BIOLOGICAL ACTIVITY OF STEROID ANALOGUES: SYNTHESIS AND RECEPTOR/ENZYME INTERACTIONS

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ABSTRACT

This thesis investigates the biological activity of selected non-steroidal analogues of sex steroid hormones by examining two different effects of analogues on endogenous sex hormone activity. Non-steroidal analogues of sex hormones were synthesised to study their biological interactions with a sex steroid receptor and a sex steroid metabolising enzyme.

Chapter One introduces the steroid hormones and their physiology, which leads to a review of the mechanisms by which steroids exert their effects. Their implication in disease is discussed, with particular emphasis on the sex steroids. As the biological activity of steroids is related to their chemical structure, the important features of steroid structure are identified, including the cyclopentanoperhydrophenanthrene nucleus, arrangement of ring substituents and ring junction conformation. The concept of non-steroidal analogues of steroids is introduced, and the harmful or beneficial effects analogues have on endogenous steroid activity are considered. Alteration of steroid activity and its consequences are focussed on two main areas; the potential adverse effects of environmental chemicals which mimic sex steroid activity, and the use of non-steroidal analogues in medicinal chemistry for treating sex steroid related disease.

Chapter Two describes an investigation into the 17β-estradiol mimicking activity of non-steroidal analogues. Exogenous chemicals that mimic estradiol are of concern as they may alter endogenous estradiol activity and disrupt endocrine systems. Firstly, an introduction to the field of research concerned with environmental chemicals that mimic steroid hormones is given. The interaction of xenoestrogens with the estrogen receptor is described, as are the methods available for assessing the estrogen mimicking activity of xenoestrogens. The concern for insecticides mimicking estrogen activity is described by reviewing reported activities of insecticides, which leads into a discussion of work carried out as part of this thesis. Metabolites of the pyrethroid insecticides permethrin and cypermethrin, **2.14**, **2.15**, and **2.16** were synthesised while others were commercially obtained. The interaction of pyrethroid insecticide metabolites with the human estrogen receptor expressed in recombinant yeast (*Saccharomyces cerevisiae*) was studied, following the establishment and validation of the assay. Metabolites **2.11**, **2.12**, and **2.14**

were found to weakly stimulate estrogen receptor-mediated estradiol responsive gene expression in the yeast assay (10^5 less active than 17β-estradiol). Since the activity of the metabolites using the yeast assay was greater than for the parent compounds, metabolic pathways need to be considered when assessing the impact of exposure to environmental estrogens. The low estrogenic activity suggests these compounds are not individually contributing significantly to the xenoestrogenic impact on humans, but will add to total xenoestrogen exposure.

Chapter Three describes the inhibition of a sex steroid metabolising enzyme, steroid 5α-reductase, by novel non-steroidal compounds. Inhibitors of this enzyme are potentially useful therapeutic agents for regulating the activity of an androgen in prostate disorders. A review of the literature on non-steroidal inhibition of 5α-reductase identified three key structural features known to enhance inhibitor potency; ring substitution, position and nature of ring unsaturation and angular methyl group presence. These features were taken into account in the design of inhibitors synthesised in this thesis (**3.55-3.57**, **3.59**, **3.61**, **3.62**, **3.110** and **3.111**). Inhibitors consisting of non-steroidal 5- or 1-aryl pyridone scaffolds were synthesised to investigate SAR for 4'-substituents. The 5-aryl 1-methyl-2-pyridone/piperidone scaffold of compounds **3.55-3.57** and **3.59** was constructed by Suzuki cross coupling methodology, while the 1-aryl 2-methyl 2,3-dihydro-4-pyridone scaffold of **3.61** and **3.62** was constructed by aza Diels-Alder methodology. Long carbon chain olefin containing tethers **3.107** and **3.108** were synthesised for conjugation to inhibitor **3.57** by cross metathesis to give conjugates **3.110** and **3.111**. Compounds **3.55-3.57**, **3.59**, **3.61**, **3.62**, **3.110** and **3.111** inhibited the type 1 5α-reductase isozyme expressed by HEK-I cells, with activities comparable to those of related literature compounds. The 1-aryl 2,3-dihydro-4-pyridone **3.62** inhibited both the type 1 and 2 isozymes (expressed by HEK-II cells) of 5α-reductase. The presence of bulky hydrophobic groups (benzoyl, long chain tethers) at the 4'position enhanced the potency of type 1 inhibition by 5-aryl pyridone type compounds in comparison to *N,N*-diisopropyl- and *N*-allylacetamide groups. This information provides further understanding of SAR within and across different classes of non-steroidal inhibitors of steroid 5α-reductase towards improved drug design.

ABBREVIATIONS

AF	activation function
AMC	ceric ammonium molybdate solution (TLC)
AR	androgen receptor
BPH	benign prostatic hyperplasia
br	broad (in NMR)
CAT	chloramphenicol acetyl transferase
cDNA	complementary deoxyribonucleic acid
CFU	colony forming units
COSY	correlation spectroscopy
CPRG	chlorophenol red β-galactopyranoside
CYP	cytochrome P450
d	doublet (in NMR)
DBD	DNA binding domain
DCM	dichloromethane
dd	doublet of doublets (in NMR)
DDE	1,1-dichloro-2,2-bis(*p*-dichlorodiphenyl)ethylene (DDT metabolite)
DDT	1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane
DES	diethylstilbestrol
DHT	dihydrotestosterone
DIPEA	*N,N*-diisopropylethylamine
DMAP	4-(dimethylamino)pyridine
DMF	*N*,*N*-dimethylformamide
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dt	doublet of triplets (in NMR)
EC_{50}	median effective concentration (calculated concentration of agonist which generates 50% maximum response from binding to receptor)
EDC	endocrine disrupting chemical
EDCI	1-[3-(dimethylamino)propyl]-3-carbodiimide hydrochloride
EDTA	ethylenediaminetetraacetic acid

EI	electron impact (in mass spectrometry)
ELISA	enzyme-linked immunosorbent assay
EPA	Environmental Protection Agency
ER	estrogen receptor
ERE	estrogen response element (DNA sequence)
ESI	electrospray ionisation (in mass spectrometry)
ESR	Institute for Environmental Science and Research
FDA	Food and Drug Administration (USA)
h	hour(s)
HEK	human embryonic kidney cell
hER	human estrogen receptor
HOBt	1-hydroxybenzotriazole
HPLC	high performance liquid chromatography
HRMS	high resolution mass spectrometry
hsp	heat shock protein
HSQC	heteronuclear single quantum correlation (in NMR)
Hz	hertz (in NMR)
IC_{50}	inhibitor concentration that decreases enzyme activity by 50%
J	coupling constant (in NMR)
K_d	receptor-ligand dissociation constant
K_i	enzyme-inhibitor dissociation constant
K_m	Michaelis-Menten constant (enzyme-substrate dissociation constant)
LBD	ligand binding domain
LRMS	low resolution mass spectrometry
m	multiplet (in NMR)
min	minute(s)
mp	melting point
mRNA	messenger ribonucleic acid
NA	no activity
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
$NADP^+$	oxidised form of above
NMR	nuclear magnetic resonance
OD	optical density

PBS	phosphate buffered saline
PCB	polychlorinated biphenyl
PCDD	polychlorinated dibenzodioxin
PCDF	polychlorinated dibenzofuran
ppm	parts per million
QSAR	quantitative structure-activity relationships
rt	room temperature
RVP	rat ventral prostate
s	singlet (in NMR)
SAM	self assembled monolayer
SAR	structure-activity relationships
SD	standard deviation
SHBG	steroid hormone-binding globulin
SRE	steroid response element
t	triplet (in NMR)
TBT	tributyltin
THF	tetrahydrofuran
TLC	thin-layer chromatography
Tris-HCl	tris(hydroxymethyl)aminomethane hydrochloride
TS	transition state
YES	yeast estrogen screen

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CHAPTER ONE

INTRODUCTION

1.1 OVERVIEW

The steroids are a group of naturally occurring compounds that possess a lipophilic cyclopentanoperhydrophenanthrene skeleton (Fig 1.1), or a derivative thereof. Steroids are hormones (chemical messengers) that play a critical role in endocrine systems which control the regulation of metabolic processes. These hormones stimulate biological responses in a wide range of tissues to influence endocrine processes such as sexual differentiation, reproductive physiology, and maintenance of salt balance and sugar metabolism. They have also been implicated in responses to stress and behaviour. The most important classes of steroids are the sex hormones (estrogens and androgens), progestins, mineralcorticoids and glucocorticoids, vitamin D and its derivatives, bile acids, and sterols found in cell membranes (Fig. 1.1). Synthetic derivatives of steroids have also attracted a good deal of attention for the purpose of developing drugs to treat hormone related disease.

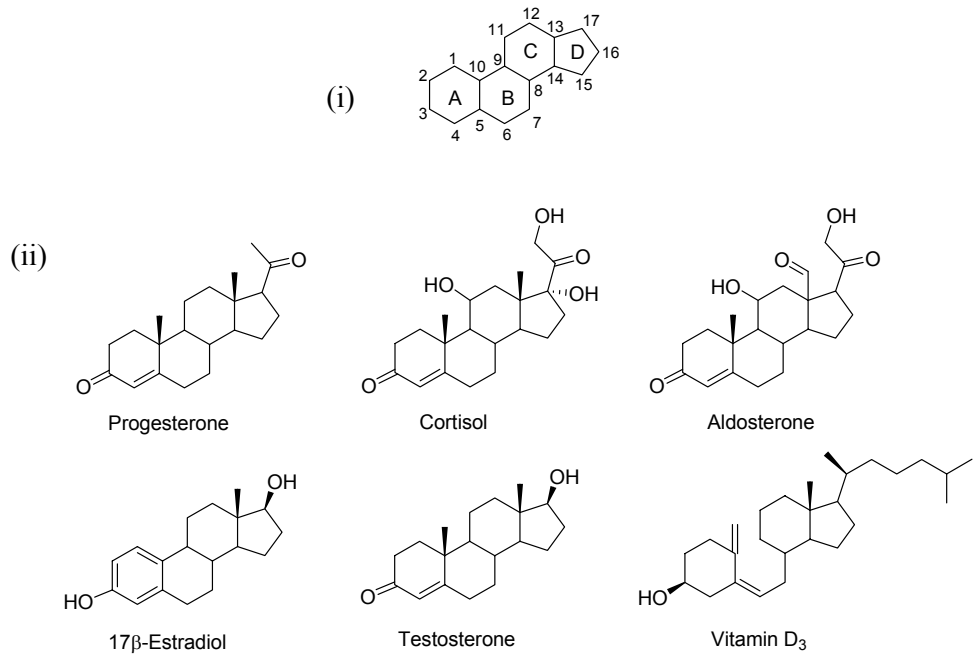
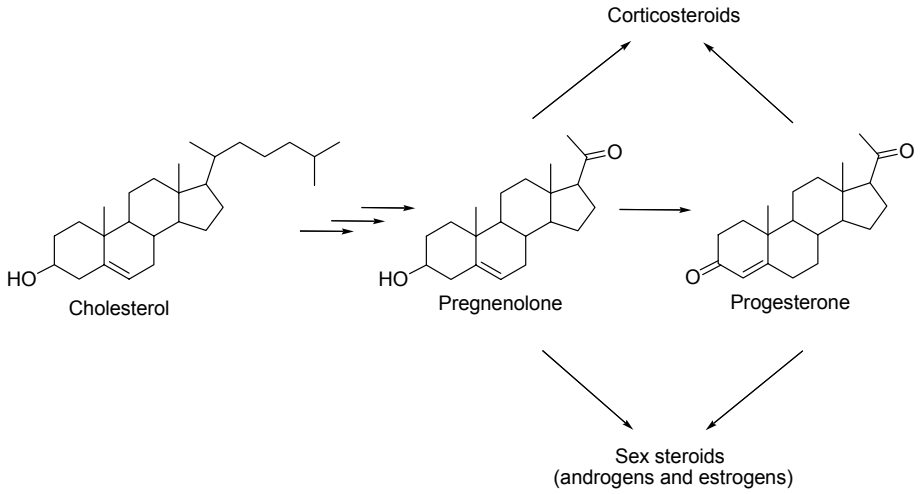


Figure 1.1. (i) Steroid skeleton numbering and ring notation.
(ii) Examples of naturally occurring steroids.

Compounds that do not possess a steroidal structure (non-steroidal analogues) can mimic the biological activity of steroids, and this is the theme of this thesis. The purpose of this chapter is to provide an overview of the biological activity of steroids (the sex steroids in particular); their role in normal physiology and their molecular and biochemical mechanisms of action. Key molecular events that steroids are involved in will be described, followed by their roles in certain disease states which can arise because of problems associated with hormone action. As the biological activity of steroids is related to their chemical structure, the chemistry of steroid structure will also be discussed. This provides the context for the study of biological effects of non-steroidal analogues, in particular, the potential adverse hormonal action that analogues may induce, and the use of non-steroidal analogues in drug design and development.

1.2 STEROID HORMONE PHYSIOLOGY

Most steroids are biosynthesised in the cell from cholesterol, the principal mammalian sterol (Scheme 1.1). Steroids are produced mainly in the adrenal gland, testis, ovary and liver, and secreted into the bloodstream where they are transported to target tissues by plasma transport proteins. Based on the distance of a target site from the site of synthesis and secretion, steroid hormones are classified as endocrine (distant target tissue), paracrine (neighbouring cells), or autocrine (same cell) factors. Because of their hydrophobic nature steroid hormones diffuse across the lipid bilayer into target cells where they play a vital role in differentiation and regulation.



Scheme 1.1. Synthesis of steroid hormones from cholesterol.

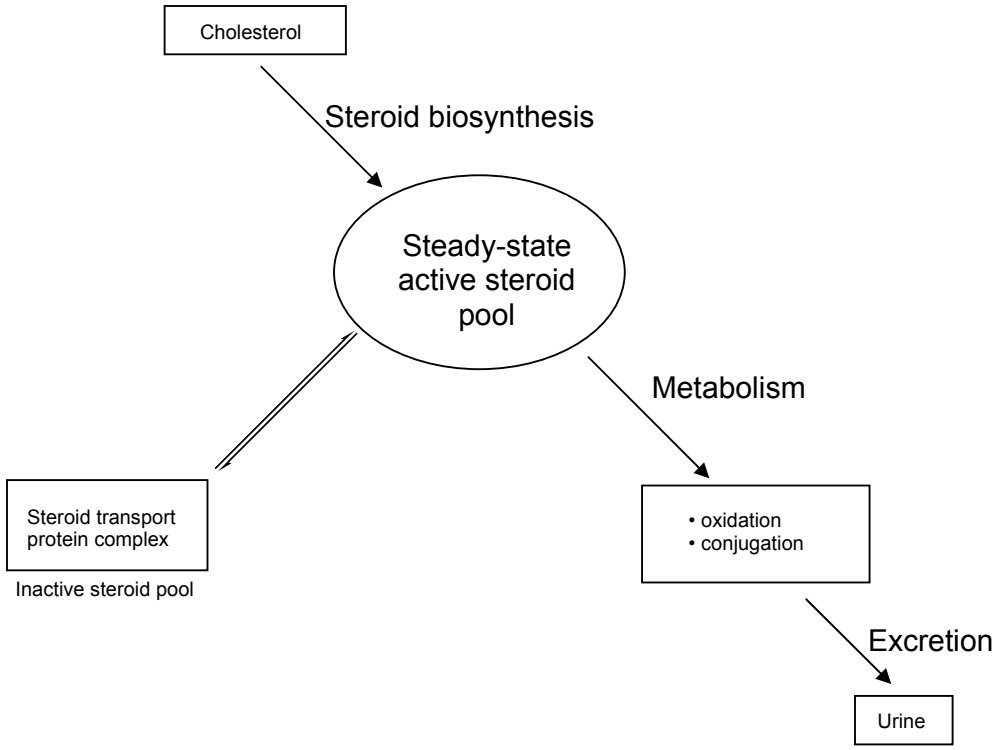
The hormonally active form of a steroid is the 'free' steroid which has dissociated from its plasma transport protein. Each transport protein binds its ligand with high affinity, with K_d values in the range of 5-500 nM.[1] The concentration of free steroid in the blood is a key determinant of steroid activity, and depends on three factors (Scheme 1.2);

i) The rate of biosynthesis of steroid and transport to body pools.

ii) The rate of catabolism which inactivates steroids and removes them from body pools. Steroids are inactivated by metabolism, and because of their hydrophobic

nature, they are metabolised to more hydrophilic compounds suitable for excretion. Conjugation with sulfate or glucuronide groups increases their water solubility.

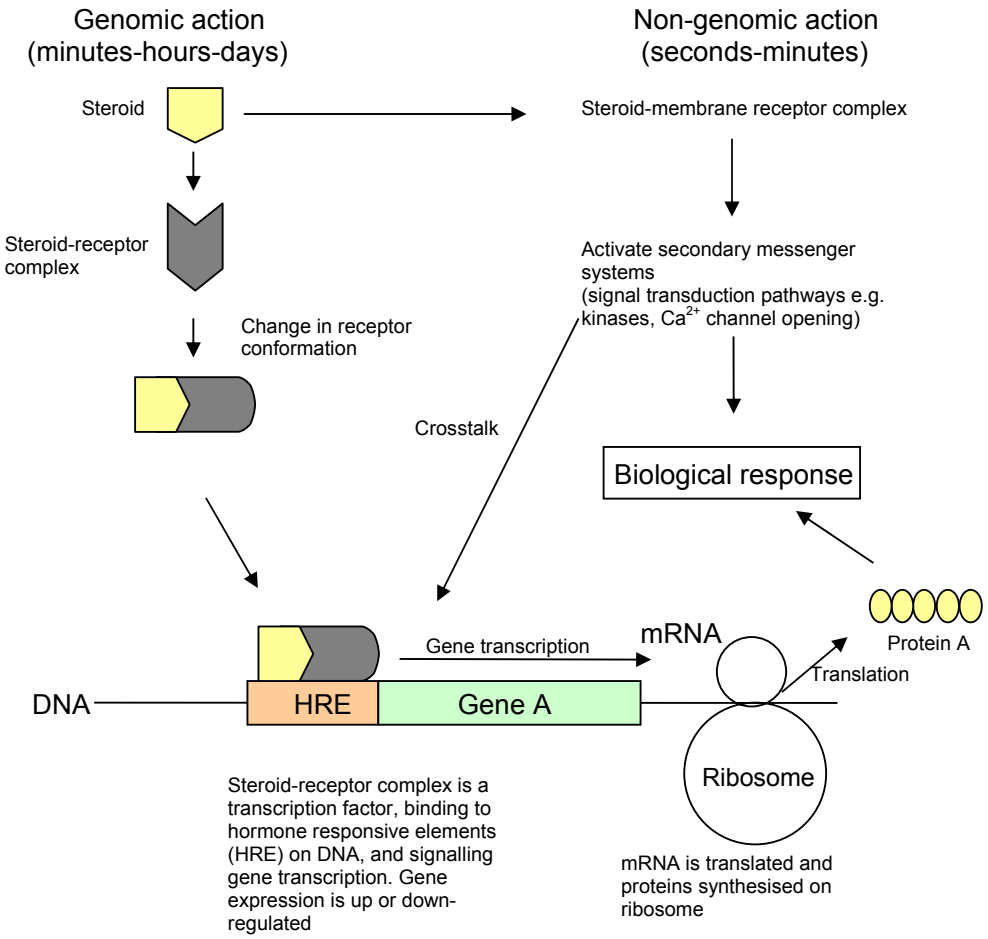
iii) The affinity (K_d) the steroid has for its plasma transport protein. For example, estrogens bind quite tightly to transport proteins, so only a small proportion (1-2%), is available to cells to exert their physiological effects.[2]



Scheme 1.2. Regulation of active steroid concentration.

1.3 STEROID MODE OF ACTION

The present understanding of the major pathways by which steroid hormones generate biological responses is shown in Scheme 1.3.[1] The biological effects of steroid hormones in cells are mediated by non-genomic and genomic mechanisms. In the non-genomic action, the rate of appearance of biological responses to the hormone is generally rapid, varying from seconds to an hour.[1] Here, plasma membrane associated receptors have been implicated in hormone action.[1] These receptors are believed to stimulate secondary messenger cascades, such as protein kinases, changes in intracellular pH and Ca^{2+} concentration.[3] However, the nature and characteristics of such membrane sites remain unclear. The genomic action of steroids is well understood, in which a steroid ligand-receptor complex acts as a transcriptional factor for gene expression. This mechanism is non-rapid in contrast to the non-genomic, with biological responses taking a few hours to days to manifest.[1] Crosstalk may occur between genomic and non-genomic mechanisms. For the remainder of this section, discussion will focus on the well characterised genomic mechanisms of action.



Scheme 1.3. Genomic and non-genomic modes of steroid hormone action (adapted from Norman *et al.*[1]).

1.3.1 Genomic action of steroids

The genomic action of steroid hormones is mediated by the formation of a complex between a steroid and a specific nuclear protein called a receptor. This steroid-receptor complex functions in the cell nucleus as a transcription factor which selectively modulates gene expression (generating 'genomic' responses). Steroid receptor mediated gene expression is well understood and is regarded as the 'classic' steroid mechanism of action.[1] Steroid receptors recognise minimal structural differences among steroid molecules to bind

their cognate steroids tightly with dissociation constant (K_d) values in the range of 0.05-50 nM.[1] Steroids have higher affinity for their receptors than plasma transport proteins (see section 1.2) which facilitates dissociation of the steroid-transport protein complex.

Steroid receptors

Steroid receptors are a subfamily of the nuclear receptor group, which also includes the thyroid hormone and retinoic acid receptors, and orphan (unknown ligand) receptors. The elucidation of steroid receptor structure and function has been made possible by the cloning of corresponding gene sequences.[4] Steroid hormone receptors are a highly conserved group of 'ligand-dependent' nuclear transcription factors, consisting of particular amino acid sequences (domains) that give rise to various receptor functions. These include ligand binding, DNA binding, and transactivation domains. Other functions such as dimerisation and translocation to the nucleus have been assigned to more complex regions of the protein. The DNA binding domain (DBD) is the most highly conserved region of the nuclear receptors, followed by the ligand binding domain (LBD) located towards the carboxy terminus of the protein.[4]

Steroid receptor mediated gene expression

In the absence of steroid hormones, steroid receptors are localised in the cell nucleus, where they are kept in an inactive state as part of complexes with heat shock proteins hsp90 and hsp70. They are also present in the cytoplasm or can be partitioned between the cytoplasm and nucleus, depending on the particular steroid hormone.[1] The mechanism of action of steroid hormones begins when they move passively from the bloodstream across cell membranes to the nucleus where they bind and activate nuclear steroid receptors.[5] Once a steroid binds to its cognate receptor, a different conformation of the receptor becomes energetically favourable, and helix-12 of the receptor closes over the ligand binding pocket.[6] This 'active' conformation promotes dissociation of the heat shock proteins. The receptor is also phosphorylated, converting it to the transcriptionally active form that binds to specific DNA sequences termed steroid response elements (SREs).

SREs are different for different steroid-receptor complexes, and are associated with promoter regions that regulate gene transcription. The activated steroid-receptor complex binds to DNA as a homodimer where it recognises individual base pairs in the major groove of DNA. Following DNA binding, transcriptional factors are recruited and stabilised at the target gene promoter, and RNA polymerase is activated to begin mRNA synthesis. As well as being stimulated, transcription rates can also be inhibited. The final product of the steroid-receptor-gene interaction is the expression of specific proteins that modulate cell and tissue function.

Regulation of steroid action

Since the cell response to steroid hormones is dependent on receptor levels, mechanisms that modify receptor concentration also control steroid action. Nuclear steroid receptors are regulated by their ligands in a feedback mechanism termed autoregulation,[7] e.g. sex steroid receptors are down-regulated by their cognate ligand (negative autoregulation). However vitamin D receptors are up-regulated (positive autoregulation).[8] The overall effect steroids have on cells is the induction and inhibition of cell growth.[5] Their effects are profound at the cellular level; it is for this reason that multifaceted active steroid level control mechanisms are important.

1.4 STEROIDS AND DISEASE

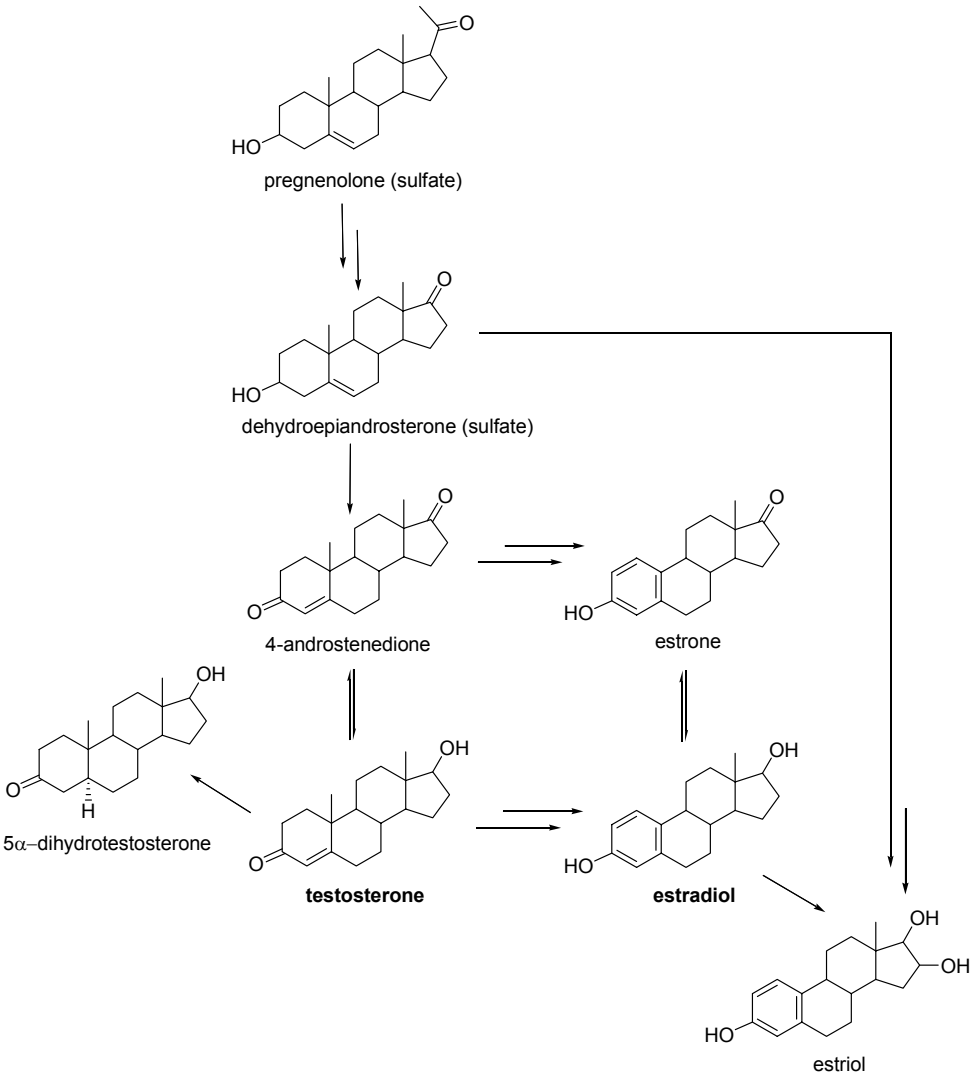
Steroids play essential and pervasive roles in the short-term and long-term regulation of endocrine processes. For this reason there are strict control mechanisms that regulate steroid activity (some of which were discussed in section 1.2). However disorders relating to steroid activity may arise when control mechanisms fail. Both overactive and underactive steroid secretion, resulting in changes to normal steroid activity, inevitably leads to diseases which are often debilitating or life threatening.

The sex steroids (estrogens and androgens) are extremely important hormones that are responsible for fundamental endocrine processes such as sexual differentiation (feminisation and masculinisation), the development of secondary sex characteristics and the control of cell growth in various tissues. Changes to normal sex steroid activity within these processes may contribute to the pathogenesis of diseases such as cancer of sex steroid target tissues and developmental and reproductive abnormalities. Estrogens and androgens are linked to breast and prostate cancer, two of the most common types of cancer in the Western world.[9,10] These prominent diseases have attracted a great deal of research into the role of sex steroids in cancer, and the development of therapeutic drugs. The following section describes the role of estrogens and androgens within the endocrine system, which lead to a discussion on their implication in breast and prostate cancer and other pathological states.

1.4.1 Sex steroids

The sex steroids consist of two major groups, the estrogens and androgens. Estrogens are responsible for the development of the female sex organs, and secondary sex characteristics. Estrogens are biosynthesised mainly in the ovaries but also in the adrenal cortex, testes and other tissues. The androgens stimulate development of the male sex organs and secondary male sex characteristics. Androgens are largely biosynthesised in Leydig cells in the testes, the adrenal cortex, and a small amount in the ovaries and placenta (Scheme 1.4). Circulating sex steroids are transported by the plasma transport protein, steroid hormone-binding globulin (SHBG). The estradiol to testosterone ratio is

important when these steroids exert their effects. For example, in females the ratio is very much in favour of estradiol, but very much in favour of testosterone for males.



Scheme 1.4. Biosynthesis of estrogens and androgens.

Estrogens

Ovarian steroid hormone pharmacology began over 40 years ago when in 1962 Jensen *et al.* showed that radiolabelled estradiol was held in cells that demonstrated a response to this hormone.[11] This finding led to the discovery of a protein that mediates the biological activity of estrogens, which was named the estrogen receptor (ER). A second type of ER was discovered in 1996, when the two receptors were named ERα and ERβ (these will be discussed further in Chapter Two). The presence of ER in a tissue usually indicates estrogen mediated biological responses at that location (Table 1.1). Such responses include the stimulation of growth and development of the vagina, uterus and Fallopian tubes. Estrogens initiate breast enlargement through the promotion of duct growth, stromal development and fat accumulation. Estrogens also control the menstrual cycle, along with progesterone, with main roles in preparing the reproductive tract for reception of a fertilised ovum, and maintaining a pregnancy. There are also effects on the hypothalamus and brain, and cardiovascular system.[12,13]

Distribution of Estrogen Receptors in Tissue			
Reproductive Tract Uterus Placenta Ovary Corpus luteum Mammary tissue	*Neuroendocrine system* Pituitary Hypothalamus Brain	*Male tissue* Testis Epididymis Prostate gland Seminal vesicles	*Visceral organs* Liver Kidney Lung

Table 1.1. The presence of estrogen receptors in tissue types.
(Taken from Norman and Litwack[14]).

The main estrogen found in a non-pregnant female is 17β-estradiol. Estradiol is biosynthesised from testosterone, via aromatisation of the A ring, or from estrone, by reduction of the ketone (Scheme 1.4). Aromatase, a cytochrome P450 enzyme, catalyses the conversion of testosterone to estradiol, and requires NADPH and O_2. These reactions occur mostly in ovarian granulosa cells, but also in the adrenal cortex, the foetal-placental

unit and adipose tissue. Small amounts are synthesised in the testes, prostate, muscle tissue, and liver. The presence of the ER in tissue cells indicates the biological actions of 17β-estradiol at that location. In these target cells, estradiol diffuses across the nuclear cell membrane where it binds to the ER. The ER is predominantly found in the cell nucleus, but constantly shuttles between the nucleus and cytoplasm. Once bound with estradiol, the ER acts as a transcriptional regulatory protein which modulates gene expression (see section 1.3.1) and ultimately cell proliferation or death. Chapter Two of this thesis investigates ER mediated gene expression stimulated by chemicals other than estradiol, to identify agents that have the potential to disrupt endocrine processes. Recent research reports that estradiol is also capable of acting through non-genomic mechanisms (described in section 1.3), including increasing intracellular Ca^{2+} levels, activating a kinase enzyme associated with cardiovascular system protection, and binding to plasma-membrane receptors to block apoptosis (for a review and references therein see Norman *et al.*[1]).

Estrogens and cancer

Estrogens are important factors in cancers of their main target organs, the breast, endometrium and ovary. Breast cancer is the most common cancer in women worldwide, with more incidence in Western countries than compared with Asian countries.[9] Estrogens are involved in the pathogenesis of breast cancer, of which the mechanisms are yet to be fully understood. The genotoxicity of estrogen and several metabolites have been suggested as factors in the development and growth of tumours.[15] Estrogens are implicated in the development of mammary cancer in rodents and aid in the proliferation of cultured human breast-cancer cells.[16] Therefore, exposure to estrogen affects the risk of breast cancer. Genetic and environmental factors are able to influence estrogen homeostasis and exposure to estrogen and its metabolites. The life-stage of a woman, exercise and diet, serum concentrations of SHBG and exposure to exogenous sex steroids contribute to individual variation in estrogen exposure. Estrogen levels may be enhanced by exposure to endocrine disrupting chemicals (EDCs), some of which mimic the chemical structure of 17β-estradiol and can activate ER mediated gene expression. Many different classes of compounds have been identified as estrogen mimics, including environmental chemicals such as pesticides. Chapter Two describes an investigation into ER mediated gene

expression stimulated by pesticide metabolites. Because of their steroid-like effects, EDCs have been linked to cancer but their relationship remains to be determined. The activity of enzymes in the metabolism of estrogen is another factor implicated in cancer, due to the generation of potentially carcinogenic estrogen metabolites.[15]

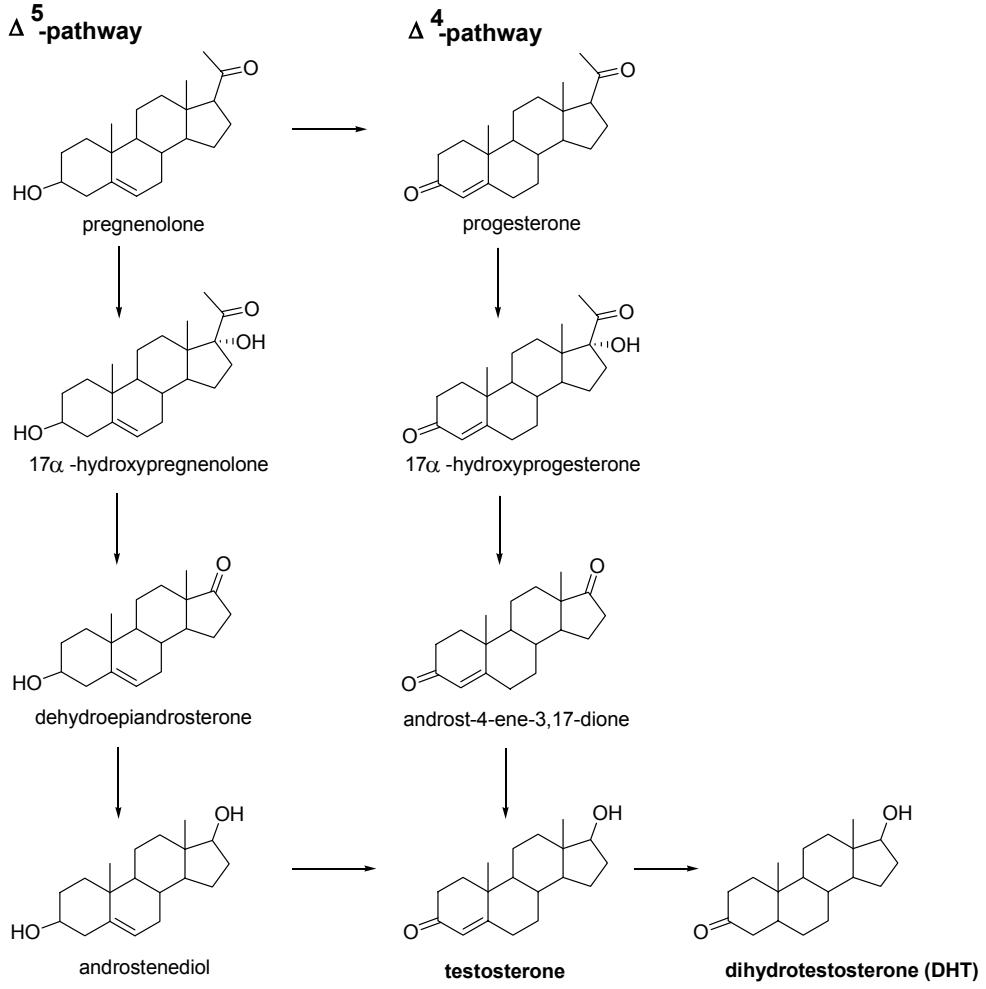
Androgens

Androgens are responsible for sex determination, foetal development, growth and sexual maturation. The two most important androgens are testosterone (the main androgen secreted in the testes) and 5α-dihydrotestosterone (DHT). Testosterone and DHT have different physiological roles in the male (see Table 1.2). At the embryonic stage, the production of testosterone regulates gonadotropin production, spermatogenesis, and formation of internal male genitalia (epididymis, seminal vesicles and vas deferens). In the embryo DHT stimulates formation of male external genitalia (penis, scrotum, urethra, and prostate). At puberty testosterone is associated with modifying external genitalia, increasing muscle mass, voice deepening, and male sexual behaviour, while DHT is responsible for increasing body and facial hair and prostate enlargement.

Biological Response	Androgen responsible
Reproductive tract- differentiation and growth of tract:	DHT
epidiymus, prostate, seminal vesicles, vas deferens, bulbourethral glands	
Stimulation of male secondary sex characteristics:	T
growth of external genitalia, voice deepening, hair growth and body distribution	
Anabolic actions: skeletal and muscle growth, subcutaneous fat distribution,	T
growth of prostate	DHT
growth of seminal vesicle	T, DHT
CNS action and brain:	
differentiation of regions	T metabolised to E
libido development	T

Table 1.2. Biological responses of testosterone (T) and DHT (E - estradiol).
(Taken from Norman and Litwack[14]).

The biosynthesis of testosterone and DHT is shown in Scheme 1.5. The Δ^5-pathway predominates in humans. Other androgens synthesised are released in low concentrations. Any steroid on the Δ^5-pathway can be converted to the corresponding steroid on the Δ^4-pathway by the enzyme 3β-steroid dehydrogenase/Δ^5,Δ^4 isomerase.



Scheme 1.5. Biosynthesis of testosterone by Δ^5- and Δ^4-pathways and conversion to DHT.

Both testosterone and DHT are produced by the testes and circulate in the plasma, but there is significant metabolism of testosterone to DHT in target cells, e.g. prostate cells. This reaction is carried out by the enzyme steroid 5α-reductase. The tissue distribution of steroid 5α-reductase determines whether testosterone or DHT is the initiating androgen in

target cells. Both bind very efficiently to the androgen receptor (AR), whilst stimulating different biological responses (Table 1.2). The AR functions, like all steroid receptors, as a transcriptional regulatory protein in gene expression when activated by androgen binding (the genomic action of steroids was discussed in detail in section 1.3.1). Of all the androgens, testosterone and DHT have the highest affinity for the AR, hence their designation as the active androgens. DHT has approximately twice the affinity for the AR compared to that of testosterone.[14]

Testosterone and DHT effect different biological responses despite the fact they bind to the same receptor. DHT amplifies the androgen signal (compared with that of testosterone) because of its higher affinity for the AR. It has been suggested that DNA binding and expression by the testosterone-AR complex is different to that of the DHT-AR complex (which is proposed to have higher affinity for DNA).[17] Another explanation is that tissue response differences are related to the activity of steroid 5α-reductase and also AR concentrations in target tissues.[18] In muscle tissue for example the activity of steroid 5α-reductase is so low that testosterone produces the hormonal effect.

Androgens and prostatic disease

Androgens are required for normal prostate growth, in particular DHT which promotes cell growth. However, androgens are also implicated in prostatic disease. Prostate cancer is one of the most common cancers in men over the age of 50 in the Western world, and 149,000 deaths occur annually worldwide.[10,19] The tumour is endocrine-responsive as DHT stimulates tumour growth. Benign prostatic hyperplasia (BPH) is a non-cancerous enlargement of the prostate which is also common in men over the age of 50, and causes bladder outlet obstruction and urination problems. Prostatectomy (removal of all or part of the prostate) is the second most common operation in men over the age of 65.[20] The presence of androgens, the AR and steroid 5α-reductase are requirements for the development of BPH (5α-reductase activity is 2-3 times higher in the prostates of BPH sufferers[21]). Although it is known that prostate cancer and BPH occur when the balance between cell growth and apoptosis (programmed cell death) is not maintained,[19] the exact

cellular mechanisms involved in upsetting this balance are not fully understood. Factors that have been implicated in initiating prostate cancer and BPH are:

i) *Increased androgen levels*: Changes in cell growth/death balance could be due to increased androgen synthesis and decreased inactivation, which is regulated by the pathway of steroidogenic and steroid-metabolising enzymes (see Scheme 1.4). As a consequence, higher than normal levels of androgens may be produced, promoting increased cell growth.

ii) *Estrogen*: Evidence suggests that exposure to estrogen can also lead to the development of changes in cell growth within the prostate, and is therefore implicated in the development of BPH and prostate cancer.[10,19] Testosterone levels decline in men with age, while estrogen levels are maintained by enhanced levels of aromatase activity, resulting in estrogen accumulation in the prostate.

Controlling androgen levels in prostate disease is the aim for therapeutic intervention. A strategy for treatment of DHT-dependent tumours and BPH is to reduce the level of DHT in the prostate. Halting the production of DHT by inhibiting steroid 5α-reductase activity is one such strategy that has been investigated. Steroidal inhibitors of 5α-reductase have been developed, two of which are in clinical use (see section 3.1.4.1, Chapter Three). However their steroidal nature is associated with side effects. For this reason, the recent focus of 5α-reductase inhibitor development has been on non-steroidal compounds. Chapter Three of this thesis describes the synthesis and assay of novel non-steroidal inhibitors of steroid 5α-reductase.

1.5 STEROID CHEMICAL STRUCTURE

Because of the key role that steroids play in health and disease, it is essential to understand the molecular details of steroid action. The biological activity of steroids is due to their chemical structure which is recognised by steroid receptors and metabolising enzymes. Examination of the molecular structures and three-dimensional shapes of endogenous steroids that stimulate biological responses provides useful information for several reasons; predicting exogenous compounds which can mimic steroids and be recognised by receptors (a subject of concern which is the focus of Chapter Two) or enzymes, and for designing analogues of steroids for the synthesis of drugs to target disease (the subject of Chapter Three).

The chemical structure of steroids is derived from the cyclopentanoperhydrophenanthrene nucleus (see Fig. 1.1), which consists of a phenanthrene molecule (rings A to C) with a five membered carbocyclic ring attached (ring D). The basic ring structure is biologically modified to produce a particular steroid hormone. Hydroxyl or carbonyl substituents and varying degrees of unsaturation are added in various positions. Another important structural feature of steroids is the presence of asymmetric carbon atoms. The three-dimensional arrangement of atoms plays important roles in the structure and therefore activity of steroids, especially in selective binding to steroid receptors and metabolising enzymes. Fusion of the rings leads to a surface (which is almost flat for many steroids) having readily discerned upper and lower faces. Functional groups can be above/in, or below the plane of rings as drawn in Figure 1.2.

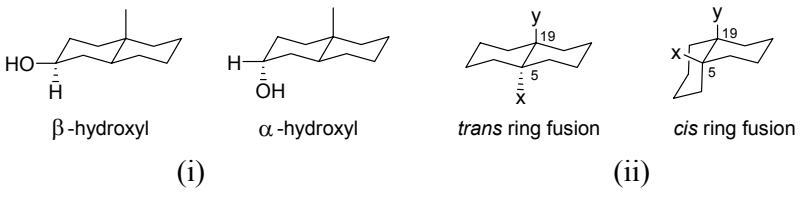


Figure 1.2. (i) Steroidal α and β nomenclature and (ii) ring junction orientations.

The three-dimensional structure of steroids varies greatly depending on the types of ring junction present. The rings in steroids can be *trans* or *cis* fused (see Fig. 1.2). The three most commonly encountered structures result from differences in the AB ring junction which may be *trans* or *cis* (Fig 1.3). The BC and CD ring junctions normally exist as the *trans* form. *Cis-trans* isomerism is not possible at the AB ring junction when the A ring possesses unsaturation as shown in Figure 1.3 (e.g. in testosterone).

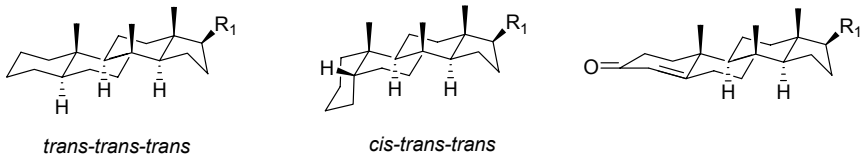


Figure 1.3. Common three-dimensional conformations of steroids.

In the estrogens the A ring is aromatic (see Scheme 1.3) so there is no *cis-trans* isomerism possible. The aromatic ring is completely planar which has significant effects on their biological activity (which will be discussed in more detail in Chapter Two).

1.6 NON-STEROIDAL ANALOGUES OF SEX STEROIDS

The structure and conformation of steroids play a vital role in recognition and binding to their cognate receptor or metabolising enzyme, and it is now widely recognised that compounds which mimic steroidal conformation can also bind to these cellular targets.[6] Steroid mimics without the steroidal cyclopentanoperhydrophenanthrene nucleus are termed non-steroidal analogues. These mimics are of interest because they may enhance or decrease steroid activity which potentially has beneficial or harmful consequences. The effects of non-steroidal analogues on steroid activity are implicated in several areas of research. Non-steroidal analogues of steroids have been developed to treat disease, for example cancer, for the purpose of reducing steroid activity within the tumour. From another perspective, unintentional exposure to non-steroidal analogues of steroids may enhance steroid activity. An area of concern is the exposure to environmental chemicals which may mimic steroid activity. The following section describes the potential adverse effects of non-steroidal analogues, and the use of non-steroidal analogues in medicinal chemistry for health benefits. This thesis focuses on these two different effects of non-steroidal analogues on steroid activity.

1.6.1 Adverse effects of non-steroidal analogues on steroid activity

While pharmaceuticals are used intentionally to alter steroid activity in disease treatment, there is increasing knowledge of natural and synthetic compounds which are suspected to have unintentional adverse effects on steroid activity. A wide range of non-steroidal compounds from environmental sources have been found to mimic endogenous steroids, in particular the sex steroids, either by binding to hormone receptors, or to enzymes involved in steroid biosynthesis and metabolism, or through other mechanisms.[6,22] The activity of these compounds is encompassed in a research field termed ‘endocrine disruption’. There is concern that steroid mimics may alter endogenous steroid activity and disrupt endocrine processes, the result of which may be disease. A widely investigated area within endocrine disruption research is that of exogenous chemicals which bind to the ER, and thus mimic 17β-estradiol activity. Genistein and bisphenol A (Fig. 1.4) are examples of natural and synthetic exogenous compounds which have been found to bind and activate the ER. An

understanding of how exogenous compounds mimic 17β-estradiol is important for predicting compounds capable of altering endogenous steroid activity. Such information could lead to further elucidation of causative agents in disorders like cancer and reproductive abnormalities. Chapter Two of this thesis will discuss endocrine disruption and estrogen mimics in further detail to introduce an investigation into non-steroidal compounds which activate ER mediated gene expression in recombinant yeast.

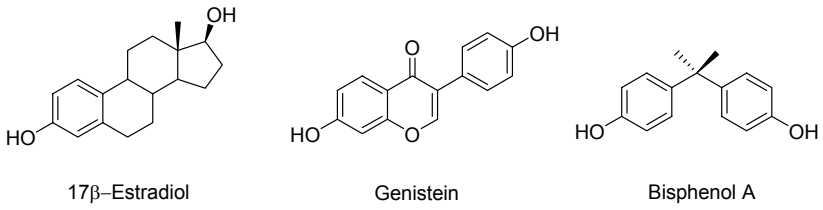
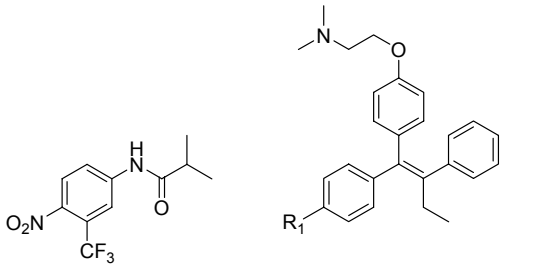


Figure 1.4. 17β-Estradiol and examples of environmental mimics.

1.6.2 Non-steroidal analogues in medicinal chemistry

Non-steroidal analogues of steroids provide the basis of various pharmaceuticals used to treat hormone related problems. The biological targets for such drugs may be steroid receptors or enzymes involved in steroid biosynthesis and metabolism. Pharmaceuticals with a steroidal based structure designed for a particular receptor (or enzyme) have the potential to bind to other steroid receptors, as there is a high degree of homology in steroid receptor LBDs. The non-selective binding of steroid drugs may lead to adverse side effects. Non-steroidal drugs offer advantages over steroidal drugs by enhancing receptor selectivity. Non-steroidal compounds also offer better pharmacokinetic properties, such as oral bioavailability and hepatic metabolism.[23] These properties make them attractive targets for novel pharmaceuticals. Non-steroidal compounds have been developed to mimic androgens and bind to the AR for the purpose of reducing the level of androgens in cancer cells. AR antagonists are used clinically in treating prostate cancer; one example is flutamide (Fig. 1.5). Flutamide competes with DHT for binding to the AR in prostate tissue. Non-steroidal mimics of estrogen are also in clinical use for the treatment of ER positive breast cancer, for example tamoxifen (Fig. 1.5). This drug is metabolised to the

more potent 4-hydroxytamoxifen which binds to the ER in breast tissue, and prevents ER mediated gene expression and growth of cancer cells.



Flutamide

R_1 = H, tamoxifen
R_1 = OH, 4-OH tamoxifen

Figure 1.5. Non-steroidal analogues of sex steroids in clinical use for cancer treatment.

Enzymes involved in the biosynthesis and metabolism of steroids are also attractive targets for the development of non-steroidal drugs. Non-steroidal compounds are of great interest for inhibiting steroid 5α-reductase, the enzyme that converts testosterone to DHT (see Scheme 1.5). Abnormal levels of DHT are implicated in several disease states, including prostate cancer, BPH, androgenetic alopecia and acne. Reducing the levels of DHT by inhibiting 5α-reductase activity is a logical strategy for treating these disorders. To date there are no non-steroidal inhibitors approved for clinical use, but some classes of compounds are currently involved in clinical trials. Chapter Three will discuss steroid 5α-reductase inhibition by non-steroidal compounds in more detail, and work towards this thesis on the design, synthesis, and testing of non-steroidal inhibitors of this enzyme.

1.7 WORK DESCRIBED IN THIS THESIS

The information provided in this introduction gives an overview of the importance of steroids and mechanisms for their biological activity within cells. The chemical structure of a steroid is recognised by steroid receptors and metabolising enzymes, interactions which are critical for mediating steroid activity within cells and tissues. However steroid receptors and metabolising enzymes also recognise and bind non-steroidal compounds, the effect of which may be the alteration of endogenous steroid activity. The consequences of altering steroid activity may be harmful, due to unintentional effects of steroid mimics (e.g. EDCs), or beneficial, for example treating disease with drugs designed to mimic steroids. This thesis investigates the effect of non-steroidal analogues on the biological activity of sex steroids from two perspectives; the interaction of non-steroidal compounds with a sex steroid receptor and an enzyme involved in sex steroid metabolism.

Chapter Two describes a study investigating the estrogen-like activity of non-steroidal compounds through their interaction with the human ER (hER). Exogenous chemicals which mimic estrogen are of concern as they may alter estrogen activity and disrupt endocrine systems. Endocrine disruption by exogenous chemicals is reviewed, including their mechanisms of action and the evidence observed in wildlife and humans that links these chemicals with adverse effects on endocrine systems. The non-steroidal chemicals investigated in this study were pyrethroid insecticide metabolites, which were proposed to interact with the ER due to structural features mimicking those of 17β-estradiol. Whilst some were available commercially, a number of metabolites were synthesised. A biological assay was established and used to determine the activation of hER-mediated gene expression by these pyrethroid metabolites relative to that of 17β-estradiol. This assay involves a recombinant yeast strain which expresses hERα and possesses a *lacZ* reporter gene construct to allow detection of estrogen stimulated gene expression. The results are put into context by considering the estrogenic activity of metabolites in relation to the likelihood of exposure to these compounds.

Chapter Three is concerned with the inhibition of a sex steroid metabolising enzyme by novel non-steroidal compounds. Steroid 5α-reductase converts testosterone to DHT and

abnormal levels of DHT are associated with prostate and skin disorders. Regulating DHT activity by 5α-reductase inhibition is a logical strategy for drug therapy. A review of non-steroidal compounds already known to inhibit this enzyme is presented, which leads to a discussion on efforts in this thesis towards the rational design of novel inhibitors, with focus on investigating structure-activity relationships (SAR). The synthesis of proposed non-steroidal inhibitors is detailed, followed by a discussion on their activity against steroid 5α-reductase expressed by transfected human embryonic kidney cells.

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CHAPTER TWO

ESTROGENIC ACTIVITY OF PYRETHROID INSECTICIDE METABOLITES IN A YEAST ESTROGEN SCREEN

2.1 INTRODUCTION

Endocrine systems of the body provide means to enable target tissue responses to hormonal signals originating in another organ. Steroid hormones play essential roles within the endocrine system, which were discussed in detail in Chapter One. The objective of endocrine systems is to maintain homeostasis within the body, avoiding significant changes in hormone levels and responses which may have detrimental effects on metabolism.[1] Increased or decreased hormone activity in endocrine systems can lead to potentially debilitating and life threatening disease involving different organs and functions. Chapter One discussed the potential adverse consequences of altering normal sex steroid hormone activity using target tissue cancers as an example (see Chapter One, section 1.4). There is significant concern for chemicals in the environment which mimic sex steroids and thus may alter endogenous sex steroid activity by interacting with sex steroid hormone receptors and enzymes. The following section discusses these endocrine disrupting chemicals (EDCs); their mechanisms of action and their history, which includes evidence for the implication of EDCs in endocrine abnormalities observed in wildlife and humans.

2.1.1 Endocrine disruption

The last two decades have witnessed growing concern about chemicals in the environment, including those that may interfere with the endocrine systems of wildlife and humans (EDCs). EDCs come from a wide range of chemical classes and sources, which include natural and synthetic hormones, plant constituents, pesticides, chemicals used for plastic and consumer product manufacture, industrial by-products and other pollutants. They are widely dispersed in the environment, where some are persistent. Others are rapidly degraded in the environment or human body, or may be present for short periods of time but at critical periods of development. Although they often display weak hormonal activity, their lipophilic nature allows them to accumulate in the fatty tissues of the body. This increases their concentration and bioavailability.

EDCs may interfere with endocrine systems by mimicking hormone action (particularly steroid hormones), preventing the action of hormones, altering hormone biosynthesis and metabolism, or by affecting the level of hormone receptors within the cell. Upon recognition of EDCs and their potential adverse health effects, laws were introduced in the United States. These laws required the Environmental Protection Agency (EPA) to develop and implement a screening strategy to assess risk from estrogenic endocrine disruptors, as the binding of molecules, other than estrogen, to the ER was already known to be a major mechanism of endocrine disruption. Androgenic and thyroid-active chemicals were also included and active chemicals were to be subjected to rigorous animal studies. Several methods have been established for screening and detecting endocrine disruptors, including the measurement of receptor-dependent responses, structure-activity relationship (SAR) based priority assessment, as well as a number of *in vitro* and *in vivo* assays. These will be discussed further in sections 2.1.7-2.1.8.

There are several critical points that must be considered when assessing the potential impact of endocrine disruptors:[1]

i) There may be no significant or observable effect if adult exposure is compensated for by normal homeostasis;
ii) Permanent change to endocrine function can occur if EDC exposure is during foetal development;
iii) Various effects may be observed if exposure to an EDC is at different life stages or seasons;
iv) Endocrine pathways are connected intimately and the effects of EDCs may be unpredictable for systems not included in the assessment;
v) Caution needs to be taken when extrapolating results from *in vitro* hormonal activity to the situation *in vivo.*

2.1.2 EDC mechanisms of action

An EDC is defined by Kavlock *et al*.[2] as "an exogenous agent that interferes with the production, release, transport, metabolism, binding, action, or elimination of natural hormones in the body responsible for the maintenance of homeostasis and regulation of developmental processes." This interference is believed to be from chemicals mimicking endogenous hormones (in particular sex steroid hormones), antagonising endogenous receptors, altering the biosynthesis and metabolism of hormones, and modifying hormone receptor levels. The focus in this thesis is on chemicals that mimic 17β-estradiol by activating the ER.

Interaction with hormone receptors

Interaction with cellular hormone receptors is widely recognised as a major EDC mode of action. The role of receptors in hormone mediated gene expression (see Fig. 2.1) was discussed in detail in Chapter One, section 1.3.1. EDCs can mimic endogenous hormones and potentially induce hormone directed effects by activating a particular receptor (agonist) or by blocking the receptor (antagonist). This results in changes to gene expression characteristics for a certain hormone. Change in gene expression is a critical step in the regulation of a biological function, which could therefore be altered by an EDC. There are several systems used to evaluate the interaction of exogenous chemicals with hormone receptors, in particular those interacting with the ER, AR, thyroid, and aryl hydrocarbon receptors.[1] Binding and activation of the ER by chemicals (other than the endogenous hormone 17β-estradiol) has been well studied and will be discussed in detail in section 2.1.6. However, there is a growing knowledge that exogenous chemicals may interact with other hormone receptor systems. These include the retinoic acid receptor, cytokine systems and orphan receptors (with unknown ligands and/or functions).

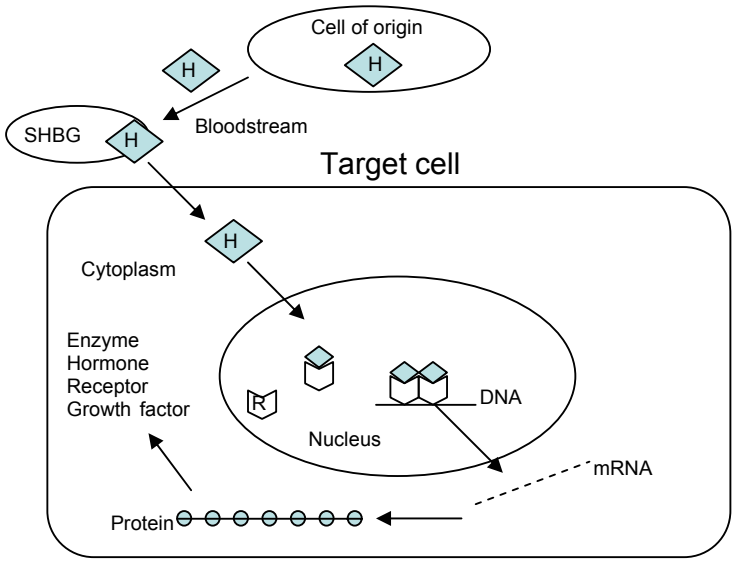


Figure 2.1. Key steps in steroid hormone action which may be sensitive to EDCs (adapted from WHO report[1]):

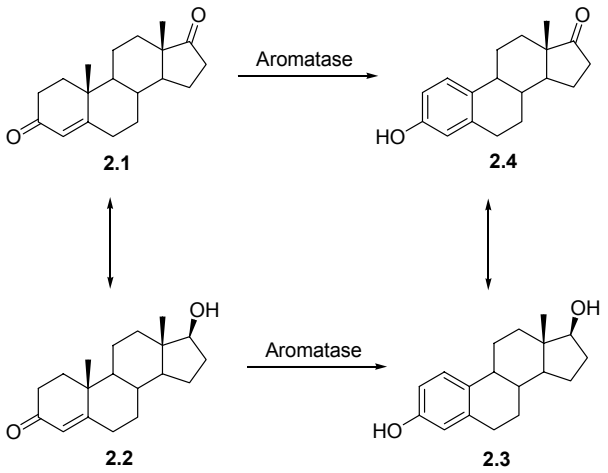
The steroid hormone (H) *is synthesised in an endocrine cell, then transported in the bloodstream bound to* SHBG *to the target cell. The steroid diffuses into the target cell and binds to nuclear receptor proteins* (R). *Steroid-receptor binding induces a conformational change in the receptor, increasing its affinity for specific DNA regions. Binding of the steroid-receptor complex to DNA initiates mRNA synthesis and translation to give proteins (enzymes, hormones, receptors and growth factors) which can initiate proliferative responses in the target cell.*

Interaction of chemicals with hormone biosynthesis and metabolism

EDC research has focussed on interactions of exogenous chemicals with sex hormone receptors, particularly the ER. However alternative modes of action for EDCs have been investigated. Some chemicals, for example PCBs, exhibit estrogenic activity in experimental animals, but have little or no affinity for the ER.[3] Therefore their estrogenic action cannot be due to ER mediated gene expression. An alternative mechanism of EDCs includes altering the function of key enzymes in steroid hormone biosynthesis and metabolism. Cytochrome P450 enzymes are of interest as they are responsible for the

highly specific reactions in steroid biosynthetic pathways, in which potent endogenous steroid hormones are formed.

A key cytochrome P450 enzyme in steroidogenesis is aromatase (CYP19). Aromatase catalyses both the conversion of testosterone (**2.2**) to estradiol (**2.3**), and androstenendione (**2.1**) to estrone (**2.4**) (Scheme 2.1). This enzyme is of particular interest as it catalyses the final rate-limiting step in estrogen formation which occurs in ovarian granulosa cells, adrenal cortex, and in tissues such as brain, adipose and placenta. Aromatase plays a critical role in sexual differentiation, reproduction, and behaviour, as well as in disease such as estrogen-dependent cancer.[4]



Scheme 2.1. Aromatase catalysed metabolism of testosterone (**2.2**) and androstenedione (**2.1**).

EDCs may interfere with the activity or expression of aromatase and disrupt processes such as the estrous cycle, sperm production and maturation, onset of puberty, development of sexual behaviour, as well as the inhibition or stimulation of breast, ovary and prostate hormone-dependent tumours. Several classes of pesticides that persist in the environment are suspected or have been shown to alter aromatase activity, including organotin compounds (used as antifouling agents on ship hulls), DDT and several metabolites, some

azole fungicides, and several triazine herbicides.[4] In particular, azole fungicides have been shown to inhibit aromatase activity in rainbow trout, which may indicate their ability to block estrogen mediated responses.[5] More experimental evidence is required to determine the exact mechanism of interference that chemicals have with aromatase activity. There has been some attempt to determine whether the enzyme is being induced or inhibited, or whether aromatase mRNA expression is altered, but this is still not completely clear. However, this area warrants further investigation to increase understanding in the complexity of the mechanisms by which EDCs may act.

Enzymes are also responsible for regulating steroid hormone metabolism, another potential target for EDC activity. Metabolism of chemicals in cells usually occurs via Phase I and II reactions.[6] Phase I metabolism, carried out by cytochrome P450 enzymes, introduces hydroxyl groups to a molecule. These hydroxyl groups are then conjugated, via Phase II metabolism, with charged groups such as sulfate, glucuronide, or amino acids. Circulating estrogens are metabolised extensively in the body, especially in the liver. They are either conjugated with sulfate alone (Phase II) or after hydroxylation by P450 enzymes (Phase I).[4] They can also be conjugated to glucuronide moieties. Both metabolic processes render them hormonally inactive. A small fraction of estrogens are hydroxylated by P450 enzymes, which convert them to catechol estrogens. Some of these Phase I metabolised estrogens have been implicated in estrogen-mediated genotoxic carcinogenesis.[7] Polyhalogenated and polycyclic aromatic hydrocarbons are capable of affecting the catalytic activity of these metabolising enzymes by inducing certain P450 enzymes that hydroxylate estrogens.[4]

2.1.3 History and evidence for endocrine disruption

Rachel Carson's book *Silent Spring*[8] first raised concerns about chemicals and their potential to cause unpredicted effects on wildlife and the environment. Since the publication of *Silent Spring* in 1962, there has been increasing awareness of chemicals in the environment that might have profound adverse effects on wildlife, even decades after their use (such as the case for DDT). A significant amount of literature has surfaced on the hormone-like effects of environmental chemicals in fish, wildlife and humans.

Unfortunately, these effects were discovered following the release of chemicals into the environment. Research into endocrine disruption has only been active over the last 15 years since Theo Colborn and coworkers presented the notion at a conference in the USA in 1991.[9] Research has since focussed on chemicals that mimic reproductive hormones, especially estrogens. The insecticide DDT was the first intentionally released chemical to be identified as an estrogen mimic years later. A link between aviation crop dusters handling DDT and reduced sperm counts was put forward in 1949.[10] Other cases of occupational exposure to pesticides gave further evidence that these compounds may be having adverse effects on workers. Further experiments in animals demonstrated that these chemicals were able to mimic the biological activity of estrogens.[11] In 1994 Guillette *et al.* published a significant paper reporting the relationship between the reproductive development of alligators and contamination in Lake Apopka, Florida.[12] This lake contained pollution from a spill of the pesticides dicofol and DDT (an intermediate in dicofol manufacture), which were implicated in their findings that male alligators had depressed plasma testosterone concentrations and abnormally smaller phalli, compared to alligators in nearby uncontaminated Lake Woodruff.

Many studies have focussed on establishing cause and effect relationships for chemicals and effects observed in wildlife, but it is difficult to prove that certain chemicals have directly caused defects. The cause is defined as the chemical suspected to induce an effect. The effect is defined as the biological response to the cause. Biological responses include those at the molecular, cellular, tissue, organ, individual, population, and ecosystems. Cause and effect relationships can have varying strengths of association. There is also awareness for the 'cocktail effect' of chemicals in the environment, where exposure to multiple chemicals may carry certain risks compared to those of a single chemical.

Wildlife effects

Adverse reproductive effects observed in wildlife have been linked to exposure from mixtures of chemicals in the environment. Fish, birds, reptiles, mammals and other species inhabiting areas contaminated with pollutants have been observed to have reproductive problems. Lower organisms such as the invertebrates exhibit anatomical and physiological

traits that are under endocrine control, so have unique susceptibilities to EDCs and are important indicators of hormonal imbalance. The following are examples of abnormalities (particularly reproductive) observed in wildlife, and whilst there are many more than mentioned here, they clearly demonstrate why chemicals in the environment are suspected of causing endocrine disruption.

i) *Invertebrates*: An important example of endocrine disruption by environmental contaminants is the exposure of molluscs to tributyltin (TBT). This compound is used as an antifouling paint applied to boat hulls. The condition of imposex (imposition of male sex organs onto females) was observed in marine gastropods exposed to TBT with increasing frequency.[13] Supporting work showed that TBT induced penis formation when injected into female dogwhelks (*Nucella lapillus*), and high levels of testosterone were measured.[14] Recent work suggests that TBT oxide interferes with hormone metabolism, presumably by inhibiting aromatase and therefore increasing androgen levels.[15]

ii) *Fish*: Impairment of reproductive functions in fish was studied in areas downstream of sewerage treatment works effluent discharge in the UK. Male fish were found to produce a female specific egg-yolk protein precursor, vitellogenin, indicating estrogenic compounds were present in the water. This was also observed in rainbow trout exposed to treated sewerage.[16] Populations of wild fish in English rivers have been found to be intersex (a phenomenon induced by estrogen exposure).[17] Many causative agents, including estrogenic compounds, have been suggested.[18] Waterborne bisphenol A has been demonstrated to affect transcription of estrogen responsive genes transfected into tadpoles and fish.[19]

iii) *Amphibians*: This class of vertebrates may be subject to EDC exposure during different life stages, and therefore be particularly at risk from EDC effects. However, while scientists agree that amphibian population levels are decreasing, there is insufficient data to link this to EDCs. Studies are now focussing on understanding amphibians as potential targets for endocrine disruption.[1] Recent laboratory studies have shown that amphibians can be feminised or demasculinised by EDCs.[20]

iv) *Reptiles*: Alligators (*Alligator mississippiensis*) inhabiting Lake Apopka in Florida provide one of the most publicised examples of EDC effects on wildlife. The lake was contaminated with dicofol and DDT , and alligators were observed to have reduced penis size, lowered testosterone levels, and elevated 17β-estradiol levels.[12] These observations were made in comparison with alligators inhabiting another Florida lake, Lake Woodruff, a relatively uncontaminated site. Further studies have led to hypotheses on the mechanism of effect that DDT has on reproductive systems in alligators.[21] Later reports suggested that the effect observed in Lake Apopka was also apparent in other Florida lakes, after further studies at lake sites and in the laboratory. [22,23]

v) *Birds*: Populations of gulls in the Canadian Great Lakes area and California have been exposed to great concentrations of organochlorines, including DDT, from the 1950s until the 1970s. Exposure to these chemicals has been implicated in observed reproductive problems, including egg shell thinning, abnormal parental behaviour and poor hatching.[1] Gull embryos exposed to organochlorines via injection into eggs showed signs of abnormal male and female reproductive system development, suggesting these chemicals may be the causal agent of defects observed in wildlife. Male chicks were found to possess oviducts and sexual organs resembling ovaries. Female chicks were found to have abnormally developed oviducts.[24] Current hypotheses for a mechanism to explain egg shell thinning in some birds involves prostaglandin inhibition, but this remains undetermined.[25]

vi) *Mammals*: Declining populations of mink and otter have also been observed in the organochlorine polluted Great Lakes Basin area, as has reproductive failure in commercially reared mink which were fed fish collected from the Great Lakes. These observations led to the suggestion that organochlorines were having an effect on these fish eating mammals.[26] Trends in the Swedish European otter population from 1968-1999 have also been examined. The observed decline in otter population during this period was associated with exposure to PCBs, due to an increase in the population since PCB concentration has decreased.[27] Reproductive failure in Baltic grey seals has also been linked to PCBs.[28] Recently sperm concentration and motility was found to be reduced in male rats exposed to xenoestrogen-contaminated fish during lactation.[29]

Human health effects

Evidence for endocrine disruption in wildlife has led to concerns for possible effects in humans. There are several examples of chemicals suspected to influence human health effects, but cause and effect relationships are difficult to ascertain, as for wildlife. Research into the relationship between EDCs and human health has focussed on particular areas; reproduction, neurobehaviour, immune function, and cancer. Health effects observed are related to the level of exposure; high human exposure to environmental chemicals can clearly demonstrate adverse effects, whereas for low levels of exposure the data is much less clear. It is difficult to draw conclusions in relating EDCs to human disease by comparing human, laboratory, and epidemiological studies. The lack of exposure data, especially during development, is also highlighted when trying to draw associations between the cause and effect of environmental chemicals. The following section describes human health effects which have been associated with EDC exposure.

i) *Reproductive effects*: The male reproductive system is of concern when considering the impact of EDCs on humans, and many studies have investigated variations in sperm count and quality. In 1992 Carlsen *et al*. examined 61 separate studies on sperm count in men in different countries and concluded the mean sperm count of healthy men had declined within a period of 50 years (between 1938 and 1991).[30] However, the paper was heavily criticised on the basis that men included in the studies could hardly be compared due to differences in location, age, and fertility. Additionally the sample sizes in some studies were very small, and thus did not permit useful comparisons. To date most studies have been retrospective and data analysed may have been collected for other reasons (e.g. infertility studies). In 1993 Sharpe and Skakkebaek proposed that environmental chemicals, interfering with the normal development of reproductive systems by an endocrine mechanism, may be related to observed increases in male reproductive disorders.[31] They argued that increased exposure to environmental estrogens *in utero* may contribute to male reproductive abnormalities. These disorders included decreased sperm count and quality, testicular cancer, cryptorchidism (undescended testes), and malformations, e.g. hypospadias (developmental abnormality where the urethra opens on the underside of the penis). In 2003 Sharpe and Skakkebaek concluded that environmental estrogens probably pose little risk to male reproductive development.[32] However when the

mechanisms by which estrogen acts are more understood the role of environmental chemicals in male reproductive development disorders could be redefined.

The effects of EDCs on fertility have been widely studied and occupational exposure to chemicals is often analysed for evidence. Studies have focussed on maternal exposure to agricultural chemicals (e.g. pesticides), for example an increased risk of infertility was associated with women working in the agricultural industry.[33] PCBs and pesticides are thought to account for this reduced fertility. To date no clear association with endocrine disruption has been demonstrated, but fertility has decreased in some occupational groups exposed to pesticides, which warrants further investigation to better characterise the association.[1]

ii) *Neurobehaviour*: The brain contains steroid receptors, so it is a potential target for EDC related effects. Epidemiological studies have implicated exposure to environmental chemicals in neurobehavioural alterations. These chemicals include pesticides (DDT and DDE, kepone), fungicides (methoxychlor, fenamirol), polychlorinated dibenzodioxins (PCDDs), PCBs and dibenzofurans.[1] Concern is especially focussed on exposure *in utero*. The mechanisms responsible for effects observed remain to be determined.

iii) *Immune function*: The immune system interacts with the neuroendocrine system, so there is potential for environmental chemicals to influence immune function by interfering with endocrine mechanisms. However, there are few compounds proven to cause immune alterations by disrupting endocrine mechanisms. Diethystilbestrol (DES) was related to weak immunological changes after *in utero* exposure in animals, as were PCBs, PCDFs and PCDDs.[1] DES was recently found to reduce the level of circulating lymphocytes in rhesus monkeys.[34]

iv) *Cancer*: Increased rates of cancer worldwide are used as evidence for the impact of of EDCs on human health. Cancer incidence in hormonally sensitive tissues has increased in Europe and North America, including cancer of the breast (since the 1940s), prostate (1980s), and testis (1960s).[1] These increased cancer rates have been associated with an increasing use and release of chemicals in the environment, with many acting as hormone mimics *in vitro* and *in vivo*. However, cause and effect relationships between chemicals

and cancer are extremely difficult to identify. While there is not enough information in the literature to completely reject endocrine disruptors as a causal factor in cancer, further research is needed, especially on the effect of EDC exposure at critical periods in relation to cancer incidence.

2.1.4 Xenoestrogens

Endogenous estrogens are involved in foetal development and organ development in the female genital tract and other tissues (see Chapter One, section 1.4.1). They are responsible for female development and reproductive processes such as maintaining the menstrual cycle, pregnancy and lactation. Other chemicals with estrogenic action have been known since the 1930s when DES was first synthesised.[35] DES is an estrogen mimic and is still one of the most potent synthetic estrogens. In 1946 it was reported by Bennetts *et al.* that breeding problems in ewes from Western Australia were associated with their diet of clover.[36] Infertility, dystocia and prolapsed uterus were linked to the phytoestrogens (plant based estrogenic compounds) formononetin, biochanin A and genistein.[37] Chemicals from many different sources are now understood to have the capability to mimic endogenous estrogens. These mimics are termed xenoestrogens. The prefix 'xeno' is derived from the Greek word 'xenos' meaning foreign.

The estrogenicity of some chemicals was accidentally discovered. Experiments conducted to study the effects of natural estrogens were disrupted by compounds exhibiting estrogenic activity. In 1991 nonylphenol (**2.6**, Fig. 2.2) was found to be estrogenic when an estrogen-like substance was being released into plasma stored in plastic centrifuge tubes.[38] Nonylphenol is used as an antioxidant in plastic manufacture. Bisphenol A (a plasticiser) (**2.5**) was accidentally discovered to be estrogenic when researchers, who were searching for an estrogen binding protein in yeast, found an estrogenic chemical was released into the yeast culture medium during autoclaving of the polycarbonate plastic-ware for sterilisation.[39]

2.5 **2.6**

Figure 2.2. Xenoestrogens bisphenol A (**2.5**) and *p*-nonylphenol (**2.6**).

The interaction of xenoestrogens with the ER is considered to be a major mechanism of endocrine disruption, and is the mechanism focussed on in this thesis. Non-genomic effects of estrogen have also recently been characterised, involving rapid modulation of signal transduction pathways.[40] Overall, estrogen action with respect to the mechanism and role of non-genomic activity remains to be determined. Other proposed mechanisms of action of xenoestrogens include:

i) Activation or suppression of the AR
ii) Activation of the aryl hydrocarbon receptor
iii) Inhibiting steroid hormone synthesis, transport, and metabolism
iv) Interaction with neurotransmitter receptors.

There is concern for the 'additive' activity of xenoestrogens in humans and wildlife as they may affect the levels of endogenous estrogens already present in target cells. The normal level of 17β-estradiol in human females ranges from 20-350 ng/L (premenopausal), 10-30 ng/L (postmenopausal), and 100-3000 ng/L (pregnant).[41] In human males the level of estradiol ranges between 10-50 ng/L. Exposure to natural estrogens is a principal risk factor in the development of breast and endometrial cancer. Adult human males exposed to estrogens can result in gynecomastia and abnormalities of the endocrine system, such as reduced libido, impotence, decreased blood levels of androgens and lowered sperm count.[11] If exposure is *in utero*, then cryptorchidism (undescended testes, from the Greek 'kryptos' - hidden, and 'orchidos' - testes), enlarged prostates, malformations of the female genital tract, and cancer of the vagina may occur as a result of over exposure to estrogens.[11] For these reasons xenoestrogens are of significant importance, as they may alter the level of exposure to estrogens and promote the onset of the aforementioned disease states.

2.1.5 Estrogen receptor

A major mechanism of endocrine disruption is considered to be via the binding of xenoestrogens to the ER. This receptor is normally activated by endogenous estrogens. Estrogens were first isolated in the 1920s,[42] and by the 1950s synthetic estrogens were used as oral contraceptives. The biochemical basis for the action of estrogens was revealed in the early 1960s when Jensen *et al* discovered a protein (subsequently named the ER) with high affinity for estrogen that migrated to the cell nucleus.[43] Incidentally this work also led to the study of other nuclear receptors. Upon its discovery, it became apparent that the ER was part of a larger protein complex before activation by estrogens.[44] This activation significantly increased the binding of the receptor to DNA,[45] providing a rationale for how the receptor has specific effects on DNA transcription. The isolation, purification and characterisation of major steroid receptors followed.

The ER belongs to the steroid/thyroid nuclear hormone receptor superfamily. There are two subtypes of ER, ERα which was discovered in 1962 and the more recently discovered ERβ, cloned from rat prostate in 1996.[46] Both receptors contain features common to this family of receptors. These include a two zinc finger motif DBD and C-terminal LBD composed of 12 alpha helical structures. Each receptor subtype contains two activation functions (AF), AF-1 in the N-terminus and AF-2 in the C-terminus within the LBD. The ERα and β receptors are only 47% identical. ERα is a slightly longer protein with 595 amino acids compared to 530 in ERβ. The most conserved region between them is the DBD (differing only by three amino acids), and the most divergent is the N-terminal AF-1.[47] The LBD shows only 59% homology. With the distinct pharmacology and differences in tissue distribution and expression levels of the two subtypes, complexity is added to the biological role of 17β-estradiol. ERβ is expressed predominantly in the urogenital tract, and the central nervous system, where ERβ seems to have great significance in brain function, and in the testis. However ERα is mainly involved in reproductive events.[48] This thesis will focus solely on the effects of ERα.

ERα pharmacology

17β-Estradiol has a molecular volume of 245Å^3, with an O-O distance of 10.9 Å. From X-ray studies, the A-ring is planar, while the B, C, and D rings are distorted. The conformational flexibility of the molecule lies in the B ring, which has a half-chair conformation.[49] The ERα is activated upon binding by estradiol within the LBD (with an approximate K_d of 0.3 nM[50]), found between amino acids 303-533. The contact sites for estradiol-ER interaction define the orientation of estradiol within the binding pocket (see Fig. 2.3). The phenolic hydroxyl of the A-ring lies between helices 3 and 6. Hydrogen bonds connect the phenolic hydroxyl to the carboxylate of glutamate 353, the guanidinium group of arginine 394, and a water molecule. The 17β-hydroxyl group of the D ring makes a single hydrogen bond with histidine 524 in helix 11.[51] The rest of the molecule participates in a number of hydrophobic contacts. Estradiol binds diagonally across this cavity between helices 3, 6, and 11 and adopts a low energy conformation.[51] Estradiol is completely enclosed by ERα, forming a hydrophobic core in the protein.

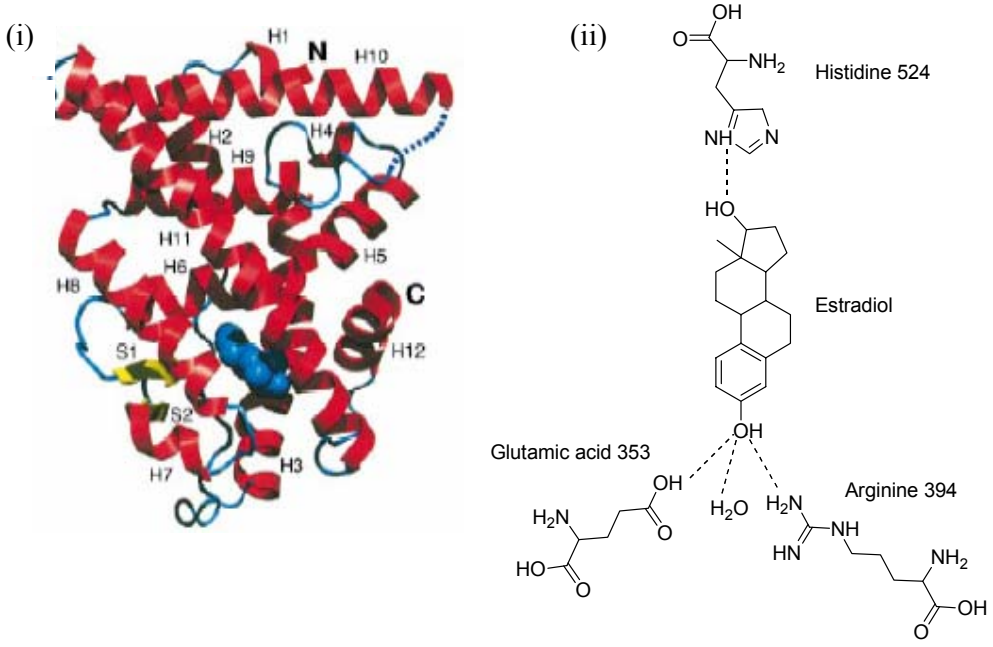


Figure 2.3. Binding of 17β-estradiol to the ERα:

i) Ribbon representation of estradiol in LBD nestled in between helices 3, 6, and 11 (taken from[51]).

ii) Amino acids involved in ER-estradiol interaction with hydrogen bonding (dotted line).

Binding of estradiol to the ER initiates specific conformational changes in the LBD which promote receptor homo-dimerisation. The ER dissociates from heat shock protein hsp90, and allows co-activator recruitment. Hyperphosphorylation of the ER follows, revealing the DBD. This region of ER recognises short (13-15 base pairs) DNA sequences named estrogen response elements (EREs) in upstream flanking regions of estrogen responsive genes. Binding to EREs stimulates gene transcription and mRNA synthesis resulting in increased or decreased expression of estrogen responsive genes (Fig. 2.4). This series of events describes the agonistic effect of estradiol, but some chemicals can act as antagonists of the receptor. Helix 12 (Fig. 2.3) is pivotal in distinguishing between agonists and antagonists. Antagonists block access to a groove located in between helices 3, 4, and 5, which is the binding site for co-activators during transcription.

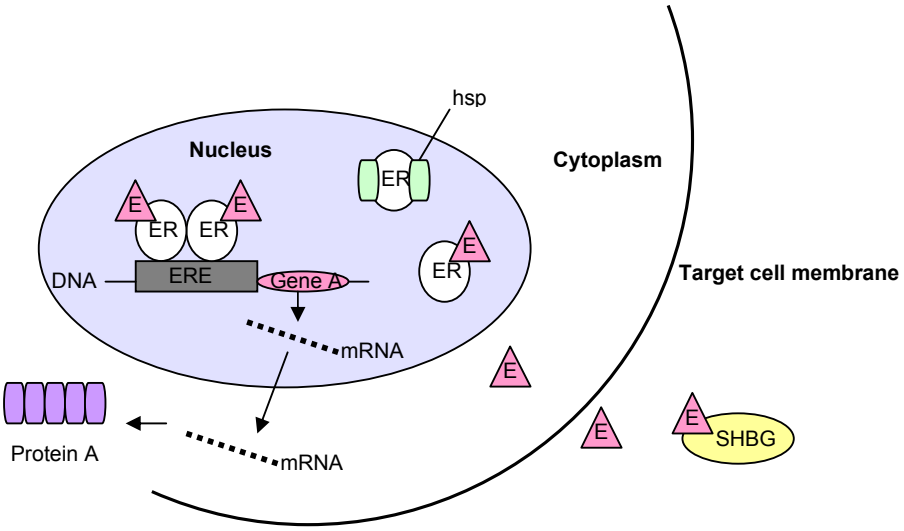


Figure 2.4. Mechanism of estradiol (E) action.

Estradiol dissociates from its transport protein (SHBG) and diffuses across the cell membrane and binds to ER. Estradiol binding causes dissociation of the hsp, which allows the estradiol-ER complex to bind as a dimer to EREs and activate transcriptional machinery to alter gene activity.

2.1.6 ER and xenoestrogens

17β-Estradiol stimulates the maximum ER response as it naturally has the best fit into the binding site of the protein, initiating maximum receptor conformational change. It is now widely recognised that other chemicals (xenoestrogens) fit into this site as they share common structural features with estradiol. However, they don't fit as well and elicit a lesser ER-mediated response compared to estradiol. The ER has a relatively accommodating binding pocket and is often termed 'promiscuous,' with respect to the nature of the binding ligand. In general planar compounds with phenolic character and two electronegative moieties approximately 11-12Å apart can fit in the ER binding pocket.[52] These include synthetic or natural compounds which can also be non-steroidal, for example, DES, PCBs, alkylphenols, triphenylethylenes, phytoestrogens, pesticides and others (Fig. 2.5). Xenoestrogens have quite different chemical structures compared to estradiol, yet they can bind to the ER and presumably activate receptor mediated processes.

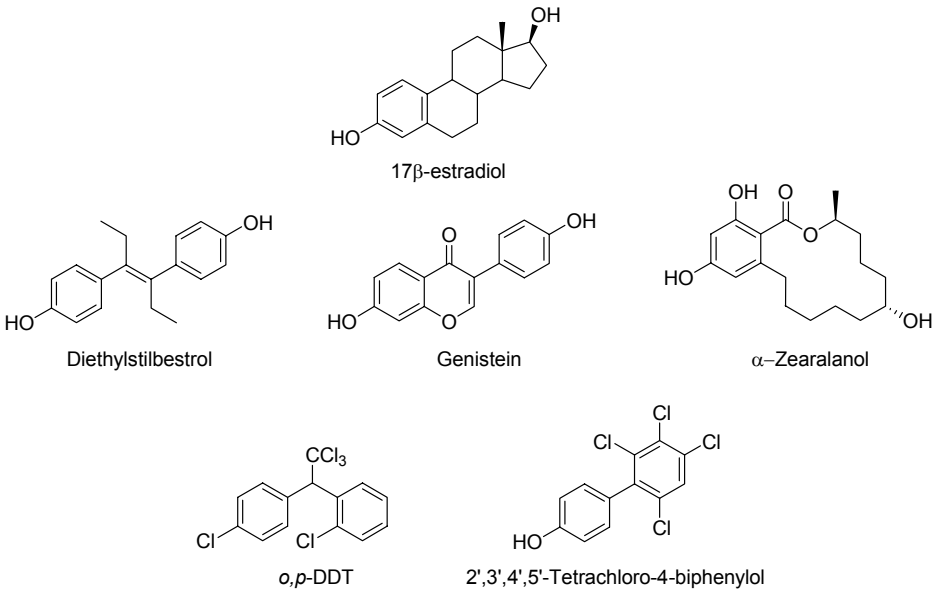


Figure 2.5. Examples of chemicals that bind to the ER.[53] All possess the main features required for ER binding, two electronegative groups separated by a hydrophobic region.

2.1.7 SAR of xenoestrogens

As a wide variety of natural and synthetic chemicals are capable of acting as hormone receptor agonists and antagonists, there is a need for screening techniques to aid in human health and environmental risk assessments. SAR could serve as a primary screening tool to prioritise chemicals, which potentially may act as estrogen mimics, for more intense and costly evaluations. There is a reality that there are not sufficient resources to carry out *in vitro* or *in vivo* assessments to screen for example, the existing 70,000 industrial organic chemicals (not including pharmaceuticals and pesticides), as well as the approximately 2000 new industrial chemicals submitted each year in the United States for evaluation under their Toxic Substances Control Act.[54] SAR and now QSAR (quantitative structure-activity relationships) are considered useful and credible tools for predicting the ecological effect and fate of chemicals when there is little experimental data available.

SAR are useful for predicting the potential estrogenicity of a chemical. Major efforts have been made to determine the structural requirements for a chemical to exhibit estrogen receptor binding, so the 'estrogenicity' of a chemical may be predicted. Fang *et al*. have completed a comprehensive SAR study into the binding of a large set of natural and synthetic xenoestrogens to the ER.[53] A total of 230 chemicals were investigated to develop structure-activity relationships. An ER binding assay was used to generate relative binding affinities for test chemicals, covering a 10^{-6} range. Five key features were deemed essential for estrogenic activity, and the more key structural features a chemical contained, the more active it was found to be. The key features were:

i) Hydrogen bonding ability of a phenolic ring mimicking 3-OH
ii) Distance between 3-OH and 17β-OH
iii) Steric hydrophobic groups at 7α- and 11β-positions
iv) Overall hydrophobicity
v) Rigid ring structure.

If a chemical contains a phenolic ring and other key features then it is likely to be an ER ligand. The binding potential for chemicals without a phenolic ring is dependent on other features, such as the presence of a heteroatom required for hydrogen bonding. Whilst this study has provided a lot of information about SAR for xenoestrogens and ER binding, it

fails to determine whether a chemical acts as an agonist or antagonist. However SAR studies, such as this, are essential in identifying important features so that potential xenoestrogens may be identified for further biological based analysis.

2.1.8 Methods for assessing estrogenicity

SAR are useful for predicting estrogenic compounds but they require further testing to determine their biological effect. Estrogens are defined by their biological activity, so bioassays must be used to identify them. There are several types of assays used to assess the estrogenicity of chemicals. These include *in-vitro* and *in-vivo* assays. Determining the different degrees of estrogenicity of xenoestrogens (potency) relative to estradiol is important in assessing their potential effects on animals, humans, and the environment.

In vitro **assays**

There are three main types of *in-vitro* assays used to determine the estrogenicity of a chemical. Each of them represents a step in the ER-mediated pathway of estrogen action.

i) Receptor binding assays which measure the affinity a chemical has for the ER.
ii) Receptor-dependent gene expression assays, which measure the ability of a chemical to stimulate gene expression and protein synthesis via binding to the ER.
iii) Cell proliferation assays, which measure a whole cell estrogenic response.

Receptor binding assays

A competitive binding assay measures the binding of radiolabelled estradiol in the presence of competing unlabelled ligand. It is a rapid method for determining the affinity of a chemical for the ER.[55] Receptors are obtained from various tissues (bovine, rat, or MCF-7 human breast cancer cell line), or as purified recombinant protein. If the test chemical competes with the labelled estradiol, then it will displace a fraction of estradiol.

A relative binding affinity is generated so the affinity of test chemicals for the receptor can be compared with each other and with estradiol.

Receptor-dependent gene expression assays

These assays are based on the ability of a test chemical to bind to the ER and activate DNA binding and transcription, leading to gene expression. Assays of this type include:

a) *Yeast based receptor assays* – yeast cells have been transfected with human ERα (hER) DNA, and EREs linked to the *lacZ* reporter gene.[56] This gene codes for the expression of β-galactosidase. If a test chemical binds to the ER and activates gene expression, then β-galactosidase is synthesised and released into the assay medium, where it cleaves a yellow galactoside dye (chlorophenol red β-D-galactopyranoside). Chlorophenol red is produced as a result, and can be measured spectrophotometrically. A luciferase reporter gene can also be used, the luminescent product of which can be measured. A yeast two-hybrid method has also been developed, which detects a test chemical-dependent interaction between ERα and a coactivator required for DNA transcription (coactivators interact with the ER LBD in the presence of estrogens).[57] β-Galactosidase activity is measured as an end-point.

(b) *Mammalian based receptor assays* – the cell either contains the ER (such as MCF-7 cells), or hER DNA is transfected into the cell via a vector, along with a reporter gene linked to EREs (such as in transfected HepG2 cells). These cells synthesise β-galactosidase or luciferase as a result of estrogen activated reporter gene expression, and their activity is measured.[58] MCF-7 cells have also been incubated with human and rat S9 or liver microsomes, which are capable of metabolising test compounds.[59]

HeLa cells have been transfected with a vector (pRSV) containing mouse ER cDNA, and an estrogen responsive vector (ERET81CAT) with EREs linked to a reporter gene coding for chloramphenicol acetyl transferase (CAT). Upon estrogenic activation, the amount of CAT produced is measured via the ELISA method.[55]

Ishikawa Var-1 human endometrial cancer cells are rich in ER and express the enzyme alkaline phosphatase in response to estrogens. This enzyme converts *p*-nitrophenyl phosphate (added to the exposed cells) to *p*-nitrophenol which is detected spectrophotometrically.[60]

The levels of pS2 mRNA in MCF-7 cells can be measured to determine estrogenic activity. The cells are exposed to test chemicals and the amount of pS2 mRNA produced in response to estrogen activity is measured via hybridising radiolabelled probes and northern analysis.[61]

Cell proliferation assays

The hallmark of physiological estrogen action is the proliferation of estrogen-responsive cells. Cell proliferation assays measure the ability of chemicals to induce cell proliferation as an end point of interaction with the ER. The proteins synthesised as a result of DNA transcription and translation effect cell growth. The E-Screen assay was developed by Soto *et al.* and is one of the most commonly used estrogenic assays.[62] In this assay MCF-7 cells (an estrogen responsive human breast cancer cell line) are used, and cells are counted upon incubation with a test chemical and compared to control cell growth.

***In vivo* assays**

Diverse animal models and assays have been utilised for determining estrogenic activity. *In vivo* assays are more meaningful as they allow the full physiological effects of estrogenicity to be observed, i.e. they allow interactions between different parts of the endocrine system in the animal, which is more comprehensive than just a particular cell type based assay. Assays of this type include:

i) Measuring the proliferative effect of estrogens in the female genital tract. For example the uterotrophic assay measures uterine weight in ovariectomised rodents.[63] Other estrogen endpoints are also measured, such as uterine and vaginal

epithelium height.[64] Ovariectomised mice or rats are used to minimise the effect of endogenous estrogens.

ii) Measuring the effect of xenoestrogens on testicular size and sperm quality in male rodents, including reduction in testicular size, reduction in ventral prostate weight, and reduction in sperm production in male rats.[65]

iii) Induction of vitellogenin in fish. Vitellogenin is a protein normally produced in response to estrogens in female fish. The induction of vitellogenin synthesis in male rainbow trout after exposure to xenoestrogens has been measured.[66]

2.1.9 Metabolism of xenoestrogens

SAR can be used to predict potential xenoestrogens for further biological analysis. However SAR do not take into account whether the chemical is an agonist or antagonist of the ER, and possible metabolic conversion in test assays and animals. For several chemicals, the products of their metabolism/environmental degradation, rather than the parent compound itself, are estrogen mimics. Compounds of this type include metabolites of phthalate plasticisers, PCBs and some insecticides.[67-69] The effect of metabolism might explain the 'hidden' estrogenicity of compounds whose activity is not detected in assays incapable of metabolising chemicals.

Information regarding a chemical's metabolism is important in assessing its toxic potential, including estrogenicity. Metabolism of chemicals occurs endogenously in animals, humans, and also in the environment (environmental degradation) via microbes. Chemicals can be converted to a more or less estrogenic form than the parent compound (see Fig. 2.6). These conversions involve Phase I oxidation reactions that are catalysed mainly by many of the CYP (cytochrome P450) family of enzymes. These oxidations are followed by Phase II conjugations of the oxidised moiety with highly polar groups such as glucose, sulphate, methionine, cysteine or glutathione, aiding in the excretion of hydrophobic molecules. These reactions occur endogenously in living organisms, as well as in the environment via microbes.

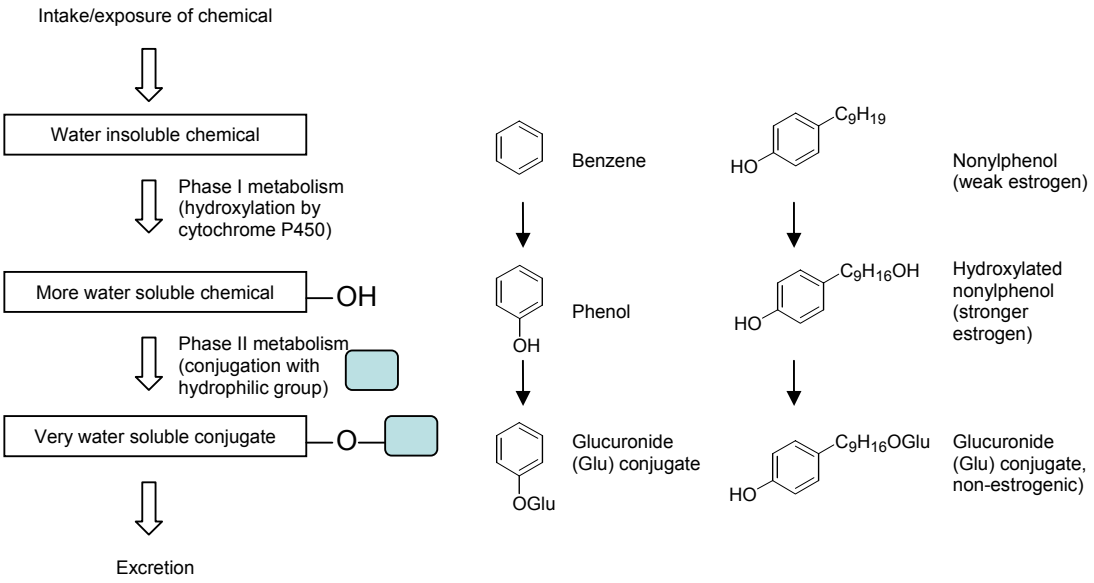


Figure 2.6. Cytochrome P450 catalysed metabolism of chemicals.

2.1.10 Pyrethroid insecticides

Insecticides are chemicals used to control insects that attack crops and other plants, and for domestic public health purposes. Concern has arisen that some insecticides may possess the ability to mimic the action of 17β-estradiol through interaction with the hER. An example is the association between the concentration of dicofol, and DDT and its metabolites, in Lake Apopka, Florida, USA, and developmental abnormalities in the lake alligators.[12,21,22]

There is evidence to indicate that exposure to pyrethroids may also cause effects linked to hER mediated responses.[60] Synthetic pyrethroids (such as those in Fig. 2.7) are commonly used insecticides around the world being the 4th major group used after organochlorines, organophosphates, and carbamates.[70] Pyrethroids are used in forestry, on vegetable and fruit crops, wheat and barley, targeting many types of insects.[71] Many of these are synthetic analogues of the naturally occurring pyrethrins found in the flowers of *Chrysanthemum cinerariaefolium*. They are highly toxic to insects, and the level of toxicity

greatly decreases in the order of amphibia, fish, mammals and birds. These compounds act on target insect nervous systems at sodium channels within lipophilic membranes. They act by stimulating Na^+ gate proteins to open and close, leading to depolarisation of the cell and therefore death by neurotransmission inhibition. In the past they have been widely regarded as safe with a favourable insect to mammalian toxicity ratio.

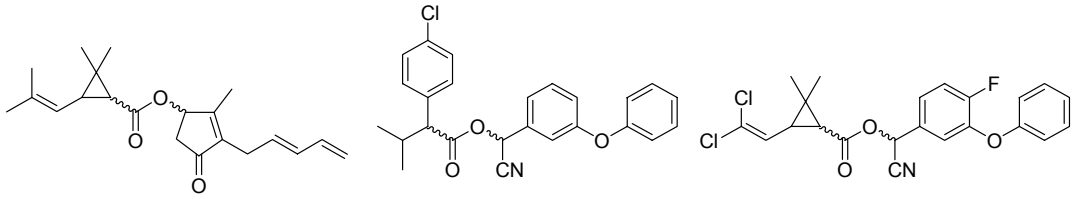


Figure 2.7. Examples of pyrethroid insecticides.

However, an increase in the awareness for xenoestrogens in the environment has led to the testing of pyrethroid insecticides for potential estrogenic activity by several different methods. Highly variable results have been reported for the activity of pyrethroids in receptor binding assays, receptor-dependent gene expression assays, or varying cell proliferation assays. Cypermethrin and permethrin are commonly used pyrethroids that have been tested by these methods; a summary of reported results is shown in Table 2.1.

	ER binding	Gene expression	Cell proliferation	*In-vivo* assay
Cypermethrin	NA[72], A[73]	NA[72], NA[74], NA[75], NA[59], NA[76], A[73]	NA[72], A[73]	
Permethrin	NA[72], A[73]	NA[72], NA[74], NA[60], NA[61], NA[59], NA[76], A[73], A[75]	NA[72], A[73], A[61]	NA[63]

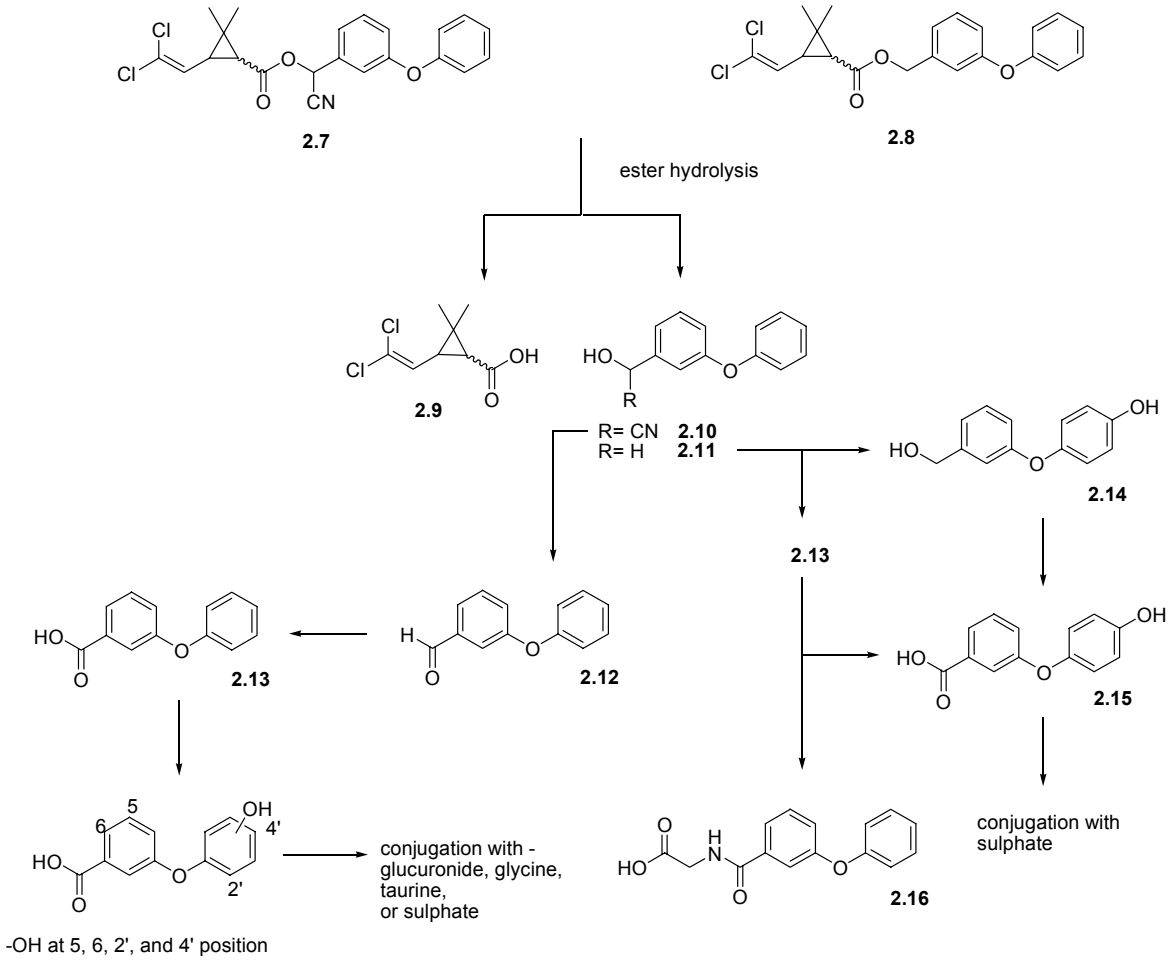
Table 2.1. Estrogenic activity of cypermethrin and permethrin in various estrogenicity assays (NA denotes no estrogenic activity observed, A denotes estrogenic activity).

Cypermethrin and permethrin were among the pyrethroids that Kim *et al*. tested using three assays (ER binding, pS2 mRNA from MCF-7 BUS cells, and E-screen).[72] Neither compound displayed any estrogenic activity compared to 17β-estradiol. Saito *et al*. also observed no significant estrogenicity when both pesticides were subjected to three *in-vitro* assays (ER binding, luciferase reporter gene, yeast two hybrid).[74] A study by Garey and Wolff revealed that permethrin did not enhance cell proliferation in an endometrial cell line.[60] Go *et al*. found that permethrin did not stimulate pS2 (estrogen responsive gene) mRNA levels in MCF-7 cells.[61] In an *in-vivo* assay (uterotrophic), Kunimatsu *et al*. saw no increase in ovariectomised rat uterine weight when orally exposed to permethrin.[63] Tyler *et al*. tested both cypermethrin and permethrin in the yeast receptor assay. Permethrin displayed weak agonistic activity while cypermethrin did not.[75] Sumida *et al*. tested both also in a reporter gene assay (MCF-7 and HeLa cells with a luciferase endpoint).[59] Neither was found to be estrogenic. Kojima *et al*. tested both pyrethroids in a luciferase reporter gene assay. Neither displayed activity in this assay. [76]

Several results reported are contrary to the above. Chen *et al*. tested both pyrethroids in three types of assay (E-screen, ER binding, and pS2 expression).[73] Both were active in all

three assays. Go *et al*. reported that permethrin had a noticeable effect on cell proliferation in MCF-7 cells.[61]

Pyrethroid insecticides are readily metabolised and environmentally degraded from the parent compound into smaller compounds. Cypermethrin (**2.7**) and permethrin (**2.8**) have similar mammalian metabolic pathways (Scheme 2.2), which produce metabolites with chemical similarity to 17β-estradiol.



Scheme 2.2. Mammalian metabolism of cypermethrin (**2.7**) and permethrin (**2.8**).[77]

The pathways of metabolism and degradation in insects, fish, birds and mammalian animals are well understood.[70,77] The metabolism of permethrin (**2.8**) and cypermethrin (**2.7**) follow a very similar route. The ester bond of the parent insecticide is enzymatically cleaved by an oxidase for the *cis* disubstituted cyclopropane of permethrin, and an esterase for the *trans*. Both isomers of cypermethrin are hydrolysed by an esterase. The resulting cyclopropyl acids in both series are then further metabolised and conjugated with glucuronide or glycine moieties to aid in excretion. The cyanohydrin **2.10** and the aromatic alcohol **2.11** are oxidised and hydroxylated in different positions as shown in Scheme 2.2. These hydroxylated compounds are finally conjugated with glucuronide, glycine, taurine or sulphate groups. This can occur at either the carboxyl or hydroxyl groups.

Although the parent compounds show little obvious structural resemblance to 17β-estradiol, and limited estrogenicity as discussed, the metabolites are potentially more estrogenic based on proposed SAR.[53] To study whether metabolites are more active than the parent compound, an assay with metabolic capability needs to be utilised, or the metabolites themselves can be tested. Kojima *et al*. recently found that permethrin had weak estrogenic activity after exposure to rat liver S9 homogenate (it was inactive before exposure), suggesting the products of permethrin metabolism were more estrogenic than permethrin itself.[76] The products of metabolism were not identified. Only one paper to date has examined the ability of three permethrin metabolites to activate the estrogen receptor.[75] The cyclopropyl acid **2.9** was inactive, whereas 3-phenoxybenzyl alcohol (**2.11**) was weakly estrogenic (10^5 fold less potent than 17β-estradiol). 3-Phenoxybenzoic acid (**2.13**) was also analysed, and found to behave as an antiestrogen (antagonist) by blocking the action of estradiol in an anti-estrogen screen. The cyclopropyl acid **2.9** also showed anti-estrogenic behaviour. However metabolites **2.14** and **2.15**, which are potentially more estrogenic than **2.9**, **2.11**, and **2.13**, were not investigated. Known SAR,[53] suggest that the 3-(4-hydroxyphenoxy)benzyl alcohol (**2.14**) metabolite of permethrin would be more active in the yeast assay than **2.11**, due to the presence of the aromatic hydroxyl group which is known to be favoured for receptor binding. 3-(4-Hydroxyphenoxy)benzoic acid (**2.15**) (metabolite of both permethrin and cypermethrin) also contains an aromatic hydroxyl, suggesting a potential greater estrogenicity than **2.13** also.

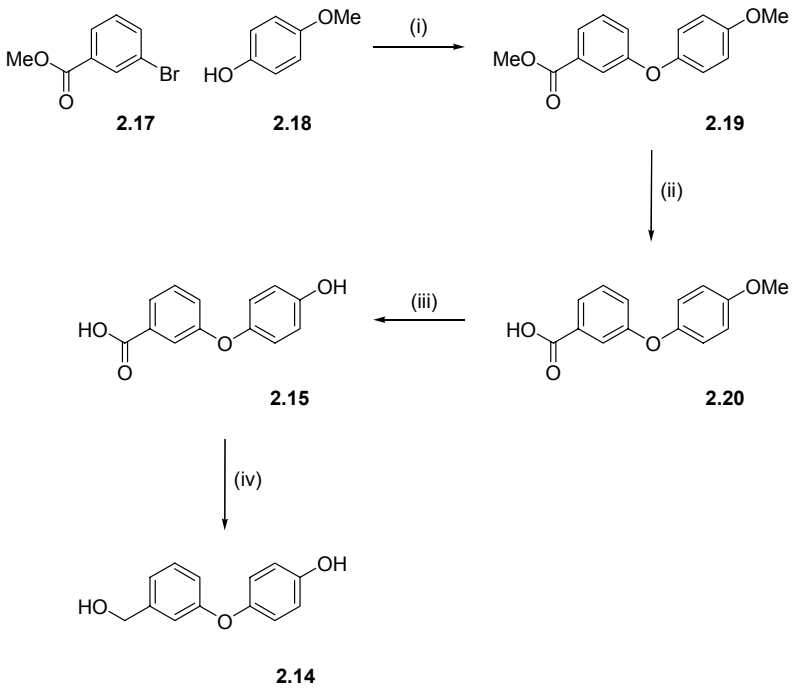
The lack of data on the estrogenicity of permethrin and cypermethrin metabolites stimulated the research described in this chapter, which concerns the synthesis and estrogenicity of other cypermethrin and permethrin metabolites not tested by Tyler *et al*. For the reasons discussed in the previous paragraph, metabolites **2.14**, **2.15**, *N*-3-(phenoxybenzoyl)glycine (**2.16**), and 3-phenoxybenzaldehyde (**2.12**) were chosen for exposure to a yeast based receptor assay (see section 2.1.8) to determine their estrogenicity, along with previously tested metabolites 3-phenoxybenzyl alcohol (**2.11**), and 3-phenoxybenzoic acid (**2.13**). Metabolite **2.16** is a glycine conjugate of **2.13**, which is biosynthesised in the final step of permethrin metabolism. Conjugation with a glycine molecule increases the molecular length of the metabolite, so it was included in the assay for investigations into the relationship between the molecular dimensions of the metabolites and their estrogenic activity. The synthesis of three metabolites, **2.14-2.16**, was required before they could be assayed. Their synthesis is discussed in section 2.2. Metabolites **2.11**, **2.12**, and **2.13** were purchased from SigmaAldrich. The estrogenicity of these six metabolites was determined with a yeast based reporter assay which detects estrogen-dependent gene expression. Details of the assay and results will be discussed in section 2.3. The estrogenicity of metabolites **2.12**, **2.14**, **2.15**, and **2.16** has not previously been reported.

2.2 SYNTHESIS OF PYRETHROID METABOLITES

The following section describes the synthesis of pyrethroid metabolites **2.14** and **2.15** (Scheme 2.3) which was guided by the synthesis of Unai and Casida.[78] The synthesis of *N*-(3-phenoxybenzoyl)glycine (**2.16**) is discussed in section 2.2.2.

2.2.1 Synthesis of metabolites 2.14 and 2.15

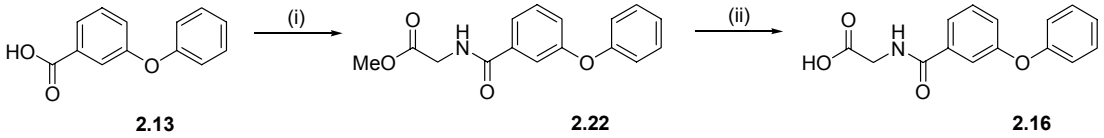
The first step in the reaction scheme (Scheme 2.3) involved an Ullman coupling between methyl-3-bromobenzoate (**2.17**) and the sodium salt of *p*-methoxyphenol (**2.18**) in DMF in the presence of copper(I)chloride to give the key biaryl ether intermediate **2.19** in 31% yield. Hydrolysis of the methyl ester of **2.19** was accomplished with potassium hydroxide in equal volumes of THF and water to give the acid **2.20** in 90% yield. The methyl aryl ether of **2.20** was cleaved with equal volumes of aqueous hydrobromic acid and acetic acid to give the product (pyrethroid metabolite) **2.15** in 88% yield. The acid group of **2.15** was reduced with lithium aluminium hydride in ether to give pyrethroid metabolite **2.14** in 33% yield.



Scheme 2.3. *Reagents and conditions:* (i) NaH, DMF, rt, 30 min, then CuCl, reflux, 6 h (31%). (ii) KOH, 1:1 THF in H_2O, reflux, 4 h (90%). (iii) 1:1 48% aq. HBr in AcOH, reflux, 7 h (88%). (iv) $LiAlH_4$, Et_2O, rt, 16 h (33%).

2.2.2 Synthesis of glycine-conjugated metabolite 2.16

Scheme 2.4 outlines the synthesis of **2.16**.[79] 3-Phenoxybenzoic acid (**2.13**) was reacted with glycine methyl ester hydrochloride in DCM under EDCI coupling conditions to give the ester **2.22** in 85% yield. Hydrolysis of the methyl ester of **2.22** was carried out in THF and water in the presence of potassium hydroxide to give the glycine conjugate **2.16** in 65% yield.



2.13 2.22 2.16

Scheme 2.4. *Reagents and conditions:* (i) DIPEA, GlyOMe.HCl, EDCI HOBt, DCM, rt, 16 h (85%). (ii) KOH, 1:1 THF in H_2O, reflux, 4 h (65%).

2.2.3 HPLC analysis of metabolites

Assessing the purity of metabolites for assay was important to avoid false results that may have arisen from the presence of estrogenic impurities. Analytical HPLC was performed to assess the purity of synthesised metabolites **2.14** and **2.15**. Samples of these compounds were run through an analytical C-18 reverse phase column eluting with various concentrations of water (containing 0.05% trifluoroacetic acid) in acetonitrile. Figure 2.8 shows the HPLC trace of metabolites **2.14** and **2.15**.

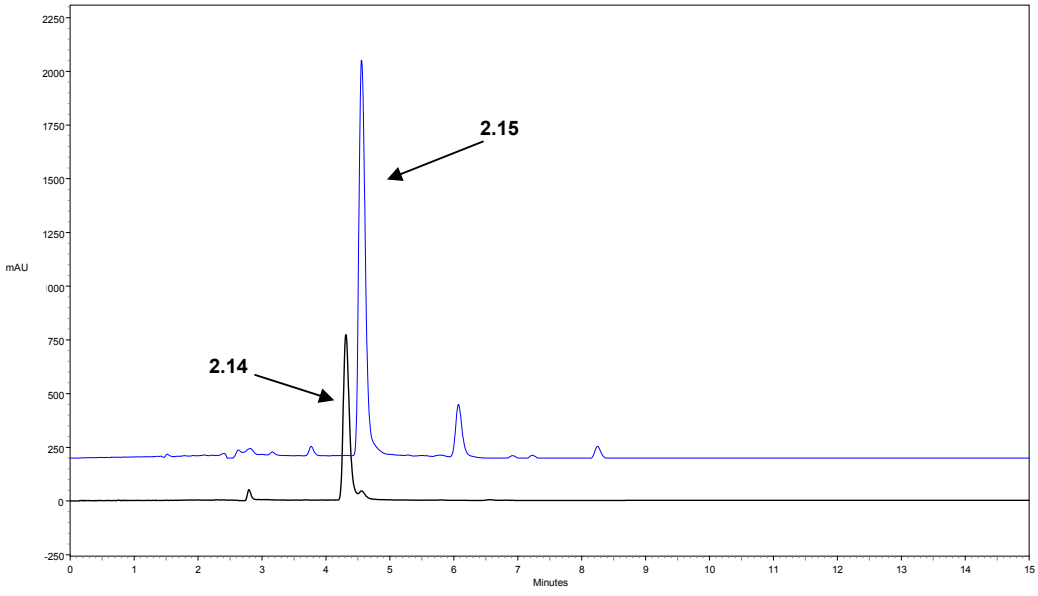


Figure 2.8. HPLC analysis of synthesised metabolites **2.14** (black trace) and **2.15** (blue trace). The metabolites were detected by absorbance at 254 nm.

Two of the synthesised metabolites, **2.14** and **2.15**, were analysed by HPLC to investigate their purity. Further purification (preparative HPLC) was planned for metabolites found to contain any impurities so the possibility of false positive results could be avoided. The black trace in Figure 2.8 is comprised of one major peak and low levels of impurities. The major peak was assumed to be metabolite **2.14** and this was confirmed by the ^{1}H and ^{13}C NMR spectra of **2.14**, in which only **2.14** was observed (see Chapter Four, section 4.2.1). The blue trace in Figure 2.8 is comprised of one major peak and several impurities. The major peak was assumed to be metabolite **2.15** and this was confirmed by the ^{1}H and ^{13}C NMR spectra of **2.15** (see Chapter Four, section 4.2.1). In the YES screen, metabolite **2.14** was active, whilst metabolite **2.15** was not (the assay and results will be discussed in the following section). The negative result for **2.15** suggests that it was not estrogenic, and thus was not purified further, but there is a possibility that the impurities present may be antagonists of the hER and thus block its activation. However, this possibility is remote as ER antagonists are usually large molecules, e.g. tamoxifen, which possesses three aromatic rings (see Chapter One, Fig. 1.5). The very slight shoulder of the peak corresponding to **2.14** was assumed to be **2.15** by comparison with the blue trace (**2.15** was the precursor in the synthesis of **2.14**). As **2.15** was inactive, **2.14** was not purified further. The purity of metabolite **2.16** was not analysed by HPLC as it was inactive in the YES screen.

2.3 YEAST ESTROGEN SCREEN (YES)

As previously discussed (in section 2.1.8) there are different assays available for assessing the estrogenicity of a chemical. *In vitro* assays include receptor binding, yeast and mammalian cell reporter gene, and cell proliferation. *In vivo* assays include the rodent uterotrophic assay, measuring the effects on male rodent testicular size and sperm quality, and the induction of vitellogenin in trout. A yeast based reporter gene assay was chosen for this research, as it not only determines whether a chemical binds to hERα, but also if estrogen-dependent gene expression is stimulated. Yeast (*Saccharomyces cerevisiae*) has proven to be a good model for studying more complex eukaryotic processes, such as steroid receptor function. The advantages of using yeast include the relatively short culture period, and the ability to use well defined culture conditions. Yeast based assays are considered robust, sensitive and relatively inexpensive to screen chemicals for *in vitro* estrogenic activity.

The yeast estrogen screen (YES) developed by Arnold *et al*.[56] (depicted in Fig. 2.9) was used in this research. The yeast is recombinant and was developed by integrating the DNA sequence for the hERα into the main chromosome of *Saccharomyces cerevisiae*. The recombinant yeast cells also contain a plasmid that possesses the *lacZ* reporter gene. This gene codes for the expression of β-galactosidase, an enzyme which modifies a galactoside dye added to the assay medium [see (i), Fig. 2.9]. The binding of a compound to hERα results in a complex that binds to EREs on the plasmid. β-Galactosidase is expressed and secreted into the assay medium, where it modifies the yellow galactoside dye, chlorophenol red β-galactopyranoside (CPRG). CPRG is converted to chlorophenol red by enzymatic activity [see (ii), Fig. 2.9], and the release of chlorophenol red can be monitored spectrophotometrically at 540nm. A background reading at 620nm is taken to record the optical density of the assay solution (reflecting the yeast growth). The absorbance of chlorophenol red measured at 540 nm is indicative of the degree of ER binding by the test chemical. Sigmoidal 'S' curves (typical for ligand-receptor binding) are obtained when the absorbance at 540 nm is plotted against a range of different concentrations of estrogenic chemical. The relative responses of different chemicals in the yeast assay can be compared by taking a concentration value at a common point on the curve. This value makes it

possible to measure the relative estrogenicity of one compound to another. Therefore, an $EC5_{50}$ value is generated for estrogenic chemicals, and is defined as the statistically derived molar concentration of a test chemical (agonist) which generates 50% of the maximum possible response for that chemical (in this case absorbance). Data points obtained from the assay are fitted to a curve determined by a computer programme, and the mathematical equation for the fit curve is used to calculate the concentration of estrogenic chemical (EC_{50}) where the absorbance is half the maximum value obtained.

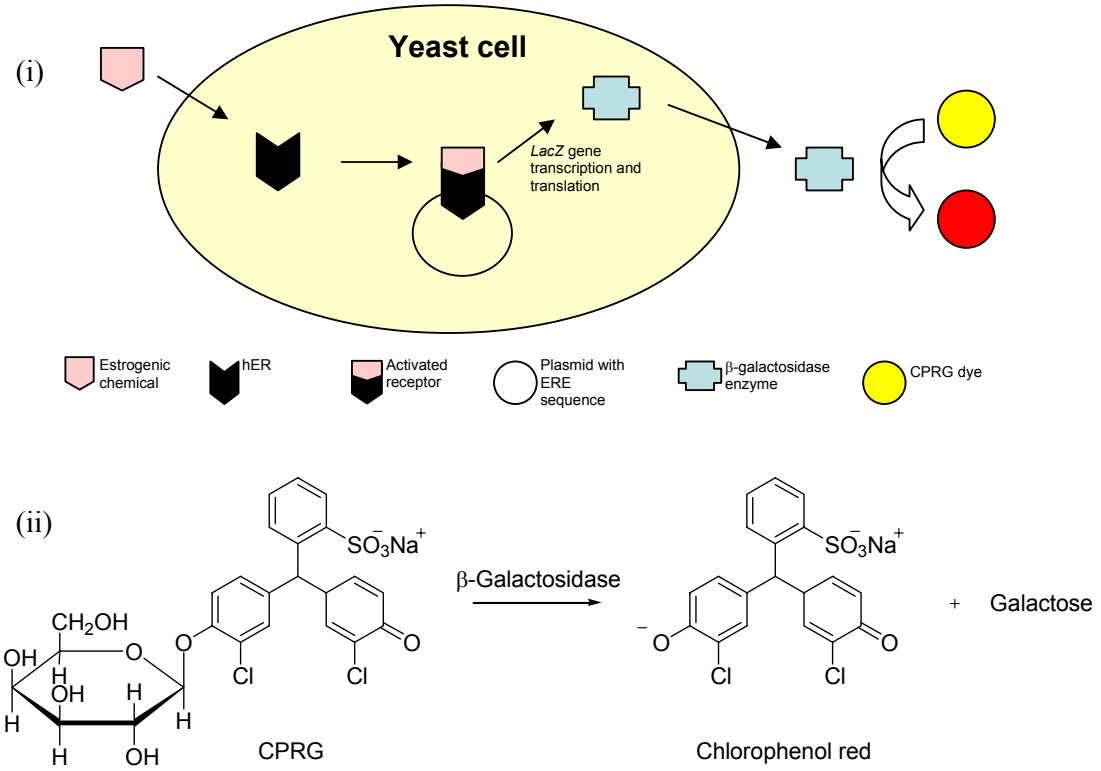


Figure 2.9. (i) Schematic of yeast estrogen screen:

Estrogenic chemical binds to hER and the complex binds to EREs on the plasmid, initiating synthesis of β-galactosidase enzyme, which cleaves the CPRG substrate in the assay medium, resulting in a yellow to red colour change.

(ii) β-galactosidase catalysed cleavage of CPRG substrate.

2.3.1 Yeast preparation

The recombinant yeast was donated by John Sumpter of Brunel University, UK. All preparation and storage of the yeast, and assay procedures were followed from those described by Routledge and Sumpter.[80] Personal communications with the Sumpter research group also provided more detailed procedures. Five vials of agar slopes containing the yeast were obtained, and they were each recultured on to agar plates, and two samples from each vial were transferred to cryovials with sterile glycerol and frozen at -80°C.

To prepare the yeast as required for assay, a 10 x concentrated yeast stock was prepared (see Chapter Four section 4.2.2). This involved growing the yeast for 3 days (when it became turbid). A small aliquot of this yeast was transferred to two flasks of yeast growth medium, in which the yeast was grown separately for a further 24 h. Both cultures were centrifuged and re-suspended in media and sterile glycerol for storage at -20°C.

2.3.2 Yeast assay establishment

The yeast assay required establishing and validation, due to differences that might arise when biological systems are in new hands. Initial assays were carried out to check results were comparable with literature values (see Chapter 4, section 4.2.2 for assay procedure). Early work on establishing the assay was carried out in collaboration with Dr Barbara Thomson at ESR (Institute of Environmental Science and Research). Briefly, the assay involves incubating test chemicals in a solution of yeast, growth media and CPRG. 17β-Estradiol was used as the standard and included on each assay plate. A blank control row was also included on each plate, each well in the row consisted of the yeast, growth media and ethanol (no test chemical). Two known xenoestrogens (genistein and daidzein) were included in initial assays, to be used for further validation of the assay. In the protocol followed, the optical density of the yeast culture in the assay plates was recorded at 620 nm. The plate reader used for this research required fixed wavelength light filters, and only several different wavelength filters existed for use. Amongst these filters was a 610 nm filter so this wavelength was used to record the optical density of the assay plates, instead

of at 620 nm as described in the protocol. A 540 nm filter was purchased specifically for this assay out of necessity. Early assay attempts were unsuccessful. Upon removal from the incubator after 48 h (our standard assay time), high absorbances at 540 nm (wells were observed to be an intense red colour) were recorded for all wells containing test chemicals, indicating significant production of chlorophenol red as a result of hERα activation. Even the row of blank wells with no test chemical present displayed estrogenic activity, suggesting there was estrogenic contamination of some sort present in the assay.

2.3.2.1 Experiments in detecting source of assay contamination

Assay runs were designed to investigate why the all the well solutions in assays performed so far were displaying a high absorbance at 540 nm. Estrogenic contamination was suspected to be the cause. The yeast minimal media was identified first as a possible source of estrogenic contamination. Therefore a new batch of yeast minimal media was prepared. An assay was then attempted using the new media, with a new control included. This control contained growth media and yeast, as well as the CPRG dye, to assess the possibility there were microbes in the media also exhibiting β-galactosidase activity. After 48 h, high absorbances at 540 nm were again recorded, except for the new control wells which remained yellow. The presence of wells displaying high absorbance at 540nm indicated there was still an estrogenic contaminant. The yellow control wells indicated that there were no microbes with β-galactosidase activity present.

Further sources of the estrogenic contamination were postulated. The yeast culture was considered next. A new 10 x concentrated yeast stock was prepared and an assay run. Again high absorbances at 540 nm were recorded for all well solutions on the plate. The next possible source of contamination considered was the CPRG dye. The dye solution was stored in plastic tubes, and it was thought that the solution may be contaminated with plasticisers. Phthalate compounds are used as plasticisers and have been shown to be estrogenic in the YES by Harris *et al*.[81] Despite awareness for this potential problem, it was thought that small plastic tubes would be suitable for storing the aqueous dye solution. We didn't think this would promote plasticiser leaching. However, some interesting results were obtained when the next assay was run with CPRG stored in glass vials (Fig. 2.10).

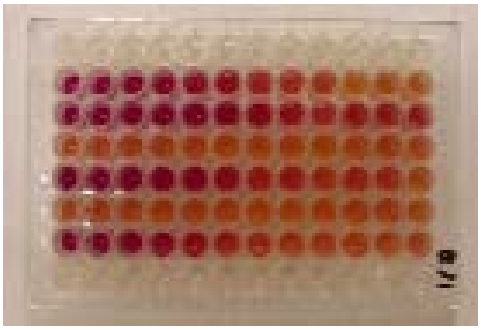


Figure 2.10. Assay plate comparing CPRG stored in plastic and glass vials.

Key to rows: A - empty

B - estradiol (CPRG in glass)

C – estradiol (CPRG in plastic)

D – ethanol blank

E – 10 x estradiol dilution (to check sensitivity; CPRG in glass)

F – genistein (CPRG in plastic)

When rows B and C were compared, it appeared that the CPRG stored in plastic vials was contaminated with an estrogenic substance, most likely plasticiser. This result also prompted us to store the 10 x concentrated yeast stock in glass tubes as well.

From the results obtained in the previous assay, it seemed the contamination source had been identified. Therefore, selected pyrethroid metabolites (**2.11**, **2.13**, and **2.14**) were introduced into the next assay run. However, high absorbances at 540 nm were recorded for all wells after 48 h again. We then tested for the possibility that the ethanol used to dissolve the test chemicals was contaminated, by incubating a sample containing only growth medium, yeast and dye in a sterilised glass bottle. This sample also displayed high absorbance at 540 nm, without ethanol, which was therefore ruled out as a contamination source.

From the results above, it became clear that the CPRG was not the only problem affecting the assay. The effect of the amount of yeast added to the assay media was investigated

next. The addition of too many yeast cells to the assay was a likely explanation for the over-activity observed in the attempted assays as described. A review of the literature associated with the assay procedure revealed that the optical density (OD) at 640 nm of the yeast assay culture was important. The optical density of a solution was used to determine the number of microbes in a culture solution. Microbes present in a solution are able to scatter light, and a spectrophotometer was used to measure the amount of light that reaches the detector upon passing through a culture solution. Therefore the number of cells present is proportional to the optical density of the culture solution (the optical density will increase as the concentration of cells increases). The optical density is measured at a particular wavelength of light that minimises absorption by biomolecules in microbial cells; around 600 nm is generally suitable. The assay protocol followed stated that between 0.5 mL and 2 mL of yeast culture or 4 x 10^7 cells was required to seed the assay medium. To investigate the concentration of yeast cells in our yeast culture, the number of yeast cells in a 24 h assay culture was counted by Nicola Turner of ESR at three time intervals over 24 h and the OD_{640} measured concurrently. The results are shown in Figure 2.11. After 24 h of growth the yeast was found to be in the stationary growth phase with an optical density of 2.5.

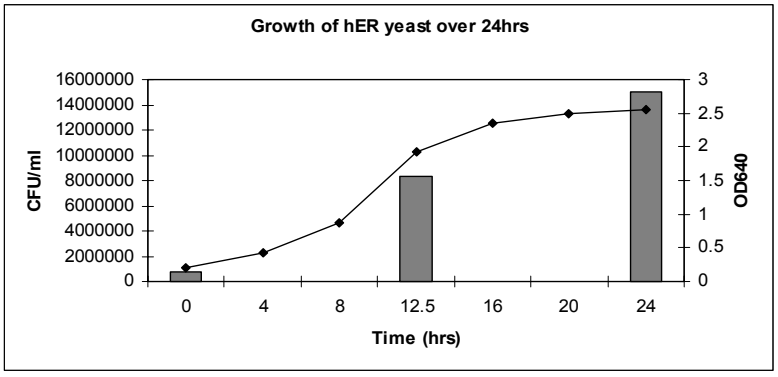


Figure 2.11. Growth of hER yeast over 24 h. Bars indicate CFU/mL and data points indicate OD at 640 nm.

The 24 h yeast culture contained 1.5 x 10^7 colony forming units (CFU) per mL which corresponded to an optical density of 2.5 at 640 nm. In the attempted assays described previously, 1 mL of 24 h yeast culture was added to the assay medium (which corresponds to about 1.5 x 10^7 cells as guided by the information in Figure 2.11). Although this is about the same number of cells as suggested by the protocol followed, high absorbances were obtained at 540 nm for all the assay well solutions (indicating significant chlorophenol red production). Therefore it was decided to add slightly fewer cells than the suggested number, so 0.25 mL of yeast culture (which corresponds to 3.75 x 10^6 cells according to the cell count in Fig. 2.11) was added to the assay medium from this point on. The addition of fewer cells to the assay medium had a profound effect on subsequent assays. For assay wells containing test chemicals, sensible absorbances at 540 nm (between 1 and 2.5) were obtained, and the blank control wells (with no test chemical present) appeared yellow and displayed low absorbance at 540 nm as desired. From this point on, the OD of the 24 h yeast culture at 640 nm was measured before each assay. By monitoring the OD of the culture solution, the number of cells being added to the assay could be controlled.

2.3.3 Activity of pyrethroid metabolites 2.11-2.16 in yeast assay

A total of 12 various assay runs were done before reproducibility was obtained and the plate wells were no longer all turning red. We were hopeful that reasonable results could now be obtained from assaying the metabolites. Standard solutions of metabolites in AnalaR ethanol were prepared at a concentration of 2 x 10^{-2} M as suggested by the protocol. Estradiol was prepared at a concentration of 2 x 10^{-7} M. As estrogenic potency is different for different compounds, varying concentrations of starting solutions are needed to generate a spread of absorbance readings. Therefore chemicals were added to the assay plates and diluted by a factor of 2 sequentially across rows, giving a range of 12 concentrations. One row was left between each chemical to prevent the creeping effect described by Beresford *et al.*[82] The ethanol was allowed to evaporate and then 200 μL assay media was added. The final concentrations for estradiol ranged from 0.0048 nM to 10 nM, and for the metabolites, 0.488 μM to 1000 μM.

Of the metabolites tested, **2.15**, **2.14**, and **2.16** were synthesised, while **2.11**, **2.12**, and **2.13** were purchased from Sigma-Aldrich. Each of these six metabolites was assayed at least 4 times to assess variation between assays and the reproducibility of data. To show variation between assays, data from four estradiol samples from different assay runs are presented in Figure 2.12.

2.3.3.1 Assay results

The following graphs (Fig. 2.13) were constructed from assay data. Plates were read in a plate reader after 48 h of growth in a 30˚C incubator. Absorbances at 540 nm and 610 nm were recorded. The 540 nm absorbance was corrected for by the following equation:
Corrected absorbance = chem. abs. (540) – [chem. abs. (610) – blank abs. (610)].
This equation corrects for absorbance arising from the turbidity of the assay solution due to the presence of yeast cells. The corrected absorbance values are plotted against the range of concentrations for each metabolite as shown in Figure 2.13 (the plots are semi-log). Typical (ligand-receptor binding) sigmoidal 'S' curves were obtained for the estradiol standard.

EC_{50} values were calculated for estradiol and the estrogenic metabolites, **2.11**, **2.12**, and **2.14**. These values were determined using SigmaPlot and Microsoft Excel (see Chapter Four, section 4.2.2). The reported EC_{50} for each chemical was an average of EC_{50} values calculated from various assay runs. A relative potency for each chemical was calculated by dividing the EC_{50} by the estradiol EC_{50} and inversing the obtained number. This was done to report the relative estrogenic activity of the metabolites as less than estradiol (Table 2.2).

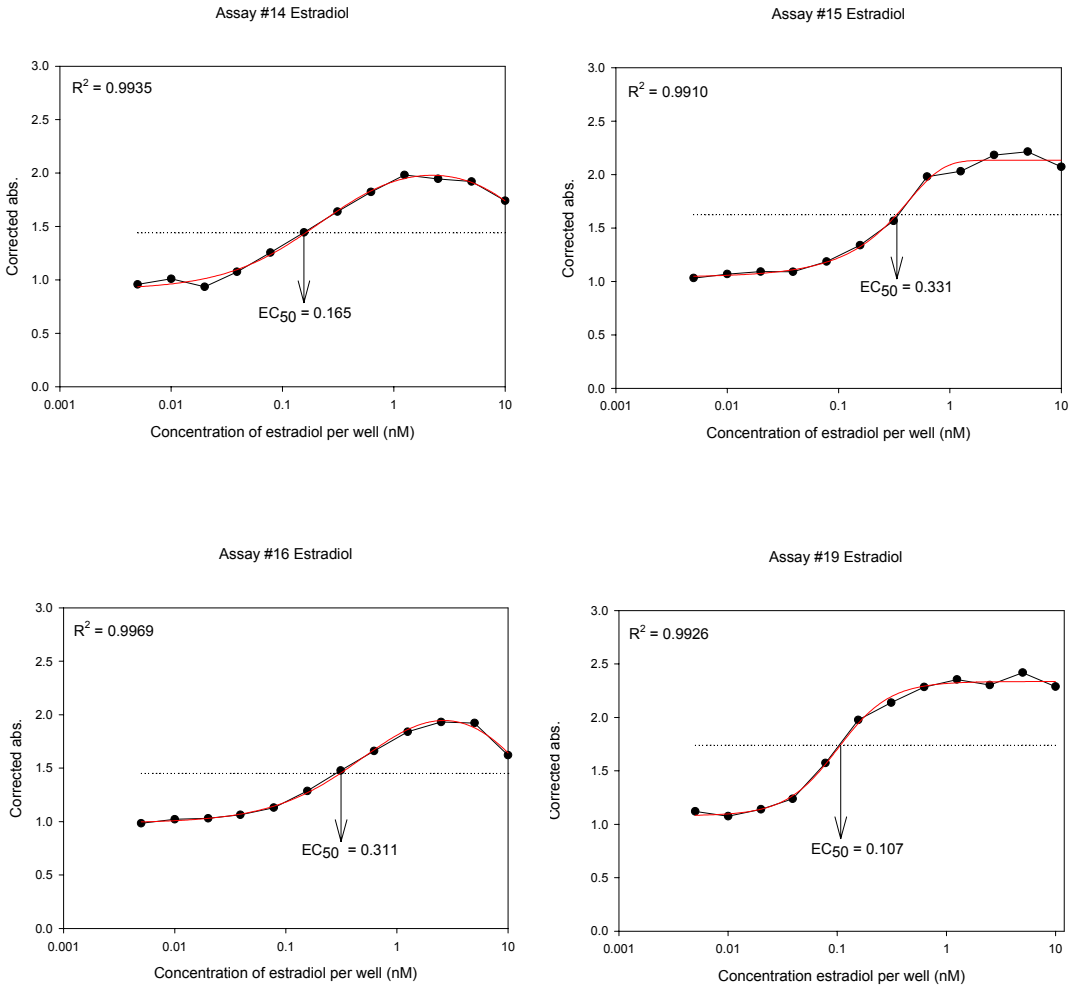


Figure 2.12. 17β-Estradiol EC_{50} values calculated from different assays.

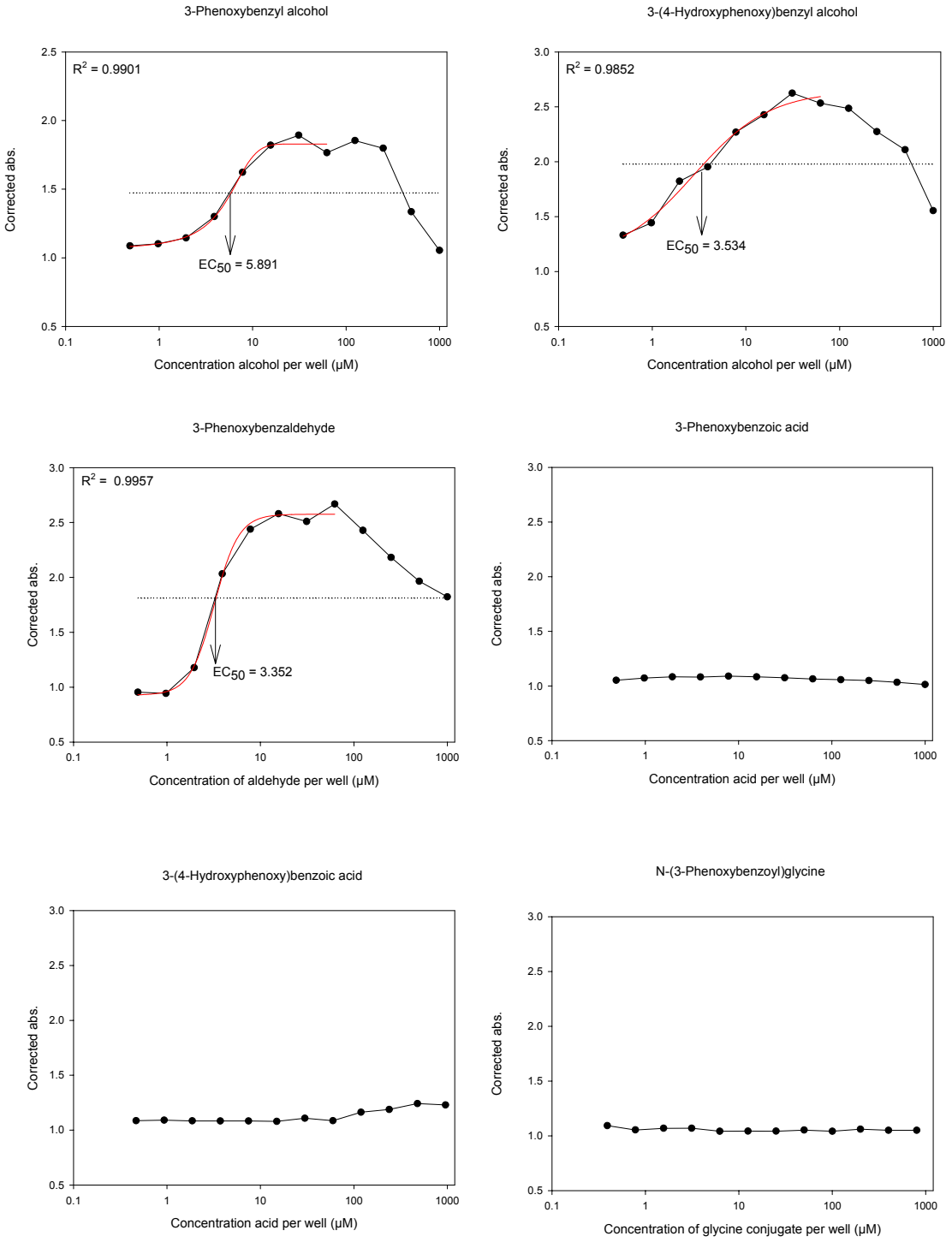


Figure 2.13. Estrogenic activity of pyrethroid metabolites determined by the YES screen (data from sample assays).

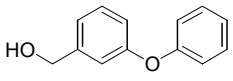
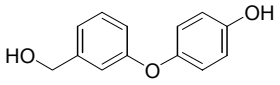
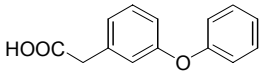
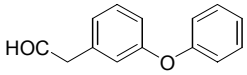
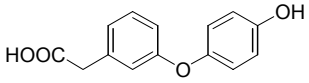
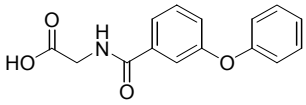
Pyrethroid Metabolite	Estrogenic Activity EC_{50} (µM)	Relative Potency (Estradiol=1)
 **2.11**	6.67 ± 3.11	$3x10^{-5}$
 **2.14**	6.75 ± 2.28	$3x10^{-5}$
 **2.13**	NA	-
 **2.12**	4.32 ± 1.70	$5x10^{-5}$
 **2.15**	NA	-
 **2.16**	NA	-

Table 2.2. Estrogenic activity (relative potency) of pyrethroid metabolites measured by calculated EC_{50} values [NA denotes no estrogenicity detected at a concentration less than the minimum concentration of 17β-estradiol (5×10^{-3} nM)]. The activity of 17β-estradiol was 0.229 ± 0.11 nM (mean ± SD).

Assay #	17β-Estradiol EC_{50} (nM)
14	0.165
15	0.331
16	0.311
19	0.107
	Mean = 0.229
	SD = 0.11

Table 2.3. Mean calculated EC_{50} for 17β-estradiol.

Yeast assay protocol	17β-Estradiol EC_{50} (nM)
This thesis	0.229
Tyler *et al.*[75]	0.21
Beresford *et al.*[82]	0.22
Gaido *et al.*[83]	0.225

Table 2.4. Comparison of 17β-estradiol EC_{50} with those from the literature.

Table 2.2 illustrates that **2.11**, **2.14** and **2.12** have weak estrogenic activity of five orders of magnitude less than estradiol. Metabolites **2.13**, **2.15**, and **2.16** show no activity (see graphs in Fig. 2.13). To validate the assay, the estradiol EC_{50} determined here was compared to values reported in the literature (Table 2.4). Our EC_{50} value is in excellent agreement with literature data.

The assay concentration for the test chemicals was quite high (the highest concentration was 1000 μM), and this is reflected in the graphical curves obtained for the estrogenic metabolites (**2.11**, **2.14**, and **2.12,** Fig. 2.13). Instead of the sigmoidal curve seen for the estradiol standard, the metabolite curves rise in absorbance and then fall as the concentration increases. This may indicate that the metabolite concentration was high

enough to kill the yeast. Yeast growth in this assay is shown by the turbidity (yeast cell concentration) of the solution, which was measured at 610 nm for all wells containing test chemicals. The absorbances measured at 610 nm for the highest concentration of metabolites were often very low due to the absence of turbidity, shown by clear yellow wells which indicated the cells were lysed. These cells are non-viable and therefore not synthesising β-galactosidase, so chlorophenol red is not produced as a result. Lysed cells present in wells containing high concentrations of the active metabolites displayed low absorbances at 540 nm, hence the observed decline in absorbance with increasing chemical concentration (e.g. **2.11**, Fig. 2.13). Therefore, in determining an EC_{50} for each of the active metabolites, only the first eight data points were fitted to a curve in SigmaPlot (as described above), as these points formed a sigmoidal curve characteristic of ligand-receptor binding.

Lysed cells in the first 2 or 3 wells of rows on the assay plates (chemicals were present in decreasing concentration across the plate) indicated that the assay concentration was too high in these wells and toxic to the yeast. This result suggested that the test chemical concentration needed to be diluted to reduce yeast cell toxicity. Each well across the row represents a subsequent decrease in concentration by 0.5. From about the third well reasonable absorbances were measured, so the three estrogenic metabolites were diluted by 0.1 to give a concentration similar to the concentration of the chemical in the third well.

2.4 DISCUSSION

In order to determine the estrogenicity of pyrethroid metabolites, either the pyrethroid needs to be tested in an assay with metabolic capability, or the actual metabolites need to be tested in an assay such as YES. Six metabolites (three of which were synthesised in this work) of cypermethrin and permethrin were tested with a recombinant YES expressing hERα. Estrogenic activity resulted from the test chemical forming a complex with hER, which binds to EREs found on the plasmid present in the yeast cell. Binding of the ligand-receptor complex to EREs induces *lacZ* expression and synthesis of β-galactosidase. Activity of this enzyme was measured by recording the absorbance of chlorophenol red at 540 nm.

The yeast assay required validation due to differences that might arise with biological systems in different circumstances. Initially the assay was not reproducible (the test chemicals and blank appeared bright red on the assay plate after 48 h) due to various problems as discussed in section 2.3.2.1; contamination of the CPRG solution stored in particular plastic tubes by an estrogenic compound (most likely plasticiser), and by the addition of what seemed to be too many yeast cells (presumably leading to over-expression of β-galactosidase, hence the high absorbance recorded at 540 nm for all well solutions). A cell count of the 24 h yeast culture was carried out and it was determined that there were 1.5×10^7 CFU per mL of culture, corresponding to an OD_{640} of 2.5. This result revealed we were not adding to many yeast cells, but due to the over-activity observed in assays performed before the cell count, it was decided to add slightly fewer cells than the protocol suggested (4×10^7 cells). In subsequent assays, 0.25 mL of 24 h yeast culture (containing 3.75×10^6 cells) was added to the assay medium, resulting in sensible absorbance responses. The yeast culture in our hands seems to be more active, as fewer cells provided similar responses to literature reports. The number of hERs being expressed by the yeast cell may be higher which might explain these observations. To validate the assay, the EC_{50} of the assay standard, 17β-estradiol, was compared to literature EC_{50} values. Our value of 0.229 nM was in excellent agreement with three other estradiol EC_{50} values from the literature (see Table 2.4).

Cypermethrin and permethrin hydrolyse under enzymatic or high UV conditions to products **2.10** or **2.11** respectively (Scheme. 2.2). Metabolite **2.11** has estrogenic activity (Table 2.3), and both are very likely to be further metabolised by hepatic first pass metabolism following ingestion with food. Results from this work show that the Phase I first pass metabolites **2.12**, (for cypermethrin) and **2.14** (for permethrin) respectively, are also both weakly estrogenic. Following oral ingestion of the estrogenic environmental degradation product (**2.11**), the first pass Phase I metabolites (**2.12** and **2.14**) are also estrogenic so increasing the risk of xenoestrogenic pharmacological effects on the consumer.

From known SAR studies, we predicted **2.14** would be more estrogenic in the assay than **2.11** due to the presence of two hydroxyl groups at both ends of the molecule (see section 2.1.10 for reasoning). This structural arrangement is well known to be preferred for binding to the estrogen receptor, as well as the presence of phenolic rings.[53] However, based on the assay results reported here, the activity of **2.14** was of the same order of magnitude as that of **2.11**. The presence of a phenol did not increase the estrogenic activity of **2.14** when compared to **2.11**. It is noted that the SAR,[53] from which our predictions were made, are based on receptor binding assays and computational calculations, whereas the yeast assay used in this work measures the effects of gene expression, further down the effect pathway than receptor binding alone. This is a likely reason as to why we see a difference in our predicted activity compared to the observed, and also reiterates the fact that determining absolute SAR for xenoestrogens is complex and dependent on the type of assay utilised as well as the mechanism of action. The yeast assay measures an estrogenic response further down the effect pathway, but only the response of a single gene in a genetically modified cell system, unlike the complex system of an animal or human. It is for this reason that this assay is considered a simple screen to prioritise candidates that might require further biological testing.

Of the two assays discussed, each has their own advantages and limitations. The receptor binding assay measures the relative binding affinity of chemicals for the estrogen receptor. They are easy and rapid to carry out, making large scale screening a possibility. However binding assays are unable to determine the difference between agonistic and antagonistic chemicals. As there is no cellular system present, there are no metabolic processes that

could produce xenoestrogens upon metabolism. Reporter gene assays (as used in this study) test the ability of a chemical to activate transcription and therefore reporter gene expression. The yeast cells are easy to grow over short time frames, possessing some metabolic capability. However, the number of receptors expressed may alter the sensitivity of the assay, as well as the type of reporter gene used. Some test chemicals may not penetrate the yeast cell to effect a response, resulting in false negatives.[84] Although the yeast assay is further along the effect pathway than receptor binding, neither of these *in vitro* assays are particularly appropriate for modelling cellular effects in wildlife or humans. The yeast assay measures the response of a single gene in a genetically engineered cell system. This is a long way from a whole animal or human. Complicating this matter further is the emergence of new mechanisms by which estrogen may act in target cells, which are not mediated by the ER.

There are two main sources of the metabolites shown to be estrogenic in this study. Microbial enzymes that can hydrolyse the parent pyrethroid to produce the metabolites are widely found in the environment (e.g. in the bacteria of aquatic silts). Therefore the metabolites are more likely to be found in the environment than the parent compound. Metabolites might then form residues in food crops and so be ingested with food.[85] The other source of these metabolites is internal mammalian metabolism, **2.13** and **2.15** have been detected in human blood and urine.[86-88] These compounds are end products of cypermethrin and permethrin metabolism after ingestion. Phase I enzyme reactions produce the estrogenic metabolites via hydroxylation (**2.11**, and **2.14**) and oxidation (**2.12**) of the inactive parent. Subsequent Phase II enzyme reactions are responsible for conjugating molecules to metabolites to lower toxicity and aid in excretion. Phase I enzymes are mostly localised on the endoplasmic reticulum, while phase II enzymes are found in the cytoplasm.[89] Thus the estrogenic insecticide metabolites produced from phase I reactions are then available for conjugation by phase II enzymes or interaction with hER in the cell. Therefore Phase II metabolism could lower the concentration of estrogenic metabolites, thus decreasing possible interaction with hER. Conjugation with glycine inactivates the metabolite (as shown by the non-activity of **2.16**).

The synthetic pyrethroid insecticides were designed to be relatively unstable to minimise their toxicological impact on both the environment and humans. For this reason food

residues are infrequent and very low even though their usage is relatively high.[90] The major environmental degradation products of the pyrethroids (Scheme. 2.2) are not normally measured as part of pesticide residues surveillance programmes and therefore there are few, if any data, from which exposure estimates, can be made. The finding that several of these breakdown products are estrogenic brings into question their impact on humans. This risk assessment cannot be carried out without food residues data. However it is likely that the breakdown products do occur as residues in food since pyrethroid insecticides are applied directly to crops, e.g. tomatoes.[85] Pyrethroid degradation occurs on/in the crop and so the breakdown products are likely to remain as residues. This reasoning suggests that there will be an impact on human health, but the magnitude of the impact cannot be assessed without detailed residue data.

2.5 CONCLUSION AND FUTURE WORK

Six metabolites of the pyrethroid insecticides, cypermethrin and permethrin, (**2.11**-**2.16**) were exposed to the YES. Metabolites **2.14-2.16** were synthesised in this thesis for this purpose. Three metabolites, **2.11**, **2.12**, and **2.14**, have been shown to be weakly estrogenic in the recombinant yeast assay. Even though the standard concentration of the metabolites was much higher than 17β-estradiol and not likely to be indicative of environmental concentrations, the yeast assay allows for estrogenic potencies to be compared for potential risk assessment. The fact that **2.11**, **2.12**, and **2.14** were about 10^5 less active than 17β-estradiol suggests that a large amount of metabolite would have to be ingested or produced internally to observe any estrogenic effect. Pyrethroids are also metabolised rapidly, being renally excreted with a half life of about 6 hours.[88] Upon evidence to date our opinion is that these insecticide metabolites are not contributing significantly to the xenoestrogenic impact on humans, but might be more significant to animals in the environment, contributing to total estrogenic exposure.

Future work in this area would involve assessing the risk posed to humans and the environment from exposure to cypermethrin and permethrin metabolites. To carry out this assessment, exposure data for cypermethrin and permethrin would be required, such as how much insecticide is used in the environment. As these insecticides are sprayed on food crops (such as grains, fruit and vegetables), ingestion by mouth is the likely exposure route for humans. Data for the average amount of crops eaten, which have been sprayed with cypermethrin and permethrin, would be needed to estimate how much insecticide is eaten. Finally, an estimate of the amount of metabolites produced by internal metabolism would be required, as it is these metabolites (such as **2.11**, **2.12**, and **2.14**) which interact with the ER.

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CHAPTER THREE

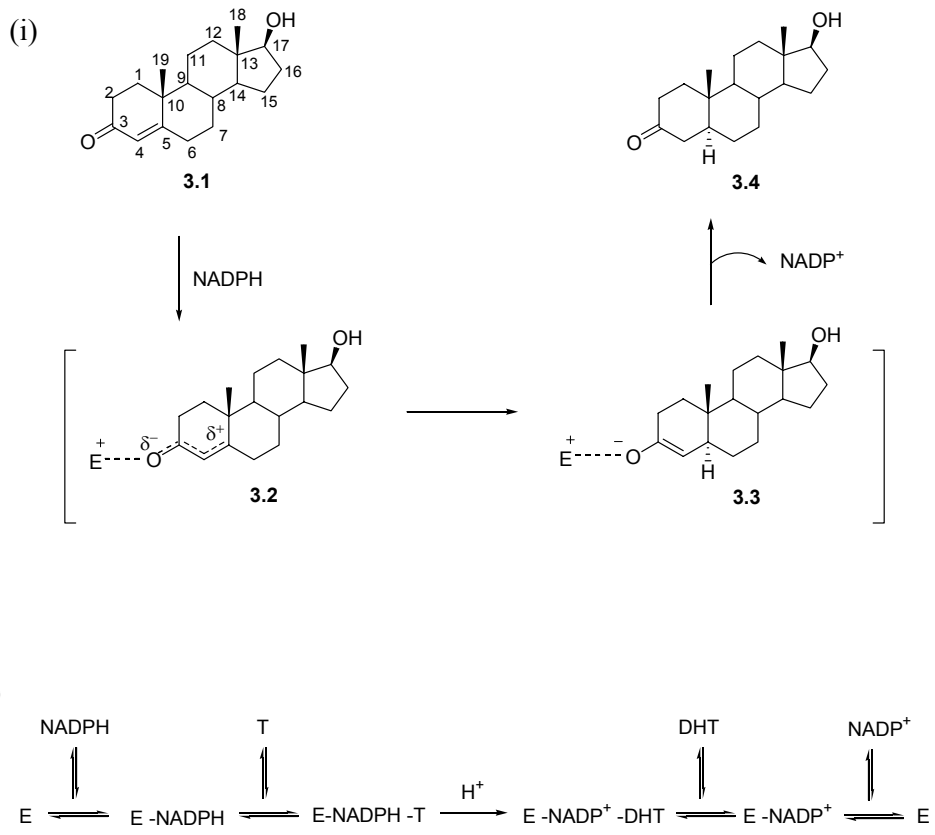
DESIGN, SYNTHESIS AND TESTING OF NON-STEROIDAL INHIBITORS OF STEROID 5α-REDUCTASE

3.1 INTRODUCTION

The major circulating androgen testosterone is metabolised to DHT in a reaction catalysed by steroid 5α-reductase. Like other steroid hormones, both testosterone and DHT activate the expression of responsive genes by binding to the AR. The genomic action of steroid hormones was discussed in Chapter One, section 1.3.1. However, the action of these two androgens differs from other steroid hormones because both interact with the AR but induce different physiological effects, which were described in Chapter One, section 1.4. DHT has been proposed as a causative factor in some disease states, so there has been intense interest in developing inhibitors of steroid 5α-reductase to lower the levels of DHT synthesised. The following section details the mechanism of steroid 5α-reductase, diseases associated with DHT, and the development of 5α-reductase inhibitors to date.

3.1.1 Mechanism of steroid 5α-reductase

Steroid 5α-reductase (3-oxo-5α-steroid:Δ^4-oxidoreductase) is a membrane bound, NADPH-dependent enzyme that catalyses the irreversible reduction of testosterone. The mechanism for this reduction has been has been investigated, and is believed to proceed via binding of the substrate followed by release of the product DHT.[1] NADPH firstly binds to the enzyme to give a binary complex that binds testosterone (**3.1**), forming a ternary complex (see Scheme 3.1).[2] Upon formation of the ternary complex, the enone system is activated by a strong interaction with an electrophilic residue present in the active site (proton or proton donor), giving a delocalised carbocation at C5. A hydride from NADPH is transferred stereospecifically to the 5α-position of testosterone (**3.1**), forming the enolate **3.3** of DHT (**3.4**). Protonation on the α-face of **3.3** gives **3.4** which is released from the active site.



Scheme 3.1. Steroid 5α-reductase catalysed conversion of testosterone (**3.1**) to DHT (**3.4**); (i) a molecular mechanism and (ii) kinetic mechanism.

3.1.2 Steroid 5α-reductase isozymes

Steroid 5α-reductase is a hydrophobic and unstable intrinsic membrane protein which is present at trace levels in tissue. These properties created difficulty in early work to study the enzyme's biochemistry and regulation. These problems have now been largely overcome with the use of expression cloning technology to isolate cDNA that encodes 5α-reductase. Several observations led to the suggestion that more than one isoform of 5α-reductase existed.[3] These were the fact that cDNA for 5α-reductase was found to be present in individuals with 5α-reductase deficiency, and the enzyme was inhibited poorly by finasteride, a known potent inhibitor of 5α-reductase in human prostate. Two isozymes

of steroid 5α-reductase have subsequently been cloned, expressed, and these are termed type 1 and type 2.[4] Type 1 is a hydrophobic protein of 259 amino acids, while type 2 consists of 254 amino acids (with 45% homology to the type 1 isozyme).[5] The DNA sequences coding for type 1 and 2 are found on separate chromosomes. The isozymes have different expression patterns in various tissues and also different enzyme kinetics. Testosterone has high affinity for the type 2 enzyme (K_m = 4-50 nM), but its affinity is lower for the type 1 isozyme (K_m = 1-5 μM).[1] These differing affinities suggest that there are sequence differences in the enzyme binding domains.

The tissue localisation of type 1 and 2 differs. When the two isozymes were first cloned in the early 1990s, type 2 was only identified in prostate tissue whilst type 1 was identified in skin and liver. However, techniques used to detect proteins or their corresponding mRNAs have since become more sensitive and reliable. Both isozymes have now been identified in almost all the target tissues with different ratios and distribution in target organs. Overall, the type 2 isozyme is found predominantly in the prostate, genital skin, seminal vesicles and dermal papilla. Type 1 is predominantly found in non genital skin, scalp, sebaceous gland, and in the liver and brain (see Occhiato *et al.* and references therein).[1]

3.1.3 DHT-related pathologies

DHT-related pathologies are associated with the tissues and organs discussed in the above paragraph. Information regarding the role of the two isozymes in DHT disorders arose from clinical evidence of type 2 deficiency. Total or part deficiency of the type 2 isozyme has been found in an inherited form of male pseudohermaphroditism.[6] At birth these male individuals have female-like external genitalia, and at puberty, the genitalia enlarge and the testes descend, but the prostate remains undeveloped. Facial and body hair growth remains similar to that of female patterns. This finding suggested the isozyme is required for differentiation of the male external genitalia in the foetus.[7]

Type 2 is the main 5α-reductase isozyme found in the prostate and it is involved in prostate pathology. Prostate cancer is one of the main causes of cancer deaths in Western males, and was discussed in Chapter One, section 1.4. DHT is implicated as a causative

factor, due to the well founded link between androgens and prostate cancer.[1] DHT is also implicated in BPH, a condition where benign prostate enlargement occurs (also discussed in Chapter One). Men with deficiencies of the type 2 isozyme do not develop BPH.

Type 1 is implicated in skin and hair conditions as these tissues predominantly express this isozyme. The hair follicle and sebaceous gland together form the pilosebaceous unit, whose development and function is dependent on the androgens. Therefore DHT is implicated in disorders of the pilosebaceous unit, such as androgenetic alopecia (male hair pattern baldness), hirsuitism, and acne.[8] It has recently been established that the rate of DHT production is higher in bald men than non-bald men.[9] However, the local production of DHT is dependent on the distribution of the two isozymes throughout the hair follicle, which is currently not fully understood.[1] In the second component of the pilosebaceous unit, the sebaceous gland, there is more indication that only the type 1 isozyme is expressed.[10] In skin areas prone to acne, androgens can cause the sebaceous gland to enlarge greatly. DHT is responsible for the hyper production of sebum, which causes acne.

As a consequence of the role of DHT in these conditions, there has been much interest in developing inhibitors of steroid 5α-reductase as potential therapeutics. Inhibition of 5α-reductase is an ideal strategy, as the enzyme catalyses the final step in DHT synthesis, avoiding a decrease in testosterone levels and its beneficial effects. By decreasing prostatic DHT levels through 5α-reductase inhibition, prostatic volume may be reduced, a desirable effect which has been shown in human trials with finasteride.[11] Finasteride is now marketed as a treatment for BPH and hair loss. The interest of 5α-reductase inhibitors in prostate cancer is in its prevention. Long term androgenic stimulation is associated with causing prostate cancer,[1] so manipulating androgen levels (by inhibiting 5α-reductase) may have the best influence at the initial stages of tumour development and progression. Lowering DHT levels through 5α-reductase inhibition is also potentially useful for treating hirsutism and acne (perhaps by selective inhibition of the type 1 isozyme).

3.1.4 Inhibitors of steroid 5α-reductase

Scientists at Merck initiated projects in the 1980s to identify inhibitors of prostatic 5α-reductase activity, after realising the significance of the finding that male pseudohermaphrodites with underdeveloped prostates were type 2 deficient (see section 3.1.3). The development of enzyme inhibitors usually requires information about the enzyme active site structure; however the two 5α-reductase isozymes have yet to be isolated and purified from tissues or cells. The only available information comes from the primary sequence derived from their cDNAs. The first inhibitors of 5α-reductase were therefore designed by modifying the structure of the natural substrates. Potent inhibitors have been identified using both rational design and compound screening approaches. Both steroidal and non-steroidal inhibitors have been reported. The following section briefly discusses steroidal inhibitors, which leads to a review of the non-steroidal inhibitors reported to date, with focus on their SAR and the key structural features responsible for inhibitory potency.

3.1.4.1 Steroidal inhibitors

Potent steroidal inhibitors of steroid 5α-reductase were discovered by Merck in the 1980s as a result of modifying the natural substrates. Such modification included substituting a carbon atom in the A or B ring (see **3.5**, Fig. 3.1) of the steroid backbone with a heteroatom. Substitution of a carbon with a nitrogen atom at the 4-position in the A ring resulted in the 4-azasteroid inhibitor class, to which finasteride (**3.5**) belongs. Finasteride (PROSCAR®) was the first 5α-reductase inhibitor approved for BPH treatment. It preferentially inhibits the type 2 isozyme (IC_{50} = 9.4 nM), and can reduce the level of prostatic DHT by 80-90%, and serum DHT by 70-80%.[12] Finasteride is also FDA approved for treating male pattern baldness by reducing the level of DHT in human scalp, and decreasing apoptosis of hair follicles. Other classes of steroidal inhibitors discovered were the 6-azasteroids (**3.6**), 10-azasteroids (**3.7**), and steroidal carboxylic acids (**3.8-3.10**). The steroidal pharmacophore provides a tether between the A ring and the C17 substituent

of the D ring. The C17 substituent aids in binding within a lipophilic pocket in the enzyme, which differs for the two isozymes.

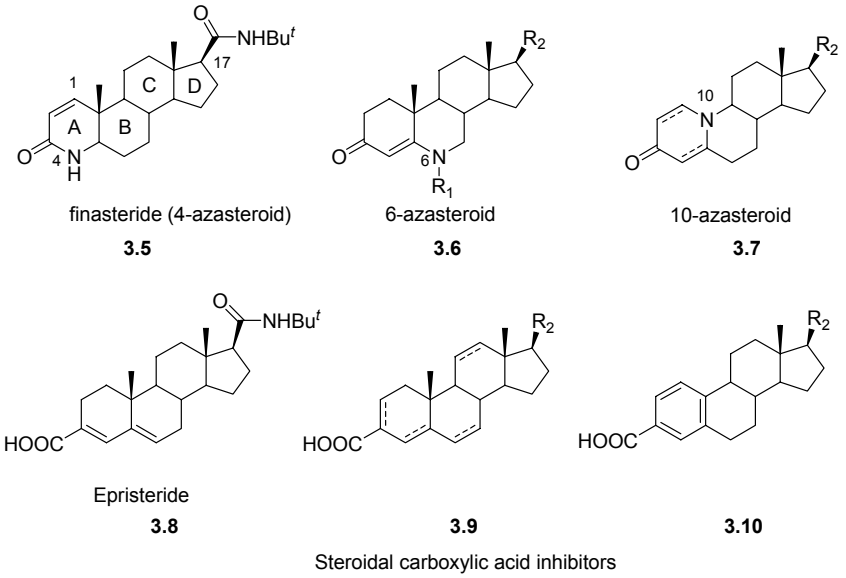
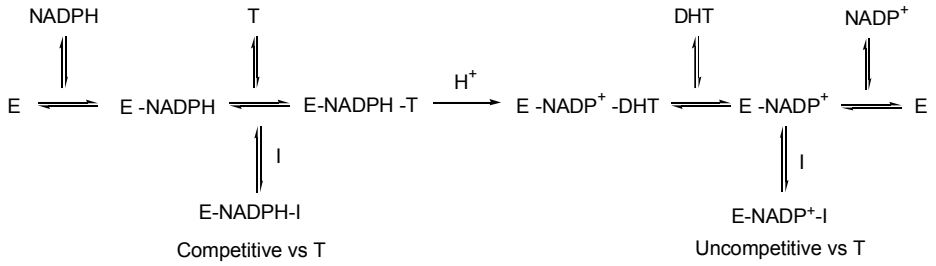


Figure 3.1. Early steroid based inhibitors of 5α-reductase.

The exact structure of the transition state in the conversion of testosterone to DHT is unknown; however inhibitors (including the above) have been designed as mimics of the proposed enolate **3.3** in the biosynthesis of DHT. This has been a major theme in the design of 5α-reductase inhibitors, where their electrostatic properties and A ring geometry mimic that of enolate **3.3**. The 4-azasteroids, 6-azasteroids, and 10-azasteroids mimic the enolate **3.3** and are competitive inhibitors with respect to testosterone as they interact with the enzyme-NADPH complex (see Scheme 3.2). The steroidal carboxylic acids are also enolate mimics but are uncompetitive inhibitors as they interact with the enzyme-$NADP^+$ complex.



Scheme 3.2. Interaction of steroid 5α-reductase inhibitors (I) with enzyme mechanism.

On the basis of the enzyme mechanism (Scheme 3.1), two possible transition states for the enzymatic process have been postulated, in considering how substrate competitive inhibitors act as transition state analogues (Fig. 3.2).[13] In the substrate-like transition state **3.11**, C5 is sp^2 hybridised like the carbocation (**3.2**, Scheme 3.1), and for the product-like transition state **3.12**, C5 is sp^3 hybridised as for the enolate of DHT (**3.3**, Scheme. 3.1). 4-Azasteroids (**3.5**) are sp^3 hybridised at C5, as in product-like transition state **3.12**, so they may be thought of as analogues of the DHT enolate. However the 6-azasteroids (**3.6**) and 10 azasteroids (**3.7**) are sp^2 hybridised at C5, so can be thought of as analogues of the substrate-like transition state **3.11**.

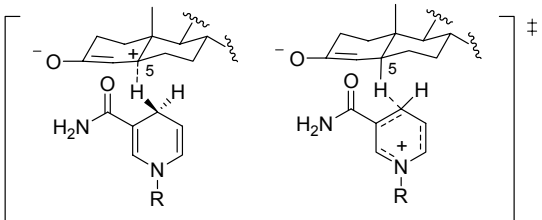
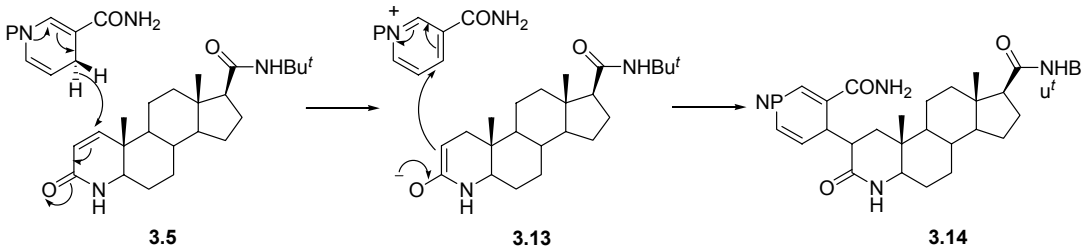


Figure 3.2. Postulated transition states (TS) in the biosynthesis of DHT (taken from Occhiato *et al.*[1]).

Glaxo and Merck have recently demonstrated how finasteride (**3.5**), and other related 4-azasteroids, behave as irreversible inhibitors (Scheme 3.3).[1] Irreversible inhibition results from alkylation by $NADP^+$of the enolate (**3.13**, Scheme 3.3), formed after 1,4-reduction of the A ring. The resulting enzyme-NADP-dihydrofinasteride complex is very stable, making finasteride extremely potent and selective. The enzyme-NADP-dihydrofinasteride complex has a dissociation constant (K_i) of less than 10^{-13} M, which ranks finasteride amongst the most potent non-covalently bound inhibitors known for any enzyme. The high affinity is due to adduct **3.14** acting as a bi-substrate inhibitor. The adduct fits in both cavity sites of the enzyme normally bound by testosterone and NADPH.

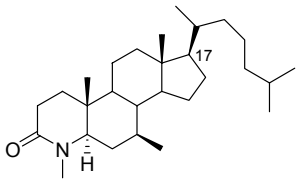


Scheme 3.3. Formation of NADP-dihydrofinasteride adduct **3.14** in the mechanism of irreversible inhibition of steroid 5α-reductase by finasteride (**3.5**). P = phosphoadenosine diphosphoribose.

The steroidal based carboxylic acid inhibitors (**3.8-3.10**, Fig 3.1) were also designed to mimic the postulated enolate intermediate (**3.3**, Scheme 3.1). The negatively charged carboxylate ion mimics the enolate oxygen binding to the enzyme active site. These charged inhibitors show uncompetitive kinetics (Scheme 3.2) as they bind to the enzyme-$NADP^+$ complex. Epristeride (**3.8**, Fig. 3.1) is of this class, and is a potent and selective inhibitor of the type 2 isozyme. The carboxylate and double bond between C3 and C4 mimic the negative charge and planarity of the enolate. Isozyme selectivity of steroidal inhibitors is apparently due to the nucleophilic potency of the substituent at the C3 position.[14] Faragalla *et al.* proposed a 5α-reductase pharmacophore from molecular modelling and suggested the type 2 isozyme requires a negatively ionisable group, while the type 1 requires only a hydrogen bond acceptor, plus hydrophobic and hydrophobic-

aromatic features.[15] This would explain the observed selective type 2 activity of the aryl carboxylic acids which can be ionised.

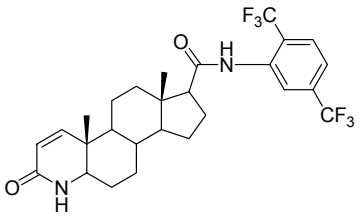
Selectivity towards the type 1 isozyme by steroidal inhibitors has been achieved with the inclusion of an appropriate substituent at C17. For the 4-azasteroids, selectivity towards the type 1 isozyme was found to increase with increasing lipophilicity at C17. The first inhibitor reported to address type 1 selectivity was MK386[16] (**3.15**, Fig. 3.3), which has the side chain present in cholesterol at C17, however further development was abandoned due to its hepatotoxicity.



3.15

Figure 3.3. Steroidal inhibitor of type 1 5α-reductase MK386 (IC_{50} = 0.9 nM).

Steroidal dual inhibitors of 5α-reductase have also been developed. These inhibitors are able to inhibit both isozymes. A drug which has this property is desirable for potentially further reducing the serum concentrations of DHT (finasteride, which is type 2 selective, reduces these levels by only approximately 70%). Dual inhibitors have the potential to enhance therapeutic effect, as they would inhibit not only the type 2 isozyme but also slight type 1 activity in the prostate. An example is that of dutasteride (GG745) (Fig. 3.4), which has been marketed by GlaxoSmithKline and is FDA approved for treating BPH. Greater reductions in serum and tissue levels of DHT compared to finasteride have been observed, but clinical efficacy is comparable to that of finasteride.[12]



3.16

Figure 3.4. Dutasteride (GG745).

3.1.4.2 Non-steroidal inhibitors

The development of non-steroidal inhibitors of steroid 5α-reductase has gained importance recently due to the side effects associated with steroidal inhibitors. It is generally accepted that some of the side effects of steroidal compounds are related to the fact that they are recognised by steroid receptors.[17] Problems relating to sexual function have been reported as side effects from taking finasteride.[18] The design of non-steroidal inhibitors is based on the steroidal inhibitors, via removal of one or more rings from the steroid structure. Examples include benzo[f]quinolinones, pyridones and quinolinones, benzo[c]quinolinones, and benzo[c]quinolizinones. Most other types of non-steroidal inhibitors are carboxylic acids also derived from steroidal equivalents. Non-steroidal inhibitors provide the most potent and selective type 1 inhibitors to date.

Benzo[f]quinolinones

A series of benzo[f]quinolinone compounds were the first reported non-steroidal mimics of steroidal inhibitors.[19] These compounds are related to the 4-azasteroids (see Fig. 3.5), the steroidal D ring is absent and the steroidal C ring is replaced with an aromatic ring. Inhibitors within this class are generally selective for type 1 steroid 5α-reductase, some of which are extremely potent.

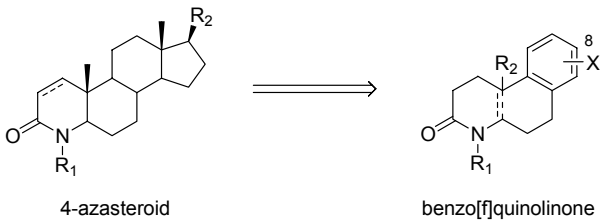
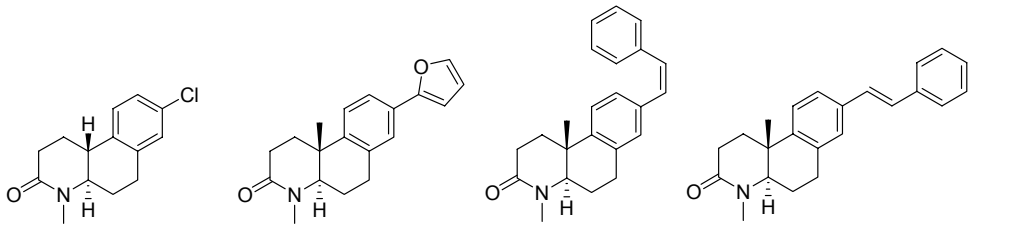


Figure 3.5. Non-steroidal benzo[f]quinolinone structure derived from the 4-azasteroid series.

For benzo[f]quinolinone inhibitors, potency is enhanced by a halogen substituent (in particular Cl, as inhibitory potency decreases with Br or F substitution) at the 8-position of the aromatic ring, and with a methyl group attached to the lactam nitrogen. This is in analogy with the 4-methyl substitution of the nitrogen in MK386 (Fig. 3.3). Potency decreases dramatically in this series with the removal of the methyl from the lactam nitrogen. A QSAR study of benzo[f]quinolinone inhibitors has investigated the effect of the 8-substituent on the aromatic ring, proposing that it interacts with a lipophilic pocket in the enzyme.[20] The property space of a Cl atom was proposed to be optimal for occupation of the lipophilic pocket.

A variety of substituents have been introduced at the 8-position to modulate isozyme selectivity. This work led to the discovery of dual inhibitors, and also potent selective type 1 inhibitors (Fig. 3.6).[19,21] Interestingly the *cis* 8-styryl compound **3.19** is a potent selective type 1 inhibitor, but the *trans* 8-styryl isomer **3.20** displays dual activity.[21] In the benzo[f]quinolinone inhibitor series, compound LY191704 (**3.17**) has progressed into the clinical trial phase of pharmaceutical development.



Type 1 IC_{50} = 8 nM — **3.17**

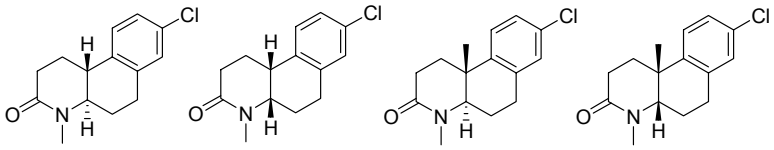
Type 1 IC_{50} = 59 nM — **3.18**

Type 1 IC_{50} = 6 nM — **3.19**

Type 1 IC_{50} = 23nM, type 2 = 180 nM — **3.20**

Figure 3.6. Benzo[f]quinolinone inhibitors.

Each of the four stereoisomers of LY191704 [(±)-**3.21** and (±)-**3.22**] and LY266111 [(±)-**3.23** and (±)-**3.24**] were separated by preparative chiral HPLC, and the inhibitory activity of each stereoisomer against both type 1 and 2 5α-reductase was determined.[22] This important study revealed that the *trans* enantiomers of **3.21** (K_i = 6 and 4 nM) and *cis* enantiomers of **3.22** (K_i = 15 and 15 nM) displayed similar and potent activity against the type 1 isozyme, as did the *trans* enantiomers of **3.23** (K_i = 9 and 10 nM). In contrast, the *cis* enantiomers of **3.24** showed much reduced type 1 activity (K_i = 4000 and 7000 nM). These results were explained by conformational analysis. The extended planar nature of the *trans* compounds **3.21** and **3.23** allowed excellent overlay of the enantiomeric pairs. The structures of the *cis* enantiomers of **3.22** also showed good overlay with the *trans* enantiomers of **3.21**. This was explained by the absence of the constraints of the angular methyl group. The poor activity of the *cis* enantiomers of **3.24** was explained by the bowl shaped *cis* ring junction decreasing the overall planarity in both enantiomers, resulting in poor overlay with the *trans* enantiomers of **3.23**. All eight stereoisomers were poor type 2 inhibitors.

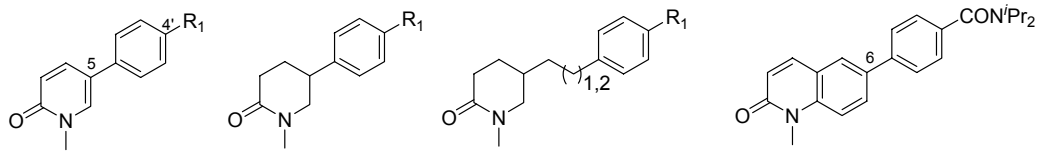


(±)-3.21 (±)-3.22 (±)-3.23 (±)-3.24

Figure 3.7. *Trans* and *cis* isomers of inhibitors LY191704 (**3.21**, **3.22**) and LY266111 (**3.23**, **3.24**).

Pyridones and derivatives

5-Aryl substituted pyridones and piperidones typified by **3.25-3.27** (Fig. 3.8) have been prepared as non-steroidal analogues of the 4-azasteroids (**3.5**, Fig. 3.1) to test for steroid 5α-reductase inhibition. Pyridone and piperidone inhibitors **3.25** and **3.26** lack the steroidal B and D rings of the 4-azasteroids. The absence of the steroidal B and D rings results in a decrease in inhibitory potency against the type 1 isozyme, when compared to the tricyclic benzo[f]quinolinones (in which only the steroidal D ring is absent) (Fig. 3.6). For compounds of type **3.25-3.26**, the highest inhibitory activity was associated to the presence of a Cl atom at the R_1 position on the aromatic ring of **3.26** (IC_{50} = 1690 nM).[23] The steroidal B and D ring of the 4-azasteroid backbone have been replaced with an acyclic linker in 5-aryl substituted piperidones such as **3.27**. These compounds display poor inhibition against both type 1 and 2 5α-reductase.[24,25] 6-Aryl substituted quinolinones such as **3.28** have also been reported to inhibit the type 1 isozyme.[26] Here only the steroidal C ring of the 4-azasteroids is absent, which is associated with better inhibitory activity compared to compounds **3.25-3.27**. An IC_{50} of 510 nM was observed for **3.28**. A methyl group on the quinolinone nitrogen is important for type 1 inhibition. When the methyl group on the quinolinone nitrogen of **3.28** is removed, there is almost a complete loss of activity against type 1, but inhibition of the type 2 isozyme was observed (83% at 10 μM).

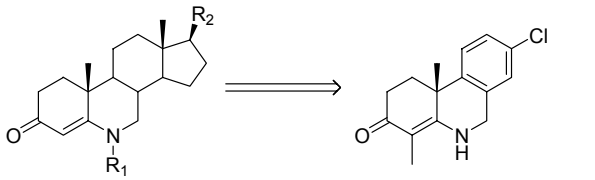


3.25 **3.26** **3.27** **3.28**

Figure 3.8. Aryl substituted pyridone/piperidone and quinolinone inhibitors.

Benzo[c]quinolinones

This class of tricyclic non-steroidal inhibitor mimics the 6-azasteroid compounds (**3.6**, Fig. 3.9). They are selective but weak type 1 inhibitors.[27] Their structure is similar to the benzo[f]quinolinones (Fig. 3.7), but they fail to show the same potent activity, even with the presence of a Cl atom on the aromatic ring. Compound **3.29** has a K_i of 920 nM towards type 1, and 49% inhibition of type 2 (at 20 μM).



6-azasteroid **3.6** phenanthridin-3-one mimic **3.29**

Figure 3.9. Non-steroidal phenanthridin-3-one mimic of 6-azasteroids.

The presence of a methyl group on the A ring is important for inhibition potency. Removal of the A-ring methyl group of **3.29** resulted in an inhibitor with a K_i greater than 10000 nM. The same effect is seen with the benzo[f]quinolinones and benzo[c]quinolizinones (Fig. 3.10), and can be accounted for by the presence of a postulated small hydrophobic

pocket in the enzyme active site. This pocket is thought to accommodate a small alkyl group at the 4-position of the A ring of these tricyclic inhibitors.[1]

Benzo[c]quinolizinones

The tricyclic benzo[c]quinolizinones are based on the 10-azasteroids (**3.7**, Fig. 3.10). Potent and selective type 1 inhibitors are found within this class (e.g. **3.31**, Fig. 3.10).[28-31] There are two major series within this class; one with the double bond located between C1 and C2 (4a*H* series, **3.30**), and the second between C4 and C4a (1*H* series, **3.31**).

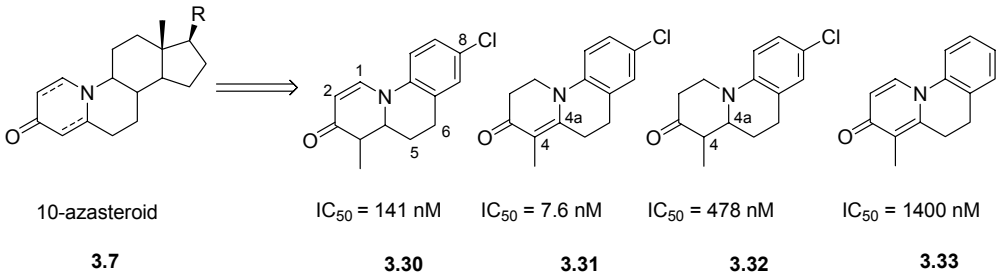
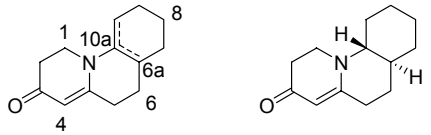


Figure 3.10. Benzo[c]quinolizinone inhibitors of type 1.

As for the 10-azasteroid inhibitors, the presence of a double bond between the carbonyl group and the nitrogen atom is essential for potency. Removal of the double bond results in a significant decrease in activity against the type 1 isozyme (**3.31** compared to **3.32**). The 1*H* series of inhibitors with the double bond between C4 and C4a generally displays higher activity (10 fold) than those with the double bond between C1 and C2 (4a*H* series). A thorough investigation into the effects of substituents on the different positions of this tricyclic system has been discussed by Occhiato *et al.*[1] A Cl atom at position 8 generally increases the potency observed for both series, in analogy with the previously mentioned tricyclic inhibitors. Substitution with a methyl group in the same position also increases potency when combined with one or two methyl groups on the aliphatic rings. A 4-methyl substituent enhances inhibitory activity, the same effect seen for the benzo[f]quinolinones

and benzo[c]quinolinones. A 6-methyl substituent also enhances potency, while a methyl group at position 1 or 5 generally reduces the potency.

The higher inhibitory activity toward the type 1 isozyme displayed by compounds of the 1*H* series (e.g. **3.31**) compared to inhibitors of the 4a*H* series (e.g. **3.30**) has been associated to their more extended planarity.[28] This effect is analogous to that discussed for the benzo[f]quinolinones (Fig. 3.7). However, molecular planarity is not the only important feature when evaluating inhibition data. For example, the large difference in potency of **3.34** and **3.35** (Fig. 3.11) cannot be explained by differences in molecular planarity. A molecular modelling study showed that the two inhibitors possessed similar overall extended planarity.[1] As a consequence Occhiato *et al*. have suggested that the difference in inhibitory activity is associated with an important interaction between the double bond between C6a and C10a of **3.34**, with an aromatic amino acid side chain in the enzyme active site.[1] This hypothesis arose from the relatively recent report that residues 26-29 (AVFA) in the type 1 isozyme sequence are involved in inhibitor-substrate binding, and contain an aromatic residue.[32]



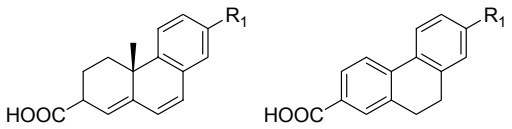
3.34 **3.35**

Figure 3.11. Inhibition of type 1 by benzo[c]quinolizinone **3.34** containing 6-10a/10-10a double bond (isomers in 10:1 ratio, IC_{50} = 58 nM for type 1) compared to the saturated *trans*-fused compound **3.35** (IC_{50} = 20000 nM).[33]

Non-steroidal acids

A range of non-steroidal aryl acids has been synthesised and tested for 5α-reductase inhibition. Tricyclic aryl acids of the type **3.36** and **3.37** (Fig. 3.12) are related to the steroidal carboxylic acid inhibitors (**3.8** and **3.10**, Fig. 3.1). The steroidal D ring is absent in this tricyclic series. In contrast to the steroidal carboxylic acid inhibitors, the tricyclic

acids are selective type 1 inhibitors.[34] The introduction of a Cl atom to the aromatic ring (**3.39**, IC_{50} = 320 nM) does not increase the potency when compared to inhibitor **3.38** (IC_{50} = 310 nM). This observation is in contrast with the enhanced potency usually seen with Cl substitution at the corresponding position in the other classes of tricyclic non-steroidal inhibitors. Instead enhanced potency is observed with a Br atom on the aromatic ring (**3.37**, IC_{50} = 26 nM). These carboxylic acid inhibitors act by a different mechanism to the aza-inhibitors, supposedly interacting with the enzyme-$NADP^+$ complex (see Scheme 3.2) in an uncompetitive manner versus testosterone (in analogy to the mechanism of the steroidal carboxylic acid inhibitors).



3.36

3.37 R_1 = Br
3.38 R_1 = H
3.39 R_1 = Cl

Figure 3.12. Tricyclic aryl acid inhibitors derived from steroidal carboxylic acids.

Other non-steroidal aryl acid inhibitors related to the steroidal carboxylic acids have been reported. While the tricyclic aryl acid inhibitors (Fig. 3.12) are selective for the type 1 isozyme, non-steroidal aryl acids (summarised in Fig. 3.13, 3.14, and 3.15) are generally selective for the type 2 isozyme. In compound **3.40** the steroidal B and D rings are absent; while in compounds **3.42** and **3.43** the steroidal B and C rings have been replaced by an acyclic linker (Fig. 3.13). Compounds with inhibition values in the nanomolar range are found within the three classes of compounds in Figure 3.13. Inhibitor **3.40** was reported by Holt *et al.* to have a K_i of 60 nM.[35] Picard *et al*. investigated the effect on inhibition by varying the substituent at the R_1 position of **3.40**.[36] Inhibitor **3.41** with a dicyclohexyl group present has an IC_{50} of 220 nM. The presence of benzyl or alkyl carbonyl groups at the 4'-position (e.g. **3.40** and **3.41**) was found to enhance potency in comparison to compounds with the corresponding amide present (for **3.40**, where R_1 = $N(cyclohexyl)_2$, IC_{50} = 4.7 μM). Hydrophobic and bulky substituents (e.g. methyldicyclohexyl and benzyl

groups) at the R_1 position were also found to increase inhibition of type 2, which supports the hypothesis for a hydrophobic pocket in the enzyme. *N*-Piperidine-4-(benzylidene-4-carboxylic acid) compounds such as **3.42** have been reported. Substitution at the R_1 position with bulky groups also enhances potency. Inhibitor **3.42** has an IC_{50} of 60 nM, but when the methyldicyclohexyl group was replaced with a *t*-butyl group, an IC_{50} of 75 μM was observed.[37]

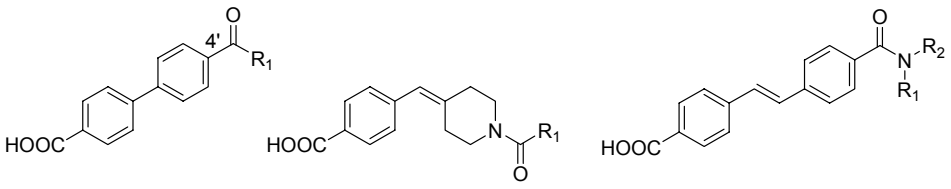


Figure 3.13. Non-steroidal biaryl acid inhibitors.

Some particularly potent aryl acid inhibitors of the type in Figure 3.14 have been reported. An IC_{50} of 53 nM was obtained for inhibitor **3.44** with selective activity against the type 2 isozyme.[38] The ether linkage of **3.44** is important for potency since substitution of the phenoxy group with the amide group as in **3.45** results in a 10 fold decrease in potency (IC_{50} = 820 nM). A very potent inhibitor containing an ether linkage was reported (**3.46**, IC_{50} = 6 nM).[39] Replacing the ether linkage in **3.46** with a methylene linker results in a 10 fold decrease in inhibition of the type 2 isozyme.

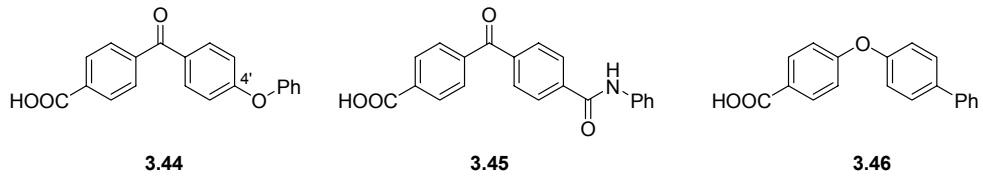
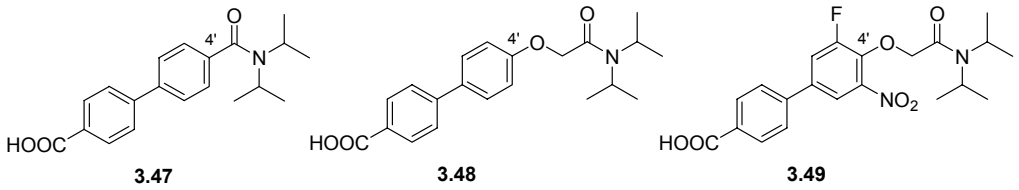


Figure 3.14. Benzophenone and biaryl ether carboxylic acid inhibitors.

Lesuisse *et al*. have reported biaryl acid inhibitors such as **3.49** (Fig. 3.15) that are among the most potent selective inhibitors of type 2 with IC_{50} values in the low nanomolar range.[40] The inhibition behaviour of compounds was studied by LeSuisse *et al.* by introducing various linkers between the aromatic ring and the amide portion (see **3.47**, Fig. 3.15). Inhibition of type 2 isozyme activity increased from 32.5% (**3.47**) to 57.5% (**3.48**) (at 10^{-6} M) with the introduction of an OCH_2 linker between the aromatic ring and the amide of **3.47**. The effect of substitution of the aromatic rings towards inhibition was also investigated. No significant effect on inhibition was observed when the carboxylic acid containing ring of **3.48** was substituted with various groups (F, Cl, Me, CF_3). However when the phenxoyacetamide ring of **3.48** was substituted with various groups *ortho* to the acetamide, a dramatic effect on inhibition was observed. A F atom and a nitro group in these positions resulted in a very potent type 2 inhibitor (**3.49**, IC_{50} = 9.8 nM). The diisopropyl amide of **3.49** is important for potency. A 10 fold decrease in inhibitory activity (IC_{50} = 92 nM) was found when a trityl amide was substituted for the diisopropyl amide of **3.49**.



3.47 **3.48** **3.49**

Figure 3.15. Biaryl carboxylic acid type 2 inhibitors.

Dual non-steroidal inhibitors

Steroidal dual inhibitors have been developed including dutasteride (see Fig. 3.4). Therefore the synthesis and inhibition profile of non-steroidal dual inhibitors is currently of interest. Streiber *et al.* have reported methyl esters of *N*-(dicyclohexyl)acetyl-piperidine-4-(benzylidene-4-carboxylic acids) (e.g. **3.50**, Fig 3.16) that were initially designed as prodrugs to enhance cell permeability, but were discovered to be potent inhibitors of type 1 in human plasma.[14] The ester **3.50** displays an IC_{50} of 1494 nM against type 1 in human plasma, but is metabolised to the acid **3.51** in human prostate tissue, where selective type 2

inhibition is observed (IC_{50} = 11 nM). Guarna *et al.* have reported dual inhibitors of the benzo[f]quinolizinone type.[31] Inhibitor **3.52** (Fig. 3.16) inhibits both isozymes with similar potency. Compounds such as **3.50** and **3.52** present the future possibility of dual inhibition of steroid 5α-reductase by administering one drug, which would potentially achieve nearly complete suppression of serum DHT levels.

3.50 R_1 = $COOCH_3$ type 1 IC_{50} 1494 nM, type 2 ca 7500
3.51 R_1 = COOH type2 IC_{50} 11nM, type 1 > 10,000

3.52 type 1 IC_{50} = 93 nM
type 2 IC_{50} = 119 nM

Figure 3.16. Dual inhibitors of type 1 and 2 isozymes.

3.1.5 SAR of non-steroidal inhibitors

Despite a diversity of structures amongst the known classes of non-steroidal inhibitors, there are general structural features which influence potency. A structural feature common to inhibitors is a lipophilic scaffold incorporating a ring which mimics the geometry and electrostatic properties of the A ring in the DHT enolate **3.3**. The potency and selectivity of non-steroidal inhibitors depends greatly on the nature and position of ring substituents. Features which influence non-steroidal inhibitor potency are summarised below.

Ring substitution

The nature of the substituent present at the 8-position (tricyclic inhibitors, see Fig. 3.5) or the 4'-position (biphenyl inhibitors, see Fig. 3.15) of non-steroidal inhibitors has a profound effect on potency against the type 1 isozyme. An 8-Cl substituent is found in some very potent inhibitors within the benzo[f]quinolinone and benzo[c]quinolizinone classes, for example **3.17** (IC_{50} = 8 nM) and **3.31** (IC_{50} = 7.6 nM). Replacement of the 8-Cl of **3.17** (for example) with other substituents decreases the potency against type 1 5α-

reductase (Me, IC_{50} = 11 nM; Br, IC_{50} = 35 nM; H, IC_{50} = 560 nM). However, in the related tricyclic aryl acids (Fig. 3.12), potency is dependent on an 8-Br substituent (**3.37**, IC_{50} = 26 nM) and activity decreases 10 fold with either an 8-Cl (**3.39**) or 8-H substituent (**3.38**). For biaryl acid inhibitors of the type depicted in Figure 3.13, hydrophobic and bulky groups (e.g. benzyl, methyldicyclohexyl) at the R_1 position of **3.40-3.43** enhance the potency of type 2 inhibition compared to compounds with smaller alkyl R_1 substituents (replacement of the methyldicyclohexyl group of **3.41** with an isopropyl group results in a decrease in IC_{50} from 220 nM to 10 μM). The same influence is seen with the biaryl acid inhibitors depicted in Figures 3.14 and 3.15, where potent type 2 inhibition is also observed for compounds with bulky groups at the 4'-position [e.g. phenyl (**3.44** has an IC_{50} of 6 nM), diisopropylacetamide (**3.49** has an IC_{50} of 9.8 nM)]. The presence of substituents at other positions on the phenxoyacetamide ring of **3.49** greatly enhances type 2 inhibition. Electron-withdrawing groups such as F and NO_2 *ortho* to the acetamide group influence inhibitory potency dramatically, as shown by the difference in activity between **3.49** and **3.48** (57.5% inhibition of type 2 activity at 10^{-6} M).

Position and nature of unsaturation

The influence of ring unsaturation on type 1 inhibitory potency for the tricyclic aza-inhibitors (benzo[f]quinolinones, benzo[c]quinolinones, and benzo[c]quinlizinones) has been widely investigated. Potent inhibitory activity for these compounds depends on the presence (and position) or absence of unsaturation. For the benzo[c]quinolinones (Fig. 3.5) potency is observed with the absence of a double bond in the lactam ring (see **3.17**, IC_{50} = 8 nM). However, for the benzo[c]quinolizinones (Fig. 3.10) the presence of conjugation between the nitrogen and the carbonyl is essential for potency (see **3.31** vs **3.32**), where a double bond between C4 and C4a (*1H* series) is preferred. Inhibitor **3.31** has an IC_{50} of 7.6 nM, in comparison to **3.30** (IC_{50} = 141 nM) which has a double bond between C1 and C2. A double bond between C6a and C10a in the benzo[c]quinolizinones (see Fig. 3.11) is also influential on inhibitory potency (see Fig. 3.11), as it supposedly interacts with an aromatic amino acid in the enzyme active site. Potency has been associated to extended molecular planarity which is present in potent isomers of benzo[f]quinolinones LY191704 (**3.21**, K_i = 6, 4 nM, and **3.22**, K_i = 15, 15 nM) and LY266111 (**3.23**, K_i =9, 10 nM).

Lactam N-alkylation and angular methyl groups

The presence of a 4-methyl group influences the potency of non-steroidal inhibitors of type 1. Methylation of the lactam nitrogen enhances the potency of compounds within the benzo[f]quinolinone, pyridone and piperidone (**3.25-3.27**, Fig. 3.8), and quinolinone (**3.28**, Fig. 3.8) classes. The most dramatic effect can be seen by comparing the activity of compound **3.28** (IC_{50} = 510 nM) with that of its derivative in which the *N*-methyl group is absent (almost no inhibition of type 1). The presence of an angular methyl group at the 4-position of the benzo[c]quinolinones (**3.29**, Fig. 3.9) and benzo[c]quinolizinones (**3.31**, Fig. 3.10) also influences the potency of these tricyclic aza-inhibitors. For example, removal of the 4-methyl group of **3.29** (K_i = 920 nM) results in a compound with a K_i greater than 10000 nM. Potent inhibition is observed for the *trans* enantiomers of LY266111 (**3.23**, IC_{50} = 9, 10 nM) in which the angular methyl group is *trans* to the H at C4. In the benzo[c]quinolizinone series of inhibitors, the presence of an angular methyl group at the 6-position either maintains or enhances potency against the type 1 isozyme. For example, incorporation of a methyl group at the 6-position of **3.31** (IC_{50} = 7.6 nM) results in an inhibitor with similar potency (IC_{50} = 8.5 nM).

3.1.6 Biological testing of steroid 5α-reductase inhibitors

SAR studies of inhibitors depend on the reliability and reproducibility of the biological inhibition assay. Methods have been developed to assess the activity of inhibitors versus human steroid 5α-reductase using either the native isozymes (sourced from tissue homogenates or human cell lines), or recombinant isozymes expressed by transfected cells. Tissue homogenates contain both isozymes in different amounts, complicating the testing procedure and hence the interpretation of results. When assessing the inhibition of a compound against both isozymes in tissue homogenates, it is necessary to selectively activate one of the isozymes in the presence of the other. Different concentrations of testosterone have been used to activate either 5α-reductase type 1 or 2 on the basis of the different affinities of the substrate (testosterone has higher affinity for the type 2 isozyme than type 1).

Human derived cell lines, which predominantly express the type 1 isozyme, can be used to test inhibitors. These include tumour cell lines originating from the prostate, such as PC3, DU145, and LnCaP, or lines from skin, like SZ95.[41-43] However, tumour lines are difficult to grow and maintain in culture. Transfected cell lines expressing recombinant isozymes have also been used to test inhibitors. These cell lines avoid the problems associated with tissue homogenates, as each isozyme is tested separately. A biological test for assessing 5α-reductase inhibitors should be chosen carefully.[1] Human isozymes are essential for determining the potency and selectivity of novel inhibitors, especially if they are clinical study candidates. The structural difference of the isozymes between species can cause different responses to inhibitors. This is often the case with rat versus human isozymes. For example, finasteride is a weak inhibitor of human type 1 (K_i = 300 nM), but a potent inhibitor of rat type 1 (K_i = 5 nM).[1]

A number of different classes of non-steroidal 5α-reductase inhibitors have appeared in the literature, which has made the definition of SAR within and across classes desirable. The aim of the work presented in this chapter was to investigate SAR across different classes of non-steroidal inhibitors of steroid 5α-reductase. Several non-steroidal inhibitor scaffolds were identified for synthesis in this thesis. A 5-aryl 1-methyl-2-pyridone scaffold [related to the 4-azasteroids (**3.5**)], was chosen so that SAR for novel inhibitors of this type could be investigated further and compared with SAR from another series of inhibitors. There is a lack of reported information which compares SAR for 4'-substituents of this pyridone scaffold (see **3.55**, Fig. 3.18) with SAR for 4'-substituents of biaryl acid inhibitors (see Fig. 3.13 and Fig. 3.15). Potent type 2 inhibitors are found within the biaryl acid series of compounds, so it was of interest to see if 4'-substituents influencing potency have a similar effect when incorporated into the pyridone scaffold. 5-Aryl 1-methyl-2-pyridone based inhibitors have been reported previously (and are generally type 1 inhibitors) however this thesis investigates SAR for substituents at the 4'-position in particular, because the nature of the 4'-substituent is known to influence potency. A 1-aryl 2-substituted 2,3-dihydro-4-pyridone scaffold [related to the 10-azasteroids (**3.7**) and benzo[c]quinolizinones (e.g. **3.30**)] was also chosen (see **3.61**, Fig. 3.20), as it is a novel non-steroidal scaffold with which to investigate 5α-reductase inhibition. SAR for substituents at the 4'-position of inhibitors of this type were also of interest, for reasons discussed for the 5-aryl 1-methyl-2-

pyridone scaffold. Activity comparison between the two different series of pyridones was also desirable.

A further aim was to conjugate an inhibitor to a long carbon chain olefin-containing tether by a novel method using cross metathesis. Conjugates such as these are useful for attaching to a gold surface, so that biological interactions (e.g. between an inhibitor and enzyme) may potentially be studied by the use of a solid support. A sulfur atom at the tether terminus is required to facilitate attachment of a conjugate to a gold surface. Self-assembled monolayers (SAMs) occur when a gold surface is modified by an organic substance, such as an inhibitor-tether conjugate.[44] SAMs provide well defined systems for investigating biomolecular recognition, for example the interaction between an enzyme and an inhibitor. Steroid 5α-reductase has not been isolated (see section 3.1.2), so we proposed that SAM technology may be a possible method to isolate the enzyme for further investigation into its molecular conformation, as well as inhibition kinetics.

3.2 DESIGN OF NOVEL NON-STEROIDAL INHIBITORS OF STEROID 5α-REDUCTASE

Novel non-steroidal inhibitors of steroid 5α-reductase, consisting of a 5-aryl 1-methyl-pyridone scaffold, were based upon particular structural features of literature inhibitors (e.g. **3.53** and **3.48**, Fig. 3.17). Pyridones of this nature have been reported to be weak inhibitors of type 1 5α-reductase (discussed in section 3.1.4.2). The 5-aryl 1-methyl-pyridone scaffold of **3.53** was chosen as a non-steroidal skeleton for the synthesis of novel inhibitors, so SAR for substituents at the 4'-position could be further investigated and compared to SAR for the biaryl acid type inhibitors. Section 3.1.5 discussed the important structural features that influence inhibitor potency, one of which was ring substitution. It was also of interest to determine whether the potency of particular 5-aryl 1-methyl-pyridones from the literature could be enhanced by incorporating particular groups from potent known biaryl acid inhibitors in the scaffold at the 4'-position. The selection of groups to attach to the aromatic ring at the 4'-position of the pyridone scaffold (see **3.53**) was based on those from inhibitors reported by Hartmann and Reichert[24] (**3.53** and **3.54**, Fig. 3.17) and Lesuisse *et al*.[40] (**3.47-3.49**, Fig. 3.17). The *N,N*-diisopropylamide group of **3.53** and **3.54** was used as it is known to influence the potency of type 1 inhibitors previously reported by Hartmann.[25] Lesuisse *et al.* reported potent inhibition of the type 2 isozyme also using an *N,N*-diisopropylamide group but on a biaryl acid scaffold, e.g. **3.49** (Fig. 3.17). However, the potency of **3.49** is also due to the presence of an OCH_2 linker between the aromatic ring and the diisopropyl amide. The presence of the OCH_2 linker results in enhanced activity for compound **3.48** compared to **3.47**.

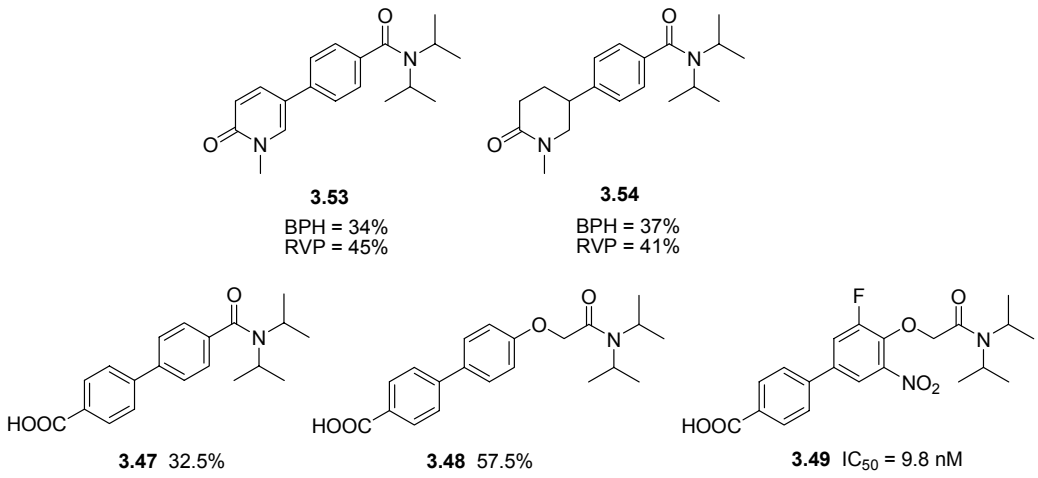
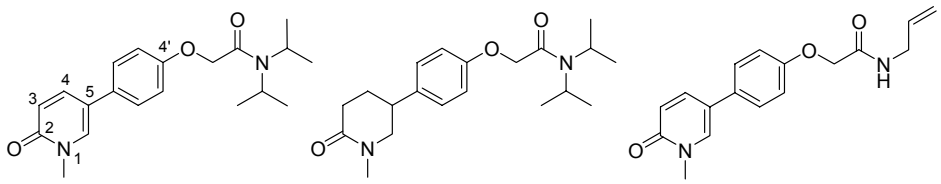


Figure 3.17. Inhibition values (% inhibition at 100 μM) for 4'-substituted benzamides **3.53** and **3.54** [BPH (type 2) and RVP (pH 6.6 type 1) denote the enzyme source, benign prostatic hyperplasia tissue, or rat ventral prostate]. % Inhibition values for **3.47** and **3.48** were obtained at 10^{-6} M.

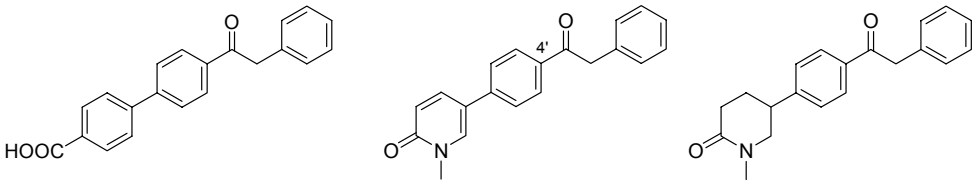
Because of the effect of the OCH_2 linker on potency for **3.48** and **3.49**, we decided to incorporate it into literature inhibitors **3.53** and **3.54** to give the novel compounds **3.55** and **3.56** (Fig. 3.18). The inhibitory activity of these novel compounds allows the evaluation of SAR for the *N,N*-diisopropylacetamide group at the 4'-position for 5-aryl 1-methyl-2-pyridone/piperidone based compounds. Compound **3.49** is a potent inhibitor of the type 2 isozyme, so inhibition data for **3.55** and **3.56** would provide useful information on whether SAR for 4'-substituents of 5-aryl 1-methyl-pyridones/piperidones correlate with SAR derived from the biaryl acid inhibitors. The *N*-allylacetamide of **3.57** was incorporated on a similar basis to the diisopropylacetamide of **3.55**. However, the allyl group was chosen specifically to facilitate conjugation with a long chain olefin-containing tether, for the potential attachment of inhibitor-tether conjugates to a gold surface (which will be discussed in further detail later in this section).



3.55 **3.56** **3.57**

Figure 3.18. Structures of initial targets for synthesis.

To evaluate SAR for substituents at the 4'-position of the pyridone scaffold, it was necessary to incorporate groups other than the acetamides already discussed. A potent 4'-benzoyl biaryl carboxylic acid inhibitor of the type 2 isozyme (**3.58**, Fig. 3.19) was reported by Holt *et al*.[35] Interest in related benzophenone aryl acid compounds stemmed from previous work which indicated potency was related to the benzophenone moiety. Therefore the benzoyl group of **3.58** was incorporated into the pyridone/piperidone scaffold under investigation in this thesis, resulting in novel compounds **3.59** and **3.60**. The presence of the bulky benzoyl group at the 4'-position (see **3.59**) allows for evaluation of SAR in relation to compounds **3.55-3.57** which have an *N*-substituted acetamide. Iinhibition data for **3.59** and **3.60** would also indicate whether SAR for the pyridone type compounds correlate with those of the biaryl carboxylic acids.



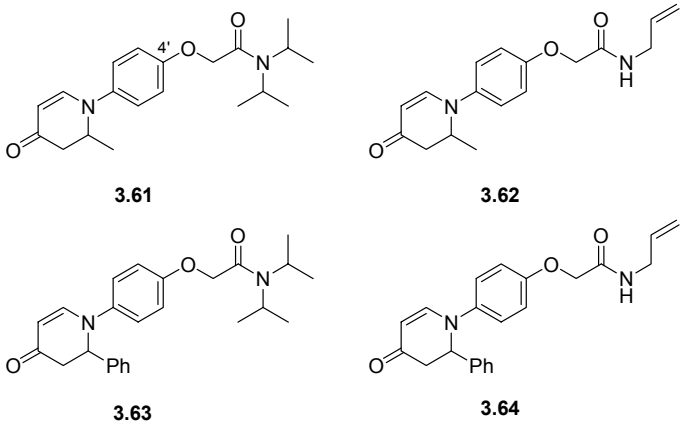
3.58

Type 2 K_i = 60 nM

3.59 **3.60**

Figure 3.19. Potent reported benzoyl inhibitor **3.58** and novel inhibitors **3.59** and **3.60**.

After the design of initial targets **3.55-3.57** and **3.59-3.60**, further compounds were developed for synthesis. The 5-aryl 1-methyl-2-pyridone type inhibitors represent non-steroidal derivatives of the 4-azasteroids, with two of the steroidal rings (B and D) missing. Inhibitors related to the 10-azasteroids (**3.7**, Fig. 3.1) with the steroidal B and D rings absent have not been reported to date. The 10-azasteroid related tricyclic benzo[c]quinolizinones (**3.30**, Fig. 3.10) have been reported, in which a steroidal D ring is absent. A 1-aryl 2-substituted 2,3-dihydro-4-pyridone (e.g. **3.61**, Fig. 3.20) represents a non-steroidal derivative of the 10-azasteroids and was used as a novel scaffold for the design of inhibitors. In analogy with compounds **3.55-3.57**, *N,N*-diisopropyl- and *N*-allylacetamide groups were incorporated at the 4'-position of the dihydro-4-pyridone scaffold to give **3.61** and **3.62**. Inhibition data for dihydro-4-pyridones **3.61** and **3.62** would allow SAR comparisons with the corresponding 5-aryl pyridones **3.55** and **3.57**, as these series differ by the position of the N atom relative to the carbonyl in the pyridone rings. Compounds **3.61** and **3.62** also have unsaturation between C2 and C3 of the pyridone ring, a structural feature which is known to influence the inhibitory potency of the related tricyclic benzo[c]quinolizinone inhibitors (see **3.30**, Fig. 3.10). The presence of angular methyl groups is also known to influence the inhibitory potency of the benzo[c]quinolizinone inhibitors, so a methyl group was incorporated at the 2-position of the pyridone ring (see **3.61**). In the design of compounds **3.63** and **3.64**, a phenyl group was incorporated at the 2-position of the pyridone ring in order to investigate the effect on inhibition that this bulky group has in comparison with a small 2-methyl group.

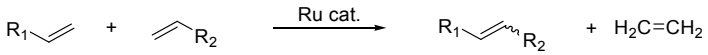


3.61 **3.62**

3.63 **3.64**

Figure 3.20. Novel non-steroidal inhibitors related to the 10-azasteroids.

Another series of potential inhibitors was also designed in which compounds **3.57**, **3.62**, and **3.64** are conjugated to a long carbon chain tether with a protected thiol at its terminus (Fig. 3.22). The purpose of conjugating a thiol-containing tether to an inhibitor is to facilitate attachment of inhibitors to a gold surface. The sulfur atom at the tether terminus is required for reaction with a gold surface to form SAMs (see section 3.1.6). Long chain tethers which are suitable for conjugating to biological ligands (e.g. inhibitors) have been reported by Svedham *et al*.[45] Poly-(ethylene glycol)-terminated alkane thiol amides (see **3.65**, Fig. 3.21) were synthesised to study the structure and stability of SAMs on gold for potential biosensing interfaces. Poly-ethylene glycol spacer units provide good anchors for biological receptors and ligands and are known to reduce the non specific binding of proteins and other bioactive molecules. In a series of tethers typified by **3.65**, the alkane chain length (x) was varied from 2C to 15C. The number of ethylene glycol units (y) was also varied to investigate the conformations induced by different chain lengths when attached to the gold surface. The approach of Svedham *et al*. was used in the design of long chain tethers **3.66** and **3.67** (Fig. 3.21). For tethers **3.66** and **3.67**, a 15C alkane chain was chosen, due to the readily available starting material, 16-mercaptohexadecanoic acid. The number of ethylene glycol units was also varied; **3.66** contains one unit, while **3.67** contains two units. An allyl group was incorporated into the terminus of these tethers as it allows conjugation to compounds **3.57**, **3.62**, and **3.64** (which also possess an allyl group) by the cross metathesis reaction. This is a novel and mild method for conjugating a biological molecule to a long carbon chain tether for the purpose of gold surface attachment. Cross metathesis is the exchange of substituents between two alkenes; terminal alkenes are particularly favourable as the reaction is driven by the release of ethylene gas (Scheme 3.4).



Scheme 3.4. Cross metathesis general reaction.

The reaction is catalysed by ruthenium compounds which tolerate a wide variety of alkene substrates. It is an ideal method for conjugating long chain tethers to biological molecules as the reaction conditions are generally mild (substrates are simply stirred in solvent in the presence of catalyst). The reaction can be done in a wide range of solvents including polar solvents (e.g. MeOH and water) without heating with an appropriate catalyst. These mild conditions are desirable for biological substrates. The inhibitor-tether conjugate targets initially designed for synthesis in this thesis are shown in Figure 3.22.

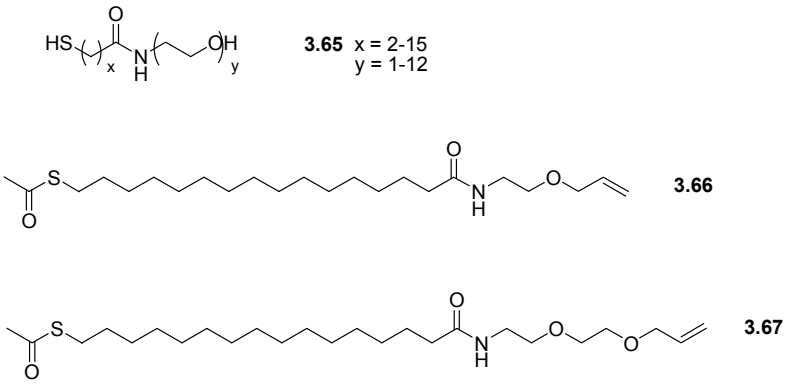


Figure 3.21. Long chain tethers **3.66** and **3.67** derived from **3.65**.

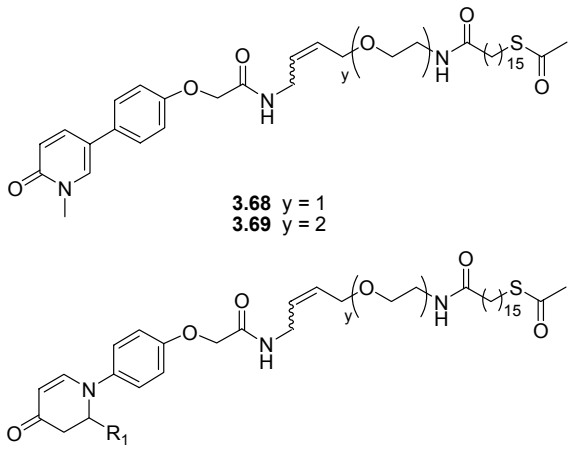


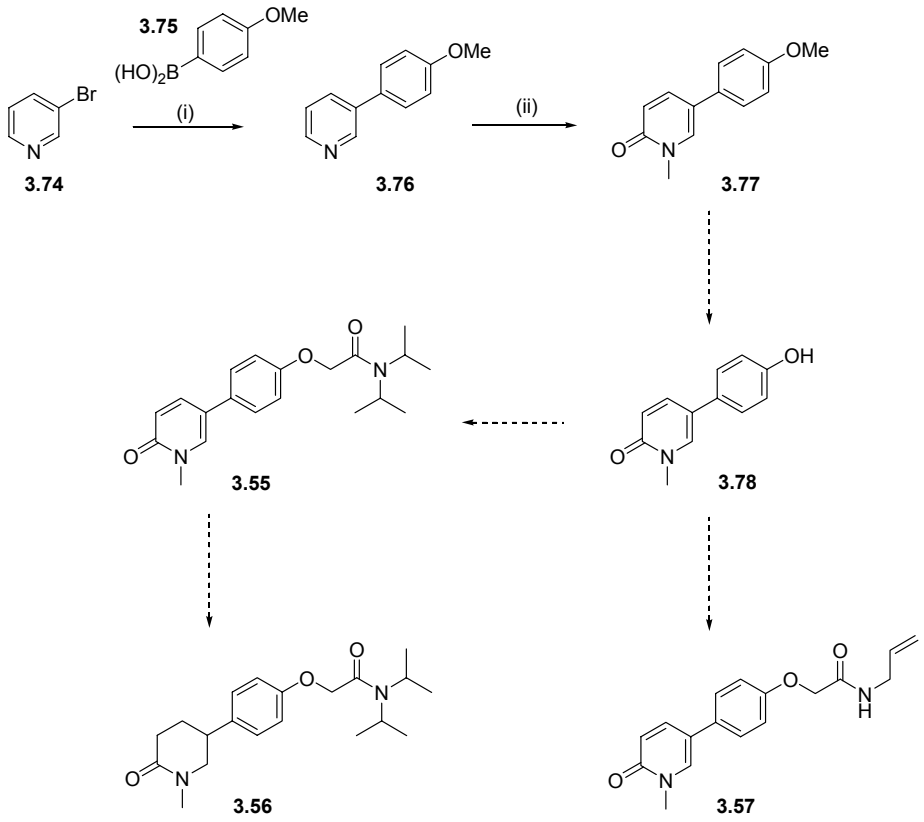
Figure 3.22. Inhibitor-tether conjugate targets.

3.3 SYNTHESIS OF 5-ARYL 1-METHYL-2-PYRIDONES/PIPERIDONE INHIBITORS 3.55-3.57

The following sections describe the synthesis of pyridones **3.55** and **3.57** and piperidone **3.56**. A key step in the scheme towards preparation of the 5-aryl 1-methyl-pyridone scaffold involved a Suzuki cross coupling to generate an aryl-substituted pyridine intermediate which could subsequently be converted into the 2-pyridone **3.77**. Two different strategies were investigated for the synthesis of **3.77**. An initial strategy is discussed in section 3.3.1, which was abandoned due to the low yielding nature of the *N*-methylation/α-oxidation reaction [Scheme 3.5, step (ii)]. The synthetic route to inhibitors **3.55-3.57** is discussed in section 3.3.2 in which 5-aryl-2-fluoropyridine (**3.80**, Scheme 3.6) was converted to the 2-pyridone **3.81** by facile acid hydrolysis. The *N,N*-diisopropyl- and *N*-allylacetamide groups of **3.83** and **3.84** (Scheme 3.7) were then introduced at the 4'-position of phenol **3.78** to give pyridone target compounds **3.55** and **3.57**. The piperidone target **3.56** was prepared by catalytic hydrogenation of **3.55**.

3.3.1 Attempted synthesis of pyridones/piperidone 3.55-3.57

The reported synthesis[24] of related literature inhibitors **3.53** and **3.54** was used for an initial attempt to prepare **3.55-3.57**. Scheme 3.5 outlines the initial strategy used for the preparation of **3.55-3.57**. A Suzuki cross coupling was utilised for the generation of key intermediate **3.76**, which would be converted to the 1-methyl-pyridinium salt and then α-oxidised to give the pyridone **3.77**. Deprotection of **3.77** to give **3.78**, followed by alkylation with an *N*-substituted bromoacetamide was envisaged to give pyridones **3.55** and **3.57**.



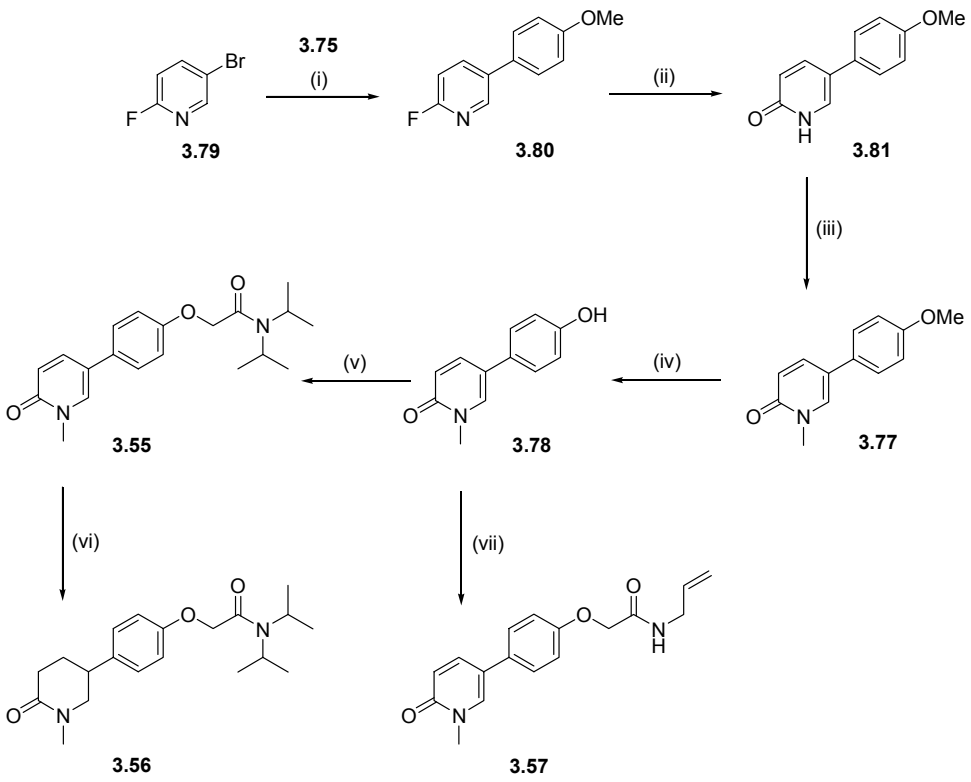
Scheme 3.5. *Reagents and conditions:* (i) KOH, *n*Bu_4NBr, $Pd(PPh_3)_4$, THF, reflux, 16 h (73%). (ii) $(MeO)_2SO_2$, 80˚C, 45 min, then $K_3[Fe(CN)_6]$, KOH (at 0˚C), DCM, rt, 16 h (8% yield).

The Suzuki cross coupling reaction of 3-bromopyridine (**3.74**) and *p*-methoxyphenyl boronic acid (**3.75**) (both commercially available) was performed in THF in the presence of tetrakis(triphenylphosphine)palladium(0) catalyst, potassium hydroxide and *n*-tetrabutylammonium bromide to give **3.76** in 73% yield. 3-Arylpyridine **3.76** was N-methylated with dimethyl sulfate, the resulting *N*-methylpyridinium salt was hydroxylated with potassium hydroxide and oxidised *in situ* with potassium ferricyanide to give the 1-methyl-pyridone **3.77** in 8% yield. This compound was difficult to purify, requiring extended chromatography resulting in a very poor yield (8%). For this reason the synthesis

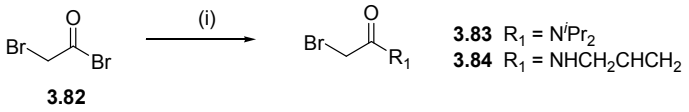
of 1-methyl-pyridone **3.77** by this method was abandoned and an alternative route to inhibitors **3.55-3.57** was sought.

3.3.2 Synthetic route to pyridones/piperidone 3.55-3.57

A revised route to inhibitors **3.55-3.57** is outlined in Scheme 3.6. Sutherland *et al*. have described the use of 5-bromo-2-fluoropyridine (**3.79**) in Suzuki cross couplings for the synthesis of 5-aryl-2-fluoropyridines.[46] These fluorinated pyridines are converted to the corresponding 2-pyridones by acid hydrolysis. Therefore **3.79** was incorporated into the synthetic strategy for preparing **3.55-3.57**. Suzuki cross coupling of **3.79** with **3.75** would give the key intermediate **3.80**, which upon acid hydrolysis would give the pyridone **3.81**. *N*-Methylation using lithium hydride and methyl iodide (coordination of lithium to the pyridone O atom supposedly promotes selective *N*-methylation),[47] is followed by deprotection to give **3.78**. Alkylation of phenol **3.78** with an *N*-substituted 2-bromoacetamide (**3.83** or **3.84**) was envisaged to give the pyridones **3.55** and **3.57**. Catalytic hydrogenation of **3.55** would produce the piperidone **3.56**.



Scheme 3.6. *Reagents and conditions:* (i) $Pd(PPh_3)_4$, KOH, $n$$Bu_4NBr$, THF, reflux, 19 h (82%). (ii) 4 M aq. HCl in dioxane, H_2O, reflux, 24 h (quantitative). (iii) LiH, DMF, 50˚C, 1.5 h, then MeI, 50˚C, 16 h (68%). (iv) 1:1 48% aq. HBr in AcOH, reflux, 16 h (83%). (v) 2 M aq. NaOH, DMSO, 80˚C, 30 min, then **3.83**, 80˚C, 2 h (46%). (vi) 10% Pd/C/H_2, EtOH, rt, 40 bar, 48 h (63%). (vii) 2 M aq. KOH, DMF, 80˚C, 1.5 h, then **3.84**, 16 h (79%).



Scheme 3.7. *Reagents and conditions:* $BnNEt_3Cl$, $ClCH_2CH_2Cl$, HN^iPr_2 or $NHCH_2CHCH_2$, NaOH, 0˚C, then $BrCH_2COBr$, rt, 16 h (**3.83** 75%, **3.84** 67%).

Scheme 3.6 details the synthesis of **3.55-3.57**. The Suzuki cross coupling reaction of 5-bromo-2-fluoropyridine (**3.79**) with *p*-methoxyphenyl boronic acid (**3.75**) (both commercially available) was performed in THF in the presence of tetrakis(triphenylphosphine)palladium(0) catalyst, potassium hydroxide and *n*-tetrabutylammonium bromide to give key intermediate **3.80** in 82% yield. Hydrolysis of 2-fluoropyridine **3.80** was carried out in 4 M aqueous hydrochloric acid in dioxane and water to give the 2-pyridone **3.81** in quantitative yield. Pyridone **3.81** was deprotonated with lithium hydride and subsequently methylated with methyl iodide to give **3.77** in 68% yield. Cleavage of the methyl aryl ether of **3.77** was achieved in equal volumes of hydrobromic acid and acetic acid to give **3.78** in 83% yield.

Scheme 3.7 outlines the synthesis of 2-bromo-*N*,*N*-diisopropylacetamide (**3.83**) and *N*-allyl-2-bromoacetamide (**3.84**)[48] required for the synthesis of **3.55** and **3.57**. Bromoacetylbromide (**3.82**) was reacted with either diisopropylamine or allylamine in the presence of sodium hydroxide and benzyltriethyl ammonium chloride in 1,2-dichloroethane to give the 2-bromoacetamides **3.83** and **3.84** in 75% and 67% yield respectively.

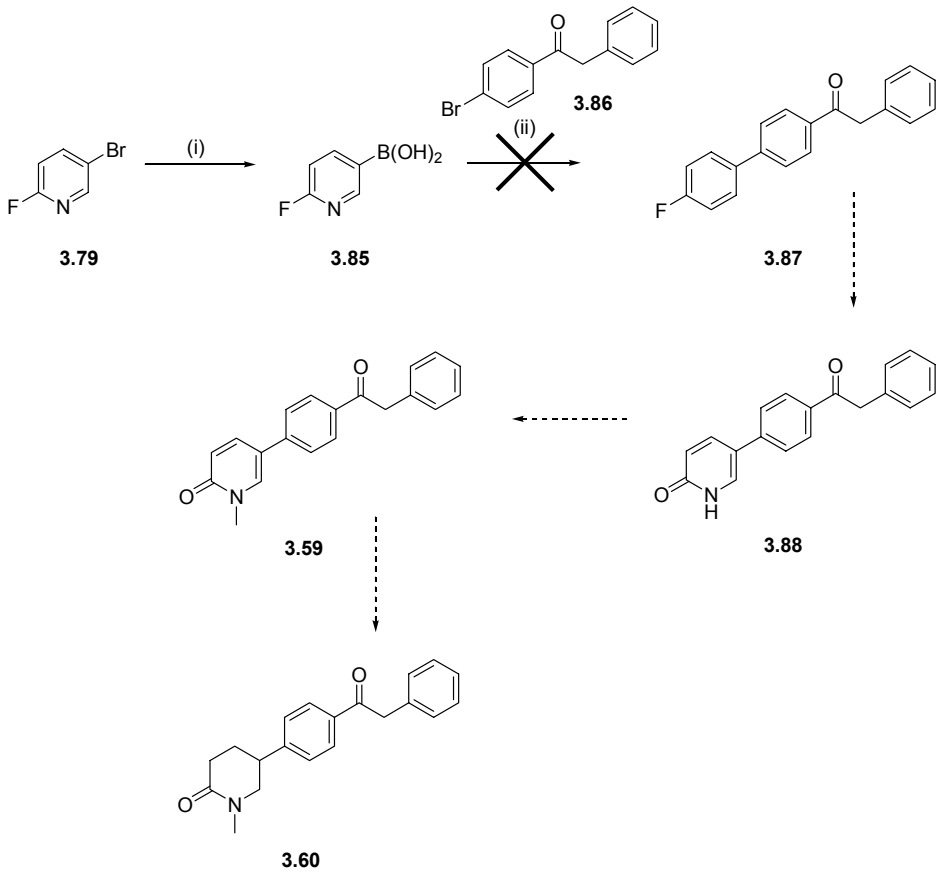
The alkylation reaction of phenol **3.78** with **3.83** was performed in DMSO in the presence of aqueous sodium hydroxide to give phenoxyacetamide **3.55** in 46% yield. Compound **3.78** was also alkylated with **3.84**, but in DMF in the presence of potassium hydroxide, to give **3.57** in 79% yield. Catalytic hydrogenation of **3.55** was carried out in ethanol in a PARR hydrogenator with H_2 at 40 bar and 10% palladium on charcoal to give the racemic piperidone **3.56** in 63% yield. The piperidone derivative of **3.57** was not synthesised due to probable hydrogenation of the allyl group.

3.4 SYNTHESIS OF BENZOYL INHIBITOR 3.59

The following section describes work in the synthesis of benzoyl inhibitor **3.59**. A key step in the synthetic strategy for preparation of the 5-aryl 1-methyl-pyridone scaffold (outlined in Scheme 3.8) was a Suzuki cross coupling of fluorinated boronic acid **3.85** and benzyl-4-bromophenyl ketone (**3.86**). Several different cross coupling conditions were investigated before the 2-fluoropyridone **3.87** was successfully synthesised. Initial attempts to synthesise key intermediate **3.87** are discussed in section 3.4.1, while section 3.4.2 describes the final synthesis of inhibitor **3.59**.

3.4.1 Attempted synthesis of key intermediate 3.87

Initially it was envisaged that inhibitor **3.59** could be synthesised via the methodology developed for the pyridones **3.55** and **3.57** [see Scheme 3.6, steps (i) to (iii)]. Scheme 3.8 outlines an initial strategy for the synthesis of benzoyl compound **3.59**. Benzyl-4-bromophenyl ketone (**3.86**) was identified as a reactant for Suzuki cross coupling to generate the key intermediate 2-fluoropyridine **3.87**. 5-Bromo-2-fluoropyridine (**3.79**) required conversion to boronic acid **3.85**, via bromine-lithium exchange and subsequent reaction with trimethyl borate, to faciliate cross coupling with **3.86**. The remaining steps in Scheme 3.8 were derived from the methodology shown in Scheme 3.6. Acid hydrolysis of 2-fluoropyridine **3.87** would give the 2-pyridone **3.88**, which could be *N*-methylated to give 1-methyl-pyridone **3.59**. Catalytic hydrogenation of **3.59** was envisaged to give the piperidone **3.60** in the final step.

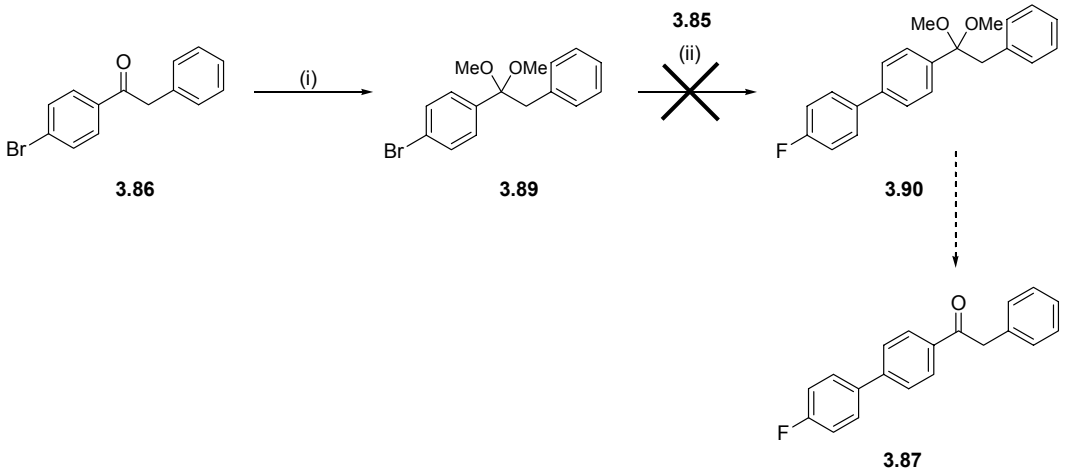


Scheme 3.8. *Reagents and conditions:* (i) *n*BuLi, Et_2O, -78˚C, 45 min, then $B(OMe)_3$, 1 h (73%). (ii) KOH, *n*Bu_4NBr, $Pd(PPh_3)_4$, THF, reflux, 16 h.

5-Bromo-2-fluoropyridine (**3.79**) was converted to an organolithium species *in situ* with *n*-butylithium and reacted with trimethylborate to give boronic acid **3.85** in 73% yield.[49] The work up for this reaction was important and involved slow addition of aqueous sodium hydroxide to the reaction mixture, followed by careful addition of aqueous hydrochloric acid at 0˚C to prevent protodeboronation. The cross coupling reaction of **3.85** and benzyl-4-bromophenyl ketone (**3.86**) was carried out in THF in the presence of tetrakis(triphenylphosphine)palladium(0), potassium hydroxide and *n*-tetrabutyl ammonium bromide. However, when the crude product was analysed by ^{1}H NMR, unidentified material was observed. From this observation it was assumed that the cross coupling of **3.85** and **3.86** did not proceed as desired.

Three equivalents of a strong base, potassium hydroxide, were used in the coupling reaction. Since **3.86** can be deprotonated α to the carbonyl, the base may have reacted with ketone **3.86**. Organoboron species (such as **3.85**) are poor nucleophiles and cross coupling with aryl halide electrophiles does not usually proceed unless a negatively charged base is present to enhance the nucleophilicity of the organic group on the boron atom.[50] Reaction of potassium hydroxide with **3.86** would reduce the amount of base available to activate **3.85**, suggesting a possible reason as to why the coupling of **3.85** and **3.86** did not proceed as desired. The low solubility of potassium hydroxide in THF observed would also reduce the amount of hydroxide ion present in solution.

As the cross coupling of **3.85** and **3.86** did not proceed under the conditions described in Scheme 3.8, a new strategy for the synthesis of key intermediate **3.87** was devised. It was envisaged that if potential α-deprotonation of **3.86** by potassium hydroxide could be prevented then cross coupling might occur. Protection of the carbonyl functional group in **3.86** would remove the possibility of α-deprotonation. Carbonyl groups are easily converted to acetal derivatives, so the carbonyl of ketone **3.86** was protected through conversion to the acetal in **3.89**. Scheme 3.9 presents a revised strategy for synthesising key intermediate **3.87**.

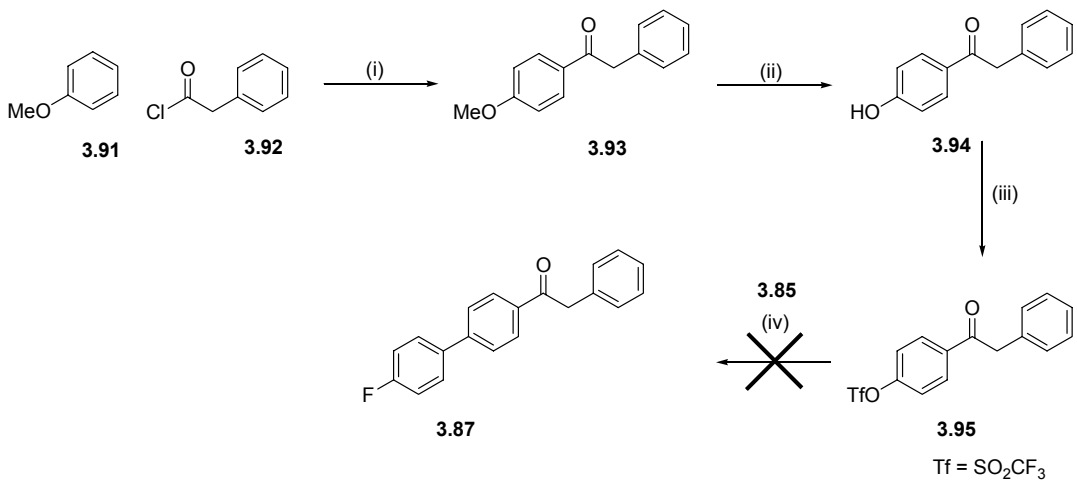


3.86 **3.89** **3.90** **3.87**

Scheme 3.9. *Reagents and conditions:* (i) $CH(OMe)_3$, CF_3SO_3H, 4Å sieves, MeOH, rt, 16 h (73%). (ii) KOH, nBu$_4$NBr, Pd(PPh$_3$)$_4$, THF, reflux, 16 h.

Benzyl-4-bromophenyl ketone (**3.86**) was reacted with trimethylorthoformate in methanol in the presence of catalytic trifluoromethane sulfonic acid and 4Å molecular sieves to give the acetal **3.89** in 73% yield. The cross coupling reaction of boronic acid **3.85** and **3.89** was performed in THF in the presence of tetrakis(triphenylphosphine)palladium(0), potassium hydroxide and *n*-tetrabutyl ammonium bromide. Analysis of the crude product by ^{1}H NMR revealed that only starting material was present, so it was assumed that the cross coupling did not proceed to give **3.90**.

Since cross coupling between the protected ketone **3.89** and boronic acid **3.85** did not proceed, a new strategy was devised to synthesise **3.87** (see Scheme 3.10). For the cross coupling depicted in Scheme 3.8 [step (ii)], a strong base (potassium hydroxide) was used for the activation of boronic acid **3.85**. A weaker base than potassium hydroxide was thought to aid coupling with the suspected base sensitive starting material **3.86**. Weakly basic conditions have been reported in the Suzuki coupling of triflates with boronic acids.[50] A triflate derivative of ketone **3.86** was therefore synthesised for coupling with boronic acid **3.85**. Different reaction conditions are required when a triflate is used for coupling with a boronic acid. The reaction of triphenylphosphine (palladium catalyst ligand) with triflates is thought to cause catalyst decomposition.[50] Potassium bromide is often added to prevent decomposition of the Pd catalyst (precipitating palladium black is observed at the early stage of reaction), which is proposed to cause failure of triflate mediated cross coupling. Addition of potassium bromide to the reaction prevents catalyst decomposition. A method using tetrakis(triphenylphosphine)palladium(0), tripotassium phosphate, and potassium bromide in dioxane[40] was identified for use in an attempt to couple boronic acid **3.85** with triflate **3.95** (Scheme 3.10).

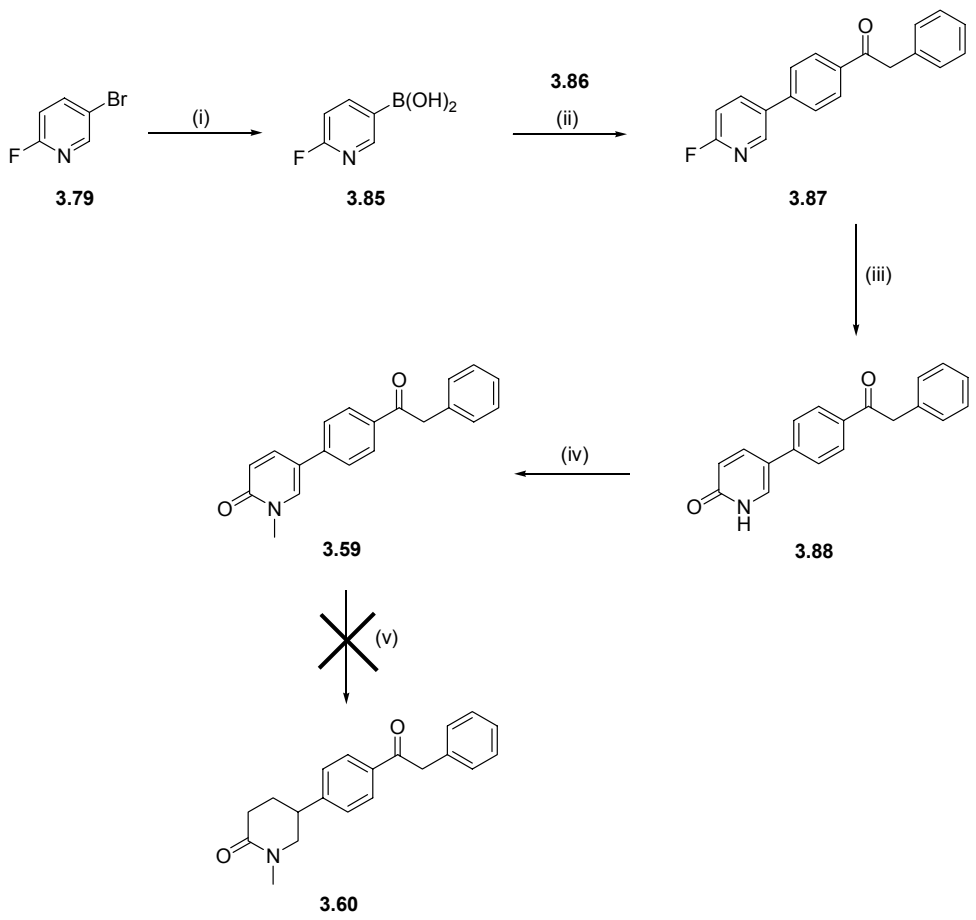


Scheme 3.10. *Reagents and conditions:* (i) $AlCl_3$, $ClCH_2CH_2Cl$, rt, 16 h (60%). (ii) 1:1 48% aq. HBr in AcOH, reflux, 7 h (57%). (iii) Pyridine, $(CF_3SO_2)_2O$, CH_2Cl_2, 0˚C, 45 min (59%). (iv) $Pd(PPh_3)_4$, K_3PO_4, KBr, dioxane, 85˚C, 16 h.

Scheme 3.10 outlines the attempted synthesis of **3.87** via coss coupling of triflate **3.95** with boronic acid **3.85**. Friedel-Crafts acylation of anisole (**3.91**) with phenylacetyl chloride (**3.92**) in 1,2-dichloroethane in the presence of aluminium trichloride gave benzoyl compound **3.93** in 60% yield.[51] The methyl ether of **3.93** was cleaved with aqueous hydrobromic acid in acetic acid to give phenol **3.94** in 57% yield. Phenol **3.94** was reacted with triflic anhydride and a catalytic amount of pyridine in DCM to give triflate **3.95** in 59% yield. The cross coupling of boronic acid **3.85** with triflate **3.95** was carried out in dioxane in the presence of tetrakis(triphenylphosphine)palladium(0), potassium bromide and tripotassium phosphate. However, when the crude product was analysed by ^1H NMR, only starting material and unidentified material were observed. This method for coupling was subsequently abandoned.

3.4.2 Synthetic route to benzoyl inhibitor 3.59

A literature search was conducted to find other possible conditions for synthesising key intermediate **3.87** via Suzuki cross coupling. A significant paper was identified in which Br-substituted aryl ketones were coupled to aryl boronic acids under mild conditions, using tetrakis(triphenylphosphine)palladium(0) with aqueous sodium carbonate in ethanol and toluene. The ketone and boronic acid starting materials for couplings described in the paper were similar to **3.86** and **3.85**, so the reported mild conditions were of interest for synthesising **3.87**. These conditions were used to couple **3.85** with **3.86** to successfully generate the desired 2-fluoropyridine **3.87** in excellent yield. Investigations into the synthesis of **3.87** described here have led to the conclusion that the choice of conditions for Suzuki cross coupling is critical. Scheme 3.11 outlines the synthesis of benzoyl inhibitor **3.59**.



Scheme 3.11. *Reagents and conditions:* (i) *n*BuLi, Et_2O, -78˚C, 45 min, then $B(OMe)_3$, rt, 1 h (73%). (ii) $Pd(PPh_3)_4$, 2 M aq. Na_2CO_3, EtOH, toluene, reflux, 18 h (85%). (iii) 4 M aq. HCl in dioxane, H_2O, reflux, 24 h (95%). (iv) LiH, DMF, rt, 1 h, then MeI, 16 h (52%). (v) 10% Pd/C/H_2, rt, 40 bar, 48 h.

The cross coupling of boronic acid **3.85** with benzyl-4-bromophenyl ketone (**3.86**) was performed in ethanol/toluene in the presence of tetrakis(triphenylphosphine)palladium(0) and aqueous sodium carbonate to give 2-fluoropyridine **3.87** in 85% yield. The ^{1}H NMR spectrum of 2-fluoropyridine **3.87** is shown in Figure 3.23. The presence of the product **3.87** could be crudely determined by observing the chemical shift of the peak which represents the methylene protons adjacent to the carbonyl. This chemical shift was compared to that of the corresponding protons in the starting material **3.86** (Scheme 3.9).

In the ^{1}H NMR spectrum of **3.86** (not shown here), a singlet representing the methylene protons was observed at 4.22 ppm, but in the spectrum of product **3.87** (Fig. 3.23), this singlet is present at 4.32 ppm. HRMS analysis also confirmed the presence of **3.87** by the accurate mass obtained for protonated product ($C_{19}H_{15}NOF$ = 292.1132 g mol^{-1}, calculated mass = 292.1138 g mol^{-1}). Hydrolysis of 2-fluoropyridine **3.87** was carried out in 4 M aqueous hydrochloric acid in dioxane and water to give the 2-pyridone **3.88** in 95% yield. Pyridone **3.88** was deprotonated with lithium hydride at rt and subsequently methylated with methyl iodide to give **3.59** in 68% yield. Catalytic hydrogenation of **3.59** in methanol in the presence of 10% palladium on charcoal was attempted, but the expected piperidone product **3.60** was not obtained. When the crude product was analysed by ^{1}H NMR, unidentified material was observed.

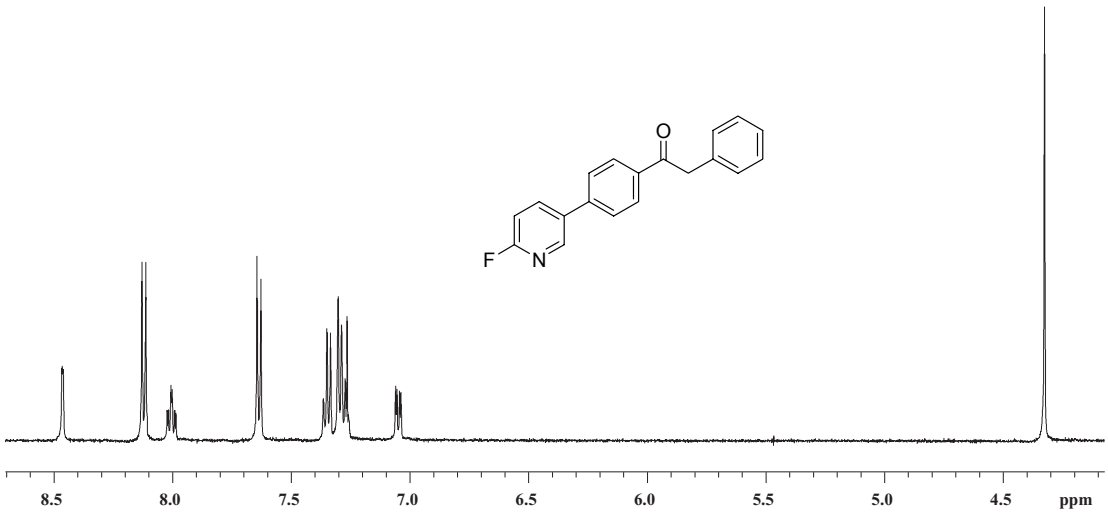
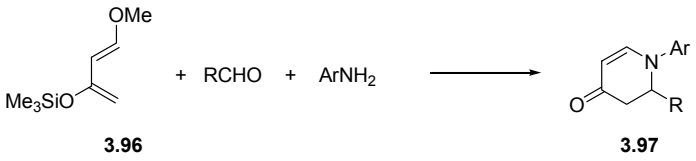


Figure 3.23. ^{1}H NMR spectrum ($CDCl_3$, 500 MHz) of cross coupling product **3.87**.

3.5 SYNTHESIS OF 1-ARYL 2-METHYL 2,3-DIHYDRO-4-PYRIDONE INHIBITORS AND ATTEMPTED SYNTHESIS OF 2-PHENYL ANALOGUES

The synthesis of 1-aryl 2-methyl 2,3,-dihydro-4-pyridone inhibitors **3.61** and **3.62** is discussed in the following section (see Scheme 3.13). A search of the literature revealed that 2-substituted 2,3-dihydro-4-pyridones (see **3.97**, Scheme 3.12) can be synthesised by the aza Diels-Alder reaction of Danishefsky's diene (**3.96**) with imines. An efficient protocol for this reaction was reported by Yuan *et al.* in which the reaction occurs in methanol at rt without acid catalysis (Scheme 3.12).[52]

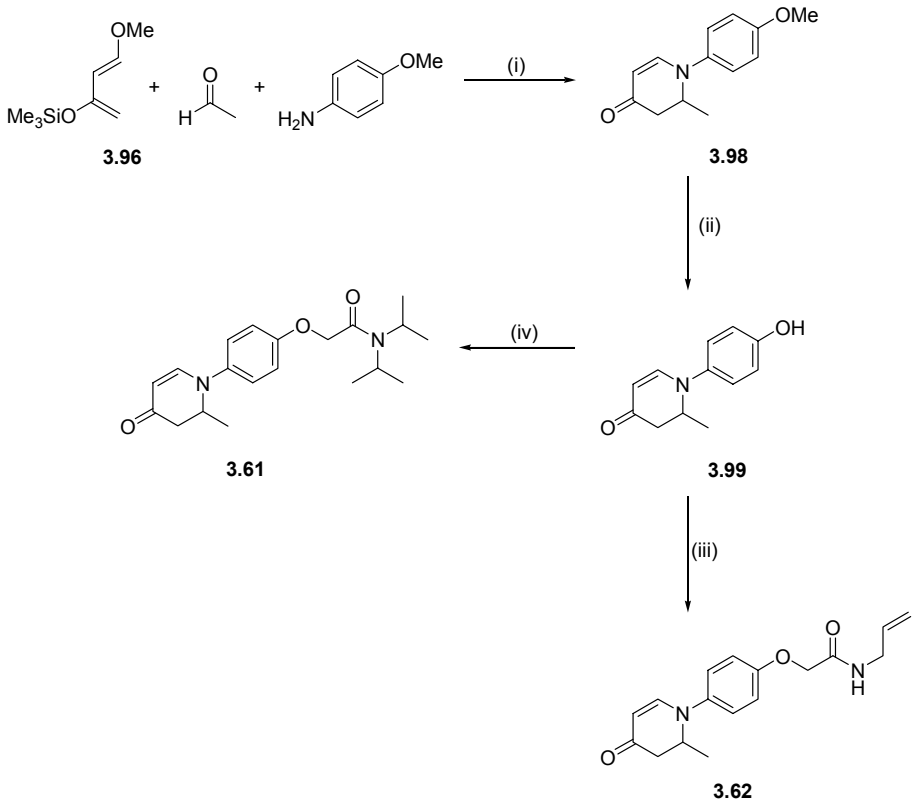


3.96 **3.97**

Scheme 3.12. Aza Diels-Alder reaction of Danishefsky's diene with imines.

3.5.1 Synthetic route to dihydro-4-pyridones 3.61 and 3.62

A one pot aza Diels-Alder procedure was used to synthesise the 1-aryl 2-methyl 2,3-dihydro-4-pyridone scaffold of **3.61** and **3.62** using an appropriate aldehyde (acetaldehyde) to incorporate the desired methyl functionality at the 2-position. Scheme 3.13 outlines the synthetic route to inhibitors **3.61** and **3.62**.

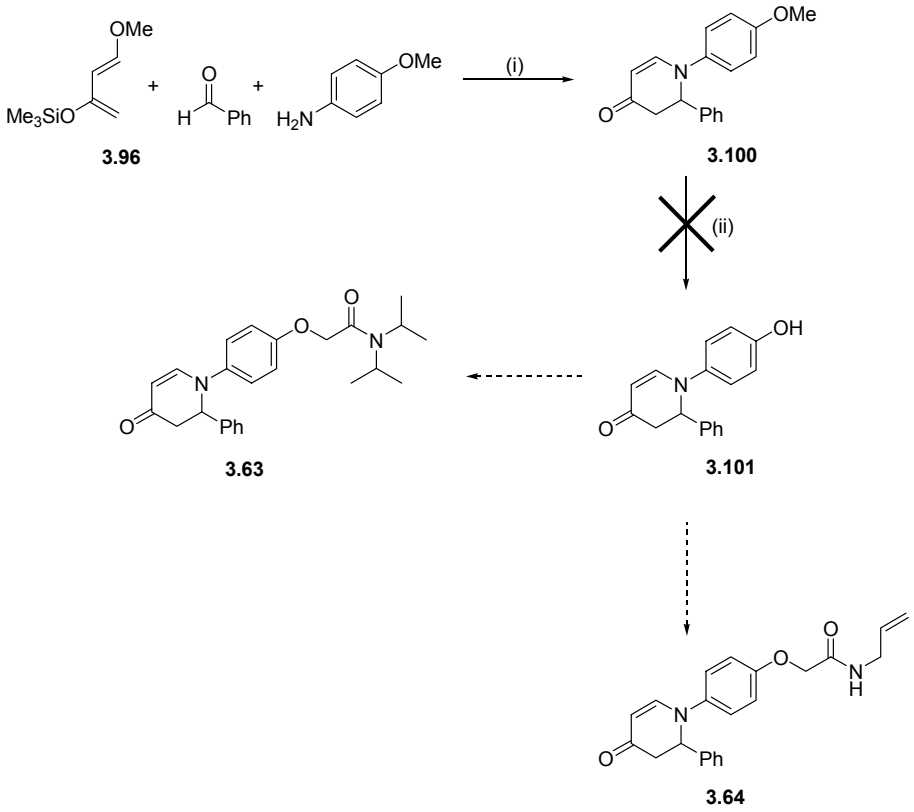


Scheme 3.13. *Reagents and conditions:* (i) MeOH, rt, 2 h (89%). (ii) 1:1 48% aq. HBr in AcOH, 80˚C, 8 h (66%). (iii) 2 M aq. KOH, DMF, 80˚C, 1.25 h, then **3.84**, 16 h (30%). (iv) 2 M aq. KOH, DMF, 80˚C, 30 min, then **3.83**, 16 h (43%).

To the imine (dienophile), generated *in situ* by reacting acetaldehyde with *p*-anisidine, was added Danishefsky's diene (**3.96**) to give the racemic dihydro-4-pyridones **3.98** in 89% yield. Cleavage of the aryl methyl ether of **3.98** to give the phenols **3.99** was accomplished with equal volumes of aqueous hydrobromic acid and acetic acid in 66% yield. Phenols **3.99** were alkylated with either bromoacetamide **3.84** or **3.83** in DMF in the presence of potassium hydroxide to give the 2-methyl 2,3-dihydro-4-pyridone inhibitors **3.62** and **3.61** in 30% and 43% yield respectively.

3.5.2 Attempted synthesis of dihydro-4-pyridones 3.63 and 3.64

The synthetic route to 2-phenyl 2,3-dihydro-4-pyridones **3.63** and **3.64** (Scheme 3.14) was devised by analogy to the synthesis of **3.61** and **3.62** (Scheme 3.13). Benzaldehyde was used for the key aza Diels-Alder step to incorporate the phenyl group at the 2-position of the dihydro-4-pyridone ring.



Scheme 3.14. *Reagents and conditions:* (i) MeOH, rt, 2 h, (64%).

The key starting material, 2-phenyl 2,3-dihydro-4-pyridone **3.100**, was synthesised via a one pot aza Diels-Alder reaction as shown in Scheme 3.14 step (i). A solution of benzaldehyde and *p*-anisidine in methanol was stirred to form the imine *in situ*, to which diene **3.96** was added to give the racemic 2-phenyl 2,3-dihydro-4-pyridones **3.100** in 64% yield. Cleavage of the methyl aryl ether of **3.100** with aqueous hydrobromic acid and acetic

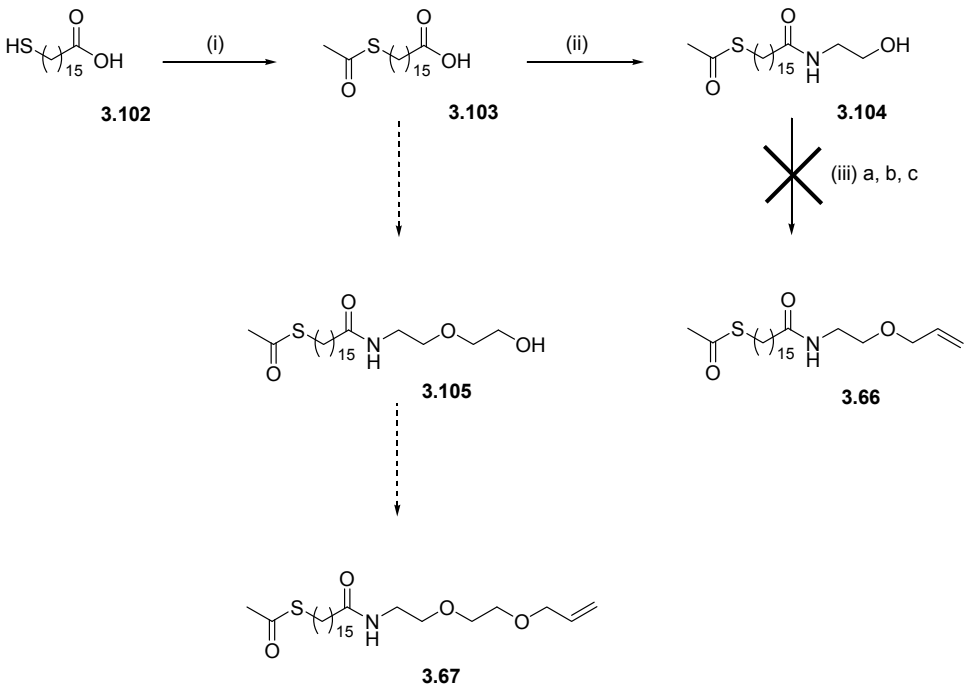
acid was attempted. When the crude product was analysed by ^{1}H NMR, an intractable mixture of compounds was observed. In order to find another method to cleave the methyl aryl ether group of **3.100**, more of this starting material needed to be synthesised. However the synthesis of **3.100** could not be reproduced, so further synthetic work in this area was discontinued.

3.6 SYNTHESIS OF LONG CARBON CHAIN TETHERS AND INHIBITOR-TETHER CONJUGATES

The design of novel steroid 5α-reductase inhibitor-tether conjugates was discussed in section 3.2. The following section describes the attempted synthesis of initial tether targets **3.66** and **3.67**. These tethers were not successfully synthesised so a different approach was required to generate alternative terminal olefin containing tethers for cross metathesis with inhibitors **3.57** and **3.62**. Section 3.6.2 describes the synthesis of alternative tethers **3.107** and **3.108**.

3.6.1 Attempted synthesis of initial tether targets

A proposed synthetic route to tethers **3.66** and **3.67** is outlined in Scheme 3.15. Due to the high nucleophilicity of sulfur, the thiol group of **3.102** required protection to avoid inter- and intramolecular thioesterification during the coupling step [Scheme 3.15 step (ii)]. Ethanolamine or 2-(2-aminoethoxy)ethanol would then be coupled to the acid **3.103** to give the alcohols **3.104** and **3.105**. Allylation of the alcohols **3.104** and **3.105** with allyl bromide was envisaged to give **3.66** and **3.67** as desired for conjugation to inhibitors **3.57** and **3.62**.

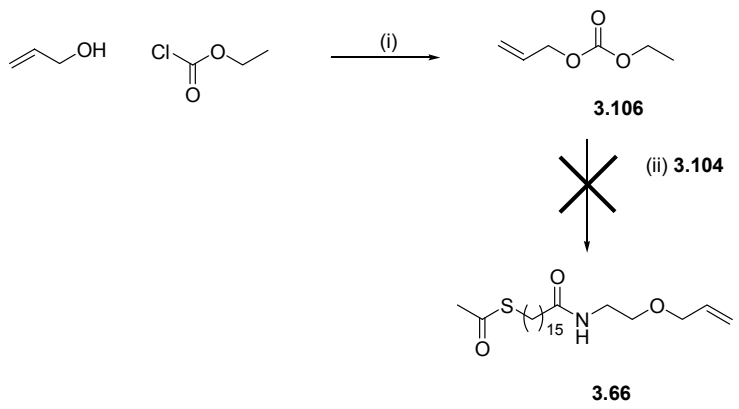


Scheme 3.15. *Reagents and conditions:* (i) Zn, CH_2Cl_2, AcOH, rt, 15 min, then AcCl, 0˚C, rt, 10 min (83%). (ii) EDCI, HOBt, ethanolamine, DCM, 16 h (93%). (iii) (a.) *n*Bu_4NBr, 10% aq. NaOH, DCM, rt, 1 h, then allyl bromide, 16 h. (b) KOH, allyl bromide, DMF, rt, 3 h. (c) NaH, THF, rt, 1 h, then allyl bromide, 16 h.

Reduction of contaminant disulfide and subsequent acetylation of **3.102** was carried out in a one-pot reaction with zinc and acetyl chloride to give the thioacetylated acid **3.103** in 83% yield. Ethanolamine was coupled to **3.103** under EDCI coupling conditions[45] to give **3.104** in 93% yield. Attempts were then made to allylate the alcohol of **3.104** with allyl bromide under various conditions. Firstly the alcohol was treated with 10% aqueous sodium hydroxide in DCM with *n*-tetrabutyl ammonium bromide, followed by the addition of allyl bromide to give a mixture of compounds by ^{1}H NMR spectroscopy. Starting material **3.104** was observed in the ^{1}H NMR spectrum, as well as unidentified material. However, analysis by electrospray LRMS indicated a mixture in which the desired protonated product was present ($C_{23}H_{43}NO_3S$ = 414.3204 g.mol^{-1}). From this observation it was assumed that product **3.66** was present as a very minor constituent of the crude ^{1}H NMR spectrum. Therefore, an alternative method was used to allylate alcohol **3.104** with

allyl bromide in DMF in the presence of potassium hydroxide. Starting material was observed in the ^{1}H NMR spectrum of the crude product, as was confirmed by TLC. The desired product **3.66** was not evidentially obtained. A third method was attempted to allylate alcohol **3.104** with allyl bromide THF in the presence of NaH. Again a mixture of starting material and unidentified material was observed by ^{1}H NMR spectroscopy.

An alternative method was sought for allylating the alcohol of **3.104**. Zumpe and Kazmaier reported a method for selective *O*-allylation of amino acid side chains with allyl ethylcarbonate (**3.106**) in the presence of π-allyl-palladium chloride dimer catalyst.[53] The hydroxyl group of the side chain of serine (CH_2OH) was selectively allylated over the NH group. Therefore, this method was attempted for allylating alcohol **3.104** (which also contains an NH group). Scheme 3.16 outlines the attempted synthesis of **3.66**.

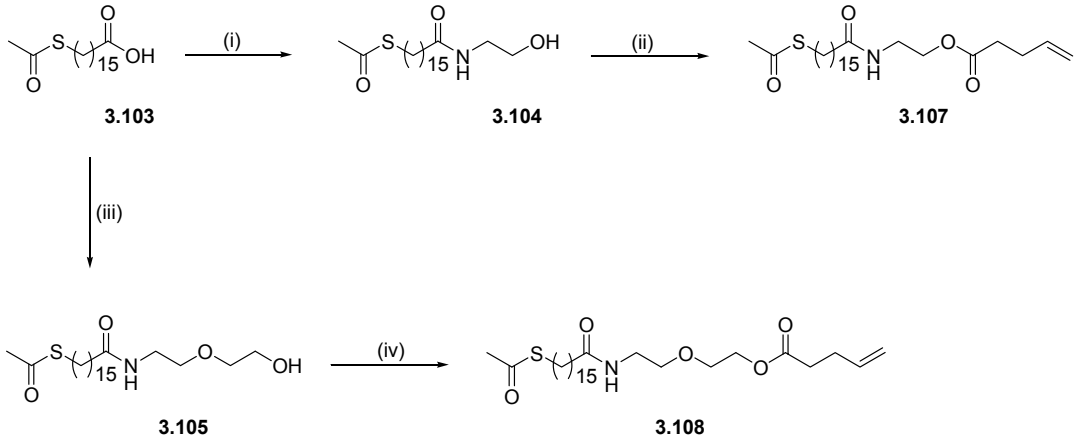


Scheme 3.16. *Reagents and conditions:* (i) Allyl alcohol, THF, 0˚C, 10 min, then ethyl chloroformate, rt, 3 h (47%). (ii) PPh_3, $[allylPdCl_2]_2$, THF, rt, 16 h.

Coupling of allyl alcohol and ethyl chloroformate gave the allyl carbonate **3.106** in 47% yield. *O*-allylation of **3.104** with the allyl carbonate **3.106** was attempted in THF with triphenylphosphine, in the presence π-allyl-palladium chloride dimer. However, only starting materials were observed in the ^{1}H NMR spectrum of the crude product, which was confirmed by the presence of two spots by TLC which correlated with those of **3.104** and **3.106**.

3.6.2 Synthesis of alternative tethers 3.107 and 3.108

Four different methods were utilised in the attempted synthesis of tether **3.66**. Allylation of the hydroxyl group in **3.104** seemed to be difficult so a different approach was investigated to introduce an allyl group into the long chain tethers, as shown in Scheme 3.17. Instead of directly allylating the hydroxyl of **3.104**, a terminal olefin could be introduced by coupling 4-pentenoic acid to the alcohol **3.104**, as depicted in Scheme 3.17. 4-Pentenoic acid was chosen as it was a readily available terminal olefin containing compound with a carboxylic acid group to facilitate esterification with the hydroxyl group of **3.104**.

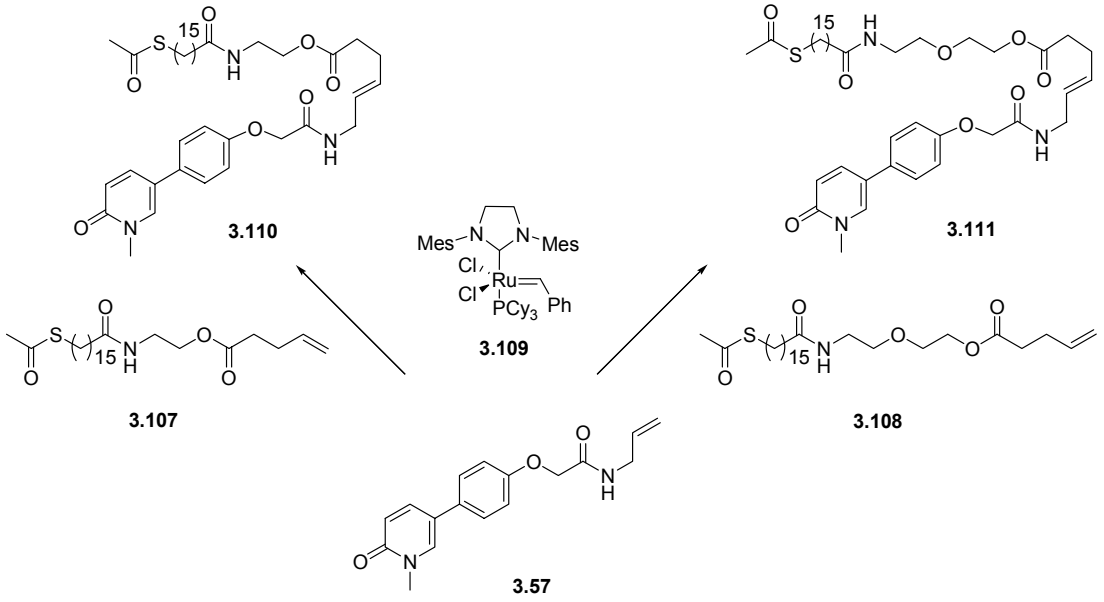


Scheme 3.17. *Reagents and conditions:* (i) EDCI, HOBt, ethanolamine, DCM, rt, 16 h (93%). (ii) DMAP, 4-pentenoic acid, EDCI, DCM, rt, 3 h (49%). (iii) 2-(2-Aminoethoxy)ethanol, EDCI, HOBt, DCM, rt, 16 h (95%). (iv) DMAP, 4-pentenoic acid, EDCI, rt, 16 h (62%).

Ethanolamine was coupled to **3.103** under EDCI coupling conditions to give **3.104** in 93% yield. Esterification of **3.104** was carried out in DCM with 4-pentenoic acid, DMAP and EDCI[54] to give the desired tether **3.107** in 49%. The acid **3.103** was also coupled to 2-(2-aminoethoxy)ethanol under EDCI conditions to give the alcohol **3.105** in 95% yield. Esterification of **3.105** was accomplished with 4-pentenoic acid in DCM in the presence of DMAP and EDCI to give the desired tether **3.108** in 62% yield.

3.6.3 Synthesis of inhibitor-tether conjugates 3.110 and 3.111

Conjugation of tethers **3.107** and **3.108** to inhibitor **3.57** and also **3.62** was attempted using cross metathesis methodology (which was discussed in section 3.2). Grubbs' second generation ruthenium catalyst **3.109** was employed for the cross metathesis outlined in Scheme 3.18. This particular metathesis catalyst was used as it is tolerant to a wide range of substrates, and is more stable to air and water than the first generation catalyst. The second generation catalyst was also readily available.



Scheme 3.18. *Reagents and conditions:* 20 mol% Grubbs' 2nd gen. catalyst **3.109**, 1,1,2-trichloroethane, rt, 16 h, **3.110** (30%), **3.111** (52%).

Tether **3.107** was reacted with inhibitor **3.57** in 1,1,2-trichloroethane in the presence of 20 mol% Grubbs' second generation catalyst **3.109** to give conjugate **3.110** in 30% yield. A flow of nitrogen was used to remove the ethylene gas side product to help drive the reaction to completion. Tether **3.108** was reacted with **3.57** in an analogous fashion to give conjugate **3.111** in 52% yield. The ^{1}H NMR spectrum of conjugate **3.111** is shown in Figure 3.24. A characteristic multiplet at 5.57 ppm, which represents the vicinal hydrogens

of the double bond, indicated the formation of a new double bond from cross metathesis. The configuration of the double bond of conjugates **3.110** and **3.111** was assumed to be *E* for steric reasons. This assumption was confirmed by calculation of the vicinal coupling constant across the double bond for **3.111**. A vicinal coupling constant of 15 Hz was extrapolated from the multiplet present at 5.57 ppm. It is possible that traces of the *Z* isomers were present but corresponding peaks were not observed in the ^{1}H NMR and ^{13}C NMR spectra of **3.110** and **3.111**. A single set of signals was observed in the ^{13}C NMR spectra of both **3.110** and **3.111**, indicating that only one isomer was present.

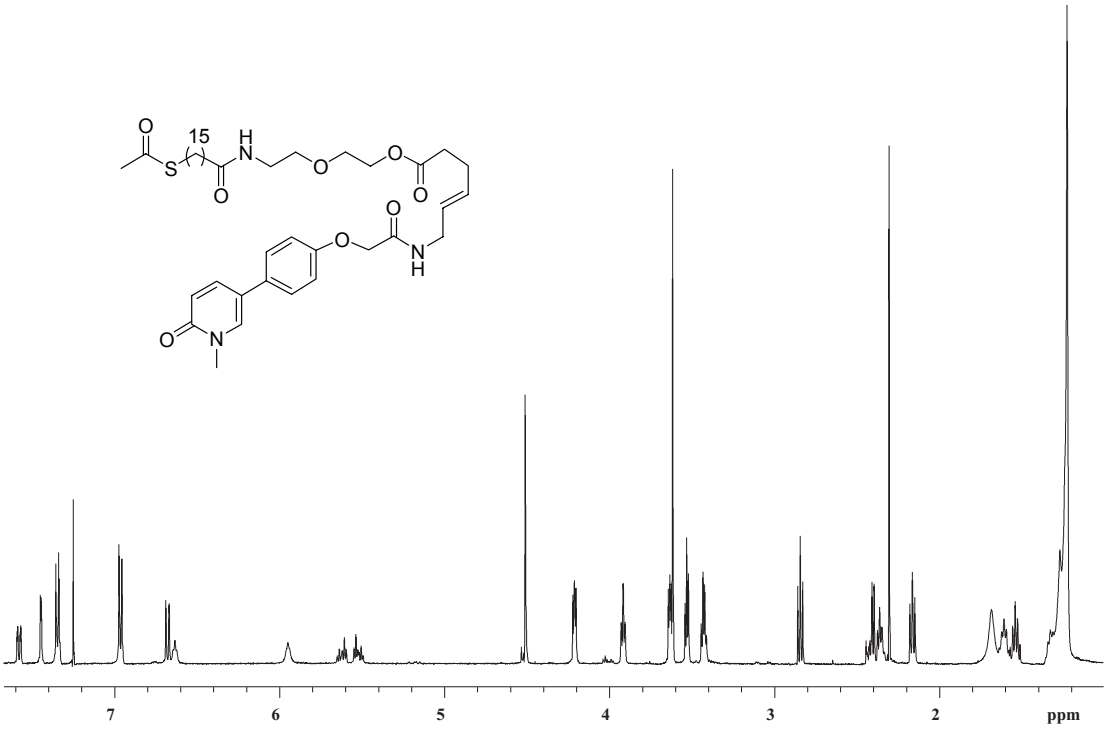
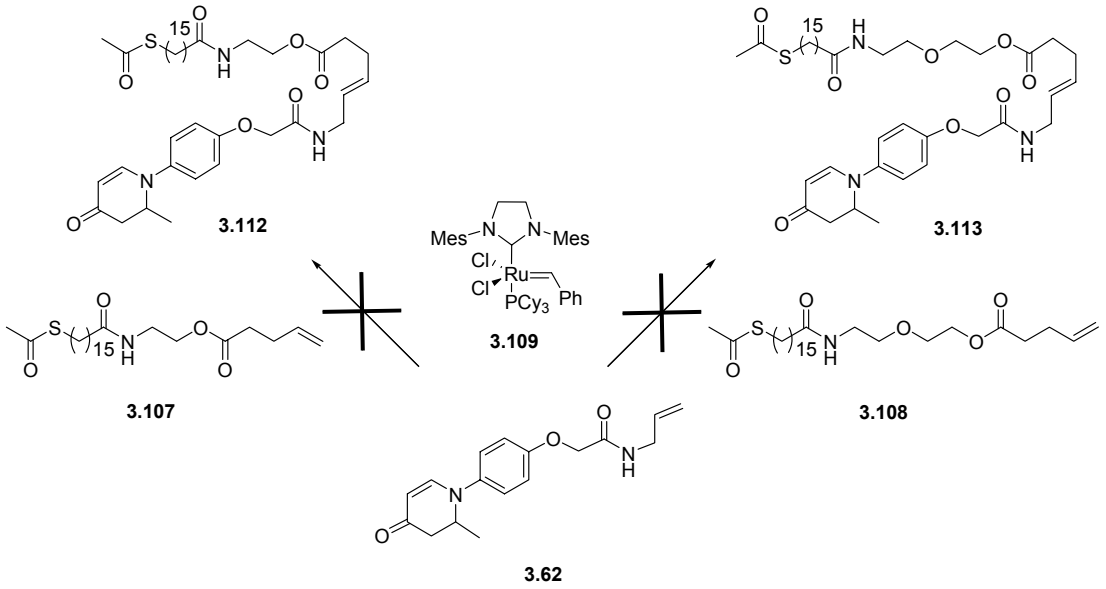


Figure 3.24. ^{1}H NMR spectrum ($CDCl_3$, 500 MHz) of inhibitor-tether conjugate **3.111**.

3.6.4 Attempted synthesis of inhibitor-tether conjugates 3.112 and 3.113

The methodology described in section 3.6.3 was used in the synthetic strategy for conjugates **3.112** and **3.113**, as shown in Scheme 3.19.



Scheme 3.19. *Reagents and conditions:* as for Scheme 3.18.

Inhibitor **3.62** was reacted with tether **3.107** or **3.108** in the presence of Grubbs' second generation catalyst **3.109** in 1,1,2-trichloroethane, but the expected conjugate products **3.112** and **3.113** were not evidentially obtained. The ^{1}H NMR spectrum of both reactions revealed that starting materials were present. The reaction of **3.62** with **3.108** was repeated, but at 50˚C rather than rt. However, when the crude product was analysed by ^{1}H NMR, starting material was again observed. From these observations it appears that cross metathesis of **3.62** with tethers **3.107** and **3.108** does not proceed under these conditions.

3.7 INHIBITION ASSAY

Methods for the biological testing of steroid 5α-reductase inhibitors were discussed in section 3.1.6. The cell lines and methodology required for testing 5α-reductase inhibitors was beyond the scope of this project. Therefore inhibitors synthesised in this thesis (**3.55-3.57**, **3.59**, **3.61**, **3.62**, **3.110**, **3.111**, as well as **3.77** for SAR purposes) were sent for assay to the research group of Professor Rolf Hartmann at Saarland University, Germany. This group has recently developed an assay method using human embryonic kidney cells (HEK 293) which have been transfected with either type 1 5α-reductase (HEK-I) or type 2 (HEK-II) cDNA. An advantage in using these cell lines is that inhibition of type 1 and 2 5α-reductase can be tested separately. All compounds were tested for inhibition against both isozymes using HEK-I and HEK-II cell free homogenates. The cells were grown, homogenised, and then incubated with $NADP^+$, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, [^{3}H]-androstenedione (substrate) and test compound. After 30 min the steroids were extracted and subsequently analysed by HPLC. Radioactivity was detected for separated substrates and products.[55]

3.7.1 Inhibition results

Results obtained for inhibition of type 1 and 2 steroid 5α-reductase from HEK cell-free homogenates, by **3.77**, **3.55-3.57**, **3.59**, **3.61**, **3.62**, **3.110**, and **3.111**, are shown in Table 3.1. These compounds were tested at a concentration of 10 μM, and results are expressed as % inhibition (calculated from the amount of enzyme reaction product recovered).

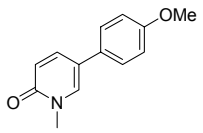
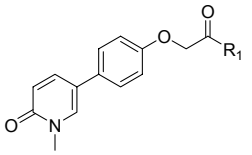
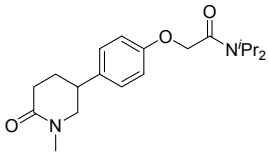
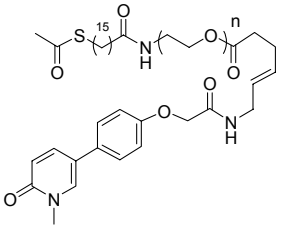
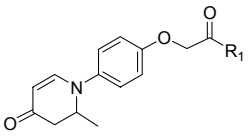
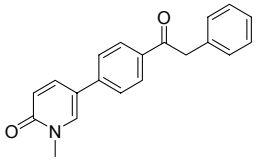
Compound	Inhibition of HEK-I homogenate (% inhibition at 10 μM)	Inhibition of HEK-II homogenate (% inhibition at 10 μM)
 **3.77**	IC_{50} = 2.5 μM	3%
 **3.55** $R_1 = N^iPr_2$ **3.57** $R_1 = NHCH_2CHCH_2$	**3.55** = 8% **3.57** = 6%	no inhibition
 **3.56**	6%	no inhibition
 **3.110** n = 1, **3.111** n = 2	**3.110** = 43% **3.111** = 33%	no inhibition
 **3.61** $R_1 = N^iPr_2$ **3.62** $R_1 = NHCH_2CHCH_2$	**3.61** = 3% **3.62** = 8%	no inhibition 12%
 **3.59**	61%	no inhibition

Table 3.1. Inhibition of type 1 and 2 5α-reductase.

HEK-I: human embryonal kidney cell homogenate containing 5α-reductase type 1; substrate: ^{3}H androstenedione 505 nM, reference compound: finasteride IC_{50} = 453 nM.
HEK-II: human embyonal kidney cell homogenate containing 5α-reductase type 2; substrate: ^{3}H androstenedione 505 nM, reference compound: finasteride IC_{50} = 25 nM.

3.7.2 Discussion of inhibition results

All compounds tested inhibited the type 1 isozyme. Weak inhibition was observed with inhibitors **3.55-3.57**, **3.61**, and **3.62**, while more potent inhibition was observed with inhibitors **3.77**, **3.110**, **3.111**, and **3.59**. There was little difference in activity between **3.55** and **3.56** (8% and 6% inhibition respectively at 10 μM) suggesting type 1 inhibition is not enhanced by the presence of a piperidone mimicking the steroidal A-ring, compared to a pyridone. This observation is consistent with the small difference in inhibition between literature compounds **3.53** and **3.54** (Fig. 3.17). There was no difference in activity when an *N*-diisopropylacetamide or *N*-allylacetamide group was present at the 4'-position of the 5-aryl substituted 1-methyl-2-pyridones (**3.55** vs **3.57**). There was also little difference in activity between the 5-aryl 1-methyl-2-pyridones (**3.55** and **3.57**) and the 1-aryl 2-methyl-2,3-dihydro-4-pyridones (**3.61** and **3.62**). The dihydro-4-pyridones **3.61** and **3.62** displayed 3% and 8% inhibition respectively, which is comparable to the activity of pyridones/piperidone **3.55**, **3.56**, and **3.57.** For the dihydro-4-pyridones **3.61** and **3.62**, the presence of an *N*-diisopropylacetamide or *N*-allylacetamide group at the 4'-position made little difference to the inhibitory activity observed with inhibition values of 3% and 8% respectively.

The two inhibitor-tether conjugates **3.110** and **3.111** were more potent inhibitors of type 1 (inhibiting type 1 activity by 43% and 33% respectively) than the pyridones **33** and **35** which have *N*-diisopropylacetamide and *N*-allylacetamide side chains at the 4'-position. This result is interesting as it provides further evidence that large hydrophobic groups are tolerated in the enzyme site not involved in reaction with the enzyme. A lipophilic group at the 4'-position is known to influence isozyme selectivity of inhibitors, so type 1 inhibitory activity by **3.110** and **3.111** correlates with that of the type 1 selective steroidal inhibitor MK386 (**3.15**, Fig. 3.3), which has a cholesterol moiety at C17 (steroidal numbering). The

presence of the long carbon chain tether in **3.110** and **3.111** enhances potency when compared to the activity of **3.57**, in which the tether is absent. Comparisons can also be made between **3.110** and **3.111** which differ in the number of ethylene glycol units (n) in the tether. Inhibitor **3.110** has one ethylene glycol unit and inhibits type 1 activity by 43%, while **3.111** has two glycol units and inhibits type 1 activity by 33%.

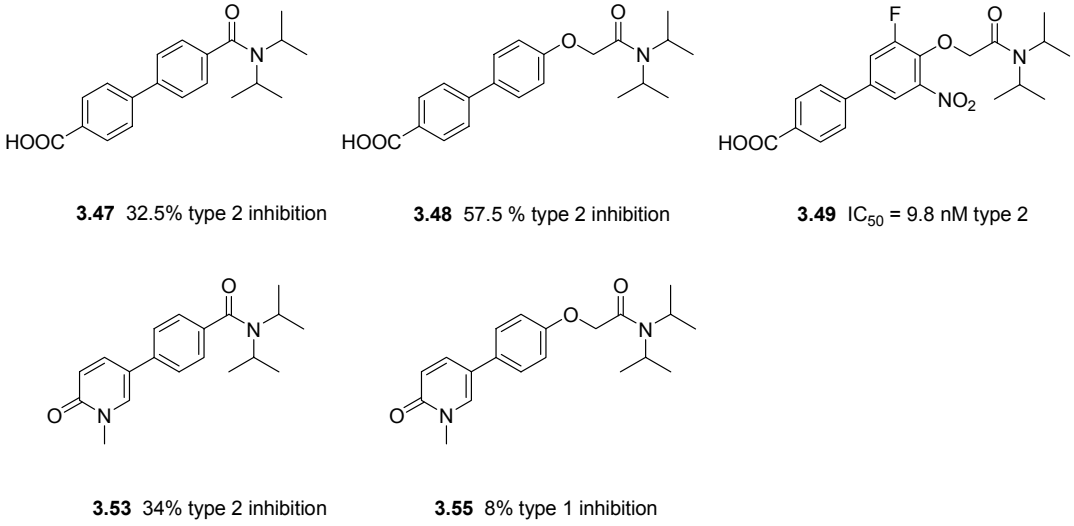
The most potent inhibitor of type 1 was the benzoyl compound **3.59** which inhibited enzyme activity by 61% (at 10 μM). The presence of the benzoyl group attached to the aromatic ring is clearly involved in enhancing potency when compared to inhibitors containing the *N*-substituted acetamide groups as in **3.55**, **3.56**, and **3.57**, and also **3.61**, and **3.62**. This observation correlates with that of Hartmann *et al.* who observed that the presence of bulky ketone groups at the 4'-position of 5-aryl substituted pyridones enhanced inhibitory potency in comparison to compounds with 4'-amide groups.[24]

5-Aryl 1-methyl-2-pyridone **3.77**, and 1-aryl 2-methyl-2,3-dihydro-4-pyridone **3.62** inhibited type 2 steroid 5α-reductase activity by 3% and 12% respectively. None of the other compounds tested inhibited this isozyme. Interestingly the dihydro-4-pyridone **3.62** showed slight dual inhibition of the isozymes, inhibiting type 1 activity by 8%, and type 2 activity by 12%. The corresponding 5-aryl 1-methyl-2-pyridone **3.57** did not inhibit type 2 at all.

Compounds **3.55** and **3.56** were designed from reported inhibitors **3.53** and **3.54** (Fig. 3.17). In these literature inhibitors, the amide is attached directly to the aromatic ring at the 4'-position. Incorporation of an OCH_2 linker into inhibitors **3.53** and **3.54** between the aromatic ring and amide resulted in novel compounds **3.55** and **3.56**. Lesuisse *et al.* found enhanced potency with the OCH_2 linker (see Fig. 3.25, **3.47** vs **3.48**) for compounds containing an aryl carboxylic acid in place of the pyridone as in **3.53** and **3.54**.[40] Strikingly this ether linker did not result in enhanced potency for inhibitors **3.55** and **3.56**, which inhibited the type 1 isozyme activity by 8% and 6% respectively.

The Lesuisse compound **3.49** (Fig 3.25) is an extremely potent inhibitor of the type 2 isozyme (IC_{50} = 9.8 nM). This compound contains a biphenyl scaffold consisting of an aryl

carboxylic acid attached to an aromatic ring with F, NO_2 and 4'-OCH_2 substitution, and was prepared after enhanced potency was seen for **3.48** compared to **3.47** (Fig. 3.25).



3.47 32.5% type 2 inhibition

3.48 57.5 % type 2 inhibition

3.49 IC_{50} = 9.8 nM type 2

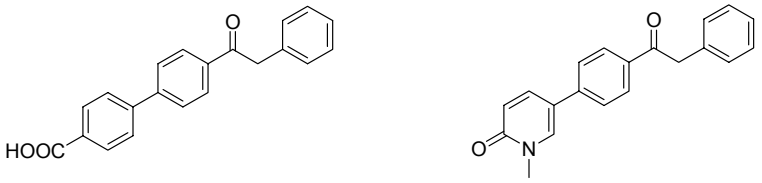
3.53 34% type 2 inhibition

3.55 8% type 1 inhibition

Figure 3.25. Effect of OCH_2 linker on inhibition (**3.47** and **3.48** at 1 μM, **3.53** at 100 μM, **3.55** at 10 μM).

The non-steroidal inhibitors focussed on (5-aryl pyridones, 1-aryl 2,3-dihydro-4-pyridones, and the biaryl carboxylic acids) possess two rings as compared to the four of the steroidal inhibitors. The substituent present at the 4'-position of non-steroidal biaryl inhibitors is influential on both type 1 and 2 inhibition potencies, as discussed in section 3.1.5. This is clearly illustrated when comparing inhibition data for **3.47** with **3.48**, and **3.49** (Fig. 3.25). The 4'-$OCH_2CON^iPr_2$ group of **3.48** and **3.49** has a positive influence on potency for type 2 inhibition, so we proposed that incorporation of the OCH_2 linker into compound **3.53** to give **3.55** might have a similar effect on type 1 potency. The finding that **3.55** did not show greater type 1 inhibition than **3.53** (and also **3.56** compared to **3.54**) is a significant result for defining SAR across different inhibitor classes. We can conclude that for the pyridone inhibitors, SAR for 4'-substituents do not correlate with SAR derived from the biaryl carboxylic acid inhibitors.

The design of inhibitor **3.59** was based on a potent compound **3.58** (K_i = 60 nM) reported by Holt *et al.* (Fig. 3.26) which possesses a benzoyl group at the 4'-position on the biaryl carboxylic acid scaffold.[35] The substituent at the 4'-position is influential on the potency of non-steroidal inhibitors, so the 4'-benzoyl group of **3.58** was incorporated into the 4'-position of the 5-aryl 1-methyl-pyridone scaffold to give **3.59**. The literature compound **3.58** had a K_i of 60 nM for the type 2 enzyme. Our compound **3.59** showed 61% inhibition of type 1 activity at a concentration of 10 μM. Whilst not as potent as **3.58**, this result shows that the benzoyl group is favoured by the enzyme active site in both isozymes, as the presence of this group translated into potent type 2 inhibition and moderate type 1 inhibition.



3.58 K_i type 2 = 60 nM

3.59 61% type 1 inhibition

Figure 3.26. 4'-Benzoyl group inhibitors (**3.59** tested at a concentration of 10 μM).

Inhibitors consisting of a 1-aryl 2-substituted 2,3-dihydro-4-pyridone scaffold (e.g. **3.61** and **3.62**) have not been reported to date, so direct comparison of inhibitory data for **3.61** and **3.62** to that of literature compounds is impossible. This non-steroidal class of compounds is related to the 10-azasteroid inhibitors and the non-steroidal tricyclic 4a*H*-benzo[c]quinolizinone inhibitors (Fig. 3.10). The pyridone and aromatic rings of **3.61** and **3.62** mimic the A and C rings respectively of the 10-azasteroids and the 4a*H*-benzo[c]quinolizinones. The 4a*H*-benzo[c]quinolizinones are good inhibitors of the type 1 isozyme. Inhibitors **3.61** and **3.62** were designed on a similar basis to pyridones **3.55** and **3.57**. The *N*-diisopropylacetamide and *N*-allylacetamide groups of **3.55** and **3.57** were incorporated into the 2,3-dihydro-4-pyridone scaffold to give **3.61** and **3.62**. The inhibition of steroid 5α-reductase by this novel class of inhibitor was of interest with focus on SAR for the substituent at the 4'-position. As for the pyridones **3.55** and **3.57**, the presence of

the OCH_2 linker in **3.61** and **3.62** did not translate into good inhibitory activity. Therefore it would seem that SAR for 4'-substituents of the 2,3-dihydro-4-pyridones do not correlate with SAR derived from the biaryl acid inhibitors.

The presence of a long carbon chain tether enhanced the inhibitory activity of inhibitor-tether conjugate **3.110** in comparison to **3.57**. The conjugate **3.110** inhibited type 1 activity by 43%, while the non-conjugated compound **3.57** only inhibited the isozyme by 6%. This finding provides further evidence for the presence of a lipophilic pocket (see section 3.1.4.2) in the enzyme active site that tolerates bulky hydrophobic groups at the end of the inhibitor not interacting with the active site region which facilitates the enzymatic reaction.

Literature compounds **3.53** and **3.54** inhibit steroid 5α-reductase sourced from human BPH tissue and RVP.[24] Compound **3.53** inhibited BPH enzyme activity by 34% and RVP enzyme activity by 45% (at 100 μM). Compound **3.54** inhibited BPH enzyme activity by 37% and RVP enzyme activity by 41% (at 100 μM). The BPH tissue was used to test for type 2 inhibition while RVP was used for type 1. In the test protocol the BPH preparation was buffered at pH 5.5 to select for type 2 activity, while the RVP preparation was buffered at pH 6.6 to select for type 1 activity. Inhibitors synthesised in this thesis were tested with transfected cell line homogenates expressing either type 1 or type 2 5α-reductase. It is not understood why the inhibitory activity of **3.55** and **3.56** was significantly lower than the activity of **3.53** and **3.54**. The observed difference in activity might be accounted for by the method used to assay **3.53** and **3.54**, where the activity of one isozyme was selected over the other in tissue homogenates by using pH 5.5 or pH 6.6 buffer. Perhaps the RVP preparation used by Hartmann *et al.* did not specifically select type 1 activity, which would suggest some of the inhibition data was due to type 2 activity. These observations highlight the difficulty in using tissue preparations which contain both isozymes, such as RVP in this case. Inhibitors synthesised in this thesis were tested versus each single isozyme in cell-free homogenates of transfected HEK-I and HEK-II cells. This avoids the problems associated with tissue homogenates, but makes comparison to results derived from tissues difficult.

3.8 CONCLUSION AND FUTURE WORK

Inhibitors of steroid 5α-reductase are of great pharmaceutical interest. Currently research is focused on developing non-steroidal inhibitors to avoid potential side effects associated with steroid based drugs. The appearance of a number of different classes of non-steroidal inhibitors of 5α-reductase has made it necessary to define the SAR within each class and across classes. SAR studies of literature compounds led to the design and synthesis of novel non-steroidal proposed 5α-reductase inhibitors in this thesis, with focus on establishing SAR. Compounds containing a non-steroidal substituted 5-aryl 1-methyl-2-pyridone/piperidone scaffold (**3.55-3.57** and **3.59**) were synthesised. This scaffold is related to that of the 4-azasteroids which are potent inhibitors. The 5-aryl pyridone scaffold of **3.55-3.57** and **3.59** was constructed under Suzuki cross coupling conditions and was subsequently reacted under various conditions to give **3.55**, **3.57**, and the piperidone **3.56**. Inhibitor **3.59** required slightly different conditions for the Suzuki cross coupling due to base sensitivity of the ketone starting material **3.86**. Synthesis of the piperidone derivative **3.60** was unsuccessful.

A novel type of steroid 5α-reductase inhibitor possessing a 1-aryl 2-substituted 2,3-dihydro-4-pyridone scaffold (**3.61** and **3.62**) was developed and synthesised. These compounds are related to the 10-azasteroids, and non-steroidal benzo[c]quinolizinones (e.g. **3.7** and **3.30** respectively). The 1-aryl 2,3-dihydro-4-pyridone scaffold of **3.61** and **3.62** was constructed using aza Diels-Alder methodology. A methyl group was incorporated at the 2-position of the pyridone ring by the use of acetaldehyde in generating an imine with *p*-anisidine. An attempt was made to incorporate a phenyl group at the 2-position of the pyridone ring (compounds **3.63** and **3.64**) for SAR purposes but the aza Diels-Alder step was not reproducible in the synthesis of **3.63** so further work in this area was abandoned.

Inhibitor **3.57** was conjugated with olefin containing tethers **3.107** and **3.108** by cross metathesis to give conjugates **3.110** and **3.111**. These conjugates possess a thiol terminus for the purpose of future attachment to a gold surface. Attempts were made to attach the

tethers to the dihydro-4-pyridone **3.62** by the cross metathesis methodology already established for **3.110** and **3.111**, but these were unsuccessful.

The target compounds synthesised in this thesis (**3.55-3.57**, **3.59**, **3.61**, **3.62**, **3.110** and **3.111**) were tested for inhibitory activity against both type 1 and 2 steroid 5α-reductase. Human embryonal kidney cells stably transfected with either type 1 or 2 cDNA (HEK-I or HEK-II) were used to evaluate inhibition of the isozymes separately. The compounds tested inhibited the type 1 isozyme to varying degrees at a concentration of 10 μM. The 5-aryl pyridones **3.55**, **3.57** and piperidone **3.56**, inhibited the enzyme weakly (6-8% inhibition of isozyme activity). The 2,3-dihydro-4-pyridones **3.61** and **3.62** also inhibited the enzyme weakly (3% and 8% inhibition respectively). Conjugates **3.110** and **3.111** were moderate inhibitors displaying 43% and 33% inhibition respectively. The benzoyl compound **3.59** was the most active, inhibiting isozyme activity by 61%. One compound, dihydro-4-pyridone **3.62** showed slight inhibition of both isozymes (type 1 8%, type 2 12%).

Incorporation of an OCH_2 linker into **3.53** and **3.54** at the 4'-position, resulting in compounds **3.55** and **3.56** did not enhance potency towards type 1 5α-reductase as predicted. Therefore SAR determined for substituents at the 4'-position of biphenyl carboxylic acid inhibitors do not correlate with the pyridone series of compounds as illustrated here. The benzoyl group of **3.59** is a more favoured substituent at this position for the type 1 isozyme.

Future work in this area would involve attaching conjugates **3.110** and **3.111** to a gold surface. The thioester of **3.110** and **3.111** would require hydrolysis *in situ* to give a free thiol which is required for interaction with a gold surface. The development of inhibitor **3.59** could be another area of future work. The synthesis of an inhibitor based on **3.59**, but with substitution of the aromatic ring attached directly to the benzoyl group, would be of interest based on the influence of the F and NO_2 groups of literature compound **3.49**.

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CHAPTER FOUR

EXPERIMENTAL

4.1 GENERAL METHODS AND EXPERIMENTAL PROCEDURES

Melting points

All melting points were obtained on an Electrothermal apparatus and are uncorrected.

Nuclear Magnetic Resonance

Proton NMR spectra were obtained on a Varian Inova spectrometer, operating at 500 MHz. Carbon NMR spectra were obtained on a Varian Unity 300 spectrometer, operating at 75 MHz, with a delay (D_1) of 1 s. All spectra were obtained at 23°C. Chemical shifts are reported in parts per million (ppm) on the δ scale. Solvents used for NMR analysis (reference peaks listed) included: DMSO-d_6 ($C\mathbf{H}D_2SOCD_3$ at δ_H 2.60 ppm, $(\mathbf{C}D_3)_2SO$ at δ_C 39.6 ppm); $CDCl_3$ ($C\mathbf{H}Cl_3$ at δ_H 7.25 ppm, $\mathbf{C}DCl_3$ at δ_C 77.0 ppm); CD_3OD ($C\mathbf{H}D_2OD$ at δ_H 3.30 ppm, $\mathbf{C}D_3OD$ at δ_C 49.3 ppm); acetone-d_6 ($C\mathbf{H}D_2COCD_3$ at δ_H 2.17 ppm, $(\mathbf{C}D_3)_2CO$ at δ_C 29.2 ppm). Two-dimensional NMR experiments included COSY and HSQC, and were obtained on the Varian Inova spectrometer operating at 500 MHz.

Small Molecule Mass Spectrometry

Electron impact mass spectra were obtained on a Kratos MS80 RFA mass spectrometer operating at 4000 V (accelerating potential) and 70 eV (ionisation energy). The source temperature was 200-250°C. Electrospray ionisation mass spectra were detected on a micromass LCT TOF mass spectrometer, with a probe voltage of 3200 V, temperature of 150°C and a source temperature of 80°C. Direct ionisation used 10 μL of a 10 μg mL^{-1} solution, using a carrier solvent of 50% acetonitrile/H_2O at a flow rate of 20 μL min^{-1}. Ionisation was assisted by the addition of 0.5% formic acid.

High Pressure Liquid Chromatography

Analytical HPLC was performed on a Shimadzu LC-10AD VP liquid chromatograph coupled to a SIL-10A VP autoinjector, a CTO-10A VP column oven set to 40˚C and a SPD-M10A VP photodiode array detector. The system was controlled by Shimadzu CLASS-VP (Version 5.02) software. For reverse phase HPLC a Phenomenex Prodigy C18 5-ODS (3μ, 250 x 4.6 mm) column was used with a flow rate of 1 mL min^{-1} with a standard HPLC solvent gradient system that was comprised of variable concentrations of H_2O (Milli-Q) containing 0.05% trifluoroacetic acid and acetonitrile (HPLC grade). The gradient consisted of a 24 min run with the following steps - 2 min of 50% water in acetonitrile, followed by a linear gradient to 75% water in acetonitrile over 18 min, then returned to 50% water in acetonitrile over 2 min, which was maintained for 2 min to allow the column to re-equilibrate.

Reagents, solvents and laboratory methodology

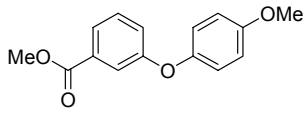
Oven dried glassware was used in all the reactions that were carried out under an atmosphere of dry nitrogen. All starting materials were obtained commercially unless otherwise stated. "Removal of the solvent (or volatiles) by evaporation under reduced pressure" refers to the process of bulk solvent removal by rotary evaporation (low vacuum pump) followed by application of high vacuum (oil pump) for a minimum of 30 min. Analytical thin layer chromatography (TLC) was performed on plastic backed Merck Keisegel KG60F_{254} silica plates, and visualised using short wave ultra violet light, or AMC dip[1]. Flash chromatography was performed using 230-400 mesh Merck Silica Gel 60 following established guidelines[2] under positive pressure. THF and diethyl ether were distilled from sodium benzophenone ketyl under an inert atmosphere immediately prior to use. Dichloromethane (DCM), 1,2-dichloroethane, and toluene were distilled from calcium hydride under an inert atmosphere. Dimethyl sulfoxide (DMSO) was distilled from calcium hydride under reduced pressure and stored over 4 Å molecular sieves. "Super-dry" methanol and ethanol were prepared according to the literature procedure.[3] Petroleum ether refers to the fraction collected between 50-70°C. Ethyl acetate, petroleum ether, and DCM were distilled from calcium hydride prior to their use in column chromatography. DMF was dried by placing over 4 Å molecular sieves, applying a high vacuum for 15 min, then flushed briefly with an inert atmosphere. This process was repeated twice over 24 h, after

which the DMF was stored over 4 Å molecular sieves under an inert atmosphere. All other reagents and solvents requiring purification prior to use were purified using literature procedures.[4]

4.2 EXPERIMENTAL WORK DESCRIBED IN CHAPTER TWO

4.2.1 Synthesis of permethrin metabolites 2.14-2.16

Methyl 3-(4-methoxyphenoxy)benzoate (2.19)[5]



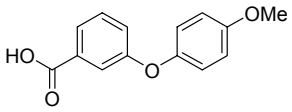
Sodium hydride (60% in oil, 270 mg, 5.58 mmol, 1.2 equiv) was added to a flask under N_2 atmosphere. This was washed with petroleum ether (stored over sieves) twice, DMF (2 mL) was added and the resulting bubbly grey solution was stirred at rt. *p*-Methoxyphenol (577 mg, 4.65 mmol, 1 equiv) (recrystallised from toluene and used immediately) was dissolved in DMF (5 mL) in another flask under N_2. The phenol solution was added dropwise over 10 min to the sodium hydride at 0˚C, resulting in a thick creamy solution which was stirred for 30 min. Methyl-3-bromobenzoate (1 g, 4.65 mmol, 1 equiv) and copper (I) chloride (23 mg, 0.23 mmol, 0.05 equiv) were stirred in DMF (5 mL) for 10 min before the phenol solution was added dropwise. The resulting dark brown mixture was heated at reflux for 6 h, and then cooled to rt. The reaction mixture was diluted with water (50 mL), and extracted with ether (3 x 50 mL). The organic phases were combined and washed with water (5 x 50mL). The ethereal solution was dried over $MgSO_4$, and the solvent removed under reduced pressure. The resulting green/yellow oil was purified by column chromatography on silica (10% ethyl acetate/petroleum ether) to give **2.19** (370 mg, 31%) as a pale yellow oil.

^{1}H NMR ($CDCl_3$, 500 MHz) δ 3.81 (s, 3H, OC**H$_3$**), 3.88 (s, 3H, COOC**H$_3$**), 6.90 (d, 2H, *J* = 9.1 Hz, Ar**H**), 6.98 (d, 2H, *J* = 8.7 Hz, Ar**H**), 7.14 (dd, 1H, *J* = 2.8, 8.3 Hz, Ar**H**), 7.36 (t, 1H, *J* = 7.9, 15.9 Hz, Ar**H**), 7.57 (d, 1H, *J* = 1.6 Hz, Ar**H**), 7.71 (d, 1H, *J* = 7.9, Ar**H**).

^{13}C NMR ($CDCl_3$, 75 MHz) δ 52.16, 55.59, 114.97, 118.14, 120.93, 122.07, 123.51, 129.58, 131.70, 149.52, 156.15, 158.62, 166.57.

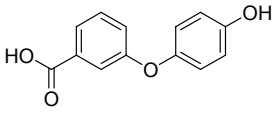
HRMS Found 258.0888 (Calcd. for $C_{15}H_{14}O_4$ 258.0892).

3-(4-Methoxyphenoxy)benzoic acid (2.20)



The aryl ester **2.19** (153 mg, 0.59 mmol, 1 equiv) was heated with potassium hydroxide (132 mg, 2.36 mmol, 4 equiv) at reflux for 4 h in THF (2.5 mL) and water (2.5 mL). After cooling to rt, the solution was acidified with 10% aqueous hydrochloric acid until neutral pH (universal indicator paper), and extracted with ethyl acetate (3 x 20 mL). The combined organic phases were dried over $MgSO_4$, and the solvent was removed under reduced pressure to give **2.20** (130 mg, 90%) as a white solid. The product was not purified further.
mp 138-140˚C (Lit.[6] 141-143˚C).
^{1}H NMR (acetone-d_6, 500 MHz) δ 3.93 (s, 3H, OC**H**$_3$), 7.12 (d, 2H, J = 9.3 Hz, Ar**H**), 7.17 (d, 2H, J = 9.3 Hz, Ar**H**), 7.34 (dd, 1H, J = 2.4, 7.8 Hz, Ar**H**), 7.61 (t, 1H, J = 7.8 Hz, Ar**H**), 7.64 (d, 1H, J = 2.4 Hz, Ar**H**), 7.85 (d, 1H, J = 7.8 Hz, Ar**H**).
^{13}C NMR (acetone-d_6, 75 MHz) δ 53.76, 113.83, 116.31, 119.91, 120.55, 122.25, 128.7, 130.95, 148.08, 155.34, 157.72, 164.93.
HRMS Found 244.0740 (Calcd. for $C_{14}H_{12}O_4$ 244.0736).

3-(4-Hydroxyphenoxy)benzoic acid (2.15)



The methyl ether **2.20** (70 mg, 0.29 mmol) was heated at reflux for 7 h in 48% aqueous hydrobromic acid (2.5 mL) and acetic acid (2.5 mL). The mixture was cooled to rt, and the solvent removed under pressure. The orange residue was dissolved in ethyl acetate (25 mL) and washed with 1 M aqueous potassium hydroxide (3 x 15 mL). The aqueous washings were combined, acidified with 6 M aqueous hydrochloric acid until neutral pH (universal indicator paper), and extracted with ethyl acetate (3 x 20 mL). The organic phases were

combined and dried over $MgSO_4$. The solvent was removed by evaporation under reduced pressure to give a brown solid which was purified by column chromatography on silica (50% ethyl acetate/petroleum ether) to give **2.15** (59 mg, 88%) as a cream solid.

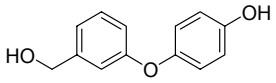
mp 169-171˚C (Lit.[6] 172-174˚C).

^{1}H NMR (acetone-d_6, 500 MHz) δ 7.02 (d, 2H, J = 9.3 Hz, Ar**H**), 7.08 (d, 2H, J = 9.3 Hz, Ar**H**), 7.31 (dd, 1H, J = 2.4, 7.8 Hz, Ar**H**), 7.58 (t, 1H, J = 7.8 Hz, Ar**H**), 7.64 (d, 1H, J = 2.4 Hz, Ar**H**), 7.83 (d, 1H, J = 7.8 Hz, Ar**H**), 8.56 (s(br), 1H, O**H**).

^{13}C NMR (acetone-d_6, 75 MHz) δ 114.61, 115.59, 119.54, 119.78, 121.5, 128.06, 130.31, 146.55, 152.43, 157.4, 164.67.

HRMS (M-H) Found 229.0502 (Calcd. For $C_{13}H_9O_4$ 229.0501).

3-(4-Hydroxyphenoxy)benzyl alcohol (2.14)



The acid **2.15** (82 mg, 0.36 mmol, 1 equiv) was dissolved in freshly distilled dry ether (5 mL) under N_2 atmosphere, and lithium aluminium hydride (33 mg, 0.90 mmol, 2.5 equiv) added at 0°C. The grey suspension was stirred at rt for 16 h at which time saturated aqueous ammonium chloride was added slowly until bubbling ceased. The mixture was extracted with ethyl acetate (3 x 20 mL). The organic phases were combined and washed with water, saturated aqueous sodium chloride, and dried over $MgSO_4$. The solvent was removed by evaporation under reduced pressure to give a clear oil which was purified by column chromatography on silica (20% ethyl acetate/petroleum ether) to give **2.14** (25 mg, 33%) as a cream solid.

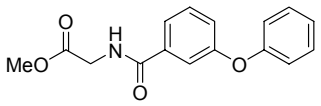
mp 106-109˚C (Lit.[5] 110-111˚C).

^{1}H NMR (acetone-d_6, 500 MHz) δ 4.43 (s, 1H, O**H**), 4.71 (d, 2H, J = 6.4 Hz, C**H$_2$**), 6.89 (dd, 1H, J = 2.4, 7.8 Hz, Ar**H**), 6.97 (d, 2H, J = 9.3 Hz, Ar**H**), 7.02 (d, 2H, J = 9.3 Hz, Ar**H**), 7.06 (d, 1H, J = 2.4 Hz, Ar**H**), 7.14 (d, 1H, J = 7.8 Hz, Ar**H**), 7.38 (t, 1H, J = 7.8 Hz, Ar**H**), 8.42 (s, 1H, O**H**).

^{13}C NMR (acetone-d_6, 75 MHz) δ 62.03, 113.68, 114.11, 114.77, 118.81, 119.58, 127.91, 143.14, 147.64, 152.38, 157.5.

HRMS Found 216.0789 (Calcd. for $C_{13}H_{12}O_3$ 216.0786).

_N_-(3-Phenoxybenzoyl)glycine methyl ester (2.22)



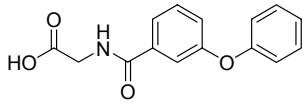
DIPEA (0.41 mL, 2.34 mmol, 1 equiv) was added to a stirring solution of glycine methyl ester hydrochloride (293 mg, 2.34 mmol, 1 equiv) in dry DCM (10 mL) under N_2 atmosphere. EDCI (448 mg, 2.34 mmol, 1 equiv), HOBt (316 mg, 2.34 mmol, 1 equiv) and 3-phenoxybenzoic acid (500 mg, 2.34 mmol, 1 equiv) were added, and the resulting clear solution stirred at rt for 3 h. The solution was diluted with ethyl acetate (50 mL) and washed successively with 10% aqueous hydrochloric acid, 10% aqueous sodium bicarbonate, and water. The organic phase was dried over $MgSO_4$ and the solvent removed by evaporation under reduced pressure to give **2.22** (596 mg, 85%) as viscous pale yellow oil. The product was not purified further.

^{1}H NMR ($CDCl_3$, 500 MHz) δ 3.71 (s, 3H, OC**H$_3$**), 4.14 (d, 2H, J = 4.8 Hz, C**H$_2$**), 6.97 (d, 2H, J = 7.8 Hz, Ar**H**), 7.08 (m, 2H, Ar**H**), 7.18 (s(br), 1H, N**H**), 7.30 (m, 3H, Ar**H**), 7.48 (m, 2H, Ar**H**).

^{13}C NMR ($CDCl_3$, 75 MHz) δ 41.53, 52.25, 117.28, 119.02, 121.45, 121.7, 123.65, 129.76, 129.8, 135.29, 156.35, 157.5, 166.88, 170.31.

HRMS Found 285.0998 (Calcd. for $C_{16}H_{15}NO_4$ 285.1001).

***N*-(3-Phenoxybenzoyl)glycine (2.16)**



The ester **2.22** (200 mg, 0.70 mmol, 1 equiv) was heated with potassium hydroxide (157 mg, 2.80 mmol, 4 equiv) at reflux for 4 h in THF (3 mL) and water (3 mL). After cooling to rt, the solution was acidified with 10% aqueous hydrochloric acid until neutral pH (universal indicator paper), and extracted with ethyl acetate (3 x 50 mL). The organic phases were combined, dried over $MgSO_4$, and the solvent removed by evaporation under reduced pressure. The resulting white solid was purified by column chromatography on silica (50% ethyl acetate/petroleum ether) to give **2.16** (124 mg, 65%) as a fluffy white solid.

mp 144-146˚C (lit.[7] 148-150˚C).

^{1}H NMR (acetone-d_6, 500 MHz) δ 4.23 (d, 2H, J = 5.9 Hz, C**H$_2$**), 7.04 (d, 2H, J = 8.3 Hz, Ar**H**), 7.18 (m, 2H, Ar**H**), 7.41 (t, 1H, J = 7.3 Hz, Ar**H**), 7.52 (m, 2H, Ar**H**), 7.69 (d, 1H, J = 9.3 Hz, Ar**H**), 7.92 (d, 1H, J = 9.7 Hz, Ar**H**), 8.09 (s(br), 1H, N**H**), 8.69 (s, 1H, COO**H**).

^{13}C NMR (acetone-d_6, 75 MHz) δ 39.41, 115.8, 117.46, 120.03, 120.4, 122.19, 128.47, 134.66, 155.3, 156.03, 164.7, 168.97.

HRMS Found 271.0853 (Calcd. for $C_{15}H_{13}NO_4$ 271.0845).

4.2.2 Yeast Estrogen Screen (YES)

Preparation of yeast for assay

The recombinant yeast required for the assay was obtained from the Sumpter group at Brunel University, London, UK. This group has frequently published results obtained with this assay, and one paper in particular was consulted for assay procedure.[8] We obtained five vials of agar slopes containing recombinant *Saccharomyces cerevisiae*. Two yeast samples from each slope were transferred to cryovials with 15% sterile glycerol, and frozen at -80˚C.

All work involving the yeast was carried out in a type II laminar flow cabinet at the Institute of Environmental Science and Research (ESR). All glassware, spatulas and stirring bars (which had no prior contact with steroids) were scrupulously cleaned and rinsed with ethanol to eliminate estrogenic contamination, and left to dry. The yeast required various solutions for storage and growth for assay use. These included:

(i) Minimal medium
(ii) 20% D-Glucose solution
(iii) L-Aspartic acid solution
(iv) Vitamin solution
(v) L-Threonine solution
(vi) Copper (II) sulfate solution

Preparation and storage of yeast minimal medium and medium components

Chemicals required for the yeast growth were obtained from Sigma Aldrich. Double distilled water was obtained from the chemistry department apparatus, and collected into large schott bottles with teflon lids. All solutions were stored in glass jars with teflon lids or aluminium foil lined lids. Autoclaving was done at 121˚C for 15 min.

Minimal media (pH 7.1)

13.61 g KH_2PO_4, 1.98 g $(NH_4)_2SO_4$, 4.2 g KOH, 200 mg $MgSO_4$, 1 mL $Fe_2(SO_4)_3$ solution (40 mg in 50 mL H_2O), 50 mg, L-leucine, 50 mg L-histidine, 50 mg adenine, 20 mg L-arginine-HCl, 20 mg L-methionine, 30 mg L-tyrosine, 30 mg L-isoleucine, 30 mg L-lysine-HCl, 25 mg L-phenylalanine, 100 mg L-glutamic acid, 150 mg L-valine, and 375 mg L-serine were added to 1 L of double distilled water. The solution was stirred with gentle heating until all solids had dissolved. Aliquots of 45 mL were dispensed into glass schott bottles, autoclaved to sterilise, and stored at rt.

D-(+)-Glucose solution

A 20% w/v solution in double distilled water was prepared, dispensed into 20 mL aliquots in glass vials, autoclaved and stored at rt.

L-Aspartic acid

A 4 mg/mL solution in double distilled water was prepared, which required sonication to dissolve the acid. Aliquots of 20 mL were dispensed into glass vials, autoclaved and stored at rt.

Vitamin solution

8 mg Thiamine, 8 mg pyridoxine, 8 mg pantothenic acid, 40 mg inositol, and 20 mL biotin solution (2 mg/100 mL H_2O) were added to 180 mL double distilled water, and sterilised by filtering through a 0.2 μm pore size sterile disposable filter in a laminar flow cabinet. Aliquots of 10 mL were transferred to sterile glass vials and stored at 4˚C.

L-Threonine

A solution of 24 mg/mL in double distilled water was prepared, dispensed into 10 mL aliquots in glass vials, autoclaved and stored at 4˚C.

Copper (II) sulfate

A 20 mM solution of copper (II) sulfate in double distilled water was prepared and sterilised as for the vitamin solution. Aliquots of 5 mL were stored in glass bottles at rt.

Chlorophenol red-β-D-galactopyranoside (CPRG)

A 10 mg/mL solution in double distilled water was prepared and sterilised by filtering (as for the vitamin solution) into sterile glass bottles in 5 mL aliquots and stored at 4˚C.

Preparation and storage of yeast stock for assay use

For short term storage of the yeast (maximum of four months), a 10 x concentrated yeast stock culture was prepared. This involved first preparing growth medium, which consisted of:

(i) 45 mL Minimal medium
(ii) 5 mL Glucose solution
(iii) 1.25 mL L-Aspartic acid solution
(iv) 0.5 mL Vitamin solution
(v) 0.4 mL L-Threonine solution
(vi) 125 µL Copper (II) sulfate solution

The growth medium was added to a sterile conical flask and 125 µL yeast stock (initially from yeast stored at -80˚C and -20˚C thereafter) added. The solution was incubated for 24 h at 28˚C on an orbital shaker, after which it was turbid. This 24 h culture was re-cultured by adding 1 mL to two sterile conical flasks containing growth medium. The two solutions were incubated at 28˚C on an orbital shaker for 24 h. Each culture was transferred to a sterile 50 mL centrifuge tube, and centrifuged at rt for 10 min at 2,000 g. The supernatant was decanted, and each culture resuspended in 5 mL minimal medium with 15% sterile glycerol (8 mL glycerol per 45 mL minimal medium). Aliquots of 0.5 mL were transferred to sterile glass tubes (those used for HPLC), and stored at -20˚C for a maximum of four months.

Assay procedure

Test chemical solutions were prepared in AnalaR 96% ethanol, and weighed directly into volumetric flasks. The assay standard, 17β-estradiol, was prepared at a concentration of 2 x 10^{-7} M (54.48 μg/L). The pyrethroid metabolites tested in this assay were prepared at a concentration of 2 x 10^{-2} M. All work involving the yeast was carried out in a type II laminar flow cabinet.

To prepare the yeast for assay, growth medium was prepared and transferred to a sterile conical flask, and 125 μL yeast (from stock stored at -20˚C) was added. The solution was incubated for 24 h on an orbital shaker at 28˚C.

Test chemical standard solutions of appropriate concentrations in ethanol were serially diluted by a factor of 2 in ethanol across selected rows in a 96 well plate. 100 μL of test chemical standard solution was added to the first well of the row and 50 μL of ethanol was added to each remaining well in the row. 50 μL of test chemical solution in the first well was transferred to the next well in the row which contained 50 μL of ethanol. This procedure was repeated for each remaining well in the row to give a range of concentrations of test chemical. 10 μL from each well in the row was transferred to the corresponding empty well in a new row, and allowed to evaporate. 17β-Estradiol (over the concentration range 0.049 to 10 nM) was used as a positive control, and ethanol used as a negative. For each assay, growth solution (50 mL) was seeded with 250 μL of 24 h yeast culture (optical density at 640 nm of 2.5, approx. 3 x 10^6 yeast cells), and CPRG (0.5 mL) was added. 200 μL of this assay medium was added to each well, and the plates were sealed with autoclave tape and shaken in a fixed wavelength plate reader (BIO-TEK EL312) for 2 min. The plates were incubated for 48 h at 30°C, until a colour degradation from red to yellow was observed across the estradiol standard. The production of chlorophenol red (indicating estrogenic activity) was monitored by measuring the absorbance at 540 nm. The absorbance at 610 nm was also measured to monitor the turbidity of the yeast culture. The negative control (ethanol blank) appeared a pale orange colour due to background β-galactosidase activity. The absorbance at 540 nm was

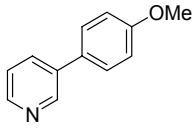
corrected for absorbance arising from the turbidity of the assay solution by applying; chem. abs. (540 nm) – [chem. abs. (610 nm) – blank abs. (610 nm)].

Absorbance values were entered into Excel to calculate the corrected absorbances (from the equation above). These values were presented graphically using SigmaPlot 8.0. An example is shown in the Appendix. For the metabolites that possessed estrogenic activity an EC_{50} was calculated (concentration of chemical required to induce 50% of the maximum estrogenic activity). This value was calculated in Excel using a mathematical equation obtained from the line fit generated in SigmaPlot. EC_{50} values of metabolites were compared to the 17β-estradiol EC_{50} in terms of a relative potency, where estradiol had a value of 1. Relative potency = estradiol EC_{50} / test chemical EC_{50}.

4.3 EXPERIMENTAL WORK DESCRIBED IN CHAPTER THREE

4.3.1 Synthesis of 5-aryl 1-methyl-pyridone/piperidone inhibitors 3.55-3.57

3-(4-Methoxyphenyl)pyridine (3.76)



3-Bromopyridine (275 μL, 2.74 mmol, 1 equiv) was stirred in dry THF (10 mL) under N_2 atmosphere. Tetrakis(triphenylphosphine)palladium(0) (158 mg, 0.14 mmol, 0.05 equiv) was added, followed by *p*-methoxyphenyl boronic acid (500 mg, 3.29 mmol, 1.2 equiv), and anhydrous potassium hydroxide (460 mg, 8.22 mmol, 3 equiv). The resulting orange solution was heated at reflux for 16 h. The reaction mixture was cooled to rt, diluted with ethyl acetate, washed with brine (2 x 20 mL), and dried over $MgSO_4$. Removal of the solvent by evaporation under reduced pressure gave a crude yellow oil which was purified by column chromatography on silica (50% ethyl acetate /petroleum ether) to give **3.76** (367mg, 73%) as a yellow solid.

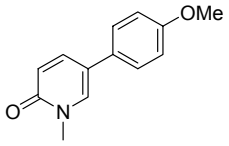
mp 60-61˚C (Lit.[9] 63-64˚C).

^{1}H NMR ($CDCl_3$, 500 MHz) δ 3.87 (s, 3H, OC**H$_3$**), 7.02 (d, 2H, *J* = 8.3 Hz, Ar**H**), 7.34 (dd, 1H, *J* = 4.9, 7.8 Hz, Ar**H**), 7.58 (d, 2H, *J* = 8.8 Hz, Ar**H**), 7.83 (dt, 1H, *J* = 2, 7.8 Hz, Ar**H**), 8.55 (dd, 1H, *J* = 1.5, 4.9 Hz, Ar**H**), 8.82 (d, 1H, *J* = 2 Hz, Ar**H**).

^{13}C NMR ($CDCl_3$, 75 MHz) δ 55.17, 114.38, 123.33, 128.03, 130.03, 133.63, 136.03, 147.70, 159.58.

HRMS (M+H) Found 186.0928 (Calcd. For $C_{12}H_{12}NO$ 186.0919).

5-(4-Methoxyphenyl)-1-methylpyridin-2(1*H*)-one (3.77) - Method 1[10]



3-Arylpyridine **3.76** (102 mg, 0.55 mmol, 1 equiv) was stirred at 80˚C for 30 min in dimethyl sulfate (0.05 mL, 0.55 mmol, 1 equiv). The dark brown mixture was cooled in ice and aqueous potassium ferricyanide (452 mg in 2 mL water, 1.38 mmol, 2.5 equiv) added, followed by the slow addition of potassium hydroxide (246 mg, 4.40 mmol, 8 equiv). DCM (2 mL) was then added and the mixture stirred at rt for 1.5 h. Further portions of aqueous potassium ferricyanide (226 mg in 2 mL water, 0.68 mmol, 1.25 equiv) and potassium hydroxide (123 mg, 2.20 mmol, 4 equiv) were added in a similar fashion as before. The mixture was stirred at rt for 16 h, then diluted with DCM and washed with water (3 x 20 mL). The organic phases were combined, dried over $MgSO_4$ and the solvent removed by evaporation under reduced pressure. The resulting crude brown/red solid was purified by column chromatography on silica (several columns were required) (50% ethyl acetate/petroleum ether) to give **3.77** (9.5 mg, 8%) as a yellow solid.

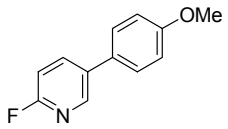
mp 99-103˚C.

^{1}H NMR (CD_3OD, 500 MHz) δ 3.63 (s, 3H, NC**H$_3$**), 3.81 (s, 3H, OC**H$_3$**), 6.61 (d, 1H, *J* = 9.3 Hz, COC**H**=CH), 6.97 (d, 2H, *J* = 8.8 Hz, Ar**H**), 7.44 (d, 2H, *J* = 8.3 Hz, Ar**H**), 7.83 (dd, 1H, *J* = 2.4, 9.3 Hz, COCH=C**H**), 7.88 (d, 1H, *J* = 2.4 Hz, NHC**H**).

^{13}C NMR (CD_3OD, 75 MHz) δ 38.60, 56.02, 115.70, 120.35, 122.62, 128.20, 129.88, 137.31, 141.55, 160.89, 164.33.

HRMS (M+H) Found 216.1014 (Calcd. For $C_{13}H_{14}NO_2$ 216.1025).

2-Fluoro-5-(4-methoxyphenyl)-pyridine (3.80)



5-Bromo-2-fluoropyridine (1 mL, 9.72 mmol, 1 equiv) was stirred in THF (30 mL) under N_2 atmosphere. Tetrakis(triphenylphosphine)palladium(0) (560 mg, 0.49 mmol, 0.05 equiv) was added resulting in a clear yellow solution. *p*-Methoxyphenyl boronic acid (1.77 g, 11.65 mmol, 1.2 equiv) was added to give an orange solution which turned yellow on further stirring. Powdered anhydrous potassium hydroxide (1.63 g, 29.16 mmol, 3 equiv) and *n*-tetrabutylammonium bromide (3.1 g, 9.72 mmol, 1 equiv) were added successively and the mixture heated at reflux for 19 h. The reaction mixture was cooled to rt, diluted with ethyl acetate, washed with water (2 x 50 mL), and dried over $MgSO_4$. Removal of the solvent by evaporation under reduced pressure gave a dark brown oil which was purified by column chromatography on silica (10% ethyl acetate/petroleum ether) to give **3.80** (1.65 g, 84%) as a fluffy white powder.

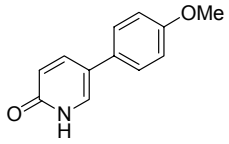
mp 66-68°C (Lit.[11] 64°C).

^{1}H NMR ($CDCl_3$, 500 MHz) δ 3.85 (s, 3H, OC**H$_3$**), 6.98 (m, 3H, Ar**H**), 7.46 (d, 2H, *J* = 8.3 Hz, Ar**H**), 7.91 (dt, 1H, *J* = 2.4, 8.7 Hz, Ar**H**), 8.36 (d, 1H, *J* = 2.4 Hz, Ar**H**).

^{13}C NMR ($CDCl_3$, 75 MHz) δ 55.24, 109.2 (d, *J* = 37.4 Hz), 114.47, 128.01, 128.96, 134.37, 139.14 (d, J = 7.8 Hz), 145.16 (d, *J* = 14.5 Hz), 156.65, 162.61 (d, *J* = 238.1 Hz).

HRMS (M+H) Found 204.0828 (Calcd. For $C_{12}H_{11}NOF$ 204.0825).

5-(4-Methoxyphenyl)pyridin-2(1*H*)-one (3.81)



2-Fluoropyridine **3.80** (504 mg, 2.48 mmol) was heated at reflux for 24 h in 4 M aqueous hydrochloric acid in dioxane (5 mL) and water (1.67 mL). The solution was cooled to rt, and the solvent removed by evaporation under reduced pressure to give **3.81** (498 mg, quantitative) as a white solid. The product was not purified further.

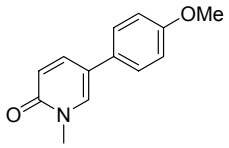
mp 186-188˚C.

^{1}H NMR (DMSO-d_6, 500 MHz) δ 3.87 (s, 3H, OC**H$_3$**), 6.81 (d, 1H, *J* = 9.3 Hz, COC**H**=CH), 7.09 (d, 2H, *J* = 8.8 Hz, Ar**H**), 7.63 (d, 2H, *J* = 8.8 Hz, Ar**H**), 7.94 (d, 1H, *J* = 2.9 Hz, NHC**H**), 8.11 (dd, 1H, *J* = 2.4, 9.3 Hz, COCH=C**H**).

^{13}C NMR (DMSO-d_6, 75 MHz) δ 55.25, 114.45, 118.03, 120.00, 126.59, 128.68, 131.91. 140.24, 158.44, 161.71.

HRMS (M+H) Found 202.0859 (Calcd. For $C_{12}H_{12}NO_2$ 202.0868).

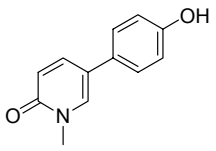
5-(4-Methoxyphenyl)-1-methylpyridin-2(1*H*)-one (3.77) - Method 2



To the pyridone **3.81** (145 mg, 0.72 mmol, 1 equiv) in DMF (2.5 mL) under N_2 atmosphere was added lithium hydride (7 mg, 0.86 mmol, 1.2 equiv) and the grey suspension stirred at 50°C for 1.5 h. Methyl iodide (67 μL, 1.08 mmol, 1.5 equiv) was added and the orange solution stirred at 50°C for 16 h. The mixture was cooled to rt, and saturated aqueous sodium bicarbonate was added to give a thick white suspension. The organic material was extracted with ethyl acetate (3 x 20 mL). The organic phases were combined and washed

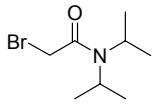
with water (4 x 20 mL), dried over $MgSO_4$ and the solvent removed by evaporation under reduced pressure. The resulting yellow oil was purified by column chromatography on silica (5% methanol/DCM) to give **3.77** (106 mg, 68%) as an orange solid.
See **3.77** - Method 1 for characterisation.

5-(4-Hydroxyphenyl)-1-methylpyridin-2(1*H*)-one (3.78)



The methyl ether **3.77** (90 mg, 0.42 mmol) was heated at reflux for 16 h in 48% aqueous hydrobromic acid (1.5 mL) and acetic acid (1.5 mL). The solution was cooled to rt, and the solvent removed by evaporation under reduced pressure to give a brown residue that was dissolved in ethyl acetate. The solution was extracted with 1 M aqueous potassium hydroxide (3 x 20 mL). The aqueous phases were combined and acidified with 6 M aqueous hydrochloric acid until neutral pH (universal indicator paper). The aqueous solution was re-extracted with ethyl acetate (3 x 50 mL). The organic phases were combined and dried over $MgSO_4$. Removal of the solvent by evaporation under reduced pressure gave an orange solid which was purified by column chromatography on silica (5% methanol/DCM) to give **3.78** (70 mg, 83%) as a cream powder.
mp 246-248°C
^{1}H NMR (DMSO-d_6, 500 MHz) δ 3.58 (s, 3H, NC**H$_3$**), 6.56 (d, 1H, *J* = 9.3 Hz, COC**H**=CH), 6.90 (d, 2H, *J* = 8.8 Hz, Ar**H**), 7.46 (d, 2H, *J* = 8.8 Hz, Ar**H**), 7.84 (dd, 1H, *J* = 2.9, 9.8 Hz, COCH=C**H**), 8.08 (d, 1H, *J* = 2.9 Hz, NCH$_3$C**H**).
^{13}C NMR (DMSO-d_6, 75 MHz) δ 37.14, 115.74, 118.56, 118.93, 126.58, 126.78, 136.07, 139.14, 156.61, 161.08.
HRMS (M+H) Found 202.0858 (Calcd. For $C_{12}H_{12}NO_2$ 202.0868).

2-Bromo-*N*,*N*-diisopropylacetamide (3.83)[12]



Benzyltriethylammonium chloride (80 mg, 0.35 mmol, 0.01 equiv) was dissolved in 1,2-dichloroethane (26 mL). Diisopropylamine (6.01 mL, 34.50 mmol, 1 equiv) was added, followed by an aqueous solution of sodium hydroxide (1.38 g in 3 mL water, 34.50 mmol, 1 equiv). The solution was cooled in ice and bromoacetyl bromide (3 mL, 34.50 mmol, 1 equiv) was added dropwise to give a thick yellow precipitate. The solution was stirred at rt for 16 h. The reaction mixture was washed with 1 M aqueous sodium hydroxide (2 x 50 mL), then 10% aqueous hydrochloric acid (2 x 50 mL), followed by brine. The organic phase was dried over $MgSO_4$ and the solvent removed by evaporation under reduced pressure. The resulting orange oil was purified by column chromatography on silica (50% ethyl acetate/petroleum ether) to give **3.83** (5.71 g, 75%) as a white solid.

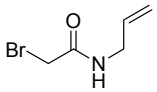
mp 60-63˚C.

^{1}H NMR ($CDCl_3$, 500 MHz) δ 1.24 (d, 6H, J = 6.4 Hz, (C**H**$_3$)$_2$), 1.38 (d, 6H, J = 6.8 Hz, (C**H**$_3$)$_2$), 3.42 (m, 1H, C**H**(CH$_3$)$_2$), 3.80 (s, 2H, C**H**$_2$), 3.95 (m, 1H, C**H**(CH$_3$)$_2$).

^{13}C NMR ($CDCl_3$, 75 MHz) δ 19.99, 20.58, 28.68, 46.14, 50.34, 165.22.

HRMS (M+H) Found 222.0497 (Calcd. For $C_8H_{17}NO^{79}Br$ 222.0494).

***N*-Allyl-2-bromoacetamide (3.84)**



Benzyltriethylammonium chloride (56 mg, 0.25 mmol, 0.01 equiv) was dissolved in 1,2-dichloroethane (16 mL). Allylamine (1.88 mL, 25 mmol, 1 equiv) was added, followed by an aqueous solution of sodium hydroxide (1 g in 5 mL water, 25 mmol, 1 equiv). The solution was cooled in ice and bromoacetyl bromide (2.18 mL, 25 mmol, 1 equiv) was added dropwise. The solution was stirred at rt for 16 h. The reaction mixture was diluted

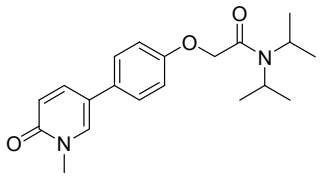
with ethyl acetate, washed with 1 M aqueous sodium hydroxide (2 x 50 mL), 10% aqueous hydrochloric acid (2 x 50 mL), and brine. The organic phase was dried over $MgSO_4$ and the solvent removed by evaporation under reduced pressure. The resulting clear oil was purified by column chromatography on silica (20% ethyl acetate/petroleum ether) to give **3.84** (3.02 g, 67%) as a clear oil.

^{1}H NMR ($CDCl_3$, 500 MHz) δ 3.92 (m, 4H, (C**H$_2$**)$_2$), 5.17 (m, 2H, CH=C**H$_2$**), 5.85 (m, 1H, C**H**=CH_2), 6.67 (s(br), 1H, N**H**).

^{13}C NMR ($CDCl_3$, 75 MHz) δ 28.98, 42.32, 116.73, 133.14, 165.42.

HRMS (M+H) Found 177.9859 (Calcd. For $C_5H_9NO^{79}Br$ 177.9868).

N,N-Diisopropyl-2-(4-(1-methyl-6-oxo-1,6-dihydropyridin-3-yl)phenoxy)acetamide (3.55)



To the phenol **3.78** (100 mg, 0.49 mmol, 1 equiv) in DMSO (6 mL) under N_2 atmosphere was added 2 M aqueous sodium hydroxide (0.37 mL, 0.75 mmol, 1.5 equiv) and the resulting brown solution stirred at 80°C for 30 min. 2-Bromo-*N*,*N*-diisopropylacetamide (**3.83**) (109 mg, 0.49 mmol, 1 equiv) was added and the solution stirred at 80°C for 16 h. The reaction mixture was cooled to rt, quenched with water (25 mL), and extracted with ethyl acetate (3 x 20 mL). The organic phases were combined and washed with water (4 x 20 mL), 2 M aqueous sodium hydroxide (20 mL) and dried over $MgSO_4$. Removal of the solvent by evaporation under reduced pressure gave a thick yellow oil which was purified by column chromatography on silica (5% methanol/DCM) to give **3.55** (78 mg, 46%) as a yellow oil.

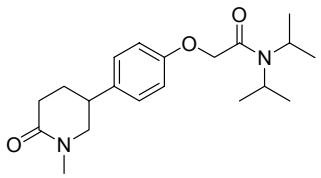
^{1}H NMR (CD_3OD, 500 MHz) δ 1.24 (d, 6H, *J* = 6.4 Hz, (C**H$_3$**)$_2$), 1.39 (d, 6H, *J* = 6.4 Hz, (C**H$_3$**)$_2$), 3.43 (m, 1H, C**H**(CH_3)$_2$), 3.61 (s, 3H, NC**H$_3$**), 4.09 (m, 1H, C**H**(CH_3)$_2$), 4.72 (s, 2H, C**H$_2$**), 6.59 (d, 1H, *J* = 9.3 Hz, COC**H**=CH), 7.00 (d, 2H, *J* = 8.8 Hz, Ar**H**), 7.43 (d,

2H, *J* = 8.8 Hz, Ar**H**), 7.80 (dd, 1H, *J* = 2.4, 9.3 Hz, COCH=C**H**), 7.86 (d, 1H, *J* = 2.4 Hz, NCH_3C**H**).

^{13}C NMR (CD_3OD, 75 MHz) δ 21.01, 21.20, 38.64, 47.68, 69.22, 116.60, 120.44, 122.69, 128.36, 130.83, 137.65, 141.75, 159.52, 164.63, 169.21.

HRMS (M+H) Found 343.2032 (Calcd. For $C_{20}H_{27}N_2O_3$ 343.2022).

_N,N_-Diisopropyl-2-(4-(1-methyl-6-oxopiperidin-3-yl)phenoxy)acetamide (3.56)



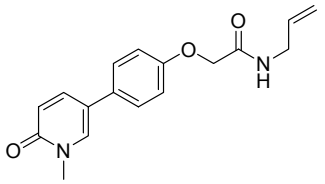
The 1-methyl pyridone **3.55** (21 mg, 0.06 mmol, 1 equiv) was dissolved in ethanol (10 mL) and 10% palladium on charcoal (20 mg) was added. The mixture was reacted with hydrogen (40 bar) in a PARR hydrogenator for 48 h. The catalyst was removed by filtration and the solvent removed by evaporation under reduced pressure. The resulting cream solid was purified by column chromatography on silica (5% methanol/DCM) to give **3.56** (13 mg, 63%) as a white solid.

^{1}H NMR (CD_3OD, 500 MHz) δ 1.24 (d, 6H, *J* = 6.4 Hz, (C**H$_3$**)**$_2$**), 1.38 (d, 6H, *J* = 6.8 Hz, (C**H$_3$**)**$_2$**), 2.02 (m, 2H, NCH_3C**H$_2$**), 2.46 (m, 2H, COC**H$_2$**CH_2), 2.94 (s, 3H, NC**H$_3$**), 3.09 (m, 1H, NCH_3CH_2C**H**), 3.41 (m, 2H, $COCH_2C$**H$_2$**), 3.54 (m, 1H, C**H**$(CH_3)_2$), 4.09 (m, 1H, C**H**$(CH_3)_2$), 4.67 (s, 2H, OC**H$_2$**), 6.92 (d, 2H, *J* = 8.8 Hz, Ar**H**), 7.23 (d, 2H, *J* = 8.3 Hz, Ar**H**).

^{13}C NMR (DMSO-d_6, 75 MHz) δ 20.43, 20.59, 28.00, 31.77, 34.00, 44.99, 47.80, 55.66, 67.59, 114.57, 127.93, 128.06, 134.65, 157.05, 166.04, 168.30.

HRMS (M+H) Found 347.2327 (Calcd. For $C_{20}H_{31}N_2O_3$ 347.2335).

***N*-Allyl-2-(4-(1-methyl-6-oxo-1,6-dihydropyridin-3-yl)phenoxy)acetamide (3.57)**



The phenol **3.78** (350 mg, 1.72 mmol, 1 equiv) was stirred in DMF (10 mL) under N_2 atmosphere to which 2 M aqueous potassium hydroxide (1.29 mL, 2.59 mmol, 1.5 equiv) was added and the mixture stirred at 80˚C for 1.5 h. *N*-Allyl-2-bromoacetamide (**3.84**) (333 mg, 1.89 mmol, 1.1 equiv) was added and the solution stirred at 80˚C for 16 h. The reaction mixture was cooled to rt, quenched with water (25 mL), and extracted with ethyl acetate (3 x 20 mL). The organic phases were combined and washed with water (4 x 20 mL), and dried over $MgSO_4$. Removal of the solvent by evaporation under reduced pressure gave an orange oil which was purified by column chromatography on silica (5% methanol/DCM) to give **3.57** (239 mg, 79%) as a yellow solid.

mp 160-162˚C.

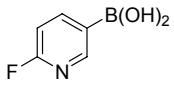
^{1}H NMR (CD_3OD, 500 MHz) δ 3.65 (s, 3H, NC**H$_3$**), 3.89 (d(br), 2H, *J* = 5.4 Hz, NHC**H$_2$**), 4.55 (s, 2H, OC**H$_2$**), 5.13 (m, 2H, CH=C**H$_2$**), 5.85 (m, 1H, C**H**=CH_2), 6.62 (d, 1H, *J* = 9.3 Hz, COC**H**=CH), 7.06 (d, 2H, *J* = 8.8 Hz, Ar**H**), 7.48 (d, 2H, *J* = 8.8 Hz, Ar**H**), 7.83 (dd, 1H, *J* = 2.4, 9.3 Hz, COCH=C**H**), 7.90 (d, 1H, *J* = 2.4 Hz, NCH_3C**H**).

^{13}C NMR (CD_3OD, 75 MHz) δ 38.66, 42.63, 68.54, 116.58, 116.70, 120.47, 122.55, 128.47, 131.29, 135.54, 137.72, 141.70, 158.97, 164.63, 171.07.

HRMS (M+H) Found 299.1390 (Calcd. For $C_{17}H_{19}N_2O_3$ 299.1396).

4.3.2 Synthesis of 5-aryl 1-methyl-pyridone inhibitor 3.59

6-Fluoropyridin-3-yl boronic acid (3.85)[11]



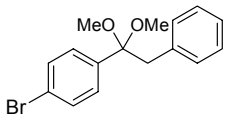
n-Butyllithium (15% in hexane, 3.6 mL, 5.76 mmol, 1.2 equiv) was added to dry ether (5 mL) in a flask under N_2 atmosphere and cooled to -78°C. A solution of 5-bromo-2-fluoropyridine (0.5 mL, 4.86 mmol, 1 equiv) in dry ether (3 mL) was added dropwise and the resulting yellow precipitate was stirred at -78°C for 45 min. Trimethylborate (0.66 mL, 5.83 mmol, 1.2 equiv) was added and the reaction warmed to rt, then stirred for 1 h. The reaction mixture was treated with 5% aqueous sodium hydroxide (25 mL), cooled in ice and acidified with 3 M aqueous hydrochloric acid until neutral pH (universal indicator paper). The solution was extracted with ethyl acetate (3 x 50 mL) and the organic phases combined and dried over $MgSO_4$. The solvent was removed by evaporation under reduced pressure to give a yellow solid which was washed with 10% ethyl acetate/petroleum ether to give **3.85** (456 mg, 73%) as a cream powder.

mp 190-191˚C (Lit.[13] 190-191˚C).

^{1}H NMR (DMSO-d_6, 500 MHz) δ 7.24 (dd, 1H, J = 2.5, 8.3 Hz, **H**5), 8.37 (dt, 1H, J = 2, 8.3 Hz, **H**4), 8.52 (s(br), 2H, (O**H)$_2$**), 8.64 (d, 1H, J = 2 Hz, **H**2).

^{13}C NMR (DMSO-d_6, 75 MHz) δ 108.75 (d, J = 35.3 Hz), 147.66 (d, J = 7.8 Hz), 153.5 (d, J = 14.5 Hz), 164.54 (d, J = 237.6 Hz).

1-Bromo-4-(1,1-dimethoxy-2-phenylethyl)benzene (3.89)



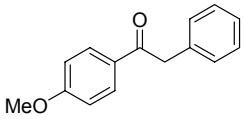
Benzyl-4-bromophenyl ketone (200 mg, 0.72 mmol, 1 equiv) and 4Å molecular sieves were added to a flask under N_2 atmosphere. Methanol (2 mL), trimethylorthoformate (4 mL), and a few drops of trifluoromethanesulfonic acid were added, and the solution heated at reflux for 19 h. The reaction mixture was cooled to rt, quenched with saturated aqueous sodium bicarbonate (15 mL), and extracted with ether (3 x 20 mL). The organic phases were combined, washed with water, and dried over $MgSO_4$. The solvent was removed by evaporation under reduced pressure to give a clear pale yellow oil which was purified by column chromatography on silica (5% ethyl acetate/petroleum ether) to give **3.89** (169 mg, 73%) as a colourless oil.

^{1}H NMR ($CDCl_3$, 500 MHz) δ 3.16 (s, 2H, OC**H$_2$**), 3.27 (s, 6H, (OC**H$_3$**)$_2$, 6.76 (d, 2H, *J* = 9 Hz, Ar**H**), 7.08 (m, 5H, Ar**H**), 7.34 (d, 2H, *J* = 8.3 Hz, Ar**H**).

^{13}C NMR ($CDCl_3$, 75 MHz) δ 43.5, 49, 103.59, 121.69, 126.2, 127.65, 129.15, 130.24, 130.7, 135.58, 139.21.

No further analytical data was obtained due to product degradation.

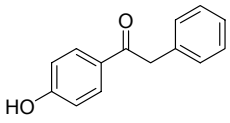
1-(4-Methoxyphenyl)-2-phenylethanone (3.93)[14]



Aluminium chloride (1.2 g, 9.07 mmol, 1.2 equiv) was added to a flask under N_2 atmosphere containing 1, 2-dichloroethane (8 mL). Anisole (0.82 mL, 7.56 mmol, 1 equiv) was added and the mixture cooled in ice. Phenylacetyl chloride (1 mL, 7.56 mmol, 1 equiv) was added dropwise, resulting in a bright fluorescent red mixture that was heated at reflux for 16 h. The reaction mixture was cooled to rt, poured into water (20 mL) and

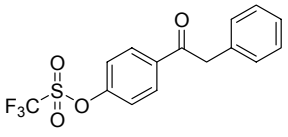
extracted with ethyl acetate (3 x 50 mL). The organic phases were combined and washed with water, brine, and dried over $MgSO_4$. The solvent was removed by evaporation under reduced pressure to give an orange oil which was purified by column chromatography on silica (10% ethyl acetate/petroleum ether) to give **3.93** (1.07 g, 60%) as yellow crystals.
mp 69-71˚C (Lit. 68-69˚C).
^{1}H NMR ($CDCl_3$, 500 MHz) δ 3.86 (s, 3H, OC**H$_3$**), 4.23 (s, 2H, C**H$_2$**), 6.92 (d, 2H, *J* = 9.1 Hz, Ar**H**), 7.28 (m, 5H, Ar**H**), 7.99 (d, 2H, *J* = 8.7 Hz, Ar**H**).
^{13}C NMR ($CDCl_3$, 75 MHz) δ 45.13, 55.33, 113.68, 126.66, 128.52, 129.28, 129.46, 130.85, 133.35, 134.862, 163.42, 176.59, 196.21.

1-(4-Hydroxyphenyl)-2-phenylethanone (3.94)



The methyl ether **3.93** (300 mg, 1.33 mmol) was heated at reflux for 7 h in 48% aqueous hydrobromic acid (3 mL) and acetic acid (3 mL). The solution was cooled to rt, and the solvent removed by evaporation under reduced pressure to give a brown residue that was dissolved in ethyl acetate. The solution was extracted with 1 M aqueous potassium hydroxide (3 x 40 mL). The aqueous phases were combined and acidified with 6 M aqueous hydrochloric acid until neutral pH (universal indicator paper). The aqueous solution was re-extracted with ethyl acetate (3 x 80 mL). The organic phases were combined and dried over $MgSO_4$. Removal of the solvent by evaporation under reduced pressure gave an orange solid which was purified by column chromatography on silica (50% ethyl acetate/petroleum ether) to give **3.94** (160 mg, 57%) as a white solid.
mp 130-132 ˚C (Lit.[15] 134-136˚C).
^{1}H NMR (acetone-d_6, 500 MHz) δ 4.38 (s, 2H, C**H$_2$**), 7.04 (d, 2H, *J* = 10.7 Hz, Ar**H**), 7.42 (m, 5H, Ar**H**), 8.11 (d, 2H, *J* = 11.2 Hz, Ar**H**), 9.35 (s(br), 1H, O**H**).

4-(2-Phenylacetyl)phenyltrifluoromethanesulfonate (3.95)



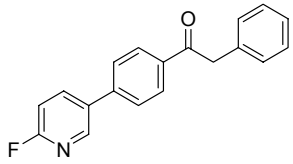
The phenol **3.94** (160 mg, 0.75 mmol, 1 equiv) was dissolved in dry ice cooled DCM (2 mL) under N_2 atmosphere and pyridine (0.5 mL) was added. Trifluoromethanesulfonic anhydride (0.19 mL, 1.13 mmol, 1.5 equiv) was added dropwise and the resulting red solution was stirred for 45 min in ice. The reaction was quenched by the addition of water (5 mL), and the organic layer was separated and diluted with DCM (20 mL). This solution was washed with 10% aqueous hydrochloric acid (3 x 20 mL), dried over Na_2SO_4, and the solvent removed by evaporation under reduced pressure. The resulting red/brown solid was purified by column chromatography on silica (50% ethyl acetate/petroleum ether) to give **3.95** (152 mg, 59%) as a pale yellow solid.

^{1}H NMR ($CDCl_3$, 500 MHz) δ 4.28 (s, 2H, C**H$_2$**), 7.31 (m, 7H, Ar**H**), 8.11 (d, 2H, *J* = 9.1 Hz, Ar**H**).

^{13}C NMR ($CDCl_3$, 75 MHz) δ 45.63, 116.51, 120.76, 121.65, 127.17, 128.81, 129.34, 130.85, 134.99 (d, *J* = 195 Hz), 152.38, 195.73.

No further analytical data was obtained due to product degradation.

1-(4-(6-Fluoropyridin-3-yl)phenyl)-2-phenylethanone (3.87)



A flask was charged with benzyl-4-bromophenyl ketone (1.05 g, 3.82 mmol, 1 equiv) and tetrakis(triphenylphosphine)palladium(0) (440 mg, 0.38 mmol, 0.1 equiv) under N_2 atmosphere. A solution of 6-fluoropyridin-3-yl boronic acid (**3.85**) (1.07 g, 8.39 mmol, 2.2

equiv) in ethanol (15 mL) was added followed by toluene (30 mL), and 2 M aqueous sodium carbonate (4.18 mL, 8.39 mmol, 2.2 equiv). After 10 min the reaction mixture was slightly black in colour so a further small amount of catalyst was added. The solution was heated at reflux for 18 h, after which it was cooled to rt, diluted with ethyl acetate and washed with water. The organic phase was dried over $MgSO_4$ and the solvent removed by evaporation under reduced pressure. The resulting yellow solid was purified by column chromatography on silica (15% ethyl acetate/petroleum ether) to give **3.87** (934 mg, 85%) as a fine white solid.

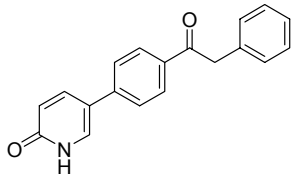
mp 134-136°C.

^{1}H NMR ($CDCl_3$, 500 MHz) δ 4.33 (s, 2H, C**H$_2$**), 7.05 (dd, 1H, *J* = 2.8, 8.3 Hz, Ar**H**), 7.31 (m, 5H, Ar**H**), 7.64 (d, 2H, *J* = 8.3 Hz, Ar**H**), 8.0 (dt, 1H, *J* = 2.4, 8.3 Hz, Ar**H**), 8.12 (d, 2H, *J* = 8.3 Hz, Ar**H**), 8.46 (d, 1H, *J* = 2 Hz, Ar**H**).

^{13}C NMR ($CDCl_3$, 75 MHz) δ 45.63, 109.73 (d, *J* = 37.9 Hz), 126.98, 127.19, 128.74, 129.38, 129.49, 133.53, 134.30, 135.96, 139.78 (d, *J* = 7.8 Hz), 141.13, 146.06 (d, *J* = 15 Hz), 163.52 (d, *J* = 240.7 Hz), 196.95.

HRMS (M+H) Found 292.1132 (Calcd. For $C_{19}H_{15}NOF$ 292.1138).

5-(-4-(2-Phenylacetyl)phenyl)pyridin-2(1*H*)-one (3.88)



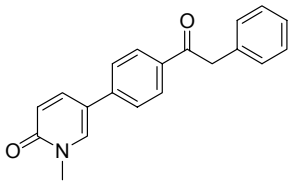
2-Fluoropyridine **3.87** (187 mg, 0.64 mmol) was dissolved in 4 M aqueous hydrochloric acid in dioxane (3 mL) and H_2O (1 mL). The solution was heated at reflux for 24 h, cooled to rt, and the solvent removed by evaporation under reduced pressure to give **3.88** (176 mg, 95%) as a cream solid. The product was not purified further.

mp 200-204°C.

^{1}H NMR (DMSO-d_6, 500 MHz) δ 4.48 (s, 2H, C**H$_2$**), 6.57 (d, 1H, *J* = 9.3 Hz, COC**H**=CH), 7.25 (m, 5H, Ar**H**), 7.84 (d, 2H, *J* = 8.3 Hz, Ar**H**), 7.99 (d, 1H, *J* = 2.4 Hz, NHC**H**), 8.04 (dd, 1H, *J* = 2.4, 9.3 Hz, COCH=C**H**), 8.16 (d, 2H, *J* = 8.8 Hz, Ar**H**).

^{13}C NMR (DMSO-d_6, 75 MHz) δ 44.77, 108.17, 116.85, 120.13, 125.25, 126.58, 128.43, 129.30, 129.75, 134.19, 135.32, 139.93, 140.75, 161.87, 197.10.
HRMS (M+H) Found 290.1168 (Calcd. For $C_{19}H_{16}NO_2$ 290.1181).

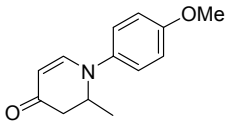
1-Methyl-5-(4-(2-phenylacetyl)phenyl)pyridin-2(1*H*)-one (3.59)



To the pyridone **3.88** (140 mg, 0.48 mmol, 1 equiv) in DMF (4 mL) under N_2 atmosphere was added lithium hydride (3.7 mg, 0.48 mmol, 1 equiv) and the grey suspension stirred at rt for 1 h. Methyl iodide (45 μL, 0.72 mmol, 1.5 equiv) was added and the yellow solution stirred at rt for 16 h. The reaction was quenched with water (10 mL), and the mixture was extracted with ethyl acetate (3 x 20 mL). The organic phases were combined and washed with water (4 x 20 mL), dried over $MgSO_4$, and the solvent removed by evaporation under reduced pressure. The resulting yellow oil was purified by column chromatography on silica (5% methanol/DCM) to give **3.59** (90 mg, 52%) as a pale yellow solid.
mp decomp.>140˚C.
^{1}H NMR (DMSO-d_6, 500 MHz) δ 3.62 (s, 3H, NC**H$_3$**), 4.49 (s, 2H, C**H$_2$**), 6.61 (d, 1H, *J* = 9.8 Hz, COC**H**=CH), 7.38 (m, 5H, Ar**H**), 7.85 (d, 2H, *J* = 8.3 Hz, Ar**H**), 8.02 (dd, 1H, *J* = 2.7, 9.7 Hz, COCH=C**H**), 8.18 (d, 1H, *J* = 8.3 Hz, Ar**H**), 8.41 (d, 1H, *J* = 2.9 Hz, NCH$_3$C**H**).
^{13}C NMR (DMSO-d_6, 75 MHz) δ 37.26, 44.77, 116.35, 119.34, 125.16, 126.59, 128.44, 129.33, 129.76, 134.38, 135.33, 138.59, 139.74, 140.85, 161.91, 197.10.
HRMS (M+H) Found 304.1340 (Calcd. for $C_{20}H_{18}NO_2$ 304.1338).

4.3.3 Synthesis of 1-aryl 2-methyl 2,3-dihydro-4-pyridone inhibitors 3.61 and 3.62

1-(4-Methoxyphenyl)-2-methyl-2,3-dihydropyridin-4(1*H*)-one (3.98)



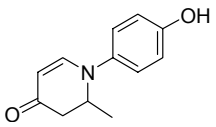
Acetaldehyde (42 μL, 0.75 mmol, 1 equiv) was added to super-dry methanol (6 mL) under N_2 atmosphere. *p*-Anisidine (93 mg, 0.75 mmol, 1 equiv) was added, followed by Danishefsky's diene (291 μL, 1.50 mmol 2 equiv). The bright yellow solution was stirred at rt for 2 h, and quenched with 1 M aqueous hydrochloric acid (1.5 mL). The mixture was diluted with ether (50 mL), dried over $MgSO_4$, and the solvent removed by evaporation under reduced pressure. The resulting yellow oil was purified by column chromatography on silica (5% methanol/DCM) to give **3.98** (145 mg, 89%) as a bright yellow oil.

^{1}H NMR ($CDCl_3$, 500 MHz) δ 1.26 (d, 3H, *J* = 6.8 Hz, CHC**H$_3$**), 2.30 (dd, 1H, *J* = 3.9, 16.6 Hz, COC**H$_a$**H$_b$), 2.94 (dd, 1H, *J* = 6.8, 16.6 Hz, COCH$_a$**H$_b$**), 3.77 (s, 3H, OC**H$_3$**), 4.19 (m, 1H, C**H**CH$_3$), 5.08 (d, 1H, *J* = 7.5 Hz, C**H**=CH), 6.86 (d, 2H, *J* = 9.1 Hz, Ar**H**), 7.03 (d, 2H, *J* = 9.1 Hz, Ar**H**), 7.18 (d, 1H, *J* = 7.5 Hz, CH=C**H**).

^{13}C NMR ($CDCl_3$, 75 MHz) δ 15.79, 42.60, 54.82, 55.52, 99.80, 114.73, 122.54, 137.66, 148.76, 157.23, 191.28.

HRMS (M+H) Found 218.1181 (Calcd. for $C_{13}H_{16}NO_2$ 218.1184).

1-(4-Hydroxyphenyl)-2-methyl-2,3-dihydropyridin-4(1*H*)-one (3.99)



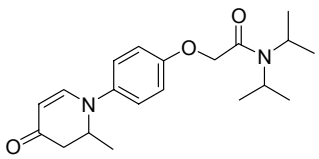
The methyl ether **3.98** (145 mg, 0.67 mmol, 1 equiv) was stirred at 80˚C for 8 h in 48% aqueous hydrobromic acid (1 mL) and acetic acid (1 mL). The solution was cooled to rt, and the solvent removed by evaporation under reduced pressure to give a brown residue that was dissolved in ethyl acetate. The solution was extracted with 1 M aqueous potassium hydroxide (3 x 40 mL). The aqueous phases were combined and acidified with 1 M aqueous hydrochloric acid until neutral pH (universal indicator paper). The aqueous solution was re-extracted with ethyl acetate (3 x 50 mL). The organic phases were combined and dried over $MgSO_4$. Removal of the solvent by evaporation under reduced pressure gave a brown oil which was purified by column chromatography on silica (5% methanol/DCM) to give **3.99** (89 mg, 66%) as a pale yellow solid.

mp 171-173˚C.

^{1}H NMR (acetone-d_6, 500 MHz) δ 1.22 (d, 3H, J = 6.8 Hz, CHC**H$_3$**), 2.29 (dd, 1H, J = 3.9, 16.6 Hz, COC**H$_a$**H$_b$), 2.97 (dd, 1H, J = 6.8, 16.6 Hz, COCH$_a$**H$_b$**), 4.30 (m, 1H, C**H**CH$_3$), 5.06 (d, 1H, J = 7.3 Hz, C**H**=CH), 6.82 (d, 2H, J = 8.8 Hz, Ar**H**), 7.11 (d, 2H, J = 8.8 Hz, Ar**H**), 7.46 (d, 1H, J = 7.8 Hz, CH=C**H**), 8.67 (s, 1H, O**H**).

^{13}C NMR (acetone-d_6, 75 MHz) δ 13.65, 41.26, 53.31, 97.79, 114.76, 121.52, 135.84, 147.07, 153.81, 188.49.

_N,N_-Diisopropyl-2-(4-(2-methyl-4-oxo-3,4-dihydropyridin-1(2_H_)-yl)phenoxy) acetamide (3.61)



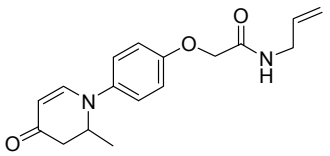
To the phenol **3.99** (30 mg, 0.15 mmol, 1 equiv) in DMF (2 mL) under N_2 atmosphere was added 2 M aqueous potassium hydroxide (110 μL, 0.22 mmol, 1.5 equiv) and the yellow/green solution stirred at 80°C for 1 h. 2-Bromo-*N*,*N*-diisopropylacetamide (**3.83**) (32.5 mg, 0.15 mmol, 1 equiv) was added and the solution stirred at 80°C for 16 h. The reaction mixture was cooled to rt, quenched with 10% aqueous sodium bicarbonate (10 mL), and extracted with ethyl acetate (3 x 10 mL). The organic phases were combined, washed with water (4 x 20 mL) and dried over $MgSO_4$. Removal of the solvent by evaporation under reduced pressure gave a pale orange oil which was purified by column chromatography on silica (5% methanol/DCM) to give **3.61** (15 mg, 30%) as a yellow oil.

^{1}H NMR (CD_3OD, 500 MHz) δ 1.25 (d, 3H, *J* = 6.3 Hz, NCHC**H$_3$**), 1.25 (d, 6H, *J* = 6.3 Hz, CH(C**H$_3$)$_2$**), 1.40 (d, 6H, *J* = 6.8 Hz, CH(C**H$_3$)$_2$**), 2.30 (dd, 1H, *J* = 3.2, 16.6 Hz, COC**H$_a$**H$_b$), 3.00 (dd, 1H, *J* = 6.8, 16.6 Hz, COCH$_a$**H$_b$**), 3.56 (m, 1H, C**H**$(CH_3)_2$), 4.08 (m, 1H, C**H**$(CH_3)_2$), 4.37 (m, 1H, NC**H**CH_3), 4.72 (s, 2H, OC**H$_2$**), 5.09 (d, 1H, *J* = 7.8 Hz, COC**H**=CH), 7.01 (d, 2H, *J* = 9.3 Hz, Ar**H**), 7.24 (d, 2H, *J* = 8.8 Hz, Ar**H**), 7.53 (d, 1H, *J* = 7.8 Hz, COCH=C**H**).

^{13}C NMR (CD_3OD, 75 MHz) δ 16.00, 21.00, 21.18, 43.10, 47.71, 48.45, 56.18, 69.33, 99.60, 117.04, 124.33, 139.545, 152.56, 158.00, 169.11, 194.44.

HRMS (M+H) Found 345.2188 (Calcd. for $C_{20}H_{29}N_2O_3$ 345.2178).

_N_-Allyl-2-(4-(2-methyl-4-oxo-3,4-dihydropyridin-1(2_H_)-yl)phenoxy)acetamide (3.62)



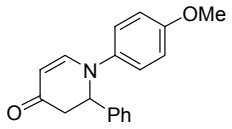
To the phenol **3.99** (35 mg, 0.17 mmol, 1 equiv) in DMF (2 mL) under N_2 atmosphere was added 2 M aqueous potassium hydroxide (129 μL, 0.26 mmol, 1.5 equiv) and the green solution stirred at 80°C for 1 h. *N*-Allyl-2-bromoacetamide (**3.84**) (30 mg, 0.17 mmol, 1 equiv) was added and the solution stirred at 80°C for 16 h. The reaction mixture was cooled to rt, quenched with 10% aqueous sodium bicarbonate (10 mL), and extracted with ethyl acetate (3 x 10 mL). The organic phases were combined, washed with water (4 x 20 mL) and dried over $MgSO_4$. Removal of the solvent by evaporation under reduced pressure gave a yellow oil which was purified by column chromatography on silica (5% methanol/DCM) to give **3.62** (22 mg, 43%) as a yellow oil.

^{1}H NMR (CD_3OD, 500 MHz) δ 1.25 (d, 3H, *J* = 6.4 Hz, CHC**H$_3$**), 2.31 (dd, 1H, *J* = 2.9, 16.6 Hz, COC**H$_a$**H$_b$), 3.01 (dd, 1H, *J* = 6.4, 16.6 Hz, COCH$_a$**H$_b$**), 3.88 (d(br), 2H, *J* = 4.9 Hz, NHC**H$_2$**), 4.39 (m, 1H, C**H**CH$_3$), 4.55 (s, 2H, OC**H$_2$**), 5.10 (d, 1H, J = 7.3 Hz, COCH=C**H**), 5.12 (m, 2H, CH$_2$CH=C**H$_2$**), 5.84 (m, 1H, CH$_2$C**H**=CH$_2$), 7.06 (d, 2H, *J* = 8.8 Hz, Ar**H**), 7.26 (d, 2H, *J* = 9.2 Hz, Ar**H**), 7.54 (d, 1H, *J* =7.8 Hz, COC**H**=CH).

^{13}C NMR (CD_3OD, 75 MHz) δ 15.70, 42.32, 42.80, 55.82, 68.45, 99.47, 116.28, 116.88, 124.00, 135.23, 139.61, 152.05, 157.06, 170.66, 194.14.

HRMS (M+H) Found 301.1546 (Calcd. For $C_{17}H_{21}N_2O_3$ 301.1552).

1-(4-Methoxyphenyl)-2-phenyl-2,3-dihydropyridin-4(1*H*)-one (3.100)[16]



Benzaldehyde (75 μL, 0.75 mmol, 1 equiv) was added to super-dry methanol (6 mL) under N_2 atmosphere. *p*-Anisidine (93 mg, 0.75 mmol, 1 equiv) was added, followed by Danishefsky's diene (291 μL, 1.50 mmol 2 equiv). The bright yellow solution was stirred at rt for 2 h, and quenched with 1 M aqueous hydrochloric acid (1.5 mL). The mixture was diluted with ether (50 mL), dried over $MgSO_4$, and the solvent removed by evaporation under reduced pressure. The resulting orange oil was purified by column chromatography on silica (5% methanol/DCM) to give **3.100** (132 mg, 64%) as a bright yellow oil.

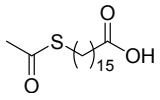
^{1}H NMR ($CDCl_3$, 500 MHz) δ 2.77 (dd, 1H, *J* = 4.0, 16.7 Hz, COC**H$_a$**H$_b$), 3.26 (dd, 1H, *J* = 7.1, 16.3 Hz, COCH$_a$**H$_b$**), 3.76 (s, 3H, OC**H$_3$**), 5.19 (m, 1H, C**H**Ph), 5.23 (d, 1H, *J* = 7.9 Hz, COC**H**=CH), 6.81 (d, 2H, *J* = 8.7 Hz, Ar**H**), 6.96 (d, 2H, *J* = 9.1 Hz, Ar**H**), 7.29 (m, 5H, Ar**H**), 7.55 (d, 1H, *J* = 7.5 Hz, COCH=C**H**).

^{13}C NMR ($CDCl_3$, 75 MHz) δ 43.19, 55.41, 62.25, 101.25, 114.52, 121.07, 126.21, 127.80, 128.84, 138.02, 138.10, 140.96, 156.86, 190.32.

HRMS (M+H) Found 280.1324 (Calcd. For $C_{18}H_{18}NO_2$ 280.1338).

4.3.4 Synthesis of long carbon chain tethers 3.107 and 3.108

16-(Acetylthio)hexadecanoic acid (3.103)[1]



16-Mercaptohexadecanoic acid (1.5 g, 5.22 mmol, 1 equiv) was dissolved in DCM (24 mL) and acetic acid (24 mL). Zinc powder (3 g) was added to the solution which was stirred for 15 min. The reaction mixture was cooled in ice and acetyl chloride (7.2 mL, 10.44 mmol, 2 equiv) was added dropwise. The mixture was warmed to rt and stirring was continued for a further 10 min. The grey suspension was filtered to remove the zinc, and the filtrate washed with portions of iced 0.1 M aqueous hydrochloric acid (2 x 50 mL). The organic phase was dried over $MgSO_4$ and the solvent removed by evaporation under reduced pressure to give **3.103** (1.43 g, 83%) as a white solid. The product was not purified further.

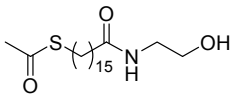
mp 64-66°C.

^{1}H NMR ($CDCl_3$, 500 MHz) δ 1.29 (m(br), 22H, (C**H$_2$**)$_{11}$), 1.55 (m, 2H, C**H$_2$**), 1.62 (m, 2H, C**H$_2$**), 2.31 (s, 3H, OC**H$_3$**), 2.34 (t, 2H, *J* = 7.5 Hz, C**H$_2$**), 2.85 (t, 2H, *J* = 7.5 Hz, C**H$_2$**).

^{13}C NMR ($CDCl_3$, 75 MHz) δ 196.17, 180.06, 34.03, 30.62, 29.59, 29.55, 29.46, 29.40, 29.21, 29.14, 29.09, 29.03, 28.79, 24.65.

HRMS (M+H) Found 331.2304 (Calcd. For $C_{18}H_{35}O_3S$ 331.2307).

_N_-2-(2-Hydroxyethyl)-16-(acetylthio)hexadecanamide (3.104)



A mixture of the acid **3.103** (300 mg, 0.91 mmol, 1 equiv), EDCI (261 mg, 1.37 mmol, 1.5 equiv), HOBt (184 mg, 1.37 mmol, 1.5 equiv), and ethanolamine (82 μL, 1.37 mmol, 1.5 equiv) was stirred in DCM (20 mL) under N_2 atmosphere at rt for 16 h. The clear solution was diluted with ethyl acetate (25 mL), washed with 0.1 M aqueous hydrochloric acid (2 x 10 mL), and water (10 mL). The organic phase was dried over $MgSO_4$ and the solvent removed by evaporation under reduced pressure to give **3.104** (316 mg, 93%) as a white solid. The product was not purified further.

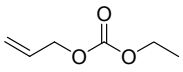
mp 65-67˚C.

^{1}H NMR ($CDCl_3$, 500 MHz) δ 1.28 (m(br), 22H, (C**H$_2$**)$_{11}$), 1.58 (m, 4H, (C**H$_2$**)$_2$), 2.19 (t, 2H, J = 7.7 Hz, C**H$_2$**), 2.31 (s, 3H, OC**H$_3$**), 2.85 (t, 2H, J = 7.3 Hz, C**H$_2$**), 3.42 (m, 2H, NHC**H$_2$**CH_2), 3.72 (t, 2H, J = 5.0 Hz, $NHCH_2$C**H$_2$**), 6.16 (s(br), 1H, N**H**).

^{13}C NMR ($CDCl_3$, 75 MHz) δ 25.71, 28.79, 29.09, 29.15, 29.23, 29.30, 29.44, 29.59, 30.64, 36.63, 42.45, 62.32, 174.94, 196.23.

HRMS (M+H) Found 374.2748 (Calcd. For $C_{20}H_{40}NO_3S$ 374.2729).

Allyl ethyl carbonate (3.106)[17]

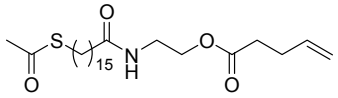


Allyl alcohol (1 mL, 0.02 mmol, 1 equiv) was stirred in freshly distilled THF (15 mL) under N_2 atmosphere. DMAP (3.6 g, 0.03 mmol, 2 equiv) was added and the solution stirred for 10 min. Ethyl chloroformate (1.4 mL, 0.02 mmol, 1 equiv) was added with ice cooling, and the cream suspension stirred at rt for 3 h. The reaction mixture was acidified with 10% aqueous hydrochloric acid until neutral pH (universal indicator paper), and extracted with ether (3 x 50 mL). The organic phases were combined, washed with brine,

and dried over $MgSO_4$. The solvent was removed by evaporation under reduced pressure to give a pale yellow oil which was purified by column chromatography on silica (10% ethyl acetate/petroleum ether to give **3.106** (900 mg, 47%) as a colourless oil.

^{1}H NMR ($CDCl_3$, 500 MHz) δ 1.95 (t, 3H, *J* = 7.1 Hz, $CH_2C\mathbf{H_3}$), 4.19 (q, 2H, *J* = 7.1, Hz, $C\mathbf{H_2}CH_3$), 4.60 (d(br), 2H, *J* = 5.9 Hz, $C\mathbf{H_2}OCO$), 5.3 (m, 2H, $\mathbf{H_2}C$=CH), 5.92 (m, 1H, CH_2=C**H**).

2-(16-(Acetylthio)hexadecanamido)ethylpent-4-enoate (3.107)



The alcohol **3.104** (100 mg, 0.27 mmol, 1 equiv) was stirred with 4-pentenoic acid (27 μL, 0.27 mmol, 1 equiv) and DMAP (16 mg, 0.14 mmol, 0.5 equiv) in ice cooled DCM (5 mL) under N_2 atmosphere. EDCI (52 mg, 0.29 mmol, 1.1 equiv) was added and the solution stirred at rt for 3 h. The reaction mixture was diluted with ethyl acetate (20 mL), washed with saturated aqueous sodium bicarbonate (10 mL) and water (10 mL). The organic phase was dried over $MgSO_4$ and the solvent removed by evaporation under reduced pressure to give **3.107** (60 mg, 49%) as a white solid. The product was not purified further.

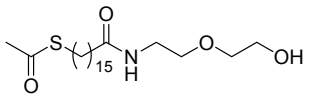
mp 70-71°C.

^{1}H NMR ($CDCl_3$, 500 MHz) δ 1.23 (m(br), 22H, $(C\mathbf{H_2})_{\mathbf{11}}$), 1.51 (m, 4H, $(C\mathbf{H_2})_{\mathbf{2}}$), 2.15 (t, 2H, *J* = 7.7 Hz, $C\mathbf{H_2}$), 2.31 (s, 3H, $OC\mathbf{H_3}$), 2.40 (m, 4H, $COC\mathbf{H_2}C\mathbf{H_2}$), 2.84 (t, 3H, *J* = 7.3 Hz, $C\mathbf{H_2}$), 3.50 (m, 2H, $NHC\mathbf{H_2}CH_2$), 4.16 (t, 2H, *J* = 5.1 Hz, $NHCH_2C\mathbf{H_2}$), 5.03 (m, 2H, $CH{=}C\mathbf{H_2}$), 5.71 (s(br), 1H, N**H**), 5.81 (m, 1H, C**H**=CH_2).

^{13}C NMR ($CDCl_3$, 75 MHz) δ 173.25, 172.96, 136.45, 115.49, 63.11, 38.54, 36.57, 33.23, 30.52, 29.50, 29.49, 29.44, 29.37, 29.35, 29.25, 29.17, 29.02, 28.99, 28.69, 28.67, 25.58.

HRMS (M+H) Found 456.3132 (Calcd. for $C_{25}H_{46}NO_4S$ 456.3148).

_N_-(5-Hydroxy-3-oxapentyl)-16-(acetylthio)hexadecanamide (3.105)



A mixture of the acid **3.103** (100 mg, 0.30 mmol, 1 equiv), EDCI (87 mg, 0.45 mmol, 1 equiv), HOBt (61 mg, 0.45 mmol, 1.5 equiv), and 2-(2-aminoethoxy)ethanol (46 μL, 0.45 mmol, 1.5 equiv) was stirred in DCM (5 mL) under N_2 atmosphere at rt for 16 h. The clear solution was diluted with ethyl acetate (10 mL), washed with 0.1 M aqueous hydrochloric acid (2 x 10 mL), and water (10 mL). The organic phase was dried over $MgSO_4$, and the solvent removed by evaporation under reduced pressure to give **3.105** (316 mg, 93%) as a white solid. The product was not purified further.

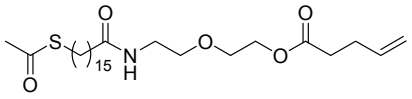
mp 78-81˚C.

^{1}H NMR ($CDCl_3$, 500 MHz) δ 1.29 (m(br), 22H, (C**H₂**)$_{11}$), 1.58 (m, 4H, (C**H₂**)$_2$), 2.17 (t, 2H, *J* = 7.5 Hz, C**H₂**), 2.31 (s, 3H, OC**H₃**), 2.85 (t, 2H, *J* = 7.3 Hz, C**H₂**), 3.47 (m, 2H, NHC**H₂**CH_2), 3.57 (m, 4H,OC**H₂**C**H₂**), 3.75 (t, 2H, *J* = 5.2 Hz, NHCH$_2$C**H₂**), 5.87 (s(br), 1H, N**H**).

^{13}C NMR ($CDCl_3$, 75 MHz) δ 25.68, 28.71, 29.01, 29.06, 29.24, 29.30, 29.37, 29.42, 29.46, 29.53, 30.55, 36.61, 39.09, 61.52, 69.89, 72.20, 173.62, 196.13.

HRMS (M+H) Found 418.2996 (Calcd. For $C_{22}H_{44}NO_4S$ 418.2991).

2-(2-(16-(Acetylthio)hexadecanamido)ethoxy)ethylpent-4-enoate (3.108)



The alcohol **3.105** (120 mg, 0.29 mmol, 1 equiv) was stirred with 4-pentenoic acid (30 μL, 0.29 mmol, 1 equiv) and DMAP (18 mg, 0.15 mmol, 0.5 equiv) in ice cooled DCM (5 mL) under N_2 atmosphere. EDCI (61 mg, 0.32 mmol, 1.1 equiv) was added and the solution stirred at rt for 3 h. The reaction mixture was diluted with ethyl acetate (20 mL) and

washed with saturated aqueous sodium bicarbonate (10 mL), and water (10 mL). The organic phase was dried over $MgSO_4$ and the solvent removed by evaporation under reduced pressure to give **3.108** (89 mg, 62%) as a white solid. The product was not purified further.

mp 57-60˚C.

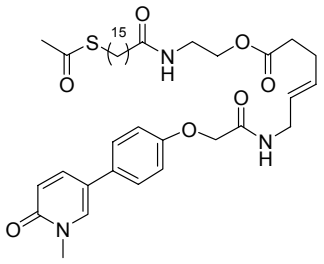
^{1}H NMR ($CDCl_3$, 500 MHz) δ 1.29 (m(br), 22H, (C**H$_2$**)$_{11}$), 1.59 (m, 4H, (C**H$_2$**)$_2$), 2.16 (t, 2H, *J* = 7.7 Hz, C**H$_2$**), 2.31 (s, 3H, OC**H$_3$**), 2.42 (m, 4H, COC**H$_2$**C**H$_2$**), 2.44 (m, 2H, C**H$_2$**), 2.85 (t, 2H, *J* = 7.5 Hz, C**H$_2$**), 3.44 (m, 2H, NHC**H$_2$**CH_2), 3.54 (t, 2H, *J* = 5.2 Hz, $NHCH_2$C**H$_2$**), 3.65 (t, 2H, *J* = 4.8 Hz, OC**H$_2$**), 4.23 (t, 2H, *J* = 4.8 Hz, OC**H$_2$**), 5.03 (m, 2H, CH=C**H$_2$**), 5.81 (m, 1H, C**H**=CH_2), 5.85 (s(br), 1H, N**H**).

^{13}C NMR ($CDCl_3$, 75 MHz) δ 25.66, 28.72, 29.02, 29.06, 29.24, 29.29, 29.41, 29.47, 29.53, 29.54, 30.55, 33.33, 36.68, 38.97, 63.15, 68.90, 69.78, 115.53, 136.41, 172.93. 173.15, 195.99.

HRMS (M+H) Found 500.3414 (Calcd. For $C_{27}H_{50}NO_5S$ 500.3410).

4.3.5 Synthesis of inhibitor-tether conjugates 3.110 and 3.111

2-(16-(Acetylthio)hexadecanamido)ethyl 6-(2-(4-(1-methyl-6-oxo-1,6-dihydropyridin-3-yl)phenoxy)acetamido)hex-4-enoate (3.110)



A solution of **3.57** (50 mg, 0.23mmol, 1 equiv) and **3.107** (73 mg, 0.23 mmol, 1 equiv) in 1,1,2-trichloroethane (4 mL) was stirred under a steady flow of N_2. Grubb's second generation catalyst (26 mg, 0.05 mmol, 0.20 equiv) was added, resulting in a reddish-brown solution which was stirred at rt for 16 h. The solvent was removed by evaporation under reduced pressure to give a brown oil which was purified by column chromatography on silica (5% methanol/DCM) to give **3.110** (35 mg, 30%) as a fine white solid.

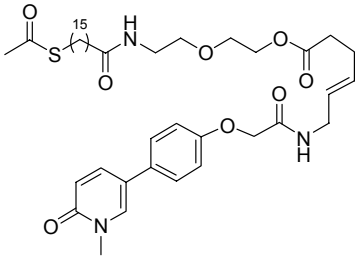
mp 69-71˚C.

^{1}H NMR ($CDCl_3$, 500 MHz) δ 1.29 (m(br), 22H, (C**H**$_2$)$_{11}$), 1.56 (m, 4H, (C**H**$_2$)$_2$), 2.16 (t, 2H, *J* = 7.5 Hz, C**H**$_2$), 2.30 (s, 3H, C**H**$_3$CO), 2.40 (m, 4H, COC**H**$_2$C**H**$_2$), 2.83 (t, 2H, *J* = 7.1 Hz, C**H**$_2$), 3.48 (m, 2H, NHC**H**$_2$CH$_2$), 3.60 (s, 3H, NC**H**$_3$), 3.90 (t, 2H, *J* = 5.9 Hz, NHC**H**$_2$CH=CH), 4.14 (t, 2H, *J* = 5.2 Hz, NHCH$_2$C**H**$_2$), 4.50 (s, 2H, OC**H**$_2$CO), 5.56 (m, 2H, CH$_2$C**H**=C**H**), 5.96 (s(br), 1H, N**H**CH$_2$CH$_2$), 6.64 (d, 1H, *J* = 9.5 Hz, COC**H**=CH), 6.71 (s(br), 1H, N**H**CH$_2$CH=CH), 6.95 (d, 2H, *J* = 8.7 Hz, Ar**H**), 7.34 (d, 2H, *J* = 8.7 Hz, Ar**H**), 7.44 (d, 1H, *J* = 2.4 Hz, NCH$_3$C**H**), 7.57 (dd, 1H, *J* = 2.4, 9.5 Hz, COCH=C**H**).

^{13}C NMR ($CDCl_3$, 75 MHz) δ 25.59, 27.185, 28.66, 28.96, 29.01, 29.17, 29.24, 29.34, 29.37, 29.41, 29.49, 30.51, 33.38, 36.50, 37.87, 38.53, 40.58, 63.16, 67.29, 115.17, 119.51, 120.45, 126.32, 126.49, 127.09, 130.29, 131.02, 135.16, 139.23, 156.50, 162.20, 167.72, 172.81, 173.49, 196.06.

HRMS (M+H) Found 726.4182 (Calcd. For $C_{40}H_{60}N_3O_7S$ 726.4152).

2-(2-(16-(Acetylthio)hexadecanamido)ethoxy)ethyl 6-(2-(4-(1-methyl-6-oxo-1,6-dihydropyridin-3-yl)phenoxy)acetamido)hex-4-enoate (3.111)



A solution of **3.57** (70 mg, 0.23 mmol, 1 equiv) and **3.108** (116 mg, 0.23 mmol, 1 equiv) in 1,1,2-trichloroethane (4 mL) was stirred under a steady flow of N_2. Grubb's second generation catalyst (38 mg, 0.05 mmol, 0.2 equiv) was added, resulting in a reddish-brown solution which was stirred at rt for 16 h. The solvent was removed by evaporation under reduced pressure to give a brown oil which was purified by column chromatography on silica (5% methanol/DCM) to give **3.111** (94 mg, 52%) as a fine white solid.

mp 60-62˚C.

^{1}H NMR ($CDCl_3$, 500 MHz) δ 1.28 (m(br), 20H, (C**H**$_2$)$_{10}$), 1.58 (m, 6H, (C**H**$_2$)$_3$), 2.16 (t, 2H, *J* = 7.5 Hz, C**H**$_2$), 2.31 (s, 3H, C**H**$_3$CO), 2.40 (m, 4H, COC**H**$_2$C**H**$_2$), 2.84 (t, 2H, *J* = 7.5 Hz, C**H**$_2$), 3.43 (m, 2H, NHC**H**$_2$CH$_2$), 3.53 (t, 2H, *J* = 5.2 Hz, NHCH$_2$ **CH**$_2$), 3.61 (s, 3H, NC**H**$_3$), 3.63 (t, 2H, *J* = 4.8 Hz, OC**H**$_2$), 3.92 (t, 2H, *J* = 5.9 Hz, NHC**H**$_2$CH=CH), 4.21 (t, 2H, *J* = 4.8 Hz, OC**H**$_2$), 4.51 (s, 2H, OC**H**$_2$CO), 5.57 (m, 2H, CH$_2$C**H**=C**H**), 5.95 (s(br), 1H, N**H**CH$_2$CH$_2$), 6.64 (d, 1H, *J* = 9.5 Hz, COC**H**=CH), 6.67 (s,(br), 1H, N**H**CH$_2$CH=CH), 6.96 (d, 2H *J* = 8.7 Hz, Ar**H**), 7.35 (d, 2H, *J* = 8.7 Hz, Ar**H**), 7.44 (d, 1H, *J* = 2.8 Hz, NCH$_3$C**H**), 7.57 (dd, 1H, *J* = 2.8, 9.5 Hz, COCH=C**H**).

^{13}C NMR ($CDCl_3$, 75 MHz) δ 25.69, 27.297, 28.74, 29.04, 29.08, 29.27, 29.32, 29.41, 29.44, 29.49, 29.57, 30.58, 33.51, 36.66, 37.96, 39.03, 40.63, 63.24, 67.37, 68.86, 69.79, 108.48, 114.53, 115.23, 119.61, 120.58, 126.60, 127.18, 130.39, 131. 27, 135.21, 139.31, 156.55, 162.28, 167.70, 172.79, 173.36, 196.10.

HRMS (M+H) Found 770.4426 (Calcd. For $C_{42}H_{64}N_3O_8S$ 770.4414).

4.3.6 Steroid 5α-reductase inhibition assay

The proposed steroid 5α-reductase inhibitors synthesised in this thesis were assayed in the laboratory of Professor Rolf Hartmann at Saarland University, Germany. The human embryonic cell line HEK293 used was stably transfected with cDNA for either of the steroid 5α-reductase isozymes (type 1 or 2).[18] HEK-I cells express the type 1 isozyme, and HEK-II express type 2. The separate cell lines allow assay of potential inhibitors separately against both isozymes. A cell free assay was used to avoid cell permeation issues.

Homogenate preparation

HEK-I and HEK-II cells were grown according to Reichert *et al.*[18] The subconfluent cells (approximately 80% of the monolayer surface covered) were washed twice with PBS buffer (pH 7.3), and harvested in cold homogenate buffer (pH 7.3) containing Tris-HCl (50 mM), EDTA (1 mM), and sucrose (300 mM). The cells were homogenised on ice using an ultrasonic rod (5 x 30 seconds at intervals of 60 seconds).

Inhibition assay

Incubation of cell homogenate and test compound was carried out for 30min at 37˚C, with a total volume of 500 μL using Tris-HCl buffer (50 mM, pH 7.3) and ETDA (1 mM). The incubation mixture contained $NADP^+$ (0.55 mM), glucose-6-phosphate (5 mM), glucose-6-phosphate-dehydrogenase (1.2 U/mL), androstenedione (505 nM) including [1β-^{3}H]-androstenedione (3.2 μCi), and 2% MeOH with or without test compound. A standard concentration of 10 μM was used for test compounds.

The reaction was started by adding the enzyme preparation to the incubation mixture, and stopped by extracting the steroids with cold diethyl ether (750 μL). The mixture was shaken for 10 min and centrifuged for 10 min at 4000 g. The water layer was frozen and the ether layer decanted into fresh tubes and evaporated to dryness. The steroids were dissolved in methanol (50 μL) prior to HPLC analysis.

For inhibition of type 1 5α-reductase, 0.3 x 10^6 HEK-I cells, and for type 2, 0.14 x 10^6 HEK-II cells, were used per test vial. As a control for conversion, a triplicate of wells without inhibitors was used.

The steroids were analysed by HPLC (Agilent 1100 Series, Agilent Chemstation for LC 3D). 12.5 μL of the steroids (androstenedione and androstanedione) were injected into the computer controlled HPLC system that was checked before use by running labelled reference controls. Radioactivity was measured with a radioflow monitor (Berthold LB509). A mobile phase of MeOH/H_2O (65:35 v/v) was used with a flow of 0.35 mL min^{-1}, as well as an additive flow of 1 mL (quickszint flow 302) with a RP18 Nucleodur column. Baseline separation of the steroid substrate and product was achieved within 15 min. Results were expressed as the amount of androstanedione formed as a percent of the control values.

In this assay finasteride was used as a positive control, which had an IC_{50} of 453 nM for type 1, and an IC_{50} of 25 nM for type 2.

4.4 REFERENCES FOR CHAPTER FOUR

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APPENDIX

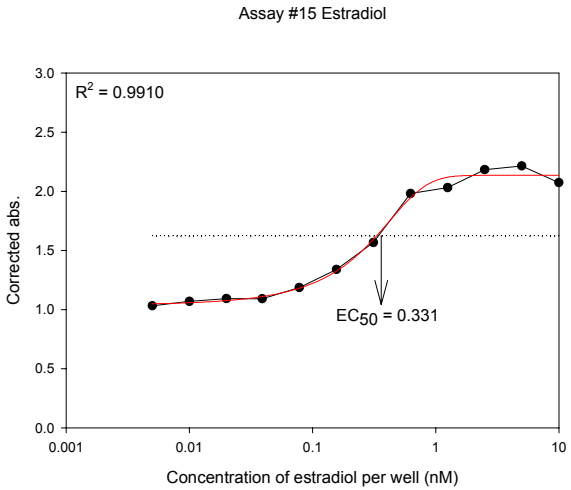
SAMPLE OF YEAST ASSAY ANALYSIS AND EC_{50} CALCULATION

Example for 17β-estradiol (Assay 15)

Well #	Absorbance 540 nm	Absorbance 610 nm	Absorbance blank 610 nm	Corrected Absorbance	Estradiol conc. nM
1	2.401	1.288	0.982	2.073	9.984
2	2.569	1.314	0.981	2.215	4.992
3	2.509	1.286	1.007	2.183	2.496
4	2.326	1.255	0.949	2.031	1.248
5	2.274	1.253	0.94	1.981	0.624
6	1.735	1.128	0.98	1.567	0.312
7	1.396	1.017	0.924	1.339	0.156
8	1.223	0.997	0.95	1.186	0.078
9	1.132	1.001	0.968	1.091	0.039
10	1.075	0.942	0.98	1.093	0.02
11	1.059	0.95	0.948	1.069	0.01
12	0.915	0.843	0.906	1.032	0.005

Avg. = 0.960

Corrected Absorbance = Abs 540 - (Abs 610 - Abs blank 610)



Equation of line determined by SigmaPlot® = f=y0+a/(1+exp(-(x-x0)/b))

Sigmoidal 4 Parameter

	Coefficient	Std. Error	t	P
a	1.785	0.6761	2.6402	0.0297
b	0.2412	0.0729	3.3085	0.0107
x0	0.1112	0.1918	0.5798	0.578
y0	0.3499	0.6679	0.5239	0.6146

x(EC50)	f
0.331	1.623072

y50 1.6235