxy-2-[1-(4-hydroxyphenyl)methylene]-3,4-dihydro-2H-naphthalen-1-one (92)

(C\(_{17}\)H\(_{14}\)O\(_3\), MW: 266.291)

\[
\begin{align*}
\text{H}_2\text{C}-\text{O} & \quad \text{OH} \\
81 & \quad 2 \text{ M HCl} \\
\text{Refluxed, 1 hours} & \quad \rightarrow \\
\text{H}_2\text{C}-\text{O} & \quad \text{OH} \\
92
\end{align*}
\]

With 6-hydroxy-2-[1-(4-hydroxyphenyl)methylene]-3,4-dihydro-2H-naphthalen-1-one (92), a yellow powder was obtained. Yield: 0.67 g (55 %), t. l. c. system: petroleum ether – ethyl acetate 3:1 v/v, Rf: 0.05, stain positive. Microanalysis (C\(_{17}\)H\(_{14}\)O\(_3\),0.1H\(_2\)O):

Calculated C = 76.04 %, H = 5.29 %; Found C = 76.16 %, H = 5.34 %. Melting point: 260 – 262 °C.
Chapter 5

\(^1\)H n.m.r. (DMSO-\(d_6\)) \(\delta\) 10.38 (s, 1H, OH), 9.90 (s, 1H, OH), 7.85 (d, J = 8.5 Hz, 1H, H-8), 7.59 (s, 1H, \(-\text{CH}_2\text{-CH}_2\text{-C}=\text{CH-Phenyl}\)), 7.38 (d, J = 8.4 Hz, 2H, Ar), 6.86 (d, J = 8.3 Hz, 2H, Ar), 6.78 (dd, J = 7.1, 8.5 Hz, 1H, H-7), 6.68 (s, br, 1H, H-5), 3.04 (m, 2H, \(-\text{CH}_2\text{-CH}_2\text{-C}=\text{CH-Phenyl}\)), 2.83 (m, 2H, \(-\text{CH}_2\text{-CH}_2\text{-C}=\text{CH-Phenyl}\)).

\(^{13}\)C n.m.r. (DMSO-\(d_6\)) \(\delta\) 185.57 (C=O), 162.38 (C, C-6), 158.47 (C, C-4'), 146.10 (C, C-4a), 135.47 (CH, \(-\text{CH}_2\text{-CH}_2\text{-C}=\text{CH-Phenyl}\)), 133.15 (C, \(-\text{CH}_2\text{-CH}_2\text{-C}=\text{CH-Phenyl}\)), 132.23 (2 x CH, CH-3' and CH-5'), 130.49 (CH, CH-8), 126.67 (C, C-8a), 125.68 (C, C-1'), 115.82 (2 x CH, CH-2' and CH-6'), 114.96 (CH, CH-7), 114.20 (CH, CH-5), 28.53 (CH\(_2\), \(-\text{CH}_2\text{-CH}_2\text{-C}=\text{CH-Phenyl}\)), 27.12 (CH\(_2\), \(-\text{CH}_2\text{-CH}_2\text{-C}=\text{CH-Phenyl}\)).

**2-(1-(2-Bromophenyl)methylidene)-6-hydroxy-3,4-dihydro-2\(H\)-naphthalen-1-one** (93)

(C\(_{17}\)H\(_{13}\)BrO\(_2\), MW: 329.188)

With 2-[1-(2-bromophenyl)methylidene]-6-hydroxy-3,4-dihydro-2\(H\)-naphthalen-1-one (93), a white solid was obtained. Yield: 0.60 g (63 %), t. l. c. system: petroleum ether - ethyl acetate 3:1 v/v, R\(_f\): 0.20, stain positive. Microanalysis (C\(_{17}\)H\(_{13}\)BrO\(_2\)): Calculated C = 62.03 %, H = 3.98 %; Found C = 62.03 %, H = 4.05 %. Melting point: 178 – 180 °C.

\(^1\)H n.m.r. (DMSO-\(d_6\)) \(\delta\) 10.51 (s, 1H, OH), 7.90 (d, J = 8.5 Hz, 1H, H-8), 7.74 (d, J = 7.7 Hz, 1H, Ar), 7.60 (s, 1H, \(-\text{CH}_2\text{-CH}_2\text{-C}=\text{CH-Phenyl}\)), 7.47 (d, J = 2.3, 2H, Ar), 7.34 (s, br, 1H, Ar), 6.81 (d, J = 8.5 Hz, 1H, H-7), 6.69 (s, br, 1H, H-5), 2.87 (s, br, 4H, \(-\text{CH}_2\text{-CH}_2\text{-C}=\text{CH-Phenyl} and \(-\text{CH}_2\text{-CH}_2\text{-C}=\text{CH-Phenyl}\)).

\(^{13}\)C n.m.r. (DMSO-\(d_6\)) \(\delta\) 185.37 (C=O), 162.87 (C, C-6), 146.66 (C, C-4a), 137.72 (C, \(-\text{CH}_2\text{-CH}_2\text{-C}=\text{CH-Phenyl}\)), 135.88 (C, C-1'), 133.44 (CH, \(-\text{CH}_2\text{-CH}_2\text{-C}=\text{CH-Phenyl}\)), 133.08 (CH, CH-3'), 131.15 (CH, CH-4'), 130.74 (CH, CH-6'), 130.63 (CH, CH-8), 127.97 (CH, CH-5'), 125.23 (C, C-8a), 124.56 (C, C-2'), 115.27 (CH, CH-7), 114.42 (CH, CH-5), 28.66 (CH\(_2\), \(-\text{CH}_2\text{-CH}_2\text{-C}=\text{CH-Phenyl}\)), 27.04 (CH\(_2\), \(-\text{CH}_2\text{-CH}_2\text{-C}=\text{CH-Phenyl}\)).
2-{1-[4-(Dimethylamino)phenyl]methylidene}-6-hydroxy-3,4-dihydro-2H-naphthalen-1-one (94)

(C_{19}H_{19}NO_{2}, MW: 293.360)

With 2-{1-[4-(dimethylamino)phenyl]methylidene}-6-hydroxy-3,4-dihydro-2H-naphthalen-1-one (94), a yellow solid was obtained. Yield: 0.82 g (70 %), t. l. c. system: petroleum ether – ethyl acetate 3:1 v/v, R_{f}: 0.05, stain positive. Melting point: 210 – 212 °C.

{\textsuperscript{1}}H n.m.r. (DMSO-\textit{d}_6) \delta 9.03 (s, br, 1H, OH), 7.84 (d, J = 8.6 Hz, 1H, H-8), 7.61 (s, 1H, –CH\textsubscript{2}-CH\textsubscript{2}-C=CH–Phenyl), 7.56 (d, J = 8.6 Hz, 2H, Ar), 7.44 (s, br, 2H, Ar), 6.81 (dd, J = 2.3, 8.6 Hz, 1H, H-7), 6.70 (d, J = 2.1 Hz, 1H, H-5), 3.08 (s, 6H, 2 x CH\textsubscript{3}), 3.01 (m, 2H, –CH\textsubscript{2}-CH\textsubscript{2}-C=CH–Phenyl), 2.82 (m, 2H, –CH\textsubscript{2}-CH\textsubscript{2}-C=CH–Phenyl) (Yoshihama et al., 1999).

{\textsuperscript{13}}C n.m.r. (DMSO-\textit{d}_6) \delta 185.41 (C=O), 162.65 (C, C-6), 146.17 (2 x C, C-4a and C-4’), 134.38 (CH, –CH\textsubscript{2}-CH\textsubscript{2}-C=CH–Phenyl), 133.38 (C, –CH\textsubscript{2}-CH\textsubscript{2}-C=CH–Phenyl), 131.76 (2 x CH, Ar), 130.51 (CH, CH-8), 125.48 (C, C-8a), 123.8 (C, C-1’), 117.50 (2 x CH, Ar), 115.11 (CH, CH-5), 114.26 (CH, CH-7), 43.36 (2 x CH\textsubscript{3}), 28.52 (CH\textsubscript{2}, –CH\textsubscript{2}-CH\textsubscript{2}-C=CH–Phenyl), 27.12 (CH\textsubscript{2}, –CH\textsubscript{2}-CH\textsubscript{2}-C=CH–Phenyl).
2-Biphenyl-4-ylmethylene-6-hydroxy-3,4-dihydro-2H-naphthalen-1-one (95)
(C_{23}H_{18}O_2, MW: 326.388)

With 2-biphenyl-4-ylmethylene-6-hydroxy-3,4-dihydro-2H-naphthalen-1-one (95), a yellow solid was obtained. Yield: 0.38 g (95 %), t. l. c. system: petroleum ether – ethyl acetate 3:1 v/v, R_f: 0.05, stain positive. Microanalysis (C_{23}H_{18}O_2): Calculated C = 84.64 \%, H = 5.56 \%; Found C = 84.71 \%, H = 5.51 \%. Melting point: 258 – 260 °C.

^1^H n.m.r. (DMSO-d_6) δ 10.47 (s, br, 1H, OH), 7.89 (d, J = 8.5 Hz, 1H, H-8), 7.75 (m, 4H, Ar), 7.62 (d, J = 8.1 Hz, 2H, Ar), 7.50 (m, 2H, Ar), 7.41 (m, 1H, Ar), 6.80 (dd, J = 1.7, 8.6 Hz, 1H, H-7), 6.70 (s, br, 1H, H-5), 3.10 (t, J = 5.9 Hz, 2H, –CH_2–CH_2–C=CH–Phenyl), 2.87 (t, J = 6.1 Hz, 2H, –CH_2–CH_2–C=CH–Phenyl).

^13^C n.m.r. (DMSO-d_6) δ 185.54 (C=O), 162.68 (C, C-6), 146.39 (C, C-1’’’’), 140.48 (C, C-2’’’’), 139.79 (C, C-4a), 136.34 (C, –CH_2–CH_2–C=CH–Phenyl), 134.98 (C, C-1’’’), 134.44 (CH, –CH_2–CH_2–C=CH–Phenyl), 130.86 (2 x CH, CH-3’’’ and CH-5’’’’), 130.65 (CH, Ar), 129.39 (2 x CH, CH-2’’’ and CH-6’’’’), 128.16 (CH, Ar), 127.04 (CH, CH-8), 127.04 (2 x CH, CH-3’’ and CH-5’’), 125.46 (C, C-8a), 115.14 (CH, CH-5), 114.30 (CH, CH-7), 28.58 (CH_2, –CH_2–CH_2–C=CH–Phenyl), 27.23 (CH_2, –CH_2–CH_2–C=CH–Phenyl).
2-Biphenyl-2-ylmethylene-6-hydroxy-3,4-dihydro-2H-naphthalen-1-one (96)

(C$_{23}$H$_{18}$O$_{2}$, MW: 326.388)

With 2-biphenyl-2-ylmethylene-6-hydroxy-3,4-dihydro-2H-naphthalen-1-one (96), a yellow solid was obtained. Yield: 0.86 g (91 %), t. l. c. system: petroleum ether – ethyl acetate 3:1 v/v, R$_f$: 0.05, stain positive. Microanalysis (C$_{23}$H$_{18}$O$_{2}$): Calculated C = 84.64 %, H = 5.56 %; Found C = 84.44 %, H = 5.51 %. Melting point: 221 – 223 °C.

$^1$H n.m.r. (DMSO-$d_6$) $\delta$ 10.46 (s, br, 1H, OH), 7.81 (d, J = 8.5 Hz, 1H, H-8), 7.41 (m, 10H, Ar), 6.78 (dd, J = 1.5, 8.5 Hz, 1H, H-7), 6.68 (s, 1H, H-5), 2.88 (m, 2H, –CH$_2$-CH$_2$-C=CH–Phenyl), 2.80 (m, 2H, –CH$_2$-CH$_2$-C=CH–Phenyl).

$^{13}$C n.m.r (DMSO-$d_6$) $\delta$ 185.48 (C=O), 162.69 (C, C-6), 146.60 (C, C-2'), 142.09 (C, C-1''), 140.48 (C, C-4a), 136.15 (C, C-1'), 134.77 (CH, –CH$_2$-CH$_2$-C=CH–Phenyl), 133.84 (C, –CH$_2$-CH$_2$-C=CH–Phenyl), 130.60 (CH, Ar), 130.29 (2 x CH, CH-2''' and CH-6'''), 130.02 (CH, Ar), 129.82 (CH, Ar), 129.09 (CH, Ar), 128.63 (2 x CH, CH-3''' and CH-5'''), 127.77 (CH, Ar), 127.57 (CH, CH-8), 125.34 (C, C-8a), 115.12 (CH, CH-5), 114.34 (CH, CH-7), 28.66 (CH$_2$, –CH$_2$-CH$_2$-C=CH–Phenyl), 27.21 (CH$_2$, –CH$_2$-CH$_2$-C=CH–Phenyl).
4-[(6-Methoxy-3,4-dihydro-2H-naphthalenyl)methyl]phenol (97)

(C_{18}H_{20}O_{2}, MW: 268.35)

A mixture of 2-[[1-(4-hydroxyphenyl)methylidene]-6-methoxy-3,4-dihydro-2H-naphthalen-1-one (91) (1.0 g, 3.56 mmol) and 10 % palladium on charcoal (75 mg) in methanol (200 mL), was shaken in an atmosphere of hydrogen at room temperature for 2 h. Palladium was removed by filtration over a bed of celite and the filtrate was concentrated in vacuo to give 4-[(6-methoxy-3,4-dihydro-2H-naphthalenyl)methyl]phenol (97), as white solid. Yield: 0.60 g (63 %), t. l. c. system: petroleum ether – ethyl acetate 3:1 v/v, R_f: 0.61, stain positive. Melting point: 84 – 86 °C. Microanalysis (C_{18}H_{20}O_{2}): Calculated C = 80.56 %, H = 7.51 %; Found C = 80.27 %, H = 7.51 %.

^{1}H n.m.r. δ 7.09 (d, J = 8.3 Hz, 2H, Ar), 6.96 (d, J = 8.3 Hz, 1H, H-8), 6.80 (d, J = 8.3 Hz, 2H, Ar), 6.69 (dd, J = 2.6, 8.3 Hz, 1H, H-7), 6.64 (d, J = 2.3 Hz, 1H, H-7), 3.79 (s, 3H, –O-CH_{3}), 2.78 (m, 3H, –CH_{2}-CH_{A}H_{B}-CH_{x}-CH_{A}H_{B}-Phenyl and CH_{2}-CH_{A}H_{B}-CH_{x}-CH_{A}H_{B}-Phenyl), 2.75 – 2.70 (s, br, 1H, OH), 2.61 (d, J = 7.1 Hz, 2H, –CH_{2}-1), 2.40 (dd, J = 10.5, 16.1 Hz, 1H, –CH_{2}-CH_{A}H_{B}-CH_{x}-CH_{A}H_{B}-Phenyl), 2.02 – 1.92 (m, 2H, –CH_{2}-CH_{A}H_{B}-CH_{x}-CH_{A}H_{B}-Phenyl and –CH_{2}-CH_{A}H_{B}-CH_{x}-CH_{A}H_{B}-Phenyl), 1.45 (m, 1H, –CH_{2}-CH_{A}H_{B}-CH_{x}-CH_{A}H_{B}-Phenyl).

^{13}C n.m.r. δ 157.79 (C, C-6), 154.19 (C, C-4'), 138.40 (C, C-4a), 133.41 (C, C-1'), 130.74 (2 x CH, CH-2’ and CH-6’), 130.36 (CH, CH-8), 129.48 (C, C-8a), 115.70 (2 x CH, CH-3’ and CH-5’), 114.06 (CH, CH-5), 112.42 (CH, CH-7), 55.87 (CH_{3}, –O-CH_{3}), 42.47 (CH_{2}, –CH_{2}-CH_{A}H_{B}-CH_{x}-CH_{A}H_{B}-Phenyl), 37.10 (CH, CH_{A}), 35.62 (CH_{2}, –CH_{2}-1), 29.92 (CH_{2}, –CH_{2}-CH_{A}H_{B}-CH_{x}-CH_{A}H_{B}-Phenyl), 29.50 (CH_{2}, –CH_{2}-CH_{A}H_{B}-CH_{x}-CH_{A}H_{B}-Phenyl).
Synthesis of saturated 3,4-dihydro-2H-naphthalen-1-one (saturated tetralone) derivatives (compound 98–104)

6-(Hydroxy-2-(4-hydroxybenzyl)-3,4-dihydro-2H-naphthalen-1-one (98)

(C₁₇H₁₆O₃, MW: 268.307)

A mixture of 6-hydroxy-2-[1-(4-hydroxyphenyl)methylidene]-3,4-dihydro-2H-naphthalen-1-one (92) (0.50 g, 1.88 mmol) and 10% palladium on charcoal (50 mg) in methanol (150 mL), was shaken in an atmosphere of hydrogen at room temperature for 1 hour. Palladium was removed by filtration over a bed of celite and the filtrate was concentrated in vacuo to give yellow syrup which was fractionated on a column of dry silica with dichloromethane – methanol 100:0 v/v increasing to 97:3 v/v. The fraction was evaporated to give crude white fluffy solid. The white fluffy solid was then triturated with acetone to give 6-(hydroxy-2-(4-hydroxybenzyl)-3,4-dihydro-2H-naphthalen-1-one (98) as a light brown solid. Yield: 30 mg (6%), t. l. c. system: petroleum ether – ethyl acetate 1:1 v/v, Rf: 0.05, stain positive. Microanalysis (C₁₇H₁₆O₃): Calculated C = 76.10%, H = 6.01%; Found C = 76.18%, H = 6.07%. Melting point: 189 – 191 °C.

¹H n.m.r. (DMSO-d₆) δ 10.27 (s, br, 1H, OH), 10.22 (s, br, 1H, OH), 7.80 (d, J = 8.6 Hz, 1H, H-8), 7.02 (d, J = 8.4 Hz, 2H, H-3’ and H-5’), 6.74 (dd, J = 2.4, 8.6 Hz, 1H, H-7), 6.70 (d, J = 8.4 Hz, 2H, H-2’ and H-6’), 6.63 (d, J = 2.4 Hz, 1H, H-5), 3.15 (dd, J = 4.0, 13.2 Hz, 1H, −CH₂−CH₃−CH₃−CH₃−CH₃−Phenyl), 2.83 (m, 2H, −CH₂−CH₃−CH₃−CH₃−(C=O)), 2.69-2.59 (m, 1H, −CH₂−CH₃−CH₃−(C=O)), 2.53 (m, 1H, −CH₂−CH₃−CH₃−Phenyl), 1.97 – 1.89 (m, 1H, −CH₂−CH₃−CH₃−(C=O)), 1.67 – 1.54 (m, 1H, −CH₂−CH₃−CH₃−(C=O)).

¹³C n.m.r. (DMSO-d₆) δ 197.51 (C=O), 162.33 (C, C-6), 155.84 (C, C-4'), 147.16 (C, C-4a), 130.43 (2 x CH, CH-2' and CH-6'), 130.26 (C, C-1'), 129.73 (CH, CH-8), 124.71 (C, C-8a), 115.36 (2 x CH, CH-3' and CH-5'), 114.70 (CH, CH-5), 114.45 (CH,
CH-7, 48.63 (CH2-CH2-CH=CH=CHx-(C=O)), 34.63 (CH2-CH2-CH=CHx-(C=O)), 28.31 (CH2-CH2-CH=CHx-(C=O)), 27.53 (CH2-CH2-CHx-CH2-Phenyl).

The following derivatives of saturated 3,4-dihydro-2H-naphthalen-1-one (saturated tetralone) (compound 99 – 104) were prepared using the same general method detailed above.

2-(4-Hydroxybenzyl)-6-methoxy-3,4-dihydro-2H-naphthalen-1-one (99)

(C18H16O3, MW: 280.318)

With 2-(4-hydroxybenzyl)-6-methoxy-3,4-dihydro-2H-naphthalen-1-one (99), a hygroscopic solid was obtained after trituration with acetone. Yield: 0.34 g (57 %), t. l. c. system: petroleum ether – ethyl acetate 1:1 v/v, Rf: 0.30, stain positive. Microanalysis (C18H16O3.0.3H2O): Calculated C = 75.27 %, H = 6.36 %; Found C = 75.14 %, H = 6.52 %.

1H n.m.r. δ 8.06 (d, J = 8.8 Hz, 1H, H-8), 7.08 (d, 2H, J = 8.4 Hz, Ar), 6.83 (m, 3H, Ar and OH), 6.68 (d, J = 2.3 Hz, 1H, H-7), 6.49 (s, br, 1H, H-5), 3.86 (s, 3H, 1-O-CH3), 3.37 (dd, J = 3.1, 12.9 Hz, 1H, -CH2-CH=CH2-CH=CHx-(C=O)), 2.90 (m, 2H, -CH2-CH=CH2-CH=CHx-Phenyl), 2.65 (m, 2H, -CH2-CH=CH2-CH=CHx-(C=O)), 2.17 – 2.04 (m, 1H, -CH2-CH=CH2-CH=CHx-(C=O)), 1.83 – 1.72 (m, 1H, -CH2-CH=CH2-CH=CHx-(C=O)).

13C n.m.r. δ 199.78 (C=O), 164.10 (C, C-6), 154.85 (C, C-4'), 147.27 (C, C-4a), 132.02 (C, C-1'), 130.74 (2 x CH, CH-2' and CH-6'), 130.53 (CH, CH-8), 126.40 (C, C-8a), 115.80 (2 x CH, CH-3' and CH-5'), 113.72 (CH, CH-5), 112.94 (CH, CH-7), 55.73 (CH, -CH2=CH2=CHx-(C=O)), 49.64 (CH3), 35.30 (CH2, -CH2=CH2=CHx-CH2-Phenyl), 29.17 (CH2, -CH2=CH2=CHx-(C=O)), 27.89 (CH2, -CH2=CH2=CHx-(C=O)).
2-[4-(Dimethylamino)benzyl]-6-hydroxy-3,4-dihydro-2H-naphthalen-1-one (100)

(C\textsubscript{19}H\textsubscript{21}NO\textsubscript{2}, MW: 295.38)

With 2-[4-(dimethylamino)benzyl]-6-hydroxy-3,4-dihydro-2H-naphthalen-1-one (100), a brown solid was obtained after trituration with acetone. Yield: 42 mg (6 %), t. l. c. system: petroleum ether – ethyl acetate 3:1 v/v, R\_F: 0.10, stain positive. Melting point: 200 – 202 °C. Microanalysis (C\textsubscript{19}H\textsubscript{21}NO\textsubscript{2}.0.1H\textsubscript{2}O): Calculated C = 76.85 %, H = 7.25 %, N = 4.62 %; Found C = 76.79 %, H = 7.19 %, N = 4.71 %.

\(^1\text{H}\) n.m.r. (DMSO-\textit{d}_6) \(\delta\) 10.20 (s, br, 1H, OH), 7.78 (d, J = 8.6 Hz, 1H, H-8), 7.04 (d, J = 8.6 Hz, 2H, H-3' and H-5'), 6.72 (dd, J = 2.4, 8.6 Hz, 1H, H-7), 6.66 (d, J = 8.7 Hz, 2H, H-2' and H-6'), 6.61 (d, J = 2.3 Hz, 1H, H-5), 3.12 (dd, J = 3.7, 13.0 Hz, 1H, \(-\text{CH}_2-\text{CH}_2\text{HB}-\text{CH}_x-\text{(C=O)})\), 2.85 (s, 6H, 2 x CH\textsubscript{3}, \(-\text{N(CH}_3)_2\)), 2.81 (m, 2H, \(-\text{CH}_2-\text{CH}_2\text{HB}-\text{CH}_x-\text{(C=O)}\)), 2.66 – 2.57 (m, 1H, \(-\text{CH}_2-\text{CH}_2\text{HB}-\text{CH}_x-\text{CH}_2\text{H}_5-\text{Phenyl}\)), 2.54 – 2.49 (m, 1H, \(-\text{CH}_2-\text{CH}_2\text{HB}-\text{CH}_x-\text{(C=O)}\)), 1.97 – 1.88 (m, 1H, \(-\text{CH}_2-\text{CH}_2\text{HB}-\text{CH}_x-\text{(C=O)}\)), 1.66 – 1.55 (m, 1H, \(-\text{CH}_2-\text{CH}_2\text{HB}-\text{CH}_x-\text{(C=O)}\)).

\(^13\text{C}\) n.m.r. (DMSO-\textit{d}_6) \(\delta\) 197.58 (C=O), 162.31 (C, C-6), 149.26 (C, C-4'), 147.16 (C, C-4a), 129.95 (2 x CH, CH-3' and CH-5'), 129.73 (CH, CH-8), 127.10 (C, C-8a), 124.74 (C, C-1'), 114.69 (CH, CH-7), 114.44 (CH, CH-5), 112.94 (2 x CH, CH-2' and CH-6'), 48.73 (CH, \(-\text{CH}_2-\text{CH}_2-\text{CH}_x-\text{(C=O)}\)), 40.70 (2 x CH\textsubscript{3}, \(-\text{N(CH}_3)_2\)), 34.50 (CH\textsubscript{2}, \(-\text{CH}_2-\text{CH}_2-\text{CH}_x-\text{Phenyl}\)), 28.29 (CH\textsubscript{2}, \(-\text{CH}_2-\text{CH}_2-\text{CH}_x-\text{(C=O)}\)), 27.51 (CH\textsubscript{2}, \(-\text{CH}_2-\text{CH}_2-\text{CH}_x-\text{(C=O)}\)).
2-[2,5-Di(trifluoromethyl)benzyl]-6-methoxy-3,4-dihydro-2H-naphthalen-1-one (101)

(C\textsubscript{20}H\textsubscript{16}F\textsubscript{6}O\textsubscript{2}, MW: 402.330)

![Chemical Structure 84](image)

\[ \text{Pd/C 10\%}, \text{H}_2 \quad \xrightarrow{1 \text{ hours}} \quad \text{H}_3\text{C}-O \]

![Chemical Structure 101](image)

The resulting residue was purified by column chromatography with petroleum ether – ethyl acetate 100:0 v/v increasing to 87.5:12.5 v/v to give white residue with two close spots on t. l. c. plate. The impure compound was further purified using preparative t. l. c. to give 2-[2,5-di(trifluoromethyl)benzyl]-6-methoxy-3,4-dihydro-2H-naphthalen-1-one (101) as white solid. Yield: 19 mg (6\%), t. l. c. system: petroleum ether – ethyl acetate 1:1 v/v, R\textsubscript{f}: 0.72, stain positive. HRMS (ES\textsuperscript{+}) m/z Calculated for C\textsubscript{20}H\textsubscript{16}F\textsubscript{6}O\textsubscript{2} [M+H]\textsuperscript{+} 403.1127; Found 435.1121. Melting point: 48 – 50 °C.

\textsuperscript{1}H n.m.r. δ 8.05 (d, J = 8.8 Hz, 1H, H-8), 7.80 (d, J = 8.3 Hz, 1H, H-3'), 7.69 (s, 1H, H-6'), 7.60 (d, J = 8.2 Hz, 1H, H-4'), 6.85 (d, J = 2.5, 8.8 Hz, 1H, H-7), 6.68 (d, J = 2.5 Hz, 1H, H-5'), 3.86 (s, 3H, –O-CH\textsubscript{3}), 3.82 (m, 1H, –CH\textsubscript{2}-CH\textsubscript{2}H-B-CH\textsubscript{3}-(C=O)), 2.93 (m, 2H, –CH\textsubscript{2}-CH\textsubscript{2}H-B-CH\textsubscript{2}H-Phenyl), 2.87 – 2.74 (m, 2H, –CH\textsubscript{2}-CH\textsubscript{2}H-B-CH\textsubscript{x}-(C=O)), 2.02 (m, 1H, –CH\textsubscript{2}-CH\textsubscript{2}H-B-CH\textsubscript{3}-(C=O)), 1.88 (m, 1H, –CH\textsubscript{2}-CH\textsubscript{2}H-B-CH\textsubscript{x}-(C=O)).

\textsuperscript{13}C n.m.r. δ 197.43 (C=O), 164.07 (C, C-6), 146.65 (C, C-4a), 141.32 (C, C-1'), 134.35, 133.92 (C, C-2'), 130.53 (CH, CH-8), 128.98, 128.93 (CH, CH-6'), 127.47, 127.26 (CH, CH-4'), 126.30, 126.03 (C, C-5'), 125.61 (C, C-8a), 123.68, 123.59 (CH, CH-3'), 122.39 (C, CF\textsubscript{3}), 121.99 (C, CF\textsubscript{3}), 113.75 (CH, CH-7), 112.89 (CH, CH-5), 55.87 (CH\textsubscript{3}), 49.27 (CH, –CH\textsubscript{2}-CH\textsubscript{2}CH\textsubscript{x}-(C=O)), 32.82 (CH\textsubscript{2}, –CH\textsubscript{2}-CH\textsubscript{2}CH\textsubscript{2}CH\textsubscript{x}-Phenyl), 29.83 (CH\textsubscript{2}, –CH\textsubscript{2}-CH\textsubscript{2}CH\textsubscript{x}-(C=O)), 28.83 (CH\textsubscript{2}, –CH\textsubscript{2}-CH\textsubscript{2}CH\textsubscript{x}-(C=O)).
6-Methoxy-2-(2-methylbenzyl)-3,4-dihydro-2H-naphthalen-1-one (102)

(C_{19}H_{20}O_{2}, MW: 280.361)

The resulting residue was purified by column chromatography with petroleum ether – ethyl acetate 100:0 v/v increasing to 80:20 v/v to give 6-methoxy-2-(2-methylbenzyl)-3,4-dihydro-2H-naphthalen-1-one (102) as yellow syrup. Yield: 0.43 g (77%), t. l. c. system: petroleum ether – ethyl acetate 3:1 v/v, R_f: 0.71, stain positive. Microanalysis (C_{19}H_{20}O_{2}.0.1H_{2}O): Calculated C = 80.88 %, H = 7.22 %; Found C = 80.98 %, H = 7.07 %.

^1H n.m.r. δ 8.07 (d, J = 8.8 Hz, 1H, H-8), 7.15 (m, 4H, Ar), 6.85 (dd, J = 2.5, 8.7 Hz, 1H, H-7), 6.64 (d, J = 2.4 Hz, 1H, H-5), 3.86 (s, 3H, -O-CH_{3}), 3.64 (dd, J = 3.4, 13.8 Hz, -CH_{2}-CH_{A}H_{B}-CH_{X}-CH_{A}H_{B}-Phenyl), 2.89 (m, 2H, -CH_{2}-CH_{A}H_{B}-CH_{X}-(C=O)), 2.66 (m, 1H, -CH_{2}-CH_{A}H_{B}-CH_{X}-(C=O)), 2.53 (dd, J = 10.38, 13.8 Hz, 1H, -CH_{2}-CH_{A}H_{B}-CH_{X}-CH_{A}H_{B}-Phenyl), 2.36 (s, 3H, CH_{3}), 2.05 – 2.14 (m, 1H, -CH_{2}-CH_{A}H_{B}-CH_{X}-(C=O)), 1.78 – 1.88 (m, 1H, -CH_{2}-CH_{A}H_{B}-CH_{X}-(C=O)).

^13C n.m.r. δ 198.66 (C=O), 163.91 (C, C-6), 146.96 (C, C-4a), 138.96 (C, C-1’), 136.92 (C, C-2’), 130.88 (CH, CH-3’), 130.50 (CH, CH-8), 130.43 (CH, CH-6’), 126.68 (CH, CH-4’), 126.56 (C, C-8a), 126.24 (CH, CH-5’), 113.64 (CH, CH-5), 112.87 (CH, CH-7), 55.86 (CH_{3}, -O-CH_{3}), 48.40 (CH, -CH_{2}-CH_{2}-CH_{X}-(C=O)), 33.45 (CH_{2}, -CH_{2}-CH_{2}-CH_{X}-(C=O)), 29.62 (CH_{2}, -CH_{2}-CH_{2}-CH_{X}-(C=O)), 28.36 (CH_{2}, -CH_{2}-CH_{2}-CH_{X}-(C=O)), 19.96 (CH_{3}).
2-Biphenyl-4-ylmethyl-6-hydroxy-3,4-dihydro-2H-naphthalen-1-one (103)

(C$_{23}$H$_{20}$O$_2$, MW: 328.404)

With 2-biphenyl-4-ylmethyl-6-hydroxy-3,4-dihydro-2H-naphthalen-1-one (103), a white solid was obtained after trituration with methanol. Yield: 0.20 g (63 %), t. l. c. system: petroleum ether – ethyl acetate 1:1 v/v, R$_f$: 0.32, stain positive. Microanalysis (C$_{23}$H$_{20}$O$_2$): Calculated C = 84.12 %, H = 6.14 %; Found C = 84.06 %, H = 6.14 %. Melting point: 220 – 222 °C.

$^{1}$H n.m.r. (DMSO-$d_6$) $\delta$ 10.45 (s, br, 1H, OH), 7.82 (d, J = 8.6 Hz, 1H, H-8), 7.65 (m, 2H, Ar), 7.58 (m, 2H, Ar), 7.45 (m, 2H, Ar), 7.34 (m, 3H, Ar), 6.74 (dd, J = 2.2, 8.6Hz, 1H, H-7), 6.63 (d, J = 1.9 Hz, H-5), 3.31 (dd, J = 3.9, 13.3 Hz, 1H, –CH$_2$-CH$_A$H$_B$-CH$_x$-CH$_y$H$_z$-Phenyl), 2.88 – 2.74 (m, 2H, –CH$_2$-CH$_A$H$_B$-CH$_x$-(C=O)), 2.66 (dd, J = 9.2, 13.3 Hz, 1H, –CH$_2$-CH$_A$H$_B$-CH$_x$-(C=O)), 1.96 (m, 1H, –CH$_2$-CH$_A$H$_B$-CH$_x$-(C=O)), 1.73 – 1.59 (m, 1H, –CH$_2$-CH$_A$H$_B$-CH$_x$-(C=O)).

$^{13}$C n.m.r. (DMSO-$d_6$) $\delta$ 197.29 (C=O), 162.40 (C, C-6), 147.19 (C, C-4a), 140.42 (C, C-1”), 139.81 (C, C-1’”), 138.20 (C, C-4”), 130.11 (2 x CH, phenyl), 129.80 (CH, CH-8), 129.25 (2 x CH, phenyl), 127.54 (CH, CH-4’”), 126.88 (2 x CH, phenyl), 126.85 (2 x CH, phenyl), 124.68 (C, C-8a), 114.75 (CH, CH-5), 114.48 (CH, CH-7), 48.37 (CH, –CH$_2$-CH$_2$-CH$_x$-CH$_2$-Phenyl), 35.18 (CH$_2$, –CH$_2$-CH$_2$-CH$_x$-CH$_2$-Phenyl), 28.46 (CH$_2$, –CH$_2$-CH$_2$-CH$_x$-(C=O)), 27.83 (CH$_2$, –CH$_2$-CH$_2$-CH$_x$-(C=O)).
2-Biphenyl-2-ylmethyl-6-hydroxy-3,4-dihydro-2H-naphthalen-1-one (104)

(C_{23}H_{20}O_{2}, MW: 328.404)

The resulting residue was purified by column chromatography with petroleum ether – ethyl acetate 90:10 v/v increasing to 65:35 v/v to give 2-biphenyl-2-ylmethyl-6-hydroxy-3,4-dihydro-2H-naphthalen-1-one (104) as light brown semi-solid. Yield: 0.50 g (83 %), t. l. c. system: petroleum ether – ethyl acetate 1:1 v/v, R_F: 0.05, stain positive. Microanalysis (C_{23}H_{20}O_{2}.0.3H_{2}O): Calculated C = 82.76 %, H = 6.22 %; Found C = 82.93 %, H = 6.14 %. Melting point: 38 – 40 °C.

^1H n.m.r. δ 8.35 (s, br, 1H, OH), 8.00 (d, J = 8.6 Hz, 1H, H-8), 7.47 – 7.31 (m, 9H, Ar), 6.85 (dd, J = 2.4, 8.6 Hz, 1H, H-7), 6.71 (d, J = 2.3 Hz, 1H, H-5), 3.66 (dd, J = 4.3, 14.0 Hz, 1H, –CH₂-CH₃H₆-H₆-C₆H₆-Phenyl), 2.79 (dd, J = 10.7, 14.0 Hz, 1H, –CH₂-CH₃H₆-H₆-C₆H₆-Phenyl), 2.71 (m, 2H, –CH₂-CH₃H₆-H₆-C₆H₆-(C=O)), 2.60 (m, 1H, –CH₂-CH₃H₆-H₆-C₆H₆-(C=O)), 1.96 – 1.87 (m, 1H, –CH₂-CH₃H₆-H₆-C₆H₆-(C=O)), 1.66 – 1.54 (m, 1H, –CH₂-CH₃H₆-H₆-C₆H₆-(C=O)).

^13C n.m.r. δ 200.60 (C=O), 162.05 (C, C-6), 147.97 (C, C-4a), 143.04 (C, C-1’), 142.13 (C, C-2’), 137.88 (C, C-1”), 130.96 (CH, Ar), 130.78 (CH, Ar), 130.54 (CH, Ar), 129.77 (2 x CH, Phenyl), 128.76 (2 x CH, Phenyl), 128.01 (CH, Ar), 127.48 (CH, Ar), 126.79 (CH, CH-8), 125.66 (C, C-8a), 115.23 (CH, CH-5), 114.99 (CH, CH-7), 48.90 (CH, –CH₂-CH₂-CHₓ-(C=O)), 33.33 (CH₂, –CH₂-CH₂-CHₓ-(C=O)), 28.97 (CH₂, –CH₂-CH₂-CHₓ-(C=O)), 27.91 (CH₂, –CH₂-CH₂-CHₓ-(C=Phenyl)).
CHAPTER 6

Inhibition of vitamin D\textsubscript{3} and all-trans retinoic acid metabolism in rat kidney mitochondria and rat liver microsomes
Chapter 6

6. Inhibition of vitamin D₃ and all-trans retinoic acid metabolism in rat kidney mitochondria and rat liver microsomes

6.1 Vitamin D₃ metabolism study

25-Hydroxyvitamin D₃ (25-(OH)-D₃), synthesised in the liver, is then oxidised in the kidney to:

- 1α,25-dihydroxyvitamin D₃ (1α,25-(OH)₂-D₃), the active metabolite, by cytochrome P450 1α-hydroxylase (CYP1α); and
- 24-hydroxyvitamin D₃ (24,25-(OH)₂-D₃), inactive metabolite, by cytochrome P450 24-hydroxylase (CYP24). This enzyme can also oxidised the active metabolite 1α,25-(OH)₂-D₃ to 1α,24,25-trihydroxyvitamin D₃ (1α,24,25-(OH)₃-D₃).

It was found by Omdahl et al. (Omdahl et al., 1972) that isolated kidney mitochondria from low calcium-fed animals metabolised 25-(OH)-D₃ to 1α,25-(OH)₂-D₃; whereas, those isolated from high calcium-fed animals metabolised 25-(OH)-D₃ to a 24,25-(OH)₂-D₃ as identified by Holick et al. (Holick et al., 1972).

A few research groups have carried out the investigation of vitamin D₃ metabolism studies in mitochondria of the:

- chick kidney (Burgos-Trinidad et al., 1986; Burgos-Trinidad et al., 1990);
- rat kidney (Ohyama and Okuda, 1991; Vieth and Fraser, 1979; Warner, 1982)
- pig kidney (Araya et al., 2003).

Ohyama et al. have developed a method for the purification of 24-hydroxylase from rat kidney mitochondria by solubilising the enzyme from the kidney mitochondria with cholate and then subjected the soluble fraction to the column chromatography (Ohyama et al., 1989; Ohyama et al., 1997; Ohyama and Okuda, 1991).

Since the amino acid sequence of the human (Chen et al., 1993) and rat (Ohyama and Okuda, 1991) CYP24 have been identified, Sakaki’s group employed the Escherichia coli expression system to reveal CYP24-dependent metabolism of vitamin D₃ in rat (Kusudo et al., 2003; Sakaki et al., 1999) and human (Kusudo et al., 2003; Sakaki et al., 2000).
6.2 All-trans retinoic acid metabolism study

The liver is recognised as an important site for P450-mediated oxidation of retinoic acid in the rat (Ahmad et al., 2000). The microsomal cytochrome P450 isozyme system is involved in the 4-hydroxylation of retinoic acid (RA) (Raner et al., 1996). The different P450 isozymes that are able to catalyse the 4-hydroxylation of RA are, for example:

- rat liver P450s 2B1, 2C7 (Leo and Lieber, 1985) and 3A4 (Martini and Murray, 1993)
- rabbit liver P450s 1A2 and 2B4 (Roberts et al., 1992)
- human liver P450s 2C8, 2C9, 3A4 (Leo et al., 1989; McSorley and Daly, 2000; Nadin and Murray, 1999), 3A7 (Marill et al., 2000) and 26 (Ray et al., 1997; White et al., 1997).

The search for the inhibitors of the CYP-mediated metabolism of RA, i.e. retinoic acid metabolism blocking agents (RAMBAs) is actively pursued by many research groups. Liarozole and ketoconazole are capable of inhibiting the CYP-dependent metabolism of RA by hamster (Van Wauwe et al., 1990) and rat (Kirby et al., 2003) liver microsomes respectively. The use of human CYP26 expressed in Candida albicans and other expression systems has been developed by some research groups to investigate the ATRA metabolism in vitro (Chithalen et al., 2002; Stoppie et al., 2000).

6.3 Aims and objectives

The aims of this study were:

- To develop a method for the vitamin D₃ metabolism study and to evaluate the inhibition of vitamin D₃ metabolism using crude mitochondria cytochrome P450s extract from rat kidney.
- To follow on the method described by our group (Ahmad et al., 2000; Kirby et al., 2003) to evaluate the inhibition of ATRA metabolism by the synthesized compounds using microsomal preparations of rat liver.
### 6.4 Materials and equipments

The following materials and equipments were used in the vitamin D₃ metabolism assay in rat kidney mitochondria and ATRA metabolism assay in rat liver microsomes:

<table>
<thead>
<tr>
<th>Material/Chemical</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>25-Hydroxy-[26,27-methyl-³H]-vitamin D₃ (0.925 MBq, 500 µCi)</td>
<td>Amersham Biosciences (Bucks, UK)</td>
</tr>
<tr>
<td>25-Hydroxyvitamin D₃</td>
<td>Fluka Chemicals (Dorset, UK)</td>
</tr>
<tr>
<td>[³H-11,12]-All trans retinoic acid (9.25 MBq, 250 µCi)</td>
<td>PerkinElmer Life Science Ltd. (Massachusetts, USA)</td>
</tr>
<tr>
<td>All-trans retinoic acid (ATRA)</td>
<td>Sigma Chemicals (Dorset, UK)</td>
</tr>
<tr>
<td>1 % Calcium and Vitamin D₃ special feed</td>
<td>Special Diets Services, Essex, UK</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>Sigma Chemicals (Dorset, UK)</td>
</tr>
<tr>
<td>HPLC grade solvents e.g. acetonitrile, methanol, butylated-hydroxyanisole, ethyl acetate, ethanol, ammonium acetate</td>
<td>Fisher Scientific (Leicestershire, UK)</td>
</tr>
<tr>
<td>Nicotinamide-adenine dinucleotide phosphate (reduced form) (NADPH) – cofactor</td>
<td>Sigma Chemicals (Dorset, UK)</td>
</tr>
<tr>
<td>All buffer (see preparation below)</td>
<td>Aldrich Chemicals, UK</td>
</tr>
<tr>
<td>OptiFlow Safe 1 liquid scintillation cocktail</td>
<td>Fisons Chemicals, UK</td>
</tr>
<tr>
<td>Borosilicate tubes (12 x 75 mm and 13 x 100 mm)</td>
<td>Corning (New York, USA)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Equipment</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Centrifuge</td>
<td>MSE Harrier 18/80, Sanyo, Japan</td>
</tr>
<tr>
<td>Ultracentrifuge (high speed 2000 – 64,000 rpm) and centrifuge tubes</td>
<td>Sorvall centrifuge, Dupont, USA</td>
</tr>
<tr>
<td>Potter-Elvejehm homogeniser (TRI-R STR-R model S63C) and homogeniser tubes</td>
<td>TRI-R Instruments, Inc., USA</td>
</tr>
<tr>
<td>10 µm C₁₈ µBondapak® 3.9 x 300 mm column</td>
<td>Waters, UK</td>
</tr>
<tr>
<td>5 µm ODS EXSIL® 4.6 x 200 mm column</td>
<td>Jones Chromatography, UK</td>
</tr>
<tr>
<td>Beta-RAM online scintillation detector</td>
<td>LKB Wallace 1217 Rackbeta</td>
</tr>
<tr>
<td>Pump</td>
<td>Milton-Roy</td>
</tr>
<tr>
<td>Water bath with shaker</td>
<td>Grant, UK</td>
</tr>
<tr>
<td>Rotating evaporator</td>
<td>Christ Alpha RVC (Germany)</td>
</tr>
<tr>
<td>Computer</td>
<td>Compaq™</td>
</tr>
<tr>
<td>Laura data acquisition and analysis software</td>
<td>Lablogic Ltd.</td>
</tr>
</tbody>
</table>
Chapter 6

- **Preparation of 25-hydroxy-[26,27-methyl-^3^H]-vitamin D₃ stock solution**
  50 µL of 0.5 mM 25-hydroxyvitamin D₃ in EtOH was diluted with 850 µL of 
  iPrOH:EtOH 1:1 v/v. To this was added 100 µL of 25-hydroxy[26,27-methyl-
  ^3^H]-vitamin D₃ (0.925 MBq, 500 µCi).
  - 1 mL of the above stock solution contains 100/500 x 0.925 MBq = 0.185 
    MBq.
  - 10 µL of the stock solution contains 0.01 µCi x 0.185 MBq/0.037 MBq = 
    0.05 µCi.

- **Preparation of [^3^H-11,12]-all-trans retinoic acid stock solution**
  100 µL of 1.2 mM ATRA in EtOH was diluted with 900 µL of iPrOH:EtOH 1:1 
  v/v. To this was added 10 µL of [^3^H-11,12]-ATRA (9.25 MBq, 250 µCi).
  - 1 mL of the above stock solution contains 10/250 x 9.25 MBq = 0.37 
    MBq.
  - 10 µL of the stock solution contains 0.01 µCi x 0.37 MBq/0.037 MBq = 
    0.10 µCi.

- **Preparation of phosphate buffer (50 mM, pH 7.4)**
  To prepare 1000 mL of phosphate buffer (50 mM, pH 7.4), 6.68 g of disodium 
  hydrogen orthophosphate dehydrate was dissolved in 750 mL of water and 1.95 g 
  of monosodium dihydrogen orthophosphate dihydrate was dissolved in 250 mL 
  of water. This mixture was then titrated to pH 7.4 with aqueous NaOH (5 M). To 
  prepare 500 mL of phosphate/sucrose buffer (250 mM), 42.75 g of sucrose made 
  up to 500 mL with phosphate buffer prepared above.

- **Preparation of Tris-acetate buffer (15 mM, pH 7.4)**
  To prepare 1000 mL of 15 mM Tris-acetate buffer, 1.82 g TRIS 
  (tris(hydroxymethyl)methylamine) was dissolved in 1000 mL of water and 
  titrated to pH 7.4 with 5 M aqueous acetic acid. To prepare 500 mL of Tris-
  acetate/sucrose buffer (250 mM), 42.75 g of sucrose made up to 500 mL with 
  Tris-acetate buffer prepared above.
6.5 **Pharmacological preparation of the rat kidney mitochondria and rat liver microsome**

The pharmacological preparation of the rat kidney mitochondria for the study of 25-hydroxyvitamin D₃ metabolism was based on a modification of the procedures described by DeLuca's group (Burgos-Trinidad *et al.*, 1986).

Whereas, the pharmacological preparation of the rat liver microsome for the study of all-*trans* retinoic acid metabolism was based on the assay previously set up by our group (Ahmad *et al.*, 2000; Kirby *et al.*, 2003).

6.5.1 **Method for the preparation of the rat kidney mitochondria**

1. Three male Wistar rats (250 g) were bought in from Harlan and were fed with calcium and vitamin D replete diet (calcium carbonate was added to the feed to achieve a 1 % calcium level and vitamin D₃ was added to achieve 2200 iu/kg in the feed). The rats were fed for two weeks to reach the weight of 300 g.

2. The rats were killed by cervical dislocation, and the kidneys were removed. The kidneys were washed with ice-cold phosphate buffer (50 mM, pH 7.4) containing 0.25 mM sucrose, to remove excess blood. The kidney was stored in the – 80 °C freezer for 5 days.

3. The kidney was defrosted in ice-cold Tris-acetate buffer (15 mM, pH 7.4) with 0.25 mM sucrose. The connective tissues were removed and the buffer was drained off.

4. The kidney was cut in to smaller pieces using scissors. The tissues were homogenized with four times their weight of Tris-acetate buffer (15 mM, pH 7.4). The Potter-Elvejhem homogeniser was used by moving the pestle up and down to create a uniform, stable emulsion (the homogenate). [NOTE: Do not move the pestle up and down more than four times, to minimise degradation of tissue enzymes.] The homogenising tube was immersed in an ice bucket at all times.

5. The above homogenate was decanted into centrifuge tubes and the homogenate was then centrifuged at 2000 rpm for 20 min at 3 °C to remove nuclei and cell debris. The supernatant was retained.

6. The pellet from 5 was resuspended in the sucrose medium and was centrifuged at 2000 rpm for 20 min at 3 °C.
7. The supernatant from 5 and 6 above were combined and centrifuged at 10,000 rpm for 20 min at 3°C.

8. The supernatant was decanted and the pellet containing the mitochondria was washed with the sucrose medium and resuspended in ice-cold 20% glycerol and 15 mM Tris-acetate pH 7.4, containing 0.6% sodium cholate.

9. This 20% w/w homogenate was stirred on ice for 1 h and the homogenate was centrifuged at 12,000 rpm for 1 h.

10. Aliquots of the above supernatant were placed into 1.5 mL capped eppendorfs and snapped frozen in liquid N₂ and stored in a –80°C freezer.

6.5.2 Method for the preparation of the rat liver microsome

1. Three male Wistar rats (275 g) were bought in from Harlan.

2. The rats were killed by cervical dislocation, and the livers were removed. The livers were washed with ice-cold phosphate buffer (50 mM, pH 7.4) containing 0.25 mM sucrose, to remove excess blood. The connective tissues were removed and the buffer was drained off.

3. The liver was cut in to smaller pieces using scissors. The tissues were homogenized with four times their weight of the above ice-cold phosphate buffer. The Potter-Elvejhem homogeniser was used by moving the pestle up and down to create a uniform, stable emulsion (the homogenate). [NOTE: Do not move the pestle up and down more than four times, to minimise degradation of tissue enzymes.] The homogenising tube was immersed in an ice bucket at all times.

4. The above homogenate was decanted into the centrifuge tubes and the homogenate was then centrifuged at 12000 rpm for 20 min at 3°C. The pellet (solid at the bottom of the centrifuge tube which contains unwanted connective tissues, cell membranes, etc.) was discarded.

5. The above supernatant was centrifuged at 37,000 rpm for 1 h at 3°C.

6. The supernatant was decanted and the pellet containing the microsome was washed with the sucrose medium and resuspended in ice-cold phosphate buffer (50 mM, pH 7.4) and homogenised with the Potter-Elvejhem homogeniser.

7. Aliquots of the above homogenate were placed into 1.5 mL capped eppendorfs and snapped frozen in liquid N₂ and stored in a –80°C freezer.
6.6 General assay for the inhibition studies of vitamin D$_3$ in rat kidney mitochondria and all-trans retinoic acid in rat liver microsome

The procedures for the general assay for the metabolism of vitamin D$_3$ and all-trans retinoic acid are summarised in the following steps. The experiments were performed in duplicate:

2. 10 μL ethanol or acetonitrile as control or synthesised compound (at various concentrations) was added to each test tube.
3. All test tubes were diluted with phosphate buffer (50 mM, pH 7.4) to make up the final volume, after addition of enzyme and co-factor, of 500 μL.
4. Enzyme which was stored at – 80 °C was defrosted prior to use. 30 μL of rat kidney mitochondria or 20 μL of rat liver microsomes was added to each test tube.
5. NADPH (16 mM, 50 μL, in pH 7.4 phosphate buffer) was added to each test tube and vortexed.
6. The reaction mixture was incubated in a shaking water bath at 37 °C.
7. After 30 mins, the reaction was stopped by the addition of 100 μL 2 % acetic acid, and the mixture was extracted from 2 mL of ethyl acetate containing butylated-hydroxyanisole 0.05 %.
8. The top organic layer was removed, and then evaporated to dryness.
9. The residue left in the test tube was redisolved in 60 μL methanol, and then subjected to HPLC analysis.

6.6.1 Set up of the high performance liquid chromatography (HPLC)

The assay used was to measure the effect of the novel compounds on the rate of conversion of radiolabelled 25-hydroxyvitamin D$_3$ or all-trans retinoic acid to their respective metabolites.

The HPLC system was equipped with a high pressure pump (Milton-Roy pump), injector with a 50 μL loop connected to a beta-RAM radioactivity detector, connected to a Compaq™ computer running Laura® data acquisition and analysis software. This enabled on-line detection and quantification of radioactive peaks. The 5 μm ODS EXSIL® 4.6 x 200 mm column (for vitamin D$_3$ assay) or 10 μm C$_{18}$ μBondapak® 3.9 x 300 mm column (for ATRA assay) operating at room temperature was used to separate
the metabolites which were eluted with 750 mL acetonitrile/250 mL 1 % ammonium acetate in water/1 mL acetic acid at a flow rate of 1.5 mL/min (for vitamin D₃ assay) or 1.9 mL/min (for ATRA assay).

6.7 Biological results and discussions

6.7.1 Metabolism of vitamin D₃ in rat kidney mitochondria

The special diet which were fed to the male Wistar rats has enhanced the activity of the metabolism of [³H]-25-(OH)-D₃. In the control set up, 40 ± 5 % of [³H]-25-(OH)-D₃ was converted into metabolites by using 30 μL of the crude enzyme of the special diet rats (section 6.5.1). Compared to 20 ± 8 % of [³H]-25-(OH)-D₃ was converted into metabolites using 50 μL of the crude enzyme in normal diet rats. This result was similar to the observation by Warner (Warner, 1982).

The HPLC separation of the [³H]-25-(OH)-D₃ metabolites were based on a modification of the method described by Kang et al. (Kang et al., 1997). The three possible metabolites of 25-(OH)-D₃ metabolism in rat kidney mitochondria are 1α,25-(OH)₂-D₃, 24,25-(OH)₂-D₃ and 1α,24,25-(OH)₃-D₃ (Ohyama et al., 1997; Warner, 1982). Due to the high cost of the radiolabelled metabolites, the 25-(OH)-D₃ metabolites were identified by matching their elution rate to the retention time of the known standards, 1α,25-(OH)₂-D₃, 24,25-(OH)₂-D₃ and 1α,24,25-(OH)₃-D₃, (generous gifts from Dr. L. Binderup, Leo Pharma, Denmark) by using a photodiode array detector to monitor UV absorption at 265 nm [Figure 6.1 (C)]. Following the same conditions described in Figure 6.1 (A), known standards of the vitamin D₃ metabolites were injected into the HPLC and the elution order detected by photodiode array detector is 1α,24,25-(OH)₃-D₃ (retention time: 2.5 min); 24,25-(OH)₂-D₃ (retention time: 3.8 min); 1α,25-(OH)₂-D₃ (retention time: 4.5 min) followed by 25-(OH)-D₃ (retention time 10.5 min) [Figure 6.1 (C)].

The separated [³H]-metabolites were quantitatively calculated from the areas under the curves. The approximate percentage metabolites of the individual [³H]-metabolites of 25-(OH)-D₃ are as following:

- 20 % of [³H]-1α,24,25-(OH)₃-D₃
- 10 % of [³H]-24,25-(OH)₂-D₃
- 10 % of [³H]-1α,25-(OH)₂-D₃
Figure 6.1 (A) Reverse-phase HPLC chromatograms showing the metabolism of $[^3$H] -25-(OH)-D$_3$ in the presence of (A) rat kidney mitochondria and ethanol with NADPH. 35 ± 5 % of $[^3$H] 25-(OH)-D$_3$ was converted into metabolites by using 30 μL of the crude kidney mitochondria enzyme from rats fed with special diet. (B) Same as (A) but with 100 μM ketoconazole. HPLC column: 5 μm ODS EXSIL® 4.6 x 200 mm; Mobile phase: 75:25:0.1 = acetonitrile:1 % w/v ammonium acetate in water:acetic acid at a flow rate of 1.5 mL/min. Retention time for $[^3$H]-25-(OH)-D$_3$ = 10.5 min.
Figure 6.1 (C) The retention time of approximately 10 µM of the vitamin D₃ metabolites, 1α,25-(OH)₂-D₃, 24,25-(OH)₂-D₃, 1α,24,25-(OH)₃-D₃, and 25-(OH)-D₃ by using a photodiode array detector to monitor UV absorption at 265 nm. Column: 5 µm ODS EXSIL® 4.6 x 200 mm; Mobile phase: 75:25:0.1 = acetonitrile:1 % w/v ammonium acetate in water:acetic acid at a flow rate of 1.5 mL/min.

Ideally, the synthesised compounds should be more selective against CYP24 than CYP1α, as CYP24 is responsible for metabolism of the biologically active hormone, 1α,25(OH)₂D₃. Due to the low level of the individual metabolites, it was not possible to study the inhibition against the specific hydroxylases enzymes, CYP1α and CYP24 present in the rat kidney mitochondria. Therefore the IC₅₀ values reported in Table 6.1
– 6.2 represent the inhibition against the metabolism of both hydroxylases, CYP1α and CYP24, i.e. inhibition against the 25-hydroxyvitamin D₃ metabolising enzymes (vitamin D₃ metabolising enzymes). The percentage inhibition of [³H]-25-(OH)-D₃ metabolism was calculated from: 100[(metabolites (control) – metabolites (inhibitor) / (metabolites control)]%.

Ketoconazole and liarozole are known to inhibit the vitamin D₃ metabolising enzymes (Kang et al., 1997; Ly et al., 1999; Reinhardt and Horst, 1989). However, liarozole was shown to enhance the metabolism of [³H]-25-(OH)-D₃ in the assay described here. Therefore, ketoconazole was used as the standard inhibitor in this assay. In this study, the inhibition of [³H]-25-(OH)-D₃ catabolism by ketoconazole and the synthesised compounds was evaluated.

Out of the six synthesised compounds 1- and 4- [benzofuran-2-yl]phenylmethyl]triazoles described in Chapter 3, only compound 16 showed comparable inhibition with ketoconazole (Table 6.1). The hydrophobic phenyl ring (16 and 18) showed improved inhibition compared with a linear alkyl chain (14).

The benzo[b]furan-2-carboxamidoethyl-imidazole and -1,2,4-triazole compounds (described in Chapter 4) showed either weak inhibition or induction at 100 µM (i.e. enhanced 25-(OH)-D₃ metabolism) in this assay. This was similar to that observed in the ATRA metabolism assay (section 6.7.2).
Table 6.1. Inhibition of 25-(OH)-D₃ metabolism using rat kidney mitochondria homogenate. The IC₅₀ values were the mean of two experiments (± 5%). These values for inhibition of [³H]-25-(OH)-D₃ metabolism were measured using 0.5 μM 25-(OH)-D₃ with 0.05 μCi [³H]-25-(OH)-D₃ as substrate.

![Chemical Structures](image)

(14) R' = CH₂CH₃  
(16) R' = Phenyl  
(18) R' = Phenyl-Cl

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC₅₀ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>15</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>16</td>
<td>10 – 20</td>
</tr>
<tr>
<td>17</td>
<td>&gt; 50</td>
</tr>
<tr>
<td>18</td>
<td>20 – 40</td>
</tr>
<tr>
<td>19</td>
<td>&gt; 50</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>20</td>
</tr>
</tbody>
</table>

The saturated form of the 6-substituted-2-(phenylmethylene)-3,4-dihydro naphthalene-1-one derivatives (Box 6.1) showed good inhibition compared with the standard inhibitor, ketoconazole (Table 6.2).

The saturated tetralone and tetralin compounds show greater inhibition activity compared with the unsaturated tetralone. The rigid structure of the unsaturated tetralone could affect the flexibility of the compound to interact with the enzyme active site. Among the saturated tetralone compounds, 102 and 104 showed better inhibition against the vitamin D₃ metabolising enzymes. From here, we can predict that there may be a hydrophobic pocket at the second position of the phenyl ring. By comparing compound 97, 98 and 99, it tells us that:

- a methoxy group at the 6-position of the tetralone, might be indicative of a hydrophilic interaction between the inhibitor and the enzyme at this position
- the oxo group in position-1 of the tetralones is not essential for P450 inhibitor activity.
Box 6.1

Unsaturated Tetralone

![Diagram of Unsaturated Tetralone]

Saturated Tetralone

![Diagram of Saturated Tetralone]

Table 6.2. Inhibition of 25-(OH)-D₃ metabolism using rat kidney mitochondria homogenate. The IC₅₀ values were the mean of two experiments. These values for inhibition of [³H]-25-(OH)-D₃ metabolism were measured using 0.5 μM 25-(OH)-D₃ with 0.05 μCi [³H]-25-(OH)-D₃ as substrate and was determined from dose-response curves (Yee and Simons, 2004).

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC₅₀ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All unsaturated tetralone</td>
<td>&gt; 20</td>
</tr>
<tr>
<td>98</td>
<td>8.9</td>
</tr>
<tr>
<td>99</td>
<td>3.5</td>
</tr>
<tr>
<td>100</td>
<td>18</td>
</tr>
<tr>
<td>101</td>
<td>4.5</td>
</tr>
<tr>
<td>102</td>
<td>0.9</td>
</tr>
<tr>
<td>103</td>
<td>0.8</td>
</tr>
<tr>
<td>104</td>
<td>2.1</td>
</tr>
<tr>
<td>97</td>
<td>2.6</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>20</td>
</tr>
</tbody>
</table>
6.7.2 Metabolism of all-trans retinoic acid in rat liver microsome

Figure 6.2 (A) shows a representative chromatogram of the radioactivity profile obtained by HPLC analysis of metabolites of [3H]-ATRA metabolism in rat liver microsome. ATRA was converted into two prominent metabolites, mainly the 4-keto-ATRA (retention time: 1.5 min) and 4-hydroxy-ATRA (retention time: 2.5 min) and other polar metabolites which eluted at the same positions as the two prominent metabolites. This result was in agreement with the previous results reported by our group (Mason, 2000). The separated [3H]-metabolites were quantitatively calculated from the areas under the curves. The percentage inhibition was calculated from: 100[(metabolites (control) − metabolites (inhibitor))/(metabolites control)]%. Typically in a control set up, 60 ± 10 % of [3H]-ATRA was converted into metabolites.

The synthesised compounds 1- and 4-[(benzo[b]furan-2-yl)phenylmethyl]triazoles (Chapter 3) showed poor activity (IC50 > 100 μM). Moreover, the synthesised compounds benzo[b]furan-2-carboxamidoethyl-imidazole and -1,2,4-triazole (Chapter 4) either showed weak inhibition or induction at 100 μM (i.e. enhanced ATRA metabolism) in this assay. In addition, liarozole which is a well-known inhibitor against retinoic acid metabolism (Freyne et al., 1998), showed induction of ATRA metabolism in this assay, which was also observed by another group (Stoppie et al., 2000). Therefore, ketoconazole was used as the standard inhibitor in this assay.
Figure 6.2. Reverse-phase HPLC chromatograms showing the metabolism of $[^3]$H-ATRA in the presence of (A) liver microsomes and acetonitrile with NADPH and (B) liver microsomes and 100 μM ketoconazole with NADPH. Column: 10 μm C$_{18}$ μBondapak® 3.9 x 300 mm; Mobile phase: 75:25:0.1 = acetonitrile:1 % w/v ammonium acetate in water:acetic acid at a flow rate of 1.9 mL/min. Retention time of $[^3]$H-ATRA = 5.5 min.

The synthesised compounds of the 6-substituted-2-(phenylmethylene)-3,4-dihydro naphthalene-1-one derivatives (Chapter 5), as summarised in Box 6.1, were evaluated for inhibition of ATRA metabolism (Table 6.3).

Generally, the reduced tetralone compounds and tetralin showed improved inhibition of ATRA metabolism (except for 101) compared with the unsaturated tetralone compounds (IC$_{50}$ > 20 μM) and ketoconazole (IC$_{50}$ = 18 μM). The tetralin compound (97) also showed comparable inhibition result with the saturated tetralone.
Table 6.3. Inhibition of ATRA metabolism in rat liver microsome. The IC\textsubscript{50} values were mean of two experiments. These values for inhibition of ATRA metabolism were measured using 2.4 μM ATRA with 0.1 μCi [\textsuperscript{3}H]-ATRA as substrate and were determined from dose-response curves (Yee et al., 2005).

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC\textsubscript{50} (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>93</td>
<td>2.4</td>
</tr>
<tr>
<td>All unsaturated tetralone except 93</td>
<td>&gt; 20</td>
</tr>
<tr>
<td>98</td>
<td>1.5</td>
</tr>
<tr>
<td>99</td>
<td>0.4</td>
</tr>
<tr>
<td>100</td>
<td>1.2</td>
</tr>
<tr>
<td>101</td>
<td>18</td>
</tr>
<tr>
<td>102</td>
<td>1.8</td>
</tr>
<tr>
<td>103</td>
<td>&gt; 20</td>
</tr>
<tr>
<td>104</td>
<td>0.5</td>
</tr>
<tr>
<td>97</td>
<td>2.2</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>18</td>
</tr>
</tbody>
</table>

6.8 General conclusions

A novel method to study the metabolism of [\textsuperscript{3}H]-25-(OH)-D\textsubscript{3} using crude enzyme from rat kidney mitochondria has been developed. An on-line radioactivity detector and reverse-phase HPLC was used to analyse the [\textsuperscript{3}H]-25-(OH)-D\textsubscript{3} metabolites. The limitation of this assay is that it only allows the study of inhibition of both vitamin D\textsubscript{3} metabolising enzymes, CYP1\textalpha{} and CYP24 rather than the individual enzymes, as the level of metabolites formed from CYP1\textalpha{} and CYP24 are insufficient to allow determination of the IC\textsubscript{50} against each enzyme.

The preparation of rat liver microsomes to evaluate the inhibition of ATRA metabolism by the synthesised compounds was carried out with slight modifications from the methods described earlier from our group (Kirby et al., 2003).

Generally, the synthesised reduced tetralone compounds and tetralin showed improved inhibition compared with ketoconazole against the ATRA and 25-(OH)-D\textsubscript{3} metabolism, as summarised in Table 6.4. The \textit{in vitro} evaluation of the tetralone compounds and tetralin revealed that compounds 97, 99, 102 and 104 exhibited good inhibition against both ATRA and 25-(OH)-D\textsubscript{3} metabolism. This showed that a hydroxyl
group at the 4-position and a hydrophobic group at the 2-position of the phenyl ring form hydrophilic interaction and hydrophobic interactions respectively at both the enzyme active sites.

The methods described above for the inhibition studies of the 25-(OH)-D₃ or ATRA metabolising enzymes are more rapid and facile assays in identifying potential inhibitors compared with the inhibition studies carried out using intact cell-lines or using yeast or bacterial cell systems that expressed specific enzyme.

**Table 6.4.** The IC₅₀ of the tetralone, tetralin and ketoconazole compounds against ATRA metabolism by rat liver microsome and against 25-(OH)-D₃ metabolism by rat kidney mitochondria.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Inhibition of 25-(OH)-D₃ metabolism IC₅₀, µM</th>
<th>Inhibition of ATRA metabolism IC₅₀, µM</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image" alt="Compound 93" /></td>
<td>&gt;100</td>
<td>2.4</td>
</tr>
<tr>
<td><img src="image" alt="Compound 97" /></td>
<td>2.6</td>
<td>2.2</td>
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<td>8.9</td>
<td>1.5</td>
</tr>
<tr>
<td><img src="image" alt="Compound 99" /></td>
<td>3.5</td>
<td>0.4</td>
</tr>
<tr>
<td>Compound</td>
<td>Structure</td>
<td>Value 1</td>
</tr>
<tr>
<td>----------</td>
<td>-----------</td>
<td>---------</td>
</tr>
<tr>
<td>100</td>
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</tr>
<tr>
<td>101</td>
<td><img src="image" alt="Structure 2" /></td>
<td>4.5</td>
</tr>
<tr>
<td>102</td>
<td><img src="image" alt="Structure 3" /></td>
<td>0.9</td>
</tr>
<tr>
<td>104</td>
<td><img src="image" alt="Structure 4" /></td>
<td>2.1</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td><img src="image" alt="Structure 5" /></td>
<td>20.0</td>
</tr>
</tbody>
</table>
CHAPTER 7

In vitro cell culture studies of vitamin D₃ and all-trans retinoic acid metabolism
7. **In vitro** cell culture studies of vitamin D₃ and all-trans retinoic acid metabolism

1α,25-(OH)₂-D₃ and ATRA have been shown to have pro-differentiation and anti-proliferation effects in both breast cancer and prostate cancer cell lines, namely the MCF-7, LNCaP, PC-3 and DU-145 cell lines (Campbell *et al.*, 1997; Campbell *et al.*, 1998; de Vos *et al.*, 1997; Guo *et al.*, 2002; Guo *et al.*, 2000; James *et al.*, 1995; Koshiuka *et al.*, 2000; Krekels *et al.*, 1997; Wang *et al.*, 2001).

*In vitro* cell line studies of 25-(OH)-D₃ and ATRA metabolism have been studies by various groups, to name a few examples:

- The use of human keratinocytes for vitamin D₃ metabolism (Kang *et al.*, 1997; Schuster *et al.*, 2001a; Schuster *et al.*, 2001b).
- The use of human colon cancer cells (Bareis *et al.*, 2001) and human non-small cell lung cancer cells (Jones *et al.*, 1999) for vitamin D₃ metabolism.
- The use of prostate cancer cells (DU-145, PC-3 and LNCaP) for the vitamin D₃ or all-trans retinoic acid metabolism (Farhan *et al.*, 2002; Farhan *et al.*, 2003; Guo *et al.*, 2002).
- The use of breast cancer cells (MCF-7, T47D) for the ATRA metabolism (Krekels *et al.*, 1997; Patel *et al.*, 2004; Van heusden *et al.*, 2002).
- The use of microsomal preparations from T47D breast cancer cells induced to express CYP26 enzyme (Mulvihill *et al.*, 2005; Stoppie *et al.*, 2000).

7.1 **Aims and objectives**

DU-145 and MCF-7 cell lines were chosen for the studies of *in vitro* metabolism of 25-(OH)-D₃ and ATRA. The MCF-7 cell line was routinely cultured at the Tenovus Cancer Research Centre at the Welsh School of Pharmacy. Whereas, DU-145 cell line was kindly donated by Dr. Moray J. Campbell (Institute of Biomedical Research, University of Birmingham) and cultured at the Tenovus Cancer Research Centre.

The aims of these studies were:

- To establish the methods and to investigate the metabolism of 25-(OH)-D₃ and ATRA in DU-145 and MCF-7 cell-lines.
- To evaluate the inhibition of 25-(OH)-D₃ and ATRA metabolism by the synthesized compounds using this *in vitro* cell culture based assay.
• To compare the metabolism of 25-(OH)-D₃ and ATRA in the two cell-lines with the metabolism of 25-(OH)-D₃ and ATRA in the rat kidney mitochondria and rat liver microsomes respectively.

7.2 Overview

7.2.1 Tissue culture

The equipments and materials used in tissue culture are as following:

<table>
<thead>
<tr>
<th>Disposable materials used in tissue culture</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterile disposable pipettes (1 mL, 5 mL and 10 mL)</td>
<td>Corning (New York, USA)</td>
</tr>
<tr>
<td>Sterile syringe (1 mL and 10 mL)</td>
<td>Becton Dickinson (BD®) UK Ltd.</td>
</tr>
<tr>
<td>25 mL Universal tube</td>
<td>L.I.P. (Equipment and Services Ltd.)</td>
</tr>
<tr>
<td>Sterile needles (BD microbalance™ 25 G 0.5 x 16 mm or 23 G 0.6 x 25 mm)</td>
<td>Becton Dickinson UK Ltd.</td>
</tr>
<tr>
<td>Tissue culture plates (6 wells) and flasks (25 cm² and 72 cm²)</td>
<td>Corning (New York, USA)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Media and supplements</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tissue culture media (RPMI 1640, RPMI 1640 phenol red-free)</td>
<td>All from Gibco Europe Ltd. (Paisley, Scotland)</td>
</tr>
<tr>
<td>0.25 % Trypsin-EDTA</td>
<td>All from Gibco Europe Ltd. (Paisley, Scotland)</td>
</tr>
<tr>
<td>Streptomycin/Penicillin</td>
<td>All from Gibco Europe Ltd. (Paisley, Scotland)</td>
</tr>
<tr>
<td>Fungizone</td>
<td>All from Gibco Europe Ltd. (Paisley, Scotland)</td>
</tr>
<tr>
<td>Foetal calf serum (FCS)</td>
<td>All from Gibco Europe Ltd. (Paisley, Scotland)</td>
</tr>
<tr>
<td>L-glutamine</td>
<td>All from Gibco Europe Ltd. (Paisley, Scotland)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Chemicals and Equipment</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trypan blue</td>
<td>Sigma Chemical (Dorset, UK)</td>
</tr>
<tr>
<td>Phosphate Buffer Saline (PBS) tablets (50 mM, pH 7.4)</td>
<td>Sigma Chemical (Dorset, UK)</td>
</tr>
<tr>
<td>Class II biological safety cabinet</td>
<td>Sorvall, Kendro Laboratory Products, Germany</td>
</tr>
<tr>
<td>Incubator (37 °C, 5 % CO₂)</td>
<td>Sanyo (Japan)</td>
</tr>
<tr>
<td>Haemocytometer</td>
<td>Neubauer (Germany)</td>
</tr>
<tr>
<td>Phase contrast microscope</td>
<td>Leitz Wetzlar (Germany)</td>
</tr>
<tr>
<td>Liarozole and R115866</td>
<td>Gift from Stiefel Laboratories, High Wycombe, UK</td>
</tr>
</tbody>
</table>
7.2.2 Cell-lines used

The two cell lines used in this research were:

1. The oestrogen responsive MCF-7 human mammary-carcinoma cell line which is oestrogen receptor-positive. This cell line is also known as the wild-type MCF-7 cell line. This cell was routinely grown in phenol-red free RPMI medium (wRPMI), supplemented with 5 % (v/v) steroid-depleted foetal calf serum (S-FCS), antibiotics (streptomycin and penicillin) and fungizone at the same concentration of 10 iU/mL.

Note: Phenol-red has been shown to have oestrogenic properties. Therefore MCF-7 cells were cultured in the absence of unwanted oestrogenic factors by using phenol-red free RPMI. In addition, it is necessary to grow the cells in the absence of steroid with the steroid-depleted foetal-calf serum (S-FCS).

2. The DU-145 human prostate cancer cell line which is androgen receptor-negative. This cell was routinely grown in RPMI medium, supplemented with 10 % (v/v) foetal calf serum (FCS), antibiotics (streptomycin and penicillin) and fungizone at the same concentration of 10 iU/mL.

7.2.3 Methods of tissue culture

7.2.3.1 Cells passaging

The MCF-7 and DU-145 cells were routinely cultured in 75 cm\(^2\) sterile flasks. Cells were passaged approximately every 5 – 7 days when the cells in the flasks reached confluence. Cells passaging were carried out as follows:

1. The medium in the flask was removed by an aspirating pump.
2. The monolayer was washed with PBS and was then removed.
3. 2 mL of trypsin (0.25 % trypsin-EDTA) was added to allow the cells in the flask to detach. Trypsin should not be left in the flask for longer than 5 minutes.
4. When cells were detached, 8 mL of the medium was added to the flask. The mixture was then transferred into a 25 mL universal tube and centrifuged at 1000 rpm for 5 min at room temperature.
5. The supernatant was removed by an aspirating pump.
6. The cells (pellet) were resuspended in 10 mL of medium by pipetting through a 10 mL sterile disposable pipette until no cell lumps could be seen.
7. 1 mL of the cell suspension above was seeded in 15 mL medium in a 75 cm² culture flask.

8. The culture flask was then kept in an incubator (5 % CO₂ at 37 °C) until needed.

7.2.3.2 Setting up of cells for vitamin D₃ and all-trans retinoic acid metabolism studies

The MCF-7 and DU-145 cell lines were seeded at 3 x 10⁶ cells and 1.5 x 10⁶ cells per well-plate (12 wells) respectively. MCF-7 cells are smaller in size compared with DU-145. Each of the cell lines was set up as follows:

1. The medium in the 75 cm² culture flask was removed by an aspirating pump and replaced by 2 mL of trypsin (0.25 % trypsin-EDTA).

2. When cells were detached (no longer than 5 minutes), 8 mL of medium was added to the flask. The mixture was then transferred into a 25 mL universal tube and centrifuged at 1000 rpm for 5 min at room temperature.

3. The supernatant was removed by an aspirating pump.

4. The cells (pellet) were resuspended in 10 mL of medium and were aspirated through a 10 mL sterile syringe attached to a 25 G 0.5 x 16 mm needle (for DU-145 a 23 G 0.6 x 25 mm needle was used instead) and then the cells were pushed through so that the cells could be separated from each other rather than forming clumps of cells when seeded on the well-plates.

5. A viable cell count was performed using a haemocytometer.

6. An adequate volume containing the appropriate number of cells needed was then mixed with the medium and seeded in the well-plates.

7.3 General assay for metabolism of vitamin D₃ and all-trans retinoic acid in cell based assay

The materials and equipments used in the general assay of vitamin D₃ and ATRA metabolism in cell culture were the same as described in section 6.4.

7.3.1 Methods

The methods described below were based on a modification of the method of Jarno (Jarno, 2003) and Kirby et al. (Kirby et al., 2003).

1. The MCF-7 cell lines were seeded at 3 x 10⁶ cells per well (12 wells) in the medium and left to settle for 24 h. The DU-145 cell line was seeded at 1.5 x 10⁶ cells per well (12 wells) in the medium and left to settle for 48 h.
2. After 24 h, the medium of the wild-type MCF-7 cells were removed and washed with 1 mL of PBS before being replaced by fresh medium plus various treatments.

3. After 48 h, the medium of the DU-145 cells were removed and washed with 1 mL of PBS before being replaced by the medium plus various treatments.

4. These treatments in each well contained either cold 25-hydroxyvitamin D₃ (10⁻⁸ M) plus 0.05 μCi 25-hydroxy-[26,27-methyl-³H]-vitamin D₃ or ATRA (10⁻⁷ or 10⁻⁸ M) plus 0.10 μCi [³H-11,12]-ATRA plus control (ethanol or acetonitrile) or inhibitor.

5. Tissue culture plates were wrapped in aluminum foil during the incubation time to prevent metabolism of the substrate.

6. Each treatment was performed in duplicate.

7. The incubation with the respective substrate was stopped by the addition of 2 % v/v acetic acid (100 μL/well).

8. The medium and acetic acid from each well was then removed and transferred to borosilicate glass tubes (13 x 100 mm) which contained 2 mL solution of ethyl acetate with 0.05 % (w/v) butylated-hydroxyanisole.

9. Distilled water (200 μL) was subsequently added to each well plate and the cells were scrapped off using the rubber end of a 1 mL syringe insert.

10. The cell suspension from each tube was transferred to the respective glass tubes.

11. Finally, each well was rinsed with distilled water (400μL) and then transferred to the respective glass tubes.

12. The glass tubes were centrifuged (3000 rpm for 15 min at room temperature).

13. The top organic layer containing the substrate and metabolites was transferred into respective borosilicate glass tubes (12 x 75 mm).

14. The medium was re-extracted with 2 mL of ethyl acetate with 0.05 % (w/v) butylated-hydroxyanisole. Steps 12 and 13 above were then repeated.

15. The tubes were placed in a rotating evaporator for 50 – 60 min.

16. The residue in each tube was redisolved in 60 μL methanol, then analysed using an on-line radioactive detector connected to a HPLC as described before under section 6.61.
7.4 Biological results and discussions

7.4.1 Metabolism of vitamin D₃ in MCF-7 and DU-145 cell-lines

After 3, 12 or 24 h incubation time, the peaks for the [³H]-25-(OH)-D₃ metabolites were not significant in both cell lines. Attempt to incubate the cells with \(10^{-8}\) M 1α,25-(OH)₂D₃ to enhance the level of CYP24 enzyme was carried out. After 12 – 48 h of pre-incubation time with 1α,25-(OH)₂D₃, the medium was removed and replaced with 25-hydroxyvitamin D₃ (\(10^{-8}\) M) plus 0.05 µCi 25-hydroxy-[26,27-methyl-³H]-vitamin D₃ in medium. The cells were then incubated for another 3, 12 or 24 h. However, the peaks for the [³H]-25-(OH)-D₃ metabolites were not significant (less than 15 % 25-(OH)-D₃ metabolites) in both cell lines. This could be due to the absence of the hydroxylase enzymes in the cells which resulted in the absence of 25-(OH)-D₃ metabolites.

A RT-PCR (reverse-transcriptase polymerase chain reaction) analysis was carried out, as described in chapter 8.2, to identify the presence of CYP24 mRNA. Since high level of CYP24 mRNA was expressed in DU-145 cells as shown in the PCR (see chapter 8.2), another method was tried to investigate the metabolism of 25-(OH)-D₃ in DU-145 cells based on the modification of various literatures (Bareis et al., 2001; Ma et al., 2004; Miller et al., 1995). DU-145 cells at approximately 80 % confluence were trypsinised to detach the cells from a 75 cm² sterile flask. 1 x 10⁶ cells in 200 µL of RMPI + 1 % FBS media were transferred into 12 x 75 mm borosilicate glass tubes. To each glass tube was added 1 µM 25-(OH)-D₃ plus 0.05 µCi 25-hydroxy-[26,27-methyl-³H]-vitamin D₃ with or without inhibitor prior to the addition of 1 x 10⁶ cells/200µL medium. The glass tubes were then incubated for 30 min at 37 °C in a shaking water bath. The reaction was stopped by adding 620 µL of chloroform:methanol (1:2) into each glass tubes. After each glass tubes was vortexed, the tubes were left standing at room temperature for 45 min. The tubes were then centrifuged at 1000 rpm for 15 min. The supernatant was then transferred into respective glass tubes containing 200 µL of chloroform and 100 µL of distilled water. After vortexing, the tubes were centrifuged at 1000 rpm for 15 min. The lower organic phase was transferred into another clean glass tube, whereas the water phase was re-extracted with 200 µL chloroform. The combined organic phases in the tubes were placed in a rotating evaporator for 20 min. The percentage of the peaks for the 25-(OH)-D₃ metabolites were 25 – 28 % in the absence of inhibitor (Figure 7.1A), compared with 10 – 11 % metabolites in the presence of the
inhibitor, (50 μM of compound 99) (Figure 7.1B). The inhibition studies of the 25-(OH)-D₃ metabolisms using the synthesised compounds were not carried out due to the low amount of metabolites in the control.

Figure 7.1 (A) Reverse-phase HPLC chromatograms showing the metabolism of [³H] 25-(OH)-D₃ in the presence of (A) suspension of DU-145 cells in RPMI and 1 % FBS medium (control) (B) suspension of DU-145 cells in RPMI and 1 % FBS medium plus 50 μM compound 99. Column: 5 μm ODS EXSIL® 4.6 x 200 mm; Mobile phase: 75:25:0.1 = acetonitrile:1 % w/v ammonium acetate in water:acetic acid at a flow rate of 1.5 mL/min. Retention time for [³H] 24,25-(OH)-D₃ = 3.8 min, [³H] 25-(OH)-D₃ = 10.5 min.
7.4.2 Metabolism of all-trans retinoic acid in MCF-7 cell-line

After 9 h incubation time, approximately 60 ± 10 % [³H]-ATRA was metabolised in the MCF-7 cell-line (Figure 7.2). Addition of 100 μM liarozole reduced the metabolism of all-trans retinoic acid to 13 % in the wild-type MCF-7 cells. This result was consistent with the result reported by Jarno (Jarno, 2003).

A RT-PCR analysis was carried out, as described in section 8.2, to identify the presence of CYP26A1 isozyme. CYP26A1 is only present after MCF-7 cells are induced with 10⁻⁷ M ATRA for 9 hours. This shows that this MCF-7 cell based assay is more relevant being a measure of human ATRA metabolism by the specific CYP26A1 enzyme.

![Diagram](image)

**Figure 7.2.** HPLC of an incubation extract of 10⁻⁷ M all-trans retinoic acid plus 0.1 μCi [³H-11,12] all-trans retinoic acid with MCF-7 cell-line. HPLC column: 10 μM C18 υBondapak® 300 x 3.9 mm column; mobile phase: 75:25:1 = Acetonitrile: 1 % w/v ammonium acetate in water: acetic acid. Pump rate: 1.9 mL/min. Retention time: 4-keto-retinoic acid and 4-hydroxy-retinoic acid (1.5 min – 3.5 min) and all-trans retinoic acid (5.5 min).
7.4.2.1 Inhibition of all-trans retinoic acid metabolism in MCF-7 cell-line

Inhibition of ATRA metabolising enzyme in MCF-7 cells was studied using the synthesised compounds described in chapter 3 – 5. Ketoconazole, liarozole and the selective CYP26 inhibitor, R115866, were used as the standard inhibitors. The IC$_{50}$ of the inhibition of ATRA metabolising enzyme in the MCF-7 cell line are illustrated in Table 7.1 – 7.4. The IC$_{50}$ values were the mean of two experiments. These values for inhibition of ATRA metabolism was measured using $10^{-7}$ M ATRA with 0.1 $\mu$Ci $[^{3}]$H-ATRA as substrate.

Table 7.1. Inhibition of ATRA metabolising enzyme in MCF-7 cells.

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC$_{50}$</th>
<th>Compound</th>
<th>IC$_{50}$</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1.png" alt="Image" /></td>
<td>4.5 $\mu$M</td>
<td><img src="image2.png" alt="Image" /></td>
<td>5 $\mu$M</td>
</tr>
<tr>
<td><img src="image3.png" alt="Image" /></td>
<td>7 $\mu$M</td>
<td><img src="image4.png" alt="Image" /></td>
<td>9 $\mu$M</td>
</tr>
<tr>
<td><img src="image5.png" alt="Image" /></td>
<td>20 – 40 $\mu$M</td>
<td><img src="image6.png" alt="Image" /></td>
<td>20 – 40 $\mu$M</td>
</tr>
<tr>
<td><img src="image7.png" alt="Image" /></td>
<td>7 $\mu$M</td>
<td><img src="image8.png" alt="Image" /></td>
<td>10 – 20 $\mu$M</td>
</tr>
<tr>
<td><img src="image9.png" alt="Image" /></td>
<td>0.005 $\mu$M</td>
<td><img src="image10.png" alt="Image" /></td>
<td>10 – 20 $\mu$M</td>
</tr>
</tbody>
</table>

Liarozole

Ketoconazole

R115866
Table 7.2. Inhibition of ARRA metabolizing enzyme in MCF-7 cells.
Table 7.3. Comparing the unsaturated and saturated tetralone compounds for inhibition of ATRA metabolising enzyme in MCF-7 cells (Yee et al., 2005).

<table>
<thead>
<tr>
<th>Compound (Unsaturated tetralone)</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt;</th>
<th>Compound (Saturated tetralone)</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image" alt="Compound 91" /></td>
<td>7 µM</td>
<td><img src="image" alt="Compound 97" /></td>
<td>25 - 50 µM</td>
</tr>
<tr>
<td><img src="image" alt="Compound 92" /></td>
<td>9 µM</td>
<td><img src="image" alt="Compound 99" /></td>
<td>5 µM</td>
</tr>
<tr>
<td><img src="image" alt="Compound 94" /></td>
<td>50 - 100 µM</td>
<td><img src="image" alt="Compound 98" /></td>
<td>20 - 40 µM</td>
</tr>
<tr>
<td><img src="image" alt="Compound 84" /></td>
<td>50 - 100 µM</td>
<td><img src="image" alt="Compound 100" /></td>
<td>25 - 50 µM</td>
</tr>
<tr>
<td><img src="image" alt="Compound 85" /></td>
<td>20 - 40 µM</td>
<td><img src="image" alt="Compound 101" /></td>
<td>50 - 100 µM</td>
</tr>
<tr>
<td><img src="image" alt="Compound 95" /></td>
<td>50 - 100 µM</td>
<td><img src="image" alt="Compound 102" /></td>
<td>20 - 40 µM</td>
</tr>
<tr>
<td><img src="image" alt="Compound 96" /></td>
<td>20 - 40 µM</td>
<td><img src="image" alt="Compound 103" /></td>
<td>10 - 20 µM</td>
</tr>
<tr>
<td><img src="image" alt="Compound 104" /></td>
<td></td>
<td></td>
<td>10 - 20 µM</td>
</tr>
</tbody>
</table>
Table 7.4. Inhibition of ATRA metabolising enzyme in MCF-7 cells.

<table>
<thead>
<tr>
<th>Compound (Unsaturated tetralone)</th>
<th>IC$_{50}$</th>
<th>Compound (Unsaturated tetralone)</th>
<th>IC$_{50}$</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image" alt="Compound 82" /></td>
<td>50 – 100 μM</td>
<td><img src="image" alt="Compound 83" /></td>
<td>9 μM</td>
</tr>
<tr>
<td><img src="image" alt="Compound 93" /></td>
<td>20 – 40 μM</td>
<td><img src="image" alt="Compound 78" /></td>
<td>50 – 100 μM</td>
</tr>
<tr>
<td><img src="image" alt="Compound 79" /></td>
<td>50 – 100 μM</td>
<td><img src="image" alt="Compound 86" /></td>
<td>25 – 50 μM</td>
</tr>
<tr>
<td><img src="image" alt="Compound 80" /></td>
<td>50 – 100 μM</td>
<td><img src="image" alt="Compound 81" /></td>
<td>25 – 50 μM</td>
</tr>
</tbody>
</table>

Table 7.1 and 7.2 show two series of compounds which have either imidazole or triazole ring moieties. The first series of compounds (14 – 17) illustrated in Table 7.1 shows comparable IC$_{50}$ results to liarozole. Introduction of a chloro atom at the 4-position of the bi-phenyl ring (compounds 18 and 19) reduces the inhibition. This may indicate that the size of the enzyme active site is not favourable for bulky groups.

However, when introducing an amide linkage between the benzofuran and the phenyl ring (Table 7.2, compounds 51 – 65), this reduces the IC$_{50}$ results compared with liarozole, except for compounds 52 and 55 which were comparable with ketoconazole (10 μM – 20 μM). This indicates that there may be a hydrogen-bonding interaction between the –OCH$_3$ (compound 52) and the amino acid of the enzyme active site and there may be a hydrophobic interaction between the Cl atom (compound 55) at the enzyme active site.

Derivatives of unsaturated and saturated tetralones were synthesised (described in chapter 5) and reported here to be less potent or equipotent with standard inhibitors, ketoconazole and liarozole, in MCF-7 cells (Table 7.3 and 7.4). Compounds 91, 92, 99 (Table 7.3) and compound 83 (Table 7.4) are equipotent to liarozole, whereas compounds 103 and 104 (Table 7.3) are equipotent to ketoconazole.
Chapter 7

Our group (Kirby et al., 2003) have demonstrated that 2-(4-aminophenylmethyl)-6-hydroxy-3,4-dihydro-2H-naphthalen-1-one (105) (Figure 7.3) to have moderate inhibition of ATRA-metabolising enzymes in mammalian cadaverous tissue microsomes and homogenates as well as ATRA-induced enzymes in cultured human genital fibroblasts and HaCat cells (transformed keratinocytes), as summarised in Table 7.5. In view of this, the compounds (+) and (-) 2-(4-aminophenylmethyl)-6-hydroxy-3,4-dihydro-2H-naphthalen-1-one, (+) 105, were tested using the above MCF-7 cell assay (Table 7.5). But they are less active than ketoconazole and liarozole.

![Chemical structure of 2-(4-aminophenylmethyl)-6-hydroxy-3,4-dihydro-2H-naphthalen-1-one, (+) 105.](image)

Figure 7.3. Chemical structure of 2-(4-aminophenylmethyl)-6-hydroxy-3,4-dihydro-2H-naphthalen-1-one, (±) 105.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Compound</th>
<th>IC₅₀ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat liver microsomes (Kirby et al., 2003)</td>
<td>(±)-105</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>(+)-105</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>(-)-105</td>
<td>10</td>
</tr>
<tr>
<td>Human liver microsomes (Kirby et al., 2003)</td>
<td>(±)-105</td>
<td>66 % at 100 µM</td>
</tr>
<tr>
<td></td>
<td>(+)-105</td>
<td>61 % at 100 µM</td>
</tr>
<tr>
<td></td>
<td>(-)-105</td>
<td>54 % at 100 µM</td>
</tr>
<tr>
<td>ATRA-induced genital fibroblast cells</td>
<td>(±)-105</td>
<td>19 ± 5</td>
</tr>
<tr>
<td>(Kirby et al., 2003)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATRA-induced HaCat cells</td>
<td>(±)-105</td>
<td>16 ± 8</td>
</tr>
<tr>
<td>(Kirby et al., 2003)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCF-7 cells</td>
<td>(+)-105</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td>(-)-105</td>
<td>39</td>
</tr>
</tbody>
</table>

Table 7.5. IC₅₀ (µM) of compound (±) 105 against ATRA metabolising enzymes in different assay systems.

The synthesised saturated and unsaturated tetralone derivatives evaluated for their retinoic acid metabolism inhibitory activity using rat liver microsomes were described in chapter 6. A summary of their inhibitory activity using rat liver microsomes and the MCF-7 cell based assay is described here in Table 7.6.
Table 7.6. A summary of the tetralone derivatives evaluated for their retinoic acid metabolism inhibitory activity using MCF-7 cell assay and rat liver microsomes. Ketoconazole, liarozone and R115866 were used as standards for comparison (Yee et al., 2005).

<table>
<thead>
<tr>
<th>Compound</th>
<th>MCF-7 IC₅₀, µM</th>
<th>Rat liver microsomes IC₅₀, µM</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1" alt="Image" /></td>
<td>9</td>
<td>&gt; 20</td>
</tr>
<tr>
<td><img src="image2" alt="Image" /></td>
<td>20-40</td>
<td>2.4</td>
</tr>
<tr>
<td><img src="image3" alt="Image" /></td>
<td>7</td>
<td>&gt; 20</td>
</tr>
<tr>
<td><img src="image4" alt="Image" /></td>
<td>9</td>
<td>&gt; 20</td>
</tr>
<tr>
<td><img src="image5" alt="Image" /></td>
<td>25-50</td>
<td>2.2</td>
</tr>
<tr>
<td><img src="image6" alt="Image" /></td>
<td>20-40</td>
<td>1.5</td>
</tr>
<tr>
<td><img src="image7" alt="Image" /></td>
<td>5</td>
<td>0.4</td>
</tr>
<tr>
<td><img src="image8" alt="Image" /></td>
<td>25-50</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>Structure</td>
<td>Activity 1</td>
</tr>
<tr>
<td>---</td>
<td>-----------</td>
<td>------------</td>
</tr>
<tr>
<td>101</td>
<td><img src="image1" alt="Structure 101" /></td>
<td>&gt; 50</td>
</tr>
<tr>
<td>102</td>
<td><img src="image2" alt="Structure 102" /></td>
<td>20 – 40</td>
</tr>
<tr>
<td>103</td>
<td><img src="image3" alt="Structure 103" /></td>
<td>10 – 20</td>
</tr>
<tr>
<td>104</td>
<td><img src="image4" alt="Structure 104" /></td>
<td>10 – 20</td>
</tr>
<tr>
<td><strong>Liarozole</strong></td>
<td><img src="image5" alt="Structure Liarozole" /></td>
<td>7</td>
</tr>
<tr>
<td><strong>Ketoconazole</strong></td>
<td><img src="image6" alt="Structure Ketoconazole" /></td>
<td>10 – 20</td>
</tr>
<tr>
<td><strong>R115866</strong></td>
<td><img src="image7" alt="Structure R115866" /></td>
<td>0.005</td>
</tr>
</tbody>
</table>
The inhibitory activity using the two assay systems, as summarised in Table 7.6 did not generally correlate. This is because, the ATRA metabolism in the rat liver microsomes involves non-specific liver CYPs whereas the ATRA metabolism in MCF-7 cells involves the ATRA-inducible CYP26. In terms of selectivity, the unsaturated tetralone compounds 83, 91 and 92 (IC₅₀: rat liver microsomes, > 20 μM; MCF-7 cells, 7 – 9 μM) would appear to be more specific for CYP26A1. In contrast, compound 99 shows good inhibitory activity in both assays (IC₅₀: rat liver microsomes, 0.4 μM; MCF-7 cells, 5 μM). Two research groups, Johnson & Johnson Pharmaceutical Research group (Stoppie et al., 2000; Van heusden et al., 2002) and OSI Pharmaceuticals Inc. (Mulvihill et al., 2005) have shown compounds with high selectivity of CYP26 compared with different CYP isoforms found in liver. However, it seems that both the non-specific liver CYPs and the ATRA-inducible CYP26 would need to be inhibited. This is because it is necessary to have initial ATRA accumulation by the inhibition of non-specific CYPs in the liver to allow induction of CYP26 (Njar et al., 2000; Patel et al., 2004).

7.4.2.2 Molecular Docking

Cytochrome P450 is a superfamily of enzymes comprising a large range of proteins with diverse functions. The 3-dimensional structures of P450s are of scientific interest. Although the sequences of different P450s proteins are known, only five crystallographic structures of P450s were identified by 2002. Four of the P450s are from bacterial sources and one from a mammalian source, they are:

- P450cam (CYP101) derived from Pseudomonas putida camphor P450 (Poulos et al., 1987)
- P450BM-3 (CYP102) derived from Bacillus megaterium P450 (Ravichandran et al., 1993)
- P450ery (CYP107) derived from Saccharaopolyspora erythreae erythromycin F P450 (Cupp-Vickery and Poulos, 1995)
- P450terp (CYP108) derived from Pseudomonas putida α-terpineol P450 (Hasemann et al., 1994)
- P450 2C5/3LVdH derived from modification to the residues and modifications to the C- and N-terminal transmembrane domain of the rabbit cytochrome P450 2C5 (to increase solubility and to promote crystallisation) (Williams et al., 2000)
In July 2003, P.A. Williams’s group reported the first crystal structure of human cytochrome P450, which is the CYP2C9 (Williams et al., 2003). This was then followed by two other crystal structures of human P450 enzymes, namely, CYP2C8 (Schoch et al., 2004) and CYP3A4 (Yano et al., 2004). Our group recently used comparative modelling to construct models of CYP26A1 using the three-dimensional structure of these three recent human cytochrome P450 as templates, i.e. CYPs 2C8 (PDB code: 1PQ2), 2C9 (1R90) and 3A4 (1TQN). The three models were generated by the available software packages (i) Molecular Operating Environment (MOE) (Molecular Operating Environment 2003.04) and (ii) SYBYL (SYBYL 7.0).

### 7.4.2.3 Docking studies

The FlexX programme (Rarey et al., 1996) interfaced with SYBYL 7.0 and the MOE-Dock programme (MOE-Dock 2003.04) were used to dock the substrate (ATRA) and CYP26A1 inhibitor (R115866) inside the active site of these three generated CYP26A1 models. The docking studies showed that the CYP26A1 model using the CYP3A4 as template gave the best active site fitting for both ATRA and CYP26A1 inhibitor after performing molecular dynamics and further active site optimisation (Figure 7.4). Figure 7.4 shows the ATRA substrate at the active site of this CYP26A1 model based on CYP3A4 as template. The carboxylic acid group of ATRA is H-bonded to the SER126 and ASN127 residues and the cyclohexyl ring of ATRA lies close to the heme (Figure 7.4). The C-4 atom of the ATRA is positioned at a distance of 4.85 Å directly above the heme iron to allow hydroxylation at the C-4 position by a water molecule. ATRA is held within the hydrophobic tunnel via multiple hydrophobic interactions with amino acid residues at the active site TRP112, PHE299, THR304, PHE374 and VAL370.

The nitrogen atom of the imidazole or triazole group of the inhibitors, R- and S-liarozole and R115866, coordinate with the heme iron transition metal, at a distance of 2.2 – 3.2 Å. H-bonding and multiple hydrophobic interactions between the inhibitor and the active site hold the inhibitor within the active site. For example, H-bonding interactions between the nitrogen atom of the liarozole benzimidazole ring and the peptide carbonyl of VAL480 and PRO113 for the R- and S-enantiomer respectively [Figure 7.5 (A) and (B)]. Similarly to ATRA, R- and S-liarozole is also held within the hydrophobic tunnel. The main amino acid residues at the active site, TRP112, PHE299, PRO113, THR304, PHE374, ALA114, VAL116 and THR118 contributed to the
hydrophobic interactions with $R$- and $S$-liarozole [Figure 7.5 (A) and (B)]. $R$- and $S$-R115866 form a H-bonding interaction with the peptide carbonyl of PRO113 in the model (Figure 7.6), as well as hydrophobic interactions with the side chains of TRP112, PHE374, THR304, VAL370, PHE84, PHE299, VAL116 and GLY300.

Docking of the synthesised compounds has been carried out using FlexX. The triazole ring of compounds 14 – 19 showed a co-ordination with the transition metal, as seen with liarozole, at the active site and only hydrophobic interactions between the inhibitor and the amino acid side-chains. Similarly, the series II compounds, 51 – 65 also form hydrophobic interactions with the side-chain amino acids and co-ordination with the heme iron transition metal. The nitrogen atom of the amide bond in compounds 51 – 65 does not form a H-bonding with the amino acid unlike R115866. This may explain why these compounds displayed poor IC$_{50}$ values compared with R115866.

![Diagram](image.png)

**Figure 7.4.** Diagram showing ATRA docked at the active site of the CYP26A1 model based on CYP3A4 as template. (A) Hydrogen bonding and hydrophobic interactions are shown as red and green dashed lines respectively. (B) The distance between the C-4 atom of ATRA and the heme iron is shown as dark green line. Colour coding of the atoms: grey=carbon; red=oxygen; blue=nitrogen, brown=iron, yellow=sulphur.
Figure 7.5. Diagram showing the inhibitor (A) R-liarozole and (B) S-liarozole docked at the active site region of CYP26A1 model based on CYP3A4 as template. Hydrogen bonding interactions are shown as red dashed lines and co-ordination with the transition metal is indicated with a purple line. Colour coding of the atoms: grey=carbon; red=oxygen; blue=nitrogen, brown=iron, yellow=sulphur.

Docking of the tetralone compounds have been carried out. The docking results show that the 4-hydroxyphenyl of compounds 91, 92, 97 – 99 forms H-bonding interaction with GLY300 and/or co-ordination with the transition metal at the active site (Figure 7.6). In addition, the 6-hydroxy group of compound 92 forms H-bonding interaction with SER115 and ASP227 besides co-ordination of the 4-hydroxy group of the phenyl ring with the transition metal at the active site (Figure 7.6). Similarly to liarozole and R115866, these compounds also form hydrophobic interactions with the side chains.

These H-bonding, hydrophobic interactions and co-ordination with the transition metal at the active site resulted in good inhibition of CYP26A1 by compounds 91, 92 and 99 in MCF-7 cells (IC$_{50}$: 5 – 9 µM). Although compounds 97 and 98 also form these interactions at the active site, they demonstrate poor IC$_{50}$ (> 20 µM). Overall, this CYP26A1 model has led to a better understanding of the key binding mode between the ligand or inhibitor with the amino acid residues at the active site.
Figure 7.6. The H-bonding interactions between the inhibitors and the side-chains at the active site as shown in red dashed line. Colour coding of the atoms: grey=carbon; red=oxygen; blue=nitrogen, brown=iron, yellow=sulphur.
7.4.3 Metabolism of all-trans retinoic acid in DU-145 cell-line

After 24 h incubation time, only 15 – 18 % of the [3H]-ATRA were metabolised in the DU-145 cell-lines. Attempt to incubate the cells with 10⁻³ M ATRA to enhance the level of CYP26 enzyme was carried out. After 24 h of incubation time with ATRA, the medium was removed and being replaced with ATRA (10⁻³ M) plus 0.10 μCi [³H]-ATRA in medium. The cells were then incubated for another 12, 24 or 48 h. After 12, 24 or 48 h incubation time, approximately 20±5 %, 40±2 % and 60±5 % of [³H]-ATRA were metabolised respectively (Figure 7.4).

![Figure 7.4](image)

**Figure 7.4.** HPLC of an incubation extract of 10⁻³ M ATRA plus 0.1 μCi [³H]-ATRA with DU-145 cell-line. HPLC column: 10 μM C18 μBondapak⁵ 300 x 3.9 mm column; mobile phase: 75:25:1 = Acetonitrile: 1 % w/v ammonium acetate in water: acetic acid. Pump rate: 1.9 mL/min. Retention time: 4-keto-retinoic acid and 4-hydroxy-retinoic acid (1.5 min – 3.5 min) and ATRA (5.5 min). DU-145 cells were incubated with 10⁻³ M ATRA for 24 h before addition of 10⁻³ M ATRA plus 0.1 μCi [³H]-ATRA for a further 24 h.

Due to time constraints, only 4 compounds (14, 15, 91 and 99) and two standard inhibitors (liarazole and R115866) at 10 μM were tested for inhibition of ATRA metabolism in DU-145 cells. The % inhibitions of ATRA metabolism in DU-145 cells using 10 μM of tested compounds are shown in Table 7.7. Unlike the inhibition of ATRA metabolism in MCF-7 cells, the percentage inhibition of the synthesised compounds (14, 15, 91 and 99) and liarazole at 10 μM did not show above 50 % inhibition. The differences could be due to the different susceptibility of the inhibitor to DU-145 cells and the different accessibility of the inhibitor to the target enzymes compared with MCF-7 cells.
Table 7.7. % Inhibition of ATRA metabolism in DU-145 cells using 10 μM of the tested compounds and 10^{-7} M ATRA plus 0.1 μCi [3H]-ATRA. DU-145 cells were incubated with 10^{-7} M ATRA for 24 h before addition of 10^{-7} M ATRA plus 0.1 μCi [3H-11,12] ATRA and the following inhibitors for a further 24 h.

<table>
<thead>
<tr>
<th>Compound</th>
<th>% Inhibition</th>
<th>Compound</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="14.png" alt="Chemical Structure" /></td>
<td>44</td>
<td><img src="15.png" alt="Chemical Structure" /></td>
<td>41</td>
</tr>
<tr>
<td><img src="91.png" alt="Chemical Structure" /></td>
<td>39</td>
<td><img src="99.png" alt="Chemical Structure" /></td>
<td>37</td>
</tr>
<tr>
<td><img src="Liarozole.png" alt="Chemical Structure" /></td>
<td>42</td>
<td><img src="R115866.png" alt="Chemical Structure" /></td>
<td>80</td>
</tr>
</tbody>
</table>

7.5 General conclusions

Despite the fact that DU-145 cells expressed a high level of CYP24 mRNA (as demonstrated by RT-PCR analysis, section 8.2), only a small amount of [3H]-25-(OH)-D₃ metabolites (10 – 15 %) were observed by HPLC analysis. Even though DU-145 cells were pre-treated with 10 nM 1α,25-(OH)₂-D₃ for 24 h to induce a higher level of CYP24 enzyme, the amount of [3H]-25-(OH)-D₃ metabolites were still low (20 – 25 %), unlike the rat kidney mitochondria assay, where 40 ± 5 % of [3H]-25-(OH)-D₃ metabolites were formed (section 6.7.1). The rate of metabolism and the rate of transport of [3H]-25-(OH)-D₃ in these two systems could be very different and resulted in the lower [3H]-25-(OH)-D₃ metabolites formed in DU-145 cells.

A novel method to study the metabolism of ATRA in MCF-7 and DU-145 cells has been described here. In the MCF-7 and DU-145 cell assays, the ATRA metabolising enzyme, CYP26A1 is induced by ATRA (10^{-7} M). The RT-PCR analysis (section 8.2) showed the presence of CYP26A1 mRNA only after 9 h and 24 h incubation with ATRA (10^{-7} M) in MCF-7 and DU-145 cells respectively. DU-145 cells required a longer incubation time with ATRA (up to 48 h) to produce the same amount of [3H]-
ATRA metabolites as in MCF-7 cells (9 h). As a result, the IC$_{50}$ of the synthesised compounds were only evaluated in the MCF-7 cells.

Compared with the rat liver microsome assay (section 6.7.2), the enzyme activity in the MCF-7 and DU-145 cell assays is more relevant being a measure of human ATRA metabolism by the specific CYP26A1 enzyme. Some of the synthesised compounds described in Table 7.6 inhibited both enzyme activities in both assays, whereas, some compounds were more selective against the non-specific CYPs in the rat liver microsomes. As mentioned earlier, it is necessary to inhibit the non-specific CYPs in the liver microsomes in order to have a sufficient level of ATRA to induce the CYP26A1 enzyme.

A molecular model for CYP26A1 based on the CYP3A4 template was constructed by our group recently. Molecular docking using the substrate, ATRA and inhibitors (standard inhibitors and some of the synthesised compounds) was carried out to identify the key interactions between the substrate and inhibitors with the enzyme residues at the active site.
CHAPTER 8

Further studies and investigations
8. Further studies and investigations

Since the DU-145 prostate cancer cells did not express significant level of 25-(OH)-D₃ metabolites after incubation of [³H]-25-(OH)-D₃ at various time point and after pre-treatment with 1,25-(OH)₂-D₃ (section 7.4.1), further investigations were pursued. The analysis that could be carried out to determine whether the DU-145 cells that were used express the CYP24 enzyme are western blotting, immunohistochemistry and reverse-transcriptase polymerase chain reaction (RT-PCR). Due to its simplicity and the available facilities, RT-PCR was used to look at the expression of CYP24 and CYP26A1 mRNA in the DU-145 and MCF-7 cells that were used in the assays described in Chapter 7. RT-PCR was carried out (section 8.2) under the supervision of Dr. Bronwen Evans and Mrs. Carole Elford at the Department of Child Health, Heath Hospital, Cardiff.

Dr. Moray J. Campbell (Institute of Biomedical Research, Birmingham University) who has done considerable research in studying vitamin D₃ receptor and vitamin D₃ gene targets in breast and prostate cancer (Campbell et al., 1997; Campbell and Koeffler, 1997; Ma et al., 2004), allowed me to spend some time in his laboratory. The aim and objectives of the work in Campbell’s laboratory were:

- To quantify the expression level of CYP24 in DU-145 with or without inhibitors using real-time quantitative RT-PCR method.
- To investigate whether co-treatment with the inhibitor and 1α,25-(OH)₂-D₃ will enhance the proliferation of the DU-145 cells compared with 1α,25-(OH)₂-D₃ treated prostate cancer cells.

8.1 Introduction to RT-PCR and real-time quantitative RT-PCR

Polymerase chain reaction (PCR) has a great variety of application in forensic sciences and in molecular biology (White, 1996). It is a method by which millions of DNA or genes can be made in a couple of hours. This method (Mullis and Fuluona, 1987) was invented by Dr. Kary B. Mullis in 1983, for which he received the Nobel Prize in Chemistry in 1993.

In this section, a brief introduction to the principle of the PCR will be discussed using CYP24 as the example of the mRNA of interest.

In order to run a PCR, cDNA is required. Total RNA from the cells (e.g. DU-145 cells) is first isolated and then all the RNA in the cells is reverse transcribed to cDNA by the enzyme reverse transcriptase (RT). Following the reverse transcription
step, PCR is then carried out with the amplification of the target cDNA (i.e. CYP24 as example) using CYP24 forward and reverse primers (Figure 8.1). In the PCR reaction, only the cDNA of interest will be amplified, as specific primers of the specific cDNA are used here. Since both strands of the cDNA are copied during the PCR (Step 2, Figure 8.1), there is an exponential increase in the number of copies of the gene. The forward and reverse primers that are chosen have to be very specific for the cDNA of interest, and it is usually 19-25 base pair long. Taq Polymerase enzyme, which was originally isolated from Thermus aquaticus (Eom et al., 1996; Saiki et al., 1988), can now be supplied as a recombinant enzyme from Escherichia coli (Lawyer et al., 1989), and is an important enzyme in the annealing of the primer to the cDNA (Step 2) and allow extension of the chain (Step 3, Figure 8.1).

The next step is the verification and detection of PCR product at the end of the PCR reaction on agarose gel. This is to check whether the cDNA is formed and whether it is of the right size.

Before introducing the quantitative real-time PCR technique, it is important to understand what happens during PCR reaction. PCR reaction is broken down to 3 phases (Figure 8.2):

- **Exponential** phase: Doubling of the product occurs every cycle.
- **Linear** phase: Reaction is slowing down since reaction components are being consumed.
- **Plateau** phase (End-point): The reaction begins to slow down and stops all together. The PCR products will degrade if the reaction is left longer.
Figure 8.1. The diagram shows the steps involved in the reverse-transcriptase polymerase chain reaction (RT-PCR). The RT reaction converts the total RNA in the cells into cDNA. In the PCR reaction, only the specific cDNA of interest (e.g. CYP24 cDNA) is amplified.

In the traditional PCR, agarose gel is used to detect the amount of the target at the **plateau phase**, which would not truly represent the initial amounts of the target. In real-time PCR, a Sequence Detection Systems (SDS) instrument is used to measure the amount of the target during the amplification cycle of the PCR (as shown in Figure 8.2). This technique is revolutionised by the use of a fluorescence dye in the reaction. The higher the starting copy number of the target (e.g. CYP24), the sooner an increase in fluorescence is observed, and thus giving a lower threshold cycle (Ct) value. Ct value is the cycle number at which logarithmic PCR plots cross a calculated threshold-line (Figure 8.2).
Figure 8.2. The three phases in a PCR reaction. The different colours of the dashed lines indicate different cDNA sample, for example, cDNAs of DU-145 cells treated at different time point with 1α,25-(OH)₂-D₃. The diagram is modified from the Applied Biosystems “Application tools and tutorials” which can be downloaded from the website www.appliedbiosystems.com.

There are two types of fluorescence dye that are used to detect PCR products using SDS instrument, namely fluorogenic 5' nuclease chemistry and SYBR Green I dye chemistry. Fluorogenic 5’ nuclease chemistry involves a fluorogenic probe, while SYBR Green I dye chemistry involves a highly specific, double-stranded DNA binding dye, to enable the detection of the PCR product as it accumulates during the PCR cycles. Fluorogenic probe was chosen for the quantitation of the PCR products due to its specificity, sensitivity and the ability to multiplex reactions. The following diagram (Figure 8.3) demonstrates the steps that are involved in the fluorogenic 5’ nuclease chemistry.

The SDS instrument will capture the fluorescence emitted by the reporter signal. The amount of reporter signal increase is proportional to the amount of product being produced for a given sample (Applied Biosystems). As a result, you will get a plot as shown in Figure 8.2. Real-time PCR provides fast, precise and accurate results for DNA or RNA quantitation. This makes the real-time PCR able to detect as little as a 2-fold change.
1. A fluorescent reporter (R) dye and a quencher (Q) are attached to the 5' and 3' ends respectively, of a dual-labeled probe. This dual-labeled probe is designed to anneal to a specific sequence of the template between the forward and reverse primers. This probe do not fluoresce when intact.

2. During the extension cycle of the template, the DNA polymerase is adding bases to the growing chain of DNA, and when it comes to the probe, it then cleaves the fluorescent reporter dye (R).

Once the dyes become separated, the reporter dye emits its characteristic fluorescence.

Figure 8.3. The steps that are involved in the quantitation of the PCR products using the fluorogenic probe (dual-labeled probe). The figure has been adapted from Applied Biosystem's website (Applied Biosystems).

The objectives of using real-time RT-PCR in this study were:

- To quantify the expression level of CYP24 mRNA in DU-145 and PC-3 androgen-independent prostate cancer cell-lines with or without 1α,25-(OH)_{2}-D_{3}.

- To quantify the expression level of CYP24 mRNA in DU-145 and PC-3 cells treated with 1α,25-(OH)_{2}-D_{3} and inhibitor, e.g. ketoconazole or inhibitor alone.

1α,25-(OH)_{2}-D_{3} inhibits growth and stimulates differentiation of cancer cells, by regulating the expression of specific genes. Two of the genes that are involved in cell growth and cell differentiation are p21^{waf1/cip1} and GADD45α genes. Previous work has demonstrated that p21^{waf1/cip1} gene, encoding the cyclin-dependent kinase inhibitor, is a target of 1α,25-(OH)_{2}-D_{3} (Prudencio et al., 2001). The magnitude of p21^{waf1/cip1} induction varies among different cell types (Prudencio et al., 2001). The expression of GADD45α (growth-arrest and DNA-damage) gene is overexpressed in cells that are associated with growth arrest (Prudencio et al., 2001). It would be interesting to investigate by the real-time PCR whether the level of p21^{waf1/cip1} and GADD45α mRNA are affected by 1α,25-(OH)_{2}-D_{3} alone or with inhibitor.
8.2 RT-PCR analysis

The following traditional RT-PCR analysis was carried out under the supervision of Dr. Bronwen Evans and Mrs. Carole Elford at the Department of Child Health, Heath Hospital, Cardiff.

DU-145 (gift from Dr. M.J. Campbell) and MCF-7 cells cultured at Tenovus Cancer Research Centre, Cardiff University, were both seeded at 1.5 x 10⁶ cells in a T75 flask. Cells at approximately 70 % confluence were then used for isolation of total RNA using Tri Reagent (Sigma-Aldrich) following the manufacturer’s instructions. Total RNA amounts were quantified by measuring absorbance at 260 nm. The A₂₆₀/A₂₈₀ nm absorption ratio was greater than 1.7. Total RNA was used to synthesise cDNA using the manufacturer’s protocol provided with an AMV reverse transcriptase (Promega, Madison, Wisconsin) and performed in a thermocycler (Perkin Elmer). Total RNA (2 μg) in the presence of 8 μL of MgCl₂ (25 mM), 2 μL of oligo(dT)₁₅ primer (500 ng/μL), 4 μL of Reverse Transcription 10 x buffer, 4 μL of dNTP mix (10 mM), 1 μL of Recombinant RNasin® Ribonuclease Inhibitor, 1.6 μL AMV reverse transcriptase and nuclease-free water (to give a total reaction volume of 40 μL) was incubated at 42 °C for 60 min in a thermocycler. The newly synthesised cDNA was then used for PCR analysis. The RNA and cDNA were stored at – 20 °C when not used.

PCR amplification kit by Promega (Promega, Madison, Wisconsin) was used for the PCR reaction. The PCR reaction mixture for CYP24 contained 2 μL of cDNA, 2.5 μL of 10 x PCR reaction buffer, 1 μL forward primer (50 pmol/μL), 1 μL reverse primer (50 pmol/μL), 0.5 μL dNTP mix (10 mM), 0.2 μL Taq DNA polymerase (5U/μL) and nuclease-free water (to give a total reaction volume of 25 μL). Reactions were cycled as follows: 94 °C for 5 min, 59 °C for 30 s, 72 °C for 1 min, then 28 cycles at 94 °C for 15 s, 59 °C for 30 s, 72 °C for 1 min.

The PCR reaction mixture for CYP26A1 or β-actin contained 2.5 μL of cDNA, 2.5 μL of 10 x Reverse Transcription 10 x buffer, 0.25 μL forward primer (100 μM), 0.25 μL reverse primer (100 μM), 0.5 μL dNTP mix (10 mM), 0.2 μL Taq DNA polymerase (5U/μL) and nuclease-free water (to give a total reaction volume of 25 μL). Taq DNA polymerase was activated at 94 °C for 5 min. The mixtures were then subjected to 30 cycles of amplification. Each cycle conditions: 94 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min. An extra extension step was included (72 °C for 10 min) at the end of the incubation. β-Actin was used as the endogenous control.
Negative control of cDNA synthesis was carried out under the same experimental conditions, but in the absence of AMV reverse transcriptase. The sequences of the forward and reverse primers are listed in Table 8.1. 20 μL of the PCR products were separated on a 1.5 % (w/v) agarose gel containing ethidium bromide and separated at 100 V. The bands were visualized under UV light illumination (Figure 8.4).

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP24 forward primer</td>
<td>5'-CCCCTAGCACCTCGTACCAAC-3'</td>
</tr>
<tr>
<td>CYP24 reverse primer</td>
<td>5'-CGTAGCCTTTTGCCTGATC-3'</td>
</tr>
<tr>
<td>(507 base pair segment)</td>
<td>(Bareis et al., 2001; Farhan et al., 2003)</td>
</tr>
<tr>
<td>CYP26A1 forward primer</td>
<td>5'-GCTGAAGAGATAGGGTTTAC-3'</td>
</tr>
<tr>
<td>CYP26A1 reverse primer</td>
<td>5'-CTTGGGAATCTGGATCCAT-3'</td>
</tr>
<tr>
<td>(184 base pair segment)</td>
<td>(Van Heudsen et al., 2002)</td>
</tr>
<tr>
<td>β-actin forward primer</td>
<td>5'-CCCAGCCATGTACGTTGCTA-3'</td>
</tr>
<tr>
<td>β-actin reverse primer</td>
<td>5'-AGGGCATACCCTCGTAGATG-3'</td>
</tr>
<tr>
<td>(800 base pair segment)</td>
<td></td>
</tr>
</tbody>
</table>

The results showed that DU-145 cells expressed significant level of CYP24 but MCF-7 cells did not express the CYP24 enzyme, in untreated condition [Figure 8.4, (A)]. Enhanced level of CYP24 mRNA is observed in both DU-145 and MCF-7 cells in the presence of 10 nM 1α,25-(OH)2-D3 for 6 h as demonstrated from the real-time quantitative RT-PCR (data not shown here).

CYP26A1 is not expressed in untreated DU-145 and MCF-7 cells, however, CYP26A1 is expressed after treatment with 10⁻⁷ M ATRA for 24 and 9 h respectively [Figure 8.4, (B)]. This demonstrated that in the MCF-7 and DU-145 cell assays (described in section 7.4.2 and 7.4.3), expression of the ATRA metabolising enzyme, CYP26A1 is induced by ATRA (10⁻⁷ M), therefore the inhibition enzyme studies in the MCF-7 and DU-145 cell assays is more relevant being a measure of the human CYP26A1.
8.3 Real-time quantitative RT-PCR analysis (qPCR)

The following experiments described below were carried out in Dr. Moray J. Campbell’s laboratory at the Institute Biomedical Research, Birmingham University.

The prostate cancer cell lines PC-3 and DU-145 were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). The cells were maintained in RPMI 1640 medium (Gibco-BRL, Paisley), supplemented with 10% fetal calf serum (Gibco-BRL), 100 units/mL penicillin and 100 μg/mL streptomycin. The cells were passaged by trypsinising with 0.25% trypsin-EDTA (Gibco-BRL). The cells were grown at 37 °C in a humidified atmosphere of 5% CO₂ in air.

DU-145 and PC-3 cells were both seeded at 3.5 x 10⁵ cells/well in a 6-well plate. Cells at approximately 70% confluence were then used for isolation of total RNA using Tri Reagent (Sigma-Aldrich) following the manufacturer’s instructions. The cells were also treated with various treatments for certain amounts of time before total RNA isolation was carried out. Total RNA amounts were quantified by
measuring absorbance at 260 nm. The $A_{260}/A_{280}$ nm absorption ratio was greater than 1.7. Total RNA was used to synthesise cDNA using the manufacturer’s protocol provided with a M-MLV reverse transcriptase (Superscript™ II Reverse Transcriptase, Invitrogen, UK) and performed in a thermocycler (Applied Biosystems Gene Amp PCR System 9700). In brief, total RNA (2 µg) in the presence of 10 mM dNTP mix (1 µL) and 250 ng/µ L random hexamer mix (0.5 µL, Promega) and nuclease-free water (to give a total reaction volume of 12 µL) was incubated at 68 °C for 5 min to allow for annealing of random primers, then placed on ice. 8 µL of the reverse transcriptase master mix (table below) was then added to the mixture above and incubated in a thermocycler (Applied Biosystems Gene Amp PCR System 9700) at 25 °C for 10 min, then 42 °C for 90 min to enable reverse transcription elongation to occur, and then 70 °C for 5 min to denature the enzyme. The newly synthesised cDNA is ready for qPCR. The cDNA were stored at – 20 °C if not used.

**Reverse transcriptase master mix**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>5X First-strand buffer (Invitrogen, UK)</td>
<td>4 µL</td>
</tr>
<tr>
<td>0.1 mM DTT (Dithiothreitol – as a stabilizer in PCR) [Invitrogen, UK]</td>
<td>2 µL</td>
</tr>
<tr>
<td>40 units/µL recombinant ribonuclease inhibitor (RNaseOUT™, Invitrogen, UK)</td>
<td>1 µL</td>
</tr>
<tr>
<td>2000 units/µL M-MLV reverse transcriptase (Superscript™ II Reverse Transcriptase, Invitrogen, UK)</td>
<td>1 µL</td>
</tr>
</tbody>
</table>

The expression of the specific mRNAs (*i.e.* CYP24, p21wafl/cip1 and GADD45α) was quantitated using the ABI PRISM 7700 Sequence Detection System. The sequences of the forward and reverse primers and the probe are listed in Table 8.2. Each sample was amplified in triplicate wells in 20 µL volumes containing 19 µL of the following qPCR master mix and 1 µL of the cDNA made. All-reactions were multiplexed with pre-optimised control primers and VIC labeled probe for 18S ribosomal RNA (TaqMan® Ribosomal RNA control reagents, Applied Biosystems, Warrington, UK). Reactions were cycled as follows: 50 °C for 2 min, 95 °C for 10 min (for activation of the QuickGoldStar DNA polymerase); then 44 cycles for 95 °C for 15s and 60 °C for 1 min (Gommersall et al., 2004; Ma et al., 2004).
**qPCR master mix**

<table>
<thead>
<tr>
<th></th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>2X qPCR™ QuickGoldStar MasterMix (5mM MgCl₂, dNTPs, QuickGoldStar DNA polymerase, Uracil-N-glycosylase) [Eurogentec Ltd., Belgium]</td>
<td>10 μL</td>
</tr>
<tr>
<td>1.25 pmol/μL FAM-labeled TaqMan probe (Eurogentec Ltd., Belgium)</td>
<td>2 μL</td>
</tr>
<tr>
<td>9 pmol/μL forward primer (Alta Bioscience, Birmingham, UK)</td>
<td>2 μL</td>
</tr>
<tr>
<td>9 pmol/μL reverse primer (Alta Bioscience, Birmingham, UK)</td>
<td>2 μL</td>
</tr>
<tr>
<td>18S ribosomal RNA forward primer (Applied Biosystems, Warrington, UK)</td>
<td>0.1 μL</td>
</tr>
<tr>
<td>18S ribosomal RNA reverse primer (Applied Biosystems, Warrington, UK)</td>
<td>0.1 μL</td>
</tr>
<tr>
<td>18S ribosomal RNA VIC labeled probe (Applied Biosystems, Warrington, UK)</td>
<td>0.1 μL</td>
</tr>
<tr>
<td>Nuclease-free water</td>
<td>2.7 μL</td>
</tr>
</tbody>
</table>

**Table 8.2.** The sequences of the forward and reverse primers and the FAM-labelled TaqMan probe used for the specific mRNAs.

<table>
<thead>
<tr>
<th>Primers and probe</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP24 forward primer</td>
<td>5’-CAAACCGTGGAAGGCTATC-3’</td>
</tr>
<tr>
<td>CYP24 reverse primer</td>
<td>5’-AGTCTTCCCCCTCCAGGATCA-3’</td>
</tr>
<tr>
<td>CYP24 probe</td>
<td>5’-ACTACCGCAAAGGAAGGTACGGCTGCTG-3’</td>
</tr>
<tr>
<td>p21 wafl/cip1 forward primer</td>
<td>5’-GCAGACCAGCATGACAGTTTC-3’</td>
</tr>
<tr>
<td>p21 wafl/cip1 reverse primer</td>
<td>5’-GGATTAGGGCTTCTCTTGG-3’</td>
</tr>
<tr>
<td>p21 wafl/cip1 probe</td>
<td>5’-CCACTCAAACGCGGCTGATCTTT-3’</td>
</tr>
<tr>
<td>GADD45α forward primer</td>
<td>5’-AAGACCGAAAGGATGGATAAGGT-3’</td>
</tr>
<tr>
<td>GADD45α reverse primer</td>
<td>5’-GTGATCGTGCGCTGACTCA-3’</td>
</tr>
<tr>
<td>GADD45α probe</td>
<td>5’-TGCTGAGCCTTCTCCAGGGCAT-3’</td>
</tr>
</tbody>
</table>

Data were expressed as, Ct values (see Figure 8.2) and used to determine ∆Ct values. ∆Ct = Ct of the target gene minus Ct of the housekeeping gene. The housekeeping gene is the 18S ribosomal RNA. The data was transformed through the equation 2^-∆∆Ct to give fold changes in gene expression (Gommersall et al., 2004; Ma et al., 2004). ∆∆Ct = ∆Ct of the target gene from treated cells minus ∆Ct of the target gene from non-treated (control) cells (Livak and Schmittgen, 2001). To exclude potential bias due to averaging of data all statistics were performed with ∆Ct values. Measurements were carried out at least two times each in triplicate wells.
8.3.1 Results and discussions of the qPCR analysis

Initial studies have focused on the effect of the inhibitors of vitamin D₃ metabolising enzymes, i.e. ketoconazole and compound 99 (2-(4-hydroxybenzyl)-6-methoxy-3,4-dihydro-2H-naphthalen-1-one), for the qPCR study to measure the regulation of the known VDR target genes, CYP24, p21^{waf1/cip1} and GADD45α in response to the following treatment conditions (1 – 4) in DU-145 and PC-3 cells.

1. DMSO solvent (control).
2. 10 nM 1α,25-(OH)₂-D₃ for 7 h.
3. Ketoconazole (100 µM) or compound 99 (100 µM) throughout the treatment.
4. For treatment with inhibitor plus 1α,25-(OH)₂-D₃, the cells were pre-treated with the inhibitor, ketoconazole (100 µM) or compound 99 (100 µM) for overnight (16 h) before the addition of this combination mixture for further 7 h.

However, it was found later from cell-proliferation assay (MTT assay, section 8.4) and direct cell-counting (by haemocytometer) that the concentration of the inhibitors used (100 µM) had direct anti-proliferative effect. Therefore, lower concentration of the inhibitors was used that do not have direct anti-proliferative effect to the cells. Due to time factor, the qPCR analysis was repeated following the above same treatment conditions (1 – 4) however using lower concentration of the inhibitors (i.e. 7.5 µM ketoconazole or 10 µM compound 99) in DU-145 cells only.

Compound 99 was chosen among all the other synthesised compounds, as it was shown to have good inhibition compared with ketoconazole and other compounds in the in vitro inhibition of 25-(OH)-D₃ metabolism in rat kidney mitochondria (Table 6.2); and ATRA metabolism in rat liver microsomes (Table 6.3) and MCF-7 cell culture assay (Table 7.3).

Each data point in the graphs here (Graphs 8.1 – 8.3) represent the mean of three separate experiments (only two separate experiments for the DU-145 cells treated with 7.5 µM ketoconazole and 10 µM compound 99 with or without 1α,25-(OH)₂-D₃) each measured in triplicate wells. The CYP24, p21^{waf1/cip1} and GADD45α mRNA levels of all samples shown in the graphs are normalized to 18S RNA and compared with the cells treated with DMSO only, which was arbitrarily set to 1. The error bars in the graphs represent ± standard error mean (SEM). All statistical analyses were performed using the Student’s t-test, p < 0.05 was considered statistically significant.
8.3.1.1 Regulation of CYP24 mRNA in DU-145 and PC-3

The untreated DU-145 and PC-3 cells expressed undetectable to low level of CYP24 mRNA. However, when the cells were treated with 10 nM 1α,25-(OH)₂-D₃ for 7 h, DU-145 cells showed 1999-fold induction, while PC-3 cells showed 1100-fold induction in CYP24 mRNA relative to the untreated cells (Graph 8.1). Ketoconazole (at 100 and 7.5 μM) or compound 99 (at 100 and 10 μM) alone did not have significant effect on the CYP24 mRNA level in DU-145 and PC-3 cells.

Ketoconazole (100 μM), when combined with 10 nM 1α,25-(OH)₂-D₃ reduced the CYP24 mRNA to 362-fold change and 266-fold change in DU-145 and PC-3 cells respectively, relative to the cells treated with 10 nM 1α,25-(OH)₂-D₃ alone. However, 7.5 μM ketoconazole when combined with 10 nM 1α,25-(OH)₂-D₃ synergistically increased the CYP24 mRNA transcription level (5340-fold change) in DU-145 compared to DU-145 treated with 1α,25-(OH)₂-D₃ alone (1999-fold change), as shown in Graph 8.1.

On the other hand, combination treatment with compound 99 (100 or 10 μM) and 10 nM 1α,25-(OH)₂-D₃ in DU-145 significantly enhanced the CYP24 mRNA (> 10000-fold change) compared to the cells treated with 1α,25-(OH)₂-D₃ alone (1999-fold change). However, combination treatment with compound 99 (100 μM) and 10 nM 1α,25-(OH)₂-D₃ in PC-3 cells (2242-fold change) did not show statistically significant difference to the PC-3 cells treated with 1α,25-(OH)₂-D₃ alone (1100-fold change) (p < 0.1).

![Graph 8.1](image_url)

**Graph 8.1.** Determinations of CYP24 mRNA fold changes in DU-145 and PC-3 cells by quantitative real-time RT-PCR. Values are given as mean ± S.E.M (n = 2 or 3). **p < 0.05 when compared to control and ***p < 0.05 when compared to cells treated with 1α,25-(OH)₂-D₃ alone.
It is known from various studies that ketoconazole inhibits the activity of vitamin D₃ metabolising enzymes, \textit{i.e.} both CYP1α and CYP24 enzymes (Kang \textit{et al.}, 1997; Peehl \textit{et al.}, 2001) and thus prevents the conversion of active metabolite, 1α,25-(OH)₂-D₃, to inactive metabolites. Farhan’s group (Farhan \textit{et al.}, 2002; Farhan \textit{et al.}, 2003) has shown that 50 μM of genistein alone reduces CYP24 mRNA expression to more than 40 % after 8 h dosing in DU-145. The group used the conventional RT-PCR and the densitometer to semi-quantitatively measured the intensity of the band on the agarose gel. In contrast, Kang’s group demonstrated using northern blot analysis that addition of ketoconazole to 1α,25-(OH)₂-D₃ on normal healthy human skin for 2 days, caused synergistic increase of the CYP24 mRNA level (RNA extracted from the normal healthy human skin biopsy specimen) (Kang \textit{et al.}, 1997). Recently, Feldman’s group (Swami \textit{et al.}, 2005) used real-time RT-PCR and demonstrated significant increase in CYP24 mRNA in DU-145 co-treated with 10 μM genistein and 10 nM 1α,25-(OH)₂-D₃.

The results here showed that compound 99 (at 100 μM and 10 μM) and ketoconazole (at 7.5 μM) enhanced the 1α,25-(OH)₂-D₃-mediated induction of CYP24 mRNA in DU-145. In the rat kidney mitochondria assay (section 6.6.1, \textit{Table 6.2}), ketoconazole and compound 99 showed inhibition of 25-(OH)-D₃ metabolising enzymes activity. As a result, this prolongs the half-life of 1α,25-(OH)₂-D₃ and enhances the genomic actions of 1α,25-(OH)₂-D₃ to induce the VDR target genes including CYP24 gene as measured by the increased in CYP24 mRNA expression as shown in here (\textbf{Graph 8.1}).

However, the reduced CYP24 mRNA level in DU-145 and PC-3 caused by combination treatment of ketoconazole (100 μM) with 10 nM 1α,25-(OH)₂-D₃ (\textbf{Graph 8.1}) could be due to the direct anti-proliferative effect of ketoconazole (at 100 μM) which resulted in a decreased in the number of cells, and thus reduced the level of CYP24 mRNA.

\textbf{8.3.1.2 Regulation of p21\textsuperscript{waf1/cip1} mRNA in DU-145 and PC-3}

The effects of the inhibitor alone or with 1α,25-(OH)₂-D₃ and the effect of 1α,25-(OH)₂-D₃ alone on expression of p21\textsuperscript{waf1/cip1} mRNA were investigated here. Studies in cancer cell-lines have shown that transcriptional induction of p21\textsuperscript{waf1/cip1} mRNA facilitates the induced differentiation of the cell-lines (Hager \textit{et al.}, 2001; Liu \textit{et al.}, 1996). The p21\textsuperscript{waf1/cip1} protein, a cell-cycle regulatory protein, inhibits the
activity of cyclin-dependent kinases in the G0/G1 cell-cycle phase, for example, resulting in cell-cycle arrest in response to 1α,25-(OH)2-D3 in prostate cancer cells (Campbell et al., 1997).

However, the degree of p21waf1/cip1 induction varies among different cell types (Prudencio et al., 2001). There were conflicting results from various studies related to the degree of p21waf1/cip1 expression. Some studies found that 1α,25-(OH)2-D3 had no effect on p21waf1/cip1 mRNA expression, others found that 1α,25-(OH)2-D3 downregulated p21waf1/cip1 mRNA, while others found that the anti-proliferative effect of 1α,25-(OH)2-D3 is linked to the over expression of p21waf1/cip1 protein (Hager et al., 2004).

The results here showed that treatment with 10 nM 1α,25-(OH)2-D3 alone had no significant effect on p21waf1/cip1 mRNA expression in DU-145 and PC-3 cells compared to untreated cells (Graph 8.2). However, when both cell-lines were treated with inhibitor alone (at 100 µM) or combination treatment of inhibitor and 1α,25-(OH)2-D3, these enhanced the p21waf1/cip1 mRNA significantly (p < 0.005). There were no significant difference when comparing the cells treated with inhibitor alone (at 100 µM) to the cells treated with both inhibitor and 1α,25-(OH)2-D3. This observation indicated that the inhibitor itself at 100 µM has a significant effect on p21waf1/cip1 mRNA.

Eichenberger et. al. (Eichenberger et al., 1989) showed that ketoconazole (1 – 50 µg/mL) has a direct anti-proliferative effect on the prostate cancer cells (DU-145 and PC-3). Moreover, the cell proliferation and cell viability assay (MTT assay, described in section 8.4) showed that ketoconazole (at 10 – 25 µM) or compound 99 (at 25 µM) alone in DU-145 and PC-3, showed anti-proliferative effect compared to untreated cells (Graph 8.4). This direct anti-proliferative effect by ketoconazole (100 µM) or compound 99 (100 µM) could result in induction of p21waf1/cip1 mRNA as observed in the qPCR here.

At lower concentration of the inhibitor alone (10 µM compound 99), this did not have an effect on the p21waf1/cip1 mRNA level in DU-145 (0.7-fold change). However, ketoconazole (7.5 µM) alone caused slightly enhanced p21waf1/cip1 mRNA (2-fold change) compared to untreated cells. It is interesting to observe the synergistic enhanced p21waf1/cip1 mRNA level in DU-145 cells co-treated with compound 99 (10 µM) and 1α,25-(OH)2-D3 compared to the cells treated with compound 99 or 1α,25-(OH)2-D3 alone (Graph 8.2).
However, this co-operative enhancement of $p21^{\text{waf1/cip1}}$ mRNA level was not observed in combination treatment with ketoconazole (7.5 μM) and 1α,25-(OH)$_2$-D$_3$, as ketoconazole (at 7.5 μM) alone also caused significant enhanced induction of $p21^{\text{waf1/cip1}}$ mRNA (2.0-fold change, Graph 8.2).

**Graph 8.2.** Determinations of $p21^{\text{waf1/cip1}}$ mRNA fold changes in DU-145 and PC-3 cells by quantitative real-time RT-PCR (qPCR). Values are given as mean ± S.E.M. ($n$ = 2 or 3).

### 8.3.1.3 Regulation of GADD45α mRNA in DU-45 and PC-3

Campbell’s group and others have demonstrated using cDNA microarray analysis that GADD45α is a vitamin D$_3$ receptor (VDR) target gene in 1α,25-(OH)$_2$-D$_3$-sensitive cell lines (Akutsu et al., 2001; Gommersall et al., 2004; Khanim et al., 2004). Over expression of GADD45α inhibits cell proliferation and it is induced by various agents that damage DNA and arrest cell growth (Akutsu et al., 2001). Induction of GADD45α contributes to the growth-inhibitory effects of 1α,25-(OH)$_2$-D$_3$ in 1α,25-(OH)$_2$-D$_3$-sensitive cell lines (Akutsu et al., 2001).

After 7 h treatment with 10 nM 1α,25-(OH)$_2$-D$_3$ alone, there is no significant elevated levels of GADD45α mRNA (1.3- and 1.7-fold change in DU-145 and PC-3 respectively compared to untreated cells) [Graph 8.3]. This showed that 1α,25-(OH)$_2$-D$_3$ alone had limited effect on GADD45α in DU-145 and PC-3 cells. This is similar to the reported studies by Campbell’s group (Gommersall et al., 2004).

However, when DU-145 and PC-3 were treated with inhibitor alone (at 100 μM) or combination treatment of inhibitor (at 100 μM) with 1α,25-(OH)$_2$-D$_3$, there is a strong induction of GADD45α mRNA ($p < 0.05$) (> 3-fold changes). There
were no significant difference when comparing the cells treated with inhibitor alone (at 100 μM) to the cells co-treated with inhibitor (at 100 μM) and 1α,25-(OH)₂-D₃. This indicated that the inhibitor alone at 100 μM significantly elevated the GADD45α mRNA in DU-145 and PC-3. The induction of GADD45α mRNA observed here could be due to the direct anti-proliferative effect in the presence of high concentration of the inhibitor (100 μM).

The synergistic enhanced GADD45α mRNA in DU-145 cells treated with compound 99 (10 μM) and 1α,25-(OH)₂-D₃ was observed here (Graph 8.3). However, this synergistic enhanced of GADD45α mRNA level was not observed in combination treatment with ketoconazole (7.5 μM) and 1α,25-(OH)₂-D₃, as ketoconazole (at 7.5 μM) alone also caused induction of GADD45α mRNA (1.9-fold change, Graph 8.3).

**Graph 8.3.** Determinations of GADD45α mRNA fold changes in DU-145 and PC-3 cells by quantitative real-time RT-PCR (qPCR). Values are given as mean ± S.E.M. (n = 2 or 3).

### 8.3.1.4 Summary of the qPCR results

Ketoconazole (7.5 μM) or compound 99 (100 and 10 μM) alone did not alter the basal CYP24 mRNA level, however, the combination of the inhibitor with 1α,25-(OH)₂-D₃ (10 nM) synergistically increased the CYP24 mRNA transcription level in DU-145 and/or PC-3 (Graph 8.1) by impeding the inactivation of 1α,25-(OH)₂-D₃ (i.e. by inhibiting the CYP24 enzyme activity).

Ketoconazole or compound 99 (at 100 μM) alone or with 1α,25-(OH)₂-D₃ (10 nM) caused significant anti-proliferative effect which resulted in the enhanced level of p21waf1/cip1 and GADD45α mRNA in DU-145 and PC-3 (Graph 8.2 and 8.3).
Compound 99 (at 10 μM) did not alter the p21waf1/cip1 and GADD45α mRNA level, however, compound 99 (at 10 μM) with 1α,25-(OH)2-D3 (10 nM) synergistically increased the p21waf1/cip1 and GADD45α mRNA level in DU-145 (Graph 8.2 and 8.3). These observations co-relate well with the result from the cell proliferation assay [section 8.4, Graph 8.4 (b)], which showed that the addition of compound 99 (at 10 μM) to 1α,25-(OH)2-D3 (10 nM) significantly reduced the growth of DU-145 cells.

8.4 Cell proliferation and cell viability assay (MTT assay)

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay is commonly used to study cell viability and proliferation in cell populations. This assay can be carried out in a microplate (96-well plates), thus reduces the amount of culture medium and number of cells required.

MTT assay is developed based on the detection of cell viability and cell proliferation (Mossman, 1983). The MTT is reduced to a blue-magenta coloured formazan precipitate only by reductase enzyme present only in metabolically active cell’s mitochondrial dehydrogenases (cofactor NADPH) (Figure 8.5). Moreover, this assay is also for the determination of cell proliferation since proliferating cells are metabolically more active than non-proliferating (resting) cells. Quantitation of MTT reduction measured directly in the microplate using an absorbance microplate reader.

![Chemical structure of MTT and its corresponding formazan product](image)

Figure 8.5. Chemical structure of MTT and its corresponding formazan product.

8.4.1 Methods for MTT assay

Cells were plated in 96-well plates (Appleton Woods, Birmingham, UK). Both the DU-145 and PC-3 cells were seeded at 2 x 10³ cells per well. Cells were allowed to attach (left at least 7 h), and treated with growth media containing varying concentrations of the inhibitor alone, varying concentrations of the inhibitor with 10 nM 1α,25-(OH)2-D3, or 10 nM 1α,25-(OH)2-D3 alone, resulting in a final volume of 100 μL per well. The plates were incubated for 96 h, with re-dosing after 48 h. After
93 h incubation, 20 μL of MTT solution (5 mg/mL in distilled water and filtered to sterilise and to remove small amount of insoluble residue present) [Sigma-Aldrich, Dorset, UK] were added to each well and the cells were left for 3 h in the incubator (37 °C and 5 % CO₂) [So in total 96 h incubation time]. The 120 μL of MTT-containing cells and medium were removed without disturbing the blue precipitate attached to the well. 100 μL of DMSO (Sigma-Aldrich, Dorset, UK) were added into each well and incubated for 15 min at room temperature. Finally, the absorption was measured at 550 nm using the absorbance microplate reader (EMax®, Molecular Devices Corporation). The growth inhibition was expressed as a percentage of control.

8.4.2 Results and discussions from the MTT assay

The action of the individual inhibitor alone (ketoconazole or compound 99) and in combination with 10 nM 1α,25-(OH)₂-D₃, or with 10 nM 1α,25-(OH)₂-D₃ alone in both DU-145 and PC-3 cells was examined using this MTT assay. The results are shown in Graphs 8.4 (a) – (d).

DU-145 and PC-3 cells responded minimally to the anti-proliferative effect of 1α,25-(OH)₂-D₃ (at 10 nM), i.e. only 5 % - 7 % growth inhibition after 96 h [Graphs 8.5 (a) – (b)]. In DU-145 cells, ketoconazole and compound 99 showed significant anti-proliferation effect at > 10 μM (> 20 % growth inhibition). However, the combination treatment with 5 μM ketoconazole or 10 μM compound 99 with 10 nM 1α,25-(OH)₂-D₃ caused more than 30 % growth inhibition in DU-145 [Graphs 8.4 (a) – (b)].

In contrast, PC-3 cells are not only recalcitrant to 1α,25-(OH)₂-D₃ (at 10 nM) but also did not show significant anti-proliferation when the inhibitor and 1α,25-(OH)₂-D₃ were used together [Graphs 8.4 (c) – (d)]. Ketoconazole and compound 99 alone showed significant anti-proliferation effects at, ≥ 10 μM and > 25 μM, respectively in PC-3.

The potentiation of the anti-proliferative effect of 1α,25-(OH)₂-D₃ by ketoconazole and compound 99 in DU-145 could be due to the inhibition of 1α,25-(OH)₂-D₃ metabolism in the cells. However, this potentiation of the anti-proliferative effect of 1α,25-(OH)₂-D₃ is not significant in PC-3 cells.
Graphs 8.4 (a) – (d). The effects of the inhibitor (ketoconazole or compound 99 at various concentrations) with or without 10 nM 1α,25-(OH)₂-D₃ on the proliferation of DU-145 (a – b) and PC-3 cells (c – d) was assessed by MTT assay after 96 h, with a re-dose after 48 h. Each data point represents the mean of three separate experiments undertaken in triplicate wells (± S.E.M). * Indicates p < 0.05 and ** indicates p < 0.01 when samples were compared to samples treated with the inhibitor alone.

From the above preliminary data, further MTT assays were carried out to study the anti-proliferative effect of other inhibitors alone and in combination with 10 nM 1α,25-(OH)₂-D₃ in DU-145 and PC-3. A total of four other tetralone analogues (compounds 91, 102, 98 and 104) and four azole compounds (compounds 14, 16, 55 and liarozole) at 20 μM, 10 μM and 2 μM concentration with or without 1α,25-(OH)₂-D₃ were used in this proliferation assay [Graphs 8.5 (a) – (d)].

Generally, the inhibitors (at 20 μM and 10 μM) with 10 nM 1α,25-(OH)₂-D₃ showed synergistic anti-proliferative effect in both DU-145 and PC-3 cells. Among the azole compounds, compound 14 maintained synergistic anti-proliferative effect with 10 nM 1α,25-(OH)₂-D₃ even at 2 μM concentration in both cell-lines, whereas, 2 μM liarozole with 1α,25-(OH)₂-D₃ only showed synergistic effect in PC-3 [Graphs 8.5 (a) and (b)].

Unlike the tetralone analogues, the azole compounds alone generally showed anti-proliferative effect at 10 and 20 μM. The potentiation of the anti-proliferative
effect of 1α,25-(OH)₂-D₃ by these tetralone analogues is more significant in DU-145 than in PC-3. However, this potentiation effect was lost when low concentration of the inhibitor (2 μM) was used together with 10 nM 1α,25-(OH)₂-D₃.

In conclusion, the azole compounds and the tetralone analogues (at 10 – 20 μM) with 1α,25-(OH)₂-D₃ (at 10 nM) showed strong combinatorial inhibition of proliferation in DU-145 and PC-3. Some inhibitors with 1α,25-(OH)₂-D₃ showed more significant anti-proliferation in DU-145 compared to PC-3. This shows that the enhancement of anti-proliferative activity of 1α,25-(OH)₂-D₃ by the inhibitor is cell-type specific, which was also observed by Zhao et al. (Zhao et al., 1996). A number of cell-proliferation assay have been carried out by other research groups which demonstrated that ketoconazole or liarozole showed synergistic growth inhibition with 1α,25-(OH)₂-D₃ (1 – 10 nM) in various cancer cell lines, e.g. HL60, MCF-7, MDA-MB-231 and DU-145 (Ly et al., 1999; Pehl et al., 2002; Rashid et al., 2001; Wang et al., 1997; Zhao et al., 1996). This showed that ketoconazole, liarozole or other potential vitamin D₃ metabolising enzymes inhibitor could potentially be used together with 1α,25-(OH)₂-D₃ for the treatment of various cancer, including prostate cancer. This combination treatment allowed the used of lower dose of 1α,25-(OH)₂-D₃ and thus the reducing the undesirable side-effects of 1α,25-(OH)₂-D₃, e.g. hypercalcemia.
The point represents the mean of two separate experiments undertaken in triplicate wells (± S.E.M.).

The effects of the inhibitory azole compounds and ketoconazole analogues at 20, 2 μM and 10 μM concentrations with or without 10 μM 1α,25-(OH)_{2}D_{3} on the proliferation of DU-145 and PC-3 cells was assessed by MTT assay after 96 h. Each data point represents the mean of two separate experiments undertaken in triplicate wells (± S.E.M.).
8.5 General conclusions

The conventional RT-PCR and real-time quantitative RT-PCR analysis demonstrated that the presence of 1α,25-(OH)₂-D₃ and ATRA induced the expression of CYP24 and CYP26A1 mRNA respectively in DU-145 and MCF-7 cells.

1α,25-(OH)₂-D₃ is an effective anti-proliferative agent at high concentration (> 100 nM), however, its use at high concentration is limited by side-effects in human, e.g. hypercalcemia and hypercalciuria. Moreover, the half-life of 1α,25-(OH)₂-D₃ is limited by the induction of CYP24 enzyme. Therefore, the combination treatment using an inhibitor of vitamin D₃ metabolising enzymes with low dose 1α,25-(OH)₂-D₃ (10 nM) could increase the half-life of 1α,25-(OH)₂-D₃. The cell-proliferation assay demonstrated that the sensitivity of DU-145 and/or PC-3 cells to the growth inhibitory actions of low dose 1α,25-(OH)₂-D₃ (10 nM) is increased by co-treatment with an inhibitor of vitamin D₃ metabolising enzymes (ketoconazole, liarozole and the synthesised tetralone analogues and azole compounds). Consistent with this cell-proliferation data, the expression of VDR target genes, i.e. p21⁰⁰⁰⁻¹ and GADD45α, associated with growth arrest showed significant increase following the combination treatment of an inhibitor of vitamin D₃ metabolising enzymes (compound 99) with 1α,25-(OH)₂-D₃ (10 nM).

It is possible that inhibitors of vitamin D₃ metabolising enzymes or selective CYP24 inhibitors can be used as combination therapies or as single agents in AIPC or other cancers.

8.6 Antifungal and antileishmanial evaluations

Dr. Marc Le Borgne and his group at the Department of Organic and Medicinal Chemistry, Faculty of Pharmacy, Nantes, France, have synthesised several series of imidazole and triazole compounds that have shown interesting antifungal and/or antileishmanial activities (Marchand et al., 2002; Na et al., 2003; Na et al., 2004; Pagniez et al., 2002). His group has collaborated with us to test some of their imidazole compounds for inhibition of retinoic acid metabolism in our rat liver microsomal enzyme assay. In return, his group at the Department of Parasitology, has performed the antifungal and antileishmanial in vitro activity of some of the compounds described in chapter 4.

Amphotericin B and the conazole compounds (e.g. ketoconazole, fluconazole, itraconazole and miconazole) are currently used in the market for both superficial and
systemic fungal infections. The function of the conazoles is to inhibit the cytochrome P-450-dependent 14α-lanosterol demethylase in the fungi and *Leishmania* species. This enzyme is responsible for the synthesis of the ergosterols (a component of the fungal and *Leishmania* cell membrane) via three sequential hydroxylations to remove the methyl group on C-14 (Aoyama *et al.*, 1989).

A total of seven compounds 51 – 55, 58 and 64 (Chapter 4) were selected to be evaluated against *Candida albicans* and *Aspergillus fumigatus*, the two most clinically important fungi responsible for the majority of systemic fungal infections; and these seven compounds were also tested against *Leishmania mexicana* promastigotes *in vitro*. The methods for the *in vitro* antifungal activity was carried out by the method based on the fluorometric properties of alamar Blue described by Le Borgne’s co-workers (Pagniez and Le Pape, 2001). The *in vitro* antileishmanial activity involved measuring the antiproliferative effect of the inhibitor against the *L. mexicana* promastigotes using a colorimetric method as described by Le Borgne’s group (Marchand *et al.*, 2002; Na *et al.*, 2004). Amphotericin B, ketoconazole, fluconazole and itraconazole were used as positive controls.

Among the seven compounds tested, compounds 51, 52, 54 and 55 showed activity against *Candida albicans* (IC$_{80}$ < 5 μM) and *Leishmania mexicana* promastigotes (IC$_{50}$ 38 – 62 μM) [Table 8.3] but no activity against *Aspergillus fumigatus*. Compounds 52 and 55 were the most active compounds among the seven compounds tested. The introduction of a methoxy group at 6-position of the benzofuran ring (compound 52) and chlorine atom at 4-position of the phenyl ring (compound 55) improved the activity slightly. Introduction of the nitro group at the 5-position of benzofuran group (compound 53) resulted in a loss of activity. The results also showed that the triazole compounds (58 and 64) were less active than the imidazole compounds. Although none of these compounds were as active as the positive controls these results will be useful for future reference in rational design of azole compounds to improve the potency against the fungal and/or leishmania species.
Table 8.3. *In vitro* antifungal and antileishmanial activity of benzofuran-2-carboxamido ethyl-imidazole and -triazole derivatives (51 – 55, 58 and 64)

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<th>Compound</th>
<th>Antifungal activity MIC (=IC&lt;sub&gt;50&lt;/sub&gt;)&lt;sup&gt;Candida albicans (CA982001)&lt;/sup&gt;</th>
<th>Antifungal activity MIC (=IC&lt;sub&gt;50&lt;/sub&gt;)&lt;sup&gt;Aspergillus fumigatus (AF988003)&lt;/sup&gt;</th>
<th>Antileishmanial activity IC&lt;sub&gt;50&lt;/sub&gt; Leishmania mexicana promastigotes</th>
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<td>&gt; 100</td>
<td>&gt; 100</td>
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<table>
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<th>Compound</th>
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CHAPTER 9

References
References


References


References


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References


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References


References


References


APPENDIX 1

X-ray crystal data
Table 1. Crystal data and structure refinement for compound 83.

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</tr>
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</tr>
<tr>
<td>Max. and min. transmission</td>
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<tr>
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<tr>
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<td>Goodness-of-fit on F²</td>
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<tr>
<td>R indices (all data)</td>
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<tr>
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Table 2. Atomic coordinates (x $10^4$) and equivalent isotropic displacement parameters (Å$^2$ x $10^3$) for compound 83. U(eq) is defined as one third of the trace of the orthogonalized U$_{ij}$ tensor.

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## Table 3. Bond lengths [Å] and angles [°] for compound 83.

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Table 4. Anisotropic displacement parameters (Å² x 10³) for compound 83. The anisotropic displacement factor exponent takes the form: -2π²[ h²a²U₁₁ + ... + 2 h k a* b* U₁₂ ].

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Table 5. Hydrogen coordinates ($x \times 10^4$) and isotropic displacement parameters ($\text{Å}^2 \times 10^3$) for compound 83.

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APPENDIX 2

Conference abstracts and publications
DESIGN AND SYNTHESIS OF BENZOFURAN DERIVATIVES AS CYP24 INHIBITORS FOR ANDROGEN-INDEPENDENT PROSTATE CANCER

S.W. Yee and C. Simons

Welsh School of Pharmacy, Cardiff University, Cathays Park, CF10 3XF

Prostate cancer is the most common malignancy among males in the US, with 220,900 new cases and 28,900 deaths for the year 2003 alone. Androgen ablation via hormonal therapy and surgical castration has a prominent role in the treatment of advanced prostate cancer. It is aimed at inhibiting prostate growth by suppressing endogenous androgen production or blocking androgen action. Unfortunately, hormonal therapy is not capable of producing durable responses in the majority of patients with advanced disease. Once the patient develops hormone-refractory prostate cancer (HRPC), his outlook is poor, with a median survival time of 9 months. Clearly, new effective treatment strategies are needed for the treatment of HRPC. One of the new therapeutic strategies is to employ a differentiating agent to suppress prostate cancer cell proliferation. 1α,25-Dihydroxyvitamin D₃ (1α,25(OH)₂D₃), a member of the steroid hormone superfamily, is involved in regulating cellular proliferation and differentiation in various target tissues that possess vitamin D receptors.

Numerous studies have shown that 1α,25(OH)₂D₃ and vitamin D₃ analogues increase differentiation and decrease proliferation of the prostate cancer cells. However, the use of 1α,25(OH)₂D₃ and vitamin D₃ analogues for prostate cancer is limited by the risk of hypercalcemia and hypercalciuria. 25-Hydroxyvitamin D₃-24-hydroxylase (CYP24) is responsible for degradation of the active vitamin D metabolite 1α,25(OH)₂D₃. CYP24 has been shown to be expressed in some prostate cancer cell lines. This suggest that the expression of CYP24 can abolish the growth regulatory effect of 1α,25(OH)₂D₃. Identification of potent inhibitors of CYP24 may be a new strategy for the treatment of androgen-independent prostate cancer.

Schuster and co-workers have identified SDZ 89-443 and VID400 as selective CYP24 inhibitor. Based on these structures, we have synthesised benzofuran-2-carboxamido ethyl-imidazole and -1,2,4-triazole derivatives. Biological assessment of CYP24 inhibition by these synthesised compounds will be established in the near future.

SDZ 89-443

VID400

Benzofuran-2-carboxamido ethyl-imidazole and -1,2,4-triazole derivatives

Design and synthesis of P450 enzymes inhibitors as differentiating agent for androgen-independent prostate cancer

Sook Wah Yee and Claire Simons

Department of Medicinal Chemistry, Welsh School of Pharmacy, Cardiff University, King Edward Avenue VII, Cardiff, UK

Prostate cancer is the most common malignancy among males in the US, with 220,900 new cases and 28,900 deaths for 2003 alone [1]. Hormonal therapy and surgical castration have prominent roles in the treatment of advanced prostate cancer. Unfortunately, hormonal therapy is not capable of producing durable responses in the majority of patients with advanced disease. Once the patient develops hormone-refractory prostate cancer (HRPC), his outlook is poor, with a median survival time of 9 months. Clearly, new effective treatment strategies are needed for the treatment of HRPC.

One of the new therapeutic strategies is to employ a differentiating agent to suppress prostate cancer cell proliferation. Vitamin D and retinoic acid have antiproliferative and differentiating effect on prostate cancer cells [2]. The P450 enzymes that are responsible for the metabolism of vitamin D and retinoic acid are cytochrome 24 (CYP24) and 26 (CYP26) respectively.

CYP 24 has been shown to be expressed in some prostate cancer cell lines [3]. Liarozole, the inhibitor of CYP26 has shown promising data in the treatment of HRPC [4]. Identification of potent inhibitors of CYP24 and CYP26 may be a new strategy for the treatment of androgen-independent prostate cancer.

Based on the known structures of liarozole and CYP24 inhibitors [5], we have synthesised a series of benzofuran and tetralone derivatives. Biological assessment of the inhibition of these P450 enzymes is being carried out in our laboratory, with promising inhibitory activity observed.

Homology models of CYP24 and CYP26 have been constructed using various templates, and molecular docking studies of substrate and inhibitors have been carried out to broaden the understanding of enzyme/inhibitor interactions.

Synthesis and evaluation of tetralone derivatives: P450 enzyme inhibitors as differentiating agents for the treatment of hormone-refractory prostate cancer

S.W. Yee and C. Simons

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Prostate cancer is the most common malignancy among males in the US, with 230,110 estimated new cases and 29,900 deaths for 2004 alone (Jemal et al 2004). Hormonal therapy and surgical castration have prominent roles in the treatment of advanced prostate cancer. Unfortunately, hormonal therapy is not capable of producing durable responses in the majority of patients with advanced disease. Once the patient develops hormone-refractory prostate cancer, his outlook is poor, with a median survival time of 9 months. Clearly, new effective treatment strategies are needed for the treatment of HRPC.

One of the new therapeutic strategies is to employ a differentiating agent to suppress prostate cancer cell proliferation. Vitamin D and retinoic acid have anti-proliferative and differentiating effect on prostate cancer cells (Peehl & Feldman 2003). The P450 enzymes that are responsible for the metabolism of vitamin D and retinoic acid are cytochrome 24 (CYP24) and 26 (CYP26) respectively.

CYP 24 has been shown to be expressed in some prostate cancer cell lines. Liarozole and ketoconazole, the inhibitors of CYP26 have shown promising data in the treatment of HRPC. Identification of potent inhibitors of CYP24 and CYP26 may be a new strategy for the treatment of androgen-independent prostate cancer. It has been shown in our own laboratory, that isoflavones and flavones, tetralones (Kirby et al 2003) and coumarins are known to affect the activity of a variety of cytochrome P450 enzymes involved in hormone biosynthesis. In view of these facts, it was of interest to investigate the inhibitory activity of these compounds against CYP24 and CYP26.

The biochemical evaluation of the synthesised tetralone compounds was undertaken using a modification of the method of Kirby et al (2003). The incubation mixtures (0.5 mL) containing NADPH generating system (50 μL) and substrate (10 μL, either [11,12-3H] retinoic acid or 25-hydroxy[26,27-methyl-3H]-vitamin D) in phosphate buffer (pH 7.4) and enzyme suspension (20 μL liver microsomal or 50 μL kidney mitochondrial fractions) were incubated at 37 °C for 30 min in a shaking water bath. The solutions were quenched by the addition of 1 % acetic acid v/v (200 μL). Then ethyl acetate containing 0.02 % butylated hydroxyl anisole (2 mL) was added and the tube vortexed for 15 s. The organic layer (1.5 mL) was transferred into a clear tube and the solvent evaporated using a centrifuge connected to a vacuum pump and a multitrap at – 80 °C. The residue was reconstituted in methanol (50 μL) and was injected into a reverse-phase HPLC connected to an online scintillation detector. The separated [3H]-metabolites were quantitatively calculated from the areas under the curves. The percentage inhibition was calculated from: 100((metabolites (control) – metabolites (inhibitor))/(metabolites control))%.

The results showed that the synthesised tetralone compounds (at 100 μM and 20 μM) displayed greater or similar inhibitory activity than the standard compound for CYP24 and CYP26, namely ketoconazole.

Homology models of CYP24 and CYP26 have been constructed using various templates, and molecular docking studies of substrate and inhibitors have been carried out to broaden the understanding of enzyme/inhibitor interactions.

Synthesis and evaluation of retinoic acid metabolism blocking agents (RAMBAs) as indirect differentiating agents for cancer therapeutics

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Differentiating agents are one of the new therapeutic strategies in treating solid tumours e.g. breast and prostate cancers. All-trans retinoic acid (ATRA), derived from vitamin A, is able to inhibit cell proliferation and to restore normal differentiation of various cancer cells. However, the use of ATRA is limited by the induction of the cytochrome P-450 enzymes that are involved in the metabolism of ATRA. In addition to CYP26, which only recognises ATRA as its substrate, different P-450 isozymes, namely CYP2C8, CYP2C9, CYP3A4 are able to catalyze this reaction. A drug which can prolong the action of endogenous retinoic acid by inhibiting the P-450 retinoic acid metabolizing enzymes could have potential use as an anti-cancer agent. The synthesis of three new series of novel compounds with improved activities compared with Ketoconazole and Liarozole in two different biological assay systems will be discussed in relation to the SAR studies and molecular docking studies using homology models of CYP26.
DESIGN AND ASSESSMENT OF NOVEL INHIBITORS OF CYP24 TO ENHANCE VDR SIGNALLING IN ANDROGEN-INDEPENDENT PROSTATE CANCER CELLS

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Epidemiological, polymorphism and in vitro studies support an antiproliferative role for the VDR in the prostate, and have justified an ongoing series of clinical trials of 1,25(OH)₂D₃, either alone or in combination with other chemotherapies. Enhanced autocrine 1,25(OH)₂D₃ metabolism via the cytochrome P450 enzyme, CYP24 (itself a VDR target gene) limits efficacy. Thus CYP24 inhibitors are therapeutically attractive.

Isoflavones, flavones and tetralones inhibit a variety of cytochrome P450 enzymes with broad spectrum. We therefore synthesised a series of tetralone derivatives to investigate the inhibition of CYP24, compared with the broad spectrum P450 inhibitor, ketoconazole. Of these tetralone derivatives 2-(4-hydroxybenzyl)-6-methoxy-3,4-dihydro-2H-naphthalen-1-one, (compound YSW87) showed potent inhibition of the metabolism of [³H]-25-(OH)-D₃ in the rat kidney mitochondrial assay (IC₅₀ = 3.5 μM, compared to ketoconazole, IC₅₀ = 20 μM). This compound was subsequently screened in proliferation and gene regulatory actions in androgen-independent prostate cancer cell lines, PC-3 and DU-145, which are recalcitrant to 1,25(OH)₂D₃.

Antiproliferative screens revealed that YSW87 alone was minimally active compared to ketoconazole, possibly reflecting the more selective targeting of CYP24. However when DU-145 cells were pre-treated with YSW87 prior to 1,25(OH)₂D₃ treatment there was a strong combinatorial inhibition of proliferation. Equally we demonstrated a combinatorial enhancement VDR gene regulation by examining CYP24 and p21⁵⁸(wad/tcip1) mRNA levels after individual and co-treatment of YSW87 and 1,25(OH)₂D₃.

These data identify a novel series of therapeutically attractive tetralone analogs as potent selective inhibitors of CYP24.
Synthesis and CYP24 inhibitory activity of 2-substituted-3,4-dihydro-2H-naphthalen-1-one (tetralone) derivatives

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Abstract—The synthesis of novel 2-benzyl- and 2-benzylidene-3,4-dihydro-2H-naphthalen-1-one (tetralone) derivatives and their inhibitory activity versus kidney mitochondrial 25-hydroxyvitamin D3 24-hydroxylase (CYP24) is described. The 2-benzylidene-tetralone derivatives were found to be very weak inhibitors (IC50 20–100 µM), whereas the 2-benzyltetralone derivatives showed promising inhibitory activity (IC50 0.9 µM for the most active derivative) compared with ketoconazole (IC50 20 µM).

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Prostate cancer, being the second leading cause of cancer death in males, is a major disease for therapeutic intervention.¹ Androgens play an important role in the development, growth and progression of prostate cancer,² therefore androgen ablation therapy by gonadotropin suppression and androgen receptor blockade are current methods of treatment.³ Although most patients respond well to this therapy, eventually many tumours recur as a result of transition of the cancer cells to androgen-independent growth.⁴ Owing to the lack of effective treatments for androgen-independent metastatic prostate cancer, alternative strategies, such as 'differentiation therapy' may be useful.⁵ Pro-differentiating agents of interest include retinoic acid (vitamin A) and vitamin D₃ (and their analogues).

1α,25(OH)₂D₃ (calcitriol) is the hormonally active metabolite of vitamin D₃, which functions as an antiproliferative and pro-differentiating agent, especially in epithelial and hematopoietic cells.⁶ The use of vitamin D analogues (VDR agonists) as differentiating agents has been studied,⁶ however the overall therapeutic activity of such analogues is uncertain owing to additional pharmacological effects such as transcalcification (elevation of intracellular calcium activation of intestinal calcium uptake).⁷ In addition the natural substrate calcitriol, and derivative VDR agonists, are rapidly metabolised into less active metabolites by the 24-hydroxylase (CYP24) resulting in a very limited use for either calcitriol or its derivatives as differentiating agents.⁸ Therefore, compounds capable of inhibiting CYP24, the cytochrome P450 enzyme that initiates calcitriol metabolism, would have the effect of increasing endogenous levels of calcitriol so enhancing its differentiating capabilities.

Known inhibitors of CYP24 include (i) the CYP26 and CYP17 (P450 17, 17,20-lyase) inhibitor liarozole⁹ and (ii) ketoconazole, the nonspecific competitive inhibitor of cytochrome P450-catalysed reactions, which inhibit both CYP24 and 1α-hydroxylase.¹⁰ More selective CYP24 inhibitors have been described such as SDZ 89-443 and VID400.¹¹ All these inhibitors have a characteristic nitrogen heterocyclic moiety capable of coordinating to the Fe²⁺-haem component of the P450 active site.

We have recently demonstrated the CYP26 inhibitory activity of a series of 2-(4-aminophenylmethyl)-tetralone derivatives,¹² therefore tetralones with varying substituents in both naphthalene and 2-aryl moieties were synthesised and evaluated for CYP24 inhibitory activity.

Keywords: 2-Substituted-3,4-dihydro-2H-naphthalen-1-one (tetralone) derivatives; Enzyme inhibition; 24-Hydroxylase (CYP24); Differentiating therapy.

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The method employed for the preparation of the 6-methoxy-2-(phenylmethylidene)-3,4-dihydro-2H-naphthalen-1-one derivatives (2a-d) involved direct condensation of the commercially available tetralone (1) with the appropriate benzaldehyde in ethanolic KOH solution. This method was successfully used in the absence of a hydroxyl group on the tetralone or benzaldehyde (Scheme 1).

The synthesis of hydroxyl derivatives (5, 8 and 14) required initial protection of one or both benzaldehyde and tetralone hydroxyl groups (see Schemes 2-4, respectively) with the tetrahydropyran (THP) protecting group, which was stable under the basic ethanolic KOH condensation conditions.

The 6-biphenyl substituted derivatives (11a–b) were prepared by Suzuki coupling with phenylboronic acid in the presence of Pd(PPh3)4 catalyst14 (Scheme 3). The reduced 2-(benzylidene)-3,4-dihydro-2H-naphthalen-1-one derivatives (3a–b, 6, 9, 12a–b and 15) were readily obtained by hydrogenation with 10% Pd/C catalyst for 1 h, at approximately 30 psi (Parr hydrogenator). When the hydrogenation reaction was allowed to proceed for 2 h, deoxygenation at C1 was found to occur (7, Scheme 2).

The dihydroxy derivative 15 was prepared from the corresponding THP-protected tetralone and benzaldehyde (Scheme 4).

The nine 2-(benzylidene) (2a–d, 5, 8, 11a, 11b and 14) and eight 2-(benzyl)-tetralone (3a–b, 6, 7, 9, 12a–b and 15) derivatives were evaluated for their inhibitory activity versus CYP24 using rat kidney mitochondria. The assay performed was based on a modification of the general

**Scheme 1.** Reagents and conditions: (i) R–C₆H₅CHO, 4% KOH/EtOH, rt, 1–72 h; (ii) 10% Pd/C, H₂, MeOH, rt, 1 h [a, R₁ = R₂ = CF₃, R₃ = H, b, R₁ = CH₃, R₂ = R₃ = H; c, R₂ = NMe₂, R₁ = R₂ = H; d, R₁ = Br, R₂ = R₃ = H].

**Scheme 2.** Reagents and conditions: (i) THP–C₆H₅CHO, 4% KOH/EtOH, rt, 12 h; (ii) 2 M HCl (aq), EtOAc/2-butanone (1:1 v/v), reflux, 1 h; (iii) 10% Pd/C, H₂, MeOH, rt, 1 h; (iv) 10% Pd/C, H₂, MeOH, rt, 2 h.

**Scheme 3.** Reagents and conditions: (i) Me₂N–C₆H₅CHO, 4% KOH/EtOH, rt, 72 h; (ii) 2 M HCl (aq), EtOAc/2-butanone (1:1 v/v), reflux, 1 h; (iii) 10% Pd/C, H₂, MeOH, rt, 1 h; (iv) Br–C₆H₅CHO, 4% KOH/EtOH, rt, 1–4 h; (v) phenylboronic acid, Pd(PPh₃)₄, toluene, 100°C, 5 h.
procedure previously described for CYP26,15 using [26,27-methyl-1H]-25-hydroxyvitamin D3 (from a stock mixture containing 100μL of [26,27-methyl-1H]-25-hydroxyvitamin D3 and 1 mL of unlabelled 25-hydroxyvitamin D3 (25μM), with a total of 5μCi of radioactivity in the 1 mL of stock mixture), NADPH, inhibitor (varying concentrations using acetonitrile as solvent) and phosphate buffer (pH 7.4). After incubation in a shaking water bath for 30 min at 37°C, the vitamin D metabolites were obtained by extraction with ethyl acetate. After evaporation of the organic solvent, the residue was analysed by a HPLC system connected to a β-RAM online scintillation detector, connected to a Compaq PC running Laura data acquisition and analysis software (LabLogic Ltd). The separated [3H]-metabolites were quantitatively calculated from the areas under the curves. Using a control with acetonitrile instead of inhibitor, these results were expressed as ‘percentage inhibition relative to control’ = 100[metabolites (control)−metabolites (inhibitor)/metabolites control]%.

Ketoconazole was used as a standard for comparison (Table 1).

The benzylidene derivatives were all poor inhibitors of CYP24, perhaps indicating the requirement for flexibility at the C2 position for optimal structure conformation with respect to interaction at the enzyme active site. In the 2-benzyltetralone series, introduction of an alkyl or aryl substituent at the 2-benzyl position, for example, 2-(2-methylbenzyl)- and 2-(2-biphenyl)-derivatives 3b and 12a (IC50 0.9 and 2.1 μM, respectively), resulted in good activity, whereas introduction of an aryl substituent at the 4-benzyl position, for example, 2-(4-biphenyl)-derivative 12b (IC50 >20 μM) substantially reduced activity. Introduction of a hydroxyl group at the 4-benzyl position was tolerated, for example 6 and 7 (IC50 3.5 and 2.6 μM, respectively), however introduction of a more bulky moiety, for example, N,N-dimethyl in compound 9 (IC50 18 μM) was not tolerated. These preliminary results suggest that the compounds may be orientated in the active site with a hydrophobic region or large pocket above the 2-position of the benzyl ring, and a small pocket containing an amino-acid residue capable of forming a hydrogen bond at the 4-benzyl position.

There would appear to be a slight preference for a 6-methoxy rather than a 6-hydroxy substituent in the naphthalene ring (cf. 6 and 15). IC50 3.5 and 8.9 μM, respectively). However, no significant difference in inhibitory activity was noted for the tetralone and 1,2,3,4-tetrahydro-naphthalene structures (cf. 6 and 15). IC50 3.5 and 2.6 μM, respectively). Compared with ketoconazole, potent inhibitory activity was observed for the 2-(2-methylbenzyl)tetralone derivatives 3b, which may

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<th>Compound</th>
<th>IC50 (μM)</th>
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<tr>
<td>2a</td>
<td>&gt;100</td>
<td>3a</td>
<td>4.5</td>
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<tr>
<td>2b</td>
<td>&gt;20</td>
<td>3b</td>
<td>0.9</td>
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<tr>
<td>2c</td>
<td>&gt;100</td>
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<td>&gt;100</td>
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<tr>
<td>14</td>
<td>&gt;20</td>
<td>Ketoconazole</td>
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IC50 values are the mean of two experiments.
be a useful lead compound for further development of potent CYP24 inhibitors.

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References and notes