The characterisation of the catalytic activity of human steroid 5α-reductase towards novel C19 substrates

by
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Thesis presented in fulfilment of the requirements for the degree of Master of Science in the Faculty of Science at Stellenbosch University

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March 2015
DECLARATION

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SUMMARY

This study describes:

- The UPLC-MS/MS analyses and quantification of novel 5α-reduced steroids using response factors.
- The kinetic characterisation of human steroid 5α-reductase type 1 (SRD5A1), expressed in HEK-293 cells, towards 11OHA4 and 11OHT and their keto derivatives by progress curve analysis.
- The subcloning, transformation and functional expression of SRD5A1 in the yeast expression system, \textit{P. pastoris}.
- The conversion of 11OHA4 and 11OHT and their keto derivatives by SRD5A1 expressed in \textit{P. pastoris}.
- The endogenous enzymatic activity in \textit{P. pastoris} towards the 5α-reduced metabolites in the 11OHA4- and alternate 5α-dione pathways.
- The potential application of \textit{P. pastoris} as a biocatalyst in the production of 5α-reduced C19 steroids.
OPSOMMING

Hierdie ondersoek beskryf:

- Die UPLC-MS/MS analise en kwantifisering van nuut-ondekte 5α-gereduseerde steroïede met behulp van responsfakte.
- Die kinetiese karakterisering van menslike steroïed 5α-reduktase tipe 1 (SRD5A1), uitgedruk in HEK-293 selle, vir 11OHA4 en 11OHT en hul ketoderivate deur middel van progressiekurwe-analise.
- Die subklonering, transformasie en funksionele uitdrukking van SRD5A1 in die gis \textit{P. pastoris}.
- Die omsetting van 11OHA4 en 11OHT en hul ketoderivate deur SRD5A1 uitgedruk in \textit{P. pastoris}.
- Die omsetting van 5α-gereduseerde steroïede in die 11OHA4 en alternatiewe 5α-dioon paaie deur endogene ensieme in \textit{P. pastoris}.
- ‘n Onderzoek na die toepassing van die gisuitdrukkingstelsel as ‘n moontlike OR potensiële biokatalis vir die produksie van 5α-gereduseerde C19 steroïede.
Dedicated to my mother, whom I can never repay and my wife, Feruska, who makes me feel like the richest man in the world.
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Almighty God, my father, You are my source of strength
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ABBREVIATIONS

11K-5α-dione 11-keto-5α-androstanedione
11KA4 11-ketoandrostenedione
11KDHT 11-keto-5α-dihydrotestosterone
11KT 11-ketotestosterone
11OH-5α-dione 11-hydroxy-5α-androstanedione
11OHA4 11β-hydroxyandrostenedione
11OHDHT 11-hydroxy-5α-dihydrotestosterone
11OHT 11-hydroxytestosterone
17OH-PREG 17-hydroxypregnenolone
5α-DHP 5α-dihydropregnenolone
5α-dione 5α-androstan-3,20-dione, androstanedione
A4 5α-androstane-3,20-dione, androstenedione
ACTH adrenocorticotropic hormone
ADT Androgen deprivation therapy
AKR1C3 17β hydroxysteroid dehydrogenase type 5
AOX Alcohol dehydrogenase gene
AR Androgen receptor
ARE Androgen response element
BMGY Buffered minimal glycerol
BMMY Buffered minimal methanol
BPH Benign prostatic hyperplasia
COS-1 Green monkey kidney cell line
CHO-K1 Chinese hamster ovary cell line
CRPC Castration resistant prostate cancer
CYP11A1 Cytochrome P450 cholesterol side-chain cleavage
CYP17A1 Cytochrome P450 17α-hydroxylase/17,20-lyase
CYP11B1 Cytochrome P450 11β-hydroxylase
CYP11B2 Cytochrome P450 aldosterone synthase
Cyt b₅ Cytochrome b5
DHEA Dehydroepiandrosterone, 5-androsten-3β-ol-17-one
DHEAS Dehydroepiandrosterone sulphate
DHT  Dihydrotestosterone
DNA  Deoxyribonucleic acid
dNTP  Nucleoside triphosphate
DMEM  Dulbecco’s modified eagles medium
DTT  Dithiothreitol
EDTA  Ethylenediaminetetraacetic acid
ESI+  Electrospray ionisation in positive mode
FDA  Food and drug agency of USA
FSB  Fetal bovine serum
G418  Geneticin
H295R  Human adrenocortical carcinoma cell line
HEK-293  Human embryonic kidney cell line
HSD11B  11β-hydroxysteroid dehydrogenase
HSD17B  17β-hydroxysteroid dehydrogenase
HSD3B  3β-hydroxysteroid dehydrogenase/Δ5-Δ4-isomerase
HSP  Heat shock protein
$K_m$  Michaelis-Menten constant
LH  Luteinizing hormone
LHRH  Luteinizing hormone releasing hormone
MD  Minimal dextrose
MRM  Multiple reaction monitoring
Mut+  Mutation positive
NADPH  Nicotinamide adenine dinucleotide phosphate
OD$_{600}$  Optical density at 600 nm wavelength
PBS  Phosphate buffered saline
PCR  Polymerase chain reaction
PEG  Polyethylene glycol
PSA  Prostate-specific antigen
PREG  Pregnenolone
RPM  Revolutions per minute
RT  Room temperature
SHBG  Sex hormone binding globulin
SRD5A  Steroid 5α-reductase
StAR  Steroid acute regulatory protein
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>SULTA2</td>
<td>Sulfotransferase</td>
</tr>
<tr>
<td>T</td>
<td>Testosterone</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin layer chromatography</td>
</tr>
<tr>
<td>UPLC-MS</td>
<td>Ultra performance liquid chromatography mass spectrophotometry</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra violet</td>
</tr>
<tr>
<td>$V_{\text{max}}$</td>
<td>Maximum velocity of enzymatic reaction</td>
</tr>
<tr>
<td>w/o</td>
<td>without</td>
</tr>
<tr>
<td>YNB</td>
<td>Yeast nitrogen base</td>
</tr>
<tr>
<td>YPD</td>
<td>Yeast peptone dextrose</td>
</tr>
</tbody>
</table>
Chapter 1

STEROID 5α-REDUCTASE AND ITS ROLE IN PROSTATE CANCER

1.1 Prostate

The prostate is a male accessory sex organ comprised of stromal components surrounding the urethra and found just below the bladder as illustrated in figure 1.1 (1). The primary function of the prostate is to contribute, together with the seminal vesicles, to the ejaculate by the synthesis and secretion of proteins and fluid. The prostate requires nutrients, oxygen and androgen stimulation for normal growth, development and function. Androgens are a class of steroid hormones required for the development and maintenance of male sexual characteristics (2). The most abundant androgen in males, testosterone (T), is synthesized primarily in the Leydig cells of the testes (3). The production and secretion of T from the testes is under the endocrine control of the luteinizing-hormone-releasing hormone (LHRH) and luteinizing hormone (LH) axis (4). Mediation of androgenic effects are by way of the androgen receptor (AR) which is a ligand-dependant transcription factor and a member of the nuclear receptor family (5). While T is the most abundant androgen in circulation, it is converted to 5α-dihydrotestosterone (DHT) in peripheral tissue, such as the prostate, by the enzyme steroid 5α-reductase (SRD5A). DHT is the most potent natural androgen (6). The relative potency of T for activation of wildtype AR is 0.1-1 nM while that of DHT is 0.01-0.1 nM (7, 8).

![Figure 1.1 Location of prostate relative to the bladder and urethra (Reproduced from http://www.cancer.gov)](image_url)

In the absence of androgens the AR is bound to heat-shock proteins, HSP70 and HSP90, as illustrated in figure 1.2. The AR proteins are prohibited from binding to DNA in the absence
of ligand rendering the receptor inactive. Ligand binding to AR releases the receptor from heat shock proteins, facilitating AR homodimerization, rapid nuclear translocation, post-translational modification and receptor stabilization (9, 10). Rapid entry into the nucleus is achieved by ligand bound AR homodimers. These homodimers bind to specific DNA-sequences called androgen responsive elements (AREs). Binding of the AR homodimers, together with a number of cofactors, to an ARE initiates the transcription of androgen regulated genes (11).

One such gene is kallikrein-3, which is better known as prostate specific antigen (PSA). PSA is a protease which assists in liquefying semen to enable sperm movement to the ovum (12). During prostate cancer and other prostate diseases PSA leaks into the surrounding stroma and vasculature due to the disruption of the basal membrane and basal epithelial layer. Serum PSA levels are therefore used as a diagnostic marker for prostate diseases (13).

Figure 1.2 Androgen dependent gene regulation. After entering the prostate epithelial cell T is converted to DHT by SRD5A. DHT binds to the AR resulting in a conformational change which facilitates the dissociation of the HSPs. Two ligand-bound ARs form a homodimer which enters the nucleus and binds to an ARE to initiate transcription. (Reproduced from (10))

The AR axis is vital to the maintenance of balance between proliferation and apoptosis, resulting in no net growth of the adult prostate while cells are continuously replaced (14). Without androgens the prostate gland would atrophy (15). The importance of DHT in the development of the prostate is evident in individuals with the inherited 5α-reductase type 2 (SRD5A2) deficiency, characterised by atrophied prostate glands (16). Similarly, the dependence of the prostate on androgens is clearly demonstrated by surgical castration and adrenalectomy removing all sources of androgen and resulting in atrophy of the prostate (17). In addition to being essential to the normal functioning of the prostate, androgens also play a
significant role in the pathogenesis of benign prostatic hyperplasia (BPH) and prostate cancer. The role of androgens in prostate cancer has been a subject of interest since the mid-20th century (14).

1.2 Prostate Cancer

Globally, prostate cancer is the second most diagnosed cancer in men and is the sixth leading cause of cancer-related deaths, with a fatality rate of 63% (18, 19). Prostate cancer has been shown to be more prevalent in developed countries, though this could be due to under-reporting in developing countries. In the United States of America, it is the second leading cause of death due to cancer amongst men (20, 21). African-American men have the highest incidence of prostate cancer in the world with a 50-fold higher incidence of prostate cancer than Japanese and Chinese men (22, 23). According to the national cancer registry, prostate cancer is the most common cancer affecting men in South Africa, with at least 4000 cases diagnosed each year (24).

Charles Brenton Huggins was awarded the Nobel Prize in Physiology in 1966 for discovering that the prostate is dependent on androgens. He demonstrated that castration led to a decrease in the prostate's size and weight (25). Based on this discovery the first line in treating advanced localised prostate cancer is the inhibition of testicular T production, which is known as androgen deprivation therapy (ADT) (25, 26). This is accomplished by either surgical (orchiectomy) or chemical castration. Chemical castration is accomplished using LHRH agonist or antagonists, which both lead to the inhibition of T biosynthesis by the testes (27). Another therapeutic strategy is the use of AR antagonists, which would normally be administered along with ADT for a synergistic effect (28).

Apoptotic regression of both benign and malignant prostate epithelial cells is triggered by ADT (29). A decrease in PSA levels as well as objective and symptomatic responses are observed in over 80% of patients treated with ADT (29). This treatment initially yields good results, but in many cases AR signalling pathways are reactivated after a 2-3 year remission period characterised by an increase in PSA expression (26). The recurring cancer is then termed castration resistant prostate cancer (CRPC) (30).
1.3 Castration Resistant Prostate Cancer

1.3.1 Castration resistant prostate cancer development

Despite the initial remission which is observed following ADT, the progression to CRPC is extremely common (31). This deadly form of prostate cancer is proposed to be the result of the selective pressure imposed by ADT favouring the growth of androgen-insensitive cells (32–34). However, the reactivation of the androgen axis is characterised by the up-regulation of PSA expression (35). Genome- wide expression profiling, has revealed that most androgen gene networks are reactivated during CRPC progression (36–38). Furthermore, recent clinical studies with the CYP17A1 inhibitor, abiraterone (39, 40) and the AR antagonist enzalutimide (41) confirm that CRPC remains androgen dependent.

Mechanisms implicated in the reactivation of the AR axis following ADT (circulating T < 20 ng/dL) include adaptations such as AR amplification, expression of AR splice variants, AR mutations and the activation of AR complex via cross-talk with other signalling pathways (42). While these mechanisms increase the sensitivity of the AR axis, they still required the presence of AR ligand (31). Tumours displaying resistance to castration are characterised by elevated intratumoural androgen levels which are high enough to elicit a response from the AR (8, 42). While surgically castrated males demonstrate a 90-95% drop in circulating T levels, intraprostatic DHT levels have been shown to decrease by only 50% (2, 36, 37). An explanation for this observation is that T is not the only precursor to DHT. The adrenal gland produces androgen precursors, dehydroepiandrosterone (DHEA), androstenedione (A4) and 11β-hydroxyandrostenedione (11OHA4) which are all released into circulation (43–45). Recent studies have shown that these weak adrenal androgen precursors can be metabolised in CRPC tissue to yield active androgens which reactivate the AR axis (46–51). Enzymes such as AKR1C3, SRD5A1 and HSD3B2 are required for the metabolism of adrenal androgen precursors and have been shown to be upregulated during CRPC (31). A number of androgen biosynthetic pathways have been suggested to play a role in CRPC. These include the classical pathway, the alternative 5α-androstenedione (5α-dione) pathway and the newly discovered 11OHA4 pathway (26, 46, 47).

1.3.2 Androgen biosynthesis by the testes and adrenal cortex

Five enzymes stand between the conversion of cholesterol to T and DHT, these include: cytochrome P450 side-chain cleavage (CYP11A1), cytochrome P450 17α-hydroxylase/17,20-
lyase (CYP17A1), 3β-hydroxysteroid dehydrogenase/Δ5,4 isomerase (HSD3B), 17β-hydroxysteroid dehydrogenase (HSD17B) and steroid 5α-reductase (SRD5A).

The first step in the production of all steroid hormones involves the side-chain cleavage of cholesterol catalysed by CYP11A1 which is found in the inner mitochondrial membrane (52) (Fig. 1.3). Cholesterol is transported from the outer mitochondrial membrane to the inner membrane by the action of the protein StAR (53). Following the conversion of cholesterol to pregnenolone (PREG) in the testes, the product is further metabolized by the 17α-hydroxylase activity of CYP17A1 yielding 17α-hydroxypregnenolone (17OH-PREG). 17OH-PREG is subsequently catalysed by the 17,20-lyase activity of CYP17A1 to produce the C19 steroid, DHEA. DHEA is converted to A4 by HSD3B2 and A4 is subsequently converted to T by HSD17B3 (53).

The Leydig cells, found in the testes, are responsible for 90-95% of the production of circulating T in males. (15). LHRH from the hypothalamus stimulates the release of LH from anterior pituitary gland which in turn stimulates T production by the Leydig cells. A negative feedback system regulates the production of T as it inhibits the release of LHRH to maintain normal circulating levels. Biosynthesis of T (Fig 1.3) takes place continually as the Leydig cells cannot store androgens. T is secreted into circulation with only 1-2% being in free form while the remainder is either bound to albumin (±54%) or sex hormone binding globulin (±44%) (15).
T is also produced in the adrenal, accounting for 5-10% of the steroid in circulation. The adrenal’s contribution under normal physiological conditions is, however, insignificant compared to the levels produced by the Leydig cells. The adrenal cortex is divided into three distinct zones, the zona glomerulosa, the zona fasiculata and the zona reticularis. These three zones produce mineralocorticoids, glucocorticoids and androgen precursors, respectively. The type of steroid produced by each zone is determined by the specific enzymes expressed in the zones. Steroidogenesis in the zona reticularis is very similar to that in Leydig cells. In humans a distinct zona reticularis forms during adrenarche which occurs between age 6-8 and is marked by the increase in synthesis and secretion of adrenal androgen precursors such as DHEA and DHEA-S, as a result of the maturation of the zona reticularis. During adrenarche
the expression profile of key steroidogenic enzymes are altered in the developing zona reticularis in order to ensure adrenal androgen production. The expression of cytochrome b5, a small hemoprotein which enhances the 17,20-lyase activity of CYP17A1 by an allosteric mechanism, is increased thus increasing DHEA production (54, 55). In contrast, HSD3B2 expression decreases during adrenarche, preventing the production of mineralocorticoids and glucocorticoids (55).

In the zona reticularis, as in the Leydig cells steroidogenesis involves the conversion of cholesterol to PREG by CYP11A1, followed by the 17α-hydroxylase and subsequent 17,20-lyase activities of CYP17A1 yielding DHEA (fig. 1.4). In the zona reticularis a large proportion of the resulting DHEA is sulfated by SULT2A1 to produce DHEAS which is released into circulation (55). DHEA itself is also released into circulation. DHEA is, however, also converted to A4 by HSD3B2 and is released into circulation. A4 can also serve as the substrate for the adrenal enzyme CYP11B1, which is responsible for the hydroxylation of the substrate at C11 yielding 11OHA4 (44) as well as AKR1C3 which reduces A4 to T.

Xing and colleagues have shown, while determining the effects of ACTH on steroid profiles in human adrenal cells, that the androgen precursors A4 and 11OHA4 are two of the most abundant steroids produced under basal conditions. The production of these steroids is further increased after incubation with ACTH (56). Using UPLC-MS/MS analysis our research group was able to quantify steroid intermediates and end products of adrenal steroidogenesis. 11OHA4, was shown to be one of the major metabolites produced in H295R (human adrenal carcinoma) cells. Upon forskolin stimulation, the basal levels of 11OHA4 was shown to increase 4.5-fold to 390 nM (44). Forskolin stimuliates steroidogenesis in H295R cells which are insensitive to ACTH. More recently, Rege et al. showed that 11OHA4 is one of the most abundant C19 steroids detected in the adrenal vein (43–45). Levels of 11OHA4 were found to be 2-fold higher than those of A4, both pre- and post-ACTH stimulation. 11OHA4 levels increased to 811 nM after ACTH stimulation (table 1.1). The levels of 11OHA4 were ~100-fold higher (under both basal conditions and ACTH stimulation) than T and its derivatives. Low levels of 11KA4, 11OHT and 11KT were also detected under both basal and stimulated conditions (table 1.1) (45). Investigations into the catalytic activity of CYP11B1 and CYP11B2, showed that both enzymes are able to catalyse the hydroxylation of T and A4. Both HSD11B1 and 2 interconvert 11OHT and 11OHA to 11KT and 11KA4 (57), however, further investigation is needed to establish whether 11KA4 and 11KT is catalysed from 11OHA4 and 11OHT by HSD11B2 in the adrenal. AKR1C3 and low levels of HSD11B1,
HSD11B2 and HSD17B3 (mRNA transcripts) have been shown to be expressed in the adrenal gland, are responsible for the low levels of 11OHT 11KT and 11KA4 detected in adrenal vein samples (45).

Table 1.1 Primary androgens produced and secreted by the adrenal after ACTH stimulation. Steroids are displayed in order of the most to the least abundant as detected in human adrenal vein samples. Modified from (45).

<table>
<thead>
<tr>
<th>Steroid metabolite</th>
<th>Post-ACTH (nmol/L)</th>
<th>Fold Change from basal</th>
</tr>
</thead>
<tbody>
<tr>
<td>DHEAS</td>
<td>18 266 ± 3842</td>
<td>4.8</td>
</tr>
<tr>
<td>DHEA</td>
<td>2659 ± 666</td>
<td>21.2</td>
</tr>
<tr>
<td>11OHA4</td>
<td>811 ± 260</td>
<td>5.2</td>
</tr>
<tr>
<td>A4</td>
<td>585 ± 199</td>
<td>7.4</td>
</tr>
<tr>
<td>Androstenediol</td>
<td>20.7 ± 6.46</td>
<td>18.0</td>
</tr>
<tr>
<td>T</td>
<td>5.71 ± 1.42</td>
<td>7.3</td>
</tr>
<tr>
<td>11KA4</td>
<td>3.18 ± 0.63</td>
<td>3.2</td>
</tr>
<tr>
<td>11OHT</td>
<td>2.62 ± 0.74</td>
<td>5.5</td>
</tr>
<tr>
<td>11KT</td>
<td>0.49 ± 0.11</td>
<td>1.3</td>
</tr>
</tbody>
</table>
**1.4 Steroid metabolism in the prostate**

*1.4.1 The classical and alternate 5α-dione pathways*

The circulating levels of T are almost completely diminished during ADT, declining to levels lower than 20 ng/dl (27). Under castrate conditions the prostate tumour is able to adapt and use adrenal androgen precursors to produce active androgens which can reactivate the AR axis. Initially, intratumoural biosynthesis of DHT using adrenal androgen precursors was thought to follow the conventional pathway whereby A4 (and DHEA which is converted to A4 by HSD3B2) is first converted to T by a 17-keto reduction catalysed by HSD17B5 (AKR1C3), followed by the 5α-reduction of T by SRD5A1 to yield DHT. Recent studies
have, however, shown that the prevailing pathway for DHT biosynthesis in CRPC is the alternate 5α-dione pathway as illustrated in figure 1.5. In this pathway T production is bypassed completely and A4 is instead reduced by SRD5A, to produce 5α-dione which is in turn converted to DHT by AKR1C3 (46, 58, 59).

![Diagram of androgen metabolism](image)

**Figure 1.5** 5α-dione pathway illustrated by the green arrows circumvents T via 5α-reduction of A4 to form 5α-dione, and subsequent HSD17B action to give rise to DHT in CRPC. Reproduced from (58).

Chang *et al* showed that A4 is the preferred substrate over T for SRD5A1, which is upregulated during CRPC (16, 59–62). Chang and colleagues (46) investigated androgen metabolism in six CRPC cell-lines and consistently found that the 5α-dione pathway was the dominant route to DHT production. This finding suggested that the pathway is common to the CRPC state. As *ex vivo* studies only provide a view of androgen concentrations limited to a moment in time (31, 63), Chang *et al* undertook further analyses of tumour biopsies from two patients and observed the same trends in these cells. The importance of the pathway in a clinical setting became evident, and the 5α-dione pathway was thus proposed by Chang *et al* to explain the increase observed in the T to DHT ratio seen in in CRPC tissue. Observations in untreated prostate cancer and BPH cells showed the T:DHT ratio to be approximately 1:10, which indicates a rapid flux from T to DHT. A general interpretation of these results was that
all DHT formed was from the conversion of T. However, clinical studies showed this ratio was reversed in CRPC. Chang et al., however, proposed that the reason for the increased ratio is that the flux to DHT formation does not readily occur through T but rather via the alternate pathway (46). This notion was supported by the increase in the T:DHT ratio observed in the prostates of normal men, when the flux from T to DHT is pharmacologically blocked (31, 63). A slight increase in T production would thus increase the T:DHT ratio as the conversion from T to DHT is not favoured. Furthermore, as AR agonists, it is important to note that androgen concentrations detected within the tumours in clinical studies disproportionately reflect androgens which are found in the interstitial space and cellular cytoplasm of the tumours (46). A more accurate representation of active androgens could be obtained by determining their internuclear concentrations as this would represent androgens bound to the AR (64). Ascertaining concentrations of DHT in the nucleus of clinical CRPC tissue have not yet been completed, but it is believed that DHT concentrations herein will exceed the sum of DHT in all other compartments of the tumour (31, 63).

1.5 11OHA4 pathway

In addition to A4, DHEA and DHEA-S the human adrenal gland also produces the C19 steroid, 11OHA4 as discussed above. After being overlooked since its discovery over half a century ago, 11OHA4 has made a comeback as an adrenal precursor to active androgens which likely play a central role CRPC (65). The initial loss of interest in this metabolite was as a result of its weak androgenic activity and the production of the steroid was thought to be a mechanism by which adrenal androgens are inactivated (66). Recent studies have, however, revealed the potential physiological importance of 11OHA4.

11OHA4 was shown to be the substrate for a novel pathway involving the enzymes HSD11B and SRD5A. The conversion of 11OHA4 to 11KA4 as well as the conversion of 11OHT to 11KT, was shown to be catalysed via the action of HSD11B2 (11-hydroxy to 11-keto form) in LNcaP cells as shown in figure 1.6. HSD11B2, unlike HSD11B1 has been shown to be expressed in the prostate (67), further experiments need to be done to rule out HSD11B1 fully. Both SRD5A1 and SRD5A2 were shown to be able to convert 11OHA4, 11KA4, 11OHT and 11KT to the novel C19 steroids, 11OH-5α-dione, 11K-5α-dione, 11OHDHT and 11KDHT, respectively. Although commercial standards are not available for these 5α-reduced steroids, their production was confirmed by accurate mass determinations (47). HSD11B2 was shown to convert 11OH-5α-dione and 11OHDHT to 11K-5α-dione and 11KDHT, respectively. HSD17B3 and HSD17B5 were shown to catalyse the conversion of 11KA4 and
11K-5α-dione to 11KT and 11KDHT, respectively, while 11OHA4 and 11OH-5α-dione were not substrates for these enzymes. In addition, the reverse reactions were all catalysed by HSD17B2 (57, 65, 68). Taking the enzymes and reactions described above into account, the metabolism of 11OHA4 would result in the formation of 11KT and 11KDHT as shown in figure 1.6. Three potential metabolic routes from 11OHA4 to 11KDHT are, however, possible. The existence of the proposed 11OHA4 pathway was confirmed in the androgen dependent prostate cancer cell line, LNCaP (57). Furthermore, all the enzymes involved in the metabolism of 11OHA4 are expressed during CRPC (31, 67).

Although 11OHA4 itself is not androgenic, the 11OHA4 pathway yields active androgens. 11KT and 11OHDHT displayed partial AR agonist activity at the physiologically relevant concentration of 1 nM (11KT was comparable to T), while 11KDHT displayed full agonist activity, comparable to DHT (47, 57).

Figure 1.6 C19 steroids on the 11OHA4 pathway. The enzymes responsible for the metabolism of 11OHA4 and downstream metabolites are HSD11B, HSD17B and SRD5A. The 5α-reduction of 11OHA4, 11OHT, 11KA4, and 11KT results in the production of novel C19 products 11OH-5α-dione, 11OHDHT, 11K-5α-dione and 11KDHT, respectively. The pathway was confirmed in the androgen dependent prostate cancer line, LNCaP, which does not possess 11βHSD1 (47).
11OHA4 therefore clearly functions as a precursor to active androgens in the prostate, which contains the necessary enzymatic machinery. Furthermore, the identification of the 11OHA4 pathway has revealed novel substrates for the enzymes involved in androgen metabolism. The metabolism of 11OHA4 and other C19 derivatives towards novel C19 steroids offers a robust mechanism which may not only have role in normal tissue which express the relevant enzymes but also in prostate diseases such as CRPC. Although numerous pathways have been identified for the metabolism of adrenal androgens within CRPC, it is likely that all the pathways make a contribution to the production of active androgens. The extent of the contribution will, however, depend on the availability of the adrenal androgen precursors as well as the preference of the specific enzymes for the different substrates. SRD5A1 expression is upregulated during CRPC (69) and plays a vital role in the conventional, 5α-dione and the 11OHA4 pathways. The aim of this study was therefore to characterise SRD5A1 towards all the potential substrates from the various pathways as to understand its substrate preference.

1.6 Steroid 5α-reductase

1.6.1 Background and history

Three SRD5A isozymes have been identified to date and have been named based on the chronological order in which the cDNA was isolated (1). The reduction of T to DHT was first discovered in 1951, and was shown to be catalysed by the enzyme SRD5A. At the time researchers were unaware that multiple isozymes of the enzyme existed. Originally SRD5A was believed to play a role in the catabolism of steroids, by terminating endocrine hormone action. In 1968, however, DHT was shown to be a more potent androgen than T (3), demonstrating that SRD5A played an anabolic role (64). In 1974 the significance of SRD5A in human physiology was confirmed by the discovery of an inborn error of male sexual differentiation, due to a SRD5A deficiency. Males deficient in SRD5A exhibit developmental defects in the formation of external genitalia and prostate as a result of a defect in the biosynthesis of DHT in the embryo (16). The external genitalia resemble those of a female, while the internal genitalia (excluding the prostate) were normal. The observation that these men demonstrated incomplete prostate development along with decreased baldness and acne, served as the rational for the development of inhibitors for the enzyme, in treating these conditions in normal men. SRD5A was shown to have an acidic pH optimum in human prostate (70), epididymis or genital skin fibroblasts (71). SRD5A activity with an acidic pH optimum was absent in genital skin fibroblasts grown from subjects with SRD5A deficiency,
however, SRD5A activity with an alkaline pH optimum was identified, suggesting that multiple molecular forms of SRD5A may exist (72–74). Further investigations into the existence of multiple isoforms were hampered, as the enzyme proved to be highly insoluble (16), due to the SRD5A isoforms being integral membrane proteins located in the nucleus and endoplasmic reticulum (75, 76). Intrinsic membrane proteins are deeply embedded in the lipid bilayer, making these hydrophobic proteins very difficult to purify. After numerous attempts, solubility of the enzyme was achieved using detergents, but resulted in the loss of enzymatic activity upon purification (77–79). In 1989, the cDNA of full length rat SRD5A was cloned and expressed in *Xenopus* oocytes offering an alternative route to study enzyme function (80). This breakthrough allowed the cross-hybridisation screening of human prostate cDNA library for human SRD5A using expressed full length rat SRD5A as a hybridisation probe (81). cDNA encoding human SRD5A was successfully obtained using this method, and rat and human cDNA was subsequently expressed in simian COS cells. The cloned human enzyme (SRD5A1) was not sensitive to inhibition by the potent SRD5A inhibitor finasteride and demonstrated an alkaline pH optimum indicating that the cloned enzyme was not the same SRD5A first identified in tissue, again suggesting the presence of multiple isoforms. Furthermore, no mutations were found in the SRD5A cDNA from patients with SRD5A deficiency (73, 81). The cloned SRD5A was named SRD5A1. Male pseudohermaphroditism due to 5α-reductase deficiency was later shown to be due to a mutation in SRD5A2 (72, 82). Human cDNA SRD5A2 was later isolated by using an expression cloning method that isolated cDNA pools which displayed SRD5A activity different from the already isolated SRD5A1. Pools of active cDNA expressed in HEK-293 cells were gradually divided into smaller groups until SRD5A2 cDNA was isolated and confirmed by the expressed enzymes’ acidic pH optimum and sensitivity to finasteride (83). Normington and Russel subsequently isolated and characterised the cDNA encoding for rat SRD5A2 after they generated a probe for SRD5A2 cDNA using PCR (72). RNA isolated from the ventral prostates of castrated rats, treated with T for 3 days, was reverse transcribed to generate cDNA which was used as a template for subsequent PCR amplification. The amplified sequence produced an oligonucleotide probe that was used to identify a positive clone from a rat testes cDNA library (72).

More recently a third SRD5A isozyme, SRD5A3, was discovered (84). SRD5A3 was originally identified in hormone refractory prostate cancer tissue via a genome-wide gene expression profile analysis of these cells (84). This isozyme has been shown to be overexpressed in CRPC, but the significance of this finding is yet to be fully elucidated (6).
Uemura and others have suggested that the third isoform of SRD5A might be responsible for the conversion of T to DHT in the presence of dutastide and finastride (84–86). Thus far, SRD5A3 has been shown to be mainly involved in polyrenol biosynthesis and it has been suggested that SRD5A3 does not play a significant role to play in intratumoral androgen biosynthesis (87). Cantegrel et al. showed, in both biochemical and clinical investigations, the proposed 5α-reductase domain of SRD5A3 reduces substrates which are not related to steroids but rather to nonsteroid lipids (87). In contrast, Titus et al. more recently showed that SRD5A3 is capable of 5α-reduction of 3-oxo-Δ4,5 containing steroid substrates (85).

1.6.2 Tissue distribution and regulation of SRD5A isozymes

SRD5A1 is expressed in very low levels in embryonic tissues, while SRD5A2 is the predominant isozyme found in these tissues as is evident by the drastic phenotypic change observed in males with SRD5A2 deficiency. In a mature adult, SRD5A1 is highly expressed in nongenital skin, liver and certain brain regions, but it is expressed at significantly lower levels in the androgen-dependent tissue (prostate, epididymis, seminal vesicle and testis) as well as genital skin, adrenal glands and the kidney. In contrast, SRD5A2 has high expression in androgen-dependent regions, genital skin, adrenal glands, kidney, and is also, to a lesser degree, expressed in the ovaries and hair follicles (88, 89). Regulation of SRD5A expression is achieved in prostate and liver tissue by hormonal control with androgen induced expression of SRD5A seemingly mediated by IGF-1 which is expressed at high levels in the prostate (82).

1.6.3 Characterisation of SRD5A

SRD5A1 exhibits a broad pH optimum, which ranges between 6.0 and 8.5, while SRD5A2 exhibits a narrow acidic pH optimum (pH 5–5.5) in the lysates of transfected cells (3, 16). SRD5A2 has been observed to have a higher \( \frac{V_{\text{max}}}{K_{m}} \) ratio than SRD5A1 when compared under optimal conditions suggesting a higher 5α-reducing activity (3). Both isozymes contain an NH\(_2\)-terminal ligand binding domain and a COOH-terminal NADPH binding domain. Both SRD5A1 and SRD5A2 have similar apparent dissociation constants for NADPH (3–10 µM) (3, 61). The apparent dissociation constant of NADPH for SRD5A3 is undetermined. Azzouni and colleagues have shown that SRD5A2 has a pH optimum between pH 6.0 and 7.0 inside intact human cells (3). Lysates of CHO cells expressing SRD5A1 from rat and human, produces an enzyme with micromolar affinity (apparent \( K_{m} = 1-5 \) µM) for substrates such as T, A4, and progesterone (81). However, under optimal conditions (pH 5.0) the apparent \( K_{m} \) of SRD5A2 for T is in the submicromolar range (0.1-1.0 µM) with a \( V_{\text{max}} \) of 2.0-5.0 nmol of
DHT per mg protein (90). Interestingly, under sub-optimal conditions (pH 7.0) the apparent $K_m$ values obtained for SRD5A2 in cell lysates, permeabilized cells, and intact cells are in the low nanomolar range (4-50 nM), while the $V_{\text{max}}$ is reduced to 0.2 nmol DHT per mg protein (61). Based on $V_{\text{max}}/K_m$ versus pH plots, the most efficient range for SRD5A2 activity was determined to be between pH 6.0 and 7.0 (61).

Table 1.2 Comparison of properties of 5α-reductase isoenzymes relevant in CRPC (91)

<table>
<thead>
<tr>
<th>Properties</th>
<th>SRD5A1</th>
<th>SRD5A2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biochemical state</td>
<td>Hydrophobic</td>
<td>Hydrophobic</td>
</tr>
<tr>
<td>Chromosome location</td>
<td>5p15</td>
<td>2p23</td>
</tr>
<tr>
<td>Molecular weight (kDa)</td>
<td>29.5</td>
<td>28.4</td>
</tr>
<tr>
<td>Optimal pH</td>
<td>6-8.5</td>
<td>5-5.5</td>
</tr>
<tr>
<td>Size</td>
<td>259 amino acids</td>
<td>254 amino acids</td>
</tr>
<tr>
<td>Tissue distribution</td>
<td>Liver, nongenital skin,</td>
<td>prostate, epididymis,</td>
</tr>
<tr>
<td></td>
<td>prostate, brain, ovary,</td>
<td>seminal vesicle,</td>
</tr>
<tr>
<td></td>
<td>testis</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>genital skin, uterus,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>breast, hair follicle,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>placenta, testis</td>
</tr>
</tbody>
</table>

Characterisation of the native enzyme has proved to be cumbersome since, as mentioned above, the SRD5A isozymes are membrane-bound proteins, embedded in the membrane bilayer of the ER. In addition the overall topology of these hydrophobic proteins have not been determined, with an earlier study, using differential permeabilization of transfected CHO cell membranes, showing that the carboxyl termini of the 5α-reductase isozymes lie on the cytoplasmic side of the endoplasmic reticulum bilayer. (80). In a previous study in the mid-1980s, solubilisation of active human prostatic SRD5A was successfully achieved from microsomal pellets, in order to facilitate further purification and characterisation (77). Even after several attempts by many laboratories SRD5A is yet to be purified in an active form (16, 74, 78, 79, 91). Detergents are required to solubilize the proteins, which leads to the loss of function of the unstable enzyme upon chromatographic purification steps (77). In addition, SDS-PAGE analysis of purified proteins have shown that the isozymes exhibit an
uncharacteristic electrophoretic mobilities (16, 61, 92), as the proteins migrate at apparent molecular masses of 21,000-27,000 instead of the predicted 28,000-29,000.

The substrates for SRD5A1 and SRD5A2 are 3-oxo Δ4-5 C19/C21 steroids. The 3-oxo refers to the oxygen moiety (keto group) at C3 and Δ4-5 refers to the double bond between C4 and C5. SRD5As catalyse an irreversible, stereo-specific double bond breakage, while inserting a hydride anion to the α face at C5 and a proton to the β face at C4 (figure 1.7) (3, 93).

**Figure 1.7** Proposed mechanism for reduction of T by SRD5A. Reproduced from (94).

In the catalytic reaction, NADPH is the first to bind the enzyme and NADP+ is the last product to leave the enzyme, thus the reaction has an ordered bi-bi kinetic mechanism as illustrated in figure 1.8. NADPH provides the hydride ion for C5 and a proton (from water) attaches to C4. There is no direct transfer of hydrogen from NADPH to the Δ4-5 double bond in the 3-oxosteroid. Unlike most reactions involving NADPH which are freely reversible, the 5α-reductase catalysed reaction is irreversible (93) with the resultant 5α-reduction of the steroid causing the molecule to have a planar structure and to be less polar due to the rearrangement of the A and B rings. The 3-oxo groups of 5α-reduced products are more easily reduced by 3α/3β hydroxysteroid dehydrogenase, sulfation and glucuronidation. The modification of 5α-reduced products facilitates excretion, by rendering these steroids more hydrophilic (16).

**Figure 1.8** Ordered bi-bi reaction scheme for SRD5A. Modified from (94).

### 1.6.4 Pharmacology of SRD5A
The 5α-reduction of C19 steroids such as T and 11KT leads to the production of active androgens capable of activating the AR. The physiological importance of SRD5A2 is clearly demonstrated by the inactivating SRD5A2 mutation which resulted in male pseudohermaphroditism and prevented the development of the prostate gland as discussed above. This observation, together with high levels of SRD5A2 expression detected in normal prostatic tissue as well as the observation that SRD5A2 has a higher affinity towards T than SRD5A1, led to the initial view that SRD5A2 was the only SRD5A isoenzyme with clinical relevance in the prostate (6). Finasteride was therefore developed for the prevention and treatment of prostate cancer. Finasteride is a potent 4-azasteroid inhibitor of human SRD5A2 which is much less effective at inhibiting human SRD5A1 (61). Finasteride treatment, however, failed to reduce intraprostatic DHT to the desired levels. Dutasteride was later developed due to the inability of finastride to effectively inhibit DHT production. Dutasteride inhibits both SRD5A1 and SRD5A2, and is more potent than finasteride (95). A greater reduction in serum and intraprostatic DHT levels was observed when using dutastride, confirming the suggestion that SRD5A1 may contribute more significantly to prostatic DHT formation than initially thought. More recent studies have confirmed that SRD5A1 is associated with the development and progression of prostate cancer. SRD5A1 mRNA expression levels are significantly higher in prostate cancer than the levels detected in BPH and normal prostate tissue (96). A growing body of evidence suggests that when a decrease in SRD5A2 activity, with a simultaneous rise in SRD5A1 mRNA expression is observed, it leads to the development of primary prostate cancer. This change in the ratio of SRD5A1 and SRD5A2 expression levels is also observed in CRPC (69, 97, 98). Clinical trials with dutastide and finastride resulted in a short remission period followed by the development of a more aggressive form of cancer in a subset of patients. For this reason dutastide and finastride are not approved by the Food and Drug Administration (FDA) for the prevention of prostate cancer (99). This decision was based on the inhibition of SRD5A resulting in the accumulation of T or other DHT precursors which are able to activate the wild type and mutated AR.

1.6.5 Characterisation of SRD5A1 towards novel substrates

The peripheral metabolism of A4, DHEA and 11OHA4 have been shown to yield androgenic products, while these steroids themselves are not significantly androgenic (17–19). The role played by SRD5A1 in the metabolism of these adrenal androgen precursors is clear. However, the enzymatic activity of SRD5A1 towards the novel substrates identified in the newly
discovered 11OHA4 pathway is yet to be determined. It is therefore the aim of this study to characterise SRD5A1 activity towards 11OHA4, 11KA4, 11OHT and 11KT and to compare these activities to the known substrates, A4 and T. These results will firstly allow us to better understand the flux through the 11OHA4 pathway and secondly, will provide insight into which of the adrenal androgen precursors are the preferred substrates for intratumoral androgen synthesis. Two approaches for the characterisation of SRD5A1 were followed during this study: (1) SRD5A1 was expressed in HEK293 cells, a human embryonic kidney cell line, as to assess the enzymatic activity of SRD5A1 in a nonsteroidogenic mammalian cell model; (2) SRD5A1 was expressed in a yeast expression system in order to produce high levels of enzyme which could be used for the biocatalytic production of the novel 5α-reduced products, 11OH-5α-dione, 11K-5α-dione and 11OHDHT (which are currently not available commercially) as well as 11KDHT.

1.7 Expression of SRD5A1 in HEK-293 cells

Characterisation of enzymes expressed in different systems each come with their own set of pros and cons. Recombinant strategies to overcome difficulties in purifying these integral membrane proteins from natural sources, have been implemented to express SRD5A in a number of expression systems (80, 81). These systems include, amongst others, *Xenopus* oocytes (80), *Saccharomyces cerevisiae* (100), insect cells (*Spodoptera frugiperda*) (101) and mammalian cell lines such as Green Monkey kidney (COS-1) cells (81), Chinese hamster ovary (CHO) (61) and HEK-293 cells (72, 83, 86, 90, 102, 103). As most of what is known about SRD5A surfaced as a result of heterologous expression of the enzyme, the characterisation of SRD5A1 towards novel C19 substrates by these methods would be a natural choice. In mammalian cell lines proper folding, post-translational modifications and product assembly is achieved easily and can thus facilitate the characterisation of an enzyme. Numerous research groups have utilised HEK-293 to express SRD5A successfully and to complete kinetic characterisation of the enzyme in the presence of novel drugs and substrates (57, 86, 102–104). The enzymatic activity of SRD5A1 towards 11OHA4, 11KA4, 11OHT and 11KT has, however, not been characterised. It is well documented that SRD5A1 has a higher affinity for A4 over T (105) and preliminary data has suggested that the same is true for the 11β-hydroxyl and 11-keto derivatives of A4 and T (47). The characterisation of SRD5A1 towards 11OHA4 11KA4, 11OHT and 11KT will elucidate the preferred flux through the 11OHA4 pathway and will allow for a better understanding of intratumoral androgen metabolism.
1.8 Expression of SRD5A1 in P. pastoris

The functional expression of rat and human SRD5A has been achieved in a number of eukaryotic systems as listed above, however, these systems are generally regarded as complex and costly systems (61, 80, 81). Yeast expression systems, on the other hand, are cost effective with the added benefit of high levels of expression (106). Yeast can be transformed with plasmids containing the gene of interest and subsequent recombination leads to the incorporation of multiple copies of the gene of interest into the yeast genome facilitating overexpression of the encoded protein (107). Yeast expression systems are regarded as invaluable biotechnology for studying the structure and function of proteins, in particular, membrane bound proteins as well as proteins that are expressed in low levels in tissue (100, 108). Numerous studies have shown the ability of yeast to achieve post-translational modifications required for the activity of proteins involved in steroid metabolism (75, 100, 109). Ordman et al. previously demonstrated the expression of catalytically active rat SRD5A in S. cerevisiae (75). T was efficiently converted to DHT confirming functional expression and uptake of hydrophobic steroid substrates. SRD5A activity was maintained even when intact yeast, spheroplasts, or lysed yeast were snap frozen and stored at -20 or -70°C (9). Interestingly, a second reaction product was observed during prolonged incubation periods with T. TLC analysis revealed that the product co-migrated with 5α-androstan-3α-17β-diol (3α-adiol) and 5α-andorstan-3β-17β-diol (3β-adiol), suggesting that yeast may express a 3α- or 3β-hydroxysteroid dehydrogenase activity which can metabolise DHT (75). The study showed that yeast not expressing SRD5A (negative control) were unable to metabolise T, while being able to metabolise DHT due to the endogenous 3α- or 3β-hydroxysteroid dehydrogenase activity (75, 100). Our laboratory has investigated the use of S. cerevisiae for the expression of CYP17A1, however, desired yields were not achieved. The yeast Pichia pastoris (also known as Komagatella pastoris (110)), however, was shown to yield desired protein expression levels (109). Hult et al. also previously demonstrated the successful expression of the steroid metabolising enzyme HSD11B1 in P. pastoris. During this study, the authors determined the substrate specificities and inhibitor constants for synthetic and naturally occurring compounds and demonstrated that recombinant human HSD11B1 expressed in P. pastoris behaves as it does in mammalian systems (111).

The P. pastoris yeast system has become one of the most popular heterologous protein expression systems since 1993 when it was released by the Phillips Petroleum Company for academic research laboratories to utilise freely (106, 112). P. pastoris is a methalotrophic...
yeast – as utilises methanol as its sole carbon source for energy. An added benefit to using *P. pastoris* as a protein expression system is the yeast’s ability to withstand relatively high levels of methanol in which most other micro-organisms could not survive (106). *P. pastoris* has the ability to produce high cell densities in relation to fermentative yeasts due to its strong preference for respiratory growth (113). Cell growth is often accompanied by the production of high levels of functional proteins, which include membrane-bound proteins. Heterologous genes can be integrated into the yeast genome downstream of an alcohol oxidase 1 promoter (AOX1p), resulting in the tightly regulated, methanol-induced expression of recombinant proteins (114).

The molecular genetic manipulations required for the integration of a heterologous gene into the genome of *P. pastoris* are similar to those required for one of the most well characterised experimental system in modern biology, *S. cerevisiae*. An integrative vector, pPIC3.5K, is used to facilitate the stable integration of exogenous DNA into the host genome. Single or multiple copy numbers can be stably integrated into the yeast genome even in the absence of selective pressure (106). The pPIC3.5K vector (figure 4.1) is designed to enable multiple integrations of the gene of interest into the genome of *P. pastoris*. Multicopy candidate strains are screened by first selecting for a HIS+ phenotype and subsequently replating positive transformants on YPD-agar plates containing increasing concentrations of G418 (Geneticin) (115).

![Figure 1.9 Map of yeast expression plasmid pPIC3.5K.](https://scholar.sun.ac.za)

**Figure 1.9** Map of yeast expression plasmid pPIC3.5K. *P. pastoris* AOX1 gene promoter, 5’AOX1; AOX1 transcription terminator, 3’ AOX1 (TT); Histidinol dehydrogenase gene, HIS4; 3’ AOX1downstream sequence, 3’ AOX1; Ampicillin resistance gene, Ampicillin; Kanamycin resistance gene, Kanamycin; pBR322, *E. coli* origin of replication (116).
*P. pastoris* was the system of choice for investigating the heterologous expression of SRD5A1 due to the success achieved in our laboratory with the stable transformation and subsequent expression of the membrane bound steroidogenic enzyme CYP17A1 in this yeast (109). The aim of this trial was to determine if functional expression of human SRD5A1 could be achieved in *P. pastoris* with the end goal of using the system to produce 11OH-5α-dione, 11K-5α-dione, 11OHDHT and 11KDHT.

1.9 Objectives

Prostate cancer is a prevalent disease which often develops into CRPC which is fatal. Recent studies, outlined above, have shown that CRPC is dependent on a number of steroid pathways which convert adrenal androgen precursors into potent androgens. The characterisation of these pathways and enzymes therein is therefore vital to understand the progression of this disease and for the identification of the most suitable drug targets.

The objective of the present investigation was therefore to characterise the activity of SRD5A1 towards the novel C19 substrates in the recently identified 11OHA4 pathway. Specific aims were:

- To characterise SRD5A1 expressed in HEK293 cells towards A4, T, 11OHA4, 11KA4, 11OHT and 11KT.
- To determine if functional expression of SRD5A1 is possible in *P. pastoris*.
- To determine if the yeast model could be used as a biocatalyst for the production of C19 androgens which are not available commercially.
Chapter 2

MATERIALS AND METHODS

2.1 Plasmids

The pCMV7 vector containing human SRD5A1 was obtained from Prof. DW Russel (Southwestern Medical School, University of Texas, Dallas, USA). The pCIneo and pPIC3.5K plasmids were available for use in the laboratory.

2.2 Reagents

HEK-293 cells were purchased from the American Type Culture Collection (Manassas, VA, USA). Penicillin-streptomycin, fetal calf serum and trypsin-EDTA were obtained from Gibco-BRL (Gaithersburg, MD, USA). 11β-hydroxyandrostenedione, 11-ketoandrostenedione, 11β-hydroxytestosterone and 11-ketotestosterone were purchased from Steraloids (Wilton, USA). Testosterone, androstenedione, 5α-androstenedione, dihydrotestosterone, as well as Geneticin disulphate (G418), Lyticase and Dulbecco’s modified Eagle’s medium was purchased from Sigma-Aldrich (St. Louis, MO, USA). Deuterated cortisol (9, 11, 12, 12- D4-cortisol) and deuterated testosterone (D2-T) were purchased from Cambridge isotopes (Andover, MA, USA). Nuclease free water was purchased from Ambion, Applied Biosystems (Austin, Texas, USA). Restriction endonucleases (EcoRI, PstI, SalI and NcoI), Pfu polymerase as well as the 1kb DNA marker were purchased from Fermentas (Burlington, Canada). X-tremeGENE HP DNA transfection reagent and Rapid DNA dephos and ligation kit was from Roche Diagnostics (Mannheim, Germany). Wizard® SV Gel Clean-Up and Zyppy® Plasmid miniprep Kit were purchased from Promega Biotech (Madison, WI, USA) and Inqaba Biotec (Pretoria, RSA), respectively. P. pastoris strain GS115 (his4) was purchased from Invitrogen (Carlsbad, CA, USA). Nucleobond® Midiprep DNA isolation kits were purchased from Macherey-Nagel (Duren, Germany). All yeast growth media components were purchased from Difco Laboratories (Detroit, MI, USA) and Zymolase™ was purchased from Zymo Research Corporation (Irvine, CA, USA). All other chemicals were of the highest analytical grade and purchased from scientific supply houses. All protocols were carried out according to the manufacturer’s instructions unless otherwise stated.
2.3 pCMV7-SRD5A1, pPIC3.5k and pCIneo plasmid preparation

Luria-Bertani (LB) medium, 100 ml, containing ampicillin, 100 µg/ml, was inoculated with *E.coli* strain JM109 containing either pCMV7-SRD5A1, pCIneo or pPIC3.5k and incubated overnight at 37°C while shaking at 200 rpm (Innova shaking incubator, New Brunswick). Plasmid DNA was isolated using a Nucleobond® Midiprep DNA isolation kit. The resulting plasmid DNA pellets were resuspended in 600 µl nuclease free water. Plasmid DNA concentration and yield was determined spectrophotometrically using a Cary 60 UV-VIS (Agilent technologies). Plasmids were subsequently analysed by restriction endonuclease digestions. The total reaction volume was 20 µl and each reaction contained: the appropriate enzyme BSA-buffer (10X), 2 µl; *Eco*RI (10 U/µl), 5U, 0.5 µl; and 1 µg plasmid DNA. Reactions were incubated at 37°C for 1 hour. Digested and undigested plasmids were subsequently analysed by 1% agarose gel electrophoresis using a 1kb DNA marker. The DNA samples (0.5 µg) were diluted in 0.2% loading buffer (0.1% Orange G (w/v), 20% Ficoll (w/v), 10 mM EDTA). Electrophoresis was carried out at room temperature in TAE buffer (40 mM Tris-acetate, 2 mM EDTA and 20 mM acetic acid) at 110V for three minutes and at 75V until the dye front was 1 cm from the bottom of the gel. The agarose gel was stained using GR green® prior to visualisation on a UV transluminator.

2.4 SRD5A1 expression in HEK-293 cells

2.4.1 Cell culture procedure

HEK-293 cells were routinely grown to confluence in 75 cm³ culture dishes at 37°C, 90% humidity and 5% CO₂ in DMEM, supplemented with 0.12% NaHCO₃, 10% fetal calf serum and 1% penicillin–streptomycin (stock containing 10 000 U/ml penicillin and 10 000 µg/ml streptomycin). Cell count and viability were determined using trypan blue and Countess® Automated cell counter (Invitrogen).

2.4.2 Enzymatic assays in transiently transfected HEK-293 cells

HEK-293 cells were grown to confluence and cells were plated into 24-well culture dishes with each well containing 2×10⁵ cells (500 µl/well) 24 hours prior to transfection. Cells were transiently transfected using X-tremeGENE HP DNA transfection reagent using a 3:1 ratio of transfection reagent (µl) to plasmid DNA (µg) in serum-free medium (DMEM without FSB supplementation). After 15 minutes at room temperature, 50 µl of the transfection cocktail was added to each well in a drop-wise manner and the cells were incubated for a further 72...
hours prior to substrate addition. The media was replaced with media supplemented with either 11OHA4, 11KA4, 11OHT, 11KT, A4 or T at a final concentration of 0.5, 5 or 30µM. T (5 µM) was included in all experiments as a positive control for enzyme expression. Aliquots, 500 µl, were collected at specific time intervals: all steroids were assayed at 0.5 µM, aliquoted at 0, 0.25, 0.5, 0.75, 1, 1.5, 2, 4 and 6 hours; 11OHA4 and 11KA4 were assayed at 5 µM, aliquoted at 0, 0.5, 1, 1.5, 2, 4, 6, 8 and 12 hours; 11OHT and 11KT were assayed at 5 µM, aliquoted at 0, 2, 4, 6, 10, 12, 14, 18 and 24 hours; A4 and T assayed at 5 µM aliquoted at 0, 0.5, 1, 1.5, 2, 3, 5, 7 and 10 hours; all steroids were assayed at 30 µM, aliquoted at 0, 1, 2, 3 and 4 hours. Samples were stored at 4°C prior to extraction and UPLC-MS/MS analysis.

2.5 Steroid extraction

Steroids were extracted from aliquotes by liquid-liquid extraction using a 10:1 volume of dichloromethane to sample collected. D4-cortisol (15 ng) and D2-T (15 ng) were added to the samples prior to extraction, after which the samples were vortexed for 15 minutes and centrifuged at 3500 rpm for 5 minutes at room temperature. The polar phase was aspirated and the non-polar dichloromethane phase transferred to a clean test tube and evaporated under a stream of nitrogen gas (N2). The dried steroids were reconstituted in methanol, 150 µl, prior to analysis by UPLC-MS/MS. Sample from the experiments containing 30 µM steroid were diluted 10x in methanol prior to analysis by UPLC-MS/MS.

2.6 UPLC-MS/MS analysis of steroids

Steroid metabolites were separated by UPLC (ACQUITY UPLC, Waters, Milford, USA) using a Phenomenex Kinetex PFP (2.1 mm × 100 mm, 2.6 µm) column as previously described (47, 57). The mobile phases consisted of solvent A (1% formic acid) and solvent B (49:49:2, methanol:acetonitrile:isopropanol) (44). The gradients used for separation are shown in table 2.1. The total run time was 5 minutes and the injection volume was 5 µl. A Xevo triple quadrupole mass spectrometer (Waters, Milford, USA) was used for quantitative mass spectrometric detection. All steroids were analysed in multiple reaction monitoring (MRM) mode (table 2.2) using an electrospray probe in the positive ionization mode (ESI+). The following settings were used: capillary voltage of 3.5 kV, source temperature 120°C, desolvation temperature 400°C, desolvation gas 900 l h⁻¹ and cone gas 50 l h⁻¹. Data was collected with the MassLynx 4.1 software. Standard curves were generated for each steroid metabolite using the following concentrations: 0.05, 0.5, 2.5 and 5.0ng/ml. The calibration
curves were linear over these concentration ranges, with regression correlation coefficients \( (r^2) \) always being greater than 0.99.

**Table 2.1** UPLC gradient specifications of the chromatographic separation of the C19 steroids (57).

<table>
<thead>
<tr>
<th>Step</th>
<th>Time (minutes)</th>
<th>% Solvent A</th>
<th>% Solvent B</th>
<th>Curve</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.00</td>
<td>85.0</td>
<td>15.0</td>
<td>6</td>
</tr>
<tr>
<td>2</td>
<td>1.00</td>
<td>60.0</td>
<td>40.0</td>
<td>6</td>
</tr>
<tr>
<td>3</td>
<td>3.50</td>
<td>45.0</td>
<td>55.0</td>
<td>6</td>
</tr>
<tr>
<td>4</td>
<td>3.60</td>
<td>0.0</td>
<td>100.0</td>
<td>6</td>
</tr>
<tr>
<td>5</td>
<td>4.00</td>
<td>0.0</td>
<td>100.0</td>
<td>6</td>
</tr>
<tr>
<td>6</td>
<td>4.01</td>
<td>85.0</td>
<td>15.0</td>
<td>6</td>
</tr>
<tr>
<td>7</td>
<td>5.00</td>
<td>85.0</td>
<td>15.0</td>
<td>6</td>
</tr>
</tbody>
</table>

**Table 2.2** Parameters for the detection and quantification of C19 steroids by UPLC–MS/MS: retention times (RT); cone voltages (CV); collision energy (CE); limit of detection (LOD) (defined as a S/N >3); limit of quantification (LOQ) (defined as a S/N >10); and regression correlation coefficient \( (r^2) \) (57).

<table>
<thead>
<tr>
<th>Steroid metabolite</th>
<th>RT (minutes)</th>
<th>Parent ion</th>
<th>CV</th>
<th>Daughter ion A</th>
<th>CE</th>
<th>Daughter ion B</th>
<th>CE</th>
<th>LOD (ng/ml)</th>
<th>LOQ (ng/ml)</th>
<th>( r^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>11KT</td>
<td>2.24</td>
<td>303.2</td>
<td>30</td>
<td>121.0</td>
<td>20</td>
<td>267.0</td>
<td>20</td>
<td>0.7</td>
<td>2</td>
<td>0.99</td>
</tr>
<tr>
<td>11OHT</td>
<td>2.25</td>
<td>305.3</td>
<td>35</td>
<td>121.0</td>
<td>20</td>
<td>269.0</td>
<td>15</td>
<td>0.07</td>
<td>0.2</td>
<td>0.99</td>
</tr>
<tr>
<td>11OHA4</td>
<td>2.36</td>
<td>303.2</td>
<td>30</td>
<td>121.0</td>
<td>30</td>
<td>267.0</td>
<td>15</td>
<td>0.07</td>
<td>0.2</td>
<td>0.99</td>
</tr>
<tr>
<td>11KA4</td>
<td>2.46</td>
<td>301.2</td>
<td>35</td>
<td>241.0</td>
<td>30</td>
<td>257.0</td>
<td>25</td>
<td>0.2</td>
<td>0.4</td>
<td>0.99</td>
</tr>
<tr>
<td>T</td>
<td>3.07</td>
<td>289.2</td>
<td>30</td>
<td>97.2</td>
<td>22</td>
<td>109.0</td>
<td>22</td>
<td>0.07</td>
<td>0.2</td>
<td>0.99</td>
</tr>
<tr>
<td>A4</td>
<td>3.23</td>
<td>287.2</td>
<td>30</td>
<td>96.9</td>
<td>15</td>
<td>108.8</td>
<td>15</td>
<td>0.07</td>
<td>0.2</td>
<td>0.99</td>
</tr>
</tbody>
</table>

**2.7 Determination of response factors**

Standards for UPLC-MS/MS analysis of the novel steroids, 11OH-5α-dione, 11K-5α-dione, 11OHDHT and 11KDHT were not commercially available and these metabolites were
therefore quantified by generating standard curves integrating data of their respective substrates. The peak areas of the 5α-reduced products were adjusted using response factors. Response factors were determined as follows: HEK-293 cells were plated as described above and co-transfected with SRD5A1 (0.5 µg) and SRD5A2 (0.5 µg) using the X-tremeGENE HP DNA transfection reagent. Control transfections were carried out using the mammalian expression vector pCIneo (1.0 µg) (no cDNA inserted). After a 72 hour incubation period, 1 µM of steroid substrate (T, A4, 11OHA4, 11OHT, 11KA4 and 11KT) was added to cells expressing SRD5A1-SRD5A2 and pCIneo transfected HEK-293 cells after which the cells were incubated for 48 hours. Samples were collected and the steroids extracted and analysed as described above. D4-cortisol (15 ng) and D2-T (15 ng) were added to the samples prior to extraction. The peak areas of the substrates and their respective 5α-reduced products were determined after ascertaining that SRD5A samples contained only 5α-reduced steroids (no substrate remaining) and pCIneo samples contained only substrate precursor. The assumption was therefore made that the substrate was fully converted to the respective 5α-reduced product (1 µM substrate = 1 µM product). Response factors were determined using the formula below:

\[
\frac{A_S}{A_{IS}} = RF\left(\frac{A_P}{A_{IS}}\right)
\]

Where: \(A_S = \) peak area of the substrate; \(A_{IS} = \) peak area of the internal standard; \(A_P = \) peak area of the 5α-reduced product; [S] = substrate concentration; [P] = product concentration.

The peak areas of each sample were divided by the area of the internal standard for each of 6 replicates. The average ratio of substrate steroid/internal standard was divided by the average ratio of 5α-reduced product/internal standard in order to obtain the response factor.

2.8 Determination of kinetic parameters

Kinetic parameters (\(V_{max}\) and \(K_m\)) were determined using progress curve analysis. The following objective function was minimised in Mathamatica (Wolfram) for the respective substrates (S), in order to estimate the \(V_{max}\) and \(K_m\) values of SRD5A1:

\[
\sum_{i=1}^{3} \left( \frac{E_S(t_i) - M_S(t_i)}{E_S(t_i)} \right)^2
\]
E represents the experimental values and M the model predicted values for S. The Michaelis-Menten equation was used as a model for the predicted change in S. The equation was numerically integrated to calculate S during the conversion. Data obtained from different experiments with different transfection efficiencies was normalised by using a control conversion of 5 μM T which was included in each experiment. All kinetic parameter determinations (from the experimental data) were carried out by Prof JL Snoep (Department of Biochemistry, University of Stellenbosch).

2.9 Subcloning of SRD5A1 into the pPIC3.5K expression vector

2.9.1 Amplification of SRD5A1 cDNA

The pCMV7-SRD5A1 construct, was used as the template for the amplification of the SRD5A1 cDNA sequence. Primers were designed to include a Kozak consensus sequence in the upstream primer (for correct initiation of translation of gene of interest) as well as to include EcoRI recognition sites on the 3’ and 5’ termini of the cDNA sequence as shown in table 2.1.

Table 2.3 SRD5A1 and AOX1 primer sequences used in sub-cloning, direct DNA sequencing and screening.

The first primer pair (SRD5A1) is complementary to the 5’ and 3’ ends of the SRD5A1 coding sequence. EcoRI recognition sites are shown in **bold** and the Kozak consensus sequence is **underlined**. The second primer pair (AOX1) is complementary to the 5’ and 3’ ends of AOX1 gene, used to identify the incorporation of the gene of interest into genome of *P. pastoris*.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Oligonucleotide sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>SRD5A1 (Sense)</td>
<td>5’-GTATCGAATTCCGCCACCATGGCAACGGCGACGGGGGTGGCG-3’</td>
</tr>
<tr>
<td>SRD5A1 (Antisense)</td>
<td>5’-CGTAGCAGAATTTCTTTAAAAACAAAAATGGAATTATAATTT-3’</td>
</tr>
<tr>
<td>AOX1 (Sense)</td>
<td>5’-GACTGGTTCAAATGACAAGC-3’</td>
</tr>
<tr>
<td>AOX1 (Antisense)</td>
<td>5’-GCAAATGGGCATTCTGACATCC-3’</td>
</tr>
</tbody>
</table>

PCR was carried out using a PCR-Sprint thermo cycler (Hybraid). Each PCR amplification mixture (50 µl) contained: the appropriate reaction buffer containing MgSO$_4$ (10X), 5 µl, 20 mM MgSO$_4$; 200 μM of each dNTP; 0.6 μM of each primer; 2.5 U Pfu-polymerase; and pCMV7-SRD5A1 plasmid DNA (750 ng). The amplification profile was as follows: (1) denaturing of the template at 95°C for 2 minutes; (2) 35 cycles of denaturing at 95°C for 30
seconds, annealing at 47.5°C for 30 seconds and extension at 72°C for 2 minutes; (3) and a final extension at 72°C for 10 minutes. The resulting amplicons were analysed on a 1% agarose gel. Four PCR amplifications were performed for the amplification of SRD5A1 cDNA and then pooled prior to cloning.

2.9.2 Cloning of SRD5A1 into the pPIC3.5K vector

SRD5A1 cDNA was purified using a Wizard® SV Gel Clean-Up kit following amplification by PCR. The concentration of the purified cDNA was determined spectrophotometrically at 260 nm. The cDNA, 1 μg, was subsequently digested with EcoRI, 10U, at 37°C for 90 minutes.

The pPIC3.5K vector was prepared for cloning as follows: The vector, 1 μg, was linearized by an EcoRI, 10 U, digest for 90 minutes at 37°C. The linearized vector, 0.75 μg, was dephosphorylated using 1 U of the rAPid Alkaline Phosphatase in a final volume of 20 μl by incubating at 37°C for 10 minutes. The enzyme was subsequently inactivated at 75°C for 2 minutes. The prepared vector and cDNA were ligated using the Rapid DNA Dephosphorylation and Ligation Kit with a 1:5 molar ratio of vector: insert DNA. Ligation reactions, 10μl, contained: reaction buffer (20X), 0.5 μl; 50 ng DNA insert; 100 ng of vector; and 1.5 U of T4 ligase. The following control reactions were included: dephosphylated plasmid DNA (100 ng) without insert (negative control); and linearized plasmid DNA (100 ng) which had not been dephosphorylated (dephosphorylation control). Competent DH5α cells were subsequently transformed with the ligated vectors.

2.9.3 Transformation of E. coli strain DH5α with pPIC3.5K-SRD5A1

Competent cells were prepared using the CAPS method (Modified Inoue method) (117), stored at -80°C and thawed when needed. E. coli strain DH5α cells were streaked on a LB-agar plate and incubated overnight at 37°C. A single colony was picked and grown overnight in 5 ml of LB medium at 37°C shaking at 200 rpm. Fresh LB medium, 200 ml, was inoculated with overnight culture, 1.5 ml, and grown for a further 3 hours to OD₆₀₀ of 0.5. The cells were subsequently centrifuged at 3000 rpm for 15 minutes at 4°C. DH5α cells were resuspended in 16 ml ice-cold CAPS buffer (60 mM CaCl₂, 15% glycerol and 10 mM PIPES). Hereafter, cells were again centrifuged in pre-cooled centrifuge tubes at 3000 rpm for 15 minutes at 4°C. The supernatant was discarded and the pellet resuspended in 4 ml ice-cold CAPS buffer. Aliquots, 200 μl, were prepared in microcentrifuge tubes and stored at -80°C until use.
Competent DH5α cells were thawed on ice for 25 minutes prior to transformation. Each ligation reaction, 20 µl, as added to 200 µl of competent cells, gently mixed and incubated on ice for 30 minutes. Transformation reactions included; dephosphorylated vector with insert; dephosphorylated vector with no insert (negative control); linearized plasmid DNA which had not been dephosphorylate (dephosphorylation control); and 5 ng of PUC19 (positive control). The cells were subsequently subjected to heatshock at 42°C for 45 seconds followed by incubation at room temperature for 2 minutes. Super optimal broth with catabolite repression medium (SOC) (2% w/v tryptone, 0.5% w/v Yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂ and 20 mM Glucose; pH7.0), 800 µl, was subsequently added and the cell suspension incubated for 1 hour at 37°C shaking at 200 rpm. Dilutions of the transformation reactions were plated on LB-agar plates supplemented with ampicillin (100 µg/ml) and incubated overnight at 37°C.

Single colonies were selected from the plates and used to inoculate 5 mL LB supplemented with ampicillin (100 µg/ml). Cultures were grown overnight at 37°C, shaking at 200 rpm. Plasmid DNA was isolated from the overnight cultures using the Zyppy® Plasmid Miniprep Kit. Potential SRD5A1 clones were digested with NcoI (10 U) in order to identify plasmids containing SRD5A1 cDNA as well as to determine the orientation of the insert. Digests were analysed on a 1% agarose gel. Plasmids containing the correct orientation of cDNA insert were subsequently subjected to direct sequence analysis at the Central Analytical Facility (CAF) of Stellenbosch University using the SRD5A1 forward and reverse primers listed in Table 2.1.

2.10 Transformation in GS115 strain of P. pastoris

2.10.1 Maintenance of P. pastoris

Routine growth of P. pastoris strain GS115 was carried out in YPD medium (1% Yeast extract, 2% peptone and 2% D-glucose) shaking at 275 rpm at 30°C. All cultures were started from a single colony which was incubated on YPD-agar (YPD medium with 15 g agar per litre) for 2 days prior to inoculation. Selection of yeast transformants was carried out on minimal dextrose plates (MD) containing 13.4 g yeast nitrogen base without amino acids, 400 µg biotin, 5 ml methanol and 15 g agar per litre and subsequently on YPD-agar containing increasing concentrations (0.25, 0.5, 0.75, 1.0 mg/ml) of Geneticin® (G418). Heterologous expression P. pastoris was achieved by culturing in buffered minimal glycerol (BMGY) medium (0.1 M potassium phosphate pH 6.0, 1.34% w/v YNB w/o amino acids,
0.00004% biotin, 1% v/v glycerol) for at least 24 hours and induced in buffered minimal methanol (BMMY) medium containing 0.5% v/v methanol replacing 1% v/v glycerol in BMGY medium.

2.10.2 Transformation

The cell wall of yeast hinders the uptake of DNA and it is thus necessary to either partially remove the cell wall or to create pores in order to allow transformation to occur. Transformation is less efficient in *P. pastoris* than in *S. cereversiae* (116, 118). Spheroplasting and electroporation have been documented to yield the highest transformation efficiency (±10⁴ transformants per µg DNA) (116, 118). A method combining PEG and LiCl has been shown to yield lower transformation efficiencies (± 10³ transformants per µg DNA), but is less laborious, uses intact cells and requires no specialized equipment (118). Spheroplasting, electroporation and the PEG/LiCl methods were all employed for the transformation of *P. pastoris* in this study.

2.10.3 Vector preparation

The pPIC3.5K-SRD5A1 expression vector and the parent vector pPIC3.5K, were both linearized with *SalI* prior to transformation, as digestion with *SalI* produces Mut+ (Methanol utilisation plus) transformants (116). Controls (no DNA and parent vector without insert) were included in all transformations reactions and were subsequently used as background controls for expression.

2.10.4 Spheroplasting

*P. pastoris* strain GS115 cells were picked from single colonies, grown for 2 days at 30°C on YPD-agar plates, and subsequently used to inoculate 10 ml of YPD medium. Starter cultures were grown at 30°C while shaking vigorously at 275 rpm overnight. The starter culture, 10 µl, was used to inoculate 200 ml YPD and the culture incubated overnight at 30°C while shaking at 275 rpm. Once an OD₆₀₀ of 0.3 was reached the cells were centrifuged at 1500 x g for 10 minutes at room temperature. The supernatant was discarded, the yeast cells were washed with 20 ml sterile water and centrifuged at 1500 x g for 5 minutes at room temperature. The pellet was washed in 20 ml SED (1 M sorbitol, 1 mM EDTA, pH8.0 and 50 mM DTT) and centrifuged at 1500 x g for 5 minutes at room temperature. The pellet was subsequently washed with 1 M sorbitol and centrifuged at 1500 x g for 5 minutes at room temperature. The pellet was resuspended in 20 ml of SCE (1M sorbitol, 1 mM EDTA and 10 mM sodium citrate buffer, pH5.8) by swirling. Zymolase™, 37.5 U, was added to 10 ml cell suspension in
a clean centrifuge tube and incubated for 30 minutes at 30°C in order to achieve 70% spheroplasting. The spheroplasts were harvested by centrifugation at 750 x g for 10 minutes at room temperature. Spheroplasts were subsequently washed with 10 ml 1M sorbitol, collected by centrifugation at 750 x g for 10 minutes at room temperature and washed once with 10mL CaS (1 M sorbitol, 10 mM Tris-HCl, pH 7.5 and 10 mM CaCl₂). The spheroplasts were harvested by centrifugation and gently resuspended in 600 µl CaS.

Spheroplasts, 100 µl, were prepared in 15 ml snap cap falcon tubes and linearized plasmid (pPIC3.5K-SRD5A1, pPIC3.5K), 10 µg, was added to the spheroplasts and to 100 µl sterile water (negative controls). Reaction mixtures were incubated at room temperature for 10 minutes. Freshly prepared PEG/CaT, 1 ml, (1:1, 40% PEG and CaT (20 mM Tris-HCl, pH 7.5; 20 mM CaCl₂)), was added to the cells (100 µl) and incubated for a further 10 minutes. Cells were centrifuged at 750 x g for 10 minutes at room temperature and the PEG/CaT removed by aspiration. SOS medium (1 M sorbitol, 0.3X YPD, 10 mM CaCl₂), 150 µl, was subsequently added and the mixture was incubated at room temperature for 20 minutes. 1 M sorbitol, 850 µl, was added to each of the transformations prior to plating.

All transformations were plated using top cell wall regeneration media (RDB) (1 M sorbitol, 2% dextrose, 1.34% YNB without amino acids, 0.00004% biotin and 0.005% amino acids (L-glutamic acid, L-methionine, L-lysine, L-leucine and L-isoleucine)) by adding 200 µl of each transformation cocktail to 10 ml molten RD agar before pouring onto a RDB agar plate. The viability and selection of positive transformants was accomplished due to the absence of histidine on these plates (116). Plates were inverted and incubated for 4 days at 30°C. Untransformed spheroplasts were plated on RDBH (regeneration plates containing 0.004% histidine) plates in order to confirm the viability of the spheroplasts.

2.10.5 Electroporation

YPD culture, 10 ml, was inoculated with a single fresh colony of *P. pastoris* GS115 grown on an YPD-agar plate, and subsequently incubated overnight at 30°C shaking at 275 rpm. Fresh YPD medium, 500 ml, was inoculated with the starter culture and incubated in a 2 l baffled flask at 30°C shaking at 275 rpm until an OD₆₀₀ of 1.5 was reached. Yeast cells were harvested by centrifugation at 1500 x g for 5 minutes at 4°C and resuspended in 500 ml of ice-cold sterile water. Cells were collected by centrifugation 1500 x g for 5 minutes at 4°C and resuspended in 250 ml of ice-cold sterile water. Cells were collected by centrifugation at 1500 x g for 5 minutes at 4°C and resuspended in 20 ml ice-cold 1 M sorbitol. Cells were again
collected by centrifugation at 1500 x g for 5 minutes at 4°C and resuspended in 1 ml ice-cold 1 M sorbitol (final volume ± 1.5 ml).

Electro-competent cells, 80 µl, were mixed with 10 µl linearized DNA (10 µg), transferred to an ice cold electroporation cuvette (0.2 cm) and incubated on ice for 5 minutes. Electroporation was subsequently carried out using savant GTF100 gene transformer which was set to 1800 V and initiated by holding the pulse button for 10 seconds. Following electroporation 1 mL ice-cold 1 M sorbitol was immediately added to the cuvette and the contents to a sterile microcentrifuge tube and incubated at 30°C without shaking for 90 minutes. Transformed cells, 100 µl, were subsequently spread on MD (His−) plates for the selection of positive transformants. Plates were inverted and incubated for 5 days at 30°C until colonies formed.

2.10.6 PEG/LiCl procedure

YPD media, 10 ml, was inoculated with a single P. pastoris strain GS115 colony grown on a YPD-agar plate (previously incubated for 2 days at 30°C), and grown overnight at 30°C while vigorously shaking at 275 rpm. Cells were centrifuged at 3000 rpm for 2 minutes before 50 µl of cells were transferred to a 1.5 ml microcentrifuge tube. Sterile deionised water, 1 ml, was added to the cells and centrifuged at 13000 rpm for 30 seconds. The water was discarded and the pellet resuspended in 100 mM LiCl, 1 ml, and incubated at 30°C for 5 minutes without shaking. A cell suspension, 50 µl, was subsequently transferred to a sterile round bottom microcentrifuge tube, centrifuged at 13000 rpm in a bench top centrifuge for 20 seconds and the supernatant discarded. The following reagents were subsequently added sequentially; 240 µl 50% PEG1000; 26 µl of 1M LiCl; 25 µl salmon sperm DNA (2 mg/ml); and 5 µg plasmid DNA in a volume of 50 µl. The cocktail was mixed thoroughly by vortexing and incubated in a water bath at 42°C for 25 minutes. Cells were centrifuged at 13000 rpm for 20 seconds and resuspended in sterile water before plating 150 µl onto MD (His−) plates. Plates were inverted and incubated at 30°C for 5 days.

2.11 Screening of P. pastoris GS115/pPIC3.5K-SRD5A1 and GS115/pPIC3.5K positive transformants

Successful transformation was achieved using the PEG/LiCl method. Positive transformants grown on MD plates, lacking histidine, were pooled and re-plated on YPD-G418 plates in a G418 concentrations range (0.25, 0.5, 0.75 and 1.0 mg/ml). The level of G418 resistance has
been shown to correlate to the number of copies of the kanamycin gene incorporated into the yeast genome (119). A single copy of pPIC3.5K plasmid integrated into the genome confers resistance to 0.25 mg/ml G418 (116). These plates were inverted and incubated at 30°C for 4 days.

The integration of the SRD5A1 cDNA into the *P. pastoris* genome was subsequently confirmed by PCR amplification analysis. Single colonies picked from YPD-G418 plates and suspended in sterile water (30 µl) prior to the addition of 100 U of lyticase (crude, from *Arthrobacter luteus*) (120). The mixture was incubated at 30°C for 12 minutes to allow digestion of the cell wall. The cells were then flash frozen in liquid nitrogen for 1 minute and the lysate was used as a template for the PCR amplification.

Each PCR amplification mixture (50 µl) contained: the appropriate reaction buffer containing MgCl$_2$ (25 mM); 200 µM of each dNTP; 0.25 µM of each primer (AOX1 and SRD5A); 2.5 U of Pfu polymerase (10 U/µl); and 5 µl crude cell lysate. The amplification profile was as follows: (1) denaturing of the template at 94°C for 1 minutes; (2) 35 cycles of denaturing at 94°C for 1 minute, annealing at 55°C for 1 minute and extension at 72°C for 1 minute; (3) and a final extension at 72°C for 7 minutes. Amplicons were separated and analysed on a 1% agarose gel by electrophoresis. Positive colonies were grown overnight at 37°C while shaking at 200 rpm and freezer stocks were prepared in 15% glycerol and stored at -80°C.

### 2.12 Endogenous steroid metabolism by *P. pastoris*

The endogenous steroid metabolism of *P. pastoris* was firstly investigated as 3α- or 3β-hydroxysteroid dehydrogenase activity has been reported in *S. cerevisiae* (75) and may, as such, interfere with analysis of SRD5A products in this study. A starter culture grown in BMGY medium for 20 hours was used to inoculate a baffled flask containing 25 ml BMMY to an OD$_{600}$ of 1 (5x10$^7$ cells/ml). The culture was incubated at 200 rpm for 48 hours at 30°C. Methanol (0.5% v/v) was added every 24 hours. The metabolism of steroid substrates, T, 11OHT, 11KT, 11OHA4, 11KA4 and DHT was subsequently assayed. Cells were harvested by centrifugation at 2000 x g for 5 minutes at RT, washed with PBS and resuspended in 1 ml fresh BMMY media to an OD$_{600}$ of 1x10$^8$. After a 15 minute pre-incubation at 30°C, steroid (1 µM final concentration) in BMMY media was added in an equal volume (1 ml). Aliquots, 500 µl, were removed at specific time intervals, 0, 30, 60 and 120 minutes to analyse substrate conversion. Steroids were extracted and analysed as described in section 2.5 and 2.6.
2.13 The selection of optimal SRD5A1 expression in *P. pastoris* by substrate conversion assays.

The pPIC3.5K-SRD5A1 vector was successfully incorporated into the yeast genome with clones demonstrating different copy numbers. Each of the transformants were screened for the ability to metabolise T to DHT to determine which clone exhibited the highest SRD5A1 activity. A single pPIC3.5K-SRD5A1 transformant colony from each of the 0.25, 0.75 and 1.0 mg/ml G418 plates (S0.25, S0.75 and S1.0), as well as a single parent vector transformant colony from at 0.25 mg/ml G418 plate (P0.25; control), were inoculated in 25 ml of BMGY medium and incubated at 30°C while shaking at 275 rpm for 18 hours. The cells were grown to an OD$_{600}$ of 4, collected by centrifugation and resuspended in BMGY to a cell density equivalent to that of an OD$_{600}$ of 1. The cultures, 200 ml each, were subsequently grown at 30°C, shaking at 275 rpm for a total of 98 hours. Aliquots, 1 ml, were taken at 0, 24 and 48 hours in order to monitor growth rate. Methanol, 0.5% v/v, was added every 24 hours.

The metabolism of T was assayed after 48 hours as follows. Cells were harvested by centrifugation, 2000 x g for 5 minutes at RT, washed with PBS and resuspended in 1 ml fresh BMGY media to an OD$_{600}$ of 1 (5x10$^7$ cells/ml). After a 15 minute pre-incubation at 30°C in a 6 well culture plate, steroid (1 µM final concentration) in BMGY media was added in an equal volume of 1 ml. Aliquots, 500 µl, were removed at specific time intervals, 30, 60, 120 and 240 minutes to analyse substrate conversion. Control reactions were performed using cells containing the parent vector. Steroids were extracted and analysed as described in section 2.5 and 2.6.

2.14 C19 steroid metabolism by *P. pastoris* expressing SRD5A1

Single colonies of *P. pastoris* strain GS115 expressing SRD5A1 were grown in 200 ml BMGY growth medium for 24 hours at 30°C in a shaking incubator at 275 rpm. Yeast cells were collected by centrifugation at 2000 x g for 5 minutes and subsequently resuspended in BMGY induction medium to an OD$_{600}$ of 1 (5x10$^7$ cells/ml). The cells were subsequently incubated at 30°C, shaking at 275 rpm, for an additional 48 hours. Methanol (0.5% v/v) was supplemented at 24 hours. The metabolism of C19 steroids was subsequently assayed as follows: Cells were harvested by centrifugation, 2000 x g for 5 minutes at RT, washed with PBS and resuspended in 1 ml fresh BMGY media to an OD$_{600}$ of 1 (5x10$^7$ cells/ml). After a 15 minute pre-incubation period at 30°C in a 6 well culture plate, steroid (30 µM final concentration) in BMGY media was added in an equal volume of 1 ml. Aliquots, 500 µl,
were removed at specific time intervals, 30, 60, 120 and 240 minutes to analyse substrate conversion. Control reactions were performed using cells transformed with the parent vector.
Chapter 3

RESULTS

3.1 Analyses of C19 steroid conversion by SRD5A1 expressed in HEK-293 cells

SRD5A1 expressed in HEK-293 cells catalysed the conversion of 1µM 11OHA4, 11KA4, 11OHT and 11KT in 48 hours with no substrate remaining, as confirmed by UPLC-MS/MS. Figure 3.1 shows a chromatogram for the conversion of 11OHA4 to 11OH-5α-dione by SRD5A1. Figure 3.2 shows the negative control in which no 11OHA4 was converted to 11OH-5α-dione.

![Figure 3.1](image)

**Figure 3.1** Conversion of 1 µM 11OHA4 (A) to 11OH-5α-dione (B) by SRD5A1 expressed in HEK-293 cells. Peak at retention time (RT) 3.55 minutes indicated on (B) shows 11OH-5α-dione formation and lack of peak at 3.12 minutes on (A) indicates full conversion of 11OHA4.
Figure 3.2 Negative control for the conversion of 1µM 11OHA4 (A) to 11OH-5α-dione (B) by pClneo (no SRD5A1 expressed) transfected HEK-293 cells. RT 3.12 minutes on (A) indicates no conversion was observed as all of the substrate remains. No product peak is seen at RT 3.55 minutes in (B).

Response factors were determined for the SRD5A products, 11OH-5α-dione, 11K-5α-dione, 11OH-DHT and 11KDHT, so that these products could be quantified using the standard curves obtained for their respective substrates. These response factors were determined by comparing the area under the peak for 1 µM substrate to that of 1 µM product. This allowed for the products to be quantified from the standard curves obtained for the substrate. The response factors are listed below in table 3.1.
Table 3.1 Response factors for the SRD5A1 products 11OH-5α-dione, 11K-5α-dione, 11OHDHT and 11KDHT. Response factors are calculated by dividing the area of the substrate over area of internal standard by the area of product over the area of internal standard. Results are shown as the average ± standard deviation, n=6.

<table>
<thead>
<tr>
<th>Products</th>
<th>11OH-5α-dione</th>
<th>11K-5α-dione</th>
<th>11OHDHT</th>
<th>11KDHT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substrates</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11OHA4</td>
<td>4.147±0.640</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11KA4</td>
<td></td>
<td>4.501±0.549</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11OHT</td>
<td></td>
<td></td>
<td>3.812±0.243</td>
<td></td>
</tr>
<tr>
<td>11KT</td>
<td></td>
<td></td>
<td></td>
<td>2.973±0.179</td>
</tr>
</tbody>
</table>

The response factors determined above allowed for the quantification of the SRD5A1 products investigated in this study. Time course experiments were first performed using HEK-293 cells expressing SRD5A1 and a single substrate concentration of 5 μM. The SRD5A1 catalysed metabolism of A4 and T to 5α-dione and DHT, respectively, is shown in figure 3.3. The metabolism of 11OHA4, 11KA4, 11OHT and 11KT by SRD5A1, as determined using the response factors (table 3.1), to 11OH-5α-dione, 11K-5α-dione, 11OHDHT and 11KDHT, respectively, is shown in figure 3.4. The data shown in figures 3.3 and 3.4 represent different experiments and cannot be compared directly to each other.

Figure 3.3 Time course for the metabolism of 5μM (A) T and (B) A4 by SRD5A1 expressed in HEK-293 cells. Three independent experiments were performed in triplicate. Representative results are from a single experiment performed in triplicate and are expressed as ±SEM.
Figure 3.4 Time course for the metabolism of 5µM (A) 11OHA4, (B) 11KA4, (C) 11OHT and (D) 11KT by SRD5A1 expressed in HEK-293 cells. The results are from a single experiment performed in duplicate and are expressed as ±SD.

A4 was utilised more readily than T within the same experiment, as seen in figure 3.3. After 2 hours 95% of A4 was converted to 5α-dione, while only 78.5% T was converted to DHT. A similar, but more pronounced trend was observed with the 11-oxygenated steroid substrates. After 5 hours, both 11OHA4 and 11KA4 were converted to 91.8% and 94.7% of their respective products. During the same period only 32.3% of 11OHDHT and 42.4% of 11KDHT was formed from the metabolism of 11OHT and 11KT, respectively (figure 3.4). While this data gave an indication of the substrate preferences of SRD5A1, kinetic parameters needed to be determined before any conclusions could be drawn.

3.2 The kinetic characterisation of SRD5A1

Time course experiments were conducted in HEK-293 cells expressing SRD5A1 as the cell line has no significant endogenous steroidogenic activity which would influence the data obtained. The substrates A4, T, 11OHA4, 11KA4, 11OHT and 11KT were assayed at 0.5, 5.0 and 30µM in order to generate progress curves which could be used for the kinetic characterisation of SRD5A1. Transfection efficiency varies in individual experiments, influencing the kinetic parameters. As the enzyme concentration is directly proportional to the initial rate of conversion for a given substrate concentration, the conversion of 5 µM T was
included in all experiments as a reference. The initial rate for the conversion of 5 µM T was used as a scaling factor for the subsequent determination of kinetic parameters.

![Graph](https://scholar.sun.ac.za)

**Figure 3.5** Determination of the initial rate for the metabolism of 5µM T by SRD5A1 expressed in HEK-293 cells.

The initial rates indicated in table 3.2 were determined for the control reactions (5µM T) in each of the progress curve experiments and were used to normalise the experiments during subsequent kinetic parameter determinations.

**Table 3.2** Initial reaction rates for the 5µM T control reactions for the respective progress curve time courses.

<table>
<thead>
<tr>
<th>Initial reaction rate (µM/h)</th>
<th>Substrates</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1949</td>
<td>0.5µM and 5µM A4</td>
</tr>
<tr>
<td>0.6494</td>
<td>0.5µM T</td>
</tr>
<tr>
<td>1.0839</td>
<td>0.5µM and 5µM 11OHA4 and 11KA4</td>
</tr>
<tr>
<td>0.9889</td>
<td>0.5µM 11OHT and 11KT</td>
</tr>
<tr>
<td>3.5517</td>
<td>5µM 11OHT and 11KT</td>
</tr>
<tr>
<td>1.4766</td>
<td>30µM all substrates</td>
</tr>
</tbody>
</table>

41
Figures 3.6 – 3.11 below show the experimental time course data (0.5, 5 and 30 µM) for each of the SRD5A1 substrates investigated in this study as well as the fitted data obtained using the objective function (S, substrates; E, experimental substrate values; M, model predicted substrate values):

\[
\sum_{i=1}^{3} \left( \frac{E_S(t)_i - M_S(t)_i}{E_S(t)_i} \right)^2
\]

The Michaelis-Menten equation was used as a model for the predicted change in S. The 30 µM assays were performed over a period of 4 hours in order to determine the \( V_{\text{max}} \) of SRD5A1 for each of its substrates. These 30 µM assays were therefore all performed in the same experiment in order to compare the \( V_{\text{max}} \) directly.
Figure 3.6 Progress curve analysis of A4 metabolism by SRD5A1 expressed in HEK-293 cells. The initial substrate concentrations were (A) 0.5 µM, (B) 5.0 µM and (C) 30 µM. Experimental data is shown by the individual data points and the fitted data is shown with solid lines. Each time course is from a single experiment performed in triplicate and is expressed as ±SD.
Figure 3.7 Progress curve analysis of T metabolism by SRD5A1 expressed in HEK-293 cells. The initial substrate concentrations were (A) 0.5µM, (B) 5.0µM and (C) 30µM. Experimental data is shown by the individual data points and the fitted data is shown with solid lines. Each time course is from a single experiment performed in triplicate and is expressed as ±SD.
Figure 3.8 Progress curve analysis of 11OHA4 metabolism by SRD5A1 expressed in HEK-293 cells. The initial substrate concentrations were (A) 0.5µM, (B) 5.0µM and (C) 30µM. Experimental data is shown by the individual data points and the fitted data is shown with solid lines. Each time course is from a single experiment performed in triplicate and is expressed as ±SD.
Figure 3.9 Progress curve analysis of 11KA4 metabolism by SRD5A1 expressed in HEK-293 cells. The initial substrate concentrations were (A) 0.5μM, (B) 5.0μM and (C) 30μM. Experimental data is shown by the individual data points and the fitted data is shown with solid lines. Each time course is from a single experiment performed in triplicate and is expressed as ±SD.
Figure 3.10 Progress curve analysis of 11OHT metabolism by SRD5A1 expressed in HEK-293 cells. The initial substrate concentrations were (A) 0.5μM, (B) 5.0μM and (C) 30μM. Experimental data is shown by the individual data points and the fitted data is shown with solid lines. Each time course is from a single experiment performed in triplicate and is expressed as ±SD.
Figure 3.11 Progress curve analysis of 11KT metabolism by SRD5A1 expressed in HEK-293 cells. The initial substrate concentrations were (A) 0.5µM, (B) 5.0µM and (C) 30µM. Experimental data is shown by the individual data points and the fitted data is shown with solid lines. Each time course is from a single experiment performed in triplicate and is expressed as ±SD.

The determined kinetic parameters are shown below in table 3.3. Interestingly a 1.8-fold difference was observed in the affinity of SRD5A1 for A4 and T, with SRD5A1 demonstrating a higher affinity for T ($K_m = 3.73 \mu M$) than A4 ($K_m = 6.76 \mu M$). This contradicts previous studies (86) performed in stably transfected HEK-293 cells in which SRD5A1 demonstrated a higher affinity for A4 ($K_m = 3.5\pm0.5 \mu M$) than for T ($K_m = 12.0\pm1.8 \mu M$). Possible reasons for this discrepancy will be discussed in detail in chapter 4. The $V_{max}$
obtained for A4 was 2-fold higher than that of T (table 3.3), which is in agreement with previous studies (81). \( V_{\text{max}}/K_m \) is a better estimation of catalytic efficiency (121). True enzyme efficiency (\( K_{\text{cat}}/K_m \)) cannot be utilised here as the determination of \( K_{\text{cat}} \) (maximum number of enzymatic reactions per second) requires purified enzyme. \( V_{\text{max}}/K_m \) shows that SRD5A1 has a similar catalytic efficiency towards A4 (\( V_{\text{max}}/K_m = 0.59 \)) and T (\( V_{\text{max}}/K_m = 0.55 \)), illustrated in figure 3.3. When considering the enzymatic activity of SRD5A1 towards the novel substrates, 11OHA4 is the preferred substrate as its catalytic efficiency is 2-fold greater than that of 11KA4. The conversion of T is more efficient than 11OHT and 11KT as the catalytic efficiency was 10-fold greater for T. The metabolism of 11OHT and 11KT was also considerably less efficient than for the C11 hydroxy and keto A4 derivatives as reflected in the 6.6 and 3.4-fold lower catalytic efficiency, respectively. This suggests that the flux through the 11OHA4 pathway would favour the reduction of 11OHA4 and 11KA4 by SRD5A1 rather than that of 11OHT and 11KT, thereby mirroring the flux through the 5α-dione pathway.

**Table 3.3** \( V_{\text{max}} \) and \( K_m \) values generated by progress curve analysis (figures 3.6 - 3.11).

<table>
<thead>
<tr>
<th>Substrates</th>
<th>( V_{\text{max}} ) (µM/h)</th>
<th>( K_m ) (µM)</th>
<th>( V_{\text{max}}/K_m )</th>
</tr>
</thead>
<tbody>
<tr>
<td>A4</td>
<td>4.0</td>
<td>6.8</td>
<td>0.59</td>
</tr>
<tr>
<td>T</td>
<td>2.1</td>
<td>3.7</td>
<td>0.55</td>
</tr>
<tr>
<td>11OHA4</td>
<td>3.1</td>
<td>9.3</td>
<td>0.33</td>
</tr>
<tr>
<td>11KA4</td>
<td>5.9</td>
<td>35.2</td>
<td>0.17</td>
</tr>
<tr>
<td>11OHT</td>
<td>3.9</td>
<td>72.1</td>
<td>0.05</td>
</tr>
<tr>
<td>11KT</td>
<td>4.8</td>
<td>100*</td>
<td>0.05</td>
</tr>
</tbody>
</table>

*No satisfactory optimal solution was found.

### 3.3 Analyses of SRD5A integration into the pPIC3.5K expression vector

The plasmids SRD5A1-pCMV7 and pPIC3.5K were successfully purified. PCR amplification of SRD5A1 cDNA from the SRD5A1-pCMV7 plasmid yielded a 850bp amplicon. SRD5A1 cDNA is 820bp in size, however, the addition of the Kozak sequence and the flanking \( \text{EcoRI} \) restriction cut sites comprised 30 bp. Figure 3.12 shows the analysis of the PCR amplification
on a 1% agarose gel indicating the SRD5A1 DNA fragment was successfully amplified. No DNA fragment was obtained in the negative control PCR amplification.

The amplified SRD5A1 cDNA was subsequently cloned into the pPIC3.5k vector and transformed into DH5α E.coli cells. Plasmid DNA was isolated from transformed colonies and subjected to restriction enzyme digestion with NcoI in order to identify positive clones as well as to analyse the orientation of the cDNA insert. Figure 3.13 shows the analysis of the restriction enzyme digest on a 1% agarose gel.

Lanes 4 show DNA fragments with sizes (2350 bp and 7400 bp) that were calculated for the NcoI digest of the pPIC3.5k vector containing the human SRD5A1 cDNA insert orientated in the 3’-5’ direction, while lane 7 shows DNA fragments with sizes (3150 bp and 6600 bp) which indicate the desired 5’-3’ orientation of the insert. DNA sequencing confirmed that the positive clone identified in lane 7 contains the cDNA encoding human SRD5A1 (GenBank...
assession number E.C 1.3.1.22) in correct orientation, and that the cDNA is in reading frame for functional expression.

![Figure 3.13](image_url)

**Figure 3.13** 1% agarose gel analysing the restriction digest analysis of pPIC3.5K vector containing the SRD5A1. **Lane 1:** 1kb DNA marker (Promega). **Lanes 2, 3, 5, 6 and 8:** *Neoi* digest of vector only; **Lane 4:** *Neoi* digest of a positive clone with containing SRD5A1 in the 3'-5' orientation; **Lane 7:** *Neoi* digest of a positive clone with containing SRD5A1 in the 5'-3' orientation.

### 3.4 Identification of SRD5A1 *P. pastoris* Transformants

Transformation of *P. pastoris* with pPIC3.5K-SRD5A1 plasmid using the spheroplasting and electroporation transformation methods proved to be unsuccessful. Successful transformation was, however, achieved using the PEG/LiCl method. Primary selection was carried out using minimal medium, His- plates. Only transformants which gained the ability to produce histidine (histidine positive (his+) phenotype) were able to grow on these plates.

Positive clones obtained from the first round of selection were subsequently pooled for a secondary selection process on G418-YPD plates containing increasing concentrations of G418. The concentration of G418 plates on which transformants survive correlate with the copy number expression cassette integration into the yeast genome (115). Resistance observed using plates containing 0.25 mg/ml G418 is indicative of a single copy of the expression cassette being integrated into the yeast genome. SRD5A1 transformants were observed on the 0.25, 0.5, 0.75 and 1.0 mg/ml G418 plates (figure 3.14).
Figure 3.14 SRD5A1 transformed *P. pastoris* plated on agar with increasing geneticin concentrations. The degree of resistance correlates to the number of SRD5A1 copies integrated into the yeast genome. (A) 0.25 mg/ml G418 indicating at least 1 copy integration. (B) 0.5 mg/ml G418, growth on this plate indicates 1-2 copy number integration. (C) 0.75 mg/ml G418, 2 or more copy integration and (D) 1.0 mg/ml G418 also indicates 2 or more copy integration.

Single colonies were selected from each plate, termed S 0.25, S 0.5, S 0.75 and S 1.0, respectively and used to inoculate starter cultures. Parent vector transformants were only able to grow on the 0.25 mg/ml G418 plates (figure 3.15). A single colony, P 0.25, was selected.

Figure 3.15 pPIC3.5K transformed *P. pastoris* plated on increasing concentrations of G418. (A) 0.25 mg/ml G418, single copy integration minimum for growth on these plates. 0.5 (B), 0.75 (C) and 1.0 (D) mg/ml G418 respectively, however no growth of parent vector was observed on these plates.
Further confirmation of the successful integration of SRD5A1 into the yeast genome was obtained by PCR analysis of crude yeast lysates. Figure 3.16 shows the successful amplification of SRD5A1 cDNA from the yeast genome of all positive transformants (Lanes 2-4 and 6-9) with exception of the transformant seen in lane 5. The size of the amplicons correlated with the amplicon obtained in the positive control (lane 10) representing the purified SRD5A1-pPIC3.5k plasmid template. Integration of SRD5A1 into the genome of GS115 strain *P. pastoris* was thus confirmed.

![Figure 3.16](image)

**Figure 3.16** 1% agarose gel analysing the direct PCR amplification screening of GS115/pPIC3.5K-SRD5A1 clones. **Lane 1**: Negative control – amplification of yeast transformed with pPIC3.5k vector containing no SRD5A1 insert; **lane 2 and 3**: PCR amplification of positive transformants grown on 1.0 mg/ml G418-YPD selection plates; **lane 4 and 5**: PCR amplification of positive transformants grown on 0.75 mg/ml G418-YPD selection plates; **lane 6 and 7**: PCR amplification of positive transformants grown on 0.5 mg/ml G418-YPD selection plates; **lane 8 and 9**: PCR amplification of positive transformants grown on 0.25 mg/ml G418-YPD selection plates. **Lane 10**: Positive control - PCR amplification of purified pPIC3.5K-SRD5A1 plasmid (100ng). **Lane 11**: 1kb DNA marker.

PCR analysis of crude yeast lysates were also performed using AOX1 primers. These primers result in the amplification of the AOX gene (214bp) and the cloned gene of interest. As the
gene of interested is inserted into the AOX gene, amplification with AOX primers can also detect the integration of multiple copy numbers. Figure 3.17, lane 2, shows the amplification of a single copy of the AOX1 gene (214 bp) from yeast transformed with the parent vector (pPIC3.5k). Lane 3 shows the amplification of a single copy of the AOX (214 bp) with the integrated SRD5A1 (850 bp) cDNA resulting in a band of approximately 1000 bp. Lanes 3-6 show bands of approximately 2200 bp (genomic AOX gene amplification product) indicating that these transformants grown on YPD plates containing 0.5, 0.75 and 1.0 mg/ml G418 have a mut+ phenotype.

Figure 3.17 1% agarose gel analysing the direct PCR amplification screening of GS115/pPIC3.5K-SRD5A1 and pPIC3.5K transformants using AOX1 gene primer set. Lane 1: 1kb DNA marker. Lane 2: PCR amplification of positive Parent vector (pPIC3.5k) transformant grown on a 0.25 mg/ml G418-YPD plate; Lanes 3 – 6 PCR amplification of positive SRD5A1 transformants grown on 0.25, 0.5, 0.75 and 1.0 mg/ml G418-YPD plates, respectively.
3.5 Analysis Determination of endogenous steroid metabolism of *P. pastoris*

The endogenous enzymatic activity of *P. pastoris* towards C19 steroids was assessed. Figure 3.18 shows that *P. pastoris* was unable to metabolise SRD5A1 substrates, T, 11OHA4, 11KA4, 11OHT and 11KT, within a 2 hour incubation period. The cells did, however, metabolise the 5α-reduced steroid, DHT, to an unknown product. These results are in agreement with studies conducted in the yeast *S. cerevisiae* which demonstrated endogenous 3α- or 3β-hydroxysteroid dehydrogenase activity which was able to metabolise DHT (75, 100).

![Figure 3.18](https://scholar.sun.ac.za)  
**Figure 3.18** Endogenous steroid metabolism by *P. pastoris* strain GS115. Cells were incubated with 1μM substrate for 2 hours. DHT was utilised by an endogenous yeast enzyme(s). The results are from a single experiment performed in triplicate and are expressed as ±SD. Data was analysed by a 1way ANOVA followed by a Bonferroni’s Multiple Comparison post-test. (***P<0.001, ns=not significant, n=3).

3.6 Analyses of experimental protocol optimisation Selection for optimal SRD5A1 activity in *P. pastoris*

The growth of SRD5A1 transformants selected in section 3.4 was assessed during an 80 hour induction period with methanol. Figure 3.19 show that the methanol induced expression of SRD5A1 does not negatively affect the growth of any of the transformants.
Figure 3.19 Growth curves of GS115 transformants during induction phase. Growth of transformants with different copy number integrations was monitored after the addition of methanol (0.5%, v/v final concentration). Methanol was added every 24 hours to maintain induction. The results are from a single experiment performed in triplicate and are expressed as ±SEM.

SRD5A1 transformants were screened for SRD5A1 activity by completing a time course assay with T as a substrate. Figure 3.20 shows that the parent vector transformant P 0.25 was unable to metabolise the T substrate. All SRD5A transformants, however, were able to metabolise T. As expected, the single copy number transformant, S 0.25, showed the least SRD5A1 activity. The S 0.75 and S 1.0 transformants which both contain two copies of the SRD5A1 cDNA (section 3.4) exhibited similar activity towards T. S 0.75 showed marginally higher activity than S 1.0 and was therefore chosen for all further experiments. Taken together these results show the successful expression of functional human SRD5A1 in the *P. pastoris* yeast expression system.
Figure 3.20 Time course of T metabolism by SRD5A1 activity in transformed P. pastoris. T (1μM) was assayed in the S 0.25, S 0.75, S 1.0 and P 0.25 transformants. The results are from a single experiment performed in duplicate and are expressed as ±SD.

3.6 Analysis of C19 steroid metabolism by P. pastoris expressing SRD5A1

Figure 3.21 shows that both T and A4 are effectively metabolised by P. pastoris expressing SRD5A1. A4 is, however, metabolised more efficiently than T when using 30μM substrate. 50% of A4 is converted within 30 minutes with negligible formation of AST, thus primarily indicating the catalytic activity of SRD5A1. At this time ±25% of T was metabolised by SRD5A1. This result, obtained under saturating substrate concentrations, is in agreement with the observations made in section 3.2 that $V_{\text{max}}$ of A4 is 2-fold that of T (table 3.3) as well as from previous studies (61). Figure 3.21(A) shows that while T was efficiently metabolised, the 5α-reduced product DHT did not accumulate, indicating that this steroid presented as a substrate for a secondary reaction catalysed by an endogenous yeast enzyme. 3α- or 3β-hydroxysteroid dehydrogenase activity has previously been observed S. cerevisiae (75, 100). Conversion of 5α-dione to androsterone (AST) was detected when A4 was used as a substrate, suggesting endogenous HSD3A activity (figure 3.21B). Interestingly, the 5α-reduced product of A4, 5α-dione, increased during the metabolism of A4 suggesting that the endogenous HSD3A activity does not favour 5α-dione as a substrate (figure 3.21B). In contrast, DHT did not accumulate with levels remaining negligible during the course of the assay, indicating that DHT is the preferred substrate for the endogenous enzymatic activity.
Figure 3.21 Metabolism of T (A) and A4 (B) by *P. pastoris* expressing SRD5A1. Two independent experiments were performed. Representative results are shown from a single experiment performed in triplicate and are expressed as ±SD.

Figure 3.22 shows the metabolism of 11OHA4, 11KA4, 11OHT and 11KT by the SRD5A1 transformant S 0.75. The A4 C11-derivatives, 11OHA4 and 11KA4, were utilised more readily by SRD5A1 than the T C11-derivatives, 11OHT and 11KT. Within 15 minutes 50% of 11KA4 was used while 50% of 11OHA4 was utilised after 30 minutes. In contrast, only 34.5% 11OHT and 52.5% 11KT were metabolised in 2 hours.

The trend observed between 11OHA4 and 11KA4 metabolism by SRD5A1 expressed in the HEK293 cells is not as prominent. The progress curves thus do not reflect what is observed for 11OHA4 and 11KA4 in the yeast model. The substantial difference between the A4 and T derivatives was, however, consistent in both models. The concentration of substrate measured after a 2 hour incubation of the C19 steroids with the P 0.25 control did not change significantly, confirming that substrate conversion was due to SRD5A1 activity.
Figure 3.22 Metabolism of 11OHA4 (A), 11KA4 (B), 11OHT (C) and 11KT (D) by SRD5A1 expressed in P. pastoris. The results are from a single experiment performed in triplicate and are expressed as ±SD.

Interestingly, the endogenous enzyme’s activity was able to utilise the 5α-reduced steroids as substrates as indicated by the lack of accumulation of product. 11K-5α-dione was more readily converted by endogenous HSD3A as the profile of the time course assay for the steroid is downward compared to that of 11OH-5α-dione after 2 hours. The 5α-reduced products of 11OHT and 11KT do not accumulate as the A4 derivatives, however, this could be a result of the lower rate of conversion of 11OHT and 11KT to their respective 5α-reduced products or due to endogenous HSD3A preference for T derivatives over the A4 derivatives. This trend is similar to observations seen for 5α-dione and DHT in figure 3.21.
Chapter 4

DISCUSSION

The SRD5A enzymes are responsible for converting androgenic steroids and androgen precursors to potent androgens (16). The most well-known reaction which SRD5A catalyses is the conversion of T to DHT. Abrogation of SRD5A2 activity during early developmental stages causes a disruption in male sexual differentiation in the embryonic urogenital tract (16, 90). DHT is not available to initiate the differentiation of male the external genitalia and prostate gland (71). The importance of SRD5A in the development and growth of the healthy prostate was clearly established as a result of this genetic disease (16, 122).

As prostate cancer and CRPC are androgen dependent, SRD5A, plays an equally important role in the development and progression of these diseases. Interestingly though, while SRD5A2 expression is predominant in the healthy prostate, studies have shown that it is SRD5A1 which is upregulated during CRPC development, while SRD5A2 is downregulated in this disease (123). Steroid conversion assays in mammalian cells expressing SRD5A have clearly demonstrated that while SRD5A1 has a lower affinity for T than SRD5A2, SRD5A1 has a substantially higher \( V_{\text{max}} \) (61). SRD5A1, however, favours the conversion A4 over T. The upregulation of SRD5A1 expression during CRPC therefore gives the tumour the ability to use the adrenal androgen precursor A4 as a substrate for DHT formation via the alternate 5α-dione pathway, which bypasses T (58). The recent identification of the 11OHA4 pathway has introduced four new substrates, 11OHA4, 11KA4, 11OHT and 11KT, for intratumoral metabolism via SRD5A1 (47, 57). SRD5A1 has been shown to convert all four substrates into novel 5α-reduced androgens (47). These were shown to be capable of activating the wild type human AR to differing degrees. The androgenic activity of 11KT was comparable to that of T, while 11KDHT demonstrated androgenic activity comparable to that of DHT, the “most potent” natural androgen (47, 65).

Other enzymes, which include HSD17B and HSD3A, are present in the healthy prostate and are upregulated during CRPC. These enzymes metabolise 5α-dione pathway metabolites and have more recently been shown to play a vital role, together with HSD11B2, in the 11OHA4 pathway (47, 57). Although SRD5A1 is therefore not the only enzyme in the 11OHA4 pathway metabolising adrenal C19 steroids, the characterisation of SRD5A1 towards
11OHA4, 11KA4, 11OHT and 11KT will provide insight into the preferred steroid flux in the 11OHA4 pathway. The subsequent characterisation of HSD11B2, HSD17B and HSD3A enzymes will allow for the development of a computational model for the conventional C19 pathway, the 5α-dione pathway and the 11OHA4 pathway. This will greatly enhance our understanding of intratumoral androgen metabolism and will aid in the identification of more suitable drug targets.

The first part of this study was conducted with HEK-293 cells, a mammalian model which has been shown to be suitable for the expression of all the relevant steroidogenic enzymes, including SRD5A1 (57, 102, 103, 124). The novel 5α-reduced metabolites produced in the 11OHA4 pathway are not, however, commercially available, creating a hurdle for the quantification of these C19 steroids. This problem was overcome by determining response factors for these 5α-reduced steroids so that they could be quantified using standard curves generated for their substrates. This was possible as multiple reaction mode (MRM) methods had previously been developed for these steroids (44) and since the reaction catalysed by SRD5A is irreversible (47, 57). The peak area for the product could therefore be related to that of the unconverted substrate in the control assay in order to generate a response factor. The accuracy of the response factors was ensured by the incorporation of internal standards which compensate for sample loss during the steroid extraction procedure. In this study the response factors were applied in the quantification of the 5α-reduced steroids produced by the heterologously expressed SRD5A1 in both mammalian and yeast systems. Response factors can in future be utilised for the quantification of novel 5α-reduced steroids metabolised by other enzymes such as HSD11B and HSD17B.

During the first part of this study, SRD5A1 was expressed in HEK-293 cells and activity towards the six substrates from the 5α-dione and the 11OHA4 pathways were assayed. SRD5A1 converted A4 more readily than T when using an initial substrate concentration of 5μM. This trend is in agreement with previous studies which have shown that SRD5A1 catalyses the conversion of A4 more readily than that of T (46). This preference is significant in the post castration environment, where T is depleted, and the adrenal C19 steroids (DHEA and A4) are used as precursors for the production of DHT by the CRPC tumour (46). SRD5A1’s preference towards substrates in the 11OHA4 pathway appear to be similar to those substrates in the alternate pathway, where the 11-hydroxy and 11-keto derivatives of A4 were more readily converted by SRD5A1 than the T derivatives. Performing conversion assays at three separate substrate concentrations permitted the determination of Michealis-
Menten kinetic parameters by progress curve analysis (125). As transfection efficiency can vary between experiments, the SRD5A1 catalysed conversion of 5µM T to DHT was included in each experiment in order to normalise enzyme expression. Using this approach all data sets could be compared even though they were generated in separate experiments. The $K_m$ value for T ($K_m = 3.73\mu M$) produced in this study is very similar to that obtained in previous studies ($K_m = 3.6\mu M$) (81, 86). In contrast, the $K_m$ obtained for A4 (6.76µM) was not only greater than the previously reported value (1.7µM) (81), but also greater than $K_m$ values for T. A possible reason for the difference in $K_m$ values obtained for A4 and T in this study, and those of previously reported, is that whole cells were used in the current study, while previous studies used cell homogenates with the addition of exogenous NADPH. As cofactors assist in the activity of enzymes, increasing or creating an environment in which cofactors are unlimited would allow enzymes to perform under optimal conditions, throughout the assay period. However, these conditions may not always be a true reflection of biologically relevant concentrations. The whole cell approach thus reflects true cellular concentrations as well as the depletion of cofactors resulting from enzymatic activity. The $V_{\text{max}}$ values acquired from this study (A4 = 4.01µM/h and T = 2.05µM/h) relates to a previous study by Andersson et al., as $V_{\text{max}}$ for A4 (1.1 -5.0nmol/(min-mg)) was substantially higher than that for testosterone (0.7 - 3.6 nmol/(min-mg)) (81).

The use of the $V_{\text{max}}/K_m$ ratio is perhaps a more effective way to compare the catalytic efficiency of an enzyme towards different substrates (121). These values suggested that SRD5A1 has similar catalytic efficiencies towards A4 (0.59) and T (0.55). Our results do, however, indicate that T is the preferred substrate at lower substrate concentrations due to the higher affinity of the enzyme for this substrate, while A4 is metabolised more efficiently at higher substrate concentrations. Under castrate conditions, the concentration of A4 is significantly higher (100-fold) than that of T (45) suggesting that A4 will be the preferred substrate (46). The results for the 11OHA4 pathway metabolites suggest that the flux through this pathway would mirror that of the 5α-dione pathway as SRD5A1 prefers 11OHA4 and 11KA4 considerably over 11OHT and 11KT. Considering the C11-hydroxy and C11-keto steroids, the $K_m$ was lowest for 11OHA4 (9.33µM), indicating that SRD5A1 has a greater affinity for this substrate over the other metabolites in the 11OHA4 pathway, 11KA4 (35.20µM), 11OHT (72.06µM) and 11KT (100µM). The $V_{\text{max}}$ values of these metabolites were consistent with the trends observed for the utilisation of the A4 derivatives (11KA4 = 5.91µM/h and 11OHA4 = 3.07µM/h) over the T derivatives (11KT = 4.84 µM/h and 11OHT
= 3.85\mu M/h). The catalytic efficiency ($V_{\text{max}}/K_m$) of 11OHA4 is highest at 0.33, followed by 11KA4 at 0.17 and finally 0.05 for both 11OHT and 11KT. This indicates that the enzymatic activity towards the metabolites in the 11OHA4 pathway is highest towards the A4 derivatives compared to the T derivatives, as is true for the 5α-dione pathway metabolites (46). 11KA4 is a better substrate at higher concentrations which approach $V_{\text{max}}$, however, SRD5A1 has a ± 2 fold higher catalytic efficiency towards 11OHA4 than towards 11KA4 and is likely the preferred substrate under cellular conditions.

The fitted data and experimental data in the progress curve analyses relate well to one another for the metabolites in the 11OHA4 pathway, but the absence of accurate quantification of product formation introduces possible errors that could culminate into less accurate kinetic parameters. The 11OHT and 11KT experimental and theoretical data seem to be the least well fitted, thus $K_m$ values obtained here are preliminary results and could be the improved if the novel steroids were available. The $K_m$ obtained for 11KA4 by progress curve analysis is substantially higher (3.8-fold) than that of 11OHA4 although the substrate utilisation trends are similar, perhaps suggesting that more accurate kinetic parameters can be obtained during future optimisations. Furthermore, future studies should be performed to investigate the ability of the current kinetic parameters to predict the metabolism of C19 substrates at various starting concentrations and with multiple starting substrate combinations. Such experiments will serve as a validation of the current kinetic model. Since the completion of this project 11KDHT has become available, but no quantitative analyses have been carried out with this steroid to date.

A second approach to overcome the unavailability of commercial standards is to use the SRD5A1 enzyme as a biocatalyst to produce sufficient quantities of the novel steroids for analytical purposes. A yeast expression system, $P$. pastoris, was therefore chosen to express functional SRD5A1 protein with the aim of facilitating purification of the enzyme and developing a biocatalyst for the production of novel C19 androgens. The GS115 strain is histidinol dehydrogenase deficient (114), which is exploited in the selection of positive transformants, as the pPIC3.5K vector introduces a selectable marker, $\text{HIS4}$, a gene which encodes histidine biosynthesis. An added selectable marker introduced to the genome of the positively transformed yeast is the $\text{Tn903kan}^r$ gene (Kanamycin resistant gene), which translates to a resistance to G418 in $P$. pastoris that proportionally increases with increase in copy number integration (119). In this study, SRD5A1 was successful cloned into the yeast expression vector and transformation of the yeast was accomplished using the PEG/LiCl
method. Two rounds of selection were carried out, favouring multicopy transformants due to the high expression rates. Transformants which possess the ability to produce histidine, as a result of plasmid integration (HIS4), were pooled post selection on medium lacking histidine and subjected to increasing concentrations of G418 containing YPD-agar plates. Positive transformants, able to resist concentrations of 0.25, 0.5, 0.75 and 1.0 mg/ml G418, were subjected to a crude DNA extraction and subsequent PCR of the genomic DNA as confirmation of the integration of the gene into yeast genome. This also allowed for the estimation of the copy number incorporated. The selection on G418 plates coupled to the PCR results indicated that a single copy number was obtained for the transformant S 0.25, while copy numbers of two were achieved for the transformants S 0.5, S 0.75 and S1.0.

Copy number integration does not, however, always guarantee the more efficient expression of a protein. A simple conversion assay was therefore employed in this study in order to select the transformants with the highest SRD5A1 activity. The transformant S 1.0 and S 0.75 demonstrated the highest SRD5A1 activity, correlating to the copy number determinations, with S 0.75 demonstrating marginally higher SRD5A1 activity. Furthermore, expression of SRD5A1 was shown not to effect the growth of the yeast during the induction phase. This study therefore marks the first report of the functional expression of a human SRD5A enzyme in the methalotrophic yeast P. Pastoris.

A previous study demonstrated the endogenous expression of HSD3A or HSD3B in the yeast S. cerevisiae as this yeast was capable of converting the 5α-reduced steroid DHT to 3α-adiol or 3β-adiol (75). HSD3A/B activity has not previously been investigated in P. pastoris and was therefore explored in this study. Untransformed, methanol induced P. pastoris were incubated with the Δ4 steroids, 11OHA4, 11KA4, 11OHT, 11KT and T as well as the 5α-reduced steroid DHT. While no conversion of the Δ4 steroids was observed, DHT was completely metabolised, indicating probable HSD3A/B activity in the yeast.

This endogenous activity would be a hindrance for the production of the novel 5α-reduced steroids as these steroids would potentially serve as substrates for the endogenous enzymatic activity. An ideal solution would therefore be to purify SRD5A1 from the yeast cells. However, due to the unstable nature of the membrane bound microsomal proteins, neither human isoenzyme has been purified in their active form to date (8). The elimination of the endogenous enzymatic activity may, however, be achieved by fractionating the yeast in order to compartmentalise the SRD5A1. SRD5A1 is expressed in the ER, while HSD is likely
cytosolic. Microsomes could therefore be prepared to obtain SRD5A1 activity in the absence of HSD activity. This approach will be further investigated in future studies. It was, however, first important to confirm that SRD5A1 expressed in the yeast demonstrated sufficient activity towards the hydroxyl and keto derivatives of A4 and T, if this system is to be used as a biocatalyst in future. The metabolism of metabolites in the 5α-dione and 11OHA4 pathways was therefore investigated and the activity of SRD5A1 expressed in yeast was comparable to the activity of SRD5A1 expressed in a mammalian system.

A4 and T metabolism displayed the same trends as seen in the HEK-293 cells (5µM, figure 3.3) as well as in previous studies (86), with the utilisation of A4 being preferred over that of T. A4 (30µM) and T (30µM) were metabolised within 2 hours and 4 hours, respectively by P. pastoris expressing SRD5A1. This data, obtained under substrate saturating conditions, is in agreement with the 2-fold difference in $V_{\text{max}}$ value obtained from the progress curve analysis studies performed in HEK-293 cells (table 3.3).

Furthermore, the conversion of the metabolites in the 11OHA4 pathway by SRD5A1 expressed in the yeast system correlated to trends observed in the mammalian expression system, with the enzyme demonstrating a preference for the A4 derivatives over the T derivatives. The yeast system thus proved to be a viable alternative in studying the enzymatic activities of mammalian enzymes. The unmodified human steroidogenic enzyme CYP17A1, was first expressed in its functional form to allow for characterisation of the enzyme in this yeast system (109). P. pastoris has also been used for the heterologous expression of vast ranges of bacterial (126) and human (109) proteins, which include intracellular (127) and secreted proteins (128).

Conversion of the C11 hydroxy and C11 keto 5α-reduced steroids by the endogenous HSD3A/B was also observed in this study. Interestingly though, the 5α-reduced A4 products (11OH-5α-dione, 11K-5α-dione and 5α-dione) were found to accumulate prior to utilisation by the endogenous enzyme, while the 5α-reduced T products (11OHDHT, 11KDHT and DHT) were rapidly metabolised by the endogenous enzyme demonstrating that the endogenous enzyme prefers the 5α-reduced T derivatives over the A4 derivatives. The yeast model therefore provides further evidence that 5α-reduced metabolites of the 11OHA4 pathway are capable of being packaged for elimination. HSD3A/B catalyses the conversion of active androgen to weak androgens, which are subsequently glucoronidated or sulphonated for elimination (94). HSD3A is thought to be the endogenous enzyme expressed in P.
as evidence of AST formation was observed when A4 was incubated with yeast expressing SRD5A1. Investigations into the inactivation of 5α-reduced C19 steroids by HSD3A and subsequently glucuronidation in the model CRPC cell line LNCaP uncovered an extension to the 11OHA4 pathway. Swart and Storbeck recently proposed that 11OHDHT and 11KDHT are substrates for HSD3A, yielding 11OH-3α-atrion and 11K-3α-adiol, respectively. Although they were only able to show the metabolism of 11KDHT by human HSD3A, this study provides evidence that both 11OHDHT and 11KDHT are metabolised by endogenous HSD3A in *P. pastoris*. Swart and Storbeck have also demonstrated that 11OH-5α-dione and 11K-5α-dione are substrates for human HSD3A, yielding 11OHAST and 11KAST, respectively (129). In this study we found that while 11OH-5α-dione and 11K-5α-dione were substrates for endogenous HSD3A, this yeast enzyme preferred 5α-reduced T derivatives. In the prostate the A4 derivatives are only glucuronidated/sulphonated and eliminated once HSD3A has metabolised them, the accumulation of these metabolites in the CRPC tumour environment may therefore elicit unfavourable receptor driven responses.

In conclusion, charactering SRD5A1, in conjunction with other enzymes involved in the 5α-dione and 11OHA4 pathways, requires innovative approaches as the 5α-reduced steroids produced in this pathway are not commercially available. Incorporating response factors thus far has yielded good results which allowed for the characterisation of SRD5A1 in HEK-293 cells. The accuracy and linearity of these response factors could be confirmed in future by repeating the experiments using multiple starting concentrations. The best method would, however, be to produce sufficient quantities of the steroids, using a biocatalyst, as to facilitate their extraction and purification. This study has shown that the yeast expression system is a strong candidate for achieving high yields of active enzyme which can be either purified or studied in its cellular environment in microsomal preparations. Higgins and Cregg previously stated that the biggest hurdle with regards to heterologous protein expression in yeast systems is achieving initial success, with the expression of protein at any level considered as such (106). This objective was successfully achieved in this study, the first in which the expression of functional human SRD5A1 in *P. pastoris* has been reported.
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